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Damipipecolin and damituricin, novel bioactive bromopyrrole alkaloids from the Mediterranean sponge *Axinella damicornis*

Anna Aiello,[a] Ernesto Fattorusso,[a,*] Antonella Giordano,[a] Marialuisa Menna,[a] Werner E. G. Müller,[b] Sanja Perović-Ottstadt[b] and Heinz C. Schröder[b]

[a]*Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli ''Federico II'', via D. Montesano 49, 80131 Napoli, Italy*
[b]*Abteilung für Angewandte Molekularbiologie, Institut für Physiologische Chemie, Johannes Gutenberg-Universität Mainz, Duesbergweg 6, 55099 Mainz, Germany*

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Abstract—Two new bromopyrrole alkaloids, damipipecolin (**1**) and damituricin (**2**), have been isolated from the Mediterranean sponge *Axinella damicornis*, and their structures established through spectroscopic methods. Compounds **1** and **2** extend the structural variety of the so far known pyrrole alkaloids; in these compounds, the 4-bromopyrrole 2-carboxylic acid is directly condensed with a non-protein cyclic α-amino acid, the (2*R*, 4*R*)-*trans*-4-hydroxypipecolic acid and (2*R*, 4*R*)-*cis*-*N*,*N*′-dimethyl-4-hydroxyproline (D-turicine) in **1** and **2**, respectively. Compounds **1** and **2** were found to display a modulating effect of the serotonin receptor activity in vitro.

1. Introduction

Bromopyrrole alkaloids constitute a family of exclusively marine alkaloids and represent a fascinating example of the large variety of secondary metabolites formed by marine sponges.[1] Most of these compounds fall under the oroidin class of alkaloids,[2] defined by the signature bromopyrrole carboxamide and aminoimidazole moieties connected through a propyl chain. Over the last 30 years, about 140 derivatives, with various structures and interesting biological activities, have been isolated from more than 20 different sponges of various genera, essentially (but not exclusively) belonging to Agelasidae, Axinellidae, and Halichondridae families. It is currently admitted that these alkaloids are taxon-specific of at least Agelasida order and play the role of chemical markers of these phylogenetically related sponges.[3] From the ecological point of view, the antipredatory role of these alkaloids can be considered their most important biological function,[4] but the variety of pharmacological activities that they show make these compounds important leads for the development of new drugs and tools for cell biology.[5–10]

In our search for bioactive natural products from Mediterranean sponges, we have investigated two *Axinella* species collected in the bay of Calvi (Corsica): *Axinella damicornis* and *Axinella verrucosa*. These sponges were shown to produce complex mixtures of structurally diverse bromopyrrole alkaloids. The analysis of *A. verrucosa* allowed us to isolate twenty pyrrole-imidazole alkaloids; among them, four were new compounds.[5] The same study performed on *A. damicornis* led to the isolation of a new bromopyrrole alkaloid, daminin, which was shown to be a potent neuroprotective agent that might represent a new therapeutic tool for the treatment of CNS diseases such as Parkinson's and Alzheimer's diseases. The synthesis of this compound has also been performed.[11]

A deeper investigation of the methanolic extract of *A. damicornis* has now led to the isolation, in addition to a number of already known bromopyrrole alkaloids (4-bromopyrrole-2-carboxylic acid,[12] 4,5-dibromopyr-

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* Corresponding author. Tel.: +39 081 678503; fax: +39 081 678552; e-mail: fattoru@unina.it

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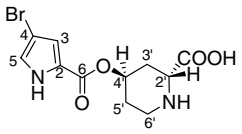
role-2-carboxylic acid,[2] oroidin,[2] and agelongine[10]), of two new bromopyrrole alkaloids, damipipecolin (**1**) and damituricin (**2**).



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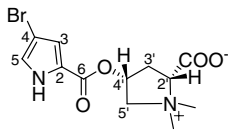

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In these compounds, the 4-bromopyrrole 2-carboxylic acid is directly condensed with a non-protein cyclic α-amino acid, the (2*R*, 4*R*)-*trans*-4-hydroxypipecolic acid and (2*R*, 4*R*)-*cis*-*N*,*N*′-dimethyl-4-hydroxyproline (D-turicine) in **1** and **2**, respectively. The D-and L-forms of the betaine turicine, where the carboxylate and hydroxyl functions are *cis* to each other, and L-betonicine, where these functions are in the *trans* relationship, are naturally occurring derivatives of 4-hydroxyproline. They have been studied in the acetylcholine esterase system and betonicine, though not turicine, is a competitive inhibitor of the same order of magnitude of choline itself.[13] Pipecolic acids (hexahydropiperidine-2-carboxylic acids) are frequently encountered in nature with their hydroxylated derivatives and often display interesting and potent biological activity.[14–18] Pipecolic acid derivatives are also useful synthetic intermediates for the preparation of medicinally important compounds such as peptides,[19] immunosuppressants,[20] enzyme inhibitors,[15,21] or NMDA antagonists.[22]

Compounds **1** and **2** were found to display a modulating effect on the serotonin receptor activity in vitro.

2. Results

2.1. Isolation of compounds 1 and 2

Specimens of *A. damicornis* (Esper, 1794) were collected in the bay of Calvi (Corsica) in November 2001. The sponge was homogenized and extracted with methanol and, subsequently, with chloroform. The combined extracts were concentrated in vacuo. The resulting aqueous suspension was partitioned between H_2O and butanol. The fractionation of the butanol layer was achieved by MPLC over a reversed-phase RP-18 column and a fraction mainly composed of polar alkaloids was obtained. Separation and purification of this fraction was achieved by repeated reverse-phase HPLC, to give compounds **1** and **2** as pure products.

2.2. Structure elucidation

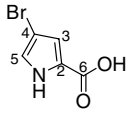
The planar structure of damipipecolin (**1**) was determined by spectroscopic methods. The ESI (positive ions) mass spectrum of **1** showed two intense pseudomolecular ion peaks at *m*/*z* 317 and 319 $[M+H]^+$ in the ratio 1:1, suggesting that **1** is a monobromo compound. The ^{13}C NMR spectrum (CD_3OD) contained 11 signals, composed of three methylenes, four methines, and four unprotonated carbons, according to DEPT and HSQC experiments. The combined NMR and mass spectral data of **1** were compatible with the molecular formula $C_{11}H_{13}N_2O_4Br$, which implied six degree of unsaturation. From ^{13}C NMR data it was evident that four of the elements of unsaturation were present as double bonds, two of them being carbonyl groups, one as part of a conjugated ester and the other as a free acid; the molecule must be thus bicyclic.

The ^1H NMR spectrum (CD_3OD) of **1** revealed the presence of two aromatic signals at δ 6.92 and 7.01, a deshielded methylene signal (δ 3.17, Ha; 3.20, Hb), two sp^3 deshielded methine signals (δ 3.74 and 5.31), and two partially overlapped methylene resonances in the region 1.9–2.4 ppm. The HSQC experiment allowed us to assign all these resonances to the relevant carbon atoms in the ^{13}C NMR spectrum. Interpretation of the COSY spectrum illustrated the proton connectivities in **1** and revealed the presence of two distinct spin systems belonging to two nitrogen-containing rings. The presence of a 4-bromopyrrole-2-carboxylic acid derivative moiety was evident from the chemical shifts and the multiplicity of the aromatic proton signals, which constituted one of the two spin systems, and from a ^{13}C NMR pattern of resonances (δ 124.5, 97.8, 117.7, 123.6, 159.9). This hypothesis was fully corroborated by comparison of the NMR data of **1** with those reported in the literature for other bromopyrrole alkaloids.[23]

The identified moiety accounted for two double bonds, the ester function and one ring. The remaining part of the molecule, consisting of $C_6H_{10}NO_2$, thus contained three methylene groups, two deshielded sp^3 methine groups, and a carboxyl function. Following the COSY correlations in the spectrum recorded in CD_3OD, starting from the deshielded methine signal at δ 3.74, the other large spin system was observed, corresponding to the segment H-2′/2H-6′ of the molecule. In the HSQC spectrum, the proton signal at δ 5.31 (H-4′) of this spin system was correlated to a signal at δ 67.8, clearly indicating that it was due to a proton linked to an oxygen-bearing carbon. A nitrogen atom was inferred to be attached to both C-2′ and C-6′ from their ^{13}C chemical shifts (δ 56.4 and 40.2), and a cross peak, present in the HMBC spectrum of **1** between the resonance for H-2′ (adjacent to N) and the carbonyl resonating at δ 179.9, suggested the carboxylic function to be located at C-2′. On the basis of the combination of these NMR results, a framework of 4-substituted piperidine-2-carboxylic acid (pipecolic acid) was identified. The HMBC spectrum showed a long-range correlation between the oxymethine proton at δ 5.31 (C-4′) with C-6 carbon (δ 159.9), thus indicating that C-4′ was connected with the bromopyrrolecarboxylic acid moiety through an ester linkage.

The relative stereochemistry of the two substituents on the pipecolic acid ring of damipipecolin could be defined by ^1H–^1H coupling constants analysis. The C-2′ proton, resonating at δ 3.74, appeared as a double doublet with a large coupling of 12 Hz and a small coupling of 3 Hz

to the protons of the adjacent C-3′ methylene, whereas H-4′ (brs) showed small J values to the protons of the adjacent C-3′ and 5′ methylenes. Assuming that the piperidine ring is in a chair-like conformation, a *trans* relative configuration of the 4-substituted pipecolic acid, where H-2′ and H-4′ occupy an axial and an equatorial position, respectively, was consistent with the observed coupling pattern. The absolute stereochemistry of the amino acid portion could be clarified through acid hydrolysis of **1** performed with 1 N HCl in H_2O at 80 °C overnight. The reaction mixture was evaporated under nitrogen and then subjected to HPLC purification, yielding 1 mg of pure 4-bromopyrrole 2-carboxylic acid (**3**) and (+)-*trans*-4-hydroxypipecolic acid (**4**). Both compounds could be easily identified by comparison of their spectroscopic data with those reported in the literature; in particular, the NMR data of 4-hydroxypipecolic acid were identical to those reported for the (2*R*, 4*R*)-*trans* isomer,[24] thus confirming the relative stereochemistry proposed for damipipecolin. Both the sign and value of the optical rotation of the amino acid **4** matched well with those of (2*R*, 4*R*)-4-hydroxypipecolic acid previously reported[18] thus indicating that the absolute stereochemistry of the amino acid portion of damipipecolin is (2*R*, 4*R*).



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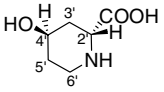

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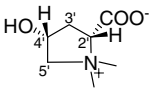

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The ESI (positive ions) mass spectrum of damituricin (**2**) showed two intense pseudomolecular ion peaks at *m*/*z* 331 and 333 $[M+H]^+$ in the ratio 1:1. The ^{13}C NMR spectrum (CD_3OD) contained 12 carbon signals, which were identified, through DEPT and HSQC experiments, as two methyls, two sp^3 methylenes, two sp^2 methines, two deshielded sp^3 methines, and four unprotonated carbons. These NMR and mass spectral data were compatible with the molecular formula $C_{12}H_{15}N_2O_4Br$, implying six elements of unsaturation. From ^{13}C NMR data, the presence of two carbon–carbon double bonds and two carbonyl groups was evident; thus, also molecule **2** must be bicyclic.

The comparison of ^1H and ^{13}C NMR spectra of **1** and **2** revealed some similarities. Particularly, the aromatic proton and carbon resonances present in the spectra of **2** (δ_C: 124.3, 97.1, 117.5, 122.2; δ_H: 6.95, 7.05) were almost identical to those of **1**, indicating in **2** the presence of the same 4-bromopyrrole-2-carboxylic acid moiety as in **1**, connected via an ester bond (δ_C 159.2) to a different cyclic unit. Analysis of ^1H NMR spectrum (CD_3OD) of **2** was integrated with data obtained from the HSQC experiment. In addition to pyrrole signals, the proton spectrum contained two three-proton-singlets at δ 3.43 and 3.34, attributable, also on the basis of their deshielded carbons (δ 52.3 and 46.7), to N-linked methyls of a quaternary ammonium compound. Additional features of ^1H NMR spectrum were two AB systems due to methylene groups ($2H_5$: δ 3.92, Ha; 3.98, Hb. $2H_3$: δ 2.64, Ha; 3.10, Hb), and two sp^3 deshielded methine signals (δ 4.19 and 5.56). These signals were connected, by interpretation of COSY spectrum, in an only large spin system belonging, also according to mass data, to an oxygenated nitrogen-containing ring, as deduced by the following HMBC results. In the HMBC spectrum, both the *N*-methyl protons were long-range correlated to the methylene carbon at δ 72.0 (C-5′) and the methine carbon at δ 75.7 (C-2′); thus, the same nitrogen atom was inferred to be linked to these carbons and to methyl carbons. A further key cross peak was observed in the HMBC spectrum between the proton resonating at δ 4.19 (H-2′) and the carbonyl resonating at δ 169.0; it clearly indicated that a carboxylate function was linked at C-2′. On the basis of these results, and according to the whole series of COSY, HSQC, and HMBC correlations, an *N*,*N*′-dimethyl-4-hydroxypyrrolidine-2-carboxylic acid (4-hydroxyproline betaine) unit was deduced to be present in **2**. An HMBC correlation between the oxymethine proton at δ 5.56 (H-4′) and the ester carbonyl at δ 159.2 suggested that this unit was connected to the bromopyrrolecarboxylic acid moiety through an ester linkage.

Crystal structures of the two diastereoisomeric betonicine (*trans*-*N*,*N*′-dimethyl-4-hydroxy-L-proline, levorotatory) and turicine (*cis*-*N*,*N*′-dimethyl-4-hydroxy-D-proline, dextrorotatory) had been previously determined as their hydrochlorides;[25] the NMR characterization of the *trans* diastereomer, isolated from the extracts of leaves of *Melaleuca* species, had been also reported.[26] Taking into account these reported data, the relative *cis*-stereochemistry to the betaine unit present in the molecule of **2** could be assigned on the basis of a ROESY experiment (CD_3OD) results. In fact, an intense cross peak was observed between H-2′ (δ 4.19) and H-4′ (δ 5.56), which was consistent only with a relative *cis*-orientation of the carboxylate group and the acylic portion. The absolute stereochemistry of the amino acidic portion of **2** could be clarified through acid hydrolysis of **2**, performed following the same procedure reported for **1**, which gave 4-bromopyrrole 2-carboxylic acid (**3**) and (+)-*cis*-*N*,*N*′-dimethyl-4-hydroxy-D-proline (turicine, **5**). The optical rotation value and sign of compound **5** were identical to those reported for turicine;[27] thus, on the basis of this result, the (2*R*, 4*R*) absolute stereochemistry was assigned to the amino acidic portion of damituricin.

2.3. Bioactivities of damipipecolin (1) and damituricin (2)

2.3.1. Cytotoxic effect. The cell viability tests using the MTT assay[28] revealed that compounds **1** and **2** are not toxic at a concentration of 10 μg/ml or less for all cell lines tested (PC12, HeLa, and L5178y cells).

2.3.2. Effect of compound 1, compound 2, and serotonin on Ca^{2+}-level in primary neurons. As shown in Figure 1A addition of 200 μM serotonin (5-hydroxytryptamine; 5-HT) and 2.5 mM $CaCl_2$ to primary neuronal cells (rat cortical cell cultures) resulted in a marked increase in

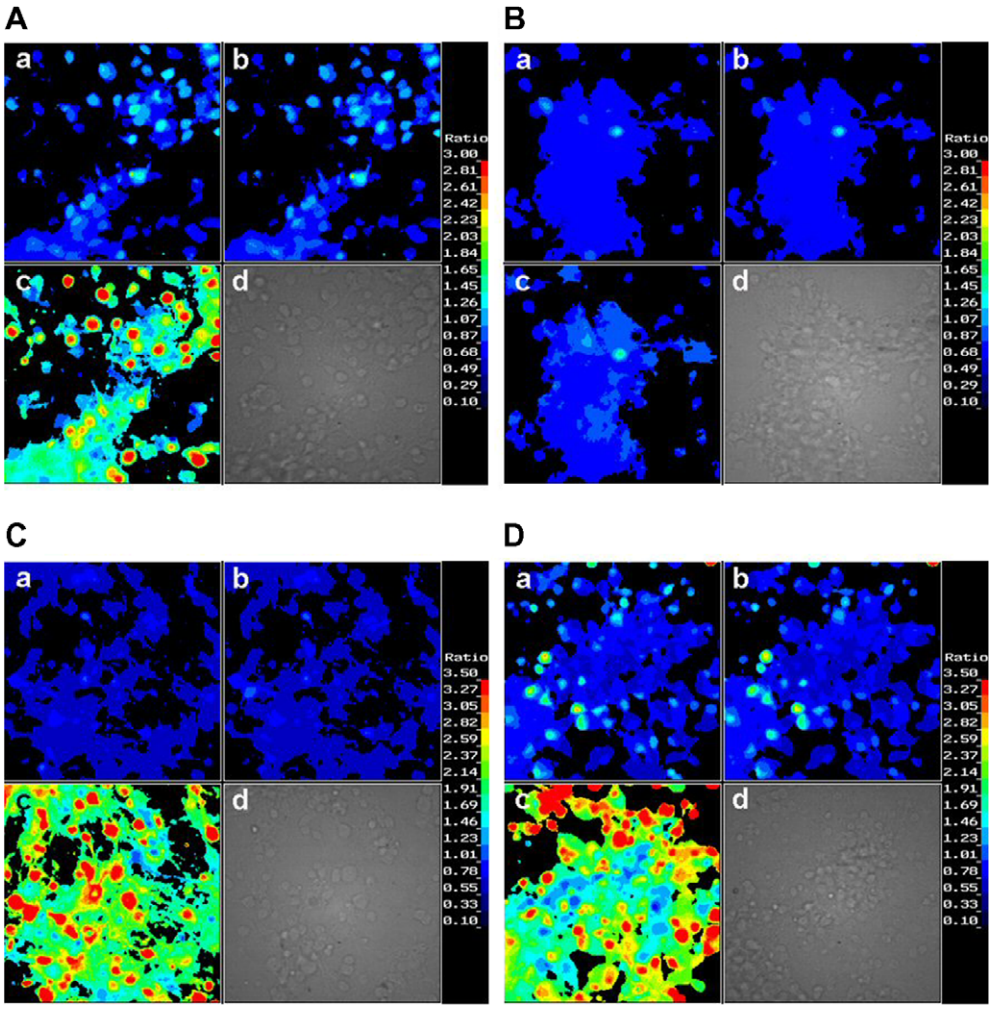

Figure 1. Effect of damipipecolin (**1**) on 5-HT (A, B) and L-Glu (C, D)-induced increase in $[Ca^{2+}]_i$ level in primary neuronal cells. Cells were loaded with fura-2-AM and analyzed by the fluorescence ratio-imaging system as described in Experimental procedures. Fluorescence images were recorded at time zero (a), after 5 (b) and 10 min (c). In (d) the cells inspected are shown by Nomarski phase contrast interference optics. (A, B) Effect on 5-HT-induced increase in $[Ca^{2+}]_i$ level. (A-a) Start of measurement (time: 0 min). (A-b) Addition of 0.1% (v/v) DMSO (control; 5 min). (A-c) Addition of 200 μM 5-HT and 2.5 mM Ca^{2+} (10 min). (B-a) Time zero. (B-b) Addition of 0.1 μg/ml of compound **1** (5 min). (B-c) Addition of 5-HT/Ca^{2+} (10 min). (C, D) Effect on L-Glu-induced increase in $[Ca^{2+}]_i$ level. (C-a) Time zero. (C-b) Addition of 0.1% (v/v) DMSO (control; 5 min). (C-c) Addition of 200 μM L-Glu and 2.5 mM Ca^{2+} (10 min). (D-a) Time zero. (D-b) Addition of 0.1 μg/ml of compound **1** (5 min). (D-c) Addition of L-Glu/Ca^{2+} (10 min). The spectrum colour scale ranges from blue (low $[Ca^{2+}]_i$) to red (high $[Ca^{2+}]_i$). Magnification, 400-fold.

intracellular free calcium level ($[Ca^{2+}]_i$), as documented by the record of the fluorescence images obtained in experiments using the Ca^{2+} indicator dye fura-2. A strong shift from dark blue to light-blue, green, orange and red was seen in all cells immediately (30 s) after addition of 5-HT/$CaCl_2$ (Fig. 1A-c), which was done 10 min after the beginning of the experiment. Addition of 0.1 μg/ml of compound **1** strongly reduced the 5-HT-induced elevation of $[Ca^{2+}]_i$ (Fig. 1B-c). In Figure 1A-d and B-d, a photo of the cells taken at the end of the experiment by Nomarski phase contrast interference optics is shown.

The strongest effect on $[Ca^{2+}]_i$ in neurons was observed after preincubation of the cells for 5 min with 0.1 μg/ml of compound **1** (Fig. 2) followed by 5-HT/$CaCl_2$ application (reduction to 11.2 ± 0.8%; mean value of three experiments). A quantitative analysis revealed that the increase in the 340/380 nm ratio was also significantly ($p < 0.001$) reduced (decrease to 29.7 ± 20.3% and 38.0 ± 19.7%, respectively; mean value of 2–5 experiments) when 0.3 or 1 μg/ml of compound **1** was added to the cells. At higher or lower concentrations, the compound became less effective (0.03 μg/ml; 71.6%); no effect on 5-HT/$CaCl_2$-induced change of $[Ca^{2+}]_i$ level was found at 0.01, 3, and 10 μg/ml of compound **1** (Fig. 2). The increase in the fluorescence ratio (340/380 nm) in the control samples after addition of 5-HT/$CaCl_2$ was set to 100%.

Compound **2** showed on primary neurons a similar effect like compound **1**. As shown in Figure 6 preincubation of the cells for 5 min with 10 μg/ml of compound **2** resulted in a small decrease in the $[Ca^{2+}]_i$ values to 79.3%. In contrast, treatment (5 min) of neurons with 10 and 100 times (1 and 0.1 μg/ml) lower doses of compound **2** induced a significant ($p < 0.001$) reduction of the $[Ca^{2+}]_i$ influx after addition of 5-HT/$CaCl_2$

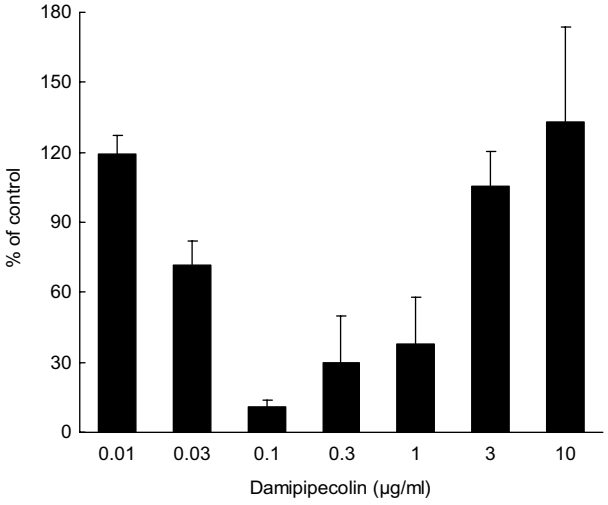

Figure 2. Incubation of neurons with 5-HT and $CaCl_2$ in the presence of various concentrations of compound **1** (damipipecolin). In the first set of experiments (control) neurons were stimulated with 200 μM 5-HT and 2.5 mM $CaCl_2$ only; the values obtained were set to 100%. In the second set of experiments the effect of 5-HT/$CaCl_2$ on the calcium level in neurons preincubated for 5 min with 0.01 to 10 μg/ml of compound **1** was determined. Experiments were performed in Locke's solution. In all assays, $[Ca^{2+}]_i$ level was measured for 20 min. The results are expressed as % of control ± standard deviation of 3–5 independent experiments. The cell number per concentration per experiment was ~90.

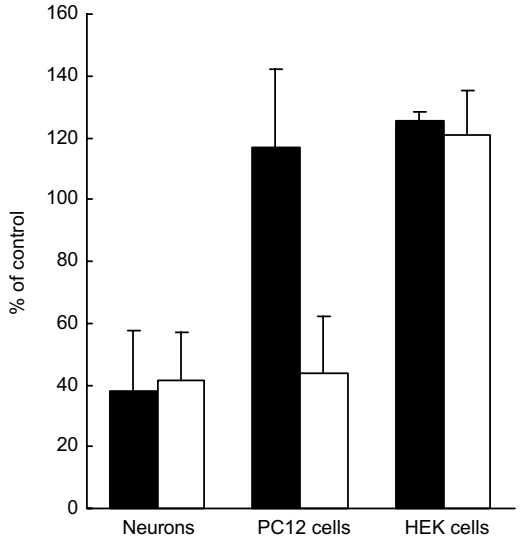

Figure 3. Incubation of primary neurons, PC12 cells and HEK cells with 200 μM 5-HT and 2.5 mM $CaCl_2$ in the presence of 1 μg/ml of compound **1** (damipipecolin; black column) and **2** (damituricin; white column). The results are expressed as % of control ± standard deviation of three independent experiments. Further details are given in Figure 2.

(Fig. 6). The $[Ca^{2+}]_i$ values decreased to 41.8 ± 4.5% (1 μg/ml) and 29.5 ± 9.5% (0.1 μg/ml), respectively.

2.3.3. Effect of compound 1, compound 2, and serotonin on Ca^{2+}-level in PC12 and HEK cells. Treatment of PC12 and HEK cells with 200 μM 5-HT and 2.5 mM $CaCl_2$ resulted only in a small increase in $[Ca^{2+}]_i$ (expressed as 340/380 nm ratio values); Δ ratio 0.096 (PC12 cells) and 0.115 (HEK cells). The control values were set to 100%.

As shown in Figure 3 preincubation of PC12 cells and HEK cells for 5 min with 1 μg/ml of **1** did not induce a significant change of $[Ca^{2+}]_i$ after addition of 5-HT/$CaCl_2$, while preincubation of primary neuronal cells with the compound under the same condition caused a strong decrease in 5-HT/$CaCl_2$-induced elevation of $[Ca^{2+}]_i$ level.

In parallel experiment preincubation of PC12 cells for 5 min with 1 μg/ml of compound **2** (Fig. 3) generated a significant change of $[Ca^{2+}]_i$ after addition of 5-HT/$CaCl_2$. The $[Ca^{2+}]_i$ decreased to 43.8 ± 18.4%. At the same time preincubation of HEK cells with compound **2** under the same conditions caused no changes in $[Ca^{2+}]_i$.

2.3.4. Effect of compound 1, compound 2, and L-glutamic acid on the Ca^{2+}-level in primary neurons. In primary neuronal cells treated with 200 μM L-glutamic acid (L-Glu) and 2.5 mM $CaCl_2$ a strong ($p < 0.001$) increase in $[Ca^{2+}]_i$ from 0.704 ± 0.012 to 2.533 ± 0.058 (Δ ratio 1.829; set to 100%) was measured. As shown in Figure 1C addition of L-Glu and 2.5 mM $CaCl_2$ to neuronal cells resulted in a strong shift from dark blue to green, orange and red (Fig. 1C-c).

As shown on Figure 4A preincubation (5 min) of the neuronal cells with 0.1, 1, and 10 μg/ml of **1** induced no significant decrease in $[Ca^{2+}]_i$ after addition of L-Glu/$CaCl_2$. The fluorescence images showed like in the control sample a fast change of the color from dark blue to red after addition of L-Glu/$CaCl_2$ following preincubation of the neurons with 0.1 μg/ml of **1** (Fig. 1D-c). In comparison with the control, $[Ca^{2+}]_i$ amounted to 83.3% (Δ ratio 1.524; 0.1 μg/ml), 90.5 (Δ ratio 1.656; 1 μg/ml), and to 96.6% (Δ ratio 1.766; 10 μg/ml), respectively.

Preincubation (5 min) of the neurons with 0.1, 1, and 10 μg/ml of compound **2** (Fig. 6) induced no changes in the $[Ca^{2+}]_i$ after addition of L-Glu/$CaCl_2$. In comparison with the control the $[Ca^{2+}]_i$ reached 109.7% (0.1 μg/ml), 100.2% (1 μg/ml), and 98.9% (10 μg/ml).

2.3.5. Effect of compound 1, compound 2, and *N*-methyl-D-aspartic acid on the Ca^{2+}-level in primary neurons. Stimulation of primary neuronal cells with 200 μM *N*-methyl-D-aspartic acid (NMDA) and 2.5 mM $CaCl_2$ resulted in a significant ($p < 0.001$) increase in $[Ca^{2+}]_i$; Δ ratio 0.868 (set to 100%).

As shown in Figure 4B preincubation (5 min) of the neurons with 10 and 0.1 μg/ml of **1** induced no significant decrease in $[Ca^{2+}]_i$ after addition of NMDA/$CaCl_2$. In comparison with the control the changes in $[Ca^{2+}]_i$ are between 105.9% (Δ ratio 0.919; 10 μg/ml) and 114.1% (Δ ratio 0.990; 0.1 μg/ml). Only after addition of 1 μg/ml of **1** a significant ($p < 0.01$) decrease in $[Ca^{2+}]_i$ was measured (70.4%, Δ ratio 0.611).

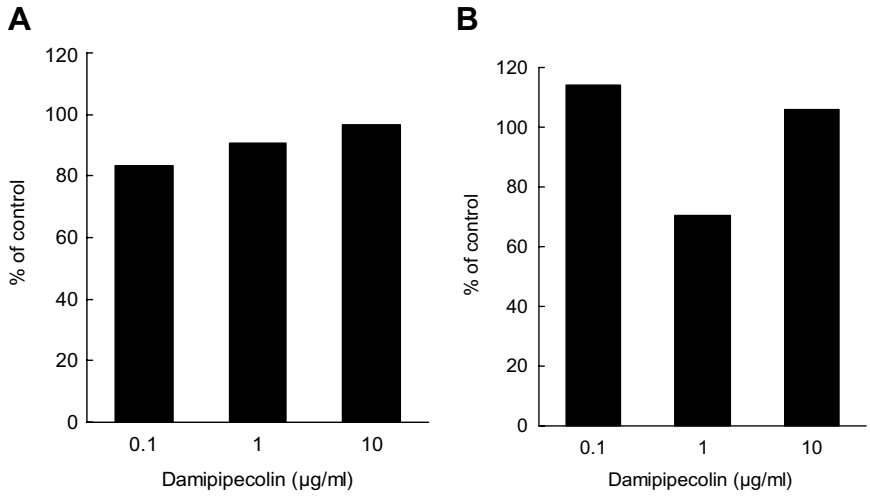

Figure 4. Incubation of neurons with (A) 200 μM L-Glu and 2.5 mM $CaCl_2$ and (B) 200 μM NMDA and 2.5 mM $CaCl_2$ in the presence of 0.1, 1, and 10 μg/ml of compound **1** (damipipecolin). Further details are given in Figure 2.

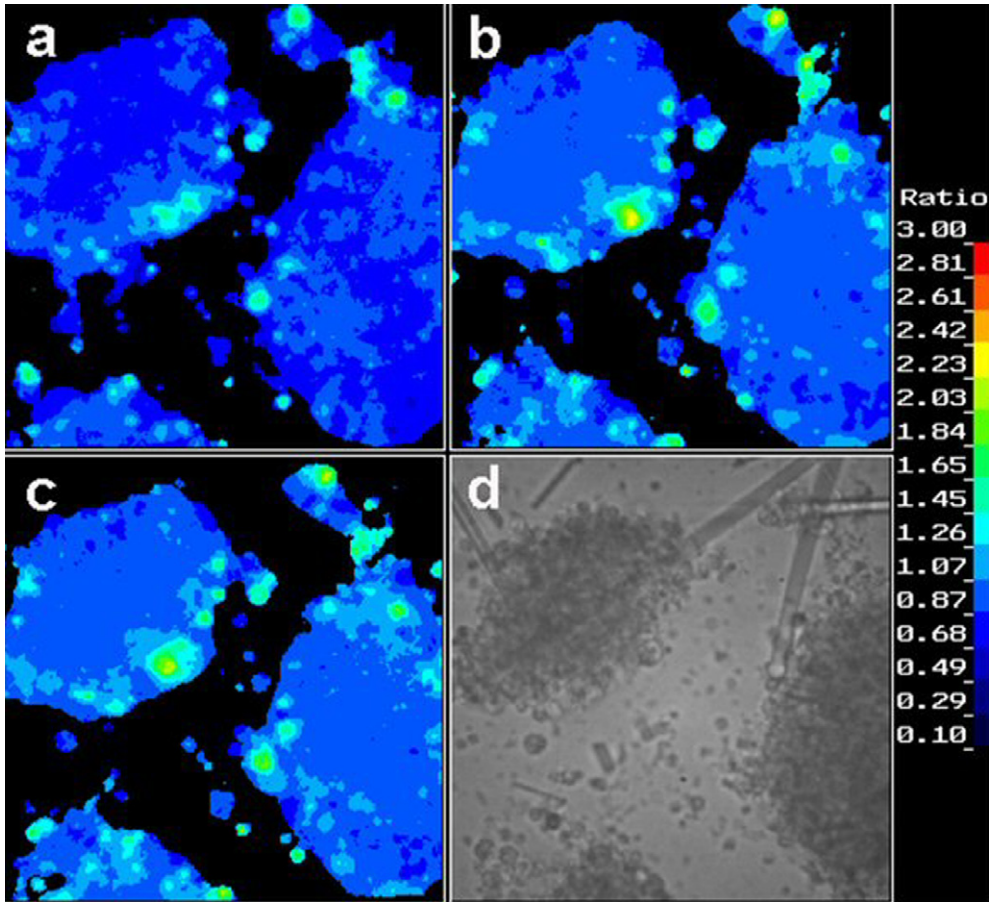

Figure 5. Effect of 5-HT on $[Ca^{2+}]_i$ level on cells of *Suberites domuncula*. Fluorescence images were recorded at time zero (a) and after 5 (b) and 10 min (c). The sponge cells were treated with 200 μM 5-HT and 10 mM $CaCl_2$. In (d) the cells inspected are shown by Nomarski phase contrast interference optics. Magnification, 400-fold.

In contrast, pretreatment of neurons with compound **2** induced only in the concentration range of 10 μg/ml a significant decrease in $[Ca^{2+}]_i$ after addition of NMDA/$CaCl_2$ (Fig. 6). The values decreased to 58.8% (Δ ratio 0.510). No changes were measured after treatment of neurons with 1 (119.52%) and 0.1 μg/ml (95.0%) of compound **2**.

2.3.6. Effect of compound 1 and serotonin on calcium level in sponge cells. In parallel to mammalian cells, the effect of 5-HT on $[Ca^{2+}]_i$ level in primmorphs (aggregates of dissociated cells) from the marine sponge *Suberites domuncula* was determined. As shown in Figure 5, addition of 200 μM 5-HT and 10 mM $CaCl_2$ to *S. domuncula* cells did not result in a significant change of $[Ca^{2+}]_i$ level

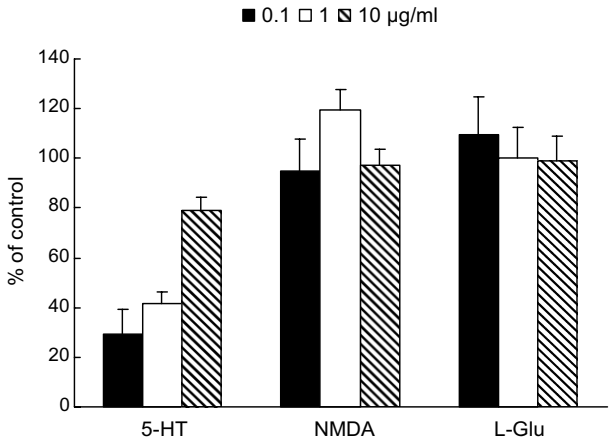

Figure 6. Incubation of primary neurons with (i) 200 µM 5-HT and 2.5 mM $CaCl_2$, (ii) 200 µM NMDA and 2.5 mM $CaCl_2$, and (iii) 200 µM L-Glu and 2.5 mM $CaCl_2$ in the presence of 0.1, 1, and 10 µg/ml of compound **2** (damituricin). The results are expressed as % of control ± standard deviation of 2–3 independent experiments. Further details are given in Figure 2.

(340/380 nm ratio) after an incubation period of 5 and 10 min. No change of $[Ca^{2+}]_i$ level was also found after addition of 0.1 µg/ml or 1 µg/ml of **1** to the cells (data not shown).

3. Discussion

The new bromopyrrole alkaloids damipipecolin (**1**) and damituricin (**2**) extend the structural variety of the so far known pyrrole alkaloids since their structures lack the commonly found short linear aliphatic segment linking the bromopyrrole 2-carboxylic acid moiety to the common imidazole nucleus. Until now, this feature has been found only in manzacidins A–C,[29] in agelongine,[10] and in its bromine-free analog daminin[11] isolated from the same sponge *A. damicornis*. Many alkaloids comprising a pyrrole ring linked to an imidazole moiety are biologically active, showing antibacterial,[6] antifungal,[6] antitumor,[30] antihistaminergic,[8] and immunosuppressive activities,[30] or acting as α-adrenergic[9] and serotonergic receptor antagonists.[10]

Damipipecolin and damituricin were found to exhibit a modulating effect on the serotonin receptor activity in vitro. Alterations of serotonin (5-hydroxytryptamine, 5-HT) levels are associated with many pathological dysfunctions of CNS, such as affective disorders, schizophrenia, and migraine.[31] The serotonergic system also plays a significant role in the development of depression. 5-HT reuptake inhibitors are effective antidepressants.[32] Some forms of phobia seem to be associated with an increase in serotonin levels; 5-HT_2 receptor antagonists seem to be efficacious in the treatment of psychosis.[33–35]

Serotonergic neurons are found in nearly every brain area. Serotonin receptors can be classified in three different groups: (i) the transporter (5-HT 'uptake site'), (ii) the ligand-gated ion-channel (5-HT_3) and (iii) the largest group of the G protein-coupled receptors.[36] Based on sequence data seven subtypes (5-HT_1–5-HT_7) have been distinguished.[37] The 5-HT_1 receptors exhibit a high affinity for serotonin, in contrast to 5-HT_2 and 5-HT_4 receptors, and have been implicated in the pathogenesis of many mental and neurological disorders; therefore they have become an important target for drug therapy.[38] 5-HT_3-receptors, on the other hand, are increasingly used as target for antiemetics to prevent chemotherapy-induced nausea and vomiting.[39]

Our studies revealed that compound **1** is a potent antagonist of serotonin in neuronal cells; the effect of serotonin on neurons was nearly completely blocked at low concentrations of 0.1 and 0.3 µg/ml of compound **1** (~88% and ~70% reduction). Higher concentrations of compound **1** were interestingly less active. At a concentration of 1 µg/ml of **1**, Ca^{2+} influx in neurons was reduced to ~62%, while preincubation of neurons with $\geqslant$3 µg/ml of compound **1** did not induce a decrease in Ca^{2+} influx after addition of 5-HT/$CaCl_2$. In contrast, compound **2** is a slightly less potent agonist of serotonin at the concentration of 1 µg/ml (~59% reduction) and 0.1 µg/ml of **2** (~71% reduction). Like compound **1** higher concentration of compound **2** (10 µg/ml) induces small (~22% reduction) decrease in Ca^{2+} influx after addition of 5-HT/$CaCl_2$.

The reason of the reduced efficiency of compounds **1** and **2** at higher concentration is not yet known. It should be noted that different types of serotonin receptors exist in brain tissue, and it has not been elucidated which receptor subtype(s) is/are involved in the mode of action of compounds.

In PC12 cells, 5-HT is known to have neuritogenic effects, although the signal transduction pathway responsible for these effects is not understood.[40] Application of 5-HT to PC12 cells enhances nerve growth factor (NGF)-induced neurite outgrowth in a dose-dependent manner.[40] Compound **1** did not exhibit a significant effect on Ca^{2+} influx in PC12 cells and HEK cells. In contrast, compound **2** was a potent antagonist of serotonin-induced Ca^{2+} entry in PC12 cells; the effect of serotonin on PC12 cells was more then 50% blocked (57% reduction). These pharmacological tests indicated that 5-HT-induced $[Ca^{2+}]_i$ increase in PC12 cells involves the mediation by a voltage-dependent Ca^{2+} channel, evoked by membrane depolarization via the activation of cation channel-type receptors, 5-HT_3 receptors.[40,41] The effect can be blocked in a dose-dependent manner with nifedipine[40,41] (voltage-dependent L-type calcium channel blocker), MDL 72222,[40] and metoclopramide[41] (selective 5-HT_3 receptor antagonist), but not by ketanserin,[40] a 5-HT(2) receptor antagonist, or thapsigargin,[40] a specific inhibitor of endoplasmic reticulum Ca^{2+}-ATPase. Our results indicate that compound **2** is a strong 5-HT_3 receptor antagonist or L-type calcium channel blocker in PC12 cells.

In addition, no evidence was obtained that compounds **1** and **2** interact with glutamate or NMDA receptors. L-Glutamate and L-aspartate are the most abundant

excitatory neurotransmitters in the CNS. The excitotoxic effect of L-Glu is associated with an influx of Na^+, K^+, and Ca^{2+} in neurons as a consequence of a sustained activation of glutamate receptors.[42,43] L-Glu stimulates ionotropic receptors, such as the NMDA receptor, and metabotropic receptors. In our experiments, preincubation of neurons with 0.1, 1, and 10 μg/ml of compounds **1** and **2** did not induce a significant decrease in Ca^{2+} entry.

Further studies could be performed on compounds **1** and **2** in order to gain information about their mode of action and structure–activity relationships. Anyway, some indication can be obtained from the data previously reported on structurally related compounds. Experiments performed on pyrrole alkaloids to evaluate the interactions with the cellular calcium homeostasis led to a structure–activity relationship underlining both the importance of the degree of bromination of the pyrrole moiety and the presence of an aminoimidazole group.[44] Particularly, these studies revealed that the 4-bromopyrrole-2-carboxylic acid moiety is not responsible alone for the biological effect of the brominated pyrrole alkaloids since both 4-bromopyrrole-2-carboxylic acid and pyrrole-2-carboxylic acid did not decrease voltage-dependent calcium elevation in PC12 cells.[44] Besides, the different potency of 4,5-bromopyrrole-2-carboxylic acid and oroidin (more active), in which this moiety is linked to the aminoimidazole group through a short linear aliphatic segment, reveals the importance of this group. This is further supported by earlier studies showing a marked antiserotonergic activity for the pyrrole alkaloids hymenidin, oroidin, and their unbrominated analog clathrodin[45,46] and the lack of activity of dispacamides, in which the aminoimidazole group is oxidized to an aminoimidazolone moiety.[8]

The bioactivity shown by compounds **1** and **2** suggests that antiserotonergic effects are associated to bromopyrrole rings when they are linked both to an aminoimidazole group and to other nitrogen heterocycle rings, such as pyrrolidine and piperidine moieties. In order to clarify the mode action of **1** and **2**, the effect of compounds **1** and **2** on Ca^{2+} level in the presence of selective agonists of 5-HT receptors [e.g., sumatriptan, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane],[47] could provide useful information.

In conclusion, damipipecolin and damituricin seem to be promising new serotonin antagonists with a potential in the therapy of disorders associated with changes of serotonin levels, such as psychosis, different phobia, and mood fluctuation disorders, as well as chemotherapy-induced emesis.

4. Experimental procedures

4.1. General aspects

ESI mass spectra were obtained by using an API 2000 mass spectrometer. High-resolution FAB mass spectra (glycerol matrix) were recorded on a VG Prospec (FISONS) mass spectrometer. Optical rotations were measured using a Perkin-Elmer 192 polarimeter. CD spectra were recorded on a J-710 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a J-710 for Windows software (Jasco). NMR experiments were performed on aVarian INOVA 500 spectrometer; chemical shifts are referred to the residual solvent signal. Medium pressure liquid chromatographies (MPLC) were performed on a Buchi 861 apparatus with SiO_2 (230–400 mesh) packed columns. High-performance liquid chromatography (HPLC) separations were achieved on a Knauer 501 apparatus equipped with an RI detector.

4.2. Collection, extraction, and isolation of compounds 1 and 2

Specimens of the sponge *Axinella damicornis* were collected and kept frozen until used. For the extraction, the fresh thawed sponge (75.2 g dry weight after extraction) was homogenized and treated at room temperature with methanol (3× 600 ml) and, subsequently, with chloroform (3× 600 ml). The extracts were combined and concentrated in vacuo to give an aqueous suspension which was then partitioned between H_2O and BuOH. The BuOH layer, after evaporation of the solvent, was subjected to a medium pressure chromatography (MPLC) over a reversed-phase (RP-18) column using a gradient elution ($H_2O \rightarrow MeOH \rightarrow CHCl_3$). Following this procedure, the fraction eluted with MeOH/H_2O 8:2 was mainly composed of polar alkaloids. This fraction was further separated by HPLC on a preparative RP-18 column (Kromasil, 10 μm, 250 × 10 mm), with H_2O/MeOH 7:3 as the eluent, and gave a mixture of compounds **1** and **2**. Final separation and purification of the two compounds was achieved by HPLC on a RP-18 column (Luna, 5 μm, 250 × 4.6 mm), using MeOH/H_2O 2:8 as the eluent, thus obtaining damipipecolin [**1**, 4 mg], and damituricin [**2**, 3 mg], both in a pure form.

Damipipecolin (1): $[\alpha]_D^{20}$ + 5.4 (H_2O, *c* 0.001). HR-FAB-MS (positive ion mode): *m/z* = 317.013694 $[M + H]^+$; the molecular formula $C_{11}H_{14}N_2O_4Br$ requires 317.013688. ^1H (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data are reported in Table 1.

Damituricin (2): $[\alpha]_D^{20}$ + 10.7 (H_2O, *c* 0.0013). HR-FAB-MS (positive ion mode): *m/z* = 331.029354 $[M + H]^+$; the molecular formula $C_{12}H_{16}N_2O_4Br$ requires 331.029338. ^1H (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data are reported in Table 1.

4.3. Hydrolysis of damipipecolin

A solution of 1.3 mg of **1** was stirred with 1 N HCl in methanol 91% at 80 °C overnight. The reaction mixture was evaporated under nitrogen and then subjected to HPLC purification using a RP-18 Luna 5 μm (250 × 3 mm) column with H_2O/MeOH 8:2 as the eluent, yielding 1 mg of pure 4-bromopyrrole 2-carboxylic acid (**3**) and (+)-*trans*-4-hydroxypipecolic acid (**4**, $[\alpha]_D^{20}$ +23, *c* 0.001, H_2O; ESI-MS : *m/z* 146 $[M+H]^+$; ^1H and ^{13}C NMR data are reported in Table 2).

Table 1. ^{1}H and ^{13}C NMR data (CD_3OD) of compounds **1** and **2**

Position	Compound **1**		Compound **2**	
	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)
2	123.6	—	122.2	—
3	124.5	7.01 (d, 1.6)	124.3	7.05(d, 1.6)
4	97.8	—	97.1	—
5	117.7	6.92 (d, 1.6)	117.5	6.95(d, 1.6)
6	159.9	—	159.2	—
2′	56.4	3.74 (dd, 12, 3.4)	75.7	4.19 (dd, 12.3, 8.5)
3′	32.9	H_a: 2.40 (bd, 14,7)	33.6	H_a: 3.10 (m)
		H_b: 1.91[a]		H_b: 2.64 (ddd, 15, 12.3, 4.1)
4′	67.8	5.31 (bs)	68.9	5.56 (m)
5′	28.5	H_a: 2.01[a]	72.0	H_a: 3.98 (bd, 13.5)
		H_b: 1.98[a]		H_b: 3.92 (dd, 13.5, 6.6)
6′	40.2	H_a: 3.17[a]	—	—
		H_b: 3.20[a]		
COOH	179.9	—	169.0	—
N–Me	—	—	Me_a: 52.3	Me_a: 3.43 (s)
			Me_b: 46.7	Me_b: 3.34 (s)

[a] Overlapped signals.

Table 2. ^{1}H and ^{13}C NMR data (D_2O) of 4-hydroxypipecolic acid (**4**) and D-turicine (**5**)

Position	Compound **4**		Compound **5**	
	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)
2′	54.1	3.82 (dd, 12, 3)	76.2	4.05 (dd, 12.5, 8.5)
3′	32.7	H_a: 2.11 (dt, 14.3, 3.3)	36.2	H_a: 2.89 (m)
		H_b: 1.86[a]		H_b: 2.25 (ddd, 15.3, 12.1, 4.0)
4′	62.0	4.12 (bs)	65.8	4.63 (m)
5′	29.0	1.81[a]	74.5	H_a: 3.62 (bd,13.2)
				H_b: 3.67 (dd, 13.2, 6.8)
6′	38.7	3.20 (m)	—	—
COOH	174.5	—	170.4	—
N–Me	—		Me_a: 53.1	Me_a: 3.26 (s)
			Me_b: 47.8	Me_b: 3.19 (s)

[a] Overlapped signals.

4.4. Hydrolysis of damituricin

A solution of 2 mg of **2** was stirred with 1 N HCl in methanol 91% at 80 °C overnight. The reaction mixture was evaporated under nitrogen and then subjected to HPLC purification using a RP-18 Luna 5 μm (250×3 mm) column with H_2O/MeOH 8:2 as the eluent, yielding 4-bromopyrrole 2-carboxylic acid (**3**) and 1.3 mg of (+)-*cis*-*N*,*N*′-dimethyl-4-hydroxy-D-proline (turicine, **5**, $[\alpha]_D^{20} + 20.3$, *c* 0.001, H_2O; ESIMS : *m*/*z* 182 $[M+Na]^+$; ^{1}H and ^{13}C NMR are reported in Table 2).

4.5. Mammalian cells and sponge primmorphs

Primary neuronal cells (rat cortical cells) were prepared from the brains of 17- to 18-day-old Wistar rat embryos, as described.[48,49] The cells were maintained in DMEM/HG (high glucose; 4.5 g/l glucose) serum-free medium supplemented with 0.1% (w/v) bovine serum albumin (BSA), 2 mM L-glutamine, 100 μg/ml transferrin, 100 mU/l insulin, 16 μg/ml putrescine, 6.3 ng/ml of progesterone, and 5.2 ng/ml Na_2SeO_3.

PC12 cells were grown in Dulbecco's modified Eagle's medium [DMEM]/10% (v/v) fetal calf serum (FCS)/5% (v/v) horse serum. HEK cells were grown in Dulbecco's modified Eagle's medium [DMEM]/10% (v/v) FCS/4.5 g/l glucose. PC12 and HEK cells were passaged twice per week at a 1:10 ratio. L5178y and HeLa cells were maintained in Roswell Park Memorial Institute medium [RPMI] 1640 supplemented with 10% (v/v) FCS. The cells were subcultured twice weekly at a 1:160 (L5178y cells) and 1:10 ratio (HeLa cells). All cell cultures were kept in a humidified atmosphere of 5% CO_2/air at 37 °C.

Specimens of the marine sponge *Suberites domuncula* (Porifera, Demospongiae, Hadromerida) were collected in the Northern Adriatic near Rovinj (Croatia) and kept in aquaria in Mainz (Germany) at a temperature of 17 °C. Sponge primmorphs were prepared from dissociated sponge cells as described.[50] The primmorphs were incubated for 1 day in Ca^{2+}- and Mg^{2+}-free artificial seawater (CMFSW)[51] at 17 °C until start of the experiments.

4.6. MTT assay

To estimate the IC_{50} values, cells were incubated for 72 h in the presence of various concentrations of compounds **1** and **2**. The viability of the cells was determined using the MTT colorimetric assay system.[28] Evaluation was performed in 96-well plates at 595 nm using an ELISA plate reader, after overnight incubation at 37 °C.

4.7. Calcium measurements

For determination of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), cells were cultivated on poly-L-lysine coated borosilicate coverglass (Nunc). An inverted-stage Olympus IX70 microscope with objective UApo40X/340 was used for the fluorescence measurements. The cells were alternately illuminated with light of wavelengths 340 and 380 nm. An additional 0.25 ND filter was used at 380 nm. The fluorescence emissions at 510 nm were monitored by an intensified CCD camera, model C2400-87 (Hamamatsu, Herrsching, Germany). Images were then digitized with a computerized imaging system (Argus-50, Hamamatsu).

The $[Ca^{2+}]_i$ was determined by measuring the fluorescence ratio of the Ca^{2+}-indicator dye fura-2-AM at

340 and 380 nm.[49] Neurons were loaded in the dark with 4 μM fura-2-acetoxymethyl (AM) ester (Molecular Probes, Leiden, The Netherlands), and PC12 and HEK cells with 10 μM fura-2-AM in DMEM/HG serum-free medium, supplemented with 1% (w/v) BSA at 37 °C for 1 h. Sponge cells (primmorphs) were loaded with 12 μM fura-2-AM in CMFSW containing 1% (w/v) BSA at 17 °C for 2 h. Subsequently, the cells were washed twice with medium supplemented with 10% FCS (mammalian cells) and incubated further at 37 °C for 1 h; sponge cells were washed with CMFSW and incubated further at 17 °C for 1 h. A calcium calibration curve was prepared according to the method of Grynkiewicz.[52]

Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose, and 10 mM Hepes; pH 7.4) without Ca^{2+} and Mg^{2+} was used as incubation medium in all set of experiments. A stock solution of 10 mg/ml of compound **1** dissolved in DMSO and of compound **2** (5 mg/ml) dissolved in water were used. Serial dilutions were done in DMSO or water. In experiments with sponges, Locke's solution with sea water osmolarity pH 8.0 was used.

In the first set of experiments (control) neurons were stimulated with 0.1% (v/v) DMSO or water after 5 min and with 200 μM 5-HT, NMDA or L-Glu and 2.5 mM $CaCl_2$ after 10 min from the beginning of the measurements. In the second set of experiments the primary neurons were first preincubated with various concentrations of compound **1** or **2** (5 min). After 10 min 5-HT/$CaCl_2$, NMDA/$CaCl_2$ or L-Glu/$CaCl_2$ was added to the neurons.

In the next set of experiments PC12 and HEK cells were first preincubated with 1 μg/ml of compound **1** or **2** (5 min) and after 10 min 5-HT/2.5 mM $CaCl_2$ was added to the cells. In the case of sponge cells, the preincubation of the cells was done with 0.1 and 1 μg/ml of compound **1** (5 min) and after 10 min 5-HT/10 mM $CaCl_2$ was added to the cells. As a control, cells treated with 0.1% (v/v) DMSO and 200 μM 5-HT with 2.5 or 10 mM $CaCl_2$ were used. In all set of experiments the $[Ca^{2+}]_i$ level was measured during the whole incubation period (for at least 20 min).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.05.074.

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