Bioactive Alkaloids from the Tropical Marine Sponge *Axinella carteri*

A. Supriyono[a,f], B. Schwarz[a], V. Wray[b], L. Witte[c], W. E. G. Müller[d], R. van Soest[e], W. Sumaryono[f] and P. Proksch[a]

[a] Julius-von-Sachs-Institut für Biowissenschaften, Lehrstuhl für Pharmazeutische Biologie, Mittlerer Dallenbergweg 64, D-97082 Würzburg, Bundesrepublik Deutschland
[b] Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Bundesrepublik Deutschland
[c] Institut für Pharmazeutische Biologie, Mendelssohnstraße 1, D-38106 Braunschweig, Bundesrepublik Deutschland
[d] Institut für Physiologische Chemie, Duesbergweg 6, D-55099 Mainz, Bundesrepublik Deutschland
[e] Instituut voor Systematiek en Populatie Biologie, Zoölogisch Museum, P.O. Box 94766, 1090 GT Amsterdam, Netherlands
[f] Life Sciences, BPPT, Jl. M. H. Thamrin No. 8, Jakarta, Indonesia

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Analysis of the tropical marine sponge *Axinella carteri* afforded six unusual alkaloids, including the new brominated guanidine derivative 3-bromo-hymenialdisine. The structure elucidation of the new alkaloid is described. The alkaloid patterns of sponges collected in Indonesia or in the Philippines were shown to be qualitatively identical suggesting *de novo* synthesis by the sponge or by endosymbiontic microorganisms rather than uptake by filter-feeding. All alkaloids were screened for insecticidal activity as well as for cytotoxicity. The guanidine alkaloids hymenialdisine and debromohymenialdisine exhibited insecticidal activity towards neonate larvae of the polyphagous pest insect *Spodoptera littoralis* (LD_{50}s of 88 and 125 ppm, respectively), when incorporated into artificial diet and offered to the larvae in a chronic feeding bioassay. The remaining alkaloids, including the new compound, were inactive in this bioassay. Cytotoxicity was studied *in vitro* using L5178y mouse lymphoma cells. Debromohymenialdisine was again the most active compound (ED_{50} 1.8 μg/ml) followed by hymenialdisine and 3-bromohymenialdisine, which were essentially equitoxic and exhibited ED_{50}s of 3.9 μg/ml in both cases. The remaining alkaloids were inactive against this cell line.

Introduction

Marine invertebrates are known as rich sources of natural products that attract interest due to unique chemical features (*e.g.* frequent occurrence of halogen substituents), as well as due to their pronounced biological activities (*e.g.* cytotoxicity) which suggest potential value as lead structures for the development of new pharmaceuticals (Munro *et al.,* 1987; Sarma *et al.,* 1993; Proksch, 1994). The most advanced marine natural product with pharmacological activity is bryostatin 1 from the bryozoan *Bugula neritina.* Bryostatin 1 shows pronounced anti-tumor activity and has recently successfully completed phase I clinical studies in the U.S.A. (Schmitz, 1994). Marine natural products, however, are not only important with regard to their pharmacological properties, but are also a promising source of new insecticides. Nereistoxin, for example, served as a model for development of the commercially available insecticide Padan.

In the present study we describe several unusual alkaloids isolated from the marine sponge *Axinella carteri* (Dendy, 1889) (Axinellidae) collected in Indonesia and the Philippines, and report on the insecticidal and cytotoxic properties of several of these secondary metabolites.

Reprint requests to Prof. Dr. P. Proksch.
Telefax: 0931-8886158.

Materials and Methods

Collection of sponges

Specimens of *A. carteri* were collected in April 1993 (island of Java, Indonesia, registration No. ZMA POR. 10611) and in April 1994 (island of Sumatra, Indonesia, registration No. ZMA POR.

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10924 and island of Mindoro, Philippines, registration No. ZMA POR. 10984) by diving or by snorkelling. Voucher specimens are on file under the given registration numbers in the Zoölogisch Museum, Amsterdam.

Isolation and identification of compounds

Freshly collected sponges were directly immersed in MeOH. Following repeated reextraction of the sponge tissue with MeOH, the respective extracts were combined, evaporated to dryness and partitioned between H_2O/EtOAc and H_2O/*n*-BuOH. Both organic fractions were taken to dryness and separately chromatographed on a silica gel column (mobile phase CH_2Cl_2/MeOH/NH_3 90:8:2 or 70:28:2 v/v). Fractions (20 ml) were collected and monitored by TLC on precoated silica gel plates (Merck, Darmstadt, Germany) using the same solvent. Alkaloids were detected by their absorbance under UV_{254} nm. Final purification was achieved by column chromatography on Sephadex LH-20 with MeOH or with mixtures of MeOH/CH_2Cl_2 as eluents and by column chromatography on reversed phase lobar columns (Merck, Darmstadt, Germany) with mixtures of MeOH, H_2O and TFA (*e.g.* 70:27:3 v/v) as eluents.

^1H NMR and ^{13}C NMR spectra were recorded on Bruker AM 300 or WM 400 NMR spectrometers, respectively. All 1D or 2D spectra were obtained using the standard Bruker software. Mass spectra (FAB, glycerol as matrix) were measured on a Finnigan MAT 8430 mass spectrometer. UV spectra were recorded in MeOH.

HPLC analysis

For HPLC analysis crude MeOH extracts were diluted with H_2O and injected into a HPLC system (Pharmacia, LKB, Sweden) coupled to a photodiode-array detector (Waters Millipore GmbH, Eschborn, Germany). The separation was achieved by applying a linear gradient from 100% A (10% MeOH, 90% H_2O adjusted to pH 2 with phosphoric acid) to 100% B (MeOH) in 30 min following an isocratic segment at 100% A during the first 5 min of each run. Routine detection was at 254 nm. The separation column (125 × 4 mm, i.d.) was prefilled with Nova-Pak C-18 (4 μm) (Waters Millipore GmbH, Eschborn, Germany). Quantification was achieved by the external standard method using previously isolated and purified compounds.

Experiments with insects

Larvae of *Spodoptera litoralis* were from a laboratory colony reared on artificial diet under controlled conditions as described previously (Srivastava and Proksch, 1991). Feeding studies were conducted with neonate larvae (n = 20) that were kept on an artificial diet which had been treated with various concentrations of the compounds under study. After 6 days, survival of the larvae and weight of the surviving larvae were protocolled and compared to controls. LD_{50}s were calculated from the dose-response curves by probit analysis.

Cytotoxicity studies

L5178y mouse lymphoma cells were grown in Eagle's minimal essential medium supplemented with 10% horse serum in roller tube culture as described previously (Kreuter *et al.*, 1992). For the dose-response experiments, 5 ml cultures were initiated by inoculation of 5×10^3 cells/ml and were incubated at 37 °C for 72 h. Controls showed a population doubling time of 10.5 h. Cell growth was determined by cell count with a Cytocomp counter (128-channel counter, system Michaelis, Mainz, Germany) with a 32-channel size-distribution plotter.

Results and Discussion

A. carteri is a thickly and irregularly flabellate orange or orange-brown sponge, occurring in reefs over a large part of the Indo-West Pacific (*e.g.* Red Sea, Madagascar, Seychelles, India, Indonesia, Australia, New Caledonia). Its grooved and corrugated surface and robust styles make it a characteristic and easily recognizable sponge. Originally *A. carteri* was described in the genus *Acanthella* (Dendy, 1889, Gulf of Manaar, India). Morphological (Hooper and Levi, 1993) and chemical (Braekman *et al.*, 1994) comparisons of true *Acanthella* species with *A. carteri* have made it, however, abundantly clear that the latter does not belong to the genus *Acanthella*.

Sponges of the genus *Axinella* are a remarkable source of unusual natural products and are espe-

cially rich in alkaloids that include guanidine, as well as pyrrole, derivatives (Sharma and Burkholder, 1971; Forenza *et al.*, 1971; Garcia *et al.*, 1973; Sharma and Magdoff-Fairchild, 1977; Sharma *et al.*, 1980; Cimino *et al.*, 1982; Kobayashi *et al.*, 1988; Pettit *et al.*, 1990). Extraction of the sponge *A. carteri* collected at the island of Java (Indonesia) and chromatographic separation of the crude extract yielded the known alkaloids debromohymenialdisine (**1**), hymenialdisine (**2**), dibromophakellin (**4**), hymenidin (**5**) and oroidin (**6**) (Fig. 1). All compounds were readily identified from their spectroscopic data and by comparison with published data (Sharma and Burkholder, 1971; Forenza *et al.*, 1971; Garcia *et al.*, 1973; Sharma and Magdoff-Fairchild, 1977; Sharma *et al.*, 1980; Cimino *et al.*, 1982; Kitagawa *et al.*, 1983; Kobayashi *et al.*, 1986; Kobayashi *et al.*, 1988; Pettit *et al.*, 1990).

In addition to the known compounds a further previously unknown guanidine-based alkaloid (**3,** Fig. 1) was isolated that was identified as the 3-bromo derivative of hymenialdisine (**2**). The new compound (**3**) showed a cluster of quasi-molecular ions $(M+H)^+$ at *m/z* 402/404/406 in the FAB mass spectrum, when recorded in the positive mode, that was typical of a dibromo compound. The online-recorded UV spectrum of **3** was identical to that of hymenialdisine (**2**) and debromohymenialdisine (**1**), indicating that **3** was an additional guanidine-based alkaloid. The ^{1}H NMR spectrum of **3** resembled those of hymenialdisine (**2**) and debromohymenialdisine (**1**) (Table I) with the excep-

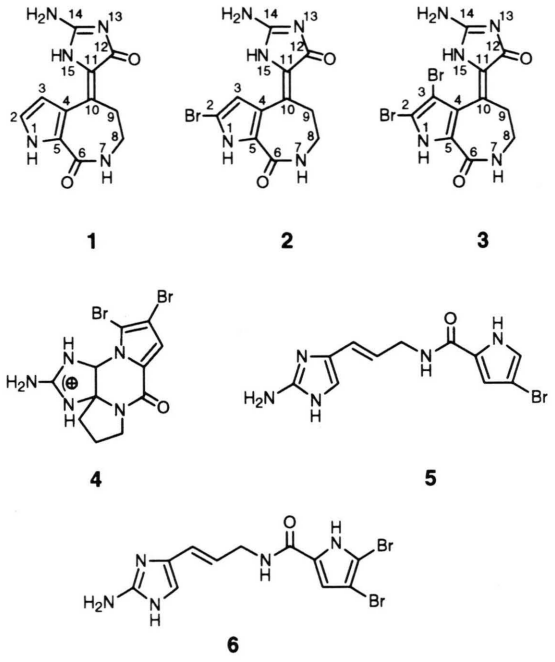

Fig. 1. Structures of alkaloids isolated from *A. carteri*. **1:** Debromohymenialdisine; **2:** hymenialdisine; **3:** 3-bromohymenialdisine; **4:** dibromophakellin; **5:** hymenidin; **6:** oroidin.

Table I. ^{1}H and ^{13}C NMR, UV and MS data of **1–3**.[a]

^{1}H NMR

^{1}H	**1**	**2**	**3**
H-2	7.11 t *J*(H-2–H-1) 2.5 Hz, *J*(H-2–H-3) 2.5 Hz		
H-3	6.51 t *J*(H-3–H-2) 2.5 Hz *J*(H-3–H-1) 2.5 Hz	6.54 s	
H-8 AB	3.30 br	3.30 br	3.27 br
H-9 AB	3.30 br	3.30 br	3.27 br
1-NH	12.1 br	12.8 br	13.4 br
7-NH	8.06 t	8.06 t	8.08 t
14-NH_2 }	9.0 br,	8.9 br,	8.6 br, 9.3 br,
15-NH }	11.8 (very br)	11.9 (very br)	11.0 br

s, singlet; d, doublet; t, triplet; br, broad.

[a] The compound numbering follows Fig. 1. NMR spectra were measured in deuterated DMSO, chemical shifts are given in ppm. Positive ion fast atom bombardment mass spectra were measured with glycerol as matrix.

^{13}C NMR

^{13}C	**1**	**2**	**3**
C-2	122.6 d	104.9 s	107.3 s
C-3	109.5 d	112.2 d	98.5 s
C-4	[b]	[b]	[b]
C-5	[b]	[b]	[b]
C-6	162.9 s	162.2 s	163.3 s
C-8	38.9 t	39.0 t	[c]
C-9	31.5 t	32.1 t	35.1 t
C-10	[b]	[b]	[b]
C-11	[b]	[b]	[b]
C-12	163.9 s	163.7 s	163.3 s
C-14	154.7 s	154.7 s	154.6 s

s, singlet; d, doublet; t, triplet.

1: UV (λ_{max}): 265, 347; FAB-MS (*m/z*, rel. int.): 246 (68) $(M+H)^+$; **2:** UV (λ_{max}): 259, 349; FAB-MS (*m/z*, rel. int.): 326/324 (42/40) $(M+H)^+$; **3:** UV (λ_{max}): 269, 332; FAB-MS (*m/z*, rel. int.): 402/404/406 (52/100/54) $(M+H)^+$.

[b] The quaternary carbon signals of C-4, C-5, C-10, C-11 could not be unambiguously assigned and were at 120.3, 120.6, 126.7 and 129.9 in **1,** 121.6, 121.6, 128.3 and 128.3 in **2,** and 120.8, 124.2, 125.3 and 127.2 in **3.**

[c] This signal was hidden under the solvent signal.

tion of the aromatic proton signals at C-2 and/or C-3 that were absent in the spectrum of **3.** On comparing the ^{13}C NMR spectrum of **3** with those obtained for **1** or **2** the only differences observed were with regard to the number of aromatic doublets and singlets (Table I). Whereas the ^{13}C NMR spectrum of **1** contained two doublets at 120 (C-2) and 109 ppm (C-3), respectively, inspection of the ^{13}C NMR spectrum of **2** indicated one singlet at 105 ppm (C-2) and one doublet at 115 ppm (C-3) whereas the ^{13}C NMR spectrum of **3** revealed the presence of two singlets at 99.5 (C-2) and 107 ppm (C-3) (Table I). Thus the structure of the new alkaloid **3** was assigned as the 3-bromo derivative of hymenialdisine (**2**).

Reversed phase HPLC proved to be an excellent tool for the separation and quantification of the alkaloids present in crude extracts of *A. carteri* (Fig. 2). The alkaloid patterns of different specimens of *A. carteri* collected along the coast of Java or Sumatra (both in Indonesia) or in the Philippines proved to be qualitatively remarkably homogeneous when analyzed by HPLC (Fig. 2). The chemical similarity of the alkaloid patterns elucidated for the different samples of *A. carteri* is remarkable in view of the geographical distance of approximately 1500–2000 km between the different collection sites. This chemical similarity argues, in our opinion, for a *de novo* synthesis either by the sponge itself or by symbiontic microorganisms, whereas filter-feeding as an alternative source of alkaloids seems less likely by comparison.

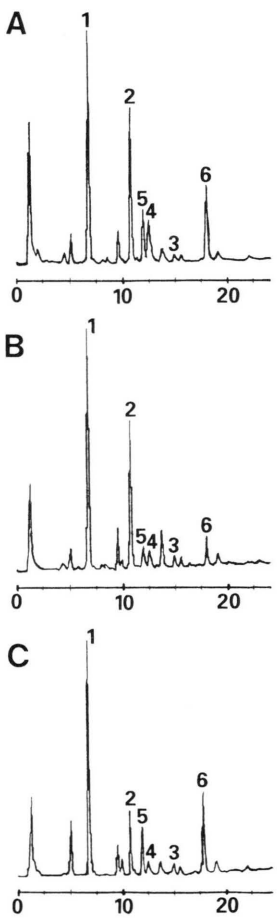

Fig. 2. HPLC charts of crude extracts from specimens of *A. carteri* collected along the coasts of: A) Java (Indonesia), B) Sumatra (Indonesia), C) Mindoro (Philippines).

All compounds isolated from *A. carteri* (**1–6**) were analyzed for insecticidal activity, as well as for cytotoxicity using mouse lymphoma cells grown *in vitro.* Insecticidal activity was studied by incorporating each compound into artificial diet at an arbitrarily chosen concentration (300 ppm) and offering the spiked diet to neonate larvae of the vigorous pest insect *S. littoralis* in a chronic feeding experiment. After 6 days of exposure, larval survival and larval weight were monitored and compared to controls. All compounds except **1** and **2** were inactive with regard to survival (80–100% larval survival compared to controls) of the larvae of *S. littoralis* (data not shown). Debromohymenialdisine (**1**) and hymenialdisine (**2**), however, caused complete larval mortality at a concentration of 300 ppm each.

In a subsequent experiment the active compounds debromohymenialdisine (**1**) as well as hymenialdisine (**2**) were analyzed for insecticidal activity at a range of concentrations (26–132 ppm). From the dose-response curves obtained (Fig. 3) the LD_{50}s of compounds **1** and **2** were calculated by probit analysis as 88 ppm and 125 ppm respectively. The ED_{50}s (doses which reduce larval weight by half) were similar for both compounds (33 and 36 ppm), as indicated by probit analysis (data not shown). Bromination of pyrrole moiety of debromohymenialdisine (**1**) apparently leads to reduced insecticidal activity as evident from the comparison of the insecticidal activity of debromohymenialdisine (**1**) compared to its bromo (**2**) and dibromo derivative (**3**) (the latter being inactive in the range of doses analyzed).

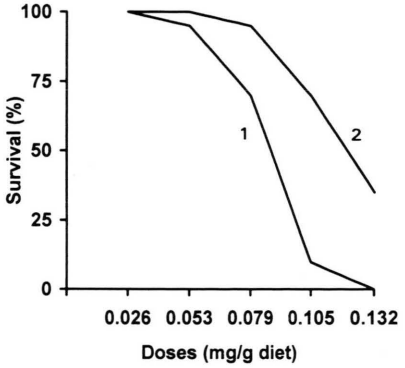

Fig. 3. Survival of neonate larvae of *S. littoralis* (n = 20) on artificial diet spiked with increasing amounts of debromohymenialdisine (**1**) and hymenialdisine (**2**). Survival was monitored after 6 days. Numbers of compounds follow Fig. 1.

The LD_{50} of the most active compound debromohymenialdisine (**1**) compares favourably, for example, to the well known natural insecticide precocene II (LD_{50} of 275 ppm towards neonate larvae of *S. littoralis*) (Srivastava and Proksch, 1991) originally isolated from *Ageratum houstonianum* (Asteraceae) (Bowers *et al.*, 1976). Recently, another insecticidal compound, ulosanthoin, was isolated from the Carribean sponge *Ulosa ruetzleri* (van Wagenen *et al.*, 1993). The LD_{50} of the phosphorylated hydantoin derivative ulosantoin towards larvae of the tobacco hornworm *(Manduca sexta)* was reported to be 6 ppm in a similar experimental set-up as used for this study (van Wagenen *et al.*, 1993). Ulosantoin thus appears to be significantly more active than debromohymenialdisine (**1**). However, on comparing the insecticidal activity of debromohymenialdisine (**1**) and ulosantoin differences in susceptibility of the test organisms used in both studies have to be taken into consideration. Whereas the tobacco hornworm is an oligophagous insect that feeds exclusively on tobacco plants the larvae of *S. littoralis* are polyphagous and adapted to cope with a much broader variety of dietary natural products (Moussa *et al.*, 1960). Thus, larvae of *S. littoralis* are a far more vigorous model for studies on natural insecticides than larvae of the tobacco hornworm, that are adapted only to the rather narrow set of natural products from tobacco and can be expected to react more sensitively to any other foreign metabolites than larvae of *S. littoralis*.

Cytotoxicity of the alkaloids from *A. carteri* was assessed *in vitro* with a mouse lymphoma cell line using the microculture tetrazolium (MTT) assay. Each alkaloid was tested for its cytotoxic activity at a range of concentrations (0.3–20 μg/ml). The guanidine alkaloids **1–3** were found to be active against the cell line chosen, whereas dibromophakellin (**4**) as well as the pyrrole derivatives hymenidin (**5**) and oroidin (**6**) were inactive (data not shown). From the dose-response curves obtained (Fig. 4), the ED_{50}s of the guanidines were calculated by probit analysis as 1.8 μg/ml for the most active compound debromohymenialdisine (**1**). Hymenialdisine and 3-bromohymenialdisine were comparable with regard to cytotoxicity and exhibited an ED_{50} of 3.9 μg/ml in both cases. Whereas this is the first report on the cytotoxic properties of 3-bromohymenialdisine (**3**), both debromohymenialdisine (**1**) and hymenialdisine (**2**) had previously been shown to be active in the murine p388 lymphocytic leukemia system with very similar ED_{50}s to those found here (Pettit *et al.*, 1990). It is interesting to note that the cytotoxicity of at least compound **3** is not paralleled by insecticidal activity towards neonate larvae of *S. littoralis* (Fig. 3). Thus, it is possible that the observed insecticidal activity of **1** and **2** (Fig. 3) is likewise not caused by general cytotoxicity but may rather be due to a different, yet unknown, mode of action.

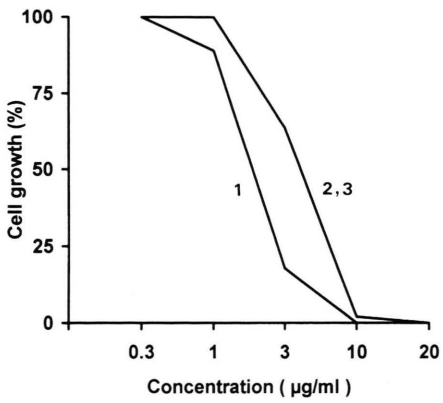

Fig. 4. Growth inhibition of L5178y mouse lymphoma cells following incubation with increasing amounts of debromohymenialdisine (**1**), hymenialdisine (**2**) and 3-bromohymenialdisine (**3**). Cell growth was monitored after 72 h. Numbers of compounds follow Fig. 1.

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