

AMPHITOXIN, A NEW HIGH MOLECULAR WEIGHT ANTIFEEDANT PYRIDINIUM SALT FROM THE CARIBBEAN SPONGE *AMPHIMEDON COMPRESSA*

STEFANIA ALBRIZIO, PATRIZIA CIMINIELLO, ERNESTO FATTORUSSO,* SILVANA MAGNO,

*Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli,
"Federico II," Via D. Montesano 49, 80131 Napoli, Italy*

and JOSEPH R. PAWLIK

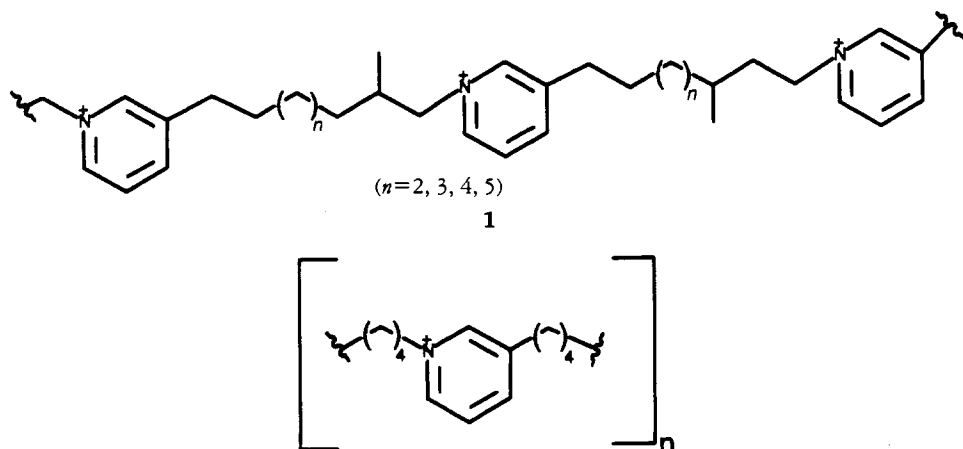
*Department of Biological Sciences, University of North Carolina at Wilmington,
Wilmington, North Carolina 28403-3297*

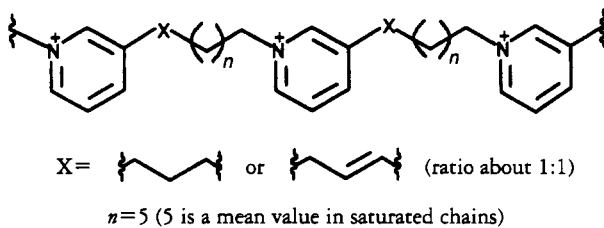
ABSTRACT.—A new polymeric pyridinium alkaloid named amphitoxin [**2**] has been isolated from *Amphimedon compressa*, and its structure determined by spectroscopic analysis. In laboratory feeding experiments, crude extracts and purified amphitoxin [**2**] from *A. compressa* at lower than natural concentration levels effectively deterred feeding of a generalist predatory Caribbean reef fish, *Thalassoma bifasciatum*.

Alkaloids containing long-chain hydrocarbons functionalized by substituted 3-pyridine or 1,3-pyridinium rings have been isolated from several marine sponges belonging to the order Haplosclerida (1). These compounds, which generally show interesting bioactivities, include halitoxin [**1**], a mixture of high-molecular-weight pyridinium salts isolated from the sponge *Haliclona rubens*, *H. viridis*, and *H. erina* (2). The chemical and spectral data of **1** indicated a complex mixture of polymeric molecules containing pyridinium rings with alkyl chains of different lengths and branched structures. Halitoxin was found to be cytotoxic, haemolytic, and toxic to fish and mice.

In examining Porifera collected along the coast of the Bahamas, we recently discovered strong antifeedant activity in a crude extract of *Amphimedon compressa*. This sponge belonging to the family Haliclonidae, whose color varied from brown with green shades to red or purple-brown, is considered to be synonymous with *H. rubens* (3).

In this paper we report the purification and the identification of the active compound, amphitoxin [**2**], which was present in relatively high quantities in an extract of *A. compressa* collected in the summer of 1990 near the coast of San Salvador Island (depth 8 m). It is a polymeric compound containing 3-alkyl- and 3-alkenyl pyridinium units. The structure of **2** resembles that of **1**, from which it differs in having a carbon-





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carbon double bond and a more ordered backbone structure without variations in the length and without branching of the alkyl chains linking the pyridinium rings. It is also similar structurally to a polymeric pyridinium alkaloid [3], recently isolated from a sponge belonging to the genus *Callyspongia* (*C. fibrosa*), which, however, lacks the carbon-carbon double bond in the side-chain, and whose mol wt has not been established exactly (4).

The structural differences between the two compounds was evident when the ^1H -nmr spectra of **2** and **1** (2) were compared. Noting these spectral features, we performed a careful nmr analysis of the chromatographic fractions of the crude extract of the sponge. This analysis excluded the possibility that our *A. compressa* specimens elaborated **1**, which was found as a major product of a sample collected near Maguayez Island (Puerto Rico), at 15 ft or less depth (2).

A second experiment carried out on a further specimen of *A. compressa* collected along the coast of Grand Bahama Island (depth 10 m) in the summer of 1992, showed that in this case the crude extract also contained remarkable quantities of **2** while it lacked **1**.

In the light of the foregoing, *A. compressa* appears to be able to accumulate two different, but structurally related compounds, both possessing very interesting bioactivities which could be ecologically relevant for the animal. At present it is not possible to establish the conditions which affect the secondary metabolism of the sponges, inducing such significant variations in their chemical constituents.

RESULTS AND DISCUSSION

Amphitoxin [**2**] was isolated from frozen specimens of *A. compressa* by extraction with MeOH-toluene (3:1). The aqueous suspension, obtained after removal of the organic solvents, was extracted successively with EtOAc and *n*-BuOH. The *n*-BuOH-soluble material, which showed strong antifeedant activity, was chromatographed on a Si gel column eluting with CHCl_3 -MeOH- H_2O mixtures (70:30:5, 65:35:8, 60:40:10), 100% MeOH, and 5% AcOH in MeOH. This led to the isolation of crude **2** as a complex mixture which gave a cluster of peaks by reversed-phase hplc using a gradient of eluents from 100% H_2O to 100% MeOH. Because of the simple features of its ^1H - and ^{13}C -nmr spectra, this result suggested a polymeric structure for **2** which was fractionated by membrane ultrafiltration of an aqueous solution to give mol wt range fractions of 1,000–3,000 (fraction A, 38%) and 3,000–10,000 (fraction B, 62%).

Elemental analysis of the two fractions gave very similar ratios of C, H, N, and Cl, consistent with an empirical formula of $\text{C}_{14}\text{H}_{21}\text{NCl}\cdot 2\text{H}_2\text{O}$. Uv, ir, and nmr features of the crude amphitoxin and of the two samples with different mol wt ranges were practically identical. Therefore, the characterization was performed on the polymer **2** before ultrafiltration.

All the spectral data characterized amphitoxin as a 1,3-dialkylpyridinium salt, possessing a C-C double bond as an additional functionality. Compound **2** exhibited a uv spectrum characteristic of an alkylpyridinium salt (λ max 266 nm),

TABLE 1. ^1H - and ^{13}C -Nmr Data of the Two Subunits **2a** and **2b**.

Position	Structure 2a		Structure 2b	
	δ_{H} (mult., J in Hz)	δ_{C}	δ_{H} (mult., J in Hz)	δ_{C}
1				
2	8.97 (s)	145.20	9.02 (s)	145.28
3		145.76		144.50
4	8.45 (d, 8.0)	146.49	8.50 (d, 8.0)	146.60
5	8.06 (t, 8.0)	128.98	8.07 (t, 8.0)	129.08
6	8.91 (d, 8.0)	143.35	8.93 (d, 8.0)	143.54
1'	4.67 (t, 7.5)	62.98	4.67 (t, 7.5)	62.98
2'	2.06 (m)	32.59	2.06 (m)	32.59
1''	2.92 (t, 7.5)	33.53	3.72 (d, 7.5)	31.10
2''	1.76 (qui, 7.5)	31.59	5.63 ^a	125.45
3''			5.74 ^a	135.30
4''			2.24 (q, 7.5)	28.36

^aFurther coupled AB system: $J_{\text{AB}}=17.5$; $J_{\text{AX}}=J_{\text{BX}}=7.5$.

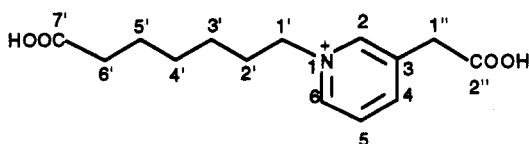
while its ir spectrum showed an absorption at 1640 cm^{-1} , typical of such a salt (5).

The ^1H -nmr and COSY spectra of **2** gave convincing evidence for the presence of the two different pyridinium moieties, **2a** and **2b**, depicted in Table 1. Our nmr assignments were based on ^1H - ^1H 2D COSY, ^1H -homo-decoupling experiments, XHCORR, and COLOC experiments. The XHCORR experiment enabled the assignment of all the proton-bearing carbons in the ^{13}C -nmr spectrum. A COLOC nmr experiment permitted the assignment of the unprotonated carbons at position 3 of the pyridinium rings. The *E* configuration of the double bond of **2b** was deduced by the large coupling constant ($J=17.5\text{ Hz}$) between H-2'' and H-3''. The remaining signal present in the ^1H -nmr spectrum was a broad band at $\delta\ 1.40$ consistent with the presence in **2** of unbranched methylene chains. These methylene protons were correlated to carbons in the ^{13}C -nmr spectrum, which resonated between $\delta\ 27.31$ and $\delta\ 30.53$.

The above data indicated that **2** is a polymeric mixture containing molecules of different sizes, composed of two very similar monomer units, which differ only in the presence of a C-C double bond. The integration of peak areas in the ^1H -nmr spectrum suggested two different subunits, in a ratio of 1:1. The two subunits contained 8 methylene groups in addition to the moieties of **2a** and **2b**.

In order to further examine its structure, amphitoxin was subjected to ozonolysis to produce information which could confirm the presence of **2a** and **2b**. Treatment of 100 mg of amphitoxin with O_3 at -50° , in MeOH solution, followed by oxidation with H_2O_2 , afforded a degradation mixture (54 mg), which was subjected to membrane ultrafiltration to give fractions in four mol wt ranges: fraction 1, <500 , 17.0 mg; fraction 2, 500–1,000, 17.7 mg; fraction 3, 1,000–3,000, 10.7 mg; and fraction 4, 3,000–10,000, 8.6 mg. Fraction 2, which by preliminary spectral and chromatographic analyses was shown to be a very complex mixture, was not further investigated.

Additional purification of fraction 1 was effected by hplc, which gave 8 mg of **4**. The molecular formula of **4**, $\text{C}_{14}\text{H}_{20}\text{NO}_4$, was deduced from its hrms ($m/z\ 266.1382$, $[\text{M}]^+$, calcd 266.1387) and ^{13}C -nmr data. Its ^1H -nmr and uv spectra ($\lambda\ \text{max}\ 266\text{ nm}$) gave evidence for a 3-substituted pyridinium ring [$\delta\ 8.91$ (1H, s, H-2), 8.81 (1H, d, $J=7.0\text{ Hz}$, H-6), 8.46 (1H, d, $J=7.0\text{ Hz}$, H-4), 8.00 (1H, t, $J=7.0\text{ Hz}$, H-5)]. The non-aromatic signals in the ^1H -nmr spectrum were assigned to the methylene protons at position 3 of the pyridinium ring [$\delta\ 3.75$ (2H, s, H_2 -1'')] and to those of the alkyl chain on the quaternary nitrogen [$\delta\ 4.60$ (2H, t, $J=8.0\text{ Hz}$, H_2 -1'), 2.34 (2H, t, $J=7.5\text{ Hz}$,



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H-6'), 2.04 (2H, m, H₂-2'), 1.63 (2H, m, H₂-5'), 1.36 (2H, br, H-3'), 1.36 (2H, br, H-4')]. The ¹H-2D COSY spectrum further established the methylene multiplicities and the connectivities in the spin system C-1'-C-6'.

Uv and ¹H-nmr data of fractions 3 and 4 (see Experimental) were identical, suggesting that they have the same basic polymeric structure but differ only in the mol wt range. The ¹H-nmr spectra of both fractions suggested a simplified amphitoxin. Both spectra displayed signals pertaining to the protons of the partial structure **2a**, while those of **2b** were completely absent. Consequently, fractions 3 and 4 were concluded to be homopolymers of the **2a** type. They could be present in the original amphitoxin or they could be derived by oxidative degradation of the larger molecules containing blocks built of **2a** subunits spaced by **2b** subunits.

All of the above data suggested that amphitoxin is a polymer containing molecules of different sizes based on the two partial structures **2a** and **2b**, in a ratio of 1:1, which are randomly sequenced. Information useful to define the length of the methylene chains, linking **2a** and **2b**, was gained from compound **4**, obtained after ozonolysis of amphitoxin; it directly indicated the number of the methylene groups linked to **2b**. As for the methylene groups linked to **2a**, a mean value of about nine can be deduced from the total number of methylenes of the two units determined from elemental analysis and the integrated peak areas in the ¹H-nmr spectrum of **2**.

As shown in Table 2, **2** deters feeding of the fish *Thalassoma bifasciatum* at a concentration of 1 mg/ml, which is 1/6th its natural concentration; this strong bioactivity suggests a role in the chemical defense of *A. compressa*. The feeding experiments were performed onboard ship near the site of sponge collection employing a common predatory fish, the bluehead wrasse *Thalassoma bifasciatum*, from the same reefs where sponges were collected. The advantages of using this species for aquarium bioassays have been detailed previously (6).

TABLE 2. Palatability of Crude Extracts and Purified **2** from a Mixed-Colony Collection of *Amphimedon compressa* in Food Pellets Offered to the Reef Fish *Thalassoma bifasciatum* in Aquarium assays.^a

Sample	Conc. mg/ml	Pellets Eaten of 10
Crude extract	100	0
(natural concentration ≈ 37 mg/ml)	10	0
	1	3
Amphitoxin ^b	100	0
(natural concentration ≈ 6 mg/ml)	10	0
	1	2
Control	—	10

^aThe activity was measured on the crude extracts obtained from ten specimens of *A. compressa* collected in the same geographic area. The obtained results were very similar; in the table we report the data obtained on the extract utilized for the isolation of amphitoxin. The extract and purified compound significantly deterred feeding at all concentrations ($p < 0.005$, Fisher's exact test). Natural concentration refers to mg of crude extract or amphitoxin contained in 1 ml of fresh sponge tissue.

^bThe experiments were performed by using the polymer **2**.

It should be noted, however, that all the fractions from extracts of *A. compressa* were not assayed for antifeedant activity, so, while amphitoxin [2] is sufficient to provide an effective chemical defense, it cannot be concluded that it was the only defensive compound in the extract.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.— ^1H - and ^{13}C -nmr spectra were performed in CD_3OD on a Bruker AMX-500 spectrometer. Methyl, methylene, and methine carbons were distinguished by a DEPT experiment. Homonuclear ^1H connectivities were determined using a COSY experiment. One-bond heteronuclear ^1H - ^{13}C connectivities were determined with an XHCORR experiment, optimized for an average C-H coupling of 135 Hz. Two- and three-bond heteronuclear ^1H - ^{13}C connectivities were determined by a COLOC experiment, optimized for $^2J_{\text{CH}}$ of 8 Hz. Hreims data were recorded on a Kratos MS-80 mass spectrometer at 70 eV. Ft-ir spectra were recorded on a Bruker IFS-48 spectrophotometer using a KBr matrix. Uv spectra were performed on a Beckman DU-70 spectrometer in aqueous solution. Mplc was performed on a Büchi 861 apparatus using a SiO_2 (230–400 mesh) column.

ANIMAL MATERIAL.—Specimens of *A. compressa* were collected in the summer of 1990 along the coast of San Salvador Island, at a depth of 8 m. They were frozen immediately after collection and kept frozen (-18°) until extraction. Reference specimens are deposited at the Istituto di Zoologia, University of Genoa, Italy.

EXTRACTION AND ISOLATION.—The collected animals (59 g, dry wt after extraction) were homogenized and extracted with MeOH-toluene (3:1) (500 ml \times 5) at room temperature. The combined MeOH/toluene solutions, after filtration, were evaporated *in vacuo* to give an aqueous suspension which was extracted with *n*-BuOH. The *n*-BuOH-soluble material, after removal of the organic solvent, afforded 10 g of a residue which was chromatographed by mplc on a Si gel column (Merck, 110 g) by stepwise elution with CHCl_3 -MeOH- H_2O mixtures (70:30:5, 65:35:8, 60:40:10), MeOH and MeOH-HOAc (95:5). A comparison of the nmr spectrum of each mplc fraction with data previously reported (2) for halitoxin excluded the presence of this compound from the sponge extract. Amphitoxin [2] (2.5 g) was contained in the fraction eluted with CHCl_3 -MeOH- H_2O (60:40:10). Other samples of *A. compressa*, collected in the summer of 1992, along the coast of Grand Bahama Island (depth 10 m), were extracted and analyzed as described above. The results were identical to those previously obtained. Crude amphitoxin (30 mg) was dissolved in H_2O and passed through an anion-exchange column [Partisil 10 SAX chloride form (4 \times 250 mm)]. The column was eluted with H_2O and the aqueous solutions were lyophilized to yield amphitoxin [2] as a brown powder.

Amphitoxin [2].—Ir ν max (KBr) 1640 cm^{-1} ; uv λ max (H_2O) 266 nm; *anal.*, calcd for $\text{C}_{14}\text{H}_{21}\text{NCl}\cdot 2\text{H}_2\text{O}$: C, 61.2, H, 9.1, N, 5.1, Cl, 12.9; found: C, 60.6, H, 9.4, N, 5.2, Cl, 13.2. The nmr data of amphitoxin, which is formed by the two subunits **2a** and **2b** in about the same ratio, are reported in Table 1.

ULTRAFILTRATION OF AMPHITOXIN [2].—A 20-mg sample of **2** was dissolved in H_2O and filtered under pressure with N_2 through increasing nominal mol wt limit membranes (Diaflo YC05, YM1, YM3, and YM10 of Amicon, Inc., Beverly, MA). At each stage of the ultrafiltration, the material retained was diluted and the ultrafiltration was repeated two times using the same membrane to insure that all materials of mol wt less than the membrane's nominal mol wt cutoff range had passed into the filtrate. Two mol wt range fractions of 1,000–3,000 (fraction A, 7.6 mg) and 3,000–10,000 (fraction B, 12.4 mg) were obtained. Both fractions exhibited very similar spectral properties (nmr, uv, ir) and elemental analysis, practically identical to those reported for amphitoxin [2].

OZONOLYSIS OF AMPHITOXIN [2].—Amphitoxin (**2**, 100 mg), dissolved in 1.5 ml of MeOH, was ozonized at -50° for 15 min. Cold H_2O and, after removal of the solvents *in vacuo*, a few drops of 30% H_2O_2 , were added to solution. Evaporation of H_2O_2 yielded 60 mg of a residue that was subjected to ultrafiltration to give four mol wt range fractions: <500 (fraction 1, 17.0 mg), 500–1,000 (fraction 2, 17.7 mg), 1,000–3,000 (fraction 3, 10.7 mg), and 3,000–10,000 (fraction 4, 8.6 mg). Fraction 1 was further purified by reversed-phase hplc using a gradient of eluents from 100% H_2O to 100% MeOH to afford 8 mg of **4**.

Compound 4.—Uv (MeOH) λ max 266 nm; ^1H nmr (CD_3OD , 500 MHz) δ 8.91 (1H, s, H-2), 8.81 (1H, d, $J=7.0$ Hz, H-6), 8.46 (1H, d, $J=7.0$ Hz, H-4), 8.00 (1H, t, $J=7.0$ Hz, H-5), 4.60 (2H, t, $J=8.0$ Hz, H_2 -1'), 3.75 (2H, s, H_2 -1''), 2.34 (2H, t, $J=7.5$ Hz, H-6'), 2.04 (2H, m, H_2 -2'), 1.63 (2H, m, H_2 -5'), 1.36 (2H, br, H-3'), 1.36 (2H, br, H-4'); hrms m/z [M] $^+$ 266.1382 (calcd 266.1387 for $\text{C}_{14}\text{H}_{20}\text{NO}_2$).

Fractions 3 and 4.—Uv (MeOH) λ max 266 nm; ^1H nmr (CD_3OD , 500 MHz) δ 8.95 (1H, s), 8.46 (1H, d), 8.06 (1H, t), 8.90 (1H, d), 4.68 (2H, t), 2.90 (2H, t), 2.05 (2H, m), 1.76 (2H, qui an intense broad band at δ 1.42).

FEEDING ASSAYS.—Groups of three fish (one blue-green, two yellow phase) were held in each of 15 40-liter divisions of three large flow-through aquaria. Groups of fish were chosen at random for feeding assays, each group given either a treated or control food pellet, followed by other choice. When a treated pellet was rejected as a second offer, a third control pellet was given to determine whether the fish had ceased feeding. Groups of fish that did not eat control pellets were considered satiated and were not used in the experiment. Pellets were considered rejected if not eaten after a minimum of three attempts by one or more fish to take them into their mouth cavity or if the pellets were approached and ignored. The significance of differences in consumption of treated vs. control pellets was evaluated with Fisher's exact test (7). Food pellets were prepared as detailed previously (8), by mixing 0.15 g alginic acid with 0.25 g freeze-dried, powdered squid mantle, followed by addition of the volumetric equivalent of a fraction or compound in a carrier solvent (for treated pellets) or solvent alone (for control pellets). The mixture was brought up to a final volume of 5 ml with distilled H₂O, vigorously stirred to remove lumps, loaded into a syringe, and extruded into a 0.25 M solution of CaCl₂ to form long strands. Pellets were prepared by cutting strands into 4-mm long pieces.

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