INVESTIGATIONS ON THE STABILITY OF THE N^{im}-BOC* PROTECTING GROUP OF HISTIDINE

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(Received January 10, 1973)

The optimal conditions of synthesizing N^{α},N^{im}-di-*t*-butyloxycarbonyl-histidine were elaborated and the stability of the N^{im}-BOC protecting group was studied under the conditions of solid phase peptide synthesis. According to our results, the conditions for using this protecting group are rather restricted.

In the solid phase peptide synthesis, the nitrogen of the histidine imidazole has to be protected in a suitable way. The protecting group should satisfy the following requirements: ready availability, stability to the removal conditions of the N-protecting groups in the solid phase peptide synthesis, and selective removal of the protecting group without racemization.

YAMASHIRO *et al.* [1] used N^{α}-BPOC-N^{im}-BOC histidine and N^{α},N^{im}-di-BOC-histidine (**II**) for synthesizing a sequence of human growth hormone (HGH). As the literature gives contradictory data concerning the synthesis of **II** and the stability of the N^{im}-BOC protecting group, it seemed justified to clear up these problems in a series of experiments.

According to literature [1—3], histidine and *t*-butyloxy-carbonyl-azide were brought into reaction, giving the following results under the conditions used:

a.) $\underset{\text{1 mmole}}{\text{H—His—OH}} + \underset{\text{2 × 2 mmole}}{\text{BOC—N}_3} \xrightarrow[\text{pH 10}]{\text{20 °C, 48 hours}} \underset{\text{I, 92\%}}{\text{BOC—His—OH}}$ [2]

b.) $\underset{\text{1 mmole}}{\text{H—His—OH}} + \underset{\text{4 mmole}}{\text{BOC—N}_3} \xrightarrow[\text{pH 8.4—9}]{\text{45 °C, 10 hours}} \underset{\text{II, 23\%}}{\text{BOC—His(BOC)OH}}$ [1]

c.) $\underset{\text{1 mmole}}{\text{H—His—OH}} + \underset{\text{3 mmole}}{\text{BOC—N}_3} \xrightarrow[\text{MgO}]{\text{20 °C, 4 days}} \underset{\text{II, 50\%}}{\text{BOC—His(BOC)OH}}$ [3]

Our experiments showed that the divergences between the quoted data could be explained by the sensibility of the N^{im}-BOC protecting group to higher temperatures and to basicity of the medium. Under the conditions given in [2] **II** always forms in small quantities, but at the pH value used it hydrolyses to **I**. Indeed, **II** can be obtained with the best yield by the method of FRIDKIN [4] in consequence

* The abbreviations used conform with the standards of IUPAC-IUB. BPOC=biphenyl-isopropyl-oxycarbonyl.

of the comparatively low temperature (20 °C) and the low basicity of the medium (MgO). YAMASHIRO's method gives **II** with a yield of only 20—25%; thus the increased temperature and basicity prove to be unfavourable for the reaction.

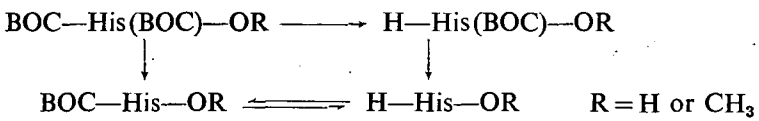
Similarly contradictory data are found in literature concerning the synthesis of **III** and **IV**. SCHRÖDER and GIBIAN[5] considered the oil obtained by the reaction of histidine methylester and BOC-azide as pure **III**, because it transforms with hydrazinehydrate into homogeneous BOC-histidine-hydrazide. According to WÜNSCH and ZWICK [6] the product formed by the reaction of histidine methylester and 2 equiv. BOC-azide consists of a mixture of **III** and **IV**, which they were able to separate by fractional crystallization. Our results show — in good accordance with HANFORD *et al.* [7] — that by the reaction of histidine methylester and 1 equiv. BOC-azide both **III** and **IV** are formed; they can be easily separated by column chromatography on silicagel.

After synthesizing compounds **I—IV**, we have studied the stability of both BOC-protecting groups of histidine under various reaction conditions. Our results can be summarized as follows:

1a). Trifluoroacetic acid (100%, 90% and 50% in CH_2Cl_2) splits both N^{α}- and N^{im}-BOC protecting groups of histidine quantitatively, without by-products, in 10 to 15 min.

1b). 2*N* HBr in acetic acid splits both N^{α}- and N^{im}-BOC groups totally in 10 to 15 min, giving, however, significant quantities of N^{im}-acetyl-histidine methylester from **III** and **IV** as by-product. Addition of 20 to 30% water to the reaction mixture prevents this acetylation.

1c). The N^{α}-BOC group of **I—IV** is cleaved with *N* HCl in acetic acid in about 60 min. The cleavage of the N^{im}-BOC protecting group of **II** and **IV** is only partial in this time, but from **II** also **I**, from **IV** also **III** are formed and the cleavage reaction is not completed even in 24 hours. The following reactions and equilibrium may be supposed:



Formation of N^{im}-BOC-histidine and N^{im}-BOC-histidine methylester can be demonstrated by thin layer chromatography.

2a). By hydrolysis both **III** and **IV** are converted into **I**. The N^{im}-BOC group is sensible to bases to a degree, that in a buffer solution of pH 9.2 **IV** transforms quantitatively into **III** in an hour. (At pH 8 no hydrolysis of the N^{im}-BOC group occurs.)

2b). The N^{im}-BOC protecting group is also cleaved by primary amines. Thus from **IV** with equiv. benzylamine at 20 °C **III** and about 5% N-benzyl-carbamic acid *t*-butylester, with equiv. *n*-butylamine **III** and 10% N-*n*-butyl-carbamic acid *t*-butylester are formed. Amino acid esters and amino acid amides split N^{im}-BOC group very slowly. **IV** transforms with NH_3 in methanol into N^{α}-BOC-histidine amide, with hydrazine hydrate to BOC-His-N_2H_3 [5, 6], by splitting off the N^{im}-BOC protecting group.

3. The N^{im}-BOC group is sensitive to higher temperatures, so about the half of **IV** transforms into **III** in 10% methanolic solution by refluxing for 1 hour. This,

as well as the sensitivity to bases mentioned above, may explain the failure in synthesizing **III** and **IV**, and the unexpectedly low yields [1]. Summing up our results, it can be stated that N^{α},N^{im}-di-*t*-butyloxycarbonyl-histidine (**II**) can be obtained with good yield only at room temperature and at pH 7.5—8.5. The cleavage of the N^{α}-BOC group is not selective, acidolysis also splits the N^{im}-BOC group partially or totally. This group is sensitive to nucleophilic attack, too, therefore under the reaction conditions of the solid phase peptide synthesis — during and after the neutralization step — it can react also with the N-terminal amino group by transforming it to a BOC-product. A further problem in the solid phase peptide synthesis is the forming of N^{im}-aminoacyl derivatives of histidine, mentioned in [1], as the N^{im}-BOC group is split by any kind of acidolysis step. Therefore the N^{α}-BOC-N^{im}--trityl-histidine [2] and/or N^{α}-BOC-N^{im}-dinitrophenyl-histidine [8] are to be used for synthesizing the ACTH sequence 5—10. This synthesis shall be dealt with in a further paper.

Experimental

Melting points were measured by a BOETIUS "Mikroheiztisch"; the values given are uncorrected. IR spectra were measured with a Unicam SP 200 spectrophotometer.

For thin layer chromatography Silicagel G (Merck) adsorbent was applied in a layer thickness of 0.25 mm. The following solvent systems were used:

System A.: *n*-butanol—acetic acid—water 4:1:1
System B.: ethylacetate—pyridine—acetic acid—water 60:20:6:11

N^{α}-t-Butyloxycarbonyl-L-histidine (**I**) *and N^{α},N^{im}-di-t-butyloxycarbonyl-L-histidine* (**II**)

a) From 24.2 g (100 mmole) L-histidine hydrochloride by the method of FRIDKIN [4] 14.2 g (40 mmole) **II**, m.p. 155—157 °C (DCHA-salt) was obtained. In this reaction also **I** forms; it can be separated from **II** by chromatography on silicagel column, with system B as solvent.

b) By the method of YAMASHIRO [1], from 15.5 g (100 mmole) L-histidine, 9.05 g (25%) **II** was obtained; m.p. 154—157 °C (DCHA-salt). From the aqueous phase, after evaporation (pH 5.6) and extraction with ethanol, 3.5 g (13.7%) **I** could be separated, m.p. 178—180 °C.

c) The method of LOSSE [2] yielded 9.1 g (51.2%) **I** from 7.5 g (50 mmole) L-histidine.

N^{α}-t-Butyloxycarbonyl-L-histidine methylester (**III**) *and N^{α},N^{im}-di-t-butyloxycarbonyl L-histidine methylester* (**IV**)

III and **IV** were synthesized as described by SCHRÖDER and GIBIAN [5], from 48.4 g (200 mmole) L-histidine methylester-dihydrochloride. A mixture of **III** and **IV** was obtained in the form of an amorphous solid; the components were separated by chromatography on silicagel column. 13.0 g (17.6%) **IV** was eluated with $CHCl_3$—acetone 7:3, and then 12.6 g (24%) **III** with $CHCl_3$—methanol 9:1. **III**: m.p. 121—122 °C, ν_{max}^{KBr} 3460 (NH), 2970 (CH), 1750 and 1700 (C=O), 1680 (urethane), 1390 and 1360 (*t*-butyl), 1150 cm^{-1} (CO) (broad) R_f^A 0.50, R_f^B 0.75. Anal.: $C_{12}H_{19}N_3O_4$

(269.32); Calc.: 53.52 H 7.11 Found: C 53.30 H 6.93. **IV**: m.p. 127—128 °C, ν_{max}^{KBr} 9390 (NH), 2980 (CH) 1760 and 1710 (C=O), 1695 (urethane) 1385 and 1340 (*t*-butyl) 1160 (broad) (CO) cm^{-1}; R_f^A 0.65, R_f^B 0.95. Anal.: $C_{17}H_{27}O_3N_6$ (369.43) Calc.: C 55.27 H 7.37 Found: C 55.52 H 7.14.

Reaction of compounds **I—IV** *with trifluoroacetic acid*

Compounds **I—IV** (50 mg each) were reacted with 1 ml trifluoroacetic acid (100%; 90% and 50%, respectively, in CH_2Cl_2). Samples were taken from the reaction mixtures in 5 min intervals and the cleavage of the protecting group determined by thin layer chromatography. After 10—15 min reaction time the BOC protecting groups of **I—IV** were quantitatively split off, evaporation of the mixtures and addition of ether gave from **I** and **II** histidine trifluoroacetate (hygroscopic crystals, R_f^A 0.10, R_f^B 0.05); from **III** and **IV** histidine methylester-di-trifluoroacetate (m.p. 112—115 °C, recrystallized from ethanol—ether, R_f^A 0.25, R_f^B 0.10).

Reaction of compounds **I—IV** *with 2N HBr in acetic acid*

a) 50 mg **I—IV** were reacted with 1 ml 2*N* HBr in acetic acid. The progress of the reaction was followed as described above. After 10—15 min the BOC protecting groups were split off. From **I** and **II**, beside histidine-hydrobromide (R_f^A 0.10, R_f^B 0.05), also a small quantity of N^{im}-acetyl-histidine (R_f^A 0.25, R_f^B 0.15); from **III** and **IV**, beside histidine methylester-dihydrobromide (m.p. 199—200 °C, R_f^A 0.20, R_f^B 0.10), a considerable amount of N^{im}-acetyl-histine methylester (R_f^A 0.50, R_f^B 0.40) were obtained. The acetylated histidine derivatives could be isolated from reaction mixtures by ethereal precipitation of the hydrobromides. Identification of the N^{im}--acetyl-histidine derivatives was based on the respective R_f values and IR spectra.

b) The same reaction in the presence of 30% water yielded from **I** and **II** only histidine-hydrobromide, from **III** and **IV** only histidine methylester-dihydrobromide; no traces of acetylated derivatives were found.

Reaction of compounds **I—IV** *with N HCl in acetic acid*

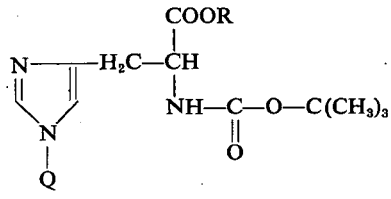
50 mg of **I—IV** was solved in 1 ml *N* HCl in acetic acid and the progress of the reaction followed by thin layer chromatography. In 60 min **I** was converted into histidine, **III** into histidine methylester, which could be isolated from the reaction mixtures as hydrochlorides. The N^{im}-BOC protecting group of **II** and **IV** was not totally cleaved in 60 min; after 24 hours standing from the reaction mixture of **II** histidine-hydrochloride (m.p. 255 °C, R_f^A 0.10, R_f^B 0.05) and **I**, from that of **IV** histidine methylester-dihydrochloride (m.p. 202—204 °C; R_f^A 0.25, R_f^B 0.10) and **III** could be isolated. As mentioned, the presence of some N^{im}-BOC-histidine and N^{im}-BOC-histidine methylester, respectively, could be detected by thin layer chromatography.

Reaction of **III** *and* **IV** *with NaOH*

1 mmole of **III** or **IV** was solved in 2 ml methanol and 1 ml NaOH was added. Thin layer chromatography of the reaction mixtures showed that both **III** and **IV** were converted into **I** in 15—20 min; this could separated from the reaction mixture with 85% yield by LOSSE's method [2].

Reaction of **III** *and* **IV** *with ammonia*

50 mg **III** or **IV** was treated with methanolic ammonia solution. In 2 hours the N^{im}-BOC group of **IV** was quantitatively cleaved and the methyl ester group partly converted into amide. After 24 hours standing, about 50% N^{α}-BOC-histidine--amide (R_f^B 0.30) could be separated from both reaction mixtures by fractional crystallization or silicagel column chromatography.



I Q=H, R=H
II Q=$(CH_3)_3C$—O—CO
R=H
III Q=H, R=CH_3
IV Q=$(CH_3)_3C$—O—CO
R=CH_3

Fig. 1. Histidine derivatives used for testing the stability of the protecting groups.

Reaction of **IV** *with primary amines*

50 mg **IV**, solved in 1 ml abs. dimethylformamide, was reacted with equivalent *n*-butylamine or benzylamine. After 2—3 hours the N^{im}-BOC-group of **IV** was cleaved and only **III**, beside a small quantity of N-benzyl-carbamic acid *t*-butyl ester or N-*n*-butyl-carbamicacid-*t*-butyl ester, could be found in the reaction mixture.

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ИССЛЕДОВАНИЕ СТАБИЛЬНОСТИ N^{im}-ТРЕТ-БУТИЛЬНОЙ ГРУППЫ ГИСТИДИНА

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Разработаны оптимальные условия синтеза N^{α},N^{im}-BOC-гистидина и исследована стабильность N^{im}-BOC защитной группы в условиях твердофазного синтеза. Нами было доказано, что эта защитная группа применяется только в определённых случаях.