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Isolation and characterisation of two degradation products derived from the peptide antibiotic nisin

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Two degradation products of nisin have been isolated and their structures have been determined by ^{1}H NMR. Nisin$^{1-32}$ [(des-ΔAla33-Lys34; Val32-NH_2)nisin] and (des-ΔAla5)nisin$^{1-32}$ [(des-ΔAla5, ΔAla33-Lys34; Ile4-NH_2, pyruvyl-Leu6, Val32-NH_2)nisin] are formed on storage or by acid treatment. Contrary to previous reports, nisin$^{1-32}$ showed potent antimicrobial activity against Gram-positive organisms comparable to that of nisin itself. (des-ΔAla5)Nisin$^{1-32}$, however, was biologically inactive, thus demonstrating the importance of ΔAla5 and/or ring A for biological activity.

Nisin; Peptide antibiotic; HPLC; NMR; Peptide structure

1. INTRODUCTION

The antibiotic nisin belongs to the unique group of post-translational modified peptide antibiotics which includes subtilin [1], epidermin [2] and pep-5 [3]. Nisin is produced by strains of *Lactococcus lactis*, and possesses antimicrobial activity against a wide spectrum of Gram-positive organisms, as well as inhibiting sporulation of *Bacilli* and *Clostridia*; it is extensively employed as a food preservative, particularly in the diary industry [4].

The chemical structure of nisin (fig.1), originally proposed by Gross and Morell [5], was recently confirmed by complete ^{1}H NMR resonance assignment [6,7]. The peptide contains *meso*-lanthionine (2*S*,3*S*,6*R*)-3-methyllanthionine and α,β-didehydroamino acids; these atypical residues are derived from serine, threonine and cysteine residues in the precursor peptide via a series of post-translational enzymatic modifications [8,9]. The lanthionine and 3-methyllanthionine residues introduce thioether bridges at various locations in the molecule, resulting in a series of cyclic units.

Commercial samples of nisin commonly contain more than one component, and it is generally observed that their antimicrobial activity decreases with the age of the sample. Berridge et al. [10] reported the isolation by counter-current distribution of two major components (nisin A and nisin B), both of which were biologically active, but they were unable to characterise them chemically. Gross and Morell [11,12] reported that treatment of nisin with strong acid liberated pyruvyllysine and yielded a biologically inactive material which they concluded was nisin$^{1-32}$.

We now report the isolation and preparation, structural characterisation (by 2D ^{1}H NMR spectroscopy) and biological activity of nisin$^{1-32}$ and (*des*-ΔAla5)nisin$^{1-32}$, two major degradation products of nisin.

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Abbreviations: HPLC, high pressure liquid chromatography; 2D NMR, two-dimensional nuclear magnetic resonance; HOHAHA, homonuclear Hartmann-Hahn experiment; NOESY, nuclear Overhauser enhancement spectroscopy

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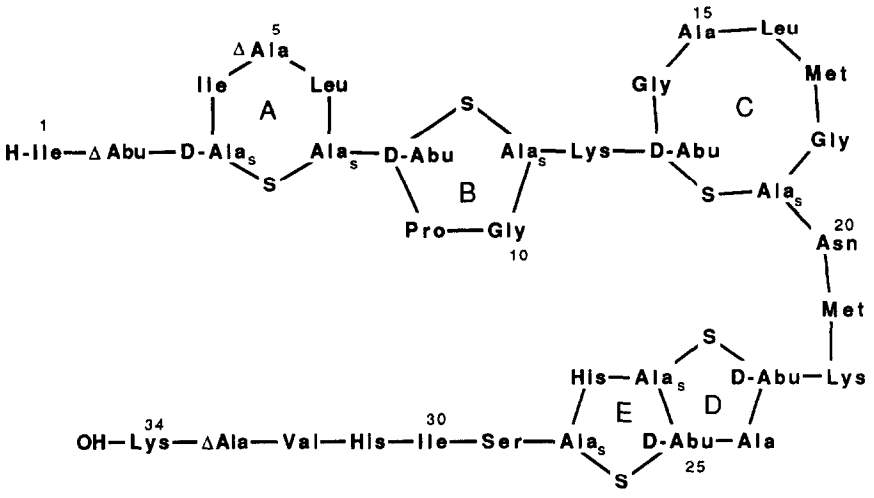


Fig.1. The schematic structure of nisin. ΔAbu, dehydrobutyrine; ΔAla, dehydroalanine; Ala$_s$, the alanine moiety of lanthionine or 3-methyllanthionine; D-Abu, the β-aminobutyric acid moiety of 3-methyllanthionine.

2. EXPERIMENTAL

Nisin$^{1-32}$ was isolated from a sample of nisin (obtained from Aplin & Barrett, Beaminster, England, expiry date July 1986) by semi-preparative HPLC on a C-18 reverse-phase column (Spherisorb S5ODS2, 8 mm × 250 mm). The solvents used were: (A) aqueous triethylammonium acetate buffer (48 mM, pH 2.9) and (B) 22.5% A in acetonitrile. Elution was with a linear gradient from 30% to 50% B in 15 min, followed by isocratic 50% B for 2 min, using a flow rate of 3.0 ml/min and monitoring the effluent at 232 nm. Fractions containing nisin$^{1-32}$ were concentrated in vacuo, dialysed against 0.2% aqueous acetic acid, and lyophilised. Nisin$^{1-32}$ was also prepared from pure nisin (37000 U/mg, 20 mg) by treating it with 1 M hydrochloric acid in 20% aqueous acetonitrile (8 ml) at room temperature for 6 days.

(*des*-ΔAla5)Nisin$^{1-32}$ was prepared from nisin as follows: a suspension of nisin (37000 U/mg, 40 mg, 11 μmol) in 1.0 M hydrochloric acid in glacial acetic acid (1.5 ml) and water (0.5 μl, 27 μmol) was vigorously stirred under nitrogen atmosphere at 50°C for 1.5 h. The mixture was concentrated, diluted with water (4 ml), dialysed against 0.2% aqueous acetic acid, and finally lyophilised to give a pale brown solid (30 mg). This was then purified by semi-preparative HPLC as described above, but with elution by isocratic 35% B for 4 min, a linear gradient from 35% to 50% B in 10 min, followed by isocratic 50% B for 2 min, to afford (*des*-ΔAla5)nisin$^{1-32}$ (12.5 mg).

Analytical HPLC was carried out on a Spherisorb S5ODS2 column (4.6 mm × 250 mm) at a flow rate of 1.20 ml/min. Antimicrobial activity was estimated by using the agar diffusion assay of Tramer and Fowler [13].

All NMR measurements were carried out on a Bruker AM 500 spectrometer operating at 500.13 MHz. Samples contained 4 mM peptide in sodium phosphate buffer (100 mM, pH 3.0, 85% H_2O/15% D_2O), and spectra were referenced to sodium 3-(trimethylsilyl)-1-propanesulphonate. 2D-NMR spectra were acquired and processed in the phase-sensitive mode using time proportional phase incrementation methods. HOHAHA, NOESY and relayed-NOESY spectra were acquired as described previously [6]. In the HOHAHA experiments a MLEV-17 mixing sequence (with durations 35, 65 and 110 ms) was used [14,15], and the H_2O resonance was suppressed using the SCUBA-pulse sequence [16]. The NOESY experiments employed mixing periods of 100, 200, 350 and 400 ms, and the relayed-NOESY experiments [17,18] were composed of a NOESY-pulse sequence (τ_m = 350 ms) followed by a MLEV-17-pulse sequence (τ = 40 ms).

All data were processed in the phase-sensitive mode using either a Gaussian window function or a sine-bell square function with a $\pi/6$ phase shift.

3. RESULTS AND DISCUSSION

HPLC analysis of an 'old' sample of nisin (expiry date July 1986), using the solvent system described in section 2, revealed the presence of a number of slow-eluting components (fig.2), whereas analysis of the culture broth from a nisin-producing *Lactococcus lactis* MG 5822 showed the presence only of nisin (data not shown). The three major components of the crude sample have now been identified by high-field 2D-NMR spectroscopy as, in the order of elution, (*des*-ΔAla5)nisin$^{1-32}$, nisin, and nisin$^{1-32}$ (see below). In addition, we have treated pure nisin with hydrochloric acid in glacial acetic acid, conditions which were reported by Gross and Morell [11] to yield nisin$^{1-32}$; we find, however, that under these

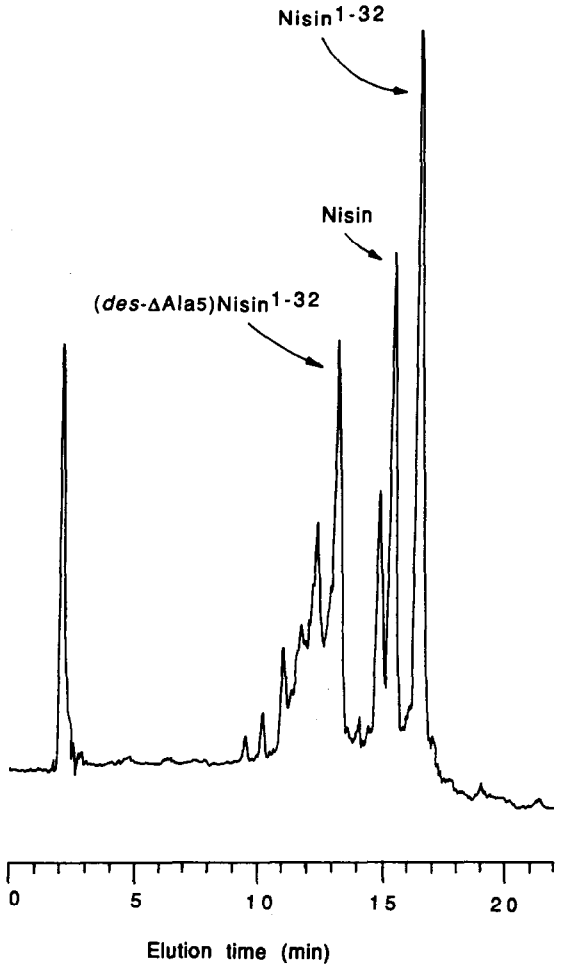


Fig.2. Analytical HPLC profile of a crude sample of nisin (expiry date July 1986), showing the presence of the three identifiable components. Gradient elution profile: linear gradient 30% to 50% B in 15 min, followed by isocratic 50% B for 7 min.

conditions the major nisin related product is in fact (*des*-ΔAla5)nisin$^{1-32}$.

The two degradation products of nisin were isolated and purified to homogeneity by semi-preparative HPLC as described in section 2. The low-field regions of the ^{1}H NMR spectra of nisin and its degradation products, nisin$^{1-32}$ and (*des*-ΔAla5)nisin$^{1-32}$, in aqueous solutions are shown in fig.3. The loss of singlet resonances in the δ5.4 to 5.8 region is indicative of the loss of ΔAla residues in the degradation products, while the quartet visible in all three spectra at ~δ6.7 shows that the ΔAbu2 residue remains intact. The spectra of the two degradation products have been completely assigned by using a sequential strategy which has been described in detail for nisin itself [6]; the assignment procedure will therefore be discussed only briefly.

Resonance was first assigned to individual types of amino acids by observation of relayed scalar connectivities from the peptide backbone amide protons to the side chain aliphatic protons. Analysis of the HOHAHA spectrum obtained with a spin-locking period of 110 ms (fig.4) revealed 27 NH-C$^\alpha$H cross peaks; the N-terminal IleNH$_3^+$-C$^\alpha$ cross peak was not observed, probably as a result of exchange with the solvent. The characteristic spin systems of glycine (ABX), alanine (A_3MX), valine ($(A_3)_2MPX$) and leucine ($A_3B_3MN_2PX$) were readily identified. For example, the NH resonance at δ8.47 showed connectivities to δ4.35, 1.80, 1.70, 0.99 and 0.97, characteristic of the NH-C$^\alpha$H-C$^\beta$H$_2$-C$^\gamma$H-(C$^\delta$H$_3$)$_2$ system of leucine (in fact Leu16). The unique A_3MPX spin system of the D-Abu moiety of 3-methyllanthionine residues were also readily identified, with characteristic chemical shifts of C$^\beta$H at ~δ3.6 and C$^\gamma$H$_3$ at δ1.3 to 1.5. Interestingly, the D-Abu8NH(δ8.89) showed connectivity to C$^\alpha$H but not to either the C$^\beta$H or C$^\gamma$H$_3$; these latter resonances were identified by the C$^\alpha$H-C$^\beta$H-C$^\gamma$H$_3$ connectivities. A similar absence of relayed connectivity from the NH beyond C$^\alpha$H is also observed for this residue in nisin [6].

The sequential assignment to individual residues was based on a systematic search for short-range NOESY cross peaks between the amide proton of the $(i+1)$ residue and the C$^\alpha$, C$^\beta$ and amide protons of the proceeding (i) residue. Confirmation of this analysis was obtained from the relayed-NOESY experiment, in which incoherent (dipolar) transfer from the NH($i+1$) to C$^\alpha$H(i) is followed by coherent (scalar) transfer to NH(i), thus giving rise to NH($i+1$)-NH(i) cross peaks asymmetric about the diagonal [17,18]. The presence of the thioether bridges was confirmed by the observation of NOE connectivities across the sulphur atom, namely the D-Ala$_s$7C$^\beta$H–Ala$_s$3C$^\beta$H connectivity, and the D-AbuC$^\beta$H–Ala$_s$C$^\beta$H NOE and D-AbuC$^\beta$H–Ala$_s$C$^\alpha$H NOE-J connectivities for the residue pairs 8-11, 13-19, 23-26 and 25-28. The complete resonance assignments of nisin$^{1-32}$ and (*des*-ΔAla5)nisin$^{1-32}$ are summarised in tables 1 and 2.

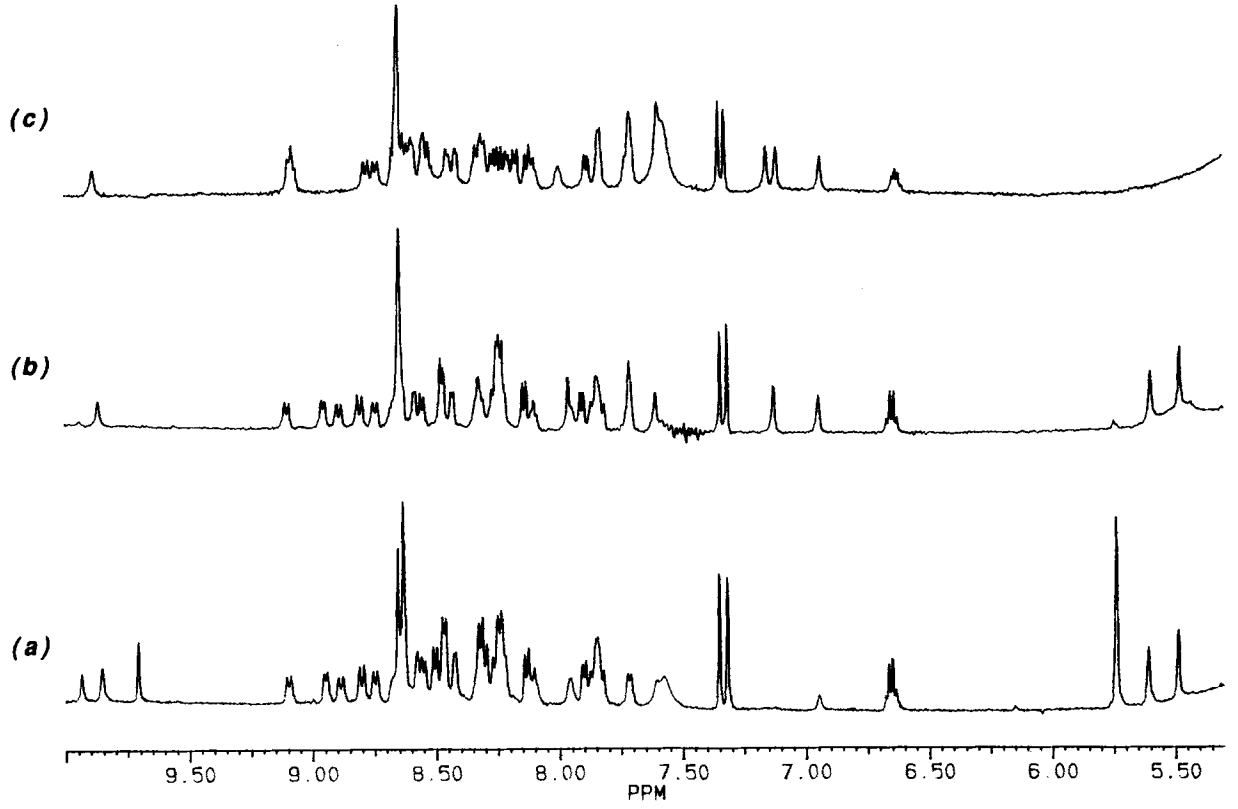
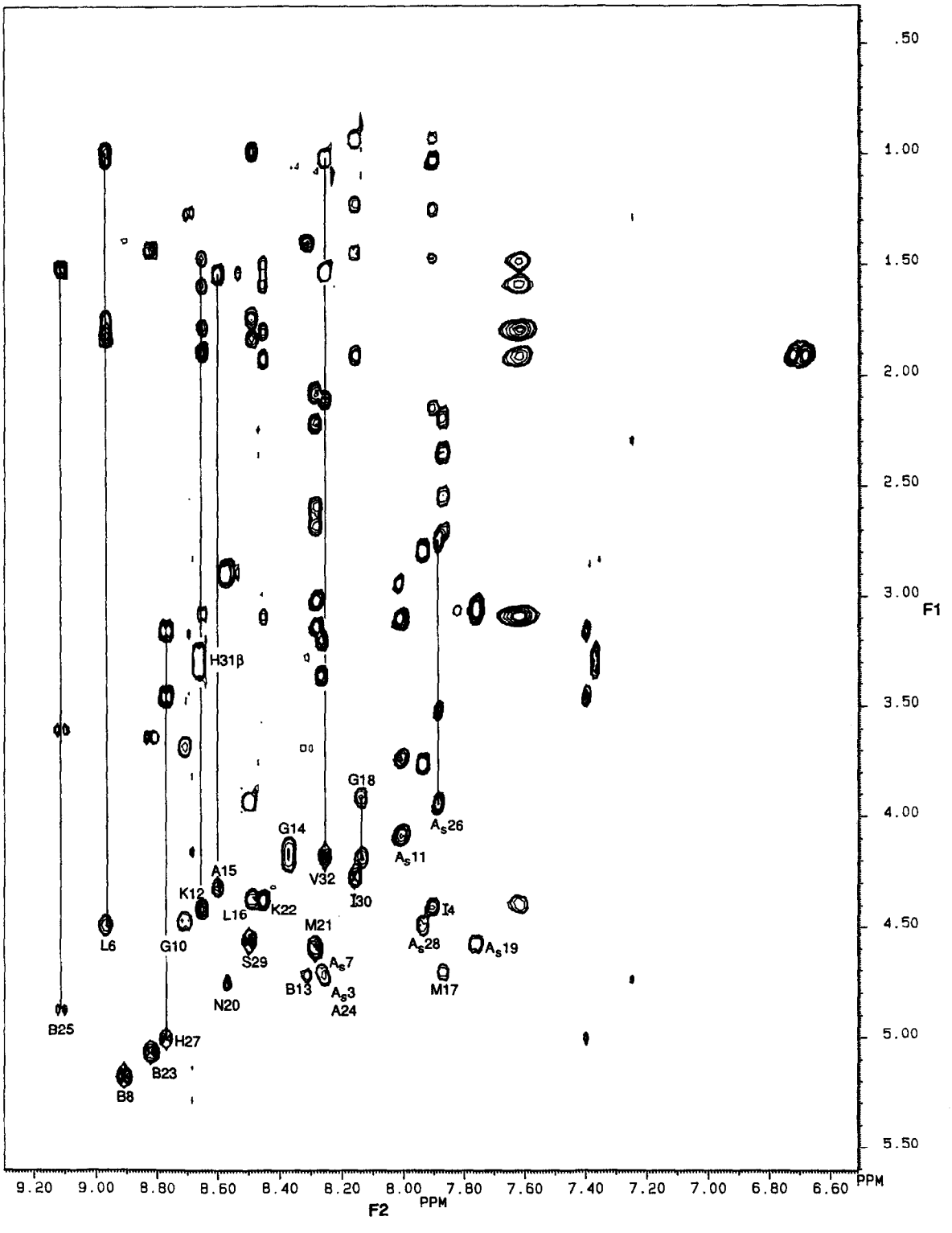


Fig.3. Down-field region of the ^1H NMR spectrum (500 MHz) of (a) nisin, (b) nisin$^{1-32}$, and (c) (*des*-ΔAla5)nisin$^{1-32}$ in aqueous sodium phosphate buffer (pH 3.0, 85 H_2O:15 D_2O) at 303 K.

The spectrum of nisin$^{1-32}$ was characterised by the absence of one of the three lysine spin systems, and one of the two ΔAla spin systems. The sequential assignment confirmed that the two missing residues are ΔAla33 and Lys34, and additional amide NH_2 signals are observed for the terminal amide of Val32. In (*des*-ΔAla5)nisin$^{1-32}$, the resonances of both ΔAla residues, as well as those of Lys34 are missing, but those of the segment Ile1–Ile4 are clearly identifiable, indicating that the D-Ala_s3–Ala_s7 side chain thioether bridge is intact. The $\alpha CONH_2$ and $C^{\beta}H_3$ resonances of Ile4 and of the *N*-pyruvyl substituent on Leu6, respectively, could be identified in the spectrum of (*des*-ΔAla5)nisin$^{1-32}$.

The likely mechanism of formation of these two degradation products, illustrated in scheme 1, involves initially the reversible acid-catalysed formation of an imine salt, which can then add an equivalent of water to form an unstable tetrahedral α-hydroxyalanyl intermediate. This readily breaks down to yield the corresponding peptide-amide and pyruvyl-peptide derivatives. It is apparent that ΔAla33 is considerably more susceptible to degradative hydrolysis than ΔAla5 under a variety of mild acidic conditions (e.g. 0.02 M aqueous HCl, 100°C, or 1 M aqueous HCl, room temperature). The dehydrobutyrine residue (ΔAbu2) appears to be unaffected by even strong acid.

Fig.4. Part of the HOHAHA spectrum (τ = 110 ms) of nisin$^{1-32}$ (4 mM) in aqueous sodium phosphate buffer (pH 3.05) at 303 K, showing relayed through-bond connectivities between amide and side chain aliphatic protons. Connectivities of selected amino acid residues are illustrated.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B13
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M17
I4
L6
G10
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B23
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9.20 9.00 8.80 8.60 8.40 8.20 8.00 7.80 7.60 7.40 7.20 7.00 6.80 6.60
PPM
F2
PPM

Table 1

^{1}H NMR (500 MHz) data of nisin$^{1-32}$ (4 mM) in aqueous solution (100 mM sodium phosphate buffer, pH 3.05, 85 H_2O:15 D_2O) at 303 K

	δ (ppm)					
	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\epsilon}H$
Ile1		4.19	2.16	1.34, 1.61; 1.16	1.04	
Δ^zAbu2	9.86		6.69	1.89		
D-Ala$_s$3	8.25	4.69	3.16, 3.32			
Ile4	7.89	4.39	2.12	1.21, 1.43; 0.99	0.88	
ΔAla5 s	9.86		5.52, 5.65			
Leu6	8.95	4.48	1.80	1.71	0.96, 1.01	
Ala$_s$7	8.27	4.60	2.97, 3.11			
D-Abu8	8.89	5.15	3.64	1.35		
Pro9		4.48	1.87, 2.50	2.00, 2.21	3.50	
Gly10 s	8.70	4.45, 3.66				
Ala$_s$11	7.99	4.07	3.08, 3.71			
Lys12	8.64	4.39	1.86	1.43, 1.55	1.75	3.05
	7.62 ($N^{\epsilon}H_3^+$)					
D-Abu13	8.30	4.70	3.65	1.36		
Gly14	8.35	4.13, 4.19				
Ala15	8.59	4.30	1.52			
Leu16 s	8.47	4.35	1.80	1.70	0.97, 0.99	
Met17	7.86	4.67	2.16, 2.32	2.51, 2.68	2.15	
Gly18	8.12	3.90, 4.16				
Ala$_s$19	7.74	4.54	3.02, 3.07			
Asn20	8.55	4.73	2.88	6.96, 7.62 ($\beta CONH_2$)		
Met21	8.27	4.57	2.05, 2.18	2.57, 2.65	2.14	
Lys22	8.44	4.36	1.90	1.47, 1.55	1.77	3.06
	7.62 ($N^{\epsilon}H_3^+$)					
D-Abu23	8.80	5.02	3.60	1.40		
Ala24 s	8.24	4.73	1.50			
D-Abu25	9.10	4.83	3.55	1.49		
Ala$_s$26	7.87	3.91	2.27, 3.48			
His27 s	8.75	4.95	3.13, 3.43	8.69 (H2), 7.38 (H5)		
Ala$_s$28	7.92	4.48	2.75, 3.73			
Ser29	8.48	4.54	3.92			
Ile30	8.14	4.24	1.88	1.19, 1.40; 0.89	0.89	
His31	8.65	4.83	3.23, 3.31	8.68 (H2), 7.35 (H5)		
Val32-NH_2	8.24	4.15	2.07	1.00		
	7.14, 7.73 ($\alpha CONH_2$)					

The antimicrobial activities of pure nisin and its degradation products are given in table 3. It is notable that nisin$^{1-32}$ is essentially as active as nisin against a number of Gram-positive organisms, while (*des*-ΔAla5)nisin$^{1-32}$ is at least 500-fold less active. It has been reported earlier that nisin$^{1-32}$ is devoid of antimicrobial activity [11,12]. Gross and Morell [11] reported that, under their conditions (HCl in glacial acetic acid, 110°C) pyruvyllysine was released, indicating cleavage at ΔAla33, and concluded that the product was nisin$^{1-32}$; they did not, however, establish whether the other dehydroamino acids had been affected. Our observation that (*des*-ΔAla5)nisin$^{1-32}$ is the major nisin related degradation product under conditions similar to theirs strongly suggests that this was in fact the inactive material formed in their experiments. The two active components

Table 2

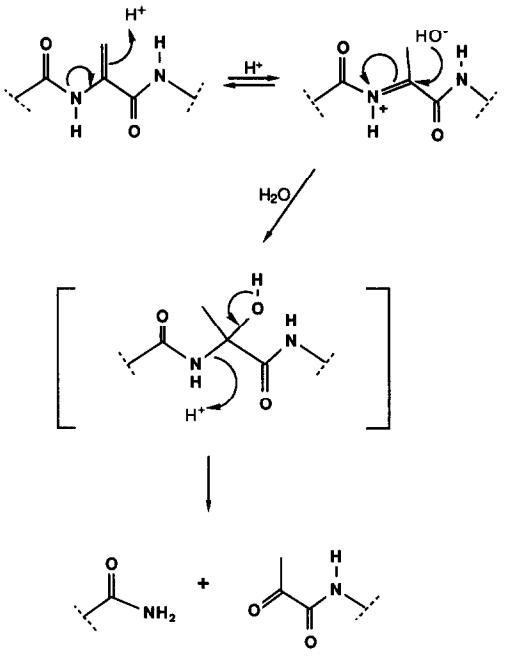
^{1}H NMR (500 MHz) data of (*des*-ΔAla5)nisin$^{1-32}$ (4 mM) in aqueous solution (100 mM phosphate buffer, pH 3.05, 85 H_2O:15 D_2O) at 303 K

	δ (ppm)					
	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\epsilon}H$
Ile1		4.18	2.15	1.38, 1.64, 1.17	1.06	
Δ^zAbu2	9.92		6.67	1.89		
D-Ala$_s$3	8.35	4.72	3.08, 3.22			
Ile4-NH_2	8.15 7.19, 7.74 ($\alpha CONH_2$) 2.51 ($PyrC^{\beta}H_3$)	4.29	2.01	1.39, 1.56, 1.04	0.96	
Pyr-Leu6	8.67	4.50	1.81	1.70	0.97, 1.01	
Ala$_s$7	8.66	[a]	3.06			
D-Abu8	9.10	5.15	3.68	1.40		
Pro9		4.50	1.96, 2.49	1.96, 2.19	3.48, 3.56	
Gly10	8.64	3.74, 4.43				
Ala$_s$11	8.05	4.12	3.11, 3.73			
Lys12	8.62 7.62 ($N^{\epsilon}H_3^+$)	4.42	1.90	1.46, 1.57	1.77	3.08
D-Abu13	8.32	4.71	3.68	1.40		
Gly14	8.38	4.14, 4.21				
Ala15	8.59	4.32	1.54			
Leu16	8.49	4.35	1.83	1.73	0.98	
Met17	7.88	4.70	2.20, 2.34	2.54, 2.73		
Gly18	8.14	3.92, 4.18				
Ala$_s$19	7.77	4.58	3.05, 3.09			
Asn20	8.58	4.77	2.88	6.97, 7.62 ($\beta CONH_2$)		
Met21	8.30	4.58	2.05, 2.20	2.59, 2.68		
Lys22	8.45 7.62 ($N^{\epsilon}H_3^+$)	4.38	1.92	1.47, 1.57	1.80	3.10
D-Abu23	8.81	5.03	3.63	1.42		
Ala24	8.24	4.73	1.52			
D-Abu25	9.11	4.87	3.60	1.51		
Ala$_s$26	7.88	3.94	2.75, 3.52			
His27	8.78	4.96	3.15, 3.45	8.69 (H2), 7.40 (H5)		
Ala$_s$28	7.92	4.48	2.80, 3.75			
Ser29	8.50	4.54	3.92			
Ile30	8.20	4.25	1.89	1.20, 1.43, 0.90	0.90	
His31	8.70	4.83	3.27, 3.34	8.68 (H2), 7.37 (H5)		
Val32-NH_2	8.27 7.15, 7.73 ($\alpha CONH_2$)	4.18	2.10	1.01		

[a] Bleached out due to solvent preirradiation

reported by Berridge et al. [10], nisin A and nisin B, were most probably nisin and nisin$^{1-32}$.

The results presented here demonstrate that ΔAla33 and Lys34 at the C-terminus are not required for antimicrobial activity. In the light of the preferential acid-susceptibility of ΔAla33, the full biological activity of nisin$^{1-32}$ must account for the observation that commercial nisin preparations can be autoclaved in dilute acid for short periods without significant loss of activity [4]. Degradation of ΔAla5, by contrast, leads to a very marked less of activity. It remains to be unambiguously established whether this residue per se is required for activity, or whether the loss of activity results from the increased conformational flexibility resulting from opening ring A.



Scheme 1.

Table 3

Minimum inhibitory concentration (MIC, μg/ml) of nisin and its degradation products determined by the agar diffusion assay

Test organism	MIC		
	Nisin	Nisin$^{1-32}$	(*des*-ΔAla5)-Nisin$^{1-32}$
Lactoc. lactis MG 1614	0.06	0.04	>100
Lactoc. cremoris NCDO 495	0.05	0.04	>300
Staph. aureus NTCC 10188	1.12	2.30	>500
Microc. luteus NCIB 8166	0.06	0.05	N.D.
E. coli ESS	>500	>500	>500

N.D., not determined

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