Title

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Journal

The Journal of Organic Chemistry, 66(21)

ISSN

0022-3263

Authors

Carroll, Jennifer

Jonsson, Erika N

Ebel, Rainer

et al.

Publication Date

2001-10-01

DOI

10.1021/jo015784t

JOC
The Journal of Organic Chemistry

VOLUME 66, NUMBER 21 OCTOBER 19, 2001

Articles

Probing Sponge-Derived Terpenoids for Human 15-Lipoxygenase Inhibitors

Jennifer Carroll,[†,‡] Erika N. Jonsson,[†] Rainer Ebel,[†,‡] Michael S. Hartman,[†] Theodore R. Holman,*[,†] and Phillip Crews*[,†,‡]

Department of Chemistry and Biochemistry and Institute for Marine Sciences, University of California—Santa Cruz, California 95064

phil@chemistry.ucsc.edu

Received May 22, 2001

A human 15-lipoxygenase (15-HLO) assay has been employed to discover new marine-sponge-derived bioactive compounds. Extracts from two different sponges, *Jaspis splendens* (order Choristida, family Jaspidae) and *Suberea* sp. (order Verongida, family Aplysinellidae), exhibited potent IC_{50} values of 0.4 and 0.1 μg/mL, respectively. Both are sources of terpenoids, and the former is a known source of (+)-jasplakinolide (**7**), which is inactive as a 15-HLO inhibitor. The terpenoids included (+)-(5*S*,6*S*)-subersin (**1**, $IC_{50} > 100$ μM), (−)-(5*R*,10*R*)-subersic acid (**2**, $IC_{50} = 15$ μM), jaspaquinol (**3**, $IC_{50} = 0.3$ μM), and (−)-jaspic acid (**4**, $IC_{50} = 1.4$ μM). Structure elucidations and lipoxygenase activity studies of these compounds are reported.

Introduction

Many bioactive marine natural products are known,[1] but few are noted as being lipoxygenase (LO) inhibitors.[2] The 5-, 12-, and 15-lipoxygenases, ubiquitous in terrestrial animals, carry out hydroperoxidation of polyenoic fatty acids. The resultant leukotrienes and lipoxins are important classes of signaling molecules that may be involved in a variety of human diseases.[3,4] Thus, the availability of selective inhibitors of the various human lipoxygenase (HLO) isoforms could provide biomolecules that are useful as pharmacological agents,[5] nutraceuticals,[4] or molecular tools.[6] The recent description of orange-peel-derived hexamethoxyflavone[7] as an inhibitor of soybean lipoxygenase (SLO) ($IC_{50} = 49$ μM) is an interesting example. More intriguing is that the small number of known marine-derived LO inhibitors have unique molecular structures. Among the first were the

* To whom correspondence should be addressed. Phone: (831) 459-2603. Fax: (831) 459-4197. E-mail (T.R.H.): holman@chemistry.ucsc.edu.
† Department of Chemistry and Biochemistry.
‡ Institute for Marine Sciences.

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10.1021/jo015784t CCC: $20.00
Published on Web 09/15/2001

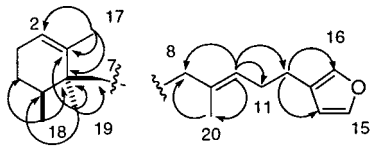
pseudopterosins and the fuscosides, diterpenoid-glycosides that were isolated from marine gorgonians. The pseudopterosins putatively possess anti-inflammatory properties that are derived from the inhibition of both cycloxygenase and LO biosynthetic pathways,[8] while the fuscosides were originally identified as 5-HLO inhibitors.[9] The only sponge-derived inhibitors described to date are the 15-HLO active pentabromo biphenyl ethers, which are isolated from cyanobacteria-containing sponges.[10] Believing that marine-derived chemotypes were an untapped source of new LO inhibitors, we began an investigation of the UCSC repository of marine sponge extracts and pure compounds. Our design employed a purified 15-HLO rather than the more common soybean 15-LO screen. Described herein are results on a series of terpenoids that were discovered in the first step of this process.

Results and Discussion

Out of an initial set of 100 crude extracts evaluated in the 15-HLO assay, one exhibited potent lipoxygenase inhibition activity at a concentration below the level of microgram per milliliter. The single potent extract of this set was from a Papua New Guinea sponge, *Suberea* sp. (order Verongida, family Aplysinellidae, coll. no. 97243), and its IC_{50} of 0.1 μg/mL (Table 1) merited a bioassay-guided followup using the 15-HLO screen. A cursory literature search revealed that the *Suberea* genus was unremarkable in its elaboration of secondary metabolites,[11] perhaps due to the relatively new characterization of this genus.[12] However, inspection of the NMR and MS data for the active crude extract and semipure fractions indicated the presence of several compounds possessing aliphatic methyls, appearing as intense ^{1}H NMR singlet resonances. At varying stages of the isolation, especially as pure substances were obtained, these extracts were first evaluated in the 15-HLO assay, and then via standard structural-dereplication procedures. Two compounds proved to be both known and strikingly potent, including jaspaquinol (**3**), $IC_{50} = 0.3$ μM, and (−)-jaspic acid (**4**), $IC_{50} = 1.4$ μM. This same pair of compounds was isolated in 1996 by our group[13] from a very different sponge that is now characterized as *Jaspis splendens*,[14] the well-known source of (+)-jasplakinolide (**7**). The two other new compounds that were isolated possessed significantly less potency and included (+)-subersin (**1**), $IC_{50} > 100$ μM, and (−)-subersic acid (**2**), $IC_{50} = 15$ μM.

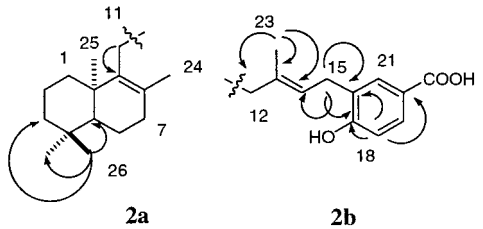
The characterization of (+)-subersin (**1**) commenced once its molecular formula of $C_{20}H_{30}O$ was established. The formula was supported by data, including positive ion HREIMS *m/z* 286.2267 $[M]^+$ (Δ 3.0 mmu of calcd) and the DEPT-135 ^{13}C NMR carbon types, $4CH_3 + 6CH_2 +$ $6CH + 4C$, giving a count of $C_{20}H_{30}$. A furan moiety was proposed because heteroatom hydrogens were lacking and characteristic ^{13}C NMR shifts could be located (δ 142.7, d, C15; 139.1, d, C16; 125.1, s, C13; 111.3, d, C14). There were also two double bonds (δ 139.6, s, C1; 122.1, d, C2; 137.2, s, C9; 123.3, d, C10). The preceding features accounted for five of the six degrees of unsaturation, indicating that a carbocyclic ring was present.

(+)-subersin (**1**)



1a **1b**

(-)-subersic acid (**2**)



2a **2b**

Figure 1. Substructures and selected gHMBC correlations. gHMBC correlations not shown are those for (+)-subersin (**1**) H7 to C8 or (−)-subersic acid (**2**) H11 to C12.

Table 1. 15-HLO Inhibition Activity of Crude Sponge Extracts

sponge	coll no.	site	IC_{50} (μg/mL)
Suberea sp.	97243	Papua New Guinea	0.1 ± 0.02
J. splendens	94541	Indonesia	150 ± 15
	95077	Papua New Guinea	45 ± 5
	96117	Papua New Guinea	0.4 ± 0.02
	96555	Indonesia	6.5 ± 3
	97238	Papua New Guinea	>300

The substructures **1a** and **1b**, shown in Figure 1, were assembled as follows. A set of ^{1}H−^{1}H COSY correlations revealed the spin systems H2−H3−H4−H5−$H_3$18, H10−H11−H12, and H14−H15. Additional important data came from a series of Me-based ^{1}H−^{13}C gHMBC correlations (Figure 1) of $H_3$17 (δ 1.63) to C1, C2, and C6; $H_3$18 (δ 0.94) to C4, C5, and C6; $H_3$19 (δ 1.03) to C1, C5, C6, and C7; and $H_3$20 (δ 1.59) to C8 and the other ^{1}H-^{13}C gHMBC correlations shown in Figure 1. The two substructures could be interconnected using the ^{1}H−^{13}C gHMBC correlation from H7′ (δ 1.34) to C8. Next, the relative stereochemistry was determined through 1D-nOe correlations from $H_3$19 (δ 1.03) to H5 and from $H_3$18 (δ 0.94) to H7. As expected, the ^{13}C NMR shifts of *trans* methyls 18 and 19 agree with those of a sponge-derived sesterterpene containing substructure **1a**.[15]

Attention was shifted next to the structure elucidation of (−)-subersic acid (**2**) which has a molecular formula of $C_{27}H_{38}O_3$. The HRTOFMS data were identical to those of (−)-jaspic acid (**4**),[13] $[M - H]^-$ *m/z* = 409.2743 (Δ 0.0

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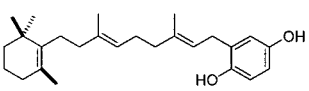
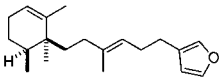
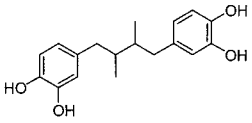
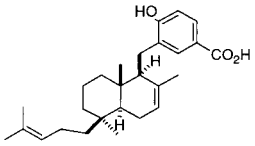
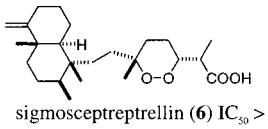
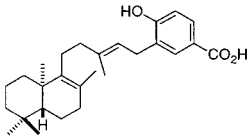
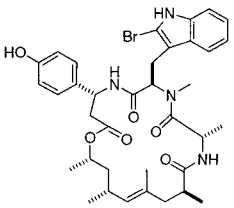
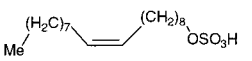
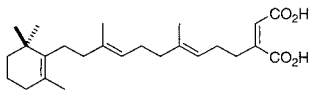
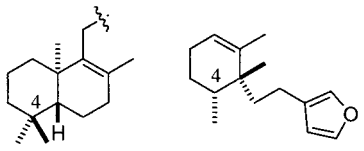
Table 2. 15-HLO Inhibition Activity (μM) of Pure Compounds

active natural products	inactive natural products	standards
 jaspaquinol (**3**) IC_{50} 0.3 ± 0.1	 (+)-subersin (**1**) IC_{50} > 100	 NDGA (**8**) IC_{50} 0.3 ± 0.1
 (-)-jaspic acid (**4**) IC_{50} 1.4 ± 0.2	 sigmosceptreptrellin (**6**) IC_{50} > 50	
 (-)-subersic acid (**2**) IC_{50} 15 ± 3	 (+)-jasplakinolide (**7**) IC_{50} > 100	 oleyl sulfate (**9**) IC_{50} 0.9 ± 0.1
	 dehydroluffariellolide diacid (**5**) IC_{50} > 100	

mmu of calcd), whereas the NMR data were dissimilar, showing that **2** and **4** were structural isomers. The DEPT-135 ^{13}C NMR data gave $5CH_3 + 7CH_2 + 7CH + 8C$, totaling to $C_{27}H_{36}$ and making it evident that two OH residues were present, with one being on an aromatic ring (δ 159.8, s), and the other being part of a carboxylic acid (δ 171.7, s). There were striking similarities in the ^{13}C NMR data between (−)-subersic acid (**2**) and (−)-jaspic acid (**4**) that allowed the two substructures **2a** and **2b** to be assembled. For example, the 4-hydroxybenzoic acid moiety of **2b** had shifts identical to those of **4** from C15 to C22, which were verified by gHMBC data (shown in Figure 1): H18 (δ 6.86) to C16 and C20; H15 (δ 3.42) to C13, C14, C16, C17, and C21; H21 and/or H19 (δ 7.91) to C17, C16, and C15; and H23 (δ 1.83) to C12, C13, and C14. The ^{13}C NMR data of the bicyclic C1−C11 portion of **2a** were identical to those published for this well-known ring system.[16] The side chain attachment was identified from the gHMBC correlations of H11 (δ 2.14) to C8, C9, and C12, with the latter correlation to C12 supporting the proposed interconnection of substructures **2a** and **2b**. The *E* geometry of position $\Delta^{13/14}$ could be deduced from the upfield ^{13}C NMR shift of methyl C23 (δ 16.7).

The final point to be established concerned the absolute stereochemistry of the ring substituents in **1** and **2**. This assignment for **2** was addressed with a detailed comparison of its molecular rotation to that of the same ring system, previously determined by Capon.[16] The measured molar rotation ([Φ] = −189) of (−)-subersic acid is comparable to the [Φ] = −191 reported by Capon for substructure **10**, shown in Figure 2. This analysis then indicates a 5*R*,10*R* stereochemistry for **2**. Using the same approach, we used the 4*R*,5*R* absolute stereochemistry known for (−)-microcionin-2 (**11**, Figure 2), assigned by total synthesis,[17] to delineate a 5*S*,6*S* stereochemistry for **1**, given that the molar rotation for **1** ([Φ] = +10) is similar in magnitude but opposite in sign to that of **11** ([Φ] = −27).[18]



substructure (**10**) (-) micrinin (**11**)

Figure 2. Compounds used for stereochemical comparison to **1** and **2**.

An unexpected outcome represented in the findings reported above deserves additional comment. We first reported on the two terpene-benzenoids, jaspaquinol (**3**) and (−)-jaspic acid (**4**), reisolated here from *J. splendens* (order Choristida, family Jaspidae). This sponge (coll. no. 96117) was chemically distinct from 18 others within this same species that were present in our repository, though all such extracts that were examined contained (+)-jasplakinolide (**7**). To further test the efficacy of our assay, we screened five *J. splendens* sponge extracts, including the unique one containing **3** and **4**. As shown in Table 2, (+)-jasplakinolide (**7**) was inactive, and indicated in Table 1 is the insignificant activity observed for three of the five *J. splendens* extracts tested. The most active extract, 96117 (IC_{50} = 0.4 μg/mL), was the source

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of **2** and **3**. Another potent extract was 96555 (IC_{50} = 6.5 μg/mL), which appears to contain additional and previously undescribed constituents. The observation of the identical pair of terpene-benzenoids **3** and **4** from sponges in two distinct orders (Choristida and Verongida, respectively) is unusual and, in this case, cannot be explained as a taxonomic anomaly.[19]

We have found the 15-HLO screen that was developed for this research is both a robust tool and a reliable reporter of lipoxygenase inhibition. It was important to avoid the inconsistent behavior of 5-HLO assays previously encountered with manoalide, a sponge-derived sesterterpene.[20] DeVries et al. initially reported manoalide to have an IC_{50} = 0.3 μM in 5-HLO assays (in both RBL-1 and HPMNL cell extracts),[21] while Cabré et al. found that it did not inhibit 5-HLO (in HPMNL cell extracts)[22] at concentrations of up to 50 μM. However, both observed nordihydroguaiaretic acid (NDGA, **8**, IC_{50} = 0.3 μM), a known LO inhibitor, to be fully potent.[21,22] To avoid this problem, we utilized purified 15-HLO and obtained an IC_{50} of 0.3 ± 0.1 μM for NDGA (**8**), consistent with previous results. Data from 15-HLO assays were also obtained for another benchmark compound, oleyl sulfate (OS, **9**), previously shown to be an allosteric inhibitor.[23] We observed its IC_{50} of 0.9 μM shown in Table 2 to be in good agreement with the K_i of 0.4 ± 0.005 μM.

Conclusions

There are multiple pathways that are currently known to inactivate lipoxygenases, ranging from competitive[24] to allosteric[23] to reductive inhibition.[25] Having a series of LO inhibitors in hand provides an opportunity to explore the requirements of such alternative pathways. On one hand, NDGA (**8**) is known to be a LO redox inhibitor that reduces the active Fe(III) enzyme to the inactive Fe(II) enzyme.[25] This mode of action is also responsible for the inhibition by jaspaquinol (**3**), the most potent 15-HLO inhibitor isolated in this study (IC_{50} = 0.3), because of the reduction of the active site ferric ion concentration as seen by fluorescence spectroscopy using soybean 15-LO.[26] It is probable that the hydroquinol residue of **3** reduces the iron, but more data will be required to prove this point. A similar mechanism could be operating for sponge-derived, polybrominated phenols and diphenyl ethers that are known 15-HLO inhibitors (IC_{50} = 1−7 μM),[10] and these are currently being re-examined in our laboratory.

It would appear likely that the other two 15-HLO inhibitors isolated here, (−)-subersic acid (**2**) and (−)-jaspic acid (**4**), operate by a nonredox inactivation mechanism. This conclusion is based on the observation that these compounds do not cause a change in the fluorescence spectroscopy of soybean 15-LO (i.e., no reduction of Fe(III)). While (−)-jaspic acid (**4**) and (−)-subersic acid (**2**) are potent inhibitors of 15-HLO, IC_{50} = 1.4 and 15 μM, respectively, they exhibit differential activity with approximately a 10-fold variation. Though the data set is insufficient at this point to draw extensive SAR conclusions, it is noteworthy that the polar headgroups are the same for **2** and **4**, indicating that their polyunsaturated spacers are critical for inhibition. Furthermore, two other sesterterpene acids evaluated here, dehydroluffarellolide diacid (**5**) and sigmosceptreptrellin (**6**), were inactive, as was (+)-subersin (**1**), which adds further support to the hypothesis that the structure of the polyunsaturated spacer is important for inhibition activity.

Work in progress will be published in due course and will address two interesting issues. First, preliminary data indicate that these inhibitors may be selective against the different types of human lipoxygenases (**12** vs **15**). Second, a structural parallelism between **4** and arachidonic acid (AA) may be of significance. Both have a similar spatial separation between the unsaturated functionality that undergoes dioxygen incorporation by 15-HLO and the carboxylic headgroup. In AA, this involves the 14,15 double bond, while in the case of **4**, our current hypothesis is that the trisubstituted side-chain double bond is bound in a similar location but cannot undergo oxygenation, possibly due to the lack of a 1,4-diene functionality.

Experimental Section

General Procedures. NMR spectra were recorded in $CDCl_3$ and CD_3OD solutions at 500 or 125.7 MHz for ^1H and ^{13}C, respectively. Optical rotation was measured on a JASCO DIP-370 digital polarimeter. UV data were obtained on a Hewlett-Packard 8452A diode array spectrophotometer, ES-IMS spectra on a VG Quattro II apparatus, and HRMS data on PE Biosystems Mariner and JEOL JMS-AX505HA mass spectrometers.

Biological Material, Collection, and Identification. The *Suberea* sp. sponge specimen was collected by SCUBA at a depth of 20 m in the Madang region of Papua New Guinea. Preliminary taxonomy was performed by M. Sanders who identified the sponge as *Hyrtios* sp. (family Thorectidae). Microscopy of the sponge fibers yielded an interesting new taxonomy. The specimen is a massive globular sponge that is dark gray externally and tan internally. The surface is conulose, and the consistency is dense and rubbery and is very hard in dry specimens. The skeleton consists only of fibers (typical of Verongids) that are 250−300 μm in diameter and concentrically layered , with a strong organic pith that is 40−50 μm in diameter. The nature of the fibers, with both pith and bark well represented, points toward the recently described genus *Suberea*.[12] Taxonomy of the *Jaspis* sp. sponges was performed by methods previously described.[14]

Extraction and Isolation. The sample was preserved by immersion in a 1:1 alcohol/seawater solution. After 24 h, this solution was decanted and discarded. The damp organism was then transported in a Nalgene bottle back to the home lab at ambient temperature and extracted with 100% MeOH to give a crude oil which was then partitioned between various solvents. The hexane and methylene chloride fractions were further purified on Biotage silica gel columns (100% $MeCl_2$ and EtOAc/hexane, respectively) to give five fractions each. These were further purified by reverse-phase gradient HPLC to give **1** (0.02% dry wt of sponge), **2** (0.01% dry wt of sponge), **3** (0.01% dry wt of sponge), and **4** (0.25% dry wt. of sponge).

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Lipoxygenase Assay. 15-HLO was expressed and purified as described previously.[27] The enzyme activity was determined by direct measurement of the product formation of a 3 μM linoleic acid solution by following the increase of absorbance at 234 nm in 25 mM Hepes (pH 7.5) and 0.2% (g/L) cholic acid. All reactions were performed in 2 mL of buffer, ≈200 nM 15-HLO, and the reaction mixtures were constantly stirred with a rotating magnetic bar (≈22 °C). IC_{50} values were determined by measuring the enzymatic rate at a variety of inhibitor concentrations (depending on the inhibitor potency) and plotting their values versus inhibitor concentration. The corresponding data were fit to a simple saturation curve, and the inhibitor concentration at 50% activity was determined. The inhibitors were typically dissolved in methanol at a concentration of ≈25 mg/mL.

(+)-(5*S*,6*S*)-Subersin (1): yellow oil; 24 mg; $[\alpha]_D$ +30° (*c* 2.6, $CHCl_3$); HREIMS *m*/*z* 286.2267 $[M]^+$ (Δ 3.0 mmu of calcd for $C_{20}H_{30}O$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34 (s, H15), 7.22 (d, J = 0.5 Hz, H16), 6.28 (s, H14), 5.40 (s, H2), 5.17 (t, J = 7.5 Hz, H10), 2.45 (t, J = 8 Hz, 2H, H12), 2.24 (q, J = 8 Hz, 2H, H11), 1.97 (m, H8), 1.96 (m, 2H, H3), 1.89 (m, H8′), 1.63 (s, H_3, H17), 1.60 (m, H4), 1.59 (s, H_3, H20), 1.57 (m, H5), 1.47 (m, H7), 1.43 (m, H4′), 1.34 (m, H7′), 1.03 (s, H_3, H19), 0.94 (d, J = 8 Hz, H_3, H18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 142.7 (d, C15), 139.6 (s, C1), 139.1 (d, C16), 137.2 (s, C9), 125.1 (s, C13), 123.3 (d, C10), 122.1 (d, C2), 111.3 (d, C14), 39.8 (s, C6), 37.8 (d, C5), 35.8 (t, C8), 34.7 (t, C7), 28.6 (t, C11), 27.5 (t, C4), 26.6 (q, C19), 25.1 (t, C12), 24.1 (t, C3), 19.7 (q, C17), 16.3 (q, C20), 16.0 (q, C18).

(−)-(5*R*,10*R*)-Subersic Acid (2): yellow oil; 12 mg; $[\alpha]_D$ −46° (*c* 0.5, $CHCl_3$); HREIMS *m*/*z* = 409.2743 $[M - H]^-$ (Δ 0.0 mmu of calcd for $C_{27}H_{38}O_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.91 (s, 2H, H21 and H19), 6.86 (d, J = 10 Hz, H18), 5.36 (t, J = 5 Hz, H14), 3.42 (d, J = 10 Hz, H_3, H15), 2.14 (m, 2H, H11), 2.12 (m, 2H, H12), 2.09 (m, 2H, H7), 1.83 (m, H1), 1.83 (s, H_3, H23), 1.62 (m, 4H, H6 and H2), 1.58 (s, H_3, H24), 1.17 (m, H3′), 1.14 (m, 2H, H1′ and H5), 0.95 (s, H_3, H25), 0.89 (s, H_3, H26), 0.84 (s, H_3, H27); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.7 (s, C22), 159.8 (s, C17), 141.0 (s, C13), 132.9 (d, C21), 130.8 (d, C19), 127.0 (s, C16), 126.3 (s, C8), 121.9 (s, C20), 120.3 (d, C14), 116.0 (d, C18), 52.1 (s, C5), 42.1 (t, C3), 40.7 (t, C12), 39.3 (s, C10), 37.3 (t, C1), 33.9 (t, C7), 33.6 (s, C4), 33.6 (q, C26), 29.9 (t, C15), 27.3 (t, C11), 21.9 (q, C27), 20.4 (q, C25), 19.8 (q, C24), 19.3 (t, C2), 19.3 (t, C6), 16.7 (q, C23).

Acknowledgment. Financial support was from Sea Grant NA06RG0142 Project E/IF-3 (P.C.), NIH Grant CA47135 (P.C.), ACS Grant RPG-00-219-01-CDD (T.R.H.), NIH Grant GM 56062-01 (T.R.H.), U.S. Department of Education GAANN Fellowship P200A000226 (J.C.), and a DAAD fellowship to R.E. We thank M. J. Beck of Applied Biosystems for the ESITOF-MS data, Dr. M. C. Diaz for the sponge taxonomy determination, Ms. M. Sanders for the assistance in sponge collection, and Mr. J. Williams for technical assistance. The cover art was prepared as a joint effort between J.C., P.C., and T.R.H.

Supporting Information Available: Spectra of **1** and **2** (^{1}H and ^{13}C NMR and MS). This material is available free of charge via the Internet at http://pubs.acs.org.

JO015784T

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