Reversal of Fluconazole Resistance in Multidrug Efflux-Resistant Fungi by the *Dysidea arenaria* Sponge Sterol 9α,11α-Epoxycholest-7-ene-3β,5α,6α,19-tetrol 6-Acetate

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The sponge sterol 9α,11α-epoxycholest-7-ene-3β,5α,6α,19-tetrol 6-acetate (ECTA) (**1**) is the first marine natural product to reverse fluconazole resistance mediated by a *Candida albicans* MDR efflux pump. The IC_{50} of fluconazole is decreased from 300 to 8.5 μM (35-fold enhancement) when combined with **1** (3.8 μM). A revised C-6 configuration of **1** is established.

For more than a decade, HIV-infected patients have been maintained on low-dose prophylactic fluconazole (FLU) therapy to prevent opportunistic fungal infections. The result was the development of azole-resistant *Candida albicans* isolates. These fungi are resistant to the classes of antifungal agents (imidazoles and triazoles) collectively known as "azoles". Azole antifungal agents block fungal ergosterol biosynthesis by inhibiting lanosterol 14α-demethylase and include major clinically useful drugs such as fluconazole, ketoconazole, and itraconazole. Other patient populations, such as those receiving bone marrow transplants, have also experienced infection by azole-resistant *C. albicans*.[1] Several molecular mechanisms of fluconazole resistance have been identified. These include point mutations in the *ERG11* gene that encodes FLU's target lanosterol demethylase, overexpression of *ERG11*, modifications of other genes involved in ergosterol biosynthesis, and decreased intracellular accumulation of FLU by membrane-associated efflux pumps.[2−4] Two families of efflux pumps found in *C. albicans* include the major facilitators (multidrug resistance, MDR) that are fueled by a proton gradient and the P-glycoprotein ABC transporters (CDR) that require ATP hydrolysis for energy. Within each family, several subtypes have been discovered (i.e., CDR1, CDR2, MDR1, FLU1).[1]

As part of an ongoing project to discover potential new drugs to treat resistant opportunistic fungal infections,[5] a bioassay system (similar to Lee et al.,[6] in which extracts derived from microbial fermentation were screened) was developed to identify natural products that reverse efflux pump-mediated azole resistance. Plasmids containing *C. albicans* genes that encode CDR1, CDR2, MDR1, and FLU1 (a member of the major facilitator family) have been incorporated into *Saccharomyces cerevisiae*, producing a phenotypic resistance to FLU.[7−9] These azole-resistant *S. cerevisiae* strains (along with a null pump strain) were used to evaluate over 5000 crude extracts of marine organisms from the NIH Open Repository for natural products that reverse FLU resistance.

A primary assay (% inhibition of fungal growth by the extract in the presence and absence of a subinhibitory concentration of FLU) was used to deselect extracts with either inherent antifungal activity (≥80% inhibition without FLU) or inability to reverse FLU resistance in MDR1 and CDR1 strains. Extracts showing ≥40% increase in % inhibition in the presence of FLU (~3%) were selected for dose−response studies (three, 5-fold dilutions) in the CDR1, MDR1, and null pump strains (to eliminate pump-independent synergy).

Through a process of bioassay-guided fractionation, the unusual epoxy sponge sterol 9α,11α-epoxycholest-7-ene-3β,5α,6α,19-tetrol-6 acetate (**1**) was isolated from the lipophilic extract of an Australian collection of *Dysidea arenaria* cf. Bergquist (family Dysideidae). Compound **1** was first isolated by Gunasekera and Schmitz from a sample of *Dysidea* sp. collected in Guam nearly 20 years ago.[10]

After confirming the activity of **1** in a three-concentration dose−response assay using the MDR1 (IC_{50} + 33 μM FLU = <4 μM, IC_{50} − FLU = >104 μM) and CDR1 (IC_{50} + 33 μM FLU = 31 μM, IC_{50} − FLU = >104 μM) *S. cerevisiae* strains, a checkerboard-type assay was employed to monitor the effect of varying the concentrations of both **1** and FLU simultaneously (Table 1 and Figure 1). Amphotericin B (AmB), inherently antifungal via binding to ergosterol in the cell membrane, is not a substrate for the efflux pumps and was included as a negative control. Certain cyclic depsipeptides are known inhibitors of ABC transporter-type efflux pumps (CDR1), and beauvericin was therefore included as a positive control.[6,11] Unfortunately, there is no known positive control for the MDR-type efflux pumps.

Analysis of the results of the checkerboard experiment may be done using several techniques to determine if the effect of the combination treatment is synergistic, additive, ineffective, or antagonistic.[12,13] One such method is the fractional inhibitory concentration (FIC),[14] which compares the effect of each test compound (**A** and **B** in the equation below) alone and in combination and quantitatively assigns activity (FIC ≤ 0.5 = synergistic; 0.51−1.0 = additive; 1.1−2.0 = indifferent; >2.0 = antagonistic):

$$\frac{\text{MIC or } IC_{50}\ \mathbf{A} \text{ in combination with } \mathbf{B}}{\text{MIC or } IC_{50}\ \mathbf{A} \text{ alone}} + \frac{\text{MIC or } IC_{50}\ \mathbf{B} \text{ in combination with } \mathbf{A}}{\text{MIC or } IC_{50}\ \mathbf{B} \text{ alone}} = \text{FIC}$$

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Table 1. IC_{50}/MIC (μM) of Fluconazole in the Presence of 3.8 μM **1**, 0.1 μM Amphotericin B, or 3.1 μM Beauvericin

sample[a]	null pump	CDR1	MDR1	CDR2	FLU1
1 (3.8 μM)	3.0/4.0	150/400	8.5/12.5	9.0/25.0	15.0/25.0
amphotericin B (0.1 μM)	3.0/4.0	300/>400	300/400	10.0/25.0	15.0/25.0
beauvericin (3.1 μM)	5.5/8.0	10.0/25.0	300/400	4.5/6.25	35.0/50.0
fluconazole alone	3.5/8.0	>400/>400	300/400	15.0/25.0	35.0/50.0

[a] Compound **1** and beauvericin were inactive alone at the highest test concentrations of 30 and 25 μM, respectively. Amphotericin B had similar activity in all strains (IC_{50} = 0.55 μM, MIC = 0.75 μM).

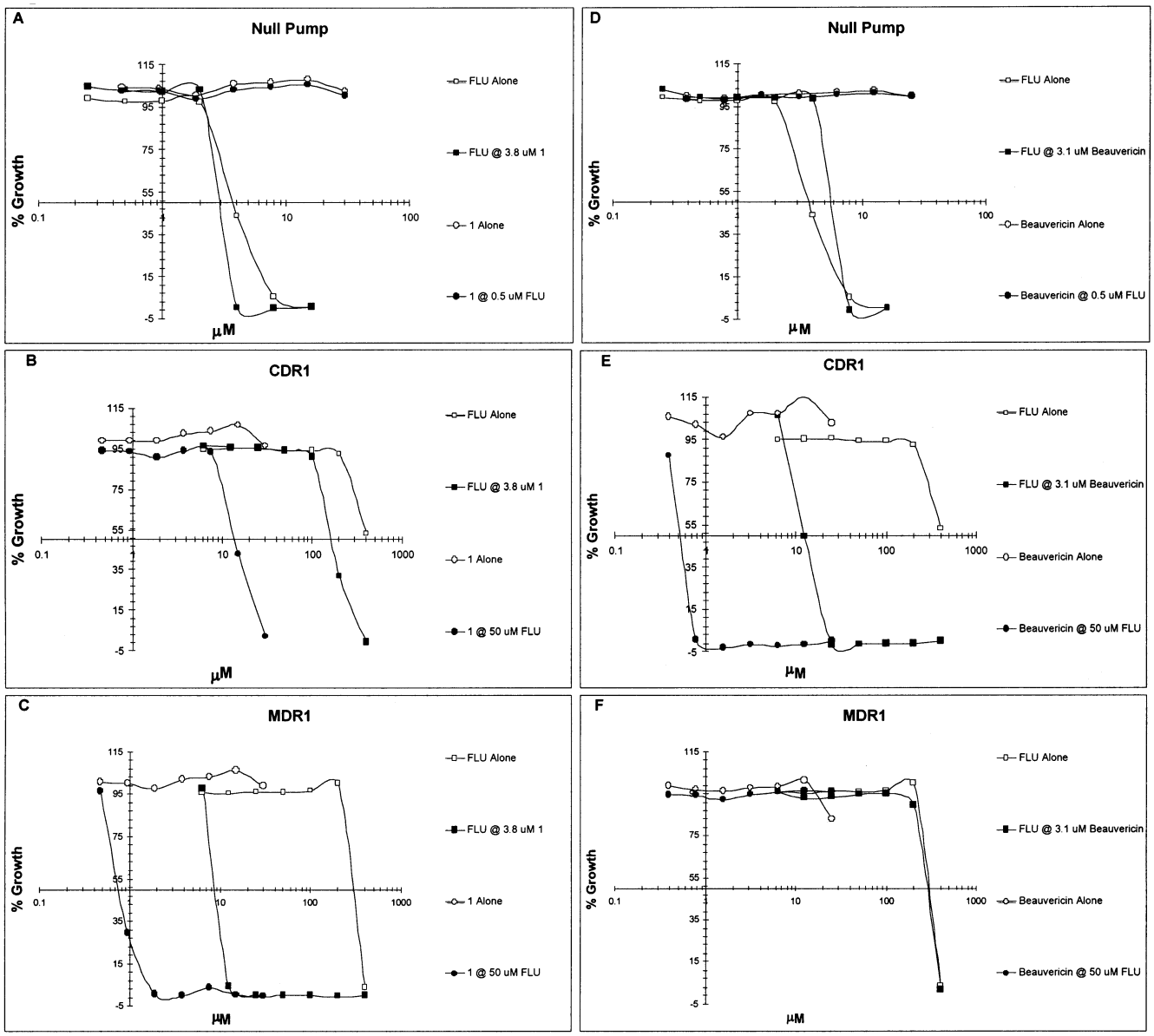


Figure 1. Dose−response curves of **1** and FLU alone and in combination in the null pump (A), CDR1 (B), and MDR1 (C) strains; dose−response curves of beauvericin and FLU alone and in combination in the null pump (D), CDR1 (E), and MDR1(F) strains.

However, by the nature of the checkerboard assay, there are several choices of concentration combinations to use to calculate the FIC. The concentration of **A** or **B** should be high enough that an effect is seen in combination, yet low enough that any inherent activity of **A** or **B** alone is minimal or nonexistent. Because **1** and beauvericin were not inherently antifungal at the highest test concentrations used (30 and 25 μM, respectively), the concentrations of **1** and beauvericin used in determining the FIC in combination with FLU were the lowest in which a significant synergistic effect was seen (3.8 and 3.1 μM, respectively). However, with AmB and FLU, an inherent antifungal effect was observed; therefore the concentration of AmB and FLU used to determine the FIC was approximately 1/10th to 1/6th of their IC_{50} alone to eliminate possible additive effects (Table 2).

An alternate method for evaluating drug combination experiments is the isobologram (Figure 2); however the same caveats for FIC determination exist. The isobologram assumes that varying ratios of **A** and **B** (i.e., 0.5 IC_{50} **A** + 0.5 IC_{50} **B**) can elicit the same response (IC_{50}) as **A** or **B** alone. All of these ratios of combinations construct the additivity line. Any significant deviation below or above this line suggests synergy or antagonism, respectively.

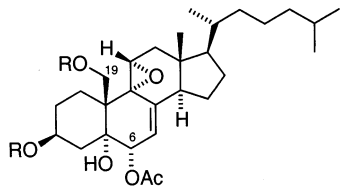
Because IC_{50}'s were not achievable for **1** (due to sample limitation), FLU versus CDR1, and beauvericin (additional

Table 2. Fractional Inhibitory Concentrations (FICs)[a] of Combination Treatment of FLU[b] with 3.8 μM **1**, 0.1 μM AmB, or 3.1 μM Beauvericin Using IC_{50} as an Endpoint

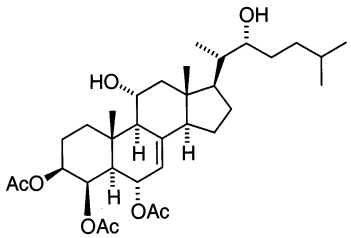
sample	null pump	CDR1	MDR1	CDR2	FLU1
1	1.85[c]	0.88[c]	0.05[c]	1.60[c]	1.26[c]
amphotericin B	1.85	0.89[c]	1.42	1.58	0.97
beauvericin	2.57[c]	0.045[c]	2.0[c]	1.30[c]	2.0[c]

[a] FIC $\leq$ 0.5 = synergistic; 0.51−1.0 = additive; 1.1−2.0 = indifferent; >2.0 = antagonistic. [b] FLU concentrations used for FIC calculations were approximately 1/10th to 1/6th of the FLU IC_{50} alone: 0.5 μM for null pump, 50 μM for CDR1 and MDR1, 1.56 μM for CDR2, and 3.13 μM for FLU1. [c] FICs are estimated due to lack of IC_{50} values for **1**, FLU vs CDR1, or beauvericin alone (see text).

tests showed that at concentrations as high as 128 μM in the CDR1 strain, no inherent antifungal effect was observed), exact FICs could not be calculated. Therefore, the IC_{50} of each compound is theoretically greater than the highest test concentration and the resulting FIC must be less than or equal to the calculated value. The level of synergistic effect of **1** (and of beauvericin) is actually conservatively represented by this reported FIC value of 0.05. In any case, it is evident from the dose−response curves, the FIC values, and the isobologram that **1** is a selective and potent inhibitor of FLU resistance in this *C. albicans* MDR1 strain of *S. cerevisiae*. No effect is seen in the null pump strain (ruling out nonspecific synergistic mechanisms such as enhanced permeability).



1 R = H
3 R = Ac



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Spurred by structural similarities between **1** and agosterol A (**2**) from the marine sponge *Spongia* sp.,[15] a known inhibitor of tumor cell MRP1 (P-glycoprotein)-mediated multidrug resistance (at $\leq$5 μM), compound **1** was treated with Ac_2O/pyridine to prepare 9α,11α-epoxycholest-7-ene-3β,5α,6α,19-tetrol-3,6,19 triacetate (**3**) for evaluation. Compound **3** is spectroscopically identical with previously published data.[10] However, **3** failed to reverse MDR1-mediated resistance at concentrations up to 90 μM (data not shown).

Compound **1** isolated from *D. arenaria* is spectroscopically identical with previously published data.[10] Compound **1** was originally assigned a β-configuration for the C-6 acetate. Fujimoto and co-workers later suggested a possible revised 6α-configuration for **1** (based on coupling constant studies with related metabolites), and several other publications followed suit, basing the assignment of the C-6 geometry in similar sterols on this proposed structural

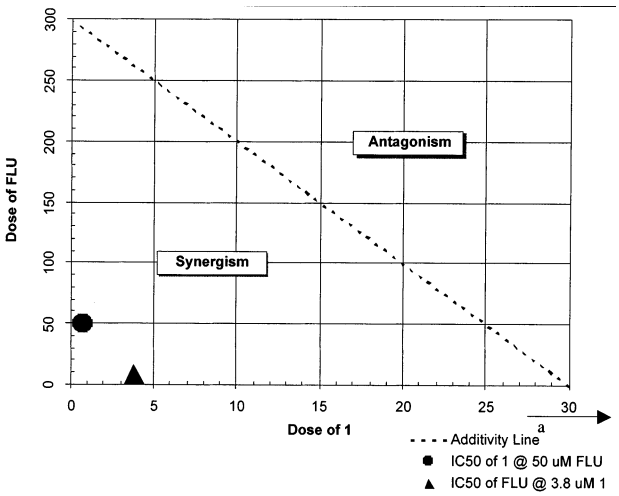

Figure 2. Isobologram of FLU/**1** combination treatment against the *S. cerevisiae* MDR1 strain. The additivity line connects the IC_{50} of **1** and FLU alone and represents all theoretical combinations that produce the same endpoint. However, the IC_{50} of **1** was greater than the highest test concentration of 30 μM (indicated by an arrow). The additivity line is therefore drawn to afford a more conservative estimate of synergy.

revision.[16,17] However, West and Cardellina reexamined the issue with a series of structurally related *D. etheria* polyhydroxlated sterols and subsequently refuted Fujimoto's proposed 6α assignment.[18] Therefore, we reexamined this issue with an authentic sample of **1**, isolated from *D. arenaria*. Clearly resolved NOESY correlations (in $CDCl_3$) observed between H-6 (5.19 bs) and H-19_a (3.76 d, J = 10.7 Hz) established an α-configuration for the C-6 acetate ester and subsequent revision of the C-6 assignment (Supporting Information). This "α" C-6 configuration of **1** is identical to the C-6 configuration of the tumor cell MRP1 inhibitor agosterol (**2**), as established through ROESY studies.[15]

Disabling efflux pumps in bacteria greatly enhance the effect of antimicrobial agents found in plants.[19−21] Few studies have examined the effects of efflux pump inhibitors on drug-resistant fungi.[6] To our knowledge, **1** is the first marine natural product that has been shown to reverse azole resistance and the first possible inhibitor of the MDR1-type efflux pump of *C. albicans*, although similar compounds are known to inhibit distinctly different efflux pumps in tumor cells.[15,22] However, it must be noted that tumor cell efflux pumps are of the ATP-driven ABC transporter family, rather than the fungal MDR1 proton gradient pumps.

Considering the ability of **1** (ECTA)[23] to inhibit MDR1 in fungi, **1** was also evaluated for cytotoxicity using human breast tumor cells. Compound **1** showed no cytotoxic activity in human ductal breast carcinoma T47D cells up to the highest concentration evaluated (10 μM).

Experimental Section

General Experimental Procedures. Optical rotation was measured on a RUDLPH Research Autopol IV automatic polarimeter. The IR spectrum was obtained using an AATI Mattson Genesis Series FTIR. The ^{1}H NMR, ^{1}H−^{1}H COSY, NOESY, and HMBC spectra were recorded on a Bruker AV 400 spectrometer. The ^{13}C NMR and HMQC spectra were recorded on a Bruker DRX 400 spectrometer. Both NMR spectrometers were operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively. The NMR spectra were recorded running gradients and using a residual $CDCl_3$ solvent peak (δ 7.26) as internal reference. The HRESIMS and ESIMS data were acquired on a Bruker BioAPEX 30es mass spectrometer. TLC were run on Merck TLC plates precoated with Si_{60} F_{254} or Si_{60}

RP18 F_{254} with visualization by spraying with 1:1 H_2SO_4 in EtOH and heating. HPLC was carried out on a Waters Millennium system with a 996 photodiode array detector.

Sponge Material. The marine sponge *Dysidea arenaria* cf. Bergquist (Dysideidae) was collected and identified by the Australian Institute of Marine Sciences on Whitsundays Reef in Australia in October 1987. The sample was identified, frozen at −20 °C, and ground in a meat grinder. A voucher specimen is stored at the Smithsonian Institute, Washington, D.C. (voucher # Q66c0875 7).

Extraction and Isolation. Ground sponge material was extracted with water. The residual sample was then lyophilized and extracted with CH_2Cl_2/MeOH (1:1), residual solvents were removed under vacuum, and the crude extract was stored at −20 °C in the NCI Open Repository at the Frederick Cancer Research and Development Center (Frederick, Maryland). The sponge extract was obtained through the NCI Open Repository Program.

The organic extract (1.0 g) was separated by Si gel VLC using a step gradient of MeOH (0−100%) in CH_2Cl_2 to give eight fractions. Fraction 4 (51 mg), which was eluted with 5% MeOH in CH_2Cl_2, showed positive activity in the reversal of fluconazole resistance assay. Fraction 4 was purified by NP-HPLC [Prodigy Silica (3), 5 μM, 21.2 × 250 mm column, 5% MeOH in CH_2Cl_2 (v/v), 13 mL min^{-1}, photodiode array detection monitored at 210 nm] to obtain **1** (7.5 mg, yield 0.8%).

Assay for Reversal of Azole Resistance in *S. cerevisiae* Strains. *S. cerevisiae* containing *C. albicans* efflux pumps CDR1 (DSY 415),[9] MDR1 (DSY 416),[9] CDR2 (DSY 417),[7] and FLU1 (DSY 426),[8] along with the null pump strain (DSY 390),[9] were generously supplied by Dominique Sanglard (Institute of Microbiology, University Hospital, Lausanne, Switzerland) and stored in 15% glycerol/YPD broth at −80 °C. One to 2 days prior to the assay, cultures were prepared from frozen stocks on YNB-URA/TRYP selection agar at 30 °C. A modified version of the NCCLS methods was used in the checkerboard assay.[24]

Serial dilutions of FLU and the test compound (dissolved in DMSO) were made in 0.9% saline in a 96-well microplate and transferred (10 μL each) to the wells of a flat-bottom 96-well microplate to afford one compound running vertically and the other horizontally (all combinations of concentrations were generated). One row or column was reserved for each test compound and saline only. The *S. cerevisiae* inoculum was prepared by picking several colonies from the YNB agar plate, suspending in 0.9% saline, comparing the OD_{630} to that of the 0.5 McFarland Standard, and adjusting with YPD broth to afford a final inoculum of 1.0×10^4 CFU/mL. The microbial inoculum (180 μL) was added to the samples to achieve a final volume of 200 μL and varying final test concentrations depending upon the microorganism and test compound. Amphotericin B (ICN Biomedicals, Aurora, OH) and beauvericin (Sigma, St. Louis, MO) were also tested in combination with FLU as a negative and positive control, respectively. Growth and blank (media only) controls were added to each test plate and used to generate the % growth of compound-containing wells. The microplates were read at 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Winooski, VT) prior to and after incubation at 30 °C for 40−48 h. The dose−response curves were generated in Excel and the IC_{50}'s (concentrations that afford 50% growth inhibition) determined. The MIC (minimum inhibitory concentration) was defined as the lowest test concentration that affords no detectable growth.

Assay for Cytotoxicity in T47D Human Breast Carcinoma Cells in Vitro. T47D cells (American Type Culture Collection) were maintained in DMEM/F12 medium (JRH Biosciences) and supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone), 50 U/mL penicillin G sodium, and 50 μg/mL streptomycin in a humidified atmosphere (5% CO_2 and 95% air) at 37 °C. Exponentially grown T47D cells were plated at 30 000 cells/well in a volume of 100 μL of DMEM/F12 medium supplemented with FBS (10%) and Penn/Strep into 96-well plates. Twenty-four hours later, test compounds were diluted in DMEM/F12 medium supplemented with Pen/Strep and added to the wells in a volume of 100 μL. The final concentrations of test compound were 0.03, 0.1, 0.3, 1, 3, and 10 μM. After incubating at 37 °C for 24 and 48 h, cell viability was determined using the Neutral Red method.[25] Briefly, conditioned medium was removed at the end of incubation and fresh medium that contained 0.15 mg/mL Neutral Red was added. After 2 h incubation at 37 °C, the medium was removed and the cells were washed once with saline solution (0.9% NaCl) and lysed with an acidified solvent (0.04 N HCl in 2-propanol). Absorbance (*A*) (540 nm) was measured and background absorbance (630 nm) was subtracted using the EL312e plate reader (Bio-Tek Instruments). Only viable cells will pick up Neutral Red from the medium, and the A_{540} value correlates with the level of cell viability. The data are presented as percentage of the control.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra of ECTA (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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