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Use of capillary electrophoresis–electrospray ionization mass spectrometry in the analysis of synthetic peptides

Kenneth J. Rosnack, Justin G. Stroh*, David H. Singleton, Bradley C. Guarino, Glenn C. Andrews

Central Research Division, Pfizer Inc., Groton, CT 06340, USA

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Abstract

We have constructed a capillary electrophoresis (CE) system with UV detection and have successfully interfaced it to an electrospray ionization mass spectrometry (ES-MS) system. A synthesized fragment of heregulin-β (212–226) was thought to be a single component by re-injection into an HPLC system, but results from CE–UV–ES-MS indicated that a dehydration product was present in the desired peptide sample. A synthetic heregulin-α (177–241) was isolated by preparative HPLC, but re-injection on an analytical system indicated a tailing peak. CE–UV–ES-MS indicated a mixture whose two major components were of the same nominal molecular mass (within experimental error), suggesting the presence of an isomer or a deamidation product. The results show that CE–UV–ES-MS can be used as an orthogonal analytical technique to solve practical problems encountered in peptide synthesis laboratories.

1. Introduction

Peptides of biological interest in the molecular mass range 500–8000 are often synthesized using an automated peptide synthesizer. Crude reaction products are usually purified using preparative reversed-phase HPLC with UV detection which in most cases produces a peptide that is 95% pure by area. When multiple peaks are observed in the chromatogram, identification entails isolation of the fraction and amino acid analysis, N-terminal sequencing, or mass spectrometry (MS). Occasionally a single HPLC peak is actually a mixture of co-eluting peptides or peptides that cannot be fully resolved using HPLC. When this is the case, capillary electrophoresis (CE) with UV detection may be employed as an orthogonal analytical method due to its superior resolving power [1–3].

While analytical CE has superior resolving power, its major drawback is that sample volumes (nanoliter injections) and sample amounts (femtomoles) are low, severely limiting the methods by which the identity of a peak may be determined. Semi-preparative CE [4,5] is possible but is difficult and time consuming. One approach to this problem is to interface CE directly with MS [6–11] to obtain the necessary

* Corresponding author.

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resolving power coupled with analytical sensitivity. In this article, we report on the interfacing of a CE system with electrospray ionization (ES) MS, and on the use of the system for the analysis of synthetic peptide mixtures.

2. Experimental

2.1. Mass spectrometer and electrospray interface

All experiments were performed on a Finnigan MAT TSQ700 triple quadrupole mass spectrometer (Finnigan, San Jose, CA, USA) equipped with an electrospray ionization source (Analytica of Branford, Branford, CT, USA). The source was operated at −3500 V and 20–50 nA in positive ion mode. A coaxial sheath liquid consisting of 2-methoxyethanol (Aldrich, Milwaukee, WI, USA) at a flow-rate of 1 μl/min was used for direct-infusion ES-MS while a 50:50 mixture of isopropanol–1% aqueous acetic acid at a flow-rate of 4 μl/min was used for the CE–ES-MS experiments. Nitrogen was used as the curtain gas and the coaxial sheath gas. The curtain gas was set at 15 p.s.i. (1 p.s.i. = 6894.76 Pa) for all experiments. For direct-infusion ES-MS experiments, the sheath gas was set at 22 p.s.i. while the CE–ES-MS experiments required 13 p.s.i. with the flow controller wide open.

The manufacturer-supplied electrospray probe was used for the direct-infusion experiments while a modified version was used for the CE–ES-MS experiments [12–14]. The modified version of the electrospray probe replaces the stainless-steel sample needle with a 375 μm O.D. polyimide fused-silica tubing used in the CE portion of the experiment. The stainless-steel tubing used for the coaxial liquid sheath was replaced with a section of 22-gauge stainless-steel tubing (0.028 in. O.D. × 0.016 in. I.D.; 1 in. = 2.54 cm; Scientific Instrument Services, Ringoes, NJ, USA) to accommodate the fused-silica CE capillary. The tip of the 22-gauge tubing was rounded and polished to improve electrospray stability. The orifice in the gas sheath probe tip was enlarged to accommodate the 22-gauge tubing. The fused-silica CE capillary terminated flush with the edge of the 22-gauge stainless-steel tubing. Many of the above design changes have been described by Thompson et al. [12].

The mass spectrometer was tuned and calibrated using horse heart cytochrome *c* and glucagon, respectively. Profile mode spectra (peaks presented in analog format) were collected at a scan time of 3.5 s, averaging 32 scans. Spectra were smoothed using a seven-point smoothing routine. Profile mode provides the best accuracy of the data but rapidly consumes disk storage space. Centroid spectra (profile data converted to bar graph format) were collected at a scan time of 2 s. These spectra were averaged over 10–25 scans and were background subtracted. Centroid data files are considerably smaller than profile data files and hence were used in collecting the CE–UV–ES-MS data. Electropherograms were smoothed using a seven-point routine and were also baseline corrected. The molecular mass as well as the multiply charged peaks in the profile and centroid spectra were identified using a "deconvolution algorithm" [15] and an "averaging algorithm" [15] using Finnigan's Biotech software. All numbers shown in the mass spectra are observed m/z values while the "observed mass" in the figure captions and text refers to the observed molecular mass of the compound.

2.2. HPLC

The HPLC analysis was performed on a Beckman System Gold (Beckman Instruments, Fullerton, CA, USA). The chromatography was performed using a linear gradient consisting of 0.1% aqueous trifluoroacetic acid (TFA) and 0.1% TFA in acetonitrile on a Waters C_{18} μBondapak 300 × 3.9 mm column (Waters, Division of Millipore, Milford, MA, USA).

2.3. Capillary electrophoresis

A CE system was constructed in our laboratory and consisted of commercially available

components and a fabricated Plexiglass housing. A Spellman CZE1000R 30 kV power supply (Spellman, Plainview, NY, USA) and a Spectra-Physics Spectra 100 UV–Vis detector (Spectra-Physics Analytical, San Jose, CA, USA) comprised the main components of the system. The CE columns were made from 75 μm I.D. × 375 μm O.D. fused-silica (Polymicro Technologies, Phoenix, AZ, USA) capillary tubing. Uncoated capillary columns were 110 cm in length while linear polyacrylamide-coated columns [16] (made in our laboratory) were 90 cm. Both the untreated and linear polyacrylamide columns were used for CE–UV and CE–UV–ES-MS analysis. The bradykinin–magainin spacer peptide (MSP) mixture and heregulin-β fragment were run on the untreated fused-silica CE columns while the heregulin-α sample was run on the linear polyacrylamide-coated CE columns. The internally coated polyacrylamide columns were found to minimize adsorption of peptides and small proteins to the capillary wall. The absence of electroosmotic flow in the polyacrylamide column did not affect the performance of the CE–ES-MS system. Approximately 1 mm of the fused silica's external polyimide coating was removed 40 cm from the inlet side of the capillary to provide a window for the UV detector.

The polyacrylamide-coated CE column was washed with 100 μl of distilled water followed by 100 μl of the background electrolyte, while the untreated capillary was washed first with 100 m*M* NaOH followed by distilled water and the background electrolyte. Samples were siphon-infused into the CE column by inserting the column inlet into the sample vial and then elevating the column inlet 25 cm above the outlet for approximately 10 s. This procedure produced an injection volume of approximately 10 nl. Bulk flow was minimized in the CE capillary by adjusting the height of the anode reservoir with respect to the electrospray probe using the procedure described by Thompson and co-workers [12]. Samples were electrophoresed at 275 V/cm with resulting currents of 1–12 μA depending on the background electrolyte. UV detection was performed at 214–220 nm, 0.005 AUFS and 0.3 s rise time.

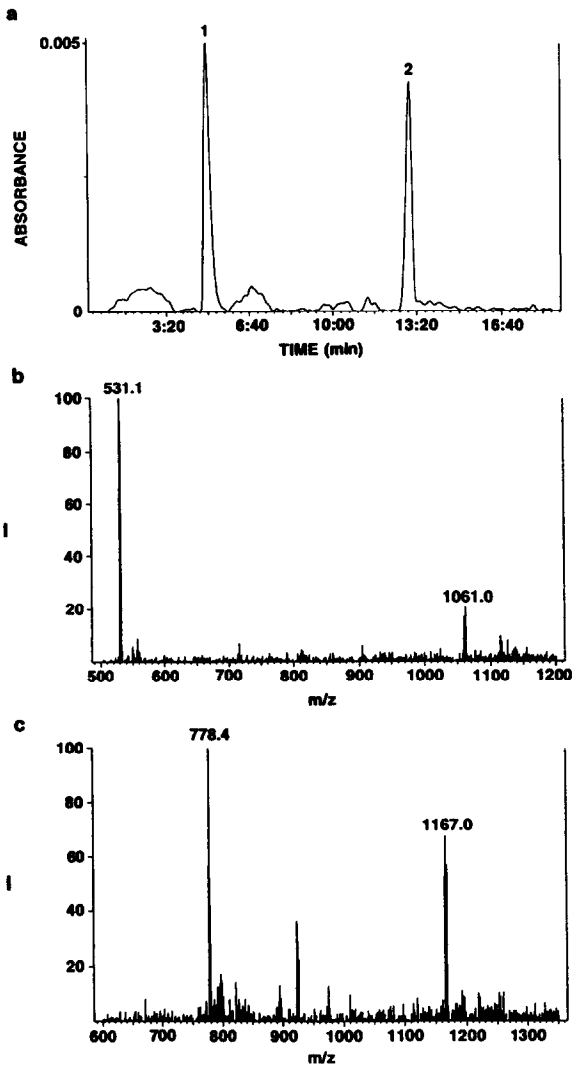

Fig. 1. (a) CE–UV electropherogram of bradykinin (peak 1, ca. 10 pmol) and magainin spacer peptide (peak 2, ca. 5 pmol) mixture. CE analysis was performed using a 110-cm untreated fused-silica column with 20 m*M* ammonium acetate (pH 5.5) as the background electrolyte. (b) Averaged mass spectrum of bradykinin (calculated M_r 1060.2) from the CE–UV–ES-MS analysis of peak 1. The observed M_r was 1059.9. (c) Averaged mass spectrum of magainin spacer peptide (calculated M_r 2332.4) from the CE–UV–ES-MS analysis of peak 2. The observed M_r was 2332.1.

2.4. Sample preparation

Cytochrome *c* (Sigma, St. Louis, MO, USA) and glucagon (Boehringer Mannheim, In-

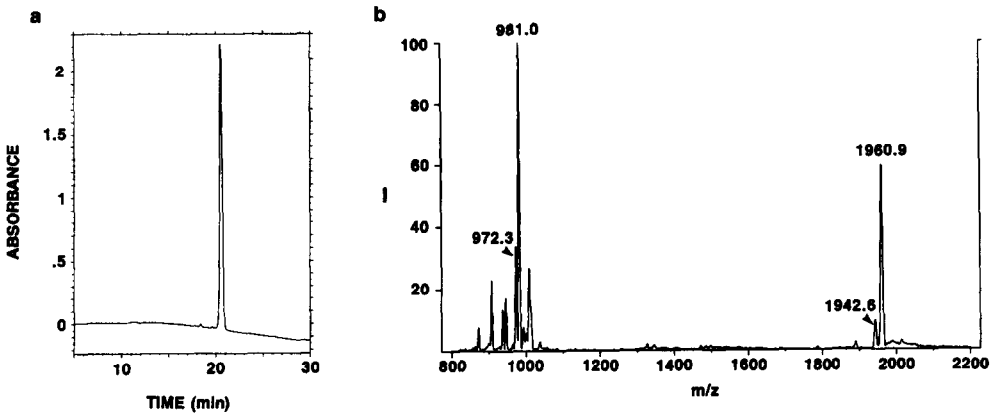

Fig. 2. (a) Reversed-phase analytical HPLC–UV chromatogram of the heregulin-β fragment (calculated M_r 1959.8). HPLC gradient was 0–80% acetonitrile (0.1% TFA) over 30 min. (b) Direct infusion ES profile mass spectrum of the heregulin-β fragment The observed M_r of 1959.9 (MH^+: 1960.9) corresponds to the intact peptide while the observed M_r of 1942.4 corresponds to a potential dehydration product.

dianapolis, IN, USA) standards, used in the tuning and calibration of the mass spectrometer, were dissolved in 0.1% TFA at 10 pmol/μl. They were infused at 1 μl/min into the mass spectrometer. Bradykinin (Sigma), MSP (Peninsula Labs., Belmont, CA, USA), and heregulin-α [17] (prepared in our laboratory), used in the CE–ES-MS experiments, were dissolved at 1 μg/μl in 0.1% TFA. A truncated synthetic heregulin-β [17] peptide Ac-C(Acm)PNEFTGDRC(Acm)QNYVM-NH_2 (prepared in our laboratory; Acm = acetamidomethyl) was dissolved to 1 μg/ml in distilled water.

The peptides were synthesized on an ABI Model 430A (Applied Biosystems, Foster City, CA, USA) solid-phase synthesizer using a *tert.*-butoxycarbonyl (Boc) protocol with capping. The ABI system employs 1-hydroxybenzotriazol (HOBT) active ester coupling using standard amino acids and N-methylpyrrolidone (NMP)-based chemistry. All samples were run as received.

The background electrolyte for heregulin-α was 20 m*M* ϵ-amino-*n*-caproic acid (EACA) [12,18] (Sigma) adjusted to pH 4.6 using glacial acetic acid. All other samples were run using 10–20 m*M* ammonium acetate (Sigma) adjusted to pH 5.5 using glacial acetic acid.

3. Results and discussion

To demonstrate a working and viable CE–ES-MS system, bradykinin (calculated molecular mass 1060.2) and MSP (calculated molecular mass 2332.4) were used as a test mixture. Fig. 1a shows the CE–UV electropherogram resulting from an approximately 10-nl injection of bradykinin (ca. 10 pmol) and MSP (ca. 5 pmol) test mixture onto a 1.1 m untreated fused-silica column using a background electrolyte of 20 m*M* ammonium acetate (pH 5.5). Bradykinin was the first peak to elute followed by MSP approximately 14 min later. The mass spectra of the bradykinin and MSP are presented in Fig. 1b and c, respectively. Both the electropherogram and mass spectra show that this particular system can be used for analysis of peptide mixtures in the low picogram range.

3.1. Heregulin-β

The heregulins [17] are specific activators of the $p180^{erbB4}$ gene [19], which has been implicated in breast and ovarian cancers. A fragment of heregulin-β (212–226), Ac-C(Acm)-PNEFTGDRC(Acm)QNYVM-NH_2 (calculated molecular mass 1959.8), was synthesized and

further purified by preparative HPLC. Although the isolate was thought to be a single component by re-injection into the HPLC system (Fig. 2a), a direct-infusion ES-MS (Fig. 2b) spectrum of the isolate indicated a possible dehydration impurity. The peaks at m/z 1960.9 and 981.0 correspond to the singly $(M+H)^+$ and doubly $(M+2H)^{2+}$ charged ions, respectively, for the desired peptide. The peaks at m/z 1942.6 and 972.3 arise from the singly and doubly charged ions, respectively, of a dehydration product $(M-18)$. It was not known whether this dehydration product was produced by the electrospray process or whether it was truly an impurity in the sample. Since a mixture was not indicated by HPLC, we performed a CE analysis to determine if the impurity was present in the truncated heregulin-β sample. A CE–UV electropherogram of the truncated heregulin-β sample is shown in Fig. 3a. Two components were found in the sample (Fig. 3a). A CE–MS mass spectrum of the minor component (peak 1) is presented in Fig. 3b. The peak at m/z 971.8 corresponds to the doubly charged ion of a dehydration product which has an observed molecular mass of 1941.6. The observed mass is in close agreement with the calculated mass of 1941.8 even though the signal obtained is very weak. Peak 2, whose mass spectrum is shown in Fig. 3c, is the desired peptide. The peak at m/z 1961.3 is the $(M+H)^+$ for the desired peptide (calculated molecular mass 1959.8) while the peak at m/z 981.2 is the doubly charged species. The observed and calculated mass agree within the expected error of a fast centroid scan analysis.

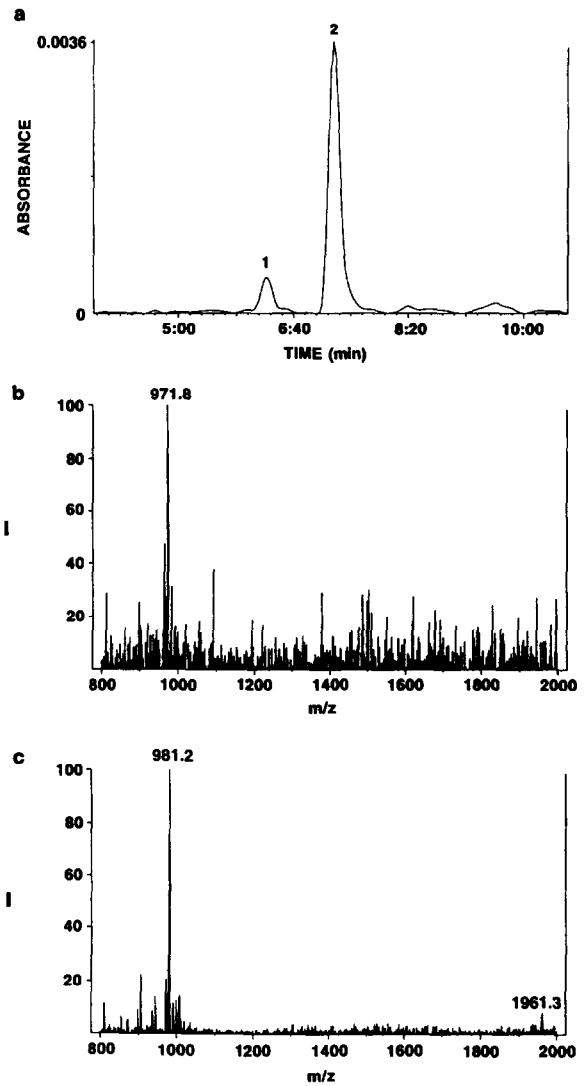

Fig. 3. (a) CE–UV electropherogram of the heregulin-β fragment (ca. 5 pmol). CE analysis was performed using a 110-cm untreated fused-silica column with 10 mM ammonium acetate (pH 5.5) as the background electrolyte. (b) Averaged mass spectrum of peak 1 from the CE–UV–ES-MS analysis of the heregulin-β fragment sample. The observed M_r based on the doubly charged ion is 1941.6 and corresponds to a dehydration product. (c) Averaged mass spectrum of peak 2 from the CE–UV–ES-MS analysis of the heregulin-β fragment sample. The observed M_r was 1960.4 and corresponds to the intact peptide.

3.2. *Heregulin-α*

A second problem that we analyzed using CE–ES-MS involved a synthetic 63 amino acid peptide, heregulin-α (177–241) which has the sequence: SHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCQPGFTGARCTENVPMKVQNQEKAEELY. This peptide was synthesized and isolated using HPLC. The HPLC–UV data indicated a single component, although there was some broadening of the peak (see Fig. 4a) which was thought to be due to overlapping peaks. Direct-infusion ES-MS indicated a single molecular mass, so any impurity present was

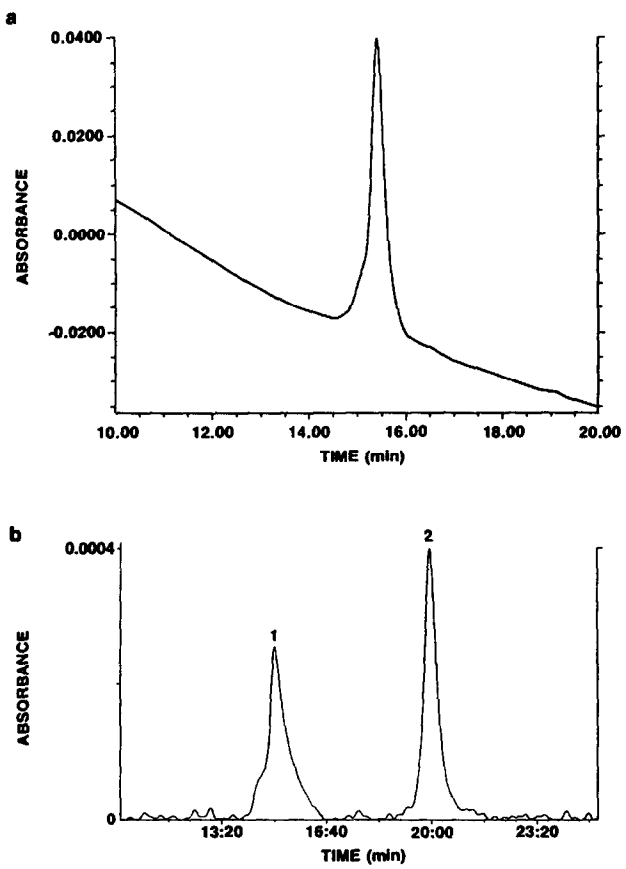

Fig. 4. (a) HPLC–UV chromatogram of the heregulin-α sample. (calculated M_r 7113.3). HPLC gradient was 10–60% acetonitrile (0.1% TFA) over 45 min. (b) CE–UV electropherogram of the heregulin-α sample. CE analysis was performed on a 90-cm linear polyacrylamide-coated capillary using 20 m*M* EACA (pH 4.6) as the background electrolyte. Peak numbers refer to Fig. 5.

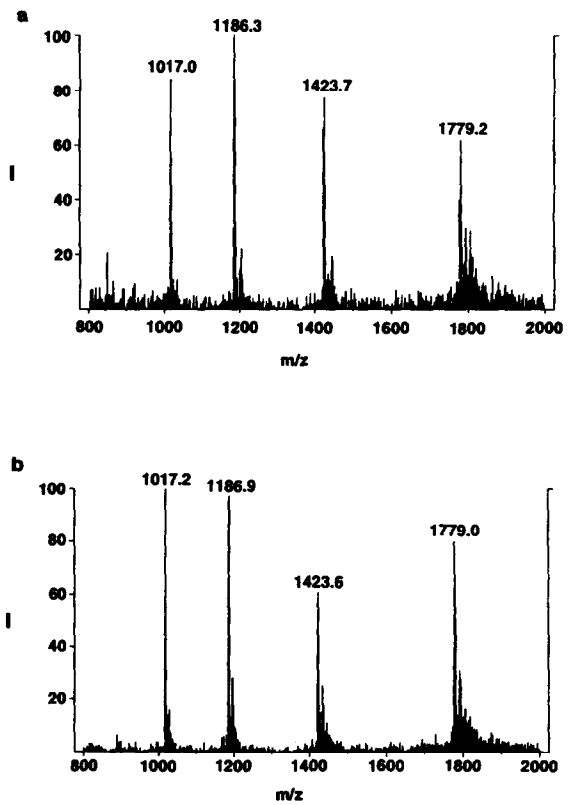

Fig. 5. (a) Averaged mass spectrum of peak 1 found in the CE–UV–ES-MS analysis of the heregulin-α sample (Fig. 4b). The observed M_r was 7113.0. (b) Averaged mass spectrum of peak 2 found in the CE–UV–ES-MS analysis of the heregulin-α sample (Fig. 4b). The observed M_r was 7112.6.

likely an isomer of the desired material. CE–UV–ES-MS was performed on this sample due to concern over the broadening of the peak in the HPLC. The CE–UV electropherogram, indicating two components, is shown in Fig. 4b. The CE–MS spectra for the two components are found in Fig. 5a and b. The two peaks give identical mass spectra, which suggests two possibilities: first, that the two components are isomeric due to differences in the disulfide bonding pattern or second, that one component contains a single deamidation (loss of 1 u) of one of the four asparagines or three glutamines in this peptide. As for the first possibility, a difference in three-dimensional structure resulting from different disulfide bonding patterns could cause differences in charge availability and hence differences in elution times in the CE analysis. As for the second possibility, a difference of 1 u at mass 7113 would appear as the same nominal mass within the error of the present instrumentation. Although the power of CE–MS in solving problems in peptide synthesis is readily apparent in this example, at the present time we cannot use it to distinguish between these two possibilities.

4. Conclusions

We have successfully interfaced a CE system to a TSQ700 mass spectrometer and have demonstrated that CE, with its superior resolving power, can separate complex synthetic peptide mixtures when reversed-phase HPLC cannot. Although low sample loading is a drawback to the CE technique, coupling CE to MS overcomes the problem and utilizes the mass-resolving capability and high sensitivity of the spectrometer for structure analysis of the mixture. CE–UV–ES-MS is a powerful complimentary analytical technique for solving practical problems encountered in a peptide synthesis laboratory.

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