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ANTIBACTERIAL TRITERPENOIDS FROM *DILLENIA PAPUANA* AND THEIR STRUCTURE–ACTIVITY RELATIONSHIPS

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IN HONOUR OF PROFESSOR ANTONIO G. GONZALEZ

Key Word Index—*Dillenia papuana*; Dilleniaceae; triterpenoids; dillenic acid D and E; betulinic acid; antibacterial activity.

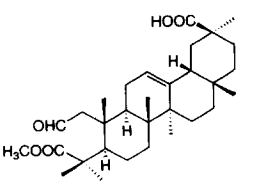
Abstract—Two new oleanene-type triterpenoids, dillenic acids D and E, have been isolated from the leaves and stems of *Dillenia papuana* together with the new natural product 3-oxoolean-12-en-30-oic acid. Together with these compounds, the known compound, betulinic acid (3β-hydroxy-20(29)-lupen-28-oic acid) was isolated as the major component of the fractions studied. Dillenic acids D and E were characterized as 2,3-*seco*-2-oxoolean-12-en-3-methylester-30-oic acid and 1α,3β-dihydroxyolean-12-en-30-oic acid and their nuclear magnetic resonance data were unambiguously assigned using two-dimensional nuclear magnetic resonance techniques. A comparison of antibacterial activities of these compounds with the earlier reported dillenic acids A–C indicated that, aside from a double bond in γ- or δ-position to a carboxylic group, a ketone function in ring A of an oleanene-skeleton may be required for the observed activity.

INTRODUCTION

In a previous paper [1], the isolation and structure elucidation of three new antibacterial oleanene-type triterpenoid acids 2α-hydroxy-3-oxoolean-12-en-30-oic acid (**2**), 2-oxo-3β-hydroxyolean-12-en-30-oic acid (**3**) and 1α-hydroxy-3-oxoolean-12-en-30-oic acid (**4**), dillenic acids A, B and C, together with the new natural product 3-oxoolean-1,12-dien-30-oic acid (**7**) from the aerial parts of the Papua New Guinean medicinal plant *Dillenia papuana* Martelli in Becc. (Dilleniaceae) were reported.

Continued investigation into the contents of the more polar extracts of this plant species has led to the isolation of a further three oleanene triterpenoids **1**, **5** and **6** together with the lupene derivative betulinic acid (**8**). The present paper deals with the isolation, structure elucidation and antibacterial activity of these compounds.

The structural variety of the seven isolated analogues of olean-12-en-30-oic acid permitted some structural requirements for the observed antibacterial activity of oleanene-type triterpenoid acids to be proposed.



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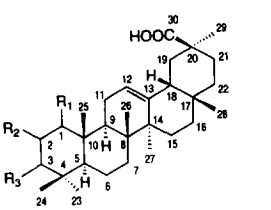
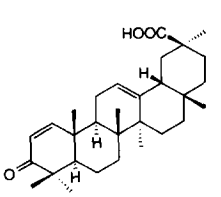


	R_1	R_2	R_3
2	H_2	α-OH	=O
3	H_2	=O	β-OH
4	α-OH	H_2	=O
5	α-OH	H_2	β-OH
6	H_2	H_2	=O



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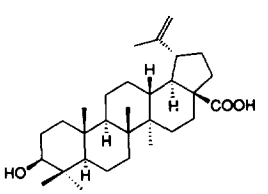

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RESULTS AND DISCUSSION

After successive extraction of the aerial parts of *Dillenia papuana* with petrol, dichloromethane and ethyl acetate, exhaustive column chromatography yielded

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three oleanene-type triterpenoids (**1**, **5** and **6**) and the known lupene derivative betulinic acid (**8**).

Dillenic acid D (**1**), a colourless amorphous powder, was shown to have the molecular formula $C_{31}H_{48}O_5$ (*m/z* 500.3492) by accurate mass measurement. Characteristic peaks at *m/z* 248 and 203 in the EI mass spectrum due to a retro-Diels–Alder fragmentation of an oleanene skeleton with a free carboxylic function either in ring D or E [2, 3] were observed. This structural type was further supported by the ^{1}H nuclear magnetic resonance (NMR) spectrum, which contained resonances for seven skeletal methyl groups (δ0.80, 0.98, 1.04, 1.13, 1.19, 1.22 and 1.28) and a broad triplet at δ5.27 for the olefinic proton (H-12). Other functional groups were a methyl ester (δ_H3.57, *s*; δ_C179.9, *s*), a carboxylic acid functionality (δ_C182.0, *s*), and an aldehyde (δ_H9.84, *br*; δ_C202.7, *d*). The carbonyl groups associated with these functionalities were also observed in the IR spectrum (1730, 1710, 1700 cm^{-1}). The ^{1}H and ^{13}C NMR data of compound **1** are given in Table 1. The assignments presented resulted from a combination of ^{1}H–^{1}H COSY, HMQC and HMBC experiments. Thus, the free carboxylic acid group was assigned to C-20, since it showed an HMBC correlation to H_3-29. Its orientation was proposed as β and axial by comparison of the ^{13}C NMR shifts with model systems [4, 5], which was in good agreement with the previously isolated dillenic acids [1]. The resonances for the protons of the methylene carbon at δ_C50.3 showed both a cross-peak to the resonance of the proton of the aldehyde group in the ^{1}H–^{1}H-COSY spectrum as well as an HMBC correlation to H_3-25. These data allowed the aldehyde moiety to be positioned at C-2. The methyl ester must be placed at C-3 as it showed long range correlations to both H_3-23 and H_3-24. Since only eight double bond equivalents were calculated for the molecular formula $C_{31}H_{48}O_5$, compound **1** was considered to be the *seco*-triterpene 2,3-*seco*-2-oxoolean-12-en-3-methylester-30-oic acid for which the trivial name dillenic acid D is proposed.

Table 1. ^{1}H (300 MHz, $CDCl_3$) and ^{13}C (75.5 MHz, $CDCl_3$) NMR spectral data of compound **1**

Carbon	δ_C*	δ_H†	HMBC (C–H) correlations
1	50.3 *t*	2.11‡, 2.26‡	H-2, H_3-25
2	202.7 *d*	9.84 *br*	H-1
3	179.9 *s*	—	H_3-23, H_3-24
4	45.4 *s*	—	H_3-23, H_3-24
5	50.8 *d*	2.24‡	H_3-23, H_3-24, H_3-25
6	19.8 *t*	1.64‡	
7	32.0 *t*	1.42‡, 1.62‡	H_3-26
8	39.9 *s*	—	H_3-26, H_3-27
9	41.8 *d*	2.12‡	H-12, H_3-25, H_3-26
10	41.9 *s*	—	H_3-25
11	23.3 *t*	1.89‡	H-12
12	122.3 *d*	5.27 *br*	
13	143.9 *s*	—	H_3-27
14	41.9 *s*	—	H_3-26, H_3-27
15	26.0 *t*	1.04‡, 1.77‡	H_3-27
16	26.9 *t*	0.91‡, 1.95‡	H_3-28
17	32.0 *s*	—	H_3-28
18	47.8 *d*	1.99‡	H-12, H_3-28
19	42.4 *t*	1.61‡, 1.86‡	H_3-29
20	44.0 *s*	—	H_3-29
21	31.1 *t*	1.37‡, 1.93‡	H_3-29
22	38.2 *t*	1.37‡	H_3-28
23	29.5 *q*	1.28 *s*	H_3-24
24	22.0 *q*	1.22 *s*	H_3-23
25	19.2 *q*	1.04 *s*	
26	16.7 *q*	0.98 *s*	
27	25.5 *q*	1.13 *s*	
28	28.2 *q*	0.80 *s*	
29	28.6 *q*	1.19 *s*	
30	182.0 *s*	—	H_3-29
3-COOMe	51.7 *q*	3.57 *s*	

*Multiplicities determined by DEPT sequence.

†^{1}H chemical shifts assigned on the basis of a ^{13}C–^{1}H COSY experiment.

‡Signal multiplicity was not asigned due to signal overlap.

4 and **5**, which have an axial hydroxyl function at C-1, have ^{13}C NMR resonances for C-5 and C-9 considerably shielded (δ_C47.7/47.4 and 37.5/37.2, respectively) from those that do not have this function (δ_C54.7–57.7 and 47.0–47.8, respectively, for **2**, **3** and **6**). As shown in Fig. 1, diagnostic NOE interactions clearly indicate rings A and B to be *trans*-fused, and hence the observed differences in chemical shifts observed for C-5 and C-9, as discussed above, can only be due to effects other than a *cis*-ring junction; i.e. the presence of an α-axial OH function at C-1.

^{13}C, ^{1}H and mass spectral data of compounds **5** and **6** showed a strong resemblance to those of compound **1**. The only differences between these three molecules could be observed in ring A. Compound **5** had the molecular formula $C_{30}H_{48}O_4$ (*m/z* 472.3535) by mass spectrometry, revealing the presence of seven degrees of unsaturation within the molecule. The ^{13}C NMR resonances at δ178.2 (*s*), 144.3 (*s*), 122.4 (*d*), 71.3 (*d*), and 70.5 (*d*) together with the HMBC correlations (Table 2), suggested **5** to be another olean-12-en-30-oic acid with all rings intact and two secondary hydroxyl groups. The first hydroxyl group was assigned to C-1, as its carbon resonance (δ_C70.5) showed an HMBC correlation to H_3-25. The second hydroxyl function was assigned to C-3, also on the basis of HMBC correlations, this time to H_3-23 and H_3-24, The relative stereochemistry of the OH group at C-1 was determined as α and axial on the basis of a NOESY cross-peak between H-1 and H_3-25. In MeOH-d_4, the resonance of this proton appeared in the ^{1}H NMR spectrum at δ_H 3.60 as a triplet, $J = 2.7$ Hz, which further supported its equatorial orientation. The configuration of the hydroxyl group at C-3 was assigned as β and equatorial on the basis of a NOESY correlation between H-3/H-5 and H-3/H_3-23, and the interproton coupling constants of this proton (δ_H3.47; *dd*, $J_1 = 3.6$, $J_2 = 12.3$ Hz). On the basis of the above findings compound **5** (dillenic acid E) was concluded to be 1α,3β-dihydroxy-12-en-30-oic acid. A similar compound (imberbic acid), but with the carboxylic group α and equatorial, has been isolated from *Combretum imberbe* [6].

It is noteworthy that the oleanene derivatives **4** and **5**, which have an axial hydroxyl function at C-1, have ^{13}C NMR resonances for C-5 and C-9 considerably shielded (δ_C47.7/47.4 and 37.5/37.2, respectively) from those that do not have this function (δ_C54.7–57.7 and 47.0–47.8, respectively, for **2**, **3** and **6**). As shown in Fig. 1, diagnostic NOE interactions clearly indicate rings A and B to be *trans*-fused, and hence the observed differences in chemical shifts observed for C-5 and C-9, as discussed above, can only be due to effects other than a *cis*-ring junction; i.e. the presence of an α-axial OH function at C-1.

Table 2. ^1H (300 MHz, DMSO-d_6) and ^{13}C (75.5 MHz, DMSO-d_6) NMR spectral data of compound **5** (values in parentheses are coupling constants J in Hz)

Carbon	δ_C*	δ_H†	HMBC (C–H) correlations
1	70.5 *d*	3.35‡	H_3-25
2	34.5 *t*	1.52‡, 1.74‡	
3	71.3 *d*	3.47 *dd* (3.6, 12.3)	H_3-23, H_3-24
4	38.6 *s*	—	H_3-23, H_3-24
5	47.4 *d*	1.12‡	H_3-23, H_3-24, H_3-25
6	18.0 *t*	1.43‡	
7	32.0 *t*	1.22‡, 1.40‡	H_3-26
8	39.6§	—	H_3-26, H_3-27
9	37.2 *d*	2.36 *dd* (6.8, 10.7)	H-12, H_3-25, H_3-26
10	40.4 *s*	—	H_3-25
11	22.6 *t*	1.83‡	H-12
12	122.4 *d*	5.15 *br*	
13	144.3 *s*	—	H_3-27
14	41.7 *s*	—	H-12, H_3-26, H_3-27
15	26.0 *t*	0.98‡, 1.72‡	H_3-27
16	26.6 *t*	0.85‡, 1.98‡	H_3-28
17	31.7 *s*	—	H_3-28
18	47.9 *d*	1.89‡	H-12, H_3-28
19	42.7 *t*	1.63‡	
20	43.3 *s*	—	H_3-29
21	30.8 *t*	1.24‡, 1.76‡	H_3-29
22	38.3 *t*	1.29‡	H_3-28
23	28.4 *q*	0.90 *s*	H_3-24
24	16.0 *q*	0.67 *s*	H_3-23
25	16.1 *q*	0.84 *s*	
26	16.8 *q*	0.90 *s*	
27	25.9 *q*	1.20 *s*	
28	28.3 *q*	0.73 *s*	
29	28.4 *q*	1.05 *s*	
30	178.2 *s*	—	H_3-29

*Multiplicities determined by DEPT sequence.

†^1H chemical shifts assigned on the basis of a ^{13}C–^1H COSY experiment.

‡Signal multiplicity was not assigned due to signal overlap.

§The chemical shift of C-8 was obscured by the solvent peak; its value was extracted from the HMBC experiment.

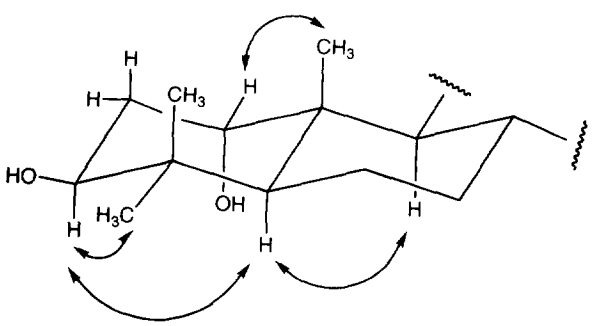

Fig. 1. Diagnostic NOE interactions observed in rings A and B of compound **5**.

The ^{13}C NMR data of the least polar triterpenoid **6**, molecular formula $C_{30}H_{46}O_3$, indicated it to be a further olean-12-en-30-oic acid derivative. The ^{13}C NMR signal at δ218.1 (*s*) confirmed the presence of a keto group, which was assigned to C-3 on the basis of HMBC correlations to H_3-23 and H_3-24. Hence, compound **6** was identified as 3-oxoolean-12-en-30-oic acid, a new natural product. Prior to this isolation, compound **6** has been reported as a semisynthetic derivative of glycyrrhetic acid and has been shown to block the urinary electrolyte effects of desoxycorticosterone in animals at the 75% dose level of spironolactone [7]. In common with **5**, the C-20 epimer (katononic acid) of **6** has been reported from *Austroplenckia populnea* [8].

After comparison of the ^{13}C NMR data with those reported in ref. [9], compound **8** was shown to be 3β-hydroxy-20(29)-lupen-28-oic acid (betulinic acid). This compound has been reported from several other genera of the Dilleniaceae [10].

Compounds **1**–**8** were tested for their effects towards the bacteria *Bacillus subtilis*, *Escherichia coli* and *Micrococcus luteus*. Minimum growth inhibition amounts on TLC are given in Table 3. Compounds **1**–**4**, **6** and **7** showed antibacterial activities, while **5** and **8** were inactive. The absence of activity for **5** suggests that not only the $\Delta^{12,13}$-double bond and the C-20 carboxylic group are important for activity, as discussed previously [1], but also an additional ketone function in ring A seems to be necessary for the observed activity in the oleanene derivatives isolated in this and the previous study. In a similar example, the reduction of the carbonyl group in ring A of 3-oxoolean-18-en-oic acid (moronic acid) also led to a loss of antibacterial activity [11]. Thus, oxidative fission or oxygen functionalization in ring A of olean-12-en-30-oic acid derivatives seems to be a simple way of introducing biological activity. Due to their biological activities and presence in the leaf or bark waxes near the plant's surface, these compounds are inferred to be part of the plant's defence against pathogenic microorganisms. In particular the chemical defences of tropical forest plants are often based on the production of large amounts of terpenoids and phenolics, since they grow on mineral-poor soils and are not able to produce nitrogen containing toxins [12].

Although most of the naturally occurring *seco*-triterpenes are 3,4-*seco*-compounds, there are some 2,3-*seco*-derivatives, e.g. 2,3-*seco*-olean-12-en-2,3,28-trioic acid from *Bursera graveolens* (Burseraceae) [13]. The family Burseraceae, just as *Dillenia papuana*, is known to con-

Table 3. Antibacterial activities* of isolated triterpenes

Compound	*B. subtilis*	*E.coli*	*M. luteus*
1	0.5	0.5	1.0
2	2.4	2.4	1.2
3	2.0	1.0	1.0
4	2.0	5.0	2.0
5	> 10	> 10	> 10
6	0.8	1.8	0.8
7	1.0	1.0	1.0
8	> 10	> 10	> 10
Chloramphenicol	0.1	0.04	0.04

*Minimum growth inhibition amount in μg on TLC.

tain triterpenoids with oxygen functionalities at C-2 and C-3. This suggests a possible biogenetic relation between these non-degraded 2,3-oxygenated triterpenes and the 2,3-*seco*-acids and/or aldehydes by means of an oxidative cleavage of ring A [14, 15]. The formation of the more common 3,4-*seco*-derivatives seems only to require a keto function at position 3 [12, 16].

In our recent publication [1], the values given for 2α-hydroxy-3-oxoolean-12-en-30-oic acid (compound **1** in Ref. [1]) for H-1α and H-1β were incorrectly reported and should be δ 1.12 (no signal multiplicity was assigned due to signal overlap) for H-1α and 2.42 (*dd*, $J = 6.5$ and 12.6 Hz) for H-1β.

EXPERIMENTAL

General. Mps uncorr. EIMS were measured using a Hitachi–Perkin-Elmer-RMUGM mass spectrometer using an ionization power of 70 eV. ^{1}H NMR at 300 MHz and ^{13}C NMR at 75.5 MHz (Bruker AMX 300) with the following internal references; ^{1}H residual $CHCl_3$ in $CDCl_3$ δ_H 7.26, DMSO δ_H 2.50, $^{13}CCDCl_3$ δ_C 77.0, DMSO δ_C 39.7 ppm. VLC (200 × 67 mm) and MPLC (460 × 15, 800 × 49 mm) were performed on silica gel 60 (Merck), particle size 40–63 and 15 μm, respectively. HPLC: 250 × 16 mm LiChrosorb Si 60, particle size 5 μm.

Plant material. Dillenia papuana Martelli in Becc. was collected near Lae in the Morobe Province, Papua New Guinea, in April 1991. The plant was identified by Dr P. Hovenkamp (University of Leiden, The Netherlands) where a voucher specimen (Hovenkamp & Katik ETH 91/22 7-04-91) is deposited.

Antibacterial activity. The test organisms used were *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), and *Micrococcus luteus* (ATCC 9341). Minimum growth inhibition amounts on TLC were determined using a bioautographic method [17].

Extraction and isolation. Air-dried and powdered leaves and stems (2.8 kg) were percolated subsequently with petrol, CH_2Cl_2 and EtOAc. Both the petrol (90.1 g) and a part of the EtOAc extract (10 g) were prefrac-

Table 4. ^{1}H (300 MHz, $CDCl_3$) and ^{13}C (75.5 MHz, $CDCl_3$) NMR spectral data of compound **6** (values in parentheses are coupling constants J in Hz)

Carbon	δ_C*	δ_H†	HMBC (C–H) correlations
1	39.5 *t*	1.90‡	H_{ax}-2, H_{eq}-2, H_3-25
2	34.4 *t*	2.37 *ddd* (3.6, 6.8, 15.9) H_{eq}-2 2.56 *ddd* (7.2, 11.1, 15.9) H_{ax}-2	
3	218.1 *s*	—	H_3-23, H_3-24
4	47.7 *s*	—	H_3-23, H_3-24
5	55.5 *d*	1.33‡	H_3-23, H_3-24, H_3-25
6	19.8 *t*	1.53‡	
7	32.3 *t*	1.38‡, 1.54‡	H_3-26
8	40.0 *s*	—	H_3-26, H_3-27
9	47.0 *d*	1.66‡	H_{ax}-2, H_{eq}-2, H_3-25
10	36.9 *s*	—	H_3-25
11	23.8 *t*	1.94‡	H-12
12	122.7 *d*	5.31 *br*	
13	144.5 *s*	—	H_3-27
14	41.8 *s*	—	H_3-26, H_3-27
15	26.3 *t*	1.01‡, 1.80‡	H_3-27
16	27.1 *t*	0.92‡, 1.94‡	H_3-28
17	32.2 *s*	—	H_3-28
18	48.2 *d*	2.01‡	H-12, H_3-28
19	42.7 *t*	1.64‡, 1.89‡	
20	44.3 *s*	—	H_3-29
21	31.3 *t*	1.37‡, 1.94‡	H_3-29
22	38.4 *t*	1.38‡	H_3-28
23	26.6 *q*	1.10 *s*	H_3-24
24	21.7 *q*	1.06 *s*	H_3-23
25	15.4 *q*	1.07 *s*	
26	16.9 *q*	1.01 *s*	
27	26.1 *q*	1.15 *s*	
28	28.4 *q*	0.82 *s*	
29	28.9 *q*	1.20 *s*	
30	183.2 *s*	—	H_3-29

*Multiplicites determined by DEPT sequence.

†^{1}H chemical shifts assigned on the basis of a ^{13}C–^{1}H COSY experiment.

‡Signal multiplicity was not assigned due to signal overlap.

tionated by VLC using a hexane–EtOAc and a hexane–EtOAc–MeOH gradient, respectively. Fr. 5 of the petrol extract, obtained by VLC with hexane–EtOAc (35:65) was subjected to MPLC (hexane–EtOAc–HCO_2H, 87:13:2) to afford 12 major frs. Subsequent HPLC (hexane–EtOAc–HCO_2H, 90:10:2 of fr. 5 yielded compounds **1** (92 mg) and **6** (19 mg). Fr. 3 of the EtOAc extract, eluted with hexane–EtOAc (1:9), was subjected to a second VLC, using $CHCl_3$ as eluent. Compound **8** (538 mg) precipitated from frs 2–4. Fr. 7 (VLC, EtOAc–MeOH, 8:2) of the EtOAc extract was further chromatographed by MPLC (hexane–EtOAc–MeOH, 80:20:3) to obtain 10 frs. Compound **5** (61 mg) precipitated from fr. 8.

Dillenic acid D (**1**). Amorphous solid. $[\alpha]_D^{20}$ + 64° ($CHCl_3$; *c* 0.12). IR ν_{max} cm^{-1}: 3450, 3020–2810, 1730, 1710, 1700; ^1H and ^{13}C NMR: Table 1; HREIMS *m/z*: 500.3492 $[M]^+$ (calcd for $C_{31}H_{48}O_5$: 500.3502); EIMS *m/z* (rel. int.): 500 $[M]^+$ (64), 455 (100), 314 (33), 248 (86).

Dillenic acid E (**5**). Amorphous solid. $[\alpha]_D^{23}$ + 107° (MeOH; *c* 0.07). IR ν_{max} cm^{-1}: 3410, 2990–2900, 1700; ^1H and ^{13}C NMR: Table 2; HREIMS *m/z*: 472.3535 $[M]^+$ (calcd for $C_{30}H_{48}O_4$: 472.3544); EIMS *m/z* (rel. int.): 472 $[M]^+$ (2), 454 $[M - H_2O]^+$ (2), 248 (100).

3-*Oxoolean*-12-*en*-30-*oic acid* (**6**). White crystalline needles (MeOH), mp 270–272°, $[\alpha]_D^{22}$ + 110°, ($CHCl_3$; *c* 0.06). IR ν_{max} cm^{-1}: 3400; 3010–2820, 1730, 1700; ^1H and ^{13}C NMR: Table 4; HREIMS *m/z*: 454.3497 $[M]^+$ (calcd for $C_{30}H_{46}O_3$: 454.3448); EIMS *m/z* (rel. int.): 454 $[M]^+$ (3), 248 (100).

3β-*Hydroxy*-20(29)-*lupen*-28-*oic acid* (*betulinic acid*) (**8**). Amorphous powder; $[\alpha]_D^{20}$ + 7° (EtOH; *c* 0.12), lit. [18] $[\alpha]_D$ + 6.3. IR ν_{max} cm^{-1}: 3475, 2940, 2860, 1685; ^1H NMR ($CDCl_3$–pyridine-d_5 1:1): δ0.44 (3H, *s*, H-26), 0.46 (3H, *s*, H-24), 0.61 (3H, *s*, H-25), 0.63 (3H, *s*, H-27), 0.67 (3H, *s*, H-23), 1.34 (3H, *s*, H-30), 4.25 and 4.42 (2H, 2 × *br s*, H-29); ^{13}C NMR ($CDCl_3$–pyridine-d_5 1:1): spectral data were identical with ref. [9]; EIMS *m/z* (rel. int.): 456 $[M]^+$ (9), 438 $[M - H_2O]^+$ (5), 220 (16), 207 (46), 203 (27), 191 (27), 190 (35), 189 (100).

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