N-Terminal Derivatization and Fragmentation of Neutral Peptides via Ion−Molecule Reactions with Acylium Ions: Toward Gas-Phase Edman Degradation?

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Abstract: The gas-phase ion−molecule reactions of neutral alanylglycine have been examined with various mass-selected acylium ions RCO^+ (R= CH_3, CD_3, C_6H_5, C_6F_5 and $(CH_3)_2N$), as well as the transacylation reagent O-benzoylbenzophenone in a Fourier transform ion cyclotron resonance mass spectrometer. Reactions of the gaseous dipeptide with acylium ions trapped in the ICR cell result in the formation of energized $[M + RCO]^+$ adduct ions that fragment to yield N-terminal b-type and C-terminal y-type product ions, including a modified b_1 ion which is typically not observed in the fragmentation of protonated peptides. Judicious choice of the acylium ion employed allows some control over the product ion types that are observed (i.e., b versus y ions). The product ion distributions from these ion−molecule reactions are similar to those obtained by collision-activated dissociation in a triple quadrupole mass spectrometer of the authentic N-acylated alanylglycine derivatives. These data indicate that derivatization of the peptide in the gas-phase occurs at the N-terminal amine. Ab initio molecular orbital calculations, performed to estimate the thermochemistry of the steps associated with adduct formation as well as product ion formation, indicate that (i) the initially formed adduct is energized and hence likely to rapidly undergo fragmentation, and (ii) the likelihood for the formation of modified b_1 ions in preference to y_1 ions is dependent on the R substituent of the acylium ion. The reaction of the tetrapeptide valine−alanine−alanine−phenylalanine with the benzoyl cation was also found to yield a number of product ions, including a modified b_1 ion. This result suggests that the new experimental approach described here may provide a tool to address one of the major limitations associated with traditional mass spectrometric peptide sequencing approaches, that is, determination of the identity and order of the two N-terminal amino acids. Analogies are made between the reactions observed here and the derivatization and N-terminal cleavage reactions employed in the condensed-phase Edman degradation method.

Introduction

The Edman degradation method for N-terminal sequence analysis of proteins[1] has been in use for over 50 years. Its advantages include well-characterized conditions for the derivatization and cleavage reactions, high stepwise yields and simple chromatographic identification of the cleaved phenylthiohydantoin amino acid. Disadvantages however, include low sensitivity, the requirement for highly purified samples and long cycle times, making the rapid identification and characterization of trace amounts of proteins difficult. Tandem mass spectrometry,[2] with demonstrated high sensitivity and rapid data acquisition capabilities, is now accepted as the method of choice for the identification and characterization of proteins and peptides.[3] Indeed, interpretation of the collision-activated dissociation product ion spectra of peptides is rapidly becoming routine, with a well-accepted sequence ion nomenclature[4] and automated data analysis programs available for sequence ion assignments.[5] The sequence analysis of novel or modified peptides by "de novo" sequencing strategies however, remains a challenge. This is commonly due to the production of "poor" product ion spectra resulting from incomplete fragmentation of many of the peptide backbone amide bonds, thereby yielding insufficient information to determine the complete amino acid sequence.[3a] In particular, the identity and order of the two N-terminal residues of peptides cannot regularly be determined due to the common absence of fragmentation at the N-terminal amide bond. As the combined

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nominal masses of many two amino acid pairs are identical, (for example, the mass of the amino acid pairs AsnAla and GlnGly, and that of the amino acid Trp all equal 186 Da), the presence of any of these combinations at the N-terminal may result in ambiguity in the amino acid sequence. Hence, a method to distinguish these possibilities is required to unequivocally assign complete amino acid sequences by mass spectrometric methods alone.

Neighboring group participation reactions based on the "mobile" proton model[6] have been proposed for the fragmentation of the amide bonds, with formation of the structurally relevant N-terminal b_n-type ions ($n \geq 2$),[7] where n equals the number of amino acid residues from the N-terminus, and complementary C-terminal y_n-type sequence ions,[8] where n equals the number of amino acid residues from the C-terminus. These mechanisms also readily explain the lack of cleavage at the N-terminal amide bond, with the general absence of b_1 ions and common lack of y_{n-1} ions, where n equals the total number of amino acid residues in the peptide. (The general absence of the b_1 ion[2] has been shown to be due to the unstable nature of simple aliphatic acylium ions, which fragment via the loss of CO to yield a_1 ions.[7] The common lack of the y_{n-1} ion[9] is likely to be due to the absence of a suitable neighboring group that would facilitate fragmentation at the N-terminal amide bond.[7,8])

Several condensed-phase methods have been employed to overcome this problem. Hunt et al. have utilized a single Edman degradation cycle to remove the N-terminal residue followed by mass determination of the truncated peptide, thus identifying the N-terminal amino acid.[2,9a] An examination of the literature reveals that fragmentation of the $[M + H]^+$ ions of N-acylated peptides often yields modified b_1 type ions indicative of the N-terminal residue.[10,11] Harrison has suggested that these b_1 ions have protonated oxazolone structures, similar to those of larger b_n ions.[7b] Recently, Gaskell et al.[12] have demonstrated that N-terminal derivatization of peptides with phenylisothiocyanate and related derivatization reagents, followed by collision-activated dissociation, results in the selective formation of modified thiazolone b_1 ions. Additionally, an analogy was made between this reaction and the condensed phase anhydrous acid cleavage step in the Edman degradation process.[12]

Several groups have explored the use of gas-phase ion−molecule reactions in mass spectrometers to gain additional structural information on biomolecules.[13−16] There are two types of ion−molecule reactions that can be employed to form modified b_1 ions. The first involves the reaction of a charged peptide ion (for example, the $[M + H]^+$ ion) with a neutral reagent. Nibbering et al.[14] have shown that the $[M + H]^+$ ions of simple peptides react with 2,5-hexanedione to yield N-terminal pyrrole derivatives, that fragment to yield N-terminal modified a- and b-type product ions, including a modified b_1 ion. Recently, O'Hair and Reid reported that the gas-phase reaction between the $[M + H]^+$ ions of simple peptides with neutral acetone, resulted in the formation of N-terminal derivatized Schiff's base adducts (Scheme 1). These derivatives were shown to fragment via an enamine intermediate to yield a modified pyrrolinone b_1 ion.[15] Unfortunately, this reaction does not occur for peptides with high proton affinities because the first step of the reaction, proton transfer from the protonated peptide to acetone, is thermodynamically unfavorable (Scheme 1).

The second type of ion−molecule reaction, that may prove useful for a wider range of peptides, is that between a neutral peptide and a reagent ion.[16] Acylium ions (RCO^+) are attractive as potential reagents for the formation of modified b_1 ions via this type of ion−molecule reaction for the following three reasons. (i) Previous studies have demonstrated that acylium ions react with simple amines (H_2NR') in Fourier transform ion cyclotron resonance, triple quadrupole, and ion trap mass spectrometers to yield adduct ions $[RC(O)NH_2R']^+$.[17] In a sector mass spectrometer, the reaction of the acetone chemical ionization[18] reagent ions CH_3CO^+ (m/z 43) and $(CH_3)_2CO(CH_3CO)^+$ (m/z 101) with simple amino acids has been shown to yield acetylated product ions[18a] (Scheme 2). Although this study did not involve mass selection of each of the chemical ionization reagent ions, it was suggested that the $[M + CH_3CO]^+$ adduct ion resulted from reaction with acetylated acetone (m/z 101) via a transacylation reaction. Such transacylation reactions have also been implicated in the gas-phase chemistry of butanedione chemical ionization reagent ions;[18b] (ii) a wide range of acylium ions can be generated in the gas-phase from readily available precursors; and (iii) solution-phase derivatives[10,11] of many of

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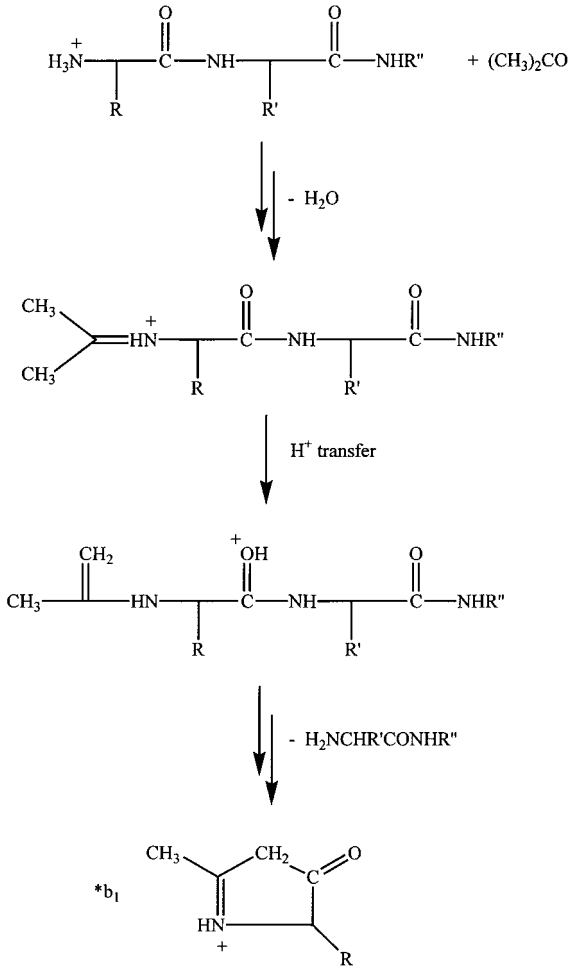
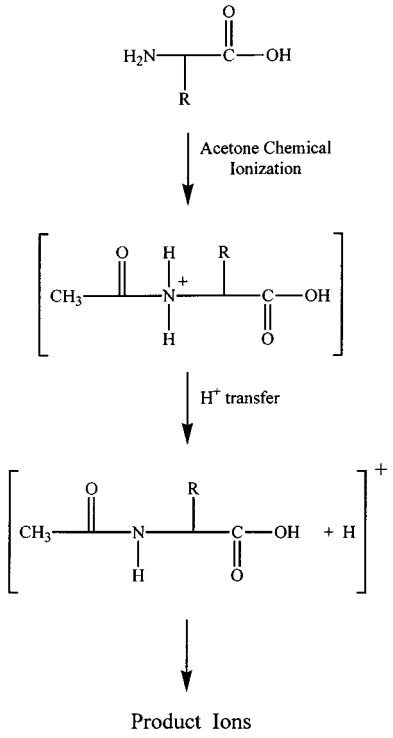
Scheme 1



the $[RC(O)NH_2R']^+$ adduct ions formed in the gas-phase can be easily synthesized. The fragmentation of these solution-phase derivatives can be used to help elucidate the mechanisms of the gas-phase reactions. Importantly, gas-phase acylation reactions also enable the formation of some acyl derivatives that may not be stable in solution. While the previous studies described above have been successful in examining some of the fundamental aspects of gaseous ion chemistry of simple peptides, there are significant difficulties associated with desorbing larger neutral biomolecules into the gas-phase due to their limited thermal stability.[19] Therefore, the ability to evaporate intact neutral peptides into the gas-phase in the absence of neutral fragments caused by thermal degradation is a key prerequisite to developing gas-phase ion−molecule reactions involving larger peptides.

Recently, Pérez et al.[20] have demonstrated that intact neutral peptides can be desorbed into a Fourier transform ion cyclotron resonance mass spectrometer using high-amplitude acoustic waves, generated by firing a laser at copper or titanium foil from the opposite side of where a sample is deposited (laser-induced acoustic desorption, LIAD). Ionization of the desorbed molecules was then performed by electron impact or chemical ionization using reagent ions trapped in the cell of the Fourier transform ion cyclotron resonance mass spectrometer. For example, the proton-transfer reaction between protonated aniline and the tetrapeptide valine−alanine−alanine−phenylalanine (VAAF), desorbed by LIAD, was shown to result in intact $[M + H]^+$ ions, whereas thermal desorption resulted in substantial degradation of the neutral peptide.[20]

Scheme 2



Here, to systematically study the reactivity of various gas-phase acylium ions toward simple peptides, the reactions of the acylium ions RCO^+ ($R = CH_3$, CD_3, C_6H_5, C_6F_5 and $(CH_3)_2N$) (Scheme 3), as well as the transacylation reagent *O*-benzoylbenzophenone (Scheme 4), with neutral alanylglycine have been examined in a Fourier transform ion cyclotron resonance mass spectrometer. To demonstrate that gas-phase acylation reactions may be used to form modified b_1 type ions, and therefore provide additional structural information to complement traditional mass spectrometric methods for de novo sequencing strategies, the reaction of the tetrapeptide VAAF with the benzoyl cation has also been examined.

Experimental Section

Materials. Alanylglycine, VAAF, and pentafluorobenzoyl chloride were purchased from Sigma (St. Louis, MO). Acetone (spectroscopic grade) was from Mallinckrodt (Paris, KY). Benzophenone was purchased from Fisher Scientific (Fair Lawn, NJ). Acetone-d_6 (99.5% D), *N*,*N*-dimethylaminocarbamyl chloride were obtained from Aldrich (Miliwaukee, WI). Acetic anhydride and d_6-acetic anhydride were purchased from Fluka (Buchs, Switzerland). Benzoyl chloride was from BDH (Poole, England). All compounds were used without further purification

Condensed-Phase Synthesis of N-Acylated Alanylglycine Derivatives. *N*-acetyl, d_3-acetyl, benzoyl, and pentafluorobenzoyl derivatives of alanylglycine were prepared by the addition of 100 μL of acetic anhydride, d_6-acetic anhydride, benzoyl chloride, or pentafluorobenzoyl chloride, respectively, to 5 mg of alanylglycine dissolved in 200 μL of 50 mM ammonium bicarbonate (pH 7.8). The reaction was allowed to proceed for 1 h at room temperature. Samples were diluted 100 fold in 1:1 CH_3OH/H_2O containing 1.0% acetic acid prior to direct mass spectrometric analysis. Attempts to synthesize the dimethylaminocarbamyl derivative of alanylglycine were unsuccessful as this compound was found to be unstable in solution.

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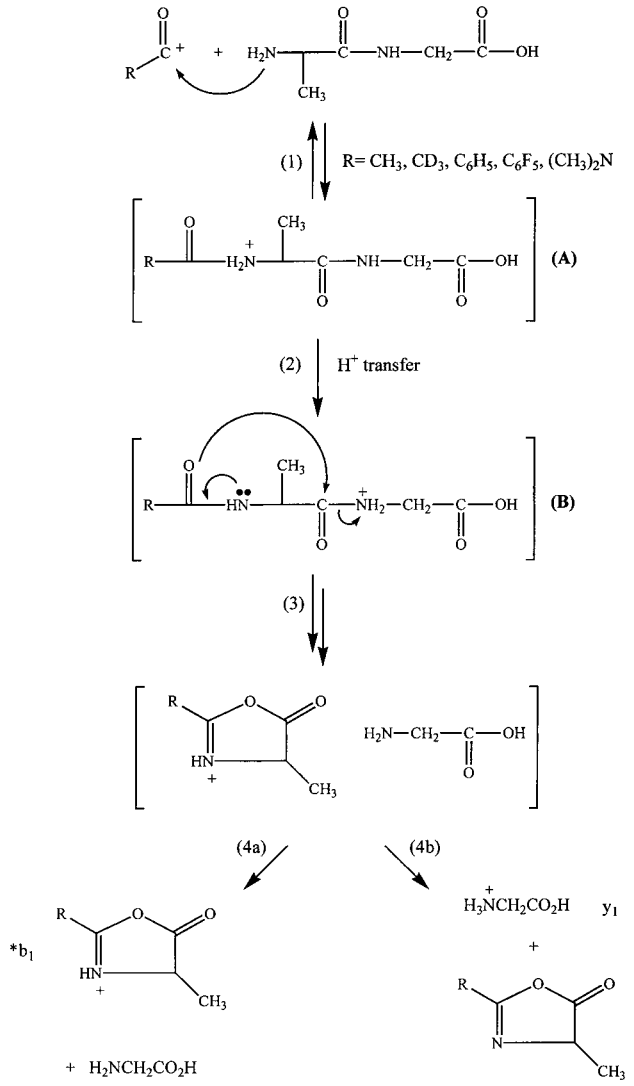
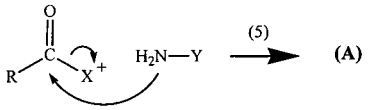
Scheme 3



Laser-Induced Acoustic Desorption Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. Ion−molecule reaction experiments were performed using an Extrel Model 2001 FTMS Fourier transform ion cyclotron resonance mass spectrometer described previously.[21] The instrument contains a differentially pumped dual cell that is placed within the magnetic field produced by a 3.0-T superconducting magnet operated at approximately 2.8 T. The nominal base pressure was less than 10^{-9} Torr, as maintained by two Balzers turbomolecular pumps (330 L/s), each backed with an Alcatel mechanical pump. The pressure was measured with two ionization gauges located on each side of the dual cell. The two cells are separated by a common wall (conductance limit) that contains a 2 mm hole in the center. Ions were transferred from one cell into the other by grounding the conductance limit plate for approximately 150 μs. Unless otherwise noted, this plate and the two end trapping plates were maintained at +2 V.

The acylium ions CH_3CO^+ (*m*/*z* 43), CD_3CO^+ (*m*/*z* 46), $C_6H_5CO^+$ (*m*/*z* 105), $(CH_3)_2NCO^+$ (*m*/*z* 72), and $C_6F_5CO^+$ (*m*/*z* 195) ions were generated in one side of the dual cell by electron ionization (typically 20 eV electron energy, 6 μA emission current, 40 ms ionization time) from acetone, d_6-acetone, benzoyl chloride, dimethylaminocarbamyl chloride, and pentafluorobenzoyl chloride (~4×10^{-8} Torr nominal reagent pressures), respectively, which resulted in an abundant signal for the ions specified. *O*-benzoylbenzophenone (*m*/*z* 287) was formed via self-chemical ionization (approximately 4 s) from benzophenone. Reactant ions were transferred into the other side of the dual cell and kinetically and internally cooled for 1 s by allowing time for IR emission and by colliding the ions multiple times with argon atoms pulsed into the cell via a pulsed valve assembly (the nominal peak pressure was $1.0-2.0 \times 10^{-5}$ Torr). Ions of interest were isolated by ejecting all unwanted ions from the cell through the application of a stored waveform inverse Fourier transform (SWIFT)[22] excitation pulse to the excitation plates of the cell (Extrel SWIFT module).

Scheme 4



R = C_6H_5, X = $(C_6H_5)_2CO$, Y = $CH(CH_3)C(O)NHCH_2CO_2H$

The neutral peptides alanylglycine and VAAF were introduced into the mass spectrometer by laser-induced acoustic desorption (LIAD) described in detail earlier.[20] Samples (1.5 mg) were dissolved in 5 μL glacial acetic acid, sonicated for 30 min and then diluted to 1.5 mL with anhydrous methanol. Then 25 μL (approximately 25 μg) of this solution was applied by electrospray deposition onto a copper foil of 9 mm diameter and 10 μm thickness at a rate of 2 μL/min using a spray voltage of 5.5−6.0 kV. After sample deposition, the foil was transferred to the sample support stage of the laser desorption probe. LIAD was performed by transmission mode laser irradiation (532 nm Continuum Minilite II Nd:YAG laser (Continuum, Santa Clara, CA)). Each experiment involved desorption of neutral molecules by 50−300 laser shots applied at 70 ms intervals (due to the 15 Hz maximum trigger rate of the laser). Each laser shot resulted in a population of neutral molecules with a resonance time of approximately 1 ms.[20] The desorbed neutral molecules were allowed to react with the reagent ion of interest stored in the ICR cell. After reaction, the ions were excited for detection using a broadband "chirp" excitation sweep (1.9 kHz to 2.6 MHz; 200V peak-to-peak; sweep rate 3200 Hz/μs). Spectra were recorded as 64 K data points at an acquisition rate of 2000 kHz. The time domain data was subjected to Hanning apodization, followed by augmentation of the data by one zero fill prior to Fourier transformation. Each spectrum shown was the result of a single scan.

For sustained off-resonance irradiation−collision-activated dissociation experiments,[23] helium was pulsed into the cell (momentary high pressure of 1.0×10^{-5} Torr) via a pulsed valve assembly and the ion of interest fragmented for approximately 300 ms using an activation frequency 1.0 kHz higher than the cyclotron frequency of the ion.

Electrospray Ionization Triple Quadrupole Mass Spectrometry. Analysis of authentic N-acylated alanylglycine derivatives synthesized in solution was performed using a Finnigan model TSQ (San Jose, CA) triple quadrupole mass spectrometer equipped with an electrospray ionization source. Peptide samples (0.05 mg/mL), dissolved in 1:1 CH_3OH/H_2O containing 1.0% acetic acid, were introduced into the mass spectrometer at 2.5 μL/min. Collision-activated dissociation experiments were performed on mass-selected ions using standard procedures.[24]

Ab Initio Molecular Orbital Calculations. To estimate the relative energies of several species involved in steps along the proposed reaction pathways (Schemes 3 and 4), ab initio molecular orbital calculations were performed at the Hartree−Fock level of theory, using GAUSSIAN 98.[25] A set of conformers for each model structure were initially optimized at the AM1 semiempirical level of theory[26] followed by further optimization of low-energy conformers at the Hartree−Fock level of theory using the standard 6-31G* basis set.[27] All optimized structures were subjected to vibrational frequency analysis with the

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Table 1. Gas-Phase Ion−Molecule Reactions between Various Reagent Ions and Neutral Alanylgylcine in a Fourier transform Ion Cyclotron Resonance Mass Spectrometer

ion	*m*/*z*	productions, *m*/z, (identity), % abundance[a]
$(CH_3)_2COH^+$	59	147, ($M + H^+$), 100; 119, ($M + H^+ - CO$), 7; 44, (a_1),5
CH_3CO^+	43	147, ($M + H^+$), 100; 114, (*b_1), 13; 86, (*a_1), 7
CD_3CO^+	46	148, ($M + D^+$), 63; 147, ($M + H^+$), 100; 120, ($M + D^+ - CO$), 12; 117, (*b_1), 14; 89 (*a_1), 44
$(CH_3)_2NCO^+$	72	173, ($M + (CH_3)_2NCO^+ - (CH_3)_2NH$), 8; 147, ($M + H^+$), 95; 143, (*$b_1$), 100; 129, ($b_2$), 18; 115, (*$a_1$), 31; 46, ($(CH_3)_2NH_2^+$), 21
$C_6H_5CO^+$	105	176, (*b_1), 90; 148, (*a_1), 33; 147, ($M + H^+$), 100; 129, (b_2), 12
$C_6F_5CO^+$	195	266, (*b_1), 13; 238, (*a_1), 45; 147, ($M + H^+$), 100; 129, (b_2), 8; 76, (y_1), 14; 44, (a_1), 9
$(C_6H_5)_2COC_6H_5CO^+$	287	251, ($M + C_6H_5CO^+$), 100; 176, (*b_1), 53; 147, ($M + H^+$), 75; 129, (b_2), 17

[a] Only those product ions observed at greater than 5% relative abundance are shown.

Table 2. Collision-Activated Dissociation Spectra of N-Acylated Alanylglycine Derivatives in a Triple Quadrupole Mass Spectrometer

species	*m*/*z*	productions, *m*/z, (identity), % abundance[a]
$[alanylglycine + H]^+$	147	129, (b_2), 1; 119, (−CO), 3; 101, (a_2), 1; 76, (y_1), 1; 44, (a_1), 100
$[CH_3CO\text{-}alanylglycine + H]^+$	189	171, (*b_2), 1; 147, (y_2), 2; 114, (*b_1), 100; 86, (*a_1), 9; 76, (y_1), 3
$[CD_3CO\text{-}alanylglycine + H]^+$	192	174, (*b_2), 1; 147, (y_2), 1; 117, (*b_1), 100; 89, (*a_1), 9; 76, (y_1), 3
$[C_6H_5CO\text{-}alanylglycine + H]^+$	251	233, (*b_2), 1; 176, (*b_1), 100; 148, (*a_1), 9; 105, ($C_6H_5CO^+$), 2
$[C_6F_5CO-alanylglycine + H]^+$	341	323, (*b_2), 5; 266, (*b_1), 100; 238, (*a_1), 32; 195, ($C_6F_5CO^+$), 1; 76, (y_1), 45

[a] Only those product ions observed at greater than 1% relative abundance are shown.

same basis set to ensure that they corresponded to minima on the potential energy surface. Calculation of the correlated energy was then performed at the MP2(FC)/6-31G* level of theory (FC = frozen core). Energies were corrected for zero-point vibrations scaled by 0.9135.[28] Throughout this paper, the energy of each structure discussed will be given at the MP2(FC)/6-31G*//HF/6-31G* level of theory. Cartesian coordinates of each of the ab initio optimized structures as well as their total and zero point vibrational energies are supplied as Supporting Information.

Results and Discussion

LIAD/Acetone Chemical Ionization of Alanylglycine Results in the Formation of Intact $[M + H]^+$ Ions. The preliminary results reported by Pérez et al.[20] demonstrated that LIAD provides a useful tool for the evaporation of intact neutral peptides for subsequent chemical ionization studies in a Fourier transform ion cyclotron resonance mass spectrometer. Thus, the LIAD approach was used here to systematically examine the reactions of the dipeptide alanylglycine, which exhibits significant degradation upon thermal desorption. Indeed, the proton-transfer reaction between protonated acetone (*m*/*z* 59) and alanylglycine desorbed by LIAD yielded the intact $[M + H]^+$ ion (*m*/*z* 147) as the major product (Table 1). Using known proton affinity values,[29] this proton-transfer reaction was predicted to be exothermic by 31.5 kcal mol^{-1}. The observation of the $[M + H]^+$ ion as the major product is also consistent with previous work by Speir and Amster[30] who reported, using both laser desorption and substrate-assisted laser desorption/chemical ionization techniques, that proton-transfer reactions on simple peptides with reaction exothermicities less than approximately 30 kcal mol^{-1} result in little fragmentation. These findings also agree with the combined results of two recent theoretical studies by Paiz et al.[8h] and Csonka et al.[8i] that indicated an overall energetic requirement for fragmentation of the amide bond in protonated *N*-formyl glycinamide to yield separated ionic and neutral products to be approximately 37 kcal mol^{-1}. Therefore, it appears that the LIAD technique results in the desorption of relatively cool neutral molecules.

Sustained off-resonance irradiation−collision-activated dissociation[23] of protonated alanylglycine was performed in the Fourier transform ion cyclotron resonance mass spectrometer to provide a reference for later comparison with the product ion spectra from the ion−molecule reactions of the acylium ions with alanylglycine. Dissociation of protonated alanylglycine resulted in formation of the a_1 ion (*m*/*z* 44, 100% relative abundance) and the $[M + H - CO]^+$ ion (*m*/*z* 119, 50% relative abundance) as the only products. Collision-activated dissociation of the $[M + H]^+$ ion of alanylglycine formed by electrospray in a triple quadrupole mass spectrometer also resulted in similar product ion yields (Table 2). It is important to note that a b_1 ion, indicative of the N-terminal amino acid, was not observed in either experiment.

LIAD/Acylium Ion Chemical Ionization of Alanylglycine Results in the Formation of Modified b_1 Ions. Examination of Table 1 reveals that the acetyl (*m*/*z* 43), d_3-acetyl (*m*/*z* 46), dimethylaminocarbamyl (*m*/*z* 72), benzoyl (*m*/*z* 105), and pentafluorobenzoyl (*m*/*z*195) acylium ions each react with neutral alanylglycine to yield a number of new product ions, including modified b_1 ions (designated hereafter as *b_1). There are four key steps associated with the proposed mechanism shown in Scheme 3 for formation of these *b_1 ions. Step 1 involves initial formation of $[M + RCO]^+$ adduct ions. The regioselectivity and exothermicity of this step will depend on the acyl cation affinity of each site within the neutral peptide. It is anticipated that the acyl cation affinities of each site will

(25) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A., Jr.; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B. G.; Chen, W.; Wong, M. W.; Andres, J. L.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. *Gaussian 98*, revision A.7; Gaussian, Inc.: Pittsburgh, PA, 1998.

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(29) (a) Values for the proton affinities of acetone (194 kcal mol^{-1}) and H_2NCH_3 (214.9 kcal mol^{-1}) were taken from Hunter, E. P.; Lias, S. G. *J. Phys. Chem. Ref. Data* **1998**, *27*, 413−457. (b) The proton affinity of alanylglycine (225.5 kcal mol^{-1}) was taken from Cassady, C. J.; Carr, S. R.; Zhang, K.; Chung-Phillips, A. *J. Org. Chem*. **1995**, *60*, 1704−1712. (c) Zhang, K.; Zimmerman, D. M.; Chung-Phillips, A.; Cassady, C. J. *J. Am. Chem. Soc*. **1993**, *115*, 10812−10822.

(30) (a) Speir, J. P.; Amster, I. J. *Anal. Chem*.. **1992**, *64*, 1041−1045. (b) Speir, J. P.; Amster, I. J. *J. Am. Soc. Mass Spectrom*. **1995**, *6*, 1069−1078.

Table 3. Gas-Phase Thermochemistry for the Adduct-Forming Reaction $RCO^+ + H_2NCH_3 \rightarrow RCONH_2CH_3^+$

species	E (HF)[a]	E (MP2)[b]	species	E (HF)[c]	E (MP2)[d]	ΔE (HF)[e]	ΔE (MP2)[e]
	RCO^+				$RCONH_2CH_3^+$		
R = CH_3	−152.01527	−152.42874	R = CH_3	−247.22133	−247.94186	−37.15	−44.09
= $(CH_3)_2N$	−246.05216	−246.75798	$(CH_3)_2N$	−341.24922	−342.26664	−31.51	−41.28
= C_6H_5	−342.49907	−343.53135	C_6H_5	−437.69041	−439.03401	−27.91	−37.52
= C_6F_5	−836.71110	−838.58540	C_6F_5	−931.92092	−934.10466	−39.51	−47.94
H_2NCH_3	−95.14686	−95.44287					

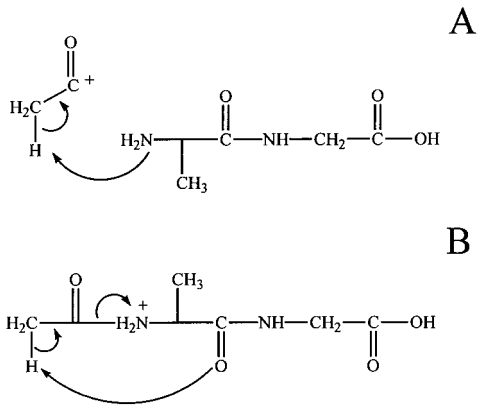
[a] Energies (in hartrees) of RCO^+ determined at the HF/6-31G* + ZPVE level of theory. [b] Energies (in hartrees) of RCO^+ determined at the MP2(fc)/6-31G*//HF/6-31G* + ZPVE level of theory. [c] Energies (in hartrees) of $RCONH_2CH_3^+$ determined at the HF/6-31G* + ZPVE level of theory. [d] Energies (in hartrees) of $RCONH_2CH_3^+$ determined at the MP2(fc)/6-31G*//HF/6-31G* + ZPVE level of theory. [e] Energy (in kcal mol^{-1}) for the reaction $RCO^+ + H_2NH_3 \rightarrow RCONH_2H_3^+$

follow their proton affinities, Therefore, we would expect that acylation will occur primarily at the N-terminal amine. Step 2 entails intramolecular proton transfer from the nitrogen at which adduct formation has occurred to the adjacent amide nitrogen or carbonyl oxygen heteroatoms, thereby generating a leaving group. (For experimental evidence supporting O-protonated peptide structures as fragmenting species in amide bond cleavage reactions, see refs 8k and 31.) Step 3 involves nucleophilic attack by the amide produced in step 1 at the adjacent protonated amide to induce amide bond cleavage by a neighboring-group mechanism. Finally, step 4 involves dissociation of the ion−neutral complex to yield either a $*b_1$ ion, or a y_1 ion, the relative abundances of which will depend on their relative proton affinities,[32,33] as well as the lifetime of the intermediate ion−neutral complex.[2,34] Several of the issues associated with steps 1 and 4 of the proposed mechanism, that is, adduct formation and relative product ion abundances, respectively, are addressed below.

Inspection of the data in Table 1 reveals several points. First, intact $[M + RCO]^+$ adduct ions (cf. step 1 in Scheme 3) were not observed. Ab initio calculations, using H_2NCH_3 as a model for alanylglycine (Table 3),[29a] predict that each of the reactions are highly exothermic (ranging from 37.5 kcal mol^{-1} for the benzoyl cation reaction to 47.9 kcal mol^{-1} for the pentafluorobenzoyl cation reactions). It is expected that the exothermicity of these adduct forming reactions will provide the driving force required to surmount the barriers for subsequent fragmentation reactions. Given the 37 kcal mol^{-1} value determined by Paizs et al.[8h,i] for amide bond cleavage, it is expected that the energized $[M + RCO]^+$ ions of alanylglycine (which has a proton affinity 10.6 kcal mol^{-1} higher than that of H_2NCH_3)[29b] formed under the low-pressure conditions employed in the Fourier transform ion cyclotron resonance mass spectrometer will undergo facile fragmentation reactions and hence will not be seen.

Second, the $[M + H]^+$ ion of alanylglycine was observed as the most abundant product (100% relative abundance) in all cases, with the exception of the dimethylaminocarbamyl cation reaction, where it was observed at 95% relative abundance. In the case of the acetyl and dimethylaminocarbamyl systems, this $[M + H]^+$ ion is most likely formed by primary proton-transfer reactions involving either intermolecular proton transfer from the acylium ion (Scheme 5A), or by intramolecular proton transfer within the $[M + RCO]^+$ adduct ion initially formed

Scheme 5



(Scheme 5B). Alternatively, secondary proton-transfer reaction processes, by intermolecular proton transfer to neutral alanylglycine from fragment ions formed by dissociation of $[M + RCO]^+$ adducts, may also lead to the formation of $[M + H]^+$ ions. To examine this further, the reaction between the d_3-acetyl cation (CD_3CO^+, m/z 46) and neutral alanylglycine was carried out. It can be seen from Table 1 that both $[M + D]^+$ ions, indicative of primary proton-transfer processes, as well as $[M + H]^+$ ions, indicative of secondary proton transfer, were observed. Therefore, it appears that both primary and secondary proton-transfer processes operate under the experimental conditions employed here. As secondary proton-transfer reactions may deplete the abundance of the primary product ions formed by fragmentation of the $[M + RCO]^+$ adduct, determination of the branching ratios, which are normally used to express the ratios of product ions formed in ion−molecule reactions, was not performed here. Instead, the percent product ion abundances, relative to the most intense product ion in each spectrum, are used throughout.

Finally, a number of new product ions that were not observed in the fragmentation spectrum of protonated alanylglycine were observed for all the acylium ion reactions examined here (Table 1). In the majority of cases, these new product ions corresponded to $*b_1$ (step 4a in Scheme 3) and $*a_1$ ions, modified by addition of the acyl group. Thus, these $*b_1$ and $*a_1$ ions are readily distinguished from b_1 and a_1 ions arising from the unmodified $[M + H]^+$ ions. Of note was the observation of the $*b_1$ ion as the most abundant product in the reaction of the dimethylaminocarbamyl cation with neutral alanylglycine (Figure 1A). The loss of $(CH_3)_2NH$ and formation of $(CH_3)_2NH_2^+$ from the energized $[M + (CH_3)_2NCO]^+$ adduct ion in this example (see Table 1) are rationalized in Scheme 6.

Interestingly, the y_1 ion was also observed in the reaction of the pentafluorobenzoyl cation with neutral alanylglycine (Figure 1B) (step 4b in Scheme 3), suggesting that the nature of the acylium ion may influence the product ions that are observed.

(31) O'Hair, R. A. J.; Styles, M. S.; Reid, G. E. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1275−1284.
(32) Nold, M. J.; Cerda, B. A.; Wesdemiotis, C. *J. Am. Soc. Mass Spectrom.* 1, 1−8.
(33) (a) Morgan, D. G.; Bursey, M. M. *Org. Mass Spectrom.* **1994**, *29*, 354−359. (b) Morgan, D. G.; Bursey, M. M. *Org. Mass Spectrom.* **1995**, *30*, 290−295.
(34) Tu, Y. P.; Harrison, A. G. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 454−462.

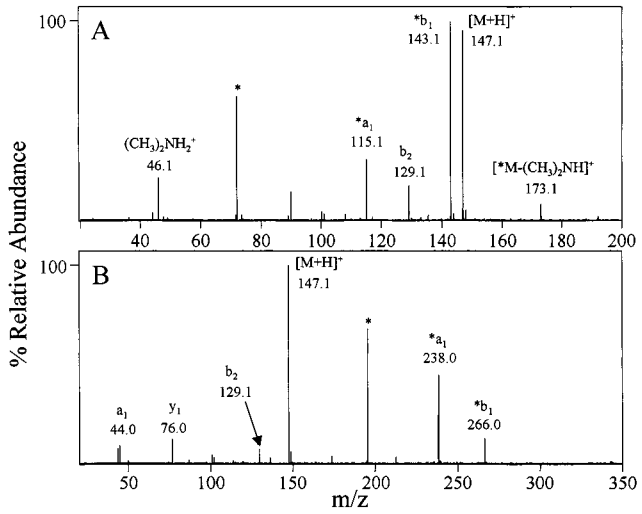
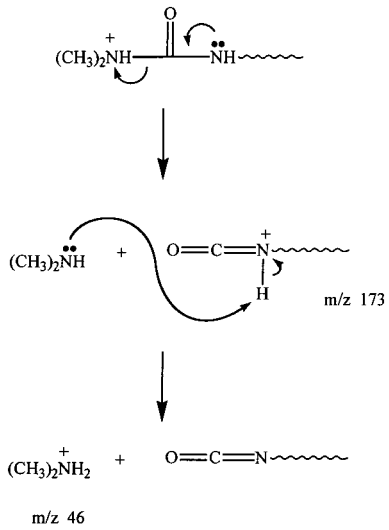


Figure 1. LIAD/chemical ionization of alanylglycine (MW 146.1) by reaction with (A) the dimethylaminocarbamyl cation ($(CH_3)_2NCO^+$) or (B) the pentafluorobenzoyl cation ($C_6F_5CO^+$). The reagent ion in each case is indicated by an asterisk. See the Experimental Section for details. The product ions are labeled according to the nomenclature described in ref 4. An asterisk on the left-hand side of the labels denotes an ion modified by the addition of $(CH_3)_2NCO$ (panel A) or C_6F_5CO (panel B).

Scheme 6



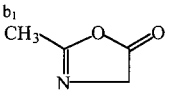
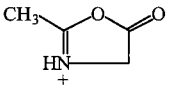
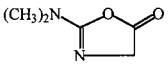
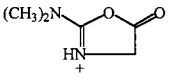
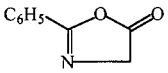
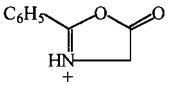
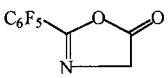
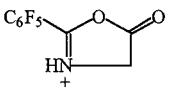
To examine the competition between $*b_1$ ion formation and y_1 ion formation, the relative proton affinities of each of the $*b_1$ ions compared to that of the y_1 ion (step 4 in Scheme 3) were determined by ab initio molecular orbital calculations. These data can be used as an indication of whether the ionizing proton will be sequestered at either the N- or C-terminal fragment following amide bond cleavage. The data obtained (Table 4) are in good agreement with the relative proton affinities determined for both glycine (213.5 kcal mol^{-1})[29c] and 2-phenyl-5-oxazolone (i.e., the N-benzoyl $*b_1$ ion) (216.7 kcal mol^{-1}).[32] As can be seen in Table 4, the $*b_1$ ion formed in the reaction between the dimethylaminocarbamyl cation and neutral alanylglycine is expected to be observed in a greater abundance compared to the y_1 ion since proton transfer from the $*b_1$ to the y_1 ion is predicted to be endothermic by 12.1 kcal mol^{-1}. In the case of the pentafluorobenzoyl cation reaction however, we would expect the y_1 ion to also be observed since proton transfer from the $*b_1$ ion to the y_1 ion is predicted to be exothermic by 4.5 kcal mol^{-1}. Indeed, these theoretical predictions (Table 4) are in qualitative agreement with the experimental observations (Figure 1).

Collision-activated dissociation of the N-acylated alanylglycine derivatives independently synthesized in solution (with the exception of the *N*,*N*-dimethylaminocarbamyl derivative which was not stable), was performed in a triple quadrupole mass spectrometer (Table 2) to determine whether the product ions observed were similar to those formed by ion−molecule reactions in the Fourier transform ion cyclotron resonance mass spectrometer. By comparing Tables 1 and 2, it can be seen that the product ions observed by fragmentation of the authentic N-acylated alanylglycine derivatives are essentially the same as the product ions resulting from fragmentation of the $[M + RCO]^+$ adducts formed in the gas-phase. Thus, these results indicate that the gas-phase ion−molecule reactions result in N-terminal derivatization of the neutral peptide.

LIAD/*O*-Benzoylbezophenone Transacylation Chemical Ionization of Alanylglycine Results in the Formation of Intact $[M + RCO]^+$ Adduct Ions. Given the exothermic nature of the acylation reactions discussed above (see Table 3), it is not surprising that intact adduct ions were not observed. Ab initio calculations predict that an S_N2 like transacylation reaction, involving the reaction of *O*-benzoylbenzophenone with the model amine H_2NCH_3 (Table 3), would be significantly less exothermic than the corresponding acylation reaction between the benzoyl cation and H_2NCH_3. Thus, the transacylation reaction was predicted to be exothermic by only 2.3 kcal mol^{-1} (Table 5) compared to 37.5 kcal mol^{-1} for the acylation reaction (Table 3). Such transacylation reactions may allow the formation of stable intact adducts, that could then be isolated for subsequent fragmentation, in the absence of other competing reaction pathways, thereby simplifying data interpretation. To determine whether such reactions could potentially be of use in forming stable $[M + RCO]^+$ adduct ions, the S_N2 like transacylation reaction between *O*-benzoylbenzophenone cation (*m*/*z* 287) and neutral alanylglycine was examined experimentally. From the data shown in Table 1, it can be seen that the benzoyl alanylglycine adduct ion (*m*/*z* 251) was observed (100% relative abundance), along with other ions corresponding to the $*b_1$ ion (*m*/*z* 176, 53% relative abundance), as well as $[M + H]^+$ (*m*/*z* 147, 75% relative abundance) and b_2 (*m*/*z* 129, 17% relative abundance) ions. The observation of the *m*/*z* 251 ion signal indicates that a significant proportion of the initial adduct ion population have internal energies smaller than that required for subsequent fragmentation, in agreement with the theoretical prediction. The appearance of the additional product ions however, which are similar to those observed in the acylation reaction between the benzoyl cation and alanylglycine (Table 1), suggests that at least some of the initial adduct ion population has sufficient internal energy to undergo subsequent fragmentation. Alternatively, these additional product ions could be formed by dissociation of the *O*-benzoylbenzophenone cation to the benzoyl acylium ion during the time frame of the experiment, which could then undergo subsequent exothermic acylation reactions with the neutral peptide as described above.

LIAD/Chemical Ionization Can Be Used To Distinguish the Order and Identity of the Two N-Terminal Amino Acids in Peptides. To determine whether the LIAD/ chemical ionization method could be used to determine the identity and order of the two N-terminal amino acids within a peptide, thus overcoming one of the major limitations of conventional mass spectrometric sequencing approaches, the reactions of the neutral tetrapeptide VAAF, desorbed into the gas-phase by LIAD, with both protonated acetone and the benzoyl cation were examined.

Table 4. Gas-Phase Thermochemistry for Formation of the Modified b_1 Ions versus y_1 ions.

Neutral Species	E(HF)[a]	E(MP2)[b]	Ionic Species	E(HF)[a]	E(MP2)[b]	PA(HF)[c]	PA(MP2)[c]
y_1 H_2NCH_2COOH	-282.75190	-283.51736	$H_3NCH_2COOH^+$	-283.09349	-283.85792	214.4(0)	213.7(0)
b_1 	-397.49266	-398.63426		-397.83717	-398.96949	216.2(+1.8)	210.4(-3.3)
	-491.50428	-492.93840		-491.87382	-493.29818	231.9(+17.5)	225.8(+12.1)
	-587.95396	-589.71807		-588.30924	-590.06227	222.9(+8.5)	216.0(+2.3)
	-1082.19504	-1084.79806		-1082.53613	-1085.13137	214.0(-0.4)	209.2(-4.5)

[a] Energies (hartrees) determined at the HF/6-31G* + ZPVE level of theory. [b] Energies (hartrees) determined at the MP2(fc)/6-31G*//HF/6-31G* + ZPVE level of theory. [c] PA = proton affinty expressed in kcal mol^{-1}. Relative proton affinities for each b_1 ion relative to the y_1 ion are given in parentheses.

Table 5. Gas-Phase Thermochemistry for the Transacylation Reaction $(C_6H_5)_2CO(C_6H_5CO)^+ + H_2NCH_3 \rightarrow C_6H_5CONH_2CH_3^+ + (C_6H_5)_2CO$

	$(C_6H_5)_2CO(C_6H_5CO)^+ + H_2NCH_3 \rightarrow C_6H_5CONH_2CH_3^+ + (C_6H_5)_2CO$					
E (HF)[a]	−915.34245	−95.14686	−437.69041	−572.79853	Δ*E* (HF)[c]	+0.2
E(MP2)[b]	−918.19327	−95.44287	−439.03401	−574.60580	Δ*E* (MP2)[c]	−2.3

[a] Energies determined at the HF/6-31G* + ZPVE level of theory. [b] Energies determined at the MP2(fc)/6-31G*//HF/6-31G* + ZPVE level of theory. [c] Energy (in kcal mol^{-1}) for the reaction $(C_6H_5)_2CO(C_6H_5CO)^+ + H_2NCH_3 \rightarrow C_6H_5CONH_2CH_3^+ + (C_6H_5)_2CO$.

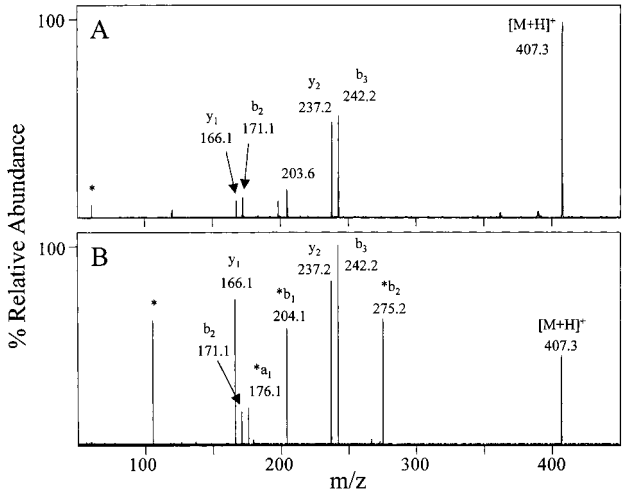


Figure 2. LIAD/chemical ionization of the tetrapeptide VAAF (MW 406.2) by (A) a proton-transfer reaction with protonated acetone (*m*/*z* 59) and (B) reaction with the benzoyl ($C_6H_5CO^+$) cation (*m*/*z* 105). The reagent ion in each case is indicated by an asterisk. See the Experimental Section for details. The product ions are labeled according to the nomenclature described in ref 4. An asterisk on the left-hand side of the labels in panel B denotes an ion modified by addition of C_6H_5CO.

(Although reactions involving the dimethylaminocarbamyl cation were predicted to result in the preferential formation of N-terminally directed products (Table 3), this reagent ion was not employed here as the $*b_1$ ion ($C_8H_{15}N_2O_2$, *m*/*z* 171) of VAAF formed using this reagent ion has the same mass as that of the unmodified b_2 ion.) It can be seen from Figure 2A, that exothermic proton transfer between protonated acetone and neutral VAAF results in the formation of y_2 (*m*/*z* 237.2) and b_2 (*m*/*z* 171.1) ions, as well as y_1 (*m*/*z* 166.1) and b_3 (*m*/*z* 242.2) ions, corresponding to cleavage between the alanine-alanine and alanine-phenylalanine residues of VAAF, respectively. Due to the lack of cleavage between the two N-terminal residues, valine−alanine, and subsequent absence of a b_1 or y_3 ion, the order of these residues could not be assigned. Similar spectra have been reported previously for protonated VAAF, using both LIAD/ chemical ionization[20] and photodissociation techniques,[35] in Fourier transform ion cyclotron resonance mass spectrometers. In contrast, the spectrum resulting from the reaction of neutral VAAF with the benzoyl cation (Figure 2B) reveals several new product ions, including a $*b_1$ ion (*m*/*z* 204), that allows the assignment of the identity and order of the valine−alanine N-terminal sequence.

Recall that several groups have employed techniques such as manual Edman degradation or solution phase acylation to obtain the additional structural information required to fully characterize an unknown peptide sequenced by mass spectrometric methods.[2,9a,10−12] However, as described above, this additional structural information can readily be obtained in the gas-phase. Therefore, it appears that LIAD, coupled with gas-phase acylation reactions, may be a useful technique in providing the additional structural information to complement traditional mass spectrometric methods for de novo peptide sequencing.

Edman Chemistry in the Gas-Phase? The gas-phase peptide acylation reactions discussed here (Scheme 3, step 1), are analogous to the initial step employed in the Edman method for formation of N-terminal phenylthiocarbamoyl derivatives.[1] Classical Edman chemistry involves the formation of a thioamide bond at the N-terminal of a peptide. However, our preliminary results (data not shown) indicate that thioacyl cations are significantly less reactive than acyl cations in the gas-phase, due to the cationic carbon of the thioacyl cation being less electrophilic than the corresponding oxygen containing cation. It is important to note however, that the modified b_1 ions formed in the gas-phase ion−molecule reactions described here, which are expected to have protonated oxazlone structures, are structurally closely related to the phenylthiocarbamoyl (PTC)

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derivatives formed by anhydrous acid cleavage in the Edman degradation method.

One of the significant differences between the collision-activated dissociation results obtained by Gaskell et al.[12] for the substituted thioacyl peptide derivatives formed in solution and the gas-phase acylium ion derivatization/fragmentation reactions described here is that the thioacyl derivatives fragmented in a directed fashion to yield a single product (either b_1 or y_{n-1}, depending on the nature of the substituent attached to the thioacyl group),[12] It is expected that this selected fragmentation is due to the enhanced nucleophilicity of the thioamide bond compared to the amides present at other sites along the peptide backbone. Similarly, it has been demonstrated that collision-activated dissociation of peptides incorporating a single thioamide along the backbone[36] results in directed fragmentation at the C-terminal residue adjacent to the thioamide bond. In contrast, the formation of an amide bond at the N-terminal, performed here by gas-phase derivatization, simply allows for fragmentation of the amide bond between the two N-terminal residues to occur in competition with all of the other amide bonds throughout the peptide backbone. However, we have demonstrated here that modification of the R substituent in the RCO^+ acylium ions does result in some control over which products (b- or y-type) are observed, which is in agreement with the results presented earlier by Gaskell et al.[12] Selective cleavage of the N-terminal residue by gas-phase derivatization/fragmentation reactions should be achievable through the introduction of a better neighboring group. While the formation of a thioamide would be attractive for this purpose, the limited reactivity of thioacylium ions in the gas-phase makes this "classical" solution-phase derivative unsatisfactory for use in the gas-phase. However, the vast scope for generating reactive reagent ions in the gas-phase means that the potential for developing useful gas-phase reactions to assist in the sequence analysis of peptides is far from exhausted.

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Supporting Information Available: Cartesian coordinates of each of the ab initio optimized structures as well as their total and zero point vibrational energies (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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