Asterlingulatosides C and D, Cytotoxic Triterpenoid Saponins from *Aster lingulatus*

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Received February 5, 1997

A further investigation of *Aster lingulatus* has led to the isolation of two additional novel triterpene saponins, asterlingulatoside C [3-*O*-*β*-D-glucopyranosyl-3*β*,16α-dihydroxyolean-12-en-28-oic acid 28-*O*-*β*-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside] (**1**) and asterlingulatoside D [3-*O*-*β*-D-glucopyranosyl-3*β*,16α-dihydroxyolean-12-en-28-oic acid 28-*O*-*β*-D-xylopyranosyl-(1→3)-*β*-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside] (**2**). Elucidation of the structures of **1** and **2** was mainly based on FABMS and 1D and 2D homonuclear and heteronuclear NMR techniques. Compounds **1** and **2** showed good inhibitory activity against DNA synthesis in human leukemia HL-60 cells with IC_{50} values of 8.8 and 6.1 μM, respectively.

Aster is a large genus of the family of Compositae, comprising more than 200 species distributed around the world. *Aster* species have been used in traditional systems of medicine for the treatment of fever, cold, tonsillitis, snake bite, and bee sting.[1] Previous phytochemical investigations on several plants of this genus have resulted in the isolation of a series of new oleanane-type triterpenoid glycosides.[2−20] More recently, we reported two new echinocystic acid saponins, asterlingulatosides A (**3**) and B (**4**), isolated from the 70% EtOH extract of the whole plant of *A. lingulatus* collected from Lijing County of Yunnan Province, China.[21] Further study of the more polar fraction led to two additional new analogs, asterlingulatosides C (**1**) and D (**2**). The present paper describes the elucidation of their structures using 2D-NMR techniques and chemical hydrolysis. In addition, their inhibitory effect on DNA synthesis in human leukemia HL-60 cells, which was used as a preliminary screen for identifying new potential antitumor natural products, was evaluated.[22]

Compounds **1** and **2** were isolated by repeated column chromatography on Diaion HP-20, Si gel, and Sephadex LH-20 from a *n*-BuOH-soluble fraction of a 70% EtOH extract of the whole plant of *A. lingulatus*. Further purification was conducted by Lobar RP-18 column chromatography. Both compounds responded positively to the Liebermann−Burchard and Molish color tests. A comparison of the NMR data of **1** and **2** with the reported values of **3** and **4** showed that they had very similar NMR profiles.[21] This observation revealed that **1**−**4** had a common aglycon, echinocystic acid, and they were 3,28-bisdemosides. Acidic hydrolysis of **1** and **2** with 2 N HCl−MeOH followed by methylation with CH_2N_2 yielded the same product identified as 3*β*,16α-dihydroxyolean-12-en-28-oic acid (echinocystic acid) methyl ester (**5**) spectroscopic and cochromatographic methods with an authentic sample. TLC and paper chromatography of the hydrolysis solution of **1** and **2** allowed the identification of the same sugar components, D-glucose, L-arabinose, D-xylose, and L-rhamnose. Analysis of the trimethylsilylated sugars by GC[23] confirmed the above results. Alkaline hydrolysis of **1** and **2** with 5% KOH−MeOH followed by esterification with CH_2N_2 gave a prosapogenin methyl ester (**6**), which was identified as 3-*O*-*β*-D-glucopyranosyl-3*β*,16α-dihydroxyolean-12-en-28-oic acid methyl ester by direct NMR comparison with an authentic sample.[21] Hence, the compounds **1**−**4** had a common prosapogenin structure and differed only in the structure of the 28-*O*-linked sugar units.

The negative-ion FABMS of **1** showed a deprotonated molecular ion peak $[M - H]^-$ at *m*/*z* 1043, and its positive-ion FABMS showed $[M + Na]^+$ at *m*/*z* 1067 and $[M + Li]^+$ at *m*/*z* 1051. Theses values were consistent with a $C_{52}H_{84}O_{21}$ molecular formula. In the ^{13}C-NMR spectrum of **1** four anomeric signals at δ 93.6, 101.0, 106.7, and 106.8 confirmed the four sugar residues. Of these, the glucose unit was attached to the C-3 of the aglycon, and the arabinose, rhamnose, and xylose units composed the 28-*O*-sugar chain. Interglycosidic linkages were established by 2D-NMR techniques. The assignments of the sugar proton resonances were achieved by a ^{1}H−^{1}H COSY spectrum. The anomeric proton resonances and some well-resolved resonances (i.e., Me-6 of rhamnose, methylene-5 of arabinose and xylose) were used as starting points for the analysis of the cross peaks of the COSY spectrum, and hence, the assignment of the ^{1}H subspectra of the individual sugar units was obtained. The ^{13}C-NMR data were assigned by the ^{13}C−^{1}H COSY spectrum and are listed in Table 1. Comparison of ^{13}C chemical shifts of the sugar units in the 28-*O*-sugar chain with those of the corresponding individual methyl glycosides[24] indicated the following glycosylation shifts: 8.5 ppm to highfield for the C-1 and 3 ppm to downfield for the C-2 of the arabinose unit, and 9.9 ppm to downfield for the C-4 of the rhamnose unit. Thus, the presence of an inner 2-substituted arabinose unit and a 4-substituted rhamnose unit was

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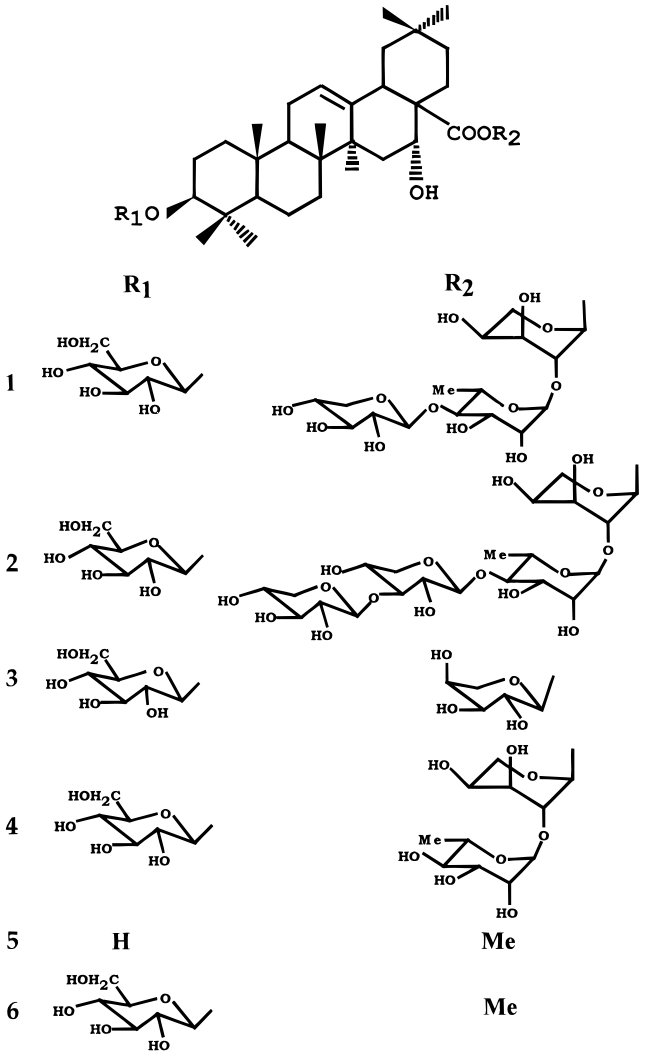
⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1997.

S0163-3864(97)00080-3 CCC: $14.00



revealed. In addition, the xylose unit could be determined to be in the terminal position because its ^{13}C-NMR data were identical to those of methyl β-D-xylopyranoside.[24] The HMBC spectrum of **1** showed cross peaks between the signals at δ 6.44 (H-1 of the arabinose) and 175.8 (C-28 of the aglycon), 6.33 (H-1 of the rhamnose) and 74.9 (C-2 of the arabinose), and 5.17 (H-1 of the xylose) and 83.5 (C-4 of the rhamnose). The above evidence allowed the establishment of a (1→28) linkage between the arabinose and the aglycon, a (1→2) linkage between the rhamnose and the arabinose, and a (1→4) linkage between the xylose and the rhamnose. The positive FABMS data of **1** showed the fragment ion peaks at *m/z* 913 [M − xylose + H]$^+$, 767 [M − xylose − rhamnose + H]$^+$ and 635 [M − xylose − rhamnose − arabinose + H]$^+$, which confirmed the link order of the sugar moieties. The anomeric configuration of the xylosyl group was determined as β based on the large coupling constant ($J_{1,2}$ = 7 Hz) in the ^{1}H-NMR spectrum. The anomeric configuration of the rhamnosyl unit could be identified from its ^{13}C-NMR chemical shifts, with C-3 and C-5 of methyl α-L-rhamnopyranoside at δ 72.5 and 69.4, and methyl β-L-rhamnopyranoside at δ 75.4 and 73.4, respectively.[24,25] The C-3 and C-5 signals of the rhamnosyl unit in **1** appeared at δ 72.8 and 68.6, indicating an α-configuration. The arabinopyranosyl unit was suggested to be in the α-configuration and ^{1}C$_4$ conformation based on the $J_{1,2}$ = 3 Hz and NMR data, which were identical to those of desacyllobatoside B.[26] Therefore, the structure of **1** was determined as 3-*O*-β-D-glucopyranosyl-3β,16α-dihydroxyolean-12-en-28-oic acid 28-*O*-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside, which is named asterlingulatoside C.

The negative-ion FABMS of **2** displayed a pseudomolecular ion peak at *m/z* 1175 ([M − H]$^-$). Its positive-ion FABMS showed [M + Na]$^+$ at *m/z* 1199 and [M + Li]$^+$ at *m/z* 1183. In combination with ^{13}C-NMR data the molecular formula of **2** was established as $C_{57}H_{92}O_{25}$. The molecular weight of **2** was 132 mass units higher than that of **1**, indicating an additional pentose unit, either arabinose or xylose, in **2**. The ^{13}C-NMR data of **2** exhibited five anomeric resonances at δ 93.5, 101.1, 106.1, 106.2, and 107.0 for the five sugar units and was consistent with FABMS data. A comparison of the ^{13}C-NMR data of the 28-*O*-sugar units in **2** with those in **1** revealed that the substitution positions of the arabinose and rhamnose units in the two compounds were the same. However, **2** exhibited five additional carbon signals as δ 106.2, 75.3, 78.4, 71.0, and 67.4 for a terminal xylose unit and a downfield shift of 9.1 ppm for the C-3 of the inner xylose unit. This showed that the terminal xylose moiety was attached at the C-3 position of the inner xylose unit. The interglycosidic linkages were confirmed by the HMBC experiment. As expected, correlations were observed between H-1 of the outer xylose unit (δ 5.19) and C-3 of the inner xylose unit (δ 87.2), H-1 of the inner xylose unit (δ 5.22) and C-4 of the rhamnose unit (δ 83.4), H-1 of the rhamnose (δ 6.40) and C-2 of the arabinose (δ 75.4), H-1 of the arabinose (δ 6.53) and C-28 of the aglycon (δ 176.0), and H-1 of the glucose (δ 4.95) and C-3 of the aglycon (δ 89.0). The fragment ions observed in the FABMS of **2** at *m/z* 1045 [M − xylose + H]$^+$, 913 [M − xylose − xylose + H]$^+$, 767 [M − xylose − xylose − rhamnose + H]$^+$, and 635 [M − xylose − xylose − rhamnose − arabinose + H]$^+$ were consistent with the sequence of sugars. The configurations of the carboyhdrate components in **2** were the same as those in **1**. Saponin **2** was accordingly identified as 3-*O*-β-D-glucopyranosyl-3β,16α-dihydroxyolean-12-en-28-oic acid 28-*O*-β-D-xylopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside and was named asterlingulatoside D.

Saponins **1** and **2** were evaluated for in vitro antitumor activity by means of their inhibitory effect on [^{3}H]-thymidine incorporation into the DNA of HL-60 cells. They inhibited the DNA synthesis in a dose-dependent manner. Comparison of IC$_{50}$ values (8.8, 6.1, 39.0, and 70.4 μM, respectively) for **1**−**4** suggested the following structure−activity relationships: (a) the presence of the rhamnosyl unit decreases activity and (b) the presence of the xylosyl unit increases the activity.

Experimental Section

General Experimental Procedures. Melting points were determined on a Kofler apparatus and are uncorrected; [α]$_D$s were measured at 28 °C on a JASCO DIP-181 polarimeter. IR spectra were obtained on a Mattson CYGNUS 100 Fourier-transform infrared spectrometer. FABMS spectra were recorded on a Finnigan MAT-90 instrument. Samples were dispersed in glycerol and bombarded with a beam of Xe atoms with an acceleration of 8 kV. ^{1}H-NMR and ^{13}C-NMR spectra were measured on a Nicolet NT-360 (360 MHz for δ_H, 90 MHz for δ_C) spectrometer. The HMBC spectra was recorded

Table 1. ^{1}H- and ^{13}C-NMR Data of Sugar Units of **1** and **2** in Pyridine-d_5[a]

	1		2	
position	δ_C (mult)	δ_H	δ_C (mult)	δ_H
3-*O*-glucose				
G-1	106.8 (d)	4.92 (d, 8)	107.0 (d)	4.95 (d, 8)
G-2	75.8 (d)	4.02	75.9 (d)	4.05
G-3	78.7 (d)	4.24	78.8 (d)	4.21
G-4	71.9 (d)	4.22	71.9 (d)	4.22
G-5	78.2 (d)	3.99	78.3 (d)	4.00
G-6	63.0 (t)	3.92, 4.56	62.8 (t)	3.96, 4.54
28-*O*-sugar arabinose				
A-1	93.4 (d)	6.44 (d, 3)	93.5 (d)	6.53 (d, 3)
A-2	74.9 (d)	4.51	75.4 (d)	4.55
A-3	70.2 (d)	4.57	69.5 (d)	4.59
A-4	66.3 (d)	4.41	65.9 (d)	4.40
A-5	63.3 (t)	4.29, 4.51	63.1 (t)	4.30, 4.55
rhamnose				
R-1	101.0 (d)	6.33 (br s)	101.1 (d)	6.40 (br s)
R-2	71.7 (d)	4.67	71.9 (d)	4.69
R-3	72.6 (d)	4.59	72.8 (d)	4.56
R-4	83.5 (d)	4.38	83.4 (d)	4.41
R-5	68.5 (d)	4.41	68.6 (d)	4.44
R-6	18.4 (q)	1.72 (d, 6.2)	18.5 (q)	1.73 (d, 6.0)
inner xylose				
X-1	106.7 (d)	5.17 (d, 7)	106.1 (d)	5.22 (d, 7)
X-2	76.0 (d)	4.03	75.0 (d)	4.03
X-3	78.5 (d)	4.13	87.2 (d)	4.04
X-4	71.0 (d)	4.17	69.1 (d)	4.08
X-5	67.4 (t)	3.46, 4.22	66.9 (t)	3.46, 4.23
outer xylose				
X′-1			106.2 (d)	5.19 (d, 7)
X′-2			75.3 (d)	4.05
X′-3			78.4 (d)	4.15
X′-4			71.0 (d)	4.16
X′-5			67.4 (t)	3.66, 4.28

[a] Spectra were recorded at room temperature at 360 MHz for δ_H and 90 MHz for δ_C. Chemical shifts are referenced on solvent peaks recorded in pyridine. *J* values are in Hz in parentheses.

on a General Electric GN Omega 500 MHz spectrometer operating at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR. ^{13}C-NMR multiplicity was determined using the DEPT experiment. GC analyses of sugars were carried out on a Varian 3400 gas chromatograph equipped with a flame ionization detector and a nonpolar fused capillary column (60 M × 0.25 mm (i.d.); 0.25-μm thickness; DB-1; J & W Scientific). The human leukemia cell line HL-60 was obtained from American Type Culture Collection (Rockville, MD). Radioactivity was measured using a Beckman LS 1701 scintillation counter.

Plant Material. The plant material of *A. lingulatus* was collected in August 1992, from Li-Jiang County, Yunnan Province, China. A voucher specimen was identified by Prof. Z. W. Lu and is deposited in the Herbarium of Kunming Institute of Botany, Academia Sinica, Kunming, China.

Extraction and Isolation. The dried, whole plants of *A. lingulatus* (7 kg) were extracted five times with 70% EtOH (each 1 week) at room temperature. The combined extracts were evaporated to dryness *in vacuo* to afford a residue (1 kg) that was suspended in H_2O and then extracted successively with petroleum ether, EtOAc, and H_2O-saturated *n*-BuOH to yield three corresponding fractions (145, 280, and 470 g, respectively). The *n*-BuOH extract was subjected to column chromatography over Diaion HP-20 eluted with H_2O and then MeOH. The MeOH eluate (425 g) was chromatographed on a column of Si gel (1.8 kg, 200−300 mesh) eluted with $CHCl_3$−MeOH−H_2O (8:1:0.1−1:1:0.1) gradient and separated into five crude fractions (fractions 1−5). Fraction 3 was repeatedly chromatographed by column chromatography over Si gel using $CHCl_3$−MeOH (4:1) as solvent and further purified over a Lichroprep RP-8 column eluted with MeOH−H_2O (6:4) to afford asterlingulatoside C (**1**) (112 mg, 0.0016% dry wt.). Fraction 2 was first chromatographed over Sephadex LH-20 and finally purified by Lichroprep RP-8 column chromatography to yield asterlingulatoside D (**2**) (66 mg, 0.00094% dry wt.).

Asterlingulatoside C (1): white amorphous powder; mp 192−194 °C; $[\alpha]_D$ −45.5° (pyridine, *c* 1.1); $C_{52}H_{84}O_{21}$; negative-ion FABMS *m/z*: 1043 $[M - H]^-$, 633 [M − xylose − rhamnose − arabinose − H]$^-$; positive FABMS *m/z* 1067 $[M + Na]^+$, 1051 $[M + Li]^+$; IR (KBr) ν_{max} 3430 (OH), 1732 (COOR), 1631 and 1567 (C=C), 1077 (C−O) cm^{-1}; ^{1}H NMR (pyridine-d_5, 360 MHz) δ aglycon unit 0.84, 0.98, 1.00, 1.07, 1.12, 1.27, and 1.80 (each 3H, each s, *tert*-Me × 7), 3.37 (1H, dd, J = 11, 4 Hz, H-3), 3.62 (1H, dd, J = 12, 4 Hz, 18-H), 5.27 (1H, br s, H-16), 5.61 (1H, br s, H-12), for the sugar residues, see Table 1; ^{13}C NMR (pyridine-d_5, 90 MHz) δ aglycon unit 38.8 (t, C-1), 26.6 (t, C-2), 88.8 (d, C-3), 39.5 (s, C-4), 55.9 (d, C-5), 18.5 (t, C-6), 33.5 (t, C-7), 40.0 (s, C-8), 47.1 (d, C-9), 37.0 (s, C-10), 23.8 (t, C-11), 122.7 (d, C-12), 144.4 (s, C-13), 42.0 (s, C-14), 36.0 (t, C-15), 74.0 (d, C-16), 49.5 (s, C-17), 41.3 (d, C-18), 47.1 (t, C-19), 30.9 (s, C-20), 36.2 (t, C-21), 32.1 (t, C-22), 28.2 (q, C-23), 17.0 (q, C-24), 15.7 (q, C-25), 17.5 (q, C-26), 27.2 (q, C-27), 175.8 (s, C-28), 33.3 (q, C-29), 24.7 (q, C-30); for the sugar units, see Table 1.

Asterlingulatoside D (2): white amorphous powder; mp 196−198 °C; $[\alpha]_D$ −63.2° (pyridine, *c* 1.0); $C_{57}H_{92}O_{25}$; negative-ion FABMS *m/z*: 1175 $[M - H]^-$, 1043 {M − xylose − H]$^-$ 633 [M − xylose − xylose − rhamnose − arabinose − H]$^-$; positive FABMS *m/z* 1199 $[M + Na]^+$, 1177 $[M + Li]^+$; IR (KBr) ν_{max} 3425(OH), 1726 (COOR),

1632 (C=C), 1077 (C−O) cm^{-1}; ^1H NMR (pyridine-d_5, 360 MHz), aglycon δ 0.86, 1.01, 1.03, 1.09, 1.17, 1.31, and 1.84 (each 3H, each s, *tert*-Me × 7), 3.40 (1H, dd, J = 11, 4 Hz, H-3), 3.64 (1H, dd, J = 12, 4 Hz, 18-H), 5.29 (1H, br s, H-16), 5.62 (1H, br s, H-12); sugar residues, see Table 1; ^{13}C NMR (pyridine-d_5, 90 MHz), δ aglycon unit 38.9 (t, C-1), 26.7 (t, C-2), 89.0 (d, C-3), 39.6 (s, C-4), 56.0 (d, C-5), 18.7 (t, C-6), 33.5 (t, C-7), 40.1 (s, C-8), 47.2 (d, C-9), 37.1 (s, C-10), 23.9 (t, C-11), 122.9 (d, C-12), 144.5 (s, C-13), 42.2 (s, C-14), 36.1 (t, C-15), 74.2 (d, C-16), 49.6 (s, C-17), 41.4 (d, C-18), 47.2 (t, C-19), 31.1 (s, C-20), 36.2 (t, C-21), 32.3 (t, C-22), 28.4 (q, C-23), 17.2 (q, C-24), 15.8 (q, C-25), 17.7 (q, C-26), 27.3 (q, C-27), 176.0 (s, C-28), 33.4 (q, C-29), 24.9 (q, C-30); for the sugar units, see Table 1.

Alkaline Hydrolysis of 1 and 2. A solution of each compound (**1** or **2**, 10 mg) in 5% KOH−MeOH (3 mL) was heated at 100 °C for 4 h. The reaction mixtures were cooled to room temperature and neutralized with 2 M HCl. After removal of MeOH, the remaining mixtures were passed through a column of Diaion HP-20 eluting initially with H_2O and then MeOH. Ethereal CH_2N_2 was added to the MeOH eluents, the mixture was allowed to stand at room temperature overnight, and then evaporated. The residues were subjected to column chromatography over Si gel using $CHCl_3$−MeOH (10:1) as solvent to afford **6** (5 mg) as colorless needles from each parent compound, identified by direct comparison of MS and NMR data with an authentic sample.[21]

Acid Hydrolysis of 1 and 2. A solution of each compound (**1** or **2**, 15 mg) in 2 N HCl−MeOH (4 mL or 3 mL) was refluxed for 3 h. After cooling to room temperature, the reaction mixture was extracted with Et_2O. The Et_2O extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to dryness and treated with ethereal CH_2N_2, and then recrystallized from MeOH to yield the methyl ester of the aglycon (**5**). The aqueous layer was neutralized with Ag_2CO_3, and the precipitates were centrifuged. The supernatant was concentrated to give the residue. The residue was silylated with hexamethyldisilazane and chlorotrimethylsilane in pyridine and analyzed by GC. The standard sugars were treated in the same way, and retention times were compared with those of methanolysates of **1** and **2**. For each sample, 0.3 μL was injected with a split ratio of 100:1. The GC was run with the following conditions: injector temperature at 270 °C, detector temperature at 300 °C, column temperature programmed from 40 to 270 °C at 4 °C min^{-1}. Retention times were 16.72 min for L-arabinose, 17.05 min for L-rhamnose, 18.96 min for D-xylose, and 23.42 min for D-glucose.

Cell Culture and Assay of [^{3}H]Thymidine Incorporation into DNA of the Cultured Cells. HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and 1% penicillin−streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C incubator. To each 1 mL of HL-60 cells (5 × 10^5 cells/mL) suspended in RPMI-1640 medium without fetal calf serum and 3 μL of [^{3}H]thymidine (50 μ *Ci*/μmol) the test sample (5, 25, or 100 μM) was added, and then incubated for 2 h at 37 °C. The reaction was terminated by addition of 1 mL of cold phosphate buffer saline solution, and then rate of DNA synthesis was determined as described methods.[22] The percentage of incorporation is expressed relative to the control incubation and represents an average of triplicate experiments. Compounds 1 and 2 inhibited the rate of DNA synthesis by 39.0 and 44.8% at concentration of 5 μM, 70.4 and 79.1% at 25 μM, or 94.9 and 97.8 μM, respectively.

Acknowledgment. The authors thank Dr. M. T. Huang of the Cancer Research Laboratory at Rutgers, The State University of New Jersey, for the provision of bioassay test facility, and Prof. Zhenwei Lu of Kunming institute of Botany, Academia Sinica, for the botanical identification. We also acknowledge the Nuclear Magnetic Resonance and Mass Spectrometry Laboratories of the Research Center, University of Illinois at Chicago, for providing assistance and the spectroscopic instrument used in this study.

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NP970080T