Anti-HIV-1 Diterpenoids from Leaves and Twigs of *Polyalthia sclerophylla*

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Abstract

Bioassay-guided fractionation and purification of the anti-HIV-1-active MeOH extract from the leaves and twigs of *Polyalthia sclerophylla* led to the isolation of two new compounds, *ent*-kaur-sclerodimer (**1**) and cyclotucanol 3-palmitate (**2**), along with the known *ent*-kaur-16-en-19-oic acid (**3**), 15*β*-hydroxy-*ent*-kaur-16-en-19-oic acid (**4**), 15*β*-acetoxy-*ent*-kaur-16-en-19-oic acid (**5**), 15-oxo-*ent*-kaur-16-en-19-oic acid (**6**), 16*α*,17-dihydroxy-*ent*-kauran-19-oic acid (**7**), 16*α*-hydroxy-*ent*-kauran-19-oic acid (xylopic acid) (**8**), a pseudodimer (15*α*-hydroxy-*ent*-kaur-16-en-19-oic acid/17-hydroxy-*ent*-kaur-15-en-19-oic acid) (**9**), ermanin, nicotiflorin, and allantoin. Among these isolates, compound **3** was the most active in both anti-syncytium (EC_{50} 13.7 µg/mL and selectivity index 3.1) and HIV-1 reverse transcriptase (IC_{50} 34.1 µg/mL) assays.

Key words

Polyalthia sclerophylla · Annonaceae · *ent*-kuarane diterpenoids · anti-HIV-1 activity

Abbreviations

SI:	selectivity index
RT:	reverse transcriptase

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

A methanol extract of the stem bark of *Polyalthia sclerophylla* Hook. F. & Thomson (Annonaceae), a tree found in many Southeast Asian countries, has been reported to possess three benzopyrans and one phenylpropene [1]. As part of our ongoing search for anti-HIV-1 agents from *Polyalthia* [2], an anti-HIV-1–active MeOH extract from leaves and twigs was separated to afford a dimeric *ent*-kaurane, *ent*-kaur-sclerodimer (**1**), and a triterpenoid ester, cyclotucanol 3-palmitate (**2**), together with the known compounds **3** [3], **4** [4], **5** [5], **6** [6], **7** [7], **8** [8], and **9** [9] (**Fig. 1**) as well as ermanin (**10**) [10], nicotiflorin (**11**) [11], and allantoin (**12**) [12]. Here we describe the isolation, structure elucidation (the copies of the original spectra are obtainable from the author of correspondence), and anti-HIV-1 effects of **1**–**12**. Our results represent the first report on the isolation of *ent*-kauranoid diterpenes from *Polyalthia*.

Ent-kaur-sclerodimer (**1**) ($C_{40}H_{58}O_5$, $[M + H]^+$ $m/z = 619.4305$ in HR-TOF-MS) was suggested to be a dimeric diterpene due to the presence of a fragment ion at $m/z = 317$ $[M - C_{20}H_{29}O_3]^+$ in the

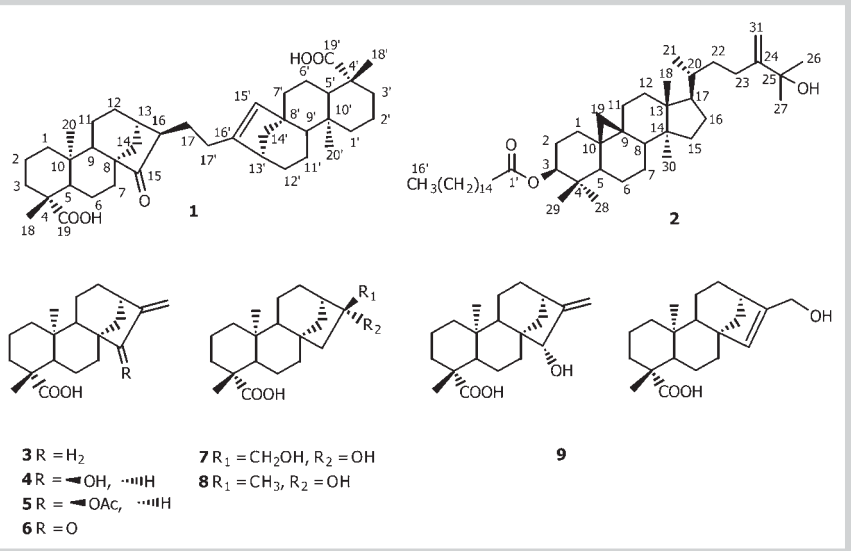

Fig. 1 Structures of **1**–**9**.

EI-MS, resulting from the loss of one diterpene unit from its molecule. The IR spectrum of **1** showed absorptions due to O-H of carboxylic acid (3690–2350 cm^{-1}), C=O of ketone (1732 cm^{-1}), C=O of carboxylic acid (1687 cm^{-1}), and C=C (1638 cm^{-1}) functionalities. The ^{1}H-NMR spectrum in pyridine-d_5 (**Table 1**) revealed the presence of four tertiary methyls at $\delta = 1.17$, 1.19, 1.32, and 1.35 (each s), a broad olefinic methine at $\delta = 5.30$ (1H), two sets of doublets at $\delta = 0.77$ and 0.88 ($J = 13.2$, 13.2, and 3.8 Hz), two broad doublets at $\delta = 1.77$ and 1.89 ($J \sim 12.7$ Hz), and other overlapping signals corresponding to the methylenes and methines in the structure. The ^{13}C-NMR signals of **1** (**Table 1**) were similar to those of the combined data of 15-oxo-*ent*-kaur-16-en-19-oic acid [6] and *ent*-kaur-15-en-19-oic acid [11], except the signals of the olefinic C-16 and C-17 in 15-oxo-*ent*-kaur-16-en-19-oic acid were replaced respectively by an sp^3 methine carbon ($\delta = 53.3$, d) and an sp^3 methylene carbon ($\delta = 24.2$, t) in **1**. According to these differences as well as the 12 degrees of unsaturation, the structure of **1** was deduced to be an asymmetric *ent*-kaurane dimer formed through the C-17–C-17′ bond. The proposed structure was further proved by analyses of 2D NMR (for HMBC data, see Supporting Information), which supported the assignments of protons and carbons in the structure. The relative configurations in compound **1** were established by analysis of its NOESY correlation spectrum (see **Fig. 1S**, Supporting Information).

The molecular formula $C_{47}H_{82}O_3$ of compound **2** was deduced from its HR-ESI-MS at $m/z = 717.6110$ $[M + Na]^+$. A prominent ion at $m/z = 438$ $[M - C_{15}H_{31}COOH]^+$, arising from McLafferty fragmentation in EI-MS, indicated the presence of a C-16 ester in the structure. Hydrolysis of compound **2** by treatment with KOH in MeOH gave cyclotucanol and palmitic acid, as analyzed by their molecular ions in the EI-MS at $m/z = 456$ and $m/z = 256$, respectively. The IR spectrum showed the alcoholic O-H, the C=O of ester, and the C=C absorptions at 3451, 1734, and 1639 cm^{-1}, respectively. The ^{1}H-NMR spectrum in $CDCl_3$ (**Table 1**) showed the signals of cyclopropyl methylene at $\delta = 0.58$ and 0.34 (each d, $J = 4.1$ Hz), together with four singlets of tertiary methyls at $\delta = 0.97$, 0.90, 0.89, and 0.84 (each s), which are compatible with those obtained for a cycloartane skeleton. The two singlets at $\delta = 5.10$ and 4.77 were assigned for the terminal methylene protons in the side chain, while the low-field multiplet at $\delta = 4.57$ was assigned to the methine proton H-3, connecting to an ester group. The assignment of carbon signals in **2** was performed through analyses of ^{13}C-NMR, DEPT (**Table 1**), and 2D NMR data (for HMBC, see Supporting Information). The NOESY spectrum of **2** (see **Fig. 1S**, Supporting Information) supported the relative configurations; the β-configuration at C-3 was confirmed by the correlations of H-3/H-5, H-3/H-28, and H-3/H-1a. Based on these data, the structure of **2** was established as 24-methylene-9,19-cyclolanostane-3β,25-diol 3-hexadecanoate.

Compounds **1**–**12** (purities 97.9–99.8%, determined by HPLC) were tested by employing an HIV-1 RT assay as described previously [13] and a syncytium assay using the $^{\Delta Tat/Rev}$MC99 and 1A2 cell line system [14, 15]. The results (**Table 2**) indicated that **2**–**5, 8**, and **9** showed significant anti-HIV-1 activities in the syncytium assay. Compound **3** was most active, with an EC_{50} of 13.7 µg/mL and an SI of 3.1, while **5** (highest SI: 6.1) exhibited an EC_{50} of 30.8 µg/mL. In the HIV-1 RT assay, **3** and **9**, which were very active against HIV-1 RT at 200 µg/mL, displayed IC_{50} values of 34.1 and 108.4 µg/mL, respectively.

Materials and Methods

Melting points (°C) were uncorrected. Optical rotations were measured on a JASCO DIP-370 digital polarimeter, 50-mm microcell (1 mL). CD spectra were measured on a JASCO J-810 spectropolarimeter. IR and UV spectra were measured on a Perkin-Elmer 2000 FTIR and a JASCO V-530, respectively. NMR spectra were measured on a Bruker AV 500, HR-TOF-MS were measured on a Micromass model VQ-Tof2, and EI-MS were measured on a Thermo Finnigan Polarlis Q. For column chromatography, silica gel 60 (Merck; 70–230 mesh) and Sephadex LH 20 (Pharmacia) were used. For preparatory layer chromatography, Kieselgel 60 PF_{254} (Merck; 0.5 mm) was used. The plant material was collected in March 2002 from Narathiwat Province, Thailand, and was identified by T. Santisuk. A voucher specimen (BKF no. 1933) is deposited at the Forest Herbarium in Bangkok, Thailand.

The dried powdered plant material (2.6 kg) was percolated with MeOH (5 × 6 L) at room temperature to give a crude MeOH extract (570 g). Sequential dissolving in MeOH–CH_2Cl_2 (1 : 1, 2 L) and

Table 1 500 MHz ^{1}H-NMR and 125 MHz ^{13}C-NMR of compounds **1** and **2** (in C_5D_5N and $CDCl_3$, respectively).

1			**2**		
Position	**H**	**C DEPT**	**Position**	**H**	**C DEPT**
1	1.77, br. d, ~ 12.7	40.2 CH_2	1	1.61, obsc.	31.6 CH_2
	0.77, ddd, 13.2, 13.2, 3.8			1.25, obsc.	
2	2.21, obsc.	19.9 CH_2	2	1.76, m	26.9 CH_2
	1.47, obsc.			1.61, obsc.	
3	2.42, obsc.	38.7 CH_2	3	4.57, m	80.4 CH
	1.03, obsc.		4	–	39.5 C
4	–	44.0 C	5	1.40, dd, 12.6, 4.3	47.2 CH
5	1.16, obsc.	57.1 CH	6	1.57, obsc.	20.9 CH_2
6	2.16, obsc.	21.2 CH_2		0.80, dd, 12.6, 2.3	
	2.08, obsc.		7	1.30, obsc.	25.8 CH_2
7	2.01, obsc.	34.9 CH_2		1.08, obsc.	
	1.44, obsc.		8	1.51, dd, 12.2, 4.7	47.8 CH
8	–	53.3 C	9	–	20.2 C
9	1.26, obsc.	52.1 CH	10	–	26.1 C
10	–	40.4 C	11	1.99, obsc.	26.5 CH_2
11	1.57, obsc	19.0 CH_2		1.13, obsc.	
	1.28, obsc.		12	1.63, obsc.	32.9 CH_2
12	1.57, obsc.	25.1 CH_2	13	–	45.4 C
13	2.43, obsc.	33.5 CH	14	–	48.9 C
14	2.39, obsc.	37.6 CH_2	15	1.30, obsc.	35.6 CH_2
	1.34, obsc.		16	1.92, obsc.	28.2 CH_2
15	–	223.1 C=O		1.30, obsc.	
16	2.28, obsc.	53.3 CH	17	1.63, obsc.	52.3 CH
17	2.25, obsc	24.2 CH_2	18	0.97, s	18.0 CH_3
	1.59, obsc.		19	0.58, d, 4.1	29.8 CH_2
18	1.32, s	29.3 CH_3		0.34, d, 4.1	
19	–	180.8 C=O	20	1.44, obsc.	36.3 CH
20	1.17, s	15.9 CH_3	21	0.91, d, 7.4	18.4 CH_3
1′	1.89, br d, ~ 12.7	41.3 CH_2	22	1.63, obsc.	35.9 CH_2
	0.88, ddd, 13.2, 13.2, 3.8			1.17, obsc.	
2′	2.27, obsc.	20.0 CH_2	23	2.19, ddd, 15.6, 11.7, 4.3	28.0 CH_2
	1.52, obsc.			1.97, obsc.	
3′	2.48, obsc	38.9 CH_2	24	–	156.9 C
	1.09, obsc.		25	–	73.6 C
4′	–	44.1 C	26	1.36, s	29.3 CH_3
5′	1.10, obsc.	56.5 CH	27	1.36, s	29.3 CH_3
6′	2.16, obsc.	21.7 CH_2	28	0.84, s	25.5 CH_3
	2.04, obsc.		29	0.89, s	15.2 CH_3
7′	1.67, obsc.	40.2 CH_2	30	0.90, s	19.3 CH_3
	1.62, obsc.		31	5.10, s	106.7 CH_2
8′	–	49.5 C		4.77, s	
9′	1.02, obsc.	48.3 CH	1′	–	173.6 C=O
10′	–	40.4 C	2′	2.30, t, 7.5	34.9 CH_2
11′	1.65, obsc.	19.5 CH_2	3′	1.63, obsc.	25.2 CH_2
	1.53, obsc.		4′	1.30, obsc.	29.2 CH_2
12′	1.50, obsc.	25.9 CH_2	5′	1.25–1.30, obsc.	*CH_2
	1.43, obsc.		6′	1.25–1.30, obsc.	*CH_2
13′	2.43, obsc.	43.9 CH	7′	1.25–1.30, obsc.	* CH_2
14′	2.09, obsc.	44.3 CH_2	8′	1.25–1.30, obsc.	* CH_2
	1.42, obsc.		9′	1.25–1.30, obsc.	* CH_2
15′	5.30, br s	135.0 CH	10′	1.25–1.30, obsc.	* CH_2
16′	–	146.6 C	11′	1.25–1.30, obsc.	* CH_2
17′	2.23, obsc.	28.9 CH_2	12′	1.25–1.30, obsc.	* CH_2
18′	1.35, s	29.5 CH_3	13′	1.25–1.30, obsc.	* CH_2
19′	–	180.0 C=O	14′	1.61, obsc.	31.9 CH_2
				1.25, obsc.	
20′	1.19, s	16.0 CH_3	15′	1.30, obsc.	22.7 CH_2
				1.25, obsc.	
			16′	0.88, t, 6.7	14.1 CH_3

Note: obsc. = obscured signal. *29.26 or 29.37 or 29.46, or 29.58 or 29.63 or 29.68

Compound	Cytotoxic and syncytium assays			Reverse transcriptase assay	
	IC_{50} (μg/mL)	EC_{50} (μg/mL)	SI	% inhibition at 200 μg/mL	IC_{50} (μg/mL)
1	> 125	I	NA	47.1	ND
2	> 125	108.7	> 1.1	3.1	ND
3	42.7	13.7	3.1	97.9	34.1
4	104.8	33.9	3.1	20.5	ND
5	189.1	30.8	6.1	11.8	ND
6	< 3.9	T	NA	66.1	ND
7	> 125	I	NA	25.2	ND
8	> 125	66.5	> 1.9	8.2	ND
9	107.5	29.6	3.6	71.1	108.4
10	5.2	T	NA	46.8	ND
11	> 125	I	NA	22.0	ND
12	> 125	I	NA	7.7	ND

Table 2 Anti-HIV-1 activities of the isolated compounds, as determined by using cell-based $^{\Delta Tat/Rev}$MC99 virus and 1A2 cell line and reverse transcriptase assays.

Note: Cytotoxic assay: IC_{50} = dose of extract, fraction, or compound that inhibited 50% metabolic activity of uninfected cells; AZT, averaged from three experiments; $IC_{50} < 2.67 \times 10^2$ μg/mL. Syncytium assay: EC_{50} = dose of extract, fraction, or compound that reduced 50% syncytium formation by $^{\Delta Tat/Rev}$MC99 virus in 1A2 cells; AZT, averaged from three experiments; EC_{50} 9.9×10^{-4} μg/mL; I, less than 50% reduction of syncytium formation at the highest nontoxic concentration; T, toxic. Selectivity index (SI): IC_{50}/EC_{50}; NA, nonapplicable or the value could not be estimated. RT assay: Compounds were prescreened at 200 μg/mL, and only those that were very active at this concentration were further determined for IC_{50}, the dose that inhibited 50% HIV-1 RT activity; ND = not determined. Positive controls were averaged from two experiments; IC_{50} fagaronine chloride, 10.1 μg/mL; IC_{50} nevirapine, 2.1 μg/mL. The 50% endpoint assay was carried out by using six concentrations in duplicate. The coefficients of determination, R^2, were 0.88–0.97, 0.91–0.99, and 0.86–0.98, respectively, for cytotoxic, syncytium, and RT assays

MeOH–EtOAc (1 : 1, 1.5 L) followed by solvent removal yielded fraction 1 (333 g), fraction 2 (61 g), and the residue (161 g), respectively.

The bioactive fraction 1 (332 g) was separated by CC (SiO_2, 1.8 kg, 16 × 18 cm), eluting with 80%, 90%, and 100% CH_2Cl_2–hexane (2 L each), then with 2%, 3%, 6%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 60%, 80%, and 100% EtOAc–CH_2Cl_2 (2 L each), followed by 1%, 10%, 20%, 30%, 50%, 70%, and 100% MeOH–EtOAc (4 L each). Fractions (500 mL each) were combined (based on TLC behavior) to give frs. A1–A12. Fr. A3 (46.5 g, eluted with 90% CH_2Cl_2–hexane) and fr. A4 (8.51 g, eluted with CH_2Cl_2) were recrystallized from EtOH–CH_2Cl_2 to give **3** (38.8 g and 3.79 g, respectively). The residue of A4 (4.54 g) yielded frs. B1–B4 after CC (SiO_2, 200 g, 5.1 × 24.5 cm, acetone–hexane gradient). Fr. B4 (1.71 g, eluted with 16–100% acetone–hexane) was rechromatographed (Sephadex LH 25 g, 2.5 × 28 cm), eluting with 90% CH_2Cl_2–hexane, followed by recrystallization (EtOH–CH_2Cl_2) to give **7** (31.8 mg). Fr. A5 (47.9 g, eluted with 2–3% CH_2Cl_2–EtOAc) afforded **5** (10.3 g) after recrystallization (EtOH–CH_2Cl_2). The residue of A5 (35.7 g) was further separated by CC (SiO_2, 700 g, 8.5 × 30.5 cm, CH_2Cl_2–hexane and MeOH–CH_2Cl_2 gradients) to yield frs. C1–C8. Fr. C2 (8.51 g, eluted with 86–90% CH_2Cl_2–hexane) was rechromatographed by CC (SiO_2, 250 g, 6 × 20.5 cm, acetone–hexane gradient) to provide frs. D1–D12. Fr. D2 (3.5 g, eluted with 4% acetone–hexane) was further separated by CC (SiO_2, 100 g, 3.5 × 26.5 cm, CH_2Cl_2–hexane gradient) to give frs. E1–E5. Fr. E3 (1.16 g, eluted with 90–100% CH_2Cl_2–hexane) afforded **2** (178.7 mg, R_f = 0.51, 20% EtOAc–hexane) after TLC (20% EtOAc–hexane) and recrystallization (EtOH–CH_2Cl_2). Fr. C4 (6.81 g, eluted with 98–100% CH_2Cl_2–hexane) was separated by CC (SiO_2, 200 g, 5.1 × 24.5 cm, acetone–hexane gradient) to yield frs. G1–G10. Fr. G6 (639.4 mg, eluted with 3.5–4% acetone–hexane) gave **6** (20.5 mg) after recrystallization (EtOH–CH_2Cl_2). Fr. A6 (24.3 g, eluted with 6–10% CH_2Cl_2–EtOAc) provided **4** (10.8 g) after recrystallization (EtOH–CH_2Cl_2). Fr A7 (13.5 g, eluted with 15–35% EtOAc–CH_2Cl_2) afforded **1** (4.41 g) after recrystallization (DMSO). The residue (9.28 g) was rechromatographed by CC (SiO_2, 250 g, 5.1 × 30.5 cm, acetone–hexane gradient) to give frs H1–H8. Fr. H6 (2.83 g, eluted with 11–18% acetone–hexane) gave **9** (101.7 mg) after recrystallizaton (MeOH). Fr. H7 (2.78 g) was further purified by CC (SiO_2, 100 g, 3.5 × 26.5 cm, EtOAc–hexane gradient) to afford frs. I1–I6. Fr. I3 (335 mg, eluted with 28% EtOAc–hexane) provided **10** (75.1 mg) after recrystallization (EtOH–CH_2Cl_2). Fr. A10 (49.7 g, eluted with 10–20% MeOH–EtOAc) was separated by CC (SiO_2, 700 g, 10 × 19 cm, MeOH–CH_2Cl_2 gradient) to give frs. J1–J7. Fr. J7 (17.7 g, eluted with 25–100% MeOH–CH_2Cl_2) was rechromatographed by CC (SiO_2, 300 g, 7 × 17.0 cm MeOH–EtOAc gradient) to give frs. M1–M7. Fr. M4 (555.7 mg, eluted with 20% MeOH–EtOAc) and fr. M5 (1.28 g, eluted with 25–40% MeOH–EtOAc) gave **11** (75.6 mg) and **12** (35.6 mg), respectively, after recrystallization (MeOH).

The bioactive fraction 2 (61 g) was separated by CC (SiO_2, 900 g, 10 × 24 cm), eluting with 50%, 60%, 80%, and 100% CH_2Cl_2–hexane (2 L each) and then with 10%, 20%, 30%, 40%, 50%, 60%, 80%, and 100% EtOAc–CH_2Cl_2 (1 L each). Fractions (500 mL each) were combined (based on TLC behavior) to give frs. N1–N9. Fr. N4 (4.4 g, eluted with 50% CH_2Cl_2–hexane) provided **3** (2.67 g) after recrystallization (EtOH–CH_2Cl_2). Fr N5 (8.77 g, eluted with 60–100% CH_2Cl_2–hexane) gave **4** (918.9 mg) after recrystallization (EtOH–CH_2Cl_2). The residue (7.6 g) was further separated by CC (SiO_2, 200 g, 5.1 × 24.5 cm, EtOAc–hexane gradient) to afford frs. O1–O6. Fr. O3 (1.91 g, eluted with 10–13% EtOAc–hexane) provided **5** (504.7 mg) after recrystallization (EtOH–CH_2Cl_2). Fr. O5 (779.7 mg, eluted with 25–40% EtOAc–hexane) was separated by CC (SiO_2, 30 g, 2.5 × 16 cm), eluting with MeOH–CH_2Cl_2–hexane 2 : 16 : 82, followed by recrystallization (MeOH–CH_2Cl_2) to give **8** (109.8 mg). Fr. N6 (2.47 g, eluted with 20–40% EtOAc–CH_2Cl_2) was further separated CC (SiO_2, 80 g, 3.5 × 28 cm, EtOAc–hexane gradient) to give frs. P1–P8. Fr. P5 (89.9 mg, eluted with 16% EtOAc–hexane) gave **9** (5.3 mg) after recrystallization (MeOH).

Ent-kaur-sclerodimer (**1**): Colorless plates; m.p. 370–372 °C (DMSO), dec.; $[\alpha]_{589}^{26}$: – 79.6 (*c* 0.5, THF). FTIR (KBr): ν_{max} = 3690–2350, 1732, 1687, 1635, 1471, 1447 cm^{-1}; ^{1}H- and ^{13}C-NMR (pyridine-d_5): see ◉ **Table 1**; EI-MS: *m/z* (rel. int.) = 618 $[M]^+$ (100),

317 (53), 298 (89), 260 (72), 215 (61), 131 (57); HR-TOF-MS (ESI positive): $m/z = 619.4305$ $[M + H]^+$ (calcd. for $C_{40}H_{59}O_5$: 619.4357).

Cyclotucanol 3-palmitate (**2**): Colorless amorphous solid; m. p. 49–50 °C (EtOH–CH_2Cl_2). $[\alpha]_{589}^{26}$: 20.3 (*c* 0.47, $CHCl_3$); FTIR (KBr): $\nu_{max} = 3451, 1734, 1639, 1470, 1377, 1177, 1099\ cm^{-1}$; ^{1}H- and ^{13}C-NMR ($CDCl_3$): see ◉ **Table 1**; EI-MS: *m/z* (rel. int.) = 694 $[M]^+$ (0.79), 438 (32) 342 (63), 164 (43), 91 (100); HR-TOF-MS (ESI positive): $m/z = 717.6110$ $[M + Na]^+$ (calcd. for $C_{47}H_{82}O_3Na$: 717.6156).

Bioassays

A detailed protocol for the bioassays is available in the Supporting Information. AZT, purity 99.8%, was kindly provided by Dr. David J. Clanton (NCI-FCRDC, Frederick, Maryland). Fagaronine chloride, purity 99.5%, was from Dr. John M. Pezzuto (previously at the College of Pharmacy, University of Illinois at Chicago), and nevirapine, anhydrous, USP, total impurities not more than 0.6%, was from the Thai Government Pharmaceutical organization.

Supporting information

HMBC and NOESY correlations observed in compounds **1** and **2** and a detailed protocol for the bioassays are available as Supporting Information.

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