Bioactive Pyridoacridine Alkaloids from the Micronesian Sponge *Oceanapia* sp.

Claudia Eder,[†] Peter Schupp,[†] Peter Proksch,*[,†] Victor Wray,[‡] Klaus Steube,[§] Christa E. Müller,[∥] Wolfram Frobenius,[∥] Markus Herderich,[∥] and Rob W. M. van Soest[⊥]

Julius-von-Sachs-Institut für Biowissenschaften, Lehrstuhl für Pharmazeutische Biologie, Mittlerer Dallenbergweg 64, D-97082 Würzburg, Germany, Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Germany, Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany, Institut für Pharmazie und Lebensmittelchemie, Lehrstuhl für Pharmazeutische Chemie, Am Hubland, 97074 Würzburg, Germany, and Instituut voor Systematiek en Populatie Biologie, Zoölogisch Museum, P.O. Box 94766, 1090 GT Amsterdam, The Netherlands

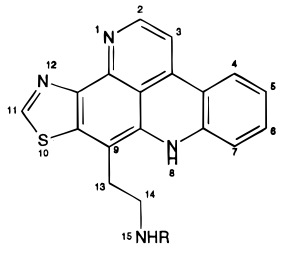
Received June 2, 1997

The Micronesian sponge *Oceanapia* sp. afforded three pyridoacridine alkaloids: the known compounds kuanoniamine C (**1**) and kuanoniamine D (**2**), as well as the new *N*-deacyl derivative (**3**) of the kuanoniamines. Compounds **1** and **2** exhibited insecticidal activity toward neonate larvae of the polyphagous pest insect *Spodoptera littoralis* (LC_{50} of 156 and 59 ppm, respectively), when incorporated into artificial diet. Both compounds also showed toxicity in the brine shrimp lethality test with a LC_{50} of 37 μg/mL (compound **1**) and 19 μg/mL (compound **2**), respectively. The *N*-deacyl derivative did not show any remarkable effect in both bioassays. Cytotoxicity of the alkaloids was studied in vitro, using two human cell lines. The new derivative (**3**) appeared to be active in the same range of concentrations as kuanoniamine C (**1**) and D (**2**). The IC_{50} of **3** was 1.2 μg/mL toward HeLa cells and 2.0 μg/mL toward MONO-MAC 6 cells. In receptor binding assays compound **2** showed affinity to A_1- and A_{2A}-adenosine receptors with K_i values of 2.94 and 13.7 μM, respectively. Compound **1** was less active than compound **2**, whereas compound **3** showed no affinity toward adenosine receptors. In addition, compounds **1**−**3** exhibited moderate affinity to benzodiazepine binding sites of $GABA_A$ receptors.

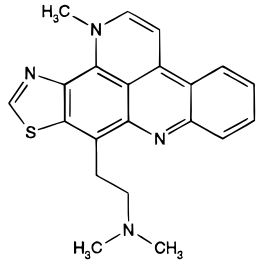
Marine invertebrates, such as sponges and ascidians, are often brightly colored due to the presence of pyridoacridine alkaloids.[1] Pyridoacridines are characterized by an 11*H*-pyrido[4,3,2-*mn*]acridine moiety and have so far only been reported from marine organisms.[1] The colors exhibited by pyridoacridine alkaloids may vary depending on their pH. This physicochemical property is correlated with the presence of at least two basic nitrogens in the aromatic ring systems.[1]

Like many other marine natural products, pyridoacridine alkaloids are known to exhibit a range of different biological activities, including cytotoxicity,[1] inhibition of topoisomerase II,[2] anti-HIV activity,[3] Ca^{2+} release activity,[4] and intercalation of DNA.[5]

In this paper, we describe the isolation of three pyridoacridine alkaloids from the Micronesian sponge *Oceanapia* sp. (order Haplosclerida, family Phloeodictyidae) and report on their insecticidal activity and cytotoxic properties as well as on their specific binding to adenosine receptors and benzodiazepine binding sites of $GABA_A$ receptors.[6] Kuanoniamines C (**1**) and D (**2**) have been previously reported from an undescribed Micronesian tunicate species and its prosobranch mollusk predator *Chelynotus semperi*[7] as well as from another tunicate of the genus *Cystodytes*.[5] Compound **1** is also known from a deep water sponge of the genus *Stelleta* collected in the Bahamas.[8] The *N*-deacyl derivative of the kuanoniamines (**3**) is a new natural product.



1: R = COC_2H_5
2: R = $COCH_3$
3: R = H



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The marine sponge *Oceanapia* sp. was collected by snorkeling (2−4 m depth) at Truk Lagoon, Micronesia. Samples were frozen immediately and freeze-dried prior to extraction. From the methanol-soluble material, compounds **1**−**3** were isolated using a fractionation scheme guided by insecticidal activity against neonate larvae of the pest insect *Spodoptera littoralis* and toxicity against the brine shrimp *Artemia salina*. Compounds **1** and **2** were identified from their spectroscopic data and comparison with published data.

Compound **3** exhibited the same UV spectrum as **1** or **2**, indicating that **3** was a further pyridoacridine alkaloid of the kuanoniamine type. From the high-resolution mass spectrum of **3** the molecular formula $C_{18}H_{14}N_4S$ was established. Complete elucidation of the

* To whom correspondence should be adressed. Tel.: 0049/931-8886174. Fax: 0049/931-8886182. E-mail: Proksch@botanik.uni-wuerzburg.de.
[†] Julius-von-Sachs-Institut für Biowissenschaften.
[‡] Gesellschaft für Biotechnologische Forschung mbH.
[§] Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH.
[∥] Institut für Pharmazie und Lebensmittelchemie.
[⊥] Instituut voor Systematiek en Populatie Biologie.

S0163-3864(97)00270-X CCC: $15.00
Published on Web 02/03/1998

Table 1. *In Vitro* Cytotoxicity of the Kuanoniamine Derivatives (IC_{50} Values, μg/mL) to HeLa and MONO-MAC 6 Tumor Cells in the [^{3}H]Thymidine Incorporation Assay (Mean ± SD, $n = 3$)

	compound		
cell line	**1**	**2**	**3**
HeLa	5.1 ± 1.3	1.4 ± 0.7	1.2 ± 0.2
MONO MAC-6	1.2 ± 0.4	0.8 ± 0.1	2.0 ± 0.5

structure of **3** was achieved by comparison of the ^{1}H and ^{13}C NMR spectra of the new compound with those of **1** and **2**. Whereas the ^{1}H NMR spectrum of **3** showed the same set of signals in the aromatic region as those reported for kuanoniamine D (**2**), it differed from that of **2** in the absence of signals originating from the *N*-acetyl substituent. This is in accordance with the ^{13}C NMR spectrum of **3**, which in contrast to those of **1** or **2** lacked carbon signals that could be ascribed to *N*-acyl substituents. The structure of **3** was confirmed by comparison of the NMR data of the isolated compound with those of the semisynthetic compound, obtained by acid hydrolysis of kuanoniamine C (**1**). Hydrolysis was carried out in order to provide a sufficient quantity of **3** for bioassays. The occurrence of the new compound (**3**) in *Oceanapia* sp. was evident from the HPLC analysis of the crude methanol extract, suggesting that **3** is a true natural product and is not formed as an artifact of **1** or **2** during isolation.

The insecticidal activity of **1**−**3** was studied by incorporating the compounds into an artificial diet over a range of five or six concentrations and offering the spiked diet to neonate larvae of the vigorous pest insect *Spodoptera littoralis* in a chronic feeding experiment. After 6 days of exposure the survival rate of the larvae was determined. From the dose−response curves obtained, LC_{50} values of 156 ppm [±0.46 (SE)] for kuanoniamine C (**1**) and 59 ppm [±0.30 (SE)] for kuanoniamine D (**2**) were calculated by probit analysis. The new compound (**3**) was tested up to a concentration of 934 ppm, but in contrast to compounds **1** and **2**, no significant insecticidal activity could be observed. Only the growth of the larvae was reduced. This can be expressed by an ED_{50} value of 141 ppm [±0.13 (SE)] for compound **3**.

Compounds **1**−**3** were also tested in the brine shrimp assay. After 24 h of exposure, the number of surviving animals was counted. LC_{50} values calculated from the dose−response curves by probit analysis were 37 μg/mL [±17 (SD)] for compound **1** and 19 μg/mL [±4 (SD)] for compound **2**. Compound **3** did not show any toxic effect up to 100 μg/mL. No LC_{50} of **3** could be obtained due to limited solubility at concentrations >100 μg/mL.

Several pyridoacridine alkaloids have been reported to exhibit significant cytotoxicity toward murine and human tumor cell lines.[1] In this study, we analyzed the effects of compounds **1**−**3** on cell growth at two different human cell lines using [^{3}H]thymidine incorporation. This cytotoxicity assay is used to determine the capability of cells to synthesize DNA during the cell cycle. The validity of the method applied in this study has been documented previously.[9] Table 1 summarizes the results obtained with the two cell lines. The small amount of ethylene glycol monomethyl ether (0.1%) used to solubilize the compounds did not affect growth of the tumor cells. Each alkaloid was tested for its cytotoxic activity at a range of concentrations (0.1−20 μg/mL).

Suppression of [^{3}H]thymidine incorporation into cells treated with compounds **1**−**3** was observed for each of the two cell lines. Intercalation with DNA has been demonstrated previously for dercitin (**4**),[10,11] a marine natural product closely related to the kuanoniamines. On the basis of the obvious structural similarities of compounds **1**−**3** with dercitin, it may be hypothesized that the kuanoniamines also interact with DNA by intercalation.

The cytotoxicity data are not paralleled by the insecticidal activity toward neonate larvae of *S. littoralis* or the toxic activity against *A. salina*; in the latter test systems the new derivative was nearly inactive, but it showed similar activity in the cytotoxicity assay to kuanoniamine C and D. Thus, it is possible that the different activities are not caused by a general cytotoxicity but may be due to a different mode of action.

A number of mostly nitrogen-containing, heterocyclic compounds including alkaloids such as the methylxanthines theophylline and caffeine[12,13] are known to bind to adenosine receptors and block them. Several classes of potent adenosine receptor antagonists share a flat aromatic bi- or tricyclic ring structure containing several nitrogen atoms and an exocyclic amino group as structural features,[14] e.g., adenine derivatives[12,13] and amino-substituted pyrrolo[2,3-*d*]pyrimidines,[14,15] pyrimido[4,5-*b*]indoles,[14,15] and triazoloquinoxalines.[13,14] On the basis of the structural similarities of the kuanoniamines to known adenosine receptor ligands we decided to test compounds **1**−**3** in radioligand binding assays with the high-affinity adenosine receptor subtypes A_1 and A_{2A} of rat brain. All compounds exhibited specific binding to A_1- as well as to A_{2A}-adenosine receptors (Table 2). The acetyl derivative kuanoniamine D (**2**) was more potent than its homologue, the propionic acid amide kuanoniamine C (**1**), at both receptor subtypes, showing a higher percentage of radioligand displacement at a test concentration of 25 μM. The amides **1** and **2** were more potent than amine **3**. Competition curves were determined for **2** in order to obtain K_i values. Compound **2** exhibited a K_i value of 2.94 μM at A_1-adenosine and 13.7 μM at A_{2A}-adenosine receptors, thus being more potent than the classical adenosine receptor antagonists theophylline and caffeine, at least at the A_1-receptor subtype.[16] Kuanoniamine D (**2**) was about 5-fold more potent at A_1- as compared to A_{2A}-adenosine receptors and thus exhibits selectivity for the A_1-receptor subtype, in contrast to the methylxanthines theophylline and caffeine, which are virtually nonselective,[16] and in contrast to compounds **1** and **3**, which were slightly A_{2A}-selective.

All adenosine receptor agonists known to date are derivatives of the physiological nucleoside adenosine,[12,13] and an intact ribose moiety is essential for agonistic activity. Adenine derivatives in which the ribose is replaced by an alkyl or aryl substituent are adenosine receptor antagonists.[12,13] Therefore, it is likely that the alkaloids of the kuanoniamine type are adenosine receptor antagonists rather than agonists.

Several classes of compounds that exhibit affinity for adenosine receptors also interact with the benzodiazepine binding sites of $GABA_A$ receptors.[16] Therefore,

Table 2. Affinity of Kuanoniamine Derivatives and Methylxanthines to Adenosine Receptors

	percent inhibition of radioligand binding ± SEM[a] at concentration indicated		K_i ± SEM[a] [μM]	
compd	A_1-receptor vs [^{3}H]CHA rat brain cortical membranes	A_{2A}-receptor vs [^{3}H]CGS21680 rat brain striatal membranes	A_1-receptor vs [^{3}H]CHA rat brain cortical membranes	A_{2A}-receptor vs [^{3}H]CGS21680 rat brain striatal membranes
1	34 ± 4 (25 μM)	43[b] (25 μM)	nd[c]	nd
2	68 ± 10 (25 μM)	56 ± 1 (25 μM)	2.94 ± 0.02	13.7 ± 0.6
3	36 ± 1 (100 μM)	46 ± 5 (100 μM)	nd	nd
theophylline	nd	nd	26[d]	22[d]
caffeine	nd	nd	17[d]	9.4[d]

[a] Results from at least three independent experiments in triplicate unless otherwise stated. [b] Single experiment (in triplicate). [c] nd = not determined. [d] Values from Müller et al. (ref 15).

compounds **1**−**3** were further investigated in benzodiazepine radioligand binding assays at rat brain cortical membranes. Compounds **1**−**3** inhibited the binding of [^{3}H]diazepam by 39%, 32%, and 30%, respectively, at a test concentration of 25 μM each. Thus, the affinity of **2** for the benzodiazepine site was weaker than its affinity for adenosine receptors. For the less potent compound **1** the affinity for adenosine receptors and for the benzodiazepine site were similar. The affinity of **3** was stronger for the benzodiazepine binding site than for the adenosine receptors. All of the compounds studied were more potent benzodiazepine ligands than the methylxanthines theophylline (K_i = 565 μM) and caffeine (K_i = 376 μM).[16]

Although compounds **1**−**3** exhibit a range of biological activities in eukaryotic cells, they did not show any growth inhibiting activity against Gram-positive and Gram-negative bacteria nor any antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum*.

Experimental Section

General Experimental Procedures. ^{1}H NMR and ^{13}C NMR spectra (chemical shifts in ppm) were measured on Bruker AM 300 and ARX 400 NMR spectrometers, respectively. Mass spectra (ESI-MS) were recorded on a Finnigan MAT TSQ-7000 triple stage quadrupole mass spectrometer. The temperature of the heated capillary (20 V) was 200 °C and the electrospray capillary voltage was set to 3.5 kV. Nitrogen served both as sheath (70 psi) and auxiliary gas; argon served as collision gas. High-resolution mass spectra were recorded on a Finnigan MAT 95 mass spectrometer. UV spectra were recorded in methanol. For HPLC analysis, samples were injected into an HPLC system equipped with a photodiode-array detector (Gynkotek, Germany). Routine detection was at 254 and 307 nm. The separation column (125 × 4 mm, i.d.) was prefilled with Eurospher C-18 (Knauer GmbH, Germany). Separation was achieved by applying a linear gradient from 100% H_2O (adjusted to pH 2 with phosphoric acid) to 100% MeOH in 40 min. TLC was performed on precoated TLC plates with Si gel 60 F_{254} and Si gel RP-18 F_{254} (Merck, Darmstadt, Germany). Compounds were detected from their UV absorbance at 254 and 366 nm. Solvents were distilled prior to use, and spectral-grade solvents were used for spectroscopic measurements.

Animal Material. The dull-red sponge has a fistular growth form, consisting of an irregular turnip-shaped main body up to 8 cm high and 4 cm in diameter, from which issue several long hollow fistules 7−8 cm long and up to 1 cm in diameter. The surface is optically smooth and slightly rough when dry. The consistency of the freeze-dried specimen is very fragile, crumbly, and dusty. The skeleton of the periphery is a halichondroid, tangential, multilayered crust of intercrossing single spicules. This is carried by thin subectosomal spicule tracts of 2−3 spicules in cross section, following a meandering course and anastomosing irregularly. The skeleton of the interior of the sponge is scanty, consisting of a loose irregular reticulation of mostly single spicules bound at the nodes with a little spongin. Spicules are exclusively curved, somewhat flexuous oxeas with variously pointed or blunt apexes that are uniform in size 260−330 × 3−6 μm. In habit, color, and general consistency the specimen appears to be closely related to *Oceanapia sagittaria*. However, that species has microscleres (both sigmas and toxas) and its oxeas are thicker. The present material cannot be matched with any description in the taxonomic literature and probably belongs to an undescribed species. The samples were frozen immediately upon collection and then freeze-dried prior to transport to the University of Würzburg, Germany. A voucher specimen is kept under registration no. ZMA POR 11007 in the Zoölogisch Museum, Amsterdam.

Extraction and Isolation. A freeze-dried sample of *Oceanapia* sp. (97 g) was extracted exhaustively with methanol. The extract was evaporated under reduced pressure to give a residue of 7.2 g. This residue was chromatographed on a silica gel column (elution with CH_2Cl_2/MeOH/NH_4OH, 70:30:3), and six major fractions were obtained. Fractions 2 and 3 contained compounds **1** (264.7 mg, 0.27%), **2** (91.3 mg, 0.09%), and **3** (3.1 mg, 0.003%), which were combined for final purification on an RP-18 silica gel column (MeOH/H_2O/TFA, 70:30:0.5). Prior to NMR spectroscopic measurements, the compounds were shaken with a buffered solution of EDTA (10 mM) and sodium citrate (50 mM) (3 × 2 mL) to remove metal ions, which cause broad signals in the NMR spectra. Finally, the compounds were washed with NaOH solution (pH 10.0; 3 × 2 mL) to remove EDTA and citrate.

***N*-Deacetylkuanoniamine C (3)**: orange amorphous powder; percent purity 91% (as estimated by HPLC analysis); UV (MeOH) λ_{max} (log ϵ) 201 (4.14), 239 (4.33), 264 (4.11), 295 (4.08), 304 (4.05), 342 (3.82), 355 (3.85); ^{1}H NMR (DMSO, 300 MHz) δ 9.08 (1 H, s, H-11), 8.67 (1 H, d, J = 5.1 Hz, H-2), 8.06 (1 H, d, J = 7.9 Hz, H-4), 7.68 (1 H, d, J = 5.1 Hz, H-3), 7.40 (2 H, m, H-6,

H-7), 7.02 (1 H, m, H-5), 3.08 (2 H, br, H-13), 2.95 (2 H, br, H-14); ^{13}C NMR (DMSO, 400 MHz) δ 150.9 (d, C-2), 148.9 (d, C-11), 143.5 (s, C-12b), 140.7 (s, C-12a), 140.0 (s, C-9a), 139.6 (s, C-7a), 139.4 (s, C-3a), 133.8 (s, C-8a), 131.8 (d, C-6), 123.9 (d, C-4), 120.8 (d, C-5), 117.9 (s, C-12c), 116.3 (d, C-7), 115.8 (s, C-3b), 108.4 (d, C-3), 106.0 (s, C-9), 39.6 (t, C-14), 34.6 (t, C-13); ESI-MS *m/z* 319 $[M + H]^+$; ESI-MS/MS (precursor ion *m/z* 319, collision energy 29 eV, 830.5 mTorr Argon) *m/z* 302 (319 − NH_3); HREIMS *m/z* 318.0930 (calcd for $C_{18}H_{14}N_4S$ 318.0939).

Acid Hydrolysis of Kuanoniamine C (1). Kuanoniamine C (**1**) (65 mg) was dissolved in 120 mL of MeOH, and the same volume of 2 N HCl was added. The reaction mixture was stirred for 48 h at 85 °C under reflux. The sample was taken to dryness, and the resulting red solid was purified by RP-18 column chromatography with the above-mentioned solvent system to yield 49 mg of compound **3**.

Experiments with Insects. Larvae of *S. littoralis* were from a laboratory colony reared on an artificial diet under controlled conditions as described previously.[17] Feeding studies were conducted with neonate larvae (n = 20) that were kept on an artificial diet that had been treated with various concentrations (13−373 ppm) of the compounds under study. After 6 days, survival of the larvae and weight of the surviving larvae were protocolled and compared to controls. LC_{50}'s were calculated from the dose−response curves by probit analysis.

Brine Shrimp Assay. *A. salina* eggs were kept for 48 h in artificial seawater as described previously,[18] and the nauplii (n = 20) were brought into vessels with brine containing various concentrations (5−100 μg/mL) of the compounds to be tested (each concentration in triplicate). DMSO (10 μL/mL brine) was added to improve solubility. After 24 h, the surviving larvae were counted and compared to controls. LC_{50}'s were calculated from the dose−response curves by probit analysis.

Cytotoxicity Studies. All cell lines used are deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cultures were mycoplasma-free and were cultivated under standardized conditions.[19] For all experiments, exponentially growing cells were used, with a viability exceeding 90%, as determined by trypan blue staining. The final concentration used in the experiments was 10^5 cells/mL. The experiments were carried out in the presence of 100 IU/mL of penicillin G and 100 mg/mL of streptomycin.

Concentrated stock solutions of the test compounds were prepared in ethylene glycol monomethyl ether and stored at −20 °C. For the cytotoxicity analysis cells were harvested, washed, and resuspended in a final concentration of 10^5 cells/mL. They were seeded in triplicate in 90 μL volumes in 96-well flat-bottom culture plates (Nunc). Test compounds in 10 μL, obtained by diluting the stock solution with a suitable quantity of growth medium, were added to each well. The cultures were incubated for 48 h at 37 °C in a humidified incubator with 5% CO_2.

Cytotoxicity was determined by incorporation of [^{3}H]-thymidine.[20] Radioactive incorporation was carried out for the last 3 h of the 48 h incubation period. A 1 μCi portion of [methyl-^{3}H]thymidine (Amersham-Buchler, Braunschweig, Germany; specific activity 0.25 mCi/μmol) was added in 20 μL volumes to each well. Cells were harvested on glass fiber filters with a multiple automatic sample harvester, and radioactivity was determined in a liquid scintillation counter (1209 Rackbeta, LKB, Freiburg, Germany). As controls, media with 0.1% ethylene glycol monomethyl ether were included in the experiments.

Adenosine Receptor Binding Assay. Inhibition of binding of [^{3}H]-N^6-cyclohexyladenosine (CHA) to A_1-adenosine receptors of rat cerebral cortical membranes and inhibition of [^{3}H]-2-[[4-(carboxyethyl)phenyl]ethyl]-amino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680) to A_{2A}-adenosine receptors of rat striatal membranes were assayed as described.[21] 2-Chloroadenosine (10 μM) was used to define nonspecific binding. Inhibition of the receptor-radioligand binding was determined over a range of five to six concentrations of the compounds in triplicate in at least three separate experiments. The Cheng−Prusoff equation[22] and K_D values of 1 nM for [^{3}H]CHA and 14 nM for [^{3}H]CGS21680 were used to calculate the K_i values from the IC_{50} values, determined by the nonlinear curve fitting program InPlot, Version 4.03 (GraphPad, San Diego, CA).

Benzodiazepine Binding Assay. The affinities of compounds **1** and **2** to benzodiazepine binding sites of $GABA_A$ receptors were tested using cortical membranes of rat brain as described previously.[16] The compounds were tested versus [^{3}H]diazepam in a single concentration of 25 μM in triplicate in three independent experiments.

Agar Plate Diffusion Assay. Susceptibility disks (5 mm diameter) were impregnated with 100 μg of the isolated compound and then placed on agar plates inoculated with the test bacterium *Bacillus subtilis* 168 and *Staphylococcus aureus* ATCC 25923 (for Gram-positive bacteria) and *Escherichia coli* ATCC 25922 (for Gram-negative bacteria). After 24 h of incubation at 37 °C, the plates were observed for zones of inhibition. No inhibition was observed with compounds **1**−**3**.

Bioautographic Detection of Fungicidal Activity. Spores of *C. cucumerinum* were cultivated from carrot-nutrient agar and were inoculated into a liquid yeast culture medium as previously described.[23] Si gel TLC plates were spotted with the isolated compounds at concentrations of 150 and 75 μg. Then the plates were sprayed with a suspension of spores of *C. cucumerinum* in liquid yeast culture medium. After 2 days of incubation at 25 °C, no inhibition zones were observed for compounds **1**−**3**.

Acknowledgment. Financial support by the BMBF (Molekulare Naturstofforschung) and by the Fonds der Chemischen Industrie (both to P.P.) is gratefully acknowledged. Furthermore, we thank Dr. Dieter Gross (Institut für Pflanzenbiochemie, Halle, Germany) for the opportunity to carry out bioassays with *C. cucumerinum*. We also thank the Coral Reef Research Foundation for assistence during collection. C. E. thanks the Studienstiftung des Deutschen Volkes for a scholarship.

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NP9702704