Bioassay-Guided Isolation and Identification of Desacetylmicrocolin B from *Lyngbya* cf. *polychroa*

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Key words
- cyanobacteria
- Oscillatoriaceae
- *Lyngbya polychroa*
- microcolins
- cytotoxins

Abstract

Bioassay-guided fractionation of a non-polar extract of *Lyngbya* cf. *polychroa* resulted in the isolation of the cytotoxic desacetylmicrocolin B (**1**) as well as the known compounds microcolins A (**2**) and B (**3**). Compound **1** was found to inhibit the growth of HT-29 colorectal adenocarcinoma and IMR-32 neuroblastoma cells with half maximal inhibitory concentration (IC_{50}) values of 14 nM for both cancer cell types. Microcolins A and B were found to have little activity against two strains of the marine fungus *Dendryphiella salina* with LD_{50} values above 200 µg/mL. Compounds **1**, **2**, and **3** were obtained by reverse-phase chromatography and their structures were determined by NMR and MS. In this paper we report the isolation, identification, and biological activity of **1**.

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Introduction

Cyanobacteria have become well-known as a rich source of biologically active natural products, including polyketide macrolides, linear and cyclic peptides, and alkaloids [1]. In particular, cyanobacteria are prolific sources of biologically active peptides with highly modified amino acid residues, including microcystins, nodularin, and microcolins. These compounds show an array of biological activities such as cytotoxicity, hepatotoxicity, neurotoxicity, antibacterial, and antifungal activities [2,3]. This paper describes the isolation, identification and biological activity of the microcolin B analogue desacetylmicrocolin B (**1**) as well as the known natural products microcolins A (**2**) and B (**3**) (**Fig. 1**). Microcolins A and B were originally isolated from a Venezuelan collection of the cyanobacterium *Lyngbya majuscula* [4]. Since their initial isolation, these compounds have been well studied as potent immunosuppressive lipopeptides [5–13]. Here we report the isolation, identification, and biological activity of desacetylmicrocolin B (**1**).

Materials and Methods

General experimental procedures

IR data was obtained on a Perkin Elmer Spectrum 100 FT-IR spectrometer and optical rotations were measured on a Perkin Elmer model 343 polarimeter. ^{1}H- and 2D-NMR spectra for **1** were recorded in $CDCl_3$ on a Bruker Avance II 600 MHz spectrometer equipped with a 1-mm triple-resonance high-temperature superconducting cryogenic probe using residual solvent signals ($\delta_H = 7.24$, $\delta_C = 77.0$ for chloroform). For microcolins A (**2**) and B (**3**), ^{1}H- and 2D-NMR spectra were recorded in $CDCl_3$ on a JEOL-600 operating at 600 MHz using residual solvent signals for reference ($\delta_H = 7.24$, $\delta_C = 77.0$ for chloroform). LC–MS/MS analysis was performed using an Agilent 1100 HPLC system equipped with a Thermo Finnigan LCQ Advantage ion trap mass spectrometer via an ESI (+) interface. HR-MS data were obtained using an Agilent LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector.

Collection

The marine cyanobacterium, *Lyngbya* sp., was collected in August 2005 by hand while scuba diving off the coast of Hollywood, Florida (26°01.1414′N, 80°05.9973′W) at a depth of 7.1 m. This species grew as red, hair-like tufts and was collected in a

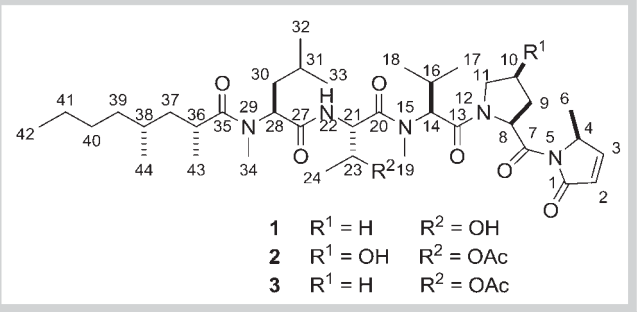

Fig. 1 Structures of compounds **1, 2**, and **3**.

large zip-lock plastic bag filled with seawater and transported back to the Smithsonian Marine Station in a cooler filled with seawater. At the Smithsonian Marine Station the sample was drained of seawater, frozen at −20 °C, and subsequently freeze dried. Voucher specimens (VPLP8_17_05) were preserved in 5% formalin/seawater and are retained at the Smithsonian Marine Station. Identification of the *Lyngbya* sp. was based on microscopic examination. Samples were identified based on morphological characteristics according to Littler and Littler [14]. The specimen most closely resembled *Lyngbya polychroa* (Meneghini) Rabenhorst 1847 with filament widths of 38.6 ± 2.5 µm, cell widths of 29.9 ± 2.3 µm, and cell lengths of 4.2 ± 0.4 µm.

Extraction and isolation

The freeze-dried cyanobacterium (195.1 g dry wt.) was extracted with 1 : 1 MeOH/EtOAc (3 × 1 L, 24 h each). The filtered extract was combined and concentrated under reduced pressure to yield 52.6 g of crude extract. The crude 1 : 1 MeOH/EtOAc extract (6 g) was fractionated by three combined separations on a C18 flash column (54 µm, 2.5 × 10 cm; Varian Mega Bond Elut, 60 mL) using a step gradient of MeOH-H_2O-acetone 20 : 80 : 0, 30 : 70 : 0, 40 : 60 : 0, 50 : 50 : 0, 75 : 25 : 0, 100 : 0 : 0, and 0 : 0 : 100 (200 mL each). The active fractions, 5 and 6 (75 : 25 MeOH/H_2O and 100% MeOH), were separated by bioassay-guided (cytotoxicity) fractionation. Fraction 5 (55 mg) was separated by RP HPLC (Econosil C18; 10 µm, 1 × 25 cm, absorbance 254 nm, flow rate 2.0 mL/min) with a gradient of 50 : 50 MeOH/H_2O to 76 : 24 MeOH/H_2O over 15 min and then from 76 : 24 MeOH/H_2O over 30 min to give nine fractions (5a – i). Fraction 5 g (t_R 30.3 min, 9.8 mg) was shown to be the known compound microcolin A (**2**). Fraction 6 (239.5 mg) was separated by RP HPLC on Econosil C18 column with 9 : 1 MeOH/H_2O to give fourteen fractions (6a – n). Fraction 6b (t_R 17.6 min, 10.8 mg) was shown to be the known compound microcolin A (**2**). Fraction 6 d (t_R 24.2 min, 11.2 mg) was the known compound microcolin B (**3**). Fraction 6c (t_R 19.9 min, 4.8 mg) was further separated by RP HPLC on Econosil C18 column with 85 : 15 MeOH/H_2O to give three fractions (6c1–6c3). Fraction 6c2 was further separated by RP HPLC on Econosil C18 column with 3 : 1 ACN/H_2O to give eleven fractions (6c2a – 6c2 k). Fraction 6c2i (t_R 24.8 min, 1.7 mg) was identified as desacetylmicrocolin B (**1**).

Desacetylmicrocolin B (**1**): Yield: 1.7 mg; Colorless oil; $[\alpha]_D^{20}$: −193 (*c* 0.01, CH_3OH); IR: ν_{max} = 2959, 2090, 1729, 1650 cm^{-1}; positive ESI-MS: *m/z* (rel. int.) = 712 $[M + Na]^+$ (100), 690 $[M + H]^+$ (4), 496 (2), 478 (18), 383 (10), 365 (92), 282 (44), 254 (4), 211 (46), 155 (2), 100 (34); HR-ESI-MS: *m/z* = 712.4614 $[M + Na]^+$ (calcd. for $C_{37}H_{63}N_5O_7Na$: 712.4625); NMR data see **Table 1**.

Conversion of microcolin B to desacetylmicrocolin B

Microcolin B was converted to desacetylmicrocolin B in order to compare the stereochemistry of the isolated desacetylmicrocolin B with that of microcolin B. This was done by reacting 4.1 mg of microcolin B dissolved in 1.8 mL of ethanol with 200 µL of 5% $NaHCO_3$ (in water) for 4 hours with constant stirring. A dilute basic solution was used because degradation of the molecule occurred at higher concentrations of base. The reaction was quenched by adding 2 N HCl dropwise to the solution until it was neutralized. The reaction solution was then dried and the salt removed through liquid-liquid partition between ethyl acetate and water. Finally, the converted product was separated by RP HPLC (Econosil C18; 10 µm, absorbance 254 nm, flow rate 2.0 mL/min) with a gradient of 3 : 1 MeOH/H_2O for 25 min and then 3 : 1 MeOH/H_2O to 100% MeOH over 10 min to give five fractions. Fraction 2 (t_R 27.8 min, 0.5 mg) was identified as the converted product, desacetylmicrocolin B. The optical rotation of the converted product was $[\alpha]_D$: −129 (*c* 0.01, CH_3OH). The ^{1}H-NMR data for the product were identical with those for compound **1** (**Table 1**).

Marine antifungal assay

Microcolins A and B (purity > 95%) were assayed against two strains of the marine fungus *Dendryphiella salina* as previously described [15,16]. Desacetylmicrocolin B was not tested in this assay due to limited amounts of material. This saprophytic fungus has been linked to disease among marine algae and seagrasses [17,18]. Therefore, inhibition of the growth of this fungus may indicate a possible ecological role of the compounds microcolins A and B. The pure compounds were dissolved in methanol and incorporated into YPM media (0.25% yeast, 0.25% peptone, 0.50% mannitol, 1 L seawater, 16 g agar) at a range of concentrations from 6.25 µg/mL to 200 µg/mL. Six hundred microliters of the treated YPM media and controls (agar and 5% methanol) were poured into 24-well plates. *D. salina* was then inoculated into the center of each well, and 12 replicates were run for each concentration. When the growth of the fungus in the control wells covered 100% of the agar, approximately 72 h later, the treated wells were scored as percent of growth relative to the controls. The known antifungal compound, amphotericin B (Sigma; 80% purity), was tested in the same way (except that 3 replicates were tested for each concentration) and was found to give 100% inhibition at concentrations of 3.13 µg/mL or higher.

Cytotoxicity assay

Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) colorimetric assay. Cancer cells (ATCC) were plated in 96-well plates (HT-29, 10 000 cells; IMR-32, 30 000 cells), and after 24 h were treated with various concentrations of compounds **1, 2**, or **3** (purity > 95%) or solvent control (1% EtOH). After 48 h of incubation, cell viability was measured using MTT according to the manufacturer's instructions (Promega). Assays were run in duplicate and dose-response curves were generated using XLfit Excel (ID Business Solutions, Ltd.). Largazole (purity > 95%), which has been shown to inhibit the cell lines HT29 (GI_{50} 12 nM) and IMR-32 (GI_{50} 16 nM), served as a positive control [19].

Supporting information

Original spectra of compound **1** and of the converted product are available as Supporting Information.

Table 1 NMR data for desacetylmicrocolin B in $CDCl_3$ (600 MHz).

Position	δ_H (J in Hz)	δ_C, mult.	HMBC	Position	δ_H (J in Hz)	δ_C, mult.	HMBC
1	–	169.9, qC	–	23	4.07 m	67.2, CH	21, 24
2	6.04 dd (6.2, 1.4)	125.3, CH	1	24	1.03 d (6.2)	14.0, CH_3	–
3	7.21 dd (6.2, 2.0)	153.8, CH	1	25	4.01 m	–	–
4	4.74 m	58.0, CH	2, 3, 6	27	–	171.4, qC	–
5	–	–	–	28	5.23 t (15.8, 7.5)	53.8, CH	27, 30, 31, 34, 35
6	1.43 d (6.2)	17.0, CH_3	–	29	–	–	–
7	–	171.7, qC	–	30a	1.66 m	35.9, CH_2	–
8	5.43 dd (8.1, 4.9)	59.5, CH	7, 9, 10, 11	30b	1.56 m		–
9a	2.37 m	28.7, CH_2	–	31	1.39 m	29.8, CH	–
9b	1.85 m		–	32	0.84 d (6.2)	21.6, CH_3	30
10a	2.04 m	24.6, CH_2	–	33	0.91 d (6.9)	23.0, CH_3	–
10b	1.94 m		–	34	2.93 s	30.4, CH_3	28, 35
11a	3.75 m	47.9, CH_2	–	35	–	178.2, qC	–
11b	3.78 m		–	36	2.81 m	33.6, CH	35, 37
12	–	–	–	37a	1.05 m	41.7, CH_2	–
13	–	169.3, qC	–	37b	1.82 m		–
14	4.99 d (11.0)	59.2, CH	13, 16, 17, 19, 20	38	1.27 m	30.5, CH	–
15	–	–	–	39	1.25 m	35.8, CH_2	–
16	2.25 m	27.0, CH	–	40	1.25 m	28.9, CH_2	–
17	0.78 d (6.9)	18.3, CH_3	–	41a	1.23 m	23.4, CH_2	–
18	0.97 d (6.9)	18.7, CH_3	–	41b	1.39 m		–
19	3.06 s	30.5, CH_3	13, 14, 20	42	0.84 d (6.2)	14.0, CH_3	40, 41
20	–	172.6, qC	–	43	1.09 d (6.9)	18.2, CH_3	36
21	4.72 m	52.0, CH	23	44	0.80 d (6.9)	19.5, CH_3	–
22	6.95 d (9.2)	–	27				

Results and Discussion

Compound **1** was isolated as a colorless oil. The molecular formula of **1**, $C_{37}H_{63}N_5O_7$, was determined from the HR-ESI-MS pseudomolecular ion peak for $[M + Na]^+$, $m/z = 712.4614$. The molecular formula defined by HR-ESI-MS indicated that compound **1** had two fewer carbon atoms than microcolin B (**3**). The IR spectrum for **1** showed the presence of an alkyl group (2959 cm^{-1}), a carbonyl group (1729 cm^{-1}), and a conjugated carbonyl group (1650 cm^{-1}).

Comparison of the ^{1}H-NMR and COSY spectra of compound **1** to those of microcolins A (**2**) and B (**3**), isolated from the same fraction, further indicated that compound **1** was similar in structure to microcolin B. The lack of a singlet for the acetyl methyl group (1.98 ppm) suggested that this group was missing from compound **1**. This was confirmed by ^{1}H-NMR and COSY analysis, which showed the proton signal for the H-β of the threonine had shifted from 5.21 ppm in microcolin B to 4.07 ppm in compound **1** (**Fig. 1**), suggesting the presence of a hydroxy instead of an acyloxy group. The proton signals for the alpha methine and methyl group of the threonine had shifted from 4.92 ppm to 4.72 ppm and from 1.15 ppm to 1.03 ppm, respectively, also indicating the loss of the acetate group. The presence of a broad singlet at 4.01 ppm confirmed that the acetate group attached to the beta carbon of the threonine had been replaced by a hydroxy group in compound **1**. The ESI-MS/MS fragmentation pattern (**Fig. 2**) is also consistent with the proposed structure for **1**. The relative and absolute configuration of compound **1** was established after base hydrolysis of microcolin B (**3**) to desacetylmicrocolin B, followed by direct comparison of NMR data and optical rotations for the hydrolysis product with data for compound **1**. The ^{1}H-NMR data for authentic sample of microcolin B (**3**) and the hydrolysis product were identical and also comparable to those reported by Andrus et. al. [5] for synthetic desacetylmicrocolin B, indicating that the relative configuration was the same. Furthermore, the same sign and also similar magnitude of the optical rotation with reported data for the microcolins [4,5] confirmed that the absolute configuration was also identical.

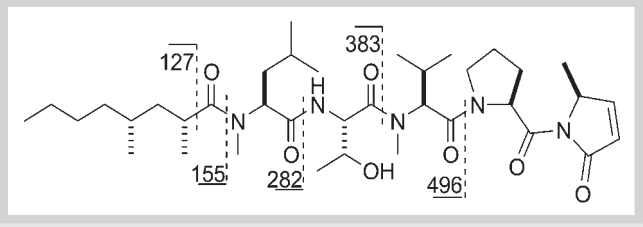

Fig. 2 ESI-MS/MS fragmentation pattern of **1**.

All compounds were tested for their ability to inhibit the growth of two solid tumor cell types: HT-29 colorectal adenocarcinoma and IMR-32 neuroblastoma cells. **Fig. 3** and the results in **Table 2** show that microcolin A had the most potent cytotoxicity in both tests, while microcolin B was less potent, and compound **1** was the least potent of the three molecules, yet still exhibited low-nanomolar activity (IC_{50} 14 nM for each). In a synthetic study done by Mattern et al. [9], the octanoyl derivative of desacetylmicrocolin B was synthesized and tested in antitumor assays against A549 and P388 cell lines. The synthesized compound had ED_{50} values of 5.0 µM in the A549 and 2.9 µM in the P388 assays. Comparison of these values to that of compound **1**, indicates that the methyl groups of the carbon chain are important for the activity of these molecules. Previously published activity results showed both microcolins A and B as potent immunosuppressive agents in a two-way murine mixed lymphocyte reaction with EC_{50} and TC_{50} values of 1.5 nM and 22.6 nM, respectively, for microcolin A and 42.7 nM and 191.0 nM, respectively, for microcolin B [4]. More recently, microcolin B has been shown as a potential

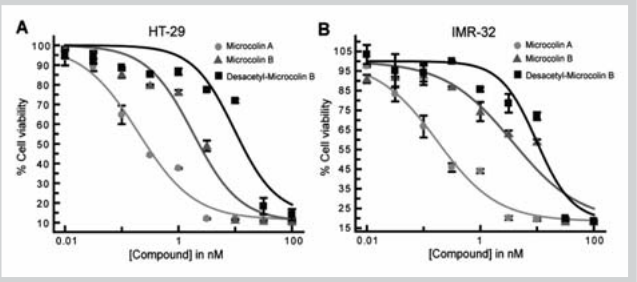

Fig. 3 Effect of microcolins **1–3** on cancer cell viability. **A** HT-29 cells, **B** IMR-32 cells. Graphs show means + 1 SD (n = 2).

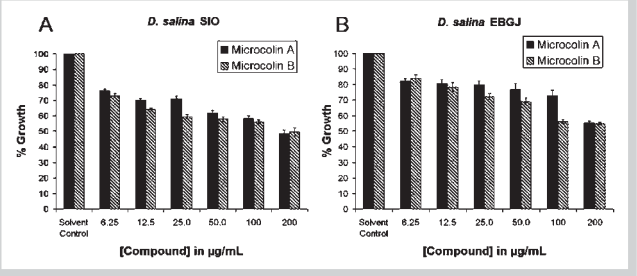

Fig. 4 Effect of microcolins A (**2**) and B (**3**) on growth of two fungal strains of *Dendryphiella salina*. **A** Strain SIO, **B** Strain EBGJ. Bars represent means + 1 SE (n = 12).

Table 2 Cytotoxicity data (IC_{50}) for compounds **1, 2**, and **3**.

	HT-29	IMR-32
Microcolin A (**2**)	0.28 nM	0.31 nM
Microcolin B (**3**)	2.3 nM	7.7 nM
Desacetylmicrocolin B (**1**)	14 nM	14 nM

inhibitor of LFA-1/ICAM-1-mediated cell adhesion with an IC_{50} value of 0.15 µM [11].

Microcolins A and B were tested for their ability to inhibit the growth of two strains of the marine fungus *Dendryphiella salina*. The purpose of this assay was to investigate a possible ecological role for the cytotoxic compounds produced by this cyanobacterium. The results of these tests are shown in **Fig. 4**. For both strains, the LD_{50} values of microcolins A and B are above 200 µg/mL. These values are 10 to 100 times higher than their cytotoxicity values in the cancer cell lines. The antifungal activities of microcolins A and B are significantly lower than that of amphotericin B, a known antifungal compound, which produced 100% inhibition in this assay at concentrations as low as 3.13 µg/mL. These results show that microcolins A and B, although potent immunosuppressive and cytotoxic substances, do not exhibit significant antifungal properties.

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