Solid-Phase Peptide Synthesis on a Novel Hydrogenolysable Linker–Resin Combination

C. S. PANDE,* N. GUPTA, and A. BHARDWAJ

Department of Chemistry, Himachal Pradesh University, Summer Hill, Shimla-171005, India

SYNOPSIS

The propensity of benzyloxycarbonylamide group towards clean and rapid hydrogenolysis was exploited in designing a linker for solid phase peptide synthesis on a copoly(styrene–divinylbenzene) resin support. Hydroxymethyl copoly(styrene–divinylbenzene) on treatment with 4-nitrophenyl chloroformate gave a resin which possessed benzyl 4-nitrophenyl carbonate pendants. These reacted with the α-amino group of Boc–Lys–OMe, resulting in the attachment of the latter through its side chain to the resin by a benzyloxycarbonylamide linkage. This was used to extend the peptide chain from the α-amino end of lysine by Boc-methodology. The peptide was released from the resin through palladium acetate–ammonium formate-based transfer hydrogenolysis. The usefulness of this linker was demonstrated by synthesizing several small peptides terminating in lysine. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Stepwise solid-phase peptide synthesis introduced by Merrifield[1] in 1963 has proved to be an extremely useful technique for rapid and convenient peptide synthesis,[2] and a large number of peptides have been successfully synthesized on polymeric supports. The C-terminal carboxyl group of the peptide to be synthesized is generally attached to the polymer through benzyl ester linkage, the cleavage of which requires the use of very strong acids, e.g., HF or HBr/TFA.[3] Not only does this treatment expose the peptide to harsh conditions and occasionally result in lower peptide yields, HF is hazardous and needs to be handled carefully under special conditions. Moreover, this is not suitable if the preparation of a protected peptide is the aim of synthesis.

Some of the methods designed to circumvent this difficulty make a modification in the polymer peptide linker. Rich and Gurwara[4] described an *o*-nitrobenzyl resin cleavable by photolysis at 3500 Å. Wang et al.[5] used a *p*-alkoxybenzyl linker which was cleaved acidolytically under much milder conditions (50% TFA in CH_2Cl_2, 30 min). Several authors[6] used the 2-methoxy-4-alkoxybenzyl linker which proved to be cleavable under still milder acidolytic conditions (1% TFA/CH_2Cl_2, 3–4 times, 15 min, room temperature). Mensi and Isied[7] devised the use of a cobalt(III) spacer between the polymer and peptide which was cleaved with mercaptoethanol in DMF (1M). Protected peptide fragments were synthesized on a polymer by a French group[8] using an allylic anchoring group which was removed with palladium-catalyzed hydrostannolytic cleavage.

The peptide assembled on a C-terminal amino acid bound to the resin through the conventional benzyl ester linkage is not quite amenable to basic or nucleophilic cleavage. Improved yield in such cleavage was made possible by using a resin where the peptide–polymer linkage was through a phenyl ester[9–11] or an *o*-nitrobenzyl ester[12] and the hydrolysis performed with a base (TMG, DBU, NaOH, Triton B, dimethylaminoethanol) or a nucleophile (Bu_4NF, KCN). Incorporating a trimethylsilyl group at an appropriate position in a specially designed spacer provided polymers[13,14] from which the peptide could be released by treatment with a fluoride ion as Bu_4NF. A cyanide ion as KCN or Bu_4NCN has been shown to be an effective nucleophile for the cleavage of phenacyl ester linkage between a peptide and a polymer.[15–17] Tam and coworkers developed several new polymeric supports[16,18] called multide-

* To whom correspondence should be addressed.

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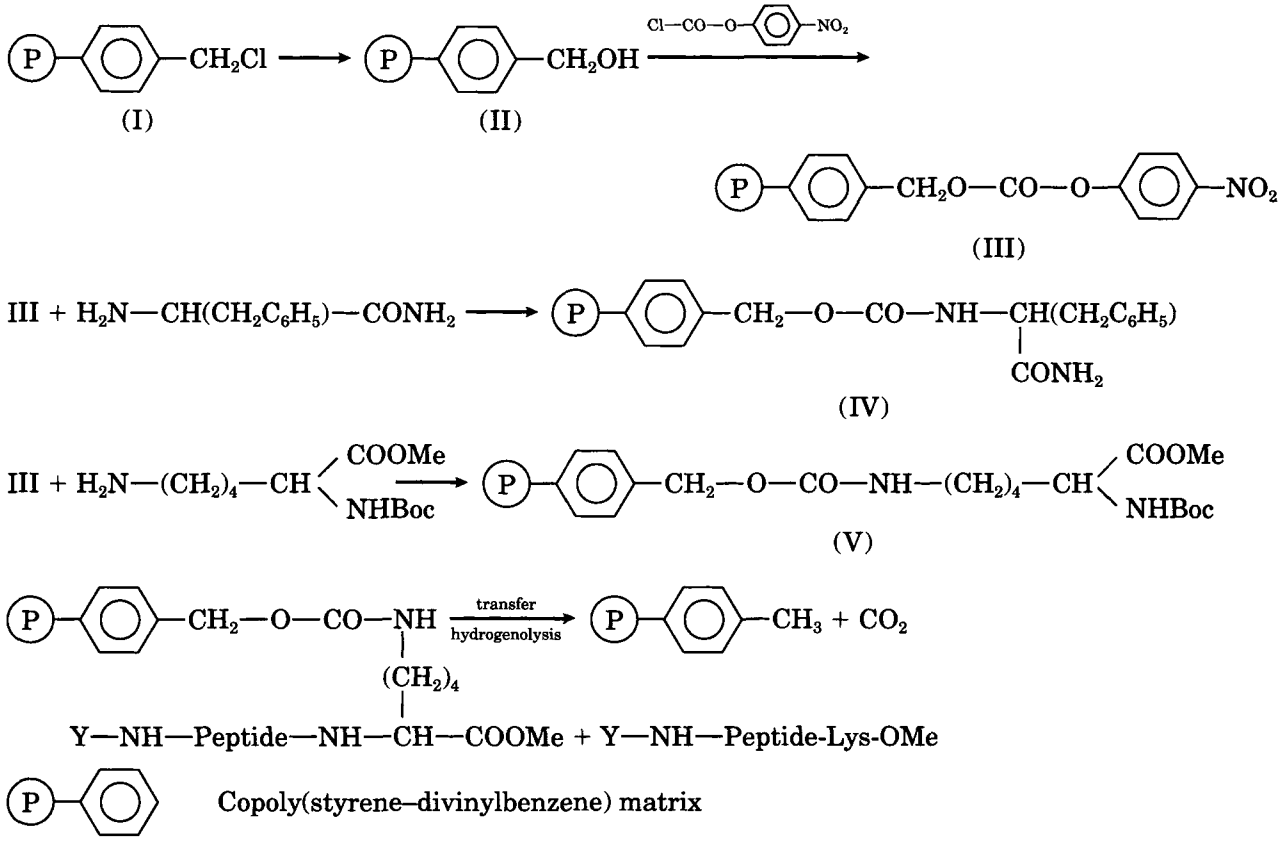
tachable resins (Pop and Pon resins) in which the spacer could be selectively cleaved at one of the two ester bonds, offering fragments usable in convergent solid-phase peptide synthesis. Peptide assembled on Pop resin could be cleaved conveniently by using a hindered, non-nucleophilic base like TMG or DBU.[19]

The propensity of a benzyloxycarbonylamide group toward clean and rapid hydrogenolysis has been exploited for the release of peptides terminating in lysine (or ornithine) from the resin at the end of polymer-supported peptide synthesis. Conventional Merrifield resin, chloromethyl copoly(styrene–divinylbenzene) I, was first converted into hydroxymethyl copoly-(styrene–divinylbenzene) II, by known methods. When treated with 4-nitrophenyl chloroformate and pyridine in methylene chloride, it afforded resin III which possessed benzyl 4-nitrophenyl carbonate pendants. Resin III reacted with the side chain amino function of an amino acid, which was thus attached through a benzyloxycarbonylamide linkage. α–Boc–Lys–OMe linked to the resin in this manner (V) was used to extend the peptide chain through the α-amino end by Boc-methodology. The peptide was released from the resin by palladium acetate–ammonium formate-based transfer hydrogenolysis.[20,21] Near-complete cleavage of the linker was demonstrated first in a model experiment in which phenylalanine amide was attached to the resin and subsequently released by hydrogenolysis.



This communication presents the preparation of 0-4-nitrophenoxycarbonyl hydroxymethyl polymer and its application in the synthesis of several small peptides terminating in lysine.

MATERIALS AND METHODS

Solvents were distilled and purified according to the literature procedure. Chloromethyl resin used was Bio-beads S–XI, 200–400 mesh (Cl = 0.42 mmol/g). IR spectra were taken on a Perkin–Elmer model 257 spectrophotometer with KBr pallets, and amino acid analyses were performed on an LKB 4150 Alpha amino acid analyzer.

Preparation of Hydroxymethyl Resin

Chloromethyl resin (20.0 g) was allowed to swell in dimethylacetamide (200 ml) and stirred gently at 80°C for 24 h with potassium acetate (8.0 g). The resin was then filtered; washed with DMF, dioxane, and methanol; and dried (IR 1725 cm^{-1}). The resulting resin was refluxed in 2N NaOH in ethanol

(200 ml) in nitrogen atmosphere for 10 h, then washed with water, DMF, 90% ethanol, and methanol (yield 19.5 g). IR showed the disappearance of the band at 1725 cm^{-1}.

Reaction of Hydroxymethyl Resin with 4-nitrophenylchloro-formate (Preparation of Resin III)

Hydroxymethyl resin (10.0 g) was suspended in dichloromethane (300 ml) containing pyridine (10 ml). 4-Nitrophenylchloroformate (1.4 g) was added and the mixture was gently stirred at 10°C for 8 h. The resin was filtered; washed with benzene, DMF, and methanol; and dried. IR showed 1530 cm^{-1}, 1350 cm^{-1} ($-NO_2$), and 1765 cm^{-1} ($-O-CO-O-$).

Reaction of Resin III with Phenylalanine Amide (Preparation of Resin IV)

Resin III (100 mg) was suspended in THF (5 ml) and was stirred with phenylalanine amide (105 mg) and aqueous 2N NaOH (0.5 ml) at room temperature for 24 h. The solution turned yellow. The resin was filtered; washed with THF, water, DMF, and methanol; and dried.

Alternatively, the NaOH solution in the above reaction was replaced by Et_3N (0.5 ml).

Both resin samples showed the absence of IR bands for $-NO_2$ and $-O-CO-O-$, but a new band at 1720 cm^{-1} appeared for $-O-CO-NH-$. Amino acid analysis gave Phe = 0.35 mmol/g.

Release of Phenylalanine Amide from Resin IV

Resin IV (20 mg) was stirred in DMF (2 ml) containing palladium acetate (5 mg). After equilibration, ammonium formate (10 mg in 0.5 ml H_2O) was added. The mixture soon turned black. After stirring for 30 min the resin was filtered and washed with DMF and water. The filtrate was dried and the resulting residue was examined on thin-layer chromatography (TLC). It contained only phenylalanine amide as ninhydrin positive organic material.

The spent resin showed less than 0.005 mmol of phenylalanine amide per g of resin.

Attachment of Boc-Lys-OMe to Resin (Preparation of Resin V)

Resin III (4.0 g) was treated with Boc-Lys-OMe (0.73 g) in the same manner as phenylalanine amide described above, using Et_3N (0.2 ml). A sample on amino acid analysis showed 0.35 mmol of lysine per g of resin.

General Method for Synthesis of a Peptide on Resin V

The solid phase synthesis was done manually using the following procedure:

(i) Deprotection of Boc-function was done by two successive treatments (5 and 30 min) of 25% TFA in CH_2Cl_2 containing 1 mg/ml of indole.[22]
(ii) DIEA in CH_2Cl_2 (5%) was used for releasing the amino function from its salt.
(iii) Two equivalents (relative to lysine, 0.70 mmol/g resin) of a *t*-Boc-amino acid was used for DCC-mediated coupling in CH_2Cl_2.
(iv) Step (iii) was repeated in CH_2Cl_2 : DMF (1 : 1).

Completion of the reaction on each coupling was checked by the ninhydrin method.[23] The side chains of Asp and Glu were protected as benzyl esters.

Release of Boc-peptide Methyl Esters from Resin

Peptidyl resins were subjected to catalytic transfer hydrogenolysis in the same manner as described for the release of phenylalanine amide, above. After resin was removed, the filtrate was dried on a rotavapor. The residue was extracted with ethyl acetate and washed with water, and the solvent was removed under reduced pressure.

Preparation of Free Peptides

(i) Saponification of methyl ester: The protected peptide was taken in ethanol and treated with ethanolic KOH (final strength 1.0 N) for 30 min at room temperature. Water was added and the solution was acidified with dilute HCl (or citric acid, in the case of a Boc-peptide).
(ii) Removal of Boc group: Boc-peptide was treated with ethyl acetate saturated with hydrogen chloride and the solution dried *in vacuo*.

Gly-Lys · HCl

Boc-Gly was attached to Resin V (1.0 g) according to the general procedure described above. Hydro-

genolysis produced Boc–Gly–Lys–OMe, which was first saponified; then the Boc-group was removed, yielding the free dipeptide (68 mg). The recrystallized product (from H_2O–EtOH), on amino acid analysis, gave Gly 1.03, Lys 1.0, $[\alpha]_D^{22}$–12.2° (c 2, in 0.5N HCl) Lit.[24] $[\alpha]_D^{20}$–13 ± 2° (2% in 0.5N HCl). Calc. for $C_8H_{18}ClN_3O_3$: C, 40.08; H, 7.51; N, 17.53. Found: C, 40.19; H, 7.65; N, 17.61.

Asp–Lys

The dipeptide was similarly prepared from Resin V (1.0 g). Aspartic acid was introduced using Z–Asp(OBz)–OH. Hydrogenolysis gave Asp–Lys–OMe which, on saponification, gave the free dipeptide. Recrystallization from aqueous methanol yielded 73 mg $[\alpha]_D^{22}$–5.5° (c 2, in 0.5N HCl). Lit.[25] $[\alpha]_D^{30}$–5.3°. Amino acid analysis gave Asp 1.01; Lys 1.0, Calc. for $C_{10}H_{19}N_3O_5$: C, 45.98; H, 7.28; N, 16.09. Found: C, 46.12; H, 7.19; N, 16.17.

Glu–Lys

The dipeptide was prepared from Resin V (1.0 g) by following the method applied for the aspartyl peptide and was recrystallized from aqueous methanol. Yield 77 mg. $[\alpha]_D^{22}$–1.9° (c 2, in 0.5N HCl). Lit.[25] $[\alpha]_D^{31}$–1.8° (0.5N HCl). Amino acid analysis gave Glu 1.02, Lys 1.0. Calc. for $C_{11}H_{21}N_3O_5$: C, 48.00; H, 7.64; N, 15.27. Found: C, 47.89; H, 7.71; N, 15.19.

Gly–Gly–Lys–HCl

The tripeptide was synthesized from Resin V (1.0 g), yield 70 mg. Amino acid analysis gave Gly 2.10, Lys. 1.0. $[\alpha]_D^{22}$–11.9° (c 2, in 0.5N HCl). Lit.[24] $[\alpha]_D^{12}$–12.1° (2% H_2O). Calc. for $C_{10}H_{21}ClN_4O_4$: C, 40.47; H, 7.08, N, 18.89. Found: C, 40.59; H, 7.05; N, 18.93.

Val–(Asp)$_4$–Lys

The hexapeptide was synthesized from Resin V (1.0 g) by the general procedure. Valine was introduced as Z–Val. The peptide methyl ester was saponified and the free peptide was desalted through a column of Bio-gel P2 in 2% AcOH. The ninhydrin positive fractions were collected and evaporated *in vacuo*. The residue was chromatographed on a column of Sephadex G-25 and eluted with 2% AcOH. Main fractions containing the peptide were pooled and lyophilized (51 mg). Amino acid analysis gave Val 0.98, Asp 3.91, Lys 1.0.

RESULTS AND DISCUSSIONS

Hydroxymethyl resin (II) was prepared from Merrifield resin (Cl substitution 0.42 mmol/g) by a two-step procedure as described by Wang.[5a] On treatment with 4-nitrophenyl chloroformate in presence of pyridine, it afforded the target resin (III). IR showed absorption at 1765 cm^{-1} for —O—CO—O— and at 1530 cm^{-1} and 1350 cm^{-1} for $-NO_2$. The resin reacted with phenylalanine amide with the release of *p*-nitrophenol, whereby phenylalanine amide was attached to the resin through its amino group by a carbobenzyloxy linker. IR revealed a urethane group at 1720 cm^{-1} and the absence of an $-NO_2$ group. Phenylalanine content of the resin was 0.35 mmol/g. When this resin was treated with palladium acetate and ammonium formate, phenylalanine amide was almost quantitatively released. The combination of palladium acetate and ammonium formate served as the best and fastest method for the hydrogenolysis of benzyloxycarbonylamide linker between the resin and amino acid.

When Resin III was treated with α–Boc–lysine methyl ester, the latter was attached to the resin through its ε-amino group. After removing the Boc-group with TFA, amino acids were successively attached by Boc-methodology using the modified procedure of Yamashiro and Li.[26] The protected peptide was released from the resin by hydrogenolytic cleavage. Saponification and removal of the Boc-group afforded the free peptide in high purity.

We have demonstrated the use of 0-4-nitrophenoxycarbonyl hydroxymethyl polymer (III) for the synthesis of a few small peptides terminating in lysine. Tryptic fragments of natural proteins yield peptides ending in lysine and are important in the study of protein sequences. The resin is constrained for the incorporation of amino acids which possess sulfur, but offers support for the solid-phase synthesis of many other peptides and requires the mildest and safest conditions for the release of the peptide, protected peptide, or peptide amide from the resin. The method is adaptable for Fmoc-methodology.

ABBREVIATIONS

Boc	*tert*-butyloxycarbonyl
DBU	1,8-diazabicyclo[5,4,0] undec-7-ene
DIEA	diisopropylethylamine
DMF	dimethylformamide
TFA	trifluoroacetic acid

THF	tetrahydrofuran
TMG	tetramethylguanidine
Z	benzyloxycarbonyl

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