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**JOURNAL
OF THE
AMERICAN
CHEMICAL
SOCIETY®**

Reprinted from
Volume 119, Number 28, Pages 6680–6681

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Received March 20, 1997

Infectious diseases due to antibiotic-resistant bacteria pose a threat to public health around the world.¹ Especially, nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals have become a serious clinical problem.² Glycopeptide antibiotics, vancomycin and teicoplanin, are the drugs of choice for the treatment of MRSA infections. However, isolation of the glycopeptide-resistant *S. aureus* and *in vitro* transfer of the vancomycin-resistant genes from *Enterococcus faecalis* to *S. aureus* have been recently reported.³ Therefore, an antibacterial agent which differs from vancomycin in mode of action is clinically desirable. In the course of our screening for antibiotics, we found that a novel antibiotic, WAP-8294A₂ produced by a Gram-negative bacterium *Lysobacter* sp. showed higher *in vivo* activity against MRSA than vancomycin. In this paper, we report the structure and activity of this antibiotic.

The *Lysobacter* sp. strain WAP-8294, isolated from a soil sample collected at Shimoda, Shizuoka, Japan, was cultivated in a production medium consisting of glucose, soybean flour, NaCl, and CaCO₃ at 30 °C for 3 days. WAP-8294A₂ (1) was isolated from the culture broth as a white amorphous powder:⁴ ninhydrin-positive; mp 215–225 °C (dec.); [α]_D²⁰ +42° (c 0.5 H₂O); IR (KBr) 3300, 1720–1715, 1636, 1541, 1404, 1207, 1137 cm⁻¹; UV λ_{\max} (nm) (ϵ) (H₂O) 275 (5000), 280 (5200), 287 (4600); HRFABMS m/z 1562.8224 (M + H)⁺, calcd for C₇₃H₁₁₂N₁₇O₂₁ Δ 0.3 ppm.⁵ The UV spectrum indicated the presence of an indole moiety, and the dominant absorptions at 1636 and 1541 cm⁻¹ in the IR spectrum indicated the peptide nature.

Amino acid analysis of the acid hydrolysate (4 M methanesulfonic acid, 110 °C, 24 h) revealed the presence of nine amino acids, Asp, Glu, Gly, Leu, Trp, 2 × Orn, and 2 × Ser.

(1) (a) Tomasz, A. *N. Eng. J. Med.* **1994**, *330*, 1247–1251. (b) Winker, M. A.; Flanagan, A. *JAMA* **1996**, *275*, 245–246.

(2) (a) Mulligan, M. E.; Murray-Leisure, K. A.; Ribner, B. S.; Standiford, H. C.; John, J. F.; Korvick, J. A.; Kauffman, C. A.; Yu, V. L. *Am. J. Med.* **1993**, *94*, 313–328. (b) Voss, A.; Kresken, M. *Chemotherapy* **1996**, *42* (supplement 2), 13–18.

(3) (a) Watanakunakorn, C. *J. Antimicrob. Chemother.* **1990**, *25*, 69–72. (b) Sieradzki, K.; Tomasz, A. *FEMS Microbiol. Lett.* **1996**, *142*, 161–166. (c) Noble, W. C.; Virani, Z.; Cree, R. G. A. *FEMS Microbiol. Lett.* **1992**, *93*, 195–198.

(4) In a typical isolation, EtOH (5 L) was added to the culture broth (5 L), which was adjusted to pH 2.5 with 6 N HCl and then centrifuged. The supernatant was passed through a column of Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan). The column was washed with water, 80% acetone (pH 11.0), and 80% acetone and was eluted with 80% acetone [0.05% trifluoroacetic acid (TFA)]. The eluate was chromatographed on a Chromatorex ODS (Fuji Silysia Chemical, Kasugai, Japan) column [30% CH₃CN (0.05% TFA)]. The column was washed with 30% CH₃CN (0.05% TFA) and 40% CH₃CN (0.05% TFA) and then eluted with 45% CH₃CN (0.05% TFA). The fraction rich in 1 (monitored by HPLC) was applied to a column of SP-TOYOPEARL 650C (Tosoh, Tokyo). The column was washed with water and eluted in a stepwise manner with 0.1–0.8 M NaCl. The active eluate was further purified by Chromatorex ODS [45% CH₃CN (0.05% TFA)] furnishing 693 mg of pure bis(trifluoroacetate) salt 1.

(5) HR-FAB mass spectra were recorded using a JEOL JMS-SX102A mass spectrometer [matrix, glycerol/*m*-nitrobenzyl alcohol (1:1)].

Additionally, three unusual amino acids were also detected by 2D-TLC from the hydrolysate and were isolated by ion-exchange chromatography. Their structures were determined as β -hydroxyaspartic acid (OHAsp), *N*-methylvaline (NMeVal), and *N*-methylphenylalanine (NMePhe) on the basis of NMR and FABMS experiments and were confirmed by direct comparison with authentic samples. The chiralities of the eight protein amino acids (except for Trp) and the two *N*-methylamino acids and Trp were determined by HPLC analysis of the (+)-1-(9-fluorenyl)ethyl chloroformate derivatives⁶ and chiral TLC analysis,⁷ respectively. The configuration of OHAsp was determined as *threo* by TLC analysis and *D* by chiral HPLC analysis.⁸ A fatty acid from the ether-extractable portion of the acid hydrolysate (6 N HCl, 110 °C, 2 h) was identified as 3-hydroxy-7-methyloctanoic acid (3-OH-7-Me-octanoic acid) on the basis of NMR spectral analysis and GC-MS analysis of its methyl ester, and its structure was finally confirmed by direct comparison with an authentic synthetic sample.⁹ The chirality of the fatty acid was determined as *D* (*R*) by HPLC of the 3,5-dinitroaniline (DA) derivatives on a chiral column.¹⁰

The determination of all constituent components from the hydrolysate and the molecular formula (C₇₃H₁₁₁N₁₇O₂₁) indicated that 1 has a cyclic peptide structure with two primary amides and one carboxylic acid in the side chains (Asx, Glx, and OHAsx). In fact, an increase of 14 mass units was observed in the FABMS of the methylation product of 1 with thionyl chloride and methanol.¹¹ Since treatment of 1 with Ac₂O/pyridine yielded a pentaacetate,¹² five out of the two amino and four hydroxyl groups (2 × Orn, and 2 × Ser, OHAsp and 3-OH-7-Me-octanoic acid) in 1 were estimated to be free. TLC analysis of the 2,4-dinitrofluorophenyl (DNP) derivative of the acid hydrolysate of 1 showed the presence of δ -DNP-Orn instead of Orn, indicating that the two Orn δ -amino groups are free. The amino acid and fatty acid analyses of the acid hydrolysate of 1 oxidized with chromic acid¹³ revealed the disappearance of three hydroxyl amino acids (2 × Ser and OHAsp) and the presence of intact 3-OH-7-Me-octanoic acid, indicating that the three hydroxyl groups of the amino acids are free. These results suggested that the hydroxyl group of 3-OH-7-Me-octanoic acid is involved in the cyclic structure formation.

Analysis of the ¹H, ¹³C, H–H COSY, and HSQC spectra of 1 (DMSO-¹²C₂, *d*₆) led to the assignment of proton signals except for the two Ser hydroxyl groups and the carbon signals

(6) Hayashi, T.; Sasagawa, T. *Anal. Biochem.* **1993**, *209*, 163–168.

(7) CHIRALPLATE (Macherey-Nagel), MeOH/H₂O/CH₃CN (50:50:200), *R_f* values: NMeVal, L-isomer 0.49, D-isomer 0.41; NMeVal from 1, 0.49; NMePhe, L-isomer 0.69, D-isomer 0.50; NMePhe from 1, 0.50; Trp, L-isomer 0.69, D-isomer 0.54; Trp from 1, 0.54.

(8) Silica gel TLC (Merck), 2-PrOH/NH₄OH/H₂O (4:2:1), *R_f* values: *threo*-OHAsp 0.52; *erythro*-OHAsp 0.39; OHAsp from 1, 0.52. HPLC, CHIRALPAK WH (4.6 × 250 mm, Daicel Chemical, Tokyo) using CH₃CN/2 mM CuSO₄ (4:96) as eluent at 254 nm. Retention time (flow rate 1.0 mL/min, 50 °C): L-isomer 9.1 min, D-isomer 5.9 min; OHAsp from 1, 6.1 min.

(9) (a) 3-OH-7-Me-octanoic acid was synthesized by the method of Fritzsche (Fritzsche, K.; Lenz, R. W.; Fuller, R. C. *Int. J. Biol. Macromol.* **1990**, *12*, 92–101). (b) The ether extract of the hydrolysate was methylated with (trimethylsilyl)diazomethane and was subjected GC-MS: GC, HP-5890II, column, J&W Scientific DB-5 (0.25 mm × 30 m); MS, JEOL JMS-AX505WA, ion mode, EI (+). For the methyl ester of authentic 3-OH-7-Me-octanoic acid: retention time, 9.68 min; *m/z* 103 (base peak), 170 (M⁺ – 18). For the methyl ester of 3-OH-7-Me-octanoic acid from 1: retention time, 9.72 min; *m/z* 103 (base peak), 170 (M⁺ – 18).

(10) (a) Nakagawa, Y.; Matsuyama, T. *FEMS Microbiol. Lett.* **1993**, *108*, 99–102. (b) DA-Derivatives were analyzed on TSK gel Enantio P2 (4.6 × 250 mm, Tosoh) using hexane/1,2-dichloroethane/EtOH (35:15:0.5) as eluent at 254 nm. Retention time (flow rate 1.0 mL/min): L-isomer 54.2 min, D-isomer 56.9 min, DA-derivative of the fatty acid from 1, 57.1 min.

(11) FABMS, JEOL JMS-AX505WA, methyl ester of 1: *m/z* 1576.8 (M + H)⁺. HRFABMS: *m/z* 1576.8326 (M + H)⁺, calcd for C₇₄H₁₁₄N₁₇O₂₁, Δ 0.8 ppm.

(12) Pentaacetate of 1: *m/z* 1773.0 (M + H)⁺.

(13) Shoji, J.; Hinoo, H.; Katayama, T.; Nakagawa, Y.; Ikenishi, Y.; Iwatani, K.; Yoshida, T. *J. Antibiot.* **1992**, *45*, 824–831.

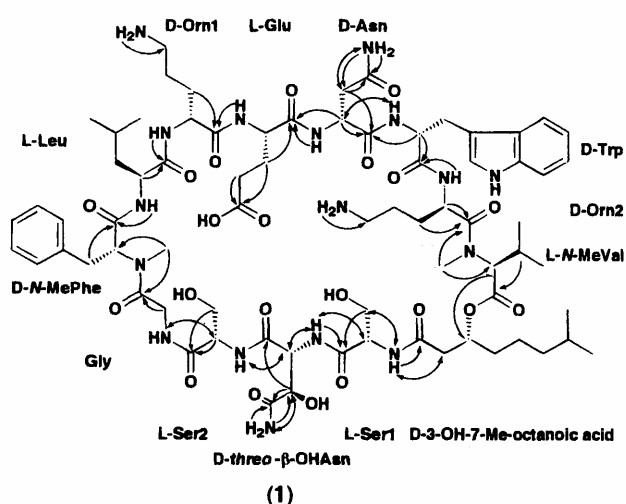


Figure 1. Structure of WAP-8294A₂ (**1**). Arrows show HMBC (→) and ROESY (↔) correlations.

except for those of the quaternary carbons.¹⁴ The assignment of the quaternary carbons was based on the HMBC correlation. The locations of the two primary amides were confirmed as Asn and OHAsn from the HMBC and ROESY data. The HMBC correlations from the α -amide protons or *N*-methyl protons to the neighboring carbonyl carbons were observed between OHAsn/Ser1, NMePhe/Gly, Leu/NMePhe, Orn1/Leu, Glu/Orn1, Asn/Glu, Orn2/Trp, and NMeVal/Orn2. The HMBC correlation from the α -methine proton of Asn to the carbonyl carbon of the neighboring Trp was also observed. In addition, the HMBC correlation of the α -amide proton of Ser1 was found to the carbonyl carbon of 3-OH-7-Me-octanoic acid, supporting the fact that the *N*-terminus of **1** is Ser1 and the amino group is acylated with 3-OH-7-Me-octanoic acid. Diagnostically important ROESY correlations from the α -methine protons to the neighboring α -amide protons were seen between Ser1/

OHAsn, OHAsn/Ser2, Ser2/Gly, and Asn/Trp. Furthermore, the ROESY correlation from the 3-methine proton of 3-OH-7-Me-octanoic acid to an α -methine proton of NMeVal indicated that the lactone linkage is formed between the 3-hydroxyl group of 3-OH-7-Me-octanoic acid and the α -carboxyl group of NMeVal. The presence of α -linked Glu, Asn, and OHAsn was shown by the HMBC and ROESY spectral analysis. The HMBC correlation from the α -amide proton of the neighboring residue to the α -carboxyl group of Glu was observed. The primary amide protons of Asn and OHAsn showed HMBC correlations with their own β -carbons. Furthermore, the α -methine protons of Asn and OHAsn showed ROESY cross peaks with the neighboring α -amide protons. Thus, the structure **1** was elucidated as shown in Figure 1.

Among the known antibiotics of bacterial origin, plusbasins (*Pseudomonas* sp.) and empedopeptin (*Empedobacter halodivium* nov. sp.) are somewhat similar to **1**, because they are cyclic depsipeptides containing one 3-hydroxy fatty acid.^{13,15} However, **1** differs from them in constituent amino acids and a fatty acid. Especially, 3-OH-7-Me-octanoic acid is the first to be isolated from antibiotics. Lysobactin, an antibiotic produced by *Lysobacter* sp. also differs from **1** in constituent amino acids and the absence of a fatty acid.¹⁶ While **1** was as active as vancomycin against MRSA clinical isolates (MIC: 0.78 $\mu\text{g}/\text{mL}$), interestingly, the activity of **1** was highly enhanced by the addition of 10% human serum (MIC: 0.1 $\mu\text{g}/\text{mL}$). No activity of **1** was observed against Gram-negative bacteria, yeasts, and fungi. *In vivo* efficacies of **1** and vancomycin were assessed in the experimental systemic MRSA infection of mice. The mean ED₅₀ values of **1** and vancomycin against nine MRSA strains were 0.38 and 5.3 mg/kg, respectively, indicating that **1** is 14 times more active than vancomycin. Very recently, vancomycin dimers with activity against vancomycin-resistant enterococci (VRE) have been reported (MIC: 19–55 $\mu\text{g}/\text{mL}$).¹⁷ Against VRE, **1** was more active than these vancomycin analogs (MIC: 6.3 $\mu\text{g}/\text{mL}$), suggesting that the mode of action of **1** is different from that of vancomycin. Moreover, **1** was active against *S. aureus* protoplasts as well as the intact cells, whereas vancomycin was not active against the protoplasts. In the disc diffusion test, the anti-MRSA activity of **1** was inhibited by the addition of phosphatidylglycerol or cardiolipin to the disc. These results suggest that **1** interacts selectively with phospholipids in the target cell membrane resulting in membrane damage.

Acknowledgment. We thank Mrs. N. Suzuki and Y. Aiba and Ms. N. Kokubo for their assistance during the course of this work. We also thank Mrs. A. Kusai and K. Nojima, JEOL and Co., Ltd., for the measurement of HR-FABMS.

Supporting Information Available: ¹H, ¹³C, H–H COSY, HSQC, HMBC, and ROESY spectra for **1** (8 pages). See any current masthead page for ordering and Internet access instructions.

JA970895O

(15) (a) Konishi, M.; Sugawara, K.; Hanada, M.; Tomita, K.; Tomatsu, K.; Miyaki, T.; Kawaguchi, H.; Buck, R. E.; More, C.; Rossomano, V. Z. *J. Antibiot.* **1984**, *37*, 949–957. (b) Sugawara, K.; Numata, K.; Konishi, M.; Kawaguchi, H. *J. Antibiot.* **1984**, *37*, 958–964.

(16) Tymiak, A. A.; McCormic, T. J.; Unger, S. E. *J. Org. Chem.* **1989**, *54*, 1149–1157.

(17) Sundram, U. N.; Griffin, J. H.; Nicas, T. I. *J. Am. Chem. Soc.* **1996**, *118*, 13107–13108.

(14) NMR spectra were taken on a JEOL A600 (600 MHz) instrument. ¹H NMR of **1**: 3-OH-7-Me-octanoic acid δ 2.31 (H2), 2.55 (H2'), 4.94 (H3), 1.50 (H4), 1.23 (H5), 1.11 (H6), 1.47 (H7), 0.81 (H8, H8'); Ser1 δ 4.82 (Ha), 3.45 (H β), 3.55 (H β'), 7.93 (NH); OHAsn δ 4.96 (Ha), 4.25 (H β), 7.34 (NH γ), 7.41 (NH γ'), 8.08 (NH), 5.65 (OH β); Ser2 δ 4.82 (Ha), 3.64 (H β), 8.29 (NH); Gly δ 3.88 (Ha), 8.19 (NH); NMePhe δ 4.41 (Ha), 2.93 (H β), 3.26 (H β'), 7.15 (H2,6), 7.26 (H3,5), 7.19 (H4), 2.56 (NMe); Leu δ 4.27 (Ha), 1.37 (H β), 1.51 (H β'), 1.38 (H γ), 0.67 (H δ), 0.70 (H δ'), 7.93 (NH); Orn1 δ 4.65 (Ha), 1.64 (H β), 1.70 (H β'), 1.55 (H γ), 2.85 (H δ), 7.72 (NH γ), 7.48 (NH); Glu δ 4.63 (Ha), 1.70 (H β), 1.82 (H β'), 2.17 (H γ), 8.51 (NH), 12.0 (OH δ); Asn δ 4.77 (Ha), 2.42 (H β), 2.60 (H β'), 6.93 (NH γ), 7.32 (NH γ'), 8.19 (NH); Trp δ 4.73 (Ha), 2.77 (H β), 3.11 (H β'), 7.13 (H2), 7.44 (H4), 6.87 (H5), 6.95 (H6), 7.30 (H7), 10.5 (NH1), 8.73 (NH); Orn2 δ 4.58 (Ha), 1.55 (H β), 1.66 (H β'), 1.45 (H γ), 2.77 (H δ), 7.72 (NH γ), 7.98 (NH); NMeVal δ 4.62 (Ha), 2.06 (H β), 0.71 (H γ), 0.86 (H γ'), 2.51 (NMe). ¹³C NMR: 3-OH-7-Me-octanoic acid δ 169.4 (CO), 38.3 (C2), 71.0 (C3), 33.1 (C4), 22.0 (C5), 38.2 (C6), 27.3 (C7), 22.3 (C8, 8'); Ser1 δ 169.6 (CO), 53.9 (Ca), 62.8 (C β); OHAsn δ 169.2 (CO), 54.7 (Ca), 71.3 (C β), 173.5 (CO γ); Ser2 δ 169.8 (CO), 54.9 (Ca), 62.1 (C β); Gly δ 169.0 (CO), 42.1 (Ca); NMePhe δ 169.4 (CO), 63.3 (Ca), 33.9 (C β), 138.4 (C1), 129.1 (C2,6), 128.3 (C3,5), 126.3 (C4), 35.1 (NMe); Leu δ 171.5 (CO), 51.2 (Ca), 39.3 (C β), 24.1 (C γ), 20.8 (C δ), 22.9 (C δ'); Orn1 δ 170.9 (CO), 50.7 (Ca), 30.3 (C β), 23.0 (C γ), 38.5 (C δ); Glu δ 169.8 (CO), 51.3 (Ca), 28.5 (C β), 29.7 (C γ), 174.0 (CO δ); Asn δ 170.5 (CO), 49.2 (Ca), 38.2 (C β), 171.8 (CO γ); Trp δ 170.7 (CO), 52.7 (Ca), 27.4 (C β), 123.3 (C2), 109.3 (C3), 118.1 (C4), 118.0 (C5), 120.7 (C6), 111.4 (C7), 127.2 (C8), 135.9 (C9); Orn2 δ 170.3 (CO), 48.3 (Ca), 28.1 (C β), 23.2 (C γ), 38.7 (C δ); NMeVal δ 169.1 (CO), 60.9 (Ca), 25.6 (C β), 18.0 (C γ), 19.1 (C γ'), 29.2 (NMe).