Lu, Z. Y. et al. Cytotoxic polyphenols from the marine-derived fungus Penicillium expansum. J. Nat. Prod. 73, 911-914

ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · APRIL 2010

Impact Factor: 3.8 · DOI: 10.1021/np100059m · Source: PubMed

Cytotoxic Polyphenols from the Marine-Derived Fungus *Penicillium expansum*

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Received January 26, 2010

Two new polyphenols containing both phenolic bisabolane sesquiterpenoid and diphenyl ether units, expansols A (**1**) and B (**2**), and two new phenolic bisabolane sesquiterpenoids, (*S*)-(+)-11-dehydrosydonic acid (**3**) and (7*S*,11*S*)-(+)-12-acetoxysydonic acid (**4**), along with two known compounds, (*S*)-(+)-sydonic acid (**5**) and diorcinol (**6**), were isolated from the metabolites of the marine-derived fungus *Penicillium expansum* 091006 endogenous with the mangrove plant *Excoecaria agallocha*. On the basis of spectroscopic analysis, chemical transformation, and theoretical calculation, the structures of **1**−**4** were elucidated as (*S*)-(+)-2-[3-hydroxy-4-(2- methoxy-6-methylheptan-2-yl)benzyl]-5-(3-hydroxy-5-methylphenoxy)-3-methylphenol, *S*-(+)-2-[3-hydroxy-4-(2-hydroxy-6-methylheptan-2-yl)benzyl]-5-(3-hydroxy-5-methylphenoxy)-3-methylphenol, (*S*)-(+)-3-hydroxy-4-(2-hydroxy-6-methylhept-6-en-2-yl)benzoic acid, and 4-[(2*S*,6*S*)-7-acetoxy-2-hydroxy-6-methylheptan-2-yl]-3-hydroxybenzoic acid, respectively. Expansol A (**1**) exhibited moderate cytotoxicity against the HL-60 cell line with an IC_{50} value of 15.7 μM, and expansol B (**2**) inhibited the proliferation of A549 and HL-60 cells with IC_{50} values of 1.9 and 5.4 μM, respectively.

Mangrove plants[1] and endophytes[2] are two prolific sources of structurally new and bioactive compounds for drug discovery. In the course of our ongoing investigations of new and bioactive natural products from microorganisms isolated from unusual or specialized ecological niches,[3–6] we investigated the metabolites of 45 endogenous fungal strains isolated from the surface-sterilized roots of the mangrove plant *Excoecaria agallocha* (Euphorbiaceae) from Wenchang, Hainan, China, by integrated chemical and bioactive screening. Among these strains, an EtOAc extract of a fungal strain 091006, authenticated as *Penicillium expansum*, showed cytotoxicity against P388 cells at a concentration of 0.1 mg/mL. The chemical constituents of *E. agallocha* and *P. expansum* have been extensively investigated. Twenty five new diterpenoids[7–11] and no fewer than 50 different secondary metabolites, such as cytochalasins,[12] communesins,[13] and tetracyclic sesquiterpene lactones,[14] were identified. We now report two new polyphenols, expansols A (**1**) and B (**2**), containing both the phenolic bisabolane sesquiterpenoid and diphenyl ether units, from an EtOAc extract of *P. expansum* 091006. Two new phenolic bisabolane sesquiterpenoids, (*S*)-(+)-11-dehydrosydonic acid (**3**) and (7*S*,11*S*)-(+)-12-acetoxysydonic acid (**4**), along with the known (*S*)-(+)-sydonic acid (**5**)[15,16] and diorcinol (**6**),[17] were also isolated and identified. Phenolic bisabolane sesquiterpenoids have mostly been found in marine sponges and only rarely sourced from marine-derived fungi.[16,18] These are the first examples of polyphenols coupled to phenolic bisabolane sesquiterpenoid and diphenyl ether units. Expansols A (**1**) and B (**2**) exhibited moderate cytotoxicity against A549 and HL-60 cell lines with IC_{50} values from 1.9 to 15.7 μM.

Results and Discussion

Expansol A (**1**) gave an HRESIMS peak at *m/z* 501.2636 [M + Na]$^+$ (calcd 501.2617), corresponding to the molecular formula $C_{30}H_{38}O_5$. Its 1D (Table 1) and 2D NMR (Figure 1) spectra displayed signals for two structural moieties, a bisabolane sesquiterpenoid moiety (**I**) similar to sydonic acid (**5**)[15,16] and a diphenyl ether moiety (**II** or **II′**) similar to diorcinol.[17] The HMBC correlations between H-15 (δ 3.96) and C-16 (δ 120.9), C-17 (δ 156.0), and C-21 (δ 139.5) indicated that moiety **I** is directly connected to **II** or **II′** via a single bond between C-15 and C-16 (Figure 1). In order to decide which structure represented expansol A, the fully methylated product was prepared by treatment with $CH_3I-K_2CO_3$.[19] The structure of the product was identified as **1a** from the key HMBC correlations between *O*-methyl protons and C-17, between H-15 and C-16, and between C-17 and C-21. Thus, the structure of expansol A may be represented as **1**.

The molecular formula of expansol B (**2**) was determined to be $C_{29}H_{36}O_5$ on the basis of the pseudomolecular ion peak at *m/z* 487.2442 [M + Na]$^+$ (calcd 487.2460) in the HRESIMS, which was "CH_2O" less than **1**. Its 1D NMR spectra were similar to those of **1** except for the lack of an *O*-methyl group, an upfield shift for C-7, and downfield shifts for C-2, C-8, and 14-CH_3. These data indicated that compound **2** was the 7-*O*-demethyl derivative of **1**, which was also confirmed by similar 2D NMR spectra (Figures S10−12).

Compounds **3** and **4** showed molecular formulas of $C_{15}H_{20}O_4$ and $C_{17}H_{24}O_6$ on the basis of HRESIMS peaks at *m/z* 265.1443 [M + H]$^+$ (calcd 265.1440) and *m/z* 347.1475 [M + Na]$^+$ (calcd 347.1471), respectively. The similar 1D NMR spectra to those of **5** indicated that both **3** and **4** were the analogues of **5**.[15] The major differences were the displacement of a methyl group by a terminal double bond at $\delta_{H/C}$ 4.60 and 4.63/110.5 (CH_2) and 146.2 (qC) in **3** and the displacement of a methyl by an acetoxymethylene at δ_C 171.0 (qC), 20.7 (CH_3), and 69.5 (CH_2) in **4**, respectively. Upfield shifts for 13-CH_3 and 10-CH_2 were observed in both **3** and **4**,

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10.1021/np100059m
Published on Web 04/23/2010

Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1**−**4** and **1a** (TMS, δ ppm)

	1[a]		**1a**[b]		**2**[b]		**3**[a]		**4**[a]	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	156.0, qC		157.2, qC		155.8, qC		157.1, qC		157.1, qC	
2	125.4, qC		130.0, qC		127.5, qC		136.5, qC		136.5, qC	
3	127.3, CH	6.97, d (7.8)	127.9, CH	7.22, d (8.0)	126.2, CH	6.88, d (7.7)	127.6, CH	7.27, d (8.2)	127.6, CH	7.28, d (8.2)
4	119.4, CH	6.70, dd (1.4, 7.8)	119.8, CH	6.66, dd (1.5, 8.0)	119.4, CH	6.66, dd (1.4, 7.7)	121.0, CH	7.45, dd (1.7, 8.2)	121.0, CH	7.44, dd (1.9, 8.2)
5	142.2, qC		140.5, qC		140.8, qC		131.2, qC		131.2, qC	
6	116.4, CH	6.63, d (1.4)	111.8, CH	6.70, d (1.5)	117.0, CH	6.63, d (1.4)	118.6, CH	7.38, d (1.7)	118.6, CH	7.38, d (1.9)
7	82.5, qC		79.2, qC		78.8, qC		78.2, qC		78.2, qC	
8	39.8, CH_2	1.75, ddd (5.0, 9.6, 14.3) 1.82, ddd (5.5, 6.4, 14.3)	39.8, CH_2	1.78, ddd (5.3, 11.3, 14.2) 1.84, ddd (6.1, 6.4, 14.2)	42.8, CH_2	1.75, ddd (4.8, 11.7, 13.9) 1.86, ddd (4.6, 9.2, 13.9)	43.0, CH_2	1.82, ddd (4.2, 12.1, 13.7) 1.97, ddd (4.9, 9.9, 13.7)	43.7, CH_2	1.82, ddd (4.6, 11.9, 13.7) 2.01, ddd (5.0, 9.1, 13.7)
9	21.6, CH_2	1.18, 1.35, m	21.7, CH_2	1.10, 1.18, m	21.7, CH_2	1.28, m	22.5, CH_2	1.40, 1.52, m	21.9, CH_2	1.26, 1.47, m
10	39.1, CH_2	1.12, m	39.3, CH_2	1.06, m	39.0, CH_2	1.14, m	38.4, CH_2	1.96, 't' like (7.1, 8.2)	34.2, CH_2	1.15, 1.35, m
11	27.7, CH	1.48, m	27.8, CH	1.46, m	27.8, CH	1.49, m	146.2, qC		33.1, CH	1.71, m
12	22.0, CH_3	0.81, d (6.9)	22.6, CH_3	0.79, d (6.6)	22.5, CH_3	0.82, d (6.6)	110.5, CH_2	4.60, d (1.1); 4.63, brs	69.5, CH_2	3.76, dd (6.8, 10.6), 3.85, dd (6.0, 10.6)
13	22.1, CH_3	0.82, d (6.9)	22.6, CH_3	0.79, d (6.6)	22.5, CH_3	0.82, d (6.6)	22.2, CH_3	1.61, s	17.0, CH_3	0.84, d (6.9)
14	22.1, CH_3	1.56, s	22.8, CH_3	1.56, s	28.9, CH_3	1.60, s	29.2, CH_3	1.66, s	29.4, CH_3	1.65, s
15	30.6, CH_2	3.96, s	31.1, CH_2	3.99, s	31.0, CH_2	3.92, s	167.2, qC		167.2, qC	
16	120.9, qC		122.7, qC		120.4, qC					
17	156.0, qC		158.7, qC		154.8, qC					
18	103.6, CH	6.45, d (2.3)	100.4, CH	6.51, d (2.2)	104.5, CH	6.31, d (2.4)				
19	156.2, qC		155.7, qC		155.6, qC					
20	112.0, CH	6.36, d (2.3)	112.9, CH	6.44, d (2.2)	113.5, CH	6.45, d (2.4)				
21	139.5, qC		139.4, qC		139.8, qC					
22	19.3, CH_3	2.18, s	20.0, CH_3	2.20, s	20.1, CH_3	2.21, s				
1-OCH_3			55.2, CH_3	3.74, s						
7-OCH_3	49.6, CH_3	3.19, s	50.1, CH_3	3.16, s						
12-$OCOCH_3$									20.7, CH_3	1.95, s
12-$OCOCH_3$									171.0, qC	
17-OCH_3			55.3, CH_3	3.76, s						
1′	158.7, qC		158.5, qC		158.2, qC					
2′	103.1, CH	6.27, t (1.8)	101.8, CH	6.39, t (2.2)	103.2, CH	6.27, t (2.0)				
3′	158.5, qC		160.7, qC		156.5, qC					
4′	110.6, CH	6.32, brs	111.6, CH	6.43, brs	111.0, CH	6.39, brs				
5′	140.4, qC		140.7, qC		141.0, qC					
6′	111.0, CH	6.43, brs	109.4, CH	6.47, brs	112.0, CH	6.43, brs				
7′	21.8, CH_3	2.22, s	21.7, CH_3	2.30, s	21.5, CH_3	2.26, s				
3′-OCH_3			55.7, CH_3	3.77, s						

[a] Recorded in acetone-d_6 and obtained at 600 and 150 MHz for ^{1}H and ^{13}C NMR, respectively. [b] Recorded in $CDCl_3$ and obtained at 600 and 150 MHz for **2** and 500 and 125 MHz for **1a**, respectively.

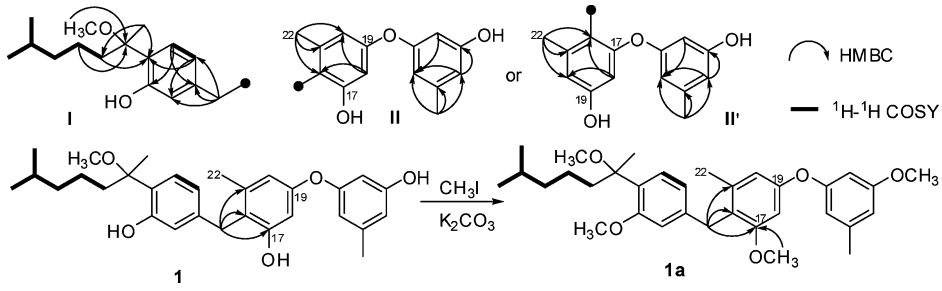

Figure 1. Key HMBC and $^1H-^1H$ COSY correlations of **1** and the methylated product **1a**.

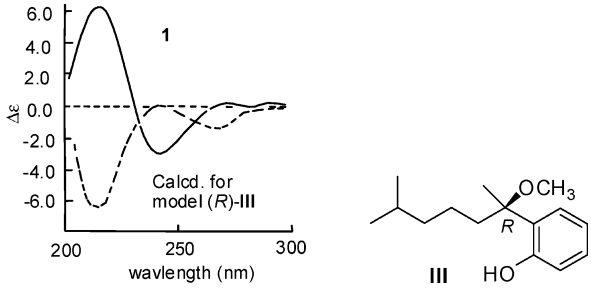

Figure 2. Measured and calculated CD spectra for **1** and the model compound (*R*)-**III**.

indicating that **3** and **4** were 11-dehydrogenated and 12-acetoxy derivatives of **5**, respectively. This deduction was further supported by the key HMBC correlations from H-12 (δ_H 3.76/3.85) to an acetyl carbonyl carbon (δ_C 171.0), C-10 (δ_C 34.2), C-11 (δ_C 33.1), and C-13 (δ_C 17.0) in **4** (Figures S19−21).

The absolute configuration of C-7 in compounds **1**−**4** and C-11 in **4** was determined by calculation of their specific rotations using the matrix model.[20] The results showed that the value of the determinant (det(*D*)) of *R*-**1**, *R*-**2**, and *R*-**3** were +9.48, +11.21, and +22.97, and the corresponding computed k_0 values were −0.46, −0.65, and −0.47, respectively. Since the k_0 value for a tertiary alcohol should be negative,[21] the real absolute configurations of **1**−**3** should be 7*S*. Compound **4** was predicted to have a (7*S*,11*S*)-configuration with a k_0 value of −1.00 instead of −4.2 for (7*S*,11*R*), which is much bigger than the average k_0 value of −0.53 of **1**−**3**. The calculations corresponded upon comparison of the specific rotation ($[\alpha]_D$) with those of the known *S*- and *R*-analogues, **1** (+4.4) and **2** (+7.3) vs (*R*)-(−)-6-methyl-2-(3-methylphenyl)heptan-2-ol (−4.8)[22] and (*S*)-(+)-sydonic acid (+2.7),[16] and **3** (+10.8) and **4** (+23) vs **5** (+2.7).[16] Furthermore, the CD spectrum of **1** was measured and calculated (Figure 2). The CD spectra of chiral compounds depend on the proximity of chromophores and stereogenic centers. Thus, the CD spectrum of **1** was calculated at the B3LYP/aug-cc-pVDZ//B3LYP/6-31G(d) level using the simplified model compound with *R*-configuration, (*R*)-**III**.[23] The calculated CD spectrum for the *R*-isomer is nearly a mirror image of the spectrum of **1** (Figure 2). Compound **1** was determined to have an *S*-configuration from the CD Cotton effect at 217 ($\Delta\varepsilon$ +6.2) nm that was opposite to that of the calculated spectrum of (*R*)-**III** at 210 ($\Delta\varepsilon$ −6.3) nm. Therefore, the structures of **1**−**4** were elucidated as (*S*)-(+)-2-(3-hydroxy-4-(2-methoxy-6-methylheptan-2-yl)benzyl)-5-(3-hydroxy-5-methylphenoxy)-3-methylphenol, (*S*)-(+)-2-(3-hydroxy-4-(2-hydroxy-6-methylheptan-2-yl)benzyl)-5-(3-hydroxy-5-methylphenoxy)-3-methylphenol, (*S*)-(+)-11-dehydrosydonic acid, and (7*S*,11*S*)-(+)-12-acetoxysydonic acid, respectively.

Compounds **1**−**4** were evaluated for their cytotoxicity against A549 and HL-60 cell lines using the SRB[24] and MTT[25] methods, respectively. Expansol A (**1**) exhibited moderate cytotoxicity against the HL-60 cell line with an IC_{50} value of 15.7 μM, and expansol B (**2**) inhibited the proliferation of A549 and HL-60 cells with IC_{50} values of 1.9 and 5.4 μM, respectively, while compounds **3** and **4** did not show cytotoxicity. There are no literature reports of the cytotoxicity of the two structure moieties, sydonol or sydonic acid and diorcinol. Few reports referred to the cytotoxicity of phenolic bisabolane-type sesquiterpenoids against tumor cells, such as (+)-curcuphenol,[26] (+)-curcudiol, parahigginine,[27] and parahigginols B−D.[28] Our experiments indicated that the condensation between phenolic bisabolane-type sesquiterpenoids and diphenyl ethers enhanced cytotoxicity.

Experimental Section

General Experimental Procedures. Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Beckman DU 640 spectrophotometer. CD spectra were obtained on a JASCO J-810 spectropolarimeter. IR spectra were taken on a Nicolet NEXUS 470 spectrophotometer in KBr disks. ^1H and ^{13}C NMR, DEPT, and 2D NMR spectra were recorded on a JEOL JNM-ECP 600 for compounds **1**−**4** and a Bruker Avance 500 spectrometer for compound **1a** using TMS as internal standard, and chemical shifts were recorded as δ values. ESIMS was measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. Semipreparative HPLC was performed using an ODS column [YMC-pack ODS-A, 10 × 250 mm, 5 μm, 4 mL/min]. TLC and column chromatography (CC) were performed on plates precoated with silica gel GF254 (10−40 μm) and over silica gel (200−300 mesh, Qingdao Marine Chemical Factory, Qingdao, China) and Sephadex LH-20 (Amersham Biosciences, Sweden), respectively. The seawater for the cultural medium of *P. expansum* was collected from the Yellow Sea near Qingdao. Me-I (AR) and K_2CO_3 (AR) were the products of Shanghai Lingfeng Chemical Reagent Co., Ltd.

Fungal Material. The endogenous fungal strain *Penicillium expansum* 091006 was isolated from the surface-sterilized roots of the mangrove plant *Excoecaria agallocha* growing in Wenchang, Hainan Province, China. It was identified according to its morphological characteristics and analyses of its 18S rRNA sequence (Supporting Information, GenBank DQ401105). A voucher specimen is deposited in our laboratory at −80 °C. The working strain was prepared on potato dextrose agar slants and stored at 4 °C.

Fermentation and Extraction. *P. expansum* 091006 was grown under static conditions at 30 °C for 28 days in one hundred 1000 mL conical flasks containing liquid medium (300 mL/flask) composed of glucose (10 g/L), maltose (20 g/L), mannitol (20 g/L), monosodium glutamate (10 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.3 g/L), corn steep liquor (1 g/L), yeast extract (3 g/L), and seawater after adjusting its pH to 7.0. The fermented whole broth (30 L) was filtered through cheesecloth to separate the filtrate from the mycelia. The filtrate was concentrated under reduced pressure to about a quarter of the original volume and then extracted three times with an equivalent volume of EtOAc to give an EtOAc solution, while the mycelia were extracted three times with acetone. The acetone solution was concentrated under reduced pressure to afford an aqueous solution. The aqueous solution was extracted three times with an equivalent volume of EtOAc to give another EtOAc solution. The EtOAc solutions were combined and concentrated under reduced pressure to give a crude extract (20 g).

Purification. The crude extract (20 g) was separated into five fractions on a Si gel column using a step gradient elution of $CHCl_3$ and MeOH (v/v 0:100−100:0). Fraction 2 (2.0 g) was rechromatographed on a Si gel column, eluted with petroleum ether/EtOAc (3:1), to provide four subfractions (fractions 2.1−2.4). Fraction 2.2 (0.5 g) was further purified on Sephadex LH-20 and semipreparative HPLC (65% MeOH) to give compound **6** (7.8 mg, t_R 10.3 min). Fraction 3 (6.5 g) was fractionated on a Si gel column, eluted with petroleum

ether/EtOAc (1:1), to provide five subfractions (fractions 3.1−3.5). Fraction 3.3 (1.8 g) was chromatographyed over Sephadex LH-20 eluting with MeOH to afford five subfractions (fractions 3.3.1−3.3.5). Compound **1** (5.0 mg, t_R 21.0 min) was obtained from fraction 3.3.4 (19 mg) by semipreparative HPLC (85% MeOH). Fraction 3.4 (0.7 g) was purified by repeated ODS CC and preparative HPLC (85% MeOH) to give compound **2** (2.4 mg, t_R 11.0 min). Fraction 4 (3.1 g) was further fractionationed on Sephadex LH-20 eluting with MeOH to give four subfractions (fractions 4.1−4.5). Fraction 4.2 (1.1 g) was purified by semipreparative HPLC (68% MeOH + 0.1% CF_3CO_2H) to give compound **5** (7.8 mg, t_R 13.8 min). Fraction 4.3 (0.7 g) was purified by semipreparative HPLC (75% MeOH + 0.1% CF_3CO_2H) to give compound **4** (7.4 mg, t_R 8.1 min). Fraction 4.4 (1.3 g) was purified by semipreparative HPLC (75% MeOH + 0.1% CF_3CO_2H) to give compound **3** (8.6 mg, t_R 6.7 min).

Expansol A (1): colorless oil; $[\alpha]^{23}_D$ +4.4 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 274 (3.55) nm; CD (MeOH), λ_{max} ($\Delta\varepsilon$) 217 (+6.2), 241 (−3.1) nm; IR (KBr) ν_{max} 3380, 2950, 2850, 1600, 1508, 1476, 1380, 1268, 1154, 1101, 980 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); HRESIMS *m*/*z* 501.2636 $[M + Na]^+$ (calcd 501.2617).

Expansol B (2): colorless oil; $[\alpha]^{23}_D$ +7.3 (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 272 (4.00) nm; IR (KBr) ν_{max} 3400, 2980, 2860, 1595, 1500, 1470, 1385, 1287, 1100, 980, 850 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); HRESIMS *m*/*z* 487.2442 $[M + Na]^+$ (calcd for $C_{29}H_{36}O_5Na$, 487.2406).

(*S*)-(+)-11-Dehydrosydonic acid (3): colorless oil; $[\alpha]^{23}_D$ +10.8 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 207 (3.95), 236 (2.75), 291 (1.45) nm; IR (KBr) ν_{max} 3340, 2975, 2860, 1695, 1595, 1500, 1460, 1380, 1160, 1015, 985 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); HRESIMS *m*/*z* 265.1440 $[M + H]^+$ (calcd for $C_{15}H_{21}O_4$, 265.1440).

(7*S*,11*S*)-(+)-12-Acetoxysydonic acid (4): colorless oil; $[\alpha]^{23}_D$ +23.0 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.30), 236 (3.21), 291 (1.95) nm; IR (KBr) ν_{max} 3330, 2950, 2860, 1740, 1700, 1600, 1505, 1386, 1150, 990 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); HRESIMS *m*/*z* 347.1471 $[M + Na]^+$ (calcd for $C_{17}H_{24}O_6Na$, 347.1471).

Methylation of 1. Expansol A (**1**) (4.7 mg, 10 μmol) was dissolved in 10 mL of acetone, and 6.2 mL (100 μmol) of CH_3I and 13.8 mg of K_2CO_3 (100 μmol) were added. The reaction mixture was refluxed for 3 h under Ar until the expansol A was consumed. Filtration of the reaction mixture followed by evaporation under vacuum and purification of the residue by chromatography on Si gel with $CHCl_3$/MeOH (9:1) gave the methylated product **1a** (5.0 mg, 96% yield).

1,17,3′-Tri-*O*-methylexpansol A (1a): colorless oil; $[\alpha]^{23}_D$ +7.0 (*c* 0.1, MeOH); ^1H and ^{13}C NMR (see Table 1); ESIMS *m*/*z* 543.4 $[M + Na]^+$.

Cytotoxicity Assays. Cytotoxicity of compounds **1**−**4** against A549 and HL-60 human tumor cells was determined by the SRB[24] and MTT[25] methods, respectively. Cells were plated in 96-well plates for 24 h before treatment and continuously exposed to different concentrations of compounds for 72 h. Inhibition rates of cell proliferation were measured compared to VP-16 (etoposide) as the positive control, with IC_{50} values of 0.63 and 0.042 μM against A549 and HL-60 cancer cells, respectively.

Acknowledgment. This work was supported by grants from National Basic Research Program of China (No. 2010CB833800), from the National Natural Science Foundation of China (Nos. 30670219 and 30770235), from the Project of Chinese National Programs for High Technology Research and Development (2007AA09Z447), from the Program for Social Profit of China Ministry of Science and Technology (2004DIB3J072), and from the Key Laboratory of Marine Drugs of Ministry of Education (No. KLMD-OUC-200607). The cytotoxicity assays were performed by Prof. M. Geng's group, Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Note Added after ASAP Publication: This paper was published on the Web on April 23, 2010, with three errors in the Purification section. The corrected version was reposted on May 7, 2010.

Supporting Information Available: The NMR spectra of **1**−**4** and the methylated product **1a**, the 18S rRNA sequence data of *Penicillium expansum* 091006, and the bioassay protocols used. These materials are available free of charge via the Internet at http://pubs.acs.org.

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NP100059M