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Microcionamides A and B, Bioactive Peptides from the Philippine Sponge *Clathria (Thalysias) abietina*

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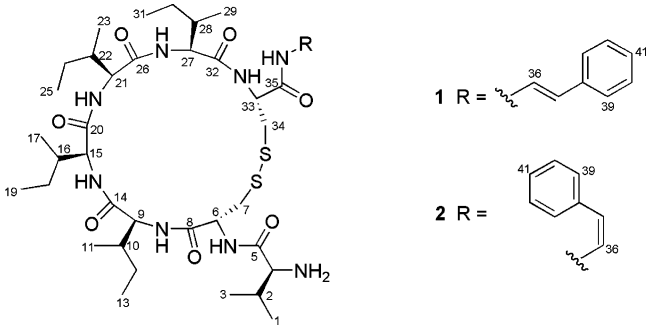
Microcionamides A (**1**) and B (**2**) have been isolated from the Philippine marine sponge *Clathria (Thalysias) abietina*. These new linear peptides are cyclized via a cystine moiety and have their *C*-terminus blocked by a 2-phenylethylenamine group. Their total structures, including absolute stereochemistry, were determined by a combination of spectral and chemical methods. Compound **1** was shown to slowly isomerize about the C-36/C-37 double bond when stored in DMSO. Microcionamides A (**1**) and B (**2**) exhibited significant cytotoxicity against the human breast tumor cells lines MCF-7 and SKBR-3 and displayed inhibitory activity against *Mycobacterium tuberculosis* $H_{37}Ra$.

Peptides have been isolated from a number of marine phyla, and many show high levels of cytotoxicity. Examples include aplidine (dehydrodidemnin B) from the ascidian *Aplidium albicans*[1,2] and dolastatin 16, which was initially isolated from the sea hare *Dolabella auricularia*,[3] but has more recently been identified in the cyanobacteria *Lyngbya majuscula*.[4] Numerous peptides have been reported from marine sponges to date.[5−10] However, apart from the eurypamides from *Clathria (Thalysias) eurypa*,[11] published under its former name *Microciona eurypa*,[12] no other peptides have been isolated from the genus *Clathria* or the family Microcionidae.[13]

As part of our continuing search for structurally and pharmacologically interesting secondary metabolites from marine invertebrates, we decided to undertake a detailed exploration of the underinvestigated marine sponge *C. (Thalysias) abietina*. Herein we report the isolation, structure elucidation, and biological activity of two new peptides that we have named microcionamides A (**1**) and B (**2**).



Results and Discussion

The crude MeOH extract of *C. (Thalysias) abietina* (Lamarck, 1814) (order Poecilosclerida, family Microcionidae) was concentrated under vacuum and then subjected to a solvent partitioning scheme resulting in hexanes and $CHCl_3$-soluble fractions. The $CHCl_3$-soluble material was chromatographed on a C_{18}-bonded silica flash column using an aqueous TFA/MeOH gradient. Further purification by C_{18} HPLC using aqueous TFA and increasing amounts of CH_3CN afforded the TFA salts of microcionamides A (**1**, 27.4 mg) and B (**2**, 19.3 mg).

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TABLE 1. NMR Data for Microcionamide A (1)[a,b]

position	^{13}C (δ)	^{1}H (δ, mult, J in Hz)	COSY	HMBC	ROESY
1	17.8	0.98 (d, 7.0)	2	2, 3, 4	2, 4
2	31.6	2.16 (dqq, 5.0, 7.0, 7.0)	1, 3, 4	1, 3, 4, 5	1, 3, 4
3	18.9	1.01 (d, 7.0)	2	1, 2, 4	2, 4
4	59.5	3.70 (d, 5.0)	2	1, 2, 3, 5	1, 2, 3, 6
5	169.6				
6	54.5	4.79 (m)	7a, 7b	5, 7, 8	4, 7a
7a	41.4	3.04 (dd, 14.0, 9.0)	6, 7b	6, 8	6
7b		3.30 (m)	6, 7a	8	
8	172.0				
9	60.0	4.30 (d, 7.0)	10	8, 10, 11, 12, 14	10, 11, 12a, 12b
10	37.7	1.95 (m)	9, 11, 12a, 12b	9	9, 11, 12a, 12b
11	16.2	0.92 (d, 7.0)	10	9, 10, 12	9, 10, 12a, 12b
12a	25.8	1.20 (m)	10, 12b, 13	13	9, 10, 11, 12b, 13
12b		1.51 (m)	10, 12a, 13		9, 10, 11, 12a, 13
13	11.8	0.88 (t, 7.0)	12a, 12b	11, 12	12a, 12b
14	174.5[c]				
15	60.2	4.13 (d, 8.5)	16	16, 17, 18, 20	16, 17, 18a, 18b
16	37.1	1.95 (m)	15, 17, 18a	15	15, 17, 18a, 18b
17	16.1	0.92 (d, 7.0)	16	15, 16, 18	15, 16, 18a, 18b
18a	26.5	1.17 (m)	16, 18b, 19	19	15, 16, 17, 18b, 19
18b		1.56 (m)	18a, 19		15, 16, 17, 18a, 19
19	11.5	0.88 (t, 7.0)	18a, 18b	17, 18	18a, 18b
20	173.5				
21	61.5	4.07 (d, 6.5)	22	22, 23, 24, 26	22, 23, 24a, 24b
22	37.4	2.02 (m)	21, 23, 24a	21	21, 23, 24a, 24b
23	16.3[d]	0.97 (d, 6.5)	22	21, 22, 24	21, 22, 24a, 24b
24a	26.4	1.20 (m)	22, 24b, 25	25	21, 22, 24b, 25
24b		1.56 (m)	24a, 25		21, 22, 24a, 25
25	11.5[e]	0.87 (t, 7.5)	24a, 24b	22, 24	24a, 24b
26	174.6[c]				
27	61.6	4.02 (d, 5.5)	28	29, 30, 32	28, 29, 30a, 30b
28	37.4	2.00 (m)	27, 29, 30a	27	27, 29, 30a, 30b
29	16.4[d]	0.95 (d, 7.0)	28	27, 28, 30	27, 28, 30a, 30b
30a	26.7	1.23 (m)	28, 30b, 31	31	27, 28, 29, 30b, 31
30b		1.56 (m)	30a, 31		27, 28, 29, 30a, 31
31	11.2[e]	0.87 (t, 7.5)	30a, 30b	28, 29	30a, 30b
32	173.7				
33	55.0	4.60 (dd, 9.0, 4.5)	34a, 34b	35	34a
34a	42.7	3.20 (dd, 13.5, 9.0)	33, 34b	33, 35	33
34b		3.27 (dd, 13.5, 4.5)	33, 34a	35	
35	169.4				
36	123.2	7.35 (d, 14.5)	37	35, 37, 38	39
37	116.4	6.38 (d, 14.5)	36	36, 39	39
38	137.6				
39	126.6	7.26 (d, 8.0)	40	37, 39, 41	36, 37, 40
40	129.7	7.22 (dd, 8.0, 7.5)	39, 41	38, 40	39, 41
41	127.8	7.11 (dd, 7.5, 7.5)	40	39	40

[a] Assignments for the four contiguous isoleucine residues may be interchangeable. [b] Spectra were recorded in CD_3OD at 26 °C. [c–e] Signals are interchangeable.

Microcionamide A (**1**) was isolated as an optically active white solid. The molecular formula of **1** was determined to be $C_{43}H_{70}N_8O_7S_2$ on the basis of an $[M + H]^+$ ion at m/z 875.48843 (Δ 0.31 ppm) in the (+)-HRESIMS. An aromatic chromophore [λ_{max} 284 nm (ϵ 16 000)] and amide group(s) (ν_{max} 1650 cm^{-1}) were evident after analysis of the UV and IR spectra, respectively. A positive ninhydrin reaction suggested the presence of an amino moiety. The ^{13}C NMR spectrum (Table 1) displayed signals for 41 unique carbons, and the DEPT experiment indicated that **1** contained 10 methyls, 6 methylenes, and 17 methine carbons. Seven of the 8 quaternary carbons resonated in the 169−175 ppm region and were consistent for amide moieties. Salient features of the ^{1}H NMR spectrum of **1** included three aromatic signals at δ 7.26 (2H, d, J = 8.0 Hz), 7.22 (2H, dd, J = 8.0, 7.5 Hz), and 7.11 (1H, dd, J = 7.5, 7.5 Hz) that were assigned to a monosubstituted phenyl ring, two olefinic resonances at δ 7.35 (1H, d, J = 14.5 Hz) and 6.38 (1H, d, J = 14.5 Hz) which belonged to an isolated *trans*-ethylene moiety, and seven signals [δ 3.70 (1H, d, J = 5.0 Hz), 4.79 (1H, m), 4.30 (1H, d, J = 7.0 Hz), 4.13 (1H, d, J = 8.5 Hz), 4.07 (1H, d, J = 6.5 Hz), 4.02 (1H, d, J = 5.5 Hz), 4.60 (1H, dd, J = 9.0, 4.5 Hz)] that were indicative of α-proton resonances for amino acids. The upfield region of the ^{1}H NMR spectrum contained a multitude of signals that integrated for 47 protons. A gCOSY experiment allowed several amino acid units to be delineated, which included four isoleucines, one valine and one cystine, or two cysteine residues. The gHSQC spectrum enabled all the proton signals to be assigned to their directly attached carbons. Several of the above-mentioned partial structures were connected using gHMBC data. These included the linkage of the isolated *trans*-ethylene system to the phenyl ring based on three-bond HMBC correlations from δ 7.35 (H-36) to the phenyl quaternary carbon at δ 137.6 (C-38) and from δ 6.38 (H-37) to the aromatic methine carbon at δ 126.6 (C-39). The

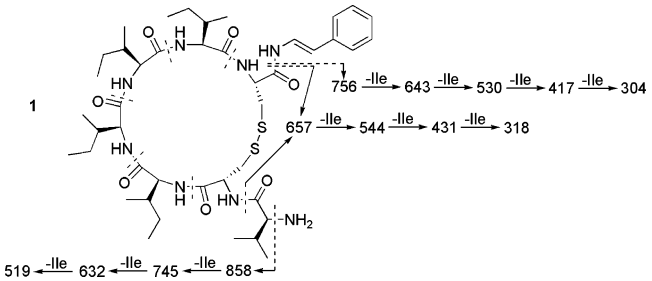


FIGURE 1. Fragmentation pathways observed in the (+)-ESI MS/MS data for **1**.

olefinic proton at δ 7.35 (H-36) also showed a $^3J_{CH}$ correlation to the amide carbonyl at 169.4 ppm and correlations to this particular carbon were also observed from both the α (H-33) and β (H-34a/H-34b) protons that constituted one of the cysteine residues. The remaining cysteine moiety was linked to the valine residue since both α protons [δ 3.70 (H-4) and 4.79 (H-6)] shared an HMBC correlation to the amide carbon at 169.6 ppm. The valine-substituted cysteine was also attached to an isoleucine based on an HMBC correlation from both α protons [δ 4.30 (H-9) and 4.79 (H-6)] to the carbonyl at 172.0 ppm (C-8). Unfortunately, due to insufficient HMBC correlations and crowding in the carbonyl region, no further connectivities across the remaining amide bonds could be made. Analysis of the ROESY spectrum did not provide any extra linkage information. The molecular formula of **1** indicated that microcionamide A contained 13 double-bond equivalents (DBE) and with the currently assigned substructures all but one DBE was accounted for; these data established a further ring system that could only be formed by linkage of the two cysteine amino acids to form a cystine. At this stage, we could not definitely say whether an isoleucine or valine residue constituted the *N*-terminus. Edman degradation of **1** followed by sequence analysis revealed that valine was the *N*-terminal amino acid. This assignment was further supported by MS/MS analysis of **1** which showed a fragmentation pathway (*m*/*z* 657 → 544 → 431 → 318) which could only be explained by the presence of valine at the *N*-terminus (Figure 1). Furthermore, high-resolution MS-MS studies showed ions for complete sequential fragmentation from both the C and N terminus (data provided in the Supporting Information). Hence, the planar peptide structure **1** was assigned to microcionamide A. The absolute stereochemistry for the amino acids in **1** was determined by acid hydrolysis of the peptide followed by treatment of the hydrolysate with Marfey's reagent.[14] Analysis of the mixture of FDAA derivatives by HPLC, using retention times and co-injections with standards, revealed the presence of only L-isoleucine and L-valine. The absolute stereochemistry for the cystine residue was also determined using Marfey's method following the desulfurization of **1** using Raney Ni in refluxing MeOH.[15,16] The linear peptide 36,37-dihydrodesthiomicrocionamide A (**3**) was shown to contain only L-alanine; hence, the cystine system of **1** was also assigned L absolute stereochemistry. No attempt was made to fully characterize 36,37-dihydrodesthiomicrocionamide A (**3**) due to the small amount of material available; however, **3** was analyzed by (+)-LRESIMS to confirm reaction success. MS data for **3** showed specific fragmentation ions that provided further proof of the amino acid sequence of **1** (Figure 2).

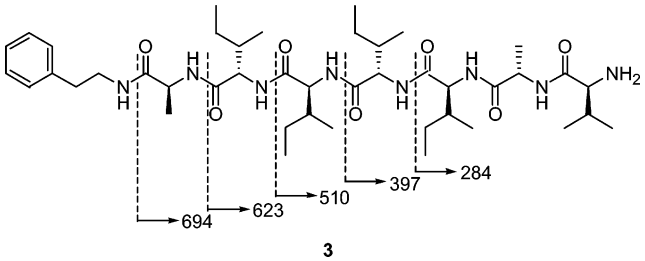
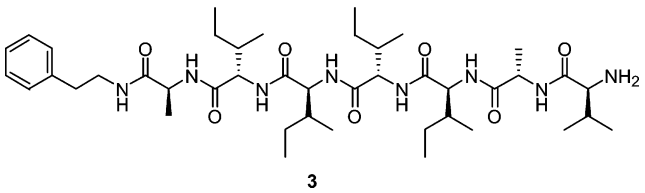


FIGURE 2. Fragmentation ions observed in the (+)-LRESIMS spectrum of **3**.



The minor metabolite, microcionamide B (**2**), was isolated as a stable, optically active white solid. The molecular formula $C_{43}H_{70}N_8O_7S_2$ (13 DBE) was determined by interpretation of the $[M + H]^+$ ion at *m*/*z* 875.48849 (Δ 0.38 ppm) in the (+)-HRESIMS. These data indicated that microcionamide B was an isomer of **1**. A positive ninhydrin reaction confirmed the presence of a free N-terminal amino acid. Comparison of the ^{13}C NMR data of **2** (Table 2) with **1** (Table 1) showed very few chemical shift discrepancies (<3.0 ppm). Likewise, the ^1H NMR spectrum for **2** showed the presence of an aromatic ring, an ethylene group, and six amino acids units. A gCOSY experiment in conjunction with the gHMBC data established the same number and type of amino acid units in **2** as had been identified in **1**. Hence, four isoleucines, one valine, and one cystine residue were attributed to **2**. A few notable differences were observed in the ^1H NMR spectrum of **2**. These included the chemical shifts and the magnitude (10.0 Hz) of the coupling constant for the ethylene protons, indicating that the C-36/C-37 double bond geometry was cis in **2**, plus a broad and complex set of multiplets in the aromatic region that appeared to display non-first-order characteristics. These aromatic signals were assigned to the phenyl system protons (H-39-H-41) following gHSQC and gHMBC analysis. Linkages for most of the amino acids and ethylene and phenyl systems of **2** were established on the basis of gHMBC analysis. However, in a manner similar to that for **1**, not all of the amide connectivities could be assigned for **2**. MS/MS analysis and Edman degradation of microcionamide B followed by sequence analysis confirmed that valine was also the N-terminal amino acid in this peptide. Although definitive positioning of each of the three remaining isoleucine residues in relation to each other could not be determined, by default they were inserted between C-14 and C-33. Hence, the planar structure **2**, which was the cis

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TABLE 2. NMR Data for Microcionamide B (2)[a,b]

position	^{13}C (δ)	^{1}H (δ, mult., J in Hz)	COSY	HMBC	ROESY
1	17.8	0.97 (d, 7.0)	2	2, 3, 4	2, 4
2	31.6	2.13 (dqq, 5.0, 7.0, 7.0)	1, 3, 4	1, 3, 4, 5	1, 3, 4
3	18.9	0.99 (d, 7.0)	2	1, 2, 4	2, 4
4	59.5	3.67 (d, 5.0)	2	1, 2, 3, 5	1, 2, 3
5	169.6				
6	54.5	4.80 (m)	7a, 7b	5, 7, 8	
7a	41.7	3.05 (dd, 14.0, 9.0)	6, 7b	6, 8	
7b		3.26 (m)	6, 7a		
8	172.1				
9	60.2	4.26 (d, 7.0)	10	8, 10, 11, 12, 14	10, 11, 12a
10	37.8	1.90 (m)	9, 11, 12a		9, 11, 12a, 12b
11	16.1	0.93 (d, 7.0)	10	9, 10, 12	9, 10, 12a, 12b
12a	26.2[c]	1.19 (m)	10, 12b, 13	13	9, 10, 11, 12b, 13
12b		1.50 (m)	12a, 13		9, 10, 11, 12a, 13
13	11.4	0.90 (t, 7.0)	12a, 12b	11, 12	12a, 12b
14	174.2[d]				
15	59.5	4.10 (d, 8.5)	16	16, 17, 18, 20	16, 17, 18a, 18b
16	36.8	1.85 (m)	15, 17, 18a		15, 17, 18a, 18b
17	15.9	0.77 (d, 7.0)	16	15, 16, 18	15, 16, 18a, 18b
18a	26.2	1.10 (m)	16, 18b, 19	19	15, 16, 17, 18b, 19
18b		1.50 (m)	18a, 19		15, 16, 17, 18a, 19
19	11.1	0.81 (t, 7.0)	18a, 18b	17, 18	18a, 18b
20	173.9				
21	61.5	4.00 (d, 7.0)	22	22, 23, 26	22, 23, 24a, 24b
22	37.0	2.10 (m)	21, 23, 24a		21, 23, 24a, 24b
23	16.3	0.95 (d, 7.0)	22	21, 22, 24	21, 22, 24a, 24b
24a	26.4[c]	1.14 (m)	22, 24b, 25	25	21, 22, 24b, 25
24b		1.50 (m)	24a, 25		21, 22, 24a, 25
25	11.6	0.87 (t, 7.0)	24a, 24b	22, 24	24a, 24b
26	174.1[d]				
27	61.3	3.92 (d, 6.5)	28	29, 30, 32	28, 29, 30a, 30b
28	37.3	2.00 (m)	27, 29, 30a		27, 29, 30a, 30b
29	16.5	0.91 (d, 6.0)	28	27, 28, 30	27, 28, 30a, 30b
30a	25.9	1.14 (m)	28, 30b, 31	31	27, 28, 29, 30b, 31
30b		1.48 (m)	30a, 31		27, 28, 29, 30a, 31
31	11.6	0.87 (t, 7.0)	30a, 30b	28, 29	30a, 30b
32	173.5				
33	54.6	4.72 (dd, 9.0, 5.0)	34a, 34b	34, 35	34a
34a	42.2	3.10 (dd, 14.0, 9.0)	33, 34b	33, 35	33
34b		3.26 (dd, 14.0, 5.0)	33, 34a	35	
35	170.1				
36	121.9	6.73 (d, 10.0)	37	35, 38	37
37	114.2	5.81 (d, 10.0)	36	36, 39	36, 39
38	136.6				
39	129.5	7.30 (br s)		37, 41	37
40	129.9	7.31 (m)	41	38, 40	
41	128.1	7.19 (br m)	40	39	

[a] Assignments for the four contiguous isoleucine residues may be interchangeable. [b] Spectra were recorded in CD_3OD at 26 °C. [c,d] Signals are interchangeable.

isomer of **1**, was assigned to microcionamide B. The absolute stereochemistry for each of the amino acids in microcionamide B (**2**) was determined using identical methods to those used for **1**.[14] L-Isoleucine, L-valine, and L-cystine were assigned to the constituent amino acids of **2**.

Interestingly, while performing some of our initial NMR studies with microcionamide A (**1**) in DMSO-d_6, we found that over a period of several weeks this peptide slowly isomerized to the cis isomer **2**. After 3 months storage in DMSO-d_6, compound **1** had quantitatively converted to microcionamide B (**2**). No isomerization was observed during the NMR studies performed in CD_3OD. The possibility that microcionamide B (**2**) may be an artifact from the extraction and isolation procedure cannot be ruled out. However, throughout our chemical investigations the two UV-active peaks corresponding to **1** and **2** were always detected in the crude MeOH extract by HPLC analysis. No enrichment of the crude extract with microcionamide B was observed over time.

Biological Activity

Microcionamides A and B showed significant cytotoxicity toward human breast tumor cell lines MCF-7 and SKBR-3. Microcionamide A (**1**) was active against MCF-7 and SKBR-3 cells with IC_{50} values of 125 and 98 nM, respectively. Microcionamide B (**2**) displayed similar activity against MCF-7 and SKBR-3 cells with IC_{50} values of 177 and 172 nM, respectively. These IC_{50} data were comparable with those of the positive control, doxorubicin (MCF-7, 257 nM; SKBR-3, 33 nM). Furthermore, both compounds were shown to induce apoptosis within 24 h in MCF-7 cells at 5.7 μM. Morphological investigations of the MCF-7 cells 24 h after drug treatment showed the hallmarks of apoptosis such as cytoplasmic membrane blebbing, cytoplasmic condensation and shrinkage, loss

of cell-to-cell contact, and formation of membrane bound vesicles.[17,18] Extensive DNA fragmentation, which is also a significant biochemical marker of apoptotic cells,[19] was detected in a TUNEL assay[20] and by Hoescht staining[21] of peptide-treated MCF-7 and SKBR-3 cells, respectively.

Peptides **1** and **2** were also tested for anti-tuberculosis activity using the microplate alamar blue assay (MABA),[22] which uses the avirulent strain *Mycobacterium tuberculosis* $H_{37}Ra$. The $H_{37}Ra$ strain has been shown to display very similar drug susceptibility profiles as the virulent strain $H_{37}Rv$. Microcionamides A and B were both shown to display MIC values of 5.7 μM toward *M. tuberculosis* $H_{37}Ra$, as compared to the positive control, rifampicin (MIC 1.52 nM).

Conclusion

Marine organisms have been the source of several peptides that contain cystine moieties such as the ulithiacyclamides[23−26] and thiocoraline;[27,28] however, microcionamides A and B are unique in that they are the first examples of sponge-derived linear peptides that are cyclized via a cystine residue.[13] In addition, compounds **1** and **2** exhibit significant cytotoxicity against the human breast tumor cells lines MCF-7 and SKBR-3 and displayed inhibitory activity against *Mycobacterium tuberculosis* $H_{37}Ra$.

Experimental Section

General Experimental Procedures. See the Supporting Information.

Animal Material. A specimen of *C. (Thalysias) abietina* (190 g wet weight) was collected in July 1998 using scuba (−10 m) at Tigtabon Island in Zamboanga, Southern Mindanao, Philippines. A small portion of the sponge was immediately steeped in MeOH as the voucher specimen, while the rest of the material was frozen. A taxonomic voucher specimen PDZ_1-98-10-138 is maintained at the University of Utah, Department of Medicinal Chemistry, Salt Lake City, UT.

Extraction and Isolation. The MeOH extract from *C. (Thalysias) abietina* (190 g wet weight) was concentrated under vacuum to yield an orange-brown gum. This material was dissolved in 90% MeOH/10% H_2O (300 mL) and partitioned with 100% hexanes (3 × 300 mL). H_2O (128 mL) was added to the aqueous phase, and the resulting 30% aqueous MeOH fraction was partitioned with 100% $CHCl_3$ (3 × 300 mL). The hexanes and $CHCl_3$ fractions were evaporated to dryness under reduced pressure and yielded 361 mg and 690 mg of material, respectively. The bioactive $CHCl_3$-soluble material was chromatographed on a C_{18}-bonded silica flash column using a 10% stepwise gradient from 100% aqueous TFA (0.5%) to 100% MeOH. The 20% aqueous TFA (0.5%)/80% MeOH fraction (64 mg) was further purified by semipreparative C_{18} HPLC using a linear gradient from 50% aqueous TFA (0.1%)/50% CH_3CN to 100% CH_3CN in 30 min at a flowrate of 1.0 mL/min. This afforded pure microcionamides A (**1**, 27.4 mg, t_R = 28.3 min) and B (**2**, 19.3 mg, t_R = 31.1 min) as their TFA salts.

Microcionamide A (1): colorless glass; $[\alpha]_D$ −36.5 (*c* 0.273, MeOH); UV (MeOH) λ_{max} 206 (sh, ϵ 20 000), 220 (sh, ϵ 12 000), 284 nm (ϵ 16 000); IR ν_{max} (NaCl) 3450−3100, 3073, 2964, 2928, 2873, 1650, 1530, 1205, 1185, 1138, 1027, 957, 840, 805, 723, 668 cm^{-1}; for ^1H and ^{13}C NMR data see Table 1; (+)-LRESIMS *m/z* (rel int) 304 (8), 318 (5), 417 (26), 431 (7), 519 (4), 530 (43), 544 (14), 632 (8), 643 (100), 657 (23), 745 (14), 756 (87), 858 (92), 875 (26); (+)-HRESIMS *m/z* 875.488 43 ($C_{43}H_{71}N_8O_7S_2$ $[M + H]^+$ requires 875.488 16).

Microcionamide B (2): colorless glass; $[\alpha]_D$ −40.3 (*c* 0.327, MeOH); UV (MeOH) λ_{max} 208 (sh, ϵ 23 000), 222 (sh, ϵ 15 000), 274 nm (ϵ 14 000); IR ν_{max} (NaCl) 3450−3100, 3073, 2964, 2928, 2873, 1658, 1512, 1203, 1185, 1138, 1027, 846, 801, 724, 688 cm^{-1}; for ^1H and ^{13}C NMR data see Table 2; (+)-LRESIMS *m/z* (rel int) 304 (5), 318 (3), 417 (9), 431 (1), 519 (3), 530 (29), 544 (9), 632 (6), 643 (100), 657 (23), 745 (9), 756 (69), 858 (59), 875 (27); (+)-HRESIMS *m/z* 875.488 49 ($C_{43}H_{71}N_8O_7S_2$ $[M + H]^+$ requires 875.488 16).

Acid Hydrolysis and Stereochemical Determination of Microcionamides A and B. Each peptide (0.5 mg) was dissolved in 6 N HCl (1.0 mL) and heated in an Ar-flushed sealed vial at 105 °C for 16 h. The resulting hydrolysate was lyophilized, dissolved in H_2O (25 μL) and 1N $NaHCO_3$ (208 μL) then derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA)[14] (6.2 mg) in acetone (620 μL) at 40 °C for 1 h. The reaction mixture was neutralized with 2 N HCl (104 μL) and then analyzed by HPLC using a C_{18} column with a linear gradient of triethylammonium phosphate (50 mM, pH 3.0)/MeCN from 90:10 to 60:40 in 40 min, then held at 60:40 for 10 min at a flowrate of 1.0 mL/min. On the basis of HPLC retention times and co-injection with standard amino acid derivatives, the hydrolysate of both **1** and **2** was shown to contain L-isoleucine and L-valine.

Desulfurization of Microcionamides A and B. Raney 2800 nickel (50% slurry in H_2O; 3.4 mg, 57 μmol) was added to each peptide (1.0 mg, 1.1 μmol) in MeOH (1 mL). The resulting black suspension was heated in a sealed vial at 85 °C for 2 h.[15,16] Upon cooling, the solution was purified on a C_{18} SPE cartridge using 90:10 MeOH/0.1% aqueous TFA as eluant. This yielded the pure TFA salt of 36,37-dihydrodesthiomicrocionamide (**3**, 0.5 mg, 54% yield) for both reactions.

36,37-Dihydrodesthiomicrocionamide (3): stable white solid; (+)-LRESIMS *m/z* (rel int) 284 (2), 397 (7), 510 (12), 623 (12), 694 (20), 734 (20), 816 (23), 838 (100).

Acid Hydrolysis and Stereochemical Determination of 36,37-Dihydrodesthiomicrocionamide. The linear peptide (**3**, 0.5 mg) was subjected to the same hydrolysis and derivatization conditions as those used for peptides **1** and **2**. On the basis of HPLC retention times and co-injection with standard amino acid derivatives, the hydrolysate of **3** was shown to contain L-isoleucine, L-valine, and L-alanine.

Culture of Breast Cancer Cell Lines. MCF-7 and SKBR-3 human breast tumor cells were obtained from the American Type Culture Collection (Rockville, MD). The MCF-7 cells were grown as a monolayer in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 1X of antibiotic-antimycotic penicillin-streptomycin and fungizone (PSF), and 0.1 mM nonessential amino acids (NEAA). SKBR-3 cells were grown in McCoy's 5A media containing 10% FBS and 1X of antibiotic−antimycotic PSF. Cell cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. All

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reagents were from Gibco Laboratories, Grand Island, NY, except for PBS, which was from Sigma Chemical Co., St. Louis, MO.

Tumor Cytotoxicity Assay. Cytotoxicity toward MCF-7 and SKBR-3 cells was assessed in an MTT-microtiter plate tetrazolium cytotoxicity assay. This assay was originally described by Mossman[29] and has since been modified by others.[30] MCF-7 and SKBR-3 (20 000 cells/well) were seeded in 200 μL of growth medium in 96-well microtiter plates (Costar) and allowed to attach for 24 h. Cells were treated with the peptides in a 5-fold serial dilution starting from 28.6 μM. The peptides were tested in quadruplicate and were solubilized in 100% DMSO with a final DMSO concentration of 1.0% or less in each well. The treated cells were incubated for 72 h. The media was removed after 72 h, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (15 μL, 5 mg/mL in PBS, pH 7.4) was added followed by a 3 h incubation. MTT is reduced by viable cells to a purple formazan product. Following removal of media the formazan product was solubilized by addition of DMSO (100 μL) to each well. The absorbance for each well at 570 nm was measured using an SLT Model Tecan Spectra III ELISA plate reader. The cell growth in the DMSO control wells was used to determine the zero inhibition growth level for each experiment. Doxorubicin was used as the positive control for both cell lines. Average absorbance for each set of quadruplicate drug-treated wells was compared to the average absorbance of the control wells to determine the percentage of growth inhibition (fractional survival) at a particular drug dose. The fractional survival values were used to compute the IC_{50} using ICPIN computer software (version 2.01).

Apoptosis Assays. MCF-7 human breast tumor cells were plated in a 96-well microtiter plate in triplicate at a density of 20 000 cells/well in 200 μL of media. Cells were incubated overnight at 37 °C at 5% CO_2 and then treated with the peptides at 5.7 μM for 24 h. After treatment, cells were subjected to a terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay. SKBR-3 human breast tumor cells, plated at the same cell density, were subjected to nuclear staining with Hoechst 33342 fluorescent dye after treatment with the peptides at 2.9 μM for 12 h.

Morphological Investigations. Cellular morphology of the MCF-7 peptide treated cells (5.7 μM) was examined using an inverted Nikon microscope equipped with a Nikon SLR camera. Cell images were captured using Kodak film ASA 400.

TUNEL Assay.[20] DNA fragmentation was investigated using a fluorescein cell death detection kit (Boeringer-Mannheim), also known as the TUNEL assay kit. After drug treatment at 5.7 μM for 24 h, the MCF-7 cells were fixed with fresh paraformaldehyde solution (4% in PBS, pH 7.4) for 30 min at rt and then rinsed with PBS [with 1% bovine serum albumin (BSA)]. Cells were incubated with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) at 4 °C for 2 min and then washed twice with PBS (with 1% BSA). TUNEL reaction mixture (50 μL) containing terminal deoxynucleotidyl transferase enzyme and fluorescein-dUTP was added to the cells, and the mixture was incubated at 37 °C in the dark for 1 h and then rinsed three times with PBS (with 1% BSA). Cells were viewed using a Nikon fluorescence microscope equipped with a Nikon SLR camera. Cell images were captured using Kodak film ASA 400.

Nuclear Staining.[21] SKBR-3 human breast tumor cells were seeded at a concentration of 20 000 cells per well in 200 μL media. After overnight incubation at 37 °C with 5% CO_2, each well was treated with 1 μL of 0.57 mM stock solutions of **1** and **2** for a final concentration of 2.9 μM per well. The cultures were incubated with the metabolite for 12 h prior to nuclear staining. Spent media was removed after incubation and replenished with 200 μL of culture medium per well. Then, 1 μL of a 2 mg/mL prepared Hoechst 33342 (Sigma) stock solution was added to each well to give a final concentration of 10 μg/mL. Cells were incubated for 90 min at 27 °C with 5% CO_2. After washing twice with PBS, 100 μL of PBS was added to each well and the plates stored at 4 °C. The cells were observed under a fluorescence microscope at 480 nm and ordinary light. Photographs were taken at a magnification of 400×.

***Mycobacterium* Strain and Culture Conditions.** *M. tuberculosis* $H_{37}Ra$, which was a gift from Dr. Scott Franzblau (Institute for Tuberculosis Research, University of Illinois, Chicago), was cultured as described by Collins and Franzblau.[22] The following media was prepared for the culturing: (1) OADC-supplemented Middlebrook 7H11 agar (100 mL): 1.9 g of 7H11 agar powder (Difco, Detroit, MI) and 0.5 mL of glycerol (Fisher Chemicals, Springfield, NJ) were dissolved in 90 mL of distilled H_2O, autoclaved for 15 min at 15 psi, and allowed to cool, and then 10 mL of OADC enrichment medium was added. (2) OADC-supplemented Middlebrook 7H9 broth (100 mL): 0.47 g of 7H9 broth powder (Difco), 0.1 g of casitone (Difco), and 0.5 mL of glycerol (Fisher) were dissolved in 90 mL of distilled water, autoclaved for 15 min at 15 psi, and allowed to cool, and then 10 mL OADC enrichment medium was added. Twenty microliters of Tween 80 (Sigma, St. Louis, MO) was added to the culture broth before autoclaving. (3) OADC enrichment media (100 mL): 0.05 g (50 μL) of oleic acid (Mallinckrodt Baker, Inc., Paris, KY), 5.0 g of BSA fraction V (Sigma), 2.0 g of dextrose (Difco), 0.004 g of beef catalase (Sigma), and 0.85 g of NaCl (Ajax Chemicals) was dissolved in 100 mL of distilled water, filter-sterilized, and dispensed in 10 mL aliquots. (4) 25% Tween 80 for MABA (20 mL): 5.0 mL of Tween 80 (Sigma) was added to 15.0 mL of 7H9 broth, the mixture was filter-sterilized, and 1.0 mL of the mixture was dispensed into 1.5 mL microfuge tubes.

M. tuberculosis $H_{37}Ra$ was grown on OADC-supplemented Middlebrook 7H11 agar slants at 35 °C. Inoculum was transferred to OADC-supplemented Middlebrook 7H9 culture broth and grown for 2−3 weeks at 35 °C up to a density of $(1-3) \times 10^7$ cfu/mL. The broth culture was diluted 1:50 with OADC-supplemented Middlebrook 7H9 broth.

Anti-tuberculosis Assay. The MABA assay was performed as described by Collins and Franzblau.[22] Using a 96-well microtiter plate, the plate layout was arranged such that perimeter wells contained only color control setups (highest sample dilution without inoculum) or sterile H_2O to prevent dehydration in wells containing samples and inoculum. The final assay volume in each well was 200 μL. Ten microliters of the sample (10 mg/mL in DMSO) was placed in wells for color controls. For the test wells, 10 μL of the sample was added for a high dose (e.g., 500 mg/mL) and 1 μL for a low dose (e.g., 50 mg/mL). One hundred ninety microliters of 7H9 broth was placed in the color control wells, 90 μL in the high-dose wells, and 99 μL in the low-dose wells. DMSO, the negative control, was added in two 10-fold dilutions. Rifampicin, the positive control, was added in at least seven 2-fold dilutions within range of its MIC. Using an 8-channel pipettor, 100 μL of inoculum ($H_{37}Ra$) was dispensed into all test wells except for color controls. Plates were sealed with Parafilm and incubated at 35 °C in a 5% CO_2 incubator for 5 days. Using a combitip and a step pipettor, 10 μL of 25% Tween-80 and 20 μL of Alamar Blue (Alamar Biosciences, Sacramento, CA) was added to the wells. Plates were resealed and incubated overnight. Color change was observed the next day. A blue color in the well was interpreted as no growth or a positive result and a pink color in the well as growth or a negative result.

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Supporting Information Available: General experimental procedures, ^{1}H and ^{13}C NMR spectra for microcionamides A (**1**) and B (**2**), and a table of MS/MS studies and (+)-HRESIMS measurements for microcionamide A. This material is available free of charge via the Internet at http://pubs.acs.org.

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