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Covalent structure of mutacin 1140 and a novel method for the rapid identification of lantibiotics

Leif Smith[1], Jan Novák[2], Jim Rocca[3], Scott McClung[4], J.D. Hillman[5] and Arthur S. Edison[3,6]

[1]*Department of Neuroscience,* [3]*University of Florida Brain Institute,* [4]*Interdisciplinary Center for Biological Research Protein Chemistry Core,* [6]*Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, and* [5]*Department of Oral Biology, University of Florida College of Dentistry, Gainesville, USA;* [2]*Department of Microbiology, University of Alabama at Birmingham, USA*

The primary structure of the *Streptococcus mutans* lantibiotic mutacin 1140 was elucidated by NMR spectroscopy, mass spectrometry, and chemical sequencing. The structure is in agreement with other closely related lantibiotics, such as epidermin. A novel method was developed in which mutacin 1140 was chemically modified with sodium borohydride followed by ethanethiol, allowing the differentiation of the thioether-containing residues from the dehydrated residues. This double-labeling strategy provides a simple method to reliably identify all modified lantibiotic residues with a minimal amount of material. While NMR spectroscopy is still required to obtain thioether bridging patterns and thus the complete covalent structure, the double-labeling technique, along with mass spectrometry, provides most of the information in a fraction of the time required for a complete NMR analysis. Thus, with these new techniques lantibiotics can be rapidly characterized.

Keywords: bacteriocin; chemical modification; double-labeling; hydrogenation; lantibiotic; mass spectrometry; NMR; structure determination.

Mutacin 1140, a member of a family of ribosomally synthesized peptide antibiotics called lantibiotics (lanthionine-containing antibiotics [1]), is produced by the Gram-positive bacterium *Streptococcus mutans* [2,3]. Because bacterial resistance to antibiotics is rapidly spreading, new antibiotics are in great demand. Mutacin 1140 has been shown to be stable, highly potent, and to inhibit a broad array of Gram-positive bacteria, including many that are responsible for human diseases [3–5]. Importantly, genetically stable resistant variants of sensitive strains have not yet been found, suggesting that the structure and chemistry of mutacin 1140 may provide important information for the development of new antibiotics. In addition, mutacin 1140 is a key feature in the development of replacement therapy for the prevention of dental caries. A genetically engineered effector strain of *S. mutans* has been constructed that has decreased virulence due to a mutation in the gene for lactate dehydrogenase [6]. By virtue of its ability to produce three-fold elevated amounts of mutacin 1140, this strain has been shown to displace disease-causing strains of *S. mutans* from the teeth of experimental animals and thereby provide lifelong protection against tooth decay. Thus, for these several reasons, it is important to obtain a comprehensive understanding of mutacin 1140.

In a previous study, Hillman and coworkers reported the genetic and biochemical analysis of mutacin 1140 [3]. In this work, they isolated the mutacin 1140 gene cluster, determined the spectrum of antibacterial activity, characterized a mutant strain of *S. mutans* (DM25) deficient in antibacterial activity, and reported the isolation and purification of mutacin 1140. Hillman's group also presented a tentative structure of mutacin 1140 [3]. This tentative structure was based primarily on Edman sequencing and mass spectrometry, but the authors noted that ''(the thioether bridge) assignment [would] have to be resolved, most probably by nuclear magnetic resonance spectroscopy'' [3]. In the work presented here, we have determined the structure of mutacin 1140 using NMR spectroscopy. Hillman and coworkers' concern proved warranted, as the correct structure differs from the tentative structure in the placement of thioether bridges. In the process of solving the correct structure, we have developed a new double-labeling strategy for Edman sequencing and a selective-labeling method for mass spectrometry. These methods allow for rapid differentiation of all types of lantibiotic residues with very small amounts of material. While developing these techniques, we also determined the problems that led to an incorrect structure. These problems are not specific to mutacin 1140 and can thus potentially mislead any lantibiotic investigation, so we describe their origin and proper interpretation.

Correspondence to A. S. Edison, Department of Biochemistry and Molecular Biology, Box 100245, University of Florida, Gainesville, Florida 32610, USA. Fax: + 1 352 392 3422, Tel.: + 1 352 392 4535, E-mail: art@ascaris.ufbi.ufl.edu

Abbreviations: Ala_S, alanyl moiety of lanthionine and 2-methyl-lanthionine; Abu, 2-aminobutyric acid; Abu_S, 2-methyl-alanyl moiety of 2-methyl-lanthionine; Dha, 2,3-didehydroalanine; Dhb, 2,3-didehydro-butyrine; ESI-MS, electrospray ionization mass spectrometry; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; MS/MS, tandem mass spectrometry; PVDF, poly(vinylidene difluoride).

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EXPERIMENTAL PROCEDURES

Chemical sequencing

All chemicals were purchased from Sigma and were the highest grade, unless otherwise stated. Mutacin 1140 was produced and purified as previously described [3].

Ethanethiol derivatization. A 10 μM solution of mutacin 1140 in 100 μL water was loaded into a prosorb column (PerkinElmer), and the peptide was drawn onto a poly(vinylidene difluoride) PVDF membrane. The ethanethiol derivatization method has been described elsewhere [7]. Briefly, after the PVDF membrane had dried, 15 μL of Solution A [280 μL methanol, 200 μL water, 65 μL 5 M sodium hydroxide, 60 μL ethanethiol (Aldrich)] was directly added to the PVDF membrane in the prosorb column. The prosorb column was then placed inside a 1.5 mL eppendorf tube, wrapped tightly with parafilm, and incubated at 50 °C for 1 h. The sample was stored for less than one day at 4 °C before sequencing.

Double labeling by sodium borohydride and ethanethiol derivatization. A 200 μM solution of mutacin 1140 in 5 μL water was added to a 0.5-mL eppendorf tube that contained 2.0 mg of sodium borohydride. This was followed by the addition of 94 μL of solution B (570 mg guanidine HCl, 100 μL *N*-ethylmorpholine and water to a final volume of 1 mL; the pH of the mixture was adjusted to 8.5 with glacial acetic acid). The reaction mixture was then added to a vial that was purged with nitrogen gas, and incubated at 37 °C for three days. At the end of the incubation period the sample was loaded into a prosorb column (PerkinElmer), and the peptide was drawn onto a PVDF membrane. The peptide was then modified with ethanethiol as described above.

Edman sequencing procedure of mutacin 1140. A glass fiber filter was pretreated with polybrene to reduce the loss of peptide during each cycle. Precycles of the actual Edman cycles were used to wash the polybrene treated glass fiber filter. The PVDF membrane was then excised from the prosorb column and dried with nitrogen gas until it turned white. The PVDF membrane was placed into the sequencer cartridge with the polybrene treated glass fiber filter. The cartridge was then placed into the sequencer (Applied Biosystems 494 Protein P.E. Biosystems, Foster City, CA), which was operated under normal pulse-liquid blot cycles. Analysis of the amino-acid sequence was done using the ABI 610 A data software. Commercially available DL-2-aminobutyric acid (Abu) (Aldrich) was used as a standard to identify the retention time using modified cycles. To prevent Abu from completely washing off the glass fiber filter, the normal pulsed-liquid blot cycles were modified to remove all of the solvent washing steps.

Mass spectrometry

A purified sample of mutacin 1140 was analyzed by electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) on a PE Sciex API III Biomolecular mass analyzer [8].

A vacuum dried sample of mutacin 1140 was chemically modified with 2-mercaptoethanol following the procedure described by Meyer *et al.* [7] under alkaline conditions. Thus, the peptide had 2-mercaptoethanol added to all of the post-translationally modified residues. We also developed a modification system using 2-mercaptoethanol under neutral pH that selectively labeled only existing dehydrated residues while thioether bridges were not disrupted. A 1 mM solution of mutacin 1140 was incubated in 12 μL of 0.1 M ammonium acetate buffer pH 7 with 3 μL 2-mercaptoethanol at 50 °C for 1 h. Samples were then analyzed by ESI-MS and MS/MS.

Mutacin 1140 was incubated for various periods of time (from 1 h to overnight) with sequencing grade trypsin (Sigma) in 0.1 M ammonium acetate buffer pH 7 or phosphate buffer pH 7, and the sample was then analyzed by ESI-MS and MS/MS.

Derivatization with 2-iminothiolane [9] was conducted to selectively label the N-terminal amino acid and lysine residue, allowing definitive identification of the b-series of ions derived from mutacin 1140 in the MS/MS spectra among other daughter ions.

NMR spectroscopy

Mutacin 1140 is not soluble in aqueous solutions at concentrations required for NMR. Therefore, 2 mM samples of mutacin 1140 were prepared in 70–90% acetonitrile-d_3 (Cambridge Isotopes) and 10–30% water in a total volume of 700 μL. The NMR data were collected on Varian Unity and Bruker Avance spectrometers, both operating at a proton frequency of 600 MHz. The ^{1}H resonances were assigned according to standard methods [10] using TOCSY [11] and NOESY [12] experiments. HMQC [13,14] and HMBC [15] experiments were used to clarify some areas of ambiguity in the TOCSY and NOESY spectra. NMR experiments were collected at 25 °C and the carrier frequency was centered on the water resonance, which was suppressed by presaturation during the 1.5 s relaxation delay. The TOCSY experiments were acquired with a 60 ms mixing time using the MLEV-17 sequence [16]. The NOESY experiments were acquired with 200 ms, 400 ms, and 450 ms mixing times. The delay times to create or refocus antiphase coherence in the HMQC and HMBC experiments were adjusted to 3.5 ms (140 Hz coupling) and 60 ms (8.5 Hz coupling), respectively. The spectral sweep width for both TOCSY and NOESY was 6588.7 Hz (11 p.p.m.) in both dimensions. The spectral sweep widths for HMQC and HMBC were 8000.0 Hz (13.3 p.p.m.) in the proton dimensions and 22500.0 Hz (149.1 p.p.m.) and 30 000.0 Hz (198.9 p.p.m.) for the carbon dimensions, respectively. All 2D data were collected with 2048 complex points in the acquisition dimension and between 256 and 512 complex points for the indirect dimensions. Phase sensitive indirect detection for all experiments was achieved using the method of States-TPPI [17]. ^{1}H chemical shifts were referenced to acetonitrile (1.93 p.p.m.). Data were processed with NMRpipe [18] by first removing the residual water signal by deconvolution, multiplying the data in both dimensions by a squared cosine function or a squared cosine function with a 60° shift (for the ^{1}H dimension of HMBC), zerofilling once, Fourier transformation, and baseline correction. Data were analyzed with the interactive computer program NMRView [19].

RESULTS

Chemical sequencing

Treatment of lantibiotics by ethanethiol results in the saturation of 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) residues and in the opening of thioether rings [7]. Subsequent Edman sequencing yields an amino-acid sequence with uncertain identification of dehydrated and thioether residues (for example, see Fig. 1A) [3]. In order to discriminate between unsaturated amino acids and residues that form thioether rings, we developed a double-labeling protocol in which we first hydrogenated the double bonds and subsequently reduced the thioether linkages with ethanethiol. Double-labeling of mutacin 1140 yielded thioethyl cysteine residues at amino-acid positions 3, 7, 11, 16, and 19 and a 2-methylthioethyl cysteine residue at position 8 (Fig. 2). Alanine and Abu residues were formed at positions 5 and 14, respectively, from

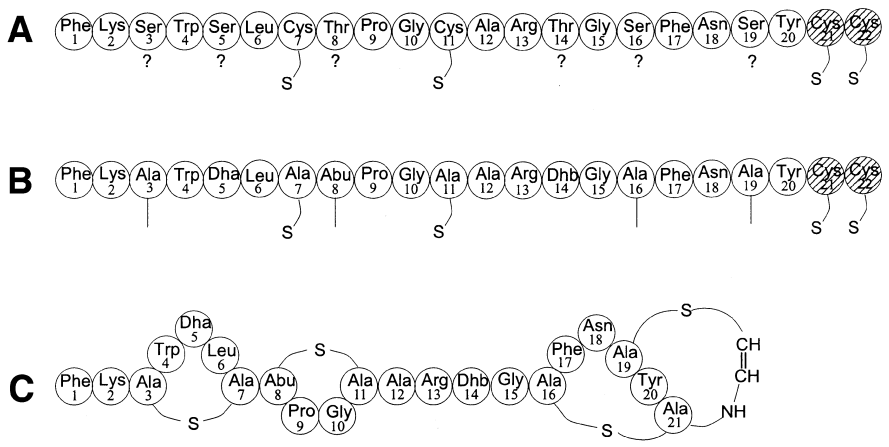

Fig. 1. Covalent structure of mutacin 11 40 derived from different sets of experiments. (A) Traditional nonselective (ethanethiol) chemical modification, mass spectrometry, and Edman sequencing. (B) Double-labeling/Edman sequencing and selective-labeling/mass spectrometry as described in this work. (C) NMR spectroscopy (which is in perfect agreement with all the other data). Question marks indicate uncertainty about the type of post-translational modification. Filled circles at the C-terminus indicate lack of Edman sequencing data. Vertical lines (B) indicate involvement in thioether bridge formation. Dha, 2,3-didehydroalanine; Dhb, 2,3-didehydrobutyrine; Abu_S, S-2-aminobutyric acid.

the initial hydrogenation. Thus, double labeling of mutacin 1140 provided definitive assignments of both the dehydrated amino acids, and the residues involved in thioether bridges (Fig. 1B).

Mass spectrometry

Purified mutacin 1140 was analyzed by ESI-MS and MS/MS. The calculated mass of 2263 Da was identical to that measured for mutacin 1140 in crude preparations [3] and indicated complete post-translational modifications. MS/MS of the doubly charged molecular ion yielded a complex spectrum of daughter ions (Table 1). The major ion was the doubly charged molecular ion, indicating the noteworthy stability of the peptide. The spectra had ions with the loss or addition of ≈ 32 or 33 mass units, suggesting the loss or addition of S or SH atoms involved with the thioether linkages. The interpretation of the b-ions was supported by an additional experiment in which the N-terminal portion (N-terminus and lysine residue) was labeled with 2-iminothiolane [9], and the doubly charged molecular ion with two additions (to the first two N-terminal amino-acid residues, Phe and Lys) was subjected again to

Table 1. Amino-acid sequences of mutacin 1140 and daughter ions. These were generated by tandem mass spectrometry of the doubly charged ion of unmodified mutacin 1140 with an *m/z* value of 1133 and of the mercaptoethanol (ME)-derivatized mutacin 1140 (under neutral pH to generate additions across existing double bonds; with two 2-mercaptoethanol additions per molecule of mutacin) with an *m/z*-value of 1211. The range was from 50–2000 *m/z*. The brackets in the amino-acid sequence column represent the thioether bridges that link the amino-acid residues based on NMR data. Ions are listed according to their corresponding *m/z* numbers observed in the mass spectra. Av, S-[aminovinyl]-cysteine.

Amino-acid position	Mutacin 1140 (native)		Derivatized mutacin 1140 (2 additions of 2-mercaptoethanol)	
	b-ions	y-ions	b-ions	y-ions
Phe1	120[a] (9.5)[b]		120[a] (2.0)	
Lys2	275 (4.6)		276 (1.6)	
Ala_S3	345/378[c] (3.0/1.6)	1987 (4.0)	345 (0.7)	
Trp4	532/564 (0.7/1.0)	1919/1887 (1.7/1.4)	534 (0.7)	[1996][d] ([2–9])
Dha5	599/633 (1.1/1.5)	1733/1700 (3.3/2.0)	[678][e] ([1–5])	[1889] ([2–4])
Leu6	714/748 (1.5/0.9)	–/1631 (–/1.0)	[791/825][c] ([0.8/2.3])	1664 [1742] (1.4 [1–4])
Ala_S7	816/784 (3.0/1.2)	1550/1519 (1.2/0.9)	[894] ([1–6])	[1630] ([1–8])
Abu_S8	899/932 (4.5/3.0)	1446 (1.8)	[978/1012] ([6–7/1,6])	[1520,1597] ([0.8, 1.6])
Pro9	997/1029 (4.8/10.4)	1364/1333 (6.2/0.8)	[1076/1108] ([3–3/5,8])	[1442,1520] ([8.0, 0.8])
Gly10	1053/1086 (5.0/4.0)	1266 (0.8)	[1131/1164] ([2–6/1,9])	[1344] ([1–7])
Ala_S11	1157/1125 (1.4/25.0)	1212/1180 (1.4/0.7)	[1232/1201] ([1.2/18.0])	[1287] ([0.7])
Ala12	1227 (1.0)	1108 (12.5)	[1303] ([1.0])	[1186] ([1–7])
Arg13	1384 (3.2)	1037 (10.5)	[1462] ([3.0])	[1115] ([3–5])
Dhb14	1465 (3.0)	880 (3.0)	[1545 [1623]] ([2–9 [1,5]])	[957] ([2–8])
Gly15	1521 (2.0)	798 (1.4)	[1600 [1679]] ([1–8 [1,6]])	799 [874] (0.8 [1–6])
Ala_S16	1591/1623 (0.7/1.2)	739 (1.3)	[1671 [1747]/1701 [1781]] ([1–6 [1,6]/1.7/[1–4]])	742 (0.18)
Phe17	–/1771 (–/2.3)	672 (1.2)	[1895/1929] ([1–3/1,4])	671 [749] (1.6[0.8])
Asn18	1853/1887 (0.8/1.4)	524 (0.3)		– (–)
Ala_S19	1922/1953 (1.8/0.5)	411/378/366 (1.6/1.5/1.4)		411 (1.6)
Tyr20		–/308 (–/1.5)		
Ala_S21		179 (203)		
Av22				

[a] Immonium ion. [b] Numbers in parentheses are relative intensities. [c] Ions with a gain or loss of ≈ 32 or 33 mass units, respectively, are indicated after the slash. [d] Daughter ions with *m/z* above 2000 are not detected by the mass spectrometer. [e] Ions with a gain of ≈ 78 mass units are indicated in square brackets.

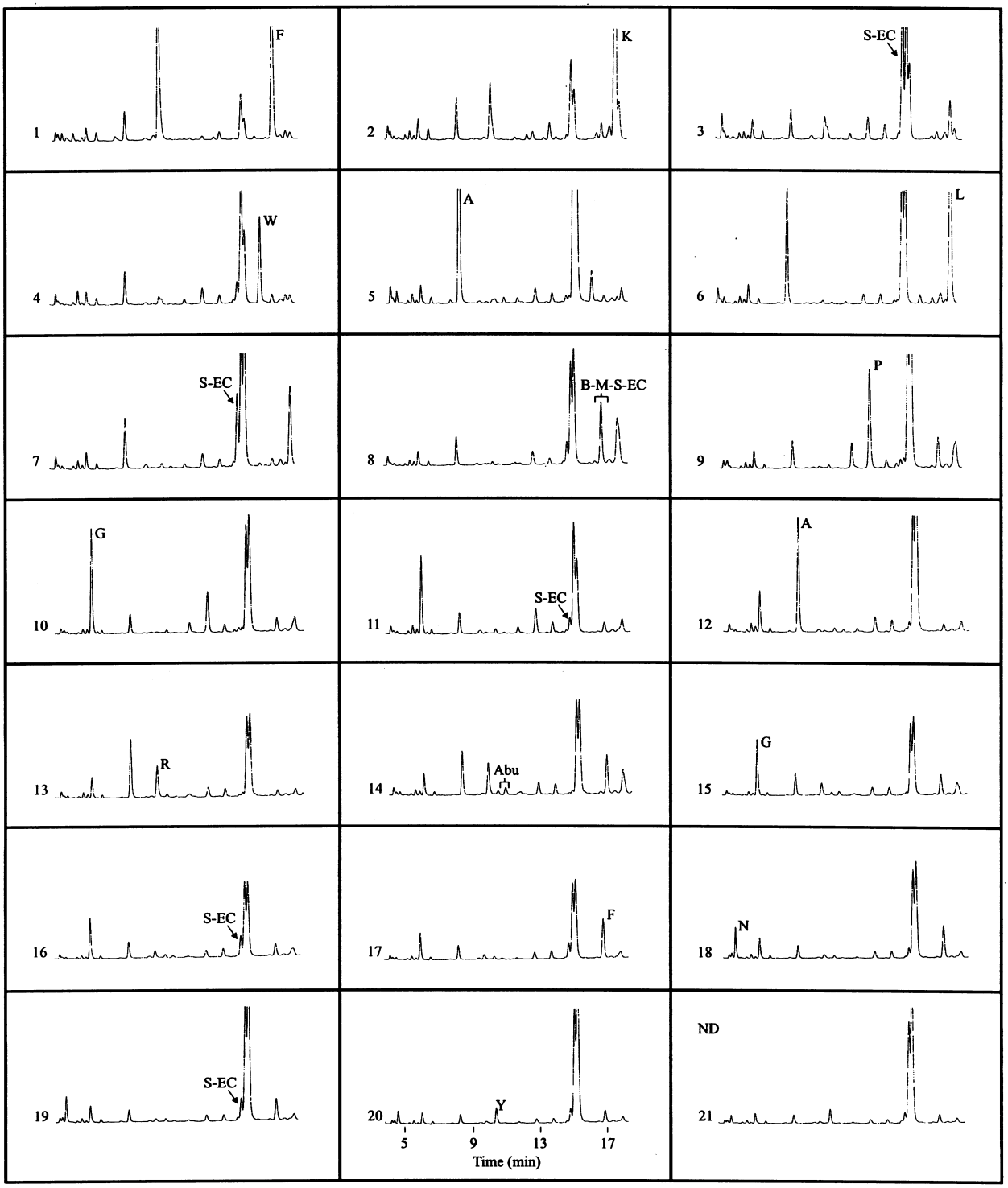

Fig. 2. Sequence analysis of the sodium borohydride/ethanethiol derivatized mutacin 1140. The chromatograms of the Edman sequence analysis are shown. Sodium borohydride derivatization of the Dha5 residue and the Dhb14 residue brought about the formation of Ala5 and Abu14. Thioethyl cysteine (S-EC) is visible in cycles 3, 7, 11–16, and 19. 2-methylthioethyl cysteine (B-M-S-EC) is visible at amino-acid position 8. Amino-acids are designated by their one letter code.

collision-induced dissociation. The corresponding increases in masses confirmed the identity of b-ions (data not shown).

These results, together with an almost complete series of b-ions and y-daughter ions indicated that thioether bonds break during collision-induced dissociation in ESI-MS/MS. Tandem mass spectrometry of a lanthionine standard indicated that the major daughter ions were generated by breaking the thioether bonds (data not shown), confirming the interpretation of the mutacin 1140 data.

To detect dehydrated residues, a reaction was developed with 2-mercaptoethanol at neutral pH that selectively labeled these groups. This chemical modification resulted in reaction products, detected in the ESI-mass spectra, that contain additions of one, two, and three molecules of 2-mercaptoethanol. This

was consistent with two dehydrated residues and an S-[aminovinyl]-cysteine [3]. The amount of the fully derivatized molecule with three additions was not sufficient for tandem mass spectrometry, but mutacin 1140 with one and two additions were subjected to collision-induced dissociation with good results. Daughter ions of the doubly charged molecule of mutacin 1140 with two additions of 2-mercaptoethanol are shown in Table 1. The 2-mercaptoethanol added 78 and 156 mass units to all b-ions following positions 5 and 14, respectively, demonstrating that these residues are Dha5 and Dhb14. The y-ion series gave similar results. Interestingly, the MS/MS spectra of mutacin 1140 indicated that a single 2-mercaptoethanol addition was localized in Dha5. This suggested good accessibility or higher reactivity of this residue compared to the other two available sites (Dhb14, Av22).

The absence of secondary daughter ions derived from b-ions or y-ions by loss or addition of S or SH atoms in the Ala12–Gly15 region (Table 1) suggested that there are no thioether bridges crossing over this region. Therefore, mutacin 1140 could be cleaved by trypsin after the Arg13 residue to yield two fragments, as described for gallidermin [20]. Mutacin 1140 tryptic digests were generated using both soluble and immobilized sequencing-grade trypsin with identical results. We observed a 1401 Da peptide that was identified by tandem mass spectrometry as the N-terminal fragment Phe1–Arg13. The C-terminal fragment was not detected, probably due to the deamination of Dhb 14 after the cleavage (similar to epidermin and gallidermin [20]). In contrast to intact mutacin 1140, collision-induced dissociation of the N-terminal tryptic fragment yielded a spectrum in which the doubly charged parent ion (*m/z* 701) was detected but was not a major signal. Apparently, structural features responsible for the remarkable stability of the mutacin 1140 peptide require an intact molecule, and are likely a result of the conformation of the whole molecule, and not just the thioether bridges in the cleaved fragment.

NMR spectroscopy

NMR is clearly the definitive method for complete lantibiotic structure determination [21]. Therefore, in order both to verify the double-labeling results and to firmly establish the correct thioether pairings, we collected TOCSY, NOESY, HMQC, and HMBC NMR data on mutacin 1140. The TOCSY/NOESY data sets provided unambiguous sequential assignments, so the HMQC/HMBC data sets were primarily used as supporting evidence to the proton-based assignments. Based on distinct spin systems, 21 amino acids and one aminovinyl group were identified in the TOCSY and NOESY experiments (Fig. 3, Table 2). Unmodified amino acids were sequentially assigned by standard methods [10] and agreed perfectly with the known mutacin 1140 prepropeptide sequence [3]. Many of the NMR connectivities described next can be seen in Fig. 3.

Residues 2 through 4 were identified through an H_i^{α} to H_{i+1}^{N} sequential walk. The residue at position 3 has a chemical shift pattern characteristic of an alanyl moiety of lanthionine and 2-methyl-lanthionine (Ala_S). Distinct vinyl proton chemical shifts, consistent with a Dha, have strong NOEs to the amide proton of Leu 6. The HMBC spectrum provided additional correlations between the Dha vinyl protons to the Leu 6 H^N by way of the Dha C′ resonance, verifying that the amino acid at position 5 is a Dha residue. Leu 6 has several H^{α} and side-chain proton NOEs to H^N of Ala_S at position 7, which in turn has H^N to H^{β} and H^{β} to H^{β} NOEs to Ala_S 3. Thus, amino acid 5 is a Dha and amino acids at positions 3 and 7 form a lanthionine.

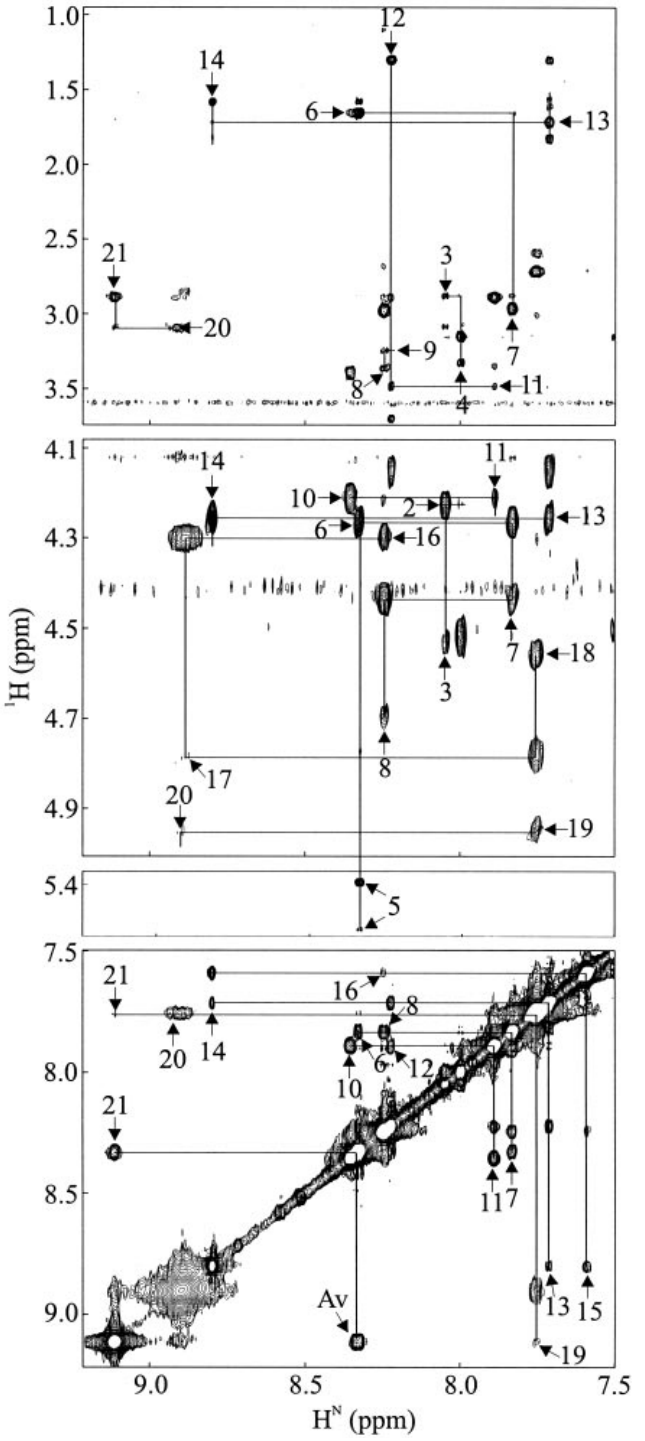

Fig. 3. NOESY NMR spectra of mutacin 1140 in acetonitrile-d_3/water (8 : 2) at 25 °C with a mixing time of 400 ms. The expansions show amide to amide, amide to vinyl, amide to alpha, and amide to side chain interactions. The numbers correspond to residue position in the peptide sequence.

Several NOEs, including H_i^{α} to H_{i+1}^{N}, exist between Ala_S 7 and the amide proton of a spin system consistent with 2-methyl-alanyl moiety of 2-methyl-lanthionine (Abu_S), which in turn has an H^{α} to H^{δ} NOE to Pro9 and establishes a *trans* Abu_s-Pro peptide bond. A complete sequential walk could be made between residues 10 and 14. Chemical shift patterns and an H_i^{α} to H_{i+1}^{N} NOE from Gly10 indicate that position 11 is Ala_S.

Table 2. ^{1}H NMR chemical shift values for mutacin 1140 in acetonitrile-d_3/water (8 : 2) at 25 °C. The brackets represent the thioether bridges that link the amino-acid residues.

Amino-acid position	H^N	H^α	H^β	Other
Phe1	–[a]	3.89	3.07, 2.82	–
Lys2	[b]	4.23	2 28., 2.22	γ-1.50, 1.24, γ -2.04, 1.86, ε-2.85
Ala_S3	8.05	4.53	3.09, 2.88	
Trp4	8.00	4.50	3.31, 3.16	2H-7.07, 4H-7.60, 5H-7.00, 6H-7.35, 7H-7.50, NH − 9.85
Dha5	–		5.69, 5.37	
Leu6	8.33	4.25	1.65 1.59	γ -1.59, δ-0.90, 0.84
Ala_S7	7.83	4.44	3.00, 2.94	
Abu_S8	8.25	4.69	3.33	γ-1.09
Pro9		4.21	2.27, 1.98	γ-1.77, 1.65, γ -3.38, 3.26
Gly10	8.36	4.20, 3.39		
Ala_S11	7.89	3.68	3.48, 2.88	
Ala12	8.22	4.15	1 30.	
Arg13	7.71	4.25	1.86, 1.71	γ-1.58, γ -3.14, NH-7.19
Dhb14	8.80		6.36	γ-1.58
Gly15	7.59	3.99, 3.85		
Ala_S16	8.25	4.30	2.98, 2.68	
Phe17	8.88	4.77	3.43, 2.85	2, 6H-6.63, 4H and/or 3,5H-7.12
Asn18	7.76	4.55	2.71, 2.59	NH_2−6.59, 7 31.
Ala_S19	7.76	4.94	3.13, 2.99	
Tyr20	8.90	4.13	3.10, 2.90	2, 6H-7.04, 3, 5H-6.67
Ala_S21	9.11	4.00	2.88	
Av22	8 34.	6 97.	5.67	

[a] Dashes indicate areas where chemical shift information is missing. [b] Amide value for lysine is not seen in this data set, but has been seen in other data sets with lower percentage of acetonitrile.

Sequential H^N NOEs correlate amino acids 14 through 16, and a spin system characteristic of a Dhb residue is between Arg13 and Gly15. Several NOEs between Abu_S8 and Ala_S11, including H^β to H^β and H^γ to H^β, show that these residues form a thioether bridge. Thus, amino acids 8 and 11 form a 2-methyl-lanthionine and the amino acid at position 14 is a Dhb.

A sequential H^α_i to H^N_{i+1} walk could be made from residues 16 through 18. Chemical shifts and several NOEs between Gly15 and Phe17 indicate that position 16 is Ala_S. A sequential walk could be made from residues 19 through 21. The residue following Tyr20 is Ala_S21, which in turn has H^β to H^β and H^β to H^N NOEs to Ala_S 16, verifying a thioether bridge between Ala_S 16 and 21. Another Ala_S residue was similarly identified at position 19. Ala_S 21 has sequential NOEs to a spin system characteristic of an amino vinyl group group, which in turn has a vinyl proton to $H^{\alpha,\beta}$ NOEs to Ala_S 19. Thus, amino acids 16 and 21 form a lanthionine, and residue 19 and the C-terminal amino vinyl group group form an S-[aminovinyl]-cysteine. The complete structure of mutacin 1140 is shown in Fig. 1C.

DISCUSSION

The findings from this study include: the complete covalent structure of mutacin 1140; identification of potential problems in lantibiotic structure determination; and the development of new techniques to rapidly and reliably identify modified lantibiotic residues.

All of the data (chemical, mass spectrometry, and NMR) presented above are in complete agreement and support the structure of mutacin 1140 shown in Fig. 1C. The chemical modification and mass spectrometry data were able to unambiguously distinguish between residues that are dehydrated or involved in thioether linkages. The NMR data verified these assignments and also allowed for the specific identification of 2-methyl-lanthionine and lanthionine residues through inter-residue NOEs. The structure of mutacin 1140 is also consistent with similar type A lantibiotics such as gallidermin [20–22], and epidermin [23]. This is in contrast to the preliminary structure [3], which proposed significantly different thioether bridging patterns.

While amino-acid sequence homology to other lantibiotics provides strong evidence for homologous thioether bridging, it cannot confirm that this bridging occurs [24,25]. Despite the fact that mutacin 1140 has high sequence similarity to other lantibiotics, misinterpretation of two experimental results led Hillman and coworkers to propose a nonhomologous structure [3]. These problems are general to lantibiotics and pose potential troubles, so we describe their origin here. First, Edman degradation of gallidermin was previously shown to stop before residue 14 (a Dhb) but not before or after the thioether bridges [7,20]. Edman degradation of mutacin 1140 stopped before residue 3, and the authors therefore postulated this to be a Dha [3]. In repeating this work with three times the amount of starting material, we found a drastic reduction, but not a complete stop, following residue 3. This suggests that steric interactions, not a dehydrated amino acid, were the source of the problem. Second, FAB mass spectrometry was previously used for the analysis of a known lantibiotic, and it was convincingly shown that fragmentation of the peptide backbone does not occur in the regions spanned by thioether bridges [26]. By analogy, Hillman *et al.* [3] postulated that MS/MS using ESI would provide comparable results. The data in Table 1 suggest that the lanthionine rings do fragment using MS/MS, and the results of the fragmentation of lanthionine presented here confirm this conclusion.

Therefore, we recognized the need for a simple, rapid, and ultra-sensitive method to discriminate between dehydrated residues and those involved in thioether bridge formation. Specifically, we wanted to develop a method that would provide most of the lantibiotic structural information with a minimal amount of sample and effort. The double-labeling method described above meets this need. By first hydrogenating double bonds and then reducing thioether bridges, all modified residues in mutacin 1140, with the exception of the C-terminus, could be distinguished and identified (Fig. 2). The two most C-terminal amino acids remained ambiguous with this method because of limitations in Edman sequencing. Thus, in just a few days and with only 1 nmol of material, we were able to determine the structure shown in Fig. 1B. These results, along with the mass spectrometry data, which indicated that residues 12–15 are not bridged by any thioethers (Table 1), provided a nearly complete structure. NMR was required to determine which thioether groups bonded together and was used to verify all the results from the double-labeling studies. The double-labeling methods described here should provide a useful starting point in any lantibiotic study.

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