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Solomonsterols A and B from *Theonella swinhoei*. The First Example of C-24 and C-23 Sulfated Sterols from a Marine Source Endowed with a PXR Agonistic Activity

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The finding of new PXR modulators as potential leads for treatment of human disorders characterized by dysregulation of innate immunity and with inflammation is of wide interest. In this paper, we report the identification of the first example of natural marine PXR agonists, solomonsterols A and B, from a *Theonella swinhoei* sponge. The structures were determined by interpretation of NMR and ESIMS data, and the putative binding mode to PXR has been obtained through docking calculations.

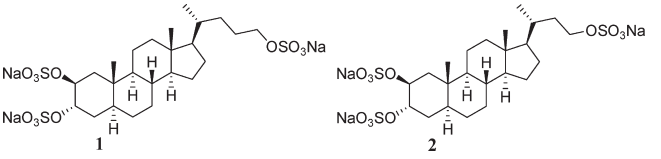
Introduction

Sulfated steroids are a family of secondary metabolites often found in sponges and echinoderms.[1−3] Since the isolation of halistanol from *Halichondriidae* sponges,[4] several sulfated polyhydroxysterols have been isolated from sponges. Most of them are characterized by the 2β,3α,6α-tri-*O*-sulfate functions with different pattern of alkylation in the side chain, whereas some show unprecedented structural features. Recent examples are pregnane ptilosterols from *Ptilocaulis spiculifer*,[5] geodisterol sulfate,[6] and iodinated and chlorinated topsentiasterol sulfates[7] from the sponge *Topsentia*, eurysterols[8] from an unclassificated sponge of the genus *Euryspongia*. Steroidal sulfates often exhibited a variety of biological activities including antiviral,[9−14] antifungal,[6,8,15] antifouling,[16] and action on specific enzymatic targets[7,17−21]

As part of an ongoing investigation of metabolites isolated from marine organisms collected off the Solomon Islands, we have studied the polar extracts of the sponge *Theonella swinhoei* Gray. Recently, we reported the isolation from this species of two new anti-inflammatory cyclopeptides, perthamides C and D.[22] Further purification of the polar extracts of the sponge afforded two new sulfated sterols **1** and **2**, named solomonsterols A and B, which exert an agonistic activity for the human nuclear receptor and xenobiotic sensor, pregnane-X-receptor (PXR). The current finding represents, to our knowledge, the first report of marine natural steroids acting as PXR-agonists.

Results

Isolation and Structure Elucidation. The initial processing of the *Theonella swinhoei* (coll. no. R3170) was conducted according to procedures described previously.[22] The *n*-BuOH extract from a solvent partitioning Kupchan procedure was chromatographed by droplet counter current chromatography (DCCC[a]). Solomonsterols A and B, eluted in the first more polar fraction, were further purified by reverse phase HPLC.



Solomonsterol A (**1**) was isolated as a colorless amorphous solid ($[\alpha]_D^{25}$ +4.6). The ^{1}H NMR spectrum showed signals typical of a sterol (two upfield Me singlets at δ_H 0.69 and δ_H 1.00; one methyl doublet at δ_H 0.95, J = 6.5 Hz). The negative ion HR ESIMS (high-resolution electrospray ionization mass spectrometry) spectrum gave a predominant pseudomolecular ion at m/z 661.1415 $[M - Na^+]^-$, corresponding to a molecular formula of $C_{24}H_{39}Na_3O_{12}S_3$. The ESI and MS/MS spectra showed pseudomolecular and fragment ions compatible with the presence of sulfate groups: 617 (M^- in hydrogen form), 308.3 (double charged species), 559 $[M - NaSO_3]^-$, 541 $[M - NaHSO_4 - Na^+]^-$, 439 $[M - 2NaHSO_4 - Na^+]^-$. ^{1}H and ^{13}C NMR spectra, together with mass data, evidenced the presence in the molecule of two secondary and one primary sulfoxy groups (δ_H 4.74, q, J = 2.9 Hz, δ_C 76.1,

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[a] Abbreviations. COSY, COrrelation SpectroscopY; CYP3A4, cytochrome P450 3A4; DCCC, droplet counter current chromatography; FXR, farnesoid-X-receptor; HMBC, heteronuclear multiple-bond correlation spectroscopy; HR ESIMS, high-resolution electrospray ionization mass spectrometry; HSQC, heteronuclear single-quantum coherence; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MDR1, multidrug resistance 1; ROESY, rotating frame nuclear Overhauser effect spectroscopy; RT PCR, real-time polymerase chain reaction; PDB, Protein Data Bank; PXR, pregnane-X-receptor; PXREs, PXR response elements; RXR, retinoid-X-receptor; TNFα, tumor necrosis factor α.

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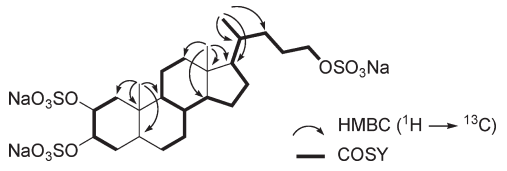

Figure 1. Key skeletal connectivities for solomonsterol A (**1**), as deduced from COSY and HMBC spectra.

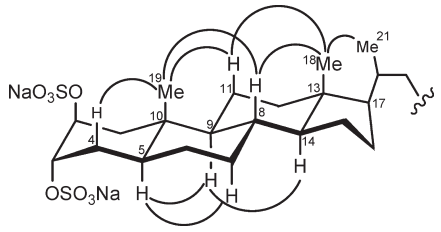

Figure 2. Key ROE correlations supporting the relative configuration of solomonsterol A (**1**).

CH; δ_H 4.70, q, J = 2.9 Hz, δ_C 75.8, CH; δ_H 3.96, t, J = 6.6 Hz, δ_C 69.3, CH_2, see Table 1 in Supporting Information).

The location of two secondary sulfoxy groups at C2 and C3 of the steroidal nucleus was determined by interpretation of ^{1}H NMR, COSY (correlation spectroscopy), HSQC (heteronuclear single-quantum coherence), and HMBC (heteronuclear multiple-bond correlation spectroscopy) spectra (Figure 1).

The 2β,3α-disulfoxy configuration was assigned on the basis of the coupling pattern of H-2 (q) and H-3 (q). The tetracyclic steroidal skeleton was considered to adopt an all-*trans* arrangement as evidenced by ROESY analysis (Figure 2). The ROESY correlations observed from H_3-19 and H_3-18 to H-8 and H-11β, from H-5 to H-9, and from H-9 to H-14 indicated the relative configuration for each ring junction to be *trans*. The constitution and configuration of steroidal nucleus was also supported by ^{1}H and ^{13}C NMR data, in agreement with those of all-*trans* 2β,3α-disulfoxy steroids reported from sponges[20] and ophiuroids.[23] The COSY spectrum revealed useful information concerning the side chain. Starting from the methine proton at δ_H 1.13 (H-17), COSY correlations could be observed to the methine proton at δ_H 1.43 (H-20), which in turn was coupled to both of the methyl protons at δ_H 0.95 (H_3-21) and the methylene protons at δ_H 1.10−1.54 (H_2-22). These latter protons were coupled to the methylene protons at δ_H 1.55−1.75 (H_2-23), which in turn were coupled to the methylene protons at δ_H 3.96 (H_2-24), suggesting a 24-sulfoxy-C24 side chain.

Hence, the new sulfated steroid **1** was established as 5α-cholan-2β,3α,24-tryl-2,3,24-sodium trisulfate and named solomonsterol A.

The molecular formula of the compound **2** was established as $C_{23}H_{37}Na_3O_{12}S_3$ through NMR (Table 1 in Supporting Information) and HR ESIMS data. The analysis of ESIMS, ^{1}H and ^{13}C NMR data suggested a close analogy between compounds **1** and **2**. In particular, the resonances relative to the steroid nucleus of solomonsterol B (**2**) were almost superimposable to those of **1**, indicating a common 2β-3α disulfoxy substitution. A shortened side chain, also suggested by MS data, was easily determined by the interpretation of 2D NMR spectra. Two downfield signals relative to a diasterotopic carbinol methylene, at δ_H 4.00 and 4.05 observed in the ^{1}H NMR spectrum, were assigned to H_2-23 on the basis of key HMBC correlations H_2-23/C20, H_2-22/C23, and H_2-22/C20. Therefore the structure of new steroid sulfate **2** was defined as 24-nor-5α-cholan-2β,3α,24-tryl-2,3,23-sodium trisulfate and named solomonsterol B.

Few examples of steroidal derivatives with a truncated side chain have been so far reported from marine organisms. They include pregnane derivatives isolated from octocorals[24−27] and sponges,[5,28−31] androstanes from a *Cliona* sponge,[32] and ergostane geodisterol, again from a sponge.[33] Whereas bile acid derivatives have been isolated from a bacterium associated to a sponge,[34] in the literature the occurrence of C23 and C22 steroids is limited to 3β-hydroxy-24-norchol-5-en-23-oic acid, isolated from *Euryspongia* n. sp.[35] and to 23,24-dinor-5α-cholane from an *Haliclona* sponge.[28]

Biological Results. Because sponge-derived steroids might have anti-inflammatory activity,[36] we have investigated whether compounds **1** and **2** exert any effect on cells of innate immunity.[37]

For this purpose, RAW264.7 cells, a murine macrophage cell line, were incubated with compounds **1** and **2** at the concentration of 10 and 50 μM in the presence of bacterial endotoxin (LPS) and expression of mRNA encoding for pro-inflammatory mediators measured by real-time (RT) polymerase chain reaction (PCR). At the concentration of 50 μM, compounds **1** and **2** effectively inhibited the expression of interleukin-(IL)-1β mRNA by 50% (Figure 3, panel A; N = 4; $P < 0.05$ versus LPS alone).[37] In contrast with their effect on IL-1β, both compounds exerted no effect on expression of tumor necrosis factor (TNFα) mRNA (Figure 3, panel B). Because expression of mRNAs encoding for IL-1β and TNFα was measured at 24 h and the time-course of cytokines expression in macrophages exposed to LPS is different, the lack of inhibition on TNFα could be explained by a different kinetic of mRNA induction.

Compounds **1** and **2** had no effect on cell viability measured at the end of the experiment by the trypan blue excluding test (viability was greater than 95%), excluding that inhibition of IL-1β production was due to a cytotoxic effect.

PXR Agonism. To identify the mechanism of action of compounds **1** and **2**, we speculated on the possibility that they could be potential ligands for an evolutionary conserved nuclear receptor, called the pregnane-X-receptor (PXR). To confirm the ability of solomosterols to activate PXR and PXR regulated genes, we performed a transactivation assay using an human hepatocyte cell line (HepG2 cells). As illustrated in Figure 4A, compounds **1** and **2** were potent inducers of PXR transactivation, boosting the receptor activity by 4−5 folds (n = 4; $P < 0.05$ versus untreated) and were at least as potent as rifaximin, a well characterized ligand for the human PXR.[38] In addition both agents effectively stimulated the expression of two PXR target genes, CYP3A4 and MDR1 in the same cell line (Figure 4B,C; n = 4; $P < 0.05$).

Docking Studies. To have details for what concerns the binding mode of **1** and **2** to PXR at atomic level, molecular docking studies were performed on **1** with PXR using Autodock Vina 1.0.3 software[39] (see Computational Details). The docking results positioned compound **1** within the PXR binding pocket, and among the nine docked conformations generated, the lowest binding energy displayed an affinity of −10.0 kcal/mol (Figure 5). In this model, the steroidal nucleus shows hydrophobic interactions with Leu206, Leu209, Val211, Ile236, Leu239, Leu240, Met243, and Met246, confirming the binding mode already reported for a set of analogous compounds.[40] Moreover, the sulfate groups exert hydrogen

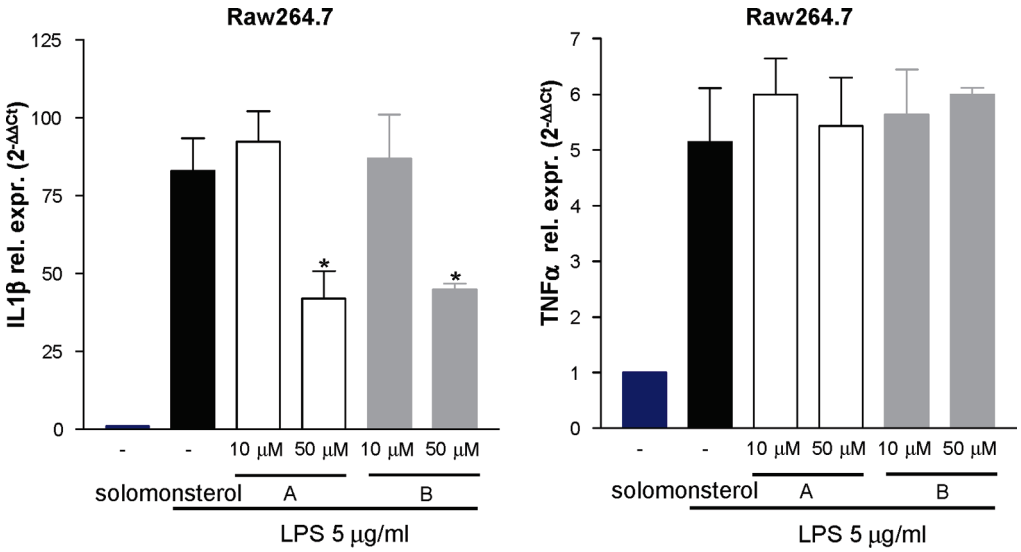

Figure 3. Relative mRNA expression of IL-1β and TNFα in RAW264.7 macrophages treated with LPS alone or with LPS in combination with **1** and **2**.

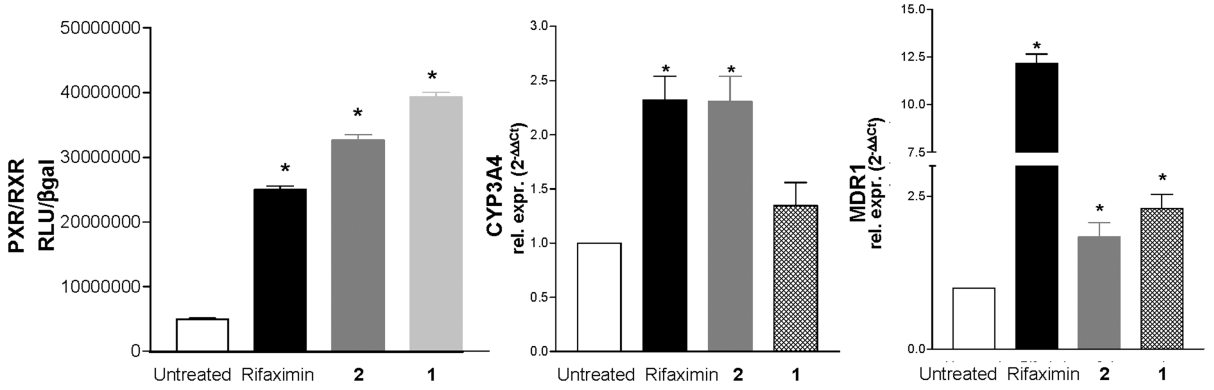

Figure 4. (A) Transactivation of PXR by compounds **1** and **2** in HepG2 cells. (B,C) Compounds **1** and **2** effectively increased the expression of CYP3A4 and MDR1, two PXR regulated genes in HepG2 cells. Expression of target genes was measured by RT-PCR.

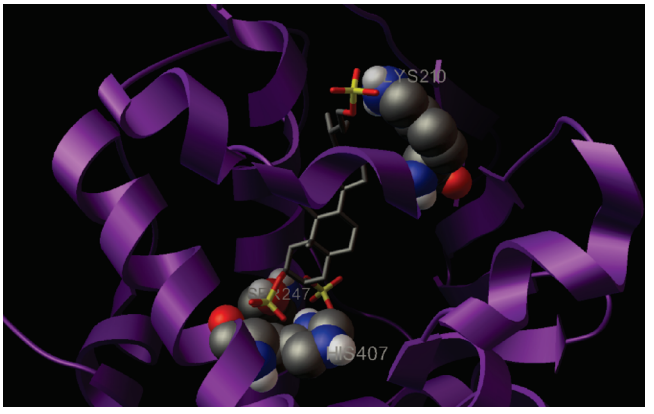

Figure 5. Docked model of **1** bound to PXR model (PDB code: 1M13, displayed as purple ribbon); **1** is displayed as sticks colored by atom type, while His407, Ser247, and Lys210 are depicted as atom type colored CPK models.

bonds with Ser247 (3-*O*-sulfate), His407 (2-*O*-sulfate), and Lys210 (24-*O*-sulfate, also protruding toward the solvent), providing the complex with an increased predicted stability fully compatible with the experimental biological assays.

Discussion and Conclusion

In the present report, we describe the isolation, structural characterization, and pharmacological profile of two novel steroids from a South Pacific specimen of the widely studied sponge *T. swinhoei*. Solomonsterols A (**1**) and B (**2**) represent the first example of 5α-cholane and 24-nor-5α-cholane from marine organisms.

The pharmacological screening demonstrated that solomonsterols A and B are endowed with an immunomodulatory potential, thus exposing RAW264.7 cells to solomonsterols inhibited IL-1β gene expression induced by LPS. Because IL-1β is a key cytokine and high in the hierarchy that drives innate immune response, these results highlight the potential for the use of solomonsterols in clinical conditions characterized by a dysregulation of innate immunity.

In the search for a molecular target(s) for solomonsterols, we have screened their effect on nuclear receptors. The results of these studies demonstrated that while solomonsterols do not activate the farnesoid-X-receptor (FXR, data not shown), both agents were effective ligands for PXR. PXR is a master gene orchestrating the expression of a wide family of genes involved in uptake, metabolism, and disposal of a number of endo- and xenobiotics, including drugs, bile acids, steroid

hormones, and metabolic intermediates in mammalian cells.[37] Following ligand binding, PXR forms an heterodimer with the retinoid-X-receptor (RXR) that binds to specific PXR response elements (PXREs), located in the 5′-flanking regions of PXR target genes, resulting in their transcriptional activation.[38,41,42] Supporting a role for solomonsterols in regulating PXR, we observed that compounds **1** and **2** effectively increased the expression of two well characterized PXR target genes, CYP3A4 and MDR1, in a human hepatocyte cell line.[42,43]

In conclusion, we report for the first time the identification of a novel class of PXR agonists, solomonsterols A (**1**) and B (**2**), isolated from *Theonella swinhoei*; such compounds could be potentially used for the treatment of human disorders characterized by dysregulation of innate immunity and with inflammation and, in light of our molecular modeling results, they can inspire the synthesis of new compounds able to target PXR. Furthermore, the discovery of solomonsterols provides insight into the mechanism of ligand recognition by PXR and reaffirms the utility of examining natural product libraries for identifying novel receptor ligands.

Experimental Section

Specific rotations were measured on a Jasco P-2000 polarimeter. High-resolution ESI-MS spectra were performed with a Micromass Q-TOF mass spectrometer. NMR spectra were obtained on Varian Inova 500 and Varian Inova 700 NMR spectrometers (^{1}H at 500 and 700 MHz, ^{13}C at 125 and 175 MHz, respectively) equipped with a Sun hardware, δ (ppm), J in hertz, and spectra referred to CD_3OH ($\delta_H = 3.31$) as internal standards. HPLC was performed using a Waters model 510 pump equipped with Waters Rheodine injector and a differential refractometer, model 401.

Droplet counter current chromatography (DCCC) was performed on a Tokyo-Japan apparatus (Tokyo RIKAKIKAI Co.) equipped with 250 glass-columns.

COSY, HSQC, HMBC, and ROESY experiments were recorded on a Varian Inova 700 NMR spectrometer (cryoprobe) using standard pulse programs. HSQC experiments were optimized for $^1J_{C-H} = 145$ Hz, and HMBC spectra were optimized for $^{2,3}J_{C-H} = 8$ Hz.

Through-space ^{1}H connectivities were evidenced using a ROESY experiment with mixing times of 400 and 200 ms, respectively.

The purities of compounds **1** and **2** were determined to be greater than 95% by HPLC, MS, and NMR.

Sponge Material and Separation of Individual Sterols. Collection and extraction of the sponge was performed as described previously.[22]

The *n*-BuOH extract (4 g) was chromatographed in two runs by DCCC using *n*-BuOH/Me_2CO/H_2O (3:1:5) in the descending mode (the upper phase was the stationary phase), flow rate 8 mL/min; 4 mL fractions were collected and combined on the basis of their similar TLC retention factors.

Fractions 2 and 3 (45.4 mg) were purified by HPLC on a C-18 column Macherey-Nagel Nucleodur 100−5 (5 μ, 250 mm × 4.6 mm, 1.0 mL/min) using 32% MeOH/H_2O (isocratic mode) as eluent to give 1.8 mg of solomonsterol B (**2**) (rt = 4.2 min) and 3.2 mg of solomonsterol A (**1**) (rt = 5.4 min).

Solomonsterol A (**1**): white amorphous solid; $[\alpha]_D^{25}$ +4.6 (*c* 0.8, methanol); ESIMS: *m*/*z* 661.2 $[M - Na]^-$, 308.3 $[M - 2Na]^{2-}$. HR ESIMS: calcd for $C_{24}H_{39}Na_2O_{12}S_3$: 661.1399; found 661.1415 $[M - Na]^-$.

Solomonsterol B (**2**): white amorphous solid; $[\alpha]_D^{25}$ +3.3 (*c* 0.1, methanol); ESIMS: *m*/*z* 647.3 $[M - 2Na]^-$, 301.2 $[M - 2Na]^{2-}$. HR ESIMS: calcd for $C_{23}H_{37}Na_2O_{12}S_3$ 647.1243; found 647.1298 $[M - Na]^-$.

Quantitative Real-Time PCR. RAW264.7 macrophages and HepG2 cells were cultured as previously described. Cells were incubated with 10 and 50 μM of compounds **1** and **2** in the presence of bacterial LPS for 24 h. At the end of incubation, cells were harvested and lysed with 1 mL of TRIZOL reagent for RNA extraction (Invitrogen). Then 50 ng template was added to the PCR mixture (final volume 25 μL) containing the following reagents: 0.2 μM of each primer and 12.5 μL of 2X SYBR Green qPCR master mix (Invitrogen, Milan, Italy). All reactions were performed in triplicate and the thermal cycling conditions were: 2 min at 95 °C, followed by 40 cycles of 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s in iCycler iQ instrument (Biorad, Hercules, CA). The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_T: cycle number at which each PCR reaction reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔC_T) between the C_T value of the sample for the target gene and the mean C_T value of that sample for the endogenous control (GAPDH). Relative expression was calculated as $2^{-\Delta\Delta C_T}$. All PCR primers were designed with PRIMER3-OUTPUT software using published sequence data from the NCBI database.[42]

Cell-Based Luciferase Assay. HepG2 cells were transfected with a PXR and RXR expressing vectors (pSG5-PXR and pSG5-RXR), with a reporter vector containing the PXR target gene promoter (CYP3A4 gene promoter) cloned upstream of the luciferase gene (pCYP3A4promoter-TKLuc) and with a β-galactosidase expressing vector as internal control of transfection efficiency (pCMV-β-gal). Twenty-four hours post transfection, the cells were stimulated 18 h with the PXR ligand rifaximin 10 μM (as positive internal control) and with 50 μM of compounds **1** and **2**. Then 5 μL of cell lysate was incubated with a substrate of luciferase gene (luciferase assay substrate; Promega) and the relative luciferase units (RLU) were measured with the Glomax 20/20 luminometer (Promega). Luciferase activities were normalized for transfection efficiencies by dividing the relative light units by β-galactosidase activity expressed from cotransfected pCMV-βgal plasmid (RLU/β-gal).

Computational Details. Molecular dynamics (MD) calculations were performed at 300K for 50 ns using the AMBER force field (MacroModel software package)[44] to give 100 structures of **1**, each of which was minimized using the Polak−Ribier conjugate gradient algorithm (PRCG, 1000 steps, maximum derivative less than 0.05 kcal/mol). These calculations provided the lowest energy minimum conformer for **1**.

Docking of the minimized energy structure of **1** to the crystal structure of the human pregame-X-receptor in complex with hyperforin, PDB accession code 1M13) was carried out with the program Autodock Vina 1.0.3. Blind docking was carried out with an exhaustiveness value of 256. The binding affinities of the 9 output structures ranged from −10.0 to −8.7 kcal/mol.

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

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