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Studies on Polypeptides, 1*

The Synthesis of C-Peptide of Porcine Proinsulin

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Summary: The synthesis of C-peptide of porcine proinsulin is described.

Untersuchungen an Polypeptiden, I. Die Synthese des C-Peptids des Schweineproinsulins

Zusammenfassung: Es wird die Synthese des C-Peptids des Schweineproinsulins beschrieben.

Proinsulin consists of A and B chains of insulin linked together by a connecting peptide[1]. The proteolytic cleavage of proinsulin yields insulin and C-peptide without the terminal basic residues[2–4]. The C-peptide is defined as the connecting peptide minus the pairs of basic residues. The comparison of C-peptide from human[5,6], porcine[7] and bovine[8] proinsulins shows considerably more differences in primary structure than occur in the insulin moiety of these species. The synthesis of the connecting peptide of porcine proinsulin has been reported[9,10]. This is the first of the series of articles dealing with the synthesis of peptides related to the C-peptide segment of proinsulin describing the total synthesis of C-peptide of porcine proinsulin.

The porcine C-peptide consists of 29 amino acids[7]. The presently accepted amino acid sequence of porcine C-peptide is shown in Fig. 1. It is devoid of basic amino acids as well as aromatic and sulfur-containing amino acids.

The synthesis of porcine C-peptide was carried out by classical methods of peptide synthesis. Four suitably protected main intermediates were prepared according to the routes illustrated in Schemes I–IV. The benzyloxycarbonyl and trityl groups were employed to protect the α-amino function of the intermediates[11]. The side-chain carboxyl functions were protected by t-butyl ester while terminal carboxyls were protected by methyl or *p*-nitrobenzyl or t-butyl ester.

The debenzyloxycarbonylation of some of the peptide fragments which were poorly soluble in acetic acid was carried out by HBr/trifluoroacetic acid treatment. This method was also found to be of advantage where the deblocked peptide derivatives were not precipitable on the addition of ether.

Abbreviations: OBu^t = t-butyl ester; ONBzl = *p*-nitrobenzyl ester; ONp = *p*-nitrophenyl ester; ONSu = *N*-hydroxysuccinimide ester; Trt = trityl; Z = benzyloxycarbonyl.

* 88th Paper on Peptides. 87th see Brandenburg, D. (1972) this J. **353**, 869–873.

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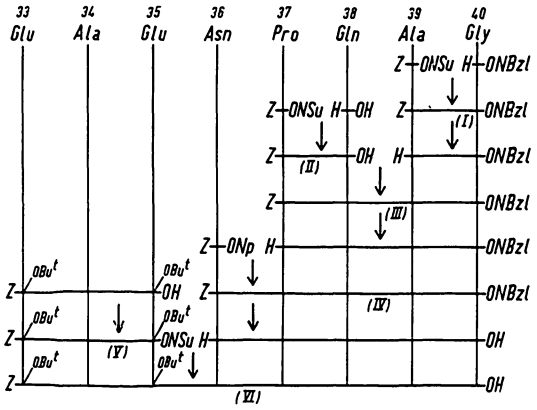
31 32 33 35 40 45 47
Arg-Arg ··· H-Glu-Ala-Glu-Asn-Pro-Gln-Ala-Gly-Ala-Val-Glu-Leu-Gly-Gly-Gly-
48 50 55 60 61 62 63
-Leu-Gly-Gly-Leu-Gln-Ala-Leu-Ala-Leu-Glu-Gly-Pro-Pro-Gln-OH ··· Lys-Arg

Fig. 1. The primary structure of porcine C-peptide.
The bold faced amino acid symbols are the part of connecting peptide.

Details of this procedure are described in the experimental part.

The various coupling methods employed in the synthesis of the C-peptide were chosen on the basis of their applicability at the particular steps to obtain the desired products in high yields and with minimum or no racemisation. The intermediate fragments were mainly prepared by *N*-hydroxysuccinimide ester[12] and mixed anhydride[13] methods. Fragment condensation was achieved essentially by the azide method[14,15]. However, one of the fragments with *C*-terminal glycine used in the synthesis of C-peptide was condensed by *N*-hydroxysuccinimide ester approach.



Scheme I: The synthesis of the protected fragment 33–40.

The Scheme I shows the synthesis of the protected *N*-terminal octapeptide (positions 33–40). Benzyloxycarbonylproline *N*-hydroxysuccinimide ester[12] was reacted with glutamine to yield dipeptide (II). The other dipeptide benzyloxycarbonylalanyl-glycine *p*-nitrobenzyl ester (I) was prepared by the condensation of benzyloxycarbonylalanine *N*-hydroxysuccinimide ester[12] with glycine *p*-nitrobenzyl ester[16]. It was then debenzyloxycarbonylated by HBr/acetic acid and the resulting derivative gave tetrapeptide (III) on condensation with the dipeptide (II) by the mixed anhydride method. The benzyloxycarbonyl group of the tetrapeptide (III) was removed with HBr/trifluoroacetic acid and the ensuing peptide, on reaction with benzyloxycarbonylasparagine *p*-nitrophenyl ester[17], gave pentapeptide (IV). The yield at this step was found to be poor. This pentapeptide was hydrogenated in acetic acid in the presence of palladium to deblock benzyloxycarbonyl and *p*-nitrobenzyl ester protecting groups. The crude product was contaminated with *p*-toluidine, the side product formed on catalytic reduction. Treatment with ethanol and ether mixture effectively removed most of the side product. The protected tripeptide benzyloxycarbonyl-γ-t-butoxyglutamyl-alanyl-γ-t-butoxyglutamate[18] was converted to *N*-hydroxysuccinimide ester (V) by dicyclohexylcarbodiimide/*N*-hydroxysuccinimide and reacted with the deblocked pentapeptide to yield the protected octapeptide (VI).

Scheme II outlines the synthesis of protected pentapeptide (positions 41–45). The mixed anhydride condensation of benzyloxycarbonyl-γ-t-butoxyglutamate[19,20] with leucyl-glycine methyl ester gave tripeptide benzyloxycarbonyl-γ-t-butoxyglutamyl-leucyl-glycine methyl ester (VII). The dipeptide

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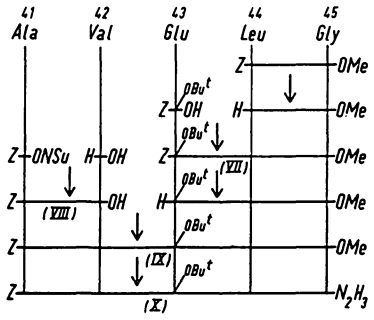


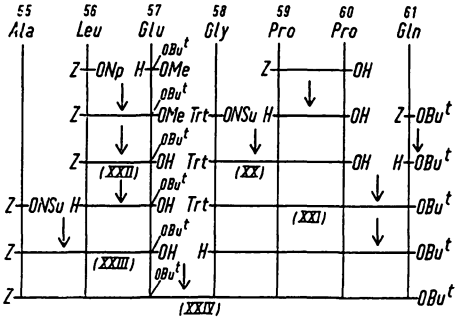
Scheme II. Synthesis of the protected fragment 41 – 45.

benzyloxycarbonylalanyl-valine (VIII) was synthesised by the condensation of benzyloxycarbonylalanine *N*-hydroxysuccinimide ester[12] with valine. Subsequently, the condensation of the dipeptide (VIII) with the peptide, obtained by catalytic reduction of protected tripeptide (VII), using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole[21] as the condensation reagent, yielded pentapeptide ester (IX). Exposure of the protected methyl ester (IX) to hydrazine hydrate yielded the hydrazide (X).

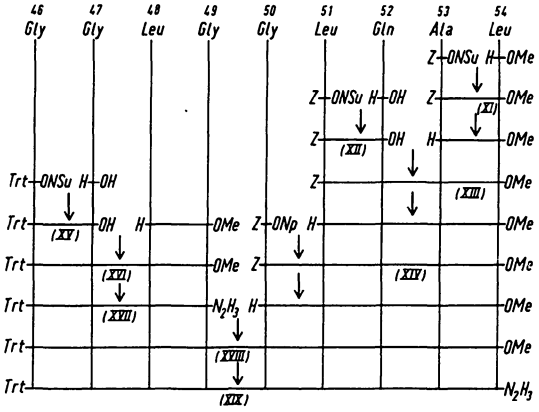
For the synthesis of the protected nonapeptide (positions 46 – 54) Scheme III was followed. Benzyloxycarbonylleucine *N*-hydroxysuccinimide ester[12] was reacted with glutamine to give benzyloxycarbonylleucyl-glutamine (XII). In another reaction, benzyloxycarbonylalanine *N*-hydroxysuccinimide ester[12] was condensed with leucine methyl ester[22] to give dipeptide (XI). This was then debenzyloxycarbonylated by catalytic reduction and condensed with the mixed anhydride of dipeptide (XII) to yield tetrapeptide (XIII).

The benzyloxycarbonyl group was removed by HBr/trifluoroacetic acid treatment and the resulting peptide was then condensed with benzyloxycarbonylglycine *p*-nitrophenyl ester[23] to give pentapeptide methyl ester (XIV).

The *N*-terminal sequence of the fragment 46 – 54 was synthesised by reacting tritylglycine *N*-hydroxysuccinimide ester[12] with glycine. The resulting dipeptide tritylglycyl-glycine (XV) was condensed with leucyl-glycine methyl ester to yield tetrapeptide methyl ester (XVI). The methyl ester (XVI) on treatment with hydrazine hydrate gave the hydrazide (XVII). This on azide condensation with the debenzyloxycarbonylated pentapeptide (XIV) yielded the trityl nonapeptide methyl ester (XVIII). Finally hydrazinolysis of the methyl ester (XVIII) gave the hydrazide (XIX).



Scheme IV. The synthesis of fragment 55 – 61.



Scheme III. The synthesis of fragment 46 – 54.

The synthesis of the *C*-terminal heptapeptide 55 to 61 was achieved as shown in Scheme IV. The tritylglycyl-prolyl-proline (XX) was next prepared by the condensation of tritylglycine *N*-hydroxysuccinimide ester[12] with prolyl-proline. The tripeptide (XX) was contaminated with tritylglycine which could not be removed by fractional crystallization. The product was ultimately purified by counter current distribution. On mixed anhydride condensation with glutamine t-butyl ester, this purified tripeptide (XX) yielded tetrapeptide tritylglycyl-

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

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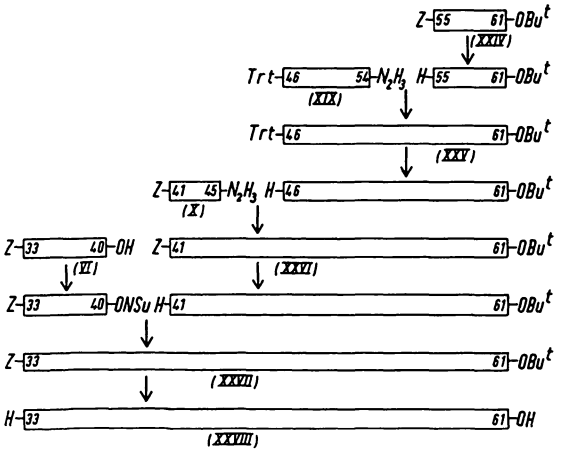
[23] Bodanszky, M. & Du Vigneaud, V. (1962) Biochem. Prep **9**, 110 – 112.

prolyl-prolyl-glutamine t-butyl ester (XXI). The protected tripeptide benzyloxycarbonylalanyl-leucyl-γ-t-butoxyglutamate (XXIII) was prepared by two stepwise condensations as shown in Scheme IV. The mixed anhydride condensation of tripeptide (XXIII) with the detritylated tetrapeptide (XXI) gave heptapeptide (XXIV).

Finally, Scheme V illustrates the synthesis of C-peptide. The benzyloxycarbonyl group of fragment (XXIV) was removed by hydrogenolysis followed by azide condensation with fragment (XIX) to yield the trityl-hexadecapeptide (XXV). On detritylation, it gave a peptide with very poor solubility.



Scheme V. The total synthesis of porcine C-peptide.

Attempts to dissolve this peptide in dimethylformamide or hexamethylphosphoric triamide or a mixture of dimethylformamide/hexamethylphosphoric triamide proved unsuccessful. The solubility problem, however, was overcome by using dimethylsulfoxide as a solvent. For azide coupling dimethylsulfoxide solution was diluted with an equal volume of dimethylformamide and the reaction was carried out at -10^0C. Fragment (X) was reacted with the detritylated hexadecapeptide (XXV) using the azide method to give benzyloxycarbonyl-uneicosapeptide (XXVI). The *N*-terminal octapeptide (VI) was converted to *N*-hydroxysuccinimide ester by dicyclohexylcarbodiimide/*N*-hydroxysuccinimide and condensed with the debenzyloxycarbonylated uneicosapeptide (XXVI) to yield the protected crude C-peptide (XXVII). It was contaminated with unreacted amine as well as with carboxylic component.

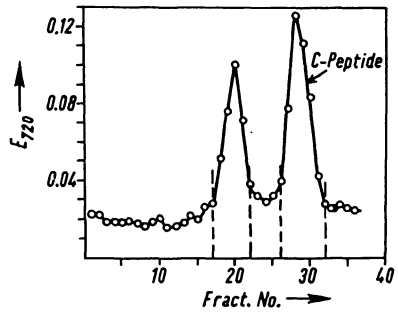


Fig. 2. The elution pattern of C-peptide.

Subsequently, the protecting benzyloxycarbonyl and t-butoxy groups from crude protected C-peptide were removed by HBr/trifluoroacetic acid treatment. The purification of C-peptide (XXVIII) was achieved by ion exchange chromatography with DEAE-cellulose using a linear gradient of 0.05M and 0.3M NH_4HCO_3 solutions, 250 ml each. The C-peptide fraction was located by the Folin-Ciocalteu reagent[24] and the elution pattern is shown in Fig. 2.

The C-peptide was shown to be homogeneous by paper electrophoresis in 30% acetic acid as well as barbital buffer, pH 8.6.

The purity of synthetic C-peptide was checked by amino acid analysis. The amino acid analysis of the C-peptide after acid hydrolysis gave a composition in molar ratios consistent with the theoretically expected values.

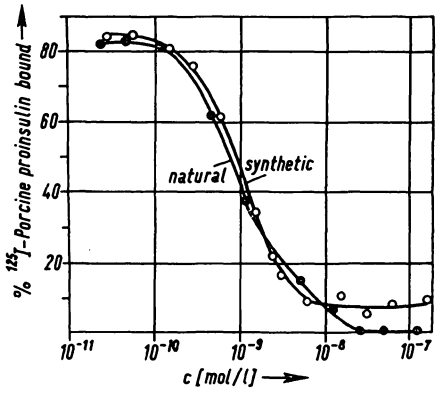


Fig. 3. The immunoassays on the synthetic porcine C-peptide (o——o) and the natural porcine C-peptide (•——•) using anti porcine proinsulin serum.

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

The identity of C-peptide was also established by immunoassay with an antiserum specific for porcine proinsulin. The corresponding data are given in Fig. 3. The synthetic C-peptide cross-reacted with the antibodies in nearly the same fashion as the natural porcine C-peptide.

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Experimental

All optically active amino acids are of L-configuration. The melting points are uncorrected. Thin-layer chromatography (Kieselgel G, Merck) was performed in the following solvent systems.

SBA: 2-butanol/formic acid/water 75:13.5:11.5 (V/V)
SBN: 2-butanol/ammonia (10%) 85:15 (V/V)
CMA: chloroform/methanol/acetic acid 95:5:3 (V/V)
HBP: n-heptane/pyridine/t-butanol 75:15:15 (V/V).

All the *N*-hydroxysuccinimide esters used in the synthesis of C-peptide were freshly prepared. Unless otherwise stated peptides were hydrolysed with 6N HCl in sealed tubes for 24 h at 110°C.

Benzyloxycarbonylalanyl-glycine p-nitrobenzyl ester (I)

To a suspension of 17.2 g (50 mmol) Z-Gly-ONBzl[16] in 30 ml acetic acid was added 30 ml solution of 40% HBr/acetic acid. After 1 h at room temperature 500 ml ether was added and the precipitated product was filtered, washed three times with ether and dried *in vacuo* over NaOH. The resulting hydrobromide was suspended in 180 ml chloroform and treated with 6.95 ml (50 mmol) triethylamine. To this solution was added 16 g (50 mmol) Z-Ala-ONSu[12] in 100 ml tetrahydrofuran and the reaction mixture was stirred overnight. The solvent was removed *in vacuo*, the solid residue was taken up in ethyl acetate which was washed successively with 1N HCl, saturated NaCl solution, $NaHCO_3$ solution and dried over Na_2SO_4. The solvent was evaporated and the product was crystallized from ethyl acetate/petrol ether. Yield 18.2 g (88%); m. p. 110 to 111°C.

$[\alpha]_D^{24}$: −19.45° (c=2.344, in methanol).
$C_{20}H_{21}N_3O_7$ (415.41)
Calcd. C 57.82 H 5.09 N 10.11
Found C 58.09 H 5.24 N 10.28

Benzyloxycarbonylprolyl-glutamine (II)

To a stirred solution of 32.12 g (220 mmol) glutamine and 36.96 g (440 mmol) $NaHCO_3$ in 500 ml water and 100 ml dioxane was added a solution of 69.2 g (200 mmol) Z-Pro-ONSu[12] in 400 ml dioxane. After 24 h the solution was concentrated under vacuum to 300 ml. Acidification with concentrated HCl gave an oil. It was extracted with ethyl acetate and the organic layer was washed with saturated NaCl solution, quickly dried over Na_2SO_4 and evaporated *in vacuo* to give a solid residue. It was filtered, washed with ether and crystallized from methanol. Yield 65.2 g (74%); m. p. 104−108°C; $[\alpha]_D^{23}$: −29.84° (c=2.912, in dimethylformamide).

$C_{18}H_{23}N_3O_6$ (377.35)
Calcd. C 57.29 H 6.14 N 11.13
Found C 56.98 H 6.60 N 10.98

Benzyloxycarbonylprolyl-glutaminyl-alanyl-glycine p-nitrobenzyl ester (III)

The benzyloxycarbonyl group was removed by HBr/acetic acid treatment of 8.3 g (20 mmol) I in the usual manner and the resulting dipeptide hydrobromide was dissolved in 30 ml absol. dimethylformamide, treated with 2.16 ml (20 mmol) *N*-methylmorpholine and stored at 0°C for coupling with the mixed anhydride.

A mixed anhydride was prepared by adding 2.6 ml (20 mmol) isobutylchloroformate at −10°C to a stirred solution of 7.54 g (20 mmol) II and 2.16 ml (20 mmol) *N*-methylmorpholine in 40 ml absol. dimethylformamide. After activation for 2 min the chilled solution of the amine component mentioned above was added and the reaction mixture was stirred for 1 h at 0°C and then for another hour at room temperature. The reaction mixture was concentrated to 20 ml and poured into 300 ml water. The product was collected by filtration, washed two times with warm methanol and crystallized from dimethylformamide/water mixture. Yield 6.9 g (52%); m. p. 229 − 230°C with sintering at 221°C; $[\alpha]_D^{22}$: −28.8 (c=0.778 in dimethylformamide).

$C_{30}H_{36}N_6O_{10} \times 1H_2O$ (658.68)
Calcd. C 54.70 H 5.81 N 12.76
Found C 54.60 H 5.80 N 12.18

Amino acid analysis

	Glu	Pro	Gly	Ala
Calcd.	1	1	1	1
Found	1	0.86	1.01	0.98

Benzyloxycarbonylasparaginyl-prolyl-glutaminyl-alanyl-glycine p-nitrobenzyl ester (IV)

13.16 g (20 mmol) III was dissolved in 25 ml trifluoroacetic acid and dry HBr was passed through the solution for 30 min. The trifluoroacetic acid and HBr were removed *in vacuo* and the residue was triturated with absol. ether. The product was washed with ether and decanted twice and dried *in vacuo* over NaOH. The hydrobromide was dissolved in 100 ml dimethylformamide and neutralised with 2.78 ml (20 mmol) triethylamine. 7.74 g (20 mmol) Z-Asn-ONp[17] was added to this reaction mixture and allowed to react for 48 h. The solvent was concentrated *in vacuo* to 25 ml and diluted with 150 ml water. An oil was separated which solidified on cooling and scratching. The product was crystallized from dimethylformamide/water/methanol mixture. Yield 6.2 g (41%); m. p. 235–237°C; $[\alpha]_D^{22}$: −63.2° (c=1.048, in dimethylformamide).

$C_{34}H_{42}N_8O_{12}$ (754.77)

Calcd. C 54.10 H 5.60 N 14.84
Found C 54.04 H 5.53 N 14.26

Amino acid analysis

	Asp	Glu	Pro	Ala	Gly
Calcd.	1	1	1	1	1
Found	1	1	0.98	1	0.98

Benzyloxycarbonyl-γ-t-butoxyglutamyl-alanyl-γ-t-butoxyglutamyl-asparaginyl-prolyl-glutaminyl-alanyl-glycine (VI)

A solution of 4.5 g (6 mmol) IV in 100 ml acetic acid was hydrogenated with 2 g Pd-catalyst for 30 h. The catalyst was filtered and the filtrate was evaporated *in vacuo* to dryness. The residue was triturated with absol. ethanol/ether 1:1, filtered, washed with ether and dried.

To a stirred solution of 3.54 g (6 mmol) Z-Glu(OBu^t)-Ala-Glu(OBu^t)-OH[18] and 0.69 g (6 mmol) *N*-hydroxysuccinimide in 35 ml tetrahydrofuran at 0°C a solution of 1.23 g (6 mmol) dicyclohexylcarbodiimide in 10 ml tetrahydrofuran was added dropwise. After 4 h the dicyclohexylurea was removed by filtration, the filtrate concentrated *in vacuo* to dryness and the residue washed three times with petrol ether by decantation. The crude active ester was dissolved in 30 ml dioxane and added to the solution of amine, mentioned above, in 30 ml water and 20 ml dioxane mixture containing 1 g $NaHCO_3$ and the reaction mixture was stirred overnight. The solution was concentrated *in vacuo* to 20 ml and acidified with dilute acetic acid. The product was filtered, washed with cold water and crystallized from a mixture of methanol/water (1:1).

Yield 3.5 g (55%); m. p. 214–216°C; $[\alpha]_D^{25}$: −104.65° (c=0.795, in acetic acid).

$C_{48}H_{72}N_{10}O_{17}$ (1061.18)

Calcd. C 54.32 H 6.80 N 13.20
Found C 53.99 H 6.80 N 13.30

Amino acid analysis

	Asp	Glu	Pro	Ala	Gly
Calcd.	1	3	1	2	1
Found	1	3.04	1.04	2.02	0.98

Benzyloxycarbonyl-γ-t-butoxyglutamyl-leucyl-glycine methyl ester (VII)

A mixed anhydride was prepared in the usual manner from 33.73 g (100 mmol) Z-Glu(OBu^t)-OH[19,20] and 10.8 ml (100 mmol) *N*-methylmorpholine in 200 ml tetrahydrofuran at −10°C with 13 ml (100 mmol) isobutylchloroformate. To this were added a solution of a hydrobromide, obtained from 33.6 g (100 mmol) Z-Leu-Gly-OMe[25] by HBr/trifluoroacetic acid treatment as described in the preparation of IV, and 10.8 ml (100 mmol) N-methylmorpholine in 60 ml absol. dimethylformamide. The mixture was stirred for 1 h at 0°C and at room temperature for 1 h. The solvent was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and the organic layer was washed in the usual manner and dried. The solvent was removed *in vacuo* and the product was crystallized from ethyl acetate/petrol ether. Yield 46.5 g (89%); m. p. 143–145°C; $[\alpha]_D^{24}$: −48.0° (c=1.058, in methanol).

$C_{26}H_{39}N_3O_8$ (521.62)

Calcd. C 59.86 H 7.53 N 8.05
Found C 60.14 H 7.59 N 8.41

Amino acid analysis

	Glu	Gly	Leu
Calcd.	1	1	1
Found	1.03	0.98	1

Benzyloxycarbonylalanyl-valine (VIII)

To a suspension of 12.88 g (110 mmol) valine and 18.48 g (220 mmol) $NaHCO_3$ in 350 ml water and 150 ml dioxane 32 g (100 mmol) Z-Ala-ONSu[12] in 150 ml dioxane was added and the reaction stirred for two days. The aqueous phase was concentrated *in vacuo* to 200 ml, then diluted with water to 500 ml and extracted with ethyl acetate. The aqueous phase was acidified with concentrated HCl to pH 2 to give an oily product. The oily product was extracted into ethyl acetate which was washed with water and dried over Na_2SO_4. The solvent was evaporated and

[25] Determann, H., Zipp, O. & Wieland, T. (1962) *Liebigs Ann. Chem.* **651**, 172–184.

the oily product was dissolved in 100 ml ether, treated with 20 ml dicyclohexylamine and kept at 4°C for 4 h. The solid product was collected by filtration and crystallized from ethyl acetate. Yield 31 g (61%); m. p. 123°C; $[\alpha]_D^{22}$: −22.8° (c = 1.064, in methanol).

$C_{28}H_{45}N_3O_8 \times {}^1/_2H_2O$ (512.71)

Calcd.	C 65.59	H 9.04	N 8.19
Found	C 65.90	H 8.81	N 7.90

5.12 g of the dicyclohexylammonium salt was suspended in 30 ml ethyl acetate, treated with dilute H_2SO_4, washed with water and dried over Na_2SO_4. The ethyl acetate layer was evaporated and the product was crystallized from ethyl acetate/petrol ether. Yield 2.5 g (77%); m. p. 152.5−153.5°C; $[\alpha]_D^{23}$: −44.82° (c = 1.932, in methanol).

$C_{16}H_{22}N_2O_5$ (322.37)

Calcd.	C 59.63	H 6.88	N 8.69
Found	C 59.99	H 6.96	N 9.06

Benzyloxycarbonylalanyl-valyl-γ-t-butoxyglutamyl-leucyl-glycine methyl ester (IX)

5.21 g (10 mmol) VII was hydrogenated in 300 ml methanol with 2 g Pd for 8 h. The catalyst was removed by filtration and the solvent evaporated *in vacuo* to dryness. The residue was dissolved in 30 ml dimethylformamide containing 1.35 g (10 mmol) 1-hydroxybenzotriazole[21] and 3.22 g (10 mmol) VIII. To this a solution of 2.06 g (10 mmol) dicyclohexylcarbodiimide in 10 ml tetrahydrofuran was added and the reaction mixture was stirred overnight. The dicyclohexylurea was filtered, washed with dimethylformamide and the combined filtrates were evaporated to dryness. The solid residue was triturated with ether, filtered and crystallized from methanol/ether. Yield 5.4 g (78%); m. p. 235−236.5°C; $[\alpha]_D^{22}$: −19.9° (c = 1.058, in dimethylformamide).

$C_{34}H_{53}N_5O_{10}$ (691.84)

Calcd.	C 59.02	H 7.72	N 10.12
Found	C 58.72	H 7.43	N 10.08

Amino acid analysis (with 6N HCl at 110°C for 36 h)

	Glu	Gly	Ala	Val	Leu
Calcd.	1	1	1	1	1
Found	1	0.95	1	1	1.03

Benzyloxycarbonylalanyl-valyl-γ-t-butoxyglutamyl-leucyl-glycine hydrazide (X)

0.69 g (1 mmol) IX was dissolved in 20 ml warm methanol and 1 ml hydrazine hydrate added. The reaction mixture was stored overnight. The solvent was evaporated, the oily residue on trituration with ether became solid. The solid was filtered and crystallized from methanol/ether. Yield 0.50 g (72%); m. p. 203−204°C; $[\alpha]_D^{24}$: −54.0° (c=0.80, in methanol).

$C_{33}H_{53}N_7O_9$ (691.84)

Calcd.	C 57.29	H 7.72	N 14.17
Found	C 57.09	H 7.33	N 13.73

Benzyloxycarbonylalanyl-leucine methyl ester (XI)

64.06 g (200 mmol) Z-Ala-ONSu[12] was added to a solution of 36.32 g (200 mmol) HCl-Leu-OMe[22] and 27.8 g (200 mmol) triethylamine in 500 ml chloroform and the reaction mixture was stirred overnight. The solvent was removed *in vacuo*, the oily residue was taken up in ether which was washed successively with solutions of 1N HCl, water, $NaHCO_3$, water and dried over Na_2SO_4. The ether was evaporated to give an oil. Yield 58 g (83%).

The dipeptide was characterised *via* its dicyclohexylammonium salt. 3.5 g (10 mmol) Z-Ala-Leu-OMe was saponified in 15 ml methanol with 1N NaOH using thymolphthaleine as an indicator. The reaction mixture was acidified with 1N HCl, extracted with ether which was washed with water and dried over Na_2SO_4. The solvent was removed *in vacuo*, the oily product was dissolved in 15 ml ether, treated with 1.96 ml dicyclohexylamin and kept at 4°C. The solid product was collected by filtration and crystallized from methanol/ether. Yield 3.2 g (62%); m. p. 163−165°C; $[\alpha]_D^{23}$: −18.2° (c = 2.318, in ethanol); Lit.[26] m. p. 163°C; $[\alpha]_D^{27}$: −16.5° (c=1, in ethanol).

$C_{29}H_{47}N_3O_5$ (517.72)

Calcd.	C 67.27	H 9.15	N 8.11
Found	C 67.14	H 9.05	N 8.18

Benzyloxycarbonylleucyl-glutamine (XII)

XII was prepared by essentially the same procedure as described for II and crystallized from ethyl acetate/isopropanol/ether. Yield 85%; m. p. 131−132°C; $[\alpha]_D^{22}$: −28.0° (c=0.846, in methanol).

$C_{29}H_{27}N_3O_6$ (393.45)

Calcd.	C 58.00	H 6.91	N 10.68
Found	C 57.54	H 6.79	N 10.70

Benzyloxycarbonylleucyl-glutaminyl-alanyl-leucine methyl ester (XIII)

17.50 g (50 mmol) Z-Ala-Leu-OMe was hydrogenated in methanol containg 3 ml acetic acid with Pd for 15 h. The catalyst was removed by filtration and the solution evaporated to dryness *in vacuo*. It was dissolved in 50 ml absol. tetrahydrofuran containing 6.95 ml (50 mmol) triethylamine and stored at 0°C for coupling with the mixed anhydride. A mixed anhydride was prepared by adding dropwise 6.5 ml (50 mmol) isobutylchloroformate to the solution of 19.65 g (50 mmol) Z-Leu-Gln-OH and 5.4 ml (50 mmol)

[26] Klieger, E., Schröder, E. & Gibian, H. (1961) *Liebigs Ann. Chem.* **640**, 157−167.

N-methylmorpholine in 80 ml dimethylformamide at −10°C and allowed to react for 2 min. To this the above mentioned amine solution was added and the reaction mixture was stirred for 1 h at 0°C and for 1 h at room temperature. The solvent was removed *in vacuo* and the solid residue triturated with ether, filtered and washed with ether. The product was crystallized from methanol/ether. Yield 20.6 g (69%); m. p. 226 to 229°C; $[\alpha]_D^{24}$: −25.37° ($c = 0.865$, in dimethylformamide).

$C_{29}H_{45}N_5O_8$ (591.72)

Calcd.	C 58.86	H 7.66	N 11.83
Found	C 58.98	H 7.66	N 11.80

Amino acid analysis

	Glu	Ala	Leu
Calcd.	1	1	2
Found	1	1	2.06

Benzyloxycarbonylglycyl-leucyl-glutaminyl-alanyl-leucine methyl ester (XIV)

The benzyloxycarbonyl group from 0.59 g (1 mmol) XIII was removed by HBr/trifluoroacetic acid treatment as described in the preparation of IV and the resulting peptide was dissolved in 15 ml dimethylformamide. To the solution were added 0.14 ml (1 mmol) triethylamine and 0.32 g (1 mmol) Z-Gly-ONp[23] and allowed to react overnight. The reaction mixture was concentrated *in vacuo* and the solid product was triturated with ether and filtered. The crude product was crystallized from methanol. Yield 0.48 g (74%); m. p. 228 to 232°C; $[\alpha]_D^{24}$: −31.4° ($c = 1.882$, in dimethylformamide).

$C_{31}H_{48}N_6O_9$ (648.77)

Calcd.	C 57.39	H 7.45	N 12.95
Found	C 57.41	H 7.39	N 13.27

Amino acid analysis

	Glu	Gly	Ala	Leu
Calcd.	1	1	1	2
Found	1	1.05	0.98	2.02

Tritylglycyl-glycine (XV)

A solution of 20.7 g (50 mmol) Trt-Gly-ONSu[12] in 150 ml dioxane was added to a solution of 7.5 g (100 mmol) glycine and 16.8 g (400 mmol) $NaHCO_3$ in 300 ml water and 150 ml dioxane. After 24 h the solution was concentrated under vacuum to 200 ml. Acidification with dilute acetic acid gave a solid product which was filtered and washed thoroughly with water and dried. The product was crystallized from acetone/petrol ether. Yield 15 g (80%); m. p. 191−193°C; Lit.[27] m. p. 180°C.

[27] Zervas, L. & Theodoropoulos, D. M. (1956) *J. Amer. Chem. Soc.* **78**, 1359−1363.

$C_{23}H_{22}N_2O_3$ (374.45)

Calcd.	C 73.77	H 5.92	N 7.48
Found	C 73.46	H 5.98	N 7.29

Tritylglycyl-glycyl-leucyl-glycine methyl ester (XVI)

A solution of 1.12 g (3 mmol) XV and 0.42 ml (3 mmol) triethylamine in 10 ml chloroform was cooled to −10°C, 0.39 ml (3 mmol) isobutylchloroformate was added and the mixture was stirred for 2 min. To this were added a solution of the amine, obtained by debenzyloxycarbonylation of 1.1 g (3.3 mmol) Z-Leu-Gly-OMe[25] by HBr/trifluoroacetic acid treatment, and 0.46 ml (3.3 mmol) triethylamine and the resulting mixture was stirred for 1 h at 0°C and at room temperature for 1 h. The solvent was removed *in vacuo*. The solid product so obtained was washed with methanol and crystallized from chloroform/ether. Yield 0.9 g (54%); m. p. 247−248°C; $[\alpha]_D^{22}$: −9.48° ($c = 0.872$, in dimethylformamide).

$C_{32}H_{38}N_4O_5$ (558.69)

Calcd.	C 68.79	H 6.85	N 10.02
Found	C 68.92	H 6.80	N 10.06

Amino acid analysis

	Gly	Leu
Calcd.	3	1
Found	2.99	1

Tritylglycyl-glycyl-leucyl-glycine hydrazide (XVII)

2 ml hydrazine hydrate was added to a solution of 5.6 g (10 mmol) XVI in 30 ml dimethylformamide and 10 ml methanol. The mixture was stored at room temperature for 48 h and the solution diluted with saturated NaCl solution and kept at 4°C for 4 h. The product was filtered, washed with water and crystallized from dimethylformamide/water. Yield 4.1 g (73%); m. p. 224−227°C; $[\alpha]_D^{23}$: 10.88° ($c = 0.90$, in dimethylformamide).

$C_{31}H_{38}N_6O_4$ (558.69)

Calcd.	C 66.64	H 6.85	N 15.04
Found	C 66.70	H 6.94	N 14.82

Tritylglycyl-glycyl-leucyl-glycyl-glycyl-leucyl-glutaminyl-alanyl-leucine methyl ester (XVIII)

The benzyloxycarbonyl group of 2.26 g (4 mmol) XIV was removed by HBr/trifluoroacetic acid treatment as described in the preparation of IV and the resulting peptide was dissolved in 10 ml dimethylformamide and treated with 0.56 ml (4 mmol) triethylamine and stored at −20°C for the azide reaction. A solution of 2.23 g (4 mmol) XVII in 20 ml dimethylformamide was cooled to −20°C. To this 0.792 ml (6 mmol) isoamylnitrite and 7 ml 2.3N HCl/tetrahydrofuran solution

were added. After 1 h the reaction mixture was cooled to -40^0C and neutralised with 2.2 ml triethylamine. The amine solution was added and the mixture stirred overnight. The solvent was removed *in vacuo*, the solid residue was triturated with ether, filtered and washed with warm ethyl acetate followed by water and dried. The product was crystallized from methanol/ether. Yield 3 g (74%); m. p. 235–237°C; $[\alpha]_D^{26}$: -20.05^0 ($c=1.22$, in dimethylformamide).

$C_{54}H_{76}N_{10}O_{11}$ (1041.28)

Calcd.	C 62.28	H 7.35	N 13.45
Found	C 61.65	H 7.28	N 13.18

Amino acid analysis

	Glu	Gly	Ala	Leu
Calcd.	1	4	1	3
Found	1	4.01	1.05	3

Tritylglycyl-glycyl-leucyl-glycyl-glycyl-leucyl-glutaminyl-alanyl-leucine hydrazide (XIX)

1 ml hydrazine hydrate was added to a solution of 2.6 g (2.5 mmol) XVIII in 5 ml dimethylformamide and 10 ml methanol. After 48 h water was added and the product filtered and dried. Yield 2.2 g (83%); m. p. 243 to 249°C (dec.); $[\alpha]_D^{26}$: -14.6^0 ($c=1.05$ in dimethylformamide).

$C_{53}H_{76}N_{12}O_{10} \times 1^1/_2\ H_2O$ (1068.31)

Calcd.	C 59.58	H 7.64	N 15.73
Found	C 59.62	H 7.44	N 14.92

Tritylglycyl-prolyl-proline (XX)

3.46 g (10 mmol) Z-Pro-Pro-OH[28] was debenzyloxycarbonylated by HBr/trifluoroacetic acid treatment as described in the preparation of IV. The crude hydrobromide and 2.5 g (30 mmol) $NaHCO_3$ were dissolved in 20 ml water and 10 ml dioxane. To this a solution of 4.14 g (10 mmol) Trt-Gly-ONSu[12] in 20 ml dioxane was added. After reacting for 24 h the mixture was acidified with dilute acetic acid and extracted with ethyl acetate. The extract was washed with water, dried over Na_2SO_4 and the solvent was removed completely *in vacuo*. The product was purified by counter current distribution (240 cycles, $K=0.36$) using the following solvent system:

chloroform/carbon tetrachloride/methanol/water 5:5:8:2. The product was crystallized from carbon tetrachloride/petrol ether. Yield 3.2 g (61%); m. p. 119–122°C; $[\alpha]_D^{24}$: -74.0^0 ($c=1.0$, in methanol).

$C_{31}H_{33}N_3O_4 \times 1/_2\ H_2O$ (520.54)

Calcd.	C 71.51	H 6.58	N 8.07
Found	C 71.22	H 6.63	N 7.83

Tritylglycyl-prolyl-prolyl-glutamine t-butyl ester (XXI)

0.67 g (2 mmol) Z-Gln-OBu^t[29] was hydrogenated in 30 ml methanol containg 0.33 ml 6N HCl in the presence of 0.5 g Pd. After 1 h a drop of pyridine was added and the catalyst was filtered. The filtrate was concentrated *in vacuo* to dryness. A mixed anhydride was prepared in the usual manner from 1.04 g (2 mmol) Trt-Gly-Pro-Pro-OH and 0.278 ml (2 mmol) triethylamine in 10 ml tetrahydrofuran at -10^0C with 0.26 ml (2 mmol) isobutylchloroformate. To this the cooled solution of the above mentioned hydrochloride and 0.278 ml triethylamine in 5 ml dimethylformamide were added. The reaction mixture was stirred for 1 h at 0°C and at room temperature for 1 h. The solvent was removed *in vacuo*, the oily residue taken up in ethyl acetate, washed with dilute acetic acid, water, dilute NH_4OH, water and dried over Na_2SO_4. The solvent was evaporated and the residue crystallized from carbon tetrachloride/petrol ether. Yield 0.82 g (59%); m. p. 102–106°C (a very ill defined melting point); $[\alpha]_D^{28}$: -87.1^0 ($c=0.702$, in methanol).

$C_{40}H_{49}N_5O_6$ (695.87)

Calcd.	C 69.03	H 7.09	N 10.06
Found	C 68.45	H 6.90	N 10.13

Amino acid analysis

	Glu	Pro	Gly
Calcd.	1	2	1
Found	1	1.99	0.90

Benzyloxycarbonylleucyl-γ-t-butoxyglutamate (XXII)

14.44 g (40 mmol) Z-Leu-ONp[17] was added to a solution of 10.12 g (40 mmol) HCl-Glu(OBu^t)-OMe[18] and 5.56 ml (40 mmol) triethylamine. After stirring for 24 h the solvent was removed *in vacuo*. The oily residue was taken up in ether which was washed with cold diluted H_2SO_4, water, $NaHCO_3$ solution, water and dried over Na_2SO_4. The ether layer was evaporated to give an impure oil. Crude yield 15.2 g (82%). The oil was dissolved in 30 ml methanol and saponified with 1N NaOH using thymolphthaleine as indicator, acidified with cold dilute H_2SO_4 and extracted with ethyl acetate. The organic layer was washed with water, dried over Na_2SO_4 and evaporated *in vacuo* to give an oily product which solidified on scratching under petrol ether. Yield 9 g (50%); m. p. 55–57°C; $[\alpha]_D^{28}$: -18.4^0 ($c=0.96$, in methanol).

$C_{23}H_{34}N_2O_7$ (450.54)

Calcd.	C 61.31	H 7.60	N 6.21
Found	C 60.70	H 7.25	N 5.79

[28] Lande, S. (1962) *J. Org. Chem.* **27**, 4558–4562.

[29] Schnabel, E. (1966) *Liebigs Ann. Chem.* **696**, 220–225.

Benzyloxycarbonylalanyl-leucyl-γ-t-butoxyglutamate (XXIII)

A solution of 4.5 g (10 mmol) XXII in 150 ml 90% methanol was hydrogenated with 1 g Pd for 10 h. The catalyst was filtered and the filtrate was concentrated *in vacuo* to dryness. The residue was dissolved in 30 ml dioxane and 30 ml water. 1.64 g $NaHCO_3$ and 3.2 g (10 mmol) Z-Ala-ONSu[12] were added and the mixture was stirred for 24 h. The dioxane was removed *in vacuo* and the aqueous phase diluted with water and extracted with ethyl acetate. The aqueous phase was acidified with cold dilute H_2SO_4 and extracted with ethyl acetate. The organic layer was washed with NaCl solution and dried over Na_2SO_4. The solvent was evaporated to give a solid residue which was crystallized from isopropyl ether/petrol ether. Yield 3.8 g (73%); m. p. 149.5–151.5°C; $[\alpha]_D^{28}$: −44.8° (c=0.944, in methanol).

$C_{26}H_{39}N_3O_8$ (521.62)

Calcd.	C 59.86	H 7.53	N 8.05
Found	C 59.85	H 7.55	N 8.38

Benzyloxycarbonylalanyl-leucyl-γ-t-butoxyglutamyl-glycyl-prolyl-prolyl-glutamine t-butyl ester (XXIV)

To a solution of 0.695 g (1 mmol) XXI in 5 ml acetic acid at room temperature 5 ml water was added dropwise over a period of 1 h. The precipitated tritylcarbinol was filtered and the filtrate was lyophylized. A mixed anhydride was prepared in the usual manner from 0.52 g (1 mmol) Z-Ala-Leu-Glu(OBu^t)-OH and 0.10 ml *N*-methylmorpholine in 5 ml absol. tetrahydrofuran at −10°C with 0.13 ml (1 mmol) isobutylchloroformate. The chilled solution of the above mentioned amine, 0.10 ml (1 mmol) *N*-methylmorpholine in 3 ml dimethylformamide and 3 ml tetrahydrofuran were added. 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 triturated under ether when the product became solid. The solid residue was filtered, washed with ether, then with water and dried. The product was crystallized from ethyl acetate. Yield 0.75 g (78%); m. p. 137–138°C; $[\alpha]_D^{22}$: −121.8° (c=0.852, in methanol).

$C_{47}H_{72}N_8O_{13}$ (957.16)

Calcd.	C 58.96	H 7.58	N 11.70
Found	C 58.96	H 7.17	N 12.36

Amino acid analysis

	Glu	Pro	Gly	Ala	Leu
Calcd.	2	2	1	1	1
Found	2.05	2.05	1	0.99	0.97

Tritylglycyl-glycyl-leucyl-glycyl-glycyl-leucyl-glutaminyl-alanyl-leucyl-alanyl-leucyl-γ-t-butoxyglutamyl-glycyl-prolyl-prolyl-glutamine t-butyl ester (XXV)

0.95 g (1 mmol) XXIV was hydrogenated in 100 ml methanol with Pd for 4 h. The catalyst was removed by filtration and the filtrate evaporated to dryness. The amine was dissolved in 10 ml dimethylformamide and stored at −20°C for azide synthesis. A solution of 1.06 g (1 mmol) hydrazide XIX in 10 ml dimethylformamide at −20°C was treated with 0.26 ml (2 mmol) isoamylnitrite and 2.5 ml 2N HCl/tetrahydrofuran and stirred for 1 h. The reaction mixture was cooled to −40°C and neutralised with 0.7 ml triethylamine. The above mentioned amine solution was added and stirred overnight. The mixture was poured into 100 ml of water and the precipitated product was filtered, extracted two times with 30 ml warm methanol and dried. Yield 1.4 g (80%); m. p. 250–253°C (dec.); $[\alpha]_D^{23}$: −92.91° (c=1.27, in formic acid).

$C_{92}H_{138}N_{18}O_{21} \times 2H_2O$ (1868.29)

Calcd.	C 59.14	H 7.66	N 13.49
Found	C 59.08	H 7.45	N 12.89

Amino acid analysis

	Glu	Pro	Gly	Ala	Leu
Calcd.	3	2	5	2	4
Found	3	1.94	4.89	2.04	4.2

Benzyloxycarbonylalanyl-valyl-γ-t-butoxyglutamyl-leucyl-glycyl-glycyl-glycyl-leucyl-glycyl-glycyl-leucyl-glutaminyl-alanyl-leucyl-alanyl-leucyl-γ-t-butoxyglutamyl-glycyl-prolyl-prolyl-glutamine t-butyl ester (XXVI)

A suspension of 0.93 g (0.5 mmol) XXV in 30 ml acetic acid was stirred at room temperature for about 30 min till a clear solution was obtained. 30 ml water was added dropwise over a period of 1 h. The tritylcarbinol precipitated during detritylation was filtered and washed three times with 10 ml 50% acetic acid. The combined filtrate was lyophylized. The product was dissolved in 10 ml dimethylsulfoxide by gentle warming and then diluted with 10 ml dimethylformamide and stored at −5°C for azide reaction. The azide was prepared by adding 0.264 ml (2 mmol) isoamylnitrite to a reaction mixture of 0.691 g (1 mmol) hydrazide X and 1.7 ml 2.4N HCl/tetrahydrofuran in 6 ml dimethylformamide at −20°C. After 1 h the temperature was lowered to −40°C, and 0.56 ml (4 mmol) triethylamine followed by amine solution was added. Then the bath temperature was maintained at −10°C for 24 h. The reaction mixture was poured into 500 ml water. The precipitated product was filtered, extracted two times with warm methanol and dried. Yield 0.85 g (75%); m. p. 248–253°C (dec.); $[\alpha]_D^{24}$: −88.25° (c=0.456, in formic acid).

Amino acid analysis

	Glu	Pro	Gly	Ala	Val	Leu
Calcd.	4	2	6	3	1	5
Found	3.75	2.19	5.72	3.12	1	5.04

Glutamyl-alanyl-glutamyl-asparaginyl-prolyl-glutaminyl-alanyl-glycyl-alanyl-valyl-glutamyl-leucyl-glycyl-glycyl-glycyl-leucyl-glycyl-glycyl-leucyl-glutaminyl-alanyl-leucyl-alanyl-leucyl-glutamyl-glycyl-prolyl-prolyl-glutamine (XXVIII)

0.45 g (0.2 mmol) XXVI was dissolved in 200 ml 90% acetic acid and hydrogenated with 0.5 g Pd for 5 h. The catalyst was removed by filtration and the filtrate evaporated *in vacuo* at 20°C. The residue was dissolved in 20 ml dimethylsulfoxide by warming. 0.22 g (1.1 mmol) dicyclohexylcarbodiimide was added to a solution of 1.06 g (1 mmol) VI and 0.12 g (1.1 mmol) *N*-hydroxysuccinimide in 10 ml dimethylformamide at 0°C and the mixture stirred for 4 h. The dicyclohexylurea was filtered and the filtrate concentrated to a small volume and added to the above mentioned amine solution. After stirring for 3 days at 40°C the reaction mixture was added to 300 ml ether and kept at 4°C for 4 h. The product was collected by centrifugation, washed with methanol and dried. Crude yield 0.452 g (71%).

60 mg of the protected peptide was dissolved in 5 ml trifluoroacetic acid and dry HBr passed into it for 10 min. Trifluoroacetic acid and HBr were removed *in vacuo*. The residue was triturated with ether and the product was collected by centrifugation and dried *in vacuo* over NaOH. Yield 43 mg (81%). The product was dissolved in 2 ml 0.05M NH_4HCO_3 solution and applied to a column (2 × 25 cm) of DEAE-cellulose equilibrated with the same solution. After application of the sample a 0.05M to 0.3M NH_4HCO_3 gradient was started and 14 ml fractions were collected. The peptide was located by Folin-Ciocalteu reagent[24] (Fig. 2). The C-peptide containing fractions were pooled and lyophilized. Yield 15.2 mg (32%); $[\alpha]_D^{23}$: -124.6^0 ($c = 0.65$, in 30% acetic acid).

Amino acid analysis

	Asp	Glu	Pro	Gly	Ala	Leu	Val
Calcd.	1	7	3	7	5	5	1
Found	0.98	7.04	2.87	7	5.27	5.02	0.84

The synthetic C-peptide moved as one band on paper electrophoresis at two different pH values: 30% acetic acid as well as barbital buffer pH 8.6, $\mu = 0.05$ at 400 Volts for 4 h. In case of barbital buffer electrophoresis was done at 2–4°C.