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Viscosaline: new 3-alkyl pyridinium alkaloid from the Arctic sponge *Haliclona viscosa*†

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Polycyclic pyridinium alkaloids are widely distributed in several sponges of the order Haplosclerida. So far, studies on *Haliclona* and related genera were mainly concentrated on warm or tropical waters. Here, we describe the chemical investigation of the Arctic sponge *Haliclona viscosa* and structure elucidation of the acyclic 1,3-dialkyl pyridinium alkaloid viscosaline. A novel structural motif of viscosaline is that β-alanine is covalently bound to one alkyl chain.

Introduction

Sponges of the genus *Haliclona* are well-known to contain chemically diverse alkaloids with heterocyclic nitrogens.[1] Many of these secondary metabolites have biological activities, *e.g.* antimicrobial, antifungal and cytotoxic activity. Isolated compounds have been identified as alkaloids such as sarains, manzamines, haliclonacyclamines, papuamines and 3-alkyl pyridine alkaloids.[2] Related metabolites from other sponges such as *Callyspongia fibrosa*, *Echinochalina* sp., *Xestospongia* sp., *Stelletta maxima* and *Amphimedon* sp. were also described. Some of these have an array of biological functions including protection from predators and invasion by fouling organisms.[3] Chemical investigations on sponges of the genus *Haliclona* were mainly focussed on tropical and temperate waters, whereas studies from Arctic waters are still under-represented in the literature.

Results and discussion

In our search for bioactive secondary metabolites of marine organisms from polar waters, *Haliclona viscosa* was collected off Blomstrandhalvøya by SCUBA diving in Kongsfjorden, Svalbard. Voucher specimens are deposited at the Zoölogisch Museum, Amsterdam, The Netherlands. Samples of *H. viscosa* were divided into portions, immediately frozen after collection and kept at −20 °C until extraction. Freeze-dried sponge tissue was extracted at room temperature with a 1 : 1 mixture of methanol and dichloromethane. The resulting crude extract was partitioned between *n*-hexane and methanol. The methanol extract was concentrated and further partitioned between ethyl acetate and water. Finally, the aqueous layer was extracted with *n*-butanol. The extracted portions were investigated by analytical HPLC. These extracts were screened for activity against 17 microorganisms which were isolated from the marine sponge *Halichondria panicea* or obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany). The antimicrobial activity of the sponge extracts were assessed using the Agar disc-diffusion test.[4] The primary screening was conducted by testing the sponge extracts in aliquots of 400 µg. Several extracts inhibited the growth of the test bacteria (see Table 1). Areas of growth inhibition were observed as clear zones surrounding the discs and recorded. Inhibition radii were measured as the radius of

† Electronic supplementary information (ESI) available: 1D proton NMR spectra as well as the MS and MS/MS spectra of viscosaline (**1**), and a more detailed description of the structure elucidation. See http://www.rsc.org/suppdata/ob/b4/b403413a/

Table 1 Relative antimicrobial activities of *Haliclona viscosa* fractions

	2000 extracts[a]					2001 extracts[a]				
Bacteria	R	H	E	B	W	R	H	E	B	W
Aquaspirillum psychrophilum	w[b]	n	**s**	**s**	n	**s**	n	w	**s**	n
Bacillus badius	m	n	w	**s**	w	m	n	m	**s**	n
Comamonas testosteroni	n	m	m	m	m	n	w	m	m	m
Cytophaga sp.	n	n	n	**s**	n	n	n	n	**s**	n
Escherichia coli	w	w	m	m	w	w	w	w	m	w
Gram-positive bacterium str. 121	m	n	**s**	**s**	n	m	n	w	**s**	n
Microbacterium barkeri	n	n	n	n	n	**s**	n	n	**s**	n
Micrococcus sp.	n	n	**s**	**s**	n	**s**	n	**s**	**s**	n
Myxococcus coralloides	w	w	w	w	w	w	w	w	w	w
Planococcus citreus	n	n	**s**	m	n	m	n	w	**s**	n
Pseudoalteromonas sp.	w	m	m	**s**	w	n	m	m	**s**	w
Pseudoalteromonas tetraodonis	w	n	w	w	w	n	n	n	w	n
Roseobacter litoralis	n	n	**s**	**s**	n	**s**	n	n	**s**	n
Ruegeria algicola	n	n	n	w	n	w	n	n	**s**	n
Streptomyces purpureus	n	n	w	m	n	w	n	n	m	n
Vibrio fischeri	n	n	n	n	n	n	n	m	m	m
Vibrio harveyi	w	m	m	—	m	w	m	m	m	m
Zobellia uliginosa	n	n	n	w	n	n	n	w	w	n

[a] R = crude extract, H = *n*-hexane extract, E = ethyl acetate extract, B = *n*-butanol extract and W = water extract. [b] n = no effect, w = weak (radius < 1 mm), m = medium (radius = 1–2 mm), s = strong (radius > 2 mm).

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Table 2 NMR data of viscosaline (**1**) and the synthetic precursor (**4**)[a,b]

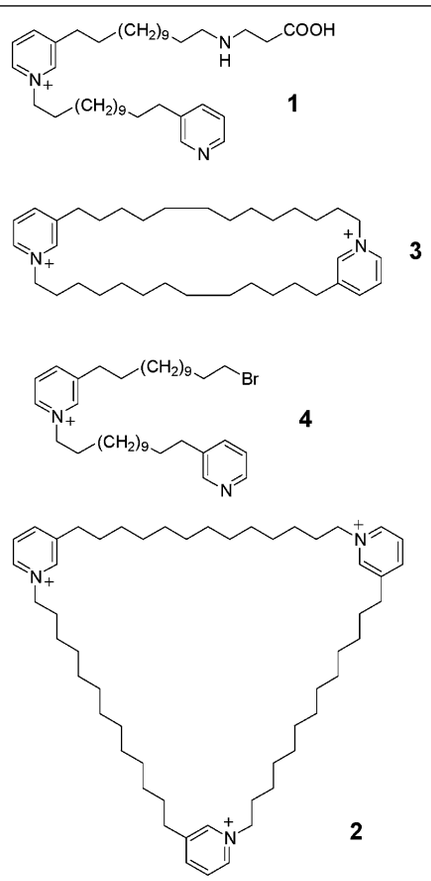
	1					**4**	
No.	$\delta(^{13}C)$	$\delta(^{1}H)$	$^{1}H,^{1}H$-COSY[d]	$^{1}H,^{13}C$-HMBC[e]	$^{1}H,^{15}N$-HMBC[f]	$\delta(^{13}C)$	$\delta(^{1}H)$
1	(214)	—	—	—	—	(215)	—
2	144.0	9.03	—	3, 4, 6, 7, 26	1	143.9	9.18
3	143.0	—	—	—	—	144.2	—
4	145.1	8.48	5	2, 6, 26	1	144.7	8.22
5	127.5	8.08	4, 6	3, 6	1	127.9	8.02
6	142.2	8.95	5	2, 4, 5, 7	—	142.5	9.29
7	60.6	4.56	8	2, 6, 8	1	61.9	4.96
8	30.6	1.90	7	7	1	31.9	2.01
9–17	–[c]	–[c]	—	—	—	–[c]	–[c]
18	30.0	1.59	19	20	—	30.4	1.60
19	31.8	2.67	18	21	—	32.7	2.58
20	139.5	—	—	—	—	137.9	—
21	145.9	8.62	—	—	22	149.9	8.42
22	(319)	—	—	—	—	(317)	—
23	143.7	8.59	—	—	22	147.1	8.41
24	128.8	7.71	25	20, 23	22	123.3	7.19
25	140.0	8.00	24	19, 21, 23	—	135.8	7.48
26	31.6	2.78	27	2, 3, 4	—	32.5	2.89
27	29.5	1.63	26	3	—	30.2	1.71
28–36	–[c]	–[c]	—	—	—	–[c]	–[c]
37	25.1	1.56	—	—	—	32.3	1.75
38	46.8	2.89	39	40	—	45.2	3.52
39	(44)	8.72	38, 40	—	—	—	—
40	42.4	3.11	39, 41	38, 41, 42	—	—	—
41	30.3	2.66	40	40, 42	39	—	—
42	171.6	—	—	—	—	—	—

[a] Chemical shifts δ are given in parts per million. ^{1}H and ^{13}C shifts are referenced to the DMSO-d_6 signal (2.50 and 39.5 ppm) for **1** and to the $CHCl_3$-d_1 signal (7.26 and 77.0 ppm) for **4**. [b] $\delta(^{15}N)$ is given in parenthesis in the $\delta(^{13}C)$ column. ^{15}N NMR spectra were not calibrated with an external standard. The δ value has an accuracy of about 1 ppm to NH_3 (0 ppm). [c] It was not possible to assign a single chemical shift value. [d] COSY correlations are given for both sides of the diagonal. [e] HMBC correlations are given from protons to carbons. The underlined HMBC correlations were only observed in MeOH-d_4. [f] HMBC correlations are given from protons to nitrogens.

the halo from the edge of the disc and evaluated. Antimicrobial activity was found in the ethyl acetate and the *n*-butanol phases whereas no activity was observed for the *n*-hexane and the water fractions. The *n*-butanol phase showed a high antimicrobial activity against several bacteria (see Table 1). Further investigations were therefore focussed on the *n*-butanol phase, which was purified by preparative HPLC using acetonitrile/water gradients. This separation step yielded 46.5 mg of an unknown compound (**1**) and 4 mg viscosamine (**2**) (see details in Experimental).[5] The molecular formula and structure were established by HR-MS techniques and two-dimensional NMR.

The molecular formula of **1** ($C_{39}H_{66}N_3O_2$) was obtained from HR ESI- and FAB-MS (ESI-MS spectrum, see electronic supplementary information). The fragment (*m*/*z* = 260.2) obtained in the MS/MS spectrum (see electronic supplementary information) of the molecular ion is already known from investigations on cyclostellettamine C (**3**) and viscosamine (**2**).[2k,5] Therefore, the structure of **1** must be closely related to **3**. The obtained masses for **1** and **3** differ by 89 mass units which corresponds to alanine. Because no methyl group was observed and two additional methylene groups are present it was concluded that **1** consists of cyclostellettamine C (**3**) and β-alanine (see Scheme 1). The NMR spectrum proves that the structure of **1** is not symmetrical in comparison to **3** because the full set of resonances is observed in the aromatic region (see Fig. 1). Two of the aromatic signals showed relatively broad line widths (8.5 ppm, see Fig. 1). The signals of one pyridine ring are all high-field shifted in comparison to **3** (see Fig. 1).

Since the NMR spectra of **1** are similar to those of a synthetic precursor of **3** (**4**),[6] it was concluded that such a system is also present for **1** (see Table 2). In DMSO-d_6 it was not possible to observe any HMBC correlations between the β-alanine part and the rest of the molecule. Measurements in MeOH-d_4 however showed sharp signals for the two "broad" methylene groups which allowed the observation of HMBC correlations from the β-alanine subunit to one alkyl chain (H-38 ⟶ C-40 and H-40 ⟶ C-38, see Fig. 2). Therefore, it is proven that the β-alanine is covalently bound to the rest of the molecule. The two broad methylene groups in DMSO-d_6 were identified



Scheme 1

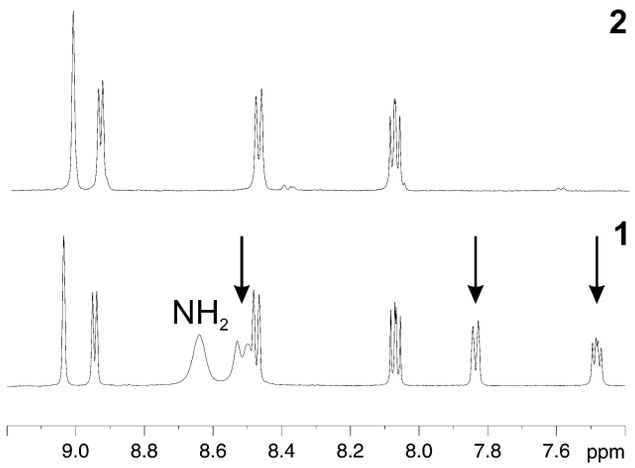


Fig. 1 Aromatic region of the 1D proton spectrum of **1** and **2**, resp. **3** (**2** and **3** have identical NMR spectra). Since the spectrum of **1** was measured in DMSO-d_6 the amino signal could be observed. The signals of the pyridine ring are indicated by arrows. The protons in *ortho*-position to the pyridine nitrogen are broad and have almost the same resonance frequency.

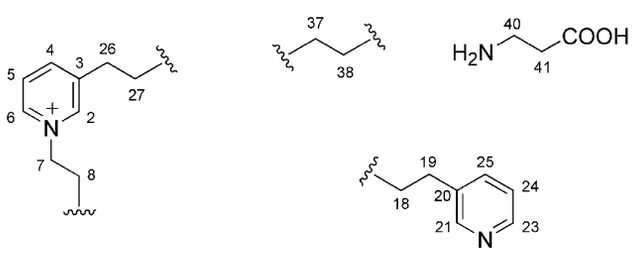


Fig. 2 Structural units of viscosaline (**1**).

as adjacent methylene groups of the amino nitrogen (N-39) of β-alanine. Due to the comparison of the spectroscopic data with **3** and **4** it can be concluded that structure **1** represents the correct constitution.

The integration ($I = 2$) of the amino signal in the 1D proton spectrum proves that the amino acid exists in the zwitter ionic form. Another proof of the betaine structure is the signal at m/z = 536.5 in the MS/MS spectrum (see electronic supplementary information). This fragment (m/z = 536.5) represents the amine of **1** which is obtained by fragmentation of CO_2 and ethene of the betaine.

By closer inspection of the structure elucidation of **1** it was realized that an alternative constitution for **1** cannot be excluded by the analytical data presented so far. The NMR data cannot distinguish between the two constitutional isomers I or II of **1** (see Fig. 3) because the functional end of the alkyl chains and the pyridinium ring are separated by 13 methylene groups. Since the methylene groups are all strongly coupled it was possible to observe a correlation in the TOCSY spectrum from H-7 to H-18 and H-19 which unambiguously proves the

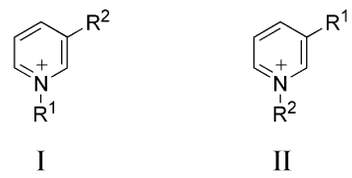


Fig. 3 Two possible constitutional isomers of viscosaline (R^1 = alkyl chain with pyridine subunit; R^2 = alkyl chain with β-alanine subunit).

existence of constitutional isomer I. This is also confirmed by the fragments in the MS/MS spectra (see electronic supplementary information) and proves that constitutional isomer I represents the correct constitution of viscosaline (**1**).

3-Alkyl pyridine alkaloids are well-known from sponges of the genus *Haliclona* and related genera. Viscosaline (**1**) is the first acyclic dimeric 3-alkyl pyridine alkaloid from natural sources. The structure of viscosaline (**1**) is related to the niphatoxins which also have a central pyridinium unit but with two alkyl chains with a residual pyridine.[7] Viscosaline (**1**) could be a biosynthetic precursor of the cyclostellettamines and related molecules.

Experimental

Haliclona viscosa was collected off the coast off Blomstrandhalvøya, near Hansneset, by SCUBA diving (15–25 m depth, June 2000 and 2001) in Kongsfjorden, which is located on the west coast of Svalbard at 79 °N, 12 °E. Voucher specimens are deposited under registration no. ZMA POR. 17008 and no. ZMA POR. 17009 at the Zoölogisch Museum, Amsterdam, The Netherlands. Sponge identification was kindly conducted by Wallie H. de Weerdt and Dr Rob W. M. van Soest, Institute for Biodiversity and Ecosystem Dynamics (Zoological Museum), University of Amsterdam, The Netherlands. Samples of *Haliclona viscosa* were immediately frozen after collection and kept at −20 °C until extraction. Freeze-dried sponge tissue was extracted at room temperature with a 1 : 1 mixture of methanol/dichloromethane (3 × 1000 mL). The resulting greenish-colored crude extract was partitioned between *n*-hexane (3 × 150 mL) and methanol (80 mL). The methanol extract was concentrated and further partitioned between ethyl acetate (3 × 150 mL) and water (80 mL) and finally the aqueous layer was extracted with *n*-butanol (3 × 150 mL). Results are shown in Table 3.

397.3 mg from the resulting *n*-butanol phase of the year 2001 were purified by using preparative HPLC yielding 46.5 mg (0.32% of dry weight) of viscosaline (**1**) and 4.0 mg of viscosamine (**2**). For HPLC analysis, samples were injected into an HPLC system (JASCO) equipped with a light scattering detector SEDEX 75 (Sedere). The analytical column (4.6 × 2500 mm, 5 μm) and the separation columm (16 × 250 mm, 10 μm) were pre-filled with Kromasil RP-18 (Knauer). Separation was achieved by applying a gradient from 5% acetonitrile (containing 0.1% trifluoroacetic acid) to 35% acetonitrile in

Table 3 Obtained weights (g) of the different fractions[a]

	2000 *Haliclona viscosa*							
Portion no.	FD[a]	R	H	E	B	W	**1**	**2**
1	20.62	2.25	—	—	—	—	—	—
2	24.64	2.53	0.7125	0.2347	0.4213	0.9938	—	—
	2001 *Haliclona viscosa*							
1	20.35	1.79	—	—	—	—	—	—
2	21.15	2.56	0.3401	0.1647	0.6429	1.1153	—	—
3	14.38	1.55	0.1979	0.1194	0.3973	0.7306	0.0465	0.0040

[a] FD = freeze-dried tissue.

40 min. For extractions, solvents were distilled prior to use. Gradient-grade solvents were used for chromatographic applications (Merck).

The NMR spectroscopy experiments were carried out at 30 °C on Bruker Avance 400 and Avance 500 spectrometers (Bruker BioSpin). The standard pulse programs from the Bruker library were used. Chemical shifts are given in ppm and are referenced to the appropriate solvent signal. Mass spectral analyses were performed on ESI-TOF (LCT, Micromass), Q-TOF (Micromass), microTOF (Bruker Daltonics) and JMS-700 (JEOL) MS spectrometers. IR spectra (KBr) were recorded on a Bruker EQUINOX 55 spectrometer (Bruker Optik). UV spectra (MeOH) were recorded on a UVIKON 810P spectrometer (KONTRON).

Viscosaline (**1**): **UV** (MeOH): λ_{max} 263.9 and 203.7 nm. **IR** (KBr): ν_{max} 3434, 2927, 2855, 1684, 1202 and 1131 cm^{-1}. **HRMS:** (ESI) m/z = 608.5151 (doubly charged m/z = 304.7), $C_{39}H_{66}N_3O_2$ Δm = 0.7 ppm; (FAB) m/z = 608.5152, $C_{39}H_{66}N_3O_2$ Δm = 0.5 ppm. NMR data is summarized in Table 2.

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