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Studies on Polypeptides, IV

The Synthesis of C-Peptide of Human Proinsulin[1]

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Summary: The synthesis of human C-peptide is described.

Untersuchungen an Polypeptiden, IV. Synthese des C-Peptids vom Human-Proinsulin

Zusammenfassung: Die Synthese des Human-C-Peptids wird beschrieben.

The synthesis of porcine C-peptide has been reported in the first publication of this series[2]. The present communication describes the total synthesis of human C-peptide. Recently Geiger *et al.*[3,4], König[5], and Jäger[6] have published the syntheses of protected fragments corresponding in sequence to the human C-peptide.

The human C-peptide consists of 31 amino acids [7,8] and the primary structure is shown in Fig. 1.

H-Glu(33)-Ala-Glu-Asp-Leu-Gln-Val-Gly(40)-Gln-Val-Glu-
-Leu-Gly-Gly-Gly-Pro-Gly-Ala(50)-Gly-Ser-Leu-Gln-Pro-
-Leu-Ala-Leu-Glu(60)-Gly-Ser-Leu-Gln(63)-OH

Fig. 1. The amino acid sequence of human C-peptide.

Abbreviations: Bu^t = *t*-butyl; OBu^t = *t*-butyl ester; ONSu = *N*-hydroxysuccinimide ester; Trt = trityl, Z = benzyloxycarbonyl.

Enzyme: Leucine aminopeptidase, L-leucyl-peptide hydrolase (EC 3.4.1.1)

* 92nd Paper on Peptides. For 91st see Brandenburg, D. & Wollmer, A. (1973) *this J.* **354**, 613–627.

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For the synthesis of human C-peptide, five suitably protected intermediate fragments were prepared according to the route illustrated in Schemes I–VI. Scheme VII describes the total synthesis of C-peptide. The protecting groups benzyloxycarbonyl, trityl, methyl ester, benzyl ester, *t*-butyl ester and *t*-butyl ether were used to protect the various functional groups[9].

The coupling methods used were the dicyclohexylcarbodiimide-1-hydroxybenzotriazole[10], *N*-hydroxysuccinimide ester[11], mixed anhydride[12] and azide[13,14].

The intermediate fragments used in the synthesis of human C-peptide were extensively purified by counter-current distribution and gel filtration over

[1] III. Commun.: Naithani, V. K. (1973) *this J.* **354**, 141–146.

[2] Naithani, V. K. (1972) *this J.* **353**, 1806–1816.

[3] Geiger, R., Jäger, G., König, W. & Treuth, G. (1973) *Chem. Ber.* **106**, 188–192.

[4] Geiger, R. & Volk, A. (1973) *Chem. Ber.* **106**, 199–205.

[5] König, W. (1973) *Chem. Ber.* **106**, 193–198.

[6] Jäger, G. (1973) *Chem. Ber.* **106**, 206–210.

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[8] Ko, A. S. C., Smyth, D. G., Markussen, J. & Sundby, F. (1971) *Eur. J. Biochem.* **20**, 190–199.

[9] Schröder, E. & Lübke, K. (1965) The Peptides Vol. 1, pp. 22–59, Academic Press, New York.

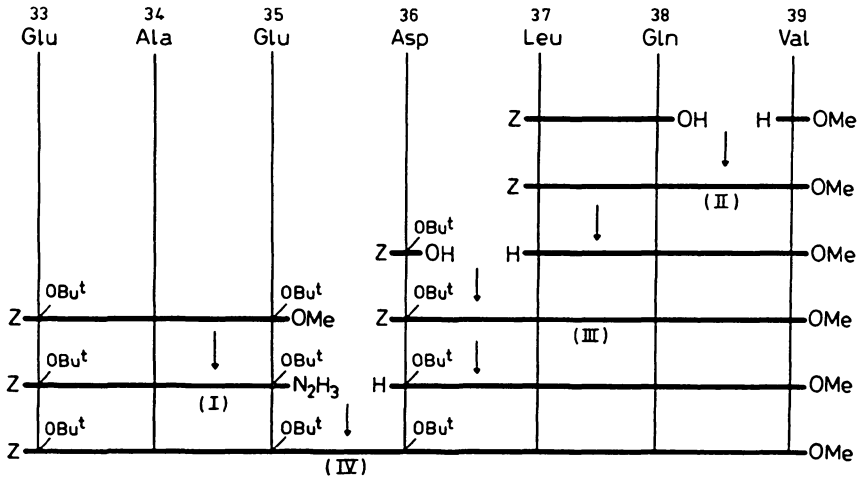
[10] König, W. & Geiger, R. (1970) *Chem. Ber.* **103**, 788–798.

[11] Anderson, G. W., Zimmerman, J. E. & Callahan, F. M. (1964) *J. Amer. Chem. Soc.* **86**, 1839–1842.

[12] Anderson, G. W., Zimmerman, J. E. & Callahan, F. M. (1967) *J. Amer. Chem. Soc.* **89**, 5012–5017.

[13] Honzl, J. & Rudinger, J. (1961) *Collect. Czech. Chem. Commun.* **26**, 2333–2344.

[14] Mazur, R. H. & Schlatter, J. M. (1964) *J. Org. Chem.* **29**, 3212–3216.



Scheme I. Synthesis of sequence 33 – 39.

Sephadex LH-20 using methanol for column development. The protected intermediates, dodecapeptide (XXVI), octadecapeptide (XXVII), tetracosipeptide (XXVIII) and hentricontapeptide (human C-peptide, XXIX), unlike the protected fragments of porcine C-peptide[2] of approximately the same chain length, were soluble in methanol and dimethylformamide. These fragments, therefore, could be purified by gel filtration over Sephadex LH-20 in methanol. The purified hentricontapeptide (XXIX) was readily soluble in dimethylformamide but had lower solubility in methanol. For the purification of deblocked human C-peptide (XXX), gel chromatography over Sephadex G-25 fine using 0.05M NH_4HCO_3 solution as an eluent was employed.

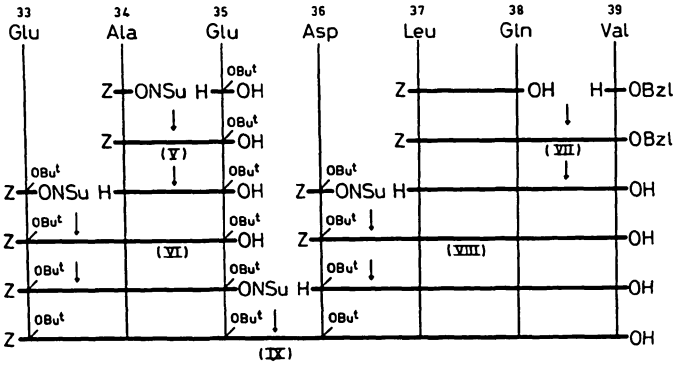
Thin-layer chromatography and amino acid analysis were used to assess the homogeneity of the intermediates. Leucine aminopeptidase digestion was also carried out on the deblocked peptides. High voltage paper electrophoresis was performed to insure the homogeneity of the intermediates octadecapeptide (XXVII) and tetracosipeptide (XXVIII) in addition to thin-layer chromatography after detritylation.

Scheme I describes the synthesis of the protected *N*-terminal heptapeptide methyl ester IV (positions 33 – 39). This was prepared by the azide condensation of benzyloxycarbonyl-γ-*t*-butoxyglutamyl-alanyl-γ-*t*-butoxyglutamyl hydrazide (I) with the debenzyloxycarbonylated tetrapeptide β-*t*-butoxyaspartyl-leucyl-glutaminyl-valine methyl ester (III) in 38% yield. The methyl ester (IV) on hydrazinolysis in dimethylformamide solution yielded a mixture of products. A preliminary investigation of the impure hydrazide mixture with leucine aminopeptidase digestion, after removal of the protecting groups with HBr/trifluoroacetic acid, revealed that α-β rearrangement of the aspartic acid residue had occured. Experiments are in progress to isolate the side products for further characterisation. The results will be published later.

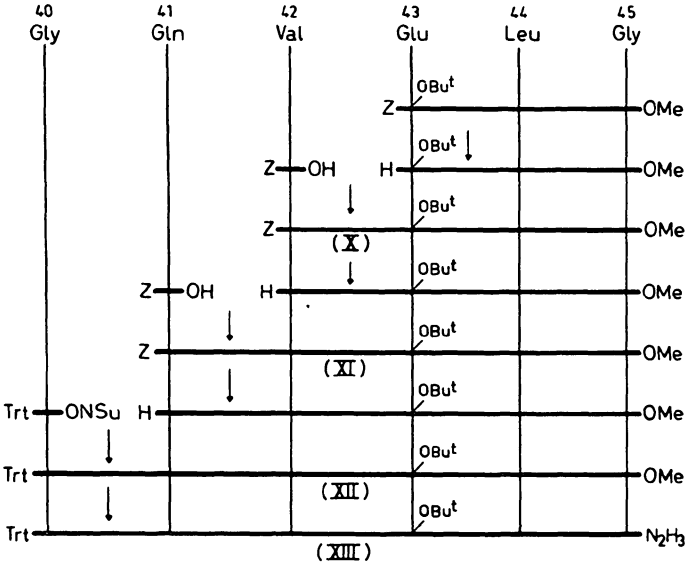
In view of the side reaction during hydrazinolysis of the methyl ester, another route (Scheme II) was developed for the synthesis of the protected *N*-terminal heptapeptide in which the carboxylic end was kept free for use in C-peptide synthesis. The mixed anhydride condensation of benzyloxycarbonylleucyl-glutamine[2] with valine benzyl ester[15] yielded tripeptide (VII). This was then catalytically reduced and condensed with benzyloxycarbonyl-β-*t*-butoxyaspartate *N*-hydroxysuccinimide ester[16] to yield tetrapeptide (VIII) in 32% yield. The benzyloxycarbonyl group was removed selectively by catalytic hydrogenation and the resulting peptide was condensed with benzyloxycarbonyl-γ-*t*-butoxyglutamyl-alanyl-γ-*t*-butoxyglutamate *N*-hydroxysuccinimide ester to give heptapeptide (IX) in 33% yield. The heptapeptide (IX) was

[15] Gibian, H. & Schröder, E. (1961) *Liebigs Ann. Chem.* **642**, 145 – 162.

[16] Hofmann, K., Haas, W., Smithers, M. J. & Zanetti, G. (1965) *J. Amer. Chem. Soc.* **87**, 631 – 639.



Scheme II. Synthesis of sequence 33 – 39.



Scheme III. Synthesis of sequence 40 – 45.

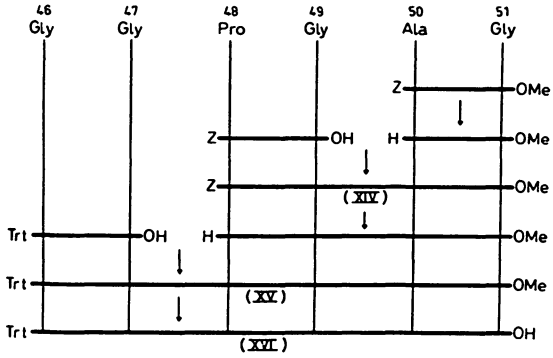
fully digestible with leucine aminopeptidase, and racemisation is therefore not thought to constitute a problem at position 35, the site of fragment coupling.

Scheme III outlines the synthesis of protected hexapeptide (positions 40 – 45). The condensation of benzyloxycarbonylvaline[17] with γ-*t*-butoxy-glutamyl-leucyl-glycine methyl ester using dicyclo-hexylcarbodiimide/1-hydroxybenzotriazole yielded tetrapeptide (X). Debenzyloxycarbonylation of the peptide (X) followed by a mixed anhydride reaction with benzyloxycarbonylglutamine[18] yielded pentapeptide (XI). This was then debenzyl-oxycarbonylated by catalytic reduction and coupled with tritylglycine *N*-hydroxysuccinimide ester[11] to give hexapeptide (XII). The methyl ester, on treatment with hydrazine hydrate, gave hydrazide (XIII).

[17] Vaughan, J. R. & Eichler, J. A. (1953) *J. Amer. Chem. Soc.* **75**, 5556 – 5560.

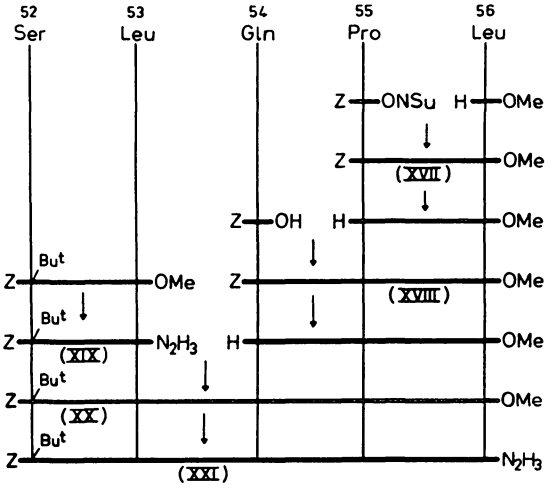
[18] Boissonnas, R. A., Guttmann, S., Jaquenoud, P. A. & Waller, J. P. (1965) *Helv. Chim. Acta* **38**, 1491 – 1501.

Scheme IV was followed for the synthesis of protected hexapeptide (position 46–51). The mixed anhydride condensation of benzyloxycarbonyl-prolyl-glycine[11] with alanyl-glycine methyl ester yielded tetrapeptide (XIV). Subsequently, the condensation of tritylglycyl-glycine[2] with the debenzyloxycarbonylated tetrapeptide (XIV) by the mixed anhydride method gave hexapeptide (XV). The saponification of the methyl ester (XV) yielded the acid (XVI).



Scheme IV. Synthesis of sequence 46–51.

Scheme V illustrates the synthesis of the pentapeptide with positions 52–56. Benzyloxycarbonyl-glutaminyl-prolyl-leucine methyl ester (XVII) was prepared by stepwise elongation. The other dipeptide, benzyloxycarbonyl-*O*-*t*-butylseryl-leucine hydrazide (XIX) was prepared from the corresponding methyl ester by treatment with hydrazine hydrate. Upon azide condensation with debenzyloxycarbonylated tripeptide (XVIII), this yielded protected pentapeptide (XX). Exposure of the methyl ester (XX) to hydrazine hydrate yielded the hydrazide (XXI).



Scheme V. Synthesis of sequence 52–56.

Scheme VI illustrates the synthesis of *C*-terminal heptapeptide (positions 57–63). Benzyloxycarbonyl-*O*-*t*-butylserine[19] was reacted with leucyl-glutamine *t*-butyl ester by dicyclohexylcarbodiimide/1-hydroxybenzotriazole to yield tripeptide (XXII). The synthesis of the tetrapeptide benzyloxycarbonylalanyl-leucyl-γ-*t*-butoxyglutamyl-glycine (XXIII) was achieved by stepwise elongation using the *N*-hydroxysuccinimide ester approach. This was then coupled with the debenzyloxycarbonylated tripeptide (XXIV) by mixed anhydride method to yield heptapeptide (XXV).

Finally, Scheme VII illustrates the synthesis of C-peptide. The benzyloxycarbonyl group from fragment (XXV) was removed selectively and the ensuing peptide was reacted with fragment (XXI) by the azide method to yield dodecapeptide(XXVI). This was then debenzyloxycarbonylated by hydrogenolysis and condensed with hexapeptide (XVI) using dicyclohexylcarbodiimide/1-hydroxybenzotriazole to yield octadecapeptide (XXVII). The next step involved the detritylation of the peptide (XXVII) by acetic acid treatment. The detritylated product was then coupled with the hexapeptide (XIII) by the azide method to yield the tetracosipeptide (XXVIII). The tetracosipeptide was detritylated and the resulting peptide was converted to the hydrochloride salt to remove acetic acid in order to prevent any possible side reaction during dicyclohexylcarbodiimide/1-hydroxybenzotriazole condensation of the carboxylic component with the amine. The hydrochloride was treated with triethylamine to liberate the free amine and this was then reacted with heptapeptide (IX) by dicyclohexylcarbodiimide/1-hydroxybenzotriazole to yield protected human C-peptide (XXIX). Subsequently, the protecting groups from protected C-peptide XXIX were removed by exposure to HBr/trifluoroacetic acid and the resulting deblocked peptide was ultimately purified by gel chromatography over Sephadex G-25 fine using 0.05M NH_4HCO_3 solution as an eluent. The peptide was located by Folin-Ciocalteu[20] reagent

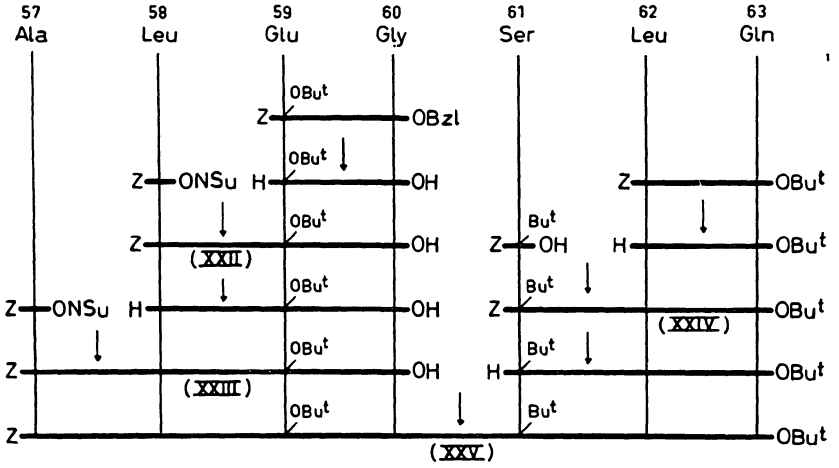
[19] Schröder, E. (1963) *Liebigs Ann. Chem.* **670**, 127–136.

[20] Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randal, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.

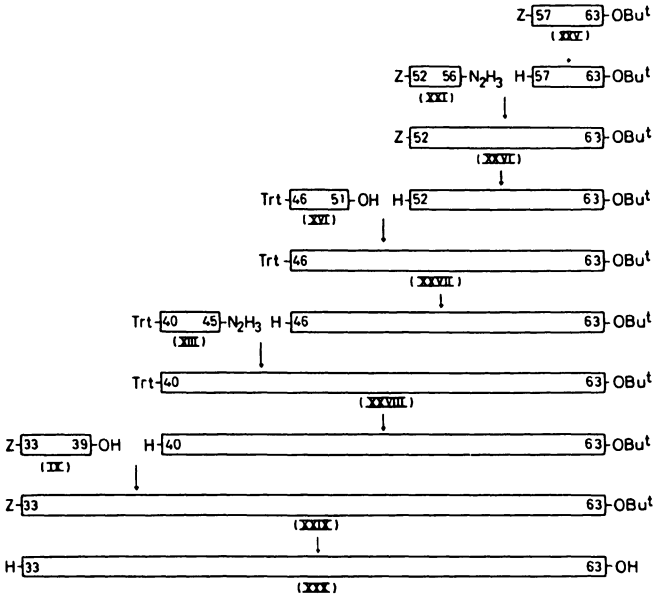
and the desired fractions were pooled and lyophilised.

The C-peptide was shown to be homogeneous by thin-layer chromatography (Fig. 2) as well as by thin-layer electrophoresis (Fig. 3). For these purposes cellulose plates (Merck) were used. For details see the experimental part.

The purity of C-peptide was checked by amino acid analysis. The amino acid analysis of the C-peptide after acid hydrolysis gave a composition consistent with the theoretically expected values. In the leucine amino peptidase digestion of C-peptide, which should proceed to the imide bond of prolyl residue at position 48, low recoveries of



Scheme VI. Synthesis of sequence 57 – 63.



Scheme VII. Synthesis of human C-peptide.

glutamine and glycine and high recoveries of glutamic acid, leucine and alanine were obtained. The lower value for glutamine, also observed in case of heptapeptides IV and IX, could be due to the instability of glutamine at pH 8.7, 40°C for 24 h. The low value for glycine could be due to the Gly-Gly sequence, which digests slowly.

Immunological investigations of this compound are now under way and the results will be published elsewhere.

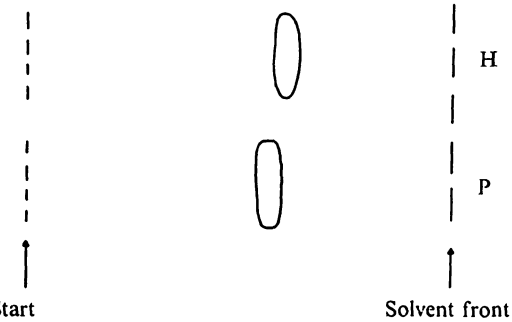


Fig. 2. The diagrammatic representation of a ninhydrin-stained thin-layer chromatogram of human C-peptide (H) in n-butanol/acetic acid/pyridine/water 30:6:20:24 [21]. The synthetic porcine C-peptide (P) has been spotted as a marker.

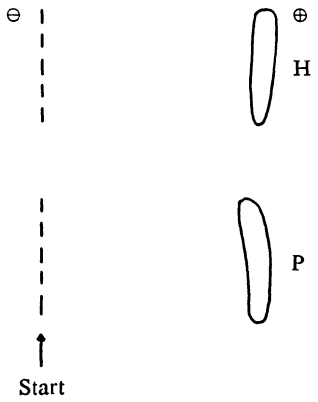


Fig. 3. The diagrammatic representation of a ninhydrin-stained thin-layer electrophoretogram of human C-peptide (H) in pyridine/acetate, pH 6.5, 40 V/cm. The synthetic porcine C-peptide (P) has been spotted as a marker.

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Finally, I thank *Deutsche Forschungsgemeinschaft* for financial support of the present work.

Experimental

All optically active amino acids are of the L-configuration. The melting points are uncorrected. All the *N*-hydroxysuccinimide esters used in the synthesis of C-peptide were freshly prepared. Unless otherwise stated, the peptides were hydrolysed with 6 N HCl in sealed tubes for 24 h at 110°C. The solvent systems used for thin-layer chromatography (Kieselgel G, Merck) of protected intermediate fragments are the same as described in the earlier publication of this series[1,2]. The solvent systems n-butanol/acetic acid/pyridine/water 30:6:20:24 (v/v)[21] and pyridine/acetate, pH 6.5 were used for thin-layer chromatography (DC-Alufolien Cellulose, thickness 0.10 mm, Merck) and thin-layer electrophoresis (DC-Fertigplatten Cellulose, thickness 0.10 mm, Merck), respectively, of human C-peptide. The gel filtration was done on a Sephadex LH-20 column (3.5 × 200 cm) using methanol as an eluent. The following solvent systems were employed for counter-current distribution:

Carbon tetrachloride system, carbon tetrachloride/chloroform/methanol/water 5:5:8:2 (v/v).

Dimethylformamide system, dimethylformamide/methanol/chloroform/cyclohexane/water 2:5:5:2:2 (v/v).

Benzyloxycarbonyl-γ-t-butoxyglutamyl-alanyl-γ-t-butoxyglutamate hydrazide (I)

To a solution of Z-Glu(OBu^t)-Ala-Glu(OBu^t)-OMe[22] (3.64 g, 6 mmol) in 15 ml methanol, 1 ml hydrazine hydrate was added. After 48 h the solvent was evaporated and the product was treated with ether to give a solid residue. The product was crystallized from methanol/water.

Yield 3.3 g (91%); m.p. 179.5–181°C; $[\alpha]_D^{21}$: −7.71° (c=0.635, in dimethylformamide).

$C_{29}H_{45}N_5O_9$ (607.72) Calcd. C 57.31 H 7.40 N 11.52
Found C 56.78 H 7.04 N 11.12

Benzyloxycarbonylleucyl-glutaminyl-valine methyl ester (II)

Isobutylchloroformate (5.2 ml, 40 mmol) was added dropwise to a stirred solution of Z-Leu-Gln-OH[2]

[21] Waley, S. G. & Watson, J. (1953) *Biochem. J.* **55**, 328–337.

[22] Kovacs, J., Giannotti, R. & Kapoor, A. (1966) *J. Amer. Chem. Soc.* **88**, 2282–2292.

(15.72 g, 40 mmol) and *N*-methylmorpholine (4.32 ml, 40 mmol) in 75 ml of a dimethylformamide and tetrahydrofuran mixture (1:2) at −10°C. After activation for 2 min, the chilled solution of HCl-Val-OMe[23] (6.68 g, 40 mmol) and triethylamine (5.56 ml, 40 mmol) in 50 ml dimethylformamide was added and the mixture was stirred for 1 h at 0°C, and then at room temperature for 1 h. The solvent was removed *in vacuo* and the residue was treated with water to give a solid and filtered. The product was crystallized from methanol/water.

Yield 14.2 g (70%); m.p. 218−222°C; $[\alpha]_D^{21}$: −17.62° (c=1.526, in dimethylformamide).

$C_{25}H_{38}N_4O_7 \times 1/2\ H_2O$ (515.62)

Calcd. C 58.23 H 7.62 N 10.86
Found C 58.58 H 6.85 N 10.62

Benzyloxycarbonyl-β-t-butoxyaspartyl-leucyl-glutaminyl-valine methyl ester (III)

II (10.30 g, 20 mmol) was dissolved in 25 ml trifluoroacetic acid and dry HBr was passed into it. After 30 min, trifluoroacetic acid and HBr were removed *in vacuo*, and the residue was washed with ether twice by decantation and dried *in vacuo* over NaOH. The hydrobromide was dissolved in 50 ml dimethylformamide and treated with triethylamine (2.78 ml, 20 mmol) and stored at 0°C for coupling with the mixed anhydride.

A mixed anhydride was prepared in the usual manner from Z-Asp(OBu^t)-OH[24] (6.46 g, 20 mmol) and *N*-methylmorpholine (2.16 ml, 20 mmol) in 35 ml absolute tetrahydrofuran at −10°C with isobutyl chloroformate (2.6 ml, 20 mmol). The solution of deblocked II was then added, and the mixture was stirred for 1 h at 0°C and then at room temperature for 1 h. The solution was poured into 500 ml water and stored at 4°C for 6 h. The precipitated product was filtered and crystallized from methanol/water.

Yield 8.2 g (61%); m.p. 210−214°C; $[\alpha]_D^{21}$: −22.66° (c=2.215, in dimethylformamide).

$C_{33}H_{51}N_5O_{10}$ (677.81) Calcd. C 58.47 H 7.58 N 10.33
Found C 58.28 H 7.42 N 10.37

Amino acid analysis

	Asp	Glu	Val	Leu
Calcd.	1	1	1	1
Found	1.09	1	0.89	0.93

Benzyloxycarbonyl-γ-t-butoxyglutamyl-alanyl-γ-t-butoxyglutamyl-β-t-butoxyaspartyl-leucyl-glutaminyl-valine methyl ester (IV)

A solution of III (3.38 g, 5 mmol) in 200 ml methanol was hydrogenated with 2 g Pd catalyst for 6 h. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo* to dryness. The amine was dissolved in 15 ml dimethylformamide and stored at −10°C for the azide reaction.

A solution of hydrazide I (3.04 g, 5 mmol) in 20 ml dimethylformamide at −20°C was treated with isoamylnitrite (0.73 ml, 5.5 mmol) and 3N HCl/tetrahydrofuran (6.66 ml, 20 mmol). After 1 h the reaction mixture was cooled to −40°C and neutralised with triethylamine (2.78 ml, 20 mmol). The solution of deblocked III was added and the mixture was stirred overnight at −10°C. The mixture was poured into 200 ml water and the precipitated product was filtered, dried and purified by counter-current distribution using the dimethylformamide system (480 cycles; K=0.13).

Yield 2.1 g (38%); m.p. 224−225°C; $[\alpha]_D^{22}$: −22.32° (c=0.73, in dimethylformamide).

$C_{54}H_{86}N_8O_7$ (1119.35)

Calcd. C 57.94 H 7.74 N 10.01
Found C 58.18 H 7.76 N 10.33

Amino acid analysis

	Asp	Glu	Ala	Val	Leu
Calcd.	1	3	1	1	1
Found	1	3.05	0.94	1.06	0.92

Amino acid analysis (after leucine aminopeptidase digestion)

	Asp	Gln	Glu	Ala	Val	Leu
Calcd.	1	1	2	1	1	1
Found	0.92	0.65	2.31	1.0	0.90	0.93

Benzyloxycarbonylalanyl-γ-t-butoxyglutamate dicyclohexylammonium salt (V)

To a solution of H-Glu(OBu^t)-OH[25] (4.06 g, 20 mmol) and $NaHCO_3$ (3.36 g, 40 mmol) in a 100 ml dioxane/water mixture (1:1) was added Z-Ala-ONSu[11] (6.4 g, 40 mmol). After the reaction mixture had been stirred overnight, the dioxane was removed *in vacuo*. The solution was diluted with water and then extracted once with ethyl acetate. The organic phase was discarded, and the aqueous phase was acidified with cold dilute H_2SO_4 solution and extracted with ethyl acetate. The organic phase was washed with saturated NaCl solution and dried over Na_2SO_4. The solvent was evaporated to give an impure oil. This was taken up in ether, treated with dicyclohexylamine (3.92 ml, 20 mmol) and stored at 4°C for 6 h. The product was filtered and crystallized from chloroform/ether.

Yield 8.4 g (72%); m. p. 171−173°C; $[\alpha]_D$: −1.6° (c=2.51, in methanol).

$C_{32}H_{51}N_3O_7$ (589.78)

Calcd. C 65.16 H 8.71 N 7.12
Found C 65.24 H 8.25 N 6.94

Benzyloxycarbonyl-γ-t-butoxyglutamyl-alanyl-γ-t-butoxyglutamate (VI)

A solution of Z-Ala-Glu(OBu^t)-OH (8.16 g, 20 mmol), obtained from V, was hydrogenated with 2 g Pd

[23] Synge, R. L. M. (1948) *Biochem. J.* **42**, 99−104.
[24] Wünsch, E. & Zwick, A. (1963) *this J.* **333**, 108−113.
[25] Schröder, E. & Klieger, E. (1964) *Liebigs Ann. Chem.* **673**, 196−207.

catalyst for 10 h. The catalyst was filtered off and the filtrate was evaporated to dryness *in vacuo*. The crude product was dissolved in 200 ml dioxane/water mixture (1:1) containing $NaHCO_3$ (3.26 g, 40 mmol) and Z-Glu(OBu^t)-ONSu[11] (8.68 g, 20 mmol) and stirred for 48 h. The dioxane was removed *in vacuo* and the solution was diluted with water and extracted twice with ethyl acetate. The aqueous phase was discarded, as the sodium salt of the tripeptide was found to be soluble in ethyl acetate. The combined organic phases were washed successively with cold dilute H_2SO_4 solution, water, and dried over Na_2SO_4. The ethyl acetate was evaporated off and the oily residue was crystallized from ether.

Yield 7.2 g (61%); m. p. 57–58°C; $[\alpha]_D^{24}$: −2.8° (c=2.5 in chloroform).

Lit.[23] m. p. 58–59°C; $[\alpha]_D^{25}$: +3.03° (c=3.0, in chloroform).

Benzyloxycarbonyl-leucyl-glutaminyl-valine benzyl ester (VII)

The tripeptide benzyl ester was prepared in essentially the same manner as described for the methyl ester (II). The product was crystallized from methanol/water.

Yield 65%; m. p. 201–205°C; $[\alpha]_D^{24}$: −20.4° (c=1.36, in dimethylformamide).

$C_{31}H_{42}N_4O_7$ (582.71)

Calcd.	C 63.89	H 7.26	N 9.61
Found	C 63.92	H 7.18	N 9.82

Benzyloxycarbonyl-β-t-butoxyaspartyl-leucyl-glutaminyl-valine (VIII)

The decarbobenzoxylation of VII (11.64 g, 20 mmol) was done in acetic acid by catalytic hydrogenation for 6 h. The acetic acid was evaporated to dryness. The product was dissolved in 300 ml dioxane/water mixture (1:1) containing $NaHCO_3$ (5.04 g, 60 mmol) and Z-Asp(OBu^t)-ONSu[16] (12.60 g, 30 mmol) and the reaction mixture was stirred for two days. The dioxane was removed *in vacuo* and the aqueous phase was acidified with cold dilute H_2SO_4. The semisolid product was taken up in ethyl acetate and the organic phase was washed with saturated NaCl solution. After a quick drying over Na_2SO_4 the ethyl acetate was evaporated to dryness to give an oil. The oily residue on trituration with ether became solid. The product was crystallized from methanol/ether mixture.

Yield 4.2 g (32%); m. p. 176–179°C; $[\alpha]_D^{23}$: −18.23° (c=0.735, in dimethylformamide).

$C_{32}H_{49}N_5O_{10}$ (663.78)

Calcd.	C 57.90	H 7.44	N 10.55
Found	C 58.26	H 7.48	N 10.39

Amino acid analysis

	Asp	Glu	Val	Leu
Calcd.	1	1	1	1
Found	0.99	1	1	0.99

Benzyloxycarbonyl-γ-t-butoxyglutamyl-alanyl-γ-t-butoxyglutamyl-β-t-butoxyaspartyl-leucyl-glutaminyl-valine (IX)

The decarbobenzoxylation of VIII (3.31 g, 5 mmol) was carried out in dimethylformamide in presence of 2 g Pd catalyst for 12 h. The catalyst was removed by filtration and the filtrate was evaporated to dryness. An active ester was prepared in the usual manner from VI (5.93 g, 10 mmol) and *N*-hydroxysuccinimide (1.15 g, 10 mmol) in 50 ml tetrahydrofuran at 0°C with a solution of dicyclohexylcarbodiimide (2.06 g, 10 mmol) in 10 ml tetrahydrofuran. After 4 h the dicyclohexylurea was filtered off, the filtrate was evaporated to dryness and the residue was washed three times with petroleum ether. The crude active ester was dissolved in 30 ml dioxane and added to the suspension of amine, deblocked VIII, in 180 ml dioxane-water mixture (1:2) containing $NaHCO_3$ (1.68 g, 20 mmol). After stirring for two days the dioxane was evaporated off *in vacuo* and the residue was acidified with dilute acetic acid and stored at 4°C for 4 h. The product was filtered and washed thoroughly with water and dried. The crude residue was extracted three times with warm ethyl acetate and finally purified over Sephadex LH-20 using methanol as an eluent.

Yield 1.8 g (33%); m. p. 217–219°C (decomposed); $[\alpha]_D^{24}$: −21.3° (c=0.84, in dimethylformamide).

$C_{53}H_{84}N_8O_7$ (1105.32)

Calcd.	C 57.59	H 7.66	N 10.13
Found	C 57.54	H 7.79	N 10.46

Amino acid analysis

	Asp	Glu	Ala	Val	Leu
Calcd.	1	3	1	1	1
Found	0.98	3.07	0.94	1.01	1

Amino acid analysis (after leucine aminopeptitase digestion)

	Asp	Gln	Glu	Ala	Val	Leu
Calcd.	1	1	2	1	1	1
Found	0.97	0.55	2.38	1.07	1.02	1

Benzyloxycarbonylvalyl-γ-t-butoxyglutamyl-leucyl-glycine methyl ester (X)

A solution of Z-Glu(OBu^t)-Leu-Gly-OMe[2] (15.63 g, 30 mmol) was hydrogenated in 400 ml methanol with 2 g Pd catalyst for 6 h. The catalyst was removed by filtration and the solvent was evaporated *in vacuo* to dryness. The residue was dissolved in 100 ml dimethylformamide containg Z-Val-OH[17] (7.53 g, 30 mmol) and 1-hydroxybenzotriazole (4.05 g, 30 mmol) and the reaction mixture was cooled to 0°C. To this was added a solution of dicyclohexylcarbodiimide (6.18 g, 30 mmol) in 15 ml dimethylformamide with stirring. After 24 h dicyclohexylurea was removed by filtration and the filtrate was evaporated to dryness. The solid residue was washed with water, filtered and crystallized from

methanol/water. The recrystallization was effected from ethyl acetate/petroleum ether.

Yield 11.8 g (63%); m. p. 176–177°C; $[\alpha]_D^{22}$: −50.3° (c=0.45, in methanol).

Lit.[4] m. p. 174–175°C; $[\alpha]_D^{22}$: −49.2° (c=1, in methanol).

$C_{31}H_{48}N_4O_9$ (620.76)

Calcd.	C 59.98	H 7.78	N 9.02
Found	C 59.56	H 7.88	N 9.72

Amino acid analysis

	Glu	Gly	Val	Leu
Calcd.	1	1	1	1
Found	1.04	1	0.90	1

Benzyloxycarbonylglutaminyl-valyl-γ-t-butoxyglutamyl-leucyl-glycine methyl ester (XI)

A mixed anhydride was prepared in the usual manner from Z-Gln-OH[18] (2.8 g, 10 mmol) and *N*-methylmorpholine (1.08 ml, 10 mmol) in 20 ml dimethylformamide at −10°C with isobutylchloroformate (1.30 ml, 10 mmol). To it the cooled amine solution, obtained from X (6.2 g, 10 mmol) by hydrogenolysis, in 30 ml dimethylformamide was added. The reaction mixture was stirred for 1 h at 0°C, and then at room temperature for 1 h. The solution was poured into 200 ml water and kept at 4°C for 4 h. The product was filtered and crystallized from dimethylformamide/water.

Yield 4.5 g (59%); m. p. 250–255°C (decomposed); $[\alpha]_D^{22}$: −19.05° (c=1.15, in dimethylformamide).

$C_{36}H_{56}N_6O_{11} \times {}^1/_2\ H_2O$ (757.91)

Calcd.	C 57.05	H 7.58	N 11.08
Found	C 56.89	H 6.98	N 11.06

Amino acid analysis:

	Glu	Gly	Val	Leu
Calcd.	2	1	1	1
Found	1.92	1	1.01	0.92

Tritylglycyl-glutaminyl-valyl-γ-t-butoxyglutamyl-leucyl-glycine methyl ester (XII)

A solution of XI (3.78 g, 5 mmol) in 200 ml dimethylformamide was hydrogenated with 2 g Pd catalyst in presence of 6N HCl (0.83 ml, 5 mmol) added in 4 lots over a period of 1 h. After 1 h a drop of pyridine was added, the catalyst was filtered, and the filtrate was concentrated *in vacuo* to dryness. The crude residue was dissolved in 25 ml dimethylformamide containing triethylamine (0.7 ml, 5 mmol) and Trt-Gly-ONSu[11] (4.14 g, 10 mmol) and the reaction mixture was stirred for two days. The mixture was poured into 200 ml water and kept at 4°C for 4 h. The product was filtered, washed with water and dried. The impure product was first purified by counter-current distribution (carbon-tetrachloride system, 240 cycles, K=0.42) followed by Sephadex LH-20 chromatography in methanol. The product was crystall. from chloroform/petroleum ether.

Yield 2.8 g (61%); m. p. 227–230°C; $[\alpha]_D^{24}$: −17.6° (c=1.175, in dimethylformamide).

$C_{49}H_{67}N_7O_{10}$ (914.13)

Calcd.	C 64.38	H 7.38	N 10.72
Found	C 64.13	H 6.97	N 10.83

Amino acid analysis

	Glu	Gly	Val	Leu
Calcd.	2	2	1	1
Found	2.08	1.98	0.99	1

Tritylglycyl-glutaminyl-valyl-γ-t-butoxyglutamyl-leucyl-glycine hydrazide (XIII)

To a solution of XII (2.5 g, 2.73 mmol) in 15 ml dimethylformamide was added 1 ml hydrazine hydrate. After 48 h the reaction mixture was poured into 100 ml water and the precipitated product was filtered and crystallized from dimethylformamide/water.

Yield 2.2 g (88%); m. p. 219–223°C (decomposed); $[\alpha]_D^{24}$: −17.3° (c=1.6, in dimethylformamide).

$C_{48}H_{67}N_9O_9$ (914.136)

Calcd.	C 63.06	H 7.38	N 13.79
Found	C 62.87	H 7.48	N 13.75

Benzyloxycarbonylprolyl-glycyl-alanyl-glycine methyl ester (XIV)

The benzyloxycarbonyl group from Z-Ala-Gly-OMe[23] (2.94 g, 10 mmol) was removed by HBr/trifluoroacetic acid treatment as described for the preparation of III. The crude hydrobromide derivative was dissolved in 20 ml dimethylformamide containing triethylamine (1.39 ml, 10 mmol) and stored at 0°C for the mixed anhydride reaction.

A mixed anhydride was prepared in the usual manner from Z-Pro-Gly-OH[11] (3.06 g, 10 mmol) and triethylamine (1.39 ml, 10 mmol) in 25 ml tetrahydrofuran at −10°C with isobutylchloroformate (1.30 ml, 10 mmol). To it the amine solution, described above, was added. The reaction mixture was stirred for 1 h at 0°C and then at room temperature for 1 h. The solvent was removed *in vacuo* and the residue was taken up in ethyl acetate and washed with $NaHCO_3$ solution, water, dilute HCl solution, water and dried over Na_2SO_4. The organic layer was evaporated *in vacuo* and the product crystallized from chloroform/ether.

Yield 3.8 g (85%); m. p. 109–112°C; $[\alpha]_D^{23}$: −49.49° (c=1.988, in methanol).

$C_{21}H_{28}N_4O_7$ (448.48)

Calcd.	C 56.24	H 6.29	N 12.49
Found	C 55.71	H 5.90	N 11.79

Amino acid analysis

	Pro	Gly	Ala
Calcd.	1	2	1
Found	1.06	2.01	1

Tritylglycyl-glycyl-prolyl-glycyl-alanyl-glycine methyl ester (XV)

The debenzyloxycarbonylation of XIV (8.96 g, 20 mmol) was done by HBr/trifluoroacetic acid treatment as described in the preparation of III. The hydrobromide was dissolved in 30 ml dimethylformamide and treated with triethylamine (2.78 ml, 20 mmol) and stored at −10°C for the mixed anhydride reaction. A mixed anhydride was prepared in the usual manner from Trt-Gly-Gly-OH[2] (7.48 g, 20 mmol) and triethylamine (2.78 ml, 20 mmol) in 45 ml tetrahydrofuran at −10°C with isobutylchloroformate (2.6 ml, 20 mmol). The amine solution described above was then added. The reaction mixture was stirred for 1 h at 0°C and then at room temperature for 1 h. The solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate and washed with NaCl solution. The solvent was removed after quick drying over Na_2SO_4 and the residue was crystallized twice from ethyl acetate.

Yield 5.5 g (41%); m. p. 140−144°C (a poorly defined m. p.); $[\alpha]_D^{21}$: −29.27° (c=0.83, in dimethylformamide).

Lit.* m. p. 133−136°C; $[\alpha]_D^{24}$: −26.6° (c=1, in dimethylformamide).

$C_{36}H_{42}N_6O_7 \times 1/2\ H_2O$ (679.79)

Calcd.	C 63.60	H 6.37	N 12.36
Found	C 63.87	H 6.34	N 12.37

Amino acid analysis (with 6N HCl at 110°C for 144 h)

	Pro	Gly	Ala
Calcd.	1	4	1
Found	0.58	3.62	1

Tritylglycyl-glycyl-prolyl-glycyl-alanyl-glycine (XVI)

A solution of XV (2.72 g, 4 mmol) in 40 ml dioxane/water mixture (1:1) was saponified with 0.1N NaOH using phthymolphthalein as an indicator. The dioxane was evaporated *in vacuo*, the solution was acidified with acetic acid and extracted with ethyl acetate. The organic phase was washed with saturated NaCl solution, dried over Na_2SO_4 and evaporated to dryness *in vacuo*. The product was crystallized from chloroform/petroleum ether.

Yield 1.4 g (52%); m. p. 147−153°C (a very poorly defined m. p.); $[\alpha]_D^{22}$: −23.16° (c=1.36, in dimethylformamide).

Lit.* m. p. 148−152°C; $[\alpha]_D^{24}$: −28.4° (c=1, in dimethylformamide).

$C_{35}H_{40}N_6O_7 \times 1\ H_2O$ (674.77)

Calcd.	C 62.30	H 6.27	N 12.49
Found	C 61.67	H 6.18	N 11.85

Amino acid analysis (with 6N HCl at 110°C for 144 h)

	Pro	Gly	Ala
Calcd.	1	4	1
Found	1.02	4	0.86

* Fehrenbach, P., unpublished results.

Benzyloxycarbonylprolyl-leucine methyl ester (XVII)

To a solution of HCl-Leu-OMe[26] (36.32 g, 200 mmol) and triethylamine (27.8 ml, 200 mmol) in 500 ml chloroform was added Z-Pro-ONSu[11] (69.26 g, 200 mmol) and the reaction mixture was stirred overnight. The solvent was evaporated *in vacuo*, the oily residue was taken up in ethyl acetate, and the organic layer was washed successively with solutions of dilute HCl, water, $NaHCO_3$, water and dried over Na_2SO_4. The ethyl acetate was evaporated and the residue was crystallized from ethyl acetate/petroleum ether.

Yield 62.5 g (83%); m. p. 72−73°C $[\alpha]_D^{22}$: −41.54° (c=1.336, in dimethylformamide).

Lit.[27] m. p. 74−75°C; $[\alpha]_D^{20}$: −42.5° (c=1 in dimethylformamide).

Benzyloxycarbonylglutaminyl-prolyl-leucine methyl ester (XVIII)

A solution of XVII (18.82 g, 50 mmol) was hydrogenated in 200 ml methanol over 3 g Pd catalyst in presence of 8.33 ml 6N HCl. After 4 h the catalyst was removed by filtration and the filtrate was evaporated *in vacuo* to dryness. The hydrochloride was dissolved in 25 ml dimethylformamide containing triethylamine (6.99 ml, 50 mmol) and stored at −10°C for use in the mixed anhydride synthesis.

A mixed anhydride was prepared in the usual manner from Z-Gln-OH[18] (14.01 g, 50 mmol) and *N*-methylmorpholine (5.4 ml, 50 mmol) in 35 ml dimethylformamide and 35 ml tetrahydrofuran at −10°C with isobutylchloroformate (6.5 ml, 50 mmol). The solution of deblocked XVII was then added and the reaction mixture was stirred for 1 h at 0°C and at room temperature for 1 h. The solvent was removed *in vacuo* and the oily residue was taken up in ethyl acetate, washed in the usual manner, and dried over Na_2SO_4. The solvent was evaporated *in vacuo* and the product was crystallized three times from ethyl acetate/petroleum ether.

Yield 9.5 g (38%); m. p. 72−78°C (a poorly defined melting point); $[\alpha]_D^{22}$: −92.5° (c=1.166, in methanol).

$C_{25}H_{36}N_4O_7$ (504.60)

Calcd.	C 59.50	H 7.19	N 11.10
Found	C 59.34	H 7.18	N 11.38

Benzyloxycarbonyl-O-t-butylseryl-leucine hydrazide (XIX)

To a solution of Z-Ser(But)-Leu-OMe* (12.66 g, 30 mmol) in 50 ml methanol was added 4 ml hydrazine hydrate and the reaction mixture was kept for 48 h.

* Föhles, J., unpublished results.

[26] Schott, H. F., Larkin, J. B., Rockland, L. B. & Dunn, M. S. (1947) *J. Org. Chem.* **12**, 490−495.

[27] Niedrich, H. (1964) *Chem. Ber.* **97**, 2527−2533.

The methanol was evaporated to dryness and the oily residue, on treatment with water, afforded a solid which was filtered and dried. The crystallization was effected from tetrahydrofuran/petroleum ether.

Yield 10.2 g (78%); m. p. 106–107°C; $[\alpha]_D^{22}$: −20.2° (c=1.512, in methanol).

$C_{21}H_{34}N_4O_5$ (422.54)

Calcd.	C 59.69	H 8.11	N 13.25
Found	C 59.78	H 7.83	N 13.23

Benzyloxycarbonyl-O-t-butylseryl-leucyl-glutaminyl-prolyl-leucine methyl ester (XX)

The benzyloxycarbonyl group was removed from XVIII (5.04 g, 10 mmol) by HBr/trifluoroacetic acid treatment as described for the preparation of III. The product was dissolved in 25 ml dimethylformamide, treated with triethylamine (1.39 ml, 10 mmol), and stored at −10°C for use in the azide synthesis.

The azide was prepared as usual from XIX (4.22 g, 10 mmol) and isoamylnitrite (1.32 ml, 10 mmol) in 35 ml dimethylformamide at −20°C with 14.18 ml 2.75N HCl/tetrahydrofuran. After 1 h the temperature was lowered to −40°C and the reaction mixture was neutralized with triethylamine (5.56 ml, 40 mmol). The solution of decarbobenzoxylated peptide XVIII was then added and the reaction mixture was stirred overnight at −10°C. The solvent was removed *in vacuo* and the residue was taken up in ethyl acetate and washed with water and dried over Na_2SO_4. The solvent was removed and the crude oily residue was purified by counter-current distribution (carbon tetrachloride system, 160 cycles, K=0.052) and crystallized from ethyl acetate/petroleum ether.

Yield 5.4 g (71%); m. p. 159–162°C; $[\alpha]_D^{22}$: −49.8° (c=0.882, in dimethylformamide).

Lit.* m. p. 152–154°C; $[\alpha]_D^{24}$: −50.7° (c=1, in dimethylformamide).

$C_{38}H_{60}N_6O_{10}$ (760.95)

Calcd.	C 59.98	H 7.95	N 11.04
Found	C 59.93	H 8.13	N 10.94

Amino acid analysis

	Ser	Glu	Pro	Leu
Calcd.	1	1	1	2
Found	0.89	1	1.04	1.92

Benzyloxycarbonyl-O-t-butylseryl-leucyl-glutaminyl-prolyl-leucine hydrazide (XXI)

To a solution of XX (3.8 g, 5 mmol) in 25 ml methanol was added 1 ml hydrazine hydrate and the mixture was kept for 48 h. The solvent was removed *in vacuo* to give an oily residue, which on trituration with ether became solid. The product was filtered and crystallized from acetonitrile/ether.

Yield 2.8 g (74%); m. p. 162–165°C; $[\alpha]_D^{23}$: −57.8° (c=1.19, in dimethylformamide).

$C_{37}H_{60}N_8O_9$ (760.95)

Calcd.	C 58.40	H 7.94	N 14.72
Found	C 57.82	H 7.86	N 14.70

Benzyloxycarbonylleucyl-γ-t-butoxyglutamyl-glycine dicyclohexylammonium salt (XXII)

Z-Glu(OBu^t)-Gly-OBzl[28] (35.33 g, 73 mmol) was hydrogenated in 200 ml 70% methanol containing 4.16 ml acetic acid. After 8 h the catalyst was removed by filtration, and the filtrate was concentrated *in vacuo* to dryness. The residue was dissolved in 400 ml aqueous dioxane (1:1) containing $NaHCO_3$ (15.39 g, 21.9 mmol). To it was added pulverised Z-Leu-ONSu[11] (26.42 g, 7.3 mmol) and the reaction mixture was stirred for two days. The dioxane was removed *in vacuo* and the mixture was acidified with cold dilute H_2SO_4. The product was extracted with ethyl acetate and the organic layer was washed with water and dried. The solvent was removed *in vacuo* to give an oil. This was dissolved in 30 ml ethyl acetate, and dicyclohexylamine (14.30 ml, 7.3 mmol) was added to it and kept at 4°C overnight. The precipitated salt was filtered and washed with ether. The product was crystallized from chloroform/ether.

Yield 37 g (74%); m. p. 170–172°C; $[\alpha]_D^{22}$: −29° (c=1.62, in methanol).

$C_{37}H_{60}N_4O_8$ (688.92)

Calcd.	C 64.50	H 8.77	N 8.13
Found	C 64.21	H 8.35	N 8.30

Benzyloxycarbonylalanyl-leucyl-γ-t-butoxyglutamyl-glycine (XXIII)

A solution of Z-Leu-Glu(OBu^t)-Gly-OH (15.21 g, 30 mmol), obtained from XXII in the usual manner by washing the dicyclohexylamine salt with dilute H_2SO_4 solution, was hydrogenated in 300 ml methanol with 4 g Pd catalyst. After 8 h the catalyst was removed by filtration, and the filtrate concentrated to dryness *in vacuo*. The residue was taken up in 300 ml aqueous dioxane (1:1) containing $NaHCO_3$ (5.04 g, 60 mmol) and Z-Ala-ONSu[11] (9.2 g, 30 mmol) and stirred overnigh . The dioxane was removed *in vacuo* and the residual oily syrup was acidified with cold dilute H_2SO_4 and extracted with ethyl acetate. The organic phase was washed with water and dried over Na_2SO_4. The ethyl acetate was evaporated to dryness and the product was crystallized from ethyl acetate.

Yield 12.5 g (72%); m. p. 101–103°C; $[\alpha]_D^{24}$: −45.8° (c=1.52, in methanol).

Lit.[28] m. p. 145–147°C with softening at 139°C; $[\alpha]_D^{22}$: −45.5° (c=1, in methanol).

* Fehrenbach, P., unpublished results.

[28] Naithani, V. K. (1973) *this J.* **354**, 67–74.

Benzyloxycarbonyl-O-t-butylseryl-leucyl-glutamine t-butyl ester (XXIV)

A solution of Z-Leu-Gln-OBu^t[2] (6.28 g, 14 mmol) was hydrogenated in 200 m*l* methanol with 2 g Pd catalyst. After 4 h the catalyst was removed by filtration and the filtrate was evaporated to dryness. The crude product was dissolved in 35 m*l* tetrahydrofuran containing Z-Ser(OBu^t)-OH[18] (4.13 g, 14 mmol) and 1-hydroxybenzotriazole (1.89 g, 14 mmol) and cooled to 0°C. To it was added dicyclohexylcarbodiimide (2.88 g, 14 mmol) in 10 m*l* tetrahydrofuran and the reaction mixture was stirred overnight. The dicyclohexylcarbodiimide was removed by filtration and the filtrate was concentrated to dryness *in vacuo*. The residue was taken up in ethyl acetate and washed with cold dilute H_2SO_4, water, $NaHCO_3$ solution, water and dried over Na_2SO_4. The ethyl acetate was evaporated and the crude product was purified by counter-current distribution (carbon tetrachloride system, 120 cycles, $K=0.41$) and crystallized from ethyl acetate/petroleum ether.

Yield 5.7 g (69%); m. p. 144–147°C; $[\alpha]_D^{23}$: −35.97° ($c=1.326$, in methanol).

Lit.[3] $[\alpha]_D^{21}$: −33.9° ($c=1$, in methanol); no m. p. given.

$C_{30}H_{48}N_4O_8$ (592.746)

Calcd.	C 60.78	H 8.16	N 9.45
Found	C 61.04	H 8.12	N 9.43

Amino acid analysis

	Ser	Leu	Gln
Calcd.	1	1	1
Found	0.92	1.03	1

Benzyloxycarbonylalanyl-leucyl-γ-t-butylglutamyl-glycyl-O-t-butylseryl-leucyl-glutamine t-butyl ester (XXV)

The benzyloxycarbonyl group from XXIV (3.46 g, 5 mmol) was removed by catalytic hydrogenation in 150 m*l* methanol with 2 g Pd. After 6 h the catalyst was removed by filtration, and the filtrate was concentrated *in vacuo* to dryness. The residue was dissolved in 15 m*l* dimethylformamide and stored at −10°C for use in the mixed anhydride reaction.

A mixed anhydride was prepared in the usual manner from XXIII (2.89 g, 5 mmol) and triethylamine (0.7 m*l*, 5 mmol) in 30 m*l* dimethylformamide and tetrahydrofuran mixture (1:2) at −10°C with isobutylchloroformate (0.65 m*l*, 5 mmol). The solution of decarbobenzoxylated peptide XXIV was then added and the reaction mixture was stirred for 1 h at 0°C, and then at room temperature for 1 h. The solvent was removed *in vacuo*. The product was triturated with ether, filtered, washed with water, dried, and crystallized from methanol/water.

Yield 3.8 g (75%); m. p. 192–195°C; $[\alpha]_D^{23}$: −36.1° ($c=1.67$, in methanol).

$C_{50}H_{82}N_8O_{14}$ (1019.27)

Calcd.	C 58.91	H 8.10	N 10.99
Found	C 58.76	H 8.10	N 11.29

Amino acid analysis

	Ser	Glu	Gly	Ala	Leu
Calcd.	1	2	1	1	2
Found	0.91	2.01	1	0.96	2.10

Benzyloxycarbonyl-O-t-butylseryl-leucyl-glutaminyl-prolyl-leucyl-alanyl-leucyl-γ-t-butoxyglutamyl-glycyl-O-t-butylseryl-leucyl-glutamine t-butyl ester (XXVI)

A solution of XXV (3.4 g, 3.3 mmol) was hydrogenated with 2 g Pd catalyst in 300 m*l* methanol. After 6 h the catalyst was removed, and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in 10 m*l* dimethylformamide and stored at −10°C for the azide reaction.

The azide was prepared from XXI (2.53 g, 3.33 mmol) and isoamylnitrite (0.66 m*l*, 5 mmol) in 25 m*l* dimethylformamide at −20°C with 4.3 m*l* 2.8N HCl/tetrahydrofuran. After 1 h the bath temperature was lowered to −40°C and the reaction mixture was neutralized with triethylamine (1.60 m*l*, 12 mmol). The solution of deblocked peptide XXV was then added and the reaction mixture was stirred overnight at −10°C. The solvent was removed *in vacuo*, and the residue was triturated with water and filtered. The purification of this peptide was effected by column chromatography over Sephadex LH-20 using methanol as an eluent. The product was crystallized from methanol/water.

Yield 4.6 g (86%); m. p. 182–185°C; $[\alpha]_D^{25}$: −57.6° ($c=1.93$, in methanol).

$C_{79}H_{132}N_{14}O_{21}$ (1614.04)

Calcd.	C 58.78	H 8.24	N 12.15
Found	C 58.77	H 8.22	N 11.50

Amino acid analysis

	Ser	Glu	Pro	Gly	Ala	Leu
Calcd.	2	3	1	1	1	4
Found	1.88	3.10	1.08	1	1	3.96

Tritylglycyl-glycyl-prolyl-glycyl-alanyl-glycyl-O-t-butylseryl-leucyl-glutaminyl-prolyl-leucyl-alanyl-leucyl-γ-t-butoxyglutamyl-glycyl-O-t-butylseryl-leucyl-glutamine t-butyl ester XXVII

To a solution of XXVI (1.61 g, 1 mmol) in 200 m*l* methanol was added 1 g Pd and the reaction mixture was hydrogenated for 6 h. The catalyst was removed by filtration and the filtrate was concentrated *in vacuo* to dryness. The product was dissolved in 10 m*l* dimethylformamide containing XVI (1.34 g, 2 mmol) and 1-hydroxybenzotriazole (0.27 g, 2 mmol) and cooled to 0°C.

To it was added (with stirring) a solution of dicyclohexylcarbodiimide (0.412 g, 2 mmol) in 2 ml dimethylformamide and stirred for 1 h at 0°C and overnight at room temperature. The solvent was removed *in vacuo* and the residue was triturated with $NaHCO_3$ solution and filtered. The product was washed with water and purified over LH-20 using methanol as the elution solvent. The purified product was rechromatographed to yield a pure peptide.

Yield 1.1 g (52%); m.p. 189–195°C (a very ill-defined m. p.); $[\alpha]_D^{24}$: −44.9° (c=0.705, in methanol).

Amino acid analysis

	Ser	Glu	Pro	Gly	Ala	Leu
Calcd.	2	3	2	5	2	4
Found	1.69	3.15	1.94	4.66	2	4.16

Tritylglycyl-glutaminyl-valyl-γ-t-butoxyglutamyl-leucyl-glycyl-glycyl-glycyl-prolyl-glycyl-alanyl-glycyl-O-t-butylseryl-leucyl-glutaminyl-prolyl-leucyl-alanyl-leucyl-γ-t-butoxyglutamyl-glycyl-O-t-butylseryl-leucyl-glutamine t-butyl ester (XXVIII)

To a solution of XXVII (900 mg, 0.42 mmol) in 10 ml acetic acid was added 10 ml water over a period of 1 h. The tritylcarbinol so precipitated during the reaction was removed by filtration and the filtrate was concentrated to dryness at 20°C. The residue was triturated with ether and filtered. The product was dissolved in 5 ml dimethylformamide and stored at −10°C for the azide synthesis. The azide was prepared from XIII (914 mg, 1 mmol) and isoamylnitrite (0.264 ml, 2 mmol) in 10 ml dimethylformamide at −20°C with 1.45 ml 2.75N HCl/tetrahydrofuran. After 1 h the reaction mixture was cooled to −40°C and neutralized with triethylamine (0.56 ml, 4 mmol). The solution of detritylated peptide XXVII was then added, and the mixture was stirred overnight at −10°C. The solvent was evaporated *in vacuo*, and the residue was treated with water, filtered and dried. The product was purified over Sephadex LH-20 column using methanol as an eluent. The desired fractions were pooled together and evaporated to dryness. Yield 800 mg (69%); m. p. 185–192°C (a poorly defined m. p.); $[\alpha]_D^{22}$: −49.9° (c=0.87, in methanol).

Amino acid analysis

	Ser	Glu	Pro	Gly	Ala	Val	Leu
Calcd.	2	5	2	7	2	1	5
Found	1.93	5	2.2	6.55	2	0.95	5.17

Benzyloxycarbonyl-γ-t-butoxyglutamyl-alanyl-γ-t-butoxyglutamyl-β-t-butoxyaspartyl-leucyl-glutaminyl-valyl-glycyl-glutaminyl-valyl-γ-t-butoxyglutamyl-leucyl-glycyl-glycyl-glycyl-prolyl-glycyl-alanyl-glycyl-O-t-butylseryl-leucyl-glutaminyl-prolyl-leucyl-alanyl-leucyl-γ-t-butoxyglutamyl-glycyl-O-t-butylseryl-leucyl-glutamine t-butyl ester (XXIX)

The detritylation of XXVIII (690 mg, 0.25 mmol) was done in 15 ml acetic acid by adding 15 ml water over a period of 1 h. The solution was concentrated to dryness *in vacuo* at 20°C. Finally 5 ml dimethylformamide was added and the solution was concentrated again to dryness to remove traces of acetic acid. The residue was triturated with ether, filtered and washed thoroughly with ether. The product was dissolved in 10 ml dimethylformamide and treated with pyridinium hydrochloride (115 mg, 1 mmol) and the solution was concentrated to dryness *in vacuo*. The residue was redissolved in 5 ml dimethylformamide and treated with triethylamine (0.14 ml, 1 mmol). A solution of IX (1.1 g, 1 mmol) and 1-hydroxybenzotriazole (270 mg, 2 mmol) in 10 ml dimethylformamide was added and the mixture was cooled to 0°C. To it was added dicyclohexylcarbodiimide (412 mg, 2 mmol), and the reaction mixture was stirred for 1 h at 0°C and at room temperature for 24 h. The solvent was removed *in vacuo*, and the residue was triturated with ether, filtered, washed with water, and dried. The crude product was dissolved in 10 ml dimethylformamide and poured into 50 ml water and kept at 4°C for 2 h. The product was filtered and dried. The final purification was done by gel chromatography over Sephadex LH-20 using methanol as the elution solvent.

Yield 530 mg (59%); m. p. 214–222°C (decomposed) a poorly defined m. p. $[\alpha]_D^{24}$: −28.2° (c=0.585, in dimethylformamide).

Amino acid analysis

	Asp	Ser	Glu	Pro	Gly	Ala	Val	Leu
Calcd.	1	2	8	2	7	3	2	6
Found	0.86	1.80	8	2.08	7.43	3.38	1.91	6.31

Glutamyl-alanyl-glutamyl-aspartyl-leucyl-glutaminyl-valyl-glycyl-glutaminyl-valyl-glutamyl-leucyl-glycyl-glycyl-glycyl-prolyl-glycyl-alanyl-glycyl-seryl-leucyl-glutaminyl-prolyl-leucyl-alanyl-leucyl-glutamyl-glycyl-seryl-leucyl-glutamine (XXX)

A 5 ml saturated solution of HBr/trifluoroacetic acid was added to the solution of XXIX (50 mg, 0.014 mmol) in 5 ml trifluoroacetic acid and kept for 5 min at room temperature. The HBr and trifluoroacetic acid were removed at room temperature under vacuum, the residue was triturated with ether, washed twice with ether by decantation, and finally dried *in vacuo* over NaOH. The product was dissolved in 3 ml 0.05M NH_4HCO_3 and applied over Sephadex G-25 fine column (1.5 × 98 cm) equilibrated with the same solution. After application of the sample, 5 ml fractions were collected. The peptide was located with Folin-Ciocalteu reagent[20]. The fractions containing C-

peptide were pooled and lyophilised. Yield 18 mg (43%) $[\alpha]_D^{22}$: -95.4^0 ($c=0.3$, in 0.05M NH_4HCO_3 solution).

Amino acid analysis

	Asp	Ser	Glu	Pro	Gly	Ala	Val	Leu
Calcd.	1	2	8	2	7	3	2	6
Found	1.01	1.79	8	1.79	7.03	3	2.03	6.31

(after leucine aminopeptidase digestion)

	Asp	Gln	Glu	Gly	Ala	Val	Leu
Calcd.	1	2	3	4	1	2	2
Found	1	1.52	3.30	2.59	1.33	1.71	2.24

The human C-peptide was shown to be homogeneous by thin-layer chromatography (Fig. 2) as well as by thin-layer electrophoresis (Fig. 3).