Structure of Subtilosin A, an Antimicrobial Peptide from *Bacillus subtilis* with Unusual Posttranslational Modifications Linking Cysteine Sulfurs to α-Carbons of Phenylalanine and Threonine

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Bacteriocins are potent antimicrobial peptides produced by bacteria which are usually active against a limited spectrum of related organisms. Such ribosomally synthesized peptides are generally cationic, typically have from 25 to 60 amino acids, and fall into one of two major classes: either unmodified peptides (except for possible disulfide bridges) or lantibiotics.[1,2] The latter, for example, nisin A which is used in over 80 countries as a food preservative, are extensively posttranslationally modified.[2] Their biosynthesis involves enzymatic dehydration of serine or threonine residues to give dehydroalanine or dehydrobutyrine moieties, some of which then undergo Michael attack at the β-position by nearby cysteine thiols to form monosulfide lanthionine bridges.[2,3] Our earlier work[4] on genes required for production of subtilosin A (**1**), a bacteriocin from *Bacillus subtilis*, indicated that the initially proposed structure[5] of this highly modified peptide required revision (Figure 1). Subsequent studies by Marx et al. suggested that thioether bridges were present: Cys13 to Phe22, Cys7 to Thr28, and Cys4 to Phe31.[6] However, the exact connectivity of these links remained uncertain, although bonds between sulfur and the aromatic rings of Phe22 and Phe31, as well as between sulfur and the β-carbon of Thr28, were proposed.[6] We now report the determination of the complete primary and three-dimensional solution structure of **1** using isotopic labeling and multidimensional NMR studies. The results demonstrate that in addition to having a cyclized peptide backbone, three cross-links are formed between sulfurs of cysteine and the α-positions of the two phenylalanines and the threonine. To the best of our knowledge, such posttranslational linkage of thiol to an α-carbon of an amino acid residue has not been previously observed in natural peptides or proteins. Thus, subtilosin A (**1**) belongs to a new class of bacteriocins.

Although **1** can be readily produced and isolated in significant quantities (5−10 mg/L) from *B. subtilis*, it is highly resistant to proteinases and defies complete sequence analysis by Edman degradation or mass spectral examination.[4,5] To allow complete structural assignment by NMR, universally [^{13}C,^{15}N]-enriched subtilosin A was prepared by fermentation of *B. subtilis* on a labeled peptone media generated from blue green algae (*Anabaena sp.*) grown on sodium [^{13}C]bicarbonate and sodium [^{15}N]nitrate as the sole carbon and nitrogen sources.[7] Levels of isotopic labeling of the peptone samples were determined to be 85−98% ^{13}C and 77−95% ^{15}N by combustion of small samples followed by atomic emission determination of ^{12}C/^{13}C and ^{14}N/^{15}N ratios.[8] Electrospray

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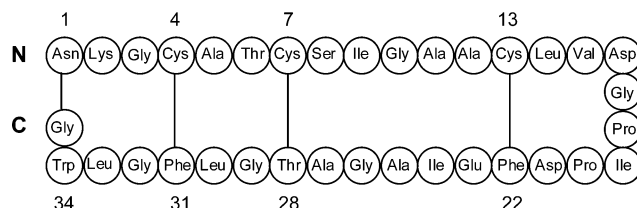
Figure 1. Linkages in subtilosin A (**1**), a cyclic peptide.
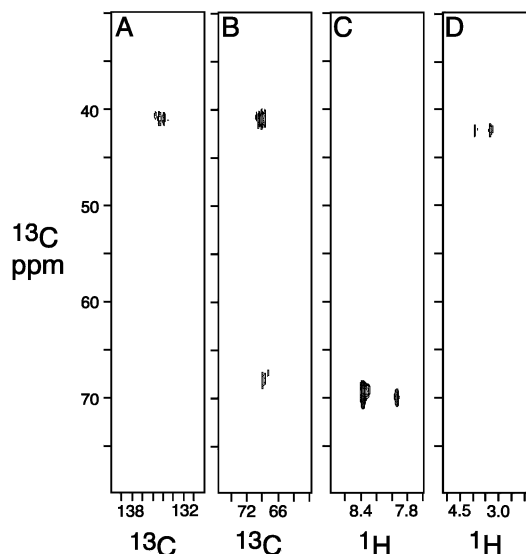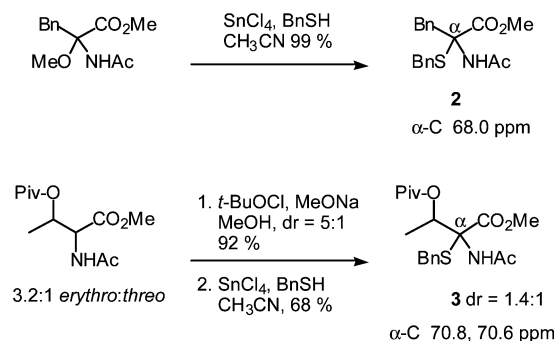

Figure 2. NMR spectra of **1** labeled by U-[^{13}C,^{15}N]Phe. Contour maps of (A) ^{13}C-COSY at 125 MHz showing Phe C$_\beta$ (*y*-axis) to aromatic carbon (*x*-axis) correlations; (B) ^{13}C-COSY of Phe C$_\beta$ (*y*-axis) to C$_\alpha$ (*x*-axis) correlations; (C) ^{1}H,^{13}C-plane from HNCA displaying intraresidue Phe C$_\alpha$ (*y*-axis) to ^{1}H (*x*-axis),^{15}N correlations. (D) ^{1}H,^{13}C-HSQC demonstrating that only the Phe C$_\beta$ (*y*-axis) have protons (*x*-axis) directly attached. The C$_\beta$ and C$_\alpha$ resonances of Phe22 and Phe31 are nearly overlapped (indistinguishable in panels A and B), but the two C$_\alpha$ to NH correlations are clearly separated in panel C.

mass spectral analysis of the labeled subtilosin A derived from this peptone indicated comparable levels of labeling.

NMR studies of labeled subtilosin A in methanol employed well-established, two- and three-dimensional techniques for protein analysis.[9] Complete assignment of all nitrogens, carbons, and hydrogens indicated that Phe22, Thr28, and Phe31 were modified at their α-carbons, which were fully substituted. To confirm this, universally labeled [^{13}C,^{15}N]-L-phenylalanine and [^{13}C,^{15}N]-L-threonine were added to separate fermentations of *B. subtilis* with standard unlabeled media. Examination of the resulting samples of **1** by ^{13}C-COSY shows the complete carbon connectivity pattern

Figure 3. Synthesis and ^{13}C chemical shifts of **2** and **3**.
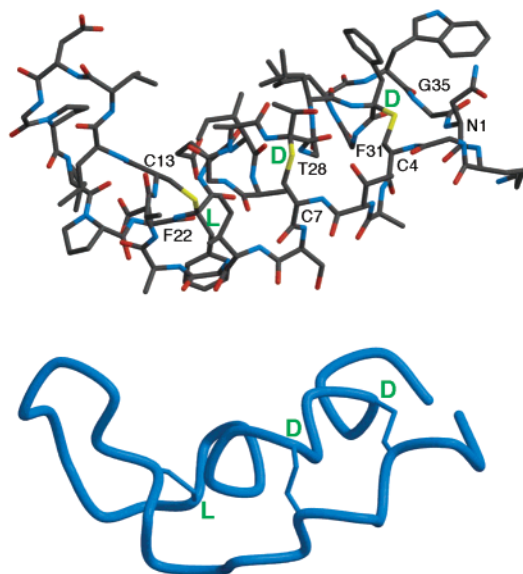

Figure 4. Top: lowest energy solution structure of **1** showing heavy atoms of all residues. Bottom: coil representation of the backbone in the same orientation with a break at the N → C ring junction for reference.

in these residues and confirms that the α-carbons appear at 69.38 (Phe22), 72.80 (Thr28), and 69.82 (Phe31) ppm (Figure 2). These chemical shifts agree with those of model compounds **2** and **3**, which were prepared as shown in Figure 3. Interresidue correlations of the three α-modified amino acids identified the attached cysteines as Cys-13, Cys-7, and Cys-4, respectively, as previously proposed by Marx et al.[6] Thus, subtilosin A (**1**) possesses three highly unusual posttranslational linkages of cysteine sulfur to α-carbons in addition to the amide link between the carboxy and amino termini (i.e., Gly35 to Asn1). Perhaps the closest analogy to such modifications occurs in fungal diketopiperazines, such as gliotoxins, aranotins, and sporidesmins.[10] However, in these eukaryotic secondary metabolites, the presence of disulfide and trisulfide bridges, as well as thiomethyl adducts, suggests that sulfur donor(s) other than cysteine as well as different mechanisms may be involved.

The stereochemistry of all residues in **1** except for the modified ones was confirmed to be L by complete desulfurization with nickel boride, acid hydrolysis to the constituent amino acids, and conversion of these to the corresponding pentafluoropropanamide isopropyl esters for chiral GC MS analysis. Although the two phenylalanines and threonine were regenerated by the desulfurization process, epimerization can occur during sulfur removal from the α-carbon (unpublished model studies). The stereochemistry at these modified quaternary carbons was assigned by extensive analysis of NOESY

spectra coupled with matching to energy minimized structures generated for all eight possible stereoisomers having different configurations at the α-carbons of Phe22, Thr28, and Phe31. The results for the 50 lowest energy structures of each isomer consistently show that the isomer with L-Phe22 (α-*R*), D-Thr28 (α-*S*), and D-Phe31 (α-*S*) has the best fit to the observed NOE data. The DDD isomer, the next best, does not match the NOE data as well, whereas the LLL isomer (i.e., hydrogen replacement by sulfur with retention of configuration for all three residues) has the worst fit. A typical structure (Figure 4) shows that subtilosin A (**1**) is a coiled loop that has a relatively rigid bowllike shape in which most amino acid side chains point outward. The genetic machinery required for production of subtilosin A (**1**) includes an *sbo* structural gene that codes for a 43 amino acid precursor, an ATP-binding cassette transporter (*albC*), two processing peptidases (*albE*, *albF*), and *albA*.[4] The peptidases may cut the leader from the prepeptide and cyclize the linear product. The *albA* product has homology to proteins that function in cofactor heme, PQQ, and molybdopterin cofactor synthesis. It may generate metalloenzyme(s) that catalyzes the ring closure of cysteine thiols onto α-carbons. This may proceed via oxidative conversion of the amino acid residue to an *N*-acyl imine[10b,11] that acts as an electrophile for a nearby cysteine, in analogy to the mechanism of thiol addition to form **2** and **3**. Further studies are in progress.

Supporting Information Available: Experimental procedures for fermentation, purification, and labeling of **1**; NMR parameters, chemical shift assignments, and NOE data; description of structural modeling; and experimental procedures for preparation of **2** and **3** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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