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Note

Problems encountered during peptide derivatization for gas chromatographic–mass spectrometric analysis

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Vapour-phase analysis of peptides is severely hampered owing to their zwitterionic character, and in order to make them amendable to gas chromatographic–mass spectrometric (GC–MS) analysis they need to be derivatized. Four major types of derivatives are used for peptide analysis: N-acyl peptide esters[1,2], permethylated peptides[3–5], Schiff base derivatives[6,7] and polyamino alcohols[8–13]. The conversion of peptides to polyamino alcohols was introduced by Biemann and co-workers, who have used it extensively for sequencing of proteins[14–16]. In our laboratory we needed to develop a method for protein sequencing and after studying all the options we decided to choose the Biemann approach, but we immediately encountered several difficulties, even with simple dipeptides. In this paper we describe these difficulties and how we finally resolved them. The scheme for reduction of peptides to polyamino alcohols introduced by Biemann and co-workers and modified by Frank and Desiderio[17] is shown in Fig. 1.

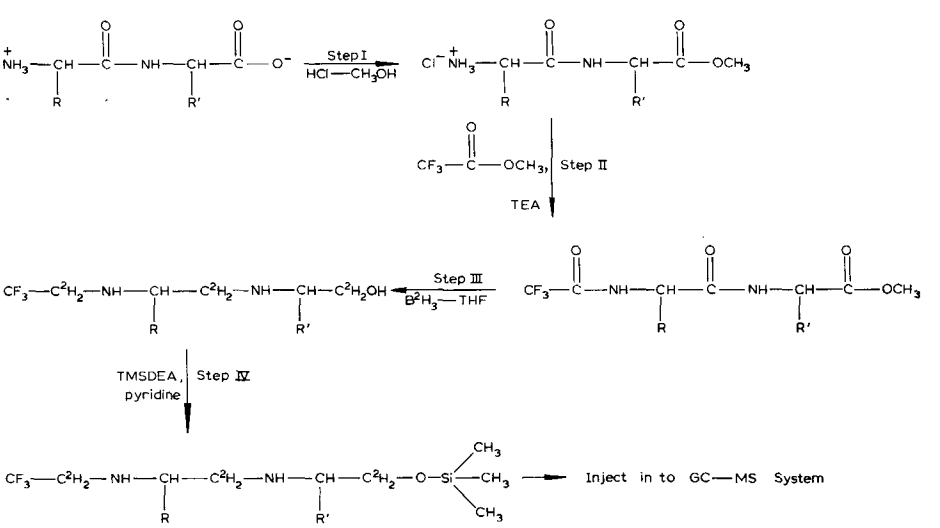

Fig. 1. Derivatization of peptides according to modified Biemann procedure.

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EXPERIMENTAL

A careful investigation of the derivatization procedure revealed that the purity of the reagent played a very important role. Hence we describe in detail below the handling of the glassware and the reagents.

Glassware cleaning and handling

All of the glassware was soaked overnight in a dilute soap solation (approximately half a tablespoon), rinsed with water, distilled water and transferred into a clean glass beaker (1000 ml) containing *ca.* 400–500 ml of 60% nitric acid. The beaker was heated (covered) on a hot-plate for 8 h at 75°C, followed by rinsing with tap water (3–4 times), and was then soaked for 1 h in distilled water and finally dried in an oven for 3–5 h at 110°C. The glassware was allowed to cool to room temperature (covered) and finally stored in zip-fastened polyethylene bags.

All glassware used was of Pyrex-Kimax brand, except for the disposable pipettes (Pasteur), which were made of soda-lime glass.

Reagents

The following reagents were needed for the Biemann procedure.

Step I

3 M hydrochloric acid–methanol. The solution was prepared by bubbling technical hydrogen chloride gas (Matheson) through concentrated sulphuric acid and freshly distilled nanograde methanol. We found it convenient to prepare about 50 ml at a time, divide it into 10-ml portions and store them under refrigeration at all times. We obtained reproducible results with a solution that was 2–3 weeks old. However, we had limited success with commercially purchased solutions.

Step II

(a) Methyl trifluoroacetate (MTFA). Generally about 6 ml were distilled and a middle fraction of about 3 ml was collected.

(b) Triethylamine (TEA). After purchase (P & B Chemicals, CT, U.S.A.), about 100 ml were stirred overnight with 1–2 g of amino acid active ester (N-*tert.*-butyloxycarbonyl-L-alanine pentachlorophenyl ester) to remove trace amounts of primary and secondary amines. The clear solution was distilled and stored in an amber-glass flask.

Step III

(a) Borontrideuteride–tetrahydrofuran. We found that the purity of this reagent was very important. Initially, about 100 ml of 1 *M* solution (Alfa Ventron, Danvers, MA, U.S.A.) were purchased, but according to our experience this must be avoided. We obtained very reproducible results when we purchased a smaller sample (*ca.* 2.5 ml in sealed glass vials) and used up the vial within 2–3 days after opening. The vials were stored under refrigeration at all times.

(b) Reagents needed for work-up of the reduced peptides.

(i) Hydrochloric acid–methanol (1 *M*). The 3 *M* solution prepared for esterification was diluted with distilled methanol to obtain a 1 *M* solution.

(ii) Potassium carbonate. About 100 ml of a 25% solution of potassium carbonate (Fisher) were prepared initially and used throughout the work.

(iii) Chloroform. This was of nanograde quality and was used without further purification.

Step IV

(a) Pyridine. Generally about 5 ml were distilled and about 0.5 ml was collected.

(b) Trimethylsilylydiethyl amine (TMSDEA). We found that it was very important to distill this reagent each time before use, as a large amount of impurities distills (*ca.* 30%) before the correct boiling point. Generally 3 ml were distilled and a middle fraction of about 0.5 ml was collected.

Derivatization

The peptides were derivatized in 100-μg amounts according to the procedure described by Herlihy[16].

Instrumentation

A Varian Model 2700 gas chromatograph interfaced via a 25 cm × 1.15 mm stainless-steel capillary tube to a Nuclide Model 12-90-G mass spectrometer, was used. The GC injection port and the flame-ionization detector (FID) oven were maintained at 280°C. The column (1% or 3% OV-17 on 100–120 mesh Suplecoport) was programmed linearly from 100 to 270°C at 6°C/min. Mass spectra were obtained at 70 eV with a trap current of 50 μA. Data were also obtained on a Varian Model 3700 gas chromatograph and on a GC–MS system at the Massachusetts Institute of Technology (MIT).

RESULTS AND DISCUSSION

The GC retention indices and the mass spectral fragmentation of the polyamino alcohols can be accurately predicted. These unique features provide unambiguous identification of the original peptides[8–16].

We found that the Biemann derivatization procedure could be easily reproduced if necessary precautions are taken. It must be emphasized that it was not necessary to distil all of the reagents each time before use as all the reagents were found to be stable if properly handled.

The importance of the freshness and purity of the reagents can be demonstrated by the following experiments.

Experiment I

The GC (FID) trace of the reduced peptide mixture (standard) shown in Fig. 2 was obtained when all of the reagents were fresh. Four major peaks corresponding to four dipeptides (confirmed by GC–MS) were recorded. The derivatization of this and other more complex mixtures could be reproduced several times.

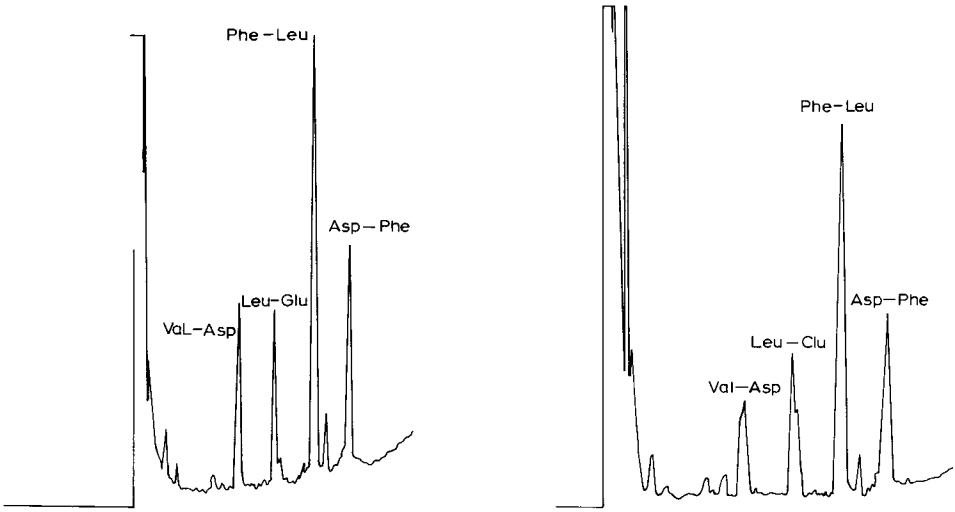

Fig. 2. Gas chromatogram of the reduced peptide mixture (standard) obtained with fresh reagents.

Fig. 3. Gas chromatogram of the reduced peptide mixture (standard) obtained with the reagents in the following states: (a) hydrochloric acid–methanol had a pale yellow colour; (b) MTFA and boron trideuteride–tetrahydrofuran were fresh; (c) TEA, TMSDEA and pyridine were not freshly distilled.

Experiment IIa

The GC (FID) trace of the reduced peptide mixture (standard) shown in Fig. 3 was obtained with the reagents under the following conditions: (1) hydrochloric acid–methanol (3 *M*) was freshly prepared but the solution had a pale yellow colour as the valve on the hydrogen chloride tank was not clean; (2) MTFA was freshly distilled and boron trideuteride–tetrahydrofuran was obtained from an unopened vial; (3) TEA, TMSDEA and pyridine were not freshly distilled.

When the chromatograms in Figs. 2 and 3 are compared, the following major differences can be seen:

(1) The height of all of the major GC peaks was reduced significantly if all the reagents were not fresh. This clearly indicates that the yields of the products are greatly affected by reagent purity.

(2) The height of the shoulder eluting along with the reduced dipeptide Leu–Glu was of much greater intensity than the same shoulder obtained in the experiment in which all the reagents were fresh. This indicates that some undesirable product was formed if impure reagents were used.

Experiment IIb

The GC (FID) trace of the reduced peptide mixture (standard) shown in Fig. 4 was obtained with the reagents under the following conditions: (1) hydrochloric acid–methanol (3 *M*) was about 2 weeks old; (2) MTFA was freshly distilled and boron trideuteride–tetrahydrofuran was obtained from a vial that had been opened 4 months earlier; (3) TEA, TMSDEA and pyriridine were not freshly distilled.

By means of control experiments, it was shown that hydrochloric acid–methanol that was about 2 weeks old gave the same results as a fresh mixture. Hence,

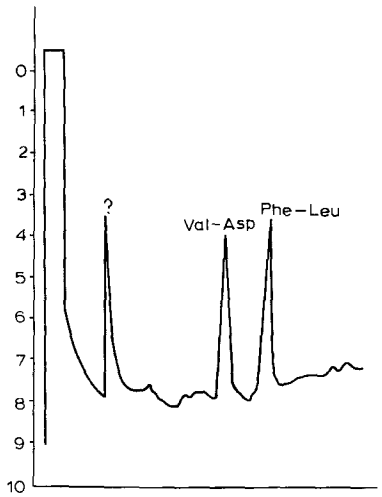

Fig. 4. Gas chromatogram of the reduced peptide mixture (standard) obtained with the reagents in the following states: (a) hydrochloric acid–methanol was about 2 weeks old; (b) MTFA was freshly distilled; (c) boron trideuteride–tetrahydrofuran was obtained from a vial that had been opened about 4 months earlier; (d) TEA, TMSDEA and pyridine were not freshly distilled.

essentially, the difference between experiments IIa and IIb was the freshness of boron trideuteride–tetrahydrofuran. When the chromatograms for experiments IIa and IIb (Figs. 3 and 4, respectively) are compared, the following major differences can be seen: (1) a major unidentifiable peak was recorded; (2) the relative height of the peak corresponding to the dipeptide Phe–Leu was substantially reduced; and (3) dipeptides Leu–Glu and Asp–Phe gave no response.

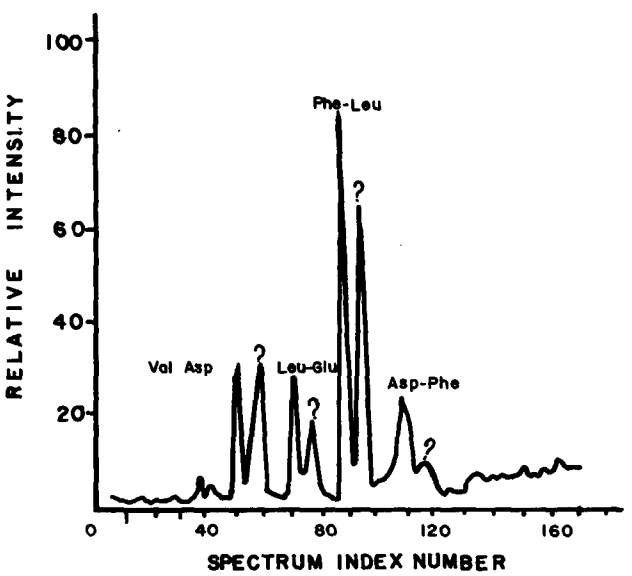

Fig. 5. Total ionization plot of the reduced peptide (standard) obtained with all of the reagents borrowed from the Biemann group but derivatization performed in our laboratory.

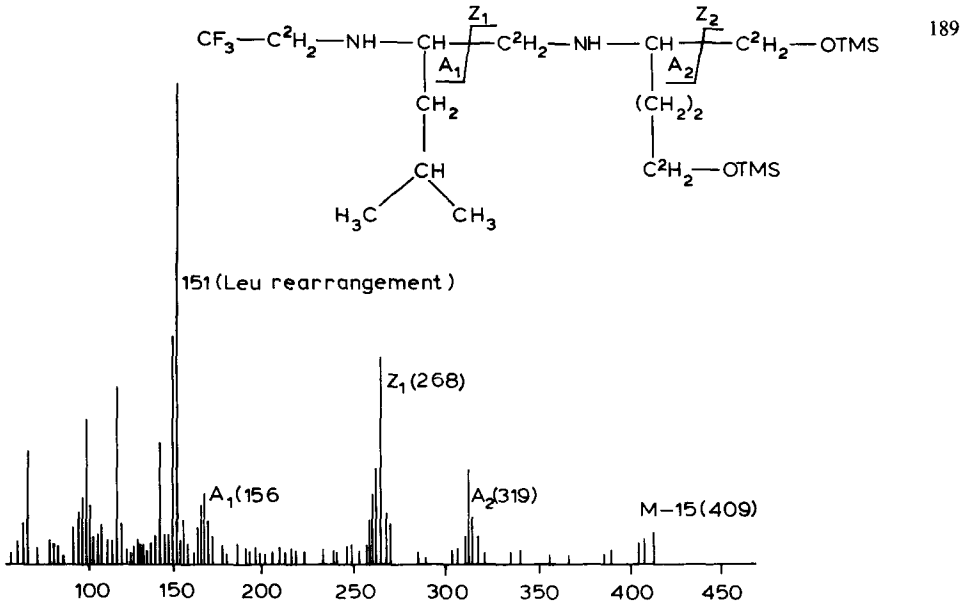

Fig. 6. Representative partial mass spectrum of reduced Leu–Glu.

Experiment IIc

The total ionization plot of the reduced peptide mixture (standard), shown in Fig. 5, was obtained with all of the reagents borrowed from the Biemann group at MIT, but the derivatization was performed in our laboratory. It can be seen that each of the dipeptides gave a major unidentifiable peak in addition to the expected peak.

At this point it became clear that the Biemann sequence was a sensitive scheme and had to be performed carefully. Hence we decided to take precautions with the reagents, and once we had purified them the derivatization could be easily reproduced.

A representative partial mass spectrum of the reduced dipeptide Leu–Glu is shown in Fig. 6.

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