SYNTHESIS OF PROTECTED PEPTIDES REPRESENTING FRAGMENTS (14-22) AND (11-17) OF YEAST CYTOCHROME c

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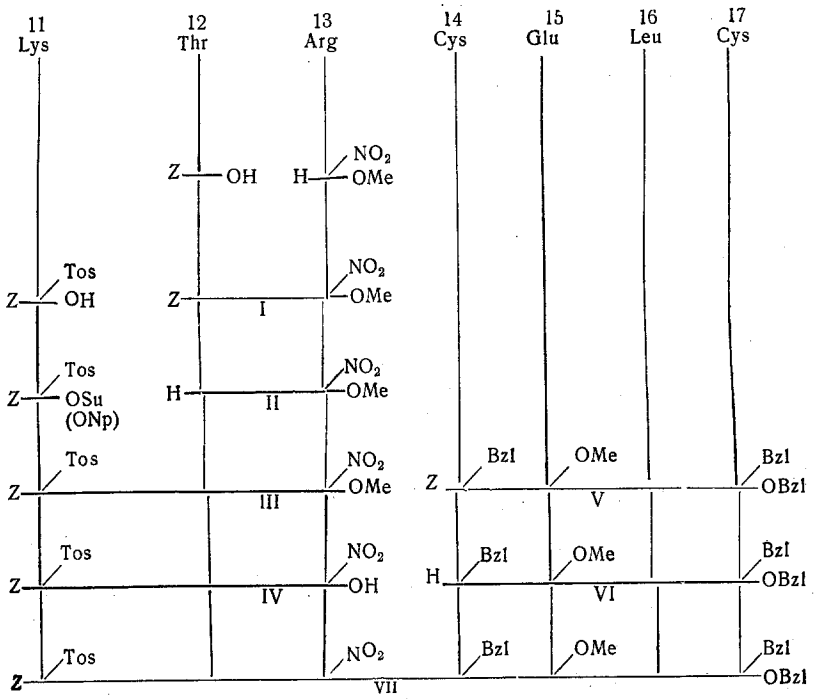
Continuing an investigation [1, 2] of the peptide fragments of cytochrome c directly connected with the prosthetic group, we have synthesized a substituted heptapeptide (VII) and a nonapeptide (XIII) corresponding to sequences 11-17 and 14-22 of the protein chain of yeast cytochrome c. Both fragments include two cysteine residues (14 and 17) which are capable of forming a covalent bond with the vinyl substituents of hemin. The choice of protective groups was governed by the possibility of their subsequent removal under the conditions of the creation of heme-peptide complexes (sodium in liquid ammonia).

The heptapeptide (VII) was obtained by condensing the tripeptide (IV) and the tetrapeptide (VI) [1] in the presence of N,N'-dicyclohexylcarbodiimide and of two equivalents of N-hydroxysuccinimide.

The dipeptide (I) was synthesized by condensing N-benzyloxycarbonyl-L-threonine [3] and the methyl ester of nitro-L-arginine [4] by the carbodiimide method. The N-benzyloxycarbonyl-N^{ε}-tosyl-L-lysine residue was introduced with the aid of the p-nitrophenyl or the N-hydroxysuccinimide ester. The tripeptide (III) was saponified with 1 N caustic soda in dioxane for 1 h. The scheme of the synthesis of the substituted heptapeptide is given below.



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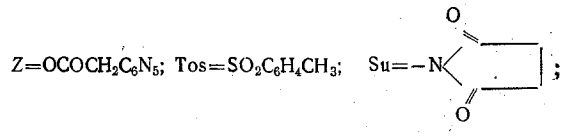
All the amino acids have the L configuration, and

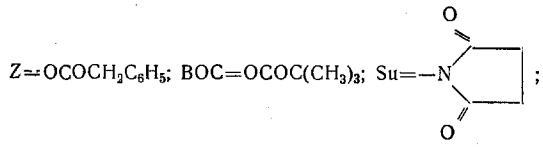


Bzl=$CH_2C_6H_5$; Me=CH_3.

The nonapeptide (XIII) was obtained by condensing the tetrapeptide (X) and the pentapeptide (XII) [2] by the carbodiimide method in the presence of two equivalents of N-hydroxysuccinimide.

The tetrapeptide (X) was synthesized by the successive growth of the chain from the C end by the mixed anhydride method. We may note that the use of this method at the stage of the preparation of the tripeptide (IX) gave unsatisfactory results. Consequently, the tripeptide (IX) was obtained in good yield (95%) by the "activated ester" (N-hydroxysuccinimide ester) method. The protection of the carboxy group of the C-terminal cysteine residue was effected by salt formation [5]. As the base for salt formation we used either triethylamine or N-methylmorpholine. Then we used the scheme of synthesis of the substituted nonapeptide given on the next page.

All the amino acids have the L configuration, and



Tos=$SO_2C_6H_4CH_3$; Bzl=$CH_2C_6H_5$.

The individuality of the compounds obtained in the course of the synthesis was shown by chromatography in a thin layer of silica gel in various systems.

The structures of the nonapeptide (XIII) and the heptapeptide (VII) was confirmed by their acid hydrolysis and by the results of the subsequent chromatography of the hydrolysate (Leningrad type B ["fast"] paper) in the water – 88% formic acid – tert-butanol (15 : 15 : 70) system. The chromatogram showed the presence of all the amino acids making up the peptides (XIII) and (VII). A quantitative amino-acid analysis was performed for compound (VII).

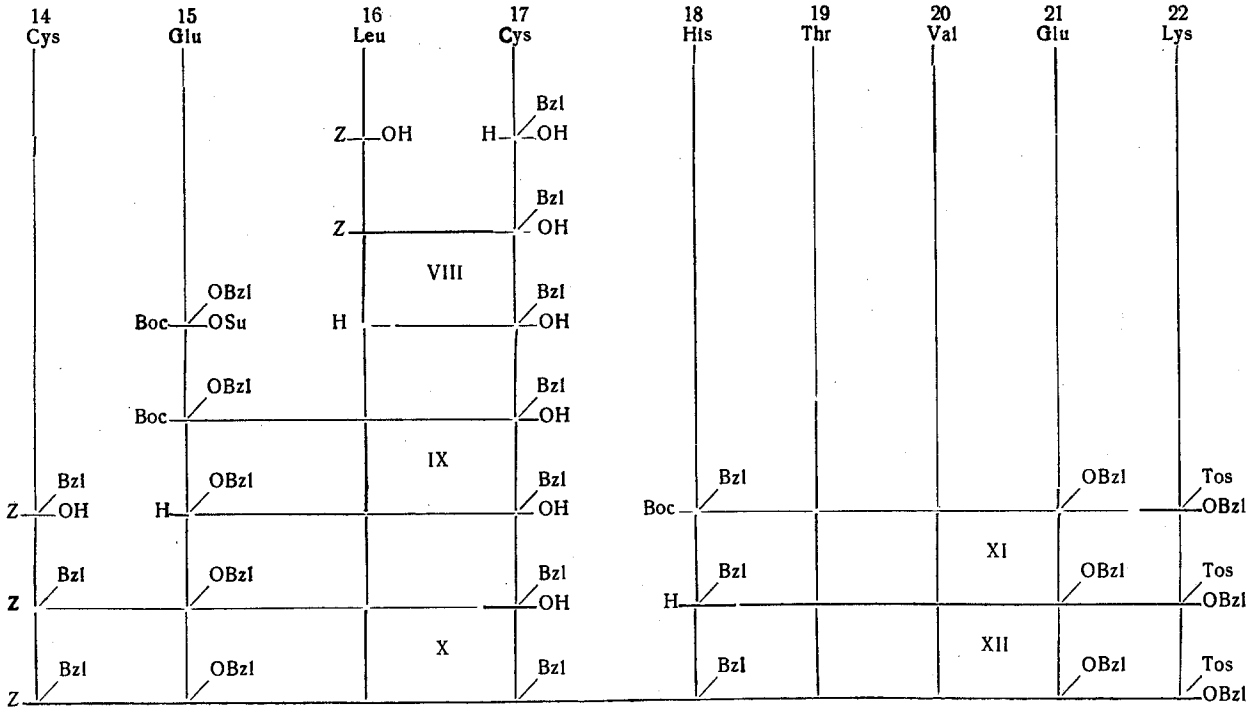
EXPERIMENTAL

The compounds were chromatographed in a nonfixed layer of silica in the following systems: 1) benzene–ethyl acetate (7 : 3); 2) benzene–ethyl acetate (2 : 1); 3) chloroform–methanol–acetone (8 : 1 : 1); and 4) butanol–acetic acid–water (4 : 1 : 1). The elementary analyses of all the compounds corresponded to the calculated figures.

Methyl Ester of N-Benzyloxycarbonyl-L-threonyl–nitro-L-arginine (I). At 40° C, 0.50 g of the hydrochloride of the methyl ester of nitro-L-arginine was dissolved in 3 ml of dimethylformamide, and 0.26 ml of triethylamine in 3 ml of methylene chloride was added, after which the mixture was cooled and, with stirring, added to a solution of 0.43 g of N-benzyloxycarbonyl-L-threonine in 3 ml of tetrahydrofuran. The resulting mixture was cooled to 0° C, and 0.39 g of N,N'-dicyclohexylcarbodiimide was added. After stirring at 0° C for 1 h and at 20° C for 20 h, the dicyclohexylurea was filtered off, and the solvent was driven off in vacuum.

The residue was dissolved in 30 ml of methylene chloride and the solution was washed with 3% sodium bicarbonate solution, water, 5% hydrochloric acid, and water again, and was dried with sodium sulfate. The solvent was distilled off in vacuum and the residue was treated with acetone (3 × 5 ml) to eliminate the dicyclohexylurea and was then recrystallized from ethyl acetate–ethanol (4 : 1). This gave a substance with the composition $C_{19}H_{28}N_6O_8$. Yield 0.60 g (70%), mp 135-136° C, $[\alpha]_D^{23}$ – 3.8° (c 1; dimethylformamide), R_f 0.60 (system 3).

Methyl Ester of N-Benzyloxycarbonyl-N^{ε}-tosyl-L-lysyl–L-threonyl–nitro-L-arginine (III). To 0.42 g of the dipeptide (II) was added 1 ml of a 36% solution of hydrogen bromide in glacial acetic acid. After 25 min, 100 ml of ether was added. The precipitate was separated off, washed with ether, and dried in a vacuum desiccator over caustic potash. To 0.39 g of the resulting hydrobromide were added 20 ml of


14 Cys
15 Glu
16 Leu
17 Cys
18 His
19 Thr
20 Val
21 Glu
22 Lys
Z—OH
H—OH
Bzl
OBzl
Boc—OSu
Boc
H
Z
VIII
IX
X
XI
XII
Tos

methanol and 1.2 ml of triethylamine. The solvent was driven off in vacuum and the residue was dissolved in 10 ml of dimethylformamide. Then 0.45 g of the p-nitrophenyl ester of N-benzyloxycarbonyl-N^{ε}-tosyl-L-lysine [5] was added and the mixture was stirred at 20° C for 24 h. The dimethylformamide was driven off in vacuum at 40° C. The residue was treated with 30 ml of chloroform and worked up as described for compound (I). The precipitate that deposited after the elimination of the solvent was recrystallized from methanol giving a substance $C_{32}H_{46}O_{11}N_8S$. Yield 0.47 g (78.4%), mp 181-182° C; $[\alpha]_D^{23}$ – 2.4° (c 1; chloroform), R_f 0.53 (system 3).

N-Benzyloxycarbonyl-N^{ε}-tosyl-L-lysyl-L-threonyl-nitro-L-arginine (IV). A solution of 0.33 g of the tripeptide (III) in a mixture of 5 ml of methanol, 1 ml of dioxane, and 3 ml of water was treated with 0.5 ml of 1 N caustic soda solution, and the mixture was stirred for 1.5 h. The solvent was evaporated off in vacuum, 5 ml of water was added to the residue, the mixture was acidified with 1 N hydrochloric acid with cooling, the substance was extracted with chloroform (3 × 10 ml), and the extract was dried with magnesium sulfate. The solvent was driven off in vacuum and the residue was treated with ether. This gave 0.28 g (86%) of the tripeptide (IV), $C_{31}H_{44}O_{11}N_8S$ in the form of an amorphous substance with $[\alpha]_D^{23}$ + 38° (c 1; dimethylformamide), R_f 0.41 (system 3).

Benzyl Ester of N-Benzyloxycarbonyl-N^{ε}-tosyl-L-lysyl-L-threonyl-nitro-L-arginyl-S-benzylcysteinyl-γ-methyl-L-glutamyl-L-leucyl-S-benzyl-L-cysteine (VII). A solution of 0.18 g of the tripeptide (IV), 0.05 g of N-hydroxysuccinimide, 0.2 ml of triethylamine, and 0.2 g of the hydrobromide of the tetrapeptide (VI) obtained from 0.26 g of the tetrapeptide (V) [1] in 4 ml of dimethylformamide was cooled to 0° C, and 0.06 g of N,N'-dicyclohexylcarbodiimide was added. The mixture was stirred at 0° C for 6 h and at 25° C for 20 h. The precipitate was filtered off, the solvent was evaporated in vacuum at 35° C, and the residue was treated as described for the dipeptide (I). Recrystallization from methanol–dioxane (10 : 1) gave 0.27 g (82.4%) of the heptapeptide (VII), $C_{70}H_{92}O_{17}N_{12}S_3$ with mp 219-220° C, $[\alpha]_D^{20}$ – 28.1° (c 1; dimethylformamide), R_f 0.64 (system 3). An amino-acid analysis gave the following ratio of the amino acids: Leu 1.14; Cys 1.93; Glu 1.00; Thr 1.07; Arg 0.87; and Lys 0.70.

N-Benzyloxycarbonyl-L-leucyl-S-benzyl-L-cysteine (VIII). A solution of 1.25 g of N-benzyloxycarbonyl-L-leucine [7] in 14 ml of tetrahydrofuran was cooled to –10° C, and 0.65 ml of triethylamine and 0.65 ml of isobutyl chloroformate were added. After 10 min, the mixed anhydride was poured into a cooled solution of the triethylammonium salt of S-benzyl-L-cysteine obtained by dissolving 1.0 g of S-benzyl-L-cysteine and 0.65 ml of triethylamine in 20 ml of water. The mixture was stirred at 0° C for 3 h. The tetrahydrofuran was distilled off in vacuum, and the aqueous solution was acidified to pH 1 with 1 N hydrochloric acid and extracted with chloroform. The chloroform layer was washed with water and with 1 N hydrochloric acid and was dried with magnesium sulfate. The solvent was driven off in vacuum, and the residue was treated with petroleum ether and recrystallized from a mixture of hexane and isopropanol (5 : 1). A substance with the composition $C_{24}H_{30}O_5N_2S$ was obtained. Yield 1.60 g (74.0%), mp 158-159.5° C; R_f 0.59 (system 1); $[\alpha]_D^{17}$ – 23.8° (c 1.4; dimethylformamide).

N-tert-Butoxycarbonyl-γ-benzyl-L-glutamyl-L-leucyl-S-benzyl-L-cysteine (IX). To 0.59 g of N-benzyloxycarbonyl-L-leucyl-S-benzyl-L-cysteine was added 1.3 ml of a 36% solution of hydrogen bromide in glacial acetic acid. After 30 min, 50 ml of ether was added. The precipitate was separated by decantation, washed with ether, and dried in a vacuum desiccator over caustic potash. The yield of hydrobromide was 0.49 g (91.5%). The hydrobromide of the dipeptide (VIII) obtained (0.38 g) was dissolved in 5 ml of dimethylformamide, and 0.28 ml of triethylamine and 0.44 g of the N-hydroxysuccinimide ester of N-tert-butoxycarbonyl-γ-benzylglutamic acid [8] were added. The mixture was stirred at 20° C for 48 h. The dimethylformamide was driven off in vacuum. The residue was treated in a similar manner to compound (I) and was recrystallized from isopropanol–hexane (5 : 1). A substance with the composition $C_{33}H_{45}N_3O_8S$ was obtained. Yield 0.4 g (68%), mp 128-129° C, R_f 0.45 (system 2) $[\alpha]_D^{20}$ – 25.7° (c 2.2; dimethylformamide).

N-Benzyloxycarbonyl-S-benzyl-L-cysteinyl-γ-benzyl-L-glutamyl-L-leucyl-S-benzyl-L-cystein (X). To 0.33 g of the tripeptide (IX) was added 0.5 ml of trifluoroacetic acid. After 30 min, 100 ml of ether was added, and the precipitate was separated off, washed with ether, and dried in a vacuum desiccator over caustic potash. The yield of trifluoroacetate was 96%, R_f 0.76 (system 4). To a solution of 0.24 g of the trifluoroacetate obtained and 0.10 ml of triethylamine in 5 ml of water was added the mixed anhydride obtained from 0.12 g of N-benzyloxycarbonyl-S-benzyl-L-cysteine, 0.05 ml of triethylamine and 0.05 ml of

isobutyl chloroformate in 4 ml of tetrahydrofuran. The mixture was stirred at 0° C for 3 h. After the working up described for compound (VIII), 0.22 g (69%) of the tetrapeptide (X) was obtained with the composition $C_{46}H_{54}N_4O_9S_2 \cdot H_2O$, mp 169-170° C, R_f 0.35 (system 2).

Benzyl Ester of N^{α}-Benzyloxycarbonyl-S-benzyl-L-cysteinyl-γ-benzyl-L-glutamyl-L-leucyl-S-benzyl-L-cysteinyl-N^{im}-benzyl-L-histidyl-L-threonyl-L-valyl-γ-benzyl-L-glutamyl-N^{ε}-tosyl-L-lysine (XIII). A solution of 0.1 g of the tetrapeptide (X) and 0.03 g of N-hydroxysuccinimide in 2 ml of dimethylformamide was poured into a solution of 0.11 g of the hydrochloride of the pentapeptide (XII) obtained from 0.15 g of the pentapeptide (XI) [1] and 0.01 ml of triethylamine in 2 ml of dimethylformamide. The mixture was cooled to 0° C, 0.03 g of N,N'-dicyclohexylcarbodiimide was added, and it was stirred at 0° C for 1 h and at 20° C for 24 h. The precipitate was filtered off, the dimethylformamide was driven off in vacuum at 40° C, and the residue was treated as described for compound (I) with recrystallization from ethanol-water (5 : 1). This gave a substance with the composition $C_{100}H_{120}N_{12}O_{19}S_3 \cdot 4H_2O$. Yield 0.09 g (44%), mp 196-198° C, $[\alpha]_D^{23}$ – 11.5° (c 2.2; dimethylformamide), R_f 0.65 (system 3).

SUMMARY

The synthesis of a nonapeptide, the benzyl ester of N^{α}-benzyloxycarbonyl-S-benzyl-L-cysteinyl-γ-benzyl-L-glutamyl-L-leucyl-S-benzyl-L-cysteinyl-N^{im}-benzyl-L-histidyl-L-threonyl-L-valyl-γ-benzyl-L-glutamyl-N^{ε}-tosyl-L-lysine (XIII), and a heptapeptide, the benzyl ester of N^{α}-benzyloxycarbonyl-N^{ε}-tosyl-L-lysyl-L-threonyl-nitro-L-arginyl-S-benzyl-L-cysteinyl-γ-methyl-L-glutamyl-L-leucyl-S-benzyl-L-cysteine (VII), corresponding to the sequences 14-22 and 11-17 of yeast cytochrome c, has been effected.

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