Preparation of γ-Glutamyl Dipeptides of Sulphur-containing Amino-acids

By **John F. Carson** * and **Francis F. Wong**, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710, U.S.A.

γ-L-Glutamyl-L-methionine, -*S*-methyl-, *S*-propyl-, -*S*-allyl-, and -*S*-benzyl-L-cysteine were prepared by condensation of α-ethyl *N*-trifluoroacetyl-L-glutamate dicyclohexylammonium salt with the corresponding amino-acid ethyl ester hydrochloride followed by alkaline hydrolysis of the intermediate *N*-trifluoroacetyl dipeptide diethyl esters. The peptides containing *S*-substituted cysteine were difficult to crystallize but were more easily obtained as the crystalline ammonium salts.

γ-L-GLUTAMYL peptides of sulphur-containing amino-acids are found in a number of plants; *e.g.* γ-glutamyl-*S*-methylcysteine is an important constituent of the non-protein fraction of kidney beans,[1] *Phaseolus vulgaris*, and of lima beans,[2] *Phaseolus lunatus*. From plants of the *Allium* genus, 21 different γ-glutamyl peptides have been characterized, 17 of which contain derivatives of cysteine of methionine.[3] This work describes the synthesis of the γ-L-glutamyl dipeptides of L-methionine and of *S*-methyl-, *S*-propyl-, *S*-allyl-, and *S*-benzyl-L-cysteine.

L-Glutamic acid was protected at the amino- and the α-carboxy-functions as the dicyclohexylammonium (DCHA) salt of α-ethyl *N*-trifluoroacetyl(TFA)-L-glutamate. For the second component the appropriate amino-acid ethyl ester was employed. DCHA α-ethyl *N*-TFA-glutamic acid was first described by Weygand and Geiger [4] in a synthesis of glutathione. Jöhl and Stoll [5] later used this derivative in a synthesis of γ-L-glutamyl hypoglycin A.

DCHA α-ethyl *N*-TFA-L-glutamate was coupled with amino-acid ethyl ester hydrochlorides in the presence of precipitated dicyclohexylammonium chloride. Peptide formation was accomplished by two procedures: (1) the application of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) [6] and (2) the combination of dicyclohexylcarbodi-imide (DCCI) and *N*-hydroxysuccinimide [7] (HOSUC). The *N*-TFA-peptide diethyl esters were obtained as crystalline solids in yields of 61—85% (Table 1). Since peptide formation generally did not go to completion, it was necessary to remove unchanged starting materials from the product with aqueous acid and a weak base to avoid contamination of the peptides, particularly with glutamic acid.

Trifluoroacetyl and ethyl ester groups were removed in one step from intermediates by hydrolysis with sodium

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[6] B. Belleau and G. Malek, *J. Amer. Chem. Soc.*, 1968, **90**, 1652.
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hydroxide in 50% aqueous ethanol or aqueous dioxan.* The pure peptides, free of salts (Table 2), were then obtained by passing the hydrolysis mixtures through a strong anion exchange (Dowex-1) in the acetate form, followed by elution with aqueous acetic acid. Alkaline hydrolysis of the *S*-substituted cysteine intermediates in many cases gave low yields of peptide, presumably due to β-elimination of thiol. Although the fully protected intermediate containing *S*-methylcysteine gave yields of 80—90% of pure peptide on hydrolysis, with the *S*-propyl derivative yields were generally 60%, with the *S*-benzyl derivative 50%, and with the corresponding allyl compound the yield on hydrolysis was only 40%. No problem was encountered in hydrolysis of the methionine intermediate. Hydrolysis under milder basic conditions as with ammonium hydroxide or sodium carbonate gave even poorer yields and it was generally advisable to add all the base in one portion rather than in small quantities. Sensitivity to base of *N*-acyl-substituted cysteine is greatly accentuated when the carboxy-group is esterified and the isolated peptides are far less prone to β-elimination than the peptide esters.

None of the isolated peptides showed the presence of sulphoxides † either when first obtained or on storage under refrigeration, but special precautions were observed to minimize contact with air during the preparation. Although γ-glutamylmethionine was easily crystallized as long needles from aqueous ethanol, the other peptides could be crystallized only with difficulty. From aqueous ethanol or aqueous acetone solutions, the peptides usually precipitated as gels which on long standing in the cold were occasionally converted into crystalline products as tiny needles. These peptides could be crystallized much more readily as the mono-ammonium salts from 80—90% ethanol as needles. This was accomplished by passing the alkaline hydrolysis mixture through a strong cation exchanger such as Dowex 50 (H^+) and elution with ammonium hydroxide followed by concentration and crystallization from aqueous ethanol. In an alternative method, the pure peptides isolated as previously described were evaporated with ammonium hydroxide.

In spite of the unsatisfactory yields frequently observed in the alkaline hydrolysis step, DCHA α-ethyl *N*-TFA-L-glutamate is a useful intermediate for the preparation of these dipeptides because of the ease of preparation of the starting materials and the small number of steps required in the synthesis. The two coupling procedures, EEDQ and DCCI + HOSUC, appeared to give comparable yields.

TABLE 1

N-TFA-γ-L-Glutamyl peptide diethyl esters

Second amino-acid component	Coupling method	Yield (%)	$[\alpha]_D^{25}$ ethanol	M.p. (°)	Analysis: Found (%) C	H	N	Calculated (%) C	H	N
L-Methionine	EEDQ [a]	77	−37·6	114·5—115	44·7	5·85	6·6	44·65	5·85	6·5
S-Methyl-L-cysteine	EEDQ	61	−45·4	111—112	43·7	5·6	6·85	43·25	5·55	6·75
	DCCI + HOSUC [b]	65								
S-Propyl-L-cysteine	EEDQ	70	−42·2	81—82	46·2	6·2	6·45	45·95	6·1	6·3
	DDCI + HOSUC	77								
S-Allyl-L-cysteine	EEDQ	70	−47·9	107—108	46·2	5·5	6·35	46·15	5·7	6·35
	DCCI + HOSUC	63								
S-Benzyl-L-cysteine	EEDQ	83	−67·2	108·5—110	51·5	5·6	5·75	51·2	5·55	5·7
	DCCI + HOSUC	85								

[a] EEDQ = 1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. [b] DCCI + HOSUC = Dicyclohexylcarbodi-imide + *N*-hydroxysuccinimide.

TABLE 2

γ-Glutamyl dipeptides prepared from *N*-TFA-γ-glutamyl peptide diethyl esters

Second amino-acid component	Yield (%) [a]	$[\alpha]_D^{25}$(°) Water	$[\alpha]_D^{25}$ (°) NH_4^+ salt	R_E(dipeptide)/R_E(alanine) BuOH–AcOH–H_2O 63 : 10 : 27	Collidine–lutidine (1 : 3)	Analysis of peptides: Found (%) C	H	N	Calculated (%) C	H	N
L-Methionine	69	−9·2	−3·7	1·95	1·30	43·1	6·5	10·1	43·15	6·5	10·05
S-Methylcysteine	51	−17·9 [b]	−19·2	1·50	1·09	40·5	6·3	10·5	40·9	6·1	10·6
S-Propylcysteine	46	−12·0	−14·0	3·27	1·41	45·1	6·85	9·6	45·2	6·9	9·6
S-Allylcysteine	28	−17·0 [c]	−18·5	1·96	1·31	45·0	6·3	9·7	45·5	6·25	9·65
S-Benzylcysteine	42	−14·1	−11·7	3·21	1·72	52·7	5·8	8·2	52·95	5·9	8·25
		−30·4 [d]									

[a] Yields based on DCHA α-ethyl *N*-TFA-L-glutamate and include coupling and hydrolysis reactions. [b] Zacharius *et al.*[1] reported $[\alpha]_D^{23}$ −19·6° for their peptide synthesized from glutathione. [c] A. I. Virtanen and E. J. Matikkala, *Suomen Kem.*, 1961, *B* **34**, 44, report $[\alpha]_D^{23}$ −17·1° (water) for this peptide isolated from garlic. [d] $[\alpha]_D$ in 50% ethanol.

* It has been reported that hydrolysis of *N*-acyl-*S*-alkyl-L-cysteine methyl esters with sodium hydroxide in absolute methanol resulted in nearly complete racemization but that no racemization occurred in 50% aqueous methanol or aqueous dioxan (L. Zervas, I. Photaki, A. Cosmatos, and N. Ghelis, in 'Peptides, Proceedings of 5th European Symposium, Oxford, September 1962,' ed. G. T. Young, Macmillan, New York, 1963, p. 30).

† Zacharius *et al.*[1] observed the formation of sulphoxides during chromatography and after prolonged storage of γ-glutamyl-*S*-methylcysteine.

EXPERIMENTAL

General.—L-Glutamic acid and L-methionine were obtained from California Biochemical Corp.,* and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ; 99%), *N*-hydroxysuccinimide, and dicyclohexylcarbodi-imide were obtained from Aldrich Chemical Co. *S*-Substituted cysteines were prepared from L-cysteine hydrochloride by the procedure of Theodoropoulos.[8] Paper chromatography was performed with Whatman No. 1 paper with two solvent systems, (I) BuOH–AcOH–H_2O (63 : 10 : 27) and (II) collidine–lutidine (1 : 3) saturated with water. Specific rotations were measured in CO_2-free water or in ethanol in concentrations of 2·5—3·5%, path length 0·2 dm except for the *S*-benzylcysteine peptide where the concentration in water was 1·2%.

Amino-acid ethyl ester hydrochlorides were prepared according to the procedure of Brenner and Huber [9] and were recrystallized from ethanol–ethyl acetate or ethanol–ether.

α-Ethyl *N*-trifluoroacetyl-L-glutamate dicyclohexylammonium salt (DCHA α-ethyl *N*-TFA-L-glutamate) was prepared in yields of 45—50% by the procedure of Weygand and Geiger.[4]

Preparation of Peptides.—Experimental details are presented for typical examples of the methods used. Reactions and filtrations were performed under argon whenever possible and water and acetic acid used with ion exchangers were oxygen-free to minimize sulphoxide formation. Analytical and physical data are recorded in Tables 1 and 2.

N-TFA-*γ*-L-*Glutamyl*-S-*methyl*-L-*cysteine diethyl ester* (DCCI + HOSUC *method*). A mixture of DCHA α-ethyl *N*-TFA-L-glutamate (8·55 g, 18·9 mmol) and *S*-methyl-L-cysteine ethyl ester hydrochloride (4·20 g, 21 mmol) in methylene chloride (300 ml) was cooled to 0°. To this mixture was added DCCI (4·77 g, 23·1 mmol) and *N*-hydroxysuccinimide (2·17 g, 18·9 mmol) and the mixture was stirred at 0° for 6 h, stored in the refrigerator for 40 h, and finally stirred at room temperature for 18 h. Excess of DCCI was destroyed by the addition of 1 ml of acetic acid. After 2 h, dicyclohexylurea and dicyclohexylammonium chloride were removed by filtration and the solution was concentrated *in vacuo* to an oil. Ethyl acetate (350 ml) was added and after filtration from a small quantity of solids, the cold ethyl acetate solution was extracted successively with cold 0·5-N-hydrochloric acid (2 × 50 ml), cold saturated brine (50 ml), cold 3% sodium hydrogen carbonate (3 × 50 ml), and again with 50 ml of brine. The organic layer was dried (Na_2SO_4) and concentrated *in vacuo* to a solid crystalline product (5·1 g, 65%). For analysis, the compound was recrystallized from ether (30 ml) at −20° to give soft fine needles (2·4 g). For hydrolysis the original product was used without recrystallization.

γ-L-*Glutamyl*-S-*methyl*-L-*cysteine*. A solution of the *N*-TFA peptide ester described above (5·26 g, 12·6 mmol) in dioxan–water (1 : 1; 200 ml) was cooled to 15° and N-sodium hydroxide (38 ml) was added. The turbid solution was stirred for 1 h at 10—15°. The solution was acidified with acetic acid (2 ml), filtered from traces of resinous material, and passed through a column of 130 cm³ of Dowex-1-acetate. The resin was washed with water (400 ml) and the compound was eluted with N-acetic acid (2 l). Concentration of the eluates *in vacuo* yielded the peptide (2·66 g, 80%) as an amorphous product which was chromatographically homogeneous. For analysis, the compound was crystallized from ethanol–water (10 : 1) or acetone–water (8 : 1) as fine needles or a gelatinous precipitate. A sample of the peptide (0·8 g) in a solution of water (3 ml) and acetone (24 ml) yielded, after long standing at 0°, crystalline *γ*-glutamyl-*S*-methyl-L-cysteine (0·67 g) as tiny needles.

N-TFA-*γ*-L-*Glutamyl*-S-*propyl*-L-*cysteine diethyl ester* (EEDQ *method*). A mixture of DCHA α-ethyl *N*-TFA-L-glutamate (8·55 g, 18·9 mmol), *S*-propyl-L-cysteine ethyl ester hydrochloride (4·78 g, 21·0 mmol), and of EEDQ (99%; 5·19 g, 21 mmol) in a mixture of tetrahydrofuran (200 ml) and methylene chloride (300 ml) was stirred for 45 h at room temperature and filtered from the precipitated dicyclohexylammonium chloride. The filtrate was concentrated *in vacuo* to a thick oil, and this was dissolved in ethyl acetate (325 ml) and extracted with acid and sodium hydrogen carbonate as previously described. Removal of solvent *in vacuo* yielded crystalline product (5·24 g). Additional product (0·7 g) was obtained by re-extracting the partially emulsified acid and hydrogen carbonate washes with fresh ethyl acetate (200 ml) (combined yield 71%). For analysis, the product (5·94 g) was recrystallized from ether (25 ml) at −20° to give fine needles (2·9 g).

γ-L-*Gutamyl*-S-*propyl*-L-*cysteine*. To a solution of the *N*-TFA-peptide diethyl ester (3·93 g, 8·8 mmol) in 50% dioxan (200 ml) at 10°, N-sodium hydroxide (32 ml) was added and the turbid solution was stirred 1 h at 10—15°. The solution was poured into ice–water (100 ml) containing acetic acid (3 ml) and the turbid suspension (thiol odour) was filtered and passed through 100 cm³ of Dowex-1-acetate. After thorough washing with water, the compound was eluted from the resin with N-acetic acid (1·3 l). Removal of solvent *in vacuo* yielded amorphous solid (1·54 g) that was homogeneous to paper chromatography. A sample (0·8 g) was crystallized from water (1 ml) and ethanol (13 ml) to give tiny needles (0·6 g).

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* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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