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Brominated pyrrole alkaloids from marine *Agelas* sponges reduce depolarization-induced cellular calcium elevation

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Abstract

Seven pyrrole alkaloids isolated from *Agelas* sponges were tested for interactions with the cellular calcium homeostasis. Brominated pyrrole alkaloids reduced voltage dependent calcium elevation in PC12 cells. Dibromosceptrin was the most potent alkaloid with a half maximal concentration of 2.8 μM followed by sceptrin (67.5 μM) and oroidin (75.8 μM). 4,5-Dibromopyrrole-2-carboxylic acid reduced calcium elevation at concentrations exceeding 30 μM but did not eliminate calcium elevation at concentrations up to 1 mM. 4-Bromopyrrole-2-carboxylic acid and pyrrole-2-carboxylic acid were not active in this respect. The aminoimidazole group appeared to have a significant effect on voltage dependent calcium elevation shown by the comparison of oroidin with 4,5-dibromopyrrole-2-carboxylic acid. The degree of bromination of the pyrrole moiety is another important factor, as was shown by the comparison of 4,5-dibromopyrrole-2-carboxylic acid with 4-bromopyrrole-2-carboxylic acid, as well as oroidin with hymenidin and dibromosceptrin with sceptrin. The previously reported feeding deterrent activity of brominated pyrrole alkaloids in *Agelas* sponges against predatory reef fish may partly be explained by a general interaction of these alkaloids with the cellular calcium homeostasis. The chemoreception of bromopyrrole alkaloids in sea water is shown using sensory neurons in the rhinophore of the sea slug *Aplysia punctata*.

Keywords: Secondary metabolite; Structure–activity relationship; Calcium imaging; PC12; *Aplysia punctata*; Olfaction

1. Introduction

Secondary metabolites from marine organisms represent a heterogeneous group of bioactive compounds. Apart from algae, conesnails, sea anemones and many other organisms sponges in particular are known to be a rich source of interesting secondary metabolites. For example, the genus *Agelas* (Agelasidae) is known to contain a variety of bromopyrrole alkaloids (reviewed in Braekman et al., 1992; Lindel et al., 2000). Several studies have shown both under laboratory and field conditions, that brominated pyrrole alkaloids like oroidin and sceptrin were the main fish antifeedant metabolites within the tissue of *Agelas* sponges at natural concentrations (Pawlik et al., 1995; Chanas et al., 1996; Assmann et al., 2000, 2001, 2004). Chemosensory cells in chemoreceptive organs are specialized cells, but share the general cell biology with other neuron and neuron-like cells. One of the possible cellular mechanisms of a brominated alkaloid for functioning as a feeding deterrent have been investigated in our previous study, where we tested 4,5-dibromopyrrole-2-carboxylic acid for effects on calcium levels in PC12 cells (Bickmeyer et al., 2004).

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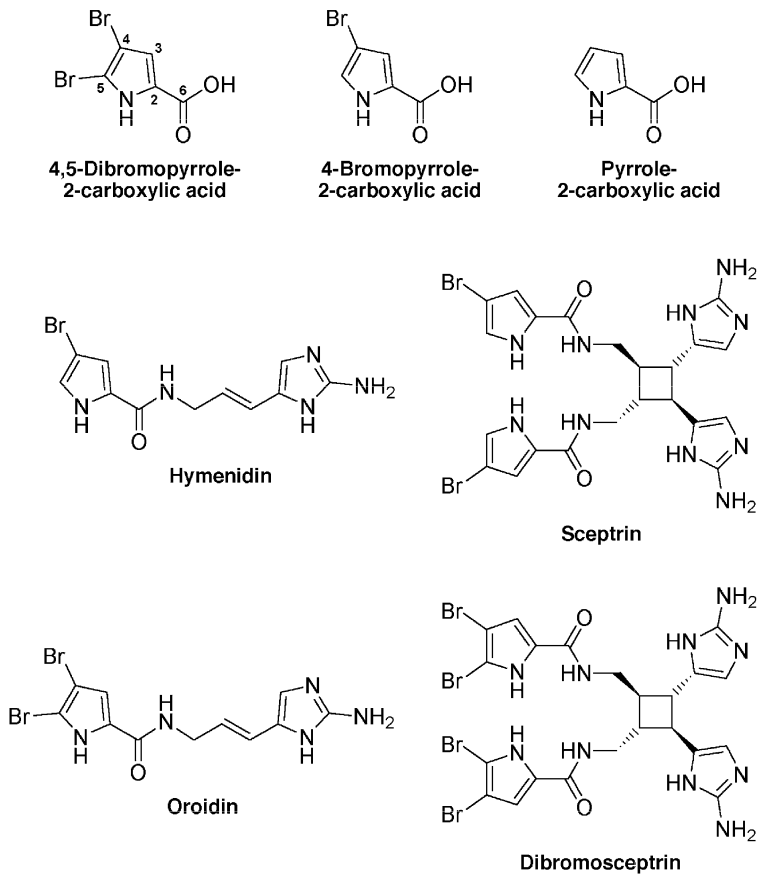

Fig. 1. Chemical structure of investigated pyrrole alkaloids.

So far, several alkaloids have been shown to affect calcium influx and calcium levels in neurons and secretory cells (Bickmeyer et al., 1994; Bennett et al., 1998; Bickmeyer et al., 1998; Khasabova et al., 2002), but no brominated pyrrole alkaloid with the exception of 4,5-dibromopyrrole-2-carboxylic acid has been tested so far.

In the present study, the efficacy of seven pyrrole alkaloids from *Agelas* sponges with respect to bromination and the addition of an aminoimidazole group was compared. To obtain an insight into the structure–activity relationship of bromopyrrole alkaloids with the cellular Ca^{2+} homeostasis in PC12 cells, hymenidin, oroidin, sceptrin, dibromosceptrin, 4-bromopyrrole-2-carboxylic acid and pyrrole-2-carboxylic acid were tested compared with 4,5-dibromopyrrole-2-carboxylic acid (Bickmeyer et al., 2004) (Fig. 1). The question as to whether these alkaloids generally act as feeding deterrents by its taste and smell still remained. Consequently the calcium response of sensory neurons in the rhinophore of *Aplysia punctata* to brominated pyrrole alkaloids in sea water was examined.

2. Material and methods

2.1. Experiments using PC12 cells

2.1.1. Culture methods

PC12 cells from the DSMZ (German collection of microorganisms and cell cultures, Braunschweig, Germany) were kept in culture medium containing RPMI 1640, 10% fetal calf serum, 5% horse serum, and 100 units penicillin/streptomycin per ml. Cells were cultivated in an incubator at 37 °C, 90% humidity and 5% CO_2. Cells grew on collagen coated cover slips and/or in collagen coated petri dishes. Cells were fed every three days and split when necessary.

2.1.2. Fluorimetric measurements of intracellular calcium levels

Cells were incubated in buffer (in mM: 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1.3 NaH_2PO_4, 30 Glucose, 26 Na HEPES) containing 5 μM Fura II acetoxymethylester for 30 min at room temperature (22 ± 2 °C). The incubation

buffer was removed and cells were washed for 20 min. Fluorescence of cells was monitored by an imaging system (Visitron, Puchheim) and a CCD camera mounted on an inverted microscope (Zeiss Axiovert 100). About 30 PC12 cells were measured simultaneously, separated using "the region of interest" function of the software (Metafluor, Meta Imaging Series). Fluorescence was obtained through an UV objective (Zeiss NeoFluar 20X). Data were obtained by division of two images, one obtained at 340 nm, the other at 380 nm excitation. For determination of R-min and R-max, 50 μM Fura II diluted in calcium buffer was used for R-max and buffer without $CaCl_2$ but with additional 10 mM EGTA for determination of R-min in calibration experiments. Fluorescence ratios were converted into calcium concentrations by the formula given by Grynkiewicz et al. (1985). Pyrrole alkaloids were stored at 50–100 mM stock solutions in methanol at 4 °C for experimental use, otherwise as freeze-dried powder at −20 °C.

2.1.3. Experimental design

The recording chamber, mounted on a microscope had a volume of 2 ml and the peristaltic pump was adjusted to 3 ml/min; exchanging the chamber volume in less then a minute. To depolarize the cells, 80 mM KCl was used (supplemented for 80 mM NaCl) in the experimental buffer. The depolarization of the cellular membrane potential therefore increased gradually in less than a minute during perfusion. Cells were depolarized three times a minute during the course of a single experiment of about 60 min. Usually 30 cells were measured simultaneously. Vehicle control experiments using methanol, the stock solution solvent of alkaloids, showed no effect at concentrations lower 1%. By using stock solutions of 50–100 mM much lower concentrations of methanol were applied during the experiments. Results are presented as the mean ± SEM, unless otherwise stated. Statistics and calculations were performed using computer software Prism (Graphpad) and Igor (WaveMetrics).

2.1.4. Experiments using rhinophores of Aplysia punctata

Specimens of *Aplysia punctata* were collected from the shallow waters around Helgoland. Rhinophores were dissected from animals, which were cooled to 0 °C. Rhinophores were cut longitudinally using a razor blade. The side comprising the ganglion was incubated for 60 min at room temperature with artificial sea water (in mM: 460 NaCl, 104 KCl, 55 $MgCl_2$, 11 $CaCl_2$, 15 Na HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid Na-salt)) containing 5 μM Fura II acetoxymethylester. In the high K^+ buffer 400 mM NaCl was replaced by 400 mM KCl.

2.1.5. Sponge secondary metabolites and chemicals

The investigated brominated pyrrole alkaloids (Fig. 1) were isolated from dichloromethane/methanol extracts of the Caribbean sponges *Agelas wiedenmayeri, A. conifera* and *A. sventres* using previously reported methods of collection, isolation and structural elucidation (Assmann et al., 2000, 2001; Assmann and Köck, 2002). Other chemicals were obtained from Sigma, Merck, Fluka and Molecular Probes.

3. Results

3.1. Measurement of the depolarization- induced calcium elevation in the presence of either 4,5-dibromopyrrole-2-carboxylic acid, 4-bromopyrrole-2-carboxylic acid, pyrrole-2-carboxylic acid, hymenidin, oroidin, sceptrin or dibromosceptrin

A significant reduction of depolarization induced calcium elevation by *4,5-dibromopyrrole-2-carboxylic acid* was obtained using concentrations exceeding 30 μM (Fig. 2). No concentration used in our experiments was sufficient to block depolarization induced calcium elevation. No complete dose-response relationship is presented as we declined the use of such high concentrations which would possibly saturate effects. To investigate the influence of the number of bromine atoms at the pyrrole moiety the following alkaloid used was 4-bromopyrrole-2-carboxylic acid, which shows one bromine atom at the pyrrole moiety (Fig. 1).

This monobrominated molecule, *4-bromopyrrole-2-carboxylic acid*, appeared to have no effect on voltage-dependent calcium entry, and was the least potent of the brominated pyrrole alkaloids (Fig. 2). There was no significant reduction of calcium elevations at 300 μM, the highest concentration used.

There also was no significant effect of *pyrrole-2-carboxylic acid* (Fig. 1) for reduction of voltage dependent calcium elevation (Fig. 2).

The addition of an aminoimidazole group to 4-bromopyrrole-2-carboxylic acid, which was not effective, results in the molecule *hymenidin*. This additional aminoimidazole moiety considerably increased the reduction potency of voltage dependent calcium elevation (Fig. 2). *Hymenidin* reduced calcium elevation significantly at a concentration of 300 μM.

Oroidin contains a second bromine atom at the pyrrole ring, which increases its potency in comparison to hymenidin. *Oroidin* fully prevents voltage-dependent calcium elevation (Fig. 2) using a concentration of 300 μM. 100 μM reduces 65% of fluorescence ratio increase in comparison to controls. The obtained data for *oroidin* were fitted by a Hille equation obtaining different fitting parameters for the best description of results. Coefficient values at ± one standard deviation for *oroidin* are: the half-maximal concentration xhalf = 75.8 ± 4.7 μM and the rate = 4.12 ± 0.89 (Fig. 3).

The bromination of the pyrrole ring seems to be an important factor for the pharmacological potency of

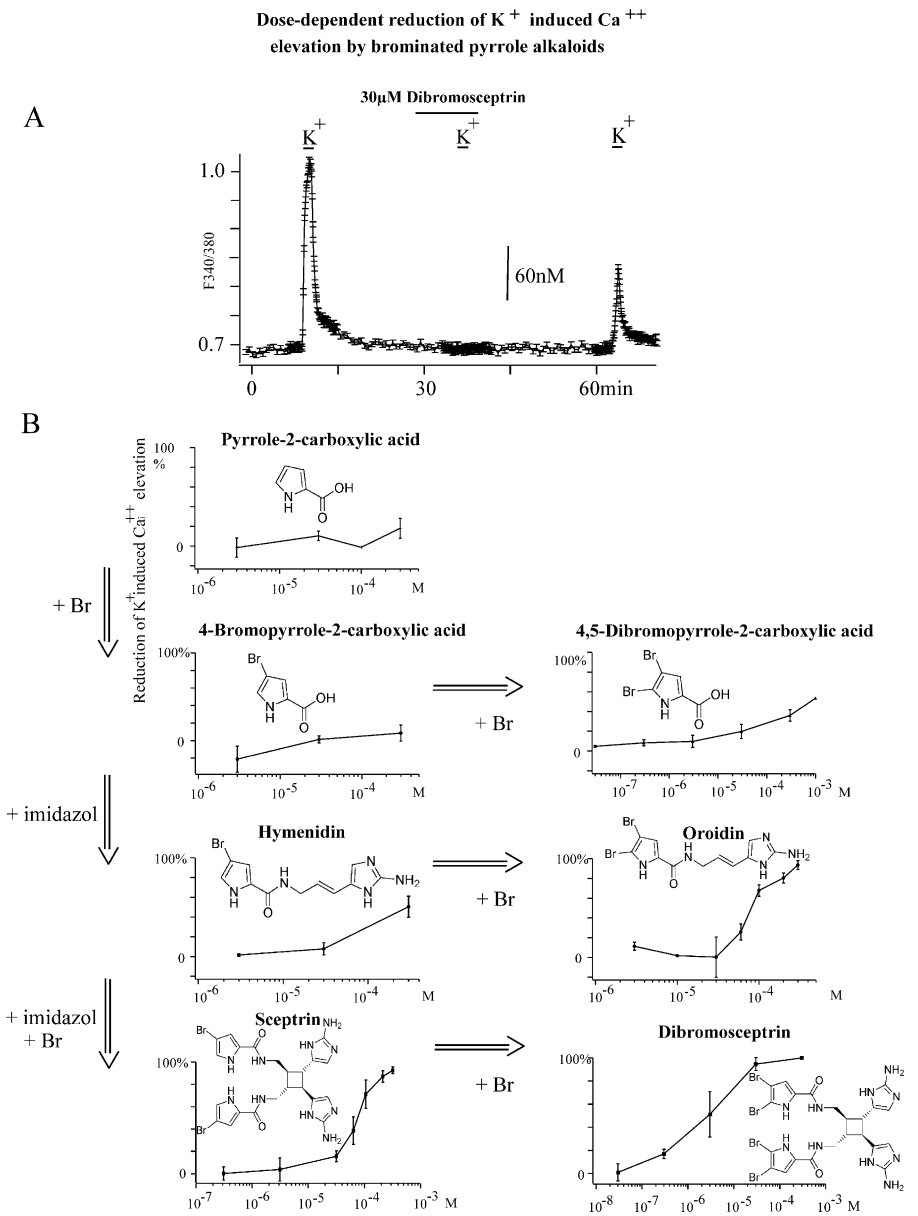

Fig. 2. Reduction of voltage dependent calcium entry by seven different pyrrole alkaloids. (A) Averaged fluorescence ratio trace of about 30 cells. This trace represents a single experiment consisting of control, application of dibromosceptrin and wash. (B) shows the dose response relationship of indicated substances; each point represents 2–7 independent experiments.

the tested alkaloids. The aminoimidazole group seems to play a similar or even major role as shown for oroidin in comparison to 4,5-dibromopyrrole-2-carboxylic acid. Thus, we chose to test the molecule *sceptrin*, which has the same degree of bromination as oroidin (two bromine atoms within the molecule), but in contrast two monobrominated pyrrole moieties each attached to an aminoimidazole group. *Sceptrin* is the formal dimerization product of the monobrominated hymenidin (Fig. 1, Fig. 2). *Sceptrin* was slightly more potent than oroidin in inhibiting evoked calcium elevation. The additional aminoimidazole group influences the half maximal concentration, but even more the steepness of the dose response function (Fig. 3). Coefficient values of ± one standard deviation for sceptrin are: xhalf = 67.5 ± 3,47 µM and the rate = 2.38 ± 0.27 (Fig. 3).

The structural of oroidin is formally represented twice in *dibromosceptrin*. The molecule comprises two dibrominated pyrrole moieties and two aminoimidazole groups (Fig. 1, Fig. 2). *Dibromosceptrin* was the most potent

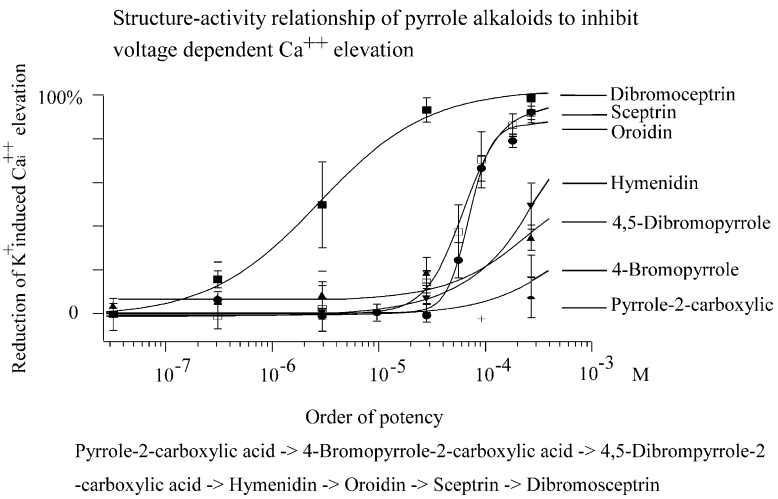

Fig. 3. Normalized dose response relationship of seven pyrrole alkaloids. The data for dibromosceptrin, sceptrin and oroidin were fitted using a Hille equation, 4,5-dibromopyrrole-2-carboxylic acid and 4-bromopyrrole-2-carboxylic acid using a single exponential function, respectively. The order of potency of examined molecules is shown below.

alkaloid tested in our study. Coefficient values of ± one standard deviation for *dibromosceptrin* are: xhalf = 2.79 ± 0.64 μM and the rate = 0.83 ± 0.16 (Fig. 3).

The outcome of the experiments is a structure–activity relationship of pyrrole alkaloids underlining both the importance of bromine and the aminoimidazole group (Figs. 1 and 3).

3.2. Calcium response of tentacle ganglion neurons in the rhinophore of Aplysia punctata to sceptrin

The original observation of the feeding deterrent activity of brominated pyrrole alkaloids stems from behavioral studies with reef fishes both in the aquarium and the field. The question remains if the alkaloids have an odour smell. We measured the pharmacological effect of these alkaloids in a mammalian cell line and came to the conclusion that brominated pyrrole alkaloids influence calcium signaling. To investigate the potency of sceptrin to activate the chemoreceptive/olfactory system of marine animals in general, we used the snail *Aplysia punctata* as a test organism. *Aplysia punctata* possess large rhinophores for olfaction in contrast to contact chemoreceptors on the oral tentacles (Emery and Audesirk, 1977). The rhinophores are usually directed into the floating water. Rhinophores were dissected and cut longitudinally. The rhinophore ganglion was brought into the focus of the microscope and calcium responses of Fura II loaded cells were measured. Excitation of the olfactory system leads to cellular calcium changes in sensory and signal processing neurons. High K^+ buffer induces distinct intracellular Ca^{++} signals, which can be measured either as Ca^{++} elevation (Fig. 4) or as Ca^{++} reduction (not shown) in rhinophore ganglion neurons due to information procession. Responses to application of

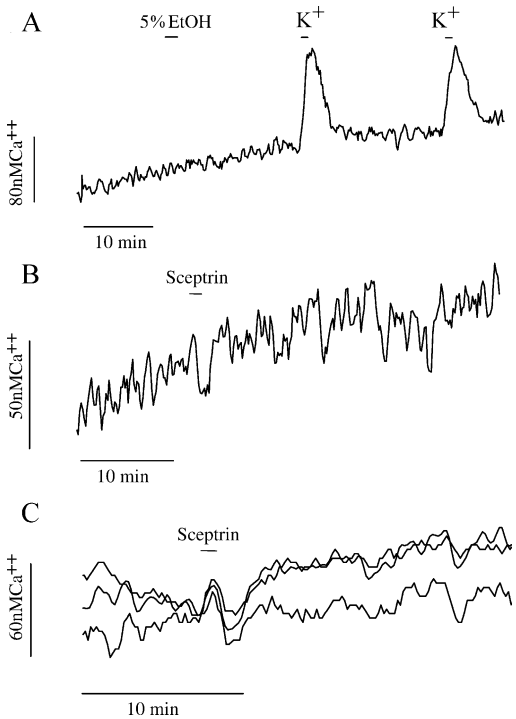

Fig. 4. Three sets of traces of optical recordings representing the calcium response of sensory neurons within the rhinophore ganglion of the sea slug *Aplysia punctata*. (A) Calcium responses to ethanol and high K^+ buffer. (B) The neurons of the olfactory pathway respond to the application of 200 μM sceptrin in sea water. The trace is an averaged trace of a 50 min experiment. (C) Three selected traces (of the experiment shown in B) with expanded time scale.

ethanol (5%) were not detected in our experiments. Fig. 4 shows a cellular calcium response to bath application of 200 μM sceptrin. Measurements were performed in the rhinophore ganglion, where an allocation of sensory information takes place. Application of sceptrin is responded by neurons of the intact olfactory pathway indicating the olfactory perception of the substance. As a control, neurons from the dissected rhinophore ganglion alone without receptor cells and receptive structures, were exposed to sceptrin and no calcium response was measured.

4. Discussion

Brominated pyrrole alkaloids, which have been isolated from marine *Agelas* sponges, interact with the cellular calcium homeostasis by reducing voltage-dependent calcium elevation. As recently shown by Iwata et al. (2001), marine-derived bromine containing gramine analogues from the bryozoan *Zoobotryon pellucidum* induce vasorelaxation in isolated rat aorta. Blockage of calcium entry induces vasorelaxation, as many hypertension drugs are calcium channel blockers. Another brominated alkaloid, a bromotryptamine derivative (Peters et al., 2002), blocks the activity of potassium currents. Interestingly, it was recently shown that brominated diphenyl ethers from industrial production of flame-retardants seem to interact with the endocrine system involving the cellular calcium homeostasis (Kodavanti and Derr-Yellin, 2002; Legler and Brouwer, 2003).

The potency of pyrrole alkaloids increased with the number of bromine atoms associated with the pyrrole ring. The different potency of 4,5-dibromopyrrole-2-carboxylic acid and oroidin apparently originates from the aminoimidazole group, which is of similar importance as the degree of bromination. An additional aminoimidazole group further increased the effects as shown by comparing oroidin with sceptrin. The main difference between oroidin and sceptrin dose–response curves was the steepness of the exponential function to describe the measured results. The addition of two bromine atoms in dibromosceptrin increases the activity to reduce voltage dependent calcium entry tremendously. The steepness of the dose response curve of active pyrrole alkaloids may suggest a non singular cellular target of the molecules such as voltage dependent calcium channels, but possibly additional adverse effects on the cellular membrane. Sceptrin and oroidin have previously been described to have antimicrobial and cytotoxic effects (Bernan et al., 1993; König et al., 1998) and to show anti-muscarinic activity (Rosa et al., 1992) with an IC50 of 50 μM for sceptrin and higher values for oroidin and dibromosceptrin. The interaction with cellular calcium entry has not been called to account as a possible basis for cell biological effects. Dibromosceptrin was shown to change the inactivation of voltage dependent sodium channels in the micromolar range in chick sympathetic ganglia (Rivera Rentas et al., 1995).

The order of potency of pyrrole alkaloids in the present work in general reflects previous results in chemical ecological studies (Assmann et al., 2000, 2004). Higher brominated pyrrole alkaloids are more potent fish feeding deterrents (Table 1) with the exception of dibromosceptrin being not as effective as suggested from the degree of bromination and the results of the present work.

Brominated pyrrole alkaloids interact with the cellular calcium homeostasis, which may be one reason for its feeding deterrence by taste and smell. The same mechanism may work in sensory cells of predatory fishes and other animals like snails. The inhibition of Ca^{++} influx into receptor cells by sceptrin may be one reason for its chemoreceptive detection. A reduction of calcium influx most probably changes the (spontaneous) activity of receptor cells leading to altered activity patterns in processing neurons. Odors can inhibit and excite olfactory receptor cells (Michel and Ache, 1994; Vogler and Schild, 1999) leading to olfactory reception. The important role of lateral inhibition in the olfactory bulb enables complex signal processing (Czesnik et al., 2003).

Neurons in the olfactory pathway of marine snails respond to sceptrin, indicating an olfactory detection of this

Table 1

Compound	PC-12	Feeding deterrent activity (μmol/ml)
Pyrrole-2-carboxylic acid	No activity	180.02
4-bromopyrrole-2-carboxylic acid	No activity	52.633
4,5-dibromopyrrole-2-carboxylic acid	(1 mM)	2.9752
Hymenidin	(300 μM)	9.6726
Oroidin	75.8 μM	1.7993
Sceptrin	67.5 μM	1.6121
Dibromosceptrin	2.8 μM	6.4259

From the seven pyrrole alkaloids listed in the table, only 4,5-dibromopyrrole-2-carboxylic acid, oroidin (*Agelas clathrodes*, *A. dispar*, *A. wiedenmayeri*, *A. cervicornis*), and sceptrin (*A. conifera*, *A. cerebrum*, *A. dilatata*, *A. sceptrum*) are each present in sponge tissue at natural concentrations sufficient to deter predatory fish. The minimum concentration of the major secondary metabolites required for feeding deterrency is as follows: 0.8 mg/ml sponge tissue for 4,5-dibromopyrrole-2-carboxylic acid, 0.7 mg/ml for oroidin, and 1.0 mg/ml for sceptrin, respectively (Assmann et al., 2000, 2004). To observe a feeding deterrent effect for the other four alkaloids much higher, non-natural amounts are required: hymenidin (3 mg/ml), dibromosceptrin (5 mg/ml), 4-bromopyrrole-2-carboxylic acid (10 mg/ml), and pyrrole-2-carboxylic acid (20 mg/ml), respectively (Assmann et al., 2000, 2001). The feeding deterrent activities of the seven pyrrole alkaloids is shown at μmol per ml sponge tissue. The results are compared with halfmaximal concentrations (if possible) measured in PC12 cells.

sponge metabolite in water. Environmentally exposed cells, such as the chemosensory cells of snails, are targets for many chemical substances and possibly several substances may produce a sensory input by an interaction with cellular membrane conductance.

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