Alkaloids from the Leaves of *Daphniphyllum subverticillatum*

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Four new alkaloids, 17-hydroxydaphnigraciline (**1**), subdaphnidine A (**2**), daphnezomine L methyl ester (**3**), and 11-hydroxycodaphniphylline (**4**), along with 24 known analogues, were isolated from the leaves of *Daphniphyllum subverticillatum*. The structures of **1**−**4** were elucidated on the basis of spectroscopic methods and from chemical evidence. Daphnilongeridine (**5**) showed cytotoxicity against several tumor cell lines at IC_{50} values in the range 2.4−9.7 μM and against the HMEC human microvascular endothelial cell line with an IC_{50} of 2.7 μM.

The genus *Daphniphyllum* (Daphniphyllaceae) is well known for biosynthesizing structurally diverse and complex *Daphniphyllum* alkaloids.[1] Several *Daphniphyllum* alkaloids have been shown to be cytotoxic against tumor cell lines.[2] *D. subverticillatum* Merr. is native to southern mainland China.[3] A preliminary study on the stems of *D. subverticillatum* with a small amount of plant material led to the isolation of three alkaloids.[1e] In a continuing phytochemical study of this plant, the leaves of *D. subverticillatum* were examined, and four new *Daphniphyllum* alkaloids, 17-hydroxydaphnigraciline (**1**), subdaphnidine A (**2**), daphnezomine L methyl ester (**3**), and 11-hydroxycodaphniphylline (**4**), along with 24 known analogues, were isolated. Some of these isolates were evaluated for cytotoxic activities against four tumor cell lines and the HMEC (human microvascular endothelial cell) cell line. Of these, only daphnilongeridine (**5**) showed cytotoxic activity. We report herein the isolation and structure elucidation of these alkaloids and their cytotoxicities.

Results and Discussion

17-Hydroxydaphnigraciline (**1**) exhibited a molecular formula of $C_{23}H_{35}NO_5$, as determined by the HREIMS peak at *m/z* 405.2511 $[M]^+$ (calcd for $C_{23}H_{35}NO_5$, 405.2515). The IR absorption bands at 3296 and 1734 cm^{-1} revealed the presence of hydroxy and ester carbonyl groups, respectively. The NMR data of **1** (Table 1) displayed the characteristic features of a yuzurine-type *Daphniphyllum* alkaloid,[4] as judged from the presence of an oxygenated methylene (δ_C 64.3, δ_H 4.23, d, J = 12.4 Hz, and 3.74, d, J = 12.4 Hz, CH_2-21), an ethyl group (δ_C 36.8, δ_H 1.58, 2H, m, CH_2-18; δ_C 8.8, δ_H 0.92, 3H, t, J = 7.3 Hz, Me-20), an N-methyl (δ_C 47.5, δ_H 2.24, 3H, s, Me-N), and a quaternary hemiketal carbon (δ_C 97.8, C-2). Comparison of the NMR data of **1** with those of daphnigraciline[4] showed that compound **1** was derived from daphnigraciline by hydroxylating C-17, which was supported by the HMBC correlations (Figure 1) from H-17 to C-9, C-10, C-11, and C-15 and from H_2-11 and H_2-16 to C-17. The coupling constant of H-17 (δ_H 4.60, brd, J = 5.7 Hz) with H-16a and H-16b suggested that H-17 is β-oriented. This was confirmed from a ROESY experiment (Figure 1), in which a correlation between H-17 and H-11b (δ_H 2.17, m) was observed, with no correlations detected between H-17 and H-11a (δ_H 2.54, m) or between H-17 and H-15 (δ_H 3.74, m). Accordingly, the structure of compound **1** was determined as shown.

Subdaphnidine A (**2**) was found to possess a molecular formula of $C_{25}H_{33}NO_5$, as established by the molecular ion peak in the HREIMS at *m/z* 427.2365 $[M]^+$ (calcd 427.2359). The ^1H and ^{13}C NMR data (Table 1) of **2** closely resembled those of daphniyunnine A,[5] except for the presence of signals for an additional oxygenated methylene and an acetoxy group, and the absence of the C-21 methyl resonance as found in daphniyunnine A suggested that an acetoxy group is linked to C-21 in alkaloid **2**. This structural assignment was confirmed by the HMBC spectrum (Figure 2), in which the correlations from H_2-21 (δ_H 4.48, d, J = 11.6 Hz and 4.32, d, J = 11.6 Hz) to C-4 (δ_C 63.9), C-5 (δ_C 56.3), C-6 (δ_C 48.1), C-8 (δ_C 62.4), and the carbonyl of the acetyl group (δ_C 173.1) were observed. The structure and relative configuration of **2** was confirmed as shown using a combination of 2D-NMR techniques, including HSQC, HMBC, and ROESY (Figure 2).

Daphnezomine L methyl ester (**3**), a colorless oil, had a molecular formula of $C_{23}H_{35}NO_2$. A comparison of the ^1H and ^{13}C NMR (Table 1) data of **3** with those of daphnezomine L[6] revealed that these substances are structurally closely related congeners with the only difference being the presence of one additional methoxy group (δ_C 52.7, δ_H 3.64, s, 3H) in **3**, suggesting it to be the methyl ester of daphnezomine L. Hydrolysis of **3** with sodium hydroxide in MeOH afforded an alkaloid, for which the ESIMS and ^1H NMR data were in agreement with those of daphnezomine L. Thus, the structure of **3** was determined as shown.

11-Hydroxycodaphniphylline (**4**) gave a molecular formula, $C_{30}H_{47}NO_4$, as determined by HREIMS at *m/z* 485.3498 $[M]^+$ (calcd 485.3505). The ^1H and ^{13}C NMR data (Table 1) showed that alkaloid **4** is an analogue of daphnilongeranin D[7] and codaphniphylline.[8] As compared with codaphniphylline, the only structural difference was the presence of an additional hydroxy group at C-11 (δ_C 70.0) of **4**. This was assigned by the HMBC correlations (Figure 3) from H-11 (δ_H 3.94, brd, J = 6.9 Hz) to C-6 (δ_C 41.8), C-9 (δ_C 51.3), C-10 (δ_C 80.1), and C-17 (δ_C 37.4) and from H_2-12 (δ_H 2.46 and 1.76, each 1H, m) to C-11. A strong ROESY correlation (Figure 3) observed between H-11 and H-9 (δ_H 2.45, m) indicated OH-11 is in the α-configuration. Thus, the structure of **4** was therefore elucidated.

Twenty-four known *Daphniphyllum* alkaloids were identified on the basis of their ^1H and ^{13}C NMR and EIMS data, some of which were also confirmed by co-TLC with authentic samples. Of these, except for deoxycalyciphylline B[1e] and deoxyisocalyciphylline B,[1e] 22 compounds, including calycinine A,[9] oldhamiphylline A,[10] daphniyunnine A,[5] daphnilongeranin A,[7] the methyl ester of yuzurimic acid B,[11] daphnilongeranin D,[7] codaphniphylline,[8] daphnilongeridine (**5**),[12] oxodaphnigraciline,[4] caldaphnidine R,[13] daphnigraciline,[4] methyl homodaphniphyllate,[14] daphgraciline,[15] the dehydrate of daphnigraciline,[4b] secodaphniphylline,[14a,16] yuzurine,[4b,17] longistylumphylline A,[1g] daphnezomine M,[6] daphnezomine A,[18] paxiphylline D,[19] paxiphylline E,[19] and daphlongamine E,[20] were isolated from *D. subverticillatum* for the first time.

The cytotoxic activities of 14 of the isolates obtained, namely, compounds **1**−**5**, deoxycalyciphylline B, calycinine A, daphnilongeranin A, daphnilongeranin D, daphnigraciline, methyl homodaph-

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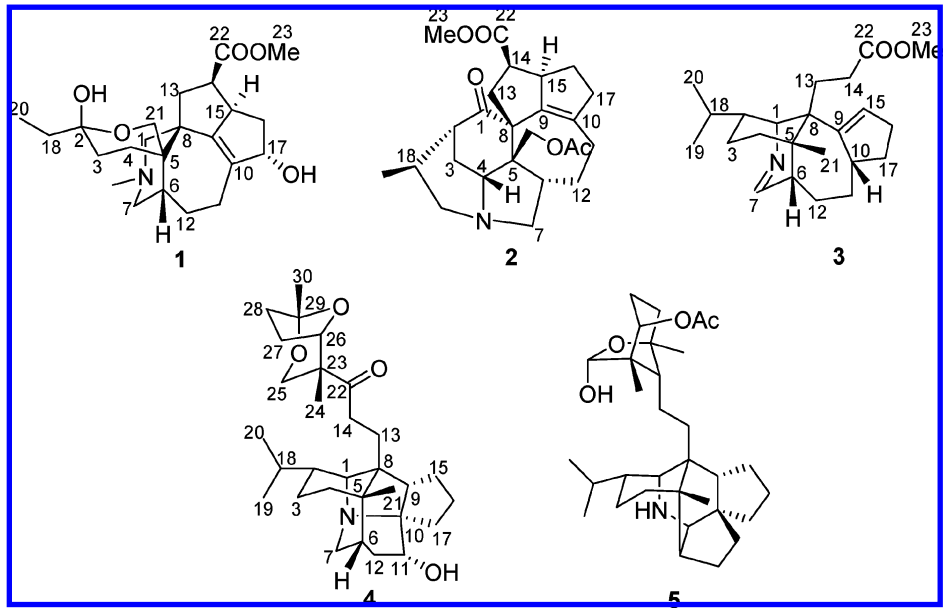
Chart 1



niphyllate, paxiphylline D, paxiphylline E, and daphlongamine E, were evaluated against the HL-60 human leukemia, P-388 murine leukemia, A-549 human lung adenocarcinoma, BEL-7402 human hepatocarcinoma, and HMEC human microvascular endothelial cell lines. Among these compounds tested, only daphnilongeridine (**5**) showed inhibitory activity, as observed against the HL-60 (IC_{50}, 9.5 μM), P-388 (IC_{50}, 2.4 μM), BEL-7402 (IC_{50}, 9.7 μM), and HMEC (IC_{50}, 2.7 μM) cell lines.

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of **1**−**4**

	1		**2**		**3**		**4**	
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)
1a	62.3	2.48 (d, 11.4)	214.3		66.9	4.34 (brs)	65.8	3.31 (brs)
1b		2.31 (d, 11.4)						
2	97.8		45.9	2.27 (m)	43.4	1.37 (m)	39.9	1.62 (m)
3a	29.1	1.74 (m)	21.5	2.23 (m)	22.7	1.62 (m)	28.0	1.96 (m, 2H)
3b		1.50 (m)		2.07 (m)		0.95 (m)		
4a	24.2	2.01 (m)	63.9	3.48 (brd, 4.1)	42.4	1.80 (m)	37.9	2.05 (m)
4b		1.62 (m)				1.22 (m)		1.55 (m)
5	38.4		56.3		38.5		38.1	
6	34.6	2.32 (m)	48.1	2.56 (m)	49.6	1.90 (m)	41.8	1.74 (m)
7a	57.4	2.82 (brd, 12.1)	58.6	2.95 (dd, 10.0, 7.7)	172.4	7.84 (s)	47.8	3.71 (brd, 13.5)
7b		2.62 (m)		2.67 (m)				3.54 (brd, 13.5)
8	48.4		62.4		44.7		49.0	
9	153.2		142.8		154.9		51.3	2.45 (m)
10	135.7		140.7		47.7	2.98 (m)	80.1	
11a	26.5	2.54 (m)	27.8	2.04 (m, 2H)	35.2	1.65 (m)	70.0	3.94 (brd, 6.9)
11b		2.17 (m)				1.36 (m)		
12a	28.2	2.28 (m)	30.0	1.89 (m)	27.6	1.95 (m)	34.4	2.46 (m)
12b		1.74 (m)		1.72 (m)		1.90 (m)		1.76 (m)
13a	41.4	2.68 (dd, 15.0, 2.3)	41.1	2.89 (dd, 14.6, 6.1)	30.6	1.89 (m, 2H)	24.9	1.45 (m, 2H)
13b		1.70 (m)		2.35 (m)				
14a	43.6	2.92 (ddd, 12.4, 10.0, 2.3)	43.5	2.80 (m)	31.5	2.38 (m)	37.7	3.01 (m)
14b						2.32 (m)		2.57 (m)
15	53.2	3.74 (m)	55.5	3.40 (m)	127.7	5.58 (brs)	30.1	1.88 (m, 2H)
16a	38.2	1.74 (m)	29.2	1.28 (m)	30.4	2.25 (m)	26.2	2.05 (m)
16b		1.68 (m)		1.89 (m)		2.14 (m)		1.85 (m)
17a	87.9	4.60 (brd, 5.7)	42.7	2.66 (m)	34.3	2.03 (m)	37.4	2.57 (m)
17b				2.37 (m)		1.49 (m)		1.40 (m)
18	36.8	1.58 (m, 2H)	34.3	2.69 (m)	31.5	1.40 (m)	32.1	1.62 (m)
19a			51.5	2.80 (m)	22.1	1.09 (d, 6.4, 3H)	21.8	1.04 (d, 5.7, 3H)
19b				2.51 (dd, 13.5, 7.8)				
20	8.8	0.92 (t, 7.3, 3H)	19.1	1.04 (d, 6.7, 3H)	21.9	0.87 (d, 6.4, 3H)	22.6	0.99 (d, 5.7, 3H)
21a	64.3	4.23 (d, 12.4)	69.0	4.48 (d, 11.6)	26.0	1.14 (s, 3H)	26.4	1.02 (s, 3H)
21b		3.74 (d, 12.4)		4.32 (d, 11.6)				
22	177.0		176.9		176.8		214.3	
23	52.0	3.61 (s, 3H)	52.4	3.63 (s, 3H)	52.7	3.64 (s, 3H)	51.5	
24							18.2	0.79 (s, 3H)
25a							66.7	4.26 (brd, 12.2)
25b								3.58 (brd, 12.2)
26							82.7	4.70 (brd, 5.6)
27							26.0	2.05 (m, 2H)
28a							35.3	2.08 (m)
28b								1.88 (m)
29							107.1	
30							24.4	1.24 (s, 3H)
N-Me	47.5	2.24 (s, 3H)						
OAc			173.1	2.07 (s, 3H)				
			21.4					

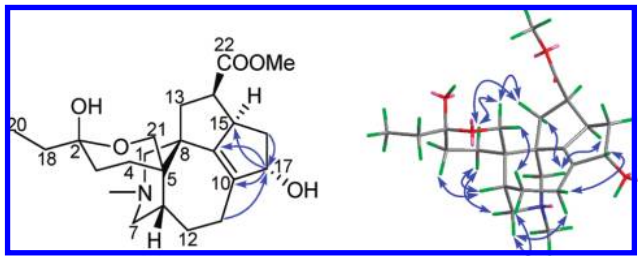


Figure 1. Selected HMBC (→) and ROESY (↔) correlations of **1**.

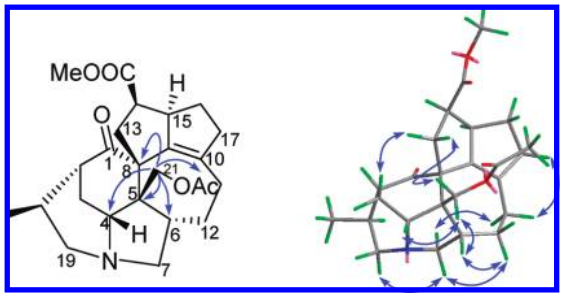


Figure 2. Selected HMBC (→) and ROESY (↔) correlations of **2**.

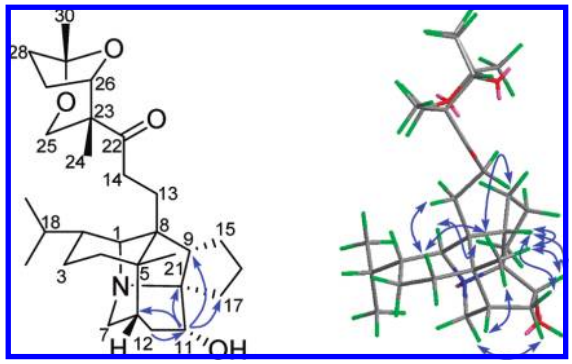


Figure 3. Selected HMBC (→) and ROESY (↔) correlations of **4**.

Experimental Section

General Experimental Procedures. Optical rotations were made on a Perkin-Elmer 341 polarimeter at room temperature. UV spectra were measured on a Shimadzu UV-2550 UV−visible spectrophotometer. IR spectra were recorded on a Perkin-Elmer 577 spectrometer. NMR spectra were measured on a Bruker AM-400 spectrometer with TMS as internal standard. EIMS (70 eV) and ESIMS were carried out on a Finnigan MAT 95 mass spectrometer and an Esquire 3000 plus LC-MS instrument, respectively. All solvents used were of analytical grade (Shanghai Chemical Plant, Shanghai, People's Republic of China). Silica gel (200−300 mesh), silica gel H60, and Sephadex LH-20 (Amersham Biosciences) were used for column chromatography, and precoated Si gel GF_{254} plates (Qingdao Haiyang Chemical Plant, Qingdao, People's Republic of China) were used for TLC.

Plant Material. Leaves of *Daphniphyllum subverticillatum* were collected in June 2004 in the town of Lutian of Guangdong Province, People's Republic of China, and authenticated by Prof. Su-Hua Shi of the Institute of Botany, School of Life Science, Zhongshan University. A voucher specimen has been deposited in the Herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences (accession number 2004-DS-2Y).

Extraction and Isolation. The powdered, dried leaves (3 kg) of *D. subverticillatum* were extracted with 95% ethanol at room temperature three times. After removal of the solvent under reduced pressure, the crude extract (700 g) was dissolved in 2 L of H_2O to form a suspension and adjusted with 2 N H_2SO_4 to pH ≈ 4. The acidic mixture was defatted with EtOAc (800 mL × 4), and the aqueous phase was basified with 30% Na_2CO_3 in water to pH ≈ 10 and extracted with $CHCl_3$ (500 mL × 5) to obtain 12.4 g of crude alkaloids. This was then subjected to passage over a silica gel column, eluted with $CHCl_3$−CH_3OH−Et_2NH (200:1:0.1 to 5:1:0.1), to give four major fractions, 1−4. Fraction 1 (8.9 g) was chromatographed on a silica gel column, eluted with petroleum ether−Et_2NH (from 150:1 to 15:1), to afford four further fractions (F1a−F1d). Fraction F1a was separated on a silica gel column, eluted with $CHCl_3$−CH_3OH (from 300:1 to 50:1), to obtain the major components, and each was then purified by separation over a Sephadex LH-20 column, eluted with CH_3OH, to yield **3** (14 mg), caldaphnidine R (3 mg), daphnigraciline (6 mg), methyl homodaphniphyllate (5 mg), the dehydrate of daphnigraciline (4 mg), secodaphniphylline (5 mg), and yuzurine (20 mg). Fraction F1b was subjected to silica gel column chromatography, eluted with petroleum−EtOAc−Et_2NH (3:1:0.03), to collect four fractions, and each was purified over a Sephadex LH-20 column, eluted with CH_3OH, to yield deoxycalyciphylline B (13 mg), deoxyisocalyciphylline B (4 mg), calycinine A (12 mg), and longistylumphylline A (3 mg). Fraction F1c was chromatographed on a silica gel column, eluted with petroleum−EtOAc−Et_2NH (2:1:0.03), and the major fraction was separated over a further silica gel column, eluted with $CHCl_3$−CH_3OH (100:1), to afford **5** (26 mg) and daphgraciline (6 mg). Fraction F1d was chromatographed over a Sephadex LH-20 column, eluted with CH_3OH, to yield paxiphylline D (11 mg), paxiphylline E (15 mg), and oxodaphnigraciline (20 mg). Fraction 2 (0.9 g) was subjected to passage over a Sephadex LH-20 column, eluted with CH_3OH, to afford three fractions (F2a−F2c). Fraction F2a was purified by silica gel column chromatography, eluted with petroleum−Et_2NH (50:1), to yield the methyl ester of yuzurimic acid B (5 mg). Fraction F2b was chromatographed over a silica gel column, eluted with petroleum−Et_2NH (from 50:1 to 5:1), and each of the major fractions was purified on a Sephadex LH-20 column, eluted with CH_3OH, to afford **2** (18 mg), **4** (6 mg), and daphlongamine E (5 mg). Fraction F2c was separated on a silica gel column, eluted with petroleum−Et_2NH (30:1), to yield daphniyunnine A (15 mg) and daphnilongeranin A (25 mg). Fraction 3 (1.9 g) was subjected to separation over a Sephadex LH-20 column, eluted with CH_3OH, to collect three fractions (F3a−F3c). Fraction F3a was chromatographed on a silica gel column, eluted with $CHCl_3$−CH_3OH (50:1), to give daphnilongeranin D (5 mg) and codaphniphylline (14 mg). Fraction F3b was chromatographed on a silica gel column, eluted with $CHCl_3$−CH_3OH (30:1), and the major alkaloid was then purified on a Sephadex LH-20 column, eluted with CH_3OH, to afford **1** (11 mg). Fraction F3c was separated over a silica gel column, eluted with $CHCl_3$−CH_3OH (30:1), to afford oldhamiphylline A (7 mg), daphnezomine M (21 mg), and daphnezomine A (4 mg).

17-Hydroxydaphnigraciline (1): colorless oil; $[\alpha]^{20}_D$ −38.6 (*c* 0.64, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.22) nm; IR (film) ν_{max} 3296, 2929, 2879, 1734, 1456, 1371, 1261, 1192, 1167, 1012, 924, 854, 750 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 405 $[M]^+$ (28), 387 (44), 374 (27), 346 (60), 320 (56), 304 (22), 84 (22), 58 (100); HREIMS *m/z* 405.2511 $[M]^+$ (calcd for $C_{23}H_{35}NO_5$ 405.2515).

Subdaphnidine A (2): colorless oil; $[\alpha]^{20}_D$ −61.2 (*c* 0.59, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.42) nm; IR (KBr) ν_{max} 2925, 1736, 1705, 1437, 1377, 1236, 1169, 1040, 754 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 427 $[M]^+$ (56), 398 (9), 368 (100), 354 (24), 326 (22), 294 (5), 110 (98), 94 (6); HREIMS *m/z* 427.2365 $[M]^+$ (calcd for $C_{25}H_{33}NO_5$ 427.2359).

Daphnezomine L Methyl Ester (3): colorless oil; $[\alpha]^{20}_D$ −123.5 (*c* 0.46, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.33) nm; IR (KBr) ν_{max} 2941, 2854, 1738, 1658, 1437, 1365, 1171, 1026 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 357 $[M]^+$ (28), 342 (18), 326 (11), 314 (20), 284 (100), 274 (60), 222 (12), 150 (30), 91 (17), 55 (16); HREIMS *m/z* 357.2665 $[M]^+$ (calcd for $C_{23}H_{35}NO_2$ 357.2668).

11-Hydroxycodaphniphylline (4): white, amorphous powder; $[\alpha]^{20}_D$ −18.0 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.11) nm; IR (KBr) ν_{max} 3423, 2929, 2870, 1707, 1633, 1456, 1388, 1180, 1138, 1055, 829 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 485 $[M]^+$ (33), 471 (59), 444 (26), 426 (9), 317 (24), 302 (100), 288 (97), 276 (32), 246 (19), 220 (19), 149 (64), 134 (24), 69 (28), 57 (32); HREIMS *m/z* 485.3498 $[M]^+$ (calcd for $C_{30}H_{47}NO_4$ 485.3505).

Hydrolysis of Daphnezomine L Methyl Ester (3). Alkaloid **3** (3 mg) was dissolved in 5 mL of MeOH, and about 5 mg of sodium hydroxide was added. The mixture was stirred at room temperature for 1 h. After neutralizing with 2 N HCl to pH ca. 7 and removal of the solvents under reduced pressure, the product was purified on a

Sephadex LH-20 column, eluted with CH_3OH, to yield an alkaloid (2 mg), which was identified as daphnezomine L[6] by spectroscopic analysis.

Cytotoxicity Assay. Cytotoxic activities were evaluated against the HL-60 and P-388 cell lines using the MTT method[21] and against the A-549, BEL-7402, and HMEC cell lines by the SRB method,[22] according to protocols described in previous literature, and pseudolaric acid B was used as the positive control[23] (for details see the Supporting Information).

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Supporting Information Available: Experimental Section: cytotoxicity assay; IR, EIMS, 1D- and 2D-NMR spectra of **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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