BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 41 182—186 (1968)

The Peptide Synthesis. II. Use of the Phenol Resin for the Peptide Synthesis*1

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(Received July 4, 1967)

The phenol resin is useful as support of the solid phase peptide synthesis. Its use is particularly advantageous for the synthesis of the *N*-protected peptide fragments, and those which have the intramolecular disulfide bond, such as oxytocin. In the latter case, the application of *S*-ethylmercapto group as the protector of thiol group of cysteine residue was combined successfully. Oxytocin and physalaemin derivatives were thus synthesized.

Recently, the solid phase peptide synthesis introduced by Merrifield[1,2] has provoked keenest interest. The essential feature of this method is protection of the carboxyl group of an amino acid by esterification with polymeric derivatives of benzyl alcohol; the products being insoluble in the usual solvents, they can be isolated and purified simply by washing with a series of appropriate solvents. Many active peptides, such as bradykinin,[3,4] angiotensin,[5] insulin[6] and others were thus synthesized simply, rapidly and automatically. Long chain peptides which were difficult to synthesize to date are further expected to be synthesized by this method. However, many problems still remain; (1) The introduction of the starting acyl amino acid to polymer requires rather drastic condition, which may give rise to racemization and other side reactions. It is difficult to introduce the acyl peptide direct to the polymer. (2) *C*-Terminal of many important active peptides is often amide, as in oxytocin, vasopressin, eledoisin, physalaemin and gastrin. Synthesis of these peptide amides as described by Bodanszky *et al.*[7] are troublesome and peptide hydrazide is also hard to prepare. (3) To obtain the acyl peptide fragments is impossible, for the removal of peptide from polymer is performed by hydrogen bromide in trifluoroacetic acid, or liquid hydrogen fluoride,[8] *etc.*

We found that the polyphenol resin is an useful

*1 This study was presented at the 20th Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1967.

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2) R. B. Merrifield, *Science*, **150**, 178 (1965).
3) R. B. Merrifield, *J. Am. Chem. Soc.*, **86**, 304 (1964).
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5) G. R. Marshall and R. B. Merrifield, *ibid.*, **4**, 2394 (1965).
6) A. Marglin and R. B. Merrifield, *J. Am. Chem. Soc.*, **88**, 5011 (1966).
7) M. Bodanoszky and J. T. Sheehan, *Chem. & Ind. (London)*, **1964**, 1423; *ibid.*, **1966**, 1597.
8) J. Lenard and A. B. Robinson, *J. Am. Chem. Soc.*, **89**, 181 (1967).

support. Conditions of its synthesis will be described in the present investigation. The phenol resin as support of the solid phase peptide synthesis has not only all the merits of Merrifield's method, but also it can solve some of the problems of Merrifield's method as described above.

The phenol resin was synthesized by reacting phenol and equimolar amount of *S*-trioxane in bis-(2-ethoxyethyl)-ether containing catalytic amount of *p*-toluenesulfonic acid at 150°C in a sealed tube for 24 hr. This phenol resin is insoluble in the usual solvents employed for the peptide synthesis but it swells in them.

The introduction of the first acyl amino acid, such as *N*-*t*-amyloxycarbonyl-,[9] *N*-*t*-butyloxycarbonyl-[10] or *N*-carbobenzoxy[11]-amino acid, to the phenol resin is carried out with *N*, *N'*-dicyclohexylcarbodiimide[12] in dimethylformamide. The resulting resinous derivative of acyl amino acid phenyl ester was isolated and purified simply by washing with a series of appropriate solvents. The acyl group was removed by hydrogen halide in suitable solvent. The resulting hydrogen halide salt was converted to the free base with triethylamine in dimethylformamide, and it was coupled with the next acyl amino acid or acyl peptide by active ester method,[13-17] or *N*, *N'*-dicyclohexylcarbodiimide method in dimethylformamide. Excess reagents and by-products were removed from the insoluble product by filtration and through washing with suitable solvents. Further elongation of peptide bond was carried out in the same way by alternate deprotecting and coupling with the appropriate acyl amino acid or acyl peptide.

Choice of solvent was important for the coupling reaction. Of the several solvents examined, dimethylformamide was the best one which had high dielectric constant and also swelled the resin. Pyridine was also satisfactory, whereas chloroform, benzene, ethyl acetate and dioxane were unfit not swelling the resin or having low dielectric constant.

Selection of the coupling methods for the formation of the peptide bond was also important. The several methods were examined, and the order of the coupling efficiency was as follows;

N, *N'*-Dicyclohexylcarbodiimide, *p*-Nitrophenyl ester[13] > 2, 4-Dinitrophenyl ester,[14] *N*-Hydroxysuccinimide ester,[15] > 2, 4, 5-Trichlorophenyl ester[16] > Pentachlorophenyl ester[17] ≫ Azide,[18] mixed anhydride.[19]

The yield was excellent, when the coupling was performed by *p*-nitrophenyl ester method and *N*, *N'*-dicyclohexylcarbodiimide method. In the active ester methods, the coupling potency depended on the bulkiness of the esters, *i. e.* the steric effect.

When the coupling was completed, the protected peptide was cleft from the resin by treatment with aqueous N sodium hydroxide in methanol, 25% ammonia in methanol or hydrazine hydrate in dimethylformamide as the corresponding acyl peptide, acyl peptide amide or acyl peptide hydrazide. The crude acyl peptide derivatives are easily purified by one or two recrystallizations, and column chromatography for the purposes of purification was not necessary. But this method was unfit to synthesize the acyl peptides which were unstable under the basic condition.*2

Some examples of acyl peptide derivatives synthesized are listed in the table.

Optical rotation of these acyl peptide derivatives nearly agreed with that of the standard samples synthesized by the usual method and racemization was scarcely observed.*3

The latter five peptides in the table are the active fragments of physalaemin and its analogues. The activities of these free peptides deprotected were compared with that of a synthetic bradykinin[20] by the hypotensive effect in rabbit.

Oxytocin was synthesized for two reasons. First, it was of interest as a further test of the applicability of the phenol resin, since oxytocin is an active peptide rather hard to prepare containing the comparatively unstable amino acids; cystine, asparagine and glutamine. Secondly, it may be expected that the two deprotected free thiol groups of cysteine residues are converted to an intramolecular disulfide bond on the phenol resin instead of forming an intermolecular disulfide bond which often occurs in usual synthetic method of oxytocin.

9) S. Sakakibara, M. Shin, M. Fujino, Y. Shimonishi, S. Inoue and N. Inukai, This Bulletin, **38**, 1522 (1965).
10) L. A. Carpino, *J. Am. Chem. Soc.*, **79**, 98 (1957); G. W. Anderson and A. C. McGregor, *ibid.*, **79**, 6180 (1957).
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18) T. Curtius, *Ber.*, **35**, 3226 (1902).
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*2 For example, the cleavages of prolyl-proline-, seryl-proline- and arginyl-proline-bonds were often observed under the basic conditions.

*3 Racemization was about 3% in the following tests; (1) Many isoleucine derivatives were synthesized on the resin using L-isoleucine, and the ratio of L-isoleucine to allo-isoleucine was determined by amino acid analyzer. (2). *N*-Carbobenzoxy-glycyl-phenylalanyl-glycine which was synthesized stepwise on the resin using L-phenylalanine, was converted to ethyl ester and it was subjected to the Anderson's racemization test.

20) S. Sakakibara and N. Inukai, This Bulletin, **39**, 1567 (1966).

TABLE 1. THE SYNTHESIS OF PEPTIDES BY THE PHENOL RESIN

Peptide[a]	Mp °C	$[\alpha]_D^{23}$ c 0.4, in DMF Found/Cited[b]	Anal. % Found/Calcd C	H	N	Activity.[c] Synthetic Bradykinin =1
Z-Glu-OH └Cys(Bz)-Gly-OH	110—120	−36.8 (c=1, EtOH)	56.36 56.49	5.71 5.50	7.79 7.91	
AOC-Phe-Phe-Phe-Gly-$NHNH_2$ 1/2 H_2O	208—210	−33.0 −32.4	64.29 64.31	6.86 6.94	12.64 12.86	
AOC-Phe-Tyr-Gly- Leu-Met-NH_2	200—202 211—212[b]	−31.5 −30.9	60.05 59.82	7.29 7.33	11.18 11.31	0.5
$(AOC)_2$-Lys-Phe-Tyr- Gly-Leu-Met-NH_2	140—145	−31.2 −33.5	59.33 59.73	7.65 7.78	11.53 11.37	100
$(AOC)_2$-Lys-Phe-Tyr(Me)- Gly-Leu-Met-NH_2	210—215	−32.0 −32.3	59.78 60.10	7.96 7.87	11.06 11.21	100
AOC-Phe-Phe-Tyr(Me)- Gly-Leu-Met-NH_2	214—215	−36.9 −38.4	62.41 62.44	7.13 7.24	10.65 10.84	10
AOC-Phe-Phe-Phe- Gly-Leu-Met-NH_2	230—232	−43.9 −43.3	63.12 63.21	7.29 7.26	11.28 11.22	1

a) The all amino acids are L-form.
b) The standard samples were prepared by the usual synthetic methods.
c) The activity of the deprotected free peptide was compared with that of a synthetic Bradykinin.[20] Z=carbobenzoxy; AOC=*t*-amyloxycarbonyl; Bz=Benzyl; Tyr(Me)=*p*-methoxytyrosine residue; DMF=dimethylformamide.

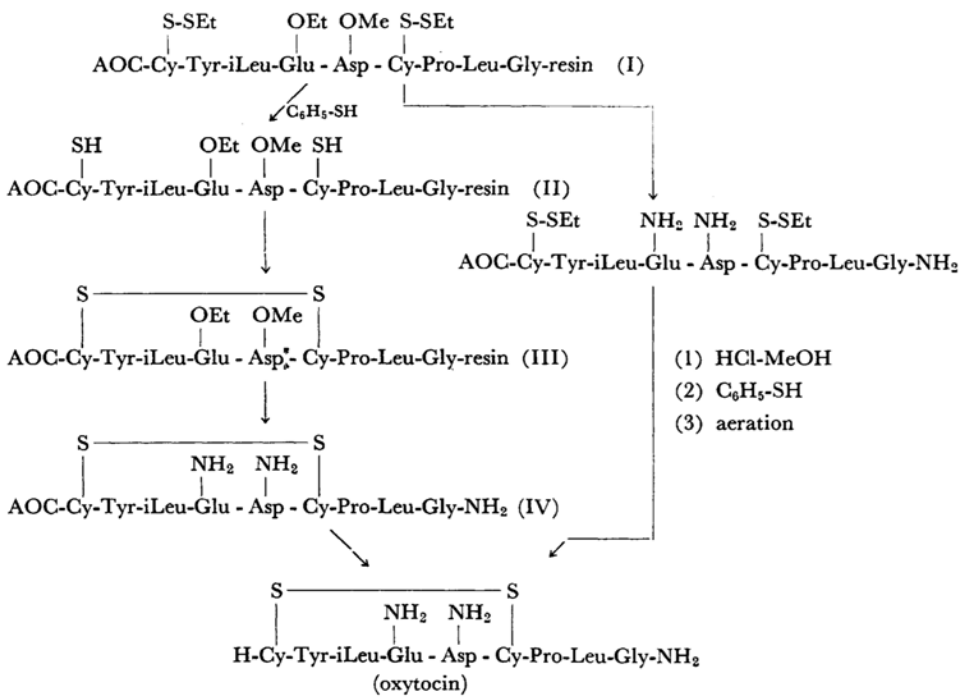

Fig. 1. The synthetic scheme of oxytocin.
AOC=*t*-amyloxycarbonyl; resin=polyphenol resin

The synthetic scheme of oxytocin was shown in the figure.

Peptide bonds were lengthened stepwise with the *N*-*t*-amyloxycarbonyl amino acids on the phenol resin. *S*-Ethylmercapto-L-cysteine which was reported in the proceeding paper[21] was used for this purpose, because the *S*-ethylmercapto group is

21) N. Inukai, K. Nakano and M. Murakami, This Bulletin, **40**, 2913 (1967).

easily removed by thiophenol.

From the completed *N-t*-amyloxycarbonyl-*S*-ethylmercapto-L-cysteinyl-L-tyrosyl-L-isoleucyl-γ-ethyl-L-glutamyl-β-methyl-L-aspartyl-*S*-ethylmercapto-L-cysteinyl-L-prolyl-L-leucylglycine phenol resin, *S*-ethylmercapto groups were removed by treating with thiophenol, the free two thiol groups of cysteine residues were converted to the intramolecular disulfide bond by aeration, and finally the peptides were cleft from the resin by treatment with 25% ammonia in methanol. By removing of the protecting group from the *N-t*-amyloxycarbonyl oxytocin thus obtained, the oxytocin could be synthesized. Its biological activity was found to be about two-thirds of that of a standard sample,[21] as determined by rat-uterine contractive activity. This result indicated that the stepwise synthesis of peptide bonds, the removal of *S*-ethylmercapto groups of cysteine residues and the formation of the intramolecular disulfide bond of cystine occured simply, rapidly and automatically on the resin without any complications.

In conclusion, the phenol resin as support of the solid phase peptide synthesis is very useful, especially so for the synthesis of the acyl peptide fragments which are stable under the basic condition. It could be employed successfully for the synthesis of the peptide derivatives which have the intramolecular disulfide bond, by the concomitant application of *S*-ethylmercapto group as protector of the thiol group of cysteine residue.

Experimental

Polyphenol Resin. The solution of phenol (18.8 g, 0.2 mol), *S*-trioxan (6.12 g, 0.068 mol) and *p*-toluenesulfonic acid (0.4 g) in bis-(2-ethoxyethyl)-ether (10 m*l*) was allowed to react at 150°C for 24 hr in the sealed tube in an oil bath. The tube is then permitted to cool to room temperature and is opened cautiously often wrapping in a towel. The amorphous solid product was repeatedly washed with acetone, dimethylformamide and ethanol and dried over phosphorus pentoxide *in vacuo*. Yield 13.8 g.

The Esterification Step. A solution of *t*-amyloxycarbonyl (AOC) amino acid or carbobenzoxyl (Z) amino acid (0.02 mol) and *N, N'*-dicyclohexylcarbodiimide (DCCD) (0.02 mol) in dimethylformamide (DMF) (20—30 m*l*) were added to the phenol resin (1 g) and the suspension was stirred at room temperature overnight. The resinous derivative was filtered and washed several times with DMF, ethanol and acetic acid to remove excess reagents and by-products, especially dicyclohexylurea was throughly removed by washing with acetic acid. 30—40% of phenolic hydroxyl group was esterified.*4 The phenolic hydroxyl group which remained was esterified with acetic anhydride.

The Deprotection Step. a) AOC-Amino acid phenol resin (containing 0.01 mol of AOC-amino aicd) was treated with 10% HCl in methanol or dioxane (20 m*l*) for 2 hr at room temperature. The suspension was filtered and washed with methanol, DMF and ethyl acetate. The resulting hydrogen chloride salt was then neutralized by shaking for 20 min in DMF (20 m*l*) containing triethylamine (0.04 mol). The solvent was removed by filtration and the resin was washed with methanol and DMF. The free base thus obtained was used to the coupling with the next acylamino acid or peptide.

b) Z-Amino acid phenol resin (containing 0.01 mol of Z-amino acid) was treated with 33% HBr in acetic acid (20 m*l*) shaking for 2 hr at room temperature. The suspension was diluted with 30 m*l* acetic acid, and filtered and washed with acetic acid, methanol and DMF. The resulting hydrogen bromide salt was neutralized with triethylamine in DMF as described above.

The Peptide-forming Step. *a) The DCCD Method.* AOC- or Z-Amino acid phenol resin (containing 0.01 mol of acylamino acid) was deprotected and neutralized as described above. The free base was suspended in DMF (20 m*l*) and the next AOC- or Z-amino acid or peptide (0.02 mol) was added and then a solution of DCCD (0.02 mol) in DMF (5 m*l*) was added. The suspension was shaken overnight. The resinous derivative was filtered and washed several times with DMF, acetic acid and methanol until dicyclohexylurea, excess reagents and byproducts are completely removed.

b) The p-Nitrophenyl Ester Method. AOC- or Z-Amino acid phenol resin (containing 0.01 mol of acylamino acid) was deprotected and neutralized as described above. The free base was suspended in DMF (20 m*l*) and the next AOC- or Z-amino acid or peptide *p*-nitrophenyl ester (0.02 mol) was added and then the suspension was shaken overnight. The resinous derivative was filtered and washed several times wiih DMF and methanol to remove excess reagents and by-products.

The Peptide Liberation Step. *a) AOC- or Z-Peptide.* The completed AOC- or Z-peptide resin (containing 0.001 mol of peptide) was saponified by shaking in a solution of 30 m*l* of ethanol-DMF (1 : 1) and 5 m*l* of aqueous N NaOH for 2 hr at room temperature. The phenol resin was removed by filtration and the filtrate was concentrated to a syrup. water (3 m*l*) was added to the syrup obtained and the solution was acidified with dilute aqueous HCl; the separated product was extracted with the suitable solvent and washed with water. The solvent was evaporated off and the crystal residue was recrystallized once or twice from the suitable solvents.

b) AOC- or Z-Peptide Amide. The completed AOC- or Z-peptide resin was treated with large excess of 25% ammonia in methanol for 2 days at room temperature in a sealed tube. The phenol resin was filtered off, the filtrate was concentrated, and the crystal residue was washed with dilute aqueous HCl, dilute aqueous sodium bicarbonate and water, and recrystallized once or twice from the suitable solvents.*5

*4 A fixed quantity of AOC- or Z-amino acid resin was saponified with aqueous N-NaOH in DMF and a ratio of the esterfication was calculated from the quantity of AOC- or Z-amino acid recovered.

*5 The rearrangement from the α-linkages of asparagine- and glutamine-residues to the β- and γ-linkages was 0—40%; it was checked by the methods of A. C. Chibnall *et al.* (*Biochem. J.*, **68**, 114 (1958)) and P. T. Lansbury (*J. Am. Chem. Soc.*, **83**, 429 (1961)).

c) AOC- or Z-Peptide Hydrazide. The completed AOC- or Z-peptide resin was treated with large excess of hydrazine hydrate in DMF overnight. The phenol resin was filtered off. Water was added to the filtrate and the precipitated crystal was collected by filtration and recrystallized from the suitable solvents.

***N*-*t*-AOC-*S*-Ethylmercapto-L-cysteinyl-L-tyrosyl-L-isoleucyl-γ-ethyl-L-glutamyl-β-methyl-L-aspartyl-*S*-ethylmercapto-L-cysteinyl-L-prol-yl-L-leucyl-glycine Resin (I).** Each peptide bonds were lengthened stepwise from the *C*-terminal with the AOC-amino acids by the DCCD method. These procedures were all carried out by the general method as described above.

***N*-*t*-AOC-L-Cysteinyl-L-tyrosyl-L-isoleucyl-γ-ethyl-L-glutamyl-β-methyl-L-asparatyl-L-cysteinyl-L-prolyl-L-leucyl-glycine resin (II) and Its Oxydated Derivative (III).** The compound I (0.5 g) was suspended in dioxane (10 m*l*) and thiophenol (0.1 m*l*) was added. The suspension was shaken for 10 hr at 45°C, and filtered and then washed with dioxane. II obtained was suspended in dioxane (5 m*l*) and methanol (5 m*l*) and CO_2 free air was bubbled for 4 hr. The oxidized derivative (III) was collected by filtration and washed with dioxane and methanol.

AOC-Oxytocin (IV). The compound III (0.4 g) was left for 2 days in 25% ammonia in methanol (20 m*l*) in a sealed tube. The phenol resin was removed by filtration and the filtrate was concentrated. The solid residue was dissolved in ethyl acetate and washed with 0.2 N aqueous HCl and water and dried over anhydrous sodium sulfate and then solvent was evaporated off. The crude amorphous solid (0.2 g) obtained was purified on a 2×30 cm Merck's silica gel G column by elution with chloroform - methanol - acetic acid (95 : 5 : 3 v/v); yield 0.1 g, mp 100—120°C, $[\alpha]_D^{23}$ −48.8 (*c* 0.5, DMF).

Found: C, 52.95; H, 6.94; N, 15.39%. Calcd for $C_{49}H_{76}N_{12}O_{14}S_2$: C, 52.48; H, 6.83; N, 14.99%.

Oxytocin. A fixed quantity of the AOC-oxytocin IV was treated with 7% aqueous HCl for 30 min, then the insoluble material was filtered off and pH of the filtrate was adjusted to 4 with dilute aqueous ammonia. The oxytocic activity of this final solution was found to be about two-thirds of that of a standard sample,[21] as determined by rat-uterine contractive activity.

The authors wish to express their thanks to Dr. Yuji Kawashima for his encouragement and valuable advice.