New Alkaloids and a Tetraflavonoid from *Cephalotaxus wilsoniana*

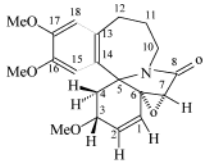
Li-Wen Wang,[†] Huey-Jen Su,[‡] Sheng-Zehn Yang,[§] Shen-Jeu Won,[⊥] and Chun-Nan Lin*[,†]

School of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan 807, Republic of China, Department of Nursing, Mei-Ho Institute of Technology, Pintung Hsien, Taiwan 912, Republic of China, Department of Forest Resource, Management and Technology, National Pintung University of Science and Technology, Pintung Hsien, Taiwan 912, Republic of China, and Department of Microbiology and Immunology, National Cheng Kung University, Taiwan 701, Republic of China

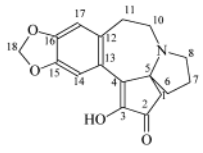
Received April 15, 2004

A new homoerythrina alkaloid, C-3-epi-wilsonione (**1**), a new tetraflavonoid, taiwanhomoflavone C (**2**), and a new stereoisomer of desmethylcephalotaxinone (**3**) have been isolated from the leaves and heartwood of *Cephalotaxus wilsoniana*, respectively. The structures were elucidated by spectroscopic methods. Compound **1** showed cytotoxic activity against a number of human cancer cell lines in vitro.

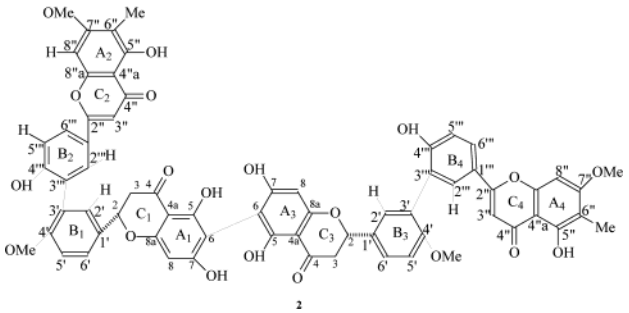
Various constituents of *Cephalotaxus wilsoniana* Hayata (Cephalotaxaceae) have been reported.[1−3] During a search for cytotoxic compounds from Formosan plants, we found that a crude $CHCl_3$ extract of the leaves and heartwood from *C. wilsoniana* showed potent cytotoxic activity against Hep G2, 212, Hep 3B, HT-29, and HCT 116 cells in vitro. Therefore, we investigated the constituents of the leaves and heartwood of *C. wilsoniana* and isolated a new homoerythrina alkaloid, C-3-epi-wilsonione (**1**), a new tetraflavone, taiwanhomoflavone C (**2**), a new stereoisomer of desmethylcephalotaxinone (**3**), and four known constituents, C-3-epi-wilsonine, taiwanhomoflavone A, ginkagetin, and apigenin. In the present paper, the structure elucidation and cytotoxicities of the three new compounds (**1**−**3**) are reported.



1



3



2

The molecular formula of **1** was determined to be $C_{20}H_{23}NO_5$ by HREIMS (*m*/*z* 357.1510 [M^+]), which was consistent with ^1H and ^{13}C NMR data (Experimental Section). The UV absorption maxima were similar to those of C-3-epi-wilsonine.[1] The IR absorption of **1** implied the presence of a carbonyl (1689 cm^{-1}) moiety. The proton signals of H-1, H-2, H-3, H_2-4, H-15, H-18, MeO-3, MeO-16, and MeO-17 of **1** were similar to those of C-3-epi-wilsonine. However, the ^1H NMR spectrum of **1** showed six aliphatic and an oxymethine proton signal. The IR absorption band at 1689 cm^{-1} together with the carbon signal at δ 167.9 confirmed that **1** contains a =N−CO moiety.[4] The HMBC correlations of H-7/C-8 and H-2/C-6 and the NOESY correlations of H-1/H-7, H-1/H-2, and H-3/H-4 further confirmed that C-3-epi-wilsonione was 1,2-didehydro-6,7-epoxy-3α,16,17-trimethoxyerythrinan-8-one (**1**). The ^{13}C NMR assignments of **1** were deduced from ^1H-decoupled, DEPT, and 2D NMR including $^1H-^1H$ COSY, HMQC, HMBC, and NOESY.

Compound **2** was obtained as an amorphous yellow solid. IR absorptions were indicative of hydroxy (3158 cm^{-1}), conjugated carbonyl (1642 cm^{-1}), and aromatic ring (1607 cm^{-1}) groups. It possesses the molecular formula $C_{66}H_{50}O_{20}$, determined from electrospray ionization mass spectrometry (ESIMS) (M + Na + 2H at *m*/*z* 1187). The UV spectrum exhibited absorption maxima at 208, 278, and 329 nm. Its ^1H NMR spectrum displayed signals for 18 aromatic protons, two oxymethine and two methylene, characteristic of two flavonone units,[5] four methoxy, and two methyl groups (Table 1). The ^1H NMR spectrum of **2** in C_5D_5N indicated crowded signals between δ 7.17 and 7.23 and between 7.77 and 7.83, while these signals in CD_3OD appeared between δ 6.76 and 6.82, between 7.30 and 7.60, and between 7.50 and 7.80, respectively, and were separable. Observing the proton signals measured in C_5D_5N and CD_3OD, repectively, and from detailed analysis of a $^1H-^1H$ COSY NMR experiment, the aromatic protons were identified as four 1,2,3,4,5-pentasubstituted and four 1,3,4-trisubstituted aromatic rings. In addition to the above evidence, the HMBC correlations of two H-2‴/C-4‴, two H-3″/C-2″, C-4″, and C-4″a, two OMe-7″/C-7″, two Me-6″/C-6″, two H-8″/C-6″, C-7″, C-8″a, and C-4″a, two H-6‴/C-4‴, and two H-5‴/C-1‴, C-3‴, and C-4‴, NOESY correlations of two Me-6″/OMe-7″ and H-8″/OMe-7″, and two hydroxy proton signals at δ 13.93 confirmed that **2** contains two 5,4′-dihydroxy-6-methyl-7-methoxy-3‴-substituted flavone moieties. The HMBC correlations of two MeO-4′/C-4′, two H-2′/C-4′ and C-6′, two H-5′/C-1′ and C-3′, two H-6′/C-2 and C-2′, two H_2-3/C-4, and two H-8/C-4a, C-7, and C-6, and two hydroxy proton signals at δ 12.69 confirmed that **2** contains two 5,7-dihydroxy-4′-methoxy-6,3′-disubstituted flavonone moieties. In addition, the UV spectrum of **2** showed bathochromic shifts upon addition of $AlCl_3$, NaOAc, and NaOMe. The above results and deshielding of the signals of substituted carbons C-6 of A_1 and A_3, C-3′ of B_1 and B_3, and C-3‴ of B_2 and B_4 rings, caused by the flavanyl substitution, compared to the monomeric flavonone or

* To whom correspondence should be addressed. Tel: +886-7-3121101∼9, ext. 2163. Fax: +886-7-5562365. E-mail: lincna@cc.kmu.edu.tw.
[†] Kaohsiung Medical University.
[‡] Mei-Ho Institute of Technology.
[§] National Pintung University of Science and Technology.
[⊥] National Cheng Kung University.

10.1021/np0498657 CCC: $27.50
Published on Web 07/08/2004

Table 1. ^{13}C NMR and ^{1}H NMR Data for **2** (C_5D_5N)

C	ring	δ_C	δ_H	C	ring	δ_C	δ_H
flavonone				flavone			
2	C_1	79.6[a]	5.63 (dd, 13.6, 2.8)	2″	C_2	164.4	
3	C_1	43.2	3.02 (dd, 17.2, 2.8)	3″	C_2	103.4	6.97 (s)
			3.43 (dd, 17.2, 13.6)	4″	C_2	183.1	
4	C_1	196.9		4″a	A_2	103.3	
4a	C_1	105.0		5″	A_2	160.8	
5	A_1	163.4		6″	A_2	105.0	
6	A_1	105.6		7″	A_2	166.0	
7	A_1	162.4		8″	A_2	91.4	6.24 (s)
8	A_1	99.7	6.92 (s)	8″a	A_2	162.1	
8a	A_1	155.5		Me-6″	A_2	7.25	2.17
OH-5	A_1		12.69	OMe-7″	A_2	55.9	3.75
1′	B_1	131.3		OH-5″	A_2		13.93
2′	B_1	132.5[b]	8.05 (d, 2.4)	1‴	B_2	123.0	
3′	B_1	123.0		2‴	B_2	128.8	7.82 (d, 2.4)
4′	B_1	159.0		3‴	B_2	122.4	
5′	B_1	111.5	7.19 (d, 8.8)	4‴	B_2	162.8	
6′	B_1	128.0	7.68 (dd, 8.8, 2.4)	5‴	B_2	116.8	7.22 (d, 8.8)
OMe-4′	B_1	55.8	3.76	6‴	B_2	128.8	7.81 (dd, 8.8, 2.4)
2	C_3	79.5[a]	5.63 (dd, 13.6, 2.8)	2″	C_4	164.4	
3	C_3	43.8	2.95 (dd, 17.2, 2.8)	3″	C_4	103.4	6.97 (s)
			3.38 (dd, 17.2, 13.6)	4″	C_4	183.1	
4	C_3	197.0		4a″	A_4	103.3	
4a	C_3	105.0		5″	A_4	163.8	
5	A_3	163.4		6″	A_4	105.0	
6	A_3	105.6		7″	A_4	166.0	
7	A_3	162.4		8″	A_4	91.4	6.21 (s)
8	A_3	99.7	6.92 (s)	8″a	A_4	162.1	
8a	A_3	155.5		Me-6″	A_4	7.25	2.16
OH-5	A_3		12.69	OMe-7″	A_4	55.9	3.61
1′	B_3	131.1		1‴	B_4	122.8	
2′	B_3	132.0[b]	8.03 (d, 2.4)	2‴	B_4	128.8	7.77 (d, 2.4)
3′	B_3	123.0		3‴	B_4	122.4	
4′	B_3	158.9		4‴	B_4	162.8	
5′	B_3	111.5	7.19 (d, 8.8)	5‴	B_4	116.8	7.19 (d, 8.8)
6′	B_3	128.1	7.66 (dd, 8.8, 2.4)	6‴	B_4	128.8	7.78 (dd, 8.8, 2.4)
OMe-4′	B_3	55.8	3.76				

[a,b] Values may be reversed.

flavone, and the NOESY correlations of H-2′ (B_1 ring)/H-2‴ (B_2 ring) and H-2′ (B_3 ring)/H-2‴ (B_4 ring) suggested that **2** contained three C−C linkages between C-6 (A_1 ring) and C-6 (A_3 ring), C-3′ (B_1 ring) and C-3‴ (B_2 ring), and C-3′ (B_3 ring) and C-3‴ (B_4 ring) (Table 1).[6,7] The ^{13}C NMR signals of **2** (Table 1) were assigned by performing ^{1}H-decoupled, DEPT, and ^{1}H, ^{13}C COSY correlation experiments and by comparison with those of corresponding data reported in the literature.[2−4,7,8] The ^{13}C NMR spectrum and the presence of significant MS peaks at *m/z* 1164 $[M + 2]^+$, 595 $[1187 + 2H - 2b]^+$, and 583$[1164 - a]^+$ also supported the structure **2**. Thus, compound **2** was characterized as di(5,7,4‴-trihydroxy-2,3-dihydro-4′,7″-dimethoxy-6″-methyl-3′,3‴-biflavanyl)-6,6-tetraflavone. The molecular formula of **3** was determined to be $C_{17}H_{17}NO_4$ by HREIMS (*m/z* 299.1155 $[M]^+$), which was consistent with the ^{1}H and ^{13}C NMR data. The UV, ^{1}H NMR, and $[\alpha]_D^{23}$ of **3** were similar to those of desmethylcephalotaxinone.[9−11] In the ^{13}C NMR spectrum of **3**, the chemical shift values were almost identical to those of corresponding data of synthetic desmethylcephalotaxinone except for C-1 to C-7 and C-10.[10] The ^{13}C NMR signals of **3** were assigned by performing ^{1}H-decoupled, DEPT, and ^{1}H, ^{13}C COSY correlation experiments and by comparison with those of synthetic desmethylcephalotaxinone reported in the literature.[10,11] The NOESY correlation of **3** showed a cross-peak between H_α-1 and H_α-6. On the basis of the above evidence, **3** was a stereoisomer of desmethylcephalotaxinone.

A microassay for cytotoxicity of the constituents isolated from *C. wilsoniana* was performed using MTT.[12−14] The cytotoxicities of **1**, **2**, **3**, and taiwanhomoflavone A were tested against a number of human cancer cell lines. Compound **1** showed cytotoxic activity against the Hep G2, MCF-7, Hep 3B, and HT-29 in a concentration-dependent manner with IC_{50} values of about 52.0, 42.0, 52.0, and 24.4 μg/mL, respectively.

Experimental Section

General Experimental Procedures. Melting points are reported uncorrected. Optical rotations were obtained on a

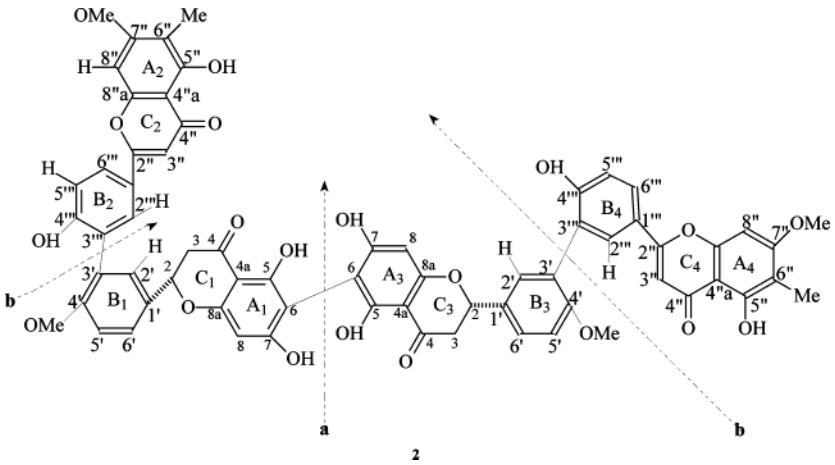


Figure 1. Major MS fragmentation pattern of **2**.

JASCO model DIP-370 digital polarimeter; UV spectra were obtained on a JASCO model 7800 UV−vis spectrophotometer; IR spectra were recorded on a Hitachi model 260-30 spectrophotometer; ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian Unity-400 spectrometer, and MS were obtained on a JMS-HX 100 mass spectrometer.

Plant Material. Whole plants of *C. wilsoniana* were collected at Taichung Hsien, Taiwan, during October 2000. A voucher specimen has been deposited in the author's laboratory.

Extraction and Isolation. The air-dried leaves (1.04 kg) of *C. wilsoniana* were extracted with $CHCl_3$ at room temperature. The $CHCl_3$ extract (35 g) was chromatographed over silica gel, and elution with CH_2Cl_2 yielded **1** (7 mg) and C-3-epi-wilsonine[9] (5 mg); elution with CH_2Cl_2−acetone (1:1) yielded **2** (10 mg), taiwanhomoflavone A[2] (9 mg), ginkagetin[3] (8 mg), and apigenin[3] (3 mg).

The heartwood (1.6 kg) was chipped and extracted with $CHCl_3$. The $CHCl_3$ extract (28 g) was chromatographed over silica gel, and elution with CH_2Cl_2−MeOH (1:1) yielded **3** (3 mg).

1,2-Didehydro-6,7-epoxy-3α,16,17-trimethoxyerythrinan-8-one (1): colorless powder; $[\alpha]_D^{28}$ +11° (*c* 0.104, $CHCl_3$); IR (KBr) ν_{max} 1689 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 280 (3.56), 225 (sh) (4.02), 206 (4.37) nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.64 (1H, m, H$_\alpha$-11), 1.99 (1H, m, H$_\beta$-11), 2.86 (1H, dd, J = 15.6, 6.8 Hz, H$_\alpha$-12), 3.18 (1H, dd, J = 15.6, 2.4 Hz, H$_\beta$-12), 3.21 (1H, dd, J = 12.4, 2.0 Hz, H$_\alpha$-10), 3.31 (3H, s, OMe-3), 3.47 (1H, m, H$_\beta$-3). 3.83 (3H, s, OMe-16), 3.83 (1H, bs, H$_\beta$-7), 3.88 (3H, s, OMe-17), 4.46 (1H, td, J = 12.4, 3.2 Hz, H$_\beta$-10) 5.76 (1H, dd, J = 10.4, 2.4 Hz, H-1), 6.26 (1H, td, J = 10.4, 1.5 Hz, H-2), 6.64 (1H, s, H-18), 7.07 (1H, s, H-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.7 (C-11), 36.3 (C-12), 38.7 (C-4), 41.2 (C-10), 55.9 (OMe-17), 56.0 (OMe-16), 56.2 (OMe-3), 59.7 (C-7), 65.9 (C-6), 67.6 (C-5), 73.6 (C-3), 112.3 (C-15), 115.3 (C-18), 124.8 (C-1), 128.3 (C-14), 133.3 (C-13), 136.8 (C-2), 146.4 (C-16), 148.0 (C-17), 167.9 (C-8); EIMS m/z 357 [M]$^+$ (100), 342 (60), 341 (38), 326 (30), 298 (23), 286 (15); HREIMS m/z 357.1510 (calcd for $C_{20}H_{23}NO_5$, 357.1576).

Di(5,7,4‴-trihydroxy-2,3-dihydro-4′,7″-dimethoxy-6″-methyl-3′,3‴-biflavanyl)-6,6-tetraflavone (2): amorphous yellow solid; $[\alpha]_D^{28}$ +44° (*c* 0.114, MeOH); IR (KBr) ν_{max} 3158, 1642, 1607 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 208 (4.85), 278 (4.55), 329 (4.31) nm; (MeOH−NaOMe) 218, 286, 386 nm; (MeOH−$AlCl_3$) 210, 256, 285, 348, 386 nm; (MeOH−NaOAc) 217, 285, 331 nm; (MeOH−NaOAc−H_3BO_3) unchanged. ^{1}H NMR (C_5D_5N, 400 MHz), see Table 1; ^{13}C NMR (C_5D_5N, 100 MHz), see Table 1; ESIMS m/z 1187 [M + Na + 2H]$^+$ (100), 1164 [M + 2H]$^+$ (6), 895 (18), 771 (18), 727 (24), 595 (23), 583 (51), 457 (15); ESIMS m/z 1187.0 [M + Na + 2H]$^+$ (calcd for $C_{66}H_{52}O_{20}Na$ [M + Na + 2H]$^+$ 1187.3).

5,6,8,9-Tetrahydro-1-hydro-4*H*-cyclopenta[*a*]-[1,3]dioxolo[4,5-*h*]pyrrolo[2,1-*b*][3]benzaepin-2(3*H*)-one (3): brownish powder; $[\alpha]_D^{28}$ +52° (*c* 0.03, MeOH); IR (KBr) ν_{max} 3422, 1718 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 212 (4.64), 288 (4.22) nm; ^{1}H NMR (C_5D_5N, 400 MHz) δ 1.82 (1H, m, H$_\alpha$-6), 1.96 (1H, m, H$_\alpha$-7), 2.00 (1H, m, H$_\beta$-7), 2.02 (1H, m, H$_\beta$-6), 2.83 (1H, d, J = 18.2 Hz, H$_\alpha$-1), 3.80 (1H, d, J = 18.2 Hz, H$_\beta$-1), 3.20 (1H, m, H$_\alpha$-8), 3.69 (1H, m, H$_\beta$-8), 3.17 (1H, m, H$_\alpha$-10), 3.67 (1H, m, H$_\beta$-10), 3.13 (1H, m, H$_\alpha$-11), 3.20 (1H, m, H$_\beta$-11), 6.00 (1H, s, H-18), 6.07 (1H, s, H-18), 6.71 (1H, s, H-17), 7.32 (1H, s, H-14); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 22.2 (C-7), 31.3 (C-11), 36.1 (C-6), 47.4 (C-1), 49.3 (C-8), 51.9 (C-10), 73.2 (C-5), 102.1 (C-18), 110.0 (C-17), 110.9 (C-14), 125.4 (C-13), 137.2 (C-4), 132.2 (C-12), 146.8 (C-16), 148.5 (C-15), 154.3 (C-3), 198.8 (C-2); EIMS m/z 299 [M]$^+$ (79), 282 (23), 271 (12), 256 (100), 228 (72); HREIMS m/z 299.1155 (calcd for $C_{17}H_{17}NO_4$, 299.1157).

Tumor Cell Growth Inhibition Assays. A microassay for cytotoxicity was performed with the MTT (3-[4,5-dimethylthiazo-2-yl]-5-[3-carboxymethoxymethoxyphenyl]-2[4-sulfophenyl]-*2H*-tetrazolium bromide) assay.[12,13] Briefly, (1−3) × 10^3 cells/100 μL were seeded in 96-well microplates (Nunck, Roskilde, Denmark) and preincubated for 6 h to allow cell attachment. The cells were incubated with each drug for 6 days and then pulsed with 10 μL of MTT (5 mg MTT/mL; Sigma, St. Louis, MO) and incubated for an additional 4 h at 37 °C. The microplates were read at 550 nm on a Multiskan photometer (MR5000; Dynatech, McLean, VA) after lysis of cells with 100 μL of 10% SDS (sodium dodecyl sulfate) in 0.01 M HCl. Control wells contained medium plus cells (total absorbance) or medium alone (background absorbance). Cell death was calculated as the percentage of MTT inhibition.

Human hepatomacellular carcinoma Hep 3B and Hep G2, human colorectal adenocarcinoma HT-29, and human breast adenocarcinoma MCF-7 cells were obtained from American Type Culture Collection (ATCC; Rockville, MD) and grown in DMEM,[13,14] containing 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. For the microassay, the growth medium was supplemented with 10 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer pH 7.3 and incubated at 37 °C in a CO_2 incubator.

Acknowledgment. This work was supported by a grant from the National Science Council of the Republic of China (NSC 92-2320-B037-017).

Supporting Information Available: Portions of ^{1}H NMR signals of **2** in C_5D_5N (A) and CD_3OD (B) are shown in Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Powell, R. G.; Mikolajczak, K. L.; Weisleder, D.; Smith, C. R., Jr. *Phytochemistry* **1972**, *11*, 3317−3320.
(2) Kuo, Y. H.; Lin, C. H.; Hwang, S. Y.; Shen, Y. C.; Lee, Y. L.; Li, S. Y. *Chem. Pharm. Bull.* **2000**, *48*, 440−441.
(3) Kuo, Y. H.; Hwang, S. Y.; Yang Kuo, L. M.; Lee, Y. L.; Li, S. Y.; Shen, Y. C. *Chem. Pharm. Bull.* **2002**, *50*, 1607−1608.
(4) Biemann, K. *Spectral Data for Structure Determination of Organic Compounds*; Springer-Verlag: Cambridge, MA, 1989; p I 150.

(5) Mabry, T. J.; Markham, K. R.; Thomas, M. B. *The Systematic Identification of Flavonoids*; Springer-Verlag: New York, 1970; p 168.
(6) Agrawal, P. K.; Rastogi, R. P. *Heterocycles* **1981**, *16*, 2181−2236.
(7) Markham, K. R.; Sheppard, C.; Geiger, H. *Phytochemistry* **1987**, *12*, 3335−3337.
(8) Agrawal, P. K. *Carbon-13 NMR of Flavonoids* **1989**, *95*−*114*, 123−148.
(9) Powell, R. G.; Mikolajczak, K. L. *Phytochemistry* **1973**, *12*, 2987−2991.
(10) Burkholder, T. P.; Fuchs, P. L. *J. Am. Chem. Soc.* **1990**, *112*, 9601−9613.
(11) Isono, N.; Mori, M. *J. Org. Chem.* **1995**, *60*, 115−119.
(12) Carmichael, J.; Mitchell, J. B.; DeGraff, W. G.; Gamson, J.; Gazdar, A. F.; Johnson, B. E.; Glatstein, E.; Minna, J. D. *Br. J. Cancer* **1988**, *57*, 540−547.
(13) Tsai, C. M.; Gazdar, A. F.; Venzon, D. J.; Steinberg, S. M.; Dedeick, R. L.; Mulshine, J. L.; Kramer, B. S. *Cancer Res.* **1989**, *49*, 2390−2392.
(14) Liu, H. S.; Scrable, H.; Villaret, D. B.; Lieberman, M. A.; Stambrook, P. *J. Cancer Res.* **1992**, *52*, 983−989.

NP0498657