Liquid-phase Peptide Synthesis by Fragment Condensation on a Soluble Polymer Support. III.[1] The Influence of the Content and the Chain Length of a Peptide Anchored to a Soluble Polymer Support on the Reactivity of the Amino-free Terminal of the Peptide

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In order to investigate the influence of the content and the chain length of a peptide anchored to a soluble polymer support on the reactivity of the amino-free terminal of the peptide, chloromethylated polystyrene, which is a starting material for peptide synthesis on a matrix, was prepared by the copolymerization of (chloromethyl)-styrene with styrene. By the copolymerization, the chloromethyl content on the resin was easily controlled and obtained as reproducible. The repetitive peptide-chain elongations of a pentapeptide, $-(Ala_2-Leu_3)-$, and a decapeptide, $-(Ala_2-Leu_3-Pro_2-Leu_3)-$, were performed by the usual method, using soluble polymer supports with various contents of Ile–Ile, which was used as the internal reference to evaluate the coupling yields by means of amino-acid analyses of the resulting peptide resins. The solubility of the peptide resin, which depends on the content of the peptide and the solubility of the peptide fragment on the matrix, was found to be one of the important factors for an efficient coupling in liquid-phase fragment condensations on a soluble polymer support.

Solid-phase peptide synthesis by fragment condensation seems to be the most promising method for the synthesis of large, homogeneous proteins.[2,3] This strategy will alleviate the difficulty[3-5] in the purification of a peptide and sequence-dependent problems[6] in the coupling and deprotecting steps. These problems are serious in solid-phase peptide synthesis by step-by-step elongation. An annoying problem in the fragment condensation method is, however, of the lower yield in the coupling step, resulting mainly from the restriction of the permeability of an *N*-protected peptide fragment into a random cross-linked polymer support which carries the amino-free terminal of peptide.[2] The use of soluble polymer supports for peptide synthesis has several advantages in carrying out peptide synthesis under homogeneous conditions. In previous papers,[1,7] we have reported that the fragment condensation on a soluble polymer support can achieve an efficient coupling of an *N*-protected tetrapeptide acid to the amino-free terminal anchored to the soluble polymer support and that the peptide-chain length of the *N*-protected tetrapeptide acid has no influence on the reactivity of the *C*-terminal amino acid in the coupling reaction using DCC[8] plus HOBt and DEPC as the coupling reagents. In this paper, we wish to report on the influence of the content and the chain-length of the peptide anchored to the soluble polymer support on the coupling reaction of Boc-peptide acids with the amino-free terminal of the peptide on the matrix.

Results and Discussion

For the above purpose, it is desirable to obtain a chloromethylated polystyrene with a controlled chlorine content. Merrifield resins which have been used in peptide synthesis generally started from the chloromethylation of a copolymer of styrene with divinylbenzene.[9] The chloromethylation of polystyrene with chloromethyl methyl ether, which is a polymer reaction, is usually accompanied by concomitant cross-linking due to the intermolecular Friedel-Crafts reaction with a tin(IV) chloride catalyst; also, it is difficult to control the chlorine content.[10,11] One could expect the cross-linking to produce sterically unfavored reaction sites, which are chemically reactive but physically inaccessible.[12] A skillful technique is also required to get a reproducible chlorine value by the chloromethylation of polystyrene with chloromethyl methyl ether.[13] The use of tin(IV) chloride as a catalyst has been further claimed to be disadvantegeous in causing a clustering of the reaction sites.[14] The preparation of a soluble chloromethylated polystyrene by the copolymerization of styrene with (chloromethyl)styrene seems, though, to have several advantages. The copolymerizations of (chloromethyl)styrene (a mixture of *m*- and *p*-isomers, 63: 28, Tokyo Kasei Co.) with styrene, using AIBN as the initiator, were, therefore, carried out in benzene at 70 °C in molar ratios of (chloromethyl)-styrene as 1 mol%, 3 mol%, 10 mol%, and 30 mol% respectively. The results are summarized in Table 1. The chlorine contents of the chloromethylated polystyrenes were determined by the Volhard method after a reaction with pyridine; they were in fair agreement with those calculated from the feed-molar ratios. The molecular weights of the polymers were measured by vapor-pressure osmometric equilibrium (VPOE) method. The reactivity ratios of (chloromethyl)styrene with styrene were not determined, since the (chloromethyl)styrene used was a mixture of *m*- and *p*-isomers. The polymer was free from an insoluble material, homo-polystyrene. This was confirmed by the fact that the pyridinium chloride derived from the reaction of 10 mol% chloromethylated polystyrene with pyridine was soluble in methanol. It could, therefore, be estimated that the chloromethyl groups were distributed at random on the resin. In comparison with the chloromethylation of soluble polystyrene,[10] which is liable to cause a heterogeneous environment due to partial

Table 1. Results of the copolymerization of (chloromethyl)styrene with styrene[a)]

Monomers to be fed (mmol)		Mol% of (chloromethyl)-styrene (mmol/g)	Product		
Styrene	(Chloromethyl)styrene		Yield (%)	Cl content[c)] (mmol/g)	Mol. wt.[d)]
396	4	1 (0.096)	84	0.096	—
582	12	3 (0.28)	99	0.27	44000
540	60	10 (0.92)	61	0.89	17000
146	63	30 (2.53)[b)]	72	2.43	21000

a) The copolymerizations were carried out using AIBN (0.5 mol %) as the initiator in benzene at 70±0.5°C for 40 h. b) One mol % of AIBN was used as the initiator. c) Determined by the Volhard method. d) Determined by the VPOE method.

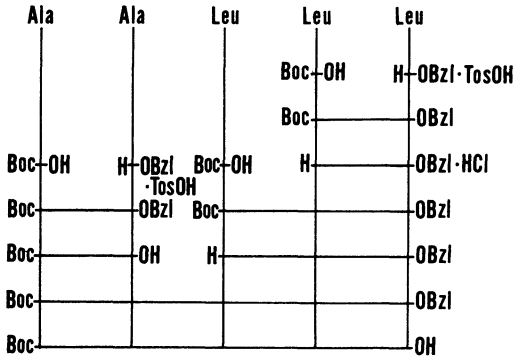
Table 2. Estimated secondary structures of Boc-peptide acids and peptides on polymer supports

Peptide	Estimated secondary structure
Boc–Ala_2–Leu_3–OH	β-Sheet
Boc–Ala_2–Leu_3–Pro_2–Leu_3–OH	Randam coil
H{Ala_2–Leu_3}$_n$$Ile_2$–$OCH_2$–resin	α-Helix ($n \geqq 2$)
H{Ala_2–Leu_3–Pro_2–Leu_3}$_n$$Ile_2$–$OCH_2$–resin	Randam coil ($n \geqq 1$)

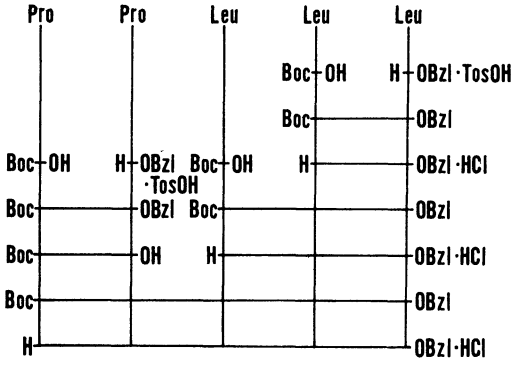
cross-linking, the copolymerization of (chloromethyl)-styrene with styrene has several advantages; 1) easy control of the chloromethyl content on the resin; 2) the absence of any cross-linking reaction, affording polymers with a nearly homogeneous structure; 3) easy preparation of polymers with a reproducible chloromethyl content; 4) the exclusion of the use of carcinogenic chloromethyl methyl ether, and 5) the exclusion of the clustering of chloromethyl groups when tin(IV) chloride is used as the catalyst.

The efficient peptide-bond formation on the polymer support is put under the influence of several factors, such as the permeability of reagents, the steric micro-environment of reactive sites on the polymer support (peptide-peptide or peptide-resin steric interactions),[5)] the secondary or tertiary structure of the *N*-protected peptide acid (reagent) and the peptide on the polymer support,[6)] and the solvation of the matrix and the peptide chain.[5,6,15)] In order to evaluate these difficulties, the repetitive elongation of a decapeptide or a pentapeptide on the polymer support was carried out using polymer supports with various Ile-Ile contents. Boc–Ala_2–Leu_3–OH and Boc–Ala_2–Leu_3–Pro_2–Leu_3–OH were used as the *N*-protected penta- and decapeptide acids respectively. The secondary structures of the sequential poly(penta- and decapeptide)s are estimated to be the α-helix and the random coil respectively by the methods of Chou and Fasman[16)] and Katakai,[17)] as is shown in Table 2.

All the peptides used here were synthesized using DCC and HOBt as coupling reagents. The deprotection of the Boc group was carried out in 3 M HCl–AcOEt, and the deprotection of Bzl group, by hydrogenolysis using 5% Pd/C as the catalyst. The amino group was liberated from the salt with NMM. The preparations of Boc–Ala_2–Leu_3–OH and H–Pro_2–Leu_3–OBzl·HCl are shown in Schemes 1 and 2. Boc–Ala_2–OBzl, Boc–Pro_2–OBzl, and Boc–Leu_2–OBzl were prepared in 93%, 76%, and 71% yields respectively by the coupling reactions of Boc-amino acids with benzyl esters. Boc–



Scheme 1.



Scheme 2.

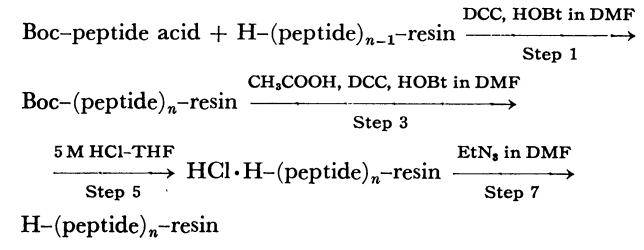
Leu_3–OBzl was prepared in a 67% yield by the step-by-step elongation, starting from H–Leu–OBzl·TosOH. Boc–Ala_2–Leu_3–OBzl and Boc–Pro_2–Leu_3–OBzl were obtained in 91% and 82% yields respectively by the coupling reactions of H–Leu_3–OBzl with Boc–Ala_2–OH and Boc–Pro_2–OH. The reaction between Boc–Ala_2–Leu_3–OH and H–Pro_2–Leu_3–OBzl gave Boc–Ala_2–Leu_3–Pro_2–Leu_3–OBzl in an 87% yield; this then provided the desired *N*-protected peptide acid, Boc–Ala_2–Leu_3–Pro_2–Leu_3–OH, in an 81% yield by hydrogenolysis.

Ile–Ile was anchored to soluble polymer supports as the internal reference by the reaction of chloromethylated polystyrenes (1 mol%, 3 mol%, and 30 mol% chlorine content) with Boc–Ile_2–OH, using potassium carbonate in DMF, followed by deprotection and neutralization, to give Ile_2–OCH_2–resins (Resins A, B, and C respectively). Residual benzylic chlorine atoms, which cause unavoidable cross-linking, were removed by heating the Boc–Ile_2–OCH_2–resins with sodium acetate in DMF. The Ile contents in acid hydrolysates of Boc–Ile_2–OCH_2–resins (Resins A', B', and C') are summarized in Table 3.

Table 3. Amino-acid analyses of Boc–Ile_2–OCH_2–resins (Resins A', B', and C')

Cl content of the starting resin (mol %)	Product	Ile_2 content (mmol/g)	Conversion[b] (%)
1	Resin A'	0.088(0.093)[a]	95
3	Resin B'	0.25(0.261)[a]	96
30	Resin C'	1.24(1.39)[a]	89

a) Theoretical values calculated from each theoretical chlorine content. b) Conversion from the chloromethyl group to the Boc–Ile_2–OCH_2 group : calculated from each theoretical Ile content.



Boc-peptide acid: Boc–Ala_2–Leu_3–OH and Boc–Ala_2–Leu_3–Pro_2–Leu_3–OH
resin: –Ile_2–OCH_2–resin (resin A, B, and C)
n: number of cycle (n=1, 2, and 3)

Scheme 3.

Each repetitive peptide-chain elongation of the penta- and the decapeptides was performed using the cycle summarized in Table 4. The reaction of each step is shown in Scheme 3. The fragment condensation on the soluble polymer support was carried out in DMF, using DCC and HOBt as coupling reagents (Step 1); this procedure had been shown to be effective for the fragment condensation.[7,18] The residual amino group was terminated by the treatment with acetic acid in DMF, using DCC and HOBt as coupling reagents (Step 3). The Boc group was removed with 5 M HCl–THF or 20% CF_3COOH–CH_2Cl_2 (v/v) (Step 5),[19] while the amino group was liberated from the salt, formed with the excess of the acid used for deprotection, by treatment with Et_3N in DMF (Step 7). In these processes, the inherent simplicity of peptide synthesis using the polymer support can be preserved for the separation and purification of intermediate resins (Steps 2, 4, 6, and 8). In these steps, the recovery of the resulting peptide resin by precipitation from the solution with a non-solvent was in a near-quantitative yield. After the termination reaction on each cycle, an aliquot of peptide resin was hydrolyzed with acid and analyzed for its amino-acid composition. The amino-acid ratios in the acid hydrolysates of the resulting peptide resins and the coupling yields in each cycle for the fragment condensations of Boc–Ala_2–Leu_3–OH and Boc–Ala_2–Leu_3–Pro_2–Leu_3–OH using the three resins, A, B, and C, are summarized in Tables 5 and 6. The yield of the coupling reaction on cycle n was calculated, by making correction by the use of the coupling yields of the n-2 and/or n-1 cycles, from the recovery of Leu in the acid hydrolysate, taking the recovery of Ile as the standard. The coupling reactions of the penta- and decapeptide acids with each resin kept a more than 90% yield in the first cycle. The yield on the reaction of the pentapeptide acid with the B and C resins in the second and third cycles decreased gradually in accordance with the decrease in the solubility of peptide resins in DMF. In the coupling reactions of the second cycles, in practice, gelation took place in the solutions of resins in which the peptide-bond formation was proceeding. Using Resin A, which bound peptides to the smallest degree, the peptide-bond formation on each cycle could proceed homogeneously and keep a high coupling yield. A tendency for the yield of the peptide-chain elongation to decrease was observed in the coupling reaction of the decapeptide acid with Resin C, which bound peptide to the highest degree. The solubility of Boc–Ala_2–Leu_3–Pro_2–Leu_3–OBzl in organic solvents is greater than that of Boc–Ala_2–Leu_3–Ala_2–Leu_3–OBzl, which is only slightly soluble in DMF, ethanol, and methanol. The solubility of the peptide fragment on the matrix directly affects that of the peptide resin.

In conclusion, the solubility of peptide resin, which is related to the solvation of the matrix and the peptide chain, the permeability of reagents, and the steric microenvironment of the reactive sites on the matrix,

Table 4. A cycle for the addition of one oligopeptide in each repetitive peptide elongation

Step	Function	Reagent, solvent, and/or nonsolvent
1	Coupling	Boc–peptide acid, DCC, and HOBt in DMF
2	Precipitation, wash, and dry	Ethanol, water, and methanol
3	Termination	Acetic anhydride and pyridine in DMF
4	Precipitation, wash, and dry	Ethanol, water, and methanol
5	Deprotection	5 M HCl–THF or 20% CF_3COOH–CH_2Cl_2
6	Precipitation, wash, and dry	Ethanol, water, and methanol
7	Neutralization	Et_3N in DMF
8	Precipitation, wash, and dry	Ethanol, water, and methanol

Table 5. The yields in each coupling step of the repetitive peptide-chain elongation of pentapeptide

Resin	Cycle 1			Cycle 2			Cycle 3		
	Amino-acid contents on resins (μmol/g)		Yield[a] (%)	Amino-acid contents on resins (μmol/g)		Yield[b] (%)	Amino-acid contents on resins (μmol/g)		Yield[c] (%)
	Ile	Leu		Ile	Leu		Ile	Leu	
A	80×2	73×3	91	75×2	63×6	87	72×2	60×9	90
B	221×2	210×3	95	207×2	178×6	81	185×2	124×9	34
C	709×2	700×3	99	512×2	423×6	67	529×2	337×9	39

a) Determined by means of the amino-acid ratios. b) Determined by means of the amino-acid ratios, making correction by the use of the coupling yields of Cycle 1. c) Determined by means of the amino-acid ratios, making correction by the use of the coupling yields of Cycles 1 and 2.

Table 6. The yields in each coupling step of the repetitive peptide-chain elongation of decapeptide

Resin	Cycle 1			Cycle 2			Cycle 3		
	Amino-acid contents on resins (μmol/g)		Yield[a] (%)	Amino-acid contents on resins (μmol/g)		Yields[b] (%)	Amino-acid contents on resins (μmol/g)		Yields[c] (%)
	Ile	Leu		Ile	Leu		Ile	Leu	
A	61×2	55×6	90	59×2	47×12	77	54×2	42×18	107
B	199×2	182×6	92	180×2	159×12	93	135×2	113×18	87
C	567×2	522×6	92	478×2	285×12	65	—	—	—

a) Determined by means of the amino-acid ratios. b) Determined by means of the amino-acid ratios, making correction by the use of the coupling yields of Cycle 1. c) Determined by means of the amino-acid ratios, making correction by the use of the coupling yields of Cycles 1 and 2.

is one of the most important factors in determining the efficient coupling yield in liquid-phase peptide synthesis on the soluble polymer support by means of fragment condensation. This technique has several advantages; 1) the reaction can easily be done in a homogeneous system and in a near-quantitative yield by controlling the peptide content on the matrix, 2) the intermediates can easily be handled for separation and purification, 3) the solubility of intermediates can easily be observed, and 4) there is a possibility of the optical analysis of the secondary structure of the peptide chain anchored to the matrix. There is, in fact, the prospect of the homogeneous synthesis of a large (50 amino acids or more) peptide or protein (100 amino acids or more).

Experimental

General. The uncorrected capillary melting points will be reported. The infrared spectra were recorded on a JASCO Model DS-403G. The molecular weights were determined by the VPOE method, using a Hitachi Model 117 molecular-weight-measurement instrument. The optical rotations were taken in a 1-dm cell on a JASCO Model ORD/UV-5 optical rotatory dispersion recorder. The elemental analyses were performed on a Perkin Elmer Model 240 elemental analyzer.[20] The amino-acid compositions of the acid hydrolysates were determined with a Hitachi Liquid Chromatograph, Model 034. The acid hydrolyses of the peptide resins were carried out on 20—40 mg samples of resins with propionic acid–12 M HCl (2: 1 v/v) for 35 h at 115 °C in sealed tubes. Ascending TLC was performed on silica gel plates (Wakogel Plate from Wako, Tokyo) using the following solvent system: chloroform–methanol–acetic acid (45: 4: 1 by vol).

Materials. The (chloromethyl)styrene (a mixture of *m*- and *p*-isomers, 63: 28) was purchased from the Tokyo Kasei Co., Tokyo, and was used without purification. The Boc-amino acids were prepared according to Nagasawa *et al.*[21] The amino acid benzyl ester tosylates[22a] and HOBt[22b] were prepared by the methods given in the literature. The DMF was purified as follows. To 1.5 l of DMF we added 75 g of phthalic anhydride, after which the mixture was kept overnight at room temperature and then distilled under reduced pressure. It was then redistilled under reduced pressure. The benzene, styrene, THF, and AcOEt were purified in the usual manners. All the other reagents were obtained from commercial sources and were used without further purification.

Soluble Chloromethylated Polystyrene. The copolymerizations of (chloromethyl)styrene with styrene, using AIBN as the initiator, were carried out in benzene for 40 h at 70 °C in molar ratios of (chloromethyl)styrene of 1 mol%, 3 mol%, 10 mol%, and 30 mol% under the conditions described previously.[7] The results are summarized in Table 1. The chlorine contents of the polymers were determined by the Volhard method after the reaction with pyridine. The 10 mol% polymer (0.5 g) was reacted in 3 ml of pyridine for 2 h at 100 °C, after which the solution was poured into water and a small amount of saturated aqueous sodium chloride was added. The precipitated polymer, pyridinium salt, was collected by filtration and dried *in vacuo*. It was soluble in methanol and free from insoluble material.

Boc–Ala$_2$–OBzl, Boc–Leu$_2$–OBzl, Boc–Pro$_2$–OBzl, and Boc–Ile$_2$–OBzl. Boc–Ala–OH (9.72 g, 51 mmol) and H–Ala–OBzl·TosOH (16.42 g, 47 mmol) were dissolved in 90 ml of THF, followed by the addition of NMM (4.72 g, 47 mmol) dissolved in 10 ml of THF and HOBt (7.20 g, 47 mmol). To the solution cooled at 0 °C we then added DCC (10.60 g, 51 mmol) dissolved in 30 ml of THF; the mixture was stirred for 2 h at 0 °C and then overnight at room temperature. After the solution had then been cooled for 30 min at 0 °C, the precipitates were filtered off and the filtrate was concentrated under reduced pressure. The residue was dissolved in 200 ml of AcOEt, and the solution was successively washed in the

usual way three times with 10% aqueous citric acid, twice with aqueous sodium chloride, three times with 4% aqueous sodium hydrogencarbonate, and twice with aqueous sodium chloride, and dried over sodium sulfate, using charcoal for decolorization. After the removal of the solvent *in vacuo*, the residue was dissolved in a mixture of AcOEt (90 ml) and petroleum ether (180 ml), and the solution was kept for 30 min at 0 °C. The precipitated *N,N'*-dicyclohexylurea was filtered off, and the filtrate was concentrated to give a crude peptide (15.3 g, 93% yield). Subsequent recrystallization from a mixture of AcOEt (20 ml) and hexane (120 ml) gave a substance, mp 69—71 °C (lit,[23] 68—70 °C); $[\alpha]_D^{22} = -60.2°$ ($c=1.0$, CH_3OH); a single spot by TLC in the solvent system (R_f, 0.65). Boc-Leu_2-OBzl (mp 92—93 °C, lit,[24] 92—92.5 °C), Boc-Pro_2-OBzl (oil), and Boc-Ile_2-OBzl (mp 89.5—91.5 °C) were similarly prepared.

Boc-Leu_3-OBzl. The preparation was performed as has been described above using Boc-Leu-OH·H_2O (18.52 g, 74.3 mmol) and H-Leu_2-OBzl·HCl (27.55 g, 74.3 mmol), prepared by the treatment of Boc-Leu_2-OBzl with 3 M HCl-AcOEt in the usual way; 84% yield. Subsequent recrystallization from a mixture of AcOEt (20 ml) and hexane (200 ml) gave a substance, mp 127—128.5 °C; $[\alpha]_D^{25} = -64.9°$ ($c=1.0$, CH_3OH); a single spot by TLC in the solvent system (R_f, 0.75). Found: C, 65.28; H, 9.06; N, 7.71%. Calcd for $C_{30}H_{49}N_3O_6$: C, 65.78; H, 9.02; N, 7.67%.

Boc-Ala_2-OH, Boc-Pro_2-OH, and Boc-Ile_2-OH. Boc-Ala_2-OBzl (14.0 g, 40 mmol) was hydrogenated at atmospheric pressure in a mixture of methanol (30 ml) and acetic acid (20 ml) in the presence of 5% Pd/C (1.0 g) as a catalyst. The reaction mixture was then stirred overnight at room temperature. After the removal of the Pd/C, the filtrate was concentrated under reduced pressure, followed by the repetition of the addition of water and its removal under reduced pressure to eliminate acetic acid, and drying *in vacuo*. The product was recrystallized from a mixture of AcOEt (80 ml) and hexane (120 ml) to give, in a near-quantitative yield, a material, mp 120—121 °C (lit, 85.5—87 °C,[25] 128—129 °C,[2d] 132—133 °C[26]); $[\alpha]_D^{22} = -39.1°$ ($c=1.0$, CH_3OH); a single spot by TLC in the solvent system (R_f, 0.34). Boc-Pro_2-OH was purified as the dicyclohexylamine salt; mp 161—162 °C, $[\alpha]_D^{23} = -72.8°$ ($c=1.0$, CH_3OH), R_f 0.40. Found: C, 65.86; H, 9.66; N, 8.67%. Calcd for $C_{27}H_{47}N_3O_5$: C, 65.69; H, 9.60; N, 8.51%.

Boc-Ala_2-Leu_3-OBzl and Boc-Pro_2-Leu_3-OBzl. The preparation of Boc-Ala_2-Leu_3-OBzl was carried out in THF in a manner similar to that described for the preparation of Boc-Ala_2-OBzl, using Boc-Ala_2-OH (11.5 g, 44 mmol) and H-Leu_3-OBzl·HCl (17.9 g, 37 mmol), prepared by the treatment of Boc-Leu_3-OBzl with 3 M HCl-AcOEt in the usual way; 91% yield. Subsequent recrystallization from a mixture of AcOEt and hexane (1:3) gave a material, mp 230—235 °C (dec); $[\alpha]_D^{22} = -70.5°$ ($c=1.0$, CH_3OH); a single spot by TLC in the solvent system (R_f, 0.53). Found: C, 62.40; H, 8.70; N, 9.93%. Calcd for $C_{36}H_{59}N_5O_8$: C, 62.68; H, 8.62; N, 10.15%.

Boc-Pro_2-Leu_3-OBzl was obtained by a similar method in an 82% yield. Subsequent recrystallization from a mixture of AcOEt and hexane (1:3) gave a material, mp 91.5—93 °C; $[\alpha]_D^{26} = -119.2°$ ($c=1.0$, CH_3OH); a single spot by TLC in the solvent system (R_f, 0.58). Found: C, 65.02; H, 8.56; N, 9.28%. Calcd for $C_{40}H_{63}N_5O_8$: C, 64.75; H, 8.56; N, 9.44%.

Boc-Ala_2-Leu_3-OH. The hydrogenolysis of Boc-Ala_2-Leu_3-OBzl in the manner described for the preparation of Boc-Ala_2-OH gave Boc-Ala_2-Leu_3-OH in an 87% yield. Subsequent recrystallization from a mixture of ethanol and AcOEt (1:6) gave a substance, mp 265—270 °C (dec); $[\alpha]_D^{23} = -69.5°$ ($c=1.0$, CH_3OH); a single spot by TLC in the solvent system (R_f, 0.46). Amino-acid ratios in acid hydrolysate: Ala 2.00, Leu 2.94. Found: C, 57.68; H, 8.73; N, 11.38%. Calcd for $C_{29}H_{53}N_5O_8$: C, 58.07; H, 8.91; N, 11.68%.

Boc-Ala_2-Leu_3-Pro_2-Leu_3-OBzl. Boc-Ala_2-Leu_3-OH (7.73 g, 12.9 mmol) and H-Pro_2-Leu_3-OBzl-HCl (7.95 g, 11.7 mmol), prepared from Boc-Pro_2-Leu_3-OBzl in the usual way, were dissolved in 100 ml of DMF, and then NMM (1.19 g, 11.7 mmol), dissolved in 20 ml of DMF and HOBt (1.98 g, 12.9 mmol), was added. To the solution cooled at 0 °C we then added DCC (2.66 g, 12.9 mmol), dissolved in 30 ml of DMF; the reaction mixture was stirred for 2 h at 0 °C and then overnight at room temperature. After the mixture had then been cooled for 1 h at 0 °C, the precipitates were filtered off and the filtrate was concentrated under reduced pressure. To the residue we added water to give a solid. On a glass filter, it was successively washed with 10% aqueous citric acid, water, 4% aqueous sodium hydrogencarbonate, and water, and dried *in vacuo*; yield, 87%. Recrystallization from AcOEt gave a material, mp 194—194.5 °C; $[\alpha]_D^{25} = -119.4°$ ($c=1.0$, CH_3OH); a single spot by TLC in the solvent system (R_f, 0.54). Found: C, 62.45; H, 8.78; N, 11.33%. Calcd for $C_{64}H_{106}N_{10}O_{13}$: C. 62.82; H, 8.73; N, 11.45%.

Boc-Ala_2-Leu_3-Pro_2-Leu_3-OH. Boc-Ala_2-Leu_3-Pro_2-Leu_3-OBzl (9.0 g, 7.4 mmol) was hydrogenated at atmospheric pressure in a mixture of methanol (100 ml) and acetic acid (20 ml) in the presence of 5% Pd/C (1.0 g) as a catalyst; then it was subjected to the work-up procedures described in the preparation of Boc-Ala_2-OH. The product was recrystallized from a mixture of ethanol and AcOEt (1:6) to give, in an 81% yield, a material, mp 187—188.5 °C; $[\alpha]_D^{25} = -134.2°$ ($c=1.0$, CH_3OH); a single spot by TLC in the solvent system (R_f, 0.48). Amino-acid ratios in acid hydrolysate: Ala 2.00, Leu 5.95, Pro 2.09. Found: C, 60.31; H, 8.61; N, 11.89%. Calcd for $C_{57}H_{100}N_{10}O_{13}$: C, 60.40; H, 8.89; N, 12.36%.

H-Ile_2-resins (Resins A, B, and C). As a typical example, 1 mol% chloromethylated polystyrene (Cl content, 0.096 mmol/g; 10.0 g) and Boc-Ile_2-OH (0.99 g, 2.88 mmol) were dissolved in 100 ml of DMF. To the solution, maintained at 40 °C, we added potassium carbonate (0.20 g, 1.44 mmol), after which the reaction mixture was stirred for 100 h at 40 °C. The addition of this mixture to ethanol (1 l) caused the precipitation of a solid, which was collected, homogenized with water, filtered off again, washed with water and methanol, and dried *in vacuo*. A solution of the product in THF (200 ml) was poured into ethanol. For the precipitate we repeated the work-up procedures described above. The yield was 10.2 g (99%). Residual benzylic chlorides were removed by a reaction with sodium acetate in DMF at 105 °C for 24 h, as has been described in the literature.[10] The Resin A' obtained was negative to a Beilstein test, indicating it to be free from chlorine. The removal of the Boc-group with the saturated HCl-THF solution and the neutralization of the amine salt with Et_3N were performed by procedures similar to those described in the literature[10] to give Resin A. Resins B and C were obtained from 3 mol% and 30 mol% chloromethylated polystyrene respectively by the same work-up procedures. The Ile content of each resin is shown in Table 3.

A Cycle of Peptide-chain Elongation on the Matrix for the Addition of One Oligopeptide. Each repetitive peptide-chain elongation of the penta- and the decapeptides was performed using the cycle summarized in Table 4. The reaction conditions are shown in Table 7. As a typical example, Resin B (amino content, 0.25 mmol/g; 4.00 g), a two-fold equivalent of Boc-Ala_2-Leu_3-OH (1.20 g, 2.00 mmol), and HOBt (0.336 g, 2.20 mmol) were dissolved in DMF (60 ml). To the solution

Table 7. Reaction conditions for the addition of one oligopeptide in each repetitive peptide-chain elongation

Step	Reagents[a]	Solvent	Reaction conditions	
			Time/h	Temp/°C
1	Two-fold equivalent each of Boc-peptide acid and DCC, and a 2.2 times equivalent of HOBt	DMF 12—20 ml/g-resin	48 24[b]	0 r.t.
3	Two-fold equivalent each of acetic acid and DCC, and a 2.2 times equivalent of HOBt	DMF 12—20 ml/g-resin	20	r.t.
5[c]	5 M HCl–THF	THF 10—40 ml/g-resin	15	0
7	4—5 times equivalent of Et_3N	DMF or THF 20—40 ml/g-resin	10 min	r.t.

a) The amounts used were based on the amount of the Ile amino-free terminal of the starting resins, A, B, and C. b) On Cycles 2 and 3, 48 h. c) In the case of Cycle 2 using Resin C, the removal of the Boc group was performed in 20% CF_3COOH–CH_2Cl_2 (v/v), 20—30 ml/g resin, for 2 h at room temperature.

cooled at 0 °C we then added DCC (0.412 g, 2.00 mmol) dissolved in DMF (20 ml) and cooled at 0 °C; the mixture was stirred for 48 h at 0 °C and then for 24 h at room temperature. The reaction mixture was near-negative to a ninhydrin test. It was then poured into ethanol (1 l), and 10% aqueous sodium chloride (30 ml) was added. The precipitated resin was filtered off and washed successively with water and methanol to remove the soluble reactant. The resin was homogenized with ethanol (200 ml) overnight at room temperature under magnetic stirring, filtered off again, washed with water and methanol, and dried *in vacuo* at 50 °C. The yield was 4.45 g (recovery yield, 97%).

The termination reaction of the unreacted N-terminal amino group of the resin was carried out as follows. The resin (4.05 g) obtained above, acetic acid (0.12 g, 2.0 mmol), and HOBt (0.34 g, 2.2 mmol) were dissolved in DMF (120 ml). To the solution we then added DCC (0.41 g, 2.0 mmol) dissolved in DMF (30 ml), after which reaction mixture was stirred for 20 h at room temperature. The reaction mixture was negative to a ninhydrin test. The addition to ethanol (1 l) caused the precipitation of a solid, which was collected by filtration, washed with water and methanol, and dried *in vacuo* at 50 °C.

The removal of Boc-group with 6 M HCl–THF and the neutralization of the amine salt with Et_3N were performed in the usual ways under the conditions described in Table 7.

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8) The abbreviations for amino acids and protecting groups are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972). The amino-acid symbols denote the L-configuration. Additional abbreviations: DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, 1-hydroxyl-1*H*-benzotriazole; DEPC, diethoxyphosphoryl cyanide; AIBN, azobisisobutyronitrile; AcOEt, ethyl acetate; NMM, *N*-methylmorpholine; DMF, *N,N*-dimethylformamide; Et_3N, triethylamine; THF, tetrahydrofuran; TosOH, *p*-toluenesulfonic acid.

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19) The Boc-protecting group on resin can be completely removed within 5 h by treatment with 6 M HCl–THF or within 1 h by treatment with 20% CF_3COOH–CH_2Cl_2 (v/v). The removal of the Boc-group was confirmed by the disappearance of IR absorption bands at 1720 and 1170 cm^{-1} due to the Boc-group.

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