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Isolation of rhododaurichromanic acid B and the anti-HIV principles rhododaurichromanic acid A and rhododaurichromenic acid from *Rhododendron dauricum*

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Abstract—Two novel chromane derivatives (**1** and **2**) and the known chromene (**3**) were isolated from the leaves and twigs of *Rhododendron dauricum*. The absolute stereostructure of **1** was established by spectroscopic examination and X-ray crystallographic analysis. The absolute stereostructures of **2** and **3** were also confirmed by photochemical conversion of **3** into **1** and **2**. Daurichromenic acid (**3**) demonstrated potent anti-HIV activity with an EC_{50} value of 0.00567 μg/mL and therapeutic index (TI) of 3,710. Rhododaurichromanic acid A (**1**) also showed relatively potent anti-HIV activity with an EC_{50} value of 0.37 μg/mL, and a TI of 91.9, whereas rhododaurichromanic acid B (**1**) displayed no anti-HIV activity.

1. Introduction

Rhododendron dauricum is distributed in the northern part of China, eastern part of Siberia, and Hokkaido. The dried leaves of this plant are known in China as 'Manshanfong,' and are used medicinally as an expectorant and to treat acute–chronic bronchitis.[1,2] During our continuing screening of plant-derived novel anti-HIV agents from natural products, the MeOH extract of the leaves and twigs of *Rhododendron dauricum* (Ericaceae) was found to display significant anti-HIV activity ($EC_{50} \leq 20$ μg/mL, TI>5). Bioassay-guided fractionation and repeated chromatography led to the isolation of two novel isomeric chromane derivatives, rhododaurichromanic acids A (**1**) and B (**2**), together with the known chromene daurichromenic acid (**3**). We report herein on the isolation and chracterization of these compounds and their anti-HIV activity.

2. Results and discussion

The MeOH extract of the leaves and twigs of *Rhododendron dauricum* was partitioned between EtOAc and water, and the anti-HIV active EtOAc-soluble fraction was subsequently separated into *n*-hexane-soluble and 90% MeOH-soluble fractions by solvent partition. Anti-HIV activity was found in the *n*-hexane-soluble portion, which was further separated by repeated chromatography on silica gel and by semi-preparative scale HPLC on YMC ODS-AM. Two novel isomeric chromane derivatives, named rhododaurichromanic acids A (**1**) and B (**2**), together with a chromene derivative (**3**) were isolated. Compound **3** was identified as daurichiromenic acid by comparison of the physical and spectral data with those reported in the literature.[3]

Rhododaurichromanic acids A (**1**) and B (**2**) had the same molecular formula $C_{23}H_{30}O_4$ as established by HRFABMS, which is identical with that of daurichiromenic acid (**3**). Compounds **1** and **2** gave similar ^{1}H NMR spectra, showing signals due to four tertiary methyl groups, an aromatic methyl group, and an aromatic proton, together with a chelated hydroxy proton signal at extreme low field, which are also comparable to those found in daurichromenic

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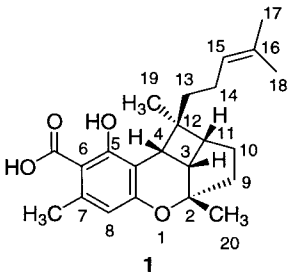

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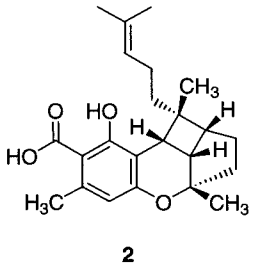

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acid (**3**). They also showed a doublet methine proton signal [δ 3.12 (d, J=9.5 Hz) in **1**; δ 3.20 (d, J=9.5 Hz) in **2**]. In contrast, one olefinic proton and a pair of *cis* coupled olefinic protons found in **3** were absent in **1** and **2**. The ^{13}C NMR spectra of **1** and **2** also resembled each other, showing nine carbon resonances in the aromatic region, including signals due to a tri-substituted double bond, a carboxylic acid group, and a penta-substituted aromatic ring group. These data indicated that **1** and **2** contained similar aromatic ring systems to that seen in **3**, but one tri-substituted double bond and a di-substituted double bond found in **3** were absent. In contrast, four additional carbon resonances, including three methine signals (δ 35.49, 38.54, 44.54 in **1**; δ 35.84, 37.86, 46.30 in **2**) and a quarternary carbon (δ 42.38 in **1**; δ 42.19 in **2**), were found upfield in both **1** and **2**. Taking their molecular formula into account, compounds **1** and **2** each have two additional rings in the molecule as compared with **3**.

Examination of the ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY spectra provided the fragment structures shown in Fig. 1. The long-range couplings in the ^{1}H–^{13}C long-range COSY spectrum established the connectivity of these fragment units, indicating that **1** and **2** have the same carbon framework, as shown in Fig. 1, but contained different stereostructures.

The coupling constants of H-4 and H-3 ($J_{3,4}$=9.5 Hz, $J_{3,11}$=7.5 Hz in each case) in the ^{1}H NMR spectra of **1** and **2**, along with the observation of NOE correlation between CH_3-20 and H-3, suggested that H-3, H-4, H-11, and CH_3-20 have the same orientations in both compounds. In **1**, CH_3-19 showed NOE enhancement with H-10, while H-4 and H-11 exhibited NOE correlations with H-13 (Fig. 2). In contrast, in **2**, CH_3-19 displayed NOE correlations with H-4 and H-11. These data indicated that CH_3-19 has

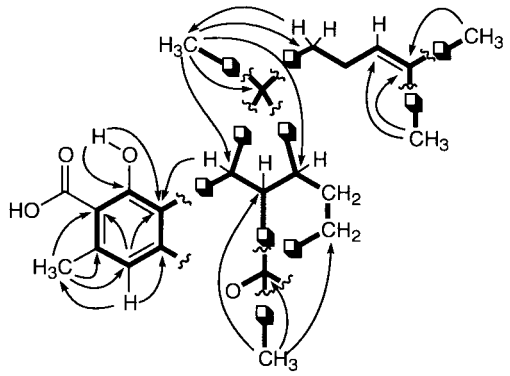


Figure 1. Partial structures and H→C long-range correlations in **1**.

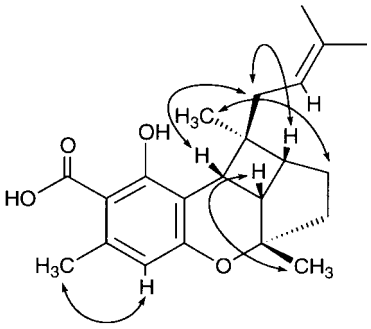
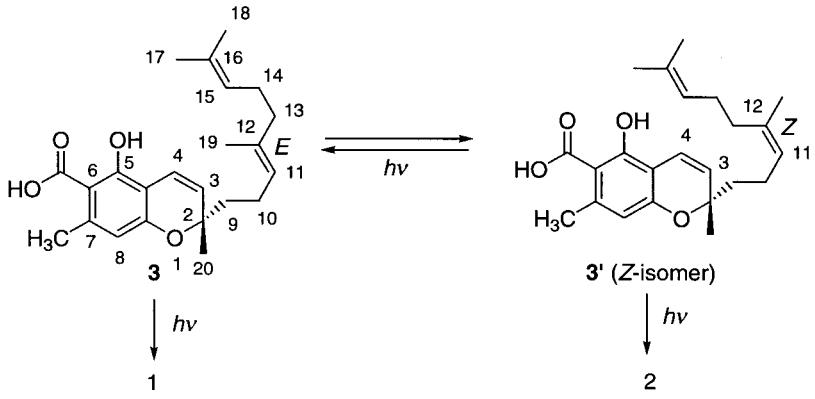


Figure 2. The NOE correlations in **1**.

the same orientation as H-4 and H-11 in **2** but the opposite orientation in **1**. These observations suggested that **1** and **2** were diasteromeric isomers at C-12. This assignment was also in good agreement with the following observation. The proton signals of the substituent located in the C-12α position were shifted upfield by the anisotropic effect of the aromatic ring; thus, CH_3-19 (δ 0.76) in **1** and H-13 (δ 1.20) in **2** were shifted upfield as compared with the corresponding isomers. In order to further support the structural assignments for **1** and **2**, the photochemical transformation of **3** was also attempted. A solution of **3** in hexane was irradiated with a low-pressure mercury lamp to give products that were separated by repeated silica gel chromatography and HPLC, and identified as **1** and **2**. *trans–cis* Isomerization of the double bond at C_{11}–C_{12} in **3** occurred,



Scheme 1.

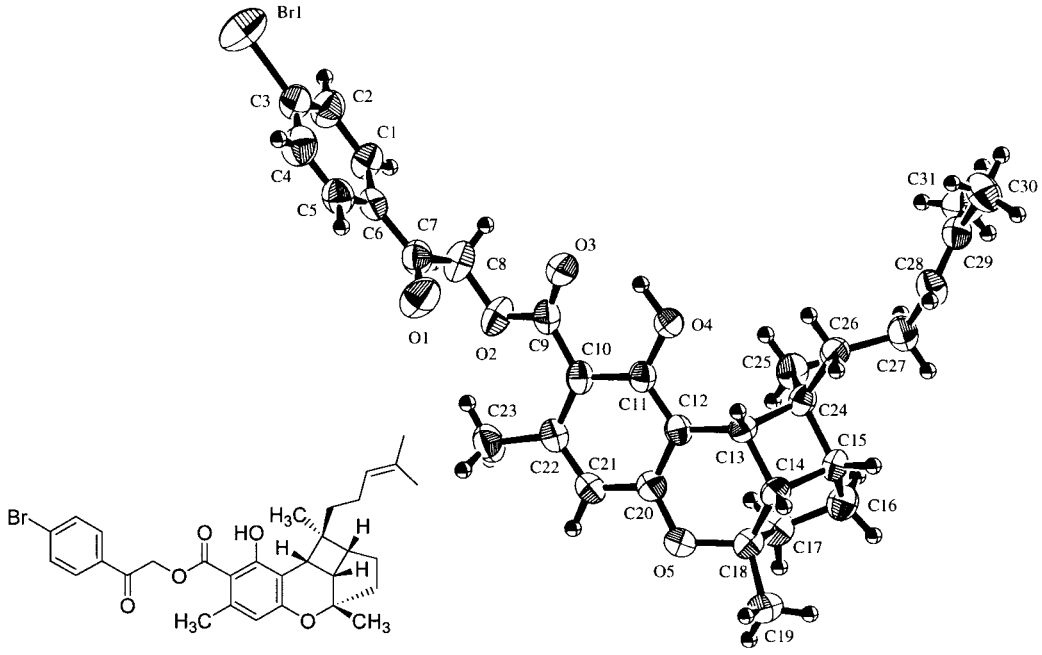


Figure 3. The crystal structure of **1a**.

followed by cyclization of C_3–C_4 and C_{11}–C_{12} to give a mixture of **1** and **2** (Scheme 1).

In order to confirm the structure of **1** unequivocally, the *p*-bromophenacyl derivative (**1a**) of **1** was prepared and analyzed using X-ray crystallography. Using anomalous scattering X-rays, the absolute stereostructure of **1a** was established as shown in Fig. 3. Because **1** and **2** were obtained by photochemical transformation of **3**, the C-2 stereochemistry in **1**, **2** and **3** must be identical. The NOE correlations in **1** and **2** described above indicated that C-3, C-4, and C-11 in both compounds also had the same absolute configurations. Therefore, the structures of **1** and **2**, including their absolute stereostructures, were established as formulae **1** and **2**. The absolute configuration of C-2 in daurichromemic acid (**3**), which had not been established previously, was also confirmed as *S*

Daurichromenic acid (**3**) demonstrated potent anti-HIV activity in acutely infected H9 cells with an EC_{50} value of

Table 1. ^{1}H (δ, J in Hz) and ^{13}C (δ) NMR data for compounds **1**–**3**

	1		**2**		**3**	
	H	C	H	C	H	C
2		84.53		84.64		80.09
3	2.58 (dd, 7.5, 9.5)	38.54	2.57 (dd, 7.5, 9.5)	37.86	5.48 (d, 10.5)	126.30
4	3.12 (d, 9.5)	35.49	3.20 (d, 9.5)	35.84	6.73 (d, 10.5)	116.65
4a		109.64		109.47		107.05
5		164.34		164.08		160.6
6		103.11		103.30		103.52
7		141.64		141.69		144.41
8	6.30 (s)	113.72	6.30 (s)	113.67	6.24 (s)	112.15
8a		159.34		159.39		158.98
9	ca. 1.7, 1.9	39.16	ca. 1.65, 2.03 (m)	40.46	1.66, 1.77 (m)	41.69
10	ca. 1.65, 1.9	25.70	ca. 1.67 (m)	26.15	2.04, 2.12 (m)	22.56
11	2.50 (m)	44.54	2.50 (m)	46.30	5.11 (t, 7)	123.71
12		42.38		42.19		135.50
13	ca. 1.7 (m) 1.85 (ddd, 4.5,12, 12)	46.25	1.20 (m)	30.80	1.95 (m)	39.66
14	ca. 1.95, 2.10	22.76	ca. 1.65, 1.86 (m)	22.74	1.97 (m)	26.67
15	5.18 (t, 6.5)	125.15	4.96 (t, 6.5)	125.27	5.08 (t, 7)	124.31
16		131.00		130.63		131.33
17	1.71 (s)	25.75	1.61 (s)	25.69	1.67 (s)	25.66
18	1.63 (s)	17.64	1.52 (s)	14.78	1.59 (s)	17.66
19	0.76 (s)	14.78	1.40 (s)	29.96	1.57 (s)	15.97
20	1.43 (s)	27.45	1.37 (s)	26.74	1.41 (s)	27.16
6-COOH		175.93		176.23		175.91
7-CH_3	2.53 (s)	24.11	2.53 (s)	23.41	2.54 (s)	24.45
5-OH	11.67 (s)		11.65 (s)		11.69 (s)	

Measured at 400 MHz (^{1}H) and 100 MHz (^{13}C) in $CDCl_3$

0.00567 μg/mL. It also inhibited uninfected H9 growth with IC_{50} value of 21.1 μg/mL, and thus, showed a good therapeutic index value of 3,710. Rhododaurichromanic acid A (**1**) also showed relatively potent anti-HIV activity with EC_{50} and TI values of 0.37 μg/mL and 91.9, respectively, whereas rhododaurichromanic acid B (**2**) displayed no anti-HIV activity. Compounds **1** and **3** represent a new class of anti-HIV natural products and are considered to be potential new leads for development of anti-HIV agents.

3. Experimental

3.1. General procedures

Melting points were measured on a Yanaco micro melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin–Elmer 241 polarimeter. NMR spectra were recorded on JEOL A-400 spectrometer. Chemical shifts were reported as δ (ppm) with tetramethylsilane (TMS) as the internal standard. FAB-MS and High resolution FAB-MS (HR-FABMS) were taken with a JEOL HX-110 spectrometer.

3.2. Plant material, extraction and isolation

The leaves and twigs of *Rhododendron dauricum* were collected in Kitami (Hokkaido, Japan) in 1996. A voucher specimen of the plant material, identified by Dr T. Yamagishi, Kitami Institute of Technology, has been deposited in the herbarium of Niigata College of Pharmacy. The air-dried leaves and twigs of *R. dauricum* (600 g), collected in Hokkaido, were extracted with MeOH (2 L) at room temperature three times. The combined MeOH extract was concentrated under reduced pressure to give a residue (175 g), which was partitioned with EtOAc and H_2O. After removal of the solvent by evaporation, the EtOAc-layer (65 g) was further partitioned with *n*-hexane and 90% aqueous MeOH to give *n*-hexane-soluble (30 g) and 90% MeOH-soluble (33 g) fractions. The aqueous layer was subsequently extracted with *n*-BuOH yielding *n*-BuOH-soluble (21 g) and H_2O-soluble (84 g) fractions.

The anti-HIV active *n*-hexane-soluble fraction was subjected to chromatography over silica gel eluting with benzene containing increasing amounts of EtOAc to give six fractions. Fraction 2 was crystallized from MeOH to give a mixture of ursolic acid and oleanolic acid, which were separated by Bondapak C_{18} yielding pure samples [ursolic acid (127 mg) and oleanolic acid (69 mg)]. The mother liquor of fraction 2 (4.23 g) was further chromatographed over silica gel eluted with $CHCl_3$ to afford daurichiromenic acid (**3**) (1.2 g). Further chromatography of fraction 3 (21 g) over silica gel (benzene) gave daurichiromenic acid (**3**) (ca. 2.0 g) and a fraction containing rhododaurichromanic acids A (**1**) and B (**2**). Crystallization of this fraction yielded **1** (52 mg). The mother liquor contained both **1** and **2**, which were separated by semi-preparative scale HPLC on Capcel-PAK ODS [MeCN–2% AcOH (9:1)] giving pure samples [**1** (75 mg) and **2** (69 mg)].

3.2.1. Rhododaurichromanic acid A (1). A white powder; mp 112–114°C; $[\alpha]_D$=+25.8° (c=0.36, $CHCl_3$); ^{1}H NMR and ^{13}C NMR: Table 1; positive FABMS m/z: 371 $(M+H)^+$. HRFABMS Calcd for $C_{23}H_{31}O_4$: 371.2222. Found m/z: 731.2234; CD (MeOH) $[\theta]_{228}$=−3900, $[\theta]_{262}$=+17 000, $[\theta]_{305}$=−1400.

3.2.2. Rhododaurichromanic acid B (2). A white powder; mp 161–162°C; $[\alpha]_D$=−118.2° (c=0.33, $CHCl_3$); ^{1}H NMR and ^{13}C NMR: Table 1; positive FABMS m/z: 370 $(M)^+$. HRFABMS Calcd for $C_{23}H_{30}O_4$: 370.2144. Found m/z: 370.2174; CD (MeOH) $[\theta]_{218}$=−26 700, $[\theta]_{262}$=+10 500, $[\theta]_{305}$=−6300.

3.2.3. Daurichromenic acid (3). Colorless syrup; $[\alpha]_D$=+30.4° (c=0.46, $CHCl_3$); ^{1}H NMR and ^{13}C NMR: Table 1; positive FABMS m/z: 731 $(M+H)^+$. HRFABMS Calcd for $C_{23}H_{31}O_4$: 370.2095. Found m/z: 730.2144; CD (MeOH) $[\theta]_{226}$=−8700, $[\theta]_{262}$=+14 200, $[\theta]_{305}$=−1500.

3.2.4. *p*-Bromophenacyl rhododaurichromanic acid A (1a). A mixture of **1** (75 mg), *p*-bromophenacylbromide (68 mg), and K_2CO_3 (27 mg) in dry Me_2CO (5 mL) was stirred at room temperature for 3 h. After removal of inorganics by filtration, the filtrate was concentrated under reduced pressure to a syrup, which was subjected to chromatography over silica gel. Elution with *n*-hexane–benzene (4:1) yielded the product (44 mg) as colorless needles: mp 172–173°C; $[\alpha]_D$=+34.8° (c=0.48, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.75 (3H, s, CH_3-19), 1.39 (3H, s, CH_3-20), 1.55 (3H, s, CH_3-18), 1.63 (3H, s, CH_3-17), 2.49 (1H, m, H-11), 2.56 (3H, s, 7-CH_3), 2.57 (1H, dd, J=7.5, 9.5 Hz, H-3), 3.12 (1H, d, J=9.5 Hz, H-4), 5.17 (1H, t, J=6.5 Hz, H-15), 5.51, 5.56 (each 1H, d, J=16.5 Hz, phenacyl H_2-2), 6.30 (1H, s, H-8), 7.66, 7.83 (each 2H, d, J=8.5 Hz, phenacyl H-2′ and -3′); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.80 (C-19), 17.62 (C-18), 22.72 (C-14), 24.11 (7-CH_3), 25.66 (C-10), 25.72 (C-17), 27.39 (C-20), 35.49 (C-4), 38.61 (C-3), 39.10 (C-9), 42.40 (C-12), 44.52 (C-11), 46.23 (C-13), 65.94 (phenacyl C-2), 84.40 (C-2), 104.04 (C-6), 109.65 (C-4a), 113.54 (C-8), 125.19 (C-15), 129.32 (2C) (phenacyl C-3′), 130.95 (C-16), 132.33 (2C) (phenacyl C-2′), 132.90 (phenacyl C-4′), 140.65 (C-7), 158.74 (C-9), 163.30 (C-5), 170.97 (6-COOH), 191.14 (phenacyl C-1); positive FABMS m/z: 567 $(M)^+$. HRFABMS Calcd for $C_{31}H_{36}O_5Br$: 567.1746. Found m/z: 567.1744.

3.3. Determination of the absolute stereochemistry of compound 1a by X-ray crystallographic analysis

Crystal data: $C_{31}H_{35}O_5Br$, F_W=567.52, monoclinic, space group $P2_1$ (#4), a=8.505 (2) Å, b=27.809 (8) Å, c=5.901 (1) Å, β=95.32 (2)° (from 25 reflections, 59.66°<2θ<59.99°), V=1389.6 (4) Å^3, Z=2, D_{Calcd}=1.356 g/cm^3, T=23°C, μ (CuKα radiation, λ=1.54178 Å)=23.2 cm^{-1}, F(000)=592.00, R=0.036, R_w=0.048; crystal dimensions: 0.2×0.2×0.2 mm^3.

All measurements were made on a Rigaku AFC7R diffractometer with a rotating anode generator. Intensity data collection was accomplished by the ω–2θ scan technique [CuKα radiation, graphite monochromator; $2\theta_{max}$ 120.1°, scan width (1.78+0.30 tan θ)°, scan rate 16.0°/min (in ω)]; 2123 unique reflections (R_{int}=0.028) were used for refinement.

The intensity data were corrected for Lorenz and polarization effect, an empirical absorption correction (transmission factors range 0.85–1.00), secondary extinction correction (coefficient=2.30023×10^{-6}).

The structure was solved by direct methods[4] and expanded using Fourier techniques.[5] The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included, but not refined. The final cycle of full-matrix least-squares refinement was based on 2025 observed reflections ($I>3.0\sigma(I)$) and 334 variable parameters, and converged (largest parameter shift was 0.02 times its esd) with unweighted and weighted agreement factors of $R=\sum||F_o|-|F_c||/\sum F_o=0.036$ $\{R_w=[\sum\omega(|F_o|-|F_c|)^2/\sum w|F_o|^2]^{1/2}=0.048\}$.

All calculations were performed using the teXscn crystallographic software package of Molecular Structure Corporation.

The absolute configuration was established by measuring reflections of 51 Friedel pairs with $|F_{c_{hkl}}|-|F_{c_{-h-k-l}}|/[\sigma^2(F_{o_{hkl}})+\sigma^2(F_{o_{-h-k-l}})]^{1/2}>4.0$. In all cases, the signs of $\Delta F_o=(|F_{o_{hkl}}|-|F_{o_{-h-k-l}}|)$ were in accord with those of $\Delta F_c=(|F_{c_{hkl}}|-|F_{c_{-h-k-l}}|)$.[6]

3.4. Photochemical conversion of 3 into 1 and 2

A solution of **3** (48 mg) in *n*-hexane (10 mL) was irradiated with a low-pressure mercury lamp for 12 h. After removal of the solvent by evaporation, the residue was separated by repeated silica gel chromatography [benzene–EtOAc (10:1)] and HPLC on Capcel-PAK ODS [MeCN–2%AcOH (9:1)] to yield **1** (7 mg) and **2** (10 mg).

3.5. Anti-HIV assay

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 dimethyl sulfoxide (DMSO). The following are the final drug concentrations routinely used for screening: 100, 20, 4 and 0.8 μg/mL. For active agents, additional dilutions were prepared for subsequent testing so that accurate EC_{50} values (see definition below) could be achieved.

As the test samples were being prepared, an aliquot of H9 cells was infected with HIV-1 (IIIB isolate) while another was mock-infected with complete medium. The mock-infected aliquot was used for toxicity determinations (IC_{50}, see definition below). The stock virus used for these studies typically had a $TCID_{50}$ value of 10^4 Infectious Units (IU)/mL. The appropriate amount of virus for a multiplicity of infection (m.o.i.) between 0.1 and 0.01 IU/cell was added to the first aliquot of cells. The other aliquot of cells received only culture medium and was then incubated under identical conditions to the HIV-infected 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-control drug). In addition, AZT was also assayed during each experiment as a positive-control drug. The plates were incubated at 37°C and 5% CO_2 for 4 d. Cell-free supernatants were collected on day 4 and tested by an in-house p24 antigen ELISA assay. p24 antigen is a core protein of HIV and, therefore, is an indirect measure of virus present in the supernatants. Toxicity was determined by performing cell counts by a Coulter counter on the mock-infected cells, which had either received culture medium (no toxicity) or test sample or AZT. If a test sample had suppressive capability and was not toxic, its effects are reported in the following terms: IC_{50}, the concentration of test sample which was toxic to 50% of the mock-infected cells; EC_{50}, the concentration of the test sample which was able to suppress HIV replication by 50%; and TI, the ratio of IC_{50} to EC_{50}.

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