SYNTHESIS OF p-NITROPHENYL GLYCOSIDES OF N-AMINOACYL DERIVATIVES OF D-GLUCOSAMINE. II

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We have previously synthesized p-nitrophenyl β-D-glucosaminides with phthalylglycine and phthalyl-D, L-alanine as substituents in the amino group of D-glucosamine [1]. The present paper describes a method for obtaining p-nitrophenyl β-D-glucosaminides acylated with glycine and L-alanine.

In the hydrazinolysis of p-nitrophenyl N-(phthalylglycyl)-β-D-glucosaminide (Ia), or its tri-O-acetyl derivative (Ib), and of the acetate of p-nitrophenyl N-(phthalyl-L-alanyl)-β-D-glucosaminide (Ic) in ethanol at the boil, the corresponding glucosaminides (IIa) and (IIb) are formed in good yield. The free bases (II) are unstable and in time are converted into compounds containing no free amino group.

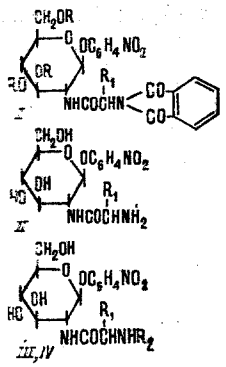
Compounds (IIa) and (IIb) were used as substrates for the investigation of β-hexosaminidases. It was found that ordinary N-acetyl hexosaminidase (EC 3.2.1.30) does not cleave compounds (IIa) and (IIb); however, we have isolated a "neutral" β-D-hexosaminidase with an optimum pH of 7 from Chaetopterus variopedatus and Ophiura sarsi which does cleave (IIa) [2, 3]. From (IIa) and (IIb) after acetylation with acetic anhydride in aqueous methanol we obtained the N-acetyl derivatives (IIIa) and (IIIb).

When (IIa) was treated with benzyloxycarbonylglycine and the methyl ester of N-benzyloxycarbonylglutamic acid in the presence of N,N'-dicyclohexylcarbodiimide, the corresponding peptide derivatives were obtained.

EXPERIMENTAL

Paper chromatography was performed on FN-15 paper in the butanol–water–acetic acid system (system 1), electrophoresis in pyridine–acetate buffer, pH 5.5 (1 ml of acetic acid and 3.5 ml of pyridine per liter of water), and thin-layer chromatography on "Silufol" plates in ether (system 2), ether–ethyl acetate (1:9) (system 3), and ethyl acetate–ethanol (4:1) (system 4). The melting points of the substances were determined on a Boétius stage, and the angles of rotation were measured on a Perkin–Elmer model 141 spectrophotometer. The compounds were hydrolyzed with 4 N HCl at 105°C for 4–16 h, and the p-nitrophenyl in the hydrolyzate was determined spectrophotometrically at 440 nm on a Spekol spectrophotocolorimeter after the hydrolyzate had been

Ia. $R = H$; $R_1 = H$
Ib. $R = CH_3CO-$; $R_1 = H$
Ic. $R = CH_3CO-$; $R_1 = CH_3$
IIa. $R_1 = H$
IIb. $R_1 = CH_3$
IIIa. $R_1 = H$; $R_2 = CH_3CO-$
IIIb. $R_1 = CH_3$; $R_2 = CH_3CO-$
IVa. $R_1 = H$; $R_2 = C_6H_5CH_2OCONHCH_2CO-$
IVb. $R_1 = H$
$R_2 = C_6H_5CH_2OCONHCHCO-$
$CH_2CH_2COOCH_3$



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made alkaline with 1 M Na_2CO_3. The solvents for the system were absolute. The analyses of all the substances corresponded to the calculated figures.

p-Nitrophenyl 2-Glycylamino-2-deoxy-β-D-glucopyranoside (IIa). Method A. A mixture of 244 mg (0.5 mmole) of p-nitrophenyl 2-phthalylglycylamino-2-deoxy-β-D-glucopyranoside (Ia), obtained as described previously [1], and 10 ml of a 0.1 M solution of hydrazine in 90% ethanol was boiled for 1 h. The reaction mixture was left at room temperature for 4 h and then the precipitate was filtered off, washed with ethanol and ether, dried, and covered with 5 ml of a 20% solution of CH_3COOH. The mixture was kept at 50-60° for 10 min, slowly cooled at room temperature, and filtered. The filtrate was evaporated to dryness, the residue was dissolved in ethanol (3 ml), ether was added until the solution had become turbid, and it was left in the refrigerator for 12 h. This gave 147 mg of the acetate of (IIa) (yield 70%), R_f 0.41 (system 1), mp 186° (from aqueous ethanol) $[\alpha]_D^{20}$ − 30° (c 1; 20% CH_3COOH); composition $C_{14}H_{19}N_3O_8 \cdot CH_3COOH$.

The hydrolysis (16 h) of (IIa) gave p-nitrophenol, glycine, and glucosamine in a ratio of 1 : 0.9 : 0.79.

Method B. A mixture of 0.31 g (0.5 mmole) of (Ib) and 10 ml of a 0.25 M ethanolic solution of hydrazine was boiled for 1 h. Then the reaction mixture was left for 5 h at room temperature, and the crystals were filtered off and and washed with ethanol and ether. This gave 120 mg (62%) of (IIa) in the form of the free base. After recrystallization from aqueous ethanol, mp 192°C and $[\alpha]_D^{20}$ −37° (c 1; 20% CH_3COOH). With time, the substance was converted into a compound electrophoretically neutral at pH 5.5 and giving no colored product with ninhydrin. The crystals of the free (IIa) were converted into the acetate as described above. This sample of (IIa) acetate had mp 182° and $[\alpha]_D^{20}$ − 29° (c 1; 20% CH_3COOH). From the mother solution of the original reaction mixture, treated similarly, another 70 mg of (IIa) acetate were obtained. The total yield of the salt of (IIa) was 85%.

2-(Phthalyl-L-alanylamino)-2-deoxy-D-glucopyranose was obtained by the method described previously [1] for the phthalyl-D-L-analyl derivative with a yield of 30%; mp 210°C (from methanol), $[\alpha]_D^{20}$ + 23.6° (c 1; H_2O, after 24 h).

3,4,6-Tri-O-acetyl-2-(phthalyl-L-alanylamino)-2-deoxy-α-D-glucopyranosyl chloride was obtained by the method described for the D,L-alanyl derivative [1]; composition $C_{23}H_{25}N_2O_{10}Cl$, yield 65% (after purification on a column of SiO_2 with elution by abs. ether), mp 99°C (from abs. ethyl acetate−abs. ether), $[\alpha]_D^{20}$ + 144° (c 1, acetone), R_f 0.6 (system 2).

p-N-Phenyl 3,4,6-tri-O-acetyl-2-(phthalyl-L-alanylamino)-2-deoxy-β-D-glucopyranoside (Ic), $C_{29}H_{29}N_3O_{13}$, was obtained in a similar manner to the D,L-alanyl derivative [1], yield 70%, mp 206°C (from ethanol), $[\alpha]_D^{20}$ − 28° (c 1; Py); R_f 0.4 (system 3). On hydrolysis, p-nitrophenol, alanine, and glucosamine were found in a ratio of 1 : 1 : 0.96.

p-Nitrophenyl 2-(L-alanylamino)-2-deoxy-β-D-glucopyranoside (IIb) was obtained from (Ic) by the method described above for obtaining (IIa) from (Ib). After the end of the reaction, the mixture was left in the refrigerator for 5-6 h. The crystals that deposited were washed with cooled ethanol and with ether. This gave the base (IIb) with mp 199°C in a yield of 70%. Treatment with 20% CH_3COOH gave the acetate of (IIb) with a yield of 3%, mp 209-210°C (from aqueous ethanol), $[\alpha]_D^{20}$ − 12° (c 1, 20% CH_3COOH), R_f 0.43 (system 1), $C_{15}H_{21}N_3O_8 \cdot H_2O$. When compound (IIb) was hydrolyzed, p-nitrophenol, alanine, and glucosamine were found in a ratio of 1 : 0.96 : 0.8.

p-Nitrophenyl 2-(Acetylglycylamino)-2-deoxy-β-D-glucopyranoside (IIIa). With cooling, 0.07 ml of triethylamine (TEA) was added to 208 mg (0.5 mole) of (IIa) in 10 ml of 10% aqueous methanol. The reaction mixture was stirred for 20 min, 0.1 ml (1 mmole) of acetic anhydride was added, and it was stirred overnight; then it was evaporated to dryness and the residue was extracted with chloroform (3 × 10 ml) and recrystallized from aqueous ethanol. Yield 60%, mp 207-208°C, $[\alpha]_D^{20}$ − 45.6° (c 0.5; DMFA); composition $C_{16}H_{21}O_9N_3 \cdot C_2H_5OH$.

p-Nitrophenyl 2-(acetyl-L-alanylamino)-2-deoxy-β-D-glucopyranoside (IIIb) was obtained from (IIb) similarly with a yield of 60%, mp 243°C, $[\alpha]_D^{20}$ − 61.5° (c 0.5; DMFA), composition $C_{17}H_{23}O_9N_3$.

p-Nitrophenyl 2-(Benzyloxycarbonylglycylglycylamino)-2-deoxy-β-D-glucopyranoside (IVa). With cooling and stirring, 0.07 ml (1 mmole) of TEA was added to 417 mg (1 mmole) of (IIa) in 15 ml of 90% aqueous pyridine. The mixture was kept for 20 min, and then 418 mg (2 mmole) of benzyloxycarbonylglycine were added and, after this had dissolved completely, 300 mg (15 mmole) of CDI, and then the mixture was stirred at room temperature for 16 h and was diluted with 100 ml of water. The filtrate was evaporated to dryness and the residue was washed with dry chloroform (3 × 10 ml) and crystallized from methanol. This gave 40% of (IVa), mp 202°C

(from methanol), $[\alpha]_D^{20} - 23°$ (c 1; DMFA), R_f 0.64 (system 3); composition $C_{24}H_{28}O_{11}N_4$. On hydrolysis, p-nitrophenyl, glycine, and glucosamine were found in a ratio of 1 : 1.96 : 0.92.

p-Nitrophenyl 2-(γ-O-methyl-N-benzyloxycarbonyl-L-glutaminoylamino)-2-deoxy-β-D-glucopyranoside (IVb) was obtained similarly from 0.5 mmole of (IIa) and 1 mmole of the γ-methyl ester of N-benzyloxycarbonyl-L-glutamic acid; yield 55%, mp 198°C (from methanol), $[\alpha]_D^{20} - 20°$ (c 1; DMFA), R_f 0.7 (system 3), composition $C_{23}H_{34}O_{13}N_4$. On hydrolysis, p-nitrophenol, glycine, glutamic acid, and glucosamine were found in a ratio of 1 : 1 : 0.96 : 0.9.

SUMMARY

1. A method has been developed for the synthesis of p-nitrophenyl β-D-glucosaminides acylated with glucine and alanine.

2. p-Nitrophenyl N glycyl-β-D-glucosaminide has been found to be a substrate of a neutral β D-hexosaminodase.

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L-ASPARAGINASE BOUND IN A POLYACRYLAMIDE GEL

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In recent years, interest has risen in the immobilization of various enzymes, including enzymes of medical significance, in order to create new medicinal forms. The immobilization of L-asparaginase (L-asparagine aminohydrolaze, EC 3.5.1.1) has been performed hitherto mainly with the aid of adsorption [1] or by covalent attachment to the matrix [2-4].

On the inclusion of the enzyme in the lattice of a polyacrylamide gel, the molecule of L-asparagine – the substrate of L-asparagine – has small dimensions in comparison with the enzyme molecule. However, there is very little information on the properties of L-asparagine included in a polyacrylamide gel.

In order to fix L-asparaginase in the structure of a polyacrylamide gel we have used a modification of the method proposed by Miller [5]. The aim of the present work was to investigate the stability and some kinetic parameters of L-asparaginase bound in a polyacrylamide gel.

To elucidate the influence of the concentration of acrylamide and of the cross-linking agent N,N'-bisacrylamide on the inclusion of L-asparaginase in the gel, we performed a series of experiments in which the amount of acrylamide was varied from 3 to 15% at a constant concentration of N,N'-methylenebisacrylamide of 1.5%, and the amount of cross-linking agent was varied from 1 to 5% in a 6% polyacrylamide gel. The best results on the binding of L-asparaginase in a polyacrylamide gel were achieved at a 6% concentration of acrylamide with 1.5% of the cross-linking agent N,N'-methylenebisacrylamide.

A distinguishing feature of L-asparaginase is its capacity for retaining its activity over a wide pH range, with a maximum at pH 7.0-8.0. The bell-shaped curve of the pH dependence of the activity of the native enzyme contracts for the enzyme included in the gel, and in place of the plateau of the optimum activity pH a well-defined sharp peak of activity appears on the curve at pH 8.5.

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