Cytotoxic Hypothemycin Analogues from *Hypomyces subiculosus*

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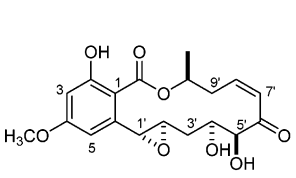
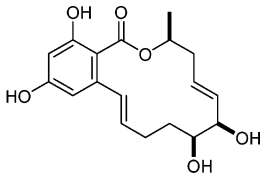
Three new hypothemycin analogues were isolated from the fungal strains *Hypomyces subiculosus* DSM 11931 and DSM 11932. The structures of these compounds were elucidated by spectroscopic methods, chemical conversion, and X-ray crystallographic analysis. One of the analogues, 4-*O*-demethylhypothemycin, exhibited potent and selective cytotoxic activity against cell lines with a BRAF mutation.

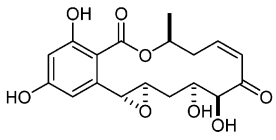
Hypothemycin is a 14-membered resorcylic acid lactone that has been isolated from the fungal fermentations of *Hypomyces trichothecoides*,[1−2] *Coriolus versicolor*,[3] and *Aigialus parvus*.[4] It has been shown to exhibit moderate antimalarial[4] and antifungal[1] activity as well as cytotoxicity against various murine and human cell lines.[3,5] It has also been demonstrated to suppress the growth of murine and human tumor cells transplanted into the backs of mice.[6] Both in vitro and in vivo studies by Zhao et al.[7] and Schirmer et al.[5] have identified hypothemycin as a potent and selective inhibitor of the threonine/tyrosine-specific kinase, MEK, and other protein kinases that contain a conserved cysteine residue in their ATP-binding site. Because many of these kinases play an important role in the signal transduction pathways that regulate cell proliferation, cell differentiation, and apoptosis,[8−10] their aberrant activation can lead to uncontrolled cell proliferation and transformation.[11−13] Hence, compounds that are specific inhibitors of these target proteins are promising candidates as anticancer agents. Here we report on the isolation, structure determination, and cytotoxicity of three new hypothemycin analogues from the fungal strains *Hypomyces subiculosus* DSM 11931 and DSM 11932.



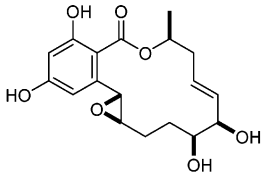
Hypothemycin



Aigialomycin D



4-*O*-Demethylhypothemycin (**1**)



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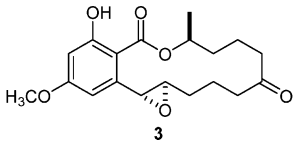

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Results and Discussion

Three new hypothemycin analogues (**1**−**3**) were isolated from the fermentations of *H. subiculosus* DSM 11931 and DSM 11932, in addition to hypothemycin, the major secondary metabolite. One of the analogues, 4-*O*-demethylhypothemycin (**1**), has a molecular formula of $C_{18}H_{20}O_8$, which is consistent with a structure having one methyl group fewer than hypothemycin ($C_{19}H_{22}O_8$). Both the ^{1}H and ^{13}C NMR spectra of **1** (Table 1) indicated the presence of a hydroxyl group at C-4 instead of an *O*-methyl group as observed in hypothemycin. Chemical conversion of the C-4 hydroxyl group of **1** to an *O*-methyl group resulted in the formation of hypothemycin.

A molecular formula of $C_{18}H_{22}O_7$ was established for **2** by HRMS and NMR spectroscopy. NMR analyses (^{1}H, ^{13}C, HSQC, HMBC, and TOCSY) (Table 2) indicated that the double bond between C-7′ and C-8′ of **2** has a *trans*-configuration ($J_{7',8'} = 15.5$ Hz) instead of the *cis*-configuration ($J_{7',8'} = 11.5$ Hz) observed in **1**. They also supported the absence of a ketone functionality at C-6′ and showed that **2** has a different oxidation state at that carbon compared to **1**. The relative configurations at C-5′ and C-6′ were determined by performing X-ray crystallographic analysis on a single crystal of **2** obtained by recrystallization from MeOH and H_2O. The analysis revealed that the alcohol moieties at C-5′ and C-6′, the epoxide at C-1′−C-2′, and the methyl group at C-10′ are positioned on the same face of the macrolactone. Because the carbon backbones of **1**, **2**, and hypothemycin are likely to be formed by the same biosynthetic pathway, the orientation of the C-10′ methyl group of these compounds is assumed to be the same. Hence, an absolute configuration of 1′*R*,2′*R*,5′*S*,6′*R*,10′*S* is proposed for **2**. The structure of **2** is very similar to that of aigialomycin D,[4] which bears a *trans*-olefinic bond between C-1′ and C-2′ instead of the epoxide functionality.

A molecular formula of $C_{19}H_{24}O_6$ was determined for **3** by HRMS and NMR spectroscopy. ^{1}H and ^{13}C NMR analyses (Table 3) revealed that **3** lacks the hydroxyl moieties at C-4′ and C-5′ that are present in hypothemycin. They also indicated a single bond instead of a double bond between C-7′ and C-8′. The HSQC, HMBC, and COSY correlations of **3** are consistent with a structure exhibiting methylene groups at C-4′, C-5′, C-7′, and C-8′. Because the formation of the carbon scaffold and epoxide of **3** are likely to be catalyzed by the same biosynthetic pathway as hypothemycin, the configurations of the epoxide carbons and C-10′ of **3** are assumed to be the same as those of hypothemycin.

The cytotoxicity of the new hypothemycin analogues was measured against three human tumor-derived cell lines: SKOV3, COLO829, and HT29. The ovarian carcinoma cell line SKOV3 expresses the wild-type *B-raf* gene product, while the melonoma cell line COLO829 and the colon cancer cell line HT29 express a mutant *B-raf* (V599E) gene product. As shown in Table 4, the cytotoxic activity of **1** was comparable to that of hypothemycin. In particular, it was more potent against the two cell lines with the BRAF mutation. Compounds **2** and **3**, however, did not exhibit significant cytotoxicity against the tested cell lines.

The RAS-RAF-MEK-ERK-MAP kinase pathway that regulates cell proliferation is constitutively activated by the mutant BRAF (V599E) that is expressed in the COLO829 and HT29 cell lines.[13−15]

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Table 1. NMR Assignments for 4-*O*-Demethylhypothemycin (**1**) in $(CD_3)_2SO$

position	δ_C, mult.	δ_H (*J* in Hz)	HMBC	TOCSY
1	102.8, C			
2	165.1, C			
3	102.3, CH	6.18, s	1, 4	
4	163.5, C			
5	103.9, CH	6.18, s	1, 4, 1′	
6	142.9, C		1′	
1′	56.7, CH	4.27, d (1.5)	5, 2′, 3	2, 5′
2′	63.1, CH	2.68, dt (9.5, 1.5)	1′, 3, 4′	1′, 3′a, 3′b, 4′, 4′-OH
3′a	33.5, CH_2	0.93, dd (14.5, 9.5)	1′, 2	2′, 3′b, 4′, 4′-OH
3′b		1.83, dd (14.5, 10.0)	1′, 2, 4′	2′, 3′a, 4′, 4′-OH
4′	68.9, CH	3.84, dd (10.0, 5.5)		2′, 3′a, 3′b, 5′, 4′-OH, 5′-OH
5′	81.1, CH	4.43, dd (5.0, 1.5)	3′, 4, 6′	4′, 4′-OH, 5′-OH
6′	201.2, C			
7′	128.1, CH	6.40, dd (11.5, 3.0)	6′, 9	8′, 9′a, 9′b, 10′, 10′-CH_3
8′	142.4, CH	6.06, td (11.5, 2.5)	6′, 10	7′, 9′a, 9′b, 10′, 10′-CH_3
9′a	36.1, CH_2	2.46, m	2′, 7, 8′	7′, 8′, 9′b, 10′, 10′-CH_3
9′b		2.88, dt (17.0, 11.0)	2′, 7, 8′, 10′	7′, 8, 9′a, 10′, 10′-CH_3
10′	73.8, CH	5.34, m		7′, 8′, 9′a, 9b, 10′-CH_3
−COO−	170.8, C			
10′-CH_3	20.4, CH_3	1.31, d (6.0)	9′, 10	7, 8′, 9′a, 9′b, 10′
2-OH		11.89, s	2, 3	
4-OH		10.46, br		
4′-OH		5.15, d (6.5)		2′, 3′a, 3′b, 4, 5′
5′-OH		4.88, d (5.0)		5′

Table 2. NMR Assignments for Compound **2** in $(CD_3)_2SO$

position	δ_C, mult.	δ_H (*J* in Hz)	HMBC	TOCSY
1	107.0, C			
2	161.8, C			
3	102.2, CH	6.20, d (2.5)	1, 2, 5	
4	161.5, C			
5	103.6, CH	6.12, d (2.5)	1, 3, 4, 1′	
6	140.8, C			
1′	53.7, CH	3.97, d (2.0)	1, 5, 6, 2′, 3	2
2′	62.7, CH	2.78, dt (5.0, 2.5)	4′	1′, 3′a, 3′b, 4′a, 4′b
3′a	25.4, CH_2	1.67, m	1′, 2′, 4′, 5′	2′, 4′a, 4′b, 5′, 5-OH
3′b		1.88, m	1′, 2′, 4′, 5′	2′, 4′a, 4′b, 5′, 5-OH
4′a	25.0, CH_2	1.31, m	2′, 3′, 5′, 6′	2′, 3′a, 3′b, 5′, 5-OH
4′b		1.52, m	2′, 3′, 5′, 6′	2′, 3′a, 3′b, 5′, 5-OH
5′	72.1, CH	3.38[a]		2′, 3′a, 3′b, 4′a, 4′b, 6′, 5′-OH, 6′-OH
6′	75.1, CH	4.03, br	5′	5′, 7′, 8′, 6′-OH
7′	134.4, CH	5.44, dd (15.5, 6.5)	6′, 8′, 9′	6′, 8′, 9′a, 9′b, 6′-OH
8′	125.1, CH	5.60, dt (15.5, 7.5)	6′, 7′, 9′, 10′	6′, 7′, 9′a, 9′b, 6′-OH
9′a	36.8, CH_2	2.25, dt (15.0, 7.5)	7′, 8, 10′, 10′-CH_3	6′, 7, 8′, 10′, 10′-CH_3
9′b		2.44, m	7′, 8′, 10′, 10′-CH_3	6′, 7′, 8′, 10′, 10′-CH_3
10′	71.8, CH	5.30, m	8′, −COO−	9′a, 9′b, 10′-CH_3
−COO−	169.1, C			
10′-CH_3	19.2, CH_3	1.28, d (6.6)	9′, 10′	9′a, 9b, 10′
2-OH		11.03, br		
4-OH		10.12, br		
5′-OH		4.49, d (5.0)	4′	5′
6′-OH		4.66, d (4.0)		5′, 6′

[a] Signal is obscured in the ^1H NMR spectrum. Chemical shift is determined from multiplicity-edited HSQC.

Previous studies have demonstrated that hypothemycin can selectively inhibit many of the kinases involved in this signaling pathway, including various mitogen receptors, MEK, and ERK, by forming a stable covalent adduct with a conserved cysteine residue in their ATP-binding site.[5,7] Moreover, they have shown that the carbonyl moiety at C-6′ together with the *cis* double bond between C-7′ and C-8′ of hypothemycin are necessary for this inhibitory activity. Resorcylic acid lactones that do not harbor the *cis*-configuration at the C-7′−C-8′ double bond or lack the α,*β*-unsaturated ketone functionality were unable to inhibit these target kinases. These findings are consistent with our observation that **1** exhibits selective cytotoxicity against COLO829 and HT29, while **2** and **3** lack cytotoxic activity against the three cell lines tested.

Although the ability to chemically convert hypothemycin into **1** has eluded us, compound **1** was successfully isolated from the fermentations of *H. subiculosus* DSM 11931. The isolation of **1** will enable us to generate new resorcylic acid lactone analogues that are more water-soluble and more amenable to formulation by chemical modification of the C-4 hydroxyl group. Because the C-4 alcohol moiety is structurally distant from the α,*β*-unsaturated ketone, the cytotoxic activity of the modified compounds may not be greatly affected.

Experimental Section

General Experimental Procedures. ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) were recorded in $(CD_3)_2SO$ solution at 300 K with a Bruker DRX 400 spectrometer equipped with a QNP *z*-axis gradient probe-head. Chemical shifts were referenced to δ 2.50 and 39.5 for ^1H and ^{13}C spectra, respectively. ^1H NMR, ^{13}C NMR, TOCSY, COSY, HMBC, and multiplicity-edited HSQC experiments were carried out. HRESIMS were obtained by manual peak matching versus internal standards by high-resolution mass spectrometry using an Applied Biosystems Mariner time-of-flight mass spectrometer configured with a turbo-ionspray source in negative-ion mode. Optical rotations were

Table 3. NMR Assignments for Compound **3** in $(CD_3)_2SO$

position	δ_C, mult.	δ_H (J in Hz)	HMBC	COSY
1	105.9, C			
2	163.3, C			
3	100.6, CH	6.30, d (2.0)	1, 2, 5	
4	163.8, C			
5	103.3, CH	6.45, d (2.0)	1, 3, 4, 6, 1′	
6	141.6, C			
1′	56.5, CH	4.24, d (1.5)	5, 6, 2′, 3	
2′	63.2, CH	2.72, d (7.5)	6, 1′, 3	
3′a	29.7, CH_2	1.30[a]	1′, 2′, 4′	2′, 3′b
3′b		1.89, m	1′, 2′, 4′, 6′	3′a, 4′b, 5′b
4′a	38.3, CH_2	2.24, dd (17.5, 5.5)	3′, 5, 6′	4′b, 5′a
4′b		2.69, s	3′, 5′, 6′	8′b
5′a	19.0, CH_2	1.51, m	6′	4′a, 5′b
5′b		1.95, m	3′, 4′, 6′	3′a, 5′b
6′	210.7, C		5	
7′a	21.2, CH_2	1.65, m	6′, 9	
7′b		1.71, m	6′	
8′a	41.5, CH_2	2.08, m	6′, 7′, 9′	8′b
8′b		2.64, m	7′, 9′	3′b, 4a, 8′a
9′a	33.9, CH_2	1.44, m	2′, 6, 8′, 10′	4′a
9′b		1.65, m	2′, 8, 10′	8′a
10′	72.9, CH	5.17, m	9′, −COO−	
−COO−	169.9, C			
10′-CH_3	20.6, CH_3	1.30, d (6.0)	9′, 10	2
4-OCH_3	55.5, CH_3	3.76, s	4	
2-OH		11.57, s	1, 2, 3	

[a] Signal is obscured in the ^{1}H NMR spectrum. Chemical shift is determined from multiplicity-edited HSQC.

Table 4. Cytotoxicty of Hypothemycin and Compounds **1**−**3** against Human Cancer Cell Lines (IC_{50}, μM)

	cell lines		
compound	COL829	HT29	SKOV3
hypothemycin	0.07	0.15	5.9
1	0.038	0.10	1.8
2	>100	>100	>100
3	>100	—[a]	>100

[a] Not determined.

determined on a Perkin-Elmer 341 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell. Infrared spectra were obtained on a Perkin-Elmer 1600 series FTIR spectrometer in ZnSe.

Inoculum Preparation. One milliliter of frozen cells of *Hypomyces subiculosus* DSM 11931 or DSM 11932 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) maintained in 20% (v/v) glycerol was inoculated into 50 mL of oat flake medium, consisting of 30 g/L Quaker oatmeal, in a 250 mL unbaffled flask. This primary seed culture was incubated at 22 °C and 190 rpm on a rotary shaker (1 in. stroke) for 4 days. Secondary seed cultures were generated by transferring 2 mL of the primary seed culture into 250 mL unbaffled flasks containing 50 mL of oat flake medium. They were grown at 22 °C and 190 rpm for 2 days and used to inoculate the production cultures.

Production of 1 and 2. Five-liter bioreactors each containing 4 L of CYS80 medium[16] were autoclaved at 121 °C for 60 min and inoculated with 160 mL of DSM 11931 secondary seed cultures. The fermentations were performed at 22 °C for 21 days with an aeration rate of 0.5 v/v/m and an initial agitation rate of 400 rpm. Dissolved oxygen was controlled at 20% of air saturation by an agitation cascade between 400 and 600 rpm. Foaming was controlled by the automatic addition of 100% UCON LB-625. Culture pH was monitored but not controlled.

Production of 3. Unbaffled 2.7 L Fernbach flasks containing 500 mL of MV8 medium[16] were autoclaved at 121 °C for 30 min and inoculated with 20 mL of DSM 11932 secondary seed cultures. The production flasks were incubated at 22 °C and 130 rpm on a rotary shaker (2 in. stroke) for 25 days.

Isolation of 1 and 2. All the solvents used in the purification process contained 0.1% (v/v) HOAc. Fifteen liters of DSM 11931 fermentation broth were extracted with an equal volume of 100% MeOH and filtered. The cell pellet was then extracted again with 7.5 L of 100% MeOH and filtered. The two filtrates were combined and chromatographed on 3 L of HP-20SS resin (Mitsubishi). The column was eluted with 3 column volumes (CV) of 60:40 (v/v) MeOH−H_2O and 3 CV of 70:30 (v/v) MeOH−H_2O. The fractions containing **1** and **2** were chromatographed on 320 mL of C_{18} sorbent (Bakerbond, 40 μm) in 25:75 (v/v) MeCN−H_2O. The resulting fractions enriched in **1** and **2** were further chromatographed on 320 mL of C_{18} in 45:55 (v/v) MeOH−H_2O to separate the two compounds. Solvent exchange was then performed on the product pool containing primarily **1** with 100 mL of C_{18}, eluting with 100% MeOH without HOAc. This step was repeated for the product pool containing primarily **2**. The eluants were dried by rotary evaporation at 40 °C. Solid-phase extraction was performed on the dried product pool containing **1** by adding 2 mL of 100% MeCN to dissolve the yellow impurities. The remaining white solids were filtered and washed with 2 mL of 100% MeCN to yield 145 mg of **1**. The dried product pool containing **2** was dissolved in 10 mL of 100% MeOH and purified by preparative HPLC using an Inertsil C_{18} column (ODS-3, 8 μm, 30 mm × 250 mm) in 65:35 (v/v) MeOH−H_2O. The preparative HPLC step was repeated to further increase the purity of **2** in the resulting product pool. Solvent exchange was then performed as described above on the enriched fractions. The eluant was dried by rotary evaporation at 40 °C to yield 75 mg of **2**.

Isolation of 3. All the solvents used in the purification process contained 0.1% (v/v) HOAc. One liter of *H. subiculosus* DSM 11932 fermentation broth was extracted with an equal volume of 100% MeOH and filtered. The cell pellet was then extracted again with 500 mL of 100% MeOH and filtered. The two filtrates were combined and chromatographed on 200 mL of HP-20SS resin using a step-gradient from 60:40 (v/v) MeOH−H_2O to 100% MeOH. The fractions eluting at 100% MeOH were chromatographed on the same HP-20SS column using a step gradient from 90:10 (v/v) MeOH−H_2O to 100% MeOH. The enriched fractions eluting at 95:5 (v/v) MeOH−H_2O and 100% MeOH were further chromatographed on 100 mL of C_{18} sorbent in 85:15 (v/v) MeCN−H_2O. Solvent exchange was performed on the product pool using the same C_{18} column, eluting with 100% MeCN without HOAc. The eluant was dried by rotary evaporation at 40 °C to yield 181 mg of **3**.

4-*O*-Demethylhypothemycin (1): colorless crystals; dec temp 203 °C; $[\alpha]^{20}_D$ +45 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ϵ) 219 (3.42), 266 (3.06), 306 (2.77) nm; IR (ZnSe) ν_{max} 3447, 3192, 1689, 1622, 1585, 1354, 1311, 1255, 1174, 1048, 1020 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 363.1074 [M − H]$^-$ (calcd for $C_{18}H_{19}O_8$, 363.1065).

Compound 2: colorless crystals; dec temp 183 °C; $[\alpha]^{20}_D$ −5 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ϵ) 217 (3.19), 264 (2.93), 303 (2.64) nm; IR (ZnSe) ν_{max} 3458, 3235, 1644, 1606, 1295, 1240, 1159, 1109, 1061, 1010 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 2; HRESIMS *m*/*z* 349.1282 [M − H]$^-$ (calcd for $C_{18}H_{21}O_7$, 349.1273).

Compound 3: colorless crystals; mp 128−130 °C; $[\alpha]^{20}_D$ +10 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ϵ) 219 (3.40), 264 (3.14), 306 (2.82) nm; IR (ZnSe) ν_{max} 1707, 1650, 1616, 1571, 1355, 1317, 1257, 1206, 1154, 1111 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 3; HRESIMS *m*/*z* 347.1489 [M − H]$^-$ (calcd for $C_{19}H_{23}O_6$, 347.1477).

Chemical Conversion of 1 to Hypothemycin. Diethyl azodicarboxylate (8.5 μL, 0.055 mmol, 4.0 equiv) was added to a stirred solution of **1** (5 mg, 0.137 mmol, 1.0 equiv), PPh_3 (14.4 mg, 0.055 mmol, 4.0 equiv), and anhydrous MeOH (3.3 μL, 0.082 mmol, 6.0 equiv) in anhydrous THF (0.5 mL) at 0 °C. After 30 min, the reaction was complete, as observed by TLC (SiO_2, 30% acetone in CH_2Cl_2). The reaction was concentrated by rotary evaporation, and the residue was purified by flash chromatography (SiO_2, 20% acetone in CH_2Cl_2) to provide 4.2 mg of hypothemycin (84% yield), which was identical in all respects to an authentic sample (400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR, and HPLC).

X-ray Crystal Structure Determination of 2. Crystallographic data: $C_{18}H_{22}O_7$, MW 350.37, orthorhombic, $P2_12_12_1$ (No. 19), a = 8.8474(5) Å, b = 12.4054(7) Å, c = 14.9524(9) Å, V = 1641.1(2) Å^3, D_{calc} = 1.418 g/cm^3, Z = 4, $F_{(000)}$ = 744, $\mu_{(MoK\alpha)}$ = 1.09 cm^{-1}. A fragment of a colorless polyhedral crystal with approximate dimensions of 0.13 × 0.20 × 0.30 mm was mounted on a glass fiber. The diffraction intensity data were acquired at −118 ± 1 °C by a Bruker APEX CCD area detector with graphite-monochromated Mo Kα radiation (λ = 0.71069 Å). Lattice determination and data collection were performed using SMART. A total of 9327 reflections were collected with 2137 unique reflections (R_{int} = 0.023). Data integration to a maximum 2θ value of 57.2° was performed using SAINT with correction for Lorentz

and polarization effects. An empirical absorption correction was applied using SADABS with $T_{max} = 1.00$ and $T_{min} = 0.87$. The crystal structure was solved by direct methods using SIR-97 and expanded using Fourier techniques. The non-hydrogen atoms were refined anisotropically for 1709 observed reflections with $I > 3.00\sigma(I)$ and 238 variable parameters by the full matrix least-squares method to yield $R = 0.029$ and $R_w = 0.040$. The hydroxyl hydrogens were located in a difference Fourier map and were refined with fixed thermal parameters.[17]

Biological Assays. All cell lines (SKOV3, COLO829, and HT29) are maintained in McCoy's 5A modified medium (Invitrogen) with 10% fetal bovine serum (Hyclone) and 2 mM L-glutamine in a humidified incubator at 37 °C with 5% CO_2. The tumor cells were plated into black 96-well microtiter plates as 50 μL aliquots at approximately 4000 cells per well. The cells were allowed to adhere overnight. Stock solutions of the hypothemycin analogues were dissolved in DMSO to a concentration of 50 mM. Serial dilutions of the compounds ranging from 100 μM to 0.1 pM were prepared in cell culture medium. Fifty microliters of each dilution was added to the wells containing cells in duplicates. After 72 h, the plates were cooled to room temperature for 30 min, and 100 μL of CellTilter-Glo luminescent reagent (Promega) was added to each well. The contents were mixed for 5 min and incubated at room temperature for 30 min. The luminescence of each well was measured by using a Wallac VICTOR multilabel counter (Perkin-Elmer).

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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