SYNTHESIS OF POLYPEPTIDES OF REGULAR STRUCTURE, CONTAINING SERINE AND GLUTAMIC ACID, AND MODELING THE NONPOLAR REGIONS OF THE COLLAGEN PROTEIN MOLECULE

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A study of the acid [1], alkaline [2], and collagenase [3] hydrolyzates of collagen disclosed that the imino acids (proline and hydroxyproline) are contained predominantly in two types of sequences: -gly-pro-R- and -gly-R-hypro-, where R is the amino acid or imino acid moiety. As is known, these sequences are concentrated in the so-called nonpolar regions of the molecule and, based on various data, constitute 25-35% of the entire primary structure of collagen [4, 5]. A high content of imino acids in collagen imparts a specific structure to the molecule, which, apparently, is very close to the hypothetical model "Collagen-II," proposed in [6]. The reality of this model was confirmed by the synthesis of the polytripeptide $(\text{-gly-pro-hypro-})_n$ [7], which, based on the x-ray structure analysis data, had the characteristic structure of the collagen type [8]. The formation of such a structure in polypeptides as a function of the arrangement of the amino acids and imino acids was studied for a series of synthesized polytripeptides [9]. The x-ray structure studies of these polymers made it possible to ascertain a number of important rules regarding the formation of a specific collagen structure and the changes in the parameters of the helices as a function of the mutual arrangement of the imino acids and amino acids in the polypeptide chain [9].

The presence in collagen hydrolyzates of the tripeptides -gly-pro-glu-, -gly-pro-ser-, -gly-pro-asp-, -gly-pro-threo-, and a number of others, makes it possible to expect that the polytripeptides of such sequences will give valuable information regarding the role that can be played by the functional groups of the third amino acid moiety in the formation of helices of the collagen type. For this purpose we synthesized two polypeptides $(\text{-gly-hypro-glu-})_n$ and $(\text{-gly-hypro-ser-})_n$. It should be mentioned that replacing the proline in the second position by hydroxyproline does not affect the realization of the ternary helix, and can only exert an influence on its parameters. In order to obtain these polytripeptides we used the method of activated 2,4,6-trichlorophenyl esters, which were obtained by the hydrogenolysis of the 2,4,6-trichlorophenyl esters of carbobenzoxytripeptides in the presence of HCl, or by hydrolysis in the presence of HCl, if the formyl group was used instead of the protective carbobenzoxy group. In this connection we failed to observe any noticeable cleavage of the ester linkage in the case of either the ester of the formylamino acid or the ester of the formyltripeptide, despite the long reaction time. The high yields and purity of the obtained product should also be mentioned.

We used the formyl protection to obtain the 2,4,6-trichlorophenyl ester of hypro-glu-(γ-benz)-gly. In this case it is quite convenient and can compete with "CBO protection." It is interesting to mention that the chlorophenyl esters of the formylpeptides vary in their sensitivity to the hydrolysis process. Thus, in contrast to the 2,4,6-trichlorophenyl esters, we were unable to synthesize the chromatographically pure pentachlorophenyl ester of glu-(γ-benz)-gly employing hydrolysis of the formyl group. In order to obtain the chromatographically pure hydrohalides of the peptide esters after hydrolysis of the formyl group it is necessary to resort to lyophilic drying. The polymerization of the hydrohalides of the 2,4,6-trichlorophenyl esters of hypro-ser-gly and hypro-glu-(γ-benz)-gly was run in dimethyl sulfoxide at 50% monomer concentration. The time of polymerization was 6-10 days. The popypeptides were purified by dialysis. The average molecular weight, when based on the N-terminal groups (Van Slyke), was respectively equal to ~ 8000

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and ~6000. It should be mentioned that the use of the Van Slyke method to determine the molecular weight is not satisfactory, since it markedly lowers the true value of the average molecular weight because of the hydrolytically labile serine – glycine peptide linkages. The determination of the N-terminal groups using the ninhydrin reaction is more accurate.

As was already mentioned earlier, when various activated peptide esters are polymerized the value of the molecular weight of the polypeptide depends not only on the nature of the ester group, but also on the purity of the monomer. This was also observed in our case. And it is not completely excluded that, using absolutely pure 2,4,6-trichlorophenyl esters, it is possible to obtain polymers of this sequence and a higher molecular weight. Preliminary studies of the polypeptide $(\text{-hypro-gly-glu-})_n$ using IR spectroscopy disclosed that structures of the collagen type are present in it.

EXPERIMENTAL

l-Amino acids were used in the synthesis. The purity of the compounds was checked by paper chromatography using paper of the "Leningrad" type, in the system water – CH_3COOH – n-butanol (5:1:4) (A); the thin-layer chromatography was run on silica gel (250 mesh), fastened layer, plate size 75 × 25 mm, in the systems water – CH_3COOH – n-butanol (30:10:100) (B) and 3% NH_3 – sec-butanol (44:100) (C).

Formyloxyproline. To a solution of 5 g of hydroxyproline in 88.5% formic acid was added, at 10°, 20 ml of acetic anhydride in 1 h. Then the mixture was stirred for another hour at 20° and at 30° for 10 min. Then 10 ml of water was added and the mixture was evaporated in vacuo. The residue represented an oily product, which was recrystallized from 20 ml of ethyl acetate by rubbing in the presence of 3 ml of ether and cooling. Then, without separating the crystals, the ethyl acetate was evaporated to a volume of 3-4 ml, followed by the addition of 20-25 ml of petroleum ether. We obtained 5.8 g (96.6%) of formyloxyproline; mp 127°; $[\alpha]_D^{21}$ −135 ± 0.5° (C 2, in methanol). Found: C 45.2; H 5.4%. $C_6H_9O_4N$. Calculated: C 44.9; H 5.52%.

2,4,6-Trichlorophenyl Ester of Carbobenzoxyglycine. To a solution of 2.1 g of carbobenzoxyglycine in 12 ml of pyridine was added 2.17 g of 2,4,6-trichlorophenol (mp 69°) and, cooling to 0° and employing vigorous stirring, a solution of 2 g of freshly distilled $POCl_3$ in 10 ml of CH_2Cl_2 was added. The reaction mass was kept at 0-2° for 1 h, after which 400 ml of ice water was added to decompose the excess $POCl_3$. Crystals deposited from the reaction mass after strenuous rubbing at −10°. We obtained 2.8 g (91%) of the 2,4,6-trichlorophenyl ester of carbobenzoxyglycine; mp 109°; R_f (B) 0.96; R_f (C) 0.92.

Hydrobromide of 2,4,6-Trichlorophenyl Ester of Glycine. To a solution of 10.7 g of the 2,4,6-trichlorophenyl ester of carbobenzoxyglycine in 25 ml of glacial acetic acid was added a solution of 18 ml of 40% HBr in glacial acetic acid. After 25 min absolute ether was added at 20° until all of the ester hydrobromide had precipitated. We obtained 8.4 g (91%) of the hydrobromide of the 2,4,6-trichlorophenyl ester of glycine, which was purified by careful reprecipitation from methanol with ether; mp 215° (decomp.); R_f (A) 0.64; R_f (B) 0.82.

2,4,6-Trichlorophenyl Ester of Formylglycine. To a solution of 4.5 g of formylglycine and 8.5 g of 2,4,6-trichlorophenol in 23 ml of acetonitrile, containing 15 ml of water and cooled to −4°, with stirring, was added 9 g of dicyclohexylcarbodiimide. The reaction mass was stirred for 1 h at a temperature ranging from −4 to 0°, then 2 h at 20°, and allowed to stand overnight. The dicyclohexylurea was filtered, the solvent was evaporated in vacuo, the oily residue was dissolved in ethyl acetate, and the residual dicyclohexylurea was separated and washed with 5% Na_2CO_3 solution. The ethyl acetate solution was dried over Na_2SO_4. After evaporation of the solvent in vacuo we obtained 12 g (93%) of the 2,4,6-trichlorophenyl ester of formylglycine; mp 105-107° (from ethyl acetate). R_f (B) 0.85; R_f (C) 0.6. Found: C 39.50; H 2.56%. $C_9H_7O_3NCl_3$. Calculated: C 39.45; H 2.57%.

Hydrochloride of 2,4,6-Trichlorophenyl Ester of Glycine. To a solution of 0.1 g of the 2,4,6-trichlorophenyl ester of formylglycine in 4 ml of absolute ethanol was added 1.5 ml of conc. HCl solution, and the mixture was allowed to stand for 56 h at 20°. Evaporation of the ethanol in vacuo at 20-25° gave an oily product as residue, which was dissolved in the minimum amount of methanol and precipitated with ether. We obtained 0.06 g (62%) of the hydrochloride of the 2,4,6-trichlorophenyl ester of glycine; mp 210° (decomp.), and R_f (B) 0.83. A small amount of glycine, with R_f (B) 0.25, was observed to be present as impurity. Found: C 34.10; H 2.50%. $C_8H_7O_2NCl_4$. Calculated: C 34.31; H 2.46%.

2,4,6-Trichlorophenyl Ester of Formyl-glu-(γ-benz)-gly. To a stirred solution of 3 g of the γ-benzyl ester of formylglutamic acid in 7 ml of dimethylformamide, cooled to −4 to −5°, were added a solution of

2.4 g of dicyclohexylcarbodiimide in 5 ml of dimethylformamide and a solution of 3.1 g of the hydrobromide of the 2,4,6-trichlorophenyl ester of glycine in 20-30 ml of dimethylformamide, containing 1.2 ml of triethylamine. The reaction mixture was stirred for 2 h at −4 to −5°, and then was allowed to stand at 20° for two days. Then several drops of acetic acid was added to decompose any residual dicyclohexylcarbodiimide. The obtained dicyclohexylurea was filtered, while the solvent was evaporated in vacuo. The residue was dissolved in ethyl acetate and again the urea derivative was filtered. The ethyl acetate solution was washed in succession with water, 5% Na_2CO_3 solution, 1 N HCl solution and again with water, and was then dried over Na_2SO_4. Evaporation of the solvent left an oily residue, which was recrystallized from a mixture of acetone and petroleum ether (2:50), with cooling and rubbing. After 12 h the product was filtered to give 4.85 g (85%) of the 2,4,6-trichlorophenyl ester of formyl-glu-(γ-benz)-gly; mp 110-112°; $[\alpha]_D^{21} = 5.3 \pm 0.5°$ (C 2, methanol); R_f (B) 0.94. Found: C 50.24; H 3.75%. $C_{21}H_{19}O_6N_2Cl_3$. Calculated: C 50.00; H 3.87%.

Hydrochloride of 2,4,6-Trichlorophenyl Ester of glu-(γ-benz)-gly. The 2,4,6-trichlorophenyl ester of formyl-glu-(γ-benz)-gly (4.5 g) was dissolved in 50 ml of absolute ethanol containing 3 ml of conc. HCl, and the solution was allowed to stand for 138 h at 20°. Then the ethanol was evaporated, while the residue was dissolved in 25 ml of water. After extraction with ether, the aqueous solution was subjected to lyophilic drying. We obtained 2.8 g of the hydrochloride of the 2,4,6-trichlorophenyl ester of glu-(γ-benz)-gly as an oil, which was dissolved in approximately 2 ml of methanol and then precipitated with ether. The crystalline product was filtered and washed with ether. We obtained 2.18 g (61.5%) of the 2,4,6-trichlorophenyl ester of formyl-glu-(γ-benz)-gly; mp 134-137°; $[\alpha]_D^{20}$ +8.7 ± 0.5° (C 3.1, methanol); R_f (A) 0.75; R_f (B) 0.73. Found: C 46.52; H 3.9%. $C_{20}H_{20}O_5N_2Cl_4$. Calculated: C 46.53; H 3.16%.

2,4,6-Trichlorophenyl Ester of Formyl-hypro-glu-(γ-benz)-gly. A mixture of 3.22 g of formylhydroxyproline (dissolved in 25 ml of DMF,* containing 4.5 g of dicyclohexylcarbodiimide), cooled to −5 to −6°, 6.4 g of the hydrochloride of the 2,4,6-trichlorophenyl ester of glu-(γ-benz)-gly and 1.82 ml of triethylamine was allowed to stand at 20° for 48 h. Then the solvent was evaporated, while the residue was dissolved in ethyl acetate, the dicyclohexylurea was separated, the filtrate was again evaporated to minimum volume, cooled to −24°, and the precipitated urea derivative was separated. After reprecipitation from ethyl acetate with petroleum ether we obtained 5.29 g (40%) of the 2,4,6-trichlorophenyl ester of formyl-glu-(γ-benz)-gly as a colorless oil, which could not be made to crystallize: $[\alpha]_D^{21}$ −25 ± 0.5° (C 1.44, methanol); R_f (B) 0.83. The obtained product was completely devoid of impurities, as was shown by electrophoresis, paper chromatography, and thin-layer chromatography. Found: C 49.80; H 4.35%. $C_{26}H_{26}O_8N_3Cl_3$. Calculated: C 49.60; H 4.23%.

Hydrochloride of 2,4,6-Trichlorophenyl Ester of hypro-glu-(γ-benz)-gly. To a solution of 5.26 g of the 2,4,6-trichlorophenyl ester of formyl-hypro-glu-(γ-benz)-gly in 20 ml of methanol was added 2.1 ml of conc. HCl solution and the mixture was allowed to stand at 20° for 47 h (the hydrolysis process was checked both chromatographically and electrophoretically at 4-5 h intervals, in which connection the formation of degradation products was not observed). The methanol was evaporated, and the aqueous residue was treated with 25 ml of water and the insoluble portion was filtered. After extraction with small portions of ether, the aqueous solution was subjected to lyophilic drying. The obtained crystalline residue was dissolved in 5 ml of methanol and precipitated with ether. The solvent was separated by decantation, and the precipitate was dried in vacuo. We obtained 3.8 g (70%) of the hydrochloride of the 2,4,6-trichlorophenyl ester of hypro-glu-(γ-benz)-gly; $[\alpha]_D^{20}$ −19.7 ± 0.5° (C 1.65, methanol). Found: C 48.62; H 4.19%. $C_{25}H_{27}O_7N_3Cl_4$. Calculated: C 48.19; H 4.33%.

Polytripeptide (-hypro-glu-(γ-benz)-gly-)$_n$. The hydrochloride of the 2,4,6-trichlorophenyl ester of hypro-glu-(γ-benz)-gly (0.9822 g) was dissolved in an ampul at 40° in 0.7304 g of dimethyl sulfoxide (57% solution). Then the ampul was cooled to 20° and 0.225 ml of triethylamine, freed of secondary amines and freshly distilled, was added and vigorously shaken with the ampul contents. After 10 days the ampul contents were dissolved in methanol and the polypeptide was precipitated by adding ether. We obtained 0.67 g (90%) of the hypro-glu-(γ-benz)-gly polytripeptide; $[\alpha]_D^{27}$ −20.5 ± 0.5° (C 0.5, methanol). The polymer has a dark color. The molecular weight, determined by the Van Slyke method, was equal to ~8500. A solution of 0.357 g of the crude polypeptide in 7 ml of water was filtered to remove some insoluble particles and the filtrate was dialyzed through cellophane against distilled water at 30-40°. After lyophilic drying we obtained 0.1488 g of the polytripeptide. The molecular weight, determined on the basis of the amine ends (ninhydrin method), was equal to 10,000-13,000.

* DMF = dimethylformamide.

Polytripeptide (-hypro-glu-gly-)$_n$. A solution of 1.7 g of the undialyzed polytripeptide [-hypro-glu-(γ-benz)-glu-]$_n$ in 18 ml of methanol was hydrogenated over metallic Pd for 1.5 h. When the hydrogen absorption had ceased the catalyst was filtered, and the solvent was evaporated in vacuo. We obtained 1.4 g of the polytripeptide (-hypro-glu-gly)$_n$. The characteristic frequency of amide A (3330 cm^{-1}) indicates the presence of a structure of the collagen type in the polypeptide.

2,4,6-Trichlorophenyl Ester of Carbobenzoxy-ser-gly. A solution of 0.5 g of carbobenzoxy-ser in 4 ml of dimethylformamide was cooled to −5° and a solution of 0.45 g of dicyclohexylcarbodiimide in 5 ml of dimethylformamide was added. After 10 min a solution of 0.72 g of the hydrobromide of the 2,4,6-trichlorophenyl ester of glycine in 10 ml of dimethylformamide, containing 0.35 ml of triethylamine, was added. The reaction mass was stirred for 2 h at −4 to −6° and then allowed to stand for 24 h. After this the dicyclohexylurea was filtered, the solvent was evaporated in vacuo, and the residue was dissolved in ethyl acetate and the residual dicyclohexylurea was filtered. The ethyl acetate solution was washed twice with water, then with 5% Na_2CO_3 solution, 1 N HCl solution, again with water, and dried over Na_2SO_4. After evaporation of the solvent the oily residue was rubbed with petroleum ether. We obtained 0.8 g (80.5%) of the crystalline 2,4,6-trichlorophenyl ester of carbobenzoxy-ser-gly; mp 124-126°; $[\alpha]_D^{20}$ −8.2 ± 0.5° (C 1.12, methanol); R_f (B) 0.93. Found: C 47.69; H 3.56%. $C_{19}H_{17}O_6N_2Cl_3$. Calculated: C 47.98; H 3.6%.

Hydrochloride of 2,4,6-Trichlorophenyl Ester of ser-gly. A solution of 3.43 g of the 2,4,6-trichlorophenyl ester of carbobenzoxy-ser-gly in methanol was hydrogenated over metallic Pd in the presence of 0.61 ml of 12 N HCl solution. The calculated amount of H_2 was absorbed in 15-30 min. The methanol was evaporated in vacuo at 30°. The residue was dissolved in the minimum amount of methanol and the product was precipitated with ether. We obtained 2.5 g (90.5%) of the hydrochloride of the 2,4,6-trichlorophenyl ester of ser-gly; mp 165-167° (decomp.); $[\alpha]_D^{21}$ −8.7 ± 0.5° (C 3.35, methanol); R_f (A) 0.54. Found: C 43.6; H 3.76%. $C_{11}H_{12}O_4N_2Cl_4$. Calculated: C 43.49; H 3.70%.

2,4,6-Trichlorophenyl Ester of Carbobenzoxy-hypro-ser-gly. The compound was prepared from 1 g of carbobenzoxy-hypro, 1.5 g of the hydrochloride of the 2,4,6-trichlorophenyl ester of ser-gly, 0.4 ml of triethylamine and 0.85 g of dicyclohexylcarbodiimide in DMF solution at −4 to −6°. The reaction was run for 24 h at 20°. After the usual workup we isolated 1.94 g (80.1%) of the 2,4,6-trichlorophenyl ester of carbobenzoxy-hypro-ser-gly; mp 153-154°; $[\alpha]_D^{21}$ −10.1 ± 0.5° (C 1.33, methanol); R_f (B) 0.93. Found: C 50.30; H 4.20%. $C_{24}H_{24}O_7N_3Cl_3$. Calculated: C 49.50; H 4.10%.

Hydrochloride of 2,4,6-Trichlorophenyl Ester of hypro-ser-gly. To a solution of the 2,4,6-trichlorophenyl ester of carbobenzoxy-ser-gly in methanol was added 0.13 ml of 12 N HCl solution and the mixture was hydrogenated over metallic Pd. After the calculated amount of H_2 had been absorbed the solvent was evaporated in vacuo at 30°. The oily residue was dissolved in the minimum amount of methanol and, with rubbing, the product was precipitated by the addition of ether. We obtained 0.51 g (68%) of the hydrochloride of the 2,4,6-trichlorophenyl ester of hypro-ser-gly; mp 180°; $[\alpha]_D^{22}$ 16.2° (C 1.67, methanol); R_f (A) 0.6. A slight amount of the tripeptide and glycine (R_f respectively 0.09 and 0.004) as impurities was detected. Found: C 39.87; H 4.18%. $C_{16}H_{18}O_5N_3Cl_4$. Calculated: C 40.55; H 3.89%.

Polytripeptide (-hypro-ser-gly-)$_n$. The hydrochloride of the 2,4,6-trichlorophenyl ester of hypro-ser-gly (0.5053 g) was dissolved at 45-50° in 0.5535 g of dimethyl sulfoxide (50% solution). Then 0.16 ml of triethylamine was added at 20° and the mixture was shaken vigorously. After 6 days, methanol was added to the ampul contents. The polypeptide, insoluble in methanol, was filtered and washed with ether. We obtained 0.093 g of the (-hypro-ser-gly-)$_n$ polytripeptide (fraction I). From the methanol solution was extracted 0.15 g of the polypeptide (fraction II), $[\alpha]_D^{27}$ −15.4° (C 0.5, methanol). The molecular weight of fraction I was 5500-5600 (Van Slyke).

CONCLUSIONS

1. It was shown that formyl protection can be used to obtain the 2,4,6-trichlorophenyl esters of amino acids and peptides.

2. Some polytripeptides were synthesized containing a sequence related to proteins of the collagen group: (-hypro-ser-gly-)$_n$ and (-hypro-glu-gly-)$_n$, of which the latter has a structure of the collagen type.

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