Pergamon

Tetrahedron 54 (1998) 4755–4760

TETRAHEDRON

# Serratiomycin, a New Antibacterial Peptolide from an Eubacterium culture, MB 5691

Y.K. Tony Lam*, Michael J. Salvatore, Charles Hirsch, Otto D. Hensens, Deborah Zink, Aurora Bernard-King, Nailin Lee, Amy Graham, and Barbara Pelak

Merck Research Laboratories, P.O. Box 2000, Rahway, NJ, USA 07065-9000

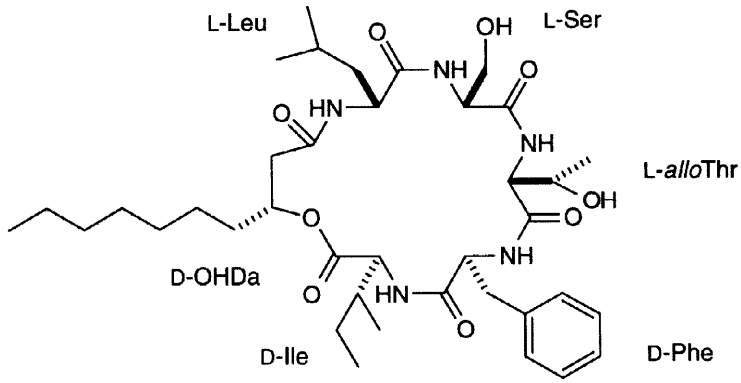
Received 15 December 1997; accepted 17 February 1998

***Abstract:*** A novel peptolide antibiotic, serratiomycin, **1**, was isolated from the culture broth of a eubacterium by solvent extraction and column chromatography. The structure of **1** was determined to be cyclo(D-β-hydroxydecanoyl-L-leucyl-L-seryl-L-*allo*threonyl-D-phenylalanyl-D-isoleucyl). **1** exhibited a weak and mostly Gram-positive antibacterial spectrum *in vitro*. 

In a non-mode-of-action screening program for new antibacterials from eubacteria, we found serratiomycin, **1**, as a metabolite of *Serratia marcescens*, MB 5691. The planar stucture of **1** was determined largely by extensive 2D-NMR and MS techniques. Chirality of the amino acid residues was determined by LC-MS analysis of the D-α-methylbenzyl thioureas of a L- or D- amino acid oxidase treated acid hydrolysate. In this paper we report the isolation, structural elucidation and absolute configuration of **1**.

## RESULTS AND DISCUSSION

The culture broth of *Serratia marcescens*, MB 5691, was extracted with methyl ethyl ketone. The organic layer was taken to dryness, dissolved in 95% MeOH (aq) and defatted with hexanes. Purification of the defatted extract in two chromatographic steps: a) Diaion HP-20 column using a stepwise gradient of 40 - 50% MeCN (aq) for elution; and then b) Hamilton PRP-1 column using 45% MeCN (aq) for isocratic elution, afforded homogeneous **1**.




**1**

* To whom correspondance should be addressed; e-mail: tony_lam@merck.com.

0040-4020/98/$19.00 
*PII:* S0040-4020(98)00187-2

Table 1. $^{1}H$ and $^{13}C$ NMR Chemical Shift Assigments of **1** in DMSO-$d_6$ (500/125 MHz, 25 °C).

| residue | position | $\delta_C$[a] (ppm) | $\delta_H$ (ppm) | residue | position | $\delta_C$ (ppm) | $\delta_H$ (ppm) |
|---|---|---|---|---|---|---|---|
| OHDa | 1 | 168.8 (s) | - | *allo*Thr | α | 58.4 (d) | 4.10 (dd, 8, 2.5) |
| | 2 | 40.1 (t) | 2.36 (dd, 14, 6) | | β | 65.5 (d) | 4.24 (m) |
| | | | 2.66 (dd, 14, 3) | | | | |
| | 3 | 71.7 (d) | 4.92 (m) | | γ | 20.2 (q) | 0.97 (d, 6.5) |
| | 4 | 31.9 (t) | 1.53 (m) | | β-OH | - | 5.11 (d, 4.5) |
| | 5 | 24.7 (t) | 1.23 (m) | | α-CO | 169.5 (s) | |
| | 6 | 28.6 (t) | 1.22 (m) | | α-NH | - | 8.03 (d, 8) |
| | 7 | 28.4 (t) | 1.21 (m) | Phe | α | 53.5 (d) | 4.45 (m) |
| | 8 | 31.1 (t) | 1.21 (m) | | β | 37.1 (t) | 2.89 (dd, 14, 7) |
| | | | | | | | 3.14 (dd, 14, 4) |
| | 9 | 22.0 (t) | 1.22 (m) | | γ | 136.7 (s) | |
| | 10 | 13.9 (t) | 0.84 (t, 7) | | δ | 128.1 (d) | 7.15 (m) |
| Leu | α | 50.6 (d) | 4.35 (m) | | ε | 129.1 (d) | 7.06 (m) |
| | β | 40.4 (t) | 1.12 (m) | | ζ | 126.4 (d) | 7.14 (m) |
| | | | 1.42 (m) | | | | |
| | γ | 23.7 (d) | 1.64 (m) | | α-CO | 170.5 (s) | - |
| | δ | 21.0 (q) | 0.82 (d, 7) | | α-NH | - | 7.46 (d, 7) |
| | | 23.3 (q) | 0.81 (d, 7) | | | | |
| | α-CO | 172.1 (s) | - | Ile | α | 57.6 (d) | 3.76 (dd, 7, 7) |
| | α-NH | - | 7.82 (d, 8.5) | | β | 34.6 (d) | 1.77 (m) |
| Ser | α | 56.6 (d) | 4.41 (m) | | γ | 24.8 (t) | 1.15 (m) |
| | | | | | | | 1.38 (m) |
| | β | 61.2 (t) | 3.55 (m) | | β-$CH_3$ | 15.3 (q) | 0.83 (d, 7) |
| | | | 3.62 (m) | | | | |
| | β-OH | - | 5.04 (t, 5.5) | | δ | 10.1 (q) | 0.80 (t, 8) |
| | α-CO | 170.6 (s) | - | | α-CO | 171.2 (s) | - |
| | α-NH | - | 8.08 (d, 8.5) | | α-NH | - | 8.49 (d, 5.5) |

[a] Multiplicities deduced from DEPT; abbreviations, s: singlet, d: doublet, t: triplet, q: quartet

Serratiomycin (**1**) was isolated as a white amorphous powder. Its UV spectrum in methanolic solution showed absorption maxima at 205 and 268 nm. The $^{1}H$ and $^{13}C$ NMR spectra were indicative of a peptide. The IR spectrum of **1** exhibited typical peptidic absorption bands at 3313, 1650, and 1529 $cm^{-1}$. EI (underivatized and silylated), $NH_3$-CI and ESI-MS agreed with a molecular ion of **1** at *m/z* 731. HR-EI-MS showed *m/z* 731.4452 ($M^+$, calcd for $C_{38}H_{61}O_9N_5$ m/z 731.4469). Total acid hydrolysis with 6N HCl at 110 °C for 3 h, followed by silylation and GC-MS analysis indicated the presence of equimolar amino acid residues: Ser, Leu, Ile, *allo*Thr and Phe, as well as a $C_{10}$-hydroxy fatty acid partial structure.

The molecular formula of $C_{38}H_{61}O_9N_5$ required eleven double bond equivalents (dbe's). 10 dbe's were accounted for by six carbonyl and one phenyl moiety. The remaining dbe suggested that the oligopeptide is cyclic. No useful sequence information was extracted from the MS data.

Complete assignments of the $^1H$ and $^{13}C$ chemical shifts derived from NMR experiments in DMSO-$d_6$ ($^1H$, $^{13}C$, DEPT, COSY, TOCSY, HMQC, HMBC and NOESY) are depicted in Table 1. The choice of this solvent is crucial at 500 MHz, to allow sufficient resolution for the assignments and the entire sequence to be unambiguously established. Other solvents attempted ($CD_3CN$, $CD_2Cl_2$) provided only partial information. The NMR results confirmed the acid hydrolysis experiment above and established the fatty acid residue as β-hydroxydecanoic acid (OHDa). In addition, a geminal coupling of 14 Hz for the H-2's in OHDa is in agreement with them being part of the cyclic structure.

Table 2. HMBC Correlations for Carbonyl Carbons of **1** ($^nJ_{CH}$ = 7 Hz).

| | | correlation with | | |
|---|---|---|---|---|
| residue | C=O | NH | α-H | β-H and others |
| OHDa | 1 | Leu | Leu | H-2,3 (OD) |
| Leu | α | Ser | Leu, Ser | - |
| Ser | α | *allo*Thr | Ser, *allo*Thr | - |
| *allo*Thr | α | Phe | *allo*Thr, Phe | - |
| Phe | α | Ile | Phe, Ile | - |
| Ile | α | Ile | Ile | - |

Table 3. Interresidue NOE Correlations[a] of **1**.

| | | correlation with |
|---|---|---|
| residue | proton | sequentially adjacent residues |
| OHDa | H-2 | NH (Leu) |
| | H-3 | α-H (Ile), NH (Leu) |
| Leu | α-H | NH (Ser) |
| | NH | NH (Ser), α-H (Ile) |
| Ser | α-H | NH (*allo*Thr) |
| | β-H | NH (*allo*Thr) |
| | NH | α-H (Leu) |
| *allo*Thr | α-H | NH (Phe) |
| | β-H | NH (Phe) |
| | NH | α-H (Ser), NH (Phe) |
| Phe | α-H | NH (Ile) |
| | β-H | NH (Ile) |
| | NH | α-H, β-H (*allo*Thr), NH (Ile) |
| Ile | α-H | H-3 (OD), NH (Leu) |
| | N-H | α-H, β-H, NH (Phe) |

[a] Observed in the NOESY spectrum; mixing time: 0.3 sec; relaxation delay: 3 sec

The sequence of the residues of amino acids and OHDa was determined by interpretation of HMBC data (Table 2) which was corroborated by NOESY evidence (Table 3). Interresidue HMBC correlations were observed through peptide bonds, i.e. between carboxyl carbon and the amide proton of adjacent residue: OHDa ($\delta_C$ 168.8 ppm) and Leu ($\delta_H$ 7.82 ppm), Leu ($\delta_C$ 172.1 ppm) and Ser ($\delta_H$ 8.08 ppm), Ser ($\delta_C$ 170.6 ppm) and *allo*Thr ($\delta_H$ 8.03 ppm), *allo*Thr ($\delta_C$ 169.5 ppm) and Phe ($\delta_H$ 7.46 ppm), and Phe ($\delta_C$ 170.5 ppm) and Ile ($\delta_H$ 8.49 ppm). Thus the whole peptide sequence of OHDa-Leu-Ser-*allo*Thr-Phe-Ile was readily established. This sequence was also supported by interresidue NOE's observed between the α-methines and their neighboring amide protons: OHDa ($\delta_H$ 2.36 and 2.66 ppm) and Leu ($\delta_H$ 7.82 ppm), Leu ($\delta_H$ 4.35 ppm) and Ser ($\delta_H$ 8.08 ppm), Ser ($\delta_H$ 4.41 ppm) and *allo*Thr ($\delta_H$ 8.03 ppm), *allo*Thr ($\delta_H$ 4.10 ppm) and Phe ($\delta_H$ 7.46 ppm), Phe ($\delta_H$ 4.45 ppm) and Ile ($\delta_H$ 8.49 ppm). An ester bond between the isoleucyl carboxyl and the β-hydroxyl of OHDa was the only possible point of attachment to complete the cyclic structure.

The absolute stereochemistry of AA residues was established by the differential action of two enzymes. L-Amino acid oxidase catalyzes the oxidative deamination of L-amino acids only and does not attack the D-amino acids[1,2]. Conversely, D-amino acid oxidase catalyzes the oxidative deamination of D-amino acids only and does not attack the L-amino acids[3]. After treatment with either enzyme, the stereochemically intact amino acids can then be derivatized and detected. Thus, a 6N HCl (aq) hydrolysate of **1**, containing the mixture of amino acids, was treated in parallel by L-amino acid oxidase type X (porcine kidney) or D-amino acid oxidase type IV (*Crotalus adamanteus* venom). The dried reaction mixture was derivatized with D-α-methylbenzyl isothiocyanate (AMBI)[4] and then analyzed by LC-MS. Comparison of the two parallel series of reactions with each other, and with enantiomerically pure amino acid standards as control, showed the presence of L-Leu, L-Ser, L-*allo*Thr, D-Phe and D-Ile in **1**. This is the first application of such a combination of enzymatic and LC-MS approach to determine the absolute stereochemistry of AA residues.

With the absolute stereochemistry of AA residues known, the only remaining stereochemistry that needs to be determined is the stereocentre in the β-hydroxydecanoyl residue. NOE's observed (Table 3) among its methine, the α-methine of D-Ile and NH of L-Leu required the configuration to be R. This was also supported by examination of a molecular model. The lipid component is therefore D-β-hydroxydecanoic acid. This lipid residue was also consistently observed in other metabolites of *Serratia* spp., such as serratamolide[5] serratamic acid[6] and (R,Z)-3-hydroxy-5-dodecenoic acid[7].

The structure of **1** was thus determined to be cyclo(D-β-hydroxydecanoyl-L-leucyl-L-seryl-L-*allo*threonyl-D-phenylalanyl-D-isoleucyl).

Compound **1** showed a weak and mostly Gram-positive antibacterial spectrum. It demonstrated MIC's of 2 μg/ml versus *Bacillus subtilis*, *Corynebacterium pseudodiptheriticum* and *Staphylococcus aureus*, 8 μg/ml versus *Enterococcus faecium*, and >128 μg/ml versuss *Proteus vulgaris*. In a mouse protection test, **1**, administered intraperitoneally at 40 mg/kg, did not protect mice against a systemic infection produced by *S. aureus*. In a study on the effect of **1** on incorporation of $^{3}H$ labelled TdR, URA, L-Leu, L-Ala and glycerol by *S. aureus*, $IC_{50}$'s of 6, 5, 17, 7.5 and 7.5 μg/ml were found.

## EXPERIMENTAL

UV spectra were recorded with a Beckman model DU-70 spectrophotometer. IR spectra were recorded as a neat deposit on a ZnSe crystal with a Perkin-Elmer model 1750 FT-IR spectrometer. Optical rotation was measured on a Perkin-Elmer model 241 polarimeter.

Mass spectra were recorded on Jeol SX-102A (electron impact, EI, 90eV) and JEOL HX110 (fast atom bombardment, FAB) and a Finnigan LCQ (LC-MS-ESI, liquid chromatography-electrospray ionization) mass spectrometers. Exact mass measurements were performed at high resolution (HR-EI) using perfluorokerosene as an internal standard.

For GC-MS analyses **1** was hydrolyzed in 6N HCl (aq) at 110 °C for 3 h. The hydrolysate residue was evaporated and derivatized with bis(trimethylsilyl)trifluoroacetamide - pyridine, 1 : 1 (v/v) at 50 °C for 30 min. GC-MS analyses were carried out using a J & W DB-5 Durabond capillary column (15 m x 0.3 mm, 25 μm film). Components were identified by interpretation of their mass spectra and by comparison to library spectra.

LC-MS analyses were carried out using a Zorbax SB-C8, 2.1 x 50 mm column, at 40 °C, with a flow rate of 0.2 ml/min. A binary solvent system, where solvent A = 10% MeCN (aq) with 0.01% TFA plus 1.3 mM ammonium formate, and solvent B = 90% MeCN (aq) with 0.01% TFA plus 1.3 mM ammonium formate, was used. The gradient was from 10% to 100% B in 15 min, hold for 10 min and return to initial conditions for 5 min. The derivatized amino acids were identified based on retention times and interpretaion of their ESI spectra versus control standards.

$^1$H-NMR and $^{13}$C -NMR spectra were recorded in 0.13 ml, 26.3 mM solutions in DMSO-$d_6$ at 25 °C, on a Varian Unity 500 NMR spectrometer, using either a standard 3 mm direct or indirect probe. Chemical shifts are given in ppm relative to TMS at zero ppm, using the solvent peaks at $\delta_H$ 2.50 ppm (for $^1$H spectra) and $\delta_C$ 39.51 ppm (for $^{13}$C spectra) as internal standard. Proton-proton chemical shift correlation spectra (COSY) were recorded using the standard pulse sequence[8]. The TOCSY experiment[9] was performed with 0.12 sec mixing time. Proton-carbon chemical shift correlations were obtained using the indirect detection methods, HMQC[10] (with $^1J_{XH}$ = 140 Hz) and HMBC[11]. The HMBC spectra were optimized for a $^nJ_{CH}$ of 7 Hz. The NOESY experiment[12] was performed with 0.3 sec mixing time and a 3 sec relaxation delay.

L-amino acid oxidase type IV (EC 1.4.3.2, from *Crotalus adamanteus*), D-amino acid oxidase type X (EC 1.4.3.3, from porcine kidney) and amino acid standards were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. D-α-methylbenzyl isothiocyanate was obtained from Trans World Chemicals, Rockville, Maryland, U.S.A.

*Fermentation*

The producing eubacterium, isolated from a soil sample collected at the base of a tree near a stream bank in Hillsborough, New Jersey, U.S.A., was identified as a strain of *Serratia marcescens*, and given the accession number, MB5691 (Merck Microbial Resources Culture Collection).

The soil was used to establish an enrichment culture in a medium containing nutrient broth, 8 g/L, glucose, 10 g/L, brom cresol purple, 15 mg/L, pH 4.5. After incubation for 3 days, the growth in the enrichment was streaked onto a medium consisting of glucose, 10 g/L, casein hydrolysate, 10 g/L, NaCl, 5 g/L, agar, 15 g/L, in 0.1M MOPS buffer, pH 7.0. Isolates were then picked and purified. Production of **1** was done by inoculating a loopful of cells of MB5691 into 50 ml production medium in a triple baffled 250 ml flask and incubating at 28 °C and 220 rpm for 24 hr. The production medium consisted of Tryptic Soy Broth (Difco) supplemented with glycerol, 5 g/L and buffered at pH 6.8 with 0.1M MOPS buffer.

*Isolation of 1*

3 L of broth was extracted with methyl ethyl ketone (2 x 4.2 L). The organic layer was flash evaporated to dryness, dissolved in 95% MeOH (aq) (50 ml), defatted with hexanes (2 x 50 ml) and flash evaporated to dryness (11.5 g). A methanolic solution of this residue was purified on an open column of Diaion HP-20 ($V_b$ = 400 ml in MeOH) equilibrated with 40% MeCN (aq) (4 L). Elution was performed using a stepwise gradient of 40% MeCN (aq) (1 L), 45% MeCN (aq) (1 L) and 50% MeCN (aq) (4 L). Compound **1** was located in 1.6 - 4.0 L elution with a dry wt. of 2.7 g upon flash evaporation.

The enriched sample above was solubilized in MeOH (8 ml) and further purified as four parallel injections on a preparative Hamilton PRP-1 column ($V_b$ = 90.8 ml). Using 45% MeCN (aq) at 15 ml/min for

isocratic elution at room temperature, 1.4 g of homogeneous **1** was obtained from an elution volume of 465 - 630 ml upon flash evaporation.

*Physical data for 1*

**1**. white amorphous powder, $C_{38}H_{61}O_9N_5$; $[\alpha]_D^{24}$= -9.4° (*c* 1.0, MeOH); UV $\lambda_{max}^{MeOH}$ nm (log ε): 205 (4.26), 268 (2.60); FTIR (ZnSe) $\nu_{max}$ $cm^{-1}$: 3313, 2932, 1650, 1529. HR-EI-MS data are detailed in the text. $^1H$ and $^{13}C$ NMR data (500/125 MHz, DMSO-$d_6$, 25 °C) are detailed in Table 1.

*Determination of chiralties of amino acid residues of 1*

Acid hydrolysate was prepared as above. Parallel 0.2 mg samples were treated with 2.55 units/0.5 ml buffer of either L- amino acid oxidase (buffer: 0.2 M Tris-HCl, pH 7.8) or D-amino acid oxidase (buffer: 0.02 M $Na_2HPO_4$, pH 8.3) and incubated at 37 °C for 6 h. Amino acid standards were treated similarly in parallel as control.

The AMBI derivatives[4] were prepared as follows. Approximately 100 μg of the amino acid oxidase treated sample was dried under vacuum. The sample was triturated with 100 μl of $H_2O$ - EtOH - $Et_3N$, 2 : 2: 1 (v/v/v) and again dried under vacuum. The AMBI derivative was then formed by adding 200 μl of fresh derivatizing reagent: EtOH - $H_2O$ - $Et_3N$ - AMBI, 7 : 1: 1 : 1 (v/v/v/v). Reaction proceeded at room temperature for 20 min in a closed vial. The reaction mixture was then dried under vacuum for 60 min. The AMBI derivatives were analyzed by LC-MS method above.

## REFERENCES


1. Malmstadt, H.V.; Hadjiioannou, T.P. *Analyt. Chem.*, **1963**, *35*, 14.
2. Van Dyke, K.; Szustkiewicz, C. *Clin. Chem.*, **1969**, *15*, 154.
3. Dixon, M.; Kleppe, K. *Biochim. Biophys. Acta*, **1965**, *96*, 368.
4. Gal, J.; Allen, J.S. *J. Chromatogr.* **1984**, *314*, 275.
5. Wasserman, H.W.; Keggi, J.J.; McKeon, J.E. *J. Am. Chem. Soc.*, **1962**, *84*, 2978.
6. Cartwright, N.J. *Biochem. J.*, **1955**, *60*, 238.
7. Ichihara, A.; Hashimoto, M.; Sakamura, S. *Agric. Biol. Chem.*, **1985**, *49*, 2207.
8. Bax, A.; Freeman, R.; Morris, G.A. *J. Magn. Reson.* **1981**, *42*, 164.
9. Bax, A.; Davis, D. *J. Magn. Reson.* **1985**, *65*, 355.
10. Bax, A.; Subramanian, J. *J. Magn. Reson.* **1986**, *67*, 375.
11. Bax, A.: Summers, M.F. *J. Am. Chem. Soc.* **1986**, *108*, 2093.
12. States, D.J.; Haberkorn, R.A.; Ruben, D.J. *J. Magn. Reson.* **1982**, *48*, 286.