ENZYMATICALLY CATALYZED SYNTHESIS OF DIPEPTIDES OF γ-CARBOXY-L-GLUTAMIC ACID FROM BENZYLOXYCARBONYL-γ-CARBOXY-DL-GLUTAMIC-ACID*

Václav ČEŘOVSKÝ and Karel JOŠT

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

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Enantioselective reaction of benzyloxycarbonyl-γ-carboxy-DL-glutamic acid with phenylhydrazides of various amino acids and with leucine, protected with various carboxy-protecting groups, has been studied.

The use of proteolytic enzymes in the synthesis of peptide bond may only hardly compete with the very well elaborated chemical methods of organic chemistry[1,2], assuming that these methods are employed only to fulfill the requirements for which they have been elaborated (*i.e.* particularly the use of at least minimal protection, optically pure starting compounds and conditions excluding racemization). The preferential domain of the enzymatically catalyzed formation of peptide bond should lay beyond the limits of these requirements and involve *e.g.* synthesis without protecting groups or from racemic compounds.

In this paper we describe the papain-catalyzed synthesis of dipeptides with amino-terminal γ-carboxy-L-glutamic acid** starting from its DL-derivative, both the γ-carboxylic groups being unprotected. Several peptides containing γ-carboxyglutamic acid are described; their classical synthesis is based on the use of tert-butyl groups for protection of the γ-carboxyl groups[5–9]. In our enzymatic synthesis we made use of the thermodynamic approach (non-esterified carboxyl of the acylating component, relatively high concentration of the substrates and the enzyme, prolonged reaction time) and followed the effect of dimethylformamide in this experimental arrangement. We described already earlier[10] the resolution of γ-carboxy-DL-glutamic acid by the papain-catalyzed condensation with phenylhydrazine. In the present work we combined the resolution of γ-carboxy-DL-glutamic acid with the peptide

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** The nomenclature and symbols of the amino acids and peptides obey the published recommendation[3]; Gla denotes a γ-carboxyglutamic acid and Xaa an unspecified amino acid moiety (ref.[4]). Unless stated otherwise, the chiral amino acids have the L-configuration.

bond formation step, *i.e.* in the enzymatically catalyzed reaction the DL-form of the non-coded acid was employed. Although enzymatic resolutions of a non-coded amino acid are not rare, there are not many cases in which the resolution of the acylating amino acid was performed in one step together with the formation of the peptide bond[11,12].

In the case of reaction of benzyloxycarbonyl-γ-carboxy-DL-glutamic acid with leucine phenylhydrazide we determined the optimal pH of the reaction mixture, the optimal ratio of both reaction components (Table I) and the effect of enzyme concentration on the yield (Table II). Table III shows a very strong dependence of the yield on the amino acid in the P_1' position[13]. Whereas leucine and phenylalanine gave very good yields, the yields with other amino acids such as valine, alanine, asparagine and methionine, were relatively low (even if one keeps in mind that the given yield involves resolution of γ-carboxy-DL-glutamic acid and synthesis of the dipeptide with unprotected carboxy groups). Addition of dimethylformamide has no unequivocal effect, decreasing the yield in some cases and increasing it in others. An operation of two effects can be assumed: a positive effect due to the lowered ionizability of the carboxy and amino groups which participate in the formation of the peptide bond[14] and a negative effect caused by enhanced solubility of the product in the reaction mixture and its subsequent enzymatic hydrolysis.

TABLE I

Reaction of Z-DL-Gla with Leu-$N_2H_2C_6H_5$; the effect of concentration of the components and pH

Concentration[a] of Leu-$N_2H_2C_6H_5$ mol l^{-1}	pH	Yield[b], %
0·05	5·0	10
0·1	5·0	21
0·2	5·0	42
0·4[c]	5·0	40
0·2	4·1	38
0·2	4·6	45
0·2	5·0	42
0·2	5·7	17·5

[a] Reaction conditions: 0·1 mol l^{-1} Z-DL-Gla, 0·2 mmol l^{-1} papain, 24 h, 10% dimethylformamide, 38°C; [b] related to the amount of the L-component in the starting DL-Gla; [c] in the presence of 40% dimethylformamide.

TABLE II

Reaction of Z-DL-Gla with Leu-$N_2H_2C_6H_5$; the effect of enzyme concentration and reaction time

Papain concentration[a] (mmol l^{-1})	Yield[b], %	
	24 h	48 h
0·01	17	24
0·05	39	43
0·2	43	43

[a] Reaction conditions: 0·1 mol l^{-1} Z-DL-Gla, 0·2 mol l^{-1} Leu-$N_2H_2C_6H_5$, pH 4·8, 10% dimethylformamide, 38°C; [b] related to the amount of the L-component in the starting DL-Gla.

TABLE III

Reaction of Z-DL-Gla with carboxyl-protected amino acids (Xaa-Y)

Xaa-Y[a]	Dimethylformamide %	Yield[b], %	
		Z-Gla-Xaa-Y	Z-Gla-Yaa-OH
Leu-$N_2H_2C_6H_5$[c]	10	42	0
	20	47·5	0
	40	54	0
Phe-$N_2H_2C_6H_5$	40	33	0
Val-$N_2H_2C_6H_5$	10	11	0
	40	3	0
Ala-$N_2H_2C_6H_5$	10	12	0
	40	7	0
Asn-$N_2H_2C_6H_5$	10	9	0
	40	2·5	0
Met-$N_2H_2C_6H_5$	40	17	0
Leu-OMe	10	2·5	10
	40	17	15
Leu-OBu^t	10	18·5	0
	40	14·5	0
Leu-OBzl	10	0	13
	40	18	15
Leu-NH_2	10	2·5	19
	40	12	24

[a] Reaction conditions: 0·1 mol l^{-1} Z-DL-Gla, 0·2 mol l^{-1} Xaa-Y, 0·2 mmol l^{-1} papain, pH 4·8, 24 h; [b] yield related to the amount of the L-component in the starting DL-Gla; [c] pH 5·0.

The protecting group of the acylated amino acid has a substantial effect on the course of the desired reaction. Except the tert-butyl ester, all other derivatives (methyl ester, benzyl ester, amide) afforded a side product (in most cases in yield higher than the desired product) with free carboxyl of the carboxy-terminal amino acid, resulting from the enzymatic cleavage of the ester or amide of the already synthesized dipeptide. Besides the tert-butyl ester, also the phenylhydrazide was not cleaved, confirming thus the already mentioned[15,16] advantage of this protecting group in enzymatically catalyzed syntheses.

Two dipeptides were prepared on the preparative scale: benzyloxycarbonyl-γ-carboxyglutamyl-leucine phenylhydrazide (40% yield) and benzyloxycarbonyl-γ-carboxyglutamyl-phenylalanine phenylhydrazide (33% yield). The free dipeptides were obtained by treatment with ferric chloride[17] followed by catalytic hydrogenation. γ-Carboxy-D-glutamyl-leucine has been already prepared by another route[5].

EXPERIMENTAL

The melting points were determined on a Kofler block and are uncorrected. Solvents were evaporated on a rotatory evaporator at bath temperature 30°C. Thin-layer chromatography was carried out on Silufol (Kavalier) silica gel plates in the following 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). Electrophoresis was performed in moist chamber on a Whatman 3MM paper at 20 V/cm for 1 h in 1 mol l^{-1} acetic acid (pH 2·4) or in a pyridine–acetate buffer (pH 5·7), in the case of amino-protected compounds after the splitting off the protecting groups. The compounds were detected with ninhydrin or by the chlorination method. Samples for amino acid analysis were hydrolyzed in 6 mol l^{-1} HCl at 105°C for 20 h. The analyses were done on an automatic amino acid analyzer type 6020 (Developmental Workshops, Czechoslovak Academy of Sciences). Optical rotations were measured on a Perkin–Elmer 141 MCA polarimeter. High performance liquid chromatography (HPLC) was carried out on a Spectra Physics SP 8700 instrument equipped with an SP 8400 UV-detector and SP 4100 integrator, using 25 × 1·27 cm (flow rate 420 ml/h) and 15 × 0·35 cm (30 ml/h) columns filled with Separon Six C-18 (Laboratory Instruments, Prague); detection at 220 nm, mobile phase methanol with 0·05% trifluoroacetic acid (the amount of methanol in % for each k' value is given in parentheses).

Benzyloxycarbonyl-γ-carboxy-L-glutamyl-leucine Phenylhydrazide

A) *On analytical scale*: Benzyloxycarbonyl-γ-carboxy-DL-glutamic acid (32·5 mg) and leucine phenylhydrazide trifluoroacetate (17 mg; 34 mg; 68 mg; 136 mg) were dissolved in a mixture of dimethylformamide (100 μl, 200 μl, 400 μl) and 0·25 mol l^{-1} NaOH. After addition of ethylenediaminotetraacetic acid (0·3 mg) and cysteine hydrochloride (1 mg) the mixture was adjusted to the required pH value with 1 mol l^{-1} NaOH, so as the total volume was 1 ml. After addition of papain (4·2 mg), the mixture was incubated for 24 h at 38°C. The course of experiments with varying enzyme concentration (0·01, 0·05, 0·2 mmol l^{-1}) was followed by HPLC. The reactions were quenched by rapid cooling and addiditon of 0·5 mol l^{-1} HCl (1 ml). Extraction into constant volume of ethyl acetate, washing with the same volume of 0·5 mol l^{-1} HCl and water, and evaporation gave a residue containing varying amounts of the dipeptide and the starting compound

(Z-DL-Gla). The yields of the dipeptide were determined by HPLC (making a correction for different absorption at 220 nm of both the compounds).

B) *Preparative experiment*: Benzyloxycarbonyl-γ-carboxy-DL-glutamic acid (325 mg) and leucine phenylhydrazide trifluoroacetate (680 mg) were dissolved in dimethylformamide (4 ml) and 0·25 mol l^{-1} NaOH (5 ml). After addition of ethylenediaminotetraacetic acid (3 mg) and cysteine hydrochloride (10 mg), the mixture was adjusted to pH 4·8 with 1 mol l^{-1} NaOH and the volume was made up to 10 ml with water. Papain (42 mg) was added and the mixture was incubated for 24 h at 38°C. The reaction was quenched with 0·5 mol l^{-1} HCl and the product was extracted into ethyl acetate. The extract was washed with 0·5 mol l^{-1} HCl, water and taken down, leaving about 300 mg of a residue containing, in addition to the dipeptide, an appreciable amount of the starting compound. The pure dipeptide was obtained: *a*) by repeated washing of the ethyl acetate solution with 0·2 mol l^{-1} Mc Ilvaine buffer, pH 3·5, evaporation of the solvent and crystallization from ethyl acetate–light petroleum which gave 110 mg (40%) of a product containing (according to HPLC) 4% of the starting compound; m.p. 106°C. Amino acid analysis: Glu 1·00, Leu 0·97; *b*) by preparative HPLC which gave 67 mg (25%) of a product m.p. 114–115°C. R_F 0·82 (S1), 0·05 (S2), 0·78 (S3), 0·63 (S4); $k' = 5{\cdot}77$ (60% methanol); $E_{2.4}^{Gly}$ 0·38, $E_{5.7}^{Glu}$ 0·63. Amino acid analysis: Glu 1·00, Leu 1·04, $[\alpha]_D$ −29·7° (*c* 0·3, methanol). For $C_{26}H_{32}N_4O_8$ (528·6) calculated: 59·07% C, 6·10% H, 10·59% N; found: 58·69% C, 6·11% H, 10·39% N.

Reaction of Benzyloxycarbonyl-γ-carboxy-DL-glutamic Acid with Xaa-$N_2H_2C_6H_5$ (Xaa = Phe, Val, Ala, Met, Asn)

The syntheses were carried out under conditions optimal for the preparation of benzyloxycarbonyl-γ-carboxyglutamyl-leucine phenylhydrazide (0·1 mol l^{-1} Z-DL-Gla, 0·2 mol l^{-1} Xaa-$N_2H_2C_6H_5$, 0·2 mmol l^{-1} papain, pH 4·8, 24 h, 38°C). The reaction mixtures (total volume 1 ml) contained 10% or 40% of dimethylformamide, the processing and evaluation being the same as described above. The desired dipeptide Z-Gla-Xaa-$N_2H_2C_6H_5$ was separated from the starting compound (Z-DL-Gla) by HPLC (25 × 1·27 cm column), the eluate was freeze-dried and the residue (after checking for homogeneity by HPLC; determination of k'; 15 × 0·32 cm column) was characterized by amino acid analysis: Xaa = Phe (Glu 1·00, Phe 1·00), $k' = 6{\cdot}63$ (60% methanol); Xaa = Val (Glu 1·00, Val 0·95), $k' = 3{\cdot}59$ (60% methanol); Xaa = Ala (Glu 1·00, Ala 0·97), $k' = 4{\cdot}91$ (50% methanol); Xaa = Met (Glu 1·00, Met 0·91), $k' = 3{\cdot}70$ (60% methanol); Xaa = Asn (Glu 1·00, Asp 1·22), $k' = 3{\cdot}33$ (50% methanol).

Benzyloxycarbonyl-γ-carboxyglutamyl-phenylalanine Phenylhydrazide

A mixture (10 ml), containing benzyloxycarbonyl-γ-carboxy-DL-glutamic acid (325 mg), phenylalanine phenylhydrazide trifluoroacetate (740 mg), ethylenediaminotetraacetic acid (3 mg), cysteine hydrochloride (10 mg), papain (42 mg) and dimethylformamide (4 ml) was incubated and worked up in the same manner as described for the preparation of the leucine dipeptide (route *a*), affording 95 mg (33%) of a product, m.p. 210–212°C which contained 1% of the starting compound (HPLC). R_F 0·82 (S1), 0·05 (S2), 0·78 (S3), 0·63 (S4); $k' = 6{\cdot}63$ (60% methanol). Amino acid analysis: Glu 1·00, Phe 0·88; $[\alpha]_D$ −35·7° (*c* 0·3, methanol). For $C_{29}H_{30}N_4O_8 \cdot 0{\cdot}5\ H_2O$ (571·6) calculated: 60·94% C, 5·47% H, 9·80% N; found: 61·24% C, 5·29% H, 9·55% N.

Reaction of Benzyloxycarbonyl-γ-carboxy-DL-glutamic Acid with Leu-Y (Y = OMe, OBu^t, OBzl, NH_2)

The quantities, reaction conditions and the work-up procedure were the same as described for the preparation of Z-Gla-Xaa-$N_2H_2C_6H_5$. In experiments where Y = OMe, OBzl, NH_2, the HPLC

analysis showed, in addition to the peak of the starting compound (Z-DL-Gla), two other peaks due to the desired product Z-Gla-Leu-Y and to the side product Z-Gla-Leu-OH which were isolated by HPLC and characterized by the amino acid analysis: Y = OMe (Glu 1·00, Leu 1·21), k' = 4·87 (60% methanol); Y = OBzl (Glu 1·00, Leu 0·98), k' = 16·7 (60% methanol); Y = NH_2 (Glu 1·00, Leu 0·96), k' = 2·21 (60% methanol), $E_{5.7}^{Glu}$ 0·85; Y = OH (Glu 1·00, Leu 1·00 ± 0·1), k' = 3·17 (60% methanol), $E_{5.7}^{Glu}$ 1·19. When Y = OBu^t, only peak of k' = 13·6, corresponding to Z-Gla-Leu-OBu^t, was obtained in addition to that of the starting compound. Amino acid analysis: Glu 1·00, Leu 1·06.

Benzyloxycarbonyl-γ-carboxyglutamyl-leucine

A solution of ferric chloride (150 mg) in water (0·5 ml) was added at 35°C to a stirred solution of the protected dipeptide phenylhydrazide (40 mg) in dioxane (1·5 ml). After 40 min the mixture was made alkaline with 2 mol l^{-1} NaOH (2 ml) and the precipitated ferric hydroxide was separated by centrifugation. The supernatant was taken down, the residue washed with ethyl acetate, acidified with cold 1 mol l^{-1} HCl and the product was taken up in ethyl acetate. After washing with water and drying over sodium sulfate, the solvent was evaporated and the residue triturated with light petroleum, affording 25 mg (75%) of the product, m.p. 85–87°C; R_F 0·82 (S1), 0·01 (S2), 0·78 (S3), 0·53 (S4), k' = 3·17; $[\alpha]_D$ −23·2° (*c* 0·15, methanol). For $C_{20}H_{26}N_3O_9.0{\cdot}5\ H_2O$ (447·5) calculated: 53·68% C, 6·08% H, 6·27% N; found: 53·66% C, 5·71% H, 5·88% N.

γ-Carboxyglutamyl-leucine

The benzyloxycarbonyl dipeptide from the preceding preparation (24 mg) was hydrogenated in 50% methanol (2·5 ml) over Pd-black for 2 h, the reaction being monitored by HPLC. After removal of the catalyst by filtration, methanol was evaporated and the residue triturated with ether, affording 12 mg (72%) of the dipeptide, m.p. 134°C. R_F 0·52 (S1), 0·00 (S2), 0·34 (S3), 0·23 (S4); k' = 0·64 (50% methanol), $E_{2.4}^{Gly}$ 0·12, $E_{5.7}^{Glu}$ 1·18; $[\alpha]_D$ +21·3° (*c* 0·15, 1 mol l^{-1} HCl). Amino acid analysis: Glu 1·00, Leu 1·03. For $C_{12}H_{20}N_2O_7.0{\cdot}5\ H_2O$ (313·3) calculated: 46·00% C, 6·76% H, 8·94% N; found: 45·72% C, 6·41% H, 8·66% N.

Benzyloxycarbonyl-γ-carboxyglutamyl-phenylalanine

This compound (87 mg; 77%), m.p. 89–90°C, was obtained from the protected dipeptide (135 mg). R_F 0·81 (S1), 0·00 (S2), 0·78 (S3), 0·52 (S4); k' = 3·33 (60% methanol); $[\alpha]_D$ +4·4° (*c* 0·2, methanol). For $C_{23}H_{24}N_2O_9.0{\cdot}5\ H_2O$ (481·5) calculated: 57·37% C, 5·23% H, 5·82% N; found: 57·58% C, 5·34% H, 5·61% N.

γ-Carboxyglutamyl-phenylalanine

Hydrogenation of the benzyloxycarbonyl dipeptide (14 mg) over Pd-black afforded 8 mg (80%) of the product, m.p. 137°C; R_F 0·48 (S1), 0·00 (S2), 0·28 (S3), 0·16 (S4); k' = 0·76 (50% methanol); $E_{2.4}^{Gly}$ 0·13, $E_{5.7}^{Glu}$ 1·18, $[\alpha]_D$ +24·7° (*c* 0·15, 1 mol l^{-1} HCl). Amino acid analysis: Glu 1·00, Phe 0·97. For $C_{15}H_{18}N_2O_7.H_2O$ (356·3) calculated: 50·56% C, 5·66% H, 7·86% N; found: 50·24% C, 5·41% H, 7·49% N.

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