ENZYMATIC RESOLUTION OF γ-CARBOXY-DL-GLUTAMIC ACID*

Václav Čeřovský and Karel Jošt

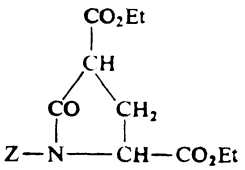
Institute of Organic Chemistry and Biochemistry,
Czechoslovak Academy of Sciences, 166 10 Prague 6

Received February 6th, 1984

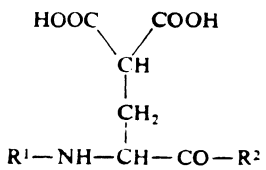
Optically active γ-carboxy-L-glutamic acid was prepared by enantioselective reaction of benzyloxycarbonyl-γ-carboxy-DL-glutamic acid with phenylhydrazine, catalyzed by papain (E.C. 3.4.22.2), and subsequent removal of the protecting groups from the obtained benzyloxycarbonyl-γ-carboxy-L-glutamic acid α-phenylhydrazide.

γ-Carboxyglutamic acid (Gla)** was found in the aminoterminal Ca^{2+}-bonding region of prothrombin and other vitamin K-dependent blood coagulation factors. Its presence was proved in bone proteins, renal stones and other tissues (for a review see ref.[2]).

Most of the syntheses of γ-carboxyglutamic acid are based on reaction of the fully protected serine with a dialkyl malonate[3-8] or on a Mannich-base condensation[9]. The racemic product was successfully resolved as the crystalline salt with quinine, ephedrine[10] or tyrosine hydrazide[11]. An attempted enzymatic resolution with kidney acylase failed[10]. Asymmetric syntheses of γ-carboxy-L-glutamic acid afford low yields and require many synthetic steps[12-14].



I



IIa, $R^1 = Z$, $R^2 = OH$ (DL)
IIb, $R^1 = H$, $R^2 = OH$ (DL)
IIc, $R^1 = Z$, $R^2 = C_6H_5N_2H_2$ (L)
IId, $R^1 = Z$, $R^2 = OH$ (L)
IIe, $R^1 = H$, $R^2 = OH$ (L)

* Part CLXXXVIII in the series Amino Acids and Peptides; Part CLXXXVII: This Journal *49*, 2557 (1984).

** Nomenclature and symbols of the amino acids and protecting groups obey the published recommendations[1]. Gla denotes a γ-carboxyglutamic acid moiety.

Our procedure represents the first successful enzymatic resolution of this acid. The synthesis started from N-benzyloxycarbonyl-O-*p*-toluenesulfonyl-DL-serine ethyl ester which on reaction with diethyl malonate afforded diethyl benzyloxycarbonyl--γ-carboxy-DL-pyroglutamate (*I*), similarly as described in ref.[4]. Alkaline hydrolysis of *I* gave benzyloxycarbonyl-γ-carboxy-DL-glutamic acid (*IIa*) which was converted into γ-carboxy-DL-glutamic acid (*IIb*) by catalytic hydrogenation. Reaction of the compound *IIa* with phenylhydrazine, catalyzed with papain, afforded benzyloxycarbonyl-γ-carboxy-L-glutamic acid α-phenylhydrazide (*IIc*) which after removal of the phenylhydrazide group with ferric chloride[15] was converted into benzyloxycarbonyl-γ-carboxy-L-glutamic acid (*IId*) and further into the desired γ-carboxy--L-glutamic acid (*IIe*) whose physico-chemical constants were identical with those reported in the literature.

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. Analytical samples were dried at room temperature at 150 Pa for 24 h. Thin-layer chromatography was carried out on silica gel (Silufol plates, Kavalier, Czechoslovakia) in the systems: 2-butanol–98% formic acid–water (75 : 13·5 : 11·5) (S1); 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5) (S2); 1-butanol–acetic acid–water (4 : 1 : 1) (S3); 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S4); benzene–ethanol (49 : 1) (S5); toluene–acetone (7 : 3) (S6); and toluene–acetone (20 : 1) (S7). Paper electrophoresis was performed in a moist chamber in 1M-acetic acid (pH 2·4) or in a pyridine–acetate buffer (pH 5·7) on a paper Whatman 3MM for 60 min at 20 V/cm. Spots were detected either with ninhydrin or by the chlorination method. HPLC was carried out on a 15 × 0·32 cm column filled with Separon SIX C-18 (Laboratorní přístroje, Prague); mobile phase methanol–trifluoroacetic acid (0·05%) (1 : 1), detection at 220 nm. Optical rotations were measured on a Perkin–Elmer 141 MCA polarimeter, amino acid analyses were done on an automatic analyzer, type 6020 (Developmental Workshops of Czechoslovak Academy of Sciences, Prague).

N-Benzyloxycarbonyl-O-*p*-toluenesulfonyl-DL-serine Ethyl Ester

p-Toluenesulfonyl chloride (14 g) was added at 0°C to a stirred solution of benzyloxycarbonyl-serine ethyl ester[16] (18 g) in pyridine (120 ml). The stirring was continued for 3·5 h at 0°C, the mixture was diluted with water, acidified with conc. hydrochloric acid and set aside for several days at 0°C. The separated crystals were filtered, dissolved in ethyl acetate, the solution was washed successively with 1M-HCl, water, 0·5M-$NaHCO_3$ and water, and dried. Evaporation of solvent and crystallization of the residue from ethyl acetate–light petroleum gave 14·5 g (51%) of the product, m.p. 62°C. R_F 0·76 (S6), 0·25 (S7). For $C_{20}H_{23}NO_7S$ (421·5) calculated: 56·99% C, 5·50% H, 3·32% N; found: 56·81% C, 5·46% H, 3·14% N.

Diethyl Benzyloxycarbonyl-γ-carboxy-DL-pyroglutamate (*I*)

Sodium hydride (2·5 g; 50% suspension in oil) was added under cooling (0°C) to a vigorously stirred solution of diethyl malonate (7 g) in benzene (80 ml). After reflux for 10 min and cooling, N-benzyloxycarbonyl-O-*p*-toluenesulfonyl-DL-serine diethyl ester (10 g) in benzene (160 ml) was added. The mixture was boiled for 8 h, neutralized with acetic acid (50%), washed successively

with water, 1M-HCl, water, 0·5M-$NaHCO_3$ and water, dried and taken down. The residue was triturated several times with light petroleum and left to crystallize in the cold under light petroleum. Filtration and crystallization from ether gave 5·5 g (57%) of the product, m.p. 63°C. R_F 0·80 (S4), 0·29 (S5). After hydrolysis with 6M-HCl for 20 h at 100°C the amino acid analysis detected only glutamic acid. For $C_{18}H_{21}NO_7$ (363·4) calculated: 59·49% C, 5·82% H, 3·85% N; found: 59·73% C, 5·79% H, 3·63% N. Mass spectrum: 363 (M^+).

N-Benzyloxycarbonyl-γ-carboxy-DL-glutamic Acid (*IIa*)

To a stirred solution of compound I (5 g) in methanol (100 ml) was added dropwise 2M-NaOH (100 ml). After standing at room temperature for 3 h, the solvent was evaporated and the cooled solution acidified with cold 2M-HCl. The product was taken up in ethyl acetate, the solution washed twice with water, dried and the solvent distilled off. Crystallization from ethyl acetate–light petroleum yielded 1·8 g (40%) of the title compound, m.p. 156°C. R_F 0·79 (S1), 0·76 (S3), 0·30 (S4); $k' = 1·16$. The analytical sample was recrystallized from ethyl acetate; m.p. 161°C. For $C_{14}H_{15}NO_8$ (325·3) calculated: 51·69% C, 4·65% H, 4·31% N; found: 51·67% C, 4·58% H, 4·58% N.

γ-Carboxy-DL-glutamic Acid (*IIb*)

The compound *IIa* (100 mg) was hydrogenated in 20% methanol (10 ml) over Pd-black. Crystallization from aqueous ethanol afforded 43 mg (73%) of the product, m.p. 167–168°C (reported[3,9] 156·5–157°C). $E^{gly}_{2.4}$ 0·11, $E^{glu}_{5.7}$ 1·50; R_F 0·16 (S1), 0·00 (S2), 0·15 (S3), 0·09 (S4). Elution time on the amino acid analyzer: 32–37 min (between cysteic and aspartic acid); colour yield for Gla is 24·8 (for Glu 56·3). For $C_6H_9NO_6$ (191·1) calculated: 37·71% C, 4·75% H, 7·33% N; found: 37·48% C, 4·70% H, 6·97% N.

N-Benzyloxycarbonyl-γ-carboxy-L-glutamic Acid α-Phenylhydrazide (*IIc*)

Phenylhydrazine (1 ml), followed by ethylenediaminotetraacetic acid (15 mg), was added to a solution of compound *IIa* (3·25 g) in 0·25M-NaOH (30 ml) and the mixture was made up to 40 ml with 1M-NaOH and water to pH 4·8–5·1. After addition of a solution of papain (0·5 g) and cysteine hydrochloride (150 mg) in water (10 ml), the resulting pH was 4·8 ± 0·1. The mixture was incubated at 38°C for 24 h, during which time a large amount of precipitate *a*) was formed. After acidification (pH ~ 3·5) with 0·2M-HCl, the precipitate was filtered, washed with 0·1M-HCl and water, suspended in ethyl acetate and washed with 0·1M-HCl and water *b*). Coloured impurities were removed by extraction of the product into 0·5M-$NaHCO_3$. The product was then liberated by acidification (with cooling) and taken up in ethyl acetate, the solution was washed with water, dried and the solvent evaporated. The residue was triturated with light petroleum and crystallized from ether, affording 0·80 g (39%) of the product, m.p. 135°C. R_F 0·89 (S1), 0·16 (S2), 0·79 (S3), 0·62 (S4); $k' = 3·58$; $[\alpha]_D$ −19° (*c* 0·3; methanol). For $C_{20}H_{21}N_3O_7$ (415·4) calculated: 57·83% C, 5·10% H, 10·12% N; found: 58·02% C, 5·07% H, 10·23% N.

A further portion of the product was obtained by ethyl acetate extraction of the combined filtrates from filtration of the precipitate *a*) and the aqueous phase from the washing *b*). The product was then taken up in 0·5M-$NaHCO_3$, liberated by acidification with 1M-HCl and extracted with ethyl acetate. The organic solution was washed many times with 0·2M McIlvain buffer, pH 3·5. Evaporation of the ethyl acetate and trituration of the residue with light petroleum gave 0·79 g of crude material, containing, according to HPLC, 67% of the desired product and 33% of the starting compound.

Benzyloxycarbonyl-γ-carboxy-L-glutamic Acid (*IId*)

A solution of ferric chloride (600 mg) in water (2 ml) was added dropwise at 35°C to a stirred solution of compound *IIc* (160 mg) in dioxane (4·5 ml). After stirring for 40 min the mixture was made alkaline with 2M-NaOH (6 ml) and the precipitated ferric hydroxide removed by centrifuging. The dioxane was evaporated, the remaining aqueous solution washed with ethyl acetate, acidified with cold 2M-HCl and the liberated material was taken up in ethyl acetate. After drying and evaporation of solvent, the product was crystallized from ethyl acetate–light petroleum; m.p. 138–139°C; yield 90 mg (72%). R_F 0·79 (S1), 0·76 (S3), 0·30 (S4); $k' = 1{\cdot}16$; $[\alpha]_D$ −6·6° (*c* 0·3; methanol). For $C_{14}H_{15}NO_8$ (325·3) calculated: 51·69% C, 4·65% H, 4·31% N; found: 51·55% C, 4·38% H, 4·51% N.

γ-Carboxy-L-glutamic Acid (*IIe*)

The compound *IId* (50 mg) was hydrogenated as described for compound *IIb*, affording 24 mg (82%) of the product, m.p. 165–167·5°C, other constants being the same as described for *IIb*; $[\alpha]_D$ +37° (*c* 1; 6M-HCl). For $C_6H_9NO_6$ (191·1) calculated: 37·71% C, 4·75% H, 7·33% N; found: 38·02% C, 4·77% H, 7·20% N. Reported[10] m.p. 167–167·5°C and $[\alpha]_D$ +35·3° (*c* 1; 6M-HCl).

We are indebted to Mrs H. Farkašová for the amino acid analyses and to Mrs Z. Ledvinová for measurement of optical rotations. Mass spectrum was taken and interpreted by Dr Trka. Elemental analyses were carried out in the Analytical Laboratory of our Institute (Dr J. Horáček, Head).

REFERENCES

1. *Biochemical Nomenclature and Related Documents.* International Union of Biochemistry, London 1978.
2. Burnier J. P., Borowski M., Furie B. C., Furie B.: Mol. Cell. Biochem. *39*, 191 (1981).
3. Bajusz S., Juhász A.: Acta Chim. (Budapest) *88*, 161 (1976).
4. Märki W., Schwyzer R.: Helv. Chim. Acta *58*, 1471 (1975).
5. Märki W., Schwyzer R.: Helv. Chim. Acta *59*, 1591 (1976).
6. Weinstein B., Watrin K. G., Loie H. J., Martin J. C.: J. Org. Chem. *41*, 3634 (1976).
7. Boggs N. T., Gawley R. E., Koehler K. A., Hiskey R. G.: J. Org. Chem. *40*, 2850 (1975).
8. Morris H. R., Thompson R., Dell A.: Biochem. Biophys. Res. Commun. *62*, 856 (1975).
9. Juhász A., Bajusz S.: Int. J. Peptide Protein Res. *15*, 154 (1980).
10. Märki W., Oppliger M., Thanei P., Schwyzer R.: Helv. Chim. Acta *60*, 798 (1977).
11. Boggs N. T., Goldsmith B., Gawley R. E., Koehler K. A., Hiskey R. G.: J. Org. Chem. *44*, 2262 (1979).
12. Oppliger M., Schwyzer R.: Helv. Chim. Acta *60*, 43 (1977).
13. Zee-Cheng R. K. Y., Olson R. E.: Biochem. Biophys. Res. Commun. *94*, 1128 (1980).
14. Danishefski S., Berman E., Clizbe L., Hirama M.: J. Amer. Chem. Soc. *101*, 4385 (1979).
15. Milne H. B., Halver J. E., Ho D. S., Mason M. S.: J. Amer. Chem. Soc. *79*, 637 (1957).
16. Hamada T., Yonemitsu O.: Chem. Pharm. Bull. *19*, 1444 (1971).

Translated by M. Tichý.