Equilibrating Isomers: Bromoindoles and a Seco-Xanthine Encountered during a Study of Nematocides from the Southern Australian Marine Sponge *Hymeniacidon* sp.

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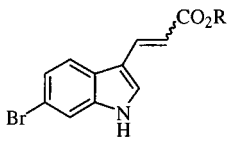
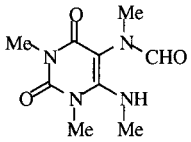
Bioassay-directed fractionation of a *Hymeniacidon* sp. yielded as nematocidal agents the equilibrating *E*/*Z* bromoindole ethyl esters **1** and **2** and corresponding methyl esters **3** and **4.** Also isolated for the first time as a natural product was an equilibrating mixture of seco-xanthine formamides, attributed the trivial name hymeniacidin (**5**). The structure for **5** was assigned on the basis of detailed spectroscopic analysis and total synthesis.

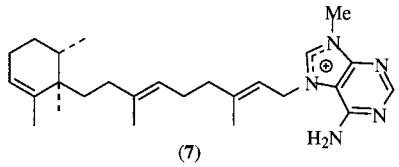
Our investigations into bioactive Australian marine natural products have revealed numerous structurally novel nematocides that are active in vitro against the commercially significant livestock parasite *Haemonchus contortus*. These metabolites have included novel lipids in the form of epoxylipids and tetrahydrofurans from the brown alga *Notheia anomala*[1] and thiocyanatins from the sponge *Oceanapia* sp.[2] Other southern Australian marine sponges have returned the nematocidal alkaloids geodin A Mg salt,[3] the amphilactams A−D,[4] and the amide onnamide F,[5] as well as the cyclic depsipeptides phoriospongin A and B.[6] In this report we describe an investigation into a *Hymeniacidon* sp. collected during scientific trawling operations in the Great Australian Bight, which returned a selection of nematocidal bromoindole esters (**1**−**4**) and the new natural product hymeniacidin (**5**).



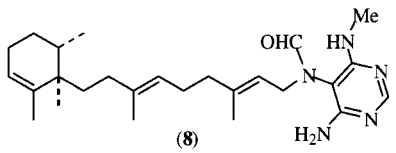
(**1**) *E* R=Et
(**2**) *Z* R=Et
(**3**) *E* R=Me
(**4**) *Z* R=Me



(**5**)



(**7**)



(**8**)

Results and Discussion

The EtOH extract of the *Hymeniacidon* sp. exhibited nematocidal activity (LD_{99} = 181 μg/mL) against the commercially significant livestock parasite *Haemonchus contortus*. In pursuit of the nematocidal agent the crude EtOH extract was decanted, concentrated in vacuo, and triturated with CH_2Cl_2, with the nematocidal activity being concentrated in the CH_2Cl_2 solubles. Subsequent fractionation of the bioactive material by silica solid-phase extraction and HPLC yielded as nematocidal agents the bromoindole ethyl esters (**1** and **2**) (LD_{99} = 42 μg/mL) and the bromoindole methyl esters (**3** and **4**) (LD_{99} = 50 μg/mL), together with an inactive alkaloid, hymeniacidin (**5**). The bromoindole ethyl ester **1** is a known synthetic compound,[7] and its isolation during this study is assumed to be due to transesterification of the natural bromoindole methyl ester **3**, brought about by storage of the sponge biomass in EtOH. The methyl ester **3** is a known sponge metabolite having first been described in 1981 by Sargent et al.[8] from a Western Australian *Iotrochota* sp., and again in 1991 by Fusetani et al.[9] from *Mycale adhaerens*, and also in 1993 by Pietra et al.[10] from *Corallistes undulates*. Although the *E* bromoindole methyl ester **3** has been isolated on several occasions as a natural product[8−10] and has been the target of successful syntheses,[8,11] no literature reports (synthetic or natural) exist for the corresponding *Z* bromoindole methyl ester **4**. Our investigations reveal that the *Z* bromoindole is very acid labile and readily interconverts to the more stable *E* isomer. The bromoindole esters **1**−**4** were well resolved by silica HPLC, with the order of elution being **2** (9.0 min), **4** (10.5 min), **1** (16.0 min), and **3** (18.0 min). In our hands, $CDCl_3$ solutions of each of the bromoindoles **1**−**4** assumed an immediate red coloration that on ^{1}H NMR analysis revealed extensive equilibration between the stereoisomeric pairs (*Z*→*E*). A plausible explanation is that this transformation is acid mediated (activated by trace amounts of acid in the $CDCl_3$) and proceeds by the mechanism outlined in Figure 1. In this proposal the iminium cation intermediate could account for the red coloration. It is worthwhile noting that failure to take precautions against exposure to acid during extraction, fractionation, handling, and/or storage would result in conversion of naturally occurring *Z* isomers to the more

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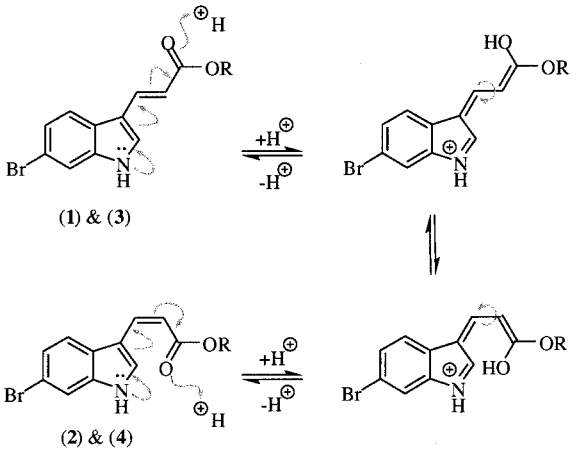

Figure 1. Proposed mechanism for equilibration of bromoindole ester isomers.

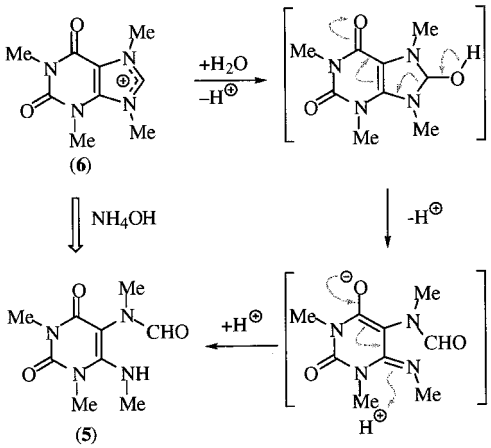

Figure 2. Proposed mechanism for the regiospecific hydrolysis of **6**.

commonly observed *E* isomers. In dry pure form the bromoindole esters **1**−**4** are white solids, while in MeOH they form colorless solutions. To the best of our knowledge this report represents the first description of nematocidal properties for the bromoindole esters **1**−**4**. Having accounted for the nematocidal agents in the *Hymeniacidon* sp., we turned our attention to the alkaloid **5**.

High-resolution ESI(+)MS analysis of **5** revealed a pseudomolecular ion [$(M + Na)^+$, *m*/*z* 249.0959] consistent with a molecular formula ($C_9H_{14}N_4O_3$, Δ mmu = −0.5) requiring five double-bond equivalents (DBE). A preliminary examination of NMR ($CDCl_3$) data for **5** indicated the presence of two equilibrating species in a ratio of ~2.5:1, an observation confirmed by HPLC analysis. Silica HPLC (EtOAc/hexane) analysis of **5** revealed two well-resolved components (10.0 and 13.4 min). However after preparative separation these species rapidly reequilibrated to the same two-component mixture, based on analytical HPLC analysis under the same chromatographic conditions. A more detailed analysis of the NMR data for **5** revealed resonances for the *major* component consistent with three tertiary *N*-methyls (^{1}H: δ 3.04 (s), 3.33 (s), and 3.48 (s); ^{13}C: 33.7 (q), 30.8 (q), and 28.4 (q) ppm), one secondary amine *N*-methyl (^{1}H: δ 2.98 (d); ^{13}C: 32.8 (q) ppm) with associated amine proton (^{1}H: δ 4.6 (m), D_2O exchangeable with associated loss of coupling to the secondary *N*-methyl), and a formamide functionality (^{1}H: δ 8.03; ^{13}C: 166.3 (d) ppm). Comparable NMR resonances were observed for the *minor* component in **5**. The observations outlined above accounted for one of the five DBE and all but the elements C_4O_2 in the molecular formula of **5**. Consideration of the remaining spectroscopic data, including 2D NMR correlations, allowed the equilibrating formamide rotamers as shown to be plausible representations for hymeniacidin (**5**). Supportive of this conclusion was the observation that the ^{1}H NMR resonances for the major and minor components in **5** coalesced in DMSO-d_6 at 150 °C, as would be expected for formamide rotamers.

To confirm this hypothesis, an authentic sample of **5** was prepared in high yield from caffeine. This synthesis involved initial methylation of caffeine to the quaternary salt **6**, which was hydrolyzed by NH_4OH to yield a material identical in all respects to hymeniacidin (**5**). The basis behind proposing this successful synthesis was an earlier observation that the quaternary adenine sponge metabolite ageline A (**7**) underwent hydrolysis to the formamide **8** during chromatography with NH_4OH.[12] The rationale for assigning regioselectivity to the hydrolysis is outlined in Figure 2, which proposes preferential cleavage of the C8−N9 bond due to resonance stabilization. After successfully confirming the structure of **5**, a literature report came to our attention in which Bredereck et al.[13] employed NaOH hydrolysis of **6** to prepare **5**. Although **5** is a known synthetic compound, no spectroscopic analysis or definitive structure determination appears in the scientific literature, and little or no interest has been expressed in **5** since it was first synthesized in 1959. To explore the possibility that the salt **6** might also be a natural product in the *Hymeniacidon* sp., a portion of the extract was treated with NH_4OH and the CH_2Cl_2 extract of the resulting product examined for production of **5**. This experiment proved negative, from which we conclude that **6** is not currently present in the sponge extract. This observation does not exclude **6** as a potential biosynthetic precursor; on the contrary, it seems highly probable that hymeniacidin (**5**) is derived from hydrolysis (enzyme mediated or otherwise) of a xanthine precusor.

Experimental Section

General Experimental Procedures. See ref 4.

Animal Material. A *Hymeniacidon* sp. (Museum of Victoria Registry Number MVF88742) (36 g dry weight) was collected during a scientific expedition to the Great Australian Bight aboard the RV *Franklin* in July 1995. The specimen was collected by beam trawl at a depth of 85 m at position 32° 58′ S; 128° 00′ E, at which point it was transported frozen to the laboratory, where it was thawed, documented, diced, and steeped in EtOH at −20 °C, prior to chemical analysis.

A description of the specimen is as follows: growth form erect, thickly flabelliform-lobate (10−30 mm thick); color in life orange-brown; color in EtOH beige; texture compressible but firm, fibrous; oscules not seen; surface opaque, detachable "skin"; spicules megascleres styles occasionally deformed (200−220 × 5−10 μm); microscleres none; ectosome a single layer of tangential spicules over subectosomal brushes which occasionally protrude the surface; choanosome meandering multispicular tracts of spicules (4−10 μm) becoming radial-plumose as they traverse the subectosomal lacunae. Collagen in the mesohyl is abundant and filled with pigment granules and lightly scattered smaller, thinner styles. Spongin is not visible.

Extraction and Isolation. The EtOH extract of a *Hymeniacidon* sp. exhibited nematocidal activity (LD_{99} = 181 μg/mL)[14] against the commercially significant endo parasite of livestock, *Haemonchus contortus*. The decanted EtOH extract was concentrated in vacuo (8.14 g), and the nematocidal components were extracted from the residue with CH_2Cl_2 (179 mg). The CH_2Cl_2-soluble material was further triturated (stepwise from 5% EtOAc/hexane to 20% EtOAc/hexane, and finally 100% EtOAc), and the combined nematocidal active fractions were subjected to Si gel SPE (10% stepwise elution from

hexane to EtOAc). Fractions eluting with 10−50% EtOAc/hexane were subjected to normal-phase HPLC (2.0 mL/min 20−30% EtOAc/hexane through a Phenomenex spherex 5μ 250 × 10 mm Si gel column) to yield the nematocidal bromoindole ethyl esters **1** and **2** (19.3 mg, LD_{99} = 42 μg/mL, 0.054% specimen dry wt) and the bromoindole methyl esters **3** and **4** (5.8 mg, LD_{99} = 50 μg/mL, 0.016% specimen dry weight). The *E* bromoindole esters **1** and **3** were identified by comparison with ^{1}H NMR published data.[7,8] The existence of the *Z*-bromoindole esters **2** and **4** was inferred from the rapid equilibration with **1** and **3** and the appearance of the following clearly resolved ^{1}H NMR resonances.

***Z*-Bromoindole ethyl ester 2:** ^{1}H NMR (400 MHz, $CDCl_3$) 1.33 (t, J = 7.2 Hz, OCH_2CH_3); 4.22 (q, J = 7.2 Hz, OCH_2-CH_3); 5.84 (d, J = 12.4 Hz, H2); 7.19 (d, J = 12.4 Hz, H3); 7.32 (dd, J = 1.8, 8.6 Hz, H5′); 7.57 (d, J = 1.8 Hz, H7′); 7.59 (obscured by *E*-H7′, H4′); 8.54 (br s, NH); 8.84 (d, J = 2.4 Hz, H2′).

***Z*-Bromoindole methyl ester 4:** ^{1}H NMR (400 MHz, $CDCl_3$,) 3.77 (s, OMe); 5.85 (d, J = 12.8 Hz, H2); 7.21 (d, J = 12.8 Hz, H3); 7.32 (dd, J = 1.8, 8.6 Hz, H5′); 7.58 (obscured by *E*-H7′, H4′, H7′); 8.54 (br s, NH); 8.85 (d, J = 3.2 Hz, H2′).

The EtOAc-soluble material from the above trituration was subjected to the same Si gel SPE fractionation with addition of a final MeOH flush. Concentration of this MeOH flush in vacuo yielded hymeniacidin (**5**) (9.9 mg, 0.028% specimen dry wt).

Hymeniacidin (5): pale yellow, viscous oil; IR ($CHCl_3$) ν_{max} 3057 (br), 1681, 1641, 1617 cm^{-1}; UV (MeOH) λ_{max} 271 nm (ϵ 11 400); ^{1}H NMR (400 MHz, $CDCl_3$) *major rotamer* δ 8.03 (s, 5-NCHO), 4.6 (m, 4-N*H*Me), 3.48 (s, 3-NMe), 3.33 (s, 1-NMe), 3.04 (s, 5-NMe), 2.98 (d, J = 5.2 Hz, 4-NH*Me*); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.3 (5-NCHO), 161.4 (C6), 153.3 (C4), 150.9 (C2), 96.6 (C5), 33.7 (5-NMe), 32.8 (4-NHMe), 30.8 (3-NMe), 28.4 (1-NMe); *minor rotamer* δ 8.25 (s, 5-NCHO), 4.4 (m, 4-N*H*Me), 3.44 (s, 3-NMe), 3.31 (s, 1-NMe), 3.18 (s, 5-NMe), 2.94 (d, J = 5.2 Hz, 4-NH*Me*); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 164.7 (5-NCHO), 159.8 (C6), 152.4 (C4), 151.1 (C2), 93.8 (C5), 37.0 (5-NMe), 31.9 (4-NHMe), 30.5 (3-NMe), 28.3 (1-NMe); ESI-(+)MS *m*/*z* 249 [(M + Na)$^+$, 83%], 227 [(M + H)$^+$, 86]; HRESI-(+)MS *m*/*z* 249.0959 [(M + Na)$^+$, calcd for $C_9H_{14}N_4O_3Na$, 249.0964].

Synthesis of Hymeniacidin (5). 1,3,5,7-Tetramethylxanthine (**6**) was prepared as described below using the method of Hori et al.[15] Caffeine (5.0 g, 0.026 mol) and dimethyl sulfate (4.9 mL, 0.052 mol) were stirred in nitrobenzene (60 mL) at 90 °C for 16 h under N_2. After cooling, Et_2O (50 mL) was poured onto the reaction mixture and the solvent decanted. The residue was further washed with Et_2O (3 × 100 mL) and hot acetone (3 × 50 mL) to return as the residue 1,3,5,7-tetramethylxanthine (**6**) (5.1 g, 93%).

1,3,5,7-Tetramethylxanthine (**6**) (5.0 g, 0.024 mol) was dissolved in a 14% NH_3(aq) solution (60 mL) and stirred at room temperature for 12 h. The resulting reaction mixture was concentrated in vacuo and extracted with CH_2Cl_2 to afford hymeniacidin (**5**) (4.9 g, 91%), which was recrystallized from EtOAc, mp 158−159 °C (lit.[13] 158−159 °C). The synthetic material was found to be spectroscopically identical to the natural hymeniacidon (**5**).

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