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Antibacterial endiandric acid derivatives from *Beilschmiedia anacardioides*

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ABSTRACT

Three endiandric acid derivatives, beilschmiedic acids A, B and C were isolated from the stem bark of *Beilschmiedia anacardioides* together with the known β-sitosterol. Their structures were established by means of modern spectroscopic techniques. The relative configuration of compound **1** was determined by single crystal X-ray analysis. The antibacterial activities of compounds A,B,C were evaluated *in vitro* against five strains of microbes. Compound C showed strong activity against *Bacillus subtilis*, *Micrococcus luteus* and *Streptococcus faecalis* (MICs below 23 μM). This Compound was more active than the reference antibiotic ampicillin against *B. subtilis* and *M. luteus*.

1. Introduction

The genus *Beilschmiedia* comprises about 200 species widely distributed in the intertropical region (Fouilloy, 1974). *Beilschmeidia anacardioides* stem bark is used in the Western Province of Cameroon to cure uterine tumours, rubella, female genital infections and rheumatisms (Tchouala, 2001). Some other species of the genus *Beilschmiedia* such as *B. mannii* are used in traditional medicine in Africa for the treatment of dysentery and headache (Iwu, 1993). *B. mannii* is also used as appetite stimulant (Iwu, 1993). Previous phytochemical investigations of plants of the genus *Beilschmiedia* reported the presence of bio-active lignans (Chen et al., 2006; 2007), flavonoids (Harborne and Mendez, 1969), triterpenoids (Chen et al., 2006); tetracyclic endiandric acids (Bandaranayake and Banfield, 1981; Banfield et al., 1994) and alkaloids (Clezy et al., 1966; Kitagawa et al., 1993). To the best of our knowledge, no phytochemical investigation has been carried out on *B. anacardioides*. As part of our continuing search for biologically active compounds from Cameroonian medicinal plants, we have examined the stem bark of *B. anacardioides* and report here the isolation and structural elucidation of three tetracyclic endiandric acid derivatives, beilschmiedic acids A (**1**), B (**2**) and C (**3**) (Scheme 1) as well as their antibacterial activity towards five strains of microbes namely *Bacillus subtilis*, *Micrococcus luteus*, *Streptococcus faecalis*, *Pseudomonas palida*, and *Escherichia coli* with respect to the zone of inhibition (ZI) and minimum inhibitory concentration (MIC).

2. Results and discussion

Air-dried and ground stem bark of *B. anacardioides* was extracted successively at room temperature with MeOH. The methanol extract was re-extracted in turn with CH_2Cl_2 and EtOAc. These extracts were concentrated to dryness under reduced pressure. The CH_2Cl_2 extract was submitted to repeated column chromatography on silica gel, yielding beilschmiedic acids A (**1**), B (**2**) and C (**3**) and the known β-sitosterol.

Beilschmiedic acid A (**1**) was obtained as white colourless crystals, mp 150–151 °C. Its HRESI mass spectrum showed a pseudomolecular ion peak at *m/z* 343.22789 $[M-H]^+$, corresponding to $C_{22}H_{32}O_3$ (calc. for 343.22743) containing 7° of unsaturations. The IR spectrum of compound **1** exhibited strong absorption at υ_{max} 3382 (OH); 2951; 2919; 1687 (C=O); 1635 (C=C) and 1202 cm^{-1}. The broad band decoupled ^{13}C NMR spectrum of compound **1** (Table 1) displayed 22 carbon signals, which were sorted by Jmod and HSQC into eight methylene groups, one methyl group, two quaternary carbons including the carboxylic group at δ 170.5 and eleven methine groups of which one oxymethine carbon resonated at δ 65.7. The ^1H NMR spectrum of compound **1** (Table 2)

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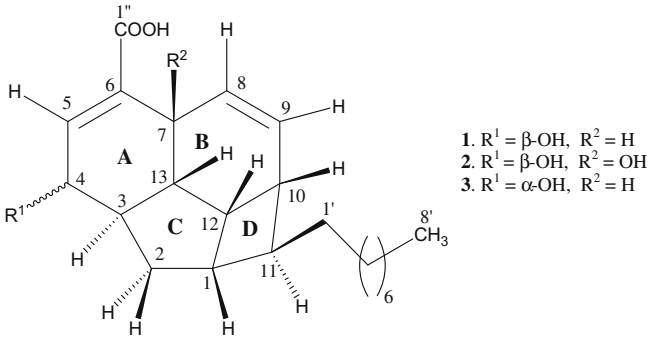
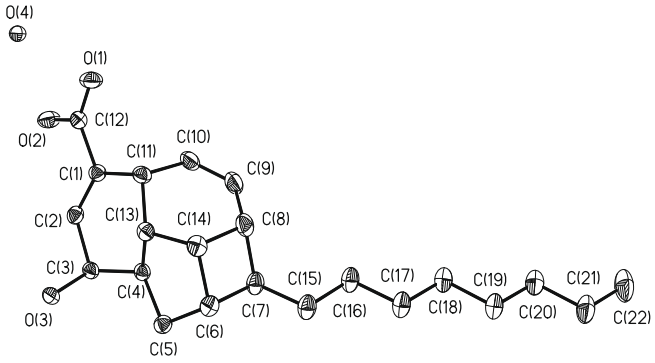


Scheme 1. Structures of compounds **1–3**.



Scheme 2. Molecular structure (relative configuration) of compound **1** according to X-ray diffraction analysis.

Table 1
^{13}C NMR (125 MHz) spectroscopic data for **1–3**.

Position	**1** (CD_3OD)	**2** (DMSO-d_6)	**3** (DMSO-d_6)
1	41.9	41.4	40.7
2	32.8	31.7	36.5
3	43.2	41.1	43.7
4	65.7	63.9	71.5
5	141.5	135.0	145.1
6	138.0	142.6	133.8
7	34.9	68.8	32.8
8	125.8	129.8	127.2
9	129.3	127.3	126.9
10	35.6	34.7	33.8
11	47.9	46.3	45.1
12	34.1	45.1	33.3
13	35.9	49.0	40.1
1′	38.4	37.1	35.1
2′	28.2	27.0	26.5
3′	30.8	29.5	29.1
4′	30.8	29.2	28.7
5′	30.5	29.6	29.1
6′	32.9	32.2	31.3
7′	23.7	22.6	22.1
8′	14.5	14.4	13.9
1″	170.5	172.0	167.3

showed signals for olefinic protons at δ 7.10 (*d*, *J* = 5.0 Hz, H-5), 5.46 (*d*, *J* = 10 Hz, H-8) and 5.64 (*dt*, *J* = 10 Hz and *J* = 3.4 Hz, H-9). In addition, a multiplet was found between *δ 1.34 and 1.53* integrating for 14 protons which correspond to seven methylene groups. One signal corresponding to an oxymethylene group appeared at δ 4.36 (H-4), together with several methine protons between δ 1.43 and 3.3 and one terminal methyl at δ 0.92 (*t*, *J* = 6.9 Hz, H-8'). These data were partially similar to some previously reported

Table 2
^{1}H NMR (500 MHz) spectroscopic data for **1–3**.

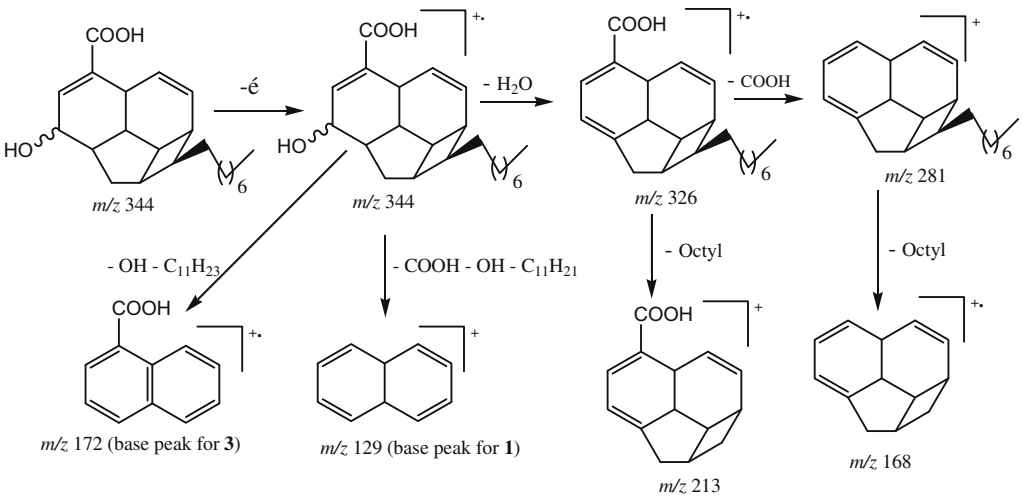
Position	**1** (CD_3OD) δ_H (*m*, *J* in Hz)	**2** (DMSO-d_6) δ_H (*m*, *J* in Hz)	**3** (DMSO-d_6) δ_H (*m*, *J* in Hz)
1	2.35 (*m*)	2.67 (*m*)	2.24 (*m*)
2	1.43 (*m*); 1.67 (*m*)	1.29 (*m*); 1.51 (*m*)	1.26 (*m*); 1.42 (*m*)
3	2.10 (*m*)	1.81 (*brs*)	1.89 (*m*)
4	4.36 (*t*, 4.0)	4.08 (*m*)	4.00 (*d*, 9.4)
5	7.10 (*d*, 5.0)	6.70 (*d*, 3.76)	6.74 (*brs*)
7	3.23 (*brs*)	–	3.04 (*brs*)
8	5.46 (*d*,10.0)	5.74 (*d*, 10)	5.46 (*d*, 10.0)
9	5.64 (*dt*, 10.0 and 3.4)	5.64 (*dd*, 10.0 and 4.3)	5.54 (*dt*, 10.0 and 3.1)
10	2.31 (*m*)	2.26 (*m*)	2.21 (*m*)
11	1.43 (*m*)	1.29 (*m*)	1.35 (*m*)
12	2.77 (*m*)	1.97 (*m*)	2.66 (*m*)
13	2.05 (*m*)	3.17 (*brs*)	1.76 (*m*)
1′	1.53 (*m*)	1.52 (*m*)	1.68 (*m*)
2′	1.34–1.36 (*m*)	1.18–1.29 (*m*)	1.16–1.24 (*m*)
3′	1.34–1.36 (*m*)	1.18–1.29 (*m*)	1.16–1.24 (*m*)
4′	1.34–1.36 (*m*)	1.18–1.29 (*m*)	1.16–1.24 (*m*)
5′	1.34–1.36 (*m*)	1.18–1.29 (*m*)	1.16–1.24 (*m*)
6′	1.34–1.36 (*m*)	1.18–1.29 (*m*)	1.16–1.24 (*m*)
7′	1.34–1.36 (*m*)	1.18–1.29 (*m*)	1.16–1.24 (*m*)
8′	0.92 (*t*, 6.9)	0.85 (*t*, 6.90)	0.83 (*t*, 6.3)

Table 3
Important HMBC (^{2}J and ^{3}J) and COSY correlation correlations in compounds **1–3**.

Compounds	H	^{2}J	^{3}J	^{1}H-^{1}H COSY
1 and **3**	H-1	C-11, C-12	C-3	H-2, H-12, H-11
	H-2	C-3, C-1	C-4	H-3, H-1
	H-3	C-4	C-7	H-4, H-13, H-2
	H-4	C-5, C-3	C-6, C-13	H-5, H-3
	H-5	C-6, C-4	C-3, C-7	H-4,
	H-7	C-8, C-13	C-9, C-1"	H-8, H-13
	H-8	C-9,	C-6, C-10,	H-7, H-9, H-11
	H-10	C-9, C-11	C-8	H-9, H-11, H-12
	H-11	C-1, C-1', C-10	C-2, C-2'	H-1, H-10
	H-12	C-10	C-3, C-11,	H-1, H-10, H-13
	H-13	C-3, C-7	C-4, C-8	H-7, H-3, H-12
	H-1'	C-11	C-1, C-10	
2	H-2	C-1, C-3		
	H-3	C-4, C-2	C-7	H-4, H-13, H-2
	H-5	C-4, C-6	C-7, C-1"	H-4
	H-8	C-7	C-10,	H-9
	H-10	C-11, C-12	C-1, C-1′	H-9
	H-11	C-1, C-1'	C-2′	
	H-12	C-10	C-7, C-11	H-10, H-1

endiandric acid derivatives (Banfield et al., 1994). The basic tetracyclic skeleton was confirmed by correlations observed in the HMBC and COSY spectra of compound **1** (Table 3). The position of the *n*-octyl moiety at C-11 was deduced from the correlations observed between the proton of the multiplet at δ 1.43 (H-11) and carbons C-1 (δ 41.9), C-1' (δ 38.4), C-2 (δ 32.8), C-2' (δ 28.2) and C-10 (δ 35.6). The HMBC correlations between H-5 (δ 7.10), H-3 (δ 2.10), H-7 (δ 3.23), H-2 (δ 1.67) and C-4 (δ 65.7) indicated that the hydroxyl group was attached to C-4. The carboxyl group was found to be located at C-6 according to the HMBC correlations between H-5 (δ 7.10), H-4 (δ 4.36), H-8 (δ 5.46) and C-6 (δ 138.0). The EI mass spectrum of **1** exhibited prominent peaks. Some of them were formed by the mechanism postulated in Scheme 3. The above data and other HMBC and COSY correlations led to structure **1**. This structure was confirmed by the single crystal X-ray analysis which also gave its relative configuration (Scheme 2). Thus compound **1** was elucidated as 4β-Hydroxy-11-n-octyltetracyclo[5.4.2.0^{3,13}.0^{10,12}]trideca-5,8-dien-6-carboxylic acid, a new endiandric acid derivative trivially named beilschmiedic acid A.

Beilschmiedic acid B (**2**) was obtained as white amorphous powder. Its HRESI mass spectrum showed a pseudomolecular ion



Scheme 3. Fragmentation mechanism of compounds **1** and **3**.

peak at *m/z* 359.22258 $[M-H]^+$, corresponding to $C_{22}H_{32}O_4$ (calc. for 359.22233) containing 7° of unsaturations. The IR spectrum of compound **2** exhibited strong absorptions at υ 3399 (OH); 2920; 2852; 1670 (C=O); 1639 (C=C) and 1026 cm^{-1}. The ^1H and ^{13}C NMR studies indicated that compound **2** was distinctively similar to that of compound **1**, with one additional hydroxyl group present in **2**. The broad band decoupled ^{13}C NMR spectrum of compound **2** (Table 1) displayed 22 carbon signals, which were sorted by Jmod and HSQC into ten methine groups including one oxymethine carbon at δ 63.9, eight methylene groups, one oxygenated quaternary carbon at δ 68.8, one methyl and three quaternary carbons including the carboxylic group at δ 172.0. All of these data confirmed that compound **2** was a hydroxyl derivative of compound **1**. The position of the second hydroxyl group in **2** was deduced from its ^{13}C NMR spectrum, in which C-7 resonates downfield at δ 68.8. In the ^1H NMR spectrum of compound **2** (Table 2), the signal of H-7 is absent, while it is the only proton to resonate between 3 and 4 ppm in the spectra of compounds **1** and **3**. Furthermore, the signal of H-8 at 5.74 (*d*, *J* = 10.0 Hz) is deshielded by this hydroxyl group at C-7, compared to **1** and **3** where it resonates at δ 5.46 (*d*, *J* = 10.0 Hz). This was confirmed by correlations observed between H-5 (δ 6.70), H-9 (δ 5.46), H-12 (δ 1.97) and C-7 on the HMBC spectrum. All of these data led us to assign the structure of compound **2** as 4β,7β-Dihydroxy-11-n-octyltetracyclo[5.4.2.$0^{3,13}$.$0^{10,12}$]trideca-5,8-dien-6-carbxylic acid, and given the trivial name beilschmiedic acid B. Its relative configuration was proposed on the basis of structural similarities to that of compound **1**.

Beilschmiedic acid C (**3**) was obtained as a white powder. Its HRESI mass spectrum showed a pseudomolecular ion peak at *m/z* 343.22789 $[M-H]^+$, corresponding to $C_{22}H_{32}O_3$ (calc. for 343.22743) containing 7° of unsaturations. The IR spectrum of compound **3** exhibited absorptions at υ 3389 (OH), 2953, 2918, 1703 (C=O), 1639 (C=C), 1233 cm^{-1}. The broad band decoupled ^{13}C NMR spectrum of compound **3** (Table 1) displayed 22 carbon signals, which were sorted by Jmod and HSQC into eleven methine groups, including one oxymethine carbon at δ 71.6, eight methylene groups, one methyl group and two quaternary carbons, including the carboxylic group at δ 167.3. The EI mass spectrum of **3** exhibited prominent peaks. Some of them were formed by the mechanism postulated in Scheme 3.

Comparison of ^1H and ^{13}C NMR chemical shifts (Tables 1 and 2) of **3** with those of **1** showed many similarities. The only difference was the slight downfield shift of carbon C-4 (δ 71.5), compared to the one of compound **1** which appeared at δ 65.7, although the different spectra were conducted in CD_3OD and DMSO-d_6 for **1** and **3**, respectively. According to the biosynthetic reactions postulated by Bandaranayake et al. (1980), it was assumed that compounds **1** and **3** differs only in the configuration at C-4 position. This change implies slight downfield and upfield shifts on carbons C-5 (δ 145.1), C-6 (δ 133.8) and C-13 (δ 40.1). This change is also confirmed by the different solubility. Based on the above data, the structure of **3** was elucidated as 4α-Hydroxy-11-n-octyltetracyclo[5.4.2.$0^{3,13}$.$0^{10,12}$]trideca-5,8-dien-6-carboxylic acid, a stereoisomer of **1**, named beilschmiedic acid C.

Compounds **1**–**3** were tested *in vitro* for their antibacterial activity. The ZI (Table 4) and the MIC (Table 5) obtained with these compounds indicated that they possessed antibacterial property with gram positive bacteria. Beilschmiedic C (**3**) demonstrated the best potency against *B. subtilis* and *M. luteus* compared to the reference antibiotic ampicillin. The MIC value of compounds **2** and **3** against *B. subtilis* and compound **3** against *M. luteus* was found to be comparable or even smaller than the one of the reference drug Ampicillin, indicating that this series of compounds might be possible candidates for antibacterial drugs. None of the tested compound was active against the gram negative microorganisms *P. palida* and *E. coli*.

Table 4
Antibacterial activity (Zone of inhibition of compounds in mm) of compounds **1**–**3** against *B. subtilis*, *M. luteus*, *S. faecalis*, *P. palida*, *E. coli*.

Compound tested	*B. subtilis*	*M. luteus*	*S. faecalis*	*P. palida*	*E. coli*
1	15	12	14	–	–
2	16	15	15	–	–
3	13	30	18	–	–
Ampicillin[a]	29	26	25	–	–

(–) inactive.
[a] Standard antibiotic used in the assay.

Table 5
Antibacterial activity (MIC in μM) of compounds **1**–**3** against *B. Subtilis*, *M. luteus*, and *S. faecalis*.

Compound tested	*B. subtilis*	*M. luteus*	*S. faecalis*
1	181.6	173.6	363.3
2	11.3	347.2	45.3
3	5.6	<0.7	22.7
Ampicillin[a]	89.5	5.58	11.1

[a] Standard antibiotic used in the assay.

Based on the skeletal features, it is difficult at this stage to define the contribution of the different functional groups with respect to activity, as compound **2** which possessed one hydroxyl group more than compounds **1** and **3**, was less active than **1** and **3**. The mechanism of action of this class of metabolite on these strains is not yet known. Further investigation will help to establish the mode of action of this particular skeleton. It have been reported that compounds with the same skeleton such as endiandric acid H, isolated from *B. fulva* and its derivatives possess antiasthmatic activity (Eder et al., 2004).

All of these interesting results partially validate the uses of *B. anacardioides* in traditional medicine and highlight the potency of this rare class of metabolites that might be further investigated in the search for new antibacterial or antiasthmatic drugs.

3. Experimental section

3.1. General experiment procedures

Melting points were determined on a Büchi B-540 melting point apparatus, and are uncorrected. Optical rotations were recorded with a Jasco DIP-360 digital polarimeter. IR spectra (KBr) were recorded on a Jasco FT/IR-410 spectrometer. The ^{1}H NMR spectra were recorded at 500 MHz and ^{13}C NMR spectra were recorded at 125 MHz, respectively, with TMS as an internal standard. The EIMS were recorded on a double focusing mass spectrometer (Varian MAT 311A). HRESIMS were recorded on a Apex III (Bruker Daltonik) 7 Tesla (ESI-FT-ICR-MS). The X-ray was recorded on a Nonius Kappa CCD.

3.2. Plant material

The stem bark of *B. anacardioides* (Lauraceae) was collected in January 2007 at Foumban, in Noun division of West province of Cameroon. The plant was identified by Paul Mezili, botanist of the Cameroon National Herbarium, where a voucher specimen has been deposited.

3.3. Antibacterial assay: measurement of ZI and MIC

In vitro antibacterial sensitivity tests of the pure compounds were determined using the agar well diffusion method (Perez et al., 1990). Agar plates with the liquid broth (LB) medium were prepared in a sterile clean bench. The medium was inoculated with 0.2 mL of the broth culture which was spread evenly on the agar surface using sterile grigalsky spatula. The wells were prepared in the agar plates with the help of a sterile cork-borer. About 75 μL of the compound dilutions and controls (ampicillin for gram positive bacteria and gentamycin for gram negative bacteria) were introduced into the wells (Parekh and Chanda, 2007; Rojas et al., 2006). The treated plates were pre-incubated in a refrigerator at 4 °C for at least 6 h to allow diffusion of the extracts and controls into the agar while arresting the growth of the test microbes. They were then transferred to an incubator for 24 h at 37 °C. Each test was carried out in duplicate. Antibacterial activity was determined by measuring the diameters of zones of inhibition in mm.

The broth micro dilution method was used to determine the MIC of the compounds. MIC is the lowest concentration which inhibits growth of the test organisms. The standard controls used were ampicillin and gentamycin (Parekh and Chanda, 2007; Rojas et al., 2006). The concentration range was between 500 μg/mL and 0.24 μg/mL. One hundred micro liter serial dilutions of the test compounds were dispensed in duplicates on 96-well microtitre plates. Positive controls of the standard drugs were included, as well as inoculated broth (LB medium) only without the test drugs. One hundred micro liter of 1×10^6 CFU/mL inoculum was added into the wells to make a volume of 200 μL for each well with the test drugs. The treated plates were incubated at 37 °C for overnight. MIC was read spectrophotometrically with a microplate reader (TECAN SPECTRA FLUOR PLUS®) using XFLUOR 4, Version 4.03 software at 560 nm.

3.4. Extraction and isolation

Dried, powdered stem bark of *B. anacardioides* (4.0 kg) was macerated for 72 h at room temperature with MeOH and concentrated to dryness and afford 275 g of extract. This extract was then re-extracted with CH_2Cl_2 (17 g), and EtOAc (34 g), respectively. The CH_2Cl_2 extract (17 g) was subjected to column chromatography over silica gel (0.063-0.200 mm) and eluted with mixtures of *n*-hexane and EtOAc with a gradient of increasing polarity. This resulted in 102 fractions of 100 mL each, which were combined on the basis of TLC analysis. Fractions 14–19 (7 g), that were eluted with a mixture of *n*-hexane and EtOAc (9:1), were concentrated and chromatographed once more on silica gel. Isocratic elution with a mixture of *n*-hexane and EtOAc (19:1) afforded β-sitosterol (15 mg). Fraction 81–89 (4.3 g) was eluted with a mixture of *n*-hexane and EtOAc (8:2) were concentrated and chromatographed a second time over silica gel. Isocratic elution with a mixture of *n*-hexane and EtOAc (17:3), afforded compound **3** (190 mg), compound **1** (160 mg), and compound **2** (50 mg).

Beilschmiedic acid A (1): colourless crystals; mp 150–151 °C; $[\alpha]_D^{20}$ – 120 (*c* 0.2, MeOH) IR (KBr) υ_{max} 3382, 2951, 2919, 1687, 1635, 1270, 1202 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD) see Table 1, ^{13}C NMR (125 MHz, CD_3OD) see Table 1. EIMS *m/z* (rel. int.,%) 344 (10), 326 (49), 281 (16), 213 (15), 189 (26), 173 (86), 172 (79), 169 (11),168 (6), 129 (100) and 91 (39). HRESIMS 343.22789, calc. 343.22743 for $C_{22}H_{31}O_3$.

X-ray crystallography data of beilschmiedic acid A (1): A colourless crystal was obtained from CH_2Cl_2–MeOH (9:1). The data were collected on a Nonius Kappa CCD. Cell parameters: $a = 54.3315(12)$ Å; $b = 7.8948$ (2) Å; $c = 9.6160$ (2) Å; $V = 4060.88$ (16) Å^3, space group monoclinic c 2/c, $Z = 8$, $D_{calc.} = 1.156$ mg/m^3, $\lambda = 0.71073$ Å, $\mu(MoK_\alpha) = 0.076$ mm^{-1}, $T = 100$ K. The complete crystallographic data for the structure of this compound have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 692579 Copies of the data can be obtained, free of charge, on application to CCDC http://www.ccdc.cam.ac.uk/deposit.

Beilschmiedic acid B (2) White amorphous powder. IR (KBr) υ_{max} 3381, 2953, 2918, 1703, 1639, 1466, 1283, 1233, 713 cm^{-1}; $[\alpha]_D^{20}$ – 80 (*c* 0.2, MeOH) ^{1}H NMR (500 MHz, DMSO-d_6) see Table 1, ^{13}C NMR (125 MHz, DMSO-d_6) see Table 1. EIMS *m/z* (rel. int.,%) 299 (8), 298 (24), 269 (6), 267 (9), 255 (16), 213 (5), 199 (11%), 143 (22), 185 (7), 129 (10), 87 (69), and 74 (100). HRESIMS 359.22258, calc. 359.22233 for $C_{22}H_{31}O_4$.

Beilsclmiedic acid C (3) white powder. IR (KBr) υ_{max} 3389, 2953, 2918, 1703, 1639, 1466, 1283, 1233, 1026, 713 cm^{-1}; $[\alpha]_D^{20}$ – 112 (*c* 0.2, MeOH) ^{1}H NMR (500 MHz, DMSO-d_6) see Table 1, ^{13}C NMR (125 MHz, DMSO-d_6) see Table 1. EIMS *m/z* (rel. int:%) 344 (10), 326 (27), 281 (8), 213 (18), 189 (37), 173 (76), 172 (100), 168 (6), 129 (90), 117 (36) and 77 (23). HRESIMS 343.22789, calc. 343.22743 for $C_{22}H_{31}O_3$.

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