# Synthesis of Some Tri-, Tetra-, and Penta-peptide Sequences of Fibrinogen by the Picolyl Ester Method

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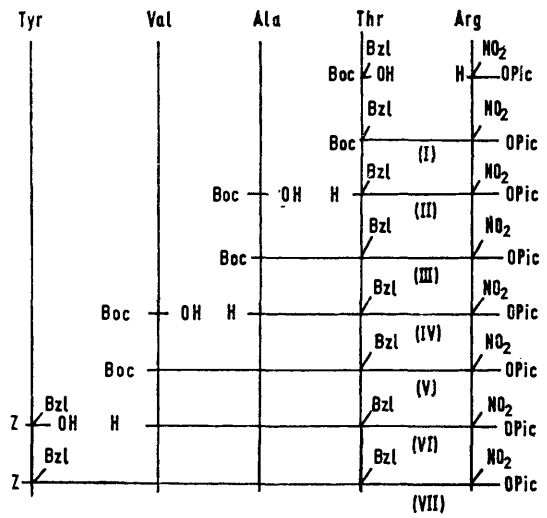
Peptide sequences corresponding to units 1—5 (C chain) and positions 13—16, 15—17, 17—19, and 20—23 (A chain) of the fibrinogen molecule have been synthesised. The use of the 4-picolyl ester group for carboxy-terminal protection has simplified the synthesis of protected intermediates.

THE fibrinogen molecule contains three distinct protein chains,[1] from which the enzyme thrombin releases two peptides (A and B) resulting in further stages in the complex clotting process. The sequences of A peptides from a range of species [2] show considerable variation except in the carboxy-terminal region, close to the point of cleavage by thrombin between residues 16 and 17. This enzyme also releases the tripeptide glycylprolylar-

[1] L. Lorand and W. R. Middlebrook, *Biochem. J.*, 1952, **52**, 196; B. Blombäck, M. Blombäck, B. Hessel, and S. Iwanaga, *Nature*, 1967, **215**, 1445.

[2] M. O. Dayhoff and R. V. Eck, 'Atlas of Protein Sequence and Structure,' National Biomedical Research Foundation, Maryland, U.S.A., 1969.




SCHEME 1 *

* Abbreviations follow the recommendations given in I.U.P.A.C. Information Bulletin No. 26; also Tcp = 2,4,5-trichlorophenyl; Pic = 4-picolyl; DCCI = *NN'*-dicyclohexylcarbodi-imide.

ginine [3] (A chain 17—19). The *N*-terminal sequence 1—5 of the C chain shows no variation in three species. Sequences corresponding to positions 1—5 (C chain)[4] and to positions 13—16, 15—17, 17—19, and 20—23 (A chain) have now been synthesised for biological testing.

The use of a 4-picolyl ester group for carboxy-terminal protection has been shown to simplify peptide synthesis by providing a 'handle' for the isolation of protected derivatives.[5] This approach has been used for most of the syntheses described here.

Scheme 1 shows the stepwise route to the pentapeptide (C chain 1—5). *NN'*-Dicyclohexylcarbodiimide was used for each coupling stage. After each coupling, the *N*-protected peptide picolyl ester [(I), (III), or (V)] was extracted into citric acid and co-reactants were washed away with ether. The pentapeptide (VII) was isolated by use of sulphoethyl-Sephadex C-25 resin ($H^+$ form); the overall yield (from $N^{\omega}$-nitro-L-arginine 4-picolyl ester dihydrobromide) was 36%.

Picolyl esters were isolated essentially as described for other syntheses using the picolyl ester method.[6,7] The 'crude' products obtained (after trituration with ether) were chromatographically and analytically pure. The reaction conditions, yields, and properties of the protected derivatives are shown in the Table. The t-butoxycarbonyl group was removed by hydrogen chloride in dioxan in each case; the resulting dihydrochlorides (usually hygroscopic) were used immediately, after characterisation only by t.l.c.

Hydrogenation of the protected derivative (VII) gave a free pentapeptide showing a single ninhydrin- and Sakaguchi-positive spot on high-voltage electrophoresis (pH 5·3 and 6·2) (apart from a trace of 4-methylpiperidine derived from the 4-picolyl ester group).

Protected peptide 4-picolyl esters [a]

| | Coupling conditions: Solvent (v/ml) | Amino-component (mmol) [b] | Acylating agents (mmol) | Time | Temp. (°C) | Yield (%) [c] | $[\alpha]_D^{25}$ (°) [d] | Found (%) C | H | N | Formula | Required (%) C | H | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (I) | $CH_2Cl_2$ (5) | 0·5 | Boc-Thr(Bzl) (1·0) DCCI (1·0) | 16 h | −10 | 96 [e] | −4·4 | 55·6 | 6·8 | 15·8 | $C_{28}H_{39}N_7O_8$ | 55·9 | 6·5 | 16·3 |
| (III) | $CH_2Cl_2$ (4) | 0·5 [f] | Boc-Ala (1·0) DCCI (1·0) | 16 h | −10 to 0 | 91 [g] | −6·9 | 55·3 | 6·9 | 16·4 | $C_{31}H_{44}N_8O_9$ | 55·35 | 6·6 | 16·7 |
| (V) | $CH_2Cl_2$ (4) | 0·35 [h] | Boc-Val (1·4) DCCI (1·4) | 16 h | −10 to 20 | 83 [i] | −3·2 | 55·6 | 6·9 | 15·6 | $C_{36}H_{53}N_9O_{10},0·5H_2O$ | 55·4 | 7·0 | 16·1 |
| VII | $CH_2Cl_2$ (5) | 0·20 [j] | Z-Tyr(Bzl) (0·8) DCCI (0·8) | 16 h | −10 to 20 | 50 [k] | −3·5 | 60·2 | 6·3 | 12·8 | $C_{55}H_{68}N_{10}O_{12},2H_2O$ | 60·3 | 6·4 | 12·8 |
| (VIII) | $Me_2N{\cdot}CHO$ (4) | 1·0 | Boc-Val (3·0) DCCI (3·0) | 16 h | 0 to 20 | 84 [l] | −12·4 | 51·5 | 6·8 | 18·8 | $C_{22}H_{33}N_7O_7$ | 51·9 | 6·9 | 19·3 |
| (X) | $Me_2N{\cdot}CHO$ (2) | 1·3 [m] | Boc-Gly(OTcp) (2·6) | 44 h | 0 | 76 [n] | −1·6 | 51·0 | 7·0 | 19·5 | $C_{24}H_{38}N_8O_8$ | 50·9 | 6·8 | 19·8 |
| (XII) | $Me_2N{\cdot}CHO$ (1) | 0·46 [p] | Boc-Gly(OTcp) (0·85) | 44 h | 20 | 81 [q] | −2·1 | 49·0 | 6·7 | 19·2 | $C_{26}H_{41}N_9O_9$ | 48·7 | 7·1 | 19·6 |
| (XIII) | $CH_2Cl_2$ (10) | 2·0 | Boc-Arg($NO_2$) (6·0) DCCI (6·0) | 16 h | −10 to 20 | 100 [r] | | 48·0 | 6·5 | 20·7 | $C_{18}H_{29}N_7O_7,0·5H_2O$ | 47·9 | 6·3 | 20·6 |
| (XV) | $CH_2Cl_2$ (10) | 2·1 [s] | Boc-Val (4·3) DCCI (4·3) | 16 h | −10 to 20 | 79 [t] | −10·9 | 50·2 | 6·9 | 18·7 | $C_{44}H_{58}N_8O_8,0·75H_2O$ | 49·7 | 6·9 | 19·3 |
| (XVI) | $CH_2Cl_2$ (10) | 1·5 | Boc-Glu(OBzl) (6·2) DCCI (3·9) | 18 h | −10 to 20 | 96 [u] | −11·8 | 55·0 | 6·5 | 15·4 | $C_{29}H_{39}N_7O_9$ | 55·3 | 6·2 | 15·6 |
| (XX) | $Me_2N{\cdot}CHO$ (4·5) Dioxan (0·8) | 0·2 [v] | Boc-Val-Val-$N_3$ (0·4) | 3 days | −5 | 72 [w] | | 56·2 | 7·0 | 15·3 | $C_{39}H_{57}N_9O_{11}$ | 56·6 | 6·9 | 15·2 |

[a] Compounds numbered as in the Schemes. [b] The amino-component was prepared from the t-butoxycarbonyl derivative [except for compounds (I), (VIII), and (XVI), where the amino-component was $N^{\omega}$-nitro-L-arginine 4-picolyl ester dihydrobromide, and compound (XIII), where it was glycine 4-picolyl ester dihydrobromide] as described under the heading 'deprotection of t-butoxycarbonyl peptides.' [c] Yields of compounds (I), (VIII), (XIII), and (XVI) are calculated with reference to the amino-acid 4-picolyl ester dihydrobromide; yields of other compounds are with reference to the amount of t-butoxycarbonyl compound taken and are of product with stated constants and analysis. [d] *c* 1 in $Me_2N{\cdot}CHO$. [e] $R_F$ 0·68 (C), 0·50 (D), 0·75 (E). [f] $R_F$ 0·06 (A2), 0·27 (B1). [g] $R_F$ 0·20 (A2), 0·70 (C), 0·52 (D), 0·67 (E). [h] $R_F$ 0·25 (B1). [i] $R_F$ 0·39 (A2), 0·59 (B1), 0·71 (C), 0·53 (D), 0·70 (E). [j] $R_F$ 0·05 (A2). [k] $R_F$ 0·60 (A2), 0·79 (B4), 0·73 (C), 0·56 (D), 0·80 (E). [l] $R_F$ 0·30 (A2), 0·63 (B1). [m] $R_F$ 0·11 (A3). [n] $R_F$ 0·21 (A2), 0·52 (A4), 0·56 (B1), 0·13 (A1), 0·24 (A3). [p] $R_F$ 0·00 (A2), 0·08 (A4). [q] $R_F$ 0·12 (A2), 0·72 (A6), 0·30 (A3). [r] $R_F$ 0·44 (B1), 0·49 (E), 0·35 (B2). [s] $R_F$ 0·10 (A6). [t] $R_F$ 0·14 (A2), 0·56 (A3), 0·70 (A4). [u] $R_F$ 0·24 (A2), 0·51 (B1), 0·52 (D). [v] $R_F$ 0·29 (B1), 0·48 (B3). [w] $R_F$ 0·20 (A2), 0·56 (B1), 0·54 (D).

[3] B. Blombäck, M. Blombäck, A. Henschen, B. Hessel, S. Iwanaga, and K. R. Woods, *Nature*, 1968, **218**, 130.

[4] H. Pirkle, A. Henschen, and A. Patapous, *Nature*, 1969, **223**, 400.

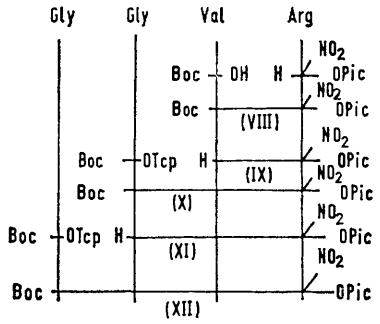
[5] R. Camble, R. Garner, and G. T. Young, *Nature*, 1968, **217**, 247; R. Garner, D. J. Schafer, W. B. Watkins, and G. T. Young in 'Peptides, 1968,' ed. E. Bricas, North Holland, Amsterdam, 1968, p. 145.

[6] D. J. Schafer, G. T. Young, D. F. Elliot, and R. Wade, *J. Chem. Soc.* (*C*), 1971, 46.

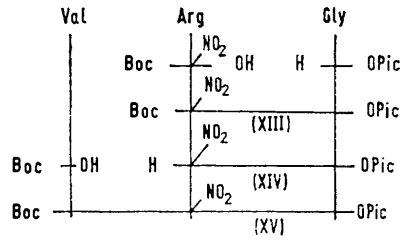
[7] R. Garner and G. T. Young, *J. Chem. Soc.* (*C*), 1971, 50.

Precipitation from ethanol with ethyl acetate removed 4-methylpiperidine although traces of impurities were detected by t.l.c. (chlorine–starch–iodide for detection).

Scheme 2 shows the synthesis of the tetrapeptide (A chain 13—16) and Scheme 3 that of the tripeptide (A chain 15—17). All the protected derivatives were isolated by the citric acid procedure. Glycine was coupled as its t-butoxycarbonyl 2,4,5-trichlorophenyl ester; otherwise *NN'*-dicyclohexylcarbodi-imide was used. Conditions for each coupling reaction and properties of the protected derivatives are given in the Table. Treatment of the protected derivatives (XII) and (XV) with hydrogen chloride followed by hydro-



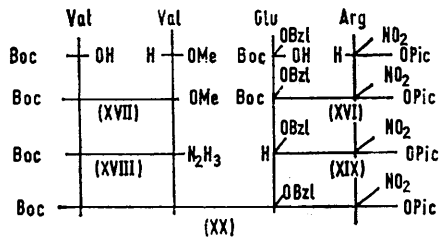

SCHEME 2




SCHEME 3

genation gave the free tetrapeptide and tripeptide, each showing one spot on t.l.c. and on high-voltage electrophoresis.

The tetrapeptide (A chain 20—23) was synthesised as shown in Scheme 4. A two-fold excess of t-butoxycarbonyl-L-valyl-L-valyl azide (prepared *in situ* from




SCHEME 4

the corresponding hydrazide) was coupled in dimethylformamide–dioxan with the dipeptide picolyl ester (XIX). All of the co-products and by-products of this reaction were easily removed by use of sulphoethyl-Sephadex to isolate the protected tetrapeptide. The resin was used in its 3-bromopyridinium form to avoid *N*-deprotection.[7] Elution was carried out with pyridine in aqueous dimethylformamide. The overall yield of protected tetrapeptide (XX) [from $N^{\omega}$-nitro-L-arginine 4-picolyl ester dihydrobromide] was 69%.

This first example of a fragment condensation by use of the azide method for coupling in conjunction with a picolyl ester demonstates further the general applicability of the picolyl ester method. Treatment of the derivative (XX) with hydrogen chloride followed by hydrogenation gave the chromatographically and electrophoretically pure tetrapeptide (98% yield).

Tripeptide (A chain 17—19) was prepared by coupling *N*-benzyloxycarbonylglycyl-L-proline 2,4,5-trichlorophenyl ester[8] with L-arginine (free base) in dimethylformamide, followed by hydrogenation of the *N*-benzyloxycarbonyl tripeptide. The free tripeptide was chromatographically and electrophoretically pure.

## EXPERIMENTAL

Thin-layer chromatograms were run on silica gel $GF_{254}$ (Merck); $R_F$ values refer to the following systems: methanol–chloroform mixtures: (A1) 1 : 19; (A2) 1 : 9; (A3) 3 : 17; (A4) 1 : 4; (A5) 1 : 3; (A6) 1 : 2; n-butanol–acetic acid–water mixtures: (B1) 10 : 1 : 3; (B2) 4 : 1 : 1; (B3) 3 : 1 : 1; (B4) 1 : 1 : 1; (C) n-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 6); (D) cyclohexane–ethyl acetate–methanol (1 : 1 : 1); (E) chloroform–methanol–acetic acid (10 : 2 : 1); (F) t-butyl alcohol–methyl ethyl ketone–ammonia (*d* 0·880)–water (5 : 3 : 1 : 1). Spots were detected by use of ninhydrin, chlorine and starch–iodide, and u.v. illumination.

Optical rotations were measured with a Perkin-Elmer 141 automatic polarimeter (1 dm cell). A Locarte high-voltage apparatus was used for electrophoresis at 100—120 V $cm^{-1}$ (Whatman 3MM paper). M.p.s were determined with a Kofler hot-stage apparatus. Organic solutions were dried over sodium sulphate. Samples for amino-acid analysis were hydrolysed at 110° for 18 h.

*General Procedures.—Deprotection of t-butoxycarbonyl-peptides.* 4N-Hydrogen chloride in dioxan was added to a vigorously stirred solution of peptide in dioxan to give a final concentration of hydrogen chloride of 3N. The solution was evaporated after 1 h and the residue triturated with ether. The resulting hydrochlorides were hygroscopic white powders and were used for the next stage immediately after characterisation by t.l.c.

*Coupling and isolation of protected derivatives.* The amino-component was liberated from its hydrochloride (or hydrobromide) by addition of just sufficient triethylamine to give an alkaline reaction to moist indicator paper held above the surface of the liquid. Acylating components and coupling conditions are given in the Table. The protected peptides were isolated when t.l.c. showed no unchanged amino-component.

*Isolation with citric acid.* The reaction mixture was filtered and the filtrate shaken with 0·67M-citric acid (50—100 ml per mmol of peptide) and ether (10—20 ml per mmol of peptide). The ether layer was washed with more citric acid (×2) and the combined citric acid extracts

[8] K. L. Agarwal, G. W. Kenner, and R. C. Sheppard, *J. Chem. Soc.* (*C*), 1969, 2213.

were washed with ether (×4). The pH was adjusted to 8 by adding solid sodium hydrogen carbonate, and the peptide was extracted into ethyl acetate (×4). Evaporation of the dried extracts and trituration with ether gave the protected peptide.

*Isolation with sulphonic acid resin.* The reaction mixture was filtered and evaporated. The residue was dissolved in aqueous 95% dimethylformamide and poured on a column of SE-sephadex C-25 resin ($H^+$ form) (10-fold excess) equilibrated with dimethylformamide. The column was washed with aqueous 95% dimethylformamide and the product eluted with 1% triethylamine in aqueous 95% dimethylformamide. Evaporation and trituration with ether gave the protected peptide.

L-*Tyrosyl*-L-*valyl*-L-*alanyl*-L-*threonyl*-L-*arginine*. The protected pentapeptide (VII) (39 mg) in 80% acetic acid (3·5 ml) was hydrogenated for 20 h over 10% palladium–charcoal (30 mg). The mixture was filtered (Celite) and evaporated and the residue triturated with ether and dissolved in water. The solution was filtered and lyophilized to give crude pentapeptide (34 mg), $R_{Arg}$ 0·55 (pH 6·2) and 0·56 (pH 5·3) on high-voltage electrophoresis, with a trace of 4-methylpiperidine, $R_{Arg}$ 1·4 (pH 6·2 and 5·3). The crude product (10 mg) was dissolved in ethanol (0·2 ml) and precipitated with ethyl acetate (1·0 ml). The precipitate was washed with ethyl acetate and dissolved in water; the solution was lyophilized to give fluffy white *pentapeptide* (10 mg), $R_F$ 0·62 (B4), 0·34 (C), 0·00 (D), 0·00 (E), with traces of impurities; amino-acid analysis: Ala 0·99; Arg, 1·03; Thr, 1·00; Tyr, 0·83; Val, 0·99.

*Glycylglycyl*-L-*valyl*-L-*arginine dihydrochloride*. Hydrogen chloride in acetic acid (3N; 25 ml) was added to a stirred suspension of the protected peptide (XII) (0·321 g, 0·5 mmol) in acetic acid (5 ml). The solution was kept at room temperature for 1 h (during which some product precipitated), the mixture was evaporated, and the residue was triturated with ether to give white, solid *glycylglycyl*-L-*valyl*-N$^{\omega}$-*nitro*-L-*arginine* 4-*picolyl ester dihydrochloride*, $R_F$ 0·12 (B2), 0·48 (C). A solution of the crude dihydrochloride in 80% acetic acid (40 ml) was hydrogenated for 5 h over 10% palladium–charcoal (30 mg). The mixture was filtered (Celite) and evaporated and the residue triturated with ethanol to give white, solid *tetrapeptide* (0·172 g), $R_F$ 0·09 (B2), 0·21 (C), 0·08 (F); amino-acid analysis: Arg, 1·05; Gly, 1·93; Val, 0·98. Electrophoresis showed one spot, $R_{Arg}$ 0·69 (pH 6·2).

L-*Valyl*-L-*arginylglycine dihydrochloride*. Hydrogen chloride in dioxan (4N; 5 ml) was added to a stirred solution of the protected tripeptide (XV) (0·207 g, 0·35 mmol) in dioxan (3 ml). After 90 min the mixture was evaporated and the residue triturated with ether and dissolved in water (5 ml); the solution was washed with ethyl acetate (×2) and ether (×2) and lyophilized to give fluffy, white L-*valyl*-N$^{\omega}$-*nitro*-L-*arginylglycine* 4-*picolyl ester dihydrochloride* (0·219 g), $R_F$ 0·08 (A3), 0·3 (A6), 0·10 (B1).

A solution of the crude dihydrochloride (0·108 g) in 80% acetic acid (3·5 ml) was hydrogenated for 24 h over 10% palladium–charcoal (75 mg). The mixture was filtered (Celite) and evaporated and the residue triturated with ethanol–ethyl acetate (1 : 2) and dissolved in water. The solution was lyophilized to give fluffy, white *tripeptide* (0·040 g), $R_F$ 0·09 (B3), 0·10 (F), 0·18 (C); amino-acid analysis: Arg, 0·93; Gly, 0·99; Val, 1·08. Electrophoresis showed one spot, $R_{His}$ 0·79 (pH 6·2).

*t-Butoxycarbonyl*-L-*valyl*-L-*valine methyl ester* (XVII). A mixture of t-butoxycarbonyl-L-valine dicyclohexylamine salt (0·40 g, 1 mmol) and L-valine methyl ester hydrochloride (0·17 g, 1 mmol) was stirred in tetrahydrofuran (3 ml) at room temperature for 30 min. The mixture was cooled to −10° and *NN'*-dicyclohexylcarbodi-imide (0·21 g, 1 mmol) was added. The mixture was filtered and the residue washed with ether (25 ml). The filtrate was washed with 2N-hydrochloric acid (×2), water (×1), saturated sodoum hydrogen carbonate (×2), and brine (×1), dried and evaporated, giving crystalline *dipeptide* (0·34 g, quantitative), m.p. 167—168° (from chloroform–ether) (Found: C, 58·2; H, 9·2; N, 8·5. $C_{16}H_{30}N_2O_5$ requires C, 58·2; H, 9·2; N, 8·5%).

*t-Butoxycarbonyl*-L-*valyl*-L-*valyl hydrazide* (XVIII). Hydrazine hydrate (2 ml) was added to a solution of the dipeptide methyl ester (XVII) (0·300 g, 0·9 mmol) in methanol (5 ml). After 24 h at room temperature the solution was diluted with water (40 ml) and set aside overnight to give the *hydrazide* as long needles (0·143 g, 48%), m.p. 204—205° (Found: C, 53·0; H, 9·5; N, 16·5. $C_{15}H_{30}N_4O_4,0{\cdot}5H_2O$ requires C, 53·1; H, 9·2; N, 16·5%).

*t-Butoxycarbonyl*-L-*valyl*-L-*valyl*-γ-*benzyl*-L-*glutamyl*-N$^{\omega}$-*nitro*-L-*arginine* 4-*picolyl ester* (XX). Hydrogen chloride in dioxan (4N; 0·303 ml) and t-butyl nitrite (0·052 ml) in dioxan (0·47 ml) were added to a stirred solution of the hydrazide (XVIII) (0·136 g, 0·4 mmol) in dimethylformamide (2·5 ml) at −20°. After 15 min at −20° this solution was added to a solution of the amino-component (prepared in the usual way; see Table) in dimethylformamide (2 ml) at −20°. The mixture was kept at −5° for 3 days and the product was isolated by use of SE-Sephadex C-25 resin as already described (general procedures) but with the 3-bromopyridinium form of the resin and 5% pyridine in aqueous 90% dimethylformamide as eluant. The product was a white solid; constants are given in the Table.

L-*Valyl*-L-*valyl*-L-*glutamyl*-L-*arginine dihydrochloride*. Hydrogen chloride in dioxan (4N; 6 ml) was added to a stirred solution of the protected tetrapeptide (XX) (60 mg, 0·072 mmol) in dioxan (2 ml) and acetic acid (1 ml). After 90 min the mixture was evaporated and the residue triturated with ether. The resulting (chromatographically pure) crude dihydrochloride was dissolved in 80% acetic acid (3 ml) and hydrogenated for 5 h over 10% palladium–charcoal (25 mg). The mixture was filtered (Celite), the filtrate evaporated, and the residue triturated with ether and dissolved in ethanol (2 ml). The product was precipitated with ethyl acetate (10 ml), giving white solid *tetrapeptide* (42 mg, 98%), $R_F$ 0·40 (B4), 0·27 (C); amino-acid analysis: Arg, 1·00; Glu, 1·09; Val, 1·58 (hydrolysed for 24 h. Electrophoresis showed one spot, $R_{Arg}$ 0·23 (pH 6·5) (Found: C, 42·9; H, 7·4; Cl, 12·4; N, 16·0. $C_{21}H_{39}N_7O_7,2HCl,H_2O$ requires C, 42·6; H, 7·3; Cl, 12·0; N, 16·55%).

*Benzyloxycarbonylglycyl*-L-*prolyl*-L-*arginine*. Benzyloxycarbonylglycyl-L-proline 2,4,5-trichlorophenyl ester [8] (1·6 g, 3·3 mmol) was added to a stirred suspension of L-arginine (0·38 g, 2·2 mmol) in dimethylformamide (2 ml) and the mixture was stirred overnight. Ether (20 ml) was added to the suspension and the while solid was collected and washed with ether giving *tripeptide* (1·1 g, quantitative), m.p. 153—157°, $R_F$ 0·24 (B1), 0·67 (B4). Electrophoresis showed one spot, $R_{Arg}$ 0·19 (pH 6·2) (Found: C, 53·7; H, 6·7; N, 17·7. $C_{21}H_{30}N_6O_6$, $0{\cdot}5H_2O$ requires C, 53·5; H, 6·6; N, 17·8%).

*Glycyl-L-prolyl-L-arginine*. A solution of benzyloxycarbonylglycyl-L-prolyl-L-arginine (0·299 g, 0·63 mmol) in 80% acetic acid (15 ml) was hydrogenated for 5 h over 10% palladium–charcoal (40 mg). The mixture was filtered (Celite) and the filtrate evaporated to give *tripeptide* (0·302 g, quantitative), m.p. 168—172° (from ethanol–ether), $R_F$ 0·03 (B1), 0·16 (C), 0·05 (F); amino-acid analysis: Arg, 1·11; Gly, 1·00; Pro, 0·89. Electrophoresis showed a single spot, $R_{Arg}$ 0·77 (pH 6·2) (Found: C, 43·8; H, 7·2; N, 18·2. $C_{13}H_{24}N_6O_4,2CH_3{\cdot}CO_2H,H_2O$ requires C, 43·8; H, 7·3; N, 18·0%).

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