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Structure Elucidation of a Potent Anti-MRSA Antibiotic, AM3, Produced by *Streptomyces* sp.

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Abstract : In order to find a potent anti-methicillin resistant *Staphylococcus aureus* (MRSA) antibiotic, actinomycetes isolated from the samples collected in Korean marine silt were screened. From the culture broth of the isolated *Streptomyces* strain AM045, a substance showing excellent biological activity against MRSA was found, isolated and named AM3. The compound showed strong activities against MRSA, *S. epidermidis, E. faecium* and *E. faecalis*, which were better than those of vancomycin and teicoplanin. Unfortunately, AM3 was identified as Actinomycin V. However, this paper reports the three dimensional study of AM3 based on high resolution nmr and Computer Aided Molecular Modeling(CAMM), and the fact that the structure of the pentapeptide lactone ring with oxo-proline in chloroform solution does not have "C conformation" any more(Received October 9, 1995; accepted December 15, 1995).

Introduction

The glycopeptides, vancomycin and teicoplanin, have been introduced in the 1980s for the therapy of serious infections caused by *Staphylococcus* spp.[1,2] However, since the glycopeptide-resistant coagulase-negative *Staphylococcus* spp. and enterococcal strains were reported,[3-8] a new antibiotic showing an activities against these strains was required.

The microorganisms of the soil samples collected in Korean marine silt were screened to find a substance with anti-methicillin resistant *Staphylococcus aureus* (MRSA) activity, and such a substance was found, isolated and named AM3. However, its structure was determined to be Actinomycin V and shown in Fig. 1.[9]

Among the derivatives of Actinomycins, Actinomycin D has usually been studied because it shows good biological activities against cancers. In addition, the structure of Actinomycin D was studied by many research groups.[10] But that of Actinomycin V was not studied and its biological activities against MRSA were not reported. This paper reports the taxonomy of the microorganism, isolation, biological activity as well as the three dimensional structure of AM3 based on high resolution NMR and Computer Aided Molecular Modeling(CAMM).

It was proposed that the pentapeptide lactone in chloroform solution adopted a "C conformation" where all peptide bond structures were trans.[11] However, in this study, it was proven when proline of the pentapeptide lactone in chloroform solution was switched to oxo-proline, the structure of the pentapeptide ring did not have "C conformation" any more.

Materials and Methods

Strains

Antibiotic producing microbial strain AM045 was isolated from a sample of silt collected in East Sea, Korea, and the strains of MRSA, *S. epidermidis, E. faecium and*

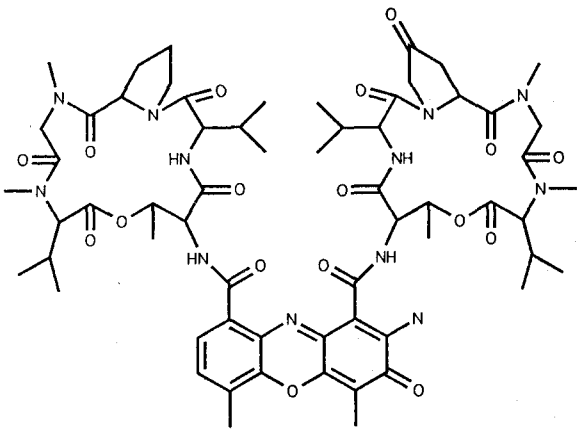

Fig. 1. The structure of AM3

Keywords : MRSA, NMR, Computer Aided Molecular Modeling
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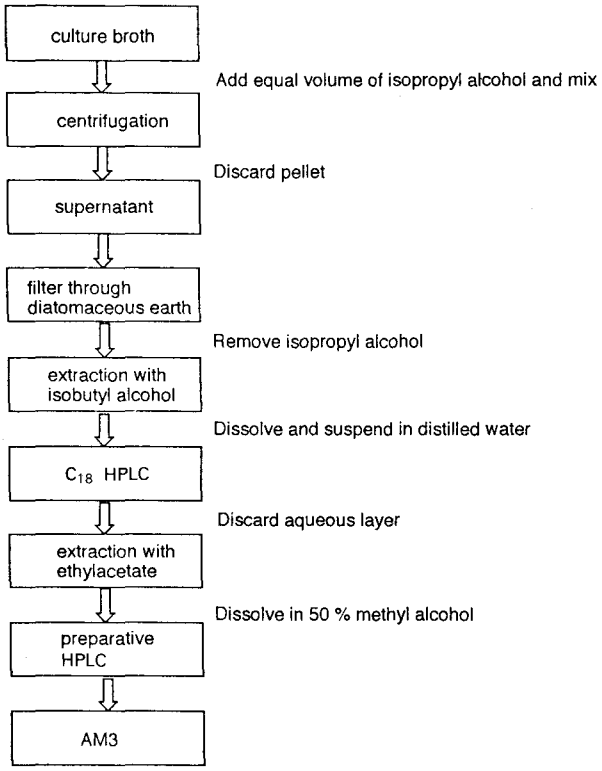

Fig. 2. The procedure for the isolation of AM3.

E. faecalis were abtained from the hospitals in seoul.

Taxonomy

Methods adopted by the International Streptomyces Projects (ISP) were used for the taxonomic studies. The cultural characteristics were determined on the media recommended by the ISP. All the cultures were incubated at 27℃ for 14 days. Cell-wall preparations were analysed by the method of Becker *et al.*,[12] and whole-cell hydrolyzates were prepared and examined using the chemotaxonomic techniques of Lechevalier.[13] The lipid compositions of cell-wall extracts were determined by the method of Lechevalier *et al.*[14]

Minimum Inhibition Concentration (MIC) test

The *in vitro* activities of AM3, vancomycin (Eli Lilly Inc., U.S.A.) and teicoplanin (Gruppo Lepetit Co., Italy) against 15 strains of MRSA, 16 strains of *S. epidermidis*, 27 strains of *E. faecium* and 27 strains of *E. faecalis* were conducted by the two fold agar dilution method. The test strains were activated in Mueller-Hinton media for 24 hours. After incubation for 18 hours at 37℃, MIC values of AM3, vancomycin and teicoplanin were determined.

Isolation

AM3 was isolated and purified with the procedure shown in Fig. 2. An equal volume of isopropyl alcohol was added to the 10 liters of the culture broth and mixed. The supernatant was centrifuged and filtered through diatomaceous *in vacuo*. The concentrated culture broth was extracted with an equal volume of isobutyl alcohol and the organic phase was concentrated to dryness under the reduced pressure. The solid residue was dissolved in the 500 milliliters of 50% isopropyl alcohol in water and concentrated *in vacuo* to remove isopropyl alcohol. The solution was applied to octadecyl silica gel column chromatography. The fraction eluted with 45% aqueous ethyl alcohol was collected and concentrated *in vacuo* to remove ethyl alcohol. The solution was extracted with an equal volume of ethyl acetate and the organic phase was concentrated to dryness under the reduced pressure. The remained precipitate was dissolved in the 100 milliliters of 50% methyl alcohol. The solution was applied to a preparative octadecyl silica gel column of μ-Bondapak C_{18} (200×25 mm, Waters) and developed with acetonitrile-water (40 : 60). The fraction with anti-MRSA activity was collected and concentrated to give AM3.

NMR spectra

All NMR spectra were recorded at 400 MHz on a Bruker ARX-400 spectrometer at a probe temperature of 25℃. AM3 was dissolved in $CHCl_3$-d.

Computer Aided Molecular Modeling

For the calculations of the nmr restraints and simulated annealing, Discover, MD-schedule and DGII supplied by Biosym Technologies were used.

Results and Discussion

Identification of strain AM045

A strain, which produces a substance showing a biological activities against MRSA, was isolated from the soil samples collected in Korean marine silt, and was named AM045. The growth of the strain AM045 was good on all the ISP media. White aerial mycelium was formed on yeast extract-malt extract agar, Bennett's agar, while yellow to brown aerial mycelium was formed on the other ISP media. Substrate mycelium color ranged yellow to brown, red to pink soluble pigments were produced on all the ISP media. The scanning electron microscopic examination of the organism revealed an exclusively branching substrate mycelium as well as abundant aerial mycelium, which is then transformed into spirals of subspherical, smooth arthrospores. No fragmentation of the substrate mycelium was noted. Acid hydrolyzates of whole-cell showed that the cell wall of the strain AM 045 contained LL-diaminopimelic acid (DAP). No sugar was detectable in whole-cell sugar test. The strain con-

Table 1. The physiological characteristics and carbon utilizations of strain AM045

morphological characteristis	
fragmentation of substrate mycelium	negative
chains of arthrospores on aerial mycelium	positive
pigmentation of substrate mycelium	yellow to brown
diffusible pigments	red to orange
cell-wall chemotype	LL-DAP
whole-cell sugar	not detectable
phospholipid type	phosphatidylethanolamine
melanin production (ISP no. 6)	negative
solubilization of	
casein	positive
tyrosine	negative
xanthine	positive
hydrolysis of starch	positive
utilization of	
D-glucose	positive
D-mannitol	positive
L-arabinose	weak positive
D-fructose	positive
sucrose	weak positive
rhamnose	weak positive
D-xylose	positive
raffinose	weak positive
I-inositol	weak positive
cellulose	weak positive

tained only phosphatidylethanolamine indicating a type II phospholipid pattern. Based on these characteristics, the strain AM045 can be assigned to the genus *Streptomyces*. The taxonomy of strain AM045 is listed in Table 1.

Biological activity

The *in vitro* activities of AM3, vancomycin, and teicoplanin against 15 strains of MRSA, 16 strains of *S. epidermidis*, 27 strains of *E. faecium* and 27 strains of *E. faecalis* are shown in Table 2. Vancomycin and teicoplanin were used to compare the activities. They have been used for the treatment of severe hospital infections caused by MRSA and other Gram-positive species, but neither vancomycin nor teicoplanin is bactericidal against coagulase-negative staphylococcal strains.[4] Recently, glycopeptide resistances have been observed amongst clinical isolates of *Enterococcus faecium* and *Enterococcus faecalis*. These resistances may be due to the genes carried on transferable plasmids which code for resistance mechanism.[15] As shown in the Table 2, AM3 exhibited strong activities against all the tested strains, which were better than those of vancomycin and teicoplanin. It is noteworthy that AM3 showed the excellent biological activities against strains of coagulase-negative *Staphylococcus epidermidis, E. faecium* and *E. faecalis*.

Structure elucidation

The structure of AM3 was identified by IR, Fab-MS, HPLC and several NMR experiments such as ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, NOESY, HOHAHA, HMQC and HMBC. The molecular weight of AM3 was determined to be 1269 by Fab-MS. The spectrum of ^{1}H-NMR provided the existence of four N-methyl groups and peptide bonds. The number of carbons in AM3 was determined to be 62 by ^{13}C-NMR. DEPT45, DEPT90, and DEPT135 experiments gave information of the types of the carbons, which were 16 methyls, 7 methylenes, 16 methines and 23 quaternary carbons.

Since ^{1}H-NMR suggested AM3 had peptide bonds, an amino acid analysis was carried out by HPLC. Proline, valines, threonines and sarcosines were detected at the molar ratio of 1 : 2 : 2 : 2. These amino acid residues were confirmed by COSY and HOHAHA. Two sets of the proton signals similar to the pattern of valine were observed in COSY. A comparison of the chemical shifts obtained from ^{1}H-NMR, ^{13}C-NMR and HMQC of valines with those of valine derivatives is listed in Table 3. In HMBC, the long-range ^{1}H-^{13}C connectivities of 26.94 (^{13}C) and 2.93 (^{1}H), 26.97 (^{13}C) and 2.94 (^{1}H) were observed. Four proton signals of 2.90, 2.91, 2.93 and 2.94 were considered to be N-methyl protons in the ^{1}H-NMR spectrum. Among the four peaks, 2.90 and 2.91 were assigned N-methyl groups of two sarcosines. As mentioned above, since two remained peaks of 2.93 and 2.94 were connected to unk1 and unk2 in Table 3, respectively, unk1 and unk2 were assigned N-methyl valines. In addition, because the ^{1}H-NMR spectrum showed only four

Table 2. The in vitro biological activity of AM3 MIC (μg/ml)

strains	AM3		Vancomycin		Teicoplanin	
	MIC range	MIC (GM[a])	MIC range	MIC (GM[a])	MIC range	MIC (GM[a])
MRSA (15[b])	0.10~0.39	0.17	0.78~1.56	1.36	0.20~0.78	0.45
S. epidermidis (16[b])	0.20~0.39	0.27	0.20~6.25	2.52	0.39~3.13	1.49
E. faecium (27[b])	0.05~0.39	0.27	0.39~3.13	1.01	0.10~0.78	0.40
E. faecalis (27[b])	0.20~0.39	0.28	0.39~3.13	1.34	0.05~0.20	0.18

[a] geometric mean, [b] No. of strains tested (clinical isolates)

Table 3. A comparison of the chemical shifts obtained from ^{1}H-NMR, ^{13}C-NMR and HMQC of valines with those of valine derivatives.

position	valine (benzenoid)		valine (quinoid)		unk1		unk2	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
α	3.57	58.54	3.71	57.19	2.68	71.33	2.68	71.52
β	2.14	31.73	2.25	31.88	2.65	26.94	2.72	26.97
γ1	1.13	18.85	1.16	18.94	0.75	19.08	0.76	19.10
γ2	0.93	19.23	0.92	19.26	0.97	21.62	1.00	21.73
NH	7.65	–	8.17	–	–	–	–	–

unit: ppm

Table 4. The ^{1}H and ^{13}C chemical shifts obtained from HMQC and the multiplicities of carbons

^{1}H	^{13}C	multiplicity[a]
6.59	54.28	d
3.88	41.95	t
2.34	41.95	t
4.58	52.88	t
3.99	52.88	t
–	208.79	s

[a] obtained from DEPT experiments, d: doublet; t: triplet; s: singlet, unit: ppm.

amide proton signals which were assigned amide protons of two threonines and two valines, the above assignments were confirmed.

Like N-methyl valines, the other unknown connectivities were observed in COSY and HOHAHA. While COSY showed the correlations among 6.59, 3.88 and 2.34, HOHAHA showed the correlations among 6.59, 3.88, 2.34, 4.58 and 3.99. In addition, HMBC had the peak correlated between 208.79 ppm (^{13}C) and 4.58 ppm (^{1}H). The chemical shifts of ^{1}H and ^{13}C obtained from HMQC are listed in Table 4. As a result, the partial structure of Fig. 3 was obtained.

IR, ^{1}H-NMR and ^{13}C-NMR suggested the existence of aromatic ring. Among the peaks between 9 ppm and 7 ppm in ^{1}H-NMR, 8.17, 7.73, 7.65 and 7.23 were assigned already and the remained peak was 7.38. The integration of ^{1}H-NMR showed the peak at 7.65 ppm included two protons. From HMQC, two peaks at 7.38 and 7.65 were correlated to 130.33 and 126.22 in ^{13}C dimension, respectively. In addition to this, 2D homonuclear experiments such as COSY and HOHAHA showed the scalar coupling of two peaks at 7.38 and 7.65. In the ^{13}C-NMR spectrum, 11 peaks were observed between 100 ppm and 150 ppm. According to DEPT experiments, all peaks were singlet carbons except 126.22 and 130.33. As a result, all protons of the aromatic ring suggested by IR were considered to be substituted except 126.22 and 130.33. From HMBC, more information was obtained.

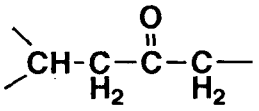

Fig. 3. The partial structure obtained from Table 4.

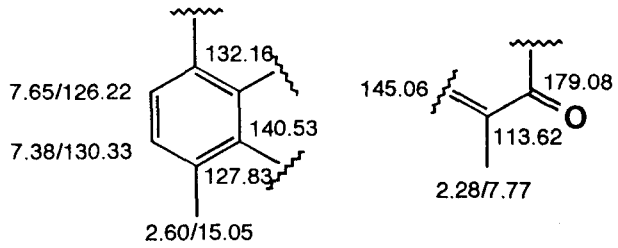

Fig. 4. The partial structures of benzenoid and quinoid.

The proton signal of 7.38 was long-range coupled to two carbon signals of 132.16 and 140.53, and the proton signal of 7.65, the carbon signal of 127.83. Two carbon signals of 127.83 and 140.53 were long-range coupled to the proton signal of 2.60 which was directly connected to the carbon at 15.05 ppm and assigned a methyl proton. Therefore, one position of the aromatic ring should be substituted with a methyl group. One of two undefined methyl protons in ^{1}H-NMR was 2.28 which was directly connected to ^{13}C peak at 7.77. HMBC showed the peak at 2.28 was long-range coupled to three carbon signals of 113.62, 145.06 and 179.08. Among these, the peak at 179.08 suggested the existence of a carbonyl carbon. Consequently, the partial structure of Fig. 4 was obtained.

As mentioned above, AM3 included one proline, two valines, two threonines, two sarcosines, two N-methyl valines and partial structures of Fig. 3 and 4. Searching these data with the database purchased from Chapman & Hall, AM3 was identified as Actinomycin V. The ^{1}H-NMR and ^{13}C-NMR spectra of Actinomycin V purchased from Sigma were exactly same as those of AM3. Actinomycin derivatives have usually been studied for the anticancer activities. The biological activities of actinomycin V against MRSA have never been reported. Therefore, even if AM3 was identified as Actinomycin V, its biological activities for the anti-MRSA can be considered to have the value reported. Besides, while the structural study of Actinomycin D by Computer Aided Molecular Modeling was reported,[11] that of Actinomycin V was not done yet so that the structural elucidation of AM3 by Computer Aided Molecular Modeling can be considered to have the value reported.

To calculate the volumes of the crosspeaks in NOESY, FELIX (Biosym Technologies) was used. This calculation was based on a $1/r^6$ dependence against proton-proton distance. Assigned crosspeaks were classified as strong,

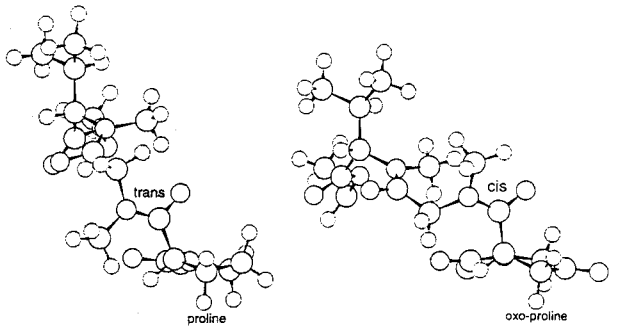

Fig. 5. The different conformations of the peptide bond between proline and sarcosine (benzenoid), and that between oxo-proline and sarcosine (quinoid)

medium or weak corresponding internuclear upper bound distances of 2.5, 3.5 and 5.0 Å, respectively. 53 distance constraints were obained. In order to apply dihedral constraints, four $^{3}J_{HNH\alpha}$ values were measured. The intial structure was lent from Available Chemical Data (Molecular Design Limited). Since the structure was two dimensional, it was minimized using Discover (Biosym Technologies). The minimized structure was put into simulated annealing with the dihedral constraints. All methyl groups were replaced by pseudo atoms. All minimization calculations were performed *in vacuo* with charges ignored. The first two phases consisted of 100 steps of steepest descent followed by 500 steps of conjugate gradient minimization against the Consitent Valence Forcefield (CVFF) potential (Biosym Technologies). The phase 3 was dynamics calculation during 30 psec at 827 ℃. Phases 4 and 5 were identical to phase 3 except the time duration of 10 psec. From phase 6 to phase 10 the temperature was cooled down to 27℃. After dynamics, resulting structures were further minimized using the identical phases to phases 1 and 2. The best 20 structures were refined. Among these, the 6th structure had the lowest total energy and satisfied all distance and dihedral constraints well. Lackner proposed the pentapeptide lactone in chloroform solution adopted a "C conformation" where all peptide bond structures were trans.[11] Since all experiments of AM3 were carried out in chloroform solution, the structure of AM3 was expected to have a "C conformation". Like an expectation, all peptide bonds of AM3 were trans except the peptide bond between oxo-proline and sarcosine (quinoid), which was cis. Fig. 5 shows different conformations of the peptide bond between proline and sarcosine (benzenoid), and that between oxo-proline and sarcosine (quinoid). In order to confirm the result, a distant geometry algorithm, DGII (Biosym Technologies), was carried out. Unlike the simulated annealing, the dihedral constraints were not applied. The best 5 structures were refined. Among these, the 4th structure satisfied all distance constraints well. Comparing the heavy atoms of the structure refined by simulated annealing with those of the structure refined by DGII, the rms difference value was 1.65 Å. Even though x-ray structure of AM3 was not known, a comparison of the results of two different algorithms gave a confidence that the refine structure was correct.

In conclusion, when proline is switched to oxo-proline, the structure of the pentapeptide ring does not have "C conformation" any more.

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방선균에 의해 생산된 항 MRSA 항생물질 AM3의 구조 연구

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초록 : 항 MRSA 물질을 찾기 위하여 한국 해양 토양을 검색하였고, 거기서 분리된 방선균의 이차 대사물질 중 항 MRSA 효능을 보이는 물질을 AM3이라고 명명하고 이에 대한 연구를 하였다.

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