HETEROCYCLES, Vol. 57, No. 7, 2002, pp. 1265 - 1272, Received, 7th March, 2002

ANTI HSV-1 ALKALOIDS FROM A FEEDING DETERRENT MARINE SPONGE OF THE GENUS *AAPTOS*

Angelica F. Coutinho[a], Brian Chanas[a,b], Thiago M. L. e Souza[c], Izabel C. P. P. Frugrulhetti[a,c], and Rosângela de A. Epifanio[a*]

[a]Programa de Pós-graduação em Química Orgânica, Instituto de Química, Universidade Federal Fluminense, 24020-150, Niterói, RJ, Brazil

[b]Núcleo de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, 21941-590, Rio de Janeiro, RJ, Brazil

[c]Departamento de Biologia Celular e Molecular, Instituto de Biologia, Universidade Federal Fluminense, 24020-150, Niterói, RJ, Brazil

Abstract – The crude extract from the marine sponge *Aaptos* sp. collected in Abrolhos, Bahia, Brazil, was unpalatable to a natural assemblage of reef fishes. Preliminary assays also indicated the presence of anti HSV-1 compounds in this crude mixture. Successive chromatographic purification afforded a new alkaloid, 8,9-dimethoxy-4-methyl-4*H*-benzo[*de*][1,6]naphthyridine (**2**,4-methylaaptamine) and the known demethyloxyaaptamine (**1**), which structures were determined mainly by 1D and 2D NMR. Both compounds showed potent antiviral activity against herpes simplex virus type 1 and low toxicity to Vero cells, suggesting that they may be selectively targets to inhibit virus replication.

It is estimate that the number of marine invertebrates and algae species living in the oceans is at least 200,000. Many of these invertebrates, including soft corals, ascidians and sponges, are sessile as adults and have evolved in habitats which are often more extreme and self limiting compared to their terrestrial counterparts.[1] Although it has been assumed that secondary metabolites are important for the success of these invertebrates in the marine environment, only in the last decade their ecological importance has been experimentally evaluated. These include, but are not limited to the defensive role against predation,

pathogens, larval settlement, and overgrowth by neighboring organisms.[2] Most of such important metabolites are of continuous interest as source of structurally unique and potentially drug leads.[1] An historical example is the pioneering research of Bergmann, over fifty years ago, with marine sponge nucleosides, spongothymidine and spongouridine, providing the scientific foundation for the known anticancer and antiviral drugs Ara-C and Ara-A.[3] Over the past quarter-century, more than 15,000 compounds have been reported from marine sources[4] and different strategies were developed to improve collection and sampling selection methods for drug leads discovery.[1]

Our research group uses to prior evaluate ecological factors such the presence of feeding deterrent compounds in crude extracts of sessile marine invertebrates, relating this to expression of bioactive secondary metabolites. In fact, we have found a higher percentage of neurotoxins and anticancer agents in feeding deterrent extracts obtained from Brazilian octocorals and ascidians.[5-9]

Among a series of crude extracts from sponges collected in the preserved area of the Marine Park of Abrolhos (Bahia State, Brazil), *Aaptos* sp. proved to contain metabolites that avoid predation in palatability field assays. The crude organic extract was avoided by co-occurring reef fishes when incorporated into carrageenan strips at its natural volumetric concentration (Figure 1, c = 0.053 g mL^{-1}, P = 0.0013). The sponge spicules, tested in the same conditions, did not deter predation (Figure 1, c = 0.237 g mL^{-1}, P = 0.0573).

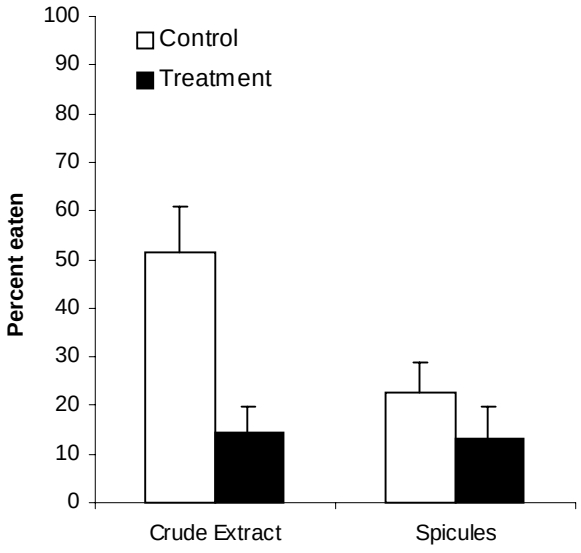

Figure 1. Consumption by co-occurring fishes of paired bait-strips with (treated) and without (control) *Aaptos* sp. crude extracts or spicules. Error bars represented SD. N = no. of paired treated and control strips used in statistical analysis (parentheses indicated no. of pairs retrieved of 20 deployed). P = probability calculated by Wilcoxon paired-sample test, one-tailed.

These results clearly indicated that metabolites in the crude mixture have feeding deterrent properties and may be responsible for the low predation rates of this sponge in the field.[10] The presence of feeding deterrent metabolites in the crude extract led us to include it in a screening of anti HSV-1 marine natural products, actually in course in our laboratories. In a preliminary screening, the Abrolhos *Aaptos* sp. extract inhibited 76 % the HSV-1 replication in Vero cells at a concentration of 2 μg mL^{-1}.

Marine sponges of the genus *Aaptos* (Hadromerida, Suberitidae) produce a unique class of alkaloids. Considered as taxonomic markers for Suberitidae family,[11] benzo[*de*][1,6]naphthyridines (aaptamines) or rearranged derivatives (5,8-diazabenzo[*cd*]azulenes) proved to have several pharmacological activities: α-adrenoreceptor blocking, antitumor, antimicrobial, and PKC or GFAT enzyme inhibitor.[12-16] Recent chemical study on *Aaptos* specimens collected in the Brazilian coast did not report the presence of such alkaloids and led to doubt about the use of these compounds as a family characteristic.[17] Nevertheless, ^{1}H NMR spectral analysis of the feeding deterrent extract from Abrolhos *Aaptos* sp. revealed aaptamines-like signals between δ_H 7.0-9.5 and δ_H 3.5-4.0. Purification of the crude extract and alkaloids enriched fractions by chromatography led to the isolation of two pure compounds (**1** and **2**).

The spectral data of compound (**1**) are in agreement with those reported for demethyloxyaaptamine, previously isolated from specimens of *Aaptos* from Okinawan[13] and Red Sea.[18,19]

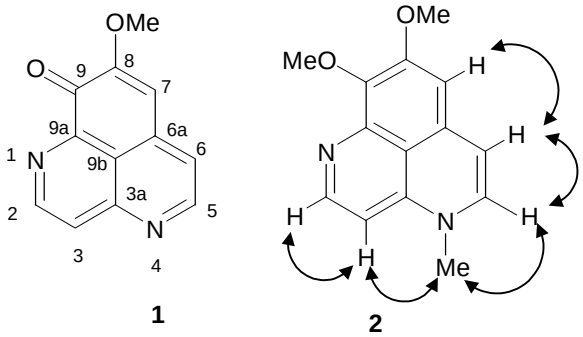

Figure 2. Alkaloids isolated from the Brazilian *Aaptos* species and NOE enhancements observed for (**2**).

The molecular formula of compound (**2**) was established as $C_{13}H_{14}N_2O_2$ by EIMS (m/z 242, M^+) and ^{13}C NMR spectral data. With slightly differences in chemical shifts and coupling constant values, the ^{1}H and ^{13}C NMR spectral data of (**2**) (Table 1) were similar to those of 8,9-dimethoxy-1-methyl-1*H*-benzo[*de*][1,6]-naphthyridine, recently synthesized.[20] The HMQC, HMBC and ^{1}H-^{1}H-COSY experiments established the direct correlation ($^1J_{CH}$) and two or three bond correlation ($^{2,3}J_{CH}$) between carbons and hydrogens in the 1*H*-benzo[*de*][1,6]naphthyridine ring system, as well as between the two methoxy groups and C-8 and C-9 (Table 1). By comparison with the synthetic alkaloid (8,9-dimethoxy-

1-methyl), the closely related natural compounds 8,9-dimethoxy (isoaaptamine) and 9-methoxy-1-methyl (aaptamine), it was observed that the methyl group at N-1 is deshielded (*ca.* 6 ppm) in relation to the isolated compound (**2**).[17-21]

The complete structural elucidation of (**2**) as the new alkaloid 8,9-dimethoxy-4-methyl-1*H*-benzo[*de*][1,6]-naphthyridine (4-methylaaptamine) was achieved using nuclear Overhauser experiments (Figure 2). NOE enhancements observed for H-2 and H-3 when the hydrogens at δ_H 3.64 (3H, s) were irradiated, placed the methyl group at N-4. Further correlations between H-7/H-6, H-6/H-5, and H-2/H-3 confirmed the proposed structure (Figure 2).

Table 1. NMR spectral data for 4-methylaaptamine (**2**)[a]

			HMBC ($H^{\#}$)[d]	
$C^{\#}/H^{\#}$	**δ ^{13}C (CH_X)[b,c]**	**δ ^{1}H (m, *J* in Hz)[b]**	**$^{2}J_{CH}$**	**$^{3}J_{CH}$**
2	142.7 (CH)	8.02 (d, 7.2)	3	
3	96.7 (CH)	6.46 (d, 7.2)	2	
3a	149.9 (C)			2, 5
5	135.5 (CH)	7.49 (d, 7.2)	6	
6	113.0 (CH)	6.93 (d, 7.5)	5	
6a	131.3 (C)			5
7	101.2 (CH)	7.17 (s)		6
8	156.3 (C)		7, 8-OC<u>H</u>$_3$	
9	131.7 (C)		9-OC<u>H</u>$_3$	
9a	133.2 (C)			2
9b	116.7 (C)			3, 6, 7
4-N<u>CH</u>$_3$	40.6 (CH_3)	3.64 (s)		
8-O<u>CH</u>$_3$	56.5 (CH_3)	4.00 (s)		
9-O<u>CH</u>$_3$	60.5 (CH_3)	3.84 (s)		

[a] In DMSO-d_6, [b] ^{1}H and ^{13}C assignments made on the basis of HMQC and ^{1}H-^{1}H COSY experiments; [d]Number of hydrogens bonded to carbons was deduced by APT experiment; [c] HMBC J_{CH} = 9 Hz.

Anti HSV-1 assays with compounds (**1**) and (**2**) at a concentration of 2 μg mL^{-1}resulted, respectively, in 78 % and 80 % of inhibition of virus yield in Vero cells. Both compounds were not cytotoxic to Vero cells in concentrations up to 20 μg mL^{-1}.

Although further studies on the metabolism of **1** and **2** in HSV-1 infected cells are necessary to elucidate their mechanism of action, the potent anti HSV-1 activity and low toxicity observed suggest that they may be selectively inhibitory targets to virus replication. This is the first report of antiviral activity of aaptamine alkaloid series.

EXPERIMENTAL

General Experimental Procedures. NMR spectra were recorded in DMSO-d_6 solution on Varian Unity Plus 300 MHz spectrometer. IR spectra were recorded on a Perkin-Elmer model 1600 (FTIR) spectrophotometer. EIMS spectra were obtained using a HP 5989A mass spectrometer. UV spectra were obtained on a Shimadzu model 160A spectrophotometer. Isolation procedures were monitored by employing TLC on pre-coated silica gel plates (Merck, Kieselgel 60 F-254).

Collection. *Aaptos* sp. (Hadromerida, Suberitidae) was collected 10 m depth by scuba diving in the National Marine Park of Abrolhos (17°30'S; 39°00'W, Bahia State, Brazil) during a cooperative program NSF-CNPq in April 1995. The sponge was stored at rt with ethanol until laboratory analyses and taxonomically identified by one of us (B. Chanas).

Crude extract and spicules. The ethanol was removed and the sponge tissue (225.8 g of dried material after extraction, corresponding to 500 mL of fresh sponge) was further extract with a mixture of methanol-methylene chloride (MeOH/CH_2Cl_2, 1:1, 3x). After evaporation of the extracts under vacuum 26.5 g of a brownish gum was obtained. A volume corresponding to 60 mL of sponge was submitted to HNO_3 treatment until completed digested, filtered and washed with distilled water to neutral pH. Both washes and residue were analyzed in the microscope, and the spicules were found only in the residue (28.4 g of inorganic matter after dry).

Isolation of pure compounds. The crude extract (12 g) was fractionated by silica gel (300 mesh) vacuum flash chromatography, employing a gradient ranging from 0 to 100 % of chloroform in methanol, to yield twelve fractions. All of them were analyzed by silica gel TLC and by ^{1}H NMR spectrometry. Part of fractions 5 and 9 were further purified by silica gel flash chromatography (0 to 100 % dichloromethane in methanol), to yield pure demethyloxyaaptamine (**1**, 10.0 mg, 0.0163 % dry weight sponge) and 4-methylaaptamine (**2**, 15.5 mg, 0.0564 % dry weight).

8-Methoxybenzo[*de*][1,6]naphthyridin-9-one (Demethyloxyaaptamine, 1).[13,18,19] Yellow amorphous solid, mp 199-201 ^{o}C (decomp); UV (H_2O) λ_{max} 234.5 (ε 15418), 374.0 nm (ε 7099); IR (film) ν_{max} 1669, 1622, 1583, 1274, 1098 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.23 (1H, d, *J* 5.4 Hz, H-2); 8.34 (1H, d, *J* 5.4 Hz, H-3); 9.26 (1H, d, *J* 4.5 Hz, H-5); 7.87 (1H, d, *J* 4.5 Hz, H-6); 7,28 (1H, s, H-7); 4.04 (1H, s, 8-OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6) 177.2 (C-9), 157.5 (CH-5), 155.9 (C-8), 149.0 (CH-2), 148.9 (C-6a), 147.5 (C-9a), 136.6 (C-3a), 126.6 (C-3), 122.4 (CH-6), 117.8 (C-9b), 109.1 (CH-7), 56.2 (CH_3O-8); EIMS (70 eV) *m/z* 212 $[M]^+$ (100), 196 (6), 183 (42), 154 (90), 171 (13), 149 (14), 141 (12), 127 (21), 114 (20), 87 (10).

8,9-Dimethoxy-4-methyl-4*H*-benzo[*de*][1,6]naphthyridine (4-Methylaaptamine, 2). Pale yellow oil; UV (MeOH) λ_{max} 216.0 (ε 1318), 239.5 (ε 13356), 258.5 (ε 11025), 269.0sh (ε 9522), 277.5sh (ε 8654), 314.0 (ε 2420), 360.5 (ε 2939), 394.0sh nm (ε 2780); ^{1}H and ^{13}C NMR (see Table 1); EIMS (70 eV) *m/z* 242 $[M]^+$ (80), 227 (91), 213 (55), 199 (25), 184 (34), 167 (30), 155 (16), 135 (14), 129 (22), 121 (21), 115 (11), 105 (31), 99 (20), 85 (23), 81 (25), 77 (34), 73 (54), 69 (75), 57 (100).

Feeding Deterrent Bioassays. Food strips were prepared using an established methodology, which involved homogenizing 2.5 g carrageenan (Sigma C-1013 type 1), 20 mL commercial tunafish puree (packed in oil) in 60 mL of water. In separate experiments, crude sponge extract and spicules were volumetrically reconstituted in a matrix of the carrageenan-based food at the same concentration as they occurred in the sponge fresh tissues (final volume of 60 mL). For each experiment, 20 treated and 20 control strips (1.0 x 0.6 x 5.0 cm each) were arranged in pairs and attached to 20 ropes. The ropes were anchored slightly above the bottom near to the same site *Aaptos* sp. was collected. Within 3 hr the ropes were retrieved and the amount of each strip eaten was measured. During the experiments, several common tropical fishes well known to occur in the studied area were observed feeding on the test strips (*e.g.* belonging to the Families Haemulidae and Labridae).[5,10,22] Field assays and underwater observations were performed with an aid of a portable surface air supply diving apparatus. The Wilcoxon paired-sample test (one-tailed) was used to analyze the results.[5,10]

Anti HSV-1 Assays. The antiviral activities were investigated according to the computation of the 50 percent endpoint method described by Reed and Muench.[23] Vero cells grown in 96-well microtiter plates were inoculated with HSV type 1. Crude extract and pure compounds were added in duplicate after virus adsortion for 2 h at 37 ^{o}C, at a concentration of 2 μg mL^{-1}. After 3 days of incubation at 37 ^{o}C in 5% CO_2 atmosphere, virus yield was determined .

ACKNOWLEDGEMENTS

The authors are grateful to the Brazilian Institute of Environmental Conservation (IBAMA) at the National Marine Park of Abrolhos (Bahia) for the permits to study this area. A. F. C. and T. L. M. S. thank CNPq for providing undergraduate fellowships. B. C. thanks NPPN (UFRJ) and CNPq for a one-year D.Sc. fellowship in Brazil. R. A. E. and I. C. C. P. F. have research fellowships from CNPq. The authors also acknowledge Prof. William Fenical (SIO-UCSD) for the sponge collection opportunity during the 1995 mission of a NSF-CNPq cooperative program with one of us (R. A. E.). This research was financially supported by the National Brazilian Research Council (CNPq, Brazil), International Foundation for Science (IFS, Sweden) and Rio de Janeiro Research Foundation (FAPERJ, Brazil).

REFERENCES AND NOTES

1. A. E. Wright, 'Biodiversity. New leads for the pharmaceutical and agrochemical industries: Marine organisms as a source of novel lead structures for drug development,' ed. by S. K. Wrigley, M. A. Hayes, R. Thomas, E. J. Chrystal and N. Nicholson, Royal Society of Chemistry, Cambridge, UK, 2000, pp. 113-139.
2. M. E. Hay, J. J. Stachowics, E. Cruz-Rivera, S. Bullard, M. S. Deal, and N. Lindquist, 'Methods in chemical ecology; Bioassay methods: Bioassays with marine and freshwater macroorganisms', ed. by K. F. Haynes and J. G. Millar, Kluwer Academic Publishers, Massachusetts, USA, 1998, pp. 39-141.
3. G. R. Pettit, J. W. Lippert III, S. R. Taylor, R. Tan, and M. D. Williams, *J. Nat. Prod.*, 2001, **64**, 883.
4. D. J. Faulkner, *Nat. Prod. Rep.*, 2001, **18**, 1 and previous reviews.
5. R. de A. Epifanio, D. L. Martins, R. Villaça, and R. Gabriel, *J. Chem. Ecol.*, 1999, **25**, 2255.
6. R. de A. Epifanio, L. F. Maia, and W. Fenical, *J. Braz. Chem. Soc.*, 2000, **11**,584.
7. L. F. Maia, R. de A. Epifanio, T. Eve, and W. Fenical, *J. Nat. Prod.*, 1999, **62**, 1322.
8. L. F. Maia, R de A. Epifanio, and W. Fenical, *J. Org. Chem.*, 2000, **65**, 782.
9. L. F. Maia, R. de A. Epifanio, and W. Fenical, *J. Nat. Prod.*, 2000, **63**,1427.
10. R. de A. Epifanio, R. Gabriel, D. L. Martins, and G. Muricy, *J. Chem. Ecol.*, 1999, **25**, 2247 and references cited therein.
11. R. Bergquist, R. C. Cambie, and M. R. Kernan, *Biochem. System. Ecol.*,1991, **19**, 289
12. Y. Ohizumi, A. Kajiwara, H. Nakamura, and J. Kobayashi, *J. Pharm. Pharmacol.*, 1984, **36**, 785.
13. H. Nakamura, J. Kobayashi, and Y. Ohizumi, *J. Chem. Soc., Perkin Trans. I*, 1987, 173.

14. A. D. Patil, J. W. Wesley, M. Mattern, A. J. Freyer, and G. A. Hoffman, *PCT Int. Appl.* WO95/0584, March, 1995.
15. SmithKline Beecham Corp. USA WO 9,505,824 A1 950302 (*Chem Abstr.*, 1995, **122**, 282233).
16. S.C. Bobzin, S. Yang, and T. P. Kasten, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, **748**, 259.
17. A. C.Granato, R. G. S. Berlinck, A. Magalhães, A. B. Schefer, A. G. Ferreira, B. Sanctis, J. C. Freitas, E. Hadju, and A. E. Migotto, *Quim. Nova*, 2000, **23**, 594.
18. A. Rudi, and Y. Kashman, *Tetrahedron Lett.*, 1993, **34**, 4683.
19. Y. Kashman, A. Rudi, S. Hirch, S. Isaacs, D. Green, D. Blasberger, and S. Carmely, *New. J. Chem.*, 1990, **14**, 729..
20. A. J. Walz, and R. J. Sundberg, *J. Org. Chem.*, 2000, **65**, 8001.
21. H. Nakamura, J. Kobayashi, and Y. Ohizumi, *Tetrahedron Lett.*, 1982, **23**, 5555.
22. G. W. Nunan, Composition, species distribution and zoogeographical affinities of the Brazilian reef-fishes fauna, PhD thesis, 1992, University of Newcastle upon Tyne, Newcastle upon Tyne, England.
23. L. J. Reed and M. A. Muench, *Am. J. Hyg.*, 1938, **27**, 493.