SYNTHESIS OF OPTICALLY PURE AMINO ACID p-NITROANILIDES

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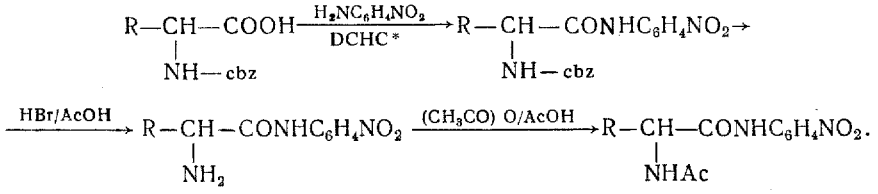
Recently, chromogenic substrates have been widely used for the study of enzymes [1]. In working with proteinases, amino acid nitroanilides have proved to be very satisfactory. They are used both in theoretical investigations and in medicine in the diagnosis of various diseases. A number of amino acid p-nitroanilides have been synthesized to determine the activity of trypsin, chymotrypsin, leucine aminopeptidase, and other enzymes [2, 3]. However, the synthesis of these compounds presents certain difficulties. It is a particularly complex matter to obtain optically pure and, at the same time, readily soluble substrates. For example, when a carbobenzoxy (cbz) residue is used to protect the amino group in the synthesis of amino acid p-nitroanilides the possibility of racemization is reduced but the solubility of the anilides decreases sharply. Nitroanilides of acetylamino acids are fairly readily soluble, but the use of a protective acetyl group during the synthesis is associated with a great danger of racemization [4].

M. M. Botvinik and E. V. Ramenskii have recently proposed the carbobenzoxy-acetyl method for synthesizing acetyl-D-phenylalanine p-nitroanilide (D-I) [5]. This method consists in the preparation of the p-nitroanilide of the cbz-amino acid with its subsequent conversion into the amino acid p-nitroanilide and acetylation of the latter with acetic anhydride in glacial acetic acid.

In the present work this method has been extended to the synthesis of acetyl-L-phenylalanine p-nitroanilide (L-I) and acetyl-L-leucine p-nitroanilide (L-II). Their preparation was effected by the following scheme.

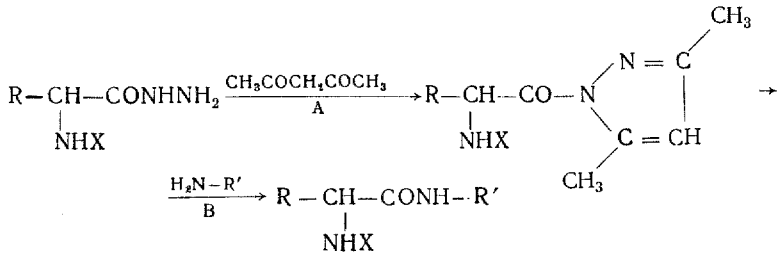


Compounds L-I and D-I had a high degree of optical purity, as was shown by two methods. The optical rotatory dispersion curves of compounds L-I and D-I proved to be symmetrical (figure).

It was also shown that L-I is completely hydrolyzed by chymotrypsin in a few hours. The enzymatic hydrolysis was monitored spectrophotometrically at 410 mμ. The sample of L-I did not contain D-I as an impurity, since D-I is scarcely hydrolyzed by chymotrypsin, and after 45 hr only fractional percentages of D-I are hydrolyzed. The small degree of hydrolysis of D-I is connected with the presence in it of traces of L-I. In a synthetic mixture containing 2% of L-I and 98% of D-I, the hydrolysis of the L-I takes place at a high rate and to completion. Consequently, D-I is practically unaffected by chymotrypsin and does not interfere with the hydrolysis of L-I.

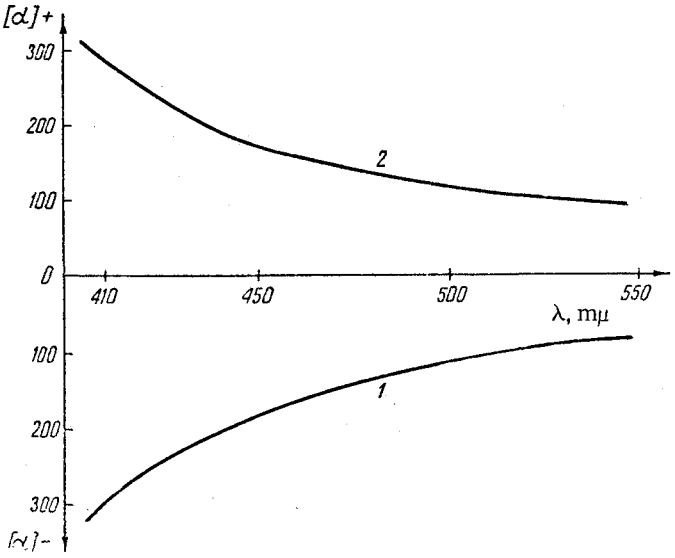
We attempted to use the pyrazole method to synthesize p-nitroanilides of acetylamino acids. This method was proposed by Ried for the synthesis of di- and tripeptides [6]. This author used cbz and tosyl protection; the specific rotation of the peptides was not measured. Condensation of the hydrazide of the acylamino acid with acetylacetone gave the 3, 5-dimethylpyrazole of the N'-acylamino acid.

The aminolysis of this compound leads to the synthesis of an amide bond.



*DCHC represents dicyclohexylcarbodiimide.

We have previously established that it is possible by means of the pyrazole method to obtain the p-nitroanilide of cbz-glycine in high yield [5]. However, the results of the present work show that the aminolysis of acetyl-D-phenylalanyl-3, 5-dimethylpyrazole (III) can be carried out only under comparatively severe conditions, when the p-nitroanilide of acetylphenylalanine is obtained in the completely or partially racemized state. Thus, fusing III with p-nitroaniline for half an hour at 130–140° C leads to an optically inactive product. With a change in the conditions (boiling in absolute ethanol for 5 hr), a compound consisting of 30% of L-I and 70% of D-I is formed. The content of L-I in the product was found by hydrolysis under the action of chymotrypsin. These results are confirmed by the specific rotation. A synthetic mixture consisting of 30% of pure L-I and 70% of pure D-I had the same optical activity. It was shown by a special experiment that keeping optically pure D-I under the conditions of the pyrazole method of synthesis did not lead to its racemization. Consequently, in this method racemization begins at reaction stage B.



Optical rotatory dispersion curves of the p-nitroanilides (c 0.5; acetone. 1) Acetyl-D-phenylalanine; 2) acetyl-L-phenylalanine.

Experimental

cbz-L-Phenylalanine p-nitroanilide

A mixture of 7.24 g (24.2 mmole) of cbz-L-phenylalanine obtained by Grassman and Wünsch's method [7], 2.7 g (19.71 mM) of p-nitroaniline, and 4.02 g (19.3 mM) of DCHC was dissolved in 100 ml of absolute tetrahydrofuran. After 20 hr, the dicyclohexylurea was filtered off and the filtrate was evaporated. The resinous mass obtained was ground with ether until it solidified and was recrystallized from 80% ethanol. The yield of cbz-L-phenylalanine p-nitroanilide was 38%, mp 158.5–159.5° C, $[\alpha]_{436}$ +141° (c 0.94; acetone). Literature data for the D analog: mp 159–159.5° C, $[\alpha]_{436}$ −154° (c 0.91; acetone) [5].

Found, %: C 65.82; H 5.19; N 9.9. Calculated for $C_{23}H_{21}N_3O_5$, %: C 65.86; H 5.05; N 10.02.

L-Phenylalanine p-nitroanilide

After 2 hr, a solution of 0.57 g (1.36 mM) of cbz-L-phenylalanine nitroanilide in 5 ml of 30% HBr in glacial CH_3COOH deposited pink crystals, which were washed with absolute ether and dissolved in 60 ml of 50% ethanol; 0.4 ml of 6.72 N NH_4OH was added and the bulk of the ethanol was distilled off. The substance that deposited was recrystallized first from ordinary ethanol and then from absolute ethanol. The yield of L-phenylalanine p-nitroanilide was 0.20 g (79%), mp 156.5–157.5° C, $[\alpha]_{436}$ −314° (c 0.79); acetone). Literature data for the D analog: mp 156.5–157.5° C, $[\alpha]_{436}$ +316° (c 0.79; acetone) [5].

Acetyl-L-phenylalanine p-nitroanilide (L-I)

A solution of 0.45 g (1.5 mM) of L-phenylalanine nitroanilide in 1 ml of boiling glacial CH_3COOH was cooled to 40° C, treated with 0.25 ml (2.5 mM) of acetic anhydride, and boiled for 2 min. With the addition of water, the solution was evaporated several times in vacuum. The yellowish powder was dried over NaOH in vacuum. The yield of acetyl-L-phenylalanine p-nitroanilide was 0.44 g (86%), mp 252–253° C (from 96% ethanol), $[\alpha]_{500}$ +117°, $[\alpha]_{436}$ +216° (c 0.5;

acetone). The optical rotatory dispersion curves are shown in the figure. Literature data for D-I: mp 251–252° C, $[\alpha]_{500}$ −117°, $[\alpha]_{436}$ +225° (c 0.5; acetone) [5].

Acetyl-L-leucine p-nitroanilide

The substance was obtained from L-leucine in a similar manner to L-I. Yield 86%, mp 192–194° C (from absolute ethanol), $[\alpha]_{546}$ −10.8° (c 0.46; acetone), UV spectrum: λ_{max} 317 mμ (log ε 4.16).

Found, %: C 57.2; H 6.7; N 14. Calculated for $C_{14}H_{19}N_3O_3$, %: C 57.33; H 6.48; N 14.33.

Acetyl-D-phenylalanine hydrazide

The substance was synthesized via the azlactone of acetyl-D-phenylalanine by the method of Simion and Morawiec [8]. Yield 1.25 g (74%), mp 166–168° C, $[\alpha]_D^{20}$ −22° (c 1.2; alcohol). Literature data for acetyl-L-phenylalanine hydrazide: mp 164–166° C, $[\alpha]_D^{20}$ +20° (c 1.2; ethanol) [8].

N'-Acetyl-D-phenylalanyl-3, 5-dimethylpyrazole

A suspension of 0.22 g (1 mM) of acetyl-D-phenylalanine hydrazide in 5 ml of absolute ethanol was treated with 0.2 ml (2 mM) of freshly-distilled acetylacetone. Then water was added and the precipitate that had deposited was recrystallized twice (from benzene/petroleum ether). The yield of N'-acetyl-D-phenylalanyl-3, 5-dimethylpyrazole was 0.215 g (77%), mp 147.5–149° C, $[\alpha]_{436}$ −296° (c 0.45; ethanol).

Found, %: C 66.89; H 6.89. Calculated for $C_{16}H_{19}N_3O_2$, %: C 67.35; H 6.70.

Acetylphenylalanine p-nitroanilide

A. A mixture of 0.15 g (0.5 mM) of acetyl-D-phenylalanyl-3, 5-dimethylpyrazole and 0.1 g (0.7 mM) of p-nitroaniline was heated at 130-140° C for 1.5 hr. The resinous mass was washed five times with ether and was recrystallized from ethanol. The yield of acetylphenylalanine p-nitroanilide was 0.12 g (80%), mp 270–272° C, $[\alpha]_{546}$ 0°. The melting point of a mixture with acetyl-D, L-phenylalanine p-nitroanilide obtained as described by Tuppy et al. [3] was 270–272° C.

B. A mixture of 0.1 g (0.33 mM) of acetyl-D-phenylalanyl-3, 5-dimethylpyrazole and 0.05 g (0.35 mM) of p-nitroanalinine in 4 ml of absolute ethanol was heated at 70–80° C for 5 hr. The solution was evaporated, and the residue was washed with ether and recrystallized from ethanol. The yield of acetylphenylalanine p-nitroanilide was 0.04 g (40%), mp 261–263° C, $[\alpha]_{546}$ −13.8° (c 0.21; dimethylformamide).

C. A mixture of 0.05 g of optically pure acetyl-D-phenylalanine p-nitroanilide and an equimolecular amount of p-nitroaniline in 3 ml of absolute ethanol was boiled for 5 hr and was then evaporated. After crystallization from ethanol, the compound obtained had mp 251–253° C; this corresponds to the melting point of the starting material.

Determination of the optical purity of L- and D-acetylphenylalanine p-nitroanilides obtained by the carbobenzoxy-acetyl method

The optical rotatory dispersion was measured on an ORD/UV-5 JASCO instrument (Japan) with automatic recording (see figure). (Great assistance in these measurements was provided by V. M. Gurevich of the Department of Macromolecular Compounds, Moscow State University.) In the performance of the enzymatic hydrolysis, sterile crystalline chymotrypsin from the Medicinal Preparations Factory of the Leningrad Meat Combine was used. The sample of chymotrypsin did not contain detectable amounts of trypsin. The activity measured by Hummel's method was 60 ± 10% [9]. The initial enzyme solution prepared in 0.001 N hydrochloric acid had a concentration of 1 mg/ml.

The solutions of the nitroanilides were prepared in dimethylformamide free from amines [10]. The concentration of the solutions was 0.5×10^{-2} M. The degree of hydrolysis of the p-nitroanilides was followed spectrophotometrically on an SFD-2 instrument fitted with a cell in which the temperature was regulated to 37 ± 0.2° C. The reaction mixture contained 0.04 M tris buffer, 0.045 M $CaCl_2$, $0.3-15 \times 10^{-4}$ M substrate, 6% of dimethylformamide, and 4% of the initial chymotrypsin solution (by volume); the pH of the mixture was 8.03, and was measured by an LPU-01 instrument.

The following characteristics of the mixture obtained were determined: 1) the dependence of the molar extinction on the wavelength in the interval from 250 to 410 mμ (the measurements were carried out in 1-cm quartz cells with the addition of 0.001 N hydrochloric acid instead of the chymotrypsin); 2) the concentration of p-nitroaniline (at 410 mμ) formed as a result of the chymotrypsin hydrolysis of the p-nitroanilides (in this case, round quartz cells were used). The results of the experiments are given in the table.

Determination of the optical purity of acetylphenylalanine p-nitroanilide obtained by the pyrazole method

The specific rotation of the material obtained, $[\alpha]_{546}$ −13.8° (c 0.21; dimethylformamide), showed that it contained

not only the D- but also the L-antipode. The concentration of the L-antipode was determined from the value of the initial rate of hydrolysis with chymotrypsin. It was known from preliminary experiments that the D-antipode does not undergo hydrolysis and does not lower the initial rate of hydrolysis of the L-antipode (L-I:D-I as 1:3). By using the graph of

p-Nitroanilides	Concentration of substrates in the mixture, M	Time of the reaction, hr	Concentration of p-nitroaniline liberated, M
Acetyl-L-phenylalanine	$1 \cdot 10^{-4}$	18	$0.98 \cdot 10^{-4}$
	$0.3 \cdot 10^{-4}$	24	$0.306 \cdot 10^{-4}$
Acetyl-D-phenylalanine	$15 \cdot 10^{-4}$*	45	$0.07 \cdot 10^{-4}$
Mixture			
Acetyl-L-phenylalanine	$0.3 \cdot 10^{-4}$	24	$0.37 \cdot 10^{-4}$
Acetyl-D-phenylalanine	$14.7 \cdot 10^{-4}$		

*10-cm cell.

the rate of hydrolysis as a function of the concentration of the substrate it was found that it contained 33% of L-I and 67% of D-I. A mixture consisting of 32% of L-I and 68% of D-I had $[\alpha]_{546}$ −14° (c 0.21; dimethylformamide).

Summary

1. Optically pure acetyl-L-leucine p-nitroanilide and acetyl-L-phenylalanine p-nitroanilide have been obtained.

2. It has been shown that the pyrazole method for the synthesis of acetyl-D-phenylalanine leads to the formation of a partially racemized compound.

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