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Alternative labeling method for peptide ladder sequencing using matrix-assisted laser desorption–ionization Fourier transform mass spectrometry

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Abstract

Following previous work, alternative terminating labels for ladder sequencing of peptides have been investigated to make ladder sequencing more compatible with analysis by matrix-assisted laser desorption–ionization Fourier transform mass spectrometry (MALDI-FT-MS). Although peptide analysis by MALDI-FT-MS using existing ladder-generating chemistry was possible, the spectra were complicated by the loss of the phenyl isocyanate (PIC) terminating label in the gas phase on the time scale of the FT-MS experiment. Therefore, in the work described here, a new reagent which would produce more stable peptide derivatives was investigated. A variety of substituted phenyl isocyanate terminating labels, using angiotensin III antipeptide (Gly–Val–Tyr–Val–His–Pro–Val) as a model peptide, were assessed for this purpose. Of the compounds investigated, *N*-carboxy phthalimide is the best alternative terminating label compatible with the ladder-generating chemistry. Upon reaction of the peptide with *N*-carboethoxy phthalimide, the peptide N-terminus is incorporated into a cyclic structure. As a consequence, the corresponding peptide derivatives are very stable and do not fragment during FT-MS analysis. Unambiguous identification of amino acids is demonstrated with an average mass error of ± 0.003 Da. © 1997 Elsevier Science B.V.

Keywords: Degradation; FT-MS; Ladder sequencing; MALDI; Peptide

1. Introduction

In a previous paper [1], we demonstrated that matrix-assisted laser desorption–ionization Fourier transform mass spectrometry (MALDI-FT-MS) is a viable alternative to MALDI time-of-flight mass spectrometry (TOF-MS) for analysis of ladder-generated peptide mixtures. However, the gas-phase stability of the blocked peptides generated by the original method of ladder sequencing [2] was incompatible with the FT-MS time scale. To overcome problems associated with metastable decay of blocked peptides, a variety of terminating labels has been investigated. Among the labels investigated, one produced derivatized peptides which possess the requisite stability. By replacing phenyl isocyanate (PIC), which was originally used as the terminating label with this new reagent, ladder sequencing becomes fully compatible with MALDI-FT-MS analysis. The use of this reagent in modified ladder-sequencing chemistry is now practical for FT-MS measurement times. Because the emphasis of the research

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was to identify improved ladder-generating reagents, relatively high levels of the model peptide were employed and the ultimate detection limits and sensitivity of the method were not investigated.

Although much effort has been put into development of modern analytical methods, Edman degradation is still the most widely used method for peptide sequence determination [3]. Approaches to sequence determination of peptides and proteins are numerous. However, a versatile and generally applicable method other than Edman degradation is still missing. Sequencing of proteins is complex because of the different chemical properties exhibited by the various amino acids. Furthermore, blocked termini and post-translational modifications often inhibit chemical or enzymatic degradation.

Since the introduction of electrospray ionization (ESI) [4,5] and matrix-assisted laser desorption–ionization (MALDI) [6,7], the mass range of mass spectrometry has been greatly extended and is now routinely utilized for bioanalysis. Mass spectrometry is now accepted as a powerful technique for accurate mass determination and structure elucidation of biomolecules. Sequence information is obtained by dissociation methods such as collision-induced dissociation (CID) [8–11], surface-induced dissociation (SID) [12–15], or photodissociation (PD) [16–18], using tandem mass spectrometry. However, dissociation patterns obtained by such techniques are often complicated and loss of neutrals or adduct formation render assignment of fragment ion peaks difficult. Furthermore, dissociation of ions is limited to an upper mass-to-charge ratio of approximately 3000 because the amount of energy deposited upon activation becomes insufficient to induce fragmentation [19]. Other mass spectrometric approaches take advantage of metastable decay of ions in the first field-free drift region of reflectron time-of-flight mass spectrometers (post-source decay) [20,21], or fragmentation of biomolecules between nozzle and skimmer of electrospray interfaces (nozzle–skimmer dissociation) [22,23].

Chemical and enzymatic degradation prior to mass analysis overcome the problem of an upper mass limit because high mass molecules are fragmented to lower mass peptides. Although acid hydrolysis results in random cleavage of peptide bonds [24], the site-specific activity of endopeptidases permits more selective degradation with well-defined cleavage positions. Among the many available endopeptidases, trypsin is the most widely used [25–27]. Tryptic digests in conjunction with mass spectrometry produce fingerprint spectra which can assist in verifying sequences of recombinant proteins. Enzymes that do not efficiently hydrolyze all possible cleavage-sites provide overlapping peptide fragments which assists in assigning the primary structure of the protein [28]. Repeated hydrolysis of fragments using enzymes with different site-specificity yields additional sequence information. Generally, only partial sequence information is obtained from such digests because lack of a full palette of endopeptidases, which would allow for residue-specific hydrolysis of peptide bonds, hinders complete sequence determination.

An alternative strategy for complete sequence analysis of unknowns is stepwise degradation from one terminus, performed by either chemical [29–31] or enzymatic methods [32,33], followed by identification of released amino acids or remaining peptide fragments. However, hydrolysis kinetics are residue-dependent, rendering exact timing of reactions problematic. For example, Patterson and co-workers [33] demonstrated C-terminal ladder sequencing using carboxypeptidase Y digestion and subsequent MALDI-TOF analysis of peptide fragments. Consecutive sets of adjacent peptide fragments were generated by varying the digestion time. Residue-dependence of hydrolysis rates was reflected by a wide range of peak abundances in the spectra.

Chemical sequencing by Edman degradation is more time-consuming, but overcomes problems associated with the kinetics of enzymatic

degradation because the terminal amino acid is released at a well-defined time. In 1993, Chait et al. [2] introduced the method of ladder sequencing based on Edman degradation chemistry which circumvents the need for continuous detection of released amino acids. In order to generate full sequence information, a small fraction of peptide fragments produced during each degradation cycle are blocked and accumulated for final analysis. Peptide ladders are generated utilizing a mixture of phenyl isothiocyanate (PITC) and phenyl isocyanate (PIC) for N-terminal labeling of peptides and proteins. The Edman-type reagent (PITC) couples to the peptide N-terminus to form a phenylthiocarbamoyl (PTC) peptide. Upon exposure to trifluoroacetic acid, the N-terminal PTC-amino acid is cleaved off, resulting in a peptide shortened by one amino acid. Reaction of the terminating reagent PIC with the peptide yields the corresponding phenylcarbamoyl (PC) peptide, which is not susceptible to cleavage. After repetitive cycles of coupling and cleavage, a mixture of sequence defining PC-peptides is generated, which subsequently can be analyzed by mass spectrometry. Mass differences between consecutive peaks of PC-peptides allows rapid determination of the amino acid sequence.

Originally, peptide mixtures generated by ladder sequencing were analyzed by linear time-of-flight (TOF) mass spectrometry [2,34]. The resulting spectra contained only peaks related to pseudomolecular ions of PC-peptides in the mixture, allowing fast read-out of the amino acid sequence. Major drawbacks of linear TOF mass analysis are its limited mass-resolving power and poor mass accuracy, which can compromise unambiguous identification of amino acids, especially in the higher m/z range where peaks of protonated and sodium-attached species overlap.

Previously, we combined ladder sequencing with MALDI-FT-MS to take advantage of the high mass accuracy and resolving power offered by FT-MS to identify amino acid sequences and demonstrate the feasibility of ladder sequencing a mixture of two amino acids simultaneously [1]. FTMS analysis of peptide mixtures generated by ladder sequencing, following the original procedure of Chait et al. [2], revealed problems associated with gas-phase stability of PC-peptides. Fragmentation from complete or partial loss of the PC label prevented simultaneous attainment of spectra that were easy to interpret and had high resolution. Problems associated with fragmentation of PC-peptides would not be noticed with linear TOF because the fragments and molecular ions arrive at the detector simultaneously. However, FT-MS measurements take place on a relatively long time scale (typically milliseconds to several seconds) and demand stability of ions throughout the experimental sequence.

To overcome problems associated with phenyl isocyanate derivatized peptides, alternative terminating labels for ladder sequencing have been investigated. Although the focus of this paper is identification of a terminating label which generates stable derivatized peptides for FT-MS analysis, this information may also prove useful for other mass spectrometry techniques which may be adversely affected by fragmentation. A variety of substituted phenyl isocyanates are evaluated as potential terminating labels. Furthermore, a novel terminating label for ladder sequencing is introduced.

2. Experimental

2.1. Materials

Angiotensin III antipeptide (Gly–Val–Tyr–Val–His–Pro–Val), trifluoroacetic acid and hexafluoroisopropanol were purchased from Sigma (St Louis, MO). 2,4-Dimethylphenyl isocyanate, 2,4,6-trimethylphenyl isocyanate, 3,4,5-trimethoxyphenyl isocyanate and 2,6-diisopropylphenyl isocyanate were obtained from Lancaster (Windham, NH). Anhydrous dimethylformamide was obtained from Pierce (Pittsburgh,

PA) and 2,5-dihydroxybenzoic acid from Fluka (Buchs, Switzerland). All other chemicals and solvents were purchased from Aldrich (Milwaukee, WI). All chemicals were used without further purification.

2.2. Preparation of phenyl isocyanate derivatives

Reactions of phenyl isocyanates with angiotensin III antipeptide were carried out according to the directions for final coupling given by Chait et al. [2] with minor modifications to solvent composition and sample preparation [1].

2.3. Preparation of N-carboethoxy phthalimide (NCP)

The terminating reagent was synthesized according to the directions given by Nefkens et al. [25]. Phthalimide potassium salt [1.85 g (1.0 mmol)] was suspended in 5 ml anhydrous dimethylformamide. The suspension was cooled in an ice bath to 5°C and 1.25 ml (1.3 mmol) of ethylchlorocarbonate was added slowly with stirring. After stirring for 2 h at room temperature, the mixture was poured into 50 ml of cold water, which led to formation of white crystals. After filtration, the crystals were washed three times with cold water, followed by one wash with cold ethanol. After drying for 24 h under vacuum 1.80 g (82%) of white product was obtained.

2.4. Ladder sequencing with NCP

All reactions were carried out under argon in 1.5 ml polypropylene vials. A mixture of phenyl isothiocyanate (PITC, 1.0 ml, 8.36 mmol), *N*-carboethoxy phthalimide (NCP, 37 mg, 0.17 mmol) and hexafluoroisopropanol (0.2 ml, 1.9 mmol) in 3.8 ml of pyridine was used as the coupling reagent. The molar ratio of NCP:PITC was 1:50. 100 μg (130 nmol) of angiotensin III antipeptide was dissolved in 100 μl pyridine–water (1:1). After adding 100 μl of coupling reagent, the reaction mixture was kept at 50°C for 20 min. Following reaction, excess reagent was extracted first with 500 μl heptane–ethylacetate (10:1) and then twice with 500 μl heptane–ethylacetate (2:1). The nonpolar phase was discarded and the remaining polar phase dried under vacuum. For the cleavage step, 80 μl of anhydrous trifluoroacetic acid (TFA) was added to the dry sample. After incubation at 50°C for 30 min, TFA was removed under vacuum. Final coupling was carried out with 100 μl of *N*-carboethoxy phthalimide (0.2 M) in pyridine under the same reaction conditions as the coupling step. After final treatment with TFA (100 μl, 50°C, 30 min), the sample was dried under vacuum. No attempt was made to optimize reaction conditions for smaller peptide quantities or for shorter reaction times.

2.5. Sample preparation

2,5-Dihydroxybenzoic acid (0.4 M in methanol) was used as the matrix and D-fructose (0.2 M in methanol) as the co-matrix. Appropriate amounts of matrix and co-matrix were added to the sample to yield an analyte: matrix:co-matrix molar ratio of 1:2000:1000. The solution was slowly sprayed onto a rotating stainless-steel probe tip as previously described [36,37].

2.6. Instrumentation

Analyses of phenylcarbamoyl-peptides were performed using a FT-MS-2000 Fourier transform mass spectrometer (Finnigan FT-MS, Madison, WI) equipped with a 3 T superconducting magnet (Oxford Cryomagnetic Systems, Oxford, England). The same experimental set-up, but using a 7 T superconducting magnet (Oxford Cryomagnetic Systems, Oxford, England), was employed for analyses of phthaloyl-peptides. Experiments were carried out on the source side of a 2 in. cubic dual cell with pressure in the range 10^{-8}–10^{-9} torr. The probe tip was located at a distance of 4 mm in

front of the front trapping plate. Ions were generated by matrix-assisted laser desorption–ionization (MALDI) using a nitrogen laser (Photon Technology International, London, ONT, Canada) with 6 ns pulse width at a wavelength of 337 nm and 1.2 mJ maximum output. The laser beam was attenuated to threshold level for desorption and focused onto the probe tip by an internal quartz lens, resulting in a spot size of 100 × 250 μm. Instrument control, data acquisition and processing were performed using a Sun Sparc station running Odyssey software version 3.1 (Finnigan FT/MS, Madison, WI).

2.7. *Fourier transform mass spectrometry parameters*

Prior to ionization, a 9.5 V potential was applied to the conductance limit (rear trapping plate of the source cell), while the front trapping plate was grounded. These potentials were maintained for 190 μs after the laser shot to allow ions, generated by the MALDI event, to enter the cell and be decelerated [34,38]. Ions were then trapped by applying a 500 mV potential to both trapping plates which was maintained until the final quench event. A relative timing diagram of the event sequence is provided in Fig. 1. A delay time between the deceleration and excitation events was imposed to allow ion relaxation. For stability studies of derivatized peptides, this delay time was varied between 500 μs and 5 s in order to investigate the extent of fragmentation. The spectrum of angiotensin III antipeptide after ladder sequencing was acquired using a delay time of 500 ms. Ions were excited by a frequency chirp from 50 Hz to 400 kHz at a rate of 190 Hz/μs. All transients were aquired in direct mode using 128 K data points. All spectra shown were generated from single laser shots. External calibration was performed using polyethyleneglycol 1000 prior to analysis.

3. Results and discussion

Various types of substituted phenyl isocyanates were investigated as potential

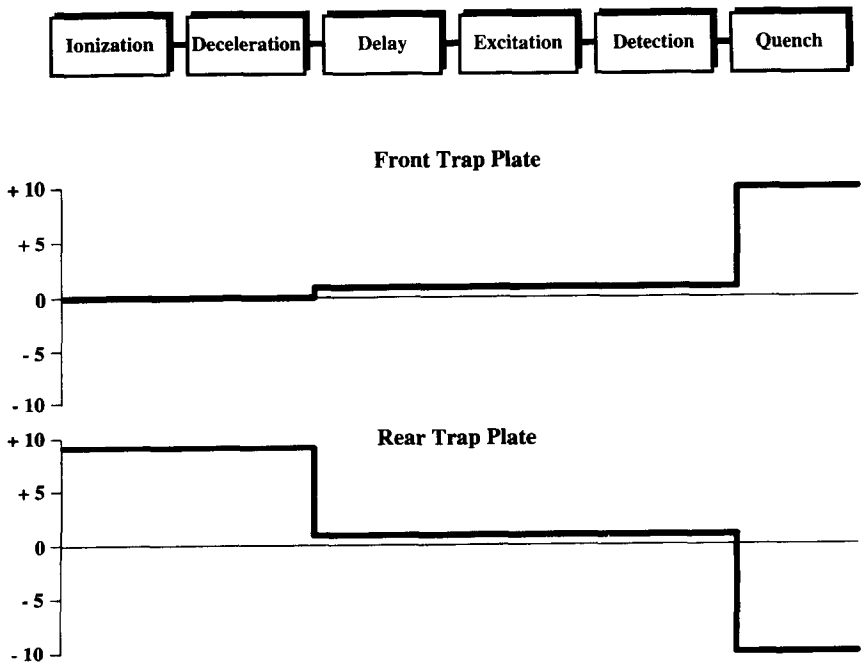


Fig. 1. FT-MS experiment event sequence. Traces show potentials applied to front and rear trapping plates.

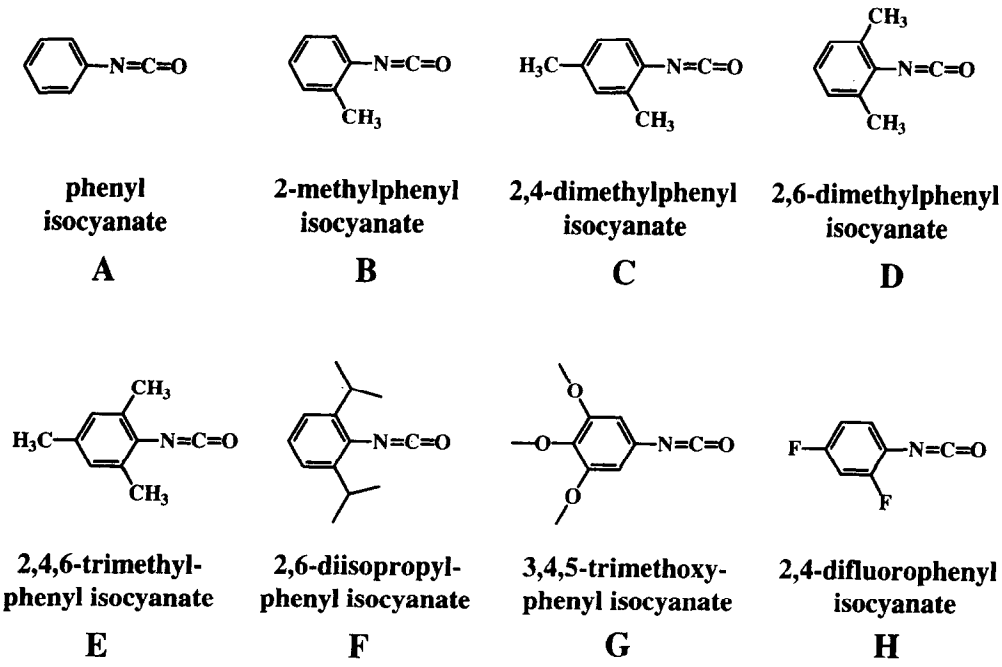


Fig. 2. Structures of substituted phenyl isocyanates investigated as alternative terminating labels for ladder sequencing.

terminating labels using angiotensin III antipeptide as a model peptide. Angiotensin III antipeptide was chosen because its gas-phase stability had previously been characterized [1]. The substituted phenyl isocyanates studied were phenyl isocyanate (PIC), 2-methyl-PIC, 2,4-dimethyl-PIC, 2,6-dimethyl-PIC, 2,4,6-trimethyl-PIC, 2,6-diisopropyl-PIC, 3,4,5-trimethoxy-PIC and 2,4-difluoro-PIC (Fig. 2). The corresponding phenylcarbamoyl-peptide (PC-peptide) was obtained upon reaction of isocyanate with the peptide. Inductive, resonance and/or steric effects from ring substituents were anticipated to influence the gas-phase stability of phenyl isocyanate derivatized peptides.

In our previous investigation [1], three fragmentation patterns of PIC derivatized peptides were observed: (1) loss of the entire label, resulting in formation of the protonated free base; (2) loss of an aniline-like neutral, resulting in a protonated peptide species with mass 26 Da greater than the protonated free base; and (3) loss of Pro–Val (214 Da) from the C-terminus. Spectra of two representative derivatized peptides, 2-methyl-PC-peptide and 2,6-di-isopropyl-PC-peptide, are shown in Fig. 3. Occurrence of fragment ions demonstrates that loss of label was not eliminated by ring substituents. Because the substituted phenyl rings are released upon fragmentation, fragment ions of identical mass-to-charge ratio (m/z of 770, 792, 796 and 818) were obtained for both derivatives. Complete loss of the label is indicated by fragment ion peaks with an m/z of 770 (protonated free base) and an m/z of 792 (sodium-attached free base). Loss of the substituted aniline-like neutral results in peaks with m/z = 796 and m/z = 818 for protonated and sodiated species, respectively. Loss of amino acids Pro–Val from the C-terminus of angiotensin III antipeptide [1] was also observed, resulting in fragment ions with m/z = 689 [Fig. 3(a)] and m/z = 759 [Fig. 3(b)].

The bar graph in Fig. 4 was compiled to compare the stability of derivatized peptides generated using the various substituted phenyl isocyanates illustrated in Fig. 2 as terminating

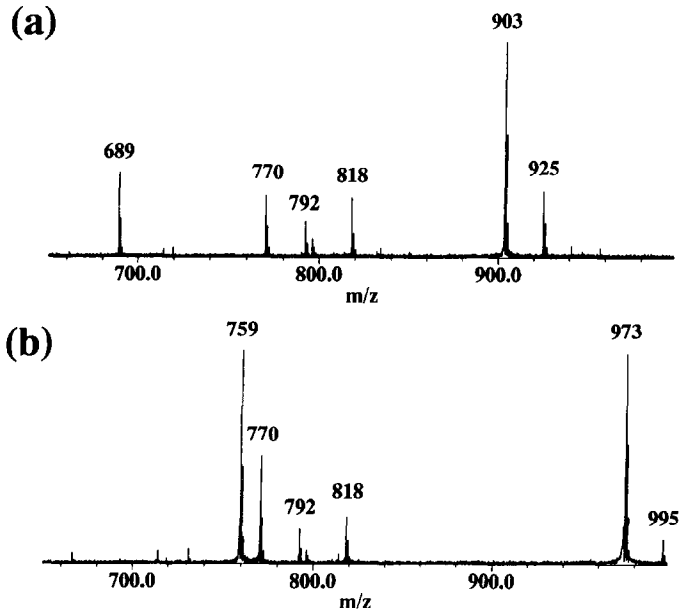


Fig. 3. MALDI-FT-MS spectra of: (a) 2-methylphenyl isocyanate; and (b) 2,6-di-isopropylphenyl isocyanate derivatives of angiotensin III antipeptide (Gly–Val–Tyr–Val–His–Pro–Val). Delay time between ion deceleration and excitation was 500 ms.

labels. For each reagent, the ratio of labeled peptide species to total peptide species is plotted for delay times of 500 μs, 5, 50 and 500 ms, and 5 s. As a result, the calculated ratio corresponds to the percentage of ions which did not lose the label. For example, a ratio of 1 indicates that loss of label did not occur, while a ratio of 0.7 indicates that 70% of all observed species are still labeled. Only monoisotopic peaks (all ^{12}C) of protonated and sodium-attached species were considered. Extensive fragmentation did not occur in experiments using a short delay time of 500 μs. For the phenylcarbamoyl-peptide (reagent A), a maximum of 83% of the ions survived a delay of 500 μs without undergoing fragmentation. However, at longer delay times, the abundance of

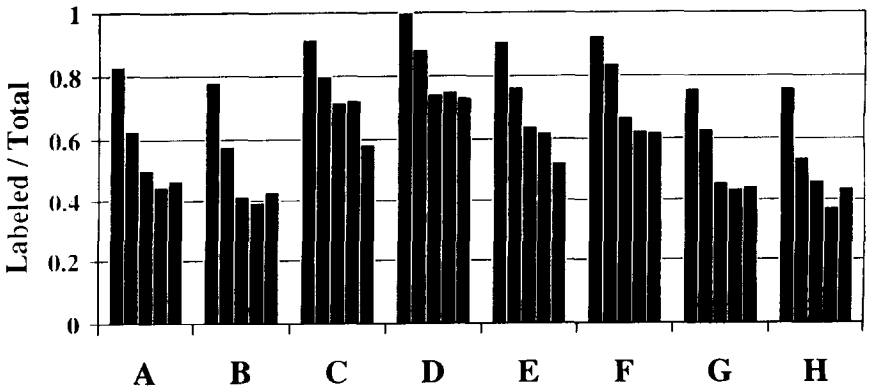


Fig. 4. Bar graph illustrating relative stability of derivatives of angiotensin III antipeptide generated using substituted phenyl isocyanate reagents A–H (see Fig. 2). For each reagent, the ratio of abundance of labeled peptides to abundance of total peptides is plotted for delay times of 500 μs, 5, 50 and 500 ms, and 5 s (from left to right for each reagent). Only monoisotopic peaks (all ^{12}C)) of protonated and sodium-attached species were considered.

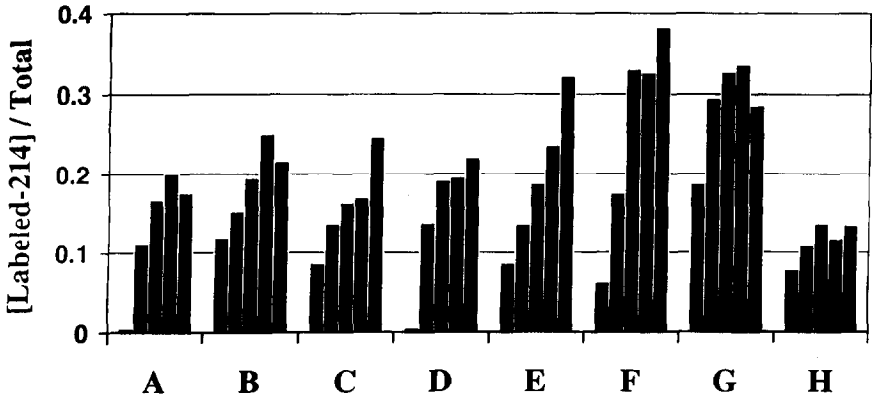


Fig. 5. Bar graph illustrating extent of *C*-terminal fragmentation (loss of Pro–Val) for various phenyl isocyanate derivatives of angiotensin III antipeptide generated using substituted phenyl isocyanate reagents A–H (see Fig. 2). For each reagent, the ratio of abundance of labeled peptides that have lost Pro–Val (214 Da) to abundance of total peptides is plotted for delay times of 500 ms, 5, 50 and 500 ms, and 5 s (from left to right for each reagent). Only monoisotopic peaks (all ^{12}C)) of protonated and sodium-attached species were considered.

labeled peptide ions dropped to about 45%, indicating extensive fragmentation. Reagents A, B, G and H exhibited similar stability. Less fragmentation was observed for reagents C, D, E and F. Double (C, D) and triple (E, F) substitution by alkyl groups seem to increase the stability of the derivatized peptides. This increased stability may be due to a combination of electronic and steric effects. The best results in this series of experiments were obtained for the 2,6-dimethylphenylcarbamoyl-peptide (reagent D). Loss of label did not occur for measurements using a short delay time of 500 μs and 75% of labeled peptide ions still survived a 500 ms delay without undergoing fragmentation.

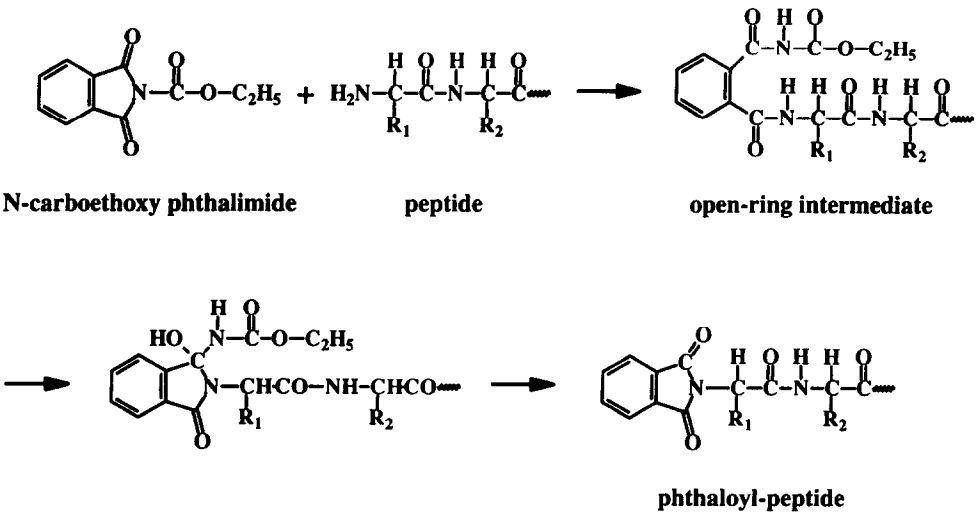
Fragmentation from the C-terminus is also observed for both labeled peptides and free bases of angiotensin III antipeptide. Cleavage of the His 5–Pro 6 bond results in loss of Pro 6–Val 7 (214 Da) with charge retention on the N-terminal fragment. According to common nomenclature [39], this type of fragment is referred to as b_5 for angiotensin III antipeptide. It has been demonstrated that such fragmentation is inherent to proline-containing peptides due to the unique structure of the proline residue, which favors cleavage of the adjacent amide bond [40–42]. An interesting observation is that the extent of fragmentation from loss of Pro–Val from the C-terminus varies depending upon the label attached to the N-terminus 15 bonds away. For each of the substituted phenyl isocyanate reagents, the bar graph in Fig. 5 plots the ratio of labeled peptides that have lost Pro–Val (214 Da) to total peptide species for delay times of 500 μs, 5, 50 and 500 ms, and 5 s. Inspection of Fig. 5 reveals that very few b_5 fragments have been formed using a delay of 500 μs for labeling reagents A and D. However, up to 18% of G labeled peptides have already lost Pro–Val at 500 μs. At longer delay times, over 30% of peptides labeled with either reagent E, F, or G have lost Pro–Val, while only approximately 13% of peptides labeled with reagent H have produced b_5 fragments. Reagents E, F and G are 2,4,6-trimethylphenyl isocyanate, 2,6-diisopropylphenyl isocyanate and 3,4,5-trimethoxyphenyl isocyanate, respectively. These three labels all have bulky substituents on the phenyl ring, which may explain the enhanced fragmentation. A possible explanation for the dependence of the C-terminal fragmentation on the N-terminal label is that the peptide may be folded in the gas phase, bringing the two ends of the peptide into closer proximity.

These data clearly demonstrate that peptides derivatized with phenyl isocyanates generally suffer from insufficient stability under FT-MS

conditions. Ring substituents may slightly enhance stability, but fragmentation is still observed with longer delay times. Any alternative reagent chosen as a substitute for phenyl isocyanate as the terminating label must be fully compatible with ladder sequencing chemistry. Therefore, choices of alternative terminating reagents are restricted by the given reaction conditions. For example, the reaction must be carried out in aqueous solution and in the presence of the Edman degradation reagent PITC. Therefore, a potential terminating label must not react with PITC and the two labels must not inhibit the other's reaction with the peptide. In addition, the kinetics of the reactions of both labeling reactions with the peptide must be within a narrow range to enable control of the product ratio. Furthermore, exposure to trifluoroacetic acid during the cleavage step, which is required for shortening the PTC-peptides by one amino acid, must not affect terminally blocked peptides. *N*-carboethoxy phthalimide (NCP) fulfills these requirements as a terminating label.

Although NCP is not commercially available, it is easily synthesized according to the directions given by Nefkens et al. [35,43]. The essential steps of the reaction of *N*-carboethoxy phthalimide with a peptide, as proposed by Nefkens et al., are shown in Scheme 1. The amino nitrogen of the peptide binds to one of the carbonyl groups of *N*-carboethoxy phthalimide, which causes the five-membered ring to open. Subsequently, formation of a bond between the peptide nitrogen and the second carbonyl group incorporates the peptide nitrogen into a cyclic structure. Ethyl carbamate is finally released upon treatment with acid, yielding the phthaloyl-peptide.

Spectra of angiotensin III antipeptide, derivatized with phenyl isocyanate and *N*-carboethoxy phthalimide are compared in Fig. 6(a and b), respectively. Both spectra were recorded using a delay time of 500 ms. Instability of the phenylcarbamoyl-peptide is clearly demonstrated in Fig. 6(a) by observation of protonated and sodiated peptide free base ions (m/z = 770 and m/z = 792, respectively) and fragment ions generated by loss of the aniline-like neutral (m/z = 796 and m/z = 818, respectively). Such fragmentation is completely absent in the case of the phthaloyl-derivative [Fig. 6(b)]. No fragmentation was observed even in experiments using extended delay times of several minutes for NCP-labeled peptides. Proton transfer from the peptide N-terminus to the isocyanate nitrogen, or



Scheme 1.

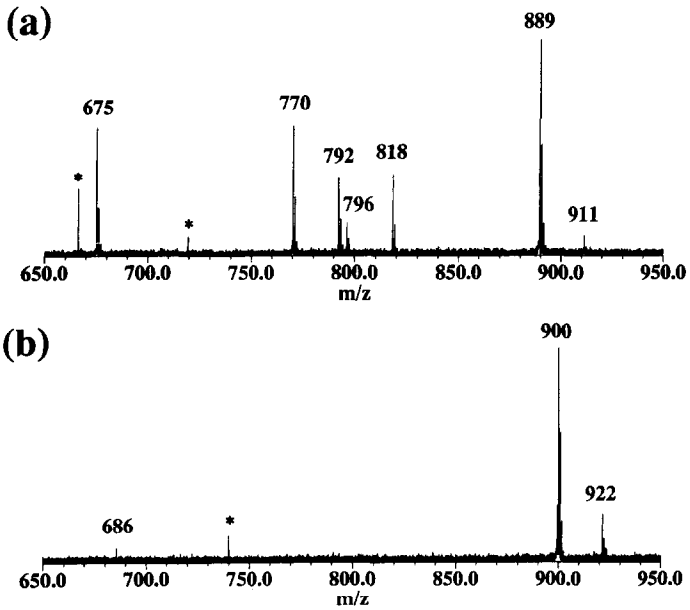


Fig. 6. MALDI-FT-MS spectra of: (a) phenylcarbamoyl-; and (b) phthaloyl-angiotensin III antipeptide obtained with 500 ms delay time between deceleration and excitation events. Peaks labeled with an asterisk are due to instrumental noise.

vice versa, was suggested as a mechanism to explain the extensive fragmentation of phenylcarbamoyl-peptides [1]. The stability of the phthaloyl-derivative apparently is based on the cyclic structure of the label which incorporates the N-terminus nitrogen atom. Formation of a tertiary amine eliminates the possibility of proton transfer, thus making fragmentation unlikely. The only fragment ion observed in the spectrum of the phthaloyl-derivatized peptide originates from cleavage of the His 5–Pro 6 bond, resulting in a peak with $m/z = 686$.

To demonstrate the compatibility of *N*-carboethoxy phthalimide with ladder generating chemistry, angiotensin III antipeptide was subjected to four cycles of ladder sequencing. Because it was found that NCP is more reactive than PITC, the ratio of both reagents in the coupling solution was adjusted to 1:50 (NCP:PITC) in order to yield a ratio of products useful for ladder sequencing. Under such conditions, approximately 15% of the peptide is converted into the phthaloyl derivative in each cycle, while the remaining peptide reacts with PITC and is available for further cycles of degradation. Side reactions were not observed. According to the original procedure described by Nefkens et al. [35], hydrochloric acid is recommended for elimination of the carboethoxy group. This elimination step can be carried out successfully by substituting trifluoroacetic acid for hydrochloric acid. Therefore, elimination of the carboethoxy group from the terminating label and release of PTC-amino acids from PTC-peptides can be performed simultaneously in one reaction step. Consequently, the original procedure for ladder sequencing, consisting of a coupling and a cleavage step, can be maintained and the risk of hydrolyzing peptides by exposure to hydrochloric acid is avoided. To obtain the maximum yield of phthaloyl-peptides, final coupling after the last cycle of degradation is carried out using an excess of approximately 20-fold of NCP. It was noticed that less-concentrated solutions did not yield complete coupling and resulted in left-over free base. Reaction times longer than 30 min and/or higher temperatures did not influence completion of the reaction.

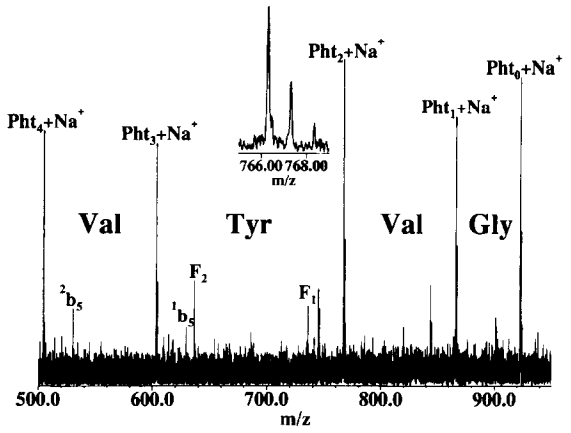


Fig. 7. Spectrum of angiotensin III antipeptide after four cycles of ladder sequencing predominantly showing peaks of sodium-attached phthaloyl-peptides [$Pht_n + Na^+$] (n indicates the number of degradation cycles). $^nb_5$ indicates C-terminal fragmentation (loss of Pro–Val). Peaks of the peptide free bases are labeled with F_n. Insert is an expanded view of $Pht_2 + Na^+$ peaks.

In Fig. 7, the MALDI-FT-MS spectrum of angiotensin III antipeptide after four cycles of ladder sequencing is displayed. Peaks of highest abundance are related to sodium-attached phthaloyl-peptides, labeled as [$Pht_n + Na^+$], where n indicates the number of cycles of degradation. A second complementary peptide ladder is generated by peaks corresponding to protonated phthaloyl-peptides, which are not labeled in the Figure due to their low abundances. Because of the high stability of the phthaloyl-derivatives, a relatively long delay time between MALDI deceleration and excitation events can be used without fragmentation occurring. A delay time of 500 ms was sufficient for obtaining spectra with average resolving power of 15 000 and mass accuracy of 4 ppm for peaks of phthaloyl-peptides. Mass differences between consecutive peaks of 57.017 Da (–0.005 Da), 99.071 Da (+0.002 Da), 163.062 Da (–0.001 Da) and 99.068 Da (–0.002 Da) unambiguously identify the four N-terminal amino acids of angiotensin III antipeptide as Gly, Val, Tyr and Val, respectively. Deviations from the calculated masses are given in parenthesis. The average mass error of ±0.003 Da is an improvement by a factor of two from results obtained with phenylcarbamoyl-peptides (±0.008 Da) [1]. Low abundance ions with m/z = 686, m/z = 629 and m/z = 530 are evidence of C-terminal fragmentation (loss of Pro–Val) and are indicated by $^nb_5$, where n refers to the number of degradation cycles [1]. However, it was noticed that for phthaloyl-derivatives, C-terminal fragmentation was not as prominent as for phenylcarbamoyl-peptides. As mentioned previously, the dependence of C-terminal cleavage on the N-terminal label suggests that the peptide is folded in the gas phase. Incorporation of the nitrogen atom of the N-terminus into the cyclic structure of the label apparently interferes with the cleavage mechanism. Peaks with m/z = 735 and m/z = 636 (labeled F_1 and F_2, respectively) are related to the peptide free bases of the first and second cycle of degradation. Small amounts of free base are present in the mixture even after final coupling with a large excess of N-carboethoxy phthalimide. Because peak abundance is independent of delay time, occurrence of peptide free base is not the result of loss of the phthaloyl-label, but due to incomplete coupling.

4. Conclusions

Of the various substituted phenyl isocyanate terminating labels investigated, 1,6-diisopropyl-phenyl isocyanate exhibits the best overall stability. Nevertheless, even this substituted isocyanate does not yield peptide derivatives with sufficient stability for best compatibility with Fourier transform mass spectrometry. However, insufficient stability of phenylcarbamoyl-peptides can be overcome by replacing phenyl isocyanate with N-carbocthoxy phthalimidc as the terminating reagent. The corresponding phthaloyl-peptides exhibit high stability under FT–MS conditions and loss of label does not occur even when extended delay times are used. Therefore, it is possible to realize the

high mass accuracy and resolving power of FT–MS. Compatibility with the original ladder generating chemistry has been demonstrated using angiotensin III antipeptide. An average mass error of ± 0.003 Da was obtained for the mass differences used for identification of the amino acid sequence. Incorporation of the new terminating label only requires minor modifications of the ladder sequencing procedure. It remains to demonstrate the generality of this reagent with other types of peptides and to determine whether it will continue to be effective at the low nanomole level used in most sequencing applications. Future research addresses these issues as well as refining the ladder generating chemistry to shorten time-consuming coupling and cleavage steps.

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