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

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Abstract

A new 1-imidazolyl-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 ¹H-¹⁵N HMBC NMR experiments that provided complementary ¹⁵N chemical shift information in support of the structure. This compound showed selective anti-proliferative 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 spongistatins¹ or altohyrtins² (macrolides), sesterstatins³ (sesterterpenes), puupehenone⁴ and 15-oxopuupehenol⁵ (mixed sesquiterpene-shikimates), and hyrtioerectines⁶ (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 south west 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 were 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 @ 10 μ g/mL).¹³ Follow-up testing of the dichloromethane extract exhibited the following IC₅₀ values for antiproliferative activity in

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

four human cancer cell lines, including HT-29 colon cancer cells (3.8 $\mu\text{g/mL}$), H522-T1 non-small-cell cancer cells (8.0 $\mu\text{g/mL}$), MDA-MB-435 melanoma (0.9 $\mu\text{g/mL}$), and U937 histiocytic lymphoma cells (2.9 $\mu\text{g/mL}$). 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 *Hyrtilios* 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**, $\text{C}_{25}\text{H}_{50}\text{O}_7$, m/z 463, $\text{M} + \text{H}$)^{9, 10} and 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (**3**, $\text{C}_{24}\text{H}_{52}\text{NO}_6\text{P}$, m/z 482, $\text{M} + \text{H}$).¹¹

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 -carboline 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 between H-4 (δ 9.19) to N-2 (δ 290.0) and 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 and N-9, δ 113.0).¹⁶ The remaining $\text{C}_4\text{H}_3\text{N}_2\text{O}$ atom count was assigned as an 4-substituted imidazolyl group attached to C-1 of the β -carboline based on 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 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 only be placed 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-9H-pyrido[3,4b]indole-3-carboxylic acid from the soft coral *Lignopsis spongiolum*.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 Several other 1-imidazolyl- β -carbolines have been reported, with the simplest being des-*N*-methylxestomanzamine A (1-imidazolyl- β -carboline),²² followed by xestomanzamine A [1-(*N*-methylimidazolyl)- β -carboline]²³ and hyrtiomanzamine [1-(4-thiomethyl-*N,N*-dimethylimidazolyl)-6-hydroxy- β -carboline].²⁴ Hyrtiocarboline (**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 of cancer cells lines, H522-T1 non-small cell lung, MDA-MB-435 melanoma, and U937 lymphoma (IC_{50} 1.2, 3.0, 1.5 $\mu\text{g/mL}$, 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**,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 μM and 300 μM , respectively, with apoptotic cell death, including highly condensed chromatin and extensive membrane blebbing.²⁵

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 $\text{TFA-}d/\text{CDCl}_3$ 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 $\times$ 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 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 five and six 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 C₁₈ 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; ¹H and ¹³C NMR (see Table 1); HRESIMS m/z 323.0766 [M + H]⁺ (calcd for C₁₆H₁₁N₄O₄, 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 – CO₂)⁺. Conversely, fragmentation of the parent ion in positive mode (pH 2) resulted in the loss of CO₂ in a two-step fragmentation, first loss of water, m/z 305 (M – H₂O)⁺, followed by loss of carbon monoxide, m/z 277 (M – H₂O – CO)⁺.

Antiproliferative Bioassays

Antiproliferative effects of compound **1** were evaluated in four cultured human cancer cell lines: BT-549 breast, HT-29 colon, NCI-H460 non-small cell lung, and DU 145 prostate cancer cells. 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 manufacturer's recommendations. Luminescence was read on a Victor²V 1420 MultiLabel HTS counter (Perkin-Elmer/Wallac). IC₅₀ 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% CO₂ 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% TritonX-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 10x magnification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References and Notes

1. Pettit GR, Cichacz ZA, Gao F, Herald CL, Boyd MR, Schmidt JM, Hooper JNA. *J Org Chem* 1993;58:1302–1304.
2. Kobayashi M, Aoki S, Sakai H, Kawazoe K, Kihara N, Sasaki T, Kitagawa I. *Tetrahedron Lett* 1993;34:2795–2798.
3. Pettit GR, Tan R, Cichacz ZA. *J Nat Prod* 2005;68:1253–1255. [PubMed: 16124771]
4. Kohmoto S, McConnell OJ, Wright A, Koehn F, Thompson W, Lui M, Snader KM. *J Nat Prod* 1987;50:336–336. [PubMed: 3655802]
5. Nasu SS, Yeung BKS, Hamann MT, Scheuer PJ, Kelly-Borges M, Goins K. *J Org Chem* 1995;60:7290–7292.
6. Youssef DTA. *J Nat Prod* 2005;68:1416–1419. [PubMed: 16180827]
7. Salmoun M, Devijver C, Daloze D, Braekman JC, van Soest RWM. *J Nat Prod* 2002;65:1173–1176. [PubMed: 12193025]
8. Ledroit V, Debitus C, Ausseil F, Raux R, Menou JL, Hill BT. *Pharm Biol* 2004;42:454–456.
9. Kim DK, Lim YJ, Kim JS, Park JH, Kim ND, Im KS, Hong J, Jung JH. *J Nat Prod* 1999;62:773–776. [PubMed: 10346968]
10. Liu Y, Lee CO, Hong J, Jung JH. *Bull Kor Chem Soc* 2002;23:1467–1469.
11. Alam N, Bae BH, Hong J, Lee CO, Shin BA, Im KS, Jung JH. *J Nat Prod* 2001;64:533–535. [PubMed: 11325244]
12. Rodriguez J, Nieto RM, Crews P. *J Nat Prod* 1993;56:2034–2040. [PubMed: 8133293]
13. Positive activity is defined as > 50% inhibition of U937 lymphoma cells @ 10 µg/mL. Initial extraction in hexane is done to de-fat the sample and the hexane extract is not tested.
14. Marine Chemistry Group. MarinLit, Marine Literature Database, version 14.3. Department of Chemistry, University of Canterbury; Christchurch, New Zealand: Feb 26. 2009
15. Pedradab S, Edrada R, Ebel R, Wray V, Proksch P. *J Nat Prod* 2004;67:2113–2116. [PubMed: 15620266]
16. Martin GE, Hadden CE. *J Nat Prod* 2000;63:543–585. [PubMed: 10785437]
17. Arai N, Shiomi K, Takamatsu S, Komiyama K, Shinose M, Takahashi Y, Tanaka Y, Iwai Y, Liu JR, Omura S. *J Antibiot* 1997;50:808–814. [PubMed: 9402984]
18. Cabrera GM, Seldes AM. *J Nat Prod* 1999;62:759–760. [PubMed: 10346963]
19. See ref. 15 for a review of 3-carboxy-beta-carbolines found from terrestrial sources.
20. Braestrup C, Nielsen M, Olsen CE. *Proc Natl Acad Sci, USA* 1980;77:2288–2292. [PubMed: 6246535]

21. Pena C, Medina JH, Novas ML, Paladini AC, De Robertis E. *Proc Natl Acad Sci, USA* 1986;83:4952–4956. [PubMed: 3014522]
22. Rao KV, Santarsiero BD, Mesecar AD, Schinazi RF, Tekwani BL, Hamann MT. *J Nat Prod* 2003;66:823–828. [PubMed: 12828469]
23. Kobayashi M, Chen YJ, Aoki S, In Y, Ishida T, Kitagawa I. *Tetrahedron* 1995;51:3727–3736.
24. Bourguet-Kondracki ML, Martin MT, Guyot M. *Tetrahedron Lett* 1996;37:3457–3460.
25. Yang YJ, Lee JJ, Jin CM, Lim SC, Lee MK. *Eur J Pharmacol* 2008;587:57–64. [PubMed: 18457825]

Table 1

NMR Data for Hyrtiocarbolone (**1**) in TFA-*d*/CDCl₃ (1:3)

position	δ_{H} (m, <i>J</i> 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			

^aInterchangeable.

Table 2Antiproliferative 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