New 3-*O*-Acyl Betulinic Acids from *Strychnos vanprukii* Craib

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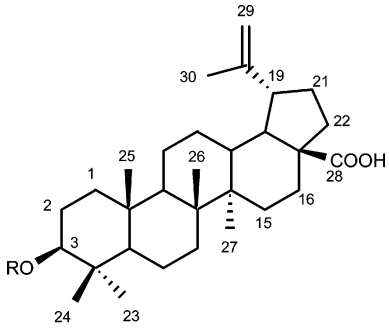
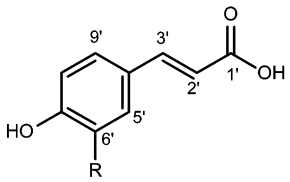
Received October 23, 2003

Three new betulinic acid derivatives, 3*β*-*O*-*trans*-feruloylbetulinic acid (**1**), 3*β*-*O*-*cis*-feruloylbetulinic acid (**2**), and 3*β*-*O*-*cis*-coumaroylbetulinic acid (**4**), along with two known triterpenes, 3*β*-*O*-*trans*-coumaroylbetulinic acid (**3**) and ursolic acid (**6**) were isolated from the leaves and twigs of *Strychnos vanprukii* Craib. All isolates showed moderate anti-HIV activity with IC_{50} values ranging from 3 to 7 μg/mL (5 to 15 μM) in an indicator cell line for HIV infectivity. The structures of the new isolates were elucidated by spectroscopic techniques including 1D and 2D NMR spectroscopy. In addition, the structure of **1** was confirmed by X-ray crystallography.

Our International Cooperative Biodiversity Group (ICBG) project was established in collaboration with institutions in the United States, Vietnam, and Laos under the auspices of the Fogarty International Center, NIH.[1] As part of the antiviral drug discovery program initiated under this ICBG, *Strychnos vanprukii* Craib (Loganiaceae) collected at the Cuc Phuong National Park in Vietnam was found to exhibit anti-HIV activity. Bioassay-guided fractionation of the MeOH extract of the dried leaves and twigs of the plant led to the isolation of five triterpenes from the $CHCl_3$-soluble fraction. Four of the isolates were determined to be betulinic acid derivatives, three of which are new [3*β*-*O*-*trans*-feruloylbetulinic acid (**1**), 3*β*-*O*-*cis*-feruloylbetulinic acid (**2**), and 3*β*-*O*-*cis*-coumaroylbetulinic acid (**4**)]. The fourth triterpene ester was identified as 3*β*-*O*-*trans*-coumaroylbetulinic acid (**3**), and the free triterpene was established to be ursolic acid (**6**). The bioassay-directed isolation, structure elucidation, and biological activity of these compounds are reported herein.



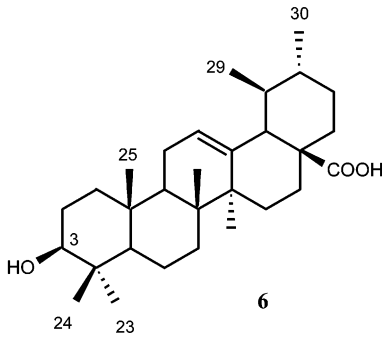
1: R = *trans*- feruloyl
2: R = *cis*- feruloyl
3: R = *trans*- coumaroyl
4: R = *cis*- coumaroyl
5: R = H



Ferulic acid: R = OMe
Coumaric acid: R = H

Results and Discussion

Compound **1** was obtained as colorless needles and was shown to have a molecular formula of $C_{40}H_{56}O_6$ according to high-resolution electrospray ionization mass spectrometry (HRESIMS). The IR spectrum of **1** exhibited absorption bands for hydroxyl (3531 and 3393 cm^{-1}), ester carbonyl (1694 cm^{-1}), conjugated olefin (1627 cm^{-1}), and aromatic functionalities (1514 cm^{-1}). The NMR spectra suggest a structure with 40 carbon atoms composed of a triterpene and a phenylpropane moiety. The acyl moiety was recognized as *trans*-feruloyl through its ^1H NMR signals at δ 7.42, 7.31, and 7.24 (two protons with *ortho*-coupling, J = 8 Hz, and one isolated proton), which is characteristic for a 1,2,4-trisubstituted benzene ring, coupled with signals at δ 6.77 (d, J = 15.8 Hz) and 8.05 (d, J = 15.8 Hz) for a *trans*-oriented double bond conjugated. The attachment of the methoxy group to C-6′ was evident by



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10.1021/np030469i CCC: $27.50
Published on Web 05/26/2004

Table 1. ^{1}H NMR Data of Compounds **1**−**4** (300 MHz, pyridine-d_5, J in Hz)

H	1	2	3	4
H-1a	1.60	1.58	1.62	1.60
H-1b		0.94	1.00	0.96
H-2a	1.85	1.80	1.83	1.82
H-2b		1.72		1.71
H-3	4.92, dd (4.5, 11.3)	4.83, m	4.89, dd (4.8, 11.8)	4.82
H-5	0.83	0.82	0.83	0.82
H-6a	1.44	1.45	1.45	1.44
H-6b		1.35	1.33	1.35
H_2−7	1.4	1.47	1.38	1.35
H-9	1.37	1.33	1.37	1.32
H-11a	1.38	1.35	1.37	1.37
H-11b		1.19	1.20	1.19
H-12a	1.9	1.94	1.93	1.92
H-12b	1.2	1.20		
H-13	2.73	2.73	2.73	2.72
H-15a	1.85	1.88	1.85	1.82
H-15b	1.25	1.29	1.26	1.26
H-16a	2.65	2.64	2.65	2.62
H-16b	1.60		1.58	1.59
H-18	1.8	1.80	1.78	1.76
H-19	3.54	3.54	3.53	3.52
H-21a	2.25	2.25	2.25	2.25
H-21b	1.60		1.55	1.55
H_2−22	2.25	2.25	2.26	2.25
Me-23	0.97, s	0.94, s	0.97, s	0.93, s
Me-24	0.95, s	0.90, s	0.97, s	0.88, s
Me-25	0.80, s	0.77, s	0.81, s	0.77, s
Me-26	1.04, s	1.03, s	1.05, s	1.05, s
Me-27	1.10, s	1.09, s	1.10, s	1.10, s
H-29a	4.95, br s	4.95, br s	4.95, br s	4.95, br s
H-29b	4.78, br s	4.78, br s	4.78, br s	4.77, br s
Me-30	1.81, s	1.80, s	1.81, s	1.79, s
H-2′	6.77, d (15.8)	6.05, d (12.9)	6.70, d (15.9)	6.02, d (12.8)
H-3′	8.05, d (15.8)	7.01, d (13.0)	8.02, d (15.9)	6.98, d (12.9)
H-5′	7.42, br s	8.34, br s	7.65, d (8.5)	8.09, d (8.6)
H-6′			7.18, d (8.5)	7.18, d (8.6)
H-8′	7.24, d (8.0)	7.23, d (9.0)	7.18, d (8.5)	7.18, d (8.6)
H-9′	7.31, d (8.1)	7.52, d (9.0)	7.65, d (8.5)	8.09, d (8.6)
6′-OMe	3.80, s	3.90, s		

Table 2. ^{13}C NMR Data of Compounds **1**−**4** (75 MHz, pyridine-d_5)

C	1	2	3	4
C-1	38.62t	38.61t	38.64t	38.63t
C-2	24.32t	24.19t	24.31t	24.14t
C-3	80.52d	80.58d	80.56d	80.67d
C-4	38.27s	38.16s	38.15s	38.12s
C-5	55.70d	55.69d	55.72d	55.71d
C-6	18.48t	18.47t	18.50t	18.48t
C-7	34.62t	34.63t	34.65t	34.62t
C-8	41.07s	41.06s	41.09s	41.07s
C-9	50.71d	50.72d	50.74d	50.71d
C-10	37.33s	37.32s	37.35s	37.33s
C-11	21.18t	21.18t	21.20t	21.18t
C-12	26.02t	26.02t	26.04t	26.01t
C-13	38.55d	38.56d	38.58d	38.55d
C-14	42.85s	42.85s	42.87s	42.84s
C-15	30.25t	30.26t	30.24t	30.24t
C-16	32.85t	32.87t	32.87t	32.85t
C-17	56.61s	56.63s	56.63s	56.61s
C-18	49.73d	49.73d	49.76d	49.75d
C-19	47.78d	47.78d	47.78d	47.76d
C-20	151.29s	151.30s	151.29s	151.27s
C-21	31.20t	31.20t	31.22t	31.21t
C-22	37.59t	37.60t	37.59t	37.57t
C-23	28.15q	28.12q	28.16q	28.13q
C-24	16.96q	16.85q	16.95q	16.86q
C-25	16.31q	16.29q	16.31q	16.27q
C-26	16.36q	16.35q	16.37q	16.35q
C-27	14.89q	14.88q	14.90q	14.88q
C-28	178.84s	178.86s	178.84s	178.81s
C-29	110.01t	110.01t	109.98t	109.96t
C-30	19.46q	19.46q	19.47q	19.46q
C-1′	167.31s	166.78s	167.29s	166.65s
C-2′	115.96d	116.59d	115.85d	116.87d
C-3′	145.36d	144.53d	144.97d	143.80d
C-4′	126.62s	127.04s	126.24s	126.70s
C-5′	111.43d	115.24d	130.70d	133.64d
C-6′	149.02s	148.11s	116.87d	115.97d
C-7′	151.11s	150.35s	161.46s	160.55s
C-8′	116.85d	116.12d	116.87d	115.97d
C-9′	123.9d	126.89d	130.70d	133.64d
6′-OMe	55.88q	55.89q		

the HMBC correlation of δ_H 3.80 (OMe) and δ_C 149.02 (C-6′) as well as by the ROESY correlation between δ_H 3.80 and δ_H 7.42 (H-5′). Characteristic UV absorption at 327 nm and the fragments at *m*/*z* 616.4283 (loss of Me), 437.3713 (loss of the acyl moiety), and 175.0618 (loss of the triterpene moiety) confirmed this partial structure. After assigning signals to the feruloyl moiety, the remaining NMR signals of **1** indicated the presence of a pentacyclic triterpene bearing six quaternary methyl, one carboxylic acid (δ_C 178.84), one oxymethine (δ_H 4.92) linked to the feruloyl moiety, and one isopropenyl group attached to a five-membered ring, with δ_H 4.78, 4.95 assigned to the vinylic methylene, δ_H 1.81 for the attached methyl, and δ_H 3.54 for the allylic carbinyl proton. This pattern suggested a lupan skeleton. Comparison of the NMR data (Tables 1 and 2) with those published for betulinic acid (**5**),[2] coupled with connectivity studies of 2D NMR spectral data, consolidated the above findings and further defined **1** as 3β-*O*-*trans*-feruloylbetulinic acid. The 3β-configuration of the feruloyl moiety was deduced from the H-3 proton signal pattern at δ_H 4.92 (dd, J = 4.5, 11.3 Hz) and from the effect of the acyl substituent upon chemical shifts of 2-CH_2, 23-CH_3, and 24-CH_3 as compared to those of betulinic acid (**5**) (Tables 1 and 2). This was further confirmed by HMBC correlations between δ_H 4.92 and δ_C 16.96 (C-24), 28.15 (C-23), and 38.27 (C-4) as well as by the ROESY correlation between δ_H 4.92 and 0.83 (H-5) and 0.97 (H_3-23). As additional confirming evidence, compound **1** was subjected to alkaline hydrolysis to yield betulinic acid (**5**) and *trans*-ferulic acid, which proved to be identical with authentic samples with respect to physical and spectroscopic properties.

In addition, a colorless crystal of **1** was selected for X-ray analysis. The X-ray data confirmed the structure of **1** and further revealed that in the crystal structure there are four distinguishable rotamers, derived from two independent units (Figure 1). The first unit consists of the major rotamer A1 and the minor rotamer A2 and has a rotamer disorder ratio of 63.1%:36.9% around the C3′−C4′ single bond. The second unit consists of the major rotamer B1 and the minor rotamer B2 and has a rotamer disorder ratio of 50.9%: 49.1% around the C1′−C2′ single bond. Analysis of the torsional angles of these rotamers (Table 3) led to the conclusion that in the crystal the preferred conformation of **1** along the C(1′)−C(2′) single bond is the *s*-*trans* conformation, and along the C(3′)−C(4′) bond the skew conformation, with the bulky half of the benzene ring close to the olefinic double bond, is the more favored one.

Compound **2** was obtained as white powder and was shown by HRESIMS to have the same molecular formula, $C_{40}H_{56}O_6$, as well as the same MS fragments as **1**. The NMR spectral data of **2** closely resembled those of **1**, except for the signals assignable to the aromatic acyl moiety. Differences in the UV absorption with a hypsochromic shift of 2 nm, along with the appearance of two neighboring olefinic proton signals at δ_H 6.05 and 7.01 with a coupling constant of 12.9 Hz instead of 15.8 as in the case of **1**, led to the deduction that **2** was the *cis*-isomer of **1**. Analysis of

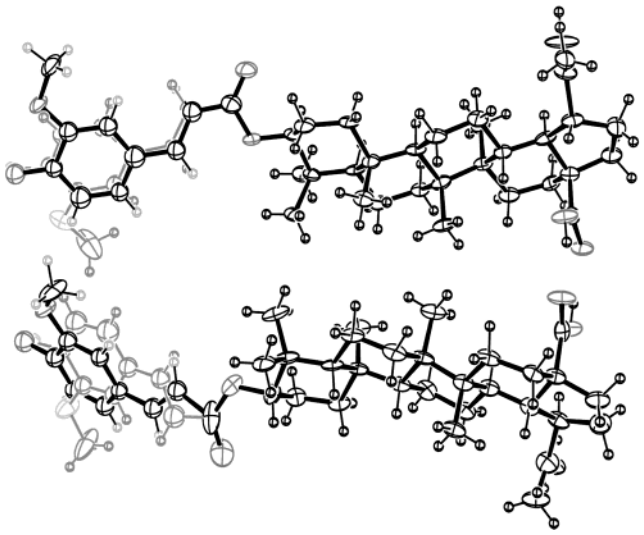

Figure 1. ORTEP drawing of the four rotamers of **1** showing two independent units in the crystal structure (top: rotamers A1 and A2; bottom: rotamers B1 and B2).

Table 3. Torsional Angles along the C1′−C2′ and the C3′−C4′ Single Bonds of the Four Rotamers of **1** in Its Crystal Structure

torsional angle	A1	A2	B1	B2
O3−C1′−C2′−C3′	−2.02°	−12.60°	167.44°	7.18°
O1′−C1′−C2′−C3′	177.36°	165.28°	−8.93°	−171.64°
C2′−C3′−C4′−C5′	−32.62°	145.73°	−21.99°	−20.35°
C2′−C3′−C4′−C9′	147.39°	−21.25°	158.02°	153.69°

the 1D and 2D NMR spectra finally defined **3** as 3*β*-*O*-*cis*-feruloylbetulinic acid.

Compound **3** was obtained as colorless needles and was evaluated to be the known 3*β*-*O*-*trans*-coumaroylbetulinic acid.[3] As ^{1}H NMR assignments for **3** are incomplete, and since ^{13}C NMR, melting point, and optical rotation data are not available, they are presented in the Experimental Section and in Tables 1 and 2.

Compound **4** was obtained as a white powder and was shown to have a molecular formula of $C_{39}H_{54}O_5$ according to HRESIMS, suggesting it to be an isomer of **3** and an analogue of **1** and **2**. A comparison of the NMR spectral data (Tables 1 and 2) of **4** with those of **1**, **2**, and **3** confirmed this observation and showed a *cis*/*trans* relationship between **4** and **3**. This led to the conclusion that **4** was 3*β*-*O*-*cis*-coumaroylbetulinic acid.

^{1}H NMR and reversed-phase HPLC have shown that under normal storage conditions at room temperature and protection from light the *trans*-isomers **1** and **3** are stable, while the *cis*-compounds **2** and **4** slowly underwent *cis*−*trans* isomerization. Samples of **2** and **4** were initially obtained in a pure state as confirmed by ^{1}H NMR and HPLC. Two and a half years of storage resulted in a mixture of 2 parts *cis*- and 1 part of the corresponding *trans*-isomer when the compounds were examined by ^{1}H NMR. It has also been observed that pyridine, which was used as solvent for NMR analyses, increased this rate of conversion. Compound **2** showed a conversion rate of 50% in approximately 3 months when stored in pyridine-d_5. However, the *trans*-isomers **1** and **3** remained pure with prolonged storage, regardless of whether they were stored dry or in pyridine-d_5.

Ursolic acid (**6**) was obtained as a white powder, and its identity was deduced by comparison of its spectroscopic data with literature information.[4]

During the initial screen, the total methanol extract of *Strychnos vanprukii* completely inhibited HIV-1 replication in the HOG.R5 reporter cell line at a concentration of 20 μg/mL with no observable toxicity (100% cell viability). Consequently, all major fractions and pure isolates from the plant were assayed for anti-HIV activity and cytotoxicity according to established protocols.[5] Compounds isolated as a result of this bioactivity-guided fractionation strategy and their activities are listed in Table 4. Anti-HIV IC_{50} values of 5−11 μM were obtained yielding SI (Selectivity Index) values of 2−3, consistent with that published for other 3-*O*-acyl derivatives of betulinic acid such as acetyl, benzoyl, crotonyl, sulfonyl, and succinyl.[6] The isolates also exhibited toxicity levels in the HOG.R5 cell line (CC_{50} = 13−22 μM) that are in agreement with literature values for similar compounds tested in H9 lymphocytes.

Table 4. Anti-HIV Activity and Cytotoxicity of Compounds **1**−**6**

	HOG.R5			CEM-SS/MTS	
compound	CC_{50}[a] (μM)	IC_{50}[b] (μM)	SI[c]	CC_{50} (μM)	% cytoprotection @ nontoxic conc
1	13.4	5.1	3	<1.6	toxic
2	22.2	11.1	2	<1.6	toxic
3	14.3	5.6	3	<1.7	toxic
4	15.9	8.0	2	<1.7	toxic
5[d]	16.7	3.1	5	5.9	2.0 μM[e] (SI = 3)
6	15.4	14.4	1	21.9	22% @ 2.7 μM
delavirdine mesylate	Nt40[f]	0.02		Nt40	0.06 μM
3TC	Nt40	1.20		Nt40	0.36 μM
calanolide A	Nt40	0.07		Nt40	0.31 μM
nelfinavir	Nt40	0.09		Nt40	0.03 μM

[a] CC_{50} = concentration mediating 50% cytotoxicity. [b] IC_{50} = concentration mediating 50% antiviral response. [c] SI = selectivity index. [d] Betulinic acid was isolated previously from *Vatica cinerea* King.[7] [e] ED_{50}, 50% cytoprotective activity against HIV-1_{IIIB} cytopathicity in vitro in μM (SI). [f] Nt40 = nontoxic @ 40 μg/mL.

Data of the parent compound, betulinic acid (**5**), was also included for comparison purposes. Betulinic acid (**5**) was reported to have an SI value of 9.3 (CC_{50} 13 μM/IC_{50} 1.4 μM) in H9 lymphocytes.[6] Previously, bioassay-guided fractionation led us to isolate betulinic acid from *Vatica cinerea* King.[7] The compound had an SI value of 5 (CC_{50} 16.6 μM/IC_{50} 3.1 μM) in the HOG.R5 assay. In addition, several standard anti-HIV agents were also tested in the HOG.R5 system for quality control purposes. The IC_{50} values of these positive control compounds were observed to be very similar to that reported in the literature using either H9 or CEM-SS cells (Table 4).

All pure compounds obtained were further tested in the CEM-SS/MTS cytoprotection assay utilizing HIV-1_{IIIB} (2 ng p24/mL and MOI = 0.4). Reagents required for this virus-induced cytopathic effect (CPE)-inhibition assay were provided by the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI). The procedure measures CEM-SS cell viability and is based on the reduction of the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS), by mitochondrial enzymes of viable host cells into MTS formazan.[8]

In all cases (except for ursolic acid, **6**), the compounds isolated were found to be more toxic to CEM-SS cells than to HOG.R5. Minimal (<20%) cytoprotection was observed for all compounds tested at nontoxic concentrations. The exceedingly toxic nature of the betulinic acid derivatives precluded the accurate assessment of their protective effects against virus-induced cytopathogenicity in CEM-SS cells. On the other hand, the antiproliferative effect of betulinic acid (**5**) on normal cells such as peripheral blood mononuclear cells (PBMCs) is reported to be mild.[9] There-

Table 5. Cytotoxic Activities of Compounds **1**−**5** in Cultured Cells[a,b]

compound	Lu1	Col-2	KB	LNCaP	HUVEC	Mel2
1	8.5	5.4	2.5	3.2	9.5	3.8
2	15.7	7.4	5.4	5.2	6.2	3.8
3	12.1	6.6	5.5	5.7	5.3	4.2
4	11.0	6.0	4.3	3.2	6.8	3.8
betulinic acid (**5**)	40.6	43.9	35.7	27.4	28.7	1.3

[a] Results are expressed as ED_{50} values (μM). [b] Key to cell lines used: Lu1 = human lung cancer; Col-2 = human colon cancer; KB = human oral epidermoid carcinoma; LNCaP = human hormone-dependent prostate cancer; HUVEC = human umbilical vein endothelial cell; Mel2 = human melanoma.

fore, in the interest of clinical relevance, the compounds are scheduled to be evaluated against HIV-1_{IIIB} in phytohemagglutinin (PHA)-stimulated PBMCs.

Numerous synthetic betulinic acid derivatives have been produced in an attempt to enhance its activity.[10] This has resulted in compounds that have shown effects in virion assembly or budding,[11] or fusion with the host cell membrane.[12,13] These findings attest to the importance of continued investigation of betulinic acid derivatives as new antiviral agents with novel mechanisms of action.

The toxicity of these betulinic acid derivatives to CEM-SS and HOG.R5 cells prompted us to extend the evaluation to include a panel of six cancer cell lines (Lu1, Col-2, KB, LNCaP, HUVEC, and Mel2). Assays were performed according to protocols established in our laboratories.[14,15] Cytotoxicity data for the six cancer cell lines tested are presented in Table 5. At an ED_{50} value of 1.3 μM, betulinic acid (**5**) demonstrated selective cytotoxicity against cultured human melanoma cells consistent with data reported in the literature.[15] Cytotoxicity obtained with the other cell lines was 20−30-fold less pronounced than that observed with Mel2. Unlike their parent **5**, compounds **1**−**4** were less active against Mel2, demonstrating ED_{50} values of approximately 3.8 μM (Table 5). In addition, no selectivity was apparent across the panel of cancer cell lines; only a 2−4-fold difference was observed between activity in Mel2 and the cytotoxic effect seen in the least susceptible cell line, Lu1.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer model 241 polarimeter. IR spectra were recorded on a Jasco FT/IR-410 spectrometer as a film on a KBr plate. 1D and 2D NMR spectra were recorded on a Bruker Avance DPX-300 MHz spectrometer. Chemical shifts (δ) are expressed in ppm with reference to internal TMS. All NMR experiments were obtained by using standard pulse sequences supplied by the vendor. Column chromatography was carried out on silica gel (230−400 mesh, Whatman). Thin-layer chromatography was performed on Whatman plates coated with 0.25 mm layers of silica gel 60. High-resolution electrospray ionization mass spectra (HRESIMS) and MS/MS spectra were recorded on a Q-ToF-2 hybrid quadrupole/time-of-flight mass spectrometer in the negative mode. X-ray crystallographic data were collected on a Bruker APEX CCD area detector at 100(1) K. Pure samples of betulinic acid and *trans*-ferulic acid were obtained from Indofine Chemical Company, Inc., Somerville, NJ, and Sigma-Aldrich, St. Louis, MO, respectively.

Plant Material. The initial collection of leaf and twig samples of *Strychnos vanprukii* Craib (Loganiaceae) was made at the Cuc Phuong National Park (CPNP), with voucher herbarium specimens represented by living collections in the park. A larger amount of the plant sample (5.5 kg) was subsequently re-collected at CPNP for complete isolation work. Voucher specimens of both collections are on deposit at both the herbaria of CPNP, Institute of Ecology and Biological Resources (National Center for Science and Technology, Hanoi), and the Field Museum of Natural History (Chicago, IL).

Anti-HIV Assay in HOG.R5 Cells. Anti-HIV and cytotoxicity assays were performed in parallel utilizing the green fluorescent protein (GFP)-based HOG·R5 reporter cell line that was constructed and developed specifically for quantitating HIV-1 infectivity. The system was validated and adapted as a moderately high-throughput procedure for screening natural products for anti-HIV activity in our laboratory.[5] Briefly, cultures in microtiter wells were infected with HIV-1_{IIIB} (2.5 ng/mL p24) in the presence of plant extracts, after which the fluorescence output was measured at the end of 4 days. Virus was omitted from parallel cultures treated with identical concentrations of plant extracts in order to monitor changes in cellular viability by a combination of microscopic and fluorometric measurements.

Inhibition of HIV-1-Induced Cytopathic Effect (CPE) in CEM-SS Cells. CellTiter 96 AQ_{ueous} One Solution Cell Proliferation kit (Promega Corporation, Madison, WI) is employed for the assessment of antiviral activity based on the protective effect of compounds or extracts on HIV-induced cytopathic effects and cell death.[8] The CellTiter 96 AQ_{ueous} One Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS(b)] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a convenient "one solution" assay format.

On the day preceding the assay, cells are split 1:2 to ensure they are in exponential growth phase at the time of infection. On the day of the experiment cells are washed, pelleted, and resuspended at (2−3) × 10^4 cells/mL in RPMI 1640 containing 10% heat-inactivated FBS, 4 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Extracts or compounds are diluted in complete RPMI1640. Test agents (50 μL) are added to cells (100 μL) in cell culture plates. Virus (50 μL) is then added to test wells and virus controls. The amount of virus to be added to each well will be the amount determined to give complete cell killing at 6 days post infection, i.e., maximum CPE is observed in untreated virus control cultures. Approximately 2 ng p24/mL virus (MOI ~0.4) is routinely used. Fresh medium is added to cell control wells, toxicity control wells, and test agent colorimetric control wells. After 6 days of incubation at 37 °C in a humidified 5% CO_2 incubator, 40 μL of MTS/PES solution is added to each well and the plate is reincubated for 4 h at 37 °C. The final culture concentrations of MTS and PES are 317 μg/mL and 50 μM, respectively. At the end of the incubation period, adhesive plate sealers are used to seal plates. Sealed plates are inverted several times to mix the soluble MTS formazan product. The plate is read spectrophotometrically at 490/650 nm with a PowerWave 200 plate reader (Bio-tek Instruments, Inc.) equipped with KC-junior software for data acquisition and analysis. The optical density (OD) value of each culture is a function of the amount of formazan produced which is proportional to the number of viable cells. Data computed include % cytoprotection (% CPE reduction), % cell viabilty, IC_{50}, and CC_{50}. Further testing is performed on active compounds using a five-concentration dose−response format.

Cytotoxicity Assay. Compounds were evaluated for cytotoxicity against a panel of human cancer cell lines that comprised Lu1, Col-2, LNCaP, HUVEC, Mel2, and KB cells. Assays involving Lu1, Col-2, LNCaP, and KB cell lines utilized established protocols,[14,15] while HUVEC were propagated and assayed in more specialized medium. HUVEC were purchased and grown in media and components supplied in the EGM-2 BulletKit (Cambrex Bio Science Walkersville, Inc., MD) with 2% fetal bovine serum (FBS). The HUVEC line constitutes a test system to identify samples with potential antiangiogenic activity.

Extraction and Isolation. The dried, milled leaves and twigs (5.5 kg) of *S. vanprukii* were extracted with MeOH and defatted with *n*-hexane. The methanolic solution was subsequently evaporated under vacuum to a syrup and partitioned

sequentially with petroleum ether, $CHCl_3$, and MeOH. The $CHCl_3$ extract (24.4 g) was dissolved in MeOH, filtered, and chromatographed over a silica gel column (1000 g), which was developed by gradient elution with $CHCl_3$ and increasing concentrations of MeOH to afford 68 fractions. The fractions were pooled into four well-resolved groups based on the presence of antiviral activity. Group 1 (fractions 13−14, 663 mg) was chromatographed over a Sephadex LH-20 column and eluted with MeOH to afford an active fraction (56.4 mg), which was then subjected to repeated column chromatography over silica gel and eluted with hexane/acetone (8:2) to yield compound **2** (8.2 mg). Similar workup of group 2 (fractions 15−18, 663 mg) led to compound **1** (39.8 mg). Group 3 (fractions 30−41, 966 mg), on treatment with $CHCl_3$, gave a precipitate, which was recrystallized from a mixture of $CHCl_3$/MeOH to afford ursolic acid (**6**) (19.1 mg). The $CHCl_3$-soluble portion was chromatographed as described above to afford compound **4** (30.4 mg). Group 4 (fractions 47−51, 2.486 g) was subjected to Sephadex LH-20 chromatography and eluted with MeOH to yield an active fraction (179.2 mg). This active fraction was sequentially chromatographed over silica gel using $CHCl_3$/MeOH (9:1) and then rechromatographed using hexane/acetone (7:3) to yield **3** (31.2 mg).

3*β*-*O*-*trans*-Feruloylbetulinic acid (1): colorless needles (hexane/acetone), mp 299−300 °C; $[\alpha]^{20}_D$ +27.0° (*c* 0.33, EtOH); UV (EtOH) λ_{max} (log ϵ) 236 (4.20), 302 (sh) (4.28), 327 (4.45) nm; IR ν_{max} (film) 3531, 3393, 2845, 1694, 1627, 1514, 1268, 1175, 756 cm^{-1}; ^1H (pyridine-d_5, 300 MHz) and ^{13}C NMR (pyridine-d_5, 75 MHz) data, see Tables 1 and 2; HRESIMS *m*/*z* 631.3996 $[M - H]^-$ (calcd for $C_{40}H_{55}O_6$, 631.3999); HRESIMS (MS/MS mode, 50 eV) *m*/*z* 616.4283, 437.3713, 175.0618.

3*β*-*O*-*cis*-Feruloylbetulinic acid (2): white powder (hexane/acetone), mp 155−160 °C; $[\alpha]^{20}_D$ +16.1° (*c* 0.37, EtOH); UV (EtOH) λ_{max} (log ϵ) 232 (3.91), 304 (sh) (3.97), 325 (4.06) nm; IR ν_{max} (film) 3533, 3414, 2942, 1695, 1595, 1514, 1173, 980, 758 cm^{-1}; ^1H (pyridine-d_5, 300 MHz) and ^{13}C NMR (pyridine-d_5, 75 MHz) data, see Tables 1 and 2; HRESIMS *m*/*z* 631.3975 $[M - H]^-$ (calcd for $C_{40}H_{55}O_6$, 631.3999); HRESIMS (MS/MS mode, 50 eV) *m*/*z* 616.4181, 437.3713, 175.0564.

3*β*-*O*-*trans*-Coumaroylbetulinic acid (3): colorless needles (hexane/acetone), mp 272−274 °C; $[\alpha]^{20}_D$ +32.4° (*c* 0.28, EtOH); UV (EtOH) λ_{max} (log ϵ) 231 (4.03), 303 (sh) (4.28), 314 (4.34) nm; IR ν_{max} (film) 3333, 2945, 1693, 1605, 1515, 1274, 1169, 831, 757; ^1H (pyridine-d_5, 300 MHz) and ^{13}C NMR (pyridine-d_5, 75 MHz) data, see Tables 1 and 2; HRESIMS *m*/*z* 601.3827 $[M - H]^-$ (calcd for $C_{39}H_{53}O_5$, 601.3893); HRESIMS (MS/MS mode, 50 eV) *m*/*z* 437.3473, 145.0328.

3*β*-*O*-*cis*-Coumaroylbetulinic acid (4): white powder (hexane/acetone), mp 180−182 °C; $[\alpha]^{20}_D$ +18.0° (*c* 0.65, EtOH); UV (EtOH) λ_{max} (log ϵ) 231 (3.93), 302 (sh) (4.18), 312 (4.22) nm; IR ν_{max} (film) 3352, 3041, 2817, 1693, 1605, 1514, 1168, 979, 758 cm^{-1}; ^1H (pyridine-d_5, 300 MHz) and ^{13}C NMR (pyridine-d_5, 75 MHz) data, see Tables 1 and 2; HRESIMS *m*/*z* 601.3901 $[M - H]^-$ (calcd for $C_{39}H_{53}O_5$, 601.3893); HRESIMS (MS/MS mode, 50 eV) *m*/*z* 437.3472, 145.0328.

Alkaline Hydrolysis of 1. A sample of **1** (9.1 mg) in 10% methanolic KOH (3 mL) and MeOH (7 mL) was refluxed for 3 h, the solvent evaporated under vacuum, and the residue neutralized and acidified with 1 N H_2SO_4 and extracted with EtOAc (10 mL × 3). The extract was evaporated to dryness and chromatographed on a silica gel column, eluted with $CHCl_3$/MeOH (95:5) to yield betulinic acid (**5**) (3.1 mg) and *trans*-ferulic acid (1.8 mg).

X- ray Crystal Structure of 3*β*-*O*-*trans*-Feruloylbetulinic Acid (1). A colorless crystal with dimensions 0.2 × 0.2 × 0.2 mm was selected for X-ray analysis. Structure analysis was performed using WinGX,[16] and the non-hydrogen atoms were found using the SIR-92 package.[17] Compound **1** crystallized in space group $P2_12_12_1$ (No. 19) with a = 11.332(2) Å, b = 14.578(3) Å, c = 41.500(7) Å, V = 6856(2) Å^3, Z = 8, D_{calc} = 1.224 g/cm^3, λ = 0.71073 Å, μ(Mo Kα) = 0.080 mm^{-1}, and $F(000)$ = 2744. A hemisphere of 33 429 reflections yielded 12 097 unique, averaged observations to 2θ = 56.08°; 5687 have intensities greater than 2σ. The structure was refined by full-matrix least-squares on F^2 with SHELXL[18] using all data to R = 0.112, $R(I > 2\sigma_I)$ = 0.054, GOF = 0.765. ORTEP was used to generate Figure 1.[19]

Acknowledgment. Sample collections, taxonomic identification of samples, bioassay-guided chemical isolation, and structure elucidation work presented in this paper were made under a grant administered by the Fogarty International Center, NIH (Grant 1 UO1-TW01015-01), as part of an International Cooperative Biodiversity Group (ICBG) program. Permits in the collection of samples of *S. vanprukii* and its export from Vietnam were granted by the Ministry of Agriculture and Rural Development, Hanoi, and from the Cuc Phuong National Park, Vietnam. The authors are grateful to the staff of the Cuc Phuong National Park for assistance with the re-collection of *S. vanprukii* samples used in the present study. The authors also wish to acknowledge Dr. D. Nicolic, Mass Spectrometry Lab, Department of Medicinal Chemistry, College of Pharmacy, UIC, for the acquisition of the MS data, and the AIDS Research and Reference Reagent Program, NIAID, NIH, for the supply of reagents critical to this study.

Supporting Information Available: X-ray crystallographic data for compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP030469I