3-Alkylpyridinium Alkaloids from the Pacific Sponge *Haliclona* sp.[§]

Agostino Casapullo,* Oscar Cobar Pinto,[†] Stefania Marzocco, Giuseppina Autore, and Raffaele Riccio

Dipartimento di Scienze Farmaceutiche, Università di Salerno, Via Ponte don Melillo, 84084, Fisciano, Italy

Received September 26, 2008

The analysis of the polar extracts of the Pacific sponge *Haliclona* sp. yielded new dimeric (**1**), trimeric (**2**), and polymeric 3-alkylpyridinium alkaloids. Their isolation and structural elucidation, based on NMR and MS data, are discussed in detail, along with their cytotoxic activity.

Sponges belonging to the genus *Haliclona* and related genera are well known to contain alkaloid-like compounds. Several 3-alkylpyridinium alkaloids have been found during the last 30 years, often characterized by interesting biological activities, such as navenones, amphitoxins, halitoxins, niphatoxins, and cyclostellettamines.[1−16]

A lyophilized sample of the Pacific sponge *Haliclona* sp., collected off the coast of Guatemala, whose crude extract was shown to be cytotoxic in preliminary tests, was extracted with MeOH, and the methanolic extract was subjected to a modified Kupchan partition procedure, thereby affording four extracts (see Experimental Section). On the basis of their chromatographic and biological profiles, the chloroform and butanol extracts were then submitted to successive fractionation steps. The chloroform residue was chromatographed by DCCC and by RP-HPLC to give pure compounds **1** and **2**. The butanol extract, partly retaining the cytotoxic properties of the crude extract, was subjected to size exclusion chromatography on a Sephadex LH-20 resin (eluent methanol), and the bioactive fraction was then submitted to membrane ultrafiltration, yielding the following different molecular weight range fractions: **A** (0.9 mg, > 300 kDa), **B** (36.5 mg, 100−300 kDa), **C** (5.6 mg, 50−100 kDa), **D** (2.8 mg, 30−50 kDa), **E** (1.5 mg, 3−10 kDa).

^{1}H and ^{13}C NMR spectra in CD_3OD of **1** and **2** were superimposable. Chemical shifts and coupling constant patterns of the ^{1}H NMR resonances were suggestive of a 1,3-disubstituted alkylpyridinium moiety (Table 1): four aromatic protons at δ 8.89 (brs), 8.86 (d, $J = 6.1$ Hz), 8.42 (d, $J = 8.0$ Hz), and 8.03 (dd, $J = 6.1$, 8.0 Hz) and the methylene protons at δ 4.61 (t, $J = 7.7$ Hz). The ^{13}C and edited-HSQC NMR spectra indicated the presence of nine aliphatic methylene and two olefinic methine carbons within the side chains. An exhaustive analysis of 2D NMR spectra (COSY, edited-HSQC, and HMBC, see Table 1) allowed the connection of the 1,3-disubstituted pyridine ring with the alkyl chains. Key HMBC correlations H7/C2 and H7/C6, along with H4/C17, H17/C2, H17/C3, and H17/C4, allowed the spin systems C7−C9 and C13−C17 to be connected to the nitrogen and C3 ring atoms, respectively. Owing to the absence of additional NMR connectivities caused by the overlap of H10, H11, and H12 resonances, C9 and C13 were tentatively connected through C10−C12 on the basis of their ^{1}H and ^{13}C chemical shifts. The *Z* geometry of the double bond was deduced from the ^{13}C chemical shift values of the allylic methylene (δ 28.3) and the H15/H16 *J* coupling (10.0 Hz).

The structural hypotheses of the monomer subunit of **1** and **2** (Figure 1) were confirmed by ESIMS and MS/MS data, which also allowed the determination of their molecular formulas. The molecular weight of 460.4 Da was attributed to **1** on the basis of the singly charged ion at *m/z* 459.4 $[M - H]^+$ and was consistent with the dimeric alkaloidic structure depicted. Pseudomolecular ion peaks corresponding to $[M - H]^+$ species have already been described for this kind of compound following a Hofmann-type rearrangement.[17] Moreover, the MS^2 spectrum contained a daughter ion at *m/z* 230.2 corresponding to the monomeric subunit, corroborating the structure assigned by NMR analysis.

Table 1. NMR Spectroscopic Data (600 MHz, CD_3OD) for **1** and **2**[a]

position	δ_C, mult.	δ_H (*J* in Hz)	HMBC[c]
1			
2	145.3, CH	8.89, br s	6, 7, 17
3	144.5, qC		
4	146.5, CH	8.42, d (8.0)	2, 6, 17
5	129.1, CH	8.03, dd (8.0, 6.1)	2, 3
6	143.5, CH	8.86, d (6.1)	4, 5
7	63.0, CH_2	4.61, t (7.7)	2, 6, 8, 9
8	32.6, CH_2	2.03, m	
9	27.2, CH_2	1.40, m	
10	30.4[b] CH_2	1.40, m	
11	30.5[b] CH_2	1.40, m	
12	30.6[b] CH_2	1.40, m	
13	30.2, CH_2	1.40, m	
14	28.3, CH_2	2.21, q (7.3)	
15	135.4, CH	5.72, dt (10.0, 7.3)	17, 13
16	125.3, CH	5.60, dt (10.0, 7.3)	14
17	31.1, CH_2	3.69, d (7.3)	2, 3, 4, 15, 16

[a] All data for **1** and **2** are superimposable. [b] Assignments may be interchanged. [c] HMBC correlations, optimized for 7.5 Hz, are from proton(s) stated to the indicated carbon.

The presence in the ESI spectrum of **2** of a Hofmann-type doubly charged ion at *m/z* 344.7 accounted for a trimeric structure with a molecular weight of 690.5 Da. When submitted to collision-induced dissociation, this species produced the same singly charged ions at *m/z* 459.4 and 230.2 previously discussed. Taken together, this evidence was indicative of a trimeric structure for **2**, formed by the same subunit as in Figure 1.

The polymeric fractions **A**−**E** covered a wide molecular weight range (3 to 300 kDa) and showed many NMR signals reminiscent of those of **1** and **2**, suggesting that also these polymers were composed of 3-alkylpyridinium monomers. In addition, ^{1}H NMR spectra of all polymeric species appeared superimposable, indicating that they differed only by the total number of monomeric subunits. The presence of two sets of signals in their ^{1}H and ^{13}C NMR spectra suggested the existence of two different types of monomeric subunits. One signal pattern was almost identical to that of **1** and **2**, while the other was consistent with the absence of the side chain double bond, replaced by two additional methylenes resonating at δ_H 2.88 and 1.73. 2D NMR spectra confirmed this deduction. Moreover, the integration of peak areas in the proton spectrum established a 1:1 ratio between the two different subunits in all

[§] Dedicated to the memory of Luigi Gomez-Paloma.

* To whom correspondence should be addressed. Tel: +39 089 969243. Fax: +39 089 969603. E-mail: casapullo@unisa.it.

[†] Facultad de Ciencias Quimicas y Farmacia, Universidad de San Carlos de Guatemala, Ciudad Universitaria, Zona 12, 01012, Ciudad de Guatemala, Guatemala.

10.1021/np800610p CCC: $40.75 © 2009 American Chemical Society and American Society of Pharmacognosy
Published on Web 01/09/2009

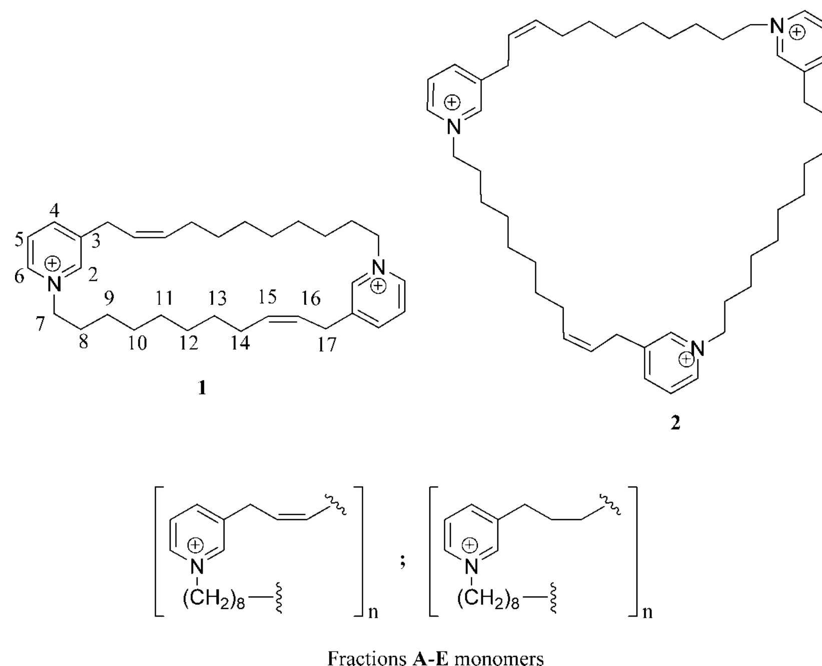

Figure 1. Chemical structures of isolated compounds **1** and **2** and polymeric fractions **A**−**E**. For the latter, saturated and unsaturated monomers coexist in an approximately 1:1 ratio.

polymeric species **A**−**E** (Figure 1). The pure compounds **1** and **2** were screened for cytotoxicicty against murine macrophage J774.A1, human epithelial kidney HEK-293, and murine fibrosarcoma WEHI-164 cell lines,[18−20] exhibiting low cytotoxic properties (IC_{50} > 20 μg/mL).

The 3-alkylpyridinium alkaloids, which these type of dimeric, trimeric, and polymeric compounds represent, have often been described from *Haliclona* species. Notably the dimeric and trimeric species (**1** and **2**) are characterized by 11 carbon spacers with a single *cis* double bond, separated from the pyridinium moiety by a single methylene rather than the more common two methylene distance. In addition, the wide group of polymeric species isolated, with molecular weights up to 300 000 Da, raise an intriguing question about the role of such large polycationic species in the cell.

Thus, even if these compounds represent minor variations of similar alkaloids of this family,[17] their proposed role as specific markers of *Haliclona* and related genera of marine sponges appears to be confirmed.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker DRX 600 [600 MHz (^{1}H) and 150 MHz (^{13}C)]. The ^{1}H and ^{13}C chemical shifts were referenced to the solvent peaks (δ_H = 3.34 ppm and δ_C = 49 ppm in methanol-d_4). IR spectra were recorded on a Shimadzu FTIR-8101 M spectrometer. UV spectra were obtained on a Beckman DU-70 spectrophotometer. HRESIMS spectra were recorded on a Waters Q-Tof Premier mass spectrometer. ESIMS and MS/MS spectra were performed on a LCQ DECA Finnigan mass spectrometer. Reversed-phase HPLC was carried out on a C_{18} Jupiter column.

Biological Material. The sponge *Haliclona* sp. was collected off the Pacific coast of Guatemala in 2002. A sample of the lyophilized specimen is kept at the Dipartimento di Scienze Farmaceutiche of the University of Salerno under the reference number 02CLO.

Extraction and Separation. The lyophilized sponge materials of *Haliclona* sp. (250 g) were extracted exhaustively with MeOH (4 × 1.5 L) at room temperature. The methanolic extract was concentrated under reduced pressure, giving a brown oil. The oily residue was successively extracted using a modified Kupchan partition procedure: the extract was dissolved in 0.7 L of a mixture of MeOH/H_2O containing 10% of H_2O and partitioned against 0.7 L of *n*-hexane. The water content (% v/v) of the methanolic fraction was adjusted to 20% and 40% and partitioned against 1 L of CCl_4 and 1 L of $CHCl_3$, respectively. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-butanol. The chloroform extract was first submitted to DCCC in the ascending mode, using $CHCl_3$/MeOH/2-propanol/H_2O (5:6:4:1). The most interesting fraction was then subjected to HPLC [Jupiter C_{18} (250 × 4.6 mm), flow rate 1.5 mL/min; detection, UV 220 nm; gradient solvent CH_3CN/H_2O (CH_3CN 5% to 95% in 50 min)] to give pure compounds **1** and **2** (7.6 and 4.5 mg, respectively). The butanol extract was submitted to size-exclusion chromatography (Sephadex LH-20 resin, eluent MeOH), and subsequently the cytotoxic fractions (60 mg), dissolved in water, were purified by membrane ultrafiltration (Amicon Ultrafiltration Cell). Ultrafiltration was performed through 1, 10, 50, 100, and 300 KDa molecular weight limit membranes. Lyophilization of both filtrate and retentate afforded residues weighing, respectively, 1.5 mg (E, 3000−10 000 molecular weight range fraction), 2.8 mg (D, 30 000−50 000 molecular weight range fraction), 5.6 mg (C, 100 000−50 000 molecular weight range fraction), 36.5 mg (B, 300 000−100 000 molecular weight range fraction), and 0.9 mg (A, >300 000 molecular weight range fraction). At each stage of the ultrafiltration, the retentate was diluted and the ultrafiltration was repeated two times using the same membrane to ensure that all material of molecular weight less than the membrane nominal molecular weight cutoff range had passed into the filtrate.

Compound 1 and 2: colorless solids; IR (KBr) ν_{max} 3030, 2855, 1630, 1200 cm^{-1}; UV (MeOH) λ_{max} 260 nm; HRESIMS (**1**) *m/z* 459.3727 [M − H]$^+$ (calcd for $C_{32}H_{47}N_2^+$ 459.3739); ESIMS *m/z* 459.4 [M − H]$^+$; ESIMS/MS *m/z* 230.2; HRESIMS (**2**) *m/z* 344.7820 [M − H]$^{2+}$ (calcd for $C_{48}H_{71}N_3^{2+}$ 344.7824); ESIMS *m/z* 344.7 [M − H]$^{2+}$; ESIMS/MS *m/z* 459.4, 230.2; NMR data are reported in Table 1.

Fractions A−E. UV λ_{max} (MeOH) 260 nm; ^{1}H NMR (CD_3OD, 600 MHz) δ 8.91 (1H, s, H-2′), 8.89 (1H, s, H-2), 8.87 (2H, m, H-6/H-6′), 8.48 (1H, d, *J* = 8.0 Hz, H-4′), 8.42 (1H, d, *J* = 8.0 Hz, H-4), 8.02 (2H, m, H-5/H-5′), 5.72 (1H, bt, H-15), 5.60 (1H, bt, H-16), 4.61 (2H, t, *J* = 7.7 Hz, H-7), 3.69 (2H, d, *J* = 7.3 Hz, H-17), 2.88 (2H, m, H-17′), 2.21 (2H, q, *J* = 7.3 Hz, H-14), 2.03 (2H, m, H-8), 1.73 (2H, m, H-16′), 1.40; ^{13}C NMR (CD_3OD, 150 MHz) δ 146.6 (C-4), 146.4 (C-4′), 145.3 (C-2), 145.1 (C-2′), 144.3 (C-3/C-3′), 143.5 (C-6), 143.3 (C-6′), 135.2 (C-15), 130.0 (C-5), 128.9 (C-5′), 125.4 (C-16), 63.1 (C-7), 32.6 (C-17), 31.5 (C-8), 30.5 (C-16), 30.4−30.2 (C-10/C-13 and C-10′/C-14′), 27.4 (C-14), 26.3 (C-9).

Cytotoxicity. Compounds **1** and **2** were submitted to a cytotoxicity assay against murine macrophage J774.A1, human kidney epitelial HEK-293, and murine fibrosarcoma WEHI-164 cell lines, using

6-mercaptopurine as a reference cytotoxic compound (IC_{50} of 5.1×10^{-6}, 7.1×10^{-7}, and 2.5×10^{-6} mg/mL, respectively). For the assay procedure see refs 18−20.

Acknowledgment. Financial support by the University of Salerno is gratefully acknowledged. The authors also acknowledge the Centre of Competence in Diagnostics and Molecular Pharmaceutics for the use of their instruments, the BIONET S.L. for specimen collection, the Laboratory of Research in Natural Products (LIPRONAT) of the Chemical Sciences School at the University of San Carlos, the Guatemala staff, and Abraham Vasquez for the initial extraction procedures.

References and Notes

(1) Sleeper, H. L.; Fenical, W. *J. Am. Chem. Soc.* **1977**, *99*, 2367–2368.
(2) Schmitz, F. J.; Hollenbeak, K. H.; Campbell, D. C. *J. Org. Chem.* **1978**, *43*, 3916–3922.
(3) Quiňoa, E.; Crews, P. *Tetrahedron Lett.* **1987**, *28*, 2467–2468.
(4) Kobayashi, J.; Murayama, T.; Ohizumi, I.; Sasaki, T.; Ohta, T.; Nozoe, S. *Tetrahedron Lett.* **1989**, *30*, 4833–4836.
(5) Carrol, A. R.; Scheuer, P. J. *Tetrahedron* **1990**, *46*, 6637–6644.
(6) Sakemi, S.; Totton, L. E.; Sun, H. H. *J. Nat. Prod.* **1990**, *53*, 995–999.
(7) Talpir, R.; Rudi, A.; Ilan, M.; Kashman, Y. *Tetrahedron Lett.* **1992**, *33*, 3033–3034.
(8) Kobayashi, J.; Zeng, C. M.; Ishibashi, M.; Shigemori, H.; Sasaki, T.; Mikami, Y. *J. Chem. Soc., Perkin Trans. 1* **1992**, 1291–1294.
(9) Spinella, A.; Avarez, L. A.; Passeggio, A.; Cimino, G. *Tetrahedron* **1993**, *49*, 1307–1314.
(10) Fusetani, N.; Asai, N.; Matsunaga, S.; Honda, K.; Yasumoro, K. *Tetrahedron Lett.* **1994**, *35*, 3967–3970.
(11) Albrizio, S.; Ciminiello, P.; Fattorusso, E.; Magno, S. *J. Nat. Prod.* **1995**, *58*, 647–652.
(12) Oku, N.; Nagai, K.; Shindoh, N.; Terada, Y.; van Soest, R. W. M.; Matsunaga, S.; Fusetani, N. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2617–2620.
(13) Wang, G. Y. S.; Kuramoto, M.; Uemura, D.; Yamada, A.; Yamaguchi, K.; Yazawa, K. *Tetrahedron Lett.* **1996**, *37*, 1813–1816.
(14) Volk, C. A.; Kock, M. *Org. Lett.* **2003**, *5*, 3567–3569.
(15) Teruya, T.; Kobayashi, K.; Suenaga, K.; Kigoshi, H. *J. Nat. Prod.* **2006**, *69*, 135–137.
(16) Xu, N. J.; Sun, X.; Yan, X. J. *Chin. Chem. Lett.* **2007**, *18*, 947–950.
(17) Davies-Coleman, M. T.; Faulkner, D. J.; Dubowchik, G. M.; Roth, G. P.; Polson, C.; Fairchild, C. *J. Org. Chem.* **1993**, *58*, 5925–5930.
(18) Kinjo, J.; Uemura, H.; Nakamura, M.; Nohara, T. *Chem. Pharm. Bull.* **1994**, *42*, 1339–1341.
(19) Setsuo, S.; Shigeya, S.; Norihiro, T.; Yiochi, N.; Keishi, N.; Madoka, I.; Isao, I. *Chem. Pharm. Bull.* **1990**, *38*, 411–414.
(20) Mosmann, T. *Methods* **1983**, *65*, 55–63.

NP800610P