Maria Fernanda de Oliveira[1]
Jaine Honorata Hortolan Luis de Oliveira[1]
Fabio C. S. Galetti[2]
Ana Olívia de Souza[3]
Célio Lopes Silva[2]
Eduardo Hajdu[4]
Solange Peixinho[5]
Roberto G. S. Berlinck[1]

Antimycobacterial Brominated Metabolites from Two Species of Marine Sponges

Abstract

A screening of 500 crude extracts of marine invertebrates against the growth of *Mycobacterium tuberculosis* H37Rv yielded MeOH extracts of the sponges *Aplysina cauliformis* and *Pachychalina* sp. with significant activity. Further bioassay-guided fractionation of both crude extracts led to the isolation of four bromine-containing metabolites. The known (+)-fistularin-3 (**1**) and 11-deoxyfistularin-3 (**2**), and the new compound 2-(3-amino-2,4-dibromo-6-hydroxyphenyl)acetic acid (**3**) were isolated from the sponge *A. cauliformis*, while the new bromotyrosine-derived 3-(3,5-dibromo-4-methoxyphenyl)-2-methoxy-*N*-methylpropan-1-ammonium (**4**) was isolated from *Pachychalina* sp. Compound **4** exhibited weak antimycobacterial activity while compounds **1 – 3** displayed activity against *Mycobacterium tuberculosis* H37Rv, with MICs of 7.1, 7.3 and 49 μM, respectively. Compounds **1** and **2** also exhibited low cytotoxicity against J744 macrophages, indicating that both **1** and **2** are interesting leads for the development of new anti-tuberculosis agents.

Key words
Antimycobacterial · marine sponge · *Aplysina cauliformis* · *Pachychalina* sp. · bromotyrosine-derived alkaloids

Introduction

Tuberculosis caused by *Mycobacterium tuberculosis* is one of the most important causes of mortality worldwide. Currently, there are about 2 billion people infected with *M. tuberculosis* and it is estimated that there are 8 million new infections/year. The number of infections is increasing, due to co-infection with HIV and the emergence of multidrug-resistant *Mycobacterium* strains. As a consequence, in 1993 the World Health Organization declared tuberculosis a global emergency disease [1], [2]. Although several drugs are available for the treatment of tuberculosis, it is a long-term therapy (6 months), which often presents side effects. Therefore, there is an urgent need for new anti-tuberculosis drugs that must be effective, less toxic and promote a short period of treatment [3], [4].

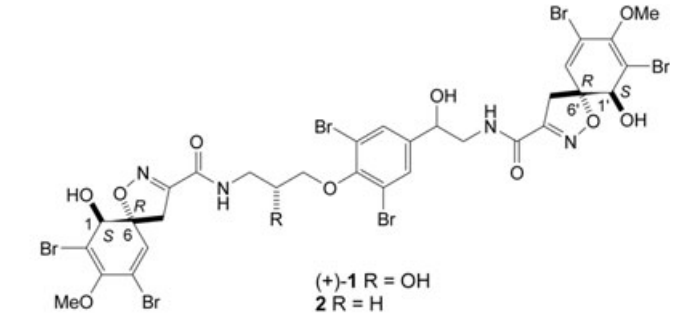
An array of natural-derived compounds display antimycobacterial activity [5]. Among these, marine natural products are emerging as a new group of secondary metabolites active against *M. tuberculosis*. These include peptides, alkaloids, terpenes and bromotyrosine-derived compounds [5], [6], [7], [8], [9]. During our current bioactivity-oriented search for new chemical entities from the marine environment, we have screened 500 crude extracts of marine invertebrates as inhibitors of *M. tuberculosis* H37Rv. Among the active extracts, those from the sponges *Aplysina cauliformis* and *Pachychalina* sp. displayed antimyco-

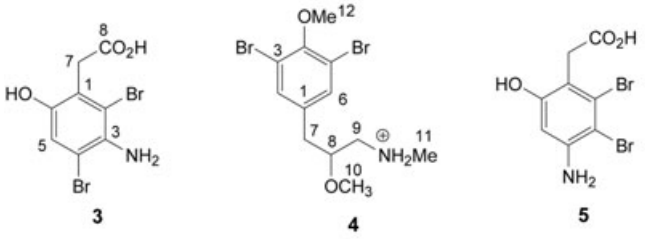
Affiliation
[1] Instituto de Química de São Carlos, Universidade de São Paulo, São Carlos, SP, Brazil
[2] Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil
[3] Laboratório de Bioquímica e Biofísica, Instituto Butantan, São Paulo, SP, Brazil
[4] Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[5] Departamento de Biologia, Universidade Federal da Bahia, Salvador, BA, Brazil

Correspondence
Roberto G. S. Berlinck · Instituto de Química de São Carlos · Universidade de São Paulo · CP 780 · CEP 13560–970 - São Carlos · SP · Brazil · Phone: +55-16-3373-9954 · Fax: +55-16-3373-9952 · E-mail: rgsberlinck@iqsc.usp.br

Received August 11, 2005 · **Accepted** October 6, 2005

Bibliography
Planta Med 2006; 72: 437–441 © Georg Thieme Verlag KG Stuttgart · New York
DOI 10.1055/s-2005-916239 · Published online January 30, 2006
ISSN 0032-0943





bacterial activity with MIC values < 200 μg/mL. We have subsequently investigated the chemistry of these animals, and isolated various alkylpiperidine alkaloids from the sponge *Pachychalina* sp., one of which displayed antimycobacterial activity [10]. Herein, we report the isolation of one new and two known bromine-containing compounds from *Aplysina cauliformis* and a new bromotyrosine-derivative from *Pachychalina* sp. active against *M. tuberculosis*. The isolated compounds were identified by analysis of spectroscopic data. Their antimycobacterial activity against *M. tuberculosis* H37Rv as well as their cytotoxicities on J774 macrophages were determined.

Materials and Methods

General experimental procedures

Optical rotations were measured on a Perkin Elmer 241 MC polarimeter at 29 °C. IR spectra (film on silica plate) were recorded on a FT-IR Bomem MB102 infrared spectrometer. The NMR spectra were recorded either on a Bruker ARX 9.4 T instrument, operating at 400.35 MHz for ^{1}H and 100.10 MHz for ^{13}C, respectively, or on a Bruker DRX500 11.7 T, operating at 500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C, respectively. All NMR spectra were obtained at 25 °C using TMS as an internal reference. Low resolution mass spectra were recorded on a VG-7070 mass spectrometer operating at a nominal accelerating voltage of 70 eV. High resolution mass spectra were recorded at a nominal resolution of 5000 or 10 000 as appropriate. LOBAR Lichroprep (Merck) separations were performed with size B (310 × 25 mm) columns. Solvents used for extraction and flash chromatography were glass distilled prior to use. HPLC-grade solvents were utilized without further purification in LOBAR and HPLC separations. TLC analyses were performed with precoated TLC sheets of silica gel on polyester, eluting with different mixtures of MeOH in CH_2Cl_2. Plates were observed under UV lamp (λ_{max} 254 and 365 nm). HPLC separations were performed either with a Waters quaternary pump 600, double beam UV detector 2487, and data module 746, or with a Waters autosampler 717, Waters 600 pump, Waters 2996 photodiode array detector monitored by Waters Millenium 32.

Animal material

The sponge *Pachychalina* sp. was collected in Ilha do Pai (Father's Island), Niterói, Rio de Janeiro state (22° 59.205′ S – 43° 05.252′ W), on May 9th, 2000 at a depth of 10 – 15 m, and immediately immersed in EtOH. The whole material was shipped to the Instituto de Química de São Carlos, Universidade de São Paulo. The sponge *A. cauliformis* was collected at the Baía de Todos os Santos (Salvador, Bahia, Brazil, at the Quebra Mar Norte station, 12° 57.7′ S, 38° 31.1′ W), on August 1st, 1999, and immediately frozen. Voucher specimens were deposited at the Museu Nacional, Universidade Federal do Rio de Janeiro (*Pachychalina* sp.: MNRJ 3098 e 3099; *Aplysina cauliformis*: MNRJ 8378).

Isolation of compounds 1, 2 and 3 from *Aplysina cauliformis*

The frozen sponge was freeze-dried to give a 164 g sample (dry weight), which was blended with CH_3CN (3 L) and re-extracted in the same solvent (2 × 2 L). The remaining solid residue was extracted with MeOH (3 L). The MeOH and MeCN extracts were separately evaporated, to give brownish gums (MeOH extract = 9.00 g; MeCN extract = 0.930 g). The MeOH extract was dissolved in 9 : 1 MeOH-H_2O and partitioned with AcOEt. After evaporation of the AcOEt, 6.14 g of a brown gum was obtained. Portions of ca. 0.9 g of the AcOEt soluble extract were subjected to chromatography on a Sephadex LH-20 column (170 × 2 cm) using MeOH. This separation yielded seven fractions (Ac-AcOEt-1 to -7). Fraction Ac-AcOEt-4 (994 mg) was subjected to silica gel flash chromatography, with a stepwise gradient of acetone in CH_2Cl_2. Six fractions were obtained (Ac-AcOEt-4A to Ac-AcOEt-4F), of which fraction Ac-AcOEt-4C (450 mg) displayed antimycobacterial activity and was further purified using silica gel flash chromatography with a stepwise gradient of AcOEt in CH_2Cl_2. Five fractions were obtained, named Ac-AcOEt-4C1 to Ac-AcOEt-4C5. Purification of fraction Ac-AcOEt-4C3 (7.3 mg) by HPLC (C_{18}, Phenomenex Synergy Fusion-RP, 4 μ, 80 Å, 250 × 4.6 mm; eluent: 1 : 1 MeCN-H_2O; λ_{max}: 254 nm; flow rate: 1 mL/min; t_R 15.3 min) yielded 1.6 mg of (+)-fistularin-3 (**1**). Fraction Ac-AcOEt-4C4 (377 mg) was separated by silica gel flash chromatography with a stepwise gradient of 95 : 5 AcOEt-MeOH in 9 : 1 CH_2Cl_2-hexanes, to give four fractions (Ac-AcOEt-4C4A to Ac-AcOEt-4C4D). The Ac-AcOEt-4C4C fraction (320 mg) was subjected to chromatography on a silica gel Waters Sep Pak (10 g) column, with a gradient of 95 : 5 *i*-PrOH-AcOEt in CH_2Cl_2, affording four fractions (Ac-AcOEt-4C4C1 to Ac-AcOEt-4C4C4). Fraction Ac-AcOEt-4C4C1 (32 mg) was purified by HPLC (phenyl-bonded silica gel, Waters μBondapak 10 μm, 125 Å, 7.8 × 300 mm; eluent: 3 : 7 MeOH-H_2O; λ_{max}: 254 nm; flow rate: 1 mL/min; t_R 25.6 min) to give 1.6 mg of 2-(3-amino-2,4-dibromo-6-hydroxyphenyl)acetic acid (**3**). Fraction Ac-AcOEt-4C4C2 (210 mg) was purified by HPLC (C_{18}, Waters μBondapak 10 μm, 125 Å, 7.8 × 300 mm; eluent: 7 : 3 MeOH-H_2O; λ_{max}: 254 nm; flow rate: 2 mL/min; t_R 40.0 min) to give 36.7 mg of (+)-fistularin-3 (**1**). Fraction Ac-AcOEt-5 (829 mg) was subjected to silica gel flash chromatography with a stepwise gradient of acetone in CH_2Cl_2, to yield six fractions (Ac-AcOEt-5A to Ac-AcOEt-5F). Fraction Ac-AcOEt-5D (34 mg) was purified by HPLC (C_{18}, Inertsil ODS-2, 125 Å, 5 μm, 250 × 9.4 mm; eluent: 75 : 25 MeOH-H_2O; λ_{max}: 254 nm; flow rate: 1.5 mL/min; t_R 22.5 min) to give 6.4 mg of 11-deoxy-(+)-fistularin-3 (**2**). Fraction Ac-AcOEt-5E (695 mg) was subjected to chromatography on a silica gel Waters Sep Pak (10 g) column with a gradient of 95 : 5 AcOEt-MeOH in CH_2Cl_2. Among the five

fractions obtained (Ac-AcOEt-5E1 to Ac-AcOEt-5E5), fraction Ac-AcOEt-5E3 (452 mg) was identified as pure (+)-fistularin-3 (**1**). Fraction Ac-AcOEt-6 was subjected to silica gel flash chromatography with a gradient of acetone in CH_2Cl_2. Of the six fractions obtained (Ac-AcOEt-6A to -6F), fraction Ac-AcOEt-6D (68 mg) was active. This later fraction was purified by HPLC (C_{18}, Inertsil ODS-2, 125 Å, 5 μm, 250×9.4 mm; eluent: 72:28 H_2O-MeOH; λ_{max}: 254 nm; flow rate: 1.5 mL/min; t_R 23.0 min) to give an additional 27 mg of (+)-fistularin-3. The MeCN extract (0.930 g) was subjected to flash chromatography on silica gel (gradient of MeOH in CH_2Cl_2), to give seven fractions. The seventh fraction was purified by HPLC (C_{18}, Inertsil ODS-2, 125 Å, 5 μm, 250×9.4 mm; eluent: 4:6 H_2O-MeOH; λ_{max}: 254 nm; flow rate: 1.5 mL/min; t_R 29.0 min) to yield 33 mg of (+)-fistularin-3 (**1**).

(+)-Fistularin-3 (**1**): $[\alpha]_D^{29}$: +147° (*c* 0.275, MeOH); UV (MeOH): λ_{max} (log ε) = 224 (4.50), 282 nm (4.11); CD (MeOH): λ = 252 ($\Delta\varepsilon$ +19.7), 286 nm (+19.7); ^{1}H- and ^{13}C-NMR data, identical with the published values [11]; ESI-MS: *m/z* = 1130.7 [M + Na^+] (calcd. for $C_{31}H_{30}Br_6N_4O_{11}Na$: 1130.7).

11-Deoxyfistularin-3 (**2**): $[\alpha]_D^{29}$: +190° (*c* 0.04, MeOH); UV (MeOH): λ_{max} (log ε) = 225 (4.50), 282 nm (4.10); CD (MeOH): λ = 255 ($\Delta\varepsilon$ +19.0), 288 nm (+18.9); ^{1}H- and ^{13}C-NMR data, identical with the published values [12]; positive HR-FAB-MS: *m/z* = 1098.70515 (calcd. for $C_{31}H_{31}{}^{79}Br_3{}^{81}Br_3N_4O_{10}$: 1098.70790).

2-(3-Amino-2,4-dibromo-6-hydroxyphenyl)acetic acid (**3**): UV (MeOH): λ_{max} (log ε) = 228 (4.10), 304 nm (3.98); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ = 9.68 (1H, s, CO_2H), 8.98 (1H, s, OH), 7.25 and 6.86 (2H, s, NH_2), 6.96 (1H, s, H-5), 3.54 (2H, s, C-7); ^{13}C-NMR (DMSO-d_6, 100 MHz): δ = 171.04 (C-8), 150.40 (C-6), 143.55 (C-3), 124.28 (C-1), 117.93 (C-2), 117.63 (C-5), 110.37 (C-4), 37.02 (C-7); positive HR-EI-MS: *m/z* = 324.87750 (calcd. for $C_8H_7{}^{79}Br^{81}BrNO_3$: 324.87722); positive EI-MS: *m/z* = 327 (3.6%)/325 (7.2%)/323 (3.6%), 310 (35.3%)/308 (71.3%)/306 (36.6%), 282 (49.7%)/280 (100.0%)/278 (51.3%), 254 (16.3%)/252 (32.7%)/250 (17%).

Isolation of compound 4 from *Pachycalina* sp.

The sponge *Pachychalina* sp. (2.0 kg, wet weight) was separated from EtOH, blended in MeOH and left overnight. After filtration of the MeOH extract, the solid material was re-extracted with MeOH. Both the EtOH and MeOH extracts were pooled and evaporated until 500 mL of an aqueous suspension was obtained. The H_2O phase was partitioned with EtOAc. The EtOAc extract was evaporated, dissolved in 95% MeOH and partitioned with hexane. After evaporation, the hexane fraction yielded 5.9 g as a light brown gum. The hexane extract (Pa-hex) was fractionated by flash chromatography on silica gel with a gradient of 1:1 MeOH/EtOAc in CH_2Cl_2. Three alkaloid fractions were obtained (Pa-hex-1 to Pa-hex-3). The Pa-hex-3 fraction (364 mg) was fractionated on a silica gel LOBAR column with a gradient of 95:5 acetonitrile/MeOH in CH_2Cl_2 and finally with 1:1 MeOH/CH_2Cl_2 to yield eight fractions (Pa-hex-3A to Pa-hex-3H). The third one (Pa-hex-3C, 24 mg) was purified by chromatography on a silica gel Sep Pak column (5 g) with a gradient of MeOH in CH_2Cl_2, to give 3.1 mg of compound **4** (0.000155% wet weight).

3-(3,5-Dibromo-4-methoxyphenyl)-2-methoxy-N-*methylpropan-1-ammonium* (**4**): glassy solid; $[\alpha]_D^{29}$: –6.4° (*c* 0.0015, MeOH); UV (MeOH): λ_{max} (log ε) = 283 (4.09) nm; IR (film on a silicon plate): ν_{max} = 3406 (OH), 2925 (CH), 2855 (CH), 1659, 1472, 1259, 1197, 1075, 1045, 740, 619 cm^{-1}; ^{1}H-NMR (MeOH-d_4, 400 MHz): δ = 7.61 (2H, s, H-2 and H-6), 3.09 (2H, m, CH_2–7), 3.55 (1H, m, CH-8), 3.64 (2H, m, CH_2–9), 3.84 (3H, s, OCH_3–12), 3.20 (3H, s, OCH_3–10), 2.69 (3H, s, NHMe); ^{13}C-NMR (MeOH-d_4, 100 MHz): δ = 136.1 (C-1), 134.7 (C-2 and C-6), 119.3 (C-3 and C-5), 155.0 (C-4), 28.8 (C-7), 73.5 (C-8), 62.3 (C-9), 61.1 (C-12), 53.7 (C-10), 39.4 (C-11); positive HR-FAB-MS: *m/z* = 351.97351 [M – CH_4 + H]$^+$ (calcd. for $C_{11}H_{14}{}^{79}Br^{81}BrNO_2$: 351.95478); positive EI-MS: *m/z* = 294 (51%)/292 (100%)/290 (52%), 279 (38%)/277 (74%)/275 (38%), 251 (8%)/249 (18%)/247 (10%).

Antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv

The antimycobacterial activity of crude extracts and isolated compounds **1**, **2**, **3** and **4** was assayed against *M. tuberculosis* H37Rv ATCC 27294 using the microplate Alamar Blue assay [13]. Crude extracts or isolated compounds were dissolved in DMSO and serially diluted in Middlebrook 7H9 broth medium before the inoculation. Crude extracts were tested in the concentration range from 0.5 to 1000 μg/mL while pure compounds were tested up to the maximum concentration of 125 μM. Rifampicin was used as a control and bioassays were performed in triplicate. The visual minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that prevented mycobacterial growth and expressed by average MIC values.

Cytotoxicity in J774 macrophages

The cytotoxicity effect was assayed on J774 macrophages by the reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [14], [15]. Stock solutions of compounds **1** and **2** were prepared in DMSO and diluted in RPMI 1640 medium without phenol red. The final concentration of the solvent in the assay was less than 0.3%. The controls received no drugs and each drug concentration was tested in six replicates, and repeated three times in separate experiments. Cells were exposed to the drugs at concentrations up to 1000 μM. Results are expressed by cellular viability of cells treated with drugs and controls and IC_{50} was defined as the drug concentration required to reduce cellular viability in 50%.

Results and Discussion

(+)-Fistularin-3 (**1**) was isolated in high amounts (ca. 550 mg, 0.33% dry weight) from the sponge *A. cauliformis* as the main antimycobacterial active compound, with an MIC value of 7.1 μM against *Mycobacterium tuberculosis* H37Rv. The 11-deoxyfistularin-3 (**2**) derivative displayed essentially the same activity, with an MIC of 7.3 μM, a result that indicated that the C-11 hydroxy group is not essential for the antituberculosis activity of (+)-fistularin-3. Moreover, since the absolute configuration of (+)-fistularin-3 (**1**) isolated in this investigation was recently established as 1(*S*), 1′(*S*), 6(*R*), 6′(*R*), 11(*R*) by circular dichroism and derivatization with Marfey's reagent [16], and since 11-deoxyfistularin-3 (**2**) herein isolated presented essentially the same CD spectrum as (+)-fistularin-3 (**1**), the same absolute stereochemistry at C-1,

C-1′, C-6 and C-6′ was assigned to compound **2**. Moreover, both compounds **1** and **2** displayed low toxicity in J774 macrophages (IC_{50} of 200 μM for **1** and of 630 μM for **2**) and their respective selectivity indexes (SI = IC_{50}/MIC) of 28.17 and 86.30, respectively, are indicative of a selective activity on *M. tuberculosis* H37Rv. Our data are indicative of a significant antimycobacterial activity of (+)-fistularin-3 (**1**) and 11-deoxyfistularin-3 (**2**), since a good drug candidate has to display an SI higher than 10 in order to justify assays for the development of a new antituberculosis drug. Therefore, compounds **1** and **2** are interesting leads for the development of new antituberculosis drug candidates.

Compound **3** was isolated as a glassy solid, with a molecular ion peak (EI) triplet at *m*/*z* = 323/325/327. An HR-EI-MS measurement at *m*/*z* = 324.87750 indicated the formula $C_8H_7{}^{79}Br^{81}BrNO_3$ (calcd. 324.87722) with 5 DBE. Analysis of its ^{13}C-NMR spectrum indicated one carbonyl group at δ = 171.04, six carbons assigned to a pentasubstituted benzene ring (δ = 150.40, 143.55, 124.28, 117.93 and 110.37) of which only one carbon at δ = 117.63 was shown to be a methine (δ_H = 6.96, s) in the HMQC spectrum. According to the ^{1}H-NMR spectrum integration, a hydrogen signal at δ = 3.54 was assigned to a methylene group (δ_C = 37.02). The ^{1}H-NMR spectrum of **3** showed six singlet signals at δ = 9.68 (carboxylic acid hydrogen), 8.98 (phenol hydrogen), 7.25 and 6.86 (NH_2), 6.96 (aromatic CH) and 3.54 (CH_2). The hydrogens at δ = 7.25 and 6.86 showed a coupling in the ^{1}H-^{1}H COSY spectrum. Since none of these hydrogens were attached to carbon (as observed in the HMQC spectrum), they were assigned to an NH_2 group. The methylene group at δ = 3.54 (δ_C = 37.01) was assigned as the connection between the carboxyl group and the benzene ring, since it showed long-range couplings in the HMBC spectra (4, 8 and 12 Hz) with carbons at δ = 171.04, 150.40, 124.28 and 117.93. The signal at δ = 124.28 was assigned to the methylene-substituted benzene carbon, in good agreement with predicted values [17]. Therefore, carbon signals at δ = 150.40 and 117.93 were assigned to quaternary carbons vicinal to carbon at δ = 124.28. The chemical shift of the carbon at δ = 150.40 is in agreement with a hydroxy-substituted carbon, while the chemical shift of carbon at δ = 117.93 agrees with a bromine substitution. Since in the HMBC spectra the aromatic hydrogen at δ = 6.96 (δ_C = 117.63) showed strong long-range couplings with carbons at δ = 150.40, 143.55 (nitrogen-substituted), 124.28 and 110.37 (bromine-substituted), and a very weak correlation with carbon at δ = 117.93, it was placed in the *para*-position to the bromine-substituted carbon at δ = 117.93. Two structures were thus considered: 2-(3-amino-2,4-dibromo-6-hydroxyphenyl)acetic acid (**3**) and 2-(4-amino-2,3-dibromo-6-hydroxyphenyl)acetic acid (**5**). Calculation of ^{13}C chemical shifts for both isomers using ^{13}C NMR charts [17] and ChemDraw 8.0 ^{13}C NMR spectrum simulation indicated a better agreement with structure **3** than with structure **5**. Therefore, the third compound isolated from *A. cauliformis* was identified as 2-(3-amino-2,4-dibromo-6-hydroxyphenyl)acetic acid (**3**). This structure is without precedent in the literature, and it presumably derives biogenetically from the condensation of four acetate units, followed by bromination, oxidation and transamination (no sequence in these events is presumed). The new compound **3** displayed antimycobacterial activity at 49 μM.

Compound **4** was obtained as an optically active glassy solid. FAB-MS analysis displayed a quasi-molecular ion [M – CH_4 + H]$^+$ 1 : 2:1 triplet cluster at *m*/*z* = 350/352/354. A high resolution measurement at *m*/*z* = 352 (obs. 351.97351; calcd. 351.95478) corresponded to a molecular formula of $C_{11}H_{14}{}^{79}Br^{81}BrNO_2$. EI-MS analysis showed a very weak molecular ion 1 : 2:1 triplet cluster at *m*/*z* = 351/353/355, along with a 1 : 2:1 triplet at *m*/*z* = 290/292/294 corresponding to the loss of a rearranged fragment of C_2H_6NO as well as a 1 : 2:1 triplet at *m*/*z* = 275/277/279. Analysis of ^{1}H, ^{13}C, HMQC and HMBC (4, 6, 8 and 12 Hz) NMR spectra of **4** clearly indicated the presence of a 1,2,4,6 symmetrically-substituted benzene ring, with chemical shifts attributable to a 1-alkyl-3,5-dibromo-4-methoxybenzene moiety which is very often present in bromotyrosine-derived secondary metabolites, commonly isolated from sponges of the order Verongida [18]. Indeed, C-1 resonates at δ = 136.1, C-3 and C-5 at δ = 119.3, C-4 at δ = 155.0 (observed through ^{1}H-^{13}C long-range coupling in the HMBC spectra), and C-2 and C-6 at δ = 134.7. Furthermore, a heteroatom-substituted alkyl chain attached to the benzene ring was identified as *N*-methyl-2-methoxypropylamine by analysis of NMR and EI-MS data. The aromatic-substituted methylene group at δ = 3.09 (m; δ_C = 28.8) showed a long-range coupling with the aromatic carbon C-1 at δ = 136.1, and the aromatic methines at δ = 7.61 showed long-range couplings with C-7 (δ_C = 28.8). The ^{1}H-^{1}H COSY spectrum showed sequential vicinal couplings between the aromatic-substituted methylene group at δ = 3.09 and the oxymethine hydrogen at δ = 3.55, as well as between this later hydrogen and a methylene at δ = 3.64. Long-range ^{1}H-^{13}C couplings observed in the HMBC spectra included correlations with the methylene group at δ = 3.09 and the oxygen substituted oxymethine carbon at δ = 73.5 (C-8), between the oxymethine hydrogen at δ = 3.55 and C-7 (δ = 28.8), C-9 (δ = 62.3, weak in the 4 Hz HMBC spectrum) and C-10 (δ = 53.7), while the methylene CH_2–9 at δ = 3.64 showed a long-range coupling with C-8 (δ = 73.5). Although no long-range correlation was observed between the methyl group at δ = 2.69 (δ_C = 39.4) in any of the HMBC spectra, it was placed at the terminal nitrogen atom connected to the methylene at δ = 3.64 (δ_C = 62.3). Such a deshielded nitrogen-bearing methylene group is explained if the nitrogen is protonated. Therefore, the structure was defined as 3-(3,5-dibromo-4-methoxyphenyl)-2-methoxy-*N*-methylpropan-1-ammonium (**4**). Although many attempts have been made to obtain a mass spectrum which could indicate the quasi-molecular ion peak [M + H]$^+$ of **4** with a triplet at *m*/*z* = 366/369/370, we have been unable to observe it in either EI, FAB or CI mass spectra. It is possible that during the MS ionization compound **4** loses methane via a Hofmann-type elimination. Compound **4** displayed very weak antimycobacterial activity (MIC of 820 μM). Cytotoxic activity was not determined for both compounds **3** and **4** due to the small amount of material available.

To the best of our knowledge, this is the first occurrence of the *N*-methyl-2-methoxypropylammonium moiety in a bromotyrosine-derived compound. Additionally, this is the first occurrence of a bromotyrosine-derived compound in a marine sponge belonging to the order Haplosclerida. In the past, dibromotyrosine alkaloids were assumed to be found only in sponges of the order Verongida [19]. However, during the last ten years compounds of this structural class have been isolated from sponges belonging to distinct taxa, including psammaplins from an association of two sponges, *Poecillastra wondoensis* (order Astrophorida, Demospongiae) and *Jaspis wondoensis* (order Astrophorida, Demos-

pongiae) [20], [21], as well as unnamed mycothiol *S*-conjugate amidase inhibitors isolated from the sponge *Oceanapia* sp. (order Petrosida, Demospongiae) [22]. Quite surprisingly, a bromotyrosine-derivative was also recently reported from a marine alga [23]. Considering that different types of marine bacteria are found in Verongida sponges, the hypothesis that these compounds may be produced by microorganisms cannot be ruled out.

In conclusion, we have been able to isolate the antimycobacterial compounds present in the crude extract of the sponge *Aplysina cauliformis*, as (+)-fistularin-3 (**1**), 11-deoxyfistularin-3 (**2**) and the novel 2-(3-amino-2,4-dibromo-6-hydroxyphenyl)acetic acid (**3**). Also, the new bromotyrosine-derived 3-(3,5-dibromo-4-methoxyphenyl)-2-methoxy-*N*-methylpropan-1-ammonium (**4**) was isolated from the sponge *Pachychalina* sp. and showed a very weak antimycobacterial activity. Although other bromotyrosine-derived metabolites displayed antimycobacterial activity [6], [22], [24], this is the first report of fistularin derivatives as antimycobacterial agents. The selectivity indexes observed for compounds **1** and **2** indicate that fistularin-like compounds are good leads for the discovery of new antituberculosis drug leads.

Acknowledgements

The authors are gratefully indebted to Dr. Norrie Pearce and Prof. Brent R. Copp (University of Auckland, Auckland, New Zealand) for their assistance in the MS analyses as well as to Prof. Gil V. J. Silva and Virginia H. B. Glass (Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo) for obtaining the NMR spectra. Financial support was provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (grant 01/03095 – 5) and American Society of Pharmacognosy Foundation Research Starter Grant (1998) to RGSB. AOS thanks PROFIX-CNPq, JHHLO also thanks FAPESP and MFO thanks CNPq for scholarships.

References

[1] Tripathi RP, Tewari N, Dwivedi N, Tiwari VK. Fighting tuberculosis: an old disease with new challenges. Med Res Rev 2005; 25: 93 – 131

[2] Lawn SD, Bekker LG, Miller RF. Immune reconstitution disease associated with mycobacterial infections in HIV-infected individuals receiving antiretrovirals. Lancet Infect Dis 2005; 5: 361 – 73

[3] Ballell L, Field RA, Duncan K, Young RJ. New small-molecule synthetic antimycobacterials. Antimicrob Agents Chemother 2005; 49: 2153 – 63

[4] Zhang Y. The magic bullets and tuberculosis drug targets. Annu Rev Pharmacol Toxicol 2005; 45: 529 – 64

[5] Copp BR. Antimycobacterial natural products. Nat Prod Rep 2003; 6: 535 – 57

[6] El Sayed KA, Bartyzel P, Shen XY, Perry TL, Kjawiony JK, Hamann MT. Marine natural products as antituberculosis agents. Tetrahedron 2000; 56: 949 – 53

[7] Rao KV, Kasanah N, Wahyuono SU, Tekwani BL, Schinazi RF, Hamann MT. Three new manzamine alkaloids from a common Indonesian sponge and their activity against infectious and tropical parasitic diseases. J Nat Prod 2004; 67: 1314 – 8

[8] Rodriguez II, Rodriguez AD. Homopseudopteroxazole, a new antimycobacterial diterpene alkaloid from *Pseudopterogorgia elisabethae*. J Nat Prod 2003; 66: 855 – 7

[9] Fetterolf B, Bewley CA. Synthesis of a bromotyrosine-derived natural product inhibitor of mycothiol-S-conjugate amidase. Bioorg Med Chem Lett 2004; 14: 3785 – 8

[10] De Oliveira JHHL, Grube A, Köck M, Berlinck RGS, Macedo ML, Ferreira AG et al. Ingenamine G and cyclostellettamines G – I, K, and L from the new Brazilian species of marine sponge *Pachychalina* sp. J Nat Prod 2004; 67: 1685 – 9

[11] Gopichand Y, Schmitz FJ. Marine natural products – fistularin-1, fistularin-2 and fistularin-3 from the sponge *Aplysina fistularis* forma *fulva*. Tetrahedron Lett, 1979: 3921 – 4

[12] Compagnone RS, Avila R, Suarez AI, Abrams OV, Rangel HR, Arvelo F et al. 11-Deoxyfistularin-3, a new cytotoxic metabolite from the Caribbean sponge *Aplysina fistularis insularis*. J Nat Prod 1999; 62: 1443 – 4

[13] Collins LA, Franzblau SG. Microplate Alamar Blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob Agents Chemother 1997; 41: 1004 – 9

[14] De Souza AO, Santos-Jr RR, Sato DN, De Azevedo MMM, Ferreira DA, Melo PS et al. Free 2-propen-1-amine derivative and inclusion complexes with β-cyclodextrin: scanning electron microscopy, dissolution, cytotoxicity and antimycobacterial activity. J Braz Chem Soc 2004; 15: 682 – 9

[15] Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986; 89: 271 – 7

[16] Rogers EW, Oliveira MF, Berlinck RGS, König GM, Molinski TF. Stereochemical heterogeneity in verongid sponge metabolites. Absolute stereochemistry of (+)-fistularin-3 and (+)-11-*epi*-(+)-fistularin-3 by microscale LCMS-Marfey's analysis. J Nat Prod 2005; 68: 891 – 6

[17] Silverstein RM, Webster FX, Kiemle D. Spectrometric identification of organic compounds; 7th edn New Jersey: John Wiley & Sons Inc, 2004

[18] Yagi H, Matsunaga S, Fusetani N. Purpuramines A-I, new bromotyrosine-derived metabolites from the marine sponge *Psammaplysilla purpurea*. Tetrahedron 1993; 49: 3749 – 54

[19] Bergquist PR, Wells RJ. In: Scheuer PJ, editor. Marine natural products: chemical and biological perspectives.; Vol. V New York: Academic Press, 1983: pp 1 – 50

[20] Jung JH, Sim GJ, Lee CO. Cytotoxic compounds from a two-sponge association. J Nat Prod 1995; 58: 1722 – 6

[21] Park Y, Liu Y, Hong J, Lee CO, Cho H, Kim DK et al. New bromotyrosine derivatives from an association of two sponges, *Jaspis wondoensis* and *Poecillastra wondoensis*. J Nat Prod 2003; 66: 1495 – 8

[22] Nicholas GM, Newton GL, Fahey RC, Bewley CA. Novel bromotyrosine alkaloids: inhibitors of mycothiol *S*-conjugate amidase. Org Lett 2001; 3: 1543 – 5

[23] Meragelman KM, McKee TC, Boyd MR. Neo-bastadin, the first bastadin analogue isolated from algae. 43rd Annual Meeting of the American Society of Pharmacognosy and 3rd Monroe Wall Symposium, New Brunswick, NJ: 2002

[24] Encarnación-Dimayuga R, Ramírez MR, Luna-Herrera J. Aerothionin, a bromotyrosine derivative with antimycobacterial activity from the marine sponge *Aplysina gerardogreeni* (Demospongia). Pharm Biol 2003; 41: 384 – 7