Capisterones A and B, which Enhance Fluconazole Activity in *Saccharomyces cerevisiae,* from the Marine Green Alga *Penicillus capitatus*

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A whole-cell-based assay using *Saccharomyces cerevisiae* strains that overexpress *Candida albicans* CDR1 and MDR1 efflux pumps has been employed to screen natural product extracts for reversal of fluconazole resistance. The tropical green alga *Penicillus capitatus* was selected for bioassay-guided isolation, leading to the identification of capisterones A and B (**1** and **2**), which were recently isolated from this alga and shown to possess antifungal activity against the marine pathogen *Lindra thallasiae.* Current work has assigned their absolute configurations using electronic circular dichroism and determined their preferred conformations in solution based on detailed NOE analysis. Compounds **1** and **2** significantly enhanced fluconazole activity in *S. cerevisiae,* but did not show inherent antifungal activity when tested against several opportunistic pathogens or cytotoxicity to several human cancer and noncancerous cell lines (up to 35 μM). These compounds may have a potential for combination therapy of fungal infections caused by clinically relevant azole-resistant strains.

The molecular mechanisms of antifungal drug resistance may involve a variety of factors such as mutation of target genes and decreased drug concentrations in the cells due to overexpression of efflux pumps.[1,2] In recent years, the rapid development of such drug resistance, particularly for azole antifungals, has highlighted the need for new strategies in antimycotic therapies.[1−5] Efflux pump inhibition has been considered as a promising approach in this regard. Two families of efflux pumps found in *Candida albicans* include the major facilitators (multidrug resistance, MDR) that are fueled by a proton gradient and the P-glycoprotein ABC transporters (*Candida* drug resistance, CDR) that require ATP hydrolysis for energy. Within each family, several subtypes have been discovered (i.e., CDR1, CDR2, MDR1).[1,2] We have established a whole-cell-based assay using *Saccharomyces cerevisiae* strains that overexpress *C. albicans* CDR1 and MDR1 efflux pumps to screen for natural products that can reverse fluconazole resistance and may not be necessarily antifungal.[6]

Results and Discussion

In a continuing effort to search for new efflux inhibitors from natural sources, we have screened over 5000 marine extracts from the National Cancer Institute Open Repository. In the presence of a subinhibitory concentration of fluconazole, an extract of the tropical green alga *Penicillus capitatus* (Halimedaceae) that had an IC$_{50}$ value of 55 μg/mL against *S. cerevisiae* DSY 415 strain (overexpressing the CDR1 efflux pump) and an IC$_{50}$ value of <6 μg/mL against *S. cerevisiae* DSY 416 strain (overexpressing the MDR1 efflux pump) was selected for bioassay-guided fractionation. Two cycloartanone triterpene sulfates (**1** and **2**) that significantly enhanced fluconazole activity in the two efflux pump-overexpressing strains were identified. Analysis of the spectroscopic data including high-resolution MS, IR, and NMR permitted identification of compounds **1** and **2** as capisterones A and B, respectively, which were recently isolated from this alga and shown to possess antifungal activity against the marine pathogen *Lindra thallasiae.* However, the absolute configuration and conformation of the two compounds were undefined.[7] This report describes the assignment

of their absolute configuration and conformation as well as their biological activities.

1 R = Ac
2 R = H
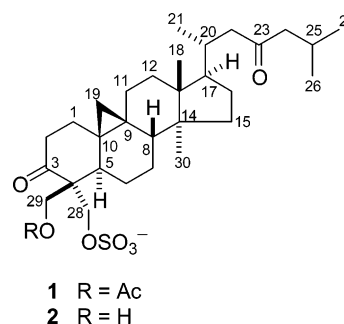
Compound **1** showed a negative specific rotation, $[\alpha]^{25}_D$ −6.3 (MeOH, *c* 0.80), in contrast to most naturally occurring cycloartan-3-ones and its derivatives, which generally have a positive specific rotation,[8−19] while compound **2** gave a small positive specific rotation, $[\alpha]^{25}_D$ +2.1 (MeOH, *c* 0.33). The above specific rotation values for the two compounds are also close to those reported by Puglisi et al.[7] This posed the question whether these compounds still possess the absolute configuration of the typical naturally occurring cycloartane skeleton. Although acetylation of **2** may induce a negative specific rotation in **1**, unambiguous evidence is needed to address the absolute configuration of the two compounds.

Circular dichroism (CD) and optical rotatory dispersion (ORD) have played important roles in determining the absolute configuration of chiral molecules. In particular, the recent advances on theoretical calculations of CD spectra and optical rotations have greatly enhanced their value in this regard.[20−22] It has been reported that 3-keto-5α-steroids and their 4α-methyl derivatives exhibit a positive Cotton effect around 300 nm in their ORD spectra, while their 4,4-dimethyl derivatives (or natural triterpenoids) displayed a negative Cotton effect in this region.[8] For cycloartan-3-ones, the introduction of the 9,19-cylopropyl functionality does not affect the sign of the Cotton effect in their ORD/CD spectra (presumably due to retention of the A-ring conformation),[8,23−25] although additional substitutions on ring B or other rings may alter the sign of the Cotton effect.[23] Compounds **1** and **2** exhibited strong negative Cotton effects around 290 nm in their CD spectra. When compared with the cycloartan-3-one compounds, which generally give a

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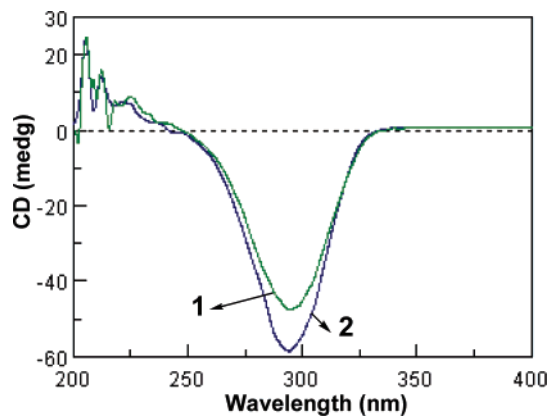
Figure 1. Experimental CD spectra of compounds **1** and **2**.

Table 1. Calculated CD Data for Compound **2**

E (eV)	λ (nm)	f^a	$R_{velocity}{}^b$	$R_{length}{}^b$
4.2315	293	0.0007	−17.7165	−18.9575
4.3083	287	0.0001	−1.7954	−5.6371
4.7717	259	0.0013	−1.1733	−0.5280

[a] Oscillator strength. [b] Rotatory strength in cgs (10^{-40} erg esu cm/ Gauss).

negative Cotton effect in the 290−300 nm range in their ORD or CD spectra,[23−25] compounds **1** and **2** should possess the same absolute configuration for the tetracyclic ABCD-ring system as in the naturally occurring cycloartane triterpenes. The very similar CD Cotton effects of **1** and **2** indicate that the acetyl substitution in **1** does not contribute to its overall CD absorption curve (Figure 1), although this derivatization did alter the sign of the specific rotation from positive in **2** to negative in **1**.

Theoretical calculation of the electronic CD spectrum (ECD) of compound **2** based on the arbitrarily assigned absolute configuration as shown in its structural formula was performed using time-dependent density functional theory (TDDFT)[26−29] with the 6-31G* basis set by the Gaussian03[30] program package. The results were in agreement with the experimental CD spectrum: a high-amplitude negative Cotton effect was obtained at 293 nm, which was attributable to the n → π^* transition of the C=O functionality. Detailed calculated data of the excitation energies, oscillator strengths, and rotational strengths for the three transition states requiring the lowest excitation energies are summarized in Table 1 for compound **2**. This is the first correlation of calculated and experimental CD results for this class of compounds. Thus, the absolute configuration of compound **2** has been firmly established as shown for typical naturally occurring cycloartanane triterpenes, which are biosynthesized in a highly stereoselective way.[31] Since a direct chemical conversion of **2** into **1** has been achieved by acetylation,[7] compound **1** should possess the same absolute configuration.

Cycloartane triterpenes and their glycosides, which are widely distributed in terrestrial plants, but rarely isolated from marine sources, are an important class of biologically active compounds.[7,8−19] Few studies have investigated the conformation of this class of compounds, although it is presumed that the conformation of the tetracyclic ABCD-ring system may be similar to those whose single-crystal structures have been determined by X-ray crystallographic analysis. Verotta et al.[32] studied the conformation of two cycloartan-3-ol glucosides by using NMR in pyridine-d_6 and molecular modeling (AM1). They proposed that the most stable conformer (focusing on the aglycone moiety) possesses a chair conformation for ring A and half-chair conformation for both rings B and C. A less stable conformation in which ring C adopts a half-boat form may also exist due to an equilibrium with the former in solution. They further demonstrated that the hydroxyl substitution at C-6 of the aglycone does not significantly affect the overall

Table 2. Selected Geometric Parameters for Compound **2**

torsion angle (deg)	2^a	2^b	ref compound^c
O3−C3−C2	116.51	119.70	119.37
O3−C3−C4	118.01	122.77	122.27
C19−C9−C10	60.11	59.36	59.44
C1−C2−C3−O3	−158.11	−133.98	−138.3
O3−C3−C4−C5	162.61	137.95	138.7
C7−C8−C9−C19	91.11	89.03	89.1
C6−C5−C10−C19	−49.11	−48.29	−49.1
C5−C10−C19−C9	108.91	108.69	109.1
C14−C13−C17−C20	−161.21	−165.03	−165.5
C15−C16−C17−C20	146.01	149.30	151.1
C20−C17−C16	111.51	112.87	112.22
C20−C17−C13	120.11	119.50	120.70

[a] Data from the conformer generated by Chem3D Pro 8.0. [b] Data from conformer optimized by B3LYP/6-31G*. [c] X-ray data of (24*R*)-24,25-dihydroxycycloartan-3-one.[33]

conformations of the tetracyclic ABCD-ring system. Horgen et al.[16] also briefly described the conformation of a cycloartan-3-ol sulfate in MeOH-d_4 on the basis of NOE evidence and molecular modeling (HyperChem, MM+ force field). A twisted-boat conformation for ring C was evident from the notable NOE correlation between H-11α and Me-30. It is important to understand the conformations of biologically active compounds in solution since they exert biological functions in aqueous media. The two simple biologically active cycloartanone compounds **1** and **2** are apparently ideal models for providing conformational information within this class of compounds.

Previous structure elucidation of capisterones A (**1**) and B (**2**) was primarily based on the analysis of their NMR spectra recorded in DMSO-d_6.[7] Here, improved resolution for the NMR spectra of both compounds was achieved in MeOH-d_4. Well-resolved NOESY spectra were obtained to assign their relative configuration and conformation in solution. For example, in the case of compound **2**, notable NOE correlations were observed between H-19β and H-6β (axial)/H-8β (axial), H-19α and H-1β (equatorial)/H-11β (equatorial), Me-30 and H-17α (axial), and Me-18 and H-20. The NOE correlation between one of the methylene protons and H-6β permitted assignment of this proton as H-29a (pro-*S*), while the H-29b (pro-*R*) showed a correlation with H-2β (axial). The assignments of the remaining geminal protons were also facilitated by the NOE correlations. All the observed NOE correlations satisfactorily supported the preferred conformation of **2** as shown in Figure 2, which was generated by Chem3D Pro 8.0 and energy-minimized by MM2, and then optimized at the B3LYP/6-31G* level with the Gaussian03 program package[30] (see geometrical data in Table 2). The three six-membered rings A−C adopted chair, twisted, and twisted-boat conformations, respectively, while the five-membered ring D was in a slightly distorted envelope conformation. The twisted-boat conformation of ring C is supported by the NOE correlation between H-11α (axial) and Me-30 and the theoretically calculated interatomic distance of 2.064 Å. This is in agreement with the finding by Horgen et al.[16] in the case of a cycloartan-3-ol compound as discussed above. The calculated torsion angles for **2** are almost identical to those of an analogue, (24*R*)-24,25-dihy-droxycycloartan-3-one, which were determined by an X-ray crystallographic analysis[33] (Table 2). This may suggest that the preferred conformation for this class of compounds in solution is generally in agreement with their crystal structures. Also, it is noted that all the calculated distances between the protons in this preferred conformation that show NOE correlations, partially indicated in Table 2, are less than 2.6 Å. To our knowledge, this is the most comprehensive NOE analysis of cycloartan-3-one derivatives,[8−19] thus leading to the assignment of a preferred conformation of compound **2** in solution with the aid of advanced computational methods (B3LYP/6-31G*). This information indeed furnished the foundation for the theoretical calculation of its ECD spectrum. Since the accuracy and reliability of a theoretically calculated CD
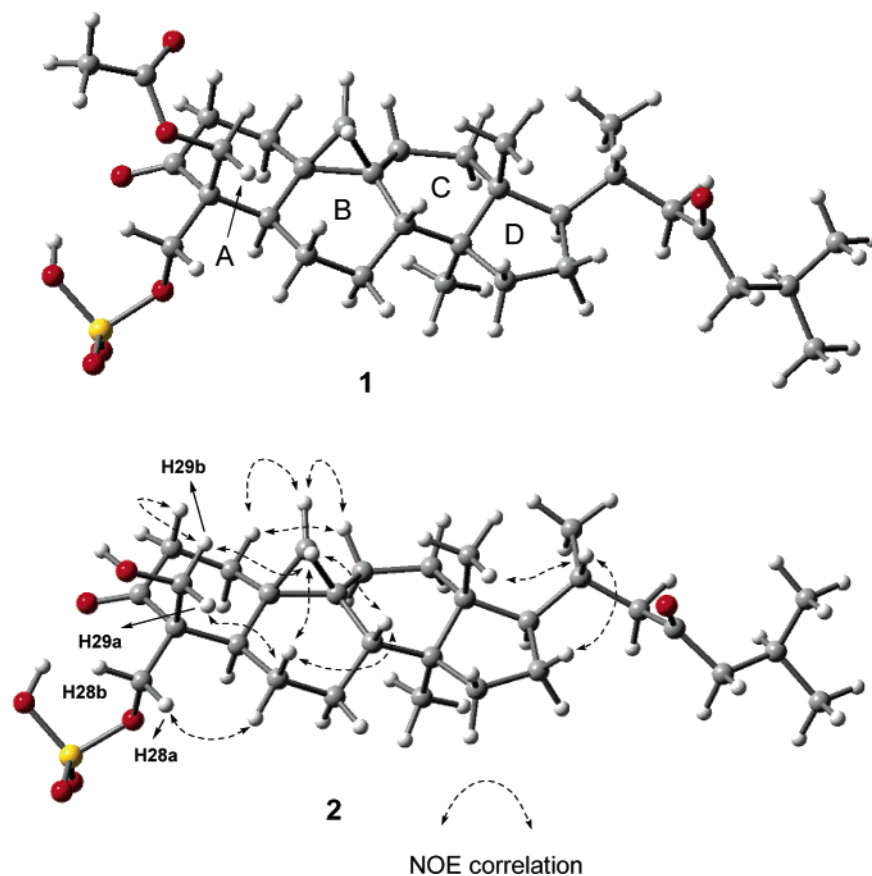
Figure 2. Preferred conformations of compounds **1** and **2** in CD₃OD.

spectrum largely depends on conformational analysis, the agreement of the calculated and experimental ECD spectra of compound **2** confirmed the presence of the assigned conformer in solution.

Similarly, the ¹H and ¹³C NMR assignments of compound **1** in MeOH-d_4 were facilitated by 2D NMR including COSY, HMQC, HMBC, and NOESY. The NOE correlation patterns in the NOESY spectrum of **1** were identical to those in **2**, indicating that both shared a similar conformation in solution as shown in Figure 2.

Compounds **1** and **2** were examined for their fluconazole reversal activity by a checkerboard assay that measures the combination treatment effect of two agents on a microbe, in which a fractional inhibitory concentration (FIC) value is used to define the effect as synergistic (FIC ≤ 0.5), additive (0.5 < FIC ≤ 1), indifferent (1 < FIC ≤ 2), or antagonistic (FIC > 2).[34] Two *S. cerevisiae* strains that overexpress *C. albicans* CDR1 (DSY 415) and MDR1 (DSY 415) efflux pumps were used in this study, along with the null pump strain (DSY 390).[6] Compound **1** alone was inactive against the above three strains (IC₅₀ > 67 μM), while compound **2** did show some inherent antifungal activity (IC₅₀, 7−11 μM) against the three strains. Both compounds significantly enhanced fluconazole activity in the CDR1 and MDR1 efflux pump-overexpressing strains (FIC < 0.5) (Figure 3, Table 3). In particular, compound **1** showed strong synergistic effects in the MDR1 strain (FIC, 0.08). It appears that the MDR efflux strain is more susceptible to the two compounds.

Compounds **1** and **2** did not show any inherent antifungal activity when tested against several opportunistic pathogens in our antifungal assay panel consisting of *C. albicans*, *C. glabrata*, *C. krusei*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*[35,36] and were not cytotoxic agaisnt several human cancer cell lines (KB, SK-MEL, BT-549, and SK-OV-3) and noncancerous Vero cells (up to 35 μM).[37] Studies on the potential of these compounds and their derivatives for combination treatment of fungal infections caused by clinically relevant azole-resistant strains are underway.

Table 3. Fractional Inhibitory Concentrations (FICs)[a,b] of Combination Treatment of Fluconazole (FLU)[c] with 22.5 μM **1**, 6.03 μM **2**, or 12.8 μM Beauvericin Using IC₅₀ as an Endpoint

sample	null pump	CDR1	MDR1
1	0.55	0.33	0.08
2	0.50	0.33	0.15
beauvericin[d]	2.67	0.05	2.00

[a] FIC ≤ 0.5 = synergistic; 0.51−1.0 = additive; 1.1−2.0 = indifferent; >2.0 = antagonistic. [b] FICs are estimated due to lack of IC₅₀ values for **1** or beauvericin alone (inactive at highest test concentrations of 67.4 and 12.8 μM, respectively). [c] FLU concentrations used for FIC calculations: 0.91 μM for null pump and 108.8 μM for CDR1 and MDR1. [d] Positive control of the CDR1 pump.

Experimental Section

General Experimental Procedures. Optical rotations were measured on an Autopol IV automatic polarimeter. CD spectra were obtained on a JASCO J-715 spectropolarimeter. IR spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer. NMR spectra were recorded on Varian Mercury-400BB or Varian Inova-600 instruments. High-resolution TOFMS were measured on an Agilent Series 1100 SL equipped with an ESI source. Column chromatography was done on silica gel (40 μm, J. T. Baker) and reversed-phase silica gel (RP-18, 40 μm, J. T. Baker). Semipreparative HPLC was conducted on an ODS (Prodigy) column (250 × 10 mm, 10 μm) using a UV detector at 220 nm. TLC was performed on silica gel sheets (silica gel 60 F₂₅₄, Merck, Germany) and reversed-phase plates (RP-18 F₂₅₄S, Merck, Germany). General procedures for antifungal and cytotoxicity assays have been described in our previous papers.[35−37]

Algal Material. The original algal material *P. capitatus* was collected in Sweetings Cay, Bahamas (Western Atlantic, Caribbean, longitude: 77°55″ W, latitude: 26°36″ N) by the Harbor Branch Oceanographic Institution in early 1988. The NCI received the material on March 18, 1988, from which a CHCl₃−MeOH (1:1) extract was prepared. A voucher for this sample is being kept at the Smithsonian Herbarium (voucher no. Q66130939).
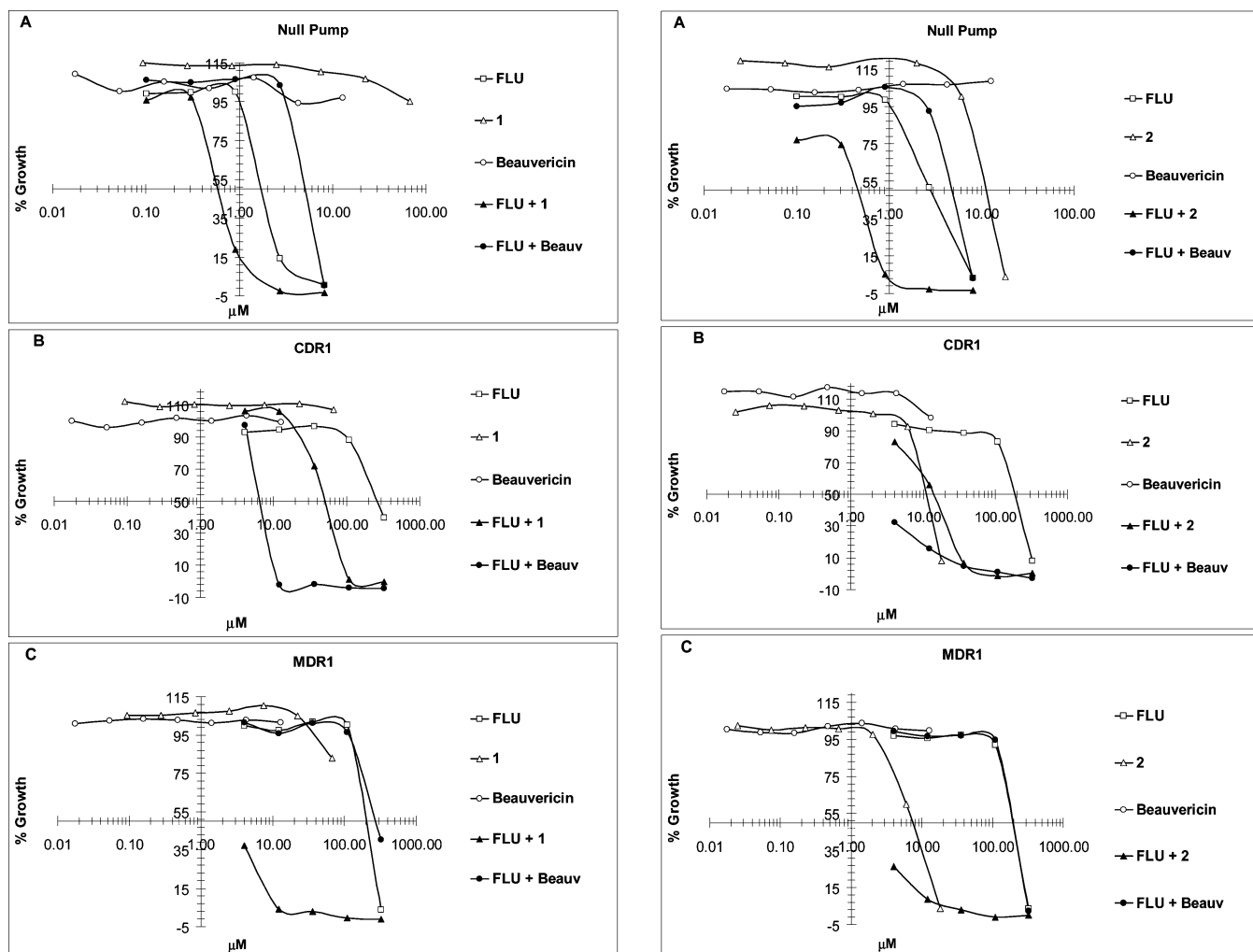
Figure 3. Dose−response curves of fluconazole (FLU) and compounds **1** and **2** alone and FLU in combination with **1** (22.5 μM), **2** (6.0 μM), and beauvericin (Beauv) (12.8 μM) in the null pump (A), CDR1 (B), and MDR1 (C) *S. cerevisiae* strains.

Isolation. The CHCl$_3$−MeOH extract (331.3 mg) was chromatographed on silica gel (42 g) eluting with CH$_2$Cl$_2$ (300 mL), CH$_2$Cl$_2$−MeOH (9:1, 750 mL; 6:1, 840 mL), and MeOH (200 mL) to afford 10 pooled fractions (A−J) according to TLC. Fractions D (29.4 mg) and F (18.9 mg), which showed enhanced activity for reversal of fluconazole resistance in our bioassay system, were purified by HPLC (ODS) using 70% MeOH to yield compounds **1** (10.5 mg) and **2** (5.3 mg), respectively.

Capisterone A (1): white powder, [α]^{25}$_D$ −6.3 (MeOH, *c* 0.80) {ref 7: [α]^{25}$_D$ −9.6 (MeOH, *c* 0.019)}; CD (*c* 0.0011 M, MeOH) λ ([θ]) 294 (−4309) nm; IR (KBr) ν_{max} 3474 (br), 2954, 2817, 1738, 1711, 1646, 1468, 1378, 1229, 1010, 813 cm^{-1} [in contrast to the sodium salt form of capisterone A in the previous report,[7] the strong IR absorption band at 3474 (br) cm^{-1} indicated the presence of a hydroxyl group in the molecule due to the free hydrogen form or at least partial hydrogen form]; NMR (CD$_3$OD) and HRTOFMS data, see Supporting Information.

Capisterone B (2): white powder, [α]^{25}$_D$ +2.1 (MeOH, *c* 0.33) {ref 7: [α]^{25}$_D$ +0.19 (MeOH, *c* 0.0052)}; CD (*c* 0.0011 M, MeOH) λ ([θ]) 294 (−5325) nm; IR (KBr) ν_{max} 3431 (br), 2955, 2872, 1709, 1469, 1377, 1266, 1219, 1065, 999, 826 cm^{-1} [in contrast to the sodium salt form of capisterone B in the previous report,[7] the broad IR absorption at 3431 cm^{-1} indicated the presence of a hydroxyl group in the molecule due to the free hydrogen form or at least partial hydrogen form]; NMR (CD$_3$OD) and HRTOFMS data, see Supporting Information.

Computational Method. Theoretical calculation of the CD spectrum for compound **2** was performed with the Gaussian03 program package.[30] The B3LYP/6-31G* method was employed to optimize the geometry of compound **2** (gas form), and vibrational analysis was done at the same level. Calculation on the electronic CD spectrum was then performed using time-dependent density functional theory (TDDFT) with the 6-31G* basis set.[26−29]

Assay for Reversal of Fluconazole Resistance in *S. cerevisiae* Strains. The detailed procedure has been described in a previous paper.[6] The growth inhibition is shown in Figure 3. The fractional inhibitory concentration (FIC) is calculated by the formula (IC$_{50}$ of test compound in combination with fluconazole (FLU)/IC$_{50}$ of test compound alone) + (IC$_{50}$ of FLU in combination with test compound/IC$_{50}$ of FLU alone). Beauvericin, a cyclic depsipeptide known to inhibit the CDR1 pump, is used as a positive control.

Supporting Information Available: NMR and MS spectral data of compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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