THE STRUCTURE OF PA48009: THE REVISED STRUCTURE OF DURAMYCIN

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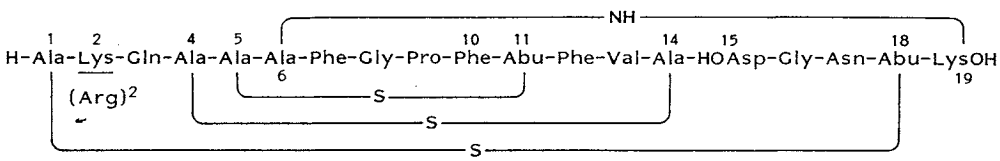
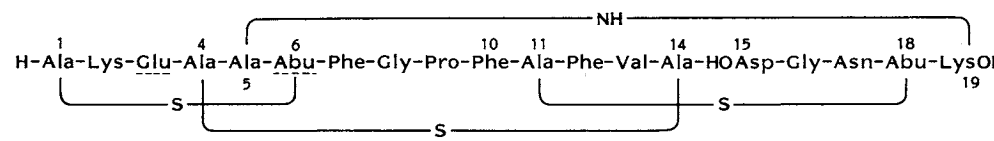
PA48009, a lanthionine-containing peptide antibiotic was isolated from the culture broth of *Streptoverticillium griseoverticillatum* PA-48009, and identified as duramycin. Determination of the structure using both Edman degradations and 2D NMR spectroscopy showed the need to revise the structure of duramycin given in literature. Duramycin (PA48009) was different from lanthiopeptin (Ro 09-0198, cinnamycin) only by a Lys/Arg exchange at position 2.

During screening of new biologically active compounds, PA48009 (**1**), a lanthionine-containing peptide antibiotic, was isolated from the fermentation broth of *Streptoverticillium griseoverticillatum* PA-48009. It exhibited weak antimicrobial activity against Gram-positive organisms. Several lanthionine-containing peptide antibiotics have been reported already[1~11]. Recent development of NMR methodology and instrumentation[12,13] make it possible to analyze NMR spectra and to determine the conformation of small proteins and oligopeptides. The structure of Ro 09-0198 was reported by KESSLER *et al.*[7,8], but the reported structure showed some discrepancies with that of lanthiopeptin (**3**) (=Ro 09-0198) reported by WAKAMIYA *et al.*[3]. We considered that this problem had arisen from the difficulty in discriminating the amino acid residue as Ala or Phe in the sequence analysis using NMR. Therefore, we employed both NMR and Edman degradation methods to analyze the structure of **1**. The discrimination of the Phe residue from the Ala residue was possible with Edman degradation, and the sulfide bridges in the lanthionine (Lan) and methyllanthionine (MeLan) moiety and the NH bridge in the lysinoalanine (LysAla) moiety were easily determined by analyzing the NOESY spectra. As PA48009 (**1**) was identified as duramycin[1,2] by physical measurements, we report here the revised structure of duramycin (**1**) as shown in Fig. 1.

Fig. 1. Structure of PA48009 and lanthiopeptin.



PA48009 (revised structure of duramycin) (**1**) Lys^2
Lanthiopeptin (Ro 09-0198, cinnamycin) (**3**) Arg^2



2 Structure of duramycin reported by GLOSS and BROWN.[2]

Structure Elucidation of PA48009 (Duramycin)

Chemical Methods

PA48009 (**1**) was purified from fermentation broth filtrate by Diaion HP-20, ion exchange resin and silica gel chromatographies. Acid hydrolysis of **1** with 6 N HCl gave several ninhydrin-positive compounds. Ten common amino acids (L-Asp, L-Glu, L-Pro, L-Gly (2), L-Val, L-Phe (3), L-Lys) and five unusual amino acids {β-hydroxyaspartic acid (HOAsp), *meso*-Lan, β-Melan (2) and LysAla} were isolated by preparative paper and ion exchange chromatographies. The structures of these four unusual amino acids were determined by MS and NMR studies and that of Lan was confirmed by X-ray analysis (T. SATO: Shionogi Res. Lab., unpublished data). The absolute configurations of their usual amino acids were determined to be all L-forms by HPLC analysis using chiral columns. The molecular formula of **1** was established as $C_{89}H_{125}N_{23}O_{25}S_3$ by MS and the elemental analysis. Compound **1** and duramycin[1,2] resembled one another. They had similar amino acids except for the replacement of the glutamine of **1** with a glutamic acid and Ala^6 vs. Abu^6. Peptide 1 differed from duramycin because the optical rotation of duramycin (HCl salts)[1] was reported to be $-6.4°$ (*c* 3.9, H_2O), while that of **1** (HCl salt) was $-91.1°$ (*c* 0.515, H_2O). Also, the third and the fifth cycle amino acids of duramycin were reported to be glutamic acid (Glu) and LysAla by GROSS and BROWN[2], but those of **1** were determined to be glutamine (Gln) and MeLan by Edman degradation. However, **1** was definitely identified with an authentic sample of duramycin[2] by comparison of physical measurements as shown in Table 1.

To determine the amino acid sequence of **1**, Edman degradations were applied to the intact molecule and several peptides obtained by chemical or enzymic modification are shown in Fig. 2.

As a result of Edman degradation, the linear sequence of **1**, except for four bridged amino acids (Lan, two MeLan and LysAla) was established as shown in Fig. 2(e). The positions of the four bridged amino acids were finally determined to be Ala^1-S-Abu^{18}, Ala^5-S-Abu^{11} for MeLan, Ala^4-S-Ala^{14} for Lan and Ala^6-Lys^{19} for LysAla by analysis of the 2D NMR spectra of **1**.

NMR Analysis

NMR analysis was conducted with the sequential information of Edman degradation as shown in Fig. 2(e). Fig. 3 shows a double quantum filtered phase sensitive (DQF)-COSY spectrum of **1** at 23°C and pH 3.0. Under these conditions, we found 13 cross peaks in the fingerprint region. Since 18 cross peaks

Table 1. Identification of PA48009 and duramycin.

HPLC	Column: Nucleosil-5-C18 (4.6 i.d. × 150 mm) Mobile phase: a) 30% CH_3CN-0.1% CF_3COOH, 1 ml/minute b) 30% CH_3CN-2% KOAc+6% AcOH, 1 ml/minute Detection: 220 nm and 240 nm (UV) Rt: a) 15.5 minutes b) 13 minutes (both compounds)
^1H NMR (in DMSO)	The spectra of both samples and of a 1 : 1 mixture were identical.
SI-MS	*m/z* 2,012 $(M+H)^+$ (PA48009 and duramycin) Calcd for $C_{89}H_{125}N_{23}O_{25}S_3+H$
Optical rotation (in H_2O)	Duramycin: $[\alpha]_D^{24}$ $-89\sim-109.0°$ (*c* 0.0514) PA48009: $[\alpha]_D^{24}$ $-97\pm4.5°$ (*c* 0.307)
CD	Both spectra were identical.
Edman degradation and amino acids analysis	Their amino acids compositions were the same and Edman degradations were identical until the 5th cycle amino acid.[a]

[a] X^1-Lys^2-Gln^3-X^4-X^5-?^{6}.

Fig. 2. Edman degradation of PA48009.

(a) H-X-Lys-Gln-X-X-X-Phe-Gly-Pro-Phe-X-?

(b) H-X-Lys-Gln-X-X-X-Phe-Gly-Pro
H-Phe-X-Phe-Val-X-X-Gly-Asn-X-X

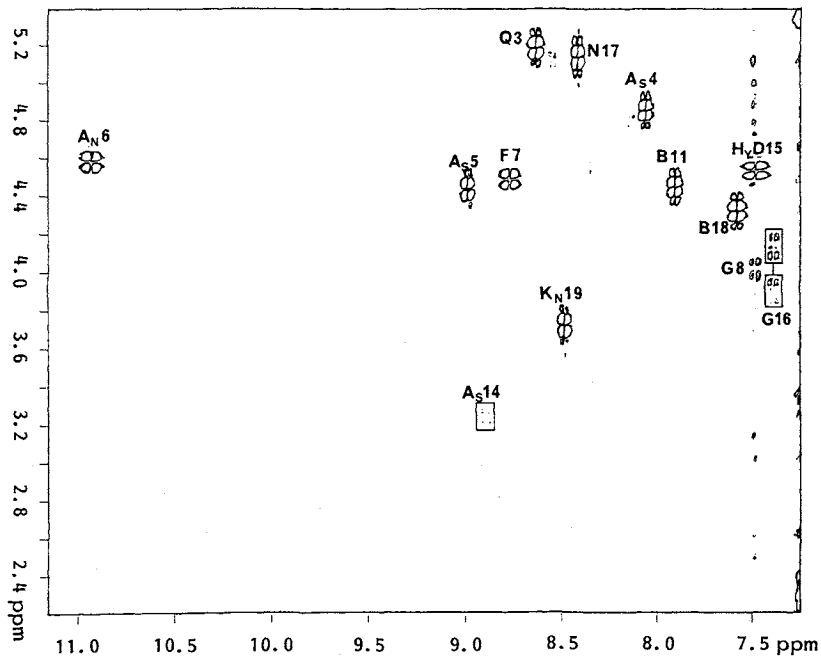
(c) -{HOAsp}-Gly-Asn-X

(d) H-MeLan-Lys-Gln-Lan-MeLan-LysAla

(e) H-(MeLan: Ala or Abu)-Lys-Gln-(Lan: Ala)-(MeLan: Ala or Abu)-(LysAla: Ala or Lys)-
Phe-Gly-Pro-Phe-X-Phe-Val-X-HOAsp-Gly-Asn-X-X

(a) The intact molecule of PA48009.
(b) The modified peptide which was obtained by treatment of PA48009 with proline specific endopeptidase.
(c) The modified peptide which was obtained by hydrolysis of PA48009 with 0.03 N HCl at 110°C, for 13.5 hours. The above three reactions were performed by automatic Edman degradation.
(d) The intact PA48009 was subjected to subtractive Edman degradation.
(e) The amino acids sequence of PA48009 was determined by Edman degradations. (X = Ala or Abu or Lys).

Fig. 3. Fingerprint region of the DQF-COSY spectrum of PA48009 at pH 3.0 and 23°C.

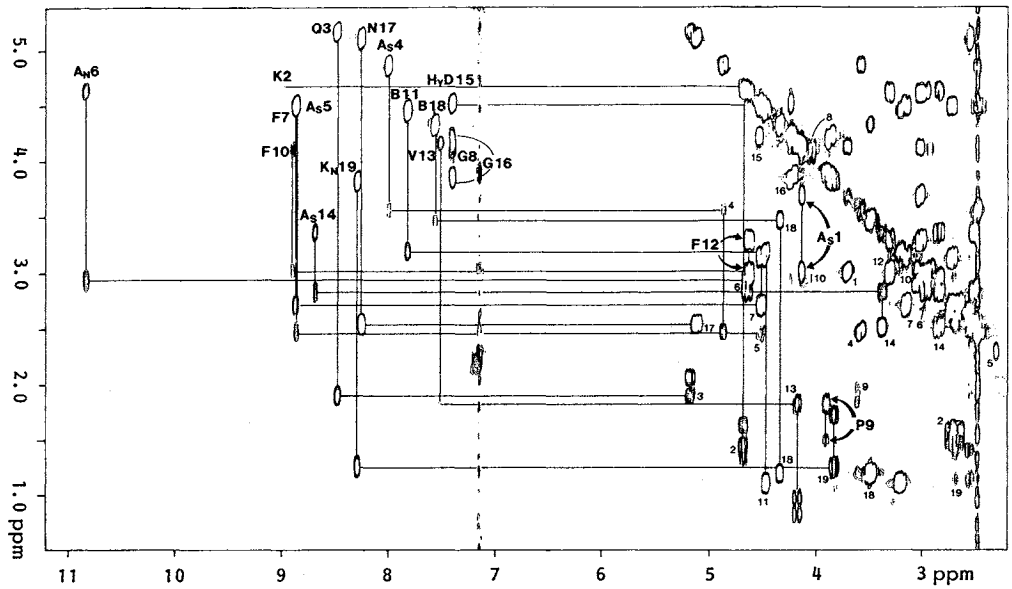


Several cross-peaks are boxed for clarity. Their assignments are also shown. Several backbone amide proton resonances were missed due to the severe line broadening under this set of conditions.

{19−1(Pro)} were expected in this region, we searched under other pH and temperature conditions.

Fig. 4 shows the single relayed coherence transferred COSY (RELAY) spectrum of **1** at 60°C and pH 3.0. When the temperature was raised to 60°C, three additional cross peaks appeared. The two other amide protons could not be detected.

Fig. 4. Portions of the single RELAY spectrum of PA48009 at pH 3.0 and 60°C.



The coupling connectivities from the backbone NH to γCH resonances are depicted as solid lines. Cross-peaks shown by arrows in the aliphatic region mean Pro or residues missing the backbone amide proton.

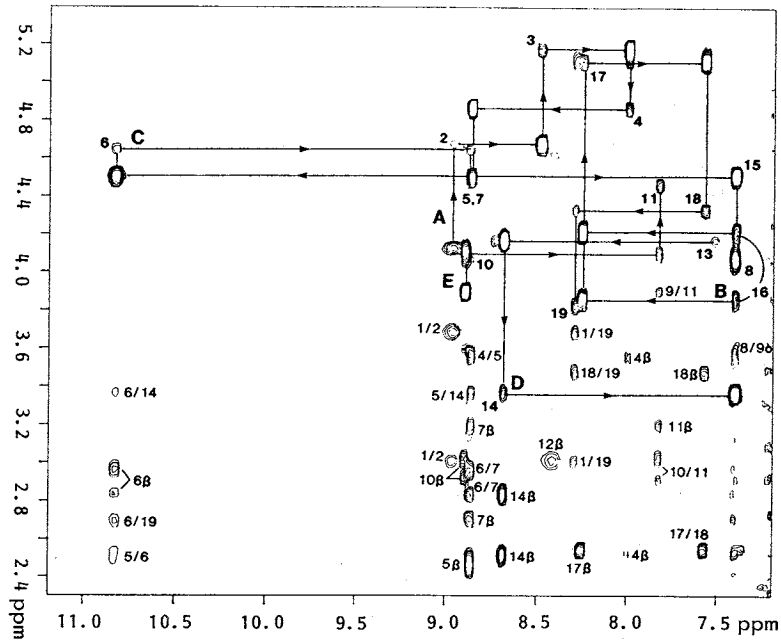
The through bond connectivities from amide NH to γCH were confirmed for all the amide proton signals. As a result, we also found three (19−16) sets of αCH-βCH cross peaks in the aliphatic region (arrows in the figure), which came from amino acid residues with no (Pro) or an undetectable amide proton of the main chain. At this stage, we could confirm the 19 amino acid residues of **1** using 2D NMR spectra.

Residue Type Assignments: Compound **1** includes amino acid residues of ten kinds. Among them, Abu and Val could be determined easily from the other residues by the coupling pattern with methyl groups, and Asn and Gln by the coupling pattern and NOE(s) with the amide protons of the side chain. The distinction between Lys and Pro was very easy because the former had an amide proton while the latter did not. Discrimination of HOAsp from Gly was a little troublesome because of the degeneration of the amide NH, αCH and βCH proton resonance shift. However, the spin system of HOAsp differs from that of Gly. Using the multiplet pattern of the cross peak in the DQF-COSY spectrum, they were discriminable. However, the distinction between Ala and Phe for a few of the residues was difficult, because of the degeneration of the αCH and the βCH proton resonance shifts and the uncertainty of NOE(s) between the aliphatic proton and ring proton signals of Phe. As described below, the use of data from both NMR analysis and Edman degradations enabled us to make the distinction.

Sequential Assignments: Fig. 5 shows the fingerprint region of the NOESY spectrum at pH 3.0 and 60°C. The peptide chain was searched sequentially by J coupling and NOE ($d_{\alpha N}$) between amide NH and αCH signals. Information on sequential NOE between the amide NH and β proton signals ($d_{\beta N}$) and between main chain amide protons (d_{NN}) were also used to confirm the sequence (data not shown).

As shown in Fig. 5, we obtained five segments A∼E. Segment A, which consists of 5 amino acid residues, starts from the $d_{\alpha N}$ cross-peak between Ala and Lys. The sequential connectivities of Lys-Gln, Gln-Ala and Ala-Ala were confirmed by the strong $d_{\alpha N}$ NOESY peaks[12,13]. Determination of the next

Fig. 5. Fingerprint region of the NOESY spectrum ($\tau_m = 350$ m) of PA48009 at pH 3.0 and 60°C.



The sequential connectivities are shown as solid lines for segment A to E. Segment A: ^{1}Ala(s)^{2}LYs^{3}Gln^{4}Ala(s)^{5}Ala(s), segment B: ^{15}HOAsp^{16}Gly^{17}Asn^{18}Abu^{19}Lys(N), segment C: ^{6}Ala(N)^{7}-Phe^{8}Gly, segment D: ^{13}Val^{14}Ala(s), segment E: ^{9}Pro^{10}Phe^{11}Abu.

residue, however, was difficult because of overlapping of the other two αCH resonances. Segment B was identified in the same way. It starts from the unequivalent intraresidue αCH-NH NOE peaks of Gly and ends at the intraresidue αCH-NH NOE peak of Lys (Gly-Asn-Abu-Lys). Moreover, an additional residue of HOAsp was found at the *N*-terminal side of the segment on analyzing the 2D spectra measured under other conditions (pH 3.0, 23°C). Thus, segment B was determined to be HOAsp-Gly-Asn-Abu-Lys. The sequence of (Ala or Phe)-Phe-Gly was found to be in segment C. On comparison with the result of Edman degradation, the sequence of Ala-Phe-Gly was determined to be segment C. This cleared any uncertainty about the distinction of residue type being Ala or Phe. Segment D contained only two residues (Val-Ala). The $d_{\alpha N}$ cross peak with the next residue could not be correlated with an individual residue because of the degeneracy of three NH resonances. Segment E was found to consist of the sequence Pro-Phe-Abu. All the residues of segments A~E could be assigned through this process. The sequential arrangement of the five segments was exclusively determined by comparison with the Edman degradations as following:

A–C–E–Phe–D–B, *i.e.*,

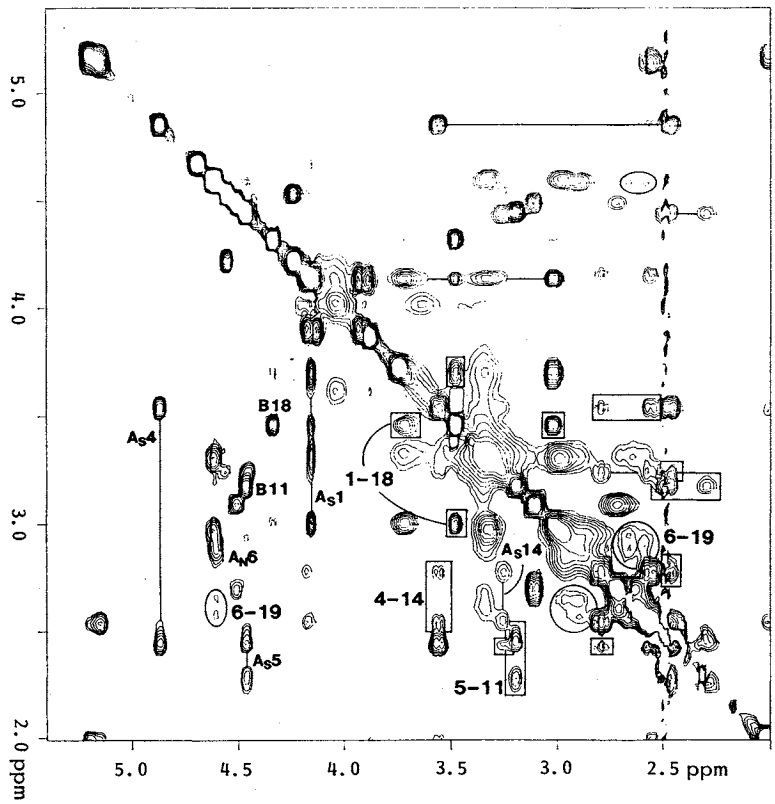
$$\overset{A}{\text{Ala}^{1}\text{-Lys}^{2}\text{-Gln}^{3}\text{-Ala}^{4}\text{-Ala}^{5}} \downarrow \overset{C}{\text{Ala}^{6}\text{-Phe}^{7}\text{-Gly}^{8}} \downarrow \overset{E}{\text{Pro}^{9}\text{-Phe}^{10}\text{-Abu}^{11}} \downarrow \text{Phe}^{12} \downarrow \overset{D}{\text{Val}^{13}\text{-Ala}^{14}} \downarrow \overset{B}{\text{HOAsp}^{15}\text{-Gly}^{16}\text{-Asn}^{17}\text{-Abu}^{18}\text{-Lys}^{19}}$$

The information obtained from NMR analysis and from Edman degradations were complementary for the sequence determination. Some kinds of residues, which were ambiguous in the Edman degradation experiment, were easily determined by 2D NMR analysis. On the other hand, the arrangement of segments and the determination of Phe or Ala were clarified by comparison with the results

Table 2. ^{1}H chemical shifts of PA48009 at pH 3.0, 23°C (δ ppm).

Residue	NH	αCH	βCH	Others
Ala[1]	—	4.16	3.73, 3.03	
Lys[2]	—	4.69	1.40, 1.56	γCH 1.30; δCH 1.46, 1.52; εCH 2.64, 2.72
Gln[3]	8.64	5.19	1.91	
Ala[4]	8.06	4.87	3.57, 2.48	
Ala[5]	8.99	4.46	2.48, 2.29	
Ala[6]	10.93	4.63	2.97, 2.90	
Phe[7]	8.77	4.51	3.31, 2.73	
Gly[8]	7.49	4.05		
Pro[9]	—	3.88	1.86	γCH 1.98, 1.86; δCH 3.64
Phe[10]	8.96	4.15	3.04, 2.95	
Abu[11]	7.91	4.48	3.22	γCH 1.09
Phe[12]	—	4.62	3.36, 2.99	
Val[13]	—	4.17	1.85	γCH 0.98, 0.83
Ala[14]	8.89	3.27	2.81, 2.59	
HOAsp[15]	7.49	4.56	4.24	
Gly[16]	7.39	4.17, 3.93		
Asn[17]	8.42	5.14	2.56	NH 7.49, 6.93
Abu[18]	7.59	4.34	3.49	γCH 1.18
Lys[19]	8.49	3.75	1.71, 1.19	γCH 1.01, 1.10; δ CH 1.10, 1.44; εCH 2.59, 2.67

Fig. 6. Aliphatic portion of the NOESY spectrum (τ_m=250 ms) of PA48009 at pH 3.0 and 23°C.



The enclosed cross-peaks in boxes indicate the NOE peaks between βCH protons in the formation of the sulfide bridge as Lan or MeLan. The encircled cross-peaks '6-19' indicate the NOE between εCH of Lys[19] and βCH of Ala[6].

of Edman degradation. All the NMR signals could be assigned, except for three phenylalanine ring protons, and are listed in Table 2.

Assignments of Sulfide Bridge and NH Bridge Connections: The NH and S bridge connections could not be determined directly by *J*-coupling analysis, because the bridged NH signal of LysAla of **1** in ^{1}H NMR was not detected under various conditions (at 23~60°C, pH 3~7) and the long-range HETCOR experiment could not be done on account of low solubility of **1** in DMSO. These bridges were confirmed by the observation of NOE connectivities across the nitrogen and sulfur atoms, namely the βCH(Ala$_{NH}$)-εCH(7Lys$_{NH}$), the βCH(Ala$_s$)-βCH(Ala$_s$) and the βCH(Abu$_s$)-βCH(Ala$_s$) connectivities. Investigation of possible conformations of Lan, MeLan and LysAla by using molecular models showed that the distances among the protons across the N or S bridges were short enough for giving clear NOESY peaks.

Fig. 6 shows the βCH and εCH region of the NOESY spectrum at 23°C. Each βCH and εCH signal showed only a pair of interresidue NOE cross peaks. Therefore, we could exclusively determine sulfide and NH bridge pairs, *i.e.*, Ala^1-S-Abu^{18}, Ala^4-S-Ala^{14}, Ala^5-S-Abu^{11} and Ala^6-NH-Lys^{19}. Thus, the primary structure of **1** shown in Fig. 1 was confirmed.

Comparison with Duramycin: The ^{1}H NMR spectrum of duramycin was regarded to be identical with that of **1**. Duramycin also exhibited the two singlet signals at 6.66 and 7.07 ppm assigned to protons of the Gln^3. Slight differences found between the isolated spectra converged into the same chemical shifts in the spectrum of the 1 : 1 mixture. This was also confirmed by 2D NMR analyses.

Conclusion

Duramycin (PA48009) differed from the recently discovered tetracyclic 19-residue peptide lanthiopeptin (Ro 09-0198, cinnamycin)[3,7,8] only in the Lys/Arg exchange at position 2. Three sulfide bonds were formed in the same way along them and the NH bridge was identical with that of lanthiopeptin. This conforms to the fact that the 2D NMR spectra of **1** showed almost the same spectral pattern as that of Ro 09-0198[7]. This probably indicates that the stereochemical configurations of Lan, MeLan and LysAla in PA48009 and lanthiopeptin are identical, leading to good sequence homology among them.

Experimental

Amino Acid Analysis and Sequence Determination

Samples were hydrolyzed with 6 N HCl for 20 hours at 110°C. Amino acid analyses were performed with a Hitachi amino acid autoanalyzer (Model 835). Automated Edman degradation was carried out with an Applied Biosystems protein sequencer 477A equipped with a phenylthiohydantoin-amino acid analyzer (Model 120A). Mass spectra were obtained with a Hitachi M-90 spectrometer. NMR spectra were measured with a Varian XL-400 spectrometer. Almost all the 2D NMR spectra were recorded in DMSO-d_6 solution of 20 mg of the sample (pH 3.0) and 0.4 ml at 23°C or 60°C. NMR spectra for comparison of duramycin[2] and **1** were obtained with solution of 4.7~4.8 mg of sample and 0.35 ml of DMSO-d_6 at 30°C. DQF-COSY and absolute mode RELAY experiments were conducted for spin system assignment. Phase sensitive 2D NOESY experiments were also employed in the main chain directed sequential assignment and the determination of the bridge structure. NMR spectra were generally recorded with 2,048 complex points and 48 to 96 scans for each free induction decay. Usually 256 t_1 increments were collected for each 2D spectrum. Spectrum width was 4,800 or 4,900 Hz in both dimensions. NOESY spectra were recorded using 250 mseconds (at 23°C) and 350 mseconds (at 60°C) for mixing times. A total mixing period of 31.3 mseconds was used in single RELAY experiments. FID sets were fourier-transformed in both dimensions after multiplication with a Lorentz-to-Gauss weighting function for phase sensitive spectra and with a pseudo-echo weighting function for absolute mode spectra. The final matrix contained 2,048 real points in the t_2 dimension and 1,024 points in the t_1 dimension.

Fermentation and Isolation of PA48009 (1)

Strain PA-48009 was cultured for 2 days at 28°C in a medium containing 0.5% of soluble starch, 0.5% of glucose, 0.5% of Polypeptone, 0.5% of beef extract, 0.5% of yeast extract, and 0.25% of NaCl. The seed was cultured for 3 days at 28°C in a medium containing 2.0% of glucose, 2.0% of potato starch, 2.0% of soybean meal, 0.5% of yeast extract, 0.25% of NaCl, 0.3% of $CaCO_3$. For isolation, the fermentation culture was filtered, and the filtrate (153 liters) was applied to a column of Diaion HP-20 (2 liters) and eluted with 0.1 M AcOH - MeOH. The eluate was lyophilized, giving a crude material (10.62 g), which was dissolved in 25% PrOH (200 ml) and chromatographed on silica gel (Merck, 70~230 mesh) (1 kg), with development using 65% PrOH (one fraction was 18 ml). The eluates from fraction 131 to fraction 230 were collected and lyophilized to give a pale yellow powder (5.5 g), which was applied to a column of Dowex 1X4(Cl^-) (150 ml) and eluted wtih H_2O. The eluate was lyophilized to give 4.28 g of a colorless amorphous powder of **1**. **1**: (a) Nature: Basic, water soluble, colorless powder, labile in dil NaOH soln (b) MP: >300°C. (c) SI-MS: *m/z* 2,012 $(M+H)^+$ Calcd for $C_{89}H_{125}N_{23}O_{25}S_3+H$. (d) *Anal* Calcd for $C_{89}H_{125}N_{23}O_{25}S_3 \cdot 16H_2O$ (MW 2,301.530): (C 46.45, H 6.88, N 14.00, S 4.18; Found: C 46.35, H 6.55, N 14.10, S 4.20. (e) UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) end absorption, 250~270 (sh, 5). (f) IR ν_{max} (KBr) cm^{-1} 3400, 3050, 2950, 1650, 1515, 1450, 1400, 1320, 1260, 1230, 1100, 740. (g) $[\alpha]_D^{23}$ (HCl salt): $-91.1 \pm 2.6°$ (*c* 0.515, H_2O). (h) HPLC, column: Nucleosil-5-C18 (4.6 i.d. × 150 mm); mobile phase: 1) 30% CH_3CN-0.1% TFA, 2) 30% CH_3CN-2% KOAc+6% AcOH, flow rate: 1 ml/minute, detection: 220 and 240 nm (UV), Rt: 1) 15.5, 2) 13 minutes. (i) TLC[a], solvent system: 1) PrOH - H_2O (6:4), Rf=0.32[b], 2) $CHCl_3$ - MeOH - H_2O (1:4:2), Rf=0.53[b], 3) BuOH - AcOH - H_2O (4:1:2), Rf=0.72[c].

Acid Hydrolysis of PA48009

A mixture of a peptide (1 mg) and 6 N HCl (0.5 ml) was heated at 110°C for 20 hours in a sealed tube. The hydrolysate, after concentration *in vacuo* to dryness on a water-bath at 60°C, was adjusted to a solution of 20 nmol/ml by adding a solution of sodium citrate (pH 2.2). The solution of 250 μl was analyzed with an amino acid analyzer.

The acid hydrolysate of **1** (165 mg) with 6 N HCl (10 ml), after concentration to dryness, was subjected to preparative paper chromatography (Toyo Roshi No 51) with BuOH - AcOH - H_2O (4:1:2). The ninhydrin positive zones were isolated and extracted with 50% MeOH as follows: Fraction 1 (Rf=0.08): Lan+LysAla (64.3 mg), Fraction 2 (0.12): Lys+MeLan (41.8 mg), Fraction 3 (0.18): HOAsp (14.7 mg), Fraction 4 (0.25): Asp+Gly (53.7 mg), Fraction 5 (0.32): Glu (30.4 mg), Fraction 6 (0.38): Pro (30.3 mg), Fraction 7 (0.52): Val (22.7 mg), Fraction 8 (0.60): Phe (42.8 mg). Moreover, the four unusual amino acids were purified by ion exchange chromatographies and recrystallization. The six common amino acids were determined to be all L-forms by HPLC on two chiral columns (Table 3).

Table 3. The absolute configurations of six common amino acids.

Condition	PA48009	L-Form	D-Form
	Rt (minutes)		
Asp (b)	9.2	9.2	7.9
Glu (a)	6.3	6.3	4.2
Pro (b)	6.6	6.7	11.0
Val (b)	9.1	9.1	7.6
Phe (b)	26.7	26.7	13.4
Lys (a)	13.7	13.8	10.9

HPLC condition: (a) Column: Crownpak CR (4.6 i.d. × 150 mm): mobile phase: 0.01 M $HClO_4$ (pH 2.2); flow rate: 0.4 ml/minute. (b) Column: TSK gel Enantio L1 (4.6 i.d. × 150 mm); mobile phase: 0.25 mM $CuSO_4$ aq; flow rate: 1.0 ml/minute; detection: 220 nm (UV).

Unusual Amino Acids

HOAsp: SI-MS *m/z* 150 $(M+H)^+$ Calcd for $C_4H_7N_2O_5+H$; ^1H NMR (D_2O) δ *ca.* 4.3, 4.6 (each 1H, br); ^{13}C NMR (DMSO-d_6) 54.9 (d), 71.9 (d), 168.1 (s), 172.0 (s). MeLan: SI-MS *m/z* 223 $(M+H)^+$ Calcd for $C_7H_{14}N_2O_4S+H$; ^1H NMR (D_2O) 1.43 (3H, d), 3.05~3.32 (2H, dd+dd), 3.65~3.71 (1H, m), 4.22~4.31 (2H, m); ^{13}C NMR (DMSO-d_6) 17.1 (q), 30.2 (t), 51.5 (d), 56.0 (d), 168.5 (s), 168.9 (s). *meso*-Lan: Its structure was unambiguously determined by X-ray analysis[13]. SI-MS *m/z* 209 $(M+H)^+$ Calcd for $C_6H_{12}N_2O_4S+H$; ^1H NMR (0.1 N

[a] Spots were detected by spraying with ninhydrin and heating at 100°C.

[b] Silica gel 60 F_{254} (Merck).

[c] Avicel SF.

DCl) 2.94~3.15 (4H, dd), 4.14~4.20 (2H, q). LysAla: SI-MS *m/z* 234 $(M+H)^+$ Calcd for $C_9H_{19}O_4N_3+H$; ^1H NMR (D_2O) *ca.* 1.5 (2H, m), *ca.* 1.7 (2H, m), *ca.* 1.9 (2H, m), *ca.* 3.01 (2H, t like), *ca.* 3.1 (2H, dd+dd), 3.61 (1H, t), 3.73 (1H, t); ^{13}C NMR (D_2O) 22.4 (t), 26.3 (t), 30.7 (t), 48.4 (t), 49.2 (t), 51.9 (d), 55.3 (d), 175.4 (s), 175.5 (s).

Edman Degradations

(a) Edman degradation was carried out as usual. (b) Proline specific endopeptidase reaction: A solution of proline endopeptidase (58 U/vial, Seikagaku Kogyo Co., Ltd.) in 0.05 M phosphate buffer (10 μl, pH 7.0) was added to a solution of PA48009 (500 μg) in dioxane (12.5 μl) and 0.1 M phosphate buffer (50 μl, pH 7.0). After the air of the reaction mixture was displaced by nitrogen gas, the mixture was incubated at 37°C for 5 days. The objective was isolated from the reaction mixture, after concentration to dryness, by HPLC (column: Nucleosil-5-C18 (4.6 i.d. × 150 mm); mobile phase: 0.1% TFA→40% CH_3CN-0.1% TFA, flow rate: 1.0 m/minute; detection: 220 nm (UV); Rt: 24 minutes). The purified material was subjected to automatic Edman degradation. The result is shown on Fig. 2(b).

Antimicrobial Activity of PA48009: (MIC μg/ml)

Bacillus subtilis NRRL B765, 0.6; *B. subtilis* NRRL B971, 0.2; *Bacillus cereus* NRRL B1877, 0.4; *Streptococcus faecalis* NRRL B537, >100; *Escherichia coli* NRRL B766, >100; *Pseudomonas aerginosa* NRRL B23, >100.

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