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Two new sesquiterpenoids and anti-HIV principles from the root bark of *Zanthoxylum ailanthoides*

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Abstract—Two new sesquiterpenes, 10β-methoxymuurolan-4-en-3-one (**1**) and 10α-methoxycadinan-4-en-3-one (**2**), were isolated from the root bark of *Zanthoxylum ailanthoides*. The structures of **1** and **2** were elucidated from spectroscopic data. Sixty-seven compounds obtained from the root bark of the same plant were evaluated for inhibition of HIV replication in H9 lymphocyte cells, and 14 compounds demonstrated significant activity. Among them, decarine, γ-fagarine, and (+)-tembamide were the most potent anti-HIV compounds, with EC_{50} values of <0.1 μg/mL and TI values of >226, >231, and >215, respectively.

1. Introduction

Zanthoxylum ailanthoides Sieb. & Zucc. (Rutaceae) is a medium to large tree with odd, pinnate leaves and conical spines in the main stem, distributed in China, Korea, Japan, Philippines, and throughout the low altitude forests of Taiwan.[1] Its leaves are used as folk medicine to treat the common cold in Taiwan.[2] In China, the bark has been prescribed for rheumatism, arthralgia, stasis, contusions, and snakebite, and to stimulate blood circulation.[3] Benzo[*c*]phenanthridines, quinolines, coumarins, lignans, flavonoids, and triterpenoids are the major constituents of this plant.[4–14] However, only two components, xanthyletin and hesperidin,[4,5] have been isolated from the root bark and, among methanolic extracts of different parts of this species, only the root bark has shown anti-HIV activity in vitro. In a previous paper, we reported two new alkylamides and 110 compounds from the root bark[15] and mentioned that, due to incorrect identification, the original plant of *Aralia bipinnata* in three papers[12–14] should be revised to *Z. ailanthoides*. A careful examination of the root bark has now resulted in the characterization of two new sesquiterpenes as additional constituents. In this article, we report the isolation and structural elucidation of these two new compounds and the anti-HIV activity of 67 compounds isolated from the root bark of *Z. ailanthoides*.[15]

2. Results and discussion

2.1. Structural determination

10β-Methoxymuurolan-4-en-3-one (**1**) was isolated as an oil. Its molecular formula, $C_{16}H_{26}O_2$, was established by EI-MS ($[M]^+$, *m*/*z* 250) and HREI-MS. UV absorption bands at 238 nm indicated an α,β-unsaturated carbonyl system, which was suggested further by measuring the absorance at 1667 and 1657 cm^{-1} in the IR spectrum. The ^{13}C NMR spectrum exhibited 16 carbon signals including one methoxy, one carbonyl, four methyl, three methylenes, two olefinic carbons, four methines, and one quaternary carbon. Four indices of hydrogen deficiency (IHD) were determined from the ^{13}C NMR spectrum, DEPT experiments, and HREI-MS. The ^1H NMR spectrum for **1** was very similar to that of the known sesquiterpene, muurolan-3-en-9β-ol-2-one, except that an OMe-16 [δ 3.17 (3H, s)] in **1** replaced the OH-16 in the latter compound. Peaks were present at δ 0.90, 0.91 (each 3H, d, J = 7.0 Hz, H-13, 14) and 1.87 (1H, br sept. J = 7.0 Hz, H-12) for an isopropyl group, a three-proton singlet at δ 1.05 (3H, s) for a methyl attached to a quaternary carbon, a broad

Keywords: *Zanthoxylum ailanthoides*; Rutaceae; Root bark; Sesquiterpene; 10β-Methoxymuurolan-4-en-3-one; 10α-Methoxycadinan-4-en-3-one; Anti-HIV activity; Decarine; γ-Fagarine; (+)-Tembamide.
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signal at 1.78 (3H, br s) due to a methyl group on a double bond, and a multiplet at δ 6.95 (1H, dq, $J = 6.4$, 1.2 Hz) for a trisubstituted olefinic proton. On the basis of the above data, **1** has a cadinan-type sesquiterpene [5-isopropyl-3,8-dimethyl-4a,5,6,7,8,8a-hexahydro-1*H*-naphthalene-2-one] skeleton. The COSY spectrum of **1** displayed connectivity between H-5 (δ 6.95) and H-6, which was also coupled to H-1 [δ 2.27 (1H, ddd, $J = 14.4$, 5.5, 4.7 Hz)] and H-7 [δ 1.46 (1H, m)]. H-1 showed a COSY correlation with H_2-protons [δ 2.35 (1H, dd, $J = 17.0$, 4.7 Hz, H-2α) and 2.41 (1H, dd, $J = 17.0$, 14.4 Hz, H-2β). H-6 [δ 2.60 (1H, m)] was coupled to the methine proton at δ 1.46 (H-7), and the coupling chain continued from H-7 to H-8 [δ 1.30–1.50 (2H, m)] and then to H-9 [δ 1.30–1.50 (2H, m)]. H-12 [δ 1.87 (1H, br sept. $J = 7.0$ Hz)] showed a COSY correlation with H-7, -13, and -14, and also a NOESY correlation with H-5. This evidence confirmed that the isopropyl group is at C-7. The relative stereochemistry of **1** was deduced from a combination of coupling constants and the NOESY spectrum. The NOESY spectrum of **1** gave proof for the proposed *cis*-fused ring system, as correlation between H-1 and H-6 was observed. The *cis*-fused **1** showed a coupling constant (6.5 Hz) between H-5 and 6. In *trans*-fused isomers, such as T-cadinol,[16] the olefinic proton [δ 5.52 (1H, br s)] appears as a broad singlet in the ^{1}H NMR, while in *cis*-fused muurolene derivatives[17] the olefinic proton resonates as a doublet. Thus, based on the above evidence, compound **1** was identified as 10β-methoxymuurolan-4-en-3-one (Fig. 1).

10α-Methoxycadinan-4-en-3-one (**2**) was isolated as an oil and shown by HREIMS to have the molecular formula $C_{16}H_{20}O_2$ [M^+, *m/z* 250.1928]. The IR spectrum of **2** displayed peaks for an α,β-unsaturated carbonyl group that was confirmed further from the UV absorptions at 241 nm. The ^{1}H NMR and ^{13}C NMR of **2** were very similar to those of 10α-hydroxycadinan-4-en-3-one,[18] except for a methoxy group at δ 3.18 (3H, s) rather than a hydroxy group, respectively. The ^{1}H NMR assignments for **2** are based on the COSY spectrum, and the relative stereochemistry of **2** was confirmed by the correlations observed in its NOESY spectrum.[19] Thus, compound **2** was identified as 10α-methoxycadinan-4-en-3-one.

2.2. Biological activity

Anti-HIV data for isolated compounds of the bark of this plant are given in Table 1. Fractionation of the $CHCl_3$-soluble fraction led to the isolation of 14 anti-HIV active principles: γ-fagarine, haplopine, decarine, *O*-methylcedrelopsin, isopimpinellin, 5,7,8-trimethoxycoumarin, (+)-platydesmine, *p*-hydroxybenzaldehyde, 4-methoxy-1-methyl-2-quinolone, (+)-hinokinin, (−)-tetrahydroberberine, (+)-tembamide, *O*-methyltembamide, and a mixture of β-sitosteryl glucoside and stigmasteryl glucoside. Among these active compounds, the benzo[*c*]phenanthridine alkaloid, decarine, showed the highest anti-HIV activity in acutely infected H9 cells, with an EC_{50} value of <0.1 μg/mL. It inhibited uninfected H9 cell growth with an IC_{50} value of 22.6 μg/mL, giving a calculated TI value of >226. Thus, in comparison with the inactive norchelerythrine, the 8-OH group in decarine seems to play an important role in anti-HIV activity. The furoquinoline, γ-fagarine, also showed a relatively potent anti-HIV activity, with EC_{50} and TI values of <0.1 μg/mL and >231, respectively. In comparison, the furoquinoline haplopine, which has a 7-OH, showed decreased activity, while replacing the 7-OH moiety with a 7-OCH_3 moiety gave skimmianine, which showed no suppression. The other furoquinoline-like compounds, dictamnine (no substituents in ring A), confusameline (7-OH), robustine (8-OH), evolitrine

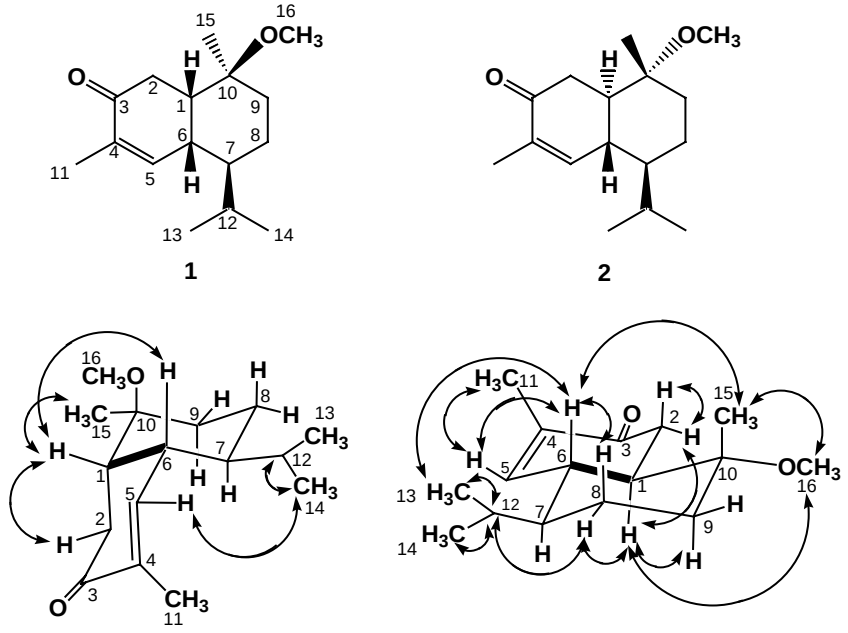

Figure 1. Selected NOESY correlations of **1** and **2**.

Table 1. Anti-HIV data of compounds isolated from the root bark of *Z. ailanthoides*

Compound	IC_{50}[a] (μg/mL)	EC_{50}[b] (μg/mL)	Therapeutic index[c]
6-Acetonyldihydrochelerythrine	2.32	NS[d]	NS
6-Acetonyldihydronitidine	0.209	<0.1	>2.09
Aesculetin dimethyl ether	26.3	NS	NS
β-Amyrin	DND[e]		
Arnottianamide	>100	NS	NS
Aurapten	2.37	NS	NS
Bergapten	>25	NS	NS
Bocconoline	20.7	NS	NS
Braylin	10.8	NS	NS
Caryophyllene oxide	2.32	NS	NS
Chelerythrine	DND		
Confusameline	23.6	NS	NS
Decarine	22.6	<0.1	>226
Dictamnine	2.17	NS	NS
Dihydroavicine	0.22	<0.1	>2.2
Dihydrochelerythrine	23.1	NS	NS
Edulitine	>25	NS	NS
Evolitrine[21]	9.41	NS	NS
γ-Fagarine	23.1	<0.1	>231
Friedelin	DND		
Haplopine	23.2	0.634	36.7
(+)-Hinokinin	1.87	<0.1	>18.7
Hydrangetin	22.1	NS	NS
p-Hydroxybenzaldehyde	22.3	3.28	6.81
Isoarnottianamide	2.12	NS	NS
Isopimpinellin	21.2	0.669	31.7
Lanyulactone	>25	NS	NS
Lupenone	DND		
Lupeol	DND		
Lupeol acetate	DND		
Luvangetin	>25	NS	NS
(±)-Lyoniresinol	>25	NS	NS
(+)-Marmesin	21.2	NS	NS
6-Methoxychelerythrine	>25	NS	NS
4-Methyl-1-methyl-2-quinolone	22.7	0.625	36.4
Methyl palmitate	>25	NS	NS
Mixture of β-sitosterol and stigmasterol	DND		
Mixture of β-sitosteryl glucoside and stigmasteryl glucoside	16.5	1.22	13.5
Nitidine	DND		
Norchelerythrine	DND		
O-Methylcedrelopsin	21.1	0.576	36.6
O-Methyltembamide	16.4	<1.49	>11.0
Oxyavicine	0.174	<0.1	>1.74
Oxychelerythrine	DND		
Oxynitidine	19.0	5.46	3.48
Phellopterin	21.7	NS	NS
Pheophytin-b	DND		
(+)-Platydesmine	>100	1.34	74.4
Pteleine[22]	10.9	NS	NS
Pregnenolone	2.01	NS	NS
Quercetin	>25	NS	NS
Robustine	101	NS	NS
(+)-Sesamin	21.3	NS	NS
Sesaminone	19.6	NS	NS
Skimmianine	22.5	7.41	3.04
Spathulenol	>25	NS	NS
Squalene	1.93	0.744	2.59
(+)-Syringaresinol	2.29	NS	NS
Syringic acid	23.4	4.90	4.76
(+)-Tembamide	21.5	<0.1	>215
Tetracosyl ferulate	DND		
(−)-Tetrahydroberberine	2.25	<0.1	>22.5
2-Tridecanone	21.6	NS	NS
5,7,8-Trimethoxycoumarin	>100	0.933	107
6,7,8-Trimethoxycoumarin	>25	NS	NS

(*continued on next page*)

Table 1 (*continued*)

Compound	IC_{50}[a] (μg/mL)	EC_{50}[b] (μg/mL)	Therapeutic index[c]
Umbelliferone	24.0	NS	NS
Vanillin	20.5	NS	NS
Xanthyletin	2.22	NS	NS
AZT[1]	500	0.022	22,727
AZT[2]	500	0.0748	6680
AZT[3]	500	<0.001	>500,000

AZT[1] for 6,7,8-trimethoxycoumarin, luvangetin, bergapten, braylin, 6-methoxychelerythrine, nitidine, isoarnottianamide, edulitine, lanyulactone, quercetin, (±)-lyoniresinol, spathulenol, lupenone, methyl palmitate.
AZT[2] for robustine.
AZT[3] for remaining compounds.
[a] Concentration that inhibits uninfected H9 cell growth by 50%.
[b] Concentration that inhibits viral replications by 50%.
[c] TI = IC_{50}/EC_{50}.
[d] NS, no suppression.
[e] DND, did not dissolve.

(7-OCH_3),[20] and pteleine (6-OCH_3),[21] showed no anti-HIV activity. Thus, these results suggested that the 8-OCH_3 in furoquinoline may be necessary for anti-HIV activity. An aromatic amide, (+)-tembamide, showed significant anti-HIV activity with EC_{50} and TI values of <0.1 μg/mL and >215. However, *O*-methyltembamide with a methoxy rather than a hydroxy group was 15-fold less active (EC_{50} = 1.49 μg/mL) than (+)-tembamide. *O*-Methylcedrelopsin, isopimpinellin, 5,7,8-trimethoxycoumarin, (+)-platydesmine, 4-methoxy-1-methyl-2-quinolone, (+)-hinokinin, and (−)-tetrahydroberberine also exhibited significant anti-HIV activity, with EC_{50} values ranging from 0.1 to 1.34 μg/mL and TI values from 18.7 to 107. The quaternary benzo[*c*]phenanthridines, fagaronine and nitidine, and protoberberine type alkaloids, berberine, palmatine, and jatrorrhizine, have been previously reported to have inhibitory activity against HIV-1 reverse transcriptase.[22] It is interesting that the tertiary phenolic benzo[*c*]phenanthridine, decarine, showed activity against HIV replication. Besides decarine, γ-fagarine and (+)-tembamide also had TI values >200 and may be useful in the development of anti-HIV agents (Fig. 2).

3. Experimental

3.1. General

Melting points were determined with a YANACO micro-melting point apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP-370 polarimeter in $CHCl_3$. IR spectra were taken on a Hitachi 260-30 spectrophotometer. UV spectra were obtained on a JASCO UV-240 spectrophotometer in EtOH. EI-MS and HREI-MS were performed on a Finnigan/Thermo Quest MAT 95XL mass spectrometer; spectra were recorded on a JEOL JMS-HX 110 mass spectrometer. ^1H NMR (600 MHz), ^{13}C NMR (150 MHz), DEPT (90, 135), ^1H–^1H COSY, ^1H–^1H NOESY, HMQC (optimized for $^1J_{HC}$ = 45 Hz), and HMBC (optimized for $^nJ_{HC}$ = 8 Hz) spectra were measured on a Varian Unity Inova 600 spectrometer in $CDCl_3$ and are given in ppm (δ) downfield from TMS used as internal standard. Silica gel 60 (Merck 70–230 mesh, 230–400 mesh, ASTM) was used for CC and silica gel 60 F_{254} (Merck) for TLC.

3.2. Plant material

The root bark of *Z. ailanthoides* was collected in Lai-I, Pingtung County, Taiwan, in December 1999. Ih-Sheng Chen, corresponding author of this paper, identified the plant, and a voucher specimen (Chen 5645) was deposited in the Herbarium of the School of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China.

3.3. Extraction and isolation

Dried root bark (9 kg) was sliced into chips, extracted with cold MeOH, and concentrated under reduced pressure. The MeOH extract (920 g) was partitioned between $CHCl_3/H_2O$ (1:1) to provide $CHCl_3$- (fraction A, 320 g) and H_2O-soluble fractions. The H_2O-soluble fraction was partitioned with EtOAc, then with *n*-BuOH to give EtOAc- (fraction B, 10 g), *n*-BuOH- (fraction C, 60 g), and H_2O-soluble (fraction D, 180 g) fractions. Part (100 g) of fraction A (320 g) was chromatographed over Si gel (2000 g) eluting with *n*-hexane, enriched with EtOAc and acetone, to furnish 15 fractions: frs. A1–A4 (each 2500 mL, *n*-hexane), frs. A5–A6 (each 2500 mL, *n*-hexane/EtOAc, 20:1), frs. A7–A9 (each 2500 mL, *n*-hexane/EtOAc, 9:1), frs. A10–A11 (each 2500 mL, *n*-hexane/EtOAc, 3:1), fr. A12 (2500 mL, *n*-hexane/EtOAc, 1:1), fr. A13 (3000 mL, EtOAc), and frs. A14–A15 (each 5000 mL, MeOH). Fr. A4 (6.0 g) was rechromatographed over Si gel (48 g) eluting with CH_2Cl_2/EtOAc (1:0–1:1) to obtain 10 fractions (each 500 mL, fr. A4-1–fr. A4-10). Fr. A4-8 (100 mg) was purified further by prep. TLC (benzene/EtOAc, 8:1) to afford **1** (1.1 mg) (R_f = 0.46) and **2** (11.1 mg) (R_f = 0.37).

3.3.1. 10β-Methoxymuurolan-4-en-3-one (1). Colorless oil; $[\alpha]_D^{25}$: −65.0° (*c* 0.015, $CHCl_3$); HREI-MS *m*/*z* 250.1928 $[M]^+$ (calcd for $C_{16}H_{26}O_2$ 250.1928); UV λ_{max} (MeOH) nm 238 (3.96); IR ν_{max} cm^{-1} 1667, 1657; ^1H NMR ($CDCl_3$) δ 0.90 (3H, d, *J* = 7.0 Hz, H-13), 0.91 (3H, d, *J* = 7.0 Hz, H-14), 1.05 (3H, s, H-15), 1.30–

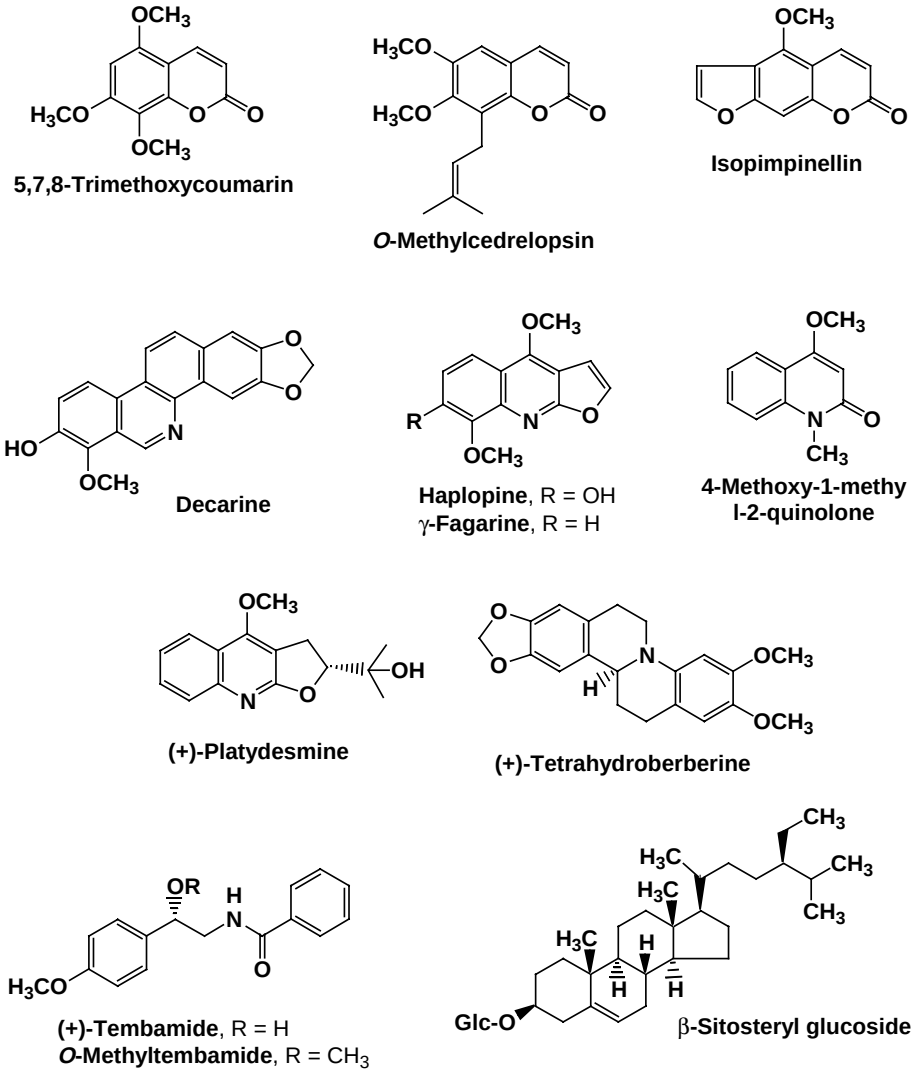

Figure 2. Active anti-HIV constituents obtained from the root bark of *Z. ailanthoides*.

1.50 (4H, m, H-8, 9), 1.46 (1H, m, H-7), 1.78 (3H, br s, H-11), 1.87 (1H, br sept., $J = 7.0$ Hz, H-12), 2.27 (1H, ddd, $J = 14.4$, 5.5, 4.7 Hz, H-1), 2.35 (1H, dd, $J = 17.0$, 4.7 Hz, H-2α), 2.41 (1H, dd, $J = 17.0$, 14.4 Hz, H-2β), 2.60 (1H, m, H-6), 3.17 (3H, s, OMe-16), 6.95 (1H, dq, $J = 6.4$, 1.2 Hz, H-5); ^{13}C NMR ($CDCl_3$) δ 15.7 (C-13), 16.0 (C-11), 19.2 (C-15), 21.3 (C-8), 21.5 (C-14), 27.8 (C-12), 30.3 (C-9), 42.6 (C-7), 43.0 (C-1), 48.1 (OMe-16), 75.0 (C-10), 134.7 (C-4), 151.0 (C-5), 199.5 (C-3); EI-MS *m/z* (rel. int.) 250 (M^+, 82).

3.3.2. 10α-Methoxycadinan-4-en-3-one (2). Colorless oil; $[\alpha]_D^{25}$: −60.0° (*c* 0.09, $CHCl_3$); HREI-MS *m/z* 250.1928 $[M]^+$ (calcd for $C_{16}H_{26}O_2$ 250.1928); UV λ_{max} (MeOH) nm 241 (3.97); IR ν_{max} cm^{-1} 1670, 1655; ^{1}H NMR ($CDCl_3$) δ 0.82 (3H, d, $J = 6.6$ Hz, H-13), 0.99 (3H, d, $J = 6.6$ Hz, H-14), 1.12 (3H, s, H-15), 1.17 (2H, m, H-7, 8β), 1.48 (1H, qd, $J = 12.0$, 4.0 Hz, H-14, H-9β), 1.71 (1H, m, H-8α), 1.80 (3H, q, $J = 1.0$ Hz, H-11), 1.87 (1H, m, H-9α), 1.97 (1H, ddd, $J = 14.4$, 10.8, 3.0 Hz, H-1), 2.06 (1H, dd, $J = 16.0$, 14.4 Hz, H-2β), 2.15 (1H, br t, $J = 10.8$ Hz, H-6), 2.23 (1H, sep d, $J = 6.6$, 2.5 Hz, H-12), 2.70 (1H, dd, J = 16.0, 3.0 Hz, H-2α), 3.18 (3H, s, OMe-16), 6.81 (1H, br s, H-5); ^{13}C NMR ($CDCl_3$): δ 15.2 (C-13), 16.0 (C-11), 17.8 (C-15), 21.0 (C-8), 21.4 (C-14), 26.2 (C-12), 35.0 (C-9), 38.3 (C-2), 40.5 (C-6), 45.1 (C-7), 47.8 (OMe-16), 48.2 (C-1), 74.8 (C-10), 135.4 (C-4), 146.1 (C-5), 200.3 (C-3); EI-MS *m/z* (rel. int.) 250 (M^+, 80).

4. Biology

4.1. Anti-HIV assay

The isolation procedure for the 67 isolates found in Table 1 was described in our previous paper.[15] The T cell line, H9, was maintained in continuous culture with complete medium RPMI 1640 with 10% fetal calf serum (FCS) supplemented with L-glutamine at 5% CO_2 and 37 °C. Aliquots of this cell line were only used in experiments when in log phase of growth. Test samples were first dissolved in dimethylsulfoxide (DMSO). The following were the final drug concentrations routinely used

for screening: 100, 20, 4, and 0.8 μg/mL, but for active agents, additional dilutions were prepared for subsequent testing so that an accurate EC_{50} value could be achieved. As the test samples were being prepared, an aliquot of the T cell line, H9, was infected with HIV-1 (IIIB isolate), while another aliquot was mock-infected with complete medium. The mock-infected aliquot was used for toxicity determinations (IC_{50}). The stock virus used for these studies typically had a $TCID_{50}$ value of 10^4 IU/mL. The appropriate amount of virus for a multiplicity of infection (moi) between 0.1 and 0.01 infections units/cell was added to the first aliquot of H9 cells. The other aliquot of H9 cells only received culture medium and then was incubated under identical conditions as the HIV-infected H9 cells. After a 4 h incubation at 37 °C and 5% CO_2, both cell populations were washed three times with fresh medium and then added to the appropriate wells of a 24-well plate containing the various concentrations of the test drug or culture medium (positive infected control/negative drug control). In addition, AZT was also assayed during each experiment as a positive drug control. The plates were incubated at 37 °C and 5% CO_2 for 4 days. Cell-free supernatants were collected on day 4 for use in our in-house p24 antigen ELISA. p24 antigen is a core protein of HIV and therefore is an indirect measure of the virus present in the supernatants. Toxicity was determined by performing cell counts by a Coulter Counter on the mock-infected H9 cells, which had either received culture medium (no toxicity) or test sample or AZT.[23]

Acknowledgments

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