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NMR studies of lantibiotics

Assignment of the ^{1}H-NMR spectrum of nisin and identification of interresidual contacts

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Nisin is a 34 residue long antimicrobial polypeptide, which contains unusual α,β-unsaturated amino acids as well as lanthionines. By making use of 2D-NMR methods, the complete ^{1}H-NMR spectrum of the polypeptide could be assigned. The NMR data indicate that the molecule adopts a well-defined three-dimensional structure.

Lantibiotic; Nisin; NMR, 2D-

1. INTRODUCTION

Nisin belongs to a class of antimicrobial polypeptides which are known as lantibiotics [1]. These polypeptides are characterized by their cationic properties and the high content of unusual amino acids, such as lanthionine, β-methyllanthionine, dehydroalanine and β-methyldehydroalanine.

All lantiobiotics which have been isolated thus far, show antimicrobial activity against Gram-positive bacteria. However, antibiotic activities against other cells and/or organisms have been reported as well [1–3].

The most prominent lantibiotic is nisin. This polypeptide is 34 amino acids long (M_r 3500) and contains five rings, closed by thio-ether bridged lanthionines. Besides, nisin contains three α,β unsaturated amino acids (fig.1).

Nisin is an important food preservative and a fundamental insight into its structure/function relationship is of major importance for the rational design of new lantibiotics with novel structures and functions. Therefore we have embarked on a systematic NMR study of nisin and related bacteriocins, aiming to unravel their spatial structure and molecular mechanisms related to their biological function.

In this paper we describe the application of two-dimensional NMR techniques which have been employed to arrive at a complete assignment of the ^{1}H NMR spectrum of nisin.

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Abbreviations: 2D-NMR, two-dimensional nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlated spectroscopy; DQF-COSY, double-quantum filtered correlated spectroscopy; TPPI, time proportional phase incrementation

2. MATERIALS AND METHODS

Nisin was purchased from Koch-Light Ltd. (lot no.00988E; spec. act. 37000 U/mg).

NMR experiments were performed both at 400 and 600 MHz on Bruker AM 400 and AM 600 spectrometers, which were interfaced to Aspect 3000 computers.

The two-dimensional ^{1}H-NMR spectra were recorded both at 7 and 25°C. Three different NMR techniques were employed at both temperatures, viz. MLEV-17 TOCSY [4] with mixing

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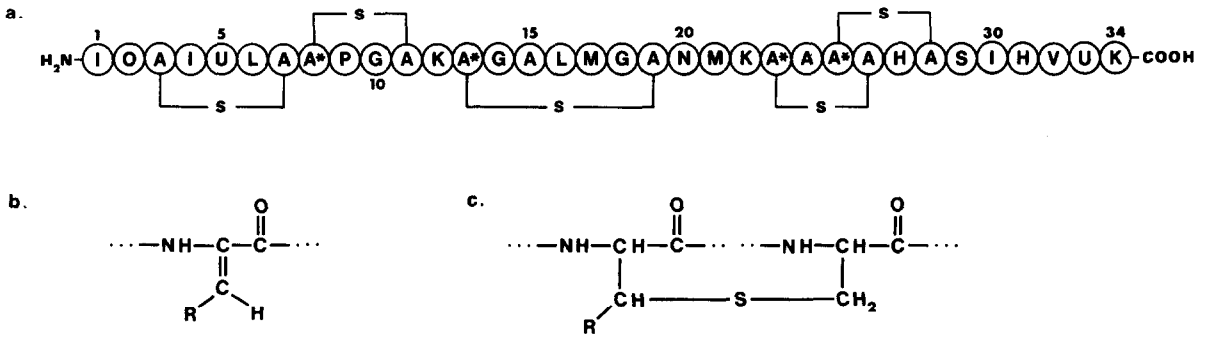


Fig.1. (a) The primary structure of nisin. The unusual amino acids occurring in nisin are indicated by the following one-letter code (the corresponding chemical structures are drawn in b and c): U, dehydroalanine (in b: R = H); O, β-methyldehydroalanine (in b: R = CH_3); A-S-A, lanthionine (in c: R = H); A*-S-A, β-methyllanthionine (in c: R = CH_3).

times varying between 30 and 60 ms, NOESY [5] with mixing times ranging from 150 to 500 ms, and DQF-COSY [6,7].

The experiments were carried out with samples containing 5–10 mM nisin at pH 3.5. For one DQF-COSY experiment the polypeptide was dissolved in 99.9% ^2H_2O; all other spectra were recorded with 90% H_2O/10% ^2H_2O solutions.

The solvent resonance was suppressed by continuous irradiation during the relaxation delays and in the NOESY experiments also during the mixing times. The carrier frequency was placed at the water resonance; phase sensitive spectra were obtained by making use of the TPPI method [6,8]. Data manipulation consisted of sine-bell window multiplication and zero filling prior to 2D Fourier transformation. The final digital resolution was 3.49 Hz/pt for the TOCSY and NOESY spectra and 0.98 Hz/pt for the DQF-COSY spectra.

3. RESULTS AND DISCUSSION

Sequence specific assignment of the resonances is a prerequisite for the elucidation of the spatial structure of the nisin molecule by NMR.

In combination with COSY spectra, the TOCSY spectra allowed identification of all amino acid patterns. The TOCSY spectra, recorded at several mixing times, were used to delineate successively direct, single relayed and multiple relayed through bond connectivities, which enabled assignment of the spin systems of long side chains, such as lysine, isoleucine and methionine. As an example the assignments of methionine (M_{17}) are discussed. This pattern can be unravelled as follows: starting at the $N^{\alpha}H$-$C^{\alpha}H$ cross peak (at 7.77–4.61 ppm) and going to the 'high-field' region, four other cross peaks of the side chain protons can be seen in the TOCSY spectrum (fig.2a). These peaks are also present at the same height in the $C^{\alpha}H$-aliphatic region (fig.2b). In the latter region of the COSY spectrum (not shown) only the two $C^{\alpha}H$-$C^{\beta}H$ cross peaks are present at 4.61–2.26 and 4.61–2.10 ppm. Thus the two peaks present in the TOCSY spectrum at 4.61–2.59 and 4.61–2.42 ppm represent the $C^{\alpha}H$-$C^{\gamma}H$ connectivities of this methionine.

The sequential assignment was performed using through space (≤5 Å) NOE connectivities involving the $N^{\alpha}H$, $C^{\alpha}H$, $C^{\beta}H$, $C^{\gamma}H$ and for proline also the $C^{\delta}H$ protons. In fig.3 a NOESY spectrum is presented, which shows the amide-aliphatic (fig.3a) and amide-amide (fig.3b) regions. All assignments could be brought into correspondence with the primary structure. In fig.4 a summary of the short range NOEs involving $N^{\alpha}H$, $C^{\alpha}H$, $C^{\beta}H$ and $C^{\gamma}H$ protons as well as the $C^{\delta}H$ protons of proline is presented.

Nisin poses some extra problems compared with conventional polypeptides when it comes to the interpretation of the NMR spectra. The amino acid residues dehydroalanine (O_2) and β-methyllanthionine (U_5 and U_{33}) have no $C^{\alpha}H$ protons. Together with proline (P_9), which lacks the $N^{\alpha}H$ proton, these amino acids will not show $N^{\alpha}H$-$C^{\alpha}H$ cross peaks in the 'fingerprint' region of the 2D spectra. However, the $N^{\alpha}H$ protons of O_2, U_5 and U_{33} were easily discernible in the 1D spectrum as singlets at about 10 ppm. The $C^{\beta}H$ proton of O_2 gave rise to a quartet (as expected) at 6.63 ppm, whereas the $C^{\beta}H$ protons of U_5 and U_{33} were obtained around 5.6 ppm, which is characteristic for olefinic protons. Thus, the combination of their unique chemical shifts, their fine structure and several specific NOEs, e.g. intra- and interresidual

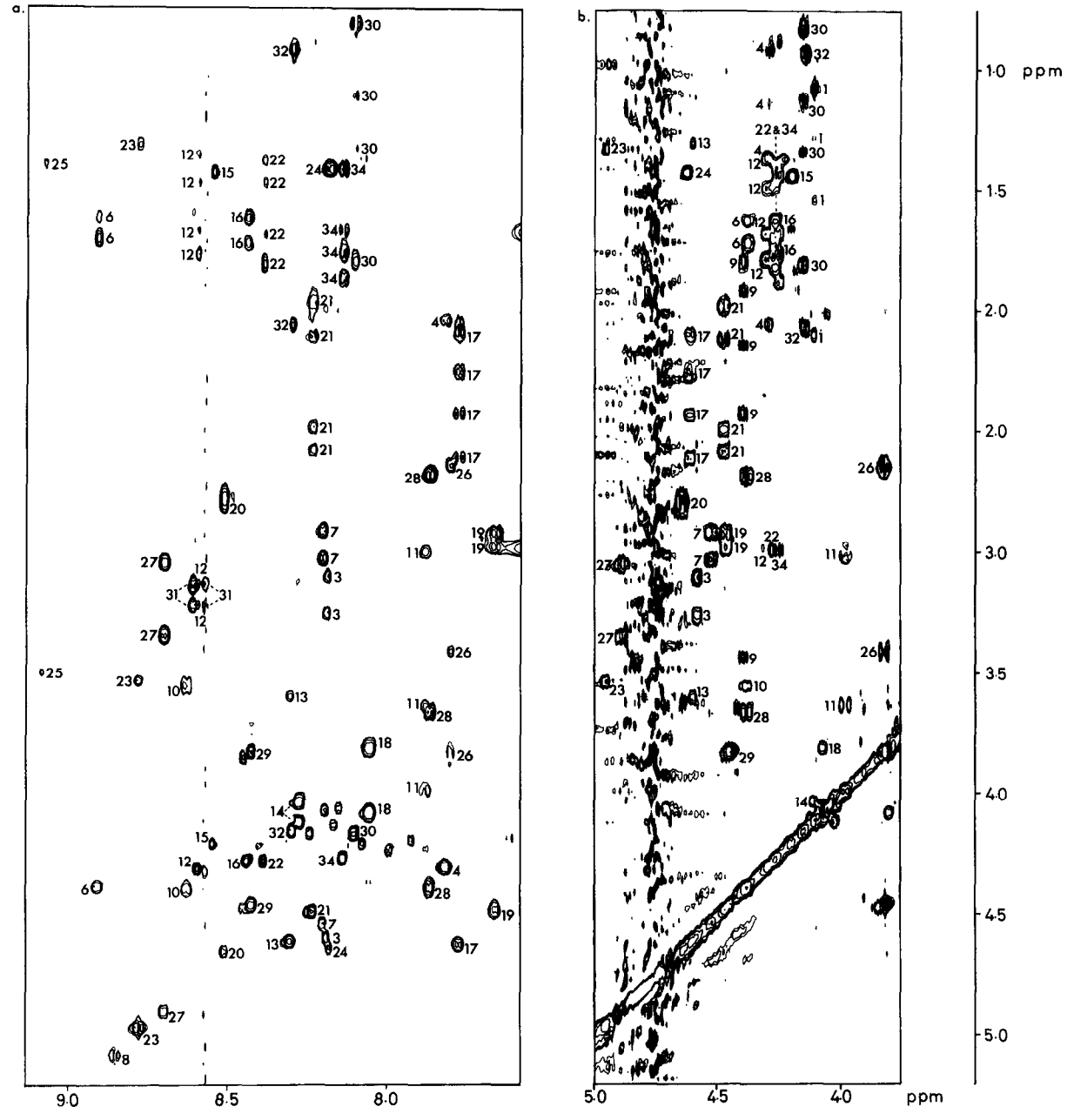


Fig.2. TOCSY spectrum of the $N^{\alpha}H$-$C^{\alpha}H$ region (a) and the $C^{\alpha}H$-aliphatic region (b). This spectrum was recorded at 25°C with a mixing time of 60 ms. The cross peaks of direct and multiple through bond connectivities are indicated by the residue numbers of the amino acids.

$N^{\alpha}H$-$C^{\beta}H$ cross peaks, allowed the unambiguous assignment of all resonances of these residues.

The spin systems of the alanine parts of the lanthionines gave rise to an 'AMX' pattern, with non-degenerated chemical shifts of the $C^{\beta}H$ protons, similar to asparagine and aspartate patterns. The $C^{\alpha}H$-$C^{\beta}H$ cross peaks arose in a region which is usually unoccupied in the 2D spectra of 'common'

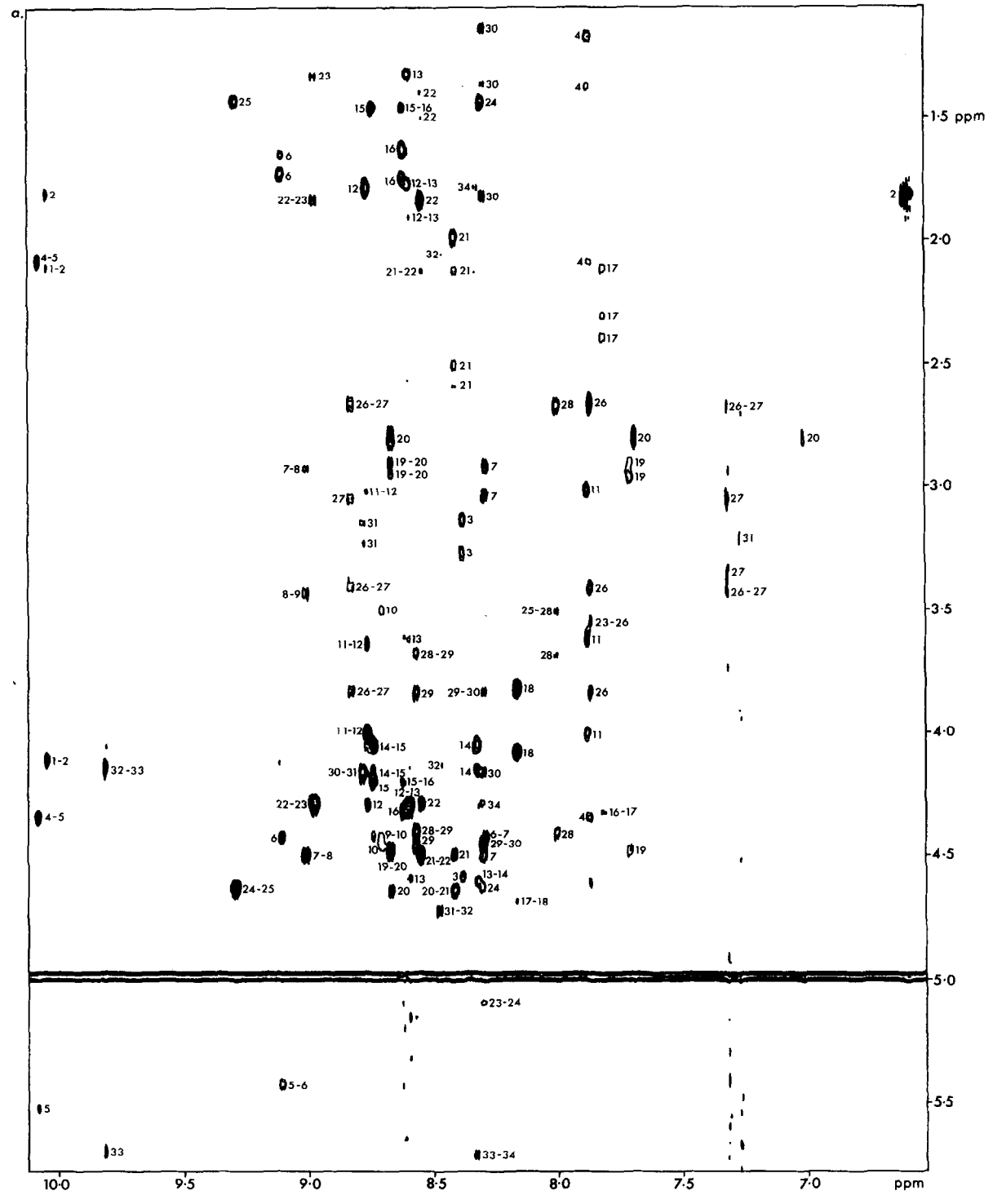
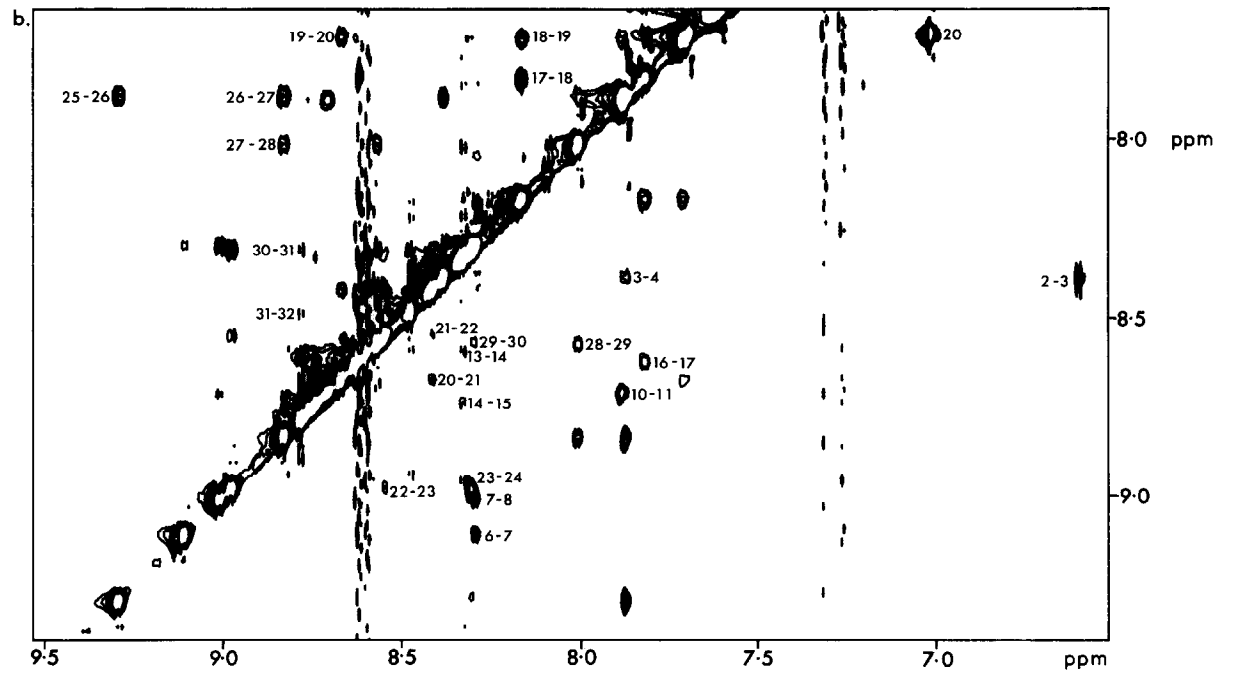


Fig.3. NOESY spectrum of the $N^{\alpha}H$-aliphatic region (a) and the $N^{\alpha}H$-$N^{\alpha}H$ region (b), recorded at 7°C, with a mixing time of 230 ms. Intraresidue connectivities are indicated by single numbers; double numbers represent the interresidue connectivities. The numbers correspond to the residue numbers of the amino acids.



amino acids: viz. the area bounded by 3.8–4.6 ppm ($C^{\alpha}H$) and 2.7–3.7 ppm ($C^{\beta}H$). The spin systems of the β-methylalanine parts of the β-methyllanthionines were also very particular and exhibited $C^{\beta}H$-$C^{\gamma}H$ cross peaks in the area bounded by 3.5–3.6 ppm ($C^{\beta}H$) and 1.3–1.5 ppm ($C^{\gamma}H$). Combined with the sequential information of the NOESY spectra, the resonances of all of the unusual amino acids could be assigned without ambiguity.

In the COSY and TOCSY spectra some cross peaks remained unidentified after the assignment of all nisin protons. These peaks are probably due to impurities, because the areas of these peaks are much smaller than the areas of the nisin proton peaks. Judging from the 1D spectrum (not shown) these impurities amount to 10% or less. Preliminary analysis of the NMR spectra of HPLC purified fractions have indicated that these impurities consist of nisin molecules which lack one

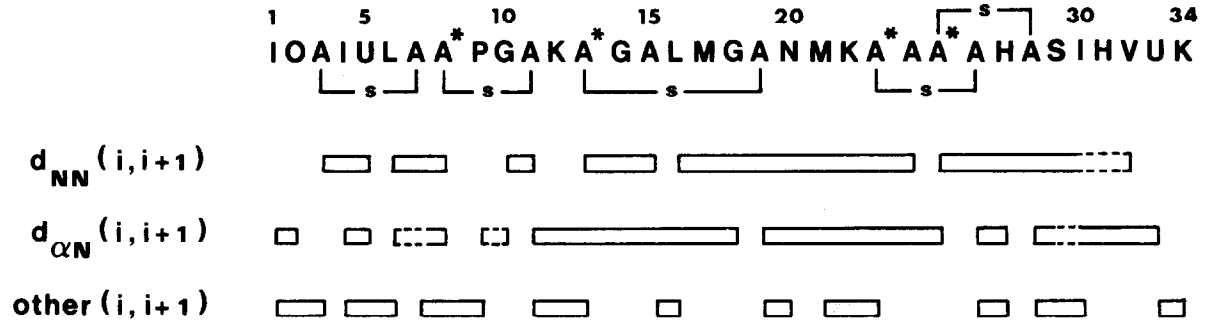


Fig.4. Summary of the sequential resonance assignments at 7°C. The different types of connectivities are indicated by blocks (the term 'other' designates the sequential $N^{\alpha}H$-$C^{\delta}H$ connectivity of the proline and the $C^{\beta}H$-$N^{\alpha}H$ and $C^{\gamma}H$-$N^{\alpha}H$ connectivities for all other residues). Sequential resonance assignments, which could not be established with certainty because of overlapping peaks, are indicated by broken lines.

Table 1

Proton resonance assignments of nisin at 7°C and pH 3.5

Residue	Chemical shifts (ppm)[a]			
	$N^{\alpha}H$	$C^{\alpha}H$	$C^{\beta}H$	Others
I1	–	4.15	2.14	$C^{\gamma}H$ 1.57, 1.31; $C^{\gamma}H_3$ 1.12; $C^{\delta}H$ 0.99
O2	10.08		6.63	$C^{\gamma}H_3$ 1.85
A3	8.42	4.61	3.31, 3.17	
I4	7.91	4.38	2.11	$C^{\gamma}H$ 1.40, 1.19; $C^{\gamma}H_3$ 0.95; $C^{\delta}H$ 0.86
U5	10.11		5.55, 5.45	
L6	9.14	4.45	1.76, 1.76	$C^{\gamma}H$ 1.69; $C^{\delta}H_3$ 0.94, 0.94
A7	8.33	4.52	3.05, 2.94	
A*8	9.04	5.16	3.62	$C^{\gamma}H_3$ 1.32
P9		4.46	2.50, 1.82	$C^{\gamma}H$ 1.97, 2.20; $C^{\delta}H$ 3.46, 3.46
G10	8.74	4.48, 3.53		
A11	7.92	4.04	3.66, 3.03	
K12	8.80	4.32	1.82, 1.73	$C^{\gamma}H$ 1.39, 1.39; $C^{\delta}H$ 1.54, 1.54; $C^{\epsilon}H$ 3.02, 3.02; $N^{\zeta}H_3$ 7.65
A*13	8.63	4.62	3.67	$C^{\gamma}H_3$ 1.36
G14	8.35	4.18, 4.08		
A15	8.77	4.24	1.49	
L16	8.66	4.36	1.78, 1.78	$C^{\gamma}H$ 1.67; $C^{\delta}H_3$ 0.91, 0.91
M17	7.85	4.71	2.34, 2.13	$C^{\gamma}H$ 2.64, 2.43; $C^{\epsilon}H_3$ 2.09/2.13[b]
G18	8.20	4.11, 3.85		
A19	7.74	4.51	3.01, 2.93	
N20	8.70	4.66	2.86, 2.82	$N^{\gamma}H_2$ 7.72, 7.05
M21	8.45	4.52	2.16, 2.01	$C^{\gamma}H$ 2.63, 2.53; $C^{\epsilon}H_3$ 2.13/2.09[b]
K22	8.58	4.31	1.87, 1.74	$C^{\gamma}H$ 1.45, 1.45; $C^{\delta}H$ 1.53, 1.53; $C^{\epsilon}H$ 3.02, 3.02; $N^{\zeta}H_3$ 7.64
A*23	9.01	5.13	3.58	$C^{\gamma}H_3$ 1.37
A24	8.34	4.65	1.46	
A*25	9.32	4.77	3.55	$C^{\gamma}H_3$ 1.47
A26	7.90	3.86	3.44, 2.70	
H27	8.86	–[c]	3.38, 3.08	$C^{\epsilon 1}H$ 7.35; $C\delta^2H$ 8.65
A28	8.04	4.44	3.72, 2.71	
S29	8.60	4.48	3.86, 3.86	
I30	8.33	4.19	1.86	$C^{\gamma}H$ 1.40, 1.18; $C^{\gamma}H_3$ 0.87; $C^{\delta}H$ 0.86
H31	8.82	4.78	3.23, 3.18	$C^{\epsilon 1}H$ 7.30; $C^{\delta 2}H$ 8.62
V32	8.50	4.18	2.09	$C^{\gamma}H_3$ 0.97, 0.97
U33	9.84		5.73, 5.73	
K34	8.34	4.32	1.85, 1.74	$C^{\gamma}H$ 1.37, 1.37; $C^{\delta}H$ 1.48, 1.48; $C^{\epsilon}H$ 3.02, 3.02; $N^{\zeta}H_3$ 7.65

[a] Chemical shifts are reported relative to 4,4-dimethyl-4-silapentane-1-sulfonate, downfield shifts are defined positive

[b] It was not possible to determine which of the $C^{\epsilon}H_3$ peaks belong to which methionine

[c] The $C^{\alpha}H$ peak of H27 is irradiated, together with water (at 5 ppm)

Table 2

Long range contacts in the NOESY spectra of nisin[a]

Interresidual contact	Protons involved
I4-P9	C^{α}-$C^{\beta 2}H$
I4-P9	$C^{\alpha}H$-$C^{\gamma 1}H$
A7-P9	$N^{\alpha}H$-$C^{\delta}H$
A7-P9	$C^{\beta 1}H$-$C^{\delta}H$
A7-P9	$C^{\beta 2}H$-$C^{\delta}H$
G10-L16	$N^{\alpha}H$-$C^{\gamma}H$
G10-L16	$N^{\alpha}H$-$C^{\delta}H$
A15-N20	$C^{\alpha}H$-$N^{\alpha}H$
A15-N20	$N^{\alpha}H$-$C^{\beta}H$
A15-N20	$C^{\beta}H$-$N^{\gamma}H_2$
M17-M21	$C^{\beta}H$-$C^{\delta}H$
M17-M21	$C^{\gamma}H$-$C^{\delta}H$
M17-M21	$C^{\delta}H$-$C^{\beta}H$
M17-M21	$C^{\delta}H$-$C^{\gamma}H$
N20-K22	$N^{\gamma}H_2$-$C^{\beta 2}H$
A*23-H27	$C^{\beta}H$-$N^{\alpha}H$
A*23-H27	$C^{\beta}H$-$N^{\epsilon 2}H$
A*23-S29	$C^{\beta}H$-$N^{\alpha}H$

[1] Only those connectivities which give direct indications of tertiary structure are presented. In addition to these, many others were observed, e.g. connectivities between residues within the thio-ether bridged rings, which allow delineation of local structural details

or two of their C-terminal amino acid residues. Complete sets of assignments were obtained at 7 and 25°C of which only the former is given in table 1.

No regular secondary structure elements could be uncovered on inspection of the short range NOEs. However, we expected from the outset that it would not be very likely to find α- or β-structure elements, since the high abundance of the thio-ether bridged rings, i.e. five rings encompassing 65% of the amino acids, were surmised to prohibit formation of standard secondary structure. It is likely that the close proximity of the thio-ether bridged rings will to a large extent determine the three-dimensional folding of the molecule. This is suggested already by a number of long-range contacts (table 2), which were observed in the NOESY spectra. These data indicate that nisin is not a random coil but is folded in a unique conformation, especially the N-terminal part is well constrained. For example, the side chain of proline (P9), which is part of the second ring, points in the direction of the first ring (i.e. the ring formed by residues A_3 to

A_7). Furthermore, the side chains of both methionines (M_{17} and M_{21}) face each other.

We have also investigated the influence of external conditions on the ^{1}H-NMR spectrum of nisin. The pH dependencies of the $C^{\epsilon 1}H$ and $C^{\delta 2}H$ resonances of the histidines, H_{27} and H_{31}, indicate pK_a values for both imidazole moieties of about 6.5, which is typical for solvent exposed histidines. Interestingly, deprotonation of the histidines causes nisin to precipitate, which indicates that the positive charge on these residues is required for solubility.

It has been suggested that nisin can form a complex with Ca^{2+} [2]. We have found no indications for such an interaction; the relatively small effects that addition of Ca^{2+} induces in the ^{1}H-NMR spectrum could also be established by addition of Na^+ and thus most likely are due to increased ionic strength.

Our data are compatible with a monomeric structure for nisin; a single set of resonances was observed and all NOEs could be accounted for as intramolecular contacts.

We are currently in the process of obtaining a detailed structure by making use of the NOE and *J*-coupling data as input for computational methods such as 'distance geometry'.

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NOTE ADDED IN PROOF

After submitting this paper we learned that similar studies have been performed by G.C.K. Roberts and co-workers. This work is in press in J. Chem. Soc. Perkin II (W.C. Chan, L.-Y. Lian, B.W. Bycroft and G.C.K. Roberts).