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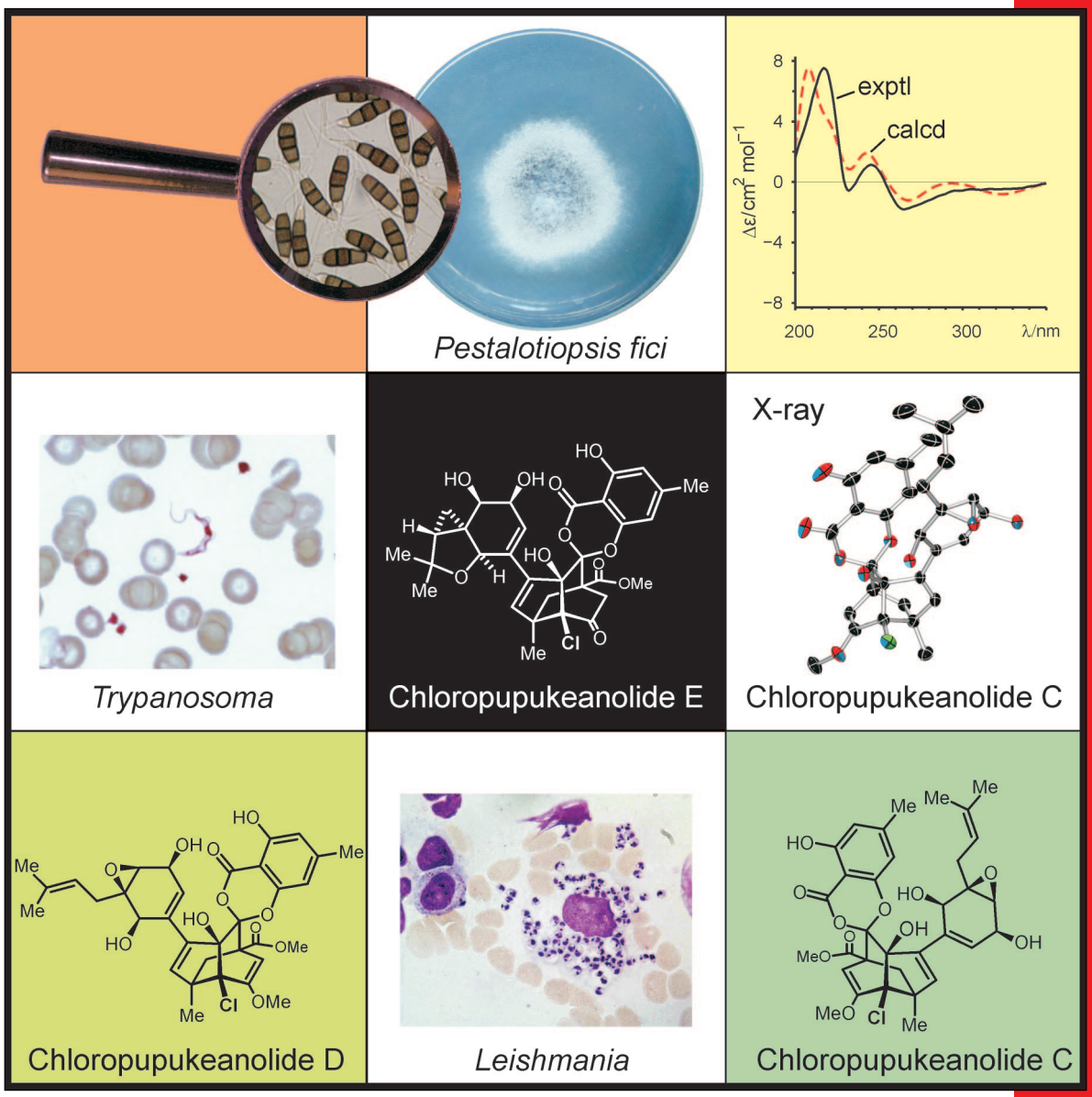

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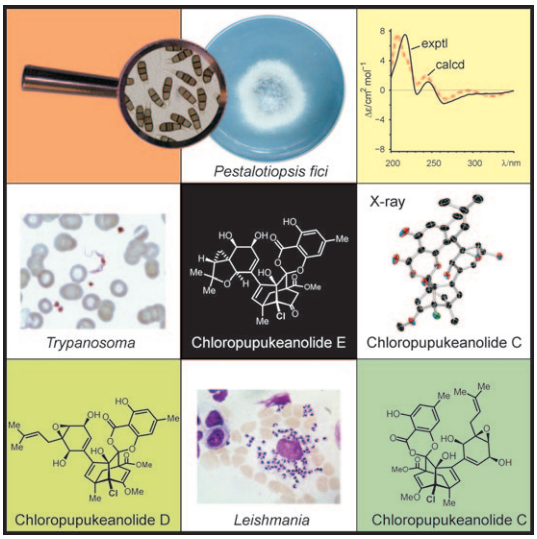
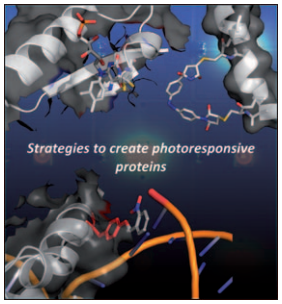
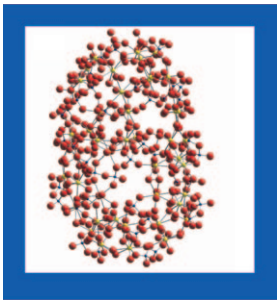
… for a novel spiroketal skeleton derived from the chlorinated tricyclo-[4.3.1.0^{3,7}]-decane (pupukeanane) and the 2,6-dihydroxy-4-methylbenzoic acid moieties were found in three highly functionalized secondary metabolites, that is, chloropupukeanolides C–E. G. Bringmann, Y. Che et al. describe the structure determination of these and other metabolites isolated from the scale-up fermentation extract of the plant endophytic fungus *Pestalotiopsis fici* in their Full Paper on page 2604 ff.

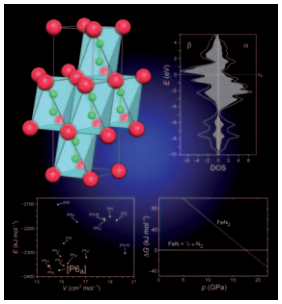




Enlightened Enzymes
In the Concept article on page 2552 ff., U. Krauss et al. describe some of the classic as well as recently developed strategies employing protein-engineering and chemical–biological hybrid methods to achieve photocontrol of biological processes. Recent cell biological examples are highlighted and possible biotechnological applications are presented.

Cage Clusters
In the Communication on page 2571 ff., P. C. Burns et al. describe how the combination of uranyl, peroxide, and pyrophosphate in aqueous solution results in the self-assembly of highly complex clusters containing 45 uranyl bipyramids and 23 pyrophosphate groups. These clusters are built from three basic structural units, and are highly unusual because they completely lack symmetry.





A New Phase in the Iron Nitrogen System
In their Full Paper on page 2589 ff., R. Dronskowski and M. Wessel discuss the rhombohedral phase of iron pernitride, FeN_2, which should be accessible at high pressures, namely above 17 GPa at a synthesis temperature of 1000 K. FeN_2 is likely to be a ferromagnetic material with a saturation moment of 1.68 μ_B per Fe atom, and the N–N bond length and the quantum-chemical bond analysis suggest the electron count to be N_2^{2-}.

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Chloropupukeanolides C–E: Cytotoxic Pupukeanane Chlorides with a Spiroketal Skeleton from *Pestalotiopsis fici*

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Abstract: Chloropupukeanolides C–E (**8**–**10**), three highly functionalized secondary metabolites featuring a novel spiroketal skeleton derived from the chlorinated tricyclo-[4.3.1.$0^{3,7}$]-decane (pupukeanane) and the 2,6-dihydroxy-4-methylbenzoic acid moieties, were isolated from the scale-up fermentation extract of the plant endophytic fungus *Pestalotiopsis fici*. The constitutions of compounds **8**–**10** were elucidated primarily by NMR experiments. Their relative configurations were deduced by analogy to metabolites **4**–**6**, which were previously isolated from the same fungus. The absolute configuration of **8** was assigned by X-ray crystallography and those of **9** and **10** by quantum-chemical CD calculations. Biogenetically, chloropupukeanolides C–E (**8**–**10**) are presumably derived from the same oxidation-induced Diels–Alder reaction pathway as compounds **1** and **4**–**7**, via the putative biosynthetic precursors **2** and **3**. The opposite configurations of the complete "Southern parts" of **8** and **9** suggests that this Diels–Alder reaction is stereochemically not very selective. Compounds **8**–**10** showed significant cytotoxicity against a small panel of human tumor cell lines and weak activities against the pathogens of tropical diseases.

Keywords: biosynthesis · cytotoxicity · fungal metabolites · natural products · structure elucidation

Introduction

The plant-endophytic strains of the fungus *Pestalotiopsis* (Amphisphaeriaceae), which is distributed widely in nature as saprobes and pathogens or endophytes to living plants,[1] have been demonstrated to be a rich source of bioactive secondary metabolites with diverse structural features.[2–14] During the course of our continuing search for new bioactive natural products from this fungal genus, a subculture of *P. fici* (AS 3.9138 = W106-1), isolated from the branches of the tea plant *Camellia sinensis* (Theaceae) in the suburb of Hangzhou, People's Republic of China, was grown in different solid-substrate fermentation cultures. Chemical studies of the resulting crude extracts had afforded structurally unique natural products showing inhibitory effects on replication of HIV-1 virus in C8166 cells[15,16] including chloropupukeananin (**1**),[15] the first chlorinated pupukeanane analogue with a highly functionalized tricyclo-[4.3.1.$0^{3,7}$]-decane skeleton and its putative biosynthetic precursors **2** and **3** (Figure 1),[15] and the pestaloficiols,[16] new 6,7-dihydroxy-5,6,7,8-tetrahydro-2*H*-chromen-4(3*H*)-ones possessing a cyclopropane moiety.

Since an HPLC chromatogram of the crude extract had revealed the presence of other components that could not be identified due to sample limitations, the fungus had been re-fermented at a larger scale on rice (1 kg). In an initial step, chloropupukeananin (**1**) had been depleted in order to readily isolate the minor components, and subsequent fractionation of the extract led to the isolation of a variety of bioactive metabolites.[17–20] Representative compounds include chloropestolide A (**4**; Figure 1),[18] a spiroketal with an unprecedented skeleton derived from a chlorinated bicyclo-[2.2.2]-oct-2-en-5-one moiety and a 2,6-dihydroxy-4-methylbenzoic acid unit, chloropupukeanolides A and B (**5** and **6**; Figure 1),[19] two novel spiroketal peroxides, and chloropupukeanone A (**7**; Figure 1),[19] an analogue of chloropupukeananin (**1**) with the C8 ketone functionality. The above-

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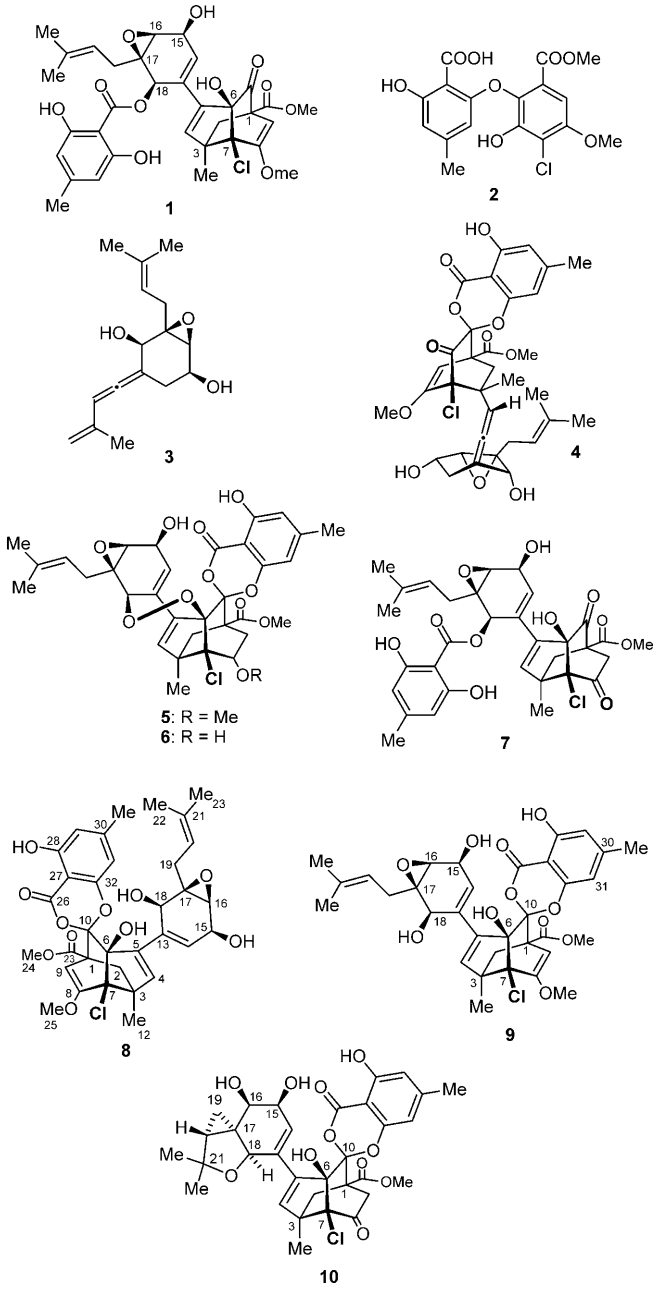


Figure 1. Metabolites **1**–**7** isolated previously from *P. fici* and chemical structures of chloropupukeanolides C–E (**8**–**10**).

mentioned metabolites **1** and **4**–**7** presumably originate from the same Diels–Alder precursors **2** and **3**.

The isolation of **4**–**7** from the same source and their possible joint biogenesis led us to postulate the presence of further minor Diels–Alder reaction analogues in the fractions from which these compounds had been isolated.

As a continuation of this work, the fungus was re-fermented (3 kg) and those particular fractions were subjected to detailed chemical studies. In the current study, three additional pupukeanane metabolites featuring a unique spiroketal skeleton derived from the chlorinated tricyclo-[4.3.1.$0^{3,7}$]-decane and 2,6-dihydroxy-4-methylbenzoic acid moieties, are presented and named chloropupukeanolides C–E (**8**–**10**). Details of the isolation, structural elucidation, cytotoxicity, bioactivity, and biogenesis of compounds **8**–**10** are reported herein. The discovery of **9** as a putative biosynthetic intermediate permits establishment of an advanced proposal for the origin of **1** in the fungus.

Results

The molecular formula of chloropupukeanolide C (**8**) was determined to be $C_{33}H_{35}ClO_{11}$ (16 degrees of unsaturation) by HR-ESIMS (*m*/*z*: calcd for: 665.1760; found: 665.1769 $[M+Na]^+$). Its ^1H and ^{13}C NMR spectroscopic data (Table 1) revealed resonances for four exchangeable protons, six methyl groups including two O-methyls, two methylenes, three oxymethines, 14 olefinic or aromatic carbons (six of which are protonated), six sp^3 quaternary carbons with four heteroatom-bonded, and two carboxylic carbons. These data hinted at a structural similarity of **8** to chloropupukeananin (**1**)[15] and chloropupukeanolides A (**5**) and B (**6**).[19] Interpretation of the $^1H,^1H$ COSY and HMBC data of **8** (Table 1) established the same isoprenylated 2,3-epoxycyclohex-5-en-1,4-diol moiety as found in **1** and **5**–**7**. However, the chlorinated tricyclo-[4.3.1.$0^{3,7}$]-decane moiety in **8** differs from that in **1** by having a ketal ($\delta_C=107.0$ ppm) instead of a ketone carbon ($\delta_C=196.4$ ppm) in position C10, thus featuring a spirocycle originating from the spirocarbon connecting a tricyclo-[4.3.1.$0^{3,7}$]-decane and a 5-hydroxy-7-methyl-4*H*-benzo[*d*][1,3]-dioxin-4-one unit, similar to the one found in **5** and **6**.[19]

In addition, this chlorinated tricyclo-[4.3.1.$0^{3,7}$]-decane moiety differed from that in **5** and **6** by having a C8–C9 double bond ($\delta_H/\delta_C=5.44/96.5$; 153.6 ppm) instead of a methine ($\delta_H/\delta_C=4.15/69.2$ ppm) and a methylene unit ($\delta_H/\delta_C=$ 2.08; 3.15/34.3 ppm). The exchangeable proton signal at $\delta_H=7.18$ ppm was correlated to the C5 non-protonated sp^2 carbon ($\delta_C=144.9$ ppm), the C6 sp^3 quaternary carbon ($\delta_C=$ 91.0 ppm), and the C10 ketal carbon ($\delta_C=107.0$ ppm) as seen from the HMBC spectrum of **8**, indicating that C6 bears a free hydroxy group. The $^1H,^1H$ COSY spectrum of **8** showed a cross-peak between H18 and 18-OH ($\delta_H=$ 5.25 ppm), indicating that the C18 oxygen of the isoprenylated 2,3-epoxycyclohex-5-en-1,4-diol is no longer acylated by a C26 carboxylic carbon of a 2,6-dihydroxy-4-methylbenzoate moiety, as in **1**,[15] and a peroxide linkage between C6 and C18 as found in **5** and **6**[19] is also absent. These results were also supported by the upfield chemical shifts for the oxygenated carbons C6 ($\delta_C=91.0$ ppm in **8** vs. 108.4 ppm in **5**) and C18 ($\delta_C=67.5$ ppm in **8** vs. 79.4 ppm in **5**). Collectively, these data allowed determination of the gross structure of **8**.

Finally, the full stereostructure of **8** was established by an X-ray crystallographic analysis (Figure 2). The presence of the chlorine atom permitted the determination of the absolute configuration of all stereogenic centers, too. Thus, the tricyclo moiety of chloropupukeanolide C (**8**) is

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data of chloropupukeanolide C (**8**) in [D$_6$]acetone.

Position	δ_C [ppm]	δ_H [ppm]	HMBC	NOESY
1	55.3 (s)			
2a	41.8 (t)	1.49 (d, 13)	1, 3, 4, 9, 10, 11, 12	9, 12
2b		2.62 (d, 13)	1, 4, 7, 9, 11	4
3	47.8 (s)			
4	141.7 (d)	6.22 (s)	3, 5, 6, 7, 12, 13	2b, 12, 14
5	144.9 (s)			
6	91.0 (s)			
7	90.4 (s)			
8	153.6 (s)			
9	96.5 (d)	5.44 (s)	1, 7, 8, 10, 11	2a
10	107.0 (s)			
11	170.4 (s)			
12	22.0 (q)	1.10 (s)	2, 3, 4, 7	2a, 4
13	134.6 (s)			
14	128.0 (d)	5.76 (dd, 2.5, 2.0)	5, 16, 18	4, 31
15	65.8 (d)	4.22 (ddd, 8.0, 2.5, 2.0)		31, 33
16	60.1 (d)	3.19 (t, 2.5)	14	19, 20
17	61.9 (s)			
18	67.5 (d)	3.36 (d, 11)		19, 20
19a	31.9 (t)	1.81 (dd, 15, 6.5)	16, 17, 18, 20, 21	16, 18
19b		2.74 (dd, 15, 6.5)	16, 17, 18, 20, 21	16, 18
20	118.5 (d)	4.86 (t, 6.5)	22, 23	16, 18
21	136.1 (s)			
22	17.9 (q)	1.58 (s)	20, 21, 23	
23	26.0 (q)	1.76 (s)	20, 21, 22	
24	52.8 (q)	3.69 (s)	11	
25	56.4 (q)	3.69 (s)	8	
26	165.0 (s)			
27	98.6 (s)			
28	160.0 (s)			
29	111.1 (d)	6.40 (s)	27, 28, 31, 33	23
30	149.1 (s)			
31	110.3 (d)	6.09 (s)	26, 27, 29, 32, 33	14, 15
32	156.3 (s)			
33	22.4 (q)	2.29 (s)	29, 30, 31	15
6-OH		7.18 (s)	5, 6, 10	
15-OH		4.12 (d, 8.0)		
18-OH		5.25 (d, 11)		
28-OH		10.2 (br s)		

1*R*,3*S*,6*R*,7*R*,10*R*-configured while the cyclohexene part has the 15*S*,16*S*,17*R*,18*R*-configuration.

Along with **1**, chloropupukeanolide D (**9**) was obtained as a mixture of the two components in a 10:1 ratio, which slowly changed to a 5:1 ratio over a period of 24 h, as determined by integration of some well-resolved ^{1}H NMR resonances (e.g., H4 and H9) for each one. Efforts to obtain pure **9** were unsuccessful due to repeated rearrangement from **9** to **1** as mentioned above. Therefore, the structure elucidation of **9** was performed on the freshly purified 10:1 mixture. On the basis of HRESIMS data (*m*/*z*: calcd for: 665.1760; found: 665.1745 $[M+Na]^+$), chloropupukeanolide D (**9**) was found to have the same molecular formula $C_{33}H_{35}ClO_{11}$ (16 degrees of unsaturation) as chloropupukeanolide C (**8**). In addition, the ^{1}H and ^{13}C NMR spectra of **9** showed resonances similar to those of **8**, except for the fact that those of the fragments near the C10 ketal carbon were significantly different. Interpretation of the ^{1}H,^{1}H COSY and HMBC data of **9** (Table S1; Supporting Information) es-

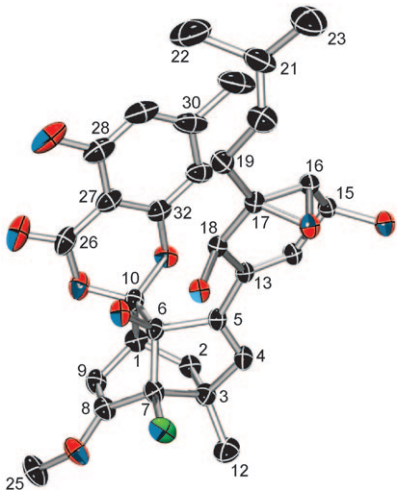


Figure 2. ORTEP plot of chloropupukeanolide C (**8**), hydrogen atoms and the CO_2Me group at C1 are omitted for reasons of clarity. The thermal ellipsoids are shown at 50% probability.

tablished the same gross structure as **8**, implying that **9** was a close analogue, that is, a stereoisomer of **8**.

The absolute configuration of chloropupukeanolide D (**9**) was established by a combination of NOESY NMR data with quantum-chemical calculations, from HPLC investigations of **9** and **1**, and by a comparison of the experimental LC-CD spectrum of **9** with the quantum-chemically simulated ones. The rigid geometry of the tricyclo-[4.3.1.$0^{3,7}$]-decane unit predetermined the relative configuration of its stereogenic centers, which can only be 1*S*,3*R*,6*S*,7*S* or, fully opposite, that is, 1*R*,3*S*,6*R*,7*R* configured in chloropupukeanolide D (**9**). The NOESY correlations between H18 and H_2-19, H_2-19 and H16, and between H16 and H15 were found in both, compounds **8** and **9** (Figure 3) and corroborated that chloropupukeanolides C and D have the same relative configuration, 15*S*,16*S*,17*R*,18*R* or 15*R*,16*R*,17*S*,18*S*, at the cyclohexene moiety. Thus, chloropupukeanolide D has two rigid chiral building blocks, in which the relative configuration is known and this significantly reduces the number of "independent" stereogenic units: the Southern tricyclic moiety, the Northern cyclohexene part, and the acetalic spirocenter at C10.

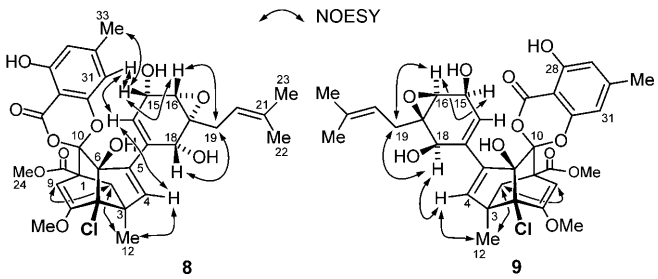


Figure 3. Key NOESY correlations for compounds **8** and **9**.

It was, however, not possible to assign the configuration of the cyclohexene moiety in chloropupukeanolide D (**9**) relative to the tricyclo-[4.3.1.$0^{3,7}$]-decane unit from NOESY interactions. Thus, the above mentioned rearrangement of **9** to chloropupukeananin (**1**) turned out to be a substantial help. Since the absolute configuration of **1** was known from X-ray crystallography,[15] the configuration of **9** was deduced as 1*S*,3*R*,6*S*,7*S*,15*S*,16*S*,17*R*,18*R*, that is, opposite to that of **8** in the Southern part of the molecule (C1–C9), leaving open only the configuration at the remaining stereogenic center, C10 (see also the proposed biosynthetic pathway below).

Based on these facts, the structures of chloropupukeanolide C (**8**) and D (**9**) were closely similar, diastereomeric, with the same relative—but opposite absolute!—configuration within the tricyclic moiety, with the same relative and absolute configuration in the northern cyclohexene part, but leaving open whether the third stereogenic unit, the acetal center at C10, was *S*, as in **8**, or *R*.

Remarkably, the two compounds showed nearly the same NOE correlations in this part. The only difference were the diagnostically most significant NOE correlations of H31 with H14 and H15, which were found for **8** exclusively and thus these correlations gave useful hints at the relative configuration of the spirocenter. To show that these correlations were only possible if C10 was *R*-configured and the tricyclo-[4.3.1.$0^{3,7}$]-decane core had the 1*S*,3*R*,6*S*,7*S*-configuration (and of course in the mirror-imaged form, 1*R*,3*S*,6*R*,7*R*,10*S*), quantum-chemical calculations were performed. Conformational analyses using the B3LYP/6-31G*[21] method with subsequent SCS-MP2/TZVP[22,23] single-point energy calculations yielded seven energetically relevant conformers for the 1*S*,3*R*,6*S*,7*S*,10*S*,15*S*,16*S*,17*R*,18*R*-configuration (in the following abbreviated as "10*S*-**9**") and 21 conformers for the 1*S*,3*R*,6*S*,7*S*,10*R*,15*S*,16*S*,17*R*,18*R* diastereomer ("10*R*-**9**"). The H31–H14 and H31–H15 distances were found to be larger than 5 Å for all conformers of 10*R*-**9** (Table 2), while they are significantly below 5 Å in the case of 10*S*-**9**. Consequently, as there has been no NOE correlations of H31 with H14 and H15 observed for the *R*-spiroacetal in combination with the 1*S*, 3*R*, 6*S*, and 7*S*-configured tricyclo-[4.3.1.$0^{3,7}$]-decane unit, this gave a good hint at an absolute stereostructure of 1*S*,3*R*,6*S*,7*S*,10*R*,15*S*,16*S*,17*R*,18*R* for chloropupukeanolide D (**9**).

To further confirm these findings, CD measurements in combination with quantum-chemical calculations were performed. As mentioned above, the new compound **9** cannot be obtained in a fully pure form since it is unstable and slowly rearranges to the known chloropupukeananin (**1**), so that the CD spectra of **9** had to be measured online, by HPLC–CD[24] coupling. For this purpose, the peaks A and B in the HPLC–UV chromatogram were first identified as chloropupukeanolide D (**9**) and chloropupukeananin (**1**), respectively, by comparison with pure isolated **1** (Figure 4a).

The measured online CD spectrum of peak A was compared with the spectra calculated for the remaining possible diastereomers, 10*S*-**9** and 10*R*-**9**. TDA B2PLYP/SV(P)[23,25] computations of the conformers found before, for the NOE interpretation (see above), yielded a CD spectrum for the *R*-configured spirocenter (Figure 4b, right) that matched quite well with the one experimentally obtained for Peak A (compound **9**), while the spectrum of the 10*S*-configured diastereomer (Figure 4b, left) was nearly opposite to the experimental one, finally proving that chloropupukeanolide D (**9**) had the 1*S*,3*R*,6*S*,7*S*,10*R*,15*S*,16*S*,17*R*,18*R*-configuration. The different relative configurations at C1, C3, C6, C7, and C10 in **8** and **9** seem to be the reason for the different stabilities of these two compounds. Only with the given configuration in chloropupukeanolide D (but not in the case of compound **8**), C26 and C18 get close enough to each other so that the rearrangement to chloropupukeananin can occur—a perfect diastereomer-differentiating reaction.

The elemental composition of the third new compound, chloropupukeanolide E (**10**), was established as $C_{32}H_{33}ClO_{11}$ (16 degrees of unsaturation) by HRESIMS (*m*/*z*: calcd for: 651.1604; found: 651.1592 $[M+Na]^+$), which is 14 mass units less than that of compounds **8** and **9**. The NMR data of **10** (Table S2, Supporting Information) revealed the presence of the same 5-hydroxy-7-methyl-4*H*-benzo[*d*]-[1,3]dioxin-4-one moiety as found in **8** and **9**. However, the

Table 2. Relative energies (SCS-MP2/TZVP//B3LYP/6-31G*), H31–H14 and H31–H15 distance, and Boltzmann factor (B_f) of the conformers of 10*S*-**9** and 10*R*-**9**.

Conformer	E [kcal mol^{-1}]	Distance H31–H14 [Å]	Distance H31–H15 [Å]	B_f [%]
10*S*-**9**_1	0.00	4.58	2.96	88.1
10*S*-**9**_2	1.81	4.58	2.93	4.2
10*S*-**9**_3	1.88	4.58	2.95	3.7
10*S*-**9**_4	2.37	4.57	2.87	1.6
10*S*-**9**_5	2.67	4.18	4.64	1.0
10*S*-**9**_6	2.78	4.19	4.64	0.8
10*S*-**9**_7	2.85	4.18	4.63	0.7
10*R*-**9**_1	0.00	6.18	4.91	23.5
10*R*-**9**_2	0.26	9.16	7.33	15.1
10*R*-**9**_3	0.52	6.15	5.01	9.7
10*R*-**9**_4	0.53	6.11	4.97	9.6
10*R*-**9**_5	0.55	6.13	5.09	9.3
10*R*-**9**_6	0.84	9.14	7.31	5.7
10*R*-**9**_7	0.88	6.15	5.07	5.3
10*R*-**9**_8	0.91	6.16	5.12	5.1
10*R*-**9**_9	1.04	9.14	7.37	4.1
10*R*-**9**_10	1.08	8.66	6.80	3.8
10*R*-**9**_11	1.28	8.65	6.79	2.7
10*R*-**9**_12	1.48	9.26	7.48	1.9
10*R*-**9**_13	1.90	9.22	7.40	0.9
10*R*-**9**_14	1.99	9.18	7.38	0.8
10*R*-**9**_15	2.12	9.24	7.47	0.7
10*R*-**9**_16	2.33	9.19	7.39	0.5
10*R*-**9**_17	2.54	8.65	6.79	0.3
10*R*-**9**_18	2.66	8.64	6.78	0.3
10*R*-**9**_19	2.66	9.19	7.39	0.3
10*R*-**9**_20	2.77	9.16	7.36	0.2
10*R*-**9**_21	2.81	9.17	7.36	0.2

resonances for the C8–C9 olefin and the C25 methoxy group in the tricyclo-[4.3.1.0^{3,7}]-decane cores of **8** and **9** were replaced by signals typical of a ketone carbon (δ_C= 199.3 ppm) and a methylene unit (δ_H/δ_C=2.80; 3.44/44.3 ppm) in the spectra of **10**, and these observations were supported by HMBC correlations from 9-CH_2 to C1, C2, C7, C8, C10, and C11. The ^{1}H,^{1}H COSY NMR data showed the presence of two isolated proton spin systems of C14–C16 (including 15- and 16-OH) and C19–C20. HMBC correlations from 22- and 23-CH_3 to C20 and C21 indicated that C20, C22, and C23 are all connected to C21. Further correlations from H18 to C17, C19, and C20, and from 19-CH_2 and H20 to C16, C17, and C18 evidenced that the cyclopropane and cyclohexene units are joined by the spirocenter C17. A key HMBC correlation from H18 to C21 established that these two ring systems are both annulated by a joint tetrahydrofuran substructure, completing the gross structure of **10**.

The NOESY data of **10** (Figure 5) indicated that the tricyclo-[4.3.1.0^{3,7}]-decane moiety has the same relative configuration as **9**, especially the NOE correlation between H9b and H31 is of importance as it directly proved the relative configuration of C10 and thus **10** had either the 1*S*,3*R*,6*S*,7*S*,10*R* or the 1*R*,3*S*,6*R*,7*R*,10*S* configuration. The 15,16-diol moiety was assigned a *cis*-configuration by analyzing the ^{1}H NMR spectrum of its acetonide (**10a**), in which the acetonyl methyl signals appeared as two singlets resonating at δ_H=1.28 and 1.41 ppm.[26] The large coupling constant of 8.0 Hz observed between H19b and H20 of the cyclopropane ring suggested their *syn*-relationship.[27] NOESY correlations of H4 with H14 and H18 confirmed their spatial proximity, while further interactions of H16 with H19b and H20, and of H18 with H19a indicated that these protons are close to each other. Taken together, these data permitted assignment of the relative 15*S*,16*R*,17*S*,18*R*,20*R*-configuration (or 15*R*,16*S*,17*R*,18*S*,20*S*) of this part of **10**. However, as in the case of **9**, it was impossible to directly correlate the sets of relative configurations of the two parts of the molecule with each other by NOE correlations.

The absolute configuration of **10** was again deduced by a comparison of the experimental CD spectrum with that of quantum-chemically calculated ones and, in this case, additionally by biosynthetic considerations. Due to the lack of a chromophore, the hexahydrobenzofuran moiety will most probably not have a significant effect on the CD spectrum. The only possibility to get a hint at the absolute configuration was the biosynthetic origin of this part of the molecule (see below). Compound **3**, the precursor of the hexahydrobenzofuran moiety, had been found enantiomerically pure in previous isolation works and this moiety had the same absolute configuration in compounds **8**, **9**, **1**, and **4**–**7**. Thus, one can expect the 15*S*,16*R*,17*S*,18*R*,20*R* configuration in compound **10**, too, so that only two possible diastereomers had to be considered for the elucidation of its full absolute configuration. A conformational analysis of 1*S*,3*R*,6*S*,7*S*,10*R*-**10** and 1*R*,3*S*,6*R*,7*R*,10*S*-**10**, both with the 15*S*, 16*R*, 17*S*, 18*R*, and 20*R* configuration, gave three relevantly populated conformers for the first diastereomer and four for the latter one. The comparison of the experimental CD curve of **10** with the spectra calculated for these configurations using the TDA B2PLYP/SV(P) method showed a nearly perfect agreement for the data calculated for 1*S*,3*R*,6*S*,7*S*,10*R*-**10** (Figure 6) and no match for 1*R*,3*S*,6*R*,7*R*,10*S*-**10**. This finally evidenced that chloropupukeanolide E (**10**) was 1*S*,3*R*,6*S*,7*S*,10*R*,15*S*,16*R*,17*S*,18*R*,20*R*-configured, provided the biosynthetic considerations for the hexahydrobenzofuran part were correct.

Compounds **8**–**10** were tested for their cytotoxicity against a small panel of human tumor cell lines including HeLa (cervical epithelium) and HT29 (colon) (Table 3). Chloropu-

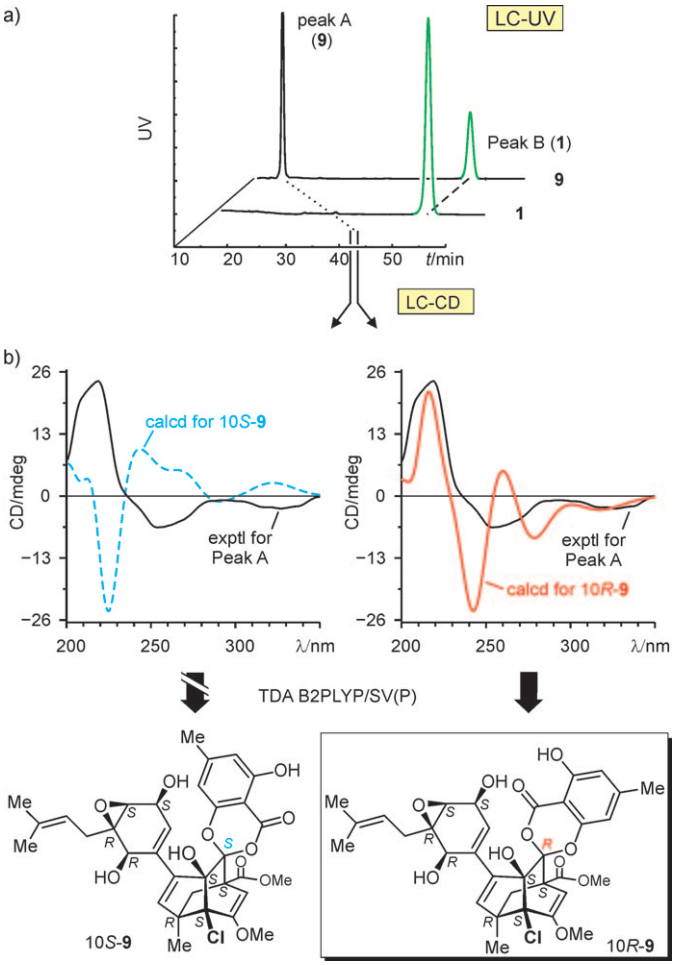


Figure 4. a) Comparison of HPLC-UV chromatograms of chloropupukeanine (**1**) and of chloropupukeanolide D (**9**) obtained on a Waters Symmetry-C18 column (2.1×150 mm); H_2O/MeOH 30:70 (flow rate: 0.8 mL min^{-1}, detection wavelength 220 nm). b) Assignment of the absolute configuration of **9** by comparison of the experimental LC-CD spectrum of peak A with calculated CD spectra.

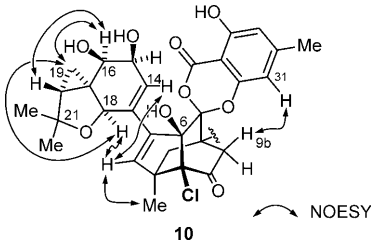


Figure 5. Key NOESY correlations for compound **10**.

pukeanolides C (**8**) and D (**9**) showed significant cytotoxicity against both cell lines, with IC_{50} values ranging from 1.2 to 7.9 μM, with a higher activity than the positive control 5-fluorouracil, which gave IC_{50} values of 10.0 and 15.0 μM.

In addition, the chloropupukeanolides C–D (**8–10**) were tested against the pathogens of the tropical diseases malaria, Chagas disease, leishmaniasis, and African sleeping sickness. All compounds showed weak activities against these pathogens (Table 4), but also exhibited cytotoxicities against rat skeletal myoblast (L6) cells at similar IC_{50} values. Compound **9** revealed a general toxicity as demonstrated by the very similar IC_{50} values for all parasites. The most interesting value was the activity of compound **10** against *Trypanosoma brucei* rhodesiense, which is modest but selective for that parasite.

Table 3. Cytotoxicity of compounds **8–10**.

Compound	IC_{50} [μM]	
	HeLa	HT29
8	2.3	7.9
9	1.2	4.2
10	31.8	>100
5-fluorouracil	10.0	15.0

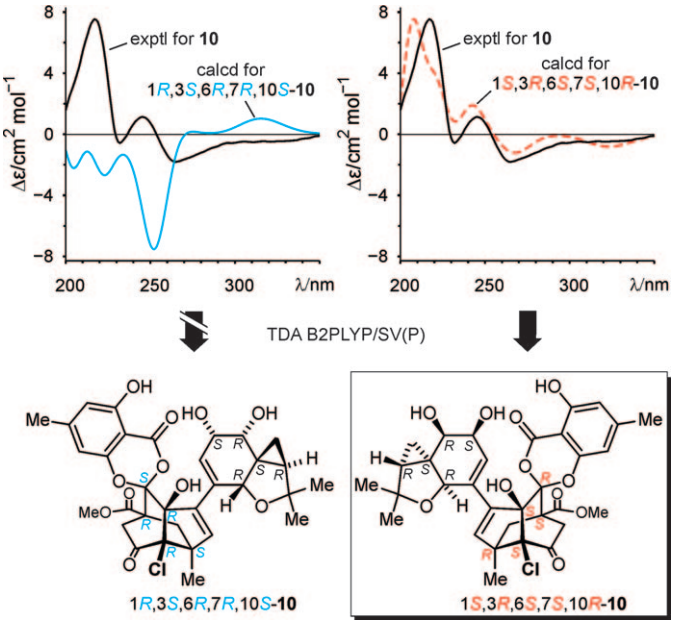


Figure 6. Assignment of the absolute configuration of chloropupukeanolide E (**10**) by comparison of the experimental CD spectrum with the ones calculated for 1*S*,3*R*,6*S*,7*S*,10*R*-**10** and 1*R*,3*S*,6*R*,7*R*,10*S* diastereomer (both with the 15*S*,16*R*,17*S*,18*R*,20*R* configuration).

Discussion

The pupukeanane sesquiterpenoids with the unique tricyclo-[4.3.1.0^{3,7}]-decane skeleton have so far mostly been isolated from marine sponges in the forms of isocyanates, thiocyanates and isothiocyanates.[28–43] Examples include: 2- and 9-isocyanopupukeanane, the marine invertebrate allomones isolated from the nudibranch *Phyllidia varicosa* and its prey, a sponge *Hymeniacidon* sp.;[27,28] the C9 epimer of 9-isocyanopupukeanane from the nudibranch *P. bourguini*;[29] 2-thiocyanatopupukeanane from a Palauan sponge of *Axinyssa aplysinoides*;[30] 9-isothiocyanatopupukeanane from the

Table 4. Bioactivities of compounds **8**–**10** against *Plasmodium falciparum* (strain K1), *Trypanosoma cruzi*, *T. brucei rhodesiense*, *T. brucei brucei*, and *Leishmania donovani*, and cytotoxicities against rat skeletal myoblast (L6) cells.

Compound	IC_{50} [μM]					
	P. falciparum	*T. cruzi*	*T. brucei rhodesiense*	*T. brucei brucei*	*L. donovani*	L6 cells (cytotoxicity)
8	5.73	60.93	19.27	–[a]	12.27	61.50
9	2.71	2.83	5.47	2.28	4.11	9.50
10	7.05	35.48	1.26	–[a]	37.52	112.35
standard	0.17[b]	1.75[c]	0.008[d]	0.003[e]	0.48[f]	0.012[g]

[a] Not measured. [b] Chloroquine. [c] Benznidazole. [d] Melarsoprol. [e] Pentamidin. [f] Miltefosin. [g] Podophyllotoxin.

sponge *Axinyssa* sp.[31] Some neopupukeananes have also been isolated from marine sponges, such as 2- and 4-thiocyanatoneopupukeanane[30,32] and 9-isocyanoneopupukeanane.[33] Prior to the discovery of chloropupukeananin (**1**),[15] chloropupukeanolides A (**5**) and B (**6**),[19] and chloropupukeanone A (**7**),[19] the only example with a tricyclo-[4.3.1.$0^{3,7}$]-decane carbon skeleton from terrestrial sources was nemorosonol, a polyisoprenylated pupukeanane from the fruits of the South-American plant *Clusia nemorosa* and the leaves of the Vietnamese plant *Garcinia bracteata* (both of the Clusiaceae family).[35,36]

Chloropupukeanolides C–E (**8**–**10**) are new chlorinated pupukeanane metabolites featuring a unique spiroketal skeleton derived from the highly functionalized tricyclo-[4.3.1.$0^{3,7}$]-decane and 2,6-dihydroxy-4-methylbenzoic acid moieties. Structurally, compounds **8** and **9** are new analogues of **5** and **6**,[15] all with a 5-hydroxy-7-methyl-4*H*-benzo[*d*]-[1,3]dioxin-4-one unit spirally joined to the tricyclo-[4.3.1.$0^{3,7}$]-decane core via C10, but differ by the absence of the six-membered peroxide moiety, and the hybridization states of C8 and C9. Compound **9** is a diastereomer of **8**, whereas **10** is a new analogue of **9** with a significant difference in the isoprenylated 2,3-epoxycyclohex-5-en-1,4-diol and the tricyclo-[4.3.1.$0^{3,7}$]-decane moieties. Specifically, a series of reactions of the isoprenyl group in **8** and **9** with the epoxycyclohexendiol moiety could afford the additional cyclopropane and tetrahydrofuran rings, leading to the formation of an unusual substructure with the 2,2-dimethyl-3-oxabicyclo[3.1.0]hexane unit *cis*-fused to the cyclohexen-diol moiety.

Biogenetically, compounds **2** and **3** isolated previously could also be the putative Diels–Alder precursors, not only for **1** and **4**–**7**,[15,18,19] but also for compounds **8**–**10** (Scheme 1). This initial, oxidation-induced Diels–Alder reaction seems to lack a high degree of stereoselectivity, since chloropestolide A (**4**) and chloropupukeanolide D (**8**) show a fully oppositely configured southern tricyclic moiety as compared to the compounds **1**, **5**, **6**, **9**, and **10**, which originate from the same pathway. Thus, once a reactive putative diene intermediate (assumedly the known[37] metabolite maldoxin, **a**) has been generated oxidatively, the Diels–Alder reaction itself may occur (nearly) spontaneously, without major enzymatic assistance.

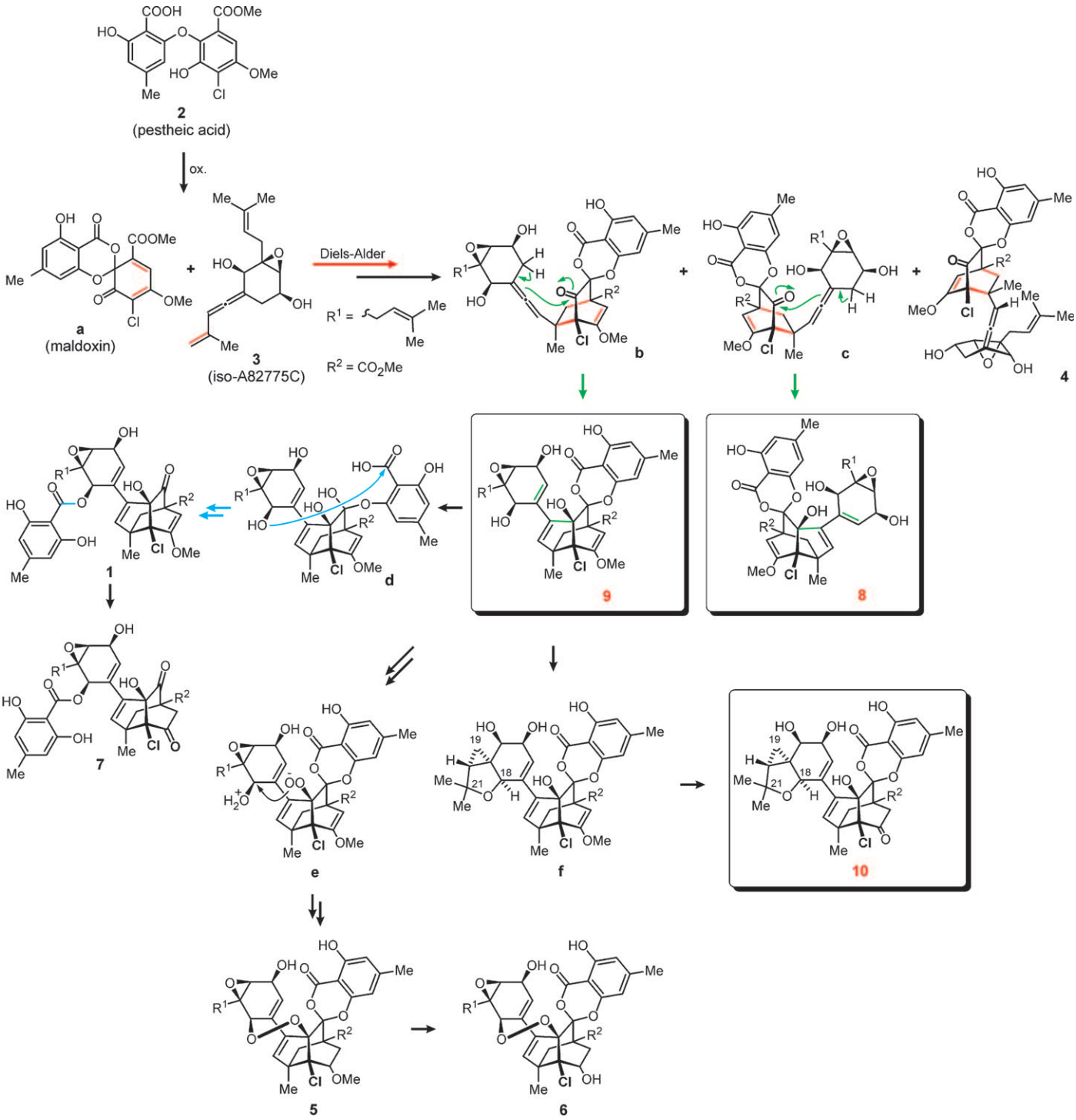
Conclusion

Indendation chloropupukeanolides C–E (**8**–**10**) are highly functionalized secondary metabolites featuring a unique spiroketal skeleton originating from the chlorinated tricyclo-[4.3.1.$0^{3,7}$]-decane and 2,6-dihydroxy-4-methylbenzoic acid moieties. The discovery of these metabolites from the plant-endophytic fungus *P. fici* provided further evidence for the biogenesis of **1** proposed in our earlier work[15] by identifying the key product or intermediate **9**, which might also be the precursor for the biosynthesis of compounds **10**, **5**, and **6** (Scheme 1). Chloropupukeanolides C (**8**) and D (**9**) do not only possess a unique spiroketal skeleton, but also display significant cytotoxicity against HeLa and HT29 cell lines, and show weak and mostly unselective activities against the pathogens of tropical diseases like malaria, African sleeping sickness, and leishmaniasis. Considering the fact that compounds **9**–**10** and **5**–**7** are presumably derived from intermediate **b**, whereas **8** is derived from intermediate **c** in the—stereochemically quite unselective—Diels–Alder cycloaddition pathway from the precursors **2** and **3** (Scheme 1), other Diels–Alder intermediates and/or products are likely to be present in this reaction cascade, but in lower concentrations. These results imply the possibility to identify further structurally related minor metabolites with possibly interesting activities, by taking advantage of the remarkable metabolic potential of the fungus in future studies.

Experimental Section

General procedures: Optical rotations were measured on a Perkin–Elmer 241 polarimeter, and UV data were recorded on a Hitachi U-2800 spectrophotometer. CD spectra were recorded on a JASCO J-715 spectropolarimeter using MeOH as solvent. IR data were recorded using a Bruker Vertex 70 spectrophotometer. ^{1}H and ^{13}C NMR data were acquired with a Bruker Avance-400 spectrometer using solvent signals ([D_6]acetone, $\delta = 2.05$, 29.8, 206.1 ppm, respectively) as references. The HMQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. ESIMS data were obtained on a Bruker Esquire 3000^{plus} spectrometer, and HRESIMS data were obtained using a Bruker APEX III 7.0 T spectrometer.

Fungal material: The culture of *P. fici* was isolated from the branches of *Camellia sinensis* (Theaceae) in a suburb of Hangzhou, People's Republic of China, in April, 2005. The isolate was identified as *P. fici* by one of the authors (L.G.) based on sequence (GenBank Accession number DQ812914) analysis of the ITS region of the ribosomal DNA and assigned the accession number AS 3.9138 (= W106-1) in the China General Microbial Culture Collection (CGMCC) at the Institute of Microbiology, Chinese Academy of Sciences, Beijing. The fungal strain was cultured on



Scheme 1. Plausible biosynthetic pathways for compounds **1**–**10**.

slants of potato dextrose agar (PDA) at 25 °C for 10 d. Agar plugs were used to inoculate Erlenmeyer flasks (250 mL), each containing 50 mL of media (0.4 % glucose, 1 % malt extract, and 0.4 % yeast extract), and the final pH of the media was adjusted to 6.5 before sterilization. Flask cultures were incubated at 25 °C on a rotary shaker at 170 rpm for 5 d. Fermentation was carried out in 36 Erlenmeyer flasks (500 mL), each containing 80 g of rice. Spore inoculum was prepared by suspension in sterile, distilled H_2O to give a final spore/cell suspension of 1×106 mL^{-1}. Distilled H_2O (120 mL) was added to each flask, and the contents were soaked overnight before autoclavation at 15 psi for 30 min. After cooling to room temperature, each flask was inoculated with 5.0 mL of the spore inoculum and incubated at 25 °C for 40 d.

Extraction and isolation: The fermented material was extracted with EtOAc (4×3 L), and the organic solvent was evaporated to dryness under vacuum to afford a crude extract (33 g). The extract was fractionated on silica gel by vacuum column chromatography (CC; 7×34 cm) using petroleum ether/EtOAc gradient elution. The fraction containing unidentified components was eluted with 32 % EtOAc (280 mg), and was fur-

ther separated by Sephadex LH-20 CC using $CHCl_3/CH_3OH$ 1:1. The resulting subfractions were combined and purified by semipreparative RPHPLC (Agilent Zorbax SB-C18 column, 5 μm, 9.4×250 mm, 65% CH_3OH in H_2O for 2 min followed by 65–75% CH_3OH in H_2O for 40 min, 2 mL min^{-1}) to afford chloropupukeanolide C (**8**; 3.0 mg, t_R = 27.81 min) and a mixture of chloropupukeanolide D and chloropupukeananin in a 10:1 ratio (**9** and **1**; 15.0 mg, t_R = 34.64 min). Another targeted fraction eluted with 42% EtOAc (120 mg) was purified by semipreparative RPHPLC (70% CH_3OH in H_2O for 2 min followed by 70–87% CH_3OH in H_2O for 30 min, 2 mL min^{-1}) to afford chloropupukeanolide E (**10**; 8.9 mg, t_R = 18.52 min).

Chloropupukeanolide C (8): Pale yellow oil; $[\alpha]_D = -36.1°$ (c = 0.1, MeOH); UV (MeOH): $\lambda_{max}(\varepsilon)$ = 219 (12100), 252 nm (8300 mol^{-1} dm^3 cm^{-1}); CD (MeOH): $\Delta\varepsilon_{308} = -1.69$, $\Delta\varepsilon_{250} = +2.16$, $\Delta\varepsilon_{223} = -19.5$ cm^2 mol^{-1}; IR (neat): ν_{max} = 3354 (br), 2957, 1736, 1702, 1640, 1464, 1365, 1205, 1035 cm^{-1}; ^{1}H, ^{13}C NMR, HMBC, and NOESY data see Table 1; HRESIMS: m/z: calcd for $C_{33}H_{35}ClO_{11}Na$: 665.1760 $[M+Na]^+$; found: 665.1769.

X-ray crystallographic analysis of 8: Upon recrystallization from MeOH/H_2O 50:1 using the vapor diffusion method, colorless crystals were obtained for **8** and a crystal (0.30×0.27×0.10 mm) was separated from the sample and mounted on a glass fiber, and data were collected using a MM007-HF CCD diffractometer with graphite-monochromated $Mo_{K\alpha}$ radiation (λ = 0.71073 Å) at 173(2) K. Crystal data: $C_{33}H_{35}ClO_{11}$, M = 643.06, space group monoclinic, $P2(1)2(1)2(1)$, unit cell dimensions: a = 7.0147 (15), b = 15.189 (3), c = 30.178 (6) Å, V = 3215.5 (12) Å^3, Z = 4, ρ_{calcd} = 1.328 mg m^{-3}, μ = 0.179 mm^{-1}, $F(000)$ = 1352. The structure was solved by direct methods using SHELXL-97[38] and refined by using full-matrix least-squares difference Fourier techniques. All non-hydrogen atoms were refined with anisotropic displacement parameters and all hydrogen atoms were placed in idealized positions and refined as riding atoms with the relative isotropic parameters. Absorption corrections were applied using the Siemens Area Detector Absorption Program (SADABS).[39] The 29366 measurements yielded 5895 independent reflections after equivalent data had been averaged and Lorentz and polarization corrections applied. The final refinement gave R_1 = 0.0654 and wR_2 = 0.1915 [$I > 2\sigma(I)$]. CCDC 785007 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Chloropupukeanolide D (9): Pale yellow oil; $[\alpha]_D = +13.2°$ (c = 0.1, MeOH); ^{1}H, ^{13}C NMR, HMBC, and NOESY data see Table S1 (Supporting Information); IR (neat): ν_{max} = 3443 (br), 2957, 1741, 1693, 1642, 1571, 1367, 1263, 1085 cm^{-1}; UV (MeOH), λ_{max} (ε) = 224 (18100), 255 nm (14300 mol^{-1} dm^3 cm^{-1}); CD (MeOH): $\Delta\varepsilon_{250} = -3.48$, $\Delta\varepsilon_{220} = +8.78$ cm^2 mol^{-1}; HRESIMS: m/z: calcd for $C_{33}H_{35}ClO_{11}Na$: 665.1760 $[M+Na]^+$; found: 665.1745.

Chloropupukeanolide E (10): Pale yellow oil; $[\alpha]_D = -25°$ (c = 0.1, MeOH); ^{1}H, ^{13}C NMR, HMBC, and NOESY data see Table S2 (Supporting Information); IR (neat): ν_{max} = 3359 (br), 2928, 1732, 1699, 1639, 1581, 1464, 1362, 1200 cm^{-1}; UV (MeOH): λ_{max} (ε) = 216 (16300), 259 nm (9600 mol^{-1} dm^3 cm^{-1}); CD (MeOH): $\Delta\varepsilon_{271} = -1.75$, $\Delta\varepsilon_{217} = +5.13$ cm^2 mol^{-1}; HRESIMS: m/z: calcd for $C_{32}H_{33}ClO_{11}Na$: 651.1604 $[M+Na]^+$; found: 651.1592.

Preparation of 10a, the acetonide derivative of 10:[40] A sample of **10** (1.0 mg) was dissolved in 1 mL of reagent (0.04 mL of 37% HCl and 20 mL of Me_2CO). The reaction was run at room temperature for 2 d and monitored by TLC. The mixture was dried in vacuo and then purified by semipreparative RP HPLC (70% CH_3OH in H_2O for 2 min followed by 70–90% CH_3OH in H_2O for 25 min, 2 mL min^{-1}) to afford compound **10a** (0.8 mg, t_R = 21.55 min): ^{1}H NMR (400 MHz, [D$_6$]acetone, 25 °C): δ = 9.88 (br s, 28-OH), 6.55 (s, H4), 6.42 (s, H29), 6.38 (s, H31), 6.05 (d, J = 2.0 Hz, H14), 5.76 (s, 6-OH), 4.59 (dd, J = 6.0, 2.0 Hz, H15), 4.22 (s, H18), 4.00 (d, J = 6.0 Hz, H16), 3.61 (s, 24-CH_3), 3.42 (dd, J = 19, 3.5 Hz, H9b), 2.80 (d, J = 19 Hz, H9a), 2.78 (dd, J = 14, 3.5 Hz, H2b), 2.30 (s, 3H, 33-CH_3), 2.06 (d, J = 14 Hz, H2a), 1.61 (dd, J = 8.0, 4.5 Hz, $H_2$0), 1.41 (s, 3′-CH_3), 1.28 (s, 2′-CH_3), 1.24 (s, 12-CH_3 and 23-CH_3), 1.11 (s, 22-CH_3), 0.79 (dd, J = 8.0, 5.5 Hz, H19b), 0.72 ppm (dd, J = 5.5, 4.5 Hz, H19a).

MTT assay:[19] The assay was run in triplicate. In 96-well plates, each well was plated with 10^4 cells. After cell attachment overnight, the medium was removed, and each well was treated with 50 μL of medium containing 0.2% DMSO, or appropriate concentrations of the test compounds (10 mg mL^{-1} as a stock solution of a compound in DMSO and serial dilutions). Cells were treated at 37 °C for 4 h in a humidified incubator at 5% CO_2 first, and were allowed to grow for another 48 h after the medium was changed to fresh Dulbecco's Modified Eagle Medium (DMEM). MTT (Sigma) was dissolved in serum-free medium or PBS at 0.5 mg mL^{-1} and sonicated briefly. In the dark, 50 μL of MTT/medium was added into each well after the medium was removed from wells, and incubated at 37 °C for 3 h. Upon removal of MTT/medium, 100 μL of DMSO was added to each well, and agitated at 60 rpm for 5 min to dissolve the precipitate. The assay plate was read at 540 nm using a microplate reader.

Anti-infective tests: Antiparasitic activities against *Plasmodium falciparum*, *Leishmania donovani*, *Trypanosoma cruzi*, *Trypanosoma brucei* rhodesiense, and *Trypansoma brucei* brucei were assessed as described earlier.[41]

Computational details: The conformational analyses for **9** and **10** were started with the MMFF94[42] force field. This yielded 120 possible conformers within 8 kcal mol^{-1} for 10*S*-**9**, 130 for 10*R*-**9**, 30 for 1*R*,3*S*,6*R*,7*R*,10*S*-**10**, and 15 conformers for 1*S*,3*R*,6*S*,7*S*,10*R*-**10**. These conformers were further optimized using Gaussian03[43] and the B3LYP/6-31G*[21] method. Single-point energy calculations at the SCS-MP2/TZVP[22,23] level and TDA B2PLYP/SV(P)[23,24] computations were performed with ORCA.[44] SpecDis 1.50[45] was used to sum up single UV or CD spectra after a Boltzmann statistical weighting, for the Gauss curve generation (using a sigma value of 0.24 eV), for the determination of the UV shift (15 nm),[46] and for comparison with the experimental data.

Acknowledgements

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