Plakinidine D, a New Pyrroloacridine Alkaloid from Two Ascidians of the Genus *Didemnum*

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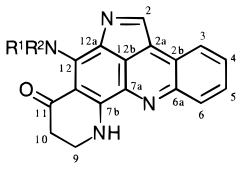
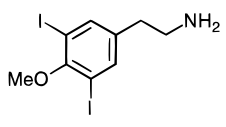
A previously undescribed red *Didemnum* sp. collected in Indonesia contained a novel pyrroloacridine, plakinidine D (**4**), along with the known compounds 3,5-diiodo-4-methoxyphenethylamine (**5**) and ascididemin (**6**), both of which had previously been isolated from ascidians of the genus *Didemnum*. Plakinidine D (**4**) and 3,5-diiodo-4-methoxyphenethylamine (**5**) were also isolated from *Didemnum rubeum* from the Republic of Palau. Interestingly, a collection of *D. rubeum* from Indonesia did not contain plakinidine D (**4**), but instead contained 3,5-diiodo-4-methoxyphenethylamine (**5**) and ascididemin (**6**). The structure of plakinidine D (**4**) was elucidated by analysis of its spectral data. Plakinidine D (**4**) is closely related to plakinidines A−C (**1**−**3**), previously isolated from the sponge *Plakortis* sp.

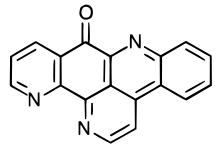
Marine sponges and ascidians have proven a rich source of heteroaromatic pigments, many of which interact with DNA[1] and/or DNA processing enzymes such as topoisomerase II.[2] Interestingly, pyridoacridine alkaloids have been reported with almost equal frequency from both sponges and ascidians.[3] A number of years ago we reported the isolation of the pyrroloacridine alkaloids plakinidines A−C (**1**−**3**) from a *Plakortis* sponge.[4] We now report the isolation of plakinidine D (**4**) from an undescribed *Didemnum* sp. ascidian (Didemnidae) collected at Barrang Lompo, Indonesia, and *Didemnum rubeum* collected at Lighthouse Channel, Republic of Palau. The major metabolite in both ascidians was 3,5-diiodo-4-methoxyphenethylamine (**5**).[5] The specimen from Indonesia also contained the pyridoacridine ascididemin (**6**).[6] A collection of *D. rubeum* from Bonne Tambon, Indonesia, contained **5** and **6** but did not contain any plakinidines.



(**4**) R^1=R^2=H
(**1**) R^1=H, R^2=CH_3
(**2**) R^1=R^2=CH_3
(**3**) R^1=H, R^2=CH_3; 9,10-didehydro



(**5**)



(**6**)

The chloroform soluble material from a solvent partition of an ethanol extraction of *Didemnum* sp. collected at Barrang Lompo in Indonesia was purified by silica chromatography ($CHCl_3$/MeOH gradient) and size exclusion chromatography (LH-20, MeOH) to give a novel red alkaloid, plakinidine D (**4**), and the known compound ascididemin (**6**).[6] The aqueous MeOH fraction of the solvent partition contained predominantly 3,5-diiodo-4-methoxyphenethylamine (**5**).[5]

The structure of the novel component, plakinidine D (**4**), was elucidated predominantly by the use of NMR and mass spectrometry and by comparison to the known plakinidines A−C (**1**−**3**).[4] A molecular formula of $C_{17}H_{12}N_4O$ was assigned by HREIMS. Interestingly, the positive ion FAB mass spectrum displayed two equally intense ions at *m*/*z* 289 and 290, suggesting an *in situ* reduction during the ionization process. Table 1 displays complete ^{1}H and ^{13}C NMR assignments obtained from analysis of one- and two-dimensional experiments. In $CDCl_3$ solution, all 17 carbons were resolved in the 125 MHz ^{13}C NMR spectrum. An HMQC experiment in combination with the integrations from the one-dimensional 500 MHz ^{1}H NMR ($CDCl_3$) established that the molecule contained 2 methylene, 5 methine, and 10 quaternary carbons. The spectrum contained signals typical of an *ortho*-disubstituted benzene ring (H3-H6), an isolated methine proton (H2) adjacent to a heteroatom, and an isolated pair of coupled methylenes ($H_2$9, $H_2$10). The assignment of H3−H6 was verified by analysis of a phase sensitive gradient double quantum COSY experiment.

Long-range ^{1}H−^{13}C correlations (GHMBC; Figure 1) from H10 to C11 and C9, and from H9 to C11, C10, and C7b indicated the presence of a dihydropyridone ring. The proton H2 showed correlations with quaternary carbons at δ 122.6, 120.4, and 113.2 (C2a, C12a, and C12b, respectively), implying that these three quaternary carbons, the methine carbon, and a nitrogen might be present in a five-membered ring. A series of correlations within the *ortho*-disubstituted benzene ring verified its structure, and a correlation from H3 to C2a established a connectivity between these five- and six-membered ring systems.

These data alone are consistent with a plakinidine ring system. Indeed after comparison to the literature

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Table 1. ^{13}C and ^{1}H NMR Data for Plakinidine D (**4**)

atom	^{13}C[a]		^{1}H[a]	HMBC correlations
2	124.09	d	8.19 (1H, s)	C2a, C12a, C12b
2a	122.58	s		
2b	125.10	s		
3	123.93	d	8.25 (1H, dd, J = 8.3, 1.1)	C2a, C4, C6a
4	128.42	d	7.81 (1H, dt, J = 7.5, 1.1)	C2b, C6
5	130.94	d	7.77 (1H, dt, J = 7.7, 1.2)	C3, C6a
6	132.19	d	8.31 (1H, dd, J = 8.0, 1.1)	C2b, C5
6a	145.17	s		
7a	140.40	s		
7b	152.40	s		
8			8.39[b] (1H, br s)	
9	40.40	t	4.05 (2H, t, J = 7.2)	C7b, C10, C11
10	35.61	t	2.89 (2H, t, J = 7.2)	C9, C11
11	193.32	s		
11a	99.83	s		
12	157.91	s		
12a	120.39	s		
12b	113.16	s		
13			10.9[b] (2H, br s)	

[a] $CDCl_3$ as solvent and internal standard. [b] Exchangeable.

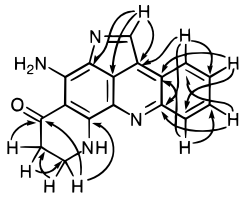

Figure 1. Long-range $^{1}H-^{13}C$ couplings observed for **4** in a GHMBC (8 Hz) experiment.

data for plakinidines A−C (**1**−**3**),[4] the structure was assigned as that shown for compound **4**. The only difference in structure between plakinidine D (**4**) and plakinidines A and B (**1**−**2**) was the absence of methylation at N12.

It is interesting to note that a sample of *D. rubeum* collected at an adjacent island in Indonesia did not contain plakinidine D (**4**) but contained both ascididemin and 3,5-diiodo-4-methoxyphenethylamine. The only difference in external appearance of the two organisms collected was the abundance of a red pigment on the surface of the organism plakinidine D was isolated from. This, coupled with the observance that plakinidines A−C were isolated from a sponge not an ascidian, poses the question as to whether the plakinidines are produced by a symbiotic microorganism.[7]

Plakinidine D showed *in vitro* cytotoxicity against the human colon tumor cell line HCT-116 at 5 μg/mL.

Experimental Section

General Experimental Procedures. UV spectra were recorded using a Hewlett-Packard 8452A diode array spectrophotometer in the solvent indicated at 25 °C. IR spectra were recorded using a Perkin-Elmer 1600 Series FTIR spectrophotometer. ^{1}H and ^{13}C NMR experiments were performed using a Varian Unity 500 MHz spectrometer with a deuterium lock in the solvent indicated at 26 °C. Spectra were referenced to residual undeuterated solvent peaks or solvent ^{13}C signals. High- and low-resolution mass spectrometry were performed on a Finnegan MAT 95 high-resolution gas chromatography/mass spectrometer. Silica gel used for flash chromatography was Merck Kieselgel 60, particle size 0.040−0.063 mm (Merck 230−400 mesh ASTM). Size exclusion chromatography was performed using Sigma Lipophilic Sephadex LH-20, bead size 25−100 μm.

Animal Materials. The ascidian *Didemnum* sp. Monniot and Monniot 1997 (sample no. CMI-96-5-1; registry no. A2 Did C 414) was collected at Barrang Lompo (5° 3.177′ S × 119° 19.898′ E at −3 to −15 m), Indonesia, in October 1996. The colonies were encrusting sheets, thin and brittle, red in life, with a smooth upper surface. The small calcareous spicules fill the whole thickness of the tunic and have few rays in optical section. The colony is crossed by large cloacal channels. The yellow zooids have sharp oral lobes, no cloacal languet, four rows of stigmata, large protruding lateral thoracic organs, a long waist, a twisted gut loop, one large testis vesicle, and the sperm duct coiled in six turns around it. No larvae were present in the colony observed. In the absence of larvae and the observance that the colony and zooids had no special original characters, this ascidian could not be identified to the species level. It belongs to the genus *Didemnum* among the family Didemnidae. It does not correspond to any already described *Didemnum* sp. from the same geographical area. The ascidian *D. rubeum* Monniot and Monniot 1996 (sample no. 95-137; registry no. MNHN: A2 Did C376) was collected by hand using SCUBA (−10 m) from Lighthouse Channel, Republic of Palau, in July 1996. The living colonies are red, encrusting rocks in large sheets that contain raised common cloacal openings. When fixed, the red color totally disappears and the tunic containing unicellular algae and the zooids turn dull green. The zooids have a wide cloacal aperture without a languet, eight stigmata in the first row, a long and stout retractor muscle, and a twisted gut with gonads in its loop. Two testis vesicles, pressed to each other, are encircled by six coils of the sperm duct. The larvae, deeply pigmented, have three adhesive papillae and four ampullae on each side. The spicules in balls are made of numerous blunt tipped rods. The ascidian *D. rubeum* Monniot and Monniot 1997 (sample no. CMI-96-27-3; registry no. A2 Did C 415) was collected at Bonne Tambun (5° 1.923′ S × 119° 16.745′ E at −3 to −15 m), Indonesia, in October 1996. Voucher specimens were deposited at the Museum national d'Histoire naturelle, Paris, France.

Isolation and Purification. The *Didemnum* sp. organism was ground and exhaustively extracted with EtOH, and the combined extracts were concentrated *in vacuo*. The dried extract (651 mg) was redissolved in 10% aqueous MeOH (50 mL) and extracted with hexane (3 × 100 mL). The concentration of the aqueous MeOH was adjusted to 30% by the addition of water (14.3 mL), and the resulting solution was extracted with $CHCl_3$ (3 × 100 mL). All three phases were concentrated *in vacuo* and inspected by ^{1}H NMR. The $CHCl_3$ extract (171.2 mg) contained predominantly 3,5-diiodo-4-methoxyphenethylamine (**5**)[5] and another compound accounting for the red color. The $CHCl_3$ extract was redissolved in 40% aqueous MeOH (50 mL) and extracted with $CHCl_3$ (3 × 100 mL). Concentration of the two phases *in vacuo* and analysis by ^{1}H NMR showed that the red compound was concentrated in the $CHCl_3$ layer and the 3,5-diiodo-4-methoxyphenethylamine (**5**) was in the aqueous MeOH phase. The resulting $CHCl_3$ soluble material (91.2 mg) was purified by gradient silica flash chromatography (10

× 180 mm, 0−50% MeOH in $CHCl_3$). Two fractions were obtained, one of which contained ascididemin (**6**)[6] (3.7 mg, identified using ^{1}H NMR and positive FABMS, $M^+ + 3H$, 286) and the other, the red pigment (4.5 mg). The latter was further purified by size-exclusion chromatography (Sephadex LH-20, 20 × 460 mm, $CHCl_3$/MeOH (1:1)) to afford plakinidine D (**4**) as a red-orange solid (3.5 mg). The *D. rubeum* organism collected at Bonne Tambun was subjected to a similar purification scheme but yielded only 3,5-diiodo-4-methoxyphenethylamine (**5**) and ascididemin (**6**).

The *D. rubeum* organism collected at Lighthouse Channel (100 g wet weight) was extracted with MeOH (3 × 150 mL) and 1:1 MeOH/CH_2Cl_2 (3 × 150 mL) with sonication. The combined extracts were concentrated and partitioned between CH_2Cl_2 (100 mL) and 15% MeOH in water (100 mL). The organic phase was evaporated, and the residue was partitioned between hexanes (100 mL) and 10% water in MeOH (100 mL). The aqueous MeOH was diluted with water (100 mL) and extracted with CH_2Cl_2 (3 × 100 mL). The organic phase was dried over anhydrous sodium sulfate and concentrated to a red oil. The oil was twice chromatographed on Sephadex LH-20 using MeOH/CH_2Cl_2 (1:1) as eluant to obtain plakinidine D (**4**) (10 mg, 0.01% wet weight) and 3,5-diiodo-4-methoxyphenethylamine (**5**) (40 mg, 0.04% wet weight). The aqueous MeOH extract was concentrated until no MeOH remained, and the residual aqueous extract was extracted with an equal volume of EtOAc. The aqueous layer contained primarily 3,5-diiodo-4-methoxyphenethylamine hydrochloride, which was not purified further.

Plakinidine D (4): UV (4:1 $CHCl_3$/MeOH) λ_{max} 250 (ϵ 4770), 282 (ϵ 3500), 328 (ϵ 2980), 388 (ϵ 1940), 436 (ϵ 820), 514 (ϵ 920); IR (film) υ_{max} 3417, 1627 cm^{-1}; ^{1}H and ^{13}C NMR data shown in Table 1; FABMS *m*/*z* 290 [M + 2H]$^+$ (100), 289 (84); EIMS *m*/*z* 288 [M]$^+$ (100), 287 (42), 259 (16), 233 (8); HREIMS *m*/*z* 288.1008 (calcd for $C_{17}H_{12}N_4O$, 288.1011).

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