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The Interaction of Analogues of the Antimicrobial Lipopeptide, Iturin A_2, with Alkali Metal Ions

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Abstract—Electrospray mass spectrometry was employed as a tool in this first study on the molecular interaction between the alkali metal ions and antifungal lipopeptide iturin A, and some analogues. Cationisation by sodium and signal intensity of lipopeptide species depended on sodium concentration, but was independent of sample solvent, carrier solvent polarity and sample pH between 4 and 11. 8-Beta, a linear analogue of iturin A_2 (8-Beta; β-aminotetradecanoyl-NYNQPNS), and its shorter linear lipopeptide analogues, associated either one or two alkali metal cations, while the N→C cyclic peptides associated with only one cation. The chirality of the β-NC_{14} residue had a limited influence on the cationisation. It was observed that 8-Beta contained at least four interaction sites for a cation of which two, the C-terminal carboxylate and the side-chain of tyrosine, can take part in ionic interaction with a cation. It is proposed that the remaining two interaction centres of alkali metal ions are within the two type II β-turns found in conformation of natural iturin A. This was corroborated by the diminished capacity of the shorter peptides, in which one of the β-turns was eliminated to bind a second larger cation. All the lipopeptides showed the same order of alkali metal ion selectivity: $Na^+ > K^+ > Rb^+$. These results indicated a size limitation in the interaction cavity or cavities. The absence of, or observation of only low abundance, di-cationised complexes of cyclic peptides the indicated association of the cation in the interior of the peptide ring. It is thus hypothesised that alkali metal ions can bind in one of the two β-turns in the natural iturin A molecule.

Introduction

The eletrostatic interactions between metal ions and biomolecules are essential for life processes and have been implicated as the most influential driving forces in their functionality.[1] Metal ions, such as Mg^{2+} in nucleotide complexes, have an important structural function whereas Zn^{2+} participates in the catalytic mechanism of many enzymes, for example carboxypeptidase A. As secondary messenger in cells Ca^{2+} interacts with several proteins, whereas the redox pair Fe^{2+}/Fe^{3+} is utilised in electron transfer. The alkali metal ions, Na^+ and K^+, are very important in the osmoregulation of cells and nervous impulses, in which Na^+/K^+-ATP-ases play a major role. Any disruption of essential ion–biomolecule interactions or new 'foreign' interactions will consequently have an influence on the functioning of the cell as a live unit. Ionophores, such as the cyclic antibiotic dodecapeptide, valinomycin, disrupt the osmotic balance by specifically complexing with K^+ [2] and transporting it over the cell membrane.[3] Valinomycin is a perfect example of a biological counterpart of the crown ethers. Crown ethers are known to complex alkali metal ions in their cyclic cavity with oxygens as the chelating atoms.[4] The biological action of other cyclic antibiotic peptides, i.e., gramicidin S, a cyclic decapeptide[5,6] and one cyclic octalipopeptide in this study, the antifungal iturin A_2,[7,8] leads to the leakage of the alkali metal ions, Na^+ and K^+, over membranes. Gramicidin S has a β-pleated sheet conformation and also contains two type II′ β-turns[5,6] similar to that of iturin A.[9] Both peptides show affinity for alkali metal cations such as Na^+ and K^+.[10,11] Iturin A, however, precipitates in the presence of divalent cations such as Mg^{2+} and Ca^{2+}.[12] It has been suggested that specific interaction between iturin A and alkali metal ions may be an important feature of its mechanism of action.[13] The investigation of these antibiotic peptides and their interaction with alkali metal ions at an atomic level is therefore important to elucidate their structure–function relationship and mechanism of action.

Nuclear magnetic resonance (NMR), ion selective electrodes and conductive methods have been used to assess peptide–ion complexation.[14,15] The use of mass spectrometry as a tool to probe gas-phase peptide–ion complexation became possible with the development of new

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desorption and ionisation techniques. These techniques include fast atom bombardment (FAB),[16] matrix-assisted laser desorption/ionisation (MALDI)[17] and electrospray ionisation (ESI).[18–23] ESI-MS was pioneered by Dole et al.,[18] but Fenn[19] recognised its potential for analysis of macromolecules. ESI-MS has been extensively reviewed elsewhere.[19–23] FAB–MS permits the study of peptide–ion interactions in the gas-phase, but because of the nature of ionisation, these studies may exclude some interactions that depend on the prior aqueous conformation of the peptide. Electrospray ionisation makes it possible to also observe weak non-covalent interactions that depend on native conformation.[24] Solution and ESI-MS instrument parameters can be optimised to balance the processes occurring in solution as well as in the gas-phase as many parameters affect ion formation during electrospray ionisation, such as solvent pH,[25–27] solvent and analyte pK_a,[28] solvent composition,[29] solvent polarity[30] and ionic strength.[30–32]

This is the first report on the molecular interaction between alkali metal ions and the antifungal lipopeptide, iturin A, as investigated with ESI-MS. The structure of the cyclic lipopeptide in this study, iturin A_2 (cyclic 8-Beta I (D)), includes a type II β-turn in each of the tetrapeptide moieties: β-D-amino tetradecanoyl-L-Asn_2-D-Tyr_3-D-Asn_4 and L-Gln_5-L-Pro_6-D-Asn_7-L-Ser_8 (refer to Table 1 for the iturin A_2 structure).[9] A linear analogue of iturin A_2, 8-Beta, with the β-aminotetradecanoic acid (β-NC_{14}) as N-terminal residue and L-Ser_8 as C-terminal residue (Table 1) was used as model peptide and Na^+ as model ion. The influence of sodium concentration in the sample, pH and pre-incubation time with sodium on the cationisation of 8-Beta during ESI-MS was investigated. ESI-MS was also used to study the interaction between alkali metal ions, Na^+, K^+ and Rb^+, and a series of iturin A_2 analogues in which one of the β-turns was sequentially eliminated. In 7-Beta and cyclic 7-Beta the L-Asn_2 residue was omitted, in 6-Beta the L-Asn_2-D-Tyr_3 dipeptide unit, and in 5-Beta the L-Asn_2-D-Tyr_3-D-Asn_4 tetrapeptide unit (Table 1). We also investigated the influence of the chirality of the β-NC_{14} residue in some of the lipopeptides on the association with alkali metal ions. The primary structures of all the iturin A_2 analogues used in this study are given in Table 1.

Results and Discussion

Interaction of the model linear iturin A_2 (8-Beta) with sodium

The influence of 10 mM NaCl on the ESI-MS spectra of linear iturin A_2 (8-Beta, the longest linear lipopeptide) is shown in Figure 1. The sodium binding curves depicted in Figure 2 show that 8-Beta is nearing 'saturation' at 10 mM NaCl. Under ESI-MS conditions, half-maximal saturation of 8-Beta with two sodiums was achieved at 2 mM NaCl.

Ion clustering[33,34] and signal suppression, as a consequence of the counter anion effect,[30–32] was observed in spectra recorded at saturating concentrations of 40 mM and 80 mM NaCl (results not shown). Signal suppression of ±26% by 10 mM NaCl was observed. Cationised species containing one sodium ($[M+Na]^+$) and two sodiums ($[M+2Na-H]^+$), with signal intensities between 60 and 90% of that of the free peptide ($[M+H]^+$), were observed in fresh samples (compare Fig. 1(A) and (B)). Molecular species, associated with three sodiums ($[M+3Na-2H]^+$, $[M+3Na-H]^{2+}$), were also observed at very low signal intensities. The singly charged species $[M+3Na-2H]^+$ was absent in most of the spectra. After a protracted incubation (three months of storage at −20 °C), a substantial increase in the signals of the multi-cationised species was observed (compare Figure 1(B) and (C)). The signal of $[M+3Na-H]^{2+}$ increased to about 50% of the free peptide signal, and a new species, associated with four sodiums $[M+4Na-2H]^{2+}$ ($m/z=575$), was also detected. These changes in the spectrum are indicative of a gradual association of 8-Beta with sodium in solution. 8-Beta also has a tendency to aggregate in solution with time, which may also influence the solution equilibrium between sodium and the lipopeptide. An 8-Beta molecular species, containing four sodiums ($[M+4Na-3H]^+$), was also detected at very low signal intensity, at pH 12 (results not shown).

Table 1. Primary structures of the iturin A_2 analogues in this study

Iturin A_2 analogue[a]	Peptide primary structure	M_r
5-Beta	β-NC_{14}-L-Gln-L-Pro-D-Asn-L-Ser	670.8
6-Beta	β-NC_{14}-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	783.9
7-Beta I (D)	β-D-NC_{14}-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	947.1
7-Beta II (L)	β-L-NC_{14}-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	
8-Beta II (D) (linear Iturin A_2)	β-D-NC_{14}-L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	1061.2
8-Beta I (L)	β-L-NC_{14}-L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	
Cyclic 7-Beta II (D)	β-D-NC_{14}-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	929.1
Cyclic 7-Beta I (L)	β-L-NC_{14}-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	
Cyclic 8-Beta I (D) (Iturin A_2)	β-D-NC_{14}-L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	1043.2
Cyclic 8-Beta II (L)	β-L-NC_{14}-L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	

[a]The number in the name refers to the number of residues, the roman I or II to the elution order from C_{18}-HPLC and the (L) or (D) to the chirality of the β-NC_{14}-residue in the peptide. Omission of I/II and L/D indicates a diastereomeric peptide mixture.

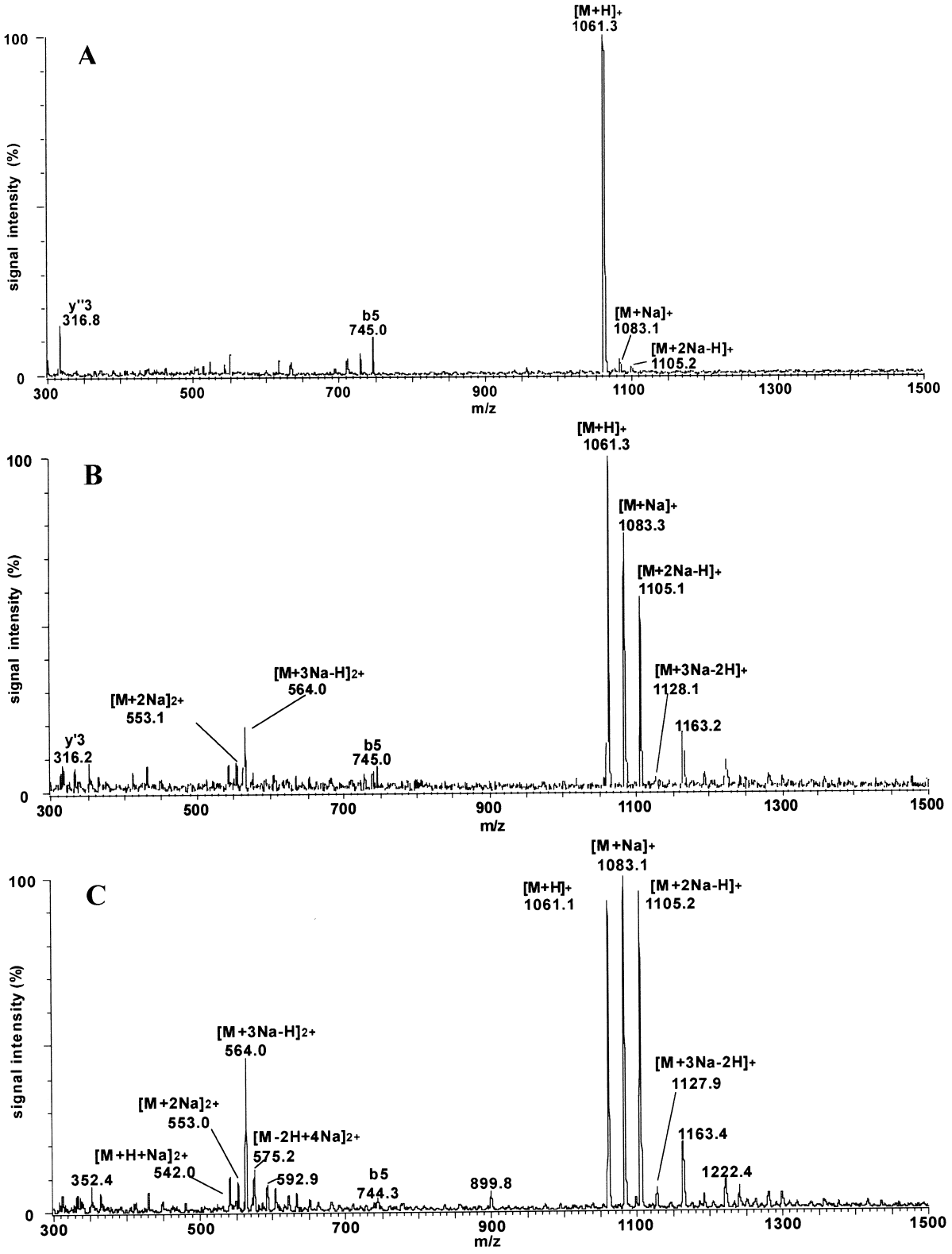

Figure 1. ESI-MS spectra of 8-Beta before (A) and after incubation with NaCl where (B) is the spectrum obtained with freshly prepared sample and (C) after 3 months cold storage. Spectra were obtained at 70 V cone voltage using 50% acetonitrile in water as carrier solvent. The sample consisted of 0.2 mg/mL peptide (without (A) or with 10 mM NaCl (B and C)) dissolved in 50% acetonitrile containing 0.05% TFA.

A sample pH between 4 and 11 had little influence on the cationisation and signal intensity of the sodium adducts of 8-Beta, whereas the signal for the free peptide decreased substantially over this pH-range (Fig. 3). Over this pH-range the relative constant signal intensity of the sodiated 8-Beta species indicated the contribution of associated sodium ions to the overall positive charge of these complexes (Fig. 3). Signal depression at the extreme pH values is the consequence of ion-pairing. Masking of the positive ions by counter anions (chloride

or hydroxide ions) in the sample eradicated the improved ionisation at lower pH values.[30–32] The deprotonation of the peptide, however, is also a major factor in the loss of signal at high pH-values (Fig. 3). The detection of the free peptide over an apparently broad sample pH-range (Fig. 3) correlated with results of Kelly et al.[27] that showed that solution pH is not the determinant for multiply charged proteins in ESI-MS. The detection of the free peptide could be ascribed to the oxidation of water in the ESI-MS capillary. This oxidation would yield protons and lower the pH of the solution[25] with up to two units under certain conditions.[26]

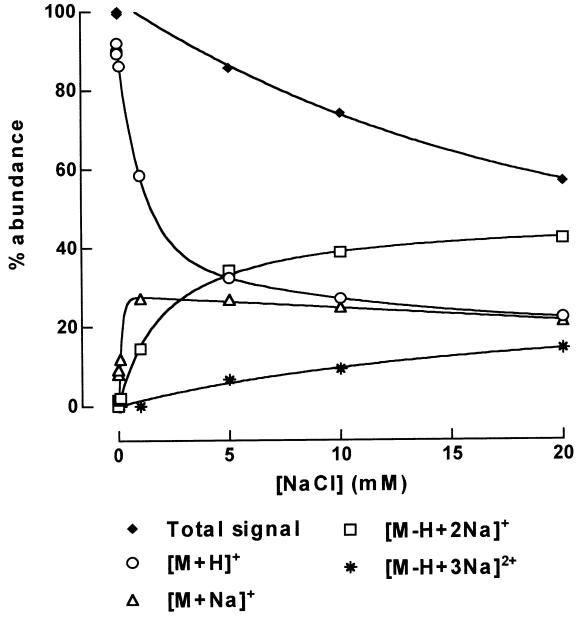

Figure 2. Influence of NaCl concentration on cationisation of 8-Beta with sodium as observed by ESI-MS at a cone voltage of 70 V. Results were calculated as a percentage of the sum of the signals of the four species under consideration. The signal values of $[M+2Na-H]^+$ were corrected with a factor of 1.7 for comparison. The influence of NaCl concentration on total signal is also depicted with the 100% signal taken as the total ion current of the spectra obtained with no added NaCl.

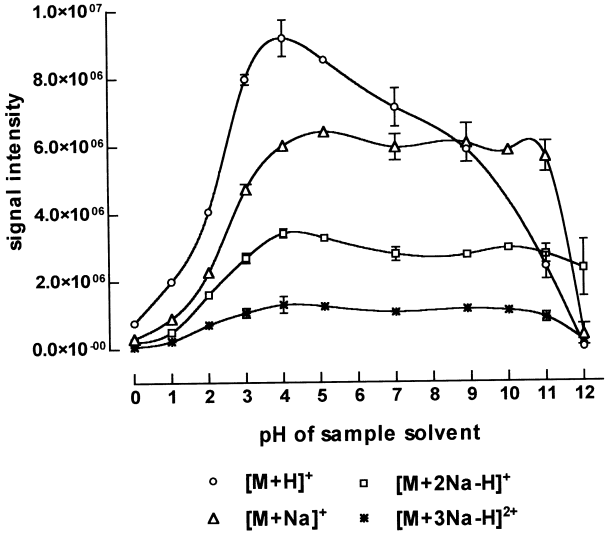

Figure 3. Influence of pH of the sample solvent on detection and cationisation of 8-Beta with sodium using ESI-MS at 70 V cone voltage. All samples contained 10 mM sodium. Duplicate samples were analysed and the standard error of the mean (SEM) is shown for each value.

From these results it can be deduced that there are at least four sodium binding sites on the linear octalipopeptide, 8-Beta, of which only two, the C-terminal carboxylate group and the tyrosyl residue side-chain (phenolate group), can take part in ionic association with sodium.

Influence of the iturin A_2 analogue's primary structure on interaction with sodium

The free molecular ions of the octa- and heptalipopeptides were found to be extremely stable, while the two shorter peptides tended to fragment between the Pro and Gln residues under the ESI-MS conditions in this study (results not shown). Highly stable sodiated molecular ions (cationised species) in all the lipopeptide samples, modified with 10 mM NaCl, were detected using positive mode ESI-MS (Figs 1, 4, 5). Yuan et al.[35] also observed high stability under their ESI-MS conditions for the sodium adducts of mycrocystins (cyclic heptapeptides produced by cyanobacteria). The ESI-MS signal of the sodiated species of the linear lipopeptides ranged between 55 and 70% (Figs 1 and 6) and that of the cyclic lipopeptides between 90 and 95% of the total peptide signal (Figs 4 and 5). For this discussion we assumed that the signal intensities of the gas-phase ions of the linear peptides are at least an indication of their concentrations in solution. This general assumption is supported by several experimental studies.[36–45] It must be noted, however, that there have been exemptions of this view.[32,46–50]

It was generally found that association with sodium increased with length (molecular mass) of the linear lipopeptides, although a linear relationship was not demonstrated. This is probably because alkali metal ions tend to associate with basic carbonyl groups.[51,52] Very little difference in sodium association between the two diastereomers of 7-Beta was found. A small difference (±4%) in association between the diastereomers of 8-Beta was detected and may be the consequence of a greater influence of the β-NC_{14} residue on the conformation of

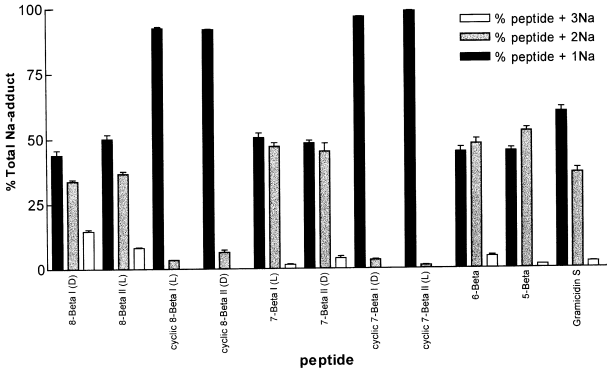

Figure 4. Preference of each of the iturin A_2 analogues and gramicidin S to either associate with one, two or three sodium ions as observed under specified ESI-MS conditions. The mean of quadruplicate values and SEM for each of the detected molecular peptide species are shown.

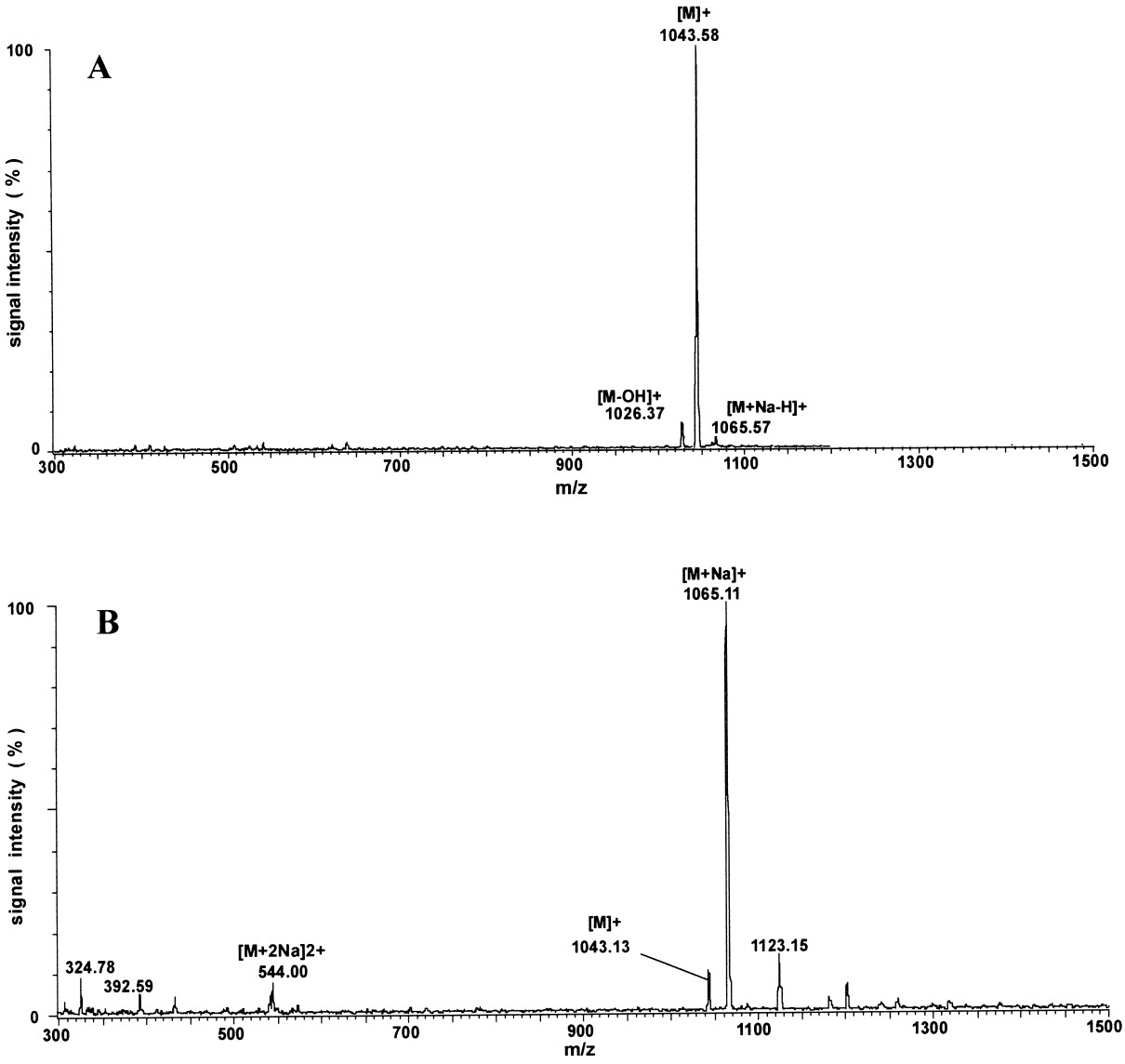

Figure 5. Positive ESI-MS spectra of cyclic 8-Beta I (D) (synthetic iturin A_2) before (A) and after incubation with NaCl (B). The sample consisted of 0.2 mg/mL peptide (without (A) or with 10 mM NaCl and 0.05% TFA (B)) dissolved in 50% acetonitrile/water.

this longer peptide. Elimination of amino acids from one of the polar β-turns lead to more hydrophobic iturin A_2 analogues. These shorter peptides were retained longer on the hydrophobic octadecanoyl silane matrices used in reverse phase HPLC (results not shown). In general the shorter linear peptides tended to associate less with sodium, although all the linear peptides, with almost equal distribution, associated with either one or two sodium ions (Fig. 4). The longest linear peptides, 8-Beta I (D) and 8-Beta II (L), also formed complexes with a third sodium ion. Higher abundance of the mono-sodiated species was detected in the longer peptides, whereas the di-sodiated species of the shorter peptides predominated. This is attributed to differences in signal intensity and/or the stability of the different sodiated species under the specified ESI-MS conditions.

The four cyclic peptides, derived from 7-Beta and 8-Beta, differed very little from each other in their tendency to associate with sodium. Cationisation by sodium improved the ESI-MS detection of these uncharged free cyclic peptides. The singly sodiated species of the four cyclic peptides predominated (>90% of total signal) (Figs 4 and 5). An inverse relationship between the molecular mass of the cyclic peptides and sodium association was found (results not shown). It thus appears as if the shorter cyclic peptides, cyclic 7-Beta diastereomers, bind sodium with higher affinity than the longer cyclic 8-Beta diastereomers, suggesting that the sodium ion binds within the cyclic peptide's cavity ('tighter' binding in a smaller cavity). The low abundance or absence of di-cationised complexes is further evidence suggesting association in the interior of the peptide ring. Electrostatic repulsion could be restricting the inclusion of a second cation. The cyclic peptide, valinomycin, is such an example, with a non-solvated alkali metal ion (especially potassium) co-ordinated to the carbonyl oxygens in the cyclic cavity.[2,3] The longer cyclic gramicidin S, used as control, bound to either one or two sodium ions, resembling the sodium

association pattern of the linear lipopeptides (Fig. 4). A previous study of the association of gramicidin S with alkali metals indicated that the alkali metal ion is bound to the exterior of the peptide ring, thus avoiding the charge repulsion of two cations in the interior of the peptide ring.[53]

Alkali metal ion selectivity of the iturin A_2 analogues

To probe the specificity and interaction cavity size of the alkali metal ion binding sites of the different iturin A_2 analogues, Na^+, K^+ and Rb^+ were incubated with these analogues (refer to Table 1 for structures and nomenclature). The influence of the peptide ring size and blocking of the N- and specifically the C-terminal, on peptide–ion interaction was investigated using the cyclic analogues of the peptides 7-Beta and 8-Beta.

Other investigators showed that natural iturin A has a tendency to associate with cations, such as the alkali metal ions, sodium and potassium.[10] We therefore investigated this again by using a competition assay in which the different lipopeptides were incubated with the chloride salts of sodium, potassium and rubidium. Difference in abundance of complexes between iturin A_2 analogues and the alkali metal ions Na^+, K^+ and Rb^+ was related to the accessibility of the interaction sites. A range of cationised peptide species, associated with either one or two alkali metal ions, was found for all the linear peptides. In contrast, only mono-cationised species were detected for the cyclic peptides. The ESI-MS spectra of 7-Beta and cyclic 7-Beta, in Figure 6, illustrate these observations. However, the major molecular species detected for all the lipopeptides, linear and cyclic, was the mono-sodiated species. Abundance of the mono-cationised species decreased with increase in size of the cation for all the peptides, indicating at least a size limitation in the 'interaction cavity' (Fig. 7). This size limitation was further demonstrated by the fact that only mono-cationised species were detected for all the cyclic

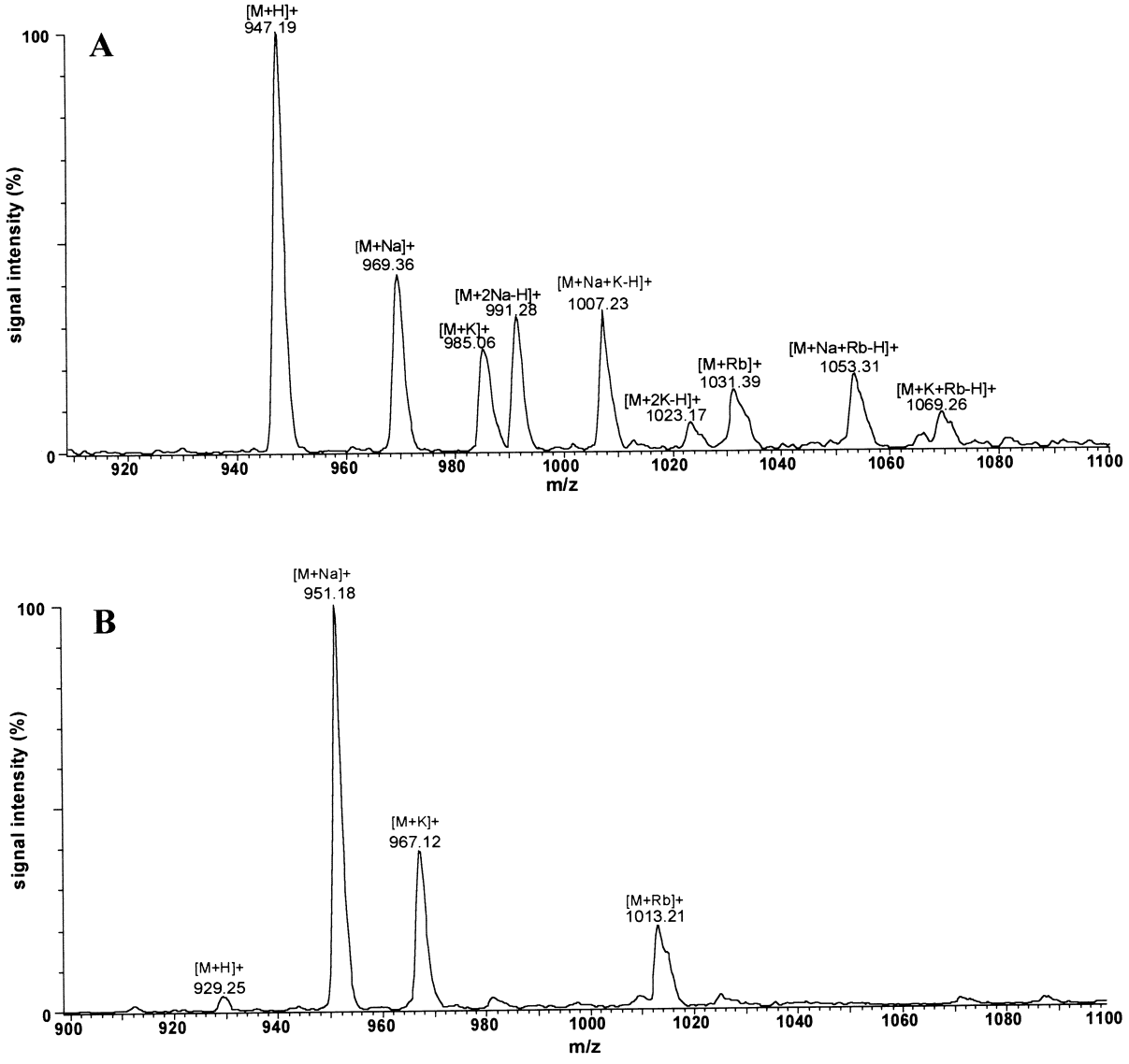

Figure 6. ESI-MS spectra of 7-Beta (A) and cyclic 7-Beta (B) incubated in the presence of NaCl, KCl and RbCl. The sample consisted of 0.2 mg/mL peptide dissolved in 50% acetonitrile/water modified with 0.05% TFA and 10 mM of each of the salts.

lipopeptides (Fig. 7). In the case of the cyclic peptides it was clear that only one alkali metal ion "interaction cavity" was accessible. All the lipopeptides showed an identical gas-phase selectivity for the alkali metals, namely: $Na^+ > K^+ > Rb^+$, which is the inverse of that observed for the longer valinomycin in methanol.[2]

Substantial amounts of di-cationised species, containing either two Na^+ or Na^+ and K^+, were detected for the longer linear peptide pairs of 8-Beta and 7-Beta (Fig. 7(a)). These results indicated that at least two "interaction cavities" are present in the linear hepta- and octa-lipopeptides. Di-cationisation of the shorter 5-Beta and

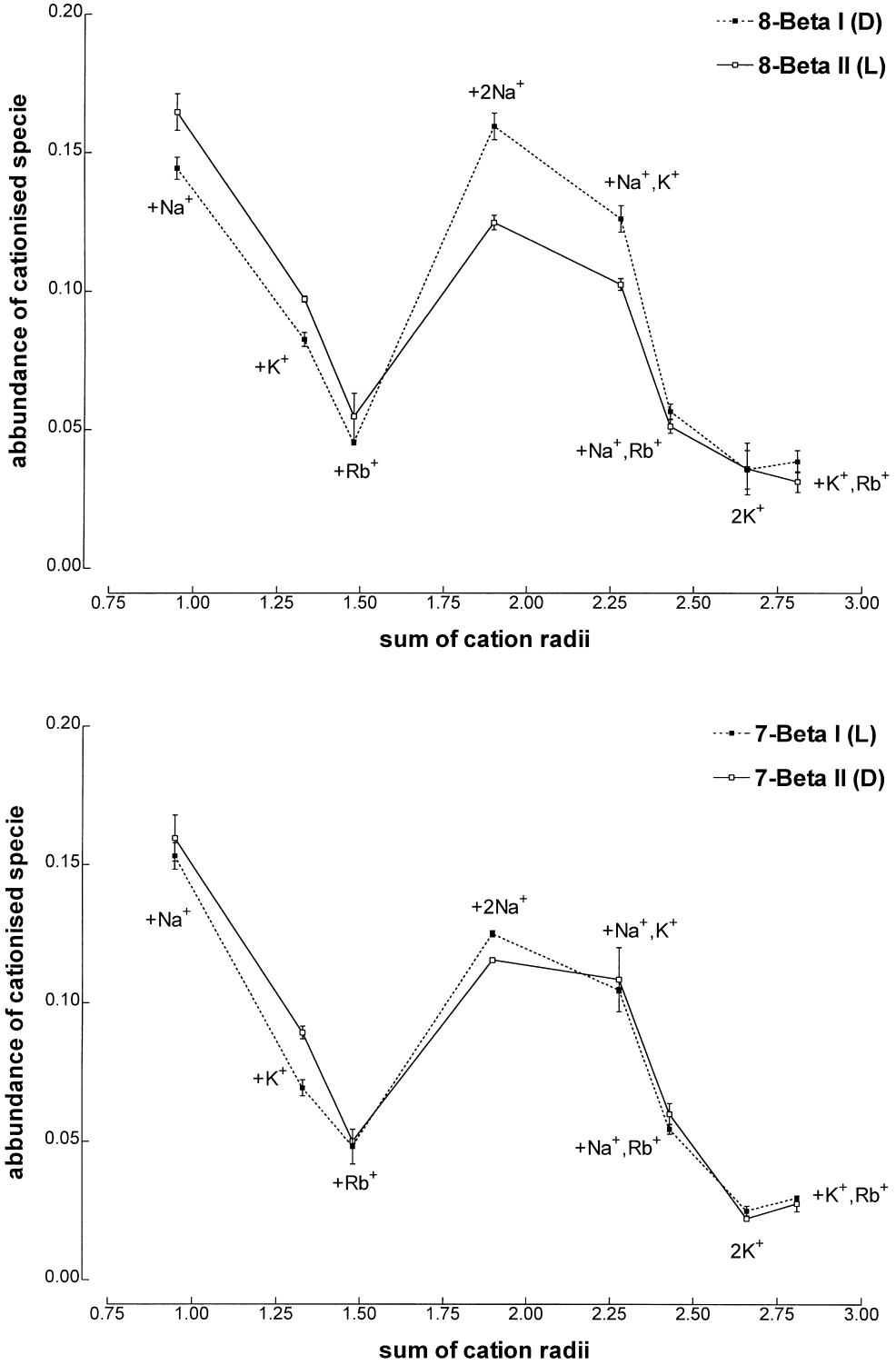

Figure 7(a). Relationship between the abundance of cationised peptide species and cation radii. The data points are the average of duplicate sample analyses. (Cation radii: $Na^+ = 0.95$ Å; $K^+ = 1.33$ Å; $Rb^+ = 1.48$ Å.[54]) (*continued on next page*)

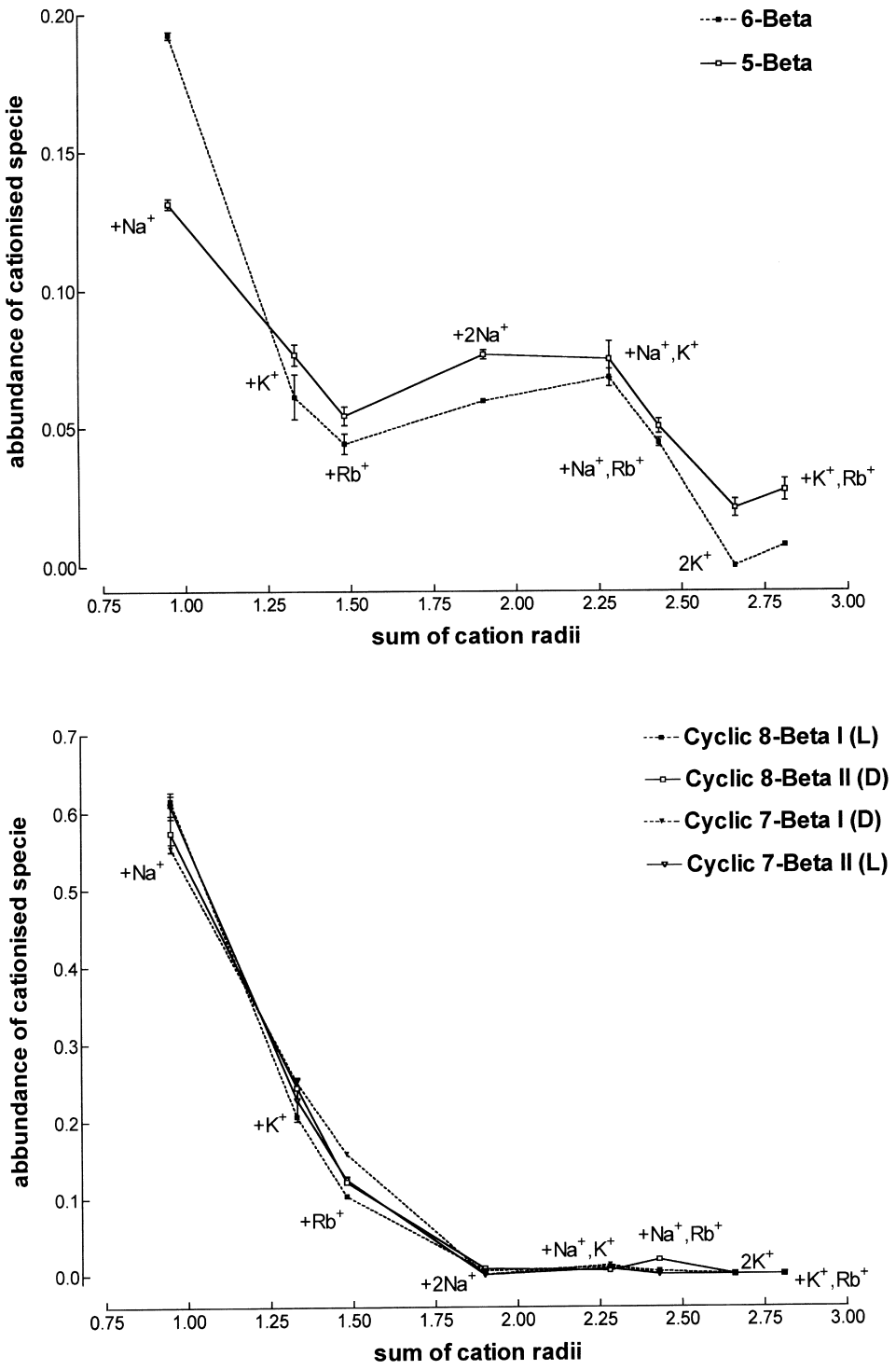

Figure 7(b). Relationship between the abundance of cationised peptide species and cation radii. The data points are the average of duplicate sample analyses. (Cation radii: $Na^+ = 0.95$ Å; $K^+ = 1.33$ Å; $Rb^+ = 1.48$ Å.[54])

6-Beta was greatly impaired (Fig. 7(b)), whereas di-cationised species of the cyclic peptides were virtually absent (Figs 6 and 7(b)).

Conclusions

The stable nature and ease of detection of iturin A_2 analogues and its cationised species allows the use of ESI-MS to study the specific interactions of these peptides with alkali metals. The cationisation of 8-Beta with sodium probably takes place in the solution and the complexation equilibrium is slow. Furthermore, under ESI-MS conditions, the lipopeptide–sodium interaction is not influenced by solvent polarity (25, 50 and 80% acetonitrile in water; results not shown) and is also independent of pH over a broad range. From the pH study it was further deduced that there are at least four

binding sites for sodium to 8-Beta of which two, the C-terminal carboxylate and the side-chain of tyrosine, can take part in ionic interaction with sodium. Results from the pH study also showed that the pH is not as important as the concentration of the counter anions in the sample solvent. We also found, as did other investigators,[30–32] that the influence of solvent counter ions on ionisation during the electrospray process must not be underestimated.

The interaction with sodium was influenced by the primary structure of the linear peptides, as a higher abundance of sodiated species of longer peptides was observed. This is probably the result of decrease in essential carbonyl oxygens by the sequential elimination of the N-terminal β-turn in the shorter peptides. The chirality of the β-NC_{14} residue had a limited influence on the cationisation under ESI-MS conditions. The cyclic peptides bound to a single sodium with a slightly higher abundance observed for sodiated cyclic 7-Beta diastereomers, having a smaller cyclic cavity than the sodiated cyclic 8-Beta diastereomers. These results indicated that cation-binding site was situated inside the peptide ring.

The order of alkali metal ion selectivity of all the lipopeptides was $Na^+ > K^+ > Rb^+$, indicating a size limitation in the interaction cavity or cavities. The omission of amino acid residues in one of these cavities, as in 5-Beta and 6-Beta, leads to a diminished capacity to bind a second larger cation. 6-Beta and 5-Beta contained only one complete β-turn sequence, in the C-terminal tetrapeptide moiety QPNS, indicating that this is one of the interaction cavities. It can therefore be hypothesised that a second cavity is situated in the N-terminal tetrapeptide moiety, β-NC_{14}-NYN. The interaction of 8-Beta with sodium in these interaction cavities has been shown to be sequence specific in an investigation using tandem MS and will be reported shortly.[55]

Association of the cation in the interior of the peptide ring of the cyclic peptides is proposed because of the absence of, or observation of only low abundance, dicationised cyclic peptide complexes, and the slightly better association of the cyclic heptalipopeptides with sodium. The inclusion of a second cation may be unfavourable because of electrostatic repulsion. This resembles valinomycin accommodating a single cation in its cyclic cavity[2,3] and not gramicidin S that binds the alkali metal cations on its exterior.[53] It is therefore hypothesised that alkali metal ions can bind in either one of the two β-turns in the peptide ring of natural iturin A molecule, with the carbonyl oxygens as chelating atoms.

Experimental

Materials

HPLC grade trifluoroacetic acid (TFA, 99.5%), NaCl (99.9%), KCl (99.9%), HCl and NaOH were from Merck (Darmstadt, Germany). Acetonitrile (HPLC-grade, UV cut-off 190 nm) was from Romil Ltd (Cambridge, UK). Gramicidin S, triethylamine (TEA) and RbCl (99.9%) were from Sigma Chemical Co. (St. Louis, USA). Analytical quality water was prepared by filtering glass distilled water through a Millipore Milli Q® water purification system. The synthetic iturin A_2 analogues were synthesised with the Fmoc-polyamide peptide synthesis protocol, HPLC purified and analysed by the BIOPEP Peptide Synthesis Laboratory, Department of Biochemistry, University of Stellenbosch.

Sample preparation for ESI-MS analysis

Lyophilised HPLC purified 8-Beta (a racemic mixture of 8-Beta II (D) and 8-Beta I (L)) was dissolved in a mixture of 50% acetonitrile in analytical quality water (glass-distilled, de-ionised) to a concentration of 5.0 mg/mL. This stock solution was diluted to 0.2 mg/mL (±0.2 μmol/mL) with 50% acetonitrile modified with 0.05% TFA in water, except when stated otherwise. To determine the influence of sodium concentration, an NaCl concentration range from 0.1 μM to 80 mM was used. All samples in the remaining investigations contained 10 mM NaCl. To investigate the influence of the pH of the sample solution, 0.2 mg/mL of 8-Beta was dissolved in analytical grade water and the pH adjusted with standardised HCl (pH 0, 1.0, 2.0, 3.0 and 4.0), TFA (pH 5.1), TEA (pH 7.0 and 8.9) and standardised NaOH (pH 10.0, 11.0 and 12.0). The sodium concentration was kept constant at 10 mM in all these samples. All samples were freshly prepared before analysis, except in the one case when the influence of cold storage (−20 °C) on cationisation of 8-Beta was examined. To investigate the structures of the cationised 8-Beta and the effect of cationisation with sodium on its stability, peptide (0.2 mg/mL) in 50% acetonitrile/water modified with 0.05% TFA and 10 mM of NaCl was used.

To investigate the influence of primary structure on cationisation, the purified diastereomers of 8-Beta, 7-Beta, cyclic 8-Beta and cyclic 7-Beta and the isomeric mixtures of 6-Beta and 5-Beta were analysed in the presence of different alkali metal ions (Na^+, K^+ and Rb^+). The purified peptide (0.2 mg/mL) in 50% acetonitrile, modified with 0.05% TFA in water, was incubated with NaCl, KCl and RbCl. In this competition assay all three of the alkali metal ions (10 mM of each chloride salt) were used.

Electrospray ionisation mass spectrometry

ESI-MS was performed using a Micromass triple quadrupole mass spectrometer fitted with an electrospray ionisation source. The carrier solvent was 50% acetonitrile in water and the solvent was delivered at a flow rate of 20 μL/minute during each analysis using a Pharmacia LKB 2249 gradient pump. Ten μL of the sample solution (±2 nmol peptide) was introduced into the ESI-MS using a Rheodyne® injector valve. A capillary voltage of 3.5 kV was applied and the source temperature was set at 80 °C. The skimmer lens offset was 5 V and the cone voltage 70 V.

Data acquisition was in the positive mode, scanning the first analyser (MS_1), through $m/z = 300$ to 1300 or 1500 at a scan rate of 100 atomic mass units/second. Representative scans were produced by combining the scans across the elution peak and subtracting the background. Signal intensity of each of the ions under investigation was determined by integrating the ion specific chromatographic peaks detected during analysis.

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