RESEARCH ARTICLE

Differentiation of Positional Isomers of Hybrid Peptides Containing Repeats of β -Nucleoside Derived Amino Acid (β -Nda-) and L-Amino Acids by Positive and Negative Ion Electrospray Ionization Tandem Mass Spectrometry (ESI-MSⁿ)

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Abstract

A new class of positional isomeric pairs of -Boc protected oligopeptides comprised of alternating nucleoside derived β -amino acid (β -Nda-) and L-amino acid residues (alanine, valine, and phenylalanine) have been differentiated by both positive and negative ion electrospray ionization ion-trap tandem mass spectrometry (ESI-MSⁿ). The protonated dipeptide positional isomers with β -Nda- at the N-terminus lose CH₃OH, NH₃, and C₂H₄O₂, whereas these processes are absent for the peptides with L-amino acids at the N-terminus. Instead, the presence of L-amino acids at the N-terminus results in characteristic retro-Mannich reaction involving elimination of imine. A good correlation has been observed between the conformational structure of the peptides and the abundance of y_n^+ and b_n^+ ions in MSⁿ spectra. In the case of tetrapeptide isomers that are reported to form helical structures in solution phase, no y_n^+ and b_n^+ ions are observed when the corresponding amide -NH- participates in the helical structures. In contrast, significant y_n^+ and b_n^+ ions are formed when the amide -NH- is not involved in the H-bonding. In the case of tetra- and hexapeptides, it is observed that abundant b_n^+ ions are formed, presumably with stable oxazolone structures when the C-terminus of the b_n^+ ions possessed L-amino acid and the β -Nda- at the C-terminus appears to prevent the cyclization process leading to the absence of corresponding b_n^+ ions.

Key words: Electrospray ionization, Tandem mass spectrometry, Hybrid peptides, Non-natural amino acid, Positional isomers

Introduction

Over the past decade there has been growing interest in peptides derived from non-natural amino acids because of their importance in pharmaceutical and foldamer chemistry

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[1–3]. Oligomers of non-natural peptidic residues [4–6], particularly with β -amino acids [7, 8] and their hybrids with natural L-amino acids have emerged as versatile structural templates (foldamers) as these exhibit predictable and well-defined secondary structures such as helices, turns, and strands, and offer a variety of possibilities for orienting functional side-chains [9–13]. The oligomers of these amino acids both in *cis*- and *trans*- conformation have exhibited diverse helical patterns in solution [12–14]. The fundamental structural element in proteins is the β -strand, which is known to be conformationally suitable for specific recognition by bimolecular receptors such

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as proteolytic enzymes, major histocompatibility complex (MHC) proteins and transferases [12–17].

There have been several reports on mass spectrometry (MS) of natural amino acid peptides [18–20]. The tandem mass spectrometry (MS/MS) of protonated [21–27], deprotonated peptides [28–33] in electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) [34–37] have been used for structure elucidation of peptides. There are very few reports in the literature on the mass spectral study of peptides derived from non-natural amino acids [38–48]. We have reported ESI-MS/MS of a series of Boc-protected carbopeptides [39–48], and differentiated both positional and diastereomeric isomers [39–46, 48]. Recently, we have reported a study on the effect of N-terminal β - and γ -carbo amino acids on the fragmentation of γ -amino butyric acid (γ -Abu) containing hybrid peptides [46]. We have studied the differentiation of four pairs of diastereomers of norbornene amino acid peptides using electrospray ionization tandem mass spectrometry [48]. We have also shown that MS/MS of some of the α,γ - γ,α -peptides can be used to identify the -NH- hydrogens that participate in the hydrogen bonding leading to helical structure in solution phase [44].

In continuation of our studies, here we report an ESI tandem mass spectrometric study on differentiation of a new series of positional isomers of hybrid peptides [49] containing repeats of β -Nda- and L-amino acids (alanine, valine, and phenylalanine) (Scheme 1).

Experimental

Mass Spectrometry

ESI mass spectra of these positional isomeric pairs of non-natural amino acid hybrid peptides (Scheme 1) were recorded using a liquid chromatography quadrupole ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The data acquisition was under the control of Xcalibur software (Thermo Finnigan). The typical source conditions were: spray voltage, 5 kV; capillary voltage, 15–20 V; heated capillary temperature, 200 °C; tube lens off-set voltage, 20 V; sheath gas (N_2) pressure, 20 psi; and helium was used as damping gas. For the ion-trap mass analyzer, the automatic gain control (AGC) settings were 2×10^7 counts for a full-scan mass spectrum and 2×10^7 counts for a full product ion mass spectrum with a maximum ion injection time of 200 ms. In the full-scan MS^2 and MS^3 modes, the precursor ion of interest was first isolated by applying an appropriate waveform across the end-cap electrodes of the ion-trap to resonantly eject all trapped ions, except those ions of the m/z ratio of interest. The isolated ions were then subjected to a supplementary alternating current (ac) signal to resonantly excite them and so cause collision induced dissociation (CID). The collision energies used were 20–40 eV. The excitation time used was 30 ms, and the isolation width used at 1–3 Da. All the spectra were recorded under identical experimental conditions, and are an average of 20–25 scans.

The samples were infused into the ESI source at a flow rate of 5 μ L/min via syringe pump. We have used the nomenclature of Cooks for MS^n spectra given in the figures [53].

Materials

Solvents and chemicals used in the present study were purchased from Merck (Mumbai, India), and used without further purification. Stock (1 mM) solutions of compounds were diluted with HPLC-grade CH_3OH to achieve a final concentration of 10 μ M each.

Synthesis of the Peptides

All the peptides were prepared from corresponding Boc-protected monomers of L-alanine, L-valine, L-phenylalanine, and nucleoside derived β -amino acid (β -Nda-) (Scheme 1). The synthesis of oligomers involved coupling of monomers in the requisite sequence using standard coupling reagents 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), 1-hydroxy benzotriazole hydrate (HOBt), and di-isopropylethylamine (DIPEA) in CH_2Cl_2 . The compounds reported were purified by column chromatography over silica gel. All the dipeptides were eluted from the column with the mobile phase ratio of 2:98 ($CH_3OH:CHCl_3$) except for the dipeptides 5 and 6, which were eluted with the mobile phase ratio of 3:7 (hexane:ethyl acetate). The tetra- and hexapeptides were eluted from the column with the mobile phase ratio of 4:96 ($CH_3OH:CHCl_3$), 6:94 ($CH_3OH:CHCl_3$), respectively.

Results and Discussion

The positional isomeric pairs of the Boc-protected di-, tetra-, and hexapeptides studied in this work are shown in Scheme 1. The positive ion ESI mass spectra of all these peptides (1–20) show abundant $[M + H]^+$, $[M + Na]^+$ ions, and low abundance $[M + H - C_4H_8]^+$, $[M + H - Boc + H]^+$ ions. As reported earlier [39–48, 54], the formation of $[M + H - Boc + H]^+$ ions can be explained by a McLafferty-type rearrangement involving a γ -H migration from the *tert*-butyl group to the carbonyl oxygen in the Boc-N- moiety followed by the loss of 2-methyl-prop-1-ene (C_4H_8 , 56 Da) and subsequent loss of CO_2 from the $[M + H]^+$ ions (Scheme 2). Fragmentation of these peptides can be explained by using the nomenclature, which was originally proposed for α -peptides by Roepstorff and Fohlman, and later modified by Biemann [18–20]. The negative ion ESI mass spectra of these peptide acids (11–20) show abundant $[M - H]^-$ ion and low abundance $[M - H - (CH_3)_3COH]^-$ ion [41, 43–45].

CID of Dipeptide Positional Isomers β -Nda-(L)-Ala-/(L)-Ala- β -Nda-

The ESI MS/MS spectra of $[M + H]^+$ ions (m/z 441) of positional isomeric peptides 1 (Boc-NH- β -Nda-(L)-Ala-OCH₃, Figure 1a) and 2 (Boc-NH-(L)-Ala- β -Nda-OCH₃, Figure 1b)

Scheme 1. Structures of studied hybrid peptides 1–20

both yield $[M + H - C_4H_8]^+$ (m/z 385) ions as the base peak, while the protonated isomer 2 shows an additional peak at m/z 341 corresponding to the $[M + H - Boc + H]^+$ ion. The direct formation of $[M + H - Boc + H]^+$ ion in 2 can also be explained

by another mechanism involving 1,5-H migration from the *tert*-butyl group to the -NH- leading to the concomitant loss of CO_2 and C_4H_8 (Scheme 3). This process may not be possible for 1 due to the N-terminal β -Nda.

Scheme 2. Proposed McLafferty type rearrangement

Figure 1. ESI MS² spectra of $[M+H]^+$ ions (m/z 441) of dipeptides **(a)** 1 and **(b)** 2 at 28 eV, ESI MS⁴ spectra of $[M+H-Boc+H]^+$ ions (m/z 341) of dipeptides **(c)** 1 and **(d)** 2 at 31 eV and ESI MS⁵ spectrum of $[M+H-Boc+H-thymine]^+$ ion (m/z 215) of dipeptide **(e)** 2 at 27 eV

Scheme 3. Proposed mechanism for the concomitant loss of C_4H_8 and CO_2

The formation of $[M + H-Boc + H]^+$ ion can also be explained by a stepwise mechanism involving the loss of CO_2 from $[M + H - C_4H_8]^+$ (Scheme 2) as evidenced by the MS^3 spectrum. To differentiate the positional isomers, we have examined the MS^4 spectra of $[M + H-Boc + H]^+$ ions of 1 and 2, which were formed via sequential losses of C_4H_8 and CO_2 from the $[M + H]^+$ precursor ions. The spectra are found to be remarkably different from one another (Figure 1c and d). The $[M + H-Boc + H]^+$ ion (m/z 341) of the dipeptide isomer 1 undergoes extensive fragmentation producing m/z 324 ($[M + H-Boc + H - NH_3]^+$), m/z 309 ($[M + H-Boc + H - CH_3OH]^+$), m/z 215 ($[M + H-Boc + H-thymine]^+$), m/z 198 ($[M + H-Boc + H-thymine - NH_3]^+$) (base peak), m/z 187 ($[M + H-Boc + H-thymine - CO]^+$), m/z 183 ($[M + H-Boc + H-thymine - CH_3OH]^+$), m/z 166 ($[M + H-Boc + H-thymine - NH_3 - CH_3OH]^+$), m/z 138 ($[M + H-Boc + H-thymine - NH_3 - C_2H_4O_2]^+$), and m/z 130 ($[M + H-Boc + H-thymine - NH_3-furan]^+$) ions (Figure 1c, Table 1). These fragmentation pathways have been confirmed by MS^n experiments. Loss of CH_3OH from $[M + H-Boc + H]^+$, $[M + H-Boc + H-thymine]^+$, and $[M + H-Boc + H-thymine - NH_3]^+$ ions can be explained by a plausible mechanism involving an intramolecular nucleophilic attack by the protonated C-terminus amide carbonyl on the carbonyl carbon of the C-terminus ester group, forming a five membered cyclic ring with the extrusion of CH_3OH [27]. The $[M + H-Boc + H-thymine - NH_3 - C_2H_4O_2]^+$ ion (m/z 138) can be formed from m/z 198 by the loss of $C_2H_4O_2$ (methyl formate) involving the migration of a hydrogen from β -methyl group to the carbonyl of carbomethoxy group. This fragmentation is absent when β -Nda- is present at the C-terminus.

Interestingly, the $[M + H-Boc + H]^+$ ion (m/z 341) of the other isomer 2 (Figure 1d) containing L-Ala at the N-terminus shows a simple spectrum with only one ion at m/z 215 as the base peak, due to the loss of thymine. This may be ascribed to greater stability of the peptide with N-terminal L-Ala. The MS^5 of m/z 215 ion of 2 (Figure 1e) shows an abundant ion at m/z 172 formed by a retro-Mannich reaction [46], involving the elimination of imine. This process is absent when β -Nda- is present at the N-terminus. The spectrum also shows c_1^+ (m/z 89) and z_1^+ (m/z 127) ions formed by the cleavage of N- C_α bond [55, 56] (Figure 1e). Thus, the dipeptide 1 can be clearly distinguished from 2 by the characteristic fragmentation observed in MS^n experiments.

β -Nda-(L)-Val-/-(L)-Val- β -Nda-

Similarly to 1 and 2, both the protonated peptides, 3 (Boc-NH- β -Nda-(L)-Val-OCH₃) and 4 (Boc-NH-(L)-Val- β -Nda-OCH₃) yield $[M + H - C_4H_8]^+$ ions (m/z 413) as the base peak and the latter shows an additional peak at m/z 369 corresponding to $[M + H-Boc + H]^+$ ion. The MS^3 of $[M + H - C_4H_8]^+$ ions of these peptides give predominantly $[M + H-Boc + H]^+$ ions (m/z 369). To differentiate the isomers, we have examined the MS^4 spectra of the $[M + H-Boc + H]^+$ ions (m/z 369) of 3 and 4, which were formed via sequential losses of C_4H_8 and CO_2 from the $[M + H]^+$ precursor ions (Supplementary Material Figure S1a and b). The former fragments extensively to produce abundant product ions at m/z 243 ($[M + H-Boc + H-thymine]^+$), m/z 226 ($[M + H-Boc + H-thymine - NH_3]^+$) (base peak), m/z 166 ($[M + H-Boc + H-thymine - NH_3 - C_2H_4O_2]^+$), m/z 158 ($[M + H-Boc + H-thymine - NH_3-furan]^+$), and low abundance ions at m/z 352 ($[M + H-Boc + H - NH_3]^+$), m/z 337 ($[M + H-Boc + H - CH_3OH]^+$), m/z 215 ($[M + H-Boc + H - thymine - CO]^+$), m/z 211 ($[M + H-Boc + H-thymine - CH_3OH]^+$), and m/z 194 ($[M + H-Boc + H-thymine - NH_3 - CH_3OH]^+$) (Supplementary Material Figure S1a). These fragmentation pathways have been confirmed by MS^n experiments.

In contrast, the MS^4 spectrum of the $[M + H-Boc + H]^+$ ion (m/z 369) of 4 (Supplementary Material Figure S1b) is dominated by an abundant ion at m/z 243 (base peak) corresponding to the loss of thymine. This is similar to the fragmentation behavior of the analogous fragment ion of dipeptide 2. The MS^5 of $[M + H-Boc + H - thymine]^+$ (m/z 243) ion of 4 (Supplementary Material Figure S1c) shows a different fragmentation pattern. Similarly to 2, the presence of L-valine at the N-terminus results in a retro-Mannich characteristic ion at m/z 172 formed by the elimination of imine (71 Da). The spectrum also shows the product ions at m/z 117 (c_1^+), m/z 127 (z_1^+), and m/z 72 (valine immonium ion) (Supplementary Material Figure S1c). Thus, the MS^n ($n=4,5$) of 3 and 4 are useful in establishing whether β -Nda or L-Val are located at the N-terminus.

β -Nda-(L)-Phe-/-(L)-Phe- β -Nda-

Similarly to the previous peptide isomers, the ESI MS/MS spectra of $[M + H]^+$ ions (m/z 517) of positional isomeric peptides 5 (Boc-NH- β -Nda-(L)-Phe-OCH₃) and 6 (Boc-NH-(L)-Phe- β -Nda-OCH₃) both display $[M + H - C_4H_8]^+$ ions (m/z 461) as the base peak, and the latter shows an additional peak at m/z 417 corresponding to $[M + H-Boc + H]^+$ ion. The major fragmentation in MS^3 of $[M + H - C_4H_8]^+$ ions is the formation of $[M + H-Boc + H]^+$ ions (m/z 417). The MS^4 of $[M + H-Boc + H]^+$ ions (m/z 417) of these dipeptides are significantly different from one another (Supplementary Material Figure S2a and b). The CID spectrum of the $[M + H-Boc + H]^+$ ion of 5 shows product ions at m/z 400 ($[M + H-Boc + H - NH_3]^+$), m/z 385 ($[M + H-Boc + H - CH_3OH]^+$), m/z 291 ($[M + H-Boc + H-thymine]^+$), m/z 274 ($[M + H-Boc +$

Table 1. Partial CID Spectra of $[M + H - \text{Boc} + \text{H}]^+$ Ions (in m/z) of Peptides 1–10

Ion	1	2	3	4	5	6	7	8	9	10
$[M + \text{H-Boc} + \text{H} - \text{NH}_3]^+$	324	-	352	-	400	-	-	-	-	-
$[M + \text{H-Boc} + \text{H} - \text{CH}_3\text{OH}]^+$	309	-	337	-	385	-	693	-	-	-
$[M + \text{H-Boc} + \text{H-thymine}]^+$	215	215	243	243	291	291	599	599	935	935
$[M + \text{H-Boc} + \text{H-thymine} - \text{CH}_3\text{OH}]^+$	183	-	211	-	259	-	567	-	-	-
$[M + \text{H-Boc} + \text{H-thymine} - \text{NH}_3]^+$	198	-	226	-	274	-	582	-	-	-
$[M + \text{H-Boc} + \text{H-thymine} - \text{NH}_3\text{-C}_2\text{H}_4\text{O}_2]^+$	138	-	166	-	214	-	-	-	-	-
c_1^+	-	89	-	117	-	165	-	-	-	-
z_1^+	-	127	-	127	-	127	-	-	-	-
Immonium ions	-	-	-	72	-	120	-	-	-	-
Retro-Mannich ions	-	172	-	172	-	172	-	-	-	-
$[M + \text{H-Boc} + \text{H} - 2 \text{ thymines}]^+$	-	-	-	-	-	-	473	473	809	809
$[M + \text{H-Boc} + \text{H} - 3 \text{ thymines}]^+$	-	-	-	-	-	-	-	-	-	683
b_1^+	-	-	-	-	-	-	238	-	-	-
b_2^+	-	-	-	-	-	-	309	-	309	-
b_3^+	-	-	-	-	-	-	-	-	-	456
b_4^+	-	-	-	-	-	-	-	-	-	-
b_5^+	-	-	-	-	-	-	-	-	693	-
y_1^+	-	-	-	-	-	-	-	-	-	-
y_2^+	-	-	-	-	-	-	417	-	369	-
y_3^+	-	-	-	-	-	-	488	-	516	-
y_4^+	-	-	-	-	-	-	-	-	753	-
y_5^+	-	-	-	-	-	-	-	-	-	-
Other ions	130, 166, 187.	-	158, 194, 215.	-	206, 242, 263.	-	707, 550, 514, 456, 441, 400, 388, 356, 326, 281, 274.	581, 564, 536, 456, 428, 410, 385, 357, 347, 330, 309, 302, 291, 273, 255, 241.	917, 900, 792, 710, 627, 610, 584, 567, 550, 522, 441, 403, 385, 369, 358, 309.	917, 900, 872, 764, 666, 649, 621, 540, 523, 505, 480, 473, 428.

H-thymine – NH₃⁺) (base peak), m/z 263 ([M + H-Boc + H-thymine – CO]⁺), m/z 259 ([M + H-Boc + H-thymine – CH₃OH]⁺), m/z 242 ([M + H-Boc + H-thymine – NH₃ – CH₃OH]⁺), m/z 214 ([M + H-Boc + H-thymine – NH₃ – C₂H₄O₂]⁺), and m/z 206 ([M + H-Boc + H-thymine – NH₃ – furan]⁺) (Supplementary Material Figure S2a and Table 1). These fragmentation pathways have been confirmed by MSⁿ experiments.

Similarly to dipeptides 2 and 4, the MS⁴ of [M + H-Boc + H]⁺ (m/z 417) ions of 6 shows m/z 291 ([M + H-Boc + H-thymine]⁺), and m/z 165 (c_1^+) ions (Supplementary Material Figure S2b). The MS⁵ of [M + H-Boc + H-thymine]⁺ (m/z 291)

ion of 6 shows product ions at m/z 172 [loss of imine (119 Da)], m/z 165 (c_1^+), m/z 127 (z_1^+), and m/z 120 (phenylalanine immonium ion) (Supplementary Material Figure S2c).

CID of Tetrapeptide Positional Isomers

The ESI MS/MS spectra of [M + H]⁺ ions (m/z 825) of isomeric tetrapeptides 7 and 8 are given in Figure 2a and b. Both the protonated peptides display the common product ions at m/z 769 ([M + H – C₄H₈]⁺), m/z 725 ([M + H-Boc + H]⁺), m/z 699 [M+H-thymine]⁺, and m/z 599 ([M + H -Boc +

Figure 2. ESI MS² spectra of [M + H]⁺ ions (m/z 825) of tetrapeptides (a) 7 and (b) 8 at 32 eV and ESI MS³ spectra of [M + H-Boc + H]⁺ ions (m/z 725) of tetrapeptides (c) 7 and (d) 8 at 25 eV

H-thymine]⁺). It can be noted that in case of 7, [M + H – C₄H₈]⁺ ion is the base peak and [M + H-Boc + H]⁺ ion is of low abundance. Whereas in case of 8, the latter ion is base peak and the former ion is low abundant. The low abundance of [M + H-Boc + H]⁺ ion in 7 may be attributed to the presence of β-Nda at the N-terminus as explained for dipeptide isomer 1 (Scheme 2). Another difference noticed between 7 and 8 is, the former shows a moderately abundant ion at *m/z* 643 ([M + H – C₄H₈-thymine]⁺), whereas it is totally absent for the latter.

The MS³ of [M + H-Boc + H]⁺ ions (*m/z* 725) of 7 shows abundant ions at *m/z* 599 ([M + H-Boc + H-thymine]⁺), *m/z* 514 (*x*₃⁺), *m/z* 473 ([M + H-Boc + H – 2 thymines]⁺), and *m/z* 309 (*b*₂⁺). Other moderately abundant ions observed are the C-terminal ions, *m/z* 417 (*y*₂⁺), *m/z* 488 (*y*₃⁺), *m/z* 400 (*z*₂⁺), *m/z* 388 (*x*₃⁺-thymine), *m/z* 356 (*x*₃⁺-thymine-CH₃OH), *m/z* 274 (*z*₂⁺-thymine), and N-terminal ions, *m/z* 238 (*b*₁⁺), *m/z* 281 (*a*₂⁺), and *m/z* 326 (*c*₂⁺) (Figure 2c). In addition, the spectrum also displays fragment ions due to loss of small neutral molecules from various fragment ions, viz., *m/z* 707 ([M + H-Boc + H – H₂O]⁺), *m/z* 693 ([M + H-Boc + H – CH₃OH]⁺), *m/z* 582 ([M + H-Boc + H-thymine – NH₃]⁺), *m/z* 567 ([M + H-Boc + H-thymine – CH₃OH]⁺), *m/z* 550 ([M + H-Boc + H-thymine – CH₃OH – NH₃]⁺), *m/z* 456 ([M + H-Boc + H – 2 thymines – NH₃]⁺), and *m/z* 441 ([M + H-Boc + H – 2 thymines – CH₃OH]⁺) (Table 1). These fragmentation pathways have been confirmed by MSⁿ experiments.

The MS³ of [M + H-Boc + H]⁺ ions (*m/z* 725) of 8 shows relatively simple spectrum. Similarly to 7, the spectrum displays the abundant product ions at *m/z* 599 ([M + H-Boc + H-thymine]⁺) and *m/z* 473 ([M + H-Boc + H – 2 thymines]⁺). The major differences noticed are the absence of *m/z* 514 (*x*₃⁺), *m/z* 417 (*y*₂⁺), *m/z* 326 (*c*₂⁺), and *m/z* 309 (*b*₂⁺) ions, which are significant for 7. The MS³ of [M + H-Boc + H]⁺ ions also shows *m/z* 581 ([M + H-Boc + H-thymine – H₂O]⁺), *m/z* 564 ([M + H-Boc + H-thymine – H₂O – NH₃]⁺), *m/z* 536 ([M + H-Boc + H-thymine-H₂O – NH₃ – CO]⁺), *m/z* 357 (*a*₂⁺), *m/z* 428 (*a*₃⁺), *m/z* 410 (*a*₃⁺-H₂O), and *m/z* 302 (*a*₃⁺-thymine) ions (Figure 2d). Interestingly, the *m/z* 473 ion also decomposes to form significant ions at *m/z* 347 (*c*₃⁺), *m/z* 309 (*z*₃⁺), *m/z* 241 (*x*₂⁺), *m/z* 456 (loss of NH₃), *m/z* 330 (*c*₃⁺-NH₃), *m/z* 291 (*z*₃⁺-H₂O), *m/z* 273 (*z*₃⁺-2 H₂O), and *m/z* 255 (*z*₃⁺-3 H₂O). Besides, it can be noted that the *y*_{*n*}⁺ and *b*_{*n*}⁺ ions, which are significant for 7, are totally absent for 8.

CID of Hexapeptide Positional Isomers

The ESI MS/MS spectra of [M + H]⁺ ions (*m/z* 1161) of isomeric hexapeptides 9 and 10 display product ions at *m/z* 1105 ([M + H – C₄H₈]⁺), *m/z* 1061 ([M + H-Boc + H]⁺) (base peak), and *m/z* 1035 ([M + H-thymine]⁺). It can be noted that *m/z* 1105 and 1035 ions are more abundant in 9 than 10. The other differences noticed are, the ions at *m/z* 1143 ([M + H – H₂O]⁺), *m/z* 979 ([M + H – C₄H₈-thymine]⁺), *m/z* 935 ([M + H-Boc + H-thymine]⁺), *b*₄⁺ (*m/z* 793), and *y*₄⁺ (*m/z* 753), which are significant in 9, are insignificant for 10. In

addition, hexapeptide 10 shows *b*₅⁺ (*m/z* 892) ion, which is totally absent for 9 (Figure 3a and b).

Further, MS³ of [M + H-Boc + H]⁺ ions (*m/z* 1061) of 9 and 10 display different fragmentation pattern (Figure 3c and d). The former shows product ions at *m/z* 1043 ([M + H-Boc + H – H₂O]⁺), *m/z* 935 ([M + H-Boc + H-thymine]⁺), and *m/z* 809 ([M + H-Boc + H – 2 thymines]⁺). The spectrum also shows peptide sequencing ions, *y*_{*n*}⁺ (*n*=2–4) at *m/z* 369, *m/z* 516, *m/z* 753, respectively, and *b*₂⁺ (*m/z* 309), *b*₄⁺ (*m/z* 693), *c*₂⁺ (*m/z* 326), *c*₄⁺ (*m/z* 710), and *a*₄⁺ (*m/z* 665). Other fragment ions observed are *m/z* 627 (*y*₄⁺-thymine), *m/z* 584 (*c*₄⁺-thymine), *m/z* 567 (*b*₄⁺-thymine), *m/z* 550 (*b*₄⁺-thymine – NH₃), *m/z* 532 (*b*₄⁺-thymine – NH₃ – H₂O), *m/z* 522 (*b*₄⁺-thymine – NH₃ – CO), *m/z* 501 (*y*₄⁺-2 thymines), *m/z* 441 (*b*₄⁺-2 thymines), *m/z* 792 (loss of NH₃ from *m/z* 809), *m/z* 423 (*b*₄⁺-2 thymines – H₂O) (Figure 3c). The MS⁴ of *m/z* 935 yields significant product ions at *m/z* 610 (*z*₄⁺), *m/z* 484 (*z*₄⁺-thymine), *m/z* 358 (*z*₄⁺-2 thymines), *m/z* 917 ([M + H-Boc + H-thymine – H₂O]⁺), *m/z* 900 ([M + H-Boc + H-thymine – H₂O – NH₃]⁺), and *m/z* 872 ([M + H-Boc + H-thymine – H₂O – NH₃ – CO]⁺).

The MS³ of [M + H-Boc + H]⁺ ions (*m/z* 1061) of hexapeptide 10 shows significant sequencing ions, *y*₃⁺ (*m/z* 606), *b*₃⁺ (*m/z* 456), *b*₅⁺ (*m/z* 792), *a*₃⁺ (*m/z* 428), and *c*₃⁺ (*m/z* 473), which are absent for 9. Other fragment ions observed are, *m/z* 764 (*b*₅⁺-CO), *m/z* 666 (*b*₅⁺-thymine), *m/z* 649 (*b*₅⁺-thymine – NH₃), *m/z* 621 (*b*₅⁺-thymine – NH₃ – CO), *m/z* 540 (*b*₅⁺-2 thymines), *m/z* 523 (*b*₅⁺-2 thymines – NH₃), *m/z* 505 (*b*₅⁺-2 thymines – NH₃-H₂O), *m/z* 480 (*y*₃⁺-thymine), *m/z* 428 (*b*₃⁺-CO), *m/z* 935 ([M + H-Boc + H-thymine]⁺), *m/z* 809 ([M + H-Boc + H – 2 thymines]⁺), *m/z* 683 ([M + H-Boc + H – 3 thymines]⁺), *m/z* 917 ([M + H-Boc + H-thymine – H₂O]⁺), *m/z* 900 ([M + H-Boc + H-thymine – H₂O – NH₃]⁺), and *m/z* 872 ([M + H-Boc + H-thymine – H₂O – NH₃ – CO]⁺) (Figure 3d).

Structure of *b_n*⁺ Ions

Over the years, there have been several studies focused on the structure and mechanism of the formation of *b_n*⁺ ions from natural peptides [27, 29, 30]. This has been mainly due to its complex and variable structures. We have also studied earlier the structure of ‘b’ ions in some of the unnatural peptides [57]. With a view to examine the effect of presence of β-Nda in the peptide backbone on the formation of ‘b’ ions from these hybrid peptides, the structures of these ions are discussed here. As discussed earlier, the MS³ spectrum of [M + H-Boc + H]⁺ of hexapeptide 9 (Figure 3c) shows abundant *b*₄⁺ (*m/z* 693) and low abundance *b*₂⁺ (*m/z* 309) ions. The *b*₁⁺, *b*₃⁺, and *b*₅⁺ ions are absent. It should be noted that *b*₄⁺ and *b*₂⁺ do not contain β-Nda- at the C-terminus whereas *b*₁⁺, *b*₃⁺, and *b*₅⁺ do. If all the *b_n*⁺ ions possessed linear acylium ion structures, their intensities would be of similar order. As this is not the case, we propose a cyclic structure for *b*₂⁺ and *b*₄⁺ ions, which can be formed through an oxazolone-type mechanism, as shown in Scheme 4. A similar mechanism that has been reported for the natural peptides [29, 30], is proposed here. In case of the formation

Figure 3. ESI MS² spectra of [M + H]⁺ ions (*m/z* 1161) of hexapeptides **(a)** 9 and **(b)** 10 at 29 eV, and ESI MS³ spectra of [M + H-Boc + H]⁺ ions (*m/z* 1061) of hexapeptides **(c)** 9 and **(d)** 10 at 29 eV

of b₁⁺, b₃⁺, and b₅⁺ ions, it appears that the initial nucleophilic attack by the carbonyl oxygen on the carbonyl carbon is probably suppressed by the β-Nda- located at the C-terminus, leading to the absence of these ions in the MS³ spectrum. In contrast, the MS³ spectrum of [M + H-Boc + H]⁺ of hexapeptide 10 (Figure 3d) shows abundant b₅⁺ (*m/z* 792) and b₃⁺ (*m/z* 456) ions. The b₂⁺ and b₄⁺ ions are absent. This may be due to the fact that b₃⁺ and b₅⁺ ions do not contain β-Nda- at the C-terminus whereas b₂⁺ and b₄⁺ do. Thus, it is likely that the formation of b₃⁺ and b₅⁺ ions may proceed through energetically favorable five-membered cyclic transition states to form oxazolone structures. The absence of b₂⁺ and b₄⁺ ions can be ascribed to the presence of β-Nda at the C-terminus, which probably suppresses the nucleophilic attack by the carbonyl oxygen which is required for the formation of oxazolone structure.

Similarly, b₃⁺ and b₁⁺ ions are insignificant and highly abundant b₂⁺ ion (Figure 2c) in the tetrapeptide 7, can be assigned an oxazolone-type structure. However, in case of 8, all the b_n⁺ (*n*=1–3) ions are absent (Figure 2d). This has been explained below in the context of effect of helical structures in these peptides (Scheme 1)

Analogy Between Solution Phase and Gas Phase Ion Chemistry of These Peptides

It has been reported by one of us that both the tetra peptide isomers 7 and 8 form novel 11/8 helical structures in solution phase (Scheme 1) [49–53]. It is observed that the MSⁿ fragmentation pattern of these protonated tetra peptides provides an opportunity to identify the amide -NH- hydrogens, which are reported to involve in H-bonding leading to

Scheme 4. Pathway to form b₄⁺ ion from protonated hexapeptide 9 through the oxazolone mechanism

helical structures in solution phase. It is known that y_n⁺ and b_n⁺ ions are formed by the cleavage of the peptide bond. It can be noticed that significant y_n⁺ and b_n⁺ ions are formed when the corresponding amide -NH- does not participate in the helical structures through H-bonding, whereas the participation of amide -NH- in the H-bonding results in the insignificant or relatively low abundance y_n⁺ and b_n⁺ ions in the CID process. For example, tetrapeptides 7 and 8 forms 11/8-helices [49–53] through H-bonds involving all the three amide -NH- and CO groups. In the former, the Boc-carbonyl also participates in hydrogen bonding whereas in the latter the Boc- carbonyl group does not participate (Scheme 1). Accordingly, b_n⁺ and y_n⁺ ions are not observed in the MS/MS of [M + H]⁺ ions of 7 and 8 (Figure 2a and b). However, the MS³ of [M + H-Boc + H]⁺ ions of 7 shows significant b₁⁺, b₂⁺, y₂⁺, y₃⁺ ions and the b₃⁺, y₁⁺ are absent (Figure 2c). This indicates that 8-helix structure through

hydrogen bonding between 4-NH and 2-CO appears to be retained and the hydrogen bond involving Boc- carbonyl is disturbed after the loss of Boc- group. In case of 8, b_n⁺ and y_n⁺ (n=1–3) are totally absent (Figure 2d) as the loss of Boc-group does not seem to be affecting the helical structure in the gas phase as the Boc- group is not involved in the hydrogen bonding (Scheme 1).

Negative Ion CID of Isomeric Peptide Acids

Peptides containing a free terminal carboxylic acid are known to readily form negative ions; hence, we examined the possibility of differentiating these peptide acids (Scheme 1) under negative ion ESI conditions. The negative ion ESI of di- (11–16) tetra- (17–18) and hexa (19–20) peptide acids give abundant [M – H][–] ions. In contrast to the formation of abundant [M + H-Boc + H]⁺ ions under positive ion conditions, the negative ion ESI mass spectra of these peptide acids show abundant [M – H-(CH₃)₃COH][–] ions [39, 41, 43–46, 54]. The formation of these ions may presumably be due to a 1,3-H migration from -NH- to the O of the butyloxy group leading to the elimination of *tert*-butanol by charge-remote fragmentation [46].

CID of Dipeptide Acids

To differentiate these isomers, the MS³ of [M – H-(CH₃)₃COH][–] ions of 11, 13, and 15 were compared with those of 12, 14, and 16, respectively (Figure 4, Supplementary Material Figure S3 and Table 2). The MS³ of [M – H-(CH₃)₃COH][–] ions of former group of isomeric peptides with β-Nda- at the N-terminus shows an abundant [M – H-(CH₃)₃COH – HNCO][–] ion due to loss of HNCO. Formation of this ion can be explained by a ‘H’ migration from the active β-methylene group to the -NCO [40, 42, 45, 46]. Further it eliminates CO₂ to form [M – H-(CH₃)₃COH – HNCO – CO₂][–] ion. Both these ions are totally absent for 12, 14, and 16. Instead, these peptides form [M – H-(CH₃)₃COH – CO₂][–] which are totally absent for 11, 13, and 15. Another important fragmentation in the peptides with L-amino acid (alanine, valine and phenylalanine) at the N-terminus is the formation of c₁[–] and z₁[–] ions which are insignificant for 11, 13, and 15. Loss of thymine from [M – H-(CH₃)₃COH – HNCO][–] is observed in 11, 13, and 15, whereas [M – H-(CH₃)₃COH – HNCO – CO₂][–] eliminates thymine in case of 12, 14, and 16. The [M – H-(CH₃)₃COH][–] ions of all these isomeric peptides display an abundant m/z 125 ion corresponding to thymine anion [58, 59].

CID of Tetrapeptide Acids

The ESI MS/MS spectra of [M – H][–] ions (m/z 809) of isomeric tetra peptide acids 17 and 18 are given in Figure 5a and b. Both these deprotonated peptide acids give ([M – H-(CH₃)₃COH][–]) (m/z 735) ions as the base peak due to loss of *tert*-butanol. The

Figure 4. ESI MS³ spectra of $[M - H-(CH_3)_3COH]^-$ ions (m/z 351) of dipeptides (a) 11 and (b) 12 at 30 eV, and ESI MS³ spectra of $[M - H-(CH_3)_3COH]^-$ ions (m/z 379) of dipeptides (c) 13 and (d) 14 at 30 eV

former ions display additional fragment ions at m/z 717 ($[M - H-(CH_3)_3COH - H_2O]^-$), m/z 709 ($[M - H-Boc]^-$), m/z 691 ($[M - H-(CH_3)_3COH - CO_2]^-$), and m/z 565 ($[M - H-(CH_3)_3COH - CO_2-thymine]^-$), which are totally absent for 18.

Further, the MS³ spectra of the $[M - H-(CH_3)_3COH]^-$ ions (m/z 735) are found to be significantly different from one another. The former shows abundant product ions at m/z 692 ($[M - H-(CH_3)_3COH - HNCO]^-$), m/z 566 ($[M - H-$

Table 2. Partial CID Spectra of $[M - H-(CH_3)_3COH]^-$ Ions (in m/z) of Dipeptides 11–16

Ion	11	12	13	14	15	16
$[M - H - (CH_3)_3COH - HNCO]$	308	-	336	-	384	-
$[M - H - (CH_3)_3COH - HNCO - CO_2]$	264	-	292	-	340	-
$[M - H - (CH_3)_3COH - CO_2]$	-	307	-	335	-	383
Thymine anion	125	125	125	125	125	125
c_1^- ion	-	113	-	141	-	189
z_1^- ion	-	237	-	237	-	237
Other ions	182, 221, 246.	181, 193, 225.	210, 249, 262, 274.	193, 209, 253.	193, 214, 258, 279, 297, 322.	193, 257, 301.

Figure 5. ESI MS² spectra of $[M - H]^-$ ions (m/z 809) of tetrapeptides **(a)** 17 and **(b)** 18 at 33 eV, and ESI MS³ spectra of $[M - H-(CH_3)_3COH]^-$ ions (m/z 735) of tetrapeptides **(c)** 17 and **(d)** 18 at 25 eV

$(CH_3)_3COH - HNCO - thymine]^-$) and low abundance ions x_3^- (m/z 498), z_2^- (m/z 384), a_1^- (m/z 236), m/z 717 ($[M - H-(CH_3)_3COH - H_2O]^-$), m/z 648 ($[M - H-(CH_3)_3COH - HNCO - CO_2]^-$), m/z 609 ($[M - H-(CH_3)_3COH - thymine]^-$), m/z 591 ($[M - H-(CH_3)_3COH - thymine - H_2O]^-$), m/z 548 ($[M - H-(CH_3)_3COH - HNCO - thymine - H_2O]^-$), m/z 522 ($[M - H-(CH_3)_3COH - HNCO - thymine - CO_2]^-$), and m/z 440 ($[M - H-(CH_3)_3COH - HNCO - 2\ thymines]^-$) (Figure 5c).

In contrast to 17, MS³ of the $[M - H-(CH_3)_3COH]^-$ ions of (m/z 735) tetrapeptide 18 shows significant product ions, c_3^- (m/z 497), z_3^- (m/z 545), z_1^- (m/z 237), m/z 691 ($[M - H-(CH_3)_3COH - CO_2]^-$), m/z 609 ($[M - H-(CH_3)_3COH - thymine]^-$), m/z 419 ($z_3^- - thymine$), m/z 648 ($[M - H-(CH_3)_3COH - CO_2 - HNCO]^-$), m/z 591 ($[M - H-(CH_3)_3COH - thymine - H_2O]^-$), m/z 566 ($[M - H-(CH_3)_3COH -$

$thymine - HNCO]^-$), m/z 522 ($[M - H-(CH_3)_3COH - CO_2 - HNCO - thymine]^-$), m/z 483 ($[M - H-(CH_3)_3COH - 2\ thymines]^-$), m/z 454 ($c_3^- - HNCO$), m/z 371 ($c_3^- - thymine$), m/z 353 ($c_3^- - thymine - H_2O$), and m/z 293 ($z_3^- - 2\ thymines$) (Figure 5d).

CID of Hexapeptide Acids

The ESI MS/MS spectra of $[M - H]^-$ ions (m/z 1145) of isomeric hexapeptide acids 19 and 20 display an abundant $[M - H-(CH_3)_3COH]^-$ (m/z 1071) ion and moderately abundant ions at m/z 1045 ($[M - H-Boc]^-$), m/z 1019 ($[M - H-thymine]^-$), and m/z 945 ($[M - H-(CH_3)_3COH - thymine]^-$). The major differences noticed are, isomer 19 shows additional ions at m/z 1028 ($[M - H-(CH_3)_3COH - HNCO]^-$), m/z 902 ($[M - H-$

(CH₃)₃COH – HNCO-thymine][−]) and m/z 776 ([M – H-(CH₃)₃COH – HNCO – 2 thymines][−]), which are totally absent for 20. In addition, hexapeptide 20 shows z_5^- (m/z 881) ion, which is totally absent for 19 (Figure 6a and b).

Further, the MS³ of [M – H-(CH₃)₃COH][−] (m/z 1071) ions of 19 shows abundant ions at m/z 1028 ([M – H-(CH₃)₃COH – HNCO][−]), m/z 902 ([M – H-(CH₃)₃COH – HNCO-thymine][−]), m/z 858 ([M – H-(CH₃)₃COH – HNCO – thymine – CO₂][−]), m/z 776 ([M – H-(CH₃)₃COH – HNCO – 2 thymines][−]), z_4^- (m/z 720), a_4^- (m/z 691), m/z 708 (x_5^- -thymine), and m/z 594 (z_4^- -thymine). In addition, the spectrum also displays low abundance ions at m/z 984 ([M – H-(CH₃)₃COH – HNCO – CO₂][−]),

m/z 945 ([M – H-(CH₃)₃COH-thymine][−]), m/z 884 ([M – H-(CH₃)₃COH – HNCO-thymine – H₂O][−]), x_4^- (m/z 763), x_5^- (m/z 834), m/z 735 (x_4^- -CO), m/z 819 ([M – H-(CH₃)₃COH – 2 thymines][−]), m/z 582 (x_5^- -2 thymines), and m/z 564 (x_5^- -2 thymines – H₂O) (Figure 6c).

The MS³ of [M – H-(CH₃)₃COH][−] (m/z 1071) ions of hexapeptide acid 20 shows the ions at m/z 1027 ([M – H-(CH₃)₃COH – CO₂][−]), m/z 945 ([M – H-(CH₃)₃COH – thymine][−]) (base peak), m/z 901 ([M – H-(CH₃)₃COH – CO₂ – thymine][−]), c_5^- (m/z 833), c_3^- (m/z 497), z_5^- (m/z 881), z_4^- (m/z 644), m/z 755 (z_5^- -thymine), m/z 629 (z_5^- -2 thymines), m/z 707 (c_5^- -thymine) and m/z 689 (c_5^- -thymine-H₂O) (Figure 6d).

Figure 6. ESI MS² spectra of [M – H][−] ions (m/z 1145) of hexapeptides (a) 19 and (b) 20 at 33 eV, and ESI MS³ spectra of [M – H-(CH₃)₃COH][−] ions (m/z 1071) of hexapeptides (c) 19 and (d) 20 at 25 eV

Conclusions

Positive and negative ion ESI-MS/MS has been shown to be very useful for differentiating positional isomers of hybrid peptides containing repeats of β -Nda- and L-amino acids (alanine, valine, and phenylalanine). In case of positive ions, di-, tetra-, and hexapeptide isomers have been clearly distinguished by the CID of their $[M + H]^+$ ions. In addition, the CID spectra of $[M + H\text{-Boc} + H]^+$ produce product ions that are different for the positional isomers of di-, tetra-, and hexapeptides. The protonated dipeptide positional isomers with β -Nda- at the N-terminus lose CH_3OH , NH_3 , and $\text{C}_2\text{H}_4\text{O}_2$. Instead, the presence of L-amino acids at the N-terminus results in characteristic retro-Mannich reaction involving elimination of imine. It is observed that, significant y_n^+ and b_n^+ ions are observed in the CID of tetra- and hexapeptides. In contrast to abundant $[M + H\text{-Boc} + H]^+$ ions observed in positive ion mode, the negative ion mode yields intense $[M - H(\text{CH}_3)_3\text{COH}]^-$ ions. It is also observed that peptides with β -Nda- at the N-terminus give rise to $[M - H(\text{CH}_3)_3\text{COH} - \text{HNCO}]^-$ and $[M - H(\text{CH}_3)_3\text{COH} - \text{HNCO} - \text{CO}_2]^-$ ions, whereas the peptides with L-amino acid form $[M - H(\text{CH}_3)_3\text{COH} - \text{CO}_2]^-$. With regards to the formation of sequence ions, it is worth noting that while b_n^- and y_n^- ions are totally absent in negative ion spectra, significant c_n^- and z_n^- ions are observed. It can be concluded that ESI tandem mass spectrometry of both the protonated and deprotonated peptides is highly useful for the structural characterization and differentiation of these isomeric di-, tetra-, and hexahybrid peptides.

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