

Full Research Article

Antimicrobial and Antimycobacterial Activity of Cyclostellamine Alkaloids from Sponge *Pachychalina* sp.

Jaine H. H. L. de Oliveira ¹, Mirna H. R. Selegim ¹, Christoph Timm ², Achim Grube ², Matthias Köck ², Gislene G.F. Nascimento ³, Ana Claudia T. Martins ³, Elissa G. O. Silva ³, Ana Olívia de Souza ⁴, Paulo R. R. Minarini ⁴, Fabio C. S. Galetti ⁵, Célio L. Silva ⁵, Eduardo Hajdu ⁶ and Roberto G. S. Berlinck ^{1,*}

¹ Instituto de Química de São Carlos, Universidade de São Paulo, CP 780, CEP 13560-970, São Carlos, SP, Brazil. (E-mail: jainehh@iris.ufscar.br for Jaine H. H. L. de Oliveira; E-mail: mirna@iqsc.usp.br for Mirna H. R. Selegim)

² Alfred-Wegener-Institut für Polar- und Meeresforschung in der Helmholtz-Gemeinschaft, Am Handelshafen 12, D-27570 Bremerhaven, Germany. (E-mail: agrube@awi-bremerhaven.de for Achim Grube; E-mail: mkoeck@awi-bremerhaven.de for Matthias Köck)

³ Faculdade de Ciências da Saúde, Universidade Metodista de Piracicaba, Piracicaba, SP, Brazil. (E-mail: ggfranco@unimep.br for Gislene G.F. Nascimento)

⁴ Laboratório de Bioquímica e Biofísica, Instituto Butantan, São Paulo, Brazil. (E-mail: olívia@butantan.gov.br for Ana Olívia de Souza)

⁵ Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil. (E-mail: galetti@cpt.fmrp.usp.br for Fabio C. S. Galetti)

⁶ Museu Nacional, Universidade Federal do Rio de Janeiro, Quinta da Boa Vista, s/n, 20940-040, Rio de Janeiro, RJ, Brazil. (E-mail: hajdu@acd.ufrj.br for Eduardo Hajdu)

* Author to whom correspondence should be addressed; E-mail: rgsberlinck@iqsc.usp.br

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Abstract: Cyclostellamines A – F (**1 – 6**) isolated from the sponge *Pachychalina* sp. and cyclostellamines G - I, K and L (**7 – 11**) obtained by synthesis were evaluated in bioassays of antimicrobial activity against susceptible and antibiotic-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* and antibiotic-susceptible *Escherichia coli* and *Candida albicans*, as well as in antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv bioassays. The results obtained indicated that cyclostellamines display different antimicrobial activity depending on the alkyl-chain size, suggesting that, if a mechanism-of action is implied, it is dependent on the distance between the two pyridinium moieties of cyclostellamines.

Keywords: alkaloids, antimycobacterial, antibacterial, *Pachychalina* sp., sponge

Introduction

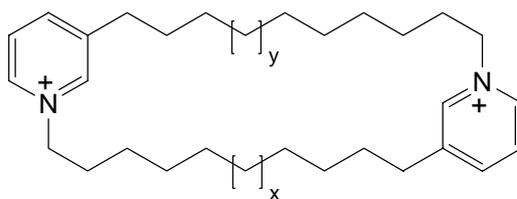
Microbial promoted infectious diseases are a major public health issue worldwide. Among other, these include infection by antibiotic-resistant microorganisms, which are considered very problematic since only few antibiotics currently available are effective against resistant microbial strains [1-3]. One of the most promising classes of antibiotics is inhibitors of bacteria cell wall synthesis, which are largely based on different classes of natural products [4]. Therefore, there is great interest in finding new classes of natural products that may be effective against antibiotic-resistant bacteria. Also, tuberculosis promoted by *Mycobacterium tuberculosis* is a major cause of mortality. It is estimated that about 2 billion people are currently infected with *M. tuberculosis*. The number of infections by *M. tuberculosis* appear to be increasing, due to co-infection with HIV and the emergence of multiple drug resistant *Mycobacterium* strains [5,6]. Although several drugs for the treatment of tuberculosis are available, it is a long-term therapy (6 months), which very frequently present 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 [7,8].

In addition to microbial promoted infectious diseases, systemic mycoses are also difficult to medicate. In particular, infections by *Candida albicans* are increasing in the group of immunologically suppressed patients, for which only a few antifungal agents are effective. Therefore, the search for new antifungal compounds active against *Candida* spp. is of special importance [9].

Marine macro- and microorganisms have proven to be an outstanding source of structurally unique biologically active natural products [10-12]. Marine sponges constitute the main biological group source of bioactive secondary metabolites from the marine environment. In particular, marine sponges of the order Haplosclerida have been known for a long time as a typical source of alkyipyridine and alkyipiperidine alkaloids [13-16]. During our current search for new bioactive compounds from marine sponges, we have recently started to investigate the chemistry of the antimycobacterial, antimicrobial and cytotoxic MeOH crude extract of the sponge *Pachychalina* sp. and isolated a new member of the ingenamine family of alkaloids as well as six known and five new cyclostellettamine alkaloids identified by LC-MS/MS analysis [17]. Herein, we report the antimycobacterial and antimicrobial activity against antibiotic-resistant bacteria of pure cyclostellettamines A – F (**1** – **6**) isolated from *Pachychalina* sp. as well as of isolated cyclostellettamines G – I, K and L (**7** – **11**) obtained by total synthesis.

Results and Discussion

Cyclostellettamines A – F (**1** – **6**) were isolated as pure entities from the n-BuOH crude extract of *Pachychalina* sp. obtained previously [17] by chromatography on Sephadex LH20 (MeOH) followed by reversed phase C₁₈ HPLC purification, while cyclostellettamines G – I, K and L (**7** – **11**) were obtained by total synthesis [18]. All compounds were unambiguously identified by HPLC-MS/MS analysis, as previously reported [17].



1 $x = 3, y = 3$	7 $x = 1, y = 3$
2 $x = 3, y = 4$	8 $x = 2, y = 3$
3 $x = 4, y = 4$	9 $x = 1, y = 4$
4 $x = 3, y = 5$	10 $x = 1, y = 5$
5 $x = 4, y = 5$	11 $x = 2, y = 5$
6 $x = 5, y = 5$	

Scheme 1. Structures of cyclostelllettamines A – F (**1** – **6**), G - I, K and L (**7** – **11**).

Since only a small amount of pure cyclostelllettamines was available, they were subjected to antibacterial, antifungal and antimycobacterial bioassays only. The results indicated (Table 1) that cyclostelllettamine A (**1**) was the less active alkaloid in all antimicrobial assays. All other cyclostelllettamines were strongly active against antibiotic-susceptible *S. aureus* ATCC 25923 (strain **Sa** in table 1) and *E. coli* ATCC 25922 (**Ec**), with exception of compound **7** against *E. coli*. Cyclostelllettamine C (**3**) was the most active against *P. aeruginosa* ATCC 27853 (strain **Pa**), cyclostelllettamines C (**3**) and F (**6**) were the most active against one oxacillin-resistant strain of *S. aureus* (strain **ORSa8**), and cyclostelllettamine C (**3**) against another ORSA strain (strain **ORSa108**). Cyclostelllettamine F was the most active against both sensitive and antibiotic-resistant *P. aeruginosa* strains (**Pa** and **Pa13**). Only cyclostelllettamines C – F (**3** – **6**) were active against *Candida albicans* ATCC 10231 (**Ca**). The results of the antifungal assay suggested that the length of the alkyl chains of cyclostelllettamines is an important criterion for activity against *Candida*. It seems likely that lipophilic alkyl chains can interact with the microbial membrane, while the hydrophilic pyridine moieties may either interact with negatively charged membrane sites. This process may initialize a disorder of the membrane lipids and perhaps an uncontrolled flux of electrolytes, where the positively charged pyridinium moieties may play a crucial role. It has been indeed observed that positively charged lipophilic peptides and cationic vesicles exert a strong antifungal activity against *Candida albicans* [19-22] through the interaction with the membrane positively charged sites. Cyclostelllettamines B (**2**), C (**3**), G (**7**) and L (**11**) were strongly active against *M. tuberculosis* H37Rv (**MtH37Rv**). Overall, cyclostelllettamine C (**3**) was the most active alkaloid. It is interesting to observe that, with exception to *S. aureus* ATCC 25923 (**Sa**) and *E. coli* ATCC 25922 (**Ec**), all other microorganisms were sensitive to the cyclostelllettamines antimicrobial activity depending on the size of the alkyl chains bridging the two alkylpyridinium groups, a result that suggests a mechanism-based activity related to the size of the alkyl chains.

In particular, the results of antimycobacterial activity against *M. tuberculosis* H37Rv were suggestive that cyclostelllettamines with larger alkyl chains, such as compounds **4**, **5** and **6**, were less active than compounds with smaller alkyl chains as linkers of the two pyridinium moieties, although cyclostelllettamine A (**1**) is an exception. A similar alkyl chain size-dependent activity was observed for cyclostelllettamines A - F (**1** – **6**) as inhibitors of the binding of methyl quinuclidinyl benzilate to muscarine acetylcholine receptors when these compounds have been originally reported from the sponge *Stelletta maxima* [23]. In this case, cyclostelllettamine A (**1**) was the most active and

cyclostelletamine F (**6**) the less active alkaloid. Apparently, a correct positioning of the charged pyridinium moieties is necessary to maximize the activity in different bioassays. The results observed in our bioassays suggest a mechanism-based antimicrobial action for the cyclostelletamine dimeric alkylpyridinium alkaloids, differently than observed for the structurally-related oligomeric and polymeric halitoxins, which display potent biological activities in several different bioassays irrespective of their average size, and are considered as nuisance compounds [24]. However, since we have not attempted to simulate the distance between the two pyridinium rings in each cyclostelletamine, our mechanism of action proposal is suggestive only. It should be also noted that since Gram-positive (*S. aureus*) and Gram-negative (*E. coli*, *P. aeruginosa*) bacteria have different membrane properties, cyclostelletamines might certainly present distinct modes of interactions with the respective bacterial membranes. Further structure-activity studies on cyclostelletamine alkaloids may clarify if there is or not a mechanism-based relationship of their biological activities.

Table 1. Minimal inhibitory concentration (MIC, in $\mu\text{g/mL}$) of the antimicrobial activity of cyclostelletamine alkaloids on sensitive and antibiotic-resistant microbial strains*.

Alkaloids	alkyl chains $\Sigma(\text{CH}_2)_n$	Microbial strains								
		Sa	Ec	Pa	ORSa8	ORSa108	Pa13	PaP1	Ca	MtH37Rv
1	24 (12 + 12)	56.3	56.3	112.5	56.3	225	112.5	450	*	32
2	25 (12 + 13)	0.5	2	31.3	62.5	7.8	31.3	62.5	*	4.0
3	26 (13 + 13)	1.2	1.2	8.6	1.8	4.7	18.8	18.8	0.12	4.0
4	26 (12 + 14)	0.3	2.1	16.4	16.4	8.2	16.4	16.4	0.7	8.0
5	27 (13 + 14)	2.3	2.4	18.8	9.4	9.4	9.4	18.8	0.7	11.0
6	28 (14 + 14)	0.6	9.4	75	0.24	75	9.4	4.7	0.7	8.0
7	22 (10 + 12)	0.6	39.1	156.3	39.1	78.1	156.3	156.3	+	4.6
8	23 (11 + 12)	2.4	9.5	312.5	78.1	625	312.5	312.5	+	9.3
9	23 (10 + 13)	1.1	8.8	40.6	35.2	35.2	140.6	17.6	+	6.6
10	24 (10 + 14)	1.7	1.7	27.3	437.5	875	13.7	27.3	+	5.3
11	25 (11 + 14)	0.8	3.2	51.6	51.6	25.8	51.6	51.6	+	4.6

* Microbial strains: **Sa.** *S. aureus* (ATCC 25923) – Gram positive; **Ec.** *E. Coli* (ATCC 25922) – Gram negative; **Pa.** *Pseudomonas aeruginosa* (ATCC 27853) – Gram negative; **ORSa8.** Oxacillin-resistant *S. aureus* strain 8 – Gram positive; **ORSa108.** Oxacillin-resistant *S. aureus* strain 108 Gram positive; **Pa13.** *P. aeruginosa* 13 (biofilm producer and resistant to several antibiotics); **PaP1.** *P. aeruginosa* P1 (biofilm producer and resistant to several antibiotics); **Ca.** *Candida. Albicans* (ATCC 10 231) – yeast; **MtH37Rv.** *Mycobacterium tuberculosis* H37Rv. +: microbial growth with no antibiotic activity detected; * not tested due to insufficient amount of material.

In conclusion, the isolation of the known cyclostelletamines A – F (**1** – **6**) from the sponge *Pachychalina* sp. and the synthesis of the new cyclostelletamines G – I, K and L (**7** – **11**) provided enough material for evaluation of antibacterial, antifungal and antimycobacterial activity of these alkaloids. The results obtained indicated that the size of the alkyl chain linker between the two pyridinium aromatic rings seems to be related to the activity level of the cyclostelletamine alkaloids,

suggesting a mechanism-of-action based on the distance of the charged moieties, possibly through interaction with ionic membrane sites. Further pharmacological and biophysical studies are necessary to clarify such hypotheses.

Experimental

General

General experimental procedures for isolation and identification of cyclostellamines have been previously reported [17].

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 10-15 m depth, and immediately immersed in EtOH. The whole material was shipped to the Instituto de Química de São Carlos, Universidade de São Paulo. Voucher specimens are deposited at the Museu Nacional (MNRJ 3098 e 3099).

Extraction and isolation

The sponge *Pachychalina* sp. was processed as previously described [17]. A further amount of the n-BuOH extract was separated exactly as described in our original procedure, to give additional amounts of the the alkyipyridinium alkaloids fractions AmNL1FN5a and AmNL4a [17]. These fractions were purified by HPLC (Inertsil ODS-2, 125 Å, 5 µm, 250 x 9.4 mm; 43:57 MeCN-H₂O + 0.1% trifluoroacetic acid; flow rate: 1 mL/min; λ_{\max} 266 nm) to give 2.5 mg of **1**, 5.4 mg of **2**, 3.4 mg of **3**, 3.6 mg of **4**, 4.1 mg of **5** and 3.0 mg of **6**. Pure compounds were identified by our previously reported HPLC-MS/MS analysis method [17]. Synthetic cyclostellamines G – I, K and L were prepared as recently described [18].

Antibacterial and antifungal assays

Procedures for the antimicrobial assay against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, oxacillin-resistant *S. aureus* strain 8, oxacillin-resistant *S. aureus* strain 108, *P. aeruginosa* strain 13, *P. aeruginosa* strain P1 and *C. albicans* ATCC 10231 were performed using the reference broth microdilution assay, adapted from the National Committee for Clinical Laboratory Standards (USA) and from Pettit et al. [25], as it follows. Isolated colonies from overnight cultures were suspended and diluted as recommended to yield final inocula of approximately 10⁵ CFU/mL. Tests were performed in sterile 96-well microplates by dispensing into each well a total volume of 100 µL (40 µL de medium + 20 µL de inocula + 40 µL substance solution dilutes in water) and incubated 24 h at 37 °C. Microorganism growths were determined by absorbance measurement at 620 nm in an automated microplate reader (Labsystem, Multiskan MS model). The MIC was defined as the lowest concentration of drug that inhibited growth. As control it was used microbial cultures growth without the addition of the tested substance and all samples were assayed in replicate.

Antimycobacterial assay against Mycobacterium tuberculosis H37Rv

The antimycobacterial activity of crude extracts was assayed against *M. tuberculosis* H37Rv ATCC 27294 by the microplate Alamar Blue assay [22]. Mycobacteria were grown on Loweinstein-Jensen medium at 37°C and the concentration adjusted to a bacterial density corresponding to 1.0 McFarland turbidity standard (1×10^7 cell/mL), that was further diluted 1:25 in Middlebrook 7H9 broth medium before the inoculation (4×10^5 mycobacteria/mL). Stock solutions of the crude extracts were diluted in DMSO at concentrations ranging from 1.0 to 80 mg/mL, sterilized by passage through a 0.22 µm PFTE filter (Millex-FG, Millipore) and diluted 1:10 in Middlebrook 7H9 broth. The diluted samples were aliquoted and all the samples were stored at -20°C until necessary. Serial dilutions (of the 1:10 solution) were performed in Middlebrook 7H9 broth medium, in a microplate of 96 wells, to obtain 100 µL of solution in each well. The higher concentration of DMSO was 2.5%. *M. tuberculosis* H37Rv was added to each well containing the samples and the microplate incubated at 37°C in a humidified chamber. Control wells consisting of either *M. tuberculosis* only (MB) or medium only (M), as well as those containing crude extract samples (100 µL) ranging from 0.01 to 2.0 mg/mL, were inoculated with 100 µL of a diluted suspension of *M. tuberculosis* (4×10^5 cell/mL). The microplate was incubated at 37°C for 6 days. Afterwards, 25 µL of a mixture 1:1 (v/v) of 10x Alamar Blue reagent and 10% Tween 80 were added to the wells and the plates were reincubated at 37°C. After 24 hours, a change in color from blue to pink was observed in the wells where the mycobacteria grew. The visual minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that prevented a color change from blue to pink. Rifampicin was used as a reference. Assays were performed in triplicate.

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Samples availability: not available.

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