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Leaving Group and Gas Phase Neighboring Group Effects in the Side Chain Losses from Protonated Serine and its Derivatives*

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The gas phase fragmentation reactions of protonated serine and its $YNHCH(CH_2X)CO_2H$ derivatives, β-chloroalanine, S-methyl cysteine, O-methyl serine, and O-phosphoserine, as well as the corresponding N-acetyl model peptides have been examined via electrospray ionization tandem mass spectrometry (MS/MS). In particular, the competition between losses from the side chain and the combined loss of H_2O and CO from the C-terminal carboxyl group of the amino acids or H_2O or CH_2CO from the N-acetyl model peptides are compared. In this manner the effect of the leaving group (Y = H or CH_3CO, vary X) or of the neighboring group can be examined. It was found that the amount of HX lost from the side chain increases with the proton affinity of X [$OP(O)(OH)_2 > OCH_3 \approx OH > Cl$]. The ion due to the side chain loss of H_2O from the model peptide N-acetyl serine is more abundant than that from protonated serine, suggesting that the N-acetyl group is a better neighboring group than the amino group. Ab initio calculations at the MP2(FC)/6-31G*//HF/6-31G* level of theory suggest that this effect is due to the transition state barrier for water loss from protonated N-acetyl serine being lower than that for protonated serine. The mechanism for side chain loss has been examined using MS^3 tandem mass spectrometry, independent synthesis of proposed product ion structures combined with MS/MS, and hydrogen/deuterium exchange. Neighboring group rather than *cis* 1,2 elimination processes dominate in all cases. In particular, the loss of H_3PO_4 from O-phosphoserine and N-acetyl O-phosphoserine is shown to yield a 3-membered aziridine ring and 5-membered oxazoline ring, respectively, and not the dehydroalanine moiety. This is in contrast to results presented by DeGnore and Qin (*J. Am. Soc. Mass Spectrom.* **1998,** *9*, 1175–1188) for the loss of H_3PO_4 from larger peptides, where dehydroalanine was observed. Alternate mechanisms to *cis* 1,2 elimination, for the formation of dehydroalanine in larger phosphoserine or phosphothreonine containing peptides, are proposed. (J Am Soc Mass Spectrom 2000, 11, 1047–1060) © 2000 American Society for Mass Spectrometry

One of the great successes of tandem mass spectrometry has been its use in the sequencing of peptides. This has prompted several groups to examine the mechanisms of the fragmentation reactions of protonated (i.e., $[M + H]^+$) ions of amino acids and peptides [1]. In our studies, aimed at developing a comprehensive understanding of the influence of various neighboring groups within a protonated peptide, we have utilized several gas phase "physical organic" tools, including multistage (MS^n) mass spectrometry experiments combined with independent synthesis of proposed product ion structures, isotopic and structural labeling, and ab initio molecular orbital calculations [2]. We have found that various functional groups present in protonated amino acids and peptides can act as intramolecular nucleophiles [2b–g] to help facilitate fragmentation reactions. In principle, there are three types of neighboring group reactions that can yield fragment ions: (1) nucleophilic side chain attack on the electrophilic (i.e., charged) backbone; (2) nucleophilic backbone attack on an electrophilic (i.e., charged) side chain; and (3) nucleophilic backbone attack on an adjacent electrophilic (i.e., charged) backbone. For peptides, further distinctions can be made depending upon whether (a) sequence or (b) nonsequence ions are formed in these neighboring group reactions.

Examples of type 1 reactions, have been demonstrated in the fragmentation reactions of protonated

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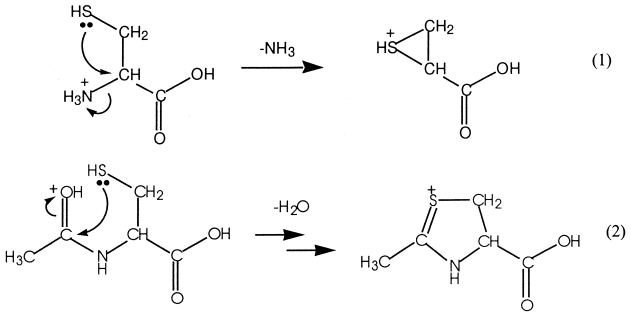
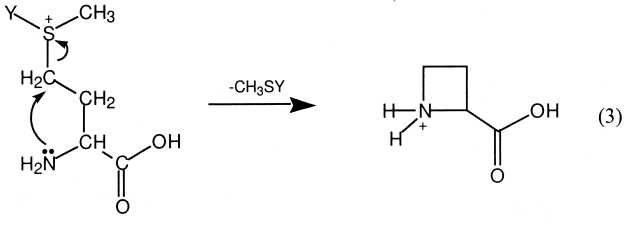
cysteine, where the thiol neighboring group facilitates loss of NH_3 (eq 1), as demonstrated by comparison of the fragmentation reactions of the proposed episulfonium ion with an independently synthesized sample [2c], and in the fragmentation of simple cysteine containing peptides, where the thiol group can displace water from an adjacent protonated peptide carbonyl (eq 2) [2b,c]. Not only are both these examples of type 1 reactions, which yield nonsequence ions, but they also have solution phase analogs [3].

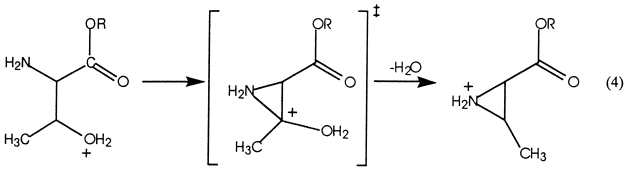


An example of a type 2 reaction has been shown recently for the side chain loss from protonated methionine and its derivatives [2f]. It was demonstrated that the competition between side chain loss and all other fragmentation reactions depends upon the ability of the methionine side chain leaving group to hold a charge (eq 3), which follows the order Y = Me (a fixed charge derivative) > OH > H. Also, the side chain loss of CH_3SOH from protonated methionine sulfoxide (which would form either a 4- or 5-membered product ion from neighboring group attack by the amine or carbonyl group, respectively) is greatly enhanced by N-acetylation, which not only introduces a better neighboring group, but enables the formation of a 6-membered product ion.



Thus, each of these neighboring group reaction types not only depend upon the ability of various groups to act as neighboring groups but also on the size of the resultant ring formed, and the nature of the neutral leaving group, the precursor for which must be charged. Therefore, it is a combination of all these factors that dictates which fragmentation processes are observed and whether neighboring group processes [4] are favored over charge remote losses [5]. (It should be pointed out that neighboring group reactions were proposed as long ago as 1973 for H_2O loss from protonated β substituted ethanols [4c].)

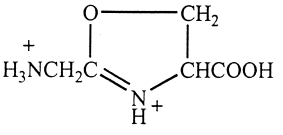
Recently, using hydrogen/deuterium exchange combined with MS/MS experiments, we have shown that the type 2 side chain loss of H_2O from protonated threonine occurs via a neighboring group mechanism (likely to involve the amino group to yield a protonated aziridine) (eq 4) [2d].



It is important to note that side chain losses which yield nonsequence ions are not merely a curiosity in tandem mass spectrometry, but can provide useful information as to the nature of a particular residue within a peptide. For example, the loss of MeSOH [2f] has been used as a diagnostic marker of methionine oxidation [6], whereas loss of H_3PO_4 is diagnostic of phosphorylated peptides, particularly those containing phosophoserine and phosphothreonine residues [7a–f]. As phosphorylation plays a key role in intracellular signaling processes and is an essential participant in protein–protein interactions [7g], to fully understand the role that phosphorylation plays in biological processes, and to design inhibitors that may perturb these interactions, characterization of the site at which phosphorylation occurs is critical. Although sequence analysis of the peptide can be employed to identify the site of phosphorylation [7b–f], few studies have been performed to probe the mechanism for this loss and to determine the structure of the resultant product ions [7c,d,h–j]. Early work by Perich et al. [7h,i] and Dass [7j] proposed that O-phosphoserine fragments by loss of H_3PO_4, via a 6-centered transition state, to yield dehydroalanine. Recent results obtained by DeGnore and Qin indicated, using MS^3 of an $[M + H - H_3PO_4]^+$ product ion generated by MS/MS, sequence specific product ions for dehydroalanine, and suggested that formation of dehydroalanine upon loss of H_3PO_4 from larger peptides occurs by *cis* 1,2 β-elimination [7c]. A recent paper by Tholey et al. [7d], using hydrogen–deuterium exchange coupled with MS/MS in a triple quadrupole mass spectrometer, also suggested *cis* 1,2 elimination by a 6-centered transition state as a possible mechanism. However incomplete exchange of all the exchangeable hydrogen atoms, and poor resolution of the product ion spectrum, makes this result inconclusive. An understanding of the structure of the product ion following side chain loss of H_3PO_4 could enable the development of tools that will assist in the identification and characterization of phosphorylated proteins of biological relevance. Therefore, it is of great interest to determine whether the loss of H_3PO_4 from phosphoserine or threonine in the gas phase does result solely in

dehydroalanine formation, or if other products also result, particularly because many solution phase studies involving elimination of leaving groups from modified serine or threonine residues [8] have shown that formation of β-lactams, azridines, and oxazolines, in addition to dehydroalanines, are all possible, depending on the reaction conditions used [8d–f,h], as well as the nature of both the neighboring [8a,c] and leaving groups [8a,b,g] present.

In order to gain further insights into the mechanisms of these side chain losses, and to determine the structure of the resultant product ions, we decided to probe the fragmentation reactions of serine and its derivatives in detail. Side chain reactions of serine and its peptides were reported as long ago as 1959, when Bovey and Tiers examined their proton NMR spectra in trifluoroacetic acid solutions [9]. They noted that water loss occurred for glycylserine and suggested that a neighboring group reaction had occurred to yield the oxazoline product (**A**). This oxazoline has also been proposed as an intermediate in N–O acyl shifts involving serine residues in peptides [10]. In the gas phase, Harrison has noted water loss from serine under chemical ionization (CI) conditions [11], whereas Gaskell has provided convincing evidence for side chain water loss from the serine residue in the $[M + H]^+$ ion of delta-sleep-inducing peptide under collision-induced dissociation (CID) conditions [1e], although in both cases the precise mechanism was not determined.

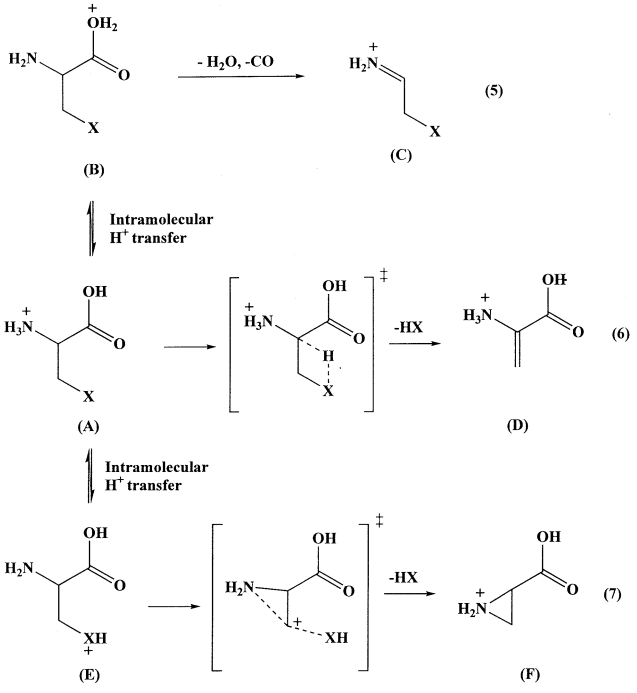


(A)

Herein, we present MS/MS, MS^3 and ab initio [7] data on the $[M + H]^+$ ions of serine and its derivatives, including O-phosphoserine, in order to examine (i) the competition between side chain loss and other fragmentation reactions (eq 5 in Scheme **1**) and (ii) whether side chain loss, particularly that of H_3PO_4, occurs via charge remote *cis* 1,2 elimination (eq 6 in Scheme **1**) or whether a type 2 neighboring group mechanism (eq 7 in Scheme **1**) operates.

Experimental

Amino acid and peptide methyl esters and their N-acetylated derivatives were prepared as described previously [2b]. N-acetyl O-phosphoserine was synthesized using a literature procedure [12]. All other reagents were commercially available and were used without further purification. $[M + H]^+$ ions and fully deuterated $[M + D]^+$ ions were formed via electrospray ionization (ESI) on a Finnigan model LCQ (San Jose, CA) quadrupole ion trap mass spectrometer. Samples (0.1 mg/mL) were dissolved in 50% H_2O/50% CH_3OH containing 0.1 M acetic acid or, for H/D exchange experiments, in 50% D_2O/50% CH_3OD containing 0.1 M CH_3CO_2D, lyophilized to dryness and then redissolved in the same deuterated solvent system to maximize hydrogen/deuteron exchange. Samples were introduced to the mass spectrometer at 3 μL/min. The spray voltage was set at −5 kV. Nitrogen sheath gas and auxiliary gas pressures were supplied at 30 lb/in.^{2} and 15 (arbitrary unit), respectively. The heated capillary temperature was set at 200 °C. MS/MS and MS^3 experiments were performed on mass selected ions in the quadrupole ion trap mass spectrometer using standard isolation and excitation procedures. ^{1}H NMR solution phase structural analysis of serine, O-phosphoserine, and N-acetyl serine (10 mg/mL) was performed in D_2O containing 1% DCl at room temperature. Resultant spectra are available from the authors upon request.



Scheme 1.

Computational Methods

Candidate transition state structures were optimized at the Hartree–Fock level of theory using the Gaussian98W molecular modeling package [13] with the standard 6-31G* basis set [14]. In each case, a set of possible rotamers was explored [15]. All optimized structures were then subjected to vibrational frequency analysis using the same basis set to determine the nature of the stationary points, followed by single point energy calculation of the correlated energy at the MP2(FC)/6-31G* level of theory (FC = frozen core). Energies were corrected for zero-point vibrations scaled by 0.9135 [16]. Intrinsic reaction coordinate (IRC) runs were performed on each transition state to locate the appropriate reactant and product ion minima. Complete structural details and lists of vibrational frequencies for each HF/6-31G* optimized structure are available from the authors upon request.

Table 1. CID MS/MS spectra of protonated serine and N-acetyl serine derivatives

Amino acid	*m/z*	MS/MS product ions; *m/z* (neutral loss) % relative abundance[a]
[Serine + H]$^+$	106	88(H_2O)24; 60(H_2O,CO)100
[Serine-OMe + H]$^+$	120	102(H_2O)100; 60(CH_3OH,CO)67
[S,S-diMe-cysteine]$^+$	150	88($S(CH_3)_2$)100
[NAc-serine + H]$^+$	148	130(H_2O)100; 106(CH_2CO)60; 102(H_2O,CO)1; 88(CH_2CO,H_2O)8
[NAc-serine-OMe + H]$^+$	162	144(H_2O)100; 130(CH_3OH)49; 120(CH_2CO)39; 102(CH_3OH,CO)3
[NAc-S,S-diMe-Cysteine]$^+$	192	130[$S(CH_3)_2$]100

[a]Only those product ions greater than 1% relative abundance are shown.

Results and Discussion

ESI CID MS/MS Studies of the Fragmentation Reactions of Protonated Serine and its Derivatives

Results of the CID MS/MS fragmentation reactions of protonated serine and the serine side chain derivatives [$YNHCH(CH_2X)CO_2H$] [where Y = H and X = Cl, SCH_3, OCH_3, $OP(OH)_2O$, or $S(CH_3)_2$] as well as their N-acetyl (Y = CH_3CO) model peptide derivatives are listed in Table 1 and Figures 1 and 2. For protonated serine (Table 1) in agreement with previous studies [1b,i,17], losses of H_2O (*m/z* 88) and the combined losses of H_2O + CO (*m/z* 60) were the only products observed. To distinguish H_2O loss from the side chain versus the carboxylic acid, the O-methyl ether derivative of serine, where the side chain loss of CH_3OH is observed in the same relative abundance as that for H_2O loss from serine, was employed. For each of the other serine side chain derivatives, only two types of product ion were observed; the combined loss of H_2O and CO and loss of HX from the side chain. The only exception to this was the case of S-methyl cysteine, where NH_3 loss (cf. eq 1) was observed as the only product (Figure 1B). For the N-acetyl amino acids, losses from the side chain were in competition with loss of H_2O from the carboxylic acid and loss of CH_2CO from the N-terminal.

In order to examine the effect of the leaving group on the product ions observed, the ratios of HX loss from the side chain and the combined loss of H_2O and CO from the carboxylate group of the protonated serine derivatives were compared (compare eq 5 with eqs 6 and 7 in Scheme **1**). The side chain losses of HCl, CH_3OH, and H_3PO_4 versus H_2O and CO loss for the β-chloroalanine, O-methyl serine, and O-phosphoserine derivatives shown in Figure 1A,C,D, respectively, were 0:1.0, 0.2:1.0, and 1.0:0. Thus, as the proton affinity of X increases [$OP(O)(OH)_2 > OCH_3 > Cl$] [18] so too does the amount of HX lost from the side chain. (This finding

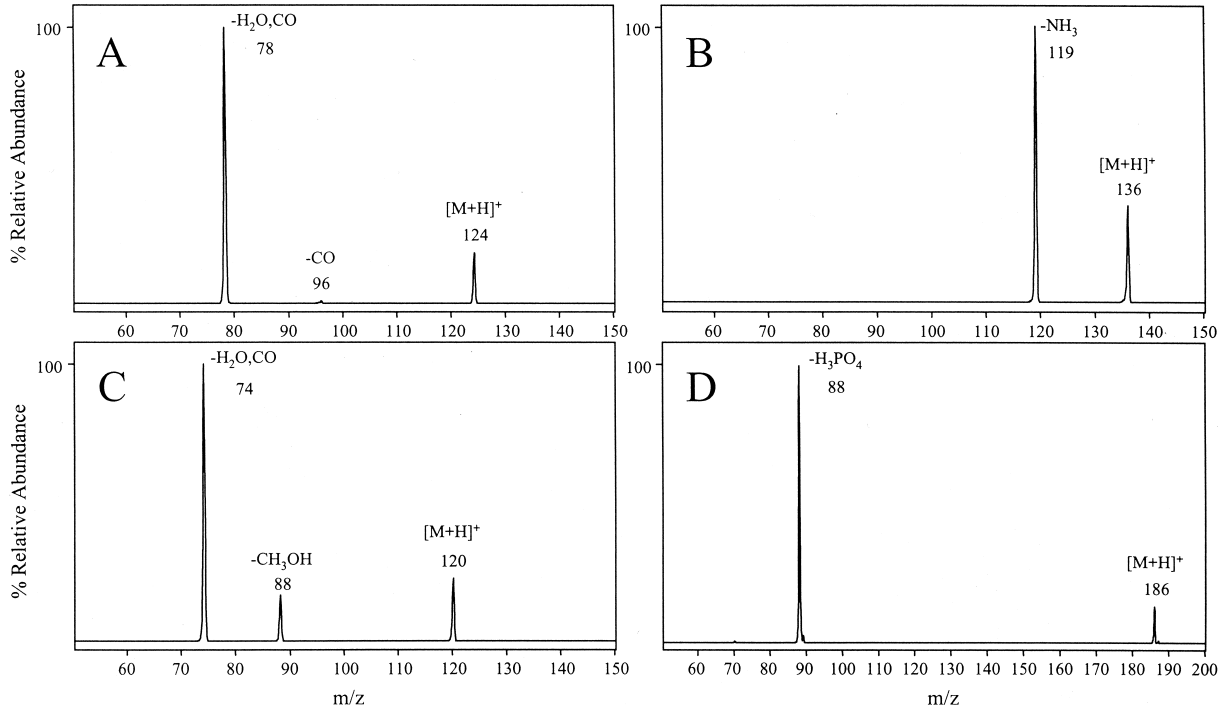


Figure 1. CID MS/MS spectra of the [M + H]$^+$ ions of (**a**) β-chloroalanine, (**b**) S-methyl cysteine, (**c**) O-methyl serine, and (**d**) O-phosphoserine.

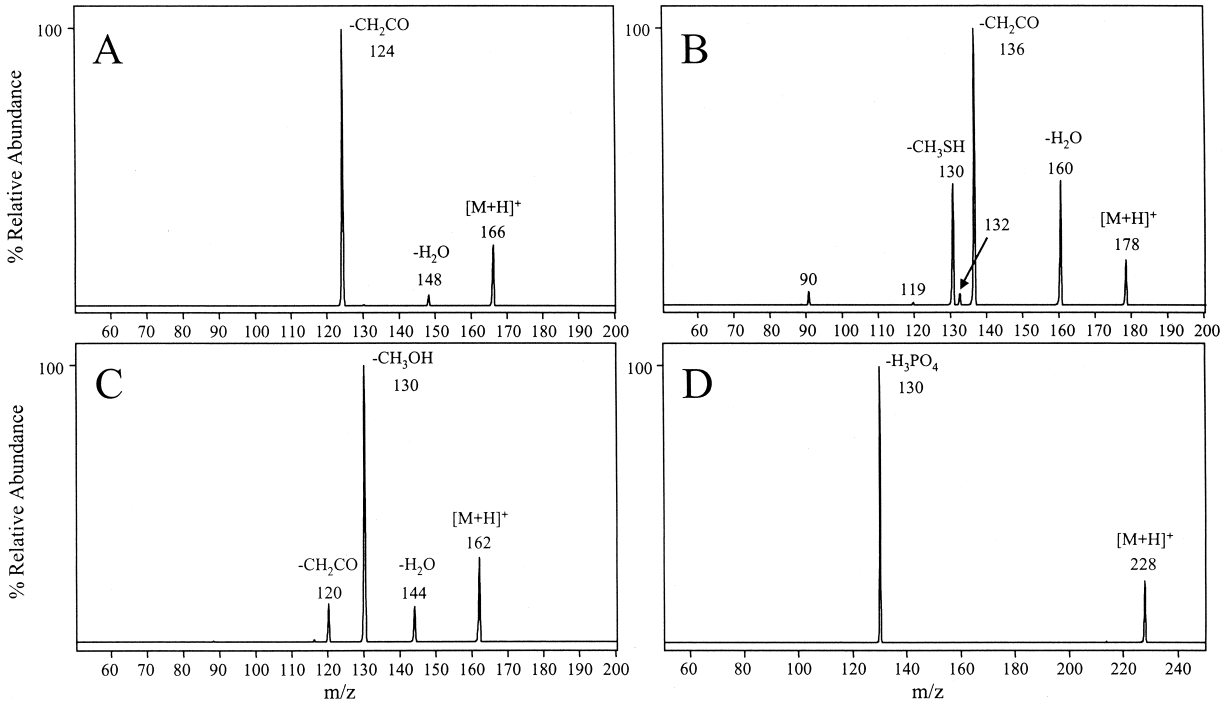


Figure 2. CID MS/MS spectra of the $[M + H]^+$ ions of (**a**) N-acetyl, β-chloroalanine, (**b**) N-acetyl, S-methyl cysteine, (**c**) N-acetyl, O-methyl serine, and (**d**) N-acetyl, O-phosphoserine.

is different to some CI studies in which species with lower PA are lost preferentially [18b]. It is important to note that in these CI studies only source decomposing $[M + H]^+$ ions account for these observations.) To assess the role of the neighboring group, the side chain losses from the amino acids were compared to those of the N-acetyl derivatives (Figure 2). In each case, where side chain loss was observed, the relative abundance was greater for the N-acetyl derivative compared to the amino acid (compare Figure 1 with Figure 2), suggesting that the N-acetyl group is a better neighboring group than the amino group.

CID MS^3 Experiments to Probe the Structures of the $[M + H - HX]^+$ Product Ions Formed by Side Chain Loss from Serine and O-phosphoserine

In order to probe the structures of the $[M + H - HX]^+$ product ions formed by side chain loss from protonated O-methyl serine and O-phosphoserine, the MS^3 spectra of each (Figure 3A,B, respectively) were compared with that of the side chain fixed charge derivative, S,S dimethyl cysteine (Figure 3C) (where only neighboring group participation reactions are expected to occur), the MS^3 spectra of dehydroalanine (Table 2), formed by the facile loss of CH_2CO from N-acetyl dehydroalanine (see Figure 4C), as well as the MS/MS spectra of protonated aziridine carboxylic acid (Table 2). Clearly, the spectra are identical to the side chain fixed charge derivative (Figure 3C) and aziridine carboxylic acid (Table 2), where H_2O loss (*m/z* 70) was observed as the major product, but markedly different to that of dehydroalanine (Table 2), where the major ion observed (*m/z* 43) corresponded to loss of $H_2O + CO$. Thus, it is apparent that the side chain loss of water from serine and O-phosphoserine occurs, as proposed earlier (cf. eq 4) for protonated threonine [2d], via attack at the protonated side chain by the amino group to yield aziridine carboxylic acid.

CID MS^3 Experiments to Probe the Structures of the $[M + H - HX]^+$ Product Ions Formed by Side Chain Loss from N-acetyl-serine and N-acetyl-O-phosphoserine

In addition to potential dehydroalanine (Scheme **2**, structure I) and aziridine (Scheme **2**, structure J) product ions as discussed above for the losses from the side chains of protonated serine derivatives, a 5-membered oxazole product ion (Scheme **2**, structure K) could be formed for the N-acetyl amino acids by nucleophilic attack on the side chain by the amide carbonyl. Therefore, the MS^3 fragmentation of the $[M + H - HX]^+$ ions for the N-acetyl derivatives of serine and O-phosphoserine (Figure 4A,B) were compared against those of the corresponding fixed charge S,S dimethyl cysteine derivative (Table 2) and the MS/MS spectrum of N-acetyl dehydroalanine (Figure 4C). As can be seen from Figure 4, the MS^3 spectra of serine and O-phosphoserine (Figure 4A,B) were found to be quite similar to each other as well as that of the fixed charge

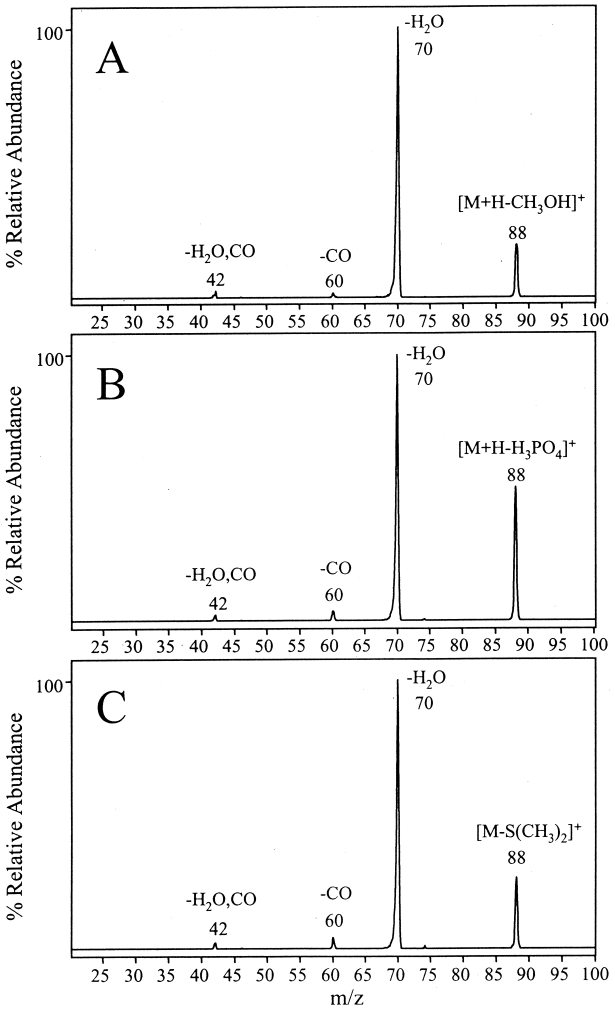


Figure 3. CID MS^3 spectra of the (**a**) $[M + H - CH_3OH]^+$ ion of O-methyl serine, (**b**) $[M + H - H_3PO_4]^+$ ion O-phosphoserine, and (**c**) $[M + H - (CH_3)_2S]^+$ ion of the fixed charged derivative S,S-dimethyl cysteine.

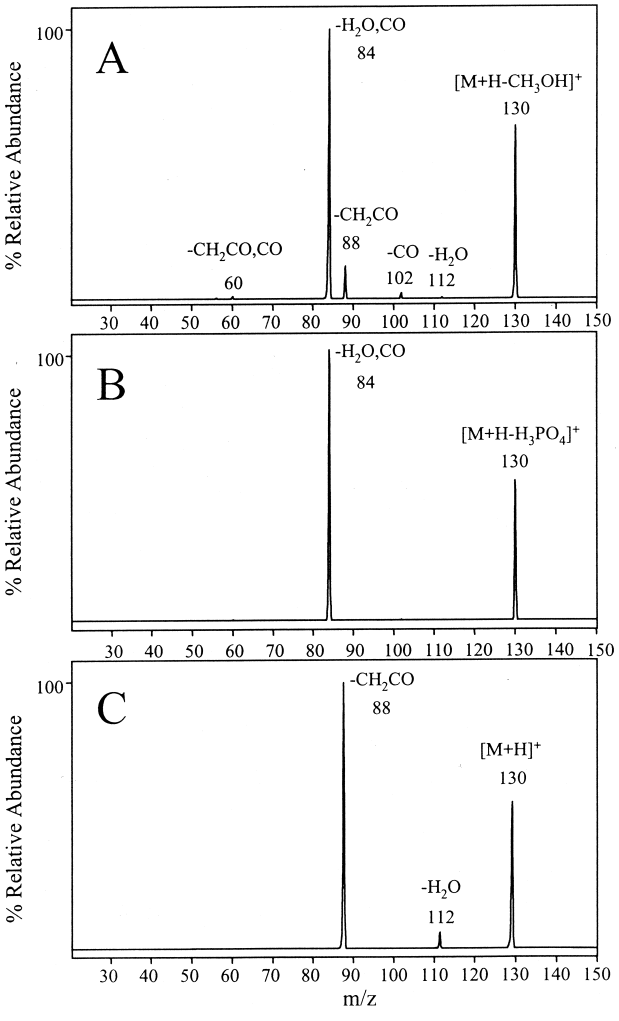


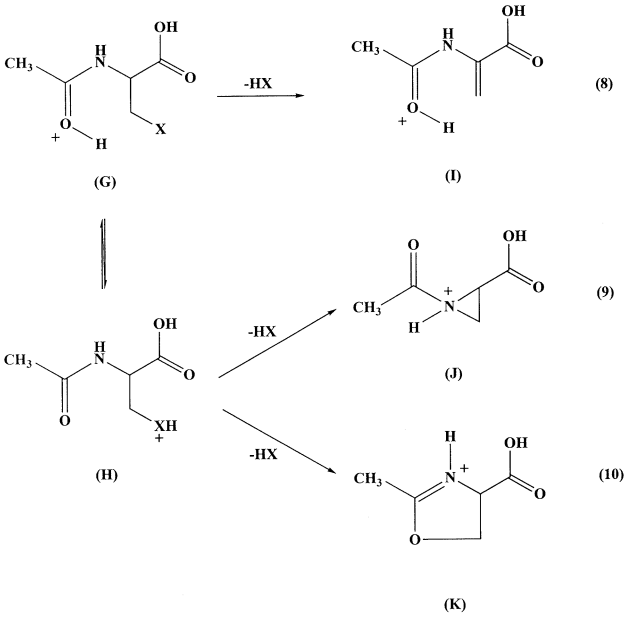
Figure 4. CID MS^3 spectra of the (**a**) $[M + H - CH_3OH]^+$ ion of N-acetyl, O-methyl serine and (**b**) $[M + H - H_3PO_4]^+$ ion of N-acetyl, O-phosphoserine. (**c**) CID MS/MS spectrum of the $[M + H]^+$ ion of N-acetyl, dehydroalanine.

derivative, where predominantly H_2O and CO loss was observed, but markedly different to the CID spectra of N-acetyl dehydroalanine, where CH_2CO loss was observed, indicating that neighboring group processes dominate. Examination of Table 2 however, reveals the loss of CH_2CO from several species, suggesting that dehydroalanine formation may occur in some cases, albeit at low abundance.

Table 2. CID MS^3 spectra of various side chain loss MS/MS product ions

Amino acid	*m/z*	MS^3 product ions; *m/z* (neutral loss) % relative abundance[a]
$[Serine + H]^+ - H_2O$	88	70(H_2O)100; 60(CO)4; 42(H_2O,CO)2
$[Serine\text{-}OMe + H]^+ - H_2O$	102	88(14)10; 74(CO)28; 70(CH_3OH)100; 60(42)75; 59(43)5; 46(56)6; 43(59)9; 42(CH_3OH,CO)2; 34(68)1
$[H_2NC(CH_2)CO_2H + H]^+$ [b]	88	70(H_2O)13; 60(CO)9; 42(H_2O,CO)100[d]
$[HN(CH_2)CHCO_2H + H]^+$ [c]	88	70(H_2O)100; 60(CO)8; 42(H_2O,CO)2[d]
$[NAc\text{-}serine + H]^+ - H_2O$	130	112(H_2O)4; 102(CO)32; 88(CH_2CO)78; 84(H_2O,CO)100; 60(CH_2CO)3
$[NAc\text{-}serine\text{-}OMe + H]^+ - H_2O$	144	126(H_2O)30; 112(CH_3OH)3; 102(CH_2CO)11; 84(CH_3OH,CO)100; 74(CH_2CO,CO)8; 56(88)3
$[NAc\text{-}SMe\text{-}cysteine + H]^+ - CH_3SH$	130	112(H_2O)7; 102(CO)1; 88(CH_2CO)100; 84(H_2O,CO)42; 60(CH_2CO,CO)1
$[NAc\text{-}S,S\text{-}diMe\text{-}cysteine]^+ - S(CH_3)_2$	130	112(H_2O)1; 102(CO)2; 88(CH_2CO)10; 84(H_2O,CO)100; 60(CH_2CO,CO)2

[a]Only those product ions greater than 1% relative abundance are shown.
[b]Formed by MS/MS of N-acetyl dehydroalanine.
[c]Formed by ESI MS of aziridine carboxylic acid.
[d]Data taken from Figure 2 of [2g].



Scheme 2.

Probing the Structures of the Side Chain Loss Product Ions Via Deuterium Labeling Experiments

Although the MS^3 experiments described above suggest that neighboring group participation reactions rather than charge remote *cis* 1,2 elimination occurs for side chain loss, hydrogen/deuterium exchange coupled with MS/MS was also performed to distinguish loss of the side chain by charge directed neighboring group reactions (where D_2O loss is expected), from charge remote *cis* 1,2 elimination (where HOD loss is expected, because the nonexchangeable α-carbon proton is involved). In agreement with the MS^3 data shown above, loss of D_2O from fully deuterated serine (Table 3), CH_3OD from serine O-methyl ether (Figure 5A), and D_3PO_4 from O-phosphoserine (Figure 5B), as well as loss of D_2O from N-acetyl serine and N-acetyl serine O-methyl ester and loss of CH_3OD from N-acetyl serine O-methyl ether indicate that neighboring group processes dominate over *cis* 1,2 elimination in all cases. Some loss of HOD and CH_3OH was observed for the N-acetyl derivatives (Table 3 and Figure 5C), confirming in the MS^3 results described above that competitive *cis* 1,2 elimination can occur in some cases, albeit at significantly reduced abundances.

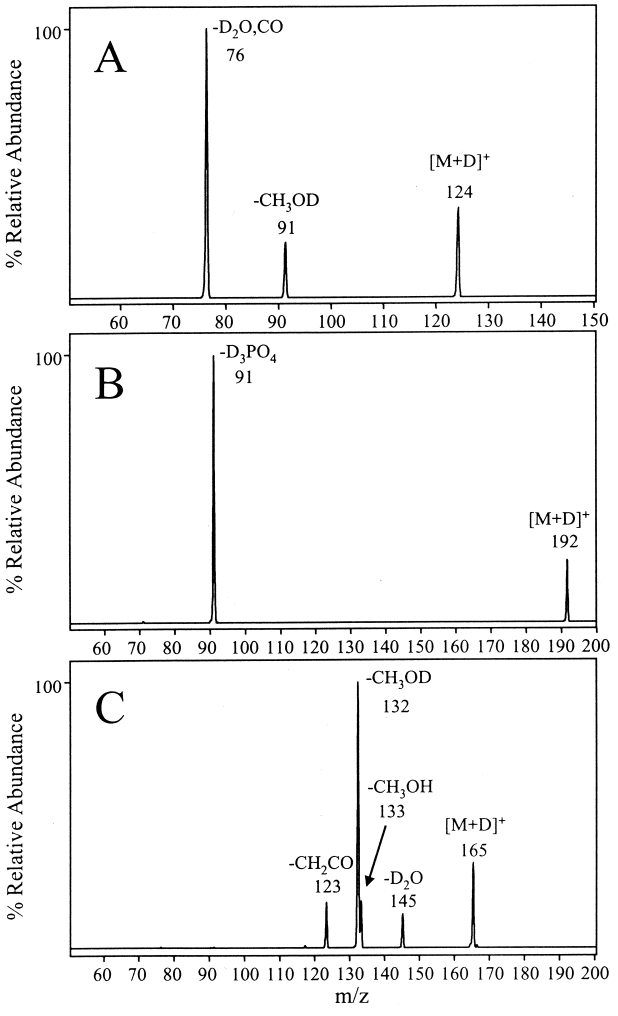


Figure 5. CID MS/MS spectra of the fully deuterated $[M + D]^+$ ions of (**a**) O-methyl serine, (**b**) O-phosphoserine, and (**c**) N-acetyl, O-methyl serine.

In order to rule out the possibility of H/D exchange of the α-carbon hydrogen atom in solution prior to the MS/MS experiments described above, we have examined the ^1H NMR spectra of serine, O-phosphoserine, and N-acetyl serine under similar H/D exchange conditions to those used for ESI. Clearly, based on both the integrated peak areas for each of the C–H signals, as well as the splitting patterns, no evidence of labilization of the αC–H bond in solution was observed.

Table 3. CID MS/MS spectra of fully deuterated serine and N-acetyl serine derivatives

Amino acid	*m/z*	MS/MS product ions; *m/z* (neutral loss) % relative abundance[a]
$[Serine + D]^+$	111	91(D_2O)28; 63(D_2O,CO)100
$[Serine\text{-}OMe + D]^+$	124	104(D_2O)100; 63(CH_3OD,CO)76
$[NAc\text{-}serine + D]^+$	152	133(HOD)50; 132(D_2O)96; 110(CH_2CO)100; 104(D_2O,CO)1; 91(61)12
$[NAc\text{-}serine\text{-}OMe + D]^+$	165	146(HOD)6; 145(D_2O)100; 132(CH_3OD)24; 123(CH_2CO)20; 104(CH_3OD,CO)2
$[NAc\text{-}SMe\text{-}cysteine + D]^+$	181	161(D_2O)36; 139(CH_2CO)100; 133(CH_3SH or D_2O,CO)35; 132(CH_3SD)7; 92(89)4

[a]Only those product ions greater than 1% relative abundance are shown.

Ab Initio Studies of Competing Side Chain Loss Mechanisms

Previously, we have shown that side chain H_2O loss from protonated threonine occurs via neighboring group participation reactions, even though the *cis* 1,2 elimination product, dehydroamino-2-butyric acid, is thermodynamically preferred over the neighboring group aziridine carboxylic acid product [2d], suggesting that the fragmentation processes are under kinetic control. Therefore in this study, the key transition state barriers for neighboring group and *cis* 1,2 elimination [19] side chain loss from protonated serine, O-phosphoserine, and N-acetyl serine have been determined by ab initio calculations at the MP2(FC)/6-31G*//HF/6-31G* level of theory. The transition states for intramolecular proton transfer have not been examined in this study as two recent papers have extensively studied these steps [20]. For example, the barrier to proton transfer from the amine to the carboxylic acid, or from the amine to the carbonyl, of protonated glycine has been calculated via ab initio MP2/6-31G* (37.2 and 35.2 kcal mol^{-1}, respectively), and hybrid density functional theory B3LYP/6-31G* methods [20a]. Thus, these energies can be used in comparison with those predicted for side chain losses from the protonated serine and O-phosphoserine, where similar proton transfer barriers are expected. Also, extensive mapping of the potential energy surface of protonated N-formyl glycinamide [20b] has determined that all the barriers to proton transfer (up to 14.9 kcal mol^{-1} for the proton transfer steps leading to amide bond cleavage) are all located below that required for amide bond cleavage (37 kcal mol^{-1}). Therefore, these energies can be compared to the barriers predicted for side chain losses from N-acetyl serine. Potential transition state structures were initially examined at the AM1 level of theory. Low energy conformers were reoptimized at the HF/6-31G* level of theory and vibrational frequency analysis performed, using the same basis set, to confirm that each of these structures represented a saddle point on the potential energy surface. Each structure was subjected to single point energy calculation of the correlated energy at the MP2(FC)/6-31G* level of theory. Energies were corrected for zero-point vibrations scaled by 0.9135. In order to locate the appropriate reactant and product ion minima connected to each transition state, IRC runs were performed followed by geometry optimization and single point energy calculations as described above.

The calculated transition state barriers for loss of H_2O from protonated serine via *cis* 1,2 elimination (TS1 in Figure 6) or neighboring group participation by the amino group (TS2 in Figure 6) are 56.7 and 40.3 kcal mol^{-1}, respectively, at the MP2(FC)/6-31G*//HF/6-31G* + ZPVE level of theory. Thus, the neighboring group process is predicted to be preferred by 16.4 kcal mol^{-1}. The activation energy predicted for this neighboring group process is in qualitative agreement with the reverse activation energy for the attack of H_2O on an unsubstituted protonated aziridine (which was determined to have a barrier of only 3.5 kcal mol^{-1}) [21].

The intermediate to the neighboring group process, O-protonated serine (OSer) was found to be 25.7 kcal mol^{-1} higher in energy than the lowest energy conformer, N-protonated serine (NSer). Note that this is not the most stable conformer of O-protonated serine, as found by Rogalewicz et al. [17], but instead is the connecting ground state minima to the transition state TS2, which is lower in energy (due to additional H bonding) than that recently proposed [17]. In agreement with that found for the product ion structures for side chain loss from protonated threonine [2d], the dehydroalanine (DHA) product ion is predicted to be more stable than the aziridine (AZI).

For protonated O-phospho serine, protonation of the P=O oxygen of the phosphate side chain is expected to be preferred over the C–O–P oxygen [22] (recent high level calculations on H_3PO_4 reveal that the proton affinity of the P=O oxygen is 196.9 kcal mol^{-1} compared to 166.8 kcal mol^{-1} for the P–OH oxygen). Thus, only the most stable conformer of P=O protonated phosphoserine was considered. The neighboring group transition state barrier for H_3PO_4 loss by anchiameric assistance from the amine was found to be 21.9 kcal mol^{-1} (TS3 in Figure 7). Two pathways for *cis* 1,2 elimination of H_3PO_4 are possible (Figures 8 and 9). The two low energy transition state structures for these are shown in TS4 of Figure 8 and TS5 of Figure 9. As the 4-membered transition state TS4 in Figure 8 was found to be significantly higher than the 6-membered TS5 shown in Figure 9 (51.8 kcal mol^{-1} compared to 20.1 kcal mol^{-1}), this pathway was not considered further. Thus, at the MP2(FC)/6-31G*//HF/6-31G* single point level of theory, the *cis* 1,2 elimination pathway is predicted to be preferred over neighboring group processes, contradicting the experimentally obtained result (see Figure 5B). If we examine the HF/6-31G*//HF/6-31G* + ZPVE energies however, the neighboring group pathway is preferred by almost 10 kcal mol^{-1}. This result indicates the danger of extrapolating geometries optimized at lower levels of theory to higher level single point energy calculations. Unfortunately, MP2 optimizations on these systems were not possible with our current computational resources. It is important to note however, that in all other cases the relative energies of the MP2 single points versus the HF optimized structures are in qualitative agreement.

Interestingly, a stable zwitterionic intermediate was found for the 6-membered *cis* 1,2 elimination pathway following transfer of the α-carbon hydrogen atom. Thus, a second transition state TS6 was found, lying 1.3 kcal mol^{-1} (at the HF/6-31G* level of theory) higher in energy than the intermediate IM5, leading to dissociation to dehydroalanine. There has been some interest in the potential role that zwitterionic structures may have in the fragmentation reactions of amino acids. Turecek [23] and Williams [24] have recently proposed zwitterionic precursors in the losses of CO_2 from N-substi-

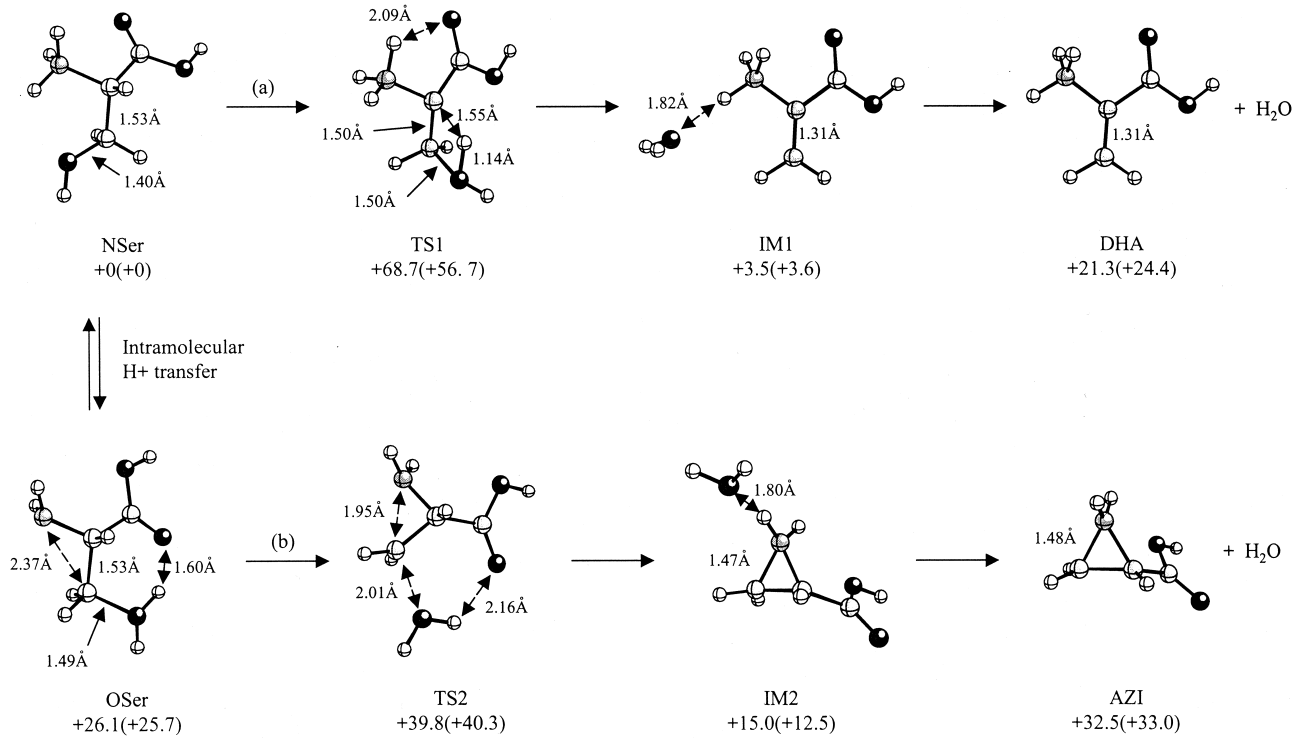


Figure 6. HF/6-31G* ab initio optimized structures for the loss of H_2O from protonated serine by (**a**) *cis* 1,2 elimination via a 4-membered transition state or (**b**) neighboring group participation reaction via a 3-membered transition state. HF/6-31G* and [MP2(FC)/6-31G*//HF/6-31G*] relative energies are given in kcal mol^{-1}.

tuted protonated histidine and for the loss of NH_3 from protonated arginine, respectively.

The potential energy surface for *cis* 1,2 elimination of H_2O from N-acetyl serine is shown in Figure 10. The transition state was found to be 53.0 kcal mol^{-1} higher in energy than the connecting ground state conformer (O1AcSer). Both 3-membered and 5-membered transition states for neighboring group losses were also examined (TS8 and TS9 in Figure 11, respectively). The energy for the 3-membered transition state (38.3 kcal mol^{-1}) leading to formation of an N-acetyl aziridine product ion was similar to that found for serine (see Figure 6, TS2). In comparison, the 5-membered transition state leading to formation of an oxazole product ion was found to be significantly lower (26.7 kcal mol^{-1}). Both 3- and 5-membered transition states were found to connect to a common ground state structure (O2ASer). In contrast to that found for the serine side chain H_2O loss product ions, the neighboring group participation oxazole product ion (OXA) was found to be more stable than the charge remote N-acetyl dehydroalanine product ion (AcDHA).

As some HOD or CH_3OD loss was observed experimentally for the N-acetyl serine derivatives (Table 3 and Figure 5C), an alternate pathway must be operating since the 4-membered *cis* 1,2 elimination transition state

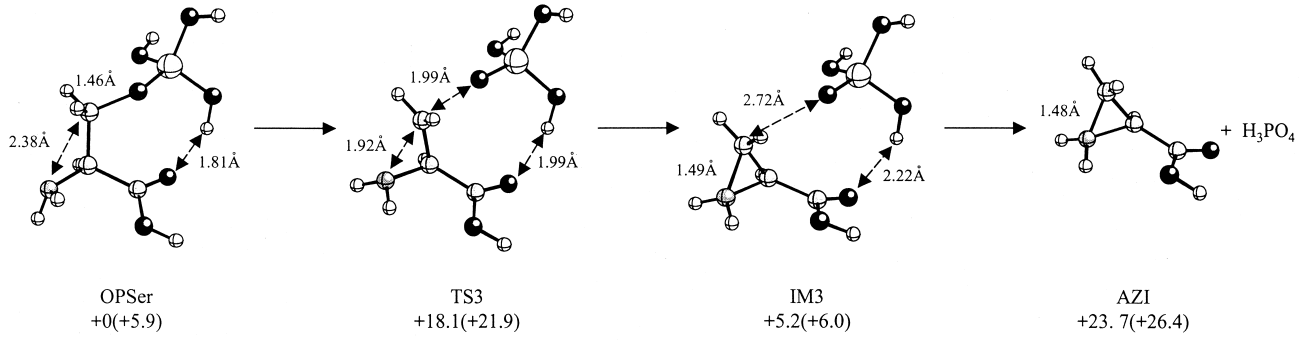


Figure 7. HF/6-31G* ab initio optimized structures for the loss of H_3PO_4 from protonated O-phosphoserine by a 3-membered transition state neighboring group participation reaction. HF/6-31G* and [MP2(FC)/6-31G*//HF/6-31G*] relative energies are given in kcal mol^{-1}.

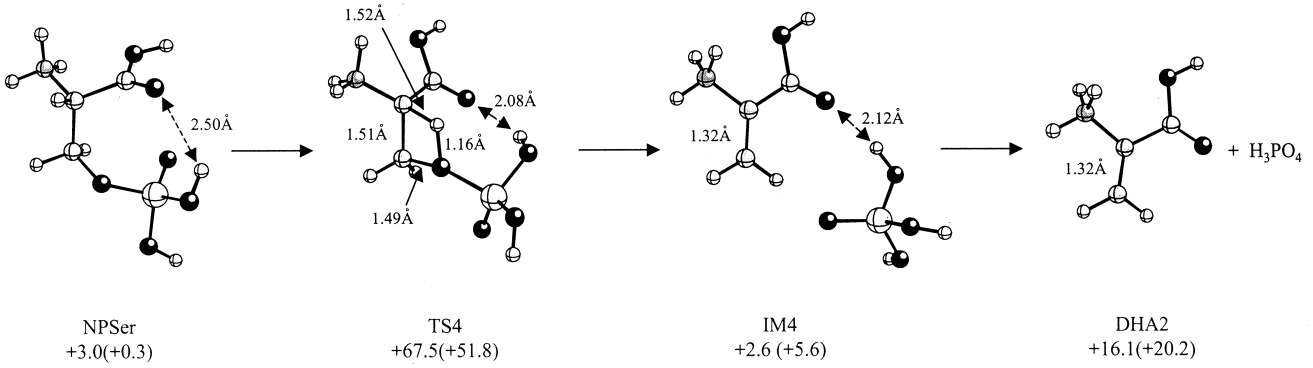


Figure 8. HF/6-31G* ab initio optimized structures for the loss of H_3PO_4 from protonated serine by a 4-membered *cis* 1,2 elimination reaction. HF/6-31G* and [MP2(FC)/6-31G*//HF/6-31G*] energies are given in kcal mol^{-1} relative to OPSer in Figure 7.

potential energy surface determined in Figure 10 was predicted to be significantly higher in energy than the neighboring group process shown in Figure 11. Therefore, an alternate low energy pathway that may be able to compete with the neighboring group processes was found (Figure 12) involving a transition state (TS10, 29.3 kcal mol^{-1}) in which transfer of the α-carbon hydrogen atom to the acetyl carbonyl group of carboxyl protonated N-acetyl serine (O3AcSer) occurs to yield the stable zwitterionic intermediate IM10. Determination of the additional steps associated with H_2O loss via this mechanism have not been carried out as it is anticipated that the step involving proton transfer to the hydroxyl with subsequent loss of H_2O will be of the same order as the proton transfers described by Csonka et al. [20b] and therefore below the level of TS10.

In order to evaluate the intrinsic neighboring group ability of an amino group versus an amide carbonyl, we

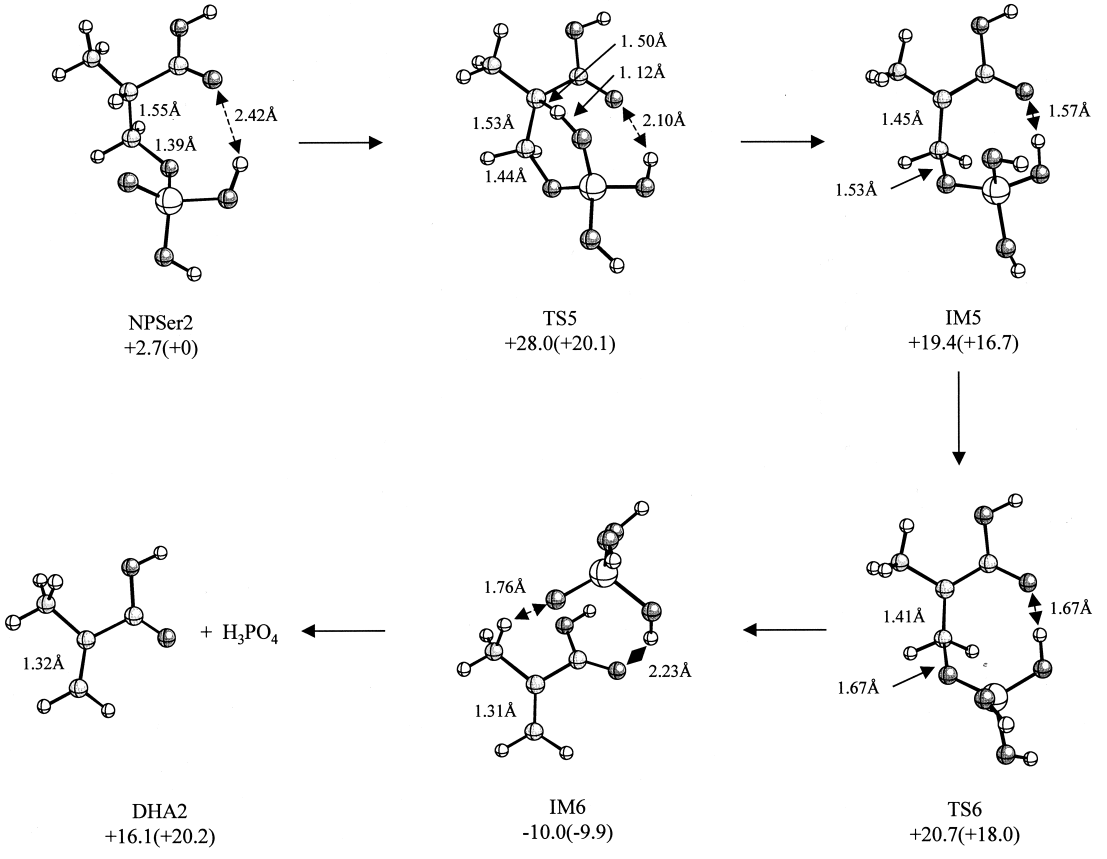


Figure 9. HF/6-31G* ab initio optimized structures for the loss of H_3PO_4 from protonated serine by a 6-membered *cis* 1,2 elimination reaction. HF/6-31G* and [MP2(FC)/6-31G*//HF/6-31G*] energies are given in kcal mol^{-1} relative to OPSer in Figure 7.

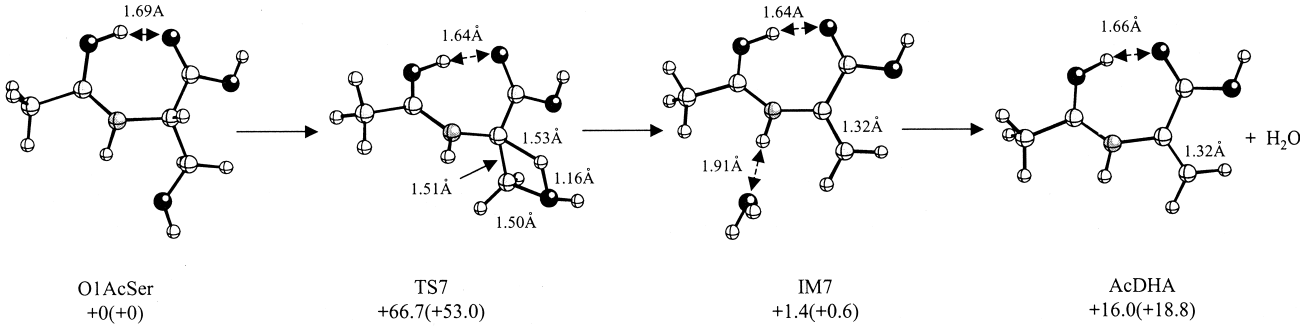


Figure 10. HF/6-31G* ab initio optimized structures for the loss of H_2O from protonated N-acetyl serine by *cis* 1,2 elimination. HF/6-31G* and [MP2(FC)/6-31G*//HF/6-31G*] relative energies are given in kcal mol^{-1}.

have compared the relative barriers for the neighboring group losses from O-protonated serine and N-acetyl serine. In complete agreement with the experimental results (compare Figures 1C and 2C), the 5-membered TS barrier for H_2O loss from N-acetyl serine (TS9 in Figure 11) of 26.7 kcal mol^{-1} is significantly lower than those for the 3-membered transition states for serine (TS2 in Figure 6) (40.3 kcal mol^{-1}) and N-acetyl serine (TS8 in Figure 11) (38.3 kcal mol^{-1}). In a similar manner, comparison of the leaving group abilities of H_3PO_4 and H_2O in O-phosphoserine and serine, respectively, can be made by examining the transition state barriers of TS3 in Figure 7 with TS2 in Figure 6. As can be seen, TS3 has a barrier 18.4 kcal mol^{-1} lower than TS2, explaining the greater abundance of H_3PO_4 over H_2O loss observed experimentally. Therefore, not only does the side chain of O-phosphoserine have a higher proton affinity than serine, therefore promoting the formation of the "reactive" isomer, but the transition state barrier is lower, facilitating its enhanced loss.

Although the results of DeGnore and Qin [7c] for the loss of H_3PO_4 via a *cis* 1,2 β-elimination mechanism are seemingly in disagreement with the results presented above, the presence of additional nucleophilic groups

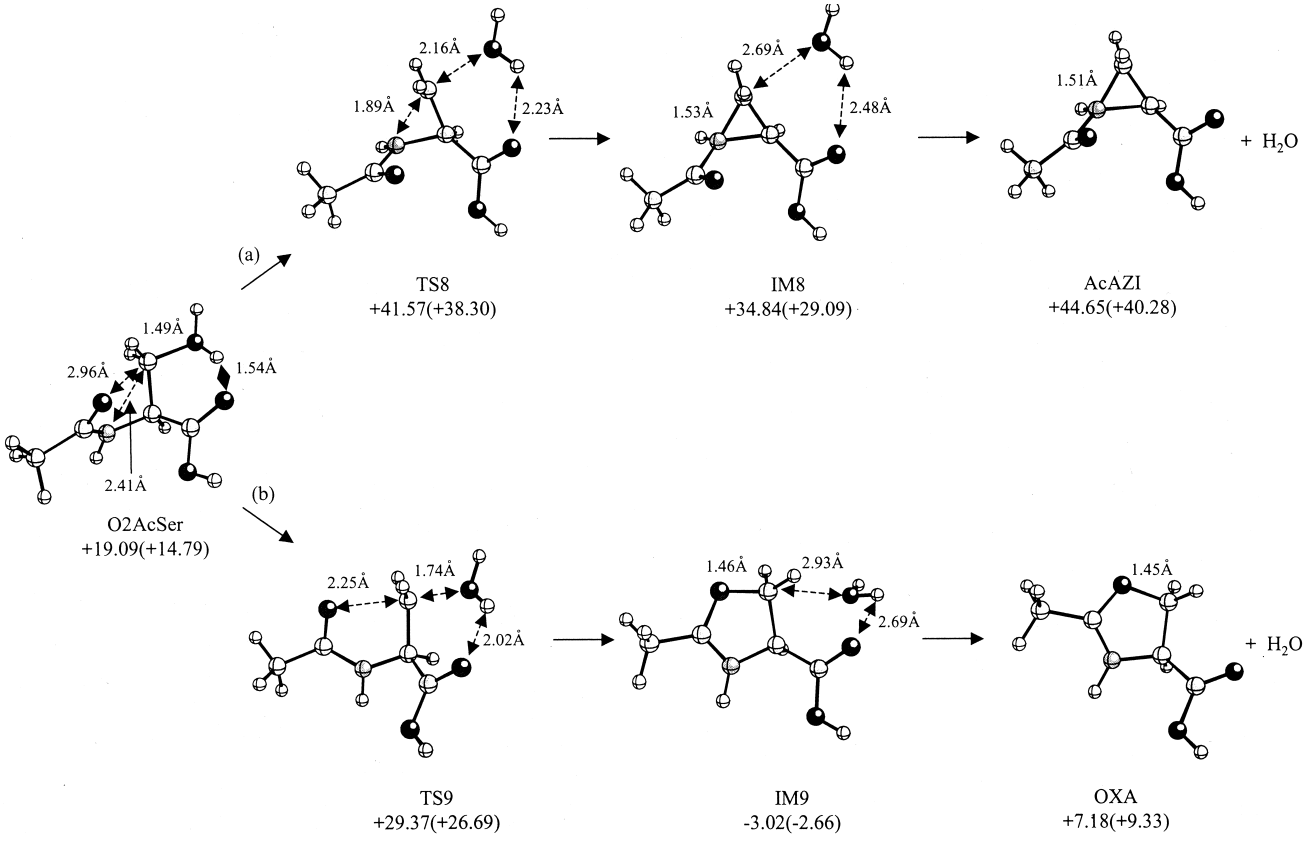


Figure 11. HF/6-31G* ab initio optimized structures for the loss of H_2O from protonated N-acetyl serine by (**a**) 3-membered or (**b**) 6-membered neighboring group participation reactions. HF/6-31G* and [MP2(FC)/6-31G*//HF/6-31G*] relative energies are given in kcal mol^{-1} relative to O1AcSer in Figure 10.

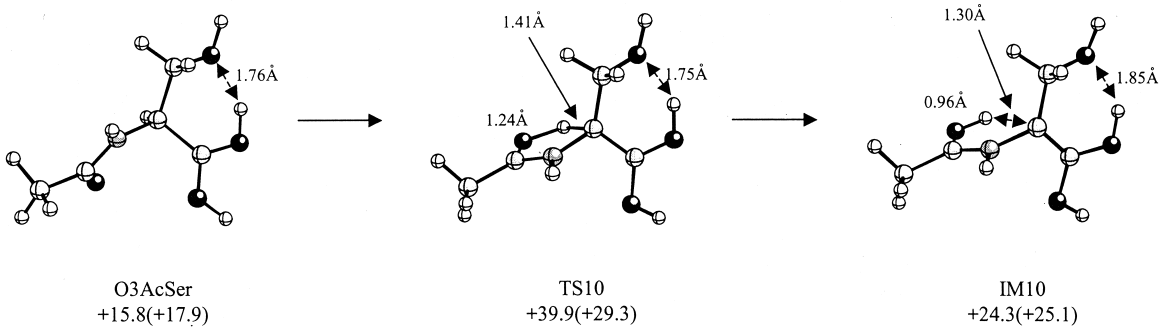
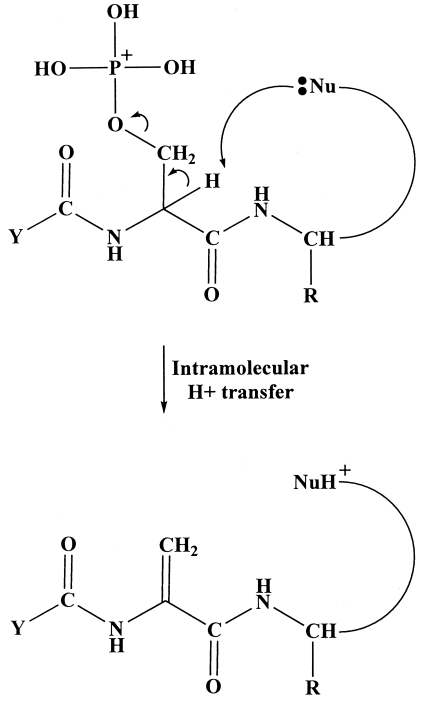


Figure 12. HF/6-31G* ab initio optimized structures for the loss of H_2O from protonated N-acetyl serine by a zwitterionic intermediate *cis* 1,2 elimination pathway. HF/6-31G* and [MP2(FC)/6-31G*//HF/6-31G*] relative energies are given in kcal mol^{-1} relative to O1AcSer in Figure 10.

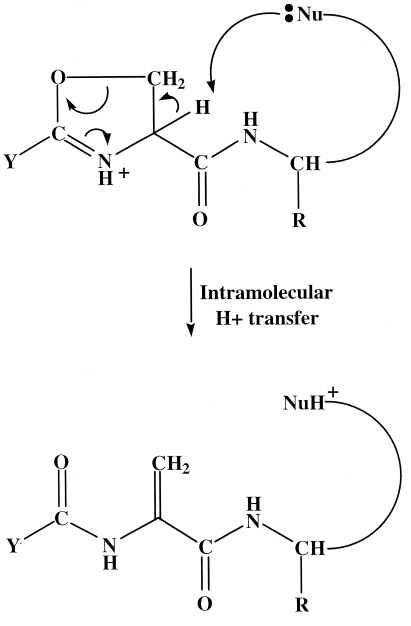
along the peptide backbone and side chains of larger peptides, may help facilitate the formation of dehydroalanine by other means. This may occur in several ways: (i) Through the direct formation of dehydroalanine by intramolecular E2 elimination of H_3PO_4 involving mobilization of the α-carbon hydrogen atom by an adjacent nucleophile (Scheme **3**). (Note that this type of elimination would result in the loss of D_3PO_4 from a fully deuterated system. This process is not expected to occur in the amino acid and model peptides studied here however due to the ring size of the transition states involved.) (ii) Through the direct formation of dehydroalanine via the low energy zwitterionic intermediate shown in Figure 12 (this may involve losses of both D_3PO_4 and HD_2PO_4 depending on the subsequent proton transfer steps prior to fragmentation). Or (iii) via ring opening of the $[M + H - H_3PO_4]^+$ ion with subsequent dehydroalanine formation by mobilization of the α-carbon hydrogen atom (also involving the initial sole loss of D_3PO_4) (Scheme **4**). A key experiment to examine these potential alternate pathways for larger peptides would involve comparison of the MS^3 spectra of the $[M + H - H_3PO_4]^+$ ions with the MS/MS spectra of independently synthesized peptides incorporating oxazole [8e,f] or dehydroalanine [8f] residues. In this manner, the product ion abundances of the amide bond cleavages either side of the dehydroalanine residues peptides could be compared with the corresponding cleavages in the MS^3 spectra of the $[M + H - H_3PO_4]^+$ ions to determine if the abundances were the same, indicative of alternate pathways (i) or (ii), or lower, indicative of a mixed population of cyclic oxazole and dehydroalanine and therefore belonging to alternate pathway (iii).

Conclusions

The side chain losses from protonated serine and O-phosphoserine as well as their corresponding N-acetyl



Scheme 3.



Scheme 4.

derivatives have been shown to occur via neighboring group participation reactions to yield 3-membered aziridine and 5-membered oxazoline product ions, respectively, and not dehydroalanine as previously postulated. These results also indicate that the products formed under gas phase reaction conditions are dependent, just as in solution phase processes, on the neighboring and leaving groups present. The importance of using several different techniques, particularly the independent synthesis of proposed product ions, when examining peptide fragmentation mechanisms is evident.

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