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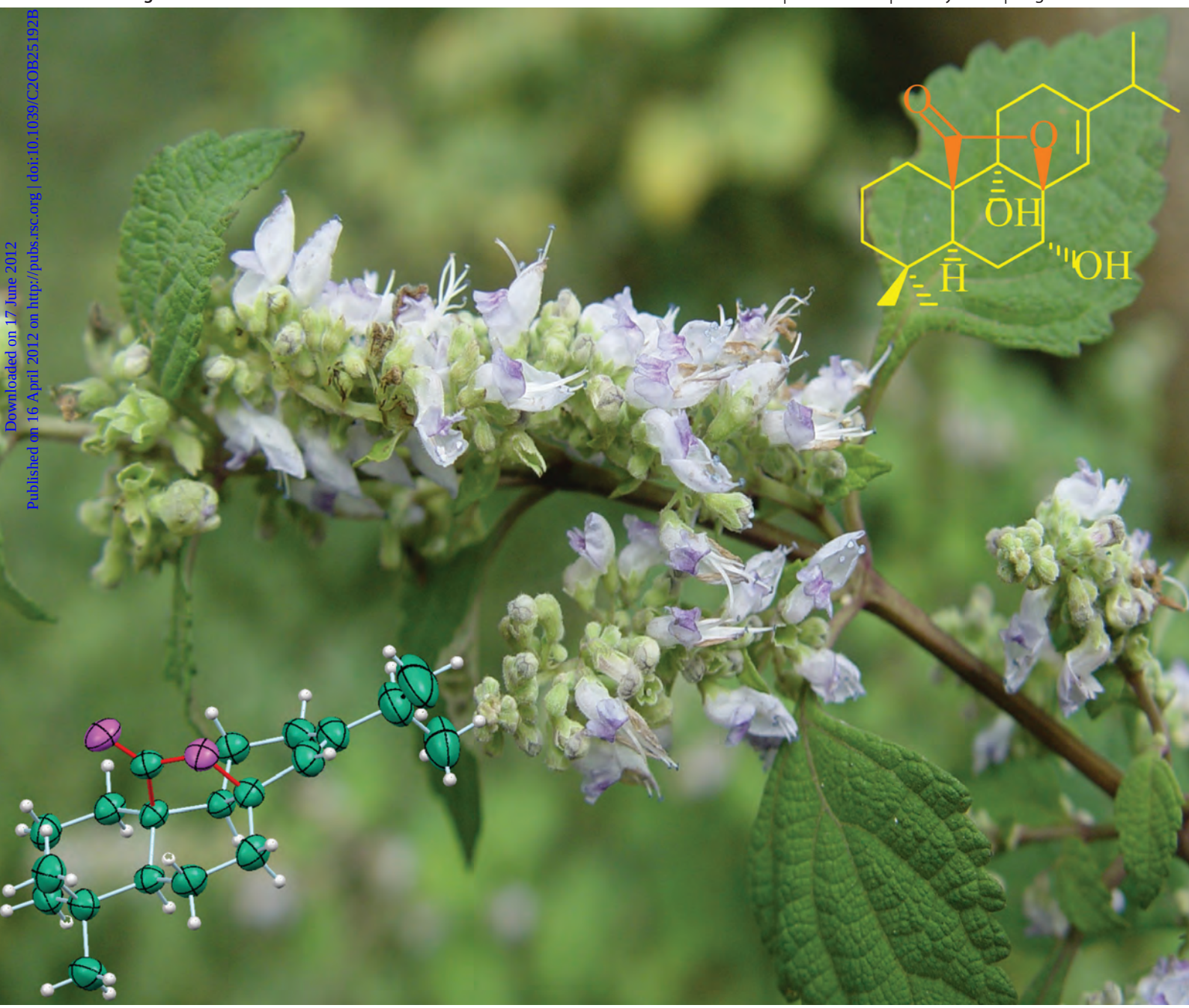

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PAPER

Rubesanolides C–E: abietane diterpenoids isolated from *Isodon rubescens* and evaluation of their anti-biofilm activity†

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Phytochemical study of the leaves of the medicinal plant *Isodon rubescens* led to the isolation of three novel abietane diterpenoids, rubesanolides C–E (**1–3**). These diterpenes contain a unique γ-lactone subgroup formed between C-8 and C-20. Their structures were determined from analysis of spectroscopic data, and were further confirmed by X-ray crystallographic data. The compounds were evaluated for their antibacterial activity, and rubesanolide D (**2**) demonstrated inhibition activity against biofilm formation of the dental bacterium *Streptococcus mutans*.

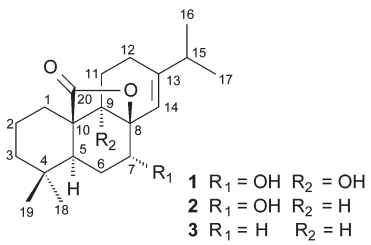
Introduction

Several plant species in the genus *Isodon* (Lamiaceae) have been used as traditional Chinese medicines, and *I. rubescens* is among the most popular ones used in China. The plant has been widely used in China for treatment of respiratory and gastrointestinal inflammation, bacterial infection and cancer diseases.[1] Phytochemical study of this species has led to the identification of more than 200 *ent*-kaurane diterpenes.[1,2] An herbal formulation, made from the extract of the whole plant of this herb has been developed as a Chinese medicine to treat sore throat and inflammation.[3] Since the plant has been extensively used as a Chinese folk medicine against bacteria, we reasoned that potent antibacterial compounds might exist in this plant. Indeed, our study showed that the extracts prepared from *I. rubescens* plant material collected in two different locations displayed more than 90% inhibition against biofilm formation by *Streptococcus mutans* at a concentration of 25 μg mL^{-1}, which prompted us to further study this plant in order to identify the antibacterial compounds.

Our previous phytochemical investigations on this plant discovered two novel rubesane diterpenoids: rubesanolides A and B, which contain an unprecedented β-lactone group formed between C-9 and C-20.[4] In our ongoing search for antibacterial and anti-biofilm compounds from this plant, we isolated three novel abietane diterpenoids, rubesanolides C–E (**1–3**), which formed a unique γ-lactone group between C-8 and C-20. This is the first time an abietane diterpene with a such group has been discovered in the genus *Isodon*. The compounds have been evaluated for cytotoxicity, antibacterial and anti-biofilm activity. Among them, rubesanolide D (**2**) demonstrated inhibitory activity against biofilm formation by the dental bacterium *Streptococcus mutans* with an MIC value at 0.5 mg mL^{-1}. The current paper reports the isolation, structure elucidation and biological evaluation of these novel compounds.

Results and discussion

A MeOH extract made from the leaves of *I. rubescens* was absorbed on silica gel and subjected to separation by a silica gel column, eluted with a petroleum ether–EtOAc gradient to yield fractions A–E. Fraction B was repeatedly chromatographed over silica gel columns and recrystallized to yield the pure compounds rubesanolides C–E (**1–3**).



1 R_1 = OH R_2 = OH
2 R_1 = OH R_2 = H
3 R_1 = H R_2 = H

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†Electronic supplementary information (ESI) available: Experimental details, NMR, MS, and IR spectra of **1–3**. Crystallographic data for the structure of **1** and **3**. CCDC 819348 and 672260. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c2ob25192b

Table 1 ^{1}H-NMR data of compounds **1–3** in $CDCl_3$

	1[a]	**2**[b]	**3**[a]
No.		δ (ppm), mult., J in Hz)	
H-1α	1.23, brtd, 13.7, 4.4	1.16, brtd, 13.3, 4.2	1.16, brtd, 13.2, 4.6
H-1β	1.77, brd, 13.4	1.79, brd, 12.7	1.90, brd, 13.6
H-2α	1.51, brdqu,[c] 13.5, 3.0	1.51, brdqu,13.6, 3.1	1.47, m
H-2β	2.02, overlap	2.09, brqt, 13.6, 3.3	2.07, brdqu, 13.5, 3.0
H-3α	1.87, brtd, 12.6, 3.1	1.15, brt, 13.5	1.15, brtd, 13.5, 3.6
H-3β	1.46, brd, 12.8	1.44, brddd, 13.3, 3.4, 1.9	1.46, brdd, 13.2, 2.7
H-5α	2.12, dd, 13.4, 4.8	1.93, dd, 13.3, 4.8	1.37, dd, 12.8, 4.5
H-6α	1.99, m	1.82, brdd, 13.6, 5.9	1.89, m
H-6β	1.88, brtd, 14.2, 4.8	1.48, brdt, 13.6, 6.0	1.63, m
H-7α		1.97, ddd, 14.2, 12.4, 6.5	2.00, m
H-7β	4.21, brd, 4.0	1.77, brdd, 13.9, 6.2	1.52, dd, 13.4, 6.2
H-9α			1.58, dd, 13.4, 4.4
H-11α	1.68, dd, 13.6, 5.0	1.69, brddd, 14.1, 5.4, 1.1	1.71, brd, 12.8
H-11β	1.40, brtd, 13.2, 5.4	1.58, ddd, 13.8, 12.1, 5.6	1.23, brqd, 12.9, 4.9
H-12α	2.37, m	2.06, m	2.03, m
H-12β	2.01, overlap	2.19, ddd, 12.2, 5.6 2.5	1.94, m
H-14	5.32, brs	5.28, brs	5.36, brs
H-15	2.29, brsep,[d] 6.9	2.24, brsep, 6.8	2.21, brsep, 6.8
CH_3-16	1.05, d, 6.9	1.03, d, 6.9	1.00, d, 6.8
CH_3-17	1.04, d, 6.8	1.03, d, 6.9	0.99, d, 6.9
CH_3-18	0.92, s	0.92, s	0.88, s
CH_3-19	0.88, s	0.91, s	0.90, s
7-OH	3.25, brs		
9-OH	5.06, brs		

[a] Data measured at 500 MHz. [b] Data measured at 400 MHz. [c] qu represents quintet. [d] sep represents septet.

Rubesanolide C (**1**) was obtained as colorless crystals. Its molecular formula was determined to be $C_{20}H_{30}O_4$ by means of analyzing its NMR spectroscopic data, and further verified by HREIMS data at m/z 334.2137 $[M]^+$ (calcd 334.2144). The molecular formula suggests the presence of 6° of unsaturation in the chemical structure of **1**. The IR spectrum of the compound displayed absorption bands at 3423 cm^{-1} and 1782 cm^{-1}, indicating the presence of hydroxyl and carbonyl groups, respectively. The ^{1}H, ^{13}C, and DEPT NMR spectra (Tables 1 and 2) showed the characteristic signals of a methine group ([δ_H 2.12 (br dd, J = 13.4, 4.8 Hz, H-5); δ_C 38.5 (d, C-5)], two tertiary methyl groups [δ_H 0.92 (s, Me-18) and 0.88 (s, Me-19)], an isopropyl group [δ_H 2.29 (br sep, J = 6.9, H-15), 1.05 (d, J = 6.9 Hz, Me-16), and 1.04 (d, J = 6.8 Hz, Me-17), one trisubstituted carbon–carbon double bond [δ_H 5.32 (br s, H-14), δ_C 115.1 (d, C-14) and 156.7 (s, C-13)], a carbonyl group [δ_C 178.8 (s, C-20)], an oxymethine carbon [δ_H 4.21 (br d, J = 4.0 Hz, H-7); δ_C 73.4 (d, C-7)], two oxyquaternary carbons [δ_C 77.6 (s, C-8), 77.5 (s, C-9)], and two quaternary carbons [δ_C 33.3 (s, C-4) and 51.8 (s, C-10)]. Six methylene groups were also clearly observed in the DEPT spectra. On the basis of these data and chemotaxonomic considerations, compound **1** was determined to be a diterpenoid.

The presence of the HMBC correlations from the proton at δ_H 5.32 (H-14) to C-7, -8, -9, -12, and -15 and from the methine proton at δ_H 2.29 (H-15) to C-13, -14, -16 and -17 indicated that the carbon–carbon double bond is located at C-13 and C-14 (Fig. 1). The presence of the HMBC correlations of H_2-1 and H-5 to the ^{13}C NMR signal at δ_C 178.8 (s) determined the carbonyl carbon at C-20. The presence of the HMBC correlations from the proton at δ_H 4.21 to the ^{13}C NMR signals at δ_C 38.5 (C-5), 77.6 (C-8), 77.5 (C-9), and 115.1 (C-14), and from H_2-6 and H-14 to the ^{13}C NMR signal at δ_C 73.4 indicated a hydroxy group at C-7. The presence of the HMBC correlations from the proton at δ_H 5.06 (br s, OH) to the ^{13}C NMR signals at δ_C 77.6 (C-8), 77.5 (C-9), and 27.1 (C-11), and from H-1α, H-5, H-7, H-12β, and H-14 to the ^{13}C NMR signal at δ_C 77.5 indicated a hydroxy group at C-9. The ^{13}C NMR chemical signal of C-5

Table 2 ^{13}C-NMR data of compounds **1–3** in $CDCl_3$

	1[a]	**2**[b]	**3**[a]
No.		δ (ppm), mult.	
C-1	24.4 CH_2	24.7 CH_2	28.4 CH_2
C-2	18.1 CH_2	18.1 CH_2	18.5 CH_2
C-3	41.2 CH_2	41.2 CH_2	41.9 CH_2
C-4	33.3 C	33.4 C	34.0 C
C-5	38.5 CH	41.4 CH	50.8 CH
C-6	29.0 CH_2	19.8 CH_2	21.5 CH_2
C-7	73.4 CH	28.8 CH_2	35.2 CH_2
C-8	77.6 C	81.1 C	80.5 C
C-9	77.5 C	75.1 C	54.7 CH
C-10	51.8 C	51.8 C	49.9 C
C-11	27.1 CH_2	26.6 CH_2	20.3 CH_2
C-12	22.8 CH_2	22.2 CH_2	25.6 CH_2
C-13	156.7 C	150.7 C	152.1 C
C-14	115.1 CH	117.7 CH	118.8 CH
C-15	35.3 CH	34.7 CH	35.1 CH
C-16	20.9 CH_3	20.2 CH_3	20.7 CH_3
C-17	21.4 CH_3	20.7 CH_3	21.3 CH_3
C-18	32.0 CH_3	32.1 CH_3	32.2 CH_3
C-19	20.3 CH_3	20.0 CH_3	20.2 CH_3
C-20	178.8 C	178.8 C	179.7 C

[a] Data measured at 125 MHz. [b] Data measured at 100 MHz.

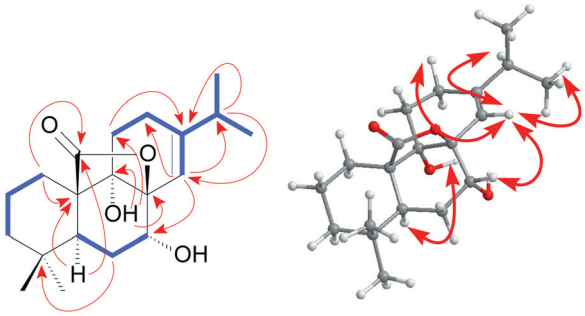


Fig. 1 Key COSY (—in blue), HMBC (→in red) and ROESY (↔in red) correlations for **1**.

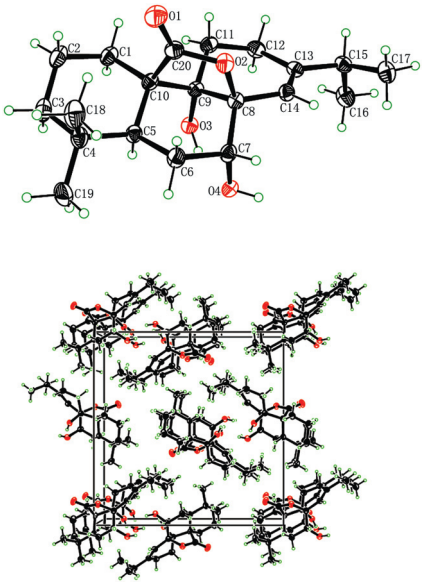


Fig. 2 Single-crystal X-ray structure of **1**.

was significantly shifted upfield by the γ-steric compression effects from 7-OH and 9-OH (see compound **3**). When the three rings and the two double bonds (the carbonyl group and the carbon–carbon double bond) were considered, an additional double-bond equivalent remained undefined in the compound. This undefined double-bond equivalent must belong to an additional ring since no other double bond was observed in the NMR spectra. The additional ring was determined as a γ-lactone group formed between C-8 and C-20. The relative stereochemistry of **1** was further determined by a ROESY experiment (Fig. 1). In the ROESY spectrum, the presence of the correlations between H-5 and H-1α/H$_3$-18/9-OH, between 9-OH and H-5α/H-12α, and between H-7 and H-14 determined H-5, 7-OH and 9-OH all as α-oriented. The CD spectrum of **1** showed a positive Cotton effect (235 nm $\Delta\varepsilon$ + 1.13, 217 nm, $\Delta\varepsilon$ − 3.05) indicative of the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ exiton of the double bond and the γ-lactone group. These split CD signals suggested C-8 and C-10 as *S* and *R* configurations respectively,[5] which, together with the large optical rotation datum, determined **1** as an abietane diterpenoid.

To confirm the structure and the stereochemistry, **1** was crystallized in MeOH to afford a crystal of the monoclinic space group $P2_12_12_1$, which was analyzed by X-ray crystallography (Fig. 2). The absolute configuration was determined by the measurement of the Flack parameter, which is calculated during the structural refinement.[5,6] In our study, the final refinement on the Cu Kα data of the crystal of **1** resulted in a Flack parameter of 0.03 (17), allowing an unambiguous assignment of the absolute structure as shown in Fig. 1.[4] The five chiral centers, C-5, C-7, C-8, C-9, C-10, were thus determined as *S*, *R*, *S*, *S*, *R*, respectively. Accordingly, the structure of **1** was established and given the trivial name rubesanolide C.

Rubesanolide D (**2**), obtained as amorphous powder, was shown to have a molecular formula of $C_{20}H_{30}O_3$ on the basis of the HREIMS ($[M]^+$, found: 318.2188, calcd: 318.2195). In the IR spectrum, absorption bands at 3449 cm^{-1} and 1737 cm^{-1} were observed, indicating the presence of hydroxyl and carbonyl groups, respectively. The ^{1}H and ^{13}C NMR spectroscopic data of **2** are very similar to those of **1** (Tables 1 and 2), indicating similar structures between the two compounds. Compound **2** differs from **1** only by the appearance of an extra methine carbon signal at δ_C 28.8, and the absence of the oxymethine carbon signal at δ_C 73.4, which resulted in the downfield shift of C-8 signal from δ_C 77.6 in **1** to δ_C 81.1 in **2**, and the upfield shift of

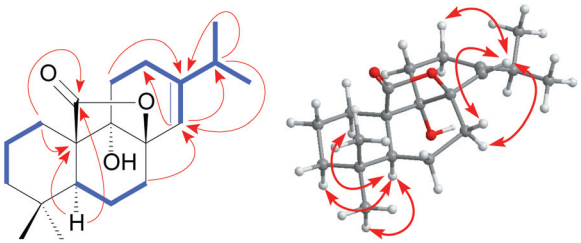


Fig. 3 Key COSY (—in blue), HMBC (→in red) and ROESY (↔in red) correlations for **2**.

C-9 signal from δ_C 77.5 in **1** to δ_C 75.1 in **2**. The methine carbon was determined to be C-7 by the presence of the HMBC correlations from H-5, H$_2$-6 and H-14 to the ^{13}C NMR signal at δ_C 28.8, the presence of the correlations from the proton signals at δ_H 1.93 (H-5) to the ^{13}C NMR signals at δ_C 41.2 (C-3), 33.4 (C-4), 28.8 (C-7), 75.1 (C-9), 51.8 (C-10), 32.1 (C-18), 20.0 (C-19) and 178.8 (C-20), and the presence of the correlations from the proton signals at δ_H 5.28 (H-14) to the ^{13}C NMR signals at δ_C 28.8 (C-7), 81.1 (C-8), 75.1 (C-9), 22.2 (C-12) and 41.4 (C-15). Similar to **1**, the γ-steric compression effect of 9-OH also resulted in the upfield shift of the C-5 signal. In the ROESY spectrum, the presence of the correlations between H-5 and H-1α/H-3α/H$_3$-18 determined H-5 as α-oriented (Fig. 3). The CD spectrum of **2** showed a split Cotton effect, indicative of exciton coupling between the $n \rightarrow \pi^*$ transition of the γ-lactone and the $n \rightarrow \pi^*$ transition of the cross conjugated dienone system with positive chirality (236 nm, $\Delta\varepsilon$ + 0.61 and 216 nm, $\Delta\varepsilon$ − 6.27) (Fig. 4). This split CD suggested the absolute stereochemistry of both C-8 and C-10 to be *R* by comparison with the CD spectral data of **1**. The other two chiral centers, C-5 and C-9, were thus both determined as *S*. Accordingly, the structure of **2** was established and given the trivial name rubesanolide D.

Rubesanolide E (**3**) was obtained as colorless cubic crystals (MeOH) with the molecular formula determined as $C_{20}H_{30}O_2$ by

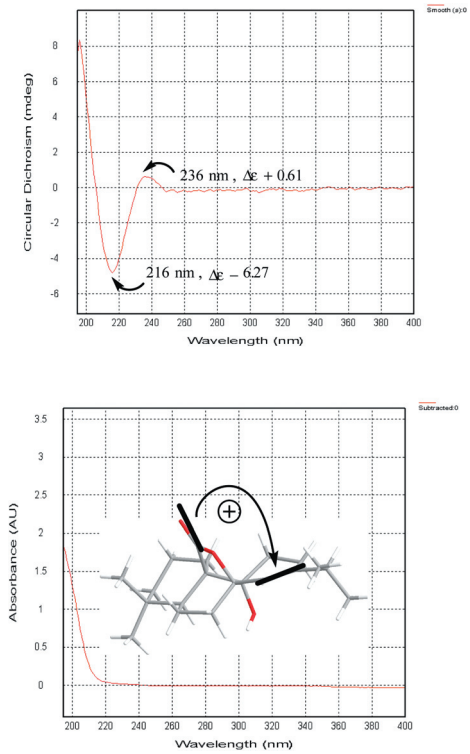


Fig. 4 CD and UV spectra of **2**. Bold lines denote the electric transition dipole of the chromophores.

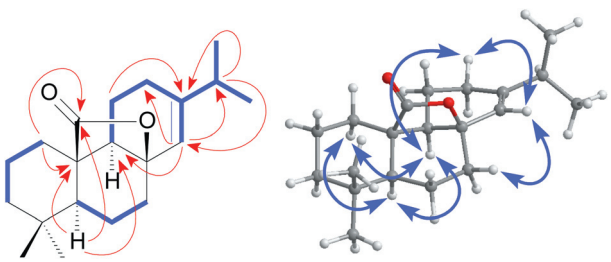


Fig. 5 Key COSY (—in blue), HMBC (→in red) and ROESY (↔in blue) correlations for **3**.

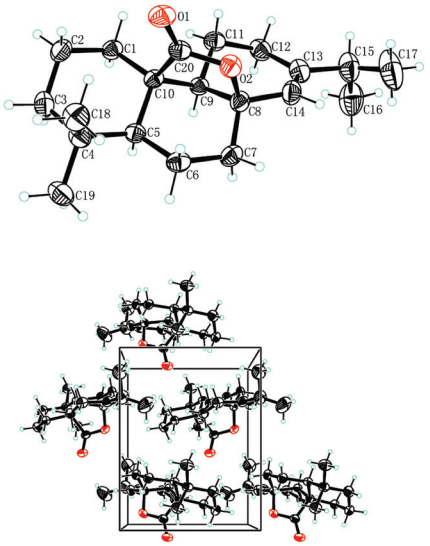
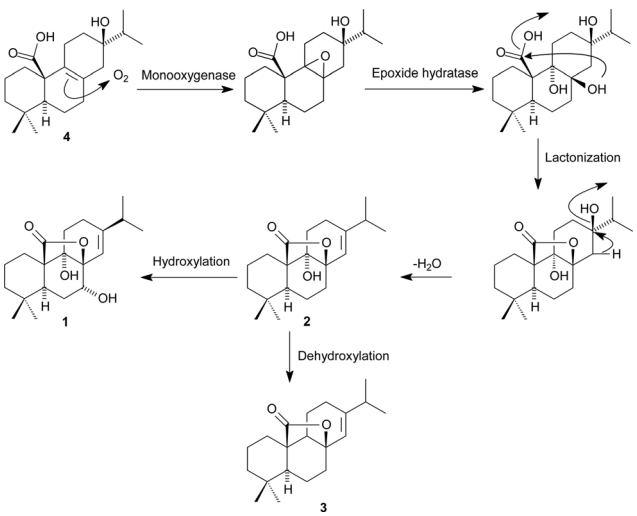


Fig. 6 Single-crystal X-ray structure of **3**.



Scheme 1 Proposed biogenetic pathway of **1**, **2** and **3**.

HREIMS ([M]$^+$, found 302.2245, calcd 302.2246). The IR spectrum of **3** showed an absorption band at 1750 cm^{-1}, indicating the presence of an ester carbonyl group. The compound showed similar ^{1}H and ^{13}C NMR spectroscopic data to those of **2** (Tables 1 and 2), suggesting structural similarity between **2** and **3**. The only difference of **3** from **2** is that **3** showed no hydroxy group at C-9, which resulted in the appearance of an additional methine carbon signal at δ_C 54.7 in **3**. The HMBC correlations from H-5, H_2-6, H-11 and H-14 to the ^{13}C NMR signal at δ_C 54.7 determined the methine carbon as C-9 (Fig. 5). In the ROESY spectrum, the presence of the correlations between H-5 and H-1α/H-3α/H_3-18 determined H-5 as α-oriented.

Finally, compound **3** was crystallized in MeOH to afford a crystal of the monoclinic space group $P2_1$, which was analyzed by X-ray crystallography (Fig. 6). The relative configuration was determined by the measurement of the Flack parameter, which is calculated during the structural refinement.[6,7] In our study, the final refinement on the Mo Kα data of the crystal of **3** resulted in a Flack parameter of 10 (10), which is too large for absolute-configuration determination, but allowing an unambiguous assignment of the relative structure. The absolute configuration can be determined by calculating the Flack parameter during the structural refinement. However, only if the parameter is refined to a value between 0–1 (as in compound **1**), can the correct absolute structure can be determined.[5,6] Accordingly, the structure of **3** was established and given the trivial name rubesanolide E.

A plausible biogenetic pathway for **1**, **2** and **3** was proposed (Scheme 1) in which the three compounds might be derived from lophanic acid (**4**).[4,7] The carbon–carbon double bond of lophanic acid (**4**) is transformed by monoxygenase to an epoxide, which is converted to dihydroxyl groups by epoxide hydratase. Lactonization of one of the hydroxyl groups (8-OH) with the C-20 carboxylic acid group then results in a γ-lactone group. The γ-lactone intermediate undergoes a series of reactions

including another hydroxylation and dehydroxylation to produce rubesanolides C (**1**), D (**2**) and E (**3**).

Compounds **1–3** were evaluated for their cytotoxicity against three human tumor cell lines, including K562, A549 and MCF7. However, no cytotoxicity against these cell lines were observed for the three compounds at a concentration of 20 μg mL^{-1}. The compounds were further evaluated for their antibacterial and anti-biofilm potential against the dental pathogen *S. mutans*. Compound **2**, at 25 μg mL^{-1}, was found to be able to inhibit biofilm formation, whereas the MIC was considerably higher at 0.5 mg mL^{-1}. This suggests that the mechanism behind prevention of biofilm formation is distinct from that which inhibits growth of the organism.

Experimental section

General

Melting points were obtained on an XRC-1 micro melting point apparatus and were uncorrected. Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. CD spectra were tested using a Chirascan Circular Dichroism spectrometer. A Tenor 27 spectrophotometer was used for scanning IR spectroscopy with KBr pellets. X-ray data were determined using a Bruker APEX DUO instrument. 1D and 2D NMR spectra were recorded on INOVA 400 and ZJEOL ECX-500 spectrometers. Unless otherwise specified, chemical shifts (δ) were expressed in ppm with reference to the solvent signals. Mass spectra were performed on a VG Autospec-3000 spectrometer at 70 eV. Column chromatography was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, People's Republic of China). Thin-layer chromatography (TLC) was carried out on silica gel 60 F254 on glass plates (Qingdao Marine Chemical Inc.) using various solvent systems and spots were visualized by heating the silica gel plates sprayed with 95–98% H_2SO_4–EtOH (v/v = 10 : 90).

Plant material

The leaves of *I. rubescens* were collected in Longli, Guizhou Province, People's Republic of China, in October, 2006. The plant was identified by Prof. Deyuan Chen and a voucher specimen (No. GM20061010) has been deposited at the Guiyang College of Traditional Chinese Medicine.

Extraction and isolation

The plant material of *I. rubescens* (8.5 kg) was powdered and exhaustively extracted with MeOH (25 L) at room temperature. The solvent was evaporated *in vacuo*, and the crude extract (1450 g) was chromatographed on a silica gel column (80–100 mesh, 2 × 120 cm, 6.0 kg) eluting with ether–EtOAc (v/v = 1 : 0, 9 : 1, 8 : 2, 2 : 1, 1 : 1, and 0 : 1, each 20 L) to yield fractions A–E. Fraction B was chromatographed on a silica gel column, eluting with petroleum ether–EtOAc (15 : 1–1 : 1) to obtain five sub-fractions (B1–B5). The fraction B3 was re-chromatographed on a silica gel column to give rubesanolides C (**1**) (13 mg), D (**2**) (17 mg) and E (**3**) (35 mg).

Rubesanolide C (1). Colorless needles (MeOH); mp 172–174 °C; $[\alpha]_D^{22}$ −48.9 (*c* 1.45 in MeOH); UV λ_{max}(MeOH)/nm (log ε) 214 (3.53); CD (MeOH) (235 nm $\Delta\varepsilon$ + 1.13, 217 nm, $\Delta\varepsilon$ − 3.05); IR (KBr) ν_{max}/cm^{-1} 3423, 2962, 2926, 1782, 1654, 1459, 1367, 1228, 1120, 1110, 964; ^1H and ^{13}C-NMR spectroscopic data, see Tables 1 and 2; HREIMS *m/z* 334.2137 ($[M]^+$, calcd for $C_{20}H_{30}O_4$, 334.2144).

Crystal data for rubesanolide C (**1**) (from MeOH): monoclinic space group $P2_12_12_1$. The crystal data: $C_{20}H_{30}O_4$, M = 334, a = 6.62460 (10), b = 16.0121 (2), c = 16.5233 (2) Å, $\alpha = \beta = \gamma$ = 90°, V = 1752.69 (4) $Å^3$, Z = 4, d = 1.267 g cm^{-3}. A crystal of dimensions 0.10 × 0.14 × 0.50 mm was used for measurements on a Bruker APEX DUO diffractometer with a graphite monochromator (θ_{max} = 67.8, $I > 2\sigma(I)$), Cu Kα radiation (296 K). The total number of independent reflections measured was 2928, of which 2885 were observed ($|F|^2 \geq 2\sigma|F|^2$). The crystal structure was solved and refined by the direct method SHELXS-97 (Sheldrich, G. M. University of Göttingen: Göttingen, Germany, 1997), expanded using difference Fourier techniques and full-matrix least-squares calculations. Final indices: R_1 = 0.0335, wR_2 = 0.0948 ($w = 1/\sigma|F|^2$), s = 1.086.

Rubesanolide D (2). Colorless cubic crystals (MeOH); mp 181–184 °C; $[\alpha]_D^{20}$ −62 (*c* 1.93 in MeOH); UV λ_{max}(MeOH)/nm (log ε) 217 (3.13); CD (MeOH) (236 nm, $\Delta\varepsilon$ + 0.61 and 216 nm, $\Delta\varepsilon$ − 6.27); IR (KBr) ν_{max}/cm^{-1} 3449, 2962, 1737, 1641, 1530, 1439, 1366, 1234, 940; ^1H and ^{13}C-NMR spectroscopic data, see Tables 1 and 2; HREIMS *m/z* 318.2188 ($[M]^+$, calcd for $C_{20}H_{30}O_3$, 318.2195).

Rubesanolide E (3). Colorless needles (MeOH); mp 142–145 °C; $[\alpha]_D^{12}$ −221 (*c* 2.53 in MeOH); UV λ_{max}(MeOH)/nm (log ε) 217 (3.25); IR (KBr) ν_{max}/cm^{-1} 3451, 2961, 1804, 1641, 1430, 1367, 1208, 1139, 1096; ^1H and ^{13}C-NMR spectroscopic data, see Tables 1 and 2; HREIMS *m/z* 302.2245 ($[M]^+$, calcd for $C_{20}H_{30}O_2$, 302.2246).

Crystal data for rubesanolide E (**3**) (from MeOH): monoclinic space group $P2_1$. The crystal data: $C_{20}H_{30}O_2$, M = 302, a = 7.85 79 (9) Å, b = 10.1629 (11), c = 11.0489 (12) Å, $\alpha = \gamma$ = 90°, β = 90.616 (4)°, V = 882.30 (17) $Å^3$, Z = 2, d = 1.138 g cm^{-3}. A crystal of dimensions 0.02 × 0.27 × 0.46 mm was used for measurements on a SMART APEX CCD SYSTEM diffractometer with a graphite monochromator (θ_{max} = 25.0, $I > 2\sigma(I)$), Mo Kα radiation (293 K). The total number of independent reflections measured was 1643, of which 1460 were observed ($|F|2 \geq 3\sigma|F|^2$). The crystal structure was solved and refined by the direct method SHELXS-86,[11] expanded using difference Fourier techniques, and refined by program pack WINGX32.[12]

Crystallographic data for the structures of compounds **1** and **3** have been deposited at the Cambridge Crystallographic Data Centre (Deposition No. CCDC 819348 for **1** and CCDC 672260 for **3**).†

Cytotoxic assay

Compounds **1–3** were evaluated for their cytotoxicity against several human tumor cell lines, including lung (A549), leukemic

(K562) and breast (MCF7) cell lines by the MTT[8] and SRB[9,10] methods as previously reported.

Antibacterial and anti-biofilm assays

Inhibition of growth was determined using a microdilution assay in sterile 96-well microtiter plates. Each well contained 50 μL twofold serially diluted test agent, 50 μL BHI growth medium and 10 μL of an overnight culture of *Streptococcus mutans* UA159, representing approximately 5×10^7 CFU mL^{-1}. The controls comprised inoculated growth medium without test agents, and sample blanks in growth medium only. The plates were incubated anaerobically for 24 h at 37 °C. 20 μL cultures from wells were then inoculated on BHI agar plates and incubated anaerobically for 48 h at 37 °C. The MIC was defined as the lowest concentration of test agent at which no growth was observed on the BHI plates. Inhibition of biofilm formation was also tested in 96-well microtiter plates. Each well contained 85 μL of chemically defined medium (SAFC Biosciences, Lenexa, KS) containing 1% sucrose, 10 μL overnight culture of *S. mutans* UA159 (OD_{600} = 0.1), and 5 μL of the test extract at 25 μg mL^{-1}. Control wells used 5 μL of the diluent DMSO. The plates were incubated 24 h at 37 °C in a CO_2 (5%) incubator to allow biofilm development. The planktonic phase was aspirated and 100 μL 0.1% crystal violet was added and allowed to stain the biofilm for 10 min at room temperature. The crystal violet was aspirated, and the plates washed 3 times by submerging in water and then air dried for 10 min. 50 μL of 33% acetic acid was added to each well and the plate placed on a rocker for 15 min to "destain" the biofilm. 25 μL was then removed to a new 96-well plate containing 25 μL water per well, and the OD measured at 550 nm. The intensities of the ODs in the test wells were compared to the OD in the control well.

Conclusions

In summary, this paper describes the isolation and structure elucidation of three novel abietane diterpenoids, rubesanolides C–E (**1–3**). The structures and absolute stereochemistry were elucidated by NMR spectroscopic data, and were confirmed by CD spectral data as well as by X-ray crystallographic analysis. No cytotoxicity was observed for the three compounds at a concentration of 20 μg mL^{-1}, which warranted further study of the compounds as biofilm inhibitors against dental pathogens. Compound **2** reduced biofilm formation by the dental pathogen *S. mutans* by over 90% at a concentration of 25 μg mL^{-1} when part of an extract. The anti-biofilm activity appeared to be distinct from growth inhibition which required a much higher dose of 500 μg mL^{-1}.

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