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Mass spectrometric analysis of amino acid/di-peptide modified gemini surfactants used as gene delivery agents: Establishment of a universal mass spectrometric fingerprint

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ABSTRACT

Lipid based gemini surfactant nanoparticles have been extensively studied as non-viral vectors for gene therapy. Novel amino acid substituted gemini surfactants have been recently developed with a molecular structure consisting of two positively charged quaternary ammonium head groups, symmetrical saturated dodecyl tails, and a spacer region containing a secondary amine group. Various amino acids were attached to the amine functional group. The purpose of this work was to confirm the molecular structure of six novel amino acid substituted gemini surfactants and to establish a universal fragmentation (MS/MS) pattern of the tested compounds (i.e., fingerprint). This was accomplished by using a hybrid quadrupole orthogonal time-of-flight mass spectrometer (QqToF-MS) and a triple quadrupole linear ion trap mass spectrometer (QqQ-LIT MS) equipped with electrospray ionization (ESI) source. The single stage QqToF-MS data obtained in the positive ion mode verified the molecular composition of all tested gemini surfactants. Tandem mass spectrometric (MS/MS) analysis showed common fragmentation behaviour among all tested compounds, allowing for the establishment of a universal fragmentation pattern. The fragmentation pathway was confirmed by MS/MS/MS experiments utilizing a Q-Trap™ 4000 LC/MS/MS system. Unique product ions, originating from the loss of one or both head groups along with the attached tail region(s), confirmed the chemical structure of the tested compounds. The established MS/MS fingerprint will be used for qualitative purposes as well as the development of future multiple reaction monitoring (MRM) HPLC–MS/MS quantification methods.

1. Introduction

Nucleic acids (DNA and RNA) have been widely investigated as therapeutic agents for the treatment of hereditary and acquired diseases in a promising medical approach known as gene therapy [1–9]. However, the great potential of gene therapy will not be fully achieved until the issue of improved gene delivery is properly addressed. Gene delivery vectors can be categorized as *viral* or *non-viral*. Viral vectors (adenovirus and retrovirus vectors) are the most effective gene delivery agents and have been utilized in several clinical trials [7–9]. However, they suffer from numerous toxicity-related drawbacks including mortality and morbidity [10]. In addition, the severe immune response caused by the viral capsid and the limited loading capacity of viral vectors significantly limit their therapeutic applications [6,11]. Conversely, non-viral vectors, such as cationic lipids, have exhibited low toxicity and no immunogenic activity [12,13]. Cationic lipids are able to condense genetic materials, through electrostatic interaction with the phosphate backbone of nucleic acid, to a nano-sized complex (*lipoplex*) [14].

One specific group of cationic lipids that have demonstrated efficient transfection activities *in vitro* and *in vivo* are the gemini surfactants. They are dimeric surfactants comprised of two hydrophobic tail regions, each of which is covalently attached to cationic head group linked to each other by a spacer region (Fig. 1) [15–17]. A wide range of gemini surfactants can be produced through chemical modifications within the head, spacer or tail regions. These modifications are intended to enhance the transfection efficiency of the lipoplex while reducing cytotoxicity [18–20]. For instance, the inclusion of secondary amine functional group in the spacer region of 1,9-bis(dodecyl)-1,1,9,9-tetramethyl-5-imino-1,9-nonanediammonium dibromide gemini surfactant, resulted in a nine-fold increase in transfection efficiency in various cell lines compared to non-substituted gemini surfactants [19]. This increase was attributed to the pH-dependent morphological changes to the DNA-gemini complex, facilitating the cytoplasmic escape of

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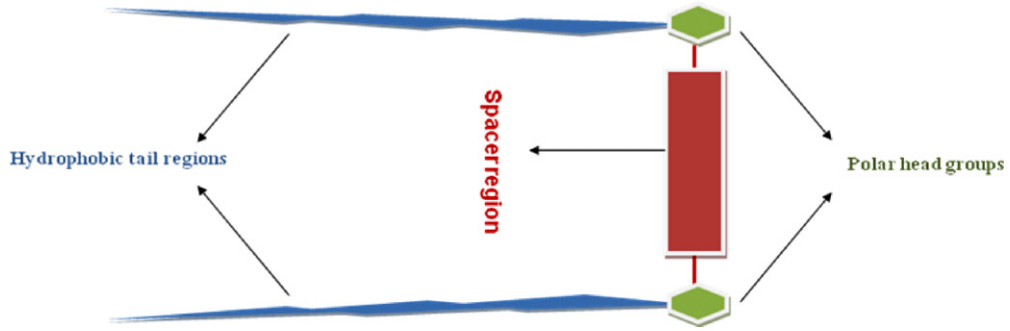


Fig. 1. General structure of gemini surfactants.

the DNA. Additional structural modifications include the covalent attachment of biocompatible and biodegradable amino acids (glycine, lysine) and dipeptides (glycyl-lysine, lysyl-lysine) to the amino group in the spacer region, enhancing transfection efficiency in epithelial cells while maintaining a low cytotoxicity profile [20,21].

The transfection efficiency of lipoplex depends on the integrity of the various components of the delivery system and their related physiochemical properties. Therefore, investigation of the physiochemical stability of the lipoplex during the manufacturing process and, furthermore its biological fate after treatment, is essential to understand and evaluate the behaviour of such complex systems. To date, most research in non-viral gene delivery has focused on the development of efficient delivery systems and less work has been done to investigate the chemical stability and biological fate of the vector. To achieve the last goal, proper analytical methods should be developed for both qualitative and quantitative applications. Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are ideally suited to achieve this goal.

Tandem mass spectrometric MS/MS studies of bioactive materials are routinely utilized for quantitative and qualitative analyses with high accuracy and precision [22–25]. For example, ESI-Qq-ToF MS/MS analysis determined the exact molecular structure of lipid A moieties isolated from mutant and wild-type *Aeromonas salmonicida* lipopolysaccharide [24]. Similarly, the analysis of saponins, naturally occurring glycosides with varieties of biological activities, using electrospray ionization multiple-stage tandem mass spectrometry (ESI-MS^n), allowed for structural differentiation between several types of saponins [25]. Subsequently, a liquid chromatography (LC)-MS/MS method was also developed for quick and precise identification of different saponins from plant extract.

In our previous work, we confirmed the molecular structure of ten un-substituted diquaternary ammonium gemini surfactants belonging to two different structural families G12-*s* and G18:1-*s* (where 12 and 18:1 correspond to the length and saturation of the alkyl tail, [-*s*] corresponds to the length of spacer region) using electrospray ionization (ESI) quadrupole time-of-flight (Qq-ToF) mass spectrometer [26]. The Q-ToF (MS/MS) analysis showed significant similarities of the fragmentation pattern for all tested geminin surfactants. Most recently, the MS/MS behaviour of 29 non-substituted diquaternary ammonium gemini surfactants, categorized into four distinct families were elucidated [27]. In the current study, we determined the tandem mass spectrometric behaviour of novel amino acid/peptide modified diquaternary ammonium gemini surfactants, specially designed for gene delivery. Mass spectrometric analysis was performed by positive ESI on time-of-flight (Q-ToF) and triple quadrupole linear ion trap (QqQ-LIT) mass spectrometers. The suggested fragmentation patterns (i.e., fingerprints) of all compounds were confirmed by means of MS/MS/MS experiments.

2. Experimental

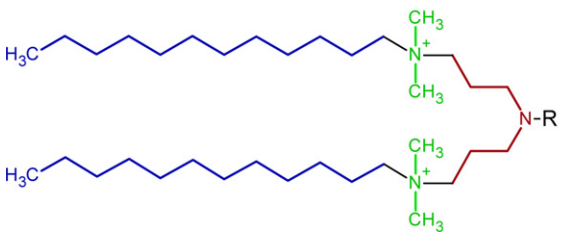
2.1. Gemini surfactant

Six novel mono-amino acid/dipeptide-substituted gemini surfactants were provided by Dr. Ronald E. Verrall's research group (Department of Chemistry, University of Saskatchewan). The synthesis of these gemini surfactants and their efficiency in gene transfer were recently reported [20,21]. Tested compounds were given the designation of 12-7N(R)-12, where (12) is the number of carbon atoms in the tail region, (7) is the length of the amine substituted spacer region and (R) represents the amino acid(s) substituent:

R = Glycine, Lysine, Histidine, Glycyl-Lysine, Lysyl-Lysine, Glycyl-Glycine

The general molecular structure of these gemini surfactants is shown in Fig. 2.

Stock solutions of 3 mM gemini surfactant were prepared in methanol/water 50:50 and 0.1% formic acid and stored at −20 °C. Samples for the MS experiment were further diluted 1000× prior to injection using the same solvent.



R= Glycine : 12-7N(Gly)-12
Lysine: 12-7N(Lys)-12
Histidine: 12-7N(His)-12
Glycyl-Lysine: 12-7N(Gly-Lys)-12
Lysyl-Lysine: 12-7N(Lys-Lys)-12
Glycyl-Glycine: 12-7N(Gly-Gly)-12

Fig. 2. General structure of amino acid/di-peptide gemini surfactant 12-7N(R)-12 where (R) is corresponding to the amino acid/di-peptide substituent.

Table 1
Mass accuracies obtained from single stage ESI-QqToF MS using internal calibration.

Gemini surfactants	Molecular formula	Mono-isotopic mass	Theoretical, *m*/*z*	Observed, *m*/*z*	Mass accuracy (ppm)
12-7N(Glycine)-12	$C_{36}H_{78}N_4O$	582.6164	291.3082	291.3094	4.1193
12-7N(Lysine)-12	$C_{40}H_{87}N_5O$	653.6899	326.8449	326.8462	3.9774
12-7N(His)-12	$C_{40}H_{82}N_6O$	662.6539	331.3269	331.3293	7.2436
12-7N(Gly-Lys)-12	$C_{42}H_{90}N_6O_2$	710.7114	355.3557	355.3571	3.9397
12-7N(Lys-Lys)-12	$C_{49}H_{99}N_7O_2$	781.7849	390.8924	390.8926	0.5116
12-7N(Gly-Gly)-12	$C_{38}H_{81}N_5O_2$	639.6379	319.8189	319.8185	1.2507

2.2. Electrospray-quadrupole orthogonal time-of-flight mass spectrometry (ESI-QqToF MS)

Gemini surfactants were analyzed in the positive ion mode by using an API QSTAR XL MS/MS hybrid QqToF tandem mass spectrometer equipped with an ESI source (Applied Biosystems Inc., CA, USA). The instrument parameters were optimized as follows: declustering potential 100 V and focusing potential of 290 V. Sample solutions were infused into the source chamber (Turbo Ionspray source) by using an integrated Harvard syringe pump (Harvard Apparatus, MA, USA) at a rate of 10 μL/min with the following parameters: spray chamber temperature 80–100 °C, needle voltage 5500 V. Nitrogen was used as the drying gas and ESI nebulizing gas. Internal calibration was used to ensure high mass accuracies and to minimize errors in mass measurements. Similar to our recent work [26], we used doubly charged standards given that the tested gemini surfactants are doubly charged species. These include [Glu1]-Fibrinopeptide B, Human (peptide EGVNDEEGFFSAR, *m*/*z* 785.4821), (BaChem Bioscience Inc., PA, USA), and the previously characterized diquaternary ammonium gemini surfactant N,N-bis(dimethyldodecyl)-1,2-ethanediammonium dibromide *m*/*z* 234.2685 [28–30]. Mass spectra acquisitions were analyzed using the Analyst software.

Tandem mass spectrometric analysis was obtained by collision-activated dissociation (CAD) using nitrogen as collision gas. The collision energy (CE) values were optimized to allow for a dissociation of the gemini surfactant while ensuring the abundance of the precursor ion (ranging from 27 to 33 eV).

2.3. Triple quadrupole linear ion trap mass spectrometry (QqQ-LIT MS)

The suggested fragmentation pathways were confirmed by performing MS/MS/MS experiments using a Q-Trap 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) a hybrid triple quadrupole linear ion trap mass spectrometer (QqQ-LIT) equipped with a "Turbo V Ion Spray" ESI source. The QqQ-LIT system provides valuable structural information because of its ability to trap ions in the LIT analyzer and, subsequently, to perform MS/MS/MS experiments [31,32].

The MS/MS/MS analysis of the precursor ion and selected product ions of the tested gemini surfactants were acquired in the MS/MS/MS mode. Stock samples were diluted 1000× and infused directly into the ionization source by using a model 11 Plus syringe pump (Harvard Apparatus, MA, USA) at a flow rate of 50 μL/min. The declustering potential (DP) was set at the range of 40–100 (compound dependent) and collision energy (CE) were optimized to obtain the greatest abundance of product ions. Excitation energy (AF2), the energy used to fragment the second precursor ion, was set at 100 mV.

3. Results and discussion

3.1. Single stage QqToF analysis

For all tested mono-amino acid and di-peptide gemini surfactants, abundant doubly charged $[M]^{2+}$ species were observed during the full scan ESI-QqToF-MS analysis providing evidence for the presence of the diquaternary ammonium head groups. In addition, the exact mass for the tested compounds were assessed; mass accuracies were less than 10 ppm mass error using internal calibration (Table 1).

3.2. Tandem mass spectrometric analysis

The variation within the substituent of the amine group of the spacer region (i.e., amino acid/di-peptide substituents) resulting in the production of gemini surfactant-specific product ions upon collision-activated dissociation (CAD) positive ESI-QqToF MS/MS analyses. The formation of these compound-specific product ions follows a similar fragmentation pattern for all mono-amino acid and dipeptide gemini surfactants, which originates from the loss of one or both quaternary ammonium head group(s) along with the attached tail region(s). This allowed for the authentication of the molecular structure, confirming the attachment of the amino acid/di-peptide moieties to the amine group of the spacer region.

The following sections include a detailed discussion of the fragmentation patterns of 12-7N(Glycyl-Lysine)-12, illustrative of a di-peptide substituted gemini surfactants with the most complex MS/MS spectra among all tested compounds. In addition, the MS/MS behaviour of 12-7N(Glycine)-12, illustrative of mono-amino acid substituted gemini surfactants, will be discussed briefly to highlight the fragmentation with mono-amino acid substituted compounds. Table 2 displays the product ions of all gemini surfactants studied herein with their corresponding molecular formula and the theoretical *m*/*z* values.

3.2.1. MS/MS fragmentation pathway of the di-peptide substituted gemini surfactants

The di-peptide gemini surfactants include three novel gemini surfactants in which a di-peptide substituent is attached to the amine group of the spacer region: 12-7N(Glycyl-Lysine)-12,12-7N(Lysyl-Lysine)-12 and 12-7N(Glycyl-Glycine)-12. Fig. 3 shows the ESI-QqToF MS/MS spectrum of 12-7N(Glycyl-Lysine)-12, as a representative for this group, and the proposed fragmentation pathway.

The fragmentation pathway of 12-7N(Glycyl-Lysine)-12 (Fig. 3a) starts with the formation of the minor diagnostic doubly charged product ion designated as $[M–C_{12}H_{24}]^{2+}$ at *m*/*z* 271.26 (ion 1, Fig. 3b) which is formed from the neutral loss of the aliphatic tail region of 168.18 Da. It can be speculated that due to the possible close proximity of the two positively charged head groups, this ion is not stable and will fragment instantly; hence, being a substantially minor ion. In fact, ion (1) was observed in the MSMS analysis of all tested compounds as a minor fragment ion. Ion (1) fragments to the major doubly charged product ion observed at *m*/*z* 248.73 (ion 2) through the neutral loss of N-methylmethanamine (i.e., head group). It is expected that the second charge within ion 2 is localized within the di-peptide (Glycyl-Lysine) terminal, possibly distant from the quaternary nitrogen; hence, enhancing their stability and abundance in comparison to (ion 1).

Subsequently, product ion (2) fragments via three different mechanisms into three diagnostic product ions (ions 3, 8^{++} and 9^{+},

Table 2
MS/MS product ion designations and corresponding theoretical mass-to-charge (m/z) values for all gemini surfactants evaluated.

Gemini surfactants		12-7N(Glycine)-12	12-7N(Lysine)-12	12-7N(Histidine)-12	12-7N(Gly-Lys)-12	12-7N(Lys-Lys)-12	12-7N(Gly-Gly)-12
Molecular formula		$C_{36}H_{78}N_4O$	$C_{40}H_{87}N_5O$	$C_{40}H_{82}N_6O$	$C_{42}H_{90}N_6O_2$	$C_{49}H_{99}N_7O_2$	$C_{38}H_{81}N_5O_2$
Precursor ion $[M]^{2+}$		291.3082	326.84495	331.32695	355.3555	390.89245	319.81895
Product ions			m/z	m/z	m/z	m/z	m/z	m/z
1	$[M–C_{12}H_{24}]^{2+}$		207.21	242.75	247.23	271.26	306.79	235.72
2	$[M–(C_{12}H_{24})–(C_2H_7N)]^{2+}$		184.68	220.22	224.70	248.73	284.26	213.19
3	$[M–C_{14}H_{32}N–(NH_3)]^{2+}$			211.70	216.19	240.22	275.75	204.68
4	$[M–C_{14}H_{32}N–(NH_3)–(C_2H_4)]^{2+}$			197.69		226.20	261.74	
5	$[M–C_{14}H_{32}N–(NH_3)–(C_2H_4)–(C_3H_5N)]^{2+}$			170.17		198.68	267.24	
6	$[C_{21}H_{44}N_2O]^{2+}$		170.17	170.17	170.17	170.17	170.17	170.17
7	$[(ION\ 6)–(CHO^+)]^+$		311.34	311.34	311.34	311.34	311.34	311.34
8^{++}	$[M–2(C_{12}H_{24}N)–(C_2H_7N)]^{2+}$		100.59	136.21	140.69	164.63	200.17	129.10
8^+	$[(ION\ 8^{++})–(H)^+]^+$		200.17	271.24	280.21	238.27	399.34	257.19
9^{++}	$[M–2(C_{12}H_{24}N)–2(C_2H_7N)]^{2+}$		78.06	113.59	118.08	142.11	177.64	106.57
9^+	$[(ION\ 9^{++})–(H)^+]^+$		155.11	226.19	235.15	283.21	354.28	212.14
10^{++}	$[M–2(C_{12}H_{24}N)–2(C_2H_7N)–(NH_3)]^{2+}$			105.15		133.59	169.13	98.06
10^+	$[(ION\ 10^{++})–(H)^+]^+$			209.16		266.18	337.25	195.10
11	$[C_{14}H_{32}N]^+$		214.25	214.25	214.25	214.25	214.25	214.25
B_1	(N-Terminal ion from peptide bond cleavage)					155.11	226.19	155.11
Y_1	(C-Terminal ion from peptide bond cleavage)					129.10	129.10	58.02

Fig. 3b). Product ion (3) at m/z 240.22 is formed by losing a (NH_3) moiety, while product ion (8^{++}) is generated by the neutral loss of the second tail region at m/z 164.63. Additionally, product ion (9^+) at m/z 283.21 is formed through the complementary loss of the singly charged ion of the tail region with the attached head group m/z 214.25 (11).

The product ion (3), designated as $[M–C_{14}H_{32}N–(NH_3)]^{+2}$, is the predominant product ion observed in the MS/MS spectrum of 12-7N(Gly-Lys)-12 and can undergo two main fragmentation process. Firstly, the loss of a (NH_3) from ion (3) yields the product ion (3′) at m/z 231.70. The second mechanism involves the loss of ethene moiety from product ion (3) producing a doubly charged product ion at m/z 226.20 (ion 4). This product ion is further fragmented to several product ions resulting from various elimination processes within the dipeptide residue. The neutral loss of (C_3H_5N) from ion (4) produces a doubly charged fragment ion at m/z 198.68 (ion 5). Subsequently, the loss of a carbon monoxide moiety from ion (5) produces a unique product ion, (ion 5′) at m/z 184.68, with a glycine residue within the spacer region. Furthermore, product ion (6) is formed by the neutral loss of a

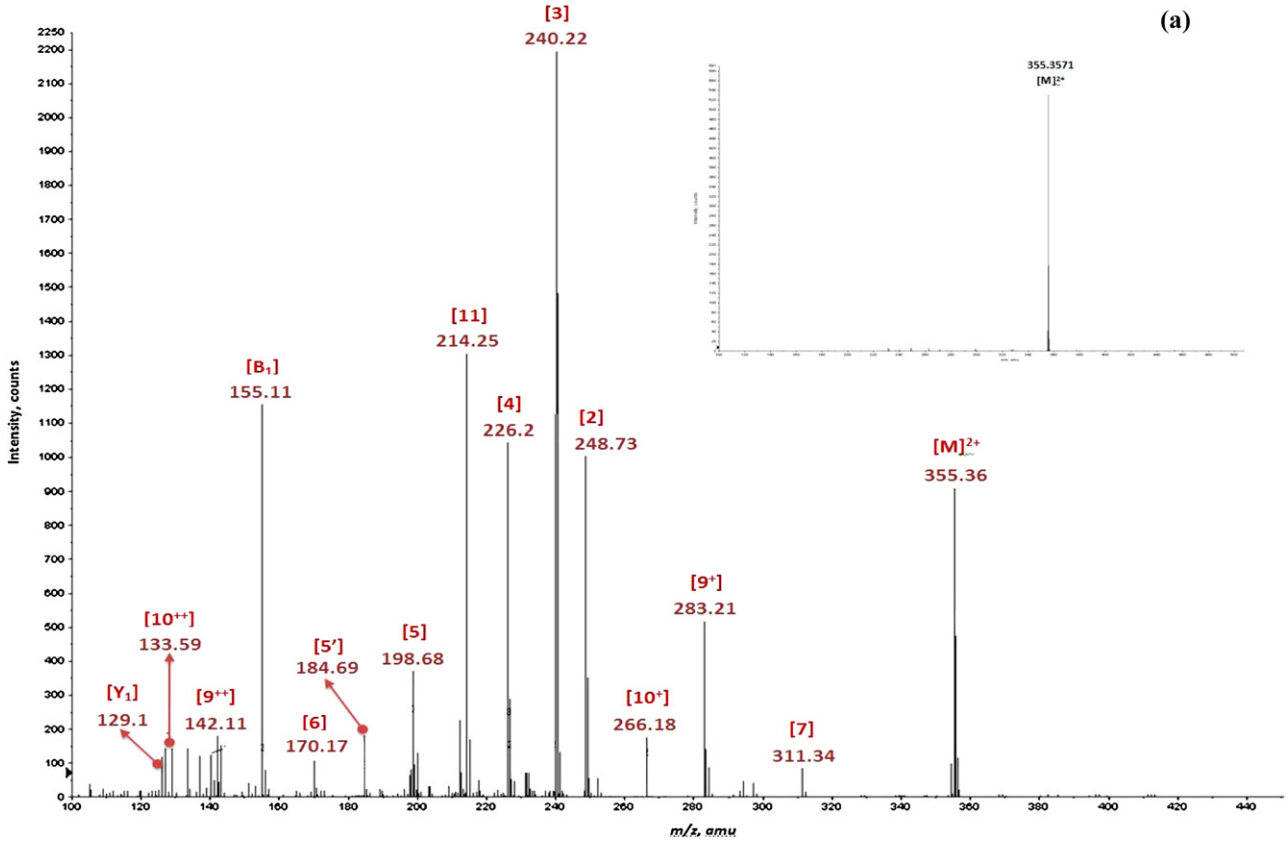


Fig. 3. (a) The ESI-QqToF MS/MS spectra of 12-7N(Glycyl-Lysine)-12 as a representative example of di-peptide gemini surfactants (full MS spectrum in the box) and (b) the MS/MS fragmentation pattern showing the most distinctive product ions, other non-diagnostic product ions are not included.

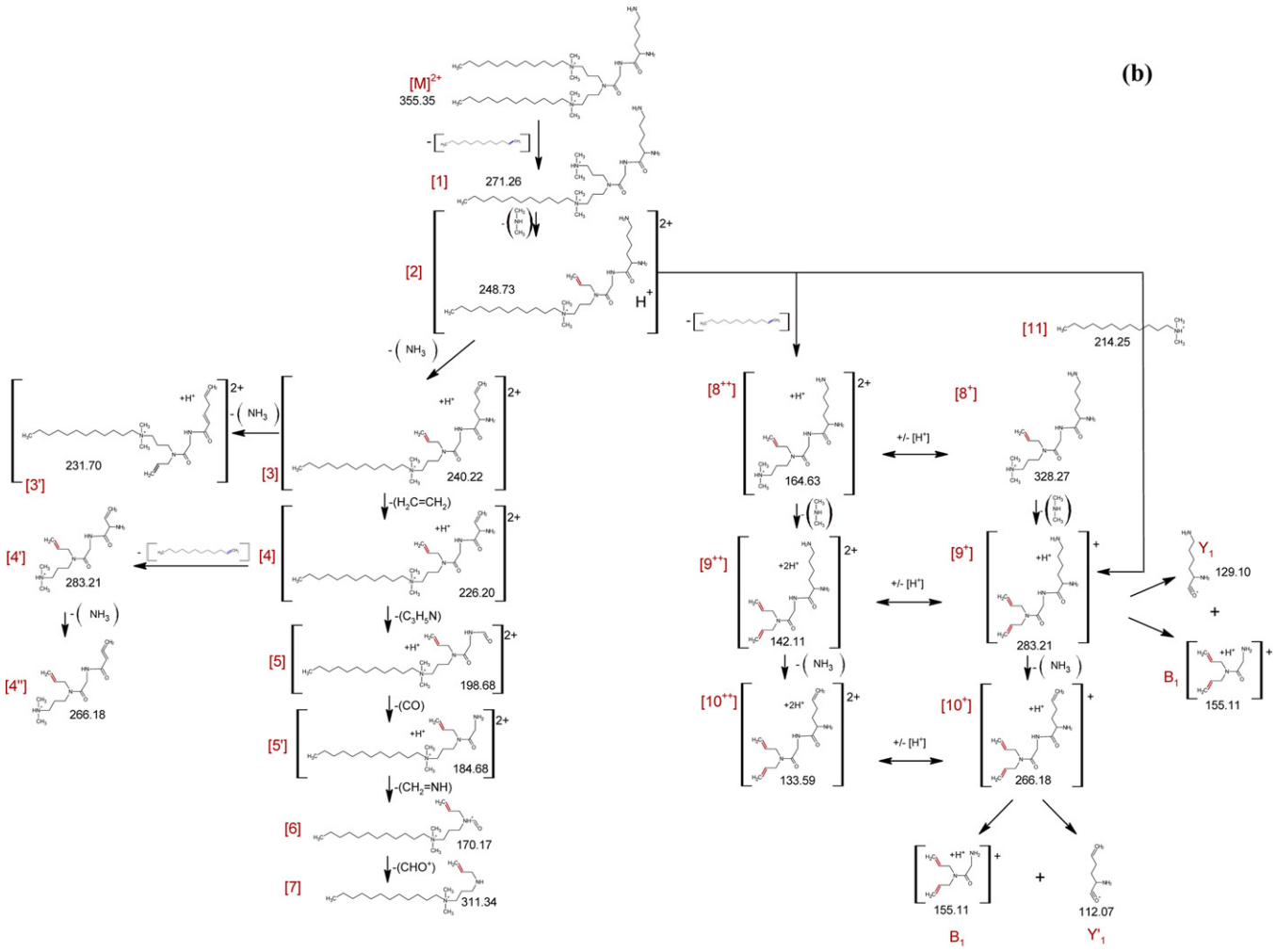


Fig. 3. (*Continued*)

methyleneimine (methanimine) moiety (–CH_2=NH) from the head group, producing a doubly charged fragment ion at *m*/*z* 170.17 (ion 6). The subsequent loss of oxomethylium (HCO^+) from this ion produces a singly charged product ion observed at *m*/*z* 311.34 (ion 7). Oxomethylium is a well identified loss in MS analysis that can occur in two isomeric forms; HCO^+ and HOC^+ [33,34]. Fragment ions (6) and (7) are common ions observed in the MS/MS analysis of all tested compounds.

The second fragmentation mechanism for ion 2 involves the formation of product ion (8) which was observed as both; singly (8^+) and doubly (8^{++}) charged ions at *m*/*z* 328.27 and *m*/*z* 164.63, respectively. The neutral loss of the second head group from product ions (8^+/8^{++}) produces the diagnostic product ions (9^+/9^{++}) at *m*/*z* 283.21 and *m*/*z* 142.11. The Q-ToF MS/MS analysis of the precursor ion and the QqQ-LIT MS/MS/MS spectrum of ion (9^+) (Table 3) indicates that this ion produces three main elimination products. The loss of (NH_3) from the di-peptide residue of product ion (9) leads to the formation of product ion (10) which was observed as doubly charged ion (10^{++}) at *m*/*z* 133.59 and singly charged ion (10^+) at *m*/*z* 266.18. The formation of singly and doubly charged product ions can be explained by the presence of multiple nitrogen centers in the spacer region of the di-peptide gemini surfactants that can easily capture a proton from other species within the collision cell.

Unique breakage of the peptide bond between the glycine and lysine amino acids produced two complementary fragment ions; ion (B_1) that corresponds to the N-terminal ion (glycine residual ion) at *m*/*z* 155.11 and ion (Y_1) corresponding to the C-terminal ion (lysine residual) at *m*/*z* 129.10. Designations for these ions follow the Roepstorff nomenclature for mass spectrometry of peptides [35]. It should be noted that ion (B_1) can also originate from ions (3) and (4) upon the loss of the tail region, which is supported by MS/MS/MS results (Table 3).

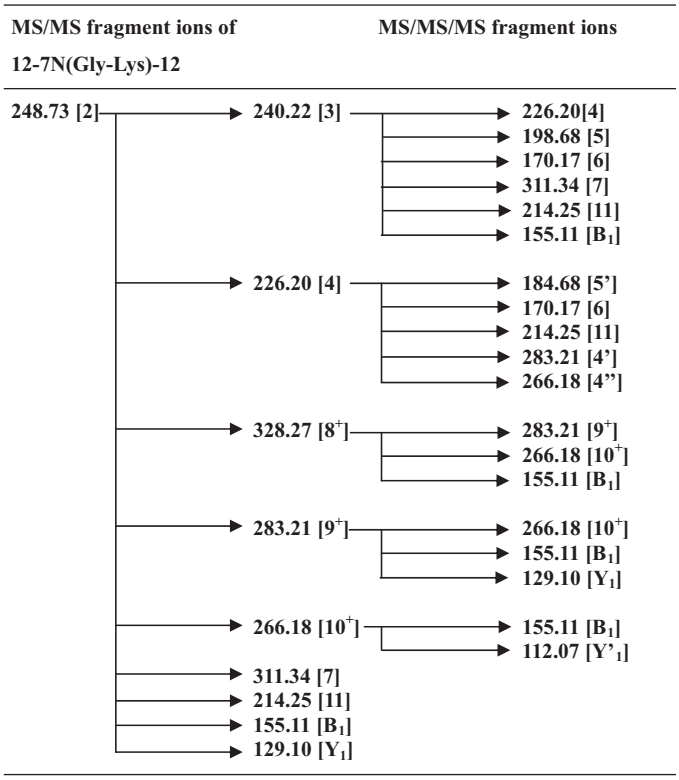
MS/MS/MS analysis was informative and assisted in the confirmation of the proposed fragmentation mechanism. For instance, the MS/MS/MS spectrum of product ion (4) shows fragment ions at *m*/*z* 283.21 and 266.18, ions (4′) and (4″), respectively (Fig. 3b and Table 3). These product ions have the same *m*/*z* values as product ions (9^+) and (10^+) which are fragments of ion (8^+). This can be explained by the fact that two isomers having the same *m*/*z* values were formed for each product ion originating by different fragmentation mechanisms (Fig. 3b). MS/MS/MS analysis allowed for the differentiation of these structural isomers.

12-7N(Lysyl-Lysine)-12 and 12-7N(Glycyl-Glycine)-12 compounds followed the same fragmentation pathway as the glycyl-lysine substituted gemini surfactant (Table 2).

3.2.2. *MS/MS fragmentation pathway of the mono-amino acid gemini surfactants*

Mono-amino acid gemini surfactants included three novel compounds in which a single amino acid is attached to the amine group of the spacer region: 12-7N(Glycine)-12,12-7N(Lysine)-12 and 12-7N(Histidine)-12. Fig. 4 shows the ESI-QqToF MS/MS spectrum of 12-7N(Gly)-12, and the corresponding fragmentation pathway.

Table 3
Summary of MS/MS/MS experiment for 12-7N(Gly-Lys)-12, using QqQ-LIT.



Similarly to the fragmentation pathway of di-peptide substituted gemini surfactants, the fragmentation pathway of 12-7N(Glycine)-12 begins with the production of the characteristic minor doubly charged product ion $[M–C_{12}H_{24}]^{2+}$ at *m*/*z* 207.21 (ion 1, Fig. 4b) formed through the neutral loss of one hydrophobic tail region as explained earlier. This fragment ion further fragments to the major doubly charged product ion observed at *m*/*z* 184.68 (ion 2) through the neutral loss of N-methylmethanamine (i.e., head group).

Consequently, product ion (2) can undergo three fragmentation pathways producing fragment ions (6), (8^{++}) and (9^{+}) (Fig. 4b). The formation of commonly observed product ions (6) and (7) was mentioned previously in the discussion of di-peptide substituted gemini surfactants. The loss of neutral methylene (CH_2) moiety from the spacer region of product ion (7) produces a singly charged fragment ion at *m*/*z* 297.32 (ion 7′).

As indicated earlier, product ion (2) also yields the formation of product ion (8) through the neutral loss of the second twelve carbon atom tail region. This ion can exist as a doubly charged species (ion 8^{++}) at *m*/*z* 100.59 or singly charged ion (ion 8^{+}) at *m*/*z* 200.17. A neutral loss of the remaining head group (i.e., CH_3NHCH_3) from product ion (8^{+}/8^{++}) results in the formation of ion (9) which also exists as a singly charged (ion 9^{+}) *m*/*z* 155.11 and doubly charged (ion 9^{++}) *m*/*z* 78.06 (not shown in the spectrum). Product ion (9) further fragments via two fragmentation mechanisms. In the first mechanism, product ion (9a) is formed through the same mechanism as fragment ion (6); i.e., via the neutral loss of the methanimine moiety producing product ion (9a) at *m*/*z* 126.09. The second mechanism involves neutral loss of ethyne (acetylene) moiety forming fragment ion (9b) at *m*/*z* 129.10.

Finally, as explained earlier in the case of the 12-7N(Gly-Lys)-12 compound, product ion (2) can also fragment through a third pathway and form product ion (9^{+}). This mechanism can occur through the loss of the remaining tail region with the attached head group from product ion (2) as a singly charged protonated ion designated as $[C_{14}H_{32}N]^{+}$ observed at *m*/*z* 214.25 (ion 11).

Similar to the 12-7N(Gly-Lys)-12 compound, the proposed fragmentation pathway for the 12-7N(Gly)-12 was confirmed via MS/MS/MS analysis using QqQ-LIT MS1. In addition, a deuterated form of glycine substituted gemini surfactant has been synthesized to be used as internal standard for the purpose of developing a quantitative multiple-reaction-monitoring LC–MS/MS method. This deuterated compound retains two deuterated tail regions of dodecayl-d_{25} and has the designation 12_{D25}-7N(Glycine)-12_{D25}. The MS/MS analysis of this compound confirms the proposed fragmentation pathway by showing an increase in the *m*/*z* values of products ions (2), (6), (7), and (11) corresponding to the presence of deuterium in the structure (Table 4). On the other hand, fragment ions bearing no tail regions were identical (in terms of structure and *m*/*z* values) to those observed on the MS/MS analyses of non-deuterated compound.

Both histidine and lysine-substituted gemini surfactants followed the same fragmentation pattern as 12-7N(Glycine)-12 with minor variations resulting from the differences in the molecular structure of the amino acid substituents. For instance fragment ions (3), (4), (5) and (10^{+}/10^{++}) (Table 2) were not observed in the MS/MS spectrum of 12-7N(Glycine)-12 compound. The formation of these product ions required the loss of the (NH_3) moiety from the terminal amino acid which is not applicable in the case of the glycine amino acid substitution. For the same reason, we could not observe product ions (4), (5) and (10^{+}/10^{++}) in the MS/MS analysis of 12-7N(His)-12. However, these product ions were detected in the MS/MS analysis of lysine-substituted gemini surfactant due to the presence of two amine groups in the structure of lysine.

In addition to this difference, 12-7N(His)-12 gemini surfactant showed unique fragmentation mechanism resulting from the presence of the heterocyclic imidazole ring: product ion (3) is formed by the neutral loss of the (NH_3) moiety from histidine, producing a doubly charged fragment ion at *m*/*z* 216.19 (ion 3, Table 2 and Fig. 5). Distinct from the fragmentation pattern of glycine and lysine substituted gemini surfactants, this product ion can undergo two fragmentation pathways that involve hydrogen relocalization. The first pathway produces the commonly observed product ion (6) at *m*/*z* 170.17 (Table 2 and Fig. 5). The second pathway results in the formation of two complementary product ions that were designated as (3a) and (3b) resulting from the cleavage between the carbon atom of carbonyl group and the adjacent carbon atom of histidine within product ion (3). Ion (3b) observed at *m*/*z* 339.57 is the singly charged form of ion (6). However, the production of ions (6) and (3b) occurs concurrently by two different mechanisms as shown in Fig. 5. Ion (6) is produced by relocalizing the second charge from the imidazole ring to the secondary amine group of the spacer region (proton transfer), while the singly charged ion (3b) bears a tertiary amine. It is worth to mention that product ion (3b) was observed only in the MS/MS analysis of 12-7N(His)-12 compound, which confirm the proposed fragmentation mechanism. This proposition is supported by the formation of a complementary ion (3a) that was observed at *m*/*z* 93.1 which was detected during scanning for product ions below *m*/*z* 100 (data not shown).

3.2.3. Universal MS/MS fragmentation pattern

Similarities in the MS/MS fragmentation behaviour of the novel mono-amino acid/di-peptide substituted gemini surfactants allowed for the establishment of a universal MS/MS fragmentation pattern. Formation of product ions observed in the universal MS/MS fragmentation (Fig. 6) starts with the homolytic cleavage of (–C–N–) bond between one of the twelve carbon atom tail region and the attached quaternary ammonium head group producing a minor doubly charged product ion $[M–C_{12}H_{24}]^{2+}$ (ion 1, Fig. 6). It

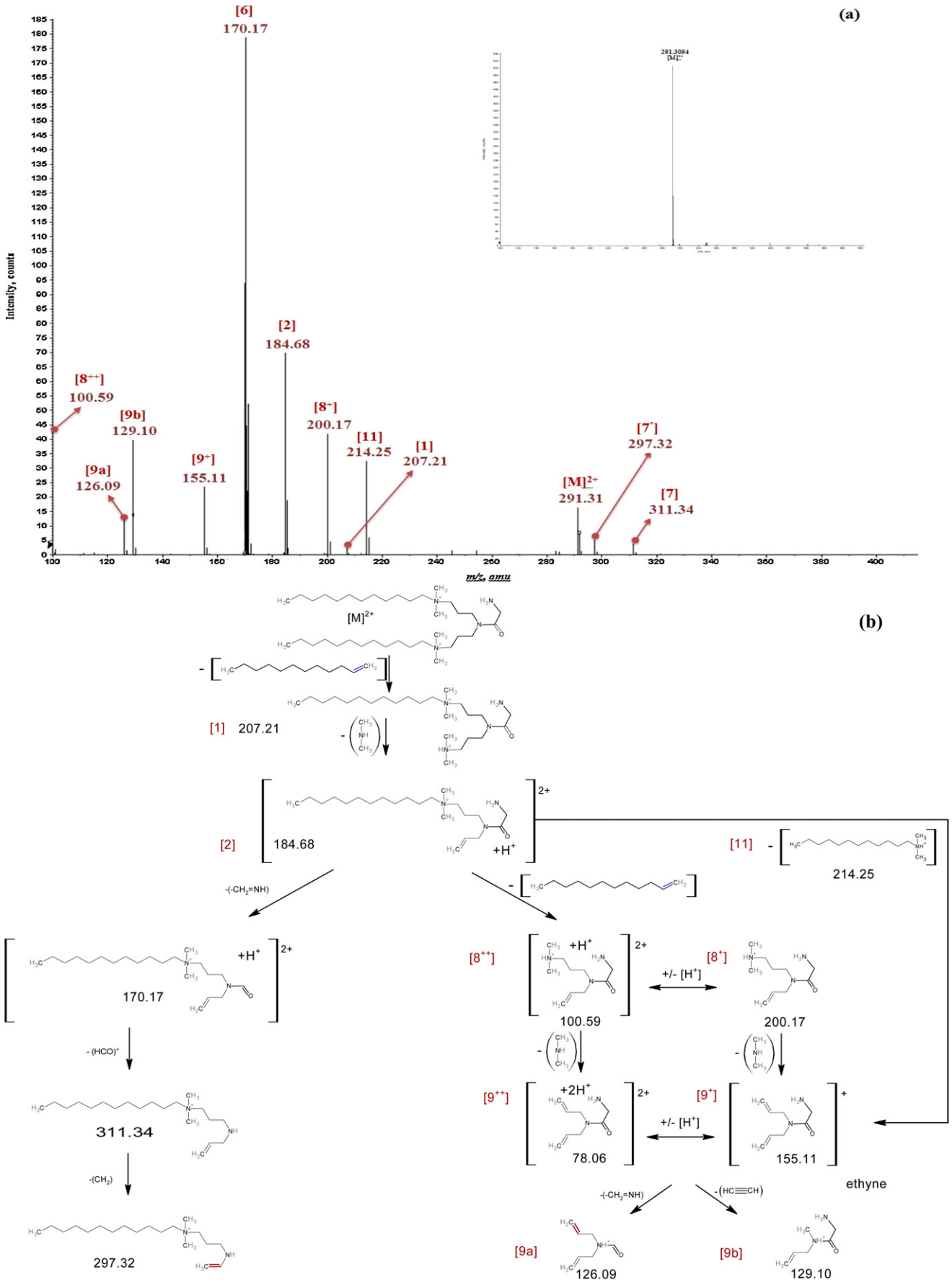


Fig. 4. (a) The ESI-QqToF MS/MS spectra of 12-7N(Glycine)-12 as a representative example of mono amino acid gemini surfactants (full MS spectrum in the box) and (b) the MS/MS fragmentation pattern showing the most distinctive product ions, other non-diagnostic product ions are not included.

Table 4
The difference in *m*/*z* values between 12-7N(Glycine)-12 and its deuterated form 12_{D25}-7N(Glycine)-12_{D25} confirm the proposed fragmentation pathway.

Product ion	12-7N(Glycine)-12	12_{D25}-7N(Glycine)-12_{D25}	*m*/*z* difference
Ion 2	184.68	197.26	12.58
Ion 6	170.17	182.75	12.58
Ion 7	311.34	336.49	25.15
Ion 11	214.25	239.41	25.15

is noteworthy that we were unable to conduct a MS/MS/MS experiment with this ion since it was always observed with very low intensity (except in the case of 12-7N(Gly)-12). Product ion (1) then produces product ion (2) and two pathways were proposed. The first mechanism was explained by the neutral elimination of the head group ($-C_2H_7N$) from ion (1) forming a diagnostic doubly charged ion $[M-(C_{12}H_{24})-(C_2H_7N)]^{2+}$ (Pathway A, ion 2). On the other hand, the second mechanism includes a neutral loss of hydrophobic tail region with the attached head group from the precursor ion $[M]^{2+}$ (Pathway B, ion 2). Product ion (2) is a predominant product ion in the MS/MS spectra of all gemini surfactants evaluated herein.

The product ion (2) in ESI-QqToF MS/MS conditions undergoes three main fragmentation processes. The elimination of (NH_3) forms the doubly charged fragment ion (3) $[M-C_{14}H_{32}N-(NH_3)]^{2+}$. This fragment ion is subjected to several fragmentation processes, producing different product ions which are shown in Fig. 6 and have been discussed with specific examples in Figs. 3b and 4b.

The second elimination process results from the heterolytic cleavage between the second tail region and the attached head group yielding the fragment ion (8^{++}). The subsequent loss of ammonium head group from ion (8^{++}) gives the product ion (9^{++}) designated as $[M-2(C_{12}H_{24}N)-2(C_2H_7N)]^{2+}$. Product ion ($9^{++}$) is further fragmented to ion (10^{++}) via the neutral loss of (NH_3).

In the case of the di-peptide gemini surfactants, peptide bond cleavage occurs in the product ion (10^{++}) producing two complementary fragment ions (B_1) and (Y_1).

Finally, in the third mechanism product ion (9^{+}) is formed directly from product ion (2) through the loss of the remaining tail region with the attached head group as a singly charged species of *m*/*z* 214.25 (product ion 11). Products ions (8^{++}, 9^{++}, 10^{++}) were always observed as minor peaks supporting the argument that the predominant singly charged form (8^{+}, 9^{+}, 10^{+}) is more stable.

Several remarkable differences are observed in the MS/MS analyses of these novel amino acid substituted gemini surfactants in comparison with our recent study evaluating the ESI-QqToF MS/MS behaviour of the non-substituted diquaternary ammonium gemini surfactants [26,27]. For instance, product ion (2) is diagnostic for the amino acid/di-peptide substituted gemini surfactants since the formation of this ion was not observed in the analysis of non-substituted compounds. This can be explained by the fact that the first generation diquaternary ammonium gemini surfactants do not have an amine group within the spacer region which can easily be charged. This ion was the source of all other fragments as shown in Figs. 3a and 4a. In addition, all diagnostic product ions formed through fragmentation within the spacer

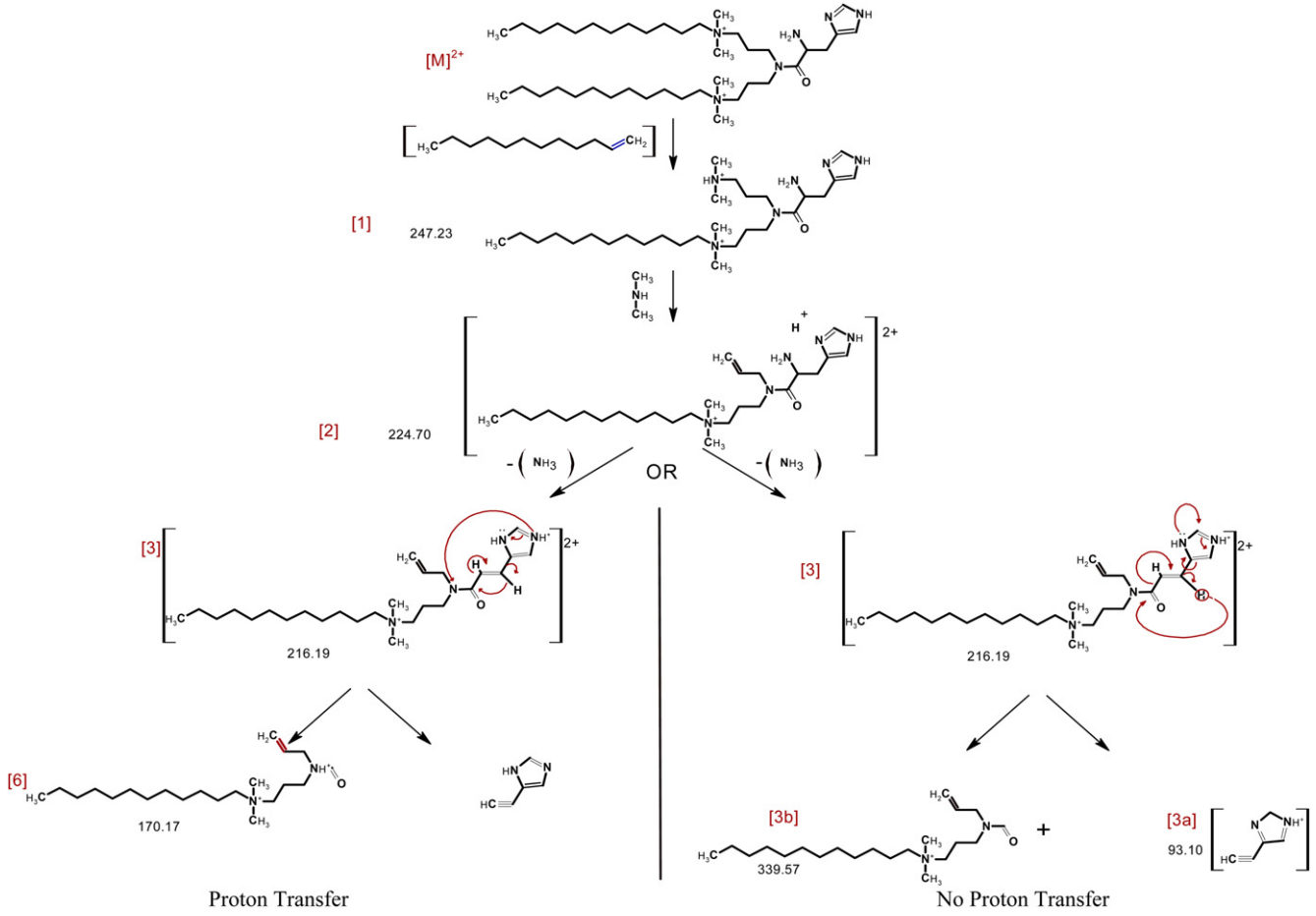


Fig. 5. Fragmentation mechanisms of product ion (3) of 12-7N(Histidine)-12.

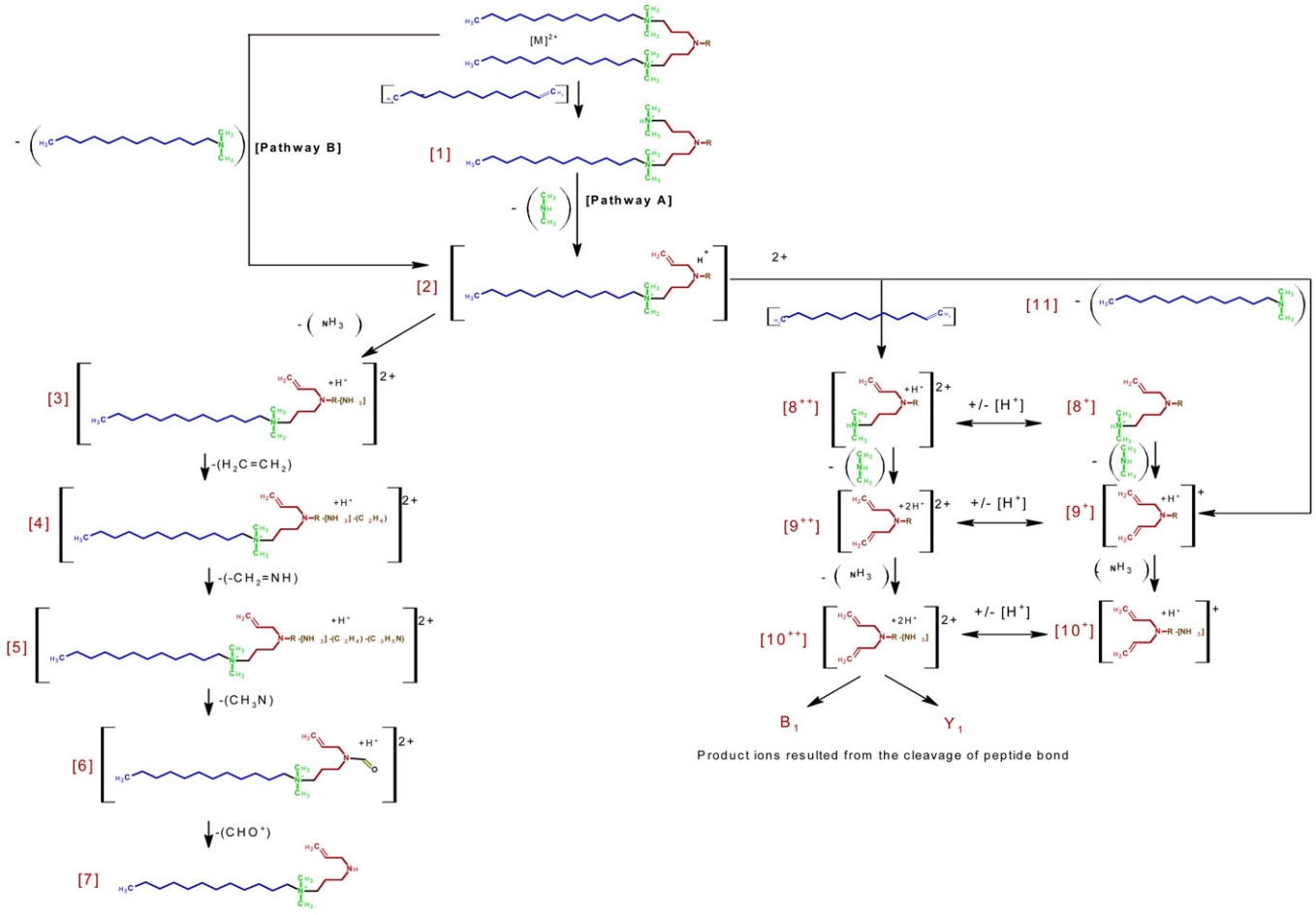


Fig. 6. Universal MS/MS fragmentation pattern for 12-7N[amino acid(s)]-12 gemini surfactants.

region are observed only in substituted gemini surfactants evaluated in this work.

4. Conclusion

In this study, the molecular structure of six novel mono-amino acid/di-peptide diquaternary ammonium gemini surfactants was confirmed using ESI-QqToF MS with internal calibration. The tandem mass spectrometric analysis (QqTof-MS/MS) showed similarities in the fragmentation patterns of all tested compounds. This allowed us to establish a universal MS/MS fragmentation pathway which was confirmed through performing MS/MS/MS experiments. In addition, we performed the MS/MS analysis for a deuterated 12_{D25}-7N(Glycine)-12_{D25} which bears deuterated tail region. It was observed that fragment ions identical to those observed when analyzing non-deuterated 12-7(Glycine)-12, were generated, differing merely by the ions bearing the deuterated tail region(s) (Table 4). This confirms the proposed universal fragmentation pathway shown in Fig. 6. The deuterated compound was synthesized as an internal standard that will be used during the development of HPLC–MS/MS quantification methods.

In summary, eleven common product ions were observed in the MS/MS analysis of almost all tested gemini surfactants. Two abundant diagnostic product ions observed in all tested gemini surfactants resulted from the loss of one tail region with attached head group (ion 2) or both tails and heads (ion 9). The proposed fragmentation pathway can be used as a "fingerprint" for rapid and accurate identification of these compounds in different biological or pharmaceutical matrices. In addition, by utilizing the MS/MS fragmentation pattern, we are currently developing a multiple reaction monitoring (MRM) HPLC–MS/MS method for the purpose of quantitation of these novel non-viral gene delivery agents.

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