

Effects of Boc-amino Acid Adsorption with Respect to Yield and Racemisation in the Merrifield Method

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The use of 2-hydroxy-1-naphthaldehyde in the Merrifield synthesis to determine the amino-groups opens possibilities for studying the reactions on the polymer in an easy way. In this work more detailed directions for amino-group determination are presented. The adsorption of α -*t*-butyloxycarbonyl(Boc)-amino acids to the aminoacyl polymer is examined. The polymer can be washed prior to adding dicyclohexylcarbodiimide and the yields are high in spite of that.

Racemisation is not detectable when using the adsorption effect for coupling (adsorption coupling) as studied with a new model peptide for testing the tendency for racemisation. When the adsorption coupling is carried out at low temperatures a considerable improvement in yield is found.

The synthesis of peptides by the solid-phase method has often failed owing to side-reactions and incomplete coupling. Since synthesis is fast new attempts can be made until the required product is obtained. The reasons for using this method of trial and error have been, to some extent, the lack of an easy method for showing the extent of the coupling reaction. The use of 2-hydroxy-1-naphthaldehyde¹ to determine the free amino groups provided a possibility of following the coupling process. Initial experiments showed that the course of the reaction was not particularly affected by dilution of the reactants. On that account it was suggested that the Boc-amino acid adsorbs to the amino acyl polymer prior to the coupling by dicyclohexylcarbodiimide. In connection with that, dicyclohexylammonium salts of *N*-blocked amino acids will be mentioned. The adsorption of Boc-amino acids to amino acyl polymer therefore seemed realistic and knowledge of the "salting-in"² process supported this hypothesis.

If the Boc-amino acids were adsorbed strongly enough to resist repeated washings with methylene chloride, the excess of Boc-amino acids could be washed off prior to coupling and used again. It should be mentioned here that triethylammonium groups have been found on the polymer.³ These groups would also bind Boc-amino acids.

The adsorption brings the carboxylic component closer to the amino group, with the possible consequence that the coupling could proceed directly without rearranging reactions. If so, intermediary azlactone formation that causes racemisation⁴ could be avoided.

We have studied the availability of the adsorbed Boc-amino acids prior to coupling and the tendency for racemisation of a new model. Furthermore we have examined the influence of the temperature on the adsorption.

MATERIALS AND METHODS

The Boc-amino acids were synthesized according to Schnabel.⁵ The dicyclohexylcarbodiimide, puriss, and 2-hydroxy-1-naphthaldehyde, purum, were bought from Fluka. The chloromethylated polystyrene was the Bio-Beads S. X-2, 200–400 mesh type. The solvents used were of pure and A. R. quality.

Reaction vessel. All reactions on polymers were carried out in a type of reaction vessel described by Kusch.⁶ To mix the reaction suspension containing analytical quantities of polymer (10–20 mg) the reaction vessel was rotated about its longitudinal axis at an angle of 45° to the horizontal (or vertical) plane.

Measurements. A Zeiss PM QII spectrophotometer was used for the absorption measurements and the polarimetric determinations were made on a Perkin-Elmer 141.

EXPERIMENTAL

(1) *2-Hydroxy-1-naphthalene-methylene-benzylimine.* 529 mg (4.9 mmoles) benzylamine and 853 mg 2-hydroxy-1-naphthaldehyde were dissolved in 10 ml absolute ethanol. The solution was left to stand for 30 min before water was added, whereupon the product solidified to a crystalline mass. After recrystallisation from ethanol/water 1.04 g (82%), m.p. 93–94°, was isolated. The molar absorptivity at 420 nm was 10 040 in 95% ethanol (Found: C 82.68; H 5.74; N 5.27. Calc. for C₁₈H₁₅NO: C 82.76; H 5.75; N 5.36).

(2) *tert.-Butyloxycarbonyl-L-leucyl-L-alanine-benzyl ester,* 1.60 g (4.9 mmoles), Boc-L-leucine-N-hydroxy-succinimide ester,⁷ 1.06 g (4.9 mmoles), L-alanine-benzylester hydrochloride, and 0.69 ml (4.9 mmoles) triethylamine were dissolved in 25 ml methylene chloride. After 90 min the product was isolated as an oil and used without further purification.

(3) *tert.-Butyloxycarbonyl-L-leucyl-L-alanine.* 1.77 g (4.5 mmoles) of the product (2) were dissolved in 75 ml 95% methanol. 200 mg 5% palladium on charcoal were added and after 60 min hydrogenation at 1.3 atm. the catalyst was removed. The oil, that was left upon vaporisation of the solvent, crystallised from diethylether/petroleum ether. Yield: 1.21 g, m.p. 132–133° [α]_D²⁵ = –32.6° (*c* = 1.01 in methanol) (Found: C 55.45; H 8.91; N 9.24. Calc. for C₁₄H₂₇N₂O₅: C 55.38; H 8.73; N 9.09).

(4) *Determination of the quantity amino groups.* The described procedure was used¹ with the modification that the reaction was carried out in a mixture of methylene chloride: absolute ethanol (1:1) instead of absolute ethanol. The described procedure was also applied to greater amounts of polymer (~1 g).

(5) *Adsorption coupling method.* Each mole of amino acid or peptide on the polymer was treated with 2–6 moles of Boc-amino acid, dissolved in methylene chloride (about 0.15–0.45 M) at room temperature for 4 h. After that the polymer was washed three times with methylene chloride, 1.5–2.0 moles of dicyclohexylcarbodiimide dissolved in methylene chloride (about 0.12–0.15 M) were poured over the polymer. The condensation reaction was interrupted after one hour and the polymer washed with methylene chloride and absolute ethanol. The unreacted amino groups were determined as described above.

(6) *L-Leucyl-D,L-alanyl-L-valine.* The tripeptide was synthesized on about 1.0 g valine-polystyrene (0.34 mmoles valine/g polymer) according to the standard method. The peptide was cleaved from the polymer by HBr(g)/trifluoroacetic acid:methylene chloride (1:1) treatment for 30 min. 137 mg crude product were chromatographed on a

column, 87.5 × 2 cm, Dowex 50-X4, 200–400 mesh, with 0.1 M pyridinium acetate buffer, pH 4.0. The elution rate was 46 ml/h and 9.0 ml fractions were collected. The distribution curve was obtained by boiling 200 μl aliquots with 1.00 ml ninhydrin reagent for 20 min. Before measuring the absorbance the solution was diluted with 5.00 ml 50 % ethanol (Fig. 1). The R_F -values for L-Leu-D-Ala-L-Val and L-Leu-L-Ala-L-Val on

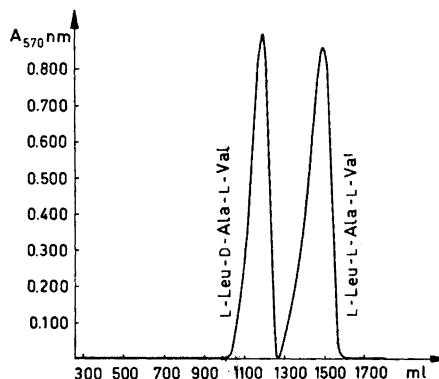


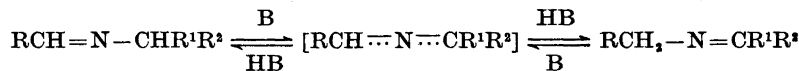
Fig. 1. The separation of L-Leu-L-Ala-L-Val and L-Leu-D-Ala-L-Val by ion-exchange chromatography. The column 87.5 × 2 cm was packed with Dowex 50-X4, 200–400 mesh, and eluted with 0.1 M pyridine-acetate buffer, pH 4.0. Elution rate was 46 ml/h and 9.0 ml samples were collected.

Merck TLC-plates, Silica gel F₂₅₄, using the system propanol:water (7:3) are 0.52 and 0.45, respectively. The amino acid compositions were 1.00:0.99:1.00 and 1.00:1.00:0.96, respectively, for valine:alanine-leucine.

(7) Separation of 2 % L-leucyl-D-alanyl-L-valine from L-leucyl-L-alanyl-L-valine. 37.0 mg L-Leu-L-Ala-L-Val and 0.8 mg L-Leu-D-Ala-L-Val were chromatographed on the same column as described under (6). The effluent rate was 44 ml/h and the fractions 9.00 ml. The distribution curve was obtained by treating 200 μl aliquots as described above. For calculating the area under the smaller peak, 1000 μl aliquots were taken instead of 200 μl ones and 3.00 ml 50 % ethanol added to dilute before measuring the absorbance. The L-Leu-D-Ala-L-Val amounted to 2.5 % of the total by integrating. According to the weighed amounts the corresponding figure is 2.1 %.

RESULTS AND DISCUSSION

Determination of free amino groups. The reaction of 2-hydroxy-1-naphthaldehyde with the free amino groups on the polymer is slow and to get reproducible values we have chosen 4 h as the minimum time for the reaction (Fig. 2). However, for a qualitative test 15–30 min reaction time is enough because even at low substitution the yellow colour change of the polymer can be observed. Racemisation can be expected by the transimination. Cram and Guthrie⁸ have showed that base catalysed tautomerism by Schiff's base with an intervening carbanion causes racemisation.



As the transimination step is carried out by using a great excess of benzylamine in methylene chloride the carbanion could be expected. It has been shown that 2-hydroxy-1-naphthalylidene-amines exist only as keto-amines.⁹ Accordingly the prototrophy would be eliminated and thereby the racemisation. Until

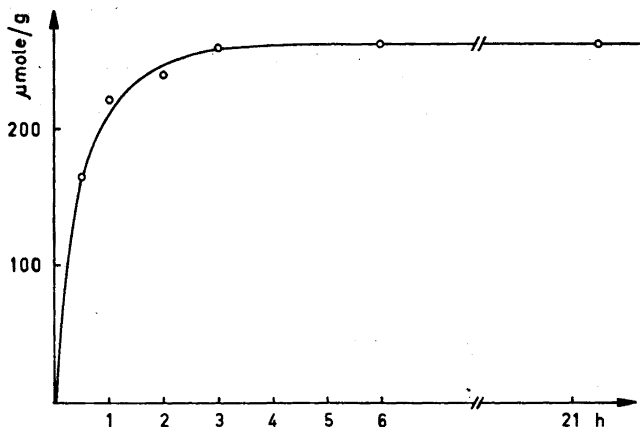


Fig. 2. The amount of free amino-groups per gram Ala-Val-polystyrene determined at different reaction times with 2-hydroxy-1-naphthaldehyde.

the tendency for racemisation caused by using 2-hydroxy-1-naphthaldehyde has been cleared up, the method must be used with discernment. It must be mentioned here that the possible racemisation caused by determining the amino groups with 2-hydroxy-1-naphthaldehyde in this work certainly does not influence the yields in the synthesis of the peptides or the degree of racemisation of the model because a second reaction, to increase the yield of Boc-Leu-Ala-Val-polystyrene was never made. The precision of the 2-hydroxy-1-naphthaldehyde method is high with a relative error of $\pm 3-4\%$. By using larger samples the relative error can be lowered.

Peptide synthesis by the adsorption coupling method. The peptide isoleucyl-leucyl-alanyl-valine was synthesized according to the adsorption coupling method. After each coupling step the content of unreacted amino groups was determined. The coupling proceeded in each step to 94% completion or

Table 1. The yields in percent by synthesizing Ile-Leu-Ala-Val-polystyrene by the adsorption coupling method.

	Polystyrene - Val - Ala - Leu - Ile		
First condensation	>99	94	98
Second condensation		98	

The yields are determined by 2-hydroxy-1-naphthaldehyde.^a Acetylation. The acetylation was carried out as described by Merrifield.¹⁰

more (Table 1). The yields clearly show that the polymer can be washed with methylene chloride prior to coupling with dicyclohexylcarbodiimide without lowering the yields to insignificance. The values in Table 2 also support that. In no sample test all but one equivalent of the Boc-amino acid

Table 2. The yields in percent by synthesizing Phe-Tyr-Ile-Ile-Ala-Val-polystyrene by the adsorption coupling method.

	Polystyrene—Val—Ala—Ile—Ile—Tyr—Phe				
First condensation	97	94	93	>99	>99
Second condensation	^a	^a	>99		

The yields are determined by 2-hydroxy-1-naphthaldehyde. ^a Acetylation. The acetylation was carried out as described by Merrifield.¹⁰

added was washed off after three washings for 15 min each with methylene chloride. These findings are based on differences in weight between Boc-amino acid added and regained. The excess amount of Boc-amino acids adsorbed can depend on the extra amino groups³ caused by the esterification. When the yields were less than 99 % the coupling step was repeated once. If, in spite of that more than 1 % amino groups were unreacted they were acetylated.¹⁰ The peptide was removed by hydrogen fluoride¹¹ and chromatographed on the column described under experimental (6) with the same buffer (Fig. 3). The amino acid analysis showed the ratio 1.04:1.00:1.02:0.98

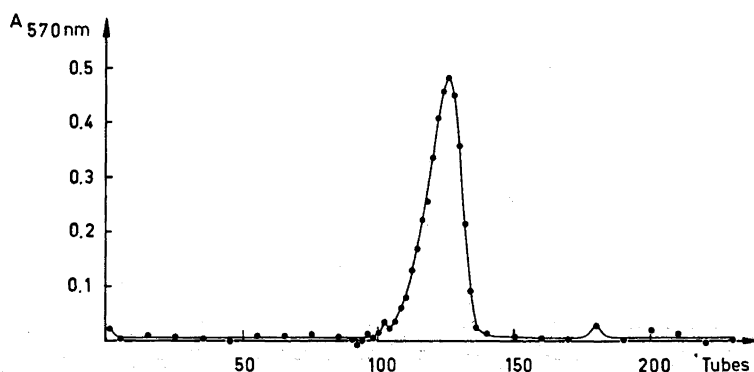


Fig. 3. The distribution on ninhydrin-positive products by chromatographing the HF¹¹ released peptide, Ile-Leu-Ala-Val, on ion-exchanger. The column 87.5 × 2 cm was packed with Dowex 50-X4, 200–400 mesh, and eluted with 0.1 M pyridine-acetate buffer, pH 4.0. Elution rate 48 ml/h and 9.6 ml samples were collected.

for valine:alanine:leucine:isoleucine. The synthesis of the hexapeptide phenyl-alanyl-tyrosyl-isoleucyl-isoleucyl-alanyl-valine was performed as described for the tetrapeptide. The sequence –Ile–Ile– in the hexapeptide did not cause any particular problem (Table 2). Lower yields have been reported for this intra chain sequence.¹² The standard method, when using a three-fold excess of Boc-isoleucine, gave a quantitative yield. Consequently the adsorption coupling method does not show any advantage with respect to yields in that case.

Tendency for racemisation with the adsorption coupling method. The diastereomers L-Leu-L-Ala-L-Val and L-Leu-D-Ala-L-Val can be separated completely

(Fig. 1) when chromatographed under conditions described under experimental. Thereby the tendency for racemisation could be studied in an easy way as no additional chemical reactions or special treatments had to be performed, after the peptide was removed from the polymer. When Boc-L-Leu-L-Ala was coupled to valine-polystyrene with dicyclohexylcarbodiimide by the standard method a 2% racemisation could be showed, *i.e.* lower than the values found by Weygand *et al.*¹³ in Leu-Phe-Val. The greater tendency for racemisation in Leu-Phe-Val can be explained by the electronegative substituent in the beta position of phenylalanine.¹⁴ The component appearing first in the chromatogram (Fig. 4) can be a third diastereomer

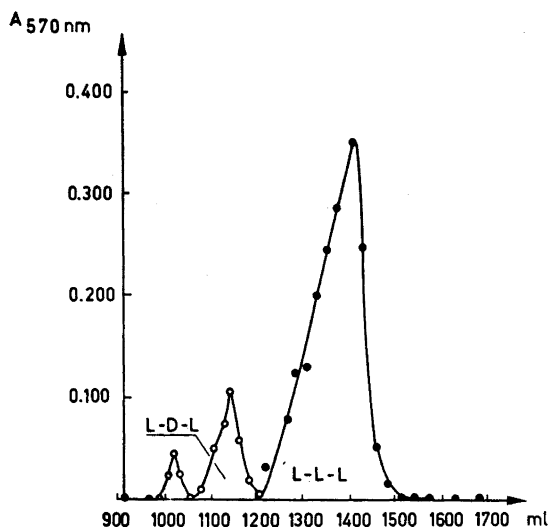


Fig. 4. The separation of L-Leu-L-Ala-L-Val, L-Leu-D-Ala-L-Val, and D-Leu-L-Ala-L-Val¹⁵ obtained by coupling Boc-L-Leu-L-Ala to Val-polystyrene by the standard method, by ion-exchange chromatography. The column, 87.5 × 2 cm, was packed with Dowex 50-X4, 200–400 mesh, and eluted with 0.1 M pyridine-acetate buffer, pH 4.0. Elution rate was 54 ml/h and 11.0 ml samples were collected. The first part of the curve (O) contains 6.2 times higher concentration of the sample than the second part (●).

D-Leu-L-Ala-L-Val¹⁵ and according to the work done on the racemisation of the preceding amino acid¹⁶ this speculation can be justified. When Boc-L-Leu-L-Ala was coupled by the adsorption method no ninhydrin-positive component could be shown where L-Leu-D-Ala-L-Val should appear. In the latter case even 2.5 times greater aliquots were used for the distribution curve. The yield of Boc-Leu-Ala-Val-polystyrene was 45–50%. The lack of racemised alanine in any detectable amounts with the adsorption coupling method can be explained thus. Owing to the adsorption the amino and carboxylic components are favourably orientated to each other for coupling. On the other hand the formation of azlactone is suppressed or completely eliminated for the same reason. If the adsorption coupling is carried out in

dimethylformamide the yield is nil, because the Boc-Leu-Ala is washed off and in addition the tendency for racemisation is greater in polar solvents.¹⁷ This supports our explanation, because in dimethylformamide the forces of adsorption are small and when using the standard method the carboxylic component is activated in the solution. Then azlactone formation with accompanied racemisation takes place.

Adsorption coupling at lower temperatures. When Boc-Ile is coupled to Val-polystyrene with the standard method the yields are not quantitative even after 21 h. After 60 min reaction the Boc-Ile-Val-polystyrene was obtained in an 84 % yield.¹ The adsorption coupling gives even lower yields than that, 65 % at room temperature. If the adsorption coupling is carried out at -12°C and the coupling step is prolonged by 1 h at $+4^{\circ}\text{C}$ the reaction goes to completion. Analogue experiments with Boc-Leu-Ala increased the yields by about 20 % to 65–70 %. It is also important that coupling is performed at low temperatures. When the polymer, after the adsorption and washing processes at -12°C , is divided into two parts and the coupling reaction is carried out at room temperature and at $+4^{\circ}\text{C}$ for each of them the yields are 42 and 65 %, respectively. These results clearly show the influence of temperature on the adsorption. The hydrogen bond seems to be responsible for the adsorption in spite of the dicyclohexylammonium salts of *N*-blocked amino acids. It is also known that the amino groups are protected by protonation.¹⁸

CONCLUSIONS

The results presented above show that the extent of the adsorption of the Boc-amino acids to amino acyl polystyrene is considerable. The polymer can be washed at least three times prior to coupling, that proceeds to give high yields. There are reasons for believing that the excess of Boc-amino acids can be regained as the coupling proceeds quantitatively even after washing when working at lower temperatures.

The tendency for racemisation is, at least in this case, not demonstrable, by using the adsorption effect. The reason for the lack of racemisation can be the unfavourable conditions for azlactone formation, where the orientation of the carboxylic group to the amino group is by adsorption.

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