A Cyclized Didemnimide Alkaloid from the Caribbean Ascidian *Didemnum conchyliatum*

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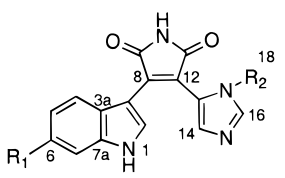
Received September 23, 1998

A novel, cyclized alkaloid of the didemnimide class, **5**, has been isolated from extracts of the Caribbean ascidian *Didemnum conchyliatum*. The structure of **5** was assigned using combined spectral methods that emphasized one- and two-dimensional NMR methods. The new alkaloid is the cyclization product of didemnimide A (**1**) formed via a C-2 indole condensation with the imidazole nitrogen.

Marine ascidians, or sea squirts (phylum Chordata, subphylum Urochordata, class Ascidiacea), have proven to be a rich source of chemically diverse secondary metabolites that often display potent pharmacological or biological activities.[1] In particular, colonial ascidians of the family Didemnidae and Polycitoridae have been studied extensively because of their unusual, cytotoxic metabolites.[2] Several studies of the chemical defenses of ascidians in these families point to an increased interest in understanding the ecological rationale for the production of these compounds.[3]

As part of our ongoing efforts to study the chemistry and biology of these animals, our attention was drawn to the Caribbean mangrove ascidian *Didemnum conchyliatum* (Sluiter, 1898, family Didemnidae). This small didemnid ascidian was found growing on the flat blades of the seagrass *Thalassia testudinum* in the mangrove channels of Grand Bahama Island, Bahamas. Although quite small in size, the abundance of the ascidian allowed us earlier to isolate and characterize four novel alkaloids, didemnimides A−D (**1**−**4**), possessing unprecedented indole−maleimide−imidazole carbon frameworks.[4] Ecological studies revealed the predator-deterrent properties of the didemnimides, which are responsible for the chemical defense of the animal.[5] During fractionation of the crude extract of *D. conchyliatum*, a less polar, deep purple metabolite was observed as a trace metabolite. This compound was subsequently isolated and characterized as **5**, a molecule possessing a novel, cyclized didemnimide carbon skeleton.

Freshly collected *D. conchyliatum* was removed from the seagrass blades and immediately extracted with methanol/dichloromethane (1:1).[4,5] The condensed wet extract was partitioned against isooctane, ethyl acetate, dichloromethane, and 2-propanol. The combined isooctane, ethyl acetate and dichloromethane fractions were subsequently fractionated by vacuum flash chromatography on silica gel, employing a 0−5% methanol in dichloromethane gradient. A fraction of intermediate polarity, containing the didemnimides C (**3**), D (**4**),[4] and **5**, was further separated by open-column, reversed-phase (C_{18}) chromatography using 30% water in methanol, followed by open column, normal-phase chromatography using 5% methanol in dichloromethane.[4] Final purification of **5** was achieved by reversed-phase HPLC using 74% acetonitrile in water. The compound subsequently quantitatively precipitated from the eluent (1 mg, ca. 10^{-3} % volume).

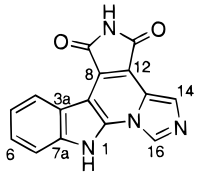


Didemnimide A (**1**): R_1=H, R_2=H

Didemnimide B (**2**): R_1=Br, R_2=H

Didemnimide C (**3**): R_1=H, R_2=CH_3

Didemnimide D (**4**): R_1=Br, R_2=CH_3



5

Compound **5** analyzed for $C_{15}H_8N_4O_2$, showing a MH^+ peak in the high-resolution FAB mass spectrum at *m*/*z* 277.0713 (Δ = 4.5 ppm). This formula required 14 degrees of unsaturation, suggesting an additional ring in comparison with didemnimides A−D (**1**−**4**). The infrared spectrum of **5** in KBr showed the presence of amide protons (3000−3450 cm^{-1}) and showed the complex absorptions derived from the symmetric and asymmetric stretching of the imide carbonyl groups (1749 (weak shoulder), 1737 (shoulder), 1719 (strong), 1696 cm^{-1} (strong).[6] In addition, **5** showed a series of characteristic infrared absorption bands at 1584 and 1567, 1484, 1367, and 750 cm^{-1}, indicating the presence of the maleimide ring.[7]

The structure of **5** was further assigned using one- and two-dimensional NMR experiments. The ^{13}C NMR spectrum of **5** in DMSO-d_6 showed 15 carbon atoms, two of which were located in the carbonyl region (δ 169.7, δ 168.7 ppm). The ^1H NMR spectrum (DMSO-d_6) showed the typical indole substitution pattern consisting of two triplets (δ 7.36, tr, 10 Hz, 1H and δ 7.44, tr, 10 Hz, 1H) and two doublets (δ 8,52, d, 10 Hz, 1H and δ 7.69, d, 10 Hz, 1H) and also displayed three singlets (δ 7.87, s, 1H, δ 8.90, s, 1H and δ 11.0, s, NH). A TOCSY NMR experiment revealed couplings consistent with a four-spin system (the aromatic portion of the indole ring, δ 8.52, δ 7.36, δ 7.44, δ 7.69 ppm) and a two-spin system with a small long-range coupling (the imidazole ring δ 7.87 ppm, H-14 and δ 8.90 ppm, H-16).

An HMQC experiment allowed the proton assignments to be transferred to the corresponding carbon nuclei. The connectivities of the aromatic portion of the indole ring and

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10.1021/np980409q CCC: $18.00
Published on Web 12/31/1998

Table 1. NMR Assignments for Alkaloid **5**

position	^{1}H (DMSO-d_6) δ (mult, J, no. of H's)[a]	^{13}C (DMSO-d_6) δ (no. of H's)[a]	HMBC (DMSO-d_6)[b]	TOCSY (DMSO-d_6)	COSY (DMSO-d_6)
1	n. o.				
2		134.8 (C)			
3		97.4 (C)			
3a		120.7 (C)			
4	8.52 (d, 10 Hz, 1H)	122.0 (CH)	C-7a, C-6, C-5, C-3a, C-7, C-3	H-5, H-6, H-7	H-5
5	7.36 (tr, 10 Hz, 1H)	121.6 (CH)	C-7a, C-6, C-4, C-3a, C-7, C-3	H-4, H-6, H-7	H-4, H-6
6	7.44 (tr, 10 Hz, 1H)	124.5 (CH)	C-7a, C-4, C-5, C-3a, C-7	H-4, H-5, H-7	H-5, H-7
7	7.69 (d, 10 Hz, 1H)	112.2 (CH)	C-7a, C-5, C-3a, C-3	H-4, H-5, H-6	H-6
7a		135.5 (C)			
8		125.9 (C)			
9		169.7 (C)[c]			
10	11.00 (s, 1H)				
11		168.7 (C)[c]			
12		113.5 (C)			
13		122.9 (C)			
14	8.90 (s, 1H)	126.6 (CH)	C-13, C-6, C-12	H-16	H-16
15					
16	7.87 (s, 1H)	121.0 (CH)	C-2, C-14, C-13	H-14	H-14
17					

[a] ^{1}H and ^{13}C NMR shifts were referenced to DMSO-d_6 (^{1}H δ 2.49 and ^{13}C δ 39.5 ppm). [b] HMBC data were optimized for $^nJ_{CH} = 5$ Hz. [c] Values may be interchanged.

the imidazole ring were fully established by a long-range heterocorrelation experiment (HMBC, performed at $J = 5$ Hz, Table 1). A strong HMBC correlation from the C-4 indole proton (δ 8.52, 10 Hz) to the quaternary carbon (δ 97.4) at C-3 was observed. A weak HMBC correlation from the imidazole proton at C-14 (δ 8.90, s) to a quaternary carbon [δ 113.5 (C)], subsequently assigned as C-12, established the connectivity of the imidazole moiety to the maleimide ring. The carbonyl carbons and the two remaining quaternary carbons were assigned by comparison of their chemical shifts with the didemnimides A−D (**1**−**4**).

Compound **5** displayed a broad absorbance centered at 487 nm in the UV/vis spectrum (diode-array HPLC), consistent with its characteristic, deep purple color. These data, together with the one- and two-dimensional NMR data and the IR data, were in full agreement with the indole−maleimide−imidazole carbon skeleton, yet possessing additional conjugation when compared to didemnimides A−D (**1**−**4**). Also, mass spectral data for **5** closely resembled those of didemnimide A (**1**, $C_{15}H_{10}N_4O_2$, 13 DBE, HREIMS $[M + H]^+$ obsd m/z 278.0793, $\Delta = 3.9$ ppm), whose structure was determined by a single-crystal X-ray experiment.[4] Metabolite **5** showed one additional degree of unsaturation (two hydrogens less in the molecular formula), when compared to this metabolite. In view of the highly conjugated nature of the latter, **5** can only be derived from **1** by ring closure connecting the imidazole and indole rings. Signals emanating from the C-2 proton of the indole ring were clearly lacking in the ^{1}H NMR spectrum of **5**. Since the protons at C-14 and C-16 were present in **5**, cyclization of the imidazole ring can only involve the N-17 nitrogen atom. Cyclization to the alternate nitrogen would create a seven-membered ring with the equivalent of a trans double bond.

Compound **5** is the first alkaloid to be isolated with this cyclized indole−maleimide−imidazole structure. The carbon skeleton is derived via a biogenetic pathway that appears to involve the coupling of two modified amino acid residues to form the central maleimide ring.[8] This pathway is supported by the occurrence of several other maleimide-containing natural products, the polycitrins[9] and the arcyriarubins,[10,11] the latter co-occurring with its dicarboxylic acid intermediate, lycogalic acid.[8,10] Cyclized alkaloid **5** appears to be derived from didemnimide A (**1**) via a mechanism involving ring closure, as was proposed for the formation of the ring-closed arcyriaflavins from the arcyriarubins.[11]

Compound **5** falls within the class of α-carbolines, which is a relatively rare structural class among natural products. α-Carbolines, both natural and synthetic, are generally considered compounds of great interest for the discovery of novel anticancer agents, due to their possible DNA intercalation properties, which depend on the planarity of the α-carboline ring system and its functional groups.[12] Two classes of α-carboline compounds (which differ in the degree of unsaturation of the pyrido-ring) have been isolated previously from natural sources. The grossularines 1 and 2, highly cytotoxic α-carbolines possessing the pyrido-[2,3-*b*]indole ring system, were isolated form the Eastern Atlantic ascidian *Dendrodoa grossularia*.[13] The closely related *N*,*N*-didesmethylgrossularine-1, which lacks significant antiproliferative activity, was isolated from the Eastern Pacific ascidian *Polycarpa aurata*.[14] The second class of α-carboline compounds, roquefortine,[15] glandicolins A and B,[16] oxaline and meleagrine,[17] and neoxaline,[18] all contain the piperidine[2,3-*b*]indole ring system.

Experimental Section

General Experimental Procedures. NMR spectra were recorded at 500 MHz using a 3 mm probe for ^{1}H and at 100 MHz using a 5 mm probe for ^{13}C. ^{1}H NMR and ^{13}C NMR spectra were referenced to solvent signals at 2.49 and 39.5 ppm for DMSO-d_6. Reversed-phase (C_{18}) HPLC was carried out on a semipreparative column (internal diameter 10 mm) by monitoring UV absorbance at 254 nm. High- and low-resolution mass measurements were provided by the Mass Spectrometry Facility at the University of California, Riverside. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR instrument. The UV spectrum was recorded on a Hewlett-Packard Series II 1090 liquid chromatograph HPLC (diode-array HPLC) using UV detection.

Animal Material. The ascidian *D. conchyliatum* (Sluiter, 1898) was collected in 1994 and 1996 at a depth of 5−6 feet from the blades of the seagrass *T. testudinum* in the mangrove channels of Sweetings Cay, Grand Bahama Island, Bahamas. The animal was identified by Dr. Françoise Monniot, and voucher specimens are preserved at the Muséum National d′ Histoire Naturelle, Paris, France.

Isolation and Purification. The freshly collected ascidians (~2100 mL) were separated from the grass blades and immediately extracted with 3 × 1.5 L portions of methanol/dichloromethane (1:1). The extracts were combined and reduced under vacuum to yield an aqueous methanol phase (~1040 mL), which was sequentially partitioned between isooctane, ethyl acetate, dichloromethane, and 2-propanol. The isooctane, dichloromethane, and ethyl acetate phases were combined, reduced to dryness, and fractionated by silica gel vacuum flash chromatography (Merck type 60) employing a gradient of 0−5% methanol in dichloromethane. Five major fractions were obtained, one containing **1** and **2** and a less polar fraction containing mixtures of **3**−**5**. The fraction containing **3**−**5** was further separated by reversed-phase (C_{18}) column chromatography (Crosfield Sorbsil C60 RP18B) using 30% water in methanol, followed by silica column chromatography (Merck Type 60) using 5% methanol in dichloromethane, and finally reversed-phase (C_{18}) HPLC (Rainin Dynamax C_{18}, 60 Å, i.d. 10 mm) using 26% acetonitrile in water. After collection from HPLC, **5** subsequently precipitated from the eluent (yield: 1 mg, ca. 10^{-3}% volume).

Compound **5** was obtained as a deep purple, crystalline solid (from acetonitrile/water), which showed the following physical and spectral properties: UV (CH_3CN/H_2O)[19] λ_{max} 487 nm (br); IR (KBr) ν_{max} 3260, 2367, 1737, 1719, 1696, 1584, 1567, 1484, 1367, 1320, 1237, 1185, 1114, 973, 920, 814, 749, 644, 515 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 1; EIMS, *m*/*z* 276 [M^+] (30.1), 205 (7.6), 193 (7.3), 178, (12.6), 166 (12.4), 153 (9.0), 151 (10.8), 139 (11.1), 125 (39.7), 111 (21.8), 99 (25.8), 97 (20.7), 95 (36.0), 83 (46.8), 81 (41.4), 71 (41.6), 69 (41.8), 67 (38.4), 57 (58.4), 55 (80.5), 45 (28.2), 44 (37.6), 43 (100.0); HREIMS [M + H]$^+$ obsd *m*/*z* 277.0713, calcd 277.0726 for $C_{15}H_9N_4O_2$, Δ = 4.5 ppm.

Acknowledgment. We thank Dr. Françoise Monniot (Muséum National d'Histoire Naturelle, Laboratoire de Biologie des Invertébrés Marins et Malacologie, Paris, France) for the identification of *Didemnum conchyliatum*. This research was funded by the National Science Foundation, Chemistry Division, under Grant No. CHE93-22776 (to W.F.), and by the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under Grant No. NA89AA-D-SG138, Project No. R/MP-54, through the California Sea Grant College (to W.F.). Our use of the research vessel *Seward Johnson* was supported by the National Science Foundation, Chemistry and Oceanography Divisions, under grant CHE90-08621 (to W.F.). We express our appreciation to the Ministry of Fisheries, Government of the Bahamas, for permission to perform research in their territorial waters.

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(19) Due to the small amount of **5** isolated, a reliable extinction coefficient could not be calculated.

NP980409Q