Volume 300, number 1, 56–62 FEBS 10849 March 1992

Sequence-specific resonance assignment and conformational analysis of subtilin by 2D NMR

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Received 16 December 1991; revised version received 5 February 1992

Subtilin, a 32-amino acid peptide with potent antimicrobial activity, has been isolated from *Bacillus subtilis* ATCC6633. The chemical structure has been confirmed by the unambiguous sequence-specific assignment of its ^{1}H NMR spectrum. Detailed NMR analysis revealed that subtilin is a rather flexible molecule; the only observed conformational contraints were those imposed by the cyclic structures created by the lanthionine and 3-methyllanthionine residues. These results suggest that in aqueous solution subtilin and the homologous peptide nisin have similar conformations.

Subtilin; Lantibiotics; HPLC; NMR; Peptide conformation

1. INTRODUCTION

Subtilin [1] belongs to a group of post-translational modified peptides known as lantibiotics [2] which includes nisin, epidermin, gallidermin and pep-5. These peptides have in common the presence, as a result of post-translational modification a number of didehydroamino acid, *meso*-lanthionine and (2*S*,3*S*,6*R*)-3-methyllanthionine residues; the latter two residues introduce thioether bridges at various locations in the molecule, resulting in a series of cyclic units. The structural gene for the precursor of subtilin has been cloned and sequenced [3]. It encodes a 56-residue peptide, comprising of a 24-residue leader sequence followed by a 32-residue sequence which corresponds to mature subtilin except it contains serine, threonine and cysteine as precursors of the above described modified amino acids. Subtilin inhibits the growth of a diverse spectrum of Gram-positive bacteria. The likely mechanism for the observed antimicrobial activity is the voltage-dependent formation of pores in the cytoplasmic membrane [4], hence resulting in the cellular efflux of electrolytes, amino acids and ATP, and subsequently cell death.

Subtilin (Fig. 1) is structurally related (> 60% identity) to nisin [5], which is used as a food preservative. The chemical structure of subtilin was proposed by Gross and his co-workers in 1973 on the basis of extensive chemical and enzymatic degradation studies [1]. However, the results of these studies have yet been confirmed by modern physicochemical methodology, e.g. 2D NMR and high resolution mass spectroscopy. As part of our ongoing programme on the biosynthesis and structure–activity relationship studies of nisin and related lantibiotics [6,7], we have now confirmed the chemical structure of subtilin by ^{1}H 2D NMR spectroscopy, and used NMR to obtain information on its solution conformation.

Abbreviations: RP-HPLC, reverse-phase high-pressure liquid chromatography; 2D NMR, two-dimensional nuclear magnetic resonance; HOHAHA, homonuclear Hartmann–Hahn; NOESY, nuclear Overhauser enhancement spectroscopy; FAB-MS, fast atom bombardment mass spectroscopy.

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2. EXPERIMENTAL

Subtilin was isolated from a 24 h *Bacillus subtilis* ATCC6633 culture supernatant as follows: the acidified (pH 2.5) supernatant (1.0 l) was extracted with butanol (2 × 250 ml), and the combined extract was concentrated *in vacuo* to 100 ml. This solution was then added dropwise to stirred acetone (50 ml) at 5°C over a period of 30 min. The resultant suspension was centrifuged, and the supernatant was added dropwise to acetone (350 ml) over a period of 30 min with stirring at 5°C. The ensuing precipitate was isolated by centrifugation.

The isolated precipitate was dissolved in 0.5% aqueous acetic acid (100 ml), filter-sterilised, and lyophilised to give a white amorphous solid. This material was then purified by semi-preparative RP-HPLC on a Kromasil KR100-5C8 column (8.0 × 250 mm). The solvents used were: (A) 0.1% aqueous trifluoroacetic acid and (B) 0.1% trifluoroacetic acid in 90% aqueous acetonitrile. Elution was with a linear gradient from 40% to 46% B in 12 min, at a flow rate of 2.60 ml/min, monitoring the effluent at 278 nm. Fractions containing subtilin were concentrated *in vacuo* at 30°C and lyophilised.

Analytical RP-HPLC was carried out on either a Kromasil KR100-5C8 or Nucleosil NC120-5C18 column (4.6 × 250 mm) at a flow rate of 1.20 ml/min, monitoring the effluent at 230 nm. Antimicrobial activities were estimated by an agar diffusion assay.

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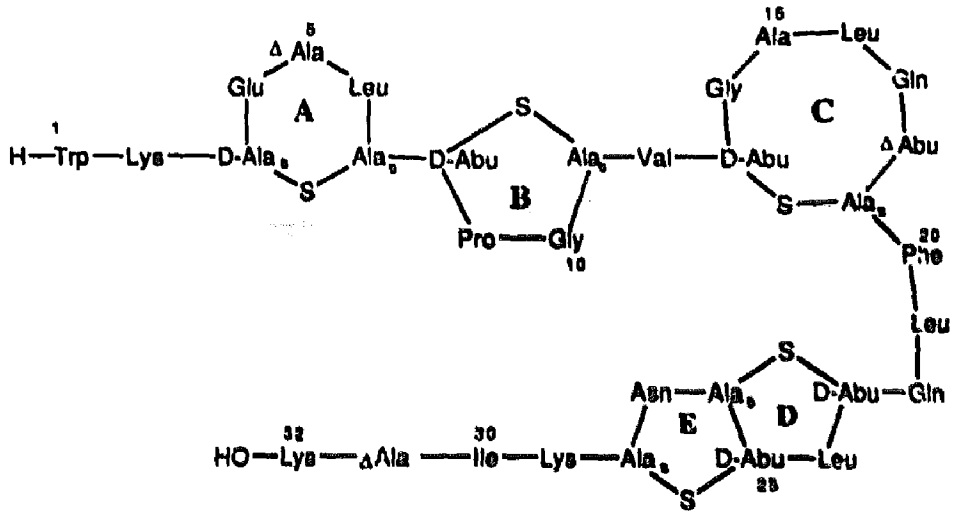

Fig. 1. The schematic chemical structure of subtilin.

All NMR measurements were carried out on a Bruker AMX 600 spectrometer operating at 600.14 MHz. Samples containing 2 mM subtilin in 85% H_2O/15% D_2O (pH 2.50, with phosphoric acid) were utilised. Use of higher concentrations led to aggregation of the peptide, with a consequent broadening of the resonance lines. 2D NMR spectra were acquired and processed in the phase-sensitive mode using time proportional phase incrementation methods. HOHAHA and NOESY spectra were acquired as described previously [6]. In the HOHAHA experiments a MLEV-17 mixing sequence (with durations 40, 80 and 120 ms) was used [8], and the H_2O resonance was suppressed using a combination of soft presaturation and a $\bar{1}$ – 1 pulse sequence [9]. The NOESY experiments employed mixing periods of 100 to 400 ms.

All data were processed in the phase-sensitive mode using either a Gaussian window function or a sine-bell square function with a $\pi/_4$ phase shift.

Structure calculations were carried out using the hybrid metric matrix distance geometry – dynamic simulated annealing approach [10]. The distance geometry calculation was performed using the program DSPACE, from which ten structures were then subjected to the dynamical simulated annealing process using the program XPLOR [11]. All calculations were carried out on a Silicon Graphics 4D/25-GT workstation and an Alliant FX40-3 computer.

3. RESULTS AND DISCUSSION

Subtilin was readily purified from the culture broth of *Bacillus subtilis* ATCC 6633 after 24 h growth at 37°C with vigorous shaking (250 r.p.m.). Briefly, the purification involves an initial butanol extraction – acetone precipitation followed by semi-preparative RP-HPLC on a Kromasil C8 column to afford ~20 mg of the desired lantibiotic in >99.5% purity from one litre culture. The integrity of the isolated peptide (Fig. 2) was established by antimicrobial assays, amino acid analysis, FAB-MS (MH^+ found 3321.9, requires 3321.968), and electrospray-MS. In the latter technique, several multiple-charge molecular ions were observed, from which a calculated molecular mass of 3320.49 ± 0.4 was obtained; this compares favourably to the required 3320.960 for subtilin ($C_{148}H_{227}N_{39}O_{38}S_5$). The lantibiotic

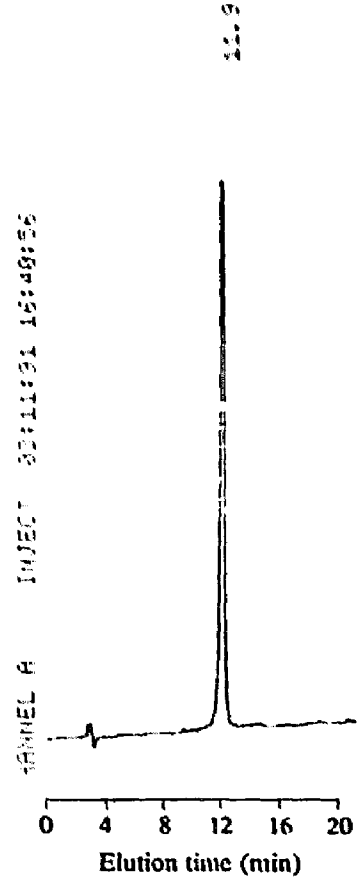

Fig. 2. RP-HPLC analysis of subtilin purified from the culture broth of *B. subtilis* ATCC6633.

Table I : ^{1}H NMR (600 MHz) chemical shifts of subtilin (2 mM) in aqueous solution (pH 2.5; H_2O,85 : D_2O,15) at 303 K.

		δ (p.p.m.)				
	NH	CαH	CβH	CγH	CδH	CεH
Trp1		4.48	3.50,3.55	7.37,7.66,7.19,7.34,7.61 (*Ind* C_6H_5) 10.40 (*Ind* NH)		
Lys2	8.87	4.37	1.86,1.92	1.42,1.48	1.78	3.06
	7.58	(NεH_3^+)				
D-Ala 3 [a]	8.69	4.86	3.11,3.32			
Glu4	7.98	4.15	1.58,1.90	2.20,2.29		
ΔAla5 S	9.71		5.50,5.66			
Leu6	8.85	4.47	1.78	1.73	0.95,1.00	
Ala_s7	8.29	4.58	3.08,3.18			
D-Abu8 [b]	8.82	5.15	3.49	1.46		
Pro9 S		4.48	2.00,2.50	1.87,2.20	3.47,3.50	
Gly10	8.77	3.68,4.42				
Ala_s11	8.03	4.08	3.09,3.72			
Val12	8.41	4.26	2.12	1.03		
D-Abu13	8.35	4.69	3.65	1.38		
Gly14	8.39	4.11,4.28				
Ala15	8.61	4.20	1.48			
Leu16 S	8.53	4.31	1.83	1.72	0.98,1.00	
Gln17	7.85	4.51	2.26	2.44,2.50	6.92,7.55 (γCONH_2)	
Δ^zAbu18	8.74		6.91	1.86		
Ala_s19	7.60	4.48	2.94,3.00			
Phe20	8.10	4.68	3.15,3.22	7.29,7.45,7.40 (C_6H_5)		
Leu21	7.97	4.40	1.68	1.51	0.93,0.98	
Gln22	8.25	4.36	2.12,2.20	2.47	6.82,7.51 (γCONH_2)	
D-Abu23	8.68	5.00	3.64	1.44		
Leu24 S	8.02	4.72	1.96	1.64	1.00,1.06	
D-Abu25	9.34	4.94	3.60	1.47		
Ala_s26	7.68	4.00	2.84,3.78			
Asn27 S	8.72	5.01	2.76,2.97		6.96,7.65 (βCONH_2)	
Ala_s28	7.74	4.32	2.81,3.70			
Lys29	8.52	4.45	1.82,1.91	1.45	1.51	3.06
	7.58	(NεH_3^+)				
Ile30	8.24	4.30	1.98	1.30,1.58; 1.04	0.96	
ΔAla31	9.67		5.79,5.82			
Lys32	8.35	4.47	1.88,2.02	1.54	1.77	3.10
	7.58	(NεH_3^+)				

[a] Alanine, and [b] α-aminobutyric acid moieties of the (2*S*,6*R*)-lanthionine and (2*S*,3*S*,6*R*)-3-methyllanthionine residues.

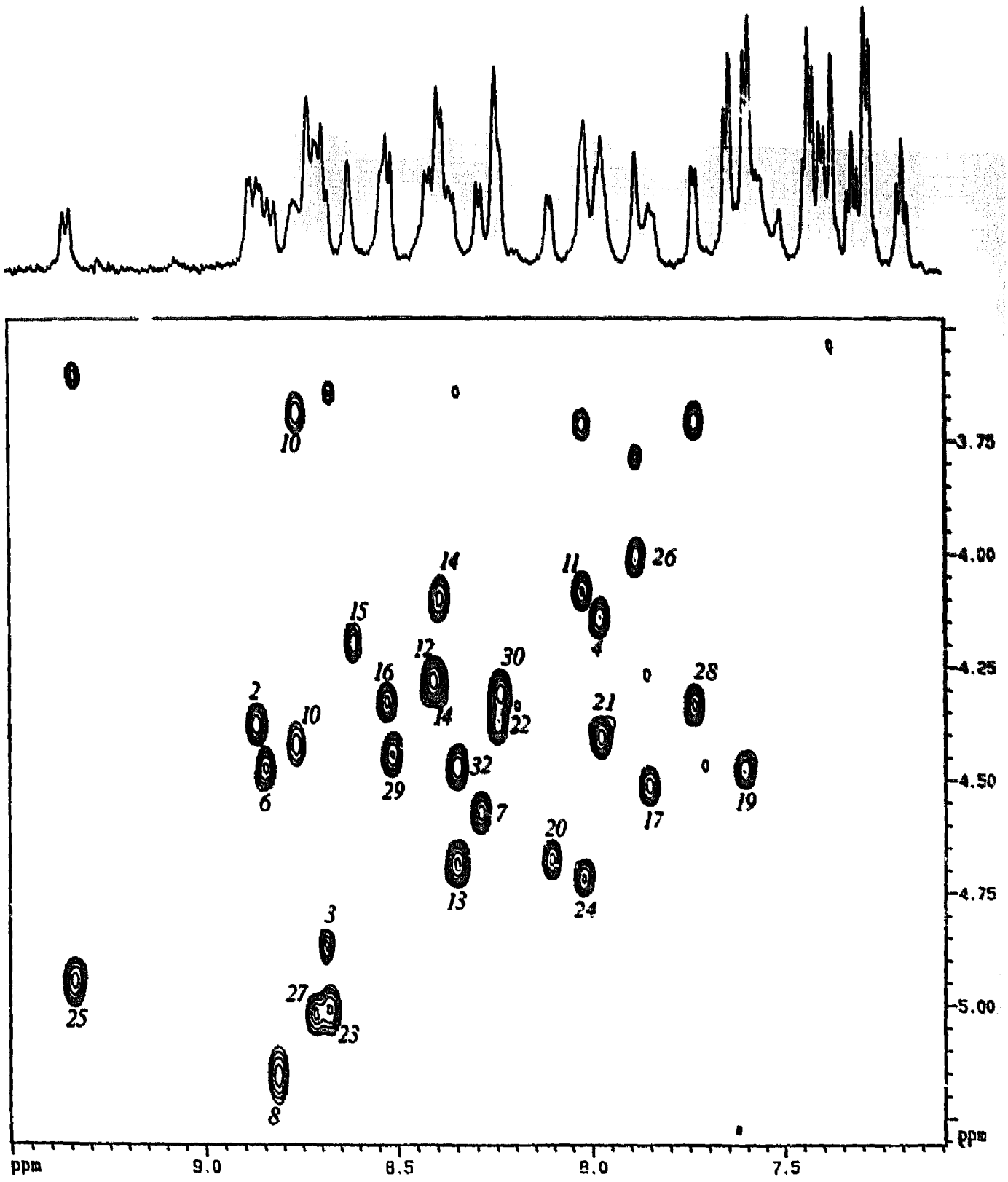

Fig. 3. Fingerprint region of the HOHAHA spectrum of subtilin (2 mM) in aqueous solution (pH 2.5) at 303 K with a spin-locking period of 80 ms. The NαH – CαH cross-peaks are indicated by the residue numbers of the amino acid.

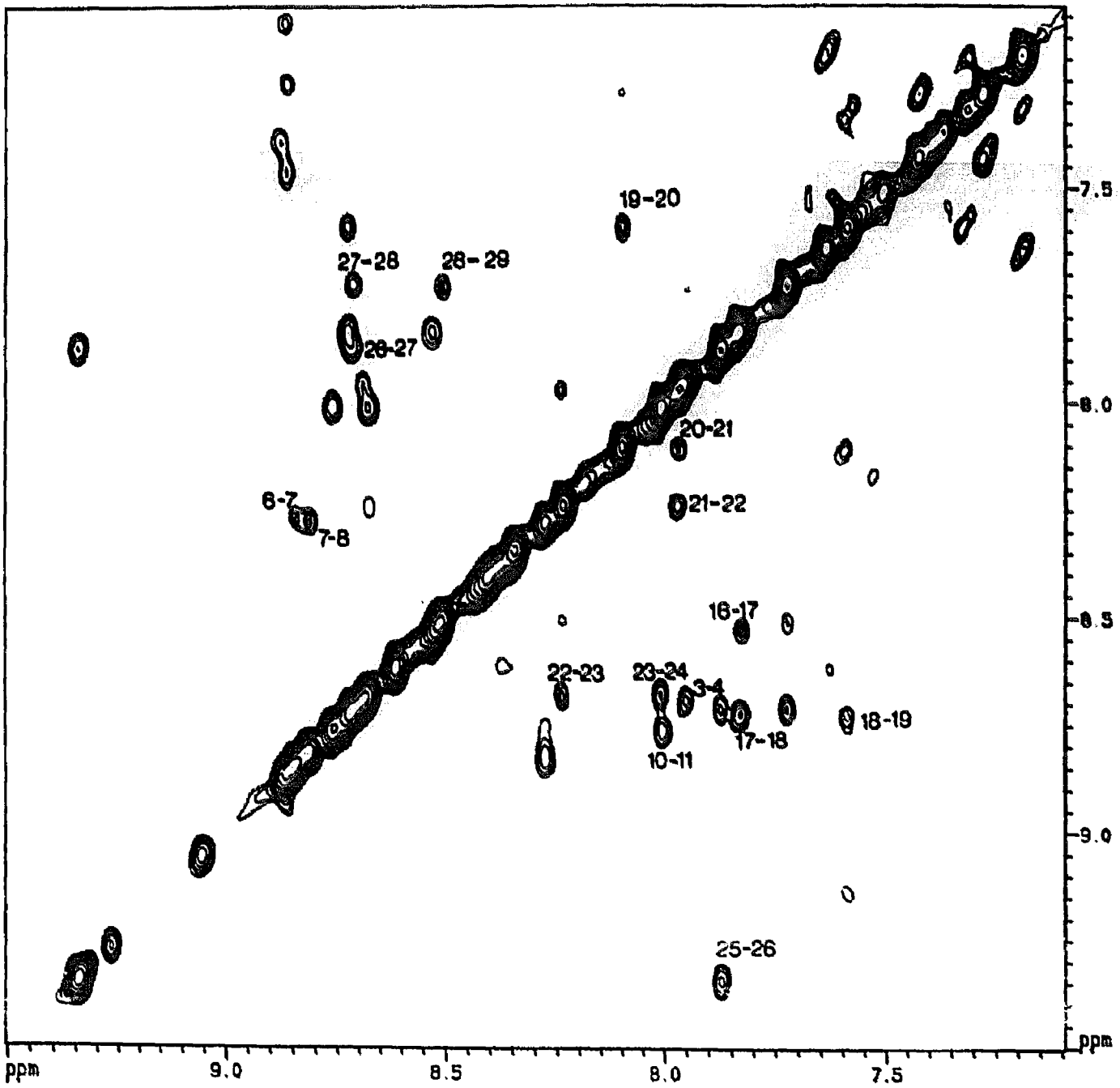

Fig. 4. NαH-NαH region of the NOESY spectrum (τ_m = 300 ms) of subtilin at 303 K. Inter-residue connectivities are indicated by the residue numbers.

was then analysed by ^{1}H NMR spectroscopy. Complete sequence-specific resonance assignment of subtilin in aqueous solution was achieved by application of several 2D NMR techniques described in detail for nisin [6], and the results are summarised in Table I.

The strategy used for the resonance assignment is based on that proposed by Wüthrich [12] for small proteins. Resonances were first assigned to individual types of amino acids by determining the relayed scalar connectivities from the backbone amide NH to the side-chain aliphatic CHs using the HOHAHA spectra (MLEV-17 pulse sequence [8], τ = 40 (Fig. 3) and 80 ms). For example, the characteristic spin systems of Gly [ABX], Ala [A_3MX] and Val [$(A_3)_2$MPX] can be readily identified. In analogy to nisin, the ABMX and the unique A_3MPX spin systems of the Ala$_x$ and Abu moieties respectively were also identified. The long-range and relayed NH-CβH-CγH_3 connectivities led to delineation of the complete spin system for the dehydrobutyrine (ΔAbu^{18}) residue.

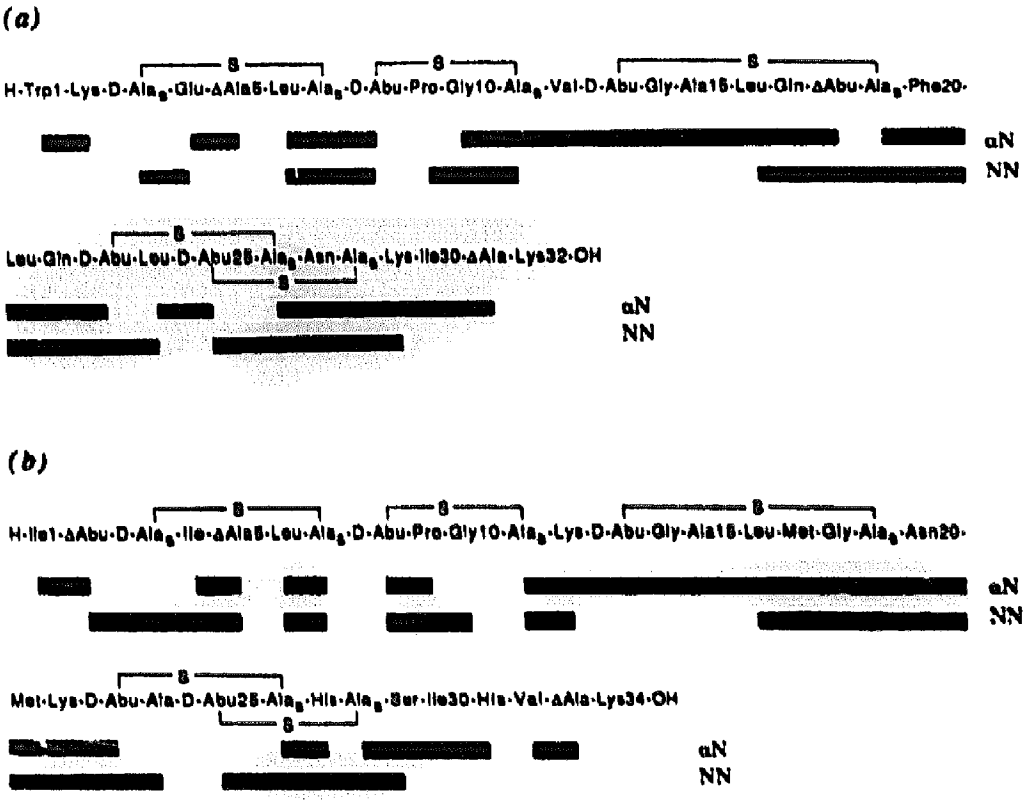

Fig. 5. Summary of the sequential resonance assignments of (a) subtilin and(b) nisin in aqueous solution. The solid bars indicate the presence of NOE connectivities between residues.

This was followed by sequential assignment, based on a search for short range NOESY cross-peaks between the CαH, CβH or NH of residue (i) and the NH of residue (i+1) in the sequence. Fig. 4 shows the NαH-NαH region of a NOESY spectrum; using the unique single occurrence of the Phe residue, the sequence 'Leu-Gln-ΔAbu-Ala$_5$-Phe-Leu-Gln-Abu-Leu' was readily established. Thus, in a similar manner, the complete sequence was determined (Fig. 5). The Z stereochemistry for the alkenic moiety in ΔAbu[18] was established by the chemical shift of the CγH_3 (δ1.86) and unambiguously confirmed by the observation of intraresidue ΔAbuNH – CγH_3 and sequential ΔAbuCβH – Ala$_6$NH NOESY cross-peaks and hence confirmation of the structure.

The temperature dependence of the amide NH chemical shifts was determined over the range 283 to 303 K. The relatively lower temperature coefficient values observed for the backbone amide NHs of Glu[4], Ala$_5$[7], Ala$_6$[11], Gly[14], Gln[17], Ala$_{19}$[19], Ala$_{26}$[26] and Ala$_{28}$[28] indicate that these protons are either solvent-shielded and/or involved in intramolecular H-bonds (Table II). The amide NαH of Ala$_6$[11], Q[17] and Ala[19] are most probably involved in stable intramolecular H-bonds, particularly the Ala$_6$[11] NαH----O=CAbu^5 stabilising a β-turn in Ring

Table II

Comparison of the temperature coefficients ($-\Delta\delta/\Delta T \times 10^{-3}$, ppm/K) of the backbone amide NH for nisin and subtilin in aqueous solution

	Nisin			Subtilin		
$-\Delta\delta/\Delta T \times 10^{-3}$	8.0–16.6	2.1–3.1	1.2–1.7	9.5–16.4	3.5–5.9	0.5–1.4
Amino acid residues	Remaining residues; G[10] (3.7), G[18] (4.3), A[24] (5.7), A$_{28}$[28] (5.9)	I[1], A$_5$[7], G[14], A$_{26}$[26]	A$_6$[11], M[13] A$_{19}$[19]	Remaining residues; G[10] (7.6), ΔB[18] (7.0), L[24] (6.5)	E[3], A$_5$[7], G[14], A$_{26}$[26], A$_{28}$[28]	A$_6$[11], Q[17], A$_{19}$[19]

Fig. 6. Superposition of 10 structures of ring C in subtilin (grey lines) and nisin (black lines), derived from NMR data using previously described protocol [13], with optimal superposition for the backbone of residues 17–19.

B. The corresponding amino acid residues in nisin were also observed to display significantly lower temperature coefficients (Table II), hence suggesting that the two lantibiotics adopt similar conformation/s in aqueous solution. Interestingly, substitution of Gly^{18} (found in nisin) by the more rigid ΔAbu in subtilin does not appear to affect the conformation of Ring C which adopts a great deal of flexibility (Fig. 6).

Analysis using distance constraints derived from observed NOEs (some of which are summarised in Fig. 5) revealed that subtilin, like nisin [13] is a rather flexible molecule and the only defined conformational features were those imposed by the lanthionine residues; only sequential and no long-range NOE was observed.

Despite the striking similarity in the conformation and chemical architecture, subtilin and nisin display quite different spectrum of antimicrobial activities against Gram-positive organisms. Indeed, subtilin inhibits the growth of nisin-producing *Lactococcus lactis*, and vice versa. Further work is currently in progress to understand the structure-activity relationship of each lantibiotics.

Acknowledgements: This work is supported by grants from SERC and AFRC, UK. We thank Drs. H. Dodd and M. Gasson (AFRC Institute of Food Research, Norwich) for supply of *Lactococcus lactis* and *Bacillus subtilis* strains. Mass spectroscopy was carried out at the Centre for Mass Spectroscopy, UMIST, Manchester.

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