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Fragmentations of $[M–H]^-$ anions of peptides containing Ser sulfate. A joint experimental and theoretical study

T. T. Nha Tran[1], Tianfang Wang[1†], Sandra Hack[2] and John H. Bowie[1*]

[1]Department of Chemistry, The University of Adelaide, South Australia, 5005, Australia
[2]Adelaide Proteomics Centre, The University of Adelaide, South Australia, 5005, Australia

RATIONALE: To determine the negative-ion cleavages from $[M–H]^-$ ions of Ser sulfate-containing peptides using experiment and theory in concert.
METHODS: Fragmentations were explored using a Waters QTOF2 mass spectrometer in negative-ion electrospray mode, together with calculations at the CAM-B3LYP/6-311++g(d,p) level of theory. Peptides used in this study were:

$GS(SO_3H)(OH)$	**1**	$GS(SO_3H)(OCH_3)$	**1a**
$GAVS(SO_3H)(OH)$	**2**	$GAVS(SO_3H)(OCH_3)$	**2a**
$GLS(SO_3H)GVA(OH)$	**3**		
$GLS(SO_3H)GDA(OH)$	**4**		
$GLS(SO_3H)GS(SO_3H)A(OH)$	**5**		

RESULTS: Previously, it has been shown that a peptide containing a Tyr sulfate group shows $[(M–H)^––SO_3]$ as the base peak. Only a small peak was observed corresponding to $HOSO_3^–$ (formed following rearrangement of the sulfate). A Ser sulfate-containing peptide, in contrast, shows pronounced peaks due to cleavage product anions $[(M–H)^––SO_3]$ and $HOSO_3^–$. Theoretical calculations at the CAM-B3LYP/6-311++g(d,p) level of theory suggest that rearrangement of a Ser sulfate to give C-terminal CO_2SO_3H is energetically unfavourable in comparison with fragmentation of the intact Ser sulfate to yield $[(M–H)^––SO_3]$ and $HOSO_3^–$. $[(M–H)^––H_2SO_4]$ anions are not observed in the spectra of peptides containing Ser sulfate, presumably because $HOSO_3^–$ is a relatively weak gas-phase base (ΔG_{acid} = 1265 kJ mol^{-1}).
CONCLUSIONS: Experimental and theoretical data suggest that $[(M–H)^––SO_3]$ and $HOSO_3^–$ product anions (from a peptide with a C-terminal Ser sulfate) are formed from the serine sulfate anion accompanied by specific proton transfer. CID MS/MS/MS data for an $[(M–H)^––SO_3]$ ion of an underivatised sulfate-containing peptide will normally allow the determination of the amino acid sequence of that peptide. The one case we have studied where that is not the case is $GLS(SO_3H)GDA(OH)$, where the peptide contains Ser sulfate and Asp, where the diagnostic Asp cleavages are competitive with the Ser sulfate cleavages. Copyright © 2013 John Wiley & Sons, Ltd.

The backbone cleavages of $[M–H]^-$ anions of peptides have been reviewed[1] as have the negative-ion cleavages of some peptide post-translational modifications, including disulfides.[2] Most of these peptide negative-ion cleavages are diagnostic with simple mechanisms.

In contrast, the negative-ion cleavages of Tyr, Ser and Thr phosphates[3,4] are complex, involving a variety of $S_Ni(P)$ rearrangements often followed by internal cyclisation/cleavage reactions.[4] The phosphate group of Tyr can transfer to Ser and Thr residues, and also to a carboxylate (or amide) moiety. In addition, a phosphate on Ser or Thr can transfer to a non-phosphorylated Ser or Thr (but not Tyr) or to a carboxylate group. In summary, if a peptide containing any or all of Ser, Thr and Tyr is not completely phosphorylated, negative-ion cleavages can determine the number of phosphorylated residues, and normally the positions of Ser, Thr and Tyr but not necessarily which specific residues are phosphorylated.[4]

We have recently initiated a study to see whether sulfated peptides undergo rearrangement of the sulfate group (like phosphates) in the negative mode and have commenced this work with a report on Tyr sulfate-containing peptides.[5] Positive-ion electrospray (and MALDI) ionisation mass spectra of Tyr and Ser sulfate-containing peptides have been widely studied. They are often devoid of MH^+ ions or alternatively have MH^+ ions of low abundance.[5–14] $[MH^+–SO_3]$ ions are the base peaks of the spectra and MS/MS/MS data (including **b** and **y** cleavages) of these ions often provide the sequence of the peptide.

* *Correspondence to:* J. H. Bowie, Department of Chemistry, The University of Adelaide, South Australia, 5005, Australia.
E-mail: john.bowie@adelaide.edu.au

† Current address: Department of Science and Education, University of the Sunshine Coast, Queensland, 4556, Australia

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The negative-ion electrospray (and MALDI) ionisation mass spectra of Tyr sulfate-containing peptides generally show $[M–H]^-$ anions together with abundant $[(M–H)^–SO_3]$ anions; MS/MS/MS data from energised $[(M–H)^–SO_3]$ anions provide sequencing data.[8,9,14]

In complete contrast to the pronounced phosphate transfers noted in the negative-ion spectra of Tyr phosphate-containing peptides, rearrangements to form the corresponding $HOSO_3^-$ (*m/z* 97) and $[(M–H)^– H_2SO_4]$ product anions produce minor peaks in the negative-ion spectra of Tyr sulfate-containing peptides.[5] Calculations at the CAM-B3LYP/6-311++g(d,p) level of theory, using Gaussian 09, complement these experimental findings.[5]

In this paper we describe, using experiment and theory in concert, the negative-ion fragmentations of $[M–H]^-$ anions from some simple synthetic Ser sulfate-containing peptides, in order to ascertain (i) the characteristic fragmentations of the Ser sulfate group, and (ii) whether rearrangement processes analogous to those reported for Ser phosphate anions[3,4] are noted in these spectra.

EXPERIMENTAL AND THEORETICAL

Peptides

All Ser-containing peptides used in this study were synthesised by Hongkong GenicBio Biotech Co., Ltd (Shanghai, China). The purities were generally better than 80% as evidenced by HPLC and MS (Shimadzu LCMS-2010) data provided by the manufacturer. These Ser-containing peptides were converted into their Ser sulfates by treatment with chlorosulfonic acid using the standard method of Burlingame *et al*.[15] No attempt was made to purify the Ser sulfate-containing peptides further because of the problem of possible hydrolysis of the sulfate-containing residues. Experimental studies utilised (i) MS/MS data from $[M–H]^-$ anions; (ii) MS/MS/MS data from $[(M–H)^–SO_3]$ product anions (or MS/MS data from CID activated source formed $[(M–H)^–SO_3]$ product anions); and (iii), when appropriate, MS/MS data from CID-activated source-formed $[(M–H)^–H_2SO_4]$ fragment anions.

Mass spectra

Electrospray ionisation mass spectra were obtained using a Micromass QTOF2 hybrid orthogonal acceleration time-of-flight mass spectrometer (Waters/Micromass, Manchester, UK) with a mass range to *m/z* 10 000. The QTOF2 is fitted with an electrospray (ES) source in an orthogonal configuration with a Z-spray interface. Samples (25 μg) were dissolved in acetonitrile/water (1:1 v/v) and infused into the ES source at a flow rate of 8 μL min^{-1}. The experimental conditions were as follows: capillary voltage 2.9 kV, source temperature 80°C, desolvation temperature 150°C, and cone voltage 50 V. MS/MS data were acquired using argon as the collision gas and the collision energy was set to 50 eV. All masses for anions shown either in figures or a table are nominal masses (i.e. the sum of the integral masses of the amino acid residues).

High-resolution MS/MS and MS/MS/MS spectra were obtained with an LTQ Orbitrap XL ETD hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an ES source. Samples were infused at 5 μL min^{-1} delivered by a built-in syringe pump and a spraying voltage of 2.3 kV. A mass resolution of 30 000 (at *m/z* 400) was used. Tandem multi-stage mass spectrometry MS^n (n = 2 or 3) experiments were performed using CID (collision-induced dissociation) with normalised collision energy set between 8 and 11%.

High-resolution MS/MS data for $[M–H]^-$ ions and MS/MS/MS data for $[(M–H)^–SO_3]^-$ (and $[(M–H)^– (H_2O + SO_3)]$ ions when appropriate) were obtained for peptides $GAVS(SO_3H)(OH)$, $GLS(SO_3H)GVA(OH)$ and $GLS(SO_3H)GDA(OH)$. The atomic compositions are consistent with those of anions described in this paper (all mass measurements were correct to the third decimal place). High-resolution data are not presented here.

Theoretical calculations

Calculations were carried out at the CAM-B3LYP/6-311++g(d,p) level of theory,[16] using Gaussian 09.[17] Stationary points were characterised as either minima (no imaginary frequencies) or transition states (one imaginary frequency) by calculation of frequencies using analytical gradient procedures. The minima connected by a given transition structure were confirmed by intrinsic reaction coordinate (IRC) calculations. Calculations were performed with supercomputing facilities from (a) eResearch (the South Australian Partnership for Advanced Computing, The University of Adelaide) and (b) the Australian Partnership for Advanced Computing (Australian National University, Canberra).

RESULTS AND DISCUSSION

The Ser sulfate-containing peptides chosen for study are listed in Table 1. Two of the peptides contain Ser sulfate in the C-terminal amino acid position, and two related peptides have C-terminal CO_2CH_3 (instead of CO_2H) groups. The remaining six-residue peptides have Ser sulfate in position 3 with Val, Asp or Ser sulfate in position 5.

Table 1. Ser sulfate-containing peptides chosen for this study

$GS(SO_3H)(OH)$	**1**	$GS(SO_3H)(OCH_3)$	**1a**
$GAVS(SO_3H)(OH)$	**2**	$GAVS(SO_3H)(OCH_3)$	**2a**
$GLS(SO_3H)GVA(OH)$	**3**		
$GLS(SO_3H)GDA(OH)$	**4**		
$GLS(SO_3H)GS(SO_3H)A(OH)$	**5**		

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Table 2. CID MS/MS of [M–H]⁻ ions from (**1**), (**1a**) and (**2a**). Mass (loss or formation) relative abundance (%). Masses listed in a table, figure or the text are nominal masses (the sum of the integral masses of amino acid residues together with sulfate groups)

1. GS(SO_3H)(OH), [M–H]⁻, *m/z* 241.

241[M–H]⁻16% Fragmentations from the [M–H]⁻ anion as follows: 223(H_2O)2; 179($H_2O + CO_2$)5; 161(SO_3)56; 131 ($SO_3 + CH_2O$)10; 97($HOSO_3^-$)100; 74($SO_3 + H_2O + G$)1.

1a. GS(SO_3H)(OCH_3), [M–H]⁻, *m/z* 255.

255[M–H]⁻12%; 97($HOSO_3^-$)100%.

2a. GAVS(SO_3H)(OCH_3), [M–H]⁻, *m/z* 425.

425[M–H]⁻15%; 97($HOSO_3^-$)100%

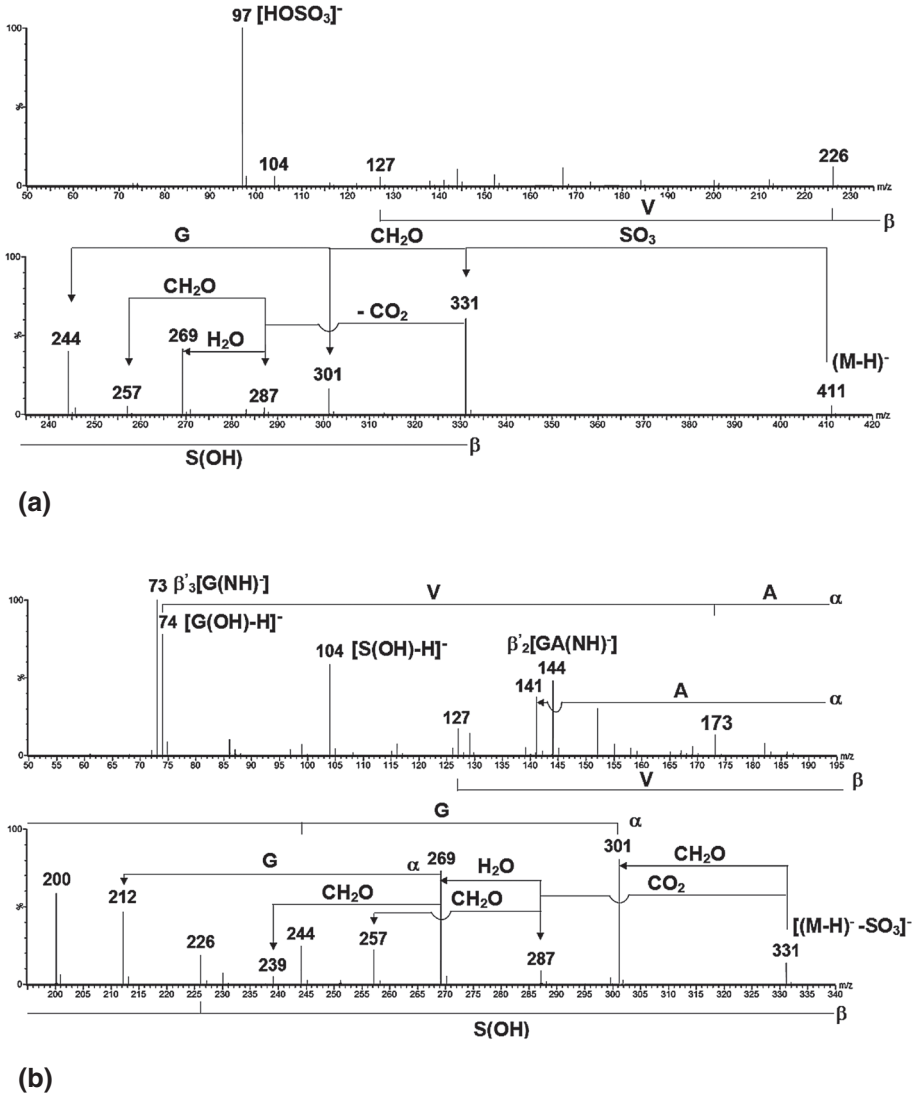

Figure 1. (a) Negative-ion electrospray CID MS/MS spectra of [M–H]⁻ of GAVS (SO_3H)(OH) (**2**). For experimental conditions, see Experimental section. Multiplication ranges: (×10), *m/z* 420–300 and 295–100. Masses are nominal masses, i.e. the sum of the integral masses of the individual amino acid residues. (b) Negative-ion electrospray CID MS/MS spectra of source-formed [(M–H)⁻–SO_3] of GAVS(SO_3H)(OH) (**2**). For experimental conditions, see Experimental section.

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A1. The fragmentations of $[M–H]^-$ ions of peptides with Ser (SO_3H) in the C-terminal position

The negative-ion spectra of activated $[M–H]^-$ ions of (**1**), (**1a**) and (**2a**) are recorded in Table 2 while those of the $[M–H]^-$ and $[(M–H)^-–SO_3]$ ions of GAVS(SO_3H)(OH) (**2**) are shown in Figs. 1(a) and 1(b), respectively.

The CID MS/MS data for the Ser sulfate-containing peptides (**1**) and (**2**) show *m/z* 97 ($HOSO_3^-$) as the base peak with $[(M–H)^-–SO_3]$ ions also giving pronounced peaks. Although $HOSO_3^-$ is the base peak in these spectra, no product ions corresponding to $[(M–H)^-–H_2SO_4]$ are observed. Thus, although there are $[(M–H)^-–H_3PO_4]^-$ ions in the negative-ion spectra of peptides containing Ser (PO_3H_2), the corresponding $[(M–H)^-–H_2SO_4]$ fragmentation is not noted in the negative-ion spectra of (**1**), (**2**) or (**1a**) and (**2a**). This is presumably a consequence of $HOSO_3^-$ being a poorer gas-phase base than $(HO)_2PO_2^-$. Namely:

$$H_3PO_4 \rightarrow H_2PO_4^- + H^+ \quad \Delta DG_{acid} = 1351 \text{ kJ mol}^{-1}$$ [18]

$$H_2SO_4 \rightarrow HSO_4^- + H^+ \quad \Delta DG_{acid} = 1265 \text{ kJ mol}^{-1}$$ [19]

Thus, $HOSO_3^-$ will be unable to deprotonate the carboxyl centres on, for example, Gly ($\Delta G_{acid} = 1406$ kJ mol^{-1}[20]) or Ser ($\Delta G_{acid} = 1363$ kJ mol^{-1}[20]) unless particular systems containing either of these amino acid residues are significantly energised.

Most other product ions in the spectra are formed from $[(M–H)^-–SO_3]$ ions. This is best seen in Figs. 1(a) and 1(b); in particular, the CID MS/MS data from *m/z* 331 (Fig. 1(b)) demonstrates how complex the negative-ion spectrum of a small peptide can be. Negative-ion backbone cleavages of peptides, for example the characteristic **α**, **β** and **β** prime backbone cleavages. (see Scheme 1 for **α** and **β** processes: endothermic by 40 and 200 kJ mol^{-1}, respectively; early calculations at the 6-31G/AM1 level of theory.[1]) The mechanism of the **β** prime fragmentation has recently been reinvestigated and corrected [a possible mechanism involves the carboxylate anion S_Ni cyclisation/cleavage to form the amide species R_1NH^- ($R_1 = CH_3CONHCH_2CO$; $R_2 = CH_3$) see Scheme 2 ($\Delta G = +150$ kJ mol^{-1})[21] cf. [1]].

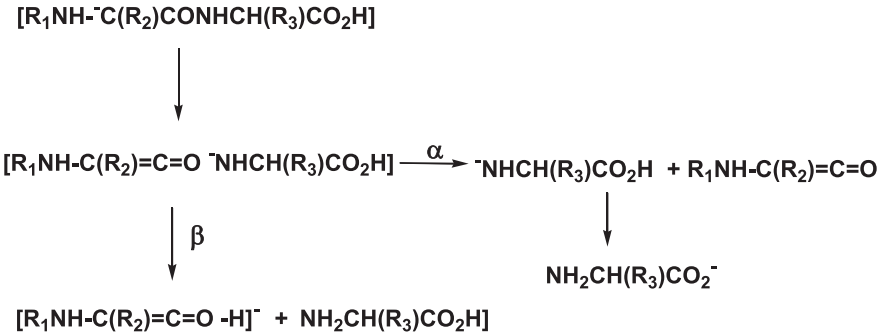
Our major interests in this study are (i) what are the basic fragmentations of the Ser sulfate group, and (ii) whether the sulfate group remains on Ser, or whether it might migrate to another position in the peptide, like the C-terminal carboxyl group, a side-chain carboxyl group of Asp or to another Ser; in other words, do sulfates behave like phosphates in undergoing extensive negative-ion rearrangements?[3,4]

Are the $[(M–H)^-–SO_3]$ and $HOSO_3^-$ ions formed directly from Ser sulfate or do they occur following migration of the sulfate to the C-terminal carboxylate position? In order to give such rearrangements the best chance of success, the Ser sulfate is placed in the C-terminal position directly adjacent to the C-terminal CO_2H group and this is the scenario we investigate first. An obvious experimental approach would be to make an ^{18}O-labelled peptide labelled either on the Ser side chain or on the C-terminal CO_2H. This is possible but complex (e.g.[22]): such an experiment would indicate the genesis of $HOSO_3^-$ (*m/z* 97) but not that of the $[(M–H)^-–SO_3]$ anion. An alternative approach would be to block any sulfate transfer to the C-terminal carboxylate, by modifying the C-terminal group so that it cannot interact in an S_Ni (S) reaction with the Ser sulfate group. We have chosen the latter procedure, in concert with *ab initio* calculations of possible reaction coordinate profiles.

The CID MS/MS data of the corresponding C-carbomethoxy derivatives (**1a**) and (**2a**) are listed in Table 2. The sulfate migration to $–CO_2^-$ is not possible in these systems. The two spectra are very simple: the major product ion is $HOSO_3^-$ (base peak, *m/z* 97): other small peaks are less than 1% abundance of the base peak. Thus *m/z* 97, for (**1a**) and (**2a**), originates directly from the Ser sulfate. Surprisingly, no loss of SO_3 is observed at all. At this stage of the investigation, the reason for the absence of SO_3 loss is equivocal. Either the original losses of SO_3 from the $[M–H]^-$ anions of (**1**) and (**2**) occur following sulfate rearrangement to the carboxylate anion centre, or the loss of SO_3 from the intact Ser sulfate moiety, *for some reason as yet undetermined*, requires the presence of an adjacent C-terminal CO_2H group.

A2. The $[(M–H)^-–SO_3]$ process from a C-terminal Ser sulfate-containing peptide

The $[(M–H)^-–SO_3]$ process which occurs from a C-terminal Ser sulfate has been explored by calculating reaction coordinate profiles for a model system at the CAM-B3LYP/6-311++g(d,p) level of theory. The results of this study are shown in Figs. 2 and 3. The process shown in Fig. 2 is the simple dissociative loss of SO_3 from the Ser sulfate anion (**1**) to yield the Ser alkoxide anion (**2**), a sequence unfavourable by 235 kJ mol^{-1}. Proton transfer within (**2**) may then form the carboxylate anion (**3**) and SO_3 (+93 kJ mol^{-1}). Alternatively, loss of CH_2O (the characteristic cleavage of Ser[1]) together with SO_3 may occur. The problem with the reaction (**1**) to (**2**) plus SO_3 is (i) it is energetically unfavourable and (ii) that it



Scheme 1.

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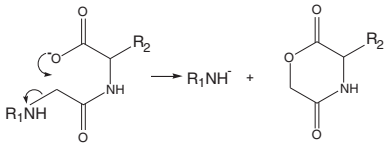

Scheme 2.

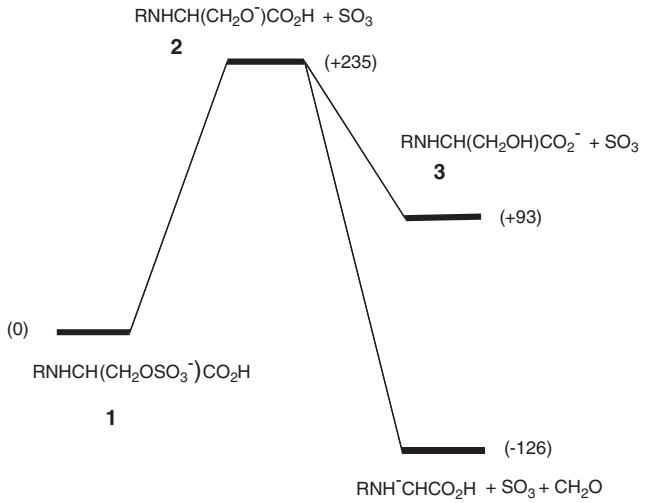

Figure 2. Reaction coordinate profile for the direct cleavage of SO_3 from $RNHCH(CH_2OSO_3^-)CO_2H$ (R = $COCH_3$). CAM-B3LYP/6-311++g(d,p) level of theory. Relative energies in kJ mol^{-1} from the sulfate anion (**1**) considered as 0 kJ mol^{-1}.

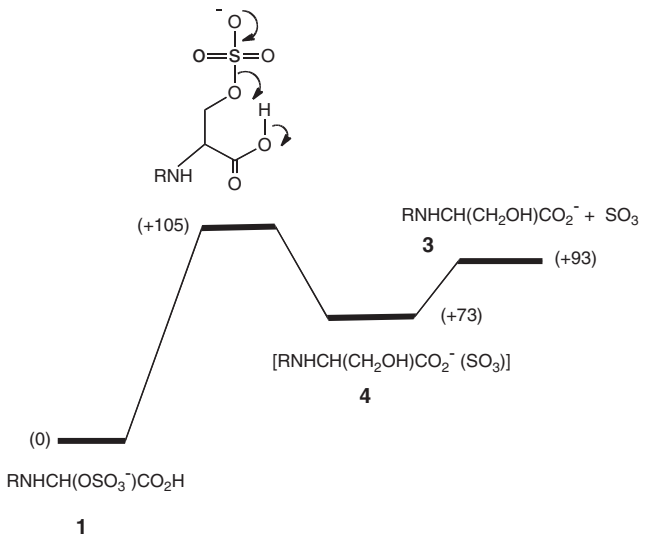

Figure 3. Reaction coordinate profile for the cleavage of SO_3 accompanied by proton transfer from the carboxyl group of $RNHCH(CH_2OSO_3^-)CO_2H$ (R = $COCH_3$). CAM-B3LYP/6-311++g(d,p) level of theory. Relative energies in kJ mol^{-1} from the sulfate anion (**1**) considered as 0 kJ mol^{-1}.

should occur irrespective of whether the C-terminus is CO_2CH_3 (for which loss of SO_3 is not noted) or CO_2H.

The scenario shown in Fig. 3 requires the presence of an intact C-terminal carboxyl group. This process is kinetically more favourable than that shown in Fig. 2. Loss of SO_3 accompanied by proton transfer involves a six-centred transition state (+105 kJ mol^{-1}) followed by the formation of reactive intermediate (**4**) (+ 73 kJ mol^{-1}) which then may decompose to carboxylate anion (**3**) and SO_3 (+93 kJ mol^{-1}). This process is (i) 142 kJ mol^{-1} more favourable than that involving direct loss of SO_3 to form the Ser alkoxide anion (**2**) shown in Fig. 2, and (ii) consistent with the absence of SO_3 loss from peptides containing $Ser(SO_3H)(OCH_3)$.

There is still the possibility that the loss of SO_3 could follow rearrangement of the sulfate from Ser to the C-terminal CO_2^- position. This scenario is shown in Fig. 4. Sulfate anion (**1**) converts into carboxylate (**5**) (+69 kJ mol^{-1}), which then effects an $S_Ni(S)$ rearrangement through a six-centred transition state concomitant with a four-centred proton transfer (barrier +181 kJ mol^{-1} from **5**) to form sulfated carboxylate (**6**) which then decomposes to products, namely carboxylate anion (**3**) and SO_3. This process requires 250 kJ mol^{-1} to reach the first transition state. As a consequence, this is an unlikely process compared with the reaction coordinate profile shown in Fig. 3.

Thus combined experimental and theoretical data suggest that loss of SO_3 from a peptide containing a C-terminal Ser sulfate anion is accompanied by proton transfer from the C-terminal carboxylic acid to yield a Ser carboxylate anion, as shown in Fig. 3.

A3. Direct $HOSO_3^-$ formation from a C-terminal Ser sulfate-containing peptide

The ion at *m/z* 97 ($HOSO_3^-$) constitutes the base peak in the negative-ion spectra of (**1**) and (**2**). That it is the only fragment anion in the corresponding spectra of the carbomethoxy analogues (**1a**) and (**2a**) suggests that it originates, at least in part, from the intact $Ser(SO_3H)$ groups of (**1**) and (**2**) (see Table 1 for sequences).

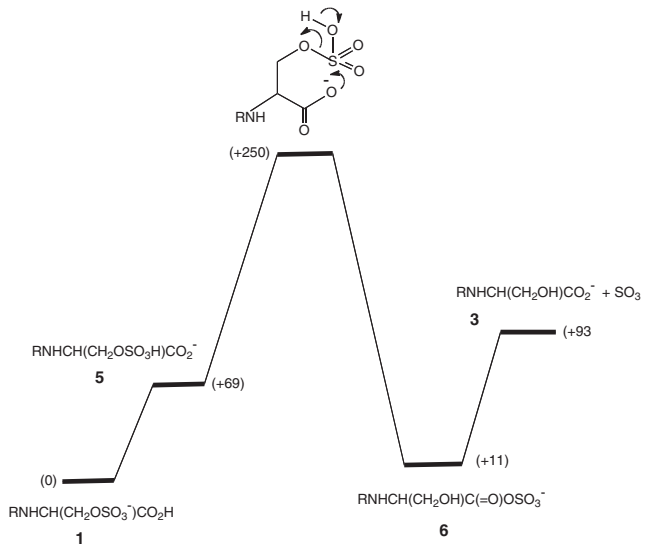

Figure 4. Reaction coordinate profile for the cleavage of SO_3 from $RNHCH(CH_2OH)C(=O)OSO_3^-$ (R = $COCH_3$) (**6**), following sulfate transfer from $RNHCH(CH_2OSO_3^-)CO_2H$ (**1**) to (**6**). CAM-B3LYP/6-311++g(d,p) level of theory. Relative energies in kJ mol^{-1} from the sulfate anion (**1**) considered as 0 kJ mol^{-1}.

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The formation of $HOSO_3^-$ by cleavage of an unstable enolate ion is shown in Fig. 5. The process occurs via synchronous proton transfer within (**7**) via an unstable enolate anion, which decomposes to form complex (**8**). Complex (**8**) may then form $HOSO_3^-$ or eliminate H_2SO_4. These processes (from sulfate **1**) require significant excess energy (+248 kJ mol^{-1}) with the formation of $HOSO_3^-$ endothermic by 27 kJ mol^{-1} while elimination of H_2SO_4 is thermodynamically unfavourable by 90 kJ mol^{-1}.

An alternative formation of $HOSO_3^-$ is shown in Fig. 6. In this case the proton transfer through an enolate anion is effected in the rate-determining step of the reaction. The process in Fig. 6 is more kinetically favourable than that shown in Fig. 5

A4. $HOSO_3^-$ formation from a C-terminal Ser sulfate-containing peptide following sulfate rearrangement to the C-terminal carboxyl

Sulfate transfer from Ser(SO_3H) to a C-terminal carboxyl anion via an S_Ni(S) rearrangement is shown in Fig. 7. The reaction is shown as initiated from the sulfate anion (to allow direct comparison with the energetics of those fragmentations considered earlier). The maximum barrier is 250 kJ mol^{-1} and the formation of $HOSO_3^-$ and loss of H_2SO_4 are endothermic by 130 and 264 kJ mol^{-1}, respectively. Loss of H_2SO_4 is not observed in any of the spectra so far considered, and the formation of $HOSO_3^-$ by this route is energetically unfavourable compared with those shown in Figs. 5 and 6.

B1. Fragmentations of a non-terminal Ser(SO_3H)

The spectra of peptides (**3**)–(**5**) which contain non-terminal Ser(SO_3H) also show peaks due to $HOSO_3^-$ and [$(M–H)^-$–SO_3] ions, with the latter usually producing the base peak. This can be seen in the negative-ion CID MS/MS data for GLS(SO_3H)GVA(OH) (**3**) shown in Fig. 8. Rearrangement of the Ser sulfate to the C-terminal carboxylate is energetically unfavourable, as described above. Calculations for a model

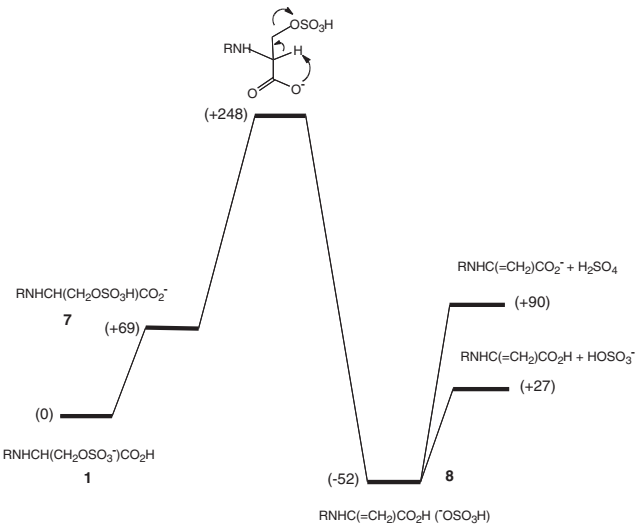

Figure 5. Reaction coordinate profile for the formation of $HOSO_3^-$ and the loss of H_2SO_4 following proton transfer from $RNHCH(CH_2OSO_3^-)CO_2H$ (R = $COCH_3$) (**1**) to the carboxylate $RNHCH(CH_2OSO_3H)CO_2^-$ (**7**). CAM-B3LYP/6-311++g(d,p) level of theory. Relative energies in kJ mol^{-1} from the sulfate anion (**1**) considered as 0 kJ mol^{-1}.

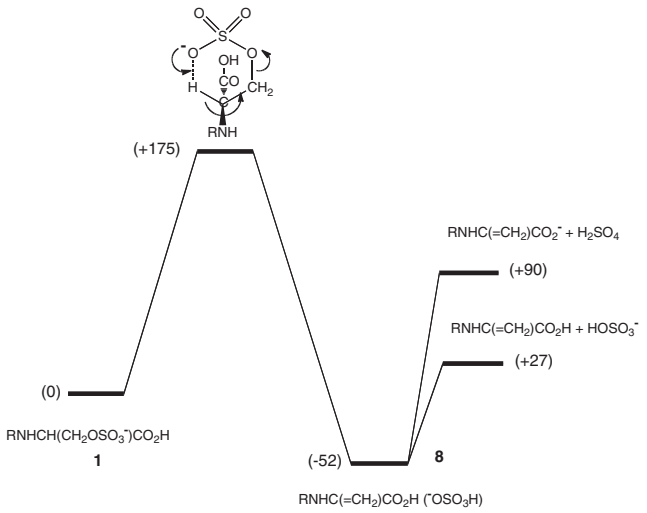

Figure 6. Reaction coordinate profile for the formation of $HOSO_3^-$ and the loss of H_2SO_4 from $RNHCH(CH_2OSO_3^-)CO_2H$ (R = $COCH_3$) (**1**) via a six-centred transition state involving proton transfer of an enolate hydrogen. CAM-B3LYP/6-311++g(d,p) level of theory. Relative energies in kJ mol^{-1} from the sulfate anion (**1**) considered as 0 kJ mol^{-1}.

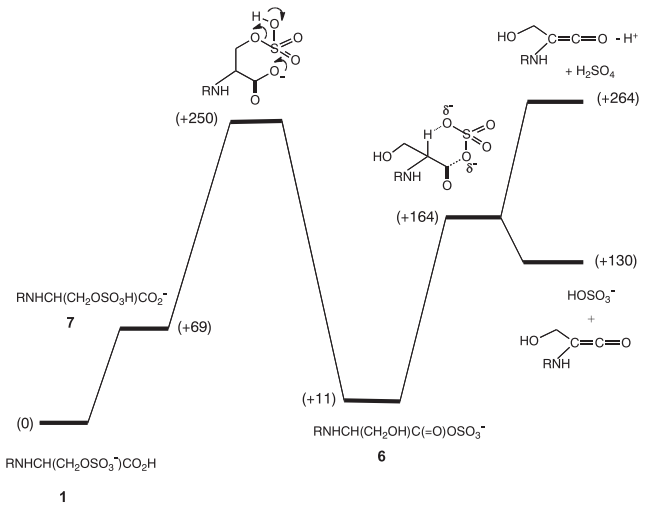

Figure 7. Reaction coordinate profile for the formation of $HOSO_3^-$ and the loss of H_2SO_4 following sulfate transfer within $RNHCH(CH_2OSO_3H)CO_2^-$ (**7**) to the carboxylate. CAM-B3LYP/6-311++g(d,p) level of theory. Relative energies in kJ mol^{-1} from the sulfate anion (**1**) considered as 0 kJ mol^{-1}.

system $RNH(CH_2OSO_3^-)CONHCH_2CO_2H$ (R = $COCH_3$) give a similar reaction coordinate profile to that shown in Fig. 7; rearrangement to $RNH(CH_2OH)CONHCH_2C(=O)OSO_3^-$ requires an excess energy of 290 kJ mol^{-1} to surmount the barrier to the S_Ni(S) reaction. Formation of $HOSO_3^-$ and loss of H_2SO_4 from the rearranged sulfate are endothermic by 122 and 256 kJ mol^{-1}, respectively, at the CAM-B3LYP/6-311++g(d,p) level of theory (details not shown). These processes are kinetically and thermodynamically unfavourable.

It has been shown in section A2 how SO_3 may be lost from a C-terminal Ser sulfate anion with accompanying proton transfer from the carboxylic acid group (Fig. 3). If the Ser sulfate is not C-terminal, the analogous loss of SO_3 might occur through a similar six-membered transition state but

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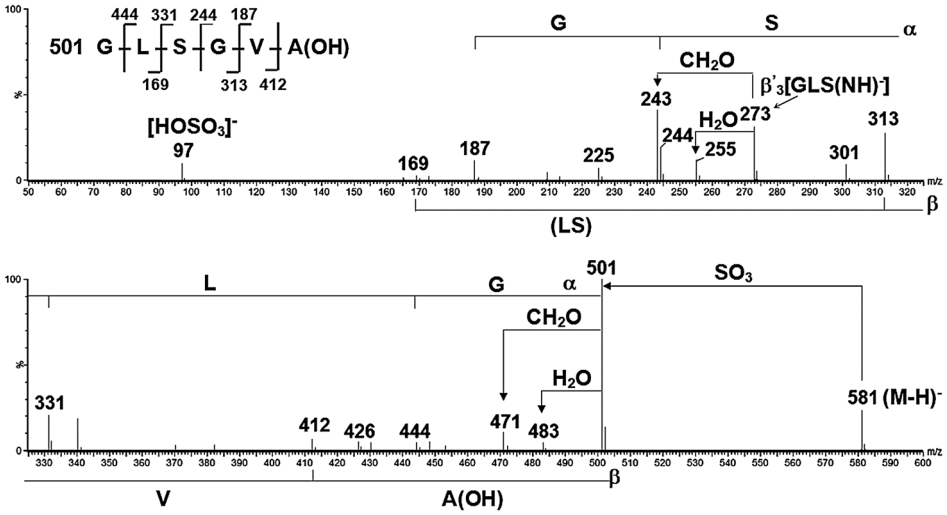


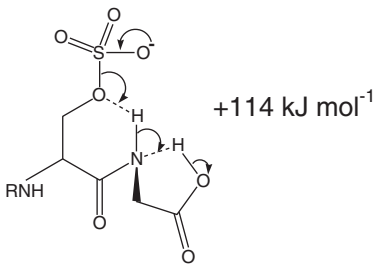
Figure 8. Negative-ion electrospray CID MS/MS spectra of $[M–H]^-$ of GLS(SO_3H)GVA (OH) (**3**). For experimental conditions, see Experimental section. Multiplication ranges: (×50), *m/z* 100–465. Masses are nominal masses, i.e. the sum of the integral masses of the individual amino acid residues.

utilizing the acidic proton of the Ser backbone amide. An extensive search of the potential surface for the overall reaction $RNHCH(CH_2OSO_3^-)CONHCH_2CO_2H \rightarrow RNHCH(CH_2OH)CO^-NCH_2CO_2H + SO_3$ (R = CH_3CO) failed to find a proton transfer to form a stable backbone amide product anion. Instead, the product is, again, the C-terminal carboxylate anion, but the mechanism is more complex than that shown in Fig. 3. The transition state and mechanism shown in Scheme 3 involve two concomitant proton transfers; from and to the adjacent backbone amide nitrogen. The barrier for this process is 114 kJ mol^{-1}, a value which increases with increasing distance between the sulfate anion and the C-terminal carboxylic acid group: for example, +200 kJ mol^{-1} for the sulfate on the third last residue (CAM-B3LYP/6-311++g(d,p) level of theory, details not shown). A prerequisite for this loss of SO_3 is that the backbone of the peptide be flexible, in order to allow approach of the adjacent amide nitrogen and the C-terminal carboxyl group.[a]

B2. Fragmentations of a non-terminal Ser(SO_3H) in the presence of Asp

The calculations described previously indicate that sulfate transfer is unlikely to occur from Ser(SO_3H) to a C-terminal carboxylate anion. Similarly, sulfate transfer from a Ser sulfate to a side-chain carboxylate anion, for example to an Asp carboxylate anion, also seems unlikely. We have not calculated the reaction coordinate profile for this rearrangement.

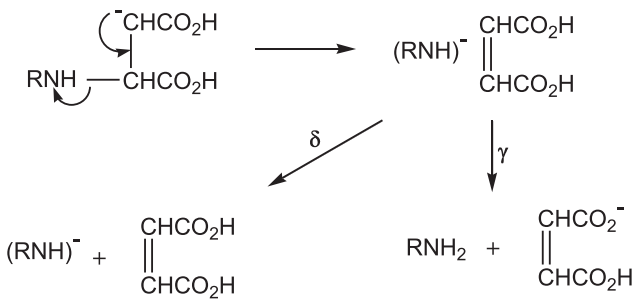
[a]The mechanism for loss of SO_3 from a C-terminal Ser sulfate anion of a peptide containing a C-terminal $CONH_2$ is analogous to that shown for the C-terminal CO_2H system depicted in Fig. 3. The barrier to the transition state for the C-terminal $CONH_2$ reaction is +218 kJ mol^{-1}. For the loss of SO_3 from $RNHCH(CH_2OSO_3^-)CONHCH_2CONH_2$, the transition state is similar to that shown in (**1**) above, but with the terminal OH replaced by NH_2. The barrier to the transition state for this process is +200 kJ mol^{-1}(full theoretical data not provided).



Scheme 3.

The CID MS/MS data for the $[M–H]^-$ ions of the Asp-containing peptide GLS(SO_3H)GDA(OH) (**4**) are shown in Fig. 9. Comparison of Figs. 8 and 9 shows domination of the classical Asp cleavages in Fig. 9, which are more favourable than the simple backbone cleavages shown in Fig. 8 (which allow sequence determination). The characteristic **δ** (*m/z* 330) and **γ** (*m/z* 186) cleavage ions of Asp[1] are formed principally from *m/z* 517. These processes are shown in the general case in Scheme 4 (the **γ** process is exothermic by 42 kJ mol^{-1}, early calculations at the HF 6-31G/AM1 level of theory[2]).

Examination of the CID MS/MS/MS data for *m/z* 517 and 499 of Fig. 9 (data not included here) indicates that *m/z* 499 is formed by the characteristic loss of H_2O from the Asp side chain (i.e. *m/z* 517 loses H_2O to form *m/z* 499)



Scheme 4.

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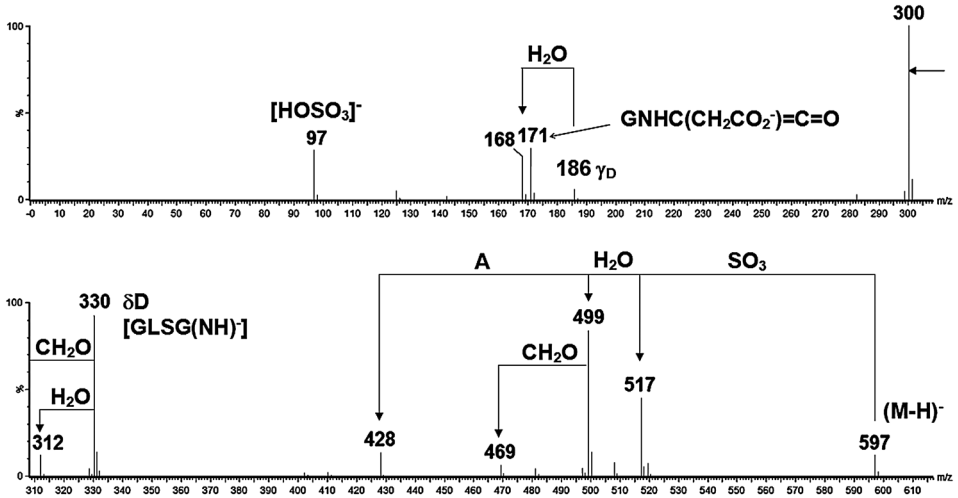
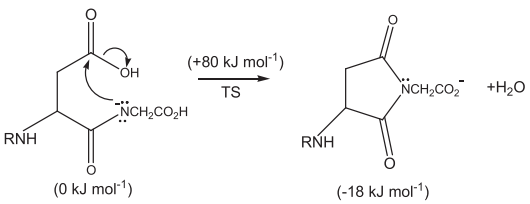
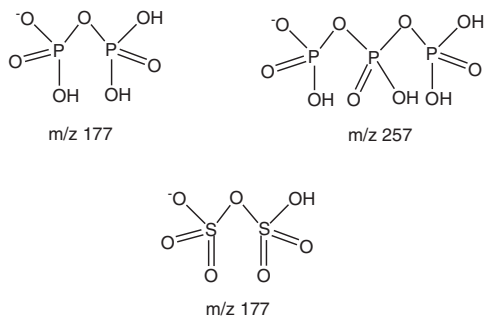


Figure 9. Negative-ion electrospray CID MS/MS spectra of $[M–H]^–$ of GLS(SO_3H)GDA (OH) (**4**). For experimental conditions, see Experimental section. Masses are nominal masses, i.e. the sum of the integral masses of the individual amino acid residues. *m/z* 171 is formed by two **α** cleavages with accompanying H^+ transfers.



Scheme 5.



Scheme 6.

as shown for a model system in Scheme 5. This should not be confused with the diagnostic loss of water from a C-terminal carboxylate position, i.e. $RNH^–CHCO_2H \rightarrow [RNHCH=C=O)\ ^–OH] \rightarrow R^–NCH=C=O + H_2O$ (R = CH_3CO). The reaction is endothermic by 92 kJ mol^{-1} and the barrier to the transition state for the first step is 156 kJ mol^{-1} at the CAM-B3LYP/6-311++g(d,p) level of theory. This reaction is less favourable than that shown in Scheme 5.

C. Do disulfates form $HS_2O_7^–$ (*m/z* 177) by sulfate $S_Ni(S)$ rearrangement?

Characteristic reactions of the $[M–H]^–$ species of di- and triphosphate-containing peptides result in the formation of $H_3P_2O_7^–$ (*m/z* 177) and $H_4P_3O_{10}^–$, respectively (*m/z* 257), following interaction of the various phosphate residues. The structures of these ions are shown in Scheme 6, and the reactions are energetically favourable. In the case of $H_3P_2O_7^–$ the reaction has a barrier of 112 kJ mol^{-1} to the $S_Ni(P)$ transition state, but the reaction is strongly exothermic (−299 kJ mol^{-1}) at the HF/6-31 + G(d)//AM1 level of theory.[23]

In order to test whether the analogous reaction occurs for a disulfate-containing peptide (to form sulfur-containing *m/z* 177, Scheme 6), the CID MS/MS data for the $[M–H]^–$ ion of GLS(SO_3H)GS(SO_3H)A(OH) (**5**) were determined; the spectrum is shown in Fig. 10. The spectrum is dominated by peaks from $HOSO_3^–$, $[(M–H)^––SO_3]$ and $[(M–H)^––2SO_3]$. No peak at *m/z* 177 ($HS_2O_7^–$) is present.

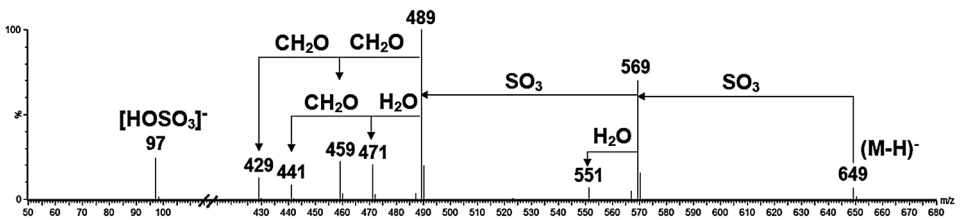


Figure 10. Negative-ion electrospray CID MS/MS spectra of $[M–H]^–$ of GLS(SO_3H)GS(SO_3H)A(OH) (**5**). For experimental conditions, see Experimental section. Masses are nominal masses, i.e. the sum of the integral masses of the individual amino acid residues.

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CONCLUSIONS

The major fragmentations occurring from energised $[M–H]^-$ ions of Ser sulfate-containing peptides, $[(M–H)^–SO_3]$ and $[M–H]^- \rightarrow HOSO_3^-$, originate by loss of SO_3 and formation of $HOSO_3^-$ each accompanied by proton transfer. For the peptides studied, the $HOSO_3^-$ ion is the base peak when the Ser sulfate is C-terminal, whereas $[(M–H)^–SO_3]$ is the base peak when the Ser sulfate is not C-terminal. The mechanisms of these fragmentations have been studied at the CAM-B3LYP/6-311++g(d,p) level of theory. No rearrangement ions corresponding to $[(M–H)^–H_2SO_4]$ are noted in these spectra. This should be contrasted to the corresponding spectra of Tyr sulfates which show $[(M–H)^–SO_3]$ as base peak, with rearrangement peaks corresponding to $HOSO_3^-$ being minor (less than 10% of the base peak), and with $[(M–H)^–H_2SO_4]$ peaks either absent or of very low abundance (≤2% of the base peak).

Sequencing information can normally be obtained using CID MS/MS/MS data of $[(M–H)^–SO_3]$ ions of the peptides studied, with the exception of the peptide that contains Asp, where the diagnostic fragmentations of Asp are more facile than those of the Ser sulfate group. This restricts the formation of most **α** and **β** product ions from the $[(M–H)^–SO_3]$ anion.

The negative-ion spectra of di- and triphosphate-containing peptides contain peaks corresponding to anions formed following cyclisation of the phosphate groups. In comparison, corresponding peaks are not detected in the spectra of energised $[M–H]^-$ ions of Ser disulfate-containing peptides.

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