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Gas-phase Fragmentation of Protonated Mono-*N*-methylated Peptides. Analogy with Solution-phase Acid-catalyzed Hydrolysis

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Fragmentation of protonated peptides with a single *N*-methylated residue was studied by low-energy collision-induced dissociation (CID) and the effects of the *N*-alkylation on the fragmentation were evaluated. Peptides with an *N*-terminal *N*-alkylated amino acid behave similarly to regular peptides except for an increased stability of the protonated molecular ion due to the increased proton affinity. On the other hand, the *N*-alkylation of an internal amino acid residue has very distinct effect on fragmentation. It causes activation of the amide bond on the *C*-terminal side of the *N*-alkylated residue resulting in preferential cleavage of this bond and formation of corresponding b ions. This is in a sharp contrast to the effect of a natural *N*-alkylated amino acid, proline, which activates its *N*-terminal amide bond providing abundant y ions upon CID. Both peptides with Ac-*N*-methylamino acid *N*-terminus and with an internal *N*-methylated amino acid exhibit the former type of fragmentation. A fragmentation mechanism is proposed that explains the observed effects based on fragmentation of model peptide analogs and isotopically labeled peptides. It was found that an interaction between carbonyl groups of neighboring amide moieties is an important factor in the formation of the b ion with a structure of a protonated *N*-methyloxazolone. AM1 calculations suggest that the stability of this ion favors its formation in the CID of *N*-methylated peptides. It was also shown that an incorporation of a basic site outside the peptide backbone does not affect the course of fragmentation of the peptides with an internal *N*-alkylamino acid. These observations provide support for the 'mobile proton' model for the initial stage of protonated peptide fragmentation in the low-energy CID. The observed phenomenon is analogous to the solution-phase acid-catalyzed hydrolysis of these peptides. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: *N*-methylated peptides; collision-induced dissociation; fragmentation mechanism; protonated oxazolone

INTRODUCTION

Peptides containing one or more *N*-alkylated amino acids are common natural products and often have specific biological activity.[1] In contrast to unmodified, non-*N*-alkylated peptides (herein referred to as regular peptides), their fragmentation has not been systematically studied so far. Before the advent of desorption techniques, mainly fast atom bombardment (FAB) in the early 1980s,[2] permethylation and peracetylation prior to mass spectral analysis were necessary in order to increase the volatility of peptides for chemical ionization analysis.[3] Later, FAB mass spectra of series of *N*-terminal blocked peptides containing one *N*-methylated amino acid residue were published.[4] This study reported enhanced fragmentation on both the *N*-and *C*-terminal sides of an *N*-methylamino acid residue (NMePhe, Sar, NMeLeu). A higher relative abundance of b ions arising from the fragmentation of an amide bond *C*-terminal to the *N*-methyl residue than of y ions from the fragmentation of the amide bond on the *N*-terminal side was noted. The same features were found in electrospray ionization/collision-induced dissociation (ESI/CID) mass spectra of these peptides.[5] This effect contrasts the effect of a natural *N*-alkylamino acid, proline, which directs the fragmentation to its *N*-terminal side resulting in an abundant y ion, while ions from its *C*-terminal side are usually suppressed (an effect frequently referred to as the 'proline effect').[6] Recently, an explanation of this discrepancy was proposed based on an unfavorable transition state and/or product ion structure in the fragmentation of a proline-containing peptide.[7]

In the low-energy collision spectra of protonated peptides, major fragment ions arise from cleavage of amide bonds between amino acid residues generally resulting in a series of *N*-terminal a- and b-type ions and *C*-terminal y type ions.[8] It is assumed that in a protonated molecule a nitrogen of a given amide bond is protonated via an intramolecular proton transfer and the bond is cleaved either directly to give a b-type ion or with an additional proton transfer from the *N*-terminal side of the bond to the *C*-terminal side providing y-type ions, where the second proton comes from the amide moiety one residue towards the *N*-terminus.[9] Mechanisms of the formation of these ions are widely accepted, but not rigorously supported by experimental data. Theoretical data from semiempirical and *ab initio*

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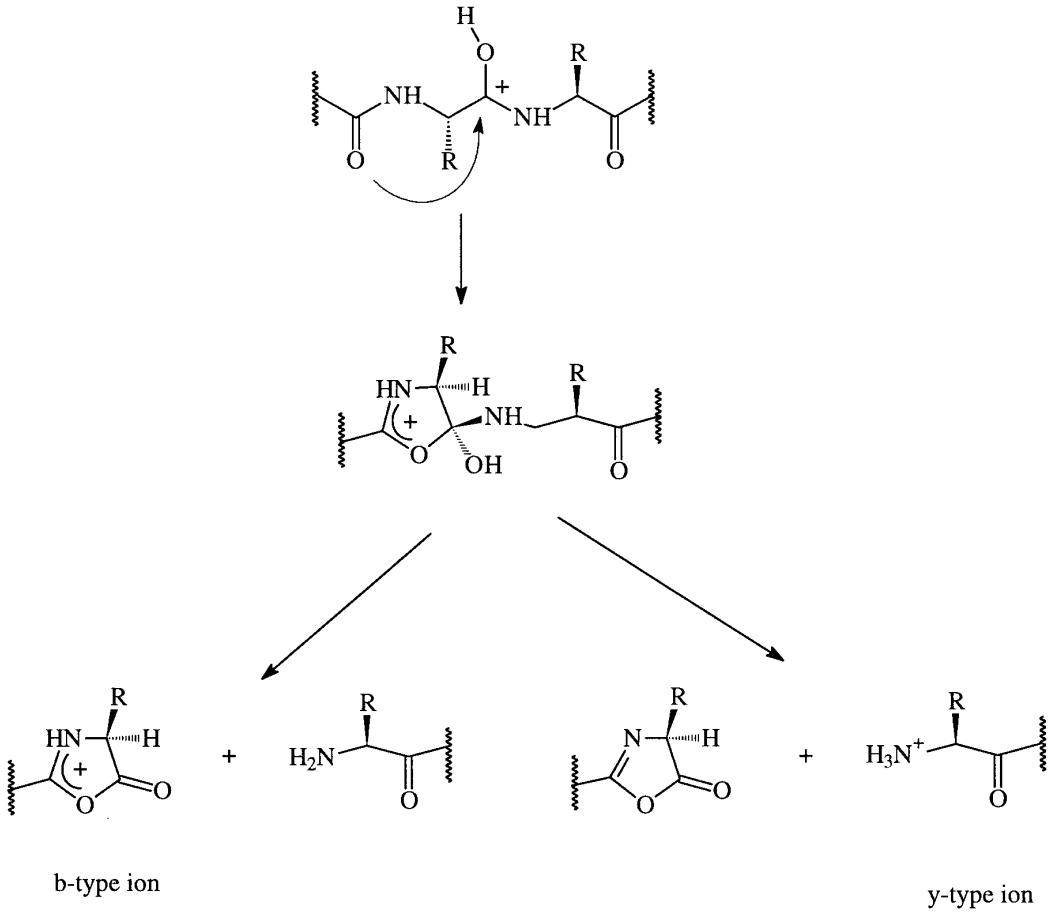
calculations clearly show that the bond order of an amide bond decreases substantially upon protonation of the nitrogen.[10]

On the other hand, the protonation at the amide nitrogen is thermodynamically unfavorable compared with the amide carbonyl oxygen.[11] Recently, Hunt and co-workers[12] proposed a different mechanism which takes this into account. According to this mechanism, after an initial intramolecular proton transfer to an oxygen of an amide bond moiety, a protonated oxazolone-like cyclic intermediate forms via nucleophilic attack of the carbonyl oxygen *N*-terminal to the protonated bond on the protonated amide bond's carbon (Scheme 1). This intermediate subsequently dissociates via a single or a double proton transfer to give b or y product ions, respectively. The structure of the b ions would be that of a protonated oxazolone. Possible structures of b ions were studied by Harrison and co-workers.[13] Fragmentation of b ions to a ions via CO loss was found accompanied by release of a substantial amount of kinetic energy. A fragmentation pathway was proposed for the process, which proceeds from a protonated oxazolone through an acylium ion transition state. This conclusion was also supported by *ab initio* calculations, finding an acylium ion only as a transition state or a reactive configuration above both the protonated oxazolone and the fragmentation products (immonium a ion and CO).[13,14] Recently, an abundant formation of b_1 ions in the low-energy CID spectra of peptide phenylthiocarbamoyl derivatives was similarly explained by the formation of protonated thiazolones.[15]

A 'mobile proton' model of an initial stage of protonated peptide fragmentation postulates that the ionizing proton is transferred prior to the fragmentation from its initial location to the site of the cleavage.[16–18] Consequently, the initial location of a proton may affect peptide fragmentation depending on the accessibility of the fragmentation site for the proton transfer. The initial location of an ionizing proton is given by the relative gas-phase basicity of possible isomers (in terms of location of the proton and also in terms of different hydrogen bonding from a single protonation site) of protonated peptides. Intrinsically, for a peptide with no basic amino acid residue, the most basic site is the *N*-terminal amino group. As revealed by *ab initio* calculations for triglycine, this proton also forms hydrogen bonds with two carbonyl oxygens.[19] Furthermore, several isomers are found close in energy with various hydrogen bonds between the internal amide protons and carbonyl oxygens. One isomer is even found where the ionizing proton is not bound to the *N*-terminus, but only to several amide bond carbonyl oxygen atoms.[19,20] The order of protonation sites found for triglycine is *N*-terminal amine > amide carbonyl > carboxylic carbonyl > amide nitrogen.[19] This can be an important factor for the elucidation of the mechanism of peptide bond cleavage.

The mobility of the ionizing proton is enabled by a substantial amount of energy imparted to the ion upon CID. It was shown that an ionizing deuteron becomes



Scheme 1. Fragmentation of protonated peptides proposed by Hunt and co-workers.[12]

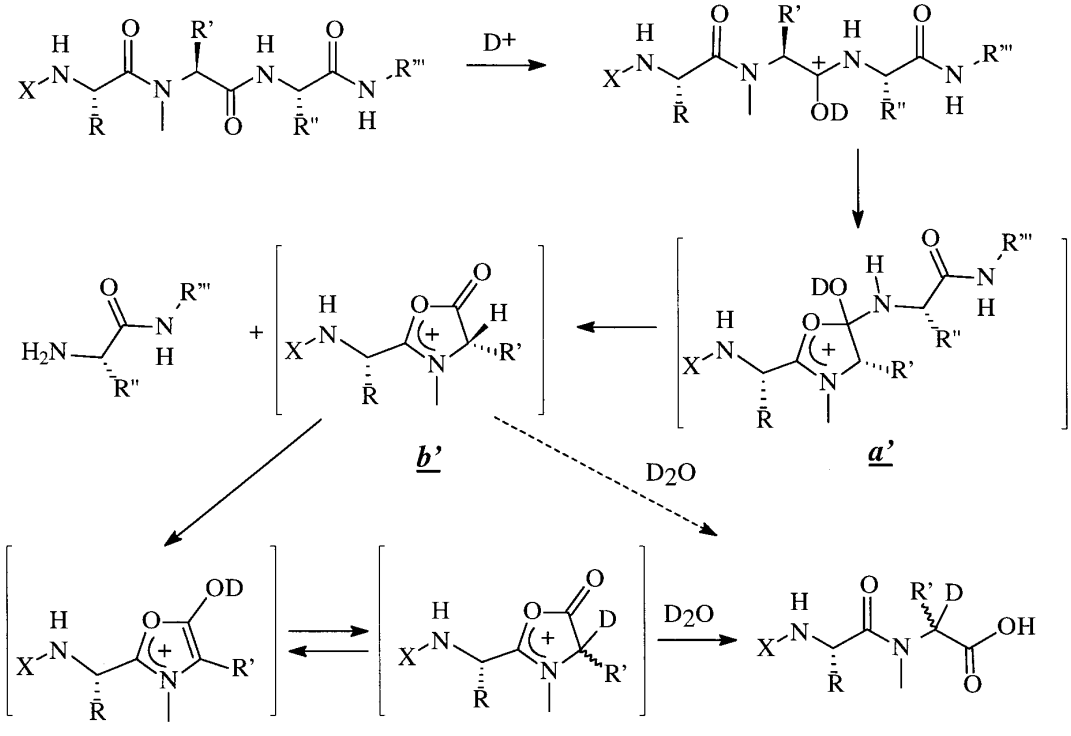
randomly distributed between product ions upon CID of a monodeuteronated molecular ion of acetylated tri-and pentapeptides.[21] On the other hand, in peptides with a basic amino acid residue (Lys, Arg), the ionizing proton, initially located at the side-chain of the basic amino acid, must be transferred to a backbone amide bond prior to fragmentation. This in turn requires more internal energy to overcome an increased activation energy barrier, as evidenced by the higher collision energy for the fragmentation onset for peptides with either *C*-or *N*-terminal Arg residues.[16] Fragmentation of protonated peptides in the gas phase can be compared with an acid-catalyzed hydrolysis in solution. A similar directing effect of Pro on peptide dissociation was observed in solution, where Pro induces acidic hydrolysis at its *N*-terminal amide bond under harsh conditions.[22] Conversely, it was found that peptides with an Ac-NMeAA moiety at the *N*-terminus hydrolyze rapidly in solution under acidic conditions.[23] Facile hydrolysis was also observed for several non-acetylated peptides with an internal *N*-methylamino acid residue. The site of the hydrolysis was assigned to the *C*-terminal amide bond of the *N*-methylamino acid residue. A reaction mechanism via an oxazolone intermediate was proposed based on evidence from H–D exchange as followed by NMR and mass spectrometry (MS) (Scheme 2).[23]

Here we report on the low-energy CID of a series of short peptides with a single *N*-methylated amino acid residue. Such compounds are interesting for several reasons: from the point of view of mass spectrometry and fragmentation mechanisms the *N*-methylation of a single peptide amide bond increases basicity of that bond's carbonyl oxygen (increased basicity of *N*,*N*-dimethylacetamide, proton affinity $PA = 908.4$ kJ mol^{-1} *vs.* *N*-methylacetamide, $PA = 888.9$ kJ mol^{-1})[24] and it also removes the proton participating in the formation of y ions. As reported earlier, this has a huge effect on the fragmentation.[25] From a structural point of view it results in the formation of distinct populations of *cis* and *trans* isomers at this bond.[26,27] This has interesting implications for the gas-phase fragmentation of protonated peptides owing to the formation of a distinct secondary structure of peptides with an *N*-methylated residue.[28] Fragmentation of both unmodified and acetylated peptides with one *N*-methylamino acid residue is presented. Comparison with their non-methylated counterparts is made to emphasize the effect of *N*-methylation on the fragmentation. Pro is included in the sequence in order to compare the directing effect of proline[6,7] with the effect of an *N*-methylated residue. The *N*-methylated peptides show distinct fragmentations which can be explained in terms of fragmentation via the oxazolone-like intermediate.[12–15] Included is a series of peptides with a basic amino acid in the sequence in order to evaluate the effect of initial protonation at the side-chain on the fragmentation of *N*-methylated peptides.

EXPERIMENTAL

Peptide synthesis

Peptides were prepared by solid-phase peptide synthesis (SPPS) on Wang resin[29] using the Fmoc strategy. No side-chain protection was used for serine; the side-chain of Lys was protected with Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl). Couplings were accomplished with a mixture of an amino acid–BOP (benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate)–HOBt (1-hydroxybenzotriazole)



Scheme 2. Reaction mechanism of the solution acid-catalyzed hydrolysis of peptides with an Ac-NMeAla moiety.[27]

–DIEA (diisopropylethylamine) (3:3:3:4 equiv.) in NMP (*N*-methylpyrrolidone). For the coupling of an amino acid following the *N*-alkylated amino acid HATU (7-azabenzotriazol-1-yl-1,1,3,3-tetramethyluronium hexafluorophosphate) was used instead of BOP and no HOBt was added. Deprotection of the Fmoc group was accomplished by treatment with 25% piperidine in DMF (dimethylformamide) for 12 min. Deprotection of Dde was accomplished by treatment with 2% hydrazine in DMF for 8 min.

Peptides containing an NMe-pyroglutamate residue were synthesized as peptides containing an NMe-Glu(OBzl) residue. (Fmoc-NMe-Glu(OBzl)-OH was prepared by the procedure described by Freidinger *et al.*[30]) After cleavage from the resin with aqueous TFA, the crude peptides were refluxed in toluene for 2 h, providing the desired NMe-Pyr (Pyr = pyroglutamic acid) peptides.

Cleavage of peptides from resin

Owing to the lability of some peptides, attention had to be paid to the method of cleavage of peptides from the resin. Peptides stable to acidic conditions were cleaved by TFA–H_2O (19:1) for 90 min. Peptides unstable under such conditions were cleaved either by ammonolysis (NH_3 in methanol, 3 days), affording amides, by alkaline hydrolysis (NaOH, 90% MeOH, 30 min), providing acids, or by methanolysis (dry MeOH, DBU (1,8-diazabicyclo[5.4.0]undec-7-ene), 60 min), providing methyl esters. Acetylated peptides were prepared in solution using acetic anhydride in dichloromethane or by acetylation with acetic anhydride on the solid phase prior to cleavage from the resin. All peptides were purified by reversed-phase high-performance liquid chromatography.

Chemicals

Fmoc-L-*N*-methylalanine, Fmoc-L-*N*-methylphenylalanine and all other protected amino acids (L-isomers) were purchased from Advanced Chemtech (Louiseville, KY, USA), Acetic anhydride-d_6 (98% D) and isobutane-d_{10} (99% D) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA).

Mass spectrometry

All mass spectra were obtained on a VG Quattro triple-stage quadrupole mass spectrometer (Micromass, Altrincham, UK). Peptides were generally ionized with an electrospray ionization under the following conditions: mobile phase methanol–water (50:50, v/v) at a flow-rate of 35 μl min^{-1}, needle voltage 2.8 kV, high-voltage lens (counter electrode) 0.1 kV and skimmer potential −10 V. For the generation of MD^+ ions chemical ionization with isobutane-d_{10} was used. The ion source was not conditioned prior to the introduction of the sample in order to achieve mainly monodeuteronation. CID experiments were accomplished in the r.f.-only hexapole with Ar introduced at a pressure of 1.3×10^{-4} mbar as read on the Pirani gauge directly attached to the hexapole region (attenuation of the main ion beam 80%). The collision energy was defined by the potential difference between the ion source and the collision hexapole and was typically set to 10 eV. For energy-resolved mass spectrometry (ERMS) the collision energy was varied from 3 to 40 eV as indicated.

Theoretical calculations

Semiempirical quantum chemical calculations were performed using the AM1 hamiltonian with EF (eigenvector following) geometry optimization procedure included in the MOPAC7 package (QCPE, QCPM130). A preliminary conformational search was performed for all structures under investigation at the molecular mechanics level using the AMBER* force field and Monte Carlo conformational search utility of the MacroModel 4.5 software package.[31] Lowest energy conformations and several other low-energy conformations were taken in the AM1 optimization. Vibrational frequency analysis of AM1-minimized structures confirmed the stationary points: minima were characterized by all real frequencies, whereas transition-state structures showed one imaginary frequency.

RESULTS

It has been shown earlier that acetylated peptides with *N*-terminal *N*-methylated amino acids exhibit a unique fragmentation, the formation of highly abundant b_1 ions.[25] The investigation of this phenomenon was the main focus of the present work. Besides this phenomenon, several peptides designed for this study showed unusual formation of y ions. This is briefly discussed in a separate, as are the opposite effects of the *N*-methylated residues and the effect of proline.

Formation of b ions

As a starting point for the investigation of the effect of *N*-alkylation of amino acid residues on the fragmentation of protonated peptides, CID spectra of protonated peptides with an *N*-methylalanine, **1a** (H-NMeAla-PLG-OH), **2a** (H-NMeAla-VPLG-OH), and *N*-terminal alanine, **1b** (H-APLG-OH), **2b** (H-AVPLG-OH), were acquired (Table 1). The peptides with a free *N*-terminal *N*-methylalanine did not show any marked difference from their alanine analogs (Fig. 1). When these peptides were acetylated (**3a** (Ac-NMeAla-PLG-OCH_3) and **3b** (Ac-APLG-OCH_3), respectively), a characteristic fragmentation was observed for the NMeAla peptide (**3a**) leading to the formation of highly abundant b ions as depicted in Scheme 3. While the NMeAla peptide **3a** (Ac-NMeAla-PLG-OCH_3) afforded primarily b_1 and y_3 fragment ions, the fragmentation of its Ala analog **3b** (Ac-APLG-OCH_3) provided mostly b_3 and y_3 ions (Fig. 2). The CID of the acetylated Ala peptide **4b** (Ac-AVPLG-OH) [analog of **2b** (H-AVPLG-OH)] produced a major y_3 ion due to the 'proline effect' at the colli-

Table 1. CID spectra of tetra- and pentapeptides with N-terminal NMeAla residues and their reference peptides[a]

			m/z (relative intensity, %)												
Peptide	No.	$[M+H]^+$	a_1	b_1	a_2	b_2	b_3	b_4	y_2	y_3	y_4	Pro	Leu	ProLeu	Other
H-**NMeA**-PLG-OH	**1a**	371(676)	(5810)		155(1)	183(10)	296(5)	n.a.	189(13)	286(46)	n.a.	70(1)	86(3)	211(3)	126(8)
H-**A**PLG-OH	**1b**	357(247)	44(1)		141(10)	169(43)	289(9)	n.a.	189(12)	286(16)	n.a.	70(1)	86(6)	211(1)	
H-**NMeA**-VPLG-OH	**2a**	470(880)				185(6)				286(94)					
H-**A**VPLG-OH	**2b**	456(166)				171(3)	268(3)	381(3)		286(89)				211(1)	
Ac-**NMeA**-PLG-OCH_3	**3a**	427(58)		**128(50)**			338(3)	n.a.		300(47)	n.a.				
Ac-**A**PLG-OCH_3	**3b**	413(179)					324(17)			300(78)		70(1)		211(1)	
Ac-**NMeA**-VPLG-OH	**4a**	512(89)		**128(12)**		227(1)		437(1)		286(45)	385(32)				
Ac-**A**VPLG-OH	**4b**	498(100)					310(3)	423(5)		286(89)	385(3)				
Ac-**NMeA**-ALG-OCH_3	**5**	401(72)		**128(74)**		199(2)	312(21)	n.a.	203(2)	274(1)	n.a.	n.a.		n.a.	

[a] ES+, skimmer 12 V, collision gas Ar at 20% transmission, E_{lab} = 10 eV. In bold: residue of interest and b ions of interest.

Table 2. CID spectra of heptapeptides[a]

			m/z (relative intensity, %)									
Peptide	No.	$[M+H]^+$	a_1	b_2	b_3	b_4	b_5	b_6	y_2	y_3	y_4	y_5
H-T-**NMeA**-VPLG-OH	**6a**	571(1811)		**187(84)**						286(16)		
H-T**A**VPLG-OH	**6b**	557(688)								286(100)		
H-VSF-**NMeA**-ALG-OH	**7**	678(249)	72(4)			**419(82)**					345(7)	492(2)
Ac-VSF-**NMeA**-ALG-OH	**8**	720(29)				**461(83)**	532(7)	645(2)			345(2)	492(3)
H-VSF**P**ALG-OH	**9**	690(240)		187(1)	334(1)	**431(2)**	502(4)	615(3)	189(1)		357(61)	505(6)
Ac-VSF**P**ALG-OH	**10**	732(53)			376(5)	**473(2)**	544(5)	657(12)	189(1)		357(49)	504(3)

[a] ES+, skimmer 12 V, collision gas Ar at 20% transmission of the main ion beam, E_{lab} = 30 eV. In bold: residue of interest and b ions of interest.

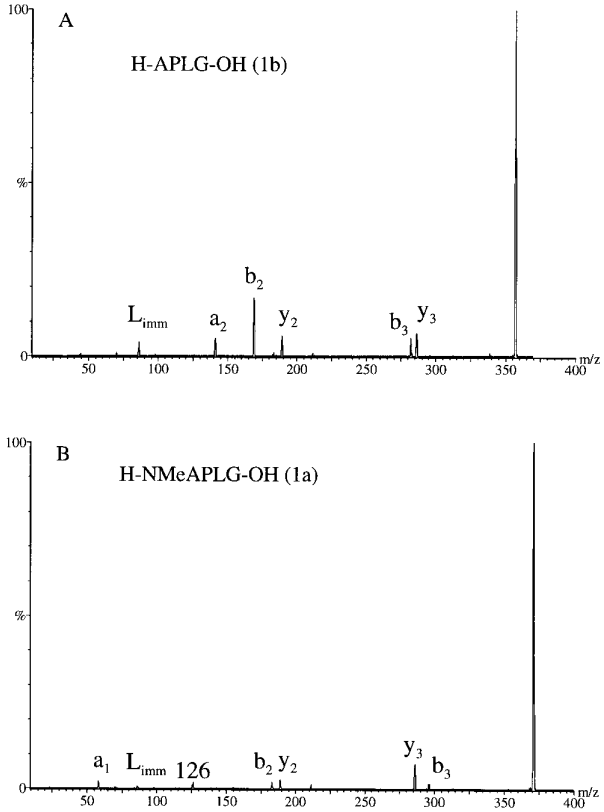


Figure 1. CID spectra of $[M + H]^+$ of peptides (A) **1b** (H-APLG-OH) and (B) **1a** (H-NMeAla-PLG-OH). 20%T Ar, 10 eV E_{lab} collision energy.

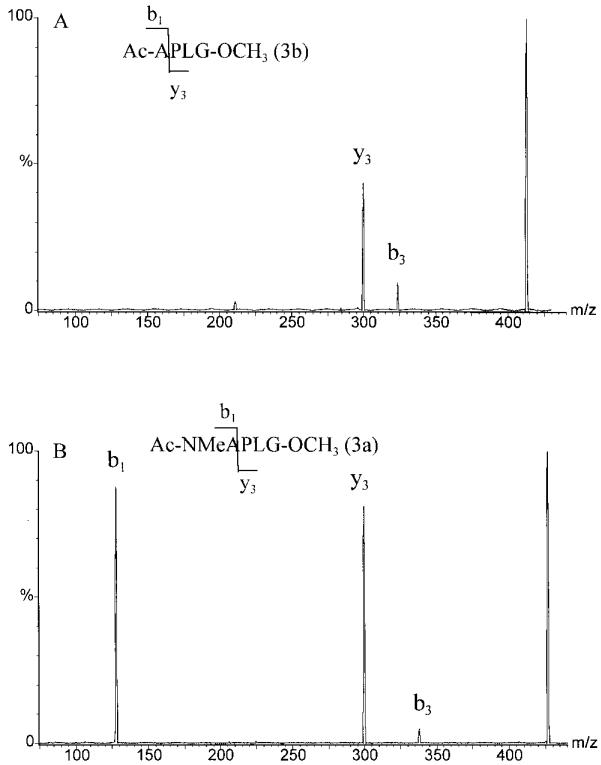
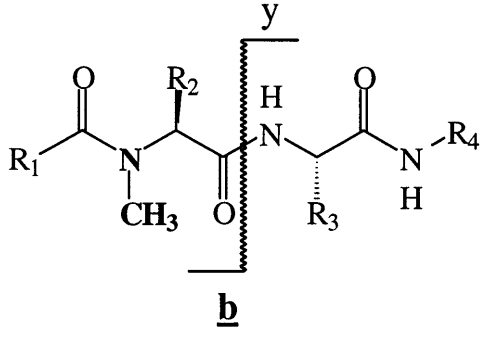


Figure 2. CID spectra of $[M + H]^+$ of peptides (A) **3b** (Ac-APLG-OH) and (B) **3a** (Ac-NMeAla-PLG-OCH$_3$). 20%T Ar, 10 eV E_{lab} collision energy.

sion energy $E_{lab} = 10$ eV (Table 1) (the formation of highly abundant y ions on the *N*-terminal side of a Pro residue is frequently referred to as a 'proline effect'). Its *N*-methylated analog **4a** (Ac-NMeA-VPLG-OH) [derived from **2a** (H-NMeAla-VPLG-OH)] at that collision energy fragmented to give abundant y_3 and y_4 ions along with b_1 ions (Table 1). Similar fragmentation was observed for an analogous peptide **5** (Ac-NMeAla-ALG-OCH$_3$) where the Pro was replaced by Ala.

In order to explore the scope of the observed phenomenon, further experiments were performed on a large series of peptides with and without an *N*-methyl residue. The emphasis was put on the effect the acetylation had on the fragmentation as described above. First, a basic *N*-terminal amino group was introduced by replacing the acetyl group with an amino acid residue, Thr. A CID spectrum of a pair of peptides, **6a** (H-T-NMeA-VPLG-OH) and **6b** (H-TAVPLG-OH), showed very distinct fragmentations. The Ala peptide **6b** (H-TAVPLG-OH) yielded exclusively a y_3 ion [in a way similar to **4b** (Ac-AVPLG-OH)], whereas CID of **6a** (H-T-NMeAla-VPLG-OH) provided an abundant b_2 ion, analogous to the b_1 ion from **4a** (Ac-NMeA-VPLG-OH), along with a weak y_3 (Table 2). Further extension of the peptide at the *N*-terminus did not change the observed effect of *N*-methylation as the CID of protonated peptide **7** (H-VSF-NMeAla-ALG-OH) yielded abundant b_4 ions [84% total fragment ion current (TFIC)] at the collision energy $E_{lab} = 30$ eV (Table 2) and only a weak y_4 ion (7%TFIC) (Table 2). This peptide was also acetylated (**8**) to examine the effect of the removal of the protonation site at the *N*-terminal amine group. The fragmentation observed did not differ from that of non-acetylated peptides. In comparison, analogous Pro peptides (**9**, **10**) yielded primarily y_3 ions.



Scheme 3. Scheme of observed b/y ion formation in peptides with an NMe-amino acid residue.

In order to investigate possible interactions of the carbonyl of the *N*-terminal acyl group (either acetyl or amino acid, henceforth referred to as '*N*-terminal carbonyl') with the carbonyl of the cleaved amide bond, two series of peptides were prepared. First, a series of peptides was prepared where the acetyl group was replaced by a formyl [**11** (For-NMeAla-PLG-OH)] or a trifluoroacetyl group [**12** (Tfa-NMeAla-GLG-OH)] to assess the effect of nucleophilicity of the '*N*-terminal carbonyl' along with a reference peptide **13** (Ac-NMeA-GLG-OH). In a second series, peptides with an *N*-methyl pyroglutamate residue, **14** (NMePyr-ALG-OH) and **15** (NMePyr-PLG-OH), were studied. In these peptides, the '*N*-terminal carbonyl' was fixed in a position preventing it from an interaction with the carbonyl of

Table 3. CID spectra of tetra- and pentapeptides[a]

			m/z (relative intensity, %)										
Peptide	No.	$[M+H]^+$	a_1	b_1	a_2	b_2	a_3	b_3	b_4	y_2	y_3	Leu	Other
For-**NMeA**-PLG-OH	**11**	399(169)		**114(22)**	183(1)	211(11)	296(4)	324(22)			286(31)		Other(10)
Ac-**NMeA**-GLG-OH	**13**	373(56)		**128(88)**				298(12)					
Tfa-**NMeA**-GLG-OH	**12**	427(241)					324(7)	352(83)		189(3)			86(7)
NMeAPyr-ALG-OH	**14**	385(146)	98(1)			197(2)	282(3)	310(32)		189(47)		86(15)	
NMeAPyr-PLG-OH	**15**	411(471)	98(1)		195(5)	223(32)	308(8)	336(50)					
Ac^t-**A**ALG-NHMe	**16a**	402(48)		**130(1)**		201(2)		314(12)	371(5)	202(1)	273(63)		185, 255, 89(16)
Ac-**A**ALG-NHMe	**16b**	386(66)					270(6)	298(48)	355(21)	202(10)	273(5)		185, 86, 368(8)
A^t**A**ALG-NHMe	**17**	431(320)				159(4)		230(2)	343(15)		273(74)		400(6)

[a] ES+, skimmer 12 V, collision gas Ar at 20% transmission, $E_{lab} = 10$ eV. In bold: residue of interest and b ions of interest.

Table 4. Effect of different residues on the formation of the b_1 fragment ions[a]

			m/z (relative intensity, %)							
Peptide	No.	$[M+H]^+$	a_1	b_1	b_2	a_3	b_3	y_2	y_3
Ac-**Sar**-ALG-OH	**18**	373(86)	86(3)	**114(38)**	185(7)	270(6)	298(46)		
Ac-**NMeF**-ALG-OH	**19**	463(49)		**204(91)**	275(4)		388(5)		
Ac-**NMeF**-SLG-OH	**20**	479(37)		**204(94)**	291(3)		404(3)		
Ac-**NMeA**-PLG-OCH_3	**3a**	427(58)		**128(50)**			338(3)		300(47)
Ac-**NMeA**-ALG-OCH_3	**5**	401(72)		**128(74)**	199(2)		312(21)	203(2)	274(1)
Ac-**NMeA**-GLG-OH	**13**	373(56)		**128(88)**			298(12)		

[a] In bold: residue of interest and b ions of interest.

the *N*-methyl residue. For both of these series, the CID yields no b_1 ions (Table 3). Thioacetyl (Ac^t-) and acetyl peptides with an Ala residue, **16a** (Ac^t-AALG-NHMe) and **16b** (Ac-AALG-NHMe), and a peptide with thio-Ala (A^t) in place of the acetyl, **17** (H-A^t-AALG-NHMe), supplemented these series. The higher nucleophilicity of thioacetyl compared with acetyl was found to induce fragmentation at the b_1/y_3 position producing abundant y_3 ions. The formation of y_3 rather than b_1, observed for Ac-NMeAla peptides, is due to the presence of the amide proton of the Ac^t-Ala moiety. Similarly, the CID of **17** produced abundant y_3 ions.

Further, a possible effect of the side-chain of the *N*-methyl residue was investigated. For this purpose, the NMeAla residue in peptide **5** (Ac-NMeAla-ALG-OCH_3) was replaced by Sar [**18** (Ac-Sar-ALG-OH)] (no side-chain) and NMePhe [**19** (Ac-NMePhe-ALG-OH)] (bulky benzyl side-chain). All three peptides yield b_1 ions upon CID with a relative abundance increasing in the order Sar (**18** (Ac-Sar-ALG-OH)) < NMeAla [**5** (Ac-NMeAla-ALG-OCH_3)] < NMePhe [**19** (Ac-NMePhe-ALG-OH)] (Table 4). This indicates possible steric and/or inductive effects of the side-chain on the fragmentation.

The relative abundance of b ions can also be affected by an adjacent amino acid residue participating in the amide bond cleaved upon formation of the b ions. Since this residue abstracts a proton from the *N*-terminal part of a peptide, it is believed that its intrinsic basicity can increase the proportion of y ions formed at the expense of b ion formation.[32] The existence of such a phenomenon is suggested by an abundant y_3 ion in the CID spectra of the peptide **3a** (Ac-NMeAla-PLG-OCH_3) with an Ac-NMeAlaPro-sequence. A peptide **20** (Ac-NMePhe-SLG-OH) with a Ser residue (PA(Ser) = 907.3 kJ mol^{-1}) complemented a series of peptides **13** (Ac-NMeAla-GLG-OH) (Gly, PA = 885.5 kJ mol^{-1}), **3** (Ac-NMeAla-ALG-OCH_3) (Ala, PA = 898.9 kJ mol^{-1}) and **3a** (Ac-NMeAla-PLG-OCH_3) (Pro, PA = 915.3 kJ mol^{-1})[24] (Table 4). Formation of the y_3 ions was only observed for the Pro peptide **3a** (Ac-NMeAla-PLG-OCH_3).

In order to investigate further the influence of the location of the protonation site on the observed specific fragmentation, a series of analogs with a Lys was studied along with their non-methylated counterparts. The CID spectra are summarized in the Table 5. In the first set of compounds a Lys residue replaced Leu in the template XXX-P*L*G of non-polar peptides **2a** (H-NMeAla-VPLG-OH), **4a** (Ac-NMeA-VPLG-OH) or **3a** (Ac-NMeAla-PLG-OH). These peptides had a distance between the NMeAla and the Lys of either one or two residues. The CID spectra were acquired of protonated peptides with both amine groups free, of peptides where the *N*-terminus was selectively acetylated and also of reference peptides with Ala residues. When the *N*-terminus sremained free, the fragmentation in all cases was qualitatively similar to that of the non-polar analogs with dominance of the y_3 ions. A major difference was found in the fragmentation efficiency, where substantially lower yields of fragment ions in the CID spectra of **21a** (H-NMeA-VPKG-OCH_3) and **21b** (abundance of the surviving molecular ion 2529% TFIC and 1404% TFIC, res.) compared with the non-polar **1a** and **1b** (molecular ion abundance 676% TFIC and 247% TFIC, res.) reflect the presence of a basic Lys residue. Contrary to the different fragmentation efficiencies of non-polar peptides **2a** (H-NMeAla-VPLG-OH) and **4a** (Ac-NMeA-VPLG-OH), a similar fragmentation efficiency is observed for the peptides with Lys **21a** (H-NMeA-VPKG-OCH_3) (surviving molecular ion 2529% TFIC) and its *N*-terminally acetylated analog **22a** (Ac-NMeA-VPKG-OCH_3) (surviving molecular ion 2707% TFIC). The formation of the b_1 ion was observed only for acetylated peptides with NMeAla residues, **22a** (Ac-NMeA-VPKG-OCH_3) and **23a** (Ac-NMeA-PKG-OCH_3). In the CID spectrum of peptide **23a** with a distance between NMeAla and Lys of only one residue (Pro), the relative abundance of the b_1 ion (64% TFIC) is higher than in the spectrum of **22a** (distance of two residues, ValPro) (43% TFIC).

This series was supplemented by a second series of peptides where an NMePhe residue was placed next to the Lys residue. In this case, either of the two free amine groups (*N*-terminal or the ω- of Lys) was selectively acetylated or the *N*-terminus was acetylated and the Lys side-chain propionylated. Propionylation instead of acetylation was chosen in order to distinguish possible fragments related to the *N*-terminus or the side-chain acylation. It was found that the formation of b_1 ions depends on the acylation of the *N*-methylated residue regardless of the Lys side-chain. The CID of non-acetylated peptides **24** (H-NMeF-KLG-OCH_3) and **25** (H-NMeF-K(propionyl)LG-OCH_3) yielded no b_1 ion, while abundant b_1 ions were observed in the CID spectra of peptides with an acetylated *N*-terminus (**26**, Ac-NMeF-KLG-OCH_3, and **27**, Ac-NMeF-K(propionyl)LG-OCH_3). CID yielded spectra qualitatively very similar to those of analogous non-polar peptides. In general, the fragmentation efficiency of these peptides is substantially higher than in the previous series of peptides with a greater distance between the *N*-methylated residue and Lys. In the acetylated peptide **26** (Ac-NMeF-KLG-OCH_3), which may be compared with peptides **22a** (Ac-NMeA-VPKG-OH) and **23a** (Ac-NMeA-PKG-OH), the relative abundance of the b_1 ion reached 90% TFIC. In the CID of peptide **27** (Ac-NMeF-K(propionyl)LG-OCH_3) the relative abundance of b_1 ion decreased (66% TFIC) compared with **26** (Ac-NMeF-KLG-OCH_3), probably as a result of 'delocalization' of the ionizing proton and accessibility of other fragmentation sites, while the overall fragmentation efficiency increases (163% TFIC for **26** and 56% TFIC for **27**).

Formation of y_{n-1} ions

Besides abundant b_1 ions, in several cases the formation of y ions resulting from the cleavage of the same amide bond was observed [**3a** (Ac-NMeAla-PLG-OCH_3), **4a** (Ac-NMeA-VPLG-OH), **23a** (Ac-NMeA-PKG-OCH_3), **22a** (Ac-NMeA-VPKG-OCH_3), **26**, and **11** (For-NMeAla-PLG-OH)] (Table 6). This is unexpected, as there is no amide proton available on the *N*-terminal side of the fragmenting bond for the formation of y ions as formally required by the accepted mechanism in regular protonated peptides.[9] To identify the source of

Table 5. CID spectra of basic peptides[a]

			m/z (relative intensity, %)												
Peptide	No.	$[M+H]^+$	a_1	b_1	a_2	b_2	b_3	y_2	y_3	y_4	Pro	Lys	ProLys, $y_3(b_2)$	Val	Other
H-**NMeA**-VPKG-OCH_3	**21a**	499(2529)	58(4)		156(4)	185(9)			315(82)						
H-**A**VPKG-OCH_3	**21b**	485(1404)			143(4)	171(5)	286(2)	218(3)	315(65)		70(2)	129(5)	226(14)		
Ac-**NMeA**-VPKG-OCH_3	**22a**	541(2707)		**128(43)**		227(3.3)			315(32)	414(11.8)			226(11)		
Ac-**A**VPKG-OCH_3	**22b**	527(3021)				213(5)			315(46)		70(4)	129(4)	226(32)	72(8)	
Ac-**NMeA**-PKG-OCH_3	**23a**	442(580)		**128(64)**					315(36)	n.a.					
Ac-**A**PKG-OCH_3	**23b**	428(1349)				211(5)		218(2)	315(47)	n.a.	70(4)	129(5)	226(38)		
H-**NMeF**-KLG-OH	**24**	478(362)	134(39)			290(31)	403(5)	189(2)	317(10)	n.a.	n.a.	129(9)	n.a.		259(4)
H-**NMeF**-K(propionyl)LG-OH	**25**	534(311)	134(48)		318(2)	346(23)	459(5)		373(3)	n.a.	n.a.		n.a.		(19)
Ac-**NMeF**-KLG-OH	**26**	520(163)	176(6)	**204(90)**			445(1)		317(4)	n.a.	n.a.		n.a.		
Ac-**NMeF**-K(propionyl)LG-OH	**27**	576(56)		**204(66)**		388(9)	501(12)		373(14)	n.a.	n.a.		n.a.		

[a] ES+, skimmer 12 V, collision gas Ar at 20% transmission of the main ion beam, E_{lab} = 20 eV. In bold: residue of interest and b ions of interest.

Table 6. Formation of the y_{n-1} ions[a]

			m/z (relative intensity, %)											
Peptide	No.	$[M+H]^+$	a_1	b_1	a_2	b_2	a_3	b_3	b_4	y_2	y_3	y_4	PL/PK	Other
Ac-**NMeAla**-PLG-OCH_3	**3a**	427(58)		**128(50)**				338(3)	n.a.		300(47)	n.a.		
Ac-**NMeAla**-VPLG-OH	**4a**	512(89)		**128(12)**		227(1)			437(1)		286(45)	385(32)		
Ac-**NMeA**-PKG-OCH_3	**23a**	442(580)		**128(64)**					n.a.		315(36)	n.a.	n.a.	
Ac-**NMeA**-VPKG-OCH_3	**22a**	541(2707)		**128(43)**		227(3.3)					315(32)	414(11.8)	226(11)	
Ac-**NMeF**-KLG-OH	**26**	520(163)	176(6)	**204(90)**			**445(1)**		**317(4)**				**n.a.**	
For-NMeA-PLG-OH	**11**	399(169)		**114(22)**	183(1)	211(11)	296(4)	324(22)	n.a.		286(31)	n.a.		other(10)
Ac-d_3-**NMeA**-PLG-OH	**28**	416(169)		**131(42)**				341(20)	n.a.	301(38)		n.a.		
Ac-d_3-**A**-PLG-OH	**29**	402(179)				214(5)		327(13)	n.a.	286(82)		n.a.		

[a] In bold: residue of interest and b inons of interest.

Table 7. CID spectra of peptides with both NMeAla and Pro in the sequence[a]

Peptide	No.	$[M+H]^+$	m/z (relative intensity, %) b_2	b_3	b_4	y_3	y_4	Other
H-P-**NMeAla**-PLG-OH	**30**	468(435)	**183(82)**		365(1)	286(1)		114(16)*N*-formylPro
Ac-P-**NMeAla**-PLG-OH	**31a**	510(59)	**225(100)**					
Ac-P**A**PLG-OH	**31b**	496(108)	211(3)	308(3)	421(8)	286(87)		
Ac-P-**NMeAla**-ALG-OH	**32**	484(59)	**225(64)**	296(22)	409(14)			
Ac-V-**NMeAla**-ALG-OH	**33**	486(56)	**227(69)**	298(15)	411(10)		345(7)	
H-T-**NMeAla**-VPLG-OH	**6a**	571(1811)	**187(84)**			286(16)		

[a] ES+, skimmer 12 V, collision gas Ar at 20% transmission, $E_{lab} = 10$ eV. In bold: residue of interest and b ions of interest.

the second hydrogen transferred, two types of experiment were performed. The CID spectrum of a monodeuteronated peptide **3a** (Ac-NMeAla-PLG-OCH_3) generated by isobutane-d_{10} CI was acquired to investigate the distribution of this 'mobile' deuteron in the fragment ions and the CID of protonated Ac-d_3-peptide **28** (Ac-d_3-NMeAla-PLG-OH) was obtained to address the behavior of the acetyl-and α-protons (Table 6). Identical experiments were also performed with reference Ala peptides **4b** (AcAPLG-OCH_3) and **29** (Ac-d_3-APLG-OH). The CID spectra of the monodeuteronated peptides showed no deuterium incorporation in the b_1 ions and one deuterium in the y_3 ions. On the other hand, in the CID spectrum of **28** (Ac-d_3-NMeAla-PLG-OH) the m/z of the y_3 ion corresponds to incorporation of one deuteron, indicating participation of the acetyl group in the fragmentation. The CID spectrum of **29** (Ac-d_3-APLG-OH) showed the y_3 without incorporation of a deuteron.

Proline versus *N*-methylamino acids

It has been shown earlier that NMeAla affects the fragmentation of protonated peptides in a very different way to Pro.[28] Whereas Pro is known to direct fragmentation to its *N*-terminus, providing abundant y ions, NMeAla directs fragmentation to its *C*-terminus, yielding abundant b ions. How Pro in the same sequence as the *N*-methyl residue affects the fragmentation was another problem to address. A series of peptides was prepared with proline either in place of or along with an NMeAla residue in the sequence. Some of the experiments have been described previously in connection with b ion formation (peptides **7**–**10**). Several other peptides were also prepared in which a Pro is put into direct competition with an NMeAla [**30** (H-P-NMeA-PLG-OH), **31a** (Ac-P-NMeA-PLG-OH), **32** (Ac-PNMeA-ALG-OH), **6a** (H-T-NMeAla-VPLG-OH)] (Tables 1 and 5). A peptide **33** (Ac-V-NMeA-ALG-OH) served as an NMeAla reference and Ala analogs **4b** (Ac-AVPLG-OH) and **31b** (Ac-PAPLG-OH) were also studied. Results are given in Table 7. Generally, the NMeAla effect governs the appearance of the CID spectrum with the exception of the acetylated peptide **4a** (Ac-NMeA-VPLG-OH), where the proline effect results in abundant y_3 ions. When the acetyl in this peptide is replaced by Thr (**6a**), the NMeAla effect dominates (Table 7).

Energy-resolved mass spectrometry

ERMS was applied to several compounds in order to obtain further information about the phenomena associated with the *N*-methylated residue in protonated peptides. The resulting energy-resolved mass spectra

Table 8. Semiempirical AM1 and PM3 calculations on isomers of protonated model compounds Ac-NMeAla-NHMe (I–VIa) and Ac-Ala-NHMe (I–VIb), their respective protonated oxazolones, neutral molecules and a methylamine[a]

	Ac-NMeAla-NHMe ΔH_f°(AM1) (kJ mol^{-1})	Ac-NMeAla-NHMe ΔH_f°(PM3) (kJ mol^{-1})	Ac-Ala-NHMe ΔH_f°(AM1) (kJ mol^{-1})	Ac-Ala-NHMe ΔH_f°(PM3) (kJ mol^{-1})
I (AcO-*trans*)	317	—[b]	292	—
II (AcO-*cis*)	303	273	296	270
III (AlaO-*cis*)	295	248	268	244
IV (AlaO-*trans*)	298	—	266	—
V (AlaN)	357	—	322	—
VI (NHMe)	328	—	299	—
Protonated oxazolone	439	365	431	381
CH_3NH_2	−31	−22	−31	−22
Neutral molecule	−342	—	−368	—
TS	344	326	315	320

[a] Ala in the first column represents either an NMeAla or an Ala residue.
[b] Dashes indicate not calculated.

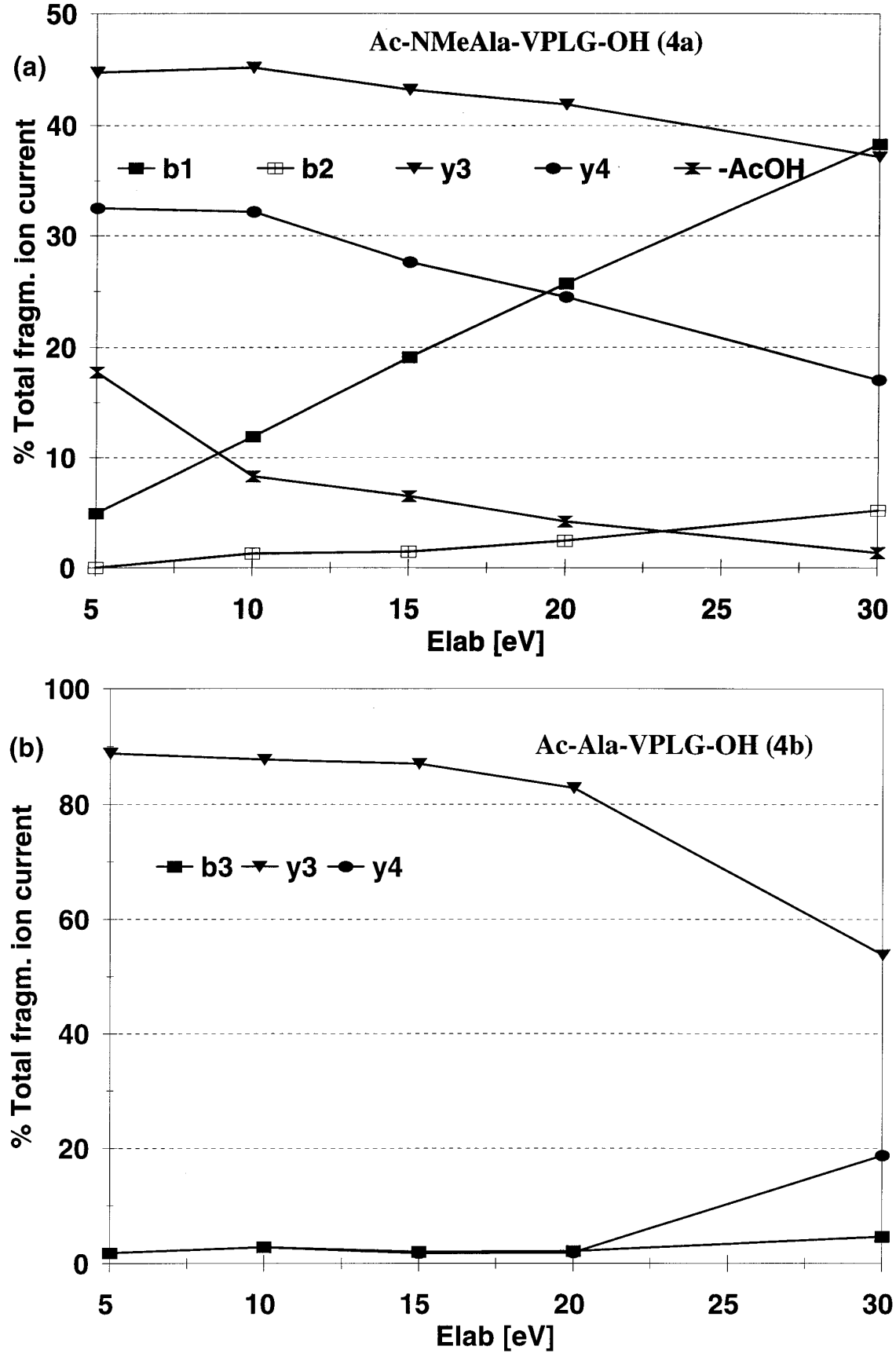
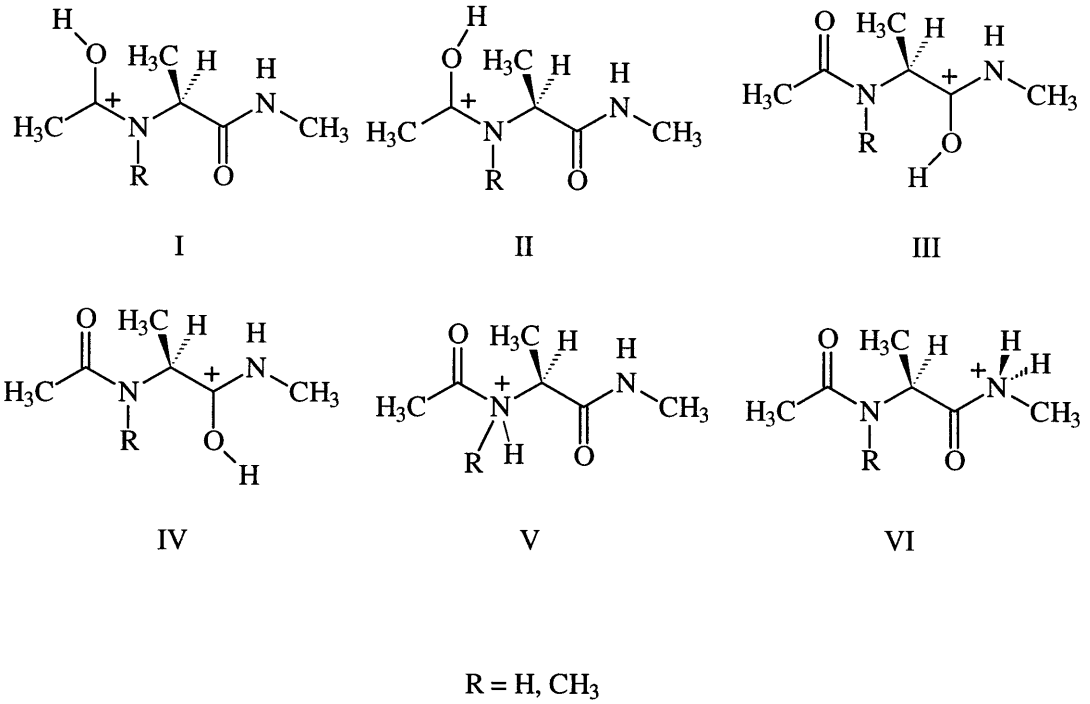


Figure 3. Breakdown graph of protonated peptides (A) **4a** (Ac-NMeA-VPLG-OH) and (B) **4b** (Ac-AVPLG-OH). 20%T Ar.



Scheme 4. Structures of conformers of model acetyl-*N*-methyl amides studied by AM1.

revealed that in general the formation of b_1 ions was not the lowest critical energy process and either a *C*-terminal b fragment or a complementary y ion appears at lower collision energies than the b_1 ion. The relative abundance of the b_1 ions rapidly increased with increasing collision energy. Thus, at the lowest collision energy applied, for a peptide **5** (Ac-NMeAla-ALG-OCH_3), it is the b_3 ion which dominates, and for **3a** (Ac-NMeAla-PLG-OCH_3) and its Lys analog **23a** (Ac-NMeA-PKG-OCH_3), it is the y_3 ion. For the peptide **4a** (Ac-NMeA-VPLG-OH), initially y_3 and y_4 ions dominate, while the b_1 ion attains similar relative abundance at E_{lab} = 30 eV [Fig. 3(A)]. In contrast, the ERMS of its Ala analog **4b** (Ac-AVPLG-OH) revealed a dominance of y_3 in the whole range of applied collision energies [Fig. 3(B)].

Calculations

AM1 semiempirical calculations were employed in order to supplement experimental results and find sup-

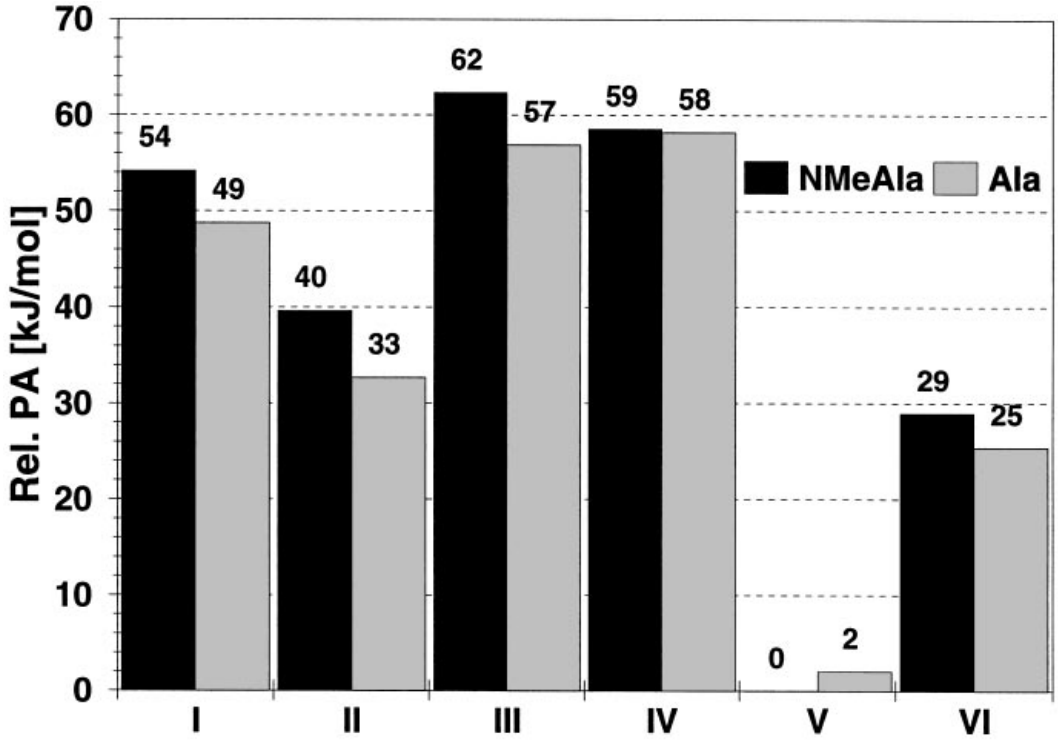


Figure 4. Calculated relative proton affinities of Ac-N(R)-Ala-$NHCH_3$ (R = H, CH_3) at various sites (structures **I–VI**). MOPAC–AM1.

porting evidence for the proposed mechanism of the observed effect of NMeAla residues on the CID fragmentation of the corresponding protonated peptides. An acetylalanine methylamide (Ac-Ala-NHMe) and acetyl-NMe-alanine methylamide (Ac-NMeAla-NHMe) were used as model compounds. Protonation at both carbonyl oxygens and both amide nitrogens was considered as depicted in Scheme 4. Both *cis* and *trans* orientations of the ionizing proton relative to the Ala nitrogen were investigated for acetyl CO protonation (**I** and **II**) and *cis* and *trans* orientation relative to the α-carbon were studied for the Ala CO protonation (**III** and **IV**). The relative *PA* results are summarized in Fig. 4. Further, the endothermicity of the reaction leading to the b ion was calculated for both model compounds from heats of formation of the lowest and highest energy reactant ions and of the expected products, a protonated oxazolone and methylamine. Results from AM1 and PM3 are summarized in Table 8.

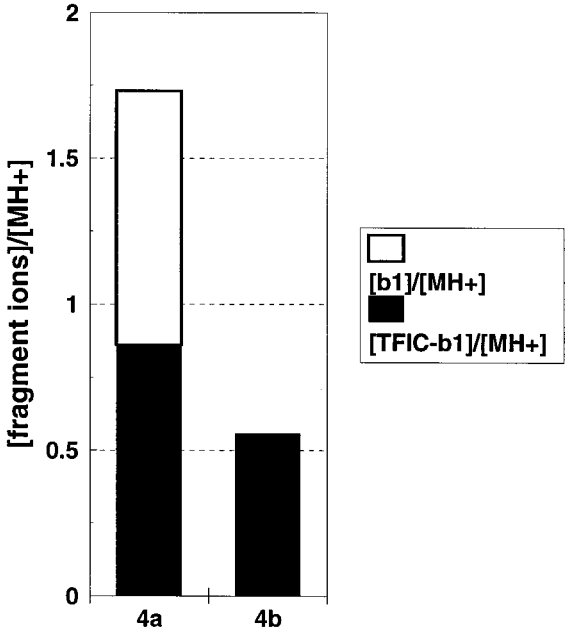


Figure 5. Graph of relative abundances of fragment ions in CID spectra of peptides **3a** (Ac-NMeAla-PLG- OCH_3) and **3b** (Ac-APLG-OH) at 10 eV E_{lab} collision energy showing relative proportion of fragment ion current of the b_1 ion to the ion current of the rest of fragment ions. (The light bar represents the ion current of b_1 ion and the black the ion current of the rest of fragment ions; the whole bar represents the extent of fragmentation)

DISCUSSION

Formation of b ions

The incorporation of an *N*-methylamino acid in the peptide has two effects on the fragmentation of a protonated molecular ion. A relatively minor effect is related to the basicity of the secondary amino group of the *N*-methylamino acid. When placed at the *N*-terminus the *N*-methylamino acid shows decreased fragmentation efficiency compared with the non-methylated analog (Fig. 1). This is expected as the *N*-methylation increases the proton affinity of the residue (e.g. $PA(\text{Gly}) = 885.5\ \text{kJ mol}^{-1}$, $PA(\text{Sar}) = 915.3\ \text{kJ mol}^{-1}$)[32] and, when located at the *N*-terminus, also of the whole peptide[33] in a way similar to a basic amino acid residue.[16,17] The fragment ions produced by CID of the complementary peptides with the free *N*-terminal NMeAla (e.g. **1a** (H-NMeAla-PLG-OH) and **1b** (H-APLG-OH), Fig. 1) do not differ remarkably and arise from fragmentations along the whole peptide chain. The major effect of *N*-methylation is related to the position of the *N*-methyl residue. In contrast to the free *N*-terminal NMe residue, an acylation of this *N*-methylamino acid residue dramatically changes the fragmentation of the corresponding protonated peptides. The predominant product of the fragmentation is an abundant b ion from the cleavage of the *C*-terminal amide bond of the *N*-methyl residue (Scheme 3). Compared with the non-*N*-methylated peptides, protonated peptides with an acetylated NMeAla residue provide abundant b_1 ions at *m/z* 128 (Table 1, Fig. 2). Unlike in the case of peptides **1a** (H-NMeAla-PLG-OH) and **1b** (H-APLG-OH), where the *N*-alkylation decreases the fragmentation efficiency, for the acetylated peptides the *N*-alkylation increases the fragmentation efficiency substantially [**3a** (Ac-NMeAla-PLG- OCH_3) *vs.* **3b** (Ac-Ala-PLG-OCH_3)]. This increase is completely due to the formation of b_1 ions [Fig. 5)].

The energy-resolved spectra reveal that b_1 ions do not dominate the CID spectra at the lowest collision energies. At these energies, other fragment ions are dominant depending on the structure of the peptide. For the peptide **5** (Ac-NMeAla-ALG-OCH_3), it is a b_3 ion, whereas for the Pro peptide **3a** (Ac-NMeAla-PLG-OCH_3) it is a y_3 ion [Fig. 6(A)]. The b_1 ions appear dominant at only slightly higher collision energies. The appearance of the CID spectra of peptides containing Pro besides an NMeAla residue is in general affected by this residue, as will be discussed below.

In the protonated molecular ion of a peptide with a free *N*-terminus, the *N*-terminal amine group is the primary site of protonation with several hydrogen bonds to other less basic sites in the molecule. In a protonated molecule of an acetylated peptide the ionizing proton is engaged via hydrogen bonds with several, mainly carbonyl, groups. This can generally affect the course of fragmentation by facilitating intramolecular hydrogen transfers to the amide bond moieties participating in the hydrogen bonds. In other words, the fragmentation pattern could possibly be affected by secondary structure of the peptide as different secondary structures also have different hydrogen bonds.[34] However, in the case of peptides with *N*-methyl residues the location of the proton is apparently not important; likewise the acetylated peptide **4a** (Ac-NMeA-VPLG-OH), the peptide where NMeAla is acylated by an amino acid Thr [**6a** (H-T-NMeA-VPLG-OH)] provides the b_2 ion as the dominant product of CID (Table 2). Similarly, in peptides where the NMeAla is in the middle of the sequence [**7** (H-VSF-NMeAla-ALG-OH), **8** (Ac-VSF-NMeAla-ALG-OH)] the b_4 ion dominates CID spectrum (Table 2). The characteristic

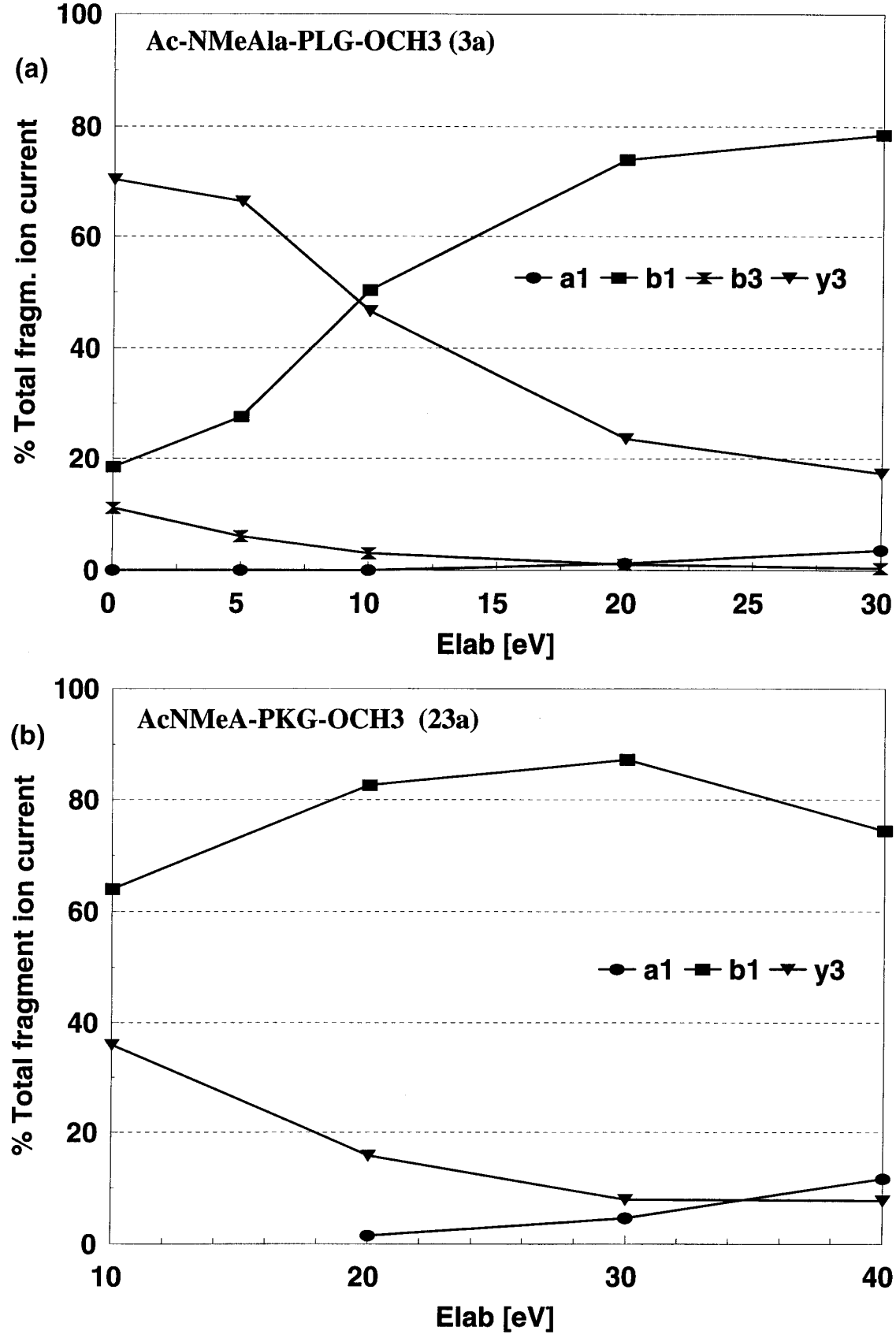
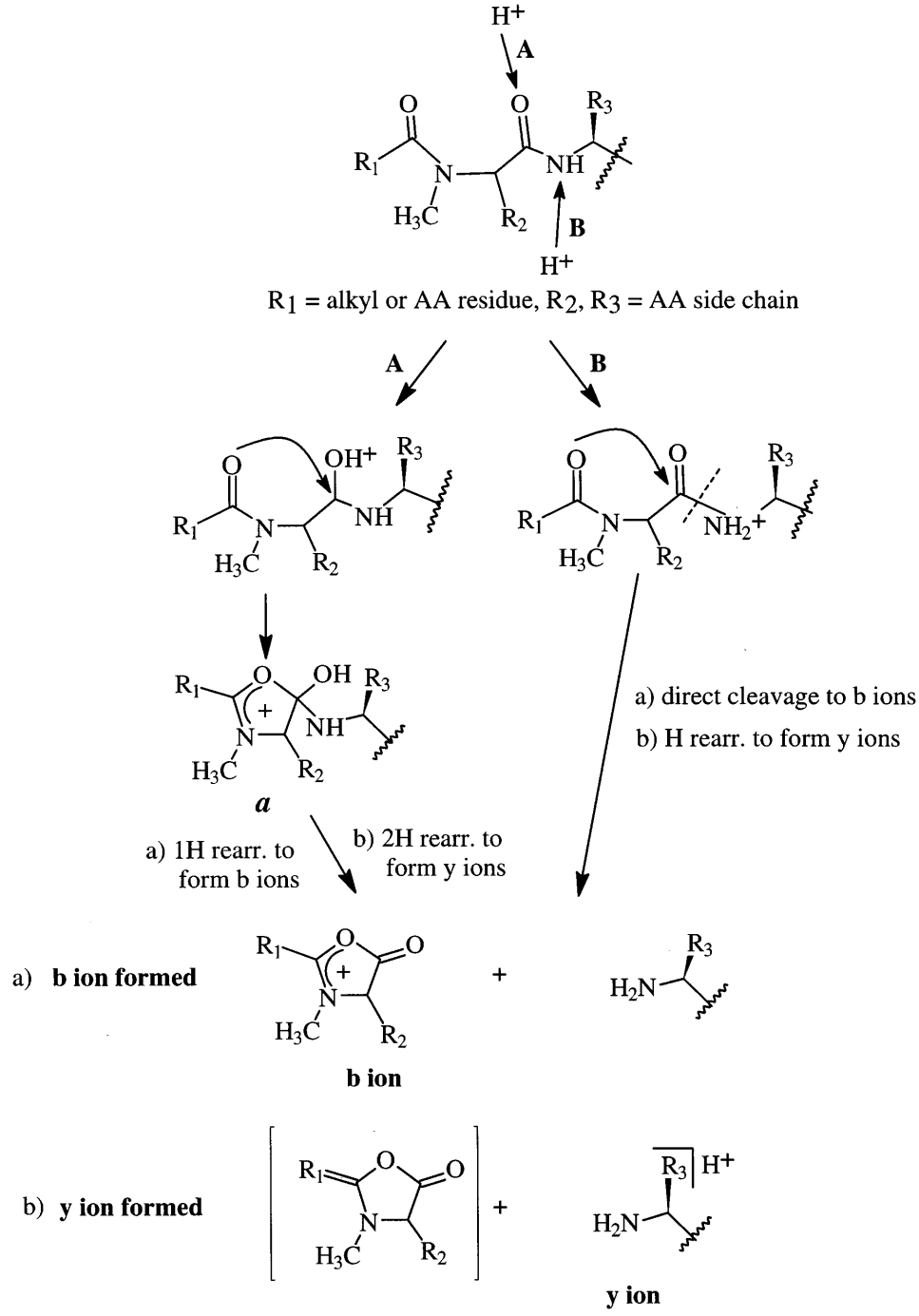


Figure 6. ERMS of (A) **3a** (Ac-NMeAla-PLG-OCH_3) and (B) **23a** (Ac-NMeA-PKG-OCH_3). 20%T Ar.

abundant formation of the b_1 ions is preserved even in peptides with a protonation site at the side-chain (Table 4). In the case of peptides **2a** (H-NMeAla-VPLG-OH) and **4a** (Ac-NMeA-VPLG-OH), the overall fragmentation efficiency increased 10-fold upon acetylation owing to the facile initial proton transfer in **4a** (Ac-NMeA-VPLG-OH) leading to b_1/y_4 ion formation. In the lysine peptides **21a** and **22a**, the acetylation of the *N*-



Scheme 5. Mechanism proposed for the formation of abundant b ions from protonated peptides with one NMe amino-acid residue.

terminus does not affect the fragmentation efficiency. The b_1 ion in the CID spectrum of **22a** (Ac-NMeA-VPKG-OCH_3) is instead formed at the expense of the y_3 ion. The increased relative abundance of the b_1 ion (43% TFIC) in **22a** (Ac-NMeA-VPKG-OCH_3) compared with the analogous Leu peptide **4a** (Ac-NMeA-VPLG-OH) (12% TFIC) (notably, a *C*-terminal basic residue usually promotes the formation of the *C*-terminal y fragment ions) is probably due to a favorable interaction of the protonated Lys side-chain with the cleaving amide bond. This is further enhanced in the peptide **23a** (Ac-NMeA-PKG-OCH_3), where the distance between the fragmentation site and the Lys is only one residue. In the protonated peptide **26** (Ac-NMeF-KLG-OCH_3), the interaction of the protonated side-chain with the equivalent amide moiety is favored even more as the relative abundance of the b_1 ion in the CID spectrum reaches 90% TFIC. In contrast, in the protonated peptide **27** (Ac-NMeF-K(propionyl)LG-OCH_3), propionylation of the side-chain results in an increased fragmentation efficiency owing to the removal of the basic site. The relative increase in the abundance of other fragment ions relative to the b_1 ion emphasizes the favorable interaction of the protonated Lys side-chain with the cleavage site in peptide **26** (Ac-NMeF-KLG-OCH_3). No change in fragmentation is observed for peptides with a free *N*-terminus, when the Lys ω-amine group is blocked [**24** (H-NMeF-KLG-OCH_3)

and **25** (H-NMeF-K(propionyl)LG-OCH_3)] (Table 5), owing to the impossibility of oxazolone formation.

These results indicate that the acylation of the *N*-methyl residue is a critical factor for the formation of the b ions. We propose that the observed abundant b ions in the CID spectra of mono-*N*-methyl peptides have structures of protonated *N*-methyloxazolone formed by a mechanism outlined in the Scheme 5. Recently, we have proposed such a structure for the b ions and of the fragmentation intermediate in order to explain the different fragmentations of NMeAla and Pro peptides.[25] The proposed mechanism is similar to the mechanism suggested by Hunt and co-workers[12] for regular peptides and there was also evidence presented supporting a protonated oxazolone as a structure of stable b ions from protonated peptides in the gas phase.[13,14] Our mechanism tentatively assumes that in the reacting ion the proton resides on the carbonyl oxygen of the *N*-methylamino acid residue. A nucleophilic attack of the '*N*-terminal carbonyl' is an initial step followed by a rearrangement of either one or two protons to yield either b or y ions, respectively (Scheme 5). The ability of the '*N*-terminal carbonyl' to undergo such an attack apparently affects the extent of b ion formation. Indeed, a variation of the attacking carbonyl nucleophilicity has a pronounced effect on the b_1 ion formation. A simple change from an acetyl, **3a** (Ac-NMeAla-PLG-OCH_3), to a formyl, **11** (For-NMeAla-PLG-OH), (Table 3) results in a decrease in the relative abundance of b_1 ions to 22% TFIC from 50% TFIC in **3a** (Ac-NMeAla-PLG-OCH_3). Also in the energy-resolved spectrum of **11** (Fig. 7), the b_1 ion domination is less apparent compared with the acetylated **3a** (Ac-NMeAla-PLG-OCH_3) [Fig. 6(A)]. A further decrease in the carbonyl nucleophilicity leads to complete disappearance of the b_1 ions in the CID spectrum of the protonated trifluoroacetyl peptide **12** (Tfa-NMeAla-GLG-OH) compared with 88% TFIC in the CID spectrum of an analogous acetylated peptide **13** (Ac-NMeA-GLG-OH) (Table 3). On the other hand, the higher nucleophilicity of the thioacetyl relative to the acetyl results in the abundant formation of a y_3 ion in CID of peptide **16a** (Ac^t-AALG-NHMe) [Fig. 8(A)] compared with the CID of its oxo analog **16b** (Ac-AALG-NHMe), where the spectrum is dominated by b_3 ions [Fig. 8(B)]. A similar result is obtained when the *N*-terminus is free amine, **17** (H-A^t-AALG-NHMe) In this case, the y_3 ion is formed by cleavage of the same bond as in the case of its NMe analog **3** (Ac-NMeAla-ALG-OCH_3). The proton of the *N*-terminal thioacetylalaninamide moiety, not present in **3**, provides for the formation of the y_3 instead of the b_1 ion.

Besides the nucleophilicity, flexibility of the peptide backbone is essential to allow the nucleophilic attack to take place. In the NMePyr analogs **14** (NMePyr-ALG-OH) and **15** (NMePyr-PLG-OH), the '*N*-terminal carbonyl' is fixed by a five-membered ring in a position 'away' from the amino acid's carbonyl. In this arrangement, any interaction between the carbonyl groups is effectively disabled, resulting in disappearance of b_1 ions in the CID spectra of protonated **14** (NMePyr-ALG-OH) and **15** (NMePyr-PLG-OH) (Table 3) compared with the acetylated peptides **5** (Ac-NMeAla-ALG-OCH_3) and **3a** (Ac-NMeAla-PLG-OCH_3).

In the proposed mechanism, the location of the proton on the NMe-amino acid's carbonyl in the reacting configuration cannot be assigned unambiguously. Alternatively, it has been suggested that in the reactive

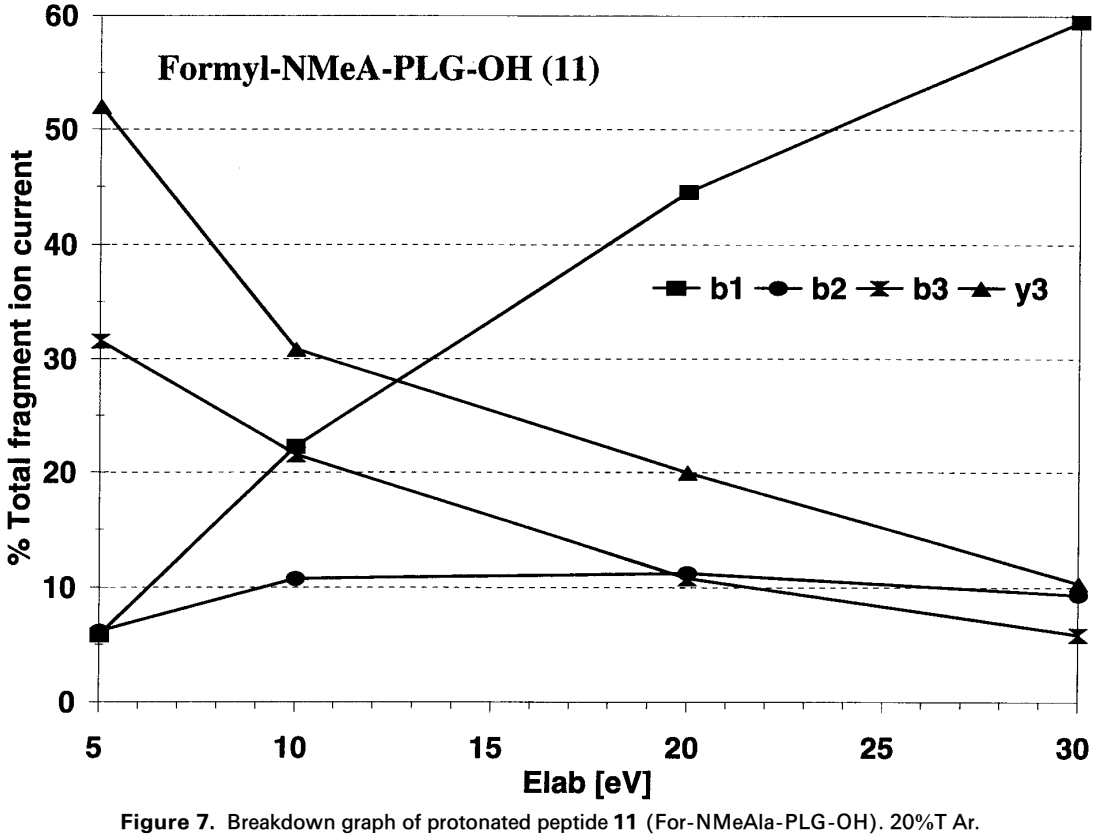


Figure 7. Breakdown graph of protonated peptide **11** (For-NMeAla-PLG-OH). 20%T Ar.

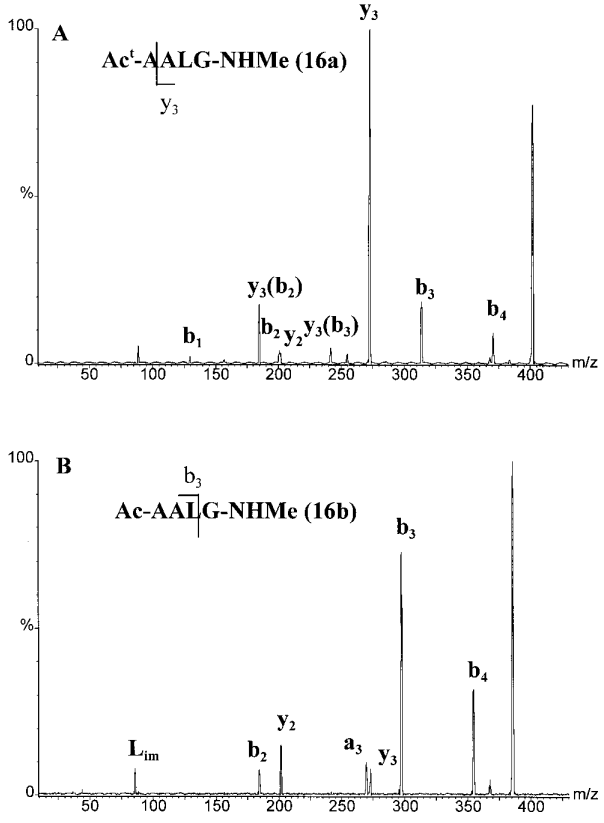


Figure 8. CID spectra of $[M+H]^+$ of peptides (A) **16a** (Ac^t-AALG-NHCH$_3$) and (B) **16b** (Ac-AALG-NHMe). 20%T Ar, 10 eV E_{lab} collision energy.

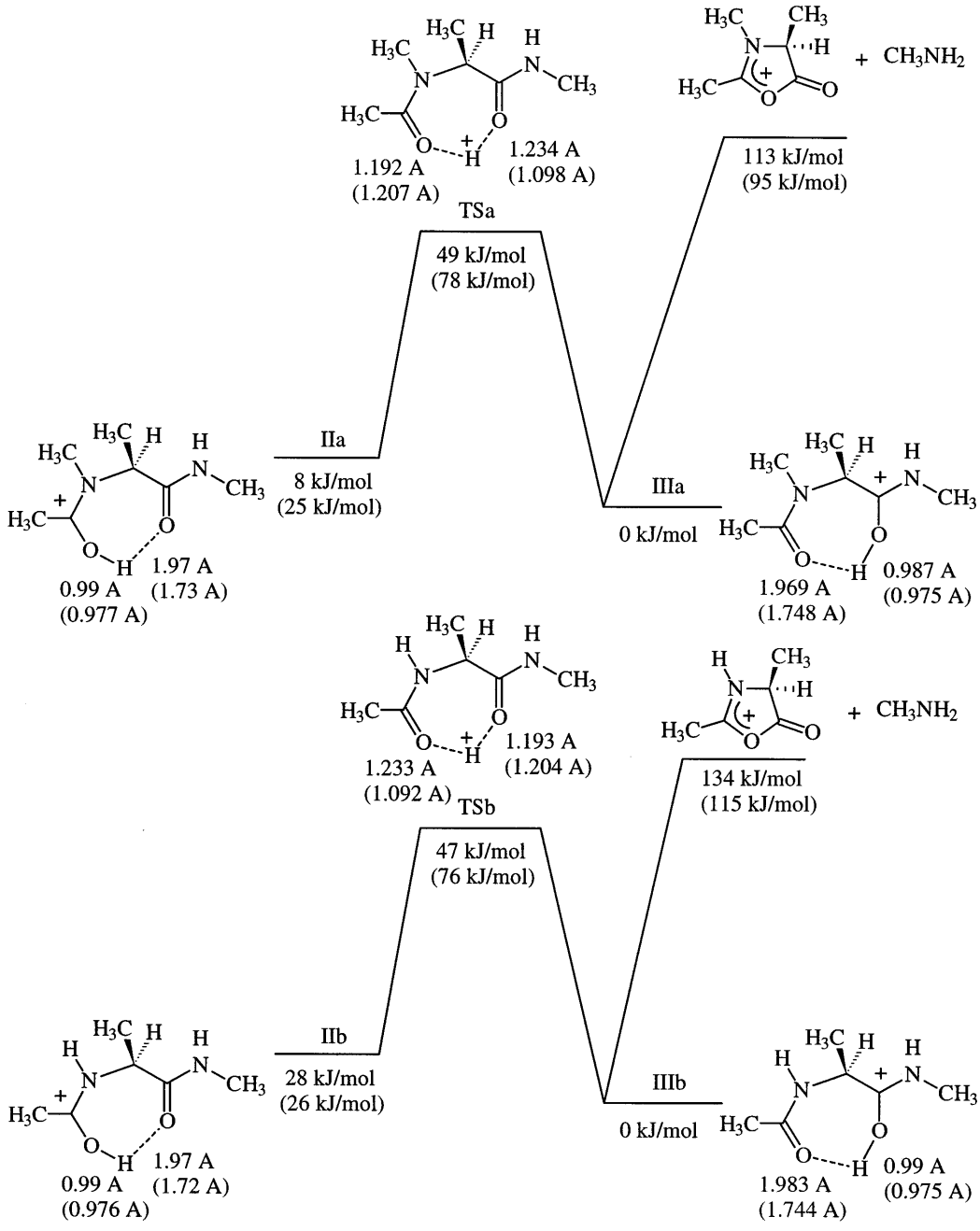
configuration for the formation of either b or y ions of a protonated peptide the amide nitrogen is protonated.[9,17] Fragmentation from this protonation isomer is favored by a decreased bond order of the amide bond. It is also conceivable that upon cleavage of this bond, a concerted nucleophilic attack takes place in a way similar to and with a similar result as in Scheme 5. The amide nitrogen is, however, intrinsically considerably less basic than the carbonyl oxygen.[11] In the proposed mechanism, the amide nitrogen in the oxazolone-like intermediate becomes a secondary amine >C(OH)—NH—, which is substantially more basic. This could stimulate an intramolecular 1,3-H^+ transfer from the neighboring hydroxyl group, as depicted in Scheme 5. Although the initial location of the proton at either of these sites is possible, it does not alter the subsequent steps in the proposed mechanism, i.e. the nucleophilic attack and the formation of an oxazolone.

Energy-resolved spectra further support the proposed mechanism. According to the mechanism, the formation of a b ion is accompanied by an intramolecular rearrangement of a single proton, whereas the formation of the complementary y ion proceeds via rearrangements of two protons. This is reflected in ERMS by an abundant formation of the y_3 ions from protonated **3a** (Ac-NMeAla-PLG-OCH$_3$) at low E_{lab}, while the b_1 ion becomes abundant at high E_{lab} [Fig. 6(A)]. Generally, a rearrangement reaction proceeds through a tight transition state with low critical energy and is accompanied by a slow increase in the rate constant with the internal energy of the precursor.[35] It can be reasoned that the formation of y ions proceeding through two hydrogen rearrangements will have a lower critical energy and therefore the y ions will appear at a lower internal energy than b_1 ions formed through a single proton rearrangement. This is shown in Fig. 6(A) for **3a** (Ac-NMeAla-PLG-OCH$_3$). The relative abundance of b_1 ions increases sharply with increasing collision energy as in the case of peptide **4a** (Ac-NMeA-VPLG-OH) mentioned earlier [Fig. 3(A)], while the abundance of y_3 ions decreases at the same rate. A similar situation is encountered in the breakdown graph of Lys peptide **23a** (Ac-NMeA-PKG-OCH$_3$) [an analog of **3a** (Ac-NMeAla-PLG-OCH$_3$)] [Fig. 6(B)].

The proposed mechanism, however, does not provide a straightforward explanation for the huge difference in the fragmentation between Ala and NMeAla peptides. One possible explanation results from an increased basicity of the *N*-methylated amide moiety, which can be expected to be substantially higher than that of the regular secondary amide (CH_3CONH_2, $PA = 864$ kJ mol^{-1}; $CH_3CONHCH_3$, $PA = 888.9$ kJ mol^{-1}; $CH_3CON(CH_3)_2$, $PA = 905$ kJ mol^{-1}).[32] AM1 calculations on model compounds Ac-Ala-NHCH$_3$ and Ac-NMeAla-NHCH$_3$ indicate that the difference in basicity between NMeAla and Ala is lower than expected from analogy with the acetamides, negligible given the precision of the AM1 calculations (Fig. 4). Interestingly, the AM1 calculations find the amino acid's carbonyl more basic than that of the *N*-alkylated amide bond moiety. The calculations also suggest that an intramolecular hydrogen bond stabilizes these species. The intramolecular proton transfer between the two carbonyls is found feasible with a barrier of ~50 kJ mol^{-1} (Scheme 6). Application of a PM3 hamiltonian qualitatively agrees with the AM1 results, providing a higher barrier of ~75 kJ mol^{-1}, probably owing to an overestimation of hydrogen bonding in AM1 calculations. The difference in the basicity due to the presence of the *N*-methyl is apparently not the cause for the difference in the fragmentation. Both AM1 and PM3 calculations yield endothermicities of the fragmentation reactions which favor the NMeAla model compound by 20 kJ mol^{-1} (Scheme 6). Assuming a close similarity of both fragmentation reactions and likely similar kinetics, this suggests that the stability of protonated oxazolone is the driving force of the observed enhanced formation of the b ions for NMe peptides.

Formation of y_{n-1} ions

Observations of y_{n-1} ions for peptides with a general sequence Ac-NMeAla-Pro or Ac-NMeAla-Val-Pro are unexpected as there is no transferable proton (i.e. amide) at the Ac-NMeAla moiety for the y_{n-1} ion formation. Both mono-deuteronation and deuterium labeling at the acetyl group of the peptides **3a** (Ac-NMeAla-PLG-OCH$_3$) and **3b** point to the acetyl group as the source of the second proton for the y_{n-1} ion formation in the NMeAla peptide **3a** (Ac-NMeAla-PLG-OCH$_3$). In contrast, in the Ala peptide **3b** the proton originates from the Ac-Ala amide bond as expected.[9] The high *PA* of the Pro residue can be considered as the driving force for the y_{n-1} ion formation. However, similar y_{n-1} ions are also observed in the CID spectra of the Ac-



Scheme 6. Intramolecular H^+ transfer model compounds by AM1 and PM3 (values in parentheses).

NMeAla-Val-Pro-XXX peptides. As the *PA* of the Val residue is comparable to the *PA* of Ser residue [in the peptide **20** (Ac-NMePhe-SLG-OH)], and substantially lower than *PA* of Pro, it appears that the *PA* of the whole y_{n-1} 'peptide' rather than the *PA* of the *N*-terminal residue of a y_{n-1} ion itself is driving the formation of these ions. A detailed account of this will be given elsewhere.

Comparison and competition of NMeAla with Pro

While both NMeAla and Pro are *N*-alkylamino acids, they exhibit very distinct effects on the fragmentation of protonated peptides.[25] Fragmentation of peptides **7** (H-VSF-NMeAla-ALG-OH) and **9** (H-VSFPALG-OH) with NMeAla and Pro, respectively, at the same position, confirm these distinct effects. Whereas in the protonated **7** (H-VSF-NMeAla-ALG-OH) the *C*-terminal bond of this residue is activated providing primarily b_4 ions in the CID spectrum, in the protonated **9** (H-VSFPALG-OH) it is activation of the *N*-terminal amide bond which provides abundant y_3 ions. The altered protonation site in the acetylated analogs [**8** (Ac-VSF-NMeAla-ALG-OH) and **10** (Ac-VSFPALG-OH)] does not affect this pattern.

When the Pro and the NMeAla are placed in the same peptide, the effect of NMeAla generally prevails over the Pro effect. In this case, the protonation site location, e.g. accessibility of the cleavage site for the proton, affects the fragmentation to some extent. The peptide **30** (H-P-NMeA-PLG-OH), where the sequence is *N*-terminally capped by a Pro residue, provides mainly b_2 ions upon CID (82% TFIC), whereas in the

peptides **3a** (Ac-NMeAla-PLG- OCH_3) and **23a** (Ac-NMeA-PKG-OCH_3), capped by an acetyl (proton at the Lys side-chain), CID provides b_1 ions at only 50% and 64% TFIC, respectively. Further, in the peptide **30** (H-P-NMeA-PLG-OH) and its acetylated analog **31a** (Ac-P-NMeA-PLG-OH), the same amide bond is activated by both NMeAla and Pro, respectively (Table 5). Regardless of the *N*-terminus, both peptides yielded dominant b_2 ions upon CID. In contrast an Ala analog of **31a** (Ac-P-NMeA-PLG-OH) [**31b** (Ac-PAPLG-OH)] provided primarily y_3 ions. Similarly, replacing the Pro^3 residue in the **31a** (Ac-P-NMeA-PLG-OH) by Ala [**32** (Ac-P-NMeA-ALG-OH)] or both Pro^1 and Pro^3 by Val and Ala [**33** (Ac-V-NMeA-ALG-OH)] results in CID spectra dominated by b_2 ions along with b_3 and b_4 ions (Table 5). From comparison of peptides with and without a Pro^3 residue, **31a** (Ac-P-NMeA-PLG-OH) (Pro^1, Pro^3), **32** (Ac-P-NMeA-ALG-OH) (Pro^1, Ala^3) and **33** (Ac-V-NMeA-ALG-OH) (Val^1, Ala^3), it is apparent that Pro, besides its 'proline effect', also affects fragmentation along the whole peptide chain. Proline is known to stabilize a β-turn secondary structure. Thereby, it can enhance/disable transfer of the 'mobile' proton to certain parts of the molecular ion, enhancing fragmentation at certain amide bonds while suppressing cleavage at others. This is reflected by the appearance of b-type ions in **32** (Ac-P-NMeA-ALG-OH) and **33** (Ac-V-NMeA-ALG-OH), which are not present in **31a** (Ac-P-NMeA-PLG-OH) (Pro^1, Pro^3).

Analogy with the solution acid-catalyzed hydrolysis

Earlier, we reported that peptides with an *N*-methylamino acid residue are prone to acid-catalyzed hydrolysis.[23] In this work, we showed that peptides with an *N*-terminal Ac-NMeAA moiety are extremely readily hydrolyzed to the Ac-NMeAA-OH and a truncated peptide, whereas peptides with an internal NMeAA residue undergo the same process at a slower rate. Based on NMR and MS data for products of the hydrolysis performed in a deuterated environment, a reaction mechanism was proposed with a protonated oxazolone as a key intermediate ***a′*** (Scheme 2). In the gas phase, CID fragmentation of the protonated peptides of the same structure discussed in the preceding sections results in ions, which are analogous to the intermediates and/or products of the solution hydrolysis (Scheme 5). Both mechanisms appear to be very similar with the reactions initiated by protonation [TFA (l), H^+ (g)] at the carbonyl of the NMe-AA followed by nucleophilic attack of the '*N*-terminal carbonyl' that produces the oxazolone-like intermediates ***a′*** (in solution, Scheme 2) and ***a*** (in the gas phase, Scheme 5). These intermediates further eliminate the *N*-terminal 'truncated' peptide to yield protonated oxazolone **b′** (solution) and **b** ions (gas phase). In solution, the reaction is concluded by keto–enol tautomerism (a stage when the α-hydrogen is exchanged for deuterium) and hydrolysis.

CONCLUSIONS

The results presented show the strong effect of *N*-methylation of an amide bond moiety on the fragmentation of protonated peptides. This effect rests in activation of the adjacent amide bond towards the *C*-terminus. An abundant b ion results from the cleavage of this bond. This effect is analogous to the solution-phase acid-catalyzed hydrolysis of these peptides.

The proposed fragmentation mechanism is triggered when the *C*-terminal amide bond of the *N*-methylated residue is protonated. The reaction further proceeds via a nucleophilic attack of the carbonyl oxygen of the *N*-methylated amide moiety on the carbon of the carbonyl of the protonated amide bond, resulting in the formation of a b-type ion with the structure of a protonated *N*-methyloxazolone. The evidence is provided by variation of the nucleophilicity of the attacking oxygen and also by conformationally restricting the attacking carbonyl moiety in the form of a *N*-methylpyroglutamic residue. The order of abundance of the b_1 ions is found to be acetyl > formyl $\gg$ trifluoroacetyl, whereas no such ion is observed for *N*-methylpyroglutamyl in the place of the acetyl-*N*-methylalanyl. Furthermore, an analogous non-methylated peptide with a thioacetyl group provides highly abundant y ions from fragmentation of the same amide bond owing to the enhanced nucleophilicity of the sulfur. A certain ambiguity exists as to where the ionizing proton is located in the reacting conformation. Two protonation sites are conceivable—the oxygen of the cleaved amide bond moiety or the nitrogen of the same moiety. This mechanism is applicable to both non-methylated and monomethylated peptides and does not explain the dramatic difference in the fragmentation of these peptides. Semiempirical calculations suggest that the specific fragmentation at the *C*-terminal side of the *N*-methyl residue is driven by stability of the b ion formed with a structure of protonated oxazolone.

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