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The unusual loss of an internal Val residue from the $(M - H)^-$ parent anions of the antimicrobial peptide citropin 1.1 and synthetically modified analogues Fragmentations which require a specific conformation of the decomposing anion

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This paper is dedicated to Dudley H. Williams on the occasion of his retirement and to celebrate his contributions to science.

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Abstract

The electrospray negative ion mass spectra of citropin 1.1 (GLFDVIKKVASVIGGL-NH_2) and of synthetically modified analogues show loss of an internal Val residue from both the $(M - H)^-$ anion and certain fragment anions. The negative ion spectra of GLFDVIQQVASVIGGL-NH_2 and of three synthetically modified versions where Val residues are successively replaced by Ile shows that it is Val12 which is eliminated. A joint experimental and theoretical study indicates that this internal rearrangement requires the decomposing anion to have an α-helical structure in the reaction region. If the conformation of the anion is randomised, either by increasing the internal energy of the decomposing $(M - H)^-$ anion, or by making the electrospray solution strongly acidic or strongly basic, the $[(M - H) - Val]^-$ fragmentation is either reduced or no longer observed. Using isopropanol (rather than water/acetonitrile) as the electrospay solvent enhances the abundances of $[(M - H) - Val]^-$ peaks (compared with normal backbone cleavage peaks) up to 30 times. The process is initiated by the C-terminal $CONH^-$ group reacting at the carbonyl centre of the amide function situated between Ser11 and Val12. The reacting groups can approach to within 2.5 Å when the peptide has an α-helical structure in the reaction region. Nucleophilic substitution followed by decomposition of the tetrahedral intermediate gives a rearranged peptide with the original Val12 now occupying the C-terminal position. Calculations at the HF/6-31G(d)//HF/6-31G(d) level of theory indicate that formation of the initial tetrahedral intermediate is exothermic (-11.9 kcal mol^{-1}) with the rearrangement sequence being endothermic (17.1 kcal mol^{-1}). The overall reaction requires the $(M - H)^-$ anion to have an excess energy of 40.8 kcal mol^{-1} at the level of theory used in this study. The $[Aspγ–H_2O–CH_2O]^-$ fragment anion in the negative ion spectrum of citropin 1.1 rearranges to a similar tetrahedral intermediate which undergoes competitive loss of Val and cleavage of the peptide backbone. These reactions require the $[Aspγ–H_2O–CH_2O]^-$ decomposing anion to have a helical structure in the vicinity of the reaction centre.
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Keywords: Citropin 1.1 and synthetic analogues; Antimicrobial peptide; Electrospray negative ion spectra; Internal loss of Val

1. Prologue

Dudley Williams and I arrived at Cambridge (UK) at about the same time in the mid 1960s, Dudley to be Assistant Director of Research and I to do postdoctoral work with Lord Todd and D.W. Cameron. For nearly 2 years, I worked with Dudley 'after hours' mainly on MS but also some NMR studies. I ran the mass spectra, did the mass measurements and interpreted some the spectra (as best I could) and Dudley wrote the papers. There were also other well known names 'moonlighting' with Dudley at this time including Graham Cooks who was then doing his (second) Ph.D. (with Peter Sykes). I learnt two very important things from Dudley, namely (i) his work ethic, and (ii) his unbounded and infectious enthusiasm for scientific research (at a time when such overt enthusiasm was frowned on by some of the then Cambridge establishment).

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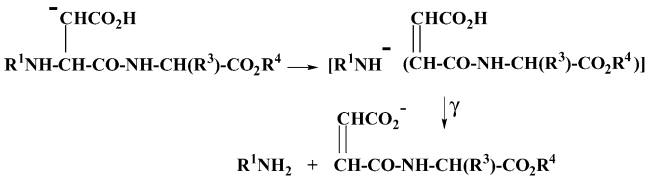
2. Introduction

We have isolated and identified more than 200 bioactive peptides from Australian amphibians in the last 15 years [1], and until recently, we have used positive ion mass spectrometry [2] along with the automated Edman degradation method [3] as the primary sequencing tools. We have also been investigating the negative ion spectra of these (and other) underivatised peptides over the same period, and within the last few years we have begun to routinely use the positive and negative ion cleavages in concert, to determine the amino acid sequence of peptides. The negative ion fragmentations of small peptides are generally complex, and only marginally useful for sequencing purposes, but for peptides of larger than say six residues, the negative ion method is very useful. We have recently reviewed our work (to 2002) on this topic [4]. In summary, MS/MS data from $(M - H)^-$ anions of peptides provide the following information: (a) α and β cleavages (see Scheme 1) provide data from backbone cleavages equivalent to those provided by B and Y + 2 cleavages of protonated peptides, except that the mechanisms of the positive and negative ion fragmentations are quite different, (b) side chain fragmentations that are characteristic of specific residues [Ser (–CH_2O), Thr (–MeCHO), Cys (–H_2S), Asp and Glu (–H_2O), Asn and Gln (–NH_3)] may dominate the spectra, and (c) particular residues which effect specific backbone cleavages initiated from anion sites on the side chain of that amino acid residue—this is most prevalent for Ser, Thr, Cys, Asp, Asn, Glu and Gln. The mechanisms of these backbone cleavages are not all the same, but they all give rise to a fragment anion that we have named a γ ion. An example of a γ cleavage is shown for Asp in Scheme 2. In principle, the negative ion spectrum may give the same information provided by the positive ion spectrum, and, in addition, give the extra information outlined in (b) and (c) above. Sometimes the fragmentations induced by particular residues (see (b) and (c) above) may be more pronounced than peaks arising from the common backbone cleavages shown in Scheme 1. A particular application worth mentioning is that low resolution negative ion spectra of peptides can often differentiate between Lys and Gln, because Gln initiates a side-chain initiated backbone cleavage whereas Lys does not.

Having studied what we thought were all the characteristic backbone cleavages of the amino acid residues of peptides,



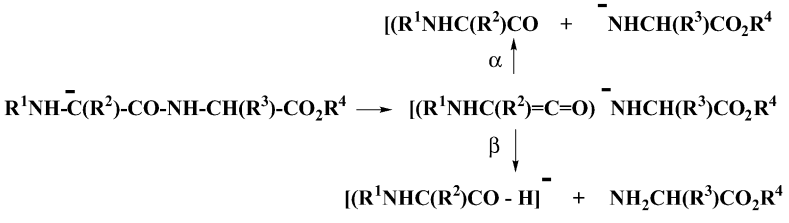
Scheme 2.

we were surprised to discover a fragmentation which can only be explained by the peptide $(M - H)^-$ anion adopting a particular conformation. The negative ion mass spectrum of caerin 1.1

caerin 1.1 GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2

shows a fragment anion formed following cyclisation of the CO_2^- group on the side chain of Glu23 with the central backbone C of Ile21 [5]. We had previously determined the secondary structure of caerin 1.1 by an NMR study in micelles, and shown it to contain N- and C-terminal helical regions separated by a flexible hinge region in the centre of the peptide (deliniated by the two Pro residues) [6]. The structure is shown in Fig. 1. A computer simulation (Fig. 2) in the Ile21–Glu23 region shows that bond rotation of the Glu side chain places the two interacting groups in an appropriate orientation in order to effect the required cyclisation/displacement reaction.

The amphibian host defence peptide citropin 1.1 (from *Litoria citropa*) has multifaceted activity. The sequence of this peptide is shown in Table 1. Citropin 1.1 exhibits wide-spectrum antibacterial action against Gram positive organisms, it shows significant anticancer activity against all the major forms of human cancers, it is a fungicide, and it also inhibits the production of nitric oxide by neuronal nitric oxide synthase by interacting with the regulatory protein Ca^{2+} calmodulin [7]. We had a number of synthetic analogues of citropin 1.1 synthesised in order to study the structure–activity relationship of this system. We have measured the negative ion spectra of all of these peptides. The $(M - H)^-$ parent anions show the expected fragmentations, in particular, α cleavages (Scheme 1), and the γ cleavage ions of Asp4 (Scheme 2) and Ser11 [4] provide sequencing information [8]. There are several unusual fragmentations of this system which have not yet been elucidated. One of these involves the loss of Val from $(M - H)^-$ anions.



Scheme 1.

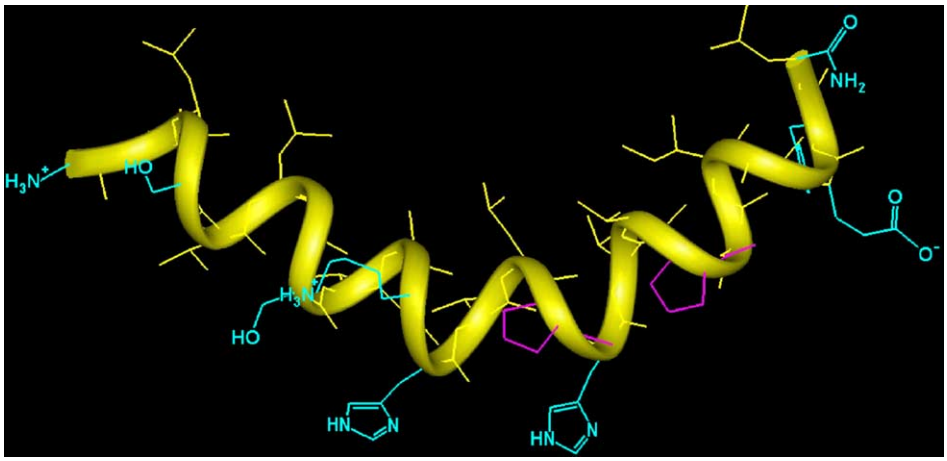


Fig. 1. The secondary structure of caerin 1.1 as determined by 2D NMR techniques [6].

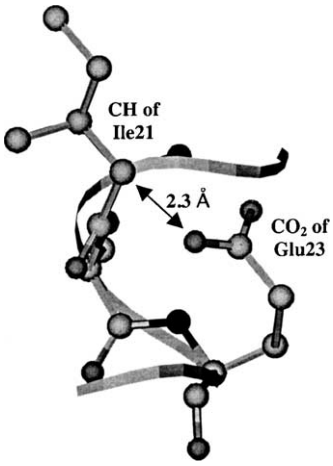


Fig. 2. The Ile21-Glu23 region of the $(M - H)^-$ anion of caerin 1.1 showing the juxtaposition of the reacting groups [5].

None of these peptides have Val at either N- or C-terminal positions [9]. Such a fragmentation is not explained by any known background cleavage that we have seen before. Which Val residue is lost, and what is the mechanism of this highly unusual fragmentation?

3. Experimental section

All amphibian peptides used in this study were synthesised by Mimotopes, Clayton, Vict., Australia.

3.1. Mass spectra

Electrospray mass spectra were determined using a Micromass QTOF2 orthogonal acceleration time-of-flight mass spectrometer with a mass range to 10 000 Da. The QTOF2 is fitted with an electrospray source in an orthogonal configuration with the ZSPRAY interface. Samples were dissolved in acetonitrile/water (1:1) (unless indicated to the contrary) and infused into the electrospray source with a flow rate of 5 μL/min. Conditions were as follows: capillary voltage 2.44 kV, source temperature 80 °C, desolvation temperature 150 °C and cone voltage 80–130 V. MS/MS data were acquired with the argon collision gas energy set to approximately 90 eV to give optimal fragmentation.

MS/MS/MS experiments were performed with a Finnigan LCQ ion-trap mass spectrometer. Samples were dissolved in acetonitrile/water (1:1) and infused into the electrospray source via a rheodyne injector with a 5 μL loop at 8 μL/min. Conditions were as follows: source voltage 4.3 kV, source current 18 μA, capillary temperature 200 °C, capillary voltage 3 V, and sheath gas flow 30 psi. MS/MS/MS data were acquired with the automatic gain control on, a maximum time of 500 ms, and using three microscans per scan, averaging over a total of 20 scans.

3.2. Calculations

Geometry optimisations were carried out using the HF/6-31G(d) level of theory using the GAUSSIAN 98 suite of programs [10]. Stationary points were characterised as either minima (no imaginary frequencies) or transition states (one imaginary frequency) by calculation of frequencies using analytical gradient procedures. Transition states were confirmed using IRC calculations. Energies of all mimima and transition states were calculated at the HF/6-31G(d)//HF/6-31G(d) level of theory unless indicated to the contrary in the text. All optimisations and transition state searches were carried on the Alpha Server SC of APAC (Australian National University, Canberra).

3.3. NMR

NMR experiments were carried out on a solution of 7.0 mg of citropin 1.1 dissolved in a mixture pf d_3-trifluoroethanol

(TFE) (350 μL) and water (350 μL), giving a concentration of 6.2 mM, at a measured pH of 2.00. The pH was later increased to 11.15 via the addition of NaOH as appropriate.

NOESY NMR experiments were acquired on a Varian Inova-600 NMR spectrometer at a ^{1}H frequency of 600 MHz. Experiments were run at 25 °C and referenced using the methylene protons of residual unlabelled TFE (3.918 ppm). NOESY [11] experiments were all collected in the phase-sensitive mode using time proportional phase incrementation in t_1 [12] over 256 increments, with 32 time-averaged scans per increment. The FID in t_2 consisted of 2048 data points over a spectra width of 5564.1 Hz. Water suppression was achieved by presaturation. The transmitted frequency was centred on the water resonance and conventional low power presaturation from the same frequency synthesiser (proton transmitter) was applied during a 1.5 s relaxation delay between scans. NOESY spectra were acquired with a mixing time of 150 ms.

NMR spectra were processed on a Sun Microsystems SunBlade 1000 workstation using VMNR software (version 6.1C). The data matrices were multiplied by a Gaussian function in both dimensions before zero-filling to 2048 data points on F_1 prior to Fourier transformation. Final processes 2D NMR matrices consisted of 2048 × 2048 real points.

4. Results and discussion

4.1. The loss of Val

The amino acid sequences of citropin 1.1 and analogues used for this study are listed in Table 1. When a fragment anion formed by the process $[(M-H)^- - Val]^-$ is observed, this is indicated in the third column of Table 1. In general, the abundance of this fragment peak can be between 5 and 50% of that of the $(M-H)^-$ anion. When the citropin 1.1 analogue contains Ser, there is sometimes loss of 99 amu (Val) from the $[(M-H)^- - CH_2O]^-$ anion. When the citropin 1.1 contains Asp4, there is sometimes loss of 99 amu from the $[(M-H)^- - H_2O]^-$ anion.

The largest $[(M-H)^- - 99]^-$ fragment peak occurs in the negative ion spectrum of modification **14** of citropin 1.1 (see Table 1 for sequence), and we have chosen this system to investigate the origin of Val loss. The negative ion spectrum of citropin 1.1 modification **14** is shown in Fig. 3. There are fragment peaks observed in this spectrum from (i) five α and two β cleavages ions, and (ii) four γ cleavage ions (from Asp4, Gln 7 and 8 and Ser11): these are shown on the formula above the spectrum shown in Fig. 3. The Asp4 γ cleavage is the dominant fragmentation in the spectrum; this ion characteristically fragments further by loss of H_2O and CH_2O (cf. [4]). Pronounced loss of 99 not only occurs from the $(M-H)^-$ anion, but also from the Aspγ, (Aspγ–H_2O) and (Aspγ–H_2O–CH_2O) ions. With the QTOF2 instrument operating in "high resolution" mode, the $(M-H)^-$ ion is shown to lose 99.068 ± 0.002 Da; Val loss is calculated to be 99.0668 Da.

Modifications **15**–**17** of citropin 1.1 have the three Val residues replaced sequentially by Ile. We chose Ile (rather than say Ala) because there was a possibility that the side chain itself may have an influence on the internal cleavage process (both Val and Ile have the structural unit Me–CH– in the side chain). In the event, the nature of the side chain was not important. The data recorded in Table 1 for modifications **15**–**17** show that Val12 is the residue lost. There are two other key pieces of information which need to be

Table 1
Losses of Val from negative ions from citropin 1.1 and synthetically modified analogues (electrospray solvent acetonitrile/water (1:1)

Name	Sequence[a]	$[(M-H)^- - 99]^-$	$[(M-H-H_2O)-99]^-$	$[(M-H-CH_2O)^- - 99]^-$	m/z = 748 or analogue
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2	x	x	x[b]	x
Mod 1	GLF**E**VIKKVASVIGGL-NH_2	x	x	x[b]	
Mod 2	GLFDVI**A**KVASVIGGL-NH_2	x	x	x[b]	m/z 691
Mod3	GLFDVIKKVASVI**K**GL-NH_2	x	x	x[b]	x
Mod 4	GLFDVIKKVASVIGG**A**-NH_2	x	x	x[b]	x
Mod 5	GLFDVIKKVA**A**VIGGL-NH_2	x	x		
Mod 6	GLFDVIKKVASVI**KK**L-NH_2	x	x	x	x
Mod 7	GLF**A**VIKKVA**C**VIGGL-NH_2	x[c]			
Mod 8	GLF**A**VIKKVASVI**K**GL-NH_2	x	x		
Mod 9	GLF**A**VIKKVA**A**VI**KK**L-NH_2	x			
Mod 10	GLF**A**VIKKVA**K**VI**KK**L-NH_2	x			
Mod 11	GLF**A**VIKKVA**K**VI**KK**L-NH_2	x			
Mod 12	**K**LF**A**VIKKVA**A**VIGGL-NH_2	x			
Mod 13	**K**LF**A**VIKKVA**A**VI**RRK**-NH_2	x			
Mod 14	GLFDVI**QQ**VASVIGGL-NH_2	x			x
Mod 15	GLFD **II**QQVASVIGGL-NH_2	x			m/z 762
Mod 16	GLFDVI**QQ I** ASVIGGL-NH_2	x			m/z 762
Mod 17	GLFDVI**QQ**VAS **I** IGGL-NH_2				x

[a] Residues modified are indicated in bold type.
[b] There is also an $[(M-H-H_2O-CH_2O)^- - 99]^-$ fragment anion.
[c] There is also an $[(M-H-H_2S)^- - 99]^-$ anion. Loss of H_2S is the characteristic fragmentation of the Cys side chain [13].

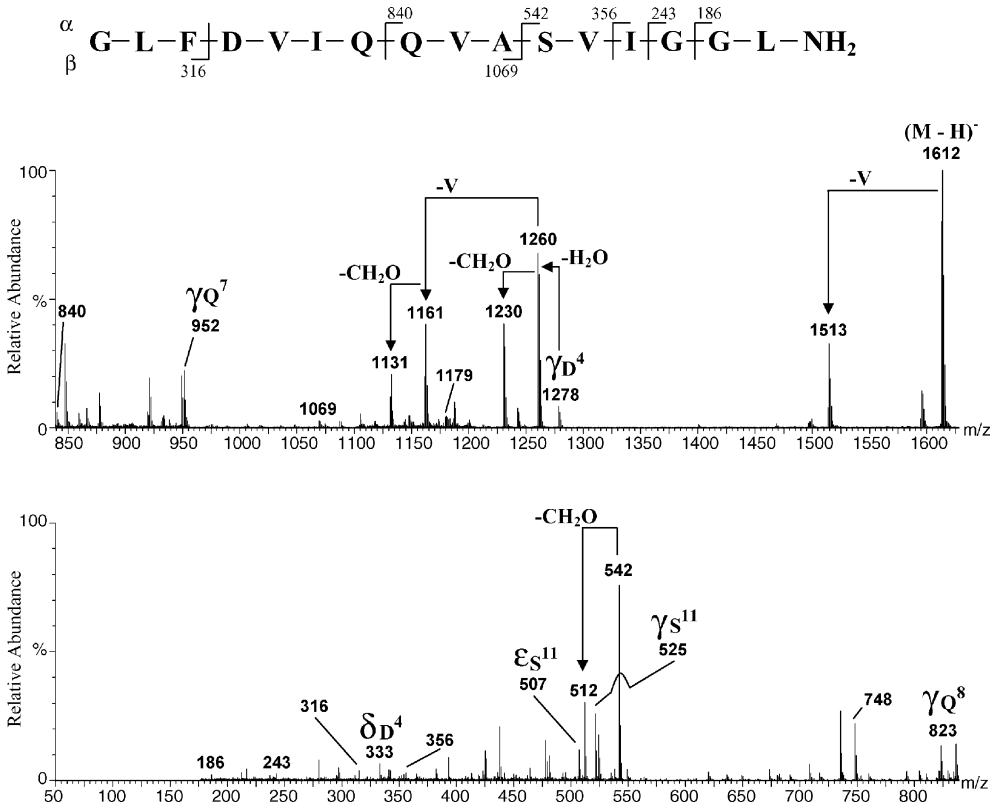


Fig. 3. The negative ion electrospray MS/MS data for the $(M - H)^-$ anion of citropin 1.1 synthetic modification **14**. Micromass QTOF2 mass spectrometer. Magnification ranges: 10×, m/z = 1168–942; × 6, m/z = 844–816; 4×, m/z = 592–492; 24×, m/z = 368–166.

explained by any mechanistic rationale proposed for loss of Val. The relative abundance of a peak corresponding to $[(M - H)^- - 99]$ is dependent on the internal energy of the $(M - H)^-$ anion. Initially, the abundance of the peak due to the fragment anion increases as the collision gas pressure in the instrument is increased, but there is a stage when any further increase in collision gas pressure significantly reduces the abundance of the fragment peak. This should be contrasted with the changes in the abundances of fragment peaks formed by normal backbone cleavage (like those shown in Schemes 1 and 2); these increase with increasing collision gas pressure. Second, and even more surprising, if the pH of the electrospray solution is increased from the value of 2 to 11 (to aid the solubility of the solute in the solvent), the $[(M - H)^- - 99]$ peak vanishes.

The data in Table 1 show that Val12 is the residue which is lost. This loss has to be preceded or accompanied by a specific cyclisation accompanied or followed by a cleavage process which places the Val residue in a terminal position [14]. Can we use the MS3 spectrum of the $[(M - H)^- - Val]^-$ ion (of Fig. 3) to provide information concerning the structure of this species? This spectrum is shown in Fig. 4. The spectrum is complex and quite unusual. There are no characteristic α and β cleavage ions originating from the parent anion. There are γ anions from Asp4, Gln 7 and 8 but not from Ser11. This suggests that there may be some structural modification in the vicinity of the Ser residue. The γ fragmentation of Asp4 dominates the spectrum, with the base peak m/z 1161 [γ(Asp4)– H_2O] fragmenting further as drawn on the spectrum. The key fragmentation in this sequence is loss of SerNH rather than Ser. These data suggest that m/z 1161 has the structure shown above the spectrum in Fig. 4, which in turn indicates that the loss of Val is preceded by cyclisation of the terminal $CONH^-$ moiety with the Ser11 carbonyl group followed by ring opening to place Val12 at the C-terminal end of the peptide.

Why does the C-terminal anion cyclise at Ser in preference to some other residue? There are only two scenarios which can reasonably explain the loss of Val. The first is that the terminal group H-bonds to the Ser OH and results in the reacting $CONH^-$ and Ser carbonyl groups being in juxtaposition. The second involves the anion adopting a specific conformation in which the C-terminal $CONH^-$ and the Ser carbonyl are in close proximity, thus allowing the nucleophilic addition to proceed.

The first of these possibilities is eliminated by consideration of the data contained in Table 1. Ser11 is not required for loss of Val: the loss of Val occurs whether residue11 is Ser, Cys or Lys. Even when Ser11 is present, the loss of Val does not require H-bonding to the Ser OH because the loss of Val can occur from the $[(M - H)^- - CH_2O]^-$ anion (this characteristic loss of CH_2O converts Ser11 to Gly11 [4]).

The second possibility requires a particular conformation of the anion in order to effect the rearrangement of the back-

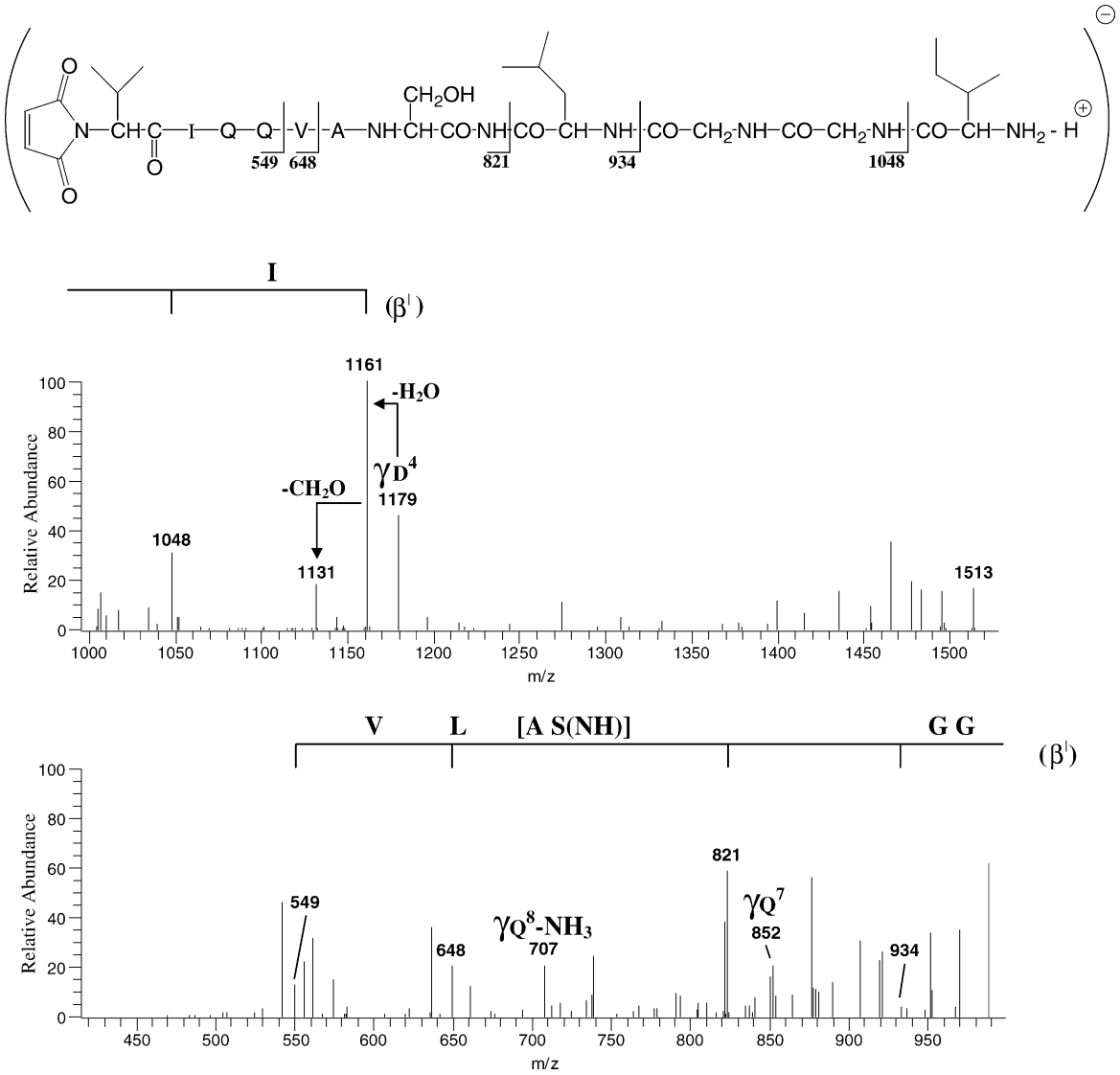


Fig. 4. MS/MS/MS data for the decompositions of the $[(M - H)^- - Val]^-$ ion of citropin 1.1 synthetic modification **14**. Finnigan LCQ mass spectrometer. Magnification ranges: 20×, m/z = 1485–1163; 50×, m/z = 1055–994; 100×, m/z = 994–847; 20×, m/z = 847–566; 100×, m/z = 566–532; 20×, m/z = 532–493.

bone, and we have already seen an example of this for a specific fragmentation of caerin 1.1 (see Section 2, also [4]). We have previously investigated the secondary structure of the bioactive citropin 1.1 in micelles using 2D nuclear magnetic resonance [4,15]. It is a simple amphipathic α helix; the structure is shown in Fig. 5. The region in the vicinity of Ser11 is enhanced in Fig. 6 to show that in the α helical form, the C-terminal group can approach the Ser11 carbonyl group to 2.5 Å in order to effect the desired nucleophilic addition reaction. The experimental data suggest that the loss of Val from the $(M - H)^-$ anion of synthetic modification **14** occurs as shown in Scheme 3 [16].

The reactions involving loss of Val do not occur when the pH of the electrospray solution is increased from 2 to 11. This was a serendipitious observation which occurred when we were attempting to solubilise the substrate by making the

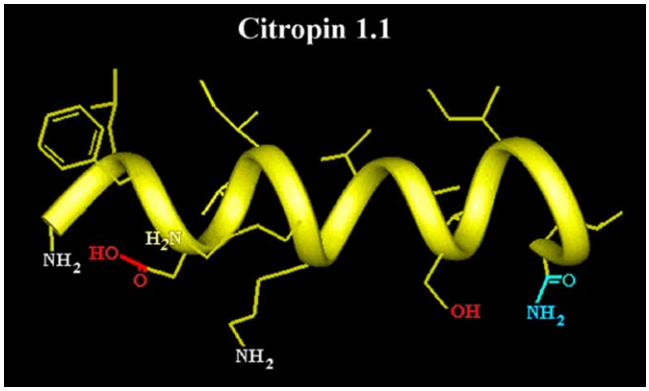


Fig. 5. The secondary structure of citropin 1.1 determined by 2D NMR techniques [4,15].

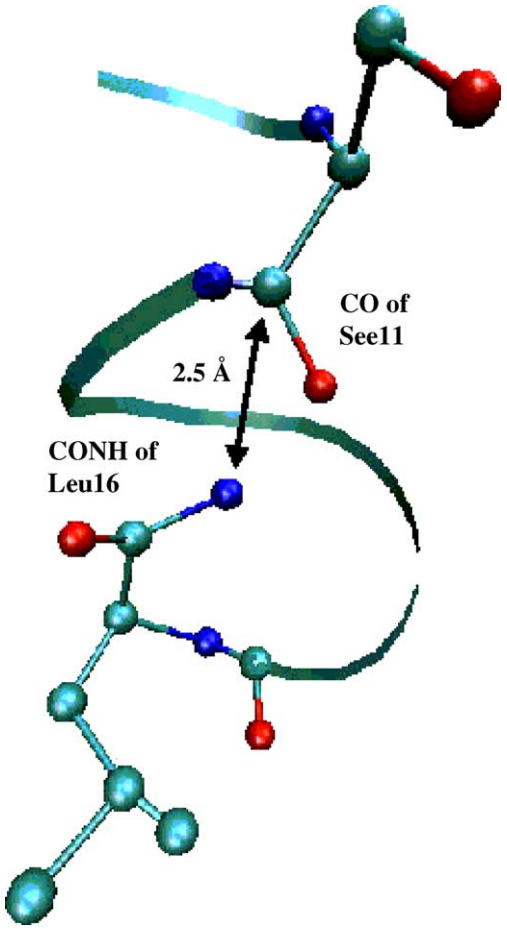
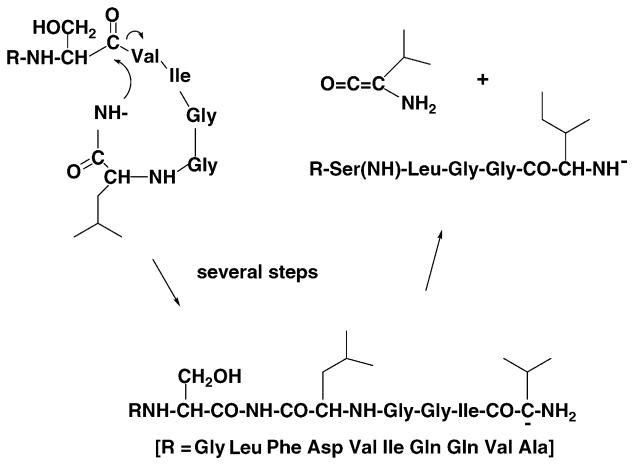


Fig. 6. Computer simulation of the Ser11 Val12 region of the α-helical structure of citropin 1.1.

electrospray solution (water:acetonitrile) more basic. This result is consistent with the peptide anion adopting a random conformation rather than the α helical conformation under basic conditions. We have done a number of experiments to support this proposal qualitatively. First, we measured the



Scheme 3.

Table 2
Ratio of $[(M - H)^- - Val]^-$ and $(M - H)^-$ under different solvent conditions

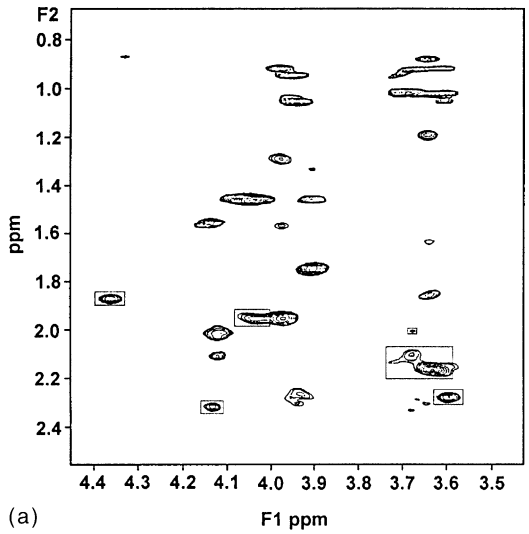
Solvent	pH	Ratio of relative abundances of $[(M - H)^- - Val]^-$:$(M - H)^-$
Acetonitrile/water (1:1)	1.5	1:16
	3.46	1:1
	7.0	1:4
	10.1	1:12
	13.4	Not present
Isopropanol		30:1

spectrum of citropin 1.1 using water/acetonitrile (1:1) and adding aqueous hydrogen chloride and aqueous sodium hydroxide to change the pH of the electrospray solvent. The results are quite dramatic and are listed in Table 2. The ratio of the abundances of the $[(M - H)^- - Val]^-$ and $(M - H)^-$ peaks is normalised to 1:1 when the solvent is at the usual pH value (3.46). At pH 1.5, the ratio has changed to 1:16, and at pH 10, the ratio is 1:12. The $[(M - H)^- - Val]^-$ peak has disappeared at pH 13.4, even though the peaks due to the α, β and γ backbone cleavages are essentially unchanged. We also measured the spectrum in pure isopropanol as the electrospray solvent. We expected the loss of Val to be enhanced in isopropanol, since the alcohol will H-bond less readily (than water) with the peptide, thus allowing a larger proportion of the α-helix in solution. The abundance of the $[(M - H)^- - Val]^-$ ion increased (relative to all other peaks [including the $(M - H)^-$ anion and the normal backbone cleavage peaks]) some 30 times in isopropanol. These results are consistent with the loss of Val occurring from an $(M - H)^-$ anion of helical structure.

We have also carried out two-dimensional NMR experiments to ascertain the effect of changing the pH on the secondary structure of the peptide. Similar experiments carried out on other short linear peptides in membrane mimicking solvents (TFE/water and DPC micelles) have shown that chemical shifts were closer to random coil values at higher pH, indicating that the peptide becomes less structured as the pH increases [17,18]. A portion of the NOESY spectra run using citropin 1.1 in TFE/water (1:1 by volume) at pH, 2.00 and 11.15 are shown in Fig. 7a and b, respectively. The inter-residue cross-peaks, indicative of α-helicity are boxed in Fig. 7a and are absent in Fig. 7b, suggesting that the α-helical conformation of the peptide is disrupted at the higher value of pH (11.15).

4.2. An unusual fragmentation from the (Aspγ–H_2O–CH_2O) anion of citropin 1.1 and analogues

There is a further fragmentation observed in the negative ion mass spectra of citropin 1.1 derivatives which contain Asp4 and Ser11 residues. This produces $m/z = 748$ in the negative ion mass spectrum of citropin 1.1: the same peak is also observed in Fig. 3. Analogous fragmentations from the



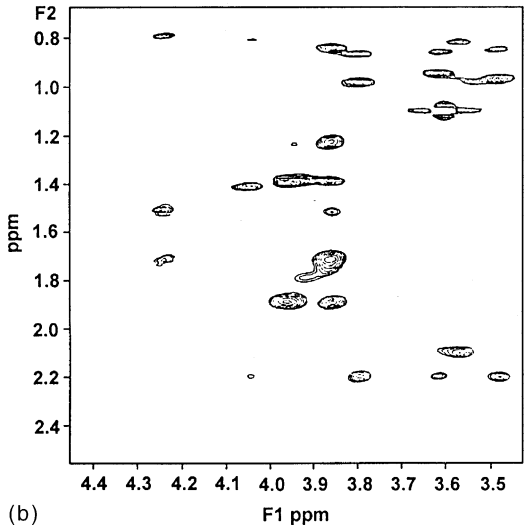
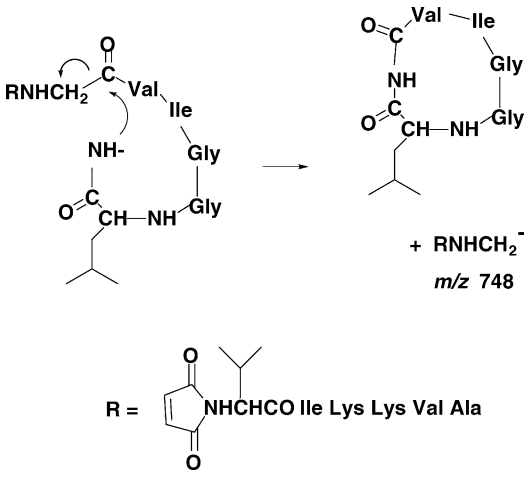


Fig. 7. (a) A region of the NOESY NMR spectra of citropin 1.1 at pH = 2.00. For full details see Section 3. Inter-residue cross-peaks are indicated in rectangles. (b) The same portion of the spectrum taken at pH = 11.15. Notice the absence of any inter-residue cross-peaks suggesting that no helical conformation is present.



Scheme 4.

other synthetic modifications are listed in Table 1. The anion *m*/*z* 748 gives a major peak in the spectrum of the citropin 1.1 analogue shown in Fig. 8. MS/MS/MS data show that it is the (Aspγ–H_2O–CH_2O) ion which fragments to form *m*/*z* = 748. The (Aspγ–H_2O–CH_2O)$^-$ anion also competitively loses Val. These two fragmentations can proceed through the same type of intermediate as that shown in Scheme 3. The formation of *m*/*z* = 748 is rationalised as shown in Scheme 4. The decomposing (Aspγ–H_2O–CH_2O)$^-$ anion must be in the α helical form in order to effect these two fragmentations.

We have carried out experiments in which the abundances of the peaks from $[(M - H)^- - Val]^-$ and *m*/*z* 748 [from citropin 1.1 synthetic modification 3 (GLFDVIKKVASVIKGL-NH_2) (Fig. 8)] are monitored as the collision gas pressure (Ar) is increased in the hexapole collision cell of the QTOF2 instrument. This effectively increases the internal energies of the decomposing $(M - H)^-$ and [Aspγ–H_2O–CH_2O]$^-$ anions. As the internal energy of the $(M - H)^-$ anion increases, the abundance of the peak corresponding to the $[(M - H)^- - Val]^-$ ion first increases. At a particular pressure of Ar, the abundance of the peak then decreases, and with a further increase in the Ar pressure, the peak disappears altogether without affecting the abundances of other peaks produced by backbone cleavage. The situation with *m*/*z* = 748 is different. This peak continues to increase in abundance at a pressure well above that necessary to produce the maximum abundance of the $[(M - H)^- - Val]^-$ ion (in other words it requires a higher internal energy of the $(M - H)^-$ anion to form a decomposing [Aspγ–H_2O–CH_2O]$^-$ anion than it does to cause elimination of Val). However, if the pressure of Ar is increased further, the abundance of the peak (*m*/*z* = 748) diminishes. These data are consistent with the required helical forms of the decomposing ions collapsing as the internal pressure of the anion becomes sufficient to break the H-bonding of the helix.

4.3. A theoretical approach

The scenario we have outlined above is complex, and we need to test our rationale for the formation of the unusual fragment anions by a theoretical study. The peptide systems under investigation are large, so we have used a simplified model system to explore both fragmentation processes outlined above. The results of this study are summarised in Fig. 9, with further details listed in Table 3. The model system we have used involves the reaction of $HCONH^-$ with the second amide centre of O=CH–NH–CH_2–CO–NH–CH_2CHO. The energies shown in Fig. 9 were obtained at the HF/6-31G(d)//HF/6-31G(d) level of theory. The geometries of the key species **A**, **B** and **C** (see Fig. 9) are listed in Table 3 at the HF/6-31G(d) level

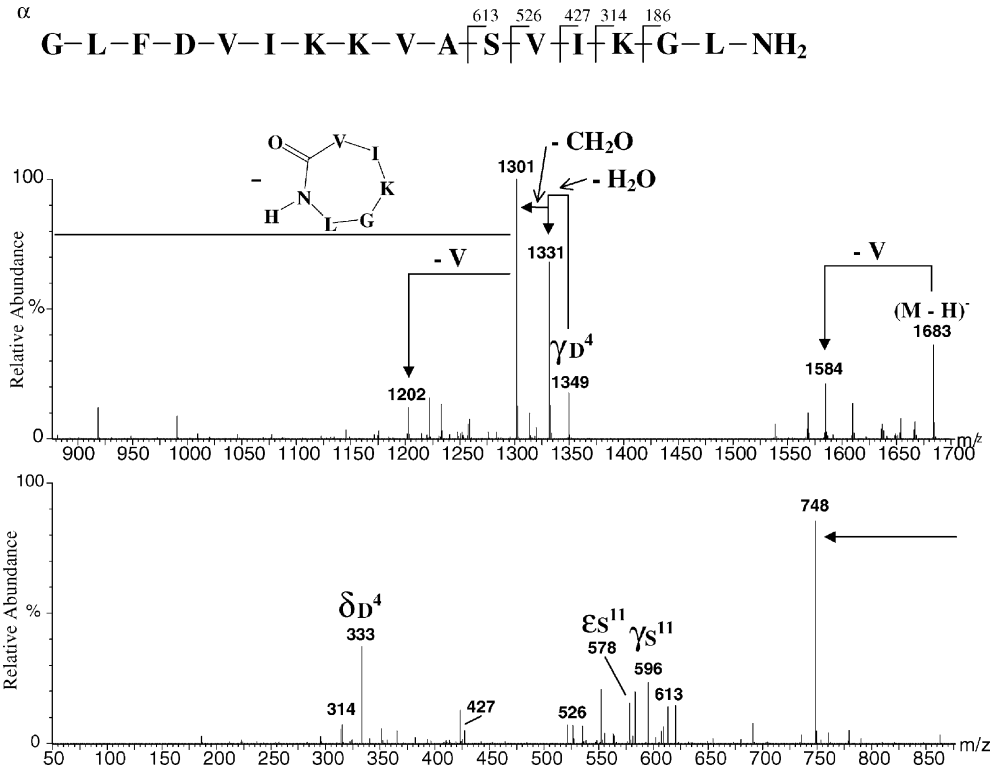


Fig. 8. The negative ion electrospray MS/MS data for the $(M - H)^-$ anion of citropin 1.1 modification 3. QTOF2 mass spectrometer. Magnification ranges: 10×, *m*/*z*1655–1552, *m*/*z* 1268–1170, *m*/*z*615–525; 16×, *m*/*z* = 431–174.

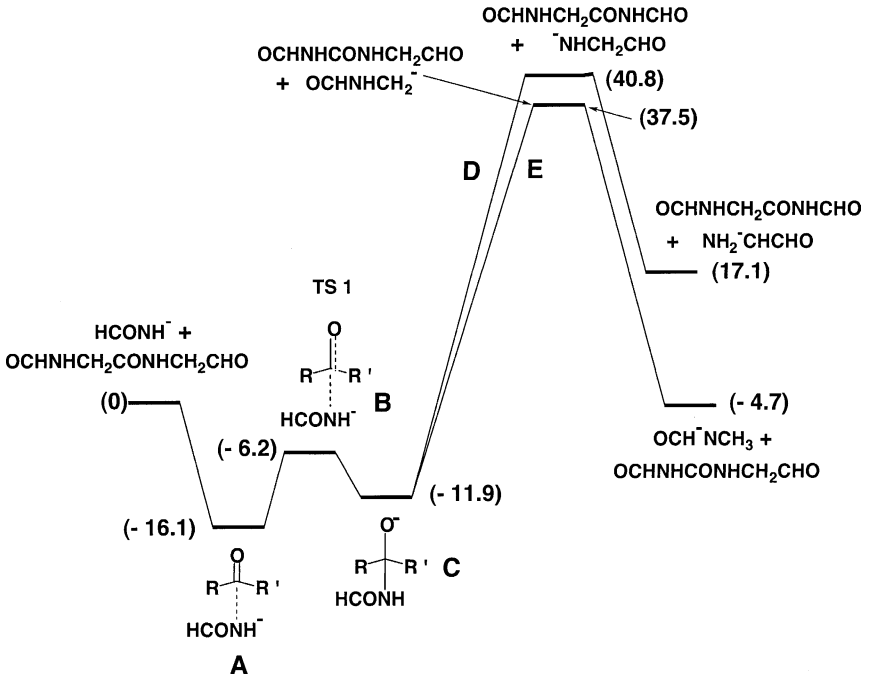
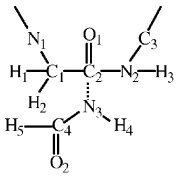
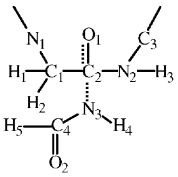
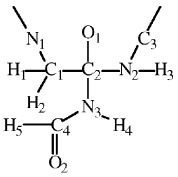


Fig. 9. Reaction coordinate profiles of the reaction of the model system $HCONH^-$ and O=CH–NH–CH_2–CO–NH–CH_2CHO. Geometries at the HF/6-31G(d) level of theory; energies at the HF/6-31G(d)//HF/6-31G(d) level of theory. Relative energies in kcal mol^{-1}. For further data concerning the geometries and energies of species **A**, **B** and **C**, see Table 3.

Table 3
Geometries and energies of key species **A**, **B** and **C** in Fig. 9

	A	**B**	**C**
			
Relative energies[a]	−16.1	−6.2	−11.9
N1C1 (Å)	1.436	1.440	1.444
C1H1	1.077	1.082	1.087
C1H2	1.089	1.087	1.084
C1C2	1.518	1.529	1.550
C2O1	1.201	1.231	1.288
C2N2	1.379	1.428	1.485
N2H3	0.997	0.999	1.001
N2C3	1.447	1.443	1.443
C2N3	2.530	1.910	1.528
N3H4	1.009	1.003	0.997
N3C4	1.310	1.315	1.325
C4O2	1.229	1.220	1.209
C4H5	1.115	1.106	1.096
N1C1C2 (°)	112.4	111.1	108.4
C1C2O1	121.3	117.5	112.0
C1C2N2	115.2	110.4	105.4
C1C2N3	86.4	98.3	108.4
C2C1H1	110.8	112.4	113.6
C2C1H2	106.3	106.4	106.5
C2N2C3	115.0	115.7	114.2
C2N2H3	110.9	111.4	111.7
C2N3C4	134.9	134.2	130.7
N3C4O2	131.7	130.2	127.3
N3C4H5	111.7	112.1	113.0
C4N3H4	107.6	112.9	117.2
N1C1C2N2 (°)	169.8	−179.8	−171.3
N1C1C2O1	−25.5	−39.2	−47.1
N1C1C2N3	82.4	73.5	72.1
C1C2N2C3	−173.0	175.4	163.6
C1C2N2H3	−45.1	−56.0	−69.9
C1C2N3C4	82.8	78.5	60.2
N2C2C1H1	46.5	56.9	66.7
N2C2C1H2	−70.7	−61.0	−52.3
C2N3C4O2	156.3	166.8	179.6
C2N3C4H5	−23.4	−13.5	−0.2
O2C4N3H4	−0.5	1.9	−1.0

HF/6-31G(d)//HF/6-31G(d) kcal mol^{-1}.

[a] Relative energies with respect to $HCONH^-$ + $OCHNHCH_2CONHCH_2CHO$ −695.8236671 hartrees (0 kcal mol^{-1}).

of theory. Several minima were also investigated at higher levels of theory: these will be described later.

When we commenced this theoretical investigation of the two mechanisms shown in Schemes 3 and 4, it was clear that there were two possible scenarios for the initial approach of the two reagents. The first is the direct approach of the two reagents to form the tetrahedral complex **C**, shown in Fig. 9. The second involves the amide anion forming an H-bonded complex with the NH of the Val amide group. This complex precedes the formation of **A**. This second possibility, although energetically favourable for our model system, is not possible for the citropins 1 because the $CONH^-$ and HN-moieties cannot approach closer than 3.7 Å when the anion is in the helical form. The proposed mechanism shown in Fig. 9, is compatible with the processes shown in Schemes 3 and 4, and with the experimental evidence already provided. Approach of the two reagents is favourable, with the formation of the first formed intermediate **A** exothermic by 16.1 kcal mol^{-1}. Intermediate **C**, the key species for both processes, is also formed in an exothermic reaction. There are two cleavage processes of intermediate **C** which occur without transition states: (i) process **D** (that which pro-

duces the precursor which loses Val). This initially forms $^{-}NHCH_2CHO$ which proton transfers to form the more stable enolate anion $NH_2{}^{-}CHCHO$, and (ii) the backbone cleavage **E** (to form $m/z = 748$ and analogues) initially giving O=CH–NH–$CH_2{}^{-}$ which then converts to the more stable amide anion O=CH–$^{-}$N–CH_3. Cleavage **E** is the more thermodynamically favoured reaction, in agreement with the experimental observation that the formation of $m/z = 748$ (from the [Aspγ–H_2O–CH_2O]$^{-}$ precursor anion in Fig. 8) is more favoured than loss of Val. The data in Fig. 9 show that intermediate **C** requires $\geq 49.4\,kcal\,mol^{-1}$ of excess energy to effect process **E**, with the full reaction sequence from the two starting reagents being exothermic by $4.7\,kcal\,mol^{-1}$. The alternative process **D** (that forming the precursor anion which loses Val), requires intermediate **C** to have an excess energy of $\geq 52.7\,kcal\,mol^{-1}$, with the overall reaction being endothermic by $17.1\,kcal\,mol^{-1}$.[1]

4.4. In conclusion

- The internal residue Val12 is lost from the $(M - H)^{-}$ (and some fragment) anions of citropin 1.1 and some synthetic analogues.
- This reaction requires the $(M - H)^{-}$ anion to be in the α-helical formation. In this structure, the C-terminal –CONH^{-} group and the carbonyl group of the amide function between residues **11** and **12** are within 2.5 Å of each other and can effect a nucleophilic reaction to form a tetrahedral intermediate which cleaves to place Val12 at the C-terminal end of a rearranged $(M - H)^{-}$ species.
- Citropin 1 derivatives which contain Asp4 and Ser11 residues give an [Aspγ–H_2O–CH_2O]$^{-}$ fragment as a major peak in the negative ion spectrum. This fragment anion undergoes the same type of rearrangement as described for the $(M - H)^{-}$ anion. The rearranged anion then undergoes two competitive cleavages, namely (i) loss of Val, and (ii) a backbone cleavage yielding a major peak in the spectrum.

[1] The relative energies of the penultimate products of the processes **D** and **E** shown in Fig. 9 differ by only $3.3\,kcal\,mol^{-1}$ at the HF/6-31G(d)//HF/6-31G(d) level of theory. As this difference is small, and is important because it determines the relative kinetics of processes **D** and **E**, we have determined the energies of these two penultimate products using different levels of theory. Results are HF/6-31G(d)/AM1 [$-15.6\,kcal\,mol^{-1}$], HF/6-31G(d)//HF/6-31G(d) [$+3.3\,kcal\,mol^{-1}$], HF/6-31+G(d)//HF/6-31G(d) [$+3.8\,kcal\,mol^{-1}$], HF/6-311G(d)//HF/6-31G(d) [$+7.1\,kcal\,mol^{-1}$], HF/6-311+G(d)//HF/6-31G(d) [$+4.3\,kcal\,mol^{-1}$], B3LYP/6-31G(d)//HF/6-31G(d) [$-3.8\,kcal\,mol^{-1}$], B3LYP/6-31+G(d)//HF/6-31G(d) [$-1.8\,kcal\,mol^{-1}$], B3LYP/6-31G(d)//B3LYP/6-31G(d) [$-5.0\,kcal\,mol^{-1}$] and B3LYP/aug-cc-pVDZ//B3LYP/6-31G(d) [$-2.5\,kcal\,mol^{-1}$]. The average of all of these results gives a difference of $-0.8\,kcal\,mol^{-1}$. The only conclusion that can be made from these data is that the energies of the penultimate products shown in sequences **D** and **E** (Fig. 9) are very similar.

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References

[1] M.A. Apponyi, T.L. Pukala, C.S. Brinkworth, V.M. Maselli, J.H. Bowie, M.J. Tyler, G.W. Booker, J.C. Wallace, J.A. Carver, F. Separovic, J. Doyle, L.E. Llewellyn, Peptides 25 (2004) 1035.
[2] K. Biemann, S. Martin, Mass Spectrom. Rev. 6 (1987) 1.
[3] M.W. Hunkapiller, R.M. Hewick, W.J. Drewer, L.E. Hood, Methods Enzymol. 91 (1983) 399.
[4] J.H. Bowie, C.S. Brinkworth, S. Dua, Mass Spectrom. Rev. 21 (2002) 87.
[5] C.S. Brinkworth, S. Dua, J.H. Bowie, Rapid Commun. Mass Spectrom. 16 (2002) 53.
[6] H. Wong, J.H. Bowie, J.A. Carver, Eur. J. Biochem. 247 (1997) 545.
[7] J. Doyle, L.E. Llewellyn, C.S. Brinkworth, J.H. Bowie, K.L. Wegener, T. Rozek, P.A. Wabnitz, J.C. Wallace, M.J. Tyler, Eur. J. Biochem. 269 (2002) 100.
[8] C.S. Brinkworth, S. Dua, J.H. Bowie, Rapid Commun. Mass Spectrom. 16 (2002) 287.
[9] For the loss of an internal residue in the mass spectrum of a doubly-protonated peptide see, X.-J. Tang, P. Thibault, R.K. Boyd, Anal. Chem. 65 (1993) 2824.
[10] M.J. Frisch, G.M. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, V.G. Zakrzewski, J.A. Montgomery, R.E. Stratmann, J.C. Burant, S. Dapprich, J.M. Millam, A.D. Daniels, K.N. Kudin, M.C. Strain, O. Farkas, J. Tomasi, V. Barone, M.Cossi, R. Cammi, B. Mennuchi, C. Pomelli, C. Adamo, S. Clifford, J. Ochterski, G.A. Pedersson, P.Y. Ayala, Q. Cui, K. Morokuma, D.K. Malick, A.D. Rabuck, K. Raghavachari, J.B. Foresman, J. Cioslowski, J.V. Ortiz, A.G. Baboul, B. Stefanov, G. Liu, M.A. Al-Latam, C.Y. Peng, A. Nanayakkara, M. Chalacombe, P.M.W. Gill, B. Johnson, W. Chem, M.W. Wong, J.L. Andres, C. Gonzales, M. Head-Gordon, E.S. Replogle, J.A. Pople, Gaussian 98, Revision A.9, Gaussian Inc., Pittsburgh, PA, 1998.
[11] J. Jenner, B.H. Meier, P. Backman, R.R. Ernst, J. Chem. Phys. 71 (1979) 4546.
[12] D. Marion, K. Wüthrich, Biochem. Biophys. Res. Commun. 113 (1983) 967.
[13] D. Bilusich, C.S. Brinkworth, A.M. McAnoy, J.H. Bowie, Rapid Commun. Mass Spectrom. 17 (2003) 2488.
[14] For examples of the formation of cyclic intermediates in the fragmentations of positively charged peptides see, W.J. Feng, S. Gronert, K.A. Fletcher, A. Warres, C.B. Lebrilla, Int. J. Mass Spectrom. 222 (2003) 117;
J.M. Farrugia, R.A.J. O'Hair, Int. J. Mass Spectrom. 222 (2003) 229;
J. Vagüe, A. Paradela, M. Ramos, S. Ogueta, A. Marina, F. Barahona, J.A.L. de Castra, R. Vazquez, Anal. Chem. 75 (2003) 1524.
[15] K.L. Wegener, P.A. Wabnitz, J.H. Bowie, J.A. Carver, J.C. Wallace, M.J. Tyler, Eur. J. Biochem. 265 (1999) 627.
[16] For folding of a peptide in a positive ion spectrum see, J.A. Loo, J.X. He, W.L. Cody, J. Am. Chem. Soc. 120 (1998) 4542.
[17] D. Coles, W. Bicknell, A.A. Watson, D.P. Fairlie, D.J. Craik, Biochemistry 37 (1998) 11064.
[18] K.J. Marcinowski, H. Shao, E.L. Clancy, M.G. Zagorski, J. Am. Chem. Soc. 120 (1998) 11082.