Polyaxibetaine, an Amino Acid Derivative from the Marine Sponge *Axinella polypoides*

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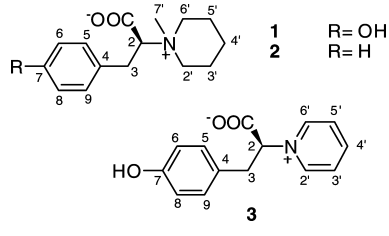
A new pyridinium derivative, polyaxibetaine (**3**), has been isolated from the marine sponge *Axinella polypoides*, together with two known modified amino acids, **1** and **2**. The planar structure of compound **3** has been elucidated by spectroscopic methods; definition of the absolute configuration of compounds **1**−**3** has been carried out through ECD studies.

Betaines and similar zwitterionic compounds are widely distributed among terrestrial plants and algae, as well as among marine invertebrates. In biological systems, these highly soluble dipolar molecules serve as organic osmolytes (osmoprotectants), substances synthesized by the organism or taken up from the environment by cells for protection against osmotic stress. Osmoprotectants can stabilize proteins and membranes when salt levels or temperatures are unfavorable; therefore, they could play important roles in the adaption of cells of marine invertebrates to various adverse environmental conditions.[1,2] Examples of betaines isolated from marine invertebrates are homarine,[3] trigonelline,[4] taurines, baikiain betaine,[5] β-stachydrine,[6] norzooanemonin,[7] aminozooanemonin,[8] and pyridine-betaines A and B.[8] Most of these compounds are amphoteric, low-molecular weight quaternary ammonium compounds derived from proteinogenic or nonproteinogenic amino acids in which the nitrogen atom is fully alkylated. During our ongoing search for new bioactive alkaloids from *Axinella* sponges, we have examined specimens of *Axinella polypoides* collected along the Sardinian coast. A recent chemical investigation performed on specimens of this sponge collected while diving off the coast of Marseille afforded two new piperidinium compounds, axiphenylalaninium (**1**) and axityrosinium (**2**), together with four known metabolites.[9] The *R* absolute configuration was suggested for compound **1**, even though some uncertainty still remained, while the configuration of **2** remained undetermined.

Our investigation resulted in the isolation of a new betaine, named polyaxibetaine (**3**), whose isolation and configurational elucidation are described here. A configurational analysis, based on ECD studies combined with quantum chemical ECD calculation, was also performed on compounds **1** and **3**, which allowed us to propose the 2*S* configuration for both compounds.

Polyaxibetaine (**3**) extends the structural variety of the known betaines; it is indeed a unique pyridinium derivative that shares the nitrogen atom with the α-amino group of a tyrosine residue.



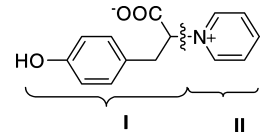


Figure 1. Subunits **I** and **II** in polyaxibetaine (**3**).

Results and Discussion

Several specimens of *A. polypoides* (Schmidt, 1862) collected from the bay of Calvi (Corsica) were homogenized and exhaustively extracted with MeOH and $CHCl_3$ successively. Combined extracts were concentrated, and the resulting aqueous residue was partitioned between *n*-butanol and H_2O. MLPC over a C-18 column of the *n*-butanol-soluble material, followed by repeated reversed-phase HPLC separation and purification steps, gave pure compounds **1**−**3**.

Compounds **1** and **2** were readily identified as axiphenylalaninium and axityrosinium, respectively, by comparison of their spectroscopic data with reported data.[9]

The ESI mass spectrum of polyaxibetaine (**3**) displayed ion peaks at *m/z* 244, 266, and 282, corresponding to $[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$, respectively. The molecular formula of **3** was established as $C_{14}H_{13}NO_3$, on the basis of its HRFAB mass data (positive ions) measured by the peak at *m/z* 244.0973 $[M + H]^+$ (calculated value: *m/z* 244.0968), and implied nine degrees of unsaturation. The ^{13}C NMR spectrum (methanol-d_4) contained 14 signals, which, on the basis of the data obtained from HSQC experiments, were sorted as one methylene, one sp^3, and nine sp^2 methines, as well as three quaternary carbons, including one carboxylic carbon. The presence of a 3-(4-hydroxyphenyl) propanoate residue (subunit **I**) and a pyridine ring (subunit **II**) (Figure 1) in structure **3** was determined on the basis of the following data.

The ^1H NMR spectrum of **3** (methanol-d_4), interpreted on the basis of 2D NMR experiments, contained two *ortho*-coupled aromatic signals, each integrating for two protons at δ 6.58 (d, J = 8.5 Hz, H-6 and H-8) and 6.88 (d, J = 8.5 Hz, H-5 and H-9), and the relevant carbons resonated at δ 116.2 and 130.4, respectively, as indicated by the HSQC spectrum. HMBC correlations of these proton signals with the unprotonated carbons at δ 127.4 and 157.7 (see Table 1) revealed a 1,4-disubstituted benzene ring. The chemical shift of the latter carbon suggested the presence of an oxygenated function on the benzene ring; this function was identified as an OH group by the ^1H NMR spectrum recorded in DMSO-d_6, which contained a further proton signal at δ 9.28 exchangeable in D_2O. Another feature of the ^1H NMR spectrum of polyaxibetaine (**3**) was two signals at δ 3.77 and 3.34 due to a methylene AB system (δ_C 39.6, C-3). They were coupled to a deshielded methine resonance at δ 5.37 (δ_C = 79.0, C-2), which, in turn, was correlated in the HMBC spectrum to the carbonyl carbon at δ 171.0 (C-1) and to the quaternary carbon at δ 127.4

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Table 1. NMR Data (Methanol-d_4) of Polyaxibetaine (**3**)

pos.	δ_H mult (*J* in Hz)	δ_C	HMBC
1		171.0	
2	5.37, dd (4.3,11.6)	79.0	1, 3, 4, 2′
3a	3.77, dd (4.3, 15.2)	39.6	2, 4, 5, 9
3b	3.34, dd (11.6, 15.2)		2, 4, 5, 9
4		127.4	
5	6.88, d (8.50)	130.4	3, 7, 9
6	6.58, d (8.50)	116.4	4, 7, 8
7		157.7	
8	6.58, d (8.50)	116.4	4, 6, 7
9	6.88, d (8.50)	130.4	3, 5, 7
2′	8.82, dd (1.2, 6.6)	146.1	2, 3′, 4′
3′	7.94, dd (6.6, 7.8)	128.1	2′, 4′, 5′
4′	8.45, dt (1.2, 7.8)	146.2	6′
5′	7.94, dd (6.6,7.8)	128.1	3′, 4′,6′
6′	8.82, dd (1.2,6.6)	146.1	2, 2′, 5′, 4′

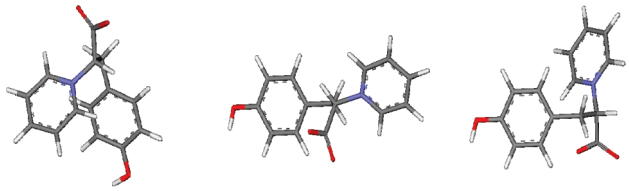

Figure 2. The three conformational families of *S*-**3**.

(C-4). A 3-(4-hydroxyphenyl)propanoate moiety (subunit **I**) was thus identified in **3**; it accounted for five of the nine unsaturation degrees and implied the molecule must contain a further aromatic ring. The ^{1}H NMR spectrum of **3** contained a further set of aromatic signals [δ 8.82 (dd, J = 6.6, 1.2 Hz, 2H), 8.45 (dt, J = 7.8, 1.2 Hz, 1H), 7.94 (dd, J = 7.8, 6.6 Hz, 2H)], which were arranged, through analysis of COSY connectivities, in a single spin system. Given the number and the chemical shifts of the remaining aromatic carbons [δ 146.1 (2C); 128.1 (2C); 146.2 (1C)] and according to MS, a pyridinium ring (subunit **II**) was thus revealed in **3**. On the basis of the diagnostic HMBC correlation between the methine proton at δ 5.37 (H-2) and the aromatic carbon resonance at δ 146.1 (2C, C-2′ and C-6′), C-2 was assumed to be attached to the nitrogen atom of the pyridinium ring. The structure of polyaxibetaine (**3**) was thus assigned as 3-(4-hydroxyphenyl)-2-(pyridinium-1-yl)propanoate.

A configurational analysis was carried out on polyaxibetaine (**3**) as well as on compounds **1** and **2**. It was based on electronic circular dichroism (ECD) measurements in combination with quantum chemical ECD *ab initio* calculations, a fast and convenient approach increasingly applied for the determination of the absolute configuration of natural compounds.[10−13] Particularly, the configuration at C-2 of each compound has been deduced by comparison of its experimental ECD spectrum with that predicted from TDDFT calculation for one of the two enantiomers. An initial conformational analysis of the *S* stereoisomer of each compound was performed using the simulated annealing procedure (INSIGHT II software package).[14] The resulting conformers were ranked on the basis of their conformational energy values and grouped into families; four minima for *S*-**1** and *S*-**2** and three minima for *S*-**3** were obtained. Conformers were optimized with the software package Gaussian 03[15] by using DFT at the RB3LYP/6-31G(d) level. The three conformational families of *S*-**3** are shown in Figure 2, the resulting relative energies (ΔE) and free energies (ΔG) are listed in Table 2, and Table 3 reports the optimized key dihedral angle for all *S*-**3** conformers. The conformational families of *S*-**1** and *S*-**2**, as well as their important dihedral angles and conformational analysis, are provided as Supporting Information.

For all conformers of *S*-**1**, *S*-**2**, and *S*-**3**, the excitation energies, as well as the oscillator and rotatory strengths of the electronic

Table 2. Conformational Analysis of *S*-**3** in the Gas Phase

	ΔE^a	ΔG^b	P%^{c}
S-**3a**	0.00	0.00	59.75
S-**3b**	1.11	0.87	24.94
S-**3c**	1.85	1.36	15.31

[a] Relative energy (kcal/mol). [b] Relative Gibbs free energy (kcal/mol). [c] Conformational distribution calculated at the B3LYP/6-31G(d) level in the gas phase.

Table 3. Key Dihedral Angles in Conformers of *S*-**3** (deg)

	S-**3a**	*S*-**3b**	*S*-**3c**
C3−C2−N−C2′	−77.50	−28.80	−18.30

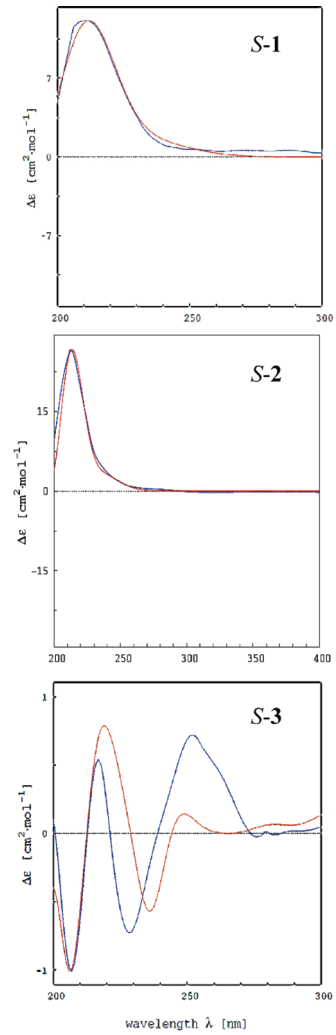

Figure 3. Theoretical CD curves (red) of *S*-**1**−**3** models vs experimental curves (blue) of compounds **1**−**3**.

excitation, were calculated, using the TDDFT methodology at the RB3LYP/6-31G(d,p) level; their ECD spectra were then simulated by the overlapping Gaussian function.[16] To obtain the final ECD spectrum of each compound, the simulated spectra of the lowest energy conformations were averaged, by following the Boltzmann statistic, and were UV corrected. The obtained theoretical curves were then compared to the experimental spectra of compounds **1**−**3**, recorded in MeOH. For all three metabolites, the calculated spectrum closely mimics the experimental spectrum (Figure 3). Therefore, these results allowed the assignment of the 2*S* absolute configuration of **1**−**3**. Our studies suggest that compound **1** possesses an absolute configuration opposite of that previously proposed by Gabant et al. by comparison of its CD spectrum with that of (*S*)-phenylalanine betaine in acidic solution.[9]

Experimental Section

General Experimental Procedures. ESI mass spectra were recorded on a hybrid quadrupole-TOF mass spectrometer in MeOH. The spectra were recorded by infusion into the ESI source using MeOH as the solvent. HRESIMS (positive mode) were performed on a Thermo LTQ Orbitrap XL mass spectrometer. Optical rotations were measured with a Perkin-Elmer 192 polarimeter at 589 nm using a 10 cm microcell. ECD spectra were recorded on an J-710 spectropolarimeter (Jasco, Tokyo, Japan) equipped with J-710 for Windows software (Jasco). ^{1}H (700 and 500 MHz) and ^{13}C (175 and 125 MHz) NMR spectra were recorded on a Varian INOVA spectrometer; chemical shifts were referenced to the residual solvent signal (methanol-d_4: δ_H 3.31, δ_C 49.0). Homonuclear ^{1}H connectivities were determined by COSY and TOCSY (mixing time 100 ms) experiments. Through-space ^{1}H connectivities were evidenced using a ROESY experiment with a mixing time of 500 ms. Two- and three-bond ^{1}H−^{13}C connectivities were determined by gradient 2D HMBC experiments optimized for a $J_{2,3}$ of 8 Hz.

All spectra were acquired at 278 K, and samples were prepared by dissolving compounds **1**−**3** in 0.5 mL of methanol-d_4 (Armar, 100% D).

Collection, Extraction, and Isolation. Specimens of *A. polypoides* were collected in the Bay of Calvi (Corsica, France), frozen immediately, and kept frozen until extraction. A reference specimen was deposited at the Dipartimento di Chimica delle Sostanze Naturali, University of Naples "Federico II". Fresh, thawed animals (74.6 g dry weight after extraction) were homogenized and extracted twice with MeOH and then twice with $CHCl_3$ (4 × 500 mL). The combined extracts were concentrated, and the resulting aqueous residue was partitioned between H_2O and *n*-BuOH. The organic phase was subjected to chromatography over a column packed with reversed-phase silica gel (RP18-MPLC) using a gradient elution (water → MeOH → $CHCl_3$). Fractions eluted with H_2O/MeOH (7:3, v/v) were combined, and the solvent was evaporated, yielding a residue (290 mg). It was subjected to chromatography by HPLC on a Synergy Polar-RP 4 μm column (250 × 4.60 mm) eluting with H_2O/MeOH (98:2, v/v), to give axityrosinium (**1**, 26.0 mg, 8.9% of dry weight), axiphenylalaninium (**2**, 10.0 mg, 3.4% of dry weight), and polyaxibetaine (**3**, 2.0 mg, 0.68% of dry weight).

Axityrosinium (1): $[\alpha]_D$ +21.0 (MeOH, *c* 0.003); ESIMS (positive ion mode) *m/z* = 264.1 $[M + H]^+$, 286.0 $[M + Na]^+$, 302.0 $[M + K]^+$; ^{1}H and ^{13}C NMR data (methanol-d_4) are identical to reported data.[9]

Axiphenylalaninium (2): $[\alpha]_D$ +22.4 (MeOH, *c* 0.0025); ESIMS (positive ion mode) *m/z* = 248.2 $[M + H]^+$, 270.1 $[M + Na]^+$, 286.1 $[M + K]^+$; ^{1}H and ^{13}C NMR data (methanol-d_4) are identical to reported data.[9]

Polyaxibetaine (3): $[\alpha]_D$ +15.6 (MeOH, *c* 0.002); ESIMS (positive ion mode) *m/z* = 244.1 $[M + H]^+$, 266.1 $[M + Na]^+$, 282.0 $[M + K]^+$; positive HR-ESIMS *m/z* 244.0974 $[M + H]^+$ (calc for $C_{14}H_{14}NO_3$ 244.0968); ^{1}H and ^{13}C NMR data (methanol-d_4), Table 1; ^{1}H NMR (DMSO-d_6) 3.25 (br s, 1H *J* = 15.1 Hz, H-3b), 3.57 (br s, 1H *J* = 15.1 Hz, H-3a), 6.51 (d, 2H *J* = 8.38 Hz, H-6 and H-8), 6.83 (d, 2H *J* = 8.38 Hz, H-5 and H-9), 7.94 (br s, 2H *J* = 6.9 Hz, H-3′ and H-5′), 8.88 (br s, 2H *J* = 5.9 Hz, H-2′ and H-6′), 9.28 (s, 1H, OH).

Computational Details. A preliminary conformational search on each couple of enantiomers was performed by simulated annealing in the INSIGHT II package. The MeOH solution phases were mimicked through the value of the corresponding dielectric constant. Using the steepest descent followed by quasi-Newton−Raphson method (VA09A) the conformational energy was minimized. Restrained simulations were carried out for 500 ps using the CVFF force field as implemented in Discover software (Accelrys, San Diego, CA). The simulation started at 1000 K, and then the temperature was decreased stepwise to 300 K. The final step was again the energy minimization, performed in order to refine the structures obtained, using the steepest descent and the quasi-Newton−Raphson (VA09A) algorithms successively. Both dynamic and mechanic calculations were carried out by using 1 (kcal/mol)/Å^2 flat well distance restraints. One hundred structures were generated. To simulate the solvent chosen for NMR analysis, a distance-dependent dielectric constant set to the value of MeOH (ε 32.63) was used during the calculations. All optimizations were performed with the software package Gaussian 03, by using the DFT functional RB3LYP and the basis set 6-31G(d). The B3LYP/6-31G(d) harmonic vibrational frequencies were further calculated to confirm their stability. Rotatory strength values for the electronic transitions from the ground state to the singly excited states for all conformers of *S*-**1**−**3** were obtained by TDDFT calculations at RB3LYP/6-31G(d,p) with Gaussian 03. The rotatory strength values were summed after a Boltzmann statistical weighting, and $\Delta\varepsilon$ values were calculated by forming sums of Gaussian functions centered at the wavelengths of the respective electronic transitions and multiplied by the corresponding rotatory strengths. The ECD spectra that were obtained were UV-corrected and compared with the experimental ones.

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Supporting Information Available: ESI, CD, and 1D and 2D NMR spectra of compounds **1**−**3**. Conformational families of *R*-**1**, *S*-**1**, *R*-**2**, and *S*-**2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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