Tridentatols D−H, Nematocyst Metabolites and Precursors of the Activated Chemical Defense in the Marine Hydroid *Tridentata marginata* (Kirchenpauer 1864)

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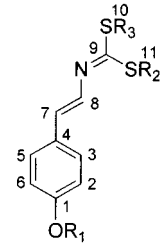
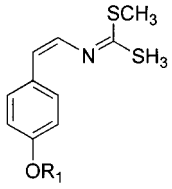
The marine hydroid *Tridentata marginata* (Kirchenpauer 1864) produces tridentatols A−C (**1**−**3**), of which **1** is a potent deterrent to fish predation. This paper reports the structures of five additional secondary metabolites, tridentatols D−H (**4**−**8**), that have been isolated from this hydroid. When hydroid tissue is crushed, as attacking predators would do, tridentatols E−H (**5**−**8**), which do not deter fish feeding, are rapidly converted to tridentatols A−D (**1**−**4**), thereby repelling potential predators. Additionally, the tridentatols were isolated from this hydroid's nematocysts (i.e., stinging organelles), representing the first report of a nonprotein venom produced by cnidarian nematocysts. The structures of **4**−**8** were determined by interpretation of their spectral data.

Marine hydroids are commonly assumed to be defended from predation by nematocysts that are capable of penetrating predators' tissues and injecting proteinaceous venom.[1] However, several recent studies suggest that small, lipophilic secondary metabolites protect many marine hydroid species against potential predators and that the lipophilic secondary metabolites and penetrating nematocysts with proteinaceous venoms typically do not co-occur within the same species.[2] Stachowicz and Lindquist[3] demonstrated that the hydroid *Tridentata marginata* (Kirchenpauer 1864; Sertulariidae)[4] is chemically defended by the novel dithiocarbamate metabolite tridentatol A (**1**). Two structurally related metabolites, tridentatols B and C (**2** and **3**),[5] and a fourth undescribed metabolite [= tridentatol D (**4**)][3] were also isolated from *T. marginata* but did not deter fish feeding.[3] A recent examination of *T. marginata* has yielded four additional metabolites, tridentatols E−H (**5**−**8**). This paper reports the structures of **4**−**8** and establishes **5**−**8** as the sulfate ester analogues of **1**−**4**. Additionally, it is reported in this paper that crushing *T. marginata* (as an attacking predator would do) results in the rapid conversion of **5**−**8** to **1**−**4**, thereby repelling potential predators. Furthermore, the tridentatols were isolated from purified nematocysts of *T. marginata*.





1 R_1 =H, R_2 = R_3 = CH_3

2 R_1 = H

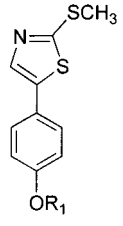
5 R_1 = SO_3Na, R_2 = R_3 = CH_3

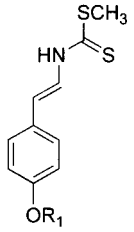
6 R_1 = SO_3Na

9 R_1 = R_2 = R_3 = CH_3

10 R_1 = R_2 = propyl, R_3 = CH_3

11 R_1 = R_3 = propyl, R_2 = CH_3





3 R_1 = H

4 R_1 = H

8 R_1 = SO_3Na

7 R_1 = SO_3Na

Results and Discussion

Tridentatol D (**4**) analyzed for $C_{10}H_{11}NOS_2$ by HREIMS in conjunction with ^1H and ^{13}C NMR data (Table 1). Thus **4** differs from the previously described **1** and **2** by the loss of one carbon and two hydrogen atoms. Of the six units of unsaturation in **4**, four were attributed to a 1,4-disubstituted benzene by the characteristic ortho coupling (8.5 Hz) between degenerate aromatic protons at δ 6.73 (2H−C-2,6) and 7.19 (2H−C-3,5). This pattern is common to compounds **1**−**8**, although the chemical shifts of these protons vary considerably among them. Compound **4** also exhibited ^1H NMR signals [δ 7.99 (C-8) and 6.32 (C-7), ^3J = 14.0 Hz] consistent with the trans carbon−carbon double bond of **1**. The λ_{max} of 342 nm (ϵ 22 500) in the UV spectrum of **4** indicates extensive conjugation of this molecule's chromophore. A bathochromic shift of 15 nm observed upon addition of 1 N NaOH to **4** in methanol confirmed the presence of a phenolic hydroxy group. Because only one upfield methyl singlet (δ 2.54, 3H) was seen in the ^1H NMR spectrum, and with this metabolite lacking CH_2 relative to **1**, **4** was proposed to have only one S-methyl as part of its dithiocarbamate functional group, in contrast to the two S-methyls in **1**. The form of the second sulfur atom of the dithiocarbamate of **4** was assigned as the thiocarbonyl on the basis of NMR data. Whereas the ^{13}C NMR spectrum of

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Assignments for Tridentatols D (**4**) and G (**7**)[a]

	4		**4**[b]	**7**	
carbon no.	δ^{13}C	δ^1H	δ^1H	δ^{13}C	δ^1H
1	158.2			153.0	
2,6	116.6	6.73 (2H, d, J = 8.5 Hz)	6.72 (2H, d, J = 8.5 Hz)	122.8	7.23 (2H, d, J = 8.5 Hz)
3,5	128.9	7.19 (2H, d, J = 8.5 Hz)	7.20 (2H, d, J = 8.5 Hz)	127.4	7.32 (2H, d, J = 8.5 Hz)
4	128.8			134.2	
7	117.9	6.32 (1H, d, J = 14 Hz)	6.36 (1H, d, J = 15.0 Hz)	116.0	6.36 (1H, d, J = 15.0 Hz)
8	126.4	7.99 (1H, d, J = 14 Hz)	7.85 (1H, bd, J = 15.0 Hz)	128.3	8.11 (1H, d, J = 15.0 Hz)
9	196.6			197.6	
10	18.0	2.54 (3H, bs)	2.60 (3H, s)	18.0	2.64 (3H, s)
O			9.53 (1H, s)[c]		
N			11.92 (1H, bs)[c]		

[a] Measured in methanol-d_4 with TMS as the internal standard except where noted. [b] Measured in DMSO-d_6 with TMS as the internal standard. [c] D_2O exchangeable.

Table 2. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Assignments for Tridentatols E (**5**), F (**6**), and H (**8**)[a]

	5		**6**		**8**
carbon no.	δ^{13}C	δ^1H	δ^{13}C	δ^1H	δ^1H
1	152.2		152.8		
2,6	122.7	7.23 (2H, d, J = 8.5 Hz)	121.8	7.21 (2H, d, J = 8.5 Hz)	7.14 (2H, d, J = 8.5 Hz)
3,5	127.4	7.37 (2H, d, J = 8.5 Hz)	131.6	7.74 (2H, d, J = 8.5 Hz)	7.53 (2H, d, J = 8.5 Hz)
4	134.8		134.8		
7	126.9	6.57 (1H, d, J = 13.5 Hz)	121.0	5.91 (1H, d, J = 8.0 Hz)	7.84 (1H, s)
8	134.3	7.61 (1H, d, J = 13.5 Hz)	132.8	6.90 (1H, d, J = 8.0 Hz)	
9	163.4		not obs.		
10	15.1	2.54 (3H, bs)	15.3	2.58 (3H, s)	2.71 (3H, s)
11	14.8	2.47 (3H, bs)	15.3	2.58 (3H, s)	

[a] Measured in methanol-d_4 with TMS as the internal standard.

1 exhibited a δ 160.5 signal assigned to C-9 of the dithiocarbamate (C═N form), for tridentatol D the C-9 resonance appeared at δ 196.6. This signal was assigned to the C═S form of the dithiocarbamate on the basis of its HMBC correlations to the S-methyl and C-8 olefinic protons and the reported δ 193.7 chemical shift for the dithiocarbamate C═S carbon of the phytoalexin, brassinin.[6] Further confirmation for the structural assignment of **4** came from its ^{1}H NMR spectrum in DMSO-d_6, which revealed two D_2O-exchangeable protons, δ 9.53 (1H, s) and 11.92 (1H, bs). The δ 11.92 proton, which appeared as a broadened doublet, was assigned to the nitrogen given its slight coupling to the C-8 proton.

Methylation of **4** with methyl iodide yielded the dimethylated product, **9**, which, by ^{1}H NMR (see Experimental Section), is identical to that obtained by methylation of **1**, further confirming the structural similarities of **1** and **4**. Additionally, when 1-iodopropane was used to alkylate tridentatol D, ^{1}H NMR revealed that the major reaction products obtained were a 2:1 ratio of dipropylated stereoisomers (**10** and **11**), each with an aromatic propyl ether but isomeric at the dithiocarbamate center with S-methyl and S-propyl substituents (see Experimental Section).

Tridentatol E (**5**) analyzed for $C_{11}H_{12}NO_4S_3$ ([M − Na]$^-$) by negative ion HRFABMS in conjunction with ^{1}H and ^{13}C NMR data (Table 2). Similarities in the UV spectra of **1** and **5** and their numbers of carbon and hydrogen atoms suggested that these two metabolites were structurally related. The ^{13}C and ^{1}H NMR spectra of **1**[5] and **5** are nearly identical except that for **5** the degenerate aromatic C-2,6 protons are shifted downfield by 0.45 ppm. The downfield shift of these protons, in conjunction with the lack of a bathochromic shift in the UV spectrum of **5** with the addition of base, indicated an alteration of the phenolic functionality. The δ 163.4 signal in the ^{13}C NMR of **5** confirmed the presence of the dithiocarbamate functional group, and the 13.5 Hz coupling constant between the two olefininc protons assigned a trans configuration to the carbon−carbon double bond of **5**. Given that, relative to **1**, **5** has an additional sulfur and three oxygen atoms, an altered phenolic functionality, and greater polarity, the structure of **5** was assigned as the sulfated analogue of **1**.

Tridentatol F (**6**) is isomeric with **5** on the basis of negative ion HRFABMS ($C_{11}H_{12}NO_4S_3$; [M − Na]$^-$) and ^{1}H and ^{13}C NMR data (Table 2). The ^{1}H NMR spectrum of **6** is remarkably similar to that of tridentatol B (**2**), except for a downfield shift of 0.43 ppm for the degenerate C-2,6 aromatic protons. Consistent with **2**, the ^{1}H NMR spectrum of **6** clearly showed a cis carbon−carbon double bond, with olefinic protons at δ 5.91 and 6.90 coupled by 8.0 Hz. The consistency of spectral differences between **2** and **6** with those between **1** and **5** indicated that **6** is the sulfated analogue of **2**.

Tridentatol G (**7**) analyzed for $C_{10}H_{10}NO_4S_3$ ([M − Na]$^-$) by negative ion HRFABMS in conjunction with ^{1}H and ^{13}C NMR data (Table 1). Again there were remarkable spectral similarities between two *T. marginata* metabolites, **4** and **7**, that differed in their molecular formulas by one sulfur and three oxygen atoms. Tridentatol D (**4**) and **7** both have (i) the trans carbon−carbon double bond, (ii) only one S-methyl, and (iii) the same HMBC correlations between their carbonyl resonance (δ 196.6 and 197.6 for **4** and **7,** respectively) and their S-methyl and C-8 olefinic protons, and the C-2,6 aromatic protons are shifted downfield in **7** by 0.51 ppm relative to **4**. Furthermore, the UV spectrum of **7** did not show a bathochromic shift with the addition of base. Thus tridentatol G (**7**) was assigned as the sulfate ester of the tridentatol D (**4**).

Tridentatol H (**8**) could not be completely separated from tridentatol G (**7**). However, it was possible to readily discern from negative ion HRFABMS of the mixture the [M − Na]$^-$ peak for **8** (*m*/*z* 301.9618), which corresponds to $C_{10}H_9NO_4S_3$. Additionally, because **7** and **8** have relatively few hydrogens and their proton signals do not overlap in the ^{1}H NMR spectrum, the resonances of **8** were readily identified. Two diagnostic ^{1}H NMR signals shared by both **8** [the singlet olefinic proton at δ 7.84 and the three-proton S-methyl signal at δ 2.71 (Table 2)] and tridentatol C (**3**),[5]

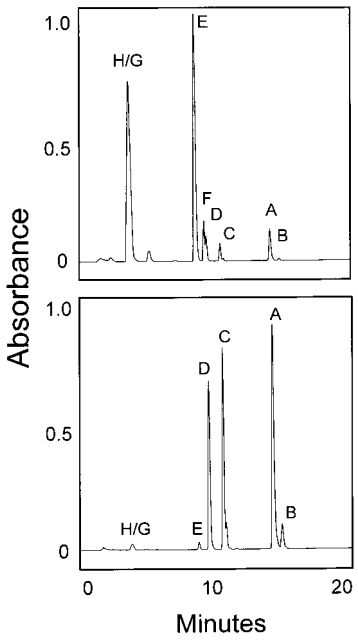

Figure 1. Tridentatol composition of *T. marginata* extracted with methanol (top) and of *T. marginata* crushed for 30 s prior to methanol extraction (bottom). Chromatograms were extracted from PDA data at 330 nm. Peaks corresponding to the various tridentatols are indicated by the letter next to each peak. Tridentatols G and H coelute at ~4 min. HPLC conditions are described in the Experimental Section.

plus the compositional difference of one sulfur and three oxygens between **8** and **3**, confirmed that tridentatol H (**8**) is the sulfate ester of tridentatol C (**3**).

The rapid conversion from a relatively palatable metabolite to a highly deterrent compound when the tissues of an organisms are physically damaged (as an attacking predator would do) has been termed "activation".[7] Hydrolysis of the phenolic sulfate of tridentatols E−H (**5**−**8**) offers a reasonable mechanism for the rapid production of tridentatols A−D (**1**−**4**), a group that includes a potent feeding deterrent (**1**). Thus, to test for activation in *T. marginata*, the tridentatol content of freshly collected, undamaged hydroid was compared to that of the hydroid briefly crushed prior to extraction. Results of this experiment (Figure 1) showed that the tridentatol profile of undamaged *T. marginata* is dominated by the sulfated tridentatols E−H, whereas that of crushed *T. marginata* shows nearly complete conversion to tridentatols A−D. Further studies indicated that the tridentatol E−H to A−D conversion does not occur spontaneously in aqueous media, but is enzyme mediated.[8]

Activation allows an organism to benefit immediately from a chemical defense when attacked, but to avoid the negative effects of maintaining active levels of a toxic chemical deterrent. Activation is common among terrestrial plants,[9] and reports of activation among marine plants are becoming more numerous.[7,10] However, among marine animals, only a few sponges of the genus *Aplysina* are known to have an activated chemical defense, converting mildly deterrent brominated isoxazoline alkaloids to the potent fish feeding deterrents aeroplysin and a related dienone.[11] The conversion of the nondeterrent tridentatol E (**5**) to the highly unpalatable tridentatol A (**1**) when *T. marginata* is physically damaged thus represents only the second known example of an activated chemical defense in a marine animal.

Additionally, because they are relatively dense, some types of nematocysts can be isolated from cnidarians with a significant proportion of them undischarged using density-gradient separation.[12] This isolation procedure was applied to a crude cell preparation of *T. marginata* to determine if its nematocysts play a role in the production and storage of the tridentatols. Although not all of the nematocysts in the cell preparation of *T. marginata* passed through the most dense layer of the gradient and settled at the bottom, the extract of those that did, representing in excess of 95% of this material, contained largely tridentatols E−H when extracted with methanol, but entirely tridentatols A−D when extracted with a 1:1 mixture of methanol/water. These results strongly suggest that *T. marginata* nematocysts are involved in the production and storage of tridentatols E−H and somehow involved in their activation to tridentatols A−D. This report represents the first example of cnidarian nematocysts producing nonproteinaceous metabolites.

Experimental Section

General Experimental Procedures. UV and IR spectra were obtained on a Beckman DU-640 and Perkin-Elmer 1600 FTIR spectrometer, respectively. 1D ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) and 2D NMR spectra were recorded on a Varian INOVA 500 spectrometer with TMS as the internal standard. MS data were recorded on a JEOL SX102 mass spectrometer.

Animal Material. Multiple collections of *T. marginata* [= *Sertularia marginata* (Kirchenpauer 1864)] were made from the rock jetty at Cape Lookout, NC, between July 1998 and July 2001. The hydroid was identified by Dr. Dale Calder of the Royal Ontario Museum, and a voucher (NL98-CLJ-H1) is on deposit at the University of North Carolina at Chapel Hill's Institute of Marine Sciences.

Extraction and Isolation. For this project, the tridentatols were isolated from multiple collections of the hydroid. As an example, the MeOH extract of freshly collected hydroid (41.2 g dry mass) was condensed by rotary evaporation and then partitioned between water and CH_2Cl_2, followed by 1-butanol extraction of the water fraction. Tridentatols D−H (**4**−**8**) were separated from the combined CH_2Cl_2/butanol soluble compounds using C_{18} reversed-phase HPLC (gradient elution with MeOH/0.1 M $AcONH_4$) to yield **4** (1.1% dry wt), **5** (2.1% dry wt), **6** (0.3% dry wt), and **7** (4.2% dry wt).

Tridentatol D (4): amorphous white solid; UV (MeOH) λ_{max} (log ϵ) 342 (4.35); IR (NaCl) ν_{max} 3594, 2958, 1725, 1609, 1513, 1261, 1171 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; HREIMS $[M]^+$ m/z 225.0265 (calcd for $C_{10}H_{11}NOS_2$, 225.0283).

Tridentatol E (5): amorphous white solid; UV (MeOH) λ_{max} (log ϵ) 326 (4.41); IR (KBr) ν_{max} 3754, 1527, 1501, 1410, 1274, 1224, 1058 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 2; neg ion HRFABMS $[M - Na]^-$ m/z 317.9918 (calcd for $C_{11}H_{12}NO_4S_3$, 317.9930).

Tridentatol F (6): amorphous white solid; IR (KBr) 3840, 1525, 1506, 1272, 1222, 1058 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 325 (3.78); ^{1}H and ^{13}C NMR, see Table 2; neg ion HRFABMS $[M - Na]^-$ m/z 317.9926 (calcd for $C_{11}H_{12}NO_4S_3$, 317.9930).

Tridentatol G (7): amorphous white powder; IR (KBr) 3856, 1642, 1604, 1497, 1412, 1341, 1264, 1217, 1051 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 289 (sh), 339 (4.32); ^{1}H and ^{13}C NMR, see Table 1; neg ion HRFABMS $[M - Na]^-$ m/z 303.9774 (calcd for $C_{10}H_{10}NO_4S_3$, 303.9773).

Tridentatol H (8): amorphous white powder; ^{1}H NMR, see Table 1; neg ion HRFABMS $[M - Na]^-$ m/z 301.9618 (calcd for $C_{10}H_9NO_4S_3$, 301.9619).

Alkylation Reactions. Methylation of Tridentatols A (1) and D (4). In separate reactions, 5 mg of **1** and 5 mg of **4** were dissolved in 10 mL of anhydrous acetone and added to a round-bottom flask sealed with a Teflon stopper. Excess K_2CO_3 and methyl iodide (500 μL) were added to the flasks, which were stirred at room temperature for 18 h. After evaporating off the acetone and excess methyl iodide, diethyl ether was used to extract any remaining starting material and reaction products, which were purified using an analytical C_{18} column

(4.6 × 100 mm) with a methanol/water gradient elution scheme. Results for both methylation reactions showed no starting material in the reaction mixture and **9** as the only reaction product, which was obtained in quantitative yield. **9** was spectroscopically identical to a synthetic sample of *O*-methyl tridentatol A obtained from PhycoGen, Inc.

Propylation of Tridentatol D (4). Five milligrams of **4** dissolved in 10 mL of anhydrous acetone was added to a round-bottom flask sealed with a Teflon stopper. Excess K_2CO_3 and 1-iodopropane (500 μL) were added to the flask, which was stirred at room temperature for 18 h. Reaction workup and compound isolation were performed as above. Results showed no starting material and **10** and **11** as the major reaction products, which were purified by C_{18} reversed-phase HPLC as described above.

***O*-Methyl tridentatol A (9)**: yellow oil; ^{1}H NMR ($CDCl_3$) δ 7.56 (1H, d, J = 14 Hz, H-8), 7.38 (2H, d, J = 8.0 Hz, H-3,5), 6.85 (2H, d, J = 8.0 Hz, H-2,6), 6.64 (1H, d, J = 14 Hz, H-7), 3.80 (3H, s), 2.60 (3H, bs, H-10), 2.52 (3H, bs, H-11).

***O*-Propyl tridentatol D (10 and 11 mixture)**: yellow oil; ^{1}H NMR (CD_3OD) δ 7.62 (d, J = 13.5 Hz), 7.57 (d, J = 13.5 Hz), 7.32 (d, J = 8.6 Hz), 6.78 (d, J = 8.6 Hz), 6.57 (d, J = 13.5 Hz), 6.53 (d, J = 13.5 Hz), 3.92 (t, J = 7.3 Hz), 3.08 (t, J = 6.9 Hz), 2.58 (bs), 2.45 (bs), 1.79 (tq, J = 7.0, 7.3 Hz), 1.68 (btq, J = 6.9, 7.0 Hz), 1.02 (t, J = 7.0 Hz).

Hydroid Crushing Experiment. Two clumps of *T. marginata* collected at Cape Lookout were carefully removed from the same colony and weighed (15−20 mg wet mass each). After being weighed, one clump was placed immediately in methanol (~5 mL) without crushing, while the other clump was placed on a small watch glass, crushed with a spatula for 30 s, and then immediately placed in methanol. After 30 min, the methanol was removed from each sample and additional methanol added. The extraction was repeated a third time. For each sample, the combined extracts were rotary evaporated to dryness. The dried extracts were redissolved in methanol with 10 μL added for each milligram of hydroid mass. Ten microliters of crude extract from crushed and uncrushed hydroids were analyzed by HPLC for tridentatol content as described above.

Nematocyst Isolation and Extraction. Tridentatols were identified by analytical HPLC (as described above) in nematocysts purified from crude cell preparations using a Percoll (Sigma) density gradient separation, all accomplished at 0° C and with solutions made with calcium/magnesium-free (CMF) seawater. The nematocysts are the most dense structures in the crude cell preparation of *T. marginata* and were virtually the only material to pass through the most dense Percoll layer (90% full strength Percoll mixed with concentrated CMF seawater to be isotonic with 35 ppt seawater) when centrifuged at 1500*g* for 3 min. The density gradient solutions were poured from replicate tubes of pelletized nematocysts, which were then rinsed with fresh CMF seawater. Once the nematocyst pellet was re-formed by centrifugation and the CMF seawater was removed, either methanol alone or a 1:1 mixture of methanol/CMF seawater was used to extract the nematocysts.

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