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Novel bioactive bromopyrrole alkaloids from the Mediterranean sponge *Axinella verrucosa*

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Abstract—The Mediterranean sponge *Axinella verrucosa* has been investigated for its alkaloid composition and has been found to produce a complex mixture of bromopyrrole alkaloids. Along with the previously isolated compounds **5**–**18**, four novel alkaloids of this class, compounds **1**–**4**, have been isolated, and their structures established through spectroscopic methods. Compounds **1**–**4** were found to display neuroprotective activity against the agonists serotonin and glutamate in vitro.

1. Introduction

Alkaloids are known to have an important role in medicinal chemistry on account of their widespread biological activity. Among the alkaloids of marine origin, especially noteworthy is the pyrrole-imidazole group, which is widely distributed in marine sponges belonging to the genera *Agelas*, *Axinella*, *Acanthella*, *Pseudoaxinyssa*, and *Hymeniacidon*.[1,2] Since the isolation of the first member of this group, oroidin, from the sponge *Agelas oroides* in 1971,[3] over the last thirty years a number of similar and/or related molecules have been encountered, and many of these compounds are reported to have intriguing biochemical activities, such as blocking α-adrenoceptors,[4] serving as antagonist of serotonergic receptors,[5] activating actomyosin ATPase,[6] inhibiting kinase activity,[7] as well as antibacterial,[8] antifungal,[8] and anti-histamine activities.[9] For example, we can mention the bisguanidine alkaloid palaua'amine isolated from the sponge *Stylotella aurantium*,[10,11] whose antifungal, antitumor, and immunosuppressive activities have been undertaken for preclinical studies.[12] The diverse biological activities of palau'amine and its apparently low toxicity make this alkaloid also an important target for total synthesis.[13] Thus, bromopyrrole derivatives could represent useful tools for chemical, pharmacological, and physiological studies oriented to discover new drugs.

During our search for bioactive substances from Mediterranean sponges, we have chemically investigated the organic extract of specimens of *Axinella verrucosa* (order Halichondrida, family Axinellidae) collected from the bay of Calvi (Corsica). The sponge extract was shown to contain a complex mixture of structurally different brominated pyrrole alkaloids (Fig. 1). Most of these alkaloids are previously described compounds and were easily identified as 4-bromopyrrole-2-carboxylic acid (**5**),[3] zooanemonine (**6**),[14] stevensine (**7**),[15] spongiacidins A and B (**8, 9**),[16] manzacidins A and C (**10**, **11**),[17] *N*-metilmanzacidin C (**12**),[18] mukanadins A–C (**13**–**15**),[19] 2-bromo aldisine (**16**),[20] 4,5-dibromopalau'amine (**17**),[11] and 2,3-dibromostyloguanidine (**18**).[21] In addition to these known compounds, the chemical analysis of *A. verrucosa* yielded four novel alkaloids, compounds **1**–**4**, all of them possessing a monobromopyrrole 2-carboxamide unit condensed with different moieties. In compound **4**, this unit is linked to a methoxymethyl group, while compounds **1**,**2**, and**3** show a short linear aliphatic segment connecting the pyrrole moiety to hydantoin, an aminoimidazolinone, and a taurine-containing imidazole ring, respectively.

Compounds **1**–**4** were tested for their biological activity and were found to display neuroprotective activity against the agonists serotonin and glutamate in vitro.

Keywords: Marine sponges; Natural products; Bromopyrrole alkaloids; *Axinella verrucosa*; Neuroprotective activity.
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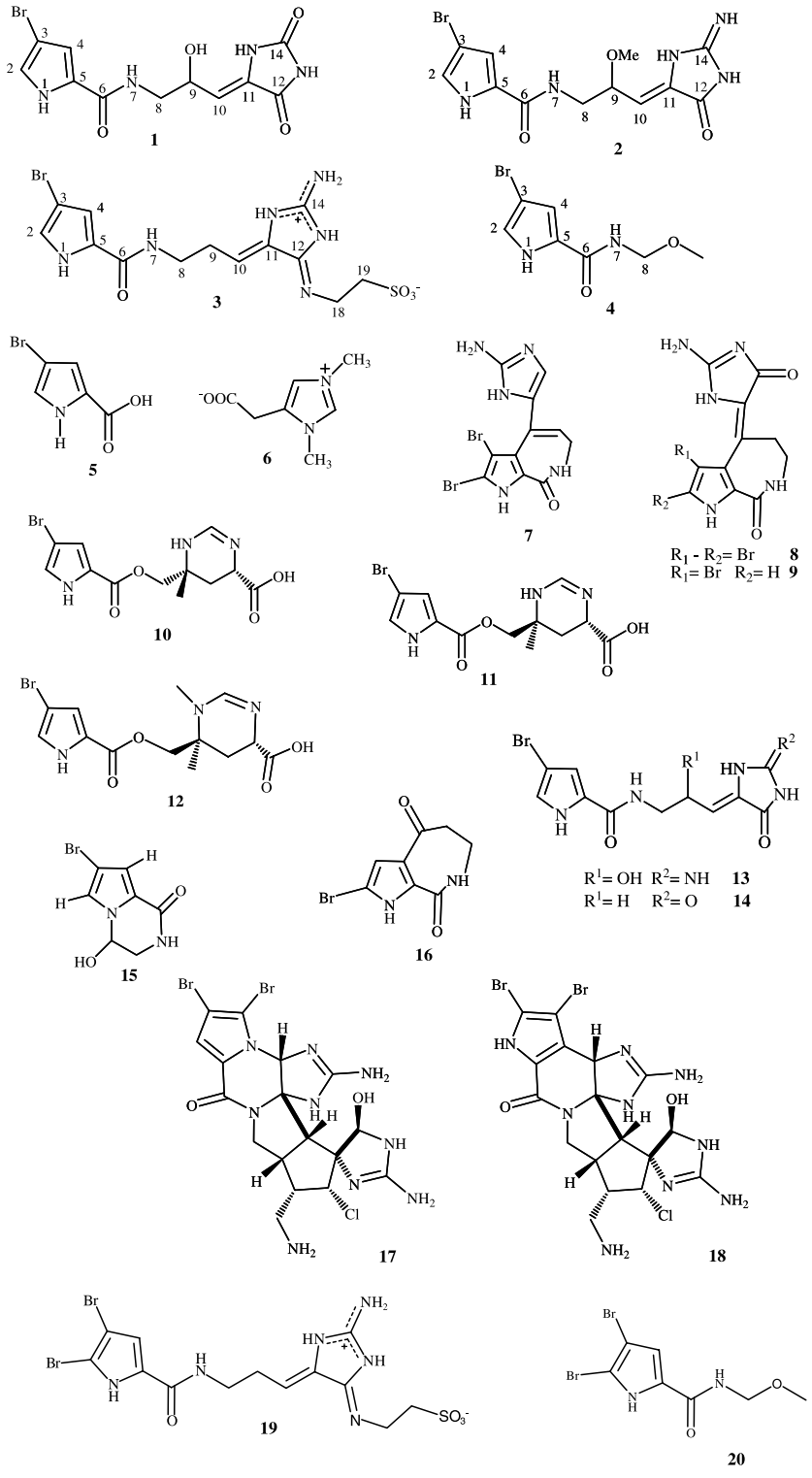


Figure 1. Bromopyrrole alkaloids isolated from *A. verrucosa*.

2. Results

2.1. Isolation of compounds 1–4

Specimens of *A. verrucosa* (Esper, 1794) were collected from the bay of Calvi (Corsica) in the winter of 2001; they were extracted with methanol, and, subsequently, with chloroform. The combined extracts were partitioned between ethyl acetate and water, and finally, the aqueous layer was subsequently extracted with butanol. First fractionation of the butanol-soluble portion was achieved by MPLC over a reversed-phase C-18 column and by gel permeation on Sephadex LH20. Several bromopyrrole alkaloids containing fractions were identified by TLC and ^{1}H NMR monitoring; separation and purification of these fractions by repeated

Table 1. List of alkaloids isolated from the sponge *Axinella verrucosa* with their relative amounts (in mg) obtained from 115 g of sponge (dry weight after extraction)

Compound	mg
4-Bromopyrrole-2-carboxylic acid (**5**)	20
Zooanemonine (**6**)	30
Stevensine (**7**)	60
Spongiacidin A (**8**)	1.2
Spongiacidin B (**9**)	1.5
Manzacidin A (**10**)	1.2
Manzacidin C (**11**)	1.5
N-metilmanzacidin C (**12**)	2.0
Mukanadin A (**13**)	2.3
Mukanadin B (**14**)	5.0
Mukanadin C (**15**)	0.7
2-Bromo aldisine (**16**)	3.5
4,5-Dibromopalau'amine (**17**)	4.0
2,3-Dibromostyloguanidine (**18**)	4.5
Compound **1**	0.8
Compound **2**	1.2
Compound **3**	1.5
Compound **4**	3.0

reversed-phase HPLCs resulted in the isolation of compounds **1**–**18** as pure products (Table 1).

The previously isolated alkaloids **5**–**18** were identified by comparison of their spectroscopic data with those previously reported in the literature.[3,11,14–21]

2.2. Structure elucidation of compounds 1–4

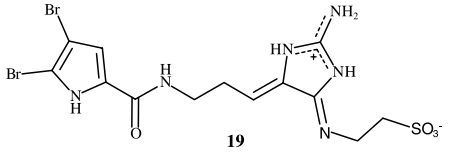
ESI mass spectrum (negative ion mode) of compound **1** showed two pseudomolecular ion peaks at *m*/*z* 341 and 343 $[M-H]^-$ in the ratio 1:1, suggesting the presence, in the molecule, of one bromine atom. The molecular formula of compound **1** was determined to be $C_{11}H_{11}BrN_4O_4$ by its positive HRFABMS measured on the peak at *m*/*z* 343.0048 $[M+H]^+$ (the calculated value for $C_{11}H_{12}{}^{79}BrN_4O_4$ is 343.0042). The ^{13}C NMR data, including DEPT experiments, revealed the presence of three carbonyls, three sp^2 quaternary and three sp^2 methine carbons, one oxymethine, and one methylene carbon (see Table 3). The presence of a 4-bromopyrrole-2-carboxamide moiety was strongly suggested by the aromatic signals present at δ 6.95 and δ 6.86 in the ^1H NMR spectrum (DMSO-d_6) and by the ^{13}C NMR pattern of resonances (δ_C 121.1, 94.7, 111.8, 126.7, and 159.9), which appeared very similar to the values reported in the literature for bromopyrrole alkaloids.[22]

The ^1H NMR spectrum of **1** contained, in addition to the above-mentioned aromatic signals, five D_2O-exchangeable signals [δ 11.86 (br s), NH-1; δ 8.20 (t, J = 5.9 Hz), NH-7; δ 10.15 (br s), NH-13; δ 10.95 (br s), NH-15; 5.42, (br s), OH]; additional features of the ^1H NMR spectrum were: a deshielded methylene signal (δ 3.34, dd, J = 13.6 Hz, 5.9 Hz) and an oxymethine signal (δ 4.45, bdd, J = 5.5 Hz, 13.6 Hz). The ^1H and ^{13}C NMR data of **1** closely resembled those of mukanadin B (**14**)[19] (see Tables 2 and 3), the main difference being the presence of a hydroxymethine signal in the NMR spectra of **1**, replacing a methylene signal in the NMR spectrum of **14**. This led to the hypothesis that **1** contained an alcohol functionality that was located at C-9, following the COSY connectivities starting from NH-7 to H-10. Thus, **1** was established to be the 9-hydroxy derivative of mukanadin B.

The ESI mass spectrum (negative ion mode) of compound **2** showed prominent pseudomolecular ion peaks at *m*/*z* 354 and 356 $[M-H]^-$ in the ratio 1:1, indicating that this compound also contained one bromine atom. The molecular formula $C_{12}H_{14}BrN_5O_3$ for compound **2** was determined by its positive HRFABMS measured on the peak at *m*/*z* 356.0363 $[M+H]^+$ (the calculated value for $C_{12}H_{15}{}^{79}BrN_5O_3$ is 356.0358). Analogous to compound **1**, a comparison of the whole of NMR data of **2** (see Tables 2 and 3) with those of the previously reported mukanadin A (**13**)[19] revealed a close resemblance between the two compounds. The only substantial difference observed in the ^{13}C NMR spectra of the two compounds was the presence, in the spectrum of **2**, of an extra signal at δ 56.5 due to a methyl carbon linked to a heteroatom. This signal was coupled, in the HSQC spectrum, with a methyl singlet resonating at δ 3.24 in the ^1H NMR spectrum. Besides this, both C-9 and H-9 chemical shifts, compared with those of mukanadin A,[19] appeared upfield shifted in **2**. This information, taking into account the mass spectral data, strongly suggested that the hydroxyl function located at C-9 in mukanadin A had been replaced by a methoxy functionality in **2**, whose structure was thus concluded to be the 9-methoxy derivative of monobromodispacamide, previously isolated from the sponges *Agela conifera* and *A. longissima*.[23]

Compounds **1** and **2** showed null optical rotations and we thus arrived at a conclusion that both compounds were racemes.

The molecular formula of compound **3** was determined to be $C_{13}H_{17}BrN_6O_4S$ by its positive HRFABMS measured on the peak at *m*/*z* 433.0295 $[M+H]^+$ (the calculated value for $C_{13}H_{18}{}^{79}BrN_6O_4S$ is 433.0299). The IR absorptions of **3** suggested the presence of NH (ν (tilde)$_{max}$ 3420 cm^{-1}), amide carbonyl (ν (tilde)$_{max}$ 1725 and 1690 cm^{-1}), and sulfonate (ν (tilde)$_{max}$ 1210 and 1040 cm^{-1}) functionalities. The ^1H and ^{13}C NMR data of **3** (in CD_3OD, see Tables 2 and 3) were almost identical to those of taurodispacamide A, a pyrrole alkaloid previously isolated from



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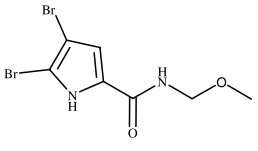

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Table 2. ^{1}H NMR spectroscopic data (500 MHz) of compounds **1**–**4**

Pos.	1 δ_H (mult., J [Hz])[a]	2 δ_H (mult., J [Hz])[a]	3 δ_H (mult.,J [Hz])[b]	4 δ_H (mult., J [Hz])[c]
1-NH	11.86 (br s)	11.82 (br s)	11.85[d] (br s)	13.82 (br s)
2	6.95 (br s)	6.95 (br s)	6.94 (s)	7.43 (s)
3	—	—	—	—
4	6.85 (br s)	6.86 (br s)	6.77 (s)	7.38 (s)
5	—	—	—	—
6	—	—	—	—
7-NH	8.18 (br s)	8.14 (t, 5.5)	8.15[d] (br s)	9.76 (t, 6.9)
8	3.43 (m)	3.43 (m)	3.49 (t, 7.0)	5.04 (d, 6.9)
9	4.42 (m)	4.19 (m)	2. 63 (dd, 7.0; 7.5)	—
10	5.35 (d, 5.5)	5.17 (d, 7.5)	5.96 (t, 7.5)	—
11	—	—	—	—
12	—	—	—	—
13-NH	10.15 (br s)	10.95 (br s)	9.65[d] (br s)	—
14	—	—	—	—
15-NH	10.95 (br s)	10.37 (br s)	9.00[d] (br s)	—
16-NH	—	8.50 (br s)	—	—
16-NH_2	—	—	5.42[d] (br s)	—
17	—	—	—	—
18	—	—	3.87 (t, 6.9)	—
19	—	—	3.14 (t, 6.9)	—
–OH	5.39 (m)	—	—	—
–OMe	—	3.21 (s)	—	3.39 (s)

Data are from the spectrum recorded in: [a]DMSO-d_6, [b]CD_3OD, [c]pyridine-d_5.
[d] Exchangeable proton resonances are from the spectrum recorded in DMSO-d_6.

Table 3. ^{13}C MNR spectroscopic data (125 MHz) of compounds **1**–**4**

Pos.	1 δ_C[a]	2 δ_C[a]	3 δ_C[b]	4 δ_C[c]
2	121.1	121.4	122.9	123.2
3	94.7	94.6	97.2	97.0
4	111.8	111.5	113.6	112.8
5	126.7	126.5	126.8	128.1
6	159.9	159.9	162.8	161.7
8	44.5	42.6	39.7	71.6
9	65.8	62.4	29.3	—
10	107.4	118.9	114.9	—
11	127.7	128.8	138.1	—
12	160.4	163.9	169.9	—
14	154.8	154.9	168.5	—
18	—	—	40.6	—
19	—	—	50.3	—
OMe	—	56.5	—	55.5

Data are from the spectrum recorded in: [a]DMSO-d_6, [b]CD_3OD, [c]pyridine-d_5.

the sponge *Agelas oroides* possessing the dispacamide skeleton and a taurine residue attached to the imidazole ring.[24] Differences between the two compounds were confined to the NMR resonances of the pyrrole moiety of **3**, which were those of a 4-bromopyrrole-2-carboxamide. This led immediately to the conclusion that **3** was the 2-debromo derivative of taurodispacamide A (**19**), well-substantiated by mass spectral data and by NMR spectral data given in Tables 2 and 3.

The molecular formula of compound **4** was determined to be $C_7H_9BrN_2O_2$ by its positive HRFABMS measured on the peak at *m/z* 232.9930 $[M+H]^+$ (the calculated value for $C_7H_{10}{}^{79}BrN_2O_2$ is 232.9924). ^{1}H and ^{13}C NMR spectra (in CD_3OD, see Section 4) showed aromatic resonances that were identical to those of compound **3** (see Tables 2 and 3), suggesting that compound **4** also contained a 3-bromo-2-carboxamide moiety. The amide carbonyl function was indicated by measuring the absorbance at 1650 cm^{-1} present in the IR spectrum and by the carbonyl resonance at δ 161.7 in the ^{13}C NMR spectrum. In addition to pyrrole signals, the ^{1}H NMR spectrum contained two singlets, at δ 4.70 (2H) and at δ 3.35 (3H), correlating, in the HSQC spectrum, the carbon signals at δ 71.6 and δ 55.5, respectively. Taking into account the molecular formula, the only way to arrange the above oxymethylene and methoxy groups is shown in formula **4**, which represents the 2-debromoderivative of compound **20**, previously isolated from a *Homoaxinella* sp.[25]

2.3. Cytotoxic effects of compounds 1–4

The cell viability tests using the MTT assay[26] revealed that compounds **1**, **2**, **3**, and **4** were not toxic at concentrations below 10 μg/ml for all tested cell lines (PC12, HeLa, and L5178y cells).

2.4. Effect of compounds 1–4 and serotonin on the Ca^{2+}-level in primary neurons

Stimulation of the neurons with 200 μM serotonin (5-HT) and 2.5 mM $CaCl_2$ resulted in a significant ($p < 0.001$) increase in intracellular free calcium level ($[Ca^{2+}]_i$); an increase from 0.810 ± 0.097 to 1.781 ± 0.112 was determined (Δ ratio 0.971; set to 100%). The changes are expressed as 340/380 nm ratio values.

Preincubation of neurons with 10 μg/ml compound **4** for 5 min did not change the free $[Ca^{2+}]_i$ caused by the addi-

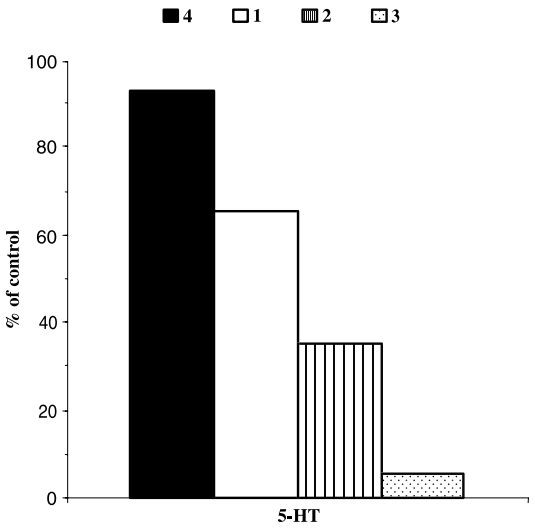

Figure 2. Incubation of the neurons with 200 μM serotonin (5-HT) and 2.5 mM of $CaCl_2$ in the presence of compound **1**, **2**, **3**, or **4**. In the first set of experiments, (control) neurons were incubated with 5-HT and $CaCl_2$ only; the values obtained were set to 100%. In the experiments, testing the effect of the compounds, the neurons were incubated with 10 μg/ml of **1**, **2**, **3**, or **4**; subsequently, the of 5-HT/$CaCl_2$-mediated change of $[Ca^{2+}]_i$ in neurons was measured, as follows. the cells were preincubated for 5 min with 10 μg/ml of the respective compound; then the agonists were added and the experiments were completed in Locke's solution. In all assays, the $[Ca^{2+}]_i$ level was measured for 25 min.

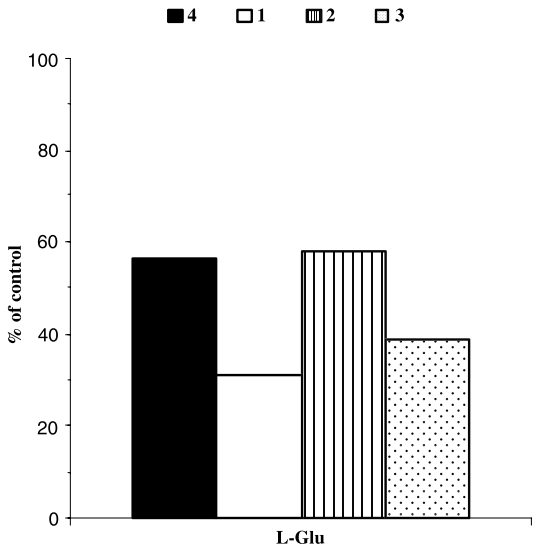

Figure 3. Incubation of the neurons with 200 μM L-glutamic acid (L-Glu) and 2.5 mM $CaCl_2$ in the presence of 10 μg/ml of compounds **1**–**4**. Further details are given in Figure 2.

tion of 5-HT and $CaCl_2$ (Fig. 2). The values increased from 0.720 ± 0.021 to 1.624 ± 0.130 (*Δ* ratio 0.904; 93.1% [control *Δ* ratio of 0.971 is set to 100%]). In experiments with compounds **1**, **2**, and **3**, respectively, neurons were preincubated with 10 μg/ml each for 5 min. As shown in Figure 2, the $[Ca^{2+}]_i$ in those pretreated neurons increased only slightly, after the addition of 200 μM of 5-HT and 2.5 mM $CaCl_2$. The changes in values obtained, in comparison to the control, are significant ($p < 0.001$). The *Δ* ratios (340/380 nm) determined were 0.638 (decrease to 65.7%) for compound **1**, 0.344 (35.4%) for **2**, and 0.054 (5.6%) for **3** (Fig. 2).

2.5. Effects of compounds 1–4 and L-glutamic acid on the Ca^{2+}-level in primary neurons

Stimulation of neurons with 200 μM L-glutamic acid (L-Glu) and 2.5 mM $CaCl_2$ also resulted in a significant ($p < 0.001$) increase in $[Ca^{2+}]_i$ from 0.828 ± 0.013 to 2.543 ± 0.081 (*Δ* ratio 1.715; 100%; control).

Preincubation (5 min) of the neurons with 10 μg/ml of compounds **4** and **2** effected a decrease in the 340/380 nm ratio after the addition of L-Glu and $CaCl_2$ (Fig. 3). The ratio values increased from 0.711 ± 0.024 to 1.682 ± 0.086 (*Δ* ratio 0.971; 56.6%; compound **4**; the *Δ* ratio of the control were 1.715) and from 0.836 ± 0.024 to 1.834 ± 0.061 (*Δ* ratio 0.998; 58.2%; **2**). As shown in Figure 3, compounds**1** and **3** caused even an stronger inhibitory effect on $[Ca^{2+}]_i$ in primary neurons. After pretreatment of the cells with 10 μg/ml of compounds **1** or **3** for 5 min, the cells responded with a significant ($p < 0.001$) decrease in free $[Ca^{2+}]_i$ after the addition of L-Glu/$CaCl_2$. The value dropped to 31.0% (*Δ* ratio 0.532; **1**) and to 38.7% (*Δ* ratio 0.663; **3**).

2.6. Effects of compounds 1–4 and quisqualic acid on the Ca^{2+}-level in primary neurons

If neurons were treated with 200 μM quisqualic acid (QUIS) and 2.5 mM $CaCl_2$, likewise a significant ($p < 0.001$) increase in $[Ca^{2+}]_i$ from 0.891 ± 0.035 to 1.263 ± 0.059 (*Δ* ratio 0.327; 100%) is observed. However, incubation of the neurons with 10 μg/ml of **4**, **1**, and **2** for 5 min protected the cells against the effect toward QUIS and $CaCl_2$ (Fig. 4). The intracellular calcium concentration increased only to 144.1% (*Δ* ratio 0.536; **4**), to 166.4 (*Δ* ratio 0.619; **1**), and to 131.7% (*Δ* ratio 0.490; **2**). In contrast, in the last series of experiments compound **3** (10 μg/ml) was shown to enhance the effect of QUIS/

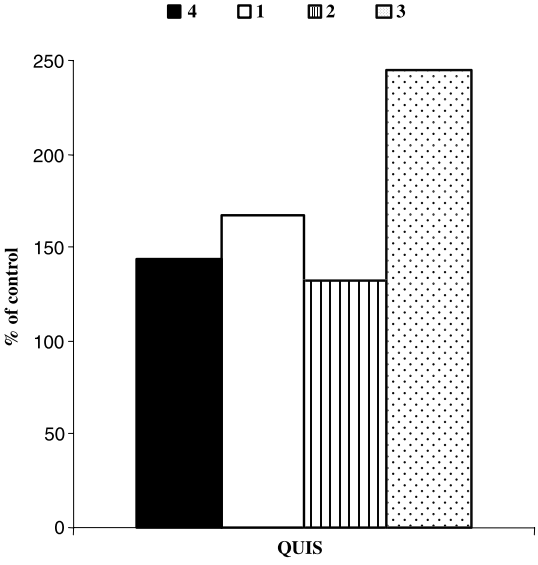

Figure 4. Incubation of the neurons with 200 μM quisqualic acid (QUIS) and 2.5 mM $CaCl_2$ in the presence of 10 μg/ml of compounds **1**–**4**. In all cases, the $[Ca^{2+}]_i$ level was measured for 35 min.

$CaCl_2$ on $[Ca^{2+}]_i$; the value increased to 243.8% ($p < 0.001$) (Fig. 4) from 0.752 ± 0.019 to 1.659 ± 0.051 (Δ ratio 0.907).

3. Discussion

The amino acids L-glutamate and L-aspartate represent one of the most powerful excitatory neurotransmitters in the central nervous system (CNS); glutaminergic neurons are especially prominent in the cerebral cortex.[27] The biological effects of these excitatory amino acids (glutamate and aspartate) are manifold. Glutamate is involved in fast synaptic transmission eliciting a postsynaptic depolarization;[28] it can also effect long-lasting activity-dependent changes in neuronal excitability, as in the case of long-term potentiation (LTP).[29] On one hand, it offers a beneficial effect on the regulation of neuronal function, growth, and differentiation, on the other, excitatory amino acids can even be injurious to brain tissue. This happens especially when large amounts of glutamate or aspartate are released into the extracellular space, leading to hyperactivation of glutamate receptors. This hyperactivation, which has been termed 'excitotoxicity' (a phenomenon that triggers the activation in the central nervous system and involves the activation of glutamate receptors), can be observed in ischemia, hypoglycemia, epileptic seizures, and in neurodegenerative diseases, such as Alzheimer's disease, parkinsonism, and amyotrophic lateral sclerosis.[30–32] The excitotoxic effect is related to the massive entry of $[Ca^{2+}]_i$ into the cells as a consequence of the sustained activation of glutamate receptors.[33,34] An excessive rise of $[Ca^{2+}]_i$ inflicts multiple cytotoxic damage to the neurons, such as perturbation of cytoskeletal proteins and activation of proteases and phospholipases. In addition to their proteolytic and lipolytic activity, these enzymes result in the formation of free radicals that damage the cells.

Addition of glutamate induces massive influx of Na^+, K^+, and Ca^{2+} into neurons.[34] Glutamate stimulates ionotropic receptors, as also the *N*-methyl-D-aspartate (NMDA) receptor, and metabotropic receptors. Metabotropic glutamate receptors are coupled to a variety of second messenger systems via G proteins. The ion channels open upon binding of glutamate and allow the entry of ions. In our experiments, preincubation of neurons with 10 μg/ml compounds **1**–**4** caused a significant decrease in Ca^{2+} entry. Thus, the data presented demonstrate that compounds **1**–**4** are acting as glutamate antagonists. A very potent neuroprotective effect (~70% reduction) was observed with compounds **1** and **3**, while compounds **4** and **2** were less potent (~55% reduction). Further studies are necessary to prove the effect of compounds **1**–**4** on the NMDA receptor. At present, we suggest that compounds **1** and **3** represent new potent glutamate antagonists. In comparison, the taurine, a newly described neuroprotective agent, displayed its activity at millimolar concentrations.[35]

Metabotropic glutamate receptors (mGluR) are coupled to G proteins and the ensuing signal transduction involves different second messenger systems.[36] The mGluR generate slow postsynaptic responses after an adequate stimulus. They can be activated by glutamate, quisqualate, etc., while at the same time being resistant to activation by NMDA, AMP, or kainate. The mGluR either interact with the adenylate cyclase system or with the protein kinase C system. Quisqualate is a selective agonist of the mGluR1. In our experiments, preincubation of neurons with 10 μg/ml of compounds **1**–**4** induced a stronger influx of Ca^{2+} into the neurons. The highest effect was measured with compound **3**. It seems that compounds **1**–**4** modulate the effect of quisqualic acid on neurons. The mode of action still remains unknown.

Serotonin belongs to the class of biogenic amines (indolamines or monoamine transmitters). Within the CNS, the serotonergic neurons can be found in nearly every brain area. Serotonin modulates several biological functions in the CNS;[37] it influences processes related to memory and learning, sexual behavior, as well as feeding behavior.[38] It also seems to be involved in regulating the aggressive behavior. Alterations in serotonin function has been linked to anxiety states, affective disorders, eating disorders, sleep disorders, depression, and migraine.[39] In the treatment of psychosis, antagonists of 5-HT_2 receptor appear to be efficacious.[40–42]

Preincubation of neurons with 10 μg/ml of compounds **1**, **2**, and **3** resulted in a significant decrease in $[Ca^{2+}]_i$ influx after that addition of 200 μM serotonin. Changes in the structure from **4** to **1** increased the activity of **1** (~35% of reduction). Compound **2** was a potent antagonist of serotonin (~65% of reduction), while the effect of serotonin on neurons was completely blocked with compound **3** (~95% of reduction). Thus, compounds **2** and **3** seem to be promising serotonin antagonists with a potential to treat psychosis, different phobia, and mood fluctuation disorders.

In conclusion, among the compounds tested compound **3** appears to be the most interesting substance since it acts as a glutamate, as well as a serotonin, antagonist and shows very potent neuroprotective effect.

4. Experimental

4.1. General remarks

ESI mass spectra were obtained on an API 2000 mass spectrometer. High-resolution FAB mass spectra (glycerol matrix) were performed 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 a Bruker AMX-500 spectrometer; chemical shifts are referred to the residual solvent signal (CD_3OD: $\delta_H = 3.31$, $\delta_C = 49.0$; DMSO-d_6: $\delta_H = 2.49$, $\delta_C = 39.5$; pyridine-d_5: $\delta_H = 8.71$, $\delta_C = 149.9$). 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 Waters 501 apparatus equipped with an RI detector. UV spectra (MeOH) were recorded on a Shimadzu UV-1204 instrument.

4.2. Extraction and isolation procedure

Specimens of the sponge *A. verrucosa* were collected in November 2001 from the bay of Calvi (Corsica) and kept frozen until used. For the extraction, the freshly thawed sponge (115 g of dry weight after extraction) was homogenized and extracted at room temperature with methanol (3×200 ml) and, subsequently, with chloroform (3×500 ml). The combined extracts were partitioned between H_2O and *n*-BuOH; the organic layer was concentrated in vacuo and afforded 30 g of a dark residue, which was chromatographed on a RP-18 silica gel column, eluting with $H_2O \rightarrow$ MeOH $\rightarrow CHCl_3$. Three alkaloid-containing fractions, eluted with H_2O/MeOH 9:1 (fraction A, 5 g), with H_2O/MeOH 6:4 (fraction B, 600 mg), and with H_2O/MeOH 2:8 (fraction C, 4 g), were obtained. Fraction A was subjected to a Sephadex LH-20 chromatography eluting with MeOH and afforded compound **6** (30 mg). Fraction B was separated and purified by subsequent HPLCs on reversed-phase columns (Synergi Polar-RP, 4 μm, 250×4.6 mm, and Luna, 3 μm, 250×4.6 mm) eluting with MeOH/H_2O 35:65 + 0.1% TFA, affording compounds **17** (4 mg) and **18** (4.5 mg). Fraction C was subjected to a further MPLC over a reversed-phase column eluting with $H_2O \rightarrow$ MeOH. The alkaloid fraction (2 g), eluted with H_2O/MeOH 6:4, was then separated by HPLC on a preparative RP-18 column (Luna, 10 μm, 250×4.6 mm) with MeOH/H_2O 3:7 as the eluent. This chromatography yielded pure compounds **7** (60 mg) and **5** (20 mg), as well as three complex alkaloid mixtures, fractions C_1–C_3. Fraction C_1 was separated by HPLC on a Synergi RP-Polar column (4 μm, 250×4.6 mm) with MeOH/H_2O 2:8 as the eluent, yielding compounds **9** (1.5 mg) and **3** (1.5 mg) in pure state. Fraction C_2 was separated and purified by subsequent HPLCs on RP-18 columns (Luna, 5 μm, 250×4.6 mm; Luna, 3 μm, 250×4.6 mm) with MeOH/H_2O 1:1 and MeOH/H_2O 3:7 as the eluent, giving pure compounds **13**–**15** (2.3, 5 and 0.7 mg, respectively), **16** (3.5 mg), **10** (1.2 mg), **11** (1.5 mg), **12** (2 mg), **8** (1.2 mg), **1** (0.8 mg), and **2** (1.2 mg). Fraction C_3 was purified by HPLC on a Synergi RP-Polar column (4 μm, 250×4.6 mm) with MeOH/H_2O 4:6 as the eluent, giving pure compound **4** (3 mg).

4.3. Cell lines

PC12 cells were grown in Dulbecco's modified Eagle's medium [DMEM]/10% (v/v) fetal calf serum (FCS)/5% (v/v) horse serum. The 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 cells were kept in an atmosphere of 95% air and 5% CO_2 at 37 °C.

4.4. MTT assay

To estimate the IC_{50} values, PC12, L5178y, and HeLa cells were incubated for 72 h in the presence of different concentrations (0.1; 0.3; 1; 3, and 10 μg/ml) of compound **1**, **2**, **3**, or **4**. The final volumes were 200 μl. All compounds were dissolved in DMSO (stock solution 10 mg/ml) and stored at −20 °C. The viability of the cells was determined using the MTT colorimetric assay system.[43] The evaluation was performed in 96-well plates at 595 nm using an ELISA plate reader, after overnight incubation at 37 °C.

4.5. Calcium measurements on primary neurons

Rat cortical cell cultures were prepared from the brains of 17- to 18-day-old Wistar rat embryos, as described earlier.[44,45] Briefly, after isolation cerebral hemispheres were placed into Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. Brain tissue was dissociated in HBSS using 0.025% (w/v) trypsin (10 min; 37 °C); the proteolytic reaction was stopped by addition of 10% FCS. The single cell suspension was centrifuged and the pellet containing dissociated neuronal cells was resuspended in DMEM/HG (high glucose; 4500 mg/L glucose), containing 2 mM L-glutamine, 100 mU/l insulin, and 10% (v/v) fetal calf serum (FCS). The cells were seeded into poly-L-lysine (5 μg/ml, 300 μl/cm^2)-coated plastic dishes at a concentration of 2.0×10^5 cells/cm^2. Two days after isolation DMEM/HG/10% (v/v) FCS was removed and the cells were cultivated further in a DMEM/HG 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.

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined by measuring the fluorescence ratio of the Ca^{2+}-indicator dye fura-2-AM at 340 and 380 nm.[46] Neurons were loaded with 4 μM fura-2-acetoxymethyl (AM) ester in DMEM/HG serum-free medium, supplemented with 1% (w/v) BSA at 37 °C for 45 min. Subsequently, the cells were washed twice with the medium and incubated further at 37 °C for 45 min. These time periods were found to be sufficient to load the cells with fura-2-AM and for hydrolysis of the acetoxymethyl ester (fura-2). A calcium calibration curve was prepared according to the method of Grynkiewicz.[46] Fluorescence images at 340 and 380 nm were obtained for each buffer and the fluorescence ratios of the images (340/380 nm) were calculated and plotted as a calibration curve. One ratio value 340/380 nm equals 228 nM $[Ca^{2+}]_i$.

For determination of $[Ca^{2+}]_i$, cells were cultivated on poly-L-lysine-coated borosilicate coverglass in 4-chamber Lab-Tek® Chamber Slide™ System (Nunc, Wiesbaden, Germany). An inverted-stage Olympus IX70 microscope with apochromatic reflected light fluorescence objective UApo40X/340 was used for the fluorescence measurements. The cells were alternately illuminated with light of wavelengths 340 and 380 nm by a computer-controlled switching of narrow-band interference filters in front of a 100 W xenon lamp. 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 as 256×256 pixels by 8-bit arrays with a computerized imaging system (Argus-50, Hamamatsu). The fluorescence ratio 340/380 nm was determined by dividing the image pairs.

In all sets of experiments, the neurons were first loaded with fura-2-AM and then stimulated with different compounds in 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+}. For the experiments, compounds **1**–**4** were used dissolved in DMSO (conc 10 mg/ml). In the first set of experiments, neurons were stimulated with 0.1% (v/v) DMSO after 5 min and with 200 μM serotonin (5-HT) L-glutamic acid (L-Glu), or quisqualic acid (QUIS) 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 10 μg/ml of compounds **1**–**4** (5 min) and after 10 min 200 μM of 5-HT or L-Glu or QUIS and 2.5 mM $CaCl_2$ were added to the neurons, as indicated. The $[Ca^{2+}]_i$ level was measured during the entire incubation period (for at least 25 min). For QUIS, the incubation period was up to 35 min.

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