

ORIGINAL ARTICLE

A new anti-MRSA antibiotic complex, WAP-8294A II. Structure characterization of minor components by ESI LCMS and MS/MS

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The anti-MRSA antibiotic, WAP-8294A, was isolated from the fermentation broth of *Lysobacter* sp. The major component, WAP-8294A2, is composed of 1 mol of Gly, L-Leu, L-Glu, D-Asn, D-Trp, D-threo- β -hydroxyasparagine, N-Me-D-Phe and N-Me-L-Val, and 2 mol of L-Ser, D-Orn and D-3-hydroxy-7-Me-octanoic acid. The structure of the WAP-8294A2 was mainly determined as a cyclic depsipeptide by 2D NMR experiments. However, it was difficult to use the NMR experiment to determine the minor components, A1, A4 and Ax13, isolated in small amounts. In the present study, ESI MS/MS was applied to the structure elucidation of these minor components. The structures of these minor components were determined on the basis of the fragmentation pattern of the product ions of WAP-8294A2 in the ESI MS/MS. As a result, it was confirmed that A1 and A4 had the same amino acid sequence as A2, while A1 and A4 had the 3-OH-octanoic acid and 3-OH-8-Me-nonanoic acid, respectively, in the place of the 3-OH-7-Me-octanoic acid in A2. In the structure of Ax13, it was found that Gly of A2 was changed to β -Ala of Ax13.

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INTRODUCTION

Nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals is still a serious clinical problem.^{1,2} The glycopeptide antibiotics, vancomycin and teicoplanin, and the aminoglycoside antibiotic, arbekacin, have been used for the treatment of infections due to MRSA. However, MRSA has been found to be resistant to teicoplanin and arbekacin. MRSA strains with an intermediate resistance to vancomycin have also been reported in clinical isolates.^{3,4} Recently, newer drugs, such as linezolid and daptomycin, have been the focus of much attention in this field.⁵ A member of the oxazolidinone class of drugs, linezolid, is active against most Gram-positive bacteria that cause disease, including streptococci, vancomycin-resistant enterococci and MRSA.^{6,7} Daptomycin is a novel lipopeptide antibiotic used in the treatment of certain infections caused by Gram-positive organisms. It is a naturally occurring compound found in the soil saprotroph *Streptomyces roseosporus*.^{8,9}

We have also found a novel anti-MRSA antibiotic, WAP-8294A, that is a complex of 20 closely related components produced by *Lysobacter* sp.¹⁰ The WAP-8294A2 (1) was isolated as the major component, while A1 (2), A4 (3) and Ax13 (4) were minor components. The major component of 1 showed a strong activity against Gram-positive bacteria including MRSA *in vitro* and *in vivo*. They are cyclic depsipeptides containing 12 amino acid residues and one 3-hydroxy-fatty acid residue.

WAP-8294A2 is composed of Gly, L-Leu, L-Glu, D-Asn, D-Trp, D-threo- β -hydroxyasparagine (β OHAsn), N-Me-D-Phe and N-Me-L-Val, and two residues of L-Ser, D-Orn and D-3-hydroxy-7-Me-octanoic acid. As shown in Figure 1, the structure of 1 was determined as the cyclic depsipeptide by 2D NMR experiments including HMBC (¹H-detected multiple-bond HMQC) and ROESY techniques.¹¹ Usually the sequence of the constituent amino acids of the peptide is mainly established with the help of 2D NMR experiments. However, these minor components were obtained in small amounts of the pure compounds. Therefore, there is a limitation to the structural determination of small amounts of samples using only NMR techniques.

ESIMS coupled with MS/MS is capable of determining the amino acid sequences of peptides and proteins.^{12,13} On the basis of the fragmentation patterns of WAP-8294A2 whose amino acid sequence was firmly determined, the amino acid sequences of the minor components were deduced under the ESI MS/MS conditions. In this paper, we report the structure characterization of the three minor components, WAP-8294A1 (2), A4 (3) and Ax13 (4) by ESI MS/MS.

RESULTS

General properties of the WAP-8294A components

These components are quite similar to each other in physico-chemical properties.¹⁰ They are soluble in water, methanol and dimethyl-

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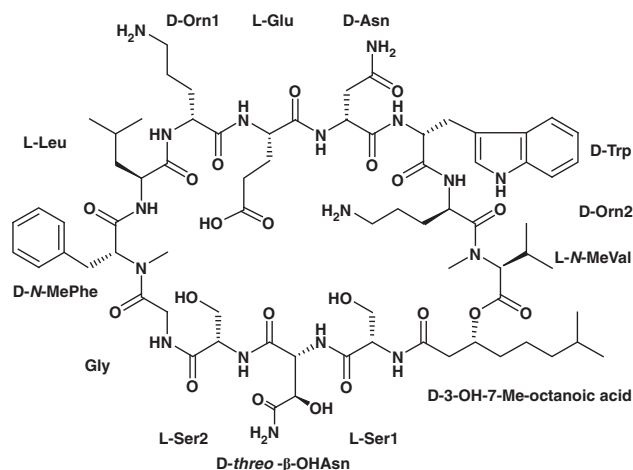


Figure 1 Structure of WAP-8294A2 (1).

sulfoxide, and insoluble in acetone, ethyl acetate and chloroform. They are positive to the ninhydrin reaction. In the IR spectra, they showed dominant absorptions at 1636 and 1541 cm^{-1} , because of the peptide bonds. The molecular formulas were determined by high-resolution FAB-MS. Acid hydrolysis of these minor components gave amino acids and hydroxy fatty acids. These constituent amino acids were determined by comparison with authentic samples using an amino acid autoanalyzer. Their absolute configurations were determined by amino acid analysis using the advanced Marfey's method.^{14–16} The mass chromatograms of the amino acid derivatives from **1** and **4** using ESI-LC/MS are shown in Figures 2A and B, respectively. The chirality of the fatty acid was determined by HPLC of the 3,5-dinitroaniline (DA) derivatives. The retention times of the authentic DA-derivative of the D- and L-3-OH-7-Me-octanoic acid were: D-isomer 56.9 min; L-isomer 54.2 min. The retention time of the DA-derivative of the fatty acid from **1** was 57.1 min. The retention times of the authentic D, L 3-OH-octanoic acid were: D-isomer 61.4 min; L-isomer 58.5 min. The retention time of the DA-derivative of the fatty acid from **2** was 61.4 min. The retention times of the authentic D, L 3-OH-8-Me-nonanoic acid were: D-isomer 52.7 min; L-isomer 50.3 min. The retention time of the DA-derivative of the fatty acid from **3** was 52.7 min. These results indicated that the chirality of the fatty acid from **1**, **2** and **3** were the D (R) form. These results are shown in Table 1.

Structure of A2 (1)

The ESIMS of **1** is shown in Figure 3. The ESIMS of **1** produced doubly protonated molecules, $[\text{M}+2\text{H}]^{2+}$, but protonated molecules, $[\text{M}+\text{H}]^+$, appeared as a weak peak. The ion at m/z 1042.6 was assigned as the triply protonated molecules of the dimer of **1**, $[\text{2M}+3\text{H}]^{3+}$. Moreover, these data were not useful fragment ions for the structure elucidation. Therefore, $[\text{M}+2\text{H}]^{2+}$ was selected as the precursor ion of **1**. Figure 4 shows the product ion spectrum for the $[\text{M}+2\text{H}]^{2+}$ of **1** and the assignment of the diagnostic ions. The fragment ions at m/z 134 and 159 correspond to the immonium ions of NMePhe and Trp, respectively. The product ion at m/z 1449.7 for $[\text{M}+2\text{H}]^{2+}$ of **1** was characterized as that the lactone linkage of **1** was opened and MeVal was consequently released from the C-terminus of the linear peptide. Moreover, the fragment ion peaks of **1** were observed at m/z 244.1, 518.3, 679.3, 792.4, 906.5, 1035.5, 1149.6 and 1335.6 as the B series and at m/z 115.1, 301.2, 415.2, 544.3, 771.4, 932.5, 989.5, 1076.6 and 1206.6 as the Y series. The detailed analysis of the

ESIMS/MS fragmentation pattern gave the following complete sequence: 3-OH-7-Me-octanoic acid-Ser- β OHAsn-Ser-Gly-NMePhe-Leu-Orn-Glu-Asn-Trp-Orn. These results reinforced the elucidated structure of **1**.

Structure of A1 (2)

The molecular formula of **2** was confirmed as $\text{C}_{72}\text{H}_{109}\text{N}_{17}\text{O}_{21}$ based on the HR FAB-MS spectral data (calcd.: 1548.8063 for $[\text{M}+\text{H}]^+$, found: 1548.8088 in the positive ion mode). This molecular formula was 14 mass units smaller than that of **1**. The constituent amino acids and their chiralities of **2** were the same amino acids as **1**. The constituent fatty acid of **2** had 3-OH-octanoic acid instead of the 3-hydroxy-7-methyloctanoic acid (3-OH-7-Me-octanoic acid) of **1**. The product ions of **2** in the ESI MS/MS were characterized as the same fragmentation as that of **1** based on a comparison of their product ion spectra. Although the common product ions at m/z 301.2, 415.2, 544.3, 771.4, 932.5, 989.5, 1076.6 and 1206.6 were observed as the Y series, the difference of 14 mass units was found in the product ions containing the 3-OH-octanoic acid moiety (Figure 5). Therefore, it was confirmed that the structure of **2** had the same amino acids sequence as **1** and that **2** possessed the 3-OH-octanoic acid in the place of the 3-OH-7-Me-octanoic acid in **1** (Figure 6).

Structure of A4 (3)

The molecular formula of **3** was confirmed as $\text{C}_{74}\text{H}_{113}\text{N}_{17}\text{O}_{21}$ based on the HRFAB-MS spectral data (calcd.: 1576.8375 for $[\text{M}+\text{H}]^+$, found: 1576.8363 in the positive ion mode). This molecular formula was 14 mass units larger than that of **1**. The constituent amino acids and their chiralities of **3** were the same amino acids as **1**. The constituent fatty acid of **3** had 3-hydroxy-8-methylnonanoic acid (3-OH-8-Me-nonanoic acid) instead of the 3-OH-7-Me-octanoic acid of **1**. The product ions of **3** in the ESI MS/MS were characterized as the same fragmentation as that of **1** based on a comparison of their product ion spectra. Although the common product ions at m/z 301.2, 415.2, 544.3, 771.4, 932.5, 989.5, 1076.6 and 1206.6 were observed as the Y series, the difference of 14 mass units was found in the product ions containing the 3-OH-8-Me-nonanoic acid moiety. These results indicated that **3** had the same amino acid sequence as **1** (Figure 5). Therefore, for the structure of **3**, it was found that the 3-OH-7-Me-octanoic acid of **1** was changed to the 3-OH-8-Me-octanoic acid of **3** (Figure 6).

Structure of Ax13 (4)

The molecular formula of **4** was determined as $\text{C}_{74}\text{H}_{113}\text{N}_{17}\text{O}_{21}$ based on the HRFAB-MS spectral data (calcd.: 1576.8375 for $[\text{M}+\text{H}]^+$, found: 1576.8324 in the positive ion mode). This molecular formula was 14 mass units larger than that of **1**. The acid hydrolysate of **4** contained 1 mol of β -Ala, Leu, Glu, Asp, Trp, β OHAsp, NMeVal and Phe, and 2 mol of Ser and Orn were confirmed. The constituent fatty acid was found to be 3-OH-7-Me-octanoic acid. These results indicated that **4** had the same fatty acid as **1**, and the difference between **4** and **1** was that between β -Ala and Gly. The product ions of **4** in the ESI MS/MS were characterized as the same fragmentation as that of **1** based on a comparison of their product ion spectra. Although the common product ions at m/z 301.2, 415.2, 544.3, 771.4 and 932.5 were observed as the Y series, the difference of 14 mass units was found in the product ions containing the β -Ala moiety as the cleavage ions of the B and Y series (Figure 5). Therefore, for the structure of **4**, it was found that Gly of **1** was changed to β -Ala of **4** (Figure 6).

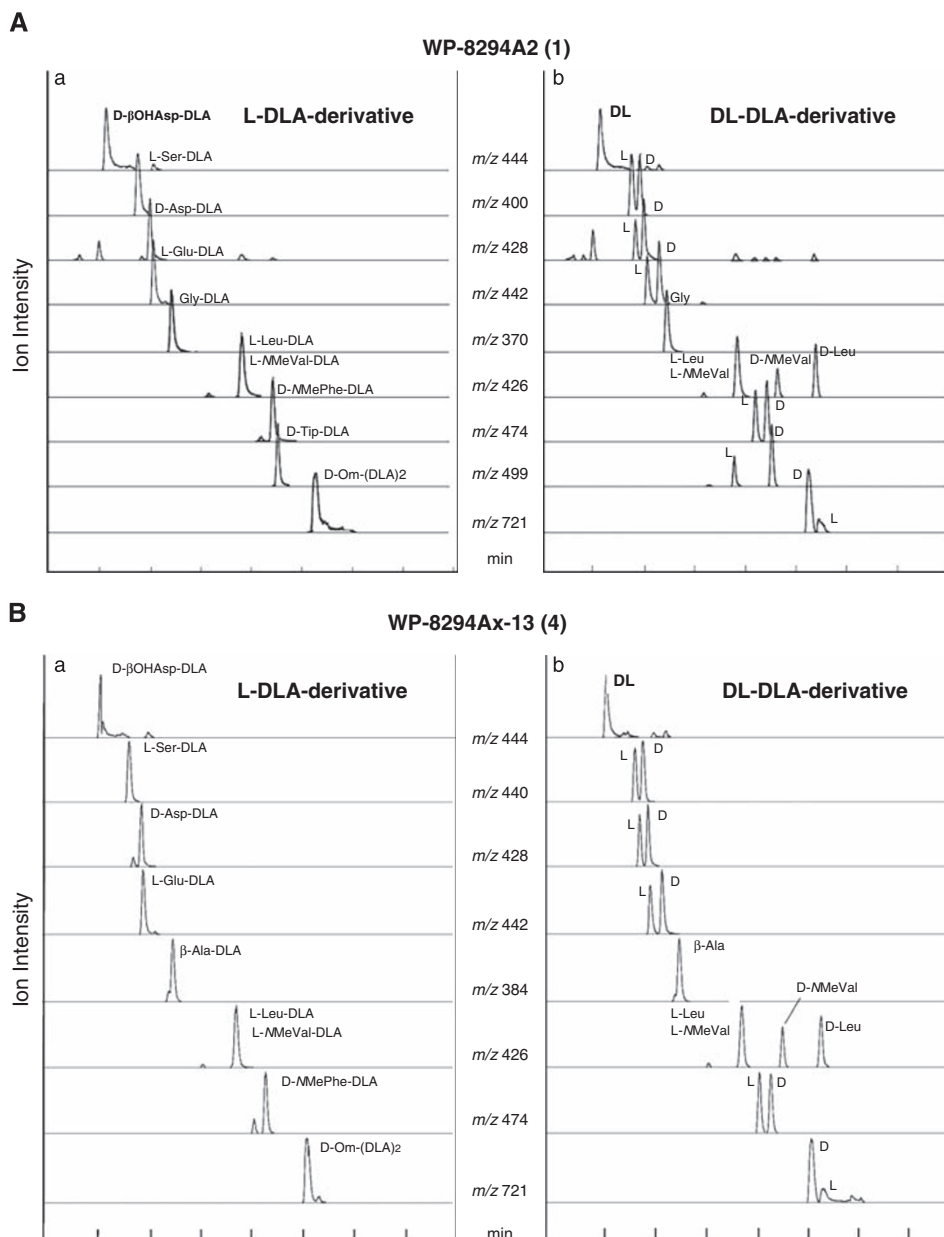


Figure 2 Mass chromatograms of each constituent amino acid. **(A)** WAP-8294A2 (1) (a) L-DLA derivative; (b) DL-DLA derivative and **(B)** WAP-8294Ax-13 (4) (a) L-DLA derivative; (b) DL-DLA derivative by advanced Marfey's method.

DISCUSSION

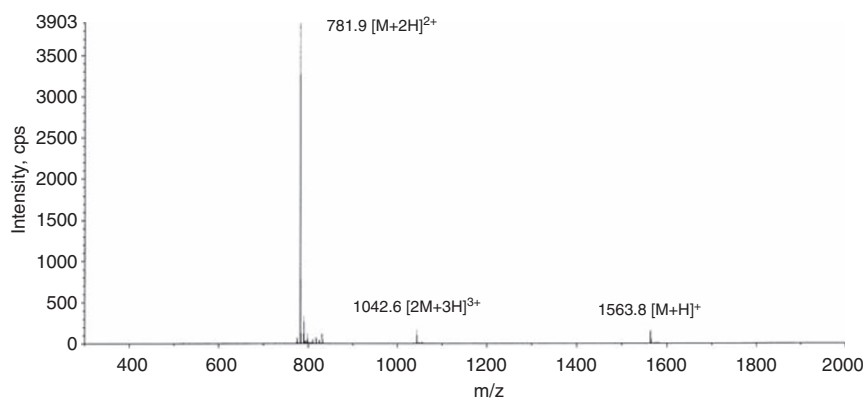
In a previous paper, we reported the taxonomy of the producing strain, isolation of the microbial active compounds, WAP-8294A, and their microbial activities.¹⁰ Originally, the producing strain, *Lysobacter* sp., produces a WAP-8294 complex that contains at least 20 components. Of these components, the main one, WAP-8294A2, has been structurally characterized mainly by NMR techniques¹¹ and WAP-8294A1, A4 and Ax13 were isolated in a pure state by chromatographic techniques. In the present study, we tried to determine the structures of WAP-8294A1, A4 and Ax13 based on the fragmentation patterns of WAP-8294A2 in the ESI MS/MS. These results are summarized in Figure 6. Thus, it was found that the ESI MS/MS method is very effective for the structural determination of closely related components derived

from the difference in a part of the constituent amino acids and fatty acids.

Jegorov *et al.*¹⁷ reported that destruxins, cyclic hexadepsipeptides, were protonated at the amide nitrogen of NMeAla or NMeVal, followed by ring opening, and subsequent fragmentation of the resulting linear peptide occurred under the FAB MS/MS conditions. Furthermore, Harada *et al.*¹⁸ reported that the lactone linkage of aeruginopeptins, cyclic hexadepsipeptides, was opened, and the C-terminus Ile was subsequently released from the protonated molecule in the FAB MS/MS.¹⁹ On the contrary, no fragmentation of glomospirin, a cyclic heptadepsipeptide, was reported in the ESI MS/MS.²⁰ In this study, we also observed the ring opening of WAP-8294A accompanied with the liberation of NMeVal and consequent fragmentation from the resulting linear peptide in the ESI MS/MS (q-TOF

Table 1 Physicochemical properties and constituent amino acids and fatty acid of WAP-8294A2 (1), A1 (2), A4 (3) and Ax13 (4)

Compound	1	2	3	4
Molecular formula	C ₇₃ H ₁₁₁ N ₁₇ O ₂₁	C ₇₂ H ₁₀₉ N ₁₇ O ₂₁	C ₇₄ H ₁₁₃ N ₁₇ O ₂₁	C ₇₄ H ₁₁₃ N ₁₇ O ₂₁
Calcd. (M+H) ⁺	1562.8219	1548.8063	1576.8375	1576.8375
Found (M+H) ⁺	1562.8224	1548.8088	1576.8363	1576.8324
Constituent fatty acid	3-OH-7-Me-octanoic acid	3-OH-octanoic acid	3-OH-8-Me-nonanoic acid	3-OH-7-Me-octanoic acid
Molecular formula	C ₉ H ₁₈ O ₃	C ₈ H ₁₆ O ₃	C ₁₀ H ₂₀ O ₃	C ₉ H ₁₈ O ₃
Constituent amino acid (chirality, molar ratio)	Asp (D, 1) Glu (L, 1) Gly (1) Leu (L, 1) Trp (D, 1) Orn (D, 2) Ser (L, 2) β-OH Asp (D, <i>threo</i> , 1) <i>N</i> -MeVal (L, 1) <i>N</i> -MePhe (D, 1)	Asp (D, 1) Glu (L, 1) Gly (1) Leu (L, 1) Trp (D, 1) Orn (D, 2) Ser (L, 2) β-OH Asp (D, <i>threo</i> , 1) <i>N</i> -MeVal (L, 1) <i>N</i> -MePhe (D, 1)	Asp (D, 1) Glu (L, 1) Gly (1) Leu (L, 1) Trp (D, 1) Orn (D, 2) Ser (L, 2) β-OH Asp (D, <i>threo</i> , 1) <i>N</i> -MeVal (L, 1) <i>N</i> -MePhe (D, 1)	Asp (D, 1) Glu (L, 1) β-Ala (1) Leu (L, 1) Trp (D, 1) Orn (D, 2) Ser (L, 2) β-OH Asp (D, <i>threo</i> , 1) <i>N</i> -MeVal (L, 1) <i>N</i> -MePhe (D, 1)

**Figure 3** ESI-MS of WAP-8294A2 (1).

type) as reported in destruxins and aeruginopeptins under the FAB MS/MS conditions mentioned above. The formed cleavage ions of the B and Y series were quite useful for the sequencing. Recently, we confirmed that ion trap LC/multi-stage MS (LC/MSⁿ) is quite effective for the sequencing of the depsipeptides.²¹

The antimicrobial activity of the minor components, WAP-8294A1, A4 and Ax13, were essentially same as that of the main one against Gram-positive bacteria including MRSA.¹⁰ WAP-8294A2 shows a more potent activity against MRSA than the clinically useful anti-MRSA antibiotic, vancomycin. Particularly, these antibiotics have the characteristic advantage that the activity is highly enhanced by the addition of human serum.¹⁰ Currently, the development of these compounds for clinical use is underway.

EXPERIMENTAL PROCEDURE

Materials

The pure compounds of 1 (major component) and 2, 3, 4 (minor components) were isolated from the fermentation broth of *Lysobacter* sp. as previously described.¹⁰ HPLC grade solvents from Wako Pure Chemical Industries (Osaka, Japan) were used. L- and D-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucinamide) were purchased from the Tokyo Kasei (Tokyo, Japan).

Determination of chirality of amino acid

Their absolute configurations were determined by amino acid analysis using the advanced Marfey's method, which was developed to non-empirically determine the absolute configuration of the constituent amino acids in a peptide using LC/MS.^{14,15} Each 500 μg sample was hydrolyzed at 110 °C for 1 h with 500 μl of 6 M HCl. This solution was divided into two portions, and each portion was derivatized with L- or D-FDLA.¹⁶ Each solution was then evaporated to dryness, and the residue was dissolved in 75 μl of water and 25 μl of methanol. To each amino acid solution, 20 μl of 1 M sodium bicarbonate and then 100 μl of 1% L- or D-FDLA in acetone were added. These solutions were vortexed and incubated at 40 °C for 1 h. These reactions were quenched by the addition of 20 μl of 1 M HCl. After dilution with 260 μl of acetonitrile, 1 μl of the L-DLA (2,4-dinitrophenyl-5-leucinamide) derivative and an equal mixture of the L- and D-DLA derivatives were analyzed by ESI LC/MS. The separation of the L- and D-DLA derivatives was performed on an TSK gel ODS-80Ts (100 × 2 mm i.d., TOHCO, Tokyo, Japan) column maintained at 40 °C. Acetonitrile-water containing 1% formic acid was used as the mobile phase in the linear gradient elution mode (acetonitrile, 40–90%, 10 min) at a flow rate of 0.2 ml min⁻¹. The MS was an API Qstar Pulsar-*i* quadrupole TOF MS (AB SCIEX, Foster City, CA, USA). A mass range of *m/z* 350–1500 was covered with a scan time of 1 s, and all data were collected in the positive ion mode. The HPLC and MS were interfaced with a laboratory-made flow splitter and an

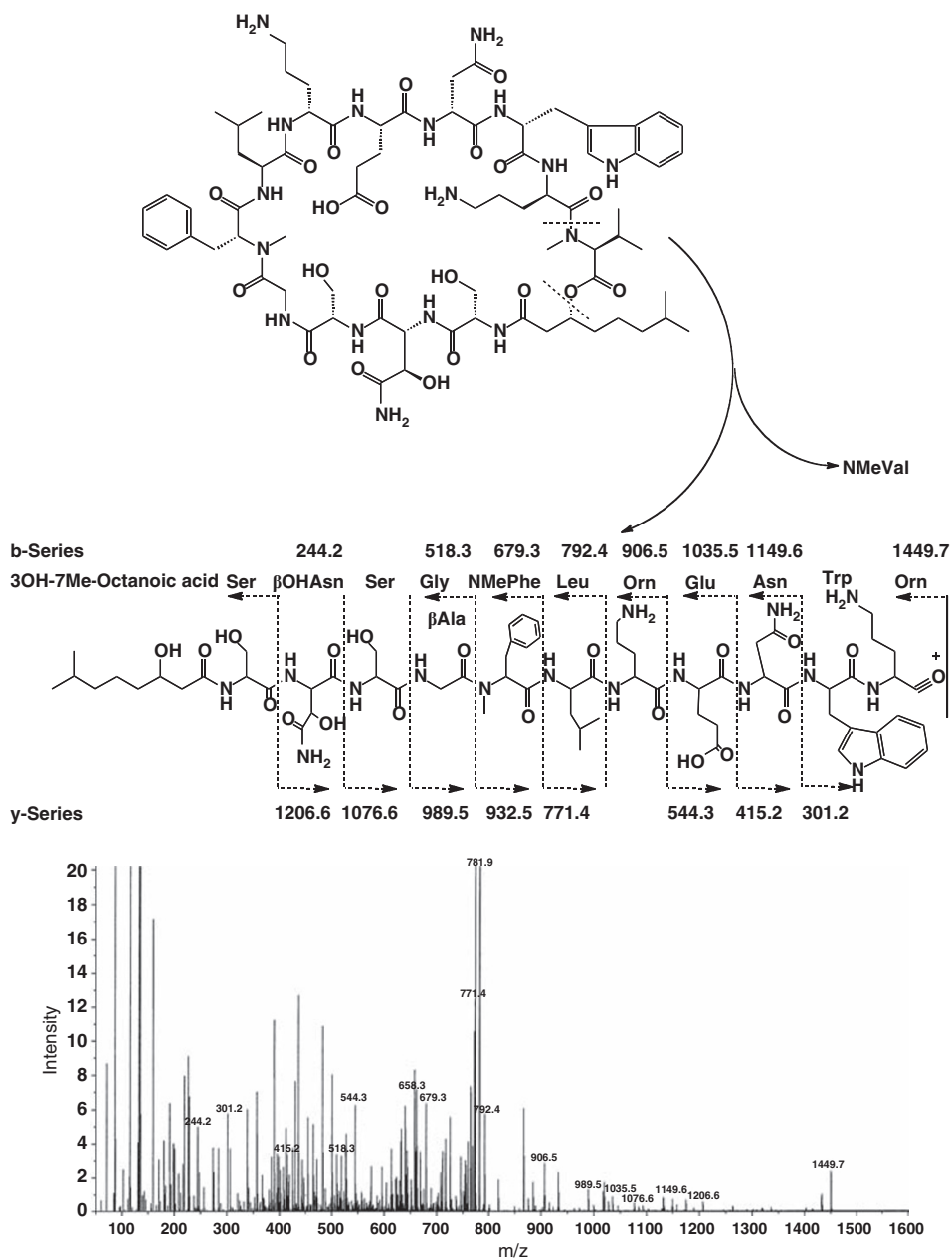


Figure 4 Product ion spectrum of $[M+2H]^{2+}$ at m/z 781.9 of WAP-8294A2 (**1**) and its fragmentation scheme.

IonSpray ion source. The effluent from the HPLC was split at a ratio of 1:40, and a smaller portion of the effluent was introduced into the ion source at the flow rate of $5 \mu\text{l min}^{-1}$. The IonSpray voltage was 5.5 kV with the nebulizer gas air pressure and curtain gas nitrogen pressure set at 20 and 25 psi, respectively.

Determination of chirality of fatty acid

Authentic D, L 3-OH-octanoic acid was purchased from Sigma-Aldrich (Prague, Czech Republic). D, L 3-OH-7-Me-octanoic acid and D, L 3-OH-8-Me-noanoic acid were synthesized by the method of Fritzsche *et al.*²² The chirality of the fatty acid was determined by HPLC of the DA derivatives on a chiral column.²³ Each 15 mg of a sample was hydrolyzed at 110°C for 2 h with 1.5 ml of 4 M HCl. The hydrolysates extracted with 1.5 ml of diethylether were dried. In all, 10 mg of the synthesized sample and the hydrolysates were dissolved in $100 \mu\text{l}$ of dioxane, then $100 \mu\text{l}$ of dioxane containing 10% *N, N'*-dicyclohexylcarbodiimide and $100 \mu\text{l}$ of

dioxane containing 10% DA (Sigma-Aldrich, St Louis, MO, USA) were added. The mixture was incubated at room temperature for 18 h. The reaction products were passed through a disposable syringe filter DISMIC-3 (Advantec Toyo, Tokyo, Japan) to remove the dioxane-insoluble co-product, *N, N'*-dicyclohexylurea. For the HPLC analysis, $10 \mu\text{l}$ of the filtrate containing the DA-derivatives of the fatty acids was injected into the column. The separation of the D, L-DA derivatives was performed on a TSK gel Enantio P2 ($250 \times 4.6 \text{ mm i.d.}$, TOHCO, Tokyo, Japan) column using a Shimadzu (Kyoto, Japan) LC-6A pump and SPD-10A detector. The detector was set at 254 nm. Elution was performed using the eluent, *n*-hexane-1,2-dichloroethane-ethanol (35-15-5).

ESI MS/MS

The positive ion ESI-MS were recorded on an API QSTAR Pulsar *i* quadrupole TOF MS (AB Sciex, Foster City, CA, USA). The analyte solutions were infused at a flow-rate of $5 \mu\text{l min}^{-1}$ into an IonSpray source maintained at 4.5 kV.

Ax13 b-Series	244.1	532.3	693.3	806.4	920.5	1049.6	1463.8
3OH-7Me-octanoic acid		(+14)	(+14)	(+14)	(+14)	(+14)	(+14)
A4 b-Series	258.2	532.3	693.3	806.5	920.5	1049.6	1163.6
3OH-8Me-nonanoic acid	(+14)	(+14)	(+14)	(+14)	(+14)	(+14)	(+14)
A1 b-Series	230.2	504.3	665.3	778.4	892.5	1021.5	1135.6
3OH-octanoic acid	(-14)	(-14)	(-14)	(-14)	(-14)	(-14)	(-14)
A2 b-Series	244.2	518.3	679.3	792.4	906.5	1035.5	1149.6
3OH-7Me-octanoic acid							

A2 y-Series	1206.6	1076.6	989.5	932.5	771.4	544.3	415.2	301.2
A1 y-Series	1206.6	1076.6	989.5	932.5	771.4	544.3	415.2	301.2
A4 y-Series	1206.6	1076.6	989.5	932.5	771.4	544.3	415.2	301.2
Ax13 y-Series	1220.2	1090.6	1003.6	932.5	771.4	544.3	415.2	301.2
	(+14)	(+14)	(+14)					

Figure 5 Fragmentation schemes of WAP-8294A2 (1), A1 (2), A4 (3) and Ax-13 (4).

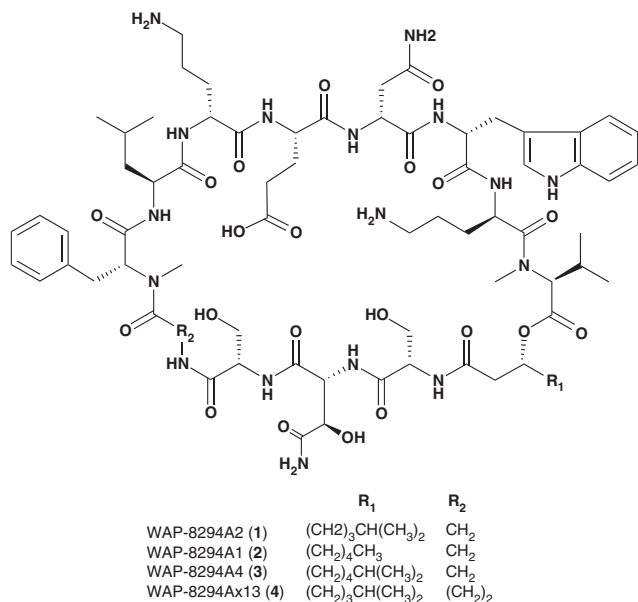


Figure 6 Structures of WAP-8294A2 (1), A1 (2), A4 (3) and Ax-13 (4) proposed by ESI MS/MS.

Nitrogen was used as the collision gas in all the experiments (collision energy at 35–55 eV). The spectra were scanned in the positive ion peak over the *m/z* range 50–2000. These samples were dissolved in 0.1% formic acid-50% methanol at a concentration of 20 μM.

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