SYNTHESIS AND PROOF OF STRUCTURE OF THE NEW AMINO ACID IN PROTHROMBIN

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Summary

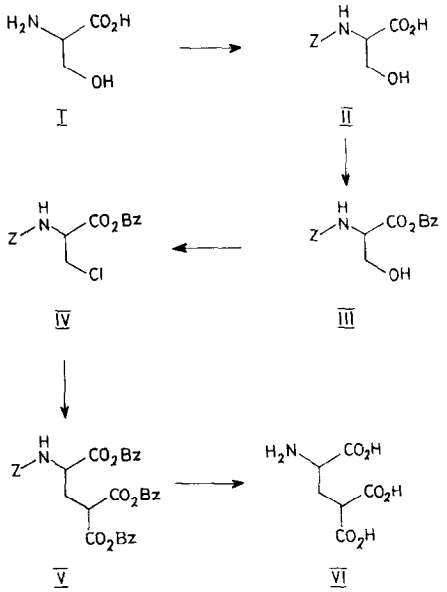
The first synthesis of the new amino acid γ-carboxyglutamic acid (Gla) found in prothrombin is reported. The structure previously proposed is thus confirmed. Part of the proof of identity with the natural product is based upon an unusual chemical modification occurring during acetylation in methanol.

Introduction

Recently, a new amino-acid has been discovered in the N-terminal region of the vitamin K - dependent coagulation factor prothrombin, and its structure has been proposed (1,2). The full sequence of the N-terminal polypeptide has been determined by classical and mass spectrometric techniques (2), and the new amino-acid has been assigned to positions 7 and 8 (1,2), and also to positions 15, 17, 20, 21, 26, 27, 30 and 33 in the sequence (2,3).

The structure assignment has been somewhat tenuous because of the small quantities of sample available for analysis, and because of some disagreement about the interpretation of spectroscopic results, on which the structure has been based (1,2).

We now wish to report the final proof of structure. A synthesis of 2-amino-4,4-dicarboxybutyric acid (γ-carboxyglutamic



Scheme 1 : Synthetic route to γ-carboxyglutamic acid (VI). Physical data on each compound were satisfactory.

acid) is described, and biochemical and spectroscopic proof of its identity with the naturally occurring amino-acid in prothrombin is given. Any details not presented here of the spectroscopic or other physical data on the prothrombin peptides or synthetic amino-acid precursors will be reported (3).

Experimental

Synthesis: The route chosen to γ-carboxyglutamic acid is shown in Scheme 1, starting from DL-serine. N-carbobenzoxyserine(II) was prepared by reaction of serine with carbobenzoxychloride in aqueous sodium bicarbonate (4). Product II was converted to the benzyl ester III by treatment of the sodium salt of the acid with benzyl bromide in DMF (5). The chloride IV was obtained by treatment of III with thionyl chloride in dry DMF (6). The displacement IV→V was achieved using dibenzyl malonate prepared by treating the acid chloride of malonic acid with benzyl alcohol (7). Treatment of IV with the anion of dibenzyl

malonate (8) generated using NaH gave V. The amino acid VI was liberated as the hydrobromide salt by hydrolysis of V in HBr/ acetic acid (9).

Acetylation: The synthetic and naturally occurring amino acids were acetylated in acetic anhydride/methanol (10) using a 1:1 ratio of CH_3OH and CD_3OD (11).

Electrophoresis and Amino-acid analysis: Paper electrophoresis was carried out at pH6.5 in pyridine, acetate (12). Automatic amino-acid analyses were made using a Locarte analyser.

Mass spectrometry: Spectra were recorded on an AEI MS902 instrument. Sample handling procedures and instrument parameters have been described previously (13).

Results and Discussion

The identity of the synthetic amino-acid, prepared as described above, with the natural product (isolated by S. Magnusson and co-workers by mild acid hydrolysis of prothrombin) is established in the following experiments.

a) The synthetic and natural product co-chromatograph on electrophoresis at pH6.5 (12) with mobility 1.35 relative to Asp=1.0.

b) The synthetic and natural product co-elute prior to aspartic acid on the Locarte amino acid analyser.

c) Hydrolysis of the synthetic product in 4M HCl at 110°C for 3hr yields mainly glutamic acid on the auto-analyser. This fully agrees with the decarboxylation of the natural product upon strong acid hydrolysis (2).

d) A derivative of the synthetic and natural product gives identical mass spectra (see below).

It was observed in our initial structural and sequence studies (2,3) that a reaction was taking place between the solvent used in the acetylation procedure, methanol, and the new amino-

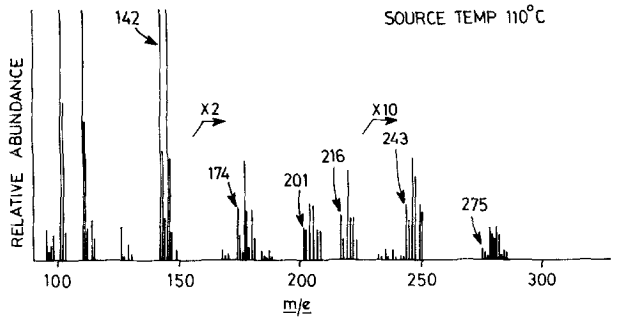


Figure 1 : Mass spectrum (above m/e 90) of a sample of synthetic γ-carboxyglutamic acid treated with acetic anhydride in methanol (see Experimental).

acid Gla. It was proven using isotope labelling methods that a di-ester derivative of the γ-carboxyl groups is formed, and although unexpected this can be rationalised mechanistically (3).

With a knowledge of the above reaction, one can apparently interpret the published mass difference of "acetylated permethylated" γ-carboxyglutamic acid of 201 mass units (1) as having arisen by esterification during acetylation, followed by an unsuccessful permethylation reaction. We find that the mass difference of acetylated permethylated γ-carboxyglutamic acid is 229 m.u. (2).

Clearly it was necessary to establish spectroscopically the occurrence of this di-ester formation in the synthetic amino-acid.

The mass spectrum of the acetylated synthetic material (see Experimental) is shown in Fig.1. The spectrum is identical with that of the natural product, and incorporation of the methanol is clearly seen from the multiplet (1:1, 1:2:1, 1:3:3:1) patterns in the spectrum. For an explanation of the implications of such patterns see (11).

A small quantity of the fully esterified product (tri-ester) was present in some but not all of the spectra taken,

whereas other signals were observed throughout vaporisation of the sample. The spectra show the major product to be the N-acetyl di-ester of γ-carboxyglutamic acid. The presence of a di-ester is evidenced by the 1:2:1 pattern of, for example, the fragment ion $M^{+\cdot}-CO_2H$ at m/e 216. The di-ester is inferred to be in the γ-position from our previous results on peptides, where a diester is also formed when no α-carboxyl group is present.

The reaction found to occur with the natural product is therefore also observed with the synthetic material, thus providing further chemical and spectroscopic evidence of identity of structure.

Acknowledgements

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