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N^{α},N^{im}-DI-tert-ALKOXYCARBONYL DERIVATIVES OF HISTIDINE

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The acylation of histidine with di-tert-butyl and di-tert-amyl pyrocarbonates has given the corresponding N^{α},N^{im}-di-tert-alkoxycarbonyl derivatives. The N^{α},N^{im}-di-tert-butoxycarbonyl derivative was obtained in the crystalline form by crystallization from benzene or carbon tetrachloride, or in the form of salts with cyclohexylamine, dicyclohexylamine, and diethylamine. N^{α},N^{im}-di-tert-amyloxycarbonylhistidine was characterized in the form of the salt with dicyclohexylamine.

In recent years, N^{α},N^{im}-di-tert-butoxycarbonylhistidine (di-Boc-histidine) has been finding ever wider use in the synthesis of histidine-containing peptides. It has been shown that this histidine derivative can be used successfully both in the classical variants of peptide synthesis [1-6] and in synthesis on a polymeric support [7-10]. The well-known methods of the selective elimination of N^{α}- or N^{im}-protective groupings are expanding the possibilities of the use of this compound even further [2, 11-13].

Di-Boc-L-histidine was first obtained by E. Schnabel et al. in 1968 [1] by acylating histidine with Boc fluoride. Later, Boc azide [2, 7, 12-14] and Boc chloride [8] were used with less satisfactory results for obtaining this compound. A variant of the synthesis of di-Boc-histidine starting from the benzyl ester of histidine, which was aclyated with Boc azide followed by the removal of the benzyl group by hydrogenation, has also been studied [8]. But, apparently, the most effective reagent for obtaining di-Boc-histidine has proved to be di-tert-butyl pyrocarbonate [15], since under mild conditions, with the minimum consumption of time and of the tert-butoxycarbonylating reagent, the desired product can be obtained in practically quantitative yield. However, in all the investigations mentioned the di-Boc-histidine was obtained either in the form of an oil or in the form of a dry film and was characterized by a low stability on storage.

In the process of studying the conditions for the synthesis of Boc derivatives of trifunctional amino acids using Boc_2O [16] we observed a capacity of di-Boc-histidine for crystallizing from benzene and carbon tetrachloride [17, 18]. Approximately 5% solutions of di-Boc-histidine are the most convenient for obtaining crystalline products. In this case, crystallization sometimes begins at room temperature, but sometimes this requires the solutions to be kept in the refrigerator. On studying the composition of the crystalline products, it was found that, even after being washed with petroleum ether and dried in vacuum, they continued to retain considerable amounts of benzene or carbon tetrachloride. Freshly-prepared samples of crystalline di-Boc-histidine obtained by crystallization from benzene, washed on the filter with petroleum ether, and dried in a vacuum desiccator over $CaCl_2$ for 16 h consist, according to PMR spectroscopy (solutions in deuterochloroform) of a solvate with benzene in a ratio of 1:1. In the IR spectrum, the benzene of crystallization is revealed only by an absorption band at 690 cm^{-1}, while the remaining bands do not appear or are masked. The solvent in the solvated crystal can be detected easily and reliably by gas chromatography (see the experimental part). To determine the amount of the main substance in the solvates it is possible also to use UV spectroscopy, since N^{im}-acylated histidine

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derivatives have a characteristic absorption maximum at 230-240 nm with an absorption coefficient of 3600 [19-21].

The capacity for crystallization of di-Boc-histidine does not depend on the configuration of the asymmetric atom. Di-Boc-D-histidine and di-Boc-DL-histidine were obtained in approximately the same yields on crystallization from benzene as di-Boc-L-histidine.

N^{α},N^{im}-di-tert-amyloxycarbonyl-L-histidine, obtained by acylating L-histidine with di-tert-amyl pyrocarbonate could not be obtained in the crystalline form from any solvent.

Another possibility for obtaining di-tert-alkoxycarbonyl derivatives of histidine in the crystalline form consists in their conversion into salts with amines. Repeated attempts have been made to obtain the salt of di-Boc-histidine with dicyclohexylamine (DCHA) [7, 9, 11, 14], but the salt crystallized with difficulty and in low yields. It was found that di-Boc-L- and -D-histidines obtained with the aid of Boc_2O form with DCHA a salt which readily crystallizes from diethyl ether with a yield greater than 90%. The salt of di-Boc-L-histidine with cyclohexylamine crystallizes just as easily, and that with diethylamine with somewhat greater difficulty. It is important that the yields of the crystalline salts of di-Boc-histidine with amines are higher than on its crystallization from benzene or carbon tetrachloride.

It has also been possible to convert di-tert-amyloxycarbonyl-L-histidine into a crystalline salt with DCHA by triturating a mixture of the components in hexane.

The salts of di-Boc-histidine with DCHA and CHA are completely stable on storage, while the stability of the salts with diethylamine is somewhat lower. The regeneration of the di-Boc-histidine from the salts with amines is done by the usual method (distribution in the ethyl acetate—dilute acid system) with a yield of 80-90%.

The nature of the UV spectra of di-Boc-histidine scarcely changes as the result of salt-formation with amines, except that the absorption maximum (in methanolic solution) shifts from 238 nm to 241 nm.

For N^{im}-substituted histidine derivatives a question of practical importance is the position of the substitutent in the imidazole ring, since there is information that in some cases the N^{τ} and N^{π} isomers [22] may differ in their capacity for racemization in the process of peptide synthesis [23]. It is known that the N^{τ} and N^{π} isomers of a N^{im}-substituted histidine differ in the magnitudes of the chemical shifts in their PMR spectra, and the spin—spin coupling constant of the proton at C-2 with the nucleus for the N^{τ} isomer is greater than 1 Hz and for the N^{π} isomer it is less than 1 Hz [24, 25].

In the PMR spectrum of di-Boc-histidine obtained by crystallization from benzene, the proton at C-2 is revealed by a narrow signal having a constant of coupling with a proton of the nucleus equal to 1.25 Hz. In the PMR spectrum of the DCHA salt of di-Boc-histidine, the proton at C-2 is also revealed by a signal with the same spin—spin coupling constant. These results permit us to consider that in the acylation of histidine with Boc_2O only N^{α},N^{τ}-di-Boc-histidine is formed. However, the question of the ratio of the isomers in a N^{im}-substituted histidine is fairly complex and deserves special study. Consequently, the results obtained should be considered as preliminary, requiring confirmation and refinement.

EXPERIMENTAL

Reanal L-histidine, Sigma D-histidine, and Soyuzreaktiv DL-histidine were used. The di-tert-butyl and di-tert-amyl pyrocarbonates were synthesized from the corresponding alkyl carbonate salts and trichlorcacetyl chloride [26]. The compositions of the reaction mixtures, the processes of isolation and purification, and the individuality of the compounds obtained were checked by thin-layer chromatography on Silufol plates. The R_f values are given in the benzene—methyl ethyl ketone—acetic acid (100:50:1) system. Melting points were determined in open capillaries and are uncorrected. IR spectra were taken on a Pye-Unicam SP-1000 spectrometer, UV spectra on a Specord spectrometer, and PMR spectra on a Varian XL-100A instrument. Angles of optical rotation were determined on a Perkin-Elmer 241 polarimeter.

To determine the amounts of benzene or carbon tetrachloride in the solvates of di-Boc-histidine by gas chromatography, we used a steel column 2 m × 4 cm containing 5% of XE-60 on Chromosorb G. The temperature of the evaporator was 150°C, that of the column 50°C, and that of the katharometer 200°C. The carrier gas was helium at the rate of 50 ml/min. A weighed

sample of solvate (5-10 mg) was dissolved in 0.1 ml of solvent (xylene in the determination of benzene, toluene in the determination of CCl_4), 1 μl of standard (toluene and benzene, respectively), and 0.5-1 μl of solution were introduced into the evaporator. The retention time of CCl_4 was 1 min, of benzene 1.5 min, and the tolene 2.5 min.

Extracts were dried with anhydrous sodium sulfate and evaporated in a rotary evaporator in vacuum at 40°C.

Amorphous N^{α},N^{im}-Di-Boc-histidine. A solution of 3.1 g of L-histidine and 3 g of K_2CO_3 in 20 ml of water and 10 ml of isopropanol was treated with 10 ml of Boc_2O, and the mixture was stirred at 30-40°C for 1-1.5 h. Then it was diluted with water to ∿80 ml and was extracted with a mixture of ether and petroleum ether (1:1, 20 ml), after which the aqueous solution was treated with 30ml of benzene, acidified with a solution of ∿15 g of citric acid in the minimum amount of water, and extracted with benzene (30 + 2 × 15 ml), and the extracts were washed with water and with NaCl solution, dried and evaporated, until the residue formed a dry foam. This gave 7-7.5 g of chromatographically homogeneous product with R_f 0.35. IR spectrum (in KBr), cm^{-1}; 1760, 1710 (C=O), 1510 (NH).

Crystalline Di-Boc-L-histidine from Benzene. The amorphous product from the preceding experiment was dissolved in 25-30 ml of benzene and the solution was left at 5-6°C until crystallization began. When a seed was introduced, crystallization began after 3-5 h. The crystallizing mixture was diluted with 40 ml of cyclohexane (or petroleum ether), and after an hour the precipitate was filtered off, washed with petroleum ether, and dried in vacuum over silica gel. This gave 6.7 g (77.3%) of di-Boc-L-HisOH·C_6H_6 with decomp. p. 82-84°C, $[\alpha]_D^{20}$ +12.5° (c, 1, CH_3OH). R_f 0.35. UV spectrum (CH_3OH): λ_{max} 238 nm (ε 3660). IR spectrum (KBr), cm^{-1}: 3460 (NH), 3000, 2960 (CH), 1760, 1710 (C=O), 1510 (NH), 1270, 1175 (tert-Bu), 690 (C_6H_6). PMR spectrum ($CDCl_3$, 20°C, TMS, δ, ppm): 1.50 s, 9 H (Boc), 1.60 s, 9 H (Boc), 3.26 d, 2 H (CH_2), 4.48 m, 1 H (CH), 5.46 m, 1 H (NH), 7.18 s, 1 H (HC-5), 7.36 s, 6 H (C_6H_6), 8.16 s, 1 H (HC-2), $J_{2,5}$ = 1.25 Hz. According to GLC results, the benzene content was 18%.

Di-Boc-D-HisOH·C_6H_6. Yield 72%, decomp. p. 85-87°C, $[\alpha]_D^{20}$ −14° (c 1; CH_3OH).

Di-Boc-DL-HisOH·C_6H_6. Yield 71.5%, decomp. p. 86-87°C.

Crystalline Di-Boc-L-histidine from Carbon Tetrachloride. The reaction of histidine with Boc_2O was carried out in the same way as in the preparation of the amorphous di-Boc-histidine, but the product was extracted with carbon tetrachloride (3 × 20 ml). The extract was washed with water and with NaCl solution, was dried for ∿1 h (with prolonged drying, the product may crystallize on the desiccant), concentrated to ∿30 ml, and allowed to stand at 5°C. After 10-20 h, the mixture was diluted with petroleum ether, and the precipitate was filtered off, washed with petroleum ether, and dried in vacuum. This gave 8.0 g of di-Boc-L-HisOH·CCl_4 with decomp. p. 88-90°C, $[\alpha]_D^{20}$ +15° (c 1; CH_3OH). UV spectrum (CH_3OH): λ_{max} 238 nm. IR spectrum (in KBr), cm^{-1}: 3480 (NH), 3000, 2940 (CH), 1760, 1710 (C=O), 1500 (NH), 1270, 1175 (tert-Bu). According to GLC, CCl_4 found, 27%; calculated, 31%.

In the preparation of amorphous di-Boc-histidine, the extraction of the product can be done with ethyl acetate. After the usual washing, drying, and evaporation of the solvent, the residue was dissolved in CCl_4 and the solution was evaporated until the residue formed a foam, and this was dissolved in CCl_4 and the solution was left for crystallization. The yield and quality of the crystalline product obtained by this variant were similar to those given above.

Dicyclohexylammonium Salt of Di-Boc-L-histidine. The amorphous di-Boc-L-histidine obtained from 1.55 g of L-histidine and 5.5 ml of Boc_2O (the product was extracted with ethyl acetate) was dissolved in 25 ml of dry ether, and 2 ml of dicyclohexylamine was added. After 15-20 min, the mixture had become converted into a dense crystalline mass, which was diluted with 30 ml of petroleum ether and left at 5°C for 1-1.5 h. The precipitate that had formed was filtered off, washed with petroleum ether, and dried in vacuum over P_2O_5 and paraffin wax. This gave 5.2 g (94%) of di-Boc-L-HisOH-DCHA, $C_{28}H_{48}N_3O_6$, mp 161-162°C [7, 14], $[\alpha]_D^{20}$ +27° (c 1; CH_3OH). R_f 0.33. UV spectrum (in CH_3OH): λ_{max} 241 nm (ε 3600). IR spectrum (in KBr), cm^{-1}: 3360 (NH), 3000, 2900 (CH), 1740, 1705, 1650, 1570, 1535, 1500, 1270, 1175. PMR spectrum ($CDCl_3$, 20°C, TMS, δ, ppm): 1.1-1.3, 2 OH (cyclohexyl), 1.38 s, 9 H (Boc), 1.56 s, 9 H (Boc), 2.75-3.10 m, 2 H (cyclohexyl CH), 3.43 m, 2 H (CH_2), 4.19 m, 1 H (CH), 5.57 m, 1 H (NH), 7.14 s, 1 H (HC-5), 7.87 s, 1 H (HC-2), $J_{2,5}$ = 1.25 Hz.

Di-Boc-D-HisOH-DCHA. Yield 81%, mp 156-157°C, $[\alpha]_D^{20}$ −26° (c 1; CH_3OH), $[\alpha]_D^{20}$ −18.3° (c 1; $CHCl_3$) [9].

Di-Boc-DL-HisOH-DCHA. Yield 79%, mp 155-155.5°C.

Cyclohexylammonium Salt of Di-Boc-L-histidine. An extract of di-Boc-L-histidine in 50 ml of benzene was obtained from 1.55 g of L-histidine and 5.5 ml of Boc_2O by the method described above. After drying, the solution was evaporated to half-volume and 1 ml of cyclohexylamine was added to the residue, whereupon a crystalline precipitate formed immediately. The mixture was diluted with ether (25 ml), petroleum ether was added, and the precipitate was filtered off, washed with hexane, and dried in vacuum. This gave 4.0 g (88%) of the cyclohexylammonium salt of di-Boc-L-histidine, $C_{22}H_{38}H_4O_6$, with mp 140-142°C, $[\alpha]_D^{20}$ +38° (c 1; CH_3OH), R_f 0.33. UV spectrum (in CH_3OH): λ_{max} 241 nm (ε 3600).

Diethylammonium Salt of Di-Boc-L-histidine. A solution of 4 g of amorphous di-Boc-L-histidine in 20 ml of ether cooled to 0°C was treated with 1.2 ml of diethylamine, and the mixture was left at 0°C. After 2 h, the crystalline precipitate was filtered off, washed with ether, and dried in vacuum over H_2SO_4. This gave 3.7 g (84%) of the crystalline diethylammonium salt of di-Boc-L-histidine, $C_{20}H_{36}N_4O_6$, mp 115-116°C, $[\alpha]_D^{20}$ +46° (c 1; CH_3OH). UV spectrum (in CH_3OH): λ_{max} 241 nm (ε 3600).

Dicyclohexylammonium Salt of Di-tert-amyloxycarbonyl-L-histidine. To a solution of 0.16 g of L-histidine and 0.2 g of K_2CO_3 in 2 ml of water and 4 ml of isopropanol was added 0.52 ml of di-tert-amyl pyrocarbonate, and the mixture was stirred at 30-35°C for 1 h. After the usual working up, di-tert-amyloxycarbonyl-L-histidine was obtained in the form of a resin This resin was dissolved in 10 ml of ether, 0.2 ml of dicyclohexylamine was added, the solution was evaporated, and the residue was triturated in small portions of hexane until it had crystallized completely. The precipitate was filtered off, washed with hexane, and dried in vacuum. This gave 0.48 g (86%) of the dicyclohexylammonium salt of di-tert-amyloxycarbonyl-L-histidine, $C_{30}H_{52}N_4O_6$, mp 119-120°C, $[\alpha]_D^{20}$ +39° (c 1; C_2H_5OH). R_f 0.45.

Isolated of Free Di-Boc-histidine from its Salts with Amines. A suspension of 3.7 g of the dicyclohexylammonium salt of di-Boc-L-histidine in 30 ml of ethyl acetate was washed in a separatory funnel with 0.5 N H_2SO_4 solution (3 × 15 ml) with water, and with NaCl solution, and then the solution was dried and evaporated in vacuum. THis gave 2.7 g of di-Boc-L-histidine in the form of a dry foam. R_f 0.35.

N. P. Potapova recorded and interpreted the PMR spectra.

SUMMARY

Methods are proposed for obtaining N^{α},N^{im}-di-tert-alkyloxycarbonyl derivatives of histidine in the crystalline form by crystallizing di-Boc-histidine from benzene or carbon tetrachloride or by converting di-Boc-histidine, and also di-tert-amyloxycarbonyl-L-histidine, into crystalline salts with amines.

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CHEMICAL CHARACTERIZATION OF THE CRYSTALLINE PROTEINS OF *Bac. thuringiensis*. CLEAVAGE WITH CYANOGEN BROMIDE

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The crystalline proteins from *Bac. thuringiensis* of the third and fourth serotypes with a molecular weight of 130,000 have been isolated and carboxymethylated, and their amino acid compositions has been determined. The CM-proteins have been found to be labile in acid media (80% CF_3COOH and 65% HCOOH). Conditions have been found for the cleavage of the CM-protein of the third serotype with cyanogen bromide. A BrCN fragment with a molecular weight of 16,000 has been isolated, the intensity of which in 5% polyacrylamide gel on scanning amounts to about half the intensity of the sum of all the fragments. The amino-acid composition of this fragment has been determined.

The crystalline protein formed by *Bac. thuringiensis* during sporulation is pathogenic for a number of insects and is therefore of great interest for chemical and biochemical investigations. In the majority of cases (this form of bacterium consists of 13 serotypes) the crystalline inclusions consist of a single protein with a molecular weight of 130-140 thousand daltons [1]. According to available literature information [2-4], these proteins differ little from one another in amino acid composition, but, nevertheless, the toxicities of the crystals of the different serotypes have not a broad, but a restricted action spectrum. The interrelationship between the features of the chemical structure and the biological activity of these proteins is the most interesting aspect of the study of the entomopathogenic crystalline inclusions of *Bac. thuringiensis*.

We have investigated the third and fourth serotypes, the crystals of which consists of a single protein with a molecular weight of 135 thousand daltons. After three days' growth at 28°C, the culture liquid was centrifuged, and the precipitate was treated with 0.1 N Na_2HPO_4 containing 8 M urea and 10^{-2} M mercaptoethanol for 7 min in the boiling water bath. The insoluble fraction was separated off by centrifuging. The protein present in the solution was carboxymethylated with the aid of iodoacetic acid. As can be seen from the results of electrophoresis in 5% polyacrylamide gel (Fig. 1a, b), the CM-protein isolated from the biomass contained as impurity a very small amount of a protein with a molecular weight of 23 thousand daltons (the accurate molecular weight and percentage of this impurity were not determined) and only in this way did it differ from the CM-protein obtained from the pure crystals.

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