SOLID-PHASE SYNTHESIS OF ANALOGS OF THE A_{17-21} FRAGMENT OF INSULIN

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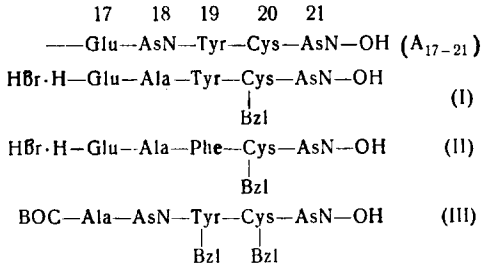
In connection with the development of methods for preparing structural analogs of insulin, we have synthesized the pentapeptides (I-III) forming structural analogs of the A_{17-21} fragment of insulin (all amino acids of the L configuration).



The synthesis was performed by Merrifield's solid-phase method [1, 2]. The polymeric support used was a chloromethylated copolymer of styrene with 1% of divinylbenzene containing 8% of chlorine.

The C-terminal amino acid was attached to the polymeric carrier in the presence of sodium iodide as reaction catalyst [3]. Under these conditions, it was also possible to perform the attachment to the polymeric carrier of a protected dipeptide with the composition BOC–Cys(Bzl)–Asn–OH (IV). In all the peptide-forming reactions, the condensing agent was dicyclohexylcarbodiimide –DCHC– (apart from the addition of an asparagine residue, which was effected by the p-nitrophenyl ester method). To eliminate the BOC groups we used a 4 N solution of HCl in dioxane. The peptides obtained were separated from the polymeric carrier by the action of HBr in trifluoroacetic acid [peptides (I) and (II)] or by alkaline saponification [peptide (III)].

Peptide (II) was synthesized by two methods: stepwise, starting from the asparaginyl-polymer and by the stepwise-block method starting from the S-benzylcysteinylasparaginyl-polymer.

EXPERIMENTAL

Analytical Methods. The ascending method of paper chromatography on paper of type "C" with the following solvent systems was used: 1) isoamyl alcohol–pyridine–water (7 : 7 : 6); 2) n-butanol–acetic acid–pyridine–water (7.5 : 1.5 : 6 : 5); and 3) isopropanol–25% aqueous ammonia–water (14 : 1 : 5). Thin-layer chromatography (TLC) was performed by the ascending method on Silufol plates coated with silica gel and starch, in the same solvent systems. Paper electrophoresis was performed at a voltage of 350 V. The electrolytes used were 0.05 M triethylammonium bicarbonate buffer solutions with pH 7.5 and 8.7.

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Compounds containing free α-amino groups were revealed on the chromatograms and electrophoregrams with ninhydrin. The benzidine reagent (PC) or a 0.05 M solution of sodium iodide (TLC) was used to reveal substances containing an imino group. The quantitative determination of the amino acids in the hydrolyzates was performed on an automatic amino-acid analyzer.*

Addition of BOC–Asn–OH to the Polymeric Carrier. To 8 g (18 meq of chlorine) of the polymeric carrier were added 40 ml of absolute dimethylformamide (DMFA), 4 g (18 mmoles) of BOC–Asn–OH, 1.4 g (9 mmoles) of sodium iodide, and 2.5 ml (18 mmoles) of triethylamine. The mixture was shaken on a machine at 40°C for 5 h. The polymeric carrier was separated off, washed with DMFA, water, and methanol, and dried in a vacuum desiccator over P_2O_5 to constant weight. The amount of asparagine attached to the carrier in various experiments was 0.35-0.50 mmole/g.

Preparation of BOC–Cys(Bzl)–Asn–OH (IV). At 5°C, with stirring, 0.68 ml (5 mmoles) of triethylamine and 0.65 ml (5 mmoles) of isobutyl chloroformate were added to a solution of 1.55 g (5 mmoles) of BOC–Cys(Bzl)–OH in 10 ml of absolute dioxane. After 5 min, a solution of 0.72 g (5.5 mmoles) of asparagine in 5 ml of 1 N NaOH was added to the reaction mixture. After another 10 min, the mixture was acidified with 50% citric acid to pH 3. A white precipitate deposited, which was filtered off. Yield 1.4 g (70% of theoretical), mp 152-153°C, $[\alpha]_D^{25}$ −25° (c 0.9; DMFA), R_{f_1} 0.60, R_{f_2} 0.89, R_{f_3} 0.93 (PC).

Found %: C 53.39; H 6.24; N 9.65. $C_{19}H_{27}N_3O_6S$ (425.4). Calculated %: C 53.64; H 6.35; N 9.88.

Addition of BOC–Cys(Bzl)–Asn–OH to the Polymeric Support. To 0.5 g (1.15 meq of chlorine) of the polymeric support were added 4 ml of absolute DMFA, 490 mg (1.15 mmole) of (IV), 174 mg (1.15 mmole) of sodium iodide, and 158 mg (1.15 mmole) of triethylamine. The mixture was shaken on a machine at 40°C for 12 h. The polymeric carrier was separated off, washed with DMFA, glacial acetic acid, and methanol, and dried in the vacuum desiccator over P_2O_5 to constant weight. The amount of dipeptide on the support was 0.32 mmole/g (from the results of the amino-acid analysis of the hydrolyzate for aspartic acid).

Preparation of BOC–Glu(OBu^t)–Ala–Tyr(Bzl)–Cys(Bzl)–Asn Polymer (V). A flask for solid-phase peptide synthesis was charged with 2 g of the BOC–Asn polymer (0.70 mmole of asparagine), 20 ml of dioxane was added, and the suspension was shaken for 10 min. The aminoacyl polymer was washed twice more with dioxane, and then the BOC groups were eliminated with 20 ml of a 4 N solution of HCl in dioxane for 30 min. After washing with dioxane (3 × 20 ml) and CH_2Cl_2 (3 × 20 ml), the reaction mixture was neutralized with 20 ml of a 10% solution of triethylamine in CH_2Cl_2 for 10 min. Then the polymer was washed with CH_2Cl_2 (3 × 20 ml), methanol (2 × 15 ml), and again with CH_2Cl_2 (3 × 20 ml); after this, 545 mg (1.75 mmole) of BOC–Cys(Bzl)–OH and, after 5 min, 360 mg (1.75 mmole) of DCHC were added. Condensation was performed with shaking for 4 h. After the end of the peptide-forming reaction, the polymeric support was washed with CH_2Cl_2 (3 × 20 ml), ethanol (3 × 20 ml), and CH_2Cl again (3 × 20 ml).

By repeating the cycle of reactions and washings described and also by subsequently using BOC–Tyr(Bzl)–OH, BOC–Ala–OH, and BOC–Glu(OBu^t)–OH we obtained (V). The substance was washed with glacial acetic acid and with ethanol and was dried in the vacuum desiccator over P_2O_5. Yield 2.6 g. Amino-acid analysis: Asp 1.04, Cys(Bzl) 0.38, Tyr 0.28, Ala 1.00, Glu 1.04.†

Preparation of BOC–Glu(OBu^t)–Ala–Phe–Cys(Bzl)–Asn Polymer (VI). A. Compound (VI) was obtained in a similar manner to the preceding case from 2 g of BOC–Asn polymer (0.70 mmole of asparagine). Yield 2.5 g. Amino-acid analysis: Asp 1.06, Cys(Bzl) 0.38, Phe 1.06, Ala 1.00, Glu 0.92.

B. Starting with 0.5 g of BOC–Cys(Bzl)–Asn polymer (0.16 mmole of dipeptide), the peptide chain was built up in the same way as in A. The yield of (VI) was 0.61 g. Amino-acid analysis: Asp 0.98, Cys(Bzl) 0.30, Phe 1.05, Ala 1.00, Glu 0.97.

Preparation of BOC–Ala–Asn–Tyr(Bzl)–Cys(Bzl)–Asn Polymer (VII). This was based on the treatment of 2 g of the BOC–Asn polymer (0.70 mmole) as described above with the exception of the stage of the introduction of an asparagine residue into the peptide chain. Compound (VII) was obtained. At the stage of the introduction of an asparagine residue to the peptide chain after the elimination of the BOC group the amino-

* The amino-acid analyses were performed by our colleagues of the chromatography division of the interfaculty laboratory of bioorganic chemistry of Moscow State University.

† The low figures for cysteine and tyrosine have a systematic nature and are explained by the decomposition of these amino acids during acid hydrolysis.

acyl polymer was washed with DMFA (3× 20 ml), neutralized with 10% triethylamine in DMFA for 10 min, and washed with DMFA (3 × 20 ml), and 990 mg (2.8 mmoles) of BOC–Asn–ONP was added. Condensation was performed with shaking for 16 h. The yield of (VII) was 2.46 g. Amino-acid analysis: Asp 1.87, Cys(Bzl) 0.53, Tyr 0.65, Ala 1.00.

Preparation of the Hydrobromide of H–Glu–Ala–Tyr–Cys(Bzl)–Asn–OH (I). To 1.65 g of (V) was added 15 ml of freshly-distilled CF_3COOH, followed by 2 ml of anisole, and a slow current of dry bromine-free HBr was passed through the suspension for 1 h. The polymeric support was separated off and washed with CF_3COOH (3 × 10 ml), and the filtrate and the wash liquids were evaporated in vacuum at 30°C. Absolute methanol was added to the oily residue and evaporated off again several times. The final residue was treated with absolute ether, and the precipitate that deposited was separated off, washed with ether, and dried in vacuum. Yield 240 mg (56% of theoretical). After recrystallization from ethanol, 160 mg of chromatographically pure compound (I) was obtained, $[\alpha]_D^{25}$ −20° (c 1; DMFA), R_{f_1} 0.31, R_{f_2} 0.68, R_{f_3} 0.68 (PC). Paper electrophoresis: $U_A + 5.8 \cdot 10^{-5}$ (2.5 relative to Ala), $U_B + 6.7 \cdot 10^{-5}$ (1.2 relative to Gln). Amino-acid analysis: Glu 0.99, Ala 1.00, Tyr 0.68, Cys(Bzl) 0.65, Asp 1.01.

Preparation of the Hydrobromide of H–Glu–Ala–Phe–Cys(Bzl)–Asn–OH (II). As described for the preceding case, 1.75 g of (VI) was treated with HBr in CF_3COOH. The yield of compound (II) was 267 mg (58% of theoretical). After recrystallization from water, chromatographically pure (II) was obtained with $[\alpha]_D^{25}$ −20° (c 1; DMFA), R_{f_1} 0.46, R_{f_2} 0.73, R_{f_3} 0.80 (PC). Paper electrophoresis: $U_A + 5.7 \cdot 10^{-5}$ (2.5 relative to Ala), $U_B + 6.6 \cdot 10^{-5}$ (1.2 relative to Gln). Amino-acid analysis: Glu 1.03, Ala 1.02, Phe 1.00, Cys(Bzl) 0.70, Asp 1.00.

Preparation of BOC–Ala–Asn–Tyr(Bzl)–Cys(Bzl)–Asn–OH (III). A mixture of 1 g of (VII), 0.6 ml of 2 N NaOH, and 6 ml of dioxane was shaken on a machine for 1 h. The polymeric support was separated off and washed with dioxane and ether. The filtrate was acidified with 10% HCl in ethanol to pH 7 and evaporated to dryness, and the residue was treated with water. The precipitate that deposited was separated off and dried in the air. Yield of (III) 200 mg (66% of theoretical), $[\alpha]_D^{20}$ −39° (c 1; DMFA), R_{f_2} 0.79, R_{f_3} 0.81 (TLC). Amino-acid analysis: Asp 1.97, Cys(Bzl) 0.53, Tyr 0.63, Ala 1.00.

CONCLUSIONS

The synthesis of the following analogs of the A_{17-21} fragment of insulin has been effected by the solid-phase method: the hydrobromides of H–Glu–Ala–Tyr–Cys(Bzl)–Asn–OH and of H–Glu–Ala–Phe–Cys(Bzl)–Asn–OH, and also BOC–Ala–Asn–Tyr(Bzl)–Cys(Bzl)–Asn–OH. The possibility of adding to a chloromethylated polymeric support a protected dipeptide with the composition BOC–Cys(Bzl)–Asn–OH has been shown.

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