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POLYMERIC REAGENT SYNTHESIS OF LUTEINIZING HORMONE-RELEASING HORMONE

Comparison With a Conventional Stepwise Preparation

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Luteinizing hormone-releasing hormone, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2,[2] was synthesized in a stepwise manner by two approaches: the use of insoluble polymeric active esters derived from (4-hydroxy-3-nitro)benzylated polystyrene and that of soluble *N,N*-dicyclohexylcarbodiimide. The overall yields of the syntheses were 40 and 7%, respectively. The efficiencies of the two synthetic routes, in which identical intermediate peptides were prepared, are compared.

INTRODUCTION

Ever since luteinizing hormone–releasing hormone (LH–RH) was identified as the linear decapeptide pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, various approaches to its synthesis have been suggested. Chief among these were classical synthesis in a homogeneous liquid phase and the use of heterogeneous polymeric supports [for review see Coy et al. (1)]. However, the synthesis of LH–RH via the polymeric reagents approach, i.e., the use of insoluble polymeric active esters as reagents for the elongation of the peptide chain (2,3), has never been described. In the present study, we report on a comparative stepwise synthesis of LH–RH by (a) polymeric reagents and (b) the use of *N,N*-dicyclohexylcarbodiimide as a coupling agent in solution (4). Clearly illustrated by this comparison are several inherent advantages of the polymeric reagents approach: simplicity of the synthetic operation, high coupling yields, and product purity.

[2]Abbreviations for amino acid derivatives and peptides follow the IUPAC–IUB Commission on Biochemical Nomenclature Recommendations. Symbols: See Eur. J. Biochem. 27:201–207 (1972).

MATERIALS AND METHODS

Amino acid analyses were performed on a Spinco-Beckman Model 120C amino acid analyzer. The blocked peptides assayed were hydrolyzed with 6 N hydrochloric acid containing 4% phenol in evacuated, sealed tubes for 36 h, at 110°C. Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter. All melting points were measured on a capillary melting point apparatus and are uncorrected. Thin-layer chromatography was performed on precoated silica gel plates (Riedel-De Haen AG, Hanover). The following solvent systems were used: chloroform–methanol (3:1 and 9:1, v/v), acetonitrile–water (9:1, v/v), 1-butanol–acetic acid–water (4:1:1, v/v), and 1-butanol–pyridine–acetic acid–water (15:10:3:12, v/v). Blocked peptides were detected by iodine vapor, by charring over a flame or under the ultraviolet lamp. Peptide derivatives possessing a free amino group were also detected by ninhydrin. Insoluble polymeric active esters derived from (4-hydroxy-3-nitro)benzylated polystyrene and from *N*-blocked amino acids (5) were prepared as previously described.

N-Benzyloxycarbonyl-L-pyroglutamyl-Im-2,4-dinitrophenyl-L-histidyl-L-tryptophyl-*O*-benzyl-L-seryl-*O*-benzyl-L-tyrosyl-glycyl-L-leucyl-ω-nitro-L-arginyl-L-prolyl-glycine benzyl ester (protected LH–RH; **IX**).

Use of Polymeric Active Esters as Coupling Reagents. To a solution of glycine benzyl ester *p*-toluenesulfonate (675 mg; 2.0 mmol) in distilled *N,N*-dimethylformamide (DMF; 25 ml) containing triethylamine (0.28 ml; 2.0 mmol), an insoluble polymeric active ester of Boc-Pro and (4-hydroxy-3-nitro)benzylated polystyrene (Boc-Pro-PHNB; 5.7 g containing 3.5 mmol proline) was added, and the suspension was gently agitated at room temperature. The coupling process was followed by thin-layer chromatography (5) and found to be completed with 2 h. The polymer was filtered and washed with dichloromethane (5 × 30 ml). The combined washings and filtrate were extracted with distilled water (3 × 50 ml), dried over Na_2SO_4, and evaporated to afford a chromatographically pure Boc-Pro-Gly-OBzl (**I**) in 99% yield. This was dissolved in anhydrous trifluoroacetic acid (10 ml), and after 10 min at room temperature, the acid was removed *in vacuo*. The oily residue was solidified by trituration with a mixture of ether and petroleum ether (b.p. 40°–60°C; 1 : 2, v/v), filtered, washed with dry ether, and dried over NaOH pellets *in vacuo*. The dipeptide trifluoroacetate salt was then dissolved in DMF (15 ml), neutralized with triethylamine (0.28 ml; 2.0 mmol), allowed to react for 5 h with Boc-Arg(NO_2)-PHNB (3.6 g containing 3.5 mmol arginine), and after working

up of reaction mixture as described for **I**, yielded the tripeptide Boc–Arg(NO_2)-Pro-Gly-OBzl (**II**) which was accompanied by a by-product (~10%), the cyclic *t*-butyloxycarbonyl nitro-L-arginine lactam. The ether-soluble cyclic lactam was separated from **II** by dissolving the mixture in ethanol and precipitating the tripeptide with ether (6). The yield of chromatographically pure **II** was 90%.

Treatment of **II** with trifluoroacetic acid afforded the corresponding trifluoroacetate salt which was coupled (12 h) in DMF with Boc-Leu-PHNB (2.0 g; 3.0 mmol leucine) to give a chromatographically pure Boc-Leu-Arg(NO_2)-Pro-Gly-OBzl (**III**) in 99% yield. Similarly, the Boc protective group of **III** was removed with trifluoroacetic acid, followed by coupling (6 h) of the deprotected peptide with Boc-Gly-PHNB (2.0 g, containing 4.0 mmol glycine). The blocked pentapeptide product Boc-Gly-Leu-Arg(NO_2)-Pro-Gly-OBzl (**IV**) was isolated (95% yield) as described for dipeptide **I**, and then crystallized from ethyl acetate–ether; m.p. 90°C; $[\alpha]_D^{23} -31.1$ (C, 0.72 $CHCl_3$). Amino acid analysis: Gly 2.1; Leu 1.1; Pro 1.0; Arg 1.0. Calculated for $C_{33}H_{51}N_9O_{10}$ (733.81): C, 54.01; H, 7.01; N, 17.18. Found: C, 52.35; H, 6.97; N, 17.18.

The synthesis was continued exactly as described for preparation of peptides **I–IV**. Successive deprotection of the Boc groups with anhydrous trifluoroacetic acid and coupling of the free α-amino ends with the corresponding polymeric reagents [Boc-Tyr(Bzl)-PHNB (4.1 g; 5.0 mmol tyrosine, 1.5 h, 96%); Boc-Ser(Bzl)-PHNB (3.9 g; 5.0 mmol serine; 3 h, 99%); Boc-Trp-PHNB (4.0 g; 6.5 mmol tryptophan; 12 h, 99%); Boc-His(Dnp)-PHNB (5.0 g; 6.0 mmol histidine; 12 h, 97%); Z-pGlu-PHNB (4.0 g; 6.0 mmol pyroglutamic acid; 12 h, 90%)] finally led to the chromatographically pure protected decapeptide Z-pGlu-His(Dnp)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-OBzl (**IX**). **IX** was crystallized from chloroform–ether (2.46 g, 68.8% overall yield, based on the amount of glycine benzyl ester *p*-toluenesulfonate salt); m.p. 134°–6°C (decomposition); $[\alpha]_D^{23}$ −27.2 (C, 0.79 DMF). Calculated for $C_{90}H_{99}N_{19}O_{22}$ (1798.84): C, 60.09; H, 5.55; N, 14.23. Found: C, 60.11; H, 5.53; N, 14.54.

Use of N,N-Dicyclohexylcarbodiimide (DCC) as a Coupling Agent. To a cooled (4°C) solution of Gly-OBzl *p*-toluenesulfonate salt (1 equiv) in dichloromethane containing 1 equiv of triethylamine, 1 equiv of Boc-Pro-OH was added. One equivalent of dicyclohexylcarbodiimide dissolved in dichloromethane was subsequently added. After standing at 4°C for 2 h and overnight at room temperature, the dicyclohexylurea formed was filtered off. The filtrate was washed consecutively with cold 10% (w/v) citric acid, 5% aqueous sodium bicarbonate (w/v), and water. After drying over Na_2SO_4, the dichloromethane solution was evaporated *in vacuo* to yield (86%) an oily residue of chromatographically pure dipeptide **I**, which

could not be crystallized. **I** was treated for 10 min with anhydrous trifluoroacetic acid, the deprotected dipeptide was then solidified by trituration with ether–petroleum ether (b.p. 40°–60°C; 1 : 2 v/v). It was then allowed to react with Boc-Arg(NO_2)-OH (1 equiv), in CH_2Cl_2–DMF (3 : 1 v/v) in the presence of 1 equiv of triethylamine, using DCC (1 equiv) as a coupling agent. The product tripeptide Boc-Arg(NO_2)-Pro-Gly-OBzl (**II**) was isolated as described for **I**, and crystallized from ethyl acetate–ether; yield 73%; m.p. 77°–82°C; $[\alpha]_D^{23}$ −27.4 (C. 1.03 DMF). Further synthetic steps were performed exactly as described previously. All couplings were carried out in CH_2Cl_2 with occasional addition of some DMF to improve reactant solubility. Trifluoroacetate salts were, as a rule, solidified by trituration with dry ether.

The consecutive peptides thus prepared are Boc-Leu-Arg(NO_2)-Pro-Gly-OBzl (**III**), crystallized from ethyl acetate–ether; yield 78%; m.p. 96°–99°C; $[\alpha]_D^{23}$ −54.8 (C, 0.81 DMF). Boc-Gly-Leu-Arg(NO_2)-Pro-Gly-OBzl (**IV**), crystallized from ethyl acetate–petroleum ether; yield 87%; m.p. 90°C; $[\alpha]_D^{23}$ − 30.1 (C, 0.88 $CHCl_3$). Amino acid analysis: Gly 2.0; Leu 1.0; Pro 0.93; Arg 1.0. Calculated for $C_{33}H_{51}N_9O_{10}$ (733.81): C, 54.01; H, 7.01; N, 17.18. Found: C, 53.80; H, 7.06; N, 17.40. Boc-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-OBzl (**V**), crystallized from ethyl acetate–ether; yield 81%; m.p. 103°–6°C; $[\alpha]_D^{23}$ −45.3 (C, 0.89 DMF). Boc-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-OBzl (**VI**), crystallized from ethyl acetate–ether; yield 86%; m.p. 112°–116°C; $[\alpha]_D^{23}$ −38.2 (C, 1.1 DMF). Boc-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-OBzl (**VII**), crystallized from ethyl acetate; yield 82%; m.p. 146°–149°C; $[\alpha]_D^{23}$ −37.8 (C, 0.89 DMF). Boc-His-(Dnp)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-OBzl (**VIII**), crystallized from ethyl acetate; yield 66%; m.p. 134°–138°C; $[\alpha]_D^{23}$ −29.2 (C, 0.91 DMF). Z-pGlu-His(Dnp)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-OBzl (**IX**), crystallized from chloroform–ether; yield 73%; m.p. 140°–143°C; $[\alpha]_D^{23}$ −26.6 (C, 1.09 DMF). Calculated for $C_{90}H_{99}N_{19}O_{22}$ (1798.84): C, 60.09; H, 5.55; N, 14.23. Found: C, 60.18; H, 5.75; N, 14.14.

L-Pyroglutamyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-glycyl-L-leucyl-L-arginyl-L-prolyl-glycine amide (LH–RH).

Thiolytic Removal of Im-2,4-dinitrophenyl Protecting Group from Decapeptide IX. A sample of decapeptide **IX** (100 mg; prepared by either the polymeric reagents or the DCC procedure) was dissolved in DMF (3 ml), and 2-mercaptoethanol (0.5 ml) was added to initiate thiolysis. The reaction (at 23°C) was followed spectrophotometrically by measuring the

increase of absorption at 340 nm (7) and was found to be complete in 45 min. The deblocked product was precipitated from solution by the addition of dry ether and collected by centrifugation. Three consecutive precipitations from DMF–ether removed most of the yellowish Dnp-S-C_2H_4OH by-product and excesses of 2-mercaptoethanol. The product was entirely devoid of parent peptide **IX**, as revealed by thin-layer chromatography.

Removal of ω-Nitro- and α-Benzyloxycarbonyl Protecting Groups. The product obtained after thiolysis was placed in a polyethylene bottle to which anisole (2 ml) and cold (−20°C) anhydrous hydrogen fluoride (10 ml) were added. The colorless solution initially obtained turned brown-red within several minutes. It was kept at 0°C for 45 min, whereupon the HF was evaporated *in vacuo*. The oily residue was triturated with dry ether (peroxide-free) and the resulting yellowish powder was collected by centrifugation, washed thoroughly with peroxide-free ether, and finally dried *in vacuo* over P_2O_5 and NaOH; yield 90% (for the two deprotection steps). Thin-layer chromatography, as well as high-voltage paper electrophoresis (pyridine–acetate buffer, pH 3.5; 60 vol/cm) indicated that product purity of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH was greater than 97%.

Amidation of Free Carboxyl Group. The carboxyl-free decapeptide (75 mg) just described was allowed to couple for 60 min at 0°C in dry DMF (3 ml) with *N*-hydroxysuccinimide (35 mg) using DCC (65 mg) as the coupling agent. Dry ammonia was then bubbled through the solution for 10 min at 0°C, and the crude LH–RH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, was obtained by evaporation of solvent. It was purified on a CMC-23 column (1 × 15 cm) using a stepwise gradient of ammonium acetate (0.001–0.1 M, pH 6.52). The LH–RH was eluted with 0.05 M NH_4OAc while unreacted carboxyl-free peptide was released using the initial buffer. The chromatographically and electrophoretically pure LH–RH had mobilities identical to those of authentic samples, the generous gifts of Ayerst Research Laboratories (Saint Laurent, Canada) and of Hoechst A.G. (Frankfurt, Germany). Yield was 65% (54 mg, for both the amidation and purification steps). About 30% of unreacted carboxyl-free starting peptide could be recovered from the column. On acid hydrolysis the LH–RH yielded a molar amino acid ratio of Glu, 1.02; His, 1.01; Ser, 0.98; Tyr, 1.01; Gly, 2.00; Leu, 1.02; Arg, 1.00; and Pro, 0.98 (tryptophan was not determined). The luteinizing hormone releasing activity of the product was assessed by a radioimmunological determination of luteinizing hormone released upon incubation of the peptide with pituitaries of female rats (8). A 27-fold increase of basal LH level was obtained by addition of 1×10^{-9} M of product, exactly the increase pro-

duced by authentic LH–RH samples. Identity of the product and markers was also established by cross-reactivity experiments using specific anti-LH–RH antibodies (9) elicited by known material.

RESULTS AND DISCUSSION

The stepwise syntheses of luteinizing hormone–releasing hormone by means of polymeric reagents as well as by utilization of *N,N*-dicyclohexylcarbodiimide as the coupling agents are summarized in Fig. 1. As can be seen, both syntheses were initiated by reacting glycine benzyl ester with the corresponding activated form of Boc-Pro and thereafter continued by application of the other appropriate Boc-amino acid derivatives. In the former procedure, the synthesis was carried out uninterrupted with only the pentapeptide **IV** and the decapeptide **IX** being characterized; in the latter synthesis, however, every intermediate peptide had to be characterized. Application of molar excesses of polymeric reagents always led to nearly quantitative couplings. The work-up of reaction mixture was a simple three-step procedure, carried out by filtration of insoluble polymer, aqueous extraction of organic solution to remove triethylammonium trifluoroacetate salts, and evaporation of solution to afford a chromatographically pure product. The formation of the cyclic lactam by-product which accompanied tripeptide **II** was an exception; it was, however, easily removed from the main product. Each coupling step by the DCC procedure, however, should properly be followed by repeated extractions of the reaction mixture with dilute citric acid and $NaHCO_3$ solutions to remove unreacted amino- and carboxyl-containing starting materials. Because these extractions become much less efficient as the peptide chain grows, we decided to purify the products by crystallization at each step; these unavoidable manipulations obviously lead to reduction in the reaction yields. The synthetic steps leading from the blocked decapeptide **IX** to the product LH–RH were identical for both procedures. It should be noted here that a direct amidation of **IX** by NH_3–methanol led to extensive opening of the pyroglutamyl ring. The indirect amidation, shown in Fig. 1, was therefore chosen. The overall yield of synthesis via the polymeric reagent route was 40%, whereas that using the DCC procedure was 7%.

The advantages of using polymeric reagents in peptide syntheses, namely, high coupling yields, high product purity, and simple synthetic operations, are clearly demonstrated by the case of the decapeptide luteinizing hormone-releasing hormone.

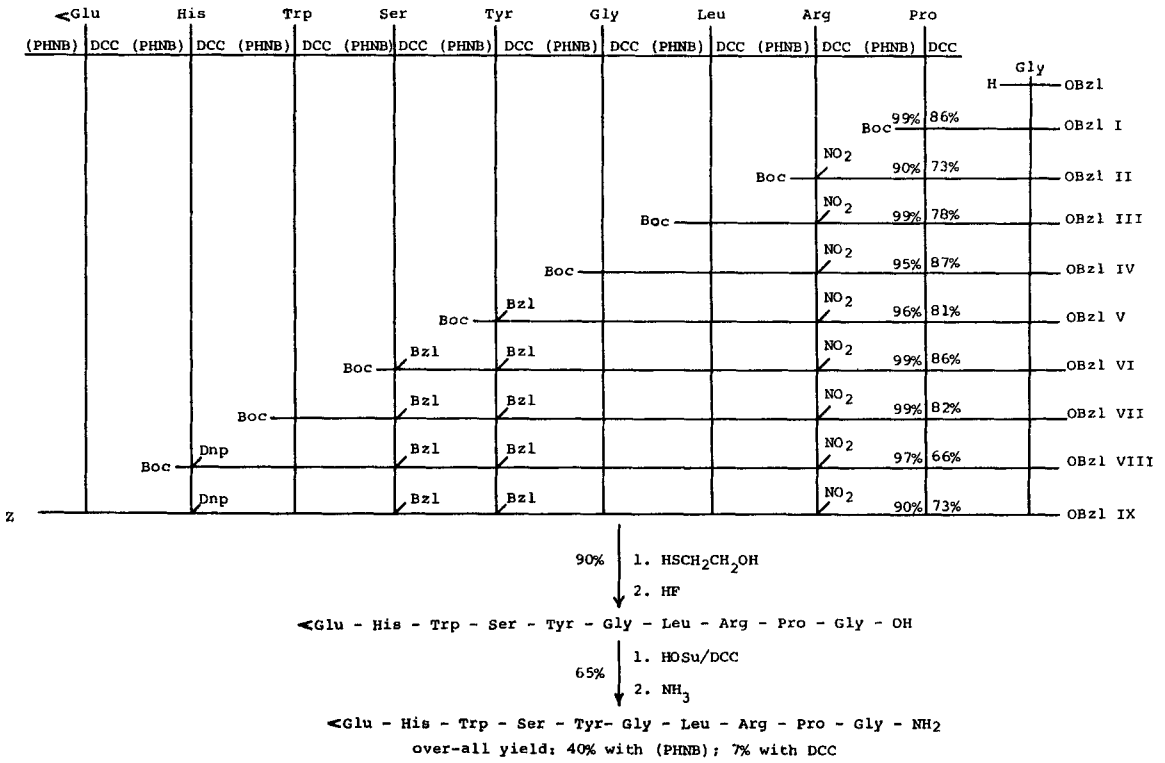

FIG. 1. Synthesis of luteinizing hormone–releasing hormone by polymeric reagents and by the *N,N*-dicyclohexylcarbodiimide procedures. The yields of the former route (left-hand column) are those obtained for both coupling and deprotection steps, thus expressing a continuous follow-up of efficiency of synthesis. Yields given for the DCC procedure (right-hand column) are only for the respective coupling step; two to four couplings were generally performed and the yields given are the optimal.

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