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Pyrrolidinones from the Ascomycete Fungus *Albonectria rigidiuscula*

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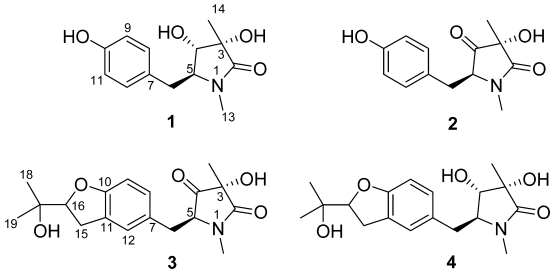
The new pyrrolidinones, rigidiusculamides A−D (**1**−**4**), have been isolated from the crude extract of the ascomycete fungus *Albonectria rigidiuscula*. The structures of these compounds were elucidated primarily by NMR experiments. The absolute configuration of the 3,4-diol moieties in **1** and **4** was assigned using Snatzke's method. Compounds **1** and **2** showed modest cytotoxicity against the human tumor cell lines HeLa and MCF-7.

Natural products incorporating the pyrrolidinone moiety have been isolated frequently from marine sponges and *Streptomyces*,[1−6] with only a limited number of derivatives from plants and fungi.[7−11] The pyrrolidin-2,4-dione moiety is commonly encountered as a 3-acyl or, less commonly, as a 4-*O*-alkyl ether derivative in naturally occurring compounds.[12] Examples of fungal secondary metabolites in this class are mostly tetramic acid derivatives including cryptocin, an antimycotic and antifungal agent isolated from the endophytic fungus *Cryptosporiopsis cf. quercina*,[9] pachydermin, an oxalylated tetramic acid from the basidiomycete *Chamonixia pachydermis*,[10] and virgineone, an antifungal glycoside from the saprotrophic *Lachnum virgineum*.[11] During an ongoing search for new cytotoxic metabolites from rarely studied fungi, the ascomycete fungus *Albonectria rigidiuscula* (Berk. & Broome) Rossman & Samuels (4367) isolated from twigs of an identified tree on Jigong Mountain, Hena Province, People's Republic of China, was subjected to chemical study. Although *A. rigidiuscula* is a common species in the tropics, it had not been identified in China until 2003.[13] The fungus was grown in a solid-substrate fermentation culture. An organic solvent extract of the culture showed cytotoxicity against HeLa and MCF-7 human tumor cell lines. Fractionation of the extract afforded four new pyrrolidinone derivatives, which we named rigidiusculamides A−D (**1**−**4**). Details of the isolation, structure elucidation, and cytotoxicity of these metabolites are reported herein.



Rigidiusculamide A (**1**) was obtained as a colorless oil. It was assigned a molecular formula of $C_{13}H_{17}NO_4$ (six degrees of unsaturation) on the basis of HRESIMS (*m*/*z* 274.1048 $[M + Na]^+$; Δ +0.2 mmu) and NMR data (Table 1). The ^1H and ^{13}C NMR spectra of **1** displayed resonances for two methyl groups including one *N*-methyl (δ_H/δ_C 2.71/28.0), one methylene, two methines (one of which is oxygenated), one oxygenated sp^3 quaternary carbon, six aromatic carbons (four of which are protonated), and one carboxylic carbon (δ_C 175.4). These data, together with three exchangeable protons, accounted for all the ^1H and ^{13}C NMR resonances and suggested that **1** was a bicyclic compound. ^1H NMR resonances for two sets of doublets (8.0 Hz each) integrating for four aromatic protons were observed at δ_H 6.76 and 7.20, respectively, suggesting the presence of a *p*-substituted aryl ring with C-10 (δ_C 156.7) being oxygenated. Interpretation of the $^1H-^1H$ COSY NMR data of **1** identified a proton spin-system corresponding to the C-4−C-6 fragment. HMBC correlations from H_2-6 to C-7, C-8, and C-12 led to the connection of C-6 to C-7, establishing the *p*-hydroxybenzyl unit. Correlations from H_3-14 to C-2, C-3, and C-4 indicated that C-2, C-4, and C-14 were all attached to the C-3 oxygenated sp^3 quaternary carbon. Key correlations from the *N*-methyl protons H_3-13 to C-2 and C-5 completed the dimethylpyrrolidin-2-one moiety. The presence of an amide bond in **1** was also confirmed by an HMBC cross-peak from the downfield methine proton H-5 (δ_H 3.66) to the C-2 amide carbon. Considering the oxygenated nature of C-3, C-4, and C-10, as well as the unsaturation requirement for **1**, the remaining three exchangeable protons were assigned to the latter carbons by default. On the basis of these data, the gross structure of rigidiusculamide A was established as shown in **1**.

The relative configuration of **1** was determined by analysis of its NOESY data. NOESY correlations of H_3-14 with H-4 and H-8 placed these protons on the same face of the dihydroxydimethylpyrrolidin-2-one ring, thereby establishing the relative configuration of **1**.

The absolute configuration of the 3,4-diol moiety in **1** was assigned using the in situ dimolybdenum CD method developed by Snatzke and Frelek.[14,15] Upon addition of dimolybdenum tetraacteate $[Mo_2(OAc)_4]$ to **1** in DMSO solution, a metal complex of chiral *vic*-diol with the achiral $Mo_2(OAc)_4$ was generated as an auxiliary chromophore. Since **1** has an inherent CD resulting from the C-2 ketone chromophore, this contribution was subtracted to give the induced CD of the metal complex to avoid its overlap (>250 nm) with those generated after addition of $Mo_2(OAc)_4$. Therefore, the observed sign of the Cotton effect in the induced spectrum originates solely from the chirality of the *vic*-diol moiety expressed by the sign of the O−C−C−O torsion angle. The positive Cotton effects observed at around 310 and 400 nm, respectively, in the induced CD spectrum (Figure 1) permitted assignment of the 3*S* and 4*S* absolute configuration on the basis of the empirical rule proposed by Snatzke, with the bulkier benzyl group pointing away from the remaining portion of the complex (Figure 2). Considering the relative configuration established by NOESY data, **1** was assigned a 3*S*, 4*S*, 5*S* absolute configuration.

Rigidiusculamide B (**2**) gave a pseudomolecular ion $[M + Na]^+$ peak at *m*/*z* 272.0900 (Δ −0.7 mmu) by HRESIMS, consistent with a molecular formula of $C_{13}H_{15}NO_4$ (seven degrees of unsaturation). Analysis of its ^1H and ^{13}C NMR data (Table 1) revealed the same *p*-substituted aryl ring as found in **1**, but the resonances for the

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Table 1. NMR Data for **1**−**4**

	1				**2**		**3**		**4**	
pos.	δ_C[a], mult.	δ_H[b] (*J* in Hz)	HMBC[a,b]	NOESY[d]	δ_C[a], mult.	δ_H[b] (*J* in Hz)	δ_C[a], mult.	δ_H[b] (*J* in Hz)	δ_C[e], mult.	δ_H[b] (*J* in Hz)
2	175.4, qC				173.3, qC		173.2, qC		175.4, qC	
3	75.1, qC				71.0, qC		71.1, qC		75.1, qC	
4	73.0, CH	3.64, d (5.0)	2, 5, 6, 14	8, 14	210.6, qC		210.7, qC		72.9, CH	3.69, d (5.0)
5	63.1, CH	3.66, dt (8.0, 5.0)	2, 4, 6, 7		68.0, CH	4.21, t (4.5)	68.1, CH	4.20, t (5.0)	63.1, CH	3.67, t (5.0)
6a	33.1, CH_2	2.99, dd (14, 5.0)	4, 5, 7, 8, 12		34.3, CH_2	2.99, dd (15, 4.5)	34.6, CH_2	3.15, dd (14, 5.0)	33.2, CH_2	2.98, dd (14, 5. 0)
6b		2.96, dd (14, 8.0)	4, 5, 7, 8, 12			2.96, dd (15, 4.5)		3.12, dd (14, 5.0)		3.00, dd (14, 5. 0)
7	129.7, qC				126.9, qC		127.8, qC		130.5, qC	
8	131.4, CH	7.20, d (8.0)	6, 10, 12	4	131.6, CH	6.89, d (8.5)	127.5, CH	6.76, d (8.0)	126.8, CH	7.12, d (8.0)
9	115.9, CH	6.76, d (8.0)	7, 11		116.0, CH	6.73, d (8.5)	109.3, CH	6.57, d (8.0)	109.2, CH	6.64, d (8.0)
10	156.7, qC				157.4, qC		160.3, qC		159.6, qC	
11	115.9, CH	6.76, d (8.0)	7, 9		116.0, CH	6.73, d (8.5)	128.9, qC		128.5, qC	
12	131.4, CH	7.20, d (8.0)	6, 8, 10		132.0, CH	6.89, d (8.5)	130.2, CH	6.86, s	129.6, CH	7.24, s
13	28.0, CH_3	2.71, s	2, 5		28.2, CH_3	3.03, s	28.1, CH_3	3.02, s	27.9, CH_3	2.76, s
14	21.7, CH_3	1.17, s	2, 3, 4	4	19.8, CH_3	0.50, s	19.9, CH_3	0.52, s	21.6, CH_3	1.21, s
15a							30.9, CH_2	3.19, dd (16, 9.0)	31.1, CH_2	3.27, dd (16, 9.0)
15b								3.11, dd (16, 9.0)		3.24, dd (16, 9.0)
16							90.2, CH	4.56, t (9.0)	90.2, CH	4.61, t (9.0)
17							71.3, qC		71.3, qC	
18							25.1, CH_3	1.16, s	25.4, CH_3	1.27, s
19							25.9, CH_3	1.17, s	26.0, CH_3	1.24, s

[a] Recorded at 125 MHz in acetone-d_6. [b] Recorded at 500 MHz in acetone-d_6. [c] HMBC correlations, optimized for 8 Hz, are from proton(s) stated to the indicated carbon. [d] Recorded at 500 MHz in DMSO-d_6. [e] Recorded at 150 MHz in acetone-d_6.

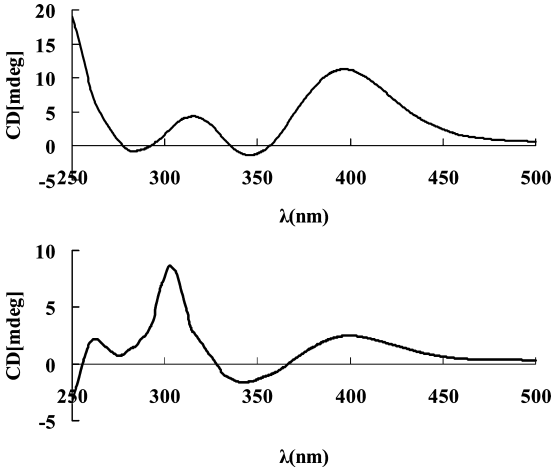


Figure 1. CD spectra of **1** (top) and **4** (bottom) in DMSO containing $Mo_2(OAc)_4$ with the inherent CD spectra subtracted.

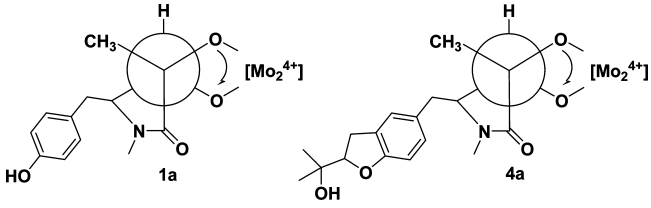


Figure 2. Conformations of the Mo_2^{4+} complexes of **1** and **4**.

dihydroxydimethylpyrrolidin-2-one moiety in **1** were different from those for the remaining portion of **2**. Specifically, the C-4 oxygenated methine unit (δ_H/δ_C 3.64/73.0) was replaced by a ketone functionality (δ_C 211.0) in **2**, and this observation was confirmed by HMBC correlations from H-5, H_2-6, and H_3-14 to C-4. Therefore, the 3-hydroxy-1,3-dimethylpyrrolidin-2,4-dione moiety was established to complete the gross structure of **2**.

The relative configuration of **2** was assigned on the basis of NOESY data (Figure S9, Supporting Information). Correlation of H_3-14 with H-8 indicated that these protons are on the same face of the pyrrolidin-2,4-dione moiety, whereas those of H-5 with H-12 and H_3-13 placed them on the opposite face of the ring. This assignment was indirectly supported by the absence of a NOESY correlation between H-5 and H_3-14. Considering the fact that **2** is the oxidative product of **1**, the stereogenic centers C-3 and C-5 in both metabolites presumably have the same absolute configuration.

The elemental composition of rigidiusculamide C (**3**) was established as $C_{18}H_{23}NO_5$ (eight degrees of unsaturation) by its HRESIMS data (*m*/*z* 356.1460 $[M + Na]^+$; Δ +0.8 mmu). The NMR spectra of **3** displayed resonances for the same 3-hydroxy-1,3-dimethylpyrrolidin-2,4-dione moiety as found in **2**, but those for the aromatic portion were significantly different. Analysis of the $^1H-^1H$ coupling patterns for aromatic protons indicated that the *p*-substituted aryl ring in **1** and **2** was replaced by a 1,2,4-trisubstituted aryl moiety in **3**. Additional resonances comprising two methyl singlets, one methylene, one *O*-methine, and one oxygenated sp^3 quaternary carbon were also observed in the spectra of **3**. The *O*-methine proton H-16 was correlated to the methylene protons H_2-15 in the COSY spectrum of **3**, revealing the connectivity of C-15 to C-16. HMBC correlations from H_2-15 to C-10, C-11, and C-12 and from H-16 to C-10 established the dihydrobenzofuran ring with connectivity of C-10 to C-16 via an oxygen atom. Other correlations from H_3-18 and H_3-19 to C-16 and C-17 indicated that the oxygenated sp^3 quaternary carbon C-17 was attached to C-16, C-18, and C-19. The remaining exchangeable proton was assigned as C-17-OH by default to complete the gross structure of **3** as depicted.

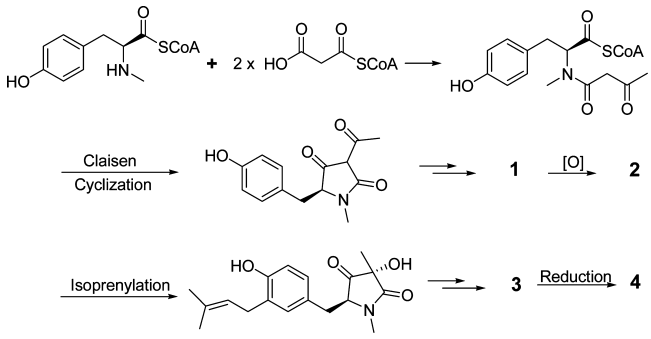
The relative configuration of the 3-hydroxy-1,3-dimethylpyrrolidin-2,4-dione ring in **3** was assigned as shown by analogy to **2**. The absolute configurations of C-3 and C-5 in **3** were assigned by comparison of the CD spectrum of **3** with that of **2** (Figures S10 and S11; Supporting Information). The CD spectra of **2** and **3** showed nearly identical positive and negative Cotton effects, suggesting the same absolute configuration for C-3 and C-5 in both compounds. The absolute configuration of the C-16 stereogenic center in **3** was not assigned.

The molecular formula of rigidiusculamide D (**4**) was determined to be $C_{18}H_{25}NO_5$ (seven degrees of unsaturation) by analysis of its HRESIMS data (*m*/*z* 358.1628 $[M + Na]^+$; Δ −0.3 mmu), two mass units more than **3**. The NMR data of **4** (Table 1) revealed nearly identical structural features to those found in **3**, except that the C-4 ketone carbon was replaced by an oxygenated methine in **4**, indicating the reduction of the ketone functionality of the pyrrolidin-2,4-dione moiety. This observation was supported by relevant $^1H-^1H$ COSY and HMBC correlations. The absolute configuration of the dihydroxydimethylpyrrolidin-2-one moiety in **4** was also assigned using Snatzke's method as described for **1**.

Table 2. Cytotoxicity of Compounds **1**−**4**

	IC_{50} (μM)	
compound	HeLa	MCF-7
1	95.6 ± 8.8	95.6 ± 7.0
2	48.2 ± 6.4	100.4 ± 17.2
3	>120.0	>120.0
4	>120.0	>120.0
5-fluorouracil	10.0	15.0

Scheme 1. Plausible Biosyntheses of **1**−**4**



Compounds **1**−**4** were evaluated for cytotoxicity against two human tumor cell lines, HeLa (cervical epithelium) and MCF-7 (breast adenocarcinoma) (Table 2). Compounds **1** and **2** showed modest cytotoxicity against HeLa cells, with IC_{50} values of 95.6 and 48.2 μM, respectively (the positive control 5-fluorouracil showed an IC_{50} value of 10.0 μM), whereas **3** and **4** did not show detectable activities when tested at 120 μM. Compounds **1** and **2** were not further evaluated for antitumor effects due to their modest cytotoxic effects.

Rigidiusculamides A−D (**1**−**4**) incorporate the pyrrolidin-2-one (**1** and **4**) and the pyrrolidin-2,4-dione moieties (**2** and **3**) as found in some tetramic acid derivatives,[12] such as the dysidamides and the tetrapetalones.[16,17] However, **1**−**4** differ significantly from these known compounds in having a stereogenic center at C-3 and different substituents (*p*-hydroxybenzyl for **1** and **2**; substituted benzofuran rings for **3** and **4**) on the pyrrolidinone moiety. Compounds **2** and **3** are the C-4 oxidation products of **1** and **4**, respectively. Biosynthetic studies of some pyrrolidinone-containing metabolites have demonstrated that these compounds originate from the cyclization of an amino acid and a polyketide.[12,18] The biosyntheses of **1** and **2** could proceed in a similar manor, whereas those for **3** and **4** could be more complex with an isoprene unit involved (Scheme 1).

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on a Shimadzu Biospec-1601 spectrophotometer. IR data were recorded using a Nicolet Magna-IR 750 spectrophotometer. ^{1}H and ^{13}C NMR data were acquired with Varian Mercury-500 and -600 spectrometers using solvent signals (acetone-d_6: δ_H 2.05/δ_C 29.8, 206.1; DMSO-d_6: δ_H 2.47) as references. The HMQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. ESIMS data were recorded on a Bruker Esquire 3000^{plus} spectrometer, and HRESIMS data were obtained using Bruker APEX III 7.0 T and APEX II FT-ICR spectrometers, respectively.

Fungal Material. The culture of *A. rigidiuscula* (4367) was isolated from twigs of an identified tree on Jigong Mountain, Hena Province, People's Republic of China, in November, 2003. The fungus was identified by one of the authors (W.Z.) based on morphology and assigned the accession No. 4367 (= HMAS 91744) in W.Z.'s culture collection at the Institute of Microbiology, Chinese Academy of Sciences, Beijing. The fungal strain was cultured on slants of potato dextrose agar (PDA) at 25 °C for 10 days. Agar plugs were used to inoculate in 250 mL Erlenmeyer flasks, each containing 50 mL of media (0.4% glucose, 1% malt extract, and 0.4% yeast extract), and the final pH of the media was adjusted to 6.5 before sterilization. Flask cultures were incubated at 25 °C on a rotary shaker at 170 rpm for five days. Fermentation was carried out in twelve 500 mL Erlenmeyer flasks each containing 80 g of rice. Spore inoculum was prepared by suspension in sterile, distilled H_2O to give a final spore/cell suspension of 1 × 10^6/mL. Distilled H_2O (100 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 lb/in.^{2} for 30 min. After cooling to room temperature, each flask was inoculated with 5.0 mL of the spore inoculum and incubated at 25 °C for 40 days.

Extraction and Isolation. The fermented material was extracted repeatedly with EtOAc (4 × 0.5 L), and the organic solvent was evaporated to dryness under vacuum to afford the crude extract (6.0 g), which was fractionated by silica gel VLC using petroleum ether−EtOAc gradient elution. The fractions eluted with 60% (150 mg) and 70% (250 mg) EtOAc were combined and separated again by Sephadex LH-20 column chromatography using 1:1 CH_2Cl_2−MeOH as eluent. Purification of the resulting subfractions with different gradients by RP HPLC (Agilent Zorbax SB-C18 column; 5 μm; 9.4 × 250 mm; 2 mL/min) afforded rigidiusculamides A (**1**; 2.0 mg, t_R 20.72 min; 25% MeOH in H_2O for 5 min, followed by 25−32% for 35 min), B (**2**; 30.0 mg, t_R 24.83 min; 22% MeOH in H_2O for 5 min, followed by 22−42% for 30 min), C (**3**; 20.0 mg, t_R 29.98 min; 35% MeOH in H_2O for 5 min, followed by 35−52% for 35 min), and D (**4**; 1.8 mg, t_R 23.65 min; 40% MeOH in H_2O for 5 min, followed by 40−52% for 35 min).

Rigidiusculamide A (1): colorless oil; $[\alpha]^{25}_D$ −16.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 230 (3.51), 278 (3.09) nm; CD (*c* 1.0 × 10^{-4} M, DMSO) λ_{max} ($\Delta\varepsilon$) 280 (+1.8), 250 (−3.2) nm; IR (neat) ν_{max} 3352, 2922, 1677, 1614, 1516, 1447, 1368, 1239, 1095 cm^{-1}; ^{1}H, ^{13}C NMR, HMBC, and NOESY data see Table 1; HRESIMS *m*/*z* 274.1048 (calcd for $C_{13}H_{17}NO_4Na$, 274.1050).

Rigidiusculamide B (2): colorless oil; $[\alpha]^{25}_D$ −19.0 (*c* 1.5, MeOH); UV (MeOH) λ_{max} (log ε) 232 (3.67), 277 (3.33) nm; IR (neat) ν_{max} 3411, 3341, 2920, 1779, 1672, 1610, 1514, 1441, 1264, 1123 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; HMBC data (acetone-d_6, 500 MHz) H-5 → C-2, 4, 6, 7, 13; H_2-6 → C-4, 5, 7, 8, 12; H-8 → C-6, 10, 12; H-9 → C-7, 11; H-11→ C-7, 9; H-12 → C-6, 8, 10; H_3-13 → C-2, 5; H_3-14 → C-2, 3, 4; NOESY correlations (DMSO-d_6, 500 MHz), H_3-14 ↔ H-8; H-5 ↔ H-12, H_3-13; HRESIMS *m*/*z* 272.0900 (calcd for $C_{13}H_{15}NO_4Na$, 272.0893).

Rigidiusculamide C (3): colorless oil; $[\alpha]^{25}_D$ −31.0 (*c* 1.2, MeOH); UV (MeOH) λ_{max} (log ε) 234 (3.51), 285 (3.30) nm; IR (neat) ν_{max} 3392, 2977, 2931, 1776, 1687, 1610, 1492, 1248, 1117 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; HMBC data (acetone-d_6, 500 MHz) H-5 → C-4, 6, 7; H_2-6 → C-4, 5, 7, 8, 12; H-8 → C-6, 7, 9, 10; H-9 → C-7, 10, 11; H-12 → C-6, 10, 11, 15; H_3-13 → C-2, 5; H_3-14 → C-2, 3, 4; H_2-15 → C-10, 11, 12, 16, 17; H-16 → C-10, 11, 18, 19; H_3-18 → C-16, 17, 19; H_3-19 → C-16, 17, 18; HRESIMS *m*/*z* 356.1460 (calcd for $C_{18}H_{23}NO_5Na$, 356.1468).

Rigidiusculamide D (4): colorless oil; $[\alpha]^{25}_D$ −17.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 230 (3.64), 285 (3.35) nm; CD (*c* 1.0 × 10^{-4} M, DMSO) λ_{max} ($\Delta\varepsilon$) 288 (+1.3), 250 (−0.21) nm; IR (neat) ν_{max} 3394, 2974, 2928, 1782, 1614, 1492, 1245, 1113 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; HMBC data (acetone-d_6, 600 MHz) H-4 → C-2, 14; H-5 → C-2, 6; H_2-6 → C-4, 5, 7, 12; H-8 → C-6, 10, 12; H-9 → C-7, 10, 11; H-12 → C-6, 8, 10, 15; H_3-13 → C-2, 5; H_3-14 → C-2, 3, 4; H_2-15 → C-10, 11, 16, 17; H_3-18 → C-16, 17, 19; H_3-19 → C-16, 17, 18; HRESIMS *m*/*z* 358.1628 (calcd for $C_{18}H_{25}NO_5Na$, 358.1625).

Absolute Configuration of the 3,4-Diol Functionalities in 1 and 4.[14,15] HPLC grade DMSO was dried with 4 Å molecular sieves. According to the published procedure,[19] mixtures of 1:1.3 diol/$Mo_2(OAc)_4$ for **1** and **4** were subjected to CD measurements at concentrations of 0.5 and 1.0 mg/mL, respectively. The first CD spectrum was recorded immediately after mixing, and its time evolution was monitored until stationary (about 10 min after mixing). The inherent CD was subtracted. The observed signs of the diagnostic bands at around 310 and 400 nm in the induced CD spectrum were correlated to the absolute configuration of the 3,4-diol moiety.

MTT Assay.[20] The assay was run in triplicate. In a 96-well plate, each well was plated with 10^4 cells. After cell attachment overnight, the medium was removed, and each well was treated with 50 μL of medium containing 0.2% DMSO, or appropriate concentrations of the test compounds and the positive control 5-fluorouracil (10 mg/mL as stock solution of a compound in DMSO and serial dilutions; the test compounds showed good solubility in DMSO and did not precipitate

when added to the cells). Cells were treated at 37 °C for 4 h in a humidified incubator at 5% CO_2 first and were allowed to grow for another 48 h after the medium was changed to fresh Dulbecco's modified Eagle medium. MTT (Sigma) was dissolved in serum-free medium or PBS at 0.5 mg/mL and sonicated briefly. In the dark, 50 μL of MTT/medium was added into each well after the medium was removed from wells, and the plate was incubated at 37 °C for 3 h. Upon removal of MTT/medium, 100 μL of DMSO was added to each well, and the plate was agitated at 60 rpm for 5 min to dissolve the precipitate. The assay plate was read at 540 nm using a microplate reader.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of **1**−**4** and CD spectra of **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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