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SYNTHESIS OF A DECAPEPTIDE FRAGMENT OF RIBONUCLEASE (64 – 73)

V. G. Degtyar and L. A. Shchukina

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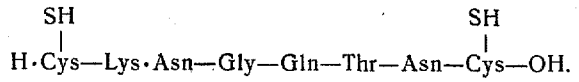
As is well known, in the preparation of peptides containing S–S bonds the main difficulty consists in the formation of these bonds between predetermined cysteine residues in the peptide chain. However, for some natural proteins and peptides it has been shown that their primary structure apparently favors the approach to one another of certain cysteine residues, as a consequence of which oxidation of the reduced forms of such proteins leads to the practically complete reconstruction of the disulfide bonds in the required sequence. This is shown by the high biological activity of the compounds formed after oxidation [1-4].

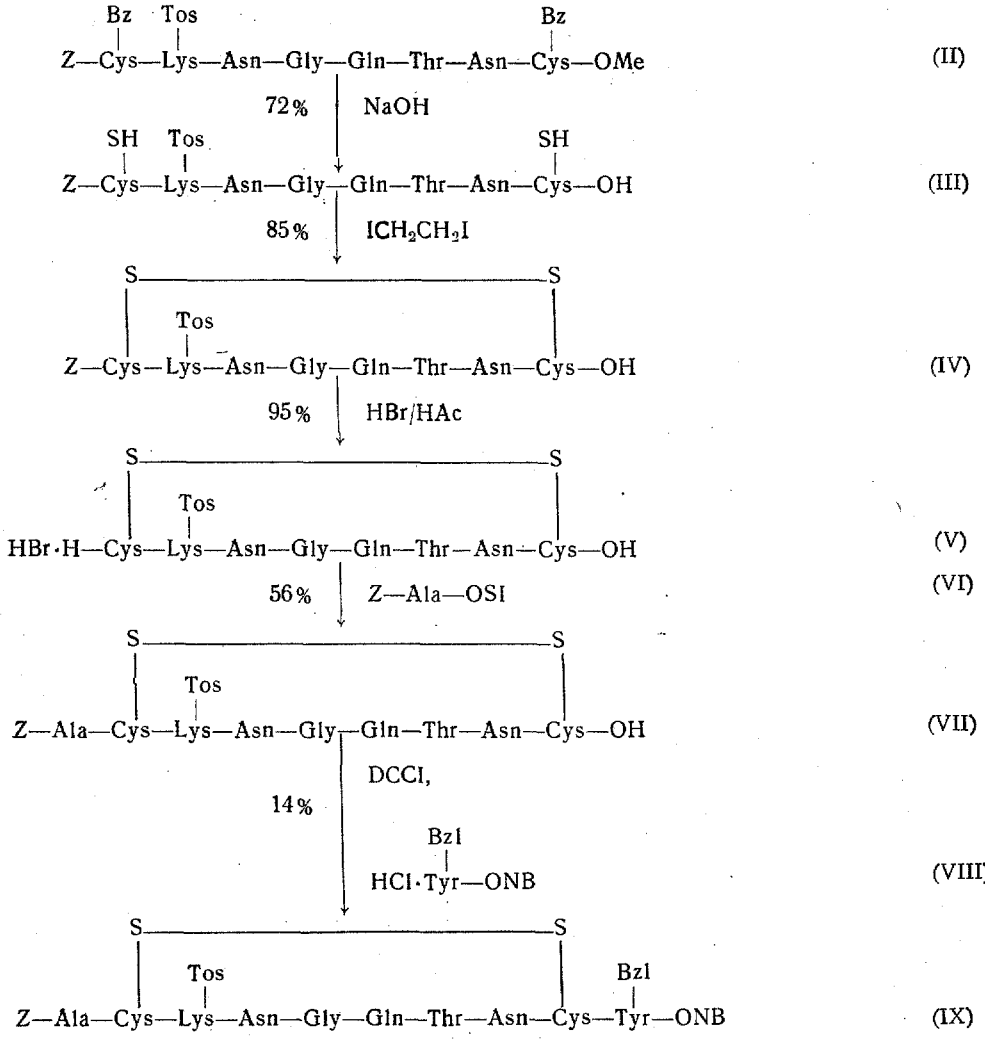
Among natural proteins of this type, particular interest is being devoted to ribonuclease, in which the four disulfide bridges link to one another the cysteine residues in positions 26 and 84, 40 and 95, 58 and 110, and 65 and 72. When reduced ribonuclease is oxidized with air at pH 8.0–8.5 more than 80% of the activity of this enzyme is regenerated [5-7].

This information has induced us to carry out a study in order to elucidate the predisposition to the formation of disulfide bonds in the synthetic fragment (I) corresponding to the sequence of amino acids 65-72 of the ribonuclease chain:



(I)

We have previously described the synthesis of the protected octapeptide (II) [8] (see scheme).



After the alkaline hydrolysis of compound (II) the partially protected peptide (III) was subjected to oxidation with diiodoethane [9], giving the cysteine-containing peptide (IV) with a yield of 85%, while when the octapeptide (III) was oxidized with air the peptide (IV) was formed with a yield of 60%. This high yield of the peptide (IV), it appears to us, confirms a definite conformational predisposition of this isolated fragment of ribonuclease (III) to create a disulfide bond.

We then studied the possibility of the further growth of the chain of the cysteine-containing peptide (V) from both the N-end and from the C-end of the molecule (see scheme).

When the peptide (V) was condensed with the N-hydroxysuccinimide ester of L-alanine (VI) [10], the peptide (VII) was isolated with a yield of 56%. However, when the p-nitrobenzyl ester of O-benzyl-L-tyrosine (VIII) was added to the C-end of the peptide (VII) with the aid of dicyclohexylcarbodiimide, the yield of the peptide (IX) was only 14%. which is perhaps due to the steric hindrance arising in the interaction of the voluminous molecule of the protected tyrosine (VIII) with the peptide (VII).

The p-nitrobenzyl ester of O-benzyl-L-tyrosine (VIII) used in this synthesis was obtained by the treatment of the p-nitrobenzyl ester of N-tert-butoxycarbonyl-L-benzyl-L-tyrosine with hydrogen chloride in ethyl acetate. The butoxycarbonyl derivative was obtained by esterifying N-tert-butoxycarbonyl-O-benzyl-O-tyrosine [11] with p-nitrobenzyl bromide.

Experimental*

For analysis, the substances were dried in vacuum for 5–6 hr. The compounds were chromatographed in thin layers of alumina and in silica with the addition of 30% of gypsum.

The R_f values were determined on Whatman paper no. 2 in the following systems: 1) 1-butanol–acetic acid–water (4:1:5) and 2) pyridine–isoamyl alcohol–water (10:10:7). The melting points are uncorrected.

Carbobenzoxy-L-cysteinyl-ε-tosyl-L-lysyl-L-asparaginyl-glycyl-L-glutaminyl-L-threonyl-L-asparaginyl-L-cysteine (III). At 27° C, in a current of hydrogen, 12 ml of 0.5 N caustic soda solution was added to a suspension of 1.34 g of the protected octapeptide (II) [8] in 30 ml of DMFA and 10 ml of methanol, and the mixture was stirred for 45 min. Then it was filtered and, with stirring, hot deoxygenated water acidified with hydrochloric acid was added to it, and then it was left in the refrigerator. The precipitate was filtered off, washed with deoxygenated water to neutrality, and dried. Yield 0.81 g (72%), mp 190–194° C (decomp.).

Carbobenzoxy-L-hemicystinyl-ε-tosyl-L-lysyl-L-asparaginyl-glycyl-L-glutaminyl-L-threonyl-L-asparaginyl-L-hemicystine (IV). A. Oxidation with diiodoethane [9]. Solutions of 0.9 g of substance (III) in 90 ml of DMFA and of 0.2 g of 1, 2-diiodoethane [12] in 90 ml of ethanol were added simultaneously in drops over an hour to 3 ml of TEA in 60 ml of DMFA and 60 ml of ethanol in a current of hydrogen. The resulting solution was stirred for another 15 min, the methanol was evaporated off, water acidified with hydrochloric acid was added to the solution, the mixture was kept in the refrigerator, and the precipitate was filtered off, washed with water, and dried. The weight of the disulfide (IVa) was 0.98 g (85%); mp 212–214° C (from acetic acid), $[\alpha]_D^{18}$ −56° (c 1; DMFA). Mol. wt. 1076 (determined by the thermistor method [13]).

Found, %: C 47.79; H 5.71; N 14.50; S 7.99. Calculated for $C_{46}H_{64}N_{12}O_{17}S_3$, %: C 47.92; H 5.60; N 14.58; S 8.34.

B. Oxidation with air. A current of air was passed through a solution of 0.12 g of substance (III) in 50 ml of DMFA until the nitroprusside reaction was negative (about 3 hr). The solution was filtered, water acidified with hydrochloric acid was added, and the mixture was kept in the refrigerator. This yielded 0.1 g of oxidized product (IVb) with mp 201–206° C.

Hydrobromide of the disulfide (V). A. One milliliter of 40% hydrogen bromide in glacial acetic acid was added to 0.6 g of the disulfide (IVa) in 2 ml of glacial acetic acid and the mixture was kept at 30° C for 30 min and evaporated to dryness; the residue was twice evaporated with methanol and was redissolved in methanol, and an excess of dry ether was added. This gave 0.52 g (95%) of the hydrobromide of the disulfide (V), R_{f_1} 0.40; R_{f_1} 0.19.

B. The oxidized product (IVb) was treated in a similar manner to (IVa). On paper chromatography, the hydrobromide gave four spots with R_{f_2} 0.08, 0.15, 0.19, and 0.25. On quantitative chromatography, the amount of the main substance (R_{f_2} 0.19) was found to be 56–60%.

Carbobenzoxy-L-alanyl-L-hemicystinyl-ε-tosyl-L-lysyl-L-asparaginyl-glycyl-L-glutaminyl-L-threonyl-L-asparaginyl-L-hemicystine (VIII). A solution of 1.1 g of substance (V), 0.14 ml of TEA, and 0.75 g of the N-hydroxysuccinimide ester of carbobenzoxy-L-alanine (VI) [10] in 10 ml of DMFA and 5 ml of methanol was kept for a day and was then

* The following abbreviations are used, in addition to those generally accepted: Asn–asparagine, Gln–glutamine, DMFA–dimethylformamide, TEA–triethylamine.

evaporated to form a sirupy residue; to this was added a large amount of acetone and the resulting mixture was acidified to pH 4 with a 1 N solution of hydrogen chloride in ether. The precipitate that deposited was filtered off, the acetone was evaporated off to dryness, the residue was treated with a mixture of ethyl acetate and ether (1:1), and the undissolved matter was filtered off and washed with the same mixture and was crystallized from DMFA–ethyl acetate. This gave 0.67 g (56%) of substance (VII), mp 195–198° C, $[\alpha]_D^{18}$ −8° (c 1; DMFA).

Found, %: C 48.19; H 5.52; N 14.78; S 7.98. Calculated for $C_{49}H_{68}N_{13}O_{16}S_3$, %: C 48.12; H 5.61; N 14.89; S 7.87.

p-Nitrobenzylester of N-tert-butoxycarbonyl-O-benzyl-L-tyrosine (X). A mixture of 3.71 g of N-tert-butoxycarbonyl-O-benzyl-L-tyrosine [11], 2.2 g of p-nitrobenzyl bromide, 2.5 ml of TEA, 20 ml of tetrahydrofuran, and 60 ml of ethyl acetate was boiled and was filtered through a layer of alumina, the alumina being washed with hot ethyl acetate and the filtrate evaporated to dryness. This provided 3.6 g (70%) of substance (X) with mp 109–110° C (from methanol); $[\alpha]_D^{19}$ −13° (c 2; DMFA).

Found, %: C 66.17; H 6.17; N 5.55. Calculated for $C_{28}H_{30}N_2O_7$, %: C 66.39; H 5.97; N 5.58.

Hydrochloride of the p-nitrobenzyl ester of O-benzyl-L-tyrosine (VIII). Two milliliters of a 3.5 N solution of hydrogen chloride in ethyl acetate was added to a solution of 2.53 g of substance (X) in 25 ml of ethyl acetate and the mixture was kept for 45 min. Then the solvent was distilled off to dryness. The weight of product was 2 g (91%), mp 202–204° C (decomp., from methanol–ether), $[\alpha]_D^{18}$ −8° (c 2; methanol).

Found, %: C 61.94, H 5.37; N 6.36; Cl 7.84. Calculated for $C_{23}H_{24}N_2O_5Cl$, %: C 62.22; H 5.48; N 6.31; Cl 8.00.

p-Nitrobenzyl ester of carbobenzoxy-L-alanyl-L-hemicystinyl-ε-tosyl-L-lysyl-L-asparaginyl-glycyl-L-glutaminyl-L-threonyl-L-asparaginyl-L-hemicystinyl-O-benzyl-L-tyrosine (IX). A solution of 0.61 g of substance (VII), 0.88 g of substance (VIII), and 0.28 ml of TEA in 5 ml of DMFA was cooled to 0° C, and, with stirring, 0.2 g of dicyclohexylcarbodiimide in 1 ml of DMFA was added in drops over 30 min; then the mixture was kept at this temperature for 2 hr and was left overnight at room temperature. After the addition of 0.5 ml of 50% acetic acid, the precipitate was filtered off and washed with 1 ml of DMFA. The solution was filtered through a layer of alumina and was acidified with dilute hydrochloric acid and kept in the refrigerator. After filtration, the residue was washed with water to neutrality and dried. This gave 1.4 g of a substance which was crystallized three times from a mixture of methanol, DMFA, and water. The weight of compound (IX) was 0.12 g (14%), mp 215–217° C (decomp.), $[\alpha]_D^{18}$ −15° (c 1; DMFA).

Found, %: C 55.21; H 5.64; N 13.29; S 6.28. Calculated for $C_{72}H_{89}N_{14}O_{22}S_3$, %: C 55.12; H 5.72; N 13.39; S 6.15.

Conclusions

1. On the oxidation of a linear partially protected octapeptide corresponding to the sequence of amino acids of the ribonuclease chain in the 65–72 segment, the corresponding disulfide is formed in high yield, which apparently shows the definite predisposition of this fragment to the formation of a disulfide bond between the terminal cysteines.

2. The growth of the cyclic disulfide of the octapeptide has been effected from both the N- and C-ends of the peptide chain.

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