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# Analogues of the Marine Immunosuppresant Microcolin A: Preparation and Biological Activity

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A series of analogs of the immunosuppressive lipopeptide microcolin A has been prepared and evaluated for in vitro activity in the human and murine two-way mixed lymphocyte reaction. The compounds tested were obtained by semisynthetic modification and chemical degradation of the natural product. The relative potencies of these analogs suggest that the hydroxyproline and 5-methyl-3-pyrrolin-2-one portion of the molecule are important for immunosuppressive activity and that other structural elements may play an ancillary role. Methanolysis of microcolin A also led to a novel immunosuppressive lactone analog.

## Introduction

Therapeutic agents which selectively exert their effects on the immune response have become increasingly important tools in the prevention of organ transplant rejection and in the treatment of autoimmune associated diseases.[1-3] Natural products have been at the forefront of these advances, as witnessed by the clinical success of the fungal-derived immunosuppressive cyclic peptide cyclosporine in organ and bone marrow transplantation.[4] Today the more recently discovered natural products, FK506[5,6] and rapamycin,[7,8] hold promise as effective new agents for liver and kidney transplantation.[9] The molecular mechanisms of cyclosporine A and FK506 in the signal transduction pathways of T-lymphocytes have been elegantly elucidated and are the subject of recent reviews.[10,11] Despite the promise held out by these and other agents, there remains an acute need for additional potent and less-toxic immunosuppressive agents.

Recently, we described the isolation and structure elucidation of a novel immunosuppressive lipopeptide, microcolin A (**1**) (Chart 1), from the marine cyanobacterium *Lyngbya majuscula*.[12] Microcolin A and its natural 10-deoxy analog microcolin B (**2**) are potent inhibitors of the murine two-way mixed lymphocyte response (MLR) in vitro, with $EC_{50}$ (effective concentration) and $TC_{50}$ (toxic concentration) values of 1.5 and 22.6 nM, respectively, for **1**, and 42.7 and 191.0 nM, respectively, for **2**.[13] These molecules posses additional biological activities as well.[14] As part of an ongoing program to discover novel immunosuppressants from marine sources, we set about to investigate the relative importance of various structural elements of the microcolin A molecule in relation to the immunosuppressive activity. We report here the preparation and in vitro immunosuppressive activities of a series of microcolin A analogs in the murine and human two-way mixed lymphocyte reaction. The compounds tested were obtained by semisynthetic modification and/or degradation of the natural product.

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**Table 1.** Immunosuppressive Activities of Microcolin Analogs in the Mixed Lymphocyte Reaction

| compound | $EC_{50}$ (nM), murine[a] | $EC_{50}$ (nM), human[a] |
|---|---|---|
| cyclosporin A | 17.8 | 23.0 |
| **1** | 0.02 | 0.02 |
| **2** | 20.5 | 4.1 |
| **3** | 19.0 | 10.2 |
| **4** | 5.4 | 5.4 |
| **5** | >668. | >668. |
| **6** | >668. | 341. |
| **7** | 0.006 | 0.04 |
| **8** | >781. | >781. |
| **9** | 380. | 380. |
| **10** | >2400. | >2400. |
| **11** | 10.0 | 8.1 |

[a] Inhibition of two-way mixed lymphocyte response, $n = 3$, as described in Experimental Section. The $EC_{50}$ value denotes that concentration of test compound which results in a 50% inhibition of murine or human splenocyte proliferation, as measured by [$^3$H]thymidine incorporation.

## Chemistry

Our efforts were initiated by consideration of the relative in vitro immunosuppressive potencies of **1** and **2**, which differ by a factor of approximately 1000 in the mouse and 200 in the human MLR (see Table 1). The limited amounts of pure natural product on hand prompted us to begin by preparing a few simple derivatives (Chart 1) to test the role of oxygen functionality on the proline ring. Microcolin A (**1**) was acetylated by treatment with acetic anhydride in pyridine at room temperature to give the corresponding C-10 acetate (**3**) in good yield.

Jones oxidation of **1** at 0 °C in acetone gave the corresponding 10-ketomicrocolin A (**4**) in 62% yield, but attempts to stereoselectively reduce **4** to the corresponding *trans*-hydroxyprolyl analog were unsuccessful. Treatment of **4** with mild reducing agents under a variety of conditions gave either **1** or a mixture of **1** and 2,3-dihydromicrocolin A (**6**).

The relative importance of the pyrrolinone double bond in **1** and **2** was investigated by the preparation of the corresponding 2,3-dihydro derivatives **5** and **6**. Catalytic hydrogenation of the olefin in both **1** and **2**

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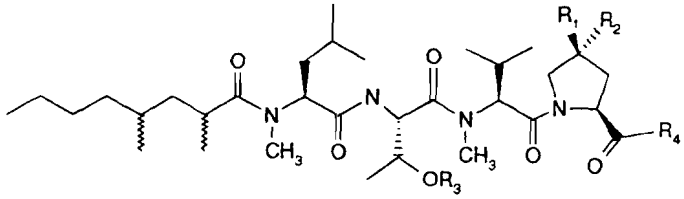
**Chart 1**



**1** $R_1$= OH, $R_2$= H, $R_3$= Ac, $R_4$= **A**

**2** $R_1$, $R_2$= H, $R_3$= Ac, $R_4$= **A**

**3** $R_1$= OAc, $R_2$= H, $R_3$= Ac, $R_4$= **A**

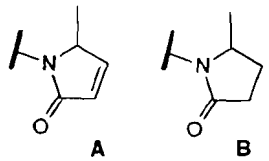
**4** $R_1$,$R_2$= [=O], $R_3$= Ac, $R_4$= **A**

**5** $R_1$= OH, $R_2$= H, $R_3$= Ac, $R_4$= **B**

**6** $R_1$, $R_2$= H, $R_3$= Ac, $R_4$= **B**

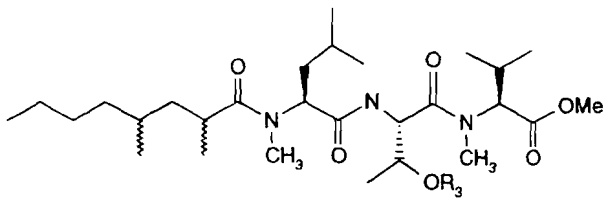
**7** $R_1$= OH, $R_2$= H, $R_3$= OH, $R_4$= **A**

**8** $R_1$= OH, $R_2$= H, $R_3$= OH, $R_4$= $OCH_3$

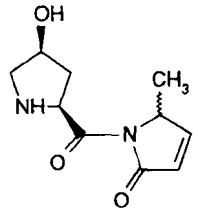


**A** **B**

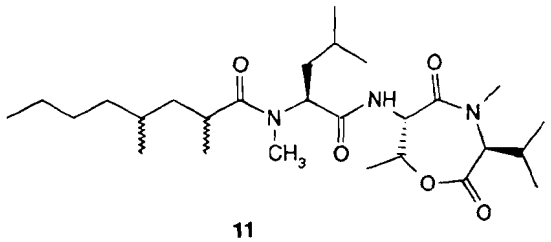
**Chart 2**



**9**



**10**



**11**

gave 2,3-dihydromicrocolin A (**5**) and 2,3-dihydromicrocolin B (**6**) in 63% and 85% yield, respectively.

Partial solvolysis of **1** in refluxing anhydrous methanol furnished five major products, which were separated by HPLC. These are shown in Charts 1 and 2; 23-desacetylmicrocolin A (**7**), 2,4-dimethyloctanoyl-NMeLeu-Thr-NMeVal-4 Hyp methyl ester (**8**), 2,4-dimethyloctanoyl-NMeLeu-Thr-NMeVal methyl ester (**9**), 4-Hyp-N-5-methyl-3-pyrrolin-2-one (**10**), and lactone **11**. Compounds **7**, **8**, and **9** were readily identified from their $^1H$ and $^{13}C$ NMR spectra and mass spectra, (see the Experimental Section). The $^1H$ NMR spectrum of **7** (0.6 mg) was virtually identical to that of **1** except for the absence of the threonine acetate signal at 1.98 ppm and the expected upfield shift of the threonine α and β proton resonances to δ 4.70 and 4.05 ppm, respectively. Compounds **8** (0.3 mg) and **9** (0.9 mg), the products of successive C terminal cleavage, were isolated by reversed-phase HPLC of the ethyl acetate soluble fraction of the methanolysis mixture and were identified by NMR and mass spectra (see the Experimental Section). The $^1H$ NMR spectra of both compounds lacked signals for the pyrrolinone group, while each contained unaltered signals for the 2,4-dimethyloctanoyl moiety, indicating the N terminus had been retained. Additional COSY experiments showed sequential proton spin systems for N-MeLeu, Thr, N-MeVal, and 4-Hyp in **8** along with the ester methyl resonance at δ 3.77 ppm. Correspondingly, the Hyp α proton in **8** showed the expected upfield shift to δ 4.46 ppm. The $^1H$ NMR spectrum of **9**, in addition to signals for the N-terminal 2,4-dimethyloctanoate, contained signals for successive N-MeLeu, Thr, and N-MeVal residues, all assigned via COSY experiment, with the αH signal of the MeVal shifted upfield to δ 4.71 ppm. Compound **10** was isolated by reversed-phase HPLC (see the Experimental Section) and its structure

established by $^1$H NMR 1D and COSY spectra, which contained signals and cross peak networks for only the 5-methylpyrrolin-2-one unit ($\delta$ 7.62, H-3; $\delta$ 6.19, H-2; $\delta$ 4.80, H-4; $\delta$ 1.48, H-5 Me) and 4-Hyp ($\delta$ 5.29, H$\alpha$: $\delta$ 4.63, H$\gamma$, $\delta$ 3.55, 3.46, H$\delta$; $\delta$ 2.79, 2.11 H$\beta$).

Lactone **11** was isolated by reversed-phased HPLC of the ethyl acetate soluble portion of the methanolysis mixture. The HRFABMS spectrum indicated a molecular formula of $C_{27}H_{49}O_5N_3$, due to loss by methanolysis of a $C_{12}H_{16}O_4N_2$ unit. The $^1$H 1D, COSY, and HOHAHA[15] spectra of **11** showed cross peak connectivities for the H$\alpha$ and side chain spin systems of N-MeLeu, N-MeVal, Thr, and 2,4-dimethyloctanoate moiety (see the Experimental Section). The upfield chemical shift of the threonine $\beta$ proton, together with correlations in HMBC[16] and NOESY[17] experiments, clearly showed the compound had lactonized via the N-MeVal carboxyl group.

## Results

**In Vitro Immunosuppressive Activity of Analogs.** Listed in Table 1 are the $EC_{50}$ values for microcolin analogues **1**−**11**, indicating their ability to inhibit the murine and human mixed lymphocyte reaction in vitro. The relative potencies of each compound in the mouse and human systems are approximately equal, with the exception of desacetylmicrocolin A (**7**), which is roughly 7 times more potent in mouse than in human.

Our first modifications of microcolin A (**1**) were prompted by the observation that the natural deoxy analog microcolin B (**2**) was roughly 3 orders of magnitude less potent than **1** in the mouse MLR. The significance of the hydroxyl functionality on the proline ring was substantiated by the reduced immunosuppressive potency in the MLR of acetate **3** and ketone **4**. We found **3** to be less potent than **1** by factors of 1000 in the murine system and 500 in the human. Correspondingly, ketone **4** was 270 times less potent than **1** in both systems. These data suggest that a free hydroxyl, as opposed to general oxygen functionality, is important for immunosuppressive activity.

A striking loss of activity occurs upon reduction of the pyrrolinone ring. The $EC_{50}$ value for dihydromicrocolin A (**5**) is greater than 0.67 $\mu$M in both murine and human MLR, as opposed to 0.02 nM for **1**, representing a loss in potency greater than $3 \times 10^4$. Similar losses in potency were measured for dihydromicrocolin B (**6**), suggesting that the pyrrolinone function is an important structural element for immunosuppressive activity. Due to the limited amounts of some analogs of low potency, it was not possible to precisely determine $EC_{50}$ values. For these compounds we list the highest dose tested, which represents an accurate *lower limit* to the $EC_{50}$ value.

Solvolysis of **1** provided several degradation products, shown in Charts 1 and 2. Desacetylmicrocolin A (**7**) is 3 times more potent than **1** in the mouse system and half as potent in the human system. Elimination of the pyrrolinone unit strongly attenuates activity, as shown by the degradation products **8** and **9**. This is consistent with the greatly diminished potencies of dihydro derivatives **4** and **5** and substantiates the role of the pyrrolinone double bond. Compound **10** incorporates both essential molecular elements implicated by the activities of analogs **1**−**9**, yet lacks immunosuppressive activity. This suggests an ancillary role for portions of the molecule which are distant from the hyroxyproline and pyrrolinone.

Lactone **11** shows relatively potent activity in both the murine and human MLR, with $EC_{50}$ values of 10.0 and 8.1 nM, respectively. This result does not correlate with the trends observed in linear peptide series **1**−**10**, given that **11** is essentially a cyclized analog of **9** and lacks the hydroxyproline and pyrrolinone units. Whether **11** should be considered a member of this series remains the subject of further investigation. Results of studies on the mechanism of action of microcolin A and derivatives will be reported elsewhere.[18]

## Summary

The marine natural product, microcolin A, is a potent immune suppressive agent in-vitro in the murine and human mixed lymphocyte reaction. From the results of semisynthetic modification and chemical degradation studies, we have identified structural elements in microcolin A needed for immunosuppressive activity. Modification of the pyrrolinone or hydroxyproline portions of the molecule result in diminished potency, indicating that these play a major role in the biological activity. The large loss in potency upon reduction of the pyrrolinone double bond is noteworthy, as we are unaware of any literature reports of immunosuppressive agents which bear this functionality. A simple degradation product incorporating these elements alone is inactive, suggesting that other portions of the lipopeptide, the hydrophobic fatty acyl chain for example, are important. In addition, a lactonized derivative of microcolin A has been identified which lacks the hydroxyproline and pyrrolinone portions but yet has potent MLR activity.

## Experimental Section

NMR spectra were measured at 300 K on either a Bruker AMX-500 at 500.13 MHz for $^1$H and 125.76 MHz for $^{13}$C or a Bruker AM360 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 chemical shifts are referenced to solvent signal at 77.0 ppm for $CDCl_3$ and 49.0 ppm for $CD_3OD$. COSY, 2D HOHAHA, and 2D NOESY experiments were measured and processed in phase-sensitive mode using time proportional phase incrementation (TPPI). HMBC spectra were measured with double quantum evolution intervals of 50 and 70 ms. HMQC spectra were measured with $^{13}$C GARP decoupling. All 2D spectra were recorded nonspinning. All samples were analyzed by positive ion liquid secondary ion mass spectrometry (LSIMS) mode using a Finnigan MAT95Q. The matrix used for all samples was 3-nitrobenzyl alcohol with a primary $Cs^+$ beam energy of 15 keV. IR spectra were obtained on an M Series FTIR (MIDAC Corp.). Optical rotations were recorded on a DIP-360 polarimeter (Japan Spectroscopic Co., Ltd. (JASCO)). Thin-layer chromatography was performed on silica gel (plastic-backed) plates precoated with Kieselgel 60 $F_{254}$ (E. Merck). Silica gel vacuum liquid chromatography (VLC) was performed on Kieselgel 60 H for thin-layer chromatography (E. Merck). Silica gel chromatography was also performed on prepacked (3 mL) silica gel columns, Supelclean LC-Si (SUPELCO, Inc.). Centrifugal countercurrent chromatography (CCC) was performed on an Ito Planetary Countercurrent chromatograph. HPLC was performed using refractive index detection, flow rates of 1.5 mL/min, and in either normal phase or reversed-phase mode (5-$\mu$m particles, 4.3 × 250 mm, Hibar LiChrosorb Si 60 (no. 50388) (E. Merck) or Vydac protein and peptide C-18 (no. 218TP54) (The Separations Group), respectively). HPLC and reaction extraction solvents were high purity HPLC grade. Solvents used for reactions were dried

over 3A molecular sieves or purchased as anhydrous reagents in sealed containers (Aldrich Chemical Co., Inc.).

**Isolation.** Lyophillized *Lyngbya* (169 g) was homogenized with 1.5 L of 100% EtOH. The homogenate was allowed to stand for 30 min and then filtered. The residue was reextracted with 1.0 L of EtOH, and the extracts were combined and evaporated under reduced pressure to bive 15.2 g of crude material. The crude extract was partitioned into $MeCl_2$/MeOH/$H_2O$ (3:2:3), and the organic layer was evaporated to give 1.9 g of crude material, 1.4 g of which was subjected to centrifugal countercurrent chromatography using a solvent system of heptane/EtOAc/MeOH/$H_2O$ (5:7:4:3) with the upper phase as mobile. The CCC column (383 mL total volume) was spun at 800 rpm under a flow rate of 5.0 mL/min, and fractions of 20 mL were collected and monitored by TLC. Fractions 15 and 16 were found to contain essentially pure **2** while fractions 20–26 contained **1** and a small amount of polar pigment material. The CCC-purified fraction of **1** (greater than 98% pure by NMR) was used directly for chemical degradation studies. Further purification of the CCC fraction of **1** by C-18 reversed-phase HPLC ($CH_3CN/H_2O$, 75:25) was performed on samples used for biological evaluation. In total, 86.5 mg of **1** (0.05%) and 46.7 mg of **2** (0.028%) were obtained.

**Microcolin A 10-Acetate (3).** A sample of of **1** (3.0 mg) was dissolved in 0.4 mL of 25% acetic anhydride/pyridine. The reaction mixture was allowed to stand at room temperature under nitrogen for 6 h and then placed into a freezer overnight. The solvent was removed under reduced pressure to give 2.6 mg of 95% (by $^1H$ NMR) pure **3**: HRLSIMS ($MH^+$) calcd. for $C_{41}H_{68}O_{10}N_5$ 790.497, found 790.497, Δ 0 mmu; $^1H$ NMR (500 MHz, $CDCl_3$) δ 7.24 (dd, 1H, *J* = 5.8, 2.1, H-3), 6.97 (d, 1H, *J* = 8.4, N*H*), 6.06 (dd, 1H, *J* = 6.3, 1.6, H-2), 5.51 (dd, 1H, *J* = 9.0, 4.7, αHyp), 5.29 (dd, 1H, *J* = 10.5, 4.7, αMeLeu), 5.25 (dd, 1H, *J* = 6.8, 2.6, βThr), 4.96 (d, 1H, = 11.0, αMeVal), 4.94 (dd, 1H, *J* = 8.9, 2.6, αThr), 4.75 (qt, 1H, 6.8, 1.6, H-4), 4.23 (dd, 1H, *J* = 11.6, 6.3, γHyp), 3.72 (dd, 1H, *J* = 11.0, 7.3, δHyp), 3.70 (dd, 1H, *J* = 11.0, 4.2, δHyp), 3.10 (s, 3H, N*Me*Val), 2.96 (s, 3H, N*Me*Leu), 2.84 (m, 1H, H-36), 2.83 (m, 1H, βHyp), 2.26 (m, 1H, βNMeVal), 2.02 (s, 3H, 10-OAc), 2.01 (s, 3H, 25-OAc), 1.98 (m, 1H, βHyp), 1.87 (ddd, 1H, *J* = 13.4, 9.0, 4.7, H-37), 1.73 (ddd, 1H, *J* = 14.6, 10.0, 4.6, βNMeLeu), 1.54 (m, 1H, H-30), 1.48 (d, 3H, *J* = 6.8, H-6), 1.42 (m, 1H, H-31), 1.27 (bm, 2H, H-38), 1.25 (bm, 4H, H-39, H-40), 1.23 (bm, 2H, H-41), 1.16 (d, 3H, *J* = 6.8, γThr), 1.14 (d, 3H, *J* = 6.8, H-44), 1.10 (m, 1H, H-37), 1.07 (m, 1H, H-39), 1.02 (d, 3H, *J* = 6.8, δNMeLeu), 0.95 (d, 3H, *J* = 6.8, γNMeVal), 0.88 (t, 3H, *J* = 6.8, H-42), 0.87 (d, 3H, *J* = 6.8, H-32), 0.84 (d, 3H, *J* = 6.8, H-43, 0.81 (d, 3H, *J* = 6.8, γNMeVal); $^{13}C$ NMR (90 MHz, $CDCl_3$) δ 177.87 (s), 171.38 (s), 170.45 (s), 170.26 (s), 169.89 (s), 169.74 (s, 2 lines), 168.58 (s), 153.91 (d), 125.43 (d), 72.14 (d), 68.25 (d), 59.07 (d), 58.14 (d, 2 lines), 53.81 (d), 52.85 (d), 51.88 (d), 41.89 (t), 37.09 (t), 35.83 (t), 34.43 (t), 33.78 (t), 30.76 (d), 30.40 (q), 30.33 (q), 29.14 (t), 27.32 (d), 24.82 (d), 23.36 (q), 22.88, (q), 21.57 (q), 20.96 (q), 20.94 (q), 19.56 (q), 18.81 (q), 18.48 (q), 18.24 (q), 17.45 (q), 17.33 (q), 14.10 (q).

**10-Ketomicrocolin A (4).** A sample of **1** (11.4 mg) was dissolved in 5.0 mL of acetone and stirred in an ice bath for for 10 min. Jones reagent (50 μL) was added dropwise, and the reaction mixture was stirred at 0 °C for 5 h. TLC analysis showed a major product of reduced polarity along with a small amount of starting material. The reaction flask was placed in a freezer overnight, and the following day another 20 μL of Jones reagent was added with continued stirring at 0 °C. After 4 h 6 drops of 2-propanol was added, and the resulting mixture was partitioned between water and EtOAc. The water layer was washed once with 10 mL of EtOAc, and the organic layers were combined, dried over $MgSO_4$, and evaporated. The crude product was chromatographed on silica gel 60 (3-mL column) with $CHCl_3$ (5.0 mL), $CHCl_3$/EtOAc (1:1) (5.0 mL), and EtOAc (9.0 mL). Evaporation of the second fraction gave 7.1 mg of pure **4** (62%). A small amount of **1** was recovered from fraction 3: HRLSIMS ($MH^+$) calcd for $C_{39}H_{64}O_9N_5$ 746.470, found 746.463, Δ 7 mmu; $^1H$ NMR (500 MHz, $CDCl_3$) δ 7.26 (dd, 1H, *J* = 6.3, 2.1, H-3), 7.02 (d, 1H, *J* = 8.9, N*H*), 6.08 (dd, 1H, *J* = 6.3, 1.6, H-2), 6.00 (dd, 1H, *J* = 10.5, 3.2, αketoPro), 5.29 (dd, 1H, *J* = 10.5, 5.3, αNMeLeu), 5.26 (qd, 1H, *J* = 6.8, 1.6, βThr), 4.93 (dd, 1H, = 8.9, 1.6, αThr), 4.90 (d, 1H, *J* = 11.0, αNMeVal), 4.75 (qt, 1H, *J* = 6.8, 2.1, H-4), 4.43 (d, 1H, *J* = 17.9, δketoPro), 4.05 (d, 1H, *J* = 17.9, δketoPro), 3.22 (dd, 1H, *J* = 18.4, 10.0, βketoPro), (s, 3H, N*Me*Val), 2.96 (s, 3H, *NMe*Leu), 2.45 (dd, 1H, *J* = 18.4, 3.2, βketoPro), 2.84 (m, 1H, H-36), 2.29 (m, 1H, βNMeVal), 2.03 (s, 3H, *OAc*Thr), 1.87 (m, 1H, H-37), 1.74 (ddd, *J* = 14.7, 10.5 4.2, H-36), 1.59 (ddd, 1H, *J* = 14.7, 8.6, 5.7, H-36), 1.42 (m, 1H, γNMeLeu), 1.09 (m, 1H, H-37), 1.27 (bm, 3H, H-39), 1.25 (bm, 4H, H-40, 41), 1.15 (d, 3H, *J* = 6.7, γThr), 1.13 (d, 3H, *J* = 6.7, H-44), 0.95 (d, 3H, *J* = 6.7, γNMeLeu), 0.95 (d, 3H, *J* = 6.7, H-33), 0.86 (d, 3H, *J* = 6.7, δNMeLeu), 0.85 (t, 3H, *J* = 6.7, H-42), 0.84 (d, 3H, *J* = 6.7, H-43), 0.82 (d, 3H, *J* = 6.7, γNMeVal); $^{13}C$ NMR (125 MHz, $CDCl_3$) δ 208.38 (s), 177.86 (s), 171.52 (s), 171.15 (s), 170.20 (s), 169.81 (s, 2 lines), 169.04 (s), 154.19 (d), 125.26 (d), 67.71 (d), 59.40 (d), 58.00 (d), 56.84 (d), 54.52 (t), 53.87 (d), 51.86 (d), 41.88 (t), 40.21 (t), 37.12 (t), 35.76 (t), 33.80 (d), 30.78 (d), 30.31 (q), 30.13 (q), 29.12 (t), 26.92 (d), 24.81 (d), 23.37 (q), 22.87 (q), 21.54 (q), 20.58 (q), 19.55 (q), 18.85 (q), 18.27 (q, 2 lines), 17.65 (q), 17.12 (q), 14.07 (q).

**3,4-Dihydromicrocolin A (5).** A suspension of palladium on carbon (10 mg) in 3.0 mL of ethanol was stirred under hydrogen for 10 min. A solution of 5.7 mg of **1** in 0.75 mL of ethanol was added, and resulting mixture was stirred at room temperature under hydrogen for 7 h. The catalyst was removed by filtration and washed once with ethanol (20 mL), and the combined solvents were evaporated to give 3.6 mg of **5**: HRLSIMS ($MH^+$) calcd for $C_{39}H_{68}O_9N_5$ 750.502, found 750.497, Δ 5 mmu; $^1H$ NMR (500 MHz, $CDCl_3$) δ 6.89 (d, 1H, *J* = 9.0 N*H*), 5.58 (dd, 1H, *J* = 10.0, 2.1, αHyp), 5.26 (1H, dd, *J* = 10.2, 5.5, αNMeLeu), 5.23 (dd, 1H, *J* = 6.6, 3.0, βThr), 4.99 (d, 1H, *J* = 11.0, αNMeVal), 4.94 (dd, 1H, *J* = 9.0, 3.0, αThr), 4.47 (m, 1H, H-4), 4.35 (m, 1H γHyp), 3.83 (dt, 1H, *J* = 11.5, 1.5, δHyp), 3.79 (1H, dd, *J* = 11.5, 4.5, δHyp), 3.07 (s, 3H, N*Me*Val), 2.95 (s, 3H, N*Me*Leu), 2.84 (m, 1H, H-36), 2.75 (ddd, 1H, *J* = 17.8, 9.3, 2.6, H-2), 2.48 (ddd, 1H, *J* = 17.8, 9.3, 3.0, H-2), 2.22 (m, 1H, H-3), 1.79 (m, 1H, H-3), 2.42 (ddd, 1H, *J* = 14.5, 10.2, 4.9, βHyp), 1.96 (m, 1H, βHyp), 2.25 (m, 1H, βNMeVal), 1.98 (s, 3H, *OAc*Thr), 1.80 (m, 1H, H-37), 1.72 (ddd, 1H, *J* = 14.6, 10.1, βNMeLeu), 1.58 (ddd, 1H, *J* = 14.6, 9.6, 5.8, βNMeLeu), 1.41 (m, 1H, γNMeLeu), 1.28 (s, 3H, H-6), 1.27 (m, 2H, H-38), 1.26 (m, 2H, H-40), 1.23 (m, 2H, H-41), 1.15 (d, 3H, *J* = 6.6, γThr), 1.12 (d, 3H, *J* = 6.7, H-44), 1.07 (m, 1H, H-39), 1.04 (m, 1H, H-37) 0.98 (d, 3H, *J* = 6.7, γNMeVal), 0.94 (d, 3H, *J* = 6.7, δNMeLeu), 0.87 (t, 3H, *J* = 6.7, H-42), 0.86 (d, 3H, *J* = 6.7, δNMeLeu), 0.84 (d, 3H, *J* = 6.7, H-43), 0.81 (d, 3H, *J* = 6.7, γNMeVal); $^{13}C$ NMR (125 MHz, $CDCl_3$) δ 177.85 (s), 175.75 (s), 175.44 (s), 171.32 (s), 169.77 (s), 169.72 (s), 168.87 (s), 71.86 (d), 68.46 (d), 59.23 (d), 59.08 (d), 56.92 (t), 53.83 (d), 53.46 (d), 51.82 (d), 41.92 (t), 37.08 (t), 36.82 (t), 35.87 (t), 33.79 (d), 31.88 (t), 30.77 (d), 30.32 (q), 29.13 (t), 27.13 (d), 25.29 (d), 24.83 (d), 23.31 (q), 22.88 (t), 21.59 (q), 21.02 (q), 19.57 (q), 19.20 (q), 18.86 (q), 18.39 (q), 18.2 (q), 17.38 (q), 14.08 (q).

**3,4-Dihydromicrocolin B (6).** A suspension of palladium on carbon (10 mg) in 3.0 mL of ethanol was stirred under hydrogen at room temperature for 10 min. A solution of **2** (4.8 mg) in ethanol (1.0 mL) was added, and the mixture was stirred under hydrogen for 24 h. The reaction mixture was filtered and the catalyst washed with 20 mL of ethanol followed by 10 mL of $CH_2Cl_2$. The original filtrate and washings were combined and evaporated to give 4.0 mg of pure (by $^1H$ NMR) **4** (85%): HRLSIMS ($MH^+$) calcd for $C_{39}H_{68}N_5O_8$ 734.507, found 734.499, Δ 8 mmu; $^1H$ NMR (500 MHz, $CDCl_3$), δ 6.94 (d, 1H, *J* = 8.9, N*H*), 5.41 (dd, 1H, *J* = 5.3, 8.7, αPro), 5.25 (dd, 1H, *J* = 10.5, 5.8, αNMeLeu), 5.23 (dd, 1H, *J* = 6.3, 3.4, βThr), 5.02 (d, 1H, *J* = 11.3, αNMeVal), 4.96 (dd, 1H, *J* = 8.9, 3.7, αThr), 4.43 (m, 1H, H-4), 3.77 (ddd, 1H, *J* = 16.5, 10.0, 6.8, δPro), 3.68 (ddd, 1H, *J* = 16.5, 10.0, 7.1, δPro), 3.10 (s, 3H, N*Me*Val), 2.95 (s, 3H, N*Me*Leu), 2.84 (m, 1H, H-36), 2.74 (ddd, 1H, *J* = 17.6, 11.0, 9.2, H-2), 2.46 (ddd, 1H, *J* = 17.6, 8.9, 2.6, H-2), 2.37 (ddd, 1H, *J* = 13.9, 12.6, 7.1, βPro), 2.26 (m, 1H, βNMeVal), 2.19 (m, 1H, H-3), 1.99 (bm, 2H, γPro), 1.99 (s, 3H, *OAc*Thr), 1.87 (m, 1H, H-37), 1.84 (m, 1H, βPro), 1.71 (ddd, 1H, *J* = 14.6, 10.0 4.5, βNMeLeu), 1.59 (ddd, 1H, βNMeLeu, obscured by residual water), 1.41 (m, 1H, γNMeLeu),

1.34 (d, 3H, $J$ = 6.3, H-6), 1.27 (m, 1H, H-38), 1.26 (m, 1H, H-39), 1.25 (bm, 4H, H-40, 41)), 1.18 (m, 1H, H-39), 1.15 (d, 3H, $J$ = 6.3, γThr), 1.12 (d, 3H, $J$ = 6.8, H-44), 0.94 (d, 3H, $J$ = 6.6, γNMeVal), 0.87 (d, 3H, $J$ = 6.6, δNMeLeu), 1.09 (m, 1H, H-37), 0.99 (d, 3H, $J$ = 6.6, δNMeLeu)), 0.88 (t, 3H, $J$ = 6.8, H-42), 0.84 (d, 3H, $J$ = 6.8, γNMeVal), 0.80 (d, 3H, $J$ = 6.6, H-43); $^{13}$C NMR (125 MHz, $CDCl_3$) δ 177.84 (s), 175.17 (s), 172.92 (s), 171.24 (s), 169.67 (s, 2 lines), 168.23 (s), 68.80 (d), 60.49 (d), 59.28 (d), 53.88 (d), 53.31 (d), 51.98 (d), 47.83 (d), 41.91 (t), 37.06 (t), 35.97 (t), 33.79 (d), 31.93 (t), 30.76 (d), 30.52 (q), 30.35 (q), 29.12 (t), 29.05 (t), 27.29 (d), 25.32 (d), 24.86 (d), 24.52 (t), 23.29 (q), 22.88 (t), 21.62 (q), 20.95 (q), 19.58 (q), 19.30 (q), 18.87 (q), 18.38 (q), 18.17 (q), 17.13 (q), 14.07 (q).

**Methanolysis of Microcolin A (1).** Microcolin A (17 mg) in MeOH (2 mL) was added to a solution of HCl in MeOH (10 mL). (The HCl was generated by adding concentrated $H_2SO_4$ (2 mL) to NaCl (2 g) and bubbling the resultant HCl through MeOH). The mixture was heated at reflux for 5 h, at which time the presence of microcolin A was not evident by TLC ($R_f$ = 0.6 in 100% EtOAc on silica gel). The solvent was then removed in vacuo, and the residue was partitioned between EtOAc and $H_2O$ to yield residues of 10 and 7 mg, respectively. Components of the EtOAc layer were then separated directly using reversed-phase HPLC (60% $CH_3CN/H_2O$).

**23-Desacetylmicrocolin A (7)** (0.6 mg): eluted with $k'$ = 2.1; HRLSIMS ($MH^+$) calcd for $C_{37}H_{63}N_5O_8$ 706.475, found 706.478, Δ −3 mmu; $^1$H NMR (500 MHz, $CDCl_3$) δ 7.26 (dd, 1H, $J$ = 1.9, 6.0, H-3), 6.94 (d, 1H, $J$ = 9.2, N*H*), 6.07 (dd, 1H, $J$ = 1.4, 6.0, H-2), 5.63 (dd, 1H, $J$ = 1.3, 9.8, αPro), 5.20 (t, 1H, $J$ = 7.8, αLeu), 5.00 (d, 1H, $J$ = 10.9, αVal), 4.79 (ddq, 1H, $J$ = 1.4, 1.9, 6.7, H-4), 4.72 (dd, 1H, $J$ = 1.4, 9.2, αThr), 4.38 (m, 1H, γHyp), 4.06 (dq, 1H, $J$ = 1.4, 6.5, βThr), 3.94 (d, 1H, $J$ = 11.7, δHyp), 3.79 (dd, 1H, $J$ = 4.2, 11.7, δHyp), 3.05 (s, 3H), 2.95 (s, 3H), 2.80 (m, 1H, H-36), 2.42, (ddd, 1H, $J$ = 4.8, 9.8, 14.0, βHyp), 2.26 (m, 1H, βVal), 2.02 (brd, 1H, $J$ = βHyp), 1.65−1.85 (m), 1.44 (d, 3H, $J$ = 6.7), 1.4 (m, 1H), 1.2−1.3 (m), 1.1 (d, 3H, $J$ = 6.5), 1.03 (d, 3H, $J$ = 6.4), 0.98 (d, 3H, $J$ = 6.5), 0.92 (d, 3H, $J$ = 6.6), 0.86 (t, 3H, $J$ − 6.3), 0.85 (d, 3H, $J$ = 6.5), 0.81 (d, 3H, $J$ = 6.8), 0.80 (d, 3H, $J$ = 7.0).

**2,4-Dimethyloctanoyl-NMeLeu-Thr-NMeVal-Hyp-OMe (8)** (0.3 mg): eluted with $k'$ = 1.8; HRLSIMS ($MH^+$) calcd for $C_{33}H_{60}N_4O_8$ 641.449, found 641.437, Δ 12 mmu; $^1$H NMR (500 MHz, $CDCl_3$) δ 6.94 (d, 1H, $J$ = 9.1, N*H*), 5.20 (t, 1H, $J$ = 8.0, αLeu), 4.95 (d, 1H, $J$ = 11.2, αVal), 4.72 (brd, 1H, $J$ =7.7, αThr, or αHyp), 4.46 (dd, 1H, $J$ = 2.0, 10.2, αThr or αHyp), 4.40 (m, 1H, γHyp), 4.04 (dq, 1H, $J$ = 2.4, 6.5, βThr), 3.85 (m, 2H, δHyp), 3.78 (s, 3H), 3.09 (s, 3H), 2.95 (s, 3H), 2.81 (m, 1H), 2.30 (m, 2H, βVal and βHyp), 2.07 (brd, 1H, $J$ = 13.9, βHyp), 1.83 (ddd, 1H, $J$ = 4.7, 9.4, 13.6), 1.65 (dt, 1H, $J$ = 1.6, 7.6), 1.2−1.45 (m), 1.10 (d, 3H, $J$ = 6.5), 1.02 (d, 3H, $J$ = 6.4), 1.00 (d, 3H, $J$ = 6.1), 0.93 (d, 3H, $J$ = 6.8), 0.86 (d, 3H, $J$ = 6.4), 0.86 (t, 3H, $J$ = 6.8), 0.82 (d, 3H, $J$ = 6.4), 0.81 (d, 3H, $J$ = 6.4).

**2,4-Dimethyloctanoyl-NMeLeu-Thr-NMeVal-OMe (9)** (0.9 mg): eluted with $k'$ = 4.2; HRLSIMS ($MH^+$) calcd. for $C_{28}H_{53}N_3O_6$ 528.401, found 528.404, Δ −3 mmu; $^1$H NMR (500 MHz, $CDCl_3$) δ 6.91 (d, 1H, $J$ = 9.2, Thr-N*H*), 5.22 (t, 1H, $J$ = 7.9, α Leu), 4.83 (d, 1H, $J$ = 10.6, αVal), 4.74 (dd, 1H, $J$ = 2.0, 9.2, αthr), 4.10 (dq, 1H, $J$ = 2.0, 6.5, βThr), 3.69 (s, 3H), 3.04 (s, 3H), 2.95 (s, 3H), 2.81 (m, 1H), 2.20 (m, 1H), 1.83 (m, 1H), 1.66 (t, 2H, $J$ = 7.5), 1.45−1.55 (m), 1.40 (m, 1H), 1.2−1.3 (m), 1.10 (d, 3H, $J$ = 6.8), 1.04 (d, 3H, $J$ = 6.4), 0.98 (d, 3H, $J$ = 6.4), 0.93 (d, 3H, $J$ = 6.6), 0.86 (d, 3H, $J$ = 6.2), 0.85 (t, 3H, $J$ = 6.7), 0.82 (d, 3H, $J$ = 6.7).

**1-Hyp-5-methyl-3-pyrrolin-2-one (10)** (1.4 mg) isolated by reversed-phase HPLC of the aqueous layer using 5% acetonitrile/$H_3O$ (with 0.1% TFA) ($k'$ = 0.5): HRLSIMS ($MH^+$) calcd for $C_{10}H_{15}N_2O_3$ 211.1082, found 211.1013, Δ 6.9 mmu; $^1$H NMR (360 MHz, $D_2O$ + MeOH-$d_4$) δ7.62 (dd, 1H, $J$ = 1.9, 6.1), 6.19 (d, 1H, $J$ = 6.1), 5.29 (dd, 1H, $J$ = 4.1, 10.9, αHyp), 4.8 (dq, 1H, $J$ = 1.9, 6.8), 4.63 (brm, 1H, αOH), 3.55 (brd, 1H, 12.2, δHyp), 3.46 (dd, 1H, $J$ = 3.2, 12.2, δHyp), 2.79 (m, 1H, γHyp), 2.11 (m, 1H, γHyp), and 1.48 (d, 3H, $J$ = 6.8).

**Lactone 11** (1.3 mg): eluted with $k'$ = 3.6; HRLSIMS ($MH^+$) calcd for $C_{27}H_{49}N_3O_5$ 496.375, found 496.374, Δ 1 mmu; $^1$H NMR (500 MHz, $CDCl_3$) δ 6.87 (brs, 1H, Thr-β*H*), 5.25 (dq, 1H, $J$ = 6.4, 9.0, βThr), 4.70 (dd, 1H, $J$ = 6.3, 8.5, αLeu), 3.87 (dd, 1H, $J$ = 3.0, 9.0, αThr), 3.59 (d, 1H, $J$ = 5.7, αVal), 3.07 (s, 3H, N*Me*-Leu), 2.98 (s, 3H, N*Me*-Val), 2.81 (m, 1H), 2.23 (m, 1H, βVal), 1.6−1.9 (m, βLeu), 1.49 (m, 1H, γLeu), 1.39 (d, 3H, $J$ = 6.4, γThr (Me)), 1.2−1.3 (m), 1.12 (d, 6H, $J$ = 7.0, dimethyloctanoyl-Me and Val-Me), 1.06 (d, 3H, $J$ = 6.8, Val-Me), 0.95 (d, 3H, $J$ = 6.6, Leu-Me), 0.90 (d, 3H, $J$ = 6.5, Leu-Me), 0.85 (d, 3H, $J$ = 6.6, dimethyloctanoyl-Me), 0.83 (t, 3H, $J$ = 6.5, dimethyloctanoyl-Me), key NOESY correlations: Hβ Thr/Hβ,Hγ MeVal; $^{13}$C NMR (125 MHz, $CDCl_3$) δ 178.5 (dimethyloctanoyl C=O), 171.6 (Leu C=O), 166.2 (Val C=O), 163.2 (Thr C=O), 72.6 (βThr), 68.4 (αVal), 59.7 (αThr), 56.6 (αLeu), 41.7 (t, C-3, dimethyloctanoic group), 37.8 (βLeu), 37.1 (t, C-5, dimethyloctanoic group), 35.6 (N*Me*-Val), 33.9 (d, C-2, dimethyloctanoic group), 32.9 (N*Me*-Leu), 32.7 (βVal), 30.7 (d, C-4, dimethyloctanoic group), 29.1 (t, C-6, dimethyloctanoic group), 25.2 (γLeu), 23.0 (t and q, C-7, dimethyloctanoic group and Leu-Me), 21.8 (Leu-Me), 19.9 (Val-Me), 19.7 (dimethyloctanoyl-Me), 19.0 (Val-Me), 17.8 (Thr-Me), 17.6 (dimethyloctanoyl-Me), 14.1 (dimethyloctanoyl-Me).

**Preparation of Cell Suspensions.** Splenocyte suspensions were prepared from 8−15 week old C57BL/6J and BALB/c mice according to established methods.[19,20] Human PBL suspensions were prepared from Leukotrap (Cutter Laboratories, Berkeley, CA) blood filters according to a previously published method.[21]

**Two-Way Mixed Lymphocyte Reaction (MRL).** The immunosuppressive properties of microcolin A and B were evaluated using the two-way MLR, utilizing murine splenocytes from C57BL/6J and BALB/c mice, or human PBL suspensions from unrelated donors. A volume of 0.100 μL of each cell suspension was added together to test and control wells of microliter plates. Wells containing 0.20 mL of each cell suspension alone served as controls. Serial 2-fold dilutions of microcolin A or B were prepared in absolute EtOH and diluted to the appropriate concentration in TCM and added directly to the wells containing splenocyte or PBL combinations. Additional solvent (EtOH) control wells were also prepared by adding the equivalent percentage of EtOH to each culture well. Plates were incubated at 37 °C in a 5% $CO_2$ humidified atmosphere for 88 h for the murine MLR or 115 for the human MLR. Following the incubation, 1 μCi of [$^3$H]-thymidine was added to each well, and the plates were returned to the incubator for 5 h. The contents of each well were harvested onto glass fiber filter strips, and the incorporation of [$^3$H]thymidine was determined by a liquid scintillation counter.

**Determination of Microcolin A and B Toxicity.** The toxicity of microcolin A and B for murine splenocytes was determined using a colorimetric assay, based on the ability of viable cells to convert the colorless tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a colored formazan product[18] and trypan blue exclusion. For the colorimetric assay, a volume of 0.075 mL of an MTT solution (550 μg/mL of MTT in TCM) was added to test and control wells containing one of the populations of splenocytes and incubated for 5−6 hr following the initial incubation period. Microtiter plates were centrifuged at 100$g$, supernatants were carefully removed, and a volume of 0.20 mL of isopropyl alcohol was added to each well to dissolve the formazan crystals. The absorbance at 570 nm was determined for each well using an ELISA plate reader. Concentrations of microcolin A or B which resulted in cell cultures exhibiting less than 90% of the control (no compound) response were considered to be toxic. As an additional parameter of toxicity, wells containing cultured lymphocytes with various dilutions of microcolin A or B were stained with trypan blue, and the percentage of dye excluding, viable cells was determined.

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(13) MLR $IC_{50}$ values for compounds **1** and **2** in Table 1 of this publication were measured using [$^3$H]thymidine incorporation, and differ from those cited in ref 12, which were measured by MTT colorimetric assay.

(14) Additional biological activities for microcolin A: P388 leukemia $IC_{50}$ = 1.3 nM. Protein kinase C, isozyme $\beta$I = 13.5 $\mu$M. Microcolin B: P388 leukemia $IC_{50}$ = 123 nM, Protein kinase C, isozyme $\zeta$ $IC_{50}$ = 25.5 $\mu$M.

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