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Biochemical Structural Analysis of the Lantibiotic Mutacin II*

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Mutacin II is a post-translationally modified lantibiotic peptide secreted by *Streptococcus mutans* T8, which inhibits the energy metabolism of sensitive cells. The deduced amino acid sequence of promutacin II is NRWWQGVVPTVSYECRMNSWQHVFTCC, which is capable of forming three thioether bridges. It was not obvious, however, how the three thioether bridges are organized. To examine the bridging, the cyanogen bromide cleavage products of mutacin II and its variants generated by protein engineering, C15A, C26A, and C15A/C26A, were analyzed by mass spectrometry. Analysis of the wild type molecule and the C15A variant excluded several possibilities and also indicated a high fidelity of formation of the thioether bridges. This allowed us to further resolve the structure by analysis (mass spectrometry and tandem mass spectrometry) of the cyanogen bromide cleavage fragments of the C26A and C15A/C26A mutants. Nuclear magnetic resonance analysis established the presence of one and two dehydrobutyrine residues in mutacin II and the C15A variant, respectively, thus yielding the final structure. The results of this investigation showed that the C-terminal part contains three thioether bridges connecting Cys residues 15, 26, and 27 to Ser/Thr residues 10, 12 and 19, respectively, with Thr^{25} being modified to dehydrobutyrine.

Lantibiotics, a subclass of bacteriocin-like inhibitory substances elaborated by many Gram-positive bacteria, are biologically active peptides that contain post-translationally modified amino acids including the thioether amino acids lanthionine and β-methyllanthionine along with the dehydrated amino acids dehydrobutyrine and dehydroalanine (Fig. 1) (1, 2). Lantibiotics are ribosomally synthesized as prepropeptides and post-translationally modified. The post-translational modifications are catalyzed by specific enzymes that are genetically organized in clusters with the respective structural genes (1–4). The prepropeptides undergo dehydration of specific hydroxyamino acids to form dehydroamino acids with a $C_\alpha=C_\beta$ double bond. This is followed by the formation of intramolecular thioether bridges via the addition of cysteine sulfur atoms to the β-carbon of the dehydroamino acid (Fig. 1). Based on structure and mode of action, lantibiotics fall into two subgroups: type A, screw-shaped peptides that disrupt membrane functions, and type B, globular peptides that act as enzyme inhibitors (1).

Mutacin II is a lantibiotic peptide secreted by *Streptococcus mutans* T8. It resembles many of the type A lantibiotics but differs in its antimicrobial mode of action (5–7). The 3,245-Da peptide was previously isolated, and its genetic determinants were cloned from genomic DNA (8). The mutacin II structural gene (*mutA*) is followed by a gene encoding a modifying enzyme (*mutM*) and by ABC transporter (*mutT*). Other genes involved in regulation and immunity are clustered within this locus (9). The complete prepropeptide consists of 53 amino acids including the 26-amino acid amphipathic leader peptide with the G^{-2}-G^{-1} sequence at the processing site. The prolantibiotic part of mutacin shows similarities with sequences of several type A II lantibiotics (2, 8). Glycine in position 6 and asparagine and serine/threonine in positions 18 and 19, respectively (numbered according to mutacin), are conserved in mutacin II, lacticin 481, salivaricin A, variacin, and SA-FF22 (8, 10–15). Also conserved are motifs T(I/V)(S/T) in positions 10–12, (E/D)C in positions 14 and 15, and a C-terminal motif of (V/L)(F/A)TCC. Mutacin II is composed of 27 amino acids, with the other four lantibiotics ranging from 22 to 27 residues. All five lantibiotics are capable of forming three thioether rings via three cysteine residues in the molecule. Unlike other lantibiotics, analysis of the amino acid sequence indicated that the N-terminal region of mutacin II, namely residues 1–8, appears uniquely able to form an amphipathic α-helix when projected on a helical wheel (16).

This paper presents the biochemical and structural characterization of mutacin II and its engineered variant peptides using a combination of Edman degradation, mass spectrometry, NMR spectroscopy, and chemical modifications.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—The *Escherichia coli* strains used for subcloning and plasmid isolation were grown in LB medium in the presence of the appropriate antibiotics (17). *S. mutans* strains and *Streptococcus sobrinus* OMZ 176 were stored frozen at −70 °C until needed and grown as described previously in TSBY medium (6). Antibiotics were added to the media when needed (for streptococci: kanamycin 500 μg/ml, tetracycline *5* μg/ml; for *E. coli:* kanamycin 50 μg/ml, tetracycline 12.5 μg/ml, ampicillin 50 μg/ml).

DNA Manipulation, Transformation, and Molecular Cloning Techniques—*E. coli* plasmid and *S. mutans* T8 chromosomal DNA were prepared as described previously (17). *S. mutans* transformation was performed using the bovine serum albumin method (18).

Site-directed Mutagenesis—The general technique used for gene replacement of the wild type mutacin gene with engineered C15A and C26A variants has been recently reported (19). The C15A/C26A double mutant was generated by the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described (19) using a plasmid with the C15A mutacin gene variant as a template and the primer set (forward

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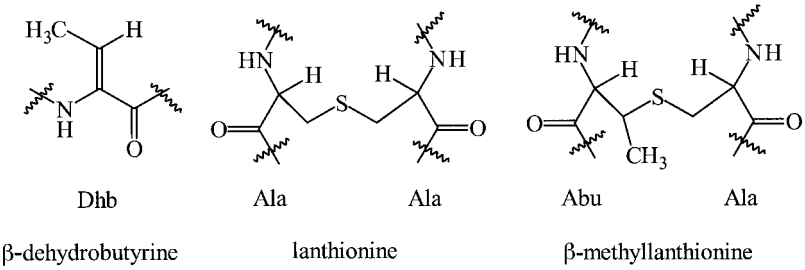

FIG. 1. **Structure of modified residues found in mutacin II.** The structure of the modified residue dehydrobutyrine is illustrated along with a lanthionine (*Ala-Ala*) bridge and a methyllanthionine (*Abu-Ala*) bridge. Dehydrobutyrine is formed by the dehydration of threonine. *Ala-Ala* represents a lanthionine bridge that originates from the nucleophilic addition of a cysteine sulfhydryl group to a dehydrated serine (dehydroalanine), and *Abu-Ala* represents a β-methyllanthionine bridge that originates from nucleophilic addition of a cysteine sulfhydryl group to a dehydrated threonine (dehydrobutyrine).

primer, CATGTTTTCACTGCTTGTTAAAAAATTAAAAATTATAACGG; reverse primer, TTTAATTTTTTAACAGCAGTGAAAACATGTTGCCATG). The identity of the introduced mutations was verified by DNA sequencing (Applied Biosystems 373 DNA sequencer). The plasmid carrying the mutated gene was digested with *Xba*I and then transformed into *S. mutans* strain CBM0 (19). The resultant transformants were analyzed for the desired reciprocal crossover by polymerase chain reaction analysis (19).

Purification and Analyses of Mutacin II and Its Analogs—Mutacin and its analogs were isolated using the ultrafiltration and selective precipitation method previously described (6). Mutacin activity was assayed on TSBY agar plates using the deferred antagonism technique with *S. sobrinus* OMZ176 as the indicator strain (20).

Edman Degradation—The purified native peptide and trypsin-, CNBr-, or ethanethiol-treated peptides were sequenced by automated Edman degradation with a gas phase microsequencing system (model PI 2090E; Porton Instruments Inc., Tarzana, CA). The system was equipped with an on-line phenylthiohydantoin-derivative analyzer.

Mass Spectrometry—Purified mutacin analogs were analyzed by electrospray ionization-mass spectrometry on a triple quadrupole mass spectrometer (API III; PE Sciex, Concorde, Ontario, Canada). MALDI-TOF[1] MS experiments were carried out in the positive mode with delayed extraction technology (Voyager Elite; PerSeptive Biosystems, Framingham, MA). The acceleration voltage was set at 25 kV, and 10–50 laser shots were summed. Sinapinic acid (Aldrich; catalog no. D13,460–0) dissolved in acetonitrile, 0.1% trifluoroacetic acid (1:1) was used as the matrix. The mass spectrometer was calibrated with bovine insulin. Samples were diluted 1:10 with matrix, and 1 μl was pipetted onto a smooth plate.

Cyanogen Bromide Cleavage—Mutacin II (0.2 mg) was dissolved in 0.5 ml of 40% formic acid in acetonitrile. To this solution approximately 0.5 mg of CNBr was added (21). Reaction times of 1, 3, and 72 h were tested simultaneously to achieve complete and selective cleavage at methionine.

NMR Analyses—Mutacin II was dissolved in 117 μl of formic acid-*d*, and a further 273 μl of acetonitrile-d_3 and 110 μl of water was added to make up a final volume of 500 μl. All NMR experiments were performed on a Bruker Instruments (Billerica, MA) WH-400 NMR spectrometer equipped with an Aspect 3000 computer. Data were collected at 303 K, and chemical shifts were referenced to internal acetonitrile. The COSY experiments were carried out in magnitude mode. All experiments used selective low power presaturation for water suppression. For all COSY experiments, 128 scans of 2,048 complex data points were collected for each of the 512 serial files. All NMR data were transferred to a Silicon Graphics Origin 200 server and processed using the Felix 97.2 program (Molecular Simulations Inc., San Diego, CA). The data were processed using different combinations of window functions to obtain the best possible representation of the transformed spectra.

RESULTS AND DISCUSSION

The primary structure of mutacin II is composed of 27 amino acids. Post-translational modifications include the formation of two lanthionine, one methyllanthionine, and one α,β-dehydroamino acid residue (16). Edman degradation stopped after eight cycles, revealing the N-terminal part of the sequence NRWWQGVV-. Next, mutacin II was linearized by reaction with ethanethiol under alkaline conditions and again looked at by Edman degradation (7). Examination of the reaction products yielded the additional sequence -P*X*V*X*YE*X*RM. Sequencing of a peptide isolated from a trypsin digest of mutacin II provided the partially overlapping sequence -MN*X*WQHVF. Overall, 20 amino acid residues were identified from the results of Edman degradation. A summary of the Edman degradation results can be seen in Table I.

TABLE I
Mutacin II sequence data

The amino acid sequence of mutacin II obtained by Edman degradation of the unmodified peptide, peptide reacted with ethanethiol, and peptide reacted with trypsin are presented. The primary structure deduced from the DNA sequence of the cloned gene is also included. For details, see "Experimental Procedures."

Modification	Sequence information
None	NRWWQGVV
Ethanethiol	NRWWQGVVPXVXYEXRM
Trypsin	NRWWQGVV MNXWQHVF
Deduced from cloned gene	NRWWQGVVPTVSYECRMNSWQHVFTCC

Interestingly, Edman degradation of the wild type molecule terminated after eight cycles. In other lantibiotics, sequencing by Edman degradation is often blocked by a dehydro residue (4). In mutacin II, a proline follows Val[8] and then is followed by a modified residue at position 10. In this case, the blocking of the sequencing cannot be attributed to a dehydro residue. A possible explanation is that proline preceding a bridging residue makes the Edman reaction sterically hindered and therefore sequencing cannot continue.

Mutacin II and the C15A variant were investigated using electrospray ionization-tandem mass spectrometry. Analysis of the doubly charged ion of mutacin II as well as the C15A variant yielded a series of *b* ions (*m/z* values 271, 457, 643, 771, 828, 928, 1027, 1124, 1207, and 1306 for *b*2 to *b*11), which corresponded to NRWWQGVVP-Dhb-V-, indicating that Thr[10] is a modified residue (Fig. 2). The 1207 signal, corresponding to N1-Dhb[10], was barely visible above the noise in the wild type compared with a very strong peak in the C15A. This indicates that the N1-Dhb[10] fragment is much less stable in the wild type and possibly results from the reduction of methyllanthionine during MS analysis. The theoretical as well as observed masses for all peptides studied can be seen in Table II. The entire sequence was later confirmed from the deduced amino acid sequence of the cloned gene (8) (Table I).

It has been previously determined that mutacin II contains

[1] The abbreviations used are: MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometry; TSBY, trypticase soy broth-yeast extract; COSY, correlation spectroscopy; Dhb, dehydrobutyrine; *b* ions, ions seen in a mass spectrum resulting from fragmentation from the N terminus; *y* ions, ions seen in a mass spectrum resulting from fragmentation from the C terminus.

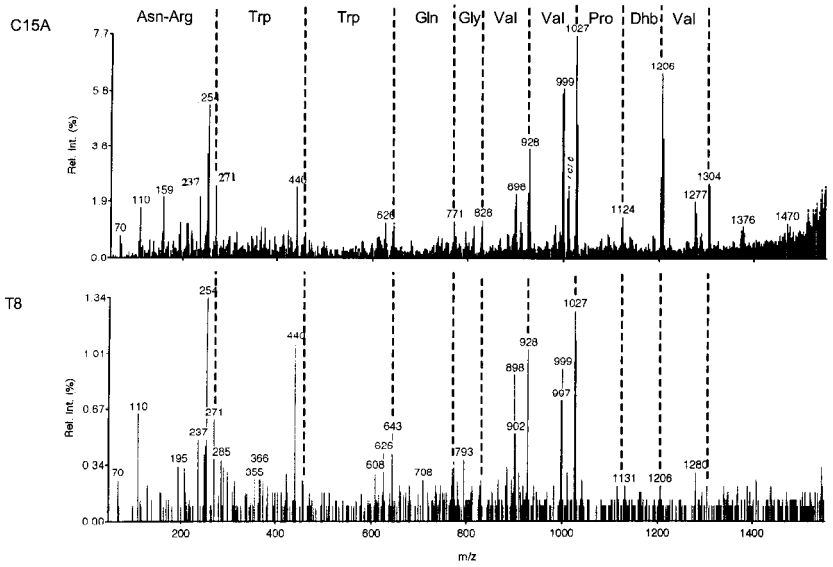

FIG. 2. **MS/MS spectra of the C15A and wild type peptides.** The MS/MS data of mutacin II and its C15A variant are presented, with the peaks representing cleavage at peptide bonds identified by the *vertical dashed lines*. The large peaks seen at 17 Da less than many of the indicated peaks are due to the loss of NH_3. Notice that the spectra are very similar up to the region surrounding Dhb[10]. The resolution in this experiment was sacrificed to increase sensitivity, and masses may be off by as many as 2 units. For details, see "Experimental Procedures."

TABLE II
Mass spectral data of cleavage reactions

The mass spectral data of the wild type, C15A, C26A, and C15A/C26A variants and products of the CNBr cleavage are shown. The experimental (Expt.) and theoretical masses (Theory) of the peptides studied are presented. Each peptide was cleaved with cyanogen bromide and studied by electrospray or MADLI-TOF mass spectrometry. The masses of the fragments obtained are shown along with the perceived number of peptide fragments. For details, see "Experimental Procedures."

Variant	Unreacted peptide mass		CNBr-cleaved peptide mass		Fragments
	Theory	Expt.	Theory	Expt.	
	Da		*Da*		
Wild type (T8)	3,245	3,245	3,214	3,214	1
C15A	3,213	3,214	3,182	3,183	1
C26A	3,213	3,215	2,028	2,028	2
			1,156	1,158	
C15A/C26A	3,181	3,181	1,995	1,995	2
			1,156	1,156	

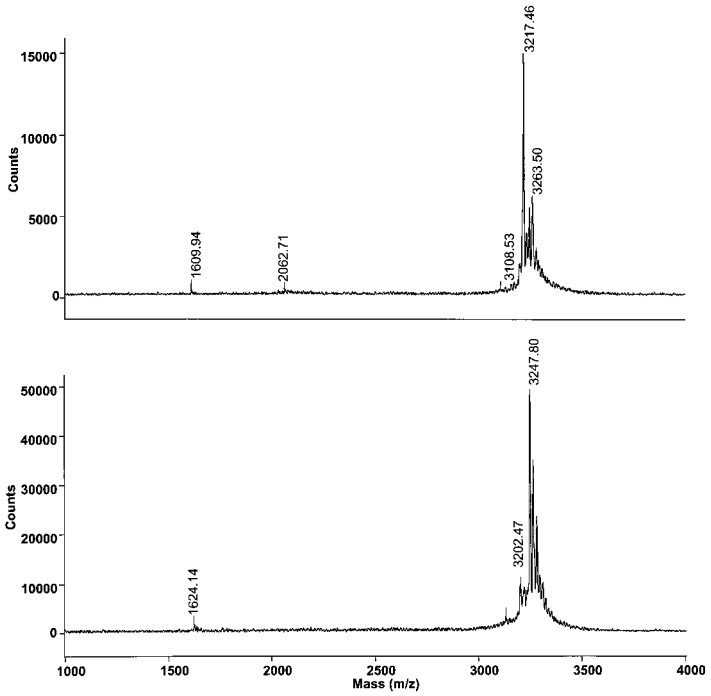

FIG. 3. **MS data of mutacin II after the cyanogen bromide cleavage reaction.** The *upper panel* shows the mass spectrum of the wild type peptide after reaction with cyanogen bromide for 3 h. The molecular mass of the peptide has been reduced by 31 Da as expected. The desired peak is seen with some possible oxygenation products. The *bottom spectrum* shows the control sample after 72 h. For details, see "Experimental Procedures."

three thioether bridges and one dehydroamino acid (7). Recently, mutagenesis studies confirmed that both the dehydrobutyrine residue and thioether bridges are required for the antimicrobial activity of mutacin II (16, 19, 22). However, the connectivity of these bridges was not obvious. The possible "partners" include cysteine residues at positions 15, 26, and 27 that pair with two serine and one threonine residues in positions 10, 12, 19, or 25.

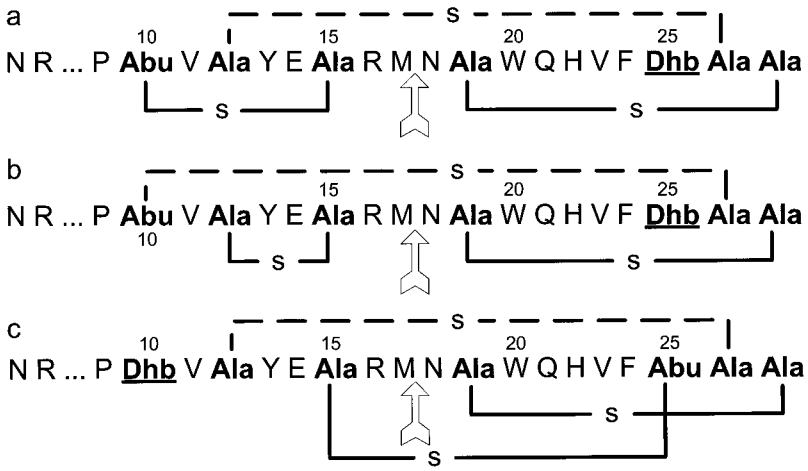

FIG. 4. **Possible bridging patterns.** The final three bridging possibilities are presented before cleavage of the C26A variant. The bridges with the *dashed lines* indicate those that would be missing in the C26A variant. The *arrow* represents the point of cleavage using cyanogen bromide. The cleavage of C26A resulting in two peptides would indicate structure *a* or *b*, while a single peptide after cleavage would indicate structure *c*.

The cleavage of a peptide containing a single methionine residue with CNBr results in the net loss of 31 Da in molecular mass from the N-terminal fragment and the formation of two peptides. However, covalent linkages that span the methionine residue, such as the thioether bridges in lantibiotics, hold the peptide fragments together. Examination of the bridging pattern of mutacin II using MS analysis was carried out on the CNBr products of mutacin II and its variants generated by protein engineering, namely C15A, C26A, and the double mutant C15A/C26A.

Analysis of the wild type molecule concluded that at least one bridge spanned the methionine. The result of cleavage by CNBr was a single peptide with molecular mass 31 Da less than the original (Table II, Fig. 3). Results from the C15A variant agreed with this finding. The cleavage product was a single molecule, thus indicating that a bridge must exist between any combination of Cys^{26} and Cys^{27} with Ser^{12} or Thr^{10} but not necessarily both (Table II). These results allowed the prediction of a series of possible structures that were then further resolved by MS analysis of the products from the CNBr cleavage of the C15A/C26A double mutant.

Reaction of the C15A/C26A variant with CNBr yielded two peptides of molecular mass 1,995 and 1,156 Da (Table II). These mass fragments were consistent with the cleavage of the 3,181-Da molecule and loss of 31 Da as expected. The results also showed that the N-terminal fragment contained two dehydro residues, Dhb^{10} and dehydroalanine 12, which indicated that these residues were processed from Thr^{10} and Ser^{12} as in the wild type peptide. The dehydro residues remained unreacted due to the unavailability of a cysteine. Apparently, the unreacted dehydroamino acid residues remain as such and do not participate in the formation of alternative bridges. Tandem mass spectra of the doubly charged ions of the two peptides confirmed their identity (results not shown). Analysis of the 1,156-Da C-terminal peptide indicated a series of at least five peptides derived from a common cyclic structure by breaking it at different sites. These fragments would indicate that a thioether bridge is likely to connect Cys^{27} with Ser^{19}. The possibility of a Cys^{27} to Ser^{19} bridge was further supported by the existence of two series of ions (*b* and *y*) from a peptide generated by sequential deletion of amino acids between Ser^{19} and Cys^{27} (results not shown).

The possible bridging patterns were narrowed down to three (Fig. 4), and the last peptide to be cleaved was the C26A variant. With Cys^{27} bridging with Ser^{19} and Cys^{26} bridging with Ser^{12} or Thr^{10}, the question at hand was the identity of Cys^{15}'s bridging partner. The next step was to determine whether its bridging partner was on the N- or C-terminal side of methionine. A product of two peptides would indicate no bridge spanning the methionine, thus Cys^{26} bridged to Tys^{10}/Ser^{12} and Cys^{15} to Thr^{10}/Ser^{12}, putting the Dhb at position 25 (Fig. 4, *a* and *b*). A product of a single peptide would indicate that Cys^{15} must be linked to Thr^{25}. Therefore, knowing the

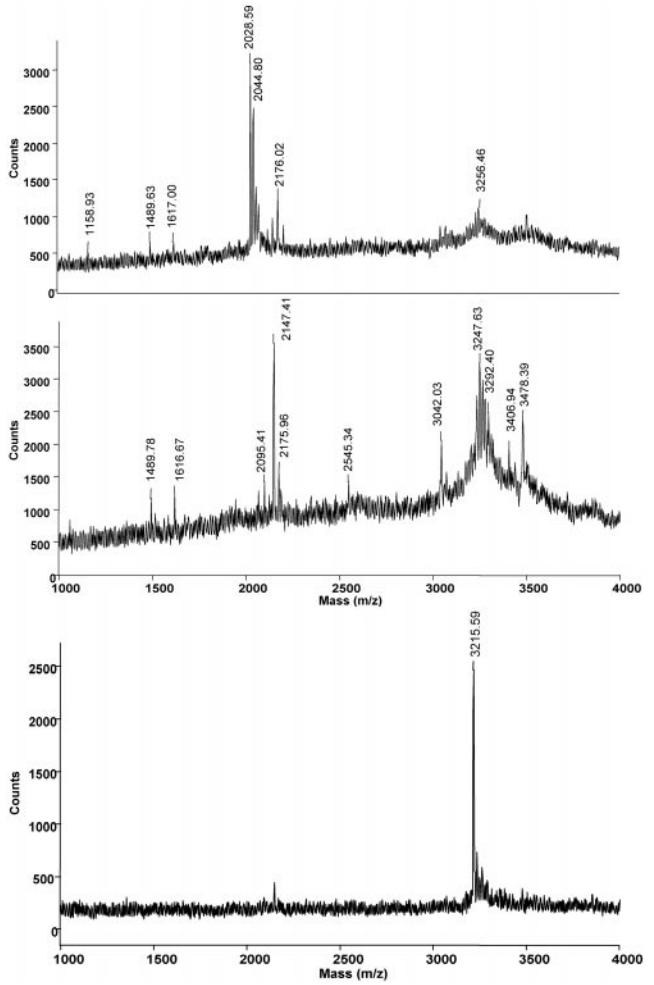

FIG. 5. **MS data of C26A after the cyanogen bromide cleavage reaction.** The *upper panel* shows the mass spectrum of the C26A variant after reaction with cyanogen bromide for 3 h. The *middle spectrum* shows the control sample after 72 h along with some oxidation products. Notice that the peaks at ~3,200 Da seen in the first spectrum are virtually gone, and the appearance of peaks at 2,028 and 1,158, which represent the two fragments. The *bottom spectrum* is the sample prior to reaction. Notice how clean it is compared with the cleaved and control samples. For details, see "Experimental Procedures."

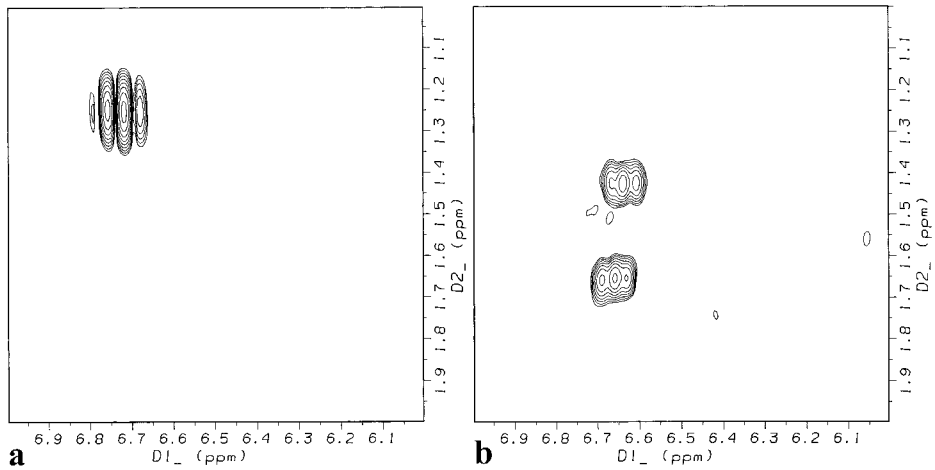

FIG. 6. **Selected COSY data for the wild type and C15A peptides.** A select region of the magnitude mode correlation spectroscopy nuclear magnetic resonance data for the wild type (*a*) and C15A (*b*) peptides is shown. The peaks represent the dehydrobutyrine H_β-H_γ interaction. Mutacin II is shown to contain one such residue, while the C15A variant is shown to have two. For details, see "Experimental Procedures."

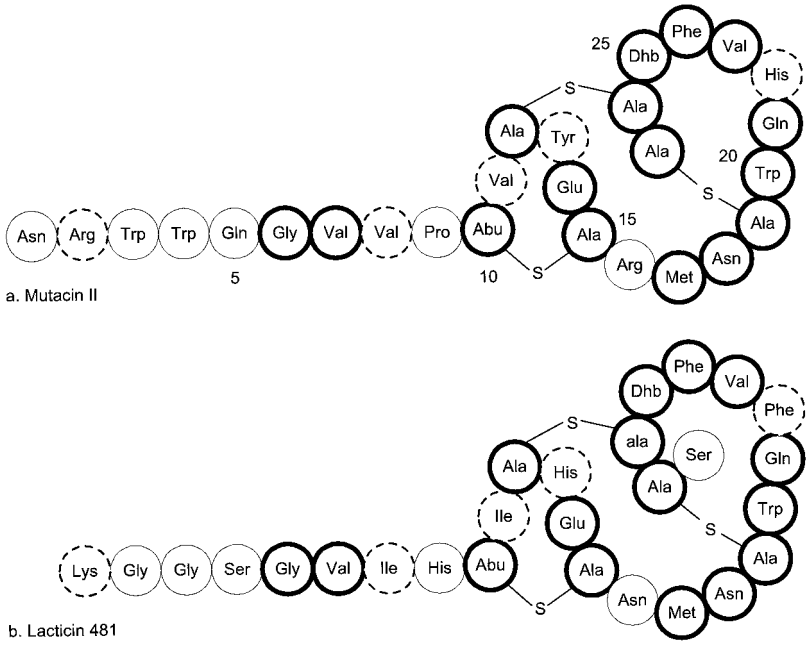

FIG. 7. **Mutacin II compared with lacticin 481.** The deduced bridging pattern of mutacin II is presented and shown to exhibit very high similarity to lacticin 481. The *thick circles* represent conserved residues, and the *dashed circles* represent residues in the same group. Thioether bridges are represented by *S*.

peptide has one Dhb, Cys^{26} must form a bridge with Ser^{12} (Fig. 4*c*). The MALDI-TOF MS spectrum of the cleaved C26A variant showed two peaks corresponding to the N-terminal and C-terminal cleavage products (Table II, Fig. 5). This indicated that two peptides were formed, narrowing down the possible structures to two: Cys^{26} linked to Ser^{12} and Cys^{15} linked to Thr^{10} (Fig. 4*a*) or Cys^{26} linked to Thr^{10} and Cys^{15} linked to Ser^{12} (Fig. 4*b*).

The last piece of evidence needed to define the bridging pattern was obtained using NMR spectroscopy. The COSY NMR spectrum of mutacin II showed a characteristic cross-peak corresponding to the H_β-H_γ interaction of Dhb (6.74 ppm, 1.26 ppm) (Fig. 6*a*). The COSY NMR spectrum of the C15A variant contained two cross-peaks corresponding to Dhb residues (6.65 ppm, 1.65 ppm and 6.64 ppm, 1.43 ppm) (Fig. 6*b*). This is indicative of the wild type mutacin containing one Dhb residue and the C15A variant containing two such residues. Since the C15A variant contained two Dhb residues, the only possibility left was that Cys^{15} is linked to Thr^{10} while Cys^{26} is linked to Ser^{12} (Fig. 4*a*). Replacing the cysteine at position 15 with alanine removed the possibility of a bridge. Dhb^{10} then remained unreacted, thus yielding two of the characteristic peaks seen in Fig. 6*b*. The sequence of mutacin II along with the location of the thioether bridges can be seen in Fig. 7*a*.

As seen in the MS data, some of the peptides undergo substantial oxygenation. Differing degrees of oxygenation were observed between the peptide variants. This is possibly an indication of the accessibility of the various sulfur atoms to oxygenation. In mutacin II, oxygenation is seen, but in the C15A variant, the oxygenated species become more prominent. This trend continues in the C26A with the doubly oxygenated species being the dominant peak in the control sample. Additionally, the data presented shows a definite trend in oxygenation being greater in the samples that were reacted for longer periods of time. For example, the C26A sample is quite clean prior to the reaction (Fig. 5). Consequently, it is believed that the thioether sulfur atoms and the methionine sulfur in the uncleaved peptides are the sites of oxygenation. This is consistent with not seeing the series of peaks for oxygenated species in the N-terminal fragments (Fig. 5).

The type A II lantibiotics include SA-FF22, lacticin 481, salivaricin A, and variacin (2). This group of peptides is thought to have the same bridging pattern but has only been proven for lacticin 481 and SA-FF22 (4, 23). The structure of one of these, lacticin 481, has been published (23). It, along with mutacin II, is comprised of 27 residues, but mutacin II lacks the C-terminal serine and has one extra residue at the N terminus (Fig. 7) (23). Interestingly, lacticin 481 and the other type A II lantibiotics kill cells by disruption of the cytoplasmic

membrane via pore formation, but mutacin II has been shown to utilize a different mechanism, one that inhibits the energy metabolism of sensitive cells (5). Also notable is the presence of proline 9 in mutacin II. Lacticin 481 does not contain this interesting structural element that has been shown to be pertinent for the biological activity of mutacin II (19). The bridging pattern of mutacin II has been found to be identical to that of lacticin 481 and SA-FF22.

The original structural study of lacticin 481 was based on NMR analysis. This study was able to narrow down the structure to two possibilities (11). The final covalent structure was then determined using a combination of methods including peptide chemistry, MS, and NMR (23). Comparatively, in this study, the structure of mutacin II was determined via a different route. In this case, the powerful tool of protein engineering was utilized to narrow down the possible bridging patterns. This worked well, but like the original study of lacticin 481 it only was able to narrow down the possibilities to two. NMR spectroscopy was then used to resolve the final structure.

Many of the structural features of mutacin II have been analyzed using a combination of protein engineering, chemical modification, mass spectrometry, and NMR. Since the thioether bridges in mutacin II appeared to be formed with high fidelity and were thus site-specific, the variant peptides provided excellent tools for structural studies. This report presents a novel and exciting approach using site-directed mutagenesis to determine the organization of thioether bridges in a lantibiotic peptide. The N-terminal portion of the molecule is coupled to the C terminus by a proline-methyllanthionine link that appears to be critical for antibacterial activity (19). The C-terminal part contains three thioether bridges connecting Cys residues 15, 26, and 27 with Ser/Thr residues 10, 12, and 19, respectively.

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