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SYNTHESIS OF DL-5,5'-DIHYDROXYLEUCINE THE REDUCTION PRODUCT OF γ CARBOXY GLUTAMIC ACID

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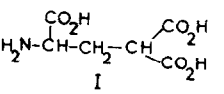
SUMMARY : The synthesis of DL-5,5'-dihydroxyleucine, by diborane reduction of N-phaloyl-DL- γ-carboxyglutamic acid- α-methylester, and the chromatographic and spectral characteristics of this amino acid are reported.

γ-carboxyglutamic acid, I, an essential component of the



I

N terminal part of prothrombin, is involved in the binding of calcium ions (1). This unusual amino acid has been identified in tryptic peptides derived from prothrombin by Stenflo (2) Nelsestuen (3) and Magnusson (4). Since its discovery and identification, several syntheses have been reported in the literature (5-10).

The research on γ-carboxyglutamic acid and its identification in enzymes and other proteins has been very extensive during the last four years and in addition to coagulation factors that are vitamin K dependent, factors VII, IX and X (1) (3), carboxyglutamic acid has been shown to occur in number of proteins : protein C (11-13) protein S (14) proteins from bones (15) proteins associated with tissue calcification (16-19) in fossil bones (20) and ribosomal proteins (21,22).

Carboxyglutamic acid is unstable and easily decarboxylated during extraction. This explains why only two methods have been used to identify this acid in proteins and tissues. The first con-

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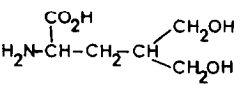
sists of alkaline hydrolysis followed by the chromatographic detection of γ-carboxyglutamic acid (23-24). In the second method, Nelsestuen et al avoided the decarboxylation problems by reducing the free acid groups of the protein with borane in tetrahydrofuran. γ-carboxyglutamic acid, if present, is reduced to 5,5'-dihydroxyleucine which can then be detected by amino acid analysis after acidic hydrolysis (25,26). Nelsestuen et al identified 5,5'-dihydroxyleucine, II, obtained after reduction of prothrombin peptides by derivatization followed by mass spectra analysis (25,26).



II

However no source of II has been described until now beyond the hydrolysis of reduced carboxyglutamic containing proteins.

Larger amounts of unequivocally identified samples of II would be useful to ascertain the chromatographic characteristics of this amino acid and to be used as reference material in the research and identification of new γ-carboxyglutamic containing proteins.

We report here the synthesis and the spectral characteristics of DL-5,5'-dihydroxyleucine, II.

MATERIAL AND METHODS

All chemicals were of the highest purity available. Borane-tetrahydrofuran (1M) and n-butyllithium in hexane were purchased from Aldrich. ^{1}H NMR spectra were recorded either at 60 MHz on a R 24 Perkin spectrometer or at 100 MHz on a HA 100 Varian spectrometer.

^{13}C NMR spectra were recorded at 20 MHz on a CFT 20 Varian spectrometer. In both cases, the chemical shifts are expressed in ppm with respect to tetramethylsilane as internal reference. Mass spectra were recorded on an AEI MS 30 mass spectrometer.

N-phthaloyl-L-serine, VII

Na_2CO_3 (10g) dissolved in H_2O (15ml) is added to L-serine (10g, 95mmoles) in H_2O (75ml). After addition of N-carbethoxyphthalimide (23g, 105mmoles) (27-28) the mixture is stirred at room temperature for 30 minutes. After filtration and extraction with methylene chloride, crystallization of the crude product in ether yields VII (10g, 45%, F = 152°C lit: 152°C (28)).

N-phthaloyl-L-Serine methylester, VIII

VII is esterified with diazomethane in ether F=115°C ^{1}H NMR ($CDCl_3$) : δ =7.9 (s,4H, aromatic protons), 5.02-5.3 (t,1H, -CH-), 4.25-4.35 (d,2H,-CH_2-O), 3.85 (s,3H,$COOCH_3$)

N-phthaloyl-L-serine methyl ester mesylate, IX

Mesyl chloride (0.5ml, 6.4mmoles) is added dropwise under argon to a cold solution (-20°C) of VIII(1.5g,5.8mmole) and triethylamine (0.9ml) in methylene chloride (20ml). After completion of the addition, the temperature is kept at -20°C for 90 minutes and then allowed to rise to 25°C in 90 minutes. After evaporation of the solvent under vacuum the crude product (1.97g) is purified by silicagel chromatography (80g, 70-230mesh, eluent : hexane/ethylacetate 1/1) and yields IX (1.32g, 78%, F=95-100°C). ^{1}H NMR ($CDCl_3$) : δ = 7.85 (s,4H, aromatic protons), 5.1-5.4 (t,1H,-CH-), 4.8-4.95 (d,2H,-CH_2-O-), 3.85 (s,3H,$COOCH_3$), 2.0 (s,3H,CH_3-S)

N-phthaloyl-dehydroalanine-methyl ester, X

IX (1.2g, 3.56mmole) and anhydrous triethylamine (0.9ml) are boiled in methylene chloride (20ml) for 20 minutes. Elimination of the solvent under vacuum and purification by silicagel column chromatography (40g, 70-230 mesh, eluent : hexane/ethylacetate 1/1) yields X (0.68g, 84%,F 108°). ^{1}H NMR ($CDCl_3$) : δ = 7.8 (d,4H,aromatic protons), 6.6 (s,1H, C=C$\lesssim^{H}_{H}$), 5.82 (s,1H, C=C$\lesssim^{H}_{H}$), 3.72(s,3H,$COOCH_3$)

N-phthaloyl-DL- γ-carboxyglutamic acid-γ,γ'- di-t-butyl-α-methyl ester, XI

1,55 N butyllithium in hexane (15.2ml) is added dropwise under argon to a cooled solution (-40°C) of di-t-butyl-malonate (6g, 28mmole) and tetra methylethylenediamine (3.9ml, 1.2eq) in anhydrous dimethoxyethane (70ml). After staying 40 minutes at -40°C, X (6.45g, 28mmoles) in dimethoxyethane (15ml) is added dropwise. After 4 hours at -40°C, the temperature is kept at -20°C overnight and then at 4°C for six days. After acidification with HCl and extraction with methylenechloride, the crude product is purified by silicagel column chromatography (330g,70-230 mesh, eluent : hexane/ethylacetate 8/2) and yields XI (10,41g, 83%, F=110°C crystallized from hexane/ethylacetate). ^{1}H NMR ($CDCl_3$): δ =7.8 (m,4H, aromatic protons), 4.97 (m,1H,-CH-N), 3.74 (s,3H, $COOCH_3$), 3.19 (m,1H,-CH-), 2.85-2.68 (m,2H,-CH_2-)1.47 (s,9H, t-Butyl), 1.37 (s,9H, t-Butyl)

N-phthaloyl-DL- γ-carboxyglutamic acid- α-methyl ester, XII

XI (1.22g, 2.7mmole) is treated at room temperature with a mixture of trifluoroacetic acid/H_2O (9/1, 6ml) for 140 minutes. The solvents are evapored under vacuum and the traces of trifluoroacetic acid and water are eliminated by distillation of heptane and benzene. XII (F=140°C) is obtained in almost quantitative yield.

N-phthaloyl-DL-5,5'-dihydroxyleucine-methylester, XIII

XII (2.7mmole) in anhydrous ethylacetate(52ml) is shared between 4 flasks cooled in an ice bath. Borane-tetrahydrofuran (1M, 55ml, 10eq/CO_2H) is added under argon. After 18 hours at room temperature, the excess of borane and the tetrahydrofuran are eliminated under vacuum. Methanol is evapored 3 times and the crude product is purified by preparative silica gel thin layer chromatography (eluent : chloroform/methanol 9/1) and yiels XIII (0.44g, 53%). ^{1}H NMR ($CDCl_3$) : δ=7.79 (m,4H, aromatic protons), 4.99 (m,1H,-CH-N) 3.79 (s,3H, $COOCH_3$), 3.79 (m,4H,CH_2OH), 2.28 (m,2H,-CH_2-) 1.66(m, 1H,CH (CH_2OH)$_2$). ^{13}CNMR ($CDCl_3$) : δ = 170.0 (-COOCH$_3$), 167.8 (N-

CO-), 134.4 ; 131.8 ; 123.7 (aromatic ring), 65.0 ; 63.13 (CH_2OH), 52.9 ($COO\underline{C}H_3$), 50.3 (CH-N) 39.2 ($\underline{C}H(CH_2OH)_2$), 27.2 ($CH-\underline{C}H_2-CH$). M.S. (70ev) 307 (M^+), 275, 259, 255, 248, 228, 219, 218, 200, 199, 190, 173, 160, 148, 105, 104.

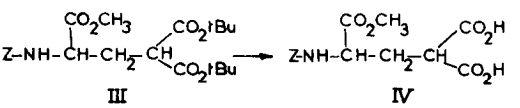
DL-5,5'- dihydroxyleucine, II

XIII (340mg, 1.1mmole) is saponified with potassium hydroxide (4N, 5.5ml) at 110°C under argon for 24 hours. After careful neutralization with perchloric acid (6N) the potassium perchlorate is eliminated by filtration and the crude product is purified on an AG1x2 column (hydroxide form). After washing with distilled water, the column is eluted with acetic acid (0.3N) and yields II (acetate form, 0.170g, 67%). ^{13}CNMR (D_2O, sodium salt) : δ = 181.7 (-COONa), 69.4 ; 69.15 (CH_2OH), 60.05 ($CH-NH_2$), 45.14 ($\underline{C}H(CH_2OH)_2$), 37.25 ($-CH_2-$).
The ^{13}CNMR spectra is recorded as sodium salt and not as acid to avoid any lactonization during the recording time.

RESULTS AND DISCUSSION

The strategy of this synthesis relies on the reductive properties of diborane which has been shown to reduce acid groups much faster than ester groups (29, 30).

Among the intermediates involved in the synthetic scheme of Schwyzer et al (8) leading to γ-carboxyglutamic acid, the triester III with two different sets of esters can be easily converted to the diacid ester IV by treatment with trifluoroacetic acid.



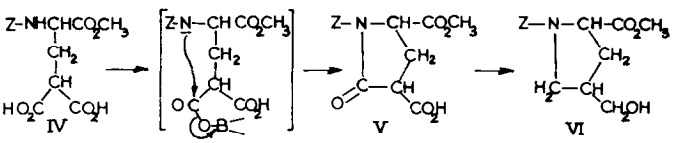
III IV

We assumed that reduction with diborane should occur selectively at the malonyl moiety and lead, after deprotection, to the desired dihydroxyleucine, II.

However no study of the reduction of malonic acid by diborane has yet been reported. We have observed, using model compounds that the reaction is slower and requires a large excess of diborane (31). The achievement of the synthesis of II (vide infra) shows that in spite of these rather drastic conditions, the selectivity remains and the carboxyl groups of a malonyl moiety can be reduced in the presence of an ester.

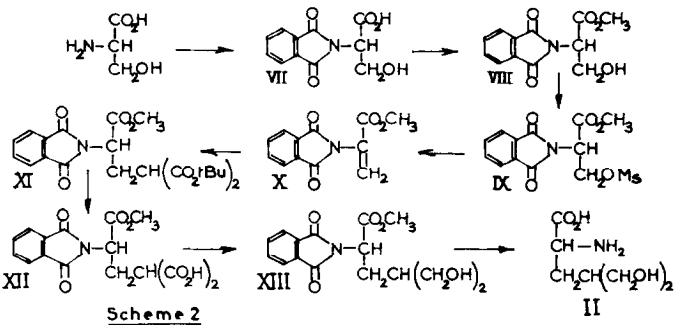
However reduction of III with borane-tetrahydrofuran under these conditions leads to mixtures of products among which we have isolated and identified (^{1}H NMR and mass spectra) a cyclized

product VI arising presumably according to the mechanism outlined in scheme 1+.



Scheme 1

We replaced the benzyloxycarbonyl protecting group by a phthalimido group which offers the advantage of blocking completely the nitrogen atom, thus preventing cyclization involving that position and we carried out the synthesis according to scheme 2



Scheme 2

We have carried out the last deblocking step by alkaline hydrolysis instead of hydrazinolysis which has always lead to complex mixtures of products arising presumably through the reaction on the ester group along with the opening of the phthalimido group.

The chromatographic behaviour of our synthetic sample is very similar to that described by Nelsestuen for the sample obtained from prothrombin (26) : paper chromatography by descending technique (R_f=0.1 eluent : Ethanol/ammonium hydroxide/water,90/5/5). Fig. 1 shows that DL-5,5'-dihydroxyleucine appears as a single peak located between aspartic acid and threonine on amino acid analyzer.

+This kind of cyclization has already been observed by Dolby during the reduction of an acid in the presence of an indolic moiety (32).

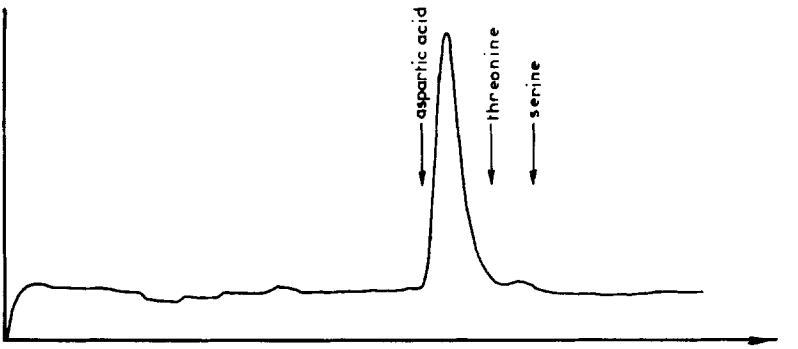


Figure 1 : Amino acid analysis of 5,5'-dihydroxyleucine recorded on an amino acid auto analyzer Technicon TSM1

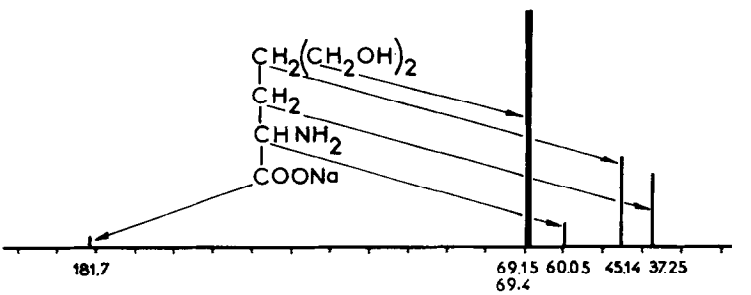


Figure 2 : ^{13}C NMR (^{1}H decoupled) spectra of DL-5,5'-dihydroxyleucine sodium salt recorded in a H_2O/D_2O mixture on a CFT 20 VARIAN spectrometer. Chemical shifts are expressed with tetramethylsilane as reference. The attributions have been made on the basis of the ^{1}H coupled spectrum.

The ^{13}C NMR spectra of DL-5,5'-dihydroxyleucine recorded as sodium salt in D_2O (Fig. 2) shows the magnetic non-equivalence of the two diastereotopic hydroxymethyl groups.
These results confirm the structure determination of Nelsestuen (12). The availability of synthetic DL-5,5'-dihydroxyleucine will help in the identification of this compound by automated amino acid analysis.

We acknowledge the skilful assistance of G. Desvages who recorded the amino acid analysis. We would like to thank L. Lacombe for recording the HA 100 ^{1}H NMR spectra and Dr G. Chassaing for recording and interpreting ^{13}CNMR spectra.

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