Solid-Phase Synthesis with Attachment of Peptide to Resin through an Amino Acid Side Chain: [8-Lysine]-Vasopressin

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Communicated by Hans T. Clarke, March 1, 1971

ABSTRACT It is proposed that the scope of solid-phase peptide synthesis could be considerably broadened by attaching peptides to the solid-phase through functional side-chain groups rather than through the commonly used α -carboxyl groups. Side-chain attachment offers the use of a large variety of chemical linkages to solid supports. Attachment through the ϵ -amino group of the lysine residue to a polystyrene resin has been applied to a solid-phase synthesis of lysine-vasopressin. N^{α} -*tert*-butoxycarbonyl-L-lysyl-glycinamide was condensed with chloroformoxymethyl polystyrene-2% divinylbenzene resin. After removal of the N^{α} -protecting *tert*-butoxycarbonyl group, the peptide chain was elongated by standard Merrifield procedures to give Tos-Cys(Bzl)-Tyr-Phe-Glu(NH₂)-Asp(NH₂)-Cys(Bzl)-Pro-Lys(Z-resin)-Gly-NH₂. Cleavage from the resin with HBr in dioxane or trifluoroacetic acid gave a partially protected nonapeptide hydrobromide. For purification, it was converted into a fully protected peptide by treatment with benzyl *p*-nitrophenyl carbonate and crystallized. Deprotection by sodium in liquid ammonia, oxidative cyclization, IRC-50 desalting, and ion-exchange chromatography gave lysine-vasopressin with high potency in a rat-pressor assay.

The solid-phase method of peptide synthesis, developed by Merrifield (1, 2), makes possible the chemical syntheses of proteins. Synthetic achievements to date include syntheses of bovine insulin (3) in 1966, an analog of horse heart cytochrome *c* (4) and bovine pancreatic ribonuclease A (5) in 1969, and human growth hormone (6) in 1970. The advantages of the solid-phase method lie in (a) unprecedented speed, (b) simplicity of operation, (c) elimination of solubility problems, (d) minimal danger of racemization, and (e) possibility for automation. However, at its present state of development, the procedure suffers from several serious shortcomings. Incomplete peptide-bond formation (a), potentially occurring in each peptide-coupling step, results in so-called failure sequences and truncated sequences (7). The reagents (b) that must be used to remove the final product from the solid support often give incomplete cleavage and (or) partial degradation of the peptide. Analytical methods (c) sensitive enough to accurately monitor the process and to assess the purity of the products are not yet available.

Some of the many efforts to improve the solid-phase method have focused on variations of the solid-phase (1, 8-16), or on facilitating formation of the commonly used benzyl-ester bond (17-19) between the starting C-terminal amino acid and the standard resin (chloromethyl polystyrene-2% divinylbenzene). In all of these investigations, the attachment to the solid-phase has been made through the α functional groups

of amino acids or peptides (2). We wish to suggest that attachment to a solid-phase through functional groups in side-chains of amino acids could, (a) considerably broaden the chemical scope of such investigations, and (b) eliminate any danger of partial racemization of the amino acid residue serving as link to the solid phase, and thereby remove certain restrictions on reaction conditions during the attachment.

We have investigated the attachment of lysine-containing peptides to a solid-phase through the ϵ -amino group of the lysine residue. For this purpose chloroformoxymethyl polystyrene-2% divinylbenzene 1 (8) was prepared from the standard Merrifield resin (chloromethyl polystyrene-2%

divinylbenzene) via acetoxymethyl and hydroxymethyl intermediates (17). Treatment of 1 with carboxyl-protected N^{α} -*tert*-butoxycarbonyl-lysine derivatives gave resin-bound N^{ϵ} -benzyloxycarbonyl-type derivatives, 4.

Side-chain attachment allows the use of the proven strategy of stepwise chain elongation, in the direction from the C-terminal toward the N-terminal*, by adding one *N*-protected amino acid at a time. Completed peptides were detached from

Abbreviations follow the rules of the IUPAC-IUB Commission on Biochemical Nomenclature, in *Biochemistry*, 5, 1445, 2485 (1966); 6, 362 (1967); *J. Biol. Chem.*, 241, 2491 (1966).

*A solid-phase synthesis of a dipeptide in opposite direction (starting from the *N*-terminal amino acid) on resin (1) was reported by Letsinger and collaborators (8). To prepare larger peptides, a completely racemization-free method would be required for peptide bond formation. The use of the azide method for this reversed solid-phase procedure was studied recently by Felix and Merrifield (8) and was judged inadequate because of marginal yields.

the resin support of type 4 under somewhat milder conditions than those required for the cleavage of the standard benzyl ester bonding (20, 21). HBr in dioxane gave good results, as did HBr in trifluoroacetic acid (20), which cleaved such bonds within one-half of the commonly required time.

To examine the practical usefulness of this type of side-chain attachment to the solid phase, the neurohypophyseal hormone [8-lysine]-vasopressin (Fig. 1) was synthesized. The C-terminal dipeptide derivative, *N*^ε-*tert*-butyloxycarbonyl-*N*^ε-benzyloxycarbonyl-L-lysyl-glycinamide (3) was catalytically hydrogenated to yield the free ε-amino group and condensed with 1. For chain elongation, the general procedure (22) for solid-phase synthesis was used except that (23) the reaction times were generally longer and the *N*-protecting groups were removed from the asparagine and glutamine components by treatment with anhydrous trifluoroacetic acid (24). Dicyclohexylcarbodiimide (25) was used as the coupling reagent for *tert*-butyloxycarbonyl-L-proline (26, 27), *tert*-butyloxycarbonyl-L-phenylalanine (26, 27), *tert*-butyloxycarbonyl-L-tyrosine (26, 27), and *S*-benzyl-*N*-tosyl-L-cysteine (28, 29). The remaining amino acid residues were introduced into the growing peptide chain via active esters, i.e., *S*-benzyl-*N*-*tert*-butyloxycarbonyl-L-cysteine *p*-nitrophenyl ester (30), *tert*-butyloxycarbonyl-L-asparagine *p*-nitrophenyl ester (31), and *tert*-butyloxycarbonyl-L-glutamine *p*-nitrophenyl ester (32). HBr in dioxane or in trifluoroacetic acid (21) was used for cleavage of the completed peptide (5) from the solid support to give the hydrobromide of *S*-benzyl-*N*-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyll-L-asparaginyll-*S*-benzyl-L-cysteinyl-L-prolyl-L-lysyl-glycinamide (6), which was purified by repeated washing and reprecipitating. Total yields, based on the dipeptide content of the starting resin, were 20–25% in several syntheses. These yields were similar to those obtained in a standard solid-phase synthesis of lysine-vasopressin (33). The hormone, prepared from 6 by deprotection and oxidative cyclization, possessed 40–130 units/mg of activity by a rat-pressor assay for different batches synthesized. Chromatographic purification gave lysine-vasopressin with rat-pressor potencies of 180–190 units/mg; the activity remained constant on repeated chromatography. To obtain highly active hormone from 6, we took advantage of the demonstrated efficacy (34) of crystallization of protected nonapeptides from organic solvents to remove impurities, previously exploited in the solid-phase syntheses of lysine-vasopressin (33) and arginine-vasopressin (23). 6 was converted into a fully protected derivative by treatment with benzyl *p*-nitrophenyl carbonate (35). The ε-amino group of the lysine residue and the hydroxyl group of the tyrosine residue were both acylated to give *S*-benzyl-*N*-tosyl-L-cysteinyl-*O*-benzyloxycarbonyl-L-tyrosyl-L-phenylalanyl-L-glutaminyll-L-asparaginyll-*S*-benzyl-L-cysteinyl-L-prolyl-*N*^ε-benzyloxycarbonyl-L-lysyl-glycinamide (7), which was purified by repeated recrystallization from dimethylformamide-formic acid 99:1 (34). Deprotection by sodium in liquid ammonia, oxidative disulfide bond formation, desalting, and purification by ion-exchange chromatography (36) on Amberlite IRC-50 in 0.5 M ammonium acetate buffer, pH 6.4 (33, 34), gave highly active [8-lysine]-vasopressin, possessing a rat-pressor potency of 260–280 units/mg.

Thus, attachment of peptides to a solid-phase through functional groups in side chains of amino acid residues can offer effective alternatives to the standard Merrifield method of solid-phase peptide synthesis. The need for improved means of

Fig. 1. The structure of [8-Lysine]-vasopressin.

bonding to the solid phase, which would allow the removal of the completed peptide by milder treatments than the acidolytic reactions presently used, is strikingly apparent in the very recent solid-phase synthesis of human growth hormone (6), in which considerable activity was lost at this penultimate step of a total of 190 operational stages. The type of bonding used in this work allowed somewhat milder cleavage conditions compared with the standard Merrifield benzyl ester cleavage, but acidolytic treatment was still required. However, side-chain attachment offers other potential alternatives, e.g., disulfide bonding through cysteine residues, or utilization of the hydroxyl functions in serine, threonine, or tyrosine residues (37).

EXPERIMENTAL

The methods used in this work have been recently described in detail (23).

Chloroformoxymethyl polystyrene-2% divinylbenzene resin (1)

Chloromethyl polystyrene-2%divinylbenzene (70 g) (1) containing 2.14 meq of Cl/g, was converted into the acetoxymethyl polymer (77.4 g) [IR (KCl) 1735 (C=O)] by treatment with potassium acetate (147 g) in benzyl alcohol (500 ml) for 50 hr at 80°C (17). The product was saponified by refluxing a suspension in benzyl alcohol (300 ml) for 5.5 hr with 6 N methanolic NaOH (300 ml) to give hydroxymethyl polystyrene-2% divinylbenzene (72.4 g; IR (KCl) no absorption at 1735] (17). Treatment [50 g] with 20% phosgene in benzene (370 ml) for 5.5 hr at 20°C, followed by washing with dry benzene and ether, gave 1, 54.1 g, containing 1.27 meq of Cl/g, IR (KCl) 1735 (C=O).

N^ε-*tert*-butyloxycarbonyl-*N*^ε-benzyloxycarbonyl-L-lysyl-glycine ethyl ester (2)

To a stirred solution of *N*^ε-*tert*-butyloxycarbonyl-*N*^ε-benzyloxycarbonyl-L-lysine (26, 38) (18.5 g, 48.6 mmol) in tetrahydrofuran (30 ml, freshly distilled from LiAlH₄) at -10°C was added triethylamine (6.8 ml, 48.6 mmol), followed by isobutyl chloroformate (6.4 ml, 48.6 mmol). After 5 min, a cooled mixture of glycine ethyl ester hydrochloride (39) (7.0 g, 50 mmol) in dimethylformamide (45 ml) containing triethylamine (7.0 ml, 50 mmol) was added. The mixture was stirred for 2 hr at -10°C and for 15 hr at room temperature, filtered, and evaporated to dryness under reduced pressure. The residual oil was taken up in ethyl acetate, washed with 1 M citric acid

(twice), with 1 N NaHCO₃, then with saturated NaCl, dried (MgSO₄), and evaporated under reduced pressure. The residual oil was crystallized twice from isopropyl ether (1500 ml) to give colorless needles (18.1 g, 80%), mp 67–68°C, $[\alpha]_D^{22} -12.9^\circ$ (*c* 1, methanol).

Anal. Calcd. for C₂₃H₃₅N₃O₇ (465.5): C, 59.3; H, 7.58; N, 9.03; O, 24.1. Found: C, 59.6; H, 7.39; N, 8.66; O, 24.3.

***N*^α-*tert*-butyloxycarbonyl-*N*^ε-benzyloxycarbonyl-L-lysyl-glycinamide (3)**

A solution of **2** (15 g, 32.1 mmol) in anhydrous ethanol (150 ml) at 0°C was saturated with ammonia. The flask was sealed and kept for 3 days at room temperature. Evaporation under reduced pressure gave an oil that crystallized from isopropanol-isopropyl ether. Yield: 13.1 g (93.5%), mp 76°C, $[\alpha]_D^{22} -3^\circ$ (*c* 1, methanol).

Anal. Calcd. for C₂₁H₃₃N₄O₆ (436.5): C, 57.8; H, 7.39; N, 12.8; O, 22.0. Found: C, 57.8; H, 7.41; N, 12.8; O, 22.0.

Attachment of Boc-Lys-Gly-NH₂ to chloroformoxymethyl polystyrene-2% divinylbenzene resin (4)

A solution of **3** (5.65 g, 13 mmol) in methanol (50 ml) was catalytically hydrogenated for 3 hr with freshly-prepared Pd black (40). The product (oil) was dissolved in distilled dimethylformamide (60 ml). Chloroformoxymethyl resin (**1**, 12 g) and triethylamine (1.32 g, 13 mmol) were added and the mixture was stirred for 24 hr at room temperature. The product was obtained by filtration and thorough repeated washings with dimethylformamide, methanol, and ether, and was dried. To inactivate any unreacted chlorocarbonyl groups, the product was suspended in benzyl alcohol (100 ml) and treated with ammonia for 17 hr as described for **3**. The product was washed with benzyl alcohol, ethanol, and ether, and dried under reduced pressure over KOH to give 12.3 g of **4**.

Amino acid analysis (41) [dioxane-12N HCl 1:1, reflux for 48 hr] gave 0.28 mmol of glycine/g and 0.29 mmol of lysine/g. Residual chlorine: 0.2 meq/g.

***S*-Benzyl-*N*-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-*N*^ε-resin-L-lysyl-glycinamide (5)**

The dipeptide resin derivative **4** (5.5–10 g in several syntheses) was placed in the glass vessel of a manual shaker (2). A cycle for the incorporation of each amino acid into the growing peptide chain involved the following washing (W, for 10 min) and reaction (R) steps (8 ml of solvent/g of starting resin was used throughout): (a) 3 × W, dioxane; (b) R, 2 N HCl in dioxane, 20 min; (c) 3 × W, dioxane; (d) 3 × W, dimethylformamide; (e) R, 10% triethylamine in dimethylformamide, 10 min; (f) 3 × W, dimethylformamide; (g) 3 × W, methylene chloride; (h) addition of 3.3–5 equivalents of *tert*-butyloxycarbonyl-amino acid in methylene chloride and mixing for 10 min; (i) R, 3–5 equivalents of dicyclohexylcarbodiimide, methylene chloride, 3–15 hr; (j) 3 × W, methylene chloride. In the active-ester cycles (Boc-Asn-ONp, Boc-Gln-ONp) step *g* was: R, 3.3–5 equivalents of active ester, dimethylformamide, 4–16 hr. The *tert*-butyloxycarbonyl protecting group was removed from the asparagine and glutamine residues with anhydrous trifluoroacetic acid (step *b*, 15 min) (24). After completion of the protected nonapeptide, the peptide-resin was removed from the reaction vessel, thoroughly washed with methylene chloride, ethanol, and ether, and dried under reduced pressure (P₂O₅ and KOH). Weight increases of the final nonapeptide-

resins over starting resins were 93–97% of the theoretical in several experiments.

Cleavage from the resin: *S*-Benzyl-*N*-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-lysyl-glycinamide hydrobromide (6)

HBr, purified by passage through a 10% solution of phenol in CCl₄ or through a 10% solution of resorcinol in anhydrous trifluoroacetic acid, was introduced for 40 min into a suspension of peptide resin **5** in dioxane (5 ml/g of **5**). The resin was removed by filtration and washed twice with dimethylformamide. Evaporation under reduced pressure and repeated trituration of the ensuing residue with ether gave a yellow solid (385 mg, 75%, from 1.5 g of **5** with 0.28 mmol of Lys-Gly/g, mp 138–144°C).—Cleavage by HBr in trifluoroacetic acid (15–20 min) and workup, as above, gave a light-brown powder (700 mg, 67%, from 3.0 g of **5**, mp 128–130°C).

Purification by repeated treatment (4 times) with hot isopropanol gave a powder (mp 185–193°C) that gave one main spot on tlc, *R_f* 0.37, and one minor spot. Precipitation of this product from glacial acetic acid-ether gave a cream-colored powder (purified **6**, mp 197–202°C, 20–25% yield, based on **4**).

Amino acid analysis (41) (6 N HCl, 101°C, 24 hr) gave the following ratios: Lys_{1.1}Asp_{0.9}Glu_{0.9}Pro_{1.0}Gly_{1.0}Tyr_{1.0}Phe_{1.1}-(NH₃)_{2.7}†.

Deprotection (Na in liquid NH₃), oxidative disulfide bond formation, and desalting on Amberlite IRC-50 (33, 34) gave crude hormone preparations (in 83–96% yields) with rat-pressor activities ranging from 40 to 130 units/mg (42) in different experiments. Hormone activities obtained ranged from 4700 to 5250 rat-pressor units/g of peptide resin **5**. Ion-exchange chromatography (33, 34, 36) gave lysine-vasopressin (15–38%, based on purified **6**) with 180–190 units/mg of rat-pressor potency.

***S*-Benzyl-*N*-tosyl-L-cysteinyl-*O*-benzyloxycarbonyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-*N*^ε-benzyloxycarbonyl-L-lysyl-glycinamide (7)**

To a solution of nonapeptide derivative **6** (120 mg, 81.5 μmol) in dimethylformamide (2 ml) containing *N*-methylmorpholine (91 μl, 815 μmol) was added benzyl *p*-nitrophenyl carbonate (35) (222 mg, 815 μmol). The mixture was stirred for 20 min at 40°C. Acetic acid (0.1 ml) was then added, followed by ether (100 ml). A solid precipitate was obtained (115 mg), which was triturated several times with hot ethanol to give a light-yellow powder (95 mg, 71%), mp 216–220°C, tlc *R_f* 0.35. The product crystallized from dimethylformamide-formic acid 99:1 yielding a colorless product (80 mg, 60%), mp 222–224°C, $[\alpha]_D^{23} -23.3^\circ$ (*c* 0.34, dimethylformamide).

Anal. Calcd. for C₃₃H₄₇N₁₃O₁₈S₃ (1661.0): C, 60.0; H, 5.89; N, 11.0. Found: C, 59.8; H, 6.12; N, 11.1.

***O*-Debenzyloxycarbonylation of the tyrosine residue (8)**

Protected nonapeptide **7** (10 mg) was dissolved in glacial acetic acid (0.9 ml). Hydrazine hydrate (0.3 ml) was added and the solution was kept at 50°C for 2 hr. Then, water (10 ml) was added; the precipitate was recovered by filtration and

† Cysteine values (not given) always were lower than expected because of incomplete hydrolysis of Tos-Cys(Bzl).

washed with ethanol, acetone, and ether. Precipitation from dimethylformamide-ether gave a colorless powder (7 mg), mp 218–220°C.

Anal. Calcd. for $C_{75}H_{91}N_{13}O_{16}S_3$ (1526.8): C, 59.0; H, 6.01; N, 11.9. Found: C, 58.6; H, 5.94; N, 11.6.

[8-Lysine]-vasopressin, L-hemicystyl-L-tyrosyl-L-phenylalanyl-L-glutaminy-L-asparaginy-L-hemicystyl-L-prolyl-L-lysyl-glycinamide 1,6-disulfide (9)

Recrystallized protected nonapeptide **7** (80 mg, 48 μ mol) was dissolved in liquid ammonia (150 ml) that had been distilled from sodium. Sodium was added in small quantities to the boiling-ammonia solution over a period of 20 min until a blue color remained for 30 sec. About 12 mg of sodium was necessary. Glacial acetic acid (3 drops) was added, the ammonia was evaporated to a small volume, and the remainder was removed from the frozen material under reduced (water pump) pressure. The residue was dissolved in deoxygenated 0.2 N acetic acid (150 ml) under nitrogen. The pH was adjusted to 7.1 with 0.5 N NH_4OH and air was passed through the solution for 1 hr, after which the pH was adjusted to 4.8 with acetic acid. The solution was desalted on an Amberlite IRC-50 column (43), as described before (33, 34), to give, after lyophilization, a colorless powder (54 mg, 87%) possessing a rat-pressor potency of 190 units/mg.

Purification of 46 mg by chromatography on an IRC-50 column in 0.5 M ammonium acetate buffer, pH 6.4 (36), as described earlier (33, 34), gave lysine-vasopressin as a colorless powder (30 mg) with rat-pressor activity of 260–280 units/mg.

Amino acid analysis (6 N HCl, 105°C, 24 hr) gave the following molar ratios: $Lys_{1.03}Asp_{1.00}Glu_{1.10}Pro_{1.02}Gly_{1.00}Cys_{1.90}^{+}Ty_{1.06}Phe_{1.04}(NH_3)_{2.90}$.

We wish to thank Dr. R. Walter, Mt. Sinai Medical and Graduate Schools, New York, N.Y., for the bioassays.

This work was reported in part at the Second Northeast Regional Meeting, American Chemical Society, Providence, R.I., October, 1970. It was supported in part by Public Health Service Research grants No. C-6516 from the National Cancer Institute and No. FR-05526 from the Division of Research Facilities and Resources, National Institutes of Health.

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