Cytotoxic Constituents of *Piper sintenense*

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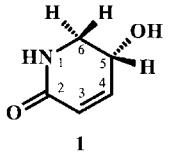
A new pyridone alkaloid, 5,6-dihydro-5-hydroxy-1*H*-pyridin-2-one (**1**), and a new ester, sintenin (= 3-(3,4-dimethoxyphenyl)propyl 3-(3,4-dimethoxyphenyl)propanoate **2**), together with three known compounds, 5,6-dihydro-1*H*-pyridin-2-one (**3**), *d*-sesamin (= 5,5′-(3a,4,6,6a-tetrahydro-1*H*,3*H*-furo[3,4-*c*]furan-1,4-diyl)bis[1,3-benzodioxole]; **4**), and (*E*)-phytol (= 3,7,11,15-tetramethylhexadec-2-en-1-ol; **5**) have been isolated from the whole plant of *Piper sintenense*. The structures of the two new compounds were determined through spectral analyses. Among twenty isolates obtained so far, four compounds exhibited effective cytotoxicities against P-388, HT-29, or A549 cell lines *in vitro*.

Introduction. – *Piper sintenense* HATUSIMA (Piperaceae) is an endemic scandent plant growing in medium altitude forests throughout Taiwan [1]. Its leaves and stems have been used as folk medicine against snake bites and wounds [2]. As an extension of our continuing studies on the cytotoxic constituents of Formosan plants, we have screened about 900 species for *in vitro* cytotoxicity, *P. sintenense* was shown to be one of the active species. In a former study of this plant, two compounds, sesamin and piperarboricoline were isolated, without testing for any biological activity [3]. In our previous study, we have reported a new alkaloid, pipersintenamide, together with 14 known compounds from this plant, including several agents cytotoxic against CCRF-CEM, HL-60, PC-3, or HA22T cell lines [4]. Continuing investigation of the minor constituents and the cytotoxic principles led to the isolation and characterization of a new pyridone alkaloid, 5,6-dihydro-5-hydroxy-1*H*-pyridin-2-one (**1**), and a new ester, sintenin (**2**), together with three known compounds, 5,6-dihydro-1*H*-pyridin-2-one (**3**), *d*-sesamin (**4**), and (*E*)-phytol (**5**). Here, we describe the structure elucidation of **1** and **2**, and the cytotoxic activity of the isolates against P-388, A549, or HT-29 cell lines *in vitro*.

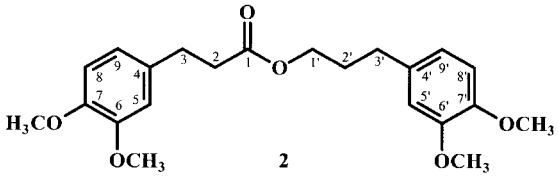
Results and Discussion. – 5,6-Dihydro-5-hydroxy-1*H*-pyridin-2-one (**1**) was isolated as a brownish oil. The EI mass spectrum afforded the molecular ion M^+ ion at *m/z* 113, implying a molecular formula of $C_5H_7O_2$, which was confirmed by the EI-HR-MS (113.0470, $[M]^+$). The presence of a conjugated C=O group was revealed by IR absorption at 1661 cm^{-1}, along with a resonance signal in the ^{13}C-NMR spectrum at δ 165.1.

The ^{1}H-NMR spectrum of **1** showed signals of two conjugated olefinic H-atoms at δ 5.89 (*dd*, $J = 10.0, 1.5$ Hz) and 6.74 (*dd*, $J = 10.0, 4.5$ Hz) corresponding to H−C(3) and H−C(4). Signals of three mutually coupling aliphatic H-atoms at δ 3.53 (*dt*, $J = 13.0$,



1

NOESY:
NH/H_α–C(6)
H–C(3)/H–C(4)
H–C(4)/H–C(3), H_β–C(5)
H_β–C(5)/H–C(4), H_β–C(5)
H_α–C(6)/NH, H_β–C(6)
H_β–C(6)/H_β–C(5), H_α–C(6)



2

NOESY:
H–C(2)/H–C(3)
H–C(3)/H–C(2), H–C(5), H–C(9)
H–C(5)/H–C(3), MeO–C(6)
H–C(8)/MeO–C(7), H–C(9)
H–C(9)/H–C(3), H–C(8)
H–C(1′)/H–C(2′), H–C(3′)
H–C(2′)/H–C(1′), H–C(3′)
H–C(3′)/H–C(1′), H–C(2′),
H–C(5′), H–C(9′)
H–C(5′)/H–C(3′), MeO–C(6′)
H–C(8′)/MeO–C(7′), H–C(9′)
H–C(9′)/H–C(3′), H–C(8′)

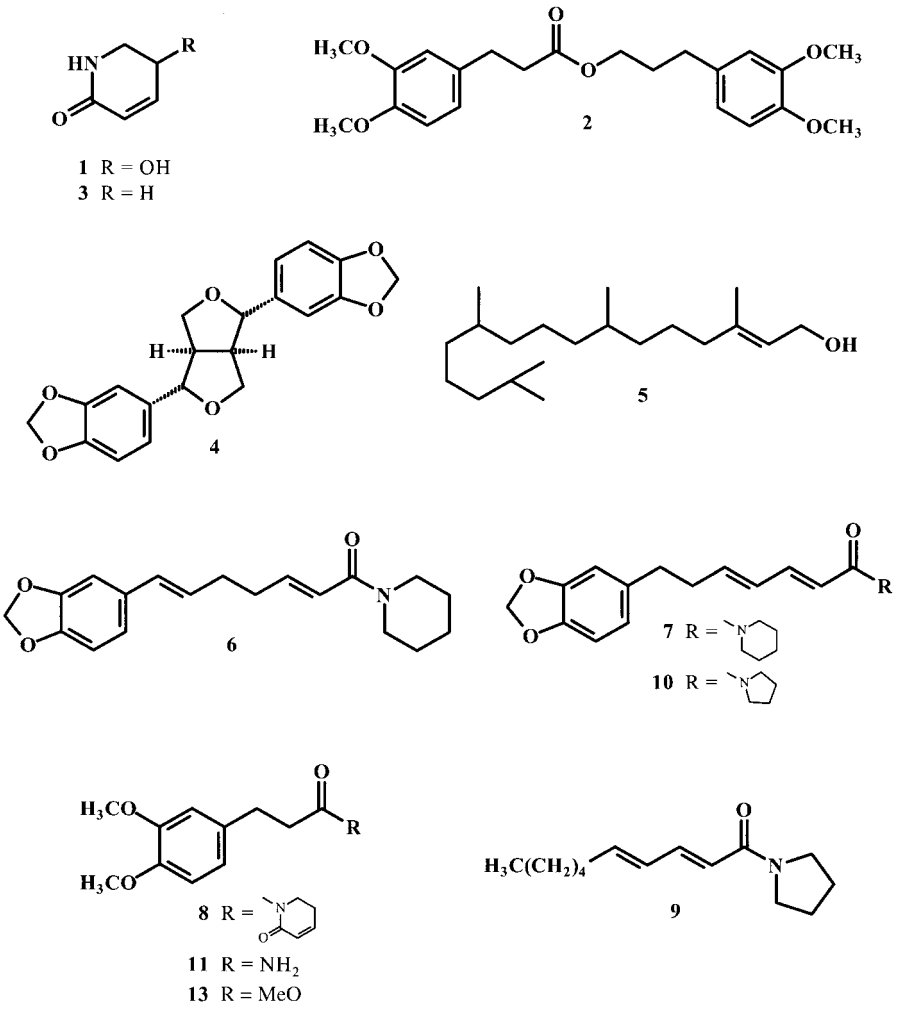
HMBC:
H–C((2)/C(1), C(3), C(4)
H–C(3)/C(1), C(2), C(4), C(5), C(9)
H–C(5)/C(4), C(7)
MeO–C(6)/C(6)
MeO–C(7)/C(7)
H–C(8)/C(6), C(7), C(9)
H–C(9)/C(3), C(5), C(7)
H–C(1′)/C(1), C(2′), C(3′)
H–C(2′)/C(1′), C(3′), C(4′)
H–C(3′)/C(1′), C(2′),C(4′), C(5′), C(9′)
H–C(5′)/C(4′), C(7′)
McO–C(6′)/C(6′)
MeO–C(7′)/C(7′)
H–C(8′)/C(6′), C(7′), C(9′)
H–C(9′)/C(3′), C(5′), C(7′)

4.5 Hz), 3.62 (*ddd*, $J = 13.0$, 4.5, 1.5 Hz), and 4.35 (*m*) were assigned to H_α–C(6), H_β–C(6), and H_β–C(5), respectively. In addition, a broad *singlet* signal at δ 6.05 (exchangeable with D_2O) and a broad *doublet* signal at δ 2.50 ($J = 8.0$ Hz, exchangeable with D_2O) were assigned to NH and HO–C(5), respectively, which could be supported by IR absorption at 3413 cm^{-1}.

On the basis of the above results and NOESY experiments, the structure of **1** was elucidated as 5,6-dihydro-5-hydroxy-1*H*-pyridin-2-one. The assignment of ^{13}C-NMR resonances were confirmed by the DEPT and HETCOR techniques, which also supported the structure of **1**.

Sintenin (**2**) was obtained as a colorless oil. The FAB-MS displayed the molecular ion M^+ at *m/z* 388, implying a molecular formula of $C_{22}H_{28}O_6$, which was confirmed by FAB-HR mass spectrum (388.1879, M^+). The presence of an ester C=O group was revealed by IR absorption at 1730 cm^{-1}, along with a resonance signal in the ^{13}C-NMR spectrum at δ 173.0.

The ^{1}H-NMR spectrum of **2** indicated the presence of a 3-(3,4-dimethoxyphenyl)-propanoyl group by two MeO *singlets* at δ 3.86 and 3.87 (each *s*, each 3 H), and showed signals of three mutually coupling aromatic H-atoms at δ 6.74 (*d*, $J = 1.5$ Hz), 6.75 (*dd*,



$J = 8.5$, 1.5 Hz), and 6.79 (d, $J = 8.5$ Hz), and of four CH_2 H-atoms at δ 2.62 and 2.91 (each t, $J = 7.8$ Hz). Three assignments are closely similar to those of methyl 3,4-dimethoxyhydrocinnamate [4], also isolated in our previous study.

In addition, in the ^{1}H-NMR spectrum of **2**, the presence of a 3-(3,4-dimethoxyphenyl)propyl group could be supported by two MeO *singlets* at δ 3.84 and 3.87 (each s), and by signals of three mutually coupling aromatic H-atoms at δ 6.69 (d, $J = 2.0$ Hz), 6.69 (dd, $J = 8.0$, 2.0 Hz), and 6.79 (d, $J = 8.0$ Hz), and of six aliphatic H-atoms at δ 4.10 (t, $J = 6.5$ Hz, $CH_2(1')$); 1.92 (m, $CH_2(2')$), and 2.60 (t, $J = 7.0$ Hz, $CH_2(3')$).

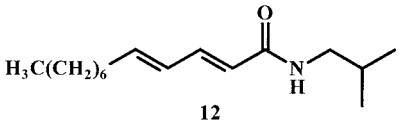
On the basis of the above data and NOESY experiments, the structure of **2** was elucidated as 3-(3,4-dimethoxyphenyl)propyl 3-(3,4-dimethoxyphenyl)propanoate, named sintenin. The assignments of ^{13}C-NMR resonances were confirmed by the HSQC, HMBC techniques, which also supported the structure of **2**.

5,6-Dihydro-1*H*-pyridin-2-one (**3**) was isolated as a brownish oil. The molecular formula C_5H_7NO was determined by EI-MS (*m/z* 97, M^+) and EI-HR-MS. The presence of a conjugated C=O group was revealed by IR absorption at 1661 cm^{-1}, along with a resonance signal in the ^{13}C-NMR spectrum at δ 165.1.

The ^1H-NMR spectrum of **3** was similar to that of **1** except that the $HO_\alpha-C(5)$ of **1** was replaced by H−C(5) in **3**. Analysis of the ^1H-NMR spectrum of **3** revealed two conjugated olefinic H-atoms at δ 5.92 (*ddd*, $J = 10.0, 4.2, 2.0$ Hz) and 6.67 (*dt*, $J = 10.0$, 4.2 Hz), which were assigned to H−C(3) and H−C(4). Four mutually coupling aliphatic H-atoms at δ 2.37 (*tdd*, $J = 7.2, 4.2, 2.0$ Hz) and 3.45 (*td*, $J = 7.2, 2.5$ Hz), which were assigned to $CH_2(5)$ and $CH_2(6)$, respectively. In addition, a broad *singlet* signal at δ 5.77 (exchangeable with D_2O) was assigned to NH, which could be supported by IR absorption at 3413 cm^{-1}.

The above assignments were further confirmed by the NOESY and ^1H, ^1H COSY experiments. On the basis of the above results, the structure of **3** was elucidated as 5,6-dihydro-1*H*-pyridin-2-one. This compound was also previously isolated from the leaves of *Piper arborescens* [3], but this finding has never before been published.

The known compounds, *d*-sesamin (**4**) [5] and (*E*)-phytol (**5**) [6], were readily identified by comparison of physical and spectroscopic data ($[\alpha]_D$, UV, IR, ^1H-NMR and mass spectrometry) with the authentic sample.



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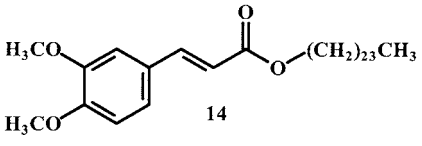

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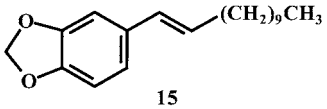

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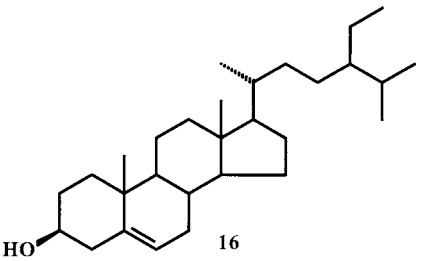

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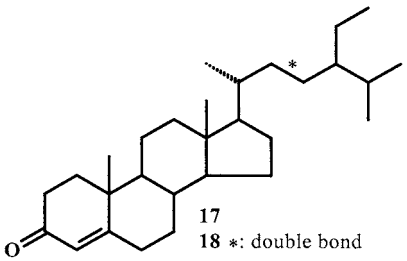
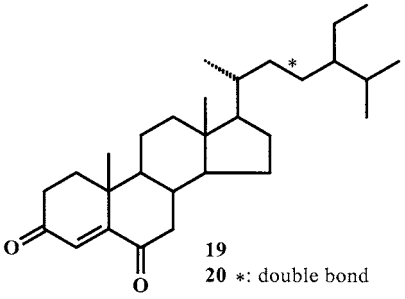


17

18 ∗: double bond



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20 ∗: double bond

Table. *Cytotoxic Effects of Compounds Isolated from* Piper sintenense *against P-388, A549, and HT-29 Cell Lines*

Compound	ED_{50} [μg ml^{-1}]		
	P-388	A549	HT-29
Mithramycin[a])	0.06	0.07	0.08
5,6-dihydro-5-hydroxy-1*H*-pyridin-2-one (**1**)	17.34	> 50	> 50
Sintenin (**2**)	0.21	> 50	> 50
5,6-Dihydro-1*H*-pyridin-2-one (**3**)	14.39	23.69	> 50
d-Sesamin (**4**)	4.58	> 50	> 50
(*E*)-Phytol (**5**)	6.36	7.88	10.71
Pipersintenamide (**6**)	3.78	10.43	7.39
Piperarboricoline (**7**)	12.55	> 50	23.40
Sintenpyridone (**8**)	0.121	0.89	0.025
Sarmentine (**9**)	2.81	> 50	> 50
Nigrinodine (**10**)	4.27	12.61	13.86
3-(3′,4′-Dimethoxyphenyl)propanamide (**11**)	40.41	> 50	> 50
(2*E*,4*E*)-*N*-Isobutyldodecadienamide (**12**)	0.167	2.05	3.36
Methyl 3,4-dimethoxyhydrocinnamate (**13**)	> 50	> 50	> 50
Tetracosyl ferulate (**14**)	5.28	> 50	> 50
(*E*)-1-[3,4-(Methylenedioxy)phenyl]dodec-1-ene (**15**)	6.00	25.43	21.07
β-Sitosterol (**16**)	> 50	> 50	46.96
Mixture of *β*-sitostenone (**17**) and stigmasta-4,22-dien-3-one (**18**)	10.09	> 50	> 50
Mixture of stigmast-4-en-3,6-dione (**19**) and stigmasta-4,22-dien-3,6-dione (**20**)	6.55	8.80	17.94

[a]) Mithramycin was used as a positive control.

The cytotoxic effects of the isolates were tested *in vitro* against P-388, A549, and HT-29 cell lines. The cytotoxicity data are shown in the *Table*, and the clinically applied anticancer agent mithramycin was used as the reference compound. Compounds **2**, **6**, **8**, **9**, and **12** exhibited effective cytotoxicities (ED_{50} values < 4 μg/ml) against P-388, A549, or HT-29 cell lines.

From the results of cytotoxic tests, the following conclusions can be drawn: *a*) Pipersintenamide (**6**) exhibited more-potent cytotoxic activity than piperarboricoline (**7**) due to the different position of the C=C bond. *b*) Among the steroids **16**–**20**, the dioxo-steroids, **19** and **20**, showed stronger cytotoxicity than other steroids against the cell lines tested. *c*) Among the new isolates, only sintenin (**2**) showed selective cytotoxicity (ED_{50} 0.21 μg/ml) against the P-388 cell line. *d*) Of the active pyrrolidine amides **9** and **10**, **9** showed selective cytotoxicity against the P-388 cell line, and the intensity of activity was in the order **9** (with deca-2,4-dienoyl) > **10** (with 7-[3,4-(methylenedioxy)phenyl]hepta-2,4-dienoyl). *e*) Sintenpyridone (**8**) showed strong cytotoxicity, but the corresponding compounds, **3** and **11**, resulting from the cleavage of **8**, were inactive.

Finally, sintenpyridone (**8**) is the most cytotoxic principle (ED_{50} < 1 μg/ml) against the P-388, A549, and HT-29 cell lines, and exhibited a more potent cytotoxicity (ED_{50} 0.025 μg/ml) against the HT-29 cell line than mithramycin (ED_{50} 0.08 μg/ml).

Experimental Part

General. TLC: silica gel *60* F_{254} precoated plates (*Merck*). Column chromatography (CC): silica gel *60* (*Merck*, 70–230 mesh, 230–400 mesh, ASTM). M.p.: *Yanaco* micro-melting-point apparatus; uncorrected.

Optical rotations: *Jasco DIP-370* polarimeter; in $CHCl_3$ or MeOH. UV Spectra: *Jasco UV-240* spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Perkin-Elmer 2000* FT-IR spectrophotometer; KBr at 26°; $\tilde{\nu}$ in cm^{-1}. ^1H-, ^{13}C-, and 2D-NMR: *Varian Unity-Plus-400* and *Varian Inova 500* spectrometers; δ in ppm rel. to Me_4Si, J in Hz. EI-MS Spectra: *VG Biotech Quatro-5022* spectrometer; m/z (rel. %). HR-EI- and HR-FAB-MS: *Jeol JMX-HX-110* mass spectrometer.

Plant Material. *Piper sintenense* was collected from Lai-I, Pingtung County, Taiwan, in August 2000 and identified by Dr. *I. S. Chen*. A voucher sample (Chen 5595) was deposited in the Herbarium of the School of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China.

Extraction and Isolation. Dried whole plant (8.2 kg) was extracted with cold MeOH, and the extract was concentrated under reduced pressure. The MeOH extract (870 g), when partitioned between $H_2O/CHCl_3$ 1:1, afforded a $CHCl_3$-soluble fraction (*Fr. A*, 320 g). Part (30 g) of *Fr. A* was chromatographed on silica gel (980 g) with hexane, gradually increasing the polarity with AcOEt to obtain 29 fractions: *Fr. A1–A6* (each 1000 ml, hexane), *Fr. A7* (1500 ml, hexane/AcOEt 19:1), *Fr. A8* and *A9* (each 1500 ml, hexane/AcOEt 9:1), *Fr. A10–A16* (each 1500 ml, hexane/AcOEt 5:1), *Fr. A17–A20* (each 1500 ml, hexane/AcOEt 5:1), *Fr. A21–A24* (each 1000 ml, hexane/AcOEt 1:1), *Fr. A25* (2000 ml, AcOEt), *Fr. A26–A29* (each 1500 ml, MeOH). *Fr. A12* (611 mg) was rechromatographed on silica gel (19.5 g) with hexane/AcOEt 10:1 to give *Fr. A12-1–A12-4*, and *Fr. A12-2* (87 mg) was further purified by prep. TLC (CH_2Cl_2) to give **5** (5.6 mg; R_f 0.60). *Fr. A20* (2.7 g) was rechromatographed on silica gel (83 g) with hexane/AcOEt 2:1 to give *Fr. A20-1–A20-7*, and *Fr. A20-4* (242 mg) was further purified by prep. TLC (CH_2Cl_2/AcOEt 20:1) to yield **4** (6.8 mg; R_f 0.76). *Fr. A24* (3.63 g) was rechromatographed on silica gel (108 g) with $CHCl_3/Me_2CO$ 10:1 to give *Fr. A24-1–A24-7*. *Fr. A24-1* (276 mg) was further purified by prep. TLC ($CHCl_3$) to provide **2** (7.3 mg; R_f 0.43). *Fr. A28* (1.34 g) was rechromatographed on silica gel (44 g) with $CHCl_3/Me_2CO$ 4:1 to give *Fr. A28-1–A28-4*, and *Fr. A28-2* (314 mg) was further purified by prep. TLC ($CHCl_3/Me_2CO$ 2:1) to yield **1** (3.8 mg; R_f 0.10) and **3** (5.9 mg; R_f 0.48).

*5,6-Dihydro-5-hydroxy-1*H*-pyridin-2-one* (**1**). Brownish oil. $[\alpha]_D^{26} = +55.7$ ($c = 0.2$, MeOH). UV ($CHCl_3$): 209 (4.24), 247 (sh, 3.52). IR (KBr): 3413 (br., NH and OH), 1661 (C=O). ^1H-NMR ($CDCl_3$, 400 MHz): 2.50 (br. *s*, $J = 8.0$, exchangeable with D_2O, HO_α–C(5)), 3.53 (*dt*, $J = 13.0, 4.5$, H_α–C(6)); 3.62 (*ddd*, $J = 13.0, 4.5, 1.5$, H_β–C(6)); 4.35 (*m*, H_β–C(5)); 5.89 (*dd*, $J = 10.0, 1.5$, H–C(3)); 6.05 (br. *s*, exchangeable with D_2O, NH); 6.74 (*dd*, $J = 10.0, 4.5$, H–C(4)). ^{13}C-NMR ($CDCl_3$, 125 MHz): 47.3 (C(6)); 61.7 (C(5)); 125.8 (C(3)); 141.9 (C(4)); 165.1 (C(2)). FAB-MS: 114 (100, $[M+H]^+$). EI-MS: 113 (10, M^+), 95 (8), 84 (66), 67 (6), 56 (36), 55 (100). HR-EI-MS: 113.0470 ($C_5H_7O_2N^+$; calc. 113.0477).

3-(3,4-Dimethoxyphenyl)propyl 3-(3,4-Dimethoxyphenyl)propanoate (= *Sintenin*; **2**). Colorless oil. UV (MeOH): 208 (4.65), 229 (4.53), 280 (4.09). IR (KBr): 1730 (C=O), 1591, 1515, 1454 (arom. ring C=C). ^1H-NMR ($CDCl_3$, 500 MHz): 1.92 (*m*, $CH_2(2')$; 2.60 (*t*, $J = 7.0$, $CH_2(3')$); 2.62 (*t*, $J = 7.8$, $CH_2(2)$); 2.91 (*t*, $J = 7.8$, $CH_2(3)$); 3.84 (*s*, MeO–C(7')); 3.86 (*s*, MeO–C(7)); 3.87 (*s*, MeO–C(6), MeO–C(6')); 4.10 (*t*, $J = 6.5$, $CH_2(1')$); 6.69 (*d*, $J = 2.0$, H–C(5')); 6.69 (*dd*, $J = 8.0, 2.0$, H–C(9')); 6.74 (*d*, $J = 1.5$, H–C(5)); 6.75 (*dd*, $J = 8.5, 1.5$, H–C(9)); 6.79 (*d*, $J = 8.5$, H–C(8)); 6.79 (*d*, $J = 8.0$, H–C(8')). ^{13}C-NMR ($CDCl_3$, 125 MHz): 30.4 (C(2')); 30.6 (C(3)); 31.7 (C(3')); 36.2 (C(2)); 55.8 (MeO–C(6)); 55.8 (MeO–C(6')); 55.9 (MeO–C(7)); 55.9 (MeO–C(7')); 63.8 (C(1')); 111.2 (C(8)); 111.2 (C(8')); 111.6 (C(5)); 111.6 (C(5')); 120.1 (C(9)); 120.2 (C(9')); 133.1 (C(4)); 133.7 (C(4')); 147.3 (C(7')); 147.5 (C(7)); 148.8 (C(6)); 148.8 (C(6')); 173.0 (C(1)). EI-MS: 388 (38, M^+), 210 (13), 179 (12), 178 (100), 163 (27), 151 (78), 147 (73), 121 (5), 107 (19), 91 (11). FAB-MS: 388 (21, M^+), 307 (22), 289 (14), 193 (9), 165 (8), 155 (25), 154 (100), 152 (10), 151 (19), 139 (14), 138 (31), 137 (56), 136 (66), 120 (11), 107 (21). HR-FAB-MS: 388.1879 ($C_{22}H_{28}O_6^+$; calc. 388.1886).

*5,6-Dihydro-1*H*-pyridin-2-one* (**3**). Brownish oil. UV (EtOH): 209 (4.24), 247 (sh, 3.52). IR (KBr): 3413 (br., NH and OH), 1661 (C=O). ^1H-NMR ($CDCl_3$, 400 MHz): 2.37 (*tdd*, $J = 7.2, 4.2, 2.0$, $CH_2(5)$); 3.45 (*td*, $J = 7.2, 2.5$, $CH_2(6)$); 5.77 (br. *s*, NH, exchangeable with D_2O); 5.92 (*ddd*, $J = 10.0, 4.2, 2.0$, H–C(3)); 6.67 (*dt*, $J = 10.0, 4.2$, H–C(4)). ^{13}C-NMR ($CDCl_3$, 125 MHz): 47.3 (C(6)); 61.7 (C(5)); 125.8 (C(3)); 141.9 (C(4)); 165.1 (C(2)). EI-MS: 97 (92, M^+), 96 (8), 86 (8), 84 (14), 78 (7), 69 (23), 68 (100), 53 (9), 51 (11), 49 (16), 42 (22), 41 (30). HR-EI-MS: 97.0528 ($C_5H_7NO^+$; calc. 97.0528).

Cytotoxicity Assays. P-388 cells were kindly provided by Prof. *J. M. Pezzuto*, University of Illinois at Chicago; A549 (human lung adenocarcinoma) and HT-29 (human colon carcinoma) were purchased from *American Type Culture Collection*.

P-388 Cells were cultured in *Fisher*'s medium supplemented with 10% heat-inactivated (56° for 30 min) fetal calf serum (FCS). A549 Cells were cultured in Eagle Minimum Essential Medium (EMEM) containing *Earle*'s salts and supplemented with 0.1 mM of nonessential amino acids and 10% heat-inactivated FCS. HT-29

Cells were maintained in *Rosewell Park Memorial Institute* (RPMI) *1640* medium containing 10% heat-inactivated FCS. All cell lines were maintained in an incubator at 37° in humidified air containing 5% CO_2.

The cytotoxic activities of compounds against P-388, A549, and HT-29 were assayed by a modification of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method [7]. For P-388 cells, 200-µl cultures were established at 1500 cells/well in 96-well tissue culture plates (*Falcon*). Compounds were dispensed to established cultures at eight concentrations in triplicate. After three days of incubation, P-388 cells were counted with MTT.

To measure the cytotoxic activities of purified compounds against A549 and HT-29 cells, each cell line was initiated at 1000 cells/well in 96-well microtiter plates. Eight concentrations (triplicate) of test compounds encompassing a 128-fold range were added to each cell line. A549 and HT-29 cells were enumerated with MTT after exposure to test compounds for 6 d, respectively. 50 µl of 1 mg/ml MTT were added to each well, and plates were incubated at 37° for a further 5 h. Formazan crystals were redissolved in DMSO (*E. Merck*) for 10 min with shaking, and the plate was read immediately on a microtiter plate reader (*Dynatech*) at a wavelength of 540 nm. The ED_{50} was defined as the concentration of the test compound resulting in a 50% reduction of absorbance compared to untreated cells in the MTT assay. All assays were repeated three times.

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