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Characterization of N^{α}-Fmoc-protected ureidopeptides by electrospray ionization tandem mass spectrometry (ESI-MS/MS): differentiation of positional isomers

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Four pairs of positional isomers of ureidopeptides, FmocNH-CH(R_1)-φ(NH-CO-NH)-CH(R_2)-OY and FmocNH-CH(R_2)-φ(NH-CO-NH)-CH(R_1)-OY (Fmoc = [(9-fluorenyl methyl)oxy]carbonyl; R_1 = H, alkyl; R_2 = alkyl, H and Y = CH_3/H), have been characterized and differentiated by both positive and negative ion electrospray ionization (ESI) ion-trap tandem mass spectrometry (MS/MS). The major fragmentation noticed in MS/MS of all these compounds is due to –N–CH(R)–N– bond cleavage to form the characteristic N- and C-terminus fragment ions. The protonated ureidopeptide acids derived from glycine at the N-terminus form protonated (9H-fluoren-9-yl)methyl carbamate ion at *m/z* 240 which is absent for the corresponding esters. Another interesting fragmentation noticed in ureidopeptides derived from glycine at the N-terminus is an unusual loss of 61 units from an intermediate fragment ion FmocNH = CH_2^+ (*m/z* 252). A mechanism involving an ion-neutral complex and a direct loss of NH_3 and CO_2 is proposed for this process. Whereas ureidopeptides derived from alanine, leucine and phenylalanine at the N-terminus eliminate CO_2 followed by corresponding imine to form (9H-fluoren-9-yl)methyl cation ($C_{14}H_{11}^+$) from FmocNH = CHR^+. In addition, characteristic immonium ions are also observed. The deprotonated ureidopeptide acids dissociate differently from the protonated ureidopeptides. The $[M - H]^-$ ions of ureidopeptide acids undergo a McLafferty-type rearrangement followed by the loss of CO_2 to form an abundant $[M - H - Fmoc + H]^-$ which is absent for protonated ureidopeptides. Thus, the present study provides information on mass spectral characterization of ureidopeptides and distinguishes the positional isomers. **Copyright © 2010 John Wiley & Sons, Ltd.**

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

Keywords: electrospray ionization; tandem mass spectrometry; ureidopeptides; structure elucidation; positional isomers

Introduction

Peptides are the ubiquitous biomolecules that have myriad roles in the life's process. Each one of their roles is due to a unique three-dimensional structure.[1] However, the extensive usage of peptides as drug candidates needs to outdo some of the unfavorable features such as low oral bioavailability, lesser permeability, poor metabolic stability *in vivo*, etc. Some of these limitations are being overcome through the modification of one or more peptide bonds into unnatural functionalities. This has led to the emergence of pseudopeptides,[1–3] a class of novel entities which are being employed as potential alternatives to peptide-based drugs as well as in *de novo* design of molecules for structural studies. In this regard, a large research attention is being bestowed upon backbone engineering and design of novel amino acid derivatives for the construction of peptidomimetics. N^{α}-Fmoc amino acid-derived isocyanates are important intermediates that were first synthesized, isolated and fully characterized by one of us and are employed for the synthesis of several classes of peptidomimetics.[4] In particular, ureidopeptides and peptidyl ureas are accessed by these synthons which are recognized as most successful variety of peptidomimetics due to the interesting metabolic and hydrogen bonding properties possessed by the urea moiety.

Tandem mass spectrometry (MS/MS) of protonated and deprotonated organic and biological analytes including peptides[5] in electrospray ionization (ESI) and MALDI[6–8] has been a well-established technique for structure elucidation and differentiation of isomers.[9–16] The applications of ESI[17] MS to various types of analytes are well documented in the literature. A literature survey revealed that there are very few reports on mass spectral studies of pseudopeptides containing urea bonds.[18,19] This has prompted us to undertake a detailed mass spectrometric study on a series

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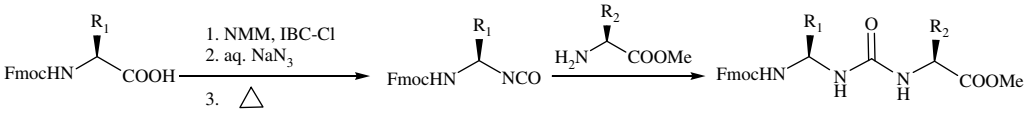


Scheme 1. Synthesis of N$^{\alpha}$-Fmoc-protected ureidopeptides.

of Fmoc-protected ureidopeptides and also the differentiation of four pairs of the positional isomeric compounds using ESI-MS/MS.

Experimental

Mass spectrometry

ESI mass spectra of ureidopeptides **1**–**20** (Scheme 1) were recorded using a quadrupole ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray source. 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 setting was 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 ac signal to resonantly excite them and thus cause collision-induced dissociation (CID). The collision energies used were 15–37 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. All the samples were infused into the ESI source at a flow rate of 5 µl/min by using an instrument's syringe pump.

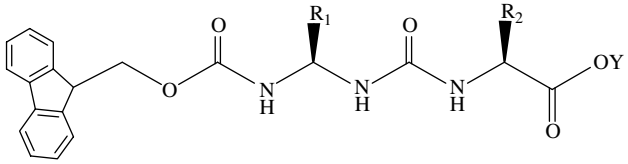
Accurate mass measurements were obtained using a quadrupole time-of-flight (QTOF) mass spectrometer (QSTAR XL, Applied Biosystems/MDS Sciex, Foster City, CA, USA), equipped with an ESI source. The data acquisition was under the control of Analyst QS software (Foster City, CA). The typical source conditions were: capillary voltage, 5.00 kV; declustering potential, 60 V; focusing potential, 260 V; declustering potential 2, 10 V; resolution 8000 (full-width half-maximum). Ultra high-pure nitrogen was used as the curtain gas and collision gas, whereas zero air was used as the nebulizer. The $[M + H]^+$ ions were selected as precursors by the quadrupole and allowed to collide with nitrogen gas in the collision cell. The product ions were then detected by a TOF analyzer. The samples were infused into the ESI source at a flow rate of 10 µl/min using an in-built 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 ureidopeptides were diluted with high-performance liquid chromatography-grade methanol to achieve a final concentration of 10 µM each.

Synthesis of the ureidopeptides

The synthesis and spectroscopic characterization of the compounds studied in this work have been reported by one of us.[20]



1. $R_1 = CH_3$	$R_2 = CH_2C_6H_5$	$Y = CH_3$
2. $R_1 = CH_3$	$R_2 = CH_2C_6H_5$	$Y = H$
3. $R_1 = CH_2C_6H_5$	$R_2 = CH_3$	$Y = CH_3$
4. $R_1 = CH_2C_6H_5$	$R_2 = CH_3$	$Y = H$
5. $R_1 = H$	$R_2 = CH_2CH(CH_3)_2$	$Y = CH_3$
6. $R_1 = H$	$R_2 = CH_2CH(CH_3)_2$	$Y = H$
7. $R_1 = CH_2CH(CH_3)_2$	$R_2 = H$	$Y = CH_3$
8. $R_1 = CH_2CH(CH_3)2$	$R_2 = H$	$Y = H$
9. $R_1 = H$	$R_2 = CH_2C_6H_5$	$Y = CH_3$
10. $R_1 = H$	$R_2 = CH_2C_6H_5$	$Y = H$
11. $R_1 = CH_2C_6H_5$	$R_2 = H$	$Y = CH_3$
12. $R_1 = CH_2C_6H_5$	$R_2 = H$	$Y = H$
13. $R_1 = H$	$R_2 = CH_3$	$Y = CH_3$
14. $R_1 = CH_3$	$R_2 = H$	$Y = CH_3$
15. $R_1 = H$	$R_2 = H$	$Y = CH_3$
16. $R_1 = H$	$R_2 = H$	$Y = H$
17. $R_1 = H$	$R_2 = CH(CH_3)_2$	$Y = CH_3$
18. $R_1 = H$	$R_2 = CH(CH_3)_2$	$Y = H$
19. $R_1 = H$	$R_2 = C_6H_5$	$Y = CH_3$
20. $R_1 = H$	$R_2 = C_6H_5$	$Y = H$

Scheme 2. Structures of the N$^{\alpha}$-Fmoc-protected ureidopeptides **(1–20)**.

General procedure for the preparation of isocyanates derived from Fmoc-α-amino acids

To a solution of *N*$^{\alpha}$-Fmoc-amino acid (1 mmol) in dry THF (5 ml) at −20 °C, *N*-methylmorpholine (NMM, 0.12 ml, 1.1 mmol) and isobutyl chloroformate (0.14 ml, 1.1 mmol) were added and the mixture was stirred at the same temperature for 20 min. It was treated with aqueous NaN_3 (98 mg, 1.5 mmol in 0.5 ml of water) and the stirring was continued for another 30 min. After completion of the reaction (TLC analysis), the organic layer was evaporated and the residue was dissolved in dichloromethane (CH_2Cl_2, 20 ml). It was washed successively thrice with 10 ml portions of 5% HCl, 5% aqueous $NaHCO_3$, water and dried over anhydrous Na_2SO_4. Evaporation of the solvent *in vacuo* affords corresponding acid azide. It was dissolved in toluene (10 ml) and heated at 65 °C under nitrogen atmosphere. After

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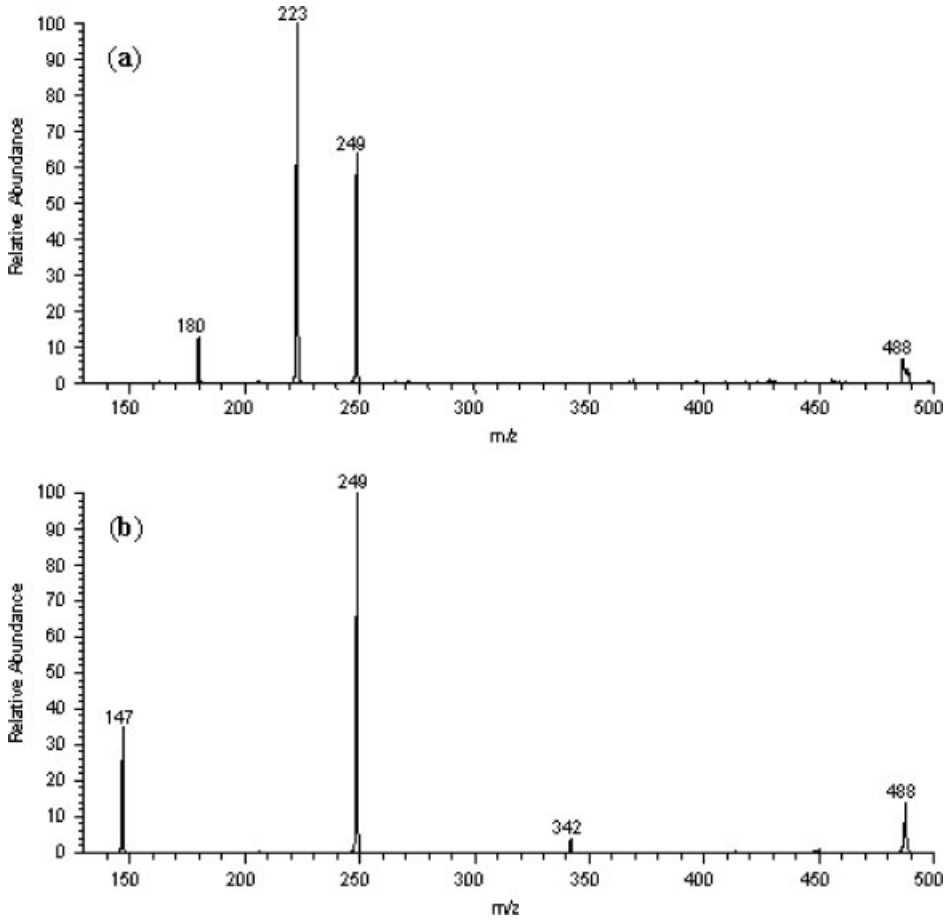
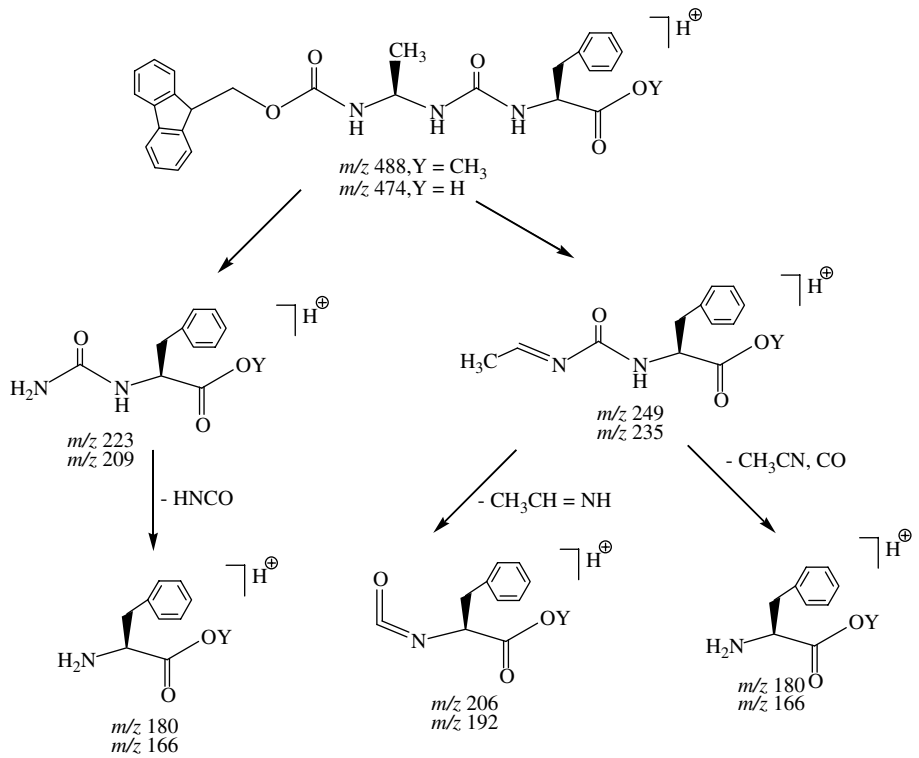
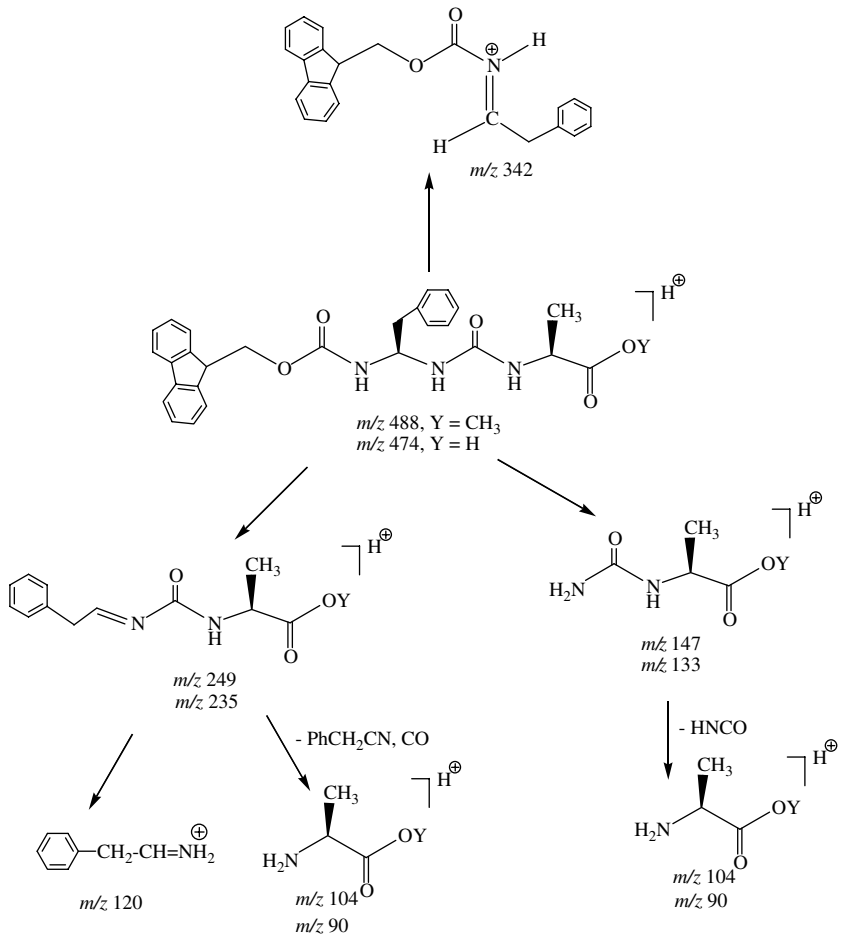


Figure 1. ESI-MS/MS spectra of $[M + H]^+$ ions (*m/z* 488) of (a) **1** and (b) **3** at 18 eV.



Scheme 3. Proposed fragmentation mechanism for protonated ureidopeptides **1**and **2**.



Scheme 4. Proposed fragmentation mechanism for protonated ureidopeptides **3** and **4**.

the completion of the reaction, the solvent was removed under reduced pressure to obtain the isocyanate, which was recrystallized using dichloromethane/*n*-hexane.

General procedure for the preparation of ureidopeptides

To a stirred suspension of amino acid methyl ester hydrochloride salt (1 mmol) in CH_2Cl_2 (5 ml), NMM (2 mmol) was added slowly and the mixture was stirred at 25 °C for few minutes. This was added to the solution of isocyanate (1 mmol) derived from Fmoc-α-amino acid, and the stirring was continued till the completion of the reaction. The precipitated solid was filtered and recrystallized using DMSO–water to obtain the uriedopeptide as crystalline white solid (Scheme 1).

Results and Discussion

The positional isomeric pairs of the N^{α}-Fmoc-protected ureidopeptides studied in this work are shown in Scheme 2. The positive ion ESI mass spectra of all these N^{α}-Fmoc-protected ureidopeptides (**1**–**20**) show abundant $[M + H]^+$, $[M + Na]^+$ and $[2M + Na]^+$ ions. To study the mass spectrometric behavior of these ureidopeptides and to investigate the possibility of distinguishing these positional isomers, we have examined the MS/MS CID spectra of the protonated positional isomers (**1**–**20**).

It can be seen from Fig. 1 that the MS^2 CID spectra (*m/z* 488) of isomers Fmoc-Ala-φ(NH-CO-NH)-Phe-OCH_3 (**1**) and Fmoc-Phe-φ(NH-CO-NH)-Ala-OCH_3 (**3**) (Fmoc = [(9-fluorenylmethyl)oxy]carbonyl)[20] are distinctly different from one another. Both the isomers show an abundant ion at *m/z* 249 corresponding to the loss of (9H-fluoren-9-yl) methyl carbamate (Scheme 3). This appears to be a diagnostic loss for the presence of Fmoc group at the N-terminus as can be seen from the fragmentation of other Fmoc-protected ureidopeptides. Isomer **1** shows additional peaks at *m/z* 223 and 180 which are absent for **3**. The former corresponds to protonated (*S*)-methyl 3-phenyl-2-ureidopropanoate which on loss of HNCO forms a low abundant ion at *m/z* 180 (Scheme 3). Whereas isomer **3** shows a low abundance ion at *m/z* 342 formed by the loss of (*S*)-methyl 2-ureidopropanoate (Scheme 4). The complementary fragment ion corresponding to protonated (*S*)-methyl 2-ureidopropanoate appears at *m/z* 147. It can be noted that the major fragmentation in these compounds does not involve the cleavage of –NH–CO–NH– bonds and it mainly involves –N–CH(R)–N– bonds.

To probe further the fragmentation of the ureidopeptides, the MS^3 CID of the common ion of *m/z* 249 from **1** and **3** was examined. It can be seen from Fig. 2 that the MS^3 of **1** is also significantly different from that of **3**. Isomer **1** forms low abundance ion at *m/z* 206 and an abundant ion at *m/z* 180 by the loss of $CH_3CH{=}NH$ and a combination of CH_3CN and CO, respectively (Scheme 3). These

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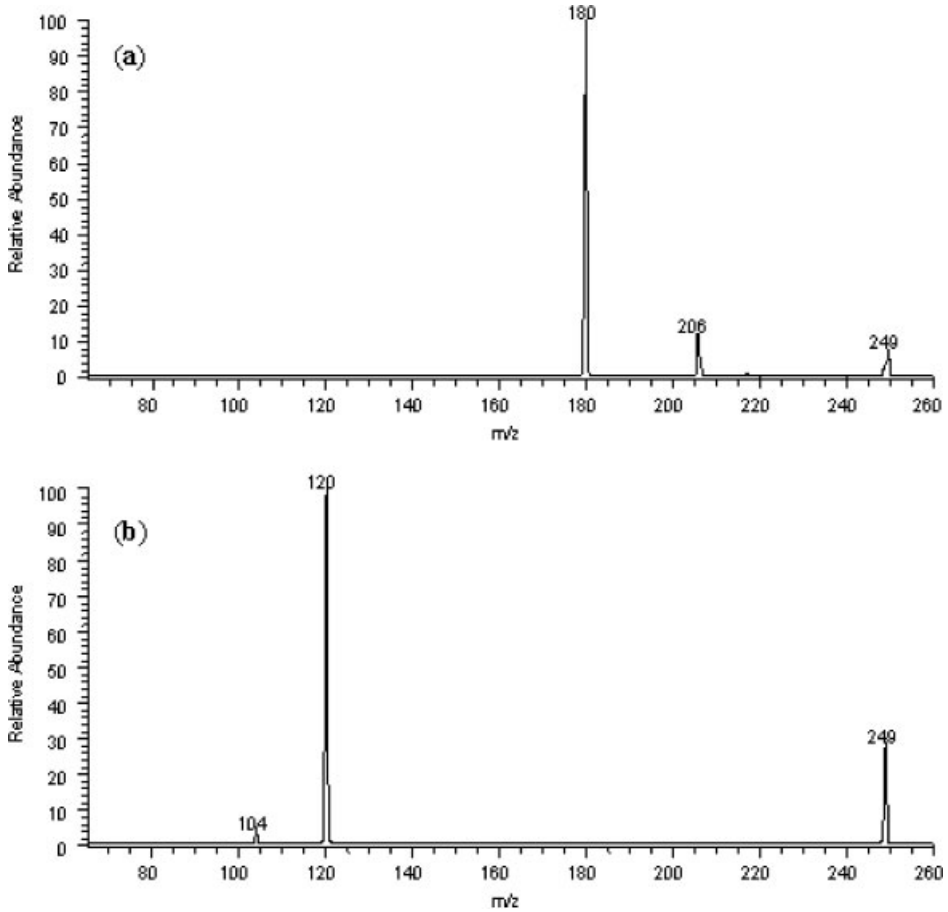


Figure 2. ESI-MS^3 spectra of *m/z* 249 of (a) **1** and (b) **3** at 22 eV.

ions are absent for **3**. Instead, the latter shows an abundant ion at *m/z* 120 corresponding to the immonium ion of phenylalanine[21] (Scheme 4), a characteristic ion for the ureidopeptides derived from phenylalanine at the N-terminus. A low abundance ion is observed at *m/z* 104 corresponding to the loss of combination of $PhCH_2CN$ and CO. Thus, the positional isomers **1** and **3** can be clearly distinguished from one another by their characteristic fragmentation in both MS^2 and MS^3 CID spectra.

Similarly, the positional isomers of corresponding acids **2** and **4** can be clearly distinguished from one another, except that the masses of the C-terminal ions are decreased by 14 Da. It is noteworthy that the intensity of the *m/z* 342 ion (Scheme 4) becomes highly abundant for acid **4** than the corresponding ester **3**.

An another pair of positional isomers, Fmoc-Gly-φ(NH-CO-NH)-Leu-OCH_3 (**5**) and Fmoc-Leu-φ(NH-CO-NH)-Gly-OCH_3 (**7**), yielded product ion spectra that are distinctly different from one another (Fig. 3). Similar to **1** and **3**, the CID mass spectra of $[M + H]^+$ ions (*m/z* 440) of **5** and **7** display an ion at *m/z* 201 corresponding to the loss of (9H-fluoren-9-yl) methyl carbamate. The major difference observed between **5** and **7** is that the former shows *m/z* 408 (loss of methanol), *m/z* 397 (loss of HNCO), *m/z* 269 (protonated (9H-fluoren-9-yl) methyl aminomethyl carbamate), *m/z* 252 (loss of (*S*)-methyl 4-methyl-2-ureidopentanoate), *m/z* 189 (protonated (*S*)-methyl 4-methyl-2-ureidopentanoate), *m/z* 158 (loss of HNCO from *m/z* 201) and *m/z* 146 (loss of HNCO from *m/z* 189), whereas these ions are totally absent for **7** (Scheme 5). Instead, the latter shows only *m/z* 308 (loss of methyl 2-ureidoacetate) and its complementary low abundance ion at *m/z* 133 corresponding to protonated methyl 2-ureidoacetate (Scheme 6). It can be noted that the ion at *m/z* 201 is relatively more abundant for **7** than for **5**. Thus, the positional isomers **5** and **7** can be readily distinguished from one another by means of their characteristic product ions.

Furthermore, the MS^3 CID of *m/z* 201 of **5** and **7** gives (Supporting Information) low abundance ion at *m/z* 158 (loss of HNCO). Besides, the former shows an abundant ion at *m/z* 172 by the loss of $CH_2 = NH$ (Scheme 5) and the latter gives abundant ions at *m/z* 90 (loss of $(CH_3)_2CHCH_2CN$ and CO) and *m/z* 86 corresponding to the immonium ion of leucine[21] (Scheme 6). Thus, the MS^3 spectra of *m/z* 201 ion of **5** and **7** are also useful to distinguish between the ureidopeptides derived from glycine and leucine at the N-terminus.

It is interesting to note that the MS^3 CID of *m/z* 252 (protonated (9H-fluoren-9-yl) methyl methylenecarbamate) ion from **5** derived from glycine at the N-terminus forms an intense product ion of *m/z* 191 that corresponds to an unusual loss of 61 units (Fig. 4). The mechanism of formation of this ion may presumably involve an ion–neutral complex (INC)[22,23] of fluorenylethaniminium ion and CO_2[24] that eliminate CO_2 and NH_3 simultaneously to form *m/z* 191 ($C_{15}H_{11}^+$) (Scheme 7). The formation of the INC seems to be triggered by a region-specific 1,2-hydride abstraction[25] by the imine nitrogen followed by migration of methylfluorenyl to the methyne carbon involving loss of CO_2 (Scheme 7). The elemental composition of *m/z* 191 ($C_{15}H_{11}^+$) ion has been

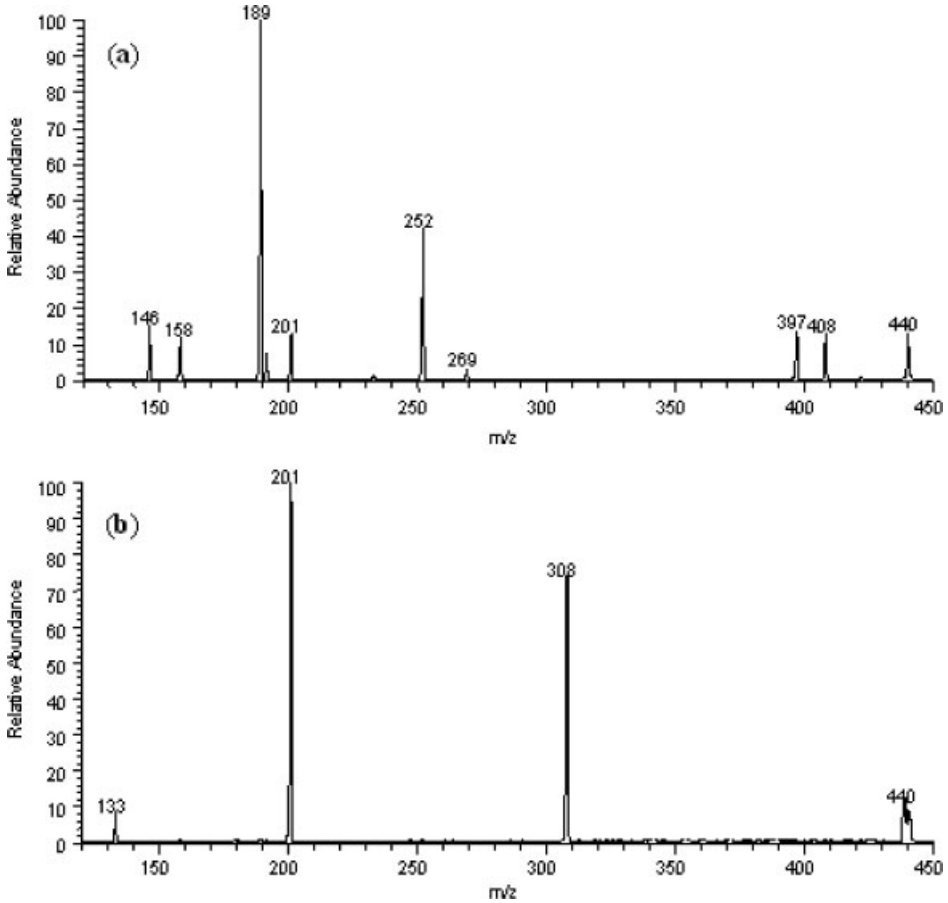


Figure 3. ESI-MS/MS spectra of $[M + H]^+$ ions (*m/z* 440) of (a) **5** and (b) **7** at 23 eV.

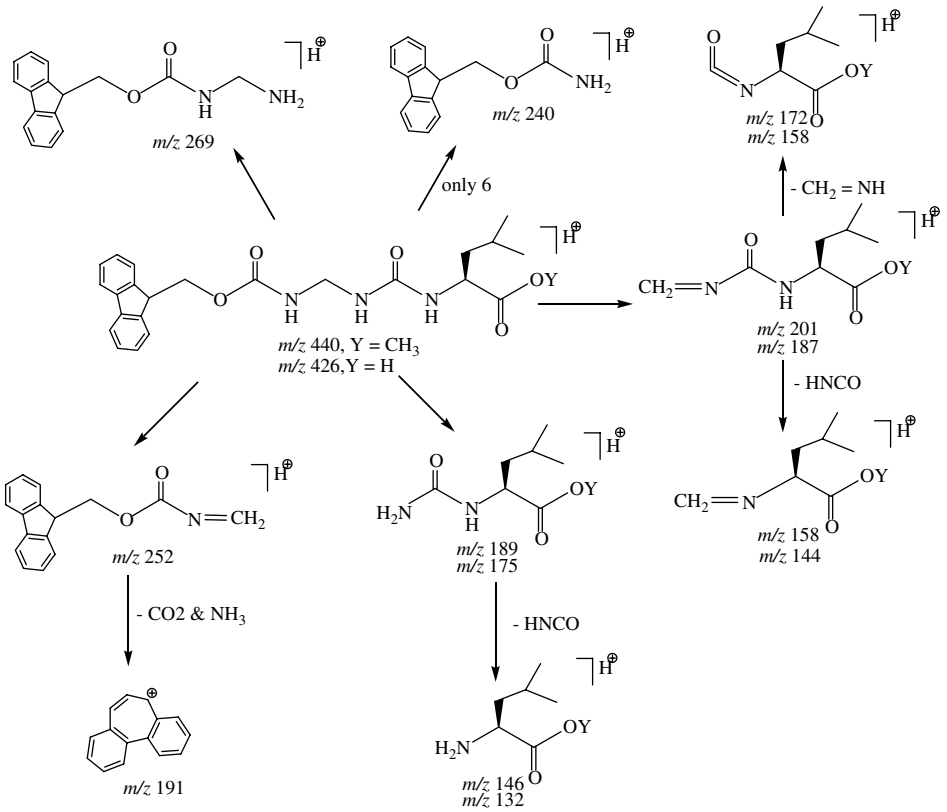
confirmed by accurate mass measurements performed on a QTOF mass spectrometer (calculated *m/z* 191.0860, observed *m/z* 191.0862 and error 0.6518 ppm). This fragmentation process has been observed for all the ureidopeptides (**9, 10, 13, 15**–**20**) derived from glycine at the N-terminus as will be discussed later. The concomitant loss of CO_2 and NH_3 (61 units) or consecutive elimination of NH_3 and CO_2 for the $[Ag–Phe]^+$ systems[26,27] through zwitter ionic structure was reported earlier.[28–30] The loss of 61 units is absent for **7** derived from leucine at the N-terminus, instead, the MS^3 spectrum of its *m/z* 308 shows *m/z* 264 (loss of CO_2) and *m/z* 179 corresponding to (9H-fluoren-9-yl)-methyl cation ($C_{14}H_{11}^+$) (Scheme 7). The elemental composition of *m/z* 179 ($C_{14}H_{11}^+$) ion also has been confirmed by accurate mass measurements (calculated *m/z* 179.0861, observed *m/z* 179.0863 and error 0.6518 ppm).

Similarly, the positional isomers of corresponding acids **6** and **8** can be clearly distinguished from one another, except that the masses of the C-terminal ions are decreased by 14 Da. In addition, the *m/z* 240 ion corresponding to protonated (9H-fluoren-9-yl) methyl carbamate (Scheme 5) is significant for acid **6** and absent for corresponding ester **5**.

The CID mass spectra of $[M + H]^+$ ions (*m/z* 474) of the third pair of isomers Fmoc-Gly-φ(NH-CO-NH)-Phe-OCH_3 (**9**) and Fmoc-Phe-φ(NH-CO-NH)-Gly-OCH_3 (**11**) yield product ion spectra that are distinctly different from one another (Table 1 and also Supporting Information). Similar to the previous isomers, both **9** and **11** display an ion at *m/z* 235 corresponding to the loss of (9H-fluoren-9-yl) methyl carbamate. Besides, **9** shows fragment ions at *m/z* 442 (loss of methanol), *m/z* 431 (loss of HNCO), *m/z* 269 (protonated (9H-fluoren-9-yl)methyl aminomethyl carbamate), *m/z* 252 (loss of (*S*)-methyl 3-phenyl-2-ureidopropanoate), *m/z* 223 (protonated (*S*)-methyl 3-phenyl-2-ureidopropanoate), *m/z* 192 (loss of HNCO from *m/z* 235) and *m/z* 180 (loss of HNCO from *m/z* 223) which are absent for **11**. Instead, the latter shows a peak at *m/z* 342 (loss of methyl 2-ureidoacetate), a characteristic ion for the ureidopeptides derived from phenylalanine at the N-terminus. It can be noted that the ion at *m/z* 235 is relatively more abundant for **11** compared with **9** (Table 1). Thus, the positional isomers **9** and **11** can be readily distinguished from one another by means of different product ions.

The MS^3 spectrum of *m/z* 235 of **9** shows (Supporting Information) *m/z* 218 (loss of NH_3), *m/z* 206 (loss of imine), *m/z* 203 (loss of methanol), *m/z* 190 (loss of CO from *m/z* 218) and abundant ion at *m/z* 192 (loss of HNCO) which are totally absent for **11**. Instead, the latter shows an abundant ion at *m/z* 120 (immonium ion) and low abundance ions at *m/z* 116 (loss of benzyl imine) and *m/z* 90 (protonated glycine ester). Thus, the MS^3 spectrum of *m/z* 235 ion of **11** is also distinctly different from that of **9**.

As explained above for **5**, the MS^3 spectrum of *m/z* 252 of **9** derived from glycine at the N-terminus shows a peak at *m/z* 191 formed by the direct loss of 61 units. Whereas the MS^3 spectrum of *m/z* 342 of **11** derived from phenylalanine at the N-terminus shows (Supporting Information) *m/z* 298 corresponding to the loss of CO_2, *m/z* 179 ((9H-fluoren-9-yl) methyl cation ($C_{14}H_{11}^+$)) (Scheme 7), low abundance ions at *m/z* 164 (loss of 9-methylene-9H-fluorene



Scheme 5. Proposed fragmentation mechanism for protonated ureidopeptides **5** and **6**.

(Fme)) and *m/z* 120 (loss of Fmoc).[31] The formation of the *m/z* 120 ion can be rationalized in terms of a McLafferty-type rearrangement involving a γ-hydrogen migration from fluorenyl moiety to the carbonyl oxygen in the Fmoc-*N*-moiety followed by the loss of 9-methylene-9H-fluorene (Fme) (178 Da) and subsequent loss of CO_2.

Similarly, the positional isomers of corresponding acids **10** and **12** can be clearly distinguished from one another, except that the masses of the C-terminal ions are decreased by 14 Da. In addition, the *m/z* 240 ion corresponding to protonated (9H-fluoren-9-yl) methyl carbamate is significant for acid **10** and absent for corresponding ester **9**.

The CID of $[M + H]^+$ ions (*m/z* 398) of isomers Fmoc-Gly-φ(NH-CO-NH)-Ala-OCH_3 (**13**) and Fmoc-Ala-φ(NH-CO-NH)-Gly-OCH_3 (**14**) yields *m/z* 355 (loss of HNCO), *m/z* 159 (loss of (9H-fluoren-9-yl) methyl carbamate) and *m/z* 116 (loss of HNCO from *m/z* 159) (Table 1 and also Supporting Information). Besides, **13** shows *m/z* 380 (loss of water), *m/z* 366 (loss of methanol), *m/z* 269 (protonated (9H-fluoren-9-yl)methyl aminomethyl carbamate), *m/z* 252 (loss of (*S*)-methyl 2-ureidopropanoate), *m/z* 147 (protonated (*S*)-methyl 2-ureidopropanoate) and *m/z* 191 (loss of 61 units from *m/z* 252) which are totally absent for **14**. Instead, the latter shows *m/z* 266 (loss of methyl 2-ureidoacetate) and *m/z* 133 (protonated methyl 2-ureidoacetate). It is noteworthy that the intensity of the *m/z* 159 ion becomes highly abundant for **14** than **13** (Table 1 and also Supporting Information). Thus, this pair of isomers can be clearly distinguished from one another by means of different product ions.

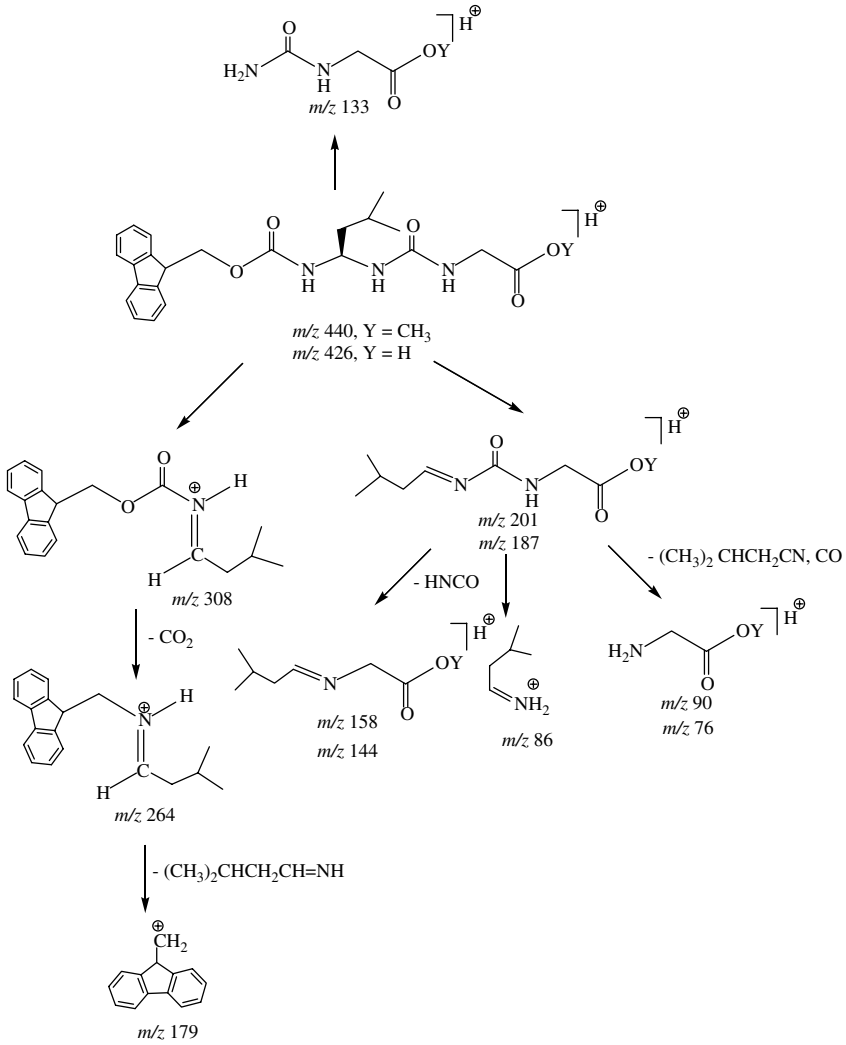
As observed in case of **5** and **9**, the MS^3 CID of *m/z* 252 from **13** also gives *m/z* 191 ($C_{15}H_{11}^+$) ions by the direct loss of 61 units. Thus, it can be generalized that protonated ureidopeptides derived from glycine at the N-terminus undergo simultaneous loss of CO_2 and NH_3 (61 units) to form $C_{15}H_{11}^+$ ions. Instead, only loss of CO_2 from *m/z* 266 leads to *m/z* 222 in case of **14** (Scheme 7). It also shows (Supporting Information) a peak at *m/z* 179 corresponding to (9H-fluoren-9-yl) methyl cation ($C_{14}H_{11}^+$).

The direct loss of 61 units from *m/z* 252 ion of ureidopeptides derived from glycine at the N-terminus has been supported by the study of some more ureidopeptides (**15–20**) derived from glycine at the N-terminus by varying C-terminus amino acid. All these compounds showed the direct loss of 61 units giving $C_{15}H_{11}^+$ in the MS^3 CID of *m/z* 252 ions. Other MS^2 fragmentations from these compounds are given in Table 1.

Negative ion CID of isomeric ureidopeptide acids

As peptide acids are known to form negative ions more readily than the esters, we examined ureidopeptide acids under negative ion ESI conditions. The negative ion ESI mass spectra of the ureidopeptide acids show $[M - H]^-$ ion as the base peak. In contrast to the positive ions that do not eliminate Fmoc, negative ion ESI CID of $[M - H]^-$ ion (*m/z* 472) of isomeric acids **2** and **4** yields (Table 2 and also Supporting Information) a low abundance ion at *m/z* 294 (loss of 9-methylene-9H-fluorene (Fme)) and an abundant ion at *m/z* 250 ($[M - H - Fmoc + H]^-$) by a loss of Fmoc.[31] The formation of the latter ion can be rationalized in terms of a McLafferty-type rearrangement involving a γ-hydrogen migration from fluorenyl moiety to the carbonyl oxygen in the FmocN-moiety followed by the loss of 9-methylene-9H-fluorene (Fme) (178 Da) and subsequent loss of CO_2 (Scheme 8).

The MS^3 spectra of isomers **2** and **4** (*m/z* 250) show distinctly different fragmentation from one another (Table 2 and also



Scheme 6. Proposed fragmentation mechanism for protonated ureidopeptides **7** and **8**.

Table 1. Partial CID of $[M + H]^+$ of ureidopeptides **9, 11, 13–20:** *m/z* with relative abundance (%) in parenthesis

Compound	$[M + H]^+$	Loss of H_2O	Loss of CH_3OH	Loss of HNCO	Other ions
9	474	–	442 (5)	431 (5)	269 (1), 252 (10), 235 (12), 223 (100), 192 (18), 180 (20)
11	474	–	–	–	342 (38), 235 (100)
13	398	380 (2)	366 (8)	355 (12)	269 (2), 252 (100), 191 (6), 159 (2), 147 (20), 116 (3)
14	398	–	–	355 (4)	266 (42), 159 (100), 133 (66), 116 (4)
15	384	366 (2)	352 (3)	341 (12)	252 (100), 191 (2), 133 (2)
16	370	352 (2)	–	327 (5)	252 (100), 240 (12), 191 (2), 179 (5)
17	426	408 (1)	394 (20)	383 (12)	269 (3), 252 (86), 191 (10), 187 (14), 175 (100), 144 (12), 132 (12)
18	412	394 (10)	–	369 (8)	269 (2), 252 (100), 240 (6), 191 (16), 179 (6), 161 (12), 118 (4)
19	460	442 (2)	428 (7)	417 (12)	269 (2), 252 (40), 221 (7), 209 (100), 191 (6), 178 (60), 166 (44), 149 (10)
20	446	428 (7)	–	403 (10)	281 (60), 269 (2), 252 (100), 240 (38), 208 (30), 195 (50), 191 (25), 179 (44), 164 (24), 152 (75), 135 (12)

Supporting Information). The CID spectra of both the isomers display an ion at *m/z* 232 (loss of H_2O). Besides, **2** shows an abundant ion at *m/z* 164 (loss of isocyanate) and low abundance ions at *m/z* 207 (loss of $CH_3CH{=}NH$) and *m/z* 190 (loss of NH_3 from *m/z* 207) which are absent for **4**. Instead, the latter shows *m/z* 131 (loss of $PhCH_2CH{=}NH$), *m/z* 113 (loss of H_2O from *m/z* 131) and an abundant ion at *m/z* 88 (loss of isocyanate) (Scheme 8). Similarly, other two pairs of isomeric acids, **6**/**8** and **10**/**12** can be clearly distinguished from each other from their characteristic fragmentation in MS^3 (Scheme 8 and Table 2). The compounds **16** and **18** also show similar fragmentation (Scheme 8) and the data are presented in Table 2.

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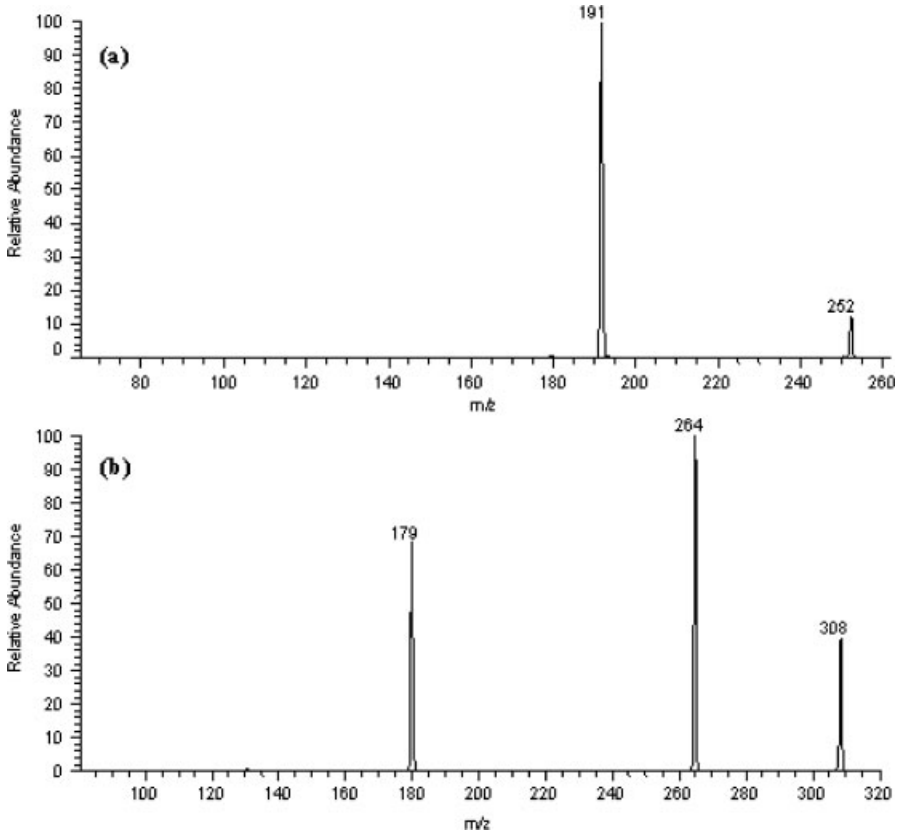
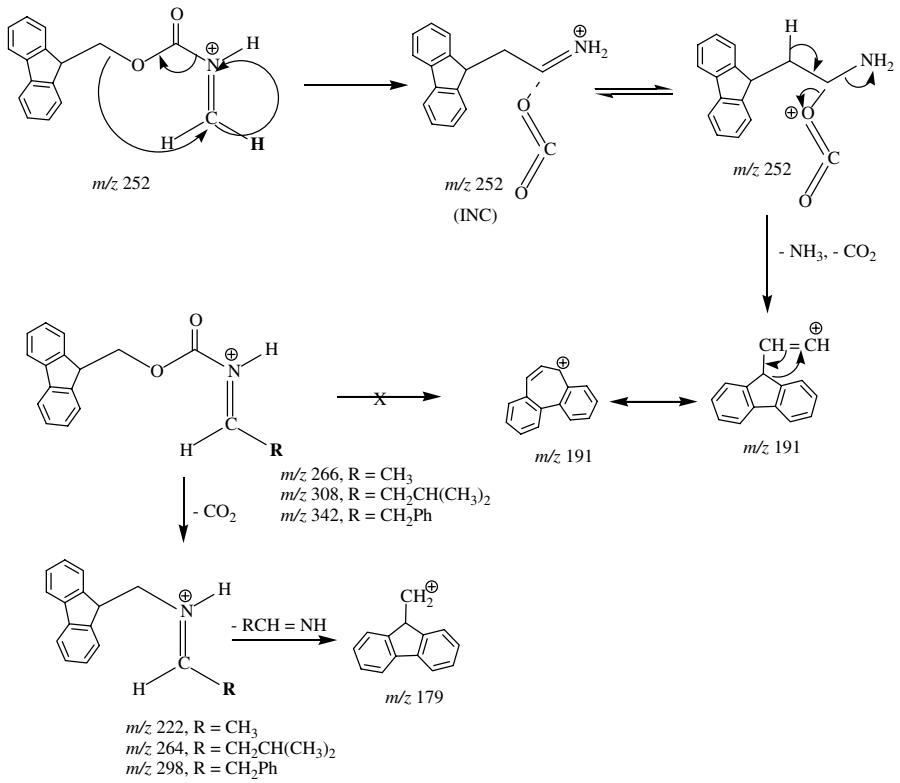


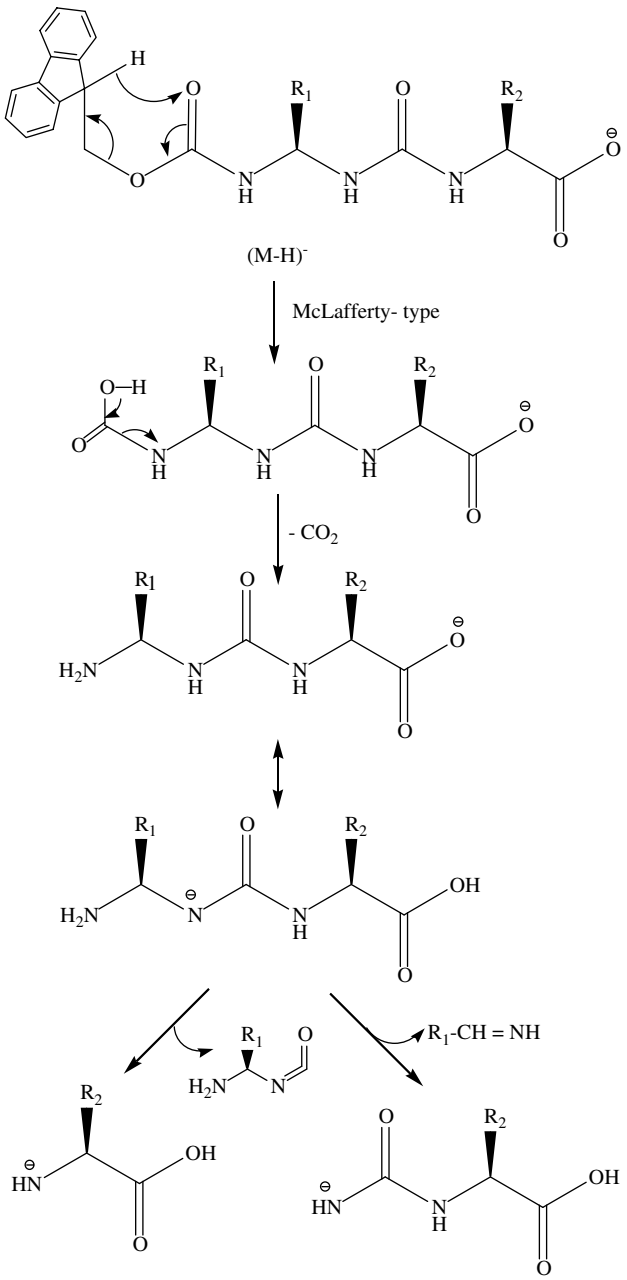
Figure 4. ESI-MS^3 spectra of *m/z* 252 of (a) **5** and *m/z* 308 of (b) **7** at 20 eV.



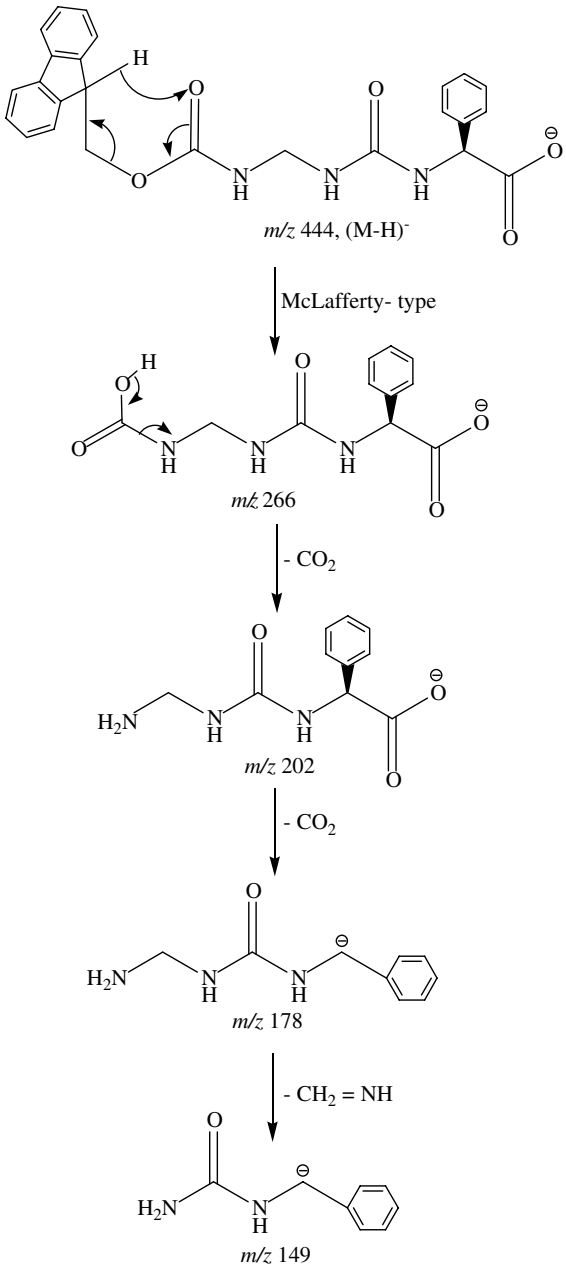
Scheme 7. Proposed fragmentation mechanism for loss of 61 units via ion–neutral complex.

Table 2. Partial CID of $[M - H]^-$ of ureidopeptide acids **2**, **4**, **6**, **8**, **10**, **12**, **16**, **18** and **20**: *m/z* with relative abundance (%) in parenthesis

Compound	$[M - H]^-$	$[M - H - Fme]^-$	$[M - H - Fmoc + H]^-$	Other ions
2	472	294 (3)	250 (100)	250→ 232 (5), 207 (8), 190 (5), 164 (100)
4	472	294 (3)	250 (100)	250→ 232 (10), 131 (50), 113 (10), 88 (100)
6	424	246 (8)	202 (100)	202→ 184 (100), 173 (15), 156 (10), 155 (6), 130 (98)
8	424	246 (8)	202 (100)	202→ 184 (5), 117 (60), 100 (10), 99 (6), 74 (100)
10	458	280 (3)	236 (100)	236→ 218 (22), 207 (3), 190 (4), 189 (2), 164 (100)
12	458	280 (3)	236 (100)	236→ 218 (12), 117 (100), 100 (14), 99 (4), 74 (72)
16	368	190 (30)	146 (100)	146→ 128 (60), 117 (40), 100 (20), 99 (3), 74 (100)
18	410	232 (3)	188 (100)	188→ 170 (100), 159 (12), 142 (8), 141 (10), 116 (90)
20	444	266 (3)	222 (100)	222→ 178 (100)→ 149 (100)



Scheme 8. Proposed fragmentation mechanism for deprotonated ureidopeptides **2, 4, 6, 8, 10, 12, 16** and **18**.



Scheme 9. Proposed fragmentation mechanism for deprotonated ureidopeptide **20**.

In contrast to the isomers discussed above, compound **20** that has a phenyl glycine at the C-terminus fragments differently. After the loss of Fmoc, the $[M - H - Fmoc + H]^-$ (*m/z* 222) eliminates an additional CO_2 from the C-terminus to form *m/z* 178 (Scheme 9). This loses imine to form *m/z* 149 as evidenced from the MS^4 spectrum of *m/z* 178 (Table 2 and also Supporting Information). Formation of these fragments may be attributed to the generation of a highly stable phenyl carbanion as shown in Scheme 9.

Conclusions

Positive and negative ion ESI-MS/MS has been shown to be very useful for the structural characterization and differentiation of four pairs of N^{α}-Fmoc-protected ureidopeptide positional isomers. The major fragmentation noticed in all these compounds is due to –N–CH(R)–N– bond cleavage. The MS^2 of $[M + H]^+$ ions of all the studied ureidopeptide acids derived from glycine at the N-terminus yield an ion at *m/z* 240 which is absent for corresponding esters. Another interesting fragmentation noticed in these compounds is the loss of 61 units from an intermediate fragment ion $FmocNH=CH_2^+$ (*m/z* 252). A mechanism involving an INC and a direct loss of NH_3 and CO_2 is proposed for this process. Whereas ureidopeptides derived from alanine, leucine and phenylalanine at the N-terminus eliminate CO_2 followed by corresponding imine to form (9H-fluoren-9-yl)methyl cation ($C_{14}H_{11}^+$) from $FmocNH{=}CHR^+$. In contrast to the positive ions, the negative ions of N^{α}-Fmoc-protected ureidopeptide acids undergo a McLafferty-type rearrangement ion involving loss of Fme and Fmoc. It is worth to note that the losses of HNCO ions are totally absent in negative ion spectra. It can be concluded that the ESI-MS/MS study of both the protonated and deprotonated ureidopeptides provides the fragmentation behavior of this new class of compounds and also useful for differentiating these isomeric ureidopeptides.

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Supporting information

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

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