Synthesis of Hapten–Polypeptide Conjugates as Antigen Models for the *N*-Terminal Region of the α-2-Chain of Rabbit Skin Collagen

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Synthesis of derivatives of the peptide sequence L-pyroglutamyl-L-phenylalanyl-L-aspartyl-glycyl-L-lysyl-glycyl-glycyl-glycine as the antigenic determinant representing the *N*-terminal non-helical region of the α-2-chain of rabbit skin collagen, and conjugation to two different polypeptide carriers, are described.

THE use of synthetic peptides for elucidation of the immunologenicity and antigenicity of naturally occurring peptides and proteins has been the subject of extensive studies.[1] Recently a non-helical peptide corresponding to the α-2-chain of rabbit skin collagen has been isolated and characterized.[2]

The present paper describes (1) the synthesis of the partially protected octapeptide □Glu-Phe-Asp(OBu^t)-Gly-Lys(Boc)-Gly-Gly-Gly-OH,† representing the *N*-terminal non-helical region of the α-2-chain of rabbit skin collagen, as the hapten (antigenic determinant), and (2) the conjugation of this octapeptide derivative to two different polypeptide carriers for immunological studies.‡

Synthesis of the Hapten.—The synthetic route is illustrated in the Scheme. The toluene-*p*-sulphonate of triglycine benzyl ester (5),[3] prepared according to Fischer[4,5] and Zervas,[6] was coupled to the lysine derivative (4)[7] by the mixed anhydride method to give the fully protected tetrapeptide (8) in high yield. Selective removal of the Bpoc group of (8) was performed with pyridine–hydrobromide in methanol[8] to yield the partially protected tetrapeptide (10). The amino-acid derivatives (1)[9] and (2)[6] were coupled *via* the mixed anhydride method to yield the fully protected dipeptide (3). Catalytic hydrogenation of (3) gave the dipeptide (7), which was coupled with the *N*-hydroxysuccinimido ester (6)[10] to yield the tripeptide (9). Condensation of (9) with (10) by the method of König and Geiger[11] afforded the fully protected heptapeptide (12). The heptapeptide (12) was hydrogenated in trifluoroethanol. Acylation of (14) with 2,4,5-trichlorophenyl pyroglutamate (13)[12] afforded the desired partially protected octapeptide (15), which was sparingly soluble in organic solvents and water.

The isolated product (15) showed a negative reaction to ninhydrin and was chromatographically homogeneous. It was deprotected to give (16). The electrophoretograms of the octapeptide derivatives (15) and (16) showed a single component. Amino-acid compositions agreed with theoretical values.

Conjugation.—Two polypeptides (Table) were utilized as carriers for conjugation attempts. To this end the

† Optically active amino-acids mentioned are all of the L-configuration.

‡ The immunological results will be published separately.

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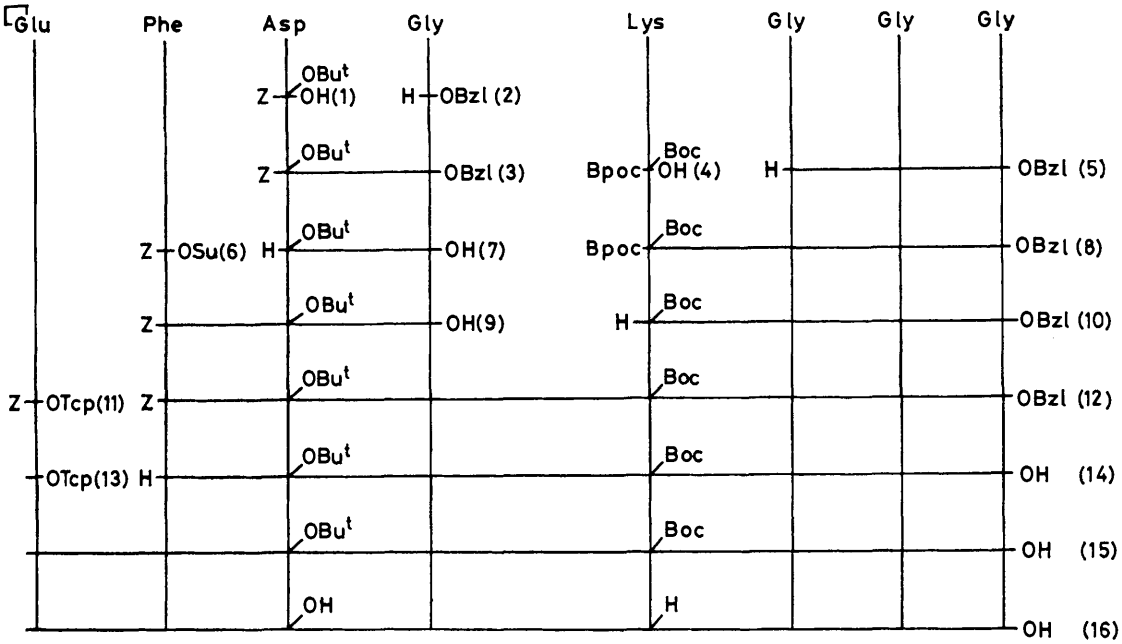
free *C*-terminal carboxy-group of partially protected octapeptide (15) was condensed to the free amino-groups of the carrier polypeptide. Owing to the poor solubility of the polymers, the conjugation reactions were attempted in suspension. The conjugate mixture was deprotected with trifluoroacetic acid, and dialysed against dilute acetic acid for removal of unconjugated octapeptide.



SCHEME OSu = *N*-hydroxysuccinimido ester; OBzl = benzyl ester; OTcp = 2,4,5-trichlorophenyl ester; Z = benzyloxycarbonyl; Boc = t-butoxycarbonyl; Bpoc = 1-*p*-biphenylyl-1-methylethoxycarbonyl

Carrier polypeptides and conjugates

Carrier	Multichain (ε-poly-DL-Ala-L-Lys)(Ala : Lys 9—10 : 1)	Copoly-L-Tyr-L-Lys-(Tyr : Lys 1 : 1)
Unit mol. weight	*ca.* 700	373
Mol. weight	50 000—10 0000	41 000 (est. from ultra-centrifugation)
Structure of carrier	H—[Lys(Ala$_n$H)]$_m$—OH	H—[Tyr(OH)—Lys($H_2$$^+Br^-$)]$_m$—OH
	$\bar{n}$ = 9—10	
Structure of conjugate	H—[Lys(Ala$_n$H)]$_m$—OH (hapten)$_1$	H—[Tyr(OH)—Lys]$_m$—OH (hapten)$_1$
	1/*m* = 0.15—0.20	1/*m* = 0.60—0.70

In the case of the multichain-Ala-Lys, conjugation with the hapten was established *via* the active ester method with an excess of 1-hydroxybenzotriazole and *NN'*-dicyclohexylcarbodi-imide;[11] whereas conjugation of the copolymer-Tyr-Lys was performed *via* the mixed anhydride method.

Amino-acid ratios of conjugates, that is, the average ratio of haptenic group to unit carrier, were obtained by amino-acid analysis.

The results of conjugation reactions are given in the Table.

EXPERIMENTAL

Thin-layer chromatograms (Merck Kieselgel 60) were performed in the following systems: CMA, chloroform–acetic acid–methanol (95 : 3 : 5); SBA, butan-2-ol–formic acid–water (75 : 13.5 : 11.5); SBN, butan-2-ol–10% ammonia (85 : 15). Amino-acid ratios were determined by hydrolysis of peptide in redistilled 6N-hydrochloric acid for 48 h at 110 °C *in vacuo*, and analysis was carried out on an LKB Biochrom Amino-acid Analyser. Tyrosine-containing peptides were hydrolysed with 6N-hydrochloric acid containing 1% phenol. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. The m.p.s were measured on a Bock Monoscop instrument.

Triton B (40% solution of benzyltrimethylammonium hydroxide in methanol) was purchased from Merck. Carrier polypeptides were obtained from Miles-Yeda Ltd. (Israel), lot and code: Allyl-17-71-181A and Lyty-5-71-160.

N$^{\alpha}$-(1-p-*Biphenylyl-1-methylethoxycarbonyl*)-N$^{\epsilon}$-*t-butoxycarbonyl-L-lysylglycylglycine Benzyl Ester* (8).—*N*$^{\alpha}$-1-*p*-Biphenylyl-1-methylethoxycarbonyl-N$^{\epsilon}$-t-butoxycarbonyl-lysine (4) [7] (2.42 g, 5.0 mmol), prepared *via* hydrogenation of *N*$^{\alpha}$-benzyloxycarbonyl-*N*$^{\epsilon}$-t-butyloxycarbonyl-L-lysine [13] and purified as a dicyclohexylammonium salt, was dissolved in dimethylformamide. *N*-Methylmorpholine (0.55 ml) and isobutyl chloroformate (0.65 ml) were added with stirring at −15 °C. After 3 min the solution of the amino-component, derived from the triglycine derivative (5) (2.26 g, 5.0 mmol), *N*-methylmorpholine (0.55 ml), and

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dimethylformamide (30 ml) and cooled to −15 °C, was added with stirring. The mixture was stirred for 1 h at −15 °C and then for 2 h at room temperature. The solvent was evaporated off under reduced pressure. A solution of the residue in ethyl acetate was washed successively with buffer solution * (pH 3) and water, dried (Na_2SO_4), stirred with basic Al_2O_3 for 2 h, filtered, and evaporated. Crystallization of the residue from aqueous methanol gave chromatographically homogeneous *product* (8) (3.04 g, 81.5%), m.p. 56—57°, $[\alpha]_D$ −5.0° (*c* 1.0 in ethyl acetate), CMA-R_F 0.72; SBA-R_F 0.81; *Gly 3.00*, Lys 0.94 (Found: C, 64.5; H, 7.1; N, 9.6. $C_{40}H_{51}N_5O_9$ requires C, 64.4; H, 6.9; N, 9.4%).

N-*Benzyloxycarbonyl*-β-*t*-*butoxy*-L-*aspartylglycine Benzyl Ester* (3).—To a solution of the aspartic acid derivative (1) [9] (4.58 g, 14.2 mmol) in dimethylformamide (30 ml) *N*-methylmorpholine (1.6 ml) and isobutyl chloroformate (1.85 ml) were added with stirring at −15 °C. After 5 min the solution was combined with a solution of the glycine derivative (2) [6] (4.80 g, 14.2 mmol) in dimethylformamide (30 ml) prepared by addition of *N*-methylmorpholine (1.6 ml) at −15 °C. The mixture was stirred for 1 h at −15 °C and for 1 h at room temperature, then evaporated. A solution of the residue in ethyl acetate was washed with buffer solution (pH 2), saturated $NaHCO_3$ solution, and water until neutral and then dried (Na_2SO_4). After removal of the solvent and then decantation from cooled n-hexane, the jelly-like *product* (3) was obtained (6.57 g, 98%), CMA-R_F 0.94, $[\alpha]_D$ −5.9° (*c* 1.32 in methanol) (Found: C, 63.75; H, 6.3; N, 6.1. $C_{25}H_{30}N_2O_7$ requires C, 63.8; H, 6.4; N, 5.95%).

N-*Benzyloxycarbonyl*-L-*phenylalanyl*-β-t-*butoxy*-L-*aspartylglycine* (9).—The fully protected dipeptide (3) (13.75 g, 29.2 mmol) was hydrogenated in absolute methanol for 4 h over palladium. Recrystallization from methanol–chloroform–ethyl acetate afforded fine white *crystals* (5.90 g, 82.0%), m.p. 156.5—157.5°, $[\alpha]_D$ +22.0° (*c* 1.02 in methanol), SBA-R_F 0.56.

With stirring at 0 °C, a solution of the active ester (6) [10] (10.0 g, 25.2 mmol) in dimethylformamide (30 ml) was combined with a solution of the dipeptide derivative (7) (5.90 g, 24.0 mmol) in dimethylformamide (50 ml) and water (20 ml), with additional *N*-methylmorpholine (2.7 ml). The mixture was stirred for 6 h at 0 °C and then overnight at room temperature, and evaporated. A solution of the residue in ethyl acetate was washed with buffer solution (pH 2) and with water, dried (Na_2SO_4), and concentrated. The residue was added to absolute ether and was kept overnight in a refrigerator. Recrystallization of the precipitate from chloroform–petroleum afforded white *crystals* (9) (8.11 g, 68.6%), m.p. 125—126°, $[\alpha]_D$ −26.6° (*c* 1.0 in methanol) CMA-R_F 0.60; Asp 1.00, Phe 0.95, *Gly 1.00* (Found: C, 61.4; H, 6.3; N, 8.05. $C_{27}H_{33}N_3O_8$ requires C, 61.5; H, 6.3; N, 8.0%).

N-*Benzyloxycarbonyl*-L-*phenylalanyl*-β-*t*-*butoxy*-L-*aspartyl*-*glycyl*-N$^{\epsilon}$-*t*-*butoxycarbonyl*-L-*lysylglycylglycyl*-*glycine Benzyl Ester* (12).—The tetrapeptide derivative (8) (2.92 g, 3.9 mmol) was dissolved in absolute methanol (50 ml) and the excess of pyridine hydrobromide (8.30 g) was added. The mixture was stirred at room temperature for 3 days. After removal of the solvent, the residue was washed with absolute ether (1.5 l). Recrystallization from chloroform–ether gave the tetrapeptide derivative (10) which still contained pyridine hydrobromide. The tripeptide derivative (9) (2.06 g, 3.9 mmol), 1-hydroxybenztriazole (0.53 g),† and *NN'*-dicyclohexylcarbodi-imide (1.20 g) were dissolved in dimethylformamide (30 ml). The solution was combined with stirring at 0 °C with a solution of the crude tetrapeptide derivative (10) in dimethylformamide (20 ml) containing *N*-methylmorpholine (5.7 ml). The mixture was stirred for 1 h at 0 °C and for 2 days at room temperature, and then was kept overnight in a refrigerator. After removal of precipitated dicyclohexylurea, the filtrate was concentrated and the residue dissolved in ethyl acetate (1 l). The solution was washed with buffer solution (pH 2), saturated aqueous $NaHCO_3$, and water, and concentrated to 1/4 volume. The *precipitate* (12) was collected (first crop; 1.68 g), m.p. 167—169°, $[\alpha]_D$ −20.7° (*c* 1.0 in dimethylformamide). The second crop was obtained from the mother liquor dried over Na_2SO_4 and was recrystallized from methanol–ether (yield 1.09 g); m.p. 166—167.5°, $[\alpha]_D$ −17.9° (*c* 1.0 in dimethylformamide) (total 69%). Both crops were chromatographically homogeneous; CMA-R_F 0.57, SBA-R_F 0.91, SBN-R_F 0.89; Lys 0.95, Asp 1.00, *Gly 4.00*, Phe 0.95 (Found: C, 60.3; H, 6.8; N, 10.9. $C_{51}H_{68}N_8O_{14}$ requires C, 60.2; H, 6.7; N, 11.0%).

L-*Pyroglutamic Acid* 2,4,5-*Trichlorophenyl Ester* (13).—The ester (13) was prepared by hydrogenolysis [12] of the *N*-benzyloxycarbonyl derivative (11); m.p. 159.5—160.5°, $[\alpha]_D$ +18.5° (*c* 0.99 in dimethylformamide) (Found: C, 42.9; H, 2.7; Cl, 34.5; N, 4.6. Calc. for $C_{11}H_8Cl_3NO_3$: C, 42.8; H, 2.6; Cl, 34.5; N, 4.5%) {lit.,[12] m.p. 161—163° $[\alpha]_D$ +14.8° (*c* 2.0 in dimethylformamide); lit.,[15] m.p. 158—159°, $[\alpha]_D$ +18.4° (*c* 1.6 in dimethylformamide) for material prepared from L-pyroglutamic acid and 2,4,5-trichlorophenol}.

L-*Pyroglutanyl*-L-*phenylalanyl*-β-*t*-*butoxy*-L-*aspartyl*-*glycyl*-N$^{\epsilon}$-*t*-*butoxycarbonyl*-L-*lysylglycylglycylglycine* (15).—Hydrogenation of the fully protected heptapeptide derivative (12) (1.22 g, 1.2 mmol) in absolute dimethylformamide over palladium took 10 h. Recrystallization from dimethylformamide–methanol–ether yielded chromatographically homogeneous heptapeptide derivative (14) (0.78 g, 85%), SBA-R_F 0.51. Hydrogenation of (12) (0.62 g, 0.61 mmol) in trifluoroethanol over palladium took only 5 h. Crystallization from ether yielded the heptapeptide derivative (14) (0.48 g, 100%). With stirring at room temperature, a solution of the active ester (13) (0.90 g, 2.9 mmol) in dimethylformamide (20 ml) was combined with a solution of the heptapeptide derivative (14) (0.78 g, 0.98 mmol) in dimethylformamide (30 ml) and *N*-methylmorpholine (0.22 ml). After stirring for 75 h at room temperature, the mixture was evaporated *in vacuo*. The jelly-like residue was washed with tetrahydrofuran. The precipitate was suspended in aqueous acetic acid (pH 4.5; 100 ml), and stirred at room temperature for 3 days. The filtrate was freeze-dried to afford the *octapeptide derivative* (15) (0.24 g, 28.1%), showing a negative ninhydrin reaction. Electrophoresis showed a single immobile component (pH 1.9, AcOH–HCO_2H, 200 V, 3 h); m.p. 135—137°, $[\alpha]_D$ −28.9° (*c* 1.12 in 1,1,1,3,3,3-hexafluoropropan-2-ol), SBA-R_F 0.44; Lys 1.01, Asp 1.00, Glu 1.05, *Gly 4.00*, Phe 0.98 (Found: C, 54.4; H, 6.9; N, 14.1. $C_{41}H_{61}N_9O_{14}$ requires C, 54.5; H, 6.8; N, 13.95%).

* Buffer solutions, pH 3 and 2, were prepared from K_2SO_4 and $KHSO_4$.[14]

† M.p. 156—157 °C (from boiling water).

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L-*Pyroglutamyl*-L-*phenylalanyl*-L-*aspartylglycyl*-L-*lysyl-glycylglycylglycine* (16).—Trifluoroacetic acid (1.5 ml) was added to the octapeptide derivative (15) (43.7 mg). The mixture was kept for 2 h at room temperature. The trifluoroacetic acid was removed and the residue was dissolved in M-acetic acid and freeze-dried (yield 35.8 mg, *ca.* 100%). The free octapeptide (16) (6.8 mg) was purified through gel filtration (G-10; eluant M-acetic acid) (yield 5.4 mg). The octapeptide (16) migrated on electrophoresis (pH 1.9, AcOH–HCO_2H, 200 V, 6 h) as a single component; m.p. 188.5—190°; Lys 1.00 Asp 1.03, Glu 1.09, Gly 3.94, *Phe 1.00*.

Conjugation to the Carrier Multichain-Ala-Lys.—Triton B (Merck) (0.1 ml) was added to the multichain-Ala-Lys (34 mg), and this mixture was evaporated 5 times *in vacuo*. Each time, absolute dimethylformamide was added until methanol and water were removed. The residue dissolved in dimethylformamide (1 ml) was used as a carrier solution. The octapeptide derivative (15) (32 mg) was dissolved in a mixture of hexamethylphosphoric triamide (0.1 ml) and dimethylformamide (1 ml). 1-Hydroxybenzotriazole (55 mg; excess) and *NN'*-dicyclohexylcarbodi-imide (74 mg; excess) were added with stirring at 0 °C. After 1 h the carrier solution was poured into the active ester solution of octapeptide. The mixture was stirred for 1 h at 0 °C and for 3 days at room temperature, then evaporated. The oily residue was washed with ether. The precipitate obtained by centrifugal separation was dissolved in trifluoroacetic acid (1 ml) and set aside for 2 h at room temperature. Some of the product was insoluble in trifluoroacetic acid. After removal of trifluoroacetic acid, 30% acetic acid was added. The soluble part in 30% acetic acid was dialysed (13.2 mg); amino-acid analysis showed *Ala 8.00*, Gly 0.69 (Asp, Glu, Phe, and Lys gave peaks too small for estimation). Approximately 17% conjugation was observed, *i.e. ca.* 17 octapeptide molecules were conjugated to 100 units of ε-poly-DL-alanyl-L-lysine.

The precipitate in 30% acetic acid was washed 3 times with hot propan-2-ol (yield 21.2 mg); Lys 1.27, *Ala 8.00*, Asp 0.20, Glu 0.12, Phe 0.13, Gly 0.86. Approximately 15—20% conjugation was observed.

Conjugation to the Carrier Copolymer-Tyr-Lys.—The amine component was prepared as follows. Triton B (Merck; 10 μl) was added to copolymer-Tyr-Lys (18.4 mg) and this mixture was evaporated 5 times *in vacuo*. Each time absolute dimethylformamide was added until methanol was removed. The residue was not completely soluble in dimethylformamide. To 1 ml of this suspension, hexafluoropropan-2-ol (1 ml) was added, but the polymer was still not completely dissolved.

The octapeptide derivative (15) (44.5 mg) was dissolved in dimethylformamide and *N*-methylmorpholine (6 μl) was added. Then the solution was cooled at −15 °C, and, with stirring, isobutyl chloroformate (7 μl) was added. After 5 min the amine component was poured in, and the mixture was stirred for 2 h at −15 °C and set aside overnight at room temperature. An insoluble jelly-like substance was obtained. The mixture was evaporated, trifluoroacetic acid (1 ml) was added to the residue, and the mixture was kept for 2 h at room temperature (to remove protecting groups). The trifluoroacetic acid was then removed, and aqueous acetic acid was poured on to the residue. The mixture containing some insoluble substance was dialysed (6 h with 5 l of aqueous acetic acid then with 5 l of water). The dialysate was filtered and the filtrate was freeze-dried (yield 25.4 mg); Asp 1.02, *Glu 1.00*, Gly 3.98, Tyr 1.38, Phe 1.08, Lys 2.70. Approximately 60—70% conjugation was observed. The precipitate was dried in a desiccator (yield 4.7 mg); Asp 1,08, *Glu 1.00*, Gly 4.67, Tyr 5.84, Phe 0.95, Lys 3.21. Approximately 45% conjugation was observed.

We thank Professor H. Zahn for encouragement, Dr. J. Föhles for amino-acid analysis, Dr. R. Timpl for polypeptide carriers and immunological advice, and the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, Deutsche Forschungsgemeinschaft, and Verband der chemischen Industrie for financial support.

[7/1257 *Received*, 14*th July*, 1977]