Novel Betaines from the Marine Sponge *Agelas dispar*

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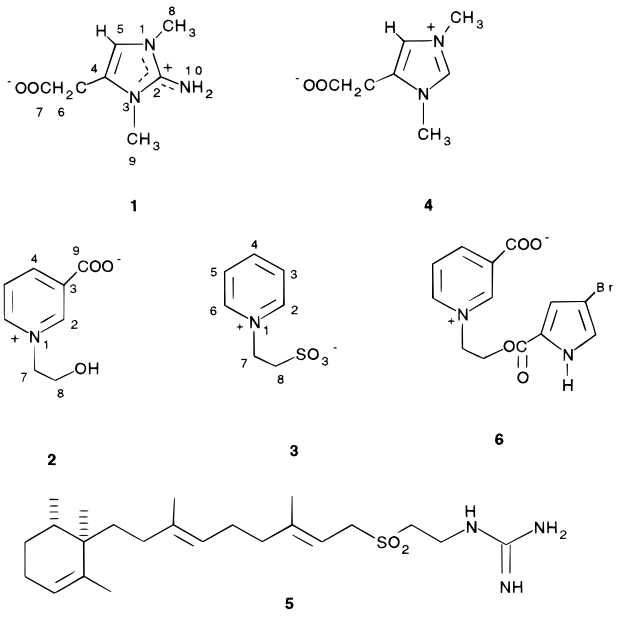
Three novel betaine alkaloids, called aminozooanemonin (**1**), pyridinebetaine A (**2**), and pyridinebetaine B (**3**), have been isolated from the Caribbean sponge *Agelas dispar*. Their structures were determined by FABMS, IR, UV, and 1D and 2D NMR spectroscopic experiments. Aminozooanemonin and pyridinebetaine A showed moderate antibacterial activity.

As part of our continuing research seeking novel biologically active secondary metabolites from marine sources, we have recently studied the Caribbean marine sponge *Agelas dispar* Duchassaing and Michelotti, 1860 (family Agelasidae, order Agelasida). Our efforts resulted in the isolation of several interesting compounds, the most important being immunostimulating α-glycosphingolipids[1] and a series of bromopyrrole alkaloids endowed with various bioactivities, for example, oroidine,[2] sceptrin,[3] and longamide B[4] possessing antibacterial activities; dispacamide A[5] and its monohydroxylated derivative dispacamide C,[6] which are potent noncompetitive antihistaminic agents; and the recently reported clathramides C and D,[4] which are antifungal compounds.

Following a bioassay-guided approach we have now examined some more polar fractions obtained from the methanolic extract. This resulted in the isolation of three novel low-molecular-weight alkaloids, named aminozooanemonin (**1**), pyridinebetaine A (**2**), and pyridinebetaine B (**3**), whose structure elucidations are the subject of the present paper. In addition, a series of nitrogenous compounds were isolated, which were readily identified as homarine,[7] trigonelline,[8] *N*,*N*,*N*-trimethyltaurine, and zooanemonin (**4**),[9] all long known and widely distributed in marine invertebrates. The polar fractions of the methanolic extract also contained the known antimicrobial spongal metabolite agelasidine C (**5**), a hypotaurocyamine derivative not previously reported from *A. dispar*. It was identified by comparison of its spectral properties with those reported in the literature.[10]

A methanolic extract of a specimen of *A. dispar* was concentrated and partitioned between water and diethyl ether, and then the polar layer was extracted with 1-butanol. The organic phases were initially subjected to chromatography over a column packed with RP18 Si gel (eluent H_2O with increasing amounts of MeOH). The fractions that eluted with MeOH−H_2O 2:8 and MeOH−H_2O 3:7 exhibited antimicrobial activity and, by ^{1}H NMR and TLC analysis, appeared to contain a number of UV-active betaine alkaloids. Further purification was achieved by repeated HPLC separations (RP18), affording aminozooanemonin (**1**), pyridinebetaine A (**2**), and pyridinebetaine B (**3**) in the pure state, as colorless amorphous solids.



The FABMS (positive ion) spectrum of **1** revealed an ion at *m*/*z* 170 $[M + H]^+$ and the high-resolution measurement indicated the molecular formula, $C_7H_{11}N_3O_2$, was compatible with the NMR data and indicated four double-bond equivalents. The IR spectrum displayed a strong carboxylate ion absorption at 1640 cm^{-1}, which shifted to 1710 cm^{-1} upon conversion of **1** to its hydrochloride. The ^{1}H NMR spectrum (DMSO-d_6) of **1** exhibited only five singlets: two methyls at δ 3.77 (H_3-8) and 3.97 (H_3-9), two signals integrating for two protons each, at δ 3.15 (H_2-6) and 8.30 (the NH_2 signal exchangeable in D_2O), and one aromatic methine signal at δ 7.63 (H-5). The ^{13}C NMR spectrum (measured in CD_3OD) showed signals of two nitrogen-linked methyls (δ 33.8 and 33.6), one methylene (δ 48.3), one methine (δ 127.0), and three nonhydrogenated sp^2 carbons (δ 134.2, 154.2, and 163.1). The above NMR data appeared compatible with an *N*,*N*-dimethylimidazolium nucleus possessing an acetate substitution. The dipolar couplings, measured by NOE difference experiments, of the signal at δ 3.97 (H_3-9) with those resonating at δ 8.30 and 3.15, and of the methyl signal at δ 3.77 (H_3-8) with the signals at δ 7.63 and 8.30, as well as the whole series of HMBC correlation peaks, allowed the unambiguous

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Table 1. ^{1}H and ^{13}C NMR Data of Pyridinbetaine A (2) and B (3) in CD_3OD

	2			3		
position	δ_H (mult., *J*)	δ_C, mult.	HMBC (^{1}H)	δ_H (mult., *J*)	δ_C, mult.	HMBC (^{1}H)
2	9.29 (s)	148.1, d	4, 6, 7	9.05 (d, 7.5)	146.1, d	3, 4, 7
3		140.5, s	4, 5	8.11 (t, 7.5)	129.5, d	2, 4
4	8.94 (d, 7.2)	146.2, d	2, 6	8.61 (t, 7.5)	148.3, d	2, 3
5	8.13 (t,7.2)	130.5, d	4, 6	8.11 (t, 7.5)	129.5, d	3, 4, 7
6	8.97 (d, 7.2)	144.4, d	4, 5, 7	9.05 (d, 7.5)	146.1, d	2, 4
7	4.78 (t, 5.2)	62.4, t	2, 6, 8	5.05 (t, 6.2)	60.2, t	2, 8
8	4.05 (t, 5.2)	64.2, t	7	3.48 (t, 6.2)	53.1, t	7
9		166.9, s	2, 4			

assignment of all the resonances in the ^{1}H NMR spectrum and to deduce structure **1**, which may be considered a zooanemonin[9] (**4**) substituted at position 2 by an amino group. It should be noted that a 2-amino-5-alkylimidazole moiety is part of the structure of almost all of the bromopyrrole alkaloids isolated from *Agelas* sponges. As far as the biogenetic origin of these structures, two alternative precursors, namely histidine or ornithine, have been hypothesized[11,12] depending on the length of the alkyl chain (two or three carbon atoms, respectively). Following this approach, we can assume that aminozooanemonin derives from histidine.

Compound **2**, named pyridinebetaine A, was shown to have the molecular formula $C_8H_9NO_3$ by accurate FABMS (positive ion). When its ^{1}H and ^{13}C NMR spectra (Table 1) were compared with those of trigonelline and homarine, the structure of a β-substituted pyridinium nucleus with a $-CH_2CH_2-$ moiety linked to the nitrogen atom appeared evident for compound **2**. Strong evidence came from the NOE between the triplet assigned to H_2-7 (δ 4.78, with scalar coupling with a triplet at δ 4.05) and the pyridine protons at δ 8.97 (H-6) and 9.29 (H-2). The presence of a carboxylate and of a hydroxyl group in the structure of **2** was inferred by IR (KBr) absorption bands at ν_{max} 1645 and 3300 cm^{-1}, respectively. This agrees with molecular formula deduced by mass spectral data. The chemical shift of H_2-8 at δ 4.05 in the ^{1}H NMR spectrum and of C-8 at δ 64.2 in the ^{13}C NMR spectrum and the HMBC correlation peaks of the carboxylate carbon (C-9) with H-2 and H-4 allowed the definition of the structure of **2**, indicating that the hydroxyl group must be linked to C-8, and the carboxylate function at C-3.

Pyridinebetaine A (**2**) may be regarded as the alcoholic portion of agelongine (**6**),[13] a specific inhibitor of serotonergic receptors isolated by our research group from the sponge *Agelas longissima*. In the structure of agelongine, the pyridinebetaine A moiety is linked to 4-bromopyrrole-2-carboxylic acid through an ester bond.

A third alkaloid, called pyridinebetaine B (**3**), was isolated, and its ^{1}H NMR spectrum (CD_3OD) (Table 1) appeared similar to that of **2** and typical of a pyridinium derivative. This was confirmed by ^{13}C NMR signals (which were associated with the proton resonances using an HMQC experiment) and by the UV spectrum, which exhibited an absorption at λ_{max} 260 (ϵ 4500) nm. The molecular formula, identified as $C_7H_9NO_3S$ by HRFABMS (positive ion) measurement, and the IR spectrum (KBr), which showed two absorptions at ν_{max} 1360 and 1175 cm^{-1}, suggested the presence of an alkylsulfonate moiety (taurine-like) as the portion linked to the pyridinium nitrogen. Two mutually coupled triplets at δ 5.05 and 3.48 in the ^{1}H NMR spectrum suggested that, as in compound **2**, the alkyl part of **3** is actually a dimethylene. In particular, the methylene resonating at δ 5.05 must be that linked to the nitrogen atom, as demonstrated by its strong NOE effect with the doublet at δ 9.05 (α protons of pyridine). As a consequence, the second triplet must link the sulfonate function, and this conclusion is further supported by the chemical shift of C-8 at δ 53.1 in the ^{13}C NMR spectrum.

Several pyridinium derivatives, often substituted at position 3 by a fatty acid chain, such as in the niphatynes,[14] nyphatoxins,[15] and cyclostellettamines,[16] have been reported from marine organisms. Moreover, the taurine residue has been found as a portion of some bromopyrrole alkaloids in the oroidin family, for example, mauritiamide A[17] and tauroacidins.[18] These latter molecules may be regarded as dispacamides C and D,[6] which link a taurine moiety.

Recently, Konig et al. reported[19] the isolation of trigonelline and norzooanemonin from *Astrosclera willeyana*, a coralline demospongia of the family Agelasidae, which belong to the same family of the metabolites presented here. These compounds, collectively called betaines and widely represented in terrestrial plants as well as in marine invertebrates, are amphoteric low-molecular-weight quaternary ammonium molecules deriving from protein or nonprotein amino acids. These include, for example, homarine,[7] trigonelline,[8] taurines, baikiain betaine,[20] and β-stachydrine,[21] and their function in the living organisms has been long debated among marine biologists. Various authors have proposed[22] that these zwitterionic compounds may play an important role in cellular osmotic activities of marine invertebrates and that their widespread occurrence could be ascribed to primary and not to secondary metabolism.

The betaine alkaloids found in *A. dispar* were screened for antibiotic activity against Gram-positive (*Bacillus subtilis* ATCC #6633 and *Staphilococcus aureus* ATCC #6538) and Gram-negative (*Salmonella typhi* ATCC #19430) bacteria. Antibacterial activities against Gram-positive bacteria were exhibited by aminozooanemonin (**1**) and pyridinebetaine A (**2**), with MICs ranging from 2.5 to 8 μg/mL.

Experimental Section

General Experimental Procedures. IR (KBr) spectra were recorded on a Bruker model IFS-48 spectrophotometer; UV spectra were obtained in MeOH using a Beckman DU70 spectrophotometer. LRFABMS and HRFABMS (CsI ion) were performed in a glycerol matrix on a Prospec (FISONS) mass spectrometer. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on a Bruker AMX-500 spectrometer; chemical shifts are referenced to the residual solvent signal (CD_3OD: $\delta_H = 3.34$, $\delta_C = 49.0$; DMSO-d_6: $\delta_H = 2.50$). The multiplicities of ^{13}C resonances were determined by DEPT experiments. One-bond heteronuclear connectivities were determined with the Bax−Subramanian HMQC pulse sequence[23] using a BIRD pulse of 0.60 s before each scan in order to suppress the signals originating from protons not directly bound to ^{13}C (interpulse delay set for $^1J_{CH} = 140$ Hz). During the acquisition time, ^{13}C broad-band decoupling was per-

formed using the GARP sequence. Two- and three-bond $^1H-^{13}C$ connectivities were determined by HMBC experiments optimized for a $^{2,3}J$ of 12 Hz. HPLC separations were achieved on a Waters 501 apparatus equipped with an UV detector (λ 240 nm) and with Hibar LiChrospher (250 × 4 mm) columns.

Animal Material, Extraction, and Isolation of Compounds 1−5. The sponge *Agelas dispar* was collected in summer 1992, in the lagoon of Little San Salvador Island, Bahamas, and identified by Prof. M. Pansini (Università di Genova). A voucher specimen has been deposited at the Istituto di Zoologia, Università di Genova, Italy. The sponge (100 g of dry wt after extraction) was extracted four times with MeOH (500 mL) at room temperature, and the resulting oily aqueous residue was partitioned against Et_2O and successively against *n*-BuOH. The organic phases were combined and concentrated *in vacuo* to give a brown viscous oil (8.8 g) that was subjected to chromatography over a column packed with RP18 Si gel (stepwise gradient elution from H_2O to MeOH). Fractions eluted with H_2O-MeOH (8:2), rechromatographed by HPLC (eluent H_2O-MeOH 95:5 with 0.5% TFA, RP18 column, flow 0.7 mL/min) furnished successively aminozooanemonin (**1**) (8.5 mg) and zooanemonin (**4**) (15 mg), and also contained homarine, trigonelline, and taurines. Fractions eluted with H_2O-MeOH 7:3 and subjected to repeated HPLC separations (eluent H_2O-MeOH, 6:4 with 0.5% TFA, RP18 column, flow 0.8 mL/min) afforded pyridinebetaine A (**2**) (1.2 mg) and pyridinebetaine B (**3**) (2.5 mg); while fractions eluted with H_2O-MeOH 3:7 gave pure agelasidine C (**5**) (25 mg).

Aminozooanemonin (1): obtained as a colorless amorphous solid, 8.5 mg; IR (KBr) ν_{max}, 3460−3000, 1780, 1640 cm^{-1}, which shifted to 1710 cm^{-1} after addition of HCl; UV (MeOH) λ_{max} 226 (ϵ 3500), 262 (ϵ 1500) nm; ^1H NMR (500 MHz, DMSO-d_6) δ 8.30 (2H, s, NH_2-10), 7.63 (1H, s, H-5), 3.97 (3H, s, H_3-9), 3.77 (3H, s, H_3-8), 3.15 (2H, s, H_2-6); ^1H NMR (CD_3OD) δ 7.75 (1H, s, H-5), 4.11 (3H, s, H_3-9), 3.92 (3H, s, H_3-8), 3.21 (2H, s, H_2-6). ^{13}C NMR (CD_3OD) δ 163.1 (C-7, s), 154.2 (C-2, s), 134.2 (C-4, s), 127.0 (C-5, d), 48.3 (C-6, t), 33.8 (C-8, q), 33.6 (C-9, q); FABMS (positive ion, glycerol matrix) *m/z* 170 $[M + H]^+$; HRFABMS *m/z* 170.0886, calcd for $C_7H_{12}N_3O_2$, 170.0852.

Pyridinebetaine A (2): obtained as a colorless amorphous solid, 1.2 mg; IR (KBr) ν_{max} 3300, 1645 cm^{-1}; UV (MeOH) λ_{max} 220 (ϵ 3000), 266 (ϵ 2500) nm; ^1H and ^{13}C NMR (CD_3OD), see Table 1; FABMS (positive ion, glycerol matrix) *m/z* 168 $[M + H]^+$; HRFABMS *m/z* 168.0547, calcd for $C_8H_{10}NO_3$, 168.0583.

Pyridinebetaine B (3): obtained as a colorless amorphous solid, 2.5 mg; IR (KBr) ν_{max} 1360, 1175 cm^{-1}; UV (MeOH) λ_{max} 222 (ϵ 6500), 260 (ϵ 4500) nm; ^1H and ^{13}C NMR (CD_3OD), see Table 1; FABMS (positive ion, glycerol matrix) *m/z* 188, 190 (95:5) $[M + H]^+$; HRFABMS *m/z* 188.0999, calcd for $C_7H_{10}NO_3S$, 188.0693.

Antibacterial Assays. Gram-positive (*B. subtilis* ATCC #6633 and *S. aureus* ATCC#6538) and Gram-negative (*S. typhi* ATCC #19430) bacteria were used for the antimicrobial assays of the isolated betaines using the Mueller−Hinton agar test. Aminozooanemonin (**1**) and pyridinebetaine A (**2**) proved active against Gram-positive bacteria, with MIC values of 2.5 μg/mL (*B. subtilis*) and 8.5 μg/mL (*S. aureus*) for **1** and 3.5 μg/mL (*B. subtilis*) and 5.0 μg/mL (*S. aureus*) for **2**. All compounds were dissolved in DMSO. Zones of inhibition were recorded after 16 h. Results presented are the average of three experiments.

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