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Do all b_2 ions have oxazolone structures? Multistage mass spectrometry and ab initio studies on protonated *N*-acyl amino acid methyl ester model systems[†]

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Abstract

The tandem mass spectrometry fragmentation reactions of 21 protonated *N*-acyl amino acid methyl esters are examined as models for more complicated peptides. Four main types of reactions are observed: loss of CH_2CO from the *N*-terminal acetyl group; loss of CH_3OH from the C-terminal ester group to yield a model system for a b_2 ion structure; loss of water from amino acids without an OH side chain group; fragmentation of the side chain by way of small molecule loss (e.g. H_2O, NH_3, and CH_3SH). CH_3OH loss is the only common reaction observed for all systems. The resultant $[M+H-CH_3OH]^+$ ions were examined in further detail by way of MS^3 experiments because previous studies have shown that the oxozolone structures liberate CO. Only lysine and arginine do not fragment by way of CO, which is suggestive of alternative cyclic structures involving the side chain. Ab initio calculations (at the MP2/6-31G*//HF/6-31G*) were carried out on isomeric b_2 ions of both types (oxazolone and that involving side-chain interaction) derived from arginine, histidine, lysine, methionine, asparagine, glutamine, and serine. For arginine, histidine, and lysine the cyclic structures involving the side chain are more stable than the oxozolone structures. Finally, solution phase data relevant to the gas phase processes are highlighted. (Int J Mass Spectrom 210/211 (2001) 71–87) © 2001 Elsevier Science B.V.

Keywords: Protonated peptide fragmentation; Structures of b ions; Multistage mass spectrometry

1. Introduction

The use of tandem mass spectrometry (MS/MS) is now the method of choice for the sequence analysis of protonated peptides due to speed, sensitivity, and availability of automated MS/MS data collection and analysis programs for sequence ion assignments [1]. In general, protonated peptides subjected to low energy collision-induced dissociation (CID) yield the complementary b_n and y_n series of sequence ions [2]. "Real world" analyses can yield a significant number of MS/MS spectra which are unassignable (i.e. product ion spectra are too complex, too low in intensity, or lack sufficient ions to enable interpretation). For example, a surprising 24% of the MS/MS spectra of peptides resulting from tryptic digestion of a protein

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Dedicated to Professor Nibbering on the occasion of his retirement and in recognition of his many seminal contributions to the physical organic chemistry of gas phase ions

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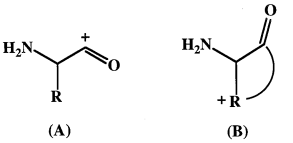
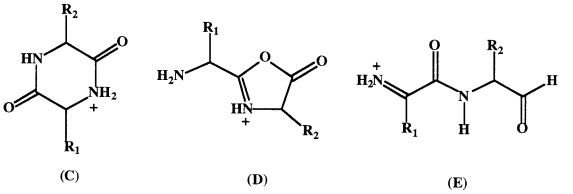
fraction from an enriched cellular membrane preparation, isolated from the human colorectal cell line LIM1215, and separated by one dimensional gel electrophoresis could not be assigned [3].

In a bid to better understand the fragmentation reactions of peptides, several groups have been examining the mechanisms for the formation of both sequence ions and nonsequence ions (typically arising from losses of small neutrals or fragmentation reactions involving the side chains of the amino acid residues). A key issue is "what are the structures of the b- and y-type sequence ions?" [4] MS^3 studies using CID and ion–molecule reactions suggest that the y_n sequence ions are truncated peptides [5]. In contrast, several different structures have been proposed for b_n sequence ions [5–10]. Although b_n sequence ions are notionally acylium ions, the fact that b_1 ions are rarely observed suggests that these structures may not be stable. Indeed over 20 years ago, Harrison and co-worker suggested [6a] that b_1 acylium ions **(A)** of simple aliphatic amino acids are unstable with respect to dissociation to an a_1 ion (formally an iminium ion) and CO. A recent study found that simple aliphatic ions of type **(A)** can be observed if they are stabilized by way of conjugation with a double bond or by being part of an aziridine ring system [6b]. Harrison and co-worker have also suggested alternative structures for the b_1 ions of nonaliphatic amino acids **(B)** which arise from neighboring group interactions involving the side chains of lysine [7a] and methionine [7b]. An examination of some of the published MS/MS spectra on amino acids and simple peptides also reveal the following potential candidates for b_1 ions with **(B)** type structures: arginine [7c,d], lysine [7c,d], histidine [7c,e], and methionine [7f].



(A) (B)

The fact that b_1 ions are rarely observed suggests that higher b-type ions do not have acylium structures. Consider the cleavage of the amide bond closest to the C-terminal of a tripeptide, resulting in the formation of a b_2 ion. A number of structures are possible, arising from participation of neighboring groups; the diketopiperazine **(C)** from nucleophilic attack by the *N*-terminal amine [5]; an oxazolone **(D)** by nucleophilic attack from the preceding amide carbonyl [8]; and an iminium ion **(E)** formed by rearrangement from the oxazolone **(D)** [9]



(C) (D) (E)

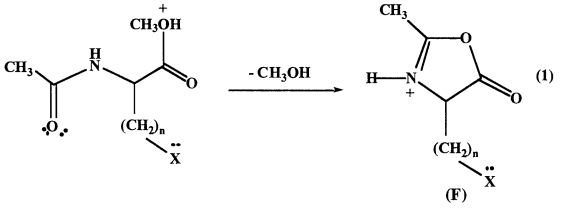
One way of determining the actual structure of the b_2 ion is to compare its CID spectrum with those of authentically synthesized **(C)**, **(D)**, and **(E)** (See Scheme 3). However, finding a gas-phase synthesis of these ions has been a challenge. In a study of the fragmentation mechanisms of the $[M+H]^+$ ions of Ala-Ala, Ala-Ala-Ala and Ala-Ala-Ala-Ala, Cordero et al. showed that the b_2 ion arising from Ala-Ala-Ala exhibits quite a different CID spectrum to that arising from the appropriate protonated diketopiperazine **(C)** [5c]. This result has been supported by a study from Harrison and co-workers [8a]. Thus, structure **(C)** can be ruled out as the structure of the b_2 ion. Harrison and co-workers have shown that the protonated *N*-benzoyl-glycyl-glycine peptide ion fragments by way of loss of the C-terminal glycine residue to form a 2-phenyl-5-oxazolone product ion [8a]. Based solely upon ab initio calculations, Eckart et al. have suggested the immonium ion **(E)** as yet another possible structure [9]. A recent paper by Harrison et al. [8c] however, has shown that the oxazolone structure **(D)** can be used to explain the fragmentation behavior of the peptides studied by Eckart et al.

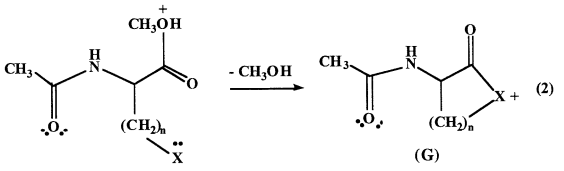
Several groups have examined the fragmentation behavior of b_n-type ions [8,10]. These studies have shown that b_n ions commonly lose CO by way of an acylium ion intermediate to produce a_n-type immonium ions ($b_n \rightarrow a_n$) [8a,b,10b]. Under favorable conditions, depending on the nature of the adjacent amino acids,

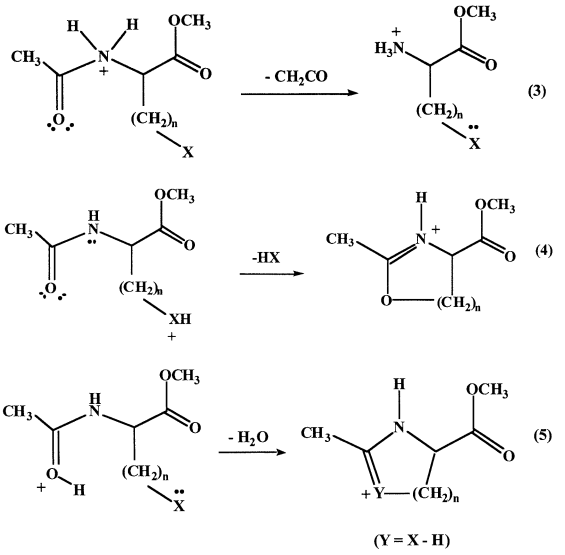
competing ($b_n \rightarrow a_{n-1}$) [8c,10b,c] or ($b_n \rightarrow b_{n-1}$) fragmentations [10c] may also occur directly from the b_n ion precursor. Additionally, a_n ions may be formed directly from the intact peptide ion ($[M+H]^+ \rightarrow a_n$) [10b]. Smaller b-type ions may also result from fragmentation of a_n ions ($a_n \rightarrow b_{n-1}$) [10b]. Other gas phase studies on the proposed oxazolone b_n ion structures include determination of proton affinities [11], H/D exchange reactions [5a], ion–molecule reactions with butylamine [12] and various ab initio studies [10d,13].

Given that b_1 ions of type **(B)** have been proposed for amino acids with nucleophilic side chains, it is surprising that related structures for higher b_n ions have received little attention in the mass spectrometry literature. Exceptions include: the C-terminal lysine systems of Harrison and co-worker [7a]; the work of Jennings and co-workers on C-terminal arginine peptides [14a]; recent work on histidine induced peptide bond cleavage [14b] and a study on the high energy CID fragmentation reactions of doubly charged bradykinin and its derivatives, in which it was suggested that serine residues yielded a lactone structure [14c].

In this paper we examine the MS/MS fragmentation reactions of protonated *N*-acyl amino acid methyl esters as models for more complicated peptides (the glycine, cysteine, methionine, and serine systems have been previously published [15]). This allows the role of side chains in the competition between sequence (Eqs. 1–3) and nonsequence ion (Eqs. 4, 5) formation to be assessed [15].







MS^3 experiments are performed on each of the b_2 ions to determine whether they arise by way of the amide neighboring group pathway [Eq. (1)] to yield ions of structure **(F)** or by way of the side chain neighboring group pathway [Eq. (2)] [16] to yield ions of type **(G)**. In addition, ab initio calculations are reported for both types of b_2 ions **(F)** and **(G)** for arginine, histidine, lysine, methionine, asparagine, glutamine, and serine to determine their relative stabilities.

2. Experimental

N-acetyl amino acid methyl esters were commercially available samples (BACHEM, Bubendorf, Switzerland) or were prepared by way of previously reported procedures [15a]. $[M+H]^+$ ions were formed by way of electrospray ionization (ESI) on a Finnigan model LCQ (San Jose, CA) quadrupole ion trap mass spectrometer. Samples (0.2 mg/mL) were dissolved in 50% H_2O/50% CH_3OH containing 0.1 M acetic acid and introduced to the mass spectrometer at 3 μL/min. The spray voltage was set at −5 kV. Nitrogen sheath gas and auxillary gas pressures were supplied at 30 psi and 15 (arbitrary unit) respectively. The heated capillary temperature was set at 200 °C. MS/MS and

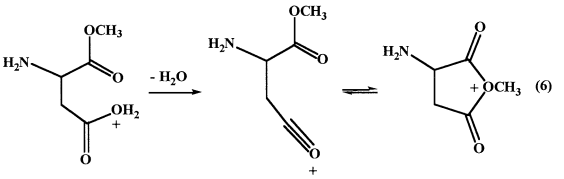
MS^3 experiments were performed on mass selected ions using standard isolation and excitation procedures

Computational methods: ground state structures were optimized at the Hartree-Fock level of theory using the GAUSSIAN98 molecular modeling package [17] with the standard 6-31G* basis set [18]. 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 [19]. Complete structural details and lists of vibrational frequencies for each HF/6-31G* optimized structure are available from the authors upon request.

3. Results and discussion

3.1. MS/MS studies on $[M+H]^+$ ions of 21 different N-acyl amino acid methyl esters

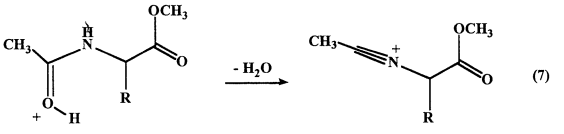
Methanol loss was observed for all 21 protonated *N*-acyl amino acid esters to yield the putative b_2 ions (Table 1). This reaction channel ranges from being the dominant pathway for alanine, valine, leucine, isoleucine, proline, lysine, histidine, methionine, phenylalanine, tyrosine, tryptophan, and cystine to being only a minor process for arginine, aspartic acid, asparagines, and glutamine. The structures of the resultant $[M+H-CH_3OH]^+$ ions are discussed in detail in sections **(B)** and **(C)**. Loss of ketene (CH_2CO) was observed as a major pathway for glycine, and only a minor pathway for all other amino acids except asparagine, glutamine, and arginine, which revealed no such loss. Loss of water was observed in several cases and can occur by way of loss of side chain oxygen, which is the likely pathway for species such as serine [see Eq. (4)], threonine, aspartic acid, and glutamic acid. Indeed, side chain water loss from protonated serine and threonine and their derivatives has been examined in detail and comparisons with analogous solution phase processes have been made [15d,e]. Harrison has proposed a mechanism for side chain water loss from aspartic acid [20] in which an acylium ion is in equilibrium with a cyclic structure [Eq. (6)].



(6)

Interestingly, solution phase neighboring group pathways are known for aspartic and glutamic acids and yield cyclic anhydrides [21].

Alternatively, loss of water can involve the carbonyl oxygen of the *N*-acetyl group and can occur by way of two pathways: a neighboring group pathway involving a side chain nucleophile as demonstrated for cysteine [Eq. (5), where X=SH) [15a,b] or by way of a retro-Ritter reaction [15a] (Eq. 7).



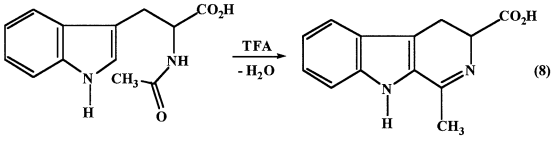
(7)

Note that the retro-Ritter reaction, which is the only possible pathway for the aliphatic systems, only occurs to a minor extent for glycine, valine, leucine, and isoleucine. This result is consistent with previous ab initio calculations on protonated *N*-formyl glycine, which reveal that this water loss channel is a relatively high energy process [15a]. Apart from the significant loss of water from cysteine previously noted [15a], the data in Table 1 reveal water loss from the following derivatives with nucleophilic side chains: lysine, histidine, phenylalanine, and tryptophan. Although we cannot rule out retro-Ritter reactions, which may be base catalyzed by way of the side chains, a more likely process involves neighboring group mechanisms in which the nucleophilic side chain induces water loss [Eq. (5)]. Indeed such a process occurs in the condensed phase where acid catalyzed water loss from the *N*-acyl tryptophan species yields carboline derivatives [22] (Eq. 8).

Table 1
CID MS/MS spectra of $[M + H]^+$ ions of N-acyl amino acid methyl esters

Ac-AA-OMe where AA =	CH_2CO	CH_3OH	H_2O	Side chain (species lost) %abund	Other ions (species lost) %abund
Glycine[a]	100	77	1	...	–
Alanine	18	100	...	...	(CH_3OH, CO) 1
Valine	2.5	100	0.5	...	(CH_3OH, CO) 2.5
Leucine	4	100	0.5	...	(CH_3OH, CO) 3.5
Isoleucine	2	100	0.5	...	(CH_3OH, CO) 4
Proline	0.5	100	...	...	(CH_3OH, CO) 11
Serine[b]	37	43		(H_2O) 100	(CH_3OH, CO) 2, (80) 0.5
Threonine				(H_2O) 100	(H_2O, CO) 4, (CH_3OH, CO) 3, (96) 0.5
Lysine	3	100	39	(NH_3) 59	(CH_3OH, NH_3) 8, (H_2O, CO) 1.5, (CH_2CO, NH_3) 3, (CH_3OH, CO) 0.5, (74) 70, (77) 5, (119) 1
Arginine	...	4	...	(NH_3) 100, (NH_3, NH_3) 10, ($(H_2N)_2C$=NH) 13, (CH_3OH, HN=C=CH) 2	(CH_3OH, NH_3) 2, (58) 1, (CH_3OH, CO) 13
Arginine (deuterated)	...	[d]	...	(ND_2H) 12, (ND_3) 100, (ND_2H, ND_2H) 5, (ND_3, ND $_2$H) 9, (ND_3, ND_3) 2, ($(D_2N)_2$ C=ND) 41, (CH_3OD, DN=C=ND) 2	(CH_3OD, ND_3) 1, (CH_3OD, CO) 3, (62) 10, (63) 15
Histidine	10	100	6	...	(CH_3OH, CO) 42, (CH_3OH, CH_2CO, CO) 3
Cysteine[a]	38	48	100	...	...
Methionine[c]	0.5	100	...	(CH_3SH) 2.5	(CH_3OH, CO) 1
Aspartic acid	6	9		(H_2O) 100	(H_2O, CO) 3
Glutamic acid	0.5	20		(H_2O) 100	(CH_3OH, CO) 14, (124) 0.5
Asparagine	...	2	...	(NH_3) 100	...
Glutamine	...	10	...	(NH_3) 100	(NH_3, CH_2CO) 1
Phenylalanine	16	100	1	...	(CH_3OH, CO) 10
Tyrosine	18	100	...	...	(CH_3OH, CO) 16
Tryptophan	14	100	2.5	...	(NH_3, CH_2CO) 2.5, (CH_3OH, CO) 4
Cystine	1	100	...	...	(CH_3OH, CO) 3, (CH_3C(O)NHCH (CH_2SH) CO_2CH_3) 1.5

[a] see [15a].
[b] see [15e].
[c] see [15c].
[d] CH_3OD is lost.

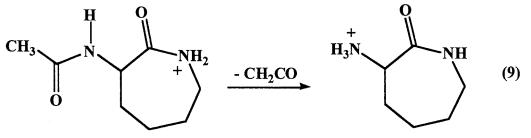


(8)

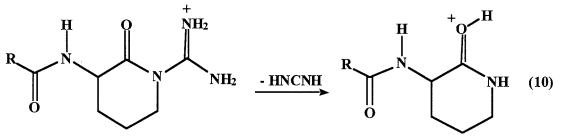
The *N*-acetyl amino acid methyl esters of lysine [7a], arginine [14a,23,24], asparagines, and glutamine [20,25] all undergo the loss of NH_3, which cannot occur from the *N*-terminus. Thus these losses must occur from the side chains. Apart from the water and ammonia losses discussed previously, other losses which occur from the side chains of *N*-acetyl amino acid methyl esters include CH_3SH loss from methionine [15c] and $(H_2N)_2C$=NH loss from arginine [14a,23,24]. In contrast, disulfide bond cleavage in the cystine derivative only occurs to a very minor extent, which is consistent with other studies on protonated peptide disulfide bridged systems [26].

3.2. MS^3 studies on $[M+H-CH_3OH]^+$ ions

As noted in Sec. 1, oxazolone b_n ions typically undergo ring opening to transient acylium ion structures which rapidly fragment by way of CO loss to form a_n ions. There is scant information however, on how the isomeric cyclic structures **(G)** involving the amino acid side chain [Eq. (2)] fragment. Exceptions include the b_1 ion of methionine [7b] and the b_n ions of histidine which also lose CO [14b], the b_2 ions of lysine, which loses CH_2CO [7a] (Eq. 9)



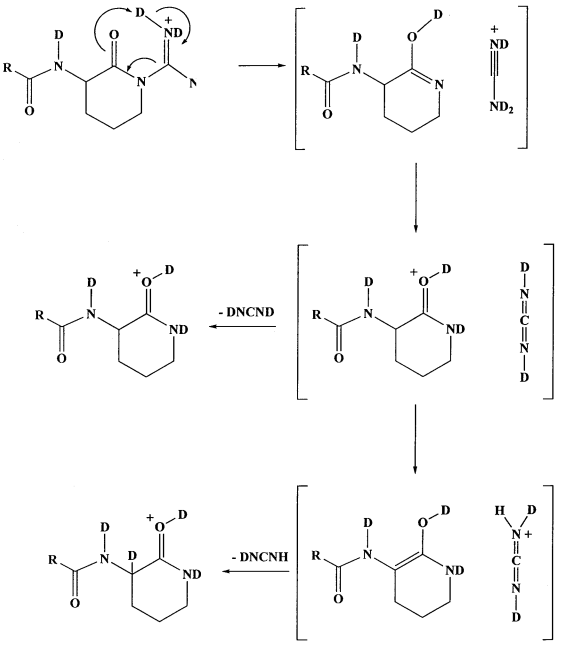
and the C-terminal b ions of arginine peptides, which lose HNCNH [14a] (Eq. 10).



From these limited studies it appears that a requirement for losses other than CO is that the heteroatom which was the original neighboring group must have an H atom attached which may be "mobilized" after cyclization in order to facilitate charge directed fragmentation. For example, in Eq. (9), the initially *N*-protonated lactam must transfer a proton to the exocylic amide to induced cleavage by way of ketene loss.

Each of the $[M+H-CH_3OH]^+$ MS/MS product ions observed in Table 1 were subjected to MS^3 to gain some insights into their potential structures. These data are summarized in Table 2. An examination of Table 2 reveals that CO loss is the dominant pathway for all amino acids except lysine and arginine which do not lose CO at all. Lysine and arginine lose a neutral mass of 42 Da as the dominant pathways [see Eqs. (9) and (10)], whereas asparagine, glutamine, phenylalanine, and tryptophan lose 42 to a minor extent. For arginine, the 42 loss could either be CH_2CO [see Eq. (9)] from the *N*-acetyl group or HNCNH from the side chain [see Eq. (10)]. MS^3 studies on the deuterium labeled derivative (formed by allowing all exchangeable hydrogens to undergo H/D exchange in solution using CH_3OD:D_2O+1% CH_3CO_2D) reveals the losses of 44 and 43 (i.e. DNCND and DNCNH, respectively) in an approximate 3:1 ratio. Note that an analogous loss is observed for the b_1 ion of arginine, which must have a structure **(B)**. Therefore, these data suggests that the loss of 42 Da occurs by way of side chain loss from a b_2 ion of structure **(G)** [Eq. (10)]. A possible mechanism for this loss, which also takes into account the scrambling of the deuterium label resulting in some DNCNH loss is shown in Scheme 1.

Unfortunately, the sole loss of CO in a MS^3 spectra is not a unique indicator of ions of structure **(F)** because ions of structure **(G)** can in principle also undergo ring opening with CO expulsion. In contrast, the losses of 42 is suggestive that ions of structure **(G)** have been formed. Given that unequivocal structural



Scheme 1.

Table 2
CID MS^3 spectra of $[M + H - CH_3OH]^+$ ions of N-acyl amino acid methyl esters

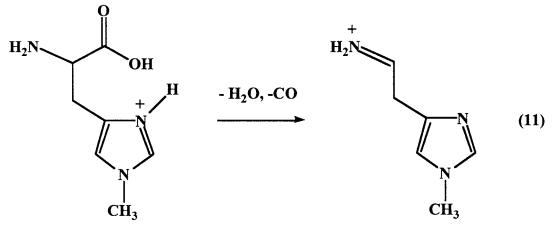
Ac-AA-OMe where AA =	CH_2CO	CO	Other ions (species lost) %abund
Glycine	...	100	...
Alanine	...	100	...
Valine	...	100	...
Leucine	...	100	...
Isoleucine	...	100	...
Proline	...	100	...
Serine	...	100	(50) 1, (CO, CH_2CO) 1
Threonine	...	100	(64) 1, (CO, CH_2CO) 12
Lysine	100	...	(H_2O) 18
Arginine	...	...	(NH_3) 6, (H_2O) 6, (HN=C=NH) 100, (($H_2N)_2$C=NH) 1, (HN=C=NH, CO) 14, (HN=C=NH, CH_2CO) 7
Arginine (deuterated)	...	...	(ND_2H) 2, (ND_3) 2, (DN=C=NH) 37, (DN=C=ND) 100, (45) 2, ($D_2N)_2$C=NH) 3, (DN=C=ND, CH_2CO) 1
Histidine	...	100	(CO, CH_2CO) 1
Cysteine	...	100	...
Methionine	...	100	(CO, CH_2CO) 0.5, (CO, CH_3SH) 1
Aspartic acid	...	100	(CO, CH_2CO) 3
Glutamic acid	...	100	(H_2O, CO) 0.5, (CO, CH_2CO) 0.5, (88) 1.5, (92) 0.5
Asparagine	50	100	...
Glutamine	26	100	(43) 1, (CO, CH_2CO) 3, (84) 2, (87) 8, (95) 1, (104) 1, (110) 2
Phenylalanine	2	100	...
Tyrosine	...	100	...
Tryptophan	11	100	(H_2O) 6.5
Cystine	...	100	...

assignment of the nature of the b_2 ions for species with reactive side chains is problematic based on MS^3 studies, in Sec. 3.3 we use ab initio calculations to examine the relative stabilities of structures **(F)** and **(G)** for several *N*-acetyl amino acid systems. Ideally we would like to do all systems with reactive side chains, but larger ones such as phenylalanine and tryptophan are computationally demanding. Based on the MS^3 studies we have elected to limit our ab initio calculations to four systems which appear to have **(G)** structures (arginine, lysine, asparagines, and glutamine) and three systems where evidence of a **(G)** structure is lacking (histidine, methionine, and serine).

3.3. Ab initio calculations on the $[M+H-CH_3OH]^+$ ions of arginine, histidine, lysine, methionine, asparagine, glutamine, and serine

We have carried out ab initio calculations in order to evaluate: whether isomeric b_2 ions of structure **(F)** [formed by way of Eq. (1)] and **(G)** [formed by way of Eq. (2)] are stable species for lysine, arginine, histidine, methionine, asparagines, and glutamine; the relative stabilities of these isomeric **(F)** and **(G)** ions. Although these reactions are likely to be under kinetic control, with both enthalpic and entropic effects playing a role, we have not carried out searches for the transition states for these competing reactions. Instead, we make the assumption that the relative ring strains which manifest themselves in the product ions should make similar contributions to the relative energies of the transition states. Complete conformational searches for each isomer are beyond the scope of the present work, although several different conformers were examined for each system. In general, conformers in which intramolecular hydrogen bonding interactions are maximized tend to be the most stable. A further complication arises for both arginine and histidine, which can have different tautomeric structures for their side chain moieties. Although the effects of tautomerization on the gas phase chemistry have not

been studied in detail, Turecek et al. have shown that methylation of the side chain of histidine can have a profound effect on the fragmentation of the resultant N1 and N3 isomeric $[M+H]^+$ ions [27] (Eqs. 11, 12).



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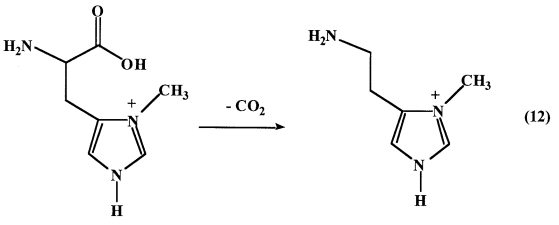


(12)

Futher, a recent theoretical article has examined side chain tautomerism for arginine and found that tautomer **(I)** is only 2.0 kcal mol^{-1} less stable than **(H)** in the gas phase [28]. Thus we examined the effect of side chain tautomerization on the two types of b_2 ions of arginine and histidine. The energies of the most stables conformers are given in Table 3 whereas their corresponding structures are shown in Figs. 1–7. The results of each of the amino acids are now discussed individually.

3.3.1. Arginine

Since arginine has such a basic side chain, the precursor to methanol loss is likely to involve side chain protonation. Proton transfer can occur either from the NH_2 groups, to give tautomers related to **(H)**, or from the NH group, to yield a tautomer related to **(I)**. It is these deprotonated tautomeric forms that

Table 3
Energies for various b_2 isomers of arginine, histidine, lysine, methionine, asparagine, glutamine, and serine

b_2 isomers[a]	HF/631G*[a] (hartrees)	MP2/631G*[b] (hartrees)	ZPVE[c]	Relative energy[d] (kcal mol^{-1})
Arg F1	−679.019 41	−681.028 39	0.265 44	0
Arg F2	−679.029 82	−681.036 72	0.265 87	−5.0
Arg G1	−679.056 73	−681.065 65	0.267 30	−22.3
Arg G2	−678.990 13	−681.013 56	0.267 32	10.4
His F1	−621.649 26	−623.485 39	0.199 44	0
His F2	−621.622 82	−623.455 45	0.198 85	18.4
His G1	−621.663 47	−623.499 00	0.199 81	−8.4
His G2	−621.583 75	−623.420 81	0.198 44	39.9
Lys F	−570.112 03	−571.808 88	0.254 88	0
Lys G	−570.113 59	−571.814 60	0.257 00	−2.4
Met F	−873.541 19	−875.057 28	0.204 28	0
Met G	−873.501 23	−875.033 64	0.202 61	13.9
Asn F	−565.762 35	−567.353 90	0.172 27	0
Asn G1	−565.699 67	−567.300 01	0.170 68	32.9
Asn G2	−565.746 96	−567.335 52	0.172 11	11.4
Gln F	−604.786 41	−606.507 46	0.202 06	0
Gln G1	−604.748 83	−606.483 05	0.202 73	15.7
Gln G2	−604.784 56	−606.505 69	0.202 88	1.6
Ser F	−472.808 09	−474.114 83	0.147 19	0
Ser F neut	−472.458 30	−473.774 97	0.134 71	0
Ser G neut	−472.456 08	−473.771 91	0.133 89	1.4

[a]Based on HF/6-31G* optimized structures shown in Figs. 1–7.
[b]Single point energies using HF/6-31G* optimized structures shown in Figs. 1–7.
[c]Zero point vibrational energies from frequency calculations on the HF/6-31G* optimized structures shown in Figs. 1–7.

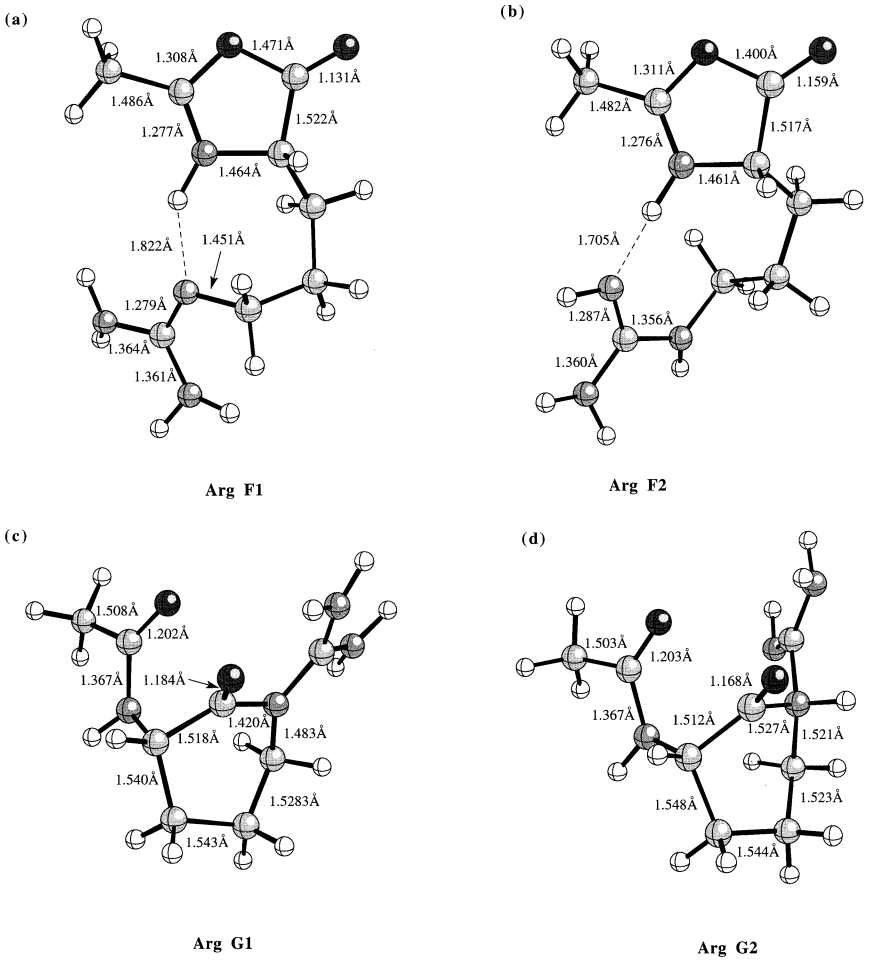
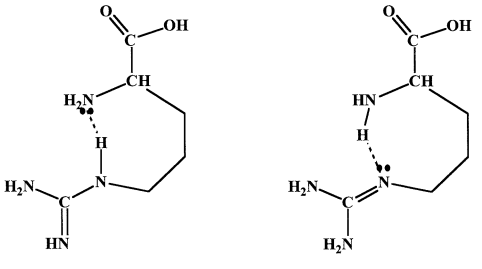


Fig. 1. HF/6-31G* optimized structures of the isomeric arginine b_2 ions: (a) Arg F1; (b) Arg F2; (c) Arg G1; and (d) Arg G2.

must be considered as playing potential roles in the formation of b_2 structures. Fig. 1 shows that both of these side chain tautomers can help stabilize the oxazolone structure **(F)** by way of intramolecular hydrogen bonding. Of these two tautomers Arg F2 is the most stable by 5 kcal mol^{-1}. In contrast, the tautomer related to **(I)** gives a stable six-membered ring structure of Arg G1 [Fig. 1(c)], which is over 30 kcal mol^{-1} more stable than the other tautomeric structure Arg G2 [Fig. 1(d)]. An examination of Table 3 reveals that the most stable b_2 ion structure found is due to side chain attack (Arg G1).



3.3.2. Histidine

Histidine is also likely to be initially protonated on the side chain. Although proton transfer is likely to

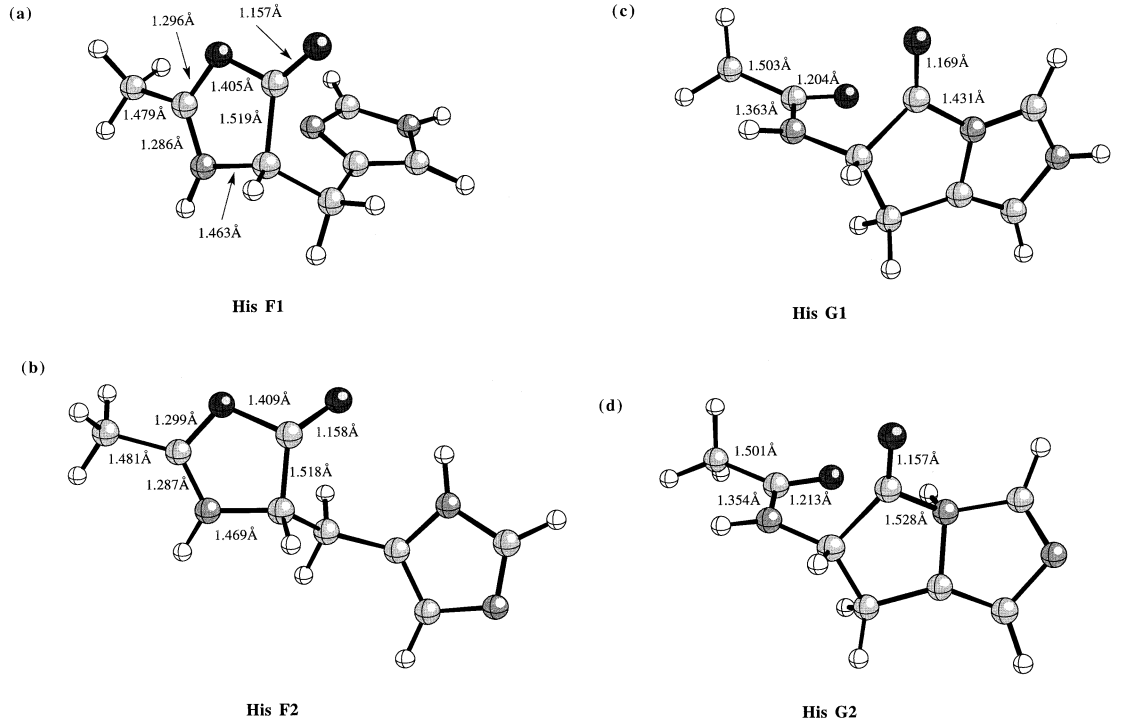
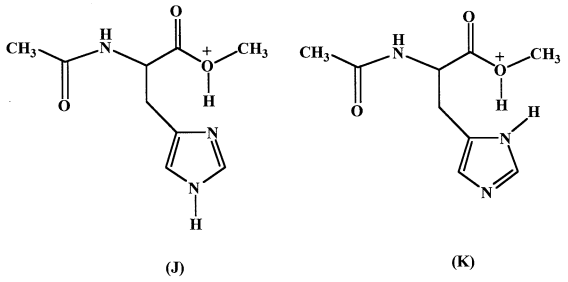


Fig. 2. HF/6-31G* optimized structures of the isomeric histidine b_2 ions: (a) His F1; (b) His F2; (c) His G1; and (d) His G2.

give a side chain tautomer **(J)**, we have also considered the other side chain tautomer **(K)**. From Table 3 it is immediately apparent that the expected side chain tautomer precursor **(J)** gives both the most stable His F1 tautomer [Fig. 2(a)] as well as the His G1 tautomer [Fig. 2(c)]. The most stable b_2 ion structure found is due to side chain attack (His G1).

3.3.3. Lysine

Lysine is also likely to be initially protonated on the side chain. However, unlike arginine and histidine proton transfer from the side chain to the acyl group does not lead to side chain tautomeric structures. This simplifies the number of potential isomeric b_2 ion structures. Once again the side chain can help stabilize the oxazolone structure **(F)** by way of intramolecular hydrogen bonding [Fig. 3(a)]. The isomer due to side chain assisted acyl bond cleavage, Lys G [Fig. 3(b)], is only 2.4 kcal mol^{-1} more stable.



3.3.4. Methionine

Both types of isomeric b_2 ions are five-membered ring structures (Fig. 4) with that arising from side chain attack (Met G) being 13.9 kcal mol^{-1} less stable.

3.3.5. Asparagine

Asparagine and glutamine are interesting since they offer two types of nucleophilic atoms (N versus O) from their side chain amide groups. Thus for

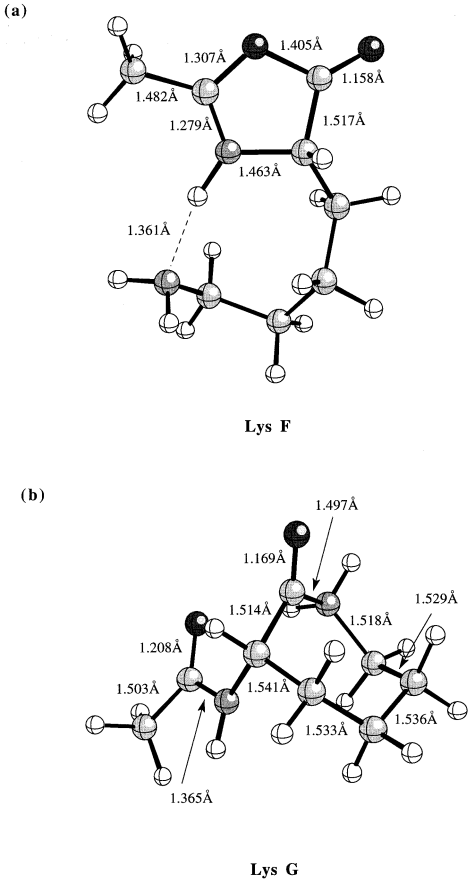


Fig. 3. HF/6-31G* optimized structures of the isomeric lysine b_2 ions: (a) Lys F and (b) Lys G.

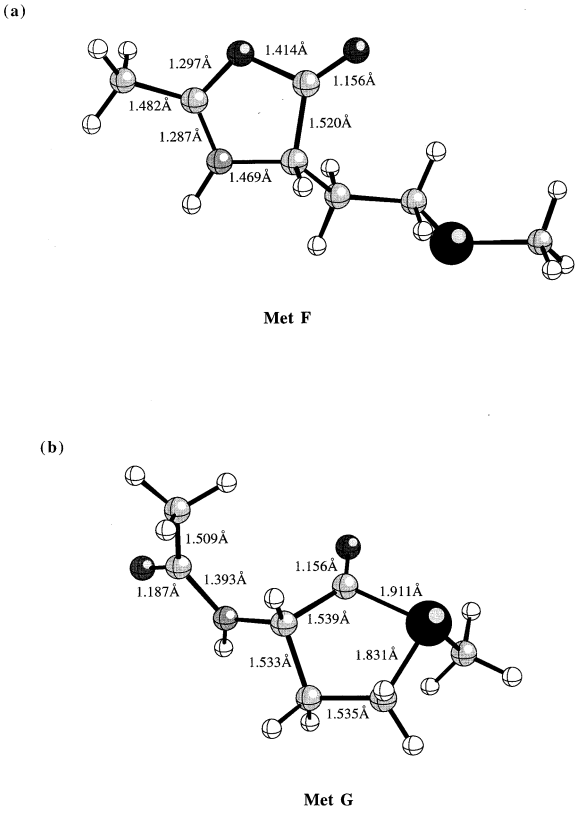


Fig. 4. HF/6-31G* optimized structures of the isomeric methionine b_2 ions: (a) Met F and (b) Met G.

asparagine there are three isomeric b_2 ions to be considered and these are shown in Fig. 5. Interestingly all three structures result in the formation of five-membered rings. The traditional oxazolone b_2 ion structure (Asn F) is the most stable, while the isomer in which the O atom of the side chain assists in acyl bond cleavage, Asn G1 [Fig. 5(b)], is the next most stable isomer. Attack by the N atom of the side chain results in the isomer Asn G2 [Fig. 5(b)] which is less stable than Asn F by some 32.9 kcal mol^{-1}. The results on the relative stabilities of Asn G1 and Asn G2 are consistent with recent studies on attack of alkyl cations onto both the N and O atoms of formamide in which the O adducts were more stable than the N adducts [29]. Finally, the loss of 42 from the b_2 ion of asparagine in its MS^3 spectrum (Table 2) is consistent with an ion of structure **(G)**, whereas the loss of CO maybe be indicative of the oxazolone b_2 ion structure **(F)**.

3.3.6. Glutamine

There are also three isomeric b_2 ions to be considered for glutamine and these are shown in Fig. 6. The most stable isomer is the oxazolone b_2 ion structure (Gln F) but Gln G2 is much closer in energy than in the analogous asparagine case. In contrast to the asparagine systems, attack from the side chain now results in two isomeric six-membered ring systems Gln G1 [Fig. 6(b)] and Gln G2 [Fig. 6(c)]. The former structure is the more stable of the two, which is consistent with the ab initio results for the asparagine system. Note that the MS^3 spectrum of the b_2 ion of glutamine shows both losses of CO and CH_2CO,

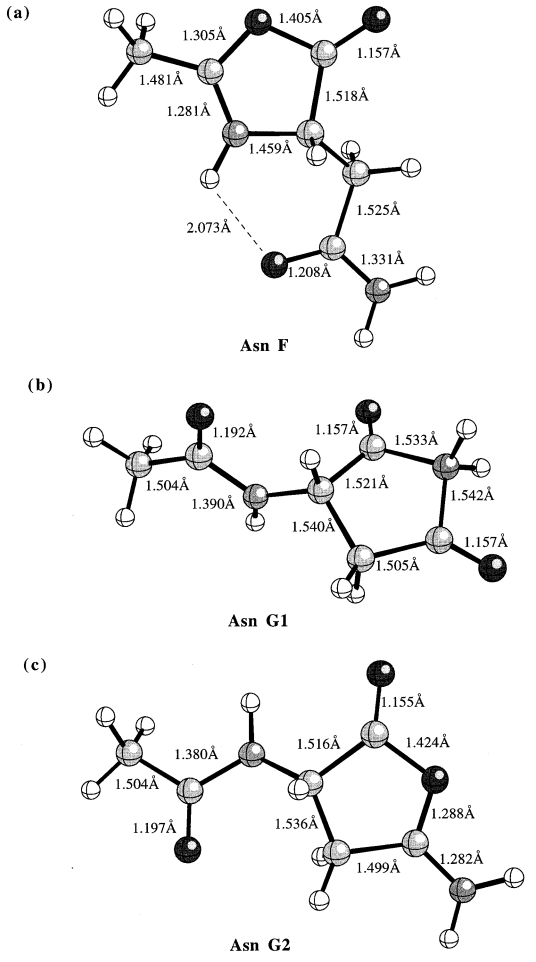


Fig. 5. HF/6-31G* optimized structures of the isomeric asparagine b_2 ions: (a) Asn F; (b) Asn G1; and (c) Asn G2.

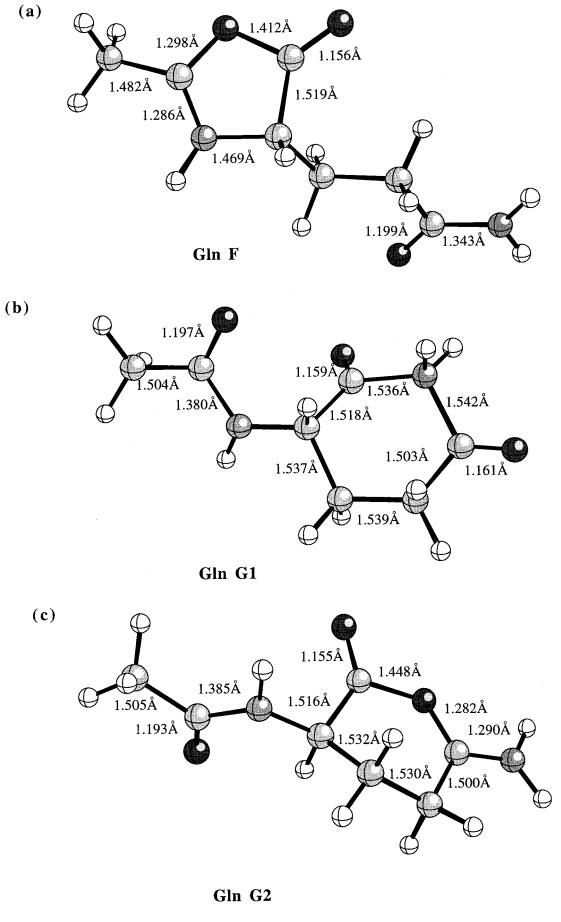


Fig. 6. HF/6-31G* optimized structures of the isomeric glutamine b_2 ions: (a) Gln F; (b) Gln G1; and (c) Gln G2.

which are consistent with a mixture of ions of structure **(F)** and **(G)**.

3.3.7. Serine

Protonated *N*-acyl serine methyl ester only yields a stable oxazolone b_2 ion structure (Ser F) shown in [Fig. 7(a)]. Extensive searches for a protonated four-membered ring lactone of structure **(G)** failed to find this species as a stable structure. In every instance the initial lactone structure underwent barrierless ring opening with subsequent ring closure to the isomeric oxazolone structure **(F)**. In larger peptides with additional basic residues, it may be possible to generate a neutral four-membered ring lactone structure, and so we have looked at the relative stabilities of this and the isomeric neutral oxazolone. In this instance a stable lactone structure was found Ser G neut [Fig. 7(c)], which is only slightly less stable than the isomeric neutral oxazolone Ser F neut [Fig. 7(b)].

3.4. Comparisons with solution phase reactions

Many of the gas-phase neighboring group reactions described previously have solution phase analogs, suggesting that there are similarities between gas and solution phase processes. Unfortunately, the literature on the solution phase involve-

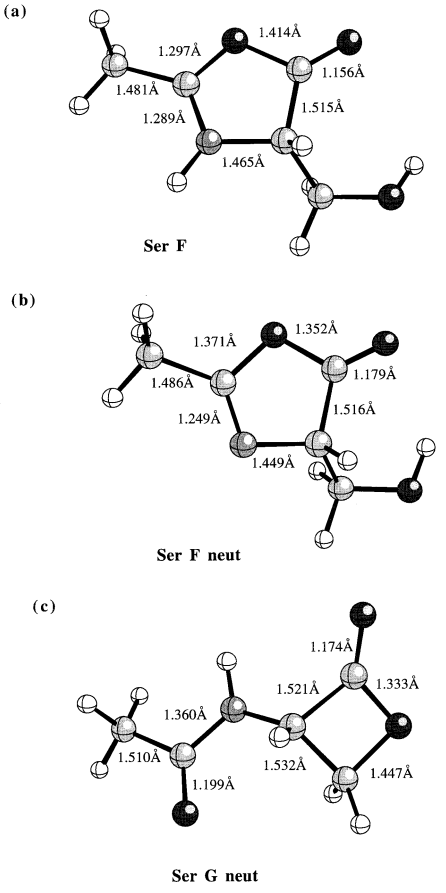


Fig. 7. HF/6-31G* optimized structures of the isomeric serine b_2 ions: (a) Ser F; (b) Ser F neut and (c) Ser G neut.

ment of side chains of amino acids and peptides is widely dispersed. Key papers from the earlier literature that are of relevance to peptide synthesis are reviewed in a comprehensive chapter of Bodanszky's book [30]. More recent studies with an emphasis on the stabilities of peptides and proteins for pharmaceutical preparations have also been reviewed [31]. Here we briefly examine the relevant solution phase literature on oxazolone structures followed by cyclic structures which are products arising from acyl bond cleavage involving reactive side chains.

3.5. Evidence for oxazolones

The structures of the *N*-terminal b_n ($n \geq 2$) product ions, now generally accepted as having oxazolone structures, have been proposed as intermediates in the racemization of acyl amino acids [32a]. One of the most comprehensive kinetic studies examined the coupling of a series of *N*-benzyloxycarbonyl-Ala-Xaa-OH peptides (with 20 different residues Xaa) to valine methyl ester [32b]. The kinetic data and the extent of epimerization were determined for the peptide synthesis and the aminolysis of isolated oxazol-5(4H) ones by means of IR spectroscopy, polarimetry, and reversed-phase HPLC.

Oxazolones have also been proposed as key intermediates in the facile acid-catalyzed hydrolysis of *N*-methyl amino acid containing peptides [32c–e] and during partial acid hydrolysis employing high concentrations of organic acids [32f]. Recently, the structures of a number of cyclic peptides containing an *N*-methyl amino acid were determined by way of x-ray crystallography in order to probe the nature of the facile amide bond cleavage site C-terminal to the *N*-methyl residue. These crystal structures clearly indicated the proximity of the carbonyl oxygen of the *N*-methyl amide bond to the adjacent C-terminal carbonyl, suggesting that fragmentation occurs by way of an oxazolone intermediate [32e].

3.6. Evidence for cleavage induced by the side chain

3.6.1. Arginine

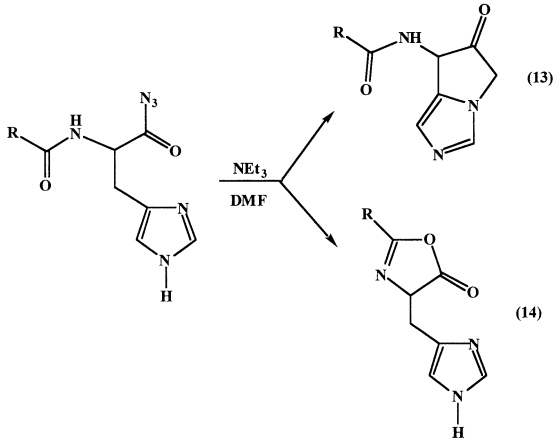
Various *N*-acylated arginine derivatives undergo cyclization under peptide coupling conditions to generate lactams which are the neutral analogues to that proposed in Eq. (2) [24,33].

3.6.2. Histidine

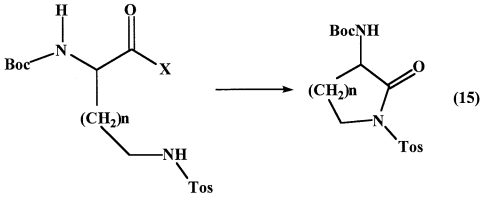
A number of *N*-acylated histidine derivatives undergo cyclization under peptide coupling conditions to generate the cyclic species shown in Eq. 13 which is the neutral analogue to that proposed in Eq. (2) [34]. No evidence for oxazolone formation via Eq. 14. was observed [34c].

3.6.3. Lysine

In a review, Geiger and Konig note that cyclization by way of a side chain amino group to form a lactam is influenced by the ring size (Eq. 15) [35]

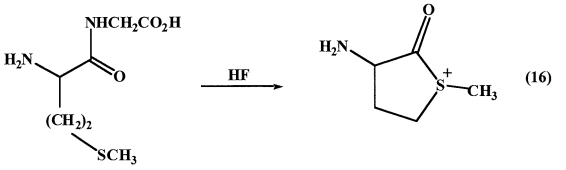


This reaction occurs for ornithine ($n = 2$) to produce a six-membered ring lactam as well as for α,γ-diaminobutyuric acid ($n = 1$) to produce a five-membered ring lactam but is not observed to any extent for lysine ($n = 3$), which requires the formation of a seven-membered ring lactam [35]. This parallels the relative rates of lactone formation which follow the ring size order: $5 > 6 > 7$ [36]. Note that Weinkam observed a related trend in relative ion abundances for the loss of water from diamino acids to form lactams under CI/MS conditions with a ring size order of: $5 > 6 > 7 > 4$ [37].



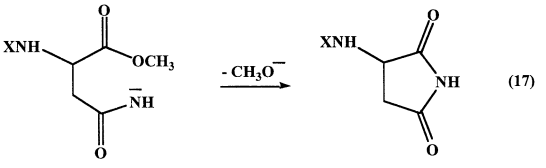
3.6.4. Methionine

Met-Gly reacts with HF to cleave the amide bond [38a]. Gross has suggested a mechanism involving the side chain to yield a b_1 intermediate [38b] (Eq. 16). In contrast, *N*-acyl methionine derivatives cyclize through involvement of the *N*-acyl group to yield oxazolones [39].

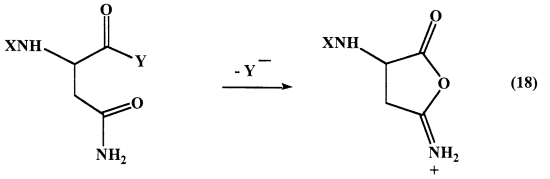


3.6.5. Asparagine

Bodansky notes that either the N or O atoms of the asparagine side chain amide can be involved in acyl bond cleavage (Eqs. 17 versus 18) depending upon the conditions used [30]. Under base conditions, the amide group is deprotonated thereby making the N atom more nucleophilic [Eq (17)]. In contrast, under neutral conditions, the amide O is a better nucleophile and it has been suggested that the intermediate shown in Eq. (18) can be used to rationalize the facile dehydration of the amide side chain under these conditions.



versus

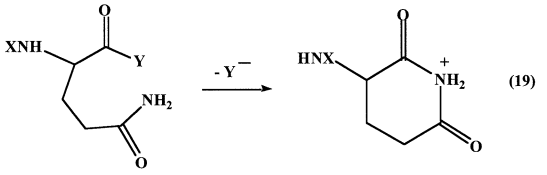


3.6.6. Glutamine

The N atoms of the glutamine side chain amide can be involved in acyl bond cleavage under either base catalyzed or neutral conditions depending upon the leaving group Y [30].

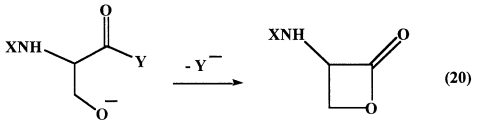
3.6.7. Serine

The only evidence for the side chain of serine acting as a intramolecular nucleophile is under basic



(19)

conditions. Presumably the alkoxide is a good enough nucleophile to yield the neutral lactone (Eq. 20) [30]



(20)

4. Conclusions

Table 4 clearly indicates that it may not be sufficient to rely on one type of data to evaluate the likelihood of direct involvement of the nucleophilic side chains in the formation of b_2 ions of structure **(G)**. Thus whereas all four of the systems which do exhibit losses other than or in addition to CO in their MS^3 spectra structures (arginine, lysine, asparagines, and glutamine) form structure related to **(G)** in solution, only two of these (arginine and lysine) are thermodynamically favoured over the oxazolone structures (based on the ab initio calculations). Of the three systems which only lose CO in their MS^3 spectra (histidine, methionine, and serine), only serine has an unstable **(G)** structure (based on the ab initio calculations). Although the methionine **(G)** structure is less stable than the **(F)** structure, the reverse situation holds for histidine. Thus there is no clear correlation between the results of the MS^3 experiments, the relative stabilities of b_2 ions predicted by the ab initio calculations and solution phase data. Further studies on peptides containing these residues may provide additional evidence for ions with structures related to **(G)**.

Although the focus of this article has been on the nature of side chain involvement in the formation of b_2 ions, it has emerged from this and other studies that the variety of reactive side chains in protonated peptides and model systems results in a rich and diverse gas phase fragmentation chemistry including acyl bond cleavage, side chain fragmentation and small molecule loss (e.g. H_2O). Although model studies have helped reveal several of these processes, database searching of the MS^2 spectra of tryptic peptides may uncover other novel fragmentation reactions involving neighboring group interactions [14b].

Note added in proof: Since the submission of this manuscript, a paper has appeared which describes the cleavage of the Gln-Gly peptide bond in protonated

Table 4
Summary of various data on the existence of side chain stabilized b_2 ions

Amino acids	Experimental MS^3 losses other than CO (Table 2) ?	Thermodynamically preferred over oxazolone structure (*ab initio* results from Table 3)?	Solution phase evidence for side chain structures?
Arginine	Yes	Yes	Yes[a]
Histidine	No	Yes	Yes[b]
Tysine	Yes	Yes	Yes, for homologs[c]
Methionine	No	No	Indirect[d]
Asparagine	Yes	No	Yes[e]
Glutamine	Yes	No	Yes[e]
Serine	No	No	Yes[e]

[a]see [24, 33].
[b]see [34].
[c]see [35].
[d]see [38, 39].
[e]see [30].

peptides [40]. A neighbouring group mechanism was proposed.

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