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Linear Energy Correlations and Failures in the Low-energy Tandem Mass Spectra of Protonated *N*-Benzoylated Tripeptides: Tools for Probing Mechanisms of CAD Processes

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The backbone cleavages for three series of protonated *N*-benzoyl tripeptide ions were studied in a hybrid tandem mass spectrometer: (i) benzoyl-Gly-Gly-Xxx, where Xxx = Gly, Ala, Val, Leu, Ile, Phe, Tyr, Met, Glu, Pro and Trp, (ii) benzoyl-Gly-Xxx-Gly, where Xxx = Gly, Ala, Leu, Phe, Tyr, Met and Trp, and (iii) benzoyl-Xxx-Gly-Gly, where Xxx = Gly, Ala, Val, Leu, Ile, Phe, Tyr, Met, Pro and Trp. *C*-Terminal *y*-type ions and *N*-terminal *a*- and *b*-type ions were noted in all three cases. For benzoyl-Gly-Gly-Xxx, a linear relationship between log (y_1/b_2) and the proton affinity of the *C*-terminal amino acid substituents was found: as the proton affinity of the *C*-terminal residue increases, the fraction of y_1 ion formation increases. A similar relationship was noted for the benzoyl-Xxx-Gly-Gly tripeptides between log (y_2/b_1) and the proton affinity of the *N*-terminal amino acid substituent: as the proton affinity of the *N*-terminal residue increases, the fraction of b_1 ion formation increases. For the series benzoyl-Gly-Xxx-Gly, these relationships did not hold true. These observations point to similar reaction pathways throughout the benzoyl-Gly-Gly-Xxx series and also similar pathways throughout the benzoyl-Xxx-Gly-Gly, but pathways that are substituent dependent for benzoyl-Gly-Xxx-Gly. The increased correlation coefficients for benzoyl-Gly-Gly-Xxx and benzoyl-Xxx-Gly-Gly when compared with the free tripeptides, suggest that fewer interfering competitive reactions exist, as fewer possibilities for internal hydrogen bonding exist in the *N*-benzoyl derivatives versus the free compounds.

INTRODUCTION

Mass spectrometry is a well established tool used for structural determination.[1] With the advent of liquid secondary ion mass spectrometry (LSIMS[1]), fast atom bombardment (FAB[2]) mass spectrometry and electrospray ionization (ESI[3]) mass spectrometry, the ionization of involatile and thermally labile compounds was achieved. However, with these techniques, not much structural information was always readily available.[1] When these ionization sources are coupled with tandem mass spectrometry (MS/MS) and product ions are forced to fragment with the input of additional energy in collisionally activated dissociation (CAD), much more structural information is made available.[4–12] Peptides and proteins are commonly subjected to this analysis as part of an ongoing quest to use this technique as a means for determining sequences[3–7] and even secondary structure.[13] With CAD, a powerful source of information on structure has come the ability to study mechanistic aspects for fragmentation.[8–16] It has been demonstrated that the acid-base properties of both the backbone[11,12] and the side groups[9] of an oligopeptide control the intensity of various sequence peaks.

Previously,[14] we have reported a relationship between log (y_1/b_2) and the proton affinity of the *C*-terminal amino acid residue for protonated tripeptides of the series Gly-Gly-Xxx, where Xxx = Gly, Ala, Val, Leu, Ile, Phe, Tyr, Met, Glu, Pro and Trp. As the proton affinity of the variable *C*-terminal acid residue increases, the fraction of y_1 ion also increases. A similar relationship was also noted for the sodiated analogues of this series of tripeptides.[15] We have also reported[16] on the protonated tripeptides of Xxx-Gly-Gly, where Xxx = Gly, Ala, Val, Leu, Ile, Phe, Tyr, Met, Pro and Trp, and Gly-Xxx-Gly, where Xxx = Gly, Ala, Leu, Phe, Tyr, Met and Trp. For the series of Xxx-Gly-Gly, we noted a relationship between log (y_2/sum of all other ions): as the proton affinity of the *N*-terminal amino acid residue increases, the fraction of y_2 decreases. A linear relationship was not obtained with the Gly-Xxx-Gly series.

In keeping with the theme[7–11] of studying simple synthetic peptide models for determining mechanistic processes, we have investigated *N*-terminal benzoylated tripeptide models. This work deals with the study of three series of benzoylated tripeptides: (i) benzoyl-Gly-Gly-Xxx, where Xxx = Gly, Ala, Val, Leu, Ile, Phe, Tyr, Met, Glu, Pro and Trp, (ii) benzoyl-Gly-Xxx-Gly, where Xxx = Gly, Ala, Leu, Phe, Tyr, Met and Trp, and (iii)

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benzoyl-Xxx-Gly-Gly, where Xxx = Gly, Ala, Val, Leu, Ile, Phe, Tyr, Met, Pro and Trp. All of the variable amino acid residues were of the L-isomer. These tripeptides were chosen to determine the effects of varying the position and substituent of an amino acid on the fragmentation of an *N*-benzoyl tripeptide backbone, and in particular to compare these effects with those found previously for the peptides with free *N*-termini.

EXPERIMENTAL

Mass spectrometry

Tandem mass spectra were obtained on a VG70-250SEQ instrument (VG Analytical, Altrincham, UK) with EBqQ geometry. Samples were added to the FAB sample probe tip in glycerol–trifluoroacetic acid. The samples were ionized using a cesium ion gun operated at 30 kV (10 μA transmission flux). Precursor ions were transmitted at 10 kV through the first two sectors and submitted to CAD in the r.f.-only quadrupole region. Precursor and product ions were mass analyzed in the quadrupole assembly. CAD experiments were performed at an E_{lab} value of 10 eV. CAD experiments were also performed at an E_{lab} value of 50 eV for the benzoyl-Gly-Gly-Xxx series only. The collision gas was argon at 2×10^{-5} mbar as read by the ion gauge (ion beam attenuation 70%).[17–19] Product-ion spectra were acquired in the multi-channel analysis (MCA) mode scanning from *m/z* 20 to 700 with the accumulation of 15 scans per sample.

Product-ion abundances are reported relative to the sum of all the products because of the transmission effects on the precursor-ion abundance as the collision energy changes.[18,20] The Roepstorff–Fohlman[21] nomenclature for peptide backbone cleavages as modified by Biemann[22] is used.

Tripeptides

Benzoyl chloride was prepared by a standard method. This was used to prepare the *N*-acylated tripeptides in the following procedure: 50 mg of the tripeptide were added to 1 ml of water and the pH was adjusted to approximately 10 with 1 M NaOH. The solution was reacted with three portions of 15 μl each of benzoyl chloride over a 15 min period while the pH was maintained near 10. The solution was allowed to stir for another 30 min, after which the pH was reduced to 4 using 6 M HCl. The solution was placed in a refrigerator for 30 min and the precipitate was collected by vacuum filtration. All tripeptides used in this study were obtained from Bachem Bioscience (Philadelphia, PA, USA), and the *N*-benzoyl derivatives were used without further purification, there being no evidence of contamination in the FAB spectra.

RESULTS

Benzoyl-Gly-Gly-Xxx

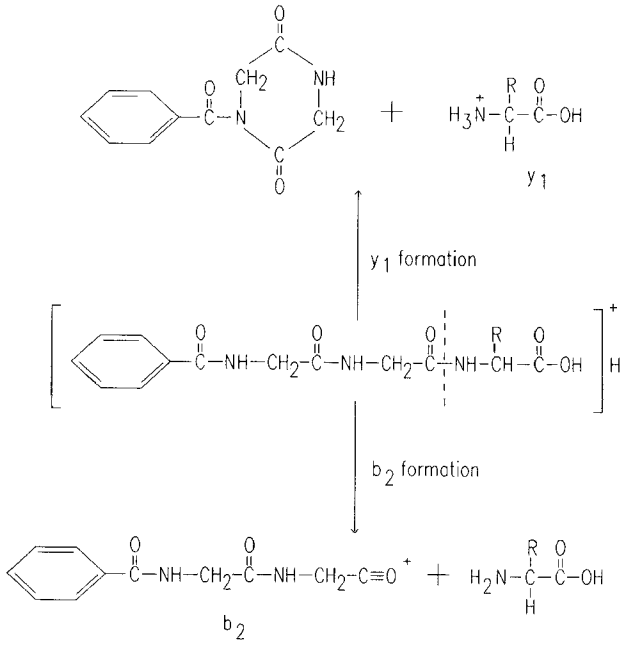
Table 1 lists the fractions of product ions in the tandem mass spectra of the protonated benzoyl tripeptide ions at a collision energy of 10 eV, expressed as percentages. The only complete set of complementary fragment ions noted in the tripeptide spectra are the b_2 and y_1 ions. This complementary aspect is demonstrated in Scheme 1.

Figure 1 shows the correlation between $\log (y_1/b_2)$ for the tripeptide based on the proton affinity of the *C*-terminal amino acid substituent at 10 eV. Proton affinity values were taken from Bojesen and co-workers' data,[23–25] which did not include values for Gly or Ala. Proton affinity values for Gly and Ala were taken from the standard source.[26] The correlation coefficient (*r*) was determined to be 0.968 for the relationship at 10 eV. For comparison, the same data obtained for protonated free Gly-Gly-Xxx gave $r = 0.959$.[14]

Table 2 shows the data for protonated benzoyl-Gly-Gly-Xxx at a collision energy of 50 eV. Figure 2 shows the linear correlation between $\log (y_1/b_2)$ and the proton affinity of Xxx. Figure 2 is the 50 eV complement of Fig. 1 at 10 eV. A correlation coefficient of 0.972, similar to the coefficient at 10 eV, is noted.

Table 1. Comparison of protonated benzoyl-Gly-Gly-Xxx CAD spectra at 10 eV collision energy

	Relative abundance (%)						
Benzoyl-Gly-Gly-Xxx	a_3	b_3	b_2	b_1	y_2	y_1	Total
Benzoyl-Gly-Gly-Gly		9.9	9.6	8.3	72.2	0.1	100.1
Benzoyl-Gly-Gly-Ala	0.5	15.4	11.4	1.1	69.7	0.3	98.5
Benzoyl-Gly-Gly-Val	5.5	31.8	9.4	0.7	51.1	1.6	100.0
Benzoyl-Gly-Gly-Leu	8.0	39.5	5.6	0.4	43.8	2.8	100.0
Benzoyl-Gly-Gly-Ile	10.0	40.6	3.6	0.4	41.4	4.1	100.0
Benzoyl-Gly-Gly-Phe		19.9	1.6		67.6	10.9	100.0
Benzoyl-Gly-Gly-Tyr	3.5	16.3	0.7		67.3	6.7	94.6
Benzoyl-Gly-Gly-Met	0.4	2.8	0.8	0.2	84.1	9.1	97.4
Benzoyl-Gly-Gly-Glu	0.3	4.5	0.7		81.9	8.3	96.7
Benzoyl-Gly-Gly-Pro		1.5	0.4		81.9	16.2	100.0
Benzoyl-Gly-Gly-Trp	3.7	13.7	0.1		74.9	7.6	100.1



Scheme 1.

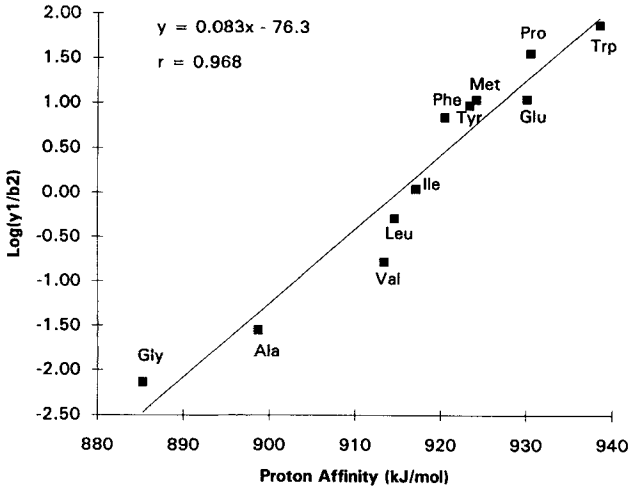


Figure 1. Correlation of the logarithm of the ratio of abundances of (y_1/b_2) in benzoyl-Gly-Gly-Xxx with proton affinity of Xxx at 10 eV collision energy.

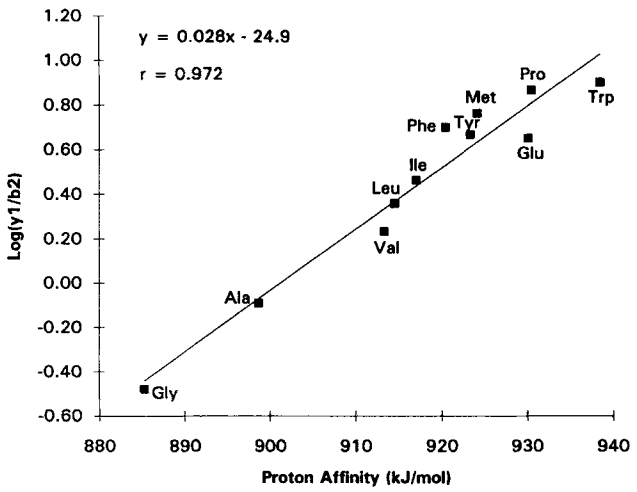


Figure 2. Correlation of the logarithm of the ratio of abundances of (y_1/b_2) in benzoyl-Gly-Gly-Xxx with proton affinity of Xxx at 50 eV collision energy.

Increasing the collision energy greatly reduces the slope of the relationship along with the *y*-intercept.

Benzoyl-Gly-Xxx-Gly

Table 3 lists the percentages of product ions for protonated benzoyl-Gly-Xxx-Gly. Previously for unprotected Gly-Xxx-Gly,[16] we had shown an extremely poor correlation for the ratio of the abundances of (b_2/all other ions) versus the proton affinity of the internal amino acid; not even a general trend of the abundance ratio could be noted from the data. Similar data are presented for the benzoyl-Gly-Xxx-Gly in Fig. 3. There is clearly no general trend in these data either. A trend was expected since a proton is transferred from the amino end of the molecule (including Xxx) to the departing carboxylate end. Even an attempt at a correlation between the ratio of abundances of (y_2/sum of all other ions) with proton affinity, Fig. 4, was not obtained.

Benzoyl-Xxx-Gly-Gly

The percentages of product ions for protonated benzoyl-Xxx-Gly-Gly are given in Table 4. Unlike the

Table 2. Comparison of Protonated benzoyl-Gly-Gly-Xxx CAD spectra at 50 eV collision energy

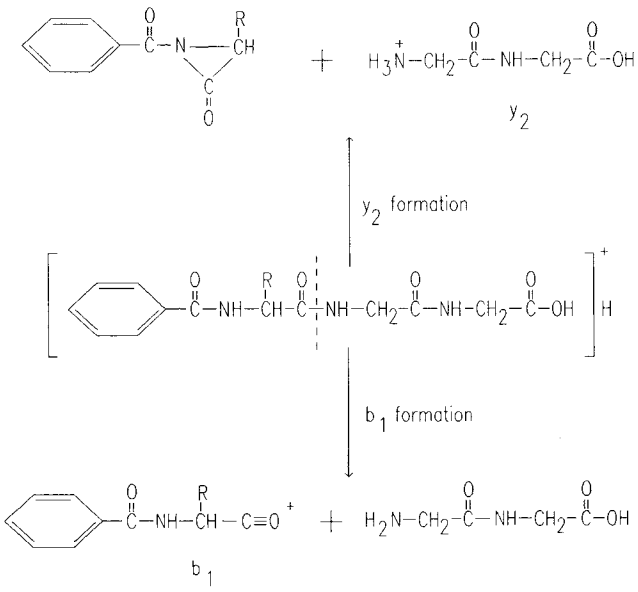
	Relative abundance (%)										
Benzoyl-Gly-Gly-Xxx	a_3	a_1	b_3	b_2	b_1	y_2	y_1	a_3y_2	Imm.[a]	Benzoyl	Total
Benzoyl-Gly-Gly-Gly		10.8	0.9	3.5	30.5	26.7	1.2			25.6	99.1
Benzoyl-Gly-Gly-Ala	0.3	8.6	1.0	5.0	28.0	32.0	4.1	0.6		17.2	97.2
Benzoyl-Gly-Gly-Val	1.0	7.1	1.7	4.8	23.5	33.0	8.3	6.4	0.9	12.8	99.3
Benzoyl-Gly-Gly-Leu	0.7	5.6	1.7	4.9	21.0	32.9	11.1	5.8	5.8	10.0	99.4
Benzoyl-Gly-Gly-Ile	1.1	7.0	1.8	4.1	19.5	30.7	11.9	8.6	5.6	9.1	99.4
Benzoyl-Gly-Gly-Phe	0.6	3.7	1.5	3.6	16.0	36.9	17.9	5.0	9.8	5.2	100.0
Benzoyl-Gly-Gly-Tyr	0.7	3.0	1.3	3.5	13.7	34.4	16.3	6.0	9.2	5.4	93.5
Benzoyl-Gly-Gly-Met		4.6	2.1	2.5	19.0	38.3	14.2	2.5	6.6	6.0	95.7
Benzoyl-Gly-Gly-Glu	0.2	4.8	2.4	2.5	20.0	40.9	11.2	0.9	3.3	6.7	92.8
Benzoyl-Gly-Gly-Pro		4.5	0.5	3.6	10.9	47.7	26.2	1.1	0.5	5.3	100.0
Benzoyl-Gly-Gly-Trp[b]	0.8	1.9	1.4	2.0	7.3	37.8	16.3	3.2	6.5	1.5	89.3

[a] Imm. = Xxx − immonium ion.
[b] z_1 Ion = 10.6%.

Table 3. Comparison of protonated benzoyl-Gly-Xxx-Gly CAD spectra at 10 eV collision energy

	Relative abundance (%)								
Benzoyl-Gly-Xxx-Gly	a_3'	a_2	b_3	b_2	b_1	y_2	y_1	z_1	Total
Benzoyl-Gly-Gly-Gly			9.9	9.6	8.3	72.2	0.1		100.1
Benzoyl-Gly-Ala-Gly			4.6	52.6	0.8	41.8		0.3	100.0
Benzoyl-Gly-Leu-Gly			3.1	50.4	0.1	41.7		0.5	95.7
Benzoyl-Gly-Phe-Gly	0.1		2.6	21.4		74.0		0.3	98.4
Benzoyl-Gly-Tyr-Gly	0.3	5.4	2.5	78.5		9.2			95.8
Benzoyl-Gly-Met-Gly			5.4	48.9		41.0			95.3
Benzoyl-Gly-Trp-Gly		0.2	2.9	42.1		51.1		1.9	98.2

protonated free tripeptide counter parts presented previously,[16] the protonated benzoyl-Xxx-Gly-Gly data contain a complete set of complementary fragment ions, y_2 and b_1. This complementary aspect is shown in Scheme 2, which assumes that structures for neutral products determined for oligoalanines[27] may be generalized to other residues.



Scheme 2.

A plot of the ion abundance ratios of y_2/b_1 with the proton affinity of Xxx is shown in Fig. 5; $r = 0.995$ was obtained for the data. In addition, in order to compare the quality of the new data with those in Fig. 1 in Ref. 16, the abundance plot of log (y_2/all other ions) versus the proton affinity of Xxx is shown in Fig. 6; this plot

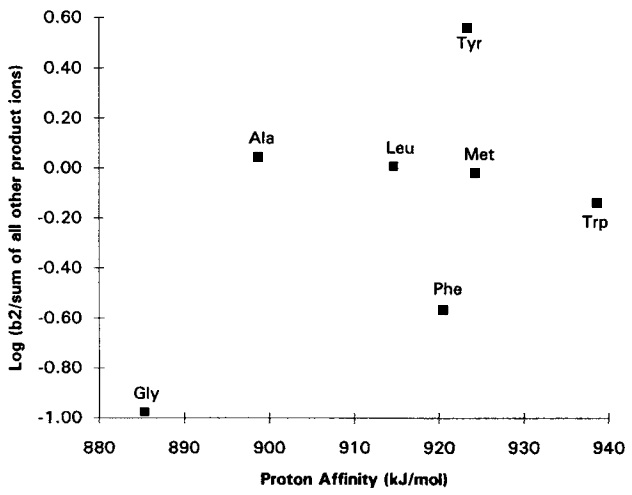


Figure 3. Correlation of the logarithm of the ratio of abundances of (b_2/sum of all other product ions) in benzoyl-Gly-Xxx-Gly with proton affinity of Xxx at 10 eV collision energy.

Table 4. Comparison of protonated benzoyl-Xxx-Gly-Gly CAD spectra at 10 eV collision energy

	Relative abundance (%)								
Benzoyl-Gly-Gly-Xxx	a_3	a_1	b_3	b_2	b_1	y_2	y_1	MH − 92	Total
Benzoyl-Gly-Gly-Gly			9.9	9.6	8.3	72.2	0.1	0.1	100.1
Benzoyl-Ala-Gly-Gly		0.4	4.9	5.8	53.2	35.8			100.0
Benzoyl-Val-Gly-Gly		1.2	1.4	1.8	88.1	5.9		1.5	100.0
Benzoyl-Leu-Gly-Gly			1.9	3.1	90.2	4.8			100.0
Benzoyl-Ile-Gly-Gly		0.2	1.1	2.1	92.4	4.2			100.0
Benzoyl-Phe-Gly-Gly		0.2	2.1	4.9	90.9	1.9			100.0
Benzoyl-Tyr-Gly-Gly	1.3	29.9	6.0	41.5	14.5	0.3		2.1	95.6
Benzoyl-Met-Gly-Gly			3.0	2.1	88.5	0.9		5.5	100.0
Benzoyl-Pro-Gly-Gly			4.5	3.6	75.1	0.2		14.3	97.7
Benzoyl-Trp-Gly-Gly		0.1	2.0	3.6	92.7	0.1			98.6

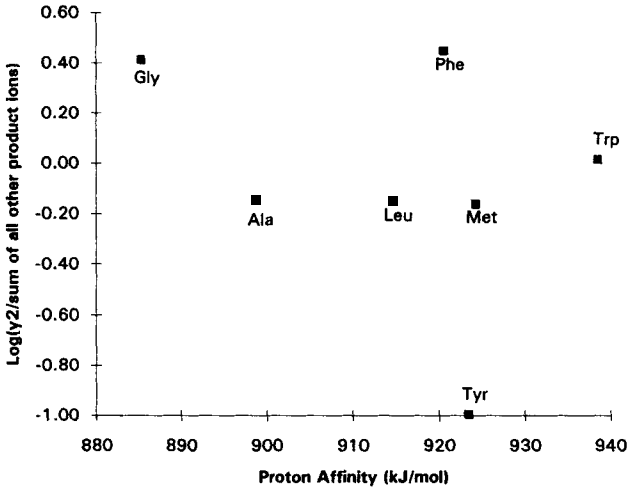


Figure 4. Correlation of the logarithm of the ratio of abundances of (y_2/sum of all other product ions) in benzoyl-Gly-Xxx-Gly with proton affinity of Xxx at 10 eV collision energy.

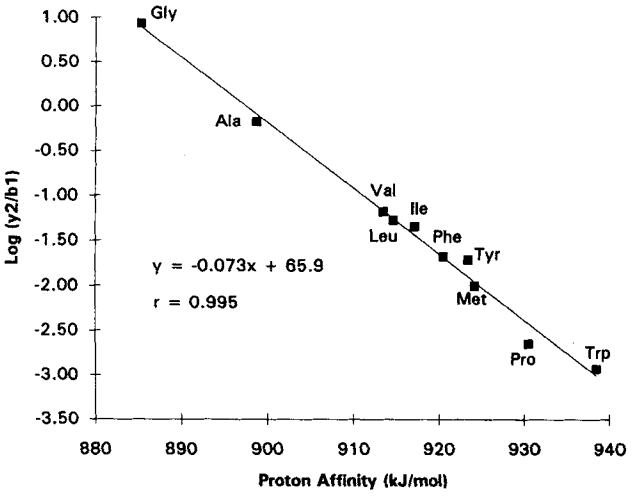


Figure 5. Correlation of the logarithm of the ratio of abundances of (y_2/b_1) in benzoyl-Xxx-Gly-Gly with proton affinity of Xxx at 10 eV collision energy.

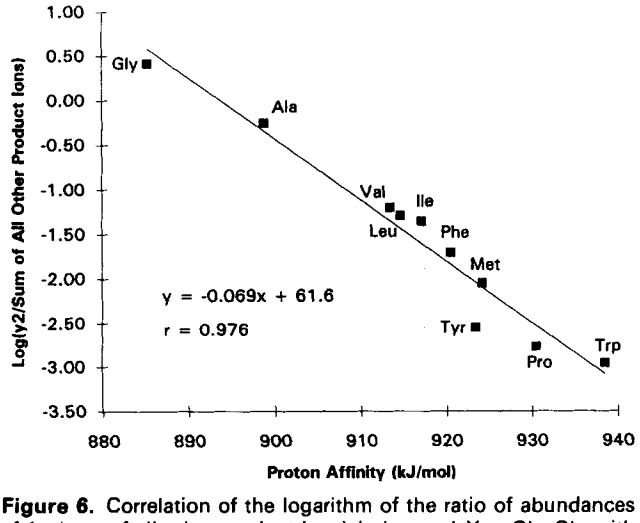


Figure 6. Correlation of the logarithm of the ratio of abundances of (y_2/sum of all other product ions) in benzoyl-Xxx-Gly-Gly with proton affinity of Xxx at 10 eV collision energy.

mimics the available data presented in Fig. 1 in our previous publication.[16] The correlation coefficient is $r = 0.976$. The correlation coefficient for the unprotected tripeptides Xxx-Gly-Gly based on similar data is $r = 0.913$.[16]

DISCUSSION

The excellent ion abundance correlation in the series benzoyl-Gly-Gly-Xxx with Xxx proton affinities suggests that the reactions to form the compared products proceed through universally similar geometries, that is, that all members of the series studied have the same characteristics as they pass from reactant to products, to the extent that the correlation is linear; there is no intrusion of structures or geometries that would proceed to products at a rate controlled by other factors. Hence there is a similar transition state for all the members of the series, and the side-chain does not influence the geometry of this structure substantially, in backbone conformation or hydrogen bonds.

The excellent ion abundance correlation in the series benzoyl-Xxx-Gly-Gly suggests the same things about structures or geometries for this series.

The lack of a correlation for the series benzoyl-Gly-Xxx-Gly suggests that reactions to form the compared products proceed through different geometries, that is, that members of the series studied have different characteristics as they pass from reactant to products; there is significant intrusion of structures or geometries that pass to products at rates controlled by other factors. The side-chain, then, has a strong influence on the energies of various conformations of different members of the series, so that different members produce products through different fractions of ion conformer populations.

Thus for *N*-benzoylated tripeptides of benzoyl-Aaa-Bbb-Ccc, the variation of the side-group in Aaa or Ccc does not significantly alter the geometry of protonated benzoyl-Aaa-Bbb-Ccc as it passes to the studied products, but the variation of Bbb does significantly alter the population of various hydrogen-bonded conformations of protonated benzoyl-Aaa-Bbb-Ccc.

Another deduction from combining these observations with previous results[14–16] concerns the similarity between correlations obtained for protonated benzoyl-Gly-Gly-Xxx and protonated Gly-Gly-Xxx. A larger correlation coefficient was noted for protonated benzoyl-Xxx-Gly-Gly than for protonated Xxx-Gly-Gly, when comparing similar ion abundance ratios with respect to the proton affinity of Xxx. When Ccc is varied, there is almost no variation in the geometry of protonated benzoyl-Aaa-Bbb-Ccc as it passes to the products, but there is slightly more variation in the geometry of protonated free Aaa-Bbb-Ccc. The difference is not obvious when Aaa is varied, since the correlation coefficients, 0.968 and 0.959, are virtually the same.

We suspect that stable geometries differ among themselves principally in the arrangement of internal hydrogen bonds, as was found, for example, in the theoretical study of *N*-protonated tetraglycine.[28] This point is even

more subtle than the differences due to different sites of protonation. In the tetraglycine study,[28] several differently hydrogen-bonded forms of similar energy were found for the *N*-protonated free peptide. When the *N*-terminal group is acylated, there are by inspection fewer hydrogen bonding arrangements that can be made than in the *N*-free ion, irrespective of where the proton may be. Regardless of what future computations may suggest, then, we propose that fewer low-energy geometries are available for the *N*-acyl ion than the *N*-free ion. The simplest picture, which may be too simple, is that one low-energy hydrogen-bonded structure is found for protonated benzoyl-Gly-Gly-Xxx and protonated benzoyl-Xxx-Gly-Gly; several low-energy forms are found for protonated free Gly-Gly-Xxx and protonated free Xxx-Gly-Gly, among which one form predominates, especially in Gly-Gly-Xxx. However, for protonated benzoyl-Gly-Xxx-Gly and protonated Gly-Xxx-Gly, there are several forms, and one form does *not* predominate, so that the distribution of precursor structures is strongly dependent upon the nature of Xxx in these cases. A tentative case can be made for as few as two forms of the precursor ions.

Is the independence of hydrogen-bonded geometry on terminal amino acids, but strong dependence of geometry on internal amino acids, carried over into larger peptides? That is a subject for future studies. Our present results indicate that *N*-acylation does not influence the dominance of the major hydrogen-bonded form negatively; if anything, it makes it more dominant. To the extent that the *N*-acyl form of the tripeptide mimics the tetrapeptide, there is some optimism that these effects will be observed in larger systems.

CONCLUSION

Once again, we have demonstrated that the proton affinity of the *N*- or *C*-terminal amino acid of a tripeptide without other side-chains controls the abundance of fragmentations containing that particular amino acid. It has been shown that benzoylation of the *N*-terminus increases the correlation of ion abundance ratios with proton affinities from the correlation found for protonated free peptides.

When the internal amino acid residue is varied in tripeptides with and without *N*-benzoylation, no correlation can be observed between ion abundance ratios and proton affinities. This failure may be due to the intervention of multiple geometries in the reactions to form the compared products.

The simplest picture consistent with all data in this series of papers[14–16] invokes a single geometry for the ions of protonated benzoyl-Aaa-Bbb-Ccc leading to y_n and b_n products when residues Aaa and Ccc are varied, and a similar geometry for the great majority of ions for protonated Aaa-Bbb-Ccc, with small fractions of other geometries. When residue Bbb is varied, a large number of possibilities for the geometry of either kind of ion exist with the most stable being dependent on the nature of Bbb itself.

Acknowledgements

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