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MICROCOLINS A AND B, NEW IMMUNOSUPPRESSIVE PEPTIDES FROM THE BLUE-GREEN ALGA *LYNGBYA MAJUSCULA*

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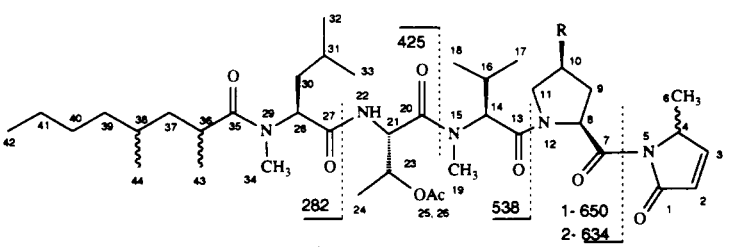
ABSTRACT.—Microcolin A [**1**] and microcolin B [**2**] are new immunosuppressive lipopeptides isolated from a Venezuelan sample of the blue-green alga *Lyngbya majuscula*. The microcolins are potent inhibitors of the murine mixed lymphocyte response and murine P-388 leukemia in vitro. Isolation and structure elucidation of **1** and **2** by nmr, mass spectral, and chemical methods are described.

Marine organisms have emerged as an abundant source of novel peptide secondary metabolites (1). Several of these, such as the didemnins (2–4), dolastatins (5–8), and discodermins (9–11), have been shown to possess striking biological activity, for the most part in the antiviral, antitumor, cytotoxic, and antimicrobial areas. Our ongoing interest in the isolation of marine-derived immunomodulatory agents (12–14) led us to examine a specimen of *Lyngbya majuscula* Gomont (Oscillatoriaceae) [=*Microcoleus lyngbyaceus* (Kützing) Crouan sensu Drouet], collected in Venezuelan waters. We report here the isolation and structure determination of two novel, potent immunosuppressive lipopeptides, microcolins A [**1**] and B [**2**]. The microcolins are related in structure to majusculamide D and deoxymajusculamide D (15), the difference being substitution of an *N*-methylleucine residue in place of the *N,O*-dimethyltyrosine in the corresponding majusculamide D compounds.

RESULTS AND DISCUSSION

Our work on *L. majuscula* began as part of an ongoing program to isolate new biologically active marine natural products. A sample of *L. majuscula* was collected by scuba near La Blanquilla, Venezuela at a depth of 12–27 m. A shipboard crude EtOH extract showed in vitro activity against murine P388 leukemia with an IC_{50} of 0.4 μg/ml and immunosuppressive activity with associated cytotoxicity in the mixed lymphocyte response (MLR) and lymphocyte viability (LCV) assay. The sample was frozen for transport and later extracted with EtOH, followed by partitioning into CH_2Cl_2/MeOH/H_2O. Bioassay-guided purification of the P388 and MLR activities from the organic soluble material by Si gel and reversed-phase hplc furnished microcolin A [**1**] (0.017% dry wt) and microcolin B [**2**] (0.013% dry wt).

Microcolin A [**1**] was obtained from reversed-phase hplc as a clear glass. For reference purposes, the atom numbering system in Figure 1 corresponds to that used previ-



1 R=OH
2 R=H

FIGURE 1. Structures and Mass Spectral Fragmentations for **1** and **2**.

ously for majusculamide D (15). The ^{1}H- and ^{13}C-nmr data for **1**, listed in Table 1, suggest a peptide structure. The ^{1}H spectrum, however, contains only one signal (H-22, δ 6.95) attributable to an amide NH, as determined by D_2O exchange and lack of a carbon correlation in an HMQC experiment, thus indicating a high degree of *N*-substitution. Resonances for two *N*-methyl groups (H-19 δ 3.06; H-34 δ 2.93) and one

TABLE 1. ^{13}C- and ^{1}H-nmr Data for Microcolin A [**1**].[a]

Position	^{13}C	^{1}H, *J* (Hz)
1	169.8 s	—
2	125.3 d	6.05 1H, dd, *J* = 6.1, 1.7
3	154.1 d	7.23 1H, dd, *J* = 6.1, 2.0
4	58.1 d	4.79 1H, qt, *J* = 6.7, 1.7
6	17.2 q	1.45 3H, d, *J* = 6.7
7	174.6 s	—
8	58.6 d	5.62 1H, dd, *J* = 7.7, 2.2
9	36.6 t	2.45 1H, ddd, *J* = 14.5, 10.2, 4.9
		2.00 1H, m
10	71.8 d	4.34 1H, bm
11	56.9 d	3.88 1H, dt, *J* = 11.7, 1.6
		3.79 1H, dd, *J* = 11.7, 4.2
13	168.9 s	—
14	59.2 d	4.98 1H, d, *J* = 11.1
16	27.1 d	2.25 1H, m
17	18.4 q	0.78 3H, d, *J* = 6.6
18	18.9 q	0.95 3H, d, *J* = 6.6
19	30.6 q	3.06 3H, s
20	169.8 s	—
21	51.8 d	4.92 1H, dd, *J* = 8.9, 2.9
22	—	6.99 1H, d, *J* = 8.9
23	68.4 d	5.21 1H, qd, *J* = 6.7, 3.3
24	17.4 q	1.15 3H, d, *J* = 6.7
25	169.7 s	—
26	21.0 q	1.98 3H, s
27	171.3 s	—
28	53.7 d	5.25 1H, dd, *J* = 10.0, 5.8
30	35.8 t	1.70 1H, ddd, *J* = 14.6, 10.0, 4.6
		1.54 1H, ddd, *J* = 14.6, 9.6, 5.8
31	24.8 d	1.42 1H, m
32	21.5 q	0.83 3H, d, *J* = 6.7
33	23.3 q	0.91 3H, d, *J* = 6.7
34	30.3	2.93 3H, s
35	177.9	—
36	33.8 d	2.80 1H, m
37	41.9 t	1.87 1H, ddd, *J* = 13.4, 9.2, 5.0
		1.09 1H, m
38	30.8 d	1.27 1H, m
39	37.1 t	1.25 1H, m
		1.07 1H, m
40	29.1 t	1.25 2H, bm
41	22.9	1.23 2H, m
42	14.1 q	0.85 3H, t, *J* = 6.6
43	18.2 q	0.82 3H, d, *J* = 6.6
44	19.5 q	1.13 3H, d, *J* = 6.6

[a]Solvent $CDCl_3$. ^{1}H 500.13 MHz, chemical shifts in ppm downfield from internal TMS. ^{13}C 125.76 MHz, chemical shifts in ppm as referenced to $CDCl_3$ at δ 77.0. Multiplicities determined by DEPT given in parentheses (s = C, d = CH, t = CH_2, q = Me). ^{1}H-^{13}C connectivities assigned by HMQC.

acetate methyl (H-26 δ 1.98) are also prominent in the ^{1}H-nmr spectrum. This was confirmed by acid hydrolysis and hplc analysis of the phenylthiocarbamyl derivatives, which showed threonine as the only standard free amino acid. Tlc analysis of the underivatized hydrolyzate both by conventional Si gel and chiral ligand exchange (16) (see Experimental) indicated the presence of *cis*-4-hydroxy-L-proline, L-threonine, *N*-methyl-L-valine, and *N*-methyl-L-leucine.

Correlations present in phase-sensitive COSY (17) and 2D HOHAHA (18) spectra, the latter run at mixing times of 55 msec and 72 msec, show cross peak connectivities for the H-α and side chain proton spin systems of 4-hydroxyproline: H-8 (δ 5.62), H-9 (δ 2.45, 2.00), H-10 (δ 4.34), H-11 (δ 3.88, 3.79); *N*-methylvaline: H-14 (δ 4.98), H-16 (δ 2.25), H-17 (δ 0.78), H-18 (δ 0.95); *n*-methylleucine: H-28 (δ 5.25), H-30 (δ 1.70, 1.54), H-31 (δ 1.42), H-32 (δ 0.83), H-33 (δ 0.91); and threonine: H-21 (δ 4.92), H-22 (δ 6.95), H-23 (δ 5.21), H-24 (δ 1.15). Also evident are two cis-coupled olefinic protons adjacent to a carbonyl: H-2 (δ 6.05) and H-3 (δ 7.23) ($J = 6.1$ Hz), with H-3 coupled to a methine quartet of triplets (H-4 δ 4.79) which in turn is coupled to the H-6 methyl (δ 1.45, $J = 6.7$ Hz), and to H-2 via allylic coupling ($J = 1.7$ Hz). Also present in the COSY and HOHAHA spectra are cross peaks which trace out the H-43–H-36–H-37–H-38–H-44 portion of the dimethyloctanoic acid moiety. Protons bound to C-40 and C-41 overlap to form a methylene envelope at 1.24 ppm and cannot be unambiguously assigned by homonuclear correlation methods. The H-42 methyl triplet signal is readily discerned at δ 0.88.

The chemical shift of H-23 (δ 5.24) argues for placement of the acetate on C-23, while correlations from H-19 (δ 3.06) to Hα-34 and H-34 (2.93) to Hα-28 in a long range COSY (19) experiment facilitate assignment of the *N*-methyl groups to the *N*-methylvaline and *N*-methylleucine residues, respectively.

The proton-decoupled ^{13}C-nmr spectrum of **1** contains 39 lines (Table 1). Multiplicities were assigned using a DEPT experiment (20), while ^{13}C-^{1}H connectivities were determined via an HMQC experiment (21). The carbonyl region contains six amide resonances, three of which partially overlap at 169.8–169.9 ppm when $CDCl_3$ is used as solvent. All six signals are readily dispersed in Me_2CO-d_6 (data not shown). We utilized HMBC (22) experiments (see Table 3 and Experimental) to provide two- and three-bond long range correlations to the amide carbonyls from flanking *N*-methyl, H-α, or NH protons on each side, thus allowing the sequence of subunits to be determined. These results are summarized as follows. Correlations from H-28, H-34, H-43, H-36, and H-37 to C-35 identify the *N*-methylleucine as the *N*-terminal amino acid that bears the dimethyloctanoic acid amide. Moving toward the carboxy terminus, the next residue must be *O*-acetylthreonine, due to correlations from C-27 to H-28, H-21, and H-22, followed by *N*-methylvaline since C-20 shows correlations to H-21, H-19, and H-14. Long range correlations from C-13 to H-14 establish C-13 as the adjacent carbonyl, and a nuclear Overhauser enhancement (3.1%) between H-14 and the C-11 protons, observed in a one-dimensional difference experiment, place the 4-hydroxyproline in the next position. Chemical shift assignment of C-7 is made on the basis of HMBC correlations to H-8 and H-9. The structure of the 5-methyl-3-pyrrolin-2-one system is established by HMBC correlations from H-2 and H-3 to C-1, and by chemical shift arguments for C-1 and C-4, when compared with values reported for 3-pyrrolin-2-one (23). The C-7 carbonyl remains as the only site for attachment of N-5 of the 5-methyl-3-pyrrolin-2-one unit, thus completing the overall connectivity.

Due to the limited quantities of pure **1** and **2** isolated, we were unable to determine by chemical degradation the absolute stereochemistry at C-4. Our attempt at ozonolysis of a small sample (4 mg) of **1** and subsequent acid hydrolysis, following the procedure of Entzeroth and Moore (15), failed to produce sufficient quantities of alanine

TABLE 2. ^{13}C and ^{1}H Nmr Data for Microcolin B [2].[a]

Position	^{13}C	^{1}H, J (Hz)
1	169.8 s	—
2	125.5 d	6.05 1H, dd, J = 6.0, 1.6
3	153.7 d	7.22 1H, dd, J = 6.0, 2.0
4	58.0 d	4.76 1H, qt, J = 6.8, 1.8
6	17.2 q	1.46 3H, d, J = 6.8
7	172.0 s	—
8	60.0 d	5.47 1H, dd, J = 8.6, 5.3
9	28.9 t	2.43 1H, ddd, J = 12.9, 11.5, 7.1
		1.86 1H, m
10	24.6 t	2.00 2H, bm
11	48.0 d	3.80 1H, ddd, J = 16.5, 10.2, 6.5
		3.70 1H, ddd, J = 16.5, 10.2, 6.5
13	168.3 s	—
14	59.3 d	5.04 1H, dd, J = 11.1
16	27.3 d	2.26 1H, m
17	18.4 q	0.81 3H, d, J = 6.6
18	18.9 q	0.94 3H, d, J = 6.8
19	30.5 q	3.11 3H, s
20	169.7 s	—
21	52.0 d	4.97 1H, dd, J = 8.8, 3.4
22	—	6.95 1H, d, J = 8.4
23	68.8 d	5.24 1H, qd, J = 6.5, 3.7
24	17.2 q	1.16 3H, d, J = 6.3
25	169.7 s	—
26	21.0 q	1.98 3H, s
27	171.3 s	—
28	53.8 d	5.26 1H, dd, J = 10.4, 5.7
30	36.0 t	1.71 1H, ddd, J = 14.6, 10.0, 4.6
		1.59 1H, ddd, J = 14.6, 9.6, 5.8
31	24.9 d	1.41 1H, m
32	21.5 q	0.87 3H, d, J = 6.6
33	23.3 q	0.94 3H, d, J = 6.8
34	30.4	2.95 3H, s
35	177.9	—
36	33.8 d	2.84 1H, m
37	41.9 t	1.87 1H, m
		1.09 1H, m
38	30.8 d	1.27 1H, m
39	37.1 t	1.26 1H, m
		1.18 1H, m
40	29.1 t	1.25 2H, bm
41	22.9	1.25 2H, m
42	14.1 q	0.88 3H, t, J = 6.8
43	18.2 q	0.84 3H, d, J = 6.8
44	19.6 q	1.12 3H, d, J = 6.6

[a]Solvent $CDCl_3$. ^{1}H 500.13 MHz, chemical shifts in ppm downfield from internal TMS. ^{13}C 125.76 MHz, chemical shifts in ppm as referenced to $CDCl_3$ at δ 77.0. Multiplicities determined by DEPT given in parentheses (s = C, d = CH, t = CH_2, q = Me). ^{1}H-^{13}C connectivities assigned by HMQC.

for unambiguous chirality determination. Comparison of ^{13}C- and ^{1}H-nmr chemical shifts with those of the corresponding majusculamide D compounds seems to suggest that the C-4 stereochemistry may also be *S* in the microcolins.

Remaining ambiguities in the chemical shift assignment of the 2,4-dimethyloctanoic acid amide moiety, caused by overlap of C-40 and C-41 proton signals in the

COSY and HOHAHA spectra, are resolved by HMBC correlations (Table 3) from H-42 to C-41 and C-40, H-44 to C-39, C-38, and C-37, and H-43 to C-37, C-36, and C-35.

The eims of **1** does not show a molecular ion, the highest mass observed being *m/z* 650. The highest measurable mass observed is 538.3843, for $C_{29}H_{52}O_6N_3$ (Δ 0 mmu), resulting from loss of the hydroxyproline-pyrrolinone unit (C-13–N-12 cleavage). The major ions observed and the corresponding fragmentations for **1** are shown in Figure 1.

Microcolin B [**2**] was eluted after **1** on reversed-phase hplc and gave a clear glass on evaporation of solvent. The hreims shows a molecular ion *m/z* 731.4817, indicating a

TABLE 3. HMBC Multiple Bond Correlations for Compounds **1** and **2**.[a]

Proton	^{13}C Correlation	
	1	**2**
H-2	C-1, C-3, C-4	C-1, C-3 C-4
H-3	C-1, C-2, C-4	C-1, C-2, C-4
H-4	C-2, C-3, C-6	C-2, C-3, C-6
H-6	C-3, C-4	C-3, C-4
H-8	7, C-9, C-10	C-7, C-9, C-10
H-9	C-7, C-8	C-7, C-10, C-11
H-10[b]	—	C-8, C-9, C-11
H-11	C-9, C-10	C-9, C-10
H-14	C-13, C-16, C-17	C-13, C-16, C-17
	C-18, C-19, C-20	C-18, C-19, C-20
H-16	C-14, C-17, C-18	C-17, C-18
H-17	C-14, C-16, C-18	C-14, C-16, C-18
H-18	C-14, C-16, C-17	C-14, C-16, C-17
H-19	C-20, C-14	C-20, C-14
H-21	C-20, C-27	C-20, C-27
H-22	C-27	C-27
H-23	C-20, C-25	C-20, C-25
H-24	C-21, C-23	C-21, C-23
H-26	C-25	C-25
H-28	C-27, C-30, C-31	C-27, C-30, C-31
	C-34, C-35	C-34, C-35
H-30	C-27, C-28, C-31	C-27, C-28, C-31
	C-32, C-33	C-32, C-33
H-31[b]	—	—
H-32	C-30, C-31, C-33	C-30, C-31, C-33
H-33	C-30, C-31, C-32	C-30, C-31, C-33
H-34	C-35, C-28	C-35, C-28
H-36	C-43, C-35	C-43, C-35
H-37	C-36, C-38, C-39	C-36, C-38, C-39
	C-43, C-44	C-43, C-44
H-38	C-40	C-40
H-39	C-40, C-43	C-40
H-40[b]	—	—
H-41[b]	—	—
H-42	C-41, C-40	C-41, C-40
H-43	C-36, C-37, C-35	C-36, C-37, C-35
H-44	C-37, C-38, C-39	C-37, C-38, C-39

[a]Spectra measured in $CDCl_3$. Correlations shown are composite of results of two experiments run with delay values of 50 msec and 70 msec for evolution of double quantum coherence.

[b]Correlations not observed or unassignable due to overlap of ^{1}H signals.

molecular formula of $C_{39}H_{65}O_8N_5$ (Δ 2.0 mmu). The ion at *m/z* 634 (see Experimental) and the match of lower mass fragments to those for **1**, suggest the presence of proline in **2** in place of the hydroxyproline in **1**. Acid hydrolysis, followed by hplc analysis of the free amino acid phenylthiocarbamate derivatives and tlc of the underivatized amino acids, showed that **2** contained threonine, proline, *N*-methylvaline, and *N*-methylleucine.

The ^{13}C-, ^{1}H-, and HMBC nmr data for **2**, listed in Tables 2 and 3, match very closely those for **1** except for the expected chemical shift and multiplicity differences for the proline ring. All assignments for **2** were confirmed by DEPT, COSY, HOHAHA, HMQC, and HMBC experiments.

The microcolins show potent immunosuppressive activity. Both microcolin A [**1**] and microcolin B [**2**] suppress the two-way murine mixed lymphocyte reaction, with EC_{50} (effective concentration) and associated TC_{50} (toxic concentration) values of 1.5 nM and 22.6 nM respectively for **1** and EC_{50} and TC_{50} values of 42.7 nM and 191.0 nM, respectively, for **2**. Results of detailed biological investigations will be reported elsewhere.

EXPERIMENTAL

GENERAL EXPERIMENTAL METHODS.—Nmr spectra were measured at 30° on either a Bruker AMX-500 at 500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C, or a Bruker AM-360 at 360.13 MHz for ^{1}H and 90.56 MHz for ^{13}C. Chemical shifts for ^{1}H are reported as ppm downfield from internal TMS. ^{13}C shifts are referenced to solvent signal at 77.0 ppm for $CDCl_3$. COSY and 2D HOHAHA spectra were measured and processed in phase-sensitive mode using time proportional phase incrementation (tppi). Mixing times for HOHAHA spectra were 55 msec and 72 msec. Phase sensitive (tppi) HMQC spectra were measured using a preceding bilinear (bird) pulse (21). Separate experiments were run with and without garp1 ^{13}C decoupling (24) during acquisition. HMBC spectra were measured using mixing times of 50 msec and 70 msec for evolution of double quantum coherence. All 2D and nOe difference spectra were run non-spinning. Amino acid standards were purchased from Sigma or Bachem. Chiralplates (Macherey-Nagel) were purchased from Alltech Associates.

COLLECTION OF ORGANISM AND ISOLATION OF **1** AND **2**.—The sample of *L. majuscula* (Harbor Branch Oceanographic Institution, Division of Biomedical Marine Research sample #3-VI-88-4-101) (25) was collected by scuba at 12–27 m on a fore reef slope, Pta. Bobos, La Blanquilla, Venezuela in June 1988. A taxonomic voucher specimen is on deposit at the Harbor Branch Oceanographic Museum Herbarium, Ft. Pierce, Florida, accession number 6205. The sample (297 g wet wt) was immediately frozen and later lyophilized. A portion of the dry organism (60.9 g) was homogenized in 100% EtOH, allowed to stand for 30 min, and filtered, and the remaining solids were reextracted. The two filtrates were combined and evaporated under reduced pressure to give 3.5 g of crude material. Partitioning of the crude extract between CH_2Cl_2 and MeOH-H_2O (3:2) gave 620 mg and 2.7 g of nonpolar and polar fractions, respectively, with the bioactivity concentrated in the CH_2Cl_2 layer. The nonpolar material was fractionated by vlc on a column of 30 g Si gel by elution with a stepwise gradient of EtOAc/hexane (10% steps from 100% hexane to 100% EtOAc). Fractions showing bioactivity were chromatographed on a column of 5 g C_{18} Si gel (Amicon) in 75% MeCN/H_2O to remove residual pigments and were subsequently purified by reversed-phase hplc (C_{18} Vydac, 10μ, 70–75% MeCN/H_2O) to give 10.6 mg of microcolin A [**1**] and 6.3 mg microcolin B [**2**].

Microcolin A [**1**].—Clear glass: $[\alpha]^{25}D$ −145.3° (*c* = 0.0026, EtOH); uv λ max (EtOH) 205 nm (ϵ 31300), 233 nm (sh); ir (KBr) 3200–3700 (br), 2935, 2360, 1740, 1640, 1240 cm^{-1}; hreims (70 eV) 538.3843, calcd for $C_{29}H_{52}O_6N_3$ $[M-C_{10}H_{13}O_3N_2]^+$, 0.0 mmu error; lreims (70 eV) *m/z* (rel. int.) 650 (0.8), 538 (7.1), 478 (9.2), 425 (14.7), 282 (46.7), 254 (29.9), 100 (100); ^{1}H and ^{13}C nmr see Table 1.

Microcolin B [**2**].—Clear glass: $[\alpha]^{25}D$ −174° (*c* = 0.005, EtOH); uv λ max (EtOH) 205 nm (ϵ 34000), 234 (sh); ir (KBr) 2935, 2335, 1740, 1639, 1240 cm^{-1}; hreims (70 eV) 731.4817, calcd for $C_{39}H_{65}O_8N_5$, $[M]^+$, 2.4 mmu error; lreims (70 eV) *m/z* (rel. int.) 731 (1.8), 671 (1.1), 634 (2.2), 538 (12.1), 510 (2.2), 478 (21.4), 425 (39.7), 282 (69.2), 100 (100); ^{1}H and ^{13}C nmr see Table 2.

DETERMINATION OF AMINO ACID STEREOCHEMISTRY.—Microcolin A [**1**] (0.5 mg) was hydrolized in 6 N HCl (100°, 24 h), and the hydrolyzate was diluted with H_2O (1.0 ml) and lyophilized. Analysis by tlc against authentic standards [Si gel, *n*-BuOH–HOAc–H_2O (4:1:1), ninhydrin] showed the presence of *N*-methylvaline, *cis*-4-hydroxyproline, *N*-methylleucine, and threonine. The hydrolyzate was

separated by hplc (C_{18} Vydac, 0.1% TFA/H_2O). Chiral tlc analysis [Chiralplates, Macherey-Nagel, Me_2CO-MeOH-H_2O (5:1:1) or MeCN-MeOH-H_2O (4:1:1)] against authentic D and L standards (Bachem) showed all amino acids to have the L configuration.

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