A β-Carboline Alkaloid from the Papua New Guinea Marine Sponge *Hyrtios reticulatus*

Wayne D. Inman,[†] Walter M. Bray,[‡] Nadine C. Gassner,[‡] R. Scott Lokey,[†,‡] Karen Tenney,[†] Young Yongchun Shen,[§] Karen TenDyke,[§] Ted Suh,[§] and Phillip Crews*[,†]

Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064, UCSC Chemical Screening Center, University of California, Santa Cruz, California 95064, and Eisai Research Institute, 4 Corporate Drive, Andover, Massachusetts 01810

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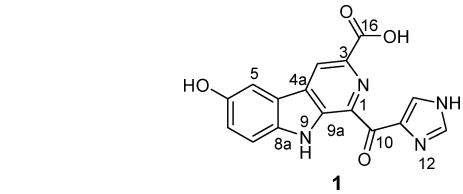
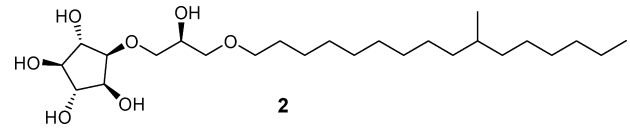
A new 1-imidazoyl-3-carboxy-6-hydroxy-β-carboline alkaloid, named hyrtiocarboline (**1**), was isolated from a Papua New Guinea marine sponge, *Hyrtios reticulatus*. The structure was elucidated from spectroscopic data, including $^{1}H-^{15}N$ HMBC NMR experiments, which provided complementary ^{15}N chemical shift information in support of the structure. This compound showed selective antiproliferative activity against H522-T1 non-small cell lung, MDA-MB-435 melanoma, and U937 lymphoma cancer cell lines.

Marine sponges of the genus *Hyrtios* (Demospongia class, Dictyoceratida order, Thorectidae family) have proven to be a rich and structurally diverse source of cytotoxic compounds as leads for new anticancer therapeutics. The list of cytotoxic compounds spanning several classes of secondary metabolites includes spongistatins[1] or altohyrtins[2] (macrolides), sesterstatins[3] (sesterterpenes), puupehenone[4] and 15-oxopuupehenol[5] (mixed sesquiterpene-shikimates), and hyrtioerectines[6] (tryptamine-derived alkaloids). Until now, only two reports on secondary metabolites from *H. reticulatus* have been published. The first report described 5-hydroxytryptamine-derived alkaloids (β-carbolines) from *H. reticulatus* collected in southwest Sulawesi (Indonesia),[7] followed by the sesterterpene heteronemin, isolated by bioassay-guided fractionation with a farnesyl transferase inhibition assay, from *H. reticulatus* obtained in Vanuatu.[8] In this paper, we report the isolation of a new 3-carboxy-β-carboline alkaloid (**1**), which we have named hyrtiocarboline, together with the known compounds sacrotride A (**2**)[9,10] and 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (**3**),[11] from the marine sponge *H. reticulatus*.

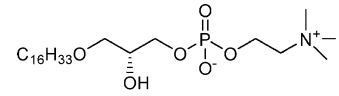
The sponge *H. reticulatus* was collected and preserved according to our standard laboratory procedures and then stored at 4 °C.[12] The preliminary extraction for bioassay and dereplication analysis was carried out using accelerated solvent extraction (ASE), where approximately 100 g of sponge was extracted with the solvent series hexanes, dichloromethane, and methanol. The dichloromethane and methanol extracts were then tested for antiproliferative activity in a primary screen against U937 histiocytic lymphoma cancer cells, and the dichloromethane extract was found to be active (98.6% inhibition at 10 μg/mL).[13] Follow-up testing of the dichloromethane extract exhibited the following IC_{50} values for antiproliferative activity in four human cancer cell lines: HT-29 colon cancer cells (3.8 μg/mL), H522-T1 non-small cell cancer cells (8.0 μg/mL), MDA-MB-435 melanoma (0.9 μg/mL), and U937 histiocytic lymphoma cells (2.9 μg/mL). Dereplication analysis of the extract was conducted using a HPLC-UV-ELSD-MS system equipped with a C_{18} HPLC column and a binary mobile phase of acetonitrile/0.1% formic acid and water/0.1% formic acid, spanning a gradient from 10% to 100% acetonitrile over 30 min. The resulting UV-DAD and MS data for the major peaks were then cross-checked for reported structures in MarinLit.[14] Three peaks with molecular ions at *m/z* 323, 463, and 482 did not correspond to any reported compounds based on mass and occurrence in *Hyrtios* and were designated as a high priority for isolation and identification (see Supporting Information, Table S1, for dereplication results and discussion). In order to isolate material for structural characterization and bioassay testing, a larger scale extraction was performed. The sponge was extracted in methanol and the methanol extract fractionated on silica followed by reversed-phase HPLC, to yield the β-carboline (**1**) and the known cytotoxic compounds sacrotride A (**2**, $C_{25}H_{50}O_7$, *m/z* 463, M + H)[9,10] and 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (**3**, $C_{24}H_{52}NO_6P$, *m/z* 482, M + H).[11]



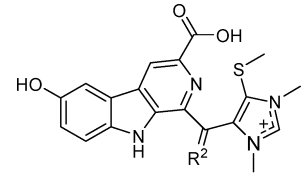
1



2



3



4 R^2 = O
5 R^2 = H, H

The molecular formula, $C_{16}H_{10}N_4O_4$, of **1** was established by HRESIMS from the pseudomolecular ion peak at *m/z* 323.0766 $(M + H)^+$. Several ^1H and ^{13}C NMR signals were absent and others were broadened when initial spectra were taken in CD_3OD or DMSO-d_6. Addition of a 1:3 mixture of TFA-*d*/$CDCl_3$ to **1** resulted in observation of all NMR signals, and the assigned values are shown in Table 1. The ^1H NMR signals for H-5 (δ 7.82, d, $J = 2.0$ Hz), H-7 (δ 7.42, dd, $J = 8.8, 2.0$ Hz), and H-8 (δ 7.74, d, $J = 8.8$ Hz) revealed the presence of an ABX spin system characteristic of a 1,3,4-trisubstituted benzene ring. The 3-carboxy-6-hydroxy β-car-

* To whom correspondence should be addressed. Tel: 831-459-2603. Fax: 831-459-2935. E-mail: phil@chemistry.ucsc.edu.
[†] Department of Chemistry and Biochemistry, University of California, Santa Cruz.
[‡] UCSC Screening Center, University of California, Santa Cruz.
[§] Eisai Research Institute.

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Table 1. NMR Data for Hyrtiocarboline (**1**) in TFA-*d*/$CDCl_3$ (1:3)

position	δ_H (m, *J* in Hz)	δ_C, type	δ_N	$HMBC_{H-C}$	$HMBC_{H-N}$
1		134.1,[a] C			
2			290.0		
3		133.2,[a] C			
4	9.19 (s)	123.5, CH		4b, 9a, 16	2
4a		134.0, C			
4b		121.9, C			
5	7.82 (d, 2.0)	108.0, CH		4a, 6, 7, 8a	
6		150.3, C			
7	7.42 (dd, 8.8, 2.0)	121.2, CH		5, 6, 8a	
8	7.74 (d, 8.8)	114.7, CH		4b, 6	9
8a		137.9, C			
9			115.9		
9a		138.5, C			
10		178.5, C			
11		131.5, C			
12			169.0[a]		
13	9.05 (s)	135.7, CH		11, 15	12, 14
14			160.0[a]		
15	8.80 (s)	127.4, CH		11, 13	12, 14
16		170.0, C			

[a] Interchangeable.

boline substructure was established by $^1H-^{13}C$ HMBC correlations of H-4 (δ 9.19) to C-4b (δ 121.9), C-9a (δ 138.5), and C-16 (δ 170.0), from H-5 (δ 7.82) to C-4a (δ 134.0), C-6 (δ 150.3), C-7 (δ 121.2), and C-8a (δ 137.9), and from H-8 (δ 7.74) to C-4b (δ 121.9) and C-6 (δ 150.3), reminiscent of correlations reported for dragmacidonamines A (**4**) and B (**5**).[15] The ^{15}N chemical shifts for N-2 and N-9 were obtained from $^1H-^{15}N$ HMBC correlations from H-4 (δ 9.19) to N-2 (δ 290.0) and from H-8 (δ 7.74) to N-9 (δ 115.9), which compared favorably to those reported for the β-carboline norharmane (N-2, δ 303.3; N-9, δ 113.0).[16] The remaining $C_4H_3N_2O$ atom count was assigned as a 4-substituted imidazoyl group attached to C-1 of the β-carboline on the basis of fulfilling the four remaining sites of unsaturation and $^1H-^{13}C$ HMBC correlations of H-13 (δ 9.05) to C-11 (δ 131.5) and C-15 (δ 127.4) along with H-15 (δ 8.80) to C-11 and C-13 (δ 135.7). The ^{15}N chemical shifts of N-12 and N-14 (δ 169.0, 160.0) obtained from $^1H-^{15}N$ HMBC correlations of H-13 to N-12 and N-14, as well as H-15 to N-12 and N-14, further substantiated the presence of the imidazole ring, and the chemical shifts are similar to values previously published for a 4-substituted imidazole (δ 178.0, 172.2) contained in amphistin.[17] Although no HMBC correlations were observed to the carbonyl at C-10, the remaining CO unit could be placed only between C-1 in the carboline and C-11 of the imidazole, extending conjugation and accounting for the extensive UV spectrum observed for **1** (λ_{max} 248, 312, 424 nm).

Natural products with a 3-carboxy-β-carboline core may originate from tryptophan rather than tryptamine and are uncommon in the marine environment. Only two examples have been reported, dragmacidonamines A (**4**) and B (**5**), isolated from a *Dragmacidon* sp. sponge (family Axinellidae, order Halichondria), and 2-methyl-9*H*-pyrido[3,4*b*]indole-3-carboxylic acid from the soft coral *Lignopsis spongiosum*.[18] Several 3-carboxy-β-carbolines have been reported from plants and microbial sources,[19] and *n*-butyl β-carboline-3-carboxylate has been implicated as an endogenous ligand for the benzodiazepine binding site in bovine brain.[20,21] Several other 1-imidazoyl-β-carbolines have been reported, with the simplest being des-*N*-methylxestomanzamine A (1-imidazoyl-β-carboline),[22] followed by xestomanzamine A [1-(*N*-methylimidazoyl)-β-carboline][23] and hyrtiomanzamine [1-(4-thiomethyl-*N*,*N*-dimethylimidazoyl)-6-hydroxy-β-carboline].[24] Hytriocarboline (**1**) was tested for antiproliferative activity against 13 cancer cell lines, and the results are shown in Table 2. Selective activity was found against three cancer cells lines: H522-T1 non-small cell lung, MDA-MB-435 melanoma, and U937 lymphoma (IC_{50} 1.2, 3.0, 1.5 μg/mL, respectively). The bioactivity profile for **1** with these three cancer cell lines was similar to the trend observed in the dichloromethane extract; however, the antiproliferative activity in the HT-29 colon cell line treated with the dichloromethane extract is not due to **1** and may be attributed in part to cytotoxic activity against colon cancer cells previously reported for **2**[9,10] and **3**.[11] Hyrtiocarboline (**1**) also exhibited 57% inhibition of HeLa cells at 230 μM after incubation for 24 h with induced cytotoxicity, disruption of the cytoskeleton, and nuclear blebbing. Likewise, harman (β-carboline) and norharman (1-methyl-β-carboline) were reported to exhibit cytotoxicity against PC12 cells at 150 and 300 μM, respectively, with apoptotic cell death, including highly condensed chromatin and extensive membrane blebbing.[25]

Table 2. Antiproliferative Activity of **1**

cancer cell line	IC_{50} (μg/mL)
H522-T1 (non-small cell lung)	1.2
MDA-MB-435 (melanoma)	3.0
U937 (lymphoma)	1.5
BT-549 (breast)	45
DU 145 (prostate)	34
H460 (large cell lung)	17−50
HCC-2998 (colon)	>50
HT-29 (colon)	>50
MCF-7 (breast)	>50
OVCAR-5 (ovarian)	>50
SF-539 (gliosarcoma)	>50
SR (lymphoma)	>50
UACC-257 (melanoma)	>50

Experimental Section

General Experimental Procedures. Small-scale extractions were carried out in a Dionex ASE 100 accelerated solvent extractor. NMR spectra were recorded on a Varian 600 spectrometer (600 MHz for ^1H and 150 MHz for ^{13}C) equipped with a cryoprobe using a 1:3 mixture of TFA-*d*/$CDCl_3$ or CD_3OD. The 7.27 and 77.2 ppm resonances for residual $CDCl_3$, and 3.31 and 49.2 ppm resonances for residual CD_3OD, were used as internal references for the ^1H and ^{13}C NMR spectra, respectively. The 112 ppm resonance for formamide was used as external reference for the ^{15}N NMR spectrum. Direct-inject HRESI mass measurements were obtained on a Mariner ESI-TOF mass spectrometer. For LCMS-DAD-ELSD analysis, samples were injected into a Waters HPLC system equipped with a 125 × 5 mm, 5 μm Luna C_{18} HPLC column (Phenomenex) and UV-DAD detector, followed by splitting the eluent between a Sedex evaporative light scattering detector (ELSD) and Mariner ESI-TOF mass spectrometer.

Sponge Description. Sponge samples (coll. no. 07325, 0.35 kg wet weight) were collected using scuba in July 2007 from Bismarck Sea, Papua New Guinea, GPS S 5°26.851, E 150°45.107, at depths of 13−20 m. The sponge was identified taxonomically as *Hyrtios reticulatus*, and a voucher specimen was deposited at the Naturalis, National Museum of Natural History, Leiden, The Netherlands, under the registration number RMNH Por 4822.

Extraction and Isolation. Samples were preserved in the field according to our standard laboratory procedures and stored in a cold room at 4 °C until extraction was performed. Initial extraction of a 100 g sample of the sponge was rapidly carried out with the solvent series hexanes (XFH), dichloromethane (XFD), and methanol (XFM) using an accelerated solvent extraction system under high pressure (1700 psi) at 110 °C. Large-scale extraction of the sponge (77 g) was carried out in 0.5 L of methanol, and 8.9 g of extract was obtained. A 2.3 g portion of the extract was fractionated into nine fractions using a Combi-Flash system equipped with a 40 g silica column and a linear gradient of methylene chloride to methanol over 50 min at 18 mL/min. The column was washed with an additional 300 mL of methanol. Fractions 5 and 6 were combined (240 mg) and purified on HPLC equipped with a 4 μm, 250 × 10 mm Synergi Hydro-RP column utilizing a 20 min gradient program of 10% to 30% acetonitrile in 0.1% formic acid in water at 4 mL/min, 280 nm detection, to yield **1** (10 mg, 0.003%). Fraction 4 (98 mg) was purified by HPLC equipped with a 10 μm, 250 × 21.2 mm Synergi MAX-RP column utilizing a 30 min gradient program of 10% to 100% methanol in 0.1% formic acid in water at 14 mL/min, ELSD detection, to yield **2** (5 mg, 0.002%). The fraction obtained from the methanol wash of the silica column (73 mg) was

purified by HPLC equipped with a 5 μm, 250 × 10 mm Luna C_{18} column utilizing a 30 min gradient program of 10% to 100% acetonitrile in 0.1% formic acid in water at 4.8 mL/min, ELSD detection, to yield **3** (1 mg, 0.0003%).

Hyrtiocarboline (1): orange oil, UV (acetonitrile/water/0.1% formic acid) λ_{max} 248, 312, 424 nm; ^{1}H and ^{13}C NMR (see Table 1); HRESIMS *m/z* 323.0766 $[M + H]^+$ (calcd for $C_{16}H_{11}N_4O_4$, 323.0775). ESIMSn fragmentation experiments in positive and negative modes substantiated the presence of the carboxylic acid. In the negative mode (pH 7) only one daughter ion was observed at *m/z* 277 $(M - CO_2)^+$. Conversely, fragmentation of the parent ion in positive mode (pH 2) resulted in the loss of CO_2 in a two-step fragmentation: first loss of water, *m/z* 305 $(M - H_2O)^+$, followed by loss of carbon monoxide, *m/z* 277 $(M - H_2O - CO)^+$.

Antiproliferative Bioassays. Antiproliferative effects of compound **1** were evaluated in 13 cultured human cancer cell lines shown in Table 2. The cells were placed into 96-well plates and grown in the absence or continuous presence of 1.5−50 000 nM test compounds for 96 h. Cell growth was assessed using the CellTiter-Glo luminescent cell viability assay (Promega) according to the manufacturer's recommendations. Luminescence was read on a Victor^{2}V 1420 MultiLabel HTS counter (Perkin-Elmer/Wallac). IC_{50} values were determined as the concentration of a compound that inhibits cell growth by 50% compared to untreated cell populations. Two separate replicate experiments were performed.

HeLa cells were plated in 384-well tissue culture-treated plates (Corning) at a density of 1500 cells per well. After incubating at 37 °C with 5% CO_2 overnight, compounds were pinned into plates using the Janus MDT (PerkinElmer). After 24 h, cells were fixed in 4% formaldehyde for 20 min, then washed with PBS using an automated plate washer (BioTek). The cells were then treated with PBS with 0.5% TritionX-100 for 10 min and washed and then blocked in PBS with 2% PBS for 20 min. Actin was stained with rhodamine-phalloidin for 20 min and then washed. Lastly, Hoechst 33342 (AnaSpec, Inc.) was used to stain the DNA; then the plate was washed once more with the automated plate washer. Images were taken using an automated fluorescence microscope (ImageXpress, MDS) at 10× magnifcation.

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Supporting Information Available: Table of dereplication results and discussion, and ^{1}H and ^{13}C NMR spectra for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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