Notes

Inhibition of the Dimerization and Active Site of HIV-1 Protease by Secondary Metabolites from the Vietnamese Mushroom *Ganoderma colossum*

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Received May 10, 2009

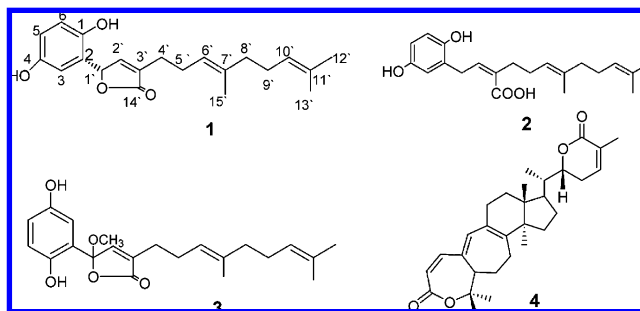
A new farnesyl hydroquinone, ganomycin I (**1**), was isolated along with ganomycin B (**2**) from the chloroform extract of the fruiting bodies of the Vietnamese mushroom *Ganoderma colossum*. These compounds inhibited HIV-1 protease with IC_{50} values of 7.5 and 1.0 μg/mL, respectively. Kinetic studies using Zhang−Poorman and Lineweaver plots revealed that compound **2** competitively inhibited the active site of the enzyme, whereas the tetracyclic triterpene schisanlactone A, previously isolated from the same fungus, was a dimerization inhibitor, with an IC_{50} value of 5.0 μg/mL. The previous findings were also confirmed by the virtual docking of both compounds with HIV-1 protease crystal structure.

Some naturally occurring phenolic compounds exhibit various antiviral activities. Hispolon and hispidin isolated from the fruiting bodies of the basidiomycete *Inonotus hispidus* (Bull.; Fr) exert antiviral activities against influenza viruses types A and B.[1] Chromones bearing hydroxy groups and their 2-phenyl-substituted derivatives (flavones) exert inhibitory activities against HIV-1 protease.[2] Curcumin inhibits HIV replication and has anti-HIV-1 and HIV-2 activities[3] through inhibiting an HIV-1 integrase capable of strand transfer and 3′-processing, with IC_{50} values of 40 and 30 μM, respectively.[4−6] In addition, curcumin and related compounds inhibit HIV-1 and HIV-2 proteases with IC_{50} values of 100 and 250 μM, respectively.[7] Biphenylcarboxylic acid compounds competitively inhibit HIV-1 protease.[8] Some phenolic diterpenes, such as carnosic acid, inhibit HIV-1 protease[9,10] with an IC_{50} of 0.24 μM.

Several phenolic compounds have been isolated from various species of *Ganoderma*. Ganomycins A and B isolated from the fruiting bodies of *Ganoderma pfeifferi* Bres. have antimicrobial activities.[11] However, other phenolics such as grifolin, grifolic acid, grifolic acid methyl ester, and grifolinol from *Albatrellus dispansus* (nonedible mushroom) are inactive against HIV-1, but have an anti-TNF-α effect.[12] Fornicins A−C obtained from the fruiting bodies of *G. fornicatum* exhibit moderate cytotoxic activity against Hep-2 cells.[13]

We previously investigated the triterpene constituents from the Vietnamese mushroom *G. colossum* and their inhibitory activities against HIV-1 protease.[14,15] During the isolation process a new farnesyl hydroquinone, ganomycin I (**1**), was isolated together with ganomycin B from the $CHCl_3$ extract of the fruiting bodies of the mushroom. The known compound was identified by comparing the spectroscopic data with reported values.[11] The anti-HIV-1 PR activity of these compounds and their mechanisms of enzyme inhibition were compared with those of the triterpenoid schisanlactone A (isolated from the same mushroom), which exerts appreciable inhibitory activity.[14]

Ganomycin I (**1**) was obtained as a yellow oil with a molecular ion peak at *m/z* 342 $[M]^+$ in ESIMS, and HRFABMS data coincided with the molecular formula $C_{21}H_{26}O_4$. The ^{1}H NMR and ^{13}C NMR spectra exhibited signals for three methyl groups, one oxymethine, four methylene groups, six sp^2 methines, six aromatic/olefinic quaternary carbons (two oxygenated), and an ester carbonyl carbon. These data suggested that a 1,2,4-trisubstituted dihydroxybenzene structure is connected to a 15-carbon side chain as in ganomycin B. Spectroscopic analyses showed that the structure of **1** was similar to that of fornicin B (**3**) isolated from *G. fornicatum*,[13] indicating a 1,4-dihydroxybenzene structure connected to a 15-carbon side chain. A comparison of the ^{1}H and ^{13}C NMR data with those of fornicin B isolated from *G. fornicatum* showed that C-1′ (δ 79.8) was oxygenated and with a similar furanone moiety. Analysis of the HMBC data revealed correlations between H-3 (δ 6.4, d, $J = 2.8$ Hz) and oxymethine carbon C-1′ (δ 79.8), indicating that the latter is attached to C-2 of the 1,4-dihydroxybenzene ring. In addition, correlations between H-1′ (δ 6.21, d, $J = 1.4$ Hz,) and an ester carbonyl carbon (δ 176.7, C-14′) as well as an olefinic methine at δ 151.4 (C-2′) suggested the existence of a γ-lactone moiety between C-1′ and C-14′. A comparison of this compound with fornicin B showed that their structures are identical except for the absence of a methoxy group at C-1′. Compound **1** structurally differed from ganomycin B, in which a free carboxylic acid group at C-14′ was replaced by an ester carbonyl carbon (δ 176.7, C-14′). Definition of the absolute configuration of compound **1** at C-1′ was done via the circular dichroic method developed for the determination of the absolute configuration of 5-substituted 2-(5*H*)-furanones.[16,17] The CD spectrum displayed Cotton effects due to the electronic transitions of the 2-(5*H*)-furanone moiety, which showed negative and positive Cotton effects at 212 and 240 corresponding to $\pi \rightarrow \pi$ and $n \rightarrow \pi$ transitions, respectively. These Cotton effects indicate *M* helicity for the five-membered α,β-unsaturated lactone moiety, and therefore the absolute configuration at C-1′ was assigned as *R*. Thus, the structure of ganomycin I (**1**) was 3-(4,8-dimethyl-3,7-nonadienyl)-(5*R*)-(2,5-dihydroxyphenyl)-2(5*H*)-furanone.



The anti-HIV protease activities of the farnesyl hydroquinones were investigated since they have not been described before. The

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10.1021/np900279u CCC: $40.75 © 2009 American Chemical Society and American Society of Pharmacognosy
Published on Web 10/08/2009

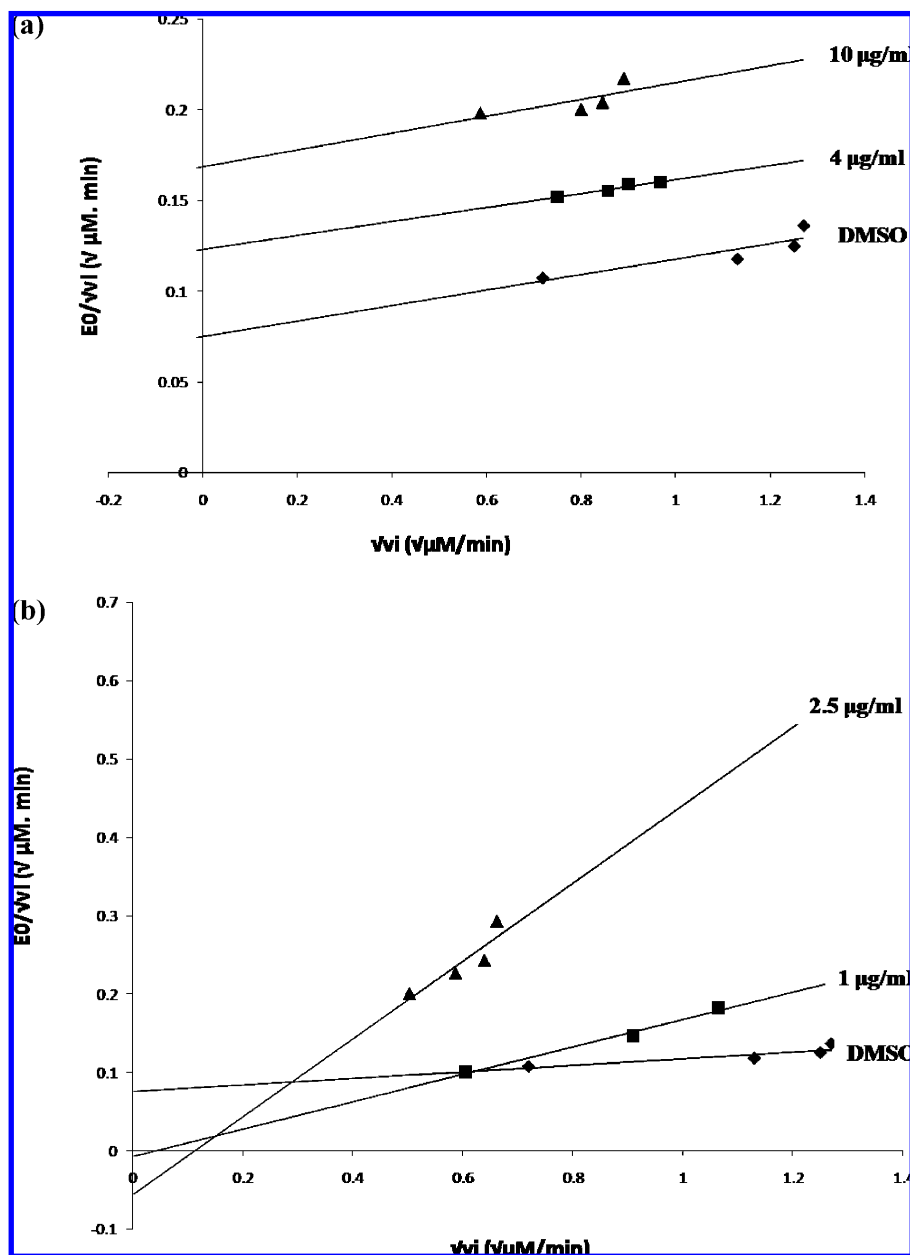

Figure 1. Zhang−Poorman plots for schisanlactone A (a) and ganomycin B (b).

farnesyl hydroquinones **1** and **2** significantly inhibited HIV-1 PR with IC_{50} values of 7.5 and 1.0 μg/mL, respectively. Previously schisanlactone A from the same mushroom was described as a highly potent inhibitor with an IC_{50} value of 5.0 μg/mL.[15]

The PR of HIV-1 is composed of two identical peptide units assembled by noncovalent interactions to form a composite active site.[18] This structural peculiarity provides the possibility of a unique inhibitory mechanism. A dimerization inhibitor of HIV-1 PR could dissociate the enzyme into inactive monomer units and thus inhibits its activity. Ursolic acid activity as a dimerization inhibitor of HIV-1 PR has been analyzed using Zhang−Poorman kinetics.[19,20] Furthermore, the ability of an oleanolic acid derivative to dissociate HIV-1 PR has been demonstrated by size-exclusion chromatography.[21]

The mechanisms of HIV-1 protease inhibition by ganomycin B and schisanlactone A were determined using Zhang−Poorman and Lineweaver−Burk plots. Zhang et al. established a method to distinguish dimerization inhibitors from active site inhibitors, especially competitive inhibitors.[19] The inhibition curves revealed a systematic variation of the intercept for schisanlactone A with straight lines having similar positive slopes to and different positive intercepts from those of noninhibitory compounds, indicating that dimerization was inhibited. The value of the inhibition constant (K_i^{dim}) with a dimerization inhibition mode was calculated from the intercepts in the presence and absence of an inhibitor. Schisanlactone A, with a K_i^{dim} value of 17.5 μM (IC_{50} = 5.0 μg/mL), altered the *y*-intercept value, but did not affect the slope (Figure 1a). This kinetic analysis indicated that schisanlactone A inhibited HIV-1 PR dimerization through disruption of the interfacial region. We also investigated the mechanism of inhibition by ganomycin B using Zhang−Poorman plots (Figure 1b). The curve of ganomycin B did not indicate specific inhibition, with intersecting lines of different slopes (indicating a large shift in the slope of the lines) and intercepts, indicating that it does not act through a dissociative mechanism. Since ganomycin B did not inhibit HIV-1 protease through a dissociative process, we further investigated its inhibitory mechanism using double Lineweaver−Burk plots of $1/\nu_i$ versus $1/s$[22−25] (Figure 2). Linear reciprocal plots showed that the respective lines intersected on the $1/\nu_i$ axis, indicating purely competitive inhibition with a K_i^{com} value of 4.0 μM.

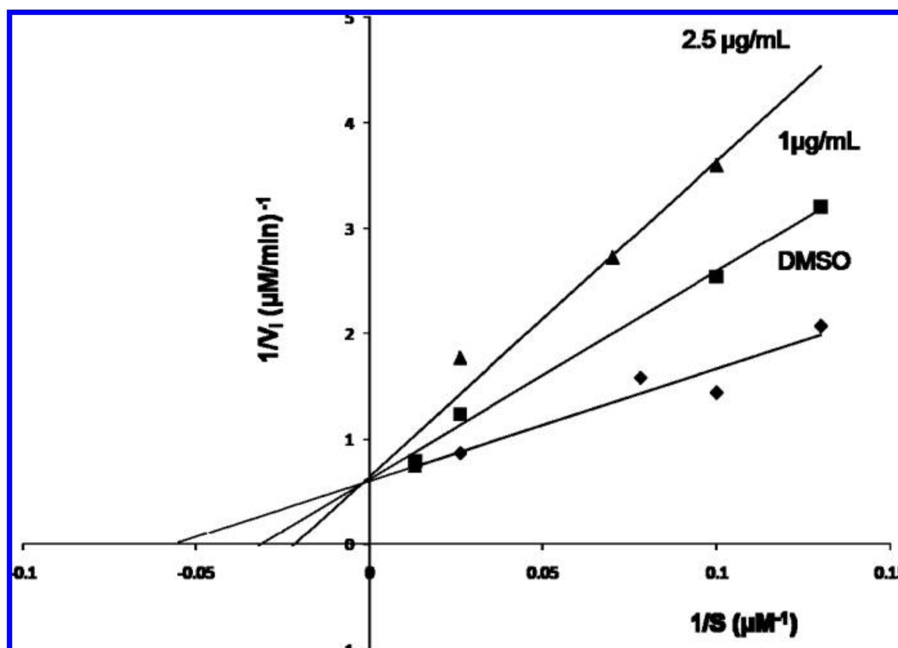

Figure 2. Lineweaver−Burk plot for ganomycin B.

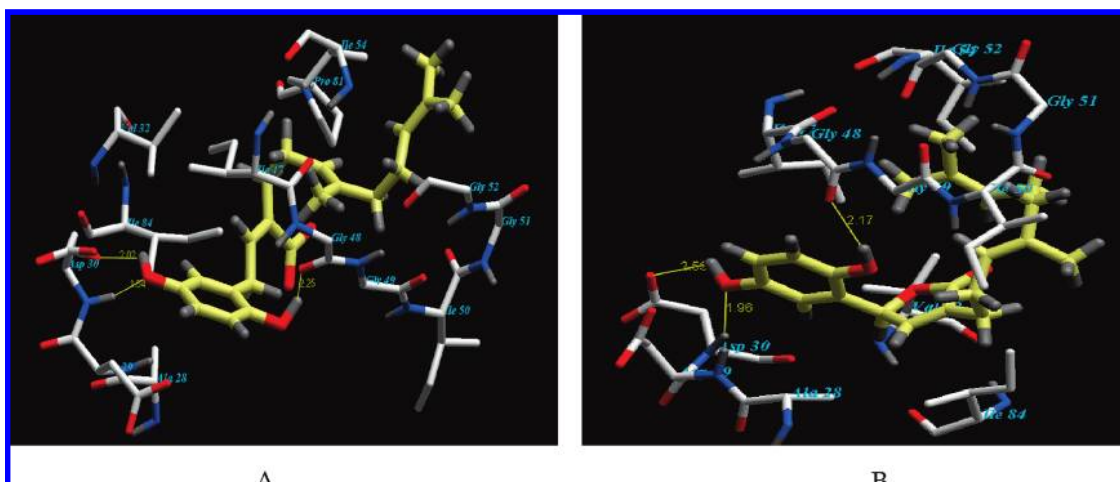

Figure 3. Docking of farnesyl hydroquinones in the active site of HIV-1 protease: (A) ganomycin B; (B) ganomycin I.

The docking results of the compounds kinetically analyzed with HIV-1 PR indicated that the more effective inhibitory activity of ganomycin B compared with ganomycin I (**1**) might be due to the stronger and shorter H-bond between the hydroxy group at C-4 and Asp30 over a distance of 1.64 Å than the H-bond in ganomycin I (**1**) (1.96 Å) in the active site region of HIV-1 protease. On the other hand, schisanlactone A forms a H-bond with the His69 residue of the HIV-1 PR monomer unit (Figure 4 A) by its carbonyl moiety at C-3, while a six-membered lactone ring does not bind and a triterpene core partially occupies the hydrophobic pocket of the interface region (cf. oleanolic acid, IC_{50} 1.0 μg/mL), which binds with Asn98 through its hydroxy group at C-3 with one H-bond (Figure 4 B) and binds through a carboxylic acid group with Pro1 with two H-bonds. Oleanolic acid also fits the hydrophobic pocket better than schisanlactone A.

It is noteworthy that this is the first report about the HIV-1 protease inhibition by farnesyl hydroquinones as well as the determination of their mechanism of inhibition. Moreover, *G. colossum* could inhibit the HIV-1 protease enzyme through a dual mechanism of action: inhibiting its dimerization by the triterpene content and its active site through the farnesyl hydroquinone content.

Experimental Section

General Experimental Procedures. The optical rotation was measured using a DIP-360 automatic polarimeter (Jasco Co., Tokyo, Japan), and UV spectra were recorded using a UV 2200/UV−vis recording spectrophotometer (Shimadzu Co., Kyoto, Japan). Infrared spectra were measured with a Fourier transform (FT)/JASCO IR-460 infrared spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded with Jeol JNA-LAA 400WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz) NMR spectrometer. We measured HRFABMS using a Jeol JMX-AX 505 HAD mass spectrometer at an ionization voltage of 70 eV, and ESIMS proceeded on an Esquire 3000 mass spectrometer (Bruker Daltanik GmbH, Bremen, Germany) system with an ESI ionization source. The MeOH extract of *G. colossum* was fractionated by column chromatography using silica gel (Kieselgel 60, 70−230 mesh, Merck, Tokyo, Japan). Fractions were then monitored by TLC on precoated silica gel 60 F_{254} and RP-18 F_{254S} plates (both 0.25 mm; Merck), and spots were detected under UV illumination and by heating after spraying with *p*-anisaldehyde/H_2SO_4.

Fungal Material. The fruiting bodies of *G. colossum* were cultivated by Drs. Co Duc Trong and Phan Thi Nhieu in Linh Chi Vi Na Co. Ltd. (Ho Chi Minh City, Vietnam) and collected in July 2002. Samples were authenticated by Drs. Co Duc Trong and Leif Ryvarden, and a

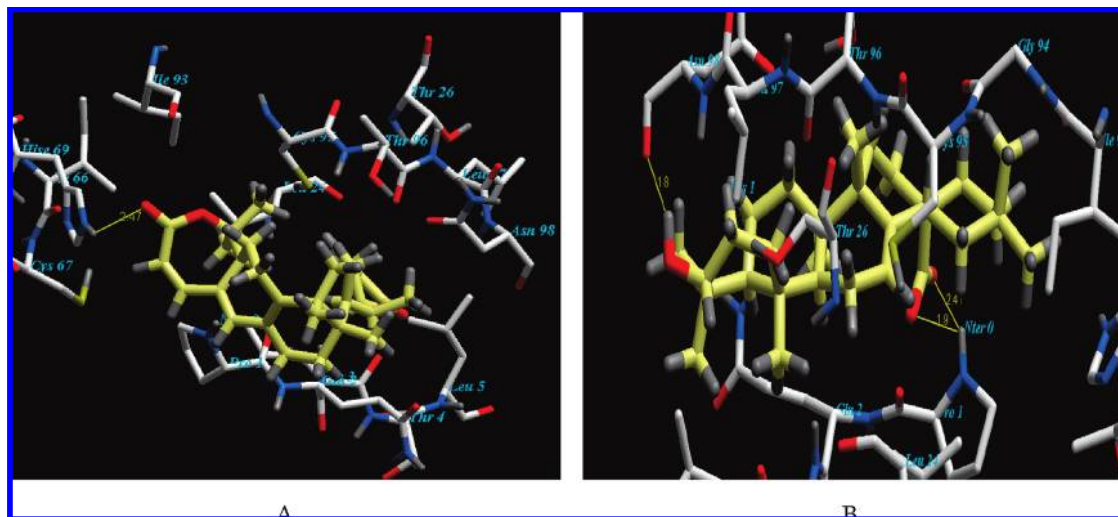

Figure 4. Docking of compounds in the dimer interface region of HIV-1 protease.**:** (A) schisanlactone A; (B) oleanolic acid.

voucher specimen (TMPW 25804) has been deposited in the Museum for Materia Medica at the Research Center for Ethnomedicine, Institute of Natural Medicine, University of Toyama, Toyama, Japan.

Enzymes and Chemicals. Rec HIV protease enzyme (expressed in *E. coli*) was purchased from Bachem A.G., Switzerland, and both HIV substrate III [His-Lys-Ala-Arg-Val-Leu-(*P*-NO_2-Phe)-Glu-Ala-Nle-Ser-NH_2] and pepstatin A were purchased from Wako Pure Chemical Co., Osaka, Japan.

Extraction and Isolation. The pulverized fruiting bodies of *G. colossum* (3.5 kg) extracted with $CHCl_3$ (8 L × 4) at room temperature for 5 days were combined, filtered, and concentrated to yield a dark brown residue of 582 g. The chloroform extract was dissolved in MeOH (1 L), defatted with *n*-hexane (2 L × 3), and then resolved by chromatography on silica gel (2 kg) with *n*-hexane−acetone mixtures (9:1 → 1:1). The homogeneity of the collected fractions (200 mL each) was monitored by TLC with solvent systems of increasing polarity (*n*-hexane−acetone, 9:1, 4:1, 7:3, and 1:1). The spots were visualized by heating the TLC plates after spraying with *p*-anisaldehyde−H_2SO_4. Fractions showing similar TLC profiles were combined to give eight pools (I−VIII). Fraction 7 was further separated by silica gel column chromatography starting with *n*-hexane−acetone (9:1 and 8.5:1.5) as the eluent to obtain fractions A−D. Fraction B (5 g) was further purified by elution with *n*-hexane−EtOAc 20% through a column containing silica gel. Fractions 10−35 were further purified on HPLC using 1% TFA in H_2O and MeOH as the mobile phase (60−100% MeOH), to yield a yellow oil (**1**) (21.9 mg), which turned green after spraying with *p*-anisaldehyde−H_2SO_4. Fractions 70−85 yielded a yellow oil (**2**) (24 mg) that turned dark violet after spraying with *p*-anisaldehyde−H_2SO_4.

Ganomycin I (1): yellow oil, $[\alpha]_D^{23}$ +36 (*c* 0.1, MeOH); UV λ_{max} (log ε) 212 (3.50), 297 (3.65) nm; CD $\Delta\varepsilon_{212}$ −0.099, $\Delta\varepsilon_{240}$ +0.028 (MeOH); IR (KBr) ν_{max} 3425, 2923, 1752, 1501, 1241,1136, 1049 cm^{-1}, HRFABMS 343.18748 (calcd for $C_{21}H_{26}O_4$, 343.18311), ^{1}HNMR (methanol-d_4, 400 MHz) 7.32 (1H, d, J = 1.4 Hz, H-2′), 6.66 (1H, d, J = 8.4 Hz, H-6), 6.58 (1H, dd, J = 2.8, 8.4 Hz, H-5), 6.46 (1H, d, J = 2.8 Hz, H-3), 6.21 (1H, d, J = 1.4 Hz, H-1′), 5.10 (1H, br t, H-10′), 5.03 (1H, br t, H-6′), 2.30 (2H, m, H-4′), 2.27 (2H, m, H-5′), 1.99 (2H, m, H-9′), 1.95 (2H, m, H-8′), 1.63 (3H, s, H-12′), 1.55 (6H, s, H-13′,H-15′); ^{13}CNMR (CD_3OD, 125 MHz) 176.7 (C-14′), 151.4 (C-4), 151.1 (C-21), 148.8 (C-1), 137.7 (C-7′), 132.9 (C-3′), 132.1 (C-11′), 125.3 (C-10′), 123.9 (C-6′), 123.4 (C-2), 117.14 (C-6),117.11 (C-3), 113.1 (C-5), 79.8 (C-1′), 40.7 (C-8′), 27.6 (C-9′), 26.8 (C-4′), 26.1 (C-5′), 25.8 (C-12′), 17.7 (C-13′), 16.2 (C-15′); positive ESIMS *m*/*z* 365 (M + Na), 342 (M), 325 (M − OH); HRFABMS 343.18748 (calcd 343.18311).

Assay of HIV-1 Protease Inhibitory Activity. HIV-protease assay buffer (10 μL; 50 mM NaOAc, pH 4.9) containing the substrate (0.1 mg/mL) was mixed with 2 μL of test compound in DMSO (100, 10, and 1 μg/mL); then 8 μL of rec HIV-protease (0.02 mg/mL) was added. The mixture was incubated for 30 min at 37 °C, and then the reaction was terminated by adding 3 μL of 10% TFA. The hydrolysates [*P*-NO_2-Phe-Glu-Ala-Nle-Ser-NH_2] and the remaining substrate were quantified by reversed-phase HPLC.[14] The HPLC conditions were as follows: column, TSK gel ODS-80Ts (21.5 × 300 mm, Tosoh Co., Tokyo, Japan); solvent, MeCN gradient (20−40%) in 0.1% TFA; flow rate, 1.0 mL/min; detector, UV 280 nm. The substrate and hydrolysate were eluted at 9.5 and 6.3 min, respectively. The HIV-protease inhibitory activity of a test compound was calculated as follows: % inhibition = $(A_{control} - A_{sample}) \times 100/A_{control}$, where A is the relative peak area of the hydrolysate. Pepstatin A, with an IC_{50} of 1 μg/mL, served as the positive control.

Kinetic Study. HIV PR inhibition was determined by monitoring spectrometric changes (280 nm) associated with cleavage of the chromogenic substrate [His-Lys-Ala-Arg-Val-Leu-(*p*-NO_2-Phe)-Glu-Ala-Nle-Ser-NH_2] by inhibitors in the presence or absence of the enzyme over time.

Zhang−Poorman Plots. Inhibition was kinetically analyzed in 0.1 M NaOAC buffer (pH 5.0) containing 1 mM EDTA at 37 °C and a constant substrate concentration of 38 μM. Enzyme and inhibitor concentrations ranged from 62 to 194 nM and from 4.0 to 10 μg/mL for schisanlactone A and from 1.0 to 2.5 μg/mL for ganomycin B, respectively. Plots of $[E]_0/\sqrt{\nu_i}$ versus $\sqrt{\nu_i}$ were constructed to determine the mechanisms of the selected compounds. For each enzyme concentration, the reaction was started by adding 10 μL of substrate followed by 2 μL of DMSO (with or without a test compound) and 8 μL of protease. We followed the reactions of all samples for at least 30 min at 37 °C, followed by 2 min intervals for 10 min and 5 min intervals for 30 min, and then the reaction was stopped by adding 3 μL of 10% trifluoroacetic acid to the mixture. By plotting a curve for the amount of products (10^{-6} M) against time (min), the initial rate ν_i was determined by linear fitting for the first 3 to 6 min. Each sample was assayed twice to determine the kinetic parameters of the enzyme. The assay was repeated in the presence of inhibitors following the same procedure.

Lineweaver−Burk Plots (double reciprocal plots). The enzyme concentration was kept constant at 133 nM in the same buffer (0.1 M NaOAc), while substrate concentrations and inhibitor concentrations varied from 7.6 to 76 μM and from 1.0 to 2.5 μg/mL, respectively. Plots of $1/\nu_i$ versus $1/S$ were constructed for ganomycin B in DMSO at concentrations of 2.5 and 1.0 μg/mL, respectively. The inhibition constant (K_i^{dim}) for Zhang−Poorman kinetics was calculated from the intercept of the Zhang−Poorman plots against inhibitor concentrations. The inhibition constant of ganomycin B (K_i^{com}) was calculated from the slope of the Lineweaver−Burk plots against the inhibitor concentrations.

Docking Study. A docking study determined the active sites of oleanolic acid, schisanlactone A, ganomycin B, and ganomycin I using Molsoft ICM version 3-8-4-C software, and the crystal structure of the inhibitor (acetyl pepstatin)/HIV-1 protease complex (5 HVP) was obtained from the protein data bank website (pdb).

Acknowledgment. The authors are grateful to Mr. Norio Ide (Reishi-Sogo Kenkyusho Co., Tokyo) for providing the fruiting bodies of *G. colossum* cultivated in Vietnam. We also offer special thanks to Dr. Nasser Ismail, Pharmaceutical Chemistry Department, Faculty of Pharmacy, Ain Shams University, Egypt, for providing the docking software and his help in doing the docking study.

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NP900279U