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Characterization of *N,N*-dimethyl amino acids by electrospray ionization–tandem mass spectrometry

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Methylation is an essential metabolic process for a number of critical reactions in the body. Methyl groups are involved in the healthy function of the body life processes, by conducting methylation process involving specific enzymes. In these processes, various amino acids are methylated, and the occurrence of methylated amino acids in nature is diverse. Nowadays, mass-spectrometric-based identification of small molecules as biomarkers for diseases is a growing research. Although all dimethyl amino acids are metabolically important molecules, mass spectral data are available only for a few of them in the literature. In this study, we report synthesis and characterization of all dimethyl amino acids, by electrospray ionization–tandem mass spectrometry (MS/MS) experiments on protonated molecules. The MS/MS spectra of all the studied dimethyl amino acids showed preliminary loss of $H_2O + CO$ to form corresponding immonium ions. The other product ions in the spectra are highly characteristic of the methyl groups on the nitrogen and side chain of the amino acids. The amino acids, which are isomeric and isobaric with the studied dimethyl amino acids, gave distinctive MS/MS spectra. The study also included MS/MS analysis of immonium ions of dimethyl amino acids that provide information on side chain structure, and it is further tested to determine the *N*-terminal amino acid of the peptides. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: *N,N*-dimethyl amino acids; protonated molecules; immonium ions; electrospray ionization; tandem mass spectrometry

Introduction

Methylation is the process of controlled transfer of methyl groups onto small molecules (amino acids, amino alcohols, hydroxyl acids, etc.), proteins/enzymes and DNA in every cell and tissue of the body that regulate a number of critical biochemical pathways. Several articles in the literature have demonstrated that the methylation process is responsive to environmental conditions and degrades with age, a process associated with a large variety of age-related disorders. Methylation process is an important dividing line between health and disease. In fact, healthy methylation processes are synonymous with good health, and inability to methylate is synonymous with symptom expressions and poor health. In biological systems, there are several approaches that theoretically could increase the availability of methyl groups. Methionine, an amino acid, contains a methyl group that is subsequently used in the process of methyl donation to many proteins and other molecules. *S*-Adenosyl-methionine (SAM) is an important carrier molecule, which donates methyl groups to a host during biological reactions. Betaines also acts as methyl donor in biological pathways and used as a dietary supplement. Lack of methyl group donors (methylation process) in human body can lead to many diseases like cancer, autism, diabetes, chronic fatigue and Alzheimer disease. In addition, methylated derivatives play an essential role in biological functions which are potential biomarkers of human physiological processes.[1,2] For example, an abnormal urinary excretion of methyl donor, betaine has been observed in many patients with diabetes. As high urinary betaine loss in diabetes mellitus patients, potentially causing a betaine insufficiency that could be expected to have complex consequences in the supply of methyl groups essential for normal metabolism. Sarcosine was identified as potential biomarker for prostate cancer,[3] while dimethylglycine was found to be increased in the plasma of chronic renal failure patients.[4] The methylated derivatives of arginine, asymmetric dimethylarginine and symmetric dimethylarginine are markers that are associated with chronic kidney disease and renal inflammation.[5–8] Kakimoto *et al.* reported isolation and identification of the symmetric/asymmetric dimethylarginines and mono-/di-/tri-methyl derivatives of lysine in human urine.[9] Identification of *N*-methylated basic amino acids[10] and *N*-methylated acidic amino acids[11] in biological tissues were also reported in the literature. Bouatra *et al.* reported methylated metabolites in human urine using nuclear magnetic resonance spectroscopy, gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) techniques.[12]

As methylation involves specific methylating enzymes, the methylated derivatives in nature are extremely diverse in association

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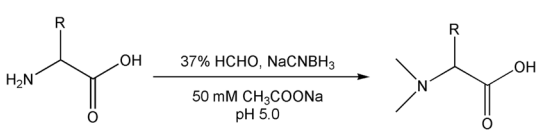
with small metabolites like amines, acids and alcohols. Amino acids are one such class of metabolites that can undergo methylation during metabolism. The methylated amino acid metabolites play an essential role in understanding the biological reactions, and thereby the changes in their levels contribute to the development of new diagnostic and therapeutic methods to diagnose specific diseases. During the analysis of biological samples, low molecular weight metabolites like amino acids and their methylated derivatives may also be encountered. For example, in the analysis of the untreated tissue of *Torpedo californica* electric organ by mid-infrared laser ablation electrospray ionization (ESI) mass spectrometric method, some of the amino acids and methylated amino acids like *N*-methyl and *N,N*-dimethyl derivatives of same nominal masses (*N*-methyl glycine or beta-alanine; dimethylglycine or GABA and dimethyllysine) were detected.[13]

Characterization of dimethylated amino acids is a part in understanding overall methylated metabolites of a cell or tissue. Moreover, nowadays the amino acids in biological matrices have been detected after converting them into *N,N*-dimethyl amino acids by methylation.[14] To the best of our knowledge, the MS/MS spectra of a few dimethyl derivatives of amino acids, i.e. glycine, histidine, arginine and lysine were available in the literature and databases (mass bank, http://www.massbank.jp/index.html; HMDB, http://www.hmdb.ca/spectra/ms/search and Metlin;http://metlin.scripps.edu/index.php). So it is important to study the mass spectral characterization of dimethyl derivatives of all the amino acids. In the previous study,[15] we have characterized all the amino acid derived betaines. The present study mainly focused on structural characterization of *N,N*-dimethyl amino acids by MS/MS and high-resolution mass spectrometry (HRMS) analyses under ESI conditions. The study is also explored to detect the *N*-terminal amino acid of peptides by applying the MS/MS of immonium ion.

Experimental

All the L-amino acids, 37% formaldehyde and sodium cyanoborohydrate ($NaCNBH_3$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All the solvents were of high performance liquid chromatography grade, and were purchased from Merck (Mumbai, India). The dipeptide (Met-Leu) and tetra peptide (Lys-Ala-Ala-Ala) were obtained from GenPro Biotech (C-39, Sector 10, Noida, Gautham Budh Nagar, Uttar Pradesh-201301, India).

All dimethyl amino acids were synthesized by methylation of free amino acids using the known procedure (Scheme 1).[16] Briefly, one equivalent of amino acid was dissolved in 50 mM sodium acetate buffer (pH 5) and immediately added 10 equivalents of 37% formaldehyde and 10 equivalents of sodium cyanoborohydride. The mixture was stirred at ambient temperature for 15–20 min. The reaction mixture was stored in a refrigerator until it was subjected to mass spectrometric analysis. The samples were diluted three times with acetonitrile and acidified with diluted HCl before injecting into the mass spectrometer. The same procedure was applied for methylation of the dipeptide (Met-Leu) and tetrapeptide (Lys-Ala-Ala-Ala).



Scheme 1. Synthetic procedure for *N,N*-dimethyl amino acids.

Mass spectral analysis of all the studied compounds (Scheme 2) were carried out on a quadrupole time-of-flight (Q-TOF) mass spectrometer (Q-Star XL, Applied Biosystems, USA), equipped with an ESI source using Analyst software. The samples were introduced into the source through a flow injection, where methanol was used as the mobile phase at a flow rate of 30 μl/min. The typical positive ESI conditions were capillary voltage 5.00 kV, declustering potential 60 V, focusing potential 220 V, and resolution 10,000 (FWHM). Collision-induced dissociation (CID) spectra were obtained by selecting the ion of interest using the quadrupole and then allowed to fragment in the collision cell by colliding with nitrogen, followed by detecting the resulted product ions by the TOF analyzer. The CID spectra were recorded at different collision energies (10, 15, 20 and 25 eV). The spectra of isomeric/isobaric ions were obtained at similar experimental conditions. For recording the CID spectra of immonium ions of dimethyl amino acids, the immonium ions were generated in the source by applying a higher declustering potential (100–160; in-source fragmentation).

Results and discussions

The chemical structures of the methylated amino acids (**1–23**) were shown in Scheme 2. The positive ion ESI mass spectra of all the methylated amino acid solutions, except lysine, showed abundant $[M+H]^+$ and $[M+Na]^+$ ions of *N,N*-dimethyl amino acids (where α-NH_2 group of the amino acids was methylated; hereafter these compounds are referred as dimethyl amino acids, **1–22**). Methylation of the lysine resulted in the tetramethyllysine (**23**), where both α-NH_2 and ε-NH_2 were methylated. The products due to methylation of other functional groups like –COOH, –OH, –SH and guanidinium were not observed. The $[M+H]^+$ ions of all the methylated amino acids (**1–23**) were further subjected to CID experiments to obtain their structural information (spectra are summarized in Table 1). The CID spectra together with HRMS data (Tables S1 and S2, see supplementary information) were used to characterize the dimethyl amino acids. In some cases, CID spectra recorded for the product ions generated by in-source fragmentation were also used. Fragmentation pattern of $[M+H]^+$ ions of dimethyl amino acids is expected to be different when compared with that of corresponding free amino acids, because methylation of amino group tends to increase the proton affinity leading to facile protonation by mobile proton. For comparison purpose, we have also recorded the CID spectra of $[M+H]^+$ ions of corresponding free amino acids (Table S3). The fragmentation pattern observed for $[M+H]^+$ ions of free amino acids was similar to those reported in the literature.[17–22]

We observed that the $[M+H]^+$ ions of all the dimethyl amino acids were fragmented at higher collision energies than those of corresponding free amino acids. The dimethyl amino acids showed primarily the elimination of (H_2O+CO) in addition to other specific product ions due to methyl groups on nitrogen as well as the substituent (–R) on the α-carbon of the amino acids. For convenience, these compounds were divided into four groups based on the nature of –R group that include aliphatic (**1–12**), aromatic (**13–16**), acidic (**17** and **18**) and amidic or basic (**19–22**) dimethyl amino acids. The isomeric dimethyl amino acids showed distinct spectra, which were also discussed subsequently. Some of the dimethyl amino acids are found to be isomeric with other free amino acids, and such cases were also included in the discussion. The possible isomeric and isobaric amino acids and their methyl derivatives were listed in Table 2.

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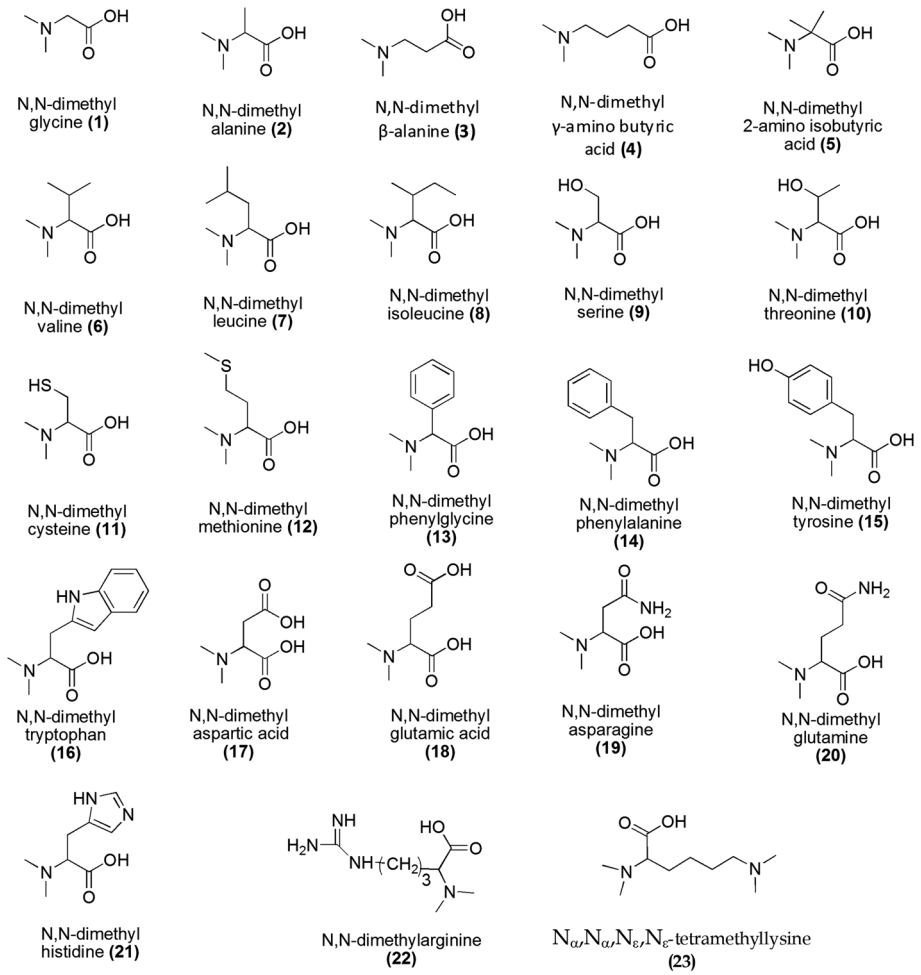

Scheme 2. The chemical structures of the studied compounds (**1–23**).

CID of $[M+H]^+$ ions

Aliphatic dimethyl amino acids (1–12)

Dimethyl glycine (**1**) is found in blood and urine samples and has a variety of biological effects.[23,24] The CID mass spectrum of $[M+H]^+$ of **1** showed the $[MH\text{-}(H_2O+CO)]^+$ (*m/z* 58) as the base peak (which is similar to that of glycine, Table S3). The spectrum of **1** included minor product ions at *m/z* 86 and 30 corresponding to $[MH\text{-}H_2O]^+$ and $[H_2C{=}NH_2]^+$ ions, respectively. In real biological samples, detection of dimethylglycine (**1**) may be challenging because it is isomeric with γ-aminobutyric acid (GABA) and 2-aminoisobutyric acid (2-AIB), which may occur in a given sample. In such cases, MS/MS analysis of their protonated species can help in peak identification. Hence, the CID spectra of GABA and 2-AIB were also recorded and compared with that of **1** (Figure S1). The spectra of $[M+H]^+$ ions of 2-AIB and **1** showed the base peak at *m/z* 58. The former included distinctive product ions at *m/z* 87, $[MH\text{-}NH_3]^+$ and *m/z* 41, $[MH\text{-}(H_2O+CO)\text{-}NH_3]^+$, which are absent for the latter. While the spectrum of $[M+H]+$ of **1** showed a specific ion at *m/z* 30, and that of GABA displayed $[MH\text{-}NH_3]^+$ ion as the major product ion in addition to loss of H_2O, (NH_3+H_2O), and (H_2O+CO) giving rise to ions at *m/z* 86, 69 and 58, respectively.

The CID spectrum of *N,N*-dimethylalanine (**2**) showed the $[MH\text{-}(H_2O+CO)]^+$ ion (*m/z* 72) as the major product ion. Other product ions were due to further loss of H_2, $^{\bullet}CH_3$ radical and CH_4 from $[MH\text{-}(H_2O+CO)]^+$ ion or corresponding to $[C_3H_8N]^+$ (*m/z* 58) and $[C_2H_6N]^+$ (*m/z* 44) ions. The CID spectrum of protonated *N,N*-dimethyl-*β*-alanine (**3**), which is isomeric to **2**, showed the base peak at *m/z* 58 corresponding to $[MH\text{-}CH_3COOH]^+$ ion. The other product ions at *m/z* 72, 44, 42 and 30 were found to be of low abundance. The compounds **2** and **3** are also isomeric to valine and glycine betaine, and they can easily be distinguished based on distinct CID spectra of their $[M+H]^+$ ions (Fig. 1). The CID spectrum of $[M+H]^+$ of valine showed the $[MH\text{-}(H_2O+CO)]^+$ (*m/z* 72) as the base peak as found in **2**, but it included a specific product ion $[MH\text{-}(H_2O+CO)\text{-}NH_3]^+$ (*m/z* 55).

The CID spectrum of $[M+H]^+$ ion of *N,N*-dimethyl GABA (**4**) is very much different from that of its isomer, *N,N*-dimethyl-2-amino isobutyric acid (**5**) (Fig. 2). The spectrum of **4** showed $[MH\text{-}HN(CH_3)_2]^+$ (*m/z* 87; 100%), $[MH\text{-}H_2O]^+$ (*m/z* 114; 28%) and other product ions at *m/z* 71, 69, 46, 45 and 43; whereas, the expected $[MH\text{-}(H_2O+CO)]^+$ ion was absent. The protonated **5** showed dominant $[MH\text{-}(H_2O+CO)]^+$ (*m/z* 86) and the characteristic product ions of **4** (*m/z* 114, 87, 86, 46 and 43) were found to be negligible. The compounds **4** and **5** are also isomeric with the free amino acids, leucine and isoleucine, but they can be easily discriminated based on the specific fragmentation of their protonated species.

The CID spectra of the protonated *N,N*-dimethylvaline (**6**) and *N,N*-dimethyl leucine/*N,N*-dimethylisoleucine (**7**/**8**) showed the $[MH\text{-}(H_2O+CO)]^+$ ion as the base peak. The anticipated $[MH\text{-}(HN(CH_3)_2)]^+$ ion, similar to $[MH\text{-}NH_3]^+$ ion found in free amino acids, was absent in the spectra of **6–8**. Between the isomeric

Table 1. Collision-induced dissociation mass spectra of $[M+H]^+$ ions of methylated amino acids (**1–23**)

Comp. Number	$[M+H]^+$ *m/z* (% RA)	Product ions, *m/z* (% RA)			
		$[M+H\text{-}H_2O]^+$	$[M+H\text{-}(H_2O+CO)]^+$	$[M+H\text{-}HN(CH_3)_2]^+$	Other ions
1	104 (46)	86 (1)	58 (100)		44 (2), 43 (1), 42 (1), 30 (2)
2	118 (100)	100 (1)	72 (40)		70 (3), 57 (2), 44 (1)
3	118 (6)	100 (2)	72 (1)		58 (100), 46 (1), 44 (2), 42 (1), 30 (1), 28 (1)
4	132 (18)	114 (28)	86 (6)	87 (100)	71 (3), 69 (5), 46 (29), 45 (16), 44 (9), 43 (28), 41 (2)
5	132 (38)	114 (1)	86 (100)	87 (2)	46 (2)
6	146 (13)		100 (100)		46 (2), 44 (4)
7	160 (13)		114 (100)		72 (3), 58 (3)
8	160 (16)		114 (100)		69 (2), 58 (1), 46 (3)
9	134 (20)	116 (46)	88 (100)		104 (2), 98 (27), 74 (25), 73 (2), 72 (17), 58 (3), 57 (6), 46 (2), 44 (13), 30 (1)
10	148 (18)	130 (100)	102 (51)		112 (4), 104 (30), 89 (2), 88 (18), 86 (19), 85 (3), 84 (52), 74 (19), 72 (8), 70 (6), 58 (8), 56 (2), 46 (4), 45 (5), 44 (11)
11	150 (12)		104 (14)	105 (21)	116 (6), 87 (32), 78 (2), 59 (16), 58 (15), 46 (100)
12	178 (8)		132 (16)	133 (100)	147 (2), 130 (14), 118 (3), 105 (2), 102 (8), 87 (7), 85 (1), 84 (15)
13	180 (3)		134 (23)	135 (80)	121 (3), 120 (1), 107 (100), 91 (2), 79 (54), 77 (19)
14	194 (36)		148 (100)	149 (2)	131 (7), 107 (3), 102 (5), 58 (3), 46 (4)
15	210 (62)		164 (52)	165 (100)	147 (28), 123 (37), 121 (1), 119 (15), 103 (1), 102 (1), 95 (1), 59 (2), 58 (5), 46 (98), 30 (1)
16	233 (5)		187 (1)	188 (63)	202 (41), 169 (3), 160 (11), 158 (4), 146 (16), 144 (5), 129 (2), 117 (2), 115 (2), 102 (100), 101 (2), 97 (4), 88 (3), 79 (13), 57 (3), 46 (5)
17	162 (6)		116 (15)		102 (100), 74 (1), 56 (1)
18	176 (23)	158 (10)	130 (100)		145 (8), 144 (10), 116 (2), 98 (44), 85 (5), 84 (4), 72 (1), 70 (6), 46 (10)
19	161 (26)		115 (6)		144 (1), 102 (100)
20	175 (8)		129 (53)	130 (100)	158 (43), 112 (3), 102 (7),85 (5), 84 (21), 46 (9)
21	184 (2)		138 (100)		140 (1), 123 (8), 102 (4),95 (74),82 (2)
22	203 (23)	185 (3)	157 (3)	158 (21)	161 (3), 144 (100), 130 (9), 125 (4), 116 (6), 114 (34), 112 (15), 100 (52), 97 (5), 72 (6), 70 (8), 58 (3), 46 (2), 43 (3)
23	203 (12)		157 (7)	158 (100)	140 (1), 130 (6), 114 (18), 112 (3), 58 (1), 46 (1)

Collision energy (CE) = 20 eV, except **19** where CE = 15 eV.
RA, relative abundance.

compounds **7** and **8**, the spectrum of latter showed specific product ions at *m/z* 69 and 46 corresponding to $[MH\text{-}(H_2O+CO)\text{-}HN(CH_3)_2]^+$ and $[HN(CH_3)_2+H]^+$, respectively. The McLafferty-type rearrangement product ions from $[MH\text{-}(H_2O+CO)]^+$ ion was observed in the spectra of **7** and **8** (*m/z* 72 and 86, respectively), but they were of low abundance. In our previous study on *N*-substituted (acetyl, benzoyl and pivaloyl) leucine and isoleucines,[25] specific product ions were observed from their $[MH\text{-}COOH]^{+\bullet}$ ions. However, the $[MH\text{-}COOH]^{+\bullet}$ ions were absent in the dimethylleucine/isoleucine (**7** and **8**).

The CID spectra of protonated *N*-dimethylserine (**9**) and *N*,*N*-dimethylthreonine (**10**) showed loss of H_2O (Scheme 3) and (H_2O+CO) due to the presence of hydroxyl group on the side chain. The spectrum of **9** included the product ions produced by the loss of H_2O (18 u), CH_2CO (42 u), NC_2H_5 (43 u), and CO_2 (44 u) from the ion $[MH\text{-}H_2O]^+$ at *m/z* 98, 74, 73 and 72, respectively, which can be explained from the proposed linear structure (Scheme 3). The *m/z* values of these product ions are shifted by +14 u in the CID spectrum of protonated **10**; it also showed an additional product ion at *m/z* 70 due to the loss of CH_3COOH from $[MH\text{-}H_2O]^+$ ion. The spectrum of protonated **9** included the product ions at *m/z* 98, 74, 73 and 72, because of further loss of H_2O (18 u), CH_2CO (42 u), NC_2H_5 (43 u), and CO_2 (44 u), respectively, from the ion $[MH\text{-}H_2O]^+$ (Scheme 3); these product ions are shifted by +14 u in the spectrum of protonated **10**. The spectrum of **10** showed an additional product ion at *m/z* 70 due to the loss of CH_3COOH from $[MH\text{-}H_2O]^+$ ion. The spectra of **9** and **10** showed a common product ion at *m/z* 104 corresponding to the loss of CH_2O and C_2H_4O, respectively, which may be formed from $[M+H]^+$ ions through a McLafferty-type rearrangement involving migration of the hydrogen from hydroxyl group to the carbonyl group. The $[M+H]^+$ ion of compound **10** is isobaric with the $[M+H]^+$ ion of glutamic acid (same nominal mass); however, these two compounds can easily be distinguished by their distinct CID spectra (Fig. 3).

The compounds *N*,*N*-dimethylcysteine (**11**) and *N*,*N*-dimethylmethionine (**12**) have sulfur atoms in their side chains. CID spectra of their protonated species generated the $[MH\text{-}(H_2O+CO)]^+$ ions. In addition, the spectra displayed specific $[MH\text{-}HN(CH_3)_2]^+$ ion in the CID spectra of **11** and **12** that may be because of sulfur atom involving cleavage of C–N bond. The compound **11** showed loss of H_2S from $[M+H]^+$ ion (6%), whereas this loss was negligible in free cysteine (<1%). Other product ions, appeared at *m/z* 87 and 59 in the spectrum of **11**, were formed by loss of H_2O from $[MH\text{-}HN(CH_3)_2]^+$ and loss of dimethylamine from $[MH\text{-}(H_2O+CO)]^+$, respectively. The spectrum of **12** showed the $[MH\text{-}CH_3SH]^+$ (*m/z* 130) and $[MH\text{-}C_3H_8S]^+$ ion (*m/z* 102) that reflect the structure of its side chain. The fragmentation pattern of the $[M+H]^+$ of **12** was found to be similar to that observed for the $[M+H]^+$ of free methionine (Figure S2). The *N*,*N*-dimethylcysteine (**11**) is isomeric with free methionine, but their CID spectra are

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Table 2. Possible isomeric (same formula) and isobaric compounds (different formula with same nominal mass) among amino acids and their methyl derivatives

m/z of $[M+H]^+$ ions (chemical formula)	Possible isomeric and isobaric compounds
m/z 104 ($C_4H_{10}NO_2$)	γ-Aminobutyric acid (GABA) ($C_4H_{10}NO_2$)
	2-Aminoisobutyric acid (2-AIB) ($C_4H_{10}NO_2$)
	N-Methylalanine ($C_4H_{10}NO_2$)
	N-Methyl-β-alanine ($C_4H_{10}NO_2$)
	N,*N*-Dimethylglycine (DMG) ($C_4H_{10}NO_2$)
m/z 118 ($C_5H_{12}NO_2$)	Valine ($C_5H_{12}NO_2$)
	N-Methyl GABA ($C_5H_{12}NO_2$)
	N-Methyl-2-AIB ($C_5H_{12}NO_2$)
	N,*N*-Dimethylalanine ($C_5H_{12}NO_2$)
	N,*N*-Dimethyl-β-alanine ($C_5H_{12}NO_2$)
	Glycine betaine ($C_5H_{12}NO_2$)
m/z 132 ($C_6H_{14}NO_2$)	Leucine ($C_6H_{14}NO_2$)
	Isoleucine ($C_6H_{14}NO_2$)
	N-Methylvaline ($C_6H_{14}NO_2$)
	N,*N*-Dimethyl GABA ($C_6H_{14}NO_2$)
	N,*N*-Dimethyl-2-AIB ($C_6H_{14}NO_2$)
	Alanine betaine ($C_6H_{14}NO_2$)
	β-Alanine betaine ($C_6H_{14}NO_2$)
m/z 134	Aspartic acid ($C_4H_8NO_4$)
($C_5H_{12}NO_3$/ $C_4H_8NO_4$)	*N*-Methylthreonine ($C_5H_{12}NO_3$)
	N,*N*-Dimethylserine ($C_5H_{12}NO_3$)
m/z 148	Glutamic acid ($C_5H_{10}NO_4$)
($C_6H_{14}NO_3$/$C_5H_{10}NO_4$)	*N*-Methylaspartic acid ($C_5H_{10}NO_4$)
	N,*N*-Dimethylthreonine ($C_6H_{14}NO_3$)
m/z 175 ($C_7H_{15}N_2O_3$/	Arginine ($C_6H_{15}N_4O_2$)
$C_8H_{19}N_2O_2$/$C_6H_{15}N_4O_2$)	*N*,*N*-Dimethylglutamine ($C_7H_{15}N_2O_3$)
	N,*N*-Dimethyllysine ($C_8H_{19}N_2O_2$)
	Asparagine betaine ($C_7H_{15}N_2O_3$)

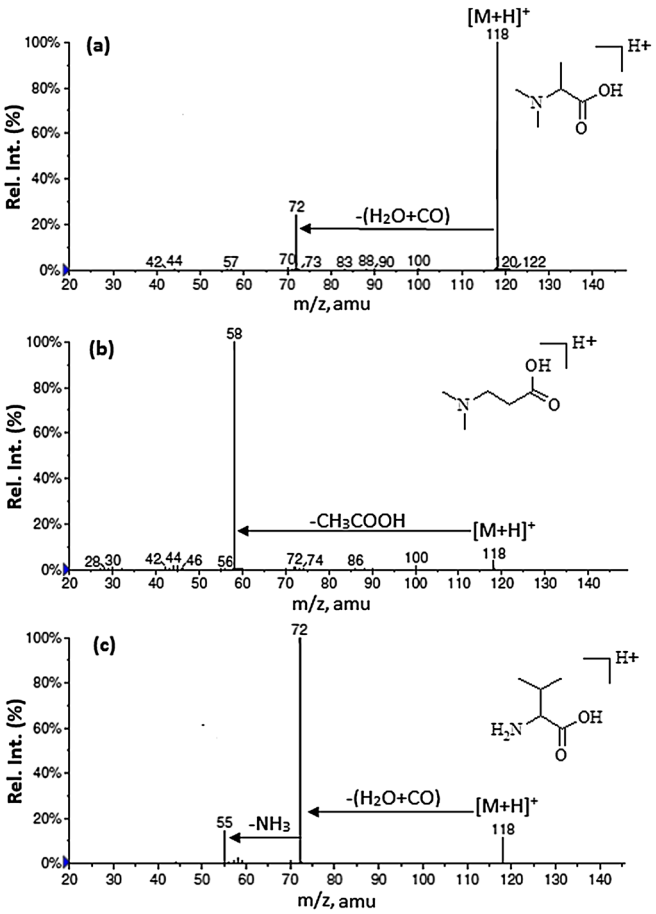


Figure 1. Collision-induced dissociation mass spectra of (a) *N*,*N*-dimethylalanine, (b) *N*,*N*-dimethyl-β-alanine and (c) valine at collision energy of 20 eV.

very much distinctive (Figure S2), where loss of NH_3 and $HN(CH_3)_2$ is characteristic to free amino acid and dimethyl amino acid, respectively.

Aromatic dimethyl amino acids (13–16)

The CID spectra of all the protonated aromatic dimethyl amino acids, i.e. *N*,*N*-dimethylphenylglycine (**13**), *N*,*N*-dimethyl phenylalanine (**14**), *N*,*N*-dimethyltyrosine (**15**) and *N*,*N*-dimethyltryptophan (**16**) showed $[MH\text{-}HN(CH_3)_2]^+$ ion (63–100%, except **14** where it was 2%), which may be well stabilized by the aromatic ring. All these compounds, except **13**, displayed the product ion at *m/z* 46 retaining charge on dimethyl amine part. The $[MH\text{-}HN(CH_3)_2]^+$ ion (*m/z* 135) from **13** showed the ions at *m/z* 107 and 79 because of consecutive losses of two CO molecules; this was further confirmed by the CID of *m/z* 135. Similarly, consecutive loss of CH_2CO and CO from $[MH\text{-}HN(CH_3)_2]^+$ ion was found in the case of **14** because of the presence of a benzyl group at the α-position. The compound **15** showed a similar fragmentation as that of **14**, where mass values of all the characteristic product ions were shifted by +16 u because of the hydroxyl group on the phenyl group in **15**. The compound **16** also showed loss of CH_2CO from $[MH\text{-}HN(CH_3)_2]^+$ ion because $-CH_2$-(imidazole ring) group is attached to an α-carbon. The compounds **15** and **16** showed the loss of CO_2 from the $[MH\text{-}HN(CH_3)_2]^+$ ion. Apart from these, $[MH\text{-}(H_2O+CO)]^+$ ion was present in all these compounds (23–100% in **13–15**, and 1% in

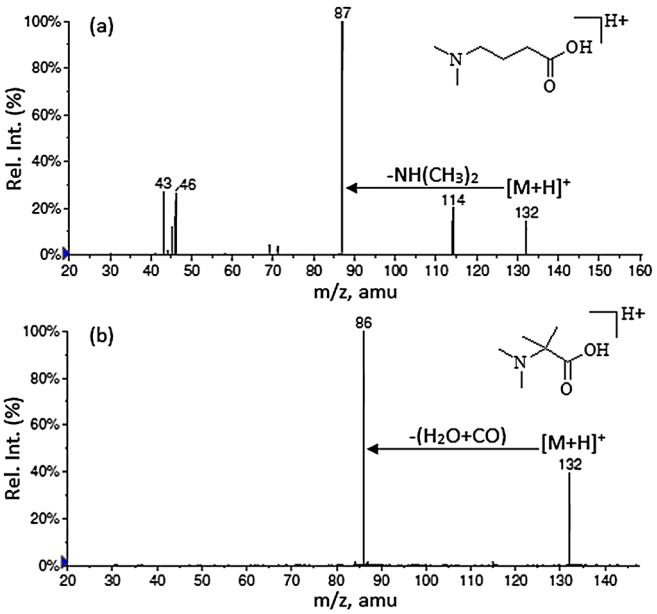
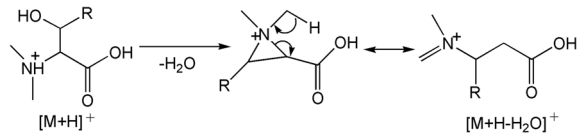


Figure 2. Collision-induced dissociation mass spectra of $[M+H]^+$ ion (a) *N*,*N*-dimethyl GABA (**4**) and (b) *N*,*N*-dimethyl 2-AIB (**5**) at collision energy of 20 eV.



Scheme 3. Plausible mechanism for the formation of $[MH\text{-}H_2O]^+$ of compounds **9** (R=H), **10** (R=CH_3).

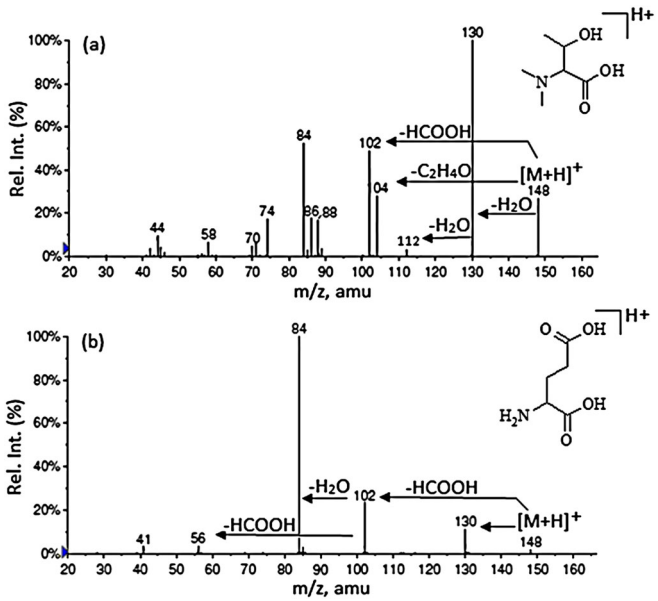


Figure 3. Collision-induced dissociation mass spectra of $[M+H]^+$ ion of (a) *N,N*-dimethylthreonine (**10**) and (b) glutamic acid at collision energy of 20 eV.

16), while $[MH\text{-}(H_2O+CO)\text{-}HN(CH_3)_2]^+$ was present in **14** and **15**. The compound **16** selectively showed an abundant product ion at *m/z* 102 corresponding to the loss of side group with abstraction of hydrogen (loss of H_3C-imadazole ring); similar product ion (*m/z* 102) was observed in **14** and **15** but low in abundance.

Acidic dimethyl amino acids (17 and 18)

The CID spectrum of $[M+H]^+$ of *N,N*-dimethylaspartic acid (**17**) showed $[MH\text{-}CH_3COOH]^+$ (*m/z* 102;100%), $[MH\text{-}(H_2O+CO)]^+$ (*m/z* 116) and further losses of H_2O, CO, (H_2O+CO) from the $[MH\text{-}CH_3COOH]^+$ ion (*m/z* 84, 74 and 56, respectively), which confirms the presence of a second carboxylic group in the structure. The *N,N*-dimethylglutamic acid (**18**) showed expected losses of H_2O (*m/z* 158), (H_2O+CO) (*m/z* 130, 100%) and CH_3COOH (*m/z* 116) from $[M+H]^+$ ion. The other product ions seen in the spectrum of **18** were due to the loss of H_2O, (H_2O+CO), and HN $(CH_3)_2$ from the $[MH\text{-}(H_2O+CO)]^+$ ion at *m/z* 112, 84, and 85, respectively, and $[MH\text{-}(CH_3COOH+H_2O)]^+$ ion (*m/z* 98).

Amidic/basic dimethyl amino acids (19–22) and tetramethyllysine

The $[M+H]^+$ ion CID spectrum of *N,N*-dimethylasparagine (**19**) showed an abundant ion at *m/z* 102 by loss of CH_3CONH_2 (59 u), which inferred the presence of amide group in the side chain in **19**. The spectrum also included the $[MH\text{-}NH_3]^+$ and $[MH\text{-}(H_2O+CO)]^+$ ions, whereas the *N,N*-dimethylglutamine (**20**) showed dominant $[MH\text{-}HN(CH_3)_2]^+$ ion (*m/z* 130), and $[MH\text{-}NH_3]^+$ (*m/z* 158) and $[MH\text{-}(H_2O+CO)]^+$ (*m/z* 129) were next two abundant product ions. The $[MH\text{-}HN(CH_3)_2]^+$ ion of **20** further fragmented to result in characteristic product ions corresponding to the loss of H_2O, CO, (H_2O+CO), and $HCONH_2$. The nominal mass of $[M+H]^+$ ion of **20** matches with that of free amino acid arginine (isobaric); but they can be easily discriminated by their distinct CID spectra, which clearly reflect the functional groups (Figure S3).

The CID spectrum of protonated *N,N*-dimethylhistidine (**21**) showed $[MH\text{-}(H_2O+CO)]^+$ ion and a specific product ion corresponding to $[MH\text{-}CO_2]^+$ ion. The spectrum also included the $[MH\text{-}CO_2\text{-}NC_2H_5]^+$ (*m/z* 95) and the *m/z* 102 formed by the loss of side chain group (H_3C-imidazole) from $[M+H]^+$ ion. The CID spectrum of *N,N*-dimethylarginine (**22**) showed the major product ions corresponding to $[MH\text{-}(HN(CH_3)_2)]^+$ (*m/z* 158) and $[MH\text{-}(HN{=}C(NH_2)_2)]^+$ (*m/z* 144; 100%) (base peak). The spectrum also included $[MH\text{-}H_2O]^+$, $[144\text{-}CO_2]^+$ and other product ions due to further losses of NH_3, CO, CO_2 and HCOOH from $[MH\text{-}HN(CH_3)_2]^+$ ion. The spectrum of **22** is found to match well with the reported spectrum by Shek *et al.*[26] Appearance of the specific product ion at *m/z* 144 in **22** rules out methylation on guanidinium group, because this ion known to be present in the CID mass spectra of asymmetric and symmetric dimethylarginines.[27]

The CID spectrum of $[M+H]^+$ ion of $N_\alpha,N_\alpha,N_\varepsilon,N_\varepsilon$-tetramethyllysine (**23**) showed loss of $HN(CH_3)_2$ and HCOOH from $[M+H]^+$ ion. Further loss of H_2O, CO, CO_2 and HCOOH were also found from $[MH\text{-}HN(CH_3)_2]^+$ ion. This spectrum well matched with the spectrum reported by Shek *et al.*[26] The tetramethyllysine (**23**) and dimethylarginine have the same nominal mass (isobaric); however, they can be well discriminated with their $[M+H]^+$ ion CID spectra.

CID of immonium ions

The MS/MS spectra of protonated peptides show specific product ions including immonium ions ($RCH{=}NH_2^+$) in the low mass range that are characteristic of the amino acids present in the peptide.[28–30] Recently, Hohmann *et al.*[31] reported that the immonium ion can be used as a quantitative indicator. The precursors to the immonium ions were examined by reaction intermediate scans sequence in peptide.[32] The selective decomposition of immonium ions from free amino acid were also used to confirm the side chain of the amino acid. Moreover, methylation is one of the post translation modifications of proteins; in this process, *N*-terminal amino acid may undergo methylation and yield mono and/or dimethylated derivative.[33] In such a case, the *N*-terminal modified protein/peptide produces corresponding immonium ion, and further fragmentation of this ion provide the structural information of the modified amino acid. Thus, it is essential to study the CID of immonium ions of all the dimethyl amino acids to enable their identification as a methylated free amino acid or amino acid located at *N*-terminal.

In this study, the immonium ions of all the dimethyl amino acids (except **3**, **4** and **16** because of their low abundance), were generated by in-source fragmentation of their $[M+H]^+$ ions. These immonium ions were further subjected to CID experiments for their structural characterization. The resulting immonium ion was denoted as **X*i***, where **X** is the dimethyl amino acid number and ***i*** refer to the immonium ion generated from **X** (for example, **1*i*** refers to immonium ion of **1**). The general structure of immonium ion formed from the dimethyl amino acids is shown in Scheme 4. The CID mass spectra of the immonium ions of all the dimethyl amino acids are tabulated in Table 3 and HRMS data is shown in Table S2. In the study of immonium ions, also a few isomeric/isobaric immonium ions can be formed from dimethyl amino acids and free

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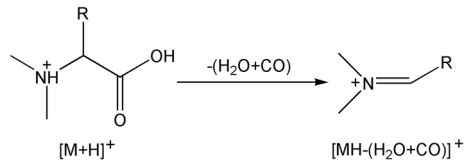

Scheme 4. Formation of immonium ions from *N,N*-dimethyl amino acids.

amino acids. Hence, we have also studied the CID of immonium ions generated from free amino acids for comparison purpose, and the spectra thus obtained were tabulated in Table S4. Characteristic ions resulting from immonium ions of dimethyl amino acids were discussed subsequently as per their *m/z* values. The isomeric/isobaric immonium ions were included at appropriate paragraph.

1i$^+$ *(m/z 58).* The CID spectrum of immonium of *N,N*-dimethylglycine (**1*i***; *m/z* 58) showed the product ions because of losses of H_2 (*m/z* 56), $^{\bullet}CH_3$ radical (*m/z* 43) and CH_4 (*m/z* 42; 100%). It also displayed other low mass product ions at *m/z* 30 $(H_2C{\equiv}NH_2)^+$, 29 ($^+C_2H_5$), 28 $(HN{\equiv}CH)^+$, and 15 ($^+CH_3$). The ion **1*i***$^+$ is isomeric with the immonium ion of 2-AIB; however, their CID spectra were distinctive (Figure S4). The immonium ion of 2-AIB showed specific losses of NH_3 and $(NH_3 + H_2)$ resulting in the product ions at *m/z* 41 and 39, respectively.

2i$^+$ *(m/z 72).* The CID spectrum of immonium ion of *N,N*-dimethylalanine (**2*i***, *m/z* 72) showed product ions at *m/z* 70, 57, 56, 44, 42 and 29 corresponding to the losses of H_2, $^{\bullet}CH_3$ radical, CH_4, C_2H_4, C_2H_6 and $(N,C_2,H_5$; may be $H_2C{=}N\text{-}CH_3)$, respectively (Fig. 4). The ion **2*i*** is isomeric to the immonium ion of valine. The immonium ion of valine showed the product ion at *m/z* 57 (loss of $^{\bullet}CH_3$) as the base peak (Fig. 4) and specific product ions ions at *m/z* 55 and 53 due to the loss of NH_3 and $(NH_3 + H_2)$, respectively.

5i$^+$ *(m/z 86).* The CID spectrum of immonium ion of *N,N*-dimethyl-2-amino isobutyric acid (**5*i***, *m/z* 86) showed product ions at *m/z* 84, 71, 70, 56, 43 and 41 corresponding to the losses of H_2, $^{\bullet}CH_3$, CH_4, C_2H_6, (N,C_2,H_5) and $(HN(CH_3)_2)$, respectively. The ion **5*i***$^+$ is isomeric to the immonium ions of leucine and isoleucine. The CID of **5*i***$^+$ and the immonium ions of leucine and isoleucine were shown in Figure S5. The spectrum of leucine immonium ion showed the product ions at *m/z* 69 (7%), 44 (base peak) and 43 corresponding to the losses of NH_3, C_3H_6 (through a McLafferty-type rearrangement), and $^{\bullet}C_3H_7$ radical, respectively. The spectrum of isoleucine immonium ion showed the product ions at *m/z* 69 (66%), 67, 58, 57, 41 and 30 corresponding to the losses of NH_3, $(NH_3 + H_2)$, C_2H_4 (through McLafferty-type rearrangement), $^{\bullet}C_2H_5$ radical, $(C_2H_4 + NH_3)$ and C_4H_8 (loss of side chain with hydrogen migration), respectively.

6i$^+$ *(m/z 100).* The CID spectrum of immonium ion of *N,N*-dimethylvaline showed the product ions at *m/z* 98, 85 (base peak), 84, 70, 58, 57 and 55 corresponding to the losses of H_2, $^{\bullet}CH_3$ radical, CH_4, C_2H_6, C_3H_6 (by McLafferty-type rearrangement), $^{\bullet}C_3H_7$ radical and $HN(CH_3)_2$, respectively. The spectrum also showed an abundant product ion at *m/z* 44 corresponding to $[C_2H_6N]^+$.

7i$^+$ *and 8i*$^+$ *(m/z 114).* The CID spectra of the immonium ions of *N,N*-dimethylleucine (**7*i***) and *N,N*-dimethylisoleucine (**8*i***) showed

Table 3. Collision-induced dissociation mass spectra of immonium ions of dimethyl amino acids

Immonium ion; *m/z* (% RA)	DP* value (V)	Collision energy (eV)	Product ions, *m/z* (% RA)
1*i*; 58 (20)	140	25	56 (2), 44 (2), 43 (42), 42 (100), 32 (2), 30 (38), 29 (8), 28 (7), 27 (4), 15 (1)
2*i*; 72 (100)	120	20	70 (16), 57 (14), 56 (6), 44 (30), 42 (29), 41 (1),30 (2)
5*i*; 86 (100)	140	15	84 (4), 71 (12), 70 (1), 56 (20), 46 (1), 44 (9), 43 (2), 42 (1)
6*i*; 100 (31)	140	25	85 (100), 84 (27), 70 (72), 58 (13), 55 (54), 53 (2), 44 (90), 43 (5), 42 (7), 41 (7)
7*i*; 114 (7)	180	25	72 (68), 71 (20), 70 (4), 58 (23), 57 (9), 56 (3), 55 (1), 44 (5), 43 (100), 41 (23), 30 (1)
8*i*; 114 (18)	140	25	99 (3), 86 (5), 85 (100), 84 (41), 72 (7), 71 (4), 70 (52), 69 (17), 58 (48), 46 (11), 44 (21), 43 (2), 42 (9), 41 (36), 30 (2)
9*i*; 88 (83)	160	20	86 (3), 73 (5), 70 (14), 59 (3), 58 (100), 56 (3), 55 (3), 46 (21), 45 (23), 44 (59), 43 (12), 42 (12), 31 (13), 30 (29)
10*i*; 102 (44)	140	20	87 (2), 84 (9), 74 (22), 72 (1), 60 (2), 58 (44), 57 (3), 56 (100), 46 (19), 45 (1), 44 (18), 42 (33), 30 (16), 29 (2), 28 (18)
11*i*; 104 (23)	100	20	89 (1), 86 (2), 78 (2), 77 (1), 71 (100), 70 (3), 58 (15), 56 (20), 46 (7), 44 (5)
12*i*; 132 (27)	150	15	114 (1), 84 (100), 61 (17), 42 (2)
13*i*; 134 (95)	140	30	132 (6), 119 (13), 118 (76), 117 (3), 116 (3), 106 (6), 105 (3), 104 (7), 103 (3), 94 (8), 93 (6), 91 (100), 88 (3), 86 (5), 77 (9), 74 (8), 74 (8), 70 (3), 67 (3), 65 (8), 63 (3), 56 (14), 53 (4), 46 (8), 44 (4)
14*i*; 148 (25)	140	30	147 (2), 146 (4), 133 (100), 132 (43), 118 (8), 117 (2), 116 (4), 115 (2), 105 (38), 104 (18), 103 (9), 91 (62), 79 (11), 77 (4), 71 (4), 67 (2), 58 (2), 56 (4), 44 (4), 42 (7), 41 (1)
15*i*; 164 (69)	150	25	162 (2), 149 (100), 148 (7), 147 (2), 135 (2), 134 (3), 132 (3), 121 (31), 119 (7), 108 (7), 107 (22), 105 (2), 103 (5), 94 (2), 93 (10), 91 (15), 77 (3), 72 (2), 71 (18), 58 (3), 56 (4), 46 (12), 45 (7), 44 (6)
17*i*; 116 (19)	130	20	98 (100), 88 (25), 84 (8), 74 (76), 73 (3), 72 (42), 71 (48), 70 (42), 58 (4), 57 (36), 56 (53), 55 (12), 46 (22), 45 (14), 44 (51), 43 (21), 42 (78), 37 (11), 33 (4), 28 (59)
18*i*; 130 (6)	150	20	98 (6), 84 (100), 71 (7), 70 (20), 58 (1), 56 (4), 46 (4), 44 (2)
19*i*; 115 (19)	160	20	98 (100), 72 (97), 70 (10), 57 (13), 44 (11)
20*i*; 129 (7)	140	20	112 (2), 86 (1), 84 (100), 83 (2), 69 (1), 55 (2), 46 (2), 42 (6)
21*i*; 138 (6)	150	25	123 (12), 122 (2), 106 (1), 96 (4), 95 (100), 81 (1), 68 (12), 44 (2), 41 (4), 30 (1)
22*i*; 157 (7)	170	20	112 (22), 111 (7), 100 (4), 98 (22), 84 (7), 70 (100), 52 (7), 44 (4), 42 (7), 41 (4)
23*i*; 157 (21)	140	20	114 (100), 112 (63), 97 (3), 84 (31), 58 (5), 46 (1), 44 (1)

RA, relative abundance.

* Declustering potential (DP) value used to generate the immonium ion in the ESI source.

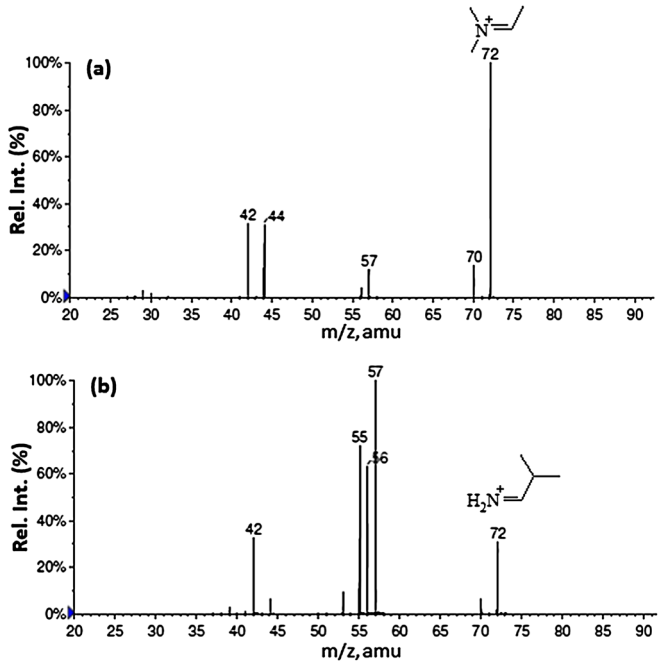

Figure 4. Collision-induced dissociation mass spectra of immonium ions of (a) *N,N*-dimethylalanine and (b) valine at collision energy of 20 eV.

distinct product ions (Fig. 5). The spectrum of **7*i*** $^{+}$ included the product ions at *m/z* 72, 71, 58 and 43 (base peak) corresponding to the losses of C_3H_6, (due to McLafferty-type rearrangement), $^{\bullet}C_3H_7$ radical, C_4H_8 and ($C_4H_8 + {}^{\bullet}CH_3$), respectively. The spectrum of **8*i*** $^{+}$ showed the specific product ions at *m/z* 99, 85 and 84 corresponding to the losses of $^{\bullet}CH_3$ radical, $^{\bullet}C_2H_5$ radical and C_2H_6, respectively. Additionally, the spectrum of **8*i*** $^{+}$ included an abundant product ion at *m/z* 70 because of the loss of a $^{\bullet}CH_3$ radical from $[\mathbf{8}i\text{-}C_2H_5]^{+\bullet}$ ion or $^{\bullet}C_2H_5$ radical from $[\mathbf{8}i\text{-}CH_3]^{+\bullet}$ ion. The spectrum of ***8i*** $^{+}$ also displayed abundant product ion because of HN

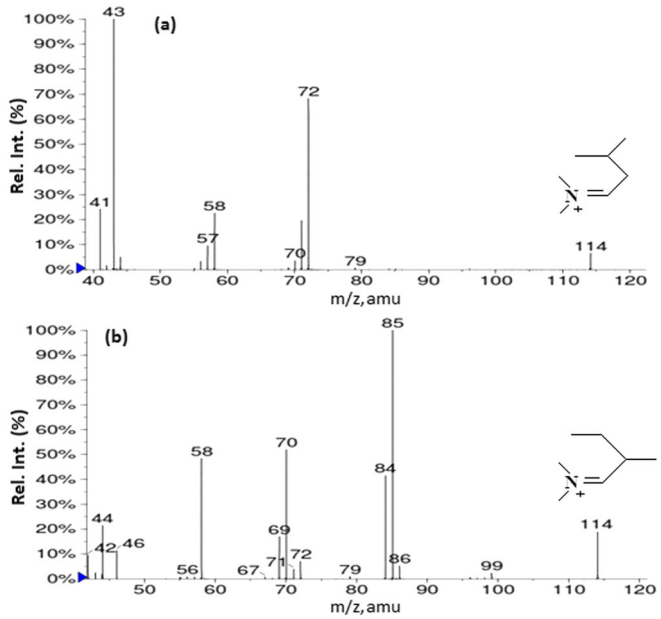

Figure 5. Collision-induced dissociation mass spectra of immonium ions of *m/z* 114 (a) *N,N*-dimethylleucine and (b) *N,N*-dimethylisoleucine at collision energy of 25 eV.

$(CH_3)_2^+$ at *m/z* 69 (17%), and the similar product ion in **7*i*** $^{+}$ was negligible (<1%).

9i^{+} *(m/z 88) and 10i*$^{+}$ *(m/z 102).* The CID spectrum of immonium of *N,N*-dimethylserine (**9*i***; *m/z* 88) showed the product ions at *m/z* 86, 73, 70, 58 (base peak), 55, 45, 44, 43 and 42 corresponding to the losses of H_2, $^{\bullet}CH_3$ radical, H_2O, CH_2O (due to McLafferty-type rearrangement), ($H_2O + {}^{\bullet}CH_3$), (N,C_2,H_5), ($H_2O + C_2H_2$), $HN(CH_3)_2$ and ($CH_2O + CH_4$), respectively. The CID spectrum of immonium ion of *N,N*-dimethyl threonine (**10*i***; *m/z*102) included the product ions at *m/z* 100, 87, 84, 74, 72, 58, 57, 56 (base peak), 44, 43 and 42 corresponding to the losses of H_2, $^{\bullet}CH_3$ radical, H_2O, CO, C_2H_6, C_2H_4O (due to McLafferty-type rearrangement), $HN(CH_3)_2$, ($H_2O + C_2H_4$), ($C_2H_6 + CO$), ($C_2H_4O + {}^{\bullet}CH_3$), and ($C_2H_4O + CH_4$), respectively. The loss of CO was specifically found from **10*i*** $^{+}$ and the immonium of threonine, while it was not found from **9*i*** $^{+}$ and the immonium of serine.

11i^{+} *(m/z 104) and 12i*$^{+}$ *(m/z 132).* The CID spectrum of immonium ion of *N,N*-dimethyl cysteine (**11*i***; *m/z* 104) showed the product ions at *m/z* 89, 71 (base peak), 70, 58 and 56 corresponding to the losses of $^{\bullet}CH_3$, $^{\bullet}SH$, H_2S, CH_2S (due to McLafferty-type rearrangement), and ($CH_2S + H_2$), respectively. The other product ions appeared in the low mass region at *m/z* 46, 44 and 43. The CID spectrum of **12*i*** $^{+}$ (*m/z* 132) showed characteristic product ions at *m/z* 84 (loss of CH_3SH), 61 $[H_2C{=}S\text{-}CH_3]^+$ and 42 $[H_2C{=}N{=}CH_2]^+$.

13i^{+} *(m/z 134), 14i*$^{+}$ *(m/z 148) and 15i*$^{+}$ *(m/z 164).* The CID spectra of immonium ions of *N,N*-dimethyl phenyl glycine (**13*i***), *N,N*-dimethyl phenyl alanine (**14*i***) and *N,N*-dimethyl tyrosine (**15*i***) showed the common product ions corresponding to the loss of H_2, $^{\bullet}CH_3$ radical, CH_4, (N,C_2,H_5) and $HN(CH_3)_2$ at respective *m/z* values. The spectrum of **13*i*** $^{+}$ showed product ions at *m/z* 91 ($^{+}C_7H_7$), 77 ($^{+}C_6H_5$) and 65 ($^{+}C_5H_5$) confirming presence of a phenyl ring. Similarly, the spectrum of **14*i*** $^{+}$ showed *m/z* 105 ($C_8H_9^+$) and 79 ($C_6H_7^+$) due to benzyl group and that of **15*i*** $^{+}$ showed *m/z* 121 ($C_8H_{10}O^+$) and 107 ($C_7H_8O^+$) due to -CH_2PhOH group.

17i^{+} *(m/z 116), 18i*$^{+}$ *(m/z 130).* The CID spectrum of immonium ion of *N,N*-dimethylaspartic acid (**17*i*** $^{+}$) showed the characteristic product ions at *m/z* 98 (base peak), 88, 74, 73, 72, 71, 70, 57, 56 and 42 corresponding to the losses of H_2O, CO, CH_2CO, (C_2, N, H_5), CO_2, $HN(CH_3)_2$, ($H_2O + CO$), ($CO_2 + {}^{\bullet}CH_3$), ($CO_2 + CH_4$), and ($CO_2 + C_2H_6$), respectively. The CID spectrum of immonium of *N,N*-dimethylglutamic acid (**18*i***) showed the product ions at *m/z* 86, 84, 71, 70, 56 and 42 corresponding to the losses of CO_2, ($H_2O + CO$), $^{\bullet}CH_2COOH$ radical, CH_3COOH, ($H_2O + CO + C_2H_4$), and ($CH_3COOH + C_2H_4$), respectively.

19i^{+} *(m/z 115), 20i*$^{+}$ *(m/z 129).* The CID spectrum of immonium ion of *N,N*-dimethyl asparagine (**19*i*** $^{+}$) showed characteristic product ions at *m/z* 98, 72, 70, 57, 55, 44 and 42 corresponding to the losses of NH_3, (O=C=NH), ($HCONH_2$; specific), ($O{=}C{=}NH + {}^{\bullet}CH_3$), ($HCONH_2 + {}^{\bullet}CH_3$), ($O{=}C{=}NH + C_2H_4$) and ($HCONH_2 + C_2H_4$), respectively. The immonium ion of asparagine showed the loss of NCO, but this loss was absent in the spectrum of **19*i*** $^{+}$. The spectrum of immonium ion of *N,N*-dimethylglutamine (**20*i*** $^{+}$) included the characteristic product ions at *m/z* 112, 101 and 84 corresponding to the losses of NH_3, CO and $HCONH_2$, respectively. The loss of $HCONH_2$ was predominant in the spectrum of **20*i*** $^{+}$, while the loss of NH_3 was dominant from the immonium ion of glutamine.

21i^{+} *(m/z 138).* The CID spectrum of immonium ion of *N,N*-dimethylhistidine (**21*i*** $^{+}$) showed product ions at *m/z* 123, 122 and 95 (dominant) corresponding to the losses of $^{\bullet}CH_3$, CH_4 and

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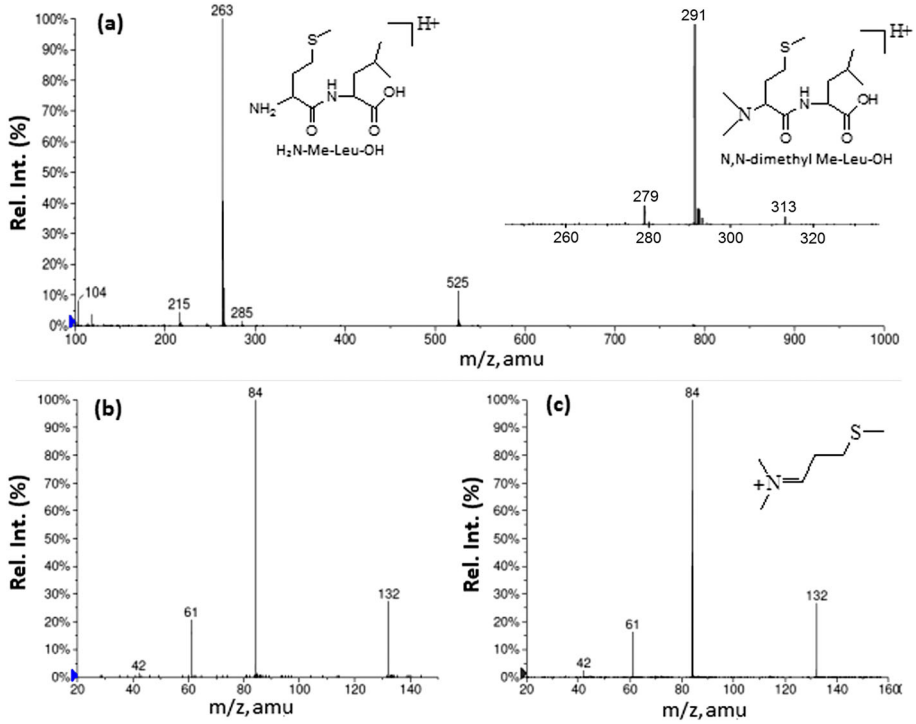

Figure 6. Collision-induced dissociation mass spectra of (a) Met-Leu dipeptide inset shows after dimethylation of dipeptide, (b) immonium ion of *m/z* 132 formed at declustering potential of 150 V from *N*,*N*-dimethyl Met-Leu dipeptide and (c) immonium ion from *N*,*N*-dimethylmethionine.

(C_2, N, H_5), respectively. Consecutive loss of two HCN moieties from the ion *m/z* 95 were also present that reflects the presence of imidazole group in the side chain. The immonium ion of histidine specifically showed loss of NH_3, HCN and (2HCN).

22i$^+$ *and 23i*$^+$ *(m/z 157).* The CID spectrum of immonium ion of *N*, *N*-dimethylarginine (**22*i***) showed product ions at *m/z* 112, 98 and 70 (base peak) corresponding to the losses of $HN(CH_3)_2$, $HN{=}C(NH_2)_2$ and $[HN(CH_3)_2 + HN{=}C{=}NH]$, respectively, due to guanidine group. The spectrum of **23*i***$^+$ showed product ions at *m/z* 114 (base peak), 112 and 84 corresponding to losses of (C_2, N, H_5), $HN(CH_3)_2$ and $(HN(CH_3)_2 + C_2H_4)$, respectively. The immonium ion of N_α,N_α,N_ε,N_ε-tetramethyllysine (**23*i***) is isobaric with the **22*i***$^+$, but their CID spectra were distinctive.

Determination of *N*-terminal amino acids of the peptides

The immonium ions generated during peptide fragmentation can also provide information about the posttranslational modifications, especially the *N*-terminal modifications. For example, *N*-terminal modified peptides (methylated/acetylated) showed the immonium ions with a mass shift of +28/+42 u, when compared with unmodified peptides.[34,35] As methylation of protein/peptide at the amino group of *N*-terminal amino acids is known in the literature, it is expected to get corresponding *N*-methylated immonium ions during the CID experiments on methylated peptides. In such cases, the CID of immonium ions of methylated amino acids discussed in the earlier section would be useful in characterization of the actual *N*-terminal amino acid methylated in a protein/peptide. In this experiment, initially the target protein/peptide is subjected to methylation by which the amino group of *N*-terminal amino acid undergoes dimethylation; in the next step, the methylated protein/peptide is subjected to in-source fragmentation in which the immonium ion of *N*-terminal modified amino acid is generated. Further, CID experiments on the immonium ions provide the information on the methylated *N*-terminal amino acid. To evaluate the methodology of methylation followed by immonium ion CID experiment for the determination of *N*-terminal amino acid in the peptides, we have selected a dipeptide and a tetrapeptide and performed the experiments. The two peptides (Met-Leu and Lys-Ala-Ala-Ala) were subjected to methylation by applying a similar procedure used for methylation of free amino acids. The ESI-MS spectrum of the original dipeptide (Met-Leu) showed the ion at *m/z* 263 corresponding to $[M + H]^+$ ion, and this ion was shifted by +28 u after methylation reaction and appeared at *m/z* 291, which indicates that the peptide was dimethylated at *N*-terminal amino acid. Similarly, the $[M + H]^+$ ion of the tetrapeptide (Lys-Ala-Ala-Ala) shifted from *m/z* 360 to *m/z* 416 (+56 u) upon methylation, which correspond to a tetramethylated peptide. As lysine is the *N*-terminal amino acid, both the amines might have undergone methylation resulting in a tetramethylated product.

When the methylated peptides were subjected to in-source fragmentation, the methylated dipeptide showed specific immonium ion at *m/z* 132, whose CID spectrum matched with the immonium ion of *N*,*N*-dimethyl methionine (Fig. 6). Similarly, the modified tetra peptide showed the specific immonium ion at *m/z* 157; the CID spectrum of this ion matched well with that of the immonium ion of tetramethyllysine. These experiments on the peptide clearly confirmed that the free amino groups of the peptide (including *N*-terminal amino acid) undergo methylation, and it is possible to determine the *N*-terminal amino acid of an unknown peptide based on the CID spectra of corresponding immonium ions (methylated).

Conclusions

In this study, dimethyl α-amino acids, including a few β-amino acids and γ-amino acids were successfully synthesized and characterized

by ESI-MS and ESI-MS/MS. The MS/MS of protonated *N*,*N*-dimethyl amino acids and their immonium ions showed structure indicative product ions. This study also shows that isomeric/isobaric natural amino acids can be clearly differentiated from dimethyl amino acids in biological matrices. The nature of amino acid (aliphatic, aromatic, acidic and basic) and methyl groups on nitrogen is very much reflected in the fragmentation of protonated *N*,*N*-dimethyl amino acids and their immonium ions. The immonium ions formed from the isomeric *N*,*N*-dimethyl amino acids and free amino acids also showed clear-cut differences in their CID spectra. This study also helps in the discrimination of isomeric immonium ions (e.g., leucine/isoleucine; lysine/glutamine) and screening of methylated peptides/proteins in post translation modification studies using MS/MS of immonium ions. The methodology can also be applied to determine the *N*-terminal amino acid of peptides.

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