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Skeleton-Rearranged Pentacyclic Diterpenoids Possessing a Cyclobutane Ring from *Euphorbia wallichii*

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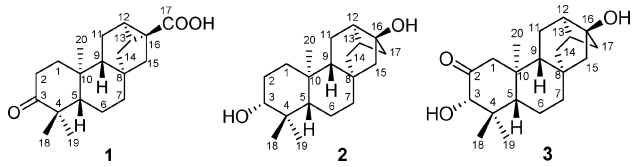
ABSTRACT



Two novel rearranged trachylobane diterpenoids, designated as wallichanol A (2) and wallichanol B (3), consisting of an unprecedented pentacyclic skeleton named wallichane with a cyclobutane ring, and a new *ent*-trachylobane diterpenoid, 3-oxo-*ent*-trachyloban-17-oic acid (1), were isolated from the roots of *Euphorbia wallichii*. Their structures were elucidated by comprehensive analysis of 2D-NMR spectroscopic data, with the stereochemistry of 1 confirmed by X-ray crystallographic study. All of these compounds potently block osteoclastogenesis in vitro, suggesting a potential therapeutic application in prevention of osteoporosis.

In continuation of our efforts to identify biologically active components from medicinal plants, we extensively investigated the constituents of the plant *Euphorbia wallichii* Hook. f. Fl., a perennial shrub growing mainly in the Himalayas, including Qinghai-Tibetan Plateau area of China, India, Nepal, and Kashmir.[1] The roots of this plant have been traditionally used in Tibetan folk medicine for treatment of edema and skin disease such as furuncle, exanthema, and cutaneous anthrax. Previous phytochemical studies have revealed the presence of diterpenoids with a variety of skeletons such as *ent*-atisane, *ent*-kaurane, *ent*-arbitane, and lathrane.[2] Herein, we report the isolation, structure elucidation, and bioactivity of a new *ent*-trachylobane acid, **1**, and two novel pentacyclic diterpenoids, **2** and **3**, consisting of an unprecedented rearranged carbon framework.

The roots of *E. wallichii* (30 kg) were collected in July 2003 in Lulang (at an altitude of 3600−4000 m) of the Tibet Autonomy Region, China. The fresh roots were cut into small pieces and then extracted with 90% EtOH. The pooled ethanolic extracts were concentrated under vacumm until most of the solvents were removed. The residue was then suspended in water and partitioned successively with EtOAc and *n*-butanol. An aliquot (180 g) of the EtOAc extract was subjected to silica gel column chromatography and eluted with a mixed solvent of $CHCl_3$/acetone with increasing polarity from 0 to 100% acetone to yield 10 fractions, denoted as Fr.1−10. Fr.4 was applied to an ODS silica gel

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Table 1. ^{13}C NMR (150 MHz, $CDCl_3$) Spectral Data for **1**−**3**

position	1	2	3
1	37.6t	37.8t	52.6t
2	33.9t	26.8t	210.7s
3	216.9s	79.1d	83.0t
4	47.6s	38.7s	45.7s
5	55.2d	55.4d	54.7d
6	20.9t	18.8t	19.0t
7	37.7t	40.7t	40.7t
8	39.9s	35.9s	36.2s
9	51.3d	53.7d	53.5d
10	37.8s	38.0s	44.3s
11	19.2t	19.5t	19.3t
12	25.3d	43.1s	42.8d
13	31.8d	25.8d	25.7d
14	32.2t	33.8t	33.3t
15	42.6t	53.5t	53.1t
16	29.8s	73.4s	73.2s
17	180.9s	47.6t	47.5t
18	26.1q	27.9q	29.3q
19	21.5q	15.4q	16.4q
20	14.2q	15.1q	15.9q

column (MeOH/H_2O, 6:4−1:0), and further purified on silica gel columns repeatedly with petroleum ether/acetone (10:1−2:1) or petroleum ether/EtOAc (10:1−1:1) as elution to afford compounds **1** (9 mg), **2** (2.0 mg), and **3** (4.2 mg), respectively.

The molecular formula of compound **1**[3] was determined as $C_{20}H_{28}O_3$ on the basis of the accurate sodiated-molecular ion peak at *m*/*z* 339.1931 $[M + Na]^+$ (calcd 339.1931) in HR-ESI-MS, indicating seven doube-bond equivelence (DBE). The IR absorptions at υ_{max} 3436, 1700, and 1671 cm^{-1} revealed the existence of carboxy and carbonyl groups. The ^1H NMR signals of **1** distributed in the relative upfield region around δ 1.0−2.6 ppm, wherein three tertiary methyl groups at δ 1.02, 1.06, and 1.10 (each 3H, s) were recognized. The DEPT ^{13}C NMR spectrum exhibited signals corresponding to three methyls, seven methylenes, four methines, and six quarternary carbons including a carboxyl at δ 180.9 and a ketone group at δ 216.9. The presence of a cyclopropane ring was deduced from the ^1H NMR spectrum, which exhibited two signals at δ 1.80 and 1.98 ppm (H-12 and H-13, respectively) and corresponding ^{13}C signals resonating at δ 25.3 (C-12) and 31.8 (C-13) ppm in the ^{13}C NMR spectrum. From these observations and by comparison with the NMR data from closely related structures,[4−6] it was concluded that compound **1** belongs to the *ent*-trachylobane family of diterpenes.

The position of the ketone group at C-3 was deduced from long-range $^1H-^{13}C$ correlations (HMBC) (key correlations

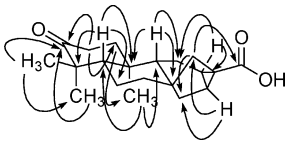

Figure 1. Key HMBC correlations of **1**.

depicted in Figure 1) between the protons at δ 2.56 (H-2α), 2.32 (H-2β), 1.06 (Me-18), and 1.02 (Me-19) and the ketone carbonyl signal at δ 216.9. Similarly, the location of carboxylic acid moiety was assigned to C-17 by HMBC correlations observed between the carboxyl carbonyl signal at δ_C 180.9 and each of the protons resonating at δ 1.80 (H-12) and 1.98 (H-13), 1.87 (H-15α) and 1.51 (H-15β). The full ^1H and ^{13}C NMR assignments were established by means of a combination of COSY, HMQC, HMBC, and NOESY spectral measurements (Tables 1 and 2). Finally, the structure including the relative stereochemistry of compound **1** was conclusively confirmed by X-ray crystallographic analysis. As shown in Figure 2, both the chair and boat conformers were coincidently adopted by ring A in the crystal lattice in a ratio 0.709:0.291. Thus, the structure of **1**

Table 2. ^1H NMR (600 MHz) Spectral Data for **1**−**3** in $CDCl_3$

position	1	2	3
1α	1.77, ddd (13.0, 7.0, 3.2)	1.56, m	2.40, d (12.0)
1β	1.29, m	0.93, m	2.06, d (12.0)
2α	2.56, ddd (15.8, 13.0, 6.9)	1.57, m	
2β	2.32, ddd (15.9, 6.1, 3.2)	1.60, m	
3		3.22, dd (11.0, 4.2)	3.90, br d (4.2)
5	1.26, br d (10.5)	0.81, br d (11.7)	1.52, br d (11.5)
6α	1.50, m	1.50, m	1.65, br d (12.9)
6β	1.50, m	1.35, m	1.42, m
7α	1.52, m	1.52, m	1.60, ddd (13.0, 3.0, 3.0)
7β	1.42, m	1.23, m	1.35, m
9	1.20, br dd (12.0, 6.5)	1.19, br dd (12.0, 6.0)	1.55m
11β	2.04, ddd (15.0, 12, 3.6)	1.76, ddd (15.0, 12.0, 3.0)	1.79, ddd (15.0, 11.5, 3.0)
11α	1.79, ddd (15, 6.5, 2.7)	1.27, ddd (15.0, 5.5, 2.4)	1.34, m
12	1.80, br d (8.7)	1.93, m	1.95, m
13	1.98, dd (8.4, 3.4)	2.04, q (6.7)	2.07, m
14a	2.17, d (12.4)	2.17, br dd (13.2, 6.6)	2.10, m
14b	1.31, dd (12, 3.6)	0.86, br d (13.4)	0.91, br d (13.2)
15α	1.87, d (12.0)	1.70, d (12.0)	1.74, d (12.2)
15β	1.51, d (12.0)	1.26, m	1.33, m
17a		1.95, m	1.97, m
17b		1.45, d (8.2)	1.48, d (8.4)
18	1.06, s	0.98, s	1.19, s
19	1.02, s	0.77, s	0.70, s
20	1.10, s	0.91, s	0.89, s

(3) Compound **1**: colorless prisms, mp 218−219 °C (MeOH); $[\alpha]^{20}_D$ −66° (*c* 0.05, $CHCl_3$); IR (KBr) ν_{max} 3436, 1700, 1671, 1456, 1433, and 1262 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m*/*z* (rel int) 317 $[M + H]^+$ (32), 339 $[M + Na]^+$ (89), and 655 $[2M + Na]^+$ (100).

(4) Arnone, A.; Mondelli, R.; Pyrek, J. St. *Org. Magn. Reson.* **1979**, *12*, 429−431.

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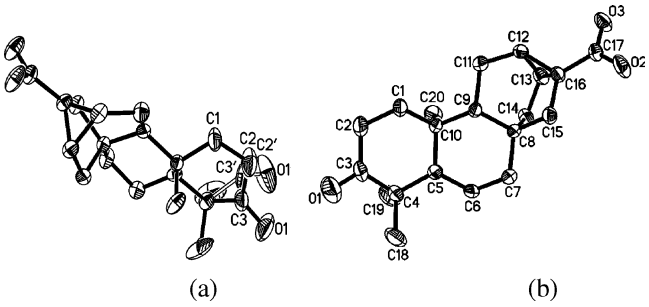

Figure 2. X-ray structure of **1**: (a) two conformations of A ring; (b) normal view.

was established as 3-oxo-*ent*-trachyloban-17-oic acid. It is notable that **1** represents the first member of *ent*-trachylobane diterpenes with a carboxylic acid group in positions other than ring A, whereas several *ent*-trachylobane-18-oic and *ent*-trachyloban-19-oic derivatives have been reported.[5−7]

Wallichanol A (**2**)[8] was shown to have a molecular formula $C_{20}H_{32}O_2$ as deduced from HRESIMS in which a sodiated molecular ion $[M + Na]^+$ was observed at *m*/*z* 327.2282 [calcd 327.2295], requiring five DBE. The DEPT ^{13}C NMR spectra (Table 1) revealed that **2** showed the following resonances: (i) four quarternary carbons including one oxygenated carbon (δ_C 73.4, C-16); (ii) five methines including one oxygenated carbon (δ_C79.1, C-3), thus accounting for both oxygen atoms of the molecular formula; (iii) eight methylenes; and (iv) three methyl groups (δ_C 27.9, 15.4, and 15.1; corresponding to δ_H 0.98, 0.91, and 0.77, each 3H, in ^1H NMR). Thus, to complete its unsaturation requirement, **2** must possess five rings.

In comparison with the data of the pentacyclic diterpenes described in the literature, the ^1H and ^{13}C NMR of **2** shared some common features with those of a known structure, trachyloban-3-ol,[9] which possesses one oxygen less in its molecular formula compared to that of **2**, suggesting the samilarity of their structures.

The failure to crystalize and limitation of sample source make compound **2** inaccessible to X-ray crystallographic analysis and chemical conversion to characterize the structure. Nevertheless, these difficulties can be overcome through the unambiguous rationalization of all of the ^1H and ^{13}C NMR resonances using a combination of COSY, HMQC, HMBC, and NOESY techniques. The ^1H and ^{13}C singals of **2** due to rings A and B are almost superimposable with those of *ent*-trachyloban-3-ol (see Supporting Information),[9] indicating both compounds have the identical partial structure on rings A and B, which is consistent with the interpretation of the data derived from 2D-NMR experiements. Based on the inspection of the COSY spectrum (with aid from HMQC for the overlapping signals) (Figure 3), four isolated spin

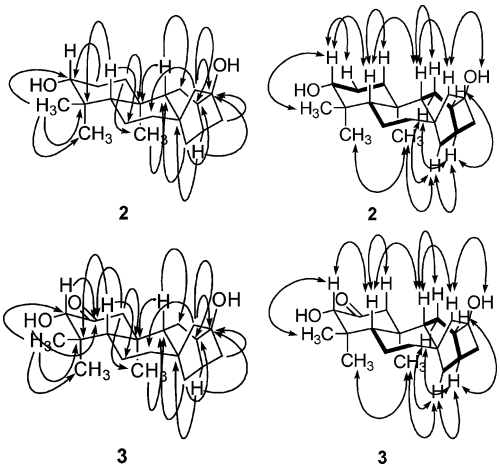

Figure 3. Key HMBC (→), $^1H-^1H$ COSY (—), and NOESY (↔) correlations of **2** and **3**.

systems were constructed: H_2-1/H_2-2/H-3 (a); H-5/H_2-6/H_2-7 (b); H-9/H_2-11/H-12/H-13/H_2-14 and H_2-17 (c); and the geminal protons H_2-15 (α, *β*) (*δ* 1.26, 1.70, each 1H), which remained as an isolated spin system with a typical AB coupling constant of 12 Hz (d). Key correlations observed in HMBC (Figure 3) between H_3-18 (*δ* 0.98, s, 3H)/C-4 (*δ* 38.7), H_3-19 (*δ* 0.77, s, 3H)/C-4; H_3-20 (*δ* 0.91, s, 3H)/C-10 (*δ* 38.0) led to the assignment of the three methyl groups. Moreover, the HMBC correlations between H-5 (*δ*0.81, br d, $J = 11.7$)/C-4, H-3 (*δ* 3.22, dd, $J = 11.0, 4.2$)/C-4; H_2-1 (*δ* 1.56 and 0.93, m, each 1H)/C-10, H-5 and H-9 (*δ* 1.19, br dd, $J = 12.0, 6.0$)/C-10; H-7 (*δ* 1.52 and 1.23, m, each 1H)/C-8 (*δ* 35.9) and H-9/C-8 allowed the establishment of connection of spin systems (a) and (b), and thus the rings A and B of compound **2** were constructed, which proved to be the same as that of trachyloban-3-ol.

Although the *ent*-trachylobane skeleton such as in *ent*-trachyloban-3-ol was taken into consideration, considerable differences were readily recognized in **2** particularly from proton and carbon signals due to rings C, D, and E. Most notably, **2** has one less methyl but one more methylene compared to trachyloban-3-ol. Since there is no carbonyl, terminal olefinic carbon, or oxygenated methylene/methine present in **2** as evident from the ^1H and ^{13}C NMR spectra, the only explanation for the "missing" methyl is that the methyl was incoporated in a ring system as part of the carbon framework of an unprecendented diterpene skeleton, which we named wallichane.

With the NMR signals due to protons and carbons from rings A and B assigned satisfactorily, a closer inspection of COSY and HMQC made it possible to construct a partial structure composed of spin system H-9/H_2-11/H-12/H-13/H_2-14 and H_2-17 (c) based on the fact that geminal protons of H-11*β* (*δ* 1.76) and H-11α (*δ* 1.27) showed coupling to

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(8) Wallichanol A (**2**): amorphous powder, $[\alpha]^{20}_D$ −40° (*c* 0.07, MeOH), IR (KBr) ν_{max} 3437, 1456, 1385, 1277, and 1089 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m*/*z* (rel int) 327 $[M + Na]^+$ (50) and 631 $[2M + Na]^+$ (100).

(9) Block, S.; Stevigny, C.; De Pauw-Gillet, M.-C.: De Hoffmann, E.; Liabres, G.; Adjakidje, V.; Quetin-Leclercq, J. *Planta Med.* **2002**, *68*, 647−649.

H-9 (δ 1.19) (J = 12.0 and 5.5 Hz) and H-12 (δ 1.93) (J = 3.0 and 2.4 Hz), respectively. Moreover, H-12 also coupled to H-13 (δ 2.04), which also coupled to one of the geminal protons of H_2-14 resonating at δ 2.17 (J = 6.6 Hz) and one of the geminal protons of H_2-17 (δ 1.95). The proton signal of H-13 appeared as a typical quartet (J = 6.7 Hz), implying that H-13 was oriented with close dihedral angles with all of the three flanked protons H-12, H-14a, and H-17a with no coupling with H-14b and H-17b as a result of the dihedral angle of nearly 90°, which provided compelling evidence for the presence of a cyclobutane ring system in **2**, which was formed by extension of the cyclopropane ring by incorporating the methyl group into the four-membered ring, instead of a cyclopropane ring as found in the *ent*-trachylobane diterpenes such as **1** and *ent*-trachyloban-3-ol. This conclusion was in full agreement with the readout of HMBC in which conclusive correlations were observed between H_2-15/C-16 (^{2}J HMBC), H_2-15/C-12, H_2-15/C-17, H_2-17 /C-12, H_2-17/C-16 (^{2}J HMBC), H_2-14/C-15 (^{2}J HMBC), H_2-14/C-17 (Figure 3). Other key correlations were detected between H_2-15/C-7, H_2-15/C-9, and H_2-15/C-8 (^{2}J HMBC); H_2-14/C-7, H_2-14/C-9, and H_2-14/C-8 (^{2}J HMBC), supporting the connection of rings A and B with the rest of this molecule.

The relative stereochemistry of **2** was established by NOESY experiement. As shown in Figure 3, The trans junction of A/B rings can be deduced by NOE correlations between between H_3-20 and H_3-19; H-5 and H_3-18. The α orientation of hydroxy group at C-3 can be deduced from the NOE correlation between H-3 and H-5, which was further suported by the large coupling value (J = 11.0) between H-3 and H-2α, indicative of a *trans*-diaxial relationship. The configuration at C-9 was also confirmed by NOE correlations between H-9/H-5, H-9/H-11β. The informative NOE correlations between H-9/H-15β, H-14/Me-20 suggested an α-orientation of bond C8−C14. To retrieve additional information for structure confirmation, we used DMSO-d_6 to replace $CDCl_3$ as the NMR solvent, which "fixed" the C_{16}-OH group at δ 4.91 as a singlet (assigned on the basis of the significant HMBC correlations bwteen C_{16}-OH /C-15, C_{16}-OH /C-16, C_{16}-OH/C-17) rendering it the "handle" for stereochemistry chracterization. The NOE correlation between C_{16}-OH and H-15β, which in turn correlated to H-9, conclusively indicated C_{16}-OH being β-oriented. Thus, the structure of **2** is established as *ent*-wallichan-3,16-diol with a trivial name wallichanol A, representing the first member of a new family of pentacyclic diterpene with a rearranged carbon framework that we named wallichane.

Wallichanol B (**3**)[10] was isolated as an amorphous powder with a molecular formula of $C_{20}H_{30}O_3$ as inferred from HRESIMS in which a sodiated molecular ion $[M + Na]^+$ was detected at *m*/*z* 341.2084 (calcd 341.2087). The IR absorptions showed an absorption band for a carbonyl group at 1704 cm^{-1}. The DEPT ^{13}C NMR spectrum grouped all of the carbon signals into the following assemblies: (i) five quarternary carbons including one ketone carbonyl resonating at δ_C 210.7 and one oxygenated carbon resonating at δ_C-73.2, (ii) five methines including one oxygenated carbon resonating at δ_C 79.1, (iii) seven methylenes, and (iv) three methyl groups. In comparing to the molecular fomula and DEPT ^{13}C NMR spectrum of **2**, it is obvious that **3** must be an analogue of **2** with the addition of a ketone group, as **3** has one more oxygen but two less hydrogens compared to **2**, and correspondingly, **3** has one more quaternary and one less methylene carbon compared to **2**.

The structure determination of **3** is straightforward by comparing its NMR data with those of **2** (Tables 1 and 2), which revealed that almost all ^{1}H and^{13}C NMR resonances corresponding to the protons and carbons except from those due to ring A are identical, suggesting their structural similarity. Thus, **3** was deduced to possess the same carbon skeleton and the same partial structure at rings B−E as that of **2** with, however, the location of the ketone carbonyl group at ring A yet to be determined. The ketone group was readily assigned to C-2 on the basis of the HMBC correlations between C═O/H_2-1 (^{2}J HMBC); and C═O/H-3 (^{2}J HMBC). Finally, the structure including the stereochemistry of **3** was further supported by NOESY, which showed correlation relationships identical with those of **2** only with the absence of those relations involved in H_2-2. Therefore, the structure of **3** was unambiguously determined to be 2-oxo-*ent*-wallichan-3,16-diol with a trivial name wallichanol B.

We propose a biogenetic pathway (see Supporting Information) to account for the plausible biosynthesis of *ent*-wallichane whereby is shown the close relationship among these diterpene skeletons of *ent*-kaurene, *ent*-trachylobane, *ent*-artisene, and *ent*-wallichane, which share a common precursor, namely, *ent*-beyerane cation.[11]

In an in vitro osteoclastogenesis assay,[12] compounds **1**, **2**, and **3** were shown to inhibit osteoclastogenesis induced by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) in a dose-response fashion with a IC_{50} values of 4, 20, and 15 μM, respectively. Since increased osteoclast-mediated bone resorption plays a central role in pathologic bone loss, our data suggest that these compounds, particularly **1**, may hold promise as therapeutic agents for the prevention of osteoporosis.

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Supporting Information Available: X-ray analysis of **1** in CIF format and 1D and 2D NMR spectra of **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Wallichanol B (**3**): $[\alpha]^{20}_D$ −69° (*c* 0.13, MeOH), IR (KBr) ν_{max} 3427, 1704, 1452, 1385, 1262, and 1097 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; NMR data in DMSO see Supporting Information. ESIMS *m*/*z* (rel int) 341 $[M + Na]^+$ (25) and 659 $[2M + Na]^+$ (100).

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