A number of nucleolus-like bodies have been found in the microspore nucleus of *Zea mays* in which the NOR is lost[16,17]. When the cultured cells of *Xenopus laevis* and of mammals are exposed to colcemid, a number of micronuclei result and many nucleolus-like bodies are formed in some of them[18-21]. No fully developed nucleoli have been found in the micronuclei with many nucleolus-like bodies. These findings seem to be well compatible with those of the present study. Inhibitors of rRNA synthesis such as ethidium bromide and cordycepin can also induce the appearance of numerous nucleolus-like bodies, but they do not result from exposure to inhibitors of the synthesis of protein or extranucleolar RNA[6,22,23]. Recently, actinomycin D has also been shown to prevent the assembly of the prenucleolar material at the NORs[24]. This means that rRNA synthesis may be involved in the mechanism which draws the prenucleolar material to the NORs. 5-Azacytidine does not inhibit RNA synthesis[15]. Therefore, reconstruction of the nucleoli in the larger nuclei suggests that the NORs still have the ability to synthesize rRNA even after they have been abnormally segregated. To clarify this, a further study is required, focusing on whether or not NORs which have been abnormally segregated can incorporate rRNA precursors.

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Confirmation of the structure of nisin and its major degradation product by FAB-MS and FAB-MS/MS

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Summary. FAB-MS has been applied to the analysis of a nisin complex and FAB-MS and FAB-MS/MS data from the major component used to provide confirmation of the amino sequence and positions of the sulphur-bridged rings in these highly modified peptides.
Key words. Nisin, modified bacterial peptide, FAB-MS, FAB-MS/MS.

The nisins are a group of several closely related modified peptides with antimicrobial activity which are produced by various strains of *Streptococcus lactis*. They have an inhibitory effect on the growth of gram-positive organisms, and also limit sporulation of a range of *Bacilli* and *Clostridia*. This latter property has been exploited by the food industry and nisin is now very widely employed as a preservative for processed foods, particularly milk products[1].

It has been known for more than 30 years that the nisin complex is a mixture of peptides and over this period various groups have investigated their structures. The most comprehensive investigation on the principal active component was carried out by Gross and Morrell who proposed[2], on the basis of extensive chemical studies, the structure shown in figure 1. This structure is based on a 34 amino acid residue peptide, incorporating a number of unusual modified residues. These consist of two dehydroalanines (Dha) and one dehydrobutyrine (Dhb), together with a lanthionine (Ala–S–Ala) residue and four β-methyl-lanthionines (Abu–S–Ala). The α-centers of the α-aminobutyric acid (Abu) moieties in the β-methyllanthionines are of the D-configuration. The remaining amino acids are all assumed to be of the L-configuration. The lanthionine and β-methyl-lanthionine residues introduce sulphur bridges at various points in the molecule, giving rise to 4.5 and 7-residue cyclic units and one very striking 4.4-residue bicyclic system.

Peptides sharing the unusual structural features of nisin, notably the presence of several sulphur-linked cyclic units, have also been isolated from a number of other micro-organisms.

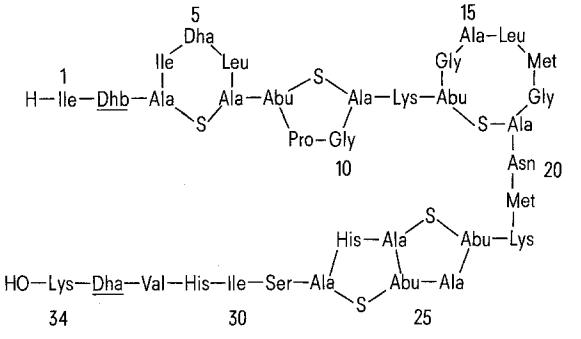


Figure 1. The structure of nisin.

Gross and Morrell[4] proposed a very similar structure for subtilin, a related peptide antibiotic produced by strains of *Bacillus subtilis* and active against gram-positive bacteria. Recently a related group of strongly basic peptides, the major component of which has been designated pep-5, containing lanthionine residues but no dehydroamino acids, but with as yet undefined structures, have been reported from *Staphylococcus epidermis*[5]. Several antibacterial peptides containing lanthionine and β-methyl-lanthionine have also been isolated from *Streptomyces* species over the past thirty years but because of their complexity the structures have remained poorly defined[6–8].

Until recently the size and complexity of modified peptides of this type were beyond the range of most physical techniques and structural information stemmed predominantly from chemical studies. The advent of FAB mass spectrometry[9] has offered a valuable new technique for not only extending the molecular weight range but also for structural studies on peptides. More recently sequence analysis of proteins by tandem mass spectrometry has been described[10]. The application of both these techniques to a nisin complex demonstrates the potential of the methodology to atypical peptides.

The FAB mass spectrum of the nisin complex was determined using as the solvent matrix water/acetonitrile/TFA 1:1:1 solution in thioglycerol. The low resolution survey scan revealed the presence of not only the protonated molecular ion species corresponding to the molecular weight of the proposed structure of nisin, but also ions corresponding to nisin + H_2O, nisin + thioglycerol, nisin + thioglycerol + H_2O (fig. 2). The major component in this particular sample, however, occurred at 3157.6 corresponding to nisin-216 mass units. A resolved spectrum of this component is shown in figure 3. Accurate mass measurements of several peaks in the isotope clusters were made using caesium and rubidium iodides as reference compounds to establish the monoisotopic molecular weights. Two measurements of the molecular weight gave a mean value of 3153.29 daltons which is in reasonable accord with the structure in figure 1 lacking the C-terminal Dha–Lys residues and containing a terminal Val–NH_2. A calculated theoretical isotope distribution was in good agreement with the observed pattern and the mass measurement of the signal was within 0.15 of a millimass unit for the molecular species $C_{134}H_{216}N_{40}O_{34}S_7$ corresponding to the hydrolysis product. The facile acid and enzymatic hydrolysis of nisin to this biologically inactive compound had already been noted[11] and is illustrated mechanistically in the scheme.

There are no fragment ions observed in the mass spectrum of the nisin complex above m/z 2179 daltons; however, below this mass there is considerable structural information. Cleavage of the α-CH–CO bonds with charge retention on the N terminal fragment being the predominant mechanism, occurring at residues 7, 8, 11–13 and 19–23, as shown in the table. Occasionally an associated peak occurs 45 daltons higher in mass resulting from cleavage of the NH–CH bond, again with charge retention on the N terminal fragment.

The masses of the lanthionine and methyllanthionine bridged rings are confirmed for the most part, by sequence ions on either side of them, but little fragmentation of the cyclic portions themselves appears to take place. Detailed information concerning the sequence of the C-terminal residue is almost non-existent. However, there are two pieces of evidence to suggest that the deficiency of Dha and Lys residues lies in the region Ala-28 to the end of the C-terminus. First, the rationalisation of the mass spectrum would indicate that the amino acid sequence and ring masses from the N-terminus to Ala-23 is as proposed by Gross, hence the

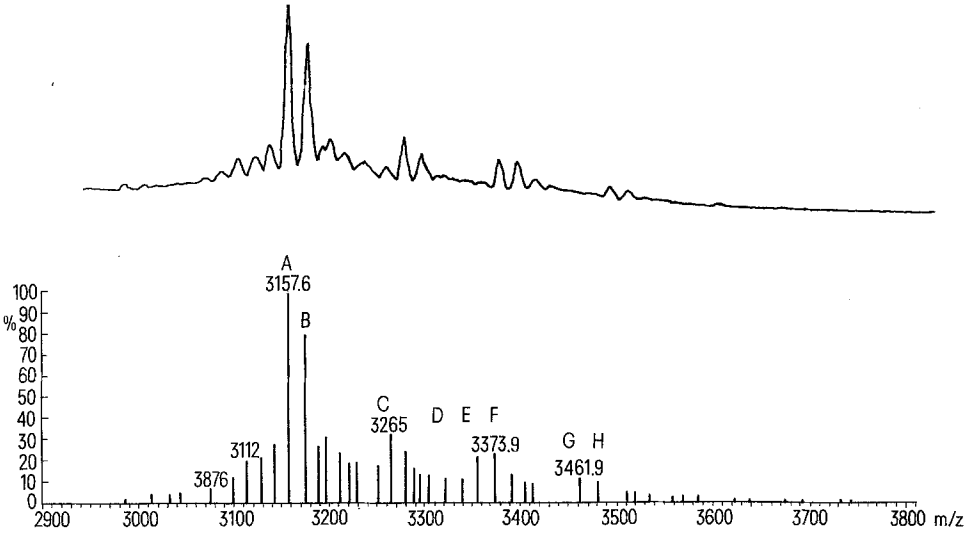


Figure 2. Low resolution FAB mass spectrum of nisin complex.
A = Nisin degradation product.
B = Nisin degradation product + H_2O.
C = Nisin degradation product + thioglycerol.
D = Nisin degradation product + H_2O + thioglycerol.
E = Nisin (as proposed by Gross and Morrell[2]).
F = Nisin + H_2O.
G = Nisin + thioglycerol.
H = Nisin + thiogylcerol + H_2O.

All data were acquired on a VG ZAB-4F tandem double focusing mass spectrometer having a B_1E_1–E_2B_2 configuration (B = magnetic sector, E = electrostatic sector). This instrument which has a mass range of 3000 dalton at 8 keV ion energy with specified parent and daughter ion resolving powers up to 100,000 and 5000 respectively, has been discussed elsewhere[10]. Mass spectra were acquired using B_1E_1 as a reverse geometry double focusing mass spectrometer, with an ion energy of 7 keV. Daughter ion mass spectra were acquired B_1E_1 to select the parent ion (MH^+) followed by collision induced decomposition (CID), using Ar as the target gas. The fragment ions produced were then analysed using E_2B_2 in a linked scanning (B_2/E_2) mode of operation. The collision gas pressure was set to give a 50% attenuation of the parent ion beam, measured at the final collector.

The atom source used was a modified saddle field gun (Ion Tech. Ltd., BIIN), operated at 8 keV and 1 mA tube current, using Xe as the bombarding gas. Samples were prepared by dissolving 5–10 μg of solid directly into 5 μl of thioglycerol.

Nisin was obtained from Aplin and Barret, Beaminster, UK.

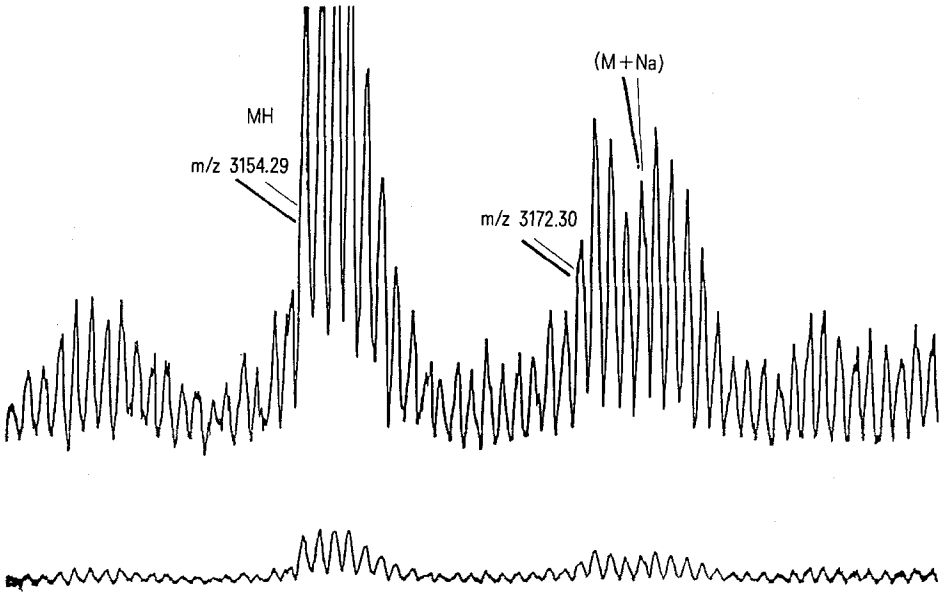
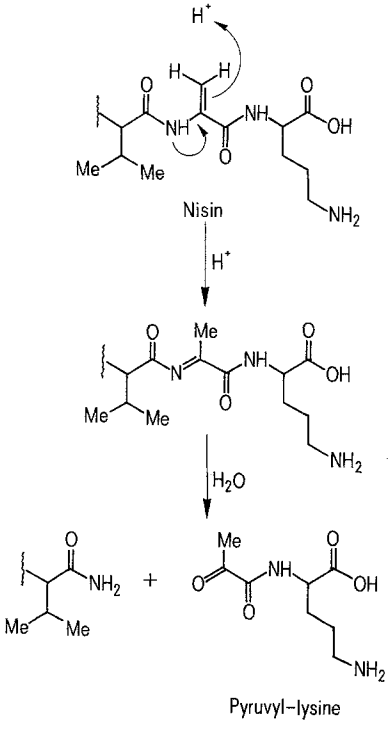


Figure 3. The molecular ion region of the positive ion FAB mass spectrum of nisin degradation product.



Scheme. Proposed mechanism for the acid hydrolysis of the C-terminal residues from nisin.

loss of the two amino acid residues lies beyond this particular Aba residue. Second, a series of ions occur at m/z 551, 523 and 508 dalton, which can be interpretated as arising by cleavage of the CH–CO, CO–NH and NH–CH bonds between His-27 and Ala-28 with the retention of sulphur on the Ala-25, the charge being retained on the C-terminal residue as illustrated in the table.

Although the resolution of the instrument should enable full unity mass resolved daughter ion spectra to be produced from a unity mass resolved parent, the paucity of ions produced by FAB at this mass precludes such an experiment. This limitation arises as a consequence of several factors including, the unavoidable trade off of sensitivity with resolution for both the parent and daughter ion analysers, and the fact that the absolute yield of MH^+ ions for peptides tends to decrease as mass increases and is distributed over a greater number isotope peaks. Furthermore, with this sample sodium adducts (M^+Na^+) and other components contribute to robbing of intensity from the species of interest. However, by operating the parent ion analyser at 1000 resolution to transmit the three major peaks in the isotope distribution of MH^+ of the hydrolysis product and the daugther ion analyser at about 400 resolution, a considerable amount of structurally significant information was obtained. The daughter ion spectrum was acquired in under 10 min from three sample introductions. After the first introduction the sample was merely refreshed with more thioglycerol, no new peptide was added. Under these conditions it should be noted, that, whilst the normal mass spectrum was calibrated manually up to m/z 2200 by counting and hence gives nominal masses (C = 12, H = 1, N = 14, O = 16 and S = 32), in the MS-MS experiment, since the unresolved isotope distribution of the MH^+ cluster is used, the masses of the parent and daughter ions are based on the chemical weights of the constituent atoms.

A line diagram of the mass spectrum is shown in figure 4, the masses correspond to the mass centroids of the daugther ion peaks. The mass scale was calibrated using caesium iodide cluster ions obtained under identical conditions and manual interpolation used. The MS-MS spectrum of the major component gives a considerable amount of information complementary to that obtained from the normal mass spectrum. However, the most important fragments are those occurring at m/z 3013, 2876, 2763 and 2676, arising by cleavage of the CH–CO bonds and confirming the C-terminal sequence to be –Ser–Ile–His–Val–NH_2. The N-terminal sequence up to the first sulphur bridge cannot be confirmed but an ion occurs at m/z 2960 corresponding to the loss of H–Ile–Dhb from the molecular ion. As with the mass spectrum, little fragmentation involving ring opening is observed, but cleavage between the cyclic portions of the molecule produces the necessary confirmation of the ring sizes. No fragmentation was apparent in the daughter ion spectrum below half the

Table 1

		FAB/MS CH–O	CO–NH	NH–CH		FAB/MS/MS CH–CO	CO–NH	NH–CH
H								
Ile								
Dhb	↓		197					
Ala	↓		300		↘		2960	
Ile								
Dha S								
Leu								
Ala	↓	636	664	681				
Abu	↓	721			↘	2549	2520	2478
Pro S								
Gly								
Ala	↓	976		1021				
Lys	↓	1104		1149	↘	2178	2150	2135
Abu	↓	1189			↘	2050	2023	2009
Gly					↘			
Ala								
Leu S								
Met								
Gly								
Ala	↓	1720		1765	↓	1721	1747	1763
Asn	↓	1834		1879	↓	1835	1863	1879
Met	↓	1967		2012	↓	1966	1993	2009
Lys	↓	2095						
Abu	↓	2179			↓	2178	2206	
Ala S								
Abu								
S Ala								
His	↘				↓	2605		
Ala		551	523	508	↓	2676	2704	
Ser					↓	2763		
Ile					↓	2876	2904	
His						3013		
Val								
NH_2								

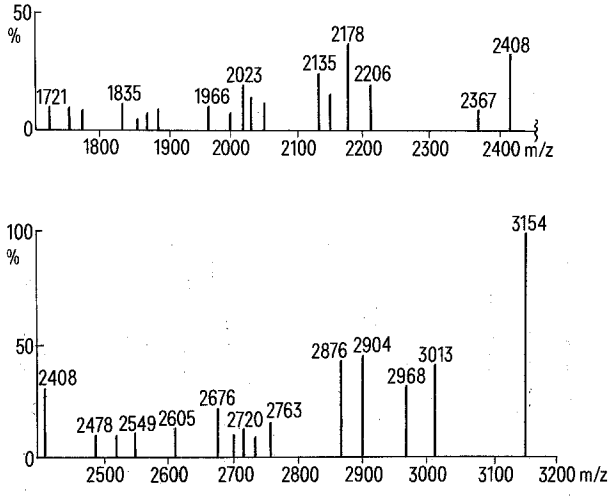


Figure 4. MS/MS spectrum of nisin degradation product.

molecular weight. The interpretation of the ions observed both in MS and MS/MS in terms of the proposed structure are shown in the table.

The MS/MS analysis represents a unique facility for examining the individual fragmentation of any defined molecular ion in a multi-component mixture, hence removing any ambiguity which may arise in attempting to interpret raw MS data. Both sets of data shown in the table are consistent with the major component of the nisin complex corresponding to the hydrolysis product. As already indicated there is evidence that the Val–Dha peptide bond is extremely labile to both mild acid and enzymatic hydrolysis and recent HPLC analysis of a number of nisin samples indicate that percentage of this component within the complex is variable depending on age and storage conditions [13]. As yet there is no evidence that this breakdown is induced by the liquid matrix (1-thioglycerol) within the mass spectrometer, nor is there evidence for the well-established addition of thiols to the dehydroamino acid residues present in the molecule. The higher mass component observed in the mass spectrum corresponds to the addition of H_2O to the nisin molecule. This could conceivably represent a similar hydrolysis to that described above for the labile Dha-5 residue within the first of the sulphur-bridged ring systems, or alternatively the replacement of either a Dha or Dhb residue by Ser or Thr respectively. Further studies are necessary to clarify this point. While there are a number of other points which remain to be resolved it has been demonstrated that the use of FAB mass spectrometry, and in particular its combination with MS/MS and collisionally induced dissociation, represents a powerful additional tool for structural studies on highly modified peptide mixtures of this type and complexity.

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o-Aminoacetophenone, a pheromone that repels honeybees (*Apis mellifera* L.)

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Summary. *o*-Aminoacetophenone is a pheromone produced by virgin honeybee queens and released with feces. In small social groups, the pheromone repels and is used to terminate agonistic interactions between queens and workers.
Key words. Pheromone; social behavior; honeybee.

The feces of virgin queen honeybees, *Apis mellifera* L., was recently reported to be a source of a pheromone that repels workers and releases autogrooming behavior[1]. When confined in small social groups, virgin queens *occasionally* release 10–30 µl of rectal fluid (feces) when workers behave agonistically toward them. They *frequently* release rectal fluid when they fight with other virgin queens. The release of feces usually terminates the agonistic behavior[1,2].

The chemical constituents of virgin queen rectal fluid have recently been identified[3] and include: 1. octyl decanoate, 2. decyl octanoate, 3. decyl decanoate, 4. tetradecyl decanoate, 5. decanoic acid, 6. dodecanoic acid, 7. octanoic acid, 8. benzoic acid, 9. 1-dodecanol, 10. octyl octanoate, 11. *o*-aminoacetophenone, and other higher molecular weight organic acids and hydrocarbons.

A 'strong floral odor' is associated with virgin queen feces and with the repellency observed[1]. Of these identified compounds *o*-aminoacetophenone (*o*-AAP) smells like grapes and is the most likely compound responsible for the floral odor. Two other compounds, octanoic acid (OA) and 1-dodecanol (1-DD) are sufficiently volatile, and unlikely to be normal metabolic constituents of feces, to warrant further investigation. Therefore, these compounds were selected as potential repellent pheromones and were assayed.

Materials and methods. Compounds were obtained from Sigma Chemical Company. Individual product numbers are C 2875, A 5158, and L 5375 for OA, *o*-AAP, and 1-DD, respectively. Each was diluted to a concentration of 1:1000 in mineral oil, Sigma product number M 3516.

Behavioral assays were identical to those previously reported[1]. Groups of 10 workers were collected from colonies into 235-ml cups. Each cup had a piece of filter paper attached to the bottom with a 1.8-cm diameter circle marked in the center. Cups were taken into the laboratory where all assays of groups of 10 bees were completed within 1 h.

For each replicate, a test and control observation arena (cup) were paired. Behavior of all bees in each arena was observed for 60 s before and after 10 µl of the specific compound diluted in mineral oil was placed in the center of the circle. The other arena of each pair received similar observational time before and after introduction of 10 µl of mineral oil. Two behavioral activities were recorded: 1) the number of times bees walked through the circle, and 2) the number of bees autogrooming after each 60-s interval. These correspond to activities that are altered significantly by virgin queen feces[1]. The responses of the workers to the tests and controls before and after addition of the test compound or the mineral oil control were summed for twenty replicate sets of trials for each compound and analyzed using a chi-square test of independence[4].

Results. Only *o*-AAP repels bees (table 1). The degree of repellency is equal to that previously shown for virgin queen feces[5]. No treatment compound increased autogrooming behavior significantly (table 2). However, mineral oil alone increased autogrooming significantly when the number of autogrooming workers before and after exposure to mineral oil (36 and 64, respectively) in all assays were considered ($\chi^2 = 7.84$, $p < 0.01$, $df = 1$). These results suggest that the higher molecular weight hydrocarbons found in virgin queen feces may be responsible for the increase in autogrooming reported previously.

As compared to the controls, octanoic acid reduced autogrooming behavior significantly. Whether this result is spurious or has real biological significance remains to be determined.

Table 1. Results and analyses of behavioral assays of different compounds for the number of worker trips through the circle on the bottom of the observation arena. Twenty replicate trials were conducted for each 60-s period before and after the introduction of control (mineral oil only) and treatment compounds.

	Number of trips	
	Control	Treatment
o-Aminoacetophenone		
Before	309	312
After	266	150
$\chi^2 = 20.29$, $p < 0.01$, $df = 1$		
Octanoic acid		
Before	348	420
After	324	355
$\chi^2 = 0.84$, $p > 0.05$, $df = 1$		
1-Dodecanol		
Before	348	340
After	306	317
$\chi^2 = 0.28$, $p > 0.05$, $df = 1$		