Massetolides A−H, Antimycobacterial Cyclic Depsipeptides Produced by Two Pseudomonads Isolated from Marine Habitats

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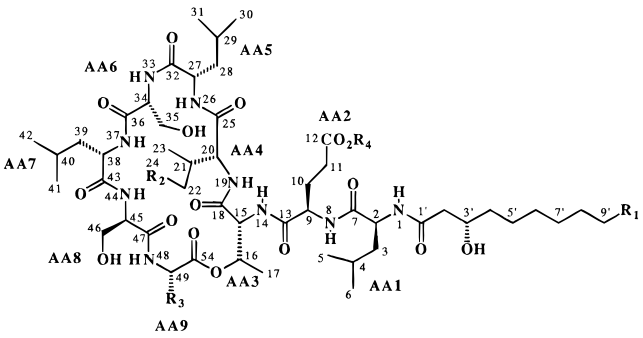
Massetolides A−H (**1**−**8**), novel cyclic depsipetides, and the known compound viscosin (**9**) have been isolated from cultures of two *Pseudomonas* sp. isolated from a marine alga and a marine tube worm, respectively. Massetolide A (**1**) and viscosin (**9**) exhibit in vitro antimicrobial activity against *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare*. Precursor-directed biosynthesis has been used to generate unnatural massetolides **11**−**13** incorporating nonprotein amino acids.

Approximately one-third of the world's population harbors *Mycobacterium tuberculosis* (MTB), the causative agent of tuberculosis (TB), and is at risk for developing the disease.[1] As a consequence, TB has become the leading cause of human death worldwide from infectious diseases. In North America, cases of TB steadily declined from 1882 until 1985 when the incidence began increasing again. Coupled with the increasing incidence of TB has been the emergence of multidrug-resistant strains. One-third of all TB cases tested in a 1991 survey in New York were resistant to one drug or more, a sobering finding because the fatality rate for TB resistant to two or more antibiotics is equivalent to that of untreated TB (40−60%). *Mycobacterium avium-intracellulare* (MAI) causes opportunistic infections that are associated with significant morbidity and mortality in AIDS patients.[2] These infections are difficult to eradicate because MAI is intrinsically resistant to most antibiotics, including those usually active against MTB. Microorganisms isolated from marine habitats represent a largely unexplored resource for the discovery of leads for the development of new drugs.[3−6] As part of an ongoing program designed to screen microorganisms isolated from the oceans for the production of new antimicrobial agents,[7−9] we have found that solid agar cultures of two marine pseudomonads produce massetolides A−H (**1**−**8**) and viscosin (**9**).[10,11] Massetolide A (**1**) and viscosin (**9**) exhibit significant selective in vitro inhibition of MTB and MAI.

Results and Discussion

Marine isolate MK90e85 was obtained from the surface of an unidentified leafy red alga collected in Masset Inlet, BC. It was shown to belong to the genus *Pseudomonas* by fatty acid analysis, but it could not be confidently identified to the species level. Cultures of MK90e85 were grown as lawns on solid agar, and they were harvested by gently scraping the cells from the surface of the agar. Fractionation of the EtOAc-soluble materials from the cell extract gave pure massetolides A (**1**), B (**2**), C (**3**), and D (**4**).



Massetolide A (1) $R_1 = CH_3$-, $R_2 = CH_3$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Massetolide B (2) $R_1 = CH_3CH_2$-, $R_2 = CH_3$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Massetolide C (3) $R_1 = CH_3CH_2CH_2$-, $R_2 = CH_3$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Massetolide D (4) $R_1 = CH_3$-, $R_2 = CH_3$, $R_3 = CH_2CH(CH_3)_2$, $R_4 = H$

Massetolide E (5) $R_1 = CH_3$-, $R_2 = H$, $R_3 = CH(CH_3)_2$, $R_4 = H$

Massetolide F (6) $R_1 = CH_3$-, $R_2 = H$, $R_3 = CH_2CH(CH_3)_2$, $R_4 = H$

Massetolide G (7) $R_1 = CH_3CH_2$-, $R_2 = H$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Massetolide H (8) $R_1 = CH_3CH_2CH_2$-, $R_2 = H$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Viscosin (9) $R_1 = CH_3$-, $R_2 = H$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Ester (10) $R_1 = CH_3$-, $R_2 = CH_3$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = CH_3$

The marine isolate MK91CC8 was obtained from an unidentified tube worm that was collected near Moira Island, BC. MK91CC8 was also shown to be in the genus *Pseudomonas* by fatty acid analysis, but it also could not be confidently identified to the species level. Cultures of MK91CC8 were also grown on solid agar and harvested by gently scraping the cells from the surface of the agar. The agar from MK91CC8 cultures was extracted by soaking in EtOAc to give a crude extracellular extract. Fractionation of the extracellular extract gave pure massetolides E (**5**), F (**6**), G (**7**), and H (**8**) along with the known compound viscosin (**9**).[10,11]

Massetolide A (**1**) was obtained as optically active ($[\alpha]_D$ −45.9°, EtOH) colorless needles that gave a $(M + H)^+$ peak at m/z 1140.7151 in the HRFABMS appropriate for a molecular formula of $C_{55}H_{97}N_9O_{16}$. The ^{1}H- and ^{13}C-NMR spectra obtained for massetolide A contained resonances that were characteristic of peptides (Table 1). Detailed analysis of the COSY, HMQC, HMBC, and HOHAHA data revealed that peptide **1** contained one glutamic acid, one threonine, two isoleucine, three leucine, and two serine residues. Hydrolysis of **1** with 6 N HCl followed by chiral GC analysis of the pentafluoropropionamide isopropyl esters of the indi-

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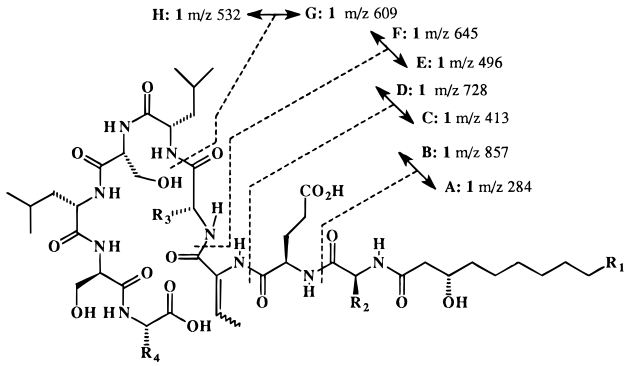
Table 1. NMR Data for Massetolide A (1)[a]

Atom	δ^{13}C	δ^{1}H	Atom	δ^{13}C	δ^{1}H	Atom	δ^{13}C	δ^{1}H
Leu1			Leu5			Ser8		
1		8.80, d, J = 6.0 Hz	26		8.50, d, J = 6.7 Hz	44		8.13, d, J = 9.2 Hz
2	53.8	4.05, m	27	53.8	3.68, dt, J = 6.7, 3.0 Hz	45	57.0	4.60, m
3	39.0	1.85, m	28	37.2	1.78, m	46	63.0	3.70, dd, J = 11.6, 6.3 Hz
4	25.4	2.0	29	25.4	2.0			3.90, dd, J = 6.6, 11.6 Hz
5	22.0	0.95, d	30	21.8	0.91, d	47	171.7	
6	24.0	0.95, d	31	23.8	0.91, d	Ile9		
7	175.5		32	171.1		48		6.64, d, J = 10.1 Hz
Glu			Ser6			49	57.0	4.60, dd, J = 10.1, 3.4 Hz
8		8.37, d, J = 4.6 Hz	33		7.18, d, J = 8.6 Hz	50	36.8	2.0
9	57.1	4.25, m	34	56.1	4.40, dt, J = 8.6, 2.9 Hz	51	25.1	2.0
10	26.5	2.08, m	35	64.8	3.85, dd, J = 2.3, 11.8 Hz	52	16.2	0.86, d
11	31.0	2.51, t, J = 7.6 Hz			4.20	53	12.2	0.92, t, J = 7.0 Hz
12	174.1		36	172.1		54	169.7	
13	176.5		Leu7			FA		
Thr			37		7.62, d, J = 5.4 Hz	1′	174.7	
14		8.18, d, J = 7.4 Hz	38	55.2	4.20, m	2′	44.6	2.63, dd, J = 4.1, 14.0 Hz
15	61.8	4.18, dd, J = 7.4, 10.7 Hz	39	42.0	1.65, m			2.56, dd, J = 4.4, 14.0 Hz
16	70.12	5.38, dq, J = 10. 7,5.9 Hz	40	25.3	2.0	3′	70.1	4.14, m
17	18.6	1.45, d, J = 5.9 Hz	41	21.4	0.93, d	4′	38.3	1.60, m
18	174.4		42	23.3	1.00, d, J = 6.3 Hz	5′	26.1	1.3
Ile4			43	174.1		6′	30.3	1.3
19		8.50, d, J = 5.8 Hz				7′	30.0	1.3
20	64.5	3.58, dd, J = 5.8, 11.5 Hz				8′	32.5	1.3
21	36.0	2.0				9′	23.3	1.3
22	26.7	1.15, m				10′	14.3	0.9, t, J = 7 Hz
22		1.52, m						
23	16.6	1.09, d, J = 6.5 Hz						
24	10.7	0.94, t, J = 6.5 Hz						
25	174.1							

[a] Recorded in Me_2CO-d_6 at 500 MHz for ^{1}H and 125 MHz for ^{13}C.

vidual amino acids confirmed the presence of D-serine, D-glutamic acid, L-isoleucine, D-alloisoleucine, L-leucine, and D-allothreonine. Subtraction of the C, N, and O atoms accounted for by the 9 identified amino acid residues ($C_{45}N_9O_{14}$) from the molecular formula of massetolide A showed that the remaining fragment of the molecule had to contain 10 carbon and two oxygen atoms. The ^{13}C/APT and HMQC data showed that the 10 carbon atoms not accounted for by amino acid residues consisted of one aliphatic methyl (CH_3), one carbinol methine (CHOH), one ester/amide carbonyl (C═O), and seven aliphatic methylene (CH_2) carbons. This suite of carbons could only be accommodated by a linear 10-carbon chain having the methyl and ester/amide carbonyl carbons at either terminus. The HMQC spectrum revealed that the carbinol methine carbon (δ 70.1) was attached to a proton that gave an ^{1}H-NMR resonance at δ 4.14 ppm. In the COSY spectrum, the carbinol methine proton resonance (δ 4.14) was correlated to a pair of geminal methylene proton resonances at δ 2.56 and 2.63. These methylene proton resonances were correlated in the HMBC spectrum to the ester/amide carbonyl carbon (δ 174.7) that was at the terminus of the linear 10-carbon chain. Taken together, the above evidence suggested the presence of a 3-hydroxydecanoyl fragment. An HMBC correlation between the Leu1-NH resonance at δ 8.80 and the 3-hydroxydecanoyl carbonyl resonance at δ 174.72 showed that the fatty acid fragment was linked to the Leu1 residue via an amide bond. ROESY correlations between the Leu1-NH resonance at δ 8.80 and the 3-hydroxydecanoyl α-proton resonances at δ 2.56 and 2.63 and the observation of an intense HRFABMS fragment at m/z 284 (Table 2) having a molecular composition of $C_{16}H_{20}NO_3$ corresponding to the FA-Leu1 unit confirmed this linkage.

Table 2. FABMS Fragment Ions for the Massetolides and Visocsin[a]



	FABMS fragment peaks (m/z)								
	(M + H)	A	B	C	D	E	F	G	H
1	1141	284	857	413	728	496	645	609	532
2	1155	298	857	427	728	510	645	623	532
3	1169	312	857	441	728	524	645	637	532
4	1141	284	857	413	728	496	645	609	532
5	1113	284	829	413	700	496	617	595	518
6	1127	284	843	413	714	496	631	595	532
7	1141	298	843	427	714	510	631	609	532
8	1155	312	843	441	714	524	631	623	532
9	1127	284	843	413	714	496	631	595	532
11	1113	284	829	413	700	496	617	581	532
12	1113	270	843	399	714	482	631	581	532
13	1125	282	843	411	714	494	631	593	532

[a] Initially the macrocyclic lactone is cleaved by elimination at threonine to give a linear peptide that then fragments as shown in the structural diagram.

The amino acid sequence in massetolide A (**1**) was determined from the HMBC data. HMBC correlations observed between the Glu-NH (δ 8.37) and the Leu1-CO (δ 175.5) resonances; between the Thr-NH (δ 8.18) and the Glu-CO (δ 176.5) resonances; and between the

Table 3. ^{1}H Chemical Shifts for the α-Methine Resonances in the Massetolides and Viscosin[a]

	α methine protons								
	chemical shift (δ ppm), multiplicity, *J* values (Hz)								
	AA1	AA2	AA3	AA4	AA5	AA6	AA7	AA8	AA9
massetolide A	4.05 m	4.25 m	4.18 dd, 7.4, 10.7	3.58 dd, 5.8, 11.5	3.68 dt, 6.7, 3.0	4.40 dt, 8.6, 2.9	4.20 m	4.60 m	4.60 dd, 10.1, 3.4
B	3.99 m	4.18 m	4.13 m	3.55 dd, 5.8, 11.1	3.67 m	4.37 m	4.18 m	4.48 m	4.57 dd, 11.5, 3.0
C	3.99 m	4.18 m	4.10 m	3.55 dd, 5.8, 11.1	3.67 m	4.37 m	4.17 m	4.48 m	4.57 dd, 10.2, 3.2
D	4.01 m	4.18 m	4.12 m	3.60 dd, 5.6, 11.1	3.69 m	4.37 dt, 8.2, 2.0	4.20 m	4.49 m	4.59 dt, 4.0, 10.0
E	4.08 m	4.26 m	4.16 m	3.47 dd, 5.9, 11.0	3.69 m	4.40 dt, 8.6, 2.0	4.20 m	4.55 m	4.55 dd, 3.4, 10.0
F	4.05 m	4.22 m	4.17 m	3.51 dd, 11.0, 5.7	3.69 m	4.39 m	4.13 m	4.52 m	4.60 dt, 9.8, 4.4
G	4.08 m	4.23 m	4.20 m	3.46 dd, 10.5, 5.5	3.67 m	4.40 br d, 8.7	4.15 m	4.51 m	4.58 dd, 10.0, 3.0
H	4.12 m	4.21 m	4.18 m	3.43 dd, 11.3, 5.4	3.66 m	4.40 m	4.14 m	4.50 m	4.57 dd, 10.2, 3.0
viscosin	4.07 dt, 14.0, 5.9	4.24 m	4.18 m	3.46 dd, 11.2, 5.8	3.69 m	4.42 dd, 8.6	4.14 m	4.57 m	4.58 dd, 10.0, 3.1

[a] Recorded in Me_2CO-d_6 at 500 MHz.

Ile4-NH (δ 8.05) and the Thr-CO (δ 174.4) resonances defined the partial sequence FA-Leu1-Glu-Thr-Ile4-CO. Reaction of massetolide A (**1**) with CH_2N_2 formed the monomethyl ester **10**. NMR and MS analysis showed that esterification had occurred at the Glu δ carboxylic acid, confirming that the Glu-Thr peptide bond involved the Glu C-1 carboxylic acid. The downfield chemical shift of the Thr carbinol methine proton resonance (δ 5.38) in massetolide A (**1**) indicated that the hydroxyl group was part of an ester linkage. An HMBC correlation between the Thr carbinol methine proton (δ 5.38) and the Ile9-CO (δ 169.7) resonances demonstrated that Ile9 was connected to the Thr via an ester linkage. The Ile9-NH resonance (δ 6.64) showed an HMBC correlation to the Ser8-CO resonance (δ 171.7) indicating that the Ile9 amino group and the Ser8 carbonyl formed a peptide bond. Additional HMBC correlations observed between the Leu7-NH (δ 7.62) and the Ser6-CO (δ 172.1) resonances and between the Ser6-NH (δ 7.18) and Leu5-CO (δ 171.1) resonances established the partial sequence HN-Leu5-Ser6-Leu7-CO. The molecular formula of massetolide A (**1**) requires 12 sites of unsaturation. Because only 11 of these could be accounted for by the carbonyl functionalities identified in the component amino acid and fatty acid residues, the remaining site of unsaturation had to be present as a ring. The only way to join the two partial structures identified from the HMBC data together to form a ring and to leave the Glu δ carboxylic acid free was to link the Leu5 amino group to the Ile4 carboxyl and the Leu7 carboxyl group to the Ser8 amino group as shown in **1**.

FABMS (Table 2) and ROESY data supported the sequence assigned from the HMBC data. ROESY correlations observed between the Glu-NH (δ 8.37) and Leu1-αCH (δ 4.05) resonances, between the Ile4-NH (δ 8.05) and Thr-βCH (δ 5.38) resonances, between the Ile9-NH (δ 6.64) and Ser8-αCH (δ 4.60) resonances, and between the Ser6-NH (δ 7.18) and Leu5-αCH (δ 3.68) resonances confirmed the HN-Leu1-Glu-CO, HN-Thr-Ile4-CO, HN-Leu5-Ser6-CO, and HN-Ser8-Ile9-CO partial sequences identified from the HMBC data. The FABMS showed peaks at *m/z* 971 [(MH − (FA)], 857 [MH - (FA-Leu1)], 728 [MH − (FA-Leu1-Glu)], 645 [MH − (FA-Leu1-Glu-Thr(−H_2O))], 609 [MH − [FA-Leu1-Glu-Thr(−H_2O)-Ile4)], 496 [FA-Leu1-Glu-Thr(−H_2O)], 413 (FA-Leu1-Glu), and 284 (FA-Leu1) that were consistent with the proposed structure **1** for massetolide A. The data for massetolide A (**1**) alone did not identify which of the stereochemical forms of isoleucine, namely L-isoleucine or D-alloisoleucine, were at the AA4 and AA9 positions. This point was clarified with the subsequent characterization of massetolide D (**4**) (see below). Once the structure of massetolide A (**1**) was in hand, it became apparent that it was related to viscosin (**9**)[10,11] and the "white line inducing principle" (WLIP)[12] isolated from *P. viscosa* and *P. reactans*, respectively. Because both viscosin (**9**) and WLIP contain D-β-hydoxydecanoyl residues, it has been assumed that the β-hydroxydecanoyl residue in massetolide A (**1**) also has the D configuration.

Massetolide B (**2**) gave a $(M + H)^+$ peak at *m/z* 1154.732 39 in the HRFABMS appropriate for a molecular formula of $C_{56}H_{99}N_9O_9$ that differed from the molecular formula of massetolide A (**1**) simply by addition of CH_2. Comparison of the ^{1}H-NMR spectrum for massetolide B (**2**) with the ^{1}H-NMR spectrum of massetolide A (**1**) (Table 3) revealed that they contained the same amino acids, and the FABMS data (Table 2) showed that they differed only in the β-hydroxy fatty acid residue. A peak at *m/z* 298 in the FABMS of **2** (Table 2), corresponding to the FA-Leu fragment A, required a fatty acid residue containing one additional methylene group compared with the β-hydroxydecanoic acid residue in **1**. Hydrolysis of **2** with 6 N HCl followed by chiral GC analysis of the pentafluoropropionamide isopropyl esters of the individual amino acids confirmed the presence of the D-serine, D-glutamic acid, L-isoleucine, D-alloisoleucine, L-leucine and D-allothreonine residues also found in **1**. The GC analysis also gave a peak corresponding to the derivative formed from authentic β-hydroxyundecanoic acid under the hydrolysis and derivatization conditions, confirming that massetolide B had structure **2**. A similar analysis of the data (Tables 2 and 3) for massetolide C (**3**) (HRFABMS *m/z* 1168.745 44, $C_{34}H_{101}N_9O_{16}$) showed that it was simply the β-hydroxydodecanoic acid homolog of massetolide A (**1**).

Massetolide D (**4**) gave a $(M + H)^+$ ion at *m/z* 1140.713 79 in the HRFABMS that was appropriate for a molecular formula of $C_{55}H_{97}N_9O_{16}$, identical with that of massetolide A (**1**). Hydrolysis of **4** with 6 N HCl followed by chiral GC analysis of the pentafluropropylamide isopropyl ester derivatives of the liberated residues identified D-alloisoleucine, D-allothreonine, D-serine, L-leucine, D-glutamic acid, and β-hydroxydecanoic acid. The hydrolysis results combined with the MS data (Table 2) suggested that massetolide D (**4**) differed from massetolide A (**1**) only by the replacement of isoleucine

Table 4. Chiral GC Retention Times (in minutes) of Pentafluoropropionamide Isopropyl Ester Derivitives[a]

	standard	**1**	**2**	**3**	**4**	**5**	**6**	**8**	**11**	**9**
D-Butyrine	10.54								10.54	
D-Thr	10.64									
D-Val	10.93					10.79	10.46	10.85		10.82
L-Thr	10.96									
L-Val	11.33					11.19				
D-allo Ile	12.75	12.80	12.61	12.65	12.67					
D-Ile	13.18									
L-Ile	13.62	13.66	13.46	13.50				13.67	13.65	13.53
D-allo Thr	13.98	13.95	13.69	13.77	13.82	13.77	13.71	13.97	13.97	13.83
D-Ser	14.32	14.22	13.93	14.02	14.09	14.01	13.96	14.17	14.18	14.10
L-allo Thr	14.40									
D-Leu	14.54									
L-Ser	14.62									
L-Leu	15.40	15.36	15.11	15.19	15.26	15.15	15.11	15.39	15.37	15.21
C10 βOH FA	20.98	21.22			21.06	21.02	20.95		21.43	21.05
C11 βOH FA	23.94		23.74							
D-Glu	25.94	25.74	25.53	25.63	25.62	25.55	25.50	25.96	25.97	25.61
L-Glu	26.31									
C12 βOH FA	26.67			26.45				26.91		

[a] All identifications were made by coinjection with an authentic standard.

with either of the equal mass residues leucine or D-alloisoleucine. Comparison of the ^{1}H NMR data (Table 3) for massetolide D (**4**) with that for massetolide A (**1**) revealed that the only detectable difference in the two spectra was in the multiplicity of the α-methine resonance assigned to the AA9 residue in the spectrum of **1**. In the spectrum of massetolide A (**1**), the isoleucine AA9 α-methine resonance (H49) appeared as a doublet of doublets at δ 4.60 (J = 10.1, 3.4 Hz) reflecting scalar coupling to the NH and β-methine protons. The corresponding resonance in the ^{1}H-NMR spectrum of massetolide D (**4**) appeared at the nearly identical chemical shift of δ 4.59, but it was a doublet of triplets (J = 4.0, 10.0 Hz) consistent with the presence of a leucine residue at the AA9 position and the D-alloisoleucine at the AA4 position.

The major antimycobacterial component from extracts of the MK91CC8 cultures was the known compound viscosin (**9**). Even though the structure of vicosin had been in the literature for some time and the compound had been quite extensively investigated, the published NMR data were not sufficiently detailed to allow a direct comparison with the data collected on **9** isolated from the MK91CC8. Thus, an independent structure elucidation involving FABMS, NMR, and hydrolytic analysis of MK91CC8 viscosin (**9**) was undertaken. This investigation confirmed that viscosin isolated from the MK91CC8 cultures indeed had the same structure as the known natural product.

Massetolide E (**5**) gave a $(M + H)^+$ ion at m/z 1126.697 08 in the HRFABMS appropriate for a molecular formula of $C_{53}H_{93}N_9O_{16}$ that differed from the molecular formula of viscosin (**9**) by the loss of CH_2. Hydrolysis of **5** with 6 N HCl followed by chiral GC analysis of the pentafluoropropionamide isopropyl esters of the individual amino acids identified the presence of L-valine, D-valine, D-serine, D-glutamic acid, L-leucine, and D-allothreonine residues. Comparison of the fragment masses in the FABMS spectrum of **5** (Table 2) with those for viscosin (**9**) indicated that the molecules were identical in the sequence between the β-hydroxydecanoic acid through AA4. Examination of the ^{1}H-NMR spectrum of **5** (Table 3) revealed that the methine resonance at δ 4.55, which could be assigned on the basis of its chemical shift to the α-methine proton (H49) of the AA9 residue by analogy with massetiolides A−D (**1**−**4**) and viscosin (**9**), was a doublet of doublets (J = 3.4, 10.0 Hz). The multiplicity of the AA9 methine resonance indicated that the second valine residue identified by the GC analysis was at this position and, therefore, that massetolide E had the structure **5**.

Massetolide F (**6**) gave a $(M + H)^+$ ion in the HRFABMS at m/z 1126.697 08 appropriate for a molecular formula of $C_{54}H_{95}N_9O_{16}$, identical to that of viscosin (**9**). Hydrolysis of **6** with 6 N HCl followed by chiral GC analysis of the pentafluoropropionamide isopropyl esters of the individual amino acids identified the presence of D-valine, D-serine, D-glutamic acid, L-leucine, and D-allothreonine residues. The only difference in the MS and ^{1}H-NMR data (Tables 2 and 3) of viscosin (**9**) and massetolide F (**6**) was in the multiplicity of the AA9 α-methine resonance. In the ^{1}H-NMR spectrum of massetolide F (**6**), the methine resonance at δ 4.60 (H49), assigned to the AA9 residue, was a doublet of triplets (J = 9.8, 4.4 Hz), which indicated the presence of a leucine at this position. Massetolides G (**7**) and H (**8**) were routinely shown to be the β-hydroxyundecanoic acid and β-hydroxydodecanoic acid analogues of visosin (**9**), respectively, by analysis of their HRFABMS, ^{1}H-NMR, and hydrolysis data (Tables 2, 3, and 4).

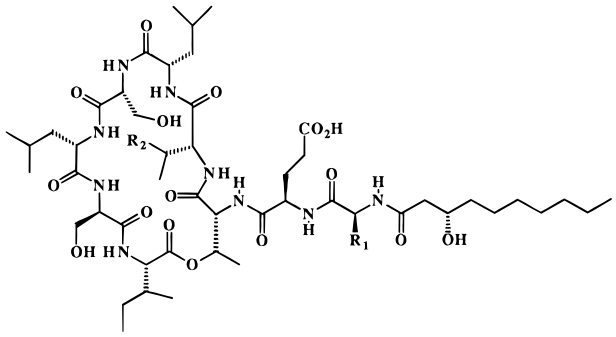
Massetolide A (**1**) and viscosin (**9**) were tested for activity against *M. tuberculosis* and *M. avium-intracellulare* by using the proportion method on Middlebrook 7H10 agar.[13] Against *M. tuberculosis*, massetolide A (**1**) had a MIC of 5−10 μg/mL, and viscosin (**9**) had a MIC of 10−20 μg/mL; against *M. avium-intracellulare*, massetolide A (**1**) had a MIC of 2.5−5 μg/mL, and viscosin (**9**) had a MIC of 10−20 μg/mL. No activity was observed for either compound against a panel of other human pathogenic bacteria including *Escherichia coli* and *Staphylococcus aureus*. A single intraperitoneal injection of 10 mg/kg of massetolide A (**1**) was found to be nontoxic to mice.

Examination of the MICs for massetolide A (**1**) and viscosin (**9**) against *M. tuberculosis* and *M. avium intracellulare* showed that variations in the nature of the aliphatic side chains of the component amino acids at the AA4 and AA9 positions resulted in a twofold change in the potency of the compounds. There appears to be a general correlation between greater lipophilicity and increased potency in families of antimycobacterial

compounds,[14] and this is consistent with the lower MICs observed for massetolide A (**1**) vs viscosin (**9**) against MTB and MAI. It has been demonstrated in several cases that it is possible to generate new analogues of microbial secondary metabolite peptides by precursor-directed biosynthesis.[15] The natural variations encountered at the positions AA4 and AA9 in the massetolide/viscosin family of cyclic depsipeptides isolated from MK90e85 and MK91CC8 suggested that analogue production by precursor-directed biosynthesis might be possible with these two organisms. Thus, several precursor-directed biosynthetic experiments were carried out with MK91CC8 in order to test the possibility of generating new analogues that might have more potent antimycobacterial activity.

Butyrine, norvaline, *tert*-leucine, and cyclopropylalanine, four nonprotein analogues of valine and leucine, were chosen as candidates for precursor-directed biosynthesis with MK91CC8. Samples of L- and D-butyrine, L- and D-norvaline, and L- and D-*tert*-leucine were available commercially, and L-cyclopropylalanine was available via a recently published synthesis.[16] Each of the nonprotein amino acids was added independently to MK91CC8 culture media at a concentration of 250 mg/L, and then solid agar cultures were grown, harvested, and the massetolides were purified as described above for the natural products. The feeding experiments with L-butyrine, L-norvaline, and L-cyclopropylalanine each gave evidence for production of new massetolide analogues that incorporated the unnatural amino acids, while the experiments with D-butyrine, D-norvaline, L-*tert*-leucine, and D-*tert*-leucine gave no evidence for the production of new analogues. Because the amounts of new analogues that were produced were typically very small, characterization was accomplished mainly by HRFABMS and chiral GC examination of derivatized hydrolysis products.

The L-butyrine feeding experiment generated massetolide I (**11**) as the major new analogue. Massetolide I (**11**) gave a parent ion in the HRFABMS at *m/z* 1112.680 29, appropriate for a molecular formula of $C_{53}H_{93}N_9O_{16}$, that differed from the molecular formula of viscosin (**9**) by the loss of CH_2. Fragments E−H in the FABMS of massetolide I (**11**) (Table 2) appeared at *m/z* 496, 617, 581, and 532, respectively, compared with *m/z* 496, 631, 595, and 532 for the corresponding fragments in the FABMS of viscosin (**9**), indicating that **11** had a butyrine residue at position AA4. Support for the butyrine for valine substitution in **11** came from GC analysis of the derivatized hydrolysis products, which showed the absence of a peak coresponding to D-valine and the presence of a peak corresponding to D-butyrine (Table 4). The L-norvaline feeding experiment generated massetolide J (**12**) as the major unnatural analogue. Massetolide J (**12**) gave a $(M + H)^+$ ion in the HRFABMS at *m/z* 1112.681 84 appropriate for a molecular formula of $C_{53}H_{93}N_9O_{16}$, that also differed from the molecular formula of viscosin (**9**) by the loss of CH_2. Analysis of the FABMS fragmentation pattern (Table 2) revealed that in massetolide J (**12**) a norvaline residue had replaced the L-leucine residue found at AA1 in viscosin (**9**). Chiral GC analysis (Table 4) of the derivatized hydrolysis products of massetolide J (**12**) showed that the norvaline residue had the L configuration. The cyclopropylalanine feeding experiment gave massetolide K (**13**) as the major unnatural analogue. Massetolide K (**13**) gave a $(M + H)^+$ ion at *m/z* in the HRFABMS appropriate for a molecular formula of $C_{54}H_{94}N_9O_{16}$. Analysis of the fragmentation pattern in the FABMS of **13** showed that the cyclopropylalanine residue had been incorporated at the AA1 position. The ^{1}H-NMR spectrum of massetolide K (**13**) showed a series of upfield resonances (δ −0.03, 0.37, and 0.80) that could be attributed to the cyclopropyl protons in the cyclopropylalanine residue.



Massetolide I (11) $R_1 = CH_2CH(CH_3)_2$, $R_2 = H$

Massetolide J (12) $R_1 = CH_2CH_2CH_3$, $R_2 = Me$

Massetolide K (13) $R_1 = CH_2\text{-}CH(CH_2)_2$, $R_2 = Me$

A number of additional unnatural massetolides were obtained from the various feeding experiments but only as inseparable mixtures of paired compounds. These analogues could not be characterized with the same degree of confidence as massetolides I−K. However, analysis of the FABMS data for the mixtures indicated that norvaline, cyclopropylalanine, and butyrine substitution had apparently occurred at the AA9 position as well. The amounts of unnatural massetolides obtained from the precursor-directed biosynthetic experiments were too small to allow antimycobacterial testing. However, these experiments have shown that it is possible to generate new massetolide analogues incorporating nonprotein amino acids at positions AA1, AA4, and AA9 by feeding the cultures nonprotein analogues of valine, leucine, and isoleucine. *tert*-Leucine appears to be too sterically demanding to be accepted by the peptide synthase, and thus it was not incorporated. It is worth noting that only the L form of butyrine was incorporated into the D-AA4 position in the massetolide skeleton. This suggests that epimerization takes place during assembly of the depsipeptide and not in the free amino acid pool. Massetolide K (**13**) appears to represent the first complex peptide incorporating cyclopropylalanine, a cyclic analogue of leucine. The dispersion of the cyclopropyl proton resonances in the ^{1}H-NMR spectrum of massetolide K (**13**) demonstrates that this substitution might be a useful tool for studying the orientation of the side-chain methyls of leucine groups or valine groups in peptides. Normally the methyl group ^{1}H-NMR resonances of leucine or valine in highly aliphatic peptides are buried in an unresolved envelope of methyl resonances. By substituting cyclopropylalanine for a particular leucine residue, a set of well-dispersed resonances are generated for the protons on the carbons that are formally equivalent to the leucine methyl carbons. These well-dispersed cyclopropyl resonances should be good NOE antennae for determining

the location of the side-chain methyls relative to the rest of the peptide structure. Experiments along these lines are in progress for **13**.

Experimental Section

NMR data were collected on a Bruker AMX500 spectrometer equipped with a 5-mm probe. All spectra were obtained in Me_2CO-d_6. Proton spectra were referenced using internal residual Me_2CO-d_5 (δ 2.04), and carbon spectra were referenced to the Me_2CO methyl carbon resonance (δ 29.8). FABMS data were collected on a Kratos Concept IIHQ hybrid mass spectrometer with cesium ion secondary ionization and a magnetic sector mass analyzer. Samples were dissolved in a MeOH−thioglycerol matrix, and spectra were obtained using a source voltage of 8 kV and a cesium ion gun voltage of 12 kV. Fragment ion peaks were confirmed via secondary MS−MS using a quadrapole mass analyzer.

The marine isolate MK90e85 was obtained from the surface of an unidentified leafy red alga collected at −15 m in Masset Inlet, BC. It was identified as a *Pseudomonas* sp. by fatty acid analysis (MIDI). MK90e85 has been cryopreserved and deposited in the marine microbial cuture collections at SeaTek and UBC. Moderate scale cultures of MK90e85 were grown for 4 days at 16 °C as lawns on the surface of solid trypticase soy agar supplemented with NaCl to a final concentration of 1.5%. The cultures were harvested by gently scraping the cells from the surface of the agar. Lyophilization of the cells (41 g dry wt) followed by repeated extraction with MeOH gave a crude cell extract that was partitioned between EtOAc and 10% MeOH in H_2O. The EtOAc-soluble materials (1.2 g) were fractionated via Sephadex LH-20 chromatography (MeOH) and isocratic reversed-phase HPLC (7:3 CH_3CN−H_2O with 0.05% TFA) to give pure massetolides A (**1**) (232 mg), B (**2**) (1 mg), C (**3**) (0.8 mg), and D (**4**) (1.9 mg).

The marine isolate MK91CC8 was obtained from an unidentified tube worm that was collected via Scuba diving near Moira Island in Moira Sound, BC. It was also identified as a *Pseudomonas* sp. by fatty acid analysis (MIDI). MK91CC8 has been cryopreserved and deposited in the marine microbial cuture collections at SeaTek and UBC. Cultures of MK91CC8 were grown for 4 days at 16 °C as lawns on the surface of solid trypticase soy agar supplemented with NaCl to a final concentration of 1.5%. The cultures were harvested by gently scraping the cells from the surface of the agar, and the agar was extracted by soaking in EtOAc, giving a crude extracellular extract (1.8 g from 20 trays). This extract was fractionated via reversed-phase flash column chromatography (gradient CH_3CN−H_2O with 0.05% TFA) and isocratic reversed-phase HPLC (8:2 CH_3CN−H_2O with 0.05% TFA) to give pure massetolides E (**5**) (6 mg), F (**6**) (4 mg), G (**7**) (0.5 mg), and H (**8**) (0.5 mg), along with the known compound viscosin (**9**) (250 mg).

Massetolide A (1): isolated as a white solid; mp 237−238 °C dec; $[\alpha]_D$ +45.9° (ETOH); HRFABMS *m/z* (formula, ΔM ppm) 1140.715 06 (M + H, $C_{55}H_{98}N_9O_{16}$, 1.67), 857.500 61 ($C_{39}H_{69}N_8O_{13}$, 2.56), 728.455 08 ($C_{34}H_{62}N_7O_{10}$, −1.01), 645.418 99 ($C_{30}H_{57}N_6O_9$, 0.45), 609.388 48 ($C_{31}H_{53}N_4O_8$, 3.51), 532.337 10 ($C_{24}H_{46}N_5O_8$, 4.62), 496.300 11 ($C_{25}H_{42}N_3O_7$, −4.36), 413.264 92 ($C_{21}H_{37}N_2O_6$, −0.59), 284.222 05 ($C_{16}H_{30}NO_3$, −1.84).

Massetolide B (2): isolated as a white solid; HR-FABMS *m/z* (formula, ΔM ppm) 1154.732 39 (M + H, $C_{56}H_{100}N_9O_{16}$, 3.11), 857.499 31 ($C_{39}H_{69}N_8O_{13}$, 1.05), 728.455 74 ($C_{34}H_{62}N_7O_{10}$, −0.10), 645.417 62 ($C_{30}H_{57}N_6O_9$, −1.67), 623.399 59 ($C_{32}H_{55}N_4O_8$, −3.85), 532.333 90 ($C_{24}H_{46}N_5O_8$, −1.39), 510.317 14 ($C_{26}H_{44}N_3O_7$, −1.55), 427.281 30 ($C_{22}H_{39}N_2O_6$, 1.15), 298.237 43 ($C_{17}H_{32}NO_3$, −2.66).

Massetolide C (3): isolated as a white solid; HR-FABMS *m/z* (formula, ΔM ppm) 1168.745 44 (M + H, $C_{57}H_{102}N_9O_{16}$, 0.84), 857.500 31 ($C_{39}H_{69}N_8O_{13}$, 2.22), 728.456 01 ($C_{34}H_{62}N_7O_{10}$, 0.26), 645.419 20 ($C_{30}H_{57}N_6O_9$, 0.77), 637.416 64 ($C_{33}H_{57}N_4O_8$, −1.58), 532.336 21 ($C_{24}H_{46}N_5O_8$, 2.95), 524.334 40 ($C_{27}H_{46}N_3O_7$, 1.56), 441.296 74 ($C_{23}H_{41}N_2O_6$, 0.64), 312.254 12 ($C_{18}H_{34}NO_3$, 0.81).

Massetolide D (4): isolated as a white solid; HR-FABMS *m/z* (formula, ΔM ppm): 1140.713 79 (M + H, $C_{55}H_{98}N_9O_{16}$, 0.56), 857.495 41 ($C_{39}H_{69}N_8O_{13}$, −3.50), 728.454 30 ($C_{34}H_{62}N_7O_{10}$, −2.08), 645.419 40 ($C_{30}H_{57}N_6O_9$, 1.09), 609.384 86 ($C_{31}H_{53}N_4O_8$, −2.43), 532.3382 ($C_{24}H_{46}N_5O_8$, −1.54), 496.304 80 ($C_{25}H_{42}N_3O_7$, 5.00), 413.265 37 ($C_{21}H_{37}N_2O_6$, 0.50), 312.254 12 ($C_{16}H_{30}NO_3$, −2.86).

Massetolide E (5): isolated as a white solid; HR-FABMS *m/z* (formula, ΔM ppm) 1112.681 73 (M + H, $C_{53}H_{94}N_9O_{16}$, −0.11), 829.466 18 ($C_{37}H_{65}N_8O_{13}$, −1.12), 700.423 28 ($C_{32}H_{58}N_7O_{10}$, −1.63)

Massetolide F (6): isolated as a white solid; HR-FABMS *m/z* (formula, ΔM ppm) 1126.697 08 (M + H, $C_{54}H_{96}N_9O_{16}$, −0.37).

Massetolide G (7): isolated as a white solid; HR-FABMS *m/z* (formula, ΔM ppm) 1140.713 46 (M + H, $C_{55}H_{98}N_9O_{16}$, 0.26), 270.242 98 ($C_{16}H_{30}NO_3$, −1.19).

Massetolide H (8): isolated as a white solid; HR-FABMS *m/z* (formula, ΔM ppm) 1154.728 34 (M + H, $C_{56}H_{100}N_9O_{16}$, −0.40).

Viscosin (9): isolated as a white solid; HRFABMS *m/z* (formula, ΔM ppm) 1126.696 95 (M + H, $C_{54}H_{96}N_9O_{16}$, −0.49), 843.483 78 ($C_{38}H_{67}N_8O_{13}$, 1.21), 714.442 49 ($C_{33}H_{60}N_7O_{10}$, 3.25), 631.403 25 ($C_{29}H_{55}N_6O_9$, 0.31), 595.371 54 ($C_{30}H_{51}N_4O_8$, 1.43), 496.301 89 ($C_{25}H_{42}N_3O_7$, −0.79), 413.265 02 ($C_{21}H_{37}N_2O_6$, −0.35), 284.221 97 ($C_{16}H_{30}NO_3$, −2.12), 256.227 45 ($C_{15}H_{30}NO_2$, −0.78).

Massetolide A Methyl Ester (10): Massetolide A (**1**) (30 mg) was dissolved in 5 mL THF and reacted with CH_2N_2 in a micro molar generator using a dry ice−Me_2CO bath to cool the THF solution. Massetolide A methyl ester (**10**) was purified using reversed-phase ODS HPLC with 7:3 CH_3CN−H_2O as eluent to yield 30 mg of massetolide A methyl ester (**10**): isolated as a white solid; HRFABMS *m/z* (formula, ΔM ppm): 1154.727 66 (M + H, $C_{56}H_{100}N_9O_{16}$, −0.99).

Hydrolysis of Massetolides. The massetolide (0.5 mg) was dissolved in 6 N HCl (1 mL) and heated at 110 °C for a period of 3 days in a glass vial. The HCl was removed under a stream of N_2 gas. *i*-PrOH−HCl (250 μL) was added to the residue, the vial sealed and heated to 110 °C for a further 45 min, and then reduced to dryness using a stream of dry N_2. The residue was dissolved in CH_2Cl_2 (250 μL), pentafluoropropionyl anhydride (100 μL) was added, and the vial was sealed and heated to 110 °C for 15 min. Excess reagent was removed, and the derivatives were stored at −20 °C

until ready for use. Standards were prepared in the same fashion using pure amino acids. Derivatized samples were dissolved in CH_2Cl_2 (200 μL), and 1 μL was injected onto a 50-m Chiralsil-Val Heliflex GC column. Elution took place over a constant temperature gradient from 90 °C to 200 °C over a 27.5-min period with the oven temperature constant at 90 °C for 4 min prior to running the gradient and either 1 or 6 min at 200 °C at the end. Helium was used as the carrier gas.

Precursor-Directed Biosynthesis. All precursor-directed biosynthetic experiments were carried out using organism MK91CC8. The cells were grown on the usual media supplemented with 250 mg of the nonprotein amino acid per liter of culture media. As in all other experiments, the cells were grown for 4 days and scraped from the agar, which was then extracted with EtOAc. The crude extract was purified in the same manner as before to give a massetolide fraction. The new peptides were identified initially by comparison of HPLC traces with those obtained when no amino acids were fed.

Massetolide I (11): isolated as a white solid; HR-FABMS *m/z* (formula, ΔM ppm) 1112.680 29 (M + H, $C_{53}H_{94}N_9O_{16}$, −1.41), 581 ($C_{29}H_{49}N_4O_8$, −5.78).

Massetolide J (12): isolated as a white solid; HR-FABMS *m/z* (formula, ΔM ppm) 1112.681 84 (M + H, $C_{53}H_{94}N_9O_{16}$, −0.01), 843.481 08 ($C_{38}H_{67}N_8O_{13}$, −1.99), 581.350 63 ($C_{29}H_{49}N_4O_8$, −7.58), 270.205 92 ($C_{15}H_{28}NO_3$, −3.71).

Massetolide K (13): isolated as a white solid; HR-FABMS *m/z* (formula, ΔM ppm) 1124.679 74 (M + H, $C_{54}H_{94}N_9O_{16}$, −1.88).

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