Anti-AIDS Agents, 11. Betulinic Acid and Platanic Acid as Anti-HIV Principles from Syzigium claviflorum, and the Anti-HIV Activity of Structurally Related Triterpenoids

Toshihiro Fujioka, Yoshiki Kashiwada, Robert E. Kilkuskie, L. Mark Cosentino, Lawrence M. Ballas, Jack B. Jiang, William P. Janzen, Ih-Sheng Chen, and Kuo-Hsiung Lee

J. Nat. Prod., **1994**, 57 (2), 243-247• DOI: 10.1021/np50104a008 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 57, No. 2, pp. 243-247, February 1994

ANTI-AIDS AGENTS, 11.[1] BETULINIC ACID AND PLATANIC ACID AS ANTI-HIV PRINCIPLES FROM *SYZIGIUM CLAVIFLORUM*, AND THE ANTI-HIV ACTIVITY OF STRUCTURALLY RELATED TRITERPENOIDS

TOSHIHIRO FUJIOKA, YOSHIKI KASHIWADA,

Natural Products Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599

ROBERT E. KILKUSKIE,[2] L. MARK COSENTINO,

Biotech Research Laboratories, 1600 East Gude Drive, Rockville, Maryland 20850

LAWRENCE M. BALLAS, JACK B. JIANG, WILLIAM P. JANZEN,

Sphinx Pharmaceuticals Corporation, Durham, North Carolina 27717

IH-SHENG CHEN,

Natural Products Research Center, Kaohsiung Medical College, Kaohsiung, Taiwan

and KUO-HSIUNG LEE*

Natural Products Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599

ABSTRACT.—Betulinic acid [**1**] and platanic acid [**2**], isolated from the leaves of *Syzigium claviflorum*, were found to be inhibitors of HIV replication in H9 lymphocyte cells. Evaluation of anti-HIV activity with eight derivatives of **1** revealed that dihydrobetulinic acid [**3**] was also a potent inhibitor of HIV replication. The C-3 hydroxy group and C-17 carboxylic acid group, as well as the C-19 substituents, contribute to enhanced anti-HIV activity. The inhibitory activity of these compounds against protein kinase C (PKC) was also examined, since a correlation between anti-HIV and anti-PKC activities has been suggested. However, there was no apparent correlation between anti-HIV activity and the inhibition of PKC among these compounds.

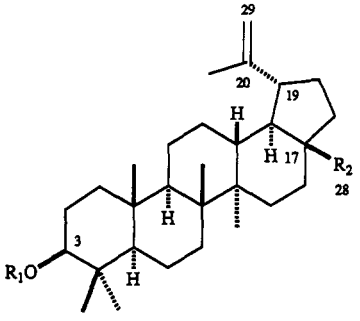
In the course of our continuing search for plant natural products as anti-AIDS agents, the MeOH extract of the leaves of *Syzigium claviflorum* (Roxb.) WALL (Myrtaceae) was found to show significant anti-HIV activity. Subsequent bioactivity-directed fractionation has resulted in the isolation of a known triterpenoid, betulinic acid [**1**] (2), and a nortriterpenoid, platanic acid [**2**] (3), as the anti-HIV principles. This finding prompted us to prepare derivatives of **1** and evaluate their anti-HIV activity. We report herein the anti-HIV activity of **1** and **2**, as well as eight derivatives of **1**.

RESULTS AND DISCUSSION

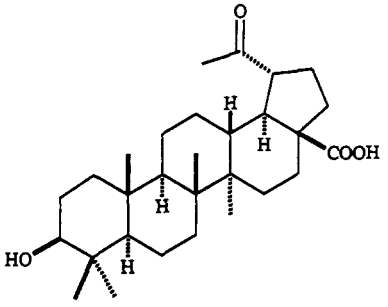
In the course of our continuing search for plant natural products as anti-AIDS agents, the MeOH extract of the leaves of *S. claviflorum* was found to show significant anti-HIV activity. Subsequent bioactivity-directed fractionation has resulted in the isolation of the known triterpenoid, betulinic acid [**1**], and the nortriterpenoid, platanic acid [**2**], as the anti-HIV principles. Compound **1** inhibited HIV replication in H9 lymphocytes with an EC_{50} value of 1.4 μM and inhibited uninfected H9 cell growth with an IC_{50} value of 13 μM. On the other hand, compound **2** showed an inhibitory effect on HIV replication with an EC_{50} of 6.5 μM, while its IC_{50} for inhibition of uninfected H9 cell growth was 90 μM. Compounds **1** and **2** are the first two identified triterpenes with a lupane skeleton to demonstrate anti-HIV activity. They are structurally related to each

[1]For part 10, see Hu *et al.* (1).

[2]Present address: Hybridon, Inc., Worcester, Massachusetts 01605.



	R_1	R_2
1	H	COOH
4	H	CH_2OH
5	H	$COOCH_2COOCH_3$
6	Ac	COOH
7	COC_6H_5	COOH
8	$COCH{=}CHCH_3$	COOH
9	SO_3K	COOH
10	$COCH_2CH_2COOH$	COOH



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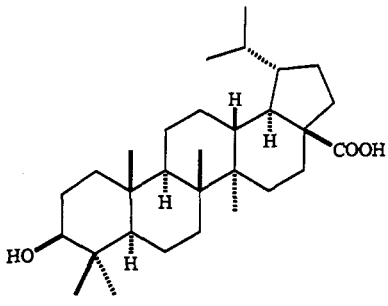

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other and differ only in the replacement of an isopropenyl group in **1** with an acetyl group in **2**. This finding prompted our preparation and evaluation of some derivatives of **1** for their anti-HIV activity.

A comparison of the anti-HIV activity of **1–10** (Table 1) clearly indicated that dihydrobetulinic acid [**3**] (4,5), with EC_{50} and IC_{50} values of 0.9 and 13 μM, respectively, is as potent or slightly more potent than **1** and **2** in inhibiting HIV replication in H9 lymphocytes. Compound **3** possesses an isopropyl group at C-19, while **2** has an acetyl group at C-19, instead of an isopropenyl group as seen in **1**. A comparison of the anti-HIV activities of **1–3** suggested that the substituents at C-19 play an important role in the demonstration of anti-HIV activity. The replacement of the COOH group at C-17 with a CH_2OH group as seen in **4** (betulin) or with an ester group as in **5** diminished the anti-HIV activity. Therefore, the carboxylic acid group at C-17 appears to be essential for the selective HIV inhibitory effect. The esterification of the C-3 hydroxyl with acetyl [**6**], benzoyl [**7**], crotonyl [**8**], sulfonyl [**9**], and succinyl [**10**] groups all led to a decrease in the anti-HIV activity. These results imply that the free hydroxy group at C-3 is essential for retaining anti-HIV activity.

The inhibitory activity of these compounds against protein kinase C (PKC) was also examined, since a correlation of anti-HIV and anti-PKC activities has been suggested (6). However, none of these compounds showed inhibitory activity against mix-R PKC.

TABLE 1. HIV Inhibitory Effects (μM) for **1–10**.

Compound No.	EC_{50}[a]	IC_{50}[b]	T.I.[c]
1	1.4	13	9.3
2	6.5	90	13
3	0.9	13	14
4	23	45	1.9
5	17	26	1.5
6	25	20	0.8
7	15	15	1.0
8	19	48	2.5
9	20	35	1.7
10	4	16	4.0
AZT	0.04	2,000	50,000

[a]Concentration which inhibits virus replication by 50%.
[b]Concentration which inhibits lymphocyte cell growth by 50%.
[c]Therapeutic index.

Further detailed examination of the inhibitory effect of these compounds against isoenzymes of PKC, such as βII-H, γ-H, δ-H, and ϵ-H, demonstrated that some of the compounds were selective inhibitors against PKC isoenzymes. Thus, compounds **3** and **10** showed inhibitory activity against δ-H PKC with IC_{50} values of 46 and 49 μM, respectively. They also showed anti-PKC activity against γ-H with IC_{50} values of 74 and 37 μM, respectively. Inhibitory activities against γ-H PKC were also observed with compounds **7** and **9**. All of the compounds tested had no inhibition against βII-H and ϵ-H PKC (IC_{50}>150 μM). However, no apparent correlation between anti-HIV activity and inhibition of PKC was observed. The mechanism of anti-HIV activity by these compounds remains to be investigated.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All melting points were determined on a Fisher-Johns micro-melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter. Mass spectra were obtained an a VG 70-250 SEQ mass spectrometer. ^{1}H- and ^{13}C-nmr spectra were measured on a Bruker AC 300 spectrometer and are given in ppm (δ) downfield from an internal TMS standard. Column chromatography was carried out with Silica 32–63 (32–63 μm, Universal Adsorbents, Inc.), MCI gel CHP 20P (75–150 μ, Mitsubishi Chemical Industries), and prep-PAK 500/C18 (Waters). Thin-layer chromatography was performed on Kieselgel 60 F_{254} plates (0.2 mm thick, Merck).

PLANT MATERIAL.—The leaves of *S. claviflorum* were collected in Lanyu, Taiwan, in February, 1990. A voucher specimen of this plant is available at the School of Pharmacy, Kaohsiung Medical College, Taiwan.

ISOLATION OF BETULINIC ACID [**1**] AND PLATANIC ACID [**2**].—The MeOH extract (120 g) of *S. claviflorum* was partitioned successively with hexane, $CHCl_3$, EtOAc, and *n*-BuOH to yield fractions soluble in hexane (6.2 g), $CHCl_3$ (25.5 g), EtOAc (5.7 g), *n*-BuOH (19.1 g), and H_2O (64.7 g). The $CHCl_3$-soluble fraction was subjected to Si gel chromatography. Elution with $CHCl_3$-EtOAc (4:1) and then $CHCl_3$-MeOH (95:5) furnished six fractions (frs. 1–6). Fraction 2 was further chromatographed over MCI gel CHP 20P [MeOH-H_2O (9:1→1:0)] and crystallized from MeOH to afford betulinic acid [**1**] (2) (612 mg) as colorless needles. Repeated chromatography of fraction 3 with MCI gel CHP 20P [MeOH-H_2O (9:1→1:0)], prep-PAK 500/C_{18} [MeOH-H_2O (85:15→1:0)], and Si gel [$CHCl_3$-MeOH (40:1)], and crystallization from $CHCl_3$-MeOH gave platanic acid [**2**] (3) (539 mg) as colorless needles.

Betulinic acid [**1**].—Colorless needles (MeOH): mp 290–293°, $[\alpha]^{25}$D +7.5° (*c*=0.37, pyridine).

Platanic acid [**2**].—Colorless needles ($CHCl_3$-MeOH): mp 279–282°, $[\alpha]^{25}$D −38.0° (*c*=0.62, pyridine); eims *m/z* 458 $[M]^+$; ^{1}H nmr (pyridine-d_5) δ 0.82, 1.02, 1.09, 1.23 (15H in total, each s, 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, and 14-CH_3), 2.24 (3H, s, 20-CH_3), 3.46 (1H, t, *J*=8 Hz, H-3), 3.70 (1H, dt, *J*=4, 11 Hz, H-19), ^{13}C nmr (pyridine-d_5) δ 14.9 (C-27), 16.4 (2C) (C-24 and C-25), 18.8 (C-6), 21.2 (C-11), 27.8 (C-12), 28.2 (C-2), 28.7 (C-23), 28.8 (C-21 or C-15), 29.7 (C-29), 30.3 (C-21 or C-15), 32.3 (C-16), 34.7

(C-7), 37.4 (C-22), 37.5 (C-10), 37.8 (C-13), 39.3 (C-1), 39.5 (C-4), 41.0 (C-8), 42.7 (C-14), 49.8 (C-18 or C-19), 50.9 (C-9), 52.1 (C-19 or C-18), 55.9 (C-5), 56.5 (C-17), 78.0 (C-3), 178.6 (C-28), 211.9 (C-20).

DIHYDROBETULINIC ACID [**3**].—Betulinic acid (60 mg) in AcOEt (10 ml) was treated wtih 10% Pd-C (20 mg) overnight under H_2 with stirring. The reaction mixture was filtered, and the filtrate was concentrated to give **3** (49 mg) as colorless needles (from MeOH): mp >300°; $[\alpha]^{25}D$ −17.6° [*c*=0.37, $CHCl_3$-MeOH (1:1)]; ^1H nmr ($CDCl_3$) δ 0.75, 0.86 (each 3H, d, *J*=7 Hz, 20-$(CH_3)_2$), 0.76, 0.83, 0.93, 0.96, 0.97 (each 3H, s, 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, and 14-CH_3), 3.20 (1H, dd, *J*=5, 11 Hz, H-3); *anal.* found C 75.22%, H 11.05%; calcd for $C_{30}H_{50}O_3 \cdot H_2O$, C 75.58%, H 10.99%.

BETULINIC ACID 28-*O*-CARBOXYMETHYLMETHYLATE [**5**].—A mixture of betulinic acid (80 mg), NaH (200 mg), and methyl chloroacetate (0.5 ml) in DMF (10 ml) was stirred overnight. The reaction mixture was then diluted with H_2O and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried over Na_2SO_4, and concentrated to a residue. The residue was subjected to Si gel column chromatography. Elution with hexane-Me_2CO (1:0→10:1) gave **5** (31 mg) as colorless needles (from MeOH): mp 257–260°; $[\alpha]^{25}D$ −16.9° (*c*=1.03, $CHCl_3$); ^1H-nmr ($CDCl_3$) δ 0.75, 0.82, 0.94, 0.96, 0.97 (each 3H, s, 4-$[CH_3]_2$, 8-CH_3, 10-CH_3, and 14-CH_3), 1.69 (3H, s, 20-CH_3), 2.99 (1H, dt, *J*=5, 11 Hz, H-19), 3.18 (1H, dd, *J*=5, 11 Hz, H-3), 3.76 (3H, s, $COOCH_3$), 4.60 (2H, s, $COOCH_2COOMe$), 4.60, 4.73 (each 1H, *s*-like; H-30); *anal.* found C 73.35%, H 9.74%; calcd for $C_{33}H_{52}O_5 \cdot 1/2H_2O$, C 74.96%, H 9.91%.

BETULINIC ACID 3-*O*-ACETATE [**6**].—Betulinic acid (80 mg) was treated with pyridine (2 ml) and Ac_2O (2 ml) overnight at room temperature. The reaction mixture was worked up as usual to give **6** (65.5 mg) as colorless needles (from MeOH): mp >300°; $[\alpha]^{25}D$ −19.5° (*c*=1.33, $CHCl_3$); ^1H-nmr ($CDCl_3$) δ 0.83, 0.84, 0.85, 0.94, 0.97 (each 3H, s, 4-$[CH_3]_2$, 8-CH_3, 10-CH_3, and 14-CH_3), 1.69 (3H, s, 20-CH_3), 2.04 (3H, s, $OCOCH_3$), 3.00 (1H, dt, *J*=4, 11 Hz, H-19), 4.47 (1H, dd, *J*=7, 9 Hz, H-3), 4.61, 4.74 (each 1H, br s, H-30); *anal.* found C 75.91%, H 10.12%; calcd for $C_{32}H_{50}O_4 \cdot H_2O$, C 75.70%, H 10.12%.

BETULINIC ACID 3-*O*-BENZOATE [**7**].—Betulinic acid (60 mg) was treated with pyridine (5 ml) and benzoyl chloride (0.5 ml) overnight with stirring. The reaction mixture was worked up as for **5** to give **7** (49 mg) as a white powder (from MeOH): mp >300°; $[\alpha]^{25}D$ −38.9° (*c*=1.2, $CHCl_3$); ^1H-nmr ($CDCl_3$) δ 0.90, 0.92, 0.97, 1.00, 1.00 (each 3H, s, 4-$[CH_3]_2$, 8-CH_3, 10-CH_3, and 14-CH_3), 1.70 (3H, s, 20-CH_3), 3.00 (1H, dt, *J*=4.5, 11 Hz, H-19), 4.62, 4.75 (each 1H, br s; H-30), 4.70 (1H, br t, *J*=5.5 Hz, H-3), 7.30–8.10 (5H in total; C_6H_5); *anal.* found C 78.13%, H 9.25%; calcd for $C_{37}H_{52}O_4 \cdot H_2O$, C 77.99%, H 9.37%.

BETULINIC ACID 3-*O*-CROTONATE [**8**].—A mixture of betulinic acid (60 mg), pyridine (5 ml), and crotonic anhydride (2.5 ml) was stirred overnight. The reaction mixture was treated in the same manner as for **5** to give **8** (25 mg) as colorless plates (from MeOH): mp >300°; ^1H nmr ($CDCl_3$) δ 0.83, 0.85, 0.86, 0.94, 0.97 [each 3H, s, 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, and 14-CH_3], 1.69 (3H, s, 20-CH_3), 1.87 (3H, dd, *J*=1.5, 7 Hz, CH_3CH=CH), 3.02 (1H, dt, *J*=4.5, 11 Hz, H-19), 4.48 (1H, m, H-3), 4.60, 4.73 (each 1H, br s, H-30), 5.1–5.2 (2/3 H in total, m; *cis*-CH=$CHCH_3$), 5.86 (2/3H, dq, *J*=1.5, 15 Hz, *trans*-CH=$CHCH_3$), 6.95 (2/3H, dq, *J*=7, 15 Hz, *trans*-CH=$CHCH_3$); the ^1H-nmr spectrum indicated that compound **8** is a mixture of *cis*- and *trans*-isomers.

BETULINIC ACID 3-*O*-SULFONATE POTASSIUM SALT [**9**].—Betulinic acid (90 mg) was treated with pyridine (5 ml) and $ClSO_3H$ (0.5 ml) overnight with stirring. The reaction mixture was alkalinized with dil. KOH and concentrated to a residue. The residue was subjected to prep-Pak 500/C18 cc. Elution with H_2O-MeOH (1:0→0:1) gave **9** (17.5 mg) as colorless needles (from MeOH-EtOH): mp >300°; $[\alpha]^{25}D$ −13.0° [*c*=0.27, $CHCl_3$-MeOH (1:1)]; ^1H nmr (CD_3OD) δ 0.80, 0.87, 0.96, 1.00, 1.01 (each 3H, s, 4-$[CH_3]_2$, 8-CH_3, 10-CH_3, and 14-CH_3), 1.68 (3H, s, 20-CH_3), 3.03 (1H, dt, *J*=4.5, 11 Hz, H-19), 3.90 (1H, dd, *J*=4.5, 12 Hz, H-3), ca. 4.60, 4.70 (H-30, the signals of H-30 were overlapped with the signal of H_2O); ir (KBr) ν max 2930, 1705, 1250, 1223, 1065 (sulfate ion, SO_3^-) cm^{-1}; *anal.* found C 59.50%, H 8.36%; calcd for $C_{30}H_{47}O_6SK \cdot 3/2H_2O$, C 59.87%, H 8.37%.

BETULINIC ACID 3-*O*-SUCCINATE [**10**].—Betulinic acid (100 mg) was treated with pyridine (2 ml) and succinic acid anhydride (200 mg) and stirred overnight at 80–90°. The reaction mixture was acidified with dil. HCl and extracted with $CHCl_3$. The $CHCl_3$ layer was worked up as for **7** to give **10** (90 mg) as colorless needles (from aqueous MeOH): mp >300°; $[\alpha]^{25}D$ −14.4° (*c*=1.09, $CHCl_3$); ^1H nmr ($CDCl_3$) δ 0.81, 0.84, 0.86, 0.94, 0.97 [each 3H, s, 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, and 14-CH_3], 1.69 (3H, s, 20-CH_3), 2.66 (4H, *s*-like; -$OOCCH_2CH_2COOH$), 3.00 (1H, dt, *J*=5, 9 Hz, H-19), 4.52 (1H, dd, *J*=7, 11 Hz, H-3), 4.61, 4.73 (each 1H, br s, H-30); *anal.* found C 72.17%, H 9.45%; calcd for $C_{34}H_{52}O_6 \cdot H_2O$, C 72.18%, H 9.44%.

HIV GROWTH INHIBITION ASSAY.—This assay was performed by incubation of H9 lymphocytes (1×10^7 cells/ml) in the presence or absence of HIV-1 (IIIB isolate) for 1 h at 37°. Cells were washed thoroughly to remove unadsorbed virions and resuspended at 4×10^5 cells/ml in culture medium. Aliquots (1 ml) were

placed in wells of 24-well culture plates containing an equal volume of test compound (diluted in test medium). After incubation for 3 days at 37°, the cell densities of uninfected cultures were determined to assess toxicity of the test compound. A p24 antigen capture assay was used to determine the level of HIV infection in HIV-treated cultures. The ability of test compounds to inhibit HIV replication was measured at four different concentrations of test compound relative to infected, untreated cultures. Test compounds were considered to be active if p24 levels were less than 70% of infected, untreated cultures (>30% inhibition). The active triterpenes did not inhibit the detection of HIV-1 p24 antigen during an ELISA assay. This assay was carried out as follows: Dilutions of the triterpenes which suppress HIV-1 replication when added to the infected H9 cells were also added to known amounts of the control p24 antigen. This p24 control antigen is used to generate a standard curve which is used during the p24 antigen ELISA. The adsorbance data indicates that the presence of the triterpenes did not interfere with the detection of a known amount of p24 in the control; therefore, the triterpenes' effect is directed at either the cell or virus level.

PROTEIN KINASE C ASSAY.—The compounds tested were solubilized in DMSO and 10 μl were added to small unilamellar vesicles consisting of 40 μg/ml phosphatidylserine (Avanti) and 1.76 μg/ml diacylglycerol (Avanti) in 20 mM HEPES buffer (pH=7.5, Sigma), 10 mM $MgCl_2$ (Sigma), 200 μg/ml histone (type HL, Worthington), 100 μM $CaCl_2$ (Sigma), 47.5 μM EGTA (Sigma), and 20 μM ^{32}P-APT (DuPont). The assay was started by addition of 20 ng of the purified PKC, incubated at 30° for 10 min, and stopped by adding 0.5 ml ice cold trichloroacetic acid (Amresco) followed by 100 μl of 1 mg/ml bovine serum albumin (Sigma). The precipitate was collected by vacuum filtration on GFC filters and quantified by counting in a beta-scintillation counter.

ACKNOWLEDGMENTS

This investigation was supported by grant AI-33066 from the National Institute of Allergy and Infectious Diseases awarded to K.-H. Lee.

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Received 1 July 1993