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Article

Antibacterial Modified Diketopiperazines from two Ascidians of the Genus *Didemnum*#

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A investigação química do extrato bruto de uma ascidia do gênero *Didemnum* levou ao isolamento das dicetopiperazinas modificadas rodriguesinas A (**1**) e B (**2**) na forma de uma mistura de homólogos, os quais puderam ser identificados pela análise de seus dados espectroscópicos inclusive experimentos MS/MS. A investigação de uma segunda ascídia do gênero *Didemnum* forneceu a *N*-acetil-rodriguesina A (**3**) e a *N*-acetil-rodriguesina B (**4**). A configuração absoluta dos compostos **1** e **2** pode ser estabelecida por hidrólise e análise de Marfey e por comparação com dados da literatura do composto **3,** previamente obtido como produto de síntese. A mistura de **1** e **2** apresentou atividade antibiótica moderada contra um isolado clínico de *Streptococcus mutans*, contra *S. mutans* UA159 e *S. aureus* ATCC6538.

The chemical investigation of the crude extract of an ascidian of the genus *Didemnum* led to the isolation of the modified diketopiperazine rodriguesines A (**1**) and (**2**) as a mixture of homologues, which could be identified by analysis of spectroscopic data including MS/MS experiments. The investigation of a second *Didemnum* sp. led to the isolation of *N*-acetyl-rodriguesine A (**3**) and *N*-acetyl-rodriguesine B (**4**). The absolute configuration of compounds **1** and **2** could be established by hydrolysis and Marfey's analysis and comparison with literature data reported for compound **3**, previously obtained as a synthetic product. The mixture of **1** and **2** displayed moderate antibiotic activity against a clinical isolate of *Streptococcus mutans* and against *S. mutans* UA159 and *Staphylococcus aureus* ATCC6538.

Keywords: ascidian, marine, diketopiperazine, absolute configuration, antibiotic

Introduction

Ascidians, or sea-squirts, are cosmopolitan, exclusively marine invertebrates, which constitute a rich source of biologically active secondary metabolites.[1] It has been estimated that *ca.* 85% of ascidian natural products are derived from amino acids, while the remaining 15% are derived from polyketide and/or terpenoid pathways.[2] Ascidians of the family Didemnidae are recognized as a particularly unique source of modified peptides and alkaloids.[3] These include the well known tamandarins,[4] didemnins, aplidine and related compounds,[5] which display potent antitumor and immunosupressive activities, as well as many bioactive alkaloids such as the G2 cell cycle checkpoint inhibitors granulatimide and isogranulatimide.[6]

During several years we have collected distinct species of colonial didemnidae ascidians, in order to expand the diversity of our library of crude extracts.[7] These animals are not easy to collect, since they frequently present

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#Dedicated to Prof. Vanderlan da Silva Bolzani, for her outstanding contributions to the development of natural product chemistry and related sciences in Brazil.

encrusting, thin and soft bodied colonies. Two such species were collected during 1999, as red *Didemnum* spp. The two Didemnidae species gave crude extracts which displayed antituberculosis and cytotoxic activities. The investigation of these extracts led to the isolation of four modified diketopiperazines, which are the subject of the present report.

Results and Discussion

The hexane-defated MeOH extract of the ascidian *Didemnum* sp. (sample BA99ASCI-05) presented aromatic, nitrogen-bearing derivatives indicated by TLC and ^{1}H NMR analyses. The MeOH extract was separated by chromatography on Sephadex LH-20 and C_{18} reversed-phase columns. Further HPLC separations using C_{18}-phenyl or cyanopropyl silica columns did not provide rodriguesines A (**1**) and B (**2**) as pure compounds, even using TFA, NH_4Cl or phosphoric acid buffers in the eluents. Subsequent analysis of 2D NMR and MS/MS analysis indicated that rodriguesines A (**1**) and B (**2**) were obtained as an inseparable mixture from the *Didemnum* sp. sample BA99ASCI-05.

NMR analysis of the fraction containing **1** and **2** indicated the presence of a phenylalanine residue (δ Hα 4.27, δ CH_2-β 3.05 and 3.20, δ aromatic Hs 7.24-7.36), a glycine residue (δ 3.08 and two doublets at δ 3.55/3.56), a linear alkyl β-amino acid and a mono-acylated diamine moiety. These structural features were evident by analysis of the HSQC, COSY and HMBC spectra, of which key correlations are shown in Figure 1. The linear alkyl β-amino acid moiety was assigned from the carbonyl substituted methylene CH_2-18 (δ 2.27 and 2.38) coupled in the COSY spectrum to the broad methine signal at δ 4.65, itself correlated with a methylene group at δ 1.55 and 1.59 (CH_2-20) substituted with the alkyl chain (multiplet at δ 1.30). The diamino moiety was assigned from the amino-substituted methylene at δ 2.97 (CH_2-14) which showed couplings with methylene signals at δ 1.87 and δ 1.69. This methylene (CH_2-15 in **1**)". showed a correlation with a methylene group at δ 3.31 and 3.28 (CH_2-16), while the methylene at δ 1.69 (CH_2-15 in **2**) was coupled to a methylene group at δ 1.55 (CH_2-15a in **2**), itself attached to a methylene at δ 3.23 (CH_2-16 in **2**). Correlations between the methylene at δ 3.28 and 3.31 (CH_2-16), as well as the methylene CH_2-18 (δ 2.27 and 2.38), with the carbonyl groups at δ 172.9 and 173.6 established the connectivity between the diamino moiety and the β-amino acid residue. Couplings between H-3 and C-2 (δ 168.5/168.4) and C-5 (δ 168.6/168.4), as well as between CH_2-6 with the same carbonyl carbons, allowed us to assign the piperazine-2,5-dione moiety. The junction between the diketopiperazine residue with the β-amino acid moiety was established by a correlation observed between the glycyl doublet hydrogens of **1** and **2** at δ 3.55/3.56 with C-19.

Considering the planar structures assigned for **1** and **2**, a search in MarinLit and SciFinder led us to discover that these compounds were closely related to etzionin (**5**),

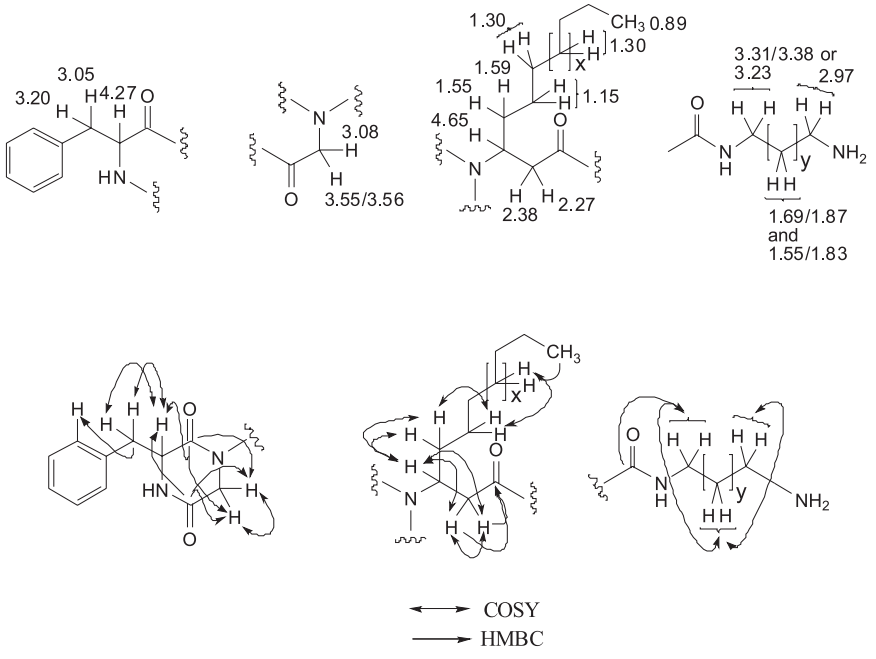
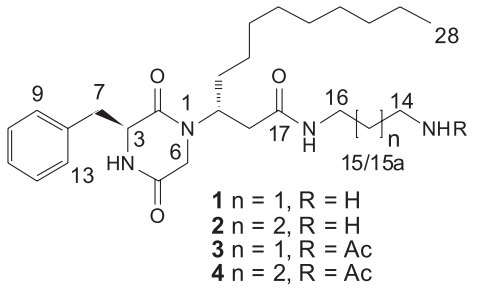
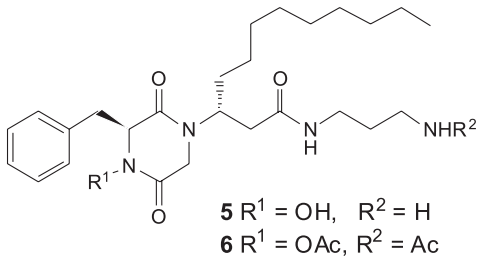


Figure 1. Structural fragments observed for **1** and **2**.

previously isolated from the ascidian *Didemnum rodriguesi*. However, the spectroscopic data of etzionin was not very informative, since only broad, poorly-defined NMR signals have been reported for **5**.[8] A distinctive feature between **1** and **2** and etzionin (**5**) was that no evidence was obtained in the infrared spectrum of **1** and **2** for the presence of the unusual etzionin hydroxamate group. We observed only a broad $\nu_{N\text{-}H}$ band at 3283 cm^{-1} but not a band associated with a $\nu_{O\text{-}H}$ at higher wavenumbers. Additionally, since the analysis of the acyl monosubstituted diamino moiety in the mixture of **1** and **2** indicated the presence of two amide carbonyls and two diamine alkyl chains, we suspected that two diamine homologues constituted the fraction isolated from the *Didemnum* sp. BA99ASCI-05. Indeed, analysis by HRMS/MS showed that the fraction containing **1** and **2** displayed two quasi-molecular ions $[M+H]^+$ at *m/z* 459.3335 ($C_{26}H_{43}N_4O_3$) and *m/z* 473.3492 ($C_{27}H_{45}N_4O_3$), which, upon fragmentation, gave an identical fragment ion at *m/z* 385.2500 ($C_{23}H_{33}N_2O_3$) corresponding to the lost of $C_3H_{10}N_2$ (1,3-diaminopropane) from **1** and of $C_4H_{12}N_2$ (1,4-diaminobutane) from **2** (Figure 2). The analysis of the MS/MS spectrum also indicated the formation of the corresponding oxazepine and oxazine ions at *m/z* 238.2181 and 252.2352, respectively, as well as the formation of a diketopiperazinium at *m/z* 343.2416 (Figure 2). Therefore, the complete planar structure of rodriguesine A (**1**) and B (**2**) was confirmed. Further support for the structures assigned for both **1** and **2** could be obtained after acetylation of this mixture and separation by HPLC. This procedure gave compounds **3** and **4** (see below) of which compound **3** showed NMR data very similar to that of bis-acetyl etzionin (**6**).[8]

In order to establish the absolute configuration of the phenylalanine residue, the mixture of **1** and **2** was subjected to hydrolysis followed by derivatization with





Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) and LC-PDA-MS analysis. Only L-phenylalanine was detected. Moreover, since the absolute configuration of etzionin (**5**) was established after the total synthesis of its 4-desoxy-*N*-acetyl derivative (**3**),[9] we decided to acetylate the mixture of **1** and **2** in order to separate both *N*-acetyl derivatives and compare the specific rotation of 4-desoxy-*N*-acetyl etzionine (**3**) derived from **1** with the literature value reported for synthetic **3**. The acetylated mixture of **3** and **4** could be separated by C_{18} HPLC. Compound **3**, obtained after acetylation of **1**, showed $[\alpha]_D$ −37.0 (*c* 0.026, $CHCl_3$), very close to the value reported for synthetic (-)-(3*S*,19*R*)-**3**, $[\alpha]_D$ −53.1 (*c* 0.06, $CHCl_3$). The ^1H NMR spectrum of **3** obtained from the acetylation of **1** was identical to that of synthetic (-)-(3*S*,19*R*)-**3**.[9] Since the absolute configuration of the phenylalanine residue in **3**

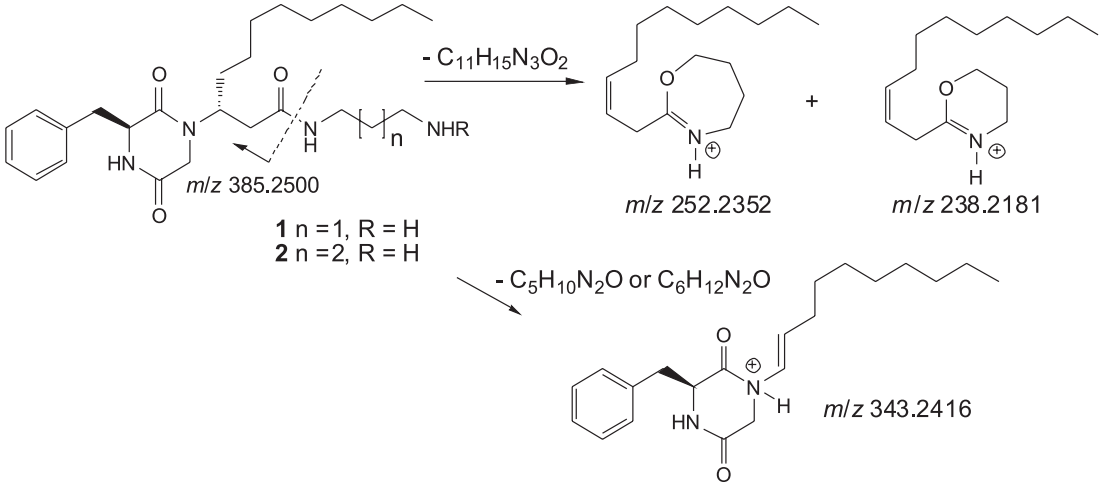


Figure 2. Major fragment ions observed in the MS/MS spectrum of **1** and **2**.

derived from the acetylation of natural **1** is *S*, the absolute configuration of C-19 must be *R*. After acetylation and purification, compound **4** obtained from **2** showed $[\alpha]_D$ −52 (*c* 0.04, $CHCl_3$). Therefore, both rodriguesines A (**1**) and B (**2**) have the 3*S*, 19*R* absolute configuration.

Sample BA99ASCI-03 of the second *Didemnum* species gave, after a similar isolation procedure, small quantities of **3** and **4** as pure compounds. Analysis of the spectroscopic data recorded for *N*-acetyl rodriguesine A (**3**) clearly assigned the presence of an acetyl group (C=O at δ_C 173.4; CH_3 at δ_C 22.7 and δ_H 1.92), corroborated by the HRESIMS analysis which showed a $[M+H]^+$ quasi-molecular ion at *m/z* 501.3483 ($C_{28}H_{45}N_4O_4$), indicating an additional C_2H_2O fragment compared to the formula of **1**. Analysis of the NMR data of the diamino moiety in **3** showed a coupling between the methylenes at δ 3.18 (t, 8.4 Hz, 6H, CH_2-14 and CH_2-16) and two carbonyl groups at δ_C 173.4 and 172.7. Since the methylene CH_2-18 (δ 2.20 and 2.32) confirmed a long-range coupling with the carbonyl group at δc 172.7 (C-17), and the methyl acetyl group showed a long range coupling with the carbonyl at δ_C 173.4, the acetyl group could be placed at the diamine terminus of compound **3**. Analogous MS and NMR data recorded for *N*-acetyl rodriguesine B (**4**) showed a quasi-molecular ion $[M+H]^+$ at *m/z* 515.3639 ($C_{29}H_{47}N_4O_4$) and quite similar NMR data for the diamide moiety (Tables 1 and 2), except for the presence of an additional methylene group (CH_2-15a at δ_H 1.50 and δ_C 27.8) in the diamino moiety. Comparison of ^1H, ^{13}C NMR and MS data recorded for natural **3** and **4** with data recorded for **3** and **4** derived from the acetylation of **1** and **2** proved them to be identical compounds. Therefore, the structure of the natural, minor *N*-acetylated compounds **3** and **4**, could be established. Compounds **3** and **4** should have the same absolute configuration as compounds **1** and **2** since their $[\alpha]_D$, HPLC t_R, ^1H and MS data are practically identical to **3** and **4** obtained from the acetylation of the mixture of **1** and **2**.

The mixture of **1** and **2** was evaluated in cytotoxic, antituberculosis and antibiotic bioassays against a panel of pathogenic bacteria. Surprisingly, rodriguesines A **1** and B **2** did not present cytotoxic or antituberculosis activities. The mixture of **1** and **2** was weakly active against the majority of the pathogen microbial strains tested (Table 3), except against *Pseudomonas aeruginosa* P1 (MIC at 4.3 μg mL^{-1}), obtained from a hospital environment. Interestingly, the mixture of **1** and **2** proved to be more active against antibiotic-resistant strains than against standard ATCC or NTCC strains (Table 3). The strains *Streptococcus mutans* clinical isolate 2.M7/4, *S. mutans* UA159, *Staphylococcus aureus* ATCC6538, *Escherichia coli* NTCC861, *Enterococcus faecalis* ATCC14506, *Streptococcus sanguinis* ATCC15300, *S. sobrinus* ATCC27607 and *Candida albicans* ATCC36801(serum type A) are all associated with muco-bucal diseases.

Table 1. ^1H data for compounds **1** and **2**, **3** and **4** in CD_3OD at 400 MHz [δ, multiplicity (*J* in Hz)]

Position	**1** and **2**	**3**	**4**
3	4.27 (t, 5.0)	4.27 (t, 6)	4.22 (t, 6.5)
6	3.08 (d, 17), 3.55/3.56 (2d, 17 Hz each)	3.00 (d, 22), 3.50 (d, 22)	2.99 (d, 22), 3.50 (d, 22)
7	3.05 (m), 3.20 (m)	3.02 (dd, 5, 17), 3.16 (m)	3.01 (dd, 5, 17), 3.16 (m)
9/13	7.24 (m)	7.21 (m)	7.20 (m)
10/12	7.36 (m)	7.31 (m)	7.28 (m)
11	7.35 (m)	7.30 (m)	7.24 (m)
14	2.97 (t, 7)	3.18 (t, 8.4)	3.16 (m)
15	1.87 (qui, 7) and 1.69 (m)	1.65 (qui, 8.4)	1.50 (m)
15a	1.55 (m)	-	1.50 (m)
16	3.28 and 3.31/3.23	3.18 (t, 8.4)	3.16 (m)
18	2.27 (dd, 7, 12), 2.38 (m)	2.20 (dd, 9, 17), 2.32 (dd, 9, 17)	2.18 (dd, 9, 17), 2.32 (dd, 9, 17)
19	4.65 (br)	4.60 (br)	4.57 (br)
20	1.55 (m), 1.59 (m)	1.52 (m)	1.53 (m)
21	1.18 (m)	1.14 (m)	1.14 (m)
22	1.30 (m)	1.26 (m)	1.26 (m)
23	1.30 (m)	1.26 (m)	1.26 (m)
24	1.30 (m)	1.26 (m)	1.26 (m)
25	1.30 (m)	1.26 (m)	1.26 (m)
26	1.30 (m)	1.26 (m)	1.26 (m)
27	1.30 (m)	1.28 (m)	1.28 (m)
28	0.93 (t, 7)	0.89 (t, 9)	0.89 (t, 8)
COMe	-	1.92 (s)	1.91 (s)

Conclusion

We reported here the isolation of four new modified diketopiperazines from two ascidians of the genus *Didemnum*. The mixture of **1** and **2** displayed moderate antibiotic activity against several human pathogenic bacteria.

Experimental

General experimental procedures

Optical rotations were measured on a Perkin Elmer 341 MC polarimeter at 20 °C. IR spectra (film on Si plate) were recorded on a FT-IR Bomem MB102 infrared

Table 2. ^{13}C data for compounds **1** and **2**, **3** and **4** in CD_3OD at 100 MHz

Position	**1** and **2**	**3**	**4**
2	168.5/168.4	168.5	168.4
3	58.4	58.2	58.2
5	168.6/168.4	168.5	168.4
6	46.4 (br)	46.0	46.3
7	41.1/41.2	41.1	41.0
8	137.12/137.10	136.9	136.8
9/13	130.0	131.4	131.4
10/12	128.6	129.8	129.8
11	128.4	128.5	128.5
14	38.5/40.5	38.0	40.1
15	28.9/26.1	30.2	27.8
15a	27.6	-	27.8
16	37.2/39.8	38.0	40.1
17	172.9/173.6	172.7	172.5
18	39.5[a]/39.7[a]	39.6	39.6
19	53.8 (br)	54.1	54.1
20	31.8	31.5	31.6
21	27.3	27.1	27.1
22	27.6	30.7	30.2
23	30.8	30.3	30.4
24	30.6	30.5	30.5
25	30.4	30.6	30.6
26	33.3	33.1	33.0
27	23.9	23.8	23.7
28	14.7	14.5	14.4
CO	-	173.4	174.0
Me	-	22.7	22.6

[a] Interchangeable assignments.

spectrometer. The NMR spectra were recorded on a Bruker ARX 9.4 T instrument, operating at 400.35 MHz for ^1H and 100.10 MHz for ^{13}C, respectively. All NMR spectra were obtained at 25 °C using TMS as an internal reference. Solvents used for extraction and column chromatography were glass distilled prior to use. HPLC-grade solvents were utilized without further purification in HPLC separations. TLC analyses were performed with precoated TLC sheets of Si gel on polyester, eluting with different mixtures of MeOH in CH_2Cl_2. Plates were observed under a UV lamp (λ_{max} 254 and 365 nm). Semi-preparative HPLC separations were performed with a Waters 600 quaternary pump and a double beam model 2487 UV detector monitored by Waters Millenium 32. High resolution mass spectra obtained for **1** and **2** were recorded on a Micromass Q-ToF Micro, in ES^+ mode, using the following experimental conditions:

Table 3. Antimicrobial activity of **1** and **2** (MIC in μg mL^{-1})

Pathogenic microbial strain	mixture of **1** and **2**
Staphylococcus aureus ATCC6538	62.5
Staphylococcus aureus ATCC259223	22.6
ORSA 8	45.3
ORSA 108	91.0
Escherichia coli ATCCNTCC861	125.0
Escherichia coli ATCC259222	45.6
Pseudomonas aeruginosa ATCC27853	22.6
Pseudomonas aeruginosa 13	45.3
Pseudomonas aeruginosa P1	4.3
Candida albicans ATCC10231	n.a.
Candida albicans ATCC36801 (serum type A)	125.0
Enterococcus faecalis ATCC14506	125.0
Streptococcus sanguinis ATCC15300	125.0
Streptococcus sobrinus ATCC27607	125.0
Streptococcus mutans UA159	62.5
Streptococcus mutans (clinical isolate 2.M7/4)	31.2

ORSA: Oxacillin-resistant *Staphylococcus aureus*; n.a.: not assigned.

capillary voltage 3.0 kV; sample cone 42.0 V; sample infused at 10 μL min^{-1} in 1: 1 MeOH/H_2O. Accurate mass measurements were made using leucine enkephalin as an internal reference at $[M+H]^+$ 556.2771. The MS/MS experiments were recorded using with a range of collision energies from 20 to 30 eV. High resolution mass spectra obtained for compounds **3** and **4** were recorded on a Bruker Daltonics equipment (UltrO-ToF, MA, USA). Samples (0.5 μg mL^{-1}) dissolved in MeOH/H_2O 1:1 were introduced in the electrospray source at 5 μL min^{-1} with a direct infusion pump. MS/MS and MS^n experiments were performed using standard isolation and excitation procedures. Nitrogen was used as nebulising and collision gas with the collision energy set at 4 eV. An accurate-mass calibration was obtained with a post acquisition application of a calibration created from the MS/MS of monensin A $[M-18+Na]^+$ under the same CID and cell conditions. The collision energy was adjusted until the intensity of the parent ion was set between 5 and 20% relative to the base peak. Capillary voltage was set to 3500 V.

Animal material

The ascidians *Didemnum* sp. BA99ASCI-03 and *Didemnum* sp. BA99ASCI-05 were collected in August/ September 1999, from the Baía de Todos os Santos, Salvador, Bahia state, Brazil and immediately frozen. Both samples were deposited in the ascidian collection of the Departamento de Zoologia, Setor de Ciências Biológicas,

Universidade Federal do Paraná. BA99ASCI-03: (DZUP DID 210) - colony is reddish orange when alive, but looses the color in formalin, zooids with the sperm duct forming ten coils around the only follicle of the testis, larval trunk oval, 0.5 mm long with four ectodermal ampullae in each side, long and close to each other. The three adhesive papillae are close to each other and present a short stalk.

BA99ASCI-05 (DZUP DID 209) - colony is red when alive, but looses the color in formalin, zooids with sperm duct forming seven coils around the only follicle of the testis, larval trunk 0.4 mm long with four to five ectodermal ampullae in each side, shorter than the ones observed in former voucher. The three adhesive papillae have long stalks.

Extraction and isolation

The frozen ascidian sample BA99ASCI-05 (308.9 g, wet) was freeze dried and exhaustively extracted with MeOH. The MeOH extract was filtered, concentrated to 400 mL and partitioned with hexane (3 × 400 mL). TLC analysis (UV and Dragendorff) of the MeOH fraction indicated the presence of intense UV-absorbing compounds with amine groups. This fraction was evaporated to dryness (5.6 g) before separation by C_{18} reversed-phase chromatography (gradient of MeOH in H_2O) in 0.5-1.0 g portions. This separation yielded eleven fractions (Da-MSP-1 to Da-MSP-11), of which the eighth (Da-MSP-8, 1.87 g) presented most of the Dragendorff and UV positive compounds. The fraction Da-MSP-8 was then subjected to chromatography on Sephadex LH-20 (MeOH). Seven fractions were obtained (Da-MSP-8A to DA-MSP-8G), of which Da-MSP-8D (0.402 g) and Da-MSP-8E (0.532 g) presented aromatic nitrogen-bearing compounds. Both fractions were similarly purified, firstly by C_{18} reversed-phase column chromatography (gradient of MeOH in H_2O) then by HPLC (phenyl-bonded silica gel, Waters μBondapak 10 μm, 125 Å, 7.8 × 300 mm; eluent: 65:35 MeOH/0.1% TFA in H_2O; λ_{max} 220 nm; flow rate: 1 mL min^{-1}; t_R 25.6 min) to give 0.148 g of the mixture of **1** and **2** (0.05%, wet).

The frozen ascidian sample BA99ASCI-03 (450.0 g) was freeze dried and exhaustively extracted with MeOH. The MeOH extract was concentrated to 400 mL and partitioned with hexane (3 × 400 mL). The alcoholic fraction was evaporated until an aqueous suspension was obtained. The H_2O fraction was first partitioned with EtOAc then with 3:2 CH_2Cl_2/MeOH. The CH_2Cl_2/MeOH fraction (2.0 g) was separated by chromatography on Sephadex LH20 (MeOH), to give five fractions (BA99AC-3A to BA99AC-3E). The last fraction obtained (Ba99AC-3E, 214 mg) was subjected to chromatography on a cyanopropyl-bonded silica cartridge (5 g), with a gradient of EtOAc in CH_2Cl_2. The first fraction obtained from this separation (BA99AC-3E1, 134 mg) was separated by chromatography on a silica-gel cartridge (2 g) with a gradient of MeOH in CH_2Cl_2, to give three fractions (Ba99AC-3E1A to BA99AC-3E1C). The second one (BA99AC-3E1B, 38.0 mg) was separated by HPLC using a phenyl-bonded silica column (Inertsil, 250 × 4.6 mm, 5 μm) using 45:55 MeCN/H_2O as eluent, to give five fractions. Fractions BA99AC-3E1C2 (7.1 mg) and BA99AC-3E1C2 (4.6 mg) were purified using a C_{18} reversed phase HPLC column (Phenomenex 5 μm), using 1:1 MeOH/0.1% HCO_2H in H_2O as eluent, to give *N*-acetyl-rodriguesine A (**3**, t_R = 19.8 min, 3.6 mg, 8.0×10^{-4}%) and *N*-acetyl-rodriguesine B (**4**, t_R = 21.2 min, 4.6 mg, 1.0×10^{-3}%).

*Rodriguesines A (**1**) and B (**2**)*

Colorless, glassy solid; UV (MeOH) λ_{max} 258 nm; ^{1}H NMR (CD_3OD, 400 MHz), see Table 1; ^{13}C NMR (CD_3OD, 100 MHz), see Table 2; Positive HRESIMS for **1** *m/z* 459.3341 $[M+H]^+$, calc. for $C_{26}H_{43}N_4O_3$ 459.3335. Positive HRESIMS for **2** 473.3499 $[M+H]^+$ calc. for $C_{27}H_{45}N_4O_3$ 473.3492. MS/MS experiments: *m/z* 385.2500, corresponding to $C_{23}H_{33}N_2O_3$ (calc. 385.24912); *m/z* 238.2181, corresponding to $C_{15}H_{28}NO$ (calc. 238.21654); *m/z* 252.2352, corresponding to $C_{16}H_{30}NO$ (calc. 252.23219); *m/z* 343.2416, corresponding to $C_{21}H_{31}N_2O_2$ (calc. 343.23800).

*N-acetyl-rodriguesine A (**3**)*

Colorless, glassy solid; $[\alpha]_D$ −37 (*c* 0.026, $CHCl_3$); UV (MeOH) λ_{max} 256 nm; ^{1}H NMR (CD_3OD, 400 MHz), see Table 1; ^{13}C NMR (CD_3OD, 100 MHz), see Table 2; Positive CID-MS/MS for **1** *m/z* 501.3483 $[M+H]^+$, calc. for $C_{28}H_{44}N_4O_4$ 501.34408.

*N-Acetyl-rodriguesine B (**4**)*

Colorless, glassy solid; $[\alpha]_D$ −52 (*c* 0.04, $CHCl_3$); UV (MeOH) λ_{max} 257 nm; ^{1}H NMR (CD_3OD, 400 MHz), see Table 1; ^{13}C NMR (CD_3OD, 100 MHz), see Table 2; Positive CID-MS/MS for **1** *m/z* 515.3639 $[M+H]^+$, calc. for $C_{29}H_{47}N_4O_4$ 515.35918.

*Acetylation of rodriguesines A (**1**) and B (**2**)*

The mixture of compounds **1** and **2** (40 mg) was stirred overnight with 1 mL of pyridine and 1 mL of acetic anhydride, at room temperature. The reaction medium was transferred to a round bottom flask and evaporated until dryness. The acetylated mixture was separated by C_{18}

reversed-phase HPLC (Inertsil, ODS 3 250 × 4.6 mm, 5 μm) with a linear gradient of MeCN in H_2O, from 7:3 to 95:5 during 30 min, to give 1.7 mg of pure *N*-acetyl-rodriguesine A (**3**) and 3.0 mg of pure *N*-acetyl-rodriguesine B (**4**), as well as 30.7 mg of an intermediate fraction containing both **3** and **4**. *N*-acetyl-rodriguesine A (**3**) showed $[\alpha]_D$ -37.0 (*c* 0.026, $CHCl_3$) and ^{1}H NMR, HPLC retention time and MS analysis identical to natural **3**. Similarly, *N*-acetyl-rodriguesine B (**4**) showed $[\alpha]_D$ −58 (*c* 0.03, $CHCl_3$) and ^{1}H NMR, HPLC retention time and MS analysis identical to natural **4**.

*Hydrolysis and Marfey's analysis of rodriguesines A (**1**) and B (**2**)*

Two milligrams of the mixture of **1** and **2** was dissolved in 0.5 mL of 6 mol L^{-1} HCl and refluxed at 105 °C for 24 h. Then the reaction medium was evaporated until dryness. The hydrolyzed product was dissolved in H_2O (40 μL) and a 0.5 mol L^{-1} $NaHCO_3$ solution was added, followed by 80 μL of a 1.4 equiv. of Marfey's reagent (2,4-dinitro-5-fluoro-phenyl-L-alaninamide) in acetone. The reaction was stirred at 40 °C for 1 h. Afterwards, 16 μL of 0.5 mol L^{-1} HCl was added to the reaction medium followed by evaporation to dryness. The reaction product was dissolved in 1 mL of MeOH. An identical derivatization procedure was employed for the preparation of a L-phenylalanine-derivatized standard. The hydrolysed and the standard-derivatized samples were analyzed by HPLC-PDA-MS using the following conditions: Phenomenex C_{18} Synergi-4 Fusion RP-80 column, 250 × 4.6 mm; eluent: gradient of MeCN in Et_3N/H_2PO_4 aqueous buffer; flow rate: 1 mL min^{-1}. The hydrolyzed and derivatized sample as well as the derivatized phenylalanine standard were injected separately and co-injected. Retention time of 2,4-dinitro-5-(L-phenylalanine)-phenyl-L-alaninamide: 14.85 min.

Antibiotic assay

Procedures for the antimicrobial assays against *Staphylococcus aureus* ATCC 6538, *S. aureus* ATCC 259223, *Escherichia coli* ATCC861, *E. coli* ATCC 259222, *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* 13, *P. aeruginosa* P1, *Candida albicans* ATCC 10231, *C. albicans* ATCC 36801 (serum type A), *Enterococcus faecalis* ATCC 14506, *Streptococcus sanguinis* ATCC 15300, *Streptococcus sobrinus* ATCC 27607, *Streptococcus mutans* UA159, *S. mutans* (clinical isolate 2.M7/4) were performed using the broth microdilution assay, adapted from the National Committee for Clinical Laboratory Standards (USA) and from Oliveira *et al*.,[10] as it follows. Briefly, bacteria were grown in BHI (Brain Heart Infusion) liquid medium at 37 °C and suspended, as recommended, to yield final inocula of approximately 10^6 CFU/mL. Tests were performed in sterile 96-well microplates by dispensing into each well a total volume of 100 μL (40 μL of medium + 20 μL de inocula + 40 μL substance solution diluted in H_2O) and incubated 24 h at 37 °C in 10% CO_2. Microorganism growths were determined by absorbance measurement at 550 nm in an automated microplate reader (Molecular Devices, Versa Max). The MIC was defined as the lowest concentration of drug that inhibited growth. As control microbial culture growths without the addition of the tested substance were used and all samples were assayed in replicate.

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Supplementary Information

^{1}H NMR, ^{13}C NMR and MS spectra of **1** and **2**, **3** and **4** are available free of charge at http://jbcs.sbq.org.br, as a PDF file.

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