Confirmation of the Structure of Nisin by Complete ^{1}H N.m.r. Resonance Assignment in Aqueous and Dimethyl Sulphoxide Solution

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The chemical structure of the peptide antibiotic nisin has been confirmed by the unambiguous sequence-specific assignment of its ^{1}H n.m.r. spectrum using a range of 2-D n.m.r. techniques. Nisin was examined in both aqueous (sodium phosphate buffer, pH 2.25) and dimethyl sulphoxide–trifluoroacetic acid solution. Amino acid spin systems were identified from observation of relayed connectivity patterns in the HOHAHA spectrum. Sequential assignments were accomplished with the aid of NOESY and relayed-NOESY spectra. In addition, the locations of the five thioether ring systems were confirmed. The detailed n.m.r. analysis provides strong evidence for the structure originally proposed on the basis of chemical degradation studies.

Nisin is a highly modified 34-amino acid residue peptide produced by certain strains of *Lactococcus lactis*. It exhibits antimicrobial activity against a wide spectrum of gram-positive organisms, and also inhibits sporulation of *Bacilli* and *Clostridia*. This latter property has been exploited by the food industry and nisin is now extensively employed as a food preservative, particularly for milk products.[1] In addition, it was recently shown that nisin has potential use in the brewing industry for growth inhibiton of 'spoilage' bacteria such as *Lactobacilli* and *Pediococci*.[2,3]

Nisin belongs to an unique group of peptide antibiotics which includes subtilin,[4] epidermin,[5,6] and gallidermin.[7] The common structural features of these antibiotics are the presence of *meso*-lanthionine, (2*S*,3*S*,6*R*)-3-methyllanthionine and α,β-didehydroamino acid residues. The chemical structure of nisin (Figure 1) was proposed by Gross and Morell[11–13] in 1971 on the basis of extensive chemical studies. Recently, support for this structure was obtained from f.a.b. mass spectroscopy[14] and total chemical synthesis.[15] The lanthionine and 3-methyllanthionine residues introduce sulphide bridges at various locations in the molecule, giving rise to cyclic units, and hence introducing considerable conformational constraints on the polypeptide backbone.

Recent molecular biological studies have shown that nisin and related antibiotics are derived from ribosomally synthesized precursors. The structural genes encoding the precursors of epidermin,[6] nisin,[8,9] and subtilin[10] have been cloned and sequenced. The encoded precursor of nisin is 57 amino acid residues long, with a 23-residue leader sequence followed by a 34-residue sequence which corresponds to mature nisin except that it contains serines, threonines, and cysteines as precursors of lanthionine, 3-methyllanthionine, and α,β-didehydroamino acid residues. It is believed that the serines and threonines undergo post-translational enzymatic dehydration to dehydroalanine and dehydrobutyrine, followed by the stereospecific addition of cysteine thiol groups to the α,β-didehydroamino acids to form the sulphide cyclic structures. Either before or, more probably, after these modifications, the leader sequence is cleaved off, and the mature nisin is secreted. A similar sequence of post-translational modification is predicted for epidermin and subtilin.

Though nisin was initially reported by Berridge *et al.*[16] to be a complex mixture, h.p.l.c. analysis of the culture broth from a producing *Lactoc. lactis* showed the presence of only one principle component[17] which exhibited an identical h.p.l.c. profile as the major component found in a high potency (37 000 units/mg) commercial preparation. This material has been isolated and its chemical structure determined by 2-D ^{1}H n.m.r. spectroscopy.

Results and Discussion

Resonance Assignments of Amino acid Residues.—500 MHz ^{1}H N.m.r.-spectra of nisin in aqueous and deuteriated dimethyl sulphoxide–trifluoroacetic acid ([$^2H_6$]-DMSO–TFA) solution are shown in Figure 2*a* and *b*. The strategy used for the resonance assignment was a modification of that proposed by Wuthrich[18] for small proteins. Resonances were first assigned to individual types of amino acid residue by observation of relayed scalar connectivities from the backbone amide protons to side-chain aliphatic protons. The HOHAHA experiment, which gives superior water suppression, was used to detect both direct and relayed through-bond connectivities,[19,20] using several spin-locking periods, from 40 to 120 ms. In addition, instead of obtaining HOHAHA spectra at different temperatures to overcome some of the bleaching effect due to solvent preirradiation, a SCUBA pulse[21] was used.

Analysis of the HOHAHA spectrum (τ = 65 ms) of a aqueous solution of nisin (5 mM, pH 2.25) at 303 K revealed 29 out of an expected 30 N*H*–Cα*H* cross-peaks (Figure 3a,b), including the N-terminal lleNH_3^+–Cα*H* cross-peak resolved. The remaining cross-peak was not observed due to bleaching by the solvent preirradiation. Subsequent analysis of the spin systems was performed in several steps, using mainly the HOHAHA spectra obtained with spin-locking periods of 65 and 110 ms.

Examination of the amino acid composition of nisin revealed five unique residues, namely ΔAbu, Pro, Asn, Ser, and Val. The N*H* resonances of Asn and Ser, located at δ 8.55 and δ 8.47 respectively, showed cross-peaks to their Cβ-protons at chemical shifts close to those of the free amino acids, that is at δ 2.86 and δ 3.90 respectively. The unique A_3B_3MPX [or in this case $(A_3)_2MPX$] spin system of Val was readily identified, with cross-peaks along the F1 axis at δ 4.24 (Cα*H*), 2.17 (Cβ*H*), and 1.01 (CγH_3). By examination of the aliphatic region of the HOHAHA spectrum, the ABC_2MX spin system of Pro could be traced; the assignment was confirmed by the characteristic down-field CδH_2 chemical shift at δ 3.50. The CβH_2 were

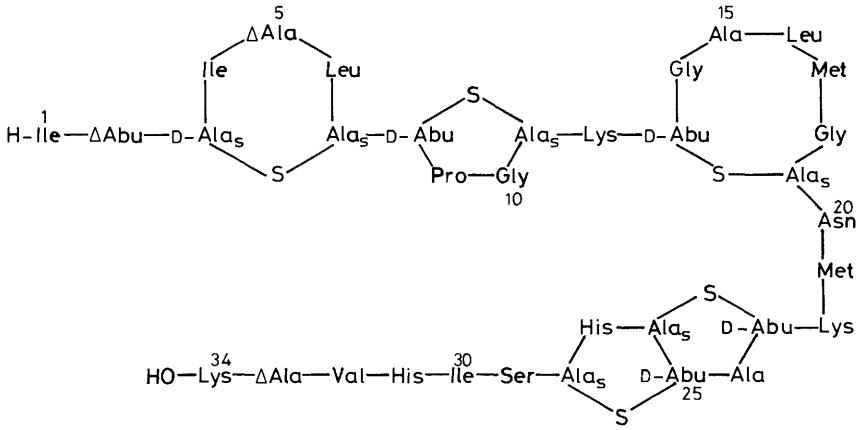


Figure 1. The structure of nisin proposed by Gross and Morell.[13] (Abbreviations: ΔAbu, dehydrobutyrine; ΔAla, dehydroalanine; Ala$_s$, the alanine moiety of lanthionine or 3-methyllanthionine; D-Abu, the 2- aminobutyric acid moiety of 3-methyllanthionine)

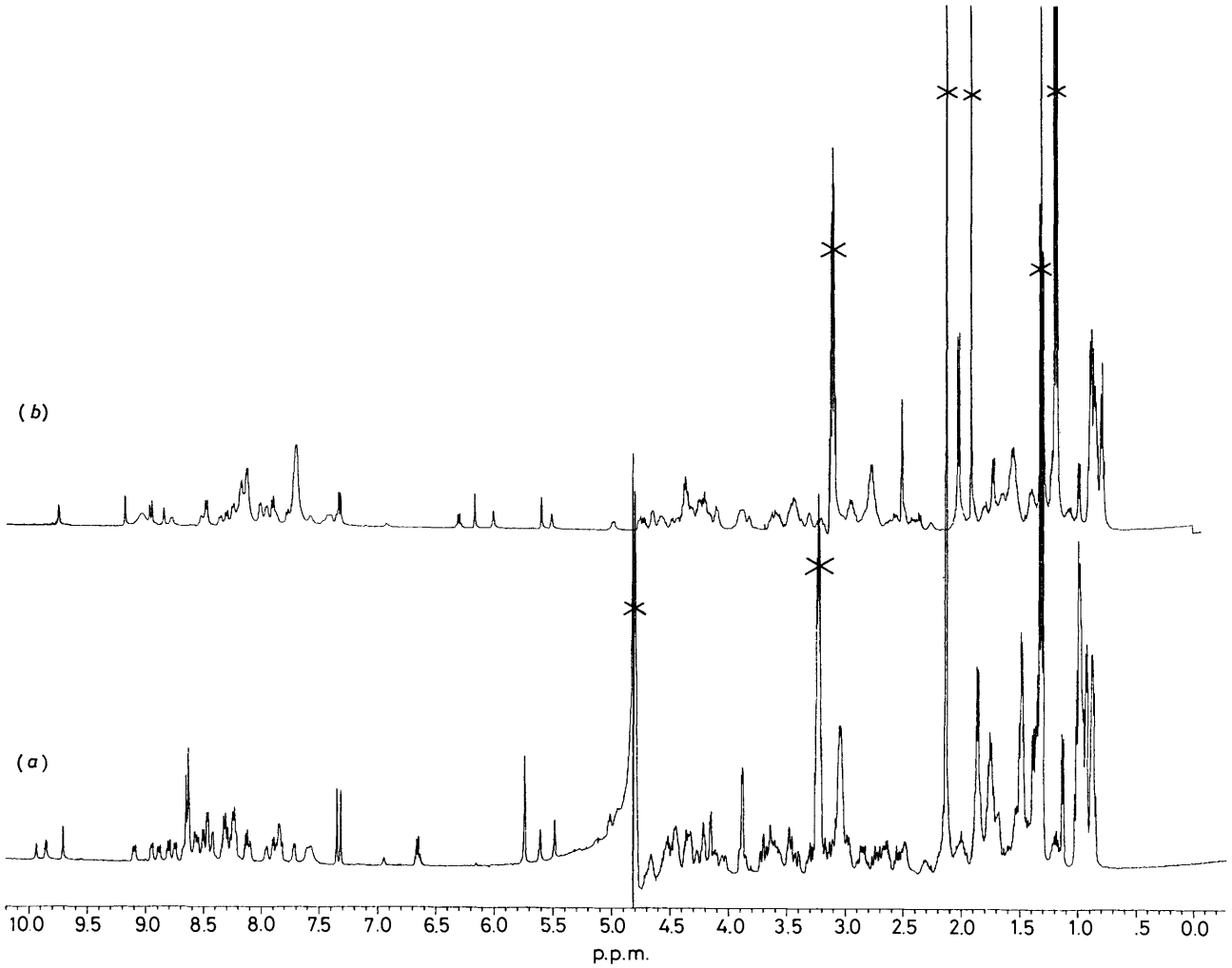


Figure 2. ^{1}H N.m.r.-spectrum (500 MHz) of nisin in (*a*) aqueous sodium phosphate butter (pH 2.25, H_2O,85:D_2O,15); (*b*) [$^2H_6$]-DMSO–TFA at 303 K

differentiated from the CγH_2 by the observation of $\alpha\beta$ and $\gamma\delta$ NOESY cross-peaks.

The readily distinguishable quartet at δ 6.65 can be specifically assigned to the CβH of ΔAbu. A long-range scalar coupling was observed between ΔAbuCβH and the proton resonating at δ 9.93, this therefore being assigned to ΔAbuNH. The vicinal coupling constant of 7.15 Hz observed for ΔAbuCβH, and the chemical shift of the coupled CγH_3 (δ 1.87) provided evidence of a *Z* stereochemistry for the alkenic moiety. This stereochemistry was unambiguously confirmed by the observation of the intraresidue ΔAbuNH–CγH and sequential ΔAbuCβH–Ala$_s$NH (Ala$_s$ is the alanine moiety of lanthionine or 3-methyllanthionine) NOESY cross-peaks.

The CβH of the two ΔAla residues resonated at δ 5.74 (2 H), δ 5.61 (1 H), and δ 5.49 (1 H), the latter two being coupled to each other. Long-range HN–Cα–CβH_2 connectivities observed in the

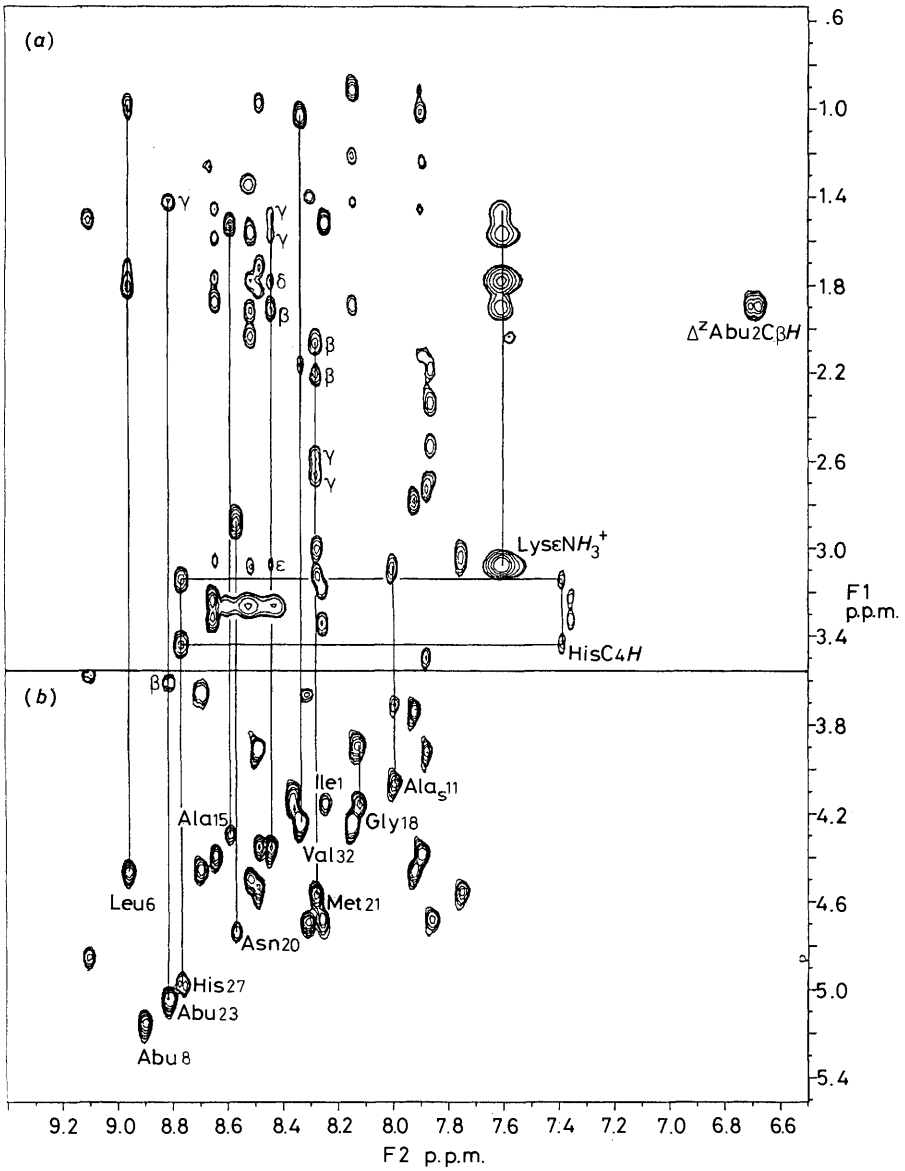


Figure 3. Part of the HOHAHA spectrum of nisin (5 mM) in aqueous sodium phosphate buffer (pH 2.25) at 303 K showing through-bond connectivities between amide and side-chain aliphatic protons. Spin-locking periods of (*a*) 110 ms, and (*b*) 65 ms were utilized. Connectivities of selected amino acid residues are shown

HOHAHA (τ = 110 ms) spectrum, as well as the intraresidue βN dipolar connectivities (NOESY, τ_m = 350 ms) led to delineation of the complete spin system for both ΔAla residues.

The broad peak at δ 7.60 was assigned to the side-chain Cε-NH_3^+ of Lys residues, and showed cross-peaks along the F1 axis of a HOHAHA spectrum for Cε-, Cγ-, Cδ-, and Cβ-methylene protons, as well as for the Cα protons at long spin-locking periods. These cross-peaks when aligned along the F2 axis are associated with cross-peaks which arise from the coherent magnetisation transfer from the individual Lys amide protons to the Cα*H* and side-chain methylene protons of each residue, hence providing complete spin system assignments for each of the three Lys residues. Similarly, assignment of the two His spin systems was confirmed by the corresponding imidazole C4*H*–CβH_2 relayed connectivities (Figure 3*a*). The N*H*–Cα*H* cross-peak of one His residue was found to be bleached out by solvent preirradiation.

Inspection of the HOHAHA spectra at 65 and 110 ms spin-locking periods provided the necessary information for assignment of amino acid residues with long hydrophobic side-chain (Ile, Leu, Met). Only two ABMNPX spin systems were present, both from the methionine residues. The identity of the Met CδH_3 resonances could be determined by the presence of n.O.e. between the CγH_2 and CδH_3 groups. Delineation of the both Leu and Ile spin systems was possible at long spin-locking periods. In the case of Ile1, the NH_3^+–Cα*H* cross-peak was only observed at short spin-locking period (τ = 65 ms). Amino proton-water exchange is expected, thereby causing broadening of the amino proton resonance. The side-chain spin system of Ile1 was identified from the aliphatic region of the HOHAHA spectrum.

The characteristic spin systems of Gly (ABX) and Ala (A_3MX) residues were also readily assigned. The resonance signals of the D-Abu moiety of the 3-methyllanthionine residues could be distinguished by the characteristic chemical shifts of Cβ*H* at ~δ 3.6 and CγH_3 at δ 1.2 to 1.5. The remaining six ABMX spin systems were assigned to the Ala_s moieties of the *meso*-lanthionine and 3-methyllanthionine residues, since the rest of the ABMX systems (Asn, Ser, His) had already been assigned.

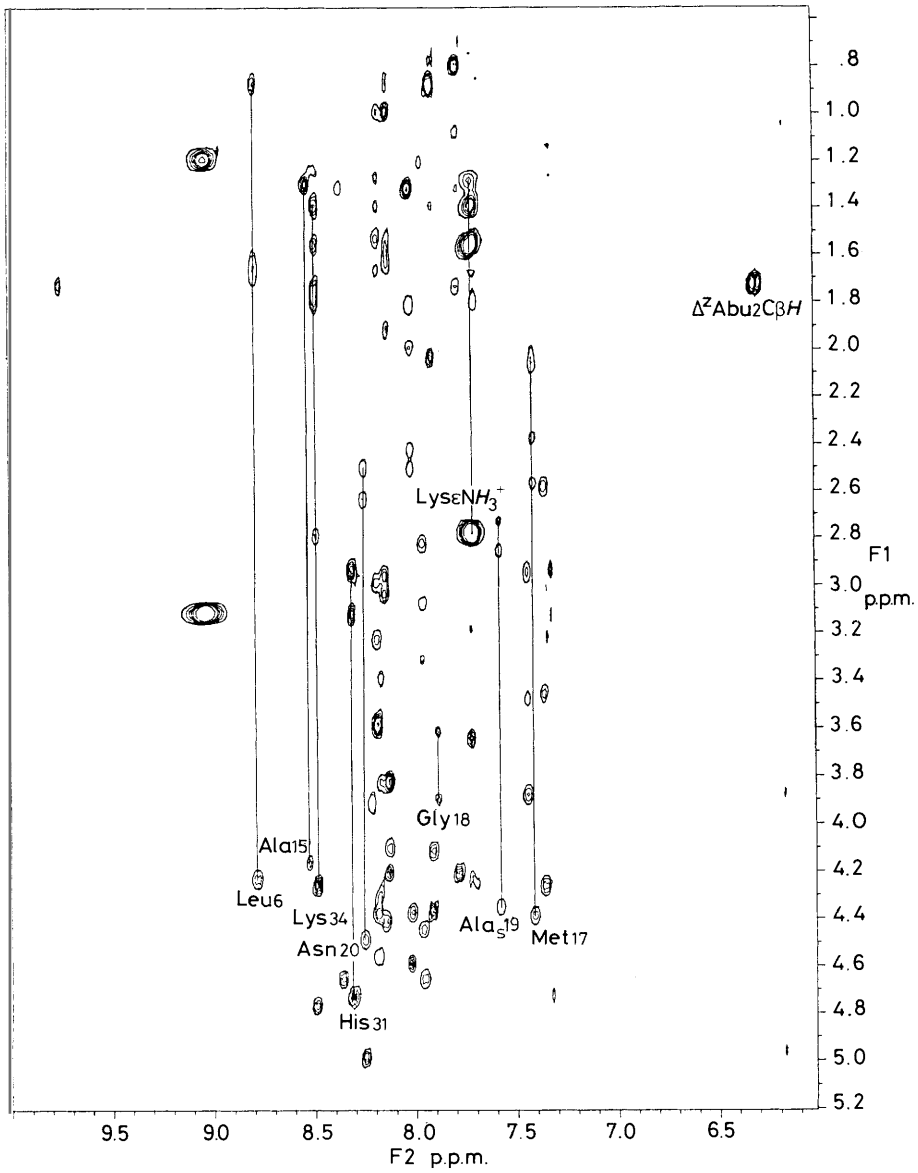


Figure 4. Part of the HOHAHA spectrum (τ = 110 ms) of nisin (2 mM) in [$^2H_6$]-DMSO–TFA at 303 K showing through-bond connectivities between amide and side-chain aliphatic protons. Connectivities of selected amino acid residues are shown

Using the approach described above, complete resonance assignment of spin systems of the constituent amino acids was also achieved with a solution of nisin in [$^2H_6$]-DMSO–TFA (Figure 4). This solvent system was chosen since nisin was found to be unstable in [$^2H_6$]-DMSO; this degradation was greatly retarded, but not entirely eliminated by the addition of TFA. There was no evidence of a similar degradation of nisin in the aqueous solution.

Sequential Resonance Assignments.—The sequential assignment procedure used was based on a search for short-range NOESY cross-peaks between the Cα, Cβ or amide proton of (*i*) residue and the amide proton of adjacent (*i* + 1) residue in the peptide sequence, denoted by αN, βN, and NN respectively.

Some details of the sequential assignment for nisin in aqueous solution will now be discussed. The three assigned low-field amide protons at δ 9.93 (ΔzAbu2), δ 9.71 (ΔAla), and δ 9.71 (ΔAla) together with the α,β-didehydroamino acids Cβ protons provided convenient starting points for the sequential assignment. Beginning with the ΔzAbu2 residue, two interresidue NOESY cross-peaks, CαH (δ 4.19)–ΔzAbu2NH and ΔzAbu2CβH–NH (δ 8.24) were easily identified (Figure 5). Since the CαH (δ 4.19) resonance is part of a $A_3MN(B_3)PTX$ spin system, and the NH (δ 8.24) is scalar coupled to an ABX spin system, these interresidue connectivities led unambiguously to the assignment of Ile1CαH and D-Ala$_s$3NH respectively. The Ala$_s$3NH also showed short-range n.O.e. connectivity to an IleNH. Following the connectivity trajectory of the scalar coupled Cβ protons (δ 5.49 and δ 5.61) of one of the ΔAla residues revealed one interresidue βN cross-peak; the associated amide proton was found to be scalar coupled to a A_3B_3MPQX spin system assigned to a leucine residue. In addition, the αN(Ile,ΔAla) and βN(Ile,ΔAla) NOESY cross-peaks were observed. Thus, a sequence Ile1–ΔzAbu2–D-Ala$_s$3–Ile4–ΔAla5–Leu6 was established, corresponding to the *N*-terminal hexapeptide of the nisin structure proposed by Gross and Morell.

By elimination, the resonance signals at δ 9.71 and 5.74 were assigned specifically to ΔAla33 NH and $C\beta H_2$ respectivity. An interresidue αN(Val,ΔAla) NOESY cross-peak was observed, hence confirming the resonance assignment of ΔAla33 and the dipeptide fragment Val32-ΔAla33.

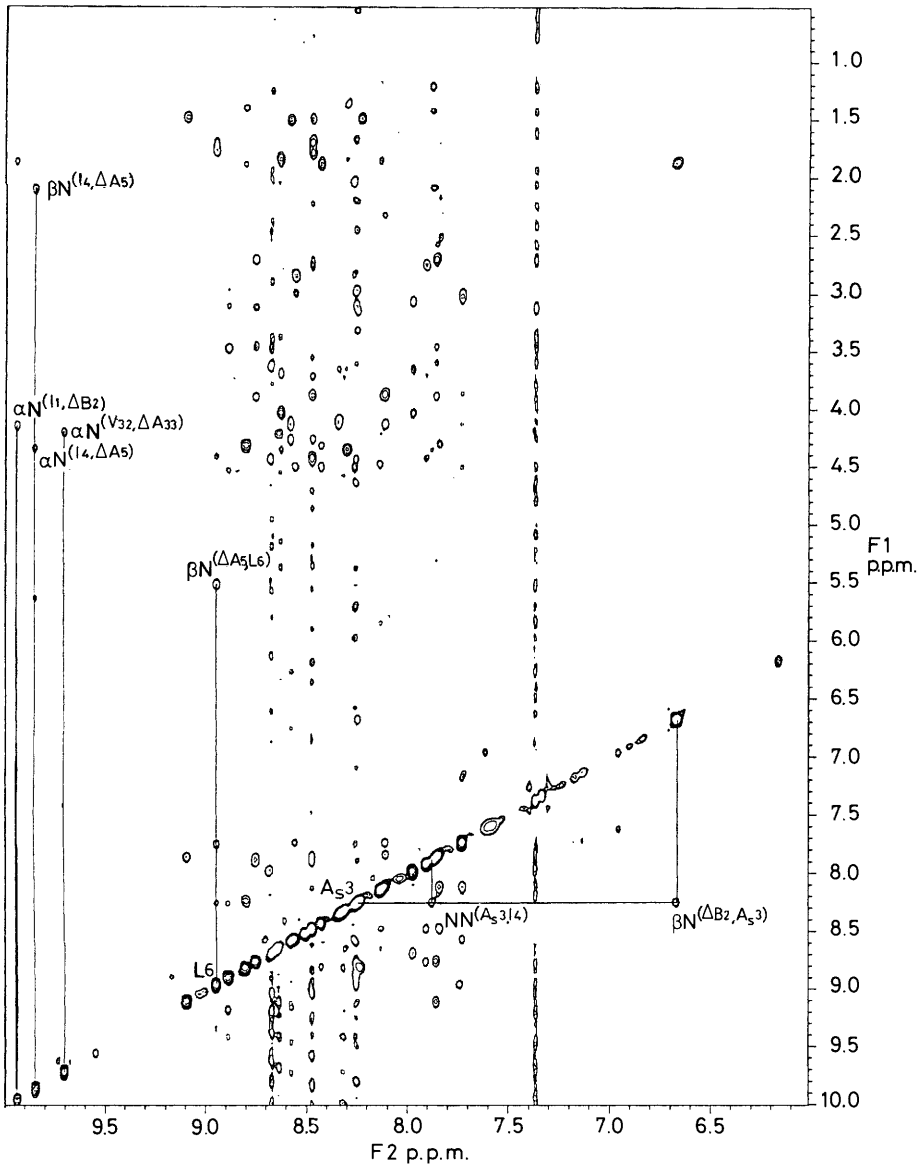


Figure 5. Regions of the NOESY spectrum (τ_m = 350 ms) of nisin in sodium phosphate buffer, used for the identification of sequential αN, βN, and NN NOE connectivities. Selected dipolar connectivities are illustrated. (Abbreviations: ΔB, ΔAbu; ΔA, ΔAla; A_s, Ala_s)

Using this approach, sequential assignment for the rest of the nisin spectrum was readily achieved. The most problematic αN NOESY cross-peaks are the ones under the water signal. These cross-peaks were either bleached out due to water preirradiation or indistinguishable from the N*H*-water exchange cross-peaks when a jump-return pulse was used for water suppression. Under these circumstances, the sequential NN and βN dipolar connectivities together with results from the relayed-NOESY experiment proved useful.

The relayed-NOESY experiment is based on an incoherent magnetisation transfer *via* n.O.e. from the N*H*(i + 1) to Cα-*H*(i), followed by the coherent magnetisation transfer to the scalar coupled N*H*(i), thus giving rise to cross-peaks asymmetrical about the diagonal.[22–24] Figure 6*a* shows the amide proton region of the relayed-NOESY spectrum where both symmetrical cross-peaks due to n.O.e. transfer and asymmetrical cross-peaks due to scalar-relayed n.O.e. transfer are observed. Using the analysis described by Kessler *et al.*,[23] based on asymmetry of cross-peaks about the diagonal, the N*H*(i)–N*H*(i + 1) n.O.e.-*J* connectivities confirmed many of the sequential assignments obtained from the NOESY spectra *via* the αN connectivities. The sequential analysis illustrated in Figure 6*a* demonstrates the ease with which the peptide sequence could be 'read' directly from the relayed-NOESY spectrum. Breaks in the sequential connectivities in the relayed-NOESY spectrum were attributed to the absence either of short-range αN n.O.e. connectivities, or of Cα(ΔAbu2 & ΔAla5,33) or amide(Pro9) protons.

The short-range connectivities observed from the NOESY spectra of nisin in phosphate buffer are shown in Figure 7, and the resonance assignments are summarised in Table 1. Using a similar approach, complete resonance assignment of nisin in [$^2H_6$]-DMSO–TFA was also achieved, and the results are summarised in Figure 8 and Table 2. For the sample in [$^2H_6$]-DMSO–TFA, the Cα*H*, Cα*H* region in the relayed-NOESY spectrum (Figure 6*b*) could be used for confirmation of the sequential assignments. In this region, n.O.e.-*J* cross-peaks derived from the magnetisation transfer Cα*H*(i) ⟶ N*H*(i + 1) ⟶ Cα*H*(i + 1) were observed.

Assignments of Side-Chains of the Lanthionine and 3-*Methyllanthionine Residues.*—The final step in the confirmation

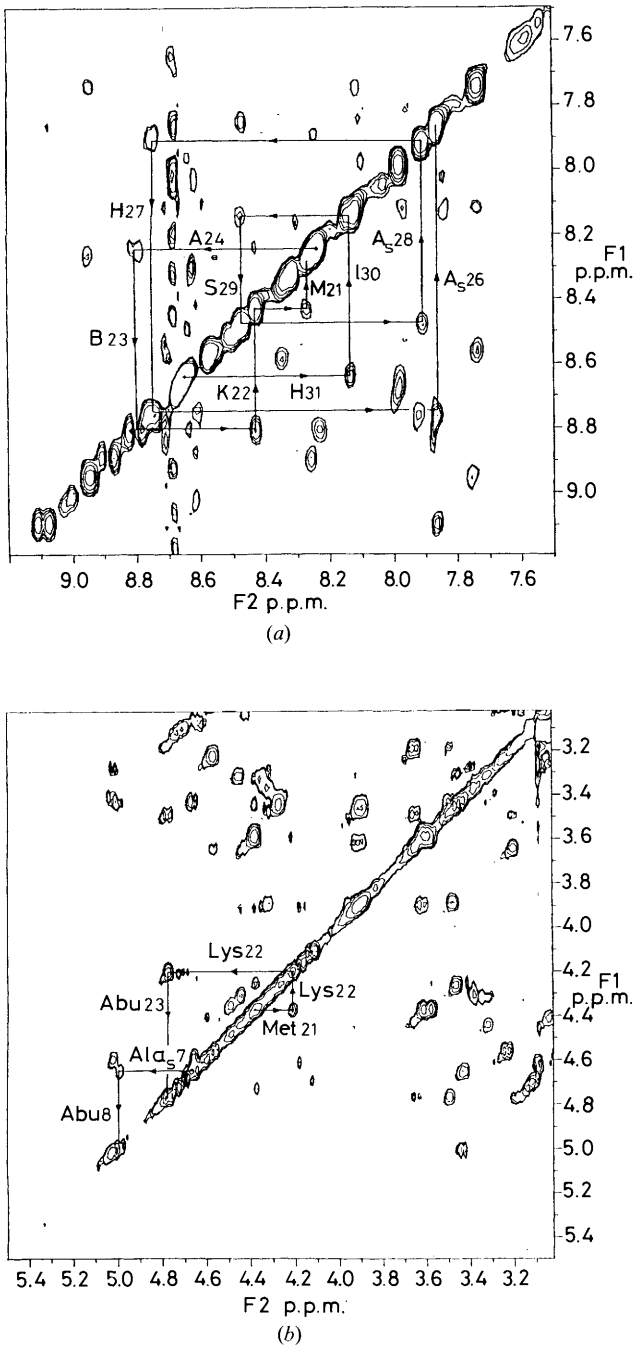


Figure 6. (*a*) N*H*, N*H* region of the relayed-NOESY spectrum of nisin in sodium phosphate buffer showing the asymmetrical n.O.e.-*J* cross-peaks used for the confirmation of sequence-specific assignments. Part of the sequence analysis is shown; break in the sequence is due to absence of A_s26CαH-B25N*H* and B25CαH-A24N*H* NOE connectivities. (Abbreviations: A_s, Ala_s; B, D-Abu). (*b*) CαH, CαH region of the relayed-NOESY spectrum of nisin in [$^2H_6$]-DMSO–TFA showing the asymmetrical n.O.e.-*J* cross-peaks used for sequence-specific assignments. Part of the sequence analysis is shown.

of the structure of nisin is the location of the five sulphide ring systems. From the Gross and Morell nisin structure the Cβ proton/s of D-Ala_s3, D-Abu8, D-Abu13, D-Abu23, and D-Abu25 are expected to exhibit n.O.e.s to the Cβ proton/s of Ala_s7, Ala_s11, Ala_s19, Ala_s26, and Ala_s28 respectively. These ββ NOESY cross-peaks were indeed observed for D-Abu13–Ala_s19, D-Abu23-Ala_s26, and D-Abu25-Ala_s28. The similarity in chemical shifts between D-Ala_s3CβH_2 and Ala_s7CβH_2 meant that any n.O.e. cross-peaks between them were too close to the diagonal to be observed. In such a case, the information available from the relayed-NOESY spectrum proved invaluable; the asymmetrical cross-peak which arose due to the incoherent magnetisation transfer from Ala_s7CβH to D-Ala_s3CβH (n.O.e. connectivity) followed by magnetisation transfer in the relayed step to the scalar coupled D-Ala_s3CαH was clearly distinguishable (Figure 9*a*).

Hence, the occurrences of the D-Ala_s3–Ala_s7, D-Abu13–Ala_s19, D-Abu23–Ala_s26, and D-Abu25–Ala_s28 sulphide ring systems were established. The assignments were confirmed by the observation of medium-range Ala_s7N*H*–D-Ala_s3CβH_2, Ala_s26N*H*–D-Abu23CβH, and Ala_s28N*H*–D-Abu25CβH n.O.e. connectivities (Figure 9*b*). Interestingly, no Ala_s19N*H*–D-Abus13CβHNOESY cross-peak was observed.

Evidence for the D-Abu8–Ala_s11 sulphide linkage was less clear. The small chemical shift separation between D-Abu8CβH (δ 3.61) and Ala_s11C$\beta'H$ (δ 3.70) made it difficult to distinguish the n.O.e.-*J* cross-peak of D-Abu8CβH–Ala_s11CαH (or Ala_s11C$\beta'H$–D-Abu8CαH) from the respective direct intra-residue scalar or dipolar connectivities. In addition, no Ala_s11CβH (δ 3.07)–D-Abu8CαH n.O.e.-*J* cross-peak was observed. The only clear evidence available from the NOESY spectrum (τ_m = 350 ms) is the presence of a long-range Ala_s11N*H*–D-Abu8CβH n.O.e. connectivity.

Secondary Structure of Nisin in Aqueous Solution.—The sequential n.O.e.s which were used for resonance assignment can also provide evidence for regions of regular secondary structure in nisin. The Ser29 to Lys34 segment showed either no or very weak NN n.O.e. connectivities, hence suggesting an extended backbone conformation. The continuous stretch of medium- or short-range sequential αN and NN connectivities from residue Leu16 to Ala24 suggest that this region may adopt a compact rather than an extended conformation. The observed strong Lys22CαH–D-Abu23N*H* NOESY cross-peak, followed by a medium intensity D-Abu23N*H*–Ala24N*H* NOESY cross-peak (τ_m = 100 ms) is indicative of β-turn about residues Lys22–D-Abu23, at the end of this region. The amide bond between D-Abu8 and Pro9 was found to exist in the *trans* configuration since a short-range D-Abu8CαH–Pro9CδH_2 n.O.e. connectivity was observed.

Further work is currently in progress to determine the overall molecular conformation of nisin using distance constraints derived from NOESY experiments.

In conclusion, the complete ^{1}H resonance assignment of nisin are in agreement with and provide strong support for the structure originally proposed by Gross and Morell, and will provide the basis for conformational analysis.

Experimental

Nisin (37 000 units/mg) was obtained from Aplin & Barrett, Beaminster, U.K., and purified by h.p.l.c.[17] to remove the previously reported major contaminant (*des*-ΔAla33-Lys34; Val32-NH_2)Nisin.[14] The h.p.l.c.-purified nisin, after dialysis and lyophilisation generally contains <1% triethylammonium acetate (buffer used in the h.p.l.c. solvent system).

All n.m.r. measurements were carried out on a Bruker AM 500 spectrometer operating at 500.13 MHz. All 2-D spectra were acquired and processed in the phase-sensitive mode using time proportional phase incrementation methods. Spectra were recorded with 2—5 mM solutions of nisin in 85%H_2O–15%D_2O (0.1M sodium phosphate, pH 2.25) and in [$^2H_6$]-DMSO (0.5 ml)–TFA (10 μl) at 303 K, and referenced to sodium

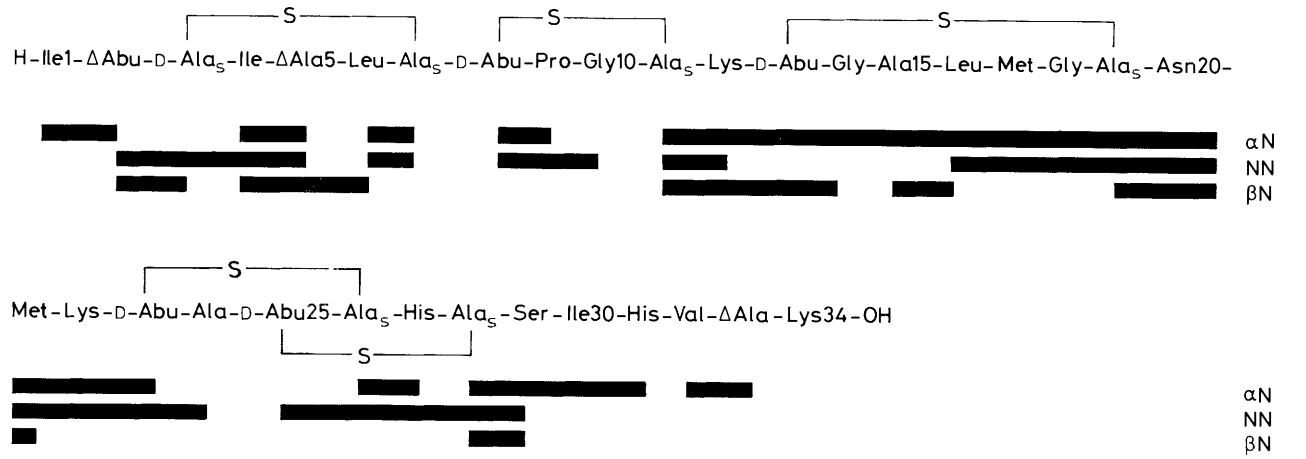


Figure 7. Summary of the sequential resonance assignments of nisin in phosphate buffer. The solid bars indicate the presence of n.O.e. connectivity between residues

Table 1. ^{1}H N.m.r. (500 MHz) data of nisin (5 mM) in aqueous solution (100 mM sodium phosphate buffer, pH 2.25, H_2O,85:D_2O,15) at 303 K

	δ (p.p.m.)					
	N*H*	Cα*H*	Cβ*H*	Cγ*H*	Cδ*H*	Cε*H*
Ile1	8.22	4.19	2.16	1.37, 1.62; 1.16	0.95	
Δ^zAbu2	9.93		6.65	1.87		
D-Ala$_S$3	8.24	4.68	3.18, 3.32			
Ile4	7.89	4.39	2.12	1.22, 1.45; 0.99	0.88	
ΔAla5 S	9.85		5.49, 5.61			
Leu6	8.94	4.47	1.78	1.69	0.94, 1.00	
Ala$_S$7	8.27	4.60	2.99, 3.11			
D-Abu8	8.88	5.16	3.61	1.40		
Pro9 S		4.48	1.77, 2.50	2.22, 1.90	3.50	
Gly10	8.70	3.66, 4.45				
Ala$_S$11	7.96	4.07	3.07, 3.70			
Lys12	8.64	4.39	1.86	1.44, 1.54	1.75	3.05
	7.60	(NεH_3^+)				
D-Abu13	8.29	4.60	3.66	1.37		
Gly14	8.35	4.12, 4.16				
Ala15	8.57	4.30	1.51			
Leu16 S	8.47	4.37	1.78	1.70	0.95, 0.98	
Met17	7.86	4.68	2.15, 2.32	2.53, 2.70	2.18	
Gly18	8.12	3.90, 4.18				
Ala$_S$19	7.72	4.55	3.01, 3.04			
Asn20	8.55	4.75	2.86			
Met21	8.27	4.57	2.04, 2.17	2.56, 2.64	2.17	
Lys22	8.43	4.37	1.90	1.48, 1.55	1.77	3.06
	7.60	(NεH_3^+)				
D-Abu23	8.80	5.05	3.60	1.40		
Ala24 S	8.24	4.72	1.50			
D-Abu25	9.09	4.85	3.57	1.48		
Ala$_S$26	7.87	3.93	2.72, 3.48			
His27 S	8.74	4.98	3.13, 3.42	8.65 (*H*2),	7.35 (*H*4)	
Ala$_S$28	7.90	4.47	2.75, 3.73			
Ser29	8.47	4.54	3.90			
Ile30	8.13	4.24	1.87	1.20, 1.38;	0.90	
His31	8.65	4.85	3.21, 3.31	8.63 (*H*2),	7.32 (*H*4)	
Val32	8.32	4.24	2.17	1.01		
ΔAla33	9.71		5.74			
Lys34	8.50	4.51	1.91, 2.01	1.54	1.77	3.07
	7.60	(NεH_3^+)				

3-(trimethylsilyl)propane-1-sulphonate in the case of the aqueous sample and tetramethylsilane for the [$^2H_6$]-DMSO samples.

For identification of spin-systems and sequential assignments, HOHAHA, NOESY, and relayed-NOESY spectra were acquired. Unless otherwise stated, the solvent resonance was suppressed using low-power (35—45 dB) preirradiation.

The H_2O resonance in the HOHAHA experiments of nisin in phosphate buffer was suppressed using the SCUBA-pulse sequence[21] (Presaturation–τ–180°$_{comp}$–τ, where τ = 20 ms,

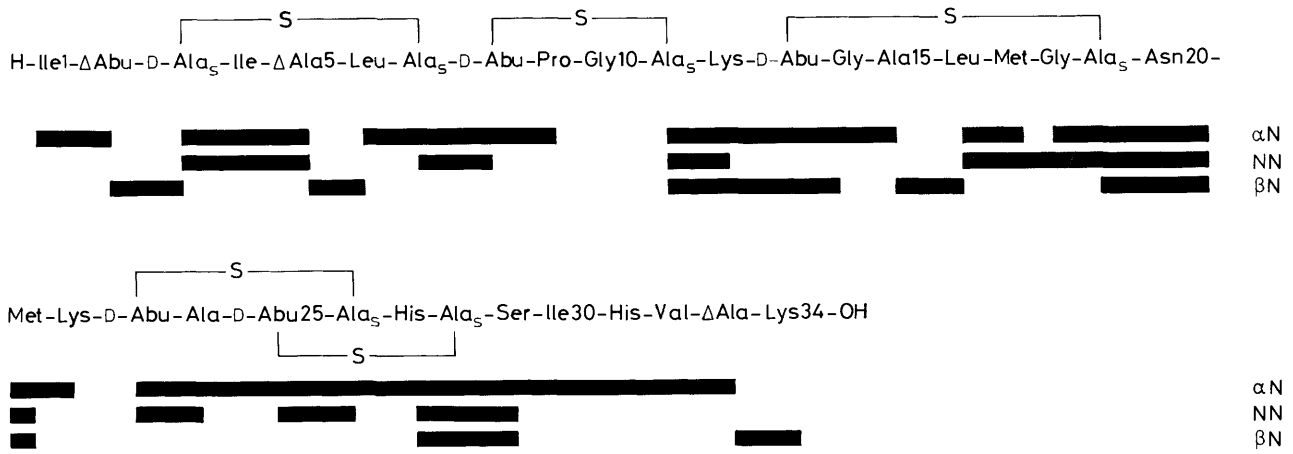


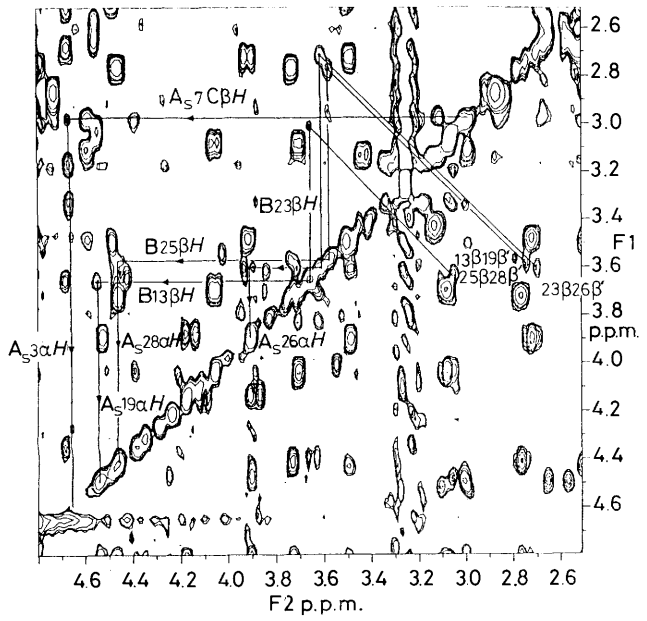
Figure 8. Summary of the sequential resonance assignments of nisin in [$^2H_6$]-DMSO–TFA. The solid bars indicate the presence of n.O.e. connectivity between residues

Table 2. ^{1}H N.m.r. (500 MHz) data of nisin (2 mM) in [$^2H_6$]-DMSO–TFA at 303 K

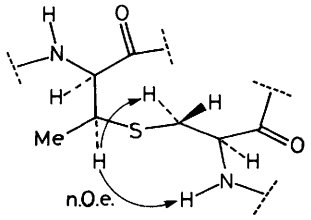
	δ (p.p.m.)					
	N*H*	Cα*H*	Cβ*H*	Cγ*H*	Cδ*H*	Cε*H*
Ile1	8.11	3.80	1.85	1.18, 1.53; 1.72	0.88	
Δ^zAbu2	9.74		6.29	1.72		
D-Ala$_s$3	8.12	4.39	2.94, 3.02			
Ile4	7.90	4.09	2.01	1.03, 1.37; 0.88	0.82	
ΔAla5 S	8.84		5.50, 6.00			
Leu6	8.77	4.21	1.65	1.58	0.84, 0.88	
Ala$_s$7	7.93	4.64	2.81, 3.07			
D-Abu8	8.23	4.98	3.41	1.16		
Pro9 S		4.31	2.25	1.80, 1.95	3.29	
Gly10						
Ala$_s$11	7.42	3.86	2.94, 3.46			
Lys12	8.16	4.28	1.51, 1.62	1.23, 1.35	1.49	2.75
	7.70	(NεH_3^+)				
D-Abu13	7.94	4.41	3.28	1.17		
Gly14	8.19	3.90				
Ala15	8.51	4.16	1.29			
Leu16 S	8.10	4.09	1.60	1.50	0.84	
Met17	7.40	4.36	2.00, 2.04	2.35, 2.54	2.04	
Gly18	7.87	3.59, 3.88				
Ala$_s$19	7.56	4.32	2.71, 2.83			
Asn20	8.23	4.48	2.48, 2.62			
Met21	8.00	4.35	1.77, 1.96	2.39, 2.47	2.03	
Lys22	8.10	4.17	1.70	1.25, 1.38	1.58	2.73
	7.70	(NεH_3^+)				
D-Abu23	8.48	4.75	3.48	1.18		
Ala24 S	8.00	4.58	1.30			
D-Abu25	8.34	4.62	3.38	1.29		
Ala$_s$26	7.70	3.63	2.80, 3.18			
His27 S	8.17	4.54	2.97, 3.22	8.97 (*H*2),	7.33 (*H*4)	
Ala$_s$28	7.34	4.24	2.57, 3.44			
Ser29	8.17	4.35	3.54, 3.58			
Ile30	7.77	4.20	1.70	1.03, 1.35; 0.86	0.79	
His31	8.30	4.72	2.90, 3.10	8.94 (*H*2),	7.30 (*H*4)	
Val32	7.90	4.34	2.00	0.84, 0.89		
ΔAla33	9.17		5.60, 6.18			
Lys34	8.48	4.22	1.67, 1.75	1.36	1.51	2.76
	7.70	(NεH_3^+)				

$180°_{comp}$ denotes a composite pulse) to allow detection of proton resonances 'under' the water resonance. The MLEV-17 mixing sequence (durations 50—120 ms) was used, with a spin locking field in the range 8—15 kHz.

For the NOESY spectra, mixing times of 100, 200, 300, 350, and 400 ms were used. When quantitative n.O.e.s were required for the aqueous sample, the final 90° observe pulse in the sequence was replaced by the jump-and-return sequence[25]



(*a*)



(*b*)

Figure 9. (*a*) High-field region of the relayed-NOESY spectrum of nisin in sodium phosphate buffer used for the identification of lanthionine and 3-methyllanthionine side-chain n.O.e. connectivities. (Abbreviations: A_s, Ala_s; B, D-Abu.) (*b*) Schematic diagram of the observed side-chain n.O.e. connectivities within a 3-methyllanthionine residue

$(90°_{\chi}–\tau–90°_{-\chi})$ with the carrier placed at the position of the solvent and a value of 125 μs for τ. This approach is taken to avoid attenuation of the amide resonance intensities due to amide proton-water exchange.

The relayed-NOESY experiment was composed of a standard NOESY pulse sequence where incoherent magnetisation was transferred during τ_m, followed by a spin-lock coherent magnetisation transfer using the MLEV-17 pulse sequence. The mixing period for the incoherent transfer was 350 ms, and that for the coherent transfer was 40 ms (optimising for *J* of 6—7 Hz).

For the majority of the 2-D spectra, 400—500 t_1 increments were collected, each with a 2 K data points over a spectral width of 6 kHz in both dimensions.

All data were processed in the phase-sensitive mode using either a gaussian window function (GB = GB1 = 0.12 to 0.15, LB = LB1 = −10 to −15 Hz) or a sine-bell square function with π/6 phase shift, together with zero-filling in F1 to give a final 1 K × 1 K data matrix.

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