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0014-4754/92/090882-04$1.50 + 0.20/0

Anti-muscarinic activity of a family of $C_{11}N_5$ compounds isolated from *Agelas* sponges

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Received 30 December 1991; accepted 27 April 1992

Abstract. In a search for potential target sites for $C_{11}N_5$ compounds obtained from marine sponges of the genus *Agelas* we evaluated their interaction with muscarinic acetylcholine receptors from rat brain membranes. In competition experiments with ^{3}H-QNB these compounds displayed the following rank order of potency: sceptrin > oroidin ≥ dibromosceptrin ≥ clathrodin. Sceptrin (50 μM) was shown to be a competitive inhibitor of ^{3}H-QNB binding as revealed by Scatchard analysis. The results demonstrate the ability of these compounds to interact with multiple target molecules in the micromolar range.
Key words. Marine sponges; muscarinic receptor; sceptrin; oroidin; dibromosceptrin; clathrodin.

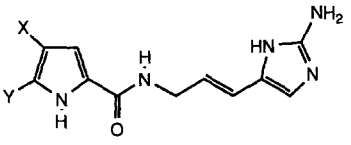
Marine organisms are good sources of compounds that act on specific sites of cell membranes. For example marine neurotoxins such as the dinoflagellate saxitoxin and the sea anemone toxin II are very selective pharmacological probes[1–5]. In our laboratory we are examining a variety of marine organisms from around Puerto Rico in an effort to identify new substances with specific biological activities.

Sponges of the genus *Agelas*, collected off-shore western Puerto Rico near Desecheo Island, were extracted in methanol and separated chromatographically. The chemical structures of compounds present in the purified fractions were elucidated using spectroscopic methods. A family of $C_{11}N_5$ compounds with different bromine substitutions in the pyrrole ring were identified: clathrodin (**1**), the only nonbrominated compound of the family, oroidin (**3**), the dibrominated analog of clathrodin, sceptrin (**4**), the 2 + 2 cycloaddition product dimer of hymenidin (**2**), and dibromosceptrin (**5**), the corresponding dimer of oroidin (fig. 1)[6–10]. Recently, Kobayashi et al. showed that some members of this family have serotonergic and adrenergic antagonist activity, while oxysceptrin is an actomyosin ATPase activator[11–13]. In this study we have specifically assessed the ability of these $C_{11}N_5$ compounds to interact with muscarinic acetylcholine receptors (mAChR) in rat brain membranes via radio receptor binding assays.

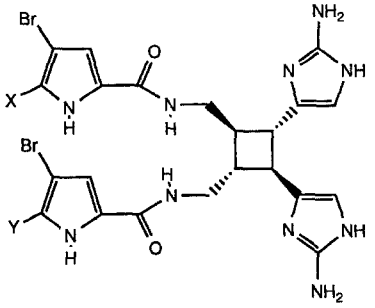
Materials and methods

Extraction and purification of alkaloids from Agelas sponges. The extraction and purification of clathrodin (**1**) was performed as described by Morales and Rodríguez[7]. The sponge was stored at 0 °C prior to freezing and lyophilization. The methanol (MeOH) extract of the sponge was suspended in H_2O and extracted succesively with chloroform ($CHCl_3$ (3 × 250 ml)) and normal butanol (n-BuOH (2 × 250 ml)). After concentration in vacuo the n-BuOH soluble portion (3.30 g) was chromatographed on a reversed phase (C_{18}, 20 g) column with water (H_2O) followed by a silica (Si) gel column (48 g) with $CHCl_3$-MeOH (4 : 1) saturated with ammonia (NH_3). Combination of like fractions on the basis of thin layer chromatography (TLC) analyses gave pure clathrodin (**1**) as a colorless semisolid (840 mg).

The extraction and purification of sceptrin (**4**) and oroidin (**3**) was performed as follows: *Agelas conifera* was



1 X = Y = H
2 X = Br, Y = H
3 X = Y = Br



4 X = Y = H
5 X = Y = Br

Figure 1. Structures of brominated and nonbrominated alkaloids identified from Puerto Rican sponges of the genus *Agelas*.

stored at 0 °C prior to freezing and lyophilization. The MeOH extract was suspended in H_2O and extracted successively with $CHCl_3$ (3 × 250 ml) and n-BuOH (2 × 250 ml). The n-BuOH soluble portion (2.50 g) was chromatographed on a Si gel column (100 g) with $CHCl_3$ / nBuOH / acetic acid (HoAc) / H_2O (3 : 12 : 2 : 2). Combination of like fractions gave oroidin (**3**) (100 mg) and sceptrin (**4**) (500 mg). In both instances the spectral analyses (^1H and ^{13}C-NMR, IR, UV and MS) agreed with those reported previously for the authentic materials[6, 8, 9].

Dibromosceptrin (**5**) was extracted as follows: *Agelas conifera* was blended with 1000 ml MeOH / $CHCl_3$ (1 : 1) and n-BuOH (1500 ml). A fraction was suction-filtered and chromatographed on a Si gel column with $CHCl_3$ / nBuOH / HoAc / H_2O (3 : 12 : 2 : 2). A fraction from this column, identified by TLC analyses, contained impure dibromosceptrin (214.3 mg) and was chromatographed successively by HPLC (MeOH (0.5% ethanolamine (Et_3N)) / H_2O, 30 : 70) and column chromatography on Si gel (6 g) with $CHCl_3$ / nBuOH / HoAc / H_2O (3 : 12 : 2 : 2) to give pure dibromosceptrin (43.6 mg) further identified by spectral analyses (^1H and ^{13}C-NMR, MS)[6]. All alkaloids extracted from *Agelas* sponges are analytically pure as determined by spectroscopic methods.

Rat brain membrane preparation and radioreceptor assays. A P_2 fraction from rat brain was obtained, all steps were done at 4 °C. The P_2 pellet enriched in rat brain synaptosomal membranes was resuspended and used directly in the binding assays. Muscarinic receptor binding assays were conducted in rat brain membranes using $^3H(-)$-QNB (sp. act. = 33.1 μCi/mol) as the labeling ligand[14]. Binding was measured by the rapid filtration method on GF/A glass fiber filters using a Millipore's filtration manifold. All equilibrium binding experiments were done at 32 °C in an assay buffer composed of 20 mM Tris HCl and 10 mM $MgCl_2$ (pH = 7.2), in a final volume of 1 ml and an incubation period of 90 min. Specific binding was determined using 1 μM atropine as the masking ligand. The $^3H(-)$-QNB saturation isotherms were obtained by incubating 0.01–2 nM $^3H(-)$-QNB in the absence and presence of 50 μM sceptrin (**4**). Competition experiments were performed under similar assay conditions with atropine ($10^{-4} - 10^{-8}$ M), sceptrin ($10^{-3} - 10^{-8}$ M), oroidin ($10^{-3} - 10^{-8}$ M), clathrodin ($10^{-3} - 10^{-8}$ M), and dibromosceptrin ($10^{-3} - 10^{-7}$ M). Competition curves and Scatchard plots were analyzed using the linear and nonlinear regression procedures of GraphPAD INPLOT.

Results and discussion

Determination of the relative potencies of sceptrin, oroidin, clathrodin and dibromosceptrin against mAChR. Atropine was used as a known mAChR antagonist, to compare the relative binding potency of the $C_{11}N_5$ family members. The IC_{50} obtained from the competition curves were atropine (0.0053 ± 0.0037)μM, sceptrin (38.77 ± 4.29)μM, oroidin (113.3 ± 38.82)μM, dibromosceptrin (267.27 ± 116.89)μM, and clathrodin (869.13 ± 603.52)μM (fig. 2). Thus, the rank order of potency is sceptrin > oroidin ≥ dibromosceptrin ≥ clathrodin. Comparing these values with those for atropine (a high affinity mAChR antagonist) it can be observed that these $C_{11}N_5$ compounds are at least 7500 times less potent than atropine (table 1), interacting with the mAChR in the low

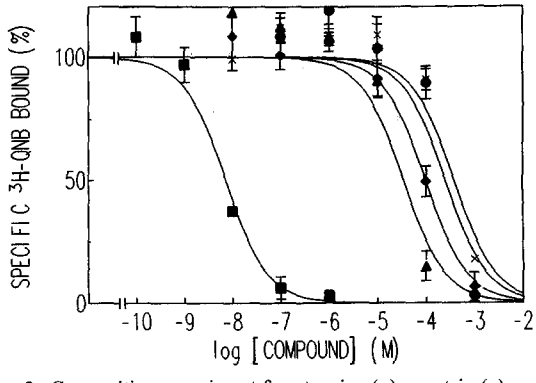


Figure 2. Competition experiment for atropine (■), sceptrin (▲), oroidin (◆), dibromosceptrin (X), and clathrodin (●) against $[^3H](-)$-QNB in rat brain membranes. The abscissa represents the logarithm of the molar concentration of each compound. The ordinate indicates the percent of $[^3H](-)$-QNB specifically bound. Each point represents the average from three different experiments and the bars their corresponding SEM.

Table 1. IC_{50} and k_i values for compounds tested for inhibition of $^3H(-)$-QNB binding to the mAChR of rat brain membranes

Compound	$IC_{50}(\mu M)$	$k_i(\mu M)$[a]
Atropine	0.0053 ± 0.0037	0.0015 ± 0.0010
Sceptrin	38.77 ± 4.29	10.68 ± 1.18
Oroidin	113.03 ± 10.69	31.13 ± 10.69
Dibromosceptrin	267.23 ± 116.95	73.61 ± 32.19
Clathrodin	869.13 ± 603.52	239.37 ± 166.22

[a] Determined according to Cheng and Prussoff[15].

Table 2. Summary of the reported biological activities of sceptrin, oroidin, dibromosceptrin and clathrodin

Compound	mAChR $IC_{50}(\mu M)$	Antimicrobial (MIC)[7]	Cytotoxicity[7]	5-HTR[10,11]
Sceptrin	38.77	16.50	—	active
Oroidin	113.03	⩾16.50	—	active
Dibromosceptrin	267.27	—	—	—
Clathrodin	869.13	—	5.2 μM	—

to high micromolar range. The rank order of potency suggests an interaction between the C-3 bromine of the pyrrole ring and the esteratic site of the muscarinic receptor and another interaction between the anionic site and the imidazole ring.

Identification of sceptrin's type of antagonism, competitive vs non-competitive. Sceptrin, the most potent member of the $C_{11}N_5$ series, was selected to determine the mechanism of inhibition of ^3H-QNB binding to rat brain mAChR by these compounds. Scatchard analysis of ^3H-QNB binding in the absence and presence of 50 μM sceptrin was performed (fig. 3). The competitive nature of the interaction with mAChR was evident from the fact that the slope ($-1/k_d$) of the control plot is significantly different from the slope obtained in the presence of 50 μM sceptrin, while no changes were obtained in the B_{max} values. The apparent k_i value for sceptrin (31.03 ± 12.90) μM, determined using competitive kinetics, was found to be similar to the IC_{50} value obtained in the competition assays.

Comparison of the reported biological activities of $C_{11}N_5$ compounds. All members of the $C_{11}N_5$ family of compounds tested in our laboratory displayed biological activity in the micromolar range (table 2). The reported activities of sceptrin and oroidin as antimicrobial agents[7] and as antagonists of serotonergic receptors[10,11], also fall within this concentration range. Other members of the family exhibit additional biological activities also in this concentration range. Oxysceptrin acts as an actomyosin ATPase activator at 30 μM[12]. Hymenidin is a serotonergic receptor blocking agent active at 15 μM[10] but with no activity against the α-adrenoreceptor. In contrast, hymenin acts as an α-adrenoreceptor blocking agent at 0.7 μM[13]. Collectively these results reveal that $C_{11}N_5$ compounds isolated from *Agelas* sponges interact with multiple target molecules in the micromolar range.

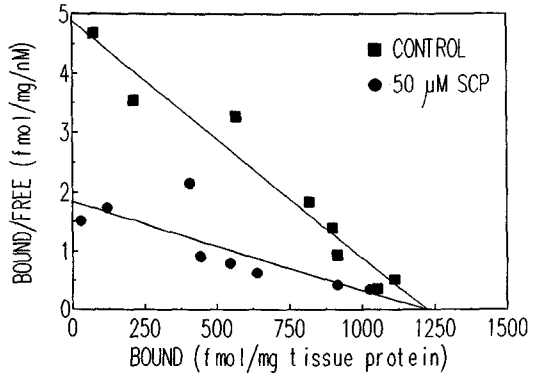


Figure 3. Scatchard plot of the specific binding of $[^3H](-)$-QNB to rat brain synaptosomes in the presence (●) and absence (■) of 50 μM sceptrin.

Acknowledgments. Supported by NIH grant GM08102, the NOAA, USDC Sea Grant College Program at U.P.R. and NIH grants NS27259 and RR03035 awarded to W.I.S.

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