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Antimycobacterial Serratamolides and Diacyl Peptoglucosamine Derivatives from *Serratia* sp.

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The cyclodepsipeptide serratamolide A (**1**) and five closely related compounds together with three new glucosamine derivatives were isolated by bioactivity-guided chromatography from the XAD adsorber resin extract of a *Serratia* sp. The structures of the compounds were elucidated by 2D NMR and MS analyses. In addition to the known serratamolide A (**1**) with two C_{10} alkyl chains, its derivatives always contained one C_{10} chain combined with either $C_{12:1}$, C_{12}, C_{11}, C_9, or C_8 chains. The glucosamine derivatives contained a common core consisting of an *N*-butyl-α-glucopyranosylamide, which was acylated at the C-1 oxygen with valine. The differences between the derivatives arise from the nature of the acyl groups attached to the N-terminus of valine, which were identified as the linear fatty acid moieties $C_{16:1}$, C_{15}, or C_{14}. Each compound was present in two isomeric forms arising from racemization of the valine moiety. All compounds showed antibiotic activity against *Mycobacterium diernhoferi* and other rapidly growing mycobacteria.

In our continuing search for new anti-infectives we have examined promising isolates found during a recent screening of bacterial cultures isolated from wheat rhizospheres in India against plant pathogenic fungi, human multidrug-resistant pathogens, and rapidly growing mycobacteria. In particular, extracts from the fermentation broth of one isolate, belonging to the *Serratia* genus, showed antibiotic activity against three species of *Mycobacterium*, namely, *M. diernhoferi*, *M. phlei*, and *M. smegmatis*. A literature search indicated there are only a few reports concerning the composition of secondary metabolites from *Serratia*. An early report established the structure of a novel cyclodepsipeptide, serratamolide,[1] and later further compounds were suggested to be produced, although their exact structures were not verified.[2] An interest in the possible antibiotic properties of serratamolide led to the production of synthetic analogues and an investigation of its solution structure.[3] However, it is only recently that a renewed interest has been shown in serratamolide, as it induces cell cycle arrest and proapoptotic effects in breast cancer cells,[4] suggesting it may represent a potential new chemotherapeutic reagent. In addition, recent reviews of depsipeptides suggest that these bio-oligomers composed of hydroxy and amino acids linked by amide and ester bonds show promising anticancer, antibacterial, antiviral, antifungal, and anti-inflammatory properties.[4,5] Consequently, our initial work suggested that structural elucidation of the bioactive secondary metabolites from this species of bacterium may afford further useful lead compounds in the fight against human pathogens.

Here we have used adsorber resin extraction of the *Serratia* supernatant broth with procedures that have proved useful over two decades for the isolation of bioactive compounds from *Myxobacteria*,[6] followed by antimycobacterial bioassay-guided fractionation of the crude extracts. Furthermore, we report the isolation and structure elucidation of two groups of compounds, namely, a series of depsipetides related to serratamolide and three diacylated peptoglucosamine derivatives.

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Results and Discussion

During previous studies a large number of bacterial isolates were recovered from the rhizosphere of wheat (*Triticum aestivum*)[7,8] taken from various representative field sites in the Indo-Gangetic region (Ujhani, District Badaun, India). A total of 60 different morphotypes were selected, two of which secreted a blood-red colored pigment into the medium and showed antibacterial activity against mycobacteria. The most promising of these, SHHRE645, was chosen for further study. The isolate formed red-pigmented mucoid colonies with a white margin, and it was characterized as a Gram-negative bacterium with very short rods ($0.86 \times 0.6\ \mu$m). The isolate was identified by 16S rDNA analysis as a *Serratia* sp. with a 99% sequence identity to *S. marcesens* accession no. AB061685; gene bank NCBI database.[8]

Cultivations and isolation procedures involved adsorption of secondary metabolites onto XAD resin followed by elution with MeOH, evaporation, and partition of the residue between EtOAc and H_2O. Initially, a small-scale cultivation (200 mL) of SHHRE645 was used to obtain a crude extract for primary screening against a broad range of target bacterial and fungal strains. The crude extract showed activity only against the rapidly growing mycobacteria *M. diernhoferi*, *M. phlei,* and *M. smegmatis* in a microdilution assay. Throughout this work the microdilution assay was performed at every isolation and purification step to identify the biologically active fractions using *M. diernhoferi* as the target strain.

An initial analytical RP-HPLC fractionation of the crude extract furnished 70 fractions, 12 of these being inhibitory toward *M. diernhoferi*. These occurred between the retention times of 19.5 and 25 min in the medium to nonpolar region of the chromatogram. HPLC-UV-MS analyses of the corresponding active fractions revealed about 15 compounds, which were provisionally recognized as serratamolides A and B and further variants for which there was no information available in all three databases used for identification. Subsequent work concentrated on the isolation and identification of the compounds found in these bioactive fractions. Fractionation, isolation, and purification of 6 L cultures through two steps of chromatography (LH-20 and RP-HPLC) gave a series of new serratamolide derivatives and glucosamine compounds.

The structures of the serratamolides (Scheme 1) were readily elucidated from the combined HRESIMS and NMR data. The ^{1}H NMR spectrum of serratamolide A (**1**) (Table 1) showed a simple set of signals, which from the cross-peaks in the COSY spectrum consisted of a serine and a β-hydroxy fatty acid moiety. The ^{13}C NMR spectrum showed only 13 signals, which from the DEPT-135 spectrum comprised two carbonyl signals at δ 172.0 and 171.3, one methyl,

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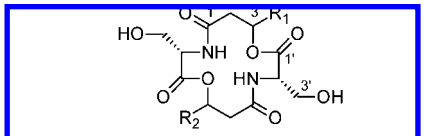
Scheme 1. Structures of Serratamolides A−F (**1**–**6**)



Serratamolide	R_1	R_2
A (**1**)	$-(CH_2)_6CH_3$	$-(CH_2)_6CH_3$
B (**2**)	$-CH_2CH{=}CH(CH_2)_5CH_3$	$-(CH_2)_6CH_3$
C (**3**)	$-(CH_2)_8CH_3$	$-(CH_2)_6CH_3$
D (**4**)	$-(CH_2)_7CH_3$	$-(CH_2)_6CH_3$
E (**5**)	$-(CH_2)_4CH_3$	$-(CH_2)_6CH_3$
F (**6**)	$-(CH_2)_5CH_3$	$-(CH_2)_6CH_3$

eight methylene, and two methine carbons that were readily correlated with the two moieties found in the ^{1}H NMR spectrum (Table 1). The HRESIMS and ^{13}C NMR data were compatible only with a symmetrical molecule. Integration of the ^{1}H NMR spectrum indicated a C-10 β-hydroxy fatty acid moiety attached to the serine unit through its hydroxy group from the low-field shift of H-3 and the three-bond HMBC correlation of this proton with C-1′ of serine in the HMBC. The cyclic nature of the molecule was necessary from the molecular weight and symmetry, and it was confirmed by the observation of a strong three-bond correlation of H-2′ with C-1 and a weaker four-bond correlation between H-2B and C-2′. The HRESIMS was compatible with a molecular formula of $C_{26}H_{46}N_2O_8$, and MS/MS of the protonated molecular ion at *m*/*z* 515.29 confirmed the symmetry of the molecule as it afforded an intense ion at *m*/*z* 240 ($C_{13}H_{22}NO_3^+$) and less intense related ions at *m*/*z* 212 (loss of CO), as well as *m*/*z* 258 and 276 before loss of water. A literature search indicated this natural product has been isolated previously from various strains of *S. marcescens*[1,2] and chemically and spectroscopically characterized as serratamolide A (**1**), a 14-membered cyclic depsipeptide incorporating L-serine and D-3-hydroxy-$C_{10:0}$ acid.[3] Our ^{1}H NMR spectroscopic, MS, and amino acid configurational data are compatible with earlier literature,[3] and we report the ^{13}C NMR data of **1** for the first time.

Both the ^{1}H and ^{13}C NMR spectra of serratamolide B (**2**) indicated that this compound was related to **1** but was unsymmetrical. Two of the four ^{1}H multiplets between δ 5.2 and 5.6 were characteristic of H-3 found in **1** and the two others of an in-chain *cis* double bond from their coupling constants. The position of the double bond at C-5/C-6 of a 3-hydroxy fatty acid unit and the presence of a saturated 3-hydroxy fatty acid unit was determined from the 2D COSY spectrum and comparison with **1**. The additive total length of the fatty acid chains, C_{22}, followed from integration of the ^{1}H NMR spectrum and the number and intensities of signals in the ^{13}C NMR spectrum. The HRESIMS confirmed the molecular formula $C_{28}H_{48}N_2O$, and MS/MS afforded two intense daughter ions at *m*/*z* 240 and 266, together with the appropriate family of related ions similar to those found in **1**, indicating the presence of a saturated $C_{10:0}$ and a unsaturated $C_{12:1}$ unit, respectively.

Four further variants, serratamolides C−F (**3**−**6**), were readily identified using the same techniques. In each case the ESIMS daughter ion spectra gave a set of peaks that afforded the length of the fatty acid chains with all possible chain lengths from C_8 to C_{12} present as one fatty acid unit together with the identical C_{10} unit. Alternative combinations without a C_{10} unit were not encountered.

After several months in CD_3OD solution some of the compounds undergo ring cleavage. Thus **2** undergoes ring opening by addition of CD_3OD to give two linear products in a ratio of 3:1. Partial analyses of the 1D and 2D TOCSY spectra, together with ESIMS data, indicated the major product has a $C_{12:1}$ unit with a free hydroxy group, an intact $C_{10:0}$ unit, and a trideuteriomethyl ester at the terminal serine, while the minor product has the $C_{12:1}$ and $C_{10:0}$ units interchanged. This slow conversion presumably depends on the pH of the solution, as it was not found in all cases. Such an explanation is compatible with the first report of the lipoamino acid, serratamic acid, isolated from alkaline extraction of *S. marcescens* cultures,[10,11] as **1** has been shown to yield serratamic acid on mild alkaline hydrolysis. In keeping with this, two ring-opened compounds each corresponding to the addition of one molecule of water to serratamolides B (**2**) and D (**4**) were characterized by mass spectrometry.

A second set of compounds with similar molecular weights and retention times were produced together with the serratamolides. Again structure elucidation followed from comprehensive NMR (Table 2) and MS data. In contrast to the serratamolides, each derivative was present as a mixture of two isomers that could not be separated and always occurred in the same proportions after various chromatographic treatments.

Glucosamine derivative A (**7**) showed an isomer ratio of ca. 1:1, and four distinct moieties were discernible in each from the 2D COSY spectrum. For the major isomer a sugar spin system could be traced starting at the anomeric proton (δ 6.26). Vicinal couplings indicated this was in an α-glucopyranosyl form with a free C-6 hydroxy group and *O*-acyl and *N*-acyl groups at C-1 and C-2, respectively, from the low-field shifts of the attached protons. The corresponding ^{13}C NMR shifts of the carbons in this unit followed from a 2D HMQC, which showed the C-2 substitutent was an amide group from the shift of δ 54.3. Further cross-peaks in the 2D COSY spectrum indicated the presence of spin systems characteristic of butyric acid and the amino acid valine. Finally, the remaining signals corresponded to those of a linear long-chain fatty acid containing one double bond. The HMQC allowed assignment of the protonated carbons belonging to each of the four moieties, and the long-range correlations in the HMBC spectrum readily differentiated the carbonyl signals of valine (δ ~172) from those of the fatty acid residues (δ 176–177). Importantly, at 14 T the shift differences of the carbonyl carbons and those of the α- and β-protons of the fatty acids were sufficiently large to distinguish these not only within the same molecule but also between isomers. The HMBC spectrum afforded the sequence of the residues from the unambiguous correlations of the anomeric proton (H-1) with the valine carbonyl carbon (C-1′), which showed internal correlations with its own α- and β-protons (H-2′ and H-3′, respectively). The same α-proton (H-2′) showed a further correlation with the carbonyl carbon (C-1″) assigned to the long chain fatty acid. Finally, H-2 of the sugar moiety correlated with the butyric acid carbonyl carbon (C-1‴), which also showed internal correlations with its own α- and β-protons (H-2‴ and H-3‴, respectively).

The HRESIMS showed the presence of only one molecular species and was compatible with the molecular formula $C_{31}H_{56}N_2O_8$. MS/MS of the protonated molecular ion supported the NMR sequence, as fragments were observed at *m*/*z* (%) 232 (100) $[C_{10}H_{18}NO_5]^+$, 214 (35) $[C_{10}H_{18}NO_5-H_2O]^+$, and 354 (7) $[C_{21}H_{40}NO_3]^+$ corresponding to cleavage at the anomeric C−O bond. Consequently, the appearance of two isomeric forms could only be rationalized by racemization of one or more of the stereogenic centers. The ^{1}H NMR chemical shift differences were most pronounced for H-1 and H-2 of the sugar moiety and for the signals of valine, hence suggesting the difference between the two species arises from the presence of L- and D-valine. Alternative acylation forms in which the fatty acids were reversed can be discounted, as the sequence of residues in the second isomer was unambiguously established from the long-range ^{13}C−^{1}H NMR correlations and MS fragmentation. Finally, amino acid configurational analysis indicated the presence of an almost 1:1 mixture of L- and D-valines.

The structures of further glucosamine derivatives, **8** and **9**, were similarly elucidated. In both cases the isomeric ratio of 2:1 made the identification of corresponding NMR signals between the two isomers easier. In **8** and **9** the short butyric acyl system was again positioned at the sugar C-2 amino group and the unbranched C_{15}

Table 1. NMR Data for Serratamolides A (**1**) and B (**2**) in CD_3OD

serratamolide A (**1**)							serratamolide B (**2**)											
							C_{10} alkyl chain						$C_{12:1}$ alkyl chain					
no.	δ_H	m	*J* in Hz	δ_C		HMBC(H→C)	no.	δ_H	m	*J* in Hz	δ_C	m	no.	δ_H	m	*J* in Hz	δ_C	m
1				172.0	s		1				171.8	s	1				172.0	s
2	A 2.72	dd	13.5, 4.9	41.2	t	1, 3, 4	2	A 2.73	dd	13.6, 4.8	41.2	t	2	A 2.70	dd	13.6, 4.8	40.4	t
	B 2.39	dd	13.5, 2.7			1, 3, 4, 2′		B 2.40	dd	13.6, 2.9				B 2.44	dd	13.6, 2.9		
3	5.33	m		73.2	d	1	3	5.33	m		73.2	d	3	5.24	m		73.1	d
4	1.71	m		33.8	t		4	A 1.75	m		33.8	t	4	A 2.53	ddd	14.4, 7.6, 7.6	31.4	t
								B 1.68	m					B 2.42	m			
5	1.34	m		27.0	t		5	1.34	m		26.9	t	5	5.42	dtt	10.8, 7.6, ∼1	125.0	d
6	1.34	m		30.5	t[a]		6	1.34	m		[b]		6	5.55	dtt	10.8, 7.3,∼1	134.2	d
7	1.34	m		30.4	t[a]		7	1.34	m		[b]		7	2.12	m		28.5	t
8	1.34	m		33.0	t		8	1.34	m		33.0	t	8	1.39	m		30.7	t
9	1.34	m		23.7	t	7, 8, 10	9	1.34	m		23.7	t	9	1.34	m		[b]	
10	0.94	t	6.7	14.4	q	8, 9	10	0.94	t	6.8	14.4	t	10	1.34	m		33.0	t
													11	1.34	m		23.7	t
													12	0.94	t	6.8	14.4	q
1′				171.3	s		1′				171.3	s	1′				171.0	s
2′	4.53	dd	3.8, 3.2	56.3	d	1, 1′, 3′	2′	4.52	dd	3.9, 3.4	56.4	d	2′	4.53	dd	4.1, 3.4	56.4	d
3′	A 4.11	dd	10.8, 3.8	63.2	t	1′, 2′	3′	A 4.11	dd	10.8, 3.9	63.1	t	3′	A 4.08	dd	10.8, 4.1	63.1	t
	B 3.87	dd	10.8, 3.2			1′, 2′		B 3.87	dd	10.8, 3.4				B 3.87	dd	10.8, 3.4		

[a] Assignments interchangeable. [b] Signals at δ_C 30.4, 30.4, 30.1.

Table 2. NMR Data of *N*-Butylglucosamine Ester Derivative A (**7**) in CD_3OD

	δ_H multiplicity (J_{HH})		δ_C	
no.	major	minor	major[b]	minor[b]
	Glucosamine			
1	6.26 d (3.6)	6.23 d (3.6)	92.93	92.86
2	4.08 dd (10.9, 3.6)	4.02 dd (10.9, 3.6)	54.29	54.54
3	3.76	3.69	72.29	72.19
4	3.50 dd (9.7, 9.1)	3.51 dd (9.8, 9.1)	71.60	71.55
5	3.79–3.68 m	3.79–3.68 m	76.82	76.24
6A	3.81 dd (12.1, 2.3)	3.80 dd (12.1, 2.4)	62.26	62.17
6B	3.79–3.68 m	3.79–3.68 m		
	Valine			
1′			172.27	171.78
2′	4.37 d (6.4)	4.36 d (6.0)	59.40	59.49
3′	2.23 m	2.30 m	31.59	31.05
4′	1.04[a] d (6.6)	1.03[a] d (6.6)	19.61	19.68
5′	1.04[a] d (6.8)	1.02[a] d (6.8)	18.58	18.40
	Butyric acid			
1‴			176.75	176.62
2‴	2.23 t (7.6)	2.23 t (7.6)	38.78	
3‴	1.64 m	1.64 m	20.24	20.14
4‴	0.94 t (7.4)	0.97 t (7.4)	14.03	
	Fatty acid (R = $^1CO-^2CH_2-^3CH_2...CH_2-CH{=}CH-CH_2...^{16}CH_3$)			
1″			176.99	176.84
2″	2.33 m		36.52	36.63
3″	1.68 m		26.98	
CH_2−CH=CH	2.07 m		28.13	
CH=CH	5.38 m		130.79/130.87	
Other CH_2's	1.40–1.32 m		30.81–30.01[c]	
16″	0.94 t (7.2)		14.40	

[a] Interchangeable between isomers. [b] Differentiation of major and minor isomer assignments was from the HMQC and HMBC spectra and signal intensities. [c] The signals of C-15 and C-14 are at δ_C 23.68 and 32.90, respectively.

Table 3. NMR Data of *N*-Butylglucosamine Ester Derivative B (**8**) in CD_3OD

	δ_H multiplicity (J_{HH})		δ_C	
no.	major	minor	major[a]	minor[a]
	Glucosamine			
1	6.26 d (3.6)	6.23 d (3.6)	92.96	92.89
2	4.08 dd (10.8, 3.6)	4.02 dd (10.9, 3.6)	54.33	54.58
3	3.78–3.73 m	3.78–3.73 m	72.31	72.23
4	3.50 dd (9.6, 9.2)	3.51 dd (9.6, 9.1)	71.63	71.63
5	3.69 m	3.69 m	76.85	76.27
6A	3.81 dd (12.1, 2.3)	3.80 dd (12.1, 2.4)	62.29	62.20
6B	3.78–3.73 m	3.78–3.73 m		
	Valine			
1′			172.31	171.82
2′	4.37 d (6.4)	4.35 d (6.1)	59.44	59.53
3′	2.23 m	2.26 m	31.64	31.09
4′	1.04 d (6.6)	1.03 d (6.6)	19.65	19.72
5′	1.04 d (6.8)	1.02 d (6.8)	18.62	18.44
	Butyric acid			
1‴			176.77	
2‴	2.23 t (7.4)		38.82	
3‴	1.63 m	1.65 m	20.28	20.19
4‴	0.94 t (7.3)	0.97 t (7.4)	14.07	
	Fatty acid (R = $^1CO-^2CH_2-^3CH_2...CH_2...^{15}CH_3$)			
1″			176.77	
2″	2.32 m		36.54	36.65
3″	1.68 m		27.03	
4″	1.4–1.3 m		33.09	
5″−12″	1.4–1.3 m		30.77–30.30	
13″	1.4–1.3 m		33.09	
14″	1.4–1.3 m		23.75	
15″	0.94 t (7.3)		14.45	

[a] Differentiation of major and minor isomer assignments was from the HMQC and HMBC spectra and signal intensities.

and C_{14} saturated fatty acids, respectively, at the valine amino group. The sequence assignment was again supported by the MS data.

The isolated compounds, serratamolides and glucosamine derivatives, exhibited antimycobacterial activities with MICs (minimum inhibitory concentrations) of ca. 0.18 mM for *M. diernhoferi*. Similar values were obtained when other strains of rapidly growing mycobacteria, namely, *M. phlei* and *M. smegmatis*, were tested. These other mycobacteria, referred to as atypical mycobacteria or nontuberculous mycobacteria, are usually saprophytes but can be opportunistic pathogens. Furthermore, the use of these strains offers the advantage of rapidly and safely screening for antituberculosis compounds. No activity was observed for either set of compounds against a panel of other human-pathogenic agents including Gram-positive as well as Gram-negative bacteria.

In summary the compounds characterized in this study possess mild but selective activity against mycobacteria and could be leads for the development of new drugs for use against *M. tuberculosis*.

Experimental Section

Strains and Media. The antibiotic-producing bacterium SHHRE645 was isolated from wheat rhizosphere obtained from the Department of Microbiology, Pantnagar (India). Primary isolation and culturing of bacterial isolates have been performed and described elsewhere (Gaur et al. 2004).[7] The 16S rDNA sequence determination and analysis were performed as described,[12] and the isolate was shown to belong to the genus *Serratia*, but it could not be confidently identified to the species level.

A large group of reference strains and clinical isolates including Gram-positive and Gram-negative bacteria, mycobacteria, yeast, and fungi was used as target for assessing the antimicrobial activity of rhizospheric bacteria, crude extracts, HPLC fractions, and isolated compounds. All organisms were maintained in glycerol broth at −80 °C.

Scheme 2. Structures of *N*-Butylglucosamine Ester Derivatives **7–9**

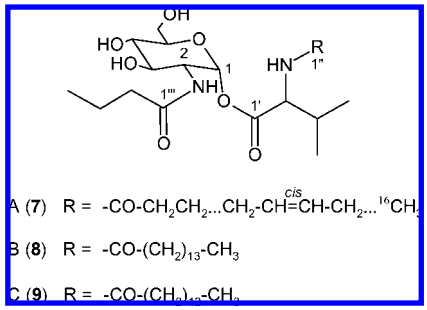


Table 4. NMR Data of *N*-Butylglucosamine Ester Derivative C (**9**) in CD_3OD

	δ_H multiplicity (J_{HH})		δ_C	
no.	major	minor	major[b]	minor[b]
	Glucosamine			
1	6.26 d (3.6)	6.23 d (3.6)	92.93	92.87
2	4.08 dd (10.9, 3.6)	4.02 dd (10.9, 3.6)	54.30	54.54
3	3.78–3.73 m	3.78–3.73 m	72.29	72.20
4	3.51 dd (9.5, 9.2)	3.51 dd (9.6, 9.1)	71.60	71.60
5	3.69 m	3.69 m	76.82	76.24
6A	3.82 dd (11.9, 2.2)	3.81 dd	62.26	62.17
6B	3.78–3.73 m	3.78–3.73 m		
	Valine			
1′			172.28	171.79
2′	4.36 d (6.2)	4.35 d	59.41	59.50
3′	2.23 m	2.26 m	31.60	31.06
4′	1.04 d (6.8)	1.03 d	19.69	19.62
5′	1.03 d (6.6)	1.02 d	18.60	18.41
	Butyric acid			
1‴			176.84[a]	177.01[a]
2‴	2.23 t (7.4)		38.79	
3‴	1.63 m	1.65 m	20.25	20.16
4‴	0.94 t (7.3)	0.97 t (7.6)	14.04	
	Fatty acid (R = $^1CO-^2CH_2-^3CH_2...CH_2...^{14}CH_3$)			
1″			176.79[a]	176.63[a]
2″	2.32 m		36.53	36.64
3″	1.68 m		27.00	
4″	1.4–1.3 m		33.06	
5″−11″	1.4–1.3 m		30.7–30.3	
12″	1.4–1.3 m		33.06	
13″	1.4–1.3 m		23.72	
14″	0.94 t (6.9)		14.42	

[a] Interchangeable within the column. [b] Differentiation of major and minor isomer assignments was from the signal intensities.

Mycobacteria target strains were grown on EBS medium containing casein peptone 0.5%; glucose 0.5%; meat extract 0.1%; yeast extract 0.1%; HEPES buffer 50 mM; and agar 1.6% (ph 7.0). The environmental *Serratia* sp. strain SHHRE645 was cultivated overnight at 28 °C on trypticase soy broth (Difco, Germany).

Extraction and isolation. Preparation and recovery of secondary metabolites were essentially a small-scale version of that described previously by Gerth et al.[6] Briefly, this involved the inoculation of 500 Erlenmeyer flasks each with 200 mL of medium trypticase soy broth (Difco, Germany) containing 3% Amberlite XAD-16 adsorbent resin (Rohm & Haas, Frankfurt, Germany), with 1 mL of preculture of SHHRE645 and incubation with shaking at 28 °C for 5 days. The XAD was recovered from the culture broths by decantation, transferred into a column, and washed with 50% aqueous MeOH. Adsorbed products were subsequently eluted with 100% MeOH, and after evaporation the aqueous mixture was extracted four times with EtOAc. Evaporation of the solvent under reduced pressure yielded 410 mg of residue from two fermentations, each with 6 L of broth.

The purification was then carried out in two steps. First, Sephadex LH-20 column chromatography (column: 890 × 50 mm; solvent MeOH; flow rate 6 mL/min; UV detection at 206 nm) was performed to separate sets of similar compounds (the serratamolides and glucosamines). The corresponding bioactive fractions were pooled and separated by preparative RP-HPLC on Nucleosil 100-10 C18EC C_{18} (column 250 × 21 mm; Macherey-Nagel, Germany), gradient of solvents A (5% ACN, 95% water, 50 mM ammonium acetate, 5 μL of HOAc per L) and B (95% ACN, 5% H_2O, 50 mM ammonium acetate, 5 μL of HOAc per L).

In the first separation targeting the serratamolides, 96 mg of residue after the LH-20 chromatography was dissolved in 0.2 mL of DMSO and subjected to the RP-HPLC step, with a gradient of 55% B for 15 min and then 70% B in 60 min (flow rate 18 mL/min; UV detection at 208 nm) to give a total of 11 HPLC fractions containing serratamolides E (**5**) (2.3 mg, t_R = 8.8 min), F (**6**) (1.8 mg, t_R= 12.6 min), A (**1**) (20.8 mg, t_R = 18.0 min), D (**4**) (2.8 mg, t_R = 25.2 min), B (**2**) (4.6 mg, t_R = 29.0 min), and C (**3**) (9.9 mg, t_R = 38.6 min). In a second purification targeting the glucosamine derivatives, 134 mg of residue after the LH-20 chromatography was dissolved in 0.2 mL of DMSO and subjected to RP-HPLC, with a gradient of 50% B for 25 min and then 70% B in 75 min (flow rate 20 mL/min; UV detection at 208 nm), to give a total of 16 HPLC fractions containing glucosamine derivatives C (**9**) (9.8 mg, t_R = 56.4 min), A (**7**) (9.1 mg, t_R = 62.6 min), and B (**8**) (5.3 mg, t_R = 70.8 min). The ring-opened serratamolide D was detected using this system (3.9 mg, t_R = 21 min).

Bioassays. For the determination of the antimicrobial activities in liquid medium, serial dilutions of the crude extracts were performed in microtiter plates (96 wells) inoculated with mycobacteria target strains in EBS medium. For the preparation of the inoculums a suspension of each mycobacteria strain was obtained by scrapping different colonies isolated in agar plates and suspending them homogenously in 1 mL of EBS medium. 100 μL of the bacterial suspension was mixed with 15 mL of medium for the inoculation of the wells.

For MIC determination of the purified compounds serial 2-fold dilutions of each compound were performed in Mueller-Hinton broth (Difco) as previously described.[13] The plates were inoculated with the bacterial suspension to yield a cell density of 5×10^5 CFU/mL. The MIC is defined as the lowest concentration that inhibited 99% of the inoculum.

Chemical Experimental Procedures. ^1H (1D, 2D COSY, TOCSY) and ^{13}C (1D, 2D HMQC, HMBC) NMR spectra of pure compounds were recorded at 300 K on Bruker DPX 300, ARX 400, or DMX 600 NMR spectrometers locked to the major deuterium signal of the solvent, CD_3OD. Chemical shifts are given in ppm and coupling constants in Hz. Positive ion elecrospray mass spectra (ESIMS) of the same were recorded on a Finnigan QTOF^2 mass spectrometer. High-resolution ESIMS were recorded on the same instrument. HPLC-UV-MS was performed on an HP model 1100 HPLC system (Agilent) with a UV diode-array detector and a PE Sciex API 2000 LC/MS/MS system with an ACI electrospray ionization device (Perkin-Elmer), using a Nucleosil 120-5 RP C_{18} column (125 × 2 mm; Macherey-Nagel, Germany). Solvents, which were degassed before used, were (A) 5 mM ammonium acetate, 5% ACN, and 30 mL of HOAc per L and (B) 5 mM ammonium acetate, 95% ACN, and 30 mL of HOAC per L with a flow rate of 0.3 mL/min at 40 °C.

Chiralities of the amino acids were determined by GC-MS analysis after hydrolysis and derivatization with pentafluoropropionic acid anhydride using a Chirasilval column (50 m) connected to a GCQ ion trap mass spectrometer.

Microfractionation of Antimycobacterial Crude Extract Using HPLC. The *Serratia* extracts were analyzed by RP-HPLC using an Agilent 1100 series HPLC system equipped with a gradient pump system, a UV diode array detector at 220–600 nm, and a fraction collector using the same solvent gradient as that for HPLC-MS analysis. Four milliliters of 800:1 concentrated MeOH extract was injected, and fractions of 150 mL were collected every 30 s in 96-well round-bottom microtiter plates for 35 min. The resulting plate with fractions was evaporated on a speed vac or dried with N_2 gas at 40 °C. The biological activity of the fractions was then analyzed against the target strains to afford the activity of each peak in the chromatogram.

The biological activity of peaks fractionated into well-plates by HPLC-UV and the molecular masses of active compounds determined by LC-MS were correlated using identical chromatographic systems. The UV and MS data were used in subsequent searches in the "Dictionary of Natural Products" database (Chapman and Hall/CRC), Antibase 2003 (VCH Wiley), and CrossFire Beilstein databases (MDL) (online).

Serratamolide A (1): (C_{10} and C_{10} alkyl chains) ^1H and ^{13}C NMR data, Table 1; (+)-ESIMS *m/z* 515.29 $[M + H]^+$, 537.29 $[M + Na]^+$, 553.28 $[M + K]^+$; fragmentation of $[M + H]^+$ 153.15 (15), 212.19 (38), 240.20 (100), 258.20 (34), 276.21 (12), 497.39 (7); (+)HRESIMS *m/z* 515.333 $[M + H]^+$ (calcd for $C_{26}H_{47}N_2O_8$, 515.3332).

Serratamolide B (2): (C_{10} and $C_{12:1}$ alkyl chains) ^{1}H and ^{13}C NMR data, Table 1; (+)-ESIMS *m/z* 541.36 $[M + H]^+$, 563.35 $[M + Na]^+$; fragmentation of $[M + H]^+$ *m/z* (%) 106.04 (12), 153.15 (8), 179.17 (11), 212.19 (25), 238.22 (38), 240.19 (100), 258.20 (49), 266.20 (100), 276.21 (18), 284.22 (52), 302.25 (19), 523.40 (18); (+)HRESIMS *m/z* 541.348 $[M + H]^+$ (calcd. for $C_{28}H_{49}N_2O_8$, 541.3489).

Methanolysis of Serratamolide B. After long exposure to CD_3OD in the NMR tube cleavage of the ring system gave two adducts in the ratio 3:1 (from NMR signal heights). After dissolution in CH_3OH (re-exchange OD to OH) subsequent (+)-ESIMS gives *m/z* 576.30 $[M + H]^+$; fragmentation of $[M + H]^+$ *m/z* (%) 123.09 (10), 153.14 (4), 179.17 (8), 212.19 (10), 230.21 (10), 238.22 (25), 240.19 (20), 256.23 (33), 257.24 (77), 275.26 (100), 284.26 (53), 293.27 (65), 301.27 (30), 404.30 (12), 540.43 (10), 558.45 (12); (+)HRESIMS of fragments using *m/z* 238.1807 ($C_{14}H_{24}NO_2$) as lock, *m/z* 275.2058 ($C_{14}H_{23}D_3NO_4$), 293.2153 ($C_{14}H_{25}D_3NO_5$), 301.2185 ($C_{16}H_{25}D_3NO_4$), 319.2302 ($C_{16}H_{27}D_3NO_5$); ^{1}H NMR (CD_3OD) as a mixture with serratamolide B (ca. 1:1, data taken from 1D and 2D TOCSY spectrum), major isomer, C_{10}-residue δ 5.29 (H-3), 2.62 (H-2), 1.71 (H-4), 1.35 (H-5), $C_{12:1}$-residue 5.55 (H-6), 5.48 (dtt; H-5; J = 11, 7.1, ~1), 4.06 (H-3), 2.48 (H-2A), 2.43 (H-2B), 2.32 (H-4), 2.11 (H-7), 1.40 (H-8); minor isomer, C_{10}-residue δ 4.02 (H-3), 2.43 (H-2), 1.53 (H-4), 1.37 (H-5).

Serratamolide C (3): (C_{10} and C_{12} alkyl chains) ^{1}H NMR data are identical with those of **1** apart from the integral of the signal at δ_H 1.34, which integrates for 12 methylene groups. Similarly the ^{13}C NMR data are identical to A apart from the six signals at δ_C 30.4, 30.4, 30.5, 30.5, 30.7, and 30.8 of half-intensity, which correspond to the central methylene carbons of the aliphatic chains. In addition the signal at δ_C 33.8 shows a fine splitting when processed without line broadening; (+)-ESIMS *m/z* 543.34 $[M + H]^+$, 565.32 $[M + Na]^+$; fragmentation of $[M + H]^+$ *m/z* (%) 153.15 (9), 181.19 (12), 212.19 (34), 240.19 (100), 258.20 (40), 268.22 (100), 276.20 (16), 286.24 (36), 304.25 (12), 525.42 (19); (+)HRESIMS *m/z* 543.365 $[M + H]^+$ (calcd. for $C_{28}H_{51}N_2O_8$, 543.3645).

Serratamolide D (4): (C_{10} and C_{11} alkyl chains) ^{1}H data are identical with those of **1** apart from the integral of the signal at 1.34, which integrates for 11 methylene groups. Similarly the ^{13}C NMR data are identical to A apart from the five signals at 30.4, 30.5, 30.5, 30.5, and 30.7 of half-intensity, which correspond to the central methylene carbons of the aliphatic chains; (+)-ESIMS *m/z* 529.33 $[M + H]^+$, 551.33 $[M + Na]^+$, 567.30 $[M + K]^+$; fragmentation of $[M + H]^+$ *m/z* (%) 153.15 (11), 167.17 (13), 212.19 (30), 226.19 (28), 240.19 (99), 254.20 (100), 258.20 (29), 272.20 (29), 276.21 (14), 290.21 (14), 511 (18); (+)HRESIMS *m/z* 529.350 $[M + H]^+$ (calcd for $C_{27}H_{49}N_2O_8$, 529.3489).

Other Serratamolides Detected Only by ESIMS in Fractions Containing Mixtures: Serratamolide E (5): (C_{10} and C_8 alkyl chains) (+)-ESIMS *m/z* 487.25 $[M + H]^+$, 509.24 $[M + Na]^+$; fragmentation of $[M + H]^+$ *m/z* (%) 153.13 (4), 184.15 (19), 212.14 (96), 230.16 (37), 240.18 (100), 248.19 (25), 258.19 (36), 276.20 (24), 469.32 (33); (+)HRESIMS *m/z* 487.304 $[M + H]^+$ (calcd. for $C_{24}H_{43}N_2O_8$, 487.3019).

Serratamolide F (6): (C_{10} and C_9 alkyl chains) mixed with serratamolide A and the ring-opened serratamolide B, (+)-ESIMS *m/z*501.27 $[M + H]^+$, 523.27 $[M + Na]^+$; fragmentation of $[M + H]^+$ *m/z* (%) 153.13 (4), 198.16 (20), 212.18 (20), 226.16(99), 240.18 (100), 244.19 (37), 258.19 (37), 262.19 (26), 276.20 (25), 483.33 (35); (+)HRESIMS *m/z* 501.319 $[M + H]^+$ (calcd for $C_{25}H_{45}N_2O_8$, 501.3178).

Ring-opened serratamolide B: mixed with serratamolide A (**1**) and serratamolide F (**6**), (+)-ESIMS *m/z* 559.31 $[M + H]^+$, 581.30 $[M + Na]^+$; Fragmentation of $[M + H]^+$ *m/z* (%) 179.16 (7), 222.17 (7), 239.21 (30), 258.20 (32), 266.20 (65), 284.22 (65), 302.23 (32), 523.38 (100), 541.4 (60); (+)HRESIMS *m/z* 559.362 $[M + H]^+$ (559.3594 calcd for $C_{28}H_{51}N_2O_9$).

Ring-opened serratamolide D: (+)-ESIMS *m/z* 547.32 $[M + H]^+$, 569.28 $[M + Na]^+$; fragmentation of $[M + H]^+$ *m/z* (%) 120.07 (10), 153.14 (4), 212.18 (13), 230.20 (37), 254.20 (58), 258.20 (50), 272.21 (92), 290.22 (100), 401.26 (16), 511.38 (7), 529.39 (23).

Glucosamine derivative A (7): ^{1}H and ^{13}C NMR data, Table 2; ESIMS *m/z* 585.40 $[M + H]^+$, 607.38 $[M + Na]^+$, 623.37 $[M + K]^+$; fragmentation of $[M + H]^+$ *m/z* (%) 214.13 (16), 232.14 (100) $[C_{10}H_{18}NO_5]^+$, 354.34 (7) $[C_{21}H_{40}NO_3]^+$; (+)HRESIMS *m/z* 623.368 $[M + K]^+$ (calcd. for $C_{31}H_{56}N_2O_8K$, 623.3674). Isomer ratio: 52:48

Glucosamine derivative B (8): ^{1}H and ^{13}C NMR data, Table 2; ESIMS *m/z* 573.36 $[M + H]^+$, 595.35 $[M + Na]^+$, 623.37 $[M + K]^+$; fragmentation of $[M + H]^+$ *m/z* (%) 214.13 (35), 232.13 (100) $[C_{10}H_{18}NO_5]^+$, 342.33 (8) $[C_{21}H_{40}NO_3]^+$; (+)HRESIMS *m/z* 595.3939 $[M + K]^+$ (calcd for $C_{30}H_{56}N_2O_8Na$, 595.3934). Isomer ratio: 71:29

Glucosamine derivative C (9): ^{1}H and ^{13}C NMR data, Table 2; ESIMS *m/z* 559.40 $[M + H]^+$, 581.35 $[M + Na]^+$, 611.37 $[M + K]^+$; fragmentation of $[M + Na]^+$ *m/z* (%) 254.10 (32) $[C_{10}H_{18}NO_5Na]^+$, 350.27 (100) $[C_{19}H_{37}NO_3Na]^+$; (+)HRESIMS *m/z* 581.3771 $[M + Na]^+$ (calcd for $C_{29}H_{54}N_2O_8Na$, 581.3778). Isomer ratio: 67:33.

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