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# Didemnolines A-D, New N9-Substituted β-Carbolines from the Marine Ascidian *Didemnum* sp.

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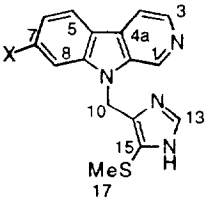
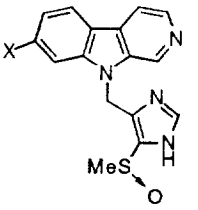
***Abstract:*** Four new β-carboline-based metabolites, didemnolines A-D (**1-4**), were isolated along with eudistomin O (**5**), β-carboline (**6**), and 2-(2',4'-dibromophenoxy)-3,5-dibromophenol (**7**) from an ascidian of the genus *Didemnum*, collected near the island of Rota, Northern Mariana Islands. These new β-carboline-based metabolites differ from most previously isolated compounds in that they are substituted at the N9 position of the β-carboline ring, rather than the C1 position.

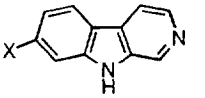
Marine ascidians have received increasing attention in recent years, in part because the first marine natural product to reach human clinical trials as an anticancer agent, the cyclic depsipeptide didemnin B,[1,2] was isolated from a didemnid ascidian, but also because they produce a wide variety of novel amino acid derived natural products.[3] Many of these compounds have been the focus of further biological studies as well as targets for synthetic studies. In addition to cyclic peptides, ascidians have yielded many heteroaromatic alkaloids, including pyridoacridines[4] and β-carbolines.[3] Ascidians of the genus *Eudistoma* have been the most common source of biologically active β-carboline derivatives, including the eudistomins,[5] the eudistomidins,[6] and woodinine.[7] We would now like to report the discovery of didemnolines A-D (**1-4**), which were isolated from an ascidian of the genus *Didemnum*, collected near the island of Rota, Northern Mariana Islands, along with the known compounds eudistomin O (**5**), β-carboline (**6**), and 2-(2',4'-dibromophenoxy)-3,5-dibromophenol (**7**), a compound previously isolated from marine sponges of the genus *Dysidea*.[8] The didemnolines differ from most other marine-derived β-carboline compounds in that they are substituted at the N9 position, rather than at the C1 position. In fact, eudistomidins D,[6b] E, and F[6c] are the only other related marine alkaloids bearing substitution at N9.



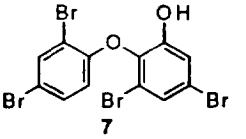

**1** R = Br
**2** R = H




**3** R = Br
**4** R = H




**5** R = Br
**6** R = H



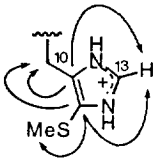

**7**

A methanolic extract, obtained by soaking homogenized, freeze-dried ascidian tissue, was subjected to a modified Kupchan solvent partition scheme.[9] The antimicrobial hexane fraction was chromatographed over silica

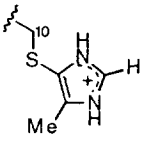
gel yielding brominated diphenyl ether **7**, which was identified through comparison of its $^1$H NMR data with that reported in the literature.[8] Fractionation of the $CCl_4$, $CHCl_3$, and MeOH fractions using silica gel flash chromatography and repeated normal phase HPLC over an amino bonded phase yielded fractions containing the following pairs of compounds: didemnolines A (**1**) and B (**2**), C (**3**) and D (**4**), eudistomin O (**5**) and β-carboline (**6**). Preparative TLC over silica gel provided pure samples of compounds **1**, **2**, **5**, and **6**, and a fraction containing approximately a 10:1 mixture of **3** and **4**. Compound **5** was identified based on a comparison its spectral data to those reported in the literature,[5c] while compound **6** was identified by comparison with an authentic standard.

The EIMS of didemnoline A (**1**) exhibited $M^+$ ions at *m/z* 374 and 372, suggesting the presence of a single bromine atom, while the HREIMS data indicated a molecular formula of $C_{16}H_{13}BrN_4S$. The UV spectrum showed maxima characteristic of a β-carboline ring, an assignment that was further supported by the formation of prominent ions in the EIMS at *m/z* 248 and 246 for the protonated bromo-β-carboline ring. The $^{13}$C NMR data for didemnoline A (**1**) (Table I) included signals for 14 $sp^2$ hybridized carbons, of which 11 belong to the β-carboline ring, and two $sp^3$ hybridized carbons. The $^1$H NMR spectrum (see Table II) contained signals assignable to a 1,2,4-trisubstituted benzene ring, two additional vicinal aromatic protons, two isolated aromatic protons, a heteroatom-substituted methylene, and a vinyl or heteroatom-substituted methyl group. Confirmation of the brominated β-carboline ring was obtained using HMBC data and by comparisons of the NMR data to those reported for eudistomin O (**5**).[5c]

Assembly of the remaining atoms ($C_5H_7N_2S$) was hampered by the lack of observable coupling between protons in the NMR spectrum. In fact, all signals not assigned to the β-carboline ring, including those for the heteroatom-substituted methylene (H10), the vinyl or heteroatom-substituted methyl group (H17), an aromatic proton (H13), and an exchangeable proton (H12), are singlets. Therefore, construction of the disubstituted imidazole ring shown in structure **1**[10] was accomplished primarily through interpretation of the long-range heteronuclear correlation data, as shown in substructure **A**, along with evaluation of chemical




**A**




**B**

shifts. The proposed heteroaromatic ring was further supported by the $^1J_{CH}$ value for the C2 position of the imidazole ring (C13). The measured value of 207 Hz matches the 206 Hz coupling constant measured for 3-methylimidazole. The presence of a thiomethyl group rather than a sulfide bridge, as shown in substructure **B**, was also confirmed by measuring the one bond carbon-proton coupling constant (142 Hz) which matched more favorably to the 141 Hz for the thiomethyl group in varamine B[11] than 127 Hz for the $CH_3$ of 3-methylimidazole.

**Table I. $^{13}$C NMR Chemical Shifts for 1-3.**

| | Compound | | |
|---|---|---|---|
| C no. | **1**[a] | **2**[b] | **3**[a] |
| 1 | 126.9 | 133.6 | 126.7 |
| 3 | 130.9 | 138.8 | 130.7 |
| 4 | 117.9 | 114.5 | 117.9 |
| 4a | 133.5 | 133.6 | 133.4 |
| 4b | 119.1 | 119.6 | 119.0 |
| 5 | 125.7 | 122.4 | 125.6 |
| 6 | 125.5 | 126.8 | 125.4 |
| 7 | 125.6 | 120.9 | 125.7 |
| 8 | 114.5 | 113.7 | 114.7 |
| 8a | 144.3 | 141.4 | 144.4 |
| 9a | 136.0 | 136.1 | 135.9 |
| 10 | 38.0 | 38.0 | 38.5 |
| 11 | 130.1 | 127.6 | 130.6 |
| 13 | 137.1 | 137.5 | 138.3 |
| 15 | 123.8 | 123.5 | 134.4 |
| 17 | 18.7 | 18.7 | 39.0 |

[a]Recorded in 5% TFA in DMSO-$d_6$. [b]Recorded in DMSO-$d_6$.

While the majority of β-carboline metabolites bear substituents at C1,[3] the connection of C10 in substructure **A** to N9 of the β-carboline ring was confirmed using HMBC data, which indicated 3-bond heteronuclear coupling from both C8a and C9a to H10. The final assignment was the placement of the bromine atom at C7. Using difference NOE techniques, irradiation of H10 caused an enhancement of the signal assigned to H8, thus supporting structure **1** for didemnoline A.

The HREIMS of didemnoline B (**2**) supported a molecular formula of $C_{16}H_{14}N_4S$, indicating that the bromine atom in didemnoline A had been replaced by a hydrogen. The NMR data corroborated this assignment. While the $^{13}C$ NMR spectrum (see Table I) was quite similar to that obtained for didemnoline A, the $^1H$ NMR spectrum included an additional aromatic signal as part of an AA'BB' spin system of an *ortho*-disubstituted benzene ring (see Table II). Based on this evidence, didemnoline B (**2**) is proposed to be debromodidemnoline A.

The mass spectrum of didemnoline C (**3**) showed molecular ions of *m/z* 390 and 388, 16 mass units greater than for didemnoline A, supporting a molecular formula of $C_{16}H_{13}BrN_4OS$. The NMR spectra are qualitatively very similar to those obtained for compound **1** (see Tables I and II). While the HMBC data indicates that didemnoline C has the same connectivity of atoms as didemnoline A, significant chemical shift differences were observed for the aromatic atoms H13/C13 (9.10/137.1 ppm for **1** vs. 8.22/138.3 ppm for **3**), the quaternary carbon C15 (123.8 ppm for **1** vs. 134.4 ppm for **3**), and the methyl group H17/C17 (2.30/18.9 ppm for **1** vs. 2.85/39.0 for **3**), all of which are associated with the imidazole ring. Therefore, to account for the additional oxygen atom indicated by the MS data, didemnoline C is proposed to bear a methylsulfoxide moiety in place of the thiomethyl group in didemnoline A. The assignment of didemnoline C as compound **3** is supported by downfield shift of the NMR signal observed for C17, as well as by the IR spectrum, which shows an absorption characteristic of sulfoxides at 1036 $cm^{-1}$, and it is optically active, as would be expected. Furthermore, treatment of **1** with $NaIO_4$ yielded a product that was racemic, but otherwise identical to didemnoline C (**3**).

**Table II. $^1H$ NMR Chemical Shifts for 1-4.**

| | δ $^1H$ (mult; $J_{HH}$, Hz) | | | |
|---|---|---|---|---|
| H no. | **1**[a] | **2**[b] | **3**[a] | **4**[b] |
| 1 | 9.63 (s) | 9.1 (s) | 9.59 (s) | 9.07 (s) |
| 3 | 8.73 (d; 6.0) | 7.91 (d; 5.1) | 8.70 (d; 5.8) | 8.13 (d; 5.2) |
| 4 | 8.86 (d; 6.0) | 8.33 (d; 5.1) | 8.86 (d; 5.8) | 8.39 (d; 5.2) |
| 5 | 8.52 (d; 8.5) | 8.11 (d; 8.0) | 8.51 (d; 8.5) | 8.26 (d; 8.2) |
| 6 | 7.66 (dd; 8.5, 1.5) | 7.60 (dt; 8.3, 1.1) | 7.66 (dd; 8.5, 1.5) | 7.58 (t; 7.5) |
| 7 | -- | 7.29 (dt; 8.3, 1.1) | -- | 7.29 (t; 7.5) |
| 8 | 8.17 (d; 1.5) | 7.80 (d; 8.0) | 8.27 (d; 1.5) | 7.75 (d; 8.0) |
| 10 | 6.03 (s) | 5.57 (s) | 6.10 (s) | 5.85 (s) |
| 13 | 9.10 (s) | 9.14 (s) | 8.23 (s) | 8.1 (s)[c] |
| 17 | 2.32 (s) | 2.16 (s) | 2.88 (s) | 2.78 (s) |

[a]Recorded in 5% TFA in DMSO-$d_6$. [b]Recorded in DMSO-$d_6$. [c]Chemical shift is estimated because signal are partially obscured by that of compound **2**.

Didemnoline D (**4**) was never obtained in pure form, but could be observed as a minor set of signals (see Table II) in the $^1H$ NMR spectrum of didemnoline C (**3**). These signals were related to those assigned to **3** in the same way as the NMR signals observed for didemnoline B (**2**) are related to those of **1**; namely, the spectrum was very similar except for the presence of an additional aromatic proton signal as part of a four-proton spin system of an *ortho*-disubstituted benzene ring. Further information was obtained during the $NaIO_4$ oxidation of a

sample of didemnoline A (**1**) that contained a small amount of didemnoline B (**2**). The minor oxidation product showed $^1$H NMR signals matching the minor signals apparent in the spectrum recorded of didemnoline **C**, thus allowing us to tentatively assign didemnoline D to structure **4**.

The didemnolines represent a new family of β-carboline metabolites. Although commonly isolated from ascidians belonging to the family Polycitoridae, members of the family Didemnidae have yielded only two other β-carboline containing metabolites, eudistomin U[12] and lissoclin C.[13] The new structures are all closely related, differing in the presence or absence of a bromine atom and on the presence of a methyl sulfide or a methyl sulfoxide. Although it is possible that didemnolines C and D are air oxidation products of didemnolines **A** and **B**, the observation that compound **2** is optically active argues against this.

Didemnolines A-C (**1-3**) are moderately cytotoxic toward human epidermoid carcinoma (KB) cells (ATCC CCL 17),[14,15] with sulfoxide-containing **3** exhibiting the greatest activity.[16] Compounds **1** and **3** also exhibit antimicrobial activity towards *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and two strains of the yeast *Saccharomyces cerevisiae*. The RS322Y strain is hyperpermeable and hypersensitive to DNA damaging agents, while the RS188N strain is hyperpermeable, but DNA repair proficient.[17] The differential activity against RS322Y vs RS188N observed for compounds **1** and **3** is consistent with DNA damaging activity; however, because samples were tested at only one concentration, additional testing is necessary to confirm this result. Compounds **1** and **3** were inactive against the yeast *Candida albicans* at 100 μg/disk. The results of these assays are summarized in Table III.

**Table III. Results of Cytotoxicity and Antimicrobial Assays for 1-3.**

| Assay/Test Organism | 1 | 2 | 3 |
|---|---|---|---|
| cytotoxicity (KB)[a] | 6.1 | 3 | 0.28 |
| *S. aureus*[b] | 13 | --[c] | 9 |
| *B. subtilis*[b] | 19 | -- | 12 |
| *E. coli*[b] | 9 | -- | 7 |
| *C. albicans*[b] | NA[d] | -- | NA[d] |
| *S. cerevisiae* (RS188N)[b] | 16 | -- | 9 |
| *S. cerevisiae* (RS322Y)[b] | 23 | -- | 18 |

[a]Results represent $IC_{50}$ values in μg/mL. [b]Results represent the diameter (mm) of the observed zones of inhibition in a disk diffusion assay run with 100 μg/6 mm disk. [c]Not tested due to insufficient sample. [d]Not active at 100 μg/disk.

The isolation of 2-(2',4'-dibromophenoxy)-3,5-dibromophenol ether **7** from an ascidian is of interest considering that recent studies have indicated that the polybrominated diphenyl ethers found in *Dysidea* sponges are produced by a cyanobacterial symbiont.[18] Considering that ascidians commonly associate with the unicellular prokaryotic alga *Prochloron* as well as with other cyanophytes,[3] it is conceivable that compound **7** is produced by an alga associated with the ascidian in this study. Alternatively, because a relatively small amount of compound was recovered, it may be possible that this compound was simply adsorbed from the water column, where it may have been exuded by sponges which were observed to be abundant in the immediate vicinity. Further studies are currently underway to ascertain whether there is sufficient biomass of alga associated with the *Didemnum* ascidian to be capable of producing the compound.

## EXPERIMENTAL SECTION

***General.*** All NMR experiments were performed on a GE Omega 500 instrument at 500 ($^1$H) and 125 MHz ($^{13}$C) operating frequencies. Chemical shifts are referenced to solvent peaks: 2.49 ppm (residual DMSO-$d_5$) and 39.0 ppm for $^{13}$C DMSO. IR spectra were recorded using a Perkin-Elmer 1600 FTIR, and the mass

spectral data were obtained on a VG-70SE mass spectrometer operating in the EI mode. UV/Vis spectra were recorded on a Milton Roy spectronic 3000 diode array spectrophotometer.

***Collection, Extraction, and Isolation.*** The tunicate *Didemnum* sp. was collected using SCUBA at a depth of 3-10 meters at Rota, Northern Mariana Islands, in December 1994. Specimens were kept frozen until extracted. The freeze-dried tunicate (28.5 g dry weight) was extracted with $CH_3OH$ to give, after concentration, a dark brown oil (4.4 g), which was solvent partitioned using a modified Kupchan technique,[9] yielding 0.43, 0.32, 0.61, and 2.8 grams of the hexane, $CCl_4$, $CHCl_3$, and $CH_3OH$ extracts, respectively. All extracts displayed antimicrobial activity against *S. aureus* and were subjected to further purification. The hexane fraction was chromatographed first over silica gel, eluting with a step-wise solvent gradient from $CHCl_3$ to $CH_3OH$, and then over Sephadex LH-20, with $CHCl_3/CH_3OH$ (1:1) as solvent, yielding 2.0 mg of 2-(2',4'-dibromophenoxy)-3,5-dibromophenol (**7**). The $CCl_4$ fraction was chromatographed sequentially over silica gel ($CHCl_3/CH_3OH$, 97:3), and an amino bonded phase column ($CHCl_3/CH_3OH$, 98:2) to give a mixture of eudistomin O (**5**) and β-carboline (**6**), which was separated using preparative TLC on silica (hexane/acetone, 7:3) to give pure **5** (1.5 mg) and **6** (1 mg). Fractionation of the $CHCl_3$ soluble material using silica gel chromatography (step-wise solvent gradient from $CHCl_3$ to 20% $CH_3OH$ in $CHCl_3$) followed by repeated HPLC over an amino bonded phase column ($CHCl_3/CH_3OH$, 95:5), provided a mixture of didemnolines A (**1**) and B (**2**), which could be separated using preparative silica gel TLC (hexane/acetone, 1:1) to give 18 mg of **1** and 8 mg of **2**. Chromatography of the $CH_3OH$ soluble material over an amino bonded phase, eluting with a step-wise gradient from $CHCl_3$ through $CH_3OH$ provided 3 mg of an inseparable mixture of didemnolines C (**3**) and D (**4**).

***Didemnoline A (1):*** UV (EtOH) $\lambda_{max}$ 240 (ε 38,700), 290 (ε 13,500), 344 (sh), 358 (ε 5,000) nm; IR (film) υ 3448, 1683, 1493, 1441, 1327, 1200, 1138, 1053, 963, 840, 802, 721 $cm^{-1}$; $^1H$ NMR see Table II; $^{13}C$ NMR see Table I; EIMS *m/z* (rel int) 374 ( 8), 372 ($M^+$,7), 249 (14), 248 (99), 247 (18), 246 (100), 169 (12), 168 (83), 167 (45), 166 (13), 141 (12), 140 (42), 139 (17), 127 (72), 111 (41), 97 (68), 71 (78); HREIMS 371.9998 ($C_{16}H_{13}BrN_4S$, Δ +4.6 mmu).

***Didemnoline B (2):*** UV (EtOH) $\lambda_{max}$ 240 (ε 36,800), 290 (ε 13,500), 344 (sh), 358 (ε 5,000) nm; IR (film) υ 3416, 1624, 1453, 1328, 1271, 1208, 1032, 804 $cm^{-1}$; $^1H$ NMR see Table II; $^{13}C$ NMR see Table I; EIMS *m/z* (rel int) 294 ($M^+$, 21), 181 (20), 169 (21), 168 (100), 167 (12), 140 (20), 127 (56), 100 (12), 73 (14), 57 (13); HREIMS 294.0937 ($C_{16}H_{14}N_4S$, Δ −0.2 mmu).

***Didemnoline C (3):*** $[\alpha]^{25}_D$ = +97.2° (c 0.1, DMSO); UV (EtOH) $\lambda_{max}$ 239 (ε 37,200), 290 (ε 14,000), 344 (sh), 358 (ε 4,000) nm; IR ($CHCl_3$) υ 3391, 1636, 1497, 1457, 1329, 1203, 1036, 1025, 800, 721 $cm^{-1}$; $^1H$ NMR see Table II; $^{13}C$ NMR see Table I; EIMS *m/z* (rel int) 400 (0.4), 388 ($M^+$, 0.4), 374 (0.2) 372 (0.3), 247 (15), 245 (16), 168 (12),84 (85), 66 (100)); HREIMS 388.0012 ($C_{16}H_{13}BrN_4OS$, Δ +1.9 mmu).

***Didemnoline D (4):*** $^1H$ NMR see Table II.

***Conversion of Didemnoline A (1) to Didemnoline C (3):*** To a solution of didemnoline A (**1**, 2 mg) in $CH_3OH/H_2O$ (9:1) was added 1.6 mg of $NaIO_4$ at 0 °C. After stirring for 24 hours an approximately 50% conversion had been obtained and the solid particulate was remove by filtration. The product mixture was chromatographed over an amino bonded column ($CHCl_3$:$CH_3OH$, 95:5) to give didemnoline C (1 mg, 50%) in addition to unreacted didemnoline A (1 mg, 50%).

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