Two New Haliclamines from the Arctic Sponge *Haliclona viscosa*

Christian A. Volk,[a] Heike Lippert,[a] Ellen Lichte,[a] and Matthias Köck*[a]

Keywords: Arctic sponges / Marine natural products / Alkaloids

There are a large number of studies on sponges from warm or tropical waters, whereas little is known about the chemistry of sponges from Arctic waters. Here, we describe the isolation and structure elucidation of two new 3-alkyltetrahydropyridine alkaloids (haliclamines C and D) from the Arctic sponge *Haliclona viscosa*. MS and 2D NMR spectroscopy were used to analyse the structure of the two new compounds. Since the haliclamines consist of long alkyl chains, MS/MS methods were necessary for the unambiguous assignment of the constitution of the new alkaloids.

Introduction

Structurally diverse polycyclic alkaloids with heterocyclic nitrogen atoms and without aliphatic methyl groups have been obtained from several sponges of the type Haplosclerida.[1−3] Especially, alkaloids containing 1,3-dialkylpyridine or -piperidine motifs are frequently isolated from marine sponges of the genera *Haliclona*,[4,5] *Xestospongia*[6] and *Amphimedon*.[7] The studies on *Haliclona* and related genera are mainly concentrated in tropical waters.[8−15] Chemical investigations on sponges from temperate and cold waters are still under-represented in the literature. We became interested in studying sponges of the genus *Haliclona* from Arctic waters because the extracts, fractions and purified compounds of *Haliclona viscosa* are active in different assays.[16,17] The feeding deterrence and antimicrobial activity are found in the ethyl acetate and the 1-butanol phase. The major metabolites of these fractions were isolated by different chromatographic methods. The structure analysis required a combination of NMR spectroscopic, MS and MS/MS techniques to unambiguously elucidate the molecular constitution of haliclamine C (**1**) and D (**2**).

Results and Discussion

A freeze-dried sample of *Haliclona viscosa* was exhaustively extracted with organic solvents and fractionated with *n*-hexane, ethyl acetate, 1-butanol and water. According to the HPLC analysis the ethyl acetate and 1-butanol phases were combined and further fractionated by size exclusion chromatography and reversed-phase HPLC. The almost identical retention times of the major metabolites **1** and **2** made the chromatographic separation difficult. The high-resolution mass spectra were obtained from FAB and ESI ionisation and propose the following molecular formulae: $C_{30}H_{55}N_2$ for **1** and $C_{31}H_{57}N_2$ for **2**. Both compounds differ by only 14 mass units. Since the NMR spectra are very similar, it was concluded that **2** has one more methylene group than **1** (see Supporting Information; for Supporting Information see also the footnote on the first page of this article). Furthermore, the proton spectra indicate a symmetric or pseudo-symmetric structure of **1** and **2** because only half of the protons from the HR-MS spectra (55 and 57 protons, respectively) appear in the NMR spectra. The difficulties in the NMR spectroscopic analysis arose from the fact that more than 50% of the protons have the same resonance frequency (see Table 1). Only two sp^2 carbon atoms (δ = 118.5 and 131.2 ppm) are identified in the ^{13}C spectrum of **1** and **2**. The DEPT spectrum indicates that all aliphatic carbon atoms are methylene groups, three of them must be adjacent to a nitrogen atom due to the observed δ(^{13}C) between 48 and 55 ppm and δ(^{1}H) between 3.0 and 3.7 ppm. Moreover, δ(^{1}H) indicates a protonation of the nitrogen atom. This is confirmed by the correlation from 10.28 ppm to 48 ppm in the $^{1}H,^{15}N$-HSQC spectrum. $^{1}H,^{1}H$-COSY, $^{1}H,^{13}C$-HMBC and $^{1}H,^{15}N$-HMBC data suggest a 3-alkyltetrahydropyridine substructure. Since both nitrogen atoms are protonated the molecule contains two charges. The doubly-charged molecular ion can be observed in the mass spectra of **1** and **2** (*m*/*z* = 222.2 for **1** and *m*/*z* = 229.2 for **2**). The singly-charged peaks result from salt formation with the TFA anion (*m*/*z* = 557.4 for **1** and *m*/*z* = 571.4 for **2**) and deprotonation of the NH by the TFA anion (*m*/*z* = 443.4 for **1** and *m*/*z* = 457.5 for **2**). TFA was introduced by chromatographic purification. Furthermore, the MS and NMR spectra show that **1** and **2** are macrocyclic compounds with two long saturated alkyl chains (see Figure 1). Similar compounds have already been

[a] Alfred-Wegener-Institut für Polar- und Meeresforschung in der Helmholtz-Gemeinschaft,
Am Handelshafen 12, 27570 Bremerhaven, Germany
Fax: (internat.) +49-471-48311425
E-mail: mkoeck@awi-bremerhaven.de
Supporting information for this article is available on the WWW under http://www.eurjoc.org or from the author.

  DOI: 10.1002/ejoc.200400026

Table 1. NMR spectroscopic data of haliclamine C (**1**) and D (**2**) in [D_6]DMSO; chemical shifts are referenced to the solvent signal (^{1}H 2.50 ppm, ^{13}C 39.5 ppm); for position 1, δ(^{15}N) is given; ^{15}N NMR spectra were not calibrated with an external standard; the δ value has an accuracy of about 1 ppm in reference to NH_3 (δ = 0 ppm); COSY correlations are given for both sides of the diagonal; HMBC correlations are given from protons to carbon atoms

Position	Haliclamine C (**1**) δ(^{13}C)	δ(^{1}H)	Int.	Mult. ($J_{H,H}$)	COSY	HMBC
1 (1′)	(48)	10.66	1	br	6	–
2 (2′)	50.1	3.69 +3.50	1 + 1	t (14.3) + d (14.3)	–	–
3 (3′)	130.8	–	–	–	–	–
4 (4′)	118.3	5.60	1	s	–	–
5 (5′)	21.3	2.36 +2.27	1 + 1	br + br	–	–
6 (6′)	48.3	3.43 +3.05	1 + 1	br + br	1	2, 4, 7
7 (7′)	54.2	3.08	2	br	8	–
8 (8′)	22.4	1.66	2	br	7	–
9-15 (9′-13′)	overlap	1.2	overlap	overlap	–	–
16 (14′)	26.3	1.35	overlap	overlap	17	3
17 (15′)	33.0	1.99	2	br	16	2, 3, 4, 16
	Haliclamine D (**2**)					
1 (1′)	(48)	10.28	1	s	2, 6	–
2 (2′)	50.3	3.71 +3.51	1 + 1	br + br	1	3, 4, 6
3 (3′)	131.1	–	–	–	–	–
4 (4′)	118.5	5.59	1	s	–	2, 5, 6, 17
5 (5′)	21.4	2.38 +2.25	1 + 1	br + br	–	–
6 (6′)	48.2	3.43 +3.04	1 + 1	br + br	1	4
7 (7′)	54.6	3.09	2	br	8	6
8 (8′)	22.6	1.68	2	br	7	–
9-15 (9′-14′)	overlap	1.2	overlap	overlap	–	–
16 (15′)	26.5	1.36	overlap	overlap	17	3
17 (16′)	33.6	1.98	2	br	16	2, 3, 4, 16

described in the literature as the haliclamines from a *Haliclona* sp.[18] and the cyclostellettamines from *Stelletta maxima* (see Figure 2).[19] The lengths of the alkyl chains are 9 and 12 (unsaturated) for the haliclamines and 12 to 14 (saturated) for cyclostellettamines.

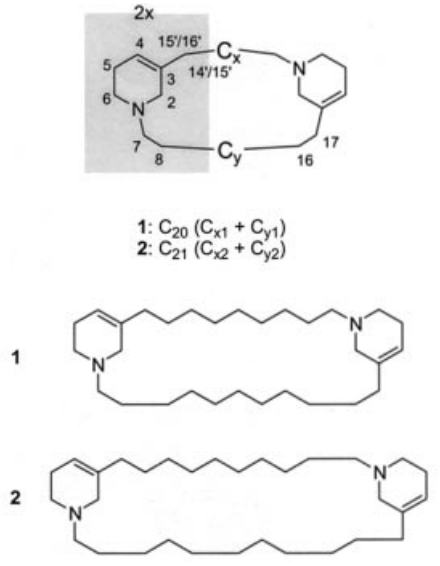

Figure 1. Schematic structure of haliclamine C (**1**) and D (**2**) derived from NMR spectroscopic and MS data

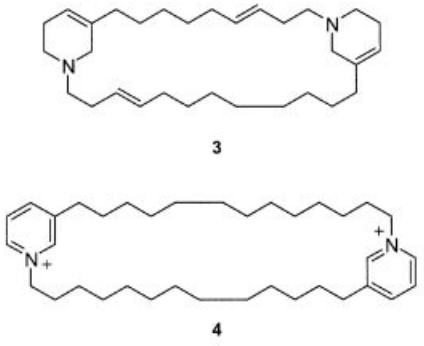

Figure 2. Structural formulae of haliclamine A (**3**) and cyclostellettamine C (**4**)

By considering the sum formula obtained from the mass spectra and the tetrahydropyridine substructure (2 × C_5H_7N) it can be concluded that both alkyl chains (C_x + C_y) consist of 20 carbon atoms for compound **1** and 21 carbon atoms for **2**. However, the distribution of the carbon atoms between the two chains cannot be solved by NMR spectroscopy. The differences in the chain length combinations is only possible by HR-MS/MS techniques. The main peaks in the MS/MS spectrum of the parent ions were observed at *m*/*z* = 208.210 and 236.240 for **1** and *m*/*z* = 222.226 and 236.241 for **2** (see Supporting Information).

The length of the alkyl chains (C_x and C_y) differ by two methylene groups for **1** and by one methylene group for **2**. The main peaks correspond to the half of each molecule as it is known from the cyclostellettamine investigations. The corresponding molecular formulae of the fragments are $C_{14}H_{26}N$ and $C_{16}H_{30}N$ for **1** and $C_{15}H_{28}N$ and $C_{16}H_{30}N$ for **2**. By subtracting the tetrahydropyridine fragment, the following chain lengths (C_x and C_y) are obtained: 9 and 11 for **1** and 10 and 11 for **2**. For further structural proof Haliclamine D (**2**) was synthesized. The spectroscopic data of the synthetic compound supports the proposed structures of **1** and **2** (for details, see Exp. Sect.).[20]

The crude extract of *Haliclona viscosa* shows feeding deterrence against the amphipod *Anonyx nugax* and the starfish *Asterias rubens* (for details, see Exp. Sect.). Further extracts and fractions as well as haliclamine C (**1**) and D (**2**) were tested for their feeding deterrence against the amphipods. The activity is observed in the hexane and 1-butanol/ethyl acetate phase, whereas no activity is found in the water fraction. The major secondary metabolites **1** and **2** show no significant feeding deterrence against *Anonyx nugax*.[21]

The extracts and fractions of *Haliclona viscosa* as well as haliclamines C (**1**) and D (**2**) were also tested for their antimicrobial activity against 5 bacterial strains isolated from the vicinity of the sponge (for details, see Exp. Sect.). The extracts and fractions were tested at natural concentrations, and the haliclamines C (**1**) and D (**2**) at a concentration of 5 mg/mL. The crude extract of *Haliclona viscosa* shows strong or very strong antimicrobial activity against all 5 bacterial strains. The activity is observed in the 1-butanol/ethyl acetate phase, whereas no activity is found in the hexane and water fraction. The activity was followed in haliclamine C (**1**) and D (**2**). The haliclamines C (**1**) and D (**2**) show a strong or very strong inhibition against two of the five bacterial strains.

The present study shows the potential of sponges from Arctic waters as a source for new, bioactive natural products. Compounds like the haliclamines are of interest as possible precursors in the biosynthesis of manzamine alkaloids. In the work of Baldwin et al., a bistetrahydropyridine was proposed as a key intermediate in the biosynthesis.[22,23] Haliclamine C (**1**) and D (**2**) were isolated from a *Haliclona viscosa* sample collected in 1999. However, the samples collected in the following years (2000 and 2001) did not contain **1** and **2** at all.[24] Currently, we are investigating *Haliclona viscosa* samples from different geographical areas as well as other samples from the same habitat to understand the diversity in the secondary metabolism.

Experimental Section

Isolation and Purification: *Haliclona viscosa* was collected off the coast of Blomstrandhalvøya, near Hansneset, by SCUBA diving (15−25 m depth, June 1999) in the Kongsfjorden, which is located at the west coast of Svalbard at 79°N, 12°E. The sponge collection was carried out during the annual summer expedition (1999) at Koldewey-Station, Ny-Ålesund, Svalbard. Samples of *Haliclona viscosa* were immediately frozen after collection and kept at −20 °C until extraction. Sponge identification was kindly conducted by Wallie H. de Weerdt and Dr. Rob W. M. van Soest. A voucher specimen was deposited under registration no. ZMA POR. 16591 at the Zoölogisch Museum, Amsterdam, The Netherlands. Freeze-dried sponge tissue (200.42 g) was extracted with a mixture of methanol and dichloromethane (1:1, 1600 mL), and dichloromethane (2 × 3500 mL) at room temperature. The resulting greenish coloured crude extract (19.901 g) was partitioned between hexane (3 × 250 mL) and methanol (75 mL). The methanol extract was concentrated and further partitioned between ethyl acetate (3 × 250 mL) and water (75 mL), and finally the aqueous layer was extracted with 1-butanol (3 × 250 mL). After HPLC analysis, the obtained 1-butanol (1.935 g) and ethyl acetate (0.715 g) phases were combined and purified by gel permeation chromatography on Sephadex® LH-20 (Pharmacia Biotech) with MeOH as eluent. Samples were injected into a HPLC system (JASCO) equipped with a light-scattering detector SEDEX 75 (Sedere). The analytical column (5 μm, 4.6 × 250 mm) was prefilled with Kromasil RP-18 (Knauer). Fractions containing major secondary metabolites were collected and monitored by HPLC. Final purification was achieved by preparative HPLC. First separation was achieved by applying a linear gradient from 10% to 50% acetonitrile (containing 0.1% TFA) and H_2O (containing 0.1% TFA) over 40 minutes. The separation column (7 μm, 16 × 250 mm) was prefilled with Kromasil RP-18 (Knauer). Second separation was achieved by a stepwise gradient from 20% acetonitrile (containing 0.1% TFA) and H_2O (containing 0.1% TFA) over 5 minutes, which was then increased to 45% acetonitrile (containing 0.1% TFA) over 30 minutes. To complete the separation these conditions were held for 5 minutes. The separation column (10 μm, 16 × 250 mm) was prefilled with Kromasil RP-18 (Knauer). Two compounds **1** (14.5 mg) and **2** (44.8 mg) were isolated by the described method. For extraction, solvents were distilled prior to use, and gradient-grade solvents were used for chromatographic applications (Merck).

NMR Spectroscopy: The NMR spectroscopy experiments were carried out at 30 °C with Bruker Avance 400 (AWI) and Avance 500 spectrometers (Bruker BioSpin). Chemical shifts in the spectra recorded in [D_6]DMSO were referenced to the solvent signal (^{1}H 2.50 ppm and ^{13}C 39.5 ppm). The pulse programs from the standard Bruker library were used.

Mass Spectrometry: Mass spectral analyses were performed with ESI-TOF (LCT, Micromass), Q-TOF (Micromass), microTOF (Bruker Daltonic) and JMS-700 (JEOL) MS spectrometers.

UV Spectroscopy: UV spectra (MeOH) were recorded with a UVIKON 810P spectrometer (KONTRON).

IR Spectroscopy: IR spectra (KBr) were recorded with a Bruker EQUINOX 55 spectrometer (Bruker Optik).

Biological Assays: The extracts were tested for the feeding deterrence against the amphipod *Anonyx nugax* and the starfish *Asterias rubens*. *A. rubens* from the North Sea was used as a model organism because the two naturally occurring starfish at Kongsfjord (*Solaster endaca* and *Crossaster papposus*) could not be collected and kept in sufficient numbers under laboratory conditions. The crude extract was not consumed by the amphipods and starfish. Further assays were conducted using the amphipods because only limited amounts of sponge tissue were available. The extracts as well as pure compounds were tested at natural concentrations which is the extract yield per g dry weight (DW) sponge tissue. Natural concentrations were used to test ecologically relevant dosages. The amphipod feeding assay was conducted as choice experiment. One test

Table 2. Comparison of the MS and MS/MS analysis of the naturally occurring and synthetic haliclamine D; all results in this table were obtained with a Bruker microTOF MS spectrometer; the mass deviations for the daughter ions **2c** and **2d** are much larger since the intensities of these peaks were much smaller than those of **2a** and **2b** (by a factor of 20)

	Haliclamine (natural product)	Haliclamine (synthetic)
HRMS (ESI)	m/z = 457.4518, $C_{30}H_{55}N_2$, Δm = 0.3 ppm	m/z = 457.4494, $C_{30}H_{55}N_2$, Δm = 4.9 ppm
MS/MS of m/z = 457		
2a	m/z = 222.2213, $C_{15}H_{28}N$, Δm = 1.6 ppm	m/z = 222.2213, $C_{15}H_{28}N$, Δm = 1.6 ppm
2b	m/z = 236.2365, $C_{16}H_{30}N$, Δm = 3.1 ppm	m/z = 236.2362, $C_{16}H_{30}N$, Δm = 4.7 ppm
2c	m/z = 288.2644, $C_{20}H_{34}N$, Δm = 14.4 ppm	m/z = 288.2639, $C_{20}H_{34}N$, Δm = 16.3 ppm
2d	m/z = 302.2842, $C_{21}H_{36}N$, Δm = 20.0 ppm	m/z = 302.2886, $C_{21}H_{36}N$, Δm = 14.5 ppm

pellet containing crude extract or pure compounds and one control pellet without extract were offered to the amphipods at the same time. The weight difference between test pellet and control pellet was monitored and statistically analysed. The haliclamines C (**1**) and D (**2**) were tested at natural concentration [1.23 mg/g(DW) for **1** and 1.29 mg/g(DW) for **2**] and showed no significant feeding deterrence against *Anonyx nugax*.[21]

The extracts and pure compounds of *Haliclona viscosa* were tested for their antimicrobial activity against 5 bacterial strains (A to E) isolated from the vicinity of the sponge. Two were isolated from the water column (A and E), two from stones (B and C) and one from the sediment (D). The five bacterial strains were taxonomically assigned by sequence analysis of 16S rDNA. The isolates A, C and D are all γ-proteobacteria, isolate B is a Gram-positive bacterium and isolate E belongs to Cytophaga. The five bacterial strains are associated to the following species: A (*Psychrobacter* spp.), B (*Planococcus* spp.), C (*Colwellia* spp.), D (*Pseudoalteromonas* spp.) and E (*Polaribacter* spp.). According to the inhibition zones of the bacterial growth, extracts and pure compounds were classified in the following inhibition zone: a) weak (0 to 1 mm), b) moderate (1 to 3 mm), c) strong (3 to 7 mm) and d) very strong (7 to 15 mm). The extracts were tested at natural concentrations (see above). The haliclamines C (**1**) and D (**2**) were tested at a concentration of 5 mg/mL. The crude extract of *Haliclona viscosa* showed strong or very strong antimicrobial activity against all 5 bacterial strains. The activity was found in the 1-butanol/ethyl acetate phase (moderate to strong), whereas no activity was found in the hexane and water fraction. The haliclamines C (**1**) and D (**2**) showed a strong inhibition against bacterial strain E and a very strong inhibition against bacterial strain B.[16]

Haliclamine C (1): 14.5 mg (0.72×10^{-2} % of dry weight) has been isolated by previous methods. **MS of 1:** HRMS (FAB), m/z = 443.4392, $C_{30}H_{55}N_2$ Δm = 6.1 ppm, HRMS (ESI): m/z = 443.4395, $C_{30}H_{55}N_2$ Δm = 6.8 ppm. **MS of 1 + TFA:** HRMS (FAB), m/z = 557.4299, $C_{32}H_{55}F_3N_2O_2$ Δm = 0.9 ppm, HRMS (ESI): m/z = 557.4379, $C_{32}H_{55}F_3N_2O_2$ Δm = 15.2 ppm. IR (KBr) of **1**: $\tilde{\nu}_{max.}$ = 3420, 2926, 2857, 2361, 1684, 1457, 1292 and 1134 cm^{-1}. There were no significant UV absorptions above 210 nm. The NMR spectroscopic data are summarized in Table 1.

Haliclamine D (2): 44.5 mg (2.24×10^{-2} % of dry weight) has been isolated by previous methods. HRMS (FAB): m/z = 457.4534, $C_{31}H_{57}N_2$ Δm = 2.6 ppm, HRMS (ESI): m/z = 457.4546, $C_{31}H_{57}N_2$ Δm = 5.2 ppm. **MS of 2 + TFA:** HRMS (FAB), m/z = 571.4489, $C_{33}H_{57}F_3N_2O_2$ Δm = 6.6 ppm, HRMS (ESI): m/z = 571.4450, $C_{33}H_{57}F_3N_2O_2$ Δm = 0.2 ppm. IR (KBr) of **2**: $\tilde{\nu}_{max.}$ = 3422, 2928, 2855, 2361, 1683, 1458, 1201 and 1134 cm^{-1}. There were no significant UV absorptions above 210 nm. The NMR spectroscopic data are summarized in Table 1. For comparison, the synthetic haliclamine D (for details, see ref.[20]) was characterised by MS, MS/MS (see Table 2) and 2D NMR techniques.

Acknowledgments

This project (03F0254A) is part of a BMBF program (Verbundprojekt V-258 "Marine Naturstofforschung") involving the research groups of Köck (AWI), Lindel (LMU München, Germany), Pörtner (AWI) and Rachor (AWI), as well as of the BASF AG (Ludwigshafen, Germany). 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). We further thank Dr. Rainer Kerssebaum (Bruker BioSpin GmbH, Rheinstetten, Germany) for NMR measurement time, Dr. Rainer Wolf (BASF AG, Ludwigshafen, Germany) for the mass spectra, and Dr. Michael Assmann (AWI) for useful comments on an earlier version of the manuscript.

[1] G. Cimino, S. D. Stefano, G. Scognamiglio, G. Sodano, E. Trivellone, *Bull. Soc. Chim. Belg.* **1986**, *95*, 783−800.
[2] R. Sakai, T. Higa, C. W. Jefford, G. Bernardinelli, *J. Am. Chem. Soc.* **1986**, *108*, 6404−6405.
[3] B. J. Baker, P. J. Scheuer, J. N. Shoolery, *J. Am. Chem. Soc.* **1988**, *110*, 965−966.
[4] M. Jaspars, V. Pasupathy, P. Crews, *J. Org. Chem.* **1994**, *59*, 3253−3255.
[5] K. Sepčić, G. Guella, I. Mancini, F. Pietra, M. D. Serra, G. Menestrina, K. Tubbs, P. Maček, T. Turk, *J. Nat. Prod.* **1997**, *60*, 991−996.
[6] S.-S. Moon, J. B. MacMillan, M. M. Olmstead, T. A. Ta, I. N. Pessah, T. F. Molinski, *J. Nat. Prod.* **2002**, *65*, 249−254.
[7] S. Albrizio, P. Ciminiello, E. Fattorusso, S. Magno, J. R. Pawlik, *J. Nat. Prod.* **1995**, *58*, 647−652.
[8] R. J. Clark, K. L. Field, R. D. Charan, M. J. Garson, I. M. Brereton, A. C. Willis, *Tetrahedron* **1998**, *54*, 8811−8826.
[9] R. M. Adlington, J. E. Baldwin, G. L. Challis, R. J. Cox, G. J. Pritchard, *Tetrahedron* **2000**, *56*, 623−628.
[10] B.-N. Zhou, C. Slebodnick, R. K. Johnson, M. R. Mattern, D. G. I. Kingston, *Tetrahedron* **2000**, *56*, 5781−5784.
[11] C. L. Blackburn, D. J. Faulkner, *Tetrahedron* **2000**, *56*, 8429−8432.
[12] S. Aoki, K. Matsui, K. Tanaka, R. Satari, M. Kobayashi, *Tetrahedron* **2000**, *56*, 9945−9948.
[13] A. Randazzo, C. Debitus, L. Gomez-Paloma, *Tetrahedron* **2001**, *57*, 4443−4446.
[14] R. Sakai, S. Kohmoto, T. Higa, C. W. Jefford, G. Bernardinelli, *Tetrahedron Lett.* **1987**, *28*, 5493−5496.
[15] E. Fahy, T. F. Molinski, M. K. Harper, B. W. Sullivan, D. J. Faulkner, L. Parkanyi, J. Clardy, *Tetrahedron Lett.* **1988**, *29*, 3427−3428.

[16] H. Lippert, R. Brinkmeyer, T. Mülhaupt, K. Iken, *Polar Biol.* **2003**, *26*, 591−600.
[17] H. Lippert, *Ber. Polarforsch. Meeresforsch.* **2003**, *465*, 1−109.
[18] N. Fusetani, K. Yasumuro, S. Matsunaga, H. Hirota, *Tetrahedron Lett.* **1989**, *30*, 6891−6894.
[19] N. Fusetani, N. Asai, S. Matsunaga, K. Honda, K. Yasumuro, *Tetrahedron Lett.* **1994**, *35*, 3967−3970.
[20] C. Timm, M. Köck, manuscript in preparation.
[21] H. Lippert, K. Iken, C. A. Volk, M. Köck, E. Rachor, *J. Exp. Mar. Biol. Ecol.*, in press.
[22] J. Baldwin, R. Whitehead, *Tetrahedron Lett.* **1992**, *33*, 2059−2062.
[23] J. Baldwin, T. Claridge, A. Culshaw, F. Heupel, V. Lee, D. Spring, R. Whitehead, *Chem. Eur. J.* **1999**, *5*, 3154−3161.
[24] C. A. Volk, M. Köck, *Org. Lett.* **2003**, *5*, 3567−3569.
Received January 14, 2004

