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ANTI-AIDS AGENTS, 21.[1] TRITERPENOID SAPONINS AS ANTI-HIV PRINCIPLES FROM FRUITS OF *GLEDITSIA JAPONICA* AND *GYMNOCLADUS CHINENSIS*, AND A STRUCTURE-ACTIVITY CORRELATION

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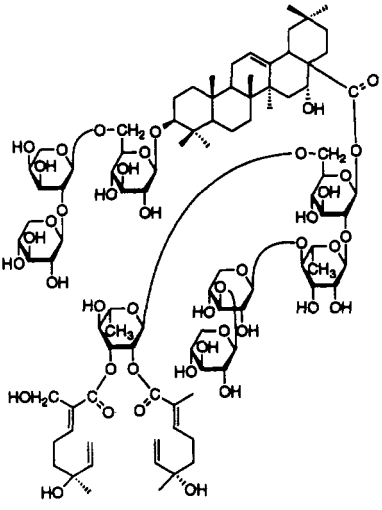
ABSTRACT.—Gleditsia saponin C [**1**] and gymnocladus saponin G [**2**] were isolated from *Gleditsia japonica* and *Gymnocladus chinensis*, respectively, as anti-HIV principles. Compounds **1** and **2** demonstrated inhibitory effects against HIV replication in H-9 cells with EC_{50} values of 1.1 and 2.7 μM, respectively. Evaluation of the anti-HIV activities of the prosapogenins of **1** and **2** suggested that the unusual monoterpenyl moieties in **1** and **2** are essential for their anti-HIV activity. Derivatives of echinocystic acid [**8**], the aglycone of compound **1**, were also prepared and evaluated for inhibitory activity against HIV replication. 3,16-Di-*O*-acetylechinocystic acid [**12**] was shown to be an anti-HIV agent with an EC_{50} value of 2.3 μM.

Gleditsia japonica Miquel (Leguminosae) and *Gymnocladus chinensis* Baillon (Leguminosae) are plants widely distributed in Japan (2), and the southern People's Republic of China, respectively. Their dried fruits have long been known in oriental medicine as saponin drugs and used, for example, as diuretics and expectorants (3). In the course of our continuing search for novel anti-HIV agents from natural products, we found that MeOH extracts of the fruits of both these species exhibited anti-HIV activity. Bioactivity-directed fractionation of the two active extracts has led to the isolation of two known saponins as the active anti-HIV principles. The compound from *Gleditsia japonica* has been identified as gleditsia saponin C [**1**] (4) and the compound from *Gymnocladus chinensis* as gymnocladus saponin G [**2**] (5). This paper describes the anti-HIV activity of **1** and **2** as well as structure-activity relationship study with these prosapogenins and related synthetic triterpenoids.

RESULTS AND DISCUSSION

The initial bioactivity-guided solvent fractionation of the MeOH extracts of the fruits of *G. japonica* and *G. chinensis* with *n*-hexane, EtOAc, *n*-BuOH, and H_2O, successively, yielded an anti-HIV active *n*-BuOH fraction in each case. Subsequent bioassay-directed repeated chromatography of these fractions on Si gel and Sephadex LH-20 and with prep. hplc led to the isolation of saponin **1**, from *G. japonica*, and saponin **2**, from *G. chinensis*, as the anti-HIV principles. The structures of **1** and **2** were assigned as gleditsia saponin C [**1**] (4) and gymnocladus saponin G [**2**] (5), respectively, by

[1]For Part 20, see Lee (1).



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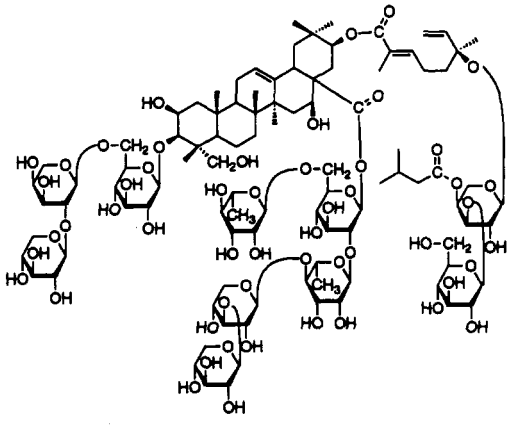

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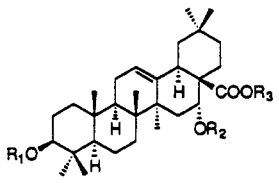
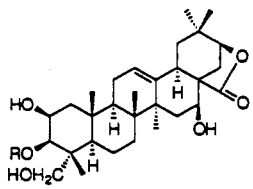
comparison of their physical and spectral data with those of authentic samples. Compound **1** inhibited HIV replication in acutely infected H9 cells with an EC_{50} value of 1.1 μM and inhibited uninfected H-9 cell growth with an IC_{50} value of 9.8 μM. The calculated therapeutic index (T.I.) value [defined as toxicity (IC_{50}) divided by anti-HIV activity (EC_{50})] was 8.9. In contrast, compound **2** showed an inhibitory effect on HIV replication with an EC_{50} value of 2.7 μM, while its IC_{50} value for inhibition of uninfected H-9 cell growth was 14 μM; thus, the T.I. value was 5.2. Compounds **1** and **2** appear to be the first examples of triterpene saponins to demonstrate significant anti-HIV activity. Therefore, the prosapogenins and related compounds of **1** and **2** were prepared and evaluated for their anti-HIV activity (Table 1).

Treatment of **1** with K_2CO_3 in EtOH and alkaline hydrolysis of **1** with KOH/EtOH both furnished inactive (EC_{50}>100 μM) prosapogenins **3** and **4**. Subsequent methylation of **4** yielded the monomethyl ester [**4a**], which was shown to be less active (EC_{50} 9.9 μM; T.I. 2.2). On acidic hydrolysis with H_2SO_4, **4** yielded the partial hydrolysates **5** and **6**, together with an aglycone (echinocystic acid) [**8**]. These compounds were shown to be less active (**5**: EC_{50} 13 μM, T.I. 3.8; **8**: EC_{50} 42 μM, T.I. 1.8) or inactive (**6**: EC_{50} 95 μM, T.I. 0.7). Alkaline hydrolysis of **2** with KOH/EtOH gave prosapogenin **7**, which exhibited no anti-HIV activity (EC_{50}>100 μM). On acidic hydrolysis with H_2SO_4, **2** yielded 2β,23-dihydroxyacacic acid lactone [**9**], which showed moderate anti-HIV activity (EC_{50} 8 μM, T.I. 5.0). Comparison of the structures of the active saponins and the inactive prosapogenins and derivatives showed that all inactive compounds lack the monoterpenyl moieties. This observation suggests that the unusual monoterpenyl moieties in compounds **1** and **2** play an important role in modulating the anti-HIV activity of these compounds.

Derivatives of echinocystic acid [**8**] were also prepared and evaluated for anti-HIV activity, since betulinic acid, maprounic acid, and their derivatives were recently shown to be anti-HIV or anti-HIV RT triterpenes (6–8). Among compounds **10**–**16**, 3,16-di-*O*-acetylechinocystic acid [**12**] (9–11) demonstrated potent anti-HIV activity (EC_{50} 2.3 μM) with relatively low toxicity (IC_{50} 13 μM). In contrast, increased toxicity was observed in methyl echinocystate [**10**] (12) and methyl 3-*O*-acetylechinocystate [**11**] (9–11). When a *n*-butyryl or valeryl group was introduced at the C-3 and/or C-17 hydroxy group(s) of **10**, the toxicity decreased compared to that of **10**; although, in each





	R_1	R_2	R_3
			glc^2—rham
3	glc^6—ara^2—xyl	H	/
			$rham^2$—xyl^3—xyl
4	glc^6—ara^2—xyl	H	H
4a	glc^6—ara^2—xyl	H	Me
5	glc^6—ara	H	H
6	glc	H	H
8	H	H	H
10	H	H	Me
11	Ac	H	Me
12	Ac	Ac	H
13	Butyryl	H	Me
14	Butyryl	Butyryl	Me
15	Valeryl	H	Me
16	Valeryl	Valeryl	Me

7 R=glc^6—ara^2—xyl
9 R=H

case the anti-HIV activity also decreased. Weak anti-HIV activities were found in **14** and **15** (EC_{50} 15 μM, T.I. >11; EC_{50} 30 μM, T.I. 5.9).

In conclusion, compounds **1** and **2** are the first identified triterpene saponins to demonstrate anti-HIV activity. They are structurally unusual compared to other saponins and possess monoterpenyl ester group(s) in their molecules.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All mps were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Ir spectra were measured on a Shimadzu IR-480 spectrometer. ^{1}H- and

TABLE 1. HIV Inhibitory Effects (μM) for **1–16**.

Compound	EC_{50}[a]	IC_{50}[b]	T.I.[c]
1	1.1	9.8	8.9
2	2.7	14	5.2
3	>100	43	—
4	>100	37	—
4a	9.9	22	2.2
5	13	50	3.8
6	95	63	0.7
7	100	>100	>1.0
8	42	74	1.8
9	8.0	40	5.0
10	3.1	4.9	1.6
11	5.1	7.6	1.5
12	2.3	13	5.4
13	27	27	1.0
14	15	>160	>11
15	30	180	5.9
16	31	54	1.8

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

^{13}C-nmr spectra were recorded on a Varian XL-300 spectrometer using TMS as internal standard. Mass spectra were determined on a Hitachi M-80 mass spectrometer using a direct inlet system at 20 eV. Si gel (Merck, type G 60, 230 mesh) was used for flash cc. Tlc was performed on precoated Kieselgel 60 (0.25-mm thick, Merck) plates and spots were detected by spraying with 10% H_2SO_4 solution containing 1% $Ce(SO_4)_2$ followed by heating. Hplc was conducted on a Shimadzu LC-6A solvent delivery system equipped with a SCL-6B system controller, a Waters R401 differential refractometer, and a μBondapak C_{18} (25.4 i.d.×300 mm) column.

PLANT MATERIAL.—*Gleditsia japonica* fruits were collected in November 1993, in Shiga Prefecture, Japan. The collection of *Gymnocladus chinensis* fruits that were used has been described previously (13). Voucher specimens are kept at Kyoto Pharmaceutical University.

EXTRACTION AND ISOLATION.—*Gleditsia Saponin C* [**1**].—The crushed dried fruits (800 g) were extracted with hot MeOH (8 liters). The extract (240 g) was concentrated *in vacuo* to a syrup, which was suspended in H_2O and partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH. The *n*-BuOH fraction (68 g) was subjected to Si gel cc using $CHCl_3$-MeOH-H_2O (8:3:1) to furnish eight fractions (fractions 1–8). Fraction 4 (4.8 g) was further chromatographed on Sephadex LH-20 using MeOH as eluent and then purified by prep. hplc with MeOH-H_2O (7:3), yielding compound **1** as a hygroscopic white powder (from MeOH-H_2O), mp 190–192°; $[\alpha]^{28}D$ −24.0° (*c*=0.90, MeOH); ir ν max 3400–3600 (OH), 1725 (COOR) cm^{-1}. Compound **1** was identified as gleditsia saponin C by comparison with an authentic sample (4).

Gymnocladus saponin G [**2**].—The crushed dried fruits (1.4 kg) were extracted with hot MeOH (7 liters) and the extract was divided by *n*-hexane, EtOAc, *n*-BuOH, and H_2O extraction in a similar manner to that described above for the purification of **1**. The *n*-BuOH fraction (54 g) was subjected to Si gel cc using $CHCl_3$-MeOH-H_2O (65:35:10) and furnished seven fractions (fractions 1–7). Fraction 3 (1.8 g) was subsequently chromatographed on Sephadex LH-20 using MeOH as eluent and purified by prep. hplc with MeOH-H_2O (68:32) yielding **2** as a hygroscopic amorphous powder: $[\alpha]^{28}D$ −4.7° (*c*=0.90, MeOH); ir ν max 3400–3600 (OH), 1680, 1725 (COOR) cm^{-1}. Compound **2** was identified as gymnocladus saponin G by comparison with an authentic sample (5).

COMPOUND **3**.—A solution of **1** (60 mg) in EtOH (10 ml) and 5% K_2CO_3 (10 ml) was refluxed for 1 h. After cooling, the reaction mixture was neutralized with Dowex 50W-X8, filtered, and concentrated to dryness. The residue was subjected to Si gel cc with $CHCl_3$-MeOH-H_2O (65:35:10) to afford **3** (41 mg) as a white powder (from MeOH-Et_2O), mp 219–221°; $[\alpha]^{28}D$ −42.2° (c=0.88, MeOH); ir ν max 3400 (OH), 1720 (COOR) cm^{-1}. The physical and spectral data were identical with those of an authentic sample (4).

COMPOUND **4**.—A solution of **1** (400 mg) in 10% KOH (40 ml) and EtOH (40 ml) was refluxed for 3 h. The reaction mixture was cooled to room temperature and neutralized with 10% H_2SO_4 under ice cooling. The neutral solution was worked up as usual and subjected to Si gel cc with $CHCl_3$-MeOH-H_2O (65:35:10) to yield **4** (100 mg) as a white powder (from MeOH/Et_2O): mp 219–222°; $[\alpha]^{28}D$ −12.0° (*c*=0.92, MeOH); ir ν max 3400 (OH), 1650 (COOH) cm^{-1}, which was identified as echinocystic acid 3-*O*-[β-D-xylopyranosyl(1→2)-α-L-arabinopyranosyl(1→6)]-β-D-glucopyranoside by comparison with an authentic sample (12).

COMPOUND **4a**.—A solution of **4** (40 mg) in MeOH was treated with ethereal CH_2N_2 for 18 h at room temperature. The reaction mixture was worked up as usual to give **4a** (25 mg) as colorless needles (from MeOH/H_2O), mp 188–190°; $[\alpha]^{28}D$ −13.0° (*c*=1.16, MeOH); ir ν max 3500 (OH), 1725 (COOMe) cm^{-1}; ^{13}C nmr (pyridine-d_5) δ 13.5 (C-6), 15.6 (C-25), 17.0 (C-24), 17.2 (C-26), 23.8 (C-11), 24.6 (C-30), 26.7 (C-2), 27.2 (C-27), 28.2 (C-23), 30.8 (C-20), 32.5 (C-22), 33.2 (C-29), 33.4 (C-7), 35.9 (C-15), 35.9 (C-21), 37.0 (C-10), 38.8 (C-1), 39.5 (C-4), 39.8 (C-8), 41.1 (C-18), 41.8 (C-14), 46.8 (C-19), 47.0 (C-9), 48.9 (C-17), 51.8 (O-Me), 55.9 (C-5), 64.2 (ara-5), 67.2 (xyl-5), 67.4 (ara-4), 69.5 (glc-6), 70.7 (xyl-4), 72.1 (glc-4), 72.5 (ara-3), 74.3 (C-16), 75.3 (xyl-2), 75.6 (glc-2), 76.0 (glc-5), 77.7 (xyl-3), 78.3 (glc-3), 80.3 (ara-2), 88.6 (C-3), 102.3 (ara-1), 106.2 (xyl-1), 106.7 (glc-1), 122.6 (C-12), 144.4 (C-13), 177.7 (C-28); *anal.*, calcd for $C_{47}H_{76}O_{17}$ H_2O, C 60.63%, H 8.44%, found C 60.65%, H 8.39%.

COMPOUNDS **5**, **6**, AND **8**.—A solution of **4** (800 mg) in 10% H_2SO_4 (60 ml) and EtOH (60 ml) was refluxed for 2.5 h. The reaction mixture was cooled to room temperature, diluted with 50 ml H_2O, and neutralized with 5% NaOH solution. The mixture was concentrated under reduced pressure to give an aqueous solution, which was extracted with *n*-BuOH. The organic layer was then washed with saturated NaCl solution, concentrated, and subjected to Si gel cc. Elution with $CHCl_3$-MeOH-H_2O (9:1:0.1) yielded **8** as colorless needles (from MeOH, 53 mg), mp 308–309°; $[\alpha]^{28}D$ +39° (*c*=1.00, EtOH); ir ν max 3400–3500 (OH), 1685 (COOH) cm^{-1}. Subsequent elution with $CHCl_3$-MeOH-H_2O (8:3:1) furnished **6** as colorless needles (from MeOH, 120 mg): mp 267–268°; $[\alpha]^{28}D$ +6.1° (*c*=1.00, MeOH); ir ν max 3400–

3600 (OH), 1670 (COOH) cm^{-1}. Further elution with $CHCl_3$-MeOH-H_2O (13:7:2) afforded **5**, colorless needles (from MeOH-H_2O, 165 mg), mp 225–227°; $[\alpha]^{28}D$ −10.7° (c=1.03, MeOH); ir ν max 3600 (OH), 1670 (COOH) cm^{-1}. The structures of **5**, **6**, and **8** were assigned by comparison with authentic samples (12).

COMPOUND **7**.—A solution of **2** (160 mg) in 10% KOH (5 ml) and EtOH (5 ml) was refluxed for 3 h. The reaction mixture was worked up in the same manner as for **4**, and the product was purified by hplc [MeOH-H_2O (66:34)] giving **7** (25 mg) as a hygroscopic white powder (from MeOH/H_2O): mp 187–189°; $[\alpha]^{28}D$ −10.0° (c=0.80, MeOH); ir ν max 3400–3600 (OH), 1760 (γ-lactone) cm^{-1}. The structural assignment was obtained by comparison with an authentic sample (5).

COMPOUND **9**.—A solution of **2** (80 mg) in 10% H_2SO_4 (10 ml) and EtOH (10 ml) was refluxed for 2.5 h. The reaction mixture was neutralized with Amberlite IRA-450, filtered, and concentrated to give an aqueous solution, which was extracted with EtOAc. The organic layer was concentrated and crystallized from MeOH/H_2O to afford **9** (20 mg) as colorless needles: mp 308–309°; $[\alpha]^{28}D$ +13.2° (c=0.60, MeOH); ir ν max 3400 (OH), 1760 (γ-lactone) cm^{-1}. Compound **9** was identified as 2β,23-dihydroxyacacic acid lactone by comparison with an authentic sample (5).

COMPOUND **10**.—Compound **8** (100 mg) was methylated by the same method as described for **4a** to afford **10** (88 mg) as colorless needles (from MeOH): mp 217–219; $[\alpha]^{28}D$ +33° (c=0.50, MeOH); ir ν max 3500 (OH), 1725 (COOMe) cm^{-1}. The physical and spectral data were identical with those of an authentic sample (12).

COMPOUND **11**.—Compound **10** (40 mg) was treated with pyridine (2 ml) and Ac_2O (2 ml) overnight at room temperature. The reaction mixture was worked up as usual and was subjected to Si gel cc with C_6H_6-EtOAc (10:1–4:1) to yield **11** (26 mg) as colorless needles (MeOH): mp 308–309°; $[\alpha]^{28}D$ +18.3° (c=0.69, $CHCl_3$); ^1H nmr ($CDCl_3$) δ 0.73, 0.86, 0.87, 0.91, 0.94, 0.97, 1.35 (3H each, s, Me), 2.05 (3H, s, COMe), 3.61 (3H, s, OMe), 4.52–4.47 (2H, m, H-3 and H-16), 5.39 (1H, t, J=3.4 Hz, H-12); *anal.*, calcd for $C_{33}H_{52}O_5$, C 74.96%, H 9.91%, found C 74.67%, H 10.01%.

COMPOUND **12**.—Compound **8** (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 and was subjected to Si gel cc with C_6H_6–EtOAc (5:1–3:1) to afford **12** (24 mg) as colorless needles (from MeOH): mp 250–252°; $[\alpha]^{28}D$ −13.3° (c=0.97, $CHCl_3$); ^1H nmr ($CDCl_3$) δ 0.70, 0.84, 0.86, 0.93, 0.95, 0.98, 1.24 (3H each, s, Me), 2.05, 2.09 (3H each, s, COMe), 4.50 (1H, t, J=8.2 Hz, H-3), 5.40 (1H, t, J=3.0 Hz, H-12), 5.63 (1H, br s, H-16); *anal.*, calcd for $C_{34}H_{52}O_6$, C 73.34%, H 9.41%, found C 73.62%, H 9.57%.

COMPOUNDS **13** AND **14**.—Compound **10** (80 mg) was treated with pyridine (2 ml) and butyric anhydride (2 ml) overnight at room temperature. The reaction mixture was worked up as usual and was subjected to Si gel cc with C_6H_6-EtOAc (10:1→5:1) to yield **13** (40 mg) and **14** (21 mg). Compound **13**: colorless needles (from MeOH), mp 211–213°; $[\alpha]^{28}D$ +30.3° (c=0.76, $CHCl_3$); ^1H nmr ($CDCl_3$) δ 0.73, 0.86, 0.91, 0.94, 0.97, 1.35 (3H each, s, Me), 0.95 (3H, t, J=7.4 Hz, CH_2CH_3), 2.28 (2H, t, J=7.4 Hz, $COCH_2CH_2$), 3.61 (3H, s, OMe), 4.50 (1H, t, J=8.0 Hz, H-16), 4.51 (1H, dd, J=10.0 and 5.6 Hz, H-3), 5.39 (1H, t, J=3.4 Hz, H-12); ms *m/z* $[M]^+$ 556 (4), 538 (6), 468 (10), 278 (15), 277 (12), 260 (100), 201 (75), 190 (40); *anal.*, calcd for $C_{35}H_{56}O_5H_2O$, C 73.13%, H 10.17%; found C 73.28%, H 10.28%. Compound **14**: oil; $[\alpha]^{28}D$ −36.8° (c=0.26, $CHCl_3$); ^1H nmr ($CDCl_3$) δ 0.71, 0.85 (×2), 0.92, 0.94, 0.97, 1.24 (3H each, s, Me), 0.95, 0.98 (3H each, t, J=7.8 Hz, CH_2CH_3), 2.28, 2.31 (2H each, t, J=7.8 Hz, $COCH_2CH_2$), 3.63 (3H, s, OMe), 4.50 (1H, t, J=7.9 Hz, H-3), 5.42 (1H, t, J=3.5 Hz, H-12), 5.68 (1H, br s, H-16); ms *m/z* 538 (20), 479 (13), 450 (8), 391 (11), 277 (3), 260 (90), 201 (100), 190 (30); *anal.*, calcd for $C_{39}H_{62}O_6$, C 74.92%, H 9.97%, found C 74.47%, H 10.06%.

COMPOUNDS **15** AND **16**.—Compound **10** (80 mg) was treated with pyridine (2 ml) and valeric anhydride (2 ml) overnight at room temperature. The reaction mixture was worked up as usual and was subjected to Si gel cc with C_6H_6-EtOAc (10:1→6:1) to yield **15** (14 mg) and **16** (29 mg). Compound **15**: colorless needles (from MeOH), mp 78–82°; $[\alpha]^{28}D$ +19.7° (c=1.35, $CHCl_3$); ^1H nmr ($CDCl_3$) δ 0.73, 0.86, 0.87, 0.91, 0.94, 1.35 (3H each, s, Me), 0.92 (3H, t, J=7.5 Hz, CH_2CH_3), 2.30 (2H, t, J=7.6 Hz, $COCH_2CH_2$), 3.61 (3H, s, OMe), 4.47–4.53 (2H, m, H-3 and H-16), 5.39 (1H, t, J=3.6 Hz, H-12); ms *m/z* $[M]^+$ 570 (4), 552 (10), 468 (13), 291 (8), 278 (15), 260 (100), 201 (80), 189 (40); *anal.*, calcd for $C_{36}H_{58}O_5H_2O$, C 73.43%, H 10.27%, found C 73.70%, H 10.31%. Compound **16**: oil; ^1H nmr ($CDCl_3$) δ 0.71, 0.85 (×2), 0.92, 0.94, 0.98, 1.24 (3H each, s, Me), 0.93, 0.94 (3H each, t, J=7.3 Hz, CH_2CH_3), 2.30, 2.33 (2H each, t, J=7.5 Hz, $COCH_2CH_2$), 3.63 (3H, s, OMe), 4.49 (1H, dd, J=6.1 and 9.7 Hz, H-3), 5.42 (1H, t, J=3.5 Hz, H-12), 5.68 (1H, t like, H-16); ms *m/z* 552 (35), 493 (28), 450 (10), 391 (13), 291 (5), 260 (100), 201 (78), 189 (40); *anal.*, calcd for $C_{41}H_{66}O_6$, C 75.18%, H 10.16%, found C 74.92%, H 10.22%.

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 (III B 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 density of uninfected cultures was determined to assess the 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 a test compound to inhibit HIV replication was measured at four different concentrations of the 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).

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