Granulatimide and 6-Bromogranulatimide, Minor Alkaloids of the Brazilian Ascidian *Didemnum granulatum*

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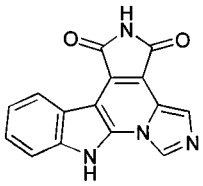
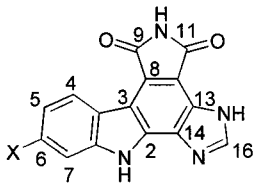
Reinvestigation of the extract of the ascidian *Didemnum granulatum* collected on the Brazilian coastline led to the isolation of two minor compounds, granulatimide (**2**) and 6-bromogranulatimide (**3**), which have been identified by analysis of their spectroscopic data. The isolation of **2** and **3** from *D. granulatum* corroborates previous assumptions about the occurrence of granulatimide as a natural product.

Ascidians (phylum Chordata, subphylum Urochordata, class Ascidiacea) are a promising source of bioactive natural products with unique structural features. Examples include the didemnins, cyclodepsipeptides isolated from the Caribbean ascidian *Tridemnum solidum*, which display potent cytotoxic, immunosupressant, and antibiotic activities, and the ecteinascidins, complex cytotoxic alkaloids obtained from the Caribbean ascidian *Ecteinascidia turbinata*. A derivative of didemnin B, dehydrodidemnin B (aplidine), and ecteinascidin 743 are presently in clinical trials as antitumor compounds.[1]

Until very recently, the Brazilian coastline remained practically unexplored toward the search for bioactive natural products from marine invertebrates.[2] In 1994 we started a program aimed at the isolation of biologically active natural products from Brazilian marine invertebrates, which ultimately led to the discovery of isogranulatimide (**1**) from the ascidian *Didemnum granulatum* (Tokioka, 1954; family Didemnidae).[3] Isogranulatimide displayed activity as an inhibitor of the G2 cell cycle checkpoint.[3,4] To confirm the structure of isogranulatimide and to obtain additional quantities of this alkaloid for the investigation of its mechanism of action, the synthesis of isogranulatimide was undertaken.[3,5] In the key step of the synthesis, a photocyclization of didemnimide A, both isogranulatimide and its constitutional isomer granulatimide (**2**) were obtained as synthetic products, a result that raised questions regarding the natural occurrence of **2** in the ascidian *D. granulatum*.



(**1**)



(**2**) X = H
(**3**) X = Br

In the present work we report the isolation of granulatimide, as well as a new alkaloid, 6-bromogranulatimide (**3**), from the ascidian *D. granulatum*. These findings confirm the hypothesis that granulatimide is in fact a naturally occurring compound.

In our previous isolation and syntheses of *D. granulatum* alkaloids,[3−5] we realized that both isogranulatimide (**1**) and granulatimide (**2**) were sparingly soluble in most organic or aqueous solvents, being soluble in DMSO and DMF only. Therefore, we envisaged an alternative extraction procedure in which the animals were first extracted with MeOH, then with DMF (see Experimental Section). Accordingly, the DMF extract of *D. granulatum* was analyzed by TLC and showed the presence of yellow, fluorescent granulatimide derivatives when compared with the synthetic compound.

Granulatimide (**2**) was isolated as a yellow solid, with spectroscopic data identical to that of the synthetic compound.[3] 6-Bromogranulatimide (**3**) was also obtained as a yellow solid, which displayed bands in the UV spectrum at 236, 282, 308, and 386 nm. The HREIMS of **3** presented a molecular ion peak at *m*/*z* 355.97319 (calcd 355.97377, $\Delta\mu$ −1.6 ppm), which was accounted for by the formula $C_{15}H_7O_2N_4{}^{81}Br$. Both ^1H and ^{13}C NMR data of **3** are shown in Table 1 and were assigned by analysis of HMQC and HMBC spectra. With the exception of H-6, the other ^1H resonances of 6-bromogranulatimide (**3**) are similar to those of granulatimide (**2**). Due to the small amount of available material, the carbon resonances of 6-bromogranulatimide were attributed by analysis of the HMQC and HMBC spectra. As in the case of isogranulatimide,[3] the signals of carbons 2, 13, and 14 were not observed, which is likely due to the isolation of **3** as its corresponding trifluoroacetate salt. We have also isolated didemnimide C as a minor compound occurring in a fraction that also contained didemnimide D. Although didemnimide C was previously isolated from the ascidian *D. conchyliatum*,[6] only didemnimides A, D, and E have been reported from *D. granulatum*.[3]

The isolation of isogranulatimide (**1**), granulatimide (**2**), and 6-bromogranulatimide (**3**) along with didemnimides A, C, D, and E from *D. granulatum* points to a common biogenetic pathway for this family of alkaloids. It is interesting to note that the synthetic photocyclization of didemnimide A yielded only granulatimide, while isogranulatimide is produced from didemnimide A via a thermal reaction.[5] This observation seems to rule out the possibility that **1** and **2** are formed in the tunicate simply by nonenzymatic photocyclization of didemnimide A via ambient sunlight.

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Table 1. ^{1}H and ^{13}C NMR Data for Granulatimide (**2**)[3] and 6-Bromogranulatimide (**3**)

	granulatimide		6-bromogranulatimide	
position	δ ^{13}C[a]	δ ^{1}H (mult, *J* in Hz)	δ ^{13}C[a]	δ ^{1}H (mult, *J* in Hz)
NH-1		12.58 (s)		12.74 (s)
C-2	135.4		n.o.	
C-3	113.0		112.0	
C-3a	121.4		118.5	
CH-4	123.8	8.89 (d, 7)	125.1	8.80 (d, 8.6)
CH-5	120.0	7.30 (dd, 7 and 7)	122.6	7.47 (dd, 8.6 and 1.5)
CH-6	126.1	7.48 (dd, 7 and 7)	120.6	
CH-7	111.6	7.61 (d, 7)	114.1	7.75 (d, 1.5)
C-7a	140.4		141.1	
C-8	122.7		122.5	
C-9	169.8		169.3	
NH-10		10.96 (s)		11.06 (s)
C-11	171.0		171.4	
C-12	109.5		109.9	
C-13	125.7		n.o.	
C-14	133.4		n.o.	
CH-16	144.5	8.50 (s)	144.7	8.56 (s)
NH-17		13.57 (bs)		13.68 (bs)

[a] Assignments by inverse detection at 500 MHz (HMQC and HMBC). n.o. = signals not observed.

Experimental Section

General Experimental Procedures. IR spectra were recorded on a FT-IR Bomem MB102 infrared spectrometer. NMR spectra were run on a Bruker AMX500 11.75 T instrument, operating at 500.0 MHz for ^{1}H and 125 MHz for ^{13}C channels, respectively. All the NMR spectra were obtained at 28 °C using the residual solvent signal as internal reference in DMSO-d_6. Low- and high-resolution FAB mass spectra were obtained on hybrid Kratos concept IIHQ equipment. Solvents employed for extraction and column chromatography were glass distilled prior to use. HPLC separations were performed with a Waters quaternary pump 600, double beam UV detector 2487, and data module 746. Chromatographic grade solvents were used in HPLC separations.

Animal Material. Specimens of *D. granulatum* (Tokioka, 1954; family Didemnidae) were collected by scuba at several sites in the São Sebastião channel and around São Sebastião Island (Ilhabela) in September 1997. An additional collection was performed in November 1998. The animals were immediately immersed in MeOH and stored at −20 °C for one month. A voucher specimen DID119 (September, 1998) is deposited in the ascidian collection of the Department of Zoology, Setor de Ciências Biologicas of Universidade Federal do Paraná.

Extraction and Isolation. The ascidian *D. granulatum* (1000 g, wet wt) was separated from the MeOH (500 mL) and re-extracted with 500 mL of dimethylformamide (DMF). The DMF extract was mixed with 1 L of a saturated NaCl solution and partitioned with 3 × 500 mL of EtOAc. The remaining DMF−saline mixture was passed through a XAD-7 column. The column was washed with H_2O, and the organic material was dessorbed with MeOH (500 mL) and with 1:1 EtOAc−MeOH (500 mL). The organic fractions were pooled and evaporated, to give 1.2 g of a brownish gum. This material was separated by Si gel flash chromatography, with a gradient of MeOH in CH_2Cl_2, to give 6 fractions named DMF-1 to DMF-6. The fractions DMF-3 and DMF-4 presented compounds with R_f similar to that of synthetic granulatimide and were further purified by normal-phase HPLC. Both fractions were dissolved in 0.2 mL of DMSO, diluted with the mobile phase (25:1 CH_2Cl_2−MeOH with 0.1% TFA), and injected in a Waters RCM (radial pak) 8 × 10 column, with a flow rate of 2.0 mL/min. Retention times: 6-bromogranulatimide = 10 min; granulatimide = 12 min. The HPLC purification provided 0.8 mg of 6-bromogranulatimide (8×10^{-5}% wet) and 0.5 mg of granulatimide (5×10^{-5}% wet).

Granulatimide (2): yellow amorphous solid; ^{1}H NMR, see Table 1 and Supporting Information.

6-Bromogranulatimide (3): yellow amorphous solid; UV λ_{max} (MeOH) 236, 282, 308, 386 nm; ^{1}H and ^{13}C NMR, see Table 1 and Supporting Information; HRFABMS *m/z* 355.97319 (MH^+, $C_{15}H_7O_2N_4{}^{81}Br$ requires 355.97377, Δ −1.6 mmu).

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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