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Characterization of N^{α}-Fmoc-protected dipeptide isomers by electrospray ionization tandem mass spectrometry (ESI-MS^n): effect of protecting group on fragmentation of dipeptides

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A series of positional isomeric pairs of Fmoc-protected dipeptides, Fmoc-Gly-Xxx-OY/Fmoc-Xxx-Gly-OY (Xxx = Ala, Val, Leu, Phe) and Fmoc-Ala-Xxx-OY/Fmoc-Xxx-Ala-OY (Xxx = Leu, Phe) (Fmoc = [(9-fluorenylmethyl)oxy]carbonyl) and Y = CH_3/H), have been characterized and differentiated by both positive and negative ion electrospray ionization ion-trap tandem mass spectrometry (ESI-IT-MS^n). In contrast to the behavior of reported unprotected dipeptide isomers which mainly produce y_1^+ and/or a_1^+ ions, the protonated Fmoc-Xxx-Gly-OY, Fmoc-Ala-Xxx-OY and Fmoc-Xxx-Ala-OY yield significant b_1^+ ions. These ions are formed, presumably with stable protonated aziridinone structures. However, the peptides with Gly- at the N-terminus do not form b_1^+ ions. The $[M+H]^+$ ions of all the peptides undergo a McLafferty-type rearrangement followed by loss of CO_2 to form $[M+H–Fmoc+H]^+$. The MS^3 collision-induced dissociation (CID) of these ions helps distinguish the pairs of isomeric dipeptides studied in this work. Further, negative ion MS^3 CID has also been found to be useful for differentiating these isomeric peptide acids. The MS^3 of $[M–H–Fmoc+H]^-$ of isomeric peptide acids produce c_1^-, z_1^- and y_1^- ions. Thus the present study of Fmoc-protected peptides provides additional information on mass spectral characterization of the dipeptides and distinguishes the positional isomers. Copyright © 2011 John Wiley & Sons, Ltd.

Peptides are ubiquitous in living organisms and play various essential roles such as protein folding, cell adhesion, cell differentiation, and tumor metastasis.[1] Peptides are also involved in important physiological and biochemical functions such as neuro transmission, neuro modulation, and act as hormones in receptor mediated signal transduction.[2] Despite their suboptimal pharmacokinetic properties, peptides demonstrate unparalleled potency and specificity against a wide array of biological targets.[3] A major portion of currently marketed drugs bear peptides in their core structure. The increasing interest about the manifold actions of the bioactive peptides has made their structural studies an important aspect of research in pharmacology and medical sciences.[4] During the drug development, identification of an 'active' part of a large peptide is important for further improving its pharmacology. This involves synthesis of a small segment, chemical modification and its biological and structural evaluation. Mass spectral analysis, together with other analytical tools, would aid the understanding of complete structural aspects. In peptide synthesis, the 9-fluorenylmethyloxycarbonyl (Fmoc) group is routinely employed as amine protector due to the various synthetic advantages associated with it.[5] Its stability over a range of reaction environments, racemization tolerance and mild deprotection conditions have made it a protecting group of choice.

Tandem mass spectrometry (MS/MS) of protonated and deprotonated organic and biological analytes including peptides[6–11] in electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)[12–14] is now a well-established technique for structural elucidation and differentiation of isomers.[15–25] Heerma and co-workers[26–28] have reported the high-energy collision-induced dissociation (CID) of a variety of protonated dipeptides and tripeptides and shown that fragmentation of the protonated species leads to formation of the y_1^+ and/or a_1^+ ions. Isa *et al.*[29] have carried out high-energy CID studies of a series of protonated dipeptides such as H-Xxx-Gly-OH, H-Gly-Xxx-OH, H-Xxx-Leu-OH and H-Leu-Xxx-OH. They have shown that for the observation of y_1^+ ion, the proton affinity of the C-terminal amino acid should be greater than that of the N-terminal amino acid. Harrison and co-workers[7,23] reported high-energy CID studies of series of protonated dipeptides.

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They have shown that fragmentation of protonated species leads to formation of the y_1^+ and/or a_1^+ ions. Fonseca *et al.*[20] also reported the formation of the y_1^+ and/or a_1^+ ions in ESI-MS/MS studies of dipeptide isomers. Hiserodt *et al.*[24] demonstrated that dipeptides containing an N-terminal basic amino acid undergo a rearrangement involving a cyclic intermediate to form the corresponding protonated molecule of the respective basic amino acid. They also reported that the $[b_1 + H_2O]^+$ ion is formed from A_1A_2 dipeptides whereas A_2A_1 dipeptides formed y_1^+ ions. Goel and Kenny[22] also reported the mechanism for the formation of the b_1-1 and corresponding a_1-1 ions in the product ion mass spectra of *N-para*-ferrocenylbenzoyl dipeptide esters. A literature survey revealed that there are very few reports on mass spectral studies of Fmoc protected peptides/amino acids.[30–37] To examine the effect of the Fmoc- group on the fragmentation of the dipeptides, we have studied a series of Fmoc-protected dipeptide positional isomers using both positive and negative ion ESI-MS/MS.

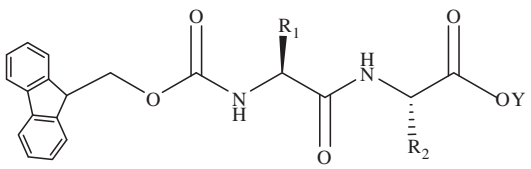
EXPERIMENTAL

Mass spectrometry

ESI mass spectra of peptides **1–24** (Scheme 1) were recorded using a liquid chromatography quadrupole (LCQ) ion trap mass spectrometer (LCQ Advantage Max, 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 offset voltage, 20 V; sheath gas (N_2) pressure, 20 psi; and helium was used as damping gas. For the ion-trap analyzer, the automatic gain control (AGC) setting 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 mode, the precursor ion of interest was first isolated by applying an appropriate wave form across the end-cap electrodes of the ion trap to resonantly eject all trapped ions, except those ions of *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 15–38 eV. The excitation time used was 30 ms. All the spectra were recorded under identical experimental conditions for isomers, and average of 20–25 scans. The methanolic solutions of samples were infused into the ESI source at a flow rate of 5 μL/min by using an instrument's syringe pump.

Materials

Solvents used in the present study were purchased from Merck (Mumbai, India), and used without further purification. Stock (1 mM) solutions of peptides were diluted with high-performance liquid chromatography (HPLC)-grade methanol to achieve a final concentration of 10 μM each.



R_1	R_2	Y	R_1	R_2	Y
1. H	CH_3	CH_3	**13.** H	$CH_2C_6H_5$	CH_3
2. H	CH_3	H	**14**. H	$CH_2C_6H_5$	H
3. CH_3	H	CH_3	**15.** $CH_2C_6H_5$	H	CH_3
4. CH_3	H	H	**16.** $CH_2C_6H_5$	H	H
5. H	$CH(CH_3)_2$	CH_3	**17.** CH_3	$CH_2CH(CH_3)_2$	CH_3
6. H	$CH(CH_3)_2$	H	**18.** CH_3	$CH_2CH(CH_3)_2$	H
7. $CH(CH_3)_2$	H	CH_3	**19.** $CH_2CH(CH_3)_2$	CH_3	CH_3
8. $CH(CH_3)_2$	H	H	**20.** $CH_2CH(CH_3)_2$	CH_3	H
9. H	$CH_2CH(CH_3)_2$	CH_3	**21.** CH_3	$CH_2C_6H_5$	CH_3
10. H	$CH_2CH(CH_3)_2$	H	**22.** CH_3	$CH_2C_6H_5$	H
11. $CH_2CH(CH_3)_2$	H	CH_3	**23.** $CH_2C_6H_5$	CH_3	CH_3
12. $CH_2CH(CH_3)_2$	H	H	**24.** $CH_2C_6H_5$	CH_3	H

Scheme 1. Structure of the studied N^{α}-Fmoc protected peptides (**1–24**).

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Synthesis of the peptides

The synthesis and spectroscopic characterization of the compounds studied in this work have been reported previously.[38]

General procedure for the preparation of N^{α}-Fmoc dipeptide methyl esters

To a stirred suspension of an amino acid methyl ester hydrochloride salt (1.0 mmol) and activated Zn dust (1.0 mmol) in tetrahydrofuran (THF) (3.0 mL) was added a solution of Fmoc amino acid chloride (1 mmol) followed by activated Zn dust (1.0 equiv) in THF and the mixture was stirred for 10–15 min at room temperature. After completion of the reaction (analyzed by thin-layer chromatography (TLC) analysis), the solution was filtered and the filtrate was evaporated. The residue was diluted with CH_2Cl_2 (15 mL), washed successively with 5% HCl (10 mL × 2), 5% aq. $NaHCO_3$ (10 mL × 2), water (10 mL × 2) and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified by column chromatography (EtOAc/hexane, 1:3) to obtain the dipeptide methyl ester as a white solid (see Supporting Information).[39]

Preparation of N^{α}-Fmoc amino acid chlorides

Under an argon atmosphere, Fmoc amino acid (1.0 mmol) was suspended in CH_2Cl_2 (5 mL), $SOCl_2$ (1 mL) was added and the mixture was sonicated at room temperature for 20–30 min. Solvent and excess of $SOCl_2$ were removed *in vacuo* and the residue was dissolved in CH_2Cl_2 (1.0 mL). Addition of hexane (10 mL) precipitated pure acid chlorides. The resulting crystals were filtered and dried (see Supporting information).[40]

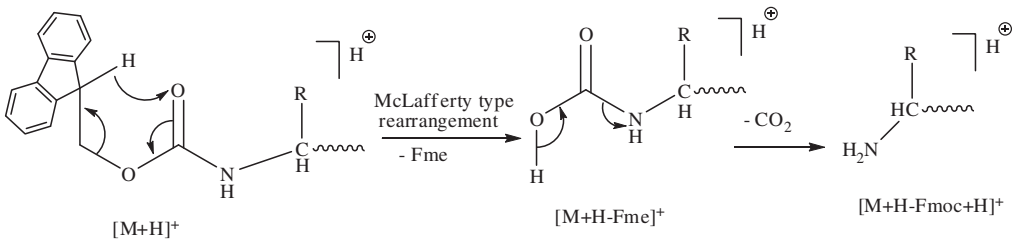
General procedure for hydrolysis of methyl esters

A solution of N^{α}-Fmoc peptide ester (1.0 mmol) in *i*PrOH/H_2O (7:3, 10.6 mL) was treated with NaOH (1.2 mmol) and 0.8 M $CaCl_2$. The reaction mixture was stirred for 3–4 h (TLC analysis). When the reaction was complete, the hydrosylate was neutralized with 1 N HCl (10 mL) and the solid residue was dissolved in MeOH (15.0 mL). Addition of H_2O (30.0 mL) resulted in a precipitate which was filtered and extensively washed with H_2O. The crude product was purified on silica gel (eluant: CH_2Cl_2/MeOH/AcOH, 1–4% MeOH, 1% AcOH) gave the pure peptide acid as a white crystalline solid (see Supporting Information).[41]

RESULTS AND DISCUSSION

A series of positional isomeric Fmoc-protected (Fmoc = [(9-fluorenylmethyl)oxy]carbonyl) dipeptides studied in this work are shown in Scheme 1. The positive ion ESI mass spectra of all these peptides (**1–24)** show abundant $[M + Na]^+$, $[2M + Na]^+$, and $[M + H]^+$ ions, and low abundance $[M + H–Fme]^+$ (Fme = 9-methylene-9*H*-fluorene), $[M + H–Fmoc + H]^+$, and *m/z* 179 (9*H*-fluoren-9-yl)-methyl cation ($C_{14}H_{11}^+$)) ions. The formation of the $[M + H–Fmoc + H]^+$ ions can be rationalized in terms of a McLafferty-type rearrangement involving a γ-hydrogen migration from the fluorenyl moiety to the carbonyl oxygen in the FmocN moiety followed by the loss of 9-methylene-9*H*-fluorene (Fme, 178 Da) and subsequent loss of CO_2[31]from $[M + H]^+$ (Scheme 2). The formation of $[M + H–Fmoc + H]^+$ ions can also be explained by a stepwise mechanism involving the loss of CO_2 from $[M + H–Fme]^+$ (Scheme 2), as evidenced by the MS^3 spectra. Fragmentation of these peptides can be explained by using the nomenclature of Roepstorff, Fohlman, and Biemann.[42–44] To study the mass spectrometric behavior of these Fmoc-protected dipeptides and to investigate the possibility of distinguishing these positional isomers, we have examined the MS/MS CID spectra of the protonated positional isomers (**1–24)**.

The ESI-MS/MS spectra of $[M + H]^+$ ions (*m/z* 383) of positional isomeric peptides Fmoc-Gly-Ala-OCH_3 (**1**) and Fmoc-Ala-Gly-OCH_3 (**3**)[34] are distinctly different from each other (Fig. 1). Both the isomers show product ions at *m/z* 351 ($[M + H–CH_3OH]^+$), 205 ($[M + H–Fme]^+$), 187 (loss of (9*H*-fluoren-9-yl)methanol), 179 (9*H*-fluoren-9-yl)-methyl cation ($C_{14}H_{11}^+$)), 173 ($[M + H–Fme–CH_3OH]^+$), 161 ($[M + H–Fmoc + H]^+$), and 129 ($[M + H–Fmoc + H\text{-}CH_3OH]^+$). Isomer **1** shows additional peaks at *m/z* 323 ($[M + H–CH_3OH–CO]^+$) and 145 ($[M + H–Fme–CH_3OH–CO]^+$) which are absent for **3**, whereas isomer **3** shows product ions at *m/z* 339 ($[M + H\text{-}CO_2]^+$), 294 (b_1^+) (Scheme 3), 133 ($[M + H–Fmoc + H–CO]^+$), and 116 (b_1^+–Fme). These fragmentation pathways have been confirmed by MS^n experiments. Loss of CH_3OH from $[M + H]^+$ and $[M + H–Fmoc + H]^+$ ions can be explained by a plausible mechanism involving an intramolecular nucleophilic attack by the protonated amide carbonyl on the carbonyl carbon of the C-terminal ester group, forming a five-membered cyclic ring with the extrusion of CH_3OH.[6,45] The formation of the b_1^+ ion in **3** containing alanine at the N-terminus is in contrast to the fragmentation behavior of the protonated unprotected dipeptides, H-Ala-Gly-OH, which mainly produce y_1^+ and/ or a_1^+ ions.[7,29] It can be noted that the absence of the b_1^+ ion for isomer **1**,



Scheme 2. Proposed McLafferty-type rearrangement.

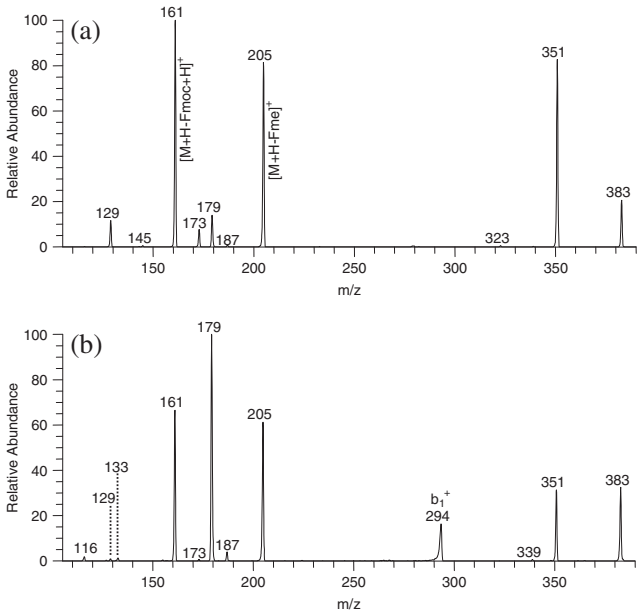


Figure 1. ESI-MS/MS spectra of $[M+H]^+$ ions (*m/z* 383) of (a) **1** and (b) **3** at 23 eV.

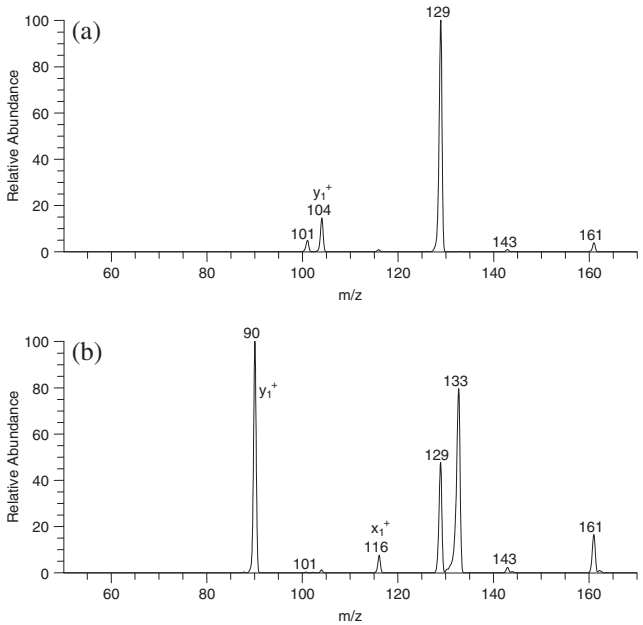
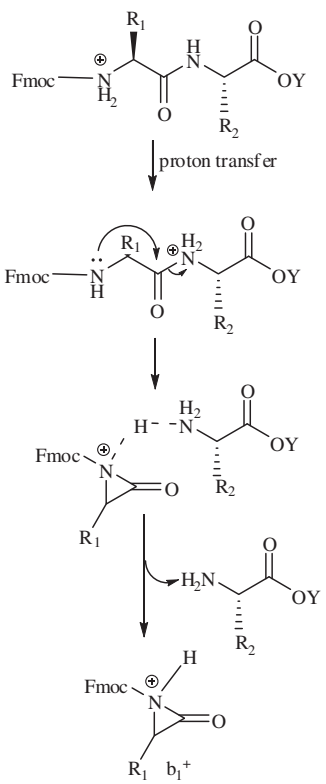


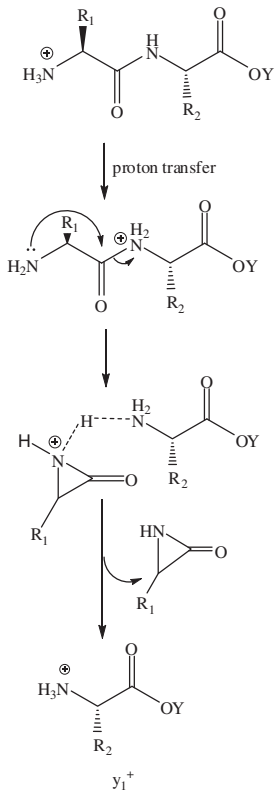
Figure 2. ESI-MS^3 spectra of $[M+H–Fmoc+H]^+$ ions (*m/z* 161) of (a) **1** and (b) **3** at 27 eV.

containing glycine at the N-terminus, may be attributed to the unstable aziridinone structure (Scheme 3).[46,47]

To probe further the fragmentation of the dipeptides, we have examined the MS^3 CID of the common ions of *m/z* 161 ($[M+H–Fmoc+H]^+$) of **1** and **3**, which were formed via sequential losses of Fme and CO_2 from the $[M+H]^+$ precursor ions. It can be seen from Fig. 2 that the MS^3 spectrum of **1** is also significantly different from that of **3**. Both the isomers show product ions at *m/z* 143 ($[M+H–Fmoc+H–H_2O]^+$), 129 ($[M+H–Fmoc+H–CH_3OH]^+$) and 101($[M+H–Fmoc+H–CH_3OH–CO]^+$). In addition, isomer **1** shows an ion at *m/z* 104 (y_1^+) (Scheme 4),[8] whereas isomer **3** shows product ions



Scheme 3. Proposed mechanism for the formation of the b_1^+ ion from $[M+H]^+$ ions.



Scheme 4. Proposed mechanism for the formation of the y_1^+ ion from $[M+H–Fmoc+H]^+$ ions.

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at *m/z* 133 ($[M + H–Fmoc + H–CO]^+$), 116 (x_1^+) and 90 (y_1^+). The formation of the y_1^+ ion from $[M + H–Fmoc + H]^+$ ion of **1** and **3** is in agreement with earlier reports of unprotected dipeptides.[7,29] Thus, the positional isomers **1** and **3** can be clearly distinguished from each another by their characteristic fragmentation in both MS^2 and MS^3 CID spectra.

Similarly, another pair of positional isomers, Fmoc-Gly-Val-OCH_3 (**5**) and Fmoc-Val-Gly-OCH_3 (**7**), yielded product ion spectra that are distinctly different from one another (Table 1 and also Supporting Information). Similarly to **1** and **3**, the CID mass spectra of the $[M + H]^+$ ions (*m/z* 411) of **5** and **7** show product ions at *m/z* 379 ($[M + H–CH_3OH]^+$), 233 ($[M + H–Fme]^+$), 215 (loss of (9*H*-fluoren-9-yl)methanol), 189 ($[M + H–Fmoc + H]^+$) and 179 ($C_{14}H_{11}^+$). Isomer **5** shows additional peaks at *m/z* 351 ($[M + H–CH_3OH–CO]^+$), 201 ($[M + H–Fme–CH_3OH]^+$), 173 ($[M + H–Fme–CH_3OH–CO]^+$), 157 ($[M + H–Fmoc + H–CH_3OH]^+$), 132 ($y_1^+$) and 129 ($[M + H–Fmoc + H–CH_3OH–CO]^+$) which are absent for isomer **7**, whereas isomer **7** shows product ions at *m/z* 322 (b_1^+), 161 ($[M + H–Fmoc + H–CO]^+$), 144 (b_1^+–Fme) and 116 (b_1^+–Fme–CO). These fragmentation pathways have been confirmed by MS^n experiments. Similarly to **1**, absence of the b_1^+ ion for isomer **5** containing glycine at the N-terminus can be attributed to the unstable aziridinone structure.[46,47] Thus, the positional isomers **5** and **7** can be readily distinguished from one another by means of their characteristic product ions.

Further, we have examined the MS^3 CID of *m/z* 189 ion ($[M + H–Fmoc + H]^+$) of **5** and **7**, which were formed via sequential losses of Fme and CO_2 from the $[M + H]^+$ precursor ions. Both the isomers show (Table 1 and also Supporting Information) an ion at *m/z* 157 ($[M + H–Fmoc + H–CH_3OH]^+$). Besides, the former shows product ions at *m/z* 171 ($[M + H–Fmoc + H–H_2O]^+$), 132 (y_1^+) and 129 ($[M + H–Fmoc + H–CH_3OH–CO]^+$) and the latter shows a low abundance ion at *m/z* 161 ($[M + H\text{-}Fmoc + H\text{-}CO]^+$) and an abundant ion at *m/z* 72 corresponding to the immonium ion (a_1^+),[23,33,45] a characteristic ion for the presence of valine at the N-terminus. The formation of the y_1^+ ion for **5** and the immonium ion (a_1^+) for **7** is in agreement with higher proton affinity of valine compared to that of glycine.[7,29] Thus, the MS^3 spectra of **5** and **7** are also useful in distinguishing the presence of glycine or valine at the N-terminus.

Similarly, another two pairs of isomeric peptides, **9/11** (Fmoc-Gly-Leu-OCH_3/Fmoc-Leu-Gly-OCH_3) and **13/15** (Fmoc-Gly-Phe-OCH_3/Fmoc-Phe-Gly-OCH_3), can be clearly distinguished from one another from their characteristic fragmentation in both MS^2 and MS^3 CID spectra (Table 1 and also Supporting Information).

The MS/MS CID mass spectra of $[M + H]^+$ ions (*m/z* 439) of a pair of positional isomers, Fmoc-Ala-Leu-OCH_3 (**17**) and Fmoc-Leu-Ala-OCH_3 (**19**), exhibit product ions that are distinctly different from one another (Fig. 3). Similarly to previous isomers, the CID mass spectra of $[M + H]^+$ ions (*m/z* 439) of **17** and **19** show product ions at *m/z* 407 ($[M + H–CH_3OH]^+$), 379 ($[M + H–CH_3OH–CO]^+$), 261 ($[M + H–Fme]^+$), 243 (loss of (9*H*-fluoren-9-yl)methanol), 229 ($[M + H–Fme–CH_3OH]^+$), 217 ($[M + H–Fmoc + H]^+$), 185 ($[M + H–Fmoc + H–CH_3OH]^+$) and 179 ($C_{14}H_{11}^+$). Isomer **17** shows additional peaks at *m/z* 294 (b_1^+), 201($[M + H–Fme–CH_3OH–CO]^+$) and 146 (y_1^+), whereas isomer **19** shows product ions at *m/z* 336 (b_1^+), 158 (b_1^+–Fme) and 130 (b_1^+–Fme–CO). These fragmentation pathways have been confirmed by MS^n experiments. Thus, the positional isomers **17** and **19** can be readily distinguished from one another by means of their characteristic product ions.

Further, MS^3 CID of *m/z* 217 ($[M + H\text{-}Fmoc + H]^+$) of **17** and **19** gives (Fig. 4) low abundance ions at *m/z* 199 ($[M + H–Fmoc + H–H_2O]^+$), 185 ($[M + H–Fmoc + H–CH_3OH]^+$) and 157 ($[M + H–Fmoc + H–CH_3OH–CO]^+$). Besides, the former shows an abundant ion at *m/z* 146 (y_1^+) and the latter gives low abundance ions at *m/z* 189 ($[M + H–Fmoc + H–CO]^+$), 104 (y_1^+) and an abundant ion at *m/z* 86 corresponding to the immonium ion (a_1^+), a characteristic ion for the presence

Table 1. Partial CID of $[M + H]^+$ ions of peptides **5**, **7**, **9**, **11**, **13**, **15**, **21** and **23**: *m/z* values with relative abundances (%) in parentheses

Compound	$[M + H]^+$	$[M + H–Fme]^+$	$[M + H–Fmoc + H]^+$	Other ions	MS^3 of $[M + H–Fmoc + H]^+$
5	411	233(32)	189(60)	379 (100), 351 (1), 215 (1), 201 (10), 179 (3), 173 (1), 157 (8), 132 (1), 129 (2)	189 → 171(1), 157(100), 132(2)[b], 129(12),
7	411	233(18)	189(41)	379 (22), 322(30)[a], 215 (5), 201 (1), 179 (100), 161(1), 144 (50), 116 (9)	189 → 161(5), 157(2), 72(100)[c],
9	425	247(38)	203(79)	393 (100), 365 (2), 229 (1), 215 (12), 187 (2), 179 (3), 171 (8), 146 (2), 143 (4)	203 → 185(1), 171(100), 146(5)[b], 143(17)
11	425	247(35)	203(98)	393 (36), 381 (1), 336 (25)[a], 229 (7), 215 (1), 179 (100), 158 (60), 130 (27)	203 → 185(1), 175(4), 171 (3), 86(100)[c]
13	459	281(30)	237(100)	427 (45), 399 (1), 263 (1), 249 (6), 221 (1), 205 (5), 180 (8), 177 (3)	237 → 219(1), 205(100), 180(32)[b], 177(47)
15	459	281(12)	237(100)	427 (15), 415 (1), 370 (6)[a], 263 (5), 221 (1), 192 (43), 179 (20), 164 (51), 148 (2), 146 (3)	237 → 219(1), 209(1), 205 (30), 177(1), 120(100)[c]
21	473	295(12)	251(72)	441(100), 413 (4), 294 (1)[a], 277 (1), 263 (2), 235 (1), 219 (1), 191 (1), 180 (28), 179 (1), 163 (1)	251 → 233(10), 219(22), 191(6), 180(100)[b]
23	473	295 (10)	251(67)	441(100), 413 (1), 370 (5)[a], 277 (3), 263 (1), 219 (1), 192 (33), 191(1), 179 (20), 164 (46), 148 (2), 146 (4)	251 → 233(1), 219(12), 191 (1), 120(100)[c], 104(1)[b]

[a] b_1^+ ion; [b] y_1^+ ion; [c] a_1^+ ion.

of leucine at the N-terminus. The formation of y_1^+ and/or a_1^+ ions from $[M+H–Fmoc+H]^+$ of **17** and **19** is in agreement with earlier reports of unprotected dipeptides.[29] Thus, the MS^3 spectra of **17** and **19** are also useful in establishing whether alanine or leucine is located at the N-terminus. Similarly, another pair of isomeric peptides, **21**/**23** (Fmoc-Ala-Phe-OCH_3/Fmoc-Phe-Ala-OCH_3), can be clearly distinguished from each other from their characteristic fragmentation in both MS^2 and MS^3 (Table 1 and also Supporting Information).

The positional isomeric pairs of corresponding acids, **2/4**, **6/8**, **10/12**, **14/16**, **18/20** and **22**/**24**, can be clearly distinguished from one another, except that the masses of the C-terminal ions are decreased by 14 Da.

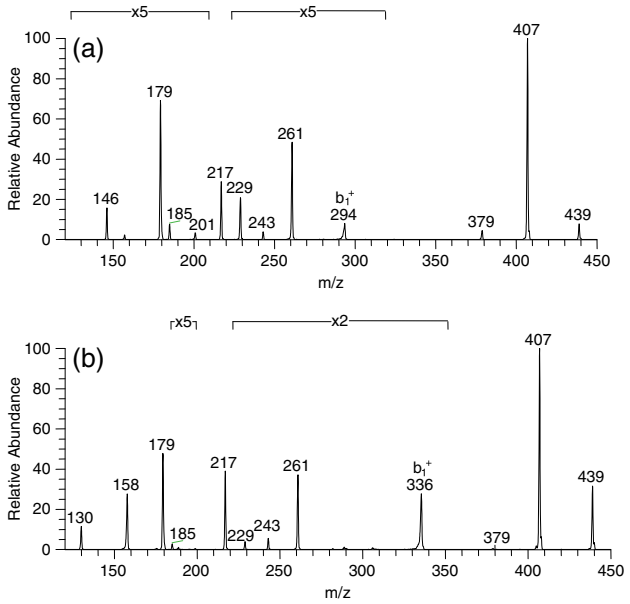


Figure 3. ESI-MS/MS spectra of $[M+H]^+$ ions (*m/z* 439) of (a) **17** and (b) **19** at 23 eV.

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 mass spectra of all the dipeptide acids show $[M–H]^–$, [M-H-9*H*-fluoren-9-yl) methanol]$^–$ and $[M–H–Fmoc+H]^–$ ions. The direct formation of $[M–H–Fmoc+H]^–$ ions and absence of $[M–H–Fme]^–$ ions under negative ion conditions can be explained by a plausible mechanism that may involve a 1,5-H migration from the fluorenyl group to the -NH- leading to the concomitant loss of CO_2 and Fme (Scheme 5).

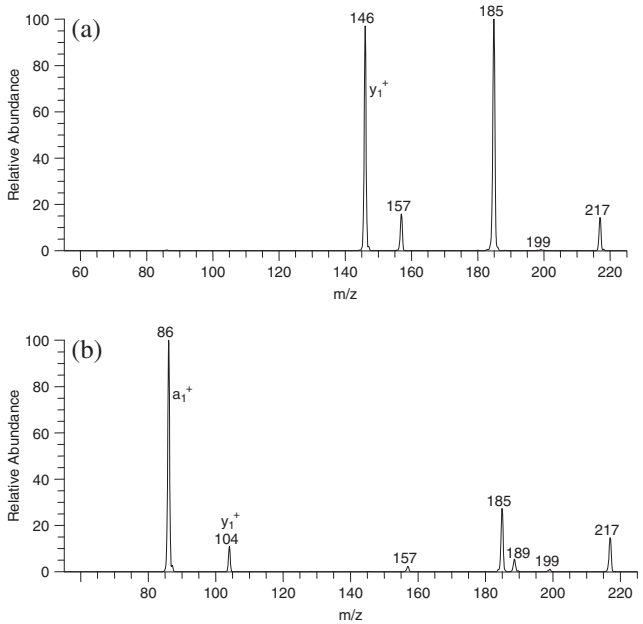


Figure 4. ESI-MS^3 spectra of $[M+H-Fmoc+H]^+$ ions (*m/z* 217) of (a) **17** and (b) **19** at 27 eV.

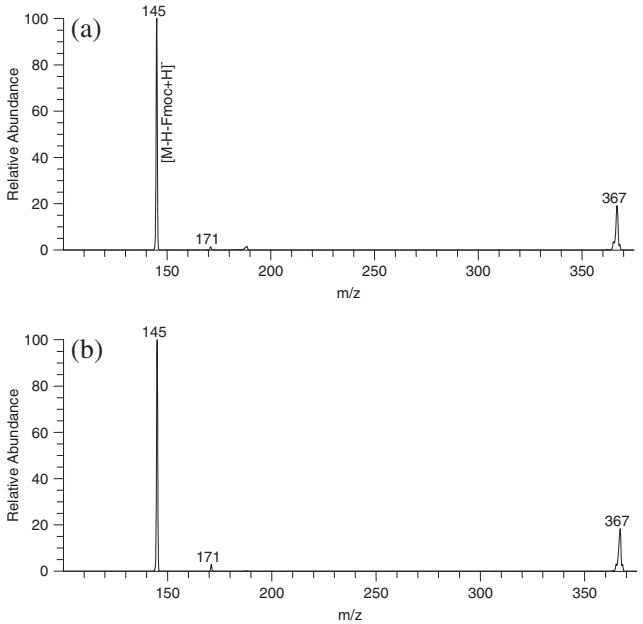
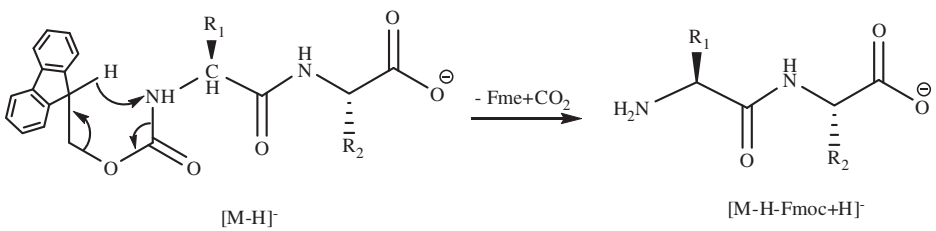


Figure 5. ESI-MS/MS spectra of $[M–H]^–$ ions (*m/z* 367) of (a) **2** and (b) **4** at 15 eV.



Scheme 5. Proposed mechanism for the concomitant loss of Fme and CO_2.

Negative ion ESI CID of the $[M–H]^-$ ion (*m/z* 367) of isomeric acids **2** and **4** yields (Fig. 5) a low abundance ion at *m/z* 171 (loss of (9*H*-fluoren-9-yl)methanol) and an abundant ion at *m/z* 145 ($[M–H–Fmoc + H]^-$). To differentiate the isomeric pair of **2** and **4**, we have examined the MS^3 of $[M–H–Fmoc + H]^-$ ions.

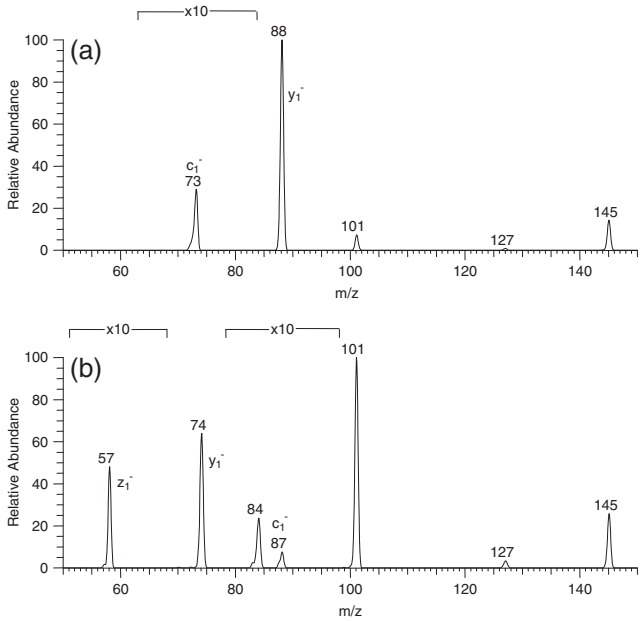
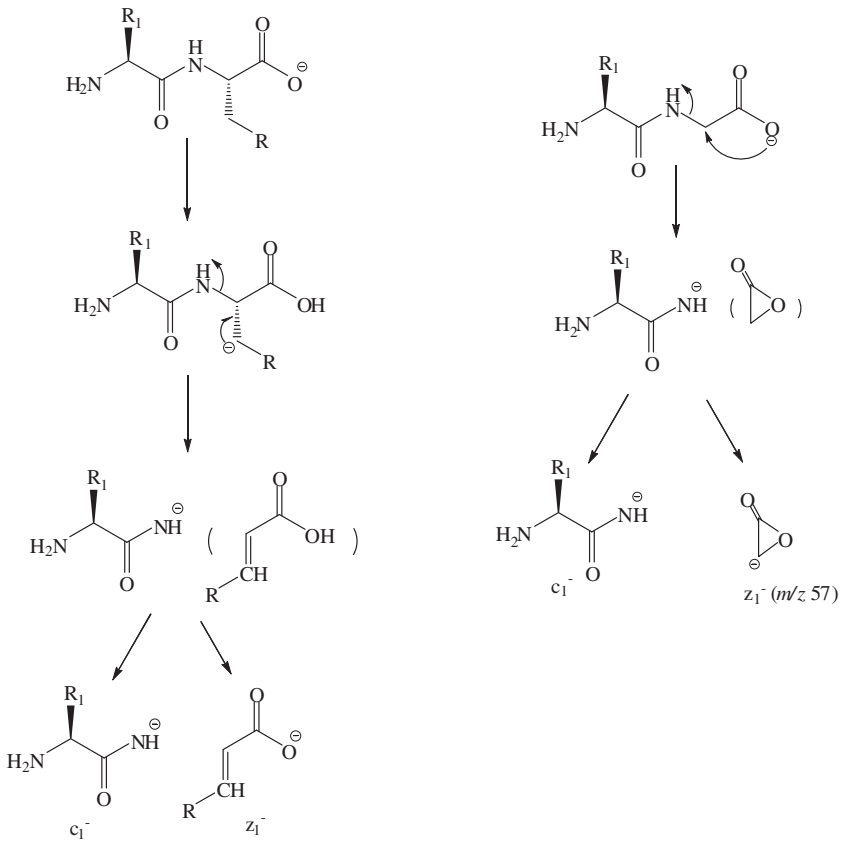


Figure 6. ESI-MS^3 spectra of $[M–H–Fmoc + H]^-$ ions (*m/z* 145) of (a) **2** and (b) **4** at 34 eV.

Figure 6 shows the MS^3 spectra of $[M–H–Fmoc + H]^-$ ions (*m/z* 145) of **2** and **4** which are significantly different from one another. The CID spectra of both the isomers display ions at *m/z* 127 ($[M–H–Fmoc + H–H_2O]^-$) and 101 ($[M–H–Fmoc + H–CO_2]^-$). In addition, **2** shows an abundant ion at *m/z* 88 (y_1^-) and a low abundance ion at *m/z* 73 (c_1^-), whereas the latter shows an abundant ion at *m/z* 74 (y_1^-) and low abundance ions at *m/z* 87 (c_1^-),84 ($[M–H–Fmoc + H–CO_2–NH_3]^-$) and 57 ($z_1^-$). The formation of c_1^- and z_1^- ions can be explained by the mechanism shown in Scheme 6.[48] It is noteworthy that N-terminal b_1^- ions are absent, instead c_1^- ions are significant which are normally reported to form under electron-capture dissociation.[49] Thus, the positional isomers **2** and **4** can be readily distinguished from one another by means of different product ions.

Similarly to **2** and **4**, negative ion ESI CID of the $[M–H]^-$ ion (*m/z* 395) of another pair of isomeric acids **6** and **8** yields a low abundance ion at *m/z* 199 (loss of (9*H*-fluoren-9-yl)methanol) and an abundant ion at *m/z* 173 ([M-H-Fmoc + H]$^-$) (Supporting Information). The MS^3 spectra of $[M–H–Fmoc + H]^-$ ions (*m/z* 173) of these isomers are distinctly different from one another (Table 2 and also Supporting Information). The spectra display ions at *m/z* 155 ($[M–H–Fmoc + H–H_2O]^-$) and 129 ($[M–H–Fmoc + H–CO_2]^-$). Besides, **6** shows an abundant ion at *m/z* 116 (y_1^-) and low abundance ions at *m/z* 99 (z_1^-) and 73 (c_1^-), whereas the latter shows an abundant ion at *m/z* 74 (y_1^-) and low abundance ion at *m/z* 57 (z_1^-). Thus, the positional isomers **6** and **8** can be readily distinguished from one another by means of different product ions.

Another four pairs of isomeric acids, **10**/**12**, **14**/**16**, **18**/**20** and **22**/**24**, can also be clearly distinguished from each other



Scheme 6. Proposed mechanism for the formation of c_1^- and z_1^- ions.

Table 2. Partial CID of $[M–H]^-$ ions of peptide acids **6**, **8**, **10**, **12**, **14**, **16**, **18**, **20**, **22** and **24**: *m/z* values with relative abundances (%) in parentheses

Compound	$[M–H]^-$	$[M–H–A^*]^-$	$[M–H–Fmoc+H]^-$	MS^3 of $[M–H–Fmoc+H]^-$
6	395	199(1)	173(100)	173 → 155 (1), 129 (85), 116 (100)[d], 99 (1)[f], 73 (5)[e]
8	395	199(6)	173(100)	173 → 155 (5), 129 (100), 74 (68)[d], 57 (4)[f]
10	409	213(1)	187(100)	187 → 169(2), 143 (38), 130 (100)[d], 113 (1)[f], 73 (16)[e]
12	409	213(4)	187(100)	187 → 169(1), 143 (100), 126 (3), 74 (64)[d], 57 (2)[f]
14	443	247(3)	221(100)	221 → 177 (5), 164 (100)[d], 147 (74)[f], 73 (25)[e]
16	443	247(15)	221(100)	221 → 204 (45), 177 (5), 163 (1)[e], 160 (30), , 129 (1)[a], 111 (1), 91 (5)[b], 85 (90)[c], 74 (100)[d]
18	423	227(4)	201(100)	201 → 183 (3), 157 (100), 140 (1), 130 (46)[d], 113 (1)[f], 87 (6)[e]
20	423	227(4)	201(100)	201 → 183 (2), 157 (40), 140 (1), 129 (2)[e], 88 (100)[d], 71 (1)[f]
22	457	261(12)	235(100)	235 → 191 (10), 164 (39)[d], 147 (100)[f], 87 (38)[e]
24	457	261(14)	235(100)	235 → 218 (22), 191 (3), 174 (7), 163(1)[e], 143 (1)[a], 99 (20)[c], 91(1)[b],88 (100)[d]

*A = (9*H*-fluoren-9-yl)methanol
[a]Loss of toluene; [b]$C_7H_7^+$; [c]loss of CO_2 from *m/z* 129/*m/z* 143 (**16** and **24**); [d]y_1^- ion; [e]c_1^- ion; [f]z_1^- ion.

by their characteristic fragmentation in MS^3 spectra (Table 2 and also Supporting Information).

CONCLUSIONS

Positive and negative ion ESI tandem mass spectrometry has been shown to be very useful for the structural characterization and differentiation of six pairs of N^{α}-Fmoc-protected dipeptide positional isomers. While MS/MS of the protonated dipeptides containing Ala-, Val-, Leu- and Phe- at the N-terminus gives rise to intense b_1^+ ions, MS^3 of $[M+H–Fmoc+H]^+$ yields y_1^+ and/or a_1^+ ions. Further, negative ion MS^3 CID has also been found to be useful for differentiating these isomeric peptide acids. In contrast to positive ions, b_1^- ions are absent, instead significant c_1^-, z_1^- and y_1^- ions are observed in the negative ion MS^n spectra.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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