
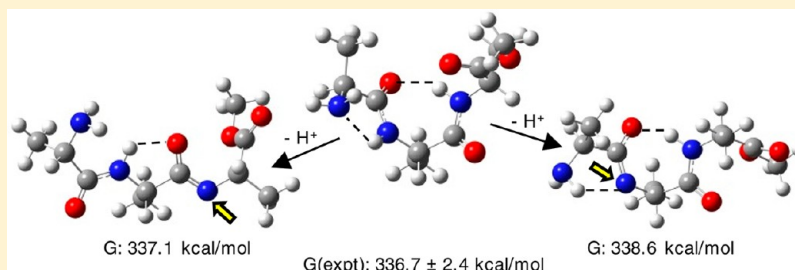


# Gas-Phase Deprotonation of the Peptide Backbone for Tripeptides and Their Methyl Esters with Hydrogen and Methyl Side Chains

Samantha S. Bokatzian-Johnson,<sup>†</sup> Michele L. Stover, David A. Dixon, and Carolyn J. Cassady\*

Department of Chemistry, University of Alabama, Tuscaloosa, Alabama 35487, United States

 Supporting Information



**ABSTRACT:** The gas-phase acidities (GAs) of six tripeptides (GlyGlyGly, GlyAlaGly, AlaGlyAla, AlaAlaAla, AibAibAib, and SarSarSar) and their methyl esters were obtained by proton transfer reactions in a Fourier transform ion cyclotron resonance mass spectrometer and G3(MP2) molecular orbital theory calculations. All six peptides have GAs in the range 321.0–323.7 kcal/mol. Their deprotonation to produce  $[M - H]^-$  occurs at the C-terminal carboxylic acid group. The tripeptides are about 10 kcal/mol more acidic than the amino acids glycine (Gly) and alanine (Ala). This is consistent with the extensive hydrogen bonding that was found in the tripeptide structures. For the methyl esters, deprotonation occurs at the peptide backbone. G3(MP2) calculations show that the most energetically favored site of deprotonation is an amide nitrogen, with the central amide being generally preferred. Nitrogen deprotonation requires 10–20 kcal/mol less energy than deprotonation at a methylene carbon. Only three of the methyl esters (GlyGlyGly-OMe, GlyAlaGly-OMe, and AlaAlaAla-OMe) deprotonate experimentally by electrospray ionization. Experimental GAs for these esters are in the range of 336.7–338.1 kcal/mol, in excellent agreement with the calculated G3(MP2) values. Experimental GAs could not be obtained for the other three methyl esters (AlaGlyAla-OMe, AibAibAib-OMe, and SarSarSar-OMe) because they did not produce sufficient deprotonated molecular ions. Trisarcosine methyl ester, SarSarSar-OMe, cannot be deprotonated at a central amide nitrogen because methyl groups are present at these sites; consequently, it has a high G3(MP2) GA value (less acidic) of 350.6 kcal/mol for deprotonation at the N-terminal nitrogen. For AlaGlyAla-OMe and AibAibAib-OMe, calculations of van der Waals and solvent accessible surfaces reveal that methyl groups are blocking the amide nitrogen sites. Therefore, conformational and steric hindrance effects are limiting the ability of these peptide methyl esters to deprotonate in the mass spectrometer.

## INTRODUCTION

Proton transfer processes are of importance to the biological activities, three-dimensional structures, and physical properties of peptides in solution.<sup>1–3</sup> Protonation and deprotonation is also important in gas-phase experiments employing mass spectrometry, which has been widely used for peptide sequencing in the past two decades.<sup>4–6</sup> Although not as commonly employed as protonated peptide fragmentation, deprotonated peptide dissociation can also be used for sequencing.<sup>7–12</sup>

Deprotonation of a peptide is generally accepted to take place at the C-terminal carboxylic acid group or other acidic sites such as the side chains of glutamic acid, aspartic acid, and cysteine residues.<sup>12–14</sup> However, gas-phase deprotonation is quite universal and even neutral and basic peptides deprotonate by electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), and fast atom bombardment (FAB).<sup>7,15–17</sup> For example, highly basic peptides without an acidic site (e.g., myosin kinase inhibiting peptide and substance

P) deprotonate readily in the gas phase.<sup>15</sup> Therefore, typical mass spectrometry ionization techniques are capable of deprotonating a peptide somewhere along the backbone, such as at the amide nitrogen or methylene carbon. Deprotonation of a peptide at a position other than the C-terminus may have a significant effect on fragmentation pathways because most dissociation in mass spectrometry is charge-directed.<sup>18,19</sup>

Exploration of alternate deprotonation sites for peptides may enhance the collective understanding of the mechanistic pathways of peptide dissociation. Various mechanisms proposed for deprotonated peptide fragmentations have involved both deprotonation at backbone methylene carbons and at backbone amide nitrogens.<sup>8,9,11,20–26</sup> For example, cleavage of the C–N bond to form negative mode c-ions has been proposed to involve nitrogen deprotonation, whereas

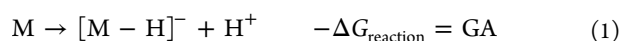
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(O=C)—C bond cleavage to form negative mode b- and y-ions has been suggested to involve carbon deprotonation.<sup>27</sup> In addition, the energetics of these deprotonation processes have not been clearly established. Using  $\Delta G_{\text{acid}}^{\circ}$  values for small molecules to estimate deprotonation of the peptide backbone suggests that deprotonation at a CH group adjacent to a carbonyl function requires  $\sim 5$  kcal/mol less than deprotonation of a NH group adjacent to a carbonyl.<sup>27</sup> Harrison and co-workers<sup>28</sup> studied the formation of "b<sub>2</sub>" ion structures derived by the loss of neutral glycine from deprotonated triglycine (GlyGlyGly) at the B3LYP and MP2 levels with the 6-31+G(d) basis set.

Determination of the gas-phase acidity (GA) for peptides that do not possess traditional acidic sites is important to understanding the energetic basis for deprotonation along the backbone. GA is defined as the negative Gibbs free energy change for the deprotonation reaction shown in reaction 1 at 298 K:



Several experimental studies have measured the GAs of the 20 standard amino acids.<sup>13,29–34</sup> The GAs of these amino acids have also been calculated at G3MP2 and lower levels.<sup>13,29–31,34–41</sup> However, GA values for amino acids are of limited value in representing residues in peptides because the acidic site on most amino acids is the C-terminal carboxylic acid group, which is absent unless the residue is located at the peptide's C-terminus.

There have been a few reports of the GAs of neutral peptides. Ren and co-workers<sup>14,42–44</sup> have performed experimental and computational studies of the GAs and structures of several small peptides containing a cysteine residue. The highly acidic cysteine side chain serves as the deprotonation site. The position of the cysteine residue in the peptide has been found to affect the GA values. In addition, the calculated structures indicate that extensive hydrogen bonding stabilizes the structures.

The goal of the current work is to explore deprotonation along the peptide backbone. Six model tripeptides and their methyl esters were studied both experimentally and computationally. The amino acid side chains contained either hydrogens or methyl groups. Hydrogens were replaced with methyl groups at specific sites along the peptide backbone to obtain information about deprotonation sites and their effects on acidity and structure.

## ■ EXPERIMENTAL AND COMPUTATIONAL METHODS

**Mass Spectrometry.** All experiments were performed on a Bruker (Billerica, MA, USA) Bruker BioApex 7e FT-ICR mass spectrometer with a 7.0 T superconducting magnet. Peptide solutions were prepared at 60  $\mu\text{M}$  in a solvent system of 50:50:1 methanol:water:ammonium hydroxide and introduced to an Apollo API source (Bruker Daltonics, Billerica, MA) using a syringe pump set to deliver  $\sim 90$   $\mu\text{L}/\text{h}$ . Electrospray ionization (ESI) employed a 3.5–4.0 kV potential with air as a heated (225 °C) counter and parallel current drying gas. Ions were allowed to accumulate in a hexapole for  $\sim 700$  ms before being transported to the ICR cell by electrostatic focusing.

Deprotonated ions,  $[\text{M} - \text{H}]^{-}$ , were isolated with correlated frequency ion ejection techniques<sup>45</sup> and then allowed to react with a reference compound that was introduced to the ICR cell

at constant pressure through a leak valve. Each of the ions selected for study was reacted with a series of reference compounds that have known GAs.<sup>46</sup> Neutral pressures were in the range  $(1\text{--}20) \times 10^{-8}$  mbar and were measured with a calibrated ionization gauge.<sup>47</sup>

Reaction rate constants,  $k_{\text{exp}}$ , were determined by observing the pseudo-first-order decay in reactant ion intensity as a function of time. In cases where deprotonation was in competition with proton-bound dimer formation,  $k_{\text{exp}}$  was determined by fitting the experimental reaction data as discussed previously.<sup>48</sup> Reported reaction efficiencies (RE) are the ratio of  $k_{\text{exp}}$  to collision rate constants that were obtained from the thermal capture trajectory calculation procedure of Su and Chesnavich.<sup>49,50</sup> An RE value of 0.269 was used as the "break point," where a reaction becomes exoergic and the GA is assigned; this selection of a break point follows the work of Bouchoux et al.<sup>51</sup> and has been discussed previously.<sup>13</sup>

**Peptide Synthesis.** All peptides were used in their L-forms. Triglycine (GlyGlyGly), trialanine (AlaAlaAla), glycylalanylglycine (GlyAlaGly), and alanylglycylalanine (AlaGlyAla) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Tri-2-methylalanine (AibAibAib) was synthesized in our laboratory by standard Fmoc procedures<sup>52</sup> on an Advanced ChemTech Model 90 peptide synthesizer (Louisville, KY, USA). Our attempts to synthesize trisarcosine (SarSarSar) in-house proved unsuccessful and this peptide was custom synthesized by Neo Bioscience (Cambridge, MA, USA).

A simple acid-catalyzed esterification with methanol was employed to generate the methyl ester forms of the six tripeptides. This equilibrium process was driven to ester formation by chemically removing water via reaction with acetic anhydride.<sup>53</sup> Typical conditions involved mixing 20  $\mu\text{L}$  of concentrated hydrochloric acid, 100  $\mu\text{L}$  of acetic anhydride, and 500  $\mu\text{L}$  of methanol and allowing the reaction to proceed for 10 min at room temperature. The product was used without purification, except for isolation of the desired deprotonated molecular ion during mass spectrometry experiments.

**Computational Details.** Calculations were performed at the density functional theory (DFT) and correlated molecular orbital (MO) theory levels with the program Gaussian-09.<sup>54</sup> The geometries were initially optimized at the DFT level with the B3LYP exchange–correlation functional<sup>55,56</sup> and the DZVP2 basis set.<sup>57</sup> The DFT calculations with this DFT optimized basis set were used to examine different conformations and to provide good starting structures for the more computationally expensive G3MP2 calculations. A range of conformers, in general up to 10, with as many hydrogen bonds as possible were examined starting from initial geometries based on our experience with the geometries of the amino acids.<sup>40</sup> In a number of cases, when there is no hydrogen bonding present due to methylation, fewer structures are possible and thus fewer were investigated. Vibrational frequencies were calculated to show that the structures are minima and to provide zero point and thermal corrections to the enthalpy and entropies so that free energies could be calculated for direct comparison to experiment. The most stable conformers were determined by optimizing a range of structures. In our previous work on the GAs of amino acids<sup>13,40</sup> and inorganic and organics acids,<sup>58</sup> the high level G3(MP2) correlated molecular orbital method<sup>59</sup> gave agreement for the acidities with the experimental values to within about  $\pm 1$  kcal/mol. G3(MP2) has an additional advantage over

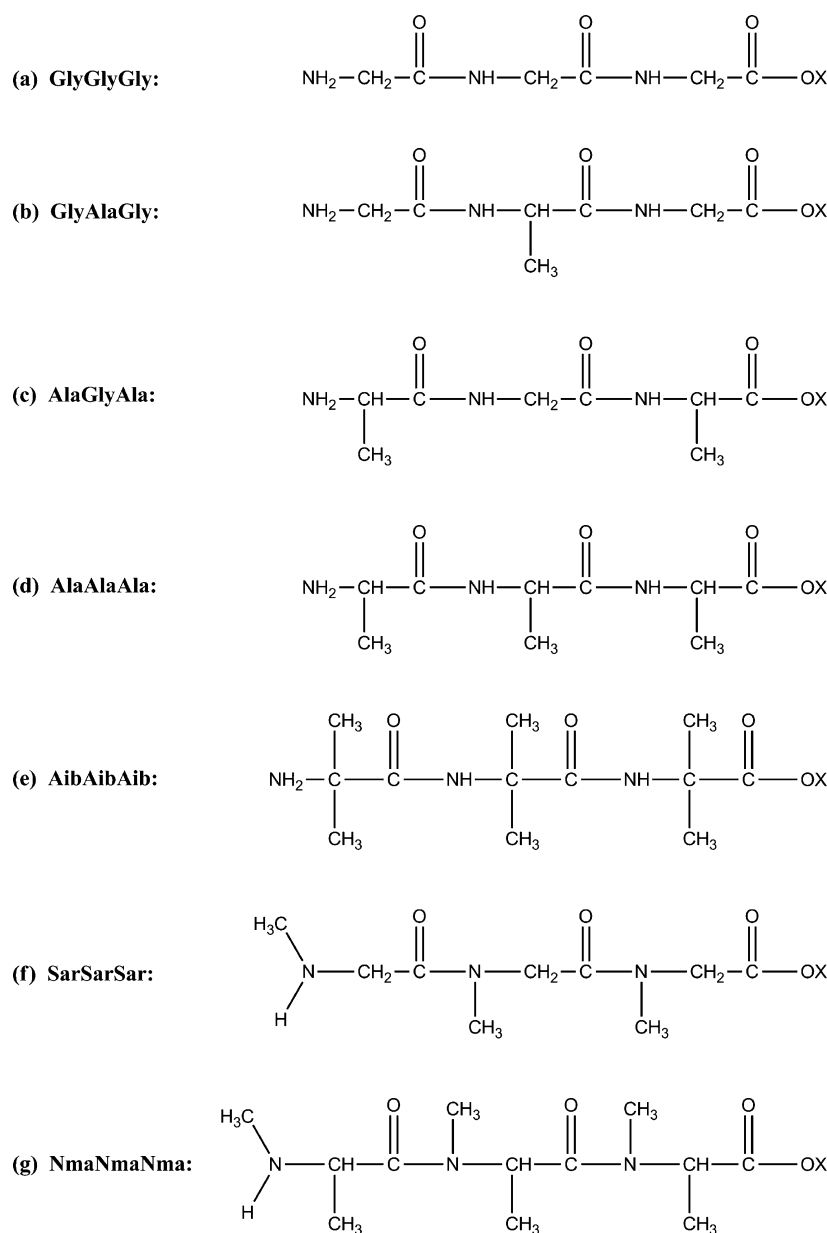


Figure 1. Structures of tripeptides (X = H) and their methyl esters (X = CH<sub>3</sub>).

Table 1. Reaction Efficiencies for the Proton Transfer Reactions of Deprotonated Tripeptides with Reference Compounds

ref compd	GA <sup>a</sup> (kcal/mol)	average reaction efficiency (±standard deviation)					
		GlyGlyGly	GlyAlaGly	AlaGlyAla	AlaAlaAla	AibAibAib	SarSarSar
trifluoropropionic acid	327.0 ± 1.1	0.05 ± 0.01	0.05 ± 0.00	0.06 ± 0.05	0.04 ± 0.00	0.04 ± 0.01	0.08 ± 0.01
difluoroacetic acid	323.8 ± 2.0	0.06 ± 0.02	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.07 ± 0.00
		BREAK <sup>b</sup>	BREAK	BREAK	BREAK	BREAK	BREAK
pentafluorophenol	320.7 ± 2.0	0.45 ± 0.05	0.48 ± 0.02	0.43 ± 0.02	0.48 ± 0.03	0.34 ± 0.02	0.57 ± 0.02
trifluoroacetic acid	317.4 ± 2.0	0.52 ± 0.06	0.53 ± 0.09	0.72 ± 0.06	0.51 ± 0.08	0.55 ± 0.11	0.72 ± 0.06

<sup>a</sup>All reference compound GAs were obtained from reference 46. <sup>b</sup>"BREAK" indicates the point where the experimental GA was assigned.

DFT methods in terms of reliable predictions for these types of compounds because the correlated molecular orbital methods in G3(MP2) perform better in the prediction of hydrogen bond energies as well as steric nonbonded interactions than do most widely used DFT exchange–correlation functionals. van der Waals surfaces and solvent accessible surfaces<sup>60–63</sup> were generated using the program Jmol.<sup>64</sup>

## RESULTS AND DISCUSSION

Six model tripeptides were chosen for this experiment: GlyGlyGly, GlyAlaGly, AlaGlyAla, AlaAlaAla, AibAibAib, and SarSarSar. Their structures are shown in Figure 1. Trisarcosine, SarSarSar, is structurally similar to triglycine, GlyGlyGly, except that each of the amide nitrogens along the peptide backbone are methylated and the N-terminal nitrogen has one methyl

Table 2. Experimental and G3(MP2) Theoretical GAs in kcal/mol for Tripeptides

peptide	experimental	G3MP2						
		N-terminus (NH <sub>2</sub> )	N-terminal C <sub>α</sub> H	central NH	central C <sub>α</sub> H	C-terminal NH	C-terminal C <sub>α</sub> H	C-terminus (COOH)
GlyGlyGly-OH	322.1 ± 2.5	364.2	350.6	334.7	344.5	329.2	344.3	323.3
GlyAlaGly-OH	322.3 ± 2.5	358.5	354.4	337.6	356.3	335.7	347.0	321.6
AlaGlyAla-OH	322.0 ± 2.5	360.3	358.4	340.3	355.3	338.1	348.9	321.0
AlaAlaAla-OH	322.2 ± 2.5	357.0	355.6	338.1	354.3	340.3	345.4	321.6
AibAibAib-OH	321.4 ± 2.5	<i>b</i>	<i>a</i>	330.4	<i>a</i>	328.9	<i>a</i>	322.1
SarSarSar-OH	322.6 ± 2.5	353.2	347.0	<i>a</i>	358.2	<i>a</i>	348.7	323.7
NmaNmaNma-OH	N/A <sup>c</sup>	322.0	322.9	<i>a</i>	329.5	<i>a</i>	337.8	322.5

<sup>a</sup>This site does not exist in the peptide. <sup>b</sup>Attempts to deprotonate at this site in the calculations resulted in a reversion back to deprotonation at the central NH. <sup>c</sup>N/A = not available. Experimental GA was not studied.

group plus one hydrogen. (Sarcosine is also known as N-methylglycine.) Tri-2-methylalanine, AibAibAib, is similar to trialanine, AlaAlaAla, except that each of the  $\alpha$  carbons along the peptide backbone has two methyl groups (i.e., no hydrogens present on any of the alpha carbons). The methyl esters of each of these peptides were also studied with the C-terminal carboxylic acid group (–COOH) converted to a methyl ester group (–COOCH<sub>3</sub>).

Electrospray ionization (ESI) produced abundant  $[M - H]^-$  for all six of the tripeptides. The peptide methyl esters produced much less abundant  $[M - H]^-$ , with the exception of AlaAlaAla-OMe which produced a  $[M - H]^-$  signal of comparable (and sometimes greater) intensity than the peptide acids. AlaAlaAla-OMe was also included in a previous study by Harrison<sup>23</sup> using deprotonated peptides with alkyl and hydrogen side chains. In that work, he reported the ability of AlaAlaAla-OMe to deprotonate readily. The fact that peptide methyl esters generally do not deprotonate as intensely as their acid forms (with the exception of AlaAlaAla-OMe) is reasonable because the peptide acids have a highly acidic carboxylic acid group at the C-terminus and this deprotonation site is lacking from the methyl esters, which must deprotonate at a backbone site.

Extremely low ESI signals were produced for  $[M - H]^-$  from AlaGlyAla-OMe, AibAibAib-OMe, and SarSarSar-OMe. This was also observed when using an ESI source on another mass spectrometer, a Bruker (Billerica, MA, USA) HCTultra high capacity quadrupole ion trap. These three methyl esters did not generate sufficient  $[M - H]^-$  for study by ion/molecule reactions and are thus not included in the experimental GA determinations. They are, however, included in our computational study.

**Experimental and Theoretical GAs and Structures of Tripeptides.** Table 1 lists the reference compounds and reaction efficiencies for deprotonation reactions with the tripeptides. Experimental GAs for each of the tripeptides are shown in Table 2 together with the G3(MP2) calculated GAs for each of the possible deprotonation sites. The lowest-energy G3(MP2) calculated structures for the neutral and deprotonated peptides are shown in Figure 2. As a benchmark of the G3MP2 method, we calculated the GAs of some of the relevant reference compounds. The calculated GA of 323.6 kcal/mol for difluoroacetic acid is in excellent agreement with the experimental value and the calculated value of 322.3 kcal/mol is within the error bars of the experimental GA of pentafluorophenol (Table 1).<sup>46</sup> As a check on this latter value, we calculated the GA of phenol to be 342.1 kcal/mol, in excellent agreement with the experimental value (Table 3).<sup>46</sup>

The GAs of all of the tripeptides were experimentally bracketed between difluoroacetic acid (GA = 323.8 kcal/mol<sup>46</sup>) and pentafluorophenol (GA = 320.7 kcal/mol<sup>46</sup>). This yields experimental GAs within a 1.2 kcal/mol range, 321.4–322.6 kcal/mol. The peptide GA values are in excellent agreement with the G3(MP2) values. As expected, the calculations predicted the most favorable site of deprotonation to be the C-terminal carboxylic acid group. These peptides are about 10 kcal/mol more acidic (lower GA values) than the amino acids glycine (G3(MP2) GA = 335.3 kcal/mol<sup>13</sup>) and alanine (G3(MP2) GA = 334.6 kcal/mol<sup>40</sup>). Thus, as the length of the peptide chain increases, the ability of the peptide to stabilize the charge increases and consequently the acidity at the C-terminus increases. This is likely due to increased hydrogen bonding stabilizing the CO<sub>2</sub><sup>−</sup> anion site that is not available in the monomer. This is analogous to basicity of the N-terminus increasing as the size of the peptide chain increases for polyglycines.<sup>48,65–67</sup> In addition, Ren and co-workers<sup>14,42–44</sup> have found that for small peptides containing cysteine residues the peptide becomes more acidic as the chain length increases; they also attributed this to enhanced hydrogen bonding for larger peptide chains.

The experimental GA of GlyGlyGly is 322.5 ± 2.3 kcal/mol. The calculated GA for GlyGlyGly of 323.3 kcal/mol is in excellent agreement and involves deprotonation at the C-terminus. The amide NH closest to the C-terminus is predicted to have a GA of 329.2 kcal/mol, which is only 5.7 kcal/mol less acidic than the carboxylic acid group of the C-terminus. The lowest energy calculated structures for neutral and C-terminally deprotonated GlyGlyGly are shown in Figure 2a. Neutral GlyGlyGly forms a wrapped structure with four hydrogen bonds. The N-terminus forms a hydrogen bond to the C-terminus (2.31 Å), the C-terminal amide forms a hydrogen bond with the N-terminal carbonyl oxygen (2.00 Å), the central amide forms a hydrogen bond with the N-terminus (2.13 Å), and the C-terminus forms a hydrogen bond between the two oxygens (2.30 Å). Deprotonated GlyGlyGly is slightly more compact than the neutral with three strong hydrogen bonds. This is due to the fact that the N-terminal carboxyl oxygen rotates away from the C-terminal amide essentially breaking that hydrogen bond and allowing the hydrogen bond between the N-terminus and the C-terminus to be shortened to 1.91 Å. The hydrogen bond between the central amide and the N-terminus is retained (2.06 Å) and a new, strong hydrogen bond forms between the C-terminal amide and the C-terminus (1.93 Å).

All of the calculated GA values are in excellent agreement with experiment to within 1.4 kcal/mol, as seen in Table 2.



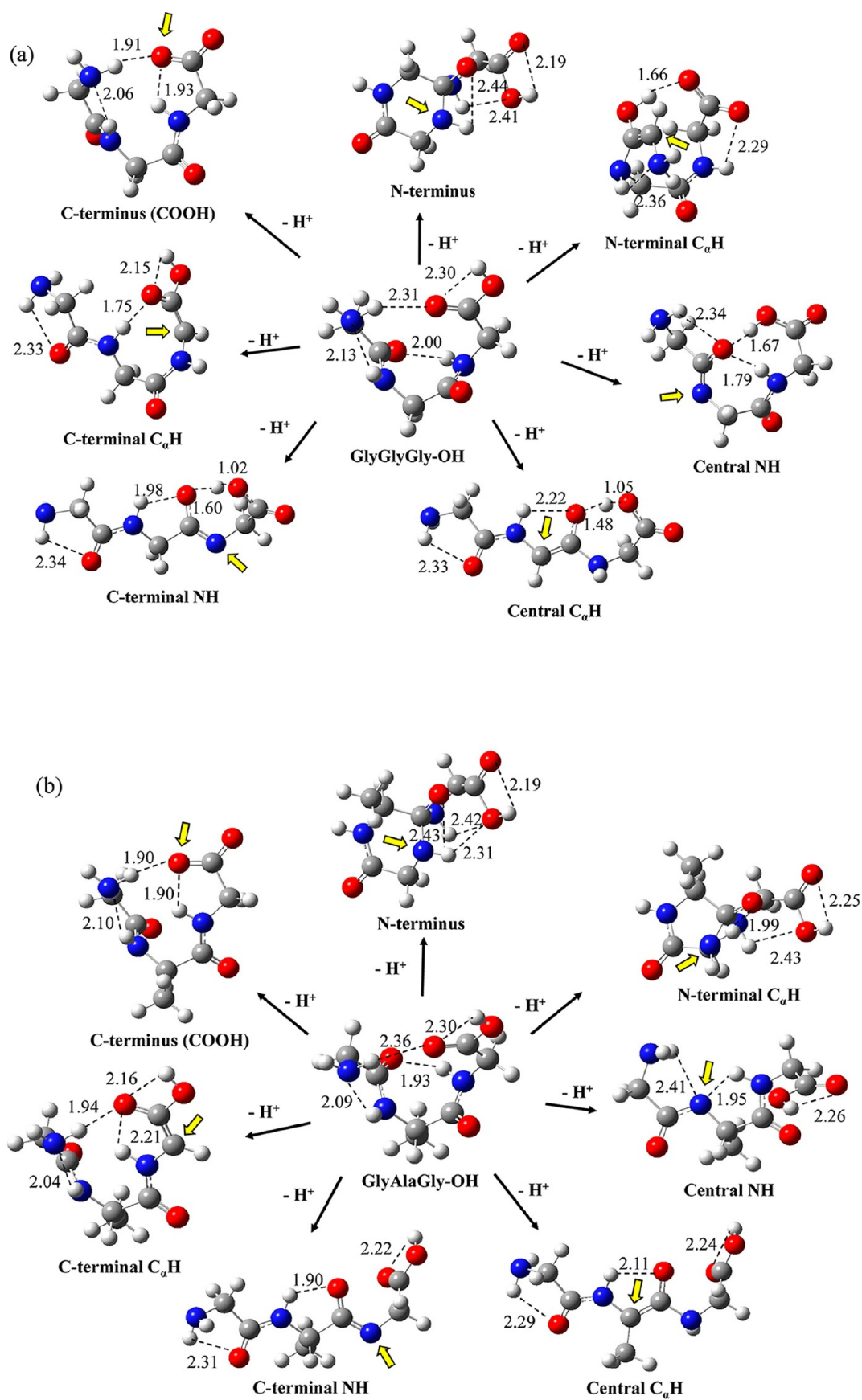


Figure 2. continued

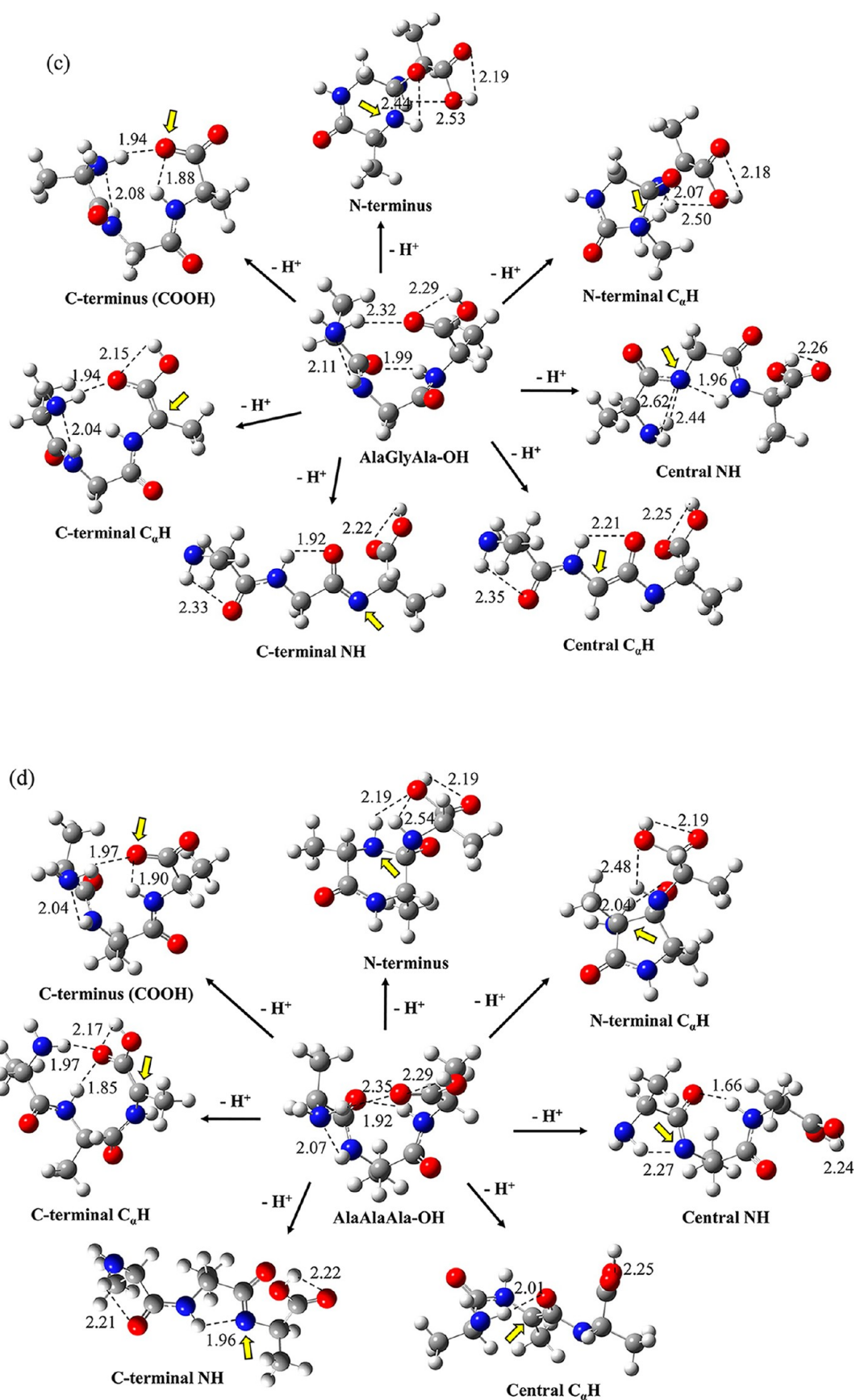
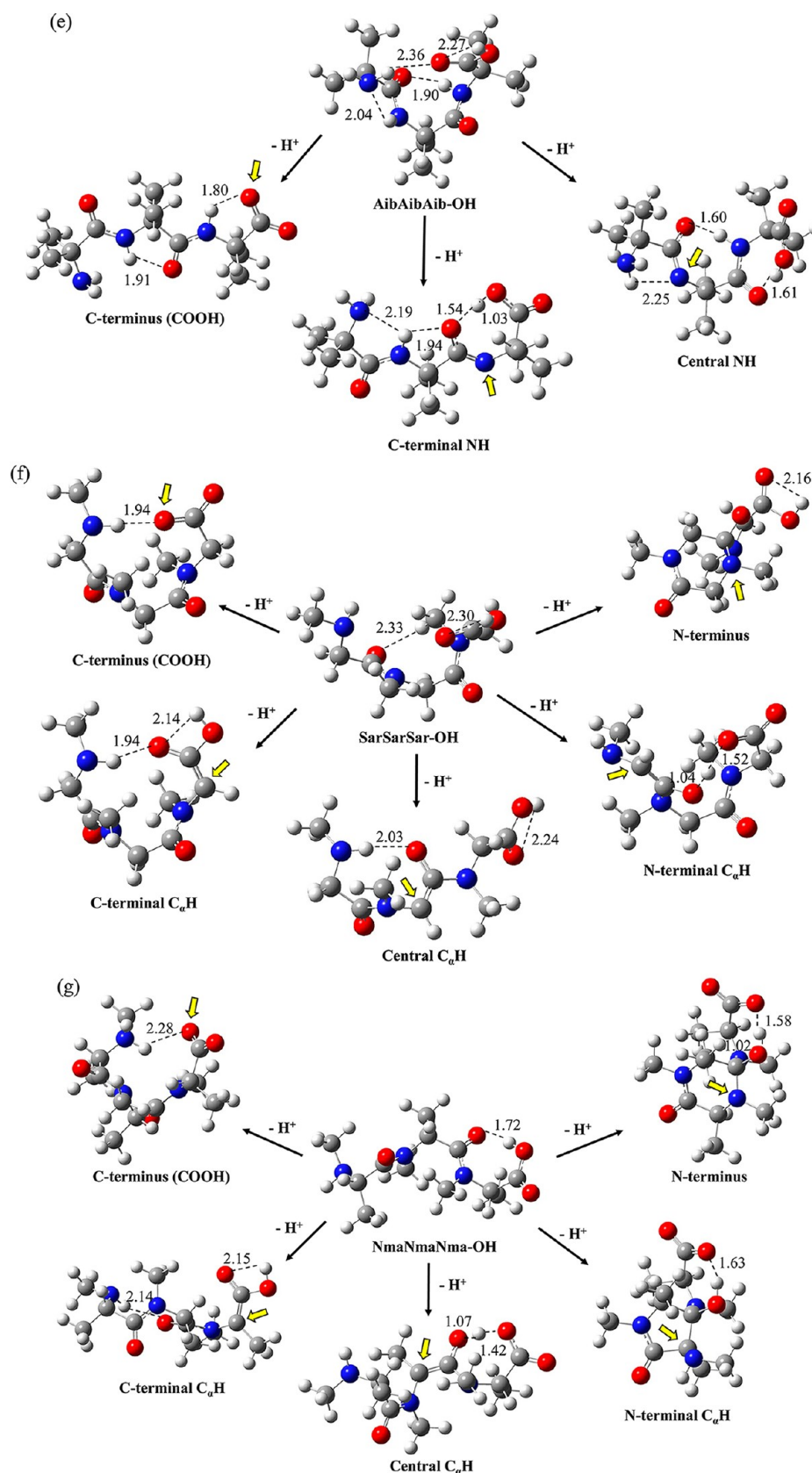


Figure 2. continued



**Figure 2.** G3(MP2) calculated structures for neutral and deprotonated tripeptide acids (a) GlyGlyGly, (b) GlyAlaGly, (c) AlaGlyAla, (d) AlaAlaAla, (e) AibAibAib, (f) SarSarSar, and (g) NmaNmaNma. Arrows indicate the deprotonation site. Bond lengths are in angstroms.

AlaGlyAla is the most acidic peptide with a calculated GA of 321.0 kcal/mol whereas AlaAlaAla and GlyAlaGly have a

slightly more basic GA of 321.6 kcal/mol. (Lower GA values are more acidic.) SarSarSar is the least acidic peptide with a

**Table 3.** Reaction Efficiencies for the Proton Transfer Reactions of Deprotonated Tripeptide Methyl Esters with Reference Compounds

ref compd	GA <sup>a</sup> (kcal/mol)	average reaction efficiency ( $\pm$ standard deviation)		
		GlyGlyGly-OMe	GlyAlaGly-OMe	AlaAlaAla-OMe
phenol	342.3 $\pm$ 2.0	0.05 $\pm$ 0.01	0.02 $\pm$ 0.004	0.04 $\pm$ 0.02
acetic acid	341.1 $\pm$ 2.0	0.025 $\pm$ 0.002	0.02 $\pm$ 0.005	0.02 $\pm$ 0.001
formic acid	339.1 $\pm$ 1.5	0.11 $\pm$ 0.06	0.10 $\pm$ 0.04	0.09 $\pm$ 0.02
isovaleric acid	338.5 $\pm$ 2.0	0.23 $\pm$ 0.04	0.09 $\pm$ 0.04	0.05 $\pm$ 0.03
trimethylacetic acid	337.6 $\pm$ 2.0	BREAK <sup>b</sup>		
		0.31 $\pm$ 0.18	0.22 $\pm$ 0.15	0.12 $\pm$ 0.02
<i>p</i> -chlorophenol	336.2 $\pm$ 2.0	0.32 $\pm$ 0.01	BREAK	BREAK
			0.41 $\pm$ 0.03	0.34 $\pm$ 0.01
3-(trifluoromethyl)phenol	332.4 $\pm$ 2.0	<sup>c</sup>	1.02 $\pm$ 0.09	1.09 $\pm$ 0.08

<sup>a</sup>All reference compound GAs were obtained from ref 46. <sup>b</sup>"BREAK" indicates the point where the experimental GA was assigned. <sup>c</sup>No experiment was performed.

**Table 4.** Experimental and G3(MP2) Theoretical GAs in kcal/mol for Peptide Methyl Esters

peptide	experimental	G3MP2					
		N-terminus (NH <sub>2</sub> )	N-terminal C <sub><math>\alpha</math></sub> H	Central NH	Central C <sub><math>\alpha</math></sub> H	C-terminal NH	C-terminal C <sub><math>\alpha</math></sub> H
GlyGlyGly-OMe	338.1 $\pm$ 2.3	363.0	358.1	340.1	358.7	342.1	348.7
GlyAlaGly-OMe	337.2 $\pm$ 2.4	358.1	354.4	338.7	357.0	339.2	350.0
AlaGlyAla-OMe	N/A <sup>b</sup>	361.8	358.2	340.7	362.3	341.0	352.2
AlaAlaAla-OMe	336.7 $\pm$ 2.4	358.4	355.0	338.6	355.4	337.1	347.9
AibAibAib-OMe	N/A <sup>b</sup>	363.2	<sup>a</sup>	339.9	<sup>a</sup>	354.9	<sup>a</sup>
SarSarSar-OMe	N/A <sup>b</sup>	350.1	354.1	<sup>a</sup>	354.7	<sup>a</sup>	351.6

<sup>a</sup>This site does not exist in the peptide. <sup>b</sup>Experimental GA could not be determined by ion/molecule reactions because peptide could not be deprotonated by ESI.

calculated GA of 323.7 kcal/mol; GlyGlyGly is only 0.2 kcal/mol more acidic. To complete this series, G3(MP2) calculations were performed on the tripeptide of *N*-methylalanine (NmaNmaNma). The GA of NmaNmaNma is essentially the same as that of C-methylalanine (AibAibAib) showing that methyl substitution on the backbone C or N affects the acidity in the same way. The lowest energy *N*-methyl neutrals SarSarSar and NmaNmaNma were compared to their corresponding lowest energy isomer C-methyl neutrals AlaAlaAla and AibAibAib, respectively. In SarSarSar versus AlaAlaAla and NmaNmaNma versus AibAibAib, both comparisons showed that the C-methyl isomers were lower in free energy than their respective *N*-methyl isomers by 30.4 and 43.5 kcal/mol, respectively.

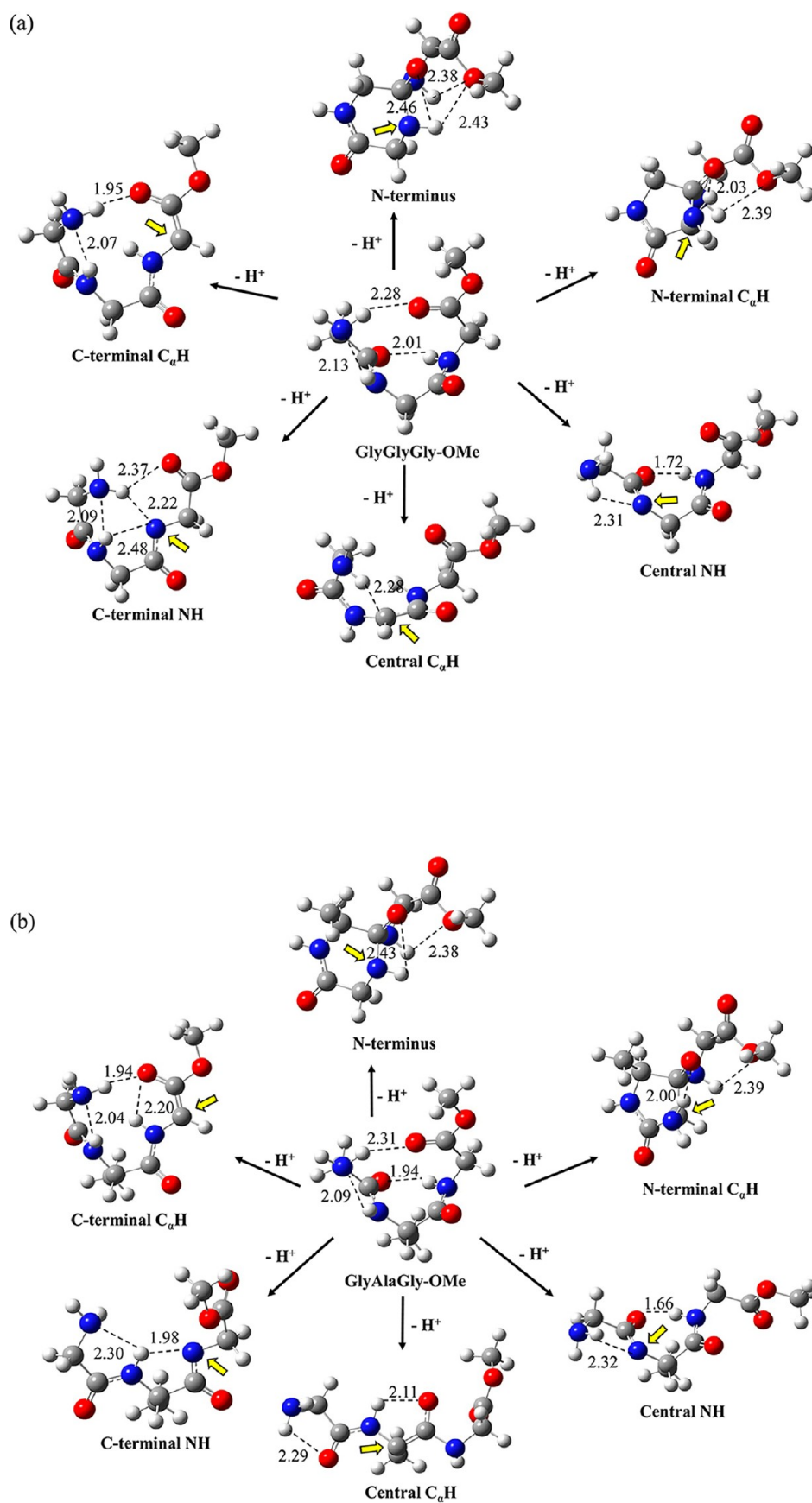
The GlyAlaGly, AlaGlyAla, AlaAlaAla, and AibAibAib lowest energy neutrals and anions basically have the same structure as neutral and anionic GlyGlyGly, respectively, including the same hydrogen bonds. This indicates that the hydrogen and methyl substituents have little effect on the structure itself. The AibAibAib anion is an exception because the additional methyl groups cause the lowest energy structure to be more linear due to steric interactions. Strong hydrogen bonds exist between the central amide and C-terminal carbonyl oxygen (1.91 Å) and the C-terminal amide and C-terminus (1.80 Å). The bond lengths and structures for these peptides are given in Figure 2b–e. Because of the methylated nitrogens in SarSarSar, the amide groups are no longer able to hydrogen bond to the carbonyl oxygens. For SarSarSar, the lowest energy neutral only contains one hydrogen bond, the C-terminus forms a bond between the two oxygens (2.30 Å). In anionic SarSarSar, a hydrogen bond forms between the N-terminus and the C-terminus (1.94 Å). For the lowest energy NmaNmaNma neutral, a very strong hydrogen bond exists between the C-terminus and the C-

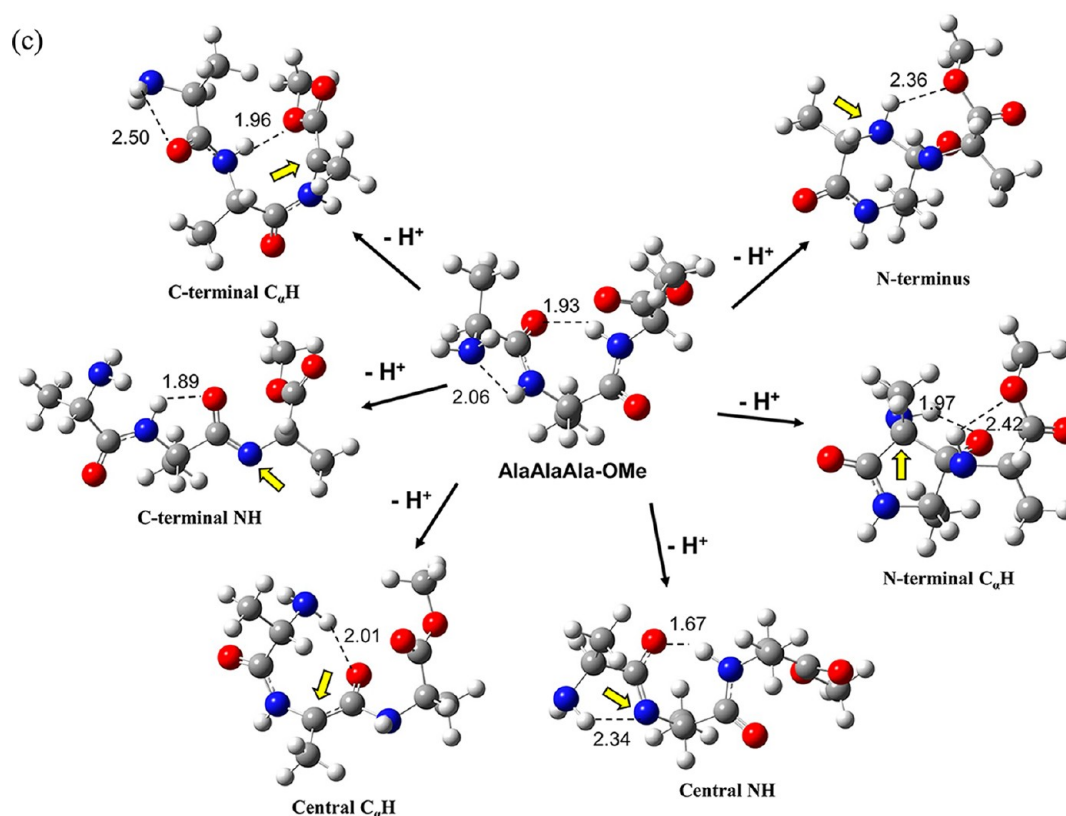
terminal carbonyl oxygen (1.72 Å). Anionic NmaNmaNma contains the same hydrogen bond as the SarSarSar anion, except the bond is longer by 0.24 Å. The structures for SarSarSar and NmaNmaNma are shown in Figure 2(f–g).

Two low energy neutral structures were predicted at the DFT and G3(MP2) levels for the neutral tripeptides. At the composite G3(MP2) level (energies based on MP2/6-31G(d) geometries), the neutral structure with three hydrogen bonds is higher in energy than the neutral structure with four hydrogen bonds for all of the tripeptides in terms of the free energy and enthalpy by 0.6–4.5 kcal/mol. As the difference in energy at the G3(MP2) level for all of the tripeptides, except for GlyGlyGly, is less than approximately 1.5 kcal/mol, both structures could be present in the gas phase. For GlyGlyGly, the energy differences are higher ( $\Delta H = 2.7$  and  $\Delta G = 4.5$  kcal/mol), so only the structure with the four hydrogen bonds should be present in the gas phase.

The DFT results can be compared to the composite G3(MP2) results to benchmark DFT's ability to predict the lowest energy neutral structures for these neutral tripeptides. The comparison showed that DFT predicted the wrong structure to be of lowest energy when compared to the G3(MP2) results. The lowest energy neutral predicted by DFT had only three hydrogen bonds, whereas the one predicted by G3(MP2) had four hydrogen bonds. At the DFT level, the neutrals with four hydrogen bonds were predicted to be higher in energy than the neutrals with three hydrogen bonds in AlaGlyAla, AlaAlaAla, and AibAibAib in terms of both the free energy and the enthalpy by 1.0–2.0 kcal/mol. For GlyAlaGly and SarSarSar, the structures with four hydrogen bonds are higher in energy than the ones with three hydrogen bonds by  $\sim 0.5$  kcal/mol for the enthalpy, but the free energy reverses the ordering with the structure with three hydrogen bonds being







**Figure 3.** G3(MP2) calculated structures for neutral and deprotonated tripeptide methyl esters (a) GlyGlyGly-OMe, (b) GlyAlaGly-OMe, and (c) AlaAlaAla-OMe. Arrows indicate the deprotonation site. Bond lengths are in angstroms. These peptide methyl esters deprotonate readily by ESI.

higher in energy by approximately 1 kcal/mol. For GlyGlyGly, the two structures are isoenergetic at the DFT level. The hydrogen bonds between the central amide and N-terminus and the C-terminal amide and the N-terminal carbonyl oxygen were retained in both the G3(MP2) and DFT structures with the difference between the two structures arising from the orientation of the hydrogen in the C-terminus. In the G3(MP2) structure, the N-terminus formed a bond to the C-terminus and the C-terminus formed a bond between the two oxygens. In the DFT structure, the hydrogen in the C-terminus was rotated away from the double bonded oxygen and formed a hydrogen bond with the C-terminal carbonyl oxygen. The structures of the higher energy neutrals at the G3(MP2) level are given in the Supporting Information.

Deprotonation from the N-terminal carbon or the N-terminus (sites 6 and 7) led to the formations of five- and six-membered rings, respectively, for all tripeptides. Deprotonation at the N-terminal carbon forms an electron rich carbon so that rotations about various bonds occur to close to a five-membered ring by bonding to the C-terminal carbonyl carbon. Deprotonation at the N-terminus leads to bond rotations so that a six-membered ring can be formed by bonding to the C-terminal carbonyl carbon. A larger free energy can also be seen in these ring molecules due to the decrease in entropy. Anions that are created from deprotonating the N-terminus that do not form a ring, as found for GlyGlyGly, have a higher GA value ( $\sim 400$  kcal/mol). The GAs of methane and ammonia, obtained by combining the experimental heats of formation with the entropy corrections calculated at the DFT level, are 409.6 and 397.0 kcal/mol, respectively.<sup>46,68</sup> The acidity calculated from deprotonating at the end terminus (i.e., the acidity of the amine group) and is comparable to that of ammonia.<sup>46</sup>

**Experimental and Theoretical GAs and Structures of Tripeptide Methyl Esters.** The GAs of the peptide methyl esters GlyGlyGly-OMe, AlaAlaAla-OMe, and GlyAlaGly-OMe have been determined for the first time. Converting the C-terminal carboxylic acid group to a methyl ester removes the most acidic site on the tripeptide, thus forcing deprotonation to occur at another site. Table 3 lists the reference compounds and reaction efficiencies for the deprotonation reactions with the methyl ester peptides. Table 4 lists the experimental GAs of each of the peptide methyl esters and the G3(MP2) calculated GAs for each of the deprotonation sites that were explored. The calculations show that the most favorable backbone deprotonation sites are the amide nitrogens. Figure 3 gives the lowest energy calculated structures for these neutral and deprotonated peptide methyl esters.

The GA of GlyGlyGly-OMe was experimentally determined to be  $338.1 \pm 2.3$  kcal/mol. G3(MP2) calculations show deprotonation of the amide nitrogens and the N-terminus to be very close in energy. The central amide nitrogen has a G3(MP2) calculated GA of 340.1 kcal/mol and the C-terminal amide nitrogen has a GA of 342.1 kcal/mol. Thus, ESI on GlyGlyGly-OMe is mostly likely causing deprotonation at the central backbone nitrogen site. The experimental and calculated GAs are within experimental error.

Figure 3a reveals that neutral GlyGlyGly-OMe adopts a conformation similar to that of GlyGlyGly except that only three hydrogen bonds are present because of methylation of the C-terminus. The N-terminus forms a hydrogen bond to the C-terminus (2.28 Å), the C-terminal amide forms a hydrogen bond with the N-terminal carbonyl oxygen (2.01 Å), and the central amide forms a hydrogen bond with the N-terminus (2.13 Å). When deprotonation occurs at the central NH of

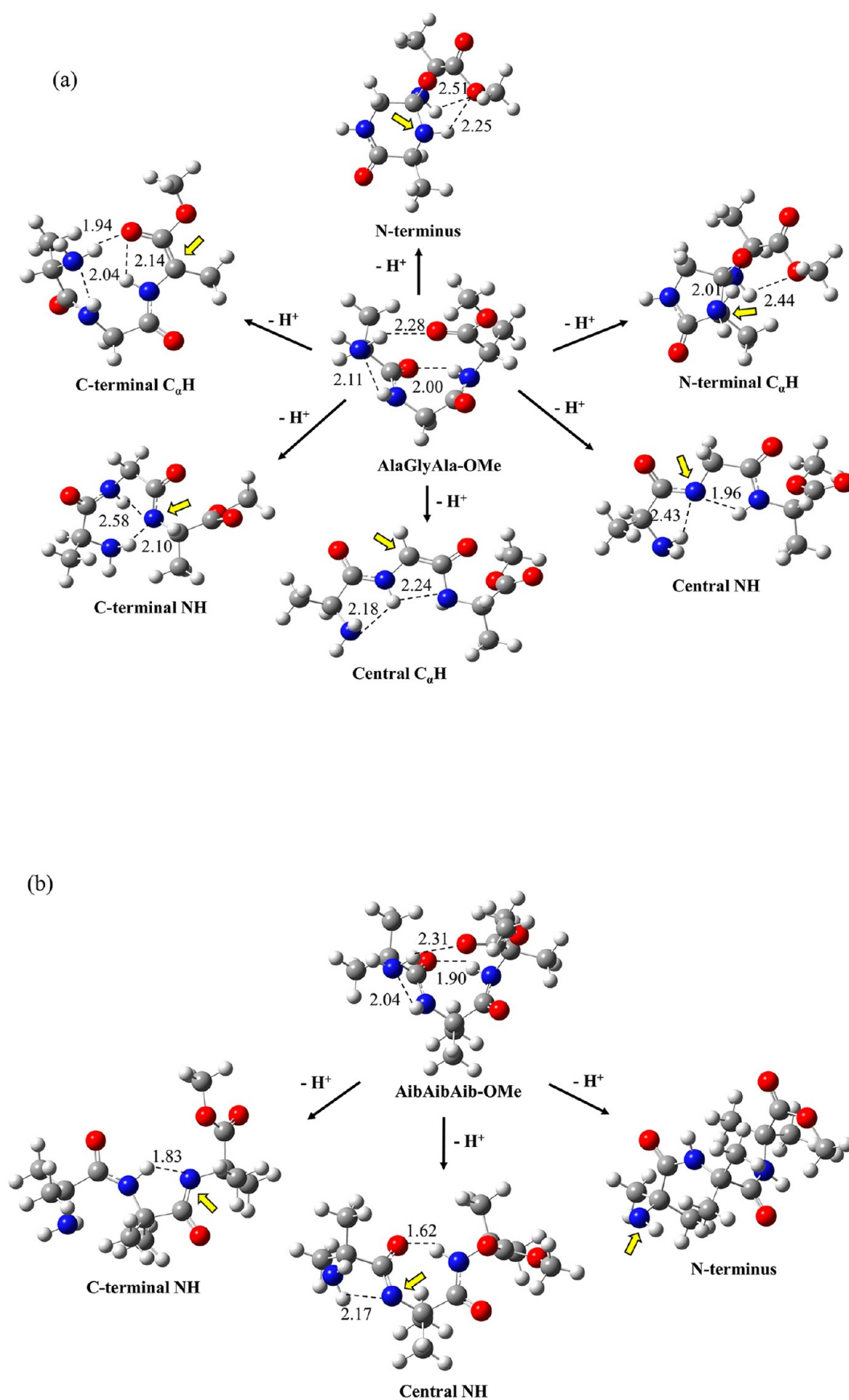
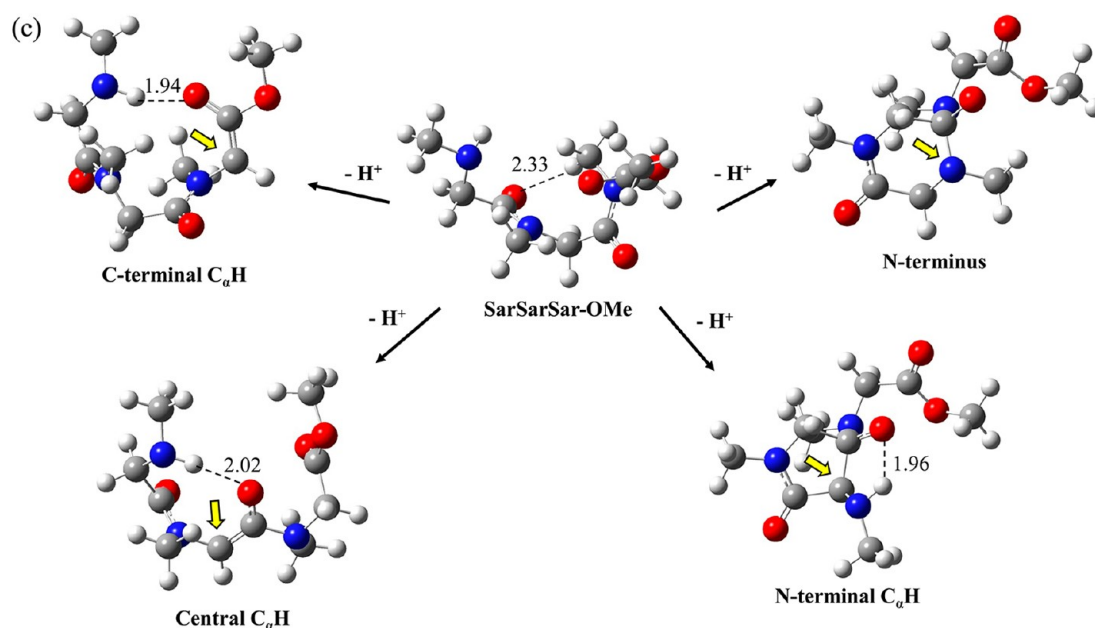


Figure 4. continued



**Figure 4.** G3(MP2) calculated structures for neutral and deprotonated tripeptide methyl esters (a) AlaGlyAla-OMe, (b) AibAibAib-OMe, and (c) SarSarSar-OMe. Arrows indicate the deprotonation site. Bond lengths are in angstroms. These peptide methyl esters do not deprotonate well by ESI.

GlyGlyGly-OMe, the resulting structure is more open than the neutral molecule due to the fact that the hydrogen bond between the N-terminus and C-terminus is broken. Hydrogen bonding is still observed between the C-terminal NH and the N-terminal carbonyl oxygen (1.72 Å), and the N-terminus has a hydrogen bond to the central nitrogen, which is the site of deprotonation (2.31 Å). Delocalization of the negative charge occurs over the central nitrogen and the adjacent carbonyl to form an amide structure given by the resonance structures  $C(=O)-N^- \leftrightarrow C(-O^-)=N$ .

The calculated GAs for GlyAlaGly-OMe and AlaAlaAla-OMe agree with experiment to within 2.0 kcal/mol for both the central and C-terminal amides (Table 4). Deprotonation from the central amide leads to a difference of only 0.1 kcal/mol between GlyAlaGly-OMe and AlaAlaAla-OMe and results in the most acidic anion for the GlyAlaGly methyl ester peptide. Deprotonation at the C-terminal amide in AlaAlaAla-OMe leads to a more acidic anion by 1.5 kcal/mol. The lowest energy structure for neutral GlyAlaGly-OMe has the same three hydrogen bonds as found for GlyGlyGly-OMe with only small changes in these bond lengths. The lowest energy structure for neutral AlaAlaAla-OMe is similar but has only two hydrogen bonds because of the loss of the hydrogen bond between the N-terminus and the C-terminus. The lowest energy GlyAlaGly-OMe anion results in the same structure as found for the GlyGlyGly-OMe anion. Anionic AlaAlaAla-OMe is still a very closed structure with a different set of hydrogen bonds. The central amide now hydrogen bonds to the C-terminus and the N-terminus is hydrogen bonded to the N-terminal carbonyl oxygen. Five- and six-membered rings are formed in the tripeptide methyl esters when deprotonation occurs at the N-terminal carbonyl carbon or the N-terminus, respectively, just as found for the nonesterified tripeptide acids.

#### Peptide Methyl Esters Not Deprotonated by ESI.

Three of the methyl esters studied, AlaGlyAla-OMe, AibAibAib-OMe, and SarSarSar-OMe, produced only extremely weak  $[M - H]^-$  by ESI and, consequently, their GAs could not be determined experimentally using ion/molecule reactions.

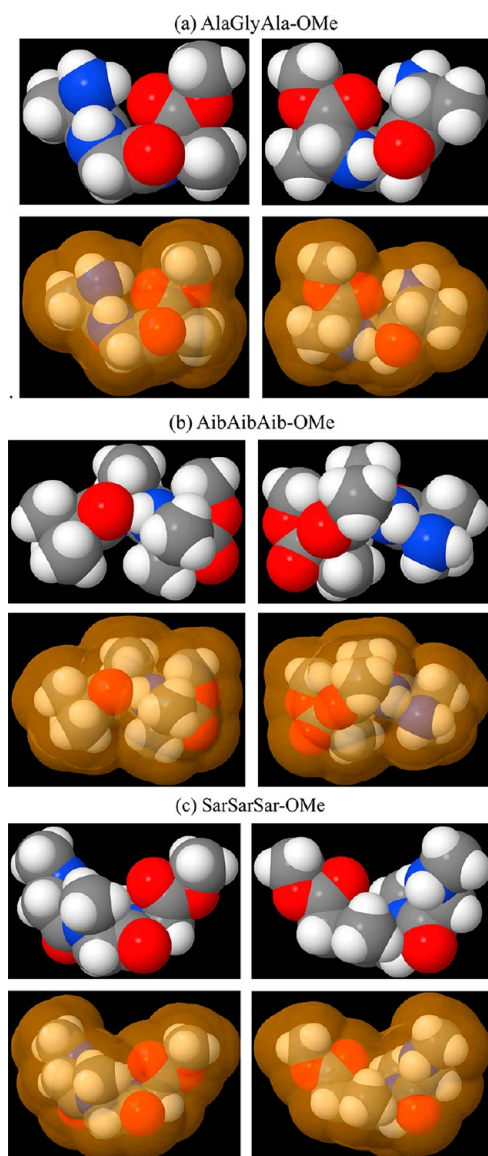
These species were studied computationally and Figure 4 shows the lowest energy structures of their neutral and deprotonated forms.

Abundant deprotonation by ESI requires that the analyte be more acidic than the solvent. This is obviously the case here. The reported GAs for the components of the solvent system used in this research are: GA of methanol =  $376.02 \pm 0.62$  kcal/mol and GA of water =  $383.74 \pm 0.06$  kcal/mol.<sup>46</sup> These GA values are much higher (less acidic) than the calculated GAs of AlaGlyAla-OMe, AibAibAib-OMe, and SarSarSar-OMe, which are included in Table 4. These methyl esters are much more acidic than the solvent system; thus solvent acidity cannot be the cause for their inability to form  $[M - H]^-$ .

The calculations show that the GAs of AlaGlyAla-OMe and AibAibAib-OMe are not the cause of the tripeptide methyl esters' inability to deprotonate by ESI. For AlaGlyAla-OMe, the calculated GAs are 340.7 and 341.0 kcal/mol for the central and C-terminal amides, respectively. These GAs are both within 1 kcal/mol of the GA for GlyGlyGly-OMe and very similar to the GAs of analogous sites in AlaAlaAla-OMe and GlyAlaGly-OMe; these latter three tripeptide methyl esters deprotonate readily by ESI. The calculated GAs of the central amides in AibAibAib-OMe show that only the central amide would deprotonate with a GA of 339.9 kcal/mol with the GA of the C-terminal amide  $\sim 15$  kcal/mol higher in energy. The GA for SarSarSar-OMe is 350.1 kcal/mol, which is  $\sim 10$  kcal/mol higher in energy than GlyGlyGly-OMe. Thus, with the potential exception of SarSarSar-OMe, the values of the GAs are not the reason that these anions are not observed.

Steric and conformational interactions within the neutral peptide are another viable reason for poor  $[M - H]^-$  formation. Conformation is known to affect the ability of ESI to protonate and deprotonate peptides (i.e., affect the charge state distribution produced by ESI).<sup>69–73</sup> More compact conformations, where potential deprotonation sites are shielded and inaccessible to solvent molecules, may not readily deprotonate.





**Figure 5.** Van der Waals surfaces and solvent accessible surfaces for neutral (a) AlaGlyAla-OMe, (b) AibAibAib-OMe, and (c) SarSarSar-OMe.

To study steric accessibility for proton removal, van der Waals surfaces and solvent accessible surfaces were generated for all of the tripeptides and their methyl esters. The surfaces for AlaGlyAla-OMe, AibAibAib-OMe, and SarSarSar-OMe are shown in Figure 5; the surfaces for the remaining peptides are in the Supporting Information. The van der Waals surface for AlaGlyAla-OMe shows that the C-terminal amide is covered by the N-terminal carbonyl oxygen because of a hydrogen bond (2.05 Å). The solvent accessible surface for AlaGlyAla-OMe shows that the orientation of the methyl groups in the terminal alanine residues and the wrapped nature of the peptide block the central amide from deprotonation. Comparison of AlaGlyAla-OMe with AlaAlaAla-OMe, which did deprotonate, shows that the orientation of the N-terminus with respect to the C-terminus can prevent deprotonation. In AlaAlaAla-OMe, the N-terminus is rotated away from the C-terminus; however, in AlaGlyAla-OMe, the end terminus has a very strong hydrogen bond to the double bonded oxygen in the C-terminus that could be preventing deprotonation. The van der

Waals surface for AibAibAib-OMe shows that the two methyl groups on each carbon in the backbone completely cover the C-terminal and central amide sites preventing deprotonation from occurring. The solvent accessible surface further justifies this conclusion. For SarSarSar-OMe, methylation of the central amide nitrogens and methylation of one hydrogen on the N-terminus essentially removes all sites of deprotonation. The van der Waals surface for SarSarSar-OMe shows that the only hydrogen available for deprotonation is blocked by the methyl group on the C-terminal carbon, consistent with its higher GA as noted above.

## CONCLUSIONS

The GAs of six tripeptides (GlyGlyGly, GlyAlaGly, AlaGlyAla, AlaAlaAla, AibAibAib, and SarSarSar) with neutral side chains and their methyl esters have been determined for the first time. Experimental and calculated GA values are in excellent agreement. The standard C-terminal acid (–COOH) peptides have very similar GAs, all falling within a 1.2 kcal/mol range. These tripeptides are about 13 kcal/mol more acidic than the amino acids glycine and alanine. Deprotonation occurs at the C-terminus, as confirmed by the computational results. The three peptide methyl esters that could be studied experimentally (GlyGlyGly-OMe, GlyAlaGly-OMe, and AlaAlaAla-OMe) also have very close GA values, all falling within a 2.0 kcal/mol range. Due to the lack of a traditional acidic site (i.e., –COOH), deprotonation of the methyl esters must be occurring at a backbone site on the peptide. High level G3MP2 computations predict that both C-terminal and central amide nitrogens (NHs) are the energetically favorable site for backbone deprotonation except for GlyGlyGly-OMe where the central NH is the most favorable. Thus, when mechanisms are proposed for the dissociation of deprotonated peptides during tandem mass spectrometry (MS/MS) processes, amide nitrogens should be considered as the most likely sites of backbone deprotonation. AlaGlyAla-OMe, AibAibAib-OMe, and SarSarSar-OMe peptides were also studied; however, ESI did not show deprotonation. The inability of ESI to deprotonate SarSarSar-OMe, which lacks amide hydrogens that can be abstracted, provides additional confirmation that amide nitrogens are the site of peptide backbone deprotonation by ESI. The calculated structures indicate that steric effects prevent deprotonation by ESI for AlaGlyAla and AibAibAib-OMe.

## ASSOCIATED CONTENT

### Supporting Information

Cartesian coordinates in angstroms of the G3MP2 (MP2(full)/6-31G(d)) optimized geometries,  $H_{298}$  and  $G_{298}$  values for all tripeptide and tripeptide methyl ester neutrals and anions at the G3MP2 level, higher energy neutral pictures with hydrogen bonds for all tripeptides at the G3MP2 level, van der Waals and solvent accessible surfaces for all of the tripeptides that readily deprotonate with ESI, and higher energy picture of GlyGlyGly N-terminus anion that did not form a ring at the G3MP2 level. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## AUTHOR INFORMATION

### Corresponding Author

\*E-mail: [ccassady@bama.ua.edu](mailto:ccassady@bama.ua.edu).

## Present Address

<sup>†</sup>Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO, USA 80521.

## Notes

The authors declare no competing financial interest.

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