ANALYTICAL

SYNTHESIS

BIO-TECHNOLOGY

BIOACTIVITY & SAR

BIODIVERSITY

NPC

PHARMACO-LOGY

BIOSYNTHESIS

MOLECULAR STRUCTURE

CHEMICAL ECOLOGY

**This Issue is Dedicated to
Professor Feng-Peng Wang
On the Occasion of his 70th Birthday**

INFORMATION FOR AUTHORS

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Professor Feng-Peng Wang

Editorial

It is my privilege and pleasure to introduce this issue, which is dedicated to Professor Feng-Peng Wang, West China School of Pharmacy, Sichuan University, China, on the occasion of his 70th birthday. The editors join me in paying tribute to Professor Wang for his outstanding contributions to natural products chemistry and diterpenoid alkaloids in particular.

He has published over 255 research papers, book chapters, reviews, and three books. He is on the Editorial Advisory Boards of several journals including *Journal of Asian Natural Products Research*, *Acta Pharmaceutica Sinica B*, the *Chinese Journal of Natural Medicines*, and *Natural Product Research and Development*.

I am extremely grateful for his contributions to *Natural Product Communications* from the very beginning. He has already published 16 articles in the journal, his first appearing in 2006 [Two new C_{18}-diterpenoid alkaloids from *Aconitum piepunense*. *Natural Product Communications*, *1*, 191-194.] and the most recent in 2015 [Recent advances in the synthesis of *Stemona* alkaloids. *Natural Product Communications*, *10*, 1093-1102]. I am also looking forward to his further publications in *NPC*, as well as his continued support.

It is a great pleasure to honor the outstanding achievements of Professor Wang on the occasion of his 70th birthday and to send him warm wishes from all his colleagues and friends. Special thanks to Professor Geoffrey A. Cordell (Natural Products Inc., Evanston, IL, USA) and Professor Qiao-Hong Chen **(**Department of Chemistry, California State University-Fresno**,** Fresno, CA, USA) for the preface. My thanks go to the authors and reviewers who have made this issue of *Natural Product Communications* possible.

We all express our appreciation for Prof. Wang's excellent contributions to natural products research and wish him all the best for his future.

Pawan K. Agrawal
Editor-in-Chief

Preface

It is a very great pleasure and honor to be invited to write a Preface to this issue of *Natural Product Communications* in recognition of the 70th birthday and the scientific achievements of my dear friend and illustrious colleague Professor Feng-Peng Wang of the West China College of Pharmacy at Sichuan University in Chengdu. He was born on May 11, 1945 in Changwu County, ShanXi Province. He received a B.S. degree in Pharmacy in 1969 from Sichuan Medical College, and was awarded a M.S. degree in 1981, and a Ph.D. degree in Medicinal Chemistry in 1984, from the Institute of Materia Medica, Peking Union Medical College & Chinese Academy of Medical Sciences. His M.S. supervisor was Dr. Qi-Cheng Fang and his Ph.D. supervisor was Academician Xiao-Tian Liang, himself a world-class natural product chemist and a pioneer in the field of natural products in China. In 1985, Professor Wang travelled to the United States and worked for two years as a postdoctoral research fellow in the laboratory of Professor S. William Pelletier at the Natural Products Institute in the University of Georgia. Professor Pelletier was a well-known alkaloid chemist with a substantial interest in the structure elucidation and chemistry of the diterpene alkaloids.

When he returned to China, Professor Wang resumed his lecturer position at the School of Pharmacy of West China University of the Medical Sciences where his talents, drive, and hard work led him through the academic and administrative ranks to become Professor and Dean of the School of Pharmacy, WCUMS, which in 2000 became assimilated with Sichuan University. He is presently a Professor of Medicinal and Natural Products Chemistry, and a highly regarded academic leader in Sichuan Province.

Professor Wang has been working in the field of natural products for over 50 years, and his research interests have consistently focused on the various groups of diterpenoid alkaloids, in particular, those which are derived from traditional Chinese medicines. His research involves the isolation, structure determination, chemistry, structural modifications, and synthesis of alkaloids, and in this process he has characterized 207 new natural products from a variety of plants. As a chemical transformation approach to the taxane diterpene system, he developed the process for the synthetic conversion of abundantly available diterpenoid alkaloids to taxane derivatives. These studies led to the development of numerous intriguing reactions, the formation of over 1000 diterpene alkaloid derivatives, and to his substantial reputation in the field of alkaloid chemistry.

He has also applied his talents in natural product chemistry to drug discovery and development, where he has made significant contributions, including four patents. Particularly, he was involved in the assessment of the effects of several traditional Chinese medicines, including *Fritillaria cirrhosa*, *Codonopsis tangshen*, and *Rhodiola rosea*. At the same time, he was responsible for the development of several standardized preparations of traditional Chinese medicines, including Huo Xiang Zheng Qi San, Wendan Decoction, Yupingfengsan, and Ziziphi Spinosae Decoction. The first three preparations have already entered the Japanese market. In collaboration with a pharmaceutical company, he has also successfully developed the first, third-generation, multidrug resistance reversal agent (XiuTaiJun) in China. This drug was approved by the Chinese FDA to enter clinical trials, and a phase I study is almost completed.

Professor Wang has assumed responsibility for the development of 18 national and provincial research projects, and has supervised 64 Masters and Doctoral students and several postdoctoral research associates. Over the years, these results have been published in 223 peer-reviewed research articles and in 23 reviews. He is the author or co-author of 11 book chapters, and is an editor of three books. He has been a consistent and outstanding contributor to *The Alkaloids, Chemistry and Biology* series, with chapters on various aspects of the diterpene alkaloids in Volumes 42, 59, 67, and 69. These contributions established him as the world expert on diterpenoid alkaloids. Indeed, his last chapter on the C_{19}-diterpenoid alkaloids was a true "tour de force", and constituted the whole volume of 578 pages! Two books which he has edited in Chinese, *The Chemistry of Alkaloids* and *Modern Chemistry of Natural Products*, are widely recognized by the natural product community in China as being essential texts.

As a result of his contributions to the chemistry of natural products, Professor Wang and his students have received a number of important awards. The doctoral dissertation of Qiao-Hong Chen, one of his graduate students, received an award as one of the Top 100 National Excellent Doctoral Dissertations in 2003. In 1991, Professor Wang received an Award for a Chinese Ph.D. with Outstanding Contributions from the Ministry of Education in China, the National Evaluation Institute of Degrees and Grade Education, and Peking Union Medical College & Chinese Academy of Medical Sciences. Since 1993, Professor Wang has received special government allocations from the State Council in China, and in 1995 he received the first prize of Wu JiePing Medical Research Award - Paul Janssen Phytochemistry Research Award. In 2002, and again in 2015, he achieved a First Place Natural Science Award from the Ministry of Education in China, and a first place Technology Invention Award from the Ministry of Education in 2004.

Professor Wang has also held a number of professional positions locally and nationally. He was the Chair of the Chengdu Pharmaceutical Association from 1995 to 2005, and the Associate Director of Medical Plant and Plant Medicine at the Botanical Society of China from 2003 to 2008. He has served as an editorial member for the *Journal of Asian Natural Products Research*, *Acta Pharmaceutica Sinica B*, the *Chinese Journal of Natural Medicines*, and *Natural Product Research and Development*. Professor Wang has dedicated his time to serve as a committee member for several key laboratories in China, including the Key Laboratory of Natural and Biomimetic Drugs at Peking University Health Science Center (1990-1996), the Key Laboratory of Phytochemistry and Sustainability of Western Plant Resources at the Kunming Institute of Botany, Chinese Academy of Sciences (1992-2007), at the Key Laboratory of Naturally Bioactive Chemicals and Their Functions, at the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (2008-2012), at currently at the Key Laboratory of Basic Substance and Resource Utilization of Chinese Herbal Medicine, Institute of Materia Medica and Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College (2012-present), and at

the Guangdong Higher Education Key Laboratory of Potential Chemical Components and Innovation of Traditional Chinese Medicines, College of Pharmacy, Jinan University (2010-present).

I have had the great pleasure of knowing Professor Wang for many years, indeed since he was with Professor Pelletier as a postdoctoral fellow, and worked with him on the chapters for *The Alkaloids* series, when I was the Editor. More personally though, I have been blessed to have had the great good fortune to have spent time in Chengdu with him on several occasions, and I have had the enormous pleasure to be the recipient of his tremendous generosity and hospitality. Together, we have traveled to many parts of Sichuan and Yunnan Provinces, as well as a highly memorable trip down the Yangtze River from Chongqing to Wuhan. We have visited many of the most spectacular sights in that part of China, including the giant panda sanctuaries in Wolong, the glorious Mt. Emei, one of the most revered mountains in Chinese Buddhism, the unparalleled scenery of Jiuzhaigou (twice!), several Tibetan autonomous areas, the ethnic communities in Lijiang and Shangri-la in Yunnan Province, and numerous wonderful temples and monasteries, as well as the remarkable water works of Dujiangyan. My life has been made significantly richer thanks to Professor Feng-Peng Wang, for which I am extremely grateful. So, at this time, it is truly and sincerely a very great pleasure to have the opportunity to honor him for his extensive contributions to natural products over the past 50 years, and to recognize the auspicious occasion of his 70th birthday.

Geoffrey A. Cordell,
Natural Products Inc.
Evanston, IL, USA

Preface

It is my great privilege and pleasure to be able to write a preface for this special issue of the *Natural Product Communications* (*NPC)* in honor of the seventieth birthday of Dr. Feng-Peng Wang, a professor of Medicinal and Natural Product Chemistry of Sichuan University in P. R. China. Professor Wang has dedicated his 45-year professional life to the advancement of natural products research and education in China.

Professor Feng-Peng Wang was born in a family of a remote village located in the northwest part of China. His illiterate parents lived on farming a small piece of land, but their vision statement was to highly inspire their children to pursuit knowledge through reading books. Professor Wang earned a B.S. degree in pharmacy in 1969 from Sichuan Medical College; he then was awarded with a M.S. degree and a P.D. degree in Medicinal Chemistry in 1981 and 1984, respectively, from the Institute of Material Medica, Peking Union Medical College & Chinese Academy of Medical Sciences. His lifetime love of natural products (especially diterpenoid alkaloids) was initiated by his master thesis and Ph.D. dissertation, especially under the guidance of Professor Xiao-Tian Liang who was a founder and pioneer of Chinese natural products chemistry. After graduation he worked for two years at the University of Georgia, USA, with Professor S. W. Pelletier as a postdoctoral associate in the field of diterpenoid alkaloids. In 1987, he returned back to his Alma mater---the West China University of Medical Sciences, where he persisted in his work on isolation, structural determination, chemical reactions, synthesis, and biological evaluation of diterpenoid alkaloids from *Aconitum* and *Delphinium* species. Since 1990, his research interests were extended to the conversional syntheses of anticancer taxane anaglogs from diterpenoid alkaloids, development of multi-drug resistance reversal agents for the combination therapy of cancer, and development of diterpenoid alkaloids for the potential treatment of cardiovascular diseases.

His 45-year affiliation with Sichuan University can be concluded as an excellent researcher and educator with productivity and high quality in both publications and training graduate students. Professor Wang has made numerous outstanding contributions to the field of Natural Products featuring the one in the field of diterpenoid alkaloids, a class of the largest and most complicated group of terpenoid alkaloids with an intriguing chemistry and numerous bioactivities. Professor Wang has 'dug out' over 200 new diterpenoid alkaloids from various *Aconitum* and *Delphinium* plants and prepared over 1000 new diterpenoid alkaloids. One diterpenoid alkaloid (bulleyaconitine A) that was first isolated by Dr. Wang *et. al*. has been developed into clinical use as non-narcotic analgesic and anti-inflammatory drug in China. Some diterpenoid alkaloids with novel skeletons (e.g. atropurpuran) discovered by his research group have become attracting and challenging synthetic targets of many excellent research groups due to their intrinsic beauty and structural complexity. Very recently, one compound designated as "WF" isolated from "Fuzi" (a famous traditional Chinese medicine) has been established by Dr. Wang's research group as a promising candidate that significantly enhance the survival rate of mice with chronic heart failure and to appreciably improve the cardiac structure and functions in mice, as compared to cedilanid. He has successfully developed the first third-generation multi-drug resistance reversal agent (XiuTaiJun) in China, which has entered human phase I clinical studies in China. During his productive and distinguished career spanning over 45 years, Professor Wang has authored or coauthored over 240 peer-reviewed scientific publications and 11 book chapters, and is an editor for 3 books. As an expert on diterpenoid alkaloids, he has persistently committed to the series collections of "The Alkaloids" edited by Professor Geoffrey A. Cordell and has compiled four chapters for volumes 42, 59, 67, and 69. Two books entitled "The Chemistry of Alkaloids" and "Modern Chemistry of Natural Products" compiled by Dr. Wang have been widely recognized by and have indeed benefited to the natural product community in China.

In addition to the high quality of his own research work, he has successfully trained 40 Master's level and 26 doctoral students, and 3 postdoctoral associates. Most of his students have highly successful careers in academies or pharmaceutical industry. His contributions continue to inspire his students and young scientists. In addition to his formal presentations in the scientific literature, Professor Wang has been and still is very active in helping and inspiring young scientists by serving as an academic committee member in a variety of universities and research institutes throughout China.

Throughout his highly rewarding tenure, Professor Wang has achieved numerous awards, including the first prize of Wu Jieping Medical Research Award – Paul Janssen Phytochemistry Research Award in 1995, two Natural Science Awards and one Technology Invention Award from the Ministry of Education in China (First Place, 2002, 2004, and 2015). Additionally, one doctoral dissertation supervised by him has achieved an Award for Top 100 National Excellent Doctoral Dissertations in China in 2003.

The author was fortunate enough to be associated with Professor Wang in the late 1990s as one of his Ph.D. students which inspired the author to be interested in natural products and initiated an over 15-year productive collaboration. The author has benefited much from his guidance.

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Contents

Continued Overleaf

Accounts/Reviews

Additions/Corrections

Manuscripts in Press

Cummulative Index

LIST OF AUTHORS

KEYWORDS INDEX

Petchienes A–E, Meroterpenoids from *Ganoderma petchii*

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Petchienes A-E (**1-5**), five new meroterpenoids, were isolated from the fruiting bodies of *Ganoderma petchii*. Their structures, including absolute configurations, were elucidated by means of spectroscopic and computational methods. Compound **4** was isolated as a racemic mixture, which was finally purified by chiral HPLC to yield individual (–) and (+)-antipodes. Biological evaluation showed that compounds **2** and (-)-**4** could increase intracellular free calcium concentration at 10 μM in HEK-293 cells.

Keywords: *Ganoderma petchii*, Ganodermataceae, Meroterpenoids, Spiro-compound.

Meroterpenoids from the genus *Ganoderma* are characterized by a 1,4-dihydroxybenzene residue conjugated with a terpenoidal moiety. Since the discovery from *G. pfeifferi* of ganomycins A and B in this class of meroterpenoids [1], few other investigations associated with this compound class from *Ganoderma* species have been reported. We isolated a novel meroterpenoid termed lingzhiol from *G. lucidum* [2a], and since then, several structurally diverse meroterpenoids with renal or neural protective effects were characterized by us [2b–2e]. In our continuing efforts focused on the search of bioactive meroterpenoids from *Ganoderma*, *G. petchii* became our research target. This fungus is distributed in China, Sri Lanka, Malaysia, Singapore and Indonesia. In China, it is used as a healthcare mushroom and seen in several markets of Chinese medical materials. Our efforts on this fungus led to the isolation of five new meroterpenoids, and here we describe their isolation, structure characterization and biological evaluation.

Figure 2: COSY and key HMBC correlations of **1**.
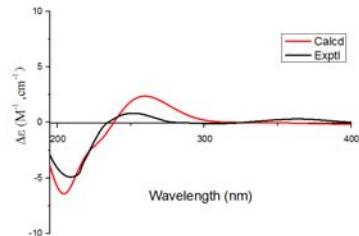
Figure 3: Calculated and experimental ECD spectra of (+)-**1** (red, at the B3LYP-SCRF (PCM)/6-311+G(2d,p)//B3LYP/6-311+G(2d,p) level in MeOH; black, experimentally observed in MeOH).

Figure 1: The chemical structures of compounds **1**–**5**.

Petchiene A (**1**) has the molecular formula $C_{16}H_{18}O_6$ derived from its HRESIMS, ^{13}C NMR and DEPT spectra. The ^{1}H NMR spectrum (Table 1) of **1** contains a typical ABX spin system [δ_H 7.32 (1H, d, *J* = 2.7 Hz, H-3), 7.07 (1H, dd, *J* = 8.9, 2.7 Hz, H-5), 6.79 (1H, d, *J* = 8.9 Hz, H-6)], suggesting the presence of a 1,2,4-trisubstituted benzene ring, along with a resonance for an olefinic proton. The ^{13}C NMR and DEPT spectra contain resonances for 16 carbons including five aliphatic methylenes (one oxygenated), four methines

(all sp[2]), and seven quaternary carbons (one ketone, one carboxylic acid, four olefinic including two oxygenated). The ^{1}H-^{1}H COSY spectrum (Figure 2) showed correlations between H-5/H-6 and H-6′/H-7′/H-8′. In the HMBC spectrum, the correlation from H-3 to C-1′ (δ_C 204.9) indicates that the phenyl unit is linked to C-1′, and from H-2′ (δ_H 3.49, 3.46) to C-2 (δ_C 120.1), C-1′, C-3′ (δ_C 44.8) indicate that C-2′ is linked to C-1′. In addition, HMBC correlations between H-7′/C-3′, C-5′ and H-4′/C-6′, C-8′, in combination with COSY correlations of H-6′/H-7′/H-8′ and degrees of unsaturation of **1**, indicate the presence of a six-membered ring. The positions of a carboxylic acid and a hydroxymethyl were assigned by the HMBC correlations between H-2′, H-4′/C-10′ and H-4′, H-6′/C-9′. Hence, the planar structure of 1 was identified as shown. **1** was isolated as a non-racemic compound indicated by its optical rotation. The absolute configuration at the single chiral center of **1** was clarified by electronic circular dichroism (ECD) calculations. For this

purpose, DFT and TD-DFT calculations were carried out at 298 K in the gas phase with Gaussian 09 [3]. The ECD spectrum of (3′*S*)-**1**, correlates well with the experimental ECD spectrum of **1**, leading to the unambiguous assignment of the absolute configurations at the stereogenic centers in **1** as 3′*S* (Figure3).

Petchiene B (**2**) was found to have the molecular formula $C_{15}H_{16}O_4$ (8 degrees of unsaturation) deduced from its HRESIMS, ^{13}C NMR and DEPT spectra. The ^{1}H and ^{13}C NMR spectra of **2** are similar to those of **1**. The only difference is that the Δ^2 double bond of **2** was formed by decarboxylation of **1** (Figure 4), which could be supported by HMBC correlations between H-2′ (δ_H 6.77)/C-1′, C-2, C-3′ and H-7′, H-8′/C-3′ (δ_C 157.7). In addition, a ROESY correlation between H-2' (δ_H 6.77)/H-4' (δ_H6.39) was observed, indicating a *trans* double bond between C-2′ and C-3′.
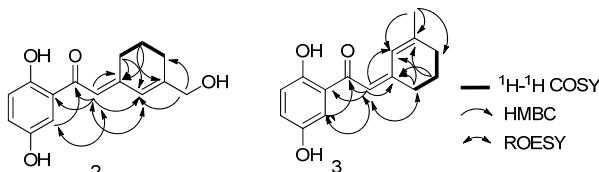
Figure 4: COSY and key HMBC correlations of **2** and **3**.

Table 1: ^{1}H NMR data of **1**–**3** (δ_H in ppm, *J* in Hz).

position	1[a]	2[b]	3[c]
3	7.32 d (2.7)	7.26 d (2.8)	7.26 overlap, 7.25 d (2.6)[d]
5	7.07 dd (8.9, 2.7)	6.97 dd (8.8, 2.8)	6.98 dd (8.8, 2.8)
6	6.79 d (8.9)	6.77overlap	6.86 d (8.8)
2′	a: 3.49 d (18.1)	6.77 overlap	6.55s
	b: 3.46 d (18.1)		6.56 s[d]
4′	5.82 s	6.39 s	6.06 s
6′	2.00 t-like (5.9)	2.20 t (5.9)	2.18t (5.8)
7′	a: 1.82 m	1.79 m	1.76 m
	b: 1.72 m		
8′	a: 2.20 m	3.02 t (6.0)	2.98 t (6.3)
	b: 1.66 m		2.48 t (5.9)[d]
9′	3.96 s	4.14 s	1.91 s

[a]600 MHz in acetone-d_6.[b]600 MHz in methanol-d_4.[c]600 MHz in CDCl$_3$.[d] in methanol-d_4.

Petchiene C (**3**), isolated as a yellow solid, has the molecular formula $C_{15}H_{16}O_3$ (8 degrees of unsaturation), based on its HRESIMS, ^{13}C NMR and DEPT spectra. The NMR data of **3** closely resemble those of **2**, indicating that they are analogues. The only difference is that a hydroxymethyl group in **2** was replaced by a methyl in **3** evidenced from HMBC correlations between H$_3$-9′ (δ_H 1.91)/C-4′, C-5′, C-6′ (Figure 4). A ROESY correlation between H-2' (δ_H 6.56 in methanol-d_4)/H-8' (δ_H 2.48) was observed, indicating the configuration of the double bond.

The molecular formula of racemic petchiene D (**4**) was determined to be $C_{15}H_{16}O_4$ (8 degrees of unsaturation) by analysis of its HRESIMS, ^{13}C NMR and DEPT spectra. Compound **4** could be generated from **2** via Michael addition reaction to construct a spiro compound. This conclusion is supported by the HMBC correlations between H-2′/C-1′, C-2, C-3′ (δ_C 79.1), C-4′ and H-8′/C-2′, C-3′, C-4′. In addition, ROESY correlations between 4-OH (δ_H 9.43 in DMSO-d_6)/H-3, H-5 were observed, indicating the position of the free hydroxyl group in the benzene ring. Thus far, the planar structure of **4** was identified as shown. Chiral HPLC analysis indicated that **4** is racemic. Subsequent separation by chiral HPLC yielded (+)-and (−)-**4** in a ratio of 1.3:1; the absolute configuration at the stereogenic center was assigned to be 3′*S* for (−)-**4** by the same methods as those of **1** (Figure 6).
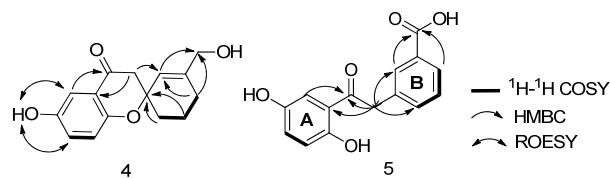
Figure 5: COSY and key HMBC correlations of **4** and **5**.

Table 2: ^{1}H NMR data of **4** and **5** (δ_H in ppm, *J* in Hz).

position	4[a]	5[a]
3	7.15 d (2.9)	7.36 d (2.9)
5	7.00 dd (8.9, 2.9)	7.10 dd (8.9, 2.9)
6	6.79 d (8.9)	6.80 d (8.9)
2′	a: 2.80d (16.5)	4.39 s
	b: 2.74 d (16.5)	
4′	5.84 s	7.95brs
6′	a: 2.09overlap	7.92 d (7.7)
	b: 1.99 m	
7′	a: 1.89 m	7.43t (7.6)
	b: 1.67 overlap	
8′	a: 2.09overlap	7.51 d (7.5)
	b: 1.67overlap	
9′	3.95s	

[a]600 MHz in methanol-d_4.

Table 3: ^{13}C NMR (150 MHz) data of **1**–**5** (δ_C in ppm).

No.	1[a]	2[b]	3[c]	4[b]	5[b]
1	156.3	157.3	157.4	152.4	156.9
2	120.1	122.3	121.1	121.8	120.2
3	115.4	115.2	114.7	110.8	115.9
4	150.2	150.4	147.2	155.0	150.7
5	125.7	125.0	123.8	126.1	126.1
6	119.5	119.6	119.2	120.6	119.8
1′	204.9	197.1	195.5	194.7	204.8
2′	48.4	119.1	116.4	49.2	45.8
3′	44.8	157.7	157.7	79.1	136.4
4′	123.7	125.7	127.1	122.1	131.9
5′	141.4	154.3	151.6	145.6	132.4
6′	25.9	27.1	31.0	26.5	129.3
7′	19.9	23.2	22.3	19.4	129.7
8′	32.4	28.8	27.2	34.1	135.3
9′	66.6	66.1	24.9	66.1	170.0
10′	177.3				

[a]In acetone-d_6.[b]In methanol-d_4.[c]In CDCl$_3$.
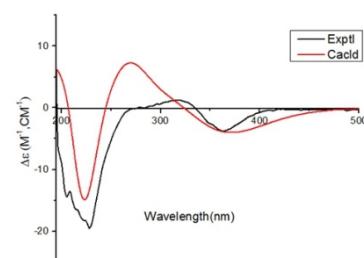
Figure 6: Calculated and experimental ECD spectra of (−)-**4** (red, at the B3LYP-SCRF (PCM)/6-311+G(2d,p)//B3LYP/6-311+G(2d,p) level in MeOH; black, experimentally observed in MeOH).

Petchiene E (**5**) was determined to have the molecular formula $C_{15}H_{12}O_5$ (10 degrees of unsaturation) on the basis of HRESIMS analysis. The[1]H NMR spectrum of **5** (Table 2) contains seven aromatic protons. The ^{13}C NMR and DEPT spectra (Table 3) contain 15 signals ascribed to one sp[3] methylene, seven methines (seven sp[2]), and seven quaternary carbon signals (one ketone, one carboxylic acid, and five sp[2]). The ^{1}H-^{1}H COSY spectrum shows the existence of fragments H-5/H-6, and H-6′/H-7′/H-8′. In the HMBC spectrum, correlations between H-3/C-1′ and H-2′/C-2, C-1′, C-3′, C-4′, C-8′ indicate that the phenyl unit A is linked to C-1′ and the phenyl unit B is linked to C-2′ (Figure 5). The HMBC
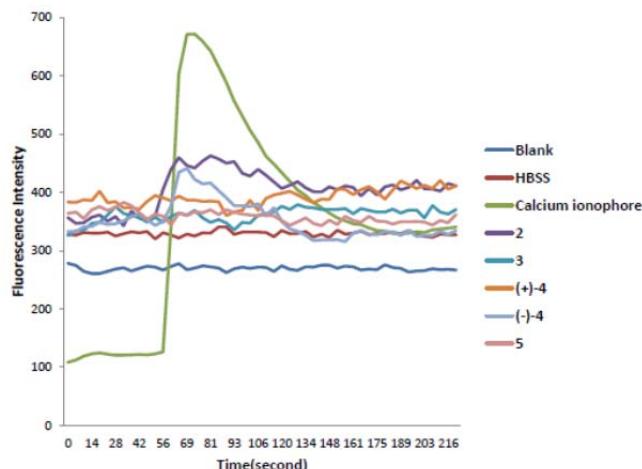
Figure 7: Effect of the isolates on intracellular Ca²⁺ concentration in HEK-293 cells.

correlations between H-4′, H-6′/C-9′ (δ_C 170.0) suggest that the carboxylic acid group is connected to C-5′. As a result, the structure of **5** was determined.

Biological evaluation: Calcium (Ca^{2+}) is a fundamental second messenger that is involved in a wide range of cellular processes and, therefore, is of potential interest in drug discovery [4]. The intracellular free Ca^{2+} concentration is regulated through multiple mechanisms in neurons, and abnormalities in Ca^{2+} signaling have been implicated in many neurological disorders [5]. Considering that many species of *Ganoderma* are used for the treatment of central nervous system associated diseases, the isolates were evaluated for their potential on the changes in Ca^{2+} in HEK-293 cells. The results showed that compounds **2** and (–)-**4** could significantly elevate intracellular Ca^{2+} concentration at 10 μM (Figure 7). Whether these two compounds are beneficial for neurological disorders needs further investigation.

Experimental

General: Column chromatography (CC) was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Inc., People's Republic of China), C-18 silica gel (40–60 μm; Daiso Co., Japan), MCI gel CHP 20P (75–150 μm, Mitsubishi Chemical Industries, Tokyo, Japan) and Sephadex LH-20 (Amersham Pharmacia, Sweden). Optical rotations were recorded on a Horiba SEPA-300 polarimeter. UV spectra were recorded on a Shimadzu UV-2401PC spectrometer, IR spectra on a Brucker Tensor-27 spectrophotometer, and CD spectra on a Chirascan instrument. Semi-preparative HPLC was carried out using an Agilent 1200 liquid chromatography; the column used was a 250 mm × 9.4 mm, i.d., 5 μm, Zorbax SB-C₁₈ and a 250 mm ×10 mm, i.d., 5 μm, Daicel Chiralpak (IC), flow rate: 3 mL/min. NMR spectra were recorded on a Bruker AV-600 spectrometer, with TMS as an internal standard. EIMS and HREIMS were determined on an AutoSpec Premier P776 spectrometer. ESIMS and HRESIMS were measured on API QSTAR Pulsar 1 spectrometer.

Fungal material: The fruiting bodies of *G. petchii* (Lloyd) Steyaert were purchased from a market of Chinese medical materials located at Zhonghao-Luoshi-Wan of Kunming, People's Republic of China, in July 2014. A voucher specimen (CHYX-0588) was authorized by Prof. Zhu-Liang Yang at Kunming Institute of Botany and deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, People's Republic of China.

Extraction and isolation: The powdered fruiting bodies of *G. petchii* (60 kg) were extracted under reflux with 70% EtOH (150 L × 2 h × 3) to give a crude extract (3.39 kg), which was suspended in H_2O followed by extraction with EtOAc to afford an EtOAc soluble extract. Separation of this extract (2.09 kg) using a MCI gel CHP 20P column eluted with a gradient of aqueous MeOH (10%–100%) produced 8 portions (Fr.1–Fr.8). Among them, fr.5 (410 g) was further separated via MCI gel CHP 20P washed with a gradient of aqueous MeOH (20%–100%) to yield 7 fractions (Fr.5.1–Fr.5.7). Fr.5.4 (3.48 g) was submitted to a RP-18 column with a gradient of aqueous MeOH (30%–80%) to afford 8 sub-fractions (Fr.5.4.1–Fr.5.4.8). Fr.5.4.4 (233 mg) was purified by preparative TLC (2 drops of formic acid in CHCl₃/MeOH, 9:1) followed by semi-preparative HPLC (MeOH/H₂O, 43%) to yield compounds **1** (6.8 mg, t_R = 23 min), **2** (9.7 mg, t_R = 18 min) and **5** (1.0 mg, t_R = 25 min). Fr. 6 (50 g) was separated using MCI gel CHP 20P (MeOH/H₂O, 10:90, 30:70, 50:50, 70:30, 80:20) to yield 4 fractions (Fr.6.1–Fr.6.4). Fr. 6.3 (12 g) was subjected to gel filtration on Sephadex LH-20 (MeOH) followed by chromatography on a RP-18 column (MeOH/H₂O, 50%–70%) to yield **3** (5.3 mg, t_R = 28 min) and **4** (6.0 mg, t_R = 26 min). Racemic compound **4** was subjected to chiral HPLC to afford (+)-**4** (2.2 mg) and (−)-**4** (2.1 mg) (*n*-hexane/ethanol, 90:10).

Petchiene A (1)
Pale yellow solid.
[α]$_D^{23.4}$: +12.3 (*c* 0.64, MeOH).
IR (KBr) ν_{max}: 3423, 2935, 2872, 1707, 1644, 1624, 1590, 1486, 1449, 1369, 1280, 1222, 1179, 998, 869, 831, 785 cm⁻¹.
UV (MeOH) λ_{max} (log ε): 365 (3.57), 256 (3.87), 224 (4.17)nm.
CD (acetone): $\Delta\varepsilon_{209}$ –4.89, $\Delta\varepsilon_{347}$ –0.80.
¹H and ¹³C NMR: Tables 1 and 3.
ESIMS: *m/z* 305 [M–H]⁻.
HRESIMS: *m/z* 305.1043 [M–H]⁻ (calcd for $C_{16}H_{17}O_6$, 305.1031).

PetchieneB (2)
Yellow solid.
UV (MeOH) λ_{max} (log ε): 380 (3.59), 320 (4.07), 220 (3.99), 202 (4.12)nm.
¹H and ¹³C NMR: Tables 1 and 3.
ESIMS: *m/z* 259 [M–H]⁻.
HRESIMS: *m/z* 259.0974 [M–H]⁻ (calcd for $C_{15}H_{15}O_4$, 259.0976).

Petchiene C (3)
Yellow solid.
UV (MeOH) λ_{max} (log ε): 384 (1.50), 327 (3.66), 226 (3.99) nm.
¹H and ¹³C NMR: Tables 1 and 3.
ESIMS: *m/z* 243 [M–H]⁻.
HRESIMS *m/z* 243.1023 [M–H]⁻ (calcd for $C_{15}H_{15}O_3$, 243.1021).

(±)-Petchiene D (4)
Pale yellow solid.
{[α]$_D^{24}$: +102.2 (*c* 0.27,MeOH); (+)-**4**}; {[α]$_D^{24}$: –123.3 (*c* 0.17, MeOH); (–)-**4** }.
UV (MeOH) λ_{max} (log ε): 362 (0.81), 255 (1.47), 228 (4.15) nm; {CD (MeOH) (+)-**4**: $\Delta\varepsilon_{230}$+18.89, $\Delta\varepsilon_{364}$+2.99; CD (MeOH) (–)-**4**: $\Delta\varepsilon_{230}$–18.89, $\Delta\varepsilon_{364}$–2.99}.
¹H and ¹³C NMR: Tables 2 and 3.
ESIMS: *m/z* 259 [M–H]⁻.
HRESIMS *m/z* 259.0974 [M–H]⁻ (calcd for $C_{15}H_{15}O_4$, 259.0970).

Petchiene E (5)
Pale yellow solid.

UV (MeOH) λ_{max} (log ε): 368 (3.51), 256 (3.76), 223 (4.20), 202 (4.34) nm.

IR (KBr) v_{max}: 3395, 3076, 2927, 1701, 1634, 1612, 1487, 1446, 1380, 1351, 1324, 1267, 1205, 1182, 784 cm^{-1}.

^{1}H and ^{13}C NMR: Tables 2 and 3.

ESIMS: *m/z* 271 [M–H]$^-$.

HRESIMS: *m/z* 271.0614 [M–H]$^-$ (calcd for $C_{15}H_{11}O_5$, 271.0612).

Calcium imaging assay: The calcium imaging assay was performed by an automated, cell-based fluorescence-imaging system (Array scan), as previously reported, with slight modification [6,7]. The HEK293 cells were seeded at a density of 2×10^4 cells/well in 96-well plate coated with poly-L-lysine and incubated for 12 h. Then, the HEK-293 cells were washed 3 times by HBSS (1.26 mM $CaCl_2$, 0.493 mM $MgCl_2$, 0.407 mM $MgSO_4$, 5.33 mM KCl, 0.441 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 137.93 mMNaCl, 0.338 mM $NaHPO_4$, 5.56 mM D-Glucose, pH 7.2-7.4) and loaded finally with 2 µM Fluo-4 AM (DOJINDO) for 80 min at 37℃ in HBSS. After incubation, the cells were washed mildly 3 times by HBSS. After the loading, calcium imaging was analyzed using Array scan VTI HCS Reader (Cellomics, The Thermo Scientific, Pittsburg, PA). Fluorescence of Fluo-4 AM was excited at 488 nm, and emitted at 543 nm and recorded as times-series mode per 5 sec. The value of the changed fluorescence was expressed as the relative intensity compared with the initial value.

References

[1] Luo Q, Tian L, Di L, Yan YM, Wei XY, Wang XF, Cheng YX. (**2015**) (±)-Sinensilactam A, a pair of rare hybrid metabolites with smad3 phosphorylation inhibition from *Ganoderma sinensis*. *Organic Letters*, **17**, 1565–1568.

[2] (a) Yan YM, Ai J, Zhou LL, Chung ACK, Li R, Nie J, Fang P, Wang XL, Luo J, Hu Q, Hou FF, Cheng YX. (**2013**) Lingzhiols, unprecedented rotary door-shaped meroterpenoids as potent and selective inhibitors of p-smad 3 from *Ganoderma lucidum*. *Organic Letters*, **15**, 5488–5491; (b) Yan YM, Wang XL, Luo Q, Jiang LP, Yang CP, Hou B, Zuo ZL, Chen YB, Cheng YX. (**2015**) Metabolites from the mushroom *Ganoderma lingzhi* as stimulators of neural stem cell proliferation. *Phytochemistry*, **114**, 155–162; (c) Luo Q, Wang XL, Di L, Yan YM, Lu Q, Yang XH, Hu DB, Cheng YX. (**2015**) Isolation and identification of renoprotective substances from the mushroom *Ganoderma lucidum*. *Tetrahedron*, **71**, 840–845; (d) Luo Q, Di L, Dai WF, Lu Q, Yan YM, Yang ZL, Li RT, Cheng YX. (**2015**) Applanatumin A, a new dimericmeroterpenoid from *Ganoderma applanatum* that displays potent antifibrotic activity. *Organic Letters*, **17**, 1110–1113; (e) Zhou FJ, Nian Y, Yan YM, Gong Y, Luo Q, Zhang Y, Hou B, Zuo ZL, Wang SM, Jiang HH, Yang J, Cheng YX. (**2015**) Two new classes of T-type calcium channel inhibitors with new chemical scaffolds from *Ganoderma cochlear*. *Organic Letters*, **17**, 3082–3085.

[3] Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Scalmani G, Barone V, Mennucci B, Petersson GA, Nakatsuji H, Caricato M, Li X, Hratchian HP, Izmaylov AF, Bloino J, Zheng G, Sonnenberg JL, Hada M, Ehara M, Toyota K, Fukuda R, Hasegawa J, Ishida M, Nakajima T, Honda Y, Kitao O, Nakai H, Vreven T, Montgomery Jr JA, Peralta JE, Ogliaro F, Bearpark M, Heyd JJ, Brothers E, Kudin KN, Staroverov VN, Keith T, Kobayashi R, Normand J, Raghavachari K, Rendell A,Burant JC, Iyengar SS, Tomasi J, Cossi M, Rega N, Millam JM, Klene M, Knox JE, Cross JB, Bakken V, Adamo C, Jaramillo J, Gomperts R, Stratmann RE, Yazyev O, Austin AJ, Cammi R, Pomelli C, Ochterski JW, Martin RL, Morokuma K, Zakrzewski VG, Voth GA, Salvador P, Dannenberg JJ, Dapprich S, Daniels AD, Farkas O, Foresman JB, Ortiz JV, Cioslowski J, Fox DJ. (**2010**) *Gaussian 09*, *Revision C.01*, Gaussian, Inc., Wallingford CT.

[4] Bonora M, Giorgi C, Pinton P. (**2015**) Novel frontiers in calcium signaling: A possible target for chemotherapy. *Pharmacological Research*, **99**, 82–85.

[5] Burgoyne RD, Haynes LP. (**2015**) Sense and specificity in neuronal calcium signaling. *Biochimica et Biophysica Acta*, **1853**, 1921–1932.

[6] Grynkiewicz G, Poenie M, Tsien RY. (**1985**) A new generation of Ca^{2+} indicators with greatly florescence properties. *Journal of Biological Chemistry*, **260**, 3440–3450.

[7] Schlag BD, Lou Z, Fennell M, Dunlop J. (**2004**) Ligand dependency of 5-hydroxytryptamine 2C receptor internalization. *Journal of Pharmacology and Experimental Therapeutics*, **310**, 865–870.

Megastigmane Glycosides from the Leaves of *Tripterygium wilfordii*

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Two new megastigmane glycosides, named wilfordonisides A and B (**1** and **2**), and four known compounds (**3-6**) were isolated from the leaves of *Tripterygium wilfordii*, and one new aglycon, named wilfordoninol A (**2a**), was acquired by enzymatic hydrolysis of **2**. The absolute stereostructures of the compounds were determined by Mosher's method and by CD. At a concentration of 10 μM, compounds **1**, **3**, and **5** inhibited STAT1 translocation by 38.1 ± 0.9%, 55.8 ± 0.8%, and 53.9 ± 1.0%, respectively.

Keywords: *Tripterygium wilfordii*, Megastigmane glycosides, Wilfordoniside, Wilfordoninol, STAT1.

Tripterygium wilfordii Hook. f., family Celastraceae, has been used in traditional Chinese medicine for the treatment of cancer, rheumatoid arthritis, and skin disorders for hundreds of years [1]. A number of studies have been focused in China on the chemistry and pharmacology of this species and hundreds of compounds have been reported [2], included triterpenoids [3], diterpenoids [4], lignins [5], and alkaloids [6]. In the course of chemical investigations of the leaves of T. wilfordii, we have isolated two new megastigmane glycosides, named wilfordonisides A and B (**1** and **2**), along with four known compounds (**3-6**) (Figure 1), and one new aglycon, named wilfordoninol A (**2a**), which was acquired by enzymatic hydrolysis of **2**. In this paper, we report the structure elucidation of the new compounds and their biological activities.

Wilfordoniside A (**1**) was obtained as a colorless amorphous powder with a molecular formula of $C_{19}H_{34}O_7$, based on HRESIMS analysis (*m/z* 397.2207 [M + Na]$^+$, calcd 397.2197). The molecular formula accounted for 3 degrees of hydrogen deficiency. The IR spectrum of **1** showed absorption bands at 3383 and 1666 cm^{-1} ascribable to hydroxyl and double bond functions, respectively. The ^{1}H and ^{13}C NMR (Tables 1 and 2) spectra of **1**, which were assigned by various NMR experiment, showed signals assignable to 1 *β*-glucose and 13 carbons of the aglycone moiety. The aglycone contained four methyls [δ_H 0.87, 0.90 (3H each, both s, H_3-11, 12; 1.24 (3H, d, J = 6.4 Hz, H_3-10), and 0.88 (3H, overlap, H_3-13)], and two methines [δ_H 3.90 (1H, m, H-3) and 4.25 (1H, m, H-9)] bearing an oxygen function and one *trans*-configuration double bond [δ_H 5.32 (1H, dd, *J* = 15.4 and 10.0 Hz, H-7) and 5.48 (1H, dd, *J* = 15.4 and 6.3 Hz, H-8)], together with two methylenes, two methines and a quaternary carbon. As shown in Figure 2, the ^{1}H-^{1}H COSY experiment on **1** indicated the presence of a partial structure, written in bold lines, and in the HMBC experiment, long range correlations were observed between the following: H_3-11 and C-1 (δ_c 36.1), C-12 (δ_c 32.2), C-6 (δ_c 58.9); H-8 and C-6, C-7 (δ_c 131.4), C-9 (δ_c 69.6), C-10 (δ_c 24.4); H_3-10 and C-8 (δ_c 138.8) and C-9; H_3-13 and C-4 (δ_c 44.2) and C-5 (δ_c 32.5). HMBC correlations from H-3(δ_H 3.90, m) to C-1' (δ_c 103.0); and from H-1' [δ_H 4.37 (1H, d, *J* = 7.8 Hz] to C-3 (δ_c 76.0) suggested that the glucose was connected to C-3. The relative stereostructure of **1**, except for the 9-position, was
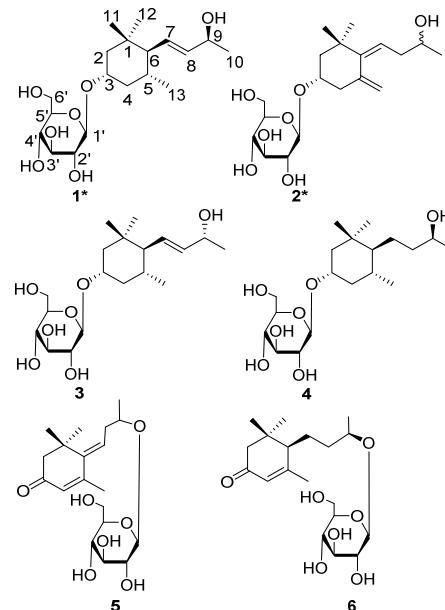
Figure 1 : Structures of compounds **1-6**.

characterized by the NOESY experiment, which showed NOE correlations between H-2*β* and H-3; H-3 and H-5; H-5 and H-4*β*; H-4*α* and H-6; and H-6 and H-2*α*, as shown in Figure 4.

Hydrolysis of **1** with snailase liberated its aglycon (**1a**), and glucose. The glucose was identified as D-(+)-glucose from its optical rotation {D-(+)-glucose had a positive optical rotation ($[\alpha]_D^{25}$ +45.7), and the optical rotation of glucose is $[\alpha]_D^{25}$ +39.8}. Finally, the absolute configuration of **1a** was characterized by application of the modified Mosher's method [7, 8].

Compound **1a** was treated with (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride and gave the 3,9-di-(*S*)-MTPA ester (**1b**) and 3,9-di-(*R*)-MTPA (**1c**) ester, respectively. As
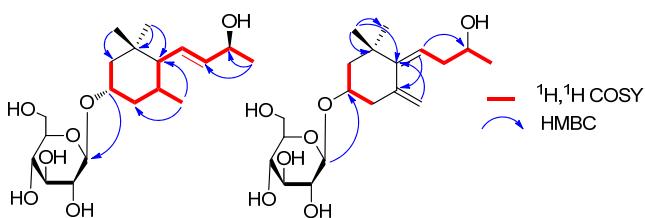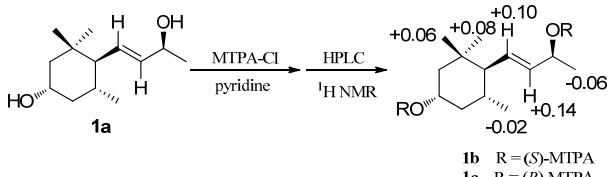
Figure 2: Key ¹H-¹H COSY and HMBC correlations of **1** and **2**.

Figure 3: Treatment of **1a** by Mosher's method

shown in Figure 3, the signals due to protons attached to the 10- and 13-position in the 3,9-di-(*S*)-MTPA ester were observed at higher fields compared with those of the 3,9-di-(*R*)-MTPA ester [Δδ: negative], while the signals due to protons of the 7-, 8-, 11- and 12-positions in the 3,9-di-(*S*)-MTPA ester were lower field compared with those of 3,9-di-(*R*)-MTPA ester [Δδ: positive]. Thus, the absolute configurations at the 3 and 9-positions of **1a** were determined to be 3*S* and 9*R*. Accordingly, the absolute configuration of 3*S*, 5*R*, 6*R*, and 9*R* was established. Meanwhile, by comparison of spectroscopic data [8], compound **1a** had the same NMR data as sarmentol F.

Wilfordoniside B (**2**) was obtained as colorless oil with a molecular formula of $C_{19}H_{32}O_7$, based on HRESIMS analysis (*m/z* 395.2046 [M + Na]⁺, calcd 395.2040). The molecular formula accounted for 4 indices of hydrogen deficiency. The IR spectrum of **2** showed absorption bands at 3395 and 1646 cm⁻¹ ascribable to hydroxyl and double bond functions, respectively. The characteristic ¹H and ¹³C NMR spectroscopic data (Tables 1 and 2) indicated that compound **2** was also a glycoside of a megastigmane derivative. As shown in Figure 2, the ¹H-¹H COSY experiment on **2** indicated the presence of a partial structure, written in bold lines, and in the HMBC experiment, long range correlations were observed between the following: H₃-11 [δ_H 0.92 (3H, s)] and C-1 (δ_c 36.8), C-12 (δ_c 28.4), C-6 (δ_c 147.6); H-7 [δ_H 5.30 (1H, m)] and C-1, C-5 (δ_c 142.6), C-9 (δ_c 66.2); H-13 [δ_H 4.61 (1H, m) and 5.11 (1H, m)] and C-6. HMBC correlations from H-3 (δ_H 3.90, m) to C-1' (δ_c 100.8) suggested that the glucose was connected to C-3. The relative configuration of the C=C double bond between C-6 and C-7 was deduced as *Z* by NOE difference experiment showing strong enhancements of H₃-11 and H₃-12 on irradiation of H-7. Hydrolysis of **2** with snailase liberated its aglycon, a new norsesquiterpene, named wilfordoninol A (**2a**), and glucose. The glucose was also identified D-(+)-glucose by the similar and positive optical rotation. The absolute configuration of C-3 was determined by application of the CD helicity rule [9, 10]. Thus, since the CD spectrum of **2a** (Figure 5) showed a positive Cotton effect at 212 nm, the 3-position was determined to have the *S* configuration.

The known compounds were identified by comparison of spectroscopic data with those reported in the literature as crotonionoside H (**3**) [11], alangioside J (**4**) [12], (*E*)-4-[3'-(*β*-D glucopyranosyloxy)butylidene] -3,5,5-trimethyl-2-cyclohexen-1-one (**5**) [13], and blumenol C glucoside (**6**) [14].

STAT1 (signal transducer and activator of transcription 1) plays a crucial role in signaling by interferons (IFNs), thereby regulating
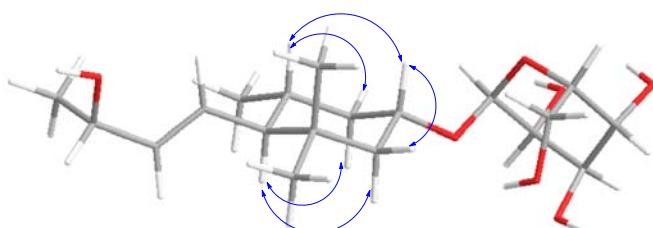
Figure 4: Key NOESY correlations of **1**.
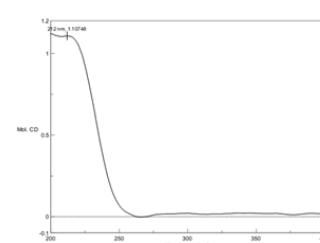
Figure 5: The CD curve of compound **2a**.

Table 1: ¹³C NMR spectral data of compounds **1-2** and **1a-2a**

NO.	**1**[a]	**1a**[b]	**2**[c]	**2a**[b]
1	36.1	35.8	36.8	39.2
2	48.1	51.1	47.6	50.8
3	76.0	67.4	72.9	69.6
4	44.2	45.6	42.6	47.3
5	32.5	32.2	142.6	144.7
6	58.9	58.5	147.6	150.0
7	131.4	131.2	117.5	118.6
8	138.8	138.5	38.0	38.1
9	69.6	69.3	66.2	69.1
10	24.4	24.1	23.1	23.1
11	22.0	21.8	27.3	27.9
12	32.2	31.9	28.4	29.0
13	22.1	21.8	113.9	114.5
1'	103.0		100.8	
2'	75.4		73.4	
3'	78.4		76.7	
4'	72.0		70.0	
5'	78.1		76.7	
6'	63.1		61.0	

a In MeOD-d_4 (125 MHz). *b* In MeOD-d_4 (150 MHz). *c* In DMSO-d_6 (125 MHz).

antiviral responses, cell proliferation, apoptosis, immune surveillance, and tumor suppression. The inhibitory effects of compounds **1** - **6** were evaluated *in vitro* for STAT1 translocation by immunofluorescence assay. Compounds **1**, **3**, and **5** inhibited STAT1 translocation by 38.1 ± 0.9%, 55.8 ± 0.8%, and 53.9 ± 1.0%, respectively, at a concentration of 10 μM; the other compounds showed weak activity.

Experimental

General experimental procedures: Optical rotations were measured on a JASCO P2000 automatic digital polarimeter, UV spectra on a JASCO V-650 spectrophotometer, and IR spectra on a Nicolet 5700 spectrometer using a FT-IR microscope transmission method. CD spectra were measured on a JASCO J-815 CD spectrometer. NMR spectra were acquired with an INOVA-500 spectrometer. HRESIMS were collected on an Agilent 1100 series LC/MSD ion trap mass spectrometer. Preparative HPLC was conducted using a Shimadzu LC-6AD instrument with a SPD-20A detector and a YMC-Pack ODS-A column (250×20 mm, 5 μm). Column chromatography (CC) was performed with silica gel (200-300 mesh, Qingdao Marine Chemical Inc., Qingdao, People's Republic of China), polyamide (60-100 mesh, Changfeng Chemical Inc., Jiangsu, People's Republic of China), D101 macroporous (Huangguang Chemical Inc., Tianjin, People's Republic of China),

Table 2: ^{1}H NMR spectral data of compounds **1-3** and **1a-3a**.

NO.	1[a]	1a[b]	2[c]	2a[b]
2α	1.18, t, (12.4)	1.11, t (12.4)	1.31, m	1.28, m
2β	1.87, br d (12.4)	1.69, br d (12.4)	1.83, m	1.95, m
3	3.90 (overlap)	3.73, m	3.89, m	3.88, m
4α	1.05, q (12.4)	0.90, q (12.4)	1.83 (overlap)	1.80, m
4β	2.13, br d (12.4)	1.98, br d (12.4)	2.75, dd (12.3, 3.4)	2.65, m
5	1.58, m	1.55, m		
6	1.33, t (10.0)	1.29, t (10.0)		
7	5.32, dd (15.4, 10.0)	5.30, dd (15.4, 10.0)	5.30, m	5.40, dd (8.0, 6.4)
8	5.48, dd (15.4, 6.3)	5.46, dd (15.4, 6.3)	2.13, m; 2.22, m	2.26, m; 2.38, m
9	4.25, m	4.23, m	3.60, dd (10.7, 5.9)	3.75, m
10	1.24, d (6.4)	1.23, d (6.5)	1.01, d (6.2)	1.15, d (6.2)
11	0.87, s	0.85, s	0.92, s	1.00, s
12	0.90, s	0.87, s	1.09, s	1.16, s
13	0.88 (overlap)	0.87 (overlap)	4.61, br s; 5.11, br s	4.68, d (2.2); 5.12, d (2.2)
1'	4.37, d (7.8)		4.25, d (7.8)	
2'	3.16, m		2.87, m	
3'	3.36, m		3.17, m	
4'	3.30, m		3.03, m	
5'	3.30, m		3.03, m	
6'	3.89, m; 3.68, m		3.65, m; 3.43, m	

[a] In MeOD-d_4 (500 MHz). [b] In MeOD-d_4 (600 MHz). [c] In DMSO-d_6 (500 MHz).

and ODS (50 μm, YMC, Japan). TLC was carried out on glass precoated silica gel GF$_{254}$ plates. Compounds were visualized either under UV light or by spraying with 10% sulfuric acid in EtOH followed by heating.

Plant material: The leaves of *Tripterygium wilfordii* (TWHF) were collected in Taining, Fujian, China, in September 2009 and identified by Professor Lin Ma from the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. A voucher specimen (No. 20090034) is deposited at the herbarium of the Institute of Material Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, China.

Extraction and isolation: Air-dried leaves of TWHF (100 kg) were reduced to a coarse powder and refluxed with 80% EtOH (400 L×2h×3). After evaporation of ethanol *in vacuo*, the aqueous residue was diluted with water, and then partitioned with EtOAc (30L×3). The water layer was subjected to passage over polyamide by elution with water and 50% EtOH-water. The water extract was subjected to passage over D101 macroporous adsorption resin by elution with water and 30%, 60%, 95% EtOH-water in sequence to give fractions A1, A2 (651.0 g), A3 (338.5 g), and A4 (12.4g). Fraction A3, with an equal weight of diatomite, was successively refluxed with EtOAc, EtOH, MeOH to obtain fractions B1 (13.62 g), B2 (151.38 g) and B3 (68.87 g). Fraction B2 was subjected to CC on silica gel (200-300 mesh) with CHCl$_3$-MeOH (15: 1 – 1: 1) to afford 7 fractions (C1 - C7). Fraction C6 (8.625 g) was passed over an RP-18 column with MeOH-water (15%-75%), and finally purified by preparative HPLC (detected at 210 nm, 8 mL/min) to give **1** (43 mg), **2** (3 mg), **3** (8 mg), **4** (7 mg), **5** (5 mg), and **6** (14 mg).

Enzymatic hydrolysis of 1 and 2 with snailase: A solution of **1** and **2** (8.0 and 3.0 mg, respectively) in H$_2$O (1.0 mL) was treated with snailase (10.0 and 5.0 mg, respectively). The solution was stirred at 37°C for 5 h; after cooling, the reaction mixture was extracted with EtOAc. The aqueous layer was subjected to CC on silica gel (200-300 mesh) with CH$_3$CN-H$_2$O (8:1) to afford glucose (1.8 mg). The EtOAc layer was purified by HPLC (25% CH$_3$CN-H$_2$O, detected at 210 nm, 8 mL/min) to furnish **1a** (2.3 mg) and **2a** (0.5 mg).

Wilfordoninol A (2a)
Colorless oil (MeOH).

^{1}H and ^{13}C NMR: Tables 1 and 2.
HRESIMS: *m/z* 233.1509 [M + Na]$^+$ (calcd for C$_{13}$H$_{22}$NaO$_2$, 233.1522).

Preparation of (R)- and (S)-MTPA esters of 1a: A solution of compound **1a** (1.0 mg) in dry CH$_2$Cl$_2$ (2.0 mL) was treated with (R)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(R)-MTPA-Cl, 10 mg] in the presence of anhydrous pyridine, and the mixture was stirred under reflux at 40°C for 6 h. After cooling, the reaction mixture was poured into ice-water and extracted with EtOAc. The EtOAc extract was washed successively with 5% HCl, NaHCO$_3$-saturated H$_2$O and brine, dried over Na$_2$SO$_4$ and filtered. Removal of the solvent from the filtrate under reduced pressure afforded a residue, which was purified by preparative HPLC (90% CH$_3$CN-H$_2$O, detected at 210 nm, 8 mL/min) to acquire the (S)-MTPA ester of **1a** (compound **1b**, 0.85 mg). Using a similar procedure, (R)-MTPA ester of **1a** (compound **1c**, 0.73 mg) was obtained from compound **1a** (1.0 mg) with (S)-MTPA-Cl (8 mg).

Compound **1b**
Colorless oil.
^{1}H NMR (600 MHz, CDCl$_3$) δ: 0.85, 0.91 (3H, each, both s, H$_3$-11, 12), 0.85 (3H, d, *J* = 6.0 Hz, H$_3$-13), 1.43 (3H, d, *J* = 6.0 Hz, H$_3$-10), 5.50 (1H, dd, *J* = 15.4, 10.0 Hz, H-7), 5.44 (1H, dd, *J* = 15.4, 7.8 Hz, H-8).

Compound **1c**
Colorless oil.
^{1}H NMR (600 MHz, CDCl$_3$) δ: 0.93, 0.97 (3H, each, both s, H$_3$-11, 12), 0.83 (3H, d, *J* = 6.0 Hz, H$_3$-13), 1.37 (3H, d, *J* = 6.0 Hz, H$_3$-10), 5.60 (1H, dd, *J* = 15.4, 10.0 Hz, H-7), 5.58 (1H, dd, *J* = 15.4, 7.8 Hz, H-8).

Biological activities: Recombinant U2OS cells (Thermo Scientific, Rorkford IL, USA) that stably express human STAT1-EGFP fusion protein were cultured in DMEM supplemented with 10% FBS and G418 (0.5 mg/mL) in a 37°C incubator with 5% CO$_2$. Cells were plated in 96-well plates (2.5 × 10^3 cell mL^{-1}) and preincubated with samples for 30 min, followed by a further 1 h treatment with IFN-γ to evaluate the nuclear translocation of STAT1-EGFP. The capacity of translocation was detected with high content analysis by Cellomics ArrayScan V^{TI} HCS Reader (Thermo Fisher Scientific Cellomics, Pittsburgh, PA, USA).

Wilfordoside A (1)
Colorless oil (MeOH).
$[\alpha]_D^{25}$: −52.3 (*c* 0.1, MeOH).
IR (microscope) ν_{max}: 3383, 2926, 1666, 1367, 1075, and 944 cm^{-1}.
^{1}H and ^{13}C NMR: Tables 1 and 2.
HREIMS: *m/z* 397.2207 ([M + Na]$^+$, calc. C$_{19}$H$_{34}$NaO$_7$, 397.2197).

Wilfordoside B (2)
Colorless oil (MeOH).
$[\alpha]_D^{25}$: − 47.3 (*c* 0.1 MeOH).
IR (microscope) ν_{max}: 3395, 2921, 1646, 1468, 1419, and 1181 cm^{-1}.

^{1}H and ^{13}C NMR: Tables 1 and 2.
HRESIMS: *m/z* 395.2046 ([M + Na]$^+$, calc. C$_{19}$H$_{32}$NaO$_7$, 395.2040).

Supplementary data: NMR, IR, and CD data are available.

References

[1] Brinker AM, Ma J, Lipsky PE, Raskin I. (**2007**) Medicinal chemistry and pharmacology of genus *Tripterygium* (Celastraceae). *Phytochemistry*, **68**, 732-766.
[2] Zhou ZL, Yang YX, Ding J, Li YC, Miao ZH. (**2012**) Triptolide: structural modifications, structure-activity relationships, bioactivities, clinical development and mechanisms. *Natural Product Reports*, **29**, 457-475.
[3] Duan H, Takaishi Y, Momota H, Ohmoto Y, Taki T, Jia Y, Li D. (**2000**) Triterpenoids from *Tripterygium wilfordii. Phytochemistry*, **53**, 805-810.
[4] Ni L, Ma J, Li C J, Guo J M, Yuan SP, Hou Q, Guo Y, Zhang DM. (**2015**) Novel rearranged and highly oxygenated abietane diterpenoids from the leaves of *Tripterygium wilfordii. Tetrahedron Letters*, **56**, 1239-1243.
[5] Cao X, Li C J, Yang JZ, Wei B X, Yuan SP, Luo Y M, Hou Q, Zhang DM. (**2012**) Four new neolignans from the leaves of *Tripterygium wilfordii. Fitoterapia*, **83**, 343-347.
[6] Duan H, Tashihisa Y, Bando M, Kido M, Imakura Y, Lee KH. (**1999**) Novel sesquiterpene esters with alkaloid and monoterpene and related compounds from *Triperygium hypoglaucum*: a new class of potent anti-HIV agents. *Tetrahedron Letters*, **40**, 2969-2972.
[7] Ohtani I, Kusumi T, Kashman Y, Kakisawa H. (**1991**) High-field FT NMR application of Mosher's method. The absolute configurations of marine terpenoids. *Journal of the American Chemical Society*, **113**, 4092-4096.
[8] Zhang Y, Nakamura S, Pongpiriyadacha Y, Matsuda H, Yoshikawa M. (**2008**) Absolute structures of new megastigmane glycosides, foloasalaciosides E$_1$, E$_2$, E$_3$, F, G, H, and I from the leaves of *Salacia chinensis. Chemical and Pharmaceutical Bulletin*, **56**, 547-553.
[9] Oritani T, Yamashita K. (**1972**) Synthesis of optical active abscisic acid and its analogs. *Tetrahedron Letters*, **25**, 2521-2524.
[10] Yoshikawa M, Shimada H, Saka M, Yoshizumi S, Yamahara J, Matsuda H. (**1997**) Medicinal foodstuffs. V. Moroherya. (1): Absolute stereostructures of corchoionosides A, B, and C, histamine release inhibitors from the leaves of Vietnamese *Corchorus olitorius* L. (Tiliaceae). *Chemical and Pharmaceutical Bulletin*, **45**, 464-469.
[11] Kawakami S, Matsunami K, Otsuka H, Shinzato T, Takeda Y. (**2011**) Crotonionosides A-G: megastigmane glycosides from leaves of *Croton cascarilloides* Rauschel. *Phytochemistry*, **72**, 147-153.
[12] Yoshikawa M, Morikawa T, Zhang Y, Nakamura S, Muraoka O, Matsuda H. (**2007**) Megastigmanes and their glucosides from the whole plant of *Sedum sarmentosum. Journal of Natural Products*, **70**, 575-583.
[13] Khan SH, Mosihuzzaman M, Nahar N, Rashid MA, Rokeya B, Ali L, Khan A. (**2003**) Three megastimane glycosides from the leaves of *Pterospermum semisagittatum. Pharmaceutical Biology*, **41**, 512-515.
[14] Matsunami K, Otsuka H, Takeda Y. (**2010**) Structural revisions of blumenol C glucoside and byzantionoside B. *Chemical and Pharmaceutical Bulletin*, **58**, 438-441.

Cytochalasans and Sesquiterpenes from *Eutypella scoparia* 1-15

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Three new compounds, an open-chain cytochalasan scoparasin C (**1**), a pyrichalasan scoparasin D (**2**), and a β-eudesmol type sesquiterpene scopararane C (**5**), along with three known compounds (**3**, **4** and **6**), were isolated from the marine fungus *Eutypella scoparia* 1-15. Their structures were determined on the basis of comprehensive NMR and MS analysis. Compound **2** exhibited potent cytotoxicities with very low IC$_{50}$ values against several cancer cell lines, including A375, A549, HepG2 and MCF-7.

Keywords: Cytochalasan, Scoparasins, Scopararanes, Cytotoxic activity.

Cytochalasans comprise a diverse group of fungal secondary metabolites with a characteristic tricyclic core structure in which an 11 to 14-membered macrocycle ring bearing either a carbocycle, a lactone or a cyclic carbonate, is fused to a highly substituted perhydroisoindol-1-one [1]. Based on the L-amino acids incorporated into the hydrogenated isoindolone, the canonical cytochalasans are divided into groups of cytochalasins (Phe), pyrichalasins (Tyr), chaetoglobosins (Trp), aspochalasins (Leu), and alachalasins (Ala) [2]. Cytochalasans exhibit a wide range of bioactivities, *e.g.*, interfering with cellular mitosis, anti-parasite, inhibition of TNF-α and anticancer [3].

In our ongoing search for bioactive compounds from marine microbes, four cytochalasans (**1-4**) and two sesquiterpenes (**5-6**) were isolated from *Eutypella scoparia* 1-15, a fungal strain from mangrove rhizosphere soil of Jimei, Fujian Province, China. Compounds **1**, **2** and **5** were proved to be new natural products named scoparasin C, scoparasin D, and scopararane C, respectively.

Scoparasin C (**1**) was obtained as colorless oil. The molecular formula was established as C$_{32}$H$_{45}$NO$_{10}$ on the basis of HR-ESI-MS (*m/z* 626.2926 [M+Na]$^+$, calcd. for C$_{32}$H$_{45}$NNaO$_{10}^+$: 626.2936), with 11 degrees of unsaturation. A strong absorption band at 1716 cm^{-1} in the IR spectrum indicated the presence of a ketone and/or a lactam carbonyl functional group, corresponding to the signal at δ 5.71 (1H, s, -NH-CO-) in the ^{1}H NMR (CDCl$_3$) spectrum. The ^{13}C NMR and DEPT spectra showed 30 resolved peaks corresponding to 32 carbons, which were classified into eight methyls (four *O*-methyl, δ$_c$ 55.3, 54.9, 53.9, and 54.4), three methylenes (δ$_c$ 43.1, 42.0, and 36.0), eleven methines (four *sp*^{2} methines, δ$_c$ 131.9, 130.1, 127.3, and 114.5), eight quaternary carbons (two olefinic, with δ$_c$ 129.4 and 158.8, two carbonyl with δ$_c$ 171.0 and 218.1, and three *sp*^{3} carbon with δ$_c$ 57.4, 77.7, and 84.9). The remaining quaternary carbon of δ$_c$ 153.9 indicated a carbonate moiety. The ^{1}H spectrum indicated a *para*-methoxybenzene ring according to the presence of the characteristic proton resonances, δ 7.14 (d, *J* = 8.5 Hz, 2H), 6.90 (d, *J* = 8.6 Hz, 2H), and 3.82 (s, 3H). Apart from the eight degrees of unsaturation due to the benzene ring, three carbonyl groups, and one olefinic bond, the remaining three degrees of unsaturation indicated that **1** possessed a tricyclic framework. By extensive
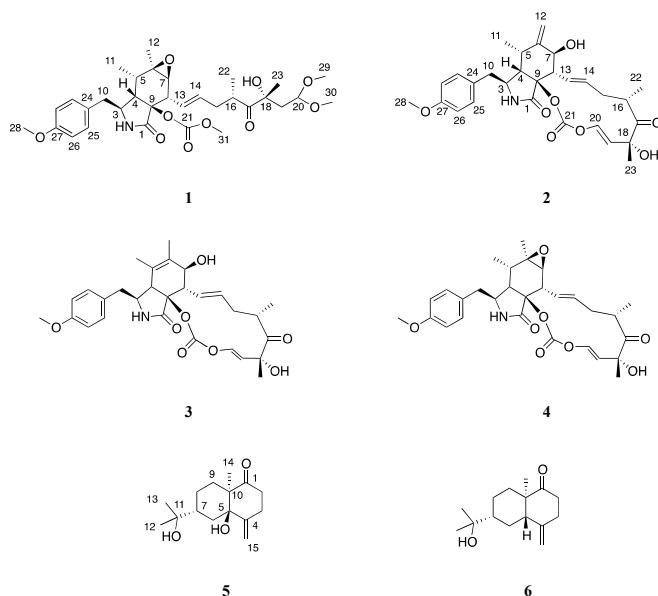
Figure 1: Structures of compounds **1-6** isolated from *Eutypella scoparia* 1-15.

analysis of the 2D NMR spectra (HMBC and ^{1}H-^{1}H COSY), the correlations from H-2 to C-3, C-4, and C-9, H-5 to C-3, C-4 and C-6, H-7 to C-8, C-9, and C-13, indicated the presence of a 10-phenyl-substitued perhydro-isoindol-1-one skeleton, the structural feature of cytochalasans. Further analysis confirmed the epoxypropane moiety, and the positions of the eight-carbon chain system and the methoxylcarbonate group at C-8 and C-9, respectively. Therefore, the structure of scoparasin C (**1**) was elucidated (Figure 1) as a new open-chain cytochalasan, a methoxyl derivative of cytochalasin Z$_{18}$ [4].

Scoparasin D (**2**) was obtained as white powder. The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1** with the exception of the absence of three methoxy groups (δ$_c$ 53.9, 54.4, and 54.9) and the presence of two olefinic carbons (δ$_c$ 120.3 and 142.2) (Table 1). The signal of H$_3$-12 (δ$_H$ 1.34) in **1** was replaced with two signals of

Table 1: ^{1}H and ^{13}C NMR spectral data of compounds **1** and **2**.

Position	1		2		Position	1		2	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C		δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	/	171.0s	/	169.0s	17	/	218.1s	/	211.8s
2-NH	5.71 (1H,s)	/	4.49 (1H,bs)	/	18	/	77.7s	/	77.2s
3	3.63 (1H, 4.1, 9.1)	54.3d	3.26 (1H,dd, 4.2, 9.4)	53.6d	19	2.24 (1H,dd, 5.7,14.5) 1.93 (1H,dd, 5.3,14.5)	42.0t	5.66 (1H,d, 11.8)	120.3d
4	2.61 (1H,t, 4.4)	49.9d	2.96 (1H,m)	48.1d	20	4.47 (1H,t, 5.5)	102.9d	5.68 (1H,bs)	142.3d
5	2.20 (1H)	35.5d	3.37 (1H,m)	32.1d	21	/	153.9s	/	149.6s
6	/	57.4s	/	148.5s	22	1.10 (3H,d, 6.8)	17.1q	1.20 (3H,d)	20.2q
7	2.81 (1H, d, 4.7)	60.1d	3.84 (1H,d, 11.3)	69.3d	23	1.32 (3H,s)	26.5q	1.54 (3H,s)	24.1q
8	2.83 (1H,d, 5.3)	47.2d	2.97 (1H,m)	40.9d	24	/	129.4s	/	128.6s
9	/	84.9s	/	86.2s	25	7.14 (2H, d, 8.7)	130.1d	7.06 (2H,d, 8.5)	130.3d
10	2.93 (1H,dd, 9.7, 13.6) 2.85 (1H,dd, 4.5, 13.9)	43.1t	2.88 (1H,dd, 9.4, 13.6) 2.61 (1H,dd, 9.4, 13.7)	43.8t	26	6.90 (2H,d, 8.7)	114.5d	6.87 (2H,d, 8.5)	114.4d
11	1.17 (3H,d, 7.4)	14.0q	1.18 (3H,d, 6.8)	14.6q	27	/	158.8s	/	158.9s
12	1.34 (3H,s)	20.4q	5.42 (1H,bs) 5.20 (1H,bs)	114.5t	28	3.82 (3H,s)	55.3q	3.82 (3H, s)	55.3q
13	5.99 (1H,dd, 7.9, 15.2)	127.3d	5.76 (1H,dd, 11.7, 15.1)	128.2d	29	3.32 (3H,s)	54.4q		
14	5.60 (1H,dd, 7.7, 15.2)	131.9d	5.39 (1H,m)	133.8d	30	3.34 (3H,s)	53.9q		
15	2.50 (1H,dd, 7.5, 14.0) 2.18 (1H,m)	36.0t	2.74 (1H,m) 2.18 (1H,m)	38.8t	31	3.81 (3H, s)	54.9q		
16	3.19 (1H,dd, 5.8, 6.8)	39.6d	2.99 (1H,m)	40.9d					

Spectra were recorded at 600 MHz for ^{1}H using TMS as internal standard in CDCl$_3$.

δ_H (5.20, bs, 1H) and δ_H (5.42, bs, 1H), characteristic of an exocyclic double bond. Further analysis of the COSY and HMBC spectra of **2** revealed the presence of a macrocyclic structure (Figure 1). This deduction was also confirmed by the molecule formula of **2**, C$_{29}$H$_{35}$NO$_8$, established on the basis of the HR-ESI-MS (*m/z* 548.2240 [M+Na]$^+$, calcd. for C$_{29}$H$_{35}$NNaO$_8$$^+$: 548.2255).

Compound **2** was previously obtained via semi-synthesis from phenochalasin B (**4**) [5], but it is the first time that it has been isolated from a natural source.

Previous isotope labeling experiments have proved that the skeletons of cytochalasans share the same stereochemistry, viz., *cis*-conjunction across the C-4/C-9, *trans*-conjunction across the C-8/C-9, and *S*-configuration at C-16. The cross-peaks at H-3/H-11 and H-5/H-8/H-10 in NOE correlations of compounds **1** and **2** suggested that both compounds had the same relative stereochemistry as previously isolated cytochalasans. The cross peak of H-16/H$_3$-23 indicated the *R*-configuration at C-18 in both compounds, which is consistent with known cytochalasans [6].

Scopararane C (**5**) had the molecular formula of C$_{15}$H$_{24}$O$_3$, deduced from HR-ESI-MS (m/z 253.1796 [M+H]$^+$, calcd. for C$_{15}$H$_{25}$O$_3$$^+$: 253.1798), which indicated four degrees of unsaturation in the molecule. The ^{13}C NMR and DEPT spectra (CDCl$_3$) displayed two sp^2 carbons (δ_c 148.4 and 110.8) and a carbonyl carbon (δ_c 214.2). The remaining two degrees of unsaturation were attributed to two rings. The ^{13}C NMR spectrum of **5** was similar to that of **6** except that δ_c 47.8 (d) in **6** was changed to δ_c77.9 (s). Extensive analysis of ^{1}H NMR and HMBC spectra revealed that scopararane C (**5**) is a new β-eudesmol sesquiterpene.

Compound **3** and compound **4** had ^{1}H and ^{13}C NMR data that were identical to those of scoparasin A [7] and phenochalasin B [8], respectively. We further confirmed the structure of **3** by X-ray diffraction (Figure 2), which also demonstrated the absolute configurations of C-16 and C-18. Compound **6** was confirmed to be ent-4(15)-eudesmen-11-ol-1-one, a known β-eudesmol sesquiterpene.

All compounds were evaluated for their cytotoxic activities against A375, A549, HepG2 and MCF-7 cell lines by the MTT method [9].
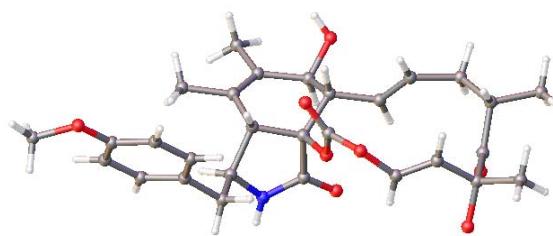
Figure 2: X-ray structure of **3**.

Table 2: IC$_{50}$ values of **2-4** against four cancer cell lines (μM).

Compds	A375	A549	HepG2	MCF-7
2	1.08	2.25	3.51	3.40
3	5.74	10.85	9.44	> 50
4	0.47	1.80	0.80	0.63

Compounds **1**, **5**, and **6** did not show any activity (IC$_{50}$ > 50 μM), while compound **4** had potent inhibitory activities against all four cancer cell lines (Table 2). These results implied that the macrocycle might be essential for the bioactivity of cytochalasans.

Experimental

General experimental procedures: Infrared spectra (IR) were determined on a Thermo Nicolet iS5 FT-IR spectrometer, ultraviolet (UV) absorption spectra, in MeOH, on a Beckman Coulter DU 800 spectrophotometer, and optical rotations on a Perkin-Elmer 341 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded in CDCl$_3$ on a 600 MHz Bruker Avance 600 spectrometer. HR-FT-MS were measured with a Bruker autoflex speed Maldi-Tof mass spectrometer. Silica GF$_{254}$ for thin layer chromatography (TLC) was produced by the Qingdao Marine Chemical Company, and Sephadex LH-20 by Pharmacia Biotech.

Fungal material: The fungal strain 1-15 was isolated from mangrove rhizosphere soil from Jimei, Fujian Province, China, in September, 2011.The fungus was identified as *Eutypella scoparia* by comparing the internal transcribed spaces (ITS) gene sequence with *Eutypella scoparia* PanM2024-1-P15 in the GenBank database, with 99% similarity (ITS1: 5'-TCCGTAGGTGAACCTGCGG -3', ITS4: 5'-TCCTCCGCTTATTGATATGC-3').

Fermentation: *Eutypella scoparia* 1-15 was cultured on potato dextrose agar (PDA) medium at 28ºC for 35 days. A total of 20 L of fungal solid culture was prepared.

Extraction and isolation: The fermented material was diced and extracted 5 times with EtOAc/MeOH/AcOH (80:15:5, v/v/v) at room temperature. After removing the solvent under vacuum, the extract was partitioned between H_2O and EtOAc. Then the EtOAc layer was concentrated and resuspended in 95% methanol (70 mL), and washed with light petroleum (PE) 5 times. The MeOH layer was concentrated to afford the defatted extract (8 g). This was fractionated by medium-pressure liquid chromatography over an RP-18 column (170 g) eluting with 2 L each of H_2O-MeOH (100:0, 70:30, 50:50, 30:70, 0:100, v/v) to give fractions A-N. Compound **5** (6.0 mg) and **6** (9.5 mg) were obtained from fraction A (1.4 g) by silica gel CC (30 ×1.5 cm) eluting with EtOAc/PE (1:25, 1:20, 1:10, 1:6, v/v). Fraction E (1.8 g) was further purified by CC over silica gel (200-300 mesh) eluted with a CH_2Cl_2-MeOH gradient (from 200:1 to 0:100, v/v) to give compounds **1** (3 mg) and **4** (20 mg). Fraction I (2.0 g) was further purified over Sephedax LH-20 eluting with MeOH to give compounds **2** (2.3 mg) and **3** (5 mg).

Scoparasin C (1)
$[\alpha]_D^{22}$: +20 (*c* 0.1, MeOH).
IR (KBr): 2967.5, 1747.2, 1716.3, 1513.2, 1263.9 cm^{-1}.
UV/Vis λ_{max} (MeOH) nm: 274, 225, 205.
MS (EI, 70 eV): *m/z* (%) = 626.44 [M+Na$^+$] (100).
HRMS-FAB: *m/z* [M+Na$^+$] calcd. for $C_{32}H_{45}NNaO_{10}$: 626.2936; found: 626.2926.
^{1}H and ^{13}C NMR: Table 1.

Scoparasin D (2)
$[\alpha]_D^{22}$: +88.0 (*c* 0.10, MeOH).
Rf: 0.58 (CHCl$_3$-MeOH, 10:1).
IR (KBr): 2967.5, 1767.5, 1716.0, 1512.7, 1248.3, 1032.9cm^{-1}.
UV/Vis λ_{max} (MeOH) nm: 274, 225, 209.
MS (EI, 70 eV): *m/z* (%) = 548.26 [M+Na$^+$] (100).
HRMS-FAB: *m/z* [M+Na$^+$] calcd. for $C_{29}H_{35}NO_8$: 548.2255; found: 548.2240.
^{1}H and ^{13}C NMR: Table 1.

Scoparasin A (3)
Rf: 0.58 (CHCl$_3$-MeOH, 10:1).
^{1}H NMR (600 MHz, CDCl$_3$): 1.21 (3H, s, Me-22), 1.53 (3H, s, Me-23), 1.55 (3H, s, Me-11), 1.70 (3H,s, Me-12), 2.17 (1H, m, Ha-15), 2.75 (1H, m, Hb-15), 2.78 (1H, m, Ha-10), 2.81 (1H, m, Hb-10), 2.85 (1H, m, H-8), 2.99 (1H, m, H-16), 3.44 (1H, t, *J* = 6.8 Hz, H-3), 3.81 (3H, s, Me-28), 3.90 (1H, m, H-7), 3.91 (1H, m, H-4), 5.41 (1H, ddd, *J* = 3.9 Hz, 10.6Hz, 15.3 Hz, H-14), 5.69 (1H, d, *J* = 11.6 Hz, H-19), 5.76 (1H, s, NH), 6.24 (1H, dd, *J* = 11.3 Hz, 16.0 Hz, H-13), 6.70 (1H, d, *J* = 11.6 Hz, H-20), 6.89 (2H, d, *J* = 8.6 Hz, H-26), 7.10 (2H, d, *J* = 8.5 Hz, H-25).
^{13}C NMR (150 MHz, CDCl$_3$): 14.0 (CH$_3$), 17.7 (CH$_3$), 20.3 (CH$_3$), 24.8(CH$_3$), 39.0 (CH$_2$), 40.9 (CH$_2$), 43.2 (CH$_2$), 48.3 (CH), 50.0 (CH), 55.3 (CH$_3$), 59.2 (CH), 70.1 (CH), 76.6 (C), 86.2 (C), 114.3 (CH), 120.4 (CH), 128.6 (C), 129.4 (CH), 125.3 (C), 130.3 (CH), 131.7 (C), 133.5 (CH), 142.5 (CH), 149.0 (C), 158.7 (C), 169.9 (C), 211.5 (C).
MS (EI, 70 eV): *m/z* (%) = 548.36 [M+Na$^+$] (100).

Crystallographic data of scoparasin A (**3**): White crystal obtained from MeOH-H_2O (1:1), orthorhombic, space group: P2$_1$2$_1$2$_1$, *a* = 11.6982(3) Å,*b* = 12.8520(5) Å, *c* = 17.2917(6) Å, *V* = 2599.73 (15) Å^3, Z = 4, D_c = 1.343 g/cm^3, μ(Cu Kα) 0.805 mm^{-1}, F(000) = 1120.0, crystal size 0.45×0.2×0.2 mm, 26153 reflections collected,

5356 independent reflections (R$_{int}$ = 0.0769), final R 0.0417 [I >2σ(I)] and wR$_2$ 0.1234 (all data), Flack parameter 0.10 (17).

Phenochalasin B (4)
Rf: 0.57 (CHCl$_3$-MeOH, 10:1).
^{1}H NMR (600 MHz, CDCl$_3$): 1.19 (3H, s, Me-22), 1.53 (3H, s, Me-23), 1.13 (3H, d, *J* = 7.1 Hz, H-11), 1.26 (3H,s, Me-12), 2.17 (1H, m, Ha-15), 2.31 (1H, m, H-5), 2.68 (1H, m, Hb-15), 2.63 (1H, m, Ha-10), 2.84 (1H, m, Hb-10), 2.66 (1H, m, H-8), 2.96 (1H, m, H-16), 3.69 (1H, brs, H-3), 3.81 (3H, s, Me-28), 2.65 (1H, m, H-7), 3.01 (1H, m, H-4), 5.25 (1H, m, H-14), 5.63 (1H, d, *J* = 11.6 Hz, H-19), 6.14 (1H, s, NH), 5.91 (1H, dd, *J* = 9.3 Hz, 14.6 Hz, H-13), 6.53 (1H, d, *J* = 11.7 Hz, H-20), 6.89 (2H, d, *J* = 7.3 Hz, H-26), 7.08 (2H, d, *J* = 7.3 Hz, H-25).
^{13}C NMR (150 MHz, CDCl$_3$): 13.2 (CH$_3$), 19.7 (CH$_3$), 20.3 (CH$_3$), 24.3 (CH$_3$), 35.8 (CH), 39.1 (CH$_2$), 44.1 (CH$_2$), 45.9 (CH), 60.4 (CH), 48.0 (CH), 53.7 (CH), 55.3 (CH$_3$), 57.3 (C), 76.7 (C), 87.0 (C), 114.3 (CH), 120.4 (CH), 127.9 (C), 128.4 (CH), 130.5 (CH), 131.6 (CH), 142.1 (CH), 149.4(C), 158.9 (C), 170.0 (C), 211.8 (C).
MS (EI, 70 eV): *m/z* (%) = 548.26 [M+Na$^+$] (100).

Scopararane C (5)
$[\alpha]_D^{22}$: -35.5 (*c* 0.24, MeOH).
Rf: 0.58 (CHCl$_3$-MeOH, 10:1).
IR (KBr): 2924.8, 1699.9 cm^{-1}.
UV/Vis λ_{max} (MeOH) nm: 234, 220, 204.
^{1}H NMR (600 MHz, CDCl$_3$): 2.70 (1H, dd, *J* = 8.2 Hz, Ha-2), 2.42 (1H, dd, *J* = 1.7Hz, 8.2 Hz, Hb-2), 2.51 (1H, dd, *J* = 7.3 Hz, Ha-3), 3.02 (1H, dd, *J* = 2.0 Hz, 7.3 Hz, Hb-3), 1.81 (2H, m, H-6), 1.80 (1H, m, H-7), 1.77 (2H, m, H-8), 1.63 (2H, m, H-9), 1.27 (3H, s, H-12), 2.07 (3H, s, H-13), 1.08 (3H, s, H-14), 5.04 (1H, m, Ha-15), 5.11 (1H, dd, *J* = 0.8 Hz, 2.0 Hz, Hb-15).
^{13}C NMR (150 MHz, CDCl$_3$): 20.1 (CH$_3$), 21.3 (CH$_2$), 26.7 (CH$_3$), 26.8 (CH$_2$), 28.1 (CH$_3$), 30.0 (CH$_2$), 30.9 (CH$_2$), 36.4 (CH$_2$), 42.9 (CH), 51.3 (C), 72.5 (C), 77.9 (C), 110.8 (CH$_2$), 148.4 (C), 214.2 (C).
MS (EI, 70 eV): *m/z* (%) = 253.18 [M+H$^+$] (100).
HRMS-FAB: *m/z* [*M* + H$^+$] calcd. for $C_{15}H_{25}O_3^+$: 253.1798; found: 253.1796.

Ent-4(15)-eudesmen-11-ol-1-one (6)
Rf: 0.77 (CHCl$_3$-MeOH, 1:1).
^{1}H NMR (600 MHz, CDCl$_3$): 2.71 (1H, m, Ha-2), 2.39 (1H, dd, *J* = 2.0 Hz, 6.2 Hz, Hb-2), 2.45 (1H, m, Ha-3), 2.62 (1H, dd, *J* = 2.2 Hz, 7.6 Hz, Hb-3), 2.15 (1H, dd, *J* = 1.4 Hz, 11.5 Hz, H-5), 1.60 (1H, m, Ha-6), 1.85 (1H, m, Hb-7), 1.33 (1H, m, H-7), 1.24 (1H, m, Ha-8), 1.77 (1H, m, Hb-8), 1.37 (1H, m, Ha-9), 1.84 (1H, m, dd, *J* = 2.5 Hz, 5.6 Hz, Hb-9), 1.24 (3H, s, H-12), 1.25 (3H, s, H-13), 1.01 (3H, s, H-14), 4.80 (1H, d, *J* =1.4 Hz, Ha-15), 5.11 (1H, d, *J* = 1.3 Hz, Hb-15).
^{13}C NMR (150 MHz, CDCl$_3$): 16.6 (CH$_3$), 21.9 (CH$_2$), 26.9(CH$_3$), 24.4 (CH$_2$), 27.5 (CH$_3$), 32.1 (CH$_2$), 34.6 (CH$_2$), 38.1 (CH$_2$), 48.4 (CH), 47.8 (CH), 48.3 (C), 72.6 (C), 109.1 (CH$_2$), 146.5 (C), 215.1 (C).
MS (EI, 70 eV): *m/z* (%) = 237.40 [M+H$^+$] (100).

References

[1]   Liu JT, Hu B, Gao Y, Zhang JP, Jiao BH, Lu XL, Liu XY. (**2014**) Bioactive tyrosine-derived cytochalansins form fungus *Eutypella* sp. D-1. *Chemistry and Biodiversity*, *11*, 800-806.
[2]   Scherlach K, Boettger D, Remme N, Christian H. (**2010**) The chemistry and biology of cytochalasans. *Natural Product Reports*, *27*, 869-886.
[3]   Liu JL, Hu ZY, Huang HY, Zheng ZH, Xu QY. (**2012**) Aspochalasin U, a moderated TNF-α inhibitor from *Aspergillus* sp. *Journal of Antibiotics*, *65*, 49-52.
[4]   Zhang Q, Xiao J, Sun QQ, Qin JC, Pescitelli G, Gao JM. (**2014**) Characterization of cytochalasins form the endophytic *Xylaria* sp. and their biological functions. *Journal of Agricultural and Food Chemistry*, *62*, 10962-10969.
[5]   Sharma SV, Oneyama C, Nakano H, Agatsuma T, Kanda Y, Tsumagari N, Ando K. From PCT Int. Appl. (**2003**), WO 2003006060 A1 20030123.
[6]   Feng YJ, Blunt JW, Cole ALJ, Munro MHG. (**2002**) Three novel cytochalasins X, Y, and Z from *Pseudeurotium zonatum*. *Journal of Natural Products*, *65*, 1274-1277.
[7]   Pongcharoen W, Rukachaisirikul V, Phongpaichit S, Rungindamai N, Sakayaroj J. (**2006**) Pimarane diterpene and cytochalasin derivatives from the endophytic fungus *Eutyppella scoparia*PSU-D44. *Journal of Natural Products*, *69*, 856-858.
[8]   Tomoda H, Namatame I, Tabata N, Kawaguchi K, Si S, Omura S. (**1999**) Structure elucidation of fungal phenochalasins, novel inhibitors of lipid droplet formation in mouse macrophages. *Journal of Antibiotics*, *52*, 857-861.
[9]   Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR. (**1998**) Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Research*, *48*, 4827-4833.

Concise Synthesis of Taiwaniaquinol B and 5-*epi*-Taiwaniaquinone G

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The natural product taiwaniaquinol B and the unnatural 5-*epi*-taiwaniaquinone G were synthesized based on a highly efficient tandem acylation–Nazarov cyclization approach to build the tricyclic skeleton.
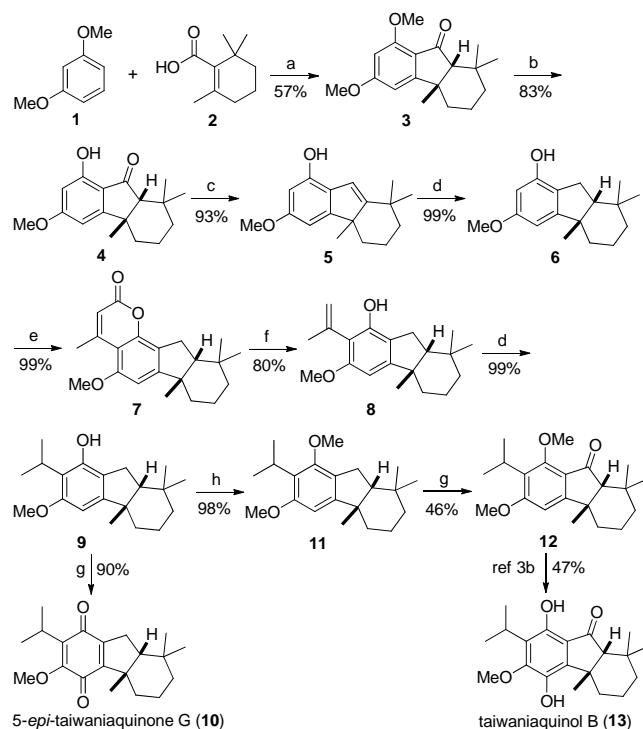
Keywords: Taiwaniaquinol B, 5-*Epi*-taiwaniaquinone G, Tandem reaction, Nazarov cyclization.

The taiwaniaquinoids are a family of tricyclic diterpenoids possessing a [6,5,6]-*abeo*-abietane skeleton isolated from *Taiwania cryptomerioides*, *Salvia dichroantha*, *Thuja standishii* and other related plants [1]. The unusual structural features and the promising biological activities [1d,2] of these compounds have attracted great interest for their chemical synthesis [3,4].

The tandem acylation–Nazarov cyclization approach for the construction of fused cyclopentenones by treatment of aromatics with α,β-unsaturated carboxylic acids in the presence of TFAA and a suitable Lewis acid catalyst [5] has been proved highly efficient in natural product synthesis [5b,6]. Herein, we report our synthetic route towards taiwaniaquinol B and the unnatural 5-*epi*-taiwaniaquinone G using this strategy as a key step.

As shown in Scheme 1, treatment of resorcinol dimethyl ether (**1**) with β-cyclogeranic acid (**2**) in the presence of TFAA and ZnCl$_2$ resulted in the formation of the desired acylation–Nazarov cyclization product **3** in 57% isolated yield [6b]. The relative stereochemistry of **3** was assigned as *cis* on the basis of NOE correlations which was the thermodynamically more stable product. Selective deprotection of the methoxy group adjacent to the carbonyl group in **3** by treatment with BBr$_3$ gave **4**, which was converted into hexahydrofluorene derivative **6** in excellent yield via hydrogenation of the intermediate tetrahydrofluorene **5**. It needs to be indicated that all attempts to transform **4** directly into **6** via Wolff–Kishner or Clemmensen reduction failed to deliver the latter in any respectable yield. Next, the requisite isopropyl group was installed through a three-step sequence including Pechmann condensation of **6** with ethyl acetoacetate (EAA) to provide coumarin derivative **7**, hydrolysis and hydrogenation, which led to the formation of **9** in excellent combined yield. Oxidation of **9** with CrO$_3$ gave 5-*epi*-taiwaniaquinone G **10**, the spectroscopic data of which were identical to those previously reported in the literature [4c]. On the other hand, protection of the phenolic hydroxyl group in **9** gave **11**, which on treatment with CrO$_3$ was oxidized to tetrahydrofluorenone **12**. Finally, compound **12** could be converted into taiwaniaquinol B **13** following known literature procedures through selective demethylation, CAN oxidation, and sodium dithionite reduction [7].

In summary, we have developed a facile route to the synthesis of taiwaniaquinol B and the unnatural 5-*epi*-taiwaniaquinone G. The

Scheme 1: *Reagents and conditions*: (a) TFAA, ZnCl$_2$, toluene, reflux; (b) BBr$_3$, DCM, -60 – 0ºC; (c) LiAlH$_4$, THF, 0ºC – rt, then 6M HCl; (d) 10% Pd/C, H$_2$, MeOH, 40ºC; (e) MsOH, EAA, Al$_2$O$_3$, rt; (f) KOH, DMSO/H$_2$O, reflux; (g) CrO$_3$, 3,5-dimethylpyrazole, DCM, -15 – -10ºC; (h) MeI, K$_2$CO$_3$, acetone, reflux.

key steps involve a tandem acylation–Nazarov cyclization reaction to build the tricyclic skeleton and a Pechmann condensation strategy to install the isopropyl group onto the aromatic ring.

Experimental

General: MP, XT4A hot-stage apparatus; IR, IFS25 FT-IR spectrometer; NMR, Agilent 400 MHz spectrometer; MS, Q-TOF micro spectrometer. Flash column chromatography was performed over silica gel 200-300 mesh.

5,6,7,8,8a,9-Hexahydro-2-isopropyl-3-methoxy-4b,8,8-trimethyl-1*H*-fluorene-1,4(4b*H*)-dione (10) [4c]: A mixture of CrO$_3$ (238 mg,

2.38 mmol) and 3,5-dimethylpyrazole (229 mg, 2.38 mmol) in DCM (6 mL) was stirred at -15ºC for 10 min. A solution of **9** (40 mg, 0.13 mmol) in DCM (6 mL) was added. The reaction mixture was allowed to warm to -10ºC and stirred for a further 1 h before being quenched with H_2O (10 mL). The separated aqueous phase was extracted with DCM (3 x 10 mL). The combined organic extracts were dried over Na_2SO_4, then filtered and evaporated *in vacuo*. The residue was purified by column chromatography (CC) on silica gel (3% ethyl acetate in light petroleum) to give 5-*epi*-taiwaniaquinone G **10** (38 mg, 90%) as a yellow oil.

IR (neat): 1648, 1592, 1460 cm^{-1}.

^{1}H NMR (400 MHz, CDCl$_3$): 3.92 (s, 3H), 3.20 (sept, 1H, J = 7.0 Hz), 2.65 (dd, 1H, J = 18.0, 8.2 Hz), 2.36 (dd, 1H, J = 18.0, 11.4 Hz), 1.89 (m, 1H), 1.74 (dd, 1H, J = 11.4, 8.2 Hz), 1.52 (s, 3H), 1.46-1.40 (m, 2H), 1.30-1.28 (m, 3H), 1.21 (d, 3H, J = 7.0 Hz), 1.19 (d, 3H, J = 7.0 Hz), 1.08 (s, 3H), 0.93 (s, 3H).

^{13}C NMR (100 MHz, CDCl$_3$): 187.5, 182.8, 156.7, 152.6, 146.3, 136.9, 61.2, 55.1, 48.1, 35.1, 34.4, 31.9, 31.2, 31.1, 29.6, 24.6, 24.4, 20.8, 20.7, 18.1.

HRMS-ESI: *m/z* [M + H$^+$] calcd for $C_{20}H_{29}O_3$: 317.2117; found 317.2113.

2,3,4,4a,9,9a-Hexahydro-7-isopropyl-6,8-dimethoxy-1,1,4a-trimethyl-1*H*-fluorene (11): Methyl iodide (68 µL, 1.1 mmol) was added dropwise to a mixture of **9** (58 mg, 0.19 mmol) and K_2CO_3 (67 mg, 0.48 mmol) in acetone (3 mL). After addition, the mixture was heated at reflux for 3 h, then cooled and evaporated *in vacuo*. The residue was purified by CC on silica gel (3% ethyl acetate in light petroleum) to give **11** (60 mg, 98%) as a colorless solid.

MP: 115-116ºC.

^{1}H NMR (400 MHz, CDCl$_3$): 6.42 (s, 1H), 3.79 (s, 3H), 3.75 (s, 3H), 3.48 (sept, 1H, J = 7.1 Hz), 2.82 (dd, 1H, J = 15.1, 7.8 Hz), 2.66 (dd, 1H, J = 15.1, 11.1 Hz), 1.83 (dd, 1H, J = 11.1, 7.8 Hz), 1.66-1.55 (m, 2H), 1.44-1.36 (m, 2H), 1.41 (s, 3H), 1.30 (d, 3H, J =

7.1 Hz), 1.29 (d, 3H, J = 7.1 Hz), 1.27-1.19 (m, 2H), 1.11 (s, 3H), 0.95 (s, 3H).

^{13}C NMR (100 MHz, CDCl$_3$): 158.4, 155.0, 154.1, 126.4, 124.7, 101.1, 60.6, 57.6, 55.9, 45.6, 36.4, 35.3, 32.3, 31.2, 30.8, 29.6, 25.6, 25.1, 21.6, 21.5, 19.1.

HRMS-ESI: *m/z* [M + H$^+$] calcd for $C_{21}H_{33}O_2$: 317.2481; found 317.2459.

2,3,4,4a-Tetrahydro-7-isopyopyl-6,8-dimethoxy-1,1,4a-trimethyl -1*H*-fluoren-9(9a*H*)-one (12) [7]: A mixture of CrO$_3$ (326 mg, 3.27 mmol) and 3,5-dimethylpyrazole (317 mg, 3.27 mmol) in DCM (7 mL) was stirred at -15ºC for 10 min. A solution of **11** (58 mg, 0.18 mmol) in DCM (7 mL) was added. The reaction mixture was allowed to warm to -10ºC and stirred for 10 h before being quenched with H_2O (15 mL). The separated aqueous phase was extracted with DCM (3 x 10 mL). The combined organic extracts were dried over Na_2SO_4, then filtered and evaporated *in vacuo*. The residue was purified by preparative TLC (3% ethyl acetate in light petroleum) to give **12** (21 mg, 46%, 71% based on recovered starting material) as a yellow oil, together with 20 mg of unreacted starting material recovered.

MP: 136-137ºC.

^{1}H NMR (400 MHz, CDCl$_3$): 6.57 (s, 1H), 3.91 (s, 3H), 3.90 (s, 3H), 3.57 (sept, 1H, J =7.1 Hz), 2.11 (s, 1H), 2.07 (m, 1H), 1.65-1.56 (m, 2H), 1.51-1.32 (m, 3H), 1.28 (d, 6H, J = 7.1 Hz), 1.25 (s, 3H), 1.23 (s, 3H), 0.70 (s, 3H).

^{13}C NMR (100 MHz, CDCl$_3$): 204.5, 164.8, 164.0, 157.1, 128.1, 121.7, 99.8, 65.7, 62.1, 55.8, 41.4, 38.6, 34.5, 34.0, 33.4, 32.6, 24.7, 24.5, 21.2, 18.4.

References

[1]   (a) Lin WH, Fang JM, Cheng YS. (**1995**) Uncommon diterpenes with the skeleton of six-five-six fused-rings from *Taiwania cryptomerioides*. *Phytochemistry*, **40**, 871-873; (b) Kawazoe K, Yamamoto M, Takaishi Y, Honda, G, Fujita T, Sezik E, Yesilada E. (**1999**) Rearranged abietane-type diterpenes from *Salvia dichroantha*. *Phytochemistry*, **50**, 493-497; (c) Ohtsu H, Iwamoto M, Ohishi H, Matsunaga S, Tanaka R. (**1999**) Standishinal, a novel carbon skeletal diterpene from the bark of *Thuja standishii* (Gord.) Carr. *Tetrahedron Letters*, **40**, 6419-6422; (d) Chang CI, Chang JY, Kuo CC, Pan WY, Kuo YH. (**2005**) Four new 6-nor-5(6→7)*abeo*-abietane type diterpenoids and antitumor cytotoxic diterpene constituents from the bark of *Taiwania cryptomerioides*. *Planta Medica*, **71**, 72-76.

[2]   (a) Iwamoto M, Ohtsu H, Tokuda H, Nishino H, Matsunaga S, Tanaka R. (**2001**) Anti-tumor promoting diterpenes from the stem bark of *Thuja standishii* (Cupressaceae). *Bioorganic & Medicinal Chemistry*, **9**, 1911-1921; (b) Minami T, Iwamoto M, Ohtsu H, Ohishi H, Tanaka R, Yoshitake A. (**2002**) Aromatase inhibitory activities of standishinal and the diterpenoids from the bark of *Thuja standishii*. *Planta Medica*, **68**, 742-745.

[3]   Majetich G, Shimkus JM. (**2010**) The taiwaniaquinoids: A review. *Journal of Natural Products*, **73**, 284-298; and references therein.

[4]   (a) Alvarez-Manzaneda E, Chahboun R, Alvarez E, Tapia R, Alvarez-Manzaneda R. (**2010**) Enantioselective total synthesis of cytotoxic taiwaniaquinones A and F. *Chemical Communications*, **46**, 9244-9246; (b) Liao X, Stanley LM, Hartwig JF. (**2011**) Enantioselective total syntheses of (-)-taiwaniaquinone H and (-)-taiwaniaquinol B by iridium-catalyzed borylation and palladium-catalyzed asymmetric α-arylation. *Journal of the American Chemical Society*, **133**, 2088-2091; (c) Tapia R, Guardia JJ, Alvarez E, Haidöur A, Ramos JM, Alvarez-Manzaneda R, Chahboun R, Alvarez-Manzaneda E. (**2012**) General access to taiwaniaquinoids based on a hypothetical abietane C7-C8 cleavage biogenetic pathway. *The Journal of Organic Chemistry*, **77**, 573-584; (d) Ozeki M, Satake M, Toizume T, Fukutome S, Arimitsu K, Hosoi S, Kajimoto T, Iwasaki H, Kojima N, Node M, Yamashita M. (**2013**) First asymmetric total synthesis of (+)-taiwaniaquinol D and (-)-taiwaniaquinone D by using intramolecular Heck reaction. *Tetrahedron*, **69**, 3841-3846; (e) Deng J, Li R, Luo Y, Li J, Zhou S, Li Y, Hu J, Li A. (**2013**) Divergent total synthesis of taiwaniaquinones A and F and taiwaniaquinols B and D. *Organic Letters*, **15**, 2022-2025; (f) Thommen C, Jana CK, Neuburger M, Gademann K. (**2013**) Syntheses of taiwaniaquinone F and taiwaniaquinol A via an unusual remote C-H functionalization. *Organic Letters*, **15**, 1390-1393.

[5]   (a) Song C, Knight, DW, Whatton MA. (**2006**) The first examples of Nazarov cyclizations leading to annulated pyrroles. *Organic Letters*, **8**, 163-166; (b) Li E, Li C, Wang J, Wang J, Dong L, Guo X, Song C, Chang J. (**2014**) Lewis acid-catalyzed tandem acylation–Nazarov cyclization for the syntheses of fused cyclopentenones. *Tetrahedron*, **70**, 874-879; (c) Huang C, Ding R, Song C, Lu J, Liu L, Han X, Wu J, Hou H, Fan Y. (**2014**) Template-induced diverse metal-organic materials as catalysts for the tandem acylation–Nazarov cyclization. *Chemistry-a European Journal*, **20**, 16156-16163.

[6]   (a) Song C, Liu H, Hong M, Liu Y, Jia F, Sun L, Pan Z, Chang J. (**2012**) Convergent formal synthesis of (±)-roseophilin. *The Journal of Organic Chemistry*, **77**, 704-706; (b) Wang J, Wang J, Li C, Meng Y, Wu J, Song C, Chang J. (**2014**) Synthesis of 5-*epi*-taiwaniaquinone G. *The Journal of Organic Chemistry*, **79**, 6354-6359.

[7]   Fillion E, Fishlock D. (**2005**) Total synthesis of (±)-taiwaniaquinol B via a domino intramolecular Friedel-Crafts acylation/carbonyl α-*tert*-alkylation reaction. *Journal of the American Chemical Society*, **127**, 13144-13145.

Effect of Enzyme Inhibitors on Terpene Trilactones Biosynthesis and Gene Expression Profiling in *Ginkgo biloba* Cultured Cells

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The biosynthetic pathway of terpene trilactones of *Ginkgo biloba* is unclear. In this present study, suspension cultured cells of *G. biloba* were used to explore the regulation of the mevalonic acid (MVA) and methylerythritol 4-phosphate (MEP) pathways in response to specific enzyme inhibitors (lovastatin and clomazone). The results showed that the biosynthesis of bilobalide was more highly correlated with the MVA pathway, and the biosynthesis of ginkgolides was more highly correlated with the MEP pathway. Meanwhile, according to the results, it could be speculated that bilobalide might be a product of ginkgolide metabolism.

Keywords: *Ginkgo biloba*, Terpene trilactones, Biosynthesis, Enzyme inhibitors, Gene expression.

Ginkgo biloba L., a traditional herb in China, is one of the oldest living plants [1]. The leaf extract of ginkgo has been employed for treating cerebrovascular and cardiovascular diseases for centuries [2]. Modern pharmacological studies showed that *G. biloba* extract could be also used for the treatment of chronic schizophrenia and Alzheimer's disease, as well as possessing antitumor activity [3-5]. Terpene trilactones, which include ginkgolides and bilobalide, are surmised to be responsible for most of the pharmacological properties of *G. biloba* extracts.

Ginkgolides are commonly supposed to be acquired via the methylerythritol 4-phosphate (MEP) pathway [6], but several experiments indicated that mevalonic acid (MVA) pathway is dominant in undifferentiated *G. biloba* cells [7]. On the other hand, bilobalide (BB) is deemed to be synthesised through the MVA pathway [8], but some authors suggest that BB is a product of ginkgolides metabolism [9]. It has been reported that 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) are the rate-limiting enzymes for the MEP pathway. Similarly, HMG-CoA reductase (HMGR) catalyzes the major rate determining steps of the MVA pathway [10].

Inhibitors of key enzymes involved in the biosynthesis pathway have been used as an additional tool to study the regulation of secondary metabolite production in plants. Lovastatin (mevinolin) can induce a specific inhibition of *HMGR* in the MVA pathway [11]. Clomazone is a soil-applied herbicide, and is the precursor of 5-keto clomazone, an inhibitor of *DXS* in the MEP pathway [12]. In the present study, we investigated the yields of terpene trilactones and the expression of key biosynthetic genes in *G. biloba* cell cultures by feeding lovastatin and clomazone to gain a better understanding of the regulation of terpene trilactones biosynthesis.

Compared with control groups, the yields of ginkgolide A (GA), ginkgolide (GB) and ginkgolide (GC) in 20 µM lovastatin-treated groups had a reduction of 12.6%, 11.4% and 16.4% at the 5th day, respectively, and that of BB had a reduction of 31.7% at the 9th day.
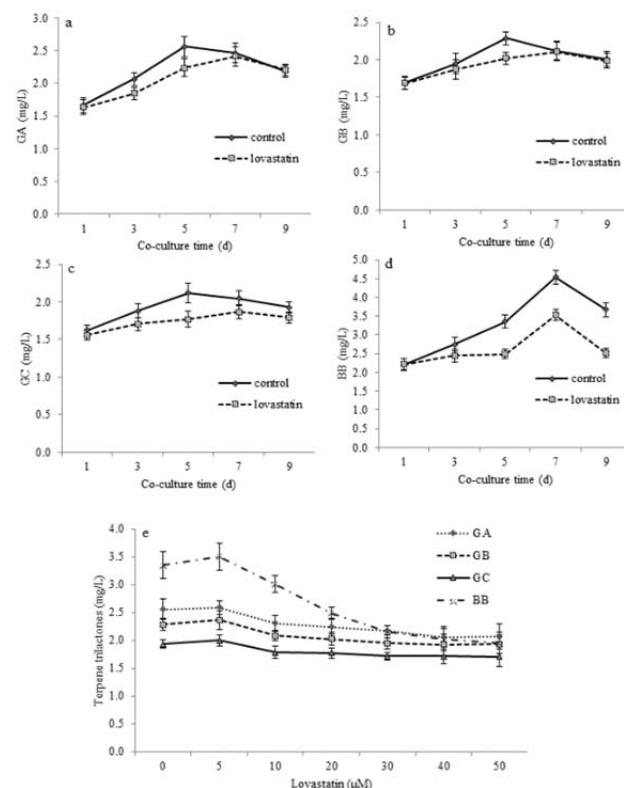
Figure 1: Effect of 20 µM lovastatin on GA (a), GB (b), GC (c), and BB (d) production in *G. biloba* suspension cultured cells, and effect of lovastatin on terpene trilactones (e) production in *G. biloba* suspension cultured cells harvested at day 5 after treatment. Lovastatin was supplied to 14-day-old *G. biloba* suspension cultured cells.

Interestingly, the yields of GA, GB and GC in lovastatin-treated groups did not have an apparent reduction on the 9th day compared with those of control groups (Figure 1). In 50 µM lovastatin-treated group, the yields of GA, GB, GC and BB had a reduction of 19.4%,
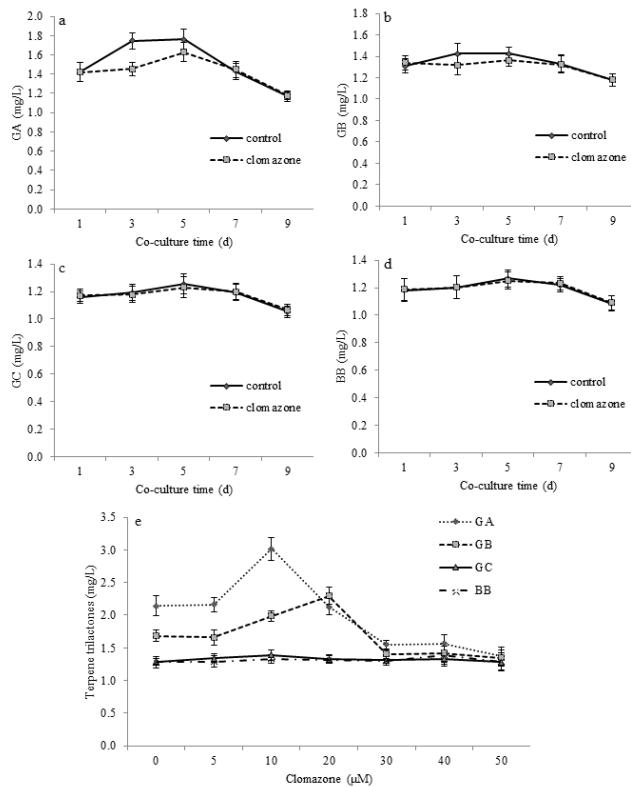
Figure 2: Effect of 40 μM clomazone on GA (a), GB (b), GC (c), and BB (d) production in *G. biloba* suspension cultured cells, and effect of clomazone on terpene trilactones (e) production in *G. biloba* suspension cultured cells harvested at day 5 after the treatment. Clomazone was supplied to 14-day-old *G. biloba* suspension cultured cells.

15.6%, 11.7% and 41.9% compared with the control group, respectively (Figure 1). These results indicated that BB was mainly synthesized from the MVA pathway, and parts of ginkgolides might be transformed into BB.

Compared with control groups, the yields of GA and GB in the 40 μM clomazone-treated groups had a reduction of 16.8% and 7.5% at the 3rd day, and that of GC and BB of 1.9% and 1.3% at the 5th day, respectively (Figure 2). In the 50 μM clomazone-treated group, the yields of GA and GB had a reduction of 36.3% and 20.0% (1.37 and 1.35 mg/L), while the yields of GC and BB were basically unchanged comparing with the control group (Figure 2). These results demonstrated that GA and GB were mainly obtained from the MEP pathway, and further indicated that the MVA pathway was the predominant one for BB.

After treatment with lovastatin (20 μM), the mRNA level of *HMGR* decreased significantly, and the transcript level of *DXS* decreased mildly. Conversely, the transcript level of *MECT* and *MVD* increased slightly. The trend in gene expressions was similar for the groups treated with different concentration of lovastatin (Figure 3). After treatment with 40 μM of clomazone, the mRNA level of *MECT* and *MVD* increased moderately. On the other hand, the *DXS* transcript level decreased slightly at the 1st day and then decreased rapidly with the increasing culture time, and *HMGR* remained stable. For the groups treated with different concentrations of clomazone, the results were similar (Figure 4). The qRT-PCR results of gene expression were consistent with the change in terpene trilactone concentrations in *G. biloba* cultured cells.

The present study provided a first attempt towards a better understanding of the regulation of the biosynthesis of terpene
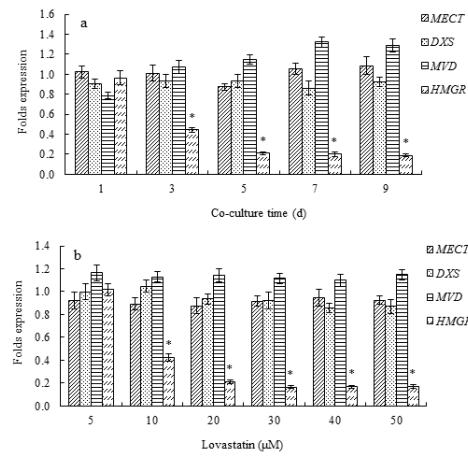
Figure 3: Effect of 20 μM lovastatin on the expression of terpene trilactones biosynthetic pathway genes (a) in *G. biloba* suspension cultured cells, and effect of lovastatin on the expression of terpene trilactones biosynthetic pathway genes (b) in *G. biloba* suspension cultured cells harvested at day 5 after the treatment. Lovastatin was supplied to 14-day-old *G. biloba* suspension cultured cells.
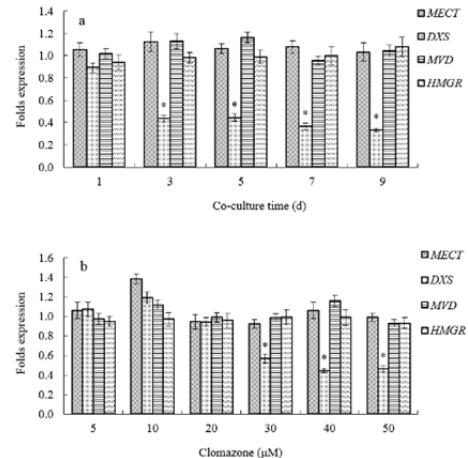
Figure 4: Effect of 40 μM clomazone on the expression of terpene trilactones biosynthetic pathway genes (a) in *G. biloba* suspension cultured cells, and effect of clomazone on the expression of terpene trilactones biosynthetic pathway genes (b) in *G. biloba* suspension cultured cells harvested at day 5 after the treatment. Clomazone was supplied to 14-day-old *G. biloba* suspension cultured cells.

trilactones in *G. biloba* cultured cells. The *in vivo* feeding experiments with two specific inhibitors, lovastatin and clomazone, showed that the biosynthesis of BB was more highly correlated with the MVA pathway, while the biosynthesis of ginkgolides was more highly correlated with the MEP pathway. Meanwhile, it could be speculated that BB might be a product of ginkgolides metabolism according to the results. This proposal requires further research by incorporation of labeled precursors and analyses of transgenic lines and mutants.

Experimental

Plant materials and methods: The cultured cells of *G. biloba* used in this research were established by our research group. Prior to the experiments, fresh 20-day-old cells (5.0 g) were transferred into 250 mL Erlenmeyer flasks and suspended in 100 mL of MS medium [13] supplemented with 30 g/L sucrose, 2.0 mg/L α-naphthalene acetic acid (NAA), 2.0 mg/L indolebutyric acid (IBA), and 5.0 mg/L ascorbic acid without agar, maintained in a rotary shaker (110 rpm) at 25°C in darkness.

Inhibitors treatment: After being filter-sterilized, the solutions of lovastatin and clomazone [14] were supplied individually to 14-day-

old suspension cultures to give final concentrations of 5, 10, 20, 30, 40 and 50 μM, respectively. Experiments for control group were made with corresponding blank solutions. 1, 3, 5, 7 and 9 days after the addition of the inhibitors, the cultured cells were harvested. The cultures were separated from liquid media by vacuum filtration, washed with distilled water, and dried at 50°C. The spent culture medium was used for analyzing terpene trilactones production. Elicitation experiments were made in triplicate.

Terpene trilactones extraction and determination: The cultured cells were separated from the culture medium by vacuum filtration and the spent medium was partitioned into ethyl acetate (v/v). The ethyl acetate extracts were combined and concentrated to dryness. The residue was dissolved in 1.0 mL of methanol and then analyzed by HPLC-ELSD. Dry cultured cells were extracted sonically with methanol 3 times. After concentration of the combined extract solutions under vacuum, the residue was dissolved in a mixture of water and ethyl acetate (1:1, v/v), and partitioning with ethyl acetate (v/v) 3 times. The ethyl acetate extracts were combined and concentrated to dryness. The residue was dissolved in 1.0 mL of methanol and then analyzed by HPLC-ELSD. For determination of ginkgolides and bilobalide, HPLC analysis was performed using a 1260 HPLC instrument (Agilent Technologies, US) equipped with an evaporative light-scattering detector (ELSD, Alltech Technologies, US). Chromatographic separations were performed

on an Agilent Hypersil C18 column (5 μm, 250 × 4.0 mm) (Agilent Technologies, US) at 25°C with gradient elution consisting of methanol and water.

Monitoring gene expression by qPCR: The extraction of total RNA from cultured cells was performed using Trizol according to reference [15]. In the present study, transcript levels of GAPDH, the genes involved in the terpene trilactones biosynthesis pathway (*HMGR*, *MVD*, *DXS* and *MECT*), were monitored in cultured cells of *G. biloba* treated with either lovastatin or clomazone. The qPCR reference manuals of PrimeScriptTM reagent kit and SYBR® Premix Ex Taq™ were used.

Statistical analysis: All values are mean ± SD. Statistical analyses were performed using an unpaired, two-tailed Student's t-test. All comparisons were made relative to untreated controls and significant difference is indicated as * $p < 0.05$.

References

[1]   Sabater-Jara AB, Souliman-Youssef S, Novo-Uzal E, Almagro L, Belchi-Navarro S, Pedreno MA. (**2013**) Biotechnological approaches to enhance the biosynthesis of ginkgolides and bilobalide in *Ginkgo biloba* . *Phytochemistry Reviews*, *12*, 191-205.

[2]   Zeng ZH, Zhu JH, Chen LL, Wen W, Yu RM. (**2013**) Biosynthesis pathways of ginkgolides. *Pharmacognosy Reviews*, *7*, 47-52.

[3]   Chen XC, Hong Y, Zheng PP. (**2015**) Efficacy and safety of extract of *Ginkgo biloba* as an adjunct therapy in chronic schizophrenia: A systematic review of randomized, double-blind, placebo-controlled studies with meta-analysis. *Psychiatry Research*, *228*, 121-127.

[4]   Liu X, Hao WL, Qin YR, Decker Y, Wang X, Burkart M, Schotz K, Menger MD, Fassbender K, Liu Y. (**2015**) Long-term treatment with *Ginkgo biloba* extract EGb 761 improves symptoms and pathology in a transgenic mouse model of Alzheimer's disease. *Brain Behavior and Immunity*, *46*, 121-131.

[5]   Liu SQ, Xu CY, Qin MB, Tan L, Zhuge CF, Mao YB, Lai MY, Huang JA. (**2015**) *Ginkgo biloba* extract enhances chemotherapy sensitivity and reverses chemoresistance through suppression of the KSR1-mediated ERK1/2 pathway in gastric cancer cells. *Oncology Reports*, *33*, 2871-2882.

[6]   Lange BM, Ghassemian M. (**2003**) Genome organization in *Arabidopsis thaliana*: a survey for genes involved in isoprenoid and chlorophyll metabolism. *Plant Molecular Biology*, *51*, 925-948.

[7]   Kang SM, Min JY, Kim YD, Park DJ, Jung HN, Karigar CS, Ha YL, Kim SW, Choi MS. (**2006**) Effect of supplementing terpenoid biosynthetic precursors on the accumulation of bilobalide and ginkgolides in *Ginkgo biloba* cell cultures. *Journal of Biotechnology*, *123*, 85-92.

[8]   Penuelas J, Munne-Bosch S. (**2005**) Isoprenoids: an evolutionary pool for photoprotection. *Trends in Plant Science*, *10*, 166-169.

[9]   Dewick PM. (**2009**) *Medicinal natural products: a biosynthetic approach*. Wiley, Chichester, England.

[10]   Shen G, Pang Y, Wu W, Liao Z, Zhao L, Sun X, Tang K. (**2006**) Cloning and characterization of a root-specific expressing gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase from *Ginkgo biloba. Molecular Biology Reports*, *33*, 117-127.

[11]   Alberts AW, Chen J, Kuron G, Huff V, Hoffman C, Rothrock J, Lopez M, Joshua H, Harris E, Petchad A, Monagan R, Currie S, Stapley E, Albers-Schönberg G, Hensens O, Hirschfield J, Hoogsteen K, Liesch J, Springer J. (**1980**). Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme a reductase and a cholesterol-lowering agent. *Proceedings of the National Academy of Sciences*, *77*, 3957-3961.

[12]   Ferhatoglu Y, Barrett M. (**2006**) Studies of clomazone mode of action. *Pesticide Biochemistry and Physiology*, *85*, 7-14.

[13]   Murashige T, Skoog F. (**1962**) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum*, *15*, 473-497.

[14]   Rodriguez-Concepcion M, Gruissem W. (**1999**). Arachidonic acid alters tomato HMG expression and fruit growth and induces 3-hydroxy-3-methylglutaryl coenzyme A reductase-independent lycopene accumulation. *Plant Physiology*, *119*, 41-48.

[15]   Liu JW, Zhu JH, Tang L, Wen W, Lv SS, Yu RM. (**2013**) Enhancement of vindoline and vinblastine production in suspension-cultured cells of *Catharanthus roseus* by artemisinic acid elicitation. *World Journal of Microbiology and Biotechnology*, *30*, 175-180.

Macrocyclic Diterpenoids from the Latex of *Euphorbia helioscopia*

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One new jatraphane diterpenoid, 7α,9β,15β-triacetoxy-3β-benzoyloxy-14β-hydroxyjatropha-5*E*,11*E*-diene (**3**), together with four known macrocyclic diterpenoids, euphoheliosnoid A (**1**), epieuphoscopin B (**2**), euphohelioscopin A (**4**) and euphoscopin C (**5**), were isolated from the stem latex of *Euphorbia helioscopia*. Their structures were established by spectroscopic analyses. In the anti-inflammatory assay, euphohelioscopin A (**4**) exhibited moderate inhibitory activity on the release of cytokine TNF-α (IC$_{50}$ = 23.7 ± 1.7 μM), IL-6 (IC$_{50}$ = 46.1 ± 1.1 μM) and chemokine MCP-1 (IC$_{50}$ = 33.7 ± 3.8 μM) in lipopolysaccharide (LPS) induced RAW 264.7 macrophages without notable cytotoxicity (IC$_{50}$ > 80 μM).

Keywords: *Euphorbia helioscopia*, Latex, Jatrophane diterpenoid, Lathyrane diterpenoid, Anti-inflammatory activity.

Plant latex is a sticky emulsion stored in laticifers and usually released immediately from damaged tissue in over 20,000 species from about 40 families of flowering plants [1]. Besides various proteins, plant latex contains a wide range of secondary metabolites, including alkaloids, terpenoids, cardenolides and phenolics [1]. These metabolites have been widely accepted to play a significant defensive role against herbivores and pathogens [1, 2], and also possess significant pharmacological activities, such as anticancer, antiviral, and anti-inflammatory effects [3, 4].

Euphorbia is the largest genus in the family Euphorbiaceae that has been extensively distributed worldwide. All species of *Euphorbia* are characteristic for the production of a white latex that is rich in secondary metabolites especially terpenoids responsible for diverse biological activities [5, 6]. For example, tirucallol, a tetracyclic triterpenoid isolated from the latex of *E. lactea*, was reported to show anti-inflammatory activity *in vivo* [7]. Diterpenoid esters that were isolated from the latex of *E. poisonii* exhibited strong and selective cytotoxic effect against human kidney carcinoma (A-498) cell line [8].

E. helioscopia L., a biennial herb with milky latex widely distributed in most parts of China, has been used as a traditional Chinese medicine to treat malaria, osteomyelitis, and bacillary dysentery [9, 10]. Previous phytochemical investigations of *E. helioscopia*, mainly focused on the whole plant, have uncovered diverse secondary metabolites, including phenols, triterpenoids and a series of structurally interesting and potentially bioactive macrocyclic diterpenoids [11-15]. However, detailed investigation of the secondary metabolites in the latex is still lacking.

To characterize the lactic metabolites, the stem latex of *E. helioscopia* was suspended in methanol to remove the macromolecular proteins and polysaccharides, and was then analyzed by reversed-phase HPLC equipped with a diode array detector and recorded at 238 nm. Five major peaks with retention times of 6.90, 7.59, 7.99, 10.38 and 12.58 min, respectively, were detected in the chromatogram (Figure 1). Subsequently, using column chromatography and reversed-phase semi-preparative HPLC, the five compounds were isolated and identified as a new (**3**)
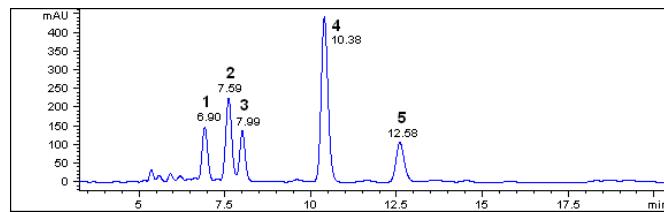

Figure 1: HPLC analysis of the secondary metabolites in stem latex of *Euphorbia helioscopia* (238 nm).
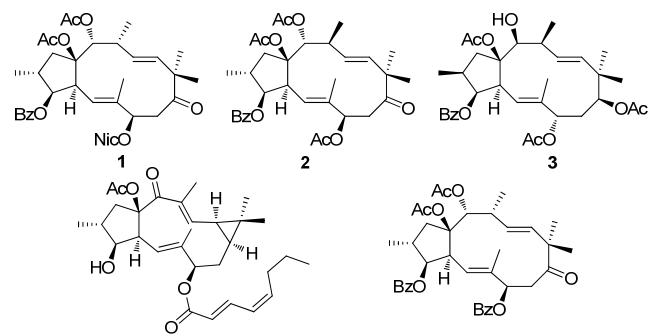

**Figure 2:** Chemical structures of compounds **1-5**.

and three known jatrophane diterpenoids (**1**, **2**, and **5**), and a known lathyrane diterpenoid (**4**) (Figure 2).

Compound **3** was obtained as a white solid. Its molecular formula was C$_{33}$H$_{44}$O$_9$ according to the ^{13}C NMR spectroscopic and HREIMS data ([M$^+$], found: *m/z* 584.2977; calcd. for 584.2985). The ^{1}H NMR spectrum showed the presence of eight methyl signals [δ$_H$ 0.89, 0.91, 0.96, 1.05, 1.09, 1.76, 1.94, and 2.38 (each 3H)], and five aromatic protons [δ$_H$ 8.05 (2H), 7.63 (1H) and 7.54 (2H)]. The ^{13}C NMR spectrum of **3** exhibited 33 carbon resonances. The carbonyl resonances (δ$_C$ 169.1, 169.7, 174.6 and 165.6) together with the aforementioned proton signals revealed the presence of three acetoxy groups and a benzoyloxy group. Apart from the above 13 signals responsible for substituents, 20 carbons including five

Table 1: NMR spectral data of **3** in acetone-d_6.[a]

Position	δ_H, (J [Hz])	δ_C	HMBC
1α	2.86 (1H, dd, 13.4, 6.8)	45.1, t	C-2, 3, 4, 15, 16
1β	2.19 (1H, t, 13.4)		
2	2.29 (1H, m)	37.9, d	C-1
3	5.39 (1H, t, 3.7)	81.3, d	C-1, 15, -C=O (3-OBz)
4	3.32 (1H, dd, 4.5, 10.2)	49.0, d	C-3, 5, 6
5	5.76 (1H, d, 10.2)	120.7, d	C-6, 7, 15, 17
6	-	133.8, s	-
7	4.89 (1H, d, 7.0)	73.8, d	C-5, 6, 8, 9, 17, -C=O (7-OAc)
8α	2.11 (1H, dd, 3.3, 16.0)	33.1, t	C-7, 9, 10
8β	1.83 (1H, ddd, 3.3, 7.0, 16.0)		
9	4.91 (1H, t, 3.2)	74.6, d	C-10, 18, -C=O (9-OAc)
10	-	40.1, s	-
11	5.09 (1H, d, 15.7)	136.1 d	C-9, 10, 18, 19
12	5.52 (1H, dd, 9.0, 15.7)	130.5, d	C-13, 14
13	2.54 (1H, m)	40.4, d	C-11, 12, 20
14	3.58 (1H, d, 5.6)	81.2, d	C-1, 12, 13, 15, 20
15	-	95.8, s	-
16	0.96 (3H, d, 6.6)	13.7, q	C-1, 2, 3
17	1.76 (3H, s)	15.9, q	C-4, 5, 6, 7
18	0.89 (3H, s)	20.4, q	C-9, 10, 11, 19
19	0.91 (3H, s)	22.9, q	C-9, 10, 11, 18
20	1.05 (3H, d, 7.0)	21.0, q	C-12, 13, 14
3-OBz C=O	-	165.6, s	-
1′	-	131.2, s	-
2′/6′	8.05 (2H, m)	130.3, d	C-2′/6′, 4′, -C=O (3-OBz)
3′/5′	7.54 (2H, t, 7.6)	129.4, d	C-1′, 3′/5′
4′	7.63 (1H, t, 7.4)	133.8, d	C-2′/6′
7-OAc	1.09 (3H, s)	19.9, q 169.1, s	-
9-OAc	1.94 (3H, s)	21.1, q 169.7, s	-
15-OAc	2.38 (3H, s)	22.4, q 174.6, s	-
14-OH	4.86 (1H, d, 4.2)	-	-

[a] ^{1}H NMR was recorded at 600 MHz; ^{13}C NMR was recorded at 150 MHz.
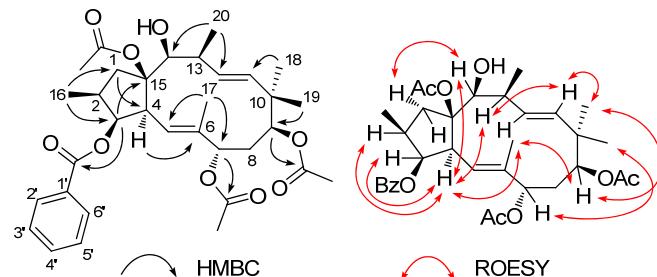
Figure 3: Key HMBC (black arrows, left), and ROESY (red arrows, right) correlations for the structure elucidation of **3**.

methyls, two methylenes, ten methines and three quaternary carbons were also observed in the ^{13}C NMR spectrum, suggesting that **3** might be a jatrophane diterpenoid [9, 11]. The jatrophane skeleton was further confirmed by 2D NMR experiments, in which a disubstituted double bond ascribable to C-11 and C-12, and a trisubstituted double bond attributable to C-5 and C-6 were observed (Figure 3). The simultaneous HMBC correlations of the proton signals at δ_H 5.39 (H-3) and 8.05 (2H, H-2′ and H-6′) with the carbonyl carbon signal at δ_C 165.6 disclosed that the benzoyloxy group was attached to C-3. Similarly, the [1]H-^{13}C long-range couplings from the proton signals at δ_H 4.89 (H-7) and 4.91 (H-9) with the carbonyl carbon signals at δ_C 169.1 and 169.7 demonstrated the presence of two acetoxy groups at C-7 and C-9, respectively. The last acetoxy group could be assigned to C-15 because no HMBC correlations were observed between the carbonyl carbon (δ_C 174.6) and any protons from the jatrophane skeleton, and further supported by the dramatically downfield

shifted C-15 (δ_C 95.8) compared with the chemical shift of the same carbon of similar jatraphane diterpenoids [9]. The ^{1}H-^{1}H COSY spectrum showed clear correlation between the proton at δ_H 3.58 (H-14) and the hydroxy resonance at δ_H 4.86, thus the hydroxy group was assignable to C-14.

The relative stereochemistry of **3** was established by analysis of the coupling constant pattern and ROESY spectrum. For the reported jatraphane diterpenoids, the angular proton H-4 is exclusively α-oriented and the C-15 acetoxy group β-oriented [5, 6]. The ROESY cross-peaks of H-4/H-3, H-4/H-13, H-4/H-14, H-1α/H-2, H-1α/H-14, and of Me-18/H-9 indicated α-orientation of these protons. The ROESY cross-peak of Me-19/H-7 revealed their β-orientation. Therefore, the structure of **3** was elucidated as 7α,9β,15β-triacetoxy-3β-benzoyloxy-14β-hydroxyjatropha-5*E*,11*E*-diene.

The four known compounds were identified as euphoheliosnoid A (**1**) [13], epieuphoscopin B (**2**) [14, 15], euphohelioscopin A (**4**) [15] and euphoscopin C (**5**) [14] by comparison of their ^{1}H and ^{13}C NMR data with those previously reported in the literatures.

The anti-inflammatory activity of euphohelioscopin A (**4**) was assayed as described previously [16]. The compound showed moderate inhibitory activity on the production of the pro-inflammatory cytokines TNF-α (IC$_{50}$ = 23.7 ± 1.7 μM), IL-6 (IC$_{50}$ = 46.1 ± 1.1 μM) and chemokine MCP-1 (IC$_{50}$ = 33.7 ± 3.8 μM) in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages, without notable cytotoxicity (IC$_{50}$ > 80 μM).

Experimental

General: Column chromatography (CC) was performed on 200-300 mesh silica gel (Qingdao Marine Chemical Factory, China), Sephadex LH-20 (Amersham Phamacia Biotech, Sweden) and MCI gel CHP-20P (75-150 μm, Mitsubishi Chemical Corp., Tokyo, Japan). Optical rotations were obtained on a Horiba-SEAP-300 spectropolarimeter, UV spectra were measured on a Shimadzu-210A double-beam spectrophotometer, and IR spectra in KBr discs were recorded on a Bruker-Tensor-27 spectrometer. NMR spectra were carried out on a Bruker Avance III 600 spectrometer (Bruker, Karlsruhe, Germany) with TMS as internal standard. MS were recorded on a VG-Auto-Spec-3000 spectrometer. Centrifugation was performed with an Eppendorf 5810R (Eppendorf, Hamburg, Germany). HPLC analysis was performed on an Agilent 1200 (Agilent, USA) series instrument equipped with a quaternary pump, a vacuum degasser, an autosampler, a thermostated column compartment and a diode array detector. Semi-preparative HPLC was also performed on the same system with a Zorbax SB-C$_{18}$, 9.4 × 250 mm column at 30°C. TLC spots were visualized under UV light and by dipping into 5% H$_2$SO$_4$ in ethanol followed by heating.

Plant material: Latex of *E. helioscopia* was collected from plants growing in Kunming Botanical Garden in February 2014.

Metabolite analysis of latex by HPLC: One hundred μL stem latex of *E. helioscopia* was suspended in 200 μL methanol in an ultrasonic bath for 10 min and then centrifuged at 12,000 rpm for 5 min. After centrifugation, the supernatant was analyzed by HPLC. At a flow rate of 1 mL/min, 10 μL of the sample was injected into a Zorbax SB-C$_{18}$ column (5 μm, 4.6 × 250 mm), and the column temperature was maintained at 30°C. A mobile phase composed of (A) water and (B) acetonitrile was used (0-20 min: isocratic 80% B, 20-25 min: linear gradient of 80-95% of B). The eluent was monitored at 200-400 nm.

Extraction and isolation: Ten mL stem latex was suspended in 50 mL methanol and centrifuged as described above. The supernatant was concentrated *in vacuo* to remove the organic solvent, and was then partitioned with EtOAc. The EtOAc fraction (205 mg) was chromatographed on a silica gel column, eluting successively with light petroleum/ethyl acetate (10: 0, 9: 1, 4: 1, 1: 1) and acetone to give 5 fractions (A-E). Fraction B was further subjected to MCI gel CC eluting with methanol/water (60-100%) to give 4 sub-fractions (2a-2d). Sub-fraction 2c (77 mg) (methanol/water, 80%) was purified by reversed-phase semi-preparative HPLC using 75% acetonitrile in water as eluent (flow rate: 3 mL/min; column: Zorbax SB-C$_{18}$, 5 μm, 9.4 × 250 mm; detection: UV 238 nm; retention times: t = 8.1, 9.4, 10.8, 12.1, 13.6, and 16.3 min) to yield **1** (3 mg), **2** (5 mg), **3** (4 mg), **4** (13 mg) and **5** (8 mg), respectively.

7α,9β,15β-Triacetoxy-3β-benzoyloxy-14β-hydroxyjatropha-5*E*,11*E*-diene (3)
White solid.
[α]$_D^{20}$: -15.6 (*c* 0.06, MeOH).
UV λ$_{max}$ (MeOH) nm (log *ε*): 201 (2.57), 228 (2.07), 271 (0.15).
IR (KBr) ν$_{max}$: 3423, 2926, 1738, 1628, 1246, 1026, 713 cm^{-1}.
^{1}H NMR (600 MHz, acetone-*d*$_6$) and ^{13}C NMR (150 MHz, acetone-*d*$_6$): Table 1.
HR-EI-MS *m/z*: 584.2977 [M]$^+$ (calcd for C$_{33}$H$_{44}$O$_9$, 584.2985).

Anti-inflammatory assay: The murine macrophage RAW 264.7 cell line was cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum in a 37°C, 5% CO$_2$ incubator. Before anti-inflammatory assay, test compounds were assessed for their cytotoxicity against the RAW 264.7 cell line and were found to be non-toxic at the tested concentrations (80, 40, 20, 10, 5, and 0 μM). Anti-inflammatory activity was assessed by enzyme-linked immunosorbent assay (ELISA) using commercial tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein 1 (MCP-1) detecting kits (BD Biosciences, Mountain View, CA, USA), as previously described [16].

Supplementary data: ^{1}H and ^{13}C NMR, HSQC, HMBC, ROESY and ^{1}H–^{1}H COSY spectra of the new compound **3**.

References

[1]   Konno K. (**2011**) Plant latex and other exudates as plant defense systems: Roles of various defense chemicals and proteins contained therein. *Phytochemistry*, **72**, 1510-1530.
[2]   Agrawal AA, Konno K. (**2009**) Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. *Annual Review of Ecology and Systematics*, **40**, 311-331.
[3]   Wang HB, Wang XY, Liu LP, Qin GW, Kang TG. (**2015**) Tigliane diterpenoids from the Euphorbiaceae and Thymelaeaceae families. *Chemical Reviews*, **115**, 2975-3011.
[4]   Feng F, Liu WY, Chen YS, Guo QL,You QD. (**2007**) Five novel prenylated xanthones from *Resina Garciniae*. *Journal of Asian Natural Products Research*, **9**, 735-741.
[5]   Shi QW, Su XH, Kiyota H. (**2008**) Chemical and pharmacological research of the plants in genus *Euphorbia*. *Chemical Reviews*, **108**, 4295-4327.
[6]   Vasas A, Hohmann J. (**2014**) *Euphorbia* diterpenes: isolation, structure, biological activity, and synthesis (2008-2012). *Chemical Reviews*, **114**, 8579-8612.
[7]   Fernandez-Arche A, Saenz MT, Arroyo M, de la Puerta R, Garcia MD. (**2010**) Topical anti-inflammatory effect of tirucallol, a triterpene isolated from *Euphorbia lactea* latex. *Phytomedicine*, **17**, 146-148.
[8]   Fatope MO, Zeng L, Ohayaga JE, Shi G, McLaughlin JL. (**1996**) Selectively cytotoxic diterpenes from *Euphorbia poisonii*. *Journal of Medicinal Chemistry*, **39**, 1005-1008.
[9]   Chen HQ, Wang H, Yang B, Jin DQ, Yang SL, Wang MC, Xu J, Ohizumi Y, Guo YQ. (**2014**) Diterpenes inhibiting NO production from *Euphorbia helioscopia*. *Fitoterapia*, **95**, 133-138.
[10]   Cateni F, Zilic J, Altieri T, Zacchigna M, Procida G, Gaggeri R, Rossi D, Collina S. (**2014**) Lipid metabolites with free-radical scavenging activity from *Euphorbia helioscopia* L. *Chemistry and Physics of Lipids*, **181**, 90-98.
[11]   Lu ZQ, Guan SH, Li XN, Chen GT, Zhang JQ, Huang HL, Liu X, Guo DA. (**2008**) Cytotoxic diterpenoids from *Euphorbia helioscopia*. *Journal of Natural Products*, **71**, 873-876.
[12]   Zhang W, Guo YW. (**2006**) Chemical studies on the constituents of the Chinese medicinal herb *Euphorbia helioscopia* L. *Chemical & Pharmaceutical Bulletin*, **54**, 1037-1039.
[13]   Zhang W, Guo YW. (**2005**) Three new jatrophone-type diterpenoids from *Euphorbia helioscopia*. *Planta Medica*, **71**, 283-286.
[14]   Tao HW, Hao XJ, Liu PP, Zhu WM. (**2008**) Cytotoxic macrocyclic diterpenoids from *Euphorbia helioscopia*. *Archives of Pharmacal Research*, **31**, 1547-1551.
[15]   Yamamura S, Shizuri Y, Kosemura S, Ohtsuka J, Tayama T, Ohba S, Ito M, Saito Y, Terada Y. (**1989**) Diterpenes from *Euphorbia helioscopia*. *Phytochemistry*, **28**, 3421-3436.
[16]   Chen ZZ, Tong L, Feng YL, Wu JZ, Zhao XY, Ruan HL, Pi HF, Zhang P. (**2015**) Ursane-type nortriterpenes with a five-membered A-ring from *Rubus innominatus*. *Phytochemistry*, **116**, 329-336.

A New Triterpenoid from the Aerial Parts of *Agrimonia pilosa*

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(1*S*,3*R*,17*R*,18*R*,19*R*,20*R*,22*R*)-1,3,19,22-tetrahydroxy-28-norurs-12-en-2-one (**1**), along with 5 known triterpenoids (**2-6**), were isolated from the aerial parts of *Agrimonia pilosa* Ledeb. Their structures were established based on extensive spectroscopic and MS analysis. The absolute configuration of compound **1** was deduced by circular dichroism (CD). Compound **1** was the first example of a 28-norursene backbone isolated from the genus *Agrimonia*. Compounds **2-6** were tested for anti-inflammatory activities against RAW 264.7 macrophages. A plausible biosynthetic pathway for compound **1** in *A. pilosa* was also proposed.

Keywords: *Agrimonia pilosa*, Ursane 28-nortriterpene, Anti-inflammatory, Biogenetic pathway.

Agrimonia, family Rosaceae, includes about a dozen species, which are perennial herbaceous flowering plants, mainly distributed in the temperate regions of the Northern Hemisphere. Some species have been used in traditional medicine. *A. pilosa* Ledeb, common name Herba Agrimoniae, (*Xianhecao* in Chinese), is a traditional Chinese medicine (TCM), and is derived from the dried aerial parts of *A. pilosa* according to the Chinese Pharmacopeia [1]. It is used for the treatment of abdominal pain, sore throat, headaches, bloody and mucoid dysentery and heat stroke. Pharmacological studies on the extracts prepared from the aerial parts of *A. pilosa* demonstrated broad biological properties, such as anti-inflammatory [2-3], anti-adipogenic [4], antioxidant [5] and antiviral [6] effects. Chemical studies on the aerial parts of *A. pilosa* showed the presence of flavonoids [7-9], 3,4-dihydroisocoumarins [2], and triterpenoids [10-11].

Recently, our research group has been examining the constituents of *A. pilosa* and has hitherto reported the isolation and identification of several flavonoids [12] and phenolic constituents [13]. Our continuous interest in bioactive constituents of this plant thus prompted us to investigate the other major chemical group in this plant - triterpenoids, and their inhibitory activities on NO and interleukin-6 (IL-6) production in RAW 264.7 macrophages induced by lipopolysaccharide (LPS). In this paper, we describe the isolation and structural elucidation of one new triterpenoid (**1**), together with five known ones (**2-6**) (Figure 1), and their *in vitro* anti-inflammatory evaluation on inhibitory activities in RAW 264.7 macrophages. The biogenetic pathway for compound **1** is also proposed (Scheme 1).

Compound **1** was obtained as a white amorphous powder. Its molecular formula was determined to be $C_{29}H_{46}O_5$, with seven degrees of unsaturation according to the positive-ion ESI-MS data
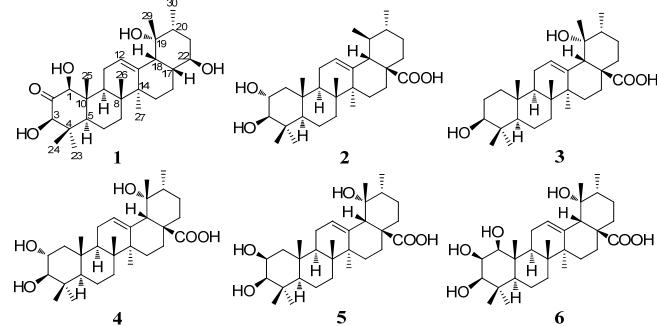
Figure 1: Structures of compounds **1-6** isolated from *Agrimonia pilosa*
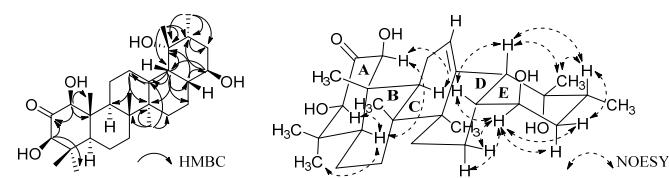
at m/z 497.4 [M+Na]+ and 473.2 [M-H]-, confirmed unambiguously from the pseudo-molecular ion peak at *m/z* 519.3340 [M+HCOO]- obtained by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). The ^{1}H NMR spectrum (Table 1) of **1**, displayed the characteristic signals of six tertiary methyl groups at δ_H 1.21 (s, H-23), 0.69 (s, H-24), 0.80 (s, H-25), 0.86 (s, H-26), 1.31 (s, H-27) and 1.19 (s, H-29), one secondary methyl group at δ_H 0.92 (d, *J* = 5.8 Hz, H-30), three oxymethine protons at δ_H 4.08 (s, H-1), 3.99 (d, *J* = 1.4 Hz, H-3) and 3.84 (like-dd, *J* = 2.0 Hz, H-22), and one tri-substituted olefinic proton at δ_H 5.35 (t, *J* = 3.6 Hz, H-12). In addition, the ^{13}C NMR spectrum (Table 1) exhibited 29 carbon signals, including characteristic signals of seven methyls at δ_C 16.3 (C-23), 29.3 (C-24), 11.8 (C-25), 17.3 (C-26), 23.4 (C-27), 27.2 (C-29) and 15.6 (C-30), three oxygenated methines at δ_C 84.2 (C-1), δ_C 81.3 (C-3) and 72.3 (C-22), one quaternary oxygen-bearing carbon at δ_C 74.0 (C-19), two olefinic carbons at δ_C 128.7 (C-12) and 138.4 (C-13), and a ketone carbonyl carbon at δ_C 211.4 (C-2). The above NMR spectroscopic data were similar to those of 1β-hydroxy-2- oxopomolic acid [14], except for the appearance of a

Table 1: The ^{1}H and ^{13}C-NMR data^{a,b} of **1** in CDCl$_3$.

No.	Carbon signals	Proton signals
1	84.2	4.08 (1H, s)
2	211.4	--
3	81.3	3.99 (1H, d, J = 1.4 Hz)
4	45.9	--
5	51.1	1.38 (1H, m)
6	18.2	1.75 (1H, d, J = 10.1 Hz)
		1.58 (1H, m)
7	32.9	1.59 (1H, m)
		1.44 (1H, m)
8	40.5	--
9	48.6	2.15 (1H, m)
10	49.7	--
11	26.3	2.48 (1H, ddd, J = 21.0, 11.0, 3.6 Hz)
		2.16 (1H, m)
12	128.7	5.35 (1H, t, J = 3.6 Hz)
13	138.4	--
14	42.7	--
15	31.8	1.59 (1H, m)
		1.23 (1H, m)
16	23.7	2.14 (1H, m)
		1.23 (1H, m)
17	45.0	1.57 (1H, m)
18	47.9	2.38 (1H, d, J = 5.6 Hz)
19	74.0	--
20	34.3	1.93 (1H, m)
21	33.9	1.84 (1H, td, J = 14.0, 2.4 Hz)
		1.40 (1H, m)
22	72.3	3.84 (1H, like-dd, J = 2.0, 2.0 Hz)
23	16.3	1.21 (3H, s)
24	29.3	0.69 (3H, s)
25	11.8	0.80 (3H, s)
26	17.3	0.86 (3H, s)
27	23.4	1.31 (3H, s)
29	27.2	1.19 (3H, s)
30	15.6	0.92 (3H, d, J = 5.8 Hz)
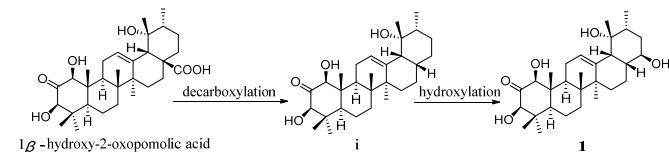
$^{a)}$ 600 MHz for ^{1}H NMR; the coupling constants (J) are in parentheses and reported in Hz; chemical shifts are given in ppm. $^{b)}$ 100 MHz for ^{13}C NMR.

hydroxyl-carrying carbon and a methine instead of a methylene and a carboxyl group, which implied that compound **1** has a 28-nor-ursene skeleton. This deduction was further supported by the HMBC correlations from δ_H 3.84 (H-22) to δ_C 47.9 (C-18) and 34.3 (C-20), and from δ_H 2.38 (H-18) to δ_C 128.7 (C-12), 138.4 (C-13), 42.7 (C-14), 23.7 (C-16), 45.0 (C-17) and 74.0 (C-19), and from δ_H 1.57 (H-17) to δ_C 72.3 (C-22) and 74.0 (C-19), and from δ_H 0.92 (Me-30) to δ_C 34.3 (C-20), 74.0 (C-19) and 33.9 (C-21), which established that a hydroxyl group was located at C-22 and a hydrogen atom instead of a carboxyl group located at C-17 of ring E (Figure 2). The location of the other functional groups and NMR data assignments of **1** were determined by HSQC and HMBC spectroscopic analysis. The strong NOESY correlations of δ_H 4.08 (H-1) with δ_H 1.38 (H-5) and 2.15 (H-9), δ_H 3.99 (H-3) with δ_H 1.38 (H-5) and 1.21 (Me-23) suggested that H-1 and H-3 were α-oriented on ring A, the same as for 1β-hydroxy-2-oxopomolic acid (Figure 2). Furthermore, the NOESY correlations of δ_H 2.38 (H-18) with δ_H 1.57 (H-17), 1.19 (Me-29) and 1.93 (H-20) suggested that ring D and E had a *cis*-configuration, and H-18 and H-20 were in the same orientation. The NOESY correlations of δ_H 1.93 (H-20) with δ_H 1.19 (Me-29) and 1.40 (H-21β), and no correlations of H-20 with H-17 and H-22 indicated that ring E possessed a chair instead of a boat conformation [15]. H-21α was axial-oriented, deduced by the triplet of doublets and vicinal coupling constant (J = 14.0, 2.4 Hz). The NOESY correlations of δ_H 3.84 (H-22) with δ_H 2.14 (H-16α), 1.84 (H-21α), 1.40 (H-21β) and 1.23 (H-16β) suggested that H-22 is equatorially-oriented (Figure 2). This is confirmed by the dd-like signal and vicinal coupling constant (J = 2.0, 2.0 Hz) that corresponds to a dihedral angle of ca. 60° from H-22 to H-17, H-21α and H-21β, respectively. The absolute configuration of **1** was established by the CD octant rule [16-17] due to the presence of a cyclohexone ring A moiety. In the CD spectrum of **1**, a positive Cotton effect at 284 nm ($\Delta\varepsilon$ = + 1.17) due to the n–π* electronic transition suggested 1*S*,3*R*,17*R*,18*R*,19*R*,20*R*,22*R* configurations of

Figure 2: Selected HMBC and NOESY correlations of compound **1**.

1. Therefore, compound **1** was established to be (1*S*,3*R*,17*R*,18*R*,19*R*,20*R*,22*R*)-1,3,19,22-tetrahydroxy-28-norurs-12-en-2-one.

The 28-norursene skeleton is unprecedented in the genus *Agrimonia* from the view point of chemotaxonomy. Compound **1** could be produced from 1β-hydroxy-2-oxopomolic acid, a key biosynthetic precursor isolated from *Rosa woodsii* (Rosaceae family), through decarboxylation [18] and hydroxylation reactions (Scheme 1).

Scheme 1: Plausible biogenetic pathway for compound **1**.

Five known triterpenoid derivatives were identified as corosolic acid (**2**) [19], pomolic acid (**3**) [20], tormentic acid (**4**) [19], *epi*-tormentic acid (**5**) [21], and 1β,2β,3β,19α-tetrahydroxyurs-12-en-28-oic acid (**6**) [11], respectively, by analysis of their spectroscopic (NMR) and MS data with those reported in the literature.

Table 2: Inhibitory effects of compounds **2-6** on NO and IL-6 production induced by LPS in mouse monocyte-macrophage RAW 264.7 cells. (n=3).

Comp.	Anti-inflammatory activity (IC$_{50}$ [μM])	
	NO	IL-6
2	33.2±2.8	42.5±3.2
3	25.6±1.9	42.2±2.7
4	52.6±4.8	76.7±5.5
5	55.6±4.2	85.1±6.9
6	59.1±4.0	>100
hydrocortisone ^{a}	56.5±4.6	63.1±5.2

$^{a)}$ Positive control

Compounds **2-6** were tested for their inhibitory activities on NO and IL-6 production in mouse monocyte-macrophage RAW 264.7 cells stimulated by LPS (Table 2). Compounds **2** and **3** showed mild inhibitory effects, while the activities of **4** and **5** were very weak. Compound **6** exhibited no significant inhibitory activity with an IC$_{50}$ value higher than 100 μM concentration. The cytotoxic activities of compounds **2-6** against RAW 264.7 macrophages were also tested by the MTT assay, but none of the compounds exhibited significant cytotoxicity at their effective concentrations. The IC$_{50}$ value of **1** was not acquired due to paucity of sample.

Experimental

Apparatus and reagents: Optical rotation was measured with a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Bruker IFS 55 spectrometer. The CD spectrum was determined with a Bio-Logic Science MOS-450 spectrometer. NMR experiments were performed on Bruker ARX-400 and AV-600 spectrometers. The chemical shifts are stated relative to TMS and expressed in δ values (ppm), with coupling constants are reported in Hz. The HRESIMS was obtained on an Agilent 6210 TOF mass spectrometer, and ESI-MS were recorded on an Agilent Series 1100 SL mass spectrometer. Silica gel GF254 prepared for TLC and

silica gel (200-300 mesh) for column chromatography (CC) were obtained from Qingdao Marine Chemical Factory (Qingdao, China). Sephadex LH-20 was a product of Pharmacia. Octadecyl silica gel was purchased from Merck Chemical Company Ltd. RP-HPLC separations were conducted using an LC-6AD liquid chromatograph with a YMC Pack ODS-A column (250 × 20mm, 5 μm, 120 Å) and SPD-10A VP UV/VIS detector. All reagents were either HPLC or analytical grade and were purchased from Tianjin Damao Chemical Company. Compounds were detected on TLC plates either under UV light or by heating after spraying with anisaldehyde-H_2SO_4 reagent.

Plant material: Dried aerial parts of *A. pilosa* were purchased from Shenyang Northeast Pharmacy, China, and identified by Professor Qishi Sun, Department of Pharmaceutical Botany, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University. A voucher specimen (AP-20061010) has been deposited in the herbarium of the Department of Natural Products Chemistry, Shenyang Pharmaceutical University.

Extraction and isolation: The dried aerial parts of *A. pilosa* (10 kg) were cut into approximately 2 cm pieces and extracted with 70% EtOH (100 L × 2 h × 2). The resulting extract (1.0 kg) was concentrated *in vacuo*, suspended in H_2O (3 L), and partitioned successively with cyclohexane, EtOAc, and *n*-BuOH (3 L × 3). The EtOAc extract (110 g) was subjected to silicagel CC (10 × 80 cm) and eluted with chloroform/MeOH (100:1, 30:1, 20:1, 10:1, 5:1, 2:1, 1:1 and 0:1 v/v) to obtain 7 fractions (EA-EG). Fraction EA (23 g) was subjected to a silica gel column (6 × 80 cm) and eluted with CH_2Cl_2/EtOAc (from 40:1 to 0:1) to produce 7 fractions (EA1-EA7). Fraction EA4 (180 mg) was chromatographed over Sephadex LH-20 (CH_2Cl_2/MeOH, 1:1; 1.5 × 30 cm) to give compound **1** (3.5 mg). Fraction ED (20.6 g) was subjected to a silica gel column (6 × 80 cm) and eluted with CH_2Cl_2/acetone (from 40:1 to 0:1) to yield ED1-ED6. Separation of ED4 (1.4 g) on a reversed-phase C_{18} silica gel column (2.5 × 30 cm) by elution with MeOH/H_2O (30:70, 50:50, 70:30 and 100:0 v/v) yielded fractions ED4-1 to ED4-5. ED4-5 (380 mg) was purified by preparative TLC (CH_2Cl_2/acetone, 3:1) to obtain **2** (11.5 mg) and **4** (22.3mg). ED5 (4.3 g) was subjected to RP-C_{18} silica gel CC (2.5 × 30 cm) and eluted with MeOH/H_2O (1:9 to 8:2) to yield ED5-1 and ED5-2. ED5-1 (100 mg) was separated by HPLC (50%MeOH/H_2O, 6mL/min) to afford compound **3** (56.2 mg, t_R= 39 min) and compound **5** (28.8 mg, t_R= 25 min). ED5-2 (120 mg) was purified by preparative TLC (CH_2Cl_2/MeOH, 7:1) to obtain **6** (13.6 mg).

(1*S*,3*R*,17*R*,18*R*,19*R*,20*R*,22*R*)-1,3,19,22-tetrahydroxy-28-norurs-12-en-2-one (3)
Amorphous white powder (MeOH).
$[\alpha]_D^{25}$: +46.0 (*c* 0.10, MeOH).
CD (MeOH): 284 (Δε = +1.17) nm.
IR v_{max} (KBr): 3448, 2928, 1715, 1618, 1459, 1384, 1021 cm⁻¹.
¹H-NMR (600 MHz, CDCl₃): Table 1.
¹³C-NMR (100 MHz, CDCl₃): Table 1.
ESI-MS (*m/z*): 497.4 [M + Na]⁺, 473.2 [M - H]⁻.
HRESIMS (*m/z*): 519.3340 [M + HCOO]⁻ (calcd. for $C_{30}H_{47}O_7$: 519.3322).

Inhibition of NO and IL-6 production bioassay: The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction. Briefly, RAW 264.7 cells were seeded into 96-well tissue culture plates at a density of $1×10^5$ cells/well, and stimulated with 1 μg/mL of LPS in the presence or absence of test compounds. After incubation at 37°C for 24 h, 100 μL of cell-free supernatant was mixed with 100 μL of Griess reagent (mixture of equal volumes of reagent A and reagent B, A: 1%, w/v, sulfanilamide in 5%, w/v, phosphoric acid, B: 0.1%, w/v, of *N*-(1-naphthyl)ethylenediamine). The test groups, LPS groups, and control groups were treated in the same way as the experiment for NO analysis, respectively. Absorbance was measured in a microplate reader at 540 nm. Nitrite concentrations and the inhibitory rates were calculated by a calibration curve prepared with sodium nitrite standards. Cytotoxicity was determined by the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay, after 24 h incubation with test compound. The cells in the 96-well plates were cultured for 6 h, and the levels of IL-6 in the cell culture supernatant were detected by using a mouse IL-6 ELISA kit according to the manufacturer's recommendations [22]. Data are presented as the mean ± standard deviation. Each experiment was performed at least 3 times.

Supplementary data: Spectral data relating to this article are available online.

References

[1]   Chinese Pharmacopeia Committee (**2010**) *Pharmacopeia of the People's Republic of China*, Chinese Medicine Industry Press, Beijing, vol. I, pp. 94–95.
[2]   Taira J, Nanbu H, Ueda K. (**2009**) Nitric oxide-scavenging compounds in *Agrimonia pilosa* Ledeb on LPS-induced RAW264.7 macrophages. *Food Chemistry*, **115**, 1221-1227.
[3]   Taira J, Ohmine W, Ogi T, Nanbu H, Ueda K. (**2012**) Suppression of nitric oxide production on LPS/IFN-γ-stimulated RAW 264.7 macrophages by a novel catechin, pilosanol N, from *Agrimonia pilosa* Ledeb. *Bioorganic & Medicinal Chemistry Letters*, **22**, 1766-1769.
[4]   Ahn EK, Lee JA, Seo DW, Hong SS, Oh JS. (**2012**) 1β-Hydroxy-2-oxopomolic acid isolated from *Agrimonia pilosa* extract inhibits adipogenesis in 3T3-L1 cells. *Biological & Pharmaceutical Bulletin*, **35**, 643-649.
[5]   Zhu LC, Tan J, Wang BC, He R, Liu YP, Zheng C. (**2009**) Antioxidant activities of aqueous extract from *Agrimonia pilosa* Ledeb and its fractions. *Chemistry & Biodiversity*, **6**, 1716-1726.
[6]   Shin WJ, Lee KH, Park MH, Seong BL. (**2010**) Broad-spectrum antiviral effect of *Agrimonia pilosa* extract on influenza viruses. *Microbiology and Immunology*, **54**, 11-19.
[7]   Jung M, Park M. (**2007**) Acetylcholinesterase inhibition by flavonoids from *Agrimonia pilosa*. *Molecules*, **12**, 2130-2139.
[8]   Kato H, Li W, Koike M, Wang YH, Koike K.(**2010**) Phenolic glycosides from *Agrimonia pilosa*. *Phytochemistry*, **71**, 1925-1929.
[9]   Su G, Su S, Zhu T. (**1984**) Studies on bacteriostic components from *Agrimonia pilosa* Ledeb. *Shenyang Yaoxue Xuebao*, **1**, 44-50.
[10]   An R, Kim H, Jeong G, Oh S, Oh H, Kim Y. (**2005**) Constituents of the aerial parts of *Agrimonia pilosa*. *Natural Product Science*, **11**, 196-198.
[11]   Kouno I, Baba N, Ohni Y, Kawano N. (**1988**) Triterpenoids from *Agrimonia pilosa*. *Phytochemistry*, **27**, 297-299.
[12]   Pan Y, Liu HX, Zhuang YL, Ding LQ, Chen LX, Qiu F. (**2008**) Studies on isolation and indentification of flavonoids in herbs of *Agrimonia pilosa*. *Zhongguo Zhongyao Zazhi*, **33**, 2925-2928.

[13]   Liu HX, Liu ZX, Jiang QH, Ding LQ, Chen LX, Qiu F. (**2010**) Phenolic constituents of whole plant of *Agrimonia pilosa* Ledeb. *Zhongguo Zhongyao Zazhi*, **27**, 286-298.
[14]   Kashiwada Y, Wang HK, Nagao T, Kitanaka S, Yasuda I, Fujioka T, Yamagishi T, Cosentino M, Kozuka M, Okabe H, Ikeshiro Y, Hu CQ, Yeh E, Lee KH. (**1998**) Anti-AIDS agents. 30. Anti-HIV activity of oleanolic acid, pomolic acid, and structurally related triterpenoids. *Journal of Natural Products*, **61**, 1090-1095.
[15]   Konoshim T, Kozuka M. (**1991**) Constituenents of Leguminous plants, XIII. New triterpenoid saponins from *Wistaria brachybotrys*. *Journal of Natural Products*, **54**, 830-836.
[16]   Kirk DN.(**1986**) The chiroptical properties of carbonyl compounds. *Tetrahedron*, **42**, 777-818.
[17]   Djerassi C, Klyne W. (**1962**) Optical rotatory dispersion: application of the octant rule to some structural and stereochemical problems. *Journal of the Chemical Society*, 4929-4950.
[18]   Solomons TWG, Fryhle CB, Snyder SA. (**2014**) *Organic chemistry*, John Wiley & Sons, Inc. pp. 744-806.
[19]   Taniguchi S, Imayoshi Y, Kobayashi E, Takamatsu Y, Ito H, Hatano T, Sakagami H, Tokuda H, Nishino H, Sugita D, Shimura S, Yoshida T. (**2002**) Production of bioactive triterpenes by *Eriobotrya japonica* Calli. *Phytochemistry*, **59**, 315-323.
[20]   Ju JH, Zhou L, Lin G, Liu D, Wang LW, Yang JS. (**2003**) Studies on constituents of triterpene acids from *Eriobotrya japonica* and their anti-inflammatory and antitussive effects. *Zhongguo Zhongyao Zazhi*, **38**, 752-757.
[21]   Delgado G, Hernandez J. (**1989**) Triterpenoid acids from *Cunila lythrifolia. Phytochemistry*, **28**, 1483-1485.
[22]   Jiao WH, Gao H, Zhao F, He F, Zhou GX, Yao XS. (**2011**) A new neolignan and a new sesterterpenoid from the stems of *Picrasma quassioides* Bennet. *Chemistry & Biodiversity*, **8**, 1163-1169.

Two New 18-Norschiartane-type Schinortriterpenoids from *Schisandra lancifolia*

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Two new 18-norschiartane-type schinortriterpenoids, namely wuwezidilactones Q (**1**) and R (**2**) were isolated from the stems of *Schisandra lancifolia*. Their structures were determined on the basis of extensive spectroscopic analysis. The absolute configurations of the new compounds were further determined by ROESY and an empirical comparison of their experimental ECD spectra with literature.

Keywords: *Schisandra lancifolia*, Schinortriterpenoids, Absolute configuration.

Plants of Schisandraceae family, contains the genera *Schisandra* and *Kadsura*, represent an important group of plants. Some of these plants were important Traditional Chinese Medicines (TCMs), and used in Chinese folk for more than two thousand years. Chemical investigation on this family was mostly focused on lignans and triterpenoids since 1970's. In 2003, Sun and co-workers reported a novel highly oxygenated nortritepenoid, micrandilactone A, from *Schisandra micrantha* [1]. This discovery revealed a new kind of natural triterpernoid, called as schinortriterpenoid (SNT). In the past decades, a series of SNTs with different novel skeletons were isolated and identified from this family [2,3], especially from *Schisandra* genus, attracting more and more attention from natural product chemists, as well as organic chemists.

Schisandra lancifolia (Rehd. et. Wils.) A. C. Smith, a species of *Schisandra* genus distributed in the southwest of China, was used to staunch, treat fractures, and eliminate stasis to reduce swelling [4]. Phytochemistry research of this species has led to the discovery of a series of SNTs with different new skeletons, such as 18-Norschiartanes [5], Norcycloartanes [6], Schisanartanes [7], 1,2,3-trinorlancishiartanes [8], 2,3-dinorlancischiartanes [9], lancifoartanes [10], 12,22-cyclopreschisanartanes [11]. Our further investigation into this plant aimed at finding more SNTs with diverse skeletons led to the isolation of two new 18-norschiartane-type shinortriterpenoids. This article deals with the isolation, structure elucidation of the new compounds.

Wuwezidilactone Q (**1**) was obtained as a white amorphous powder. Its molecular formula $C_{28}H_{36}O_8$ was determined by HRESIMS at *m/z* 523.2298 [M+Na]$^+$ (calcd for $C_{28}H_{36}O_8Na$ 523.2302) with 11 indices of hydrogen deficiency. ^1H, ^{13}C NMR and DEPT spectra of **1** displayed three methyl singlets and one methyl doublet, six methylenes, four sp^3 methines, four oxygenated sp^3 methines, two trisubstituted double bonds, four sp^3 quaternary carbons and two ester groups. All of the above data of **1** suggested that **1** was a 18-norschiartane-type nortriterpenoid with 28 carbons. Comparison of NMR data of **1** with those of lancifodilactone A indicated that the acetoxy group at C-12 in lancifodilactone A was replaced by a hydroxyl group in **1** without causing significant chemical
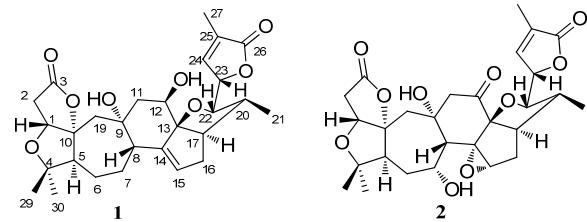
Figure 1: The structures of compounds **1** and **2**.

shifts at C-11, C-12, and C-13. This was confirmed by ^1H-^1H COSY correlation of H-11 with H-12 and HMBC correlations from H-12 to C-9, C-13, and C-14. Furthermore, compound **1** differed from lancifodilactone A at C-7 (δ_C 69.8) in ring C, the hydroxyl group at C-7 in lancifodilactone A was replaced by a methylene (δ_C 26.4) in **1**. In addition, a downfield shift of C-5 in **1** (δ_C 60.7) compared with that in lancifodilactone A (δ_C 52.3) could ascribe to the absence of γ-steric compression between the oxygen atom at C-7 and H-5 in **1** [5,12]. The change at C-7 was further proved by ^1H-^1H COSY correlations of H-7 with H-6, and H-8 and HMBC correlations from H-7 to C-5, C-8, C-9, and C-14. The relative configuration of **1** was deduced to be the same as that of lancifodilactone A from the similar carbon chemical shifts and ROESY correlations. The ROESY correlations of H-23 with Me-21, H-12 with H-17, and H-20 suggested that H-12 was α-oriented. The absolute configuration of C-23 could be determined by intense positive or negative cotton effects (CEs) around 210nm. The experimental ECD spectrum of **1** showed Negative CE at 212 nm, indicated that the absolute configuration of C-23 was "*S*" [12]. Thus, the absolute configuration of **1** was finally determined as shown in Figure 1 by an empirical comparison the experimental ECD with that of wuweizidilactones J-P.

Wuwezidilactone R (**2**) was obtained as a white amorphous powder. Its molecular formula $C_{28}H_{34}O_{10}$ was deduced by HRESIMS at *m/z* 553.2047 [M+Na]$^+$ (calcd for $C_{28}H_{34}O_{10}Na$ 553.2044) with 12 indices of hydrogen deficiency. Careful Analysis of the ^1H, ^{13}C NMR and DEPT spectra of **2** disclosed that **2** was also a 18-norschiartane-type nortriterpenoid. Structure of **2** was almost the

Table 1: ^{1}H and ^{13}C NMR data (acetone-d_6) of compounds **1** and **2**.

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1	4.29, d (4.8)	81.8 d	4.28, d (5.4)	82.1 d
2α	2.44, d (18.0)	36.0 t	2.95, overlapped	35.6 t
2β	2.92, overlapped		2.44, overlapped	
3		174.7 s		174.8 s
4		85.1 s		84.8 s
5	2.29, overlapped	60.7 d	2.76, dd (13.2, 4.8)	52.9 d
6α	1.47, overlapped	27.6 t	2.03, overlapped	32.7 t
6β	1.81, overlapped		1.76, overlapped	
7α	1.81, overlapped	26.4 t		69.6 d
7β	2.00, m		4.32, br t	
8	2.25, overlapped	49.3 d	2.73, br s	46.4 d
9		77.2 s		80.1 s
10		99.9 s		98.7 s
11α	1.81, m	43.1 t	2.21, d (13.2)	54.0 t
11β	1.91, dd (14.4, 2.4)		2.92, overlapped	
12	3.57, br d	72.9 d		202.4 s
13		97.8 s		93.7 s
14		143.4 s		74.3 s
15	5.58, m	128.0 d	3.96, br s	57.5 d
16α	2.26, overlapped	31.9 t	2.09, overlapped	26.6 t
16β	2.37, s		1.79, overlapped	
17	3.04, dt (9.0, 4.2)	45.7 d	2.59, m	41.6 d
19α	2.09, overlapped	44.6 t	2.11, overlapped	45.2 t
19β	2.18, d (15.6)		2.43, overlapped	
20	2.31, overlapped	37.9 d	2.51, m	38.2 d
21	1.01, d (6.6)	12.6 q	1.06, d (7.2)	11.9 q
22	3.52, dd (10.2, 3.6)	83.1 d	3.91, dd (9.6, 3.0)	85.3 d
23	5.01, m	81.0 d	4.88, m	81.0 d
24	7.28, m	147.8 d	7.23, m	147.2 d
25		130.3 s		130.9 s
26		174.4 s		174.0 s
27	1.84, br s	10.7 q	1.83, br s	10.8 q
29	1.14, s	22.4 q	1.14, s	22.3 q
30	1.29, s	29.2 q	1.27, s	28.7 q

δ_H were recorded at 600 MHz, δ_C were recorded at 150 MHz.
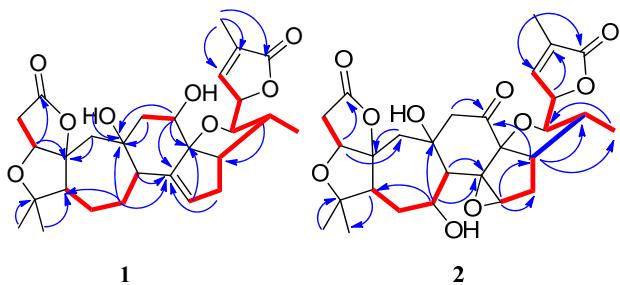
Figure 2: Selected HMBC (blue arrows H→C) and ^{1}H-^{1}H COSY (red lines) correlations of **1** and **2**.
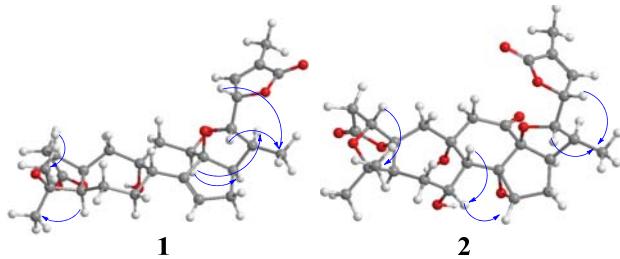
Figure 3: Key ROESY correlations (blue arrows) of **1** and **2**.

same as that of wuweizidilactone M except for a hydroxyl group at C-7 in **2** rather than an acetoxy group. This could be confirmed by the chemical shift at C-7 (δ_C 69.6), and the ^{1}H-^{1}H COSY correlations of H-7 with H-6, and H-8 and HMBC correlations from H-5, H-6, and H-8 to C-7. The ROESY experiment of **2** was similar to wuweizidilactone M, revealed that they had a same relative configuration. The ROESY correlation of H-7 with H-15 suggested that H-7 was *β*-oriented. The absolute configuration of 23*S* was proved by Negative CE at 209 nm in ECD spectrum. At last, the absolute configuration of **2** was finally determined as shown in Figure 1 by the same way as that of **1**.

Experimental

General: 1D and 2D NMR spectra were recorded on Bruker DRX 600 and DRX 500 spectrometers using TMS as the internal standard. Chemical shifts (*δ*) are expressed in ppm relative to the TMS signals. HRESIMS was performed on an API QSTAR Pulsar i spectrometer. UV spectra were obtained on a Shimadzu UV-2401PC spectrophotometer. IR spectra were obtained on a Bruker Tensor-27 FT-IR spectrometer using KBr pellets. Optical rotations were measured in MeOH with Horiba SEPA-300 and JASCO P-1020 polarimeters. Experimental ECD spectra were measured on a Chirascan instrument. Column chromatography (CC) was performed with silica gel (100~200 mesh; Qingdao Marine Chemical, Inc., Qingdao, People's Republic of China), MCI gel (CHP20P, 75~150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). Preparative HPLC was performed on an Agilent 1260 preparative liquid chromatograph with a Zorbax SB-C18 (21.2 mm × 250 mm) column. Semipreparative HPLC was performed on an Agilent 1200 liquid chromatograph with a Zorbax SB-C18 (9.4 mm × 250 mm) column. Fractions were monitored by thin layer chromatography, spots were visualized by UV light (254 nm and 365 nm) and by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH. All solvents used in column chromatography were distilled including petroleum ether (60-90°C).

Plant Material: The stems of *S. lancifolia* were collected in Shangri-La of Yunnan Province, People's Republic of China, in October 2013 and identified by Prof. Xi-Wen Li at Kunming Institute of Botany. A voucher specimen has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation: The air-dried and powdered stems of *S. lancifolia* (7.0 Kg) were extracted with aqueous acetone (4 × 8L, 3 days each) at room temperature. The extracted solution was distilled under reduced pressure to remove acetone, and then was partitioned between EtOAc and H_2O to afford EtOAc part. The EtOAc extract (250 g) was eluted with $CHCl_3$/Me_2CO (1:0, 9:1 8:2, 6:4, 1:1, 0:1) on silica gel column chromatography to give 6 fractions A-F. Fraction C (8:2) was decolorized on MCI gel with 90% MeOH/H_2O and was separated on Rp-18 with MeOH/H_2O (3:7, 4:6, 6:4, 7:3, 8:2, 1:0) to afford fractions C1-C6. Fraction C3 was separated on silica gel CC with petroleum ether/acetone (5:1, 3:1, 2:1, 1:1, 0:1) to obtain C31-C38. C33 was purified by Sephadex LH-20 CC, eluting with $CHCl_3$/MeOH (1:1), and by preparative HPLC to afford **1** (1.9 mg).

Fraction D (6:4) was successfully separated on MCI gel with 90% MeOH/H_2O to remove pigments and then on Rp-18 with MeOH/H_2O (3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, 1:0) to afford fractions D1-D8. D3 was separated over silica gel CC with petroleum ether/acetone (5:1, 3:1, 2:1, 1:1, 0:1) to afford D31-D36. D36 was purified by repeated Sephadex LH-20 CC, eluting with $CHCl_3$/MeOH (1:1), and by Semipreparative HPLC to afford **2** (4 mg).

Wuweizidilactone Q (1)

$[α]^{23}_D$: +23 (*c* 0.12, MeOH).
IR (KBr) v_{max}: 3343, 2924, 1760, 1629, 1434, 1385, 1326, 1197, 1109, 1082, 1037.
UV (MeOH) λ_{max} nm (log *ε*): 204 (3.88).
ECD (*c* 0.05 MeOH) λ_{max} nm: 209, 251.
^{1}H NMR and ^{13}C NMR: Table 1.
HRESIMS: *m/z* 523.2298 [M+Na][+] (calcd for $C_{28}H_{36}O_8Na$, 523.2302).

Wuweizidilactone Q (2)

$[\alpha]^{23}_D$: +34 (*c* 0.24, MeOH).

IR (KBr) v_{max}: 3442, 2925, 1762, 1629, 1384, 1221, 1175, 1106, 1067, 957 cm^{-1}.

UV (MeOH) λmax nm (log *ε*): 208 (3.95), 275 (2.79).

ECD (*c* 0.07 MeOH) λmax nm: 212, 302.

^{1}H NMR and ^{13}C NMR: Table 1.

HRESIMS: *m/z* 553.2047 [M+Na]$^+$ (calcd for $C_{28}H_{34}O_{10}Na$ 553.2044).

Supplementary data: 1D and 2D NMR, HRESIMS, UV, IR, and CD spectra of **1** and **2** are available.

References

1. Li RT, Zhao QS, Li SH, Han QB, Sun HD, Lu Y, Zhang LL, Zheng QT. (**2003**) Micrandilactone A: a Novel Triterpene from *Schisandra micrantha*. *Organic Letters*, **5**, 1023-1026.
2. Xiao WL, Li RT, Huang SX, Pu JX, Sun HD. (**2008**) Triterpenoids from the Schisandraceae family. *Natural Product Reports*, **25**, 871-891.
3. Shi YM, Xiao WL, Pu JX, Sun HD. (**2015**) Triterpenoids from the Schisandraceae family: an update. *Natural Product Reports*, **32**, 367-410.
4. Wu ZY. (**1988**) *A Compendium of new China Herbal Medicine*; Shanghai Science and Technology Press: Shanghai; Vol. 1.
5. Li RT, Li SH, Zhao QS, Lin ZW, Sun HD, Lu Y, Wang C, Zheng QT. (**2003**) Lancifodilactone A, a novel bisnortriterpenoid from *Schisandra lancifolia*. *Tetrahedron letters*, **44**, 3531-3534.
6. Xiao WL, Li RT, Li SH, Li XL, Sun HD, Zheng YT, Wang RR, Lu Y, Wang C, Zheng QT. (**2005**) Lancifodilactone F: a novel nortriterpenoid possessing a unique skeleton from *Schisandra lancifolia* and its anti-HIV Activity. *Organic Letters*, **7**, 1263-1266.
7. Xiao WL, Zhu HJ, Shen YH, Li RT, Li SH, Sun HD, Zheng YT, Wang RR, Lu Y, Wang C, Zheng QT. (**2005**) Lancifodilactone G: a unique nortriterpenoid isolated from *Schisandra lancifolia* and its anti-HIV activity. *Organic Letters*, **7**, 2145-2148.
8. Luo X, Chang Y, Zhang XJ, Pu JX, Gao XM, Wu YL, Wang RR, Xiao WL, Zheng YT, Lu Y, Chen GQ, Zheng QT, Sun HD. (**2009**) Schilancidilactones A and B: two novel tetranortriterpenoids with an unprecedented skeleton from *Schisandra lancifolia*. *Tetrahedron Letters*, **50**, 5962-5964.
9. Luo X, Shi YM, Luo RH, Luo SH, Li XN, Wang RR, Li SH, Zheng YT, Du X, Xiao WL, Pu JX, Sun HD. (**2012**) Schilancitrilactones A-C: three unique nortriterpenoids from *Schisandra lancifolia*. *Organic Letters*, **14**, 1286-1289.
10. Shi YM, Wang XB, Li XN, Luo X, Shen ZY, Wang YP, Xiao WL, Sun HD. (**2013**) Lancolides, antiplatelet aggregation nortriterpenoids with tricyclo[6.3.0.0(2,11)]undecane-bridged system from *Schisandra lancifolia*. *Organic Letters*, **15**, 5068-5071.
11. Shi YM, Yang J, Xu L, Li XN, Shang SZ, Cao P, Xiao WL, Sun HD. (**2014**) Structural characterization and antioxidative activity of lancifonins: unique nortriterpenoids from *Schisandra lancifolia*. *Organic Letters*, **16**, 1370-1373.
12. Shi YM, Wang LY, Zou XS, Li XN, Shang SZ, Gao ZH, Liang CQ, Luo HR, Li HL, Xiao WL, Sun HD. (**2014**) Nortriterpenoids from *Schisandra chinensis* and their absolute configurational assignments by electronic circular dichroism study. *Tetrahedron*, **70**, 859-868.

Terpenoids and Steroids from *Euphorbia hypericifolia*

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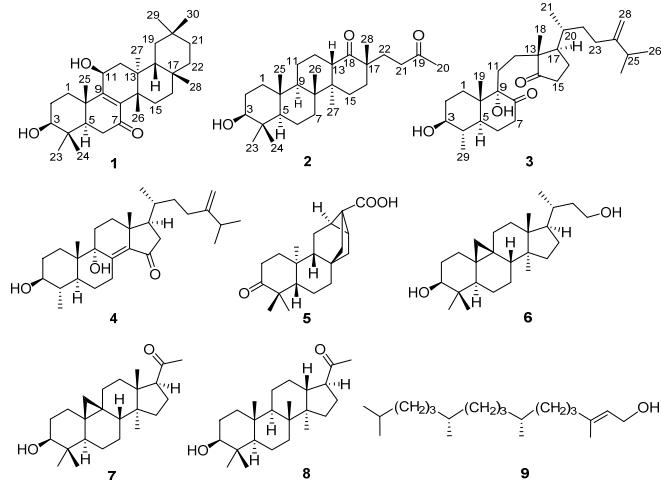
Two new triterpenoids and two new sterols, named euphyperins A–D (**1**–**4**), including an oleanane-type triterpenoid (**1**), a lupane-type nortriterpenoid (**2**), and two cholestane-type steroids (**3** and **4**), along with five known compounds (**5**–**9**) were isolated from the twigs and leaves of *Euphorbia hypericifolia*. Euphyperin B (**2**) represents a rare lupane-type nortriterpenoid, and euphyperin C (**3**) is a novel 8,14-secocholestane-type steroid. Euphyperin A (**1**) exhibited moderate PTP1B inhibitory activity with an $IC_{50} = 17.05 \pm 1.12$ μg/mL.

Keywords: *Euphorbia hypericifolia*, Triterpenoid, Steroid, PTP1B inhibitor.
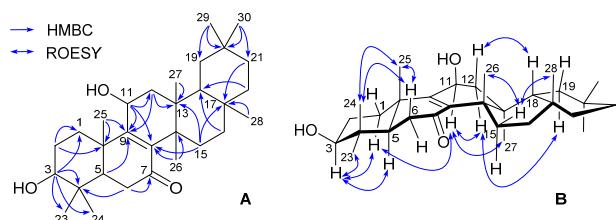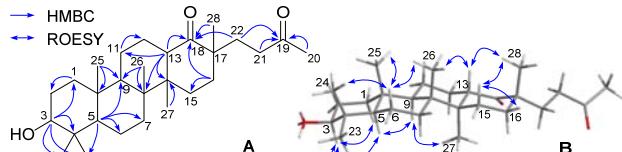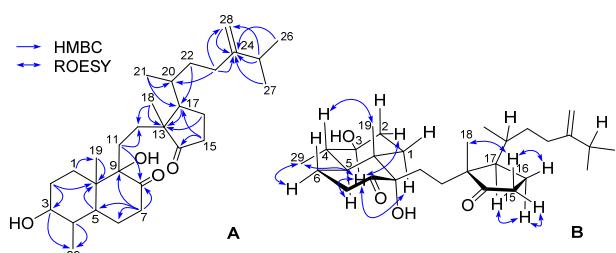
The genus *Euphorbia*, with more than 2000 species, is one of the largest genera of angiosperms [1]. Diterpenoids in this genus are the focus of natural product research, because of their wide range of potentially valuable bioactivities [2]. Our great interest in structural and biological diversities of this genus resulted in some exciting research findings, such as the first secolathyrane diterpenoid with an unprecedented skeleton [3], ingol-type diterpenoids with 11β-HSD1 inhibitory activities [4], and abietane-type diterpenoids with anti-HIV activities [5]. *E. hypericifolia* Linn. (Euphorbiaceae) has long been used as a traditional herbal medicine in China for promoting lactation [6]. Previous chemical studies on this plant only afforded some flavonoids and ellagic acid [7]. In the continuing search for structurally diverse and bioactive compounds from the genus *Euphorbia*, four new compounds, named euphyperins A–D, including one oleanane-type triterpenoid, one degraded lupane-type triterpenoid, and two sterols, along with five known compounds were isolated from the twigs of *E. hypericifolia*. Among them, euphyperin B (**2**) represents a rare class of lupane-type nortriterpenoid [8], and euphyperin C (**3**) possesses a rare 8,14-secocholestane-type steroid skeleton, which has only been found previously in a marine sponge [9]. Euphyperin A (**1**) exhibited PTP1B inhibitory activity with an $IC_{50} = 17.05$ μg/mL. Herein we describe the isolation, structure characterization, and biological activities of these compounds.

Euphyperin A (**1**) was isolated as a white, amorphous powder, and its molecular formula was determined as $C_{30}H_{48}O_3$ (seven indices of hydrogen deficiency) based on an HR-EIMS ion at *m/z* 456.3606 [M+] (calcd 456.3603), as well as comprehensive analysis of its ^{1}H and ^{13}C NMR spectra. The UV absorption band at 253 nm exhibited a conjugated unsaturated carbonyl system, consistent with the IR absorption (Supporting Information Figure S10) at 1649 cm^{-1}. The ^{13}C and DEPT spectra (Table 1) displayed 30 signals of carbons including eight methyls, nine methylenes, four methines (two oxygenated at δ_C 67.3 and 78.5) and nine quaternary. Three overlapped signals at δ_C 30.9 (C-17) and 39.9 (C-10 and C-14) were distinguished by HMBC correlations. The signals at δ_C 144.7, 158.8, and 201.1 confirmed the existence of the aforementioned enone system, which accounted for two indices of hydrogen deficiency, and the remaining five thus required **1** being pentacyclic. The above data with the aid of analysis of its 2D NMR spectra suggested that it was an oleanane-type triterpenoid bearing

an α,β-enone system and two hydroxyls. The attachments of these two hydroxyls were at C-3 and C-11, as determined by the chemical shifts of the relevant protons and carbons as well as the HMBC correlations (Figure 1A) from H-3 to C-1, C-4, C-23, and C-24, and from H-11 to C-8, C-9, and C-12. The tetrasubstituted Δ^8 double bond in the α,β-enone system was located by the multiple correlations from H-11, H_2-15, and H_3-26 to C-8 at δ_C 144.7, and from H-5, H-11, H_2-12, and H_3-25 to C-9 at δ_C 158.8, respectively. The location of the carbonyl group was assigned at C-7 as deduced from the HMBC correlations from H_2-6 to C-7 at δ_C 201.1. The above deduction unambiguously suggested that **1** was an analogue of 7-oxoisomultiflorenol [10], and the only difference was the presence of a hydroxy group at C-11 (δ_C 67.3).

The relative configuration of **1** was almost identical to that of 7-oxoisomultiflorenol by comparing their ^{1}H and ^{13}C NMR spectra, as well as analysis of the ROESY spectrum (Figure 1B) of **1**, in which the cross-peaks of H-1α/H-3, H-3/H-5, and H-3/H_3-23 revealed that they were cofacial and assigned to be α-oriented randomly. In consequence, the ROESY cross-peaks of H-11/H-1α, H-11/H-12α, and H-11/H_3-27 suggested H-11 was also α-oriented. Thus, the structure of **1** was assigned.

Figure 1: Selected HMBC (**A**) and ROESY (**B**) correlations of **1**.

Figure 2: Selected HMBC (**A**) and ROESY (**B**) correlations of **2**.

Figure 3: Selected HMBC (**A**) and ROESY (**B**) correlations of **3**.

Table 1: ^{1}H and ^{13}C NMR data of compounds **1** and **2** in CDCl$_3$.

No.	δ_H (mult., J in Hz)[a]	δ_C[b]	δ_H (mult., J in Hz)[a]	δ_C[b]
	1		**2**	
1	α 1.58, m	33.8	α 0.95 td (12.4, 4.3)	39.0
	β 2.46, dd (13.2, 3.2)		β 1.73 m	
2	1.75, m (2H)	27.5	1.62 m (2H)	27.5
3	3.32, m	78.5	3.20 dd (11.3, 4.9)	78.9
4		39.1		39.0
5	1.64, dd (13.2, 5.9)	48.7	0.69 dd (11.4, 2.1)	55.6
6	α 2.41, dd (19.1, 5.9)	36.9	α 1.37 m	18.4
	β 2.45, dd (19.1, 13.2)		β 1.55 m	
7		201.1	1.31 m	34.2
			1.48 m	
8		144.7		41.1
9		158.8	1.21 m	51.0
10		39.9		37.4
11	4.60, q (8.3)	67.3	1.20 m	20.1
			1.56 m	
12	β 1.39, m	42.5	1.30 m	22.3
	α 2.15, dd (12.7, 8.0)		1.64 m	
13		39.7	2.70 dd (11.8, 3.6)	48.1
14		39.9		46.6
15	α 1.41, m	29.5	α 1.30 m	27.0
	β 2.29, dt (15.9, 9.0)		β 2.04 td (13.4, 4.5)	
16	1.35, m	35.6	β 1.52 m	34.2
	1.52, m		α 1.69 m	
17		30.9		46.4
18	1.59, m	42.1		217.9
19	α 1.19, dd (13.9, 5.6)	35.5		209.5
	β 1.35, m			
20		28.6	2.15 s	30.0
21	1.27, m	33.0	2.47 m (2H)	39.1
	1.45, m			
22	0.91, m	38.4	1.67 m (2H)	32.3
	1.46, m			
23	0.99, s	27.7	0.96 s	28.1
24	0.91, s	15.3	0.76 s	15.5
25	1.21, s	19.7	0.86 s	16.8
26	1.51, s	27.4	1.11 s	16.1
27	0.96, s	19.1	0.76 s	16.2
28	1.14, s	30.9	1.14 s	24.4
29	0.96, s	34.3		
30	0.98, s	32.0		

[a,b] Data were measured at 400 and 125 MHz, respectively.

Euphyperin B (**2**), a white, amorphous powder, gave a molecular formula C$_{28}$H$_{46}$O$_3$ as determined by the ^{13}C NMR data and an HR-ESI(+)MS ion at *m/z* 453.3351 [M + Na]$^+$ (calcd 453.3345), requiring six indices of hydrogen deficiency. The ^{1}H NMR spectrum (Table 1) of **2** showed signals of six singlet methyl groups (δ_H 0.76, 0.76, 0.86, 0.96, 1.11, and 1.14), one acetyl group (δ_H 2.15, s, H$_3$-20) and one oxygenated methine (δ_H 3.20, dd, J = 11.3, 4.9 Hz, H-3). The ^{13}C NMR and DEPT spectra (Table 1) displayed 28 carbon signals for seven methyls, ten methenes, four methines and seven quaternarys, including two carbonyls (δ_C 209.5 and 217.9). The two keto groups accounted for two indices of hydrogen deficiency, and the remaining four thus indicated **2** being tetracyclic. The aforementioned analysis obviously suggested that the structure of **2** resembled that of 29,30-dinor-3β-acetoxy-18,19-dioxo-18,19-secolupane [8], and the only difference was the presence of a 3-OH in **2** replacing the 3-OAc in the latter as judged by the shielded H-3 ($\Delta\delta_H$ 1.25). This conclusion was confirmed by the HMBC correlations (Figure 2A) from H-3 to C-1, C-4, C-23, and C-24. The locations of the two keto groups at C-18 and C-19 were confirmed by the HMBC correlations from H-13, H$_2$-16, H$_2$-22, and H$_3$-28 to C-18 (δ_C 217.9), and from H$_3$-20, H$_2$-21, and H$_2$-22 to C-19 (δ_C 209.5).

The relative configuration of **2** was assigned in the same way as that of 29,30-dinor-3β-acetoxy-18,19-dioxo-18,19-secolupane, mainly by comparing their NMR data, which were corroborated by the ROESY spectrum (Figure 2B), in which the α-axial orientation of H-3 was determined by the cross-peaks of H-3/H-1α, H-5, and H$_3$-23. The correlations for H-6β/H$_3$-24, H-6β/H$_3$-25, H-6β/H$_3$-26, H-13/H$_3$-26, H-13/H$_3$-28, H-15β/H-16β, and H-15β/H$_3$-28 revealed that they were all β-oriented. The structure of **2** was therefore determined.

Euphyperin C (**3**) gave a molecular formula C$_{29}$H$_{48}$O$_4$ with six indices of hydrogen deficiency based on the ^{13}C NMR data and an HR-ESI(+)MS ion at *m/z* 483.3436 [M + Na]$^+$ (calcd 483.3450).

Interpretation of its ^{1}H NMR spectrum (Table 2) showed the presence of two methyl singlets (δ_H 0.74 and 0.86, each 3H), four methyl doublets (δ_H 1.00, 3H, d, J = 6.6 Hz; and three overlapped δ_H 1.03, each 3H, d, J = 6.7 Hz), one oxygenated methine (δ_H 3.16, 1H, td, J = 10.4, 5.1 Hz), and one terminal methine group (δ_H 4.68 and 4.74, each 1H, s). The ^{13}C NMR data (Table 2), with the aid of the HMBC spectrum (Figure 3A), displayed signals of 29 carbons, suggesting the existence of several characteristic functional groups including two keto groups (δ_C 215.4 and 224.5), one oxygenated methine (δ_C 75.9), one oxygenated quaternary carbon (δ_C 83.5), and one terminal methene group (δ_C 106.5 and 156.5). The above data indicated that **3** likely possessed a steroidal structure which was highly similar to that of (24R)-3β-hydroxy-24-methyl-4-methylene-5α-8,14-secocholestane-8,14-dione [9], except for the presence of a $\Delta^{24(28)}$ double bond and in the concomitant absence of a $\Delta^{4(29)}$ double bond. This deduction was further verified by the HMBC spectrum, in which the $\Delta^{24(28)}$ double bond was verified by the key correlations from H$_2$-23, H-25, H$_3$-26, H$_3$-27, and H$_2$-28 to C-24 at δ_C 156.5, and from H$_2$-23 and H-25 to C-28 at δ_C 106.5. In addition, CH$_3$-29 was located by the correlations from H-3 and H-4 to C-29 at δ_C 15.6.

The relative configuration of **3** was assigned by comparison of NMR data with those of (24R)-3β-hydroxy-24-methyl-4-methylene-5α-8,14-secocholestane-8,14-dione [9] and the ROESY experiment (Figure 3B). In particular, H-3 and CH$_3$-29 were assigned to be α-oriented on the basis of the ROESY cross-peaks of H-1/H-3, H-2α/H-3, H-3/H-5, H-3/H$_3$-29, and H-6α/H$_3$-29. The structure of **3** possessing a rare 8,14-secosteroid skeleton was thus elucidated as depicted.

Table 2: ^{1}H and ^{13}C NMR data of compounds **3** and **4** in $CDCl_3$.

No.	**3** δ_H (mult., *J* in Hz) [a]	δ_C [c]	**4** δ_H (mult., *J* in Hz) [b]	δ_C [c]
1	α1.27 m	29.6	α 1.47 m	29.7
	β 1.58 m		β 1.75 m	
2	β 1.51 m	30.4	β 1.47 m	30.7
	α 1.89 m		α 1.91 m	
3	3.16 td (10.4, 5.1)	75.9	3.16 m	75.9
4	1.35 m	40.2	1.28 m	40.0
5	1.51 m	44.1	1.69 m	41.8
6	β 1.39 m	26.4	β 1.12 m	25.0
	α 2.08 m		α 1.81 m	
7	2.48 m	36.8	α 1.96 m	22.8
	2.52 m		β 3.96 m	
8		215.4		148.5
9		83.5		74.7
10		45.7		42.2
11	1.47 m	27.5	α 1.62 m	28.0
	1.71 m		β 1.96 m	
12	1.14 td (13.3, 3.4)	30.2	α 1.51 m	33.9
	1.51 m		β 2.02 m	
13		52.1		43.3
14		224.5		141.3
15	α 2.08 m	37.5		208.5
	β 2.40 dd (18.7, 8.7)			
16	β 1.51 m	23.8	β 2.07 m	42.7
	α 2.16 m		α 2.43 dd (18.9, 7.5)	
17	1.85 m	46.3	1.57 m	50.4
18	0.86 s	18.7	0.97 s	17.4
19	0.74 s	14.2	0.84 s	16.9
20	1.55 m	34.2	1.57 m	34.5
21	1.00 d (6.6)	18.3	1.04 d (6.6)	19.3
22	1.26 m	33.3	1.23 m	34.3
	1.65 m		1.64 m	
23	1.94 m	31.4	1.91 m	30.6
	2.17 m		2.09 m	
24		156.5		156.3
25	2.23 m	33.9	2.21 m	33.9
26	1.03 d (6.7)	22.0	1.02 d (6.7)	22.0
27	1.03 d (6.7)	22.1	1.02 d (6.7)	22.1
28	4.68 s	106.5	4.65 s	106.6
	4.74 s		4.73 s	
29	1.03 d (6.7)	15.6	1.02 d (6.7)	15.5

[a–c] Data were measured at 400, 500, and 125 MHz, respectively.

Euphyperin D (**4**) was determined to possess a molecular formula $C_{29}H_{46}O_3$, as deduced from the ^{13}C NMR data and an HREIMS ion at *m/z* 442.3440 [M][+] (calcd 442.3447). The ^{13}C NMR spectrum (Table 2) showed the presence of two double bonds (δ_C 106.6, 141.3, 148.5, and 156.3) and two oxygenated carbons (δ_C 74.7 and 75.9), but no carbonyl groups. Further analysis of its ^{1}H and 2D NMR spectra indicated that **4** was a C29 steroid, structurally similar to **3** and 9α-hydroxy-15-oxoconicasterol [11] and shared the same A ring and side chain from C-20 to C-28 with **3** and the same B, C, and D rings with 9α-hydroxy-15-oxoconicasterol based on the similar NMR patterns of the involved protons and carbons, which were reaffirmed by the HMBC spectrum (Supporting Information Figure S30). The relative stereochemistry of **4** was established to be the same as that of **3** by NMR comparison and the biosynthetic reasoning that **4** undergoes reduction of the carbonyl at C-15 and subsequently oxidative cleavage of the $\Delta^{8(14)}$ double bond to form compound **3**. Accordingly, the structure of **4** was unequivocally characterized.

Five known compounds, 3-oxo-*ent*-trachyloban-17-oic acid (**5**) [12], 9,19-cyclo-24-nor-5α,9β-cholane-3β,23-diol (**6**) [13], 9β,19-cyclo-4,4,14α-trimethyl-3β-hydroxy-5α-pregnan-20-one (**7**) [14], 3β-hydroxy-22,23,24,25,26,27-hexanordammarane-20-one (**8**) [15], and phytol (**9**) [16] were also isolated. Their structures were identified on the basis of the NMR and ESIMS data.

Protein tyrosine phosphatase 1B (PTP1B) plays a major role in metabolic signaling pathways, making it ideal as a therapeutic drug target for type 2 diabetes and obesity. In addition, triterpenoids as PTP1B inhibitors have recently been reported as potential agents for

the chemoprevention and therapy of breast cancer [17]. Euphyperins A (**1**) and B (**2**) were tested *in vitro* for their inhibitory effect on PTP1B activity [18], using oleanolic acid (purity > 97%; Sigma-Aldrich Co., St Louis, MO, USA) as the positive control (IC_{50} value: 1.05 μg/mL). Euphyperin A (**1**) displayed moderate PTP1B inhibitory activity with an IC_{50} value of 17.05 ± 1.12 μg/mL. Euphyperins A (**1**) and B (**2**) were also evaluated for their *in vitro* inhibition against XBP1 mRNA splicing, which is an important therapeutic target in cancer treatment, but neither was active.

Experimental

General experimental procedures: Optical rotations, Perkin-Elmer 341 polarimeter; UV, Shimadzu UV-2550 spectrophotometer; IR, Perkin Elmer 577 IR spectrometer; NMR, Bruker AM-400 or AM-500 NMR spectrometers; ESIMS and HR-ESIMS were performed on a Bruker Daltonics Esquire 3000 plus and a Waters-Micromass Q-TOF Ultima Global mass spectrometer, respectively. EIMS and HR-EIMS were obtained on a Finnigan MAT-95 mass spectrometer. Semi-preparative HPLC was carried out on a Waters 1525 binary pump system with a Waters 2489 UV detector and a YMC-Pack ODS-A column (250 × 10 mm, S-5 μm). Silica gel (300 × 400 mesh, Qingdao Marine Chemical Co. Ltd), C_{18} reversed-phase silica gel (150 × 200 mesh, Merck), CHP20P MCI gel (75−150 μm, Mitsubishi Chemical Industries, Ltd.), D101-macroporous absorption resin (Sinopharm Chemical Reagent Co. Ltd, Shanghai, People's Republic of China), and Sephadex LH-20 gel (Amersham Biosciences) were used for column chromatography (CC), and precoated silica gel GF_{254} plates (Qingdao Marine Chemical Co. Ltd) for TLC. All solvents used for CC were of analytical grade (Shanghai Chemical Reagents Co. Ltd), and all solvents used for HPLC were of HPLC grade (J&K Scientific Ltd.).

Plant material: The twigs and leaves of *Euphorbia hypericifolia* were collected in September 2011 from Guangxi Province, People's Republic of China, and were authenticated by Professor Shao-Qing Tang of Guangxi Normal University. A voucher specimen (accession number: EuH-2011-1Y) has been deposited in Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Extraction and isolation: Air-dried powder of *E. hypericifolia* (5 kg) was extracted with 95% EtOH, 3 times, and the resulting residue was suspended in H_2O and partitioned between H_2O and EtOAc to give a crude extract (630 g). This was subjected to CC (D101-macroporous absorption resin, eluted with 30%, 80%, and 90% EtOH in H_2O) to obtain the major part (240 g) as monitored by TLC. This part was subjected to an MCI gel column (MeOH/H_2O, 50 to 100%) to give 5 major fractions, A−E. Fraction D was subjected to silica gel CC eluted with light petroleum/acetone (20:1 to 1:1, v/v) to afford 8 fractions (D1−D8). Fraction D4 (10:1 to 8:1) was separated over a column of C_{18} reversed-phase silica gel (MeOH/H_2O, 60% to 85%) to give 5 sub-fractions (D4a−D4e). Fraction D4e was purified over a silica gel column eluted with light petroleum/ethyl acetate (5:1 to 3:1) to give 2 fractions D4e1 and D4e2, each of which was further purified on a column of Sephadex LH-20 eluted with ethanol to produce compounds **2** (10 mg) and **3** (3 mg). In similar procedures, fraction D4b yielded compound **5** (3 mg). Of fraction D5 (8:1 to 5:1), sub-fraction D5c2 was purified over a column of Sephadex LH-20 to yield compound **4** (4 mg), and sub-fraction D5c3 by semi-preparative HPLC with a mobile phase of 80% CH_3CN in H_2O to yield compound **1** (5 mg). Fraction D2 (16:1 to 12:1) was chromatographed on a column of C_{18} reversed-phase silica gel (MeOH/H_2O, 65% to 85%) to give 3 major fractions, each of which was separated by semi-preparative HPLC

(75% CH$_3$CN in H$_2$O) to produce compounds **6** (12 mg), **7** (5 mg), and **8** (4 mg). Fraction E was purified over a column of silica gel (light petroleum/ethyl acetate, 100:1 to 20:1) to afford the major compound **9** (4.2 g).

Euphyperin A (1)
White amorphous powder.
[α]$^{27}_D$: +1.5 (*c* 0.14, MeOH).
IR (KBr) v_{max}: 3423, 2952, 2925, 2866, 1649, 1458, 1381, 1308, 1269, 1038 cm^{-1}.
UV (MeOH) λ_{max} (log ε): 253 (3.74) nm.
^{1}H and ^{13}C NMR: Table 1.
ESI(+)MS *m/z*: 457.5 [M + H]$^+$, 936.1 [2M + Na]$^+$; ESI(−)MS *m/z* 501.8 [M + HCO$_2$]$^-$; EIMS *m/z*: 456 [M]$^+$ (52), 438 (28), 423 (44), 302 (30), 387 (37), 252 (93), 191 (100), 95 (30).
HR-EIMS *m/z*: 456.3606 [M$^+$] (calcd for C$_{30}$H$_{48}$O$_3$, 456.3603).

Euphyperin B (2)
White amorphous powder.
[α]$^{27}_D$: +24.9 (*c* 0.33, MeOH).
IR (KBr) v_{max}: 3500, 2937, 2864, 1701, 1448, 1381, 1362, 1286, 1047, 1003 cm^{-1}.
UV (MeOH) λ_{max} (log ε): 208 (4.10) nm.
^{1}H and ^{13}C NMR: Table 1.
ESI(+)MS *m/z*: 431.2 [M + H]$^+$, 883.5 [2 M + Na]$^+$.
HR-ESI(+)MS *m/z*: 453.3351 [M + Na]$^+$ (calcd for C$_{28}$H$_{46}$O$_3$Na, 453.3345).

Euphyperin C (3)
White amorphous powder.
[α]$^{27}_D$: −6.8 (*c* 0.18, MeOH).

IR (KBr) v_{max}: 3479, 3359, 2960, 2964, 2870, 1734, 1697, 1645, 1466, 1454, 1381, 1261, 1061, 1024, 802 cm^{-1}.
UV (MeOH) λ_{max} (log ε): 207 (4.11) nm.
^{1}H and ^{13}C NMR: Table 2.
ESI(+)MS *m/z*: 483.2 [M + Na]$^+$.
HR-ESI(+)MS *m/z*: 483.3436 [M + Na]$^+$ (calcd for C$_{29}$H$_{48}$O$_4$Na, 483.3450).

Euphyperin D (4)
White amorphous powder.
[α]$^{25}_D$: +74.6 (*c* 0.18, MeOH).
IR (KBr) v_{max}: 3462, 2924, 2852, 1689, 1630, 1468, 1092 cm^{-1}.
UV (MeOH) λ_{max} (log ε): 255 (3.86) nm.
^{1}H and ^{13}C NMR: Table 2.
ESI(+)MS *m/z*: 443.4 [M + H]$^+$, 907.8 [2M + Na]$^+$; EIMS *m/z* 442 [M]$^+$ (48), 424 (97), 406 (18), 391 (28), 302 (23), 272 (29), 177 (70), 123 (100).
HR-EIMS *m/z*: 442.3440 [M$^+$] (calcd for C$_{29}$H$_{46}$O$_3$, 442.3447).

Bioassays: The PTP1B inhibitory activity was measured according to the reported protocol [18]. XBP1 mRNA splicing inhibitory activity was also determined using a reported protocol [19].

Supporting information: IR, ESIMS/EIMS, HR-ESIMS/HR-EIMS, 1D and 2D NMR spectra of compounds **1**−**4** are provided.

References

[1]   Ma JS. (**1997**) In *Zhongguo Zhiwu Zhi*. Science Press, Beijing, China, **44**, p. 26.
[2]   Vasas A, Hohmann J. (**2014**) *Euphorbia* diterpenes: Isolation, structure, biological activity, and synthesis (2008–2012). *Chemical Reviews*, **114**, 8579–8612.
[3]   Liao SG, Zhan ZJ, Yang SP, Yue JM. (**2005**) Lathyranoic acid A: First secolathyrane diterpenoid in nature from *Euphorbia lathyris*. *Organic Letters*, **7**, 1379–1382.
[4]   Qi WY, Zhang WY, Shen Y, Leng Y, Gao K, Yue JM. (**2014**) Ingol-type diterpenes from *Euphorbia antiquorum* with mouse 11β-hydroxysteroid dehydrogenase type 1 inhibition activity. *Journal of Natural Products*, **77**, 1452–1458.
[5]   Zhao JX, Liu CP, Qi WY, Han ML, Han YS, Wainberg MA, Yue JM. (**2014**) Eurifoloids A–R, structurally diverse diterpenoids from *Euphorbia neriifolia*. *Journal of Natural Products*, **77**, 2224–2233.
[6]   Ma JS. (**1997**) In *Zhongguo Zhiwu Zhi*. Science Press, Beijing, China, **44**, p. 41.
[7]   Rizk AM, Rimpler H, Ismail SI. (**1977**) Flavonoids and ellagic acid from *Euphorbia hypericifolia* L. (= *E. indica* Lam.). *Fitoterapia*, **48**, 99–100.
[8]   Chiang YM, Kuo YH. (**2002**) Novel triterpenoids from the aerial roots of *Ficus microcarpa*. *The Journal of Organic Chemistry*, **67**, 7656–7661.
[9]   Umeyama A, Shoji N, Enoki M, Arihara S. (**1997**) Swinhosterols A–C, 4-methylene secosteroids from the marine sponge *Theonella swinhoei*. *Journal of Natural Products*, **60**, 296–298.
[10]   Akihisa T, Yasukawa K, Kimura Y, Takido M, Kokke WCMC, Tamura T. (**1994**) Five D:C-friedo-oleanane triterpenes from the seeds of *Trichosanthes kirilowii* Maxim. and their anti-inflammatory effects. *Chemical & Pharmaceutical Bulletin*, **42**, 1101–1105.
[11]   Zhang HJ, Yi YH, Lin HW. (**2010**) Oxygenated 4-methylidene sterols from the South China sea sponge *Theonella swinhoei*. *Helvetica Chimica Acta*, **93**, 1120–1126.
[12]   Pan L, Zhou P, Zhang X, Peng S, Ding L, Qiu SX. (**2006**) Skeleton-rearranged pentacyclic diterpenoids possessing a cyclobutane ring from *Euphorbia wallichii*. *Organic Letters*, **8**, 2775–2778.
[13]   Corsano S, Piancatelli G. (**1967**) Correlation of acetylacteol with cycloartenol. *Ricerca Scientifica*, **37**, 360–365.
[14]   Rodriguez-Hahn H, Romo de Vivar A, Ortega A, Aguilar M, Romo J. (**1970**) Structure of argentines A, B, and C from *Parthenium argentatum*. *Revista Latinoamericana de Quimica*, **1**, 24–38.
[15]   Crabbe P, Ourisson G, Takahashi T. (**1958**) Preparation of 8,14-dimethyl-18-nortestosterone. *Tetrahedron*, **3**, 279–302.
[16]   Gellerman JL, Anderson WH, Schlenk H. (**1975**) Synthesis and analysis of phytyl and phytenoyl wax esters. *Lipids*, **10**, 656–661.
[17]   Uddin MN, Sharma G, Yang JL, Choi HS, Lim SI, Kang KW, Oh WK. (**2014**) Oleanane triterpenes as protein tyrosine phosphatase 1B (PTP1B) inhibitors from *Camellia japonica*. *Phytochemistry*, **103**, 99–106.
[18]   Zhang W, Hong D, Zhou Y, Zhang Y, Shen Q, Li J, Hu L, Li J. (**2006**) Ursolic acid and its derivative inhibit protein tyrosine phosphatase 1B, enhancing insulin receptor phosphorylation and stimulating glucose uptake. *Biochimica et Biophysica Acta-General Subjects*, **1760**, 1505–1512.
[19]   Zhang H, Zhang CR, Zhu KK, Gao AH, Luo C, Li J, Yue JM. (**2013**) Fluevirosines A–C: A biogenesis inspired example in the discovery of new bioactive scaffolds from *Flueggea virosa*. *Organic Letters*, **15**, 120–123.

A Fragmentation Study of Six C_{21} Steroidal Aglycones by Electrospray Ionization Ion-Trap Time-of-Flight Tandem Mass Spectrometry

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The fragmentation patterns of six C_{21} steroidal aglycones, metaplexigenin (**1**), caudatin (**2**), qingyangshengenin (**3**), penupogenin (**4**), 20-cinnamoylsarcostin (**5**), and gagamine (**6**), were analyzed by high-resolution electrospray ionization ion-trap time-of-flight tandem mass spectrometry (HR-ESI-IT-TOF-MS^n). The $[M-H]^+$ ions of steroids **1-3** that contain a carbonyl functional group at C-20 (Type I) and $[M+H]^+$ ions of steroids **5-6** that possess a hydroxyl group at C-20 (Type II) were readily observed in MS analyses. The fragmentation pathways and diagnostic fragment ions for these six steroidal aglycones were proposed on the basis of their MS^n analyses. The common fragmentation pathways for type I steroidal aglycones include the neutral loss of the ester group at C-12 and the hydroxyl moieties on the steroid skeleton, as well as the cleavage of ring D. Their diagnostic fragment ions were identified as *m/z* 361(**B**), 343 (**C**), 325 (**D**), 307 (**F**), 283 (**G**), 259 (**E**), and 243 (**H**). The fragmentation behavior of penupogenin (**4**) in type II was similar to those of type I, with *m/z* 363 (**B'**), 345 (**C'**), 327 (**D'**), 309 (**F'**), 283 (**G**), and 243 (**H**) as its diagnostic fragment ions. The ester group at C-20 was difficult to cleave in the MS^n analyses of 20-cinnamoylsarcostin (**5**) and gagamine (**6**) so that the loss of this ester group was slower than that at C-12 and hydroxyl groups; the key ions at *m/z* 329 (**I**), 311 (**J**), 293 (**K**), and 275 (**L**) were characteristic for **5** and **6**. The base ion peaks were derived from the loss of the substituent group at either C-12 or C-17 for both type I and type II steroidal aglycones.

Keywords: ESI-IT-TOF-MS^n, Fragmentation pathways, Diagnostic ions, C_{21} Steroidal aglycones.

C_{21} steroids, the major chemical components of the family Asclepiadaceae, have been proved to possess prolific bioactivities, such as antitumor [1], immunosuppressive [2], appetite-suppressant [3], and anti-hepatitis B virus [4,5]. Attention has been paid to the rapid detection and capture of C_{21} steroids for bioactivity screening and new drug development. Nevertheless, the isolation and identification of C_{21} steroids from natural materials are difficult due to their thermal instability and low abundance in natural materials. It is thus imperative to establish highly sensitive and accurate methods to analyze C_{21} steroids.

Recently, electrospray ionization (ESI) techniques linked with multi-stage tandem mass spectrometry (MS^n) have been extensively applied to analyze natural products including alkaloids [6], steroidal saponins [7], and triterpenoid saponins [8]. ESI-MS^n techniques make it possible to determine the relationship between a precursor and its fragment ions, by which the fragmentation patterns can be sorted out and natural products in plants may be precisely analyzed [9,10].

Since most naturally-occurring C_{21} steroids exist as glycosides, the MS^n techniques have mainly been used to test the presence of C_{21} steroidal glycosides in plants and to establish online methods to analyze the glycoside structures [11-16]. However, no systematic study of the ESI-MS^n fragmentation of C_{21} steroidal aglycones has been reported so far. Additionally, some limitations may arise from the low resolution of mass spectrometers utilized in previous molecular formula predictions [7, 13-16]. In this study, the fragmentation of six C_{21} steroidal aglycones was explored using a LC/MS-IT-TOF mass spectrometer equipped with an electrospray ionization source and ion-trap and time-of-flight mass analyzers (ESI-IT-TOF). This mass spectrometer enables fast acquisition of multistage tandem spectra (MS$^{1-10}$) with high accuracy and
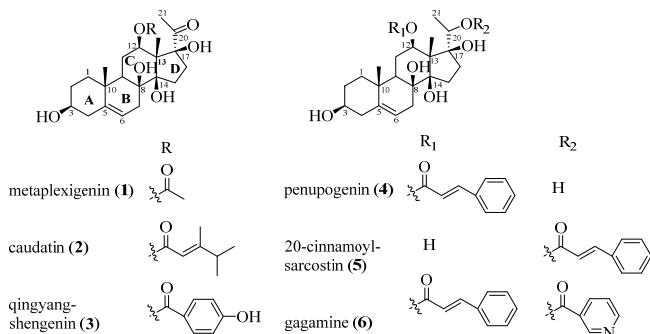
Figure 1: Structures of compounds **1-6**

resolution. The six steroidal aglycones, metaplexigenin (**1**), caudatin (**2**), qingyangshengenin (**3**), penupogenin (**4**), 20-cinnamoyl-sarcostin (**5**), and gagamine (**6**) (as shown in Figure 1), are classified into type I that contain a carbonyl group at C-20 (steroids **1-3**) and type II that possess a hydroxyl group at C-20 (steroids **4-6**). A high resolution mass technique was first applied to investigate the fragmentation patterns of C_{21} steroidal aglycones that may be used for the characterization of other structure-related steroids.

Prior to the MS^n analyses, the full-scan mass spectra of steroids **1-6** in both positive and negative ion modes were acquired in automatic pattern. The $[M−H]^−$ ions for metaplexigenin (**1**), caudatin (**2**), qingyangshengenin (**3**), and penupogenin (**4**) were easily obtained. In contrast, only $[M+H]^+$ ions for 20-cinnamoylsarcostin (**5**) and gagamine (**6**) were observed. Therefore, the MS^n investigations for steroids **1-4** in negative mode and for steroids **5-6** in positive mode were chosen, as shown in Tables 1-6. The mass spectra of steroids **1-6** are shown as Figures S1-S6 in the supporting information.

Table 1: Data for accurate masses and elemental composition of steroid **1** observed from tandem mass spectra in negative mode
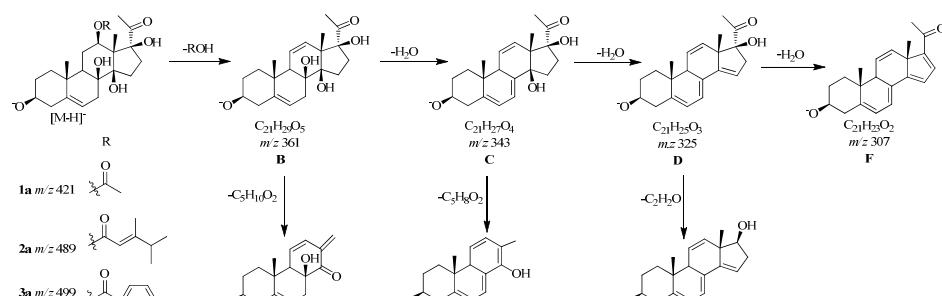
MS^n	Precursor ion (m/z)	Product ion (m/z)	Elemental composition	Measured (m/z)	Calculated (m/z)	Error (mDa)	Intensity (%)	Ion name	Assignment
(−)MS	422	421	$C_{23}H_{33}O_7$	421.2214	421.2232	−1.8	100	**1a**	$[M-H]^-$
MS^2	421	361	$C_{21}H_{29}O_5$	361.2008	361.2020	−1.2	100	**B**	$1a-C_2H_4O_2$
		343	$C_{21}H_{27}O_4$	343.1890	345.1915	−2.5	43	**C**	$B-H_2O$
		325	$C_{21}H_{25}O_3$	325.1775	325.1809	−3.4	32	**D**	$C-H_2O$
MS^3	361	343	$C_{21}H_{27}O_4$	343.1891	345.1915	−2.4	100	**C**	$B-H_2O$
		325	$C_{21}H_{25}O_3$	325.1787	325.1809	−2.2	83	**D**	$C-H_2O$
		259	$C_{16}H_{19}O_3$	259.1323	259.1340	−1.7	21	**E**	$B-C_5H_{10}O_2$
	343	325	$C_{21}H_{25}O_3$	325.1787	325.1809	−2.2	100	**D**	$C-H_2O$
		307	$C_{21}H_{23}O_2$	307.1670	307.1704	−3.4	16	**F**	$D-H_2O$
		283	$C_{19}H_{23}O_2$	283.1676	283.1704	−2.8	23	**G**	$D-C_2H_2O$
		243	$C_{16}H_{19}O_2$	243.1371	243.1391	−2.0	44	**H**	$C-C_5H_8O_2$
	325	307	$C_{21}H_{23}O_2$	307.1672	307.1704	−3.2	45	**F**	$D-H_2O$
		283	$C_{19}H_{23}O_2$	283.1686	283.1704	−1.8	100	**G**	$D-C_2H_2O$
MS^4	307	291	$C_{20}H_{19}O_2$	291.1373	291.1391	−1.8	100	**1k**	$F-CH_4$
		265	$C_{19}H_{21}O$	265.1582	265.1598	−1.6	56	**1l**	$F-C_2H_2O$
	283	265	$C_{19}H_{21}O$	265.1574	265.1598	−2.4	100	**1l**	$G-H_2O$

Table 2: Data for accurate masses and elemental composition of steroid **2** observed from tandem mass spectra in negative mode

MS^n	Precursor ion (m/z)	Product ion (m/z)	Elemental composition	Measured (m/z)	Calculated (m/z)	Error (mDa)	Intensity (%)	Ion name	Assignment
(−)MS	490	489	$C_{28}H_{41}O_7$	489.2853	489.2858	−0.5	100	**2a**	$[M-H]^-$
MS^2	489	361	$C_{21}H_{29}O_5$	361.1993	361.2020	−2.7	100	**B**	$2a-C_7H_{12}O_2$
		343	$C_{21}H_{27}O_4$	343.1878	345.1915	−3.7	32	**C**	$B-H_2O$
		325	$C_{21}H_{25}O_3$	325.1771	325.1809	−3.8	23	**D**	$C-H_2O$
MS^3	361	343	$C_{21}H_{27}O_4$	343.1882	345.1915	−3.3	100	**C**	$B-H_2O$
		325	$C_{21}H_{25}O_3$	325.1776	325.1809	−3.3	93	**D**	$C-H_2O$
		259	$C_{16}H_{19}O_3$	259.1318	259.1340	−2.2	20	**E**	$B-C_5H_{10}O_2$
	343	325	$C_{21}H_{25}O_3$	325.1778	325.1809	−3.1	100	**D**	$C-H_2O$
		307	$C_{21}H_{23}O_2$	307.1664	307.1704	−4.0	12	**F**	$D-H_2O$
		283	$C_{19}H_{23}O_2$	283.1678	283.1704	−2.6	29	**G**	$D-C_2H_2O$
		243	$C_{16}H_{19}O_2$	243.1360	243.1391	−3.1	33	**H**	$C-C_5H_8O_2$
	325	307	$C_{21}H_{23}O_2$	307.1664	307.1704	−4.0	66	**F**	$D-H_2O$
		283	$C_{19}H_{23}O_2$	283.1669	283.1704	−3.5	100	**G**	$D-C_2H_2O$
MS^4	307	291	$C_{20}H_{19}O_2$	291.1415	291.1391	+2.4	100	**2i**	$F-CH_4$
		265	$C_{19}H_{21}O$	265.1580	265.1598	−1.8	56	**2j**	$F-C_2H_2O$
	283	265	$C_{19}H_{21}O$	265.1515	265.1598	−8.3	88	**2j**	$G-H_2O$

Table 3: Data for accurate masses and elemental composition of steroid **3** observed from tandem mass spectra in negative mode

MS^n	Precursor ion (m/z)	Product ion (m/z)	Elemental composition	Measured (m/z)	Calculated (m/z)	Error (mDa)	Intensity (%)	Ion name	Assignment
(−)MS	500	499	$C_{28}H_{35}O_8$	499.2344	499.2337	+0.7	100	**3a**	$[M-H]^-$
MS^2	499	361	$C_{21}H_{29}O_5$	361.2005	361.2020	−1.5	26	**B**	$3a-C_7H_6O_3$
		343	$C_{21}H_{27}O_4$	343.1885	345.1915	−3.0	85	**C**	$B-H_2O$
		325	$C_{21}H_{25}O_3$	325.1781	325.1809	−2.8	100	**D**	$C-H_2O$
MS^3	361	343	$C_{21}H_{27}O_4$	343.1887	345.1915	−2.8	100	**C**	$B-H_2O$
		325	$C_{21}H_{25}O_3$	325.1777	325.1809	−3.2	52	**D**	$C-H_2O$
		259	$C_{16}H_{19}O_3$	259.1324	259.1340	−1.6	61	**E**	$B-C_5H_{10}O_2$
	343	325	$C_{21}H_{25}O_3$	325.1786	325.1809	−2.3	100	**D**	$C-H_2O$
		307	$C_{21}H_{23}O_2$	307.1675	307.1704	−2.9	14	**F**	$D-H_2O$
		283	$C_{19}H_{23}O_2$	283.1683	283.1704	−2.1	40	**G**	$D-C_2H_2O$
		243	$C_{16}H_{19}O_2$	243.1371	243.1391	−2.0	45	**H**	$C-C_5H_8O_2$
	325	307	$C_{21}H_{23}O_2$	307.1675	307.1704	−2.9	100	**F**	$D-H_2O$
		283	$C_{19}H_{23}O_2$	283.1684	283.1704	−2.0	31	**G**	$D-C_2H_2O$
		281	$C_{19}H_{21}O_2$	281.1534	281.1547	−1.3	10	**3i**	$D-C_2H_4O$
MS^4	283	265	$C_{19}H_{21}O$	265.1558	265.1598	−4.0	50	**3j**	$G-H_2O$
	281	265	$C_{18}H_{17}O_2$	265.1233	265.1234	−0.1	100	**3j'**	$3i-CH_4$
	259	231	$C_{15}H_{19}O_2$	231.1363	231.1391	−2.8	100	**3k**	$E-CO$

Figure 2: Proposed common fragmentation pathways for steroids **1-3**.

Common fragmentation pathways for type I steroids: In the single-stage mass spectra, the $[M-H]^-$ ions for type I C_{21} steroidal aglycones **1-3** were observed at m/z 421.2214 (**1a**), 489.2853 (**2a**), and 499.2344 (**3a**), corresponding to the molecular formulas of $C_{23}H_{33}O_7$, $C_{28}H_{41}O_7$ and $C_{28}H_{35}O_8$, respectively. These three $[M-H]^-$ ions were selected as precursor ions to perform MS^2 experiments, from which the common ion at m/z 361 (**B**) was acquired as the base ion peak due to the loss of ROH (R= acetyl, tigloyl and *p*-hydroxylbenzoyl) at C-12. Additionally, fragment ions **C** (m/z 343) and **D** (m/z 325) were also detected, which may be
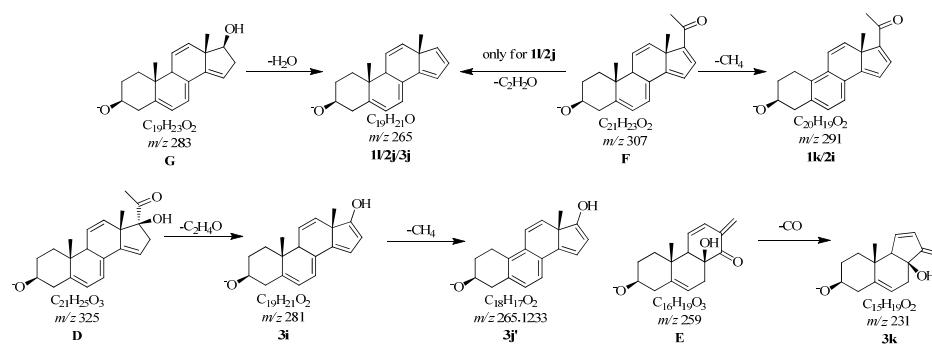
Figure 3: Other fragmentation pathways of compounds **1-3**.

Table 4: Data for accurate masses and elemental composition of steroid **4** observed from tandem mass spectra in negative mode

MS^n	Precursor ion (m/z)	Product ion (m/z)	Elemental composition	Measured (m/z)	Calculated (m/z)	Error (mDa)	Intensity (%)	Ion name	Assignment
(−)MS	512	511	C$_{30}$H$_{39}$O$_7$	511.2663	511.2701	−3.8	100	**4a**	[M−H]$^-$
MS^2	511	363	C$_{21}$H$_{31}$O$_5$	363.2148	363.2177	−2.9	100	**B'**	**4a**−C$_9$H$_8$O$_2$
MS^3	363	345	C$_{21}$H$_{29}$O$_4$	345.1960	345.2071	−11.1	17	**C'**	**B'**−H$_2$O
		327	C$_{21}$H$_{27}$O$_3$	327.1895	327.1966	−7.1	29	**D'**	**C'**−H$_2$O
		309	C$_{21}$H$_{25}$O$_2$	309.1812	309.1860	−4.8	29	**F'**	**D'**−H$_2$O
		299	C$_{19}$H$_{23}$O$_3$	299.1630	299.1653	−2.3	100	**4i**	**B'**−C$_2$H$_6$O$_2$
		283	C$_{19}$H$_{23}$O$_2$	283.1670	283.1704	−3.4	54	**G**	**D'**−C$_2$H$_4$O
		243	C$_{16}$H$_{19}$O$_2$	243.1316	243.1391	−7.5	17	**H**	**C'**−C$_5$H$_{10}$O$_2$
MS^4	299	281	C$_{19}$H$_{21}$O$_2$	281.1519	281.1547	−2.8	100	**4j**	**4i**−H$_2$O

ascribed to the sequential elimination of 8-OH and 14-OH from **B**. To investigate further the fragmentation patterns of ions **B**, **C** and **D**, they were chosen as precursor ions in MS^3 analyses to generate ions **C** to **H**. Ions **B-H** were identified as the key fragment ions for steroids **1-3**, of which ion **E** at m/z 259 was derived from ion **B** through ring D cleavage of the C$_{21}$ skeleton as C$_5$H$_{10}$O$_2$ segment. Ion **F** at m/z 307 was generated from ion **D** via the removal of one molecule of H$_2$O from 17-OH and 16-H. Ion **G** also originated from ion **D** through the elimination of 17-OAC as a C$_2$H$_2$O segment and ion **H** could be generated by the cleavage of ring D as a C$_5$H$_8$O$_2$ segment in precursor ion **C** (Figure 2).

Other fragmentation pathways for steroids 1-3: The other fragmentation pathways of steroids **1-3** were mainly involved in methyl group loss on the C$_{21}$ steroidal skeleton. Ions at m/z 291 (**1k/2i**) and m/z 265 (**1l/2j**) were both obtained in MS^4 experiments of precursor ions **F** and **G** for metaplexigenin (**1**) and caudatin (**2**). The 10-Me and 17-OAc in ion **F** were eliminated as CH$_4$ and C$_2$H$_2$O segments to produce **1k/2i** at m/z 291 and **1l/2j** at m/z 265, respectively. The latter ion can also be generated from ion **G** by losing 17-OH. The ion **3j** at m/z 265 for qingyangshengenin (**3**) was also generated from ion **G** in the MS^4 analysis. It was also found that precursor ion **D** can give rise to a peculiar ion at m/z 281 (**3i**) corresponding to the departure of 17-OAc as a C$_2$H$_4$O segment in MS^3 investigation. Removal of the methyl group at C-10 in ion **3i** as a CH$_4$ segment yielded a fragment at m/z 265.1233 (**3j'**), ascribed with the molecular formula C$_{18}$H$_{17}$O$_2$, which was different from the ion **3j** (m/z 265.1558, C$_{19}$H$_{21}$O). Precursor ion **E** lost one molecule of CO to afford ion **3k** at m/z 231 in MS^4 experiment, and such a fragmentation pattern demonstrated the mechanism that ring D cleavage in ion **B** generated ion **E** by forming a carbonyl on ring C (Figure 3).

Fragmentation pathways for steroid 4: The fragmentation pathways for penupogenin (**4**) were similar to that of type I C$_{21}$ steroid aglycones. Its single-stage MS analysis led to the detection of a [M−H]$^-$ ion at m/z 511.2663 (**4a**) as the base ion peak in negative mode with the chemical composition of C$_{30}$H$_{39}$O$_7$. Ion **4a** lost the cinnamoyl group at C-12 as one molecule of cinnamic acid to afford ion **B'** at m/z 363 in MS^2 analysis. The mass of fragment **B'** was 2 Da more than that of ion **B** owing to the presence of a
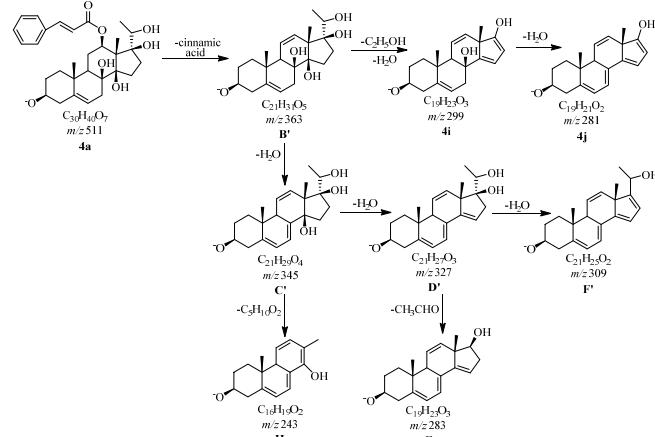
Figure 4: Proposed fragmentation pathways for compound **4**.

hydroxyl group at C-20, and so the product ions of **B'** were ions **C'** at m/z 345, **D'** at m/z 327, and **F'** at m/z 309 in MS^3 experiment, which could be interpreted by the same fragmentation routes as ions **C** (m/z 343), **D** (m/z 325) and **F** (m/z 307). Furthermore, ions **G** at m/z 283 and **H** at m/z 243 were also found whose molecular formula and fragmentation pathways were similar to the above explanations, as described in Figure 2. In addition to ions **C'**, **D'**, **F'**, **G**, and **H**, ion **4i** at m/z 299 was obtained as well due to the loss of the substituent group at C-17 as CH$_3$CH$_2$OH and of the hydroxyl group at C-14 as H$_2$O. Ultimately, **4i** eliminated 8-OH to produce ion **4j** at m/z 281 in the MS^4 experiment (Figure 4).

Fragmentation pathways of steroids 5-6: Fragmentation of 20-cinnamoylsarcostin (**5**) and gagamine (**6**) gave rise to [M+H]$^+$ ions **5a** at m/z 513.2789 (C$_{30}$H$_{41}$O$_7$) and **6a** at m/z 618.3006 (C$_{36}$H$_{44}$NO$_8$), in the first-stage mass spectrum. Due to the lack of an ester group at C-12, **5a** departed 8-OH to yield ion **5b** at m/z 495 in MS^2 investigation. Additionally, ions **5c** at m/z 477, **5d** at m/z 459, **I** at m/z 329, **J** at m/z 311, and **K** at m/z 293 were also detected. **5c** was generated from **5b** because of the loss of 7-OH, while **5d** was yielded from **5c** due to the loss of one molecule of H$_2$O at C-14 and C-15. The loss of the cinnamoyl substituent at C-20 as cinnamic acid in **5c** and **5d** led to the generation of ions **I** and **J**, respectively.
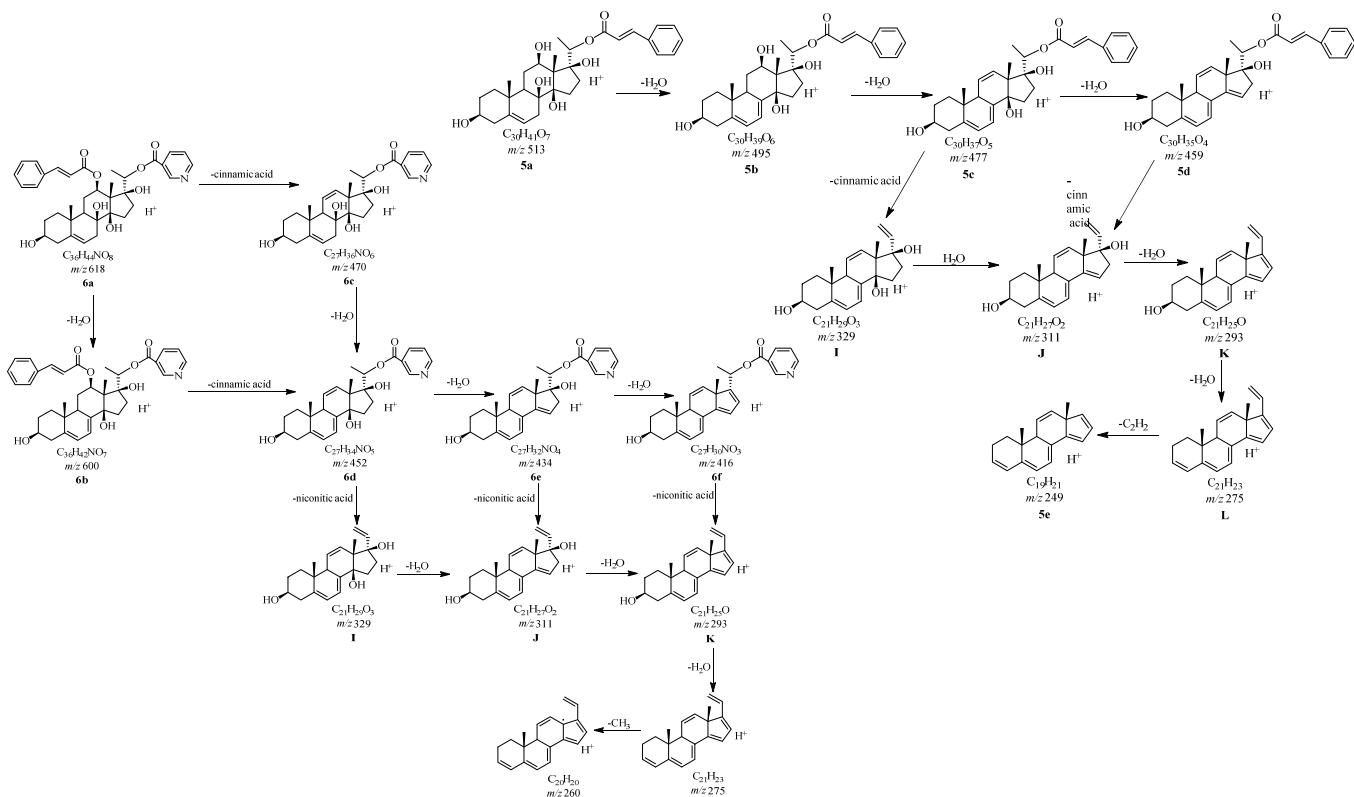
Figure 5: Proposed fragmentation pathways of ss **5-6**

Table 5: Data for accurate masses and elemental composition of steroid **5** observed from tandem mass spectra in positive mode

MS^n	Precursor ion (m/z)	Product ion (m/z)	Elemental composition	Measured (m/z)	Calculated (m/z)	Error (mDa)	Intensity (%)	Ion name	Assignment
(+)MS	512	513	$C_{30}H_{41}O_7$	513.2798	513.2847	−4.9	100	**5a**	[M+H]$^+$
MS^2	513	495	$C_{30}H_{39}O_6$	495.2675	495.2741	−6.6	43	**5b**	**5a**−H_2O
		477	$C_{30}H_{37}O_5$	477.2572	477.2636	−6.4	43	**5c**	**5b**−H_2O
		459	$C_{30}H_{35}O_4$	459.2476	459.2530	−5.4	17	**5d**	**5c**−H_2O
		329	$C_{21}H_{29}O_3$	329.2074	329.2111	−3.7	100	**I**	**5c**− cinnamic acid
		311	$C_{21}H_{27}O_2$	311.1981	311.2006	−2.5	83	**J**	**I**−H_2O or **5d**− cinnamic acid
		293	$C_{21}H_{25}O$	293.1867	293.1900	−3.3	26	**K**	**J**−H_2O
MS^3	477	459	$C_{30}H_{35}O_4$	459.2454	459.2530	−7.6	28	**5d**	**5c**−H_2O
		329	$C_{21}H_{29}O_3$	329.2074	329.2111	−3.7	100	**I**	**5c**−CINN
		311	$C_{21}H_{27}O_2$	311.1970	311.2006	−3.6	100	**J**	**I**−H_2O or **5d**− cinnamic acid
		293	$C_{21}H_{25}O$	293.1872	293.1900	−2.8	45	**K**	**J**−H_2O
	459	311	$C_{21}H_{27}O_2$	311.1987	311.2006	−1.9	100	**J**	**5d**− cinnamic acid
		293	$C_{21}H_{25}O$	293.1885	293.1900	−1.5	80	**K**	**J**−H_2O
	329	311	$C_{21}H_{27}O_2$	311.2000	311.2006	−0.6	100	**J**	**I**−H_2O
		293	$C_{21}H_{25}O$	293.1864	293.1900	−3.6	54	**K**	**J**−H_2O
MS^4	311	293	$C_{21}H_{25}O$	293.1855	293.1900	−4.5	100	**K**	**J**−H_2O
		275	$C_{21}H_{23}$	275.1821	275.1794	+2.7	44	**L**	**K**−H_2O
		249	$C_{19}H_{21}$	249.1547	249.1638	−9.1	43	**5e**	**L**−C_2H_2

The ion **J** might also be originated from ion **I** through the elimination of one molecule of H_2O at C-14 and C-15. The ion **K** at m/z 293 could be interpreted by the hydroxyl group departure at C-17 in ion **J**. Similarly, ions **5d** and **I-K** were all observed in MS^3 analyses of **5c** (m/z 477), **5d** (m/z 459), and **J** (m/z 329). Finally, the MS^4 experiment of ion **J** (m/z 311) afforded ions **K** at m/z 293, **L** at m/z 275, and **5e** at m/z 249. Among them, fragment **L** arose from ion **K** because of the departure of 3-OH, and ion **5e** was generated by the elimination of the ethylene moiety at C-17 in ion **L** as a C_2H_2 segment.

Two dissociation pathways were observed from ion **6a** (m/z 618) in MS^2 investigation, leading to the fragments at m/z 600 (**6b**) and m/z 470 (**6c**) due to the loss of H_2O and cinnamic acid segments. Further elimination of 20-cinnamoyl and 8-OH in **6b** and **6c** yielded ion **6d**

at m/z 452. Elimination of 14-OH in **6d** led to the generation of ion **6e** at m/z 434, and the subsequent departure of 17-OH in **6e** produced ion **6f** at m/z 416 with a more stable conjugated structure. The above dissociation routes were confirmed by MS^3 experiments of precursor ions **6b**, **6c** and **6d**, in which ions **6d-6f** were all obtained. Apart from ions **6e** and **6f**, two non-nitrogen ions **I** at m/z 329 ($C_{21}H_{29}O_3$) and **J** ($C_{21}H_{27}O_2$) at m/z 311 were also found from precursor ion **6d**. In addition, the MS^3 analyses of ion **6f** provided ions **K** at m/z 293 and **L** at m/z 275. Ions **I** to **L** were the common product ions for 20-cinnamoylsarcostin (**5**) and gagamine (**6**), which could be regarded as the diagnostic ions for C_{21} steroidal aglycones with the same skeleton, and 14-OH, 17-OH, and 3-OH were eliminated one by one from ions **I** to **L,** resulting in a more stable conjugated system. In MS^4 investigations, precursor ions **6e** and **K** gave rise to versatile fragments, **6f**, **J-L,** and **6g** at m/z 260,

Table 6: Data for accurate masses and elemental composition of steroid **6** observed from tandem mass spectra in positive mode

MS^n	Precursor ion (m/z)	Product ion (m/z)	Elemental composition	Measured (m/z)	Calculated (m/z)	Error (mDa)	Intensity (%)	Ion name	Assignment
(+)MS	617	618	C$_{36}$H$_{44}$NO$_8$	618.3006	618.3061	−5.5	100	**6a**	[M+H]$^+$
MS^2	618	600	C$_{36}$H$_{42}$NO$_7$	600.2879	600.2956	−7.7	8	**6b**	**6a**−H$_2$O
		470	C$_{27}$H$_{36}$NO$_6$	470.2472	470.2537	−6.5	16	**6c**	**6a**− cinnamic acid
		452	C$_{27}$H$_{34}$NO$_5$	452.2359	452.2431	−7.2	74	**6d**	**6c**−H$_2$O or
									6b−cinnamic acid
		434	C$_{27}$H$_{32}$NO$_4$	434.2269	434.2326	−5.7	85	**6e**	**6d**−H$_2$O
		416	C$_{27}$H$_{30}$NO$_3$	416.2189	416.2220	−3.1	31	**6f**	**6e**−H$_2$O
MS^3	600	452	C$_{27}$H$_{34}$NO$_5$	452.2359	452.2431	−7.2	24	**6d**	**6b**− cinnamic acid
		434	C$_{27}$H$_{32}$NO$_4$	434.2287	434.2326	−3.9	24	**6e**	**6d**−H$_2$O
		416	C$_{27}$H$_{30}$NO$_3$	416.2121	416.2220	−9.9	16	**6f**	**6e**−H$_2$O
	470	452	C$_{27}$H$_{34}$NO$_5$	452.2387	452.2431	−4.4	100	**6d**	**6c**−H$_2$O
		434	C$_{27}$H$_{32}$NO$_4$	434.2295	434.2326	−3.1	67	**6e**	**6d**−H$_2$O
		416	C$_{27}$H$_{30}$NO$_3$	416.2165	416.2220	−5.5	13	**6f**	**6e**−H$_2$O
	452	434	C$_{27}$H$_{32}$NO$_4$	434.2274	434.2326	−5.2	65	**6e**	**6d**−H$_2$O
		416	C$_{27}$H$_{30}$NO$_3$	416.2181	416.2220	−3.9	20	**6f**	**6e**−H$_2$O
		329	C$_{21}$H$_{29}$O$_3$	329.2072	329.2111	−3.9	6	**I**	**6c**−niconitic acid
		311	C$_{21}$H$_{27}$O$_2$	311.1979	311.2009	−3.0	69	**J**	**I**−H$_2$O or
									6e−niconitic acid
	416	293	C$_{21}$H$_{25}$O	293.1867	293.1900	−3.3	100	**K**	**6f**−niconitic acid
		275	C$_{21}$H$_{23}$	275.1768	275.1794	−2.6	43	**L**	**K**−H$_2$O
MS^4	434	416	C$_{27}$H$_{30}$NO$_3$	416.2221	416.2220	+0.1	14	**6f**	**6e**−H$_2$O
		311	C$_{21}$H$_{27}$O$_2$	311.1982	311.2009	−2.7	36	**J**	**6e**−niconitic acid
		293	C$_{21}$H$_{25}$O	293.1869	293.1900	−2.1	100	**K**	**J**−H$_2$O or
									6f−niconitic acid
	293	275	C$_{21}$H$_{23}$	275.1768	275.1794	−2.6	8	**L**	**K**−H$_2$O
		260	C$_{20}$H$_{20}$	260.1538	260.1560	−2.2	100	**6g**	**L**−CH$_3$

among which **6f** and **J** to **L** have been clarified in the MS^3 experiments and the ion **6g** could be explained by the loss of 13-Me as a CH$_3$ segment (Figure 5).

In conclusion, high resolution MS^n fragmentation studies of C$_{21}$ steroidal aglycones showed characteristic fragmentation patterns different from those of C$_{21}$ steroidal glycosides. [M−H]$^-$ ions for metaplexigenin (**1**), caudatin (**2**), qingyangshengenin (**3**) and penupogenin (**4**), and [M+H]$^+$ ions for 20-cinnamoylsarcostin (**5**) and gagamine (**6**) were obtained in their single-stage mass spectra. The loss of ROH (R= acetyl, tigloyl, *p*-hydroxybenzoyl, cinnamoyl and nicotinoyl), H$_2$O, C$_2$H$_4$O, C$_2$H$_2$O and CH$_4$ was the characteristic fragmentation from the precursor ions due to the presence of ester groups, hydroxyl, 1-*O*-ethyl and methyl groups. In particular, the most active 12-ester group was always first eliminated to give rise to the ion at [M−H−ROH]$^-$ as the base ion peak for compounds **1-4**. Ions at *m/z* 361 (**B**), 343 (**C**), 325 (**D**), 307 (**F**), 283 (**G**), 259 (**E**), and 243 (**H**) were the common product ions for steroids **1-3**. Steroid **4** displayed similar fragmentation pathways as steroids **1-3** to yield ions at *m/z* 363 (**B**'), 345 (**C'**), 327 (**D'**), 309 (**F'**), 283 (**G**), and 243 (**H**). All these ions could be recognized as the diagnostic ions for structural characterization. The substituent group at C-12 in compounds **5-6** was also the active group, leading to the departure of either a H$_2$O or cinnamic acid segment, with the key fragments being *m/z* 329 (**I**), 311(**J**), 293 (**K**), and 275 (**L**). Besides, the substituent group loss at either C-12 or C-17 gave rise to high abundance ions, even as the base ion peak for all six steroids. These findings demonstrate the possible online analytical ability of ESI-IT-TOF tandem mass spectrometry for establishing an effective method to analyze C$_{21}$ steroidal alycones.

Experimental

General: Acetonitrile, HPLC grade, was purchased from Merck Co. Ltd., Germany. Deionized water was purified using a MingCheTM-

D 24UV Merck Millipore system (Merck Millipore, Shanghai, China).

Steroids **1-6** were isolated in our laboratory from *Cynanchum auriculatum* Royle ex Wight., *Gymnema yunnanense* Tsiang, and *Marsdenia incisa* P. T. Li et Y. P. Li. Their structures were confirmed by the analysis of their spectroscopic data. Sample solutions with a final concentration of 0.25 mg·mL^{-1} were prepared by dissolving each sample in 80% CH$_3$CN in H$_2$O. The samples were loaded onto the spectrometer via a syringe pump at the flow rate of 5 μL·min^{-1}.

Apparatus and analytical conditions: MS^n analyses were conducted on a LCMS-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan). Precise mass was corrected by calibration using the sodium trifloroacetate clusters. The mass resolution was about 10 000 full width at half maximum (FWHM). The Shimadzu Composition Formula Predictor was used to speculate the molecular formula. MS experiments were achieved in automatic pattern, and MS2–5 experiments were performed in manual mode. The ESI-MS analytical conditions were as follows: drying gas pressure, 100.0 kPa; nebulizing gas (N$_2$) flow, 0.5 L min^{-1}; spray voltage, +4.50/−3.50 kV; detector voltage, 1.60 kV; equipment temperature, 40.0°C; heat block temperature, 200.0°C; curved desolvation line (CDL) temperature, 200.0°C; collision energy, 50%; collision gas (Ar), 50%; precursor ion selected width, *m/z* ±3.0 Da, and selected time, 20 ms; collision induced dissociation (CID) collision time, 30 ms; ion accumulation time, 10 ms; and q = 0.251; scan range, *m/z* 100–1000 for MS.

Reference

[1]   Shan L, Zhang WD, Zhang C, Liu RH, Su J, Zhou Y. (**2005**) Antitumor activity of crude extract and fractions from root tuber of *Cynanchum auriculatum* Royle ex Wight. *Phytotherapy Research*, **19**, 259-261.
[2]   Li XY, Sun HX, Ye YP, Chen FY, Pan YJ. (**2006**) C-21 steroidal glycosides from the roots of *Cynanchum chekiangense* and their immunosuppressive activities. *Steroids*, **71**, 61-66.
[3]   Heerden FR, Horak RM, Maharaj VJ, Vleggaar R, Senabe JV, Gunning PJ. (**2007**) An appetite suppressant from *Hoodia* species. *Phytochemistry*, **68**, 2545-2553.

[4]   Wang LJ, Geng CA, Ma YB, Huang XY, Luo J, Chen H, Guo RH, Zhang XM, Chen JJ. (**2012**) Synthesis, structure–activity relationships and biological evaluation of caudatin derivatives as novel anti-hepatitis B virus agents. *Bioorganic &. Medicinal Chemistry*, **20**, 2877-2888.

[5]   Wang LJ, Geng CA, Ma YB, Luo J, Huang XY, Chen H, Zhou NJ, Zhang XM, Chen JJ. (**2012**) Design, synthesis, and molecular hybrids of caudatin and cinnamic acids as novel anti-hepatitis B virus agents. *European Journal of Medicinal Chemistry*, **54**, 352-365.

[6]   Wang Y, Song FR, Xu QX, Liu ZQ, Liu SY. (**2003**) Characterization of aconitine-type alkaloids in the flowers of *Aconitum kusnezoffii* by electrospray ionization tandem mass spectrometry. *Journal of Mass Spectrometry*, **38**, 962-970.

[7]   Piaz FD, Leo MD, Braca A, Simon FD, Morelli I, Tommasi ND. (**2005**) Electrospray ionization mass spectrometry for identification and structural characterization of pregnane glycosides. *Rapid Communications in Mass Spectrometry*, **19**, 1041-1052.

[8]   Han LF, Pan GX, Wang YF, Song XB, Gao XM, Ma BP, Kang LP. (**2011**) Rapid profiling and identification of triterpenoid saponins in crude extracts from *Albizia julibrissin* Durazz. by ultra high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, **55**, 996-1009.

[9]   Geng CA, Ma YB, Zhang XM, Yao SY, Xue DQ, Zhang RP, Chen JJ. (**2012**) Mulberrofuran G and isomulberrofuran G from *Morus alba* L.: antihepatitis B virus activity and mass spectrometric fragmentation. *Journal of Agricultural and Food Chemistry*, **60**, 8197-8202.

[10]   Geng CA, Chen H, Chen XL, Zhang XM, Lei LG, Chen JJ. (**2014**) Rapid characterization of chemical constituents in *Saniculiphyllum guangxiense* by ultra fast liquid chromatography with diode array detection and electrospray ionization tandem mass spectrometry. *International Journal of Mass Spectrometry*, **361**, 9-22.

[11]   Liu YB, Su EN, Li JB, Zhang JL, Yu SS, Qu J, Liu J, Li Y. (**2009**) Steroidal glycosides from *Dregea sinensis* var. *corrugata* screened by liquid chromatography-electrospray ionization tandem mass spectrometry. *Journal of Natural Products*, **72**, 299-237.

[12]   Avula B, Wang YH, Pawar RS, Shukla YJ, Smillie TJ, Khan IA. (**2008**) Identification and structural characterization of steroidal glycosides in *Hoodia gordonii* by ion-trap tandem mass spectrometry and liquid chromatography coupled with electrospray ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, **22**, 2587-2596.

[13]   Zheng ZG, Zhang WD, Kong LY, Liang MJ, Li HL, Lin M, Liu RH, Zhang C. (**2007**) Rapid identification of C_{21} steroidal saponins in *Cynanchum versicolor* Bunge by electrospray ionization multi-stage tandem mass spectrometry and liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, **21**, 279-285.

[14]   Liang MJ, Zheng ZG, Yuan Y, Kong LY, Shen YH, Liu RH, Zhang C, Zhang WD. (**2007**) Identification and quantification of C_{21} steroidal saponins from radix *Cynanchi atrati* by high-performance liquid chromatography with evaporative light scattering detection and electrospray mass spectrometric detection. *Phytochemical Analysis*, **18**, 428-435.

[15]   Tai YP, Cao XJ, Li XY, Pan YJ. (**2006**) Identification of C-21 steroidal glycosides from the roots of *Cynanchum chekiangense* by high-performance liquid chromatography/tandem mass spectrometry. *Analytical Chimica Acta*, **572**, 230-236.

[16]   Cao XJ, Tai YP, Li XY, Ye YP, Pan YJ. (**2006**) Screening for pregnane glycosides with immunological activities from the stems of *Stephanotis mucronata* by high-performance liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, **20**, 403-411.

Three New Cytotoxic Withanolides from the Chinese Folk Medicine *Physalis angulata*

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Physagulides M-O, three new withanolides (**1-3**), were isolated from the aerial parts of *Physalis angulata* L. Their structures were elucidated through extensive spectroscopic techniques, including 1D and 2D NMR, and HRESIMS. The absolute configurations (22-*R*) of these new compounds were determined by CD analysis. Compounds **1** and **3** showed significant selective cytotoxic activities on the MG-63 cell line, with IC_{50} values of 4.28 and 5.44 μM, respectively.

Keywords: *Physalis angulata*, Withanolides, Cytotoxic activity.

Withanolides are a group of C_{28} ergostane-type steroids with a δ-lactone oxidized at C-22 and C-26, distributed mainly in plants belonging to the genera *Physalis*, *Withania*, *Datura*, *Hyoscyamus*, *Jaborosa*, *Nicandra* and *Tubocapsicum* of the family Solanaceae [1]. In recent years, withanolides have gained significant scientific interest due to their various structures and notable biological properties [2], including immunosuppression [3], quinine reductase induction [4], antiproliferative [5], anti-inflammation [6], and antitumor activities [7a, b]. *Physalis angulata* L., a widely distributed species throughout the east and southwest regions of China [8], is commonly used as a traditional Chinese medicine for antipyretic, anti-inflammatory and diuretic purposes [9]. Phytochemical research has indicated that withanolides, with diverse structures and significant antitumor activities, are the main constituents of this plant [10]. As a part of our research program on bioactive constituents from Chinese folk medicines, three new withanolides (**1-3**) were isolated from the aerial parts of the title plant. Their structures were elucidated through extensive spectroscopic techniques, including 1D and 2D NMR, and HRESIMS. The absolute configuration (22-*R*) of these new compounds was determined by CD analysis. Anantiproliferative activities screen indicated that compounds **1** and **3** had selective significant cytotoxic activity on the MG-63 cell line with IC_{50} values of 4.28 and 5.44 μM, respectively [11]. Herein, we report the isolation, structure elucidation and cytotoxic activity of these new compounds.

Physagulide M (**1**) was obtained as a white amorphous solid, and its HRESIMS showed a $[M+NH_4]^+$ peak at *m/z* 578.3326 (calcd 578.3324) corresponding to a molecular formula of $C_{31}H_{44}O_9$. The strong IR absorptions at 3444 and 1707 cm^{-1} indicated the presence of hydroxy groups and an α, β-unsaturated ketone [12], which was supported by an UV absorption at 227 nm [13]. The whole features of the ^{1}H and ^{13}C NMR (Table 1) data of **1,** especially the five methyl signals at δ_H 1.08, 1.30, 1.01, 1.86, and 1.93, and a set of carbon signals of an α, β-unsaturated ketone (δc 166.5, 122.2 and 149.1), indicated that compound **1** was a withanolide derivative, such as physagulin N [9]. Two characteristic olefinic protons of the α, β-unsaturated ketone in ring A seen in the ^{1}H NMR spectra of many normal withanolides [14] were absent in the spectrum of **1** (Table 1); instead of methylene protons at δ_H 3.08 (dd, *J* = 15.1, 5.3
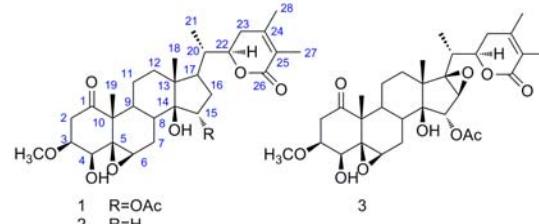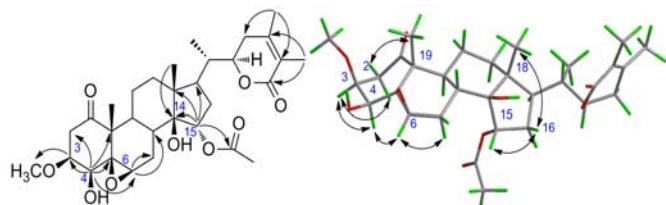
Figure 1: The structures of compounds **1-3**.

Hz, H-2β), and δ_H 2.57 (dd, *J* = 15.2, 3.7 Hz, H-2α), one more oxymethine proton at δ_H 3.73 (ddd, *J* = 5.3, 3.7, 3.4 Hz, H-3), and an additional methoxyl signal (δ_H 3.32, s, 3H) were observed. Moreover, an HMBC correlation between a methoxyl signal at δ_H 3.32 (s, 3H) and C-3 (δ_C 77.3) was observed. The aforementioned evidence suggested that the typical $\Delta^{2,3}$ of normal withanolides was absent and a methoxyl group was located at C-3. The signal at δ_H 3.51 (d, *J* = 3.4 Hz, H-4) and HMBC correlations between H-4 and C-2 (δ_C 39.1), C-3 (δ_C 77.3), C-5 (δ_C 64.3), and C-10 (δ_C 50.7) suggested that a hydroxyl group was located at C-4. Signals at δ_H 3.29 (s, H-6), δ_C 64.3 (C-5) and 61.8 (C-6) could be assigned for an epoxy group linked at C-5/6 [15], which was proved by the HMBC correlations from H-4 to C-5 and C-6, from H-6 to C-7 (δ_C 24.5) and C-8 (δ_C 35.1) (Figure 2). Two olefinic methyl signals at δ_H 1.86 (3H, s, Me-27) and δ_H 1.93 (3H, s, Me-28) and a double doublet at δ_H 4.32 (ddd, *J*=13, 4.6, 3.5 Hz, H-22) indicated the presence of a typical α, β-unsaturated-δ-lactone in the side chain, which was confirmed by the HMBC correlations shown in Figure 2 [16]. HMBC correlations between H-15 (δ_H 4.99) and C-14 (δc 84.5), C-13 (δc 46.3), C-17 (δc 52.5) and the carbonyl group (δc 169.8) suggested that an acetoxyl group was located at C-15 (δc 80.6) (Figure 2). The remaining hydroxy group was placed at C-14 (δc 84.5) by the key HMBC correlations from Me-18 and H-15 to C-14 (Figure 2). Thus, the planar structure of **1** was elucidated as shown in Figure 1.

The relative stereochemistry of **1** was established by analysis of the ROESY spectrum [17]. Correlations of H-3 with H-4 and H-2α, of H-4 with H-6, and of H-2β with Me-19 indicated the α-orientations of H-3, H-4 and H-6. The β-orientation of H-15 was supported by the ROESY correlations between H-15, Me-18 and H-16β (Figure 2).

Table 1: ^{1}H (500MHz) and ^{13}C NMR (125 MHz) data for compounds **1-3** in CDCl$_3$ (δ in ppm, *J* in Hz).

Position	1		2		3	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		209.9		210.2		210.2
2	3.08, dd (15.1, 5.3)	39.1	3.12, dd (14.6, 4.8)	38.9	3.12, dd (15.0, 4.7)	38.9
	2.57, dd (15.2, 3.7)		2.57, dd (14.6, 4.1)		2.57, dd (15.0, 3.8)	
3	3.73, ddd (5.3, 3.7, 3.4)	77.3	3.75, ddd(4.8, 4.1, 3.3)	77.7	3.74, ddd (4.7, 3.8, 3.5)	77.3
4	3.51, d (3.4)	75.0	3.50, d (3.3)	75.2	3.51, d (3.5)	75.0
5		64.3		64.6		64.1
6	3.29, br s	61.8	3.32, br s	62.7	3.27, s	62.1
7	2.51, m	25.9	2.48, m	27.1	2.56, m	25.6
	1.49, m		1.58, m		1.40, m	
8	1.60, m	36.3	1.58, m	35.3	1.58, m	34.4
9	1.82, m	38.5	1.50, m	39.0	1.90, m	37.3
10		50.7		51.0		50.4
11	1.93, m	21.4	1.50, m	21.7	1.59, m	20.6
	1.43, m		1.41, m		1.20, m	
12	1.81, m	41.2	1.44, m	41.5	1.60, m	31.6
	1.59, m		1.31, m		1.59, m	
13		46.3		46.9		46.6
14		84.5		84.8		81.4
15	4.99, d (4.5)	80.6	1.70, m	32.8	5.03, s	77.6
			1.54, m			
16	2.01, m	33.6	1.26, m	29.8	3.48, s	59.0
	1.67, m		1.25, m			
17	1.70, m	52.5	1.51, m	53.1		76.3
18	1.08, s	17.1	1.04, s	16.4	1.12, s	15.6
19	1.30, s	16.0	1.35, s	16.6	1.30, s	16.3
20	2.09, m	37.5	2.10, m	37.9	2.56, m	33.7
21	1.01, d (6.6)	16.1	0.99, d (6.6)	15.8	1.00, d (7.1)	13.4
22	4.32, ddd (13.0, 4.6, 3.5)	78.3	4.48, ddd (13.0, 4.3, 3.5)	78.5	4.50, dt (12.5, 4.0)	76.8
23	2.47, m	31.6	2.46, m	31.5	2.38, m	32.5
	1.96, m		2.03, m		2.13, m	
24		149.1		149.2		149.2
25		122.2		122.1		122.2
26		166.5		166.8		166.3
27	1.86, s	12.5	1.87, s	12.6	1.87, s	12.6
28	1.93, s	20.6	1.93, s	20.6	1.93, s	20.7
OAc	2.05, s	169.8			2.10, s	169.6
		21.8				21.0
OMe	3.32, s	56.8	3.35, s	56.8	3.32, s	56.7

Figure 2: The key HMBC and ROESY correlations of compound **1**.

The obvious positive Cotton effect at 249 nm in the ECD spectrum of **1** indicated that the absolute configuration of C-22 was assigned as *R* [18]. On the basis of all the above evidence, the structure of **1** was established as (20*S*, 22*R*)-15α-acetoxy-5β, 6β-epoxy-4β, 14β-dyhydroxy-3β-methoxy-1-oxowitha-24-en-26, 22-olide, and named physagulide M.

The spectral characteristics of physagulide N (**2**) were very similar to those of **1**, with the major difference being the absence of signals corresponding to the acetoxy group in **1**, consistent with the 58.0061 mass units less than **1** in the HRESIMS. The upfield shifts of the C-15 from δ_C 80.6 in **1** to 32.8 suggested that there was no acetoxyl group linked at C-15. The similar ROESY and ECD spectra indicated that the relative and absolute configuration of **2** were the as same as those of **1**. Thus, compound **2** was established as (20*S*, 22*R*)-5β, 6β-epoxy-4β, 14β-dyhydroxy-3β-methoxy-1-oxowitha-24-en-26, 22-olide, and named as physagulide N.

Physagulide O (**3**) gave a molecular formula of C$_{31}$H$_{42}$O$_{10}$ by HRESIMS (*m/z* 592.3117 [M+NH$_4$]$^+$, calcd. for 592.3116). Comparison of the NMR data of **3** and **1** indicated that they have

identical A-C rings and side chains. The key difference was the substitution pattern in ring D. A secondary epoxy group linked at C-16/17 was proposed by the NMR signals at δ_H 3.48 (br s, H-16), δ_C 59.0 (C-16) and δ_C 76.3 (s, C-17). This was consolidated by the HMBC spectral analysis, in which H-15 (δ_H 5.03) was correlated to C-14 (δ_C 81.4), C-13 (δ_C 46.6) and C-17 (δ_C 76.3), and H-16 (δ_H 3.48) to C-15 (δ_C 77.6). The orientation of the epoxy group was deduced to be β based on the ROESY cross-peaks for H-16 (δ_H 3.48) with Me-21 (δ_H 1.00). The absolute configuration of C-22 was established as *R* through the same ECD Cotton effects of **3** compared with **1** [18]. Thus, the structure of **3** was established as (20*S*, 22*R*)-15α-acetoxy-5β, 6β:16β, 17β-diepoxy-4β, 14β-dyhydroxy-3β-methoxy-1-oxowitha-24-en-26, 22-olide, and named as physagulide O.

Cytotoxic activities of all compounds were examined against three human cancer cell lines (MG-63, HepG-2, and MDA-MB-231); doxorubicin was used as the positive control with IC$_{50}$ values of 0.46, 3.72, and 3.70 μM, respectively. Compounds **1** and **3** demonstrated significant selective cytotoxic activity on MG-63 with IC$_{50}$ values of 4.28 and 5.44 μM, respectively (Table 2). From the results of this cytotoxicity evaluation, these compounds may be valuable for cancer therapy.

Table 2: Cytotoxicity of compounds **1–3** against three human cancer cell lines.

Compound	IC$_{50}$ (μM)		
	MG-63	HepG-2	MDA-MB-231
1	4.28	>20	>20
2	>20	>20	>20
3	5.44	>20	>20
Doxorubicin[a]	0.46	3.72	3.70

[a]Doxorubicin was used as a positive control.

Experimental

General: Optical rotations were measured with a JASCO P-1020 polarimeter. IR data were obtained on a Bruker Tensor 27 spectrometer, UV spectra on a Shimadzu UV-2501 PC spectrophotometer, and 1D and 2D NMR spectra, using CDCl$_3$ as solvent, on a Bruker Avance III NMR spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). HRESI mass spectra were collected with an Agilent 6520B UPLC-Q-TOF mass spectrometer. Circular dichroism(CD) spectra were recorded on a JASCO 810 spectropolarimeter. HPLC analysis was run on an Agilent 1200 instrument equipped with a multiple wavelength diode array detector (DAD). Preparative HPLC was performed on a Shimadzu instrument equipped with a Shim-pack RP-C$_{18}$ column (20×200 mm^2, 10 μm), and a flow rate of 10.0 mL/min. Column chromatography (CC) was carried out using macroporous resin D-101 (pore size B 13-14 nm, 26–60 mesh, Qingdao Marine Chemical Co. Ltd., Qingdao, China), Silica gel (100-200 mesh and 200-300 mesh, Qingdao Marine Chemical Co. Ltd., Qingdao, China) and ODS RP-C$_{18}$ (40-63μm, FuJi, Japan).

Plant material: Whole plants of *Physalis angulata* were collected in August 2014, in Lin Yi, Shan Dong Province, China, and were identified by Professor Zhang Mian of the Research Department of Pharmacognosy, China Pharmaceutical University. A voucher specimen (No.PA-201407-LY) is deposited in the department of Natural Medicinal Chemistry, China Pharmaceutical University.

Extraction and isolation: The air-dried aerial parts of *P. angulata* (1 kg) were powdered and extracted with CH$_2$Cl$_2$-MeOH (1:1) at room temperature, 3 times. After removing the solvent under vacuum, the residue (70 g) was subjected to column chromatography (CC) on D-101 macroporous resin and eluted with a step gradient of EtOH-H$_2$O (20:80, 40:60, 60:40, 80:20, 95:5, v/v) to yield 5 fractions: Fr. A-E. Fr. C (7g) was then chromatographed over silica gel with increasing polarities of CH$_2$Cl$_2$-MeOH (40:1, 20:1, 10:1, 0:100 v/v) to obtain 4 sub-fractions (Fr. C1-C4). Fr. C3 was applied to ODS MPLC eluted with isocratic MeOH-H$_2$O (50:50 v/v) to afford 8 sub-fractions (Fr.C3A-H). Fr.C3C was chromatographed over ODS with acetonitrile-H$_2$O (35:65, v/v) to give 2 sub-fractions (Fr.C3C 1-2). Fr.C3C1 was applied to preparative HPLC with MeOH-H$_2$O (55:45 v/v) to yield **1** (6.2 mg) and **3** (14.7 mg). Fr.C3H was chromatographed over Sephadex LH-20 with MeOH to yield 2 sub-fractions (Fr.C3H 1-2). Fr.C3H1 was subjected to preparative HPLC with MeOH-H$_2$O (60:40, v/v) to yield **2** (1.6 mg).

PhysagulideM (1)
White amorphous solid.
$[\alpha]_D^{20}$:+10.5 (*c* 0.42, MeOH).
CD: Δε$_{250}$ +4.92 (*c* 0.3, MeCN).
IR (KBr): 3444, 2925, 1707, 1383, 1256, 1095 cm^{-1}.
UV/Vis λ$_{max}$ (MeOH) nm (log ε): 227 (3.95), 206 (3.93).
^{1}H and ^{13}C NMR data: see Table 1.
HRESIMS: *m/z* 578.3326 [M+NH$_4$]$^+$ (calcd for C$_{31}$H$_{48}$NO$_9$, 578.3324).

Physagulide N (2)
White amorphous solid.
$[\alpha]_D^{20}$:-1.5 (*c* 0.18, MeOH).
CD: Δε$_{250}$ +5.30 (*c* 0.3, MeCN).
IR (KBr): 3449, 2932, 1702, 1383, 1137 cm^{-1}.
UV/Vis λ$_{max}$ (MeOH) nm (log ε): 227 (4.21), 207 (4.20).
^{1}H and ^{13}C NMR data: see Table 1.
HRESIMS: *m/z* 520.3265 [M+NH$_4$]$^+$ (calcd for C$_{29}$H$_{46}$NO$_7$, 520.3269).

Physagulide O (3)
White amorphous solid.
$[\alpha]_D^{20}$:+21.6 (*c* 0.13, MeOH).
CD: Δε$_{250}$ +4.84 (*c* 0.3, MeCN).
IR (KBr): 3489, 2929, 1707, 1381, 1225, 1097 cm^{-1}.
UV/Vis λ$_{max}$ (MeOH) nm (log ε): 227(4.58).
^{1}H and ^{13}C NMR data: see Table 1.
HRESIMS:*m/z* 592.3117 [M+NH$_4$]$^+$ (calcd for C$_{31}$H$_{46}$NO$_{10}$, 592.3116).

Cytotoxicity assay: The cytotoxicity bioassay of all new compounds against human cancer cells (MG-63, HepG-2, MDA-MB-231) was determined *in vitro* with the MTT assay, as described previously [19]. Doxorubicin was used as the positive control [20]. All experiments were carried out in triplicate.

Supplementary data: NMR spectra (^{1}H and ^{13}C NMR, HSQC, HMBC and ROESY), HRESIMS and CD spectra for the new compounds (**1-3**).

References

[1] Chen LX, He H, Qiu F. (**2011**) Natural withanolides: an overview. *Natural Product Reports*, **28**, 705-740.
[2] Ye Y, Li XQ, Tang CP. (**2010**) Natural products chemistry research 2008's progress in China. *Chinese Journal of Natural Medicines*, **41**, 68-80.
[3] Sun LJ, Liu JW, Liu P, Yu YJ, Ma L, Hu LH.(**2005**) Immunosuppression effect of withangulatin A from *Physalis angulata* via heme oxygenase 1-dependent pathways. *Process Biochemistry*, **46**, 482-488.
[4] Ding H, Hu ZJ, Yu LY, Ma ZJ, Ma XQ, Chen Z, Wang D, Zhao XF. (**2014**) Induction of quinone reductase (QR) by withanolides isolated from *Physalis angulata* L. var. *villosa* Bonati (Solanaceae). *Steroids*, **86**, 32-38.
[5] Zhang HP, Bazzill J, Gallagher RJ, Subramanian C, Grogan PT, Day VW, Kindscher K, Cohen MS, Timmermann BN. (**2013**) Antiproliferative withanolides from *Datura wrightii. Journal of Natural Products*, **76**, 445-449.
[6] Kaileh M, Berghe WV, Heyerick A, Horion J, Piette J, Libert C, Keukeleire DD, Essawi T, Haegeman G. (**2007**) Withaferin A strongly elicits IκB kinase *β* hyperphosphorylation concomitant with potent inhibition of its kinase activity. *Journal of Biological Chemistry*, **282**, 4253-4264.
[7] (a) He QP, Ma L,Luo GY, Hu LH. (**2007**) Cytotoxic withanolides from *Physalis angulata* L. *Chemistry & Biodiversity*, **4**, 443-449; (b) Alali FQ, Amrine CSM, El-Elimat T, Alkofahi A, Tawaha K, Gharaibah M, Swanson SM, Falkinham III JO, Cabeza M, Sánchez A, Figueroa M, Oberlies NH. (**2014**) Bioactive withanolides from *Withania obtusifolia. Phytochemistry Letters*, **9**, 96-101.
[8] Yang YJ, Chen MG, Hu L, Zhu DN. (**2013**) Chemical constituents of whole plant of *Physalis angulataL.. Chinese Pharmaceutical Journal*, **48**, 1715-1718.

[9]   Abe F, Nagafuji S, Okawa M, Kinjo J. (**2006**) Trypanocidal constituents in plants 6. Minor withanolides from the aerial parts of *Physalis angulata*. *Chemical & Pharmaceutical Bulletin*, *54*, 1226-1228.

[10]   Schirmer Pigatto AG, Mentz LA, Gonçalves Soares GL. (**2014**) Chemotaxonomic characterization and chemical similarity of tribes/genera of the Solanoideae subfamily (Solanaceae) based on occurrence of withanolides. *Biochemical Systematics and Ecology*, *54*, 40-47.

[11]   Cordero CP, Morantes SJ, Páez A, Rincón J, Aristizábal FA. (**2009**) Cytotoxicity of withanolides isolated from *Acnistus arborescens. Fitoterapia*, *80*, 364-368.

[12]   Zhang HP, Samadi AK, Gallagher RJ, Araya JJ, Tong X, Day VW, Cohen MS, Kindscher K, Gollapudi R, Timmermann BN. (**2011**) Cytotoxic withanolide constituents of *Physalis longifolia*. *Journal of Natural Products*, *74*, 2532-2544.

[13]   Kim KH, Choi SU, Choi SZ, Son MW, Lee KR. (**2011**) Withanolides from the rhizomes of *Dioscorea japonica* and their cytotoxicity. *Journal of Agricultural and Food Chemistry*, *59*, 6980-6984.

[14]   Hsieh PW, Huang ZY, Chen JH, Chang FR, Wu CC, Yang YL, Chiang MY, Yen MH, Chen SL, Yen HF, Lübken T, Hung WC, Wu YC. (**2007**) Cytotoxic withanolides from *Tubocapsicum anomalum*. *Journal of Natural Products*, *70*, 747-753.

[15]   Fang ST, Liu JK, Li B. (**2012**) Ten new withanolides from *Physalis peruviana*. *Steroids*, *77*, 36-44.

[16]   Guan YZ, Shan SM, Zhang W, Luo JG, Kong LY. (**2014**) Withanolides from *Physalis minima* and their inhibitory effects on nitric oxide production. *Steroids*, *82*, 38-43.

[17]   Nagafuji S, Okabe H, Akahane H, Abe F. (**2004**) Trypanocidal constituents in plants. 4. Withanolides from the aerial parts of *Physalis angulata*. *Biological & Pharmaceutical Bulletin*, *27*, 193-197.

[18]   Shingu K, Yahara S, Nohara T, Okabe H. (**1992**) Three new withanolides, physagulins A, B and D from *Physalis angulate* L.. *Chemical & Pharmaceutical Bulletin*,*40*, 2088-2091.

[19]   Mosmann T. (**1983**) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*, 55-63.

[20]   Choudhary MI, Hussain S, Yousuf S, Dar A, Mudassar, Atta ur R. (**2010**) Chlorinated and diepoxywithanolides from *Withania somnifera* and their cytotoxic effects against human lung cancer cell line. *Phytochemistry*, *71*, 2205-2209.

Diterpenoid Alkaloids from *Aconitum soongaricum* var. *pubescens*

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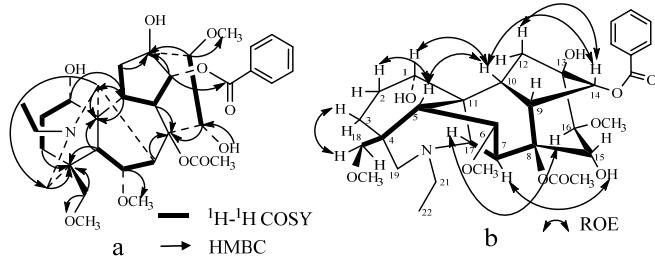
One new diterpenoid alkaloid, pubescensine (**1**), along with nine known diterpenoid alkaloids (**2–10**) were isolated from the roots of *Aconitum soongaricum* var. *pubescens*. Their structures were elucidated by spectroscopic analyses and comparison with previously reported data. All the compounds were evaluated for their antifeedant activities. The aconitine-type diterpenoid alkaloids (**1–6**) showed considerably potent antifeedant activity ($EC_{50} < 1$ mg/cm^2), while the activities of napelline-type diterpenoid alkaloids (compds. **7**, **9** and **10**) were not significant ($EC_{50} > 50$ mg/cm^2).

Keywords: *Aconitum soongaricum* var. *pubescens*, Diterpenoid alkaloids, Antifeedant activity.

Much attention has been paid to diterpenoid alkaloids not only for their complex structures, but also for their biological activities such as anti-inflammatory, analgesic, anti-arrhythmia, antifungal, and cytotoxic properties [1–3], as well as insecticidal and antifeedant activities [4–6]. Recently, in the course of our investigation of new bioactive diterpenoid alkaloids from plants of the genera *Aconitum* and *Delphinium*, we discovered that the crude alkaloid extract from *A. soongaricum* var. *pubescens* roots possesses significant feeding deterrent activity against *Pieris rapae* Linne. Chemical investigation of the active extract led to the isolation of ten diterpenoid alkaloids: pubescensine (**1**), 3-deoxyaconitine (**2**), aconitine (**3**), 15-α-hydroxyneoline (**4**), taurenine (**5**), bullatine B (**6**), songorine (**7**), 15-acetylsongorine (**8**), songoramine (**9**) and 12-*epi*-napelline (**10**) (Figure 1). Among these compounds, **1** is a new diterpenoid alkaloid. In this paper, we report the isolation, structure elucidation and antifeedant activities of these alkaloids.

Figure 2: Key ^{1}H-^{1}HCOSY, HMBC (a) and ROESY (b) correlations of **1**.

(δ_C 39.6, 43.3, 43.6, 44.0, 62.9, 71.9, 78.7, 79.1, 83.7, 89.8), and four quaternary carbons (δ_C 38.1, 49.2, 74.0, 91.9). In addition, three methoxyl groups [δ_H 3.17(s), 3.31(s), 3.77(s)], an acetyl group [δ_H 1.42 (s)], a benzoyl group [δ_H 7.46 (t), 7.58 (t), 8.02 (d)] and a *N*-ethyl group [δ_H 1.15 (t), 2.44 (m), 2.82 (overlapped)] were present in the structure according to the ^{1}H NMR spectrum. The above-mentioned data revealed that compound **1** is an aconitine-type diterpenoid alkaloid [7].

Comparison of the 1D-NMR data of **1** with those of the known alkaloid 3-deoxyaconitine (**2**) [8] indicated that there was a hydroxyl group at C(1) in **1** instead of the methoxyl group in **2**, which was also confirmed by the difference of 14 mass units between the two compounds. The hydroxyl group at C(1) was assigned an α-orientation based on the signal of H-1 at δ_H 3.67 (broad singlet) and the resonance of C-1 at δ_C 71.9 in the NMR spectra [7], which was further supported by the cross-peaks between H-C(1) with H-C(10) and H-C(10) with H-C(14) in the ROESY spectrum. Their chemical shifts and multiplicity (Table 1) suggested that ring A possesses a boat conformation [7]. The complete planar structure of **1** was further verified by the analyses of the HMBC and ^{1}H-^{1}H COSY spectra (Figure 2). The configuration of **1** could be assigned as H$_\beta$-C(1), H$_\beta$-C(6), H$_\beta$-C(10), H$_\beta$-C(14), H$_\beta$-C(15) and H$_\alpha$-C(16) based on the observation of the related cross-peaks in its ROESY spectrum (Figure 2). Thus, the structure of pubescensine was assigned to be **1**. The structures of compounds **2-10** were identified by comparison of their spectral data with those described in the literature [8-16].

Figure 1: Structures of compounds **1–10**.

Pubescensine (**1**) was obtained as a white amorphous powder. Its molecular formula was determined to be $C_{33}H_{45}NO_{10}$ from the [M+H]$^+$ peak at *m/z* 616.3123 (calcd. for $C_{33}H_{46}NO_{10}$ 616.3122) in the HR-ESI-MS. The IR spectrum indicated that **1** possesses hydroxyl (3485 cm^{-1}) and carbonyl (1723 cm^{-1}) groups. The ^{13}C-NMR and DEPT spectra of **1** (Table 1) exhibited the presence of five methylenes (δ_C 29.5, 30.0, 36.3, 56.2, 79.8), ten methines

Table 1: ¹H and ¹³C NMR data for compound **1**.

Position	δ_C	δ_H (mult., J = Hz)	Position	δ_C	δ_H (mult., J = Hz)
1	71.9 d	3.67 br s	18	79.8 t	a 3.11 ABq (8.4)
2	30.0 t	α 1.62 m			b 3.55 ABq (8.4)
		β 1.52 m	19	56.2 t	a 2.30 d (10.8)
3	29.5 t	α 1.26 m			b 2.61 d (10.8)
		β 1.90 m	21	48.7 t	a 2.44 m
4	38.1 s	-			b 2.82ᵃ
5	44.0 d	2.26 d (6.4)	22	13.0 q	1.15 t (7.2)
6	83.7 d	3.98 d (6.4)	6-OCH₃	58.1 q	3.17 s
7	43.6 d	2.82ᵃ	16-OCH₃	61.5 q	3.77 s
8	91.9 s	-	18-OCH₃	59.1 q	3.31 s
9	43.3 d	2.82ᵃ	8-OAc	21.4 q	1.42 s
10	39.6 d	2.16 m		172.4 s	-
11	49.2 s	-	14-OBz	165.9 s	-
12	36.3 t	α 2.20ᵃ	1'	129.7 s	-
		β 2.27ᵃ	2', 6'	129.6 d	8.02 d (7.2)
13	74.0 s	-	3', 5'	128.7 d	7.46 t (7.2)
14	79.1 d	4.89 d (4.8)	4'	133.3 d	7.58 t (7.2)
15	78.7 d	4.49 dd (2.7, 5.4)	13-OH	-	3.94ᵇ s
16	89.8 d	3.41 d (5.4)	15-OH	-	4.45ᵇ d (2.7)
17	62.9 d	2.91 s			

ᵃ Overlapped signals. ᵇ The signal disappeared after exchange with D₂O

The antifeedant activities of the isolated compounds **1-10** were evaluated against *Pieris rapae* Linne (Table 2). The most antifeedant activity was found for compounds **3**, **1** and **2** (EC_{50} < 0.05 mg/cm²), respectively, followed by **6**, **4** and **5** (EC_{50} < 1 mg/cm²). The antifeedant activities of napelline-type diterpenoid alkaloids (**7**, **9** and **10**) were not significant (EC_{50} > 50 mg/cm²). When the antifeedant activities of the compounds were compared for different classes, the napelline-type diterpenoid alkaloids were less active than the aconitine-type diterpenoid alkaloids.

Table 2: Antifeedant activities of the compounds isolated from *A. soongaricum* var. *pubescens* against *Pieris rapae* (n=3).

Compds.	EC_{50} (mg/cm²) (95% confidence limits)	Compds.	EC_{50} (mg/cm²) (95% confidence limits)
1	0.03 (0.01, 0.11)	**6**	0.41 (0.09, 2.10)
2	0.05 (0.01, 0.34)	**7**	>50
3	0.02 (0.01, 0.11)	**8**	nt
4	0.47 (0.33, 0.70)	**9**	>50
5	0.66 (0.32, 1.38)	**10**	>50

nt: not test (insufficient compound available)

Experimental

General: Optical rotations, Perkin-Elmer 341 polarimeter; NMR, Bruker AV600; IR, Thermo Fisher Nicolet 6700; HR-ESI-MS, Waters ACQUITY UPLC/Xevo G2-S QTOF mass spectrometer.

Plant material: The roots of *A. soongaricum* Stapf var. *pubescens* were collected in Houxia, Xinjiang Uygur Autonomous Region of China, in August 2014, and were identified (voucher specimen: C. Ren & L. Wang 705) by Prof. Qing-Er Yang of the Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation: Dried and powdered roots of *A. soongaricum* var. *pubescens* (5.3 kg) were extracted with 95% EtOH 3 times at rt, for 3 days each time. After removal of the solvent, the extract (2000 g) was suspended in water (3 L) and adjusted to pH 2 with hydrochloric acid solution, and then successively extracted with light petroleum (4×1 L) and ethyl acetate (4×1 L). Then, 28% aq. NH₄OH soln. (2 L) was added to the aq. soln. to bring it to pH 10. The solutions were extracted with CH₂Cl₂ (4×1 L). The CH₂Cl₂ extracts were concentrated to produce the crude alkaloid extract (20 g). Column chromatography (CC) of the crude alkaloid extract over silica gel, using a CH₂Cl₂/MeOH

(60:1, v/v) mixture with increasing polarity afforded fractions A–E based on TLC analysis. Fraction A was separated by silica gel CC (light petroleum/Me₂CO/Et₂NH, 8:1:0.1, v/v/v) to obtain **2** (3-deoxyaconitine, 23 mg) and **9** (songoramine, 15 mg). CC (silica gel, light petroleum /Me₂CO/Et₂NH, 6:1:0.1, v/v/v) of fraction B afforded **7** (songorine, 1.5 g) and **8** (15-acetylsongorine, 2 mg). Fraction C was chromatographed on a silica gel column and eluted with light petroleum/Me₂CO/Et₂NH (3:1:0.1-0:1:0.1, v/v/v) to afford **1** (pubescensine, 14 mg), **5** (taurenine, 25 mg) and **3** (aconitine, 300 mg). Fr. D was subjected to CC on silica gel and eluted with light petroleum/CH₂Cl₂ (1:1- 0:1, v/v) to give **6** (bullatine B, 460 mg). Fr. E was subjected to CC on silica gel and eluted with CH₂Cl₂/MeOH (10:1-1:1, v/v) to give **10** (12-epinapelline, 200 mg) and **4** (15-α-hydroxyneoline, 120 mg).

Pubescensine
White amorphous powder.
$[\alpha]_D^{20}$: -0.7 (c 0.3, CH₂Cl₂).
HR-ESI-MS *m/z* [M+H]⁺: 616.3123 (calcd. for C₃₃H₄₆NO₁₀ 616.3122).
¹H (CDCl₃, 600 MHz) and ¹³C NMR (CDCl₃, 150 MHz): Table 1.
IR (KBr) νmax: 3485, 2927, 2823, 2584, 1723, 1602, 1452, 1382, 1315, 1279, 1240, 1180, 1108, 1027, 984, 960, 919, 897, 710 cm⁻¹.

Antifeedant bioassays: A *Pieris rapae* colony was reared on cabbage foliage and maintained at 24 ± 1°C, > 70% relative humidity with a photoperiod of 16:8 h (L: D) in a growth chamber.

The antifeedant properties of the test compounds were evaluated using the choice leaf-disc method described by González-Coloma *et al.* [17, 18]. Choice experiments were conducted with newly-emerged third-instar larvae of *P. rapae*. Fresh cabbage leaves were cut into leaf discs (2 cm diameter) and then treated on the upper surface with 15 μL of either the test substance emulsions or deionized water containing acetone and Tween-20 (10: 0.012, v/v) for control. After air drying for 1 h, 2 treated leaves and 2 control leaves were arranged alternatively on 2% agar beds (2-3 mm) in 15 cm diameter Petri dishes. Four healthy and starved 3 h instars were placed in each dish and allowed to feed in a growth chamber (environmental conditions as described above). Three replicates were prepared for each treatment. Feeding was terminated after consumption of 50−70% of the control disks, and then the area of leaves consumed was examined with a LI-3000 portable area meter (American Lincoln Co. Ltd). Percent feeding reduction (%FR) was determined for each arena by the equation:

$$\%FR = (CK - T) / CK \times 100$$

Where *CK* and *T* are control leaf disc areas eaten and treated leaf disc areas eaten, respectively.

Compounds with a FR > 50% were tested in a dose-response experiment to calculate their relative potency (EC_{50} values, the effective dose for 50% feeding reduction), which was determined from linear regression analysis (%FR on log dose).

Supplementary data: NMR, IR and HR-ESI-MS for compound **1**.

References

[1]   Dzhakhangirov FN, Sultankhodzhaev MN, Tashkhodzhaev B, Salimov BT. (**1997**) Diterpenoid alkaloids as a new class of antiarrthythmic agents. Structure-activity relationship. *Chemistry of Natural Compounds*, **33**, 190–202.

[2]   Ameri A. (**1998**) The effects of aconitum alkaloids on the central nervous system. *Progress in Neurobiology*, **56**, 211–235.

[3]   Panter KE, Manners GD, Stegelmeier BL, Lee S, Gardner DR, Ralphs MH, Pfister JA, James LF. (**2002**) Larkspur poisoning: toxicology and alkaloid structure-activity relationships. *Biochemical Systematics and Ecology*, **30**, 113–128.

[4]   Ulubelen A, Mericli AH, Meriçli F, Kilincer N, Ferizli AG, Emekci M, Pelletier SW. (**2001**) Insect repellent activity of diterpenoid alkaloids. *Phytotherapy Research*, **15**, 170–171.

[5]   Liu ZL, Cao J, Zhang HM, Lin LL, Liu HJ, Du SS, Zhou LG, Deng ZW. (**2011**) Feeding deterrents from *Aconitum episcopale* roots against the red flour beetle, *Tribolium castaneum*. *Journal of Agricultural and Food Chemistry*, **59**, 3701–3706.

[6]   Reina M, González-Coloma A. (**2007**) Structural diversity and defensive properties of diterpenoid alkaloids. *Phytochemistry Reviews*, **6**, 81–95.

[7]   Pelletier SW, Mody NV, Joshi BS, Schramm LC. (**1984**) *Alkaloids: Chemical and Biological Perspectives*, Vol. **2**, Pelletier SW. (Ed.). Wiley, New York, 205-210.

[8]   Pelletier W, Mody NV, Katsui N. (**1977**) The structures of sachaconitine and isodelphinine from *Aconitum miyabei* Nakai. *Tetrahedron Letters*, **46**, 4027-4030.

[9]   Hanuman JB, Katz A. (**1993**) Isolation and identification of four norditerpenoid alkaloids from processed and unprocessed root tubers of *Aconitum ferox*. *Journal of Natural Products*, **56**, 801–809.

[10]   Takayama H, Hasegawa S, Sakai S, Haginiwa J, Okamoto T. (**1981**) Structure elucidation of a new aconite alkaloid, 15-α-hydroxyneolin. *Chemical & Pharmaceutical Bulletin*, **29**, 3078-3080.

[11]   Tel'nov VA, Vaisov ZM, Yunusov MS, Gorelova AP. (**1992**) Alkaloids of the cultivated species *Aconitum tauricum*. *Chemistry of Natural Compounds*, **28**, 91-94.

[12]   Shim SH, Kim JS, Kang SS, Son KH, Bae KH. (**2003**) Alkaloidal constituents from *Aconitum jaluense*. *Archives of Pharmacal Research*, **26**, 709-715.

[13]   Wiesner K., Itô S, Valenta Z. (**1958**) The structure of napelline and songorine. *Experientia*, **14**, 167-169.

[14]   Fan ZC, Zhang ZQ. (**2008**) Molecular and crystal structure of 15-acetylsongorine and songoramine isolated from *Aconitum szechenyianum* Gay. *Structural Chemistry*, **19**, 413-419.

[15]   Csupor D, Forgo P, Csedó K, Hohmann J. (**2006**) C_{19} and C_{20} Diterpene alkaloids from *Aconitum toxicum* R_{CHB}. *Helvetica Chimica Acta*, **89**, 2981-2986.

[16]   Zhapova Ts, Semenov AA. (**1993**) 12-*epi*-Napelline and its *N*-oxide from *Aconitum baicalense*. *Chemistry of Natural Compounds*, **29**, 791-794.

[17]   González-Coloma A, Reina M, Cabrera R, Castanera P, Gutierrez C. (**1995**) Antifeedant and toxic effects of sesquiterpenes from *Senecio palmensis* to Colorado potato beetle. *Journal of Chemical Ecology*, **21**, 1255-1270.

[18]   González-Coloma A, Terrero D, Perales A, Escoubas P, Fraga BM. (**1996**) Insect antifeedant ryanodane diterpenes from *Persea indica*. *Journal of Agricultural and Food Chemistry*, **44**, 296-300.

Two New C₁₈-Diterpenoid Alkaloids from *Delphinium anthriscifolium*

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Two new C₁₈-diterpenoid alkaloids, anthriscifoltine A (**1**) and anthriscifoltine B (**2**), along with three known diterpenoid alkaloids, deoxydelcorine (**3**), anthriscifolcine A (**4**) and anthriscifolcine G (**5**), were isolated from the whole herbs of *Delphinium anthriscifolium* var. *majus*. Their structures were elucidated by spectroscopic methods, including 1D, 2D NMR, and HR-ESI-MS.

Keywords: *Delphinium anthriscifolium* var. *majus*, Diterpenoid alkaloids, Anthriscifoltine, NMR.

Delphinium anthriscifolium var. *majus* is an herbaceous plant belonging to the family Ranunculaceae. It is widely distributed in Guizhou, Sichuan, Hubei and Shanxi provinces in China, but currently is also cultivated in other regions. It contains many diterpenoid alkaloids having biological activities such as anti-inflammatory, analgesic, anti-arrhythmia, antifungal, and cytotoxic properties [1,2]. As a part of our efforts to study the chemical composition of *D. anthriscifolium*, we have now isolated and identified two C₁₈-diterpenoid alkaloids, anthriscifoltine A (**1**) and anthriscifoltine B (**2**), along with three known compounds, deoxydelcorine (**3**), anthriscifolcine A (**4**) and anthriscifolcine G (**5**). In this paper, we report the extraction, isolation, and structure elucidation of these alkaloids.

The molecular formula of anthriscifoltine A (**1**), was determined as C₃₀H₄₅NO₉ from the HR-ESI-MS ion at *m/z* 564.3185 [M+H]⁺ (calcd. for C₃₀H₄₆NO₉, 564.3173). The IR spectrum indicated that **1** possesses hydroxyl (3462 cm⁻¹) and carbonyl (1736 cm⁻¹) groups. The ¹H NMR and ¹³C NMR data (Table 1) of **1** indicated the presence of the signals of a 2-methylbutanoyloxy group (MbO) at [(δ_H 2.38 (1H, m), 1.47 (2H, m), 0.91(3H, t, *J* = 7.2 Hz), 1.14 (3H, d, *J* = 6.6 Hz) and δ_C 177.0 (s), 41.2 (d), 26.6 (t), 11.6 (q), 16.5 (q)] [3], an *N*-ethyl group [δ_H 1.07 (3H, t, *J* = 7.2 Hz), 2.75 (2H, m), δ_C 14.0 (q) and 50.8 (t)], two methoxyl groups [δ_H 3.27 (3H, s) and 3.28 (3H, s), δ_C 55.8 (q) and 55.9 (q)], an acetyl group [δ_H 2.04 (3H, s), δ_C 21.8 (q) and 170.5 (s)] and a methylenedioxy group [4.89 (1H, br s) and 4.94 (1H, br s), δ_C 94.0 (t)]. The remaining 18 carbons were assigned based on 1D- and 2D-NMR data. The presence of only one non-oxygenated quaternary carbon signal (δ_C 55.5 s) indicates that compound **1** is a C₁₈-diterpenoid alkaloid [4a,b]. The locations of the acetoxyl group at C-6 and the 2-methylbutanoyloxy group at C-14 were determined by the correlations in the HMBC experiment. Besides the two ester groups, the two methoxyl groups were attributed to C-1 and C-16, respectively, and the methylenedioxy group was assigned to be at C-7 and C-8 as revealed by the long-range HMBC correlations. The existence of seven oxygenated carbons deduced from its ¹³C NMR spectrum suggests that **1** has one hydroxyl group, in addition to two methoxy groups, two ester groups, and a methylenedioxy group. The location of the hydroxyl group at C-10 was further confirmed by the HMBC correlations.
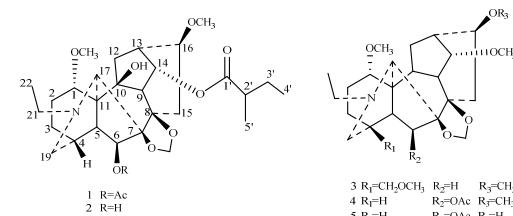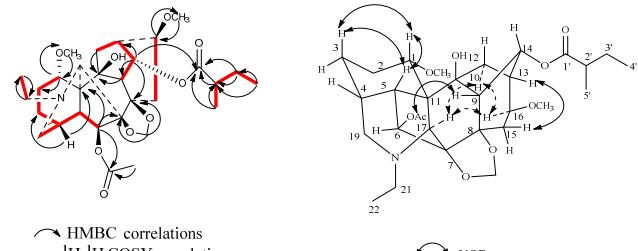
Figure 1: Structures of compounds **1**–**5**.

3 R₁=CH₂OCH₃ R₂=H R₃=CH₃
4 R₁=H R₂=OAc R₃=CH₃
5 R₁=H R₂=OAc R₃=H

⌇ HMBC correlations
— ¹H-¹H COSY correlations
⌒ NOE
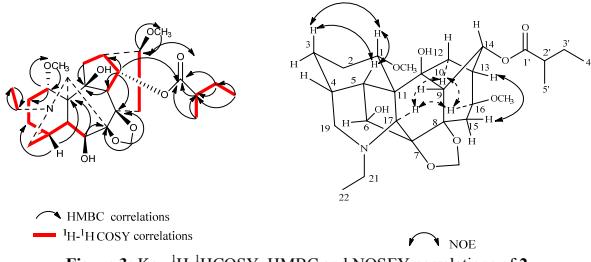
Figure 2: Key ¹H-¹HCOSY, HMBC and NOESY correlations of **1**.

The relative configuration of anthriscifoltine A was deduced from the vicinal coupling constants (Table 1) and a NOESY experiment. In the NOESY spectrum of **1**, the cross-peak between H-17 and H-16 proved that 16-OCH₃ has a β-position. The acetoxyl group at C-6 was determined to have a β-orientation as well based on the multiplicity of H-6 (singlet) in the ¹H NMR spectrum [5a]. The coupling constants of H-1 at δ_H 3.55 (1H, t, *J* = 8.4 Hz) and H-14 at δ_H 5.28 (1H, t, *J* = 4.8 Hz) confirmed the β-position of axial H-1 [5b] and the α-position of H-14, respectively [5c]. Therefore, the structure of anthriscifoltine A (**1**) was determined as shown in Figure 1. The full assignment of anthriscifoltine A (**1**) was based on the 1D- and 2D NMR spectral data (Table 1, Figure 2).

The molecular formula of anthriscifoltine B (**2**) was determined to be C₂₈H₄₃NO₈ from the [M+H]⁺ peak at m/z 522.3084 (calcd. for C₂₈H₄₄NO₈ 522.3067) in the HR-ESI-MS. The IR absorption spectrum indicated that **2** possesses hydroxyl (3445 cm⁻¹) and carbonyl (1731 cm⁻¹) groups. The ¹³C NMR data of anthriscifoltine B (**2**) were very similar to those of **1** except for lacking a signal for an acetyl group. The proton signal of H-6 at δ_H 5.27 in compound **1** was shifted upfield to δ_H 4.27 in compound **2** suggesting that 6-OAc

Table 1: ^{1}H and ^{13}C NMR data for compounds **1** and **2**.

Position	1 δ_C	1 δ_H (mult., J (Hz))	2 δ_C	2 δ_H (mult., J (Hz))
1	77.4 d	3.55 t (8.4)	77.5 d	3.62 t (9.0)
2	26.4 t	2.06 α m, 2.12 β m	26.5 t	2.06 α m, 2.12 β m
3	28.9 t	1.43 β m, 1.80 α m	29.8 t	1.40 β m, 1.73 α m
4	34.1 d	2.09 m	34.7 d	2.08 m
5	44.8 d	1.87 m	46.1 d	1.81 m
6	81.3 d	5.27 s	82.4 d	4.27 s
7	91.9 s	-	92.8 s	-
8	81.7 s	-	82.6 s	-
9	50.4 d	3.49 d (5.4)	51.0 d	3.55 d (5.4)
10	83.4 s	-	83.0 s	-
11	55.5 s	-	55.8 s	-
12	35.5 t	1.71 α m, 2.56 β m	34.9 t	1.74 α m, 2.57 β m
13	37.5 d	2.80 m	36.6 d	2.74 m
14	74.3 d	5.28 t (4.8)	74.3 d	5.28 t (4.8)
15	39.1 t	1.84 β dd (7.8, 16.2) 3.13 α d (16.2)	38.5 t	1.83 β dd (7.8, 16.2) 3.01 α d (16.2)
16	81.7 d	3.23 d (8.4)	81.3 d	3.21 d (8.4)
17	64.8 d	3.08 d (1.8)	64.4 d	3.03 d (1.8)
19	50.7 t	2.83 m	51.0 t	2.75 m
21	50.6 t	2.75 m	50.8 t	2.68 m
22	14.0 q	1.07 t (7.2)	14.0 q	1.06 t (7.2)
1-OCH₃	55.8 q	3.27 s	55.8 q	3.25 s
16-OCH₃	55.9 q	3.28 s	55.9 q	3.26 s
O-CH₂-O	94.0 t	4.89 br s 4.94 br s	93.5 t	5.05 br s 5.13 br s
6-OAc	170.5 s	-	-	-
	21.8 q	2.04 s	-	-
14-MbO 1'	177.0 s	-	176.8 s	-
2'	41.2 d	2.38 m	41.2 d	2.35 m
3'	26.6 t	1.47 m	26.5 t	1.47 m
4'	11.6 q	0.91 t (7.2)	11.7 q	0.90 t (7.2)
5'	16.5 q	1.14 d (6.6)	16.4 q	1.14 d (7.2)

HMBC correlations
^{1}H-^{1}H COSY correlations
NOE

Figure 3: Key ^{1}H-^{1}HCOSY, HMBC and NOSEY correlations of **2**.

in **1** was substituted by a hydroxyl group, which was confirmed by the difference of 42 mass units between those two compounds. Thus, the structure of anthriscifoltine B was determined as compound **2**. The full assignment of anthriscifoltine B (**2**) was based on the 1D- and 2D-NMR spectral data (Table 1, Figure 3). The structures of compounds **3–5** were identified by comparison of their spectral data with those described in the literature [5a,b,d].

Experimental

General: Optical rotations, Perkin-Elmer 341 polarimeter; NMR, Bruker AV600; IR, Thermo Fisher Nicolet 6700; HR-ESI-MS, Waters ACQUITY UPLC/Xevo G2-S QTOF mass spectrometer.

Plant material: The whole herbs of *D. anthriscifolium* var. *majus* were collected in Longshanwa, Zhuxi county, Hubei province of China, in April 2015, and were identified (voucher specimen: L H. Shan & J X. Wang 801) by Prof. Qing-Er Yang of the Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation: Dried and powdered whole herbs of *D. anthriscifolium* (21.5 kg) were extracted with 95% EtOH, 4 times at room temperature, for a week each time. After removal of the solvent, the extract (2000 g) was suspended in water (3 L) and adjusted to pH 2 with HCl, and then successively extracted with light petroleum (4×1 L) and ethyl acetate (4×1 L). The pH value of the aqueous layer was adjusted to 10 with aqueous ammonium hydroxide solution and the subsequent mixture was extracted with CH_2Cl_2 (4×1 L). The CH_2Cl_2 extracts were concentrated to produce the crude alkaloid extract (28.5 g). Column chromatography of the crude alkaloid extract over silica gel, using a CH_2Cl_2:MeOH (80:1, v/v) mixture with increasing polarity afforded fractions A–G based on TLC analysis. Fraction A was separated by silica gel CC (light petroleum /Me₂CO/Et₂N 50: 1: 0.1, v/v/v) to obtain compounds **1** (25 mg), **2** (30 mg), **3** (8 mg), **4** (16 mg) and **5** (20 mg).

Anthriscifoltine A (1)

White amorphous powder.
$[\alpha]_D^{20}$: -15.3 (*c* 0.56, CHCl₃).
IR (KBr) v_{max}: 3462, 2958, 2924, 2873, 2854, 2821, 1736, 1670, 1461, 1368, 1242, 1199, 1156, 1130, 1091, 1057, 962 cm⁻¹.
[1]H (CDCl₃, 600 MHz) and ^{13}C NMR (CDCl₃, 150 MHz): Table 1.
HR-ESI-MS *m/z*: [M+H]⁺ 564.3185 (calcd. for $C_{30}H_{46}NO_9$ 564.3173).

Anthriscifoltine B (2)

White amorphous powder.
$[\alpha]_D^{20}$: -4.9 (*c* 0.56, CHCl₃).
IR (KBr) v_{max}: 3445, 2956, 2924, 2854, 2823, 1731, 1668, 1463, 1379, 1239, 1199, 1156, 1128, 1091, 1058, 964 cm⁻¹.
[1]H (CDCl₃, 600 MHz) and ^{13}C NMR (CDCl₃, 150 MHz): Table 1.
HR-ESI-MS *m/z*: [M+H]⁺ 522.3084 (calcd. for $C_{28}H_{44}NO_8$ 522.3067).

Supplementary data: NMR, IR and HR-ESI-MS for compounds **1** and **2**.

References

[1] (a) Dzhakhangirov FN, Sultankhodzhaev MN, Tashkhodzhaev B, Salimos BT. (**1997**) Diterpenoid alkaloids as a new class of antiarrhythmic agents. Structure-activity relationship. *Chemistry of Natural Compound*, **33**, 190–202; (b) Ulubelen A, Mericli A, Kilincer N, Ferizli AG, Emecki M, Pelletier SW. (**2001**) Insect repellent activity of diterpenoid alkaloids. *Phytotherapy Research*, **15**, 170–171.

[2] Ameri A. (**1998**) The effects of aconitum alkaloids on the central nervous system. *Progress in Neurobiology*, **56**, 211–235.

[3] Pelletier SW, Oliver D. Dailey, Jr., Mody NV. (**1981**) Isolation and structure elucidation of the alkaloids of *Delphinium glaucescens* Rybd. *The Journal of Organic Chemistry*, **46**, 3284-3293.

[4] (a) Pelletier SW, Mody NV, Joshi BS, Schramn LC. (**1984**) *Alkaloids: Chemical and Biological Perspectives*, Vol. **2**, Pelletier SW. (Ed). John Wiley, New York, 205-213; (b) Wang FP. (**1982**) [13]C Nuclear magnetic resonance of diterpenoid alkaloids. *Organic Chemistry*, **3**, 161-169.

[5] (a) Song L, Liang XX, Chen DL, Wang FP. (**2007**) New C₁₈-diterpenoid alkaloids from *Delphinium anthriscifolium* var. *savatieri*, *Chemical & Pharmaceutical Bulletin*, **55**, 918–921; (b) Song L, Liu XY, Chen QH, Wang FP. (**2009**) New C₁₉- and C₁₈-diterpenoid alkaloids from *Delphinium anthriscifolium* var. *savatieri*. *Chemical & Pharmaceutical Bulletin*, **57**, 158–161; (c) Díaz JG, Ruiza JG, Herz W. (**2005**). Norditerpene and diterpene alkaloids from *Aconitum variegatum*. *Phytochemistry*, **66**, 837-846; (d) Salimov BT, Yunusov MS, Abdullaev ND, Vaisov ZM. (**1985**) Corumdephine- a new alkaloid from *Delphinium corumbosum*. *Khimiya Prirodnykh Soedinenii*, **1**, 91–94.

Majusine D: A New C$_{19}$-diterpenoid Alkaloid from *Delphinium majus*

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A new C$_{19}$-diterpenoid alkaloid, designated as majusine D (**1**), has been isolated from *Delphinium majus* W. T. Wang. The structure was elucidated by detailed NMR-spectroscopic studies.

Keywords: *Delphinium majus*, C$_{19}$-diterpenoid alkaloid, Majusine D.

Diterpenoid alkaloids are believed to be the major bioactive components of the genus *Delphinium* [1–4], a large genus within the Ranunculaceae family. *D. majus* W. T. Wang is distributed mainly in southwest Sichuan and northwest Yunnan of mainland China, especially around the Jinsha River basin [5]. In our previous papers, three new C$_{19}$-diterpenoid alkaloids and six new C$_{20}$-diterpenoid alkaloid have been reported [6]. Continuing investigations seeking new bioactive compounds of *D. majus* have now led to the isolation of one other new C$_{19}$-diterpenoid alkaloid, majusine D (**1**). This paper deals with the separation and structural elucidation of this new alkaloid.

Compound **1** was obtained as an amorphous powder. Its positive-ion HRESI-MS showed a quasi-molecular ion peak at *m/z* 466.2812, corresponding to the molecular formula, C$_{25}$H$_{39}$NO$_7$. Its NMR spectra exhibited the presence of an *N*-ethyl group [δ$_H$ 1.03 (3H, t, *J* 7.6 Hz); δ$_C$ 13.7 q, 50.3 t], four methoxyl groups [δ$_H$ 3.33, 3.35, 3.36, 3.42 (each 3H, s); δ$_C$ 56.3 q, 59.3 q, 59.2 q, 51.3 q], two unsaturated methines [δ$_H$ 5.39, 5.66 (each 1H); δ$_C$ 131.5d, 125.0 d]. Its ^{13}C NMR spectrum displayed seven oxygenated carbon signals (δ$_C$ 72.2d, 74.7 d, 78.5d, 81.0 s, 82.2 d, 90.5 d, 91.2s), suggesting that **1** possessed three hydroxyl groups in addition to four methoxyl groups. All of the available evidence revealed that **1** was a lycoctonine-type C$_{19}$-diterpenoid alkaloid [2]. A triplet signal at δ$_H$ 3.95 (*J* = 5.6 Hz) was attributed to H-14β, implying the presence of an oxygen substituted group at the C-14 position. Comparison of the NMR data of **1** (Table 1) with those of deltatsine (**2**) [7] revealed that they were similar (Figure 1). The major difference between them was that methylenes at C-2 and C-3 in deltatsine (**2**) were replaced by two unsaturated methines in **1**. Correlations between OCH$_3$-6 and C-6, OCH$_3$-8 and C-8, OCH$_3$-16 and C-16, OCH$_3$-18 and C-18 in the HMBC spectrum (Figure 2) suggested that the methoxyl groups could be assigned to C-6, and C-8, and C-16, and C-18, respectively. The occurrence of a Δ$^{2(3)}$ double bond was corroborated by the correlations from H-2 to C-4 and from H-19 to C-3 in the HMBC spectrum (Figure 2). The secondary hydroxyl group was located at C-1 on the basis of the correlation with H-2 in the COSY spectrum, while the α-orientation of OH-1 was confirmed in the NOEDS spectrum by the coupling between H-1β (3.62 d) and H-10β (2.27 m). The molecular framework of majusine D is elucidated as Figure 1. Moreover, the ^{13}C NMR spectrum showed that the compound contain an ester carbonyl (δ$_C$ 172.4s), three oxygenated carbon signals (δ$_C$ 74.1 d, 71.4 d, 64.5 t),
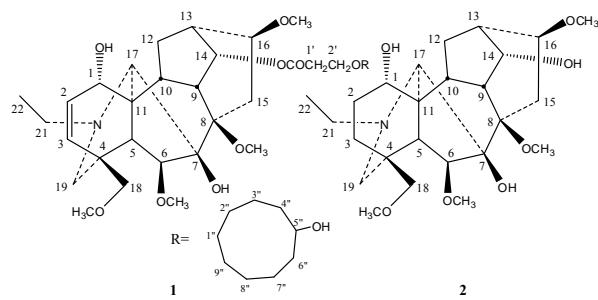
Figure 1: The structures of compounds **1** and **2**.
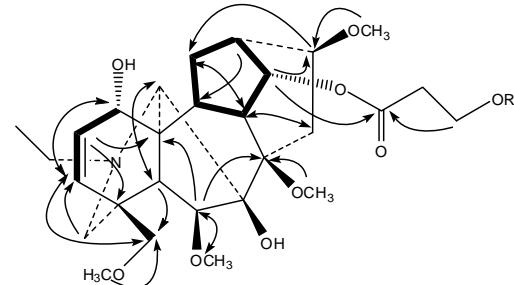
Figure 2: Key ^{1}H - ^{1}H COSY (——) and HMBC (→) correlations of **1**.
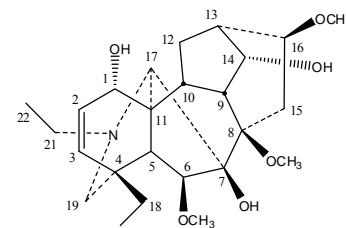
Figure 3: The hydrolysis product of the C$_{14}$-ester group from **1**.

eight secondary carbons (δ$_C$ 33.9, 32.4, 29.1, 28.3, 27.0, 26.8, 25.5, 20.2). In the HMBC spectrum, there are connections of H-14β, H-1′ (δ$_H$ 2.43 2H), and H-2′ (δ$_H$ 4.20, 2H, t, *J* = 5.2 Hz) to ester carbonyl (δ$_C$172.4). In the IR spectrum, a peak at 726 cm^{-1} displayed that the compound contained a series of four connected secondary carbons. Therefore, the structure of majusine D may be deduced as **1** (Figure 1). Compound **1** is a new C$_{19}$-diterpenoid alkaloid, and the hydrolysis product of the C$_{14}$-ester group (Figure 3) from compound **1** is also a new alkaloid.

Table 1: ^{1}H and ^{13}C NMR spectroscopic data for compounds **1** and **2**.

Position		1	2
	δ_H	δ_C	δ_C
1	3.62 d (4.4)	72.2 d	72.2 d
2	5.66 dd (9.2, 4.4)	125.0 d	27.0 t
3	5.39 d (9.2)	131.5 d	29.2 t
4	-	37.0 s	37.1 s
5	1.65 br s	48.7 d	48.8 d
6	3.79 br s	90.5 d	90.6 d
7	-	91.2 s	91.2 s
8	-	81.0 s	81.1 s
9	2.01 m	45.0 d	45.1 d
10	2.27 m	39.7 d	39.8 d
11	-	48.4 s	48.5 s
12	1.61 m, 1.41 m	28.5 t	28.4 t
13	2.26 m	39.7 d	39.8 d
14	3.95 t (5.6)	74.7 d	74.6 d
15	2.58 m, 1.78 m	31.2 t	30.9 t
16	3.38 (hidden)	82.2 d	82.2 d
17	2.82 br s	66.4 d	66.4 d
18	3.11, 3.27 ABq, (8.8)	78.5 t	78.6 t
19	2.37, 2.39 ABq, (7.6)	57.2 t	57.2 t
21	2.77 m, 2.91 m	50.3 t	50.2 t
22	1.03 t (7.6)	13.7 q	13.7 q
6-OCH₃	3.36 s	59.2 q	59.2 q
8-OCH₃	3.42 s	51.3 q	51.3 q
16-OCH₃	3.33 s	56.3 q	56.2 q
18-OCH₃	3.35 s	59.3 q	59.1 q
CO	-	174.2 s	
1'	2.43 (hidden)	33.9 t	
2'	4.20 t (5.2)	64.5 t	

(400 MHz for ^{1}H NMR, 50 MHz for ^{13}C NMR, CDCl₃, δ in ppm, *J* in Hz)

Experimental

General: Optical rotation was measured on a Autopol Automatic Polarimeter. IR spectra were obtained on a Perkin-Elmer FT-IR Spectrum Two. ^{1}H and ^{13}C NMR spectra were taken on a Varian Unity INOVA 400/45 NMR spectrometer in CDCl₃ with TMS as the internal standard. The ESIMS and HRESIMS were recorded on either a VG Auto Spec 3000 or a Finnigan-MAT 90 instrument. Silica gel H (Qingdao Sea Chemical Factory, Qingdao, People's Republic of China) was used for column chromatography. Zones on TLC (silica gel G) were detected using modified Dragendorff's reagent.

Plant material: *Delphinium majus* was collected in Yanbian County, Sichuan Province, People's Republic of China, in June 2012. The plant was authenticated by associate professor Qi Zhao of the Key Laboratory of Medicinal and Edible Plant Resources, Development of Sichuan Education Department, Chengdu University, where a voucher specimen (201201) has been deposited.

Extraction and isolation: Air-dried and powdered whole herbs of *Delphinium majus* (2.0 kg) were percolated with 0.1 M HCl (7 L). The obtained acid aqueous solution was basified with 10% aqueous NH₄OH to pH 9-10 and then extracted with ethyl acetate (4 L × 3). Removal of the solvent under reduced pressure afforded the total crude alkaloids (8.2 g) as a yellowish amorphous powder, which was chromatographed over a silica gel column, eluting with cyclohexane-acetone (8:1→1:1) gradient system, to give fractions A (105 mg), B (2.3 g), C (1.8 g), and D (2.9 g). Fraction D was separated over a silica gel H column, eluting with CHCl₃-CH₃OH (40:1 → 10:1), to yield majusine D (**1**) (11 mg).

Majusine D (1)

White amorphous powder.

$[\alpha]_D^{20}$: +22.6 (*c* 1.0, CHCl₃).

IR (KBr): 3478, 2939, 1636, 1233,726 cm⁻¹.

^{1}H NMR: Table 1.

^{13}C NMR: Table 1.

ESI-MS *m/z* (%): 466 (100), 678[M+H]⁺ (1).

HR-ESI-MS *m/z*: found 466.2812 (100), calcd. for C₂₅H₃₉NO₇, 466.2804; 678.4166 (1.2)[M+H]⁺, calcd.for.C₃₇H₆₀NO₁₀, 678.4139.

References

[1] Wang, FP, Liang XT. (**1992**) Chemistry of the diterpenoid alkaloids. In *The Alkaloids: Chemistry and Physiology*, Vol. **42**, Cordell GA (Ed.), Academic Press, New York, 151–247.

[2] Wang FP, Chen QH, Liang XT. (**2009**) The C19-diterpenoid alkaloids. In *The Alkaloids: Chemistry and Biology*, Vol. **67**, Cordell GA (Ed.), Elsevier Science, New York, 101-298.

[3] Wang FP, Chen QH. (**2010**) The C19-diterpenoid alkaloids. In *The Alkaloids: Chemistry and Biology*, Vol. **69**, Cordell GA (Ed.), Elsevier Science, New York, 1-577.

[4] Wang FP, Chen QH, Liu XY. (**2010**) Diterpenoid alkaloids. *Natural Product Reports*, **27**, 529-570.

[5] Chinese Academy of Science China Flora Editorial board. (**1979**) Flora *Repubulicae Popularis Sinicae*, Vol, **27**. Beijing, Science Press, 442.

[6] Chen FZ, Chen DL, Chen QH, Wang FP. (**2009**) Diterpenoid Alkaloids from *Delphinium majus*. *Journal of Natural Products*, **72**, 18-23.

[7] Joshi BS, Glinski JA, Chokshi HP. (**1984**) Deltatsine, a new C19-diterpenoid alkaloid from *Delphinium tatsienense* Franch. *Heterocycles*, **22**, 2037-2042.

Epoxide Opening of a 7,17-*Seco*-7,8-Epoxy-C$_{19}$-Diterpenoid Alkaloid

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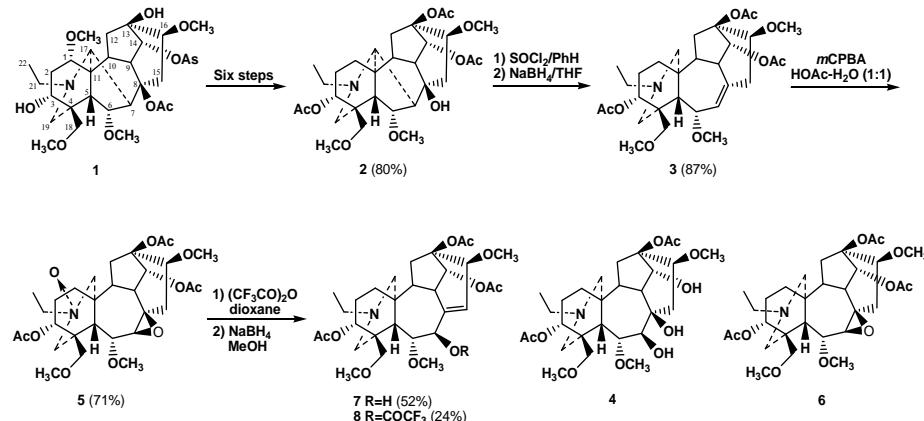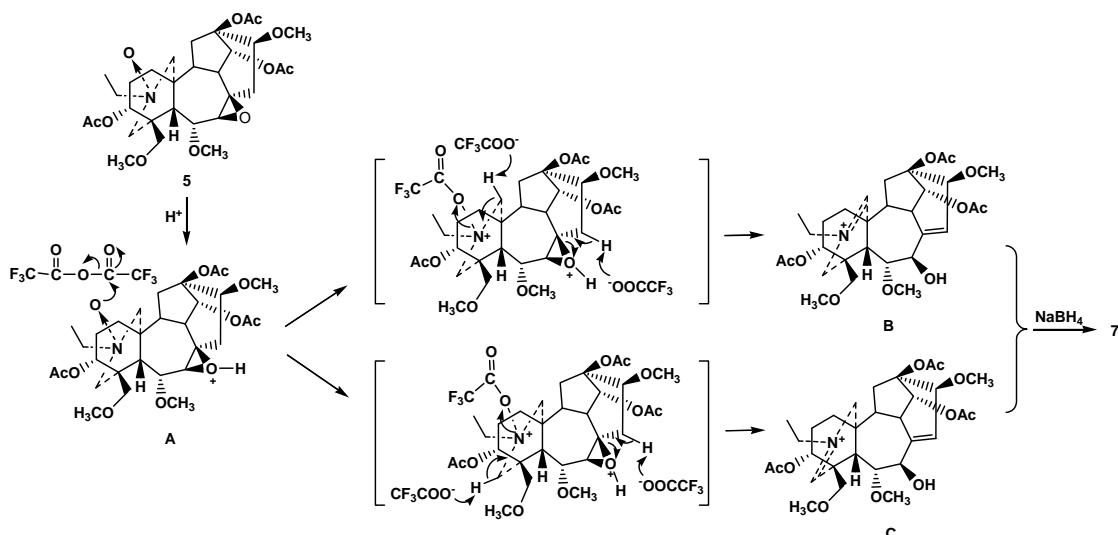
A new and effective approach toward epoxide opening of a 7,17-*seco*-7,8-epoxy-C$_{19}$-diterpenoid alkaloid is herein described. The starting epoxide was prepared from naturally occurring yunnaconitine via a nine-step transformation. Treatment of this epoxide with trifluoroacetic anhydride in dioxane at 110°C followed by reduction with sodium boron hydride generated two epoxide opening compounds **7** and **8**. Each of their structures is characteristic of a $\Delta^{8,15}$ bridgehead double bond and a 7β-oxygen-substituted group.

Keywords: C$_{19}$-diterpenoid alkaloid, Epoxide-opening.

Numerous C$_{19}$-diterpenoid alkaloids have been isolated from a variety of *Aconitum* and *Delphinium* (Ranunculaceae). Certain species have long been used as traditional Chinese medicines for the treatment of various diseases [1-2]. It has been demonstrated that C$_{19}$-diterpenoid alkaloids exhibited a plethora of biological activities [3-4]. We started to develop conversional synthesis of taxoids starting from C$_{19}$-diterpenoid alkaloids since early 1990s. During the course, several novel approaches to the taxane ABC ring system and numerous intriguing reactions of C$_{19}$-diterpenoid alkaloids have been reported [5-13]. We have found that modifications of ring B, including cleavage of C7–C17, enlargement of ring B, functionalization of C-7 and C-8 for assembly of the $\Delta^{8,15}$ bridgehead double bond, and introduction of functional groups with specific configurations, were part of the critical steps to our successful conversions from aconitine-type C$_{19}$-diterpenoid alkaloids [5-7] to the core structure of taxoids. During the B-ring modifications of yunnacotinine [11], an efficient and convenient approach to the 7,8-epoxide opening was found. We herein describe this epoxide-opening reaction, together with the structure characterization of the novel C$_{19}$-diterpenoid alkaloid derivatives resulted from this reaction.

Naturally occurring yunnaconitine (**1**) was readily converted to its derivative **2** through a six-step procedure in 80% overall yield according to the procedure as previously described by us [7]. In hope to facilitate the functionalization of C-7 and C-8, the C7–C17 bond in alkaloid **2** was cleaved by reacting with thionyl chloride in benzene followed by reducing with NaBH$_4$ to furnish compound **3** (Figure 1) [7]. The oxygenated groups at C-7 and C-8 was desired for the construction of the $\Delta^{8,15}$ bridgehead double bond in taxoid ABC ring system. Several attempts to introduce oxygenated groups at C-7 and C-8 were unfortunately failed. For example, addition of the $\Delta^{7,8}$ double bond in **3** through oxymercuration reaction did not occurred. The attempted addition with HOBr or Br$_2$ was also not successful probably due to the participation of the nitrogen atom. Dihydroxylation of 7,17-*seco*-diterpenoid alkaloid **3** produced expected 7,8-oxygenated alkaloid **4**. As compared with **3**, the NMR spectra of **4** showed the absence of the $\Delta^{7,8}$ double bond as well as an acetyl group, and the presence of two additional oxygenated carbons (δ_H 3.75 d and δ_C 71.3 d for a tertiary carbon; δ_C 76.0 s for a quaternary carbon), indicating the structure of compound **4** as described in Figure 1. However, we had to give up this synthetic method due to the extremely low yield of **4**.

Figure 1

Scheme 1: A Plausible Mechanism from **5** to Compounds **7** and **8**

Table 1: NMR spectral data of compound **7** in CDCl₃ (400 MHz for ¹H, 100 MHz for ¹³C).

No.	δ_C	δ_H mult. (J in Hz)	HMBC (H→C)
1	27.7 t	1.59 m	C-2, C-3, C-5, C-11
		1.77 m	C-2, C-3, C-5, C-11
2	34.9 t	1.37 m	C-1, C-4, C-11
		1.82 m	C-1, C-3, C-4, C-11
3	72.5 d	5.02 dd (10.8, 6.8)	C-2, C-4, C-18, C-19
4	42.5 s	–	–
5	39.4 d	1.71 brs	C-3, C-4, C-6, C-11, C-18
6	81.0 d	3.60 d (5.2)	C-4, C-5, C-7, C-8, C-11, 6-OCH₃
7	74.1 d	3.97 d (5.2)	C-6, C-8, C-9
8	137.1 s	–	–
9	47.5 d	2.62 m	C-7, C-8, C-11, C-12, C-15
10	38.5 d	2.47 m	C-8, C-9, C-11, C-12, C-14
11	37.4 s	–	–
12	45.5 t	1.23 m	C-10, C-14
		1.94 m	C-9, C-11, C-13, C-14
13	85.1 s	–	–
14	74.8 d	5.47 d (3.6)	C-8, C-10, C-13
15	122.3 d	5.78 d (5.2)	C-8, C-9, C-13, C-16
16	80.9 d	4.45 d (5.2)	C-8, C-12, C-14, 16-OCH₃
17	52.2 t	2.12 m	C-5, C-10, C-11
		2.38 m	C-5, C-11
18	73.2 t	3.37 ABq (9.2)	C-3, C-4, C-5, C-19, 18-OCH₃
		3.52 ABq (9.2)	C-3, C-4, C-5, C-19, 18-OCH₃
19	57.3 t	2.32 m	C-3, C-5, C-18
		2.50 m	C-3, C-4, C-5, C-18
21	50.9 t	2.42 q (6.8)	C-22
22	12.1 q	0.98 t (6.8)	C-21
6-OCH₃	56.1 q	3.26 s	C-6
16-OCH₃	58.8 q	3.46 s	C-16
18-OCH₃	59.2 q	3.33 s	C-18
3-OAc	171.0 s	–	–
	21.1 q	2.03 s	3-OCO
13-OAc	170.9 s	–	–
	21.9 q	2.07 s	13-OCO
14-OAc	170.3 s	–	–
	21.2 q	2.06 s	14-OCO

In order to avoid the possible oxidation of nitrogen and subsequently to improve the yield, acidic conditions were selected for the epoxidation of alkaloid **3**. Exposure of compound **3** to 30% H₂O₂ in formic acid for 2 h generated epoxides **5** (59%) and **6** (10%). The molecular formulas of compounds **5** and **6** (C₃₀H₄₅NO₁₁ and C₃₀H₄₅NO₁₀) were determined by their HR-MS data. In the NMR spectra of **6**, the signals for the Δ⁷,⁸ double bond disappeared while the characteristic signals at δ_H 3.75 (1H, d, J = 8.8 Hz), and δ_C 65.6 d and 60.4 s were observed. Compound **5** possesses the same unsaturated degree as **6**, but with 16 more mass units as compared with **6** as determined by their mass spectra, indicating that epoxide **5** is the *N*-oxidation derivative of **6**. The NMR spectra of **5** exhibited characteristic signals at δ_H 1.61 (3H, t,

J = 7.0 Hz) and δ_C 8.7 q for an *N*-ethyl group [14]. The comparison of the ¹³C-NMR spectra of **5** and **6** showed different δ values at C-1, C-2, and C-3, especially at C-17, C-19, C-21, and C-22, due to the *N*-oxidation effect [14]. This led to the determination of the structure for epoxide **5**. Treatment of **3** with an excess *m*CPBA in HOAc-H₂O (1:1) under refluxing conditions also afforded compound **5** in 71% yield. Considering that the nitrogen oxidation was inevitable and that attempt to reduce **5** into **6** failed, epoxide **5** was directly used as a substrate for the subsequent epoxide ring-opening reaction. We attempted to treat **5** with acid activated silica gel or various kinds of acids (98% formic acid, 20% H₂SO₄ and trifluoroacetic acid *etc.*) respectively, but no reactions occurred in such cases. An acetic anhydride instead of acids was used in the reaction, resulting in an unidentified product. We then tried to use trifluoroacetic anhydride which yielded two inseparable, highly polar products. The ¹H NMR spectrum of the crude product suggested that they are the corresponding iminium salts containing the Δ⁸,¹⁵ double bond. Without further purification, the crude product was directly reduced with NaBH₄ in MeOH, leading to two expected 7-oxygenated-7,17-*seco*-diterpenoid alkaloids **7** and **8**. The molecular formulas for these two desired products are C₃₀H₄₅NO₁₀ and C₃₂H₄₄F₃NO₁₁, respectively, as determined by their HR-MS data. The NMR spectra of **7** displayed the absence of the signals for the epoxide moiety but the presence of characteristic signals at δ_H 5.78 (1H, d, J = 5.2 Hz), and δ_C 137.1 s and 122.3 d for a trisubstituted double bond. This double bond was attributed to C-8 and C-15 due to the multi-bond ¹H-¹³C correlations of H-15 with C-8, C-9, C-13 and C-16 in its HMBC spectrum. In the NMR spectra of **7**, in addition to the distinctive upfield shifts of C-17, C-19, and C-21, the chemical shifts for *N*-ethyl group at δ_H 0.98 (3H, t, J = 6.8 Hz) and δ_C 12.1 q were also greatly changed as compared with those of **5**, suggesting that the *N*-oxide in **5** has been reduced back to tertiary amine. The structure of **7** was confirmed by its 2D NMR data, as shown in Table 1. The NMR spectra of **8** are very similar to those of **7**, except for the existence of signals for an additional trifluoroacetyl group. The structure of **8** was thus determined as illustrated in Figure 1. The plausible mechanism for the formation of 7-oxygenated compounds **7** and **8** was proposed. As shown in Scheme 1, in the presence of trifluoroacetic anhydride and a trace amount of H₂O in dioxane, protonation of epoxide oxygen in **5**, followed by ring opening and elimination resulted in the formation of the Δ⁸,¹⁵ double bond. Additionally, esterification of *N*-oxide in **5** with trifluoroacetic anhydride and subsequent

Polonovski-like process can produce a pair of regioisomeric immonium salts **B** and **C**. Reduction of immonium salts may yield compound **7** while reduction of the immonium salts followed by acylation may generate compound **8**.

Table 2: ^{13}C NMR spectral data of compounds 4-6 and 8 (50 MHz, CDCl$_3$).

No.	4	5	6	8
1	27.9 t	23.2 t	26.4 t	26.8 t
2	32.5 t	36.7 t	38.0 t	34.3 t
3	74.4 d	72.5 d	74.5 d	71.9 d
4	42.7 s	41.6 s	42.5 s	41.0 s
5	44.2 d	45.0 d	45.3 d	37.7 d
6	81.8 d	79.9 d	79.6 d	77.7 d
7	71.3 d	65.6 d	65.6 d	76.1 d
8	76.0 s	60.4 s	60.5 s	137.6 s
9	42.6 d	54.2 d	53.8 d	47.9 d
10	39.1 d	38.8 d	39.3 d	42.2 d
11	37.4 s	38.5 s	37.4 s	37.4 s
12	34.5 t	37.7 t	37.4 t	43.8 t
13	84.3 s	84.2 s	83.8 s	85.0 s
14	80.9 d	78.9 d	79.2 d	74.5 d
15	46.4 t	46.1 t	46.7 t	121.9 d
16	81.3 d	81.2 d	80.9 d	79.3 d
17	58.4 t	69.6 t	54.7 t	51.6 t
18	73.5 t	72.4 t	72.0 t	70.0 t
19	52.3 t	65.2 t	48.1 t	57.7 t
21	50.8 t	62.4 t	51.5 t	50.5 t
22	12.1 q	8.7 q	13.3 q	12.2 q
6-OCH$_3$	56.3 q	56.2 q	55.8 q	56.1 q
16-OCH$_3$	58.1 q	57.3 q	57.6 q	58.6 q
18-OCH$_3$	59.2 q	59.1 q	58.9 q	59.1q
3-OAc	170.8 s	170.7 s	170.6 s	170.7 s
	21.2 q	21.2 q	21.1 q	21.1 q
13-OAc	170.2 s	170.5 s	170.4 s	170.9 s
	21.7 q	21.6 q	21.4 q	21.5 q
14-OAc	–	170.1 s	170.1 s	170.3 s
	–	21.4 q	21.2 q	21.3 q
7-OCOCF$_3$	–	–	–	159.3 s
	–	–	–	114.7 s

In conclusion, a convenient and effective epoxide-opening method of 7,17-*seco*-7,8-epoxy-C$_{19}$-diterpenoid alkaloid was found. The products **7** and **8** achieved from this study serve as critical intermediates for the assembly of a taxoid ring system bearing a $\Delta^{8,15}$ bridgehead double bond and an oxygenated group at 7β position. Higher reactivity of trifluoroacetic anhydride and the rigid structure of the C$_{19}$-diterpenoid alkaloid derivatives may be the main reasons for the formation of products **7** and **8** with the $\Delta^{8,15}$ bridgehead double bond and with the hydroxyl group at the 7β position.

Experimental

General: Melting points were determined on a Kofler block (uncorrected); IR spectra were recorded on a Nicolet FT-IR 200 SXV spectrometer; Optical rotations were measured in a 1.0-dm cell by a PE-314 polarimeter at 20 ± 1 °C; ^{1}H- and ^{13}C-NMR spectra were acquired on a Varian INOVA 400/54 or a Bruker AC-E 200 spectrometer in CDCl$_3$ with TMS as internal standard; MS spectra were obtained on Finnigan LCQ and Micromass Auto Spec Ultima-Tof spectrometer; Silica gel GF$_{254}$ and H (10-40 μm, Qingdao Sea Chemical Factory, China) were used for TLC, Chromatotron, and CC.

Compound 4: A solution of KMnO$_4$ (131 mg, 0.83 mmol) in H$_2$O (13 mL) was added dropwise to a solution of compound **3** (235 mg, 0.41 mmol) and NaCO$_3$ (470 mg 5.66 mmol) in dioxane-H$_2$O (1:1, 20 mL). The mixture was stirred at room temperature for 20 min. The precipitate was removed by vacuum filtration and washed with dioxane. The filtrate and washings were combined, and the solvent was removed under reduced pressure. This residue was suspended in H$_2$O (20 mL) and extracted with ethyl acetate (20 mL×3), the combined organic extracts were dried with anhydrous NaSO$_4$, and the solvent was removed under reduced pressure. Chromatography

of the residue (200 mg) on silica gel (6 g) using cyclohexane–acetone (4:1) as eluent afforded compound **4** (white amorphous powder, 30 mg, 13%).
MP: 166-168ºC.
$[\alpha]_D^{20}$: -32.5 (*c* 1.20, CHCl$_3$).
IR (KBr): 3462, 1742, 1451, 1253 cm^{-1}.
^{1}H NMR (400 MHz, CDCl$_3$): 0.99 (3H, t, *J* = 7.2 Hz, NCH$_2$*CH$_3$*), 2.04, 2.10 (each 3H, s, 2×OAc), 3.26, 3.37, 3.38 (each 3H, s, 3×OMe), 3.75 (1H, d, *J* = 5.2 Hz, H-7α), 3.89 (1H, d, *J* = 5.2Hz, H-14β), 4.31 (1H, t, *J* = 4.8 Hz, H-16α), 4.35 (1H, d, *J* = 6.0 Hz, H-6β), 4.99 (1H, dd, J_1 = 11.2 Hz, J_2 = 7.2 Hz, H-3β), 5.56 (1H, d, *J* = 4.8 Hz, OH-7).
^{13}C NMR: Table 2.
ESI-MS: *m/z* (%) = 556 [M + H$^+$] (100).
HRMS-ESI: *m/z* [M + H$^+$] calcd for C$_{28}$H$_{46}$NO$_{10}$: 556.2998; found: 556.2989.

Compound 5 and 6: A solution of compound **3** (135 mg, 0.24 mmol) in HCOOH (3 mL) was cooled in an ice-water bath, and 30% H$_2$O$_2$ (2 mL) was added dropwise. After stirring for 10 min, the mixture was allowed to warm to room temperature and stirred for 2 h. After pouring into ice water (5 mL), the solution was basified with conc. NH$_4$OH to pH 10. The aqueous layer was extracted with CHCl$_3$ (8 mL×3). The combined extracts were dried (Na$_2$SO$_4$) and concentrated. The residue (153 mg) was subjected to column chromatography (silica gel H, 3 g), eluted with CHCl$_3$–MeOH (18:1) to afford **5** (white amorphous powder, 84 mg, 59%) and **6** (white amorphous powder, 14 mg, 10%).

Compound **5**
MP: 116-118ºC.
$[\alpha]_D^{20}$: +18.6 (*c* 2.30, CHCl$_3$).
IR (KBr): 1738, 1261, 892, 811 cm^{-1}.
^{1}H NMR (200 MHz, CDCl$_3$): 1.61 (3H, t, *J* = 7.0 Hz, NCH$_2$*CH$_3$*), 2.08, 2.11, 2.13 (each 3H, s, 3×OAc), 3.25, 3.35, 3.44 (each 3H, s, 3×OMe), 3.91 (1H, t, *J* = 8.0 Hz, H-16α), 4.49 (1H, d, *J* = 6.2 Hz, H-6β), 4.67 (1H, brs, H-7), 4.84 (1H, d, *J* = 5.0 Hz, H-14β), 5.18 (1H, dd, J_1 = 10.8 Hz, J_2 = 7.2 Hz, H-3β).
^{13}C NMR: Table 2.
ESI-MS: *m/z* (%) = 596 [M + H$^+$] (100).
HRMS-ESI: *m/z* [M + H$^+$] calcd for C$_{30}$H$_{46}$NO$_{11}$: 596.3075; found: 596.3087.

Compound **6**
MP: 132-134ºC.
$[\alpha]_D^{20}$: -27.3 (*c* 0.68, CHCl$_3$).
IR (KBr): 1738, 1245, 1193, 936, 797 cm^{-1}.
^{1}H NMR (400 MHz, CDCl$_3$): 1.10 (3H, t, *J* = 7.2 Hz, NCH$_2$*CH$_3$*), 2.04, 2.06, 2.09 (each 3H, s, 3×OAc), 3.21, 3.25, 3.32 (each 3H, s, 3×OMe), 3.75 (1H, d, *J* = 8.8 Hz, H-7), 3.82 (1H, t, *J* = 8.0 Hz, H-16α), 4. 01 (1H, d, *J* = 6.4 Hz, H-6β), 4.89 (1H, d, *J* = 5.2 Hz, H-14β), 4.93 (1H, dd, J_1 = 12.4 Hz, J_2 = 5.6 Hz, H-3β).
^{13}C NMR: Table 2.
ESI-MS: *m/z* (%) = 580 [M + H$^+$] (100).
HRMS-ESI: *m/z* [M + H$^+$] calcd for C$_{30}$H$_{46}$NO$_{10}$: 580.2995; found: 580.2982.

Compound 5: To a solution of compound **3** (135 g, 0.24 mmol) in HOAc-H$_2$O (1:1, 5 mL) was added *m*CPBA (165 mg, 0.96 mmol). The mixture was refluxed for 2 h and cooled prior to being poured into ice water (5 mL). The solution was basified with conc. NH$_4$OH solution to pH 10 and extracted with CHCl$_3$ (8 mL×3). The extracts were dried (Na$_2$SO$_4$) and concentrated. Column chromatography

(silica gel H, 3 g) of the residue (148 mg) using CHCl$_3$–MeOH (18:1) as eluent afforded **5** as a white amorphous powder (102 mg, 71%).

Compound 7 and 8: To a solution of compound **5** (142 mg, 0.24 mmol) in dioxane (10 mL) was added trifluoroacetic anhydride (1 mL). The mixture was refluxed for 3 h and then concentrated under reduced pressure. The residue was diluted with H$_2$O (10 mL), basified with conc. NH$_4$OH solution to pH 10, and extracted with CHCl$_3$ (8 mL×3). The extracts were dried (Na$_2$SO$_4$) and concentrated to give a crude. The crude was dissolved in MeOH (5 mL), to which was added NaBH$_4$ (36 mg, 0.96 mmol). The solution was stirred at room temperature for 4 h. After removal of the solvent, the residue was diluted with water (5 mL). The subsequent mixture was extracted with ethyl acetate (4 mL×3), the extracts were dried (Na$_2$SO$_4$), and the organic solvent was removed *in vacuo*. Chromatography of the residue (138 mg) on silica gel (4 g) using cyclohexane–acetone (6:1) as eluent afforded **7** (a white amorphous powder, 71 mg, 52%) and **8** (a white amorphous powder, 39 mg, 24%).

Compound **7**
MP: 123-124ºC.

[α]$_D^{20}$: +23.3 (*c* 0.82, CHCl$_3$).

IR (KBr): 3468, 1740, 1452, 1366, 1092 cm^{-1}.
^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
ESI-MS: *m/z* (%) = 580 [M + H$^+$] (100).
HRMS-ESI: *m/z* [M + H$^+$] calcd for C$_{30}$H$_{46}$NO$_{10}$: 580.2995; found: 580.2981.

Compound **8**
MP: 137-139ºC.

[α]$_D^{20}$: +10.5 (*c* 0.24, CHCl$_3$).

IR (KBr): 1788, 1525, 1374, 1078 cm^{-1}.
^{1}H NMR (400 MHz, CDCl$_3$): 0.98 (3H, t, *J* = 6.8 Hz, NCH$_2$*CH$_3$*), 2.02, 2.04, 2.08 (each 3H, s, 3×OAc), 3.10, 3.41, 3.47 (each 3H, s, 3×OMe), 3.85 (1H, d, *J* = 5.2 Hz, H-6β), 4.04 (1H, d, *J* = 4.8Hz, H-16α), 4.94 (1H, dd, *J$_1$* = 11.2 Hz, *J$_2$* = 7.2 Hz, H-3β), 5.47 (1H, d, *J* = 3.6 Hz, H-14β), 5.57 (1H, d, *J* = 4.8 Hz, H-7), 5.96 (1H, d, *J* = 5.2 Hz, H-15).
^{13}C NMR: Table 2.
ESI-MS: *m/z* (%) = 698 [M + Na$^+$] (100).
HRMS-ESI: *m/z* [M + Na$^+$] calcd for C$_{32}$H$_{44}$F$_3$NO$_{11}$Na: 698.2259; found: 698.2272.

References

[1] Amiya T, Bando H. (**1988**) *The Alkaloids*. Vol. **34**, Brossi A. (Ed). Academic Press, San Diego, 95-179.
[2] Wink M. (**1998**) *Alkaloids: Biochemistry, Ecology, and Medicinal Applications*. Roberts MF, Wink M. (Ed). Plenum Press, New York. 11-44.
[3] Wang FP, Chen QH. (**2010**) The C$_{19}$-diterpenoid alkaloids. In *The Alkaloids, Chemistry and Biology*. Vol. **69**, Cordell, GA. (Ed). Elsevier, London UK. 1-577.
[4] Wang FP, Chen QH, Liu XY. (**2010**) Diterpenoid alkaloids. *Natural Product Reports*, **27**, 529-570.
[5] Wang FP, Xu L. (**2005**) To seek an approach toward the chemical conversion of C$_{19}$-diterpenoid alkaloids to taxoids. *Tetrahedron*, **61**, 2149-2167.
[6] Zou CL, Cai L, Ji H, Xie GB, Wang FP. (**2008**) A novel approach to the taxane ABC ring system through chemical conversion from C$_{19}$-diterpenoid alkaloid deltaline. *Tetrahedron*, **64**, 7594-7604.
[7] Ji H, Chen QH, Zhu M, Wang FP. (**2010**) Conversional studies towards taxoids from C$_{19}$-diterpenoid alkaloids by the BAC sequence. *Journal of Asian Natural Product Research*, **12**, 968-977.
[8] Cheng H, Xu L, Chen DL, Chen QH, Wang FP. (**2012**) Construction of functionalized B/C/D-ring system of C$_{19}$-diterpenoid alkaloids via intramolecular Diels-Alder reaction followed by Wagner-Meerwein rearrangement. *Tetrahedron*, **68**, 1171-1176.
[9] Liang XX, Tang P, Chen QH, Wang FP. (**2012**) Synthesis of taxane ABC tricyclic skeleton from lycoctonine. *Natural Product Communications*, **7**, 697-703.
[10] Liu ZG, Xu L, Chen QH, Wang FP. (**2012**) Construction of A/E/F ring systems of C$_{19}$-diterpenoid alkaloids with both C-1 and C-6 oxygen functions. *Tetrahedron*, **68**, 159-165.
[11] Ji H, Chen QH, Wang FP. (**2009**) A novel conversion of C$_{19}$-diterpenoid alkaloids into aconane-type diterpenes with eight-membered ring system *via* skeletal rearrangement of corresponding diazonium derivatives. *Chemical and Pharmaceutical Bulletin*, **57**, 233-239.
[12] Wang FP, Chen QH. (**2003**) Further studies on the synthesis of 7,17-*seco* norditerpenoid alkaloids. *Journal of Asian Natural Products Research*, **5**, 43-48.
[13] Wang L, Chen QH, Wang FP. (**2012**) Unusual reactions of a 7,17-*seco*-type C$_{19}$-diterpenoid alkaloid derived from deltaline. *Natural Product Communications*, **7**, 721-724.
[14] Bai YL, Desai HK, Pelletier SW. (**1995**) *N*-oxides of some norditerpenoid alkaloids. *Journal of Natural Products*, **58**, 929-933.

Further Studies on Structure-Cardiac Activity Relationships of Diterpenoid Alkaloids

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The cardiac effect of thirty-eight diterpenoid alkaloids was evaluated on the isolated bullfrog heart model. Among them, twelve compounds exhibited appreciable cardiac activity, with compounds **3** and **35** being more active than the reference drug lanatoside. The structure-cardiac activity relationships of the diterpenoid alkaloids were summarized based on our present and previous studies [2]: i) 1α-OMe or 1α-OH, 8-OH, 14-OH, and NH (or *N*Me) are key structural features important for the cardiac effect of the aconitine-type C_{19}-diterpenoid alkaloids without any esters. C_{18}-diterpenoid alkaloids, lycoctonine-type C_{19}-diterpenoid alkaloids, and the veatchine- and denudatine-type C_{20}-diterpenoid alkaloids did not show any cardiac activity; ii) the presence of 3α-OH is beneficial to the cardiac activity; iii) the effect on the cardiac action of 6α-OMe, 13-OH, 15α-OH, and 16-demethoxy or a double bond between C-15 and C-16 depends on the substituent pattern on the nitrogen atom.
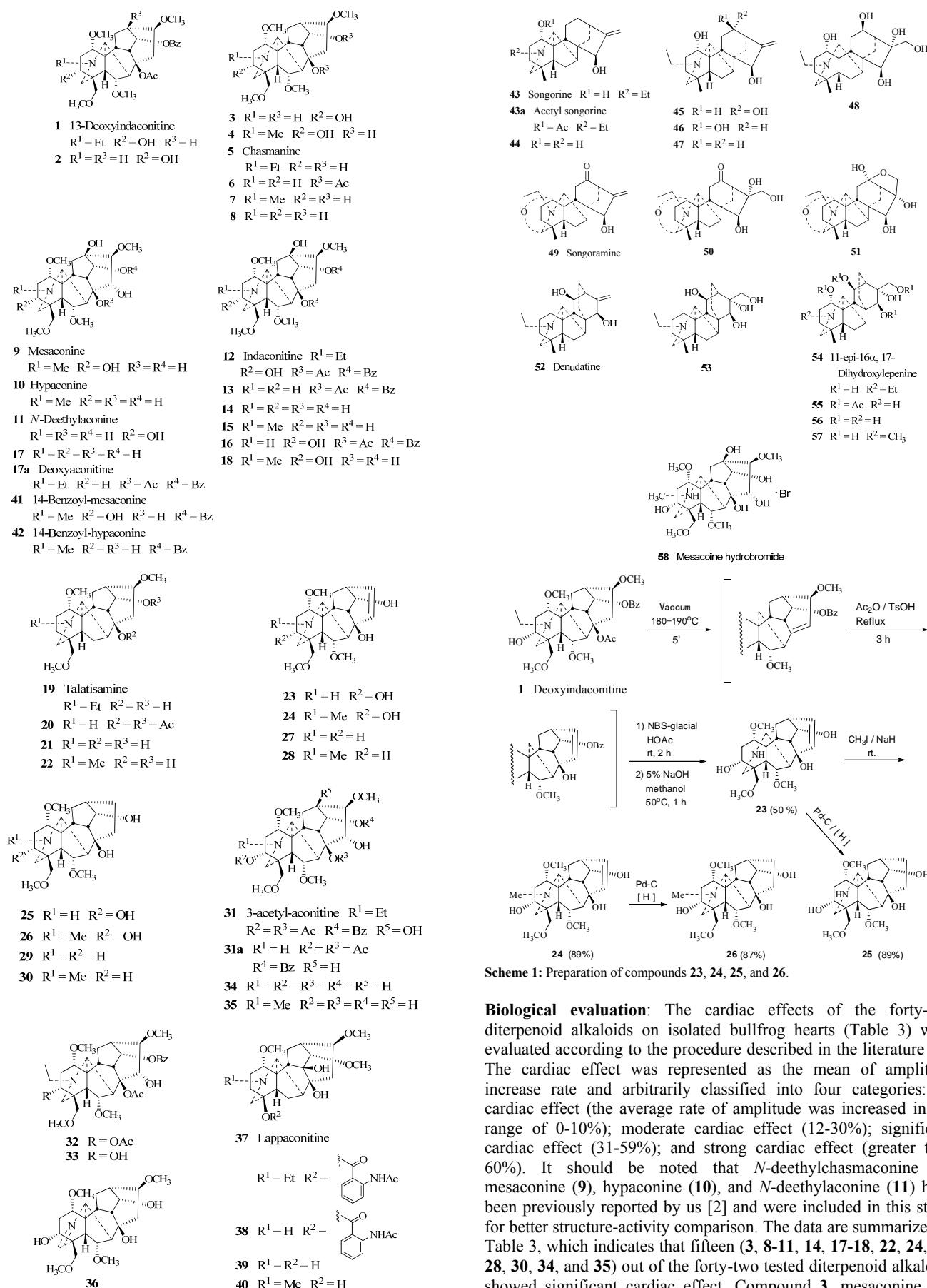
Keywords: Diterpenoid alkaloid, Cardioactive evatuation, Structure-activity relationships.

"Fuzi" (*Aconitum carmichaeli*) is a profound folk medicine throughout China, Japan, and Korea, as well as other regions in Southeast Asia. In search of diterpenoid alkaloids with considerable cardiac effect, we have previously isolated C_{19}-diterpenoid alkaloids as its chemical components with cardiac activity [1] and initiated the associated structure-activity relationships [2]. For further in-depth structure-activity studies and search for possible candidates for clinical development, thirty-eight C_{18}, C_{19}, and C_{20}-diterpenoid alkaloids were designed and synthesized using either mesaconine and hypaconine or lappaconitine, songorine, acetylsongorine, denudatine, and 11-epi-16α,17-dihydroxylepenine as the starting materials. Their cardiac effect was assessed on the isolated bullfrog heart model. The preparation, biological evaluation, and systematic structure-activity relationships of these alkaloids are presented herein.

The known and naturally occurring diterpenoid alkaloids, 13-deoxyindaconitine, chasmanine, 14-benzoylmesaconine, 14-benzoylmesaconine, indaconitine, deoxyaconitine, talatisamine, 3-acetylaconitine, lappaconitine, songorine, acetylsongorine, songoramine, denudatine, 11-epi-16α,17-dithydroxylepenine, and mesaconine hydrobromide, were extracted from either *Aconitum* or *Delphinium* spp. by one of our laboratories.

All *N*-deethylation of diterpenoid alkaloids in this paper was achieved according to the method reported by us [3, 4]; all *N*-methylation was accomplished by reacting with methyl iodide in the presence of sodium hydride. Compound **3** was prepared by *N*-deethylation of 13-deoxyindaconitine (**1**) followed by hydrolysis. Compound **4** was prepared by methylation of **2** followed by hydrolysis. Compound **6** was made by acylation of chasmanine (**5**), followed by *N*-deethylation. Compounds **7** and **8** were obtained by methylation of **6** followed by hydrolysis. Compound **13** was prepared by treatment of indaconitine (**12**) with thionyl chloride, followed by hydrogenation (H₂/Pd). Compounds **14** and **15** were

made from **13** using a similar procedure as that illustrated for the preparation of compounds **3** and **4**, respectively. Similarly, compounds **17** and **18** were prepared from **17a** and **12**, respectively. Compounds **21** and **22** were produced from talatisamine (**19**) via a three-step procedure including acetylation, *N*-deethylation, and hydrogenation. Compounds **23**, **24**, **25**, and **26** were prepared from 13-deoxyindaconitine (**1**) through a reaction sequence as shown in Scheme 1, including pyrolysis, isopyro rearrangement, *N*-deethylation, *N*-methylation or Pd/C-catalyzed hydrogenation. Similarly, compounds **27**, **28**, **29**, and **30** were obtained from chasmanine (**5**) through diacetylchasmanine (**6**). Compound **34** was prepared from 3-acetylaconitine (**31**) by a three-step reaction sequence including Barton's oxygenation [5], *N*-deethylation, and hydrolysis. Methylation of **31a** (prepared from **31**) with CH₃I/NaH, followed by hydrolysis, gave compound **35**. Compound **36** was prepared from 13-deoxyindaconitine (**1**) by *N*-deethylation and hydrolysis. Compound **38** was produced by *N*-deethylation of lappaconitine (**37**). Compounds **39** and **40** were prepared from compound **38** via hydrolysis, and methylation and hydrolysis, respectively. Compound **44** was prepared from songorine (**43**) according to the procedure described in the literature [3, 4]. Reduction of songorine (**43**) with NaBH₄ resulted in compounds **45** and **46**, while reduction of songorine (**43**) using the Huang-Minlon method with modification led to compound **47**. Oxidation of compound **46**, songoramine (**49**), and denudatine (**52**) with H₂O₂-MeOH produced compounds **48**, **50** and **51**, as well as **53**, respectively. Treatment of **55** [prepared from 11-epi-16α-17-dihydroxylepenine (**54**)] with 5% NaOH solution in methanol followed by CH₃I-NaH gave compounds **56** and **57**. Their structures were established by comparison of their ¹H (¹³C) NMR data with those reported in the literature [6], as well as the high resolution mass data. The ¹³C NMR spectral data for all compounds (Tables 1, 2) were assigned based on comparing them with those of analogues.

1 13-Deoxyindaconitine
R¹ = Et R² = OH R³ = H
2 R¹ = R³ = H R² = OH

3 R¹ = R³ = H R² = OH
4 R¹ = Me R² = OH R³ = H
5 Chasmanine
R¹ = Et R² = R³ = H
6 R¹ = R² = H R³ = Ac
7 R¹ = Me R² = R³ = H
8 R¹ = R² = R³ = H

43 Songorine R¹ = H R² = Et
43a Acetyl songorine
R¹ = Ac R² = Et
44 R¹ = R² = H

45 R¹ = H R² = OH
46 R¹ = OH R² = H
47 R¹ = R² = H

48

9 Mesaconine
R¹ = Me R² = OH R³ = R⁴ = H
10 Hypaconine
R¹ = Me R² = R³ = R⁴ = H
11 *N*-Deethylaconine
R¹ = R³ = R⁴ = H R² = OH
17 R¹ = R² = R³ = R⁴ = H
17a Deoxyaconitine
R¹ = Et R² = H R³ = Ac R⁴ = Bz
41 14-Benzoyl-mesaconine
R¹ = Me R² = OH R³ = H R⁴ = Bz
42 14-Benzoyl-hypaconine
R¹ = Me R² = R³ = H R⁴ = Bz

12 Indaconitine R¹ = Et
R² = OH R³ = Ac R⁴ = Bz
13 R¹ = R² = H R³ = Ac R⁴ = Bz
14 R¹ = R² = R³ = R⁴ = H
15 R¹ = Me R² = R³ = R⁴ = H
16 R¹ = H R² = OH R³ = Ac R⁴ = Bz
18 R¹ = Me R² = OH R³ = R⁴ = H

49 Songoramine

50

51

52 Denudatine

53

54 11-epi-16α, 17-
Dihydroxylepenine
R¹ = H R² = Et
55 R¹ = Ac R² = H
56 R¹ = R² = H
57 R¹ = H R² = CH₃

58 Mesacoine hydrobromide

19 Talatisamine
R¹ = Et R² = R³ = H
20 R¹ = H R² = R³ = Ac
21 R¹ = R² = R³ = H
22 R¹ = Me R² = R³ = H

23 R¹ = H R² = OH
24 R¹ = Me R² = OH
27 R¹ = R² = H
28 R¹ = Me R² = H

25 R¹ = H R² = OH
26 R¹ = Me R² = OH
29 R¹ = R² = H
30 R¹ = Me R² = H

31 3-acetyl-aconitine R¹ = Et
R² = R³ = Ac R⁴ = Bz R⁵ = OH
31a R¹ = H R² = R³ = Ac
R⁴ = Bz R⁵ = H
34 R¹ = R² = R³ = R⁴ = R⁵ = H
35 R¹ = Me R² = R³ = R⁴ = R⁵ = H

32 R = OAc
33 R = OH

37 Lappaconitine

36

R¹ = Et R² = 〔structure〕NHAc **38**

R¹ = H R² = 〔structure〕NHAc

39 R¹ = R² = H
40 R¹ = Me R² = H

1 Deoxyindaconitine

Vaccum
180–190°C
5'

Ac₂O / TsOH
Reflux
3 h

1) NBS-glacial
HOAc
rt, 2 h
2) 5% NaOH
methanol
50°C, 1 h

23 (50 %)

CH₃I / NaH
rt.

Pd-C
[H]

24 (89%)

Pd-C
[H]

26 (87%)

25 (89%)

Scheme 1: Preparation of compounds **23**, **24**, **25**, and **26**.

Biological evaluation: The cardiac effects of the forty-two diterpenoid alkaloids on isolated bullfrog hearts (Table 3) were evaluated according to the procedure described in the literature [7]. The cardiac effect was represented as the mean of amplitude increase rate and arbitrarily classified into four categories: no cardiac effect (the average rate of amplitude was increased in the range of 0-10%); moderate cardiac effect (12-30%); significant cardiac effect (31-59%); and strong cardiac effect (greater than 60%). It should be noted that *N*-deethylchasmaconine (**8**) mesaconine (**9**), hypaconine (**10**), and *N*-deethylaconine (**11**) have been previously reported by us [2] and were included in this study for better structure-activity comparison. The data are summarized in Table 3, which indicates that fifteen (**3**, **8-11**, **14**, **17-18**, **22**, **24**, **25**, **28**, **30**, **34**, and **35**) out of the forty-two tested diterpenoid alkaloids showed significant cardiac effect. Compound **3**, mesaconine (**9**),

hypaconine (**10**), and compound **35** exhibit better cardiac effect than the reference drug lanatoside C.

A broad range (potent-to-inactive) of cardiac effects has been observed for the forty-two diterpenoid alkaloids with the following structure-activity relationships:
1) C_{18}-diterpenoid alkaloids (e.g. **39**, **40**) or the aconitine-type alkaloids containing a monoester [e.g. 14-benzoylmesaconine (**41**) and 14-benzoylhypaconine (**42**)], the veatchine-type C_{20}-diterpenid alkaloids (e.g. **43-48**, **50**, and **51**), and the denudatine-type C_{20}-diterpenoid alkaloids (e.g. **53**, **56** and **57**) did not exhibit any apparent cardiac effect. Additionally, no considerable effect was observed for the aconitine-type alkaloids that contain an imine moiety (e.g. **36**).
2) Consistent with the results that we previously reported [2], the aminoalcohol group of aconitine-type C_{19}-diterpenoid alkaloids bearing both hydroxyl groups at C-8 and C-14 are most essential structure features for the cardiac activity. The substituent pattern on the nitrogen atom (NH, *N*Me, or *N*Et), the hydroxyl group at C-3, C-15, and C-13, and the methoxyl group at C-6 can appreciably contribute to or abolish the activity. These can be specified as below: i) the alkaloids with a 3-OH (e.g. **3**, **4** and **18**) are more potent than those without it (e.g. **8**, **7**, and **15**), suggesting the presence of a 3-OH is beneficial to the cardiac effect, which is consistent with the conclusions in our previous paper [2]; ii) in addition to alkaloids **15** and **28**, the alkaloids that possess a methyl group on the nitrogen atom [e.g. mesaconine (**9**), **22**, **28**, and **35**] are more active than the corresponding compounds containing a hydrogen instead [e.g. *N*-deethylamine (**11**), **21**, **27**, and **34**]. The similar effect of the *N*Me on the cardiac activity can also be concluded by comparing the activity of **24/23**, **22/21**, and **30/29**. However, alkaloids that contain a NH moiety but no 13, 15-OH (e.g. **3**, **8**, and **29**) have greater activity than those with a *N*Me moiety (e.g. **4**, **7**, and **30**); iii) except for compound **7**, an alkaloid possessing a 16-OMe (e.g. **4**) is more active than the corresponding one without it (e.g. **26**) and the one with a double bond between C-15 and C-16 (e.g. **24**); iv) the alkaloids **7** and **8** that contain a 13-OH have significantly improved activity compared with their corresponding alkaloids **15** and **14** that lack the 13-OH, suggesting that this group is also a determining factor for the cardiac action. V) the effect of 6-OMe on the cardiac activity depends on the substituent pattern on the nitrogen atom. Both *N*Me-containing compounds **7** and **22** did not show any cardiac activity, no matter with either the presence or absence of a 6-OMe. However, introduction of 6-OMe into the NH-containing compound **21** led to compound **8** with enhanced cardiac effect.

In conclusion, the structure-cardiac activity relationships of diterpenoid alkaloids are summarized as below based on our previous and current studies: i)1-OMe or 1-OH, 8-OH, 14-OH, and NH (or *N*Me) are critical structural features necessary for the cardiac effect of the aconitine-type C_{19}-diterpenoid alkaloids without any esters. C_{18}-diterpenoid alkaloids, lycoctonine-type C_{19}-diterpenoid alkaloids, and the veatchine- and denudatine-type C_{20}-diterpenoid alkaloids did not possess any significant cardiac activity; ii) the existence of 3-OH can significantly improve the cardiac activity; iii) the effects on the cardiac activity of 6-OMe, 13-OH, 15-OH, and 16-demethoxy or a double bond between C-15 and C-16 heavily depend upon the substituent pattern on the nitrogen atom; iv) interestingly, mesaconine hydrobromide (**58**) without showing any *in vitro* cardiac activity exhibited significant and quick *in vivo* efficacy that is comparable with the free base mesaconine (**9**) [8].

Experimental

General methods: Melting points were determined on a Koffler block (uncorrected). IR spectra were obtained on a Nicolet FT-IR 200 SXV spectrophotometer. ^{1}H and ^{13}C NMR spectra were taken on a Varian Unity INOVA 400/54 NMR spectrometer in CDCl$_3$ with TMS as the internal standard. The ESIMS and HRESIMS were recorded on either a VG Auto Spec 3000 or a Finnigan-MAT 90 instrument. Silica gel H (Qingdao Marine Chemical Factory, Qingdao, China) was used for column chromatography (CC). Zones on TLC (silica gel G) plates were detected with modified Dragendorff's reagent.

Table 1: ^{13}C NMR spectral data of compounds **2-4**, **6**, **7**, **13-15**, **17**, **18**, and **20-25** (100 MHz, CDCl$_3$).

No.	2[a]	3	4	6	7	13[b]	14	15	17	18	20[c]	21	22	23	24	25
1	83.2	75.2	83.1	83.7	82.1	83.1	83.0	85.7	83.4	84.1	83.1	84.6	86.2	81.8	82.7	82.3
2	37.5	34.7	33.9	28.9	34.7	35.1	29.3	34.8	28.4	35.3	28.0	30.5	32.5	35.3	34.0	34.3
3	71.4	71.5	72.3	23.3	25.8	23.4	23.6	25.8	22.8	72.3	24.9	26.1	25.8	71.6	71.8	71.5
4	43.6	43.6	43.5	38.9	39.4	39.0	39.1	39.4	38.6	43.4	37.8	38.3	38.2	43.8	43.5	43.7
5	47.5	47.9	48.4	43.8	48.5	44.0	49.2	50.4	51.5	50.1	44.3	38.5	37.5	54.9	47.9	57.0
6	81.3	82.1	81.9	82.4	82.1	82.5	82.6	82.1	82.2	82.8	24.6	27.0	24.6	81.3	81.5	81.3
7	55.5	56.3	51.2	53.4	51.4	43.6	57.3	51.1	47.4	50.8	47.4	52.7	45.1	47.5	47.5	48.2
8	85.5	81.7	71.9	85.8	72.4	85.6	74.3	72.8	76.1	72.3	85.9	73.2	72.7	73.8	73.8	75.1
9	42.6	47.7	48.0	43.8	49.2	43.6	44.8	49.1	42.7	47.8	42.4	46.3	46.8	46.6	47.1	47.2
10	39.5	38.5	37.8	39.0	37.8	40.2	41.1	42.3	41.0	41.6	38.8	44.0	44.7	38.5	38.5	34.8
11	49.7	49.4	50.1	50.4	50.2	50.3	48.9	50.0	48.8	49.9	37.8	48.2	48.6	49.2	49.8	50.1
12	27.5	27.2	27.8	28.5	28.0	29.0	35.9	35.6	36.7	33.7	28.5	24.9	27.4	31.4	32.6	30.5
13	44.6	45.2	45.1	43.4	45.4	74.5	76.3	76.7	78.2	76.5	41.1	45.6	45.6	45.6	45.2	45.3
14	75.5	72.6	75.3	75.4	75.4	78.9	78.9	79.4	78.4	79.2	75.3	75.4	75.4	74.0	74.1	74.7
17	56.6	58.4	63.2	57.0	63.5	57.7	59.1	63.4	61.8	63.1	56.4	55.8	63.7	57.6	64.4	59.0
18	77.5	77.7	77.1	79.8	80.4	79.9	80.1	80.4	79.8	77.0	78.8	79.1	79.2	77.8	77.1	78.1
19	40.8	40.7	49.7	*	56.3	*	50.0	56.1	50.1	49.5	49.0	47.2	*	66.9	49.8	48.9
*N*CH₃	-	-	42.1	-	42.3.	-	-	42.1	-	42.0	-	-	42.5	-	42.2	-
1-OMe	55.1	55.7	56.3	55.2	56.3	55.3	55.3	56.5	55.3	56.3	55.6	56.3	56.4	55.7	56.3	55.6
6-OMe	56.3	56.3	57.2	56.4	56.4	57.5	57.4	57.2	56.2	57.0	-	-	-	57.5	57.4	57.3
16-OMe	57.5	57.2	56.3	57.9	57.3	58.8	57.6	57.6	58.0	57.5	56.9	57.0	56.5	-	-	-
18-OMe	59.1	59.1	59.1	59.0	59.1	59.0	58.0	59.1	59.0	59.0	59.3	59.3	59.4	59.2	59.1	59.2
15	34.6	38.9	38.8	37.5	38.8	39.7	42.6	39.6	80.7	39.5	37.5	39.0	38.5	129.6	129.6	30.1
16	82.3	81.7	81.8	82.2	81.8	83.0	82.4	84.3	91.0	81.8	82.4	82.0	82.1	131.4	131.5	23.8

a: OAc: 169.5 s, 21.5 q; OBz: 166.1 s (COO), 130.1 s (1"), 129.5 d (2", 6"), 128.4 d (3", 5"), 133.0 d (4"); *b*: OAc: 169.5 s, 21.3 q; OBz: 166.0 s, (COO), 129.8 s (1"), 129.5 d (2", 6"), 128.4 d (3", 5"), 139.1 d (4"); *c*: OAc×2: 170.8 s, 169.3 s, 22.2 q, 21.2 q.).

Table 2: ^{13}C NMR spectral data of compounds **27-30**, **34-36**, and **38-40** (100 MHz, CDCl$_3$)

NO	26	27	28	29	30	34	35	36	38^a	39	40
1	82.7	83.1	85.4	82.6	85.8	82.2	83.1	82.7	82.4	82.5	82.7
2	34.0	24.1	26.2	25.6	25.8	30.4	31.0	33.3	29.5	34.1	36.8
3	71.8	29.8	34.8	31.8	30.6	70.6	70.5	72.3	26.2	25.7	26.8
4	43.5	39.3	39.4	38.8	39.5	44.8	44.7	49.5	77.3	70.4	70.8
5	47.9	53.9	48.5	57.4	52.4	53.3	50.2	57.0	36.7	52.1	49.6
6	81.5	82.0	81.7	82.4	82.4	85.2	84.7	81.8	23.6	23.4	26.1
7	47.5	44.8	48.1	44.7	49.8	45.6	45.7	45.7	52.3	36.4	35.8
8	73.7	74.4	73.9	76.3	75.1	79.4	79.3	71.2	83.5	75.5	75.4
9	47.1	46.8	47.8	47.4	47.9	49.4	49.0	47.7	76.0	77.9	78.5
10	38.5	38.3	38.7	34.6	34.9	42.0	41.7	37.3	49.1	49.4	46.4
11	49.5	49.9	49.8	50.2	50.7	50.8	51.4	51.4	52.7	52.7	50.7
12	32.6	31.5	32.9	32.3	34.9	33.9	34.0	26.9	24.4	25.6	23.4
13	45.2	44.8	45.6	44.8	45.4	46.5	46.5	44.1	44.2	47.5	49.5
14	74.1	74.1	74.2	75.4	74.8	76.3	76.1	75.2	90.0	89.9	89.9
15	129.6	128.3	129.7	29.0	30.4	79.9	79.9	37.1	44.0	43.6	44.4
16	131.5	132.8	131.6	23.3	22.9	91.9	92.2	81.5	82.2	82.5	84.8
17	64.4	58.7	64.4	58.3	63.8	57.5	65.3	62.0	57.1	57.7	62.9
18	77.1	80.3	80.5	80.0	80.6	77.3	76.1	78.8	-	-	-
19	49.8	49.3	56.2	49.0	56.2	41.6	42.5	166.3	50.8	52.0	60.1
NCH$_3$	42.2	-	42.4	-	42.4	-	49.6	-	-	-	42.2
1-OMe	56.3	55.4	56.4	55.3	56.5	55.5	55.9	55.8	55.8	55.6	55.9
6-OMe	57.4	57.6	57.5	57.7	57.3	57.3	57.1	57.0	-	-	-
14-OMe	-	-	-	-	-	-	-	-	56.0	56.3	57.7
16-OMe	-	-	-	-	-	59.3	59.2	56.5	57.8	55.8	56.7
18-OMe	59.1	59.1	59.1	59.1	59.2	58.3	58.3	59.3	-	-	-

a: NHAc: 171.3 s (N*C*O), 25.4 (25.2) q (*C*H$_3$CON); 169.0 (167.2) s (*C*OO), 141.5 (140) s (2''), 134.3 (133.1) d (4''), 130.9 (127.3) d (6''), 122.4 (122.2) d (5''), 121.3 (120.1) (3''), 115.5 s (1'').

Table 3: ^{13}C NMR spectral data of compounds **44-48**, **50**, **51**, **53**, **56** and **57** (100 MHz, CDCl$_3$)

NO	44	45	46	47	48^a	50	51^a	53^a	56^b	57^a
1	67.3	69.8	69.6	69.6	69.2	67.8	67.8	26.3	68.4	69.1
2	29.6	29.8	30.6	20.4	27.9	29.8	28.9	19.7	31.1	29.8
3	30.6	31.1	31.4	31.5	28.9	24.1	23.2	39.3	38.0	36.7
4	33.5	33.6	33.5	34.6	35.9	37.7	36.9	33.4	34.4	34.4
5	46.4	48.1	47.9	49.6	48.9	48.7	49.0	50.6	51.3	49.7
6	21.8	23.3	19.1	22.3	22.7	23.9	22.9	21.8	24.2	23.0
7	45.6	43.3	44.0	44.1	45.8	45.7	45.5	41.9	45.8	44.5
8	48.2	50.5	50.7	47.6	49.8	50.7	49.5	41.9	44.5	42.7
9	36.0	37.0	36.2	35.3	39.1	30.4	31.6	52.3	52.8	51.8
10	51.0	52.4	52.9	48.9	53.3	51.5	50.0	44.6	51.9	52.1
11	36.0	32.3	23.4	37.2	31.2	29.6	28.4	71.0	71.4	69.8
12	210.3	67.3	31.9	210.2	79.7	213.0	107.7	38.8	46.7	44.9
13	53.0	43.7	40.6	53.1	47.3	53.8	53.4	20.0	21.2	20.0
14	36.8	35.5	35.3	40.1	37.2	37.4	32.3	25.2	28.1	27.3
15	77.5	76.8	77.6	77.0	82.3	75.4	81.1	75.3	85.2	83.8
16	148.4	153.8	164.8	150.2	88.4	78.8	86.9	77.5	80.2	79.3
17	111.3	111.9	106.1	114.4	71.4	68.1	68.6	65.3	68.8	67.8
18	24.4	26.3	26.6	26.2	25.6	18.8	17.7	25.4	25.7	25.1
19	46.1	58.2	58.6	57.4	57.9	92.8	92.5	56.7	47.7	58.1
20	57.7	66.3	66.3	69.3	66.2	65.6	65.6	71.2	61.3	67.5
21	-	50.9	50.7	51.1	54.6	48.2	47.7	50.3	-	42.5
22	-	13.2	13.4	13.5	13.7	14.1	13.0	12.1	-	-

a: CDCl$_3$+CD$_3$OD, *b*: CD$_3$OD.

Table 4: Cardiac effects of 42 diterpenoid alkaloids and lanatoside on isolated bullfrog hearts

Compound	Rate of amplitude increase (%)	Result^a	Compound	Rate of amplitude increase (%)	Result^a
control^b	0	0	30	17.0	(+)
3	85.0	(+++)	34	31.5	(++)
4	31.0	(++)	35	176.7	(++++)
7	-57.0	(-)	36	-42.0	(-)
8	23.9	(+)	38	-50.0	(-)
9	82.0 [2]	(+++)	39	-27.0	(-)
10	118.0 [2]	(+++)	40	-53.0	(-)
11	28.0 [2]	(+)	41	-27.0	(-)
14	45.0	(++)	42	-18.0	(-)
15	0	(-)	43	-55	(-)
17	36.0	(++)	44	-29	(-)
18	23.6	(+)	45	-34	(-)
18a	0 [2]	(-)	46	(-)	(-)
21	0	(-)	47	-56	(-)
22	19.0	(+)	48	(-)	(-)
23	8.0	(-)	50	-5	(-)
24	25.0	(+)	51	-30	(-)
25	20.0	(+)	53	-40	(-)
26	-48.0	(-)	56	-26	(-)
27	8.0	(-)	57	-43%	(-)
28	31.0	(++)	58	0	(-)
29	-29.0	(-)	Lanatoside C	73.3	(+++)

a: The results were judged based on the average rate of amplitude increase: (-): 0-10%, (+): 12-30%, (++): 31-59%, (+++): 60-120%; *b*: distilled water; c: the cardioactive duration of alkaloids can reach 30'; the concentration of the tested compounds: 0.01mol/mL ; 0.0002 mol/mL for lanatoside C.

Preparation of compound 2*:* To a solution of 13-deoxyindoconitine (**1**, 680 mg, 1.10 mmol) in glacial acetic acid (20 mL) was added NBS (600 mg, 3.4 mmol), and the mixture was kept stirred at room temperature for 3 h prior to being basified to pH 10 with conc. ammonium hydroxide. The mixture was extracted with chloroform; the combined extracts were dried and concentrated. The residue was purified by CC on silica gel, eluting with light petroleum: acetone (3:1, saturated with conc. ammonium hydroxide) to give compound **2** (476 mg, 74%).

Compound 2
^{1}H NMR (400 MHz, CDCl$_3$): 8.05 (2H, d, *J* = 7.2 Hz, H-2", 6"), 7.55 (1H, t, *J* = 7.6 Hz, H-4"), 7.49 (2H, t, *J* = 7.6 Hz, H-3", 5"), 5.07 (1H, t, *J* = 4.8 Hz, H-14β), 4.13 (1H, d, *J* = 6.8 Hz, H-6), 3.82 (1H, dd, *J* = 10.0, 5.6 Hz, H-3), 3.85, 3.32, 3.29, 3.19 (each 3H, s, OMe × 4), 1.33 (3H, s, OAc-8).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
ESIMS: *m/z* 586 [M+H]$^+$.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{32}$H$_{44}$NO$_9$: 586.3016, found: 586.3016

Preparation of compound 3*:* Compound **2** (320 mg, 0.55 mmol) was dissolved in a solution of sodium hydroxide in methanol (5%, 10 mL), and the reaction solution was stirred at 60°C for 1 h. After concentration *in vacuo*, the residue was subjected to CC on silica gel eluting with CHCl$_3$: MeOH: diethylamine (97:7:1) to yield compound **3** (242 mg, 77%).

Compound 3
^{1}H NMR (400 MHz, CDCl$_3$): 3.17 (1H, d, *J* = 6.4 Hz, H-1), 1.97, 2.51 (each 1H, m, H$_2$-2), 3.71 (1H, t, *J* = 5.6 Hz, H-3), 1.95 (1H, t, *J* = 6.8 Hz, H-5), 4.25 (1H, d, *J* = 6.8 Hz, H-6), 3.17 (1H, hidden, H-7), 2.13 (1H, t, *J* = 5.2 Hz, H-9), 1.64 (1H, m, H-10), 1.65, 1.97 (each 1H, m, H$_2$-12), 2.29 (1H, m, H-13), 4.10 (1H, t, *J* = 4.8 Hz, H-14β), 1.78, 2.39 (each 1H, m, H$_2$-15), 3.38 (1H, hidden, H-16), 1.88 (1H, s, H-17), 3.69, 3.81 (each 1H, ABq, *J* = 9.2 Hz, H$_2$-18), 2.93, 3.38 (each 1H, ABq, *J* = 13.2 Hz, H$_2$-19), 3.31 (3H, s, OCH$_3$-1), 3.32 (3H, s, OCH$_3$-6), 3.24 (3H, s, OCH$_3$-16), 3.29 (3H, s, OCH$_3$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{23}$H$_{38}$NO$_7$: 440.2648, found: 440.2650.

Preparation of compound 4*:* To a solution of compound **2** (150 mg, 0.26 mmol) in anhydrous THF (5 mL) was added sodium hydride (80%, 7 mg, 0.23 mmol), and the mixture was stirred at room temperature before the addition of methyl iodide (18 μL). The reaction was allowed to proceed with stirring for 2 h. Removal of the solvent afforded a residue, to which was added a solution of sodium hydroxide in methanol (5%, 6 mL). A general work-up afforded a crude product, which was purified by CC (silica gel, CHCl$_3$-MeOH, 9:1) to give compound **4** (100 mg, 85%).

Compound 4
^{1}H NMR (400 MHz, CDCl$_3$): 4.57 (1H, brs, OH), 3.02 (1H, dd, *J* = 10.0, 6.8 Hz, H-1), 4.23 (1H, d, *J* = 6.8 Hz, H-6), 4.07 (1H, t, *J* = 4.8 Hz, H-14β), 3.66 (1H, dd, *J* = 11.6, 6.0 Hz, H-3β), 3.33, 3.24 (each 3H, s, OMe × 2), 3.31 (6H, s, OCH$_3$ × 2), 2.30 (3H, s, *N*CH$_3$).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HR-ESI: *m/z* [M+H]$^+$ calcd for C$_{24}$H$_{40}$NO$_7$: 454.2805; found: 454.2800.

Preparation of compound 6*:* To a solution of chasmanine (**5**, 400 mg, 0.88 mmol) in acetic anhydride (6 mL) was added TsOH (400 mg), and the reaction was allowed to proceed with stirring at room

temperature for 16 h. A general work-up provided a white solid, to which was added glacial acetic acid (20 mL) and NBS (710 mg). The subsequent reaction mixture was stirred at room temperature for 3 h before being concentrated. The white residue was subjected to CC on silica gel eluting with light petroleum: acetone:diethylamine (80:20:1) to furnish compound **6** (250 mg, 65%).

Compound 6
^{1}H NMR (400 MHz, CDCl$_3$): 4.81 (1H, t, *J* = 4.8 Hz, H-14β), 4.06 (1H, d, *J* = 5.6 Hz, H-6), 3.01, 3.58 (each 1H, ABq, *J* = 8.4 Hz, H$_2$-18), 3.32, 3.31, 3.26, 3.22 (each 3H, s, OMe × 4), 2.04, 1.97 (each 3H, s, OAc × 2).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HR-ESI: *m/z* [M+H]$^+$ calcd for C$_{24}$H$_{40}$NO$_6$: 438.2856; found: 438.2859.

Preparation of compound 7*:* To a solution of compound **6** (114 mg, 0.26 mmol) in anhydrous THF (20 mL) was added sodium hydride (80%, 6 mg, 0.2 mmol), and the mixture was stirred at room temperature for 15 min before the addition of methyl iodide (14 μL). The reaction was allowed to proceed for 2 h at room temperature. A general work-up procedure gave a residue, to which was added a solution of sodium hydroxide in methanol (5%, 3 mL). The subsequent reaction mixture was stirred at 60°C for 1 h and the solvent was removed *in vacuo*. The white foam was purified by CC eluting with CHCl$_3$: MeOH: diethylamine (97:3:1) to yield compound **7** (83 mg, 74%).

Compound 7
^{1}H NMR (400 MHz, CDCl$_3$): 4.60 (1H, brs, OH), 4.20 (1H, d, *J* = 6.8 Hz, H-6), 4.13 (1H, t, *J* = 4.5 Hz, H-14*β*), 3.60, 3.70 (each 1H, ABq, *J* = 8.0 Hz, H$_2$-18), 3.40 (1H, d, *J* = 9.2 Hz, H-16), 3.34, 3.31, 3.30, 3.25 (each 3H, s, OMe × 4), 2.31 (3H, s, *N*CH$_3$).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{24}$H$_{40}$NO$_6$: 428.2856; found: 438.2859.

Preparation of compound 8: To a solution of chasmanine (**5**, 600 mg, 1.32 mmol) in acetic anhydride (60 mL) was added TsOH (40 mg) and the reaction was allowed to proceed for 16 h. After a general work-up, the white residue was dissolved in glacial acetic acid (20 mL). To this solution was added NBS (713 mg, 4,00 mmol), and the subsequent reaction solution was stirred at room temperature for an additional 3 h. After the second standard work-up, the crude product was purified by CC (silica gel, light petroleum: acetone, 4:1 with 1% diethylamine) to give compound **8** (440 mg, 65 %).

Compound 8
^{1}H NMR (400 MHz, CDCl$_3$): 3.23, 3.31, 3.33, 3.35 (each 3H, s, OMe × 4), 3.65 (1H, ABq, *J* = 8.4 Hz, H-18), 4.18 (1H, t, *J* = 5.2 Hz, H-14*β*), 4.23 (1H, t, *J* = 6.8 Hz, H-6).
^{13}C NMR (100MHz, CDCl$_3$): 81.7 (C-1), 23.2 (C-2), 28.6 (C-3), 38.7 (C-4), 44.7 (C-5), 82.3 (C-6), 40.1 (C-7), 73.9 (C-8), 47.6 (C-9), 44.4 (C-10), 48.9 (C-11), 28.9 (C-12), 57.1 (C-13), 75.3 (C-14), 41.4 (C-15), 82.4 (C-16), 57.4 (C-17), 79.8 (C-18), 49.9 (C-19), 55.2 (C-1'), 56.1 (C-6'), 57.6 (C-16'), 59.0 (C-18').
HRMS-ESI: *m/z* [M+H]$^+$ cald for C$_{23}$H$_{38}$NO$_6$, 424.2699; found: 424.2705.

Preparation of compound 13: To a solution of indoconitine (**12**, 4 g, 6.4 mmol) in THF (12 mL) was added thionyl chloride (6 mL), and the reaction mixture was stirred at 40°C for 2 h. Removal of volatile solvent gave a white solid, which was dissolved in ethanol

(95%, 10 mL) and glacial acetic acid (2 mL). To the mixture was added 10% Pd-C and the reaction was allowed to proceed in the presence of hydrogen. A general work-up gave white foam, which was dissolved in glacial acetic acid (9 mL). To the subsequent mixture was added NBS (260 mg), and the reaction mixture was stirred at room temperature for 3 h. After a general work-up, the crude residue was purified by CC eluting with cyclohexane: acetone: diethylamine (75:25:1) to yield compound **13** (2.73 g, 71%).

Compound 13
^{1}H NMR (400 MHz, CDCl$_3$): 8.05 (1H, d, J = 7.2 Hz, H-2", 6"), 7.56 (1H, t, J = 7.6 Hz, H-4"), 7.43 (2H, t, J = 7.6 Hz, H-3", 5"), 4.93 (1H, d, J = 5.2 Hz, H-14β), 4.03 (1H, brs, NH), 4.00 (1H, d, J = 6.8 Hz, H-6), 3.55, 3.28, 3.24, 3.18 (each 3H, s, OMe × 4), 1.31 (3H, s, OAc-8).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{32}$H$_{44}$NO$_9$: 586. 3016; found: 586.3021

Preparation of compound 14: Compound **13** (95 mg, 0.16 mmol) was dissolved in a solution of sodium hydroxide in methanol (1%, 3 mL). The reaction mixture was stirred at 60°C for 1 h before removal of solvent. The crude residue was purified by CC on silica gel eluting with CHCl$_3$: MeOH: diethylamine (95:5:1) to provide compound **14** (51mg, 73%).

Compound 14
^{1}H NMR (400 MHz, CDCl$_3$): 4.13 (1H, d, J = 6.4 Hz, H-6), 3.99 (1H, d, J = 4.8 Hz, H-14β), 3.81 (1H, brs, OH), 3.64 (1H, ABq, J = 8.4 Hz, H-18), 3.42, 3.34, 3.31, 3.21 (each 3H, s, OCH$_3$ × 4).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{23}$H$_{38}$NO$_7$: 440.2648; found: 440.2649.

Preparation of compound 15: To a solution of compound **13** (95 mg, 0.16 mmol) in anhydrous THF (5 mL) was added sodium hydride (80%, 5 mg), and the mixture was stirred for 15 min before the addition of methyl iodide (11 μL). The reaction was allowed to proceed for an additional 2 h. A standard work-up provided a white solid, to which was added a solution of sodium hydroxide in methanol (5%, 5 mL). The subsequent reaction mixture was stirred at 60°C for 1 h prior to another standard work-up. The crude residue was purified by CC eluting with CHCl$_3$: MeOH: diethylamine (97:3:1) to give compound **15** (24 mg, 71%).

Compound 15
^{1}H NMR (400 MHz, CDCl$_3$): 4.11 (1H, d, J = 7.2 Hz, H-6), 4.02 (1H, brs, OH), 3.97 (1H, t, J = 4.8 Hz, H-14β), 3.69, 3.33 (each 1H, ABq, J = 8.4 Hz, H$_2$-18), 3.42, 3.30, 3.30, 3.25 (each 3H, s, OCH$_3$ × 4), 2.99 (1H, dd, J = 10.8, 6.8 Hz, H-1), 2.31 (3H, s, NCH$_3$).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{24}$H$_{40}$NO$_7$: 454.2805; found: 454.2806.

Preparation of compound 17: To a solution of dexoyaconitine (**17a**, 315 mg, 0.50 mmol) in glacial acetic acid (10 mL) was added NBS (267 mg, 1.5 mmol), and the reaction mixture was kept stirred at room temperature for 3 h. A standard work-up procedure gave a crude residue, which was subjected to CC eluting with light petroleum: acetone: diethylamine (66:33:1) to furnish *N*-deethyl-deoxyaconitine (24 mg, 80%). This compound was dissolved in a solution of sodium hydroxide in methanol (5%, 10 mL) and refluxed for 30 sec. After removing the solvent, the residue was dissolved in CHCl$_3$: MeOH (9:3, 10 mL) and filtered. The filtrate

was concentrated and subjected to CC eluting with CHCl$_3$: MeOH (95:5, saturated with conc. NH$_4$OH) to yield compound **17** (115 mg, 76%).

Compound 17
^{1}H NMR (400 MHz, CDCl$_3$): 4.61 (1H, d, J = 6.8 Hz, H-15), 4.20 (1H, brs, OH), 3.85 (1H, d, J = 6.4 Hz, H-14β), 4.14 (1H, d, J = 6.4 Hz, H-6), 3.70, 3.35, 3.32, 3.19 (each 3H, s, OCH$_3$ × 4), 3.07, 3.60 (each 1H, ABq, J = 8.4 Hz, H$_2$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{23}$H$_{38}$NO$_8$: 456.2597; found: 456.2599

Preparation of compound 18: To a solution of indaconitine (**12**, 440 mg, 0.7 mmol) in glacial acetic acid (12 mL) was added NBS (375 mg, 2.1 mmol), and the reaction was allowed to proceed at room temperature for 4 h prior to being basified with conc. ammonium hydroxide to pH 10. The subsequent mixture was extracted with CHCl$_3$ (20 mL × 3); the combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was purified by CC eluting with light petroleum: acetone: diethylamine (75:25:1) to yield *N*-deethylindeaconitine (329 mg, 78%). To the solution of this compound (300 mg, 0.5 mmol) in THF (10 mL) was added sodium hydride (80%, 15 mg, 0.5 mmol) followed by methyl iodide (35 μl, 0.6 mmol) under argon. The subsequent reaction was allowed to proceed for 2 h before the solvent was removed. To the residue was added a solution of sodium hydroxide in methanol (15 mL), and the reaction was allowed to proceed with refluxing for an additional 1 h. The reaction mixture was first acidified with conc. HCl to pH 2 then basified with conc ammonium hydroxide to pH 10. The mixture was extracted with chloroform, and the combined extracts were dried over anhydrous sodium sulfate and concentrated. The crude residue was purified by CC eluting with CHCl$_3$: MeOH (95:5, saturated with conc. ammonium hydroxide) to give compound **18** (185 mg, 78%).

Compound 18
^{1}H NMR (400 MHz, CDCl$_3$): 4.15 (1H, d, J = 6.8 Hz, H-6), 3.66 (1H, dd, J = 11.2, 6.8 Hz, H-3β), 3.41, 3.32, 3.31, 3.25 (each 3H, s, OCH$_3$ × 4), 2.32 (3H, s, NCH$_3$).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{24}$H$_{40}$NO$_8$:470.2754; found: 470.2750.

Preparation of compound 20: To a solution of talasamine (**19**, 1.1 g, 2.61 mmol) in acetic anhydride (6 mL) was added TsOH (600 mg), and the reaction was allowed to proceed at room temperature for 16 h. After a standard work-up, the white residue was dissolved again in glacial acetic acid (40 mL). To this solution was added 7 equivalents of NBS, and the reaction was continued with stirring at room temperature for 4 h. After the second standard work-up, the white foam was subjected to CC eluting with cyclohexane: acetone: diethylamine to furnish an imine (230 mg). Hydrogenolysis of this imine catalyzed by Pt$_2$O at 60°C for 24 h yielded a white residue, which was purified by CC eluting with CHCl$_3$: MeOH: diethylamine (90:10:1) to furnish compound **20** (250 mg, 20%).

Compound 20
^{1}H NMR (400 MHz, CDCl$_3$): 4.80 (1H, t, J = 4.8 Hz, H-14β), 3.31, 3.28, 3.27 (each 3H, s, OCH$_3$ × 3), 2.03, 1.94 (each 3H, s, OAc).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{26}$H$_{40}$NO$_7$: 478.2805; found: 478.2804.

Preparation of compound 21: Compound **20** (100 mg, 0.20 mmol) was dissolved in a solution of sodium hydroxide in methanol (5%, 10 mL), and the reaction mixture was stirred at 60°C for 1 h. After a general work-up procedure, the white residue was purified by CC eluting with CHCl$_3$: MeOH: diethylamine (90:10:1) to generate compound **21** (24 mg, 30%).

Compound 21
^{1}H NMR (400 MHz, CDCl$_3$): 4.56 (1H, brs, OH), 4.18 (1H, d, J = 4.5 Hz, H-14β), 3.34, 3.29, 3.27 (each 3H, s, OMe × 3), 3.95, 3.09 (each 1H, ABq, J = 8.8 Hz, H$_2$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{36}$NO$_5$: 394.2593; found: 394.2591.

Preparation of compound 22: To a solution of compound **20** (105 mg, 0.22 mmol) in THF (5 mL) was added sodium hydride (80%, 7 mg, 0.22 mmol), and the mixture was stirred for 15 min before the addition of methyl iodide (15 µL). The reaction was allowed to proceed with stirring at room temperature for 2 h. A general work-up gave a white solid, to which was added a solution of sodium hydroxide in methanol (5%, 3 mL). The subsequent reaction mixture was stirred at 60°C for an additional 1 h and then concentrated under reduced pressure. The crude residue was purified by CC on silica gel eluting with CHCl$_3$: MeOH (97:3) to yield compound **22** (59 mg, 65%).

Compound 22
^{1}H NMR (400 MHz, CDCl$_3$): 4.81 (1H, brs, disappeared with D$_2$O, OH), 4.13 (1H, t, J = 4.8 Hz, H-14β), 3.34, 3.29, 3.27 (each 3H, s, OMe × 3), 2.26 (3H, s, *N*CH$_3$).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{33}$H$_{38}$NO$_5$: 408.2750; found: 408.2755.

Preparation of compounds 23, 24, 25 and 26: Pyrolysis of 13-deoxyindaconitine (**1**, 1.2 g, 1.95 mmol) at 180-190°C under vacuum for 5 min followed by CC (light petroleum: acetone: diethylamine, 80:16:1) provided a white powder (650 mg, 62%). This was dissolved in acetic anhydride (25 mL), to which was added TsOH (200 mg). The subsequent solution was refluxed for 3 h prior to being cooled to 0°C. The mixture was basified with conc. NH$_4$OH to pH > 10, and extracted with chloroform 3 times. The combined extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The crude residue was subjected to CC eluting with light petroleum: acetone (5:1) to give a rearranged product (white amorphous powder, 590 mg, 80%). To a solution of this product in glacial acetic acid (10 mL) was added NBS (450 mg, 3 eq), and the reaction mixture was stirred at room temperature for 2 h. The mixture was cooled to 0°C and basified with conc ammonium hydride to pH > 10, which was extracted with chloroform 3 times. The combined extracts were dried over anhydrous sodium sulfate and concentrated. The crude residue (520 mg) was dissolved in a solution of sodium hydroxide in methanol (5%) and stirred at 50°C for 1 h. After removal of solvent, the residue was partitioned between water and ethyl acetate. The organic layers were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by CC eluting with light petroleum: acetone: diethylamine (60:30:1) to yield compound **23** (white amorphous powder, 400 mg, 50%). To a solution of compound **23** (200 mg, 0.24 mmol) in THF was added sodium hydride (8.0 mg) and methyl iodide (20 µL), and the reaction was allowed to proceed at room temperature for 2 h. After removal of solvent, the residue was purified by CC eluting with chloroform: methanol (50:1) to give compound **24** (white amorphous

powder,180 mg, 89%). To a solution of compound **23** (100 mg, 0.23 mmol) in 95% ethanol (3 mL) was added 2 drops of glacial acetic acid and Pt$_2$O (10 mg). The reaction was allowed to proceed in the presence of hydrogen under 30 MPa for 20 h. The mixture was basified with conc. ammonium hydroxide to pH > 10. A standard work-up procedure followed by CC [chloroform: methanol (50:1)] yielded compound **25** (white amorphous powder, 90 mg, 89%). According to a similar procedure, hydrogenation of **24** (100 mg, 0.23 mmol), followed by CC (silica gel, CHCl$_3$: MeOH = 100:1) gave compound **26** (87 mg, 87 %).

Compound 23
^{1}H NMR (400 MHz, CDCl$_3$): 5.89 (1H, d, J = 9.6 Hz, H-16), 5.72 (1H, d, J = 9.6 Hz, H-15), 4.34 (1H, d, J = 6.8 Hz, H-6), 4.01 (1H, t, J = 4.8 Hz, H-14β), 3.36, 3.31, 3.24 (each 3H, s, OCH$_3$ × 3).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{34}$NO$_6$: 408.2386; found: 408.2381.

Compound 24
^{1}H NMR (400 MHz, CDCl$_3$): 5.87 (1H, d, J = 9.6 Hz, H-16), 5.63 (1H, d, J = 9.6 Hz, H-15), 4.30 (1H, d, J = 6.8 Hz, H-6), 4.03 (1H, t, J = 4.8 Hz, H-14β), 3.34, 3.32, 3.23 (each 3H, s, OMe × 3), 2.26 (3H, s, *N*CH$_3$).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{23}$H$_{36}$NO$_6$: 422.2543 ; found: 422.2540

Compound 25
^{1}H NMR (400 MHz, CDCl$_3$): 4.27 (1H, d, J = 6.4 Hz, H-6), 4.02 (1H, t, J = 4.8 Hz, H-14β), 3.34, 3.31, 3.29 (each 3H, s, OCH$_3$ × 3).
^{13}C NMR (100 MHz, CDCl$_3$): Table 1.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{36}$NO$_6$: 410.2543; found: 4 10.2560.

Compound 26
^{1}H NMR (400 MHz, CDCl$_3$): 4.23 (1H, d, J = 6.8 Hz, H-6), 4.03 (1H, t, J = 4.8 Hz, H-14β), 3.32, 3.31, 3.27 (each 3H, s, OCH$_3$ × 3), 3.01 (1H, dd, J = 9.2, 6.0 Hz, H-1), 2.36 (3H, s, *N*CH$_3$). 2.90, 2.59 (each 1H, ABq, J = 11.2 Hz, H$_2$-19).
^{13}C NMR (100 MHz, CDCl$_3$): Table 2.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{23}$H$_{38}$NO$_6$: 424.2699; found: 424.2700.

Preparation of compounds 27, 28, 29 and 30: These 4 compounds were prepared from chasmaning (**5**, 2.0 g, 4.45 mmol) using the same procedure described for the preparation of compounds **23-26**.

Compound 27
White amorphous powder, 300 mg, 17% yield.
^{1}H NMR (400 MHz, CDCl$_3$): 5.84 (1H, m, H-16), 5.76 (1H, d, J = 9.6 Hz, H-15), 4.30 (1H, d, J = 6.8 Hz, H-6), 3.97 (1H, t, J = 5.2 Hz, H-14β), 3.33, 3.29, 3.16 (each 3H, s, OCH$_3$ × 3).
^{13}C NMR (100 MHz, CDCl$_3$): Table 2.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{34}$NO$_5$: 392.2437; found: 392.2435.

Compound 28
White amorphous powder, 140 mg, 93% yield.
^{1}H NMR (400 MHz, CDCl$_3$): 5.89 (1H, m, H-16), 5.64 (1H, d, J = 9.6 Hz, H-15), 4.25 (1H, d, J = 6.8 Hz, H-6), 4.04 (1H, t, J = 4.4 Hz, H-14β), 3.33, 3.31, 3.23 (each 3H, s, OCH$_3$ × 3), 3.01 (1H, dd, J = 10.0, 6.8 Hz, H-1), 2.26 (3H, s, *N*CH$_3$).
^{13}C NMR (100 MHz, CDCl$_3$): Table 2.

HRMS-ESI: *m/z* [M+H]$^+$ calcd for $C_{23}H_{36}NO_5$: 406.2593; found: 406.2591.

Compound 29

White amorphous powder, 65 mg, 91% yield.
^{1}H NMR (400 MHz, CDCl$_3$): 4.22 (1H, d, *J* = 6.4 Hz, H-6), 4.06 (1H, t, *J* = 4.8 Hz, H-14β), 3.35, 3.31, 3.22 (each 3H, s, OCH$_3$ × 3).
^{13}C NMR (100 MHz, CDCl$_3$): Table 2.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for $C_{22}H_{36}NO_5$: 394.2593; found: 394.2599.

Compound 30

White amorphous powder, 63 mg, 91% yield.
^{1}H NMR (400 MHz, CDCl$_3$): 4.18 (1H, d, *J* = 6.8 Hz, H-6), 4.04 (1H, t, *J* = 4.8 Hz, H-14β), 3.31, 3.30, 3.28 (each 3H, s, OCH$_3$ × 3), 3.01 (1H, dd, *J* = 10.4, 6.4 Hz, H-1), 2.72, 2.41 (each 1H, ABq, *J* = 10.4 Hz, H$_2$-19), 2.31 (3H, s, H$_3$-21).
^{13}C NMR (100 MHz, CDCl$_3$): Table 2.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for $C_{23}H_{38}NO_7$: 408.2648 ; found: 408.2642.

Preparation of compound 31a: To a solution of 3-acetylaconitine (**31**, 600 mg, 0.87 mmol) in THF (72 mL) was added imidazole (8 mg), NaH (696 mg), CS$_2$ (7.2 mL) and CH$_3$I (5.4 mL, 0.08 mmol), and the reaction was allowed to proceed at room temperature for 3 h. A general work-up yielded a residue (400 mg), to which was added benzene (20 mL) and *n*-Bu$_3$SnH (0.24 mg), and the reaction mixture was refluxed for 1 h. After removal of solvent, a general work-up gave a residue (300 mg). To a solution of this residue (180 mg) in glacial acetic acid (5.5 mL) was added NBS (144 mg, 0.81 mmol), and the reaction mixture was stirred at room temperature for 1.5 h. After removal of the solvent, the residue was diluted with water (15 mL), basified with ammonium hydroxide to pH > 10, and extracted with CHCl$_3$ (10 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified by CC eluting with light petroleum: acetone: diethylamine (75:15:1) to produce compound **31a** (150 mg, 26 %).

Compound 31a

^{1}H NMR (400 MHz, CDCl$_3$): 8.02 (2H, d, *J* = 7.6 Hz), 7.56 (1H, t, *J* = 7.2 Hz), 7.44 (2H, t, *J* = 7.6 Hz), 5.20 (1H, t, H-3), 5.06 (1H, t, *J* = 4.4 Hz, H-14β), 4.38 (1H, d, *J* = 5.2 Hz, H-15), 4.18 (1H, d, *J* = 6.4 Hz, H-6), 3.73 (1H, d, *J* = 8.8 Hz, H-18), 3.53, 3.28, 3.26, 3.21 (each 3H, s, OCH$_3$ × 4), 2.06 (3H, s, OAc-3), 1.42 (3H, s, OAc-8).
HRMS-ESI: *m/z* [M+H]$^+$ calcd for $C_{34}H_{46}NO_{11}$: 644. 3071; found: 644.3123.

Preparation of compound 34: Compound **31a** (70 mg, 0.11 mmol) was dissolved in a solution of sodium hydroxide (5%, 2 mL), and the reaction was allowed to proceed at room temperature overnight. A general work-up gave a residue, which was subjected to CC on silica gel eluting with CHCl$_3$: MeOH (10:1, saturated with conc. NH$_4$OH) to yield compound **34** (40 mg, 81%).

Compound 34

^{1}H NMR (400 MHz, CDCl$_3$): 8.02 (2H, d, *J* = 7. 2 Hz), 7.56 (1H, t, *J* = 7.2 Hz), 7.44 (2H, t, *J* = 7.6 Hz), 5.04 (1H, t, *J* = 4.4 Hz, H-14β), 4.91 (1H, dd, *J* = 12.0, 6.0 Hz, H-3), 4.40 (1H, d, *J* = 2.4 Hz, OH-15), 4.34 (1H, dd, *J* = 6.0, 2.4 Hz, H-15), 4.13 (1H, d, *J* = 6.4 Hz, H-6), 3.82 (1H, d, *J* = 8.8 Hz, H-18), 3.53, 3.29, 3.22, 3.20 (each 3H, s, OCH$_3$ × 4), 2.34 (3H, s, *N*CH$_3$), 2.06 (3H, s, OAc-3), 1.44 (3H, s, OAc-8);
^{13}C NMR (100 MHz, CDCl$_3$): Table 2.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for $C_{23}H_{38}NO_7$: 440.2648 ; found: 440.2650.

Preparation of compound 35: To a solution of compound **31a** (110 mg, 0.17 mmol) in THF (5.5 mL) was added sodium hydride (4.1 mg, 0.17 mmol) and methyl iodide (10.7 uL, 0.17 mmol), and the reaction mixture was stirred at room temperature for 2 h. A general work-up gave a crude residue, to which was added a solution of sodium hydroxide in methanol (5%, 2 mL). The subsequent reaction mixture was stirred overnight and concentrated under reduced pressure. The crude residue was subjected to CC on silica gel eluting with CHCl$_3$: MeOH (20:1) to yield compound **35** (34 mg, 84.1%).

Compound 35

^{1}H NMR (400 MHz, CDCl$_3$): 3.82 (1H, br.s, H-3), 4.27 (1H, d, *J* = 6.4 Hz, H-6), 4.04 (1H, d, *J* = 4.8 Hz, H-14β), 3.45, 3.77 (each 1H, ABq, *J* = 8.4 Hz, H$_2$-18), 3.32, 3.31, 3.37, 3.39 (each 3H, s, OMe × 4).
^{13}C NMR (100 MHz, CDCl$_3$): Table 2.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for $C_{24}H_{40}NO_8$: 470.2754; found: 470.2750.

Preparation of compound 36: To a solution of 13-deoxyindaconitine (**1**, 200 mg, 0.28 mmol) in glacial acetic acid (10 mL) was added NBS (310 mg, 1.22 mmol), and the reaction was allowed to proceed at 130°C for 10 h. A general work-up provided a residue, to which was added a solution of sodium hydroxide in methanol (5%, 10 mL). The subsequent reaction mixture was stirred at 50°C for 1 h before removing the methanol. The crude residue was purified by CC eluting with CHCl$_3$: MeOH (40:1) to give compound **36** (115 mg, 70%).

Compound 36

^{1}H NMR (400 MHz, CDCl$_3$): 7.47 (1H, s, H-19), 3.67 (1H, dd, *J* = 10.4, 6.4 Hz, H-1), 3.38, 3.36, 3.25, 3.20 (each 3H, s, OCH$_3$ × 4).
^{13}C NMR (100 MHz, CDCl$_3$): Table 2.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for $C_{23}H_{36}NO_7$: 438.2492; found: 438.2496.

Preparation of compound 38: To a solution of lappaconitine (**37**, 2.336 g, 4 mmol) in glacial acetic acid (70 mL) was added NBS (2.136 g, 12 mmol), and the reaction was allowed to proceed at room temperature for 2 h. The reaction was quenched by pouring the reaction mixture into ice water, which was basified with conc ammonium hydroxide to pH 10 and extracted with chloroform (20 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated. The residue (2.3 g) was purified by CC on silica gel eluting with light petroleum: acetone: diethylamine (75:25:1) to give *N*-deethyllappaconitine (**38**, 1.87 g, 84%).

Compound 38

^{1}H NMR (400 MHz, CDCl$_3$): 8.63 (8.56) (1H, d, *J* = 8.4 Hz, H-6"), 7.90 (1H, d, *J* = 8.0 Hz, H-3"), 7.50 (7.44) (1H, t, *J* = 8.0 Hz, H-4"), 7.01 (6.99) (1H, t, *J* = 8.0 Hz, H-5"), 6.51 (6.66) (each 1H, brs, NH), 3.37 (3H, s, OCH$_3$), 3.27, 3.26 (each 3H, s, OCH$_3$ × 2), 2. 91 (3H, s, NCO*CH$_3$*).
^{13}C NMR (100 MHz, CDCl$_3$): Table 2.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for $C_{30}H_{41}N_2O_8$: 557.2863; found: 557.2959

Preparation of compound 39: Compound **38** (300 mg, 0.54 mmol) was dissolved in a solution of sodium hydroxide in methanol (10%), and the mixture was stirred at 60°C for 1 h and then basified with conc. NH$_4$OH to pH 11. After removing the solvents, the residue was purified by CC eluting with CHCl$_3$: MeOH (95:5 - 9:1, saturated with conc. ammonium hydroxide) to furnish compound **39** (168 mg, 79%).

Compound 39
^{1}H NMR (400 MHz, CDCl$_3$): 3.43 (1H, ABq, *J* = 8.4 Hz, H-18), 3.41, 3.30, 3.28 (each 3H, s, OCH$_3$ × 3).
^{13}C NMR (100 MHz, CDCl$_3$): Table 2.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{21}$H$_{34}$NO$_6$: 396.2386; found: 396.2388.

Preparation of compound 40: To a solution of compound **38** (420 mg, 0.755 mmol) in THF (10 mL) was added sodium hydride (18 mg, 0.755 mmol) under argon, and the mixture was stirred for 15 min before the addition of methyl iodide (50 μl, 0.801 mmol). The reaction was allowed to proceed with stirring at room temperature for 2 h, and the solvent was removed under reduced pressure. To the residue was added a solution of sodium hydroxide in methanol (5%, 15 mL), and the subsequent mixture was stirred at 60°C for 1 h prior to being basified with conc. ammonium hydroxide to pH 11. The solvent was removed *in vacuo*, and the residue was subjected to CC on silica gel eluting with chloroform: methanol (95:5, saturated with conc. ammonium hydroxide) to yield compound **40** (263 mg, 85%).

Compound 40
^{1}H NMR (400 MHz, CDCl$_3$): 3.40, 3.30, 3.29 (each 3H, s, OCH$_3$ × 3), 2.29 (3H, s, *N*CH$_3$).
^{13}C NMR (100 MHz, CDCl$_3$): Table 2.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{36}$NO$_6$: 410.2543 ; found: 410.2541.

Preparation of compound 44: To a solution of acetylsongorine (**43a**, 600 mg, 1.38 mmol) in isopropanol (15 mL) was added NBS (740 mg, 4.17 mmol), and the reaction mixture was stirred at 30°C for 2 h. After being basified with conc. ammonium hydroxide to pH > 10, the mixture was extracted with chloroform: methanol (10:1, 30 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated. The crude residue was subjected to CC eluting with CHCl$_3$: MeOH (20:1) to provide a white amorphous powder (280 mg). This was dissolved in a solution of sodium hydroxide in methanol (5%, 10 mL) and the consequent mixture was stirred at 50°C for 2 h. After removal of solvent, the residue was subjected to CC on silica gel eluting with CHCl$_3$: MeOH (20:1) to yield compound **44** (150 mg, 25%).

Compound 44
^{1}H NMR (400 MHz, CDCl$_3$): 5.19, 5.14 (each 1H, s, H$_2$-17), 4.28 (1H, s, H-15), 3.57 (1H, s, H-20), 0.67 (3H, s, H$_3$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 3.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{20}$H$_{28}$NO$_3$: 330.2069; found: 330. 2072.

Preparation of compounds 45 and 46: To a solution of songorine (**43**, 420 mg, 1.18 mmol) in methanol (30 mL) was added NaBH$_4$ (100 mg, 2.63 mmol), and the reaction mixture was stirred at room temperature for 35 min. The mixture was basified with conc. ammonium hydroxide to pH 10 and extracted with chloroform (20 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The crude residue was purified by CC on silica gel eluting with CHCl$_3$: MeOH (30:1) to yield compounds **45** (160 mg, 40%) and **46** (250 mg, 60%).

Compound 45
^{1}H NMR (400 MHz, CDCl$_3$): 5.16, 5.14 (each 1H, s, H$_2$-17), 4.17 (1H, s, H-15), 3.93 (1H, dd, *J* = 7.6, 6.4 Hz, H-12), 3.52 (1H, dd, *J* = 9.6, 6.8 Hz, H-1), 3.36 (1H, s, H-20), 1.06 (3H, t, *J* = 7.2 Hz, H$_3$-22), 0.77 (3H, s, H$_3$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 3.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{34}$NO$_3$: 360.2539; found: 360.2538.

Compound 46
^{1}H NMR (400 MHz, CDCl$_3$): 5.33, 5.12 (each 1H, s, H$_2$-17), 4.21 (1H, s, H-15), 4.18 (1H, dd, *J* = 9.2, 5.6 Hz, H-12), 3.87 (1H, dd, *J* = 8.8, 6.4 Hz, H-1), 3.47 (1H, s, H-20), 1.14 (3H, t, *J* = 7.2 Hz, H$_3$-22), 0.80 (3H, s, H$_3$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 3.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{34}$NO$_3$: 360.2539; found: 360.2540.

Preparation of compound 47: To a solution of songorine (**43**, 50 mg, 0.14 mmol) in 2 ml TEG was added NH$_2$NH$_2$ H$_2$O (0.4 mL, 7 mmol) and KOH (40 mg, 0.7 mmol), and the reaction was allowed to proceed under reflux at 160°C for 1 h. After removal of water and hydrazine, the reaction was allowed to proceed at 180°C for an additional 1 h. After cooling to room temperature, the reaction mixture was diluted with water and extracted with ethyl acetate (20 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was purified by CC eluting with light petroleum: ethyl acetate: diethylamine (75:25:1) to furnish compound **47** (40 mg, 82%).

Compound 47
^{1}H NMR (400 MHz, CDCl$_3$): 5.01 (2H, s, H$_2$-17), 4.19 (1H, d, *J* = 8.4 Hz, H-15), 3.97 (1H, t, *J* = 6.0 Hz, H-1), 3.34 (1H, s, H-20), 1.06 (3H, t, *J* = 7.2 Hz, H$_3$-22), 0.78 (3H, s, H$_3$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 3.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{34}$NO$_2$: 344.2590; found: 344.2585.

Preparation of compound 48: To a solution of compound **46** (90 mg, 0.25 mmol) in formic acid (85%, 10 mL) was added hydrogen peroxide (30%, 0.4-5 mL), and the reaction was allowed to proceed at room temperature for 5 h. The reaction mixture was basified with conc. ammonium hydroxide to pH > 10 and extracted with chloroform: methanol (5:1, 10 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was purified by CC eluting with CHCl$_3$: MeOH (20:1, saturated with conc.NH$_4$OH) to give compound **48** (30 mg, 35%).

Compound 48
^{1}H NMR (400 MHz, CDCl$_3$): 4.41 (1H, d, *J* = 13.2 Hz, H-12), 3.97, 3.36 (each 1H, ABq, *J* = 8.4 Hz, H$_2$-17), 3.73 (1H, s, H-15), 0.74 (3H, s, H$_3$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 3.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{36}$NO$_5$: 394. 2593; found: 374.2585.

Preparation of compounds 50 and 51: To a solution of songoramine (**49**, 100 mg, 0.28 mmol) in formic acid (85%, 10 mL) was added hydrogen peroxide (30%, 0.5 mL), and the reaction was allowed to proceed at room temperature for 20 h prior to being basified with 25% ammonium hydroxide to pH > 10. The subsequent mixture was extracted with ethyl acetate (20 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo*. CC of the crude residue on silica gel eluting with CHCl$_3$: MeOH (30:1) furnished compounds **50** (60 mg, 85%) and **51** (35 mg, 32%).

Compound 50
^{1}H NMR (400 MHz, CDCl$_3$): 4.10, 3.70 (each 1H, ABq, *J* = 8.0 Hz, H$_2$-17), 4.03 (1H, d, *J* = 5.2 Hz, H-1), 3.83 (1H, s, H-19), 3.70 (1H, s, H-15), 1.03 (3H, t, *J* = 7.2 Hz, H$_3$-22), 0.82 (3H, s, H$_3$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 3.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{32}$NO$_5$: 390.2280; found: 390.2279.

Compound 51
^{1}H NMR (400 MHz, CDCl$_3$): 3.97 (1H, d, *J* = 5.2 Hz, H-1), 3.70 (1H, s, H-15), 3.55, 3.50 (each 1H, ABq, *J* = 7.6 Hz, H$_2$-17), 1.02 (3H, t, *J* = 7.2 Hz, H$_3$-22), 0.84 (3H, s, H$_3$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 3.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{32}$NO$_5$: 390. 2280; found: 390.2279.

Preparation of compound 53: To a solution of denudatine (**52**, 20 mg, 0.06 mmol) in methanol (1 mL) was added hydrogen peroxide (0.1 mL), and the reaction was allowed to proceed at 60°C for 20 h prior to being quenched with 25% ammonium hydroxide to pH > 10. The mixture was extracted with chloroform: methanol (5:1, 10 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was purified by CC on silica gel, using CHCl$_3$: MeOH (50:1) as eluent, to provide compound **53** (13 mg, 60%).

Compound 53
^{1}H NMR (400 MHz, CDCl$_3$): 3.82 (1H, d, *J* = 9.2 Hz, H-11), 3.38, 3.43 (each 1H, ABq, *J* = 12.0 Hz, H$_2$-17), 3.26 (1H, s, H-15), 1.06 (3H, t, *J* = 7.2 Hz, H$_3$-22), 0.73 (3H, s, H$_3$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 3.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{22}$H$_{36}$NO$_4$: 378.2644; found: 378.2648.

Preparation of compound 55: To a solution of compound **54** (70 mg, 0.12 mmol) in CH$_2$Cl$_2$ (2 mL) was added *m*CPBA (70 mg, 0.39 mmol), and the reaction mixture was kept stirred at room temperature for 30 min prior to being concentrated. To the residue was added Fe$_2$SO$_4$·7H$_2$O (130 mg, 0.46 mmol) and the subsequent mixture was stirred at room temperature for 3 h. After general workup, the crude product was purified by CC over silica gel eluting with CHCl$_3$: MeOH (100:1) to furnish compound **55** (23 mg, 35%).

Compound 55
^{1}H NMR (400 MHz, CDCl$_3$): 5.16 (1H, s, H-15), 5.12 (1H, m, H-1), 5.00 (1H, m, H-11), 3.91 (1H, m, H-20), 2.05, 2.05, 1.98, 1.99 (each 3H, s, OAc × 4), 1.03 (3H, t, *J* = 7.2 Hz, H$_3$-22), 0.72 (3H, s, H$_3$-18).
^{13}C NMR (100 MHz, CDCl$_3$): Table 3.

HRMS-ESI: *m/z* [M+H]$^+$.cald for C$_{28}$H$_{40}$NO$_9$: 534.2703; found: 534.2740.

Preparation of compound 56: Compound **55** (30 mg, 0.06 mmol) was dissolved in a solution of sodium hydroxide in methanol (5%, 10 mL), and the subsequent reaction solution was stirred at 60°C for 2 h. The reaction mixture was concentrated *in vacuo*, and the residue was purified by CC over silica gel, eluting with CHCl$_3$: MeOH (20:1, saturated with 25% ammonium hydroxide) to furnish compound **56** (7 mg, 35%).

Compound 56
^{1}H NMR (400 MHz, CDCl$_3$): 4.55 (1H, d, *J* = 9.2 Hz, H-11), 4.21 (1H, dd, *J* = 10.4, 6.8 Hz, H-1), 4.16 (1H, s, H-15), 3.80, 3.77 (each 1H, ABq, *J* = 12.0 Hz, H$_2$-17), 3.86 (1H, s, H-20), 0.83 (3H, s, H$_3$-18).
^{13}C NMR (100 MHz, CDCl$_3$+CD$_3$OD): Table 3.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{20}$H$_{32}$NO$_5$: 366.2280; found: 366.2276.

Preparation of compound 57: To a solution of compound **55** (58 mg, 0.10 mmol) in THF (2 mL) was added sodium hydride (3.0 mg, 0.13 mmol), followed by methyl iodide (7 μL, 0.10 mmol). The reaction mixture was stirred at room temperature for 3 h prior to removing THF *in vacuo*. To the residue was added a solution of sodium hydroxide (5%) in methanol (10 mL), and the subsequent reaction mixture was stirred at 60°C for 2 h. After removing methanol, the residue was subjected to CC, eluting with CHCl$_3$: MeOH (50:1, v/v, saturated with 25% ammonium hydroxide) to yield compound **57** (35 mg, 92 %).

Compound 57
^{1}H NMR (400 MHz, CDCl$_3$): 4.38 (1H, d, *J* = 9.2 Hz, H-11), 4.06 (1H, dd, *J* = 10.4, 6.8 Hz, H-1), 2.25 (3H, s, H$_3$-21), 0.65 (3H, s, H$_3$-18).
^{13}C NMR (100 MHz, CD$_3$OD): Table 3.
HRMS-ESI: *m/z* [M+H]$^+$ calcd for C$_{21}$H$_{32}$NO$_5$: 378.2280; found: 378.2271.

References

[1]   Liu XX, Jiang XX, Cai, XF, Chao RB, Chen, QH, Chen DL, Wang XL, Wang FP. (**2012**) Cardioactive C$_{19}$-diterpenoid alkaloids from the lateral roots of *Aconitum carmichaeli* "FuZi". *Chemical & Pharmaceutical Bulletin*, **60**, 144-149.
[2]   Jiang XX, Tang P, Liu XX, Chao RB, Chen, QH, She XK, Chen DL, Wang XL, Wang FP. (**2012**) Structure-cardiac activity relationship of C$_{19}$-diterpenoid alkaloids. *Natural Product Communications*, **7**, 713-720.
[3]   Wang FP. Li ZB, Yang JS, Li BG. (**1999**) Modification of norditerpenoid alkaloids: II. A simple and convenient preparation of the imine derivatives of norditerpenoid alkaloids. *Chinese Chemical Letters*, **10**, 453-456.
[4]   Wang FP. Xu L. (**2005**) To seek an approach toward the chemical conversion of C$_{19}$-diterpenoid alkaloids to taxoids. *Tetrahedron*, **61**, 2149-2167.
[5]   Barton DHB, McCombie SW. (**1975**) New method for the deoxygenation of secondary alcohols, *Journal of the Chemical Society, Perkin Transactions I*, 1574-1585
[6]   Pelletier SW, Mody NV. (**1984**) in *Alkaloids: Chemical and Biological Perspective*, Pelletier SW. (Ed), Vol. **2**, John Wiley & Sons, Chichester, 205-462.
[7]   Ding H. (**2008**) In *Experiemental Pharmacology*, Ding H. (Ed), Science Press, Beijing, China. 504-506.
[8]   Wang FP. Unpublished data.

Monoterpenoid Indole Alkaloids from *Catharanthus roseus* Cultivated in Yunnan

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A new monoterpenoid indole alkaloid, 15,20-dehydro-3α-(2-oxopropyl) coronaridine (**1**), along with sixteen analogues (**2–17**) were isolated from the leaves of *Catharanthus roseus* cultivated in Yunnan. The new alkaloid was elucidated on the basis of extensive spectroscopic analysis, and the known alkaloids were identified by comparison with the reported spectroscopic data. Among them, alkaloid **16** was isolated from *Catharanthus* for the first time.

Keywords: *Catharanthus roseus*, Apocynaceae, Monoterpenoid indole alkaloids, 15,20-Dehydro-3α-(2-oxopropyl) coronaridine.

Catharanthus roseus (L.) G. Don. (Apocynaceae), a perennial evergreen herb with medicinal and ornamental values, is widely distributed in continental Africa, America, Asia, Australia, Europe, and in some islands of the Pacific Ocean [1]. *C. roseus* has been studied widely in view of its remarkable anticancer properties, especially the bisindole alkaloids, vinblastine and vincristine [1]. In previous investigations, we found that plant secondary metabolites would be influenced significantly by the ecological environments [2]. *C. roseus* has also been cultivated widely in China as an outside ornamental, which inspired us to search for structurally diverse indole alkaloids from the same plant in different ecological environments. Phytochemical investigation of the alkaloids of *C. roseus* cultivated in Xishuangbanna, a tropical rainforest zone, afforded a new alkaloid 15,20-dehydro-3α-(2-oxopropyl) coronaridine (**1**), along with sixteen analogues (**2–17**), coronaridine (**2**) [3], tetrahydroalstonine (**3**) [4], ajmalicine (**4**) [4], ajmalicinine (**5**) [5], serpentine (**6**) [6], 19-*epi*-alstonine (**7**) [7], yohimbine (**8**) [8], 19,20-dehydro-16-*epi*-yohimbine (**9**) [9], 16*R*-sitsirikine (**10**) [10], isositsirikine (**11**) [10], 16*R*-dihydrositsirikine (**12**) [11], pericyclivine (**13**) [12], akuammidine (**14**) [12], 17-acetyllochnerine (**15**) [13], 21-carbonylnormavacurine (**16**) [14], and 19*S*-*epi*-misiline (**17**) [15]. Among them, alkaloid (**16**) was isolated from this plant for the first time.

Alkaloid **1** was isolated as a white amorphous powder. Its molecular formula was deduced as $C_{24}H_{28}N_2O_3$ based on ^{13}C NMR and HRESIMS data. The ^{1}H NMR spectrum of **1** showed characteristic signals of an unsubstituted indole alkaloid moiety at δ_H 7.47 (1H, d, 7.8 Hz, H-9), 7.08 (1H, td, 7.2 Hz, H-10), 7.14 (1H, td, 7.2 Hz, H-11), and 7.23 (1H, d, 7.8 Hz, H-12). Additionally, the ^{1}H spectrum displayed signals for an ethyl side chain at δ_H 2.25/2.10 (2H, m, H-19) and 1.07 (3H, t, Me-18), a bridgehead proton adjacent to a nitrogen at δ_H 4.15 (1H, br s, H-21), a methoxy group at δ_H 3.71 (3H, s), a –COCH_3 group (δ_H 2.13, 3H, s), and an olefinic proton at δ_H 5.77 (1H, d, 6.0 Hz, H-15). The ^{1}H–^{1}H COSY spectrum suggested two main spin systems: δ_H 2.76/1.85 (dd/dd, H_2-17)/δ_H 2.66 (m, H-14) [δ_H 3.36 (m, H-3/N)]/δ_H 2.48/2.33 (dd/dd, H_2-22)/H-15 and H_2-19/Me-18. The HMBC correlations from H-3 to δ_C 51.5 (t, C-5) and δ_C 62.1 (d, C-21) revealed that C-3, C-21, and C-5 originated from carbons attached to N-4. Likewise, the HMBC cross-peaks of H-21/ C-2, δ_C 54.8 (s, C-16), δ_C 38.7 (t, C-17) and
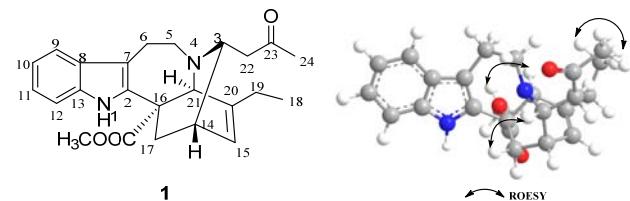
Figure 1: Structure and ROESY correlations of compound **1**.

Table 1: ^{1}H and ^{13}C NMR data for **1** (CDCl$_3$, 600 and 150 MHz, respectively).

position	δ_H, mult. (J in Hz)	δ_C, type	HMBC
1	7.62, br s		7, 8
2		136.2, s	
3	3.36, m	55.9, d	5, 21
5	3.44, m; 3.32, m	51.5, t	7, 21
6	3.26, m; 2.90, m	21.5, t	
7		110.7, s	
8		128.9, s	
9	7.47, br d (7.8)	118.2, d	
10	7.08, td (7.2)	119.5, d	
11	7.14, td (7.2)	121.9, d	
12	7.23, br d (7.8)	110.4, d	
13		134.9, s	
14	2.66, m	34.7, d	16, 20
15	5.77, d (6.0)	121.7, d	3, 21
16		54.8, s	
17	2.76, dd (13.2, 3.6)	38.7, t	
	1.85, dd (13.2, 2.4)		
18	1.07, t (7.2)	11.2, q	
19	2.25, m; 2.10, m	26.3, t	15
20		150.1, s	
21	4.15, br s	62.1, d	
22	2.48, dd (16.2, 4.8)	49.3, t	14
	2.33, dd (16.2, 7.8)		
23		209.1, s	
24	2.13, s	31.1, q	
-COOCH$_3$		174.0, s	
-COOCH$_3$	3.71, s	52.3, q	

H-17/C-2, C-16, C-21, δ_C 174.0 (s, -COOCH$_3$) implied that CH-21, CH$_2$-17 and the methoxycarbonyl group were connected *via* C-16, H-14/δ_C 49.3 (t, C-22), C-16, δ_C 150.1 (s, C-20) and H-15/δ_C 26.3 (t, C-19), δ_C 55.9 (d, C-3); C-21 showed a double bond located at C-15/C-20 and a 3α-(2-oxopropyl). These observations suggested that **1** possessed an ibogan-type monoterpenoid indole alkaloid carbon skeleton, similar to that of 3-(2-oxopropyl) coronaridine [3]. The differences between them were that a methylene and a methine in 3-(2-oxopropyl) coronaridine [3] were replaced by a double bond (δ_C 150.1, s, C-20; 127.1, d, C-15) in **1**. In its ROESY spectrum, the

NOE correlations between H-21/δ_H 3.71 (-COOCH_3), placed H-21 and COOCH_3 on the same face. Biogenetically, they were assigned with an α-orientation, and H-14 with a β-orientation of coronaridine derivatives isolated from Apocynaceae [16]. Moreover, NOE correlations between H-14 and H-3 suggested that H-14 and H-3 were on the same face, whereas the correlations between Me-18 and Me-24 positioned them on the opposite side (Figure 1). The specific rotation of **1** {$[\alpha]_D^{21}$ −11 (c 0.13, CHCl$_3$)} was also similar to that observed for ervatamine G {$[\alpha]_D^{20}$ −23 (c 0.3, CHCl$_3$)} [16]. Based on the above evidence, the structure of **1** was elucidated as shown.

Experimental

General experimental procedures: Optical rotation, JASCO P-1020 digital polarimeter; UV, Shimadzu UV-2401 PC spectrophotometer; IR, Bruker Tensor-27 infrared spectrophotometer; 1D and 2D NMR, Bruker AM-400 and DRX-600 spectrometers; ESIMS, Bruker HTC/Esquire spectrometer; HREIMS, Waters AutoSpec Premier P776 spectrometer; Semipreparative HPLC was performed on an Agilent 1260 HPLC with a ZORBAX SB-C18 (9.4 × 250 mm). TLC were visualized by Dragendorff`s reagent and 10% H$_2$SO$_4$ in ethanol.

Plant material: Air-dried stems and branches of *C. roseus* were collected in November 2012 from Xishuangbanna, Yunnan province, P. R. China. The plant was identified by Mr Hai-Chuan Tai. A voucher specimen (No. Tai2012110503) has been deposited in KIB, CAS.

Extraction and isolation: The air-dried and powdered stems and branches of *C. roseus* (9.0 kg) were extracted with MeOH under reflux conditions, and the solvent was evaporated *in vacuo*. The residue was dissolved in 0.37% HCl (pH 2-3) and the solution was subsequently basified using 10% ammonia to pH 9-10. The basic solution was partitioned with EtOAc, affording a two-phase mixture. The EtOAc fraction (190 g) was subjected to CC over silica gel (Fr. A-F). Fr. A (6.5 g) was subjected to silica gel CC

(light petroleum/acetone, 9:1-0:1, v/v) and recrystallization to yield **3** (700 mg), and **4** (26.5 mg). Fr. B (9.8 g) was eluted *via* silica gel CC under isocratic conditions (light petroleum/acetone, 7:1, v/v) to afford **1** (1.8 mg), **2** (5.6 mg), **8** (1.5 mg), **10** (4.7 mg), and **17** (10.0 mg). Compounds **14** (6.3 mg), **5** (10.3 mg), **9** (4.5 mg), and **11** (3.0 mg) were obtained from Fr. C (11.2 g) by silica gel CC with isocratic elution using light petroleum/acetone, 5:1. Fr. D (35.5 g) was subjected to silica gel CC (CHCl$_3$/MeOH, 20:1-0:1) and further resolved by semi-HPLC to yield compounds **15** (2.4 mg), **12** (1.7 mg), **13** (159.6 mg), and **16** (30.7 mg). Fr. E (95.3 g) was chromatographed on a RP-18 column with MeOH/H$_2$O (20:1-1:0, v/v) gradient system to give fractions E1-E8. Fractions E3 (6.3 g) and E5 (9.6 g) were separated on a Sephadex LH-20 column eluting with CHCl$_3$/MeOH (1:1, v/v) and further by PTLC to yield **6** (10.8 mg), and **7** (4.8 mg).

15, 20-Dehydro-3α-(2-oxopropyl) coronaridine (1)
White amorphous powder.
$[\alpha]_D^{21}$: −11 (c 0.13, CHCl$_3$).
Rf: 0.5 (light petroleum-ethyl acetate, 10:1).
IR (KBr): 3425, 2962, 2925, 1714, 1632, 1461, 1383, 1262, 1095, 1028, 804 cm^{-1}.
UV/Vis λ_{max} (MeOH) nm (log ε): 240 (3.70), 284 (3.59).
^{1}H and ^{13}C NMR (CDCl$_3$): Table 1.
ESIMS : m/z (%) = 393 [M + H]$^+$ (100).
HRESIMS : found m/z 393.2181 (Calcd. for 393.2178).

Supplementary data: NMR and mass spectra of the new compound **1**, and ^{13}C NMR data of compounds **2–17**.

References

[1]   Mujib A, Ilah A, Aslam J, Fatima S, Siddiqui ZH, Maqsood M. (**2012**) *Catharanthus roseus* alkaloids: application of biotechnology for improving yield. *Plant Growth Regulation*, **68**, 111-127.

[2]   Liu YP, Lai R, Yao YG, Zhang ZK, Pu ET, Cai XH, Luo XD. (**2013**) Induced furoeudesmanes: a defense mechanism against stress in *Laggera pterodonta*, a Chinese herbal plant. *Organic Letters*, **15**, 4940-4943.

[3]   Okuyama E, Gao LH, Yamazaki M. (**1992**) Analgesic components from Bornean medicinal plants, *Tabernaemontana pauciflora* Blume and *Tabernaemontana pandacaqui* Poir. *Chemical & Pharmaceutical Bulletin*, **40**, 2075-2079.

[4]   Wenkert E, Chang CJ, Chawla HPS, Cochran DW, Hagaman EW, King JC, Orito K. (**1976**) General methods of synthesis of indole alkaloids. 14. Short routes of construction of yohimboid and ajmalicinoid alkaloid systems and their carbon-13 nuclear magnetic resonance spectral analysis. *Journal of the American Chemical Society*, **98**, 3645-3655.

[5]   Madinaveitia A, Reina M, Fuente Gdl, Gonzalez AG. (**1996**) Obovamine, a new indole alkaloid from *Stemmadenia obovata*. *Journal of Natural Products*, **59**, 185-189.

[6]   Wachsmuth O, Matusch R. (**2002**) Anhydronium bases from *Rauvolfia serpentina*. *Phytochemistry*, **61**, 705-709.

[7]   Dinda B, De UC. (**2002**) 19-Epialstonine from *Amphicome emodi* roots. *Indian Journal of Chemistry, Section B: Organic Chemistry Including Medicinal Chemistry*, **41B**, 2698-2700.

[8]   Itoh A, Kumashiro T, Yamaguchi M, Nagakura N, Mizushina Y, Nishi T, Tanahashi T. (**2005**) Indole alkaloids and other constituents of *Rauwolfia serpentina*. *Journal of Natural Products*, **68**, 848-852.

[9]   Dos Santos Torres ZE, Silveira ER, Rocha e Silva LF, Lima ES, Carvalho de Vasconcellos M, Uchoa DEdA, Braz Filho R, Pohlit AM. (**2013**) Chemical composition of *Aspidosperma ulei* Markgr. and antiplasmodial activity of selected indole alkaloids. *Molecules*, **18**, 6281-6297.

[10]   Zhang BJ, Yan JM, Wu ZK, Liu YP, Bao MF, Cheng GG, Luo XD, Cai XH, Li Y. (**2013**) Alkaloids from *Ochrosia borbonica*. *Helvetica Chimica Acta*, **96**, 2288-2298.

[11]   Robert GMT, Ahond A, Poupat C, Potier P, Jolles C, Jousselin A, Jacquemin H. (**1983**) *Aspidosperma* from Guiana: alkaloids from *Aspidosperma marcgravianum*. *Journal of Natural Products*, **46**, 694-707.

[12]   Farnsworth NR, Loub WN, Blomster RN, Gorman M. (**1964**) Pericyclivine, a new *Catharanthus* alkaloid. *Journal of Pharmaceutical Sciences*, **53**, 1558.

[13]   Kam TS, Iek IH, Choo YM. (**1999**) Alkaloids from the stem-bark of *Alstonia macrophylla*. *Phytochemistry*, **51**, 839-844.

[14]   Liu L, Chen YY, Qin XJ, Wang B, Jin Q, Liu YP, Luo XD. (**2015**) Antibacterial monoterpenoid indole alkaloids from *Alstonia scholaris* cultivated in temperate zone, *Fitoterapia*, **105**, 160-164.

[15]   Atta ur Rahman, Silva WSJ, Alvi KA, De Silva KT. (**1987**) Isolation and structural studies on the alkaloids of *Petchia ceylanica*. *Phytochemistry*, **26**, 543-545.

[16]   Zhang DB, Yu DG, Sun M, Zhu XX, Yao XJ, Zhou SY, Chen JJ, Gao K. (**2015**) Ervatamines A-I, anti-inflammatory monoterpenoid indole alkaloids with diverse skeletons from *Ervatamia hainanensis*. *Journal of Natural Products*, **78**, 1253-1261.

Two New Oxindole Alkaloid Glycosides from the Leaves of *Nauclea officinalis*

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Two new oxindole alkaloid glycosides, nauclealomide A and (3*S*,7*R*)-javaniside, were isolated from the leaves of *Nauclea officinalis*. Their structures and absolute configurations were elucidated by means of NMR, HRESIMS, X-ray diffraction, acid hydrolysis and quantum chemical CD calculation. Nauclealomide A is a novel monoterpenoid oxindole alkaloid possessing a rare tetrahydro-2*H*-1,3-oxazine ring.

Keywords: *Nauclea officinalis*, Rubiaceae, Oxindole alkaloid, Tetrahydro-2*H*-1,3-oxazine ring.

The genus *Nauclea* (Rubiaceae) comprises thirty-five species which are mainly distributed in *Africa and* tropical regions of Asia and Australia [1]. The plants of this genus are rich in indole alkaloids, which exhibit antimalarial, antiproliferative and renin-inhibitory activities [2-5]. To date, phytochemical investigations have led to the isolation of more than 70 indole alkaloids from the genus. The bark and twigs of *Nauclea officinalis* Pierre ex Pitard is used as a traditional medicine in China for treatment of colds, swelling of the throat, pink eye and other ailments [1]. During the course of an ongoing search for new alkaloids from medicinal plants in China [6-12], the EtOH extract of the leaves of *N. officinalis* was further investigated, which led to the isolation of two new oxindole alkaloid glycosides, nauclealomide A (**1**) and (3*S*,7*R*)-javaniside (**2**) (Figure 1). The structures of indole alkaloids **1** and **2** were elucidated by NMR, HRESIMS, X-ray diffraction, acid hydrolysis, as well as CD analysis. Herein, the isolation and structural elucidation of the new indole alkaloids are described.

Nauclealomide A (**1**) showed a molecular formula of $C_{26}H_{30}N_2O_{10}$ based on the HRESIMS (*m/z* 553.1791 [M + Na]+; calculated for $C_{26}H_{30}N_2O_{10}Na$: *m/z* 553.1793). The UV spectrum of **1** showed absorption maxima at 211 and 242 nm. The IR spectrum exhibited absorptions at 3394 and 1648 cm⁻¹, which suggested the presence of amino and α,β-unsaturated carbonyl functional groups. The ¹H and ¹³C NMR spectra of **1** displayed signals due to two carbonyl groups (δ_C 180.1 and 166.1), a trisubstituted double bond [δ_H 7.44 (1H, d, *J* = 2.4 Hz); δ_C 149.7 and 109.0], a terminal vinyl group [δ_H 5.56 (1H, dt, *J* = 17.0, 10.1 Hz), 5.29 (1H, dd, *J* = 17.0, 1.8 Hz) and 5.24 (1H, dd, *J* = 10.1, 1.8 Hz); δ_C 134.1 and 120.7] and an *ortho*-substituted benzene ring [δ_H 7.25 (1H, br d, *J* = 7.5 Hz), 7.25 (1H, br t, *J* = 7.5 Hz), 7.02 (1H, br t, *J* = 7.5 Hz) and 6.84 (1H, br d, *J* = 7.5 Hz); δ_C 142.3, 131.4, 131.1, 125.4, 123.9 and 111.0]. In addition, the NMR spectra of **1** displayed signals due to a sugar unit [δ_H 4.69 (1H, d, *J* = 7.9 Hz), 3.87 (1H, dd, *J* = 11.8, 1.7 Hz), 3.66 (1H, dd, *J* = 11.8, 5.3 Hz), and 3.37-3.20 (4H, overlapped); δ_C 100.4, 78.3, 78.2, 74.7, 71.5 and 62.6]. Acid hydrolysis of **1** afforded D-glucose, which was identified by HPLC analysis using authentic samples as references.
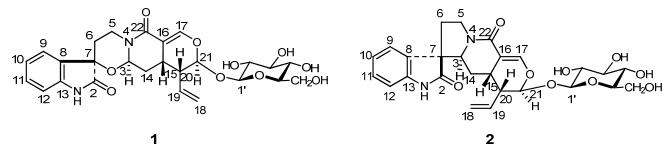
Figure 1: Structures of **1** and **2**.

In addition, the β-configuration for D-glucose was further determined based on the large ${}^3J_{H1,H2}$ coupling constant (*J* = 7.9 Hz) of the anomeric proton. Taken together, these spectral data suggested that **1** was a monoterpenoid oxindole alkaloid with a β-D-glucose unit [13-15].

With the aid of ¹H-¹H COSY, HSQC, HMBC, and ROESY experiments, all of the ¹H and ¹³C NMR signals of **1** were assigned as shown in Table 1. The ¹H-¹H COSY spectrum of **1** revealed the presence of three spin coupling systems (C-9 to C-12, C-5 to C-6, and C-3 to C-21/C-18, Figure 2). In the HMBC spectrum, the correlations between H-17 and C-15/C-16/C-21/C-22, and between H-3 and C-22 revealed the presence of ring E, which was fused to ring D. In addition, the HMBC correlation between H-21 and C-1' indicated that the glucose was located at C-21. Moreover, HMBC correlations between Ha-5 and C-3/C-7/C-22, as well as between Ha-6 and C-2/C-7/C-8 were also observed (Figure 2).
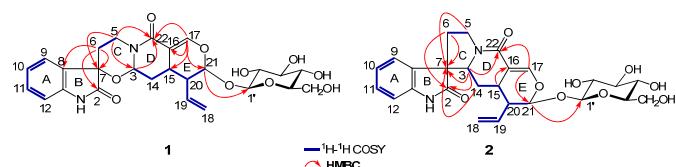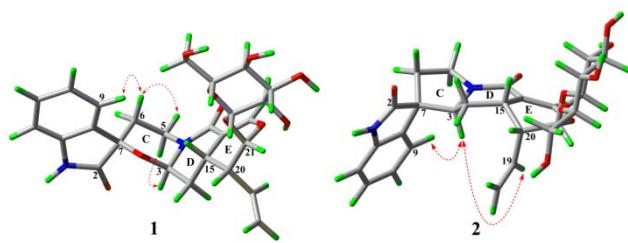
Figure 2: ¹H-¹H COSY and selected HMBC correlations of **1** and **2**.

Comparison of the NMR data of **1** with those of javaniside [13,14] indicated that they were very similar, except for the obvious down-field shift of H-3 (from δ_H 4.09 to 6.28), C-3 (from δ_C 65.5 to 80.0) and C-7 (from δ_C 58.0 to 77.6). Furthermore, according to the
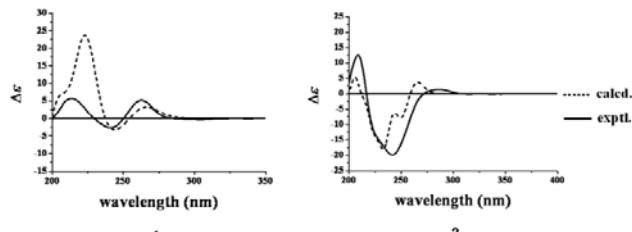
Table 1: NMR spectroscopic data for **1** and **2** in CD₃OD (400 MHz for ¹H, 100 MHz for ¹³C, δ in ppm, *J* in Hz).

Position	1		2	
	δ_C	δ_H	δ_C	δ_H
2	180.1	-	181.5	-
3	80.0	6.28 t (2.8)	63.8	3.95 m
5	39.3	a 4.60 ddd (13.0, 4.9, 1.8)	44.9	a 3.93 m
		b 3.75 dd (13.0, 3.0)		b 3.83 m
6	32.4	a 2.11 td (14.1, 5.4)	34.3	a 2.36 dt (13.0, 9.8)
		b 1.77 m		b 2.21 ddd (13.0, 7.5, 1.6)
7	77.6	-	57.7	-
8	131.4	-	129.8	-
9	125.4	7.25 br d (7.5)	124.0	7.25 br d (7.5)
10	123.9	7.02 br t (7.5)	123.7	7.05 td (7.5, 1.0)
11	131.1	7.25 br t (7.5)	130.0	7.24 td (7.5, 1.0)
12	111.0	6.84 br d (7.5)	111.0	6.91 dt (7.5, 1.0)
13	142.3	-	143.4	-
14	29.7	1.76 m	24.7	a 1.77 dt (13.7, 9.4)
				b 1.51 dt (13.7, 4.5)
15	23.4	3.27 m	24.9	3.18 m
16	109.0	-	109.9	-
17	149.7	7.44 d (2.4)	147.5	7.14 d (2.6)
18	120.7	a 5.29 dd (17.0, 1.8)	120.6	a 5.11 dd (10.1, 1.9)
		b 5.24 dd (10.1, 1.8)		b 5.06 dd (17.0, 1.9)
19	134.1	5.56 dt (17.0, 10.1)	133.8	5.59 dt (17.0, 10.1)
20	44.3	2.61 ddd (10.1, 5.8, 1.7)	45.7	2.41 ddd (10.1, 5.3, 1.6)
21	98.1	5.46 d (1.7)	97.4	5.37 d (1.6)
22	166.1	-	167.7	-
1'	100.4	4.69 d (7.9)	99.9	4.65 d (7.9)
2'	74.7	3.20 m	74.6	3.15 dd (9.0, 7.9)
3'	78.2	3.37 m	77.8	3.35 t (9.0)
4'	71.5	3.30 m	71.5	3.25 t (9.0)
5'	78.3	3.31 m	78.3	3.28 dd (5.7, 2.0)
6'	62.6	a 3.87 dd (11.8, 1.7)	62.6	a 3.84 dd (12.0, 2.0)
		b 3.66 dd (11.8, 5.3)		b 3.62 dd (12.0, 5.7)

Figure 3: Selected ROESY correlations of **1** and **2**.

molecular formula and the degree of unsaturation, the remaining oxygen atom could be assigned as a bridge between C-3 and C-7 to form a tetrahydro-2*H*-1,3-oxazine ring (Figure 2).

The relative configuration of **1** was deduced from coupling constants and ROESY data. The coupling constants of $^3J_{20,21}$ (J = 1.7 Hz) and $^3J_{20,15}$ (J = 5.8 Hz) suggested the presence of β/β/α orientation for H-15, H-20 and H-21 [13-15], while the coupling constant of $^3J_{3,14}$ (J = 2.8 Hz) indicated the presence of α configuration of H-3. In the ROESY spectrum, the correlations between H-3 and Hb-5, as well as between Ha-6 and H-9/Ha-5 indicated the presence of β/α/β configuration of Ha-5, Hb-5 and Ha-6 (Figure 3). The absolute configuration of C-15 in these indole alkaloids was determined as *S* biosynthetically based on secologanin [16]. Thus, the absolute configuration of **1** was elucidated as 3*R*,7*R*,15*S*,20*R*,21*S*, which was consistent with the results obtained by the following quantum chemical CD calculation experiment (Figure 4).

Figure 4: Calculated and experimental CD spectra of **1** and **2**.

The molecular formula of (3*S*,7*R*)-javaniside (**2**) was established as $C_{26}H_{30}N_2O_9$ (*m/z* 537.1823 [M + Na]⁺; calculated for $C_{26}H_{30}N_2O_9Na$: *m/z* 537.1844) by HRESIMS. Acid hydrolysis of **2** also afforded D-glucose. Similar to **1**, the NMR spectra of **2** revealed the presence of two carbonyl groups (δ_C 181.5 and 167.7), a trisubstituted double bond [δ_H 7.14 (1H, d, *J* = 2.6 Hz); δ_C 147.5 and 109.9], a terminal vinyl group [δ_H 5.59 (1H, dt, *J* = 17.0, 10.1 Hz), 5.11 (1H, dd, *J* = 10.1, 1.9 Hz) and 5.06 (1H, dd, *J* = 17.0, 1.9 Hz); δ_C 133.8 and 120.6], and an *ortho*-substituted benzene ring, which suggested that **2** was also a monoterpenoid oxindole alkaloid with a glucose moiety [13-15].

With the aid of 1D and 2D NMR experiments, all of the ¹H and ¹³C NMR signals of **2** were assigned (Table 1). The ¹H-¹H COSY spectrum of **2** revealed the presence of three spin coupling systems in bold (Figure 2). In the HMBC spectrum, the correlations between H-17 and C-21/C-22, between H-15 and C-16, as well as between H-3 and C-22 revealed the presence of ring E, which was fused to ring D. Moreover, the HMBC correlations between H₂-6/H-5b/H-3/H₂-14 and C-7, and between H-3/H₂-6 and C-2 were observed, which indicated that ring C was fused to ring D and then linked to ring B via the spiro-carbon (C-7). In addition, the HMBC correlation between H-21 and C-1' indicated that the glucose was located at C-21.

The relative configuration of **2** could be established by a ROESY experiment. In the ROESY spectrum, the correlation between H-3 and H-19 indicated the α orientation of H-3. In addition, the ROESY correlation between H-9 and H-3 suggested that the carbonyl group at C-2 was above the C/D/E plane (Figure 3). Considering the biogenetic pathway of these indole alkaloids, the absolute configuration of C-15 was determined as *S* [16]. Thus, the absolute configuration of **2** was elucidated as 3*S*,7*R*,15*S*,20*R*,21*S*, which was consistent with the results obtained by the single crystal X-ray diffraction experiment (Figure 5) and the quantum chemical CD calculation experiment (Figure 4).
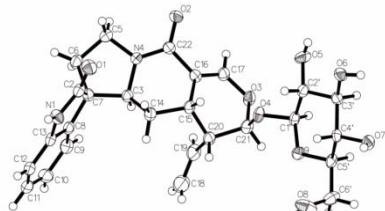
Figure 5: Perspective view of the crystal structure of **2**.

Experimental

General experimental procedures: Optical rotations were measured on a JASCO P-1020 polarimeter, UV spectra on a JASCO V-550 UV/VIS spectrophotometer with a 1 cm length cell, and IR spectra on a JASCO FT/IR-480 plus FT-IR spectrometer. ^{1}H, ^{13}C, and 2D NMR spectra were recorded on a Bruker AV-400 spectrometer and HRESIMS on an Agilent 6210 ESI/TOF mass spectrometer. CD spectra were obtained using a JASCO J-810 circular dichroism spectrometer. For column chromatography, silica gel (300-400 mesh; Qingdao Marine Chemical Group Corporation) and Sephadex LH-20 (Pharmacia) were used. TLC analyses were carried out using precoated silica gel GF$_{254}$ plates (Yantai Chemical Industry Research Institute). Analytical high-performance liquid chromatography (HPLC) was performed on an Agilent chromatograph equipped with a G1311C pump and a G1315D diode-array detector (DAD) with a Cosmosil 5C$_{18}$-MS- II column (4.6 × 250 mm, 5 μm). Preparative HPLC was carried out on an Agilent instrument equipped with a G1310B pump and a G1365D detector with a Cosmosil 5C$_{18}$-MS- II Waters column (10 × 250 mm, 5 μm). X-ray crystallographic analysis was carried out on an Agilent Gemini S Ultra diffractometer with Cu Kα radiation (λ = 1.5418 Å).

Plant material: The leaves of *N. officinalis* were collected in Sanya city, Hainan province of P. R. China, in July of 2008, and authenticated by Professor Wei-ping Chen (Hainan Branch Institute of Medicinal Plants, Chinese Academy of Medical Science). A voucher specimen (No. 20090223) was deposited in the Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, P. R. China.

Extraction and isolation: Air-dried and powdered leaves of *N. officinalis* (4.8 kg) were extracted with 95% EtOH (50 L × 3, 3 days each) at room temperature. The EtOH solution was evaporated under reduced pressure to afford a residue (250.0 g), which was suspended in water and partitioned successively with light petroleum and EtOAc. The basic solution was then partitioned with *n*-BuOH to afford *n*-BuOH and emulsion fractions. The emulsion fraction (5.0 g) was subjected to silica gel CC, eluting with CHCl$_3$ - MeOH (100:0→80:20) to give 9 fractions (Fr. 1-9). Fr. 7 (669.0 mg) were further purified on a Sephadex LH-20 column using MeOH as eluent, then nauclealomide A (**1**, 10.0 mg) and (3*S*,7*R*)-javaniside (**2**, 15.0 mg) were finally obtained by preparative HPLC using MeOH-H$_2$O (43:57, 3 mL/min) as the mobile phase.

Nauclealomide A (1)
yellowish amorphous solid.
$[\alpha]_D^{27}$: -55 (*c* 0.17, MeOH).
IR (KBr): ν$_{max}$ 3394, 1707, 1648, 1482, 1072 cm^{-1}.
UV (MeOH) λ$_{max}$ (log ε): 211 (4.32), 242 (4.10) nm.
^{1}H and ^{13}C NMR data (CD$_3$OD, 400 and 100 MHz): Table 1.
HRESIMS *m/z*: 553.1791 [M + Na]$^+$ (calcd for C$_{26}$H$_{30}$N$_2$O$_{10}$Na: 553.1793).

(3*S*,7*R*)-Javaniside (2)
colourless plates.
$[\alpha]_D^{27}$: -212 (*c* 0.25, MeOH).
IR (KBr): ν$_{max}$ 3394, 1703, 1652, 1587, 1470, 1072, 1015 cm^{-1}.
UV (MeOH) λ$_{max}$ (log ε): 211 (4.50), 244 (4.33) nm.
^{1}H and ^{13}C NMR data (CD$_3$OD, 400 and 100 MHz): Table 1.
HRESIMS *m/z*: 537.1823 [M + Na]$^+$ (calcd for C$_{26}$H$_{30}$N$_2$O$_9$Na: 537.1844).

Crystal data of (3S,7R)-javaniside (2): The X-ray data of indole alkaloid **2** were collected using a Sapphire CCD with a graphite monochromated Cu Kα radiation, λ = 1.54184 Å at 173.00(10) K. The structure was solved by direct methods using the SHELXS-97 program, and refined by the SHELXL-97 program and full-matrix least-squares calculations [17]. C$_{26}$H$_{32}$N$_2$O$_{10}$, M_r = 532.54, *Orthorhombic*, *a* = 10.99153 (12) Å, *b* = 13.26367 (14) Å, *c* = 17.28232 (16) Å, *α* = *β* = *γ* = 90.00°, *V* = 2519.56 (4) Å^3, space group *P2(1)2(1)2(1)*, *Z* = 4, *μ* (Cu Kα) = 0.912 mm^{-1}, 20419 reflections collected, 4036 independent reflections (R_{int} = 0.0270). The final R_1 value was 0.0297 [$I > 2\sigma(I)$]. The final $wR(F^2)$ value was 0.0769 [$I > 2\sigma(I)$]. The final R_1 value was 0.0310 (all data). The final $wR(F^2)$ value was 0.0781 (all data). The goodness of fit on F^2 was 1.069. Flack parameter = -0.09 (15). Crystallographic data of (3*S*,7*R*)-javaniside have been deposited at the Cambridge Crystallographic Data Centre (deposition number: CCDC 937396).

Acid hydrolysis: Indole alkaloids **1** and **2** (2 mg each) in 2 N HCl (5 mL) were heated at 80°C for 2 h, after which the residue was dissolved in pyridine (1 mL) and stirred with L-cysteine methyl ester hydrochloride (2 mg) at 60°C for 1 h. *O*-tolyl isothiocyanate (5 μL) was then added to the mixture and heated at 60°C for an additional 1 h. The reaction mixture was subsequently analyzed by HPLC and detected at 250 nm. Analytical HPLC was performed on a Cosmosil 5C$_{18}$-MS- column (4.6 × 250 mm, 5 μm) at 20°C using CH$_3$CN-0.05% CH$_3$COOH in H$_2$O (25:75, 1.0 mL/min) as the mobile phase. Peaks were detected with a G1315D DAD. D-Glucose (t_R 16.4 min) was identified as the sugar moiety of indole alkaloids **1** and **2** by comparison with authentic samples of D-glucose (t_R 16.4 min) and L-glucose (t_R 14.9 min) [18].

Supplementary data: HRESIMS, UV, IR, 1D and 2D NMR spectra of indole alkaloids **1** and **2**, and quantum chemical CD calculations of **1** and **2**.

References

[1]   Editorial Committee of Chinese Herbs. (**1999**) *Chinese Herbs*, Vol. **6**. Shanghai Science and Technology Press, Shanghai, China. 456.
[2]   He ZD, Ma CY, Zhang HJ, Tan GT, Tamez P, Sydara K, Bouamanivong S, Southavong B, Soejarto DD, Pezzuto JM, Fong HHS. (**2005**) Antimalarial constituents from *Nauclea orientalis* (L.) L. *Chemistry & Biodiversity*, **2**, 1378-1386.
[3]   Sun JY, Lou HX, Dai SJ, Xu H, Zhao F, Liu K. (**2008**) Indole alkaloids from *Nauclea officinalis* with weak antimalarial activity. *Phytochemistry*, **69**, 1405-1410.
[4]   Erdelmeier CAJ, Regenass U, Rali T, Sticher O. (**1992**) Indole alkaloids with *in vitro* antiproliferative activity from the ammoniacal extract of *Nauclea orientalis*. *Planta Medica*, **58**, 43-48.

[5]   Agomuoh AA, Ata A, Udenigwe CC, Aluko RE, Irenus I. (**2013**) Novel indole alkaloids from *Nauclea latifolia* and their renin-inhibitory activities. *Chemistry & Biodiversity*, *10*, 401-410.

[6]   Wu ZL, Zhao BX, Huang XJ, Tang GY, Shi L, Jiang RW, Liu X, Wang Y, Ye WC. (**2014**) Suffrutines A and B: a pair of Z/E isomeric indolizidine alkaloids from the roots of *Flueggea suffruticosa*. *Angewandte Chemie International Edition*, *53*, 5796-5799.

[7]   Liu ZW, Yang TT, Wang WJ, Li GQ, Tang BQ, Zhang QW, Fan CL, Zhang DM, Zhang XQ, Ye WC. (**2013**) Ervahainine A, a new cyano-substituted oxindole alkaloid from *Ervatamia hainanensis*. *Tetrahedron Letters*, *54*, 6498-6500.

[8]   Zhao BX, Wang Y, Li C, Wang GC, Huang XJ, Fan CL, Li QM, Zhu HJ, Chen WM, Ye WC. (**2013**) Flueggedine, a novel axisymmetric indolizidine alkaloid dimer from *Flueggea virosa*. *Tetrahedron Letters*, *54*, 4708-4711.

[9]   Zhao BX, Wang Y, Zhang DM, Huang XJ, Bai LL, Yan Y, Chen JM, Lu TB, Wang YT, Zhang QW, Ye WC. (**2012**) Virosaines A and B, two new birdcage-shaped Securinega alkaloids with an unprecedented skeleton from *Flueggea virosa*. *Organic Letters*, *14*, 3096-3099.

[10]   Zhao BX, Wang Y, Zhang DM, Jiang RW, Wang GC, Shi JM, Huang XJ, Chen WM, Che CT, Ye WC. (**2011**) Flueggines A and B, two new dimeric indolizidine alkaloids from *Flueggea virosa*. *Organic Letters*, *13*, 3888-3891.

[11]   Ouyang S, Wang L, Zhang QW, Wang GC, Wang Y, Huang XJ, Zhang XQ, Jiang RW, Yao XS, Che CT, Ye WC. (**2011**) Six new monoterpenoid indole alkaloids from the aerial part of *Gelsemium elegans*. *Tetrahedron*, *67*, 4807-4813.

[12]   Fan L, Fan CL, Wang Y, Zhang XQ, Zhang QW, Zhang JQ, Ye WC. (**2010**) Alkaloids from the leaves of *Nauclea officinalis*. *Acta Pharmaceutica Sinica*, *45*, 747-751.

[13]   Ma J, Hecht SM. (**2004**) Javaniside, a novel DNA cleavage agent from *Alangium javanicum* having an unusual oxindole skeleton. *Chemical Communications*, *40*, 1190-1191.

[14]   Pham VC, Ma J, Thomas SJ, Xu ZD, Hecht SM. (**2005**) Alkaloids from *Alangium javanicum* and *Alangium grisolleoides* that mediate Cu^{2+}-dependent DNA strand scission. *Journal of Natural Products*, *68*, 1147-1152.

[15]   Wu M, Wu P, Xie HH, Wu GJ, Wei XY. (**2011**) Monoterpenoid indole alkaloids mediating DNA strand scission from *Turpinia arguta*. *Planta Medica*, *77*, 284-286.

[16]   Cong HJ, Zhao Q, Zhang SW, Wei JJ, Wang WQ, Xuan LJ. (**2014**) Terpenoid indole alkaloids from *Mappianthus iodoides* Hand.-Mazz.. *Phytochemistry*, *100*, 76-85.

[17]   Dolomanov OV, Bourhis LJ, Gildea RJ, Howard JAK, Puschmann H. (**2009**) *OLEX2*: a complete structure solution, refinement and analysis program. *Journal of Applied Crystallography*, *42*, 339-341.

[18]   Tanaka T, Nakashima T, Ueda T, Tomii K, Kouno I. (**2007**) Facile discrimination of aldose enantiomers by reversed-phase HPLC. *Chemical & Pharmaceutical Bulletin*, *55*, 899-901.

Lycopodium Alkaloids from *Diphasiastrum complanatum*

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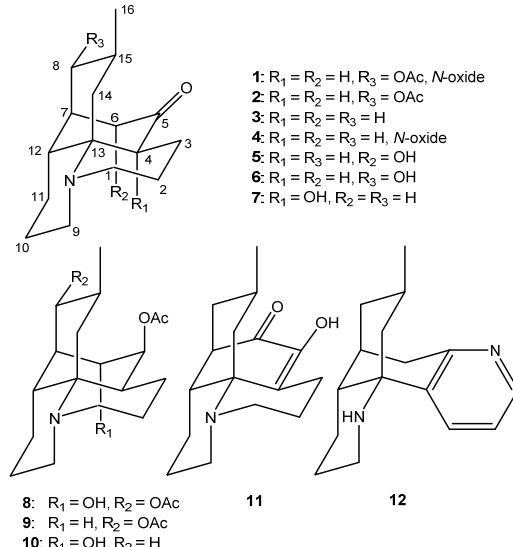
One new lycopodine-type *Lycopodium* alkaloid, dehydroisofawcettiine *N*-oxide (**1**) and eleven known analogues (**2−12**) were isolated from the whole plant of *Diphasiastrum complanatum*. The new structure was established on the basis of spectroscopic methods, including 2D NMR techniques. The absolute configurations of **2** and its new *N*-oxide derivative (**1**) were deduced by chemical transformation combined with Cotton effects in their electronic circular dichroism (ECD) spectra.

Keywords: *Lycopodium* alkaloids, *Diphasiastrum complanatum*, Absolute configuration, Chemical transformation, ECD.

Diphasiastrum complanatum (L.) Holub (syn.: *Lycopodium complanatum* L.) belonging to the family Lycopodiaceae and commonly known as "Guo-Jiang-Long" in Chinese, is a club moss mainly distributed in temperate and subtropical areas. This plant has been used as a folk medicine for the treatment of arthritic pain, quadriplegia, contusion, and blood stasis [1]. Previous investigations of this plant led to the isolation of a number of *Lycopodium* alkaloids [2] with interesting bioactivities, such as enhancement of mRNA expression for nerve growth factor (NGF) [2d,2i], cytotoxicity against murine leukemia L1210 cells [2i], and antimicrobial activity [2i]. During the continuing program of discovery of novel bioactive alkaloids from club mosses [3], the chemical constituents of *D. complanatum* were reinvestigated. Herein described are the isolation and structural determination of one new (**1**) and eleven known (**2–12**) *Lycopodium* alkaloids (Figure 1) from the title plant.

The air-dried and pulverized whole plant of *D. complanatum* was extracted with 90% MeOH at room temperature and then worked up as usual [3,4] to give the crude alkaloid-containing extract. From this, 12 *Lycopodium* alkaloids were isolated and characterized (**1–12**, Figure 1). Comparing their spectroscopic data and physicochemical properties observed and reported, the known alkaloids were identified as dehydroisofawcettiine (**2**) [5], lycopodine (**3**) [6], lycopodine *N*-oxide (**4**) [7], L.20 (= 6α-hydroxylycopodine, **5**) [8], clavolonine (**6**) [9,10], flabelliformine (**7**) [10], 6-*epi*-8β-acetoxylycoclavine (**8**) [11], acetylfawcettiine (**9**) [6], lycoclavine (**10**) [12], 12-deoxyhuperzine O (**11**) [13], and lycodine (**12**) [14]. Among them, alkaloids **2**, **4**, **6**, **8** and **9** are reported here for the first time from the title plant.

Compound **1** showed an [M+H]+ ion peak at *m/z* 322.2016 (calcd 322.2013) in its positive mode HRESIMS, corresponding to the molecular formula $C_{18}H_{27}NO_4$. The IR absorption bands at 1707 and 1733 cm^{-1} implied the presence of ester and ketone carbonyl groups. The ^{1}H and ^{13}C NMR data of **1** (Table 1), with the aid of an HSQC NMR experiment, showed the presence of one methyl doublet at δ_H 0.94 (3H, d, *J* = 6.2 Hz, Me-16; δ_C 19.0), eight *sp*^{3} methylenes, five *sp*^{3} methines (including one oxymethine at δ_H 4.62 (1H, dd, *J* = 11.0, 4.1 Hz, H-8), δ_C 79.2), one *sp*^{3} (δ 74.4) quaternary carbon, and one carbonyl group (δ_C 207.9) for the skeleton, along with signals assignable to an acetyl group [δ_H 2.06

1:	$R_1 = R_2 = H$, $R_3 = OAc$, *N*-oxide
2:	$R_1 = R_2 = H$, $R_3 = OAc$
3:	$R_1 = R_2 = R_3 = H$
4:	$R_1 = R_2 = R_3 = H$, *N*-oxide
5:	$R_1 = R_3 = H$, $R_2 = OH$
6:	$R_1 = R_2 = H$, $R_3 = OH$
7:	$R_1 = OH$, $R_2 = R_3 = H$

8:	$R_1 = OH$, $R_2 = OAc$
9:	$R_1 = H$, $R_2 = OAc$
10:	$R_1 = OH$, $R_2 = H$
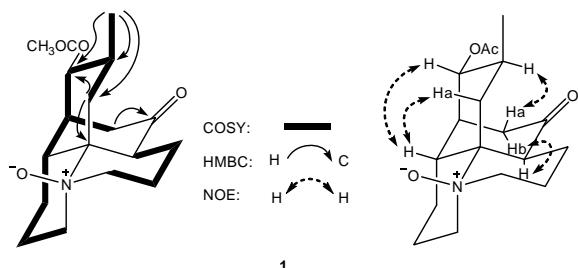
Figure 1: Chemical structures of compounds **1–12**.

(3H, s); δ_C 21.3 and 170.4]. The above spectroscopic data showed high similarity to those of a known lycopodine-type alkaloid, dehydroisofawcettiine (**2**) [5]; however, the complete ^{1}H and ^{13}C NMR data of **2** have never been reported until the present study. Differing from **2**, the molecular formula of **1** contains one more oxygen atom. Meanwhile, the ^{13}C NMR shifts of C-1 (δ_C 64.4), C-9 (δ_C 59.8) and C-13 (δ_C 74.2) neighboring the N-atom in **1** were all shifted to lower field compared with the corresponding carbons [δ_C 47.0 (C-1), 46.9 (C-9) and 59.3 (C-13)] of compound **2** (see Experimental section). Thus, compound **1** was assumed to be the *N*-oxide derivative of **2**, which was confirmed by further detailed 2D NMR (COSY and HMBC) spectroscopic analyses of **1** (Figure 2). The relative configuration of **1** was then found to be the same as that of dehydroisofawcettiine (**2**) from the magnitudes of $J_{H-8, H-15}$ (11.2 Hz) and $J_{H-14a, H-15}$ (13.0 Hz), and diagnostic NOE correlations of H-8/H-12 (δ 2.85, br d, *J* = 13.8 Hz), H-12/H$_a$-14 (δ 2.63, dd, *J* = 14.4, 14.4 Hz), H-15 (δ 1.56, m)/H$_a$-6 (δ 2.57, br d, *J* = 16.4 Hz), and H$_b$-6 (δ 2.35, dd, *J* = 16.4, 6.3 Hz)/H-4 (δ 3.01, dd, *J* = 12.2, 3.7 Hz) (Figure 2).

Table 1: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data [a, b] for alkaloid **1**.
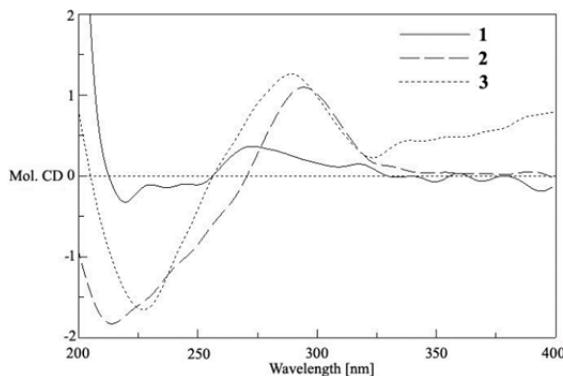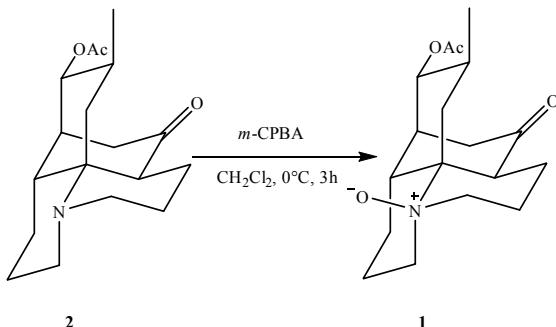
No.	δ_H, mult (J in Hz)	δ_C
1a	3.60, ddd (13.5, 13.5, 4.4)	64.4 CH$_2$
1b	3.19, br d (13.5)	
2a	1.92, m	21.3 CH$_2$
2b	1.85, m,	
3a	2.22, br d (14.6)	17.5 CH$_2$
3b	1.70, m, m	
4	3.01, dd (12.2, 3.7)	48.8 CH
5		207.9 C
6a	2.57, br d (16.4)	36.2 CH$_2$
6b	2.35, dd (16.4, 6.3)	
7	2.44, m	39.1 CH
8	4.62, dd (11.0, 4.1)	79.1 CH
9a	3.96, ddd (12.6, 12.6, 3.0)	59.8 CH$_2$
9b	3.08, br d (12.6)	
10a	2.78, m	20.1 CH$_2$
10b	1.78, m	
11a	1.91, m	23.3 CH$_2$
11b	1.68, m,	
12	2.85, br d (13.8)	36.4 CH
13		74.2 C
14a	2.63, dd (14.4, 14.4)	33.1 CH$_2$
14b	2.10, dd (14.4, 5.0)	
15	1.56, m	30.4 CH
16	0.94, d (6.2)	19.0 CH$_3$
8-OAc	2.06, s	21.3 CH$_3$
		170.4 C

[a]Assignments were made by a combination of 1D and 2D NMR experiments; [b]Recorded in CDCl$_3$.

Figure 2: COSY, key HMBC and NOE correlations of compound **1**.

As previously described by Ayer and Altenkirk [15], the lycopodine-like alkaloids with a carbonyl group at C-5 and in which the nitrogen lone pair is equatorial to ring A consistently display two Cotton effects above 200 nm, a positive around 288 nm due to n→π transition and a negative centered at 223 nm associated with σ-coupled p interaction. In good agreement with this assumption, the electronic circular dichroism (ECD) spectrum of dehydroisofawcettiine (**2**) exhibited a positive Cotton effect at 295 nm (Δε +1.09) and a negative one at 214 nm (Δε −1.85), which matched well with that of lycopodine (**3**) [15], as depicted in Figure 3. Thus, the absolute configuration of **2** could be unequivocally determined as 4*S*,7*S*,8*R*,12*R*,13*R*,15*S*. In the case of alkaloid **1**, due to the disappearance of the nitrogen lone pair after oxidation, it is hard to judge the related Cotton effects since its ECD curve almost flattened (Figure 3). Nevertheless, the absolute configuration of **1** was established to be the same as that of **2** by chemical transformation. In a supplementary oxidation experiment, compound **2** was treated with one equivalent of *m*-CPBA to produce its *N*-oxide derivative (Scheme 1), which was identical to **1** by means of HPLC, optical rotation, and spectroscopic analyses. Accordingly, the structure of **1** was defined as (4*S*,7*S*,8*R*,12*R*,13*R*,15*S*)-dehydroisofawcettiine *N*-oxide. Indeed, naturally occurring *N*-oxide derivatives of *Lycopodium* alkaloids have often been encountered [2h,7,16].

Similar to casuarinines A–J, lycodine-type alkaloids from *Lycopodiastrum casuarinoides* [3], all the isolates were evaluated for their neuroprotective and anti-acetylcholinesterase (AChE) effects, but none of them were active. They also did not show any significant cytotoxicity against human A-549 and NCI-H460 cancer cell lines.

Figure 3: ECD spectra of compounds **1–3**.

Scheme 1: Chemical transformation from **2** to **1**.

Experimental

General experimental procedures: Optical rotations were measured on an Autopol IV automatic polarimeter, IR spectra on an Avatar 360 ESP FTIR spectrometer, ECD spectra on a JASCO-810 spectropolarimeter, and NMR spectra on a Bruker Avance III 400 MHz spectrometer. Chemical shifts are expressed in δ (ppm), and referenced to the residual solvent signals. ESIMS were measured on an Agilent 1100 series mass spectrometer, and HRESIMS on an AB SCIEX Triple TOF 5600+ spectrometer. Semi-preparative HPLC was performed on a Waters e2695 system coupled with a Waters 2998 Photodiode Array Detector and an ODS column (5 μm, 250 × 10 mm, SunFire). Column chromatography (CC) was performed using silica gel (200-300 mesh, Kang-Bi-Nuo Silysia Chemical Ltd., Yantai, China), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Silica gel-precoated plates (GF254, 0.25 mm, Kang-Bi-Nuo Silysia Chemical Ltd., Yantai, China) were used for TLC. Compounds were visualized using UV light (254 and/or 365 nm) and by spraying with Dragendorff's reagent.

Plant materials: The whole plant of *D. complanatum* was collected in October 2013 from Bijie in Guizhou Province of China. A voucher specimen (No. 20131007) was deposited at the Herbarium of the Department of Natural Products Chemistry, School of Pharmacy at Fudan University. The plant was identified by Prof. Qiang Luo (Guizhou University of Engineering Science, Bijie, Guizhou Province of China).

Extraction and isolation: The air-dried and pulverized whole plant of *D. complanatum* (1.6 kg) was extracted with 90% MeOH (5 × 8 L) at room temperature, and the MeOH extract (170 g) was partitioned between EtOAc and 3% tartaric acid. The water-soluble portion, adjusted to pH 9 with sat. Na$_2$CO$_3$, was partitioned with CHCl$_3$. The CHCl$_3$-soluble portion (3.9 g) was loaded on a silica gel column, eluted with a gradient of CH$_2$Cl$_2$/MeOH (1:0–0:1) to

afford fractions 1–8. Fraction 2 (40 mg) was chromatographed on Sephadex LH-20 (CH$_2$Cl$_2$/MeOH, 2:1) to afford **2** (1.9 mg) and **11** (6.1 mg). Fraction 3 (90 mg) was subjected to silica gel CC (CH$_2$Cl$_2$/MeOH, 50:1) and then purified by semi-preparative HPLC [MeOH-H$_2$O (containing 0.05% Et$_2$NH, v/v) 60:40, v/v; flow rate, 3.0 mL/min] to furnish **1** (1.7 mg, t_R = 10.4 min) and **4** (5.6 mg, t_R = 12.0 min). Compounds **3** (16.0 mg, t_R = 13.1 min) and **12** (2.7 mg, t_R = 10.7 min) were isolated from fraction 4 (80 mg) by semi-preparative HPLC [MeOH-H$_2$O (containing 0.05% Et$_2$NH, v/v) 50:50, v/v; flow rate, 3.0 mL/min]. Fraction 5 (200 mg) was subjected to gel permeation chromatography (GPC) on Sephadex LH-20 (CH$_2$Cl$_2$/MeOH, 2:1) to afford **9** (29.5 mg). Compound **5** (7.2 mg, t_R = 14.4 min) was obtained from fraction 6 (210 mg) by semi-preparative HPLC [MeCN-H$_2$O (containing 0.05% Et$_2$NH, v/v) 30:70, v/v; flow rate, 3.0 mL/min]. Fraction 7 (50 mg) was chromatographed on Sephadex LH-20 (MeOH) to furnish **6** (9.4 mg,). Fraction 8 (300 mg) was separated by Sephadex LH-20 (MeOH) and further purified by semi-preparative HPLC [MeCN-H$_2$O (containing 0.05% Et$_2$NH, v/v) 45:55, v/v; flow rate, 3.0 mL/min] to afford **7** (6.1 mg, t_R = 16.0 min), **8** (2.1 mg, t_R = 10.8 min), and **10** (1.7 mg, t_R = 20.4 min).

Dehydroisofawcettiine *N*-oxide (**1**)

$[\alpha]_D^{25}$: +30.0 (*c* 0.1, MeOH).
IR (film): 3417, 2923, 1733, 1707, 1627, 1377, 1244, 1048 cm^{-1}.
^{1}H and ^{13}C NMR: Table 1.
HRESIMS: *m/z* [M+H]$^+$ calcd for C$_{18}$H$_{27}$NO$_4$: 322.2013; found: 322.2016.

m-CPBA oxidation of dehydroisofawcettiine (**2**): Similar to the reported literature [7], compound **2** (4.0 mg, 0.01312 mmol) was dissolved in CH$_2$Cl$_2$ (2 mL) to which *m*-CPBA (2.9 mg, 0.0142 mmol) was added. The reaction mixture was left at 0°C for 3 h and then evaporated to give a residue, which was applied to semi-preparative HPLC [MeOH-H$_2$O (containing 0.05% diethylamine, v/v) 60:40, v/v; flow rate, 3.0 mL/min], giving 3.4 mg (yield 80%)

of the *N*-oxide product of **2**. All the spectroscopic data (^{1}H NMR, ESIMS and $[\alpha]_D^{25}$) were identical with those of natural **1**.

Dehydroisofawcettiine (**2**)

$[\alpha]_D^{25}$: +34.0 (*c* 0.1, MeOH).
ECD (*c* 6.56 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$): 214 (−1.85), 295 (+1.09) nm.
^{1}H NMR (400 MHz, CDCl$_3$): δ 4.59 (1H, dd, *J* = 11.0, 4.2 Hz, H-8), 3.31 (1H, ddd, *J* = 14.2, 14.2, 3.7 Hz, H-1a), 3.14 (1H, ddd, *J* = 12.4, 12.4, 2.7 Hz, H-9a), 2.93 (1H, dd, *J* = 11.8, 3.1 Hz, H-4), 2.67 (1H, dd, *J* = 13.9, 4.9 Hz, H-14a), 2.63 (1H, br d, *J* = 12.4 Hz, H-9b), 2.55 (1H, dd, *J* = 14.2, 4.9 Hz, H-1b), 2.49 (1H, dd, *J* = 17.9, 3.6 Hz, H-6a), 2.33 (1H, d, *J* = 17.9 Hz, H-6b), 2.32 (1H, m, H-3a), 2.10 (1H, m, H-7), 2.07 (3H, s, C*H*$_3$CO), 1.88 (1H, m, H-2a), 1.84 (1H, m, H-10a), 1.77 (1H, m, H-12), 1.72 (1H, m, H-10b), 1.65 (1H, m, H-11a), 1.63 (1H, m, H-13b), 1.58 (1H, m, H-11b), 1.53 (1H, m, H-15), 1.39 (1H, br d, *J* = 13.8 Hz, H-2b), 1.09 (1H, dd, *J* = 13.9, 13.9 Hz, H-14b), 0.89 (3H, d, *J* = 6.2 Hz, Me-16);
^{13}C NMR (100 MHz, CDCl$_3$): δ 212.6 (C-5, C), 170.8 (CH$_3$CO, C), 80.4 (C-8, CH), 59.3 (C-13, C), 47.0 (C-9, CH$_2$), 46.9 (C-1, CH$_2$), 43.3 (C-12, CH), 42.9 (C-4, CH), 41.6 (C-14, CH$_2$), 39.6 (C-7, CH), 37.0 (C-6, CH$_2$), 30.1 (C-15, CH), 25.9 (C-10, CH$_2$), 24.7 (C-11, CH$_2$), 21.1 (*C*H$_3$CO, CH$_3$), 19.2 (C-3, CH$_2$), 18.9 (C-16, CH$_3$), 18.5 (C-2, CH$_2$)
ESIMS: *m/z* 306 [M+H]$^+$.

Supplementary data: NMR spectra of compounds **1** and **2** are available in electronic form on the publisher's website.

References

[1]   (a) Zhang X-C, Zhang L-B. (**2004**) *Flora of China*. Science Press. Beijing, Vol. 6, pp. 76-79; (b) Jiangsu New Medical College. (**1986**) *A Dictionary of the Traditional Chinese Medicine*. Science and Technology Press, Shanghai, pp. 872-873.

[2]   (a) Li H, Huang G, Zhan R, Jiang J, Liu Y, Chen Y. (**2014**) Studies on alkaloids of *Diphasiastrum complanaturn* (L.) Holub. *Journal of Hainan Normal University* (Natural Science), **27**, 39-41; (b) Cheng J-T, Liu F, Li X-N, Wu X-D, Dong L-B, Peng L-Y, Huang S-X, He J, Zhao Q-S. (**2013**) Lycospidine A, a new type of *Lycopodium* alkaloid from *Lycopodium complanatum*. *Organic Letters*, **15**, 2438-2441; (c) Ishiuchi K, Kubota T, Ishiyama H, Hayashi S, Shibata T, Kobayashi J. (**2011**) Lyconadins C and F, new *Lycopodium* alkaloids from *Lycopodium complanatum*. *Tetrahedron Letters*, **52**, 289-292; (d) Ishiuchi K, Kubota T, Ishiyama H, Hayashi S, Shibata T, Mori K, Obara Y, Nakahata N, Kobayashi J. (**2011**) Lyconadins D and E, and complanadine E, new *Lycopodium* alkaloids from *Lycopodium complanatum*. *Bioorganic & Medicinal Chemistry*, **19**, 749-753; (e) Wu X-D, He J, Xu G, Peng L-Y, Song L-D, Zhao Q-S. (**2009**) Diphaladine A, a new *Lycopodium* alkaloid from *Diphasiastrum complanatum* (Lycopodiaceae). *Acta Botanica Yunnanica*, **31**, 93-96; (f) Ishiuchi K, Kubota T, Hayashi S, Shibata T, Kobayashi J. (**2009**) Lycopladine H, a novel alkaloid with fused-tetracyclic skeleton from *Lycopodium complanatum*. *Tetrahedron Letters*, **50**, 6534-6536; (g) Ishiuchi K, Kubota T, Hayashi S, Shibata T, Kobayashi J. (**2009**) Lycopladines F and G, new C$_{16}$N$_2$-type alkaloids with an additional C$_4$N unit from *Lycopodium complanatum*. *Tetrahedron Letters*, **50**, 4221-4224; (h) Kubota T, Yahata H, Ishiuchi K, Obara Y, Nakahata N, Kobayashi J. (**2007**) Lycopladine E, a new C$_{16}$N$_1$-type alkaloid from *Lycopodium complanatum*. *Heterocycles*, **74**, 843-848; (i) Ishiuchi K, Kubota T, Mikami Y, Obara Y, Nakahata N, Kobayashi J. (**2007**) Complanadines C and D, new dimeric alkaloids from *Lycopodium complanatum*. *Bioorganic & Medicinal Chemistry*, **15**, 413-417; (j) Ishiuchi K, Kubota T, Morita H, Kobayashi J. (**2006**) Lycopladine A, a new C$_{16}$N alkaloid from *Lycopodium complanatum*. *Tetrahedron Letters*, **47**, 3287-3289; (k) Ishiuchi K, Kubota T, Hoshino T, Obara Y, Nakahata N, Kobayashi J. (**2006**) Lycopladines B-D and lyconadin B, new alkaloids from *Lycopodium complanatum*. *Bioorganic & Medicinal Chemistry*, **14**, 5995-6000; (l) Morita H, Ishiuchi K, Haganuma A, Hoshino T, Obara Y, Nakahata N, Kobayashi J. (**2005**) Complanadine B, obscurumines A and B, new alkaloids from two species of *Lycopodium*. *Tetrahedron*, **61**, 1955-1960; (m) Ma X, Gang DR. (**2004**) The *Lycopodium* alkaloids. *Natural Product Reports*, **21**, 752-772; (n) Kobayashi J, Hirasawa Y, Yoshida N, Morita H. (**2001**) *Journal of Organic Chemistry*, **66**, 5901-5904; (o) Kobayashi J, Hirasawa Y, Yoshida N, Morita H. (**2000**) Complanadine A, a new dimeric alkaloid from *Lycopodium complanatum*. *Tetrahedron Letters*, **41**, 9069-9073.

[3]   Tang Y, Fu Y, Xiong J, Li M, Ma G-L, Yang G-X, Wei B-G, Zhao Y, Zhang H-Y, Hu J-F. (**2013**) Casuarinines A-J, lycodine-type alkaloids from *Lycopodiastrum casuarinoides*. *Journal of Natural Products,* **76**, 1475-1484.

[4]   Ma G-L, Yang G-X, Xiong J, Cheng W-L. Cheng K-J, Hu J-F. (**2015**) Salicifoxazines A and B, new cytotoxic tetrahydro-1,2-oxazine-containing tryptamine-derived alkaloids from the leaves of *Chimonanthus salicifolius*. *Tetrahedron Letters*, **56**, 4071-4075.

[5]   Burnell RH, Taylor DR. (**1961**) *Lycopodium* alkaloids-V, the structure and stereochemistry of fawcettiine, clavolonine and related alkaloids. *Tetrahedron*, **15**, 173-182.

[6]   Halldorsdottir ES, Palmadottir RH, Nyberg NT, Olafsdottir ES. (**2013**) Phytochemical analysis of alkaloids from the Icelandic club moss *Diphasiastrum alpinum. Phytochemistry Letters*, **6**, 355-359.

[7]   Pan K, Luo J-G, Kong L-Y. (**2014**) A new *Lycopodium* alkaloid from *Phlegmariurus fargesii. Chinese Journal of Natural Medicines*, **12**, 373-376.

[8]   Takayama H, Katakawa K, Kitajima M, Yamaguchi K, Aimi N. (**2003**) Ten new *Lycopodium* alkaloids having the lycopodane skeleton isolated from *Lycopodium serratum* Thunb. *Chemical and Pharmaceutical Bulletin, 51*, 1163-1169.

[9]   Evans DA, Scheerer JR. (**2005**) Polycyclic molecules from linear precursors: stereoselective synthesis of clavolonine and related complex structures. *Angewandte Chemie International Edition*, **44**, 6038-6042.

[10]   Nakashima TT, Singer PP, Browne LM, Ayer WA. (**1975**) Carbon-13 nuclear magnetic resonance studies of some *Lycopodium* alkaloids. *Canadian Journal of Chemistry*, **53**, 1936-1942.

[11]   He J, Wu X-D, Liu F, Liu Y-C, Peng L-Y, Zhao Y, Cheng X, Luo H-R, Zhao Q-S. (**2014**) Lycopodine-type alkaloids from *Lycopodium japonicum*. *Natural Products and Bioprospecting*, **4**, 213-219.

[12]   Ayer WA, Law DA. (**1962**) *Lycopodium* alkaloids IV. The constitution and stereochemistry of lycoclavine, an alkaloid of *Lycopodium clavatum* var. *megastachyon. Canadian Journal of Chemistry*, **40**, 2088-2100.

[13]   Yang Y-F, Qu S-J, Xiao K, Jiang S-H, Tan J-J, Tan C-H, Zhu D-Y. (**2010**) *Lycopodium* alkaloids from *Huperzia serrata*. *Journal of Asian Natural Products Research*, **12**, 1005-1009.

[14]   (a) Tong X-T, Tan C-H, Ma X-Q, Jiang S-H, Zhu D-Y. (**2003**) *Lycopodium* alkaloids from *Huperzia miyoshiana. Natural Product Research and Development*, **15**, 383-386; (b) Anet FAL, Rao MV. (**1960**) The structure of lycodine. *Tetrahedron Letters*, **1**, 9-12.

[15]   Ayer WA, Altenkirk B, Burnell RH, Moinas M. (**1969**) Alkaloids of *Lycopodium lucidulum* Michx. structure and properties of alkaloid L.23. *Canadian Journal of Chemistry*, **47**, 449-455.

[16]   (a) Pan k, Luo J-G, Kong L-Y. (**2013**) Two new *Lycopodium* alkaloids from *Lycopodium obscurum. Helvetica Chimica Acta*, **96**, 1197-1201; (b) Katakawa K, Mito H, Kogure N, Kitajima M, Wongseripipatana S, Arisawa M, Takayama H. (**2011**) Ten new fawcettimine-related alkaloids from three species of *Lycopodium. Tetrahedron*, **67**, 6561-6567; (c) Wang H-B, Tan C-H, Tan J-J, Qu S-J, Chen Y-L, Li Y-M, Jiang S-H, Zhu D-Y. (**2009**) Two new *N*-oxide *Lycopodium* alkaloids from *Huperzia serrata. Natural Product Research*, **23**, 1363-1366; (d) Yin S, Fan C-Q, Wang X-N, Yue J-M. (**2006**) Lycodine-type alkaloids from *Lycopodium casuarinoides. Helvetica Chimica Acta*, **89**, 138-143.

Effects of Adding Vindoline and MeJA on Production of Vincristine and Vinblastine, and Transcription of their Biosynthetic Genes in the Cultured CMCs of *Catharanthus roseus*

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Vincristine and vinblastine were found by Liquid Chromatography-Mass Spectrometry (LC-MS) in *Catharanthus roseus* cambial meristem cells (CMCs) jointly treated with 0.25 mM vindoline and methyl jasmonate (MeJA), suggesting that *C. roseus* CMCs contain a complete set of the enzymes which are in response to convert vindoline into vincristine and vinblastine. Based on the facts that the transcript levels of vindoline-biosynthetic genes (*STR*, *SGD* and *D4H*) were up-regulated instead of being down-regulated by adding itself to the culture, and that the transcriptional factor *ORCA3* was up-regulated simultaneously, we further confirmed that the transcription of *STR*, *SGD*, *D4H* was manipulated by ORCA3.

Keywords: *Catharanthus roseus*, CMCs, TIAs, Quantitative RT-PCR, Precursor feeding, Gene expression profiling.

The terpene indole alkaloids (TIAs) from *Catharanthus roseus* are important secondary metabolites with diverse structures and biological activities [1,2]. In the complex biosynthetic process of TIAs, strictosidine aglycone is considered as a key intermediate from which at least two biosynthetic pathways are derived. From these two pathways, vindoline and cathenamine are synthesized, respectively. The coupling of vindoline and cathenamine finally leads to the production of bisindole alkaloids vincristine and vinblastine, and cathenamine is also hypothesized as the precursor of ajmalicine and serpentine. Previous results have proved that vindoline can be detected from CMCs of *C. roseus*, but not from either suspension cells or hairy roots due to deficiency of the corresponding enzymes in the latter two cells / tissues [3,4]. Unfortunately, vincristine and vinblastine are not found in the CMCs cultures which was possibly due to the low concentration of vindoline. Since the enzymes necessary for converting vindoline into vincristine and vinblastine have not been characterized yet, the levels of their transcription or expression could not be directly analyzed by biological methods, such as quantitative reverse transcription (RT)-PCR and Western blot. To clarify whether CMCs of *C. roseus* contain a complete set of response system of enzymes for converting vindoline into vincristine and vinblastine, vindoline was fed to the culture of CMCs of *C. roseus* in either the presence or absence of methyl jasmonate (MeJA). Also, the effects of excess vindoline on the transcription of the genes involved in its biosynthesis were investigated.

In the presence or absence of 250 mM MeJA, CMCs of *C. roseus* were treated with 0.5 mM vindoline for 12 h, 24 h and 36 h, respectively. Vinblastine and vincristine were observed by LC-MS. As time went on, the change in accumulation of both vindoline and catharanthine was not significantly observed, suggesting that the production and consumption of the two compounds were in a state of equilibrium (Figure 1). Interestingly, the treatment of vindoline enhanced the accumulation of catharanthine (Figure 1A). In general, additional feeding of one product can inhibit the transcription and translation of genes located at its up-stream steps due to a negative
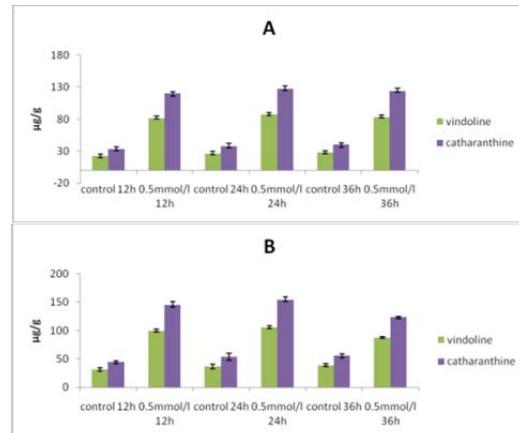
Figure 1: The accumulation of vindoline and catharanthine in *C. roseus* CMCs (A) treated with vindoline or (B) jointly treated with both vindoline and MeJA for 12, 24 and 36 h.
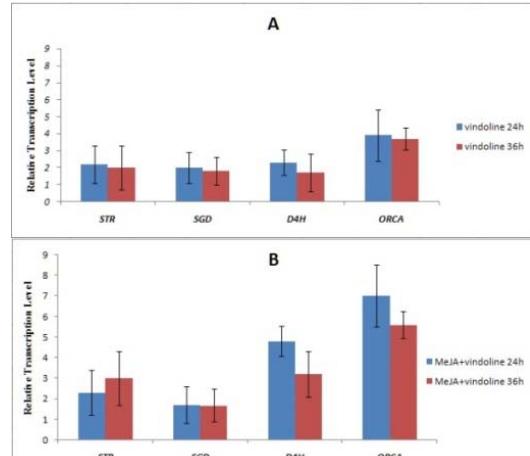
Figure 2: In comparison with untreated *C. roseus* CMCs, the relative transcription levels of *STR*, *SGD*, *D4H* and *ORCA3* in *C. roseus* CMCs (A) treated with either vindoline or (B) jointly treated with both vindoline and MeJA for 24 and 36 h, respectively.

feedback mechanism, which proposed that the transcription levels of the genes involved in the synthesis of vindoline were reduced due to the addition of vindoline. We monitored the transcription levels of *STR*, *SGD* and *D4H* in both vindoline-treated and vindoline-untreated CMCs by quantitative real time reverse transcription PCR (qRT-PCR) [5,6]. Unexpectedly, the transcription levels of *STR*, *SGD* and *D4H* were up-regulated by treated with vindoline. Eleven-day old CMCs were treated with either ethanol or 0.5 mM vindoline in ethanol, respectively. After 24 or 36 h, the CMCs were harvested and the transcript levels of *STR*, *SGD* and *D4H* were analyzed. For 24-h cells treated with vindoline enhanced the transcript levels of *STR*, *SGD* and *D4H* by 2.2, 2.0 and 2.3 folds of those of the ethanol-treated CMCs; for 36-h cells treated with vindoline led to 2.1-, 1.9- and 1.9-fold increases, respectively (Figure 2).

It has been reported that the transcriptional regulator ORCA3 is capable of manipulating the transcription of *STR*, *SGD* and *D4H* [7]. We monitored the change in *ORCA3* transcript level. The result indicated that in the CMCs with 24-h and 36-h treatment with vindoline, the transcription levels of *ORCA3* increased by 3.9 and 3.8 folds of those of the control cells, respectively (Figure2A). Therefore, we supposed that vindoline may firstly enhance *ORCA3* transcription which not only overcomes the negative feedback effect caused by feeding vindoline, but also improves the transcription of *STR*, *SGD* and *D4H*. Transcription of genes responsible for biosynthesis of catharanthine might be up-regulated by ORCA3 as well, which could be the reason that the treatment with vindoline enhanced the accumulation of catharanthine (Figure 1A). Regretfully, no enzyme involved in conversion of strictosidine aglycone to catharanthine has been characterized, so this assumption could not be verified.

MeJA can be used as an elicitor to enhance production of TIAs and transcription of their biosynthetic genes [3]. By jointly treating with 250 mM MeJA and 0.5 mM vindoline, enhancement of the accumulation of vindoline was not observed (Figure 1) and thetranscript levels of *STR*, *SGD* and *D4H* increased by magnitudes comparable with those of treating solely with vindoline, while the transcript level of *ORCA3* was induced by 7 folds of that of the control cells (Figure 2). These results indicated that MeJA, in combination with vindoline, was not capable of enhancing the accumulation of vindoline and the transcript levels of its biosynthetic genes. However, in the presence of both vindoline and

MeJA, the further increase of the transcript level of *ORCA3*, together with the fact that vincristine and vinblastine were observed only in the CMCs treated with both MeJA and vindoline, indicated that ORCA3 also improved the transcription of genes locating at downstream of vindoline, although these genes have not been characterized as yet. In conclusion, with treatment by both vindoline and MeJA, CMCs of *C. roseus* produced vinblastine and vincristine, suggesting that CMCs of *C. roseus* contain a complete set of the enzymes involved in conversion of vindoline to vinblastine and vincristine. However, CMCs cannot produce enough vindoline to yield vinblastine and vincristinein detectable amounts, and so feeding with vindoline is necessary. The transcriptions of the genes responsible for synthesizing vindoline, such as *STR*, *SGD* and *D4H*, are up-regulated by ORCA3, which may play a key role in manipulating the transcription of the genes involved in transforming vindoline into vinblastine and vincristine, as well.

Experimental

Plant material: CMCs of *C. roseus* were prepared in our research group [8]. Initially, these CMCs were cultured in solid MS medium with 1-naphthalene acetic acid (NAA, 2.0 mg/L), sucrose (10.0 g/L) and gelrite (4.0 g/L); the pH was regulated to 5.8. After that, cultures were sub-cultured every 12 days in 250 mL Erlenmeyer flasks in liquid MS medium. The cells were cultured at 25°C with a 12/12 h light / dark cycle, agitated at 100 rpm.

Extraction procedure: Alkaloids were extracted from medium and cells, respectively, following the reported method [9].

Analysis of TIAs: HPLC and LC-MS experiments were performed as previously report [3].

Monitor gene expression: The quantitative real time reverse transcription PCR (qRT-PCR) experiments were conducted as previously report [3].

References

[1]   Dutta A, Sen J, Deswal R. (**2013**) New evidences about strictosidine synthase (*Str*) regulation by salinity, cold stress and nitric oxide in *Catharanthus roseus*. *Journal of Plant Biochemistry and Biotechnology*, **22**, 124-131.
[2]   Wang CT, Liu H, Gao XS, Zhang HX. (**2009**) Overexpression of G10H and ORCA3 in the hairy roots of *Catharanthus roseus* improves catharanthine production. *Plant Cell Reports*, **29**, 887-894.
[3]   Zhou P, Yang J, Zhu J, He S, Zhang W, Yu R, Zi J, Song L, Huang X. (**2015**) Effects of β-cyclodextrin and methyl jasmonate on the production of vindoline, catharanthine, and ajmalicine in *Catharanthus roseus* cambial meristematiccell cultures. *Applied Microbiology Biotechnology*. 99:7035-7045.
[4]   Zhao J, Verpoorte R. (**2007**) Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering. *Phytochemistry Review*, **6**, 435-457.
[5]   Peebles CA, Hughes EH, Shanks JV, San KY. (**2009**)Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of ORCA3 along with jasmonic acid elicitation of *Catharanthus roseus* hairy roots over time. *Metabolic Engineering*, **11**, 76-86.
[6]   Guo Z-G, Liu Y, Gong M-Z, Chen W, Li W-Y. (**2012**) Regulation of vinblastine biosynthesis in cell suspension cultures of *Catharanthus roseus*. *Plant Cell Tissue and Organ Culture*, **112**, 43-54.
[7]   Pan Q, Wang Q, Yuan F, Xing S. (**2012**) Overexpression of ORCA3 and G10H in *Catharanthus roseus* plants regulated alkaloid biosynthesis and metabolism revealed by NMR metabolomics. *Plos One*, **7**, 3038-3049.
[8]   Lee EK, Jin YW, Park JH, Yoo YM, Hong SM, Amir R. (**2010**) Cultured cambial meristematic cells as a source of plant natural products. *Nature Biotechnology*,**28**, 1213-1217.
[9]   Hisiger S, Jolicoeur M. (**2007**) Analysis of *Catharanthus roseus* alkaloids by HPLC. *Phytochemistry Reviews*, **6**, 207-234.

Structures and Chemotaxonomic Significance of Stemona Alkaloids from *Stemona japonica*

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A pair of new alkaloid stereo-isomers, stemocochinin (**1**) and isostemocochinin (**2**), was obtained from the roots of *Stemona japonica* Miq., along with seven known alkaloids, stemonamine (**3**), isostemonamine (**4**), maistemonine (**5**), isomaistemonine (**6**), croomine (**7**), stemonine (**8**), and protostemonine (**9**). The complete structure and stereochemistry of the pair of isomers were established by extensive analysis of the spectral data. Furthermore, our results indicated that *S. japonica* is chemically closer to *S. sessilifolia* than *S. tuberosa*, which are consistent with our previous DNA study on *Stemona* species.

Keywords: *Stemona japonica*, Stemona alkaloid, Chemotaxonomic significance, DNA phylogenetics.

Plants of the genus *Stemona*, belonging to the medicinally important family Stemonaceae, are a rich source of a class of structurally unique pyrrolo[1,2-α]azepine alkaloids known as Stemona alkaloids [1]. Three species in this genus, i.e. *S. tuberosa*, *S. japonica* and *S. sessilifolia,* are collectively recorded as Radix Stemonae ('Bai-Bu' in Chinese) in the Chinese Pharmacopeia [2]. This herb is often used as an antitussive drug to treat respiratory disorders, such as cough and tuberculosis, and is also used as an anthelmintic agent for domestic animals [3]. The prominent clinical and pharmacological properties of these plants have prompted many phytochemical studies, and over one hundred Stemona alkaloids have been isolated from these herbs [4].

Our group has been engaged in this class of intriguing alkaloids for many years [5-8]. A series of alkaloids were identified from *S. tuberosa*, and some of them were found to show antitussive activities [9,10]. Interestingly, significant chemical diversity was observed for *S. tuberosa* collected from different places of production [5]. During our further systematic investigation of alkaloids from the genus *Stemona*, the chemical constituents of *S. japonica* were studied. A pair of stereoisomers, stemocochinin (**1**, Figure 1) and isostemocochinin (**2**), was isolated together with seven known alkaloids (**3-9**). Their chemical structures and stereochemistry were established by spectroscopic analysis. The chemotaxonomic significance of these alkaloids is discussed based on the distribution of these alkaloids in the genus and a DNA phylogenetic study.

A 95% ethanol extract of the herb was acidified with dilute hydrochloric acid. The acid soluble fraction was adjusted to pH 9 with ammonia solution, and then extracted with CH₂Cl₂. Compounds **1-9** were obtained from the CH₂Cl₂ fraction by silica gel column chromatography.
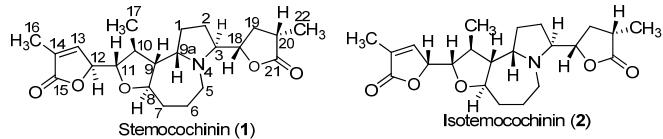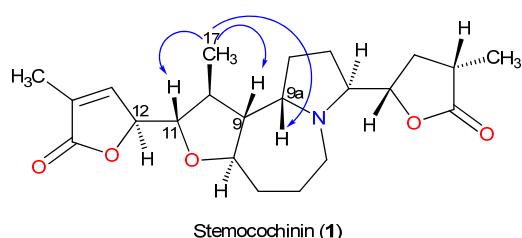
Figure 1: Chemical structures stemocochinin (**1**) and isostemocochinin (**2**).

Compound **1**, an amorphous powder, had the molecular formula $C_{22}H_{31}NO_5$, based on the quasimolecular ion [M+H]⁺ at *m/z* 389 in its EI-MS. The base peak at *m/z* 290 [M-C₅H₇O₂], a characteristic cleavage fragment of Stemona alkaloids, indicated the presence of an α-methyl-γ-lactone ring annexed to C-3. Another prominent peak at *m/z* 292 [M-C₅H₅O₂] can be attributed to the loss of the unsaturated α-methyl-γ-lactone ring from the molecular ion.

The ¹H NMR spectrum (Table 1) shows the presence of two primary methyl groups at δH 1.06, d, *J* = 6.6 Hz, H-17) and δH 1.25 (3H, d, *J* = 7.1 Hz, H-22), an olefinic methyl at δH 1.92 (3H, d, *J* = 2.4 Hz, H-16), four oxymethines at δH 3.89 (1H, m, H-8), 3.72 (1H, m, H-11), 4.83 (1H, dd, *J* = 6.4, 1.8 Hz, H-12) and 4.17 (1H, m, H-18), a *sp²* methine at δH 7.14 (1H, dq, *J* = 1.9, 1.5 Hz, H-13), a methine and two geminal protons attached to carbon atoms bearing a nitrogen function at δH 3.24 (1H, m, H-3), δH 3.45 (1H, dd, *J* = 15.6, 5.5 Hz, H-5β) and δH 2.89 (1H, dd, *J* = 15.6, 11.7Hz, H-5α). The ¹³C NMR and DEPT spectra of **1** show 22 carbon atoms: two lactonic carbonyl atoms (δC 179.5 and 174.1), two olefinic carbon atoms conjugated with carbonyl group (δC 146.9 and 130.7), four carbon atoms bearing oxygen (δC 80.1, 85.1, 83.2 and 83.1), six methylene groups (δC 26.8, 26.9, 47.3, 20.2, 35.2 and 34.3), three methyl groups (δC 10.7, 16.7 and 14.9) and four general methine carbons (δC 55.4, 58.9, 40.7 and 34.9). These spectroscopic data are reminiscent of the pentacyclic Stemoamide-type alkaloids bearing two lactone rings [11].
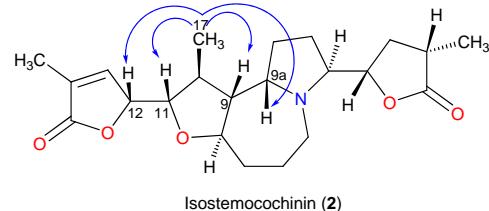
Table 1: The ^{1}H and ^{13}C NMR data of compounds **1** and **2** (*J* in Hz).

	Compound 1		Compound 2	
	^{1}H	^{13}C	^{1}H	^{13}C
1	1.75, m	26.8, t	1.81, m	26.8, t
	1.58, m		1.56, m	
2	1.89, m	26.9, t	1.90, m	26.9, t
	1.41, m		1.37, m	
3	3.24, m	64.5, d	3.21, m	64.4, d
5	3.45, dd (15.6,5.5)	47.3, t	3.44, dd, (15.6,5.2)	47.4, t
	2.89, dd (11.7,15.6)		2.88, dd (11.4,15.6)	
6	1.55, m	20.2, t	1.54, m	20.2, t
	1.33, m		1.33, m	
7	2.11, m	35.2, t	2.05, m	35.4, t
	1.28, m		1.26, m	
8	3.89, ddd (5.6,5.6,1)	80.1, d	3.79, m	80.5, d
9	1.99, ddd (4.7,10.2)	55.4, d	1.98, dd (5.2,10.6)	55.1, d
9a	3.65, m	58.9, d	3.64, dd (5.0,10.3)	59.1, d
10	1.82, m	40.7, d	2.19, m	39.6, d
11	3.52, m	85.1, d	3.77, m	83.4, d
12	4.83, m (6.4,1.8)	83.2, d	4.89, m (2.2. 1.8)	80.6, d
13	7.14,dq (1.9,1,5)	146.9, d	7.00, dq (1.8,1.5)	146.1, d
14		130.7, s		131.0, s
15		174.1, s		174.2, s
16	1.92,dd (1.5,1.8)	10.7, q	1.94, dd (1.8,1.8)	10.8, q
17	1.06,d, (6.6)	16.7, q	1.08, d (6.6)	15.9, q
18	4.17 ,m	83.1, d	4.15, m.	83.5, d
19	2.37, m	34.3, t	2.36, m	34.4, t
	1.53, m		1.52, m	
20	2.62, m	34.9, d	2.69, m	35.0, d
21		179.5, s		179.4, s
22	1.25, d (6.9)	14.9, q	1.25,d (6.9)	14.9, q

Stemocochinin (**1**)

Figure 2: Key ROESY correlations of **1**.

The full assignments and connectivities were determined by ^{1}H - ^{1}H COSY, HMQC and HMBC spectra. The ^{1}H - ^{1}H COSY spectrum established spin systems involving H-5, H-6, H-7, H-8, H-9, H-10, H-11, H-12, H-13 and H$_3$-16, and H-1, H-2, H-3, H-19, H-18, H-19, H-20 and H$_3$-22. The HMQC spectrum revealed a signal at δ_H 4.83 (H-12) attached to a carbon at δ_C 83.2 (C-12), and the HMBC spectrum showed H-12 correlated to C-11, C-13, C-14 and C-15, suggesting that the γ-lactone was formed by ring closure involving the oxygen atoms bridged to C-12 and C-15. Furthermore, the HMBC correlation H-12→C-11 suggested the location of this lactone at C-11. The HMBC correlation of the characteristic olefinic signal at δ 7.14 (H-13) to C-12, C14, C15 and C16 suggested that the double bond is within the lactone ring and conjugated with the carbonyl group at C-15. Similarly, the HMQC spectrum revealed that the signal at δ_H 4.17 (H-18) was correlated with a carbon at δ_C 80.4 (C-18), and the HMBC spectrum showed that H-18 correlated with C-3, C-19, C-20 and C-21, suggesting that another γ-lactone attached to C-3 was formed by ring closure involving the oxygen atoms bridged to C-18 and C-21.

The stereochemistry of compound **1** was determined from the ROESY spectrum in which the protons of Me-17 had correlations with H-9a, H-11 and H-9, but not with H-12, indicating that Me-17, H-9a, H-11 and H-9 are on the same side and are β-orientated, whereas H-12 should be α-orientated (Figure 2). Accordingly, compound **1** was determined to be stemocochinin. This alkaloid has been reported before [11], but the full assignment of NMR data and detailed stereochemistry were not determined.

Isostemocochinin (**2**)

Figure 3: The key ROESY correlations of **2**.

Compound **2** had the same quasimolecular ion as that of **1** at *m/z* 389. It also showed the same prominent peaks at *m/z* 290 and 292 as alkaloid **1** in its ESI-MS. The ^{1}H-, ^{13}C- and DEPT-NMR spectra of **2** were similar to those of **1** except that the chemical shifts of H-10 and H-11 are slightly different (Table 1). Comparison of the ROESY spectra of alkaloids **1** and **2** revealed a significant difference. Clear correlation was observed between methyl-17 and H-12 in the ROESY spectrum (Figure 3) of alkaloid **2**, suggesting that H-12 should be β-orientated. Thus, the only difference between alkaloids **1** and **2** was the relative configuration of C-12, (*S* in **1** and *R* in **2**).

Accordingly, compound **2** was determined to be an isomer of stemocochinin (**1**) and was accorded the trivial name isostemocochinin (**2**).

The known alkaloids were identified as stemonamine (**3**) [13, 14], isostemonamine (**4**) [13], maistemonine (**5**) [13-16], isomaistemonine (**6**) [14, 17, 18], croomine (**7**) [12, 19, 20], stemonine (**8**) [21, 22] and protostemonine (**9**) [15, 21, 22] by comparing their MS and NMR spectral data with those reported in literature (Figure 1).

Six of the seven known alkaloids have been identified from *S. sessilifolia*, while only compound **7** has been isolated from *S. tuberosa* (Table 2), indicating that *S. japonica* is chemically closer to *S. sessilifolia* than to *S. tuberosa*. Current phytochemical results are consistent with our previous DNA study on *Stemona* species [23], which showed that the 5S rRNA sequence of *S. tuberosa* shares a similarity of 76% and 78.5% with those of *S. japonica* and *S. sessilifolia*, respectively. In contrast, the similarity between *S. japonica* and *S. sessilifolia* is 84.5%. Thus, both phytochemical and DNA phylogenetic data confirm a closer relationship between *S. japonica* and *S. sessilifolia* than *S. tuberosa*.

Table 2: Distribution of compounds **1-9** in the three *Stemona* species recorded in Chinese Pharmacopeia.

Compound	Name	Source	Reference
1	Stemocochinin	*S. japonica*	12
2	Isostemocochinin	*S. japonica*	Not found
3	Stemonamine	*S. japonica*	13
		S. sessilifolia	14
4	Isostemonamine	*S. japonica*	13
5	Maistemonine	*S. japonica*	13,15
		S. sessilifolia	16,14
6	Isomaistemonine	*S. sessilifolia*	14,17
		S. japonica	18
7	Croomine	*C. japonica*	19,12
		S. tuberosa	20
8	Stemonine	*S. japonica*	21,22
9	Protostemonine	*S. japonica*	15,21,22
		S. sessilifolia	14,17

Experimental

General procedure: TLC was performed on pre-coated silica gel GF254 plates (Qingdao Marine Chemical Factory, Qingdao, China). The NMR spectra were obtained on a Bruker 300 spectrometer with chemical shifts reported in ppm using TMS as an internal standard.

ESIMS were recorded on a Finnigan MAT TSQ 7000 instrument. Column chromatography (CC) was performed on silica gel (200–400 mesh, Qingdao Marine Chemical Plant, Qingdao, People's Republic of China). All solvents used in CC and high-performance liquid chromatography (HPLC) were of analytical grade (Shanghai Chemical Plant, Shanghai, People's Republic of China).

Plant material: The roots of *S. japonica* were collected in Guangxi Province, China in September 2004. The herbs (voucher specimen No. SJ-1) were identified at the Institute of Traditional Chinese Medicine and Natural Products, Jinan University.

Isolation of compounds 1-9: One kg of dried roots of *S. japonica* were ground and percolated with 95% EtOH at room temperature for 3 days, and then filtered and concentrated under reduced pressure. The residue was acidified with 500 mL of 4% HCl solution, and then extracted with Et_2O (400 mL x 3). The Et_2O was evaporated to give a crude non-alkaloid extract (6 g). The pH of the water layer was adjusted to 9-10 with 35% NH_4OH, and extracted with diethyl ether to give the total alkaloids (4.5 g). Part of these (4.0 g) was chromatographed on a silica gel column and eluted with CH_2Cl_2: MeOH: NH_4OH (94: 6: 0.04). The eluates were monitored by TLC and grouped into 6 fractions (Fr. 1 - Fr. 6). Fr. 1 was re-chromatographed on a silica gel column using *n*-hexane: EtOAc (6:4) as eluent to yield alkaloid **3** (10 mg). Fr. 2 was chromatographed on a silica gel column eluted with *n*-hexane:

EtOAc (5: 5) to give alkaloids **4** (12 mg), **5** (20 mg) and **6** (20 mg). Fr. 4 was chromatographed on a silica gel column eluted with *n*-hexane: EtOAc: acetone (2: 2: 0.5) to give alkaloids **7** (20 mg), **8** (26 mg) and **9** (93 mg). Fr. 6 was separated by silica gel CC eluting with *n*-hexane: EtOAc: acetone (2: 2: 1) to afford alkaloids **1** (20 mg) and **2** (40 mg).

Stemochocochinin (1)
Amorphous powder.
^{1}H and ^{13}C NMR: Table 1.
FAB-MS: *m/z*: 390 [M+H]$^+$.
EI-MS: *m/z*: 389 [M]$^+$ (3), 292 [M-$C_5H_5O_2$]$^+$ (100), 290 [M-$C_5H_7O_2$]$^+$ (75), 278, 164, 134.

Isostemocochinin (2)
Amorphous powder.
^{1}H and ^{13}C NMR: Table 1.
FAB-MS: *m/z*: 390 [M+H]$^+$.
EI-MS: *m/z*: 389 [M]$^+$ (2), 292 [M-$C_5H_5O_2$]$^+$ (75), 290 [M-$C_5H_7O_2$]$^+$ (100), 278, 164,136.

References

[1]   (a) Pilli RA, Rossoa GB, de Oliveira Mda C. (**2010**) The chemistry of Stemona alkaloids: An update. *Natural Product Reports*, **27**, 1908-1937; (b) Wang FP, Chen QH. (**2014**) *Stemona* alkaloids: biosynthesis, classification, and biogenetic relationships. *Natural Product Communications*, **9**, 1809-1822.

[2]   Pharmacopoeia Commission of People's Republic of China. (**2005**) *The Pharmacopoeia of the People's Republic of China, Part 1*. Chemical Industry Publishing House, Beijing, P.R. China, 100.

[3]   Jiangsu New Medical College. (**1977**) *Dictionary of Chinese Traditional Medicine*. Shanghai People's Publishing House, P.R. China, 858-861.

[4]   Greger H. (**2006**) Structural relationships, distribution and biological activities of Stemona alkaloids. *Planta Medica*, **72**, 99-113.

[5]   Jiang RW, Hon PM, Xu YT, Chan YM, Xu HX, Shaw PC, But PPH. (**2006**) Isolation and chemotaxonomic significance of tuberostemospironine-type alkaloids from *Stemona tuberosa*. *Phytochemistry*, **67**, 52-57.

[6]   Jiang RW, Hon PM, Zhou Y, Chan YM, Xu YT, Xu HX, Greger H, Shaw PC, But PPH. (**2006**) Alkaloids and chemical diversity of *Stemona tuberosa*. *Journal of Natural Products*, **69**, 749-754.

[7]   Jiang RW, Hon PM, But PPH, Chung HS, Lin G, Ye WC, Mak TCW. (**2002**) Isolation and stereochemistry of two new alkaloids from *Stemona tuberosa* Lour. *Tetrahedron*, **58**, 6705-6712.

[8]   Jiang RW, Ye WC, Shaw PC, But PPH, Mak TCW. (**2010**) Absolute configuration of neostenine. *Journal of Molecular Structure*, **966**, 18-22.

[9]   Xu YT, Shaw PC, Jiang RW, Hon PM, Chan YM, But PPH. (**2010**) Antitussive and central respiratory depressant effects of *Stemona tuberosa*. *Journal of Ethnopharmacology*, **128**, 679-684.

[10]   Xu YT, Hon PM, Jiang RW, Cheng L, Li SH, Chan YP, Xu HX, Shaw PC, But PPH. (**2006**) Antitussive effects of *Stemona tuberosa* with different chemical profiles. *Journal of Ethnopharmacology*, **108**, 46-53.

[11]   Kaltenegger E, Brem B, Mereiter K, Kalchhauser H, Kählig H, Hofer O, Vajodaya S, Greger H. (**2003**) Insecticidal pyrido[1,2-a]azepine alkaloids and related derivatives from *Stemona* species. *Phytochemistry*, **63**, 803-816.

[12]   Tang CP, Chen T, Velten R, Jeschke P, Ebbinghaus-Kintscher U, Geibel S, Ye Y. (**2008**) Alkaloids from stems and leaves of *Stemona japonica* and their insecticidal activities. *Journal of Natural Products*, **71**, 112-116.

[13]   Ye Y, Qin GW, Xu RS. (**1994**) Alkaloids of *Stemona japonica*. *Journal of Natural Products*, **57**, 665-669.

[14]   Yang XZ, Zhu JY, Tang CP, Ke CQ, Lin G, Cheng TY, Rudd JA, Ye Y. (**2009**) Alkaloids from roots of *Stemona sessilifolia* and their antitussive activities. *Planta Medica*, **75**, 174-177.

[15]   Ye Y, Xu RS. (**1992**) Studies on new alkaloids of *Stemona japonica*. *Chinese Chemical Letters*, **3**, 511-514.

[16]   Cong X, Zhao H, Guillaume D, Xu G, Lu Y, Zheng Q. (**1995**) Crystal structure and NMR analysis of the alkaloid protostemotinine. *Phytochemistry*, **40**, 615-617.

[17]   Guo A, Lin L, Deng Z, Cai S, Guo S, Lin W. (**2008**) New Stemona alkaloids from the roots of *Stemona sessilifolia*. *Chemistry & Biodiversity*, **5**, 598-605.

[18]   Zou CY, Fu HZ, Lei, HM, Li J, Lin W-H. (**1999**) New alkaloids from the roots of *Stemona japonica* Miq. *Journal of Chinese Pharmaceutical Sciences*, **8**, 185-190.

[19]   Lin WH, Cai MS, Ying BP, Feng R. (**1993**) Studies on the chemical constituents of *Croomia japonica* Miq. *Acta Pharmaceutica Sinica*, **28**, 202-206.

[20]   Lin LG, Leung HPH, Zhu JY, Tang CP, Ke CQ, Rudd JA, Lin G, Ye Y. (**2008**) Croomine- and tuberostemonine-type alkaloids from roots of *Stemona tuberosa* and their antitussive activity. *Tetrahedron*, **64**, 10155-10161.

[21]   Götz M, Strunz GM. (**1975**) Tuberostemonine and related compounds: The chemistry of Stemona alkaloids. In *Alkaloids MTP International Review of Sciences Organic Chemistry Series One* Vol. **9** Giesner G. (Ed). Butterworths, London, UK, 143-160.

[22]   Ye Y, Qin GW, Xu RS. (**1994**) Alkaloids of *Stemona japonica*. *Phytochemistry*, **37**, 1205-1208.

[23]   Chan YM (**2003**) Molecular authentication and taxonomy of Radix Stemonae. MPhil thesis, the Chinese University of Hong Kong, Hong Kong SAR, China, 1-127.

Chemical Constituents of *Euonymus glabra*

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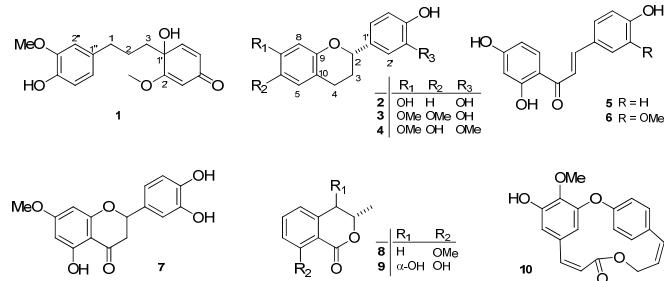
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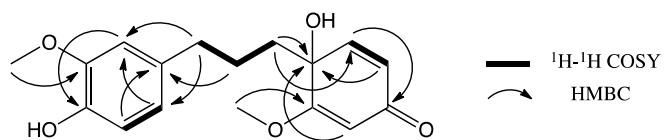
One new phenolic compound (**1**) and one new flavan (**2**), together with eight known compounds (**3–10**) were isolated from the stems and twigs of *Euonymus glabra* Roxb. Their structures were elucidated mainly on the basis of 1D and 2D spectroscopic methods and circular dichroism analysis. In addition, compounds **1–10** were tested for their inhibitory effects against LPS-induced NO production in RAW264.7 macrophages. Compounds **1–5** and **7** exhibited moderate inhibitory activities with IC_{50} values ranged from 5.1 to 11.9 μM.

Keywords: *Euonymus glabra*, Euonyphenylpropane A, Flavan, NO production.

Euonymus glabra Roxb. (Celastraceae) is a deciduous shrub, which is mainly distributed in Xishuangbanna Dai Autonomous Prefecture in Yunnan province at an elevation of 900−1800 meters. It has been recorded that the twigs and leaves of this species are poisonous [1]. Previous investigations showed that *Euonymus* species exhibited a high diversity of both secondary metabolites (including sesquiterpenes, alkaloids, terpenoids, flavonoids) and biological activities (such as anti-tumor, anti-diabetes, and insecticidal effects [2]. In the continuing search for further bioactive natural products from this genus, phytochemical investigations of *E. glabra* were carried out and led to the isolation of one new phenolic compound euonyphenylpropane A (**1**), one new flavan (2*R*)-3′,4′,7-trihydroxyflavan (**2**), along with eight known compounds, griffinoid C (**3**) [5], griffinoid B (**4**) [5], isoliquiritigenin (**5**) [6], 2′,4,4′-trihydroxy-3-methoxy-chalcone (**6**) [7], 7-*O*-methyl-eriodictyol (**7**) [8], (*R*)-(−)-mellein methyl ether (**8**) [9], *trans*-4-hydroxymellein (**9**) [10], and combretastatin D-3 (**10**) [11]. Here, we report the isolation and structural elucidation of these compounds, as well as their inhibitory activities against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 macrophages.

Figure 1: Structures of compounds **1−10**.

Compound **1** was obtained as a light yellow solid. Its molecular formula was determined to be $C_{17}H_{20}O_5$ by HRESIMS analysis (*m/z* 305.1392 [M+H]+), indicating eight degrees of unsaturation. The IR absorptions suggested the presence of hydroxyl groups (3448 cm⁻¹) and a conjugated carbonyl group (1445 cm⁻¹). The ¹H and ¹³C NMR spectral data showed great similarities to those of broussonone A [3], except for an additional methoxyl group in compound **1** (Table 1). HMBC correlation between δ_H 3.81 and C-3″ (δ_C 148.8)
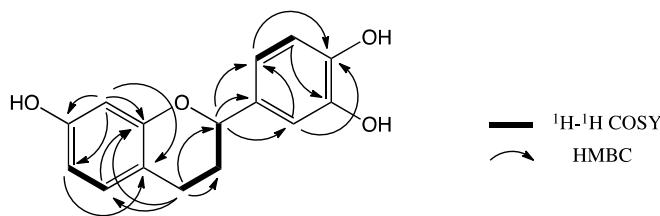
Figure 2: Key ¹H–¹H COSY and HMBC correlations of compound **1**.

Table 1: ¹³C and ¹H NMR data [δ_H (*J* in Hz)] for compound **1**.

No.	δ_C	δ_H
1	36.1	2.5 m
2	26.7	1.26 m; 1.40 m
3	38.9	1.71 m; 1.95 m
1′	72.2	
2′	178.5	
3′	102.1	5.53 d (1.7)
4′	190.3	
5′	128.1	6.08 dd (10.0, 1.7)
6′	150.1	6.63 d (10.0)
1″	134.6	
2″	113.1	6.68 d (1.9)
3″	148.8	
4″	145.6	
5″	116.1	6.66 d (8.0)
6″	121.8	6.54 dd (8.0, 1.9)
2′-OMe	56.7	3.76 s
3″-OMe	56.4	3.81 s

¹³C and ¹H NMR data were measured in CD₃OD at 100 and 400 MHz, respectively.

was observed, which indicated that the additional methoxyl group was attached at C-3″ (Figure 2). Thus, the chemical structure of **1** was confirmed and this compound was named as euonyphenylpropane A.

Compound **2** was obtained as a light yellow solid. Its molecular formula $C_{15}H_{14}O_4$ was determined from the positive HR-ESIMS ion at *m/z* 259.0951 [M+H]+, indicating nine degrees of unsaturation. The IR spectrum exhibited absorption bands ascribed to hydroxy groups (3445 cm⁻¹) and benzene rings (1644 cm⁻¹). The ¹H NMR data of **2** exhibited two sets of ABX-substituted aromatic protons [ring A: δ_H 6.25 (1H, d, *J* = 2.4 Hz), 6.31 (1H, dd, *J* = 8.1, 2.4 Hz), 6.85 (1H, d, *J* = 8.1 Hz) and ring B: δ_H 6.72 (1H, dd, *J* = 8.0, 1.8 Hz), 6.75 (1H, d, *J* = 8.0 Hz), 6.85 (1H, d, *J* = 1.8 Hz)]. In addition, signals of an oxymethine [δ_H 4.80 (1H, dd, *J* = 10.0, 2.5 Hz), δ_C 79.0] and two methylenes [δ_H 2.65, 2.80 (2H, m), δ_C 25.4; δ_H 1.90, 2.10 (2H, m), δ_C 31.3] were observed, which suggested compound **2** as a flavan.

Figure 3: Key ¹H–¹H COSY and HMBC correlations of compound **2**.

Table 2: ¹³C and ¹H NMR data [δ_H (*J* in Hz)] for compound **2**.

No.	δ_C	δ_H
2	79.0	4.80 dd (10.0, 2.5)
3	31.3	1.90 m; 2.10 m
4	25.4	2.65 m; 2.80 m
5	130.9	6.85 d (8.1)
6	109.0	6.31 dd (8.1, 2.4)
7	157.5	
8	104.0	6.25 d (2.4)
9	157.1	
10	114.3	
1′	135.0	
2′	114.4	6.85 d (1.8)
3′	146.3	
4′	146.0	
5′	116.1	6.72 dd (8.0, 1.8)
6′	118.7	6.75 d (8.0)

¹³C and ¹H NMR data were measured in CD_3OD at 100 and 400 MHz, respectively.

The corresponding carbon signals were assigned by HMQC experiment (Table 2). Analysis of the ¹H–¹H COSY spectrum revealed the spin system of H-2/H₂-3/H₂-4 as shown in Figure 3. The chemical structure of compound **2** was further confirmed by the HMBC spectrum (Figure 3). In the CD spectrum, a positive Cotton effect observed at 280 nm indicated a 2*R* configuration, in comparison with published data [4] and **2** was defined as (2*R*)-3′,4′,7-trihydroxyflavan.

Compounds **1**−**10** were tested for their inhibitory effects against LPS-induced NO production in RAW264.7 macrophages with aminoguanidine as positive control. As shown in Table 2, compounds **1**−**5** and **7** exhibited moderate inhibitory activities with IC₅₀ values ranged from 5.1 to 11.9 µM.

Table 3: Inhibitory effects of compounds **1**–**10** isolated from *E. glabra* against LPS-induced NO production in RAW264.7 macrophages.

Compounds	IC₅₀^a (µM)
1	10.3
2	8.1
3	11.9
4	5.1
5	11.9
6	20.1
7	5.4
8	21.8
9	19.3
10	19.1
aminoguanidine ^a	21.7

^a Positive control

Experimental

General procedures: TLC analysis utilized HSGF₂₅₄ silica gel plates (10–40 µm, Yantai, China). Column chromatography (CC) was performed using silica gel (100–200, 200–300 mesh, Yantai, China), silica gel H (10–40 µm, Qingdao, China) and Sephadex LH-20 (Pharmacia Co. Ltd.). Preparative HPLC (Shimadzu LC-6AD) was performed on a preparative column (Shimadzu PRC-ODS EV0233). 1D and 2D NMR spectra were recorded on Bruker Avance-400 spectrometers in either CD_3OD or DMSO-d_6 with TMS as internal standard. ESIMS were recorded on an Agilent LC/MSD Trap XCT spectrometer (Waters, USA), and HR-ESIMS on a Q-Tof micro YA019 mass spectrometer (Waters, USA). Optical rotations were recorded on a JASCO P-2000 polarimeter. IR spectra were measured on a Bruker FTIR Vector 22 spectrometer with KBr pellets. CD spectra were determined on a JACSO J-815 spectrometer.

Plant material: The stems and twigs of *E. glabra* Roxb. were collected in Xishuangbanna Dai Autonomous Prefecture, Yunnan province, PR China, in August 2010, and were authenticated by Prof. Haiou Yang, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. 201008SDWM) was deposited at the School of Pharmacy, Shanghai Jiao Tong University.

Extraction and isolation: Dried stems and twigs of *E. glabra* (4.5 kg) were powdered and extracted with 95% EtOH 3 times at room temperature and then evaporated under reduced pressure to give crude extract (350.0 g). This was further partitioned with light petroleum (PE), EtOAc and *n*-BuOH to obtain 3 fractions. The EtOAc fraction (90.7 g) was subjected to silica gel CC, and eluted with a step gradient of CH_2Cl_2-MeOH (100:0–0:100) to yield 7 fractions (EA-A−EA-G). EA-A (3.9 g) was chromatographed on macroporous resin MCI (MeOH-H_2O, 4:1) and a silica gel column eluting with a step gradient of CH_2Cl_2-MeOH (100:0–20:1), followed by a Sephadex LH-20 column (MeOH) to give 10 sub-fractions (EA-A1−EA-A10). EA-A5 was purified by preparative HPLC (MeOH-H_2O, 55:45) to yield compound **10** (5.0 mg). EA-A8 and EA-A9 were purified by preparative HPLC (MeOH-H_2O, 65:35) to obtain compounds **8** (5.0 mg) and **9** (3.5 mg), respectively. EA-B (7.0 g) was subjected to macroporous resin MCI (MeOH-H_2O, 4:1) and a silica gel column eluting with a step gradient of CH_2Cl_2-MeOH (100:1−5:1) followed by Sephadex LH-20 column (MeOH) to give 9 sub-fractions (EA-B1−EA-B9). EA-B6 was further purified by silica gel CC eluting with CH_2Cl_2-MeOH (100:1) to afford compounds **5** (5.3 mg) and **6** (3.2 mg). EA-B7 was purified by preparative HPLC (MeOH-H_2O, 60:40) to yield compound **3** (11.8 mg). EA-B8 was subjected to preparative HPLC (MeOH-H_2O, 60:40) to obtain compound **7** (1.6 mg). EA-C (7.1 g) was chromatographed on macroporous resin MCI (MeOH-H_2O, 4:1) and a silica gel column eluting with a step gradient of CH_2Cl_2-MeOH (50:1−0:100) followed by a Sephadex LH-20 column (MeOH) to give 7 sub-fractions (EA-C1−EA-C7). EA-C7 was purified by preparative HPLC (MeOH-H_2O, 60:40) to yield compounds **1** (6.1 mg), **2** (5.2 mg) and **4** (7.8 mg).

Euonyphenylpropane A (1)
Light yellow solid.
[α]²⁰_D: −3.7 (*c* 0.10, MeOH).
IR (KBr): 3448, 1620, 1505, 1645, 1455, 1383, 1189, 1121, 1075, 1032 cm⁻¹.
¹H and ¹³C NMR: Table 1.
HR-ESIMS (positive) *m/z*: 305.1392 [M+H]⁺ (calcd for $C_{17}H_{21}O_5$, 305.1384).

(2R)-3′,4′,7-Trihydroxyflavan (2)
Light yellow solid.
[α]²⁰_D: +8.1 (*c* 0.10, MeOH).
CD (1.0×10^{-4} M, MeOH): λ (Δ ε) 280 (2.1).
IR (KBr): 3445, 1644, 1383, 1189, 1153, 1119, 1077, 1022, 610 cm⁻¹.
¹H and ¹³C NMR: Table 2.
HR-ESIMS (positive) *m/z*: 259.0951 [M+H]⁺ (calcd for $C_{15}H_{15}O_4$, 259.0965).

Determination of nitric oxide (NO) production: The assay was performed using the MTT method, as previously described [12-14].

Briefly, RAW264.7 cells grown on a 100 mm culture dish were harvested and seeded in 96-well plates (2×10^5 cells/ well) for NO production. The plates were pretreated with various concentrations of samples for 30 min and incubated with LPS (1 µg/mL) for 24 h. The amount of NO was determined by the nitrite concentration in the cultured RAW264.7 macrophage supernatants with the Griess reagent. The cell viability was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich] reduction [15].

References

[1]　Xishuangbanna Tropical Botanical Garden and Department of Ethnobotany, Kunming Institute of Botany, the Chinese Academy of Sciences. (**1996**) List of plants in Xishuangbanna. *The Nationalities Publishing House of Yunnan*, *6*, 299.

[2]　Ren J, Zhang YY, Jin HZ, Yu J, Zhou YY, Wu F, Zhang WD. (**2014**) Novel inhibitors of human DOPA decarboxylase extracted from *Euonymus glabra* Roxb. *ACS Chemical Biology*, *9*, 897-903.

[3]　Ahn JH, Liu Q, Lee C, Ahn MJ, Yoo HS, Hwang BY, Lee MY. (**2012**) A new pancreatic lipase inhibitor from *Broussonetia kazinoki. Bioorganic & Medicinal Chemistry Letters*, *22*, 2760-2763.

[4]　Yang Y, Zhang T, Xiao L, Chen RY. (**2010**) Two novel flavanees from the leaves of *Morus alba* L. *Journal of Asian Natural Products Research*, *12*, 194-198.

[5]　Moosophon P, Kanokmedhakul S, Kanokmedhakul K, Buayairaksa M, Noichan J, Poopasit K. (**2013**) Antiplasmodial and cytotoxic flavans and diarylpropanes from the stems of *Combretum griffithii.. Journal of Natural Products*, *76*, 1298-1302.

[6]　Yu HW, Hua J, Qin L, Xu Q. (**2012**) Study on chemical constituents of *Premna yunnanensis. Chinese Traditional Patent Medicine*, *34*, 300　303.

[7]　Bandeira PN, de Farias SS, Lemos TLG, Braz-Filho R, Santos HS, Albuquerque MRJR, Costa SMO. (**2011**) New isoflavone derivative and other flavonoids from the resin of *Amburana cearensis. Journal of the Brazilian Chemical Society*, *22*, 372-375.

[8]　Yang WX, Huang HY, Wang YJ, Jia ZY, Li LL. (**2005**) Study on chemical constituents in total saponin from *Trigonella foenum-graecum. China Journal of Chinese Materia Medica*, *30*, 1428-1430.

[9]　Klaiklay S, Rukachaisirikul V, Sukpondma Y, Phongpaichit S, Buatong J, Bussaban B. (**2012**) Metabolites from the mangrove-derived fungus *Xylaria cubensis* PSU-MA34. *Archives of Pharmacal Research*, *35*, 1127-1131.

[10]　Djoukeng JD, Polli S, Larignon P, Abou-Mansour E. (**2009**) Identification of phytotoxins from *Botryosphaeria obtusa*, a pathogen of black dead arm disease of grapevine. *European Journal of Plant Pathology*, *124*, 303-308.

[11]　Vongvanich N, Kittakoop P, Charoenchai P, Intamas S, Danwisetkanjana K, Thebtaranonth Y. (**2005**) Combretastatins D-3 and D-4, new macrocyclic lactones from *Getonia floribunda. Planta Medica*, *71*, 191-193.

[12]　Qin JJ, Jin HZ, Zhu JX, Fu JJ, Zeng Q, Cheng XR, Zhu Y, Shan L, Zhang SD, Pan YX, Zhang WD. (**2010**) New sesquiterpenes from *Inula japonica* Thunb. with their inhibitory activities against LPS-induced NO production in RAW264.7 macrophages. *Tetrahedron*, *66*, 9379-9388.

[13]　Nie LY, Qin JJ, Huang Y, Yan L, Liu YB, Pan YX, Jin HZ, Zhang WD. (**2010**) Sesquiterpenoids from *Inula lineariifolia* inhibit nitric oxide production. *Journal of Natural Products*, *73*, 1117-1120.

[14]　Schmidt HHHW, Kelm M. (**1996**) In *Methods in Nitric Oxide Research*. John Wiley & Sons, Ltd., London. *Chapter 33*, 491-497.

[15]　Denizot F, Lang R. (**1986**) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological Methods*, *89*, 271-277.

Isoprenylated Flavonoids with PTP1B Inhibition from *Ficus tikoua*

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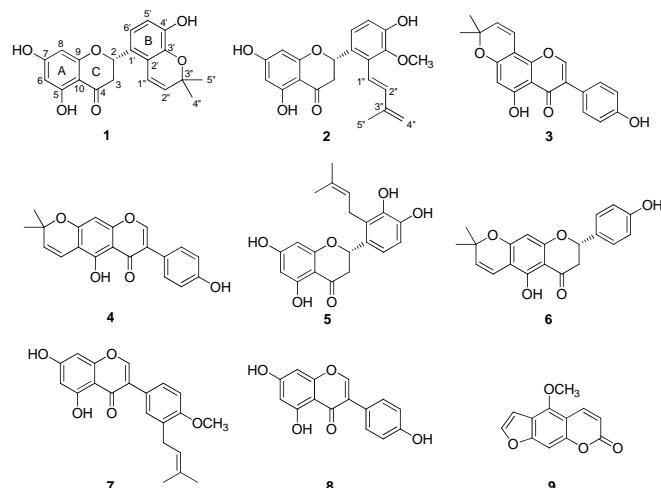
Two new isoprenylated flavanones, ficustikousins A and B (**1** and **2**), together with seven known compounds (**3–9**) were isolated from the whole plant of *Ficus tikoua* (Moraceae). The structures of the new compounds were elucidated on the basis of spectroscopic methods. Compounds **1–7** exhibited moderate inhibitory activities against PTP1B *in vitro*.

Keywords: *Ficus tikoua*, Moraceae, Isoprenylated flavonoids, Protein tyrosine phosphatase 1B.

Ficus tikoua Bur. belongs to the family Moraceae and is a creeping vine plant mainly distributed in south China, India, Vietnam and Laos [1]. This plant is used as traditional Chinese medicine and ethnomedicine in more than ten nationalities in China for the treatment of edema, jaundice, amenorrhea, and bruise [2,3]. Previous phytochemical studies on this plant resulted in the isolation of some flavonoids and phenolic glycosides, some of which showed antifungal and cytotoxic activities [4]. In the course of discovering structurally and biologically interesting compounds from the family Moraceae [5], we investigated the chemical constituents of *F. tikoua*.

Fractionation of an ethanol extract of this plant afforded eight flavonoids and one coumarin, including two new isoprenylated flavanones, ficustikousins A (**1**) and B (**2**), along with seven known compounds, derrone (**3**) [6a], alpinumisoflavone (**4**) [6b], (*S*)-5,7,3′,4′-tetrahydroxy-2′-(3-methylbut-2-enyl)flavanone (**5**) [6c], (*S*)-paratocarpin K (**6**) [6d], 3′-(3-methylbut-2-enyl)biochanin A (**7**) [6e], genistein (**8**) [6f], and bergapten (**9**) [6g]. The known compounds **3–7** were isolated from this plant for the first time. Protein tyrosine phosphatase 1B (PTP1B) plays an important role in regulating the sensitivity of insulin signaling and fat metabolism and is considered to be a significant target in treating type 2 diabetes and obesity [7]. Recently, a series of isoprenylated flavonoids with PTP1B-inhibiting activities were isolated from the family Moraceae by our group [5a, d]. As a continuing research on the discovery of effective PTP1B inhibitors, compounds isolated from *F. tikoua* were evaluated *in vitro* for the inhibition on PTP1B enzyme activity. Compounds **1–7** showed moderate inhibitory effects. We herein report the structural elucidation and biological evaluation of these compounds.

Ficustikousin A (**1**), an optically active compound ($[\alpha]_D^{25} = -8.7$), was obtained as white amorphous powder. Its molecular formula was determined as $C_{20}H_{18}O_6$ by HRESIMS (*m/z* 353.1033 [M − H]⁻; calcd. 353.1031). The IR spectrum of **1** indicated the presence of hydroxyl (3421 cm⁻¹), carbonyl (1653 cm⁻¹), and benzene ring (1539 and 1467 cm⁻¹) moieties. The UV spectrum showed absorption maxima at λ_{max} 212 (sh), 231, 282 and 331 (sh) nm, which were similar to those of flavanones [8]. The ¹H NMR
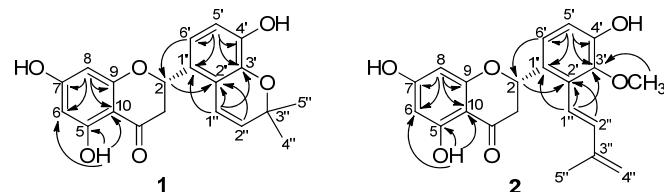
Figure 1: Structures of compounds **1–9**.

spectrum (Table 1) revealed signals of an ABX spin system at δ_H 5.73 (1H, dd, *J* = 13.2, 2.8 Hz, H-2), 3.21 (1H, dd, *J* = 17.2, 13.2 Hz, H-3), and 2.70 (1H, dd, *J* = 17.2, 2.8 Hz, H-3), characteristic of ring C on a flavanone skeleton. It also showed resonances for a hydrogen-bonded hydroxyl group at δ_H 12.20 (1H, s), two *ortho*-coupled aromatic protons at δ_H 6.99 (1H, d, *J* = 8.4 Hz, H-6′) and 6.79 (1H, d, *J* = 8.4 Hz, H-5′), two *meta*-coupled aromatic protons at δ_H 5.96 (2H, br s), and a 2,2-dimethylpyran ring at δ_H 6.81 (1H, d, *J* = 10.0 Hz, H-1″), 5.85 (1H, d, *J* = 10.0 Hz, H-2″), and 1.42, 1.43 (each 3H, s, H₃-4″, 5″). The ¹³C NMR spectrum (Table 1) exhibited 20 carbon signals attributable to one carbonyl group, eight quaternary sp², one quaternary sp³, six methine sp², one methine sp³, one methylene sp³, and two methyl carbon atoms. These spectroscopic data suggested that **1** is a monoprenylated flavanone. The two *ortho*-coupled protons at δ_H 6.99 and 6.79 were assigned to H-6′ and H-5′, respectively, as established by the HMBC correlations from H-6′ (δ_H 6.99) to C-2 and from H-5′ (δ_H 6.79) to C-1′, C-3′, C-4′, and C-6′ (Figure 2). The HMBC correlations from H-1″ (δ_H 6.81) to C-1′, C-2′, and C-3′ and from H-2″ (δ_H 5.85) to C-2′ indicated that the 2,2-dimethylpyran ring was fused at C-2′ and

Table 1: ¹H and ¹³C NMR Data (400 and 100 MHz, resp.) of compounds **1** and **2** in acetone-d_6 (δ in ppm, J in Hz).

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
2	5.73, dd (13.2, 2.8)	77.3	5.65, dd (13.6, 2.8)	77.7
3	2.70, dd (17.2, 2.8)	43.5	2.68, dd (17.2, 2.8)	43.3
	3.21, dd (17.2, 13.2)		3.31, dd (17.2, 13.6)	
4		197.8		198.0
5		165.9		165.9
6	5.96, br s	97.4	5.96, d (2.0)	97.5
7		167.9		167.8
8	5.96, br s	96.4	5.99, d (2.0)	96.3
9		164.9		164.9
10		103.8		103.8
1′		126.3		129.3
2′		121.3		133.1
3′		141.5		146.9
4′		147.6		151.9
5′	6.79, d (8.4)	116.4	6.93, d (8.8)	123.2
6′	6.99, d (8.4)	120.0	7.32, d (8.8)	124.6
1″	6.81, d (10.0)	120.5	6.73, d (16.4)	116.5
2″	5.85, d (10.0)	133.3	6.81, d (16.4)	139.6
3″		77.0		143.7
4″	1.42, sᵃ	28.0	5.06, br s	118.8
5″	1.43, sᵃ	28.0	1.94, s	18.9
OH-5	12.20, s		12.17, s	
OMe-3′			3.71, s	60.9

ᵃ Assignments are exchangeable.

Figure 2: Key HMBC (H→C) correlations of compounds **1** and **2**.

C-3′. The substitution of ring A was further supported by the HMBC correlations shown in Figure 2. The stereochemistry at C-2 was assigned as *S*-configuration by the ECD data with Cotton effects at 288 (−) and 330 (+) nm [8]. Thus, the structure of **1** was determined and named ficustikousin A.

Ficustikousin B (**2**), obtained as white amorphous powder, was assigned the molecular formula $C_{21}H_{20}O_6$ by HRESIMS (*m/z* 367.1202 [M − H]⁻; calcd. *m/z* 367.1187). The NMR spectroscopic data of **2** (Table 1) suggested a flavanone skeleton with the same rings A and C as those of **1**. It differed from **1** in the substituents on the ring B, as deduced from the NMR data of a 3-methylbutadienyl group [δ_H 6.73 (1H, d, *J* = 16.4 Hz, H-1″), 6.81 (1H, d, *J* = 16.4 Hz, H-2″), 5.06 (2H, br s, H₂-4″), and 1.94 (3H, s, H₃-5″); δ_C 116.5 (C-1″), 139.6 (C-2″), 143.7 (C-3″), 118.8 (C-4″), and 18.9 (C-5″)] and a methoxyl group [δ_H 3.71 (3H, s); δ_C 60.9 (OMe-3′)]. In the HMBC spectrum (Figure 2), the correlations from H-1″ to C-1′, C-2′ and C-3′ and from H-2″ to C-2′ indicated that the 3-methylbutadienyl group was attached to C-2′. The methoxyl group was located at C-3′ according to the HMBC correlation from the methoxyl protons to C-3′. The (*E*)-configuration of the C(1″)=C(2″) bond was indicated by the coupling constant of H-1″ and H-2″ (*J* = 16.4 Hz). Compound **2** showed no Cotton effect in the ECD spectrum. The stereochemistry at C-2 was proposed to be *S*-configuration on the basis of the negative specific optical rotation ($[\alpha]_D^{25}$ = −5.0) and the *trans* diaxial coupling constant of H-2 and H-3 (*J* = 13.6 Hz) [9]. Thus, the structure of **2** was assigned and named ficustikousin B.

The structures of compounds **3–9** were identified by comparison of their spectral data with those described in the literature.

Table 2: Inhibitory Effects of Compounds **1–7** against PTP1B.

Compound	IC_{50} ± SD (μM)
1	40.37 ± 4.66
2	16.33 ± 1.47
3	24.64 ± 0.57
4	11.16 ± 1.88
5	19.27 ± 3.26
6	25.12 ± 2.28
7	18.55 ± 0.74
Oleanolic acid	2.61 ± 0.59

The isolated compounds (**1–9**) were examined *in vitro* for the inhibition on PTP1B. Oleanolic acid is used as positive control in this test. The isoprenylated flavonoids (**1–7**) showed moderate PTP1B inhibitory activities (10 μM <IC_{50}<50 μM) (Table 2).

In summary, two new isoprenylated flavanones and seven known compounds including five isoprenylated flavonoids were isolated from the whole plant of *F. tikoua*. Although isoprenylated flavonoids from *F. tikoua* had been reported previously [4a, b, c], the flavanones with 2,2-dimethylpyran moiety (**1** and **6**) and methylbutadienyl group (**2**) were found for the first time from this plant. Furthermore, isoprenylated flavonoids (**1–7**) were found to inhibit PTP1B, while flavonoid and coumarin without isoprenoid group (**8** and **9**) were inactive.

Experimental

General: Optical rotations were measured on Autopol IV. UV spectra were taken on a Hitachi U-2900 spectrophotometer. IR spectra were measured on a Nicolet Avatar 360 spectrometer with KBr pellets. ECD spectra were manipulated on a Jasco J-810 automatic digital polarimeter. NMR spectra were recorded on a Bruker DRX-400 and Varian Mercury Plus 400 instruments. ESIMS and HRESIMS were performed on an Agilent 1100 LC/MSD and on a Bruker Daltonics Apex 7.0 TESLA FTMS mass spectrometer, respectively. Column chromatography (CC) was performed on silica gel (200–300 mesh; Yantai Institute of Chemical Technology, Yantai, PR China), Sephadex LH-20 gel (GE Healthcare Bio-Science AB, Uppsala, Sweden), and Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan). TLC analysis was run on precoated silica gel GF254 plates (10–40 μm, Yantai Institute of Chemical Technology, Yantai, PR China), and spots were visualized by spraying with 10% H_2SO_4 in EtOH, followed by heating. Semi-preparative HPLC was run on an Agilent 1200 and a Promosil C_{18} column (10 × 150 mm, 5 μm, Agela Technologies, China), using a UV detector set at 210 nm.

Plant material: The whole plant of *F. tikoua* was collected from Nanchong, Sichuan province, PR China, in August 2012. The plant material was identified by Dr. Yun Kang, Fudan University, and a voucher specimen (TCM 12-08-25 Hou) has been deposited in the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Fudan University.

Extraction and isolation: The milled and air-dried plant material (4.7 kg) was percolated with 95% EtOH (20 L × 5 times) at room temperature. The filtrate was concentrated under reduced pressure to yield a residue (400 g), which was suspended in H_2O and extracted with EtOAc (2 L × 3 times). The EtOAc extract (96 g) was subjected to column chromatography (CC) on Diaion HP-20 eluted with 85% EtOH and then was applied to CC over silica gel eluted with a gradient system of CH_2Cl_2/MeOH (100:1, 20:1, 9:1, 5:1, 2:1, v/v) to provide fractions 1–5. Fraction 1 (11 g) was purified by silica gel CC (petroleum ether (PE)/ Me_2CO 30:1, v/v) to give **9** (3.1 mg). Fraction 2 (8 g) was subjected successively to Sephadex LH-20 CC (MeOH), silica gel CC (PE/Me_2CO 50:1, v/v),

and semi-preparative HPLC (MeOH/H$_2$O 7:3, v/v, flow rate 1 mL/min) to give **6** (7 mg, t_R 70 min) and **7** (6 mg, t_R 50 min). Purification of fraction 3 (3.3 g) by Sephadex LH-20 CC (MeOH) and semi-preparative HPLC (MeOH/H$_2$O 6:4, v/v, flow rate 1 mL/min) yielded **3** (20 mg, t_R 56 min) and **8** (4 mg, t_R 10 min). Fraction 4 (8.4 g) was subjected to Sephadex LH-20 CC (MeOH) to give fractions 4.1–4.5. Fraction 4.1 was isolated by semi-preparative HPLC (MeOH/H$_2$O 7:3, v/v, flow rate 1 mL/min) to provide **4** (3.9 mg, t_R 27 min) and **5** (8 mg, t_R 29 min). Fraction 4.3 was separated by semi-preparative HPLC (MeOH/H$_2$O 6:4, v/v, flow rate 1 mL/min) to give **2** (3.5 mg, t_R 67 min). Fraction 4.4 was purified by semi-preparative HPLC (MeOH/H$_2$O 5.5:4.5, v/v, flow rate 1 mL/min) to provide **1** (4 mg, t_R 46 min).

Ficustikousin A (1)
White amorphous powder.
$[\alpha]_D^{25}$: −8.7 (*c* 0.2, MeOH).
IR (KBr) v_{max}: 3421, 1653, 1539, 1467, 1327, 1287, 1157, 1087 cm^{-1}.
UV (MeOH) λ_{max} (log ε): 212 (sh 3.35), 231 (3.51), 282 (3.42), 331 (sh 2.95) nm.
ECD (MeOH) λ_{max} (Δε): 260 (+1.27), 288 (−1.39), 330 (+0.46) nm.
^{1}H and ^{13}C NMR: Table 1.
HRESIMS: *m/z* 353.1033 [M − H]$^-$; calcd for C$_{20}$H$_{17}$O$_6$: 353.1031.

Ficustikousin B (2)
White amorphous powder.
$[\alpha]_D^{25}$: −5.0 (*c* 0.2, MeOH).
IR (KBr) v_{max}: 3421, 1653, 1579, 1467, 1411, 1331, 1301, 1255, 1163, 1087 cm^{-1}.
UV (MeOH) λ_{max} (log ε): 212 (sh 3.80), 228 (3.82), 282 (3.67), 332 (sh 2.87) nm.
^{1}H and ^{13}C NMR: Table 1.
HRESIMS: *m/z* 367.1202 [M − H]$^-$; calcd for C$_{21}$H$_{19}$O$_6$: 367.1187.

Assay of PTP1B activity: The procedure was the same as that reported previously [5d, 10]. The result of PTP1B inhibition was expressed as IC$_{50}$, which was calculated with Prism 4 software (Graphpad, San Diego, CA).

Supplementary data: ESIMS, HRESIMS, NMR (^{1}H and ^{13}C NMR, HMBC) and ECD spectra for compounds **1** and **2** are available.

References

[1] Editorial Committee of Flora of China, Chinese Academy of Sciences. (**1998**) *Flora of China.* Science Press, Beijing, **23**, 156.
[2] Wu JR, Qiu DW. (**1993**) *Colored illustration of commonly used Chinese traditional and herbal drugs.* Guizhou Science and Technology Publishing House, Guiyang, 170.
[3] Guan YX, Yang XS, Tong LH, Yang B, Hao XJ. (**2007**) Chemical constituents in *Ficus tikoua* of Miao nationality. *Chinese Traditional and Herbal Drugs*, **38**, 342-344.
[4] (a) Wei SP, Lu LN, Ji ZQ, Zhang JW, Wu WJ. (**2012**) Chemical constituents from *Ficus tikoua*. *Chemistry of Natural Compounds*, **48**, 484-485; (b) Wei SP, Wu WJ, Ji ZQ. (**2012**) New antifungal pyranoisoflavone from *Ficus tikoua* Bur.. *International Journal of Molecular Sciences*, **13**, 7375-7382; (c) Jiang ZY, Li SY, Li WJ, Guo JM, Tian K, Hu QF, Huang XZ. (**2013**) Phenolic glycosides from *Ficus tikoua* and their cytotoxic activities. *Carbohydrate Research*, **382**, 19-24; (d) Wei SP, Luan JY, Lu LN, Wu WJ, Ji ZQ. (**2011**) A new benzofuran glucoside from *Ficus tikoua* Bur.. *International Journal of Molecular Sciences*, **12**, 4946-4952.
[5] (a) Wang M, Gao XL, Wang J, Li JY, Yu MH, Li J, Hou AJ. (**2015**) Diels–Alder adducts with PTP1B inhibition from *Morus notabilis*. *Phytochemistry*, **109**, 140-146; (b) Hu X, Wu JW, Zhang XD, Zhao QS, Huang JM, Wang HY, Hou AJ. (**2011**) Isoprenylated flavonoids and adipogenesis-promoting constituents from *Morus nigra*. *Journal of Natural Products*, **74**, 816-824; (c) Hu X, Wu JW, Wang M, Yu MH, Zhao QS, Wang HY, Hou AJ. (**2012**) 2-Arylbenzofuran, flavonoid, and tyrosinase inhibitory constituents of *Morus yunnanensis*. *Journal of Natural Products*, **75**, 82-87; (d) Wang M, Yu BW, Yu MH, Gao LX, Li JY, Wang HY, Li J, Hou AJ. (**2015**) New isoprenylated phenolic compounds from *Morus laevigata*. *Chemistry & Biodiversity, 12*, 937-945.
[6] (a) Chibber SS, Sharma RP. (**1980**) Derrone, a new pyranoisoflavone from *Derris robusta* seeds. *Phytochemistry*, **19**, 1857-1858; (b) Stewart M, Bartholomew B, Currie F, Abbiw DK, Latif Z, Sarker SD, Nash RJ. (**2000**) Pyranoisoflavones from *Rinorea welwitschii*. *Fitoterapia*, **71**, 595-597; (c) Chauhan P, Saxena VK. (**1987**) A new prenylated flavanone from *Erythrina suberosa* roots. *Planta Medica*, **53**, 221-222; (d) Hano Y, Itoh N, Hanaoka A, Nomura T. (**1995**) Paratocarpins F-L, Seven new isoprenoid-substituted flavonoids from *Paratocarpus venenosa* Zoll. *Heterocycles*, **41**, 2313-2326; (e) Yenesew A, Midiwo JO, Heydenreich M, Peter MG. (**1998**) Four isoflavones from the stem bark of *Erythrina sacleuxii*. *Phytochemistry*, **49**, 247-249; (f) Krishnamurty HG, Prasad JS. (**1980**) Isoflavones of *Moghania macrophylla*. *Phytochemistry*, **19**, 2797-2798; (g) El-Fishawy A, Zayed R, Afifi S. (**2011**) Phytochemical and pharmacological studies of *Ficus auriculata* Lour. *Journal of Natural Products*, **4**, 184-195.
[7] (a) Zhang W, Li JY, Li J. (**2006**) Advance in the study about the inhibitors of PTP1B-a novel target for diabetes and obesity therapy. *Chinese Bulletin of Life Sciences*, **18**, 462-466; (b) Goldstein BJ, Bittner-Kowalczyk A, White MF, Harbeck M. (**2000**) Tyrosine dephosphorylation and deactivation of insulin receptor substrate-1 by protein tyrosine phosphatase 1B: Possible facilitation by the formation of a ternary complex with the Grb2 adaptor protein. *The Journal of Biological Chemistry*, **275**, 4283-4289.
[8] Hano Y, Nomura T. (**1983**) Constituents of the Chinese crude drug "Sang-Bai-Pi" (Morus root barks) IV. Structures of four new flavonoids, Sanggenon H, I, J and K. *Heterocycles*, **20**, 1071-1076.
[9] (a) Ma JP, Qiao X, Pan S, Shen H, Zhu GF, Hou AJ. (**2010**) New isoprenylated flavonoids and cytotoxic constituents from *Artocarpus tonkinensis*. *Journal of Asian Natural Products Research*, **12**, 586-592; (b) Kanokmedhakul S, Kanokmedhakul K, Nambuddee K, Kongsaeree P. (**2004**) New bioactive prenylflavonoids and dibenzocycloheptene derivative from roots of *Dendrolobium lanceolatum*. *Journal of Natural Products*, **67**, 968-972.
[10] Shi L, Yu HP, Zhou YY, Du JQ, Shen Q, Li JY, Li J. (**2008**) Discovery of a novel competitive inhibitor of PTP1B by high-throughput screening. *Acta Pharmacologica Sinica*, **29**, 278-284.

Phenolic Derivatives from *Hypericum japonicum*

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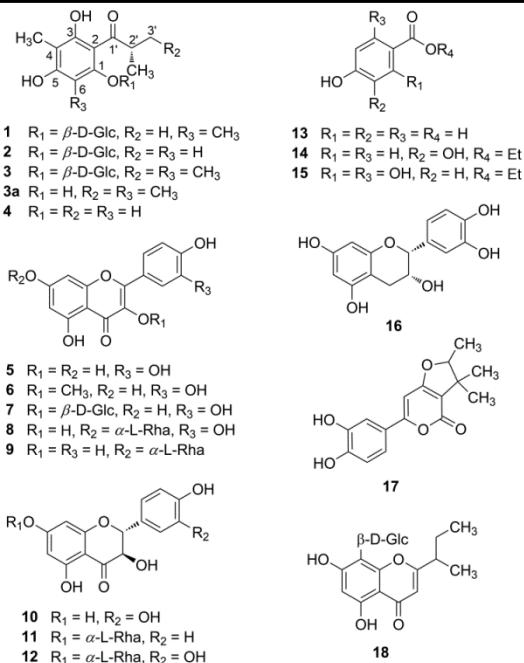
Three new acylphloroglucinol glycosides, hypericumols A - C, together with fifteen known phenolic derivatives, were isolated from the total phenolic extract of *Hypericum japonicum*. Hypericumols A, B, and C were characterized as 4,6-dimethyl-2-methylpropanoylphloroglucinol-1-*O*-β-D-glucopyranoside (**1**), 4-methyl-2-methylpropanoylphloroglucinol-1-*O*-β-D-glucopyranoside (**2**), and (2′*S*)-4,6-dimethyl-2-methylbutyrylphloroglucinol-1-*O*-β-D-glucopyranoside (**3**), respectively, on the basis of spectroscopic data interpretation and chemical degradation reaction.

Keywords: *Hypericum japonicum*, Hypericumol, Acylphloroglucinol, Flavonoid, Phenol.

Hypericum japonicum Thunb., di er cao or tian ji huang in Chinese, is an annual / perennial herbal plant [1]. It is widely distributed in Asia, Oceania, and North America [1]. The whole plant material has been used in traditional Chinese medicine for heat-relieving, detoxification, hemostasis, and detumescence [1]. Phytochemical and pharmacological investigations showed that the chemical components of *H. japonicum* have diverse biological activities such as antioxidant [2], antivirus [3], antitumor [4], antimicrobial [5], and hepatoprotection [2a, 6]. Flavonoids and chromones are the primary chemical components of *H. japonicum*, whereas phloroglucinols and xanthones are two characteristic metabolites present in *H. japonicum* [7]. Recent studies demonstrated that the total phenolic extract of *H. japonicum* showed potential application to cure chronic kidney disease (CKD), which may be attributed to its effects on interfering with renal fibrosis, and enhancing cellular and humoral immunity [8]. As part of our continuing interests in the chemistry of traditional Chinese medicines [9], here we report the isolation and structure characterization of three new acylphloroglucinol glycosides (**1 - 3**), together with fifteen known compounds, including an acylphloroglucinol (**4**), nine flavonoids (**5 - 12, 16**), three benzoic acid derivatives (**13 - 15**), a pyrone (**17**), and a chromone glycoside (**18**), from the phenolic extract of *H. japonicum* (Figure 1).

Compound **1** was isolated as a yellowish gum. Its molecular formula $C_{18}H_{26}O_9$ was calculated from the $[M+Na]^+$ ion at *m/z* 409.1480 in its HRESIMS (positive ion mode). The hydroxyl and conjugated carbonyl groups were deduced from the IR bands at 3410 and 1615 cm^{-1}, respectively. Besides the ^{1}H NMR signals for a hexose moiety, two singlet methyls at δ_H = 2.03 (3H, s) and 2.20 (3H, s), two doublet methyls at δ_H = 0.99 (3H, d, *J* = 6.9 Hz) and 1.16 (3H, d, *J* = 6.9 Hz), and a heptet methine group at δ_H = 4.05 (1H, h, *J* = 6.9 Hz) were recognized from the ^{1}H NMR spectrum (Table 1). In addition to the ^{13}C NMR signals for the groups mentioned above, seven downfield quaternary carbon signals (δ_C > 109) were observed in the ^{13}C NMR spectrum (Table 1), which indicated the presence of a phenyl and a conjugated keto carbonyl group. Compound **1** might be an acylphloroglucinol derivative in view of its characteristic UV bands at 290 and 329 nm for acylphloroglucinol [10]. A 2-methyl propionyl group was established from the two doublet methyls, the
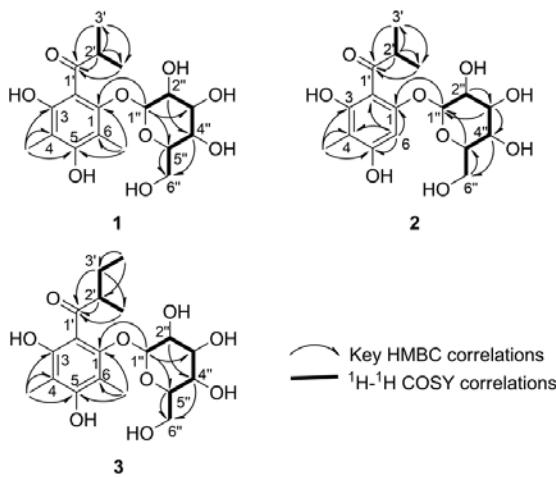
Figure 1. Chemical structures of phenolic derivatives from *Hypericum japonicum*.

heptet methine, and the keto carbonyl group on the basis of ^{1}H-^{1}H COSY correlations of H-3′ / H-2′ / H-2′-CH_3 and the key HMBC correlations of H-2′ / C-1′, C-3′, C-2′-CH$_3$; H-3′ / C-1′, C-2′-CH$_3$; and H-2′-CH_3 / C-1′ shown in Figure 2. A 2,4-dimethyl-1,3,5-trihydroxyphenyl moiety was established from the key HMBC correlations of H-4-CH_3 / C-3, C-4, C-5 and H-6-CH_3 / C-1, C-5, C-6 (Figure 2). Acid hydrolysis of compound **1** afforded an acylphloroglucinol and D-glucose with β-orientation in view of the ^{1}H NMR signal of the anomeric proton at δ_H = 4.38 (1H, d, *J* = 7.7 Hz). The 1-OH was glucosylated in view of the key HMBC correlation of H-1″ / C-1 (Figure 2). Thus compound **1**, named as hypericumol A, was characterized as 4,6-dimethyl-2-methylpropanoylphloroglucinol-1-*O*-β-D-glucopyranoside (Figure 1).

Table 1: NMR spectroscopic data of compounds 1 - 3 in CD$_3$OD.

	1		2		3	
	$\delta_H{}^a$	$\delta_C{}^b$	$\delta_H{}^a$	$\delta_C{}^b$	$\delta_H{}^a$	$\delta_C{}^b$
1		153.72, C		159.29, C		153.83, C
2		112.71, C		105.57, C		112.45, C
3		158.52, C		165.38, C		159.08, C
4		109.30, C		106.30, C		109.20, C
5		160.41, C		163.31, C		160.50, C
6		111.13, C	6.23, s	94.50, CH		111.05, C
4-CH$_3$	2.03, s	8.35, CH$_3$	1.95, s	7.48, CH$_3$	2.05, s	8.33, CH$_3$
6-CH$_3$	2.20, s	9.43, CH$_3$			2.21, s	9.64, CH$_3$
1'		214.98, C		212.12, C		214.29, C
2'	4.05, h (6.9)	40.65, CH	4.05, h (6.7)	40.32, CH	3.92, m	47.38, CH
3'	0.99 d (6.9)^c	21.16, CH$_3{}^c$	1.14, d (6.7)^c	19.55, CH$_3{}^c$	1.90, m	25.94, CH$_2$
					1.41, m	
2'-CH$_3$	1.16 d (6.9)^c	17.87, CH$_3{}^c$	1.15, d (6.7)^c	20.42, CH$_3{}^c$	1.02, d (7.1)	18.31, CH$_3$
3'-CH$_3$					0.97, t (7.5)	12.36, CH$_3$
1''	4.38 d (7.7)	105.64, CH	5.00, d (7.5)	101.60, CH	4.48, d (7.7)	105.51, CH
2''	3.48 dd (9.2, 7.7)	75.56, CH	3.51, dd (9.1, 7.5)	74.76, CH	3.52, dd (9.2, 7.7)	75.63
3''	3.40 (9.2)	77.67, CH	3.46, m	78.79, CH	3.43, t (9.2)	77.68, CH
4''	3.27 t (9.2)	72.04, CH	3.38, m	71.23, CH	3.27, t (9.2)	72.24, CH
5''	3.10 ddd (9.2, 6.2, 2.7)	77.81, CH	3.44, m	78.37, CH	3.14, ddd (9.2, 6.4, 2.8)	77.87, CH
6''	3.73 dd (11.9, 2.7)	63.15, CH$_2$	3.94, dd (12.0, 2.1)	62.56, CH$_2$	3.76, dd (11.9, 2.8)	63.18, CH$_2$
	3.56 (11.9, 6.2)		3.71, dd (12.0, 5.7)		3.57, dd (11.9, 6.4)	

^{a} Recorded at 400 MHz and reported in ppm. The coupling constants are reported in Hz; s, singlet; d, doublet; t, triplet; h, heptet; m, multiplet; ^{b} Recorded at 100 MHz; ^{c} Data in the same column are exchangeable.

Figure 2. ^{1}H-^{1}H COSY and HMBC correlations of compounds 1 - 3.

Compound **2** showed similar IR and UV bands to that of compound **1**, which suggested that **2** might be an acylphloroglucinol derivative. The molecular formula of **2** was determined to be C$_{17}$H$_{24}$O$_9$ from the [M+Na]$^+$ ion at *m/z* 395.1305 from the HRESIMS (positive ion mode). Comparison of the NMR data of compound **2** with that of compound **1** (Table 1) showed that a singlet methyl group of **1** was replaced by an aromatic proton at δ_H = 6.23 (1H, s) to form **2**, which was consistent with the molecular formula of **2**. As shown in Figure 2, the spectroscopic data interpretation confirmed that the 6-methyl of **1** was replaced by the proton mentioned above to form **2**. Thus compound **2**, named as hypericumol B, was characterized as 4-methyl-2-methyl propanoylphloroglucinol-1-*O*-β-D-glucopyranoside (Figure 1).

Compound **3** had similar spectroscopic characteristics to that of compounds **1** and **2**. The molecular formula of **3**, C$_{19}$H$_{28}$O$_9$, was determined from the [M+Na]$^+$ ion at *m/z* 423.1614 in its HRESIMS (positive ion mode). This indicated an additional CH$_2$ group present in the structure of **3**, relative to that of **1**. Comparison of the NMR signals of **3** with those of **1** showed that a doublet methyl of **1** was replaced by a triplet methyl at δ_H = 0.97 (3H, t, *J* = 7.5 Hz) in **3**. A CH$_2$ group at δ_H = 1.90 (1H, m) and 1.41 (1H, m) / δ_C = 25.94 was deduced from the NMR spectroscopic data (Table 1) and it was confirmed to be adjacent to the triplet methyl group by the ^{1}H-^{1}H COSY and HMBC correlations (Figure 2). A 2-methylbutyryl group was established from a doublet methyl, a triplet methyl, a methine, a methylene, and a keto carbonyl group on the basis of detailed spectroscopic data interpretation (Figure 2). Acid hydrolysis of **3**

led to the identification of D-glucose and 4,6-dimethylmultifidol (**3a**) [11]. The absolute configuration of C-2' in **3a** was determined to be *S* by its optical rotation ([α]$_D{}^{20}$ +2.4), according to the empirical rule established by Pei *et al.* to determine the absolute configuration of 2-methylbutyrylphloroglucinols [12]. Thus, the structure of compound **3** was elucidated as (2'*S*)-4,6-dimethyl-2-methylbutyrylphloroglucinol-1-*O*-β-D-glucopyranoside. Compound **3** was a product of the acid hydrolysis of 4,6-dimethyl-1-*O*-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl]multifidol [13]. However, no spectroscopic data were reported and assigned to the product [13]. Here we report the compound as a naturally occurring product from the plant with detail spectroscopic data and named it as hypericumol C (**3**) (Figure 1).

The known compounds isolated from the phenolic extract were identified as 2-(2-methylpropionyl)-4-methylphloroglucinol (**4**) [14], quercetin (**5**) [15], 3-*O*-methylquercetin (**6**) [16], isoquercitrin (**7**) [17], quercetin-7-*O*-α-L-rhamnopyranoside (**8**) [18], kaempferol-7-*O*-α-L-rhamnopyranoside (**9**) [19], dihydroquercetin (**10**) [2c], dihydrokaempferol-7-*O*-α-L-rhamnopyranoside (**11**) [20], (2*R*,3*R*)-taxifolin-7-*O*-α-L-rhamnopyranoside (**12**) [21], *p*-hydroxybenzoic acid (**13**) [22], ethyl 3,4-dihydroxybenzoate (**14**) [23], ethyl 2,4,6-trihydroxybenzoate (**15**) [24], epicatechin (**16**) [25], saropyrone (**17**) [26], and 5,7-dihydroxy-2-(1-methylpropyl)chromone-8-*β*-D-glucopyranoside (**18**) [27] on the basis of spectroscopic analyses.

In summary, three new acylphloroglucinol glycosides and fifteen known phenolic natural products were isolated and identified from the phenolic extract of *H. japonicum*. Among them, quercetin (**5**), isoquercitrin (**7**), and dihydroquercetin (**10**) are the major chemical components of the extract. Quercetin (**5**) has been reported to recover renal amyloidosis and collagen deposition [28] and exhibit anti-fibrotic and kidney protection activities related to renal dysfunction [29], indicating its therapeutic potential in treating CKD. Meanwhile, oxidative stress induced by free radicals play a vital role in the progression of kidney diseases such as renal fibrosis and chronic renal failure [30], which suggested that antioxidant therapy using phenolic derivatives might be a practical strategy for CKD. The chemical investigations presented here support, in part, the potent application of the phenolic extract of *H. japonicum* in the treatment of CKD.

Experimental

General: Optical rotations were recorded on a Perkin-Elmer 341 automatic polarimeter. UV-Vis spectra were collected on a Perkin-Elmer Lambda 35 UV-Vis spectrometer with λ$_{max}$ given in nanometers. IR spectra were recorded on a Perkin-Elmer Spectrum One spectrometer with ν$_{max}$ given in cm^{-1}. NMR spectra were recorded on a Bruker Ascend 400 spectrometer with chemical shifts δ in ppm. HRESIMS were measured on a Bruker Bio TOF IIIQ (quadrupole time of flight) mass spectrometer and ESIMS on a Waters Xevo TQ (tandem quadrupole) mass spectrometer. Column chromatography (CC) was performed on silica gel (Qindao Haiyang Chemical Co., Ltd., P. R. China (QHCC)), silica gel 60 (Merck, German), ODS gel (YMC Co., Ltd, Japan), or Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden). Thin layer chromatography (TLC) was conducted on plates precoated with 10 - 40 μm of silica gel GF254 from QHCC. Preparative HPLC separation was carried out on a LC3000 liquid chromatograph (Beijing Chuangxin Tongheng Science and Technology Co., Ltd) with a YMC C$_{18}$ column (20 × 250 mm; 10 μm), and semi-preparative HPLC on a Perkin-Elmer 200 liquid chromatograph with a Welch C$_{18}$ column (10 × 250 mm; 5 μm), and detected at 208 nm. All solvents were

commercially purchased and distilled under normal atmospheric pressure prior to use.

Extraction and isolation: The crude phenolic extract was provided by Chengdu Nanshan Pharmaceutical Co., Ltd. The crude extract was prepared, according to the following procedure. The whole plant material of *H. japonicum* was air-dried and extracted with 65% aqueous EtOH with refluxing. The filtrates were concentrated to remove EtOH under reduced pressure. The aqueous residue was subjected to CC on HPD100 macroporous resin eluted with H_2O, 30% and 80% EtOH, respectively. The eluents of 80% EtOH were collected and concentrated to afford the crude phenolic extract. This (350 g) was separated by CC over silica gel (160-200 mesh) eluted with light petroleum-acetone (2:1, 1:1, 0:1; v/v) to afford 15 sub-fractions S1 - S15. Fraction S3 (3.9 g) was subjected to preparative HPLC eluted with CH_3OH-H_2O (60:40, v/v; 15 mL/min) to give compound **14** (t_R = 7.6 min; 23 mg) and three sub-fractions S3B - S3D. Fraction S3B was purified with semi-preparative HPLC with CH_3CN-H_2O (31:69, v/v; 5 mL/min) as eluents to afford compound **15** (t_R = 30 min; 6 mg). Fraction S3D was separated by semi-preparative HPLC eluted with CH_3CN-H_2O (31:69, v/v; 5 mL/min) to afford compound **4** (t_R = 32 min; 70 mg). Fraction S4 was separated by preparative HPLC eluted with CH_3OH-H_2O (54:46, v/v; 20 mL/min) to give compounds **6** (t_R = 30 min; 60 mg) and **17** (t_R = 32 min; 6 mg), and four sub-fractions S4A - S4D. Fraction S4A was separated by semi-preparative HPLC eluted with CH_3OH-H_2O (38:62, v/v; 4 mL/min) to afford a sub-fraction S4A1 (t_R = 8 min) that was then subjected to CC on silica gel 60 (15 - 40 μm) eluted with $CHCl_3$-MeOH (10:1, v/v) to afford compound **13** (2 mg). Compound **5** (9.4 g) was precipitated from the light petroleum-acetone (2:1) solution of fraction S6. Compound **10** (3.8 g) was precipitated from the light petroleum-acetone (2:1) solution of fraction S7. The remaining sub-fraction of S7 was separated by CC over silica gel (160-200 mesh) with $CHCl_3$-MeOH (15:1, v/v) as solvent to give 4 sub-fractions S7A - S7D. Fraction S7B was subjected to preparative HPLC eluted with CH_3OH-H_2O (50:50, v/v; 15 mL/min) to afford compound **3** (t_R = 29 min; 63 mg) and 3 sub-fractions S7B1 - S7B3. Fraction S7B3 was separated by semi-preparative HPLC eluted with CH_3OH-H_2O (50:50, v/v; 3 mL/min) to afford subfraction S7B3C (t_R = 20 min) that was subjected to semi-preparative HPLC eluted with CH_3CN-H_2O (32:68, v/v; 3 mL/min) to give compounds **1** (t_R = 11 min; 463 mg) and **2** (t_R = 26 min; 6 mg). Compound **8** (88 mg) was crystallized from the methanol solution of fraction S9. Fraction S10 was separated over a self-packed ODS column using CH_3OH-H_2O (40:60, v/v) as solvent to give 4 sub-fractions S10A - S10D. Fraction S10C was subjected to semi-preparative HPLC with CH_3CN-H_2O (30:70, v/v; 4 mL/min) to afford compound **9** (t_R = 24 min; 26 mg) and a sub-fraction (t_R = 4 min) that was separated by semi-preparative HPLC with CH_3OH-H_2O (31:69, v/v; 3 mL/min) to afford compounds **11** (t_R = 40 min; 11 mg) and **18** (t_R = 44 min; 8 mg). Compound **7** (3.3

g) was precipitated from the methanol solution of fraction S15. The remaining sub-fraction of S15 was further subjected to CC over ODS eluted with CH_3OH-H_2O (28:72, v/v) to give a sub-fraction that was separated by semi-preparative HPLC with CH_3CN-H_2O (11:89, v/v; 5 mL/min) to afford compounds **16** (t_R = 16 min; 9 mg) and **12** (t_R = 24 min; 80 mg).

Hypericumol A [4,6-dimethyl-2-methylpropanoylphloroglucinol-1-*O*-β-D-glucopyranoside] (1)
Yellowish gum.
$[α]_D^{20}$: +79 (*c* 0.23, MeOH).
UV (MeOH) $λ_{max}$ (log ε): 290 (4.10), 329 (3.68) nm.
IR (KBr) $ν_{max}$: 3410, 2969, 2931, 2875, 1615, 1382, 1180, 1150, 1073, 1039 cm⁻¹.
¹H NMR and ¹³C NMR data: Table 1.
ESIMS (positive ion mode) *m/z*: 409.22 [M+Na]⁺, 425.12 [M+K]⁺.
ESIMS (negative ion mode) *m/z*: 385.31 [M-H]⁻, 223.19 [M-H-Glc]⁻.
HRESIMS (positive ion mode) *m/z*: 409.1480 (cacld for $C_{18}H_{26}NaO_9$, 409.1469, error -2.8 ppm).

Hypericumol B [4-methyl-2-methyl propanoylphloroglucinol-1-*O*-β-D-glucopyranoside] (2)
Yellowish gum.
$[α]_D^{20}$: -93 (*c* 0.32, MeOH).
UV (MeOH) $λ_{max}$ (log ε): 289 (4.00), 329 (3.49) nm.
IR (KBr) $ν_{max}$: 3430, 2974, 2933, 2880, 1622, 1384, 1242, 1100, 1080, 1042 cm⁻¹.
¹H NMR and ¹³C NMR data: Table 1.
ESIMS (positive ion mode) *m/z*: 395.19 [M+Na]⁺, 411.11 [M+K]⁺.
ESIMS (negative ion mode) *m/z*: 371.27 [M-H]⁻, 209.19 [M-H-Glc]⁻.
HRESIMS (positive ion mode) *m/z*: 395.1305 (cacld for $C_{17}H_{24}NaO_9$, 395.1313, error -1.9 ppm).

Hypericumol C [(2′*S*)-4,6-dimethyl-2-methylbutyrylphloroglucinol-1-*O*-β-D-glucopyranoside] (3)
Yellowish gum.
$[α]_D^{20}$: +67 (*c* 0.22, MeOH).
UV (MeOH) $λ_{max}$ (log ε): 290 (3.87), 329 (3.60) nm.
IR (KBr) $ν_{max}$: 3418, 2965, 2931, 2876, 1615, 1378, 1177, 1148, 1074, 1039 cm⁻¹.
¹H NMR and ¹³C NMR data: Table 1.
ESIMS (positive ion mode) *m/z*: 423.16 [M+Na]⁺, 439.09 [M+K]⁺.
ESIMS (negative ion mode) *m/z*: 399.25 [M-H]⁻, 237.18 [M-H-Glc]⁻.
HRESIMS (positive ion mode) *m/z*: 423.1614 (cacld for $C_{19}H_{28}NaO_9$, 423.1626, error -2.6 ppm).

References

[1]   Editorial committee of Flora of China, the Chinese Academy of Sciences. (**1990**) *Flora Reipublicae Popularis Sinicae*. Science Press, Beijing, 47.
[2]   (a) Zhang YQ, Huang MQ, Li H, Xu W, Chu KD, Zheng HY, Sha M, Chen LD. (**2012**) Hepatoprotective and antioxidant activity of the total flavonoids extraction from *Hypericum japonicum* by response surface methodology. *Latin American Journal of Pharmacy*, **31**, 1270-1278; (b) Samaga PV, Rai VR. (**2013**) Evaluation of pharmacological properties and phenolic profile of *Hypericum japonicum* Thunb. from Western Ghats of India. *Journal of Pharmacy Research*, **7**, 626-632; (c) Wang XW, Mao Y, Fan M, Ding AS, Wang NL, Yao XS. (**2009**) Isolation, identification and activity determination on the anti-hypoxic components of *Hypericum japonicum* Thunb. *Journal of Shenyang Pharmaceutical University*, **26**, 701-704.
[3]   (a) Zheng C, Liu M, Li J. (**2014**) Study on anti-HBV active ingredient of *Hypericum japonicum*. *Natural Product Research and Development*, **26**, 1350-1356; (b) Pan XJ, Yang K, Zeng JQ, Wei ZY, Chen C. (**2009**) Experimental study on anti-hepatitis B and anti-hepatoma effect of different extracts in serum of *Hypericum japonicum* Thunb. *in vitro*. *Lishizhen Medicine and Materia Medica Research*, **20**, 1076-1078; (c) Liu N, Hu XL, Meng YR, Zhu YT, Huang BS, Lin PZ. (**2008**) Effect of anti-influenza virus H3N2 of *Hypericum japonicum in vivo*. *Journal of Chinese Medicinal Materials*, **31**, 1022-1024.

[4]   (a) Zhang SD, Yin J, Li X, Zhang JG, Yue RC, Diao YY, Li HL, Wang H, Shan L, Zhang WD. (**2014**) Jacaredhyperol A induced apoptosis in leukaemia cancer cell through inhibition the activity of Bcl-2 proteins. *BMC Cancer*, **14**, 689-699; (b) Liang S, Su WW, Wang YG, Peng W, Nie YC, Li PB. (**2013**) Effect of quercetin 7-rhamnoside on glycochenodeoxycholic acid-induced L-02 human normal liver cell apoptosis. *International Journal of Molecular Medicine*, **32**, 323-330.

[5]   (a) Li XF, Fu ZR, Wei Y, Yang T, Ouyang YZ. (**2014**) Research on antibacterial activity of total flavone in *Hypericum japonicum*. *Applied Chemical Industry*, **43**, 432-434; (b) Zuo GY, An J, Han J, Zhang YL, Wang GC, Hao XY, Bian ZQ. (**2012**) Isojacareubin from the Chinese herb *Hypericum japonicum*: potent antibacterial and synergistic effects on clinical methicillin-resistant *Staphylococcus aureus* (MRSA). *International Journal of Molecular Sciences*, **13**, 8210-8218; (c) An J, Zuo GY, Hao XY, Wang GC, Li ZS. (**2011**) Antibacterial and synergy of a flavanonol rhamnoside with antibiotics against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). *Phytomedicine*, **18**, 990-993.

[6]   Wang N, Li PB, Wang YG, Peng W, Wu Z, Tian SY, Liang SL, Shen X, Su WW. (**2008**) Hepatoprotective effect of *Hypericum japonicum* extract and its fractions. *Journal of Ethnopharmacology*, **116**, 1-6.

[7]   Liu LS, Liu MH, He JY. (**2014**) *Hypericum japonicum* Thunb. ex Murray: phytochemistry, pharmacology, quality control and pharmacokinetics of an important herbal medicine. *Molecules*, **19**, 10733-10754.

[8]   (a) Wang Q, Mi J, Li XP, Liu JY, Bai XL. (**2013**) Effects of *Hypericum japonicum* total flavonoids on chronic renal failure rats induced by 5/6 nephrectomy. *Pharmacology and Clinics of Chinese Materia Medica*, **29**, 61-64; (b) Gao QL, Mei X, Yang DQ, Mi J, Liu WW, Zhao J, Zhang BC, Gao YX. (**2014**) The influence of herba *Hypericum japonicum* extract (MSN) on renal function and immune status of chronic renal failure model rats with five sixths kidney removal. *Asia-Pacific Traditional Medicine*, **10**, 7-9; (c) Liu WW, Gao YX, Zhao J, Tang SQ, Li XP. (**2014**) Research on influence of correlation genes on kidney fibrosis of 5/6 nephrectomized rats CRF model by MSN. *Pharmacy and Clinics of Chinese Materia Medica*, **5**, 28-30; (d) Zhao J, Liu WW, Yang DQ, Mei X, Li XP, Mi J, Gao YX. (**2014**) Study on interference of renal fibrosis by NSF in 5/6 nephrectomize rats. *Journal of Chengdu University of TCM*, **37**, 35-38.

[9]   (a) Luo YG, Zhou M, Ye Q, Pu Q, Zhang GL. (**2012**) Dihydroagarofuran derivatives from the dried roots of *Tripterygium wilfordii*. *Journal of Natural Products*, **75**, 98-102; (b) Luo YG, Pu X, Luo GY, Zhou M, Ye Q, Liu Y, Gu J, Qi HY, Li GY, Zhang GL. (**2014**) Nitrogen-containing dihydro-β-agarofuran derivatives from *Tripterygium wilfordii*. *Journal of Natural Products*, **77**, 1650-1657; (c) Luo GY, Yang Y, Zhou M, Ye Q, Liu Y, Gu J, Zhang GL, Luo YG. (**2014**) Novel 2-arylbenzofuran dimers and polyisoprenylated flavanones from *Sophora tonkinensis*. *Fitoterapia*, **99**, 21-27.

[10]   (a) Coskun M, Sakushima A, Nishibe S, Hisada S. (**1982**) Phloroglucinol derivatives of *Dryopteris abbreviata*. *Chemical and Pharmaceutical Bulletin*, **30**, 4102-4106; (b) Chang XL, Li W, Koike K, Wu LJ, Nikaido T. (**2006**) Phenolic constituents from the rhizomes of *Dryopteris crassirhizoma*. *Chemical and Pharmaceutical Bulletin*, **54**, 748-750.

[11]   Cheng Q, Snyder JK. (**1991**) Two new phloroglucinol derivatives with phosphodiesterase inhibitory activity from the leaves of *Eucalyptus robusta*. *Zeitschrift für Naturforschung B - A Journal of Chemical Sciences*, **46b**, 1275-1277.

[12]   Pei YH, Li X, Zhu TR. (**1989**) An empirical correlation between optical rotation and absolute configuration of optical active α-methylbutyrylphloroglucinols and its synthesis. *Acta Pharmaceutica Sinica*, **24**, 413-421.

[13]   Wang XW, Mao Y, Wang NL, Yao XS. (**2008**) A new phloroglucinol diglycoside derivative from *Hypericum japonicum* Thunb. *Molecules*, **13**, 2796-2803.

[14]   (a) Roelens F, Heldring N, Dhooge W, Bengtsson M, Comhaire F, Gustafsson JÅ, Treuter E, De Keukeleire D. (**2006**) Subtle side-chain modifications of the hop phytoestrogen 8-prenylnaringenin result in distinct agonist/antagonist activity profiles for estrogen receptors α and β. *Journal of Medicinal Chem*istry, **49**, 7357-7365; (b) Bohr G, Gerhäuser C, Knauft J, Zapp J, Becker H. (**2005**) Anti-inflammatory acylphloroglucinol derivatives from hops (*Humulus lupulus*). *Journal of Natural Products*, **68**, 1545-1548.

[15]   Mok SY, Lee S. (**2013**) Identification of flavonoids and flavonoid rhamnosides from *Rhododendron mucronulatum* for. *albiflorum* and their inhibitory activities against aldose reductase. *Food Chemistry*, **136**, 969-974.

[16]   Fu P, Li TZ, Liu RH, Zhang W, Zhang C, Zhang WD, Chen HS. (**2004**) Studies on the flavonoids of *Hypericum japonicum* Thunb. ex Murray. *Chinese Journal Natural Medicines*, **2**, 283-284.

[17]   Zhang J, Chu CJ, Li XJ, Yao S, Yan B, Ren HL, Xu NY, Liang ZT, Zhao ZZ. (**2014**) Isolation and identification of antioxidant compounds in *Vaccinium bracteatum* Thunb. by UHPLC-Q-TOF LC/MS and their kidney damage protection. *Journal of Functional Foods*, **11**, 62-70.

[18]   Wang PP, Luo J, Yang MH, Kong LY. (**2013**) Chemical constituents of *Lobelia chinensis*. *Chinese Traditional and Herbal Drugs*, **44**, 794-797.

[19]   Rho HS, Ahn SM, Lee BC, Kim MK, Ghimeray AK, Jin CW, Cho DH. (**2010**) Changes in flavonoid content and tyrosinase inhibitory activities in kenaf leaf extract after far-infrared treatment. *Bioorganic Medicinal Chemistry Letters*, **20**, 7534-7536.

[20]   (a) Cook RG, Haynes HF. (**1960**) Colouring matters of Australian plants VII. Flavonoid glycosides from *Exocarpus cupressiformis* Labill. *Australian Journal of Chemistry*, **13**, 150-155; (b) Wu W, Wang CZ, Li X, Li XR, Xu QM, Yang SL. (**2011**) Chemical constituents of antitumor active fraction of *Lysimachia clethroides*. *Chinese Traditional and Herbal Drugs*, **42**, 38-41.

[21]   Ishiguro K, Nagata S, Fukumoto H, Yamaki M, Takagi S, Isoi K. (**1991**) A flavanonol rhamnoside from *Hypericum japonicum*. *Phytochemistry*, **30**, 3152-3153.

[22]   Zheng JX, Wang NL, Chen HF, Yao XS. (**2007**) Isolation and identification of phenolic constituents from *Selaginella uncinata* (Desv.) Spring. *Chinese Journal of Medicinal Chemistry*, **17**, 302-305.

[23]   Qiu YK, Dou DQ, Pei YP, Youshikawa M, Matsuda H, Chen YJ. (**2005**) Chemical constituents of *Opuntia dillenii*. *Journal of China Pharmaceutical University*, **36**, 213-215.

[24]   Sun ZH, Zhang CF, Zhang M. (**2010**) A new benzoic acid derivative from *Eclipta prostrata*. *Chinese Journal of Natural Medicines*, **8**, 244-246.

[25]   Shang XY, Li S, Wang YH, Wang SJ, Yang YC, Shi JG. (**2007**) Dihydroflavonol glycosides and flavan-3-ols from *Bauhinia aurea*. *China Journal of Chinese Materia Medica*, **32**, 815-818.

[26]   Ishiguro K, Nagata S, Fukumoto H, Yamaki M, Isoi K, Yamagata Y. (**1994**) A 2-pyrone derivative from *Hypericum japonicum*. *Phytochemistry*, **37**, 283-284.

[27]   Wu QL, Wang SP, Du LJ, Zhang SM, Yang JS, Xiao PG. (**1998**) Chromone glycosides and flavonoids from *Hypericum japonicum*. *Phytochemistry*, **49**, 1417-1420.

[28]   Hsieh CL, Peng CC, Chen KC, Peng RY. (**2013**) Rutin (quercetin rutinoside) induced protein-energy malnutrition in chronic kidney disease, but quercetin acted beneficially. *Journal of Agriculture and Food Chemistry*, **61**, 7258-7267.

[29]   (a) Hu Q, Noor M, Wong YF, Hylands PJ, Simmonds MSJ, Xu Q, Jiang D, Hendry BM, Xu QH. (**2009**) *In vitro* anti-fibrotic activities of herbal compounds and herbs. *Nephrology Dialysis Transplantation*, **24**, 3033-3041; (b) Satyanarayana PSV, Singh D, Chopra S. (**2001**) Quercetin, a bioflavonoid, protects against oxidative stress-related renal dysfunction by cyclosporine in rats. *Methods and Findings in Experimental and Clinical Pharmacology*, **23**, 175-181.

[30]   (a) Rodrigo R, Rivera G. (**2002**) Renal damage mediated by oxidative stress: a hypothesis of protective effects of red wine. *Free Radical Biology and Medicine*, **33**, 409-422; (b) Qin J, Tao LJ. (**2014**) Progress in study on reactive oxygen species and renal fibrosis. *Journal of Clinical and Pathological Research*, **34**, 182-189.

Synthesis and Anti-Proliferative Effects of Quercetin Derivatives

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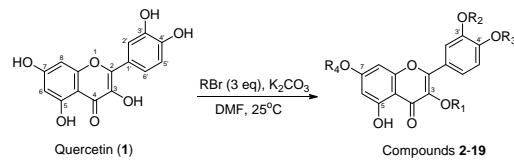
Prostate cancer is the most common diagnosed invasive cancer in American men and is the second leading cause of cancer-related deaths. Although there are several therapies successful in treating early, localized stage prostate cancer, current treatment of advanced metastatic castration-resistant prostate cancer remains ineffective due to inevitable progression of resistance to first-line treatment with docetaxel. The natural product quercetin (3,3',4',5,7-pentahydroxyflavone), a flavonoid compound ubiquitous in dietary plants, possesses evidenced potential in treating advanced metastatic castration-resistant prostate cancer. However, its poor bioavailability and moderate potency hinder its advancement into clinical therapy. In order to engineer quercetin derivatives with improved potency and pharmacokinetic profiles for the treatment of advanced metastatic prostate cancer, we started this study with creating a small library of alkylated derivatives of quercetin for in vitro evaluation. The biological data and chemical reactivity of quercetin and its derivatives reported in literature directed us to design 3,4',7-O-trialkylquercetins as our first batch of targets. Consequently, nine 3,4',7-O-trialkylquercetins, together with four 3,7-O-dialkylquercetins, four 3,3',4',7-tetraalkylquercetins, and one 3,3',4'-O-trialkylquercetin, were prepared by one step O-alkylation of commercially available quercetin mediated by potassium carbonate. Their structures were determined by 1D and 2D NMR data, and HRMS. Their anti-proliferative activities towards both androgen-refractory and androgen-sensitive prostate cancer cells were evaluated using WST-1 cell proliferation assay. The acquired structure-activity relationships indicate that 3,7-O-dialkylquercetins rather than 3,4',7-O-trialkylquercetins were much more potent than quercetin towards prostate cancer cells.

Keywords: Quercetin, Dietary natural product, Prostate cancer, Derivatives, Cell proliferation.

Prostate cancer, the most common diagnosed invasive cancer in American men, is the second leading cause of cancer-related deaths. Even though most patients can recover from localized androgen-sensitive prostate cancer after an appropriate therapy, the prostate cancer will unfortunately relapse in around 2-3 years after recovery from the early, androgen-sensitive stage [1]. At this point, prostate cancer is no longer responding to hormone therapy and/or has metastasized to different areas of the body. This was referred to as Hormone-Refractory Prostate Cancer or Androgen-Independent Prostate Cancer, and recently was designated as Castration-Resistant Prostate Cancer (CRPC). The US FDA did approve numerous drugs for the treatment of both androgen-dependent and castration-resistant prostate cancer. Sadly, there are no FDA-approved drugs that can actually cure advanced metastatic CRPC. The current first-line chemotherapy for CRPC, docetaxel, can barely increase survival by an average of 2.4 years [2]. The patients' inevitable progression of resistance to docetaxel makes it become an even less effective chemotherapeutic for advanced metastatic CRPC. Very recently, FDA approved cabazitaxel as a second-line treatment for the patients refractory to the first-line docetaxel chemotherapy due to the fact that carbazitaxel is the only available chemotherapeutic that can improve survival (even though by only 2.4 months) for docetaxel-resistant patients [3]. There is thus an urgent need to search for effective chemotherapeutics for clinical treatment of advanced metastatic castration-resistant prostate cancer.

Quercetin, 3,3',4',5,7-pentahydroxyflavone (1 in Scheme 1), is a flavonoid compound ubiquitous in plant food sources [4]. Quercetin was capable of inhibiting cell growth in the mouse prostate cancer cell line TRAMP-C2 with an IC$_{50}$ of 20 μM [5-6]. Numerous studies demonstrated that quercetin was also capable of suppressing

Scheme 1: Synthesis of alkylated derivatives of quercetin.

Table 1: Alkyl groups for compounds 2-19.

Compds	R_1	R_2	R_3	R_4
2	Methyl	H	H	Methyl
3	Methyl	H	Methyl	Methyl
4	Methyl	Methyl	Methyl	Methyl
5	Ethyl	H	H	Ethyl
6	Ethyl	H	Ethyl	Ethyl
7	Ethyl	Ethyl	Ethyl	Ethyl
8	Ethyl	Ethyl	Ethyl	H
9	Propyl	H	H	Propyl
10	Propyl	H	Propyl	Propyl
11	Butyl	H	H	Butyl
12	Butyl	H	Butyl	Butyl
13	Butyl	Butyl	Butyl	Butyl
14	Pentyl	H	Pentyl	Pentyl
15	Hexyl	H	Hexyl	Hexyl
16	Isopropyl	H	Isopropyl	Isopropyl
17	Isopentyl	H	Isopentyl	Isopentyl
18	Benzyl	H	Benzyl	Benzyl
19	Benzyl	Benzyl	Benzyl	Benzyl

the growth of both androgen-sensitive (LNCaP) and androgen-refractory human prostate cancer cell lines (PC-3 and DU145) and that quercetin has no apparent toxicity against normal prostate epithelial cells [7-8]. Maggiolini and co-workers first demonstrated that quercetin functioned as agonists for the mutant androgen receptor (AR) T877A so that it could inhibit the proliferation of LNCaP cells at low concentration. However, high concentration of quercetin may independently cause significant cytotoxicity without involving hormone receptor expression [9].

Wang *et al.* reported that quercetin exhibited potential *in vivo* anti-prostate tumor efficacy in an androgen-sensitive LAPC-4 xenograft prostate tumor mouse model using severe combined immunodeficiency (SCID) mice. It inhibited tumor growth by 3% when 0.2% was administered as a supplement in diet and by 15% when 0.4% was taken [10]. Quercetin can also significantly reduce PC-3 tumor volume and weight in 6-week old BALB/cA nude mice. The average weight of prostate tumors decreased from 0.242 g for the control group to 0.099 g for the quercetin treated group, implying the *in vivo* anti-prostate tumor potential of quercetin [11]. The above-described cell-based and animal-based studies have evidenced that quercetin could be a good candidate for prostate cancer treatment. However, its poor bioavailability and moderate potency hinder its advancement into clinical therapy. In order to engineer quercetin derivatives with improved potency and a better pharmacokinetic profile for the treatment of advanced metastatic prostate cancer, we started this project by creating a small panel of alkylated derivatives of quercetin for *in vitro* evaluation. The structure-activity relationships will be used to direct our further structure modulation.

The structure-cytotoxicity relationships of methylquercetins summarized by Beutler *et al.* [12] showed that 3,4',7-trimethylquercetin possessed better cytotoxicity than quercetin and other methylquercetins. Intriguingly, Rao *et al.* [13] summarized that methylation of the five phenolic hydroxyl groups in quercetin occurred gradually with the following sequential positions order: 4'

> 7 > 3 > 3' > 5. The superposition of cytotoxic potency and chemical reactivity of quercetin derivatives directed us to synthesize a group of new 3,4',7-*O*-trialkylquercetins as our first batch of targets. Different alkyl groups were introduced to investigate the effect of their length and steric hindrance on the cell proliferation. The targets were proposed to be achieved by one-step alkylation of quercetin.

As shown in Scheme 1, we planned to synthesize our expected 3, 4',7-*O*-trialkylquercetins by treating one equivalent of quercetin with three equivalents of alkyl halide using DMF as solvent and potassium carbonate as base. Reaction of quercetin with the appropriate alkyl halide did provide us with the expected 3,4',7-*O*-trialkylquercetins as the major products, but in moderate yields (less than 25%). Assignments of three alkyl groups were based on the key HMBC correlations between CH$_2$ protons of ethyl groups (δ 4.10, 4.10, and 4.22) and C-3, C-7, and C-4' in derivative **6** as summarized in Figure 1. Four 3,7-*O*-dialkylquercetins, four 3,3',4',7-*O*-tetraalkylquercetins, and one 3,3',4'-*O*-trialkylquercetin were also collected for structure-activity relationship studies. Similarly, the locations of the alkyl groups in 3,7-*O*-dibutylquercetin (**11**) and in 3,3',4'-*O*-triethylquercetin (**8**) were also confirmed by the existence of key correlations in their HMBC spectra (Figure 1). As shown in Table 2, all signals in the ^{1}H- and ^{13}C-NMR spectra for compounds **6**, **8**, and **11** have been fully assigned based on the extensive interpretation of their COSY, HMQC, and HMBC data.
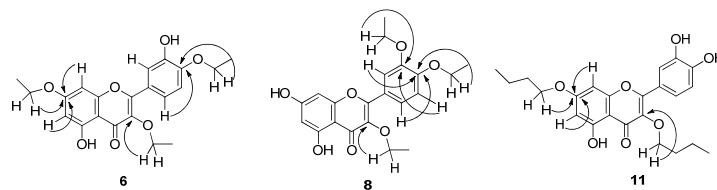
Figure 1: Key HMBC correlations in compounds **6**, **8**, and **11**.

Table 2: NMR data for derivatives **6**, **8**, and **11** (^{1}H NMR: 300 MHz; ^{13}C NMR: 75 MHz).

Position	6 (CDCl₃)		8 (acetone-d₆)		11 (acetone-d₆)	
	δ$_C$, type	δ$_H$, *J* in Hz	δ$_C$, type	δ$_H$, (*J* in Hz)	δ$_C$, type	δ$_H$, (*J* in Hz)
2	156.5, C		155.7, C		156.3, C	
3	138.7, C		137.5, C		137.7, C	
4	179.6, C		178.7, C		178.8, C	
4a	106.6, C		104.9, C		105.6 C	
5	162.6, C		162.3, C		162.0, C	
6	98.8, CH	6.33, d (1.8)	98.6, CH	6.26, s	97.9, CH	6.30, d (2.1)
7	165.4, C		164.3, C		165.1, C	
8	93.1, CH	6.42, d (1.8)	93.7, CH	6.51, s	92.2, CH	6.62, d (2.1)
8a	157.4, C		157.8, C		156.8, C	
1'	124.3, C		123.0, C		122.3, C	
2'	115.1, CH	7.72, br.s	113.8, CH	7.79, d (1.8)	115.7, CH	7.74, d (2.1)
3'	146.1, C		148.4, C		144.9, C	
4'	148.6, C		151.4, C		148.1, C	
5'	111.6, CH	6.94, d (9.3)	112.6, CH	7.12, d (8.4)	115.2, CH	7.00, d (8.4)
6'	122.2, CH	7.73, dd (9.3, 1.8)	122.2, CH	7.74, dd (8.4, 1.8)	121.4, CH	7.61, dd (8.4, 2.1)
3-O-CH₂	64.8, CH₂	4.10, q (6.9)	67.9, CH₂	4.14, overlapped	-	-
CH₃	15.2, CH₃	1.45, t (6.9)	14.3, CH₃	1.32, t (6.9)	-	-
7-O-CH₂	65.3, CH₂	4.10, q (6.9)	-	-	-	-
CH₃	15.4, CH₃	1.51, t (6.9)	-	-	-	-
7-O-CH₂	-	-	-	-	68.3, CH₂	4.14, t (6.6)
CH₂	-	-	-	-	30.9, CH₂	1.85-1.67, m
CH₂	-	-	-	-	18.9, CH₂	1.56-1.42, m
CH₃	-	-	-	-	13.15 CH₃	0.92, t (7.2)
3'-O-CH₂	-	-	64.1, CH₂	4.17, overlapped	-	-
CH₃	-	-	14.2, CH₃	1.43, t (6.9)	-	-
4'-O-CH₂	69.2, CH₂	4.22, q (6.9)	64.4, CH₂	4.18, overlapped	-	-
CH₃	16.2, CH₃	1.36, t (6.9)	14.3, CH₃	1.43, t (6.9)	-	-
3-O-CH₂	-	-	-	-	71.9, CH₂	4.05, t (6.6)
CH₂	-	-	-	-	31.9, CH₂	1.85-1.67, m
CH₂	-	-	-	-	18.9, CH₂	1.56-1.42, m
CH₃	-	-	-	-	13.19 CH₃	0.99, t (7.2)
3'-OH	-	5.81, s	-		-	8.50, brs
5-OH	OH	12.68, s		12.81, s		12.80, s
4'-OH	-	-	-	-	-	8.50, brs

Among the eighteen quercetin derivatives that we prepared, five of them (**8**, **14-17**) are new compounds. Derivatives **2-7**, **9-13**, and **18-19** are known, but there are no reports available on their anti-proliferative activity against prostate cancer cell lines, even though their *in vitro* effects have been investigated on other cancer cell models [12, 14]. It should be pointed out that the 4',7-dialkylquercetins reported in Ref. [14] should be corrected as 3,7-dialkylquercetins based on our HMBC data of **11** (Figure 1).

The anti-proliferative effect of eighteen synthetic quercetin derivatives (at 50 µM) was evaluated against both androgen-sensitive (LNCaP) and androgen-refractory (DU145 and PC-3) human prostate cancer cell lines. WST-1 cell proliferation assay was selected for this evaluation because of its easy operation in a microtiter plate without washing steps. The procedure is described in the Experimental Section and the absorbance was measured using a microplate reader (Synergy HT) at a wavelength of 430 nm. As shown in Table 3, alkylation of the hydroxyl groups at C-3, C-4', and C-7 in quercetin with short, linear alkyl groups (such as methyl groups in **3** and ethyl groups in **6**) slightly increases the anti-proliferative activity towards three human prostate cancer cell lines. However, potency was rapidly diminished when either branched alkyl groups (isopropyl in **16** and isopentyl in **17**) or linear lengthy groups (propyl in **10**, butyl in **12**, pentyl in **14**, and hexyl in **15**) were introduced into these three positions. Incorporation of ethyl groups to 3,3',4'-hydroxyl groups in quercetin (**8**) led to neither gain nor loss in its inhibitory rate. Surprisingly, all four 3,7-*O*-dialkylquercetins (**2**, **5**, **9**, and **11**) exhibited significantly higher inhibitory rate than quercetin against the three prostate cancer cell lines at both concentrations. They were over 2-11 times more potent than quercetin in the cell culture based on the comparison of their IC$_{50}$ values with that of quercetin (Table 3).

Table 3: Anti-proliferative activity of quercetin and its derivatives towards three cancer cell lines.

Compds	Inhibitory rate (%) @ 50 µM			IC$_{50}$ (µM)		
	PC-3	DU145	LNCaP	PC-3	DU145	LNCaP
Quercetin	8	13	44	>100	>100	45.46 ± 1.31
2	64	40	80	22.27 ± 6.83	46.82 ± 3.69	13.23 ± 4.75
3	66	36	77	–	–	–
4	10	25	39	–	–	–
5	79	80	91	13.74 ± 1.62	12.59 ± 0.96	4.20 ± 0.96
6	25	32	50	–	–	–
7	0	16	9	–	–	–
8	6	8	48	–	–	–
9	95	91	96	17.68 ± 0.96	19.25 ± 2.21	6.42 ± 2.72
10	41	29	50	–	–	–
11	97	97	97	11.95 ± 1.12	18.63 ± 6.86	6.46 ± 1.10
12	0	0	0	–	–	–
13	0	0	0	–	–	–
14	0	19	7	–	–	–
15	0	5	0	–	–	–
16	25	30	40	–	–	–
17	3	13	0	–	–	–
18	0	19	11	–	–	–
19	0	3	16	–	–	–

Each experiment was performed at least thrice in duplicate.

In conclusion, eighteen alkylated derivatives of quercetin were prepared by a semi-synthesized approach. Their anti-proliferative activity was evaluated against three human prostate cancer cell lines using WST-1 cell proliferation assay. The structure-activity relationships acquired show i) that simultaneous introduction of three bulky or lengthy alkyl groups to C-3, C-4' and C-7 hydroxyl groups of quercetin led to a major loss of anti-proliferative activity in prostate cancer cells; and ii) that incorporation of two methyl groups in **2**, two ethyl groups in **5**, two propyl groups in **9**, and two butyl groups in **11** to C-3 and C-7 hydroxyl groups resulted in much more potent derivatives. Chemical manipulation of one phenolic hydroxyl group might be the best direction for the future.

Experimental

General: NMR spectra were obtained on a Bruker Fourier 300 spectrometer in CDCl$_3$ and CD$_3$COCD$_3$. The chemical shifts are given in δ (ppm) referenced to the respective solvent peak, and coupling constants are reported in Hz. All reagents and solvents were purchased from commercial sources and were used without further purification. Column chromatography was performed using silica gel (32-63 µm). Preparative thin-layer chromatography (PTLC) separations were carried out on 1000 µm AnalTech thin layer chromatography plates (Lot No.13401).

General procedure for the synthesis of alkylated quercetin derivatives: To a solution of quercetin hydrate (1.0 equiv.) in DMF was added anhydrous K$_2$CO$_3$ (3 equiv.), followed by the appropriate alkyl halide (3.0 equiv.). The reaction mixture was allowed to stir at room temperature for 12 to 48 h prior to being diluted with diethyl ether and ethyl acetate (300 mL in total, 1:1, v/v). The consequent mixture was rinsed with brine (30 mL × 5), and the organic layer was dried over anhydrous MgSO$_4$, filtered and concentrated *in vacuo* to give the corresponding crude product.

Preparation of methylated derivatives of quercetin (2, 3, and 4): These derivatives were prepared according to the general procedure from quercetin hydrate (320 mg, 1 mmol) in DMF (1 mL). PTLC purification of crude product, using 30% ethyl acetate in hexane as eluent, yielded compounds **2** (24 mg, 7% yield), **3** (83 mg, 24% yield), and **4** (21 mg, 6% yield).

3,7-*O*-Dimethylquercetin (2)
MP: 180-183ºC.
IR (neat): 3244, 2923, 2852, 1653, 1605, 1559, 1506, 1456 cm^{-1}.
^{1}H NMR (300 MHz, CD$_3$COCD$_3$): 12.77 (s, 1H), 7.73 (d, *J* = 2.1 Hz, 1H), 7.61 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.00 (d, *J* = 8.4 Hz, 1H), 6.64 (d, *J* = 2.4 Hz, 1H), 6.31 (d, *J* = 2.4 Hz, 1H), 3.93 (s, 3H), 3.88 (s, 3H).
^{13}C NMR (75 MHz, CDCl$_3$): 178.7, 165.7, 161.9, 156.8, 156.1, 148.3, 145.0, 138.6, 122.1, 121.3, 115.5, 115.4, 105.7, 97.6, 91.9, 59.5, 55.5.

3,4',7-*O*-Trimethylquercetin (3)
MP: 151-153ºC.
IR (neat): 3396, 2929, 1652, 1592, 1494 cm^{-1}.
^{1}H NMR (300 MHz, CDCl$_3$): 12.64 (s, 1H), 7.73 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.70 (d, *J* = 2.1 Hz, 1H), 6.98 (d, *J* = 8.7 Hz, 1H), 6.46 (d, *J* = 2.1 Hz, 1H), 6.37 (d, *J* = 2.1 Hz, 1H), 5.73 (s, 1H), 4.00 (s, 3H), 3.89 (s, 6H).
^{13}C NMR (75 MHz, CDCl$_3$): 179.5, 166.1, 162.6, 157.4, 156.2, 149.4, 146.2, 139.8, 124.3, 122.2, 115.0, 111.0, 106.7, 98.5, 92.8, 60.8, 56.7, 56.4.

3,3',4',7-*O*-Tetramethylquercetin (4)
MP: 136-137ºC.
IR (KBr): 3580, 2933, 2839, 1652, 1589, 1496 cm^{-1}.
^{1}H NMR (300 MHz, CDCl$_3$): 12.64 (s, 1H), 7.73 (dd, *J* = 8.7, 1.8 Hz, 1H), 7.70 (s, 1H), 7.00 (d, *J* = 8.7 Hz, 1H), 6.45 (d, *J* = 1.8 Hz, 1H), 6.36 (d, *J* = 1.8 Hz, 1H), 3.98 (s, 6H), 3.88 (s, 3H), 3.87 (s, 3H).
^{13}C NMR (75 MHz, CDCl$_3$): 179.4, 166.1, 162.7, 157.4, 156.5, 152.0, 149.4, 139.6, 123.6, 122.8, 111.9, 111.5, 106.7, 98.5, 92.9, 60.8, 56.7, 56.6, 56.5.

Preparation of ethylated quercetin derivatives (5, 6, 7, and 8): These derivatives were synthesized from quercetin hydrate (640 mg, 2 mmol) in DMF (2 mL). The crude product was purified through a

pad of silica gel, eluting with 33% ethyl acetate in hexanes, followed by repetitive PTLC, eluting with 33% ethyl acetate in hexanes and 5% methanol in DCM in hexane, to yield compounds **5** (56 mg, 8% yield), **6** (137 mg, 18% yield), **7** (57 mg, 7% yield), and **8** (15 mg, 2% yield).

3,7-*O*-Diethylquercetin (5)

MP: 155-156ºC.

IR (KBr): 3210, 2980, 2929, 1654, 1590, 1496, 1339, 1206 cm^{-1}.

^{1}H NMR (300 MHz, CD$_3$COCD$_3$): 12.76 (s, 1H), 8.50 (br.s, 2H), 7.76 (d, J = 2.1 Hz, 1H), 7.68 (dd, J = 8.4, 2.1 Hz, 1H), 7.00 (d, J = 8.4 Hz, 1H), 6.55 (d, J = 2.1 Hz, 1H), 6.24 (d, J = 2.1 Hz, 1H), 4.15 (q, J = 6.9 Hz, 2H), 4.11 (q, J = 6.9 Hz, 2H), 1.40 (t, J = 6.9 Hz, 3H), 1.32 (t, J = 6.9 Hz, 3H).

^{13}C NMR (75 MHz, CD$_3$COCD$_3$): 181.4, 167.5, 164.5, 159.4, 158.8, 150.7, 147.5, 140.1, 124.9, 124.0, 118.2, 117.9, 108.1, 100.5, 94.8, 70.4, 66.7, 17.5, 16.6.

3,4',7-*O*-Triethylquercetin (6)

MP: 123-124ºC.

IR (neat): 3397, 2980, 2931, 2887, 1654, 1592, 1497 cm^{-1}.

^{1}H and ^{13}C NMR: see Table 3.

HRMS-FAB: *m/z* [M + H]$^+$ calcd for C$_{21}$H$_{23}$O$_7$: 387.1444; found: 387.1397; [M + Na]$^+$ calcd for C$_{21}$H$_{22}$O$_7$Na: 409.1258; found: 409.1261.

3,3',4',7-*O*-Tetraethylquercetin (7)

MP: 121-122ºC.

IR (neat): 2981, 2932, 1654, 1589, 1497 cm^{-1}.

^{1}H NMR (300 MHz, CDCl$_3$): 12.67 (s, 1H), 7.74 (d, J = 1.8 Hz, 1H), 7.71 (dd, J = 8.7, 1.8 Hz, 1H), 6.96 (d, J = 8.7 Hz, 1H), 6.40 (d, J = 2.1 Hz, 1H), 6.31 (d, J = 2.1 Hz, 1H), 4.18 (q, J = 6.9 Hz, 2H), 4.16 (q, J = 6.9 Hz, 2H), 4.08 (q, J = 6.9 Hz, 2H), 4.06 (q, J = 6.9 Hz, 2H), 1.500 (t, J = 6.9 Hz, 3H), 1.498 (t, J = 6.9 Hz, 3H), 1.44 (t, J = 6.9 Hz, 3H), 1.33 (t, J = 6.9 Hz, 3H).

^{13}C NMR (75 MHz, CDCl$_3$): 180.8, 166.7, 163.9, 158.6, 158.1, 153.0, 150.0, 139.8, 124.9, 124.0, 115.5, 114.1, 107.8, 100.0, 94.4, 70.4, 66.7, 66.4, 66.1, 17.6, 16.7, 16.6, 16.5.

3, 3',4'-*O*-Triethylquercetin (8)

MP: 170-172ºC.

IR (neat): 3210, 3084, 2978, 2925, 1649, 1600, 1576, 1499, 1170 cm^{-1}.

^{1}H and ^{13}C NMR: see Table 3.

Preparation of propylated derivatives of quercetin (9 and 10):

These derivatives were synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (0.6 mL). The crude product was purified through a pad of silica gel, eluting with 33% ethyl acetate in hexanes, followed by repetitive PTLC, eluting with 33% ethyl acetate in hexanes, to yield compounds **9** (9 mg, 4% yield) and **10** (9 mg, 3.6 % yield).

3,7-*O*-Dipropylquercetin (9)

MP: 143-144ºC.

IR (neat): 3352, 2965, 2878, 1654, 1590, 1496, 1205 cm^{-1}.

^{1}H NMR (300 MHz, CD$_3$COCD$_3$): 12.80 (s, 1H), 7.74 (d, J = 2.1 Hz, 1H), 7.61 (dd, J = 8.4, 2.1 Hz, 1H), 7.00 (d, J = 8.4 Hz, 1H), 6.62 (d, J = 2.1 Hz, 1H), 6.30 (d, J = 2.1 Hz, 1H), 4.09 (t, J = 6.6 Hz, 2H), 4.00 (t, J = 6.6 Hz, 2H), 1.84-1.71 (m, 4H), 1.05 (t, J = 7.2 Hz, 3H), 0.97 (t, J = 7.2 Hz, 3H).

^{13}C NMR (75 MHz, CD$_3$COCD$_3$): 178.8, 165.1, 162.0, 156.8, 156.3, 148.2, 145.0, 137.7, 122.3, 121.4, 115.7, 115.2, 105.6, 97.9, 92.3, 73.8, 70.0, 23.1, 22.1, 9.9, 9.7.

3,4',7-*O*-Tripropylquercetin (10)

MP: 80-81ºC.

IR (neat): 3320, 2965, 2877, 1654, 1597, 1497, 1207 cm^{-1}.

^{1}H NMR (300 MHz, CDCl$_3$): 12.69 (s, 1H), 7.73 (dd, J = 8.4, 2.1 Hz, 1H), 7.72 (s, 1H), 6.95 (d, J = 8.7 Hz, 1H), 6.45 (d, J = 2.1 Hz, 1H), 6.42 (d, J = 2.1 Hz, 1H), 5.73 (s, 1H), 4.12 (t, J = 6.6 Hz, 2H), 4.00 (t, J = 6.6 Hz, 2H), 3.98 (t, J = 6.6 Hz, 2H), 1.98-1.72 (m, 6H), 1.10 (t, J = 7.5 Hz, 3H), 1.07 (t, J = 7.5 Hz, 3H), 0.98 (t, J = 7.5 Hz, 3H).

^{13}C NMR (75 MHz, CDCl$_3$):179.0, 165.0, 161.9, 156.8, 155.7, 148.1, 145.5, 138.3, 123.7, 121.7, 114.5, 110.9, 106.0, 98.2, 92.5, 74.5, 70.5, 70.1, 23.4, 22.5, 22.3, 10.5, 10.43, 10.41.

Preparation of butylated derivatives of quercetin (11, 12, and 13):

These derivatives were synthesized from quercetin hydrate (426 mg, 1.26 mmol) in DMF (1.3 mL). The crude product was purified through a pad of silica gel, eluting with 33% ethyl acetate in hexanes, followed by repetitive PTLC, eluting with 33% ethyl acetate in hexanes, to yield compounds **11** (22 mg, 4% yield), **12** (42 mg, 7% yield), and **13** (16 mg, 2.4% yield).

3,7-*O*-Dibutylquercetin (11)

MP: 158-159ºC.

IR (KBr): 3210, 2958, 2931, 2873, 1656, 1586, 1492, 1169 cm^{-1}.

^{1}H and ^{13}C NMR: see Table 3.

3,4',7-*O*-Tributylquercetin (12)

MP: 66-68ºC.

IR (neat): 3431, 2958, 2933, 2872, 1655, 1597, 1497, 1206 cm^{-1}.

^{1}H NMR (300 MHz, CDCl$_3$): 12.69 (s, 1H), 7.71 (d, J = 9.3 Hz, 1H), 7.69 (s, 1H), 6.93 (d, J = 9.3 Hz, 1H), 6.42 (d, J = 1.8 Hz, 1H), 6.32 (d, J = 2.1 Hz, 1H), 5.81 (br.s, 1H), 4.14 (t, J = 6.6 Hz, 2H), 4.02 (t, J = 6.6 Hz, 2H), 4.00 (t, J = 6.6 Hz, 2H), 1.91-1.69 (m, 6H), 1.60-1.38 (m, 6H), 1.02 (t, J = 7.2 Hz, 3H), 1.00 (t, J = 7.2 Hz, 3H), 0.93 (t, J = 7.2 Hz, 3H).

^{13}C NMR (75 MHz, CDCl$_3$): 178.9, 165.0, 161.9, 156.8, 155.8, 148.1, 145.5, 138.3, 123.6, 121.6, 114.5, 110.9, 105.9, 98.2, 92.4, 72.6, 68.8, 68.4, 32.1, 31.1, 31.0, 19.21, 19.16, 19.12, 13.84, 13.83, 13.79.

3,3',4',7-*O*-Tetrabutylquercetin (13)

MP: 59-60ºC.

IR (neat): 2957, 2933, 2873, 1661, 1589, 1500 cm^{-1}.

^{1}H NMR (300 MHz, CDCl$_3$): 12.71 (s, 1H), 7.73 (d, J = 2.1 Hz, 1H), 7.69 (dd, J = 8.4, 2.1 Hz, 1H), 6.98 (d, J = 8.4 Hz, 1H), 6.44 (d, J = 2.1 Hz, 1H), 6.35 (d, J = 2.1 Hz, 1H), 4.11 (t, J = 6.6 Hz, 2H), 4.09 (t, J = 6.6 Hz, 2H), 4.04 (t, J = 6.6 Hz, 2H), 4.00 (t, J = 6.6 Hz, 2H), 1.92-1.68 (m, 8H), 1.63-1.40 (m, 8H), 1.02 (t, J = 7.2 Hz, 6 H), 1.00 (t, J = 7.2 Hz, 3 H), 0.92 (t, J = 7.2 Hz, 3H).

^{13}C NMR (75 MHz, CDCl$_3$): 178.9, 164.9, 162.0, 156.8, 156.2, 151.6, 148.5, 130.3, 123.0, 122.2, 114.1, 112.5, 105.9, 98.1, 92.6, 72.8, 69.2, 68.7, 68.4, 32.3, 31.3, 31.2, 31.0, 19.24, 19.17, 19.15, 13.89, 13.85, 13.79.

Preparation of 3,4',7-O-tripentylquercetin (14): This derivative

was synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (10 mL). The crude product was subjected to PTLC purification eluting with 15% ethyl acetate in hexanes to yield compound **14** (19 mg, 7% yield).

3,4',7-*O*-Tripentylquercetin (14)

MP: 55-56ºC.

IR (KBr): 3540, 2956, 2871, 1654, 1593, 1496 cm^{-1}.

^{1}H NMR (300 MHz, CDCl$_3$): 12.69 (s, 1H), 7.71 (dd, J = 9.3, 2.1 Hz, 1H), 7.70 (d, J = 2.1 Hz, 1H), 6.94 (d, J = 9.3 Hz, 1H), 6.43 (d, J = 2.1 Hz, 1H), 6.33 (d, J = 2.1 Hz, 1H), 5.77 (s, 1H), 4.14 (t, J = 6.6 Hz, 2H), 4.02 (t, J = 6.6 Hz, 2H), 4.00 (t, J = 6.6 Hz, 2H), 1.91-1.73 (m, 6H), 1.51 - 1.31 (m, 12H), 0.99-0.93 (overlapped, 6H), 0.90 (t, J = 6.6 Hz, 3H).
^{13}C NMR (75 MHz, CDCl$_3$): 179.0, 165.0, 161.9, 156.8, 155.8, 148.1, 145.5, 138.3, 123.6, 121.6, 114.5, 110.9, 105.9, 98.2, 92.5, 73.0, 69.1, 68.7, 29.8, 28.8, 28.7, 28.12, 28.08, 28.06, 22.45, 22.43, 22.40, 13.4.

Preparation of 3,4',7-O-trihexylquercetin (15): This derivative was synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (10 mL). The crude product was subjected to PTLC purification eluting with 15% ethyl acetate in hexanes to yield compound **15** (20 mg, 6% yield).

3,4',7-*O*-Trihexylquercetin (15)

MP: 48-50ºC.
IR (KBr): 3550, 2954, 2929, 2858, 1654, 1595, 1497 cm^{-1}.
^{1}H NMR (300 MHz, CDCl$_3$): 12.67 (s, 1H), 7.69 (d, J = 7.5 Hz, 1H), 7.68 (s, 1H), 6.93 (d, J = 7.5 Hz, 1H), 6.42 (d, J = 2.1 Hz, 1H), 6.32 (d, J = 2.1 Hz, 1H), 5.73 (s, 1H), 4.12 (t, J = 6.6 Hz, 2H), 4.01 (t, J = 6.6 Hz, 2H), 3.99 (t, J = 6.6 Hz, 2H), 1.89 – 1.70 (m, 6H), 1.42 – 1.20 (m, 18H), 0.81-0.98 (m, 9H).
^{13}C NMR (75 MHz, CDCl$_3$): 179.1, 165.1, 162.1, 156.9, 156.0, 148.2, 145.6, 138.4, 123.8, 121.8, 114.6, 111.0, 106.1, 98.4, 92.6, 73.2, 69.2, 68.8, 31.74, 31.65, 30.2, 29.2, 29.1, 25.7, 22.7, 14.2.
HRMS-FAB: *m/z* [M + Na]$^+$ calcd for C$_{33}$H$_{46}$O$_7$Na: 577.3136; found: 577.3127.

Preparation of 3,4',7-O-triisopropylquercetin (16): This derivative was synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (10 mL). PTLC purification of the crude product eluting with 15% ethyl acetate in hexanes generated compound **16** (18 mg, 7% yield).

3,4',7-*O*-Triisopropylquercetin (16)

MP: 62-64ºC.
IR (KBr): 3428, 2976, 2932, 1652, 1589, 1492 cm^{-1}.
^{1}H NMR (300 MHz, CDCl$_3$): 12.73 (s, 1H), 6.76 (d, J = 8.6 Hz, 1H), 7.74 (s, 1H), 6.95 (d, J = 8.6 Hz, 1H), 6.42 (s, 1H), 6.33 (s, 1H), 5.78 (s, 1H), 4.79-4.69 (m, 1H), 4.69-4.54 (m, 2H), 1.45 (d, J = 7.0 Hz, 6H), 1.40 (d, J = 7.0 Hz, 6H), 1.23 (d, J = 7.0 Hz, 6H).
^{13}C NMR (75 MHz, CDCl$_3$): 179.4, 164.0, 162.1, 157.0, 156.5, 146.8, 146.1, 136.9, 124.0, 122.1, 115.0, 112.1, 105.8, 99.0, 93.4, 75.3, 71.8, 70.8, 22.5, 22.2, 22.0.
HRMS-FAB: *m/z* [M + Na]$^+$ calcd for C$_{24}$H$_{28}$O$_7$Na: 451.1733; found: 451.1727.

Preparation of 3,4',7-O-triisopentylquercetin (17): This derivative was synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (10 mL). The crude product was purified by repetitive PTLC eluting with 15% ethyl acetate in hexanes to yield compound **17** (24 mg, 8% yield).

3,4',7-*O*-Triisopentylquercetin (17)

MP: 75-76ºC.
IR (KBr): 3540, 3054, 2957, 2871, 1653, 1594, 1464 cm^{-1}.
^{1}H NMR (300 MHz, CDCl$_3$): 12.67 (s, 1H), 7.69 (d, J = 8.6 Hz, 1H), 7.68 (s, 1H), 6.93 (d, J = 8.6 Hz, 1H), 6.42 (d, J = 2.4 Hz, 1H), 6.33 (d, J = 2.4 Hz, 1H), 5.72 (s, 1H), 4.15 (t, J = 6.4 Hz, 2H), 4.05 (t, J = 6.4 Hz, 2H), 4.02 (t, J = 6.4 Hz, 2H), 1.88 – 1.79 (m, 3H), 1.78 – 1.75 (m, 2H), 1.72 – 1.68 (m, 2H), 1.65 – 1.62 (m, 2H),

1.00 (d, J = 6.8 Hz, 6H), 0.97 (d, J = 6.8 Hz, 6H), 0.90 (d, J = 6.8 Hz, 6H).
^{13}C NMR (75 MHz, CDCl$_3$): 179.1, 165.0, 162.0, 156.9, 155.9, 148.2, 145.6, 138.5, 123.7, 121.8, 114.6, 111.0, 106.1, 98.3, 92.6, 71.6, 67.6, 67.2, 39.0, 37.9, 37.7, 25.2, 25.1, 25.0, 22.7.
HRMS-FAB: *m/z* [M + Na]$^+$ calcd for C$_{30}$H$_{40}$O$_7$Na: 535.2666; found: 535.2678.

Preparation of benzylated derivatives of quercetin (18 and 19): These two derivatives were synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (10 mL). PTLC purification of the crude product, using 15% ethyl acetate in hexanes as eluent, generated **18** (73 mg, 22% yield) and **19** (58 mg, 15% yield).

3,4',7-*O*-Tribenzylquercetin (18)

MP: 147-148ºC.
IR (KBr): 3651, 3089, 3064, 3032, 2926, 2870, 1653, 1592, 1494 cm^{-1}.
^{1}H NMR (300 MHz, CDCl$_3$): 12.74 (s, 1H), 7.66 (s, 1H), 7.65 (d, J = 8.4 Hz, 1H), 7.45–7.28 (m, 15H), 6.96 (d, J = 8.4 Hz, 1H), 6.51 (s, 1H), 6.46 (s, 1H), 5.88 (s, 1H), 5.20 (s, 2H), 5.14 (s, 2H), 5.10 (s, 2H).
^{13}C NMR (75 MHz, CDCl$_3$): 178.8, 164.5, 162.0, 156.7, 156.3, 148.0, 145.7, 137.7, 136.5, 135.9, 135.8, 128.94, 128.89, 128.75, 128.65, 128.4, 128.3, 128.2, 127.9, 127.5, 123.9, 121.9, 115.0, 111.6, 106.2, 98.7, 93.0, 74.3, 71.1, 70.5.

3,3',4',7-*O*-Tetrabenzylquercetin (19)

MP: 135-136ºC.
IR (KBr): 3034, 2930, 1654, 1595, 1495 cm^{-1}.
^{1}H NMR (300 MHz, CDCl$_3$): 12.70 (s, 1H), 7.71 (d, J = 2.0 Hz, 1H), 7.55 (dd, J = 8.6, 2.0 Hz, 1H), 7.48–7.22 (m, 20H), 6.96 (d, J = 8.6 Hz, 1H), 6.46 (d, J = 2.0 Hz, 1H), 6.44 (d, J = 2.0 Hz, 1H), 5.25 (s, 2H), 5.13 (s, 2H), 5.04 (s, 2H), 4.99 (s, 2H).
^{13}C NMR (75 MHz, CDCl$_3$): 178.9, 164.6, 162.2, 156.8, 151.2, 148.4, 137.0, 136.8, 136.5, 135.9, 128.94, 128.89, 128.75, 128.65, 128.5, 128.4, 128.2, 128.1, 127.6, 127.5, 127.3, 123.6, 122.8, 115.4, 113.8, 106.3, 98.7, 93.2, 74.5, 71.2, 71.0, 70.6.

Bioassay

Cell culture: All cell lines were initially purchased from American Type Culture Collection (ATCC^{TM}). The PC-3 prostate cancer cell line and the LNCaP prostate cancer cell line were routinely cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cultures were maintained in 5% carbon dioxide at a temperature of 37°C. The DU-145 prostate cancer cells were routinely cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS and 1% penicillin/ streptomycin.

WST-1 cell proliferation assay: PC-3, DU-145, or LNCaP cells were plated in 96-well plates at a density of 3200 in each well in 200 μL of culture medium. The cells were then treated with either quercetin or synthesized quercetin derivatives separately at 2 different doses of 50 μM for 3 days, while equal treatment volumes of DMSO (0.25%) were used as vehicle control. The cells were cultured in a CO$_2$ incubator at 37ºC for 3 days. Ten μL of the premixed WST-1 cell proliferation reagent (Clontech) was added to each well. After mixing gently for 1 min on an orbital shaker, the cells were incubated for an additional 3 h at 37ºC. To ensure homogeneous distribution of color, it is important to mix gently on an orbital shaker for 1 min. The absorbance of each well was measured using a microplate reader (Synergy HT) at a wavelength of 430 nm. The IC$_{50}$ value is the concentration of each compound that inhibits cell proliferation by 50% under the experimental

conditions and is the average from at least triplicate determinations that are reproducible and statistically significant. For calculating the IC$_{50}$ values, a linear proliferative inhibition was made based on at least 5 dosages for each compound.

References

[1]   Richter E, Srivastava S. Dobi A. (**2007**) Androgen receptor and prostate cancer. *Prostate Cancer and Prostatic Diseases*, *10*, 114-118.
[2]   Roviello G, Petrioli R, Francini E. (**2013**) Time for a revision on the role of PSA response rate as a surrogate marker for median overall survival in docetaxel-based first-line treatment for patients with metastatic hormone-refractory prostate cancer. *International Journal of Biological Markers*, *28*, 326-328.
[3]   Paller CJ, Antonarakis ES. (**2011**) Cabazitaxel: a novel second-line treatment for metastatic castration-resistant prostate cancer. *Drug Design, Development and Therapy*, *5*, 117-124.
[4]   Sampson L, Rimm E, Hollman PC, de Vries JH, Katan MB. (**2002**) Flavonol and flavone intakes in US health professionals. *Journal of the American Dietetic Association*, *102*, 1414-1420.
[5]   Foster BA, Gingrich JR, Kwon ED, Madias C, Greenberg NM. (**1997**) Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. *Cancer Research*, *57*, 3325-3330.
[6]   Slusarz A, Shenouda NS, Sakla MS, Drenkhahn SK, Narula AS, MacDonald RS, Besch-Williford CL, Lubahn DB. (**2010**) Common botanical compounds inhibit the Hedgehog signaling pathway in prostate cancer. *Cancer Research*, *70*, 3382-3390.
[7]   Shenouda NS, Zhou C, Browning JD, Ansell PJ, Sakla MS, Lubahn DB, MacDonald RS. (**2004**) Phytoestrogens in common herbs regulate prostate cancer cell growth *in vitro*. *Nutrition and Cancer*, *49*, 200-208.
[8]   Aalinkeel R, Bindukumar B, Reynolds JL, Sykes DE, Mahajan SD, Chadha KC, Schwartz SA. (**2008**) The dietary bioflavonoid, quercetin, selectively induces apoptosis of prostate cancer cells by down-regulating the expression of heat shock protein 90. *Prostate* (Hoboken, NJ, United States), *68*, 1773-1789.
[9]   Maggiolini M, Vivacqua A, Carpino A, Bonofiglio D, Fasanella G, Salerno M, Picard D, Ando S. (**2002**) The mutant androgen receptor T877A mediates the proliferative but not the cytotoxic dose-dependent effects of genistein and quercetin on human LNCaP prostate cancer cells. *Molecular Pharmacology, 62*, 1027-1035.
[10]   Wang P, Vadgama JV, Said JW, Magyar CE, Doan N, Heber D, Henning SM. (**2014**) Enhanced inhibition of prostate cancer xenograft tumor growth by combining quercetin and green tea. *Journal of Nutritional Biochemistry*, *25*, 73-80.
[11]   Pratheeshkumar P, Budhraja A, Son Y-O, Wang X, Zhang Z, Ding S, Wang L, Hitron A, Lee J-C, Xu M, Chen G, Luo J, Shi X. (**2012**) Quercetin inhibits angiogenesis mediated human prostate tumor growth by targeting VEGFR- 2 regulated AKT/mTOR/P70S6K signaling pathways. *PloS one, 7*, e47516.
[12]   Beutler JA, Hamel E, Vlietinck AJ, Haemers A, Rajan P, Roitman JN, Cardellina JH, 2nd, Boyd MR. (**1998**) Structure-activity requirements for flavone cytotoxicity and binding to tubulin. *Journal of Medicinal Chemistry*, *41*, 2333-2338.
[13]   Rao KV, Owoyale JA. (**1976**) Partial methylation of quercetin: direct synthesis of tamarixetin, ombuin and ayanin. *Heterocyclic Chemistry*, *13*, 1293-1295.
[14]   Shi Z-H, Li N-G, Tang Y-P, Shi Q-P, Zhang W, Zhang P-X, Dong Z-X, Li W, Zhang X, Fu H-A, Duan J-A. (**2014**) Synthesis, biological evaluation and SAR analysis of *O*-alkylated analogs of quercetin for anticancer. *Bioorganic & Medicinal Chemistry Letters*, *24*, 4424-4427.

Compounds with Antifouling Activities from the Roots of *Notopterygium franchetii*

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In antifouling screening, the extract of *Notopterygium franchetii* de Boiss showed obvious activity. Two new phenylpropanoids (**1-2**) and five known coumarins (**3-7**) were isolated from the methanol extract of the roots of this species. The structures of the isolated compounds were determined on the basis of spectroscopic analysis. Compounds **1-2** showed definite antifouling activity against larval settlement of *Bugula neritina*.

Keywords: *Notopterygium franchetii*, Umbelliferae, Chemical constituent, Phenylpropanoids, Antifouling.

Notopterygium franchetii de Boiss, family Umbelliferae, is a traditional Chinese medicine. Its main constituents include essential oils, coumarins, amino acids, and organic acids [1]. Extracts of *N. franchetii* and some other species of *Notopterygium* have been reported to possess anti-inflammatory [2], antibacterial [3], antivirus [4], anti-thrombus [5], anti-arrhythmia [6] and anti-allergy [7] properties. We have carried out a study of the chemical constituents and their activities on marine fouling resistance, and now report the isolation and structure elucidation of two new phenylpropanoids (**1-2**), together with five known coumarins (**3-7**) from the dried roots of *N. franchetii*, and their antifouling activities against larval settlement of the barnacles *Balanus amphitrite* and *Bugula neritina*.

The methanol extract of the roots of the title plant was subjected to column chromatography to give two new phenylpropanoids (**1-2**), and five known coumarins, nodakenetin (**3**) [8], notopterol (**4**) [9], notoptol (**5**) [10], 5-[(2*E*,5*Z*)-7-hydroxy-3,7-dimethyl-2,5-octadienoxy]psoralen (**6**) [11] and 5-[(2,5)-epoxy-3-hydroxy-3,7-dimethyl-6-octadienoxy]psoralen (**7**) [11]. The structures were elucidated by NMR, IR and UV spectroscopy and MS.

Compound **1** was isolated as a white amorphous powder with a molecular formula of $C_{20}H_{26}O_5$ (calcd. 346.1780) as determined by the [M]$^+$ ion at *m/z* 346.1773 in the HR-EI-MS, together with eight degrees of unsaturation [12]. The IR spectrum showed hydroxyl at 3432 cm^{-1} and a double bond at 1596 cm^{-1}, 1516 cm^{-1}, while the UV spectrum showed absorption maxima at λmax 193, 217, 235 and 327 nm [13]. The ^{1}H NMR spectrum (Table 1) displayed three methyl singlet signals at δ_H 1.15 (3H, s), 0.91 (3H, s) and 0.89 (3H, s), a tri-substituted benzene ring signals at δ_H 7.35 (1H, d, *J* = 1.8), δ_H 7.15 (1H, dd, *J* = 8.1, 1.8) and δ_H 6.87 (1H, d, *J* = 8.1), a methoxyl group at δ_H 3.91 (3H, s), and a double bond at δ_H 7.59 (1H, d, *J* = 15.6), δ_H 6.42 (1H, d, *J* = 15.6); these last two protons had a *trans* form from the coupling constant (*J* = 15.6). The ^{13}C NMR spectrum (Table 1) showed 20 carbon signals (four primary, two secondary, eight tertiary, and six quaternary), including one
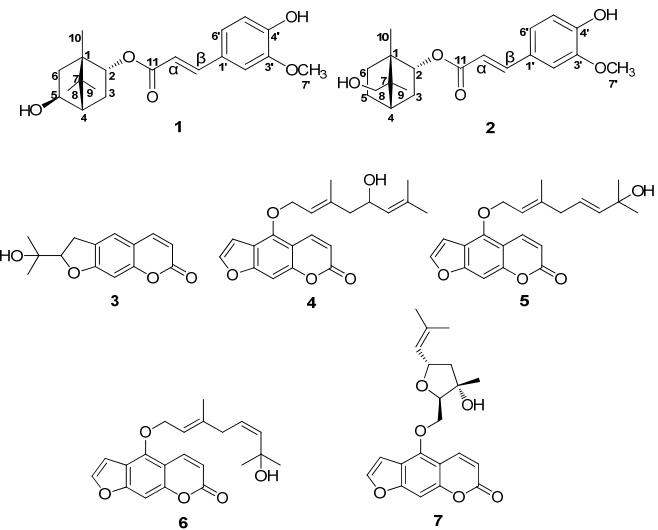
Figure 1: Isolated compounds from the roots of *Notopterygium franchetii*.

methoxyl carbon (δ_C 56.2), one carboxylic carbon (δ_C 167.6), eight aromatic carbons (δ_C 116.1, 145.4, 127.4, 111.1, 148.7, 150.0, 116.0, and 124.0). The HMBC correlation of H-7′ (δ_H 3.91) with C-3′ (δ_C 148.7) indicated that this methoxy group was at C-3′ [14]. The correlations of H-β (δ_H 7.59) with C-2′ (δ_C 111.1), C-6′ (δ_C 124.0) and C-11 (δ_C 167.6) indicated that C-β (δ_C 145.4) was connected to C-1′ (δ_C 127.4), and C-α (δ_C 116.1) with C-11 (δ_C 167.6). Analysis of the NMR data (Tables 1 and 2) of compound **1** revealed nearly identical structural features to those of (-)-bornyl ferulate [15], except that a methylene at C-5 was replaced by an oxygen-substituted methine, indicating that compound **1** is an analogue of (-)-bornyl ferulate. This was further confirmed by relevant ^{1}H–^{1}H COSY and HMBC data. The ^{1}H–^{1}H COSY correlations of a proton signal at δ_H 3.86 with H-4 (δ_H 1.74) and H-6a (δ_H 2.41) indicated this hydroxyl group at C-5 (δ_C 75.0). The planar structure of compound **1** was, therefore, determined as shown in Figure 1.

Table 1: NMR spectral data of compound **1** in CD₃COCD₃.

Position	δ_H (mult, J Hz) (600MHz)	δ_C (150 MHz)	HMBC
1		48.1 (s)	
2	4.88 (m)	78.6 (d)	C-6, C-10, C-11
3a	2.35 (m)	35.0 (t)	C-5, C-7
3b	0.85 (dd, 14.0, 3.3)		C-1, C-5
4	1.74 (d, 5.1)	53.4 (d)	
5	3.86 (m)	75.0 (d)	
6a	2.41 (m)	40.3 (t)	
6b	1.45 (dt, 13.4, 2.2)		
7		50.2 (s)	
8	1.15 (s)	21.1 (q)	C-1, C-4, C-9
9	0.91 (s)	20.2 (q)	C-1, C-4, C-8
10	0.89 (s)	13.4 (q)	C-2, C-6, C-9
11		167.6 (s)	
α	6.42 (d,15.6)	116.1 (d)	
β	7.59 (d, 15.6)	145.4 (d)	C-2´, C-6´, C-11
1´		127.4 (s)	
2´	7.35 (d, 1.7)	111.1 (d)	
3´		148.7 (s)	
4´		150.0 (s)	
5´	6.87 (d, 8.1)	116.0 (d)	
6´	7.15 (dd, 1.8, 8.1)	124.0 (d)	C-2´, C-4´, C-β
7´	3.91 (s)	56.2 (q)	C-3´

Table 2: NMR spectral data of compound **2** and (-)-bornyl ferulate.

	(**2**) (CD₃COCD₃)			(-)-bornyl ferulate (CDCl₃)	
Position	δ_H (mult, J Hz) (600MHz)	δ_C (150 MHz)	Position	δ_H (mult, J Hz) (300 MHz)	δ_C (75 MHz)
1		50.3 (s)	1		48.9 (s)
2	4.00 (m)	76.3 (d)	2	5.02 (ddd, 10.0, 3.3, 1.8)	79.8 (d)
3a	2.23 (m)	39.3 (t)	3a	2.41 (dddd, 13.8, 10.0, 4.0, 3.5)	36.8 (t)
3b	0.97 (dd, 13.6, 3.6)		3b	1.05 (dd, 13.3, 3.3)	
4	1.89 (t, 4.5)	43.0 (d)	4	1.71 (m)	44.9 (d)
5a	1.77 (m)	28.4 (t)	5a	1.78 (m)	28.0 (d)
5b	1.30 (m)		5b	1.29 (m)	
6a	2.18 (dd, 9.1, 3.2)	26.6 (t)	6a	2.06 (m)	27.2 (t)
6b	1.27 (m)		6b	1.31 (m)	
7		51.8 (s)	7		47.8 (s)
8a	4.23 (d, 11.2)	68.0 (t)	8	0.90 (s)	19.7 (q)
8b	4.03 (d, 11.2)				
9	1.01 (s)	14.2 (q)	9	0.94 (s)	18.8 (q)
10	0.93 (s)	14.3 (q)	10	0.88 (s)	13.5 (q)
11		167.7 (s)	11		167.0 (s)
α	6.43 (d,15.9)	115.9 (d)	α	6.31 (d,15.9)	116.1 (d)
β	7.61 (d, 15.9)	145.5 (d)	β	7.59 (d, 15.9)	144.3 (d)
1´		127.4 (s)	1´		127.0 (s)
2´	7.35 (d, 1.7)	111.1 (d)	2´	7.05 (d, 1.5)	109.3 (d)
3´		148.7 (s)	3´		146.8 (s)
4´		150.0 (s)	4´		147.8 (s)
5´	6.86 (d, 8.2)	116.0 (d)	5´	6.91 (d, 8.2)	114.7 (d)
6´	7.15 (dd, 1.8, 8.2)	123.9 (d)	6´	7.08 (dd, 2.2, 8.2)	123.0 (d)
7´	3.91 (s)	56.2 (q)	7´	3.93 (s)	55.9 (q)

The ROESY correlations of H-2 (δ_H 4.88) with H-3a (δ_H 2.35) and H-9 (δ_H 0.91) indicated that H-2, H-3a and H-9 were β-oriented. The correlation of H-5 (δ_H 3.86) with H-3b (δ_H 0.85) indicated that H-5 and H-3b were α-oriented. Herein, the OH at C-5 was β-oriented. Thus, the relative configuration of compound **1** was determined as shown in Figure 1.

Compound **2** was isolated as a white amorphous powder with a molecular formula of C₂₀H₂₆O₅ (calcd. 346.1780), as determined by the [M]⁺ peak at *m/z* 346.1779 in the HR-EI-MS, together with eight degrees of unsaturation. The IR spectrum showed hydroxyl at 3442 cm⁻¹ and double bond at 1631cm⁻¹, 1516 cm⁻¹, while the UV spectrum showed absorption maxima at λmax 202, 234 and 326 nm. Analysis of the NMR data (Table 2) of compound **2** revealed nearly identical structural features to those of (-)-bornyl ferulate [15]**,** except that a methyl at C-8 was replaced by a hydroxymethyl,

indicating that compound **2** is an analogue of (-)-bornyl ferulate. This was further confirmed by relevant ¹H–¹H COSY and HMBC data. The HMBC correlations of H-8a (δ_H 4.23) and H-8b (δ_H 4.03) with C-9 (δ_c 14.2) indicated that the hydroxymethyl was at C-8 (δ_C 68.0). The planar structure of compound **2** was, therefore, determined as shown (Figure 1).

The ROESY correlation of H-2 (δ_H 4.00) with H-9 (δ_H 1.01) indicated H-2 and H-9 to be β-oriented, while H-8 should be α-oriented. Thus, the relative configuration of compound **2** was determined as shown in Figure 1.

Compounds **1**, **2** and **3-7** were evaluated for their antifouling activity against larval settlement of the barnacles *Balanus amphitrite* and *Bugula neritina*. Testing was performed in accordance with the method described by Kawahara [16]. Compounds **1** and **2** had activity against larval settlement of *Bugula neritina* with an EC₅₀ value of 25 μg/mL, which indicated that the –OH group of the bornyl moiety had no effect on the larval settlement. The attachment rate of the negative control group was 100%. The test results were read 12 hours after dosing. Specific results are shown in Table 3.

Experimental

General: The optical rotation data were measured on a JASCO P-1020 digital polarimeter (Jasco, Tokyo, Japan), UV-Vis spectra on a Shimadzu UV-2401 PC spectrophotometer, IR spectra on a Bruker Tensor-27 infrared spectrophotometer with a KBr disk, and ESI-MS and HR-EI-MS on Bruker HCT/E squire 3000 and Waters Autospec Premier P776 spectrometers, respectively. The NMR spectra were recorded on a Bruker Avance III-600 spectrometer with trimethylsilyl as internal standard. Column chromatography was performed on silica gel (200–300 and 300–400 mesh; Qingdao Marine Chemical Inc.), MCI gel CHP 20P (75–150 μm; Mitsubishi Chemical Corporation), Sephadex LH-20 (40–70 μm; Amersham Pharmacia Biotech AB), and Chromatorex Rp-C₁₈ gel (20–45 μm; Merck). Compounds on thin layer chromatograms (TLC plates; Qingdao Marine Chemical Inc.) were visualized under UV light and by dipping into 5% H₂SO₄ in EtOH followed by heating.

Plant material: The dried roots of *Notopterygium franchetii* de Boiss were bought in Kunming Juhua market, Yunnan Province of China, in September 2012, and were identified by Mr Yu Chen (Kunming Institute of Botany, Chinese Academy of Sciences). A voucher specimen (H20120902) was deposited at the Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation: The air-dried roots (15.0 kg) of *N. franchetii* were powdered and extracted 3 times with MeOH under reflux. The combined MeOH extracts were concentrated using a rotary evaporator to give the crude extract, which was suspended in water and then partitioned successively with light petroleum, ethyl acetate and *n*-butyl alcohol, sequentially.

The ethyl acetate portion (1.08 kg) was subjected to silica gel CC and eluted with light petroleum–acetone mixtures of increasing polarity (from 10:0 to 4:6) to yield 5 fractions (Fr.1-5) on the basis of TLC. Fr. 3 (22.5 g) was then separated on a silica gel column (light petroleum/ethyl acetate from 7.5:2.5 to 7:3) to obtain 13 fractions (Fr.3A–M). Fr.3H (4.0 g) was separated over a MCI-gel column (MeOH/H₂O from 4:6 to 10:0) to obtain 11 fractions (Fr.3H1–3H11). Fr.3H4 (95.8 mg) was then separated on a silica gel column (light petroleum/ethyl acetate from 9.5:0.5) to obtain **7**

(15.7 mg) [11]. Fr.3H6 (1.81 g) was then separated on a silica gel column (light petroleum/ethyl acetate, 9.5:0.5) to obtain 8 fractions (Fr.3H6A–3H6H). Fr.3H6B (200 mg) was purified by HPLC (MeOH/H$_2$O, 55:45) to obtain **1** (7.3 mg) and **2** (5.2 mg). Fr.3H7 (491.2 mg) was then separated on a silica gel column (light petroleum/ethyl acetate, 9.5:0.5) to obtain 9 fractions (Fr.3H7A–3H7I). Fr.3H7E (70 mg) was purified by HPLC to obtain **5** (15.7 mg) [10] and **6** (4.8 mg) [11]. Fr.3H7G (120 mg) was purified by HPLC to yield **3** (65.9 mg) [8] and **4** (14.0 mg) [9].

Compound (1)
White amorphous powder.
$[\alpha]_D^{16}$: -20.63 (*c* 0.34, MeOH).
UV (CH$_3$OH) λ_{max} (log ε): 327 (0.39).
^{1}H and ^{13}C NMR: Table 1.
ESI-MS *m/z*: 369 [M+Na]$^+$
HR-EI-MS *m/z*: 346.1773, C$_{20}$H$_{26}$O$_5$ (calcd. 346.1780).

Compound (2)
White amorphous powder.
$[\alpha]_D^{17}$: -19.05 (*c* 0.175, MeOH).
UV (CH$_3$OH) λ_{max} (log ε): 203 (0.43), 326 (0.40).
^{1}H and ^{13}C NMR: Table 2.

ESI-MS *m/z*: 369 [M+Na]$^+$
HR-EI-MS *m/z*: 346.1779, C$_{20}$H$_{26}$O$_5$ (calcd. 346.1780).

Marine fouling resistance assay: Activity testing was performed in accordance with the method described by Kawahara [16,17]. We used the larvae of the barnacles *Balanus amphitrite* and *Bugula neritina* as test larva. First, the larvae were cultured to the state of pre-attachment, and then the sample and a certain number of larvae were cultured together. Twelve h later, the number of non-attached and attached larvae was observed and counted under a microscope. Finally, the attachment rate of larvae was calculated. The experiment was conducted by Peiyuan Qian's research group at Hong Kong University of Science and Technology.

Table 3: Activities of resistance to marine fouling of compounds **1** and **2**.

Settlement bioassay of *Bugula neritina* larvae (EC$_{50}$)	
Compounds	EC$_{50}$ (μg/mL)
1	**25**
2	**25**

References

[1]   Jin PP. (**2011**) Research progress of *Notopterygium incisun* Ting ex H. T. Chang. *Journal of Anhui Agricultural Sciences*, **39**, 815-816.
[2]   Li ZY, Zhang XS, Wang JL. (**2003**) Progress in researches on *Notopterygium* root. *Journal of Shaanxi College of Traditional Chinese Medicine*, **26**, 56-59.
[3]   Jin SF, Lin WM, Qiao J, Li WZ. (**1981**) Study on the pharmacological effects of *Notopterygium* injection. *Chinese Traditional Patent Medicine*, **4**, 41-41.
[4]   Guo YH, Sha M, Meng XS, Cao AM. (**2005**) The anti-viral studies of *Notopterygium incisum*. *Lishizhen Medicine and Materia Medica Research*, **16**, 198-199.
[5]   Lv EW, Ni ZM, He WJ, Ren ZH. (**1981**) Antithrombosis effect of nine kinds of traditional Chinese medicine. *Chinese Journal of Integrated Traditional and Western Medicine*, **1**, 101-103.
[6]   Cheng NZ, Shan ZY, Chen YP. (**1998**) Comparison antiarrhythmia effect of different water-soluble of *Notopterygium incisum*. *Chinese Journal of Basic Medicine in Traditional Chinese Medicine*, **4**, 43-43.
[7]   Sun YP, Xu Q. (**2003**) Effects of aqueous extract from rhizoma *Notopterygii* on the delayed-type hypersensitivity and inflammatory reactions and its mechanisms. *Journal of China Pharmaceutical University*, **34**, 51-54.
[8]   Zhao DF, Islam MN. (**2012**) *In vitro* antioxidant and anti-inflammatory activities of *Angelica decursiva*. *Archives of Pharmacal Research*, **35**, 179-192.
[9]   Kou GF, Zhang YB, Yang XW, Rong R. (**2010**) *O*-methylnotopterol, a new natural product from the roots and rhizomes of *Notopterygium incisum*. *China Journal of Chinese Materia Medica*, **35**, 1134-1136.
[10]   Kozawa M, Fukumoto M, Matsuyama Y. (**1983**) Chemical studies on the constituents of the Chinese crude drug "Qiang Huo". *Chemical and Pharmaceutical Bulletin*, **31**, 2712-2717.
[11]   Wu SB, Zhao Y, Fan H, Hu YH, Hamann MT, Peng JN, Starks CM, O´Neil-Johnson M, Hu JF. (**2008**) New guaiane sesquiterpenes and furanocoumarins from *Notopterygium incisum*. *Planta Medica*, **74**, 1812-1817.
[12]   Su ZS, Wang P, Yuan W, Li SY. (**2014**) Chemical constituents from *Pterocarpus soyauxii*. *Natural Product Communications*, **9**, 1483-1486.
[13]   Raksata A, Laphookhieoa S, Cheenprachab S, Ritthiwigromc T, Maneerata W. (**2014**) Antibacterial compounds from the roots of *Cratoxylum formosum* spp. *pruniflorum*. *Natural Product Communications*, **9**, 1487-1489.
[14]   Li RX, Niu SB, Guo LD, Zhang Y. (**2014**) Two new pyrone derivatives from the plant endophytic fungus *Exserohilum* sp. *Natural Product Communications*, **9**, 1497-1498.
[15]   Maldonado E, Apan MTR, Pérez-Castorena A L. (**1998**) Anti-inflammatory activity of phenyl propanoids from *Coreopsis mutica*. *Planta Medica*, **64**, 660-661.
[16]   Kawahara H, Tamura R, Ajioka S, Shizuri Y. (**1999**) Convenient assay for settlement-inducing substances of barnacle. *Marine Biotechnology*, **1**, 98–101.
[17]   Qi SH, Zhang S, Qian PY, Xiao ZH, Li MY. (**2006**) Ten new antifouling briarane diterpenoids from the South China Sea gorgonian *Junceella juncea*. *Tetrahedron*, **62**, 9123–9130.

New Isochromane Derivatives from the Mangrove Fungus *Aspergillus ustus* 094102

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Four new isochromane derivatives (**1–4**) along with the known peniciphenol (**5**) and (*R*)-2-(hydroxymethyl)-3-(2-hydroxypropyl)phenol (**6**) were isolated from the EtOAc extract of the fermentation broth of the mangrove fungus, *Aspergillus ustus* 094102. The structures of the new compounds including the absolute configuration were elucidated on the basis of spectroscopic analysis, CD and ECD calculation. Compounds **1** and **2** exhibited α-glucosidase inhibition and anti-oxidation against DPPH radical with IC$_{50}$ values of 1.4 mM and 25.7 μM, respectively.

Keywords: *Aspergillus ustus*, Isochromane derivatives, Anti-oxidation, α-Glucosidase inhibition.

Microorganisms in special niches have proved to be an important source of bioactive compounds. The mangrove is a typically special ecosystem at the junction of land and sea, in which there are abundant microorganisms that may produce structurally new and bioactive natural products [1-6]. Previously we identified cytotoxic drimane sesquiterpenes and isochromane derivatives from *Aspergillus ustus* 094102 isolated from the mud around the roots of the mangrove plant *Bruguiera gymnorrhiza* [1]. Continuous study resulted in the isolation and identification of four new isocoumarin derivatives (**1–4**), as well as two known phenolic compounds, peniciphenol (**5**) [7] and (*R*)-2-(hydroxymethyl)-3-(2-hydroxypropyl) phenol (**6**) [8]. The new compound **1** exhibited α-glucosidase inhibition while new compound **2** showed anti-oxidative activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical with IC$_{50}$ values of 1.41 mM and 25.7 μM, respectively.

Compound **1** was obtained as a colorless solid with a molecular formula of C$_{11}$H$_{12}$O$_6$ from the HRESIMS peak at *m/z* 239.0558 [M–H]$^-$ (calcd 239.0556 for C$_{11}$H$_{11}$O$_6$). The IR spectrum indicated the presence of hydroxyl (3226 cm^{-1}), ester carbonyl groups (1751 cm^{-1}) and an aromatic ring (1595, 1482 cm^{-1}). The ^{1}H and ^{13}C NMR along with DEPT spectra (Table 1, Figures S1-S3) exhibited signals for a methoxyl (δ$_{H/C}$ 3.89/56.1), two oxygenated methylenes (δ$_{H/C}$ 4.36 & 4.52/72.2, δ$_H$ 4.46/50.9), one oxygenated methine (δ$_{H/C}$ 4.75/62.6), one aromatic methine (δ$_{H/C}$ 6.72/101.0), six sp^2 quaternary carbons (δ$_C$ 100.5, 144.0, 163.8, 115.7, 160.4, 169.0), and three exchangeable protons (δ$_H$ 4.58, 5.95 & 11.36), indicating a penta-substituted benzopyrone skeleton in **1**. HMBC correlations (Figures 2 and S4) of H-3 (δ$_H$ 4.36 & 4.52) to C-1 (δ$_C$ 169.0), C-4 (δ$_C$ 62.6) and C-4a (δ$_C$ 144.0), along with HO-4 (δ$_H$ 5.95) to C-3 (δ$_C$ 72.2), C-4 and C-4a confirmed the skeleton as isochromane. In addition, the HMBC spectrum showed long distance ^{1}H-^{13}C correlations from the methoxy proton (δ$_H$ 3.89) to C-6 (δ$_C$ 163.8), from H-5 (δ$_H$ 6.72) to C-4, C-4a, C-1a (δ$_C$ 100.5), C-6 (δ$_C$ 163.8) and C-7 (δ$_C$ 115.7), and from HO-9 (δ$_H$ 4.58) to C-6, C-7 and C-8 (δ$_C$ 160.4), locating the methoxy, hydroxymethyl and hydroxy groups at C-6,
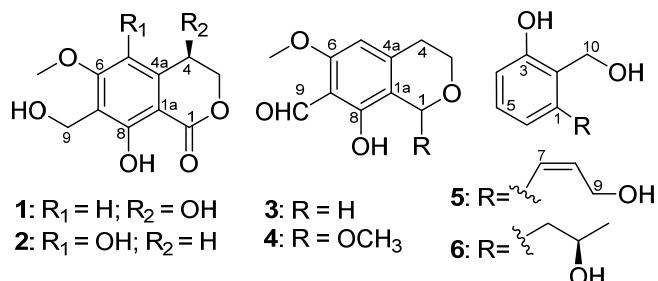
Figure 1: Chemical structures of compounds **1-6**.

1: R$_1$ = H; R$_2$ = OH
2: R$_1$ = OH; R$_2$ = H
3: R = H
4: R = OCH$_3$
5: R=
6: R=

C-7 and C-8, respectively. The absolute configuration was determined as 4*R*- by ECD calculations using the time-dependent density functional theory (TD-DFT) method at the B3LYP/6-31G(d) level [9]. The results showed that the CD curve of **1** is matched well with the calculated ECD for *R*-**1** and opposite to that of *S*-**1** (Figure 3), indicating *R*-configuration. Thus, compound **1** was identified as (*R*)-4,8-dihydroxy-7-(hydroxymethyl)-6-methoxyisochroman-1-one.
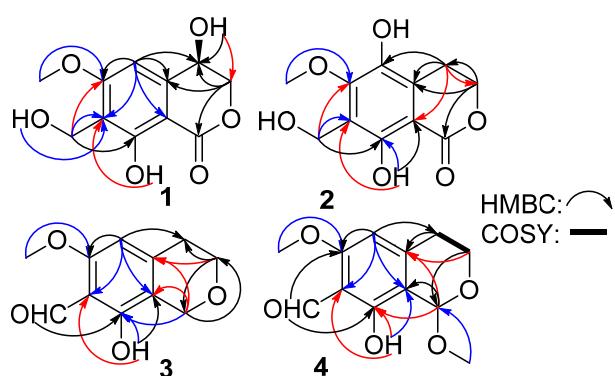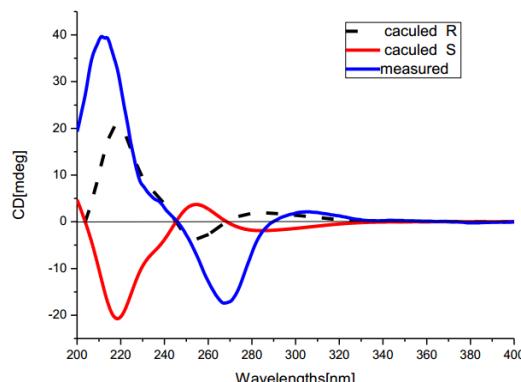
Figure 2: HMBC and ^{1}H-^{1}H COSY correlations of compounds **1-4**.

Table 1: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for compounds **1–3** in DMSO-d_6 and compound **4** in CDCl$_3$ (δ, ppm).

position	1 δ$_C$	1 δ$_H$ (J in Hz)	2 δ$_C$	2 δ$_H$ (J in Hz)	3 δ$_C$	3 δ$_H$ (J in Hz)	4 δ$_C$	4 δ$_H$ (J in Hz)
1	169.0, qC		169.6, qC		62.4, CH$_2$	4.54, s	93.8, CH	5.55, s
1a	100.5, qC		103.6, qC		114.8, qC		115.6, qC	
3	72.2, CH$_2$	4.52, dd (11.5, 3.8); 4.36, dd (11.5, 5.5)	67.7, CH$_2$	4.52, t (6.0)	63.5, CH$_2$	3.83, t (5.5)	56.3, CH$_2$	3.86, dd (15.4, 6.1); 4.18, ddd (15.4, 12.1, 3.3)
4	62.6, CH	4.75, m	21.5, CH$_2$	2.95, t (6.0)	29.0, CH$_2$	2.78, t (5.5)	29.1, CH$_2$	2.58, dd (17.6, 3.3 Hz); 3.00, ddd (17.6, 12.2, 6.1)
4a	144.0, qC		126.5, qC		146.0, qC		146.1, qC	
5	101.0, CH	6.72, s	138.3, qC		102.2, CH	6.45, s	100.7, CH	6.12, s
6	163.8, qC		154.4, qC		160.0, qC		161.7, qC	
7	115.7, qC		120.8, qC		108.2, qC		109.2, qC	
8	160.4, qC		154.4, qC		158.3, qC		161.4, qC	
9	50.9, CH$_2$	4.46, br d (3.5)	51.7, CH$_2$	4.46, s	193.9, CH	10.18, s	193.7, CH	10.24, s
6-OMe	56.1, CH$_3$	3.89, s	61.5, CH$_3$	3.84, s	56.1, CH$_3$	3.86, s	55.8, CH$_3$	3.86, s
1-OMe							55.4, CH$_3$	3.56, s
4-OH		5.95, d (5.3)						
5-OH				8.77, s				
8-OH		11.36, s		10.99, s		12.16, s		12.45, s
9-OH		4.58, t (3.5)		4.75, br s				

Figure 3: CD curve of **1** and the calculated ECD curves of *R*-**1** and *S*-**1**.

Compound **2** was obtained as a white solid with the same molecular formula as **1** from the HRESIMS peak at *m/z* 239.0549[M–H]$^-$. The presence of hydroxyl, ester carbonyl groups and a benzene ring could be deduced from the IR absorptions at 3257, 1755, 1609, and 1465 cm^{-1}, respectively. The ^{1}H and ^{13}C NMR data classified by DEPT experiments (Table 1, Figures S5-S7) were closely related to compound **1**, except that the oxygenated sp^3-methine and aromatic methine were replaced by a methylene (δ$_{H/C}$ 2.95/21.5) and an aromatic quaternary carbon (δ$_C$ 138.3), respectively. In addition, obvious up-field shifts for C-3, C-4a, C-6 and C-8 were observed while C-1a, C-7 and C-6-OMe shifted down-field. These data indicated that the HO- group at C-4 in **1** was moved to C-5 in compound **2**. This deduction was further supported by HMBC correlations of H-3 (δ$_H$ 4.52) to C-1 (δ$_C$ 169.6), C-4 (δ$_C$ 21.5) and C-4a (δ$_C$ 126.5), H-4 (δ$_H$ 2.95) to C-1a (δ$_C$ 103.6), C-3 (δ$_C$ 67.7), C-4a and C-5 (δ$_C$ 138.3), along with HO-9 (δ$_H$ 4.75) to C-7 (δ$_C$ 120.8), C-6 and C-8 (δ$_C$ 154.4) (Figures 2 and S9). Thus, compound **2** was elucidated as 5,8-dihydroxy-7-(hydroxymethyl)-6-methoxy-isochroman-1-one.

Compound **3** was obtained as a white solid with a molecular formula of C$_{11}$H$_{12}$O$_4$ from the HRESIMS peak at *m/z* 209.0839 [M+H]$^+$ (calcd 209.0830 for C$_{11}$H$_{13}$O$_4$). The IR spectrum showed characteristic absorptions of –CHO at 2848, 2736 and 1695 cm^{-1}. The ^{1}H and ^{13}C NMR along with the DEPT spectra (Table 1, Figures S10-S12) exhibited signals for one methoxyl (δ$_{H/C}$ 3.86/56.1), one aldehyde group (δ$_{H/C}$ 10.18/193.9), one penta-substituted benzene nucleus (δ$_{H/C}$ 6.45/102.2, 114.8, 146.0, 160.0, 108.2, and 158.3), two oxygenated methylenes (δ$_{H/C}$ 4.54/62.4, 3.83/63.5), one methylene (δ$_{H/C}$ 2.78/29.0), and a phenolic hydroxyl (δ$_H$ 12.16), probably suggesting an isochroman skeleton. This deduction was confirmed by the HMBC correlations (Figures 2 and

S14) from H-1 (δ$_H$ 4.54) to C-3 (δ$_C$ 63.5), C-1a (δ$_C$ 114.8) and C-4a (δ$_C$ 146.0), from H-3 (δ$_H$ 3.83) to C-1 (δ$_C$ 62.4), C-4 (δ$_C$ 29.0) and C-4a, and from H-4 (δ$_H$ 2.78) to C-1a, C-4a, C-3 and C-5 (δ$_C$ 102.2). In addition, the HMBC spectrum also showed long-distance ^{1}H-^{13}C correlations from the aldehyde proton (δ$_H$ 10.18) to C-7 (δ$_C$ 108.2) and C-8 (δ$_C$ 158.3), from the hydroxy proton (δ$_H$ 12.16) to C-1a, C-7 and C-8, from the methoxy proton (δ$_H$ 3.86) and C-6 (δ$_C$ 160.0), and from H-5 (δ$_H$ 6.45) to C-1a, C-4, C-6 and C-7, locating the methoxy, aldehyde and hydroxy protons at C-6, C-7 and C-8, respectively. Thus, compound **3** was determined as 8-hydroxy-6-methoxyisochromane-7-carbaldehyde.

Compound **4** was obtained as a white solid with a molecular formula of C$_{12}$H$_{14}$O$_5$ according to a HRESIMS peak at *m/z* 239.2445 [M+H]$^+$ (calcd 239.2437 for C$_{12}$H$_{15}$O$_5$). The ^{1}H and ^{13}C NMR data (Table 1 and Figures S16-S18) were closely related to compound **3**, except for an additional methoxy (δ$_{H/C}$ 3.56/55.4) and the replacement of the oxygenated methylene in **3** by a hemiacetal methine (δ$_{H/C}$ 5.55/93.8). The key HMBC correlation of the methoxy proton to the hemiacetal methine carbon (C-1) located the methoxy group at C-1, indicating that compound **4** was a C-1 methoxylated derivative of **3** that was further confirmed by ^{1}H-^{1}H COSY between H$_2$-3 and H$_2$-4 (Figures 2 and S20), along with almost the same HMBC pattern between **4** and **3** (Figures 2 and S21). The small [α]$^{20}_D$ value –5.6) suggested that compound **4** might be a racemic mixture, which led us to carry out a chiral analysis. Unfortunately, compound **4** was not resolved by chiral HPLC analysis over chiralpak IA and chiralpak IC chiral columns (Figure S23). Thus, compound **4** was elucidated as (±)-8-hydroxy-1,6-dimethoxyisochro-mane-7-carbaldehyde.

Compounds **1–6** were assayed for their anti-oxidative activity against DPPH radical [10] and α-glucosidase inhibitory effects using *p*-nitrophenyl-α-glucopyranoside (pNPG) as substrate [11]. The results showed that compound **2** displayed anti-oxidation with an IC$_{50}$ value of 25.7 μM, while compounds **1** and **3–6** were inactive (IC$_{50}$ > 400 μM) compared with L-ascorbic acid (positive control, IC$_{50}$ 16.5 μM) (Table 2, left). Compound **1** exhibited moderate α-glucosidase inhibition with an IC$_{50}$ value of 1.41 mM compared with acarbose (positive control, IC$_{50}$ 0.95 mM) (Table 2, right).

Experimental

General: Optical rotations were obtained on a JASCO P-1020 digital polarimeter. CD data were collected on a JASCO J-715 spectropolarimeter. UV spectra were recorded on a Beckman DU 640 spectrophotometer, and IR spectra on a Nicolet NEXUS 470 spectrophotometer as KBr discs. ^{1}H/^{13}C NMR, DEPT, and

Table 2: DPPH radical scavenging activity (left) and α-glucosidase inhibition (right) of compounds **1**–**6**.

Compound	IC$_{50}$, μM	Compound	IC$_{50}$, mM[a]
1	>417	**1**	1.4
2	25.7	**2**	6.9
3	>481	**3**	-
4	>420	**4**	7.0
5	>556	**5**	-
6	>549	**6**	12.7
ascorbic acid	16.5	acarbose	0.9

[a] - untested.

2D-NMR spectra were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as internal standard; chemical shifts were recorded as δ values. ESI-MS were measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. HPLC was performed on an ODS RP-18 column [YMC-pack ODS-A, 10×250 mm, 5 μm, 4 mL/min].
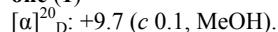
Fungal material: The fungus *Aspergillus ustus* 094102 was isolated and identified by Prof. Dr Kui Hong [1]. A reference culture of *A. ustus* 094102 is maintained at –80°C. Working stocks were prepared on Potato Dextrose agar slants and stored at 4°C.

Fermentation and extraction: *A. ustus* 094102 was incubated under static conditions at 30°C for 28 d in 100 × 1000 mL Erlenmeyer flasks containing liquid medium (300 mL/flask) composed of maltose (20 g/L), mannitol (20 g/L), glucose (10 g/L), monosodium glutamate (10 g/L), yeast extract (3 g/L), corn steep liquor (1 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.3 g/L), and seawater, after adjusting its pH to 7.0. The fermentation broth (30 L) was filtered through cheesecloth to be separated into supernatant and mycelium. The supernatant was concentrated under reduced pressure to about 25% of the original volume and then extracted 3 times with EtOAc to give an EtOAc solution, while the mycelium was extracted 3 times with acetone. After removing the acetone by evaporation under vacuum, the obtained aqueous acetone solution was extracted 3 times with equal volumes of EtOAc. Both EtOAc solutions were combined and dried under vacuum to give 70.0 g of extract.

Purification: The EtOAc extract (70.0 g) was separated into 8 fractions on a silica gel column eluting with a stepwise gradient of light petroleum-CHCl$_3$-MeOH. Fraction 7 (2.2 g) was subjected to CC over an ODS column eluting with 30–80% MeOH/H$_2$O to provide 5 sub-fractions (Fr7.1-Fr7.5). Fr7.5 (0.2 g) was further purified on an ODS column with 60% MeOH/H$_2$O followed by semi-preparative HPLC to give compound **6** (7.7 mg, t$_R$ 4.7 min, 55% MeOH/H$_2$O). Fraction 8 (5.3 g) was separated into 9 sub-fractions (Fr8.1-Fr8.9) on a silica gel column eluting with a stepwise gradient of CHCl$_3$-MeOH. Fr8.1 (0.1 g) was further purified by semipreparative HPLC with 55% MeOH/H$_2$O to give compound **5** (4.0 mg, t$_R$ 7.5 min). Fr8.2 (1.1 g) was further separated on a Sephadex LH-20 column eluting with MeOH followed by semipreparative HPLC to give compound **2** (27.7 mg, t$_R$ 4.4 min, 55% MeOH/H$_2$O). Fr8.3 (2.7 g) was further separated into 5 sub-fractions (Fr8.3.1-Fr8.3.5) on a RP-18 column eluting with 60% MeOH/H$_2$O. Fr8.3.4 (0.8 g) was then subjected to

chromatography over a Sephadex LH-20 column eluting with MeOH to afford 3 fractions (Fr8.3.4.1-Fr8.3.4.3). Fr8.3.4.1 (0.1 g) was further purified by semi-preparative HPLC with 55% MeOH/H$_2$O to give compound **3** (5.2 mg, t$_R$ 14.6 min), while Fr8.3.4.2 (0.2 g) gave compounds **1** (12.6 mg, t$_R$ 12.2 min) and **4** (9.3 mg, t$_R$ 6.8 min) purified by semi-preparative HPLC with 55% MeOH/H$_2$O.

(*R*)-4,8-Dihydroxy-7-(hydroxymethyl)-6-methoxyisochroman-1-one (1)
[α]$^{20}_D$: +9.7 (*c* 0.1, MeOH).
IR (KBr): 3226, 2910, 1751, 1595, 1482, 1359, 1195, 1004 cm^{-1}.
UV/Vis λ$_{max}$(MeOH) nm (log ε): 305 (1.10), 270 (1.47), 219 (3.55).
CD λ$_{max}$ (*c* 0.4, MeOH) (Δε) nm: 212 (+4.8), 268 (–2.1) and 306 (+0.26).
^{1}H and ^{13}C NMR; Table 1.
HRESIMS: *m/z* [M – H]$^-$ calcd for $C_{11}H_{11}O_6$: 239.0556; found: 239.0558.

5,8-Dihydroxy-7-(hydroxymethyl)-6-methoxyisochroman-1-one (2)
IR (KBr): 3257, 2926, 1755, 1609, 1465, 1361, 1206 cm^{-1}.
UV/Vis λ$_{max}$(MeOH) nm (log ε): 305 (1.21), 270 (1.47), 219 (3.65).
^{1}H and ^{13}C NMR; Table 1.
HRESIMS: *m/z* [M – H]$^-$ calcd for $C_{11}H_{11}O_6$: 239.0556; found: 239.0549.

8-Hydroxy-6-methoxyisochromane-7-carbaldehyde (3)
IR (KBr): 3254, 2925, 2848, 2736, 1695, 1602, 1458, 1323, 1193, 1085 cm^{-1}.
UV/Vis λ$_{max}$(MeOH) nm (log ε): 349 (1.10), 284 (2.89), 206 (3.38).
^{1}H and ^{13}C NMR; Table 1.
HRESIMS: *m/z* [M + H]$^+$ calcd for $C_{11}H_{13}O_4$: 209.0830; found: 209.0839.
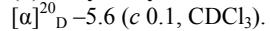
(±)-8-Hydroxy-1,6-dimethoxy isochromane-7-carbaldehyde (4)
[α]$^{20}_D$ –5.6 (*c* 0.1, CDCl$_3$).
IR (KBr): 3228, 2947, 2851, 2746, 1705, 1599, 1448, 1356, 1202, 997 cm^{-1}.
UV/Vis λ$_{max}$(MeOH) nm (log ε): 349 (1.10), 284 (2.89), 206 (3.38).
^{1}H and ^{13}C NMR; Table 1.
HRESIMS: *m/z* [M + H]$^+$ calcd for $C_{12}H_{15}O_5$: 239.2447; found: 239.2445.

Supporting information: Bioassay protocols used, the NMR spectra of compounds **1**−**4**, the chiral separation picture of compound **4**.These materials are available free of charge via the Internet at http://www.naturalproduct.us/.

References

[1]   Lu Z, Wang Y, Miao C, Liu P, Hong K, Zhu W. (**2009**) Sesquiterpenoids and benzofuranoids from the marine-derived fungus *Aspergillus ustus* 094102. *Journal of Natural Products*, **72**, 1761-1767.
[2]   Lu Z, Zhu H, Fu P, Wang Y, Zhang Z, Lin H, Liu P, Zhuang Y, Hong K, Zhu W. (**2010**) Cytotoxic polyphenols from the marine-derived fungus *Penicillium expansum*. *Journal of Natural Products*, **73**, 911-914.
[3]   Peng X, Wang Y, Sun K, Liu P, Yin X, Zhu W. (**2011**) Cerebrosides and 2-pyridone alkaloids from the halotolerant fungus *Penicillium chrysogenum* grown in a hypersaline medium, *Journal of Natural Products*, **74**, 1298-1302.

[4] Wang J, Lu Z, Liu P, Wang Y, Li J, Hong K, Zhu W. (**2012**) Cytotoxic polyphenols from the fungus *Penicillium expansum* 091006 endogenous with mangrove plant *Excoecaria agallocha*. *Planta Medica*, *78*, 1861-1866.

[5] Fan Y, Wang Y, Liu P, Fu P, Zhu T, Wang W, Zhu W. (**2013**) Indole-diterpenoids with anti-H1N1 activity from the aciduric fungus *Penicillium camemberti* OUCMDZ-1492. *Journal of Natural Products*, *76*, 1328-1336.

[6] Kong F, Wang Y, Liu P, Dong T, Zhu W. (**2014**) Thiodiketopiperazines from the marine-derived fungus *Phoma* sp. OUCMDZ-1847. *Journal of Natural Products*, *77*, 132-137.

[7] Kuramochi K, Tsubaki K, Kuriyama I, Mizushina Y, Yoshida H, Takeuchi T, Kamisuki S, Sugawara F, Kobayashi S. (**2013**) Synthesis, structure, and cytotoxicity studies of some fungal isochromanes. *Journal of Natural Products*, *76*, 1737-1745.

[8] Trisuwan K, Rukachaisirikul V, Sukpondma Y, Phongpaichit S, Preedanon S, Sakayaroj J. (**2010**) Furo [3, 2-h] isochroman, furo [3, 2-h] isoquinoline, isochroman, phenol, pyranone, and pyrone derivatives from the sea fan-derived fungus *Penicillium* sp. PSU-F40. *Tetrahedron*, *66*, 4484-4489.

[9] Stephens PJ, Pan JJ, Krohn KJ (**2007**) Determination of the absolute configurations of pharmacological natural products via density functional theory calculations of vibrational circular dichroism: the new cytotoxic iridoid prismatomerin. *Journal of Organic Chemistry*, *72*, 7641-7649.

[10] Wang WL, Lu ZY, Tao HW, Zhu TJ, Fang YC, Gu QQ, Zhu WM. (**2007**) Isoechinulin-type alkaloids, variecolorins A-L, from halotolerant *Aspergillus variecolor*. *Journal of Natural Products*, *70*, 1558-1564.

[11] Nampoothiri SV, Prathapan A, Cherian OL, Raghu KG, Venugopalan VV, Sundaresan A. (**2011**) *In vitro* antioxidant and inhibitory potential of *Terminalia bellerica* and *Emblica officinalis* fruits against LDL oxidation and key enzymes linked to type 2 diabetes. *Food and Chemical Toxicology*, *49*, 125-131.

Pericocins A−D, New Bioactive Compounds from *Periconia* sp.

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One new dihydroisocoumarin, pericocin A (**1**), one new chromone, pericocin B (**2**), and two new α-pyrone derivatives, pericocins C−D (**3−4**), together with two known compounds, 3-(2-oxo-2*H*-pyran-6-yl)propanoic acid (**5**) and (*E*)-3-(2-oxo-2*H*-pyran-6-yl)acrylic acid (**6**), were isolated from the culture of the endolichenic fungus *Periconia* sp.. Their structures were elucidated by spectroscopic methods. All these compounds are derived from the polyketone biosynthetic pathway. Compound **1** was obtained as a mixture of enantiomers. The antimicrobial activity of compounds **1−5** was tested against *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger*, and *Candida albicans*. Compounds **1−5** showed moderate antimicrobial activity against *A. niger* and weak activity against *C. albicans*.

Keywords: *Periconia* sp., Endolichenic fungus, Pericocin, Antimicrobial activity.

The *Periconia* genus is a rich source of bioactive compounds, such as cell-adhesion inhibitors (macrosphelides and peribysins) [1a-1d], antimicrobial agents (periconicins) [2a,2b], cytotoxic cytochalasans (periconiasins) [3a], and anti-inflammatory agents (periconianones) [3b]. In our early chemical investigation of a strain of this genus (19-4-2-1), some sesquiterpenes were isolated [3c,3d]. To search for more new bioactive secondary metabolites, a continuing chemical investigation of the strain (19-4-2-1) was carried out, which led to the isolation of four new compounds, pericocins A−D (**1−4**), along with two known compounds, 3-(2-oxo-2*H*-pyran-6-yl)propanoic acid (**5**) and (*E*)-3-(2-oxo-2*H*-pyran-6-yl)acrylic acid (**6**) (Figure 1). Herein, we report the isolation and structure elucidation, together with the antimicrobial activity of **1−5**.

Compound **1** was obtained as a white powder. The HR-ESI-MS showed a quasi-molecular ion peak at *m/z* 271.0587 [M + Na]$^+$, which indicated that the molecular formula should be $C_{13}H_{12}O_5$ (eight degrees of unsaturation). The ^{13}C NMR spectrum showed 13 carbons signals, which was consistent with the deduction of the HRESIMS. Combined with the DEPT-135 experiment, the carbons can be classified into six sp^2 quaternary carbons [including two ester carbonyl/carboxyl carbons (δ_C 171.0, 169.6)], four sp^2 methine carbons, one oxygenated sp^3 methine carbon (δ_C 79.0), one sp^3 methylene carbon (δ_C 33.1), and one methyl carbon (δ_C 15.4). The ^1H NMR spectrum displayed four olefinic/aromatic proton signals [δ_H 7.35 (1H, d, *J* = 7.5 Hz), 6.95 (1H, dd, *J* = 15.7, 4.7 Hz), 6.72 (1H, d, *J* = 7.5 Hz), and 6.16 (1H, d, *J* = 15.6 Hz)], one oxymethine signal [δ_H 5.32 (1H, m)], one methylene signal [δ_H 3.17 (1H, dd, *J* = 16.3, 3.8 Hz), 3.02 (1H, dd, *J* = 16.3, 10.6 Hz)], and one methyl signal [δ_H 2.21 (3H, s)]. All the proton resonances were assigned to relevant carbon atoms through the HSQC experiment. An analysis of the ^1H-^1H COSY spectrum revealed the presence of two isolated spin-systems corresponding to the C-4−C-3−C-9−C-10 and C-5−C-6 subunits. Combined with the ^1H-^1H COSY analysis and
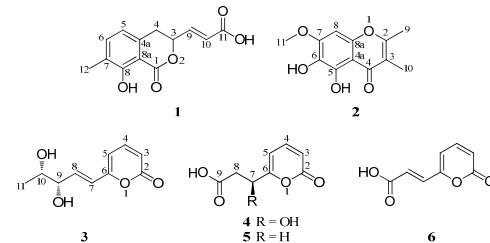
Figure 1: Chemical structures of **1−6**.
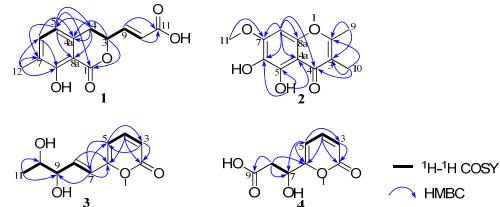
Figure 2: ^1H-^1H COSY and key HMBC correlations of **1−4**.

the molecular formula, the HMBC correlations from H-3 to C-1/C-4a, from Ha-4/Hb-4 to C-4a/C-5/C-8a, from H-5 to C-1/C-4/C-7/C-8a, from H-6 to C-8/C-4a, from H-9/H-10 to C-11, and from H$_3$-12 to C-6/C-7/C-8 deduced the planar structure of **1**, as shown in Figure 2, and the assignments of all proton and carbon resonances are shown in Table 1. In an optical rotation experiment, compound **1** showed almost no optical activity. Compound **1** displayed a single peak in achiral analytical HPLC, but two peaks (20.8 min with relative peak area of 22.3% and 24.0 min with relative peak area of 77.7%) in chiral HPLC analysis, which indicated that **1** is a mixture of enantiomers. The geometry of the Δ^9 double bond was determined as 9*E* by the value of $^3J_{H-9/H-10}$ (15.7 Hz) observed in the ^1H NMR spectrum. Based on the above analysis, the structure of **1** was established as (*E*)-3-(8-hydroxy-7-methyl-1-oxoisochroman-3-yl)acrylic acid, and named pericocin A.

Compound **2** was obtained as a white powder. The HR-ESI-MS showed a quasi-molecular ion peak at *m/z* 237.0769 [M + H]$^+$, which indicated that the molecular formula should be $C_{12}H_{12}O_5$ (seven degrees of unsaturation). The ^{13}C NMR spectrum showed 12 carbons signals, which was consistent with the deduction of the HRESIMS. Combined with the DEPT-135 experiment, the carbons can be classified into eight sp^2 quaternary carbons [including one α,β-conjugated keto carbonyl carbon (δ$_C$ 181.4)], one sp^2 methine carbon (δ$_C$ 90.3), one methoxyl carbon (δ$_C$ 56.2), and two methyl carbons (δ$_C$ 18.3, 8.8). The ^1H NMR spectrum displayed one olefinic/aromatic proton signal [δ$_H$ 6.68 (1H, s)], one methoxyl signal [δ$_H$ 3.87 (3H, s)], two methyl signals [δ$_H$ 2.39 (3H, br s), 1.92 (3H, br s)], and two exchangeable hydrogen proton signals [δ$_H$ 12.71 (1H, s), 8.61 (1H, s)]. All the non-exchangeable proton resonances were assigned to relevant carbon atoms through the HSQC experiment. Combined with the molecular formula, the HMBC correlations from H-8 to C-4/C-6/C-7/C-4a/C-8a, from H$_3$-9 to C-2/C-3, from H$_3$-10 to C-2/C-3/C-4, from H$_3$-11 to C-7, and from 5-OH to C-5/C-6/C-4a deduced the structure of **2** as shown in Figure 2; the assignments of all proton and carbon resonances are shown in Table 1. Based on the above analyses, the structure of **2** was established as 5,6-dihydroxy-7-methoxy-2,3-dimethyl-4*H*-chromen-4-one, and named pericocin B.

Compound **3** was obtained as a brown oil. The HR-ESI-MS showed a quasi-molecular ion peak at *m/z* 197.0809 [M + H]$^+$, which indicated that the molecular formula should be $C_{10}H_{12}O_4$ (five degrees of unsaturation). The ^{13}C NMR spectrum showed 10 carbons signals, which was consistent with the deduction of the HRESIMS. Combined with the DEPT-135 experiment, the carbons can be classified into two sp^2 quaternary carbons, five sp^2 methine carbons, two sp^3 oxygenated methine carbons (δ$_C$ 76.6, 71.5), and one methyl carbon (δ$_C$ 18.9). The ^1H NMR spectrum displayed five olefinic/aromatic proton signals [δ$_H$ 7.50 (1H, dd, *J* = 9.2, 6.7 Hz), 6.74 (1H, dd, *J* = 15.7, 5.4 Hz), 6.38 (1H, d, *J* = 15.7 Hz), 6.30 (1H, d, *J* = 6.7 Hz), and 6.22 (1H, d, *J* = 9.3 Hz)], two oxymethine signals [δ$_H$ 4.09 (1H, br t, *J* = 5.0 Hz), 3.72 (1H, quint, *J* = 6.3 Hz)], and one methyl signal [δ$_H$ 1.18 (3H, d, *J* = 6.4 Hz)]. All the proton resonances were assigned to relevant carbon atoms through the HSQC experiment. An analysis of the ^1H-^1H COSY spectrum revealed the presence of two isolated spin-systems corresponding to the C-3–C-4–C-5 and C-7–C-8–C-9–C-10–C-11 subunits. When **3** was tested in DMSO-*d$_6$* (Table S4, Supporting Information), exchangeable protons were observed and two additional spin-systems (H-9 and 9-OH, H-10 and 10-OH) were revealed. Combined with the ^1H-^1H COSY analysis, the molecular formula, and the HMBC correlations from H-3 to C-2/C-5, from H-4 to C-2/C-6, from H-5 to C-3/C-7, from H-7 to C-5/C-6/C-9, from H-8 to C-6, and from H$_3$-11 to C-9/C-10 deduced the planar structure of **3** as shown in Figure 2; the assignments of all proton and carbon resonances are shown in Table 1.

The geometry of the Δ^7 double bond was determined as 7*E* by the value of $^3J_{H\text{-}7/H\text{-}8}$ (15.7 Hz) observed in the ^1H NMR spectrum. In the ROESY experiment in DMSO-*d$_6$* (Table S4, Supporting Information), the observed correlations between H-9 and 10-OH/H$_3$-11, between H$_3$-11 and H-8, and between H-10 and 9-OH/H-8 signified that the relative configuration of **3** was *threo* (Figure 3), combined with the value of $^3J_{H\text{-}9/H\text{-}10}$ (6.1 Hz). The absolute configuration of the 9,10-diol moiety in **3** was determined by the *in situ* dimolybdenum CD method. On addition of Mo$_2$(OAc)$_4$ to a solution of **3** in DMSO, a metal complex as an auxiliary chromophore was produced. The induced CD of the complex was obtained by subtracting the inherent CD. The observed sign of the
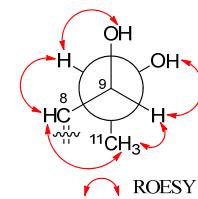
Figure 3: key ROESY correlations in DMSO-*d$_6$* of **3**.
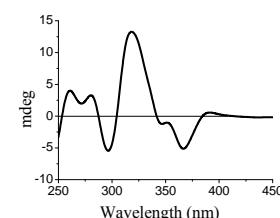
Figure 4: CD spectrum of **3** in DMSO containing Mo$_2$(OAc)$_4$ with the inherent CD spectrum subtracted.

Cotton effect in the induced spectrum comes from the chirality of the vic-diol moiety expressed by the sign of the O-C-C-O torsion angle. In this study, positive Cotton effects were observed at 310 and 400 nm, respectively. The induced CD spectrum (Figure 4) permitted the assignment of 9*S* and 10*S* on the basis of the empirical rule proposed by Snatzke [4a,b]. Thus, the structure of **3** was established as 6-((3*S*,4*S*,*E*)-3,4-dihydroxypent-1-en-1-yl)-2*H*-pyran-2-one, and named pericocin C.

Compound **4** was obtained as a brown oil. The HR-ESI-MS showed a quasi-molecular ion peak at *m/z* 185.0447 [M + H]$^+$, which indicated that the molecular formula should be $C_8H_9O_5$ (five degrees of unsaturation). The ^{13}C NMR spectrum showed 8 carbon signals, which was consistent with the deduction of the HRESIMS. Combined with the DEPT-135 experiment, the carbons can be classified into three sp^2 quaternary carbons, three sp^2 methine carbons, one oxygenated sp^2 methine carbon (δ$_C$ 68.3), and one sp^3 methylene carbon (δ$_C$ 40.9). The ^1H NMR spectrum displayed three olefinic/aromatic proton signals [δ$_H$ 7.52 (1H, dd, *J* = 9.3, 6.6 Hz), 6.45 (1H, d, *J* = 6.6 Hz), and 6.22 (1H, d, *J* = 9.3 Hz)], one oxymethine signal [δ$_H$ 4.80 (1H, dd, *J* = 8.6, 4.4 Hz)], and one methylene signal [δ$_H$ 2.82 (1H, dd, *J* = 15.7, 4.4 Hz), 2.63 (1H, dd, *J* = 15.7, 8.6 Hz)]. All the proton resonances were assigned to relevant carbon atoms through the HSQC experiment. An analysis of the ^1H-^1H COSY spectrum revealed the presence of two isolated spin-systems corresponding to the C-3–C-4–C-5 and C-7–C-8 subunits. Combined with the ^1H-^1H COSY analysis and the molecular formula, the HMBC correlations from H-3 to C-2, from H-4 to C-2/C-6, from H-5 to C-3/C-6/C-7, and from Hb-8 to C-6/C-7/C-9 deduced the planar structure of **4** as shown in Figure 2; the assignments of all proton and carbon resonances are shown in Table 1.

The absolute configuration at C-7 of compound **4** was assigned by comparison of the Cotton effect and specific rotation with those of nodulisporipyrone A [5a], whose absolute configuration at C-7 was *R*. The CD spectrum of nodulisporipyrone A showed a positive Cotton effect at 280 nm, whereas a negative Cotton effect was seen in the CD spectrum of **4**. Likewise, nodulisporipyrone A showed a positive specific rotation ([α]$_D$^{23} +67.6), whereas **4** gave a negative value ([α]$_D$^{27} –49.7). Therefore, the absolute configuration of **4** was assigned as 7*S*. Based on the above analyses, the structure of **4** was established as (*S*)-3-hydroxy-3-(2-oxo-2*H*-pyran-6-yl)propanoic acid, and named pericocin D.

Table 1: ^{1}H NMR and ^{13}C NMR data of **1**–**4** (δ in ppm, *J* in Hz).

No.	1 (CD₃OD) δ$_C$ (150 MHz)	δ$_H$ (600 MHz)	2 (DMSO-d_6) δ$_C$ (100 MHz)	δ$_H$ (400 MHz)	3 (CD₃OD) δ$_C$ (100 MHz)	δ$_H$ (400 MHz)	4 (CD₃OD) δ$_C$ (100 MHz)	δ$_H$ (400 MHz)
1	171.0							
2			163.3		164.1		164.1	
3	79.0	5.32, m	113.6		114.9	6.22, d (9.3)	114.9	6.22, d (9.3)
4	33.1	3.17, dd (16.3, 3.8), Ha; 3.02, dd (16.3, 10.6), Hb	181.4		146.4	7.50, dd (9.2, 6.7)	146.1	7.52, dd (9.3, 6.6)
4a	137.7		104.0					
5	118.8	6.72, d (7.5)	146.0		106.1	6.30, d (6.7)	103.2	6.45, d (6.6)
6	138.4	7.35, d (7.5)	129.5		160.6		167.5	
7	126.3		154.2		123.1	6.38, d (15.7)	68.3	4.80, dd (8.6, 4.4)
8	161.5		90.3	6.68, s	138.7	6.74, dd (15.7, 5.4)	40.9	2.82, dd (15.7, 4.4), Ha; 2.63, dd (15.7, 8.6), Hb
8a	108.6		149.6					
9	144.1	6.95, dd (15.7, 4.7)	18.3	2.39, br s	76.6	4.09, br t (5.0)	173.9	
10	124.9	6.16, d (15.6)	8.8	1.92, br s	71.5	3.72, quint (6.3)		
11	169.6		56.2	3.87, s	18.9	1.18, d (6.4)		
12	15.4	2.21, s						
5-OH				12.71, s				
6-OH				8.61, s				

The structures of 3-(2-oxo-2*H*-pyran-6-yl)propanoic acid (**5**), and (*E*)-3-(2-oxo-2*H*-pyran-6-yl)acrylic acid (**6**) were confirmed by comparison of their NMR data with literature values [5b,6a]. Compounds **1**–**5** were tested for antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger*, and *Candida albicans*, using tobramycin (bacteria) and cycloheximide (fungi) as the positive controls. Compounds **1**–**5** displayed moderate activities against *A. niger* and weak activities against *C. albicans* (Table 2).

Table 2: Antimicrobial activity of **1**–**5** (MIC, μg/mL).

compounds	Bacteria *S. aureus*	*E. coli*	Fungi *A. niger*	*C. albicans*
1	>1000	>1000	31	500
2	>1000	>1000	31	500
3	>1000	>1000	31	500
4	>1000	>1000	31	500
5	>1000	>1000	31	500
Tobramycin [a]	<16	<16	nt	nt
Cycloheximide [b]	nt	nt	<16	<16

[a] Positive control (bacteria). [b] Positive control (fungi). nt: not tested

This work reports the isolation of six metabolites, including four new compounds {one dihydroisocoumarin (**1**), one chromone (**2**), and two α-pyrone derivatives (**3**–**4**)}, and two known compounds (α-pyrone derivatives (**5**–**6**)). To our best knowledge, compounds **3**–**6** are the first α-pyrone derivatives from *Periconia* fungi.

Experimental

General: UV, JASCO V-550 UV/Vis spectrophotometer; IR, JASCO FT/IR-480 plus Fourier transform infrared spectrometer; HR-ESI-MS, Waters Synapt G2 TOF mass spectrometer; NMR, Bruker AV-400 and Bruker AV-600 spectrometers; CC, Sephadex LH-20 (Pharmacia) and ODS (60–80 mm, YMC); TLC, precoated silica gel plates (SGF$_{254}$, 0.2 mm, Yantai Chemical Industry Research Institute). The analytical HPLC was performed on a Shimadzu HPLC system equipped with a LC-20AB pump, and a SPD-20A diode array detector (Shimadzu) using a Phenomenex Gemini C18 column (5 μm, 4.6 mm × 250 mm, Phenomenex Inc.). The chiral analytical HPLC was performed on a Shimadzu HPLC system equipped with a LC-20AB pump, and a SPD-20A diode array detector (Shimadzu) using a Phenomenex Lux Amylose-2 column (5 μm, 4.6 mm × 250 mm, Phenomenex Inc.) at 1.0 ml/min under 60%MeOH-H$_2$O (0.1%HCOOH). The semi-preparative HPLC was performed on a Shimadzu LC-6AD system equipped with an LC-6AD pump and a SPD-M20A detector (Shimadzu), using a YMC Park ODS-A column (5 μm, 10 mm × 250 mm, YMC).

Fungal material: The strain of *Periconia* sp. (No.19-4-2-1) was isolated by one of the authors (L.D. Guo) from the lichen *Parmelia* sp. collected from Changbai Mountain, Jilin Province, China, in August 2006. The fungus strain was identified as *Periconia* sp. based on the morphological characteristics and sequence analysis of the internal transcribed spacer (ITS) regions ITS1-5.8S-ITS2 (GenBank accession No. KP873157). The strain was assigned the accession number 19-4-2-1 in the culture collection at the Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou. The fungus was cultured on slants of potato dextrose agar at 25°C for 5 days. Agar plugs were used to inoculate 4 Erlenmeyer flasks (250 mL), each containing 100 mL of potato dextrose broth. Four flasks of the inoculated media were incubated at 25°C on a rotary shaker at 200 rpm for 5 days to prepare the seed culture. Fermentation was carried out in 20 Erlenmeyer flasks (500 mL), each containing 70 g of rice. Distilled H$_2$O (105 mL) was added to each flask, and the rice was soaked overnight before autoclaving at 120°C for 30 min. After cooling to room temperature, each flask was inoculated with 5.0 mL of the spore inoculum and incubated at room temperature for 45 days.

Extraction and isolation: The culture was extracted thrice with EtOAc, and the organic solvent was evaporated to dryness under vacuum to afford a crude extract (33.4 g). This was dissolved in 90%, v/v, aqueous MeOH (500 mL) and partitioned against the same volume of cyclohexane to afford a cyclohexane fraction (C, 24.5 g) and an aqueous MeOH fraction (W, 8.7 g). The aqueous MeOH fraction (W, 8.7 g) was separated by ODS CC eluting with MeOH–H$_2$O (30:70, 50:50, 70:30, and 100:0, v/v) to afford 4 fractions (W1 to W4). Fraction W2 (2.3 g) was subjected to ODS CC with a gradient of MeOH–H$_2$O (35:65, 40:60, 45:55, 50:50, 55:45, and 100:0 v/v) to give 8 sub-fractions (W2a–W2h). Sub-fraction W2e (533.6 mg) was separated by Sephadex LH-20 (MeOH) and semi-preparative HPLC (55% MeOH–H$_2$O) to yield **1** (5.3 mg). Purification of sub-fraction W2d (199.9 mg) was carried out by Sephadex LH-20 (MeOH) and semi-preparative HPLC (45% MeOH–H$_2$O) to yield **2** (6.9 mg). Fraction W1 (1.96 g) was subjected to ODS CC with a gradient of MeOH–H$_2$O (10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 40:60, 45:55, and 100:0 v/v) to give 6 sub-fractions (W1a-W1f). Sub-fraction W1a (392.2 mg) was purified by semi-preparative HPLC (15% MeOH–H$_2$O) to yield **3** (29.9 mg), **4** (13.1 mg), **5** (35.5 mg), and **6** (3.5 mg).

Pericocin A (1)
White powder.
IR (KBr): 1698, 1676, 1427, 1287, 1138, 974 cm^{-1}.
UV (MeOH) λ_{max} (log ε): 210 (4.20), 252 (3.58), 320 (3.36) nm.

¹H and ¹³C NMR: Table 1.
HR-ESI-MS: *m/z* 271.0587 [M + Na]⁺ (calcd for $C_{13}H_{12}O_5Na$, 271.0582).

Pericocin B (2)

White powder.
IR (KBr): 3566, 3472, 1671, 1484, 1250, 1094, 803 cm⁻¹.
UV (MeOH) λ_{max} (log ε): 211 (4.27), 240 (4.05), 295 (3.75) nm.
¹H and ¹³C NMR: Table 1.
HR-ESI-MS: *m/z* 237.0769 [M + H]⁺ (calcd for $C_{12}H_{13}O_5$, 237.0763).

Pericocin C (3)

Brown oil.
$[\alpha]_D^{27}$: +9.9 (*c* 1.0, MeOH).
IR (KBr): 3429, 2979, 1712, 1650, 1538, 1378, 1110, 804 cm⁻¹.
UV (MeOH) λ_{max} (log ε): 205 (3.98), 229 (3.96), 327 (3.90) nm.
¹H and ¹³C NMR: Table 1.
HR-ESI-MS: *m/z* 197.0809 [M + H]⁺ (calcd for $C_{10}H_{13}O_4$, 197.0814).

Pericocin D (4)

Brown oil.
$[\alpha]_D^{27}$: −49.7 (*c* 1.0, MeOH).
IR (KBr): 3401, 1716, 1634, 1558, 1402, 813 cm⁻¹.
UV (MeOH) λ_{max} (log ε): 204 (3.22), 297 (3.41) nm.
CD (*c* 2.0×10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$): 231 (−0.85), 286 (−3.39).
¹H and ¹³C NMR: Table 1.
HR-ESI-MS: *m/z* 185.0447 [M + H]⁺ (calcd for $C_8H_9O_5$, 185.0450).

Antimicrobial assay: Compounds were tested for antimicrobial activity using a paper disk-diffusion assay [6b,c]. Seed cultures of two bacteria (*S. aureus* 209P, *E. coli* ATCC0111) and two fungi (*A. niger* R330, *C. albicans* FIM709) were prepared by incubating the organism for 12 h at either 37°C (bacteria) or 32°C (fungi). Aliquots of the overnight cultures (80 μL) were spread onto the surfaces of either nutrient agar (bacteria) or Sabouraud's dextrose agar (fungi). Sterile filter disks (6 mm diameter) infused with 6 μL of test solution (in DMSO), positive control and vehicle only (DMSO) were added to the plates. The plates were left upright for 30 min at room temperature before being placed in an incubator at either 37°C (bacteria) or 32°C (fungi) for 12 h, and then the diameter of the zone of growth inhibition for each disk was recorded. The continuous twofold dilution methods were used to evaluate the minimal inhibitory concentrations (MICs). The MICs were defined as the lowest concentration at which no microbial growth could be observed.

References

[1]   (a) Yamada T, Iritani M, Doi M, Minoura K, Ito T, Numata A. (**2001**) Absolute stereostructures of cell-adhesion inhibitors, macrosphelides C, E-G and I, produced by a *Periconia* species separated from an *Aplysia* sea hare. *Journal of the Chemical Society, Perkin Transactions 1*, 3046-3053; (b) Yamada T, Iritani M, Minoura K, Numata A. (**2002**) Absolute stereostructures of cell adhesion inhibitors, macrosphelides H and L, from *Periconia byssoides* OUPS-N133. *The Journal of Antibiotics*, **55**, 147-154; (c) Yamada T, Iritani M, Minoura K, Kawai K, Numata A. (**2004**) Peribysins A-D, potent cell-adhesion inhibitors from a sea hare-derived culture of *Periconia* species. *Organic and Biomolecular Chemistry*, **2**, 2131-2135; (d) Yamada T, Doi M, Miura A, Harada W, Hiramura M, Minoura K, Tanaka R, Numata A. (**2005**) Absolute stereostructures of cell-adhesion inhibitors, peribysins A, E, F and G, produced by a sea hare-derived *Periconia* sp. *The Journal of Antibiotics*, **58**, 185-191.
[2]   (a) Kim S, Shin DS, Lee T, Oh KB. (**2004**) Periconicins, two new fusicoccane diterpenes produced by an endophytic fungus *Periconia* sp. with antibacterial activity. *Journal of Natural Products*, **67**, 448-450; (b) Shin DS, Oh MN, Yang HC, Oh KB. (**2005**) Biological characterization of periconicins, bioactive secondary metabolites, produced by *Periconia* sp. OBW-15. *Journal of Microbiology and Biotechnology*, **15**, 216-220.
[3]   (a) Zhang D, Ge H, Xie D, Chen R, Zou JH, Tao X, Dai J. (**2013**) Periconiasins A–C, new cytotoxic cytochalasans with an unprecedented 9/6/5 tricyclic ring system from endophytic fungus *Periconia* sp. *Organic Letters*, **15**, 1674-1677; (b) Zhang D, Ge H, Zou JH, Tao X, Chen R, Dai J. (**2014**) Periconianone A, a new 6/6/6 carbocyclic sesquiterpenoid from endophytic fungus *Periconia* sp. with neural anti-inflammatory activity. *Organic Letters*, **16**, 1410-1413; (c) Wu YH, Chen GD, Wang CX, Hu D, Li XX, Lian YY, Lin F, Guo LD, Gao H. (**2015**) Pericoterpenoid A, a new bioactive cadinane-type sesquiterpene from *Periconia* sp. *Journal of Asian Natural Products Research*, **17**, 671-675; (d) d) Wu YH, Chen GD, He RR, Wang CX, Hu D, Wang GQ, Guo LD, Yao XS, Gao H. (**2015**) Pericolactines A-C, a new class of diterpenoid alkaloids with unusual tetracyclic skeleton. *Scientific Reports*, **5**, 17082.
[4]   (a) Bari LD, Pescitelli G, Pratelli, C, Pini D, Salvadori P. (**2001**) Determination of absolute configuration of acyclic 1,2-diols with $Mo_2(OAc)_4$. 1. Snatzke's method revisited. *Journal of Organic Chemistry*, **66**, 4819-4825; (b) Gorecki M, Jablonska E, Kruszewska A, Suszczynska A, Urbanczyk-Lipkowska Z, Gerards M, Morzycki JW, Szczepek WJ, Frelek J. (**2007**) Practical method of the absolute configuration assignment of tert/tert 1,2-diols using their complexes with $Mo_2(OAC)_4$. *Journal of Organic Chemistry*, **72**, 2906-2916.
[5]   (a) Zhao Q, Wang CX, Yu Y, Wang GQ, Zheng QC, Chen GD, Lian YY, Lin F, Guo LD, Gao H. (**2015**) Nodulisporipyrones A–D, new bioactive α-pyrone derivatives from *Nodulisporium* sp. *Journal of Asian Natural Products Research*, **17**, 567–575; (b) Poole PR, Whitaker G. (**1997**) Biotransformation of 6-pentyl-2-pyrone by *Botrytis cinerea* in liquid cultures. *Journal of Agricultural and Food Chemistry*, **45**, 249-252.
[6]   (a) Cooney JM, Lauren DR. (**1999**) Biotransformation of the *Trichoderma* metabolite 6-*n*-pentyl-2*H*-pyran-2-one (6PAP) by selected fungal isolates. *Journal of Natural Products*, **62**, 681-683; (b) Groblacher B, Maier V, Kunert O, Bucar F. (**2012**) Putative mycobacterial efflux inhibitors from the seeds of *Aframomum melegueta*. *Journal of Natural Products*, **75**, 1393-1399; (c) Shen CC, Syu WJ, Li SY, Lin CH, Lee GH, Sun CM. (**2002**) Antimicrobial activities of naphthazarins from *Arnebia euchroma*. *Journal of Natural Products*, **65**, 1857-1862.

New Benzenoids from the Roots of *Lindera aggregata*

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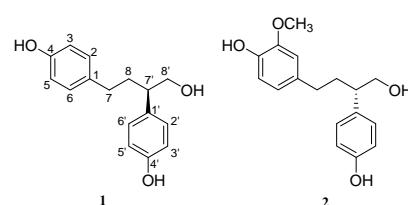
Two new benzenoids, linderagatin A and B (**1-2**), were isolated from the roots of *Lindera aggregata*. Their structures were elucidated on the basis of 1D (^{1}H, ^{13}C) and 2D NMR (COSY, NOESY, HSQC and HMBC) spectra. Moreover, their absolute configurations were established from ECD spectra compared with previous reports.

Keywords: *Lindera aggregata*, Lauraceae, Benzenoids, NMR spectroscopy, ECD.

Lindera aggregata (Sims) Kosterm (Lauraceae), also known as Wu Yao, a famous traditional Chinese medicine, has been used to treat abdominal distension and pain, acute asthma, and rheumatic diseases [1, 2]. In addition, recent studies showed the plant has other pharmacological effects, such as antioxidant [3], increased insulin sensitivity [3], anti-tumor [4] and anti-inflammatory [5]. Previous phytochemical studies on Wu Yao have led to the isolation of sesquiterpenes [6], alkaloids [7, 8], flavonoids [9] and lignans [10]. In this study, two new benzenoids, linderagatin-A (**1**) and linderagatin-B (**2**), were isolated from *L. aggregata*, as well as 27 known compounds, *N-trans*-feruloyltyramine (**3**) [11], *N-cis*-feruloyltyramine (**4**)[11], *N-trans*-feruloylmethoxytyramine (**5**) [12], thalifoline (**6**) [13], yuzirine (**7**) [14], linderaggrine-A (**8**) [8], northalifoline (**9**) [15], (+)-boldine (**10**) [16], (+)-*N*-methyllaurotetanine (**11**) [17], (+)-isoboldine (**12**) [11], 3-hydroxy-1-(4-hydroxyphenyl)propan-1-one (**13**) [18], *p*-hydroxybenzoic acid (**14**) [12], 4-hydroxy-3-methoxy acetophenone (**15**) [19], methyl 3,5-dimethoxy-4-hydroxy-benzoate (**16**) [20], vanillic acid (**17**) [12], tyrosol (**18**) [21], 2-(4-hydroxy-3-methoxyphenyl)ethanol (**19**) [22], 2-(4-hydroxy-3,5-dimethoxyphenol)ethanol (**20**) [23], 3-hydroxy-1-(4-hydroxyphenyl)propan-1-one (**21**) [18], rel-(2α, 3β)-7-*O*-methylcedrusin (**22**) [24], (-)-lyoniresinol (**23**) [25], hydroxylindestrenolide (**24**) [6], 2,6-dimethoxy-*p*-quinone (**25**) [26], evofolin-B(**26**) [27], (-)-boscialin (**27**) [28], methyl dihydrophaseate (**28**) [29], and 6′-*O*-vanilloyltachioside (**29**) [30]. Here, we report the structure elucidation of the two new benzenoids.

Linderagatin-A (**1**) was obtained as a colorless powder. Its molecular formula was determined as $C_{16}H_{18}O_3$ on the basis of its positive HRESIMS (m/z 281.1153 [M + Na]$^+$; calc. 281.1154). The complete ^{1}H and ^{13}C NMR data are shown in Table 1. The ^{1}H NMR spectrum showed two sets of AA′XX′ systems at δ_H 7.07 (2H, d, $J =$ 8.4 Hz, H-2′, 6′), 6.79 (2H, d, $J =$ 8.4 Hz, H-3′, 5′), as well as signals at 6.94 (2H, d, $J =$ 8.4 Hz, H-2, 6) and 6.72 (2H, d, $J =$ 8.4 Hz, H-3, 5). Furthermore, two methylene groups at δ_H 3.60 (2H, m, H-8′) and 2.37 (2H, m, H-7), and a methine group at δ_H 2.64 (1H, m,

Figure 1: Structure of compounds **1-2**.

Table 1: ^{1}H and ^{13}C NMR spectroscopic data of compounds **1** (acetone-d_6) and **2** (CDCl$_3$).

position	**1**[a]		**2**[b]	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	134.1		133.9	
2	130.0	6.94 d (8.4)	111.0	6.59 br s
3	115.8	6.72 d (8.4)	146.3	
3-OCH$_3$			55.9	3.85 s
4	156.2		143.6	
5	115.8	6.72 d (8.4)	114.2	6.80 d (8.0)
6	130.0	6.94 d (8.4)	120.9	6.60 dd (8.0, 1.6)
7	33.3	2.37 m	33.1	2.44 m
8a	35.2	2.11 m	33.9	1.97 m
8b		1.76 m		1.84 m
1′	134.7		134.0	
2′	129.8	7.07 d (8.4)	129.3	7.10 d (8.4)
3′	115.9	6.79 d (8.4)	115.6	6.83 d (8.4)
4′	156.6		154.4	
5′	115.9	6.79 d (8.4)	115.6	6.83 d (8.4)
6′	129.8	7.07 d (8.4)	129.3	7.10 d (8.4)
7′	48.3	2.64 m	47.1	2.74 m
8′	67.9	3.60 m	67.7	3.69 m

H-7′) were found, while methylenes at δ_H 2.11 (1H, m, H-8a) and 1.76 (2H, m, H-8b) were observed in the ^{1}H NMR and HSQC spectra. Moreover, the COSY spectrum showed correlations that indicated the presence of the spin systems H-7, H-8, H-7′ and H-8′ with a partial -CH$_2$CH$_2$(CH)CH$_2$OH structure. In the HMBC experiment, the signal at δ_H 6.94 (H-2, 6) correlated with the carbons at δ_C 33.3 (C-7), and the signal at 7.07 (H-2′, 6′) correlated with δ_C 48.3 (C-7′). The above results showed that C-7

and C-7′ are substituted with a *p*-hydroxybenzyl group. Furthermore, the absolute configuration of **1** was determined from the ECD spectrum, in which positive Cotton effects at 206, 212 and 217 nm suggested that the absolute configuration at C-7′ of **1** is opposite to that of *S*-(+)-2-phenyl-1-butanol. Therefore, the absolute configuration at C-7′ were determined as 7′*R*. [31].

Linderagatin-B (**2**) was isolated as colorless syrup and its molecular formula was determined to be $C_{17}H_{20}O_4$ on the basis of its positive HRESIMS (*m/z* 311.1261 [M + Na]$^+$; calc. 311.1259). The ^{1}H and ^{13}C NMR spectra of **2** (Table 1) were similar to those of **1**, except for the H-3 proton being replaced by a methoxyl group, the signal for which was observed at δ_H 3.85 (s, 3-OCH$_3$) in the ^{1}H NMR spectrum and at δ_C 55.9 (3-OCH$_3$) in the ^{13}C NMR spectrum. In the HMBC spectrum, the proton signal at δ_H 6.59 (H-2) correlated with carbons at δ_C 33.1 (C-7), and HMBC correlations of peaks at δ_H 7.10 (H-2′) with δ_C 47.1 (C-7′) were also found. The above results showed that C-7 and C-7′ are substituted with a benzyl group. In addition, the proton signals at δ_H 6.59 (H-2) and 6.60 (H-6) were correlated with carbon at δ_C 143.6 (C-4) in the HMBC spectrum. And the proton signals at δ_H 3.85 (3-OCH$_3$) and 6.80 (H-5) were correlated with carbon at δ_C 146.3 (C-3). The methoxy group was thus assigned at C-3 by the NOESY correlation between δ_H 6.59 (H-2) and the methyl signal at δ_H 3.85 (3H, s, 3-OCH$_3$). The absolute configuration of **2** at C-7′ was assigned from the ECD spectrum, which showed negative Cotton effects at 207, 212, and 219 nm similar to those of *S*-(+)-2-phenyl-1-butanol in ethanol, indicating a 7′*S* configuration. [31]

Experimental

General: Optical rotations were measured with a JASCO P-2000 digital polarimeter in a 0.5 dm cell. UV spectra were obtained with a Hitachi UV-3210 spectrophotometer, and IR spectra with a Shimadzu FTIR Prestige-21 spectrometer. The ECD spectra were recorded on a JASCO J-720 spectrometer, and the ^{1}H and ^{13}C NMR spectra on a Bruker AVIII-400 with TMS as the internal reference, and chemical shifts expressed in δ (ppm). The ESIMS and HRESIMS were taken on a Bruker Daltonics APEX II 30e spectrometer. Silica gel (70–230 and 230–400 mesh; Merck) and Diaion HP-20 resin (Mitsubishi, Chemical, Tokyo, Japan) were used for column chromatography (CC), and silica gel 60 F$_{254}$ (Merck) for TLC.

Plant material: The roots of *Lindera aggregata* were collected from Huisun Forest Area, Ren Ai Township, Nantou County, Taiwan. The plant material was identified and authenticated by Assoc. Prof. Dr Chang-Sheng Kuoh, Department of Life Sciences, National Cheng Kung University. A voucher specimen (TSWu-20100601) was deposited in the Department of Chemistry, Natural Cheng Kung University, Tainan, Taiwan.

Extraction and isolation: The ground air-dried whole plants of *L. aggregata* (12.5 kg) were extracted with MeOH at 60°C by refluxing for 8 h. The MeOH extracts were combined and evaporated under reduced pressure to give *ca.* 1160 g residue.

The residue was suspended in water and then partitioned with EtOAc. After removing the solvent, the EtOAc-soluble portion (340 g) underwent partition extraction with 5% aqueous HCl solution. The HCl-aqueous solution was treated with 5% NH$_4$OH$_{(aq.)}$ to obtain a phase with pH 9 and then extracted with EtOAc. The organic layer was evaporated under reduced pressure to yield an alkaloid fraction residue (40 g). This was subjected to CC over silica gel and eluted with *n*-hexane and acetone by step gradients to afford 16 fractions on the basis of TLC analysis. Fraction 11 was separated by CC with benzene–ethyl acetate (19:1) to afford **15** (1.4 mg), **16** (7.7 mg), **24** (4.9 mg), and **25** (4.9 mg). Separation of fraction 13 was performed by silica gel chromatography with CHC1$_3$–MeOH (20:1) to give **1** (4.0 mg), **2** (2.3 mg), **3** (6.5 mg), **4** (2.2 mg), **5** (13.1 mg), **6** (1.4 mg), **7** (3.2 mg), **14** (0.8 mg), **17** (3.5 mg), **18** (5.3 mg), **19** (2.6 mg), **20** (2.7 mg), **21** (1.6 mg), **22** (8.6 mg), **26** (2.1 mg), **27** (14.9 mg), and **28** (3.0 mg). Fraction 14 was purified by CC with CHCl$_3$–MeOH (15:1) to afford **9** (4.7 mg) and **23** (3.4 mg). Fraction 15 was purified by CC with CHCl$_3$–MeOH (9:1) to give **10** (93.2 mg), **11** (5.4 mg), **12** (6.0 mg) and **29** (2.1 mg).

Linderagatin-A (1)
Colorless powder;
[α]$_D$: +99.0 (*c* 0.20, MeOH)
CD[θ]: [θ]$_{196}$+405, [θ]$_{199}$−61, [θ]$_{203}$−91, [θ]$_{206}$ +71, [θ]$_{210}$−413, [θ]$_{212}$ +329, [θ]$_{215}$ +423, [θ]$_{217}$+337, [θ]$_{224}$−133, [θ]$_{231}$ +175, [θ]$_{236}$+163, [θ]$_{240}$−113, [θ]$_{251}$+123, [θ]$_{267}$+153, [θ]$_{280}$+1380, [θ]$_{287}$+1602 (*c* 7.4 ×10^{-4}, EtOH)
IR ν$_{max}$ (KBr) cm^{-1}: 3356, 2924, 2854, 1652,1458 cm^{-1}.
UV (MeOH): λ_{max} (log ε): 218 (3.6), 229 (3.5, sh),273 (3.5),282 (3.4, sh) nm.
^{1}H and ^{13}C NMR: Table 1.
ESI-MS *m/z* (rel. int. %): 281([M+Na]$^+$)
HR ESI-MS *m/* (rel. int. %): 281.1153 ([M+Na]$^+$) (calcd for $C_{16}H_{18}O_3Na$, 281.1154).

Linderagatin-B (2)
Colorless powder.
[α]$_D$: -58.3 (*c* 0.11, MeOH).
CD[θ]:[θ]$_{196}$+300, [θ]$_{199}$−191, [θ]$_{202}$+167, [θ]$_{207}$−148, [θ]$_{210}$−413, [θ]$_{212}$−295, [θ]$_{219}$−64, [θ]$_{224}$+532, [θ]$_{229}$+671, [θ]$_{241}$−369, [θ]$_{252}$+161, [θ]$_{256}$+159, [θ]$_{269}$+300, [θ]$_{284}$+300, [θ]$_{293}$+300 (*c* 4.9 ×10^{-4}, EtOH)
IR ν$_{max}$ (KBr): 3333, 2928, 2855,1608, 1454 cm^{-1}
UV (MeOH): λ_{max} (log ε): 217 (3.8), 232 (3.7, sh),277 (3.6)nm.
^{1}H and ^{13}C NMR: Table 1.
ESI-MS *m/z* (rel. int. %): 331([M+Na]$^+$)
HR ESI-MS *m/z* (rel. int. %): 311.1261 ([M+Na]$^+$) (calcd for $C_{17}H_{20}O_4Na$, 311.1259).

Supplementary data: NMR spectra (^{1}H and ^{13}C NMR, COSY, NOESY, HSQC, and HMBC) for compounds **1** and **2**.

References

[1] Gan LS, Zheng YL, Mo JX, Liu X, Li XH, Zhou CX. (**2009**) Sesquiterpene lactones from the root tubers of *Lindera aggregata*. *Journal of Natural Product*, **72**, 1497-1501.
[2] Cheng XL, Ma SC, Wei F, Wang GL, Xiao XY, Lin RC. (**2007**) A new sesquiterpene isolated from *Lindera aggregata* (Sims) Kosterm. *Chemical and Pharmaceutical Bulletin*, **55**, 1390-1392.
[3] Wang F, Gao Y, Zhang L, Bai B, Hu YN, Dong ZJ, Zhai QW, Zhu HJ, Liu JK. (**2010**) A pair of windmill-shaped enantiomers from *Lindera aggregata* with activity toward improvement of insulin sensitivity. *Organic Letters*, **12**, 3196-3199.

[4]   Lin CT, Chu FH, Chang ST, Chueh PJ, Su YC, Wu KT, Wang SY. (**2007**) Secoaggregatalactone-A from *Lindera aggregata* induces apoptosis in human hepatoma hep G2 cells. *Planta Medica*, **73**, 1548-1553.

[5]   Zhang CF, Sun QS, Wang ZT, Chou GX. (**2001**) Studies on constituents of the leaves of *Lindera aggregata* (Sims) Kosterm. *Zhongguo Zhong Yao Za Zhi*, **26**, 765-767.

[6]   Kouno I, Hirai A, Jiang ZH, Tanaka T. (**1997**) Bisequiterpenoid from the root of *Lindera strychnifolia*. *Phytochemistry*, **46**, 1283-1284.

[7]   Kazuka M, Yoshikawa M, Sawada T. (**1984**) Alkaloids From *Lindera strychnifolia*. *Journal of Natural Products*, **47**, 1063-1063.

[8]   Kuo PC, Li YC, Hwang TL, Ma GH, Yang ML, Lee EJ, Wu TS. (**2014**) Synthesis and structural characterization of an anti-inflammatory principle purified from *Lindera aggregata*. *Tetrahedron Letters*, **55**, 108-110.

[9]   Zhang CF, Sun QS, Zhao YY, Zheng W. (**2001**) Studies on flavonoids from leaves of *Lindera aggregata* (Sims) Kosterm. *Chinese Journal of Medicinal Chemistry*, 274-276.

[10]   Ichino K, Tanaka H, Ito K. (**1988**) A lignan from *Lindera praecox*. *Phytochemistry*, **27**, 1906-1907.

[11]   Chang YC, Chen CY, Chang FR, Wu YC. (**2001**) Alkaloids from *Lindera glauca*. *Journal of the Chinese Chemical Society*, **48**, 811-815.

[12]   Chen CY, Wang YD, Wang HM. (**2010**) Chemical constituents from the roots of *Synsepalum dulcificum*. *Chemistry of Natural Compounds*, **46**, 448-449.

[13]   Lia Q, Zhanga SQ, Wanga SC, Zhoua MZ. (**2009**) Efficient synthesis of thalifoline and its analogs. *Synthetic Communications*, **39**, 1752-1758.

[14]   Ziyaev R, Irgashev T, Israilov IA, Abdullaev ND, Yunusov MS, Yunusov SY. (**1977**) Alkaloids of *Ziziphus jujuba* the structure of juziphine and juzirine. *Chemistry of Natural Compounds*, **13**, 204-207.

[15]   Chou CJ, Lin LC, Chen KT, Chen CF. (**1994**) Northalifoline, a new isoquinolone alkaloid from the pedicels of *Lindera megaphylla*. *Journal of Natural Products*, **57**, 689-694.

[16]   Jackman LM, Trewella JC, Moniot JL, Shamma M, Stephens RL, Wenkert E, Leboeuf M, Cavé A. (**1979**) The carbon-13 NMR spectra of aporphine alkaloids. *Journal of Natural Products*, **42**, 437-449.

[17]   Chen CY, Chang FR, Wu YC. (**1997**) The constituents from the stems of *Annona cherimola*. *Journal of the Chinese Chemical Society*, **44**, 313-319.

[18]   Achenbach H, Waibel R, Addae-mensah I.. (**1983**) Lignans and other constituents from *Carissa edulis*. *Phytochemistry*, **22**, 749-753.

[19]   Asghar SF, Rehman HU, Rahman AU. (**2010**) Phytochemical investigations on *Iris germanica*. *Natural Product Research*, **24**, 131-139.

[20]   Hristea EN, Covaci-Cîmpeanu IC, Ioniţǎ G, Ioniţǎ P, Draghici C, Caǎproiu MT, Hillebrand M, Constantinescu T, Balaban AT. (**2009**) Reactions of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with two syringylic phenols or one aroxide derivative. *European Journal of Organic Chemistry*, **2009**, 626-634.

[21]   Takaya Y, Furukawa T, Miura S, Akutagawa T, Hotta Y, Ishikawa N, Niwa M. (**2007**) Antioxidant constituents in distillation residue of Awamori spirits. *Journal of Agricultural and Food Chemistry*, **55**, 75-79.

[22]   Kodaira H, Ishikawa M , Komoda Y, Nakajima T. (**1983**) Isolation and identification of anti-platelet aggregation principles from the bark of *Fraxinus japonica* BLUME. *Chemical and Pharmaceutical Bulletin*, **31**, 2262-2268.

[23]   Yang F, Lian G, Yu B. (**2010**) Synthesis of raphanuside, an unusual oxathiane-fused thioglucoside isolated from the seeds of *Raphanus sativus* L. *Carbohydrate Research*, **345**, 309-314.

[24]   Seidel V, Bailleul F, Waterman PG. (**2000**) Novel oligorhamnosides from the stem bark of *Cleistopholis glauca*. *Journal of Natural Products*, **63**, 6-11.

[25]   Hanawa F, Shiro M, Hayash Y. (**1997**) Heartwood constituents of *Betula maximowicziana*. *Phytochemistry*, **45**, 589-595.

[26]   Chang YC, Chang FR, Wu YC. (**2000**) The Constituents of *Lindera Glauca*. *Journal of the Chinese Chemical Society*, **47**, 373-380.

[27]   Wu TS, Yeh JH, Wu PL. (**1995**) The heartwood constituents of *Tetradium glabrifolium*. *Phytochemistry*, **40**, 121-124.

[28]   Wattanapiromsakul C, Forster PI, Waterman PG. (**2003**) Alkaloids and limonoids from *Bouchardatia neurococca*: systematic significance. *Phytochemistry*, **64**, 609-615.

[29]   Cui B, Nakamura M, Kinjo J, Nohara T. (**1993**) Chemical constituents of *Astragali semen*. *Chemical and Pharmaceutical Bulletin*, **64**, 178-182.

[30]   Yang XW, Wang JS, Ma YL, Xiao HT, Zuo YQ, Lin H, He HP, Li L, Hao XJ. (**2007**) Bioactive phenols from the leaves of *Baccaurea ramiflora*. *Planta Medica*, **73**, 1415-1417.

[31]   Cymerman CJ, Pereira Jr. WE, Halpern B, Westley JW. (**1971**) Optical rotatory dispersion and absolute configuration—XVII α-Alkylphenylacetic acids. *Tetrahedron*, **27**, 1173-1184.

12-Membered Resorcylic Acid Lactones Isolated from *Saccharicola bicolor*, an Endophytic Fungi from *Bergenia purpurascens*

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Two new resorcylic acid lactones, 13-hydroxyhidroresorcylide (**1**) and 12-hydroxyhidroresorcylide (**2**), along with four known congeners (**3-6**) were isolated from *Saccharicola bicolor*, an endophytic fungus from *Bergenia purpurascens*. Their structures were elucidated by interpretation of the spectroscopic evidence.

Keywords: *Saccharicola bicolor*, *Bergenia purpurascens*, Resorcylic acid lactone.

Resorcylic acid lactones (RALs) are a class of fungal polyketides that have a core structure of a β-resorcylic acid chromophore bearing a side-chain in the form of a lactone ring. The first isolated RAL is radicicol in 1953 [1]. Since then, more than 40 naturally occurring RALs have been reported. These have showed antifungal [2], antimalarial [3] and cytotoxic [4] activities. Due to their attractive biological activities, RALs had provoked interest in drug development. In the course of our ongoing research on the discovery of unique compounds from Tibetan plant endophytes, *Saccharicola bicolor*, an endophytic fungus, has been isolated from *Bergenia purpurascens*. The chemical investigation of its fermentation broth led to the isolation of two new resorcylic acid lactones namely 13-hydroxyhidroresorcylide (**1**) and 12-hydroxyhidroresorcylide (**2**), and four known congeners (**3-6**). Details of the isolation and structure elucidation of these compounds are discussed below.

Compound **1** was isolated as a yellow gum. Its molecular formula was assigned as $C_{16}H_{20}O_6$ on the basis of HRESIMS by the pseudo-molecular ion peak at *m/z* 309.1331 [M+H]$^+$ (calcd. 309.1333). The IR spectrum showed the presence of phenolic hydroxyl (3502 cm^{-1}), methylene (2925.6, 2854.6 cm^{-1}), and lactone (1640.6 cm^{-1}) groups. ^{1}H, ^{13}C, H-H COSY and HSQC NMR spectral data indicated the presence of a *m*-dihydroxybenzene unit [$δ_H$ 6.25 (d, *J* = 2.5 Hz), 6.11 (d, *J* =2.5 Hz); $δ_C$ 166.6, 163.9, 140.5, 114.0, 106.3, 102.9], a $CH_3CHCH_2CH(CH_2)_3$ system [$δ_H$ 5.08 (1H, m), 3.65 (1H, m), 2.85 (1H, m), 2.33 (1H, m), 2.08 (2H, m), 1.88 (1H, m), 1.62 (2H,m), 1.58 (1H, m), 1.34 (3H, d, *J* = 6.1 Hz); $δ_C$ 73.3, 68.6, 44.1, 43.7, 38.2, 21.3, 20.7], a lactone carbonyl ($δ_C$ 172.4), a methylene [$δ_H$ 4.79 (1H, d, *J* = 18.6Hz), 3.60 (1H, d, *J* = 18.6Hz); $δ_C$ 50.7] and a ketone group ($δ_C$ 211.6). HMBC correlations between H-10 and C-9 were observed and the aliphatic system was linked to the ketone. Both HMBC cross peaks between H-8 (δ 4.79, 3.60) and C-9 (δ 211.6), C-7(δ 140.5), and between H-6 (δ 6.11) and C-8 (δ 50.7) placed the insular methylene between the aromatic ring and the ketone. HMBC correlations from H-6 (δ 6.11), H-4 (δ 6.25) and H-15 (δ 5.08) to the lactone carbonyl ($δ_C$ 172.4) were observed and located the lactone between C-2 and C-15.
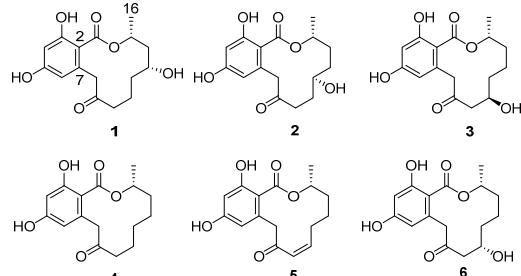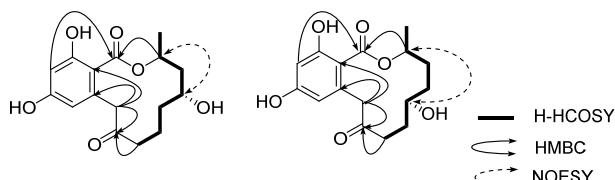
Figure 1: Compounds **1-6** isolated from *Saccharicola bicolor*.

The relative stereochemistry of **1** was established by NOESY correlations of relevant protons as in Figure 2. Both presence of the NOE interaction between H-15 (δ 5.08) and H-13 (δ 3.65) and the absence of the NOE correlation between 15-methyl (δ 1.34) and H-13 indicated that H-15 and H-13 were in the same orientation. Therefore, the structure of **1** (13-hydroxyhidroresorcylide) was determined as shown in Figure 1.

Compound **2** was obtained as a yellow gum, and its molecular formula was determined as $C_{16}H_{20}O_6$ from the [M+H]$^+$ ion peak at *m/z* 309.1333 (calcd. 309.1333) in the HRESIMS with seven degrees of unsaturation. The NMR data of **2** were similar to those of **1** with the difference of a hydroxyl located on the saturated chain system $CH_3CH(CH_2)_2CH(CH_2)_2$ [$δ_H$ 5.22 (1H, m), 3.73 (1H, m), 2.94 (1H, m), 2.20 (1H, m), 2.18 (1H, m),1.94 (1H, ddd, *J* =13.8, 9.0, 4.5 Hz), 1.67 (2H, m), 1.49 (1H, m), 1.30 (3H, d, *J* = 6.3 Hz); $δ_C$ 73.7, 70.5, 36.9, 30.3, 30.0, 29.9, 19.0]. The final structure was established by the 2D NMR experiments including HMQC and HMBC spectra. The relative stereochemistry of **2** was established by NOESY correlations of relevant protons as in Figure 2. Both the presence of a NOE interaction between H-15 and H-12 and the absence of the NOE correlation between 15-methyl and H-12 indicated that H-15 and H-12 were on the same side of the twelve-membered ring. The structure of **2** (12-hydroxyhidroresorcylide) was thus elucidated as shown in Figure 1.

Figure 2: The H-H COSY, key HMBC, and NOESY correlations of **1** and **2**.

Table 1: [1]H (400 MHz) and ^{13}C (100 MHz) NMR data in CD$_3$OD of **1** and **2**.

Position	1		2	
	δ$_H$ (*J* in Hz)	δ$_C$	δ$_H$ (*J* in Hz)	δ$_C$
1	-	172.4	-	172.3
2	-	106.3	-	106.6
3	-	166.6	-	166.6
4	6.25, d (2.5)	102.9	6.25, d (2.5)	103.0
5	-	163.9	-	163.9
6	6.11, d (2.5)	114.0	6.12, d (2.5)	113.7
7	-	140.5	-	140.0
8	4.79, d (18.6), 3.60, d (18.6)	50.7	4.73, d (18.8), 3.78, d (18.8)	52.3
9	-	211.6	-	211.0
10	2.08, 1.58 m	44.1	2.94, 2.20, m	36.9
11	1.62 (2H), m	38.2	2.18, m, 1.94, ddd (13.8, 9.0, 4.5)	29.9
12	2.08, 1.88, m	20.7	3.73, m	70.5
13	3.65, m	68.6	1.67 (2H), m	30.0
14	2.33, 2.85, m	43.7	1.49, 1.67, m	30.3
15	5.08, m	73.3	5.22, m	73.7
16	1.34, d (6.1)	21.3	1.30, d (6.3)	19.0

Experimental

General: Optical rotations were determined on a JASCO P-1020 polarimeter at room temperature. UV spectra were recorded on a Perkin-Elmer Lambda 35 UV-VIS spectrophotometer, and IR spectra on a Perkin-Elmer FT-IR spectrometer (KBr). 1D and 2D NMR were carried out on a Bruker-Ascend-400 MHz instrument at 300 K, with TMS as internal standard. HRESIMS were recorded on a Bruker MicrO TOF-Q II mass spectrometer. Preparative HPLC was performed on a Waters 2545 equipped with a Waters 2489 detector on Kromasil RP-C18 column (10 × 250 mm). Column chromatography (CC) was performed with silica gel and Sephadex LH-20. All the solvents used were of analytical grade.

Fungal material: The title strain was isolated from the root of *Bergenia purpurascens*, collected from a suburb of Lhasa, Tibet Autonomous Region, People's Republic of China. The culture was grown on potato dextrose agar (PDA) and distinguished morphologically as *Saccharicola* sp., which was further reinforced by 18S rDNA sequence with a 99% identity to *Saccharicola bicolor*. The strain (GenBank accession no. KT367526) has been preserved at Chengdu Institute of Biology, Chinese Academy of Sciences, China.

Fungal culture and extraction: This fungus was cultivated on a 4.8 L scale using 500 mL Erlenmeyer flasks containing 200 mL of the seed PDA medium for 3 days. The fermentations were carried out in 60 Fernbach flasks each containing 200 g rice. Distilled water (200 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 121°C for 30 min. The flasks were cooled to room temperature, inoculated with 3.0 mL of spore inoculum, and incubated for 21 days at 25°C. The fermented substrate in each flask was first fragmented with a spatula and then extracted 3 times with light petroleum, followed by EtOAc. The EtOAc solution was dried under vacuum and yielded 14 g extract.

Fractionation and isolation: The EtOAc residue (14 g) was separated into 4 fractions by CC on silica gel (300-400 mesh), eluting stepwise with a CHCl$_3$/MeOH gradient (CHCl$_3$, CHCl$_3$/MeOH: 10:1 (v/v), CHCl$_3$/MeOH: 3:1 (v/v), MeOH). LC-MS analysis was performed on these fractions. The third fraction (eluted with MeOH) was slected. Sephadex LH-20 separation of this fraction (CHCl$_3$/MeOH: 1:1, v/v) afforded 3 sub-fractions (Fr.1-Fr.3). Fr.2 was further purified on a Waters preparative HPLC equipped with a Kromasil RP-C18 column (10×250 mm, ID×L; MeOH/H$_2$O: 55:45, v/v), which led to the separation of **1** (10.3 mg), **2** (4.5 mg), **3** (4.6 mg), **4** (11.3 mg), **5** (7.3mg) and **6** (4.7 mg).

13-Hydroxyhidroresorcylide (1)
[α]$_D^{20}$: -19.4 (*c* 0.1, MeOH).
IR (KBr): 3502.2, 2925.6, 1640.6, 1260.8 cm^{-1}.
UV/Vis λ$_{max}$ (MeOH) nm (log *ε*): 302.6 (3.48), 263.8 (3.74), 221.1(4.08).
^{1}H and ^{13}C NMR (CD$_3$OD): Table 1.
HRESIMS: *m/z* [M + H][+] calcd. for C$_{16}$H$_{20}$O$_6$: 309.1333; found: 309.1331.

12-Hydroxyhidroresorcylide (2)
[α]$_D^{20}$: +25.1 (*c* 0.1, MeOH).
IR (KBr): 3383.9, 2936.3, 1645.9, 1622.1, 1261.8 cm^{-1}.
UV/Vis λ$_{max}$ (MeOH) nm (log *ε*): 302.6 (3.52), 263.8 (3.76), 221.1 (4.10).
^{1}H and ^{13}C NMR (CD$_3$OD): Table 1.
HRESIMS: *m/z* [M + H][+] calcd. for C$_{16}$H$_{20}$O$_6$: 309.1333; found: 309.1333.

References

[1]   Delmotte P, Delmotte-Plaquee J. (**1953**) A new antifungal substance of fungal origin. *Nature*, **171**, 344.
[2]   Nair MSR, Carey ST. (**1980**) Metabolites of pyrenomycetes XIII: Structure of (+) hypothemycin, an antibiotic macrolide from *Hypomyces trichothecoides*. *Tetrahedron Letters*, **21**, 2011-2012.
[3]   Isaka M, Suyarnsestakorn C, Tanticharoen M, Kongsaeree P, Thebtaranonth Y. (**2002**) Aigialomycins A−E, new resorcylic macrolides from the marine mangrove fungus *Aigialus parvus*. *The Journal of Organic Chemistry*, **67**, 1561-1566.
[4]   Isaka M, Yangchum A, Intamas S, Kocharin K, Jones EBG, Kongsaeree P, Prabpai S. (**2009**) Aigialomycins and related polyketide metabolites from the mangrove fungus *Aigialus parvus* BCC 5311. *Tetrahedron*, **65**, 4396-4403.

Phenylpropanoid Glycosides from the Leaves of *Ananas comosus*

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jimh66@163.com; chgying123@163.com
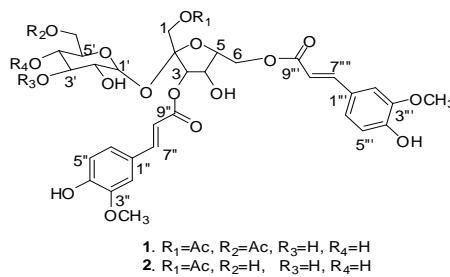
Two new phenylpropanoid glycosides, named β-D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl α-D-6′-*O*-acetylglucopyranoside (**1**) and β-D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl α-D-glucopyranoside (**2**), along with two known analogues (**3-4**) and four glycerides (**5-8**), were isolated from the EtOAc extract of the leaves of *Ananas comosus*. Their structures were elucidated on the basis of 1D- and 2D-NMR analyses, as well as HR-ESI-MS experiments. Compounds **1-4** showed significant antibacterial activities against *Staphylococcus aureus* and *Escherichia coli*.

Keywords: *Ananas comosus*, Bromeliaceae, Phenylpropanoid glycosides, Antibacterial activities.

Ananas comosus L. (family Bromeliaceae) is a perennial herbaceous plant that is employed in a traditional Chinese medicinal preparation to treat stomach upsets and as an anti-diarrheal [1]. Modern pharmacological studies on *A. comosus* leaves indicated that they could have anti-oxidant, blood sugar lowering, blood lipid reducing and antibiotic activities [2]. Previous chemical studies on the leaves of *A. comosus* have led to the isolation of an array of structurally interesting compounds, including phenylpropanoids, glycerides, amides and triterpenes [3].

The ongoing investigation, seeking stronger antibacterial compounds from *A. comosus*, led to the isolation, from the EtOAc extract of the leaves, of two new phenylpropanoid glycosides, named β-D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl α-D-6′-*O*-acetylglucopyranoside (**1**) and β-D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl α-D-glucopyranoside (**2**), together with two known analogues and four glycerides. The known compounds were identified as 2-feruloyl-*O*-α-D-glucopyranosyl-(1′→2)-3,6-*O*-feruloyl-β-D-fructofuranoside (**3**) [4], β-D-(1-*O*-acetyl-3,6-*O*-diferuloyl)fructofuranosyl α-D-2′,4′,6′-triacetyglucopyranoside (**4**) [5], 1-*O*-*p*-coumaroyl-3-*O*-caffeoylglycerol (**5**) [6], 1-*O*-feruloyl-3-*O*-*p*-coumaroylglycerol (**6**) [7], 1-*O*-feruloyl-3-*O*-caffeoylglycerol (**7**) [6], and 1,3-*O*-diferuloylglycerol (**8**) [7] (Figure 1). We present herein the isolation, structural elucidation, and antibacterial activity of these compounds.

Compound **1** was obtained as a colorless amorphous solid with the elemental composition $C_{36}H_{42}O_{19}$, established by high-resolution MS. The ^{1}H NMR spectrum (Table 1) suggested that **1** contained two feruloyl moieties, represented by signals of two sets of *trans* olefinic protons [$δ_H$ 6.40 (1H, d, *J* = 16.0 Hz), 7.66 (1H, d, *J* = 16.0 Hz); 6.46 (1H, d, *J* = 16.0 Hz), 7.72 (1H, d, *J* = 16.0 Hz)], two sets of 1,3,4-trisubstituted aromatic ring protons [$δ_H$ 7.20 (1H, d, *J* = 1.8 Hz), 6.80 (1H, d, *J* = 8.0 Hz), 7.10 (1H, dd, *J* = 1.8, 8.0 Hz); 7.25 (1H, d, *J* = 1.8 Hz), 6.83 (1H, d, *J* = 8.0 Hz), 7.12 (1H, dd, *J* = 1.8, 8.0 Hz)], and two methoxy groups [$δ_H$ 3.89 (3H, s), 3.90 (3H, s)]. Signals in the ^{13}C NMR spectrum (Table 1) at $δ_C$ 90.2, 74.34, 72.03, 71.8, 71.9, 65.0; 65.2, 105.3, 81.1, 73.8, 78.3, and 64.8 suggested the presence of a disaccharide moiety [5]. Alkaline and

1. R₁=Ac, R₂=Ac, R₃=H, R₄=H
2. R₁=Ac, R₂=H, R₃=H, R₄=H
3. R₁=H,   R₂=H,   R₃=H, R₄=H
4. R₁=Ac, R₂=Ac, R₃=Ac, R₄=Ac

5. R₁=H,   R₂=OH
6. R₁=H,   R₂=OCH₃
7. R₁=OH,   R₂=OCH₃
8. R₁=OCH₃, R₂=OCH₃
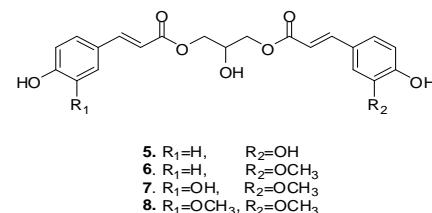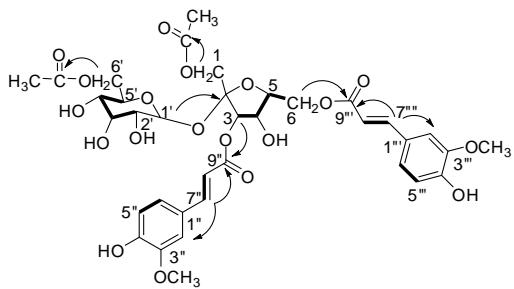
Figure 1: Structures of compounds **1-8**.

acid hydrolysis of **1** gave sucrose and a mixture of glucose and fructose, which were identified by direct comparison with authentic samples on TLC. A characteristic doublet signal with a small coupling constant at $δ_H$ 5.66 (1H, d, *J* = 3.7 Hz) in the ^{1}H NMR spectrum that was ascribed to the anomeric proton in the α-glucopyranose unit [8, 9] also supported the presence of a sucrose moiety in **1**. Furthermore, ^{1}H- and ^{13}C-NMR spectra (Table 1) revealed the presence of two acetyl groups [$δ_H$ 2.07 (3H, s), 2.05 (3H, s); $δ_C$ 20.9, 20.8] located in the sucrose moiety. The position of bond conjugation of the feruloyls and the acetyl groups on the sucrose was assigned by HMBC experiment. This HMBC spectrum (Figure 2) enabled the assignments of two feruloyl moieties located at positions 3 and 6 on the fructose unit of sucrose, since the methine proton ($δ_H$ 5.56) of position 3 and one *trans* olefinic proton ($δ_H$ 7.72) of position 7″ on a feruloyl moiety gave cross-peaks with the same carbonyl carbon ($δ_C$ 168.3), and one set of the methylene protons ($δ_H$ 4.50, 2H) of position 6 and another *trans* olefinic proton

Table 1: ¹H- and ¹³C NMR data for compounds **1** and **2** in CD₃OD (δ in ppm, *J* in Hz)[a,b].
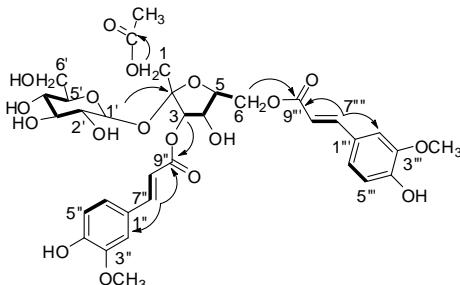
NO.	1		2	
	δ_H	δ_C	δ_H	δ_C
1	4.54, 4.60 (m)	65.2, CH₂	4.19, 4.45 (m)	65.6, CH₂
2		105.2, C		104.9, C
3	5.56 (m)	78.3, CH	5.50 (d, 8.0)	78.9, CH
4	4.50 (m)	81.1, CH	4.50 (m)	81.2, CH
5	3.45 (m)	73.8, CH	3.45 (m)	74.3, CH
6	4.10, 4.50 (m)	64.8, CH₂	4.10, 4.50 (m)	65.7, CH₂
1′	5.66 (d, 3.6)	90.2, CH	5.49 (d, 3.6)	92.9, CH
2′	3.65 (m)	71.8, CH	3.65 (m)	71.9, CH
3′	4.16 (m)	74.3, CH	4.16 (m)	74.8, CH
4′	3.82 (dd, 9.0, 9.0)	72.0, CH	3.82 (dd, 9.0, 9.0)	73.0, CH
5′	3.34 (m)	71.9, CH	3.34 (m)	72.1, CH
6′	4.14, 4.20 (m)	65.0, CH₂	4.10, 4.19 (m)	65.5, CH₂
1″		127.5, C		127.5, C
2″	7.25 (d, 1.8)	111.8, CH	7.25 (d, 1.8)	111.9, CH
3″		149.3, C		149.4, C
4″		150.8, C		150.8, C
5″	6.82 (d, 8.2)	115.2, CH	6.82 (d, 8.2)	115.1, CH
6″	7.12 (dd, 1.6,7.6)	124.4, CH	7.12 (dd, 1.6,7.6)	124.3, CH
7″	7.72 (d,16.0)	147.9, CH	7.72 (d, 16.0)	147.9, CH
8″	6.48 (d,16.0)	116.4, CH	6.39 (d, 16.0)	116.5, CH
9″		168.3, C		168.3, C
1‴		127.7, C		127.6, C
2‴	7.19 (d, 1.8)	111.7, CH	7.19 (d, 1.8)	111.6, CH
3‴		149.3, C		149.3, C
4‴		150.6, C		150.7, C
5‴	6.80 (d, 8.2)	114.6, CH	6.80 (d, 8.2)	114.4, CH
6‴	7.08(dd, 1.6,7.6)	124.1, CH	7.08 (dd, 1.6,7.6)	124.1, CH
7‴	7.67 (d, 16.0)	147.0, CH	7.67 (d, 16.0)	147.1, CH
8‴	6.41 (d, 16.0)	116.4, CH	6.41 (d, 16.0)	116.4, CH
9‴		168.8, C		168.8, C
3″-OCH₃	3.90 (s)	56.5, CH₃	3.90 (s)	56.4, CH₃
3‴-OCH₃	3.89 (s)	56.5, CH₃	3.89 (s)	56.4, CH₃
1-CO		172.4, C		173.0, C
6′-CO		172.8, C		
1-OAc	2.07 (s)	20.8, CH₃	2.08 (s)	20.9, CH₃
6′-OAc	2.05 (s)	20.9, CH₃		

[a] The ¹H NMR were measured at 400 MHz and ¹³C NMR at 100 MHz; [b] The assignments were based upon DEPT135, ¹H-¹H COSY, HSQC and HMBC spectra.

Figure 2: Selected ¹H-¹H COSY (▬) and HMBC (→) correlations of compound **1**.

(δ_H 7.67) of position 7‴ with the same carbonyl carbon (δ_C 168.8). Also, the methylene protons of position 6′ (δ_H 4.14 and 4.20) on the glucose and methylene protons of position 1 (δ_H 4.54 and 4.60) on the fructose showed cross-peaks with respective acetyl carbonyl carbons (δ_C 172.8, 172.4). On the basis of these spectroscopic data and chemical evidence, the structure of compound **1** was determined to be β-D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl α-D-6′-*O*- acetylglucopyranoside.

Compound **2**, obtained as a colorless amorphous solid with elemental composition C₃₄H₄₀O₁₈, was also a phenylpropanoid glycoside. The ¹H and ¹³C NMR spectra (Table 1) suggested that **2** possesses a structure similar to **1**, containing glucose (Glc) and fructose (Fru) units, and two feruloyl moieties [*trans* olefinic protons: δ_H 6.39 (1H, d, *J* = 15.6 Hz), 7.65 (1H, d, *J* = 15.6 Hz); 6.44 (1H, d, *J* = 16.0 Hz), 7.72 (1H, d, *J* = 16.0 Hz); 1,3,4-trisubstituted aromatic ring protons: δ_H 7.19 (1H, d, *J* = 1.6 Hz), 6.80 (1H, d, *J* = 8.0 Hz), 7.08 (1H, dd, *J* = 1.6, 8.0 Hz; 7.25 (1H, d, *J* = 1.6 Hz), 6.82 (1H, d, *J* = 8.0 Hz), 7.12 (1H, dd, *J* = 1.6, 8.0 Hz);

Figure 3: Selected ¹H-¹H COSY (▬) and HMBC (→) correlations of compound **2**.

two methoxy groups: δ_H 3.89 (3H, s) and 3.90 (3H, s), but only one acetate group (δ_H 2.07, 3H, s) was present in **2**. Acid and alkaline hydrolysis, followed by TLC, indicated that **2** contained a sucrose moiety. The HMBC spectrum (Figure 3) of **2** revealed long range couplings between the oxygenated methine and methylene protons at positions 3 and 6 of the fructose, as well as the *trans* olefinic protons at positions 8″ and 8‴ of two feruloyl groups, and their respective carbonyl carbons (¹H/¹³C/¹H: δ 5.50/168.3/6.39 for the C-3 group), so these two feruloyl groups were located at C-3 and C-6, the same as **1** [10]. Analogously, couplings between the methyl protons of one acetyl groups, as well as methylene protons at 1 of the sucrose core and its carbonyl carbon (¹H/¹³C/¹H: 2.08/173.0/4.19) indicated that the acetyl group was at C-1 [11]. These data confirmed that the substantial differences between **2** and **1** were in the interchange of the C-6′ acetyl group and hydroxy group, so the structure of **2** was β-D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl α-D-glucopyranoside.

The structures of compounds **3-8** were identified by comparison of their spectral data with those described in the literature. As stated before, the occurrence of natural phenolic diglycerides is restricted to species of Gramineae, Liliaceae, Sparganiaceae and Bromeliaceae [6]. The presence of the phenylpropanoid glycosides (**1-4**) and glycerides (**5-8**) could contribute to chemotaxonomic studies of the *Ananas* genus.

The antibacterial activities of all the isolates (**1-8**) from the leaves of *A. comosus* were evaluated using the 96-well plate method [13] and the results are given in terms of the concentration of the sample decreasing minimal inhibitory concentration (MIC). All the tested samples had different activity against different bacteria. Preliminary antibacterial activity screening revealed that four of the isolated phenylpropanoid glycosides (**1-4**) exhibited significant activity against *S. aureus* and *E. coli,* with MIC values ranging from 0.16 to 0.62 μg/mL; while **6** and **7** showed good inhibition of *S. aureus* and *M. luteus*, as shown in Table 2.

Table 2: Minimal inhibitory concentration (MIC) of compounds **1-8**.

Compds	MIC (μg/mL)				
	S. aureus	*S. albus*	*E. coli*	*B. subtilis*	*M. luteus*
1	0.16	5.00	0.31	0.62	2.50
2	0.16	10.00	0.16	0.31	1.25
3	0.16	2.50	0.62	10.00	1.25
4	0.16	1.25	0.16	10.00	5.00
5	0.62	0.62	10.0	10.00	N
6	0.31	1.25	5.00	2.50	0.62
7	0.62	2.50	5.00	1.25	0.62
8	0.62	0.62	1.25	10.00	N
CPFX[a]	0.16	0.31	0.16	0.16	0.31

[a] CPFX (ciprofloxacin) was used as positive control.

Experimental

General experimental procedure: NMR spectra: Bruker AV 400 spectrometer {400 (¹H) and 100 MHz (¹³C)}; δ in ppm rel. to TMS

as internal standard, *J* in Hz. ESI-MS: Bruker Esquire 6000 Ion Trap mass spectrometer and HR-ESI-MS: Bruker Daltonics Apex-Ultra 7.0 T mass spectrometer; in m/z. Column chromatography (CC): silica gel (SiO$_2$, 200–300 mesh; Qingdao Haiyang Chemical Group Co.), and sephadex LH-20 (Pharmacia). TLC: Precoated SiO$_2$ GF-254 (10–40 μm) plates (Qingdao Haiyang Chemical Group Co.).

Plant material: *A. comosus* leaves were collected in Wanning, Hainan Province of China, and identified by Professor Qiong-xin Zhong of Hainan Normal University. A voucher specimen has been deposited at the Key Laboratory of Tropical Medicinal Plant Chemistry of the Ministry of Education, Hainan Normal University.

Extraction and isolation: Air-dried leaves (20.0 kg) of *A. comosus* were finely cut and extracted 3 times (each for 7 days) with 95% EtOH at room temperature. Evaporation of the solvent under reduced pressure produced the EtOH extract. This was dissolved in H$_2$O and partitioned with light petroleum and EtOAc. The EtOAc soluble fraction (300 g) was initially subjected to column chromatography (CC) on silica gel, and then eluted with a step gradient of light petroleum/EtOAC (10/0-0/10, v/v) to give 10 fractions. Fraction 7 (15.6 g) was decolorized on a Sephadex LH-20 (CHCl$_3$-MeOH, 2:3, v/v) column and then chromatographed on a silica gel column (CHCl$_3$-MeOH, 6:1-1:1) to afford 6 fractions (fractions 7.1-7.6). Compounds **1** (7.0 mg) and **3** (7.4 mg) were obtained from fraction 7.2 by reversed-phase silica gel CC, and compounds **2** (7.2 mg) and **4** (6.8mg) from fraction 7.4 by reversed-phase silica gel CC. Fraction 4 (14.6g) was decolorized on Sephadex LH-20 (CHCl$_3$-MeOH, 1:1, v/v) to obtain a sub-fraction, which was further isolated and purified by silica gel CC (CHCl$_3$-Me$_2$CO, 4:1-1:1, v/v), and Sephadex LH-20 (CHCl$_3$-MeOH, 2:3, v/v) to afford compounds **5** (10.5 mg), **6** (12.1 mg), 7 (9.0 mg) and **8** (7.5 mg)。

β-D-(1-*O*-Acetyl-3,6-*O*-diferuloyl) fructofuranosyl α-D-6′-*O*-acetylglucopyranoside (1)
[α]$_D$: + 16.9 (*c* 0.1, DMSO)
^{1}H and ^{13}C NMR: Table 1.
HRESI-MS: *m/z* 777.2241 [M-H]$^-$, calcd. for C$_{36}$H$_{41}$O$_{19}$: 777.2248

β-D-(1-*O*-Acetyl-3,6-*O*-diferuloyl)fructofuranosyl-α-D-glucopyranoside (2)
[α]$_D$: + 19.5 (*c* 0.1, DMSO)
^{1}H and ^{13}C NMR: Table 1.
HRESI-MS: *m/z* 735.2149 [M-H]$^-$, calcd. for C$_{34}$H$_{39}$O$_{18}$: 735.2152

Antibacterial activity assay: Five bacterial strains, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Micrococcus luteus*, and *Staphylococcus albus*, were used for the assay using standard agar diffusion tests [13]. Ciprofloxacin (CPFX) was the positive control. The results are presented as minimum inhibitory concentrations (MIC) in Table 2.

Supplementary data: NMR (^{1}H and ^{13}C NMR, DEPT135, HSQC, HMBC, ^{1}H-^{1}H COSY and NOESY) and IR spectroscopic, and HR-ESI-MS data for the new compounds (**1-2**).

References

[1]   Song LL. (**1999**) *Chinese Herbs*. SST Press, 2000 Shanghai, PRC, Chapter 8, p. 7357 (in Chinese).
[2]   (a) Xie WD, Xing DM, Sun H, Wang W, Ding Y, Du LJ. (**2005**) The effects of *Ananas comosus* L. leaves on diabetic-dyslipidemic rats induced by alloxan and a high fat/high-cholesterol diet. *The American Journal of Chinese Medicine*, *33*, 95-105; (b) Xie WD, Wang W, Sun H, Xing DM, Cai GP, Du LJ. (**2007**) Hypolipidemic mechanisms of *Ananas comosus* L. leaves in mice: different from fibrates but similar to statins. *Journal of Pharmacological Sciences*, *103*, 267-274.
[3]   (a) Wang W, Ding Y, Xing DM, Wang JP, Du LJ. (**2006**) Studies on phenolic constituents from leaves of pineapple (*A. comosus*). *China Journal of Chinese Materia Medica*, *31*, 1242-1244; (b) Wang JP, Wang HY, Du LJ, Ding Y, Xing DM, Wang W. (**2007**) New cerebroside from leaves of pineapple. *China Journal of Chinese Materia Medica*, *32*, 401-403; (c) Ma C, Xiao SY, Li ZG, Wang W, Du LJ. (**2007**) Characterization of active phenolic components in the ethanolic extract of *Ananas comosus* L. leaves using high-performance liquid chromatography with diode array detection and tandem mass spectrometry. *Journal of Chromatography A*, *1165*, 39-44.
[4]   Yan LL, Gao WY, Zhang YJ, WangY. (**2008**) A new phenylpropanoid glycosides from *Paris polyphylla* var. *yunnanensis*. *Fitoterapia*, *79*, 306-307.
[5]   Osamu S., Setsuko S., Motoyoshi S, Yan N, Hua WY. (**1996**) Chemical constituents of Chinese folk medicine "San Leng", *Sparganium stoloniferum*. *Journal of Natural Products*, *59*, 242-245.
[6]   Delaporte RH, Guzen KP, Laverde Jr A, dos Santos AR, Sarragiotto MH. (**2006**) Phenylpropanoid glycerols from *Tillandsia streptocarpa* Baker (Bromeliaceae). *Biochemical Systematics and Ecology*, *34*, 599-602.
[7]   Shimomura HK, Sashida YK, Mimaki YR. (**1987**) Phenolic glycerides from *Lilium auratum*. *Phytochemistry, 26*, 844-875.
[8]   Binkley WW, Horton D, Bhacca NS. (**1969**) Physical studies on oligosaccharides related to sucrose. I. N.M.R. studies on the peracetates of sucrose, 1-ketose and nystose. *Carbohydrate Research*, *10*, 245-248.
[9]   Linscheid M, Wendisch D, Strack D. (**1980**) The structures of sinapic acid esters and their metabolism in cotyledons of *Raphanus sativus*. *Zeitschrift fuer Naturforschung*, *35c*, 907-914.
[10]   Yao HK, Ya WH, Chia CL. (**2005**) Cytotoxic phenylpropanoid glycosides from the stems of *Smilax china*. *Journal of Natural Products*, *68*, 1475-1478.
[11]   Sun XZ, Zimmermann ML, Campagne JM, Sneden AT. (**2000**) New sucrose phenylpropanoid esters from *Polygonum perfoliatum*. *Journal of Natural Products*, *63*, 1094-1097.
[12]   Solis PN, Wright CW, Anderson MM, Gupta MP, Phillipson JD. (**1993**) Antimicrobial activity and brine shrimp lethality bioassay of the leaves extract of *Dillenia indica* Linn. *Planta Medica*, *59*, 250-252.

Tannins and Antioxidant Activities of the Walnut (*Juglans regia*) Pellicle

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The total phenolic content and antioxidant activities of the acetone extract and derived fractions from the walnut (*Juglans regia*) pellicle were estimated. The BuOH fraction exhibited the strongest antioxidant activity with the highest phenolic content. A phytochemical investigation of this fraction led to the isolation of three tannins, 2,3-hexahydroxydiphenoylglucose (**1**), pedunculagin (**2**) and 2,3,4,6-tetragalloylglucose (**3**). Pedunculagin showed high content and powerful activity, which implied that this compound plays an important role in the antioxidant activity of the walnut pellicle.

Keywords: *Juglans regia*, Pellicle, Antioxidant, Phenolic, Tannin, Pedunculagin.

Walnut, the fruits of *Juglans regia* L. (Juglandaceae), is a highly nutritious food, which has been found to be an excellent source of multiple health-beneficial components, such as polyunsaturated fatty acids, tocopherols, phenolics, hormone and melatonin [1-3]. The increased consumption of walnuts has been correlated with reduced risk of cardiovascular disease, cancer and diabetes [4, 5]. The walnut kernel, which is officially listed in the Chinese Pharmacopoeia, has long been utilized to treat cough, stomachache and cancer in traditional Chinese medicine. Though rich in unsaturated fatty acids, walnuts are readily preserved from oxidation, which may be attributed to the protective property of the pellicle. Previous studies revealed that a large amount of phenolics possessing significant antioxidant activities exist in the walnut pellicle [6, 7]. In addition, the total phenolic content and antioxidant activity of the walnut pellicle were much higher than the kernel [8]. The antioxidant ingredients in the pellicle probably aid in stabilizing the walnut kernel against decomposition. Therefore, this investigation was conducted to elucidate the protective mechanism of the pellicle and to supply more scientific evidence of antioxidant activity for the further research and development of walnut.

Several studies concluded that walnuts possess higher antioxidant capacity than any other nuts [9], and this antioxidant capacity is presumably a product of phenolics, especially hydrolysable tannins located mainly in the pellicle [10]. The total phenolic content (TPC) of the fractions from the walnut pellicle followed the order BuOH fraction > EtOAc fraction > crude extract > light petroleum (PE) fraction > water fraction (Table 1). It revealed that phenolics in the walnut pellicle were more extractable with highly polar solvents [11, 12]. Combined with the previous studies, the major phenolics in the walnut pellicle were assigned to be tannins.

Plant phenolics, particularly phenolic acids, tannins and flavonoids, are known to be potent antioxidants, which could effectively scavenge free radicals, break radical chain reactions and chelate metal ions [13]. Three methods were utilized to evaluate the antioxidant activities of the fractions from the walnut pellicle.
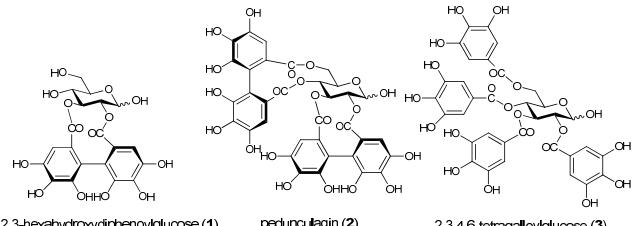
Figure 1: Structures of tannins from the walnut pellicle.

2,2'-Diphenyl-1-picrylhydrazyl (DPPH) has been widely used to estimate the radical scavenging activity of natural antioxidants since it can accommodate a large number of samples in a short period and is sensitive enough to detect active principles at low concentrations. As observed in Table 1, the BuOH fraction of the walnut pellicle showed the highest radical scavenging activity, followed by the EtOAc fraction, crude extract, PE fraction and water fraction. The IC_{50} value of the BuOH fraction (2.09 ± 0.05 μg/mL) was very close to that of the positive control, gallic acid (1.42 ± 0.02 μg/mL).

Tannins function as primary antioxidants and secondary antioxidants, which could chelate metal ions and interfere with one of the reaction steps in the Fenton reaction and thereby retard oxidation [14]. The Fe^{2+} chelating activities decreased in the order BuOH fraction > EtOAc fraction > crude extract > water fraction > PE fraction, which were similar to those of the DPPH assay (Table 1). The result revealed that the BuOH fraction from the walnut pellicle could chelate Fe^{2+} effectively, which was probably due to the presence of tannins.

The hydroxyl radical has been reported to be the most harmful reactive oxygen species that is responsible for the oxidative injury of biomolecules. It is generated from the Fenton reaction in the presence of transition metals such as Fe^{2+} and Cu^{2+} [15]. Similarly, the BuOH fraction showed the highest hydroxyl radical scavenging (HRS) activity, followed by the EtOAc fraction, crude extract, PE fraction and water fraction (Table 1). It can be concluded that the

extracts showed strong hydroxyl radical scavenging activity probably due to their Fe^{2+} chelating capability [16].

To demonstrate that the extracts' strong antioxidant activity was attributed to the large amount of phenolics, the correlation analysis between total phenolic content and antioxidant activity was carried out. The correlation between IC_{50} of DPPH and TPC was very strong ($y = 63.8698 - 0.2029x$, $R^2 = 0.8282$), and so was that between the IC_{50} of HRS and TPC ($y = 418.7180 - 1.2384x$, $R^2 = 0.7578$). The correlation between the IC_{50} of Fe^{2+} chelating activity and TPC was also remarkable ($y = 77.6002 - 0.2538x$, $R^2 = 0.6945$). The results suggested the strong antioxidant activity of walnut pellicle could be attributed to its high phenolic content.

Table 1: Total phenolic content and antioxidant activity of acetone extract and its derived soluble fractions from the walnut pellicle.

Sample	IC_{50} (µg/mL)			
	TPC	DPPH	HRS	Fe^{2+}
Crude extract	122.5 ± 5.7	32.1 ± 1.7	146.7 ± 5.5	21.9 ± 0.8
PE fraction	19.7 ± 2.1	87.8 ± 2.9	453.1 ± 15.4	81.9 ± 1.3
EtOAc fraction	188.9 ± 8.7	14.9 ± 1.0	123.6 ± 3.5	15.5 ± 0.5
BuOH fraction	344.1 ± 13.5	2.09 ± 0.05	74.6 ± 2.6	6.42 ± 0.27
Water fraction	95.4 ± 7.9	55.6 ± 2.0	369.3 ± 9.8	37.3 ± 1.1
Gallic acid	–	1.42 ± 0.02	15.9 ± 2.3	–
EDTA	–	–	–	5.32 ± 0.19

To obtain the tannin compounds, which might be the main contributors to the high antioxidant activity of the walnut pellicle, a phytochemical investigation of the BuOH fraction was performed. Three hydrolyzable tannins were isolated and identified as 2,3-hexahydroxydiphenoylglucose (**1**) [17], pedunculagin (**2**) [18], and 2,3,4,6-tetragalloylglucose (**3**) [19], by comparison of the MS, ^{1}H and ^{13}C NMR spectral data with those reported in the literature (Figure 1). Compound **3** was isolated from this species for the first time. The isolated tannins exhibited significant high radical scavenging activity and had a decreasing order **1** (5.10 ± 0.09 µM) > **2** (5.69 ± 0.10 µM) > gallic acid (7.51 ± 0.06 µM) > **3** (11.35 ± 0.14 µM). Compounds **1** and **2** were more effective than gallic acid in terms of DPPH radical-scavenging activity, which was attributed to the galloyl groups present in the structure [20].

According to the UPLC chromatogram, pedunculagin (**2**) (6.97 ± 0.09 mg/g sample) is the major compound in walnut pellicle. The quantities of compounds **1** (0.57 ± 0.03 mg/g sample), **3** (0.96 ± 0.03 mg/g sample), and gallic acid (1.05 ± 0.05 mg/g sample) in walnut pellicle were also quantified. The result revealed that pedunculagin (**2**) may play a vital role in the antioxidant activity of the walnut pellicle, while the other tannins and gallic acid may also contribute to the enhanced activity. Additionally, the stabilizing or protective effect of the pellicle to the walnut kernel may mainly be attributed to the preferential oxidation of these easily oxidizable tannins [6].

Experimental

General experimental procedures: A Shimadzu UV–Vis 2550 spectrometer (Shimadzu, Kyoto, Japan) was used for colorimetric measurements and collection of UV spectra. ESI-MS analyses were recorded with an Agilent G3250AA (Agilent, Santa Clara, USA) and Auto Spec Premier P776 spectrometer (Waters, Milford, USA). NMR spectra were acquired with a Bruker AM-500 spectrometer (Bruker, Karlsruhe, Germany) using TMS as the internal reference. A Waters semipreparative HPLC system equipped with a Waters 2487 dual λ absorbance detector, a Waters delta 600 quaternary pump, a Waters Spherisorb ODS2 (C_{18}) Semi-Prep column (S10, 20 mm × 250 mm, 5 µm, Waters, Milford, USA), and an Empower 3

HPLC workstation (Waters) was used for semipreparative HPLC. Silica gel (200-300 mesh and 300-400 mesh; Qingdao Marine, Qingdao, China), middle chromatogram isolated gel (MCI, Greenherbs, Beijing, China), and Sephadex LH-20 (GE Healthcare, Fairfield, USA) were used for column chromatography (CC). GF254 plates (Qingdao Marine, Qingdao, China) were used for thin layer chromatography, and compounds were visualized either under UV light or by spraying with 10% H_2SO_4 in ethanol followed by heating. DPPH and ferrozine were obtained from J&K Scientific Ltd (Beijing, China), gallic acid from Aladdin-Reagent (Shanghai, China), CD_3OH from Sigma-Aldrich (Shanghai, China), and CH_3CN and CH_3OH (HPLC grade) from Fisher Chemicals (New Jersey, USA). Deionized water (resistivity ≥ 18.25 MΩ•cm) was purified with a You Pu purity water system (You Pu, Chengdu, China).

Plant materials: The pellicle of *J. regia* was collected from Kunming City in Yunnan Province, China, in August 2011. The samples were identified by Professor Shu-gang Lu at the School of Life Sciences at Yunnan University in Kunming City, China. A voucher specimen (No. LCS-02) is deposited at the Key Laboratory of Medicinal Chemistry for Natural Resources, Ministry of Education in Kunming City of China.

Extraction and isolation: Air-dried and powdered pellicle of *J. regia* (1.8 kg) was extracted with 80% aqueous acetone at room temperature (3 × 5 L, each extraction 24 h). Removal of the solvent under reduced pressure afforded the crude extract (450 g), which was partitioned successively with PE, EtOAc, and BuOH to yield soluble fractions in PE (210 g), EtOAc (44 g), BuOH (144 g), and water (52 g), respectively. Five g samples were collected from each fraction for the antioxidant experiments. The remainders were used to study the fractions' chemical components.

The BuOH fraction was subjected to silica gel CC, elution with a $CHCl_3$–CH_3OH gradient system (200:1 to 1:1, *v/v*) to give 8 fractions (FrC1–FrC8). FrC5 (1.2 g) was subjected to MCI gel CC (CH_3OH–H_2O, 1:1, v/v), Sephadex LH-20 CC (CH_3OH) and semipreparative HPLC (CH_3OH–H_2O, 3:2, v/v) to provide **1** (40 mg). FrC6 (1.5 g) was subjected to silica gel CC (EtOAc-CH_3OH, 10:1, v/v) and then purified by MCI CC (CH_3OH–H_2O, 1:1, v/v) to provide **2** (252 mg) and **3** (28 mg).

Total phenolic content: The TPC was determined according to the Folin-Ciocalteau method [21]. Folin-Ciocalteau reagent was prepared according to a previously described method [22]. A total of 15 µL of extract solution was added to 2.25 mL Folin-Ciocalteau reagent, which had been prediluted by a factor of 10 with deionized water. Five min later, 3.0 mL of Na_2CO_3 (7.5%, w/v) solution was added and the mixture was allowed to stand for 30 min at room temperature. The absorbance was measured at 765 nm. TPC was expressed as mg gallic acid equivalents/g dry extract (mg GAE/g Ex) and calculated according to the calibration curve obtained from a standard solution of gallic acid at various concentrations.

DPPH assay: The DPPH assay was assessed by using a previously published method [23]. A total of 3.9 mL DPPH (0.075 mM) was mixed with 0.1 mL sample at various concentrations. The mixture was shaken and incubated at 25°C in the dark, and the absorbance of the reaction mixture was measured at 515 nm after 30 min. The DPPH radical scavenging activity was calculated by the following equation: inhibition = $(1 - A_s/A_o) \times 100\%$; where A_o is the absorbance of DPPH without any sample, and A_s is the absorbance of a sample with DPPH in it. Inhibition was the scavenging effect, and the 50% absorbance reduction (IC_{50}) was measured from a

curve relating concentration to the absorbance of a sample. Gallic acid was used as a reference standard.

Fe^{2+} chelating activity: The Fe^{2+} chelating assay was assessed by using a previously published method [24, 25]. Briefly, samples at various concentrations in CH_3OH (0.4 mL) were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), and the total volume was adjusted to 4 mL with CH_3OH. The mixture was shaken vigorously and left standing at room temperature for 10 min; absorbance of the solution was then measured at 562 nm. The Fe^{2+} chelating activity was calculated by the following equation: inhibition = $(1 - A_s/A_o) \times$ 100%; where A_o is the absorbance of the control which contains $FeCl_2$ and ferrozine, and A_s is the absorbance in the presence of the sample or standard. EDTA was used as a reference standard.

HRS assay: The HRS assay was conducted by a Fenton reaction method [26]. Briefly, the reaction mixture containing 0.2 mL of acidic chrome blue K (0.2 mM), 1.2 mL of $FeSO_4$ (0.4 mM), 1.0 mL of H_2O_2 (0.03%, *v/v*), 1.2 mL of phosphate buffer (0.1 mM, pH = 5.5) and 1.0 mL of samples at various concentrations was incubated at room temperature for 15 min, and the absorbance was measured at 520 nm. Hydroxyl radical scavenging activity was calculated by the following equation: inhibition = $[(A_s - A_o) / (A - A_o)] \times$ 100%; where A_s is the absorbance in the presence of the sample, A_o is the absorbance of the control in the absence of the sample, and A is the absorbance without the sample and Fenton reaction system. Gallic acid was used as a reference standard.

Tannins determined by UPLC: Air-dried and powdered pellicle of *J. regia* (1.0000 g) was extracted 3 times with 80% aqueous acetone at room temperature, for 24 h each time. The resulting crude extract

and the authentic samples were prepared as solutions (10.00 mg/mL and 1.00 mg/mL, respectively). All sample solutions were filtered through a 0.45 μm filter before injection into the ACQUITY UPLC system equipped with a QSM (quaternary solvent manager), a UPL PDA (photodiode array) detector, a CHA column thermostat, a SDI sample manager, a reversed phase Symmetry1 C_{18} column (T3, 2.1 mm × 100 mm, 1.8 μm; Waters, Milford, USA), and an Empower 3 UPLC workstation (Waters). The optimal mobile phase for the analysis was a gradient elution system consisting of solvent A (H_2O, 1.0% acetic acid) and solvent B (CH_3CN). The gradient program was as follows: 0-3 min, 0-5% solvent B; 3-6 min, 5-10% solvent B; 6-10 min, 10%-15% solvent B; 10-20 min, 15%-100% solvent B; 20-21 min, 100%-0 solvent B. The flow rate was 0.5 mL/min, the column temperature was set at 30 °C, and the injection volume was 5 μL. The UV detection wavelength was monitored at 280 nm. The identification of peaks was confirmed by the UV absorptions and retention times of authentic samples. The sample analyte content (C_s, mg/g dry sample) was calculated according to the equation $C_s = (C_a \times V) / M$, where C_a is the analyte content (mg/mL) based on the calibration curve, V (mL) is the final volume of sample, and M is the sample weight (g).

Statistical analysis: The results were determined as the mean ± SD for at least 3 replicates for each sample. Statistical analysis was performed using SPSS. A significant difference was evaluated at a level of $p < 0.05$.

References

[1] Grace MH, Warlick CW, Neff SA, Lila MA. (**2014**) Efficient preparative isolation and identification of walnut bioactive components using high-speed counter-current chromatography and LC-ESI-IT-TOF-MS. *Food Chemistry*, **158**, 229-238.

[2] Matławska I, Bylka W, Widy-Tyszkiewicz E, Stanisz B. (**2015**) Determination of the juglone content of *Juglans regia* leaves by GC/MS. *Natural Product Communications*, **10**, 1239-1242.

[3] Paudel P, Prabodh Satyal P, Dosoky NS, Maharjan S, Setzer NW. (**2013**) *Juglans regia* and *J. nigra*, two trees important in traditional medicine: a comparison of leaf essential oil compositions and biological activities. *Natural Product Communications*, **8**, 1481-1486.

[4] Pan A, Sun Q, Manson JE, Willett WC, Hu FB. (**2013**) Walnut consumption is associated with lower risk of type 2 diabetes in women. *The Journal of Nutrition*, **143**, 512-518.

[5] Feldman EB. (**2002**) The scientific evidence for a beneficial health relationship between walnuts and coronary heart disease. *The Journal of Nutrition*, **132**, 1062-1101.

[6] Jurd L. (**1956**) Plant polyphenols. I. The polyphenolic constituents of the pellicle of the walnut (*Juglans regia*). *Journal of the American Chemical Society*, **78**, 3445-3448.

[7] Jurd L. (**1958**) Plant polyphenols. III. The isolation of a new ellagitannin from the pellicle of the walnut. *Journal of the American Chemical Society*, **80**, 2249-2252.

[8] Arranz S, Pérez-Jiménez J, Saura-Calixto F. (**2007**) Antioxidant capacity of walnut (*Juglans regia* L.): contribution of oil and defatted matter. *European Food Research and Technology*, **227**, 425-431.

[9] Li L, Tsao R, Yang R, Liu C, Zhu H, Young JC. (**2006**) Polyphenolic profiles and antioxidant activities of heartnut (*Juglans ailanthifolia* var. *cordiformis*) and Persian walnut (*Juglans regia* L.). *Journal of Agricultural and Food Chemistry*, **54**, 8033-8040.

[10] Pellegrini N, Serafini M, Salvatore S, Del Rio D, Bianchi M, Brighenti F. (**2006**) Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different *in vitro* assays. *Molecular Nutrition and Food Research*, **50**, 1030-1038.

[11] Liu J, Wang CN, Wang ZZ, Zhang C, Lu S, Liu JB. (**2011**) The antioxidant and free-radical scavenging activities of extract and fractions from corn silk (*Zea mays* L.) and related flavone glycosides. *Food Chemistry*, **126**, 261-269.

[12] You Q, Chen F, Wang X, Sharp JL, You YR. (**2012**) Analysis of phenolic composition of Noble muscadine (*Vitis rotundifolia*) by HPLC-MS and the relationship to its antioxidant capacity. *Journal of Food Science*, **77**, 1115-1123.

[13] Kaisoon O, Siriamornpun S, Weerapreeyakul N, Meeso N. (**2011**) Phenolic compounds and antioxidant activities of edible flowers from Thailand. *Journal of Functional Foods*, **3**, 88-99.

[14] Amarowicz R. (**2007**) Tannins: the new natural antioxidants. *European Journal of Lipid Science and Technology*, **109**, 549-551.

[15] Kao TH, Chen BH. (**2006**) Functional components in soybean cake and their effects on antioxidant activity. *Journal of Agriculture and Food Chemistry*, **54**, 7544-7555.

[16] Li S, Zhao Y, Zhang L, Zhang X, Huang L, Li D, Niu C, Yang Z, Wang Q. (**2012**) Antioxidant activity of *Lactobacillus plantarum* strains isolated from traditional Chinese fermented foods. *Food Chemistry*, **135**, 1914-1919.

[17] Okuda T, Yoshida T, Hatano T, Yazaki K, Ashida M. (**1980**) Ellagitannins of the Casuarinaceae, Stachyuraceae and Myrtaceae. *Phytochemistry*, **21**, 2871-2874.

[18]   Okuda T, Yoshida T, Ashida M, Yazaki K. (**1983**) Tannins of *Casuarina* and *Stachyurus* species. Part 1. Structures of pendunculagin, casuarictin, strictinin, casuarinin, casuariin, and stachyurin. *Journal of the Chemical Society, Perkin Transactions 1*, 1765-1772.

[19]   Khanbabaee K, Lötzerich K. (**1997**) Efficient total synthesis of the natural products 2,3,4,6-tetra-*O*-galloyl-D-glucopyranose, 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucopyranose and the unnatural 1,2,3,4,6-penta-*O*-galloyl-*α*-D-glucopyranose. *Tetrahedron*, *53*, 10725-10732.

[20]   Cai YZ, Mei S, Jie X, Luo Q, Corke H. (**2006**) Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. *Life Science*, *78*, 2872-2888.

[21]   Singleton VL, Rossi JA. (**1965**) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16*, 144-158.

[22]   Huang D, Ou B, Prior RL. (**2005**) The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, *53*, 1841-1856.

[23]   He JM, Yin TP, Chen Y, Cai L, Tai ZG, Li ZJ, Liu CS, Wang YR, Ding ZT. (**2015**) Phenolic compounds and antioxidant activities of edible flowers of *Pyrus pashia*. *Journal of Functional Foods*, *17*, 371-379.

[24]   Miceli N, Trovato A, Dugo P, Cacciola F, Donato P, Marino A, Bellinghieri V, La Barbera TM, Guvenc A, Taviano MF. (**2009**) Comparative analysis of flavonoid profile, antioxidant and antimicrobial activity of the berries of *Juniperus communis* L. var. *communis* and *Juniperus communis* L. var. *saxatilis* Pall. from Turkey. *Journal of Agricultural and Food Chemistry*, *57*, 6570-6577.

[25]   Yin TP, Cai L, Fang HX, Fang YS, Li ZJ, Ding ZT. (**2015**) Diterpenoid alkaloids from *Aconitum vilmorinianum. Phytochemistry*, *116*, 314-319.

[26]   Fu FE, Wang XH, Li F, Cao QE. (**2008**) A method for screening of antioxidant activities of resina draconis *in vitro* and its applications. *Chinese Journal of Analysis Laboratory*, *28*, 2009-2011.

Chemical Constituents of *Cordyceps cicadae*

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One new bifuran derivative (**1**), together with fourteen known compounds, were isolated from *Cordyceps cicadae* X. Q. Shing. The known compounds included nine nucleosides, uracil (**2**), uridine (**3**), 2'-deoxyuridine (**4**), 2'-deoxyinosine (**5**), guanosine (**6**), 2'-deoxyguanosine (**7**), thymidine (**8**), adenosine (**9**), and 2'-deoxyadenosine (**10**); three amino acids tryptophan (**11**), phenylalanine (**12**), and tyrosine (**13**); and two dopamine analogues *N*-acetylnoradrenaline (**14**) and its dimer, *trans*-2-(3',4'-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2''-amino-ethylene)-1,4-benzodioxane (**15**). Their structures were decisively elucidated by spectroscopic analysis, including 1D and 2D NMR techniques.

Keywords: *Cordyceps cicadae*, Bifuran derivative, Nucleoside, Amino acid, Dopamine analogue.

Cordyceps cicadae X. Q. Shing (Clavicipitaceae, Ascomycotina), named 'Chan Hua', an anamorph of *Isaria cicadae* Miq, is a major parasitic fungus growing on the nymphs of *Cicada flammata* Distant, *Platypleura kaempferi* Fabricius, *Crytotympana pustulata* Fabricious, *Platylomia pieli* Kato and *Oncotympana maculatieollis* Motsch [1]. As a traditional Chinese medicine used for thousands of years, 'Chan Hua' is reported to possess antitumor, antibacterial, immunoregulatory and sedative effects, and is widely used in the treatment of fever, infantile convulsion, palpitation and dizziness [2]. Some bioactive constituents, for example, polysaccharides, galactomannan, ISP-1 (myriocin) and cyclodepsipeptides have been isolated from *C. cicadae* [3]. Cordycepin, adenosine derivatives, aromatic 4-*O*-methylglucosides, cyclopentenone and furan derivative were reported from the cultivated mycelia of *C. cicadae* [4]. As part of our investigation on biologically active constituents from Chinese traditional medicines [5], *C. cicadae* was exhaustively studied. A crude extract of the drug was chromatographed repeatedly on the MCI gel CHP 20P, HW-40F, normal phase silica gel, Sephadex LH-20, and reversed phase silica gel. As a result, one new heterocyclic compound, 5,5'-di (2-ethyl-hexyloxy)-5,5'-bifuran (**1**) (Figure **1**), was obtained, together with fourteen known compounds. Herein, we report the isolation and structural elucidation of compound **1**.

Compound **1** was obtained as colorless oil. The positive ESI-MS showed quasimolecular ion peaks at m/z 391 [M + H]$^+$ and 803 [2M + Na]$^+$, indicating a molecular weight of 390. The molecular formula of **1** was determined to be $C_{24}H_{38}O_4$ from the HR-ESI-MS ion peak at *m/z* 413.2659 [M + Na]$^+$ (calcd 413.2662), and ^{1}H and ^{13}C NMR spectroscopic data. The low-field part of the ^{1}H NMR spectrum (Table 1) indicated the presence of four olefinic protons at δ 7.734, 7.726, 7.628, and 7.620 (d, *J* = 5.6 Hz). The high-field region of the spectrum showed two oxygenated methylenes at δ 4.229 (4H, m), two methines at δ 1.681 (2H, m), eight methylenes at δ 1.392-1.423 (8H, m), and four methyl groups at δ 0.961 (6H, t, *J* = 7.5 Hz) and 0.932 (6H, t, *J* = 7.5 Hz). The ^{13}C NMR spectrum showed two quaternary carbons at δ 169.23 and 133.59, and two methine carbons at δ 132.37 and 129.86, which suggested an oxygenated furan ring. There was evidence of an oxygenated methylene carbon at δ 69.02, one methine carbon at δ 40.13, four methylene carbons at δ 31.60, 30.12, 24.04, and 24.92, and two
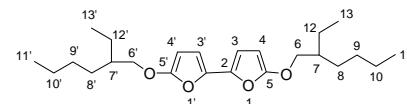
Figure 1: The structure of compound **1**.

Table 1: NMR data of compound **1**.

position	^{1}H^a	^{13}C^b	^{1}H-^{1}H COSY	HMBC (H→C)
2, 2'		133.59 s		
3, 3'	7.734, 7.726 d (5.6)	129.86 d	H-4	C-2, 4, 5
4, 4'	7.628, 7.620 d (5.6)	132.37 d	H-3	C-2, 3
5, 5'		169.23 s		
6, 6'	4.229 m	69.02 t	H-7	C-5, 7, 8, 12
7, 7'	1.681 m	40.13 d	H-6, 8, 12	C-6, 8, 9, 12, 13
8, 8'	1.421 m	31.60 t	H-7, 9	C-6, 7
9, 9'	1.392 m	30.12 t	H-8, 10	C-10
10, 10'	1.395 m	24.04 t	H-9, 11	C-9, 11
11, 11'	0.932 t (7.5)	14.45 q	H-10	C-9, 10
12, 12'	1.423 m	24.92 t	H-7, 13	C-6, 7, 8, 13
13, 13'	0.961 t (7.5)	11.44 q	H-12	C-7, 12

^{a} 400 MHz, CD$_3$OD; chemical shifts in ppm relative to TMS; coupling constant (*J*) in Hz. ^{b} 100 MHz, CD$_3$OD; multiplicity was established from DEPT data.

terminal methyl carbons at δ 14.45 and 11.44, which were derived from the alkoxy moiety. Based on the ESI-MS, compound **1** was proved as a dimer of a furan derivative. The 2-ethyl-hexyloxy moiety was established unambiguously by ^{1}H-^{1}H COSY, HMQC and HMBC experiments, in which long-range correlations were observed not only between H-7 (δ 1.681) and five carbons (C-6, C-8, C-9, C-12 and C-13), but also between H-11 (δ 0.932) and two carbons (C-9 and C-10). Moreover, the long-range correlations between the oxygenated methylene H-6, 6' (δ 4.229) and oxygenated quaternary olefin carbons C-5, 5' (δ 169.23) indicated that 2-ethyl-hexyloxy was substituted at C-5 and C-5' of each furan ring. The two furan rings were linked at C-2 and C-2' by a C-C bond, because the olefin methine signals at δ 7.734 and 7.726 (H-3, 3') showed cross peaks with a quaternary olefin carbon at δ 133.59 (C-2, 2') by *J*2 and *J*3 correlation. Consequently, compound **1** was identified as 5,5'-di (2-ethyl-hexyloxy)-5,5'-bifuran.

It is worth mentioned that Baek *et al.* reported a bifuran derivative from *Chrysanthemum coronarium* L. [6] and Guo *et al.* reported bifuran derivatives from *Cyathula officinalis* Kuan [7]. However, the peak of H-4 and H-4' of the furan was assigned as dd peaks with

coupling constants of either 5.6 Hz or 5.6 and 3.3 Hz. In fact, the peak of H-4 (H-3) was not completely overlapped by H-4' (H-3') of the furan because of a steric effect. Therefore, the aromatic protons of the furan should be assigned as four doublet peaks with a coupling constant of 5.6 Hz, respectively.

Fourteen known compounds were also isolated, including nine nucleosides, uracil (**2**), uridine (**3**), 2'-deoxyuridine (**4**), 2'-deoxyinosine (**5**), guanosine (**6**), 2'-deoxyguanosine (**7**), thymidine (**8**), adenosine (**9**), 2'-deoxyadenosine (**10**); three amino acid tryptophan (**11**), phenylalanine (**12**), and tyrosine (**13**); and two dopamine anologues, *N*-acetylnoradrenaline (**14**), and its dimer, *trans*-2-(3',4'-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2''-amino-ethylene)-1,4-benzodioxane (**15**) were isolated from *C. cicadae*.

Experimental

General: Optical rotation, Perkin-Elmer 241 automatic digital polarimeter; UV, Shimadzu UV-260 instrument; IR, Perkin-Elmer 599B instrument; NMR, Bruker DRX-400 spectrometer; (^{1}H 400 MHz and ^{13}C 100 MHz). ESI-MS, Quattro instrument. Reverse-phase chromatography utilized TSK gel Toyopearl HW-40F (30-60 μm, Toso Co., Ltd.), MCI gel CHP 20P (75-150 μm, Mitsubishi Chemical Industries Co., Ltd.) and Cosmosil 75 C18-OPN (42-105 μm, Nacalai Tesque Inc.) columns. TLC was performed using precoated silica gel 60 F254 plates (0.2 mm, Merck).

Plant material: *Cordyceps cicadae* X. Q. Shing was collected in Zhejiang Province, People's Republic of China in 2014, and authenticated by Dr Xie H. A voucher specimen has been deposited in the Herbarium of our lab (DNPC 2014006).

Extraction and isolation: *C. cicadae* (2 kg) was extracted 3 times with 70% aqueous acetone at room temperature (3 × 10 L). The solvent was evaporated under reduced pressure to 1 L and filtered through celite. The filtrate was concentrated *in vacuo* to yield 65 g of a gummy residue. This was dissolved in 400 mL water, and subjected to MCI gel CHP 20P (8 × 60 cm) eluting with a MeOH/H$_2$O gradient with a flow rate of 15 mL/min to obtain fractions 1 [1.0 L, H$_2$O], 2 [0.6 L, MeOH/H$_2$O (10:90)], 3 [0.6 L, MeOH/H$_2$O (30:70)], 4 [0.6 L, MeOH/H$_2$O (50:50)], 5 [0.7 L,

MeOH/H$_2$O (70:30)], 6 [1.0 L, MeOH], 7 [1.0 L, MeCOMe/H$_2$O (60:40)], and 8 [1.0 L, MeCOMe]. Fraction 2 (6.4 g) was chromatographed on Toyopearl HW-40F (6 × 60 cm) using water as eluent to obtained 6 fractions 2A-2F (eluent volume: 200 mL/fraction). Fraction 2C (0.3 g) was further purified by MCI gel CHP 20P (5 × 40 cm, eluted with H$_2$O→30% MeOH) and Cosmosil 75 C$_{18}$-OPN (4 × 30 cm, eluted with H$_2$O→30% MeOH) to give **13** (26 mg). Fraction 3 (5.8 g) was chromatographed on Toyopearl HW-40F (6 × 60 cm) using water as eluent to obtained 5 fractions 3A-3E (eluent volume: 200 mL/fraction). Fraction 3C (0.8 g) was purified by MCI gel CHP 20P (5 × 40 cm, eluted with 10-50% MeOH) and Cosmosil 75 C$_{18}$-OPN (4 × 30 cm, eluted with H$_2$O→50% MeOH) to give **3** (10 mg), **4** (15 mg), **5** (6 mg), **6** (11 mg), **7** (8 mg), and **9** (25 mg). Fraction 4 (6.2 g) was chromatographed on Toyopearl HW-40F (6 × 60 cm) using water as eluent to obtained 6 fractions 4A-4F (eluent volume: 200 mL/fraction). Fraction 4C (0.8 g) was further purified by MCI gel CHP 20P (5 × 40 cm, eluted with 20-60% MeOH) and Cosmosil 75 C$_{18}$-OPN (4 × 30 cm, eluted with H$_2$O→60% MeOH) to give **2** (6 mg), **8** (9 mg), **10** (18 mg), **11** (45 mg), **12** (30 mg) and **14** (6 mg). Fraction 8 (6.8 g) was chromatographed on LH-20 (4 × 200 cm) using methanol as eluent to obtained 6 fractions 8A-8F (eluent volume: 200 mL/fraction). Fraction 8C (0.4 g) was subjected to CC on silica gel to give **1** (15 mg) and **15** (11 mg).

5, 5'-Di (2-ethyl-hexyloxy)-5, 5'-bifuran (1)
Colorless oil.
$[\alpha]_D^{20}$: 0 (*c* 0.10, MeOH).
IR (KBr) ν_{max}: 3100, 1660, 1245, 1230 cm^{-1}.
UV (MeOH) λ_{max} (log ε): 241 (3.89), 274 (3.14) nm;
^{1}H and ^{13}C NMR: Table 1.
ESI-MS: *m/z* 391 [M + H]$^+$, 803 [2M + Na]$^+$.
HR-ESI-MS: *m/z* 413.2659 [M + Na]$^+$ (calcd. for C$_{24}$H$_{38}$O$_4$Na, 413.2662).

References

[1] Zeng WB, Yu H, Ge F, Yang JY, Chen ZH, Wang YB, Dai YD, Adams A. (**2014**) Distribution of nucleosides in populations of *Cordyceps cicadae*. *Molecules*, **19**, 6123-6141.

[2] Nanjing University of Chinese Medicine. (**2006**) *The Dictionary of Chinese Traditional Medicine*. Shanghai Press of Science and Technology, Shanghai, 3597-3598.

[3] (a) Kiho T, Ito M, Nagai K, Hara C, Ukai S. (**1988**) Polysaccharides in fungi. XXII. a water soluble polysaccharide from the alkaline extract of the insect-body portion of Chan hua (fungus: *Cordyceps cicadae*). *Chemical & Pharmaceutical Bulletin*, **36**, 3032-3037; (b) Ukai S, Matsuura S, Hara C, Kiho T, Hirose K. (**1982**) Structure of a new galactomannan from the ascocarps of *Cordyceps cicadae* Shing. *Carbohydrate Research*, **101**, 109-116; (c) Fujita T, Inoue K, Yamamoto S, Ikumoto T, Sasaki S, Toyama R, Chiba K, Hoshino Y, Okumoto T. (**1994**) Fungal metabolites. Part II. A potent immunosuppressive activity found in *Isaria sinclairii* metabolite. *Journal of Antibiotics*, **47**, 208-215; (d) Kuo YC, Lin LC, Don MJ, Liao HF, Tsai YP, Lee GH, Chou CJ. (**2002**) Cyclodepsipeptide and dioxomorpholine derivatives isolated from the insect-body portion of the fungus *Cordyceps cicadae*. *Journal of Chinese Medicine*, **13**, 209-219; (e) Wang J, Zhang DM, Jia JF, Peng QL, Tian HY, Wang L, Ye WC. (**2014**) Cyclodepsipeptides from the ascocarps and insect-body portions of fungus *Cordyceps cicadae*. *Fitoterapia*, **97**, 23-27.

[4] (a) Furuya T, Hirotani M, Matsuzawa M. (**1983**) N6-(2-hydroxyethyl) adenosine, a biologically active compound from cultured mycelia of *Cordyceps* and *Isaria* species. *Phytochemistry*, **22**, 2509-2512; (b) Zhang SW, Xuan LJ. (**2007**) Five aromatics bearing a 4-*O*-methylglucose unit from *Cordyceps cicadae*. *Helvetica Chimica Acta*, **90**, 404-410; (c) Zhang SW, Xuan LJ. (**2008**) Cyclopentenone and furan derivative from the mycelia of *Cordyceps cicadae*. *Journal of Antibiotics*, **61**, 43–45.

[5] (a) Jiang K, Bai JQ, Chang J, Tan JJ, Qu SJ, Luo HF, Tan CH, Zhu DY. (**2014**) Three new 24-nortriterpenoids from the roots of *Ilex asprella*. *Helvetica Chimica Acta*, **97**, 64-69; (b) Chang J, Chu ZB, Song J, Jin L, Sun X. (**2015**) Two novel isoquinoline alkaloids from the seedling of *Corydalis decumbens*. *Tetrahedron Letters*, **56**, 225-228.

[6] Song MC, Yang HJ, Jeong TS, Kim KT, Baek NI. (**2008**) Heterocyclic compounds from *Chrysanthemum coronarium* L. and their inhibitory activity on hACAT-1, hACAT-2, and LDL-oxidation. *Archives of Pharmacal Research*, **31**, 573-578.

[7] Liu J, Xu J, Zhao XJ, Gao WY, Zhang SZ, Guo YQ. (**2010**) A new heterocyclic compound from *Cyathula officinalis* Kuan. *Chinese Chemical Letters*, **21**, 70–72.

A New Bithiophene from the Root of *Echinops grijsii*

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A new bithiophene, 5-(4-hydroxy-3-methoxy-1-butyny)-2,2'-bithiophene (**1**), and sixteen known thiophenes: 2-(3,4-dihydroxybut-1-ynyl)-5-(penta-1,3-diynyl)thiophene (**2**), α-terthienyl (**3**), 5-(3,4-dihydroxybut-1-ynyl)-2,2'-bithiophene (**4**), 5-acetyl-2,2'-bithiophene (**5**), 5-formyl-2,2'-bithiophene (**6**), methyl 2,2'-bithiophene-5-carboxylate (**7**), 5-(but-3-en-1-ynyl)-2,2'-bithiophene (**8**), 5-(4-isovaleroyloxybut-1-ynyl)-2,2'-bithiophene (**9**), cardopatine (**10**), isocardopatine (**11**), 5-(3-hydroxy-4-isovaleroyloxybut-1-ynyl)-2,2'-bithiophene (**12**), 5-(3-hydroxymethyl-3-isovaleroyloxyprop-1-ynyl)-2,2'-bithiophene (**13**), 5-(4-hydroxy-1-butynyl)-2,2'-bithiophene (**14**), 5-(4-acetoxy-1-butynl)-2,2'-bithiophene (**15**), 2,2'-bithiophene-5-carboxylic acid (**16**) and 2-(4-hydroxybut-1-ynyl)-5-(penta-1,3-diynyl)thiophene (**17**) were isolated from the roots of *Echinops grijisii* Hance. Among them, compounds **6**, **7** and **16** were isolated from a natural source for the first time. Compounds **2**, **4** and **14** exhibited significant anti-inflammatory activity against nitrite of LPS-stimulated production in the RAW 264.7 cell line.

Keywords: *Echinops grijisii*, Bithiophene, 5-(4- Hydroxy -3-methoxy-1-butynyl)-bithiophene, Anti-inflammatory.

Echinops grijisii Hance (Compositae) is a perennial medicinal herb and the only native species of *Echinops* from Taiwan [1]. The roots of this plant have been used to clear heat, expel miasma and stimulate milk secretion. It was recorded in the Chinese Pharmacopoeia (2005 edition) as Yuzhou Loulu [2]. Previous chemical investigation of the roots of *E. grijissi* demonstrated the presence of thiophenes, steroids, triterpenes and essential oils [3]. Thiophenes have been proven to possess anti-inflammatory [2], anti-tumor [4] and anti-viral [5] activities. The MeOH extract of the roots showed a significant anti-inflammatory activity. Thus, the constituents of *E. grijissi* were investigated. This paper deals with the structure elucidation of a new compound and the anti-inflammatory activity of the isolates.

Silica gel column chromatography of the MeOH extract of dried roots of *E. grijisii* led to the isolation of a new compound: 5-(4-hydroxy-3-methoxy-1-butyny)-2,2'-bithiophene (**1**), as well as sixteen known compounds: 2-(3,4-dihydroxybut-1-ynyl)-5-(penta-1,3-diynyl)thiophene (**2**) [6], α-terthienyl (**3**), 5-(3,4-dihydroxybut-1-ynyl)-2,2'-bithiophene (**4**), 5-acetyl-2,2'-bithiophene (**5**) [1], 5-formyl-2,2'-bithiophene (**6**) [7], methyl 2,2'-bithiophene-5-carboxylate (**7**) [8], 5-(but-3-en-1-ynyl)-2,2'-bithiophene (**8**), 5-(4-isovaleroyloxybut-1-ynyl)-2,2'-bithiophene (**9**), cardopatine (**10**), isocardopatine (**11**), 5-(3-hydroxy-4-isovaleroyloxybut-1-ynyl)-2,2'-bithiophene (**12**) [1], 5-(3-hydroxymethyl-3-isovaleroyloxyprop-1-ynyl)-2,2'-bithiophene (**13**) [9], 5-(4-hydroxy-1-butynyl)-2,2'-bithiophene(**14**) [1], 5-(4-acetoxy-1-butynl)-2,2'-bithiophene (**15**)
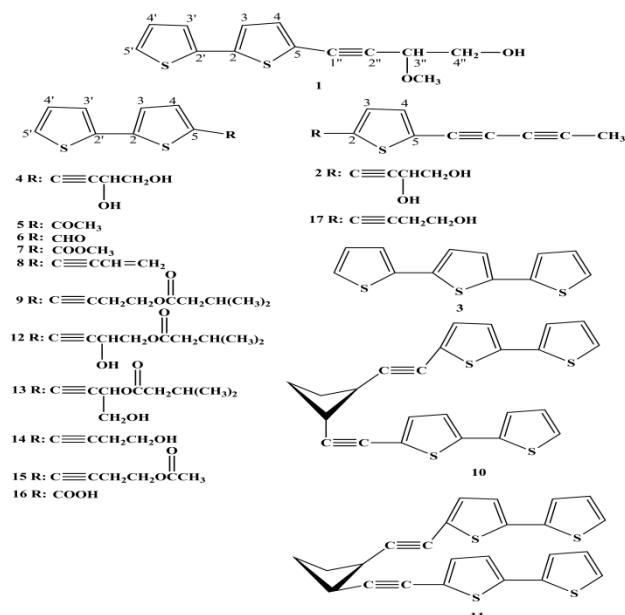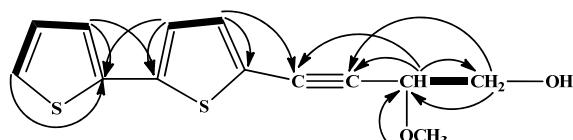
Figure 1: The chemical structures of compounds **1–17**.

[10], 2,2'-bithiophene-5-carboxylic acid (**16**) [11] and 2-(4-hydroxybut-1-ynyl)-5-(penta-1,3-diynyl)thiophene (**17**) [12] (Figure 1).

Table 1: ^{1}H- and ^{13}C-NMR data (CDCl$_3$) of **1**. δ in ppm, *J* in Hz.

Position	^{1}H NMR (400 MHz, δ, CDCl$_3$)	^{13}C NMR (50 MHz, δ, CDCl$_3$)
1		
2		139.5
3	7.13 (1H, d, *J*=3.8 Hz)	133.8
4	7.00 (1H, d, *J*=3.8 Hz)	128.2
5		122.3
1'		
2'		136.5
3'	7.17 (1H, d, *J*=3.6 Hz)	124.6
4'	7.02 (1H, dd, *J*=5.1,3.6 Hz)	123.6
5'	7.24 (1H, d, *J*=5.1 Hz)	125.4
1"		90.0
2"		80.7
3"	4.31 (1H, t, *J*=5.7 Hz)	73.0
4"	3.82 (1H, d, *J*=5.7 Hz)	65.3
OCH$_3$	3.52 (3H,s)	57.3

The numbers in parentheses are *J* values in Hz

Figure 2: ^{1}H-^{1}H COSY (▬▬) and key HMBC ([1]H → [13]C) correlation of **1**.

Compound **1** was obtained as a yellow oil. Its molecular formula C$_{13}$H$_{12}$O$_2$S$_2$ was determined by the pseudomolecular ion peak at *m/z* 262.9758 [M-2H]$^+$ in the HR-ESIMS. The structure of **1** was established by analysis of ^{1}H and ^{13}C NMR, HMQC, COSY and HMBC spectral data. The UV absorption maximum at λ$_{max}$ 333 nm showed the presence of a bithiophene group conjugated with an acetylene functionality [13]. The IR spectrum showed the presence of hydroxy (3431 cm^{-1}), alkyne (2214 cm^{-1}), and bithiophene (802, 696 cm^{-1}).

The ^{1}H NMR spectrum showed characteristic signals of 5-substituted 2,2'-bithiophene protons at δ 7.24 (1H, d, *J*=5.1 Hz, H-5'), 7.17 (1H, d, *J*=3.6 Hz, H-3'), 7.13 (1H, d, *J*=3.8 Hz, H-3), 7.02 (1H, dd, *J*=5.1, 3.6 Hz, H-4') and 7.00 (1H, d, *J*=3.8 Hz, H-4) [13]. Additionally, signals of CH$_2$CH protons appeared at δ 4.31 (1H, t, *J*=5.7 Hz, H-3") and δ 3.82 (2H, d, *J*=5.7 Hz, H-4"); proton signals that appeared at δ 3.52 (3H, s) indicated the presence of a methoxy group. The ^{13}C NMR spectrum of **1** exhibited 13 carbon signals including eight bithiophene carbons appearing at δ 139.5, 136.5, 133.8, 128.2, 125.4, 124.6, 123.6 and 122.3, along with the ynyl carbons at δ 90.0 and 80.7. Two oxygenated carbons appeared at δ 73.0 and 65.3, and one methoxy carbon at δ 57.3 (Table 1).

The COSY and HMBC experiments gave [1]H-^{1}H and [1]H-^{13}C direct correlations of **1** (Figure 2). The correlation between H-4 (δ 7.00) / C-5 (δ 122.3) and C-1" (δ 90.0), H-3" (δ 4.31) / C-1" (δ 90.0), C-2" (δ 80.7) and C-4" (δ 65.3) indicated that the CHCH$_2$OH group was connected with the ynyl group, which was connected to the bithiophene at C-5. The ^{1}H NMR and ^{13}C NMR spectral data of compound **1** were very similar to those of **4.** The only difference was that the correlation between the methoxy group (δ 3.52) with C-3" (δ 73.0) indicated that the methoxy group was connected at C-3". From the above evidence, the structure of **1** was established as 5-(4-hydroxy -3-methoxy-1-butyny)-2,2'-bithiophene.

Compounds **1-17** were tested for anti-inflammatory activity against the RAW 264.7 cell line. Compounds **2**, **4** and **14** exhibited significant activity, with IC$_{50}$ values of 2.5, 20.0 and 6.7 µg/mL (Table 2).

Table 2: Cell viability and *in vitro* anti-inflammatory activities of compounds from *E. grijsii* against nitrite of LPS-stimulated production in RAW 264.7 cell.

Compds	IC$_{50}$, (µg/mL)	Cell viability (% of control)
2	2.5	98.5 ± 3.4
4	20.0	82.7 ± 4.0
14	6.7	93.1 ± 2.7
indomethacin	65.4	

Experimental

Plant material: The roots of *E. grijsii* were bought from the Shan You herb store in Nantou country in September 2010. The plant material was identified by assistant professor Shyh-Shyun Huang from the school of Pharmacy, China Medical University.

Extraction and isolation: The roots of *E. grijsii* (10 kg) were crushed into pieces and extracted with methanol (50 L×3) at ambient temperature and concentrated under vacuum to yield the MeOH extract (2.5 kg). Part of this (1 kg) was partitioned between EtOAc-H$_2$O to give EtOAc-soluble (100 g) and H$_2$O-soluble fractions. The EtOAc-soluble fraction was chromatographed over silica gel eluted with *n*-hexane and a gradient of *n*-hexane-EtOAc The eluent was collected in constant volumes, and combined into 32 fractions based on TLC results. Fraction 10 (obtained with *n*-hexane: EtOAc = 90:10, amount 2 g) was re-separated by HPLC to yield **1** (1.46 mg), **5** (2.15 mg), **6** (13.75 mg), **7** (2.42 mg), **8** (24.74 mg), **9** (2.89 mg), **10** (4.53 mg), **11** (17.54 mg), **12** (3.82 mg), **13** (5.67 mg), 5-(4-hydroxy-1-butynyl)-2,2'-bithiophene (**14**) (3.1 mg), **15** (2.6 mg) and **17** (1.99 mg). Fraction 16 (obtained with *n*-hexane: EtOAc = 70:30, amount 1.2 g) was re-separated by Sephadex LH-20 and HPLC to yield **2** (63.6 mg), **3** (43.49 mg), **4** (77.64 mg) and **16** (112.07 mg).

General: UV spectra were obtained with a Shimadzu Pharmaspec-1700 UV-Visible spectrophotometer, optical rotations with a Jasco P-1020 polarimeter, and infrared spectra with a Shimadzu IR prestige-21 Fourier transform infrared spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded with Bruker DRX-400 and DRX-200 FT-NMR spectrometers. HRESIMS data were generated at the Mass Spectrometry Laboratory of the Chung Hsing University. Column chromatography was performed using LiChroCART Si gel (5 µM) and Sephadex^{TM}LH-20. HPLC details: detector: IOTA 2, pump: P230 HPLC PUMP, column: Phenomenex 250 × 10.00 mm 5 micron. TLC analysis was carried out using aluminum pre-coated Si plates and the compounds were visualized using a UV lamp at λ = 254 nm and λ = 365 nm .

Cell viability: Cells (2 × 105) were cultured in a 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with compounds **1–17** in the presence of 100 ng/mL LPS (lipopolysaccharide) for 24 h. After that, the cells were washed twice with DPBS and incubated with 100 µL of 0.5 mg/mL MTT for 2 h at 37°C. The medium was then discarded and 100 µL dimethyl sulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader (Molecular Devices, Sunnyvale, CA, USA)

Measurement of nitric oxide/nitrite: NO production was indirectly assessed by measuring the nitrite levels in the cultured media and serum determined by a colorimetric method based on the Griess reaction. The cells were incubated with compounds **1–17** (2.5, 5.0, 10.0 and 20 µg/mL) in the presence of LPS (100 ng/mL) at 37°C for 24 h. Then, cells were dispensed into 96-well plates, and 100 µL of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 5% phosphoric acid) and incubated at room temperature for 10

min; the absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices). Serum samples were diluted 4 times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at $10,000 \times g$ for 5 min at room temperature, 100 μL supernatant was applied to a microtiter plate well, followed by 100 μL of Griess reagent. After 10 min of color development at room temperature, the absorbance was measured at 540 nm with a Micro-Reader. By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured from the absorbance at 540 nm.

5-(4-Hydroxy -3-methoxy-1-butyny)-2,2'-bithiophene (1)
Yellow oil

MP: 101-103ºC.
$[\alpha]_D$: +0.78 (*c* 0.23, CHCl$_3$).
IR (KBr): 3431, 2214, 802,696 cm^{-1}.
UV λ_{max} (MeOH) nm (log ε): 234 (3.80), 280 (4.52), 324 (3.45).
^{1}H and ^{13}C NMR: Table 1.
HR-ESI-MS: *m/z* 262.9758 [M-2H]$^+$,calcd for C$_{13}$H$_{12}$O$_2$S$_2$.

References

[1]   Lin YL, Huang RL, Kuo YH, Chen CF. (**1999**) Thiophenes from *Echinops grijsii* Hance. *The Chinese Pharmaceutical Journal*, **51**, 201-211.
[2]   Jin W, Shi Q, Hong C, Cheng Y, Ma Z, Qu H. (**2008**) Cytotoxic properties of thiophenes from *Echinops grijissi* Hance. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*, **15**, 768-774.
[3]   Lin CC, Lin CH. (**1993**) Pharmacological and pathological studies on Taiwan folk medicine (IX): The hepatoprotective effect of the methanolic extract from *Echinops grijisii*. *The American Journal of Chinese Medicine*, **21**, 33-44.
[4]   Lambert JDH, Campbell G, Arnason JT, Majak W. (**1991**) Herbicidal properties of alpha-terthienyl, a naturally occurring phototoxin. *Canadian Journal of Plant Science*, **71**, 215-218.
[5]   Menelaou MA, Fronczek FR, Hjortso MA, Morrison AF, Foroozesh M, Thibodeaux TM, Flores HE, Fisher NH. (**1991**) NMR spectral data of benzofurans and bithiophenes from hairy root cultures of *Tagetes patula* and the molecular structure of isoeuparin. *Spectroscopy Letters*, **24**, 1405-1413.
[6]   Shi J, Zhang X, Jiang H. (**2010**) 2-(Penta-1,3-diynyl)-5-(3,4-dihydroxybut-1-ynyl)thiophene, a novel NQO1 inducing agent from *Echinops grijsii* Hance. *Molecules*, **15**, 5273-5281.
[7]   Lu Z, Li C, Fang T, Lia G, Bo Z. (**2013**) Triindole-cored star-shaped molecules for organic solar cells. *Journal of Materials Chemistry A*, **1**, 7657–7665.
[8]   Klingstedt T, Shirani H, Mahler J, Wegenast-Braun BM, Nyström S, Goedert M, Jucker M, Nilsson KPR. (**2015**) Distinct spacing between anionic groups: An essential chemical determinant for achieving thiophene-based ligands to distinguish β-amyloid or tau polymorphic aggregates. *Chemistry (Weinheim an Der Bergstrasse, Germany)*, **21**, supporting Information, 7-8.
[9]   Wang Y, Li X, Meng DL, Li ZL, Zhang P, Xu J. (**2006**) Thiophenes from *Echinops latifolius*. *Journal of Asian Natural Products Research*, **8**, 585-588.
[10]   Norton RA, Finlayson AJ, Towers GHN. (**1985**) Thiophene production by crown galls and callus tissues of *Tagetes patula*. *Phytochemistry*, **24**, 719-722.
[11]   Kilbinger AFM, Schenning APHJ, Goldoni F, Feast WJ, Meijer EW. (**2000**) Chiral aggregates of α,ω-disubstituted sexithiophenes in protic and aqueous media. *Journal of the American Chemical Society*, **122**, 1820-1821.
[12]   Abegaz BM. (**1991**) Polyacetylenic thiophenes and terpenoids from the roots of *Echinops pappii*. *Phytochemistry*, **30**, 879-881.
[13]   Zhang P, Jin WR, Shi Q, He H, Ma ZJ, Qu HB. (**2008**) Two novel thiophenes from *Echinops grijissi* Hance. *Journal of Asian Natural Products Research*, **10**, 977-981.

Cyclic Lipopeptides with Herbicidal and Insecticidal Activities Produced by *Bacillus clausii* DTM1

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Seven cyclic lipopeptide biosurfactants (**1-7**) were isolated for the first time from the fermentation broth of endophytic *Bacillus clausii* DTM1 and were identified as anteisoC$_{13}$[Val7] surfactin-(L-Glu)-O-methyl-ester (**1**), anteisoC$_{12}$[Val7] surfactin (**2**), anteisoC$_{15}$[Val7] surfactin (**3**), isoC$_{14}$[Leu7] surfactin (**4**), anteisoC$_{12}$[Leu7] surfactin (**5**), nC$_{13}$[Leu7] surfactin (**6**), and anteisoC$_{14}$[Leu7] surfactin-(L-Glu)-O-methyl-ester (**7**); **1** has not been isolated before as a natural product from any source. Plate-based herbicide and insecticide bioassays showed that all compounds exhibited interesting insecticidal and herbicidal activities.

Keywords: *Bacillus clausii*, Surfactin, Insecticidal, Herbicidal.

The secondary metabolites of *Bacillus* species are known for their ability to control plant diseases through various mechanisms [1]. It is also documented that *Bacillus* species have the potential to synthesize a wide variety of metabolites with antibacterial and/or antifungal activity, which is one determinant of their ability to control plant diseases when applied as a biological control agent [1-4]. For instance, *B. subtilis*, the most studied species, is known to have strong antifungal activity by producing fungicidal and fungistatic peptides [3]. However, to the best of our knowledge, there have been limited studies on the activity of these peptides for controlling insects and weeds [5-7]. *B. clausii*, as one of the *Bacillus* species, has rarely been studied.

In this study, seven cyclic lipopeptides were isolated from the fermentation broth of *B. clausii* DTM1, and were identified as surfact in isomers. Of these seven, compound **1** was isolated as a natural product for the first time (although it had been prepared before by esterification of valine-7 surfactin isomer with a chain length of 13 carbons, but its spectral data have not been previously reported [8]). The complete assignments of ^{1}H and ^{13}C NMR spectral data have been achieved by various 2D NMR experiments including ^{1}H-^{1}H COSY, HSQC, HMBC and ROESY.

Compound **1** was obtained as a white gum. Its molecular formula was assigned as C$_{51}$H$_{89}$N$_7$NaO$_{13}$ on the basis of the HRESIMS. In the ^{1}HNMR spectrum, seven NH and seven α-H signals showed the presence of seven amino acid units. A strong signal at δ1.21 indicated the presence of a fatty acid chain. The ^{13}C NMR spectrum showed 10 ester/amide carbonyl signals and 7α-C signals. Amino acid units were identified as glutamic acid (Glu×1), aspartic acid (Asp×1), valine (Val×2) and leucine (Leu×3) on the basis of ^{1}H-^{1}H COSY analysis. The HMBC correlation between the signals at δ3.57 (the ^{1}H signal of OCH$_3$) and 173.8 (the residual CO of Glu1) established that the carboxyl group of Glu 1 was esterified. The substitution type of the branching methyl in the hydroxyl fatty acid chain was identified as anteiso by a ^{13}C NMR experiment using the method described by Lin *et al.* [9]. The sequence of the amino acid units and the fatty acid unit were assigned by a ROSEY experiment.

	R$_1$		R$_2$
1	—CH$_2$CH(CH$_3$)CH$_2$CH$_3$		—Me
2	—CH(CH$_3$)CH$_2$CH$_3$		—H
3	—CH$_2$CH$_2$CH$_2$CH(CH$_3$)CH$_2$CH$_3$		—H

	R$_1$		R$_2$
4	—CH$_2$CH$_2$CH$_2$CH(CH$_3$)$_2$		—H
5	—CH(CH$_3$)CH$_2$CH$_3$		—H
6	—CH$_2$CH$_2$CH$_2$CH$_3$		—H
7	—CH$_2$CH$_2$CH(CH$_3$)CH$_2$CH$_3$		—Me
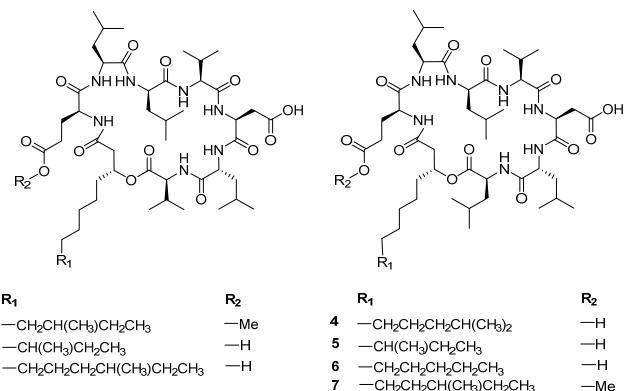
Figure 1: Structures of compounds **1-7**.
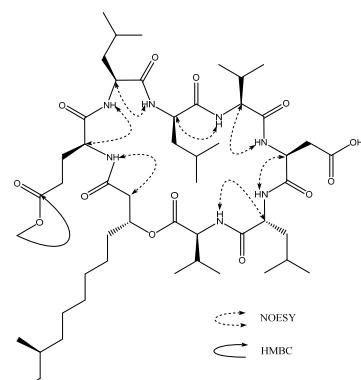
Figure 2: Key HMBC and NOESY of compound **1**.

Thus, the primary structure was established. The configurations of the amino acid units were confirmed as L-Glu (×1), L-Val (×2), L-Leu (×1), L-Asp(×1), and D-Leu (×2) by Marfey's reaction [10], and assigned as L-, L-, D-, L-, L-, D-, and L-, from N- to C-terminal according to previous biogenesis research. The absolute stereochemistry of the β-hydroxyl function of the fatty acid chain

Table 1: ^{1}H and ^{13}C-NMR spectral data of compound **1**[a].

Name	Position	δH	δC	Name	Position	δH	δC
Glu1	NH	7.82 (d, 6.6)			α-C	4.55(m)	49.6
	C=O		170.71		β-C	2.73(dd,5.10,16.6)/2.58(dd,8.8,16.6)	35.7
	α-C	4.17(m)	52.39		δC=O		171.6
	β-C	1.79(m)/1.93(m)	27.1	Leu6	NH	7.62(d, 8.5)	
	γ-C	2.24(m)	29.7		C=O		171.5
	δ-C=O		173.8		α-C	4.51(m)	50.7
	δ-OCH3	3.57	51.3		β-C	1.52(m)	41.1
Leu2	NH	7.99(d, 5.7)			γ-C	1.52(m)	24.2
	C=O		172.8		δ-C	0.84(m)	21.6/22.0
	α-C	4.17(m)	51.9	Val7	NH	8.23 (d, 8.2)	
	β-C	1.52(m)	39.4		C=O		171.7
	γ-C	1.52(m)	24.2		α-C	4.09(m)	50.7
	δ-C	0.84(m)	22.9/22.8		β-C	2.07(m)	29.4
Leu3	NH	8.29(d,7.1)			γ-C	0.84(m)	17.8/19.0
	C=O		172.60	Fatty acid	1		169.9
	α-C	4.17(m)	51.8		2	2.48(m)/2.33(m)	41.6
	β-C	1.52(m)	39.4		3	5.06(m)	71.4
	γ-C	1.52(m)	24.2		4	1.60(m)	33.5
	δ-C	0.84(m)	22.8/22.6		5	1.22(m)	24.3
Val4	NH	7.74(d, 8.3)			6-8	1.21(m)	28.6-29.0
	C=O		171.0		9	1.05(m)/1.25(m)	35.9
	α-C	4.02(m)	58,6		10	1.28(m)	33.6
	β-C	1.99(m)	30.1		11	1.21(m)	26.3
	γ-C	0.84(m)	17.8/19.0		12	0.82(m)	11.1
Asp5	NH	8.15(d,7.4)			13	0.83(m)	19.0
	C=O		169.9				

[a]Measured in DMSO-d_6 at 400MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR.

was (R) according to previous biogenesis research [11] and confirmed by comparing the specific rotation with known compounds [12]. The structure of **1** was determined to be a methylated product of [Val7] surfactin isomer with a chain length of 13 carbons.

The structures of compounds **2-7** were identified by comparison of their spectral data with those described in the literature [12, 13].

The bioassay, arranged by Sygenta, has been described in earlier work [14, 15] and the results are given in Table 2. All the compounds showed activity against aphid species. It has been speculated that surfactin isomers interact with cuticle lipid molecules of aphids and induce cuticle membrane perturbation [5]. Compound **6** was also active against *Plutella xylostella*. Compounds **2-6** were active on *Poa annua*, and this is the first report of surfactin's activity against this plant species. Compounds **1** and **7** showed weaker activity against *P. annua*, which may be due to the methyl esterification of [Glu-1].

Due to the development of resistant mutants and new physiological races of pests and pathogens, many synthetic pesticides are gradually becoming ineffective. This creates a need to find alternative ways to control farm pests and pathogens. Natural products such as surfactins, which have activity against not only plant pathogens but also pests and weeds, make them an ideal biocontrol agent. *B. clausii* might be a valuable source which could be commercialized as mentioned above.

Table 2: Herbicidal and insecticidal activities of compounds **1-7**.

Compd.	Herbicidal activity		Insecticidal activity		
	Poa annua	*Arabidopsis thaliana*	*Aphid species*	*Plutella xylostella*	*Diabrotica balteata*
1	49[a]	0	99	0	0
2	99	0	99	0	0
3	99	0	99	0	0
4	99	0	99	0	0
5	99	0	99	0	0
6	99	0	99	99	0
7	49	0	99	0	0

a. Data are presented as the means of the assessment scores across two replicates for the herbicide assays and three replicates for the insecticide assays.

Experimental

General: Perkin-Elmer-241 polarimeter; Perkin-Elmer Lambda 35 UV-VIS spectrophotometer; Perkin-Elmer one FT-IR spectrometer (KBr); Bruker-Ascend-400 MHz instrument at 300 K, with TMS as

internal standard; preparative HPLC utilized a Waters equipped with a Kromasil RP-C18 column(10 × 250 mm, ID×L) and a UV detector; columns were of either silica gel (300-400 mesh) or Sephadex LH-20; all the solvents used were of analytical grade.

***Material and cultivation of* Bacillus strain:** The bacterial strain was isolated using PDA from the plant *Dracocephalum tanguticum* Maxim., and was identified as *Bacillus clausii* by characterization and complete 16S rRNA gene sequence. The strain (Genbank accession number was KR632489) has been preserved at Chengdu Institute of Biology, Chinese Academy of Sciences, China. This bacterium was cultivated on a 30 L scale using 1L Erlenmeyer flasks containing 400 mL of the seed PDA medium for 3 days and fermentation medium (soluble starch 0.8%, peptone 0.5%, NaCl 0.2%, CaCO3 0.2%, MgSO4·7 H2O 0.05%, K2HPO4 0.05%) for 14 days at 28°C on a rotary shaker (250rpm).

Extraction and isolation of compounds: The fermentation broth (30 L) of *B.clausii* was filtered. The filtrate was extracted with EtOAc and the solvent was removed under vacuum. The EtOAc residue (18 g) was separated into 4 fractions by CC on silica gel (300-400 mesh), eluting stepwise with a CHCl3/MeOH gradient (CHCl3, CHCl3/MeOH: 10:1, v/v, CHCl3/MeOH: 3:1, v/v, MeOH). The fourth fraction (eluted with MeOH) was separated by Sephadex LH-20 (CHCl3/MeOH: 1:1, v/v) to afford 3 fractions (Fr.1-Fr.3). Fr.1 was further purified on a Waters preparative HPLC equipped with a Kromasil RP-C18 column (10 × 250 mm, ID × L; MeOH/H2O: 90:10, v/v) to afford **1** (8.3 mg), **2** (12.5 mg), **3** (82 .6 mg), **4** (53.3 mg), **5** (34.7 mg), **6** (28.4 mg) and **7** (18.5 mg).

Herbicide assays: The compounds were tested for herbicidal activity against *Arabidopsis thaliana* at 10 ppm and *Poa annua* at 32 ppm. Test plates were stored for 7 days in a controlled environment cabinet. They were scored as 0 or 99, where 99 = herbicidal effect, and 0 = no effect.

Insecticide assays: The compounds were tested for activity against an aphid species at 1000 ppm on a leaf-piece based assay, and against *Plutella xylostella* and *Diabrotica balteata* at 500 ppm in artificial diet assays. Compounds were applied to feeding aphids, or prior to infestation with *P. xylostella* and *D. balteata* larvae.

Positive control compounds were included in each test: thiamethoxam and indoxacarb for insecticide assays and norflurazon for herbicide assays (S21 and S22 in supplementary information). Test plates were stored in a controlled environment cabinet for 6 days for the aphid species, 5 days for *D. balteata* and 9 days for *P. xylostella*. Mortality was assessed relative to control wells using a 2 band system (0 or 99 where 99 = significant mortality($\geq$ 70%), 0 = mortality not significant/no effect($\leq$70%)).

Compound **1**
MP: 137-139°C.
$[\alpha]_D^{20}$: -14 (c 1.00, MeOH).
IR (KBr): 3421, 2957, 2927, 1657, 1534, 1205, 1018,592 cm^{-1}.
UV/Vis λmax (MeOH) nm (log ε): 206 (1.38).
^{1}H NMR and ^{13}C NMR: Table 1.
HR-ESI-MS *m/z*: 1030.6359 (calcd. 1030.6411 for $[C_{51}H_{89}N_7NaO_{13}]^+$)

Appendix - Supplementary data associated with this article including UV, IR, HR-ESIMS, ^{1}H, ^{13}C NMR spectra and Positive control data can be found on line.

References

[1]   Ramarathnam R, Bo S, Chen Y, Fernando WGD, Gao XW, de Kievit T. (**2007**) Molecular and biochemical detection of fengycin- and bacillomycin D-producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. *Canadian Journal of Microbiology*, **53**, 901-911.

[2]   Moyne AL, Shelby R, Cleveland TE, Tuzun S. (**2001**) Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. *Journal of Applied Microbiology*, **90**, 622-629.

[3]   Liu C, Sheng JP, Chen L, Zheng YY, Lee DYW, Yang Y, Xu MS, Shen L. (**2015**) Biocontrol activity of *Bacillus subtilis* isolated from *Agaricus bisporus* mushroom compost against pathogenic fungi. *Journal of Agricultural and Food Chemistry*, **63**, 6009-6018.

[[4]   Zhao ZZ, Wang QS, Wang KM, Brian K, Liu CH, Gu YC. (**2010**) Study of the antifungal activity of *Bacillus vallismortis* ZZ185 *in vitro* and identification of its antifungal components. *Bioresource Technology*, **101**, 292-297.

[5]   Yun DC, Yang SY, Kim YC, Kim IS, Kim YH. (**2013**) Identification of surfactin as an aphicidal metabolite produced by *Bacillus amyloliquefaciens* G1. *Journal of the Korean Society for Applied Biological Chemistry*, **56**, 751-753.

[6]   Ghribi D, Elleuch M, Abdelkefi L, Ellouze-Chaabouni S. (**2012**) Evaluation of larvicidal potency of *Bacillus subtilis* SPB1 biosurfactant against *Ephestia kuehniella* (Lepidoptera: Pyralidae) larvae and influence of abiotic factors on its insecticidal activity. *Journal of Stored Products Research*, **48**, 68-72.

[7]   Geetha I, Paily KP, Manonmani AM. (**2012**) Mosquito adulticidal activity of a biosurfactant produced by *Bacillus subtilis* subsp. *subtilis*. *Pest Management Science*, **68**, 1447-1450.

[8]   Kowall M, Vater J, Kluge B, Stein T, Franke P, Ziessow D. (**1998**) Separation and characterization of surfactin isoforms produced by *Bacillussubtilis* OKB 105. *Journal of Colloid and Interface Science*, **204**, 1-8

[9]   Lin SC, Minton MA, Sharma MM, Georgiou G. (**1994**) Structural and immunological characterization of a biosurfactant produced by *Bacillus licheniformis* JF-2. *Applied and Environment Microbiology*, **60**, 31-38.

[10]   Marfey P. (**1984**) Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. *Carlsberg Research Communications*, **49**, 591-596.

[11]   Nagai S, Okimura K, Kaizawa N, Ohki K, Kanatomo S. (**1996**) Study on surfactin, a cyclic depsipeptide. II. synthesis of surfactin B2 produced by *Bacillus natto* KMD 2311. *Chemical & Pharmaceutical Bulletin*, **44**, 5-10.

[12]   Tang JS, Gao H, Hong K, Yu Y, Jiang MM, Lin HP, Ye WC, Yao XS. (**2007**) Complete assignments of ^{1}H and ^{13}C NMR spectral data of nine surfactin isomers. *Magnetic Resonance in Chemistry*, **45**, 792-796.

[13]   Liu XY, Yang SZ, Mu BZ. (**2009**) Production and characterization of a C15-surfactin-O-methyl ester by alipopeptide producing strain *Bacillus subtilis* HSO121. *Process Biochemistry*, **44**, 1144-1151.

[14]   Zhang MZ, Chen Q, Mulholland N, Beattie D, Irwin D, Gu YC, Yang GF, Clough J. (**2012**) Synthesis and fungicidal activity of novel pimprinine analogues. *European Journal of Medicinal Chemistry*, **53**, 283-291.

[15]   Chen JL, Tang W, Che JY, Chen K, Yan G, Gu YC, Shi DQ. (**2015**) Synthesis and biological activity evaluation of novel α-amino phosphonate derivatives containing a pyrimidinyl moiety as potential herbicidal agents. *Journal of Agricultural and Food Chemistry*, **63**, 7219-7229.

Synthesis of (6*R*,12*R*)-6,12-Dimethylpentadecan-2-one, the Female-Produced Sex Pheromone from Banded Cucumber Beetle *Diabrotica balteata*, Based on a Chiron Approach

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Herein we describe a synthesis of (6*R*,12*R*)-6,12-dimethylpentadecan-2-one (**5**), the female produced sex pheromone of banded cucumber beetle *Diabrotica balteata* Le Conte, from (*R*)-4-methyl-δ-valerolactone, a methyl-branched chiron.

Keywords: Sex pheromone, (6*R*,12*R*)-6,12-dimethylpentadecan-2-one, Methyl-branched chiron, (*R*)-4-Methyl-δ-valerolactone, Synthesis.

Pheromones are species-specific biofunctional molecules that are used for communication between individuals within the same species, and thus are useful tools for pest control. Among various pheromones, those with one or more chiral methyl-branched units [1]—such as compounds **1**–**5**, Figure 1—attract us most, for we have developed several methyl chirons from industrial waste and been interested in applying them to the syntheses of natural products [2]. Herein we report a synthesis of (6*R*,12*R*)-6,12-dimethylpentadecan-2-one (**5**) from (*R*)-4-methyl-δ-valerolactone (**6**).
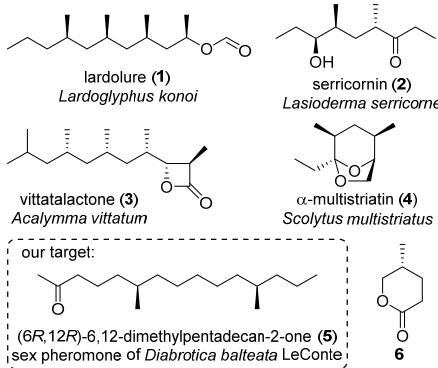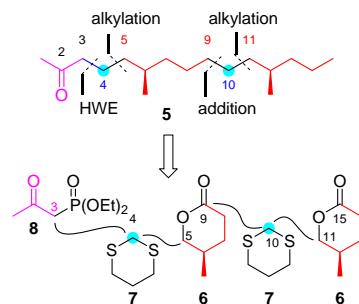
Figure 1: Chemical structures of chiral methyl-branched pheromones **1**–**5**.

Ketone **5** is the female produced sex pheromone of banded cucumber beetle (BCB) *Diabrotica balteata* Le Conte, the larvae of which are serious pests of a variety of agricultural crops such as curbits and sweet potatoes. The structure of **5** was elucidated by spectroscopic analyses and confirmed by synthesis [3]. The stereochemistry was provided by Mori's synthesis of all the four possible isomers and further affirmed by two other stereoselective syntheses [4].

Our synthetic plan is depicted in Scheme 1. Both lactone **6** and 1,3-dithiane **7** were to be used twice. The former was to form the C5–C9 and C11–C15 units in **5**, and the latter to act as linking point for fragments **6** and diethyl (2-oxopropyl)phosphonate (**8**).
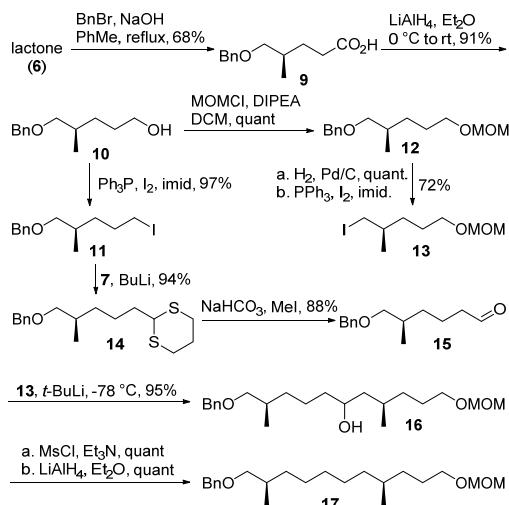
Scheme 1: Synthetic plan for (6*R*,12*R*)-6,12-dimethylpentadecan-2-one (**5**).

As shown in Scheme 2, our synthesis commenced with opening the lactone ring of **6**. Treatment of **6** with NaOH and BnBr in refluxing toluene successfully delivered acid **9** in 68% yield on a 0.2 mol scale. After reducing the acid with LiAlH$_4$, removal of the exposed hydroxyl group in resultant **10** was investigated and found to be low-yielding due to the low boiling point of the product (*ca* 142ºC). Therefore we decided to postpone this step to a later stage of the synthesis. Compound **10** was first protected as the MOM ether, then the benzyl group was removed by catalytic hydrogenation and the newly exposed OH was converted to iodide, giving **13** in 72% overall yield. In another direction, **10** was directly converted to iodide **11**, which was then treated with litho dithiane to afford **14** in high yield.
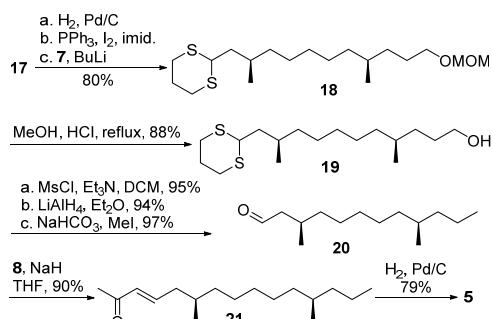
With **13** and **14** in hand, we first tried to couple them directly by preparing the anion of **14** and then reacting with **13**, but found this reaction to be low-yielding and irreproducible. Therefore, dithiane **14** was unmasked (NaHCO$_3$, MeI, MeCN, water) [5] and the resultant aldehyde **15** was coupled with the lithio derivative of **13** [6] to give **16** in high yield as a mixture of epimers. The unneeded hydroxyl group on **16** was removed by transforming to mesylate and reducing it with LiAlH$_4$ [7], affording **17** in nearly quantitative yield.

Then the benzyl ether of **17** was cleaved and the exposed hydroxyl group was transformed to iodide and coupled with 1,3-dithiane anion again to deliver **18** in 80% overall yield. The unneeded hydroxyl group at the right side was again removed in good yield

Scheme 2: Synthesis of **17**.

via cleaving the MOM ether with HCl, preparing the corresponding mesylate of **19**, and reducing it with LiAlH$_4$. The dithiane moiety was unmasked to give aldehyde **20** in high yield and then underwent Horner–Wadsworth–Emmons olefination with **8** to give enone **21**, which was subjected to catalytic hydrogenation to deliver **5** in 79% yield. The spectroscopic properties of the synthetic **5** are consistent with those reported ([α]$_D^{24}$: −0.395 (*c* 0.41, CHCl$_3$); reported in reference 4d: [α]$_D^{23}$: −0.40 (*c* 0.40, CHCl$_3$)).

Scheme 3: Completion of the synthesis of (6*R*,12*R*)-6,12-dimethylpentadecan-2-one (**5**)

In conclusion, we have achieved a synthesis of (6*R*,12*R*)-6,12-dimethylpentadecan-2-one (**5**), the female produced sex pheromone of BCB *Diabrotica balteata* Le Conte, in 17 steps with an overall yield of 20% from (*R*)-4-methyl-δ-valerolactone (**6**). Although the synthesis is relatively longer than previous ones, the reactions used herein are simpler and easier to handle.

Experimental

General: All reactions sensitive to air or moisture were performed in flame-dried round bottom flasks with rubber septum under a positive pressure of either argon or nitrogen atmosphere, unless otherwise noted. Air and moisture-sensitive liquids and solutions were transferred via syringe and stainless steel cannula. Reactions were monitored by thin-layer chromatography (TLC) carried out on silica gel plates using UV light as visualizing agent and an ethanolic solution of phosphomolybdic acid, and heat as developing agents. NMR spectra were recorded on a Bruker AMX-300 instrument and calibrated using TMS or residual undeuterated solvent as an internal reference [^{1}H NMR: TMS (0.00); ^{13}C NMR: CDCl$_3$ (77.16)]. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, br = broad.

(*R*)-5-(Benzyloxy)-4-methylpentanoic acid (9): To a solution of lactone **6** (23.0 g, 0.20 mol) in toluene (400 mL) were added NaOH (24 g, 0.60 mol) and BnBr (37.6 g, 0.22 mol). The flask was equipped with a Dean–Stark trap, heated to reflux for 20 h, cooled, added water (200 mL), and acidified with HCl aqueous solution. The mixture was separated and the aqueous layer was extracted with diethylether (150 mL×2). The combined organic layers were washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. Purification via flash column chromatography on silica gel (PE/EA: 3/1) afforded acid **10** (30.4 g, 68%) as a colorless liquid.
[α]$_D^{27}$: +1.68 (*c* 1.26, CHCl$_3$).
IR (KBr): 3033, 2962, 1708, 1496, 1454, 1276, 1100, 938, 738, 714 cm^{-1}.
^{1}H NMR (300 MHz, CDCl$_3$): 7.26-7.35 (m, 5H), 4.50 (s, 2H), 3.31 (d, 2H, *J* = 5.7 Hz), 2.36-2.43 (m, 2H), 1.78-1.88 (m, 2H), 1.47-1.55 (m, 1H), 0.95 (d, 3H, *J* = 5.7 Hz)
MS (EI, 70 eV): *m/z* (%) = 222 [M$^+$] (100)
Anal. Calcd for C$_{13}$H$_{18}$O$_3$: C, 70.24; H, 8.16. Found C, 70.28; H, 8.27.

(*R*)-5-(Benzyloxy)-4-methylpentan-1-ol (10): To a suspension of LiAlH$_4$ (1.23 g, 32 mmol) in dry diethylether (50 mL) was added dropwise a solution of acid **9** (6.0 g, 27 mmol) in diethylether (20 mL) at 0ºC. The mixture was then stirred at room temperature and quenched with Na$_2$SO$_4$•10H$_2$O after ~~which~~ TLC showed complete consumption of **9**. The mixture was filtered and washed with diethylether. The filtrate was concentrated and purified via flash column chromatography on silica gel (PE/EA: 5/1) to give **10** (5.09 g, 91%) as a colorless liquid.
[α]$_D^{28}$: +2.03 (*c* 1.26, CHCl$_3$).
IR (KBr): 2934, 2864, 1497, 1454, 1364, 1099, 1073, 1029, 736, 698 cm^{-1}.
^{1}H NMR (300 MHz, CDCl$_3$): 7.26-7.35 (m, 5H), 4.50 (s, 2H), 3.60-3.64 (m, 2H), 3.25-3.35 (m, 2H), 2.27 (bs, 1H), 1.47-1.81 (m, 4H), 1.16-1.21 (m, 1H), 0.93 (d, 3H, *J* = 6.6 Hz).
MS (EI, 70 eV): *m/z* (%) = 208 [M$^+$] (100).
Anal. Calcd for C$_{13}$H$_{20}$O$_2$: C, 74.96; H, 9.68. Found C, 74.92; H, 9.73.

(2*R*)-1-Benzyloxy-5-iodo-2-methylpentane (11): To a solution of PPh$_3$ (393 mg, 1.5 mmol) and imidazole (204 mg, 3.0 mmol) in dry CH$_2$Cl$_2$ (15 mL) was added I$_2$ (381 mg, 1.5 mmol) at 0ºC. The mixture was stirred for 30 min before a solution of **10** (104 mg, 0.50 mmol) in CH$_2$Cl$_2$ (2 mL) was added. The mixture was stirred at ambient temperature and quenched with water after TLC showed complete consumption of **10**. The mixture was diluted with CH$_2$Cl$_2$ (50 mL), washed with a saturated solution of Na$_2$S$_2$O$_3$ (20 mL) and brine (20 mL) sequentially, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 80/1) afforded **11** (154 mg, 97%) as a colorless liquid.
[α]$_D^{28}$: +4.55 (*c* 1.03, CHCl$_3$).
IR (KBr): 3064, 3030, 2957, 2854, 1496, 1454, 1363, 1222, 1179, 1099, 1029, 843, 736, 697, 611, 415 cm^{-1}.
^{1}H NMR (300 MHz, CDCl$_3$): 7.25-7.38 (m, 5H), 4.49 (s, 2H), 3.23-3.32 (m, 2H), 3.12-3.18 (m, 2H), 1.75-1.88 (m, 3H), 1.49-1.57 (m, 1H), 1.21-1.29 (m, 1H), 0.92 (d, 3H, *J* = 6.9 Hz).
^{13}C NMR (75 MHz, CDCl$_3$): 138.52, 128.24, 127.43, 127.39, 75.44, 72.90, 34.57, 32.69, 31.09, 17.05, 7.21
HRMS-EI: *m/z* [M$^+$] calcd for C$_{13}$H$_{19}$OI: 318.0481; found: 318.0480.

(2*R*)-1-Benzyloxy-5-methoxymethoxy-2-methylpentane (12): To a solution of **10** (15.0 g, 72 mmol) in dry CH$_2$Cl$_2$ (150 mL) were added *i*Pr$_2$NEt (23.9 mL, 144 mmol) and MOMCl (11.3 mL, 144

mmol) at 0ºC. The mixture was stirred at ambient temperature and quenched with water after TLC showed that **10** was completely consumed. The mixture was diluted with CH_2Cl_2, separated, washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 30/1) afforded **12** (18.8 g, quantitative) as a colorless liquid.

$[\alpha]_D^{24}$: +0.24 (*c* 0.80, CHCl₃).

IR (KBr): 2932, 2876, 1497, 1454, 1207, 1152, 1110, 1048, 919, 737, 698, 415, 410, 402 cm⁻¹.

¹H NMR (300 MHz, CDCl₃): 7.26-7.39 (m, 5H), 4.62 (s, 2H), 4.51 (s, 2H), 3.24-3.61 (m, 7H), 1.48-1.83 (m, 4H), 1.19-1.27 (m, 1H), 0.97 (d, 3H, *J* = 6.9 Hz).

¹³C NMR (75 MHz, CDCl₃): 138.71, 128.24, 127.35, 96.33, 75.63, 72.91, 68.01, 65.80, 55.01, 33.30, 30.10, 27.15, 17.03.

MS (EI, 70 eV): *m/z* (%) = 252 [M⁺] (100).

HRMS-EI: *m/z* [M+Na⁺] calcd for $C_{15}H_{24}O_3$: 275.1623; found: 275.1625.

(*R*)-5-(Methoxymethoxy)-2-methylpentan-1-ol: A reaction flask containing **12** (18.6 g, 74 mmol), Pd/C (5%, 3.0 g), and MeOH (150 mL) was evacuated and back-filled with hydrogen (1 atm). The reaction mixture was stirred at ambient temperature under hydrogen for 3 days and then filtered over a plug of silica gel topped with Celite (MeOH eluent). The filtrate was concentrated and purified by flash column chromatography on silica gel (PE/EA: 10/1) to give the title compound (11.3 g, quantitative) as a colorless liquid.

$[\alpha]_D^{24}$: +10.5 (*c* 1.04, CHCl₃).

IR (KBr): 2935, 2878, 1466, 1386, 1216, 1153, 1111, 1046, 920, 414, 402 cm⁻¹.

¹H NMR (300 MHz, CDCl₃): 4.62 (s, 2H), 3.38-3.55 (m, 4H), 3.36 (s, 3H), 2.71 (t, 1H, *J* = 5.4 Hz), 1.43-1.72 (m, 4H), 1.11-1.23 (m, 1H), 0.92 (d, 3H, *J* = 6.6 Hz).

¹³C NMR (75 MHz, CDCl₃): 96.15, 67.92, 67.61, 54.90, 35.37, 29.43, 26.92, 16.40.

MS (ESI): *m/z* = 185 [M+Na⁺].

(*R*)-1-Iodo-5-(methoxymethoxy)-2-methylpentane (13): To a solution of PPh₃ (19.65 g, 75 mmol) and imidazole (10.2 g, 75 mmol) in dry CH_2Cl_2 (100 mL) was added I₂ (19.1 g, 75 mmol) at 0ºC. The mixture was stirred for 20 min before a solution of (*R*)-5-(methoxymethoxy)-2-methylpentan-1-ol (4.0 g, 24 mmol) in CH_2Cl_2 (15 mL) was added. The mixture was stirred at ambient temperature and quenched with water after TLC showed complete consumption of the starting material. The mixture was diluted with CH_2Cl_2 (150 mL), washed with a saturated solution of $Na_2S_2O_3$ (100 mL) and brine sequentially, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 20/1) afforded **13** (4.9 g, 72%) as a colorless liquid.

$[\alpha]_D^{24}$: +0.18 (*c* 1.82, CHCl₃).

IR (KBr): 2931, 2882, 1458, 1379, 1196, 1145, 1111, 1044, 919, 415, 409, 402 cm⁻¹.

¹H NMR (300 MHz, CDCl₃): 4.56 (s, 2H), 3.45-3.50 (m, 2H), 3.31 (s, 3H), 3.08-3.20 (m, 2H), 1.36-1.58 (m, 4H), 1.18-1.1.30 (m, 1H), 0.94 (d, 3H, *J* = 6.6 Hz).

¹³C NMR (75 MHz, CDCl₃): 96.39, 67.64, 55.14, 36.18, 34.55, 27.13, 20.49, 17.40.

MS (EI, 70 eV): *m/z* (%) = 211 [M-C₂H₅O₂]⁺ (100).

(*R*)-2-(5-(Benzyloxy)-4-methylpentyl)-1,3-dithiane (14): To a solution of 1,3-dithiane (**7**) (151 mg, 1.26 mmol) in anhydrous THF (8 mL) at 0ºC under argon was added a solution of *n*-BuLi (1.5 M in pentane, 0.86 mL, 1.38 mmol). The mixture was stirred for 30 min and added to a solution of **11** (200 mg, 0.63 mmol) in THF (3.0 mL)

and warmed to room temperature. After **11** was fully consumed, the reaction was quenched with water, extracted with EtOAc (30 mL × 3), the extracts combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 50/1) afforded **14** (184 mg, 94%) as a colorless liquid.

$[\alpha]_D^{24}$: +0.89 (*c* 0.92, CHCl₃).

IR (KBr): 3030, 2934, 2901, 2856, 1496, 1454, 1423, 1363, 1276, 1180, 1100, 1028, 909, 845, 737, 698, 414 cm⁻¹.

¹H NMR (300 MHz, CDCl₃): 7.22-7.34 (m, 5H), 4.48 (s, 2H), 4.03 (t, 1H, *J* = 6.9 Hz), 3.19-3.31 (m, 2H), 2.72-2.89 (m, 4H), 2.05-2.17 (m, 1H), 1.68-1.92 (m, 4H), 1.40-1.61 (m, 3H), 1.04-1.19 (m, 1H), 0.92 (d, 3H, *J* = 6.3 Hz).

¹³C NMR (75 MHz, CDCl₃): 138.60, 128.17, 127.37, 127.27, 75.60, 72.80, 47.44, 35.54, 33.15, 32.99, 30.33, 25.89, 23.87, 16.90.

MS (ESI): *m/z* = 349 [M+K⁺].

HRMS-MALDI: *m/z* [M+Na⁺] calcd for $C_{17}H_{26}OS_2$: 333.1323; found: 333.1316.

(*R*)-6-(Benzyloxy)-5-methylhexanal (15): To a solution of **14** (8.01 g, 25.8 mmol) in MeCN/water (80 mL/24 mL) were added MeI (16 mL, 258 mmol) and NaHCO₃ (21.7 g, 258 mmol) at room temperature. The mixture was stirred for 12 h, concentrated under reduced pressure, dissolved in water, extracted with EtOAc (100 mL × 3), the extracts combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 20/1) afforded **15** (4.98 g, 88%) as a colorless liquid.

$[\alpha]_D^{24}$: +3.54 (*c* 1.08, CHCl₃).

IR (KBr): 3035, 2856, 2721, 1725, 1496, 1455, 1363, 1100, 1029, 738, 698, 463, 451, 415 cm⁻¹.

¹H NMR (300 MHz, CDCl₃): 9.74 (t, 1H, *J* = 1.5 Hz), 7.25-7.35 (m, 5H), 4.49 (s, 2H), 3.23-3.33 (m, 2H), 2.39-2.44 (m, 2H), 1.44-1.81 (m, 4H), 1.14-1.18 (m, 1H), 0.93 (d, 3H, *J* = 6.6 Hz).

¹³C NMR (75 MHz, CDCl₃): 202.60, 138.61, 128.26, 127.46, 127.40, 75.52, 72.96, 44.06, 33.31, 33.10, 19.47, 16.88.

MS (EI, 70 eV): *m/z* (%) = 221 [M+H⁺] (100).

HRMS-MALDI: *m/z* [M+Na⁺] calcd for $C_{14}H_{20}O_2$: 243.1361; found: 243.1360.

(2*R*,8*R*)-1-(Benzyloxy)-11-(methoxymethoxy)-2,8-dimethyl-undecan-6-ol (16): Under argon, to a solution of **13** (2.48 g, 9.10 mmol) in dry diethylether/pentane (20 mL/30 mL) was added a solution of *t*-BuLi (1.5 M in pentane, 12.7 mL, 19.1 mmol) at −78ºC. The mixture was stirred at −78ºC for 5 min and then at room temperature for 1 h. At −78ºC, to the resultant solution was added a solution of **15** (800 mg, 3.64 mmol) in diethylether/pentane (8 mL/12 mL). The mixture was stirred at room temperature and quenched with water after **15** was consumed completely. The mixture was separated and the aqueous layer was extracted with diethylether (60 mL × 3). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 5/1) gave **16** (1.27 g, 95%) as an inseparable mixture of epimers.

IR (KBr): 3031, 2930, 2873, 1497, 1455, 1213, 1153, 1111, 1045, 919, 736, 698, 434, 416, 403 cm⁻¹.

¹H NMR (300 MHz, CDCl₃): 7.12-7.20 (m, 5H), 4.48 (s, 2H), 4.36 (s, 2H), 3.46-3.51 (m, 1H), 3.35-3.40 (m, 1H), 3.22 (s, 3H), 3.08-3.19 (m, 2H), 0.98-1.64 (m, 15H), 0.76-0.80 (m, 6H).

¹³C NMR (75MHz, CDCl₃): 138.74, 128.26, 127.49, 127.38, 96.37, 75.84, 72.92, 69.43, 55.12, 45.14, 38.61, 35.55, 35.41, 29.59, 28.42, 27.02, 22.91, 19.20, 17.05, 16.53.

HRMS-MALDI: *m/z* [M+Na⁺] calcd for $C_{22}H_{38}O_4$: 389.2668; found: 389.2670.

(2*R*,8*R*)-1-(Benzyloxy)-11-(methoxymethoxy)-2,8-dimethyl-undecan-6-yl methanesulfonate: To a solution of **16** (290 mg, 0.79 mmol) in dry CH_2Cl_2 (5 mL) were added MsCl (0.10 mL, 1.2 mmol) and Et_3N (0.17 mL, 1.2 mmol) at 0ºC. Then the mixture was stirred at room temperature for 3 h and diluted with CH_2Cl_2 (50 mL). The solution was washed with water and brine sequentially, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 20/1) afforded the title compound (355 mg, inseparable mixture of epimers, 100%) as a colorless liquid.
$[\alpha]_D^{24}$: +1.68 (*c* 1.26, $CHCl_3$).
IR (KBr): 3031, 2935, 2872, 1497, 1455, 1356, 1336, 1175, 1110, 1030, 971, 906, 792, 739, 699, 610, 528, 415, 409 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): 7.26-7.39 (m, 5H), 4.79-4.82 (m, 1H), 4.61 (s, 2H), 4.49 (s, 2H), 3.49-3.56 (m, 2H), 3.35 (s, 3H), 3.24-3.30 (m, 2H), 2.98 (t, 3H, *J* = 2.1 Hz), 1.14-1.80 (m, 14H), 0.92-0.98 (m, 6H).
^{13}C NMR (75MHz, $CDCl_3$): 138.67, 128.27, 127.48, 127.41, 96.36, 82.42, 82.35, 75.41, 72.96, 67.89, 67.86, 65.48, 65.28, 55.14, 55.08, 41.82, 41.72, 38.72, 35.55, 34.90, 33.69, 33.39, 33.31, 33.05, 29.28, 28.92, 27.00, 26.95, 22.22, 19.69, 19.24, 17.00.
MS (EI, 70 eV): *m/z* (%) = 467 [M+Na$^+$] (100).

(2*R*,8*S*)-1-Benzyloxy-11-methoxymethoxy-2,8-dimethylundecane (17): To a suspension of LiAlH$_4$ (600 mg, 1.58 mmol) in dry diethylether (10 mL) was added dropwise a solution of the above prepared mesylate (350 mg, 0.79 mmol) in diethylether (5 mL) at 0ºC. The mixture was then stirred at room temperature for 12 h and quenched with $Na_2SO_4 \cdot 10H_2O$ after TLC showed complete consumption of **9**. The mixture was filtered and washed with diethylether. The filtrate was concentrated and purified via flash column chromatography on silica gel (with hexane) to give **17** (285 mg, 100%) as a colorless oil.
$[\alpha]_D^{24}$: −1.20 (*c* 0.82, $CHCl_3$).
IR (KBr): 3032, 2957, 2928, 2856, 1497, 1455, 1378, 1261, 1208, 1111, 1047, 920, 801, 736, 697, 417, 404 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): 7.25-7.38 (m, 5H), 4.62 (t, 2H, *J* = 1.5 Hz), 4.50 (s, 2H), 3.48-3.55 (m, 2H), 3.37 (s, 3H), 3.20-3.35 (m, 2H), 1.72-1.76 (m, 1H), 1.54-1.64 (m, 2H), 1.08-1.19 (m, 13H), 0.85-0.91 (m, 6H)
^{13}C NMR (75 MHz, $CDCl_3$): 138.84, 128.27, 127.48, 127.36, 96.38, 76.02, 72.93, 68.25, 55.07, 36.96, 33.66, 33.45, 33.39, 32.62, 30.25, 27.29, 27.01, 26.96, 19.59, 17.15
MS (EI, 70 eV): *m/z* (%) = 305 [M-C$_2$H$_5$O]$^+$ (100)
HRMS-MALDI: *m/z* [M-C$_2$H$_5$O]$^+$ calcd for $C_{22}H_{38}O_3$: 305.2481; found: 305.2487.

(2*R*,8*S*)-11-(Methoxymethoxy)-2,8-dimethylundecan-1-ol: A reaction flask containing **17** (2.30 g, 6.57 mmol), Pd/C (10%, 300 mg), and MeOH (30 mL) was evacuated and back-filled with hydrogen (1 atm). The reaction mixture was stirred at ambient temperature under hydrogen for 24 h and then filtered over a plug of silica gel topped with Celite (MeOH eluent). The filtrate was concentrated and purified by flash column chromatography on silica gel (PE/EA: 5/1) to give the title compound (1.67 g, 98%) as a colorless liquid.
$[\alpha]_D^{28}$: +6.54 (*c* 0.99, $CHCl_3$).
IR (KBr): 2928, 2857, 1465, 1379, 1214, 1153, 1112, 1046, 921, 418. 410 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): 4.62 (d, 2H, *J* = 0.9 Hz), 3.38-3.56 (m, 4H), 3.36 (s, 3H), 1.73-1.78 (m, 1H), 1.52-1.66 (m, 3H), 1.07-1.19 (m, 12H), 0.86-0.92 (m, 6H).
^{13}C NMR (75 MHz, $CDCl_3$): 96.33, 68.30, 68.23, 66.07, 55.04, 36.90, 35.72, 33.33, 33.11, 32.56, 30.19, 27.25, 26.94, 19.56, 16.56.
MS (EI, 70 eV): *m/z* (%) = 215 [M-C$_2$H$_5$O]$^+$ (100).

HRMS-MALDI: *m/z* [M-MeO]$^+$ calcd for $C_{15}H_{32}O_3$: 229.2168; found: 229.2164.

(2*R*,8*S*)-1-Iodo-11-(methoxymethoxy)-2,8-dimethylundecane: To a solution of PPh$_3$ (487 mg, 1.86 mmol) and imidazole (253 mg, 3.72 mmol) in dry CH_2Cl_2 (10 mL) was added I$_2$ (473 mg, 1.86 mmol) at 0ºC. The mixture was stirred for 20 min before a solution of alcohol (160 mg, 0.62 mmol) in CH_2Cl_2 (2 mL) was added. The mixture was stirred at ambient temperature and quenched with water after TLC showed complete consumption of the starting material. The mixture was diluted with CH_2Cl_2 (40 mL), washed with a saturated solution of $Na_2S_2O_3$ (20 mL) and brine sequentially, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 20/1) afforded iodide (210 mg, 92%) as a colorless liquid.
$[\alpha]_D^{24}$: −2.76 (*c* 0.77, $CHCl_3$).
IR (KBr): 2928, 2855, 1460, 1378, 1195, 1153, 1112, 1046, 920, 417, 404 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): 4.63 (s, 2H), 3.51 (d, 2H, *J* = 7.2 Hz), 3.36 (s, 3H), 3.13-3.26 (m, 2H), 1.49-1.61 (m, 2H), 1.12-1.43 (m, 14H), 0.97 (d, 3H, *J* = 6.3 Hz), 0.86 (d, 3H, *J* = 6.6 Hz).
^{13}C NMR (75 MHz, $CDCl_3$): 96.37, 68.22, 66.04, 55.06, 36.88, 36.42, 34.71, 33.37, 32.58, 29.94, 27.28, 26.82, 20.59, 19.58, 17.95.
MS (EI, 70 eV): *m/z* (%) = 325 [M-MOM]$^+$ (100).

2-((2*R*,8*S*)-11-(Methoxymethoxy)-2,8-dimethylundecyl)-1,3-dithiane (18): To a solution of 1,3-dithiane **7** (2.27 g, 18.9 mmol) in anhydrous THF (40 mL) at 0ºC under argon was added a solution of *n*-BuLi (2.5 M in hexane, 7.56 mL, 18.9 mmol). The mixture was stirred for 30 min and added to a solution of previously prepared iodide (1.40 g, 3.78 mmol) in THF (10 mL) and warmed to room temperature. After the starting material was fully consumed, the reaction was quenched with water, extracted with EtOAc (30 mL × 3), combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 50/1) afforded **18** (1.22 g, 89%) as a pale yellow liquid.
$[\alpha]_D^{24}$: −6.49 (*c* 0.55, $CHCl_3$).
IR (KBr): 2928, 2856, 1465, 1423, 1378, 1276, 1243, 1153, 1111, 1046, 919, 417, 404 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): 4.63 (s, 2H), 4.08-4.13 (m, 1H), 3.51 (t, 2H, *J* = 6.9 Hz), 3.37 (s, 3H), 2.78-2.92 (m, 4H), 2.08-2.17 (m, 1H), 1.43-1.92 (m, 6H), 1.11-1.39 (m, 13H), 0.85-0.92 (m, 6H).
^{13}C NMR (75 MHz, $CDCl_3$): 96.36, 68.24, 55.06, 45.57, 42.55, 36.93, 36.75, 33.37, 32.60, 30.57, 30.36, 30.13, 29.57, 27.28, 26.99, 26.75, 26.12, 19.58, 19.39.
MS (EI, 70 eV): *m/z* (%) = 362 [M$^+$] (100)
HRMS-EI: *m/z* [M$^+$] calcd for $C_{19}H_{38}O_2S_2$: 362.2313; found: 362.2322.

(4*S*,10*R*)-11-(1,3-Dithian-2-yl)-4,10-dimethylundecan-1-ol (19): A solution of **18** (90 mg, 0.25 mmol) in acidified MeOH (5 mL, two drops of HCl) was heated at reflux for 5 h and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (PE/EA: 10/1) to give **19** (70 mg, 88%) as a colorless liquid.
$[\alpha]_D^{24}$: −9.59 (*c* 0.45, $CHCl_3$).
IR (KBr): 2927, 2855, 1461, 1423, 1378, 1276, 1057, 908, 411 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): 4.05-4.10 (m, 1H), 3.60 (t, 2H, *J* = 6.6 Hz), 2.74-2.88 (m, 4H), 2.07-2.13 (m, 1H), 1.07-1.83 (m, 20H), 0.80-0.92 (m, 6H).
^{13}C NMR (75 MHz, $CDCl_3$): 63.37, 45.55, 42.51, 36.88, 36.72, 32.87, 32.55, 30.56, 30.34, 30.30, 30.07, 29.52, 26.93, 26.85, 26.71, 26.10, 19.60, 19.38.

MS (EI, 70 eV): m/z (%) = 318 [M$^+$] (100).
HRMS-EI: m/z [M$^+$] calcd for $C_{17}H_{34}OS_2$: 318.2051; found: 318.2053.

(4S,10R)-11-(1,3-Dithian-2-yl)-4,10-dimethylundecyl methanesulfonate: To a solution of **19** (770 mg, 2.42 mmol) in dry CH_2Cl_2 (30 mL) were added MsCl (0.28 mL, 3.63 mmol) and Et_3N (0.50 mL, 3.63 mmol) at 0°C. Then the mixture was stirred at room temperature for 5 h and diluted with CH_2Cl_2 (40 mL). The solution was washed with water and brine sequentially, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 10/1) afforded the title compound (910 mg, 95%) as a colorless liquid.
$[\alpha]_D^{24}$: −5.81 (c 0.43, $CHCl_3$).
IR (KBr): 2928, 2854, 1465, 1423, 1356, 1276, 1176, 919, 823, 528, 410 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): 4.22 (t, 2H, J = 6.6 Hz), 4.08-4.13 (m, 1H), 3.02 (s, 3H), 2.79-2.92 (m, 4H), 2.09-2.18 (m, 1H), 1.63-1.88 (m, 5H), 1.12-52 (m, 14H), 0.83-0.92 (m, 6H).
^{13}C NMR (75 MHz, $CDCl_3$): 70.50, 45.54, 42.52, 37.34, 36.72, 35.93, 32.50, 32.32, 30.57, 30.35, 30.03, 29.93, 29.53, 29.31, 26.90, 26.72, 26.11, 19.43, 19.38, 19.24.
MS (EI, 70 eV): m/z (%) = 396 [M$^+$] (100).
HRMS-EI: m/z [M$^+$] calcd for $C_{18}H_{36}O_3S_3$: 396.1827; found: 396.1821.

2-((2R,8R)-2,8-Dimethylundecyl)-1,3-dithiane: To a suspension of $LiAlH_4$ (239 mg, 6.3 mmol) in dry diethylether (30 mL) was added, dropwise, a solution of mesylate (830 mg, 2.1 mmol) in diethylether (10 mL) at 0°C. The mixture was then stirred at room temperature and quenched with Na_2SO_4•$10H_2O$ after TLC showed complete consumption of **9**. The mixture was filtered and washed with diethylether. The filtrate was concentrated and purified via flash column chromatography on silica gel (with hexane) to give the title compound (592 mg, 94%) as a colorless oil.
$[\alpha]_D^{24}$: −9.10 (c 0.63, $CHCl_3$).
IR (KBr): 2956, 2927, 2855, 1465, 1423, 1378, 1275, 1243, 1185, 909, 418 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): 4.08-4.13 (m, 1H), 2.79-2.92 (m, 4H), 2.10-2.16 (m, 1H), 1.67-1.89 (m, 3H), 1.47-1.52 (m, 1H), 1.05-1.36 (m, 15H), 0.82-0.93 (m, 9H).
^{13}C NMR (75 MHz, $CDCl_3$): 45.59, 42.58, 39.39, 37.04, 36.78, 32.46, 30.59, 30.39, 30.18, 29.60, 27.03, 26.78, 26.15, 20.12, 19.66, 19.41, 14.40.
MS (EI, 70 eV): m/z (%) = 302 [M$^+$] (100).
HRMS-EI: m/z [M$^+$] calcd for $C_{17}H_{34}S_2$: 302.2102; found: 302.2099.

(3R,9R)-3,9-Dimethyldodecanal (20): To a solution of dithiane (44 mg, 0.15 mmol) in MeCN/water (3 mL/1 mL) were added MeI (0.19 mL, 3 mmol) and $NaHCO_3$ (252 mg, 3 mmol) at room temperature. The mixture was stirred for 10 h, concentrated under

reduced pressure, dissolved in water, extracted with diethylether (20 mL × 3), the extracts combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 10/1) afforded **20** (30 mg, 97%) as a colorless liquid.
$[\alpha]_D^{24}$: +5.54 (c 0.81, $CHCl_3$).
IR (KBr): 2985, 2928, 2857, 2711, 1729, 1464, 1379, 1261, 1018, 804, 471, 430, 413 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): 9.75 (t, 1H, J = 2.1 Hz), 2.17-2.44 (m, 2H), 2.03-2.07 (m, 1H), 1.02-1.38 (m, 15H), 0.95 (d, 3H, J = 6.6 Hz), 0.82-0.89 (m, 6H).
^{13}C NMR (75 MHz, $CDCl_3$): 203.14, 51.09, 39.40, 37.03, 36.94, 32.46, 30.07, 28.20, 27.00, 20.13, 19.99, 19.65, 14.40.
MS (EI, 70 eV): m/z (%) = 212 [M$^+$] (100).
HRMS-EI: m/z [M$^+$] calcd for $C_{14}H_{28}O$: 212.2140; found: 212.2143.

(6R,12R,E)-6,12-Dimethylpentadec-3-en-2-one (21): To a suspension of NaH (60%, 9.0 mg, 0.22 mmol) in anhydrous THF (2 mL) was added a solution of phosphonate **8** (42 mg, 0.22 mmol) in THF (1 mL) at 0°C. The mixture was stirred for 10 min and added to a solution of aldehyde **20** (30 mg, 0.14 mmol) in THF (1 mL). The solution was stirred at room temperature for 6 h, quenched with water, extracted with EtOAc (20 mL × 3), combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 30/1) afforded enone **21** (32 mg, 90%) as a colorless oil.
$[\alpha]_D^{24}$: −3.41 (c 0.42, $CHCl_3$).
IR (KBr): 2958, 2928, 2872, 2856, 1701, 1678, 1629, 1465, 1379, 1361, 1252, 1180, 980, 616, 543 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): 6.74-6.82 (m, 1H), 6.05 (d, 1H, J = 15.9 Hz), 1.98-2.23 (m, 5H), 1.03-1.35 (m, 16H), 0.80-0.89 (m, 9H).
^{13}C NMR (75 MHz, $CDCl_3$): 198.61, 147.52, 132.39, 39.98, 39.41, 37.05, 36.73, 32.64, 32.48, 30.17, 27.06, 26.89, 20.14, 19.66, 14.41.
MS (EI, 70 eV): m/z (%) = 253 [M+H$^+$] (100).

(6R,12R)-6,12-Dimethylpentadecan-2-one (5): A reaction flask containing **21** (30 mg, 0.13 mmol), Pd/C (10%, 30 mg), and MeOH (5 mL) was evacuated and back-filled with H_2 (1 atm). The reaction mixture was stirred at ambient temperature under hydrogen for 2 days and then filtered over a plug of silica gel topped with Celite (MeOH eluent). The filtrate was concentrated and purified by flash column chromatography on silica gel (PE/EA: 100/1) to give **5** (24 mg, 79%) as a colorless liquid.
$[\alpha]_D^{24}$: −0.395 (c 0.41, $CHCl_3$).
IR (KBr): 2957, 2928, 2856, 1720, 1464, 1409, 1378, 1362, 1164, 726, 435, 419 cm^{-1}.
^{1}H NMR (300 MHz, $CDCl_3$): 2.38 (t, 2H, J = 7.8 Hz), 2.12 (s, 3H), 1.43-1.62 (m, 2H), 1.01-1.40 (m, 18H), 0.80-0.89 (m, 9H).
^{13}C NMR (75 MHz, $CDCl_3$): 209.36, 44.14, 39.41, 37.08, 36.90, 36.64, 36.51, 32.65, 32.47, 30.33, 29.84, 29.48, 27.09, 27.06, 21.43, 20.13, 19.67, 19.54, 19.22, 14.40.
MS (EI, 70 eV): m/z (%) = 254 [M$^+$] (100).

References

[1]   Ando T, Yamakawa, R. (**2015**) Chiral methyl-branched pheromones. *Natural Product Reports*, **32**, 1007-1041.
[2]   (a) Tian WS, Shi Y. (**2010**) The study of resource chemistry. *Progress in Chemistry*, **22**, 538-556; (b) Huang XG, Li T, Lin JR, Jin RH, Tian WS. (**2007**) Synthesis of the C(7)–C(13) fragment of matsuone, a sex pheromone of the matsucoccus pine bast scales. *Chinese Journal of Organic Chemistry*, **27**, 428-430; (c) Li T, Huang XG, Lin JR, Jin RH, Tian WS. (**2007**) Total synthesis of (2R,6R)-2,6,10-trimethylundecan-1-ol with (R)-5-methyl-delta-valerolactone from the industrial waste. *Acta Chimica Sinica*, **65**, 1165-1171; (d) Zhang SJ, Dong HD, Gui JH, Tian WS. (**2012**) Stereoselective synthesis of the insect growth regulator (S)-(+)-hydroprene through Suzuki–Miyaura cross-coupling. *Tetrahedron Letters*, **53**, 1882-1884; (e) Wang C, He CY, Shi Y, Xiang H, Tian WS. (**2015**) Synthesis of tribolure, the common aggregation pheromone of four *Tribolium* flour beetles. *Chinese Journal of Chemistry*, **33**, 627-631; (f) Zhang SJ, Shi Y, Tian WS. (**2015**) Synthesis of C1–C9 domain of the nominal didemnaketal A. *Chinese Journal of Chemistry*, **33**, 663-668.
[3]   (a) Guss PL, Tumlinson JH, Sonnet PE, McLaughlin JR. (**1983**) Identification of a female-produced sex pheromone from the southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber. *Journal of Chemical Ecology*, **9**, 1363-1375; (b) Chuman T, Guss PL, Doolittle RE,

McLaughlin JR, Krysan JL, Schalk JM, Tumlinson JH. (**1987**) Identification of female-produced sex pheromone from banded cucumber beetle, *Diabrotica balteata* Leconte (Coleoptera: Chrysomelidae). *Journal of Chemical Ecology*, *13,* 1601-1616.

[4]   (a) Mori K, Igarashi Y. (**1988**) Pheromone synthesis, CIX. Synthesis of the four stereoisomers of 6,12-dimethyl-2-pentadecanone, the sex pheromone of *Diabrotica balteata* Le Conte. *Liebigs Annalen der Chemie*, 717-720; (b) Enders D, Jandeleit B, Prokopenko OF. (**1995**) Convergent synthesis of (*R*,*R*)-6,12-dimethylpentadecan-2-one, the female sex pheromone of the banded cucumber beetle by iron mediated chirality transfer. *Tetrahedron*, *51*, 6273-6284; (c) Chow S, Kitching W. (**2001**) Hydrolytic kinetic resolution of mono- and bisepoxides as a key step in the synthesis of insect pheromones. *Chemical Communications*, 1040-1041; (d) Chow S, Kitching W. (**2002**) Hydrolytic kinetic resolution of terminal mono- and bis-epoxides in the synthesis of insect pheromones: routes to (−)-(*R*)- and (+)-(*S*)-10-methyldodecyl acetate, (−)-(*R*)-10-methyl-2-tridecanone, (−)-(*R*)-(*Z*)-undec-6-en-2-ol (Nostrenol), (−)-(1*R*,7*R*)-1,7-dimethylnonyl propanoate, (−)-(6*R*,12*R*)-6,12-dimethylpentadecan-2-one, (−)-(2*S*,11*S*)-2,11-diacetoxytridecane and (+)-(2*S*,12*S*)-2,12-diacetoxytridecane. *Tetrahedron: Asymmetry*, *13*, 779-793.

[5]   Takahashi S, Ogawa N, Koshino H, Nakata T. (**2005**) Total synthesis of the proposed structure for pyragonicin. *Organic Letters*, *7*, 2783-2786.

[6]   Krebs O, Taylor RJK. (**2005**) Synthesis of the eastern portion of ajudazol a based on Stille coupling and double acetylene carbocupration. *Organic Letters*, *7*, 1063-1066.

[7]   Semmelhack MF, Jiang Y, Ho D. (**2001**) Synthesis of the amino sugar from C-1027. *Organic Letters*, *3*, 2403-2406.

A Rapid Study of Botanical Drug–Drug Interaction with Protein by Re-ligand Fishing using Human Serum Albumin–Functionalized Magnetic Nanoparticles

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A great many active constituents of botanical drugs bind to human serum albumin (HSA) reversibly with a dynamic balance between the free- and bound-forms in blood. The curative or side effect of a drug depends on its free-form level, which is always influenced by other drugs, combined dosed or multi-constituents of botanical drugs. This paper presented a rapid and convenient methodology to investigate the drug-drug interactions with HSA. The interaction of two steroidal saponins, dioscin and *pseudo*-protodioscin, from a botanical drug was studied for their equilibrium time and equilibrium amount by re-ligand fishing using HSA functionalized magnetic nanoparticles. A clear competitive situation was obtained by this method. The equilibrium was reached soon about 15 s at a ratio of 0.44 : 1. Furthermore, the interaction of pseudo-protodioscin to total steroidal saponins from DAXXK was also studied. The operation procedures of this method were faster and more convenient compared with other methods reported.

Keywords: Drug–drug interaction, Re-ligand fishing, Dioscin, *Pseudo*-protodioscin, Human serum albumin–functionalized magnetic nanoparticles.

Botanical drugs are often mixtures of many active ingredients that could bind to human serum albumin (HSA) reversibly with a dynamic balance of the free- and bound-form in blood. The drug distribution of the free- or bound-form will probably be affected by the competitive binding with HSA, which is directly related to the curative or side effect. Therefore, it is of great importance to study botanical drug–drug interactions.

There has been much research on interactions between drugs and HSA using classical methods such as equilibrium dialysis [1], ultrafiltration [2], X-ray crystallography [3], and mass spectrometry [4], as well as some spectroscopic methods [5]. However, these methods are only suitable for pure compound study, not being able to detect multiple ingredients in a complex matrix. As for herb-drug interaction, the present studies concentrate mainly on the change in the curative effect of the drug, as well as the activity of CPY450 [6-8], while very few studies are involved in the interaction between each single component. Herein we demonstrate that re-ligand fishing could be applied to study the drug-drug interactions, which is based on ligand fishing experiments with HSA functionalized magnetic nanoparticles (HSA–MNPs) used in our previous research [9-12]. Ligand fishing based on receptor theory had been proposed as a new method for drug-protein interaction using a protein immobilized on the surface of MNPs for the isolation of ligands from a mixture of compound extracts [13]. Correspondingly, re-ligand fishing was defined as HSA-MNPs saturated binding with one drug in advance and then ligand fish other drugs in the second ligand fishing experiment.

"Di Ao Xin Xue Kang" capsule (DAXXK), manufactured from the total steroid saponins extract of *Dioscorea nipponica* and *D. panthaica*, is a popular botanical medicine in China for prevention and treatment of coronary heart disease by inhibiting platelet aggregation [14]. It is an interesting phenomenon that a single saponin either inhibits or induces platelet aggregation [15, 16], while the total steroid saponins has an obvious platelet aggregation

inhibition activity due to multicomponent effective ingredients synergy and/or antagonistic action [17, 18]. There has been some research on the interaction between certain proteins with saponins from *D. nipponica* [19-21]. However, the interactions with HSA by individual steroid saponins have never been reported. Thus, in the present work, the drug–drug interaction with HSA between the two main constituents of DAXXK, dioscin and *pseudo*-protodioscin (ppd), were studied by re-ligand fishing using *pseudo*-protodioscin pre-saturated HSA-MNPs (ppd-HSA-MNPs). Mass spectrometry (MS) using an electrospray ionization source (ESI) was chosen for detection.

Different drugs, even with few structural differences, have different binding capacity to HSA. As shown in Fig 1, dioscin is an isospirostanol type steroid saponin with F ring closed, and *pseudo*-protodioscin (ppd) is a furostanol type steroid saponin with F ring opened. It was reported that dioscin is the inhibitor of platelet aggregation while *pseudo*-protodioscin is not. Dioscin and *pseudo*-protodioscin are two main ingredients of DAXXK. Thus, it is of great interest to investigate the dioscin–*pseudo*-protodioscin interaction with HSA by re-ligand fishing experiment.
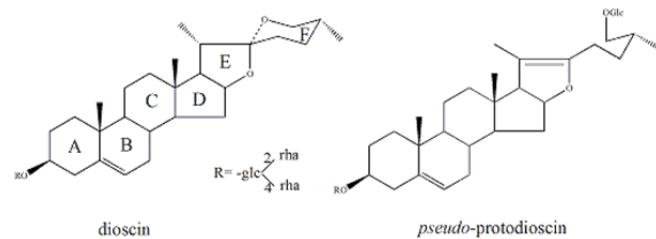
Figure 1: Chemical structures of dioscin and *pseudo*-protodioscin.

Our previous study revealed that dioscin had a greater affinity to HSA than *pseudo*-protodioscin [12]. Thus, HSA-MNPs pre-saturated with *pseudo*-protodioscin (ppd-HSA-MNPs) was used to ligand fish dioscin in this study. An excessive amount of *pseudo*-

protodioscin was used to ensure that all the HSA binding sites were saturated with *pseudo*-protodioscin. The de-binding wash of the ppd-HSA-MNPs was performed in 1 mL buffer containing 50% acetonitrile by vigorous shaking. The supernatant was carefully removed and saved for MS analysis. The existence of *pseudo*-protodioscin de-bound from ppd-HSA-MNPs was revealed by the peak at m/z 1029 [M–H]⁻, which could easily be assigned as *pseudo*-protodioscin; this was confirmed by MS².

Dioscin and *pseudo*-protodioscin were competitively bound with HSA at the same site. The dynamic interaction process and equilibrium amount of these two constituents could be rapidly reached using the ppd-HSA-MNPs re-ligand fish dioscin. It was anticipated that the ratio of dioscin/*pseudo*-protodioscin would be increased as the re-ligand fishing time increased, until the equilibrium point arrived. The re-ligand fishing time in five experiments, 3 s, 5 s, 10 s, 15 s and 25 s, was set as the different interaction time points for these two drug constituents. Their de-binding wash solutions were named as A2-1, A2-2, A2-3, A2-4 and A2-5, respectively. The peak at m/z 867 [M–H]⁻ in the mass spectrum could be easily assigned to dioscin, which was confirmed by MS². The mass spectra of A2-1, A2-2, A2-3, A2-4 and A2-5 showed the peaks of dioscin and *pseudo*-protodioscin, the height ratios of which correspond to their competitive binding; the results are summarized in Table 1. It was obvious that the height ratio of dioscin/*pseudo*-protodioscin was gradually increased with the re-ligand fishing time.

To calculate the amount of dioscin and *pseudo*-protodioscin, an eleven-point calibration curve was obtained with standard solutions at concentration ratios ranging from 8：1 to 1：128. Peak heights were used for the calculation. Linear regression analysis of the results yielded the following equations for the ratio of dioscin/*pseudo*-protodioscin: Y = 8.4247 X + 0.0157, r² = 0.998, where Y was the peak height ratio of dioscin/*pseudo*-protodioscin, X was the concentration ratio of dioscin/*pseudo*-protodioscin, and r² was the correlation coefficient. The concentration ratio of dioscin/*pseudo*-protodioscin calculated by peak heights was determined in solutions A2-1, A2-2, A2-3, A2-4 and A2-5, as shown in Table 1, which reflected the dynamic interaction process intuitively. The equilibrium was reached after about 15 s at a ratio of 0.44：1.

Among the steroidal saponins exhibiting activity for the prevention and treatment of coronary heart disease in DAXXK, there are two categories, the furostanol type and the isospirostanol type. Dioscin is the most important isospirostanol type and *pseudo*-protodioscin the most important furostanol type; both are determined in the Chinese Pharmacopoeia assay [14]. In this current study, further studies of the total steroidal saponins from DAXXK were conducted by comparison of ligand fishing results (de-binding wash solution B1) using HSA-MNPs and re-ligand fishing using ppd-HSA-MNPs (de-binding wash solution B2).

Table 1: Height ratios and concentration ratios of dioscin/pseudo-protodioscin at the different interaction times.

Solution	Re-ligand fishing time(s)	Height Ratio (dioscin/*pseudo*-protodioscin)	Con. Ratio (dioscin/*pseudo*-protodioscin)
A 1	0	0	0
A 2-1	3	1.03	0.12
A 2-2	5	1.72	0.20
A 2-3	10	3.57	0.42
A 2-4	15	3.70	0.44
A 2-5	25	3.70	0.44

Although dozens of steroidal saponins are present in DAXXK, only three, i.e. dioscin (m/z 687 [M–H]⁻), *pseudo*-protodioscin (m/z 1029 [M–H]⁻) and gracillin (m/z 883 [M–H]⁻) were bound to HSA significantly in the ligand fishing experiment. The ratio of dioscin/*pseudo*-protodioscin in the B1 solution was 0.54: 1, calculated by peak heights. By contrast, the ratio changed to 0.07: 1 in the re-ligand fishing experiment (B2 solution). This result indicated that the equilibrium was changed significantly in the presence of some outside source of *pseudo*-protodioscin and the drug-drug interaction of each constituent in DAXXK was serious. Further, it was reported that both dioscin and *pseudo*-protodioscin were degradation products of 26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-en-3β,22ξ,26-triol-3-*O*-β-D-glucopyrannosyl-(1→3)-β-D-glucopyrannosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside in *D. panthaica* [22]. The assay standard for DAXXK in the Chinese Pharmacopoeia is the content of *pseudo*-protodioscin and total saponins [14]. However, unlike modern therapeutic drugs that are single active pure compounds, DAXXK is an herbal medicine that contains many active ingredients, and the chemical compositions always vary depending on the variety, origin, harvesting time and processing technique. Hence, the strong binding ingredients such as dioscin should be integrated into the quality control standard. It is necessary to monitor the drug blood concentration in clinical use, especially with combined use with narrow therapeutic index drugs (for example warfarin, digoxin). Furthermore, this work can be important for the evaluation of the pharmaceutical effect of a herb medicine containing multi-components.

In summary, a rapid and convenient methodology was presented to determine the balance ratio and balance time of dioscin/pseudo-protodioscin interaction with HSA by re-ligand fishing using human serum albumin–functionalized magnetic nanoparticles. The equilibrium was reached soon after 15 s at the amount ratio of 0.44：1. Furthermore, the interaction of pseudo-protodioscin to total steroidal saponins from DAXXK was also studied. The procedures with the aid of mass spectrometry detection were very sensitive, quick and convenient. These results revealed that re-ligand fishing coupled with MS detection could be applied to investigate the botanical drug-drug interactions.

Experimental

Preparation of HSA-MNPs: HSA functionalized MNPs (HSA-MNPs) were prepared following the procedures reported previously [9-12]. Briefly, magnetic nanoparticles were prepared by co-precipitation with a molar ratio of Fe^{2+}: Fe^{3+} = 1: 2 at pH 10. MNPs were first coated with SiO_2 using TEOS. Secondly, the particles were dispersed in APTMS solution to add -NH_2 to the SiO_2; the resultant particles were then dispensed in the GD solution to provide -CHO to the surface. Finally, the -CHO coated MNPs were incubated with HSA to obtain HSA functionalized MNPs. HSA-MNPs were suspended in NH_4Ac solution and kept at 4°C before use.

Re-ligand fishing experiments

Saturated binding pseudo-protodioscin to HSA-MNPs (ppd-HSA-MNPs): A 225 μg/mL solution of *pseudo*-protodioscin was prepared using ammonium acetate buffer solution (10 mM/L, pH 7.4). One mL pseudo-protodioscin solution and 100 μL HSA-MNPs were added to a 4 mL Eppendorf tube. The tube was vigorously shaken for 2 min using a vortex oscillator, and then put aside for magnetic separation for 1 min. The supernatant was removed. The HSA-MNPs were washed 3 times with 1 mL of buffer solution, each by vigorously shaking for 1 min, and then the supernatants

were removed after magnetic separation. Hence, the pseudo-protodioscin saturated binding HSA-MNPs (ppd-HSA-MNPs) were obtained.

Detection binding amount of pseudo-protodioscin in ppd-HSA-MNPs: The de-binding wash of the ppd-HSA-MNPs was performed in 1 mL buffer containing 50% acetonitrile by vigorous shaking for 2 min. The supernatant was carefully removed and saved (A1) for mass spectrometric analysis.

Re-ligand fish dioscin using ppd-HSA-MNPs for different times: A 0.55 μg/mL solution of dioscin was prepared using ammonium acetate buffer solution (10 mM/L, pH 7.4). One mL dioscin solution was added to a 4 mL Eppendorf tube containing 100 μL ppd-HSA-MNPs. The tube was vigorously shaken for 3 s (re-ligand fish time investigated) using a vortex oscillator, and then put aside for magnetic separation for 1 min. The supernatant was removed. The ppd-HSA-MNPs were washed 3 times with 1 mL of buffer solution each by vigorous shaking for 1 min, and then the supernatants were removed, after magnetic separation. Finally, the de-binding wash was performed in 1 mL buffer containing 50% acetonitrile by vigorous shaking for 2 min. The supernatant was carefully removed and saved (A2-1).

Another 4 experiments were undertaken using the parallel procedures above as well. Four de-binding solutions A2-2, A2-3,

A2-4 and A2-5 were obtained for mass spectrometric analysis by prolonging the re-ligand fishing time from 3 s (A2-1) to 5 s (A2-2), 10 s (A2-3), 15 s (A2-4) and 25 s (A2-5), respectively.

Re-ligand fish total steroidal saponins using ppd-HSA-MNPs: First, a comparative ligand fishing experiment was made using HSA-MNPs. One mL DAXXK solution and 100 μL HSA-MNPs were added to a 4 mL Eppendorf tube. After vigorous shaken, magnetic separation, and buffer wash, the 50% acetonitrile de-binding wash solution (B1, ligand fishing) was obtained for comparison with B2 below.

Then, a 52 μg/mL solution of the DAXXK solution was prepared using ammonium acetate buffer solution (10 mM/L, pH 7.4). One mL dioscin solution was added to a 4 mL Eppendorf tube containing 100 μL ppd-HSA-MNPs. The 50% acetonitrile de-binding wash solution (B2, re-ligand fishing) was obtained following the same procedure. All the experiments above were repeated 3 times.

References

[1]   Taboada P, Mosquera V, Ruso JM, Sarmiento F, Jones MN. (**1999**) Interaction between penicillins and human serum albumin: A thermodynamic study of micellar-like clusters on a protein. *Langmuir*, *16*, 934-938.
[2]   Zlotos G, Oehlmann M, Nickel P, Holzgrabe U. (**1998**) Determination of protein binding of gyrase inhibitors by means of continuous ultrafiltration. *Journal of Pharmaceutical and Biomedical Analysis*, *18*, 847-858.
[3]   Zhu L, Yang F, Chen L, Meehan EJ, Huang M. (**2008**) A new drug binding subsite on human serum albumin and drug–drug interaction studied by X-ray crystallography. *Journal of Structural Biology*, *162*, 40-49.
[4]   Schmidt AC, Steier S. (**2010**) Some critical aspects in the determination of binding constants by electrospray ionisation mass spectrometry as the example of arsenic bindings to sulphur-containing biomolecules. *Journal of Mass Spectrometry*, *45*, 870-879.
[5]   Mandeville JS, Froehlich E, Tajmir-Riahi HA. (**2009**) Study of curcumin and genistein interactions with human serum albumin. *Journal of Pharmaceutical and Biomedical Analysis*, *49*, 468-474.
[6]   Qiu Z, Wang L, Dai Y, Ren W, Jiang W, Chen X, Li N. (**2015**) The potential drug-drug interactions of ginkgolide B mediated by renal transporters. *Phytotherapy Research*, *29*, 662-667.
[7]   Milić N, Milosević N, Golocorbin Kon S, Bozić T, Abenavoli L, Borrelli F. (**2014**) Warfarin interactions with medicinal herbs. *Natural Product Communications*, *9*, 1211-1216.
[8]   Miladinović DL, Ilić BS, Mihajilov-Krstev TM, Jović JL, Marković MS. (**2014**) *In vitro* antibacterial activity of *Libanotis montana* essential oil in combination with conventional antibiotics. *Natural Product Communications*, *9*, 281-286.
[9]   Qing LS, Shan XQ, Xu XM, Xue Y, Deng WL, Li BG, Wang XL, Liao X. (**2010**) Rapid probe and isolation of bioactive compounds from *Dioscorea panthaica* using human serum albumin functionalized magnetic nano-particles (HSA-MNPs)-based ligand fishing coupled with electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry*, *24*, 3335-3339.
[10]   Qing LS, Tang N, Xue Y, Liang J, Liu YM, Liao X. (**2012**) Identification of enzyme inhibitors using therapeutic target protein-magnetic nanoparticle conjugates. *Analytical Methods*, *4*, 1612-1615.
[11]   Qing LS, Xue Y, Deng WL, Liao X, Xu XM, Li BG, Liu YM. (**2011**) Ligand fishing with functionalized magnetic nanoparticles coupled with mass spectrometry for herbal medicine analysis. *Analytical and Bioanalytical Chemistry*, *399*, 1223-1231.
[12]   Qing LS, Xue Y, Zheng Y, Xiong J, Liao X, Ding LS, Li BG, Liu YM. (**2011**) Ligand fishing from *Dioscorea nipponica* extract using human serum albumin functionalized magnetic nanoparticles. *Journal of Chromatography A*, *1217*, 4663-4668.
[13]   Moaddel R, Marszałł MP, Bighi F, Yang Q, Duan X, Wainer IW. (**2007**) Automated ligand fishing using human serum albumin-coated magnetic beads. *Analytical Chemistry*, *79*, 5414-5417.
[14]   Chinese Pharmacopoeia Commission. (**2010**) Pharmacopoeia of the People's Republic of China. China Medical Science Press, Beijing, China, 671.
[15]   Fu YL. Master's thesis. (**2007**) The characteristic of steroidal saponins on platelet aggregation through structure-activity assay and mechanism study. Military Medical Science Academy of the Chinese People's Liberation Army.
[16]   Zhao NX, Han YM, Zhang SJ. (**2011**) Antithrombotic components in *Dioscorea nipponica. Chinese Traditional and Herbal Drugs*, *42*, 652-655.
[17]   Su W, (**2012**) DAXXK intervention clinical trials of aspirin resistance and its mechanism. *China Pharmaceuticals*, *21*, 26-28.
[18]   Li ZM., Xu XJ, Yin CH, (**2002**) Experimental research of DAXXK on thrombois. *Lishizhen Medicine and Materia Medica Research*, *13*, 390-391.
[19]   Ali Z, Smillie TJ, Khana IA. (**2013**) 7-Oxodiscin, a new spirostane steroid glycoside from the rhizomes of *Dioscorea nipponica. Natural Product Communications, 8*, 319-321.
[20]   Yu H, Zheng L, Xu L, Yin L, Lin Y, Li H, Liu K, Peng J. (**2015**) Potent effects of the total saponins from *Dioscorea nipponica* Makino against streptozotocin-induced type 2 diabetes mellitus in rats. *Phytotherapy Research*, *29*, 228-240
[21]   Lu F, Liu L, Yu DH, Li XZ, Zhou Q, Liu SM. (**2014**) Therapeutic effect of Rhizoma *Dioscoreae Nipponicae* on gouty arthritis based on the SDF-1/CXCR 4 and p38 MAPK pathway: an *in vivo* and *in vitro* study. *Phytotherapy Research*, *28*, 280-288.
[22]   Geng, Y, Tan NH, Zhou J, Kong LY. (**2004**) Isolation and identification of steroid saponins from the fresh rhizomes of *Dioscorea panthaica. Chinese Journal of Natural Medicines*, *2*, 25-27.

Serum Metabolomic Profiling of Rats by Intervention of *Aconitum soongaricum*

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To understand the toxic mechanism and to find the changes in the endogenous metabolites of *Aconitum soongaricum* Stapf for clinical detection, a combination of ^{1}H NMR spectroscopy and multivariate statistical analysis was applied to examine the metabolic profiles of the blood serum samples collected from the rat model. In total, thirteen biomarkers of *A. soongaricum* were found and identified. It turned out that *A. soongaricum* treatment may partially disorder the metabolism. The study has shown the potential application of NMR-based metabolomic analysis in providing further insights into the toxicity caused by *A. soongaricum*.

Keywords: Ranunculaceae, *Aconitum soongaricum*, Metabolomic Profiling, Biomarkers, Nuclear Magnetic Resonance.

Aconitum soongaricum Stapf, family Ranunculaceae, is well known for its anti-inflammatory and analgesic effect, and is widely used in the north of China as an analgesic for neuralgia, toothache, arthritis and other pains [1a-d]. However, as this plant contains *Aconitum* alkaloids, it has been reported that it has resulted in several poisoning accidents [2a,b]. To understand the toxic mechanism and to find the changes in endogenous metabolites for clinical detection, NMR based metabolomics was conducted to investigate the intervention effects of *A. soongaricum* on mice. Metabolomics analysis was carried out on serum samples, while orthogonal partial least squares-discriminant analysis (OPLS-DA) was employed to investigate the toxic effects of *A. soongaricum* and to detect related potential biomarkers [3a-c].

In this study, rats were fed with *A. soongaricum* and the biochemical variations in the serum from these rats were investigated by high resolution ^{1}H NMR spectroscopy. This procedure has provided an insight into the systematic metabolism after use of *A. soongaricum*. Results of this study have validated the applicability of NMR based metabolomics in the study of toxicity and biomarkers detection. In total, thirteen biomarkers were found and identified (Table 1). It turned out that *A. soongaricum* treatment may partially disorder the metabolism. Besides, NMR based metabolomics proved to be a powerful tool for investigating the pharmacodynamic effects of natural products and underlying mechanisms.

OPLS-DA analysis of endogenous metabolites in rat serum at different times after administration showed that there were significant differences in serum metabolites in each time group. It can be seen from the OPLS-DA analysis of different time groups that the 2d group is far away from the others in the space, but the 2d and 5d groups were closer in the plane and space, while the 2d group can be separated completely from the 9d and 13d groups in the score plot. The 9 d and 13 d groups are gradually approaching the blank group, the results showing that the effects of *A. soongaricum* on rats occurred within two days after the administration, but then they gradually recovered.

Table 1: Potential biomarkers of *A. soongaricum* detected by NMR.

No.	Metabolites	Reference value, ^{1}H NMR, δ	Measured value, ^{1}H NMR, δ
1	β-Glucose	3.24(dd)	3.24(dd)
		3.40(t)	3.40(t)
		4.64(d)	4.64(d)
2	α-Glucose	3.53(dd)	3.53(dd)
		3.72(dd)	3.72(dd)
		3.83(dd)	3.83(dd)
		5.23(d)	5.23(d)
3	VLDL	0.85(m)	0.85(m)
		0.88(m)	0.88(m)
		1.27(m)	1.27(m)
4	LDL	1.26(m)	1.26(m)
		4.25(m)	4.25(m)
5	Lactic acid	1.33(d)	1.32(d)
		4.11(q)	4.11(q)
6	Creatine	3.03(s)	3.03(s)
		3.93(s)	3.92(s)
7	Trimethylamine oxide	3.27(s)	3.26(s)
8	β-Hydroxybutyric acid	1.19(d)	1.19(d)
9	Glycine	3.57(s)	3.55(s)
10	Alanine	1.47(d)	1.47(d)
		3.76(q)	3.76(q)
11	Glutamic acid	2.13(m)	2.13(m)
12	Pyruvic acid	2.38(s)	2.36(s)
13	Acetoacetic acid	2.27(s)	2.27(s)

The variable metabolites in the serum of different time groups were VLDL, LDL, glucose, lactic acid, creatine, trimethylamine oxide, glycine, glutamic acid, alanine, β-hydroxybutyric acid, pyruvic acid and acetoacetic acid. The levels of glucose, glycine, and glutamic acid were decreased in the 2 d, 5 d and 9 d groups comparing with the blank group; these metabolites were obviously decreased in the day after administration, but were restored to normal levels in 13 d. The levels of VLDL, LDL and lactic acid were decreased 2 d after administration, but increased significantly in 9 d. The levels of creatine and trimethylamine oxide were increased compared with the blank group and trimethylamine oxide was obviously increased in 9 d. The levels of β-hydroxybutyric acid and acetoacetic acid

Table 2: The change of metabolites at different times.

No.	Metabolites	2d	5d	9d	13d
1	β-Glucose	↓↓	↓	↓	—
2	α-Glucose	↓↓	↓	↓	—
3	VLDL	↓	↓	↑↑	↑
4	LDL	↓	↓	↑↑	↑
5	Lactic acid	↓	↓	↑↑	↑
6	Creatine	↑	↑	↑	↑
7	Trimethylamine oxide	↑	↑	↑↑	↑
8	β-Hydroxybutyric acid	↑↑	↑	↑	—
9	Glycine	↓↓	↓	↓	—
10	Alanine	↓↓	↓	↓	↓
11	Glutamic acid	↓↓	↓	↓	—
12	Pyruvic acid	—	—	↓	—
13	Acetoacetic acid	↑↑	↑	↑	—

Concentration compared with the blank group was not obvious; ↑ and ↓ concentration increase or decrease compared with the blank group; ↑↑ and ↓↓concentration increase or decrease compared with the blank group was significant.

were increased obviously in 2d and higher than the blank group, while levels of pyruvic acid were decreased in 9d compared with the blank group (Table 2).

In this study, metabolomics have been used to highlight the metabolic effect of *A. soongaricum* treatment in the rat model. NMR based metabolomics has been shown to be an efficient technique to investigate the metabolic perturbation due to *A. soongaricum* poisoning. The changes in endogenous metabolites in serum samples from models of different groups of rats were identified by OPLS-DA. The toxicity of *A. soongaricum* was proven to be selective and affected a number of metabolic pathways. To complement the current study, future proteomic and immunohistochemistry studies may provide further insights into the metabolic mechanism and discover more effective treatments for toxicity detection. In summary, the current results indicate that ^{1}H NMR based metabolomics have the potential to uncover the metabolic response of *A. soongaricum* toxicity that could provide better understanding of the metabolic biomarkers.

Experimental

Plant material:The fresh roots of *A. soongaricum* were collected in Narat Grassland, Xinjiang Province of China in July 2012 and were identified by Prof. Fan Zhang (North Sichuan Medical University, Sichuan, P. R. China) [4a]. Avoucher specimen (ZGEWT20120801) was deposited at the Xinjiang Museum of Traditional Chinese Medicine and Ethnomedicine.

Animal handling and sample collection: Sixty-six healthy SPF Sprague-Dawley rats (200±20g weight) were provided by North Sichuan Medical University. All rats were housed individually in metabolic cages. The animal room was maintained under controlled condition (a 12 h light-dark cycle with constant temperature and humidity). All efforts were made to minimize animal suffering. After acclimatization for one week, the rats were randomly separated into 11 groups, i.e. 2 h group, 6 h group, 2 d group, 3 d group, 5 d group, 7 d group, 9 d group, 11 d group, 13 d group, 14 d group and blank group (n=6). The blank group was given normal diet and clean water throughout the experiment, while the model groups were fed with the dose of 170 mg/kg, which was confirmed byacute toxicity test[4b]. After 2 h, 6 h, 2 d, 3 d, 5 d, 7 d, 9 d, 11 d, 13 d and14 d, respectively, the blood was drawn from orbit using a syringe and the blood was left to clot at room temperature for 30 min. Serum samples were centrifuged (12,000 rpm) for 15 min at 4°C to remove particulate contaminants and then stored at -80°C until further analysis.
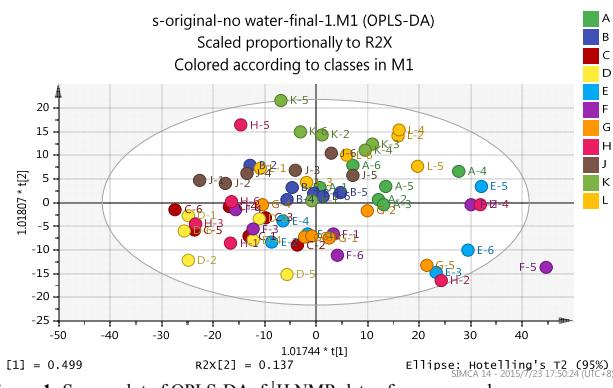
Figure 1: Scores plot of OPLS-DAof ^{1}H NMR data of serum samples.
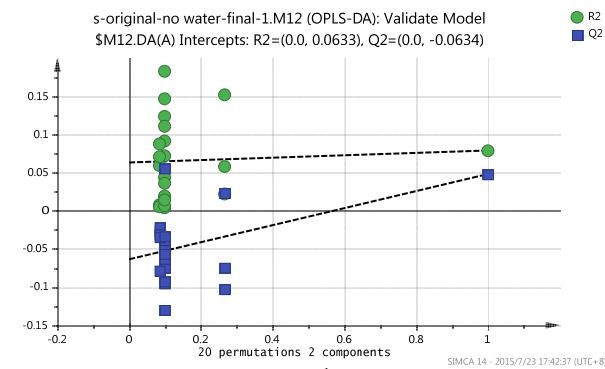
Figure 2: Permutations plot of OPLS-DA of ^{1}H-NMR data of serum samples.

Sample preparation and ^{1}H NMR spectroscopy: For ^{1}H NMR analysis, 500 μL of serum samples was mixed with 200 μL phosphate buffer solution (0.2M Na_2HPO_4/NaH_2PO_4, pH 7.4, 20% D_2O) to reduce pH variation across samples. In addition, $1×10^{-3}$mol/L TSP (3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt) was used as an internal reference standard at δ 0.0. The mixture was then transferred into a 5 mm NMR tube. The ^{1}H NMR measurements of the serum samples were performed using a Varian NMR system 600 MHz spectrometer equipped with a triple resonance probe. The experimental temperature was set to 298 K and the 90° pulse length was calibrated individually for each sample. For the serum samples, an additional Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse train was incorporated into the NOESYPR sequence with a relaxation delay-90°-(τ-180°-τ) n-acquire. A total of 128 scans with a spectral width of 10 kHz were collected for all NMR experiments. The acquired signals were zero filled to 32k before Fourier transformation. For data analysis, metabolites in the Metabolomic Responses to *A. soongaricum* rat serum ^{1}H NMR spectra were assigned with reference to published data and HMDB database (http://www.hmdb.ca/).

Data preprocessing of NMR spectra and pattern recognition: The collected NMR spectra were phased and baseline corrected using the software Topspin 2.0 (Bruker, Germany)(Figure 1). All NMR spectra were also peak-aligned manually using in-house software to minimize variation due to peak shift. The chemical shift regions of δ 4.6-6.0 (water resonances), δ 0.0-0.2 (TSP resonance) and the regions which are peak-free for all spectra were excluded from further analysis. Table S1 shows the raw spectral data of serumsamples. Then each spectrum was binned into segments by adaptive binning method. To account for variations in sample concentration, the spectra were normalized using group aggregating normalization technique. The NMR spectral data were converted to Microsoft Excelformat and imported into SIMCA software (version 14.0, Umetrics, Sweden) for multivariate analysis.
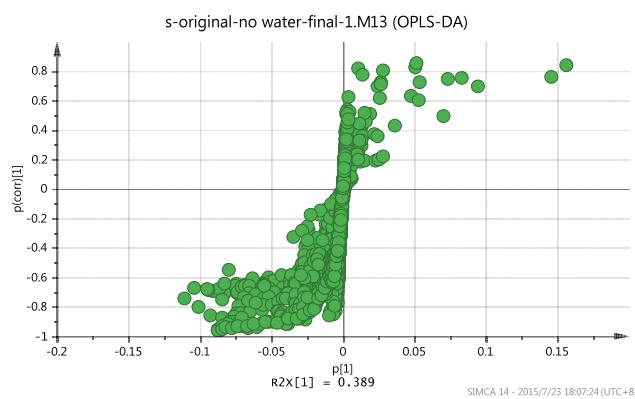
Figure 3: S-plots of OPLS-DA of ^{1}H-NMR data of serum samples.

Orthogonal partial least squares discriminant analysis (OPLS-DA) is a regression method that uses binary variables Y to indicate class membership. The OPLS-DA projects the original variables X (metabolic concentrations) to latent variables that focus on class separation, thus obtaining a better classification. The quality of the models is described by R^2X and Q^2 values. R^2X is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability. The metabolites with high variable importance in the projection values in each OPLS-DA models are selected to investigatefurther their quantitative changes in all groups.

OPLS-DA of blood serum samples: OPLS-DA analysis of blood serum samples showed that the samples clustered based on studied groups (Figure 1). To investigate further the metabolic patterns and identify potential characteristic metabolites, group comparisons were carried out using OPLS-DA (Figures 2 and 3).

Supplementary data: S1 Table. Raw spectral data of serum samples.

References

[1]   (a) Chinese Academy of Sciences of Editorial Committee of flora of China. (**1979**) In *The flora of China* (*ZhongguoZhiwuzhi*). Sciences Publishing House, Beijing, *27*, 308; (b) Xu X, Huangerhan B. (**2009**) In *Plant records of Kazakh medicine* (*HasakeYaozhi*). Ethnic Publishing House, Beijing, *1*, 45; (c) Cai DM, Liu Y, Zhang F. (**2011**) Research survey of *Aconitum* grown in Xinjiang. *Xinjiang Journal of Traditional Chinese Medicine*, *29*, 74-75; (d) Zhang F, Yang LJ, Cai DM, Liu Y. (**2012**) Analysis of the diterpenoid alkaloids in *Aconitum soongaricum* Stapf by HPLC and TLC. *Natural Product Research & Development*, *24*, 924-927.
[2]   (a) Jia XG, Zhou Q, Wang GP, Li XJ, He J. (**2012**) Toxic symptom and clinicalrescue of *Aconitum*. *Xinjiang Journal of Traditional Chinese Medicine*, *30*, 67-70; (b) Li H, Chen WM, Chen X, Wang H, Li XQ. (**2010**) Poisonous weed species and their harmfulness in the grassland of Yili regions, Xinjiang Uygur Autonomous region of China. *Pratacultural Science*, *27*, 171-173.
[3]   (a) Nicholson JK, Connelly J, Lindon JC, Holmes E. (**2002**) Metabonomics: a platform for studyingdrug toxicity and gene function. *Nature Reviews*, *1*, 153-161; (b) Zhao LC, Zhang XD, Wang HY, Lin DH. (**2011**) ^{1}H NMR-basedmetabonomic analysis of metabolic changes of serum and liver in zucker obese rats. *Analytical Letters*, *44*, 1579-1590; (c) Fages A, Pontoizeau C, Jobard E, Lévy P, Bartosch B, Elena-Herrmann B. (**2013**) Batch profiling calibration for robust NMR metabonomic data analysis. *Analytical & Bioanalytical Chemistry*, *405*, 8819-8827.
[4]   (a) Cai DM, Liu Y, WUFUER H, Zhang F. (**2011**) Morphological and histological identification of Kazak medicinal herb *Aconitum soongaricum* Stapf. *Journal of Chinese Medicinal Materials*, *34*, 876-878; (b) Yang LJ, Zhang F, Cai DM, Liu Y. (**2012**) Analysis on the acute toxicity of *Aconitum soongaricum* Stapf in mice. *Lishizhen Medicine & Materia Medica Research*, *23*, 2542-2543.

Re-evaluation of ABTS•+ Assay for Total Antioxidant Capacity of Natural Products

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2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS•+) is a stable free radical frequently used for estimating the total antioxidant capacity (TAC) of natural products. The existing methods for ABTS•+ radical-scavenging activity assays are diverse in pre-diluting solvents and reaction time, which lead to errors in the TAC estimations. To develop an effective and universal method for estimating the ABTS•+ capacity accurately and reasonably, five pre-dilution solvents [methanol, ethanol, phosphate buffer (Na_2HPO_4-NaH_2PO_4, 200 mM, pH = 7.4), PBS buffer (Na_2HPO_4-NaH_2PO_4-NaCl, 200 mM, pH = 7.4), and distilled water] and different reaction times were investigated in ABTS•+ assays of five typical antioxidants. The results showed that the solvent effects were very significant. When using different pre-diluting solvents, different detection wavelengths should be selected. ABTS•+ assay could be measured within 2-10 min to obtain a rough result, which was mostly 6 min in the literature. However, full and accurate evaluation of antioxidant reactivity rather than capacity requires recording ABTS•+ loss continuously during the whole reaction period. The present study makes a recommendation for estimating the ABTS•+ capacity accurately and reasonably.

Keywords: ABTS, Solvent, Reaction time, Phenolic compound, Recommendation.

Reactive oxygen species (ROS), a collective term often used to include oxygen radicals [superoxide ($O_2^{•-}$), hydroxyl ($OH^•$), peroxyl ($RO_2^•$), and alkoxyl ($RO^•$)] and certain nonradicals that either are oxidizing agents and/or are easily converted into radicals, such as HOCl, ozone (O_3), peroxynitrite (ONOO−), singlet oxygen ($^1O_2$), and H_2O_2 [1], may cause cell aging, cardiovascular diseases, mutagenic changes, and cancerous tumor growth [2]. Antioxidants are compounds that can delay the oxidation of other molecules by inhibiting either the initiation or propagation of oxidizing chain reactions caused by free radicals and thereby may reduce oxidative damage to the human body [3]. Phenolic substances, which exist in plant tissues as secondary metabolites, can be categorized into simple phenols, phenolic acids (both hydroxybenzoic acids and hydroxycinnamic acids), flavonoids, lignins and tannins. These substances are responsible for color, bitterness, acerbic taste, flavour, odor and antioxidant properties [4].

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS•+), a stable free radical, is frequently used for estimating the total antioxidant capacity (TAC) of natural products, including crude extracts [5-9], polyphenols [10], phenolic acids [11], flavonoids [12], and others [13]. The original ABTS•+ radical-scavenging assay was measured according to the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of antioxidants [14]. With the improving technology for determination of the blue ABTS•+ chromophore produced through the reaction between ABTS and potassium persulfate (Figure 1), a feasible method was established according to a maximal absorption of ABTS+ at wavelengths of 645 nm, 734 nm, and 815 nm [15, 16]; the original method measured at 415 nm [17, 18].

Until 1999, the applicable and universal ABTS•+ method for the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants, was

proposed by Re *et al*. [14]. This ABTS•+ assay was measured by a colorimetric method at 734 nm, and the extent of decolorization as a percentage inhibition of the ABTS•+ radical cation was determined as a function of concentration and time and calculated relative to the reactivity of Trolox, as a standard, under the same conditions. Recently, the LC-ABTS•+ assay is frequently used for ABTS•+ radical-scavenging activity. He *et al*. [19, 20] identified the phenolic compounds from pomegranate (*Punica granatum* L.) seed residues and investigated their antioxidant capacities by HPLC-ABTS•+ assay; Kalili *et al*. [21] reported that the combination of LC × LC separation with an on-line radical scavenging assay increased the likelihood of identifying individual radical scavenging species in comparison with the conventional LC-ABTS•+ assay.
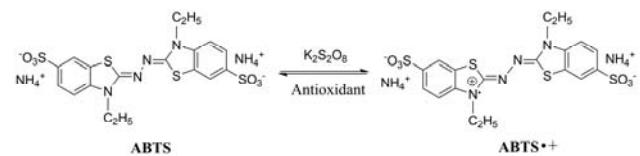
Figure 1: Reaction mechanism of ABTS and the oxygen radical (ABTS•+).

However, in the course of the application of the ABTS•+ method, some problems appeared. First, the pre-diluting solvents in ABTS•+ assay are various in the existing literature. For example, Bangoura *et al*. [22] and Shao *et al*. [23] selected PBS buffer solution (Na_2HPO_4-NaH_2PO_4-NaCl, 200 mM, pH = 7.4) as the solvent and the absorbance of ABTS•+ assay measured at 734 nm. Gursoy *et al*. [24] reported that ABTS•+ free radical-scavenging assay was measured after ABTS•+ dilution with phosphate buffer (0.1M, pH 7.4). Luo *et al*. [10] diluted the ABTS•+ with 95% ethanol, and determined the absorbance at 734 nm. Siddhuraju [25] reported the ABTS•+ solution diluted in ethanol (1:89, v/v) and used an absorbance at 734 nm. However, our previous research revealed that the ABTS•+ solution diluted with ethanol had maximal absorption at 753 nm [26]. It is inaccurate to measure the absorbance at 734 nm in either ethanol or the other solvents.

Although Dawidowicz and Olszowy discussed the importance of solvent type in estimating antioxidant properties of phenolic compounds by ABTS•+ assay [27], only three solvents (methanol, ethanol and propanol) were discussed in detail. The solvents frequently-used in ABTS•+ assay, including PBS and phosphate buffer, were not reported and discussed to our knowledge. Secondly, the reaction time is an important factor causing errors in estimating the ABTS•+ free radical-scavenging activity. ABTS•+ can be quenched by both electron (fast) and hydrogen atom transfer (slow) like DPPH [28]. That is to say, the reaction time of different antioxidants with ABTS•+ is not same. In the literature, 6 min of reaction time were employed universally, which could cause error in the ABTS•+ radical-scavenging, especially for complex samples (e.g. crude extracts). Therefore, it is necessary to investigate the proper reaction time for the ABTS•+ capacity determination of different antioxidants.

This paper deals with the effects of different solvents and different reaction times on ABTS•+ assay. In this study, five pre-diluting solvents [methanol, ethanol, phosphate buffer (PB, Na_2HPO_4-NaH_2PO_4, 200 mM, pH = 7.4), PBS buffer, and distilled water] and different reaction times (0-30 min) were investigated in ABTS•+ assays for five antioxidants.

Effects of the solvents: ABTS•+ has a maximal absorption at 734 nm according to the literature [14, 22]. However, the maximal absorption wavelengths of ABTS•+ in different solvents are not the same, which might be ascribed to a red or blue shift caused by solvent effects. Although Dawidowicz and Olszowy [27] have reported the methanol, ethanol, and propanol effects, other solvents are frequently used to pre-dilute ABTS•+. In the present study, ABTS•+ solution was diluted with PBS buffer, PB, H_2O, MeOH, and EtOH, respectively. The UV-Vis spectra of pre-diluting ABTS•+ solutions were scanned in the range of 500-1000 nm. Figure 2 shows that the ABTS•+ solutions diluted with PBS buffer, PB, and H_2O have the same maximal absorption at 734 nm, but MeOH pre-dilution of ABTS•+ has a maximal absorption at 745 nm, and the EtOH pre-dilution, a maximal absorption at 753 nm. Therefore, it is unsuitable for the literature methods [10, 24] to measure ABTS•+ capacity at the same wavelength (734 nm) without regard to the solvent effects. Additionally, the linearity coefficients between absorptions and concentrations were analyzed and are shown in Table 1. The results reveal that all the above pre-diluting ABTS•+ have good linearity coefficients ($r^2 \geq 0.998$), indicating that all of the above solvents could be used for pre-diluting ABTS•+. However, when using different pre-diluting solvents, the corresponding maximal absorption wavelengths for detection were varied.

Effects of reaction time: The same as with the DPPH free radical, ABTS•+ could be quenched by both electron (fast) and hydrogen atom transfer (slow) [28, 29]. That is to say, the time of various antioxidants quenching ABTS•+ is different. However, most existing methods used for ABTS•+ assay are measured after 6 min
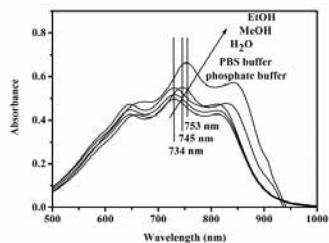
Table 1: Linearity analysis of ABTS•+ solutions at different concentrations (2.2-140 μM).[a]

Pre-diluting solvents	Linear equation (correlation coefficient)
PBS	$A = -0.00717 + 0.00748\ C$ ($r^2 = 0.99999$)
PB	$A = -0.01641 + 0.00769\ C$ ($r^2 = 0.99848$)
H_2O	$A = -0.00477 + 0.00781\ C$ ($r^2 = 0.99987$)
MeOH	$A = -0.01316 + 0.00806\ C$ ($r^2 = 0.99985$)
EtOH	$A = 0.00261 + 0.00936\ C$ ($r^2 = 0.99962$)

[a] Abbreviation: *A*, the absorbance of ABTS•+ solution at different concentrations; *C*, the concentration of ABTS•+ solution.

of reaction time, which provide little chemical information about the antioxidants. In the present study, the ABTS•+ scavenging activity of five familiar antioxidants, gallic acid, ferulic acid, caffeic acid, phenol, and glutathione were determined under different reaction times, as shown in Figure 3A. The reaction rate (k_1) at different times is shown in Figure 3B. The results reveal that ABTS•+ could be quenched very rapidly by phenol, but very slowly by glutathione. Gallic acid, ferulic acid, and caffeic acid also quench ABTS•+ very rapidly, but it takes them a longer time to reach a steady state. The ABTS•+ scavenging activity of glutathione is very weak, especially within a short reaction time, indicating that glutathione quenches ABTS•+ according to hydrogen atom transfer. That is to say, the antioxidants like glutathione need longer time to accomplish the reaction. According to our experiments, the six minutes of reaction time used in the literature is too long for reaching a slow reaction state, and it is difficult for the majority of compounds to obtain a steady state within a limited time. It can be concluded from Figure 3 that ABTS assay could be measured within 2-10 min to obtain a rough result. To get more accurate and reliable results, a time-varying measurement is recommended.
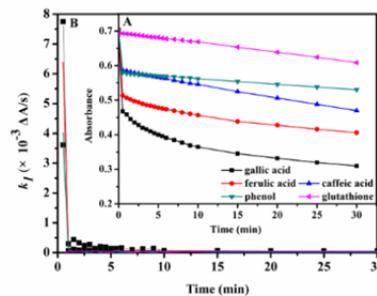
Figure 3: Absorbance (**A**) and reaction rate (k_1) (**B**) of ABTS•+ solution mixed with antioxidants (5 μM) at different time.
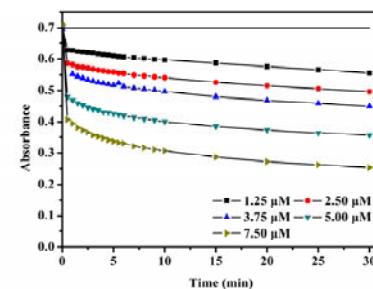
Figure 4: Absorbance of ABTS•+ solution mixed with gallic acid at different concentrations.

Effects of concentration of antioxidants: The reaction needs more time when the antioxidants have a high concentration, according to a previous report by Xie and Schaich [28]. In the present study, gallic acid was selected to determine the effects of the concentration of the antioxidant on the reaction time. The concentrations of gallic acid ranged from 1.25 μM to 7.50 μM. The results in Figure 4 show that the time of reaching a stationary state becomes longer with higher concentrations. The stationary state could not be reached within a stationary time for different concentrations of antioxidants.

Figure 2: UV-Vis spectra (500-1000 nm) of ABTS•+ solution diluted with different solvents.

Therefore, the absorbance of the ABTS·+ assay could not be measured at a stationary reaction time, and a time-varying measurement is encouraged to obtain accurate results.

Antioxidant assay: The ABTS·+ scavenging activity of five antioxidants, gallic acid, ferulic acid, caffeic acid, phenol, and glutathione were measured using five different solvents (PBS buffer solution, PB, H₂O, MeOH, and EtOH) to pre-dilute ABTS·+ radical solution, respectively. The absorbance was recorded at the corresponding maximum absorption wavelength. The results in Table 2 (IC₅₀ [APR] and I₇.₅] reveal that phenolic acids and phenol exhibit similar ABTS·+ radical-scavenging capacity in the five solvents. Additionally, good linear relationships between inhibition and antioxidant concentrations of three phenolic acids and phenol could be obtained at an inhibition of about 50%. For a limited range of concentrations, a reliable linear relationship does exist between antioxidant concentration and percentage inhibition according to the literature [30, 31]. Carmona-Jiménez *et al.* [32] reported that the IC₅₀ can be obtained accurately when a reliable linear relationship exists. Therefore, the five different solvents might be alternative solvents for pre-diluting ABTS·+ radical solution in the ABTS·+ radical-scavenging assay.

Table 2: The compounds' properties and their ABTS·+ radical-scavenging capacity.

Compound		gallic acid	ferulic acid	caffeic acid	phenol	glutathione
# -OH [a]		3	1	2	1	0
E₇ (V) [b]		0.55	0.60	0.54	0.90	0.24
IC₅₀ (APR) of	PBS	4.5 (0.22)	5.7 (0.18)	7.9 (0.13)	8.9 (0.11)	nd [g]
ABTS·+ [c]	PB	3.9 (0.26)	5.3 (0.19)	7.7 (0.13)	8.7 (0.12)	nd [g]
	H₂O	4.5 (0.22)	4.2 (0.24)	9.7 (0.10)	11.6 (0.09)	nd [g]
	MeOH	4.9 (0.20)	5.7 (0.17)	8.1 (0.12)	nd [g]	nd [g]
	EtOH	5.6 (0.18)	5.5 (0.39)	8.4 (0.12)	10.0 (0.10)	nd [g]
IC₅₀ (APR) of DPPH [d]	-	9.5 (0.10)	84 (0.012)	21 (0.05)	nr [f]	nd [g]
I₇.₅ (%) [e]	PBS	78.5	64.1	49.6	42.3	14.6
	PB	86.3	67.1	49.3	42.9	11.6
	H₂O	76.5	73.6	40.6	32.0	2.4
	MeOH	75.6	60.1	47.4	36.5	1.9
	EtOH	69.4	65.3	44.9	38.0	1.1

[a] Number of reactive −OH groups; [b] Redox potential at pH 7; [c] IC₅₀ of ABTS·+ is expressed as µM, APR=1/IC₅₀; [d] IC₅₀ of DPPH is obtained from the literature [28] and expressed as µM, APR=1/IC₅₀, [e] Inhibition of ABTS·+ at the concentration of 7.5 µM. [f] no reaction; [g] response too low to determine stoichiometry accurately.

It is interesting that phenol (monohydric phenol) exhibited moderate ABTS·+ radical-scavenging activity, but exhibited no DPPH radical-scavenging activity (Table 2). Ferulic acid has stronger APR than caffeic acid in the ABTS·+ assay, which might be caused by the higher redox potential of ferulic acid (E₇: ferulic acid > caffeic acid); this further supported the view that ABTS·+ can be quenched by both electron and hydrogen atom transfer. The above results imply that accurate ABTS·+ antioxidant capacity could not be obtained from a stationary time measurement because of the existence of diverse samples, especially for mixture samples and crude extracts of plants.

Recommendations for the use of ABTS·+ assay in the evaluation of total antioxidant activity: The ABTS·+ assay has been used extensively to compare and rank the antioxidant effectiveness of a wide range of natural extracts in thousands of studies. Nonetheless, results reported here raise serious questions about its validity for quantitating and comparing the activity of antioxidants with different sizes and structures, and especially for ranking their relative reactivity. Hence, some recommendations for the use of the ABTS·+ assay according to the results of the present study are listed as follow: (1) When using different pre-diluting solvents, the corresponding maximal absorption wavelengths for detection should be selected; (2) Although ABTS·+ antioxidant capacity could be measured within 2-10 min to obtain a rough result, to obtain more accurate and reliable results, a time-varying measurement is recommended, with calculation of reaction kinetics from early response.

Experimental

Reagents and instruments: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from J&K Scientific Ltd. (Beijing, China), gallic acid and ferulic acid from Sigma-Aldrich Co. LLC (Shanghai, China), and caffeic acid, phenol, and glutathione from Aladdin Chemistry Co. Ltd (Shanghai, China). The water (resistivity ≥ 18.25 MΩ/cm) used was purified with a purity water system (Chengdu, China). A Shimadzu UV-Vis 2401 PC spectrophotometer was used for recording the UV-Vis spectra and absorbance in colorimetric methods.

Reagent preparation: ABTS (38.4 mg) was dissolved in 10 mL 2.5 mM potassium persulfate and the mixture was reacted in the dark at room temperature for 12-16 h to prepare ABTS·+ solution. This was diluted with methanol, ethanol, PB (Na₂HPO₄-NaH₂PO₄, 200 mM, pH = 7.4), PBS buffer (Na₂HPO₄-NaH₂PO₄-NaCl, 200 mM, pH = 7.4), and distilled water, respectively, to obtain an absorbance of 0.70 ± 0.02 for ABTS·+ colorimetric method.

Sample preparation: Stock solutions (2 mM) were prepared in methanol, and then diluted with methanol to obtain serial concentration solutions. In the present study, all antioxidant sample concentrations noted are final reaction concentrations.

Effects of the solvents: The ABTS·+ was diluted 50 times with methanol, ethanol, PB (Na₂HPO₄-NaH₂PO₄, 200 mM, pH = 7.4), PBS buffer (Na₂HPO₄-NaH₂PO₄-NaCl, 200 mM, pH = 7.4), and distilled water, respectively. The absorbance of the ABTS·+ solutions pre-diluted to different concentrations with the above solvents was measured in the wavelength range from 500 nm to 1000 nm.

Effects of reaction time: ABTS·+ was diluted with PBS buffer solution to obtain an absorbance in the range of 0.70 ± 0.02. Ten µL of 2 mM antioxidant samples (5 µM, final concentration) and 90 µL distilled water were added to the tube, then 3.9 mL of diluting ABTS·+ solution was added and vibrated vigorously. The mixture was transferred to the cuvette, and the absorbance at 734 nm was measured rapidly. The absorbance was recorded successively every 30 s (0-6 min), 1 min (6-10 min), and 5 min (10-30 min), respectively. The initial absorbance was recorded by replacing the sample with methanol. The reaction rate (k_l) was obtained from the absorbance drop (ΔA_i) for every time unit (Δt) and calculated as follows: $k_l = \Delta A_i/\Delta t$ (ΔA/s)

Effects of concentration of the antioxidants: Gallic acid solutions at different concentrations were selected to determine the effects of the concentration of the antioxidants on ABTS·+ assay. The absorbance was measured as above.

ABTS free radical-scavenging activity assays: One hundred µL of each sample in methanol at various concentrations (2.5, 5.0, 7.5, 10.0, and 15.0 µM, final concentration) was added to 3.9 mL of pre-diluting ABTS·+ (absorbance at 0.70 ± 0.02) and vibrated vigorously. Then the reaction mixture was incubated at room temperature for 2-10 min, and the absorbance was recorded at the corresponding maximum absorption wavelength (734 nm for PBS, PB solution, and H₂O, 745 nm for MeOH, and 753 nm for EtOH). The ABTS free radical-scavenging activity was expressed by inhibition ($I\%$), calculated as follows: $I\% = (A_{blank} - A_{sample})/A_{blank} \times 100$; where A_{sample} was the absorbance of ABTS solution mixed with sample and A_{blank} was the absorbance of ABTS solution mixed with solvent. The IC₅₀ value was defined as the effective concentration at which the DPPH radical was scavenged by 50% and the antiradical power (ARP) was calculated as follow: ARP = 1/IC₅₀.

References

[1]　Cekic SD, Cetinkaya A, Avan AN, Apak R. (**2013**) Correlation of total antioxidant capacity with reactive oxygen species (ROS) consumption measured by oxidative conversion. *Journal of Agricultural and Food Chemistry*, *61*, 5260-5270.

[2]　Ozyurek M, Guclu K, Apak R. (**2011**) The main and modified CUPRAC methods of antioxidant measurement. *Trends in Analytical Chemistry*, *30*, 652-664.

[3]　Namiki M. (**1990**) Antioxidants/anti-mutagens in food. *Critical Reviews in Food Science and Nutrition*, *29*, 273-300.

[4]　Kartika H, Li QX, Wall MM, Nakamoto ST, Iwaoka WT. (**2007**) Major phenolic acids and total antioxidant activity in Mamaki leaves, *Pipturus albidus*. *Journal of Food Science*, *72*, 696-701.

[5]　Dong JW, Cai L, Xiong J, Chen XH, Wang WY, Shen N, Liu BL and Ding ZT. (**2015**) Improving the antioxidant and antibacterial activities of fermented *Bletilla striata* with *Fusarium avenaceum* and *Fusarium oxysporum*. *Process Biochemistry*, *50*, 8-13.

[6]　Dong JW, Cai L, Zhu XF, Huang X, Yin TP, Fang HX, Ding ZT. (**2014**) Antioxidant activities and phenolic compounds of cornhusk, corncob and stigma maydis. *Journal of the Brazilian Chemical Society*, *25*, 1956-1964.

[7]　Cretu E, Salminen J-P, Karonen M, Miron A, Charalambous C, Constantinou AI, Aprotosoaie AC. (**2014**) *In vitro* antioxidant activity and phenolic content of *Cedrus brevifolia* bark. *Natural Product Communications*, *9*, 481-482.

[8]　Hung PV, Maeda T, Miyatake K, Morita N. (**2009**) Total phenolic compounds and antioxidant capacity of wheat graded flours by polishing method. *Food Research International*, *42*, 185-190.

[9]　Sacchetti G, Di Mattia C, Pittia P, Martino G. (**2008**) Application of a radical scavenging activity test to measure the total antioxidant activity of poultry meat. *Meat Science*, *80*, 1081-1085.

[10]　Luo W, Zhao M, Yang B, Shen G, Rao G. (**2009**) Identification of bioactive compounds in *Phyllenthus emblica* L. fruit and their free radical scavenging activities. *Food Chemistry*, *114*, 499-504.

[11]　Wang X, Li X, Chen D. (**2011**) Evaluation of antioxidant activity of isoferulic acid *in vitro*. *Natural Product Communications*, *6*, 1285-1288.

[12]　Khanama UKS, Obab S, Yanaseb E, Murakamic Y. (**2012**) Phenolic acids, flavonoids and total antioxidant capacity of selected leafy vegetables. *Journal of Functional Foods*, *4*, 979-987.

[13]　Zan LF, Bao HY, Bau T, Li Y. (**2015**) A new antioxidant pyrano[4,3-c][2]benzopyran-1,6-dione derivative from the medicinal mushroom *Fomitiporia ellipsoidea*. *Natural Product Communications*, *10*, 315-316.

[14]　Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. (**1999**) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine*, *26*, 1231-1237.

[15]　Miller NJ, Rice-Evans CA, Davies MJ, Gopinathan V, Milner A. (**1993**) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science*, *84*, 407-412.

[16]　Miller NJ, Rice-Evans CA. (**1994**) Total antioxidant status in plasma and body fluids. *Methods in Enzymology*, *234*, 279-293.

[17]　Liu YR, Li WG, Chen LF, Xiao BK, Yang JY, Yang L, Zhang CG, Rong-Qing Huang RQ, Dong JX. (**2014**) ABTS+ scavenging potency of selected flavonols from *Hypericum perforatum* L. by HPLC-ESI/MS QQQ: Reaction observation, adduct characterization and scavenging activity determination. *Food Research International*, *58*, 47-58.

[18]　Nakano S, Tanaka K, Oki R, Kawashima T. (**1999**) Flow-injection spectrophotometry of manganese by catalysis of the periodate oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid). *Talanta*, *49*, 1077-1082.

[19]　He L, Xu HG, Liu X, He WH, Yuan F, Hou ZQ, Gao YX. (**2011**) Identification of phenolic compounds from pomegranate (*Punica granatum* L.) seed residues and investigation into their antioxidant capacities by HPLC-ABTS+ assay. *Food Research International*, *44*, 1161-1167.

[20]　He L, Zhang XF, Xu HG, Xu C, Yuan F, Knez Z, Novakb Z, Gao YX. (**2012**) Subcritical water extraction of phenolic compounds from pomegranate (*Punica granatum* L.) seed residues and investigation into their antioxidant activities with HPLC-ABTS+ assay. *Food and Bioproducts Processing*, *90*, 215-223.

[21]　Kalili KM, De Smet S, van Hoeylandt T, Lynen F, de Villiers A. (**2014**) Comprehensive two-dimensional liquid chromatography coupled to the ABTS radical scavenging assay: a powerful method for the analysis of phenolic antioxidants. *Analytical and Bioanalytical Chemistry*, *406*, 4233-4242.

[22]　Bangoura ML, Nsor-Atindana J, Ming ZH. (**2013**) Solvent optimization extraction of antioxidants from foxtail millet species' insoluble fibers and their free radical scavenging properties. *Food Chemistry*, *141*, 736-744.

[23]　Shao Y, Wu Q-n, Duan J-a, Yue W, Gu W, Wang X. (**2014**) Optimisation of the solvent extraction of bioactive compounds from *Lophatherum gracile* Brongn. using response surface methodology and HPLC-PAD coupled with pre-column antioxidant assay. *Analytical Methods*, *6*, 170-177.

[24]　Gursoy N, Sarikurkcu C, Cengiz M, Solak MH. (**2009**) Antioxidant activities, metal contents, total phenolics and flavonoids of seven *Morchella* species. *Food and Chemical Toxicology*, *47*, 2381-2388.

[25]　Siddhuraju P. (**2006**) The antioxidant activity and free radical-scavenging capacity of phenolics of raw and dry heated moth bean (*Vigna aconitifolia*) (Jacq.) Marechal seed extracts. *Food Chemistry*, *99*, 149-157.

[26]　Dong JW, Zhao LX, Cai L, Fang HX, Chen XH, Ding ZT. (**2014**) Antioxidant activities and phenolics of fermented *Bletilla formosana* with eight plant pathogen fungi. *Journal of Bioscience and Bioengineering*, *118*, 396-399.

[27]　Dawidowicz AL, Olszowy M. (**2013**) The importance of solvent type in estimating antioxidant properties of phenolic compounds by ABTS assay. *European Food Research and Technology*, *236*, 1099-1105.

[28]　Xie J, Schaich KM. (**2014**) Re-evaluation of the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay for antioxidant activity. *Journal of Agricultural and Food Chemistry*, *62*, 4251-4260.

[29]　Dawidowicz AL, Olszowy M. (**2012**) Mechanism change in estimating of antioxidant activity of phenolic compounds. *Talanta*, *97*, 312-317.

[30]　Buenger J, Ackermann H, Jentzsch A, Mehling A, Pfitzner I, Reiffen KA, Schroeder KR, Wollenweber U. (**2006**) An interlaboratory comparison of methods used to assess antioxidant potentials. *International Journal of Cosmetic Science*, *28*, 135-146.

[31]　Locatelli M, Gindro R, Travaglia F, Coïsson JD, Rinaldi M, Arlorio M. (**2009**) Study of the DPPH'-scavenging activity: Development of a free software for the correct interpretation of data. *Food Chemistry*, *114*, 889-897.

[32]　Carmona-Jimenez Y, Garcia-Moreno MV, Igartuburu JM, Garcia Barroso C. (**2014**) Simplification of the DPPH assay for estimating the antioxidant activity of wine and wine by-products. *Food Chemistry*, *165*, 198-204.

Chemical Synthesis of the Echinopine Sesquiterpenoids

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As new members of the sesquiterpenoid family, echinopines A and B were found to possess an unprecedented [3/5/5/7] carbon framework, which has stimulated considerable interest among the chemistry community since their isolation. This review article documents chronologically the steps towards chemical synthesis of the echinopine sesquiterpenoids, showcasing different strategies by resorting to the toolkit of organic chemistry.

Keywords: Echinopine, Natural product, Sesquiterpenoid, Total synthesis.

1. Introduction

Throughout the long research history of natural products, the terpenoids constitute the largest family, with the most diversified structures created by Mother Nature. Notable features associated with their chemical structures and therapeutic applications have made them a permanently pursued theme among the synthetic chemists worldwide [1,2].

Echinopines A (**1**) and B (**2**) (Figure 1), two closely related sesquiterpenoids with remarkable architectural novelties, were isolated from the roots of *Echinops spinosus* by Shi, Kiyota, and co-workers in 2008 [3]. Based upon spectroscopic analyses, the structures of echinopines A (**1**) and B (**2**) were established as shown in Figure 1, which represents the first example of a unique [3/5/5/7] carbon framework. Biogenetically, the unprecedented skeleton of echinopine sesquiterpenoids was proposed to originate from a guaiane-type precursor **3** (Scheme 1) [3]. Key steps concerning their biosynthetic pathways involved a C11→C13 rearrangement followed by successive C15–C13 and C13–C4 bond formations.

In spite of the lack of reported biological activities, echinopines attracted many synthetic interests immediately by their novel structures. Thus far, five successful total syntheses [4-8], together with two interesting formal syntheses [9,10], have been achieved. To the best of our knowledge, a logical summary concerning this topic is missing in the literature. This review aims to highlight these accomplishments described to date towards the fascinating echinopine sesquiterpenoids.

2. Chemical synthesis of echinopines
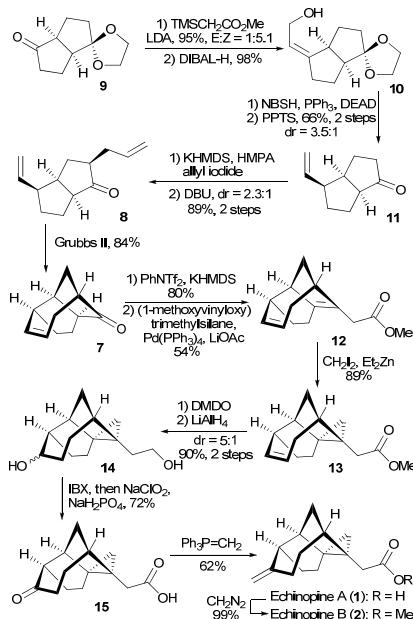
2.1 Magauer-Tiefenbacher's total synthesis of (+)-echinopines A and B

The first total synthesis of (+)-echinopines A and B was reported by Magauer, Mulzer, and Tiefenbacher in 2009 [4]. Their approach features an intermolecular cyclopropanation to form the cyclopropane, as well as a ring closing metathesis (RCM) to construct the seven-membered ring. As illustrated in Scheme 2, the precursor **8** could be synthesized from the known optically pure bicyclic intermediate **9**.

Figure 1: Structures of echinopines A (**1**) and B (**2**).

Scheme 1: Proposed biosynthesis of the echinopine sesquiterpenoids.

Scheme 2: Retrosynthetic analysis by Magauer *et al*.

The synthesis commenced with the preparation of bicycle **9** from 1,5-cyclooctadiene based on the literature method [11]. Subsequent Peterson olefination [12] and reduction of the ester afforded allylic alcohol **10** (Scheme 3). Transformation of **10** to the requisite terminal alkene was secured by a Myers' [3,3]-rearrangement protocol [13], which was followed by removal of acetal protection, resulting in the unmasked ketone as a separable diastereomeric mixture (3.5:1). The major and desired isomer **11** was subjected to allylation and epimerization to furnish the key intermediate **8**. Ring closing metathesis of the latter under the conditions of Grubbs II catalyst generated the [5/5/7]-tricyclic framework **7** (84%).
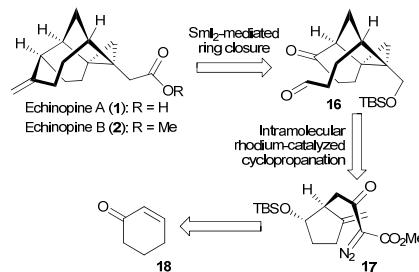
Scheme 3: The first total synthesis of (+)-echinopines A and B.

The remaining task was to introduce the side chain and cyclopropane unit. To this end, the authors developed two routes to the advanced intermediate **14**; the shorter one is shown in Scheme 3. Specifically, palladium-catalyzed coupling reaction of the corresponding vinyl triflate derived from **7** with (1-methoxyvinyloxy)trimethylsilane [14] provided ester **12**. Regioselective cyclopropanation [15,16], followed by epoxidation and LiAlH$_4$ reduction, delivered the tetracyclic diol **14**. After global oxidation, Wittig methylenation of the resulting ketone ultimately led to the production of (+)-echinopine A (**1**), which could be converted to (+)-echinopine B (**2**) on exposure to diazomethane. The absolute configuration of echinopines was determined through the synthesis, together with a single crystal X-ray diffraction analysis of **1**. Thus, the first synthesis of enantiomerically pure echinopine sesquiterpenoids was achieved starting from the known bicyclic compound **9**. The highlight of this pioneering approach was embodied in forging of the seven-membered ring by RCM to access the bridged core structure.

2.2 Nicolaou-Chen's total synthesis of echinopines A and B
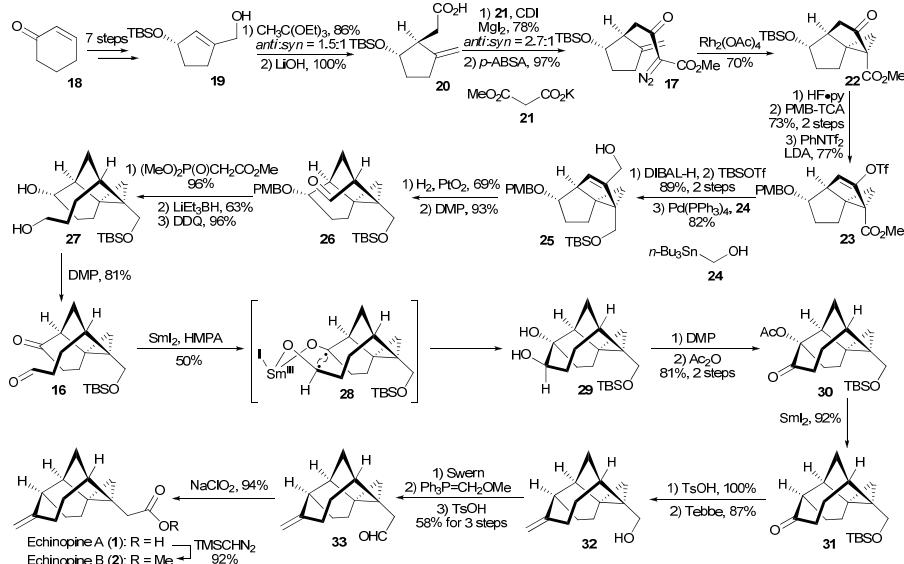
Shortly after the first total synthesis, Nicolaou, Chen and co-workers reported their endeavors on echinopine synthesis [5]. The retrosynthetic analysis is outlined in Scheme 4, which relied on an intramolecular cyclopropanation and a SmI$_2$-mediated [17] ring closure to establish the crucial cyclopropane and cycloheptane, respectively.

Scheme 4: Nicolaou-Chen's bond disconnection towards echinopines.

The functionalized cyclopentyl allylic alcohol **19** was prepared by a ring contraction process from the commercial cyclohexenone (**18**) at the beginning stage of the synthesis (Scheme 5). Compound **19** could be further transformed into the unsaturated carboxylic acid **20** through Jonson-Claisen rearrangement [18], followed by saponification. The latter was then subjected to homologation and subsequently reacted with *p*-ABSA to give the key intermediate **17**. As anticipated, the intramolecular cyclopropanation of diazo **17** took place smoothly with catalytic Rh$_2$(OAc)$_4$, affording the [3/5/5] tricyclic system (**22**) as a single isomer in 70% yield.

Further functional group manipulations of **22**, including Stille coupling of enol triflate with hydroxymethylstannane **24** and a stereocontrolled hydrogenation, furnished aldehyde **26**. Next, Horner-Wadsworth-Emmons reaction of the aldehyde followed by reduction of the resultant α,β-unsaturated ester and deprotection of PMB afforded diol **27**. After oxidation of diol to keto aldehyde **16**, the stage was set for the intramolecular pinacol coupling. Consequently, the core structure **29** was successfully established (as a single diastereoisomer) under the conditions of SmI$_2$-HMPA, through the proposed transition state **28**.
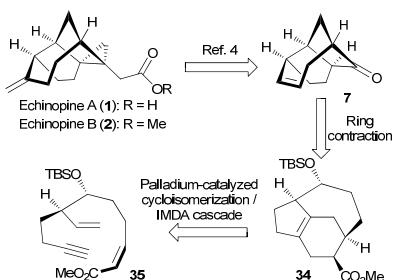
Scheme 5: Total synthesis of echinopines by Nicolaou, Chen and co-workers.

Having the tetracyclic framework **29** secured, the final stage of the synthesis was relatively easy. Oxidation of the secondary alcohol followed by removal of the tertiary acetate mediated by SmI_2 gave rise to ketone **31**. Prior to homologation of the side chain, an exocyclic olefin was installed through Tebbe olefination. A routine procedure was then applied to convert alcohol **32** to aldehyde **33** with homologation of one more carbon. Finally, echinopine A (**1**) was obtained after oxidation of the aldehyde to acid by using $NaClO_2$, while echinopine B (**2**) was realized by further esterification. The synthesis was achieved in both forms employing enantiomeric or racemic intermediate **19** [5].
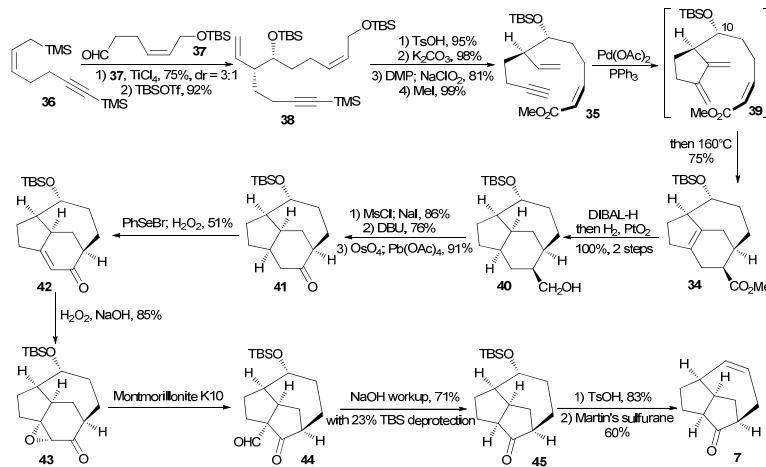
2.3 Chen's synthesis of echinopines A and B

Continuous studies by Chen and co-workers led to a conceptually different approach [9] to the intriguing sesquiterpenoids compared with the aforementioned synthesis. Inspired by the biosynthetic proposal [3], a [5/6/7] carbocyclic framework (i.e., the hypothetical intermediate **6**, Scheme 1) was associated with the planned chemical synthesis of echinopines A (**1**) and B (**2**). In this context, a ring contraction event could enable the central [5/5/7] core of the natural products. As shown in Scheme 6, the desired [5/6/7] tricyclic system **34** was envisioned to be constructed from the linear substrate **35** by a palladium-mediated cycloisomerization/ intramolecular Diels-Alder cycloaddition sequence.

Scheme 6: Palladium-catalyzed cascade strategy from Chen group.

To access the acyclic precursor **35**, a crucial Hosomi-Sakurai reaction [19] was realized between allylsilane **36** and aldehyde **37** in the presence of $TiCl_4$; and the resulting alcohol (dr = 3: 1) was silylated to afford **38** (Scheme 7). Subsequent functional group manipulations could smoothly give rise to the enyne **35**. Upon treatment of **35** with $Pd(OAc)_2/PPh_3$ at 80ºC, the diene enoate **39** was detected, which could be further converted into the tricycle **34**
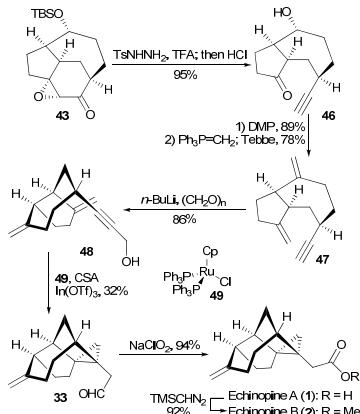
(75%) in one-pot with prolonged heating at 160ºC by intramolecular Diels-Alder cycloaddition. A selective *endo*-cycloaddition was observed, while the configuration at C10 proved to be insignificant for the stereochemical outcome of the sequence.

Further elaboration commenced with a two-step reduction (DIBAL-H; H_2/PtO_2) of **34** with high efficiency. The hydroxymethyl group in compound **40** was converted to ketone **41** through the sequence of iodination, elimination, and oxidative cleavage. Preparation of an epoxy ketone would be the next task, which was envisaged to serve as the appropriate substrate for the corresponding ring contraction process. After introduction of enone *via* the well-established α-selenation/oxidative elimination sequence, **42** underwent epoxidation by treatment with $H_2O_2/NaOH$, furnishing epoxy ketone **43**. Based on the method of Sheldon [20], keto aldehyde **44** was obtained from **43** in the presence of montmorillonite K10, which after basic aqueous workup, resulted in deformylation to provide tricyclic ketone **45** (71%) together with the TBS deprotection product (23%). Finally, global desilylation (TsOH, 83%) followed by dehydration using Martin's sulfurane delivered the known intermediate **7** [4], constituting a formal synthesis of echinopines A (**1**) and B (**2**). In addition, an auxiliary-based aldol reaction strategy provided the same intermediate **38** in its optically active form, thus enabling an asymmetric version of this synthetic approach [9].

Alternatively, Chen's group developed a novel endgame towards echinopines starting from intermediate **43** [6], which for the first time showcased the [5/7]→[3/5/5/7] ring-forming sequence by a bio-inspired late-stage intramolecular cyclopropanation strategy (Scheme 8). With an improved synthesis of epoxy ketone **43**, an Eschenmoser fragmentation [21] was envisioned to realize the bond cleavage. As such, alkynyl ketone **46** was obtained *via* treatment with $TsNHNH_2$ followed by acidic workup, setting the stage for [5/7]→[3/5/5/7] skeletal conversion. Prior to that, oxidation of the hydroxyl group followed by subsequent double methylenation of the diketone afforded diene-yne **47**. The cycloisomerization precursor **48** was produced by a lithium chemistry to introduce the terminal hydroxymethyl group. After extensive investigations, the conditions reported by the group of Trost [CpRu(PPh₃)₂Cl, CSA, In(OTf)₃] [22] was revealed to be successful to furnish the tetracycle **33** with a modest yield. Lastly, echinopines A (**1**) and B (**2**) could be formed through the same procedures as described before.

Scheme 7: Chen's formal synthesis of echinopines.

Central to Chen's novel approach towards echinopines was the use of two transition-metal-catalyzed enyne cycloisomerizations. Especially the ruthenium-mediated intramolecular cyclopropanation was successfully demonstrated in a complex molecular system, which resembled the late-stage bond formations in the proposed biosynthetic pathway for the echinopine framework.

Scheme 8: Synthesis of echinopines by a bio-inspired strategy.
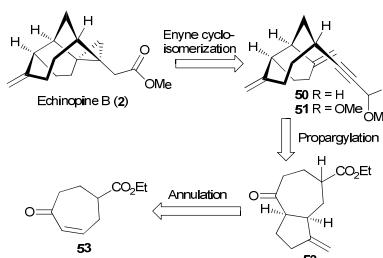
2.4 Vanderwal's synthesis of echinopine B

Another synthesis of echinopine B (**2**), employing a bio-inspired conversion of a *cis*-guaiane precursor bearing a [3/5]-bicycle into the [3/5/5/7] skeleton, was achieved by the team of Vanderwal in 2012 [7]. While the core of echinopines was established through an enyne cycloisomerization [23] from **50/51**, the bicyclic **52** was accessible by an annulation of cycloheptenone **53** (Scheme 9).

Starting from ketone **54**, a standard ring expansion process was carried out to generate the δ-substituted cycloheptenone **53** with a 46% overall yield (Scheme 10). Since the one-step annulation by Piers protocol [24] was unsuccessful, the group adopted a stepwise procedure, beginning with installation of a vinyl chain through conjugate addition of cuprate **55** (dr = 6.5:1). The relative stereochemical outcome between the two centers could not be fully determined by NMR spectroscopy at this stage. The synthesis was continued by transforming the silyl ether into tosylate ester **56** before the annulation took place. Under the condition of LDA, the *cis*-fused bicyclo[5.3.0]decane **52** was prepared in 25~40% yield, along with a near 30% recovery of the starting material. After Wittig olefination of compound **52**, the resultant ester was subjected to DIBAL-H reduction, followed by treatment with Ohira-Bestmann reagent [25], leading to the diene-yne **58** as a mixture of diastereomers (4.5:1). A control experiment indicated epimerization occurred under the Ohira-Bestmann conditions.

With bicyclic **58** in hand, the terminal alkyne was then functionalized to propargylic ether **50** upon exposure to *n*-BuLi/MOMCl. The key cycloisomerization was realized in the

presence of $PtCl_2$ [26], delivering the tetracyclic core of echinopine in 56% yield. On the basis of the successful cyclopropanation, it could be concluded that the conjugate addition (**53**→**56**) might give the undesired stereoisomer predominantly, which was corrected during the alkynylation to enable the desired cycloisomerization. Since the two-carbon chain was attached to the core as an enol ether, a direct oxidation using PCC formed the methyl carboxylate [27],

Scheme 9: Retrosynthetic analysis of echinopine B by Vanderwal *et al.*
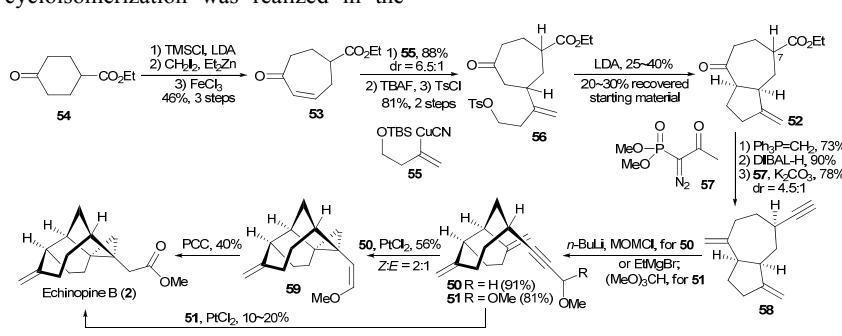
providing racemic echinopine B. Alternatively, a propargylacetal **51** was introduced from **58**, and its cycloisomerization could straightforwardly afford echinopine B, although with less efficiency (10~20% yield).

By use of the enyne cycloisomerization, together with a facile synthesis of [5/7]-bicyclic system **52**, Vanderwal's accomplishments provided a very concise solution to the synthetic issue of echinopines.
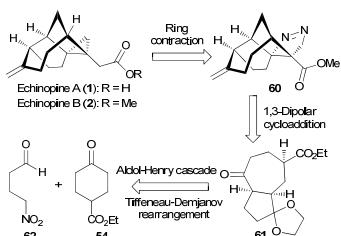
2.5 Liang's synthesis of echinopines

Liang and co-workers described a new synthesis of echinopines in 2013 [8], taking advantage of an intramolecular 1,3-dipolar cycloaddition and ring contraction sequence for the core construction. From a retrosynthetic viewpoint, the *cis*-fused bicyclo[5.3.0]decane system (**61**) could be formed through a tandem aldol-Henry reaction, followed by a Tiffeneau-Demjanov rearrangement (Scheme 11) [28].

In the forward synthesis, an aldol-Henry cascade between 4-nitrobutanal **62** and ketone **54** was employed as the launch point, with subsequent oxidation of the resulting secondary alcohol, giving access to *trans*-decaline **63a** and **63b** as a pair of isomers (Scheme 12). The undesired **63a** could be epimerized to **63b** upon treatment with Et_3N, which offered a scalable and efficient preparation of **63b** from the commercially available **54**. After ketone protection and nitro reduction, subjecting amine **64** to the Tiffeneau-Demjanov rearrangement conditions [29] delivered *cis*-fused [5/7]-bicycle **61** (77%) through the diazo intermediate **65**. The short sequence for supplying the bicyclo[5.3.0]decane system set the stage for the following chemistry.
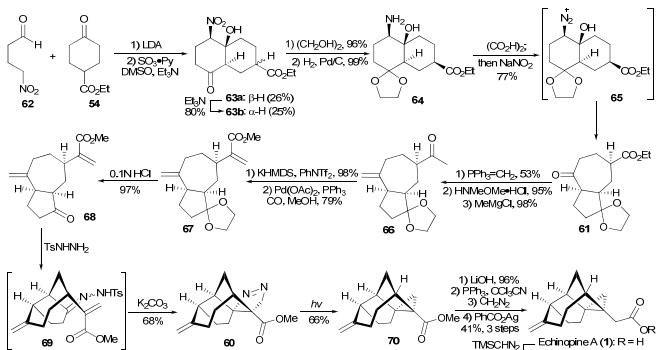
Scheme 10: A concise synthesis of echinopine B *via* a cycloisomerization strategy.

Scheme 11: Liang's bond disconnection towards echinopines.
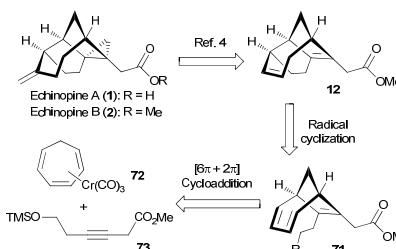
Several conventional transformations were involved before the designed 1,3-dipolar cycloaddition took place. Specifically, Wittig methylenation of **61** followed by a Weinreb ketone synthesis [30] furnished the olefinic ketone **66**. The latter was converted into unsaturated ester **67** efficiently *via* a palladium-catalyzed carbonylation of the vinyl triflate. After cleavage of the dioxolane, tosylhydrazone **69** was formed and ready for the 1,3-dipolar cycloaddition. Gratifyingly, upon exposure to K₂CO₃, **69** underwent the cycloaddition smoothly to give the advanced intermediate **60** [31]. The anticipated ring contraction was realized by a light-induced decomposition of pyrazoline to cyclopropane [32], leading to the echinopine framework **70** in 66% yield. After hydrolysis of the ester to carboxylic acid in **70**, the synthesis of echinopine A (**1**) was completed through an Arndt-Eistert homologation process [33], while echinopine B (**2**) was obtained by further methylation.

Scheme 12: Total synthesis of echinopines by Liang *et al.*
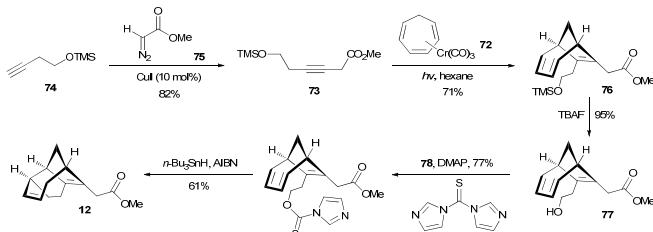
The Liang group demonstrated a new entry to the *cis*-fused bicyclo[5.3.0]decane system from an aldol-Henry cascade reaction followed by a Tiffeneau-Demjanov rearrangement. This critical building block was ultimately elaborated to echinopines by a 1,3-dipolar cycloaddition with subsequent light-induced ring contraction. The crucial 1,3-dipolar cycloaddition between diazo alkane and alkene functionalities established the structural complexity rapidly, representing its second application in natural product synthesis [34].

2.6 Misra's formal synthesis of echinopines

For a given molecule, there can be various ways for bond disconnection. In the case of echinopines, aside from the aforementioned synthesis, Misra *et al* disclosed a distinct strategy very recently [10]. The target sesquiterpenoids could be accessed from intermediate **12** according to Magauer *et al* [4], in which the five-membered ring could be forged by a radical cyclization of compound **71** (Scheme 13). The crucial bicyclo[4.2.1]nonane core could in turn be assembled via a [6π + 2π] cycloaddition of cycloheptatriene tricarbonyl chromium complex **72** and alkyne **73** [35].

Scheme 13: Retrosynthetic analysis of echinopines by Misra and co-workers.

Scheme 14: Formal synthesis of echinopines by Misra and co-workers.

After a successful model study showing the feasibility of the [6π+2π] cycloaddition/radical cyclization sequence, the authors initiated their efforts towards echinopines A and B. As depicted in Scheme 14, the substituted alkynoate **73** was easily prepared on a multigram scale from alkyne **74** and diazoester **75**, by harnessing Fu's C–H insertion method in the presence of catalytic CuI [36]. Under photolytic conditions, the key [6π + 2π] cycloaddition between **72** and **73** proceeded with excellent stereo- and regioselectivity, leading to the bicyclic intermediate **76** in 71% yield. Next, desilylation followed by treatment with 1,1'-(thiocarbonyl)diimidazole (**78**) afforded thioxoester **79** smoothly. Thus, the stage was set for the radical cyclization. After screening of the conditions, it was found that slow addition of the *n*-Bu₃SnH/AIBN mixture to the refluxing solution of **79** ultimately gave the desired product **12** in 61% yield. Synthesis of the known tricyclic intermediate **12**, which was previously prepared by Magauer and co-workers, represents a new formal synthesis of the echinopine sesquiterpenoids.

As mentioned above, Misra *et al.* have accomplished the formal synthesis of echinopines A and B in an extremely concise manner, by reaching the known intermediate **12** in only five steps. Remarkably, the utility of a photo-induced [6π + 2π] cycloaddition to construct the bicyclo[4.2.1]nonane system was of great significance to this synthesis.

3 Conclusions

The echinopine sesquiterpenoids have attracted the attention of synthetic laboratories worldwide, being reflected in the chemical synthesis published almost each year since the report of their isolation. The major achievements have been highlighted in this review, which illustrate the evolution of creative strategies and applications of modern chemical methodologies. We hope the endeavors in this field will not only serve to enrich our education in synthesis design and execution, but also as the launching point for a better understanding of the biological activities and medicinal profiles of these intriguing compounds.

References

[1]   Maimone TJ, Baran PS. (**2007**) Modern synthetic efforts toward biologically active terpenes. *Nature Chemical Biology*, **3**, 396–407.

[2]   Jansen DJ, Shenvi RA. (**2014**) Synthesis of medicinally relevant terpenes: reducing the cost and time of drug discovery. *Future Medicinal Chemistry*, **6**, 1127–1148.

[3]   Dong M, Cong B, Yu SH, Sauriol F, Huo CH, Shi QW, Gu YC, Zamir LO, Kiyota H. (**2008**) Echinopines A and B: sesquiterpenoids possessing an unprecedented skeleton from *Echinops spinosus*. *Organic Letters*, **10**, 701–704.

[4]   Magauer T, Mulzer J, Tiefenbacher K. (**2009**) Total syntheses of (+)-echinopine A and B: determination of absolute stereochemistry. *Organic Letters*, **11**, 5306–5309.

[5]   Nicolaou KC, Ding H, Richard JA, Chen DYK. (**2010**) Total synthesis of echinopines A and B. *Journal of the American Chemical Society*, **132**, 3815–3818.

[6]   Peixoto PA, Richard JA, Severin R, Chen DYK. (**2011**) Total synthesis of echinopines A and B: exploiting a bioinspired late-stage intramolecular cyclopropanation. *Organic Letters*, **13**, 5724–5727.

[7]   Michels TD, Dowling MS, Vanderwal CD. (**2012**) A synthesis of echinopine B. *Angewandte Chemie International Edition*, **51**, 7572–7576.

[8]   Xu W, Wu S, Zhou L, Liang G. (**2013**) Total syntheses of echinopines. *Organic Letters*, **15**, 1978–1981.

[9]   Peixoto PA, Richard JA, Severin R, Tseng CC, Chen DYK. (**2011**) Formal asymmetric synthesis of echinopine A and B. *Angewandte Chemie International Edition*, **50**, 3013–3016.

[10]   De S, Misra S, Rigby JH. (**2015**) Formal total synthesis of echinopines A and B via Cr(0)-promoted [6π + 2π] cycloaddition. *Organic Letters*, **17**, 3230–3232.

[11]   Zhong YW, Lei XS, Lin GQ. (**2002**) Amino alcohols with the bicyclo[3.3.0]octane scaffold as ligands for the catalytic enantioselective addition of diethylzinc to aldehydes. *Tetrahedron: Asymmetry*, **13**, 2251–2255.

[12]   Peterson DJ. (**1968**) Carbonyl olefination reaction using silyl-substituted organometallic compounds. *The Journal of Organic Chemistry*, **33**, 780–784.

[13]   Myers AG, Zheng B. (**1996**) An efficient method for the reductive transposition of allylic alcohols. *Tetrahedron Letters*, **37**, 4841–4844.

[14]   Carfagna C, Musco A, Sallese G, Santi R, Fiorani T. (**1991**) Palladium-catalyzed coupling reactions of aryl triflates or halides with ketene trimethylsilyl acetals. A new route to alkyl 2-arylalkanoates. *The Journal of Organic Chemistry*, **56**, 261–263.

[15]   Tang P, Qin Y. (**2012**) Recent applications of cyclopropane-based strategies to natural product. *Synthesis*, **44**, 2969–2984.

[16]   Chen DYK, Pouwer RH, Richard JA. (**2012**) Recent advances in the total synthesis of cyclopropane-containing natural products. *Chemical Society Reviews*, **41**, 4631–4642.

[17]   Nicolaou KC, Ellery SP, Chen JS. (**2009**) Samarium diiodide mediated reactions in total synthesis. *Angewandte Chemie International Edition*, **48**, 7140–7165.

[18]   Johnson WS, Werthemann L, Bartlett WR, Brocksom TJ, Li TT, Faulkner DJ, Petersen MR. (**1970**) Simple stereoselective version of the Claisen rearrangement leading to trans-trisubstituted olefinic bonds. Synthesis of squalene. *Journal of the American Chemical Society*, **92**, 741–743.

[19]   Hosomi A, Endo M, Sakurai H. (**1976**) Allylsilanes as synthetic intermediates. II. Syntheses of homoallyl ethers from allylsilanes and acetals promoted by titanium tetrachloride. *Chemistry Letters*, **5**, 941–942.

[20]   Elings J, Lempers H, Sheldon R. (**2000**) Solid-acid catalyzed rearrangement of cyclic α,β-epoxy ketones. *European Journal of Organic Chemistry*, 1905–1911.

[21]   Eschenmoser A, Felix D, Ohloff G. (**1967**) Eine neuartige fragmentierung cyclischer α,ß-ungesättigter carbonyl- systeme; synthese von exalton und rac-Muscon aus cyclododecanon vorläufige mitteilung. *Helvetica Chimica Acta*, **50**, 708–713.

[22]   Trost BM, Breder A, O'Keefe BM, Rao M, Franz AW. (**2011**) Propargyl alcohols as β-oxocarbenoid precursors for the ruthenium-catalyzed cyclopropanation of unactivated olefins by Redox isomerization. *Journal of the American Chemical Society*, **133**, 4766–4769.

[23]   Watsonand IDG, Toste FD. (**2012**) Catalytic enantioselective carbon-carbon bond formation using cycloisomerization reactions. *Chemical Science*, **3**, 2899–2919.

[24]   Piers E, Karunaratna V. (**1989**) Organotin-based bifunctional reagents: 4-chloro-2-lithio-1-botene and related substances: Methylenecyclopentane annotations. Total synthesis of (±) -$\delta^{9(12)}$-capnellene. *Tetrahedron*, **45**, 1089–1104.

[25]   Müller S, Liepold B, Roth GJ, Bestmann HJ. (**1996**) An improved one-pot procedure for the synthesis of alkynes from aldehydes. *Synlett*, 521–522.

[26]   Ye L, Chen Q, Zhang J, Michelet V. (**2009**) PtCl₂-catalyzed cycloisomerization of 1,6-enynes for the synthesis of substituted bicyclo[3.1.0]hexanes. *The Journal of Organic Chemistry*, **74**, 9550–9553.

[27]   Piancatelli G, Scettriand A, D'Auria M. (**1977**) Pyridinium chlorochromate in the organic synthesis: a convenient oxidation of enol-ethers to esters and lactones. *Tetrahedron Letters*, **18**, 3483–3484.

[28]   Weller T, Seebach D, Davis RE, Laird BB. (**1981**) 3-Hydroxy-4-nitro-cyclohexanone aus ketonen und 4-nitrobuttersäurechlorid. eine ringerweiternde fünfringanellierung. *Helvetica Chimica Acta*, **64**, 736–760.

[29]   Fattori D, Henry S, Vogel P. (**1993**) The Demjanov and Tiffeneau-Demjanov one-carbon ring enlargements of 2-aminomethyl-7-oxabicyclo[2.2.1]heptane derivatives. The stereo- and regioselective additions of 8-oxabicyclo[3.2.1]oct-6-en-2-one to soft electrophiles. *Tetrahedron*, **49**, 1649–1664.

[30]   Nahmand S, Weinreb SM. (**1981**) *N*-methoxy-*n*-methylamides as effective acylating agents. *Tetrahedron Letters*, **22**, 3815–3818.

[31]   Taber DF, Guo P. (**2008**) Convenient access to bicyclic and tricyclic diazenes. *The Journal of Organic Chemistry*, **73**, 9479–9481.

[32]   Van Aukenand TL, Rinehart Jr. KL. (**1962**) Stereochemistry of the formation and decomposition of 1-pyrazolines. *Journal of the American Chemical Society*, **84**, 3736–3743.

[33]   Kirmse W. (**2002**) 100 Years of the Wolff rearrangement. *European Journal of Organic Chemistry*, 2193–2256.

[34]   Schultz AG, Puig S. (**1985**) The intramolecular diene-carbene cycloaddition equivalence and an enantioselective Birch reduction-alkylation by the chiral auxiliary approach. Total synthesis of (±)- and (−)-longifolene. *The Journal of Organic Chemistry*, **50**, 915–916.

[35]   Rigby JH, Henshilwood JA. (**1991**) Transition metal template controlled cycloaddition reactions. An efficient chromium(0)-mediated [6π + 2π] cycloaddition. *Journal of the American Chemical Society*, **113**, 5122−5123.

[36]   Suarez A, Fu GC. (**2004**) A straightforward and mild synthesis of functionalized 3-alkynoates. *Angewandte Chemie International Edition*, **43**, 3580−3582.

Synergistic Effects of Dietary Natural Products as Anti-Prostate Cancer Agents

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This review is to describe synergistic effects of various combinations of dietary natural products including curcumin, quercetin, soybean isoflavones, silibinin, and EGCG that have potential for the treatment of prostate cancer. These data can provide valuable insights into the future rational design and development of synergistic and/or hybrid agents for potential treatment of prostate cancer.

Keywords: Dietary natural product, Synergy, Combination therapy, Prostate cancer.

1. Introduction

Dietary habit has been identified as one of the risk factors for prostate cancer as evidenced by several epidemiological studies. For example, Asian traditional food has been demonstrated to be correlated with the low incidence of prostate cancer in Asian men in contrast to soaring risk of prostate cancer in the Asian immigrants who live in the United States [1]. A variety of dietary agents (e.g. curcumin, quercetin, and silibinin) with diverse chemical structures (Figure 1) have been verified by *in vitro* cell-based and *in vivo* animal studies to possess the potential for preventing and treating prostate cancer [2-3]. The safety profiles enable these dietary natural products to be qualified as drug candidates. However, their moderate potency and poor bioavailability impede them in becoming effective chemotherapeutics when taken alone [4-5]. Combination therapy might be an important strategy to overcome this problem in order to achieve enhanced overall clinical efficacy with acceptable clinical toxicity. Indeed, synergistic inhibition of growth, proliferation, and apoptosis of prostate cancer cells has been evident when using combinations of one dietary natural product with another natural product or with well-established chemotherapeutics. This review summarizes the synergistic effects of dietary anti-prostate cancer agents including curcumin, quercetin, soybean isoflavones, silibinin, and (-)-epigallocatechin-3-gallate (EGCG, Figure 1). These data can provide valuable insights into future rational design and development of synergistic and/or hybrid agents for potential treatment of prostate cancer.
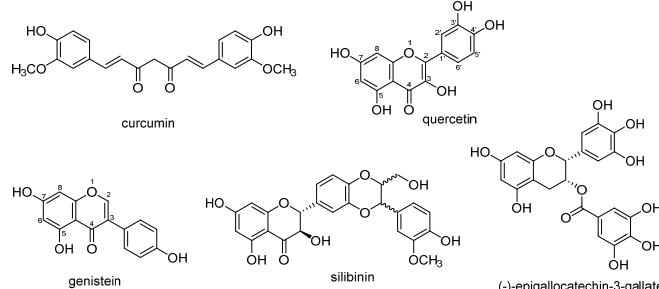
Figure 1: Structures of curcumin, quercetin, genistein, silibinin, and EGCG.

2. Synergistic effects of curcumin as anti-prostate cancer agents

Curcumin (Figure 1), derived from *Curcuma longa* (turmeric) that has long been used for food flavoring and Ayurvedic medicine, represents a dietary natural product possessing potential in preventing and treating prostate cancer [2]. As summarized below, several studies have established the synergistic effects of curcumin in combination with either other natural products or clinical used chemotherapeutics as anti-prostate cancer agents.

2.1 Synergistic effects of curcumin with isoflavones: Genistein (4',5,7-trihydroxyisoflavone, Figure 1) and daidzein (4',7-dihydroxyisoflavone, Figure 2) are two chief isoflavones that were exclusively obtained from soybeans. When they were used for the respective single agent treatment, soybean milk, genistein, and daidzein were capable of inhibiting prostate cancer cell growth *in vitro* and tumor xenograft growth *in vivo* [6].

Combined ingestion of soy isoflavones and curcumin has been unveiled by Ide and co-workers to substantially downregulate serum prostate-specific antigen levels for men who received biopsies in one clinical study [7]. Further investigation by the same research group indicated that the combination treatment of isoflavones and curcumin led to additive suppression of cell proliferation through activation of DNA damage response in LNCaP prostate cancer cells [8]. The synergism was also observed recently between curcumin and genistein in growth suppression of androgen-insensitive human prostate cancer cells [9]. Additionally, combined administration of curcumin and genistein exhibited more noticeable of the capability to inhibit cell proliferation and to induce apoptosis in androgen-insensitive PC-3 human prostate cancer cells than the respective single-agent treatment [10].
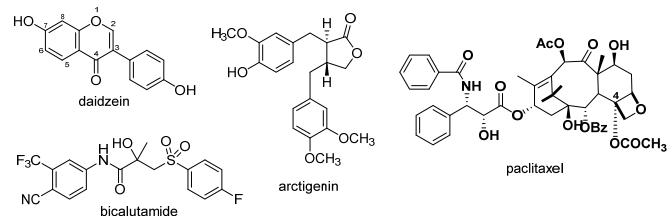
Figure 2: Structures of daidzein, bicalutamide, arctigenin, and paclitaxel.

2.2 Synergistic effects of curcumin with EGCG: EGCG stands for (-)-epigallocatechin-3-gallate (Figure 1) and is the most abundant flavan-3-ol in green tea. EGCG has been shown by various *in vitro* and animal studies to possess potential for preventing and treating prostate cancer [11]. The combination of 5-10 μM curcumin and 40 μM EGCG has been revealed by one study to improve synergistically the *in vitro* antiproliferative effects by 40% in androgen-sensitive LNCaP prostate cancer cells as compared with their individual treatments [12]. The synergism between curcumin and EGCG was linked to enhanced cell apoptosis induction and cell cycle perturbation through upregulation of multiple critical carcinogenesis-involved signaling pathways (e.g. PI3k/Akt and nuclear factor kappa B).

2.3 Synergistic effects of curcumin with arctigenin: Arctigenin (Figure 2) is a plant lignan derived mainly from *Arctium lappa* of the Asteraceae family that is commonly used in traditional Chinese medicine for the treatment of inflammation-associated diseases. The *in vitro* antiproliferative property of arctigenin has been evidenced in several cancer cell lines [12]. The cell proliferation inhibition and cell apoptosis induction of the combination treatment of curcumin and arctigenin towards androgen-sensitive LNCaP human prostate cancer cells was appreciably enhanced as compared with their individual treatment [12]. Additionally, the effect of curcumin and arctigenin in combination and alone on several vital signaling pathways relevant to cell proliferation and apoptosis has been examined. The data showed that combination treatment was capable of enhancing the ratio of Bax (pro-apoptotic protein) to Bcl-2 (anti-apoptotic protein), increasing the phosphorylation suppression of nuclear factor kappa B, and reducing the level of p-IκB.

2.4 Synergistic effects of curcumin with TRAIL: TRAIL, also designated as Apo2L, is a cytokine member of the tumor necrosis factor family and plays as an extracellular signal that activates apoptosis in a variety of cancer cell lines, but exhibits little toxicity to normal cells [13]. In combination with its *in vivo* antitumor efficacy towards human tumor xenografts in nude mice, TRAIL is regarded as an appealing cytokine for the treatment of advanced cancers, such as advanced, metastatic prostate cancer [14]. However, TRAIL alone is not efficient enough for the treatment due to its drug resistance and moderate potency. The combination of TRAIL and dietary natural products with safety profiles might provide an alternative to chemotoxic agents to treat prostate cancer.

Curcumin has been shown in both *in vitro* and *in vivo* studies to sensitize both androgen-sensitive (LNCaP) and androgen-insensitive (PC-3) human prostate cancer cells to TRAIL-induced apoptosis [15-16]. This sensitization was demonstrated by a follow-up study to be associated with downregulation of Akt-regulated nuclear factor-κB (NF-κB) and NF-κB dependent anti-apoptotic proteins, including Bcl-2, Bcl-xL, and XIAP [17]. The in-depth *in vivo* animal experiment verified that curcumin and TRAIL combination appreciably reduced the growth of PC-3 prostate tumor xenografts in Balb/c nude mice by reducing the tumor growth rate to 139% and 147% for TRAIL-treated and curcumin-treated group, respectively, to 59% for the combined treatment group [16].

2.5 Synergistic effects of curcumin with adenoviral mutants: Adenoviruses have been identified as oncolytic virotherapeutic agents that can selectively replicate in and destroy cancer cells, but causing no harm to normal cells. Numerous viral mutants have been established as potential anti-prostate cancer agents, featuring clinical safety profiles. However, the clinical efficacy when administered alone needs to be improved due to its moderate potency. Combination administration of curcumin and tumor selective adenoviral mutant (Ad5) can amplify the cytotoxicity three to eight times towards both virus-sensitive 22Rυ1 and virus-insensitive PC-3 human prostate cancer lines. This suggests that curcumin and Ad5 served as a potential combination of chemotherapy and oncolytic virotherapy [18].

2.6 Synergistic effects of curcumin with bicalutamide: Treatment of androgen-sensitive LNCaP prostate cancer cells with bicalutamide (Figure 2), a nonsteroidal androgen receptor antagonist, at 10 μM for 24 hours resulted in notable suppression of cell proliferation. Significant antiproliferative effects of bicalutamide towards androgen-insensitive human prostate cancer cell lines (PC-3 and DU145) can only be observed at higher concentration and prolonged exposure time (30 μM, 72 hours). Intriguingly, a profound synergy has been observed between curcumin at low doses (10-20 μM) and bicalutamide in both androgen-sensitive and androgen-insensitive prostate cancer cell lines [19]. The potential underlying mechanism was correlated with phosphorylation escalation of extracellular signal-regulated kinase 1/2 (ERK1/2) and stress-activated protein kinase (SAPK)/Jun amino-terminal kinase (JNK).

2.7 Synergistic effects of curcumin with paclitaxel: Curcumin has been demonstrated *in vitro* to considerably potentiate the cytotoxicity of paclitaxel (Figure 2), a naturally occurring microtubule stabilizer and well-known clinically used anticancer drug, towards two androgen-insensitive DU145 and PC-3 human prostate cancer cell lines [20]. Intriguingly, this synergy can be affected by the treatment schedule. Sequential treatment with curcumin followed by paclitaxel yielded more significant synergism than concurrent treatment with curcumin/paclitaxel and sequential treatment with paclitaxel followed by curcumin [21]. The synergism between curcumin and paclitaxel in PC-3 prostate cancer cells was evidenced to be correlated with upregulation of p21WAF1/CIPI expression and downregulation of paclitaxel-induced nuclear factor kappa B.

2.8 Synergistic effects of curcumin with radiotherapy: The limitation of radiotherapy, a frequent therapy for prostate cancer, is radio resistance. Radiotherapy failure has been more often observed in the prostate cancer patients with abnormal p53. Sensitization of curcumin on radiotherapy has been examined in one study [22]. It was concluded that the combination of curcumin and radiation can synergistically reduce cell viability and proliferation relative to either curcumin or radiation alone in both wild type p53-containing LNCaP and mutant p53-containing PC-3 human prostate cancer cell lines. Additionally, a synergistic effect independent of p53 status was also observed between curcumin and radiation towards clonogenic cell death in both LNCaP and PC-3 human prostate cancer cell lines.

3. Synergistic effects of quercetin as anti-prostate cancer agents

Quercetin, 3,3',4',5,7-pentahydroxyflavone (Figure 1), is an ubiquitous flavonol widely occurring in a variety of edible vegetables and fruits. Several *in vitro* cell based evaluations indicate that quercetin processes cytotoxic and antiproliferative effects against a panel of prostate cancer cell lines, but not obvious toxicity towards normal prostate epithelial cells [3a]. The *in vivo* anti-prostate tumor efficacy of quercetin has also been attested to by several animal experiments [23]. Combination treatment of quercetin with either other natural products or well-known chemotherapeutics was believed to be one good strategy to overcome its moderate potency and poor bioavailability.
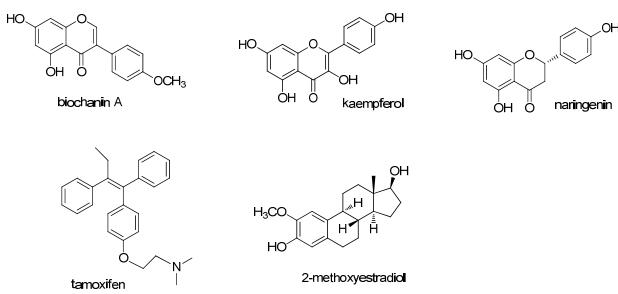
Figure 3: Structures of biochanin A, kaempferol, naringenin, tamoxifen, and 2-methoxyestradiol.

3.1 Synergistic effects of quercetin with genistein: One clinical trial (ClinicalTrials.gov Identifier: NCT01538316) is investigating the effectiveness of the combination of genistein and quercetin in reducing the level of prostate-specific antigen (PSA) in patients with rising PSA.

3.2 Synergistic effects of quercetin with genistein and biochanin A: It is believed that combinations of different subclasses of flavonoids might provide either additive or synergistic activity *in vitro* and *in vivo* when compared with single-flavonoid treatment. Kumar *et al*. systematically investigated the synergistic effects of two-flavonoid and three-flavonoid combinations among quercetin, genistein, and biochanin A (Figure 3) on suppressing cell proliferation of three prostate cancer cell lines (PC-3, DU145, and LNCaP). The findings of this study showed (i) the two-flavonoid combinations at 25 µM (12.5 µM of each flavonoid) only exhibited additive effects in inhibiting prostate cancer cell proliferation; and (ii) the three-flavonoid combination (8.33 µM of quercetin, genistein, and biochanin A, respectively) can synergistically repress cell proliferation in three prostate cancer cell lines [24]. The androgen-insensitive PC-3 and DU145 human prostate cancer cell lines are more responsive to the combination treatments than the androgen-sensitive LNCaP cell line. Both two-flavonoid and three-flavonoid combinations can cause synergistic apoptosis and cell cycle arrest at the G_1 phase of PC-3 prostate cancer cells as evidenced by flow cytometric analysis [24]. Further investigation of the molecular mechanism revealed that the synergistic effects in PC-3 cells are associated with (i) the downregulation of c-myc, PCNA, mitogen activated protein kinase (MAPK)/ERK-1/2 signaling, and PI3K/AKT signaling pathway, and (ii) upregulation of ER-β gene and MAPK/c-Jun *N*-terminal kinase signaling. The synergistic inhibition of cell-cycle in PC-3 cells is correlated with gene expression downregulation of cyclins D1 and E. The synergistic apoptosis contributed by the flavonoid combinations is related to the cooperative upregulation of caspase-3 and pro-apoptotic protein Bax, and to the collaborative downregulation of anti-apoptotic protein Bcl-2 in PC-3 cells [24].

3.3 Synergistic effects of quercetin with green tea catechins: The combination of quercetin with epigallocatechin gallate (EGCG, Figure 1) only exerts additive instead of synergistic effects in controlling proliferation of CWR22Rυ1 and PC-3 prostate cancer cells [25-26]. Intriguingly, a three-flavonoid combination consisting of quercetin, EGCG, and genistein can synergistically inhibit the CWR22Rυ1 prostate cancer cell proliferation [25]. In contrast, androgen-sensitive LNCaP prostate cancer cells are more responsive to the treatment of quercetin combined with EGCG. The combination treatment acts synergistically in inhibiting proliferation, apoptosis, and cell cycle regulation of LNCaP cells [26]. Combination treatment of PC-3 and LNCaP prostate cancer cells with quercetin plus EGCG led to synergistically increased cellular absorption and decreased methylation of both EGCG and

quercetin [26]. Additionally, the combination of quercetin and EGCG synergistically suppresses the self-renewal activities of prostate cancer stem cells, activates apoptosis, and prevents cancer stem cells' migration and invasion [27].

The *in vivo* antitumor efficacy of green tea catechins has been shown to be improved by quercetin. A Phase I clinical study (ClinicalTrials.gov Identifier: NCT01912820) is designated to translate these preclinical results into a clinical setting by assessing the possible bioavailability improvement of quercetin and green tea catechins in combination.

3.4 Synergistic effects of quercetin with kaempferol: The only structural difference between kaempferol (3,4',5,7-tetrahydroxyflavone, Figure 3) and quercetin is that kaempferol lacks the 3'-OH group in quercetin. Kaempferol has been revealed to have antiproliferative effects towards prostate cancer cells with IC_{50} values ranging from 10 to 55 µM [28]. Quercetin and kaempferol have been demonstrated to inhibit synergistically proliferation of androgen-sensitive LNCaP and androgen-insensitive PC-3 human prostate cancer cells [29].

3.5 Synergistic effects of quercetin with naringenin: Naringenin (Figure 3) is a naturally occurring flavonoid belonging to the flavanone subclass, which only exhibits weak antiproliferative effects towards prostate cancer cells [29b]. Its synergistic effect on cell proliferation suppression towards the androgen-sensitive LNCaP prostate cancer cell line has been identified [29a].

3.6 Synergistic effects of quercetin with arctigenin: As mentioned in section 2.3, arctigenin is a naturally occurring lignan with promising antiproliferative activity towards prostate cancer cells. Arctigenin has been demonstrated to exhibit 10- to 20-fold stronger antiproliferative potency than quercetin in both LAPC-4 and LNCaP prostate cancer cell lines. LNCaP cells were more refractory to arctigenin than LAPC-4 cells. The treatment with arctigenin in combination with quercetin synergistically inhibits the cell proliferation in both prostate cancer cell lines. Cell proliferation suppression was enhanced by 30% in both cell lines by a combination treatment with arctigenin (1 µM) and quercetin (20 µM) as compared with the treatments with the individual compounds. The combination index values were in a range of 0.5-0.7 for LAPC-4 cells, and 0.2-0.8 for LNCaP cells [30]. The combination of quercetin plus arctigenin also led to the synergistic phosphorylation inhibition of multiple signaling molecules in LAPC-4 prostate cancer cells as determined by the intracellular signaling array analysis, as well as by Western blot analysis [30]. The synergistic downregulation of androgen receptor and prostate specific antigen in LAPC-4 cells was generated by the combination treatment as examined by qRT-PRC analysis and Western blot analysis. Additionally, the synergistic migration suppression of LAPC-4 and LNCaP prostate cancer cells was noticeably enhanced by the combination treatment [30].

3.7 Synergistic effects of quercetin with tamoxifen: Tamoxifen (Figure 3), a specific antagonist of the estrogen receptor, has been demonstrated to be capable of potentiating the cytotoxic effect of chemotherapeutic agents in prostate cancer cell lines [31]. The *in vivo* synergistic efficacy of combined tamoxifen (10 mg/kg/week) plus quercetin (200 mg/kg/day) has been examined in CWR22 prostate tumor xenografts in severe combined immune deficient (SCID) mice. The results showed that combined tamoxifen with quercetin appreciably prolonged the appearance time of tumors by four days, reduced the final tumor volume by 73%, and lowered the tumor weight at the endpoint by 67%. As compared with the

individual agent treatment, a statistically significant synergism between quercetin and tamoxifen was established. The synergistic antitumor efficacy is linked with the modulation of angiogenesis [32].

3.8 Synergistic effects of quercetin with 2-methoxyestradiol: 2-Methoxyestradiol (Figure 3), an endogenous metabolite of 17β-estradiol, has been revealed to inhibit growth and activate apoptosis of both androgen-sensitive and androgen-insensitive human prostate cancer cells by arresting cell cycle regulation in the G2/M phase [33]. One clinical study identified that 2-methoxyestradiol has a good safety profile for humans, but poor bioavailability as a drug candidate [34]. Wang *et al.* examined the growth inhibitory effects of sixteen combinations of quercetin (5, 10, 20, 40 μM) and 2-methoxyestradiol (0.5, 1, 3, 5 μM) towards androgen-sensitive LNCaP and androgen-insensitive PC-3 human prostate cancer cell lines. Combination index values were in a range of 0.36-2.18 for LNCaP cells, and 0.32-1.77 for PC-3 cells. The combination index values indicated that synergistic effects can only be achieved when combining lower doses of quercetin (5 and 10 μM) with higher doses of 2-methoxyestradiol (3 and 5 μM) for LNCaP cells, and mixing higher doses of quercetin (20 and 40 μM) with higher doses of 2-methoxyestradiol (3 and 5 μM) for PC-3 cells [35]. The combination treatment for 48 hours significantly enhanced the apoptosis induction through regulation of anti-apoptotic Bcl-2 and proapoptotic Bax as compared with two individual treatments [35]. Additionally, the synergistic cell cycle regulation in the G2/M phase was also observed by treating LNCaP and PC-3 cells with quercetin plus 2-methoxyestradiol.

3.9 Synergistic effects of quercetin with TRAIL: Combination treatment of quercetin (100 μM) and the death ligand TRAIL (10-100 ng/mL) for 24 hours generated strong synergistic cytotoxicity and apoptosis induction in androgen-insensitive DU-145 and PC-3 human prostate cancer cell lines, as determined by Annexin V and PI staining assays. The PC-3 cell line was illustrated to be more sensitive to the synergy of quercetin with TRAIL than the DU145 cell line even though these two cell lines are both tumorigenic and independent of wild-type p53. This disparity was assumed to be caused by an inherent dissimilarity in TRAIL sensitivity [36]. Further Western blot analysis displayed that the quercetin-induced sensitization to TRAIL-mediated DU145 cell apoptosis is linked to the upregulation of caspases 3, -8, and -9 [36-38]. Downregulation of survivin (anti-apoptotic protein) gene expression in both DU145 and PC-3 cell lines in an ERK-MSK1 dependent pathway was suggested to be a vital molecular mechanism associated with the apoptosis synergism between quercetin and TRAIL. Upregulation of DR5 (decoy receptor 5) protein expression by quercetin through enhancing transcription and protein stability has also been demonstrated by Jung *et al.* to be associated with its potentiating effect on the apoptosis of prostate cancer cells induced by TRAIL [37]. However, Kim and Lee did not observe any alteration of DR5 in their study when treating prostate cancer cells with quercetin in combination with TRAIL [38]. The combination of quercetin plus TRAIL has been revealed to exhibit a stronger synergistic cytostatic effect than synergistic cytotoxicity. The overriding feature of the combination is that the synergistic apoptosis is triggered by a mixture of an apoptotic "sensitizer" (quercetin) and an apoptotic "inducer" (TRAIL) [36].

2. Synergistic effects of soybean isoflavones as anti-prostate cancer agents

4.1 Synergistic effects of genistein with daidzein: As indicated previously, genistein and daidzein are two isoflavones exclusively isolated from soybeans [6]. The only structural difference between

genistein (Figure 1) and daidzein (Figure 2) is that daidzein lacks the hydroxyl group at C-5 of genistein. Genistein has been elicited to be more potent than daidzein in suppressing cell growth and proliferation of various prostate cancer cells [39]. Interestingly, the combination of daidzein and genistein exhibited greater anti-proliferative and apoptotic effects than the respective single agent treatment towards androgen-sensitive LNCaP and its derived C4-2B human prostate cancer cell lines [40]. It is especially worth noting that an apparent apoptosis induction in C4-2B prostate cancer cells can be caused by the combination of genistein (50 μM) and daidzein (25-50 μM), but not individual genistein or daidzein. The synergism between genistein and daidzein might be contributed by their shared molecular mechanism of actions through modulating cell cycle and angiogenesis related genes and by the multi-target mechanism underlying the anti-proliferative effect of genistein.
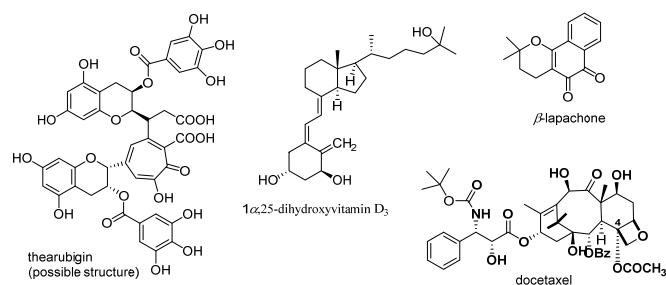
Figure 4: Structures of thearubigin, 1α,25-dihygroxyvitamin D_3, β-lapachone, and docetaxel.

4.2 Synergistic effects of soybean isoflavones with tea: In one *in vivo* animal study carried out by Zhou and co-workers in 2003, it was concluded that prostate tumor progression and metastasis can be synergistically suppressed by soy isoflavones (with genistein as its major chemical component) and tea (both green and black tea) in androgen-sensitive LNCaP human prostate tumor xenografts in severe combined immune deficient (SCID) mice [41]. They observed that (i) treatment of mice with soy phytochemicals and black tea in combination led to a 1.46-times greater effect on tumorigenicity rate than their additive effect; and (ii) combination treatment of soy phytochemicals and tea (both black and green tea) synergistically suppressed tumor metastasis to lymph nodes with ratios of 2.13 and 1.87. The synergistic effects might be linked to effective reduction of both testosterone and DHT in serum.

4.3 Synergistic effects of genistein with thearubigin: Thearubigin (Figure 4) is synonymous with black tea polyphenols, which are transformed from green tea catechins (flavan-3-ols) catalyzed by polyphenol oxidase during tea leaf fermentation [42]. Thearubigin alone (up to 25 μg) neither showed any apparent cell growth inhibition nor DNA distribution change of cell cycle modulation in an androgen-insensitive prostate cancer cell line. However, a combination of thearubigin and genistein (1:40) appreciably enhanced cell growth suppression and cell cycle arrest at the G2/M phase as compared with the genistein-treated group [43]. Additionally, exposure of PC-3 prostate cancer cells to a combination of 2.5 μg/mL of genistein and 0.0625 μg/mL of thearubigin resulted in cell growth suppression by 33%. No cell growth inhibition was observed from either thearubigin-treated (0.0625 μg/mL) or genistein-treated (2.5 μg/mL) groups. This result thus confirmed the synergism between thearubigin and genistein. The molecular mechanism underlying the synergism is still elusive. Sakamoto *et al.* pointed out that genistein, but not thearubigin, can directly regulate the cell cycle. However, thearubigin in conjugation with genistein can synergistically cause cell cycle arrest at the G2/M phase and therefore repress cell growth.

4.4 Synergistic effects of genistein with 1α,25-dihydroxyvitamin D₃: Vitamin D deficiency has been hypothesized to be one of the primary risks for prostate cancer [44]. This hypothesis was not only corroborated by the correlation between the low incidence of prostate cancer and the vitamin D-rich Asian diet, but also confirmed by the antiproliferative effect of 1α,25-dihydroxyvitamin D₃ (the most bioactive metabolite of vitamin D, Figure 4) against androgen-sensitive LNCaP human prostate cancer cells [45]. The inhibition of 1α,25-dihydroxyvitamin D₃ on prostate cancer cell proliferation is linked to the nuclear vitamin D receptor. The soy isoflavone genistein (0.1 to 1 μM) and 1α,25-dihydroxyvitamin D₃ (0.1 to 0.5 nM) have been demonstrated by Cramer and co-workers to possess synergistic effects on suppressing the growth of primary human prostatic epithelial cells and androgen-sensitive LNCaP human prostate cancer cells through cell cycle arrest [46]. The further mechanism exploration from the same research group demonstrated that 1α,25-dihydroxyvitamin D₃ and genistein can act jointly to enhance the stability and subsequently the protein level of vitamin D receptor; and to escalate the levels of cell cycle inhibitor p21 that is essential to the anti-proliferative effects of both genistein and 1α,25-dihydroxyvitamin D₃. Accordingly, modulation of nuclear vitamin D signaling was concluded as one mechanism for the synergism between genistein and 1α,25-dihydroxyvitamin D₃ [47].

4.5 Synergistic effects of genistein with β-lapachone: β-Lapachone (Figure 4) is an abundant naturally occurring quinone and can be readily obtained from *Tabebuia avellanedae*, the lapacho tree native to South America. β-Lapachone has been shown to be a distinct Topo I inhibitor that suppresses the relevant enzymatic activity through directly binding to Topo I. Additionally, β-lapachone has been revealed to be capable of suppressing cell growth and promoting cell apoptosis in both androgen-sensitive and insensitive human prostate cancer cells [48]. The androgen-insensitive PC-3 human prostate cancer cells have been shown to be significantly more responsive to the combined administration of genistein and β-lapachone when compared with the respective single-agent treatment [49]. Additionally, genistein and β-lapachone have been demonstrated to act synergistically to induce apoptosis in the PC-3 cell line.

4.6 Synergistic effects of genistein with sodium selenite: The anti-prostate cancer potential of selenite has been established based on various *in vitro* cell-based assays, *in vivo* animal experiments, and human clinical studies [50]. In search of a combination of anti-prostate cancer agents with different underlying mechanisms, Zhao *et al.* evaluated the synergistic effects of genistein and selenite. The data revealed that the combination of genistein and selenite can synergistically induce cell apoptosis, cause cell cycle arrest at the G₂/M phase, and modulate relevant signaling pathways in both androgen-sensitive LNCaP (p53-positive) and insensitive PC-3 (p53-negative) human prostate cancer cells [50]. The synergism was assumed to be partly due to their distinct molecular mechanisms. For example, selenite was shown to activate cell apoptosis but was not involved in cell cycle modulation. In contrast, genistein can cause both apoptosis activation and cell cycle modulation at the G₂/M phase, which was contributed in part by downregulation of AKT [50].

4.7 Synergistic effects of GCP with docetaxel: GCP, with the isoflavones genistein and daidzein as the main chemical components, stands for a genistein combined polysaccharide that is marketed as a nutritional supplement. GCP with an enhanced absorption rate of genistein in the small intestine is generated from soybean extract catalyzed by mycelia. Docetaxel (Figure 4),

working by mainly promoting the polymerization of tubulin to microtubules and microtubule stabilization, is the current FDA-approved gold standard, first-line treatment for castration-resistant prostate cancer. The limitation of docetaxel's clinical efficacy is that it can barely prolong a maximum 2-3 years of survival for a prostate cancer patient [51]. Even worse, approximately 30,000 US men die each year of castration-resistant prostate cancer due to the inevitable progression of resistance to first-line treatment with docetaxel [52]. To explore the possible advantage of GCP in combination with docetaxel for the treatment of castration-resistant prostate cancer, the *in vitro* antiproliferative and apoptotic effects of the combination of GCP and docetaxel were examined by Burich *et al.* in one androgen-sensitive LNCaP prostate cancer cell line and three androgen-insensitive CWR22Rυ1, PC-3, and LNCaP-R273H prostate cancer cell lines. The results showed that GCP can potentiate docetaxel in inhibiting cell proliferation and promoting cell apoptosis in the four prostate cancer cell lines [53]. However, the degree of synergism between GCP and docetaxel is dependent on the administration schedule. Much greater synergism was observed in the LNCaP cell model when docetaxel rather than GCP was administered first in the sequential treatment.

4.8 Synergistic effects of GCP with bicalutamide: GCP could also synergistically enhance the *in vitro* antiproliferative effects of androgen receptor antagonist bicalutamide (Figure 2) in androgen-dependent LNCaP and androgen-independent LNCaP-R273H prostate cancer cell lines [53]. The LNCaP-R273H model, established from its parental LNCaP cell line by constitutive expression of the p53 gain-of-function mutation R273H, may better represent patient prostate tumor cells after progression to androgen insensitive. Parental LNCaP prostate cancer cells have been demonstrated to be far more responsive than the variant LNCaP-R273H subline to individual treatment with either GCP or bicalutamide. Intriguingly, both prostate cancer cell lines were well-sensitized to the combination of GCP and bicalutamide.

3. Synergistic effects of silibinin as anti-prostate cancer agents

Silymarin is a commercially available herb preparation that is extracted from the seeds of *Silybum marianum* (L.) Gaertn. Seven flavonolignans compose the critical chemical components of silymarin, with silibinin (a 1:1 mixture of diastereomers silybin A and silybin B, Figure 1) as the most abundant one [54]. The medicinal values of silymarin and silibinin as chemotherapeutics to treat hepatotoxicity have been extensively explored, especially in Europe and Asia [55]. Since 1998, the anti-prostate cancer potential of silymarin and silibinin has been evidenced by several *in vitro* cell-based evaluations and *in vivo* animal studies [56]. Their liver protective function, antiproliferative effects, and antitumor efficacy suggest the potential of silymarin and silibinin for clinical use, along with currently established cancer therapies, to improve synergistically the efficacy while alleviating toxicity for prostate cancer patient treatment [57]. Silymarin has already been used by cancer patients to eliminate the hepatotoxicity caused by several chemotherapies [58]. The synergistic effects of silibinin and chemotherapeutic drugs on cell proliferation, cell cycle modulation, and apoptosis activation in various cancer cell lines have been reviewed by Raina and Agarwal [59]. As described in the following subsections, silymarin and silibinin have been reported to be synergized with well-established chemotherapeutic agents including cisplatin, carboplatin, doxorubicin, docetaxel, and mitoxantrone as anti-prostate cancer agents.

5.1 Synergistic effects of silibinin with cisplatin/carboplatin: Cisplatin and carboplatin belong to two platinum complexes that act as cytotoxic chemotherapeutics with molecular mechanism

underlying cytotoxicity similar to that of alkylating agents. Cisplatin [*cis*-diamminedichloroplatinum(II)] is a well-known and commonly used cytotoxic chemotherapy drug that was introduced into clinical trials in 1972. Its capability to form DNA adducts mainly contributes to the cytotoxicity of cisplatin [60]. However, its clinical efficacy is largely compromised by its high toxicity [61]. Carboplatin is another cytotoxic platinum complex with its full name as *cis*-diammine[1,1-cyclobutane-dicarboxylato]platinum. Compared with cisplatin, carboplatin has a better safety profile but no considerable effect towards castration-resistant prostate cancer [62]. Silibinin has been reported to potentiate cisplatin and carboplatin in inhibiting cell proliferation and activating cell apoptosis in the androgen-insensitive DU145 human prostate cancer cells. The synergistic effects on apoptosis were further confirmed by the results that the cleaved levels of PARP and Caspases 3, 7, and 9 were also synergistically upregulated by the combination regimen. The combination of silibinin (50-100 μM) with either cisplatin (2 μg/mL) or carboplatin (20 μg/L) has also been reported to cause synergistic cell cycle arrest at the G_2-M phase and downregulate the relevant proteins of Cdc2, cyclin B1, and Cdc25C. It is worth noting that exposure of DU145 cells to cisplatin, carboplatin, and silibinin alone resulted in cell cycle arrest at G_2-M, S, and G_1 phases, respectively. Both immunocytochemical and Western immunoblotting analyses indicated that combination treatment with silibinin and platinum complex led to a noticeable increase in the levels of cytosolic cytochrome *c* [63].
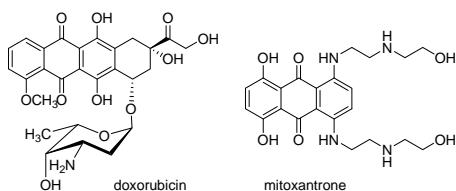
Figure 5: Structures of doxorubicin and mitoxantrone.

5.2 Synergistic effects of silibinin with doxorubicin: Doxorubicin (Figure 5) is a well-known chemotherapeutic for a variety of cancers targeting topoisomerase II; its clinical efficacy, however, is limited by its high systemic toxicity. The synergism between silibinin and doxorubicin has been examined against both androgen-insensitive DU145 and androgen-sensitive LNCaP human prostate cancer cell lines [64]. The results showed that treatment with silibinin (25-100 μM) and doxorubicin (15-25 nM) alone or in combination led to considerable cell growth suppression in a dose-dependent manner in both prostate cancer cell lines. However, DU145 cells are much more sensitive to the synergistic effects of silibinin and doxorubicin than LNCaP cells. It was established that the combination treatment of silibinin (100 μM) and doxorubicin (25 nM) generated a profound synergistic effect on inhibition of DU145 cell growth with combination index values in a range of 0.23-0.59. In contrast, almost no synergistic effect of silibinin (25 μM) and doxorubicin (15 nM) was observed on suppression of LNCaP cell growth with a combination index of 0.929. The perceived synergistic effects on growth suppression of DU145 cells were linked to intense cell cycle perturbation at the G_2-M phase. The combination treatment caused up to 88% cell arrest in the G_2-M phase while silibinin treatment alone caused G_1 arrest, and doxorubicin alone only caused moderate G_2-M arrest (41%). Mechanistically, the combination of silibinin and doxorubicin can synergistically downregulate the G2-M cell cycle related protein expression, including cdc25C, cdc2/p34 and cyclin B1, and cdc2/p34 kinase activity affiliated with histone H1. Additionally, the combination treatment can also act synergistically in promoting DU145 cell apoptosis [65].

5.3 Synergistic effects of silibinin with docetaxel/mitoxantrone: Docetaxel (Figure 4) is a well-established anticancer drug and inhibits cell division mainly through binding to and stabilizing microtubules. Docetaxel represents the first cytotoxic chemotherapeutic that offers survival benefit for patients with castration-resistant prostate cancer. For this reason, the US Food and Drug Administration (FDA) approved docetaxel in combination with predisone in 2004 for the first-line treatment in patients with castration-resistant prostate cancer [66]. However, an average 2.4 years of survival improvement and drug resistance make docetaxel less effective in the clinical use [67]. Chemotherapy with mitoxantrone (Figure 5), an inhibitor of topoisomerase II, has been shown to alleviate symptoms, but not prolong survival rate of patients with advanced castration-resistant prostate cancer [68]. In one study, only additive or even antagonistic effects on cell proliferation inhibition were observed when treating three human prostate cancer cell lines with silibinin in combination with docetaxel, with combination index values of 0.898-2.54 for DU145 cells, 0.921-2.32 for LNCaP cells, and 0.895-4.47 for PC-3 cells [69]. In contrast, the combination treatment of silibinin and mitoxantrone has been demonstrated to have noticeable synergy with combination index values in a range of 0.515–0.929 for DU145 cells, 0.521–0.967 for LNCaP cells, and 0.413–2.65 for PC-3 cells. The only exception is that antagonism was observed in the treatment of PC-3 cells with a low dose of mitoxantrone (e.g. 25 nM) in combined with silibinin [69]. To explore the molecular mechanism underlying the synergy between silibinin and mitoxantrone, the levels of caspase-3 and caspase-7 were evaluated. The results revealed that the combination treatment synergistically and significantly induced the apoptosis of three prostate cancer cell lines.

4. Synergistic effects of green tea catechins as anti-prostate cancer agents

Green tea crude extracts have been designated as green tea catechins that consist of a group of naturally occurring flavan-3-ols, with (-)-epigallocatechin-3-gallate (EGCG) as the most abundant one. Green tea catechins and EGCG have been demonstrated by several *in vitro* cell-based assays and *in vivo* animal studies to have potential in preventing and treating prostate cancer [11]. Several studies as shown in the following subsections have suggested that either green tea catechins or EGCG might be even more effective as anti-prostate agents when they synergistically act with other natural or non-natural compounds. The synergistic apoptosis induced by combinations of EGCG with anti-cancer drugs in various cancer cells (including prostate cancer cells) has been systemically reviewed [70].
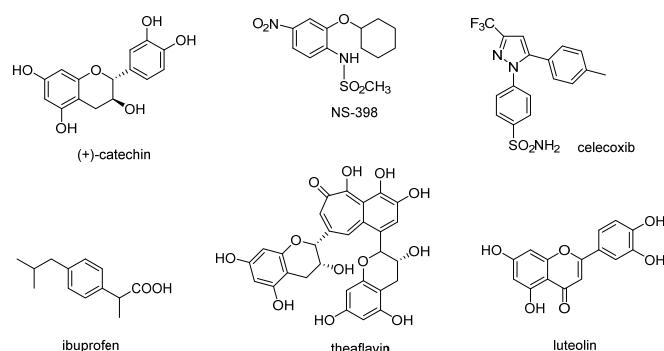
Figure 6: Structures of catechin, NS-398, celecoxib, ibuprofen, theaflavin, and luteolin.

6.1 Synergistic effects of EGCG with selective COX-2 inhibitors [71]: Cyclooxygenase (COX) is a critical enzyme that catalyzes the

transformation of arachidonic acid to prostaglandins. Highly expressed in prostate cancer tissue, prostaglandins are believed to serve as an essential role in the cell proliferation of prostate cancer. COX-2 is an inductive isoform and has been revealed to affect the proliferation and apoptosis of prostate cancer cells [72]. Consequently, COX-2 inhibitors have been proposed to be useful for potential chemotherapy for prostate cancer. (+)-Catechin (Figure 6) is the simplest flava-3-ol derived from green tea, which showed certain cell proliferation suppression at a high concentration (100 μM) toward LNCaP and DU145 (but not PC-3) prostate cancer cell lines. Zaslau and co-workers reported that catechin and NS398 (a selective COX-2 inhibitor, Figure 6) would act synergistically to inhibit prostate cancer cell proliferation [72a]. Adhami and co-workers also explored the combinatorial effect of (-)-epigallocateocatechin-3-gallate (EGCG) and selective COX-2 inhibitors (NS-398 and celecoxib, Figure 6) in both *in vitro* human prostate cancer cell models and *in vivo* prostate cancer CWR22Rv1 xenografts in athymic nude mice [71]. The *in vitro* synergism between EGCG and NS-398 was established because the combination treatment enhanced cell growth suppression and apoptosis activation, which was also supported by increased expression of Bax, pro-caspase-6, and pro-caspase-9, and poly (ADP)ribose polymerase cleavage. Additionally, the suppression of proliferator activated receptor γ and nuclear factor-κB were also enhanced as compared with their additive effects. Combination administration of EGCG and celecoxib in *in vivo* tumor xenografts led to improved anti-tumor efficacy, and decreased PSA levels and insulin-like growth factor-1 levels.

6.2 Synergistic effects of EGCG with ibuprofen: Ibuprofen (Figure 6) is a well-established non-steroidal anti-inflammatory drug (NASID) that functions *via* inhibiting cyclooxygenase (COX) enzymes. Administration of ibuprofen was associated with low incidence of prostate cancer probably due to its ability to suppress COX enzymes [73]. The *in vitro* study has revealed that ibuprofen can inhibit cell proliferation and activate cell apoptosis in LNCaP prostate cancer cells [74]. The synergism between EGCG and ibuprofen has been established by *in vitro* cell-based assays, in which they can act synergistically in inhibiting cell proliferation and inducing cell apoptosis in both androgen-insensitive and sensitive (PC-3, DU145, and LNCaP) prostate cancer cell lines [75]. The further underlying mechanism investigation indicated that combined treatment of PC-3 cells can synergistically upregulate protein phosphatase 1 activity and subsequently activate alternative splicing of anti-apoptotic proteins Bcl-X and Mcl-1 [75b].

6.3 Synergistic effects of green tea catechins with black tea theaflavin: As mentioned previously, EGCG represents the most copious flavan-3-ol in green tea while theaflavin (Figure 6) is the major flavonoid polymer in black tea. Interestingly, EGCG and theaflavin can function synergistically to decrease the growth rate of prostate cancer as compared with either EGCG or theaflavin alone. This synergism may be caused by functionally supplemented mechanisms of EGCG and theaflavin [76].

6.4 Synergistic effects of green tea catechins with luteolin: It has been demonstrated that prostate cancer progression can be driven by the existence of myofibroblasts and that EGCG may suppress the myofibroblast differentiation relevant to prostate cancer [77]. Luteolin, 3',4',5,7-tetrahydroxyflavone (Figure 6), is another ordinary flavone and can be readily obtained from a variety of vegetables and fruits, as well as traditional Chinese medicines [78]. Several *in vitro* and *in vivo* studies have shown its potential in preventing and treating prostate cancer [79]. Luteolin can potentiate EGCG to upregulate fibronectin expression in prostate fibroblast

cell lines [77]. Consequently, myofibroblast phenotypes and extracellular matrix contraction (a promoter of cancer cell invasion) mediated by TGF-β can be synergistically suppressed by combinations of EGCG and luteolin at micromolar concentrations. Mechanistically, EGCG and luteolin can individually downregulate ERK and Rho that are downstream signalings induced by TGF-β and are essential for fibronectin expression [77]. Additionally, EGCG and luteolin have been shown to reverse synergistically fibronectin expression induced by TGF-β. These findings suggested that the combination of EGCG and luteolin might be clinically useful in preventing and reversing prostate cancer progression *via* targeting the tumor microenvironment rather than the tumor itself.

6.5 Synergistic effects of EGCG with TRAIL: TRAIL is known to inhibit cell proliferation in PC-3 instead of LNCaP prostate cancer cells. The synergism between EGCG and TRAIL has been identified according to the following experimental findings: (i) combination treatment of EGCG and TRAIL enhanced antiproliferative effects towards TRAIL refractory LNCaP cells as compared with the single-agent treatments; (ii) the combination treatment led to escalated apoptosis of LNCaP cells relative to effects caused by the single treatments; and (iii) the combination treatment resulted in a synergistic suppression of the invasion and migration of LNCaP cells [80]. The improved apoptosis contributed by the combined treatment is linked to both intrinsic and extrinsic pathways through several molecular mechanisms. For example, the apoptosis was found to be accompanied by the increased poly(ADP-ribose)polymerase cleavage and pro-apoptotic proteins, together with reduced anti-apoptotic proteins.

6.6 Synergistic effects of EGCG with taxanes: The combination of ECGC and either paclitaxel (Figure 2) or docetaxel (Figure 4) generated synergistic efficacy *in vitro* and *in vivo* [81]. The *in vitro* cell-based studies have unveiled that EGCG (30 μM) in combination with either paclitaxel (6.25 nM) or docetaxel (3.12 nM) exhibited synergistic effects in inhibiting proliferation and activating apoptosis of PC-3ML human prostate cancer cells. The *in vivo* animal studies indicated that the synergistic effects in suppressing PC-3ML tumor xenograft growth were generated for the combined treatment with EGCG and docetaxel in severe combined immunodeficient mice. In addition to attenuating the preexisting PC-3ML tumor xenografts, the combined treatment increased disease-free survival rates to greater than 90%. EGCG combined with either paclitaxel or docetaxel through intraperitoneal injection (i.p.) promoted a considerable enhancement in apoptosis rates, as determined by TUNEL assays. The *in vitro* cell-based assays and *in vivo* mechanism studies in mice have also revealed that EGCG and either paclitaxel or docetaxel can act synergistically to upregulate certain apoptotic genes [81].

6.7 Synergistic effects of EGCG with doxorubicin: Doxorubicin is a well-known chemotherapeutic for a variety of cancers. Low doses of doxorubicin have been demonstrated by *in vitro* studies to act synergistically with EGCG in inhibiting cell proliferation and colony forming of PC-3ML human prostate cancer cells. The synergism between EGCG and doxorubicin was also verified by *in vivo* animal studies in mice implanted with advanced, metastatic PC-3ML human prostate cancer cells. The data showed that the combined treatment led to synergistic suppression of tumor xenograft growth, elimination of tumors, and enhancement of mouse survival rates [82]. Additionally, EGCG was also demonstrated to escalate doxorubicin retention by the prostate cancer cells *in vitro* and by the metastatic tumor xenografts *in vivo*.

6.8 Synergistic effects of EGCG with cisplatin: As described in section 5.1, cisplatin is a well-known and commonly used cytotoxic chemotherapy drug that processes promising efficacy but high toxicity. EGCG (25 μM) has been evidenced to induce synergistically apoptosis of PC-3 prostate cancer cells in combination with cisplatin (2.5 μM) as compared with the effects caused by treating the individual agent alone [83].

6.9 Synergistic effects of EGCG with free Zn^{2+}: Zinc is an essential trace element. The elevated level of intracellular zinc has been demonstrated to be linked with decreased proliferation and increased apoptosis of prostate cancer cells [84]. The antiproliferative activities of Zn^{2+}/EGCG mixture and zinc-EGCG complex were examined on an androgen-insensitive PC-3 human prostate cancer cell line. It was revealed that free Zn^{2+} can potentiate the antiproliferative effect of EGCG on PC-3 cells [85].

5. Conclusion

These data can provide valuable insights into future design and development of multi-targeting and synergistic agents for potential treatment of prostate cancer. However, most currently available data are only derived from *in vitro* evaluations. The molecular mechanism of the underlying synergism is still elusive. The in-depth mechanism investigation of the synergism of dietary natural products and the rational application of the synergism to the design of hybrid molecules would thus be a good future direction for the development of potential anti-prostate cancer agents.

References

[1]   (a) Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, Mack TM. (**1991**) Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. *British Journal of Cancer*, *63*, 963-966; (b) Mills PK, Beeson WL, Phillips RL, Fraser GE. (**1989**) Cohort study of diet, lifestyle, and prostate cancer in Adventist men. *Cancer*, *64*, 598-604.

[2]   Chen Q-H. (**2015**) Curcumin-based anti-prostate cancer agents. *Anti-Cancer Agents in Medicinal Chemistry*, *15*, 138-156.

[3]   (a) Aalinkeel R, Bindukumar B, Reynolds JL, Sykes DE, Mahajan SD, Chadha KC, Schwartz SA. (**2008**) The dietary bioflavonoid, quercetin, selectively induces apoptosis of prostate cancer cells by down-regulating the expression of heat shock protein 90. *Prostate (Hoboken, NJ, United States)*, *68*, 1773-1789; (b) Singh RP, Raina K, Sharma G, Agarwal R. (**2008**) Silibinin inhibits established prostate tumor growth, progression, invasion, and metastasis and suppresses tumor angiogenesis and epithelial-mesenchymal transition in transgenic adenocarcinoma of the mouse prostate model mice. *Clinical Cancer Research*, *14*, 7773-7780.

[4]   Prasad S, Tyagi AK, Aggarwal BB. (**2014**) Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment golden spice. *Cancer Research and Treatment*, *46*, 2-18.

[5]   Ali BH, Marrif H, Noureldayem SA, Bakheit AO, Blunden G. (**2006**) Some biological properties of curcumin: a review. *Natural Product Communications*, *1*, 509-521.

[6]   van Die MD, Bone KM, Williams SG, Pirotta MV. (**2014**) Soy and soy isoflavones in prostate cancer: a systematic review and meta-analysis of randomized controlled trials. *BJU International*, *113*, E119-E130.

[7]   Ide H, Tokiwa S, Sakamaki K, Nishio K, Isotani S, Muto S, Hama T, Masuda H, Horie S. (**2010**) Combined inhibitory effects of soy isoflavones and curcumin on the production of prostate-specific antigen. *Prostate (Hoboken, NJ, United States)*, *70*, 1127-1133.

[8]   Ide H, Yu J, Lu Y, China T, Kumamoto T, Koseki T, Muto S, Horie S. (**2011**) Testosterone augments polyphenol-induced DNA damage response in prostate cancer cell line, LNCaP. *Cancer Science*, *102*, 468-471.

[9]   Aditya NP, Shim M, Lee I, Lee Y, Im M-H, Ko S. (**2013**) Curcumin and genistein coloaded nanostructured lipid carriers: *in vitro* digestion and antiprostate cancer activity. *Journal of Agricultural and Food Chemistry*, *61*, 1878-1883.

[10]   Aditya NP, Shim M, Yang H, Lee Y, Ko S. (**2014**) Antiangiogenic effect of combined treatment with curcumin and genistein on human prostate cancer cell line. *Journal of Functional Foods*, *8*, 204-213.

[11]   Henning SM, Wang P, Heber D. (**2011**) Chemopreventive effects of tea in prostate cancer: Green tea versus black tea. *Molecular Nutrition & Food Research*, *55*, 905-920.

[12]   Wang P, Wang B, Chung S, Wu Y, Henning SM, Vadgama JV. (**2014**) Increased chemopreventive effect by combining arctigenin, green tea polyphenol and curcumin in prostate and breast cancer cells. *RSC Advances*, *4*, 35242-35250.

[13]   Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. (**1996**) Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *Journal of Biological Chemistry*, *271*, 12687-12690.

[14]   Ashkenazi A, Dixit VM. (**1999**) Apoptosis control by death and decoy receptors. *Current Opinion in Cell Biology*, *11*, 255-260.

[15]   (a) Deeb D, Xu YX, Jiang H, Gao X, Janakiraman N, Chapman RA, Gautam SC. (**2003**) Curcumin (diferuloyl-methane) enhances tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in LNCaP prostate cancer cells. *Molecular Cancer Therapeutics*, *2*, 95-103; (b) Deeb DD, Jiang H, Gao X, Divine G, Dulchavsky SA, Gautam SC. (**2005**) Chemosensitization of hormone-refractory prostate cancer cells by curcumin to TRAIL-induced apoptosis. *Journal of Experimental Therapeutics and Oncology*, *5*, 81-91.

[16]   Andrzejewski T, Deeb D, Gao X, Danyluk A, Arbab Ali S, Dulchavsky Scott A, Gautam Subhash C. (**2008**) Therapeutic efficacy of curcumin/TRAIL combination regimen for hormone-refractory prostate cancer. *Oncology Research*, *17*, 257-267.

[17]   Deeb D, Jiang H, Gao X, Al-Holou S, Danyluk AL, Dulchavsky SA, Gautam SC. (**2007**) Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1-6-heptadine-3,5-dione; C21H20O6] sensitizes human prostate cancer cells to tumor necrosis factor-related apoptosis-inducing ligand/Apo2L-induced apoptosis by suppressing nuclear factor-κB via inhibition of the prosurvival Akt signaling pathway. *Journal of Pharmacology and Experimental Therapeutics*, *321*, 616-625.

[18]   Adam V, Ekblad M, Sweeney K, Mueller H, Busch KH, Johnsen CT, Kang NR, Lemoine NR, Hallden G. (**2012**) Synergistic and selective cancer cell killing mediated by the oncolytic adenoviral mutant AdΔΔ and dietary phytochemicals in prostate cancer models. *Human Gene Therapy*, *23*, 1003-1015.

[19]   Li J, Xiang ST, Zhang QH, Wu JJ, Tang Q, Zhou JF, Yang LJ, Chen ZQ, Hann SS. (**2015**) Combination of curcumin and bicalutamide enhanced the growth inhibition of androgen-independent prostate cancer cells through SAPK/JNK and MEK/ERK1/2-mediated targeting NF-κB/p65 and MUC1-C. *Journal of Experimental & Clinical Cancer Research*, *34*, 1-21.

[20]   Hour T-C, Chen J, Huang C-Y, Guan J-Y, Lu S-H, Pu Y-S. (**2002**) Curcumin enhances cytotoxicity of chemotherapeutic agents in prostate cancer cells by inducing p21WAF1/CIP1 and C/EBPβ expressions and suppressing NF-κB activation. *Prostate (New York, NY, United States)*, *51*, 211-218.

[21]   Cabrespine-Faugeras A, Bayet-Robert M, Bay J-O, Chollet P, Barthomeuf C. (**2010**) Possible benefits of curcumin regimen in combination with taxane chemotherapy for hormone-refractory prostate cancer treatment. *Nutrition and Cancer*, *62*, 148-153.

[22]   Nayak BK, Krishnegowda NK, Galindo CA, Meltz ML, Swanson GP. (**2010**) Synergistic effect between curcumin (diferuloylmethane) and radiation on clonogenic cell death independent of p53 in prostate cancer cells. *Journal of Cancer Science & Therapy*, *2*, 171-181.

[23]   (a) Firdous A, Sharmila G, Balakrishnan S, Raja Singh P, Suganya S, Srinivasan N, Arunakaran J. (**2014**) Quercetin, a natural dietary flavonoid, acts as a chemopreventive agent against prostate cancer in an *in vivo* model by inhibiting the EGFR signaling pathway. *Food & Function*, *5*, 2632-2645; (b) Poyil P, Budhraja A, Son Y-O, Wang X, Zhang Z, Ding S, Wang L, Hitron A, Lee J-C, Xu M, Chen G, Luo J, Shi X. (**2012**) Quercetin inhibits angiogenesis mediated human prostate tumor growth by targeting VEGFR-2 regulated AKT/mTOR/P70S6K signaling pathways. *PLoS One*, *7*, e47516; (c) Wu K, Ning Z, Zhou J, Wang B, Fan J, Zhu J, Gao Y, Wang X, Hsieh J-T, He D. (**2014**) 2'-Hydroxyflavanone inhibits prostate tumor growth through inactivation of AKT/STAT3 signaling and induction of cell apoptosis. *Oncology reports*, *32*, 131-138.

[24]   Kumar R, Verma V, Jain A, Jain RK, Maikhuri JP, Gupta G. (**2011**) Synergistic chemoprotective mechanisms of dietary phytoestrogens in a select combination against prostate cancer. *Journal of Nutritional Biochemistry*, *22*, 723-731.

[25]   Hsieh T-C, Wu JM. (**2009**) Targeting CWR22Rv1 prostate cancer cell proliferation and gene expression by combinations of the phytochemicals EGCG, genistein and quercetin. *Anticancer Research*, *29*, 4025-4032.

[26]   Wang P, Heber D, Henning SM. (**2012**) Quercetin increased the antiproliferative activity of green tea polyphenol (-)-epigallocatechin gallate in prostate cancer cells. *Nutrition and Cancer*, *64*, 580-587.

[27]   Tang S-N, Singh C, Nall D, Meagher D, Shankar S, Srivastava RK. (**2010**) The dietary bioflavonoid quercetin synergizes with epigallocathechin gallate (EGCG) to inhibit prostate cancer stem cell characteristics, invasion, migration and epithelial-mesenchymal transition. *Journal of Molecular Signaling*, *5*, 14.

[28]   (a) Haddad AQ, Venkateswaran V, Viswanathan L, Teahan SJ, Fleshner NE, Klotz LH. (**2006**) Novel antiproliferative flavonoids induce cell cycle arrest in human prostate cancer cell lines. *Prostate cancer and prostatic diseases*, *9*, 68-76; (b) Tsimplouli C, Demetzos C, Hadzopoulou-Cladaras M, Pantazis P, Dimas K. (**2012**) *In vitro* activity of dietary flavonol congeners against human cancer cell lines. *European Journal of Nutrition*, *51*, 181-190.

[29]   (a) Campbell JK, King JL, Harmston M, Lila MA, Erdman JW. (**2006**) Synergistic effects of flavonoids on cell proliferation in Hepa-1c1c7 and LNCaP cancer cell lines. *Journal of Food Science*, *71*, S358-S363; (b) Knowles LM, Zigrossi DA, Tauber RA, Hightower C, Milner JA. (**2000**) Flavonoids suppress androgen-independent human prostate tumor proliferation. *Nutrition and Cancer*, *38*, 116-122.

[30]   Wang P, Phan T, Gordon D, Chung S, Henning SM, Vadgama JV. (**2015**) Arctigenin in combination with quercetin synergistically enhances the antiproliferative effect in prostate cancer cells. *Molecular Nutrition & Food Research*, *59*, 250-261.

[31]   Lin C-C, Hsu C-H, Chen J, Tsai T-C, Cheng A-L, Pu Y-S. (**2001**) A pilot study of AFL-T (doxorubicin, 5-fluorouracil, leucovorin, and tamoxifen) combination chemotherapy for hormone-refractory prostate cancer. *Anticancer Research*, *21*, 1385-1390.

[32]   Ma Z, Nguyen TH, Huynh TH, Do PT, Huynh H. (**2004**) Reduction of CWR22 prostate tumor xenograft growth by combined tamoxifen-quercetin treatment is associated with inhibition of angiogenesis and cellular proliferation. *International Journal of Oncology*, *24*, 1297-1304.

[33]   Kumar AP, Garcia GE, Slaga TJ. (**2001**) 2-Methoxyestradiol blocks cell-cycle progression at G2/M phase and inhibits growth of human prostate cancer cells. *Molecular Carcinogenesis*, *31*, 111-124.

[34]   Sweeney C, Liu G, Yiannoutsos C, Kolesar J, Horvath D, Staab MJ, Fife K, Armstrong V, Treston A, Sidor C, Wilding G. (**2005**) A phase II multicenter, randomized, double-blind, safety trial assessing the pharmacokinetics, pharmacodynamics, and efficacy of oral 2-methoxyestradiol capsules in hormone-refractory prostate cancer. *Clinical Cancer Research*, *11*, 6625-6633.

[35]   Wang G, Song L, Wang H, Xing N. (**2013**) Quercetin synergizes with 2-methoxyestradiol inhibiting cell growth and inducing apoptosis in human prostate cancer cells. *Oncology Reports*, *30*, 357-63.

[36]   Kim YH, Lee DH, Jeong JH, Guo ZS, Lee YJ. (**2008**) Quercetin augments TRAIL-induced apoptotic death: involvement of the ERK signal transduction pathway. *Biochemical Pharmacology*, *75*, 1946-1958.

[37]   Jung YH, Heo J, Lee YJ, Kwon TK, Kim YH. (**2010**) Quercetin enhances TRAIL-induced apoptosis in prostate cancer cells via increased protein stability of death receptor 5. *Life Sciences*, *86*, 351-357.

[38]   Kim YH, Lee YJ. (**2007**) TRAIL apoptosis is enhanced by quercetin through Akt dephosphorylation. *Journal of Cellular Biochemistry*, *100*, 998-1009.

[39]   Xiong P, Wang R, Zhang X, DeLa Torre E, Leon F, Zhang Q, Zheng S, Wang G, Chen Q-H. (**2015**) Design, synthesis, and evaluation of genistein analogues as anti-cancer agents. *Anti-Cancer Agents in Medicinal Chemistry*, *15*, 1197-1203.

[40]   Dong X, Xu W, Sikes RA, Wu C. (**2013**) Combination of low dose of genistein and daidzein has synergistic preventive effects on isogenic human prostate cancer cells when compared with individual soy isoflavone. *Food Chemistry*, *141*, 1923-1933.

[41]   Zhou J-R, Yu L, Zhong Y, Blackburn GL. (**2003**) Soy phytochemicals and tea bioactive components synergistically inhibit androgen-sensitive human prostate tumors in mice. *Journal of Nutrition*, *133*, 516-521.

[42]   Haslam E. (**2003**) Thoughts on thearubigins. *Phytochemistry*, *64*, 61-73.

[43]   Sakamoto K. (**2000**) Synergistic effects of thearubigin and genistein on human prostate tumor cell (PC-3) growth via cell cycle arrest. *Cancer Letters (Shannon, Ireland)*, *151*, 103-109.

[44]   Schwartz GG, Hulka BS. (**1990**) Is vitamin D deficiency a risk factor for prostate cancer? (Hypothesis). *Anticancer Research*, *10*, 1307-1311.

[45]   Zhuang S-H, Burnstein KL. (**1998**) Antiproliferative effect of 1α,25-dihydroxyvitamin D_3 in human prostate cancer cell line LNCaP involves reduction of cyclin-dependent kinase 2 activity and persistent G1 accumulation. *Endocrinology*, *139*, 1197-1207.

[46]   Rao A, Woodruff RD, Wade WN, Kute TE, Cramer SD. (**2002**) Genistein and vitamin D synergistically inhibit human prostatic epithelial cell growth. *Journal of Nutrition*, *132*, 3191-3194.

[47]   Rao A, Coan A, Welsh J-E, Barclay WW, Koumenis C, Cramer SD. (**2004**) Vitamin D receptor and p21/WAF1 are targets of genistein and 1,25-dihydroxyvitamin D_3 in human prostate cancer cells. *Cancer Research*, *64*, 2143-2147.

[48]   Planchon SM, Wuerzberger S, Frydman B, Witiak DT, Hutson P, Church DR, Wilding G, Boothman DA. (**1995**) β-Lapachone-mediated apoptosis in human promyelocytic leukemia (HL-60) and human prostate cancer cells: a p53-independent response. *Cancer Research*, **55**, 3706-3711.

[49]   Kumi-Diaka J, Saddler-Shawnette S, Aller A, Brown J. (**2004**) Potential mechanism of phytochemical-induced apoptosis in human prostate adenocarcinoma cells: Therapeutic synergy in genistein and β-lapachone combination treatment. *Cancer Cell International*, *4*, 5.

[50]   Zhao R, Xiang N, Domann FE, Zhong W. (**2009**) Effects of selenite and genistein on G2/M cell cycle arrest and apoptosis in human prostate cancer cells. *Nutrition and Cancer*, *61*, 397-407.

[51]   (a) Corcoran C, Rani S, O'Brien K, O'Neill A, Prencipe M, Sheikh R, Webb G, McDermott R, Watson W, Crown J, O'Driscoll L. (**2012**) Docetaxel-resistance in prostate cancer: evaluating associated phenotypic changes and potential for resistance transfer via exosomes. *PLoS One*, *7*, e50999; (b) Yap TA, Zivi A, Omlin A, de Bono JS. (**2011**) The changing therapeutic landscape of castration-resistant prostate cancer. *Nature Reviews Clinical Oncology*, *8*, 597-610.

[52]   Torre Lindsey A, Bray F, Siegel Rebecca L, Ferlay J, Lortet-Tieulent J, Jemal A. (**2015**) Global cancer statistics, 2012. *CA: A Cancer Journal for Clinicians*, *65*, 87-108.

[53]   Burich RA, Holland WS, Vinall RL, Tepper C, de Vere White RW, Mack PC. (**2008**) Genistein combined polysaccharide enhances activity of docetaxel, bicalutamide and Src kinase inhibition in androgen-dependent and independent prostate cancer cell lines. *BJU International*, *102*, 1458-1466.

[54]   Kim N-C, Graf TN, Sparacino CM, Wani MC, Wall ME. (**2003**) Complete isolation and characterization of silybins and isosilybins from milk thistle (*Silybum marianum*). *Organic & Biomolecular Chemistry*, *1*, 1684-1689.

[55]   Flora K, Hahn M, Rosen H, Benner K. (**1998**) Milk thistle (*Silybum marianum*) for the therapy of liver disease. *American Journal of Gastroenterology*, *93*, 139-143.

[56]   Zi X, Agarwal R. (**1999**) Silibinin decreases prostate-specific antigen with cell growth inhibition via G1 arrest, leading to differentiation of prostate carcinoma cells: implications for prostate cancer intervention. *Proceedings of the National Academy of Sciences of the United States of America*, *96*, 7490-7495.

[57]   Comelli MC, Mengs U, Schneider C, Prosdocimi M. (**2007**) Toward the definition of the mechanism of action of silymarin: activities related to cellular protection from toxic damage induced by chemotherapy. *Integrative Cancer Therapies*, *6*, 120-129.

[58]   Werneke U, Earl J, Seydel C, Horn O, Crichton P, Fannon D. (**2004**) Potential health risks of complementary alternative medicines in cancer patients. *British Journal of Cancer*, *90*, 408-413.

[59]   Raina K, Agarwal R. (**2007**) Combinatorial strategies for cancer eradication by silibinin and cytotoxic agents: efficacy and mechanisms. *Acta pharmacologica Sinica*, *28*, 1466-1475.

[60]   Bellon SF, Coleman JH, Lippard SJ. (**1991**) DNA unwinding produced by site-specific intrastrand crosslinks of the antitumor drug cis-diamminedichloroplatinum(II). *Biochemistry*, *30*, 8026-8035.

[61]   Drelichman A, Oldford J, Al-Sarraf M. (**1985**) Evaluation of cyclophosphamide, adriamycin, and cis-platinum (CAP) in patients with disseminated prostatic carcinoma. A phase II study. *American Journal of Clinical Oncology*, *8*, 255-259.

[62]   Trump DL, Marsh JC, Kvols LK, Citrin D, Davis TE, Hahn RG, Vogl SE. (**1990**) A phase II trial of carboplatin (NSC 241240) in advanced prostate cancer, refractory to hormonal therapy. An Eastern Cooperative Oncology Group pilot study. *Investigational New Drugs*, *8 Suppl 1*, S91-S94.

[63]   Dhanalakshmi S, Agarwal P, Glode LM, Agarwal R. (**2003**) Silibinin sensitizes human prostate carcinoma DU145 cells to cisplatin- and carboplatin-induced growth inhibition and apoptotic death. *International Journal of Cancer*, *106*, 699-705.

[64]   Tyagi AK, Singh RP, Agarwal C, Chan DCF, Agarwal R, (**2002**) Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth inhibition, G2-M arrest, and apoptosis. *Clinical Cancer Research*, *8*, 35123519.

[65]   Singh RP, Agarwal R. (**2004**) Prostate cancer prevention by silibinin. *Current Cancer Drug Targets*, *4*, 1-11.

[66]   McKeage K. (**2012**) Docetaxel: a review of its use for the first-line treatment of advanced castration-resistant prostate cancer. *Drugs*, *72*, 1559-1577.

[67]   Bahl A, Masson S, Birtle A, Chowdhury S, de Bono J. (**2014**) Second-line treatment options in metastatic castration-resistant prostate cancer: A comparison of key trials with recently approved agents. *Cancer Treatment Reviews*, *40*, 170-177.

[68]   Gravis G, Salem N, Bladou F, Viens P. (**2007**) Prostate cancer and chemotherapy. *Bulletin du Cancer*, *94* (Suppl. FMC, 6), F21-F28.

[69]   Flaig TW, Su LJ, Harrison G, Agarwal R, Glode LM. (**2007**) Silibinin synergizes with mitoxantrone to inhibit cell growth and induce apoptosis in human prostate cancer cells. *International Journal of Cancer*, *120*, 2028-2033.

[70]   Fujiki H, Suganuma M. (**2012**) Green tea: An effective synergist with anticancer drugs for tertiary cancer prevention. *Cancer Letters (New York, NY, United States)*, *324*, 119-125.

[71]   Adhami VM, Malik A, Zaman N, Sarfaraz S, Siddiqui IA, Syed DN, Afaq F, Pasha FS, Saleem M, Mukhtar H. (**2007**) Combined inhibitory effects of green tea polyphenols and selective cyclooxygenase-2 inhibitors on the growth of human prostate cancer cells both *in vitro* and *in vivo*. *Clinical Cancer Research*, *13*, 1611-1619.

[72]   (a) Farivar-Mohseni H, Kandzari SJ, Zaslau S, Riggs DR, Jackson BJ, McFadden DW. (**2004**) Synergistic effects of Cox-1 and -2 inhibition on bladder and prostate cancer *in vitro*. *American Journal of Surgery*, *188*, 505-510; (b) Kamijo T, Sato T, Nagatomi Y, Kitamura T. (**2001**) Induction of apoptosis by cyclooxygenase-2 inhibitors in prostate cancer cell lines. *International Journal of Urology*, *8*, S35-S39.

[73]   Platz EA, Rohrmann S, Pearson JD, Corrada MM, Watson DJ, De Marzo AM, Landis PK, Metter EJ, Carter HB. (**2005**) Nonsteroidal anti-inflammatory drugs and risk of prostate cancer in the Baltimore longitudinal study of aging. *Cancer Epidemiology, Biomarkers & Prevention*, *14*, 390-396.

[74]   Andrews J, Djakiew D, Krygier S, Andrews P. (**2002**) Superior effectiveness of ibuprofen compared with other NSAIDs for reducing the survival of human prostate cancer cells. *Cancer Chemotherapy and Pharmacology*, *50*, 277-284.

[75]   (a) Kim MH, Chung J. (**2007**) Synergistic cell death by EGCG and ibuprofen in DU-145 prostate cancer cell line. *Anticancer Research*, *27*, 3947-3956; (b) Kim MH. (**2008**) Protein phosphatase 1 activation and alternative splicing of Bcl-X and Mcl-1 by EGCG + ibuprofen. *Journal pf Cellular Biochemistry*, *104*, 1491-1499.

[76]   Kobalka Andrew J, Keck Rick W, Jankun J. (**2015**) Synergistic anticancer activity of biologicals from green and black tea on DU 145 human prostate cancer cells. *Central-European Journal of Immunology*, *40*, 1-4.

[77]   Gray AL, Stephens CA, Bigelow RL, Coleman DT, Cardelli JA. (**2014**) The polyphenols (-)-epigallocatechin-3-gallate and luteolin synergistically inhibit TGF-beta-induced myofibroblast phenotypes through RhoA and ERK inhibition. *PLoS One*, *9*, e109208.

[78]   Lin Y, Shi R, Wang X, Shen H-M. (**2008**) Luteolin, a flavonoid with potential for cancer prevention and therapy. *Current Cancer Drug Targets*, *8*, 634-646.

[79]   Zhou Q, Yan B, Hu X, Li X-B, Zhang J, Fang J. (**2009**) Luteolin inhibits invasion of prostate cancer PC3 cells through E-cadherin. *Molecular Cancer Therapeutics*, *8*, 1684-1691.

[80]   Siddiqui IA, Malik A, Adhami VM, Asim M, Hafeez BB, Sarfaraz S, Mukhtar H. (**2008**) Green tea polyphenol EGCG sensitizes human prostate carcinoma LNCaP cells to TRAIL-mediated apoptosis and synergistically inhibits biomarkers associated with angiogenesis and metastasis. *Oncogene*, *27*, 2055-2063.

[81]   Stearns Mark E, Wang M. (**2011**) Synergistic effects of the green tea extract epigallocatechin-3-gallate and taxane in eradication of malignant human prostate tumors. *Translational Oncology*, *4*, 147-156.

[82]   Stearns ME, Amatangelo MD, Varma D, Sell C, Goodyear SM. (**2010**) Combination therapy with epigallocatechin-3-gallate and doxorubicin in human prostate tumor modeling studies inhibition of metastatic tumor growth in severe combined immunodeficiency mice. *American Journal of Pathology*, *177*, 3169-3179.

[83]   Hagen RM, Chedea VS, Mintoff CP, Bowler E, Morse HR, Ladomery MR. (**2013**) Epigallocatechin-3-gallate promotes apoptosis and expression of the caspase 9a splice variant in PC3 prostate cancer cells. *International Journal of Oncology*, *43*, 194-200.

[84]   Huang L, Kirschke Catherine P, Zhang Y. (**2006**) Decreased intracellular zinc in human tumorigenic prostate epithelial cells: a possible role in prostate cancer progression. *Cancer Cell International*, *6*, 10.

[85]   Sun S-l, He G-Q, Yu H-N, Yang J-G, Borthakur D, Zhang L-C, Shen S-R, Das UN. (**2008**) Free Zn^{2+} enhances inhibitory effects of EGCG on the growth of PC-3 cells. *Molecular Nutrition & Food Research*, *52*, 465-471.

Ligustrum lucidum and its Constituents : A Mini-Review on the Anti-Osteoporosis Potential

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Osteoporosis is a metabolic bone disorder commonly occurred in aging populations, particularly postmenopausal women and patients who undergo long-term steroid or anti-estrogen therapies. Given the rapid growth of the aging population, the prevalence of bone loss, and the huge medical and healthcare cost involved, demand for alternative approaches for the promotion of bone health is pressing. With the advent of global interest in complementary and alternative medicine and natural products, Chinese medicine serves as a viable source that offers benefits to improve and maintain bone health. This review summarizes the scientific information on the Chinese medicinal herb *Ligustrum lucidum* and its chemical components as potential therapy for osteoporosis.

Osteopenia (low bone density) and osteoporosis ("porous bone") are metabolic bone disorders commonly occurred in aging populations, particularly postmenopausal women and patients who undergo long-term steroid or anti-estrogen therapies. Although postmenopausal women are at greater risk, osteopenia/osteoporosis can strike at any age of both genders. The disease is characterized by thinning of bones, with reduction in bone mass and bone mineral density, as well as microarchitectural deterioration of the bone tissue due to depletion of calcium and bone protein. The clinical result is loss of bone strength, thus making bone more fragile and vulnerable to fracture, which often happens in the hip, spine, and wrist. Worldwide, osteoporosis is estimated to affect 200 million women, approximately one-tenth of women aged 60 and one-fifth of women aged 70 [1]. Although the overall prevalence of fragility fractures is higher in women, men generally have higher rates of fracture related mortality. The National Osteoporosis Foundation projected that by 2020, 14 million Americans over the age of 50 are expected to have osteoporosis and another 47 million to have low bone mass, accounting for 55% of the population 50 years of age and older [2].

A comprehensive bone health management plan usually includes non-pharmacologic measures such as balanced diet, adequate calcium and vitamin D intake, exercise and fall prevention, together with pharmacologic therapy [3]. For osteoporosis prevention and treatment, less than ten FDA-approved drugs are currently available. They fall into two major classes, the anti-resorptive and the anabolic drugs. Anti-resorptive medications such as the bisphosphonates (alendronate, ibandronate, risedronate, and zoledronic acid), calcitonin, denosumab (a receptor activator of nuclear factor-κB ligand [RANKL] inhibitor), selective estrogen receptor modulator (raloxifene), and estrogen (with or without progesterone) slow down the process of bone loss; and the anabolic drug such as the recombinant form of parathyroid hormone (teriparatide) enhances bone formation [4-6]. However, the long-term safety of many of these anti-osteoporosis drugs has posted some concerns; for example, potentially serious adverse effects of bisphosphonate therapy have been reported [7-12]. Only a small number of investigational drugs are currently in the pipeline of development [13, 14].

Apart from drug therapy, hormone replacement therapy is effective in increasing bone density and reducing the risk of fracture, and it is an effective regimen for the prevention and treatment of postmenopausal osteoporosis. Nevertheless, the use of estrogenic hormones has been severely limited by concerns for the increased risk of breast, endometrial and ovarian cancers, heart attack, and stroke [15]. Dietary supplementation of calcium and vitamin D is often included as part of the treatment plan, yet calcium and vitamin D alone or in combination are ineffective in reducing fractures in the absence of pharmacologic agents [16, 17].

Studies in Canada [18] and Australia [19] indicated that over 50% of osteoporosis patients go for complementary and alternative medicines, with up to 10% seeking treatment from herbal therapy or Chinese medicine. Soy isoflavone [20-23], red clover [24] and black cohosh [25] are commonly used by postmenopausal women with or without osteoporosis. Indeed, botanicals prepared as dietary supplements hold promise for use and there is considerable interest because the general population is receptive to the use of natural products for long-term treatment, and the cost for dietary supplements is more affordable to the elderly population.

The dried fruit of *Ligustrum lucidum* Ait. (Oleaceae), known in herbal medicine as "Ligustri Lucidi Fructus" and in Chinese medicine as "Lu-Zhen-Zi", is a tonic drug ("good-for-health") often included in herbal prescriptions for vitalizing the "liver and kidney" functions as well as nourishing the "Yin component" in these organs. It is indicated for "weakness of the loin and knees" [26], which may represent a symptom of bone deterioration. Indeed, the Chinese medical theory states that "the kidney serves to strengthen the bone" [27] and, therefore, the use of *Lucidum* fruits as a kidney-tonifying agent among the elderly to improve skeletal strength is supported by substantial ethnomedical evidence.

To the best of our knowledge, the first reports on the osteoprotective activity of *L. lucidum* were published by Zhang *et al.* [28, 29]. In the studies, an extract of *L. lucidum* fruits was given to three-month-old ovariectomized rats four weeks after surgical operation and continued for fourteen weeks. Results showed that

both bone turnover and calcium balance were improved. Thus, treatment with the plant extract suppressed bone turnover markers such as serum osteocalcin and urinary deoxypyridinoline. It also decreased urinary and fecal calcium excretion as well as increased the bone calcium content. In addition, the mRNA expressions of renal calbindin-D9K and calbindin-D28K were up-regulated. These findings suggested the involvement of both vitamin D metabolism and calcium reabsorption. Indeed, in aged (eleven-month old) female rats treated with the ethanol extract of *L. lucidum* fruits, serum levels of 1,25-dihydroxyvitamin D_3 and the mRNA expression of duodenal calcium binding protein CaBP-9k were increased; renal CaBP-28K mRNA expression was also up-regulated [30]. In a study to investigate whether the plant extract could improve bone properties in aged animals, both sham- and ovariectomized ten-month-old female rats were treated with the extract for 12 weeks, at three levels of dietary calcium contents. The *L. lucidum* extract significantly improved bone mineral density and content at tibial and femoral diaphysis as well as the lumbar vertebrae (LV-2) in rats fed with diets containing either low calcium (0.1%) or medium calcium (0.6%) level; the biomechanical strength of the tibial diaphysis was also improved [31]. In a similar experiment, eight-week treatment in ovariectomized aged rats also prevented the loss of bone mineral density [32].

In a retinoic acid-induced osteoporosis rat model, treatment with *L. lucidum* led to an increase of serum calcium and phosphorus contents, together with lowered alkaline phosphatase and tartrate-resistant acid phosphatase levels, as well as improved bone microarchitecture [33].

When the osteoblast-like UMR-106 cells were treated with the plant extract, increased formation of calcified matrix and increased extracellular calcium and phosphorus deposition were observed. These results suggested that *L. lucidum* extract could improve bone properties possibly via the enhancement of differentiation and mineralization of osteoblasts [31]. Indeed, *in vitro* study using human bone marrow mesenchymal stem cells treated with an ethanol extract of *L. lucidum* displayed stimulatory activity on osteogenic differentiation, evidenced by an increase in alkaline phosphatase activity and shortening of time needed for mineralization. The mRNA expressions of osteoblast differentiation regulatory genes involved in the Wnt signaling, such as β-catenin, bone morphogenetic protein (BMP)-2, cyclin D1, MT1-MMP, osteoprotegerin (OPG) and TBX3, were up-regulated, thus enhancing osteogenesis [34]. The aqueous extract of the same plant materials also demonstrated promoting effects on both human and rat mesenchymal stem cells as shown by enhanced alkaline phosphatase activity, mineralization level, and the expression of osteopontin [35]. In osteoblast-like MC3T3-E1 cells, an extract of *L. lucidum* increased cell viability and promoted cell differentiation by up-regulating the OPG and RANKL mRNA and protein expressions [36].

The improved calcium balance after treatment was found to associate, at least in part, with an increase of circulating levels of 1,25-dihydroxyvitamin D_3 via up-regulating the 25-hydroxyvitamin D 1α-hydroxylase activity [37]. At the same time, the renal mRNA expression of 25-hydroxyvitamin D 24-hydroxylase was suppressed [38]. These findings suggested that the protective effects of the ethanol extract on bone could be accounted for by its direct actions on the vitamin D system, including the biosynthesis of 1,25-dihydroxyvitamin D_3 and the expression of vitamin D-dependent calcium transport protein expression. In a subsequent study, the plant extract was separated into ethyl acetate-soluble and water-soluble fractions by solvent extraction. The results indicated that the

Table 1: Secondary metabolites in the fruit of *Ligustrum lucidum* known prior to 2011 (Adopted and translated based on the information available in [44])

Compound
PHENYLETHANOIDS
Salidroside
TRITERPENOIDS
α-Amyrin
β-Amyrin
Betulin
3-*O*-Acetyl-12-hydroxydammar-24-ene
(20*S*)-3-*O*-Acetyl-dammar-24-en-3β ,20-diol
3-*O*-Acetyl- 20,25-epoxydammaran-3β ,24α-diol
(20*S*,24*R*)-3-*O*-Acetyl-24-hydroperoxyldammar-25-en-3β ,20-diol
3β,12-Dihydroxydammar-24-ene
20,25-Epoxydammaran-3β,24α-diol
(20*S*)-25-Hydroperoxyldammar-23(*E*)-en-3β,20-diol
3β-*O*-Palmitoyl-dammar-24-en-12-ol
Fouquierol
Ligustrin
Lupeol
Ocotillol II 3-*O*-palmitate
Oleanolic acid
Oleanolic acid 3-acetate
Oleanolic acid ethyl acetate
2α-Hydroxyoleanolic acid
2α-Hydroxy-3β-*O*-(trans-*p*-coumaroyl)-oleanolic acid
Oliganthas A
Tormentic acid
Ursolic acid
Ursolic acid 3-acetate
3-*O*-Acetyl-19-hydroxyursolic acid
2α-Hyroxyursolic acid
3β-Hydroxyursolic acid ethyl ester
α-Ursonic acid methyl ester
SECOIRIDOIDS
Ligustroside
10-Hydroxyligustroside
Ligustrosidic acid
Ligustaloside A
Ligustaloside B
Lucidumoside A
Lucidumoside B
Nu(e)zhenide
10-Hydroxynuezhenide
Nuezhenidic acid
Nuezhengalaside
Isonuezhenide
Neonuezhenide
Oleoside dimethyl ester
Oleoside 7-β-D-glucosyl-11-methyl diester
10-Hydroxyoleoside 7,11-dimethyl ester
Oleuropein
10-Hydroxyoleuropein
Oleuropeinic acid
Specnuezhenide
FLAVONOIDS
Apigenin
Apigenin 7-*O*-β-D-glucopyranoside
Apigenin 7-*O*-(6"-acetyl)-β-D-glucopyranoside
Apigenin-7-*O*-β-D-rutinoside
Cosmosiin
Eriodictyol
Kaemoterol
Luteolin
Luteolin 7-*O*-β-D-glucopyranoside
Quercetin
Taxifolin

water fraction inhibited urinary and fecal calcium excretion in mature female rats. However, it is noteworthy that the enhancement of calcium absorption by the water fraction was not associated with an increase in serum 1,25-dihydroxyvitamin D_3 levels, but serum parathyroid hormone levels [39].

Apart from the aged and/or ovariectomized animals, the extract of *L. lucidum* was found to benefit bone mass acquisition during early life of the animals. Thus, when an ethanol extract of *L. lucidum* was given to one-month old male and female rats for 4 months, improvements in bone mineral density, bone microarchitecture and bone mechanical properties were observed (compared with control).

Table 2: Additional secondary metabolites found in the fruit of *Ligustrum lucidum*

Compound	Reference
PHENYLETHANOIDS	
Acteoside (verbacoside)	[59]
Cimidahurinine	[60]
2-(3,4-Dihydroxyphenyl)-ethanol	[60]
2-(3,4-Dihydroxyphenyl)-ethyl-*O*-β-D-glucopyranoside	[60]
Osmanthuside H	[60]
Tyrosol	[59, 61]
Tyrosol acetate	[61]
Hydroxytyrosol	[59, 61]
TRITERPENOIDS	
Crataegolic acid	[60]
Dammarendiol-II	[60]
Dammarendiol-II 3-*O*-palmitate	[60]
(20*S*)-Dammar-23-en-3β,20,25-triol	[62]
(20*S*,24*R*) 24-Hydroperoxydammar-25-en-3β,20-diol	[62]
(20*S*,24*R*) 3-*O*-Acetyl-24-hydroperoxydammar-25-en-3β,20-diol	[62]
(20*S*,24*R*)-24-Hydroperoxyldammar-25-en-3*β*,20-diol	[60]
3-*O*-*cis*-*p*-Coumaroylmaslinic acid	[63]
3-*O*-*trans*-*p*-Coumaroylmaslinic acid	[63]
Oleanolic acid methyl ester	[63]
3-Keto-oleanolic acid	[63]
Oliganthas A	[60]
19α-Hydroxyursolic acid	[63]
19α-Hydroxyursolic acid 3-acetate	[63]
SECOIRIDOIDS	
p-Hydroxyphenethyl 7-β-D-glycosylelenolic acid ester	[64]
1'''-*O*-β-D-glucosylformoside	[65]
GI 3	[59, 61, 65, 66]
Jaspolyside methyl ester	[66]
6'-*O*-*trans*-Cinnamoyl-8-epikingisidic acid	[67]
6'-*O*-*cis*-Cinnamoyl-8-epikingisidic acid	[67]
Liguside A	[65]
Liguside B	[65]
Isoligustrosidic acid	[67]
8(*Z*)-Nuezhenide A	[65]
Nuzhenal A	[67]
Nuzhenal B	[67]
Oleonuezhenide	[65, 66]
Oleopolynuezhenide	[67]
Iso-oleonuezhenide	[66]
Nicotiflorine	[64]
6'''-Acetylnicotiflorine	[64]
6'-Elenolylnicotiflorine	[64]
Oleonin	[68]
Methyloleoside 7-ethyl ester	[66]
Oleoside 11-methyl ester	[64]
Oleoside7-ethyl-11-methyl diester	[64]

Table 3: Osteo-active secondary metabolites in the fruit of *Ligustrum lucidum*.

Compound	Biological activity	Reference
PHENYLETHANOIDS		
Tyrosol (**3**)	Protect UMR-106 cells against H$_2$O$_2$-induced injury	[59]
Hydroxytyrosol (**4**)	Increase alkaline phosphatase activity in UMR-106 cells; Protect UMR-106 cells against H$_2$O$_2$-induced injury	[59, 61]
Salidroside (**5**)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59, 61]
Acteoside (**6**)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59]
SECOIRIDOIDS		
Oleoside dimethyl ester (**7**)	Promote UMR-106 cell proliferation	[61]
Oleoside7-ethyl-11-methyl diester (**8**)	Promote UMR-106 cell proliferation	[61]
Oleuropein (**9**)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59]
Nu(e)zhenide (**10**)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[61]
Specnuezhenide (**11**)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59]
G13 (**12**)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59, 61]
FLAVONOIDS		
Luteolin 7-*O*-β-D-glucopyranoside (**13**)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59]
Apigenin (**14**)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells; Protect UMR-106 cells against H$_2$O$_2$-induced injury	[59]
Apigenin 7-*O*-β-D-glucopyranoside (**15**)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells; Protect UMR-106 cells against H$_2$O$_2$-induced injury	[59]
Apigenin 7-*O*-acetyl-β-D-glucopyranoside (**16**)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59]

In addition, *L. lucidum* treatment increased bone formation marker osteocalcin (OCN), decreased the bone-resorbing marker CTX-1, while increasing serum 25-hydroxyvitamin D$_3$. The vitamin D metabolism- and calcium absorption-related gene expressions (1α-vitamin D hydroxylase, vitamin D receptor, calcium transporter calbindin-D9K, and transient receptor potential vanilloid 6) were up-regulated. These results suggested that the extract had beneficial effects on peak bone mass acquisition during early life of the animals and the improvement of bone mechanical properties by favoring calcium metabolism [40, 41].

In diabetic mice, feeding with an aqueous extract of *L. lucidum* was found to protect against excessive urinary calcium excretion and trabecular bone deterioration. The underlying mechanism might be attributed to the regulation of duodenal calcium transporting proteins and renal calcium-sensing receptor [42].

The volatile oil obtained from *L. lucidum* fruits exhibited *in vitro* osteoblastic activity by stimulating the proliferation and alkaline phosphatase activity of rat calvarial osteoblasts [43]. The main components in the volatile oil included (*Z,Z*)-9,12-octadecadienoic acid, *n*-hexadecanoic acid, (*E*)-9-octadecenoic acid, α-cadinol, 4-hexyl-2,5-dihydro-2,5-dioxo-3-furanacetic acid, and (*E*)-8-octadecenoic acid methyl ester [43]. A number of phenylethanoids, secoiridoids, flavonoids and triterpenoids have been identified from

the fruit of *L. lucidum* and they were summarized in several review articles [44-47]. Since most of these review articles were published in the Chinese language, the chemical ingredients as highlighted by Huang and Wang [44] are listed in Table 1.

In recent years, further phenylethanoids, triterpenoids and secoiridoids have been reported and they are included in Table 2. Several ingredients of *L. lucidum* fruits have been demonstrated to display potential anti-osteoporosis activities in cell-based and/or animal models. Thus, oleanolic acid (**1**) and its glycosidic and synthetic derivatives have been known to be inhibitors of osteoclast formation [48-51]. They inhibited the formation of osteoclast-like multinucleated cells induced by 1α, 25-dihydroxyvitamin D$_3$. Oleanolic acid acetate was demonstrated to inhibit receptor activator of nuclear factor-κB (RANKL)-induced osteoclast differentiation; and it attenuated lipopolysaccharide-induced bone erosion in mice [52]. Apart from the anti-osteoclastogenic activity, oleanolic acid was found to be able to promote osteoblastic differentiation and change the gene expression profile of bone marrow stromal cells obtained from rats with corticosterone-induced osteoporosis [53]. In ovariectomized rats, oleanolic acid exerted osteoprotective effect by increasing the population of osteoblasts as well as the levels of osteocalcin and the Runt-related protein-2. It also stimulated the osteoblastic differentiation of bone mesenchymal stem cells *in vitro*. Gene expression profile analysis suggested that the effect might be related to the Notch signaling pathway [54].

Another triterpene ingredient of *L. lucidum*, ursolic acid (**2**), has been found to stimulate osteoblast differentiation and mineralization by activating osteoblast-specific genes such as mitogen-activated protein kinases, nuclear factor-κB, and activator protein-1 [55]. It also promoted bone formation in a mouse calvarial bone formation model [55]. In addition, recent studies showed that ursolic acid was able to inhibit RANKL-induced osteoclast differentiation [56-58] and down-regulate the NFATc1-regulated osteoclast marker genes [58]. Using a mouse model of titanium particle-induced osteolysis, ursolic acid protected calvarial bone loss and decreased the population of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts [58].

Two screening and bioactivity-guided isolation reports on the osteoprotective activity of *L. lucidum* have identified fourteen active compounds [59, 61]. While the screening results are considered to be preliminary at this time, the active compounds were found to promote the proliferation of osteoblast-like UMR-106 cells, increase the alkaline phosphatase activity, and/or protect the cells from hydrogen peroxide-induced damage (Table 3). The chemical structures of these osteo-active compounds are shown in Figure 1.

Among the four active phenylethanoids, tyrosol (**3**) and hydroxytyrosol (**4**) are also present in olive oil [69-71], which has been reported to possess anti-osteoporosis property when tested in ovariectomized rats [72]. Salidroside (**5**) is a major bioactive ingredient of *Rhodiola* plants and is well known for its adaptogenic potential for use in high-altitude environment [73, 74]. The inhibitory effect of this compound on diabetes-related osteoporosis has been described [75]. Acteoside (**6**), also known as verbascoside, is a phenylethanoid glycoside possessing pharmacologically beneficial properties such as antioxidant, anti-inflammatory, antitumor, and neuroprotective activities [76, 77].

For the secoiridoids, oleuropein (**9**) is also present in olive oil [69-71]. Together with the phenylethanoids, it is considered to be the active principle. The structure of specnuezhenide (**11**) differs from nu(e)zhenide (**10**) by replacing the glucose with galactose. Both compounds were demonstrated to possess proliferative activity in the osteoblast-like UMR-106 cells and they enhanced the alkaline phosphatase activity [59, 61]. Compound GI 3 (**12**) can be considered as a dimeric derivative of nu(e)zhenide; it displayed similar *in vitro* activities. It is noteworthy that nu(e)zhenide (**10**), specnuezhenide (**11**) and GI 3 (**12**) contain the salidroside moiety in their structures. It will, therefore, be of interest to determine whether the salidroside portion of the molecule acts as a pharmacophore for the osteoprotective activity.

The flavonoids apigenin (**14**) and two glucoside derivatives (**15** and **16**), as well as luteolin 7-*O*-β-D-glucopyranoside (**13**), were found to be active in UMR-106 cells [59, 61].

All in all, available information strongly suggests that the fruit of *Ligustrum lucidum* possesses osteoprotective property and it may be useful in promoting bone health. The beneficial effects seem to be associated with improved calcium balance, modulation of vitamin D metabolism, as well as osteoblastogenic and anti-osteoclastogenic activities. Yet the exact mechanisms remain to be elucidated. Further studies on the potentials of this plant drug and its chemical ingredients for use in the improvement of age-related changes in calcium homeostasis and development of anti-osteoporosis remedies are warranted.
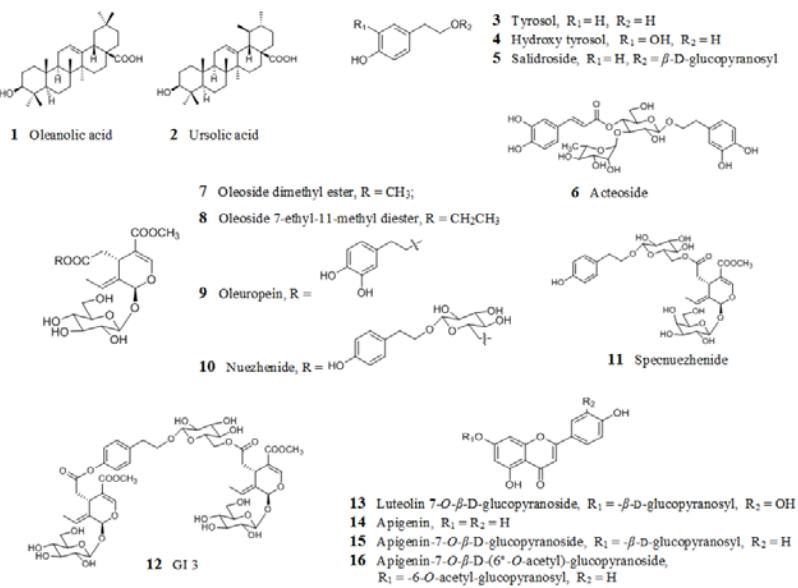
Figure 1: Chemical structures of osteo-active compounds from *Ligustrum lucidum* fruits.

References

[1]   Anon. (**2015**) International Osteoporosis Foundation: Facts and statistics about osteoporosis and its impact. International Osteoporosis Foundation.

[2]   Anon. (**2015**) National Osteoporosis Foundation Prevalence Report. National Osteoporosis Foundation.

[3]   Tella SH, Gallagher JC. (**2014**) Prevention and treatment of postmenopausal osteoporosis. *Journal of Steroid Biochemistry and Molecular Biology*, ***142***, 155-170.

[4]   Sandhu SK, Hampson G. (**2011**) The pathogenesis, diagnosis, investigation and management of osteoporosis. *Journal of Clinical Pathology*, *64*, 1042-1050.
[5]   Sweet MG, Sweet JM, Jeremiah MP, Salazka SS. (**2009**) Diagnosis and treatment of osteoporosis. *American Family Physician*, *79*, 193-200.
[6]   Rachner TD, Khosla S, Hofbauer LC. (**2011**) Osteoporosis: now and the future. *Lancet*, *377*, 1276-1287.
[7]   Hollick RJ, Reid DM. (**2011**) Role of bisphosphonates in the management of postmenopausal osteoporosis: an update on recent safety anxieties. *Menopause International*, *17*, 66-72.
[8]   Park-Wyllie LY, Mamdani MM, Juurlink DN, Hawker GA, Gunraj N, Austin PC, Whelan DB, Weiler PJ, Laupacis A. (**2011**) Bisphosphonate use and the risk of subtrochanteric or femoral shaft fracture in older women. *The Journal of the American Medical Association*, *305*, 783-789.
[9]   Lewiecki EM. (**2011**) Safety of long-term bisphosphonate therapy of the management of osteoporosis. *Drugs*, *71*, 791-814.
[10]   Watts NB, Diab DL. (**2010**) Long-term use of bisphosphonates in osteoporosis. *Journal of Clinical Endocrinology and Metabolism*, *95*, 1555-1565.
[11]   Kos M. (**2011**) Bisphosphonates promote jaw osteonecrosis through facilitating bacterial colonization. *Medical Hypotheses*, *77*, 214-215.
[12]   Knopp-Sihota JA, Cummings GG, Homik J, Voaklander D. (**2013**) The association between serious upper gastrointestinal bleeding and incident bisphosphonate use: a population-based nested cohort study. *BMC Geriatrics*, *13*, 36.
[13]   Honig S. (**2010**) Osteoporosis, new treatment and updates. *Bulletin of the New York University Hospital for Joint Diseases*, *68*, 166-170.
[14]   Silva BC, Bilezikian JP. (**2011**) New approaches to the treatment of osteoporosis. *Annual Review of Medicine*, *62*, 307-322.
[15]   Bowring CE, Francis RM. (**2011**) National Osteoporosis Society's position statement on hormone replacement therapy in the prevention and treatment of osteoporosis. *Menopause International*, *17*, 63-65.
[16]   Spangler M, Phillips BB, Ross MB, Moores KG. (**2011**) Calcium supplementation in postmenopausal women to reduce the risk of osteoporotic fractures. *American Journal of Health-System Pharmcy*, *68*, 309-318.
[17]   Quesada-Gomez JM, Blanch-Rubio J, Diaz-Curiel M, Diez-Perez A. (**2011**) Calcium citrate and vitamin D in the treatment of osteoporosis. *Clinical Drug Investigation*, *31*, 285-298.
[18]   Chong CA, Diaz-Granados N, Hawker GA, Jamal S, Josse RG, Cheung AM. (**2007**) Complementary and alternative medicine use by osteoporosis clinic patients. *Osteoporosis International*, *18*, 1547-1556.
[19]   Mak JCS, Faux S. (**2010**) Complementary and alternative medicine use by osteoporotic patients in Australia (CAMEO-A): a prospective study. *Journal of Alternative and Complementary Medicine*, *16*, 579-584.
[20]   Newton KM, Grady D. (**2011**) Soy isoflavones for prevention of menopausal bone loss and vasomotor symptoms. *Archives in Internal Medicine*, *171*, 1369-1370.
[21]   Levis S, Strickman-Stein N, Ganjei-Azar P, Xu P, Doerge DR, Krischer J. (**2011**) Soy isoflavones in the prevention of menopausal bone loss and menopausal symptoms. A randomized, double-blind trial. *Archives in Internal Medicine*, *171*, 1363-1369.
[22]   Taku K, Melby MK, Nishi N, Omori T, Kurzer MS. (**2011**) Soy isoflavones for osteoporosis: An evidence-based approach. *Maturitas*, *70*, 333-338.
[23]   Castelo-Branco C, Cancelo-Hidalgo MJ. (**2011**) Isoflavones: effects on bone health. *Climacteric*, *14*, 204-211.
[24]   Lethaby LE, Marjoribanks J, Kronenberg F, Roberts H, Eden J. (**2013**) Phytoestrogens for menopausal vasomotor symptoms. *Cochrane Database of Systematic Reviews*, (*12*):CD001395.
[25]   Depypere HT, Comhaire FH. (**2014**) Herbal preparation for the menopause: Beyond isoflavones and black cohosh. *Maturitas*, *77*, 191-194.
[26]   Anon. (**2010**) Ligustri Lucidi Fructus. In *Chinese-Pharmacopoeia, 2010 Chinese Edition, Part 1*, Chinese Medical and Pharmaceutical Press, Beijing (China), p. 43.
[27]   Kong YC. (**2010**) *Huangdi Neijing: a synopsis with commentaries*, The Chinese University Press, Hong Kong, p.131.
[28]   Zhang Y, Lai WP, Leung PC, Wu CF, Yao XS, Wong MS. (**2006**) Effects of Fructus Ligustri Lucidi extract on bone turnover and calcium balance in ovariectomized rats. *Biological Pharmaceutical Bulletin*, *29*, 291-296.
[29]   Zhang Y, Huang W, Chen B, Leung PC, Wu CF, Yao XS. (**2006**) Effect of Fructus Ligustri Lucidi on calcium metabolism and vitamin D-dependent gene expressions in ovariectomized rats. *Zhongcaoyao*, *37*, 558-561.
[30]   Zhang Y, Lai WP, Leung PC, Che CT, Wong MS. (**2008**) Improvement of Ca balance by Fructus Ligustri Lucidi extract in aged female rats. *Osteoporosis International*, *19*, 235-242.
[31]   Zhang Y, Leung PC, Che CT, Chow HK, Wu CF, Wong MS. (**2008**) Improvement of bone properties and enhancement of mineralization by ethanol extract of Fructus Ligustri Lucidi. *British Journal of Nutrition*, *99*, 494-502.
[32]   Ko CH, Siu WS, Lau CP, Lau CBS, Fung KP, Leung PC. (**2010**) Osteoprotecticve effects of Fructus Ligustri Lucidi aqueous extract in aged ovariectomized rats. *Chinese Medicine*, *5*, 39.
[33]   Kang X, Li Z, Zhang WH, Zhou Q, Liu RH, Wang XJ. (**2013**) Study on the effect of combination of Epimedii Folium and Ligustri Lucidi Fructus on osteoporosis rats induced by retinoic acid. *Zhongguo Zhongyao Zazhi*, *38*, 4124-4128.
[34]   Li G, Zhang XA, Zhang JF, Chan CY, Yew DTW, He ML, Lin MCM, Leung PC, Kung HF. (**2010**) Ethanol extract of Fructus Ligustri Lucidi promotes osteogenesis of mesenchymal stem cells. *Phytotherapy Research*, *24*, 571-576.
[35]   Li G, Chan CY, Zhang JF, Zhang XA, Yew DTW, He ML, Lin MCM, Leung PC, Kung HF. (**2008**) The aqueous extract of Fructus Ligustri Lucidi regulates the differentiation of mesenchymal stem cells. *International Journal of Integrative Biology*, *3*, 182-190.
[36]   Li Q, Fan YS, Gao ZQ, Fan K, Liu ZJ. (**2015**) Effect of Fructus Ligustri Lucidi on osteoblastic like cell-line MC3T3-E1. *Journal of Ethnopharmacology*, *170*, 88-95.
[37]   Dong XL, Zhang Y, Favus MJ, Che CT, Wong MS. (**2010**) Ethanol extract of Fructus Ligustri Lucidi increases circulating 1,25-dihydroxyvitamin D3 by inducing renal 25-hydroxyvitamin D-1alpha hydroxylase activity. *Menopause*, *17*, 1174-1181.
[38]   Zhang Y, Dong XL, Leung PC, Che CT, Wong MS. (**2008**) Fructus Ligustri Lucidi extract improves calcium balance and modulates the calciotropic hormone level and vitamin D-dependent gene expression in aged ovariectomized rats. *Menopause*, *15*, 558-565.
[39]   Dong XL, Zhao M, Wong KK, Che CT, Wong MS. (**2012**) Improvement of calcium balance by Fructus Ligustri Lucidi extract in mature female rats was associated with the induction of serum parathyroid hormone levels. *British Journal of Nutrition*, *108*, 92-101.
[40]   Lyu Y, Feng X, Zhao P, Wu Z, Xu H, Fang Y, Hou Y, Denney L, Xu Y, Feng H. (**2014**) Fructus Ligustri Lucidi ethanol extract increases bone mineral density and improves bone properties in growing female rats. *Journal of Bone Mineral Metabolism*, *32*, 616-626.
[41]   Feng X, Ying L, Wu Z, Fang Y, Xu H, Zhao P, Xu Y, Feng H. (**2014**) Fructus ligustri lucidi ethanol extract improves bone mineral density and properties through modulating calcium absorption-related gene expression in kidney and duodenum of growing rats. *Calcified Tissue International*, *94*, 433-441.
[42]   Zhang Y, Diao TY, Wang L, Che CT, Wong MS. (**2014**) Protective effects of water fraction of Fructus Ligustri Lucidi extract against hypercalciuria and trabecular bone deterioration in experimentally type 1 diabetic mice. *Journal of Ethnopharmacology, 158 (Pt A)*, 239-245.
[43]   Wu YB, Lin XH, Wu JG, Yi J, Zheng YZ, Wu JZ. (**2011**) Volatile components of fruits of *Ligustrum lucidum* Ait. stimulate proliferation and differentiation of rat calvarial osteoblasts. *African Journal of Biotechnology*, *10*, 8662-8668.
[44]   Huang XP, Wang WC. (**2011**) Chemical constituents of *Ligustrum lucidum* fruits: research advances. *Guoji Yaoxue Yanjiu Zazhi*, *38*, 47-51.
[45]   Zhao L, Feng Y. (**2012**) Advances in research on triterpenoids and iridoids of Ligustri Lucidi Fructus. *Guangdong Yaoxeuyuan Xuebao*, *28*, 107-110.

[46]   Zhang J, Wang X, Deng Z, Li X. (**2013**) Advances in research on chemical components and pharmacological effects of Chinese medicine *Ligustrum lucidum* fruit. *Xiandai Zhongxiyi Jiehe Zazhi*, **22**, 4100-4101.

[47]   Gao L, Li CY, Wang Z, Liu X, You Y, Wei H, Guo T. (**2015**) Ligustri Lucidi Fructus as a traditional Chinese medicine: a review of its phytochemistry and pharmacology. *Natural Products Research*, **29**, 493-510.

[48]   Li JX, Hareyama T, Tezuka Y, Zhang Y, Miyahara T, Kadota S. (**2005**) Five new oleanolic acid glycosides from *Achyranthes bidentata* with inhibitory activity on osteoclast formation. *Planta Medica*, **71**, 673-679.

[49]   Zhang Y, Li JX, Zhao J, Wang SZ, Pan Y, Tanaka K, Kadota S. (**2005**) Synthesis and activity of oleanolic acid derivatives, a novel class of inhibitors of osteoclast formation. *Bioorganic and Medicinal Chemistry Letters*, **15**, 1629-1632.

[50]   Li JF, Zhao YJ, Cai MM, Li XF, Li JX. (**2009**) Synthesis and evaluation of a novel series of heterocyclic oleanolic acid derivatives with anti-osteoclast formation activity. *European Journal of Medicinal Chemistry*, **44**, 2796-2806.

[51]   Li JF, Chen SJ, Zhao Y, Li JX. (**2009**) Glycoside modification of oleanolic acid derivatives as a novel class of anti-osteoclast formation agents. *Carbohydrate Research*, **344**, 599-605.

[52]   Kim JY, Cheon YH, Oh HM, Rho MC, Erkhembaatar M, Kim MS, Lee CH, Kim JJ, Choi MK, Yoon KH, Lee MS, Oh J. (**2014**) Oleanolic acid acetate inhibits osteoclast differentiation by downregulating PLCgamma2-Ca(2+)-NFATc1 signaling and suppresses bone loss in mice. *Bone*, **60**, 104-111.

[53]   Bian Q, Huang JH, Yang Z, Ning Y, Zhao YJ, Wang YJ, Shen ZY. (**2011**) Effects of active ingredients in three kidney-tonifying Chinese herbal drugs on gene expression profile of bone marrow stromal cells from a rat model of corticosterone-induced osteoporosis. *Zhongxiyi Jiehe Xuebao*, **9**, 179-185.

[54]   Bian Q, Liu SF, Huang JH, Yang Z, Tang DZ, Zhou Q, Ning Y, Zhao YJ, Lu S,Shen ZY. (**2012**) Oleanolic acid exerts an osteoprotective effect in ovariectomy-induced osteoporotic rats and stimulates the osteoblastic differentiation of bone mesenchymal stem cells *in vitro*. *Menopause*, **19**, 225-233.

[55]   Lee SU, Park SJ, Kwak HB, Oh J, Min YK, Kim SH. (**2008**) Anabolic activity of ursolic acid in bone: stimulating osteoblast differentiation *in vitro* and inducing new bone formation *in vivo*. *Pharmacological Research*, **58**, 290-296.

[56]   Tan H, Furuta S, Nagata T, Ohnuki K, Akasaka T, Shirouchi B, Sato M, Kondo R, Shimizu K. (**2014**) Inhibitory effects of the leaves of loquat (*Eriobotrya japonica*) on bone mineral density loss in ovariectomized mice and osteoclast differentiation. *Journal of Agricultural and Food Chemistry*, **62**, 836-841.

[57]   Tan H, Ashour A, Katakura Y and Shimizu K. (**2015**) A structure-activity relationship study on antiosteoclastogenesis effect of triterpenoids from the leaves of loquat (*Eriobotrya japonica*). *Phytomedicine*, **22**, 498-503.

[58]   Jiang C, Xiao F, Gu X, Zhai Z, Liu X, Wang W, Tang T, Wang Y, Zhu Z, Dai K, Qin A, Wang J. (**2015**) Inhibitory effects of ursolic acid on osteoclastogenesis and titanium particle-induced osteolysis are mediated primarily via suppression of NF-kappaK signaling. *Biochimie*, **111**, 107-118.

[59]   Huang Y, Wu Y, Wu J, Yi J, Zhang Q, Chen T, Wu J. (**2014**) Chemical constituents from *Ligustrum lucidum* differentially promote bone formation and prevent oxidative damage in osteoblastic UMR-106 cells. *Latin American Journal of Pharmacy*, **33**, 258-265.

[60]   Huang XJ, Yin ZQ, Ye WC, Shen WB. (**2010**) Chemical constituents from fruits of *Ligustrum lucidum*. *Zhongguo Zhongyao Zazhi*, **35**, 861-864.

[61]   Chen Q, Yang L, Zhang G, Wang F. (**2013**) Bioactivity-guided isolation of antiosteoporotic compounds from *Ligustrum lucidum*. *Phytotherapy Research*, **27**, 973-979.

[62]   Xu XH, Yang NY, Qian SH, Xie N, Duan JA. (**2008**) Dammarane triterpenes from *Ligustrum lucidum*. *Journal of Asian Natural Product Research*, **10**, 33-37.

[63]   Feng J, Feng Z, Wang J, Cui Y. (**2011**) Triterpenoids from the fruits of *Ligustrum lucidum*. *Zhongyaocai*, **34**, 1540-1544.

[64]   Yang NY, Xu XH, Ren DC, Duan JA, Xie N, Tian LJ, Qian SH. (**2010**) Secoirirdoid constituents from the fruits of *Ligustrum lucidum*. *Helvetica Chimica Acta*, **93**, 65-71.

[65]   Huang XJ, Wang Y, Yin ZQ, Ye WC. (**2010**) Two new dimeric secoiridoid glycosides from the fruits of *Ligustrum lucidum*. *Journal of Asian Natural Product Research*, **12**, 685-690.

[66]   Fu G, Ip FCF, Pang H, Ip NY. (**2010**) New secoiridoids from *Ligustrum lucidum* induce ERK and CREB phosphorylation in cultured cortical neurons. *Planta Medica*, **76**, 998-1003.

[67]   Aoki S, Honda Y, Kikuchi T, Miura T, Sugawara R, Yaoita Y, Kikuchi M, Machida K. (**2012**) Six new secoiridoids from the dried fruits of *Ligustrum lucidum*. *Chemical and Pharmaceutical Bulletin*, **60**, 251-256.

[68]   Huang XJ, Wang L, Shao M, Hu SZ, Jiang RW, Yao XS, Li YL, Yin ZQ, Wang Y, Ye WC. (**2013**) Oleonin, the first secoiridoid with alpha-configuration from *Ligustrum lucidum*. *RSC Advances*, **3**, 16300-16303.

[69]   Barbaro B, Toietta G, Maggio R, Arciello M, Tarocchi M, Galli A, Balsano C. (**2014**) Effects of the olive-derived polyphenol oleuropein on human health. *International Journal of Molecular Sciences*, **15**, 18508-18524.

[70]   Sacchi RP, A., Savarese M, Vitaglione P, Fogliano V. (**2014**) Extra virgin olive oil: from composition to "molecular gastronomy". *Cancer Treatment and Research*, **159**, 325-338.

[71]   Melliou E, Zweigenbaum JA, Mitchell AE. (**2015**) Ultrahigh-pressure liquid chromatography triple-quadrupole tandem mass spectrometry quantitation of polyphenols and secoiridoids in California-style black ripe olives and dry salt-cured olives. *Journal of Agricultural and Food Chemistry*, **63**, 2400-2405.

[72]   Saleh NK, Saleh HA. (**2011**) Olive oil efffectively mitigates overiectomzy-induced osteoporosis in rats. *BMC Complementary and Alternative Medicine*, **11**, 10.

[73]   Panossian A, Wikman G, Sarris J. (**2010**) Rosenroot (*Rhodiola rosea*): traditional use, chemical composition, pharmacology and clinical efficacy. *Phytomedicine*, **17**, 481-493.

[74]   Choe KI, Kwon JH, Park KH, Oh MH, Kim MH, Kim HH, Cho SH, Chung EK, Ha SY, Lee MW. (**2012**) The antioxidant and antiinflammatory effects of phenolic compounds isolated from the root of *Rhodiola sachalinensis* A. Bor. *Molecules*, **17**, 11484-11494.

[75]   Zhang SC, Wang SQ, Zhao S. (**2009**) The effect of salidroside on leptin expression of diabetic rats with osteoporosis. *Chinese Journal of Pathophysiology*, **25**, 787-788.

[76]   He J, Hu XP, Zeng Y, Li Y, Wu HQ, Qiu RZ, Ma WJ, Li T, Li CY, He ZD. (**2011**) Advanced research on acteoside for chemistry and bioactivities. *Journal of Asian Natural Product Research*, **13**, 449-464.

[77]   Alipieva K, Korkina L, Orthan IE, Georgiev MI. (**2014**) Verbascoside: a review of its occurrence, (bio)synthesis and pharmacological significance. *Biotechnology Advances*, **32**, 1065-1076.

Alice, Benzene, and Coffee: The ABCs of Ecopharmacognosy

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The sesquicentennial celebrations of the publication of "Alice's Adventures in Wonderland" and the structure of benzene offer a unique opportunity to develop a contemporary interpretation of aspects of Alice's adventures, illuminate the symbolism of benzene, and contextualize both with the globalization of coffee, transitioning to how the philosophy and sustainable practices of ecopharmacognosy may be applied to modulating approaches to the quality, safety, efficacy, and consistency (QSEC) of traditional medicines and dietary supplements through technology integration, thereby improving patient-centered health care.

Keywords: Alice in Wonderland, Benzene, Coffee, Ecopharmacognosy, Traditional medicines, Quality control, Technology integration, Global health care.

"*Everything's got a moral, if only you can find it.*" said the Duchess to Alice.

We begin our allegoric thoughts with Alice and Benzene. The year 2015 marks the sesquicentennial of two, apparently disparate, events. In 1865, August Kekulé published an iconic paper in French (for he was then a Professor in Ghent, Belgium) suggesting that the structure of benzene contained a six-membered ring of carbon atoms with alternating single and double bonds, each with an attached hydrogen [1, 2]. Describing the origin of his idea twenty-five years later, Kekulé indicated that the benzene ring shape had come to him from a dream of a snake seizing its own tail, mimicking the ancient Egyptian symbol, the ouroboros.

Coincidentally, at about the same time, across the North Sea in England, the Oxford University mathematician Charles Lutwidge Dodgson, writing under the pseudonym Lewis Carroll, was publishing the most famous and widely-translated "children's" books, "Alice's Adventures in Wonderland" and "Through the Looking Glass" [3]. The audacities of Kekulé's and Carroll's vivid imaginations have survived and thrived, and in 2015 are being celebrated following 150 years of global impact. Molecular architecture, rabbit holes, and dreams…… What can their connections be with our contemporary challenges in traditional medicine on this disappearing Earth? This brief contribution attempts to tie these two "events" together in an allegoric manner, offering a contemporary interpretation of aspects of Alice's adventures, and illuminating the symbolism of benzene, with a relationship to the globalization of coffee, and transitioning to the global need for the philosophy and practices of ecopharmacognosy as integral to a patient-centered approach for advancing the status of traditional medicines (TMs) in integrated health care systems.

Alice's story begins with curiosity after she meets the White Rabbit wearing a waistcoat and looking at a pocket watch ("..this watch is exactly two days slow."). As if on a shamanistic journey, she shrinks in size and follows him deep down a hidden hole into a world where her orderly, mundane, upper-class existence becomes completely discombobulated. Since the beginning of the industrial revolution about 260 years ago in England, we have inexorably descended our own "rabbit-hole", accelerating in the past 150 years, to our contemporary life on Earth. Aspects of that incredible

progress make abundant sense, and the continuously evolving scientific realities have dramatically and spectacularly extended our human and technological capacities throughout all aspects of society, including the extension of life expectancy which has risen from 47 years in 1950-1955 to 69 years in 2005-2010 [4].

Alice in her "Wonderland" of endless surprises and quirks represents us in ours; the wholeness of the single human race in a continuously changing, almost unpredictable world. In that wondrous and alien world, she almost completely loses both her orientation and her power of independent decision-making. Her leader in the unexpected and bizarre environment of Wonderland is the White Rabbit; for us it is burgeoning technology. We are challenged to ask whether we have now lost control of the applicability of our technology over the past 150 years [5], and the recent movie "Ex Machina", directed by Garland, provides a fascinatingly evocative and dramatic cautionary tale of creating truly humanistic, artificial intelligence systems as our pathway progresses. How did Alice get out of the deep rabbit hole and of Wonderland and return to her mundane and structured upper-class English life? She was woken up by a whirling pack of cards…..it was all a dream. Can we be awakened from our dream-like stupor and take charge of global health care for the majority? Or are we already too deeply somnolent and transfixed by our technological prowess to consider serving the minority? One situational difference is that the rabbit-hole into which Alice fell was animal-created, "zoogenic", whereas *our* "hole" is anthropogenic, a human-created, altered environment. Alice didn't have to change her habits to restore balance to her environment; we must.

Benzene, a distillate of coal tar [6], results from the decay, over hundreds of millions of years, of Earth's primordial forests. The availability and continued use of benzene as a synthetic precursor, and in gasoline fuels (at the 0.62% level in the US), epitomizes human civilization taking from the Earth, and not returning to the Earth. It recognizes our deep societal dependence on depleting Earth's finite gifts, such as coal and oil. Benzene is the synthetic base for styrene, phenol, nylon fibers, lubricants, dyes, detergents, drugs, and pesticides, etc. It is an integral (now essential) aspect of an evolving global lifestyle; one which has turned into a continuing destructive saga of the very source of everything that supports us. Ironically, like the ouroboros, we are continuously "eating" our own

tails! As such, benzene represents the antithesis of sustainability; for its primary source, bituminous coal, may only last another 100 years [7]. Ironically though, in the obverse context, we could equally now deploy the ouroboros symbol, as it was originally used in Egypt, to represent the reincarnation (recycling) of the human state. We can extend that concept to the recycling of waste to reduce the rate of depletion of our planetary resources for future generations.

A similar dichotomy of philosophy and practice exists with coffee. The coffee bean (berry) is derived from either *Coffea arabica* L. or *C. canephora* Pierre ex A. Froehner (Rubiaceae). Beginning in Ethiopia in the 15th century, and moving into Yemen [8], and then to Arabia and Persia, coffee as a consumed beverage spread to Venice, where it was deemed a Christian drink by Pope Clement VIII in 1600. The first coffee house in England appeared in 1650, and within 25 years, 3,000 existed [9]. Brazil, where cultivation began in 1822, is by far the world's largest producer (33.1%). Coffee is now grown in more than 70 countries, and engages over 100 million people [8]. It is the second-most valuable commodity exported by developing countries [10].

Coffee serves as a representative example of the philosophical culmination of the concept of transitioning a plant from the forest into a global commodity (globalization). The continued, expanding daily consumption of coffee by billions of people illuminates the underlying assumptions of the global evolution of coffee as *the* marketed beverage in four ways: i) that the coffee contents are of good quality and not contaminated; ii) that they are safe when taken on a chronic basis, iii) that they are effective on continued use, and iv) that they are consistent on a day-to-day basis. These are the fundamental principles of quality, safety, efficacy, and consistency (QSEC) which should, at the global level, underpin the sciences of patient-centered traditional medicines. In addition, because it has been globalized as a stimulant beverage over hundreds of years [9], coffee is produced in a sustainable, and in many places organic, manner, through widespread cultivation, at a level of approximately 12 billion pounds per year.

Coffee also serves as a classic example of a non-sustainable and controversial commodity. Because of issues related to the susceptibility of *C. arabica* to infestation, particularly by coffee leaf rust *Hemileia vastatrix* and the coffee berry borer beetle (*Hypothenemus hampei*), a variety of pesticides is required for cultivation. Growing in open-fields compared with shaded, under canopy, cultivation [11], the need for extensive deforestation, as well as the extreme water requirements (est. 140 L) to produce the beans for one cup of coffee [12], provide important lessons through which to lessen the environmental impact as traditional medicines are transitioned into cultivated global commodities.

Alice, **B**enzene, and **C**offee, each provide important background concepts as we look at the future development of traditional medicines. The last aspect to be introduced is the term "ecopharmacognosy", a word 25 years in development [13] over the course of numerous articles [13-34], including some on that precise topic [30, 32, 34]. "Pharmacognosy" is defined as "the study of biologically active natural products" [17]. Therein, the constituent sciences of pharmacognosy are unlimited by the source material (plant, marine, microbial, insect, mammalian, etc.), or the research area (information systems, botanical, analytical, chemical, biosynthetic, biological, pharmacological, clinical, economic, legal, regulatory, etc.). This inherently suggests that to be successful in bringing focus to the implications for, and outcomes of, pharmacognosy research, a highly integrated and collaborative approach is needed.

In London, in March 2012, the "State of the Planet Declaration" was promulgated by the "Planet under Pressure" Conference [35]. It sought support for a societal contract to encompass: i) global sustainability analyses based in science, ii) integrated, international, and solutions-oriented research, implemented and involving government, society, scientists, and the private sector, and iii) enhanced dialog on issues of global sustainability. More recently, in July 2015, the Rockefeller Foundation–*Lancet* Commission on planetary health released a report on Planetary Health [4], calling for societies to "address the drivers of environmental change by promoting sustainable and equitable patterns of consumption, reducing population growth, and harnessing the power of technology for change."

As a series of global sciences requiring, as a fundamental aspect of success, the continuing availability and judicious use of natural resources, it was astounding and distressing to acknowledge that pharmacognosy and its practices had barely considered "sustainability" as a conscious and evident component in our daily scientific research ideas and practices. There was a discussion sponsored by the World Health Organization on the conservation of medicinal plants in 1988 [36] and more recently aspects of "sustainable medicines" were discussed [25-28]; the need for awareness is much greater. Based on this situation, it was deduced that the profile and consciousness of the environmental and sustainability issues surrounding TMs needed to be raised, and hence a new term to integrate philosophy and practice was developed.

Thus "ecopharmacognosy" was born and introduced at a scientific meeting in Lublin, Poland in May, 2012. It is defined as "the study of sustainable, biologically active natural products" [30,32,34]. It offers a philosophical approach and a system of practices which stresses environmental concerns in natural product research from the perspective of the types of materials which should be studied, to opportunities for changing the practices used for their botanical, chemical, biological, and clinical assessment, to assuring a high QSEC product for the patient and the practitioner. It also embraces aspects of naturally-resourced agricultural practices, and the development of marine, bacterial, and fungal systems for scientific and economic development.

This brief and somewhat droll essay will attempt to coalesce some of the most well-known quotes and concepts from *Alice's Adventures in Wonderland* (*AAIW*) as a personal allegory for the considered use of our natural resources, with a more eco-centric view of developing Earth's natural medicinal resources, considering the patient-centered view of coffee as a pathway to develop a broad vision for traditional medicine enhancement within more integrated health care systems. Perhaps for some readers who are not familiar with "Alice's Adventures in Wonderland" this may be initially confusing. "*"Then you should say what you mean," the March Hare went on."* I will.

We all are aware of the Doomsday clock…..since 1947 a philosophical construct which "indicates" how close Earth is to nuclear disaster, based on the levels of war and conflict in its various forms around the world, and since 2007 to the impending disaster from climate change. It is presently set at 11.57 pm [37]. When Alice first meets the White Rabbit in her real world, he is concerned about how late it is as he scurries off down the hole. The White Rabbit is OUR timepiece for getting things done in time (his watch is two days slow remember!), suggesting that we look now at how late it is becoming to restore our world towards a semblance of sanity. For the truth is, as the grinning Cheshire cat states, *"We are all mad here"*, in the way we use Earth's resources.

Since 2000, the World Health Organization has been promoting the evidence-based assessment of traditional medicine practices [38], and the Beijing Declaration of 2008 encouraged countries to: i) respect, preserve, and promote knowledge and practices of TM; ii) formulate national policies, regulations and standards, as part of integrated health systems to ensure the appropriate, safe and effective use of TMs, and iii) promote TM research and innovation [39]. Further support has come from "The Regional Strategy for Traditional Medicine in the Western Pacific (2011-2020)" [40] and the "WHO traditional medicine strategy: 2014-2023" [41], which have also placed significant emphasis on the sustainability aspects of developing traditional medicines. These strategy documents challeng countries to introduce policies and practices which will ensure the availability and improve the status of TM in their respective health-care systems, based on a continuum of evidence-based approaches, in order to narrow the overt gaps in health care practices.

The economic and health care outcome gaps between traditional medicine and allopathic medicine globally are vast [30, 42-47]. The additional challenges for enhancing TM based on an evidence-based, patient-centered approach entail an exploration from a scientific perspective of the many myths which are associated with, and in some respects inhibit, unbiased TM research and development [32-34], to address the desperate need for quality control systems for TMs [13, 29, 31, 33], and to examine the profound necessity to assure the supply of critically-needed traditional medicines through sustainable sourcing [25, 26, 29, 32, 33] for the majority of patients in the world. That those situations even exist in the 21st century reflect another deep-seated "madness", a failure to bring even a semblance of equality to our health-care priorities globally, and an irrational view of natural resource sustainability, namely, the enduring "myth" that the plants will always be there.

When we speak of "sustainable medicines", the reference is to both natural and synthetic medicinal agents [25-28]. For the patient, accessibility to treatment is critical for healing, and must embrace both affordability and sustainability. In addition, the patient requirement is for a safe and effective regimen for healing; the source of the medicine is irrelevant. The costs of allopathic medicines have risen well beyond the index of inflation [48]. Stunningly, there are a number of medical treatment protocols which have been approved by the USFDA in recent years where the annual patient/insurance company cost is in excess of $200,000 [49]. Meanwhile, at the global level, we are unable to deliver the simple drug aspirin (at less than 5 cents per dose) to those in pain. Drug resistance against cancer chemotherapeutics, antibiotics, antimalarials, anti-AIDS, and other infectious diseases is increasing steadily, but is not being addressed as a priority by the global pharmaceutical drug companies for economic reasons. Similarly, systematic drug discovery for the global major killer diseases and for tropical (orphan) diseases is not on the radar of Big Pharma, or even WHO; although the Gates Foundation and a recent Drugs for Neglected Diseases Initiative (www.dndi.org) will hopefully be impactful over time. Typically, and quite absurdly, these health care issues have been deemed to be addressed by the countries where the disease is prevalent; a truly inhumane and unethical decision with unrealistic expectations when only a handful of countries has the capacity to "discover" new medicinal agents for therapeutic use. Their typical resource is their plants and the medicinal knowledge associated with them, accumulated over time.

Let us return to our allegoric tale and to the sustainability of plant-based traditional medicine systems. It is fascinating that the dodo is one of the animals mentioned and depicted in *AAIW*; speculated to

be because of Do-do-Dodgson's stammer [3]. The dodo, endemic to Mauritius, in 1662 became the first recorded extinction of a species caused by the actions of humans. It serves here as a profound reminder of the fragility of nature, and of the facile detrimental outcomes of the irrational urge for human dominance over nature. Today, there is a global system in place actively reviewing and recording the status of disappearing animals, plants, and other species, under the auspices of the International Union for Conservation of Nature. On these Red Lists of threatened and endangered species are (as of 2011) an estimated 9,568 plants of 61,900 recognized medicinal plant species. One of the priority activities recommended by WHO [40, 41, 50] is for countries to invest in assessing the availability and projected future access and supply chain for the needed TMs for their respective systems; some have done so [51, 52]. In the former instance, 39 of the most widely-used medicinal plant species were classified as threatened. The European Union has also recently issued a Red List of medicinal plants [53]. It was estimated that 31% of the medicinal plant species were in decline, principally due to wild-crafting.

The most powerful biological effects in *AAIW* are those produced by the mushroom, which is introduced to Alice by the caterpillar, who sits atop it, smoking a hookah. For us, Caterpillar is the local medicine practitioner, curandero, hakim, shaman, etc. The left part of the mushroom causes Alice to grow larger, while the right part causes her to diminish in size. She eats these parts in turn to "control" her height. The mushroom represents what in pharmacognosy are known to be the very powerful attributes of biologically active natural products; on one hand to promote disease, to cause profound toxicity, even death, and on the other to overcome toxicity, treat disease, and restore homeostasis.

The mushroom is also an allegory for the impact of what we ingest (physically and metaphorically) on global population dynamics. Over the past two centuries, humanity has been taking the left part of the mushroom, which has allowed the global population to grow larger (to 7.4 billion in 2015), stay healthier, have more mothers and children surviving, and to live longer. Disease, famine, war, natural events, and some naturally-derived products for male and female fertility regulation, and social and personal reassessments of family size have caused a modulated population growth, or even a diminishing population, as seem in countries like Russia, Japan, Germany, and Bulgaria [54].

Those are effects derived from the right part of the mushroom. The mushroom also suggests that, through nature, an acceptable balance for Earth's population can be found. It is distressingly clear that such a balanced view of humanity is not on the agenda of any of the major international agencies or religions at this time. Maybe their leaders have not yet met any White Rabbits with watches indicating how behind the current time they are! For the dream seems to be of an Earth filled beyond sustainable capacity with healthy people. In this regard, Caterpillar asks Alice, *"What size do you want to be?"* As a human species, what is our answer for Earth's population? We have already passed, or are close to passing, several tipping points for a sustainable population of Earth [55-57], such is the measure of our "overshoot". This occurs when the population exceeds the carrying capacity that an environment can maintain indefinitely. Even optimistic estimates for what population Earth could support for extended periods are low (ca. 4 billion; passed in July, 1974) based on an ecological footprint, and much lower (ca. 1 billion; passed in August, 1804) based on a thermodynamic footprint [58].

Alice takes the left part of the mushroom that makes her grow taller to a point where she completely fills the little room she is in and then can't move. *"There's no room to grow up any more here"* she

cries out. Some of the major cities in the world (Beijing, Manila, Delhi, Sao Paolo, Dhaka) are like that now, houses are cramped, streets are thronged with people, cars cause endless traffic jams, and combined with industrialization, produce stunning levels of pollution, and in war zones, refugees live with many families to a single tent. Like Alice, we too are losing our freedom of movement. Earth's population is projected to rise to 10 billion by 2045. The oceans are rising, and are becoming distinctly more acidic, as a result of climate modulation (global warming) [59-61]. Major cities, such as Bangkok [62] and Venice [63], are sinking at alarming rates, and the future fate of needed medicinal plants in low-lying regions of the world is poorly studied [64, 65]. We can all shed tears, buckets of them, as we travel and observe what has happened to the state of our planet in our lifetimes. So when Alice swims in the pool of her tears with the dormouse (now an endangered species in England, by the way) she does so reflecting our tears for our environmental and biodiversity losses, now, and for the future generations. In terms of health care and medicinal agents, there are no solutions at present for delivering traditional and synthetic medicines in a sustainable manner to this exploding patient base.

Interestingly, and as a slight diversion from the main discussion, there are a number of direct associations with pharmacognosy present in the original drawings for *AAIW* by John Tenniel. There is for example a foxglove depicted in the meeting with the Cheshire Cat, and also when Alice is carrying the piglet. Then there is the powerful mushroom upon which the caterpillar sits, and chamomile is also cited. There is also the reference to the children down a well living on treacle, which was originally an extract of medicinal plants. And there is the warning on the first bottle that Alice encounters which is labeled *"Drink me"*. *"Better read it first, for if one drinks too much from a bottle marked "Poison", it's almost certain to disagree with one sooner or later"*, says Alice. This is an admirable admonition (read the label!) to the patient taking any medicine, particularly one where the plant material is known to have toxic effects at higher doses. It is a reminder too that the notion of "more is better", does not apply to the dosing of traditional medicines, a topic in itself of great controversy. With the differential effects of the mushroom evident, the important relationship between stereochemistry (handedness) and biological activity is also raised, where one enantiomer may have a completely different, even opposite biological effect, than the other enantiomer [66-68].

At one point in the narrative, Alice ponders the question, *"What is a "mustard?"* Is it a mineral, an animal, or a vegetable? A reasonable question for a young, sheltered girl from an "upstairs" environment, for which the answer is actually not trivial, beyond being "vegetable" [69]. Her question though is a reminder to consider, and be grateful for, the items in our daily lives which we take for granted, and which are derived from nature without us thinking about them. In a contemporary context, consider, for example, how many readers of this article are aware of the origins and processing of cochineal and its uses or its' potential for sustainable development to provide the natural rouges and lipsticks for the faces of an expanding world? [70, 71].

Speaking of red, Alice witnesses the presaging of GMOs, for there in the garden are three playing cards (spades, of course) painting the white roses red at the behest of the Queen. Biotechnology has revealed approaches to a new scenario, where colors and biologically useful metabolites can be modulated and potentiated in biosynthetic terms, and deleterious compounds minimized or eliminated in content; a highly effective demonstration of the

principles of ecopharmacognosy, since expensive methods of separation and purification can be reduced.

Gryphons (griffins) are, according to legend, half eagle, and half lion. They are characterized by their power, extreme alertness and being stirred into rapid and fatally aggressive action at the slightest sound or movement, so as to alert the community. In *AAIW*, Carroll's gryphon is dozing, in spite of everything going on around. In our allegory, this is a clear reference to our lack of awareness and active concern, of not seeing and hearing the long standing, and increasingly loud indications from Nature itself, and those humanistic scientists concerned about nature, that aggressive corrective action in our behavior is needed.

Perhaps the most relevant scene to Earth's contemporary situation in *AAIW* is the Mad Hatters' Tea Party, where the time never changes (it is always six o'clock, and thus the time for tea). At the tea party, the participants (the Mad Hatter, the March Hare, and Dormouse) move around the table at each tea time, and always a new tea service is used. It is a classic example of our profligate consumption, as we move around our substantially disposable, non-reusable world, and of how, only very slowly, is global thinking at the political level concerning the sustainability and re-using of our resources (washing the dishes in *AAIW*) evolving. This scene can also be viewed as illustrating that changing time, actually moving through time as the world turns, requires the continuous reassessment of the *status quo*, whereas if time stands still, so does the *status quo*. For traditional medicines, it is this very *status quo* situation, which must shift to a patient-centered perspective for the benefit of humanity; we are already two days too slow (!), and need to move to a different time where the product and science and technology coalesce for the benefit of the patient. We, in consideration of future generations, have to wake up, "smell the coffee", and change our habits and practices to improve the role of traditional medicine in global health care.

The Preamble to the Constitution of the World Health Organization [72] states that "the highest attainable standard of health is one of the fundamental rights of every human being without distinction of race, religion, political belief, economic or social condition". Only when a population is healthy and not ravaged by disease, whether acute (Ebola) or chronic (malaria), can social and economic development be linked to effective and appropriate resource use [73]. About 80% of the plant resources used for traditional medicines in commerce are wild-crafted, and many are typically offered in an open manner in a local market, with little or no quality control, marginal concerns for safety, and almost no consideration afforded as to the age of the plant, and its changing efficacy and consistency. For most of the population of the world, this scene *is* their primary health care *status quo*, and has been for at least 4,000 years since the times of the early Chinese and Persian physicians until the present day [28]. As a global natural product health care community, we should be deeply embarrassed and ashamed by this appalling patient care situation.

In developed countries, the status of a patient-centric system for dietary supplements and traditional medicines is no better (and in some respects is even worse). A recent action by the Attorney General for New York State offers a glimmer of hope that even in the United States, where dietary supplement quality control is notoriously poor, and that of traditional medicines even worse, positive movement is possible [74]. In this instance, four large companies, GNC, Walgreen's, Walmart, and Target, were determined (through some not particularly well-conducted science) to have sold fraudulent dietary supplements; indeed 80% of the

package contents were not as labeled. Stunningly, several industry-related groups [75], not the retailers, went on the defensive, rather than acknowledging that, irrespective of the quality of the science, this is a huge, long-standing, industry-wide issue. Numerous instances of fraud in dietary supplements have been identified recently [76-83]. When products are recalled by the FDA, they may remain on the market for over four years and still contain synthetic adulterants, including those products from US manufacturers [84]. However, it seems unlikely that, absent US Congressional action, concerted industry reform in terms of research investment and an enhanced commitment to real quality control (QSEC) will follow voluntarily. The present system is based entirely on the trust between the manufacturer and the patient; a trust which is now essentially dissipated. From the patient perspective, the opportunity for the dietary supplement manufacturing industry to change effectively the *status quo* as an evidence-based commitment to US patient health care has been lost. Similarly to when Alice is "lost" in Wonderland, the situation begs the very core question for the global traditional medicine and the dietary supplement industry:

"Would you tell me, please, which way I ought to go from here?" asked Alice. "That depends a good deal on where you want to get to," said the Cat.

Louis Sullivan, perhaps the most famous early American architect, who was based in Chicago, wrote, in an essay in 1896 concerning the design of tall buildings, that "….form ever follows function. That is the law." [85]. In practical terms, therefore, know what the building is for, and then design its form to meet the functional requirements. So what then is to be the perceived function of (eco)pharmacognosy? For what, as a conglomeration of diverse and integrated scientific applications, will it accept responsibility in terms of meeting the needs of those patients who choose to take natural products for their health care? Will it redefine itself, as this author has urged for more than 25 years, or will it forever be engulfed at the junction of *status quo* and societal health care responsibility; frozen at, indeed by, the abyss of change?

Traditional medicines, TM practitioners, and their patients around the world are in a state of deep flux, moving down the long pathway of information and experimentation from an inherently knowledge-based system, to an experience-based system, and eventually to an evidence-based system. It is well-known that the existing frameworks for the development, regulation, quality control, and sale of traditional medicines, dietary supplements and phytotherapeuticals, in spite of burgeoning sales, are not scientifically or societally acceptable because they are not patient-centered. The patient, who we as individuals all are, should be assured of the continued availability of quality, safe, effective, consistent (QSEC), and affordable traditional medicine products based on the results of evidence-based research and sustainability.

In the mid-1950s, before the global Japanese brands were renowned for their product quality, W. Edwards Deming, an American management expert, introduced a focus on continuous quality improvement to enhance the performance and product quality of Japanese industry [86]. One of his aphorisms from that time is "Quality begins with intent". Another dictum is that "We have lived in a world…… of defective products. It is time to adopt a new philosophy." [86]. In terms of the global quality control of traditional medicines and dietary supplements, we are still stuck living in a time of defective products. Moreover, the manufacturing industry, as the New York case showed, cannot grasp that it is their responsibility to change voluntarily their practices and their products to lead the improvements that are anticipated by the

patient. The *status quo* places the patient in the unacceptable position of "buyer beware", with no reliable resources to consult. Now is indeed the time for a new philosophy, a new intent for the quality of traditional medicines and dietary supplements; the existing paradigm must change [13, 28, 29, 31, 32, 33]. The patient must be assured of QSEC in a trustworthy product as an essential moral and ethical responsibility. Less than that is an ethical misstep by the manufacturer, the provider, and a moral dilemma for the practitioner. Therefore, evidence-based research, focused on the development of sustainably-resourced, standardized traditional medicines and dietary supplements, as well as those essential oils used in aromatherapy and cosmeceutical products, is fundamental. In this way, appropriate biological and clinical studies can be conducted for the health benefit of the patient, and the assurances of the practitioner. There is a need also to label products, in an appropriate manner, to reflect the standards being applied to placing the product in the market place, and how sustainable is its sourcing.

There are numerous practical examples of ecopharmacognosy. Long-term resourcing of traditional medicines (and those for drug discovery) is an early stage development question. Consideration of a bark or a root material offers very different long-term challenges for sustainability than the corresponding development of a leaf or fruit. Following the principles of "green chemistry", ecopharmacognosy could embrace six applicable and practical principles [26, 87], so that the global use of plants in traditional medicine can transition a "forest economy" to a "field economy", just as coffee has done [88-95]. Some other examples of the practical application of ecopharmacognosy include: i) the improved use of information systems to delineate what has been done and what needs to be done (not wasting resources by reinventing the wheel!); ii) reducing the energy requirements for plant material extraction; iii) reducing the reliance on non-recyclable chromatographic supports and solvents; iv) determining the significance of individual plants in complex plant matrices to conserve species use; v) using network pharmacology [96, 97] to develop new uses for established and sustainable plants, vi) studying the waste products of the industrial processing of plants for new applications; vii) developing natural pesticides and insecticides from renewable resources, viii) assessing whole organisms, plants, and microbial systems as renewable catalytic reagents showing high yield and high enantioselectivity for organic chemical processes [98-101]; ix) re-energizing the development of natural dyestuffs from sustainable resources; and x) investigating well-established commercial crops, such as turmeric, ginger, oregano, garlic, cinnamon, caraway, etc. for new biological responses. One recent study found that an aqueous extract of caraway seed could have an obesity-reducing effect [102, 103]. Finally, microfluidic, lab-on-a-chip approaches to the chemical and biological assessment of plant samples in the field (previously referred to as "pharmacognosy in a suitcase") [25-27, 29, 30, 34] would save significantly on the drying, transportation, and macro-analytic processes required to evaluate the optimal time for accessing plant materials for therapeutic purposes based on the levels of their bioactive agents. Similarly, hyperspectral imaging using drone technology and/or remote sensing devices based on Raman technology could play a significant role in the field determination of important biological molecules, as has already been achieved with collagen [104, 105].

Conclusions
Using the allegories based on *Alice's Adventures in Wonderland*, the dreamt discovery of the structure of benzene, and the globalization of coffee, new insights regarding the future, evidence-based directions of traditional medicine, are presented from the perspective of patient needs and expectations.

References

[1] Kekulé FA. (**1865**) Sur la constitution des substances aromatiques. *Bulletin de la Societé Chimique de Paris*, **3**, 98-110.

[2] Kekulé FA. (**1866**) Untersuchungen über aromatische Verbindungen. *Liebigs Annalen der Chemie und Pharmacie*, **137**, 129-136.

[3] Carroll, L. (**1999**) *The Annotated Alice. The Definitive Edition*. Gardner M. (Ed). W.W. Norton Company, Inc. New York, NY, USA, pp. 352.

[4] The Rockefeller Foundation-*Lancet* Commission on Planetary Health. *Safeguarding human health in the Anthropocene epoch: report of The Rockefeller Foundation–Lancet Commission on planetary health*. July 16, 2015, pp. 56. Available at http://www.thelancet.com/commissions/planetary-health. Accessed on July 17, 2015.

[5] Cordell GA, Colvard MD. (**2012**) Natural products and traditional medicine - turning on a paradigm. *Journal of Natural Products*, **75**, 514-525.

[6] Hofmann AW. (**1845**) Über eine sichere Reaction auf Benzol. *Annalen der Chemie und Pharmacie*, **55**, 200-205.

[7] http://riverbasinenergy.com/pages/the-future-of-coal/how-long-will-coal-last.php. Accessed on July 9, 2015.

[8] Vega FE. (**2008**) The rise of coffee. *American Scientist*, **96**, 138-145.

[9] http://www.history.co.uk/study-topics/history-of-london/londons-coffee-houses. Accessed July 9, 2015.

[10] Talbot JM. (**2004**) *Grounds for Agreement: The Political Economy of the Coffee Commodity Chain*. Rowman & Littlefield, Lanham, MD, USA, pp. 50.

[11] Janzen DH. (Ed) (**1983**) *Costa Rican Natural History*. University of Chicago Press, Chicago, IL, USA, pp. 823.

[12] http://environment.nationalgeographic.com/environment/freshwater/change-the-course/water-footprint-calculator/. Accessed July 9, 2015.

[13] Cordell GA. (**2015**) Ecopharmacognosy and the globalization of traditional medicines. *International Journal of Traditional Knowledge*, in press.

[14] Cordell GA. (**1987**) Pharmacognosy: far from dead. *American Druggist,* March, 96-98.

[15] Cordell GA. (**1987**) Pharmacognosy: far from dead. *Thai Journal of Pharmaceutical Sciences*, **12**, 221-224.

[16] Cordell GA. (**1990**) Pharmacognosy - a high tech pharmaceutical science. *Pharmacia*, **30**, 169-181.

[17] Cordell GA. (**1993**) Pharmacognosy - new roots for an old science. In *Studies in Natural Products Chemistry, Volume 13. Bioactive Natural Products (Part A)*, Atta-ur-Rahman, Basha FZ, (Eds), Elsevier, Amsterdam, The Netherlands, 629-675.

[18] Cordell GA. (**1995**) Changing strategies in natural products chemistry. *Phytochemistry*, **40**, 1585-1612.

[19] Cordell GA. (**2000**) Biodiversity and drug discovery - a symbiotic relationship. *Phytochemistry*, **55**, 463-480.

[20] Cordell GA. (**2001**) The yin and yang of natural products in the new millennium, *Acta Manilana*, **49**, 1-4.

[21] Cordell GA. (**2004**) Accessing our gifts from nature, now and in the future. Part III., *Revista Química, 19*, 33-41.

[22] Cordell GA, Colvard MD. (**2005**) Some thoughts on the future of ethnopharmacology. *Journal of Ethnopharmacology*, **100**, 5-14.

[23] Cordell GA, Colvard MD. (**2007**) Natural products in a world out-of-balance., *Arkivoc **vii***, 97-115.

[24] Cordell GA. (**2008**) Natural products research - a view through the looking glass. *Science and Culture*, **74**, 11-16.

[25] Cordell GA. (**2009**) Sustainable drugs and global health care. *Química Nova*, **32**, 1356-1364.

[26] Cordell GA. (**2011**) Sustainable medicines and global health care. *Planta Medica*, **77**, 1129-1138.

[27] Cordell GA. (**2011**) Phytochemistry and traditional medicine - a revolution in process. *Phytochemistry Letters*, **4**, 391-398.

[28] Cordell GA. (**2011**) Plant medicines key to global health. *Chemical & Engineering News*, June 27, 52-56.

[29] Cordell GA. (**2012**) New strategies in traditional medicine. In *Medicinal Plants: Diversity and Drugs*. Rai M, Cordell GA, Martinez JL, Marinoff M, Rastrelli L. (Eds). CRC Press, Boca Raton, FL, USA, 1-45.

[30] Cordell GA. (**2014**) Ecopharmacognosy: exploring the chemical and biological potential of nature for human health. *Journal of Biology and Medicinal Natural Product Chemistry*, **4**, 1-21.

[31] Cordell GA. (**2014**) Phytochemistry and traditional medicine - the revolution continues. *Phytochemistry Letters*, **10**, 28-40.

[32] Cordell GA. (**2015**) Ecopharmacognosy - the responsibilities of natural product research to sustainability. *Phytochemistry Letters*, **11**, 332-346.

[33] Guo DA, Wu W-Y, Ye M, Liu X, Cordell GA. (**2015**) A holistic approach to the quality control of traditional Chinese medicines. *Science*, **347**, S29-S31.

[34] Cordell GA. (**2015**) Ecopharmacognosy. In *Ethnopharmacology – A Reader*. Heinrich M, Jäger A. (Eds). Wiley Science Publishers, Chichester, UK, in production.

[35] Brito L, MS Smith MS. (**2012**) State of the planet declaration. In *Proceedings of the 'Planet under Pressure' Conference, London*. 26-29.

[36] Akerele O, Heywood, VH. (**1991**) *The Conservation of Medicinal Plants: Proceedings of an International Consultation, 21-27 March 1988 held at Chiang Mai, Thailand*. Cambridge University Press, Cambridge, UK, pp. 384.

[37] Doomsday Clock. (**2015**) https://en.wikipedia.org/wiki/Doomsday_Clock. Accessed July 9, 2015.

[38] WHO. (**2002**) http://whqlibdoc.who.int/hq/2002/WHO_EDM_TRM_2002.1.pdf. Accessed on July 9, 2015.

[39] WHO. (**2008**) http://www.who.int/medicines/areas/traditional/congress/beijing_declaration/en/. Accessed on July 9, 2015.

[40] WPRO. (**2011**) http://www.wpro.who.int/traditional_medicine/about/en/. Accessed on July 9, 2015.

[41] WHO. (**2014**) http://www.who.int/medicines/publications/traditional/trm_strategy14_23/en/. Accessed on July 9, 2015.

[42] World Health Organization. (**1998**) *The World Health Report 1998: Life in the 21st Century A Vision for All*. World Health Organization, Geneva, Switzerland, pp. 241.

[43] Evans T, Whitehead M, Diderichsen F, Bhuiya A, Wirth M, Whitehead M. (**2001**) *Challenging inequities in health: from ethics to action*. Oxford University Press, Oxford, UK, pp. 368.

[44] Lee J.-W. (**2003**) Global health improvement and WHO: shaping the future. *The Lancet*, **362**, 2083-2088.

[45] Frenk J, Chen L. (**2011**) Overcoming gaps to advance global health equity: a symposium on new directions for research. *Health Research Policy and Systems*, **9**, 11.

[46] Balarajan Y, Selvaraj S, Subramanian SV. (**2011**) Health care and equity in India. *The Lancet*, **377**, 505-515.

[47] Holtz C. (Ed) (**2013**) *Global Health Care: Issues and Policies*. Jones & Bartlett Learning Publishers, Burlington, MA, USA, pp. 614.

[48] Thomas K. (**2012**) Brand name drug prices rise sharply, report says. http://www.nytimes.com/2012/11/29/business/cost-of-brand-name-prescription-medicines-soaring.html. Accessed on July 9, 2015.

[49] Williams S. (**2014**) The 5 most expensive drugs in the world. http://www.fool.com/investing/general/2014/10/26/the-5-most-expensive-drugs-in-the-world.aspx. Accessed on July 9, 2015.

[50] IUCN/WHO/WWF. (**1993**) Guidelines for the Conservation of Medicinal Plants. IUCN, Gland, Switzerland, pp. 38.

[51] Reddy KN, Reddy CS. (**2008**) First red list of medicinal plants of Andhra Pradesh, India - conservation assessment and management planning. *Ethnobotanical Leaflets*, **12**, 103-107.

[52] Williams VL, Victor JE, Crouch NR. (**2013**) Red listed medicinal plants of South Africa: status, trends, and assessment challenges. *South African Journal of Botany*, **86**, 23-35.

[53] Allen D, Bilz M, Leaman DJ, Miller RM, Timoshyna A, Window J. (**2014**) European Red List of Medicinal Plants. European Union, Luxembourg, pp. 75.

[54] https://en.wikipedia.org/wiki/List_of_countries_by_population_growth_rate. Accessed on July 11, 2012.

[55] Murray J, King D. (**2012**) Climate policy: oil's tipping point has passed. *Nature*, **481**, 433-435.

[56] Kiron D, Kruschwitz N, Haanaes K, von Streng Velken I. (**2012**) Sustainability nears a tipping point. *MIT Sloan Management Review*, **53**, 69-74.

[57] Boettiger C, Hastings A. (**2013**) Tipping points: from patterns to predictions. *Nature*, **493**, 157-158.

[58] Chefurka PS. (**2013**) Sustainability. http://www.paulchefurka.ca/Sustainability.html. Accessed on July 10, 2015.

[59] Orr JC, Fabry VJ, Aumont O, Bopp L, Doney SC, Feely RA, Gnanadesikan A, Gruber N, Ishida A, Joos F, Key RM, Lindsay K, Maier-Reimer E, Matear R, Monfray P, Mouchet A, Najjar RG, Plattner G-K, Rodgers KB, Sabine CL, Sarmiento JL, Schlitzer R, Slater RD, Totterdell IJ, Weirig M-F, Yamanaka Y, Yool A. (**2005**) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*, **437**, 681-686.

[60] Doney SC, Fabry VJ, Feely RA, Kleypas JA. (**2009**) Ocean acidification: the other CO_2 problem. *Annual Review of Marine Science*, **1**, 169-192.

[61] McCulloch M, Falter J, Trotter J, Montagna P. (**2012**) Coral resilience to ocean acidification and global warming through pH up-regulation. *Nature Climate Change*, **2**, 623-627.

[62] Phien-Wej N, Giao PH, Nutalaya P. (**2006**) Land subsidence in Bangkok, Thailand. *Engineering Geology*, **82**, 187-201.

[63] Lewis T. (**2013**) Venice's gradual sinking charted by satellites. http://www.livescience.com/39979-venice-gradual-sinking-charted-by-satellites.html. Accessed on July 11, 2015.

[64] Kirilenko AP, Sedjo RA. (**2007**) Climate change impacts on forestry. *Proceedings of the National Academy of Sciences*, **104**, 19697-19702.

[65] Cavaliere C. (**2009**). The effects of climate change on medicinal and aromatic plants. *Herbal Gram*, **81**, 44-57.

[66] Waldeck B. (**1993**) Biological significance of the enantiomeric purity of drugs. *Chirality*, **5**, 350-355.

[67] Biellmann JF. (**2003**) Enantiomeric steroids: synthesis, physical, and biological properties. *Chemical Reviews*, **103**, 2019-2034.

[68] Garrison AW. (**2006**) Probing the enantioselectivity of chiral pesticides. *Environmental Science & Technology*, **40**, 16-23.

[69] Antol MN. (**1999**) *The Incredible Secrets of Mustard: The Quintessential Guide to the History, Lore, Varieties, and Healthful Benefits of Mustard*. Avery Publishing Group, Knoxville, TN, USA, pp. 228.

[70] Eisner T, Nowicki S, Goetz M, Meinwald J. (**1980**) Red cochineal dye (carminic acid): its role in nature. *Science*, **208**, 1039-1042.

[71] Campana MG, Robles García NM, Tuross N. (**2015**) America's red gold: multiple lineages of cultivated cochineal in Mexico. *Ecology and Evolution*, **5**, 607-617.

[72] WHO (**1946**) The Constitution of the World Health Organization. http://www.who.int/governance/eb/who_constitution_en.pdf. Accessed on July 11, 2015.

[73] Mitchell RJ, Bates P. (**2011**) Measuring health-related productivity loss. *Population Health Management*, **14**, 93-98.

[74] O'Connor A. (**2015**) New York Attorney General Targets Supplements at Major Retailers. New York Times, February 3. http://well.blogs.nytimes.com/2015/02/03/new-york-attorney-general-targets-supplements-at-major-retailers/?_r=0. Accessed on July 12, 2015.

[75] http://www.nutraceuticalsworld.com/contents/view_breaking-news/2015-02-03/new-york-ag-targets-herbal-supplements-at-major-retailers/. Accessed on July 12, 2015.

[76] Cohen PA, Benner C, McCormick D. (**2012**) Use of a pharmaceutically adulterated dietary supplement, Pai You Guo, among Brazilian-born women in the United States. *Journal of General Internal Medicine*, **27**, 51-56.

[77] Harel Z, Harel S, Wald R, Mamdani M, Bell CM. (**2013**) The frequency and characteristics of dietary supplement recalls in the United States. *JAMA Internal Medicine*, **173**, 929-930.

[78] Posadzki P, Watson L, Ernst E. (**2013**) Contamination and adulteration of herbal medicinal products (HMPs): an overview of systematic reviews. *European Journal of Clinical Pharmacology*, **69**, 295-307.

[79] Wheatley VM, Spink J. (**2013**) Defining the public health threat of dietary supplement fraud. *Comprehensive Reviews in Food Science and Food Safety*, **12**, 599-613.

[80] Campbell N, Clark JP, Stecher VJ, Thomas JW, Callanan AC, Donnelly BF, Goldstein I, Kaminetsky JC. (**2013**) Adulteration of purported herbal and natural sexual performance enhancement dietary supplements with synthetic phosphodiesterase type 5 inhibitors. *The Journal of Sexual Medicine*, **10**, 1842-1849.

[81] Song F, Monroe D, El-Demerdash A, Palmer C. (**2014**) Screening for multiple weight loss and related drugs in dietary supplement materials by flow injection tandem mass spectrometry and their confirmation by liquid chromatography tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, **88**, 136-143.

[82] Zhu Q, Cao Y, Cao Y, Chai Y, Lu F. (**2014**) Rapid on-site TLC–SERS detection of four antidiabetes drugs used as adulterants in botanical dietary supplements. *Analytical and Bioanalytical Chemistry*, **406**, 1877-1884.

[83] Gilard V, Balayssac S, Tinaugus A, Martins N, Martino R, Malet-Martino M. (**2015**) Detection, identification and quantification by ^{1}H NMR of adulterants in 150 herbal dietary supplements marketed for improving sexual performance. *Journal of Pharmaceutical and Biomedical Analysis*, **102**, 476-493.

[84] Cohen PA, Maller G, DeSouza R, Neal-Kababick J. (**2014**) Presence of banned drugs in dietary supplements following FDA recalls. *Journal of the American Medical Association*, **312**, 1691-1693.

[85] Sullivan LH. (**1896**) The tall office building artistically considered, *Lippincott's Magazine*, March.

[86] Deming WE. (**2000**) *Out of the Crisis*. MIT Press, Cambridge, MA, USA, pp. 507.

[87] Cordell GA, Michel J. (**2007**) Sustainable drugs and women's health. In *Proceedings of the Third Women's Health and Asian Traditional Medicine Conference and Exhibition*. Rao AN. (Ed), Kuala Lumpur, Malaysia, 15-27.

[88] Schippmann UWE, Leaman D, Cunningham AB. (**2006**) A comparison of cultivation and wild collection of medicinal and aromatic plants under sustainability aspects. *Frontis*, **17**, 75-95.

[89] Lange D. (**2004**) Medicinal and aromatic plants: trade, production and management of botanical resources. *Acta Horticulturae*, **629**, 177-197.

[90] Anastas PT, Warner JC. (**1998**) *Green Chemistry: Theory and Practice*. Oxford University Press, New York, NY, USA, pp. 152.

[91] De Silva T. (**1997**) Industrial utilization of medicinal plants in developing countries. In *Medicinal Plants for Forest Conservation and Health Care*. FAO, Rome, Italy, 34-44.

[92] Dubey NK, Kumar R, Tripathi P. (**2004**) Global promotion of herbal medicine: India's opportunity. *Current Science*, **86**, 37-41.

[93] Hamilton AC. (**2004**) Medicinal plants, conservation and livelihoods. Biodiversity *and Conservation*, **13**, 1477-1517.

[94] Canter PH, Thomas H, Ernst E. (**2005**) Bringing medicinal plants into cultivation: opportunities and challenges for biotechnology. *Trends in Biotechnology*, **23**, 180-185.

[95] Lubbe A, Verpoorte R. (**2011**) Cultivation of medicinal and aromatic plants for specialty industrial materials. *Industrial Crops and Products*, **34**, 785-801.

[96]   Li S, Zhang B. (**2013**) Traditional Chinese medicine network pharmacology: theory, methodology and application. *Chinese Journal of Natural Medicines*, **11**, 110-120.

[97]   Zheng CS, Xu XJ, Ye HZ, Wu GW, Li XH, Xu HF, Liu XX. (**2013**) Network pharmacology-based prediction of the multi-target capabilities of the compounds in Taohong Siwu decoction, and their application in osteoarthritis. *Experimental and Therapeutic Medicine*, **6**, 125-132.

[98]   Sousa JSN, Machado LL, de Mattos MC, Solange S, Lemos TLG, Cordell, GA. (**2006**) Bioreduction of aromatic aldehydes and ketones using *Manihot* species, *Phytochemistry*, **67**, 1637-1643.

[99]   Cordell GA, Lemos TLG, Monte FJQ, de Mattos MC. (**2007**) Vegetables as chemical reagents. *Journal of Natural Products*, **70**, 478-492.

[100]   Assunção JCC, Machado LL, Lemos TLG, Cordell GA, Monte FJQ. (**2008**) Sugar cane juice for the reduction of carbonyl compounds, *Journal of Molecular Catalysis B – Enzymatic*, **52-53**, 194-198.

[101]   Fonseca AM, Monte FJQ, Braz-Filho R, de Mattos MC, Oliveira MCF, Cordell GA, Lemos TLG. (**2009**) Coconut juice (*Cocos nucifera*) - a new biocatalyst system for organic synthesis. *Journal of Molecular Catalysis B – Enzymatic*, **57**, 78-82.

[102]   Kazemipoor M, Radzi CJM, Hajifarji M, Hearian B, Mosadegh M, Cordell, GA. (**2013**) Anti-obesity effect of caraway extract on overweight and obese women: a randomized triple-blind, placebo-controlled clinical trial. *Evidence-based Complementary and Alternative Medicine*, id 928582, 8 pp.

[103]   Kazemipoor M, Radzi CJM, Hajifarji M, Cordell GA. (**2014**) Preliminary safety evaluation and biochemical efficacy of *Carum carvi*: a triple-blind, placebo-controlled clinical trial. *Phytotherapy Research*, **28**, 1456-1460.

[104]   Pestle WJ, Ahmad F, Vesper B, Cordell GA, Colvard MD. (**2014**) Ancient bone collagen assessment by hand-held vibrational spectroscopy. *Journal of Archaeological Science*, **42**, 381-389.

[105]   Pestle WJ, Brennan V, Sierra R, Smith EK, Vesper BJ, Cordell GA, Colvard MD. (**2015**) Hand-held Raman spectroscopy as a prescreening tool for archaeological bone. *Journal of Archaeological Science*, **58**, 113-120.

Additions and Corrections

Title: Cytotoxic and Antimalarial Alkaloids from the Twigs of *Dasymaschalon obtusipetalum*

Authors: Atchara Jaidee, Thanika Promchai, Kongkiat Trisuwan, Surat Laphookhieo, Roonglawan Rattanajak, Sumalee Kamchonwongpaisan, Stephen G. Pyne and Thunwadee Ritthiwigrom*

- After publishing the above paper we became aware of the following reference indicating that the correct name for the plant species that we studied is Dasymaschalon yunnanense and the name D. obtusipetalum is now regarded regarded as a heterotypic synonym.

  Guo, X., Wang, J., Xue, B., Thomas, D.C., Su, Y. C. F., Tan, Y. H., Saunders, R. M. K., "Reassessing the taxonomic status of two enigmatic *Desmos* species (Annonaceae): Morphological and molecular phylogenetic support for a new genus, *Wangia*," *Journal of Systematics and Evolution*, **52**, 1-15 (2014).

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Volume 10, Number 12 (2015)

Natural Product Communications

2015   **Volume 10**

Natural Product Communications

Contents of Volume **10**
2015

Number 1

10th Anniversary issue

Accounts/Reivews

Number 2

NPC-CSPR issue

Original Paper

Number 3

Flavonoids: Colors, Functions and Activities
(Guest Editor: Tsukasa Iwashina)

Number 4

Number 5

Metabolism and Biological Function of Natural Products in Plants
(*Guest editor: Hiroshi Ashihara*)

Number 8

Accounts/Reviews

Number 9

Number 10

Natural Products from Endophytes
(Guest Editors: John A. Johnson and Christopher A. Gray)

Original Paper

Accounts/Reviews

Accounts/Reviews

Number 11

Re-Discovery of the Plant Kingdom as a Valuable Source of Novel Drugs
(Guest Editor: Francesco Epifano)

Additions/Corrections

Number 12

Accounts/Reviews

Additions/Corrections

Natural Product Communications

Volume **10** (1-12)
2015
Author Index

Natural Product Communications

Volume **10** (1-12)
2015
Keywords Index

Accounts/Reviews

Additions/Corrections

Cummulative Index

Natural Product Communications
2015

Volume 10, Number 12

Contents