Three-Dimensional Solution Structure of Bottromycin A۲: A Potent Antibiotic Active against Methicillin-Resistant \( \textit{Staphylococcus aureus} \) and Vancomycin-Resistant \( \textit{Enterococci} \)

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The three-dimensional (3D) structure of bottromycin A۲, a natural anti-methicillin-resistant \( \textit{Staphylococcus aureus} \) (MRSA) and anti-vancomycin-resistant \( \textit{Enterococci} \) (VRE) agent consisting of seven amino acids, has been investigated through NMR spectroscopy. On the basis of 57 experimental constraints, a total of 34 converged structures were obtained. The average pairwise atomic root mean square difference is 0.74±0.59 Å for all heavy atoms. The resulting structure indicates an interesting feature in that the three C-terminal residues of bottromycin A۲ fold back on the 12-membered cyclic skeleton made by the four N-terminal residues. Thus, MePro(2) and Thia-β-Ala-OMe(7), modification of which significantly affects the antibacterial activities of bottromycin A۲, are located on one side of its 3D structure. These distinct structural features might be important for the binding of bottromycin A۲ with the bacterial ribosome.

Key words bottromycin A۲; solution structure; NMR; antibiotic; methicillin-resistant \( \textit{Staphylococcus aureus} \); vancomycin-resistant \( \textit{Enterococci} \)

The increasing prevalence of multidrug-resistant Gram-positive bacteria, including methicillin-resistant \( \textit{Staphylococcus aureus} \) (MRSA) and vancomycin-resistant \( \textit{Enterococci} \) (VRE) strains, has created serious problems worldwide.1,2 In particular, MRSA has been recognized as one of the major pathogens causing nosocomial infections. Therefore, there is an urgent and growing need for development of new antibiotics with novel modes of action to overcome the increasing threat posed by pathogens rapidly evolving drug resistance. During the screening for new types of anti-MRSA and anti-VRE agents from the natural product library in our institute, bottromycin A۲ was found to exhibit potent antibiotic activity against clinically-isolated MRSA and VRE strains, exhibiting minimum inhibitory concentration (MIC) values of 1.0 μg/mL and 0.5 μg/mL, respectively.3 Bottromycin A۲ was first isolated from the fermentation broth of \( \textit{Streptomyces bottropensis} \) by Waisvisz et al. in 1957, and found to be an antibacterial peptide active against Gram-positive bacteria and mycoplasma.4–8 Bottromycin A۲ consists of seven amino acids, includes four unusual residues, and possesses a 12-membered cyclic skeleton made by the Gly(1)-tert-Leu(4) region (Fig. 1). The most significant structural characteristic is an amidine moiety formed through condensation of the cyclic tetrapeptide and linear tripeptide units.9 As for the mode of action, bottromycin A۲ inhibits bacterial protein synthesis by blocking the binding of aminoacyl-tRNA to the A site on the 50S ribosome, but it does not inhibit peptide bond formation and translocation steps.10–12 This function is different from that seen in the commonly used antibiotics, such as erythromycin and linezolid.13,14 Therefore, bottromycin A۲ represents a promising compound for development of novel antibiotics. Recently, we have accomplished the first asymmetric total synthesis and determination of the absolute stereochemistry of bottromycin A۲.3 However, the exact three-dimensional (3D) structure of bottromycin A۲, which would be useful for facilitating the design and creation of novel anti-MRSA and anti-VRE agents, remained undetermined. We herein report the 3D solution structure of bottromycin A۲ identified using NMR spectroscopy.

For analyses, a sample of 10 mg bottromycin A۲ was dissolved in 0.6 mL of CDCl۳, because it is not very soluble in D۲O. All NMR spectra were recorded on a Varian INOVA600 spectrometer operating at 600 MHz for \( ^{1}H \) at 25°C. Sequence specific assignments were established using double-quantum filtered-correlation spectroscopy (DQF-COSY),15 total correlation spectroscopy (TOCSY),16 and transverse rotating-frame Overhauser effect spectroscopy (TROESY) results from a previous study.17 The \( ^{1}H \) chemical shifts of bottromycin A۲ are provided in the supplementary data. The dihedral constraints were obtained by applying the Karplus equation to vicinal proton–proton scalar coupling constants obtained by the 1D proton spectrum with high resolution.18 tert-Leu(4) and MePhe(6) have \( ^{3}J_{\text{H}N\text{C}}\) coupling constants larger than 8 Hz. Therefore, the \( \varphi \) angles (C۳–C۴–N۵–C۶–C۷) for tert-Leu(4) and MePhe(6) were constrained in the range of \(-120°\pm 40°\). As Val(3) has a \( ^{3}J_{\text{H}H\text{P}}\) coupling constant larger than 10 Hz, it was treated as indicating an anti \( \mu \)-H۳–H۶ orientation and a dihedral-angle estimate of \(180°\pm 40°\). A total of 3 torsional constraints were obtained. For extraction of proton–proton distance constraints, a set of 2D-TROESY spectra was measured with mixing times of 100, 150, 200, 250, and 300 ms. A plot of the volume of the cross-peak \textit{versus} mixing time showed linearity up to 250 ms. Therefore, the proton–proton distance constraints were based on the integrated cross-peaks from the 250 ms spectrum. Volumes of the four geminal proton cross-peaks (2-H۲, 5-H۲, 6-H۴, 49-H۳) were averaged and used for calibrating measured ROE volumes. A distance of 1.8 Å was used as a distance reference for geminal proton cross-peaks. This calibration yielded the theoretically expected value, \( ca. 2.7 \) Å, for the ROE volumes. A distance of 2.2 Å was used as a distance reference for non-geminal proton cross-peaks. This calibration yielded the theoretically expected value, \( ca. 2.7 \) Å, for the ROE volumes.

Fig. 1. Chemical Structure and Atom Numbering for Bottromycin A۲

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distance between 46-H and 47-H. Distance constraints were classified into three categories corresponding to <2.8, <3.5, and <5.0 Å, indicating strong, medium, and weak ROEs, respectively. Pseudoatoms were used for non-stereospecifically assigned methylene, methyl, and aromatic protons.20) In addition, 0.5 Å was added to the distance constraints involving methyl protons.21) A total of 54 distance constraints were obtained.

We determined the 3D structure of bottromycin A2 using the dynamical simulated annealing protocol of the CNS (Crystallographic and NMR System) program, version 1.1.22) The topology files for the unusual residues, i.e., MePro(2), tert-Leu(4), tert-Leu(5), and Thia-β-Ala-OMe(7), were prepared in the CNS calculation by modifying topology files for standard amino acids. The topology file for the entire structure of bottromycin A2 is available upon request. A set of 100 individual structures was calculated on the basis of experimental NMR constraints, which consisted of 54 distance constraints and 3 dihedral constraints. These calculations provide 34 converged structures, which have no distance violations greater than 0.2 Å and no dihedral angle violations. Structural statistics for the 34 converged structures are given in Table 1.

![Fig. 2. Stereopairs of the Superposition of the Resulting 34 3D Structures of Bottromycin A2, Which Were Determined in CDCl3](image2)

![Fig. 3. Stereo View of Surface Representation of the Lowest Energy Structure of Bottromycin A2](image3)

MePro(2) and Thia-β-Ala-OMe(7) are colored green. This view is obtained by 90° rotation of Fig. 2 around the vertical axis.

tert-Leu(5) could help the C-terminal residues to be located over the cyclic skeleton made by the N-terminal residues. Although our experimental condition using CDCl3 does not necessarily mimic the aqueous environment, there is a report indicating that the structural features of a 12-membered cyclic peptidomimetic with a similar size to bottromycin A2 were retained in both water and chloroform.23) Therefore, bottromycin A2 could be expected to retain this interesting structural feature even in the aqueous environment. The thiazole ring of Thia-β-Ala-OMe(7) was found to be in close contact with Val(3), because several critical ROEs were observed between these two residues. Therefore, the Hα resonance of Val(3) ap-

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### Table 1. Structural Statistics

<table>
<thead>
<tr>
<th>Structural parameter</th>
<th>34 converged structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>R.M.S. deviations from experimental distance constraints (Å)</td>
<td></td>
</tr>
<tr>
<td>All (54)</td>
<td>0.018±0.004</td>
</tr>
<tr>
<td>Intraresidue (22)</td>
<td>0.024±0.003</td>
</tr>
<tr>
<td>Sequential (</td>
<td>i−j</td>
</tr>
<tr>
<td>Medium range (</td>
<td>i−j</td>
</tr>
<tr>
<td>R.M.S. deviations from experimental dihedral angle constraints (deg.) (3)</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>Energetic statistics (kcal mol⁻¹)⁹</td>
<td></td>
</tr>
<tr>
<td>ENOE</td>
<td>1.30±0.64</td>
</tr>
<tr>
<td>Etor</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>E_L-J</td>
<td>−18.89±3.13</td>
</tr>
<tr>
<td>R.M.S. deviations from idealized geometry</td>
<td></td>
</tr>
<tr>
<td>Bonds (Å)</td>
<td>0.005±0.0002</td>
</tr>
<tr>
<td>Angle (deg.)</td>
<td>0.975±0.031</td>
</tr>
<tr>
<td>Improper (deg.)</td>
<td>1.132±0.039</td>
</tr>
<tr>
<td>Average pairwise R.M.S. difference (Å)</td>
<td></td>
</tr>
<tr>
<td>Gly(1)-tert-Leu(5)</td>
<td>0.09±0.06</td>
</tr>
<tr>
<td>Gly(1)-Thia-β-Ala-OMe(7)</td>
<td>0.74±0.59</td>
</tr>
</tbody>
</table>

⁹ ENOE, Etor, and E_L-J are the energies related to the NOE violations, the torsion angle violations, and van der Waals term, respectively.
pears at a higher field (2.36 ppm) due to the ring current effect of the thiazole ring. The phenyl ring of MePhe(6) showed several weak ROEs to the residues, including Gly(1), MePro(2), and C-terminal methoxy groups, which could not be simultaneously satisfied in the individual structures. This suggests the presence of a few different orientations of the phenyl ring of MePhe(6) in solution, as shown in Fig. 2. It was reported that two bottromycin A₂ analogues, bottromycin B₂ and C₂, show less antibacterial activity against Mycoplasma gallisepticum.⁸ They have proline and dimethyl-proline residues respectively at the position corresponding to the MePro(2) of bottromycin A₂. In addition, our previous study showed that chemical modifications of the methyl-ester group of Thia-β-Ala-OMe(7) of bottromycin A₂ affects its antibacterial activity against several Gram-positive bacteria, including MRSA and VRE.²⁴ Figure 3 shows the surface representation of the lowest energy structure of bottromycin A₂. Interestingly, MePro(2) and Thia-β-Ala-OMe(7) are both located on one side of bottromycin A₂. These results suggest that the surface region shaped by MePro(2) and Thia-β-Ala-OMe(7) might be involved in the binding of bottromycin A₂ to the A site on the 50S ribosome. These findings could prove useful in helping to design new agents with bioactivity against MRSA and VRE, to improve understanding of structure/activity relationships, and to facilitate discovery of antibiotics with novel modes of action.

References