Liquid Phase Peptide Synthesis by the Fragment Condensation on Soluble Polymer Support. I. Efficient Coupling and Relative Reactivity of a Peptide Fragment with Various Coupling Reagents

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The fragment condensation on the soluble polymer support using dicyclohexylcarbodiimide (DCCI) plus 1-hydroxy-1*H*-benzotriazole (HOBt), diethoxyphosphoryl cyanide (DEPC), and triphenylphosphine (Ph_3P) plus di-2-pyridyl disulfide ($(PyS)_2$) as coupling reagents gave rise to efficient coupling (86—99% yield) of the peptide fragment, *t*-butyloxycarbonyl-L-*O*-benzyl-tyrosylglycylglycyl-L-phenylalanine [Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH], to the amino free terminal of Leu anchored to the soluble polymer support, H-L-Leu-OCH_2-resin. Relative reactivity ratios of Boc-amino acids and Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH with H-L-Leu-OCH_2-resin in the coupling reaction using the three coupling reagent systems could be easily determined by use of amino acid ratios in the acid hydrolysates of the resulting peptide resins in which they were taken. The peptide chain length of Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH has no influence on the reactivity of *C*-terminal amino acid in the coupling reaction using DCCI plus HOBt or DEPC as a coupling reagent when Boc-L-Phe-OH was replaced with Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH. The coupling reagent, Ph_3P plus $(PyS)_2$, brought about a marked decrease in the reactivity of the fragment peptide acid.

Through progress in peptide chemistry the synthesis of several large proteins has been achieved by means of solid phase[1] and classical solution methods.[2] However, difficulties arise when these methods are applied to large molecule peptides and proteins. Heterogeneous reaction conditions in solid phase peptide synthesis cause the formation of deletion and truncated sequences,[3] diffusion, slow reaction rates[4] of coupling and removal of protecting groups, solvation problems,[5] and formidable separation of the desired peptide from a large number of similar products. There is a drastic decrease in the overall yield of synthesis, and further progress in peptide synthesis along classical lines is hindered mainly because of the low solubility of polypeptides in organic solvents and the difficulty of removing the side products in the process of synthesis.[2]

In these embarrassing situations, it is evident that a considerable modification of present techniques is necessary for the synthesis of homogeneous proteins of a defined structure. Solid phase peptide synthesis using the fragment condensation procedure is expected to be of value for the synthesis of large molecule peptides and proteins.[6] This strategy will alleviate sequence-dependent problems in coupling and deprotecting steps, formation of failure sequences, and the difficulty in purification of the desired peptide. The serious problem in this method is, however, of low yield in the coupling steps due mainly to restriction of permeability of the peptide fragment into the random closs-linked polymer support anchored amino free terminal of peptide. It can be expected that use of a soluble polymer support in the fragment condensation procedure eliminates it to attain efficient coupling.

Soluble polymer supports were used in the stepwise elongation of peptide chains.[4,7] Kinetic studies of the coupling reaction using the soluble polymer support indicate that the rate is of the same order of magnitude as that of classical liquid phase peptide synthesis,[4a] approximately 100 times faster than that of solid phase peptide synthesis.[4b]

In this paper, we wish to report that the new technique described here can achieve the efficient coupling of the fragment peptide acid using dicyclohexylcarbodiimide (DCCI) plus 1-hydroxy-1*H*-benzotriazole (HOBt),[8] triphenylphosphine (Ph_3P) plus di-2-pyridyl disulfide ($(PyS)_2$),[9] and diethoxyphosphoryl cyanide (DEPC)[10] as coupling reagents. The relative reactivity of a tetrapeptide fragment is practically the same as that of carboxylic acid of the amino acid corresponding to the carboxyl terminal of the fragment.

Results and Discussion

Fragment Condensation of Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH on the Soluble Polymer Support Using DCCI plus HOBt, DEPC, and Ph_3P plus $(PyS)_2$ as Coupling Reagents. Three systems of coupling reagents, DCCI plus HOBt, DEPC, and Ph_3P plus $(PyS)_2$, were investigated for the fragment condensation of Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH with the amino free terminal of Leu anchored to the soluble polymer support, H-L-Leu-OCH_2-resin. Soluble chloromethylated polystyrene (Cl content: 0.91 mmol/g) was prepared by the copolymerization of (chloromethyl) styrene (a mixture of *m*- and *p*-isomers 7 : 3) with styrene (molar ratios 1 : 9) using azobisisobutyronitrile as an initiator. H-L-Leu-OCH_2-resin (Leu content: 0.62 mmol/g) and Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH were prepared in the usual way.[7b,11]

On the basis of the amino component attached to the polymer, three equivalent each of the fragment peptide carboxylic acid and a coupling reagent are employed in order to pursue an efficient coupling. The reaction was performed in *N,N*-dimethylformamide (DMF) at 0—5 °C for 2 h and then at room temperature for 3 h. In the case of Ph_3P plus $(PyS)_2$, the coupling reaction was carried out with a threefold excess of the carboxyl component and a 30-fold excess each of Ph_3P and $(PyS)_2$. The reaction mixture was poured into methanol followed by the addition

Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH

$$+ \text{H-L-Leu-OCH}_2\text{-resin} \xrightarrow[\text{in DMF, 0—5 °C, 2 h and then r. t., 3 h}]{\text{coupling reagent}}$$

Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-L-Leu-OCH_2-resin

of a small amount of saturated aqueous sodium chloride. The precipitated peptide resin was filtered off and washed successively with water and methanol in order to remove the soluble reactant. In each case, recovery of the resulting peptide polymer by precipitation from the solution in DMF with methanol was more than 93% on the basis of yields. Amino acid analyses of the resulting peptide resins and coupling yields with various coupling reagents are summarized in Table 1. The average yield of the coupling reaction was calculated from the recovery of Phe and Gly in the acid hydrolysate taking the recovery of Leu as the standard. The fragment condensation on the soluble polymer support, especially when DCCI plus HOBt and DEPC were used as coupling reagents, could achieve efficient coupling (96—99% yield) of the tetrapeptide acid to the amino free terminal of Leu on the soluble polymer support.

Relative Reactivities of Boc-Amino Acids and Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH with H-L-Leu-OCH_2-resin in the Coupling Reaction. In order to find out to what extent the peptide chain length influences the reactivity of the *C*-terminal amino acid in the fragment condensation reaction, and the effect of the coupling reagent on the reactivity of the tetrapeptide acid when Boc-L-Phe-OH is replaced by Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH, the technique as given in the following was used. Relative reactivities of Boc-amino acids with H-L-Leu-OCH_2-resin were first determined with use of various coupling reagents. The results are summarized in Table 2. As a typical example (run 1), starting with 200 mg of H-L-Leu-OCH_2-resin containing 0.124 mmol of Leu, the coupling reaction was carried out with a three-fold excess each of Boc-Gly-OH, Boc-L-Val-OH, and Boc-L-Phe-OH and a nine-fold excess (an equimolar amount with the total of the

TABLE 1. AMINO ACID ANALYSES OF Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-L-Leu-OCH_2-resins AND COUPLING YIELDS WITH VARIOUS COUPLING REAGENTS

Coupling reagent	Amino acid content (mmol/g)				Average yield of coupling[a] (%)
	Tyr[b]	Gly	Phe	Leu	
DCCI + HOBt[c]	0.38	0.85	0.42	0.44	96
DCCI + HOBt[d]	0.39	0.89	0.45	0.46	97
DEPC[c]	0.42	0.88	0.45	0.45	99
$Ph_3P + (PyS)_2$[c]	0.31	0.71	0.35	0.41	86

a) Calculated from the recovery of Phe and Gly in the acid hydrolysate taking the recovery of Leu as a standard. b) Acid hydrolysis of tyrosyl peptides usually gave low recovery of Tyr (K. Watanabe and K. Inouye, *Bull. Chem. Soc. Jpn.*, **50**, 201 (1977)). c) Reaction carried out with stirring at 0—5 °C for 2 h and then at room temperature for 3 h. d) Reaction carried out with stirring at 0—5 °C for 2 h and then at room temperature for 20 h.

TABLE 2. AMINO ACID ANALYSES OF PEPTIDE RESINS AND RELATIVE REACTIVITIES OF BOC-AMINO ACIDS AND Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH IN THE COUPLING REACTION WITH H-L-Leu-OCH_2-resin USING VARIOUS COUPLING REAGENTS

Run No.	Coupling reagent	Acid components[b]	Amino acid content (mmol/g)				Relative reactivities[a] (Amino acid ratios)		
			Gly	Val	Leu	Phe	Gly	Val	Phe
1	DCCI+HOBt[c]	A	0.209	0.054	0.552	0.281	3.9	1.0	5.2
2	DEPC[c]	A	0.263	0.063	0.545	0.203	4.2	1.0	3.2
3	$Ph_3P+(PyS)_2$[d]	A	0.308	0.012	0.561	0.199	25.7	1.0	16.6
4	DCCI+HOBt[c]	B	—	0.131	0.544	0.401	—	1.0	3.1
5	DEPC[c]	B	—	0.136	0.572	0.397	—	1.0	2.9
6	$Ph_3P+(PyS)_2$[d]	B	—	0.019	0.531	0.467	—	1.0	24.6
7	DCCI+HOBt[c]	C	0.722	0.118	0.467	0.346	(3.1)	1.0	2.9
8	DEPC[c]	C	0.711	0.118	0.484	0.353	(3.0)	1.0	3.0
9	$Ph_3P+(PyS)_2$[d]	C	0.224	0.246	0.470	0.112	(0.46)	1.0	0.46

a) Determined with use of the amino acid ratios of resulting peptide resins taking the recovery of Val as a standard. b) Abbreviations; A: mixture of an equivalent each of Boc-Gly-OH, Boc-L-Val-OH, and Boc-L-Phe-OH, B: mixture of an equivalent each of Boc-L-Val-OH and Boc-L-Phe-OH, C: mixture of an equivalent each of Boc-L-Val-OH and Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH. c) A three-fold excess each of carboxyl components and an equimolar amount of the total of the carboxyl components of a coupling reagent were used. d) The coupling reaction was carried out with a three-fold excess each of carboxyl components and a two-fold excess of the total of the carboxyl components each of Ph_3P and $(PyS)_2$.

carboxyl components) of DCCI and HOBt each in 20 ml of DMF at 0—5 °C for 2 h and at room temperature for 13 h. The resulting peptide resin by precipitation from the solution in DMF with methanol was subjected to acid hydrolysis with propionic acid–12 M HCl (2: 1 v/v) at 115 °C for 35 h followed by the amino acid analysis. Relative reactivities of Boc–amino acids were determined using the amino acid ratios of the resulting peptide resin taking the recovery of Val as a standard. Relative reactivity of Boc–L-Tyr(Bzl)–Gly–Gly–L-Phe–OH was also determined by a similar procedure using a combination of carboxyl components.

It seems that in each coupling reagent (except DCCI plus HOBt), peptide bonds involving Boc–L-Phe–OH and Boc–L-Val–OH which have bulky side chains are not formed as readily as Boc–Gly–OH forms peptide bond. A marked influence of bulky side chains on a decrease in the rate of the reaction was observed when Ph_3P plus $(PyS)_2$ was used as a coupling reagent (run Nos. 1—3, Table 2).

Relative reactivities using DCCI plus HOBt as a coupling reagent:

Boc-L-Phe-OH > Boc-Gly-OH > Boc-L-Val-OH

Relative reactivities using DEPC as a coupling reagent:

Boc-Gly-OH > Boc-L-Phe-OH > Boc-L-Val-OH

Relative reactivities using Ph_3P plus $(PyS)_2$ as a coupling reagent:

Boc-Gly-OH > Boc-L-Phe-OH $\gg$ Boc-L-Val-OH

The values reflect the reactivities of the intermediates formed by the reactions of coupling reagents with Boc–amino acids (Fig. 1).

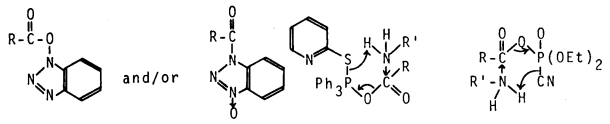

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Fig. 1. Intermediates for the corresponding coupling reagents (DCCI+HOBt,[12] $Ph_3P+(PyS)_2$,[9] and DEPC[10]).

The data runs 4—9, Table 2 show that the relative reactivities of Boc–L-Val–OH, Boc–L-Phe–OH, and Boc–L-Tyr(Bzl)–Gly–Gly–L-Phe–OH are as follows:

Coupling reagents: DCCI + HOBt and DEPC

Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH

= Boc-L-Phe-OH > Boc-L-Val-OH

Coupling reagent: Ph_3P + $(PyS)_2$

Boc-L-Phe-OH $\gg$ Boc-L-Val-OH >

Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH

The results indicate that the peptide chain length of Boc–L-Tyr(Bzl)–Gly–Gly–L-Phe–OH has no influence on the reactivity of the *C*-terminal amino acid in the coupling reaction using DCCI plus HOBt and DEPC as coupling reagents. A marked decrease in the reactivity of the *C*-terminal amino acid of the tetrapeptide acid was observed when Ph_3P plus $(PyS)_2$ was used as a coupling reagent. The differences due to coupling reagents can be attributed to the steric hindrance effect of the peptide chain on the intermediates I, II, and III.

Fragment condensation on the soluble polymer support could achieve efficient coupling of the tetrapeptide acid to the amino free terminal anchored to the soluble polymer support. Relative reactivities of Boc–amino acids and the peptide fragment could be easily determined by use of amino acid ratios in the acid hydrolysates of the resulting peptide resins in which they were taken. Thus it was found that the peptide chain length of Boc–tetrapeptide acid has no influence on the reactivity of the *C*-terminal amino acid in the coupling reaction using DCCI plus HOBt and DEPC as coupling reagents. However, a marked decrease in the reactivity of the *C*-terminal amino acid of the tetrapeptide was observed when Ph_3P plus $(PyS)_2$ was used as a coupling reagent.

The versatility of this new approach eliminates shortcomings of the solid phase and the classical liquid phase peptide syntheses, their merits, namely the inherent simplicity of synthesis using polymer supports and the advantage of carrying out the peptide synthesis under homogeneous conditions, being preserved. It promises the possibility of unequivocal chemical synthesis of homogeneous proteins of defined structures.

Experimental

Boc–amino acids were prepared according to Nagasawa *et al.*[13] HOBt, DEPC, and $(PyS)_2$ were prepared by the methods given by Konig and Geiger,[8] Takamizawa *et al.*[14] and Matsueda *et al.*,[18] respectively. All other reagents were commercial products. DMF was purified as follows. To benzene-dried DMF was added phenyl isothiocyanate (1% by volume) followed by distillation under reduced pressure. It was then redistilled under reduced pressure. Optical rotations were taken in a 1-dm cell on a Jasco Model ORD/UV-5 optical rotatory dispersion recorder. Amino acid analyses were performed on a Hitachi Liquid Chromatograph, Model 034. Hydrolyses of peptide resins were carried out on 20—40 mg samples of resins with propionic acid–12 M HCl (2 : 1 v/v) at 115 °C for 35 h.

Soluble Chloromethylated Polystyrene. Styrene, 28.12 g (0.27 mol), 4.58 g (0.03 mol) of (chloromethyl)styrene (Tokyo Kasei Co., a mixture of *m*- amd *p*-isomers (7 : 3)), and 0.25 g (1.5 mmol) of azobisisobutyronitrile were dissolved in 100 ml of benzene. The solution in a polymerization tube was sealed off *in vacuo* and heated in a bath maintained at 70±0.5 °C for 38 h. The solution was poured into methanol to obtain a soluble chloromethylated polystyrene. The product was dried *in vacuo* at 75—80 °C for 20 h. The yield was 20.3 g. The chlorine content of the polymer was determined by the Volhard method to get a value of 0.91 mmol per 1 g of resin.

H–L-Leu–OCH$_2$–resin. Esterification of the chloromethylated polystyrene with Boc–L-Leu–OH was carried out at room temperature for 7 days.[7b] Boc–L-Leu–OCH$_2$–resin: Leu content, 0.61 mmol/g (amino acid analysis); Cl content, 0.20 mmol/g (titrated by Volhard method). After attachment of Leu to the polymer support, residual benzylic chlorides were removed by the reaction with sodium

acetate in DMF at 105 °C for 24 h. The final chlorine content was less than 0.02 mmol/g. H-L-Leu-OCH_2-resin was prepared by a similar procedure.[7b] H-L-Leu-OCH_2-resin: Leu content, 0.62 mmol/g (amino acid analysis).

Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OCH_3. Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OCH_3 was prepared with use of DCCI plus HOBt as a coupling reagent,[11] mp 114—116 °C, $[\alpha]_D^{21}=+13.1°$ ($c=2.0$, CH_3OH) (lit,[11] mp 108—110 °C, $[\alpha]_D=+26.3°$ ($c=1.0$, CH_3OH)). It was also prepared by the following procedure. To a mixture of 3.73 g (0.01 mol) of Boc-L-Tyr(Bzl)-OH and 3.63 g (0.011 mol) of H-Gly-Gly-L-Phe-OCH_3·HCl in 30 ml of DMF was added 1.80 g (0.011 mol) of DEPC in 10 ml of DMF at 0 °C, followed by the addition of 2.1 g (0.021 mol) of triethylamine in 10 ml of DMF during 5 min. The mixture was strired at 0 °C for 1 h and then at room temperature overnight. The reaction mixture was diluted with 300 ml of ethyl acetate, successively washed three times with 5% hydrochloric acid, twice with 10% aqueous sodium chloride, three times with saturated aqueous sodium hydrogencarbonate, and twice with 10% aqueous sodium chloride, and dried over sodium sulfate. Removal of solvent *in vacuo* afforded the crude peptide (6.10 g). Recrystallization from ethyl acetate gave a substance, mp 114—116 °C, $[\alpha]_D^{21}=+12.9°$ ($c=1.0$, CH_3OH). Amino acid ratios in acid hydrolysate: Tyr 0.93, Gly 2.00, Phe 1.01 (Found: C, 64.27; H, 6.42; N, 8.87%).

Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH. To the tetrapeptide methyl ester, 3.5 g(5.0 mmol) in 60 ml of methanol, was added 12 ml of 0.5 M aqueous sodium hydroxide and stirred overnight at room temperature. Removal of solvent *in vacuo* afforded the crude peptide sodium salt, which was treated with 10% citric acid to give the tetrapeptide acid, 2.85 g. Recrystallization from water-methanol (3 : 1) gave a pure material, mp 104—107 °C, $[\alpha]_D^{21}=+21.0°$ ($c=1.0$, CH_3OH) (lit,[11] mp 95—96 °C, $[\alpha]_D=+34.3°$ ($c=0.95$, CH_3OH)). Amino acid ratios in acid hydrolysate: Tyr 0.91, Gly 2.00, Phe 1.00 (Found: C, 63.33; H, 6.15; N, 9.02%).

Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-L-Leu-OCH_2-resin. H-L-Leu-OCH_2-resin, 200 mg(Leu content: 0.124 mmol), was dissolved in 10 ml of DMF. To the solution was added 224 mg (0.37 mmol) of Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH, followed by the addition of 0.37 mmol of a coupling reagent at 0—5 °C. The reaction mixture was stirred at 0—5 °C for 2 h and then at room temperature for 3 h. To DCCI and DEPC, used as coupling reagents, were added 0.37 mmol of HOBt and 0.37 mmol of triethylamine, respectively. In the case of Ph_3P plus $(PyS)_2$, the coupling reaction was carried out with a three-fold excess of the carboxyl component and a 30-fold excess each of Ph_3P and $(PyS)_2$. The solution was poured into 200 ml of methanol followed by 30 ml of saturated aqueous sodium chloride. The precipitated peptide resin was filtered off, and washed successively with water and methanol to remove the soluble reactant. The yields were 253 mg (DCCI plus HOBt), 277 mg (DEPC), and 243 mg (Ph_3P plus $(PyS)_2$) (dried *in vacuo* for 2 days).

Relative Reactivities of Boc-Amino Acids and Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH in the Coupling Reaction. H-L-Leu-OCH_2-resin, 200 mg (Leu content: 0.124 mmol), and a three equivalent each of carboxyl components were dissolved in 20 ml of DMF. To the solution was added a coupling reagent, an equivalent mole with the total of the carboxyl components, under cooling with ice-cold water. When DCCI and DEPC were used as coupling reagent, an equivalent each of HOBt and triethylamine was added, respectively, after addition of the coupling reagent. In the case of Ph_3P plus $(PyS)_2$, the coupling reaction was carried out with a three-fold excess each of carboxyl components and a two-fold excess of the total of the carboxyl components each of Ph_3P and $(PyS)_2$. The reaction mixture was stirred for 2 h in an ice bath and 13 h at room temperature. The work-up was essentially the same as described above. The peptide resins were subjected to acid hydrolyses.

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