Defensive 2-Alkylpyrrole Sulfamates from the Marine Annelid *Cirriformia tentaculata*

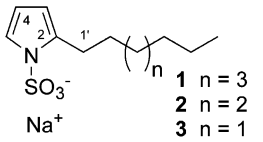
Todd Barsby,[†] Cynthia E. Kicklighter,[†] Mark E. Hay,[†] M. Cameron Sullards,[‡] and Julia Kubanek*[,†,‡]

School of Biology, Georgia Institute of Technology, Atlanta, Georgia 30332-0230, and School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332-0400

Received April 2, 2003

Three novel 2-alkylpyrrole sulfamates (**1**−**3**) were isolated from the marine worm *Cirriformia tentaculata*. The structures were elucidated by the interpretation of spectral data obtained on inseparable mixtures of the unstable compounds. This suite of metabolites deterred feeding by the generalist predatory fish *Thalassoma bifasciatum*.

A small array of natural products have been identified from segmented marine worms, consisting predominately of peptides,[1] pigments,[2] halogenated aromatics,[3] amino acids,[4] nucleosides,[4] and fatty acid derivatives.[5] Some of these metabolites act as sex pheromones,[4,5] whereas others have been hypothesized to defend worms against predators.[6] However, direct tests of annelid natural products in feeding assays have not previously been reported. Herein we report the isolation and structure elucidation of three novel and unstable 2-alkylpyrrole sulfamates from the segmented marine worm *Cirriformia tentaculata* Montagu (Cirratulidae): 2-*n*-octylpyrrole sulfamate (**1**), 2-*n*-heptylpyrrole sulfamate (**2**), and 2-*n*-hexylpyrrole sulfamate (**3**), which to our knowledge comprise the first example of a confirmed chemical defense system of a marine annelid and only the second example of a pyrrole sulfamate natural product.[7,8]



The bright red annelid *C. tentaculata* was collected at a depth of about 1 m within beds of the sea grass *Thalassia testudinum* around Rodriquez Key, near Key Largo, FL. Fractionation of *C. tentaculata* crude extracts was guided by feeding assays using the predatory fish *Thalassoma bifasciatum*, as previously described.[9] Crude extracts were subjected to solvent partitioning and reversed-phase flash column chromatography to yield a single active but labile fraction (5.5% by dry weight) that appeared by TLC and ^{1}H NMR spectroscopy to consist of one compound. However, analysis by HPLC-MS and ^{13}C NMR spectroscopy revealed the presence of three apparently homologous compounds that possessed molecular weights of 258, 244, and 230 corresponding to parent ions [M] $^-$ for compounds **1**, **2**, and **3**, respectively. From UV absorbances and total ion counts acquired by HPLC-MS, it appeared that **1**, **2**, and **3** were present in an approximate ratio of 1:2:1. Further attempts to purify each individual compound within this mixture permitted the isolation of a 1:1 mixture of **1** and **2** that was stable enough to complete a series of ^{1}H, COSY, HMQC, and HMBC NMR spectral experiments and high-resolution mass measurements. Another fraction composed predominantly of **3** decomposed too rapidly for acquisition of NMR or high-resolution mass spectral data.[10]

High-resolution mass measurements of the 1:1 mixture of **1** and **2** provided molecular formulas of $C_{12}H_{20}NO_3S$ ([M]$^-$ 258.1163, calcd 258.1164) for **1** and $C_{11}H_{18}NO_3S$ ([M]$^-$ 244.1025, calcd 244.1007) for **2**. Loss of sulfite from both **1** and **2** was evident from MS-MS daughter ions representing $SO_3^{\bullet -}$ and the alkyl pyrrole moieties (see Experimental Section). Additionally, low-intensity mass peaks offset by 2 amu, representing ^{34}S at natural abundance, were observed by MS-MS for both parent ions and sulfite daughter ions (but not for the alkyl pyrrole daughter ions), and thus confirmed the presence of sulfur in these natural products.

Examination of the ^{1}H and ^{13}C NMR spectral data for the 1:2:1 mixture of **1**−**3** revealed a suite of coincidental downfield resonances indicative of a heteroaromatic moiety common to all three compounds. In addition, a group of aliphatic resonances in the ^{13}C NMR methylene region (30−33 ppm) were sufficiently resolved to support a homologous relationship of **1**−**3**.

The ^{1}H, COSY, HMQC, and HMBC data of the 1:1 mixture of **1** and **2** permitted the assignment of the heteroaromatic group as a substituted pyrrole. Three aromatic protons, with chemical shifts δ 5.82 (H-3; correlated by HMQC to δ_C 109.9), 5.89 (H-4; correlated by HMQC to δ_C 108.5), and δ 7.04 (H-5; correlated by HMQC to δ_C 122.3) all showed COSY correlations to each other. HMBC correlations from H-5 to C-2 (δ_C 137.3) and C-3, from H-3 to C-2 and C-5, and from H-4 to C-2, -3, and -5 supported a pyrrole monosubstituted at C-2, the only quaternary aromatic carbon. Proton couplings, including a pseudotriplet (J = 3.2 Hz) for H-4, confirmed this substitution pattern. If the pyrrole had instead been substituted at C-3, H-4 would have possessed one J^4 coupling, which for pyrroles is approximately 1−2 Hz,[11] rather than the 3.2 Hz observed.

Analysis of the remaining NMR resonances suggested an alkyl chain, attached to the quaternary carbon C-2. A methylene triplet (δ_H 2.88, H-1′; correlated by HMQC to δ_C 29.3) showed HMBC correlations to C-2 and C-3 in the pyrrole ring, and to C-2′ (δ_C 31.3) of the alkyl chain. COSY correlations between H-1′ and H-2′ (δ_H 1.68), and between H-1′ and H-3, further confirmed this linkage. A spin system, evident by COSY and HMBC correlations extending from H-2′ into a complex methylene region (δ_H 1.31−

* Corresponding author. E-mail: julia.kubanek@biology.gatech.edu. Tel: (404)-894-8424. Fax: (404)-385-4440.
[†] School of Biology.
[‡] School of Chemistry and Biochemistry.

10.1021/np030149z CCC: $25.00
Published on Web 07/23/2003

1.42; δ_C 29.3−34.6), eventually terminated at a methyl group (δ_H 0.90, δ_C 15.9). This methyl group showed HMBC and COSY correlations to the penultimate methylene group (δ_H 1.33, δ_C 25.1). However, the exact assignment of the methylene resonances from positions 3′ to 6′ for **1** (or 3′ to 5′ for **2**) was confounded by overlapping resonances associated with the mixture. Nevertheless, it could be logically concluded from the lack of methine resonances within this region, and by the presence of a solitary methyl signal, that the alkyl chains of **1** and **2** were not branched or otherwise substituted.

Because the alkyl pyrrole accounted for all carbons and hydrogens in these compounds, the sulfite group had to be attached to the pyrrole nitrogen as a sulfamate. This assignment was further supported by the deshielded nature (∼+5 ppm) of the ^{13}C NMR chemical shifts of the pyrrole region, indicative of the presence of an electron-withdrawing group attached to the pyrrole nitrogen.[12]

From mass spectral data and by comparing NMR spectral features of the tertiary mixture of **1**−**3** with the binary mixture of **1** and **2**, it appeared that the structure of **3** was a simple *n*-hexyl homologue. Thus, the structures of **1**−**3** were determined to be 2-alkylpyrrole sulfamates with the attachment of either an *n*-octyl, *n*-heptyl, or *n*-hexyl chain for **1**, **2**, and **3**, respectively. There is only one other published report[7]of a pyrrole sulfamate natural product, also from a marine worm, leading to questions regarding their potentially widespread occurrence and ecological functions among marine worms.

When incorporated at natural concentration into squid-based food that nutritionally mimicked the worm, the mixture of **1**−**3** reduced feeding by the predatory reef fish *T. bifasciatum* by 80% ($p = 0.0007$).[13] Throughout the bioassay-guided fractionation of *Cirriformia* extracts, only fractions containing these three compounds were deterrent, and recombined fractions containing all components of the worm extract except for **1**−**3** were not deterrent. Thus, it appears that **1**−**3** act as chemical defenses in this worm.

Experimental Section

General Experimental Procedures. ^1H NMR spectra were acquired on a Varian Gemini 300 MHz and a Bruker Avance DRX 500 MHz spectrometer; ^{13}C NMR spectra were acquired on a Bruker AMX-400 spectrometer at 75 MHz; and HMQC, HMBC, and COSY experiments were conducted on a Bruker Avance DRX 500 MHz spectrometer. NMR spectra were acquired in CD_3OD and referenced to CD_2HOD (δ^1H 3.31; $\delta^{13}C$ 49.0). High-resolution mass spectra including MS-MS were acquired on a QSTAR-XL hybrid QqTOF tandem mass spectrometer. Intact molecular ions were formed via negative-ion ESI. Product ion analyses were performed by MS1 to select the precursor ion of interest, and nitrogen was used to induce dissociations by collision. Subsequently formed product ions were then detected with high resolution and mass accuracy using the TOF mass analyzer as MS2. HPLC-MS was performed on a Waters-Micromass system (Waters Alliance 2695 pump; 2996 photodiode array detector; Micromass ZQ2000 mass spectrometer run in negative electrospray mode; MassLynx v.3.5 software). HPLC-MS utilized a Waters Xterra C18 column (2.1 mm × 50 mm) with a gradient of acetic acid/acetonitrile/water. HPLC separation was performed with a Waters 2690 separations module coupled to a Waters 996 photodiode array detector, utilizing a Zorbax SB-C18 column (9.4 mm × 250 mm).

Collections. *Cirriformia tentaculata* was collected from seagrass beds around Rodriguez Key, off of Key Largo, FL, in July, August, and December 2002. Identification was based on microscopic examination of worm setae and dorsal tentacular cirri, compared with published accounts.[14] Worms were distinguished from their congener *Cirriformia punctata* by the lack of an irregular pattern of dark, transverse bars on the dorsal part of the body.[15] A voucher specimen is stored at the School of Biology, Georgia Institute of Technology.

Feeding Assays. Aquarium feeding assays using the predatory reef fish *T. bifasciatum* were performed as previously described,[9] and data from these assays are reported elsewhere.[13] Briefly, worm extracts were incorporated at natural concentrations into an artificial food matrix consisting of squid meat and sodium alginate, which was extruded through a syringe into a calcium chloride bath to form a solidified strand, which was then cut into small pellets. Consumption or rejection of treated (with worm extracts) and control (without worm extracts) foods by ≥10 individual fish was analyzed using the Fisher's exact test. This bioassay was used to guide fractionation of worm extracts, leading to pure, deterrent compounds as described below.

Extraction and Isolation. Worms (24.9 g wet) were extracted twice each with acetone, methanol, and ethyl acetate. Combined extracts were filtered and dried on a rotary evaporator. Crude extract was partitioned between 9:1 methanol/water and hexanes, then the aqueous fraction was partitioned between water and ethyl acetate. The deterrent ethyl acetate fraction was fractionated with a 10 g C_{18} silica Waters Sep-Pak, eluting with aqueous methanol of decreasing polarity. Only the materials that eluted with methanol/water (4:1) deterred fish feeding. This fraction contained a mixture of three compounds (251.0 mg, 5.5% by dry weight) in an approximate ratio of 1:2:1, as determined by LC-MS. Further purification by reversed-phase HPLC of a portion of the mixture, eluting with a gradient of methanol and water, yielded a 1:1 mixture of **1** and **2**.

2-*n*-Octylpyrrole sulfamate (1): colorless glass in a mixture with **2**; ^1H NMR (CD_3OD, 500 MHz) δ 7.04 (1H, dd, J = 3.2, 2.0, H-5) 5.89 (1H, dd, J = 3.2, H-4), 5.82 (1H, b m, H-3), 2.88 (2H, t, J = 7.9, H-1′), 1.68 (2H, p, J = 7.3, H-2′), 1.42−1.31 (b m, H-3′, H-4′, H-5′, H-6′), 1.33 (b m H-7′), 0.90 (3H, t, J = 6.6, H-8′); ^{13}C NMR (75 MHz and HMQC, HMBC extrapolations) δ 137.3 (C, C-2), 122.3 (CH, C-5), 109.9 (CH, C-3), 108.5 (CH, C-4), 31.3 (CH_2, C-2′), 29.3 (CH_2, C-1′), 34.6−29.3 (CH_2, C-3′, C-4′, C-5′, C-6′), 25.1 (CH_2, C-7′), 15.9 (CH_3, C-8′); HRESIMS [M] $^-$ m/z 258.1163 (calcd for $C_{12}H_{20}NO_3S$, 258.1164); MS-MS of m/z 258.1163 [M − SO_3]$^-$ m/z 178.1652; [$SO_3^{\bullet}$]$^-$ m/z 79.9640; LRESIMS m/z 258.4 (100).

2-*n*-Heptylpyrrole sulfamate (2): colorless glass in a mixture with **1**; ^1H NMR (CD_3OD, 500 MHz) δ 7.04 (1H, dd, J = 3.2, 2.0, H-5) 5.89 (1H, dd, J = 3.2, H-4), 5.82 (1H, b m, H-3), 2.88 (2H, t, J = 7.9, H-1′), 1.68 (2H, p, J = 7.3, H-2′), 1.42−1.31 (m, H-3′, H-4′, H-5′), 1.33 (b m H-6′), 0.90 (3H, t, J = 6.6, H-7′); ^{13}C NMR (75 MHz and HMQC, HMBC extrapolations) δ 137.3 (C, C-2), 122.3 (CH, C-5), 109.9 (CH, C-3), 108.5 (CH, C-4), 31.3 (CH_2, C-2′), 29.3 (CH_2, C-1′), 34.6−29.3 (CH_2, C-3′, C-4′, C-5′), 25.1 (CH_2, C-6′), 15.9 (CH_3, C-7′); HRESIMS [M]$^-$ m/z 244.1025 (calcd for $C_{11}H_{18}NO_3S$, 244.1007); MS-MS of m/z 244.1025 [M − SO_3]$^-$ m/z 164.1463; [$SO_3^{\bullet}$]$^-$ m/z 79.9608; LRESIMS m/z 244.4 (100).

2-*n*-Hexylpyrrole sulfamate (3): colorless glass in a mixture with **1** and **2**; ^1H NMR (CD_3OD, 300 MHz) δ 7.04 (1H, dd, J = 2.9, 1.7, H-5) 5.89 (1H, dd, J = 2.9, H-4), 5.83 (1H, b m, H-3), 2.88 (2H, t, J = 8.4, H-1′), 1.68 (2H, p, J = 8.4, H-2′), 1.44−1.26 (m, H-3′, H-4′, H-5′), 0.90 (3H, m, H-6′); ^{13}C NMR (75 MHz) δ 135.2 (C, C-2), 121.5 (CH, C-5), 108.7 (CH, C-3), 107.3 (CH, C-4), 32.9−28.0 (CH_2, C-1′, C-2′, C-3′, C-4′), 23.8 (CH_2, C-5′), 14.5 (CH_3, C-6′); LRESIMS m/z 230.4 (100).

Acknowledgment. This research was supported by the Harry and Linda Teasley endowment to Georgia Institute of Technology and by NSF grant OCE-0134843 to J.K. C.K. was supported by an NSF predoctoral fellowship and as an NSF IGERT fellow. We thank Alex Chequer for technical assistance and Deron Burkepile, Brock Woodson, and Emily Prince for help with collections. Collections and assays in the Florida Keys were facilitated by NOAA's National Undersea Research Center in Key Largo.

Supporting Information Available: HPLC, MS, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Takahashi, T.; Furukawa, Y.; Muneoka, Y.; Matsushima, O.; Ikeda, T.; Fujita, T.; Minakata, H.; Nomoto, K. *Comp. Biochem. Physiol.* **1995**, *110C*, 297−304.

(2) Pelter, A.; Ballantine, J. A.; Murray-Rust, P.; Ferrito, V.; Psaila, A. F. *Tetrahedron Lett.* **1978**, *21*, 1881−1884.

(3) (a) Higa, T.; Scheuer, P. J. *J. Am. Chem. Soc.* **1974**, *96*, 2246−2248. (b) Goerke, H.; Emrich, R.; Weber, K.; Duchene, J.-C. *Comp. Biochem. Physiol.* **1991**, *99B*, 203−206. (c) Emrich, R.; Weyland, H.; Weber, K. *J. Nat. Prod.* **1990**, *53*, 703−705.

(4) Zeek, E.; Harder, T.; Beckmann, M. *Chemoecology* **1998**, *8*, 77−84.

(5) Bartels-Hardege, H. D.; Hardege, J. D.; Zeeck, E.; Muller, C.; Wu, B. L.; Zhu, M. Y. *J. Exp. Mar. Biol. Ecol.* **1996**, *201*, 275−284.

(6) Cowart, J. D.; Fielman, K. T.; Woodin, S. A.; Lincoln, D. E. *Mar. Biol.* **2000**, *136*, 993−1002.

(7) Fielman, K. T.; Targett, N. M. *Mar. Ecol. Prog. Ser.* **1995**, *116*, 125−136.

(8) For other examples of non-pyrrole natural product sulfamates see: Meragelman, K. M.; West, L. M.; Northcote, P. T.; Pannel, L. K.; McKee, T. C.; Boyd, M. R. *J. Org. Chem.* **2002**, *67*, 6671−6677, and references therein.

(9) (a) Pawlik, J. R.; Fenical, W. *Mar. Ecol. Prog. Ser.* **1992**, *87*, 183−188. (b) Hay, M. E.; Stachowicz, E.; Cruz-Rivera, E.; Bullard, S.; Deal, M. S.; Lindquist, N. In *Methods in Chemical Ecology*; Haynes, K. F., Millar, J. G., Eds.; Chapman and Hall: New York, 1998; Vol. 2, *Bioassay Methods*, Chapter 2, pp 39−141.

(10) Compound resolution by HPLC-MS was obtained with the addition of acetic acid to the mobile phase (see Experimental Section); however, the acid lability of **1**−**3** impeded the use of acid on a semipreparative scale.

(11) (a) Silverstein, R. M.; Bassler, G. C.; Morill, T. C. *Spectrometric Identification of Organic Compounds*, 5th ed.; John Wiley & Sons: New York, 1991; p 222. (b) Jiang, Z. D.; Gerwick, W. H. *J. Nat. Prod.* **1991**, *54*, 403−407.

(12) Thompson, A.; Gao, S.; Modzelewska, G.; Hughes, D. S.; Patrick, B.; Dolphin, D. *Org. Lett.* **2000**, *2*, 3587−3590.

(13) Kicklighter, C. E.; Kubanek, J.; Barsby, T.; Hay, M. E. *Mar. Ecol. Prog. Ser.* **2003**, submitted.

(14) Fauchald, K. *The Polychaete Worms. Definitions and Keys to the Orders, Families and Genera*; Natural History Museum of Los Angeles County: Los Angeles, 1977; pp 29−30.

(15) Fauchald, K. *Polychaetes from Intertidal Areas in Panama, with a Review of Previous Shallow-Water Records*; Smithsonian Institution Press: Washington, DC, 1977; pp 49−51.

NP030149Z