SYNTHESIS OF Z-L-Phe-L-His-L-Leu, A SUBSTRATE OF CARBOXYCATHEPSIN

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Carboxycathepsin (peptidyl dipeptidase, EC. 3.4.15.1) [1] is apparently one of the regulators of the vasoactive peptide antagonists (angiotensin and bradykinin) in the blood [2]. A simple and sensitive method of determining the activity of this enzyme is based on the fluorometric determination of the histidylleucine liberated as the result of the enzymatic cleavage of the substrate. As the substrate is used the N-benzyloxycarbonyl derivative of the C-terminal tripeptide of angiotensin — Z-Phe-His-Leu (I) [2, 3]. The information in the literature on the synthesis of (I) and its esters is sparse [3, 4] and the proposed methods are unsatisfactory in the preparative aspects. We have tested some variants of the synthesis of (I) both some described previously [3] and some now proposed for the first time.

The specific difficulties of the synthesis of histidine-containing peptides are due to the presence of the imidazole nucleus, which requires a N^{im}-protective grouping to be introduced in the activation of the carboxy group, or the laborious azide method of condensation to be used [5]. In building up the peptide chain from the C-end at the stage of the synthesis of histidylleucine we started from N^{α}, N^{im}-di-tert-butyloxycarbonylhistidine (II) and its N'-hydroxysuccinimide ester (III) [7] and the hydrazide of N^{α}-tert-butyloxycarbonylhistidine (IV). To obtain the tert-butyloxycarbonyl derivatives of histidine we used di-tert-butyl dicarbonate [6, 7].

The azide of N^{α}-tert-butyloxycarbonylhistidine was obtained from (IV) by the method of Bailey and Booth [8] and condensed with the methyl ester of leucine to reform the methyl ester of N^{α}-tert-butyloxycarbonylhistidylleucine (V), which was obtained in the crystalline state with a yield of about 60%. The methyl ester of N^{α}, N^{im}-di-tert-butyloxycarbonylhistidylleucine (VI), obtained from (II) by the mixed-anhydride method with propyl carbonate consisted of a noncrystallizing oil (see [9]). It is known that the N^{im}-tert-butyloxycarbonyl grouping is stable under the conditions of peptide synthesis [9] but can be eliminated by the action of ammonia [10]. We found that it is also comparatively readily removed in aqueous methanolic solutions of potassium carbonate. By means of this method, the oily ester (VI) was converted into the crystalline ester (V) with a yield of 40%. In this process, the ester group also partially saponified. The esters of the N-protected dipeptides (V) and (VI) were converted by treatment with hydrogen chloride in acetic acid into the dihydrochloride of the methyl ester of histidylleucine (VII), which was obtained from (V) in the analytically pure form with a high melting point [11].

The free histidylleucine (VIII) obtained after the saponification of the ester (VII) or the debutoxycarbonylation of N^{α}, N^{im}-di-tert-butoxycarbonylhistidylleucine (IX) — the product of the condensation of the N-hydroxysuccinimide ester of N^{α}, N^{im}-di-tert-butoxycarbonylhistidine (III) with the sodium salt of leucine. In both cases, the dipeptide (VIII) was isolated from the reaction mixture by chromatography on IR-45 ion-exchange resin in the OH form.

The synthesis of the methyl ester of the tripeptide (I) has been described previously [3, 4]. The yield of the ester (Ia) isolated from the p-nitrophenylester of benzyloxycarbonylphenylalanine and the methyl ester of histidylleucine was about 40% [4] and increased when the azide method [3] was used. We have found the first method gives reproducible results and the yield of the ester (Ia) can be raised to 79% by using the N'-hydroxysuccinimide

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ester of N-benzyloxycarbonylphenylalanine. The condensation of the azide of the N-terminal dipeptide with the ester of leucine (second method) is laborious and does not give constant yields.

Piquilloud et al., [3] performed the saponification of the ester (Ia) under standard conditions with alkali in aqueous methanolic solution. On checking it was found that under these conditions the saponification of the ester is accompanied by the formation of a byproduct which differs considerably from (I) in its chromatographic mobility. A special experiment showed that in an alkaline solution with a high pH the tripeptide (I) is converted completely into the unknown substance (X), and this was then isolated and was characterized by elementary analysis. An acid hydrolyzate of the unknown substance contained all the amino acids of the tripeptide (I). Apparently, substance (X) is a hydantoin formed as the result of a splitting out of the benzyloxy group, which agrees with the results of elementary analysis. The formation of hydantoins in the saponification of esters of N-benzyloxycarbonyl derivatives of peptides has been mentioned repeatedly [5].

Complications in saponification can be avoided by lowering the pH of the reaction solution. When we saponified (Ia) in aqueous methanolic solution with potassium carbonate we observed no formation of the byproduct (X) and obtained (I) with a high yield and a melting point 10°C higher than that reported by Piquilloud et al., [3]. The method of synthesizing (I) from the N-hydroxysuccinimide ester of N-benzyloxycarbonylphenylalanine and the sodium salt of histidylleucine proved to be even better. This variant of the synthesis is characterized by being less laborious and giving constant high yields of the end product.

The samples of the substrate (I) obtained by various methods did not differ in their capacity for being cleaved by carboxycathepsin.

EXPERIMENTAL

The compositions of the reaction mixtures, the processes of isolation and purification, and the individualities of the substances obtained were checked by thin-layer chromatography on "Silufol" plates in the following solvent systems: 1) acetonitrile–NH_4OH (conc.) (9:1) (R_{f_1}); 2) acetonitrile–NH_4OH (conc.) (4:1) (R_{f_2}); 3) benzene–methyl ethyl ketone–acetic acid (80:40:1) (R_{f_3}); 4) ethyl acetate–pyridine (9:1) (R_{f_4}); 5) chloroform–methanol–NH_4OH (conc.) (5:3:1) (R_{f_5}).

The analyses of the compounds obtained corresponded to the calculated figures.

Hydrazide of N^{α}-tert-Butoxycarbonylhistidine (IV). To a solution of the dihydrochloride of the methyl ester of histidine (4.8 g, 20 mmole) in a mixture of 15 ml of water and 25 ml of tert-butanol were added 6 ml of triethylamine and 10 ml of BOC_2O. A vigorous reaction with the evolution of CO_2 began immediately. The mixture was stirred at 20°C for 1.5 h and was then diluted with water to 100 ml and extracted with benzene (2 × 30 ml). The extract was washed with water (3 × 20 ml) and with brine and was dried with $MgSO_4$. After the benzene had been distilled off in vacuum the residue (the methyl ester of N^{α}, N^{im}-di-tert-butoxycarbonylhistidine (7.5 g) was dissolved in 30 ml of methanol, and then 5 ml of hydrazine hydrate was added and the mixture was boiled for 2 h. The methanol was distilled off in vacuum and the residue was dried by the distillation of isobutanol and then of toluene and was triturated in ether. The crystalline residue was filtered off, washed with ether, and dried in vacuum. This gave 5 g (93%) of the hydrazine (IV) with mp 105-110°C; after reprecipitation from isopropanol with ether, mp 143-144°C [8, 12].

Methyl Ester of N^{α}-Tert-Butoxycarbonylhistidylleucine (V). A. Azide Method. The hydrazine (IV) (2.7 g, 10 mmole) was dissolved at −10°C in a mixture of 15 ml of 1 N HCl and 5 ml of brine, and then a solution of 700 mg of $NaNO_2$ in a 50% solution of K_2CO_3 was added. The mixture was stirred for 3 min, the ethyl acetate layer was separated off, the aqueous layer was extracted with cooled ethyl acetate (2 × 15 ml), the combined extracts were washed with brine and were cooled to −10°C, and a solution of the methyl ester of leucine (10 mmole) in 15 ml of tetrahydrofuran was added. The mixture was left at −5°C for 2 h and at ∿ 20°C for 16 h and was then washed with water (3 × 15 ml), and the peptide was extracted with a 10% solution of citric acid (2 × 20 ml). The acid extract was neutralized with solid Na_2CO_3 and was saturated with $NaHCO_3$. The peptide was extracted with ethyl acetate (3 × 15 ml). The extract was washed with water and brine, dried with $MgSO_4$, and evaporated in vacuum. The residue was crystallized from a mixture of dioxane and cyclohexane. This gave 2.1 g (57%) of (V), $C_{18}H_{30}N_4O_5$, mp 115-117°C, $[\alpha]_D^{20}$ −30.2 (c 1; C_2H_5OH) R_{f_1} 0.58; R_{f_4} 0.32.

On recrystallization from a mixture of dioxane, ether, and hexane, the substance crystallized with a molecule of ether, $C_{18}H_{30}N_4O_5 \cdot C_4H_{10}O$, mp 127-128°C, R_{f_4} 0.32.

B. Mixed Anhydride Method. To a solution of N^{α}, N^{im}-di-tert-butoxycarbonylhistidine (II) [7] (3.5 g, 10 mmole) in 20 ml of THF was added 1.1 ml of methylmorpholine, and the solution was cooled to −10°C and 1.3 ml of n-propylfluoroformate was added. The mixture was stirred for 15 min (−10°C), and a solution of 1.8 g of the hydrochloride of the methyl ester of leucine and 1.1 ml of methylmorpholine in 15 ml of dimethylformamide was added. The resulting mixture was stirred at −5 to 0°C for 30 min and then at 20°C for 1 h. Then it was diluted with water to 100 ml and was extracted with ethyl acetate (3 × 25 ml).

The extract was washed with water (3 × 15 ml), 0.2 N HCl (20 ml), water again, and brine, and was dried with $MgSO_4$. The ethyl acetate was distilled off in vacuum, giving 4.3 g of the amorphous methyl ester of N^{α}, N^{im}-di-tert-butoxycarbonylhistidylleucine (VI) (Rf_1 0.95; R_{f_3} 0.55). It was dissolved in 10 ml of methanol, 3 ml of a 1 M solution of K_2CO_3 was added, and the mixture was stirred at 18°C for 1 h. Then it was evaporated to half its volume, made up with water to 50 ml, and extracted with ethyl acetate (3 × 20 ml). The extract was washed with water and with brine, dried with $MgSO_4$, and evaporated in vacuum. The residue (3.0 g) was crystallized from a mixture of dioxane and cyclohexane. This gave 1.8 g (47%) of the methyl ester of N^{α}-tert-butoxycarbonylhistidylleucine (V) with mp 112-115°C, $[\alpha]_D^{20}$ −30.0 (c 1; C_2H_5OH).

Dihydrochloride of the Methyl Ester of Histidylleucine (VII). A. The methyl ester of the peptide (V) (1 g, 2.6 mmole) was dissolved in 20 ml of 1 N HCl in acetic acid, and the solution was left at 20°C for 1 h. Then it was evaporated in vacuum, the residue was dissolved in 5 ml of methanol, and the peptide was precipitated with ether. After 1 h, the precipitate was filtered off and washed with ether, giving 0.9 g (98%) of the chromatographically homogeneous dihydrochloride of the ester of the peptide (VI), $C_{13}H_{22}N_4O_3 \cdot 2HCl$, with mp 138-141°C, R_{f_1} 0.32 [11].

B. The methyl ester of N^{α}, N^{im}-di-tert-butoxycarbonylhistidylleucine (VI) (3.8 g, 7.9 mmole) was dissolved in 60 ml of 1 N HCl in acetic acid and the solution was left at 20°C. After 1 h it was evaporated in vacuum, and the oily residue was triturated in ether. After drying in vacuum, 2.4 g (85%) of the chromatographically homogeneous dihydrochloride of the ester (VII) was obtained with R_{f_1} 0.33.

Histidylleucine (VII). A. The dihydrochloride of the ester (VI) (2.4 g) was dissolved in 30 ml of methanol, 10 ml of 4 N NaOH was added, and the mixture was stirred at 20°C for 3 h. Then the methanol was distilled off in vacuum, the residue was neutralized with 1 N HCl, and the solution was passed through a column (25 × 2 cm) with JR-45 resin in the OH^- form, and the column was washed with water. The filtrate was evaporated. The residue (in the form of a syrup) was acidified with acetic acid. After a day, the precipitate of histidylleucine (0.3 g) was filtered off. The column was washed with 300 ml of 1 N acetic acid and 200 ml of water. The filtrate was evaporated to dryness. The residue was shaken with ethanol, and another 1.0 g of histidylleucine was obtained. The two precipitates were combined and heated to the boil in a mixture of 15 ml of ethanol and 5 ml of water. Then the solid matter was filtered off and was washed with ethanol and ether. Yield 0.9 g, mp 214-215°C [13], $[\alpha]_D^{20}$ −40.1 (c 1, 0.1 N NaOH).

B. Leucine (1.3 g; 10 mmole; was dissolved in 10 ml of 1 NaOH, the solution was diluted in 10 ml of tert-butanol, CO_2 was passed in and, with stirring, a solution of the N-hydroxysuccinimide ester of N^{α}, N^{im}-di-tert-butoxycarbonylhistidine (III) (10 mmole) in 20 ml of DMFA was added in portions over 30 min. The mixture was stirred for 2 h, diluted with water to 100 ml, treated with 3 ml of glacial acetic acid, and extracted with ethyl acetate (3 × 20 ml). The extract was washed with water and with brine, dried with $MgSO_4$, and evaporated to dryness. The residue [N^{α}, N^{im}-di-tert-butoxycarbonylhistidylleucine (IX)] was dissolved in 35 ml of a 1 N solution of HCl in acetic acid. After 1 h, the solution was evaporated in vacuum (40°C) and the residue was dissolved in water (20 ml) and extracted with ethyl acetate. The aqueous solution was passed through a column of JR-45 resin in the OH^- form, and the column was washed with water (100 ml) and 1 N acetic acid (100 ml).

The acetic acid eluate was evaporated to dryness, the crystalline residue was heated in ethanol, the mixture was cooled, and the solid matter was filtered off, washed with ethanol and ether, and dried in vacuum. This gave 1.8 g (67%) of histidylleucine with mp 215-217°C, $[\alpha]_D^{20}$ −40.3 (c 1, 0.1 N NaOH) R_{f_2} 0.2; R_{f_5} 0.50.

Methyl Ester of Benzyloxycarbonylphenylalanylhistidylleucine (Ia). A. Activated Ester Method. To a solution of 1.6 g of the N'-hydroxysuccinimide ester of N-benzyloxycarbonylphenylalanine and 1.4 g of the dihydrochloride of the methyl ester of histidylleucine in 20 ml of chloroform was added 1.1 ml of triethylamine, and the mixture was stirred at room temperature for 12 h. The chloroform was distilled off in vacuum, the residue was dissolved in ethyl acetate (50 ml), and the solution was washed with water, 5% $NaHCO_3$, and water again (2 × 20 ml). The peptide was extracted with a 10% solution of citric acid (3 × 20 ml) and the extract was neutralized with dry Na_2CO_3 and saturated with $NaHCO_3$. After 3 h, the precipitate was filtered off, washed with water, and dried in vacuum over $CaCl_2$. This gave 1.7 g (79%) of (Ia) with mp 189-190°C, R_{f_1} 0.70; R_{f_4} 0.35 [3, 4].

N-Benzyloxycarbonylphenylalanylhistidylleucine (I). A. To a solution of 0.90 g (1.6 mmole) of the ester (Ia) in 15 ml of methanol was added 10 ml of a 1 M solution of K_2CO_3, and the mixture was stirred at 20°C for 4 h; a clear solution was formed. The methanol was distilled off in vacuum and the residue was diluted with water to 50 ml and extracted with ethyl acetate (2 × 10 ml). The solvent was blown off with air, and 1.5 ml of glacial acetic acid was added. The peptide that deposited was filtered off after 3 h, washed with water and with ether, and dried in vacuum over $CaCl_2$ and then over P_2O_5. This gave 0.80 g (91%) of (I), $C_{29}H_{35}N_5O_6$, mp 216-217°C [3], $[\alpha]_D^{20}$ −27.3 (c 1, 0.1 N NaOH); −15.2 (c 1, DMFA), R_{f_1} 0.15; R_{f_2} 0.44; R_{f_5} 0.95.

B. Histidylleucine (1.1 g, 4.1 mmole) and 1 g of $NaHCO_3$ were dissolved in 10 ml of water, and 10 ml of isopropanol was added, and then, with stirring a solution of the N'-hydroxysuccinimide ester of benzyloxycarbonylphenylalanine (2.0 g; 5 mmole) in 10 ml of dioxane was added over 1.5 h. The mixture was stirred for 12 h, the alcohol and dioxane were distilled off in vacuum, the residue was diluted with a 5% solution of $NaHCO_3$ to 70 ml and extracted with ethyl acetate (20 ml), and the peptide was isolated as in experiment A. This gave 2.1 g (90%) of (I) with mp 202-206°C; for purification, it was triturated in hot ethanol. Yield 1.7 g (75.5%), mp 214-216°C, R_{f_1} 0.17.

Preparation of the Hydantoin (X). A. A solution of 1.42 g of the ester (Ia) in 10 ml of dioxane, 3 ml of methanol, and 2.5 ml of 2 N NaOH was stirred at 20°C for 4 h. Then it was evaporated, and the residue was diluted with water (30 ml), extracted with ethyl acetate, and acidified to pH 4 with 1 N HCl. The precipitate was filtered off, washed with water, and dried in vacuum over $CaCl_2$. This gave 880 mg of substance (X), $C_{22}H_{28}N_5O_5$, with mp 218-219°C, R_{f_1} 0.00; R_{f_2} 0.25.

B. A solution of 0.3 g of (I) in a mixture of 2.5 ml of dioxane, 1 ml of methanol, and 1.5 ml of 2 N NaOH was stirred at 20°C for 6 h. The reaction mixture was evaporated in vacuum, the residue was dissolved in water (30 ml), and the solution was acidified with acetic acid. The precipitate was filtered off, washed with water, and dried. This gave 0.15 g (64%) of substance (X) with mp 219-220°C, R_{f_5} 0.30.

SUMMARY

It has been shown that in the synthesis of the peptide Z-L-Phe-L-His-L-Leu by various methods, the best results are obtained by extending the peptide chain from the C-end by the method using activated N-hydroxysuccinimide esters.

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