SYNTHESIS OF N-(L-β-ASPARTYL)-D-GLUCOSAMINE, METHYL N-(O-METHYL-L-β-ASPARTYL)-α-D-GLUCOSAMINIDE, AND THEIR DERIVATIVES

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The character of the carbohydrate-protein linkage is an important question in present-day biochemistry associated with the elucidation of the specificity of proteins. Numerous investigations have shown that in many carbohydrate-containing proteins (glycoproteins) the polysaccharide complex is attached to the protein by a covalent bond. This bond may be very varied in type: from an ester [1], glucoside-ether [2], or ether [3] type to bonds of an acylamino character, in the formation of which the NH_2 group of a hexosamine may take part [4]. In some glycoproteins the link between the polysaccharide and the protein is effected through the COOH group of aspartic acid; these include egg albumin [5-9], the glycoprotein from the submaxillary gland of sheep [2], human γ-globulin [10], and the γ-globulin of rabbits. In many proteins (the casein of milk [12], fetuin [13], etc.) aspartic acid has been found in the peptide part of the glycopeptide. From the γ-globulin of human blood Rothfus [3] isolated a fragment made up from glucosamine and aspartic acid, and he suggested that these are joined with an amide linkage. From ovomucoid Tanaka [14] succeeded in obtaining a dinitrophenyl derivative of aspartylglucosamine after the partial hydrolysis of the polysaccharide-peptide isolated from the pancreatic hydrolyzate of this protein. The acylamino linkage is the most probable also in the case of egg albumin. The linkage in this protein is not split under the action of hydroxylamine [13], but is broken under hydrazinolysis conditions [8] (10 h, 100°, anhydrous hydrazine, or 16 h, 60°, hydrazine sulfate).

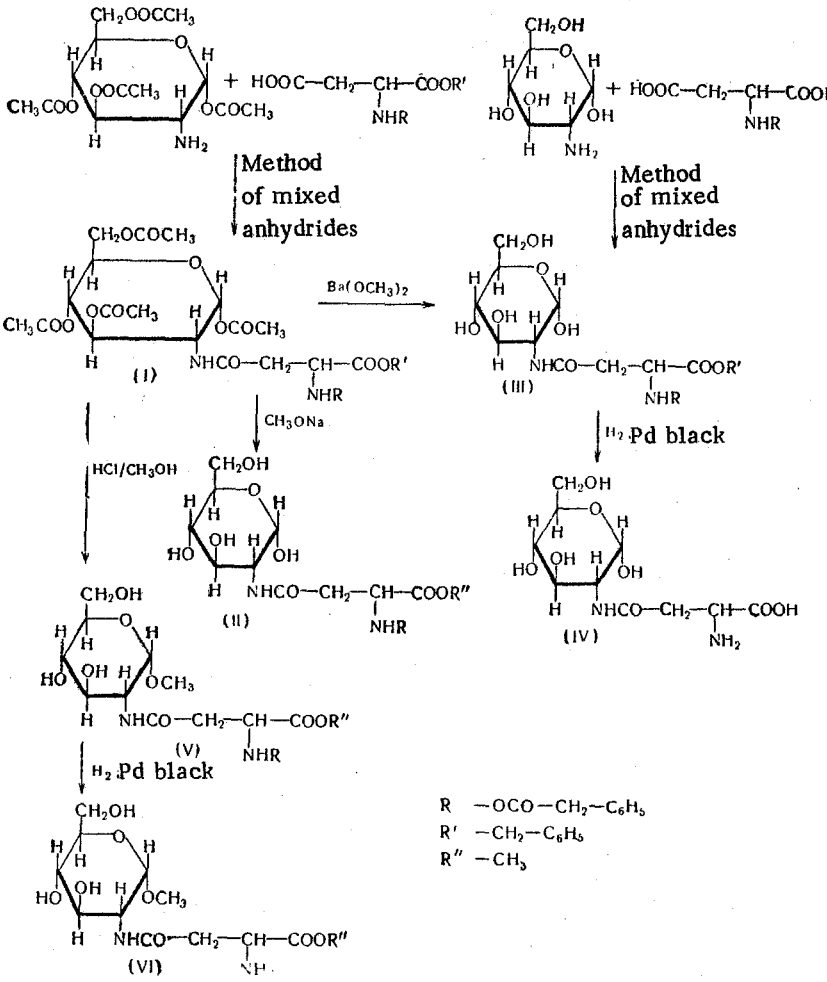
It is possible that the synthesis of products of the addition of amino acids to amino sugars at the NH_2 group and a study of their properties would facilitate the determination of the character of the carbohydrate-protein linkage in some glycoproteins. For the condensation of glucosamine with certain amino acids, the following methods have been proposed: the acid chloride [16], anhydride [17], and carbodiimide [18] methods and the method of activated ethers (p-nitrophenyl ethers) [19]. For the synthesis of N-(L-β-aspartyl)-D-glucosamine (IV) we made successful use of the method of mixed anhydrides (by the use of chloroformic esters in accordance with Boissonnas' method) [20]. In addition to N-(L-β-aspartyl)-D-glucosamine, it was of interest to prepare also derivatives of this in which the 1-COOH group of aspartic acid was blocked and also in which the glucosidic hydroxyl was blocked, for in most glycoproteins a reducing group is absent.

We synthesized the required derivatives by the scheme given in the next page.

From 1,3,4,6-tetra-O-acetyl-β-D-glucosamine and the 1-benzyl ester of N-BOC-L-aspartic acid* by the method of mixed anhydrides we prepared 1,3,4,6-tetra-O-acetyl-N-(O-benzyl-N-BOC-L-β-aspartyl)-D-glucosamine (I) in good yield. On removal of acetyl groups from (I) with sodium methoxide, together with some breakdown of the acylamino linkage, transesterification of the 1-COOH groups of the aspartic acid occurred (replacement of benzyl by methyl) with formation of N-(N-BOC-O-methyl-L-β-aspartyl)-D-glucosamine (II). On hydrolysis of (I) with barium methoxide we succeeded in isolating N-(O-benzyl-N-BOC-L-β-aspartyl)-D-glucosamine (III) from the reaction mixture, but it was difficult to purify. It was found to be simple to carry out the condensation with free glucosamine. In this reaction the hydroxyls of the glucosamine take scarcely any part. By subsequent hydrogenation we succeeded in removing the benzyl and benzyloxycarbonyl groups quantitatively and obtain (IV). In the preparation of the glucosaminide the removal of acetyl groups from (I) was carried out by boiling the substance with 2% HCl/CH_3OH. It is known [21] that under these conditions all the acetyl groups of pentaacetylglucosamine are removed except that attached to nitrogen, and a methyl glucoside derivative is formed. With such a treatment the

* BOC = benzyloxycarbonyl.

Scheme of Synthesis of N-(L-β-Aspartyl)-D-glucosamine, Methyl N-(O-Methyl-L-β-aspartyl)-α-D-glucosaminide, and Their Derivatives



acetyl groups were removed from (I) and the glucosidic hydroxyl was blocked with a methyl group. Simultaneously, the transesterification of the 1-COOH of aspartic acid occurred and we obtained methyl N-(N-BOC-O-methyl-L-β-aspartyl)-α-D-glucosaminide (V). By hydrogenation over palladium black (V) was converted into methyl N-(O-methyl-L-β-aspartyl)-α-D-glucosaminide (VI).

EXPERIMENTAL

1-Benzyl Ester of N-BOC-L-aspartic Acid. 5.4 g (0.0217 mole) of N-BOC-L-aspartic anhydride was heated at 100° for four hours with 3.42 ml of freshly distilled benzyl alcohol in a sealed tube. The sirup was dissolved in ethyl acetate, and the solution was extracted with 6% sodium bicarbonate solution in portions of 40-50 ml. On acidification of the fractions with 10% hydrochloric acid an oil was precipitate, and this crystallized out in the cold. The total yield of unpurified ester was 6.31 g. After three crystallizations from four parts of toluene we obtained 2.36 g of the 1-benzyl ester of N-BOC-L-aspartic acid; m.p. 85-85.2° (from toluene); $[\alpha]_D^{19} - 14.0°$ (c 2; acetone). The literature gives: m.p. 84-85° [22]; $[\alpha]_D^{17} - 14.8°$ (c 5.0; acetone) [23]; $[\alpha]_D^{23} - 14.2°$ (c 2.5; acetone) [18].

1,3,4,6-Tetra-O-acetyl-N-(O-benzyl-N-BOC-L-β-aspartyl)-D-glucosamine (I). 0.24 ml of ethyl chloroformate was added to a solution of 0.93 g of the 1-benzyl ester of N-BOC-L-aspartic acid and 0.354 ml of triethylamine in 40 ml of dry freshly distilled tetrahydrofuran cooled to −10°. After 10-15 minutes we added a solution of 1 g (0.0026 mole) of 1,3,4,6-tetra-O-acetyl-D-glucosamine: m.p. 225-227° (decomp.); $[\alpha]_D^{23}$

+ 28.5 ± 1.5° (c 1; water). The literature [24] gives: m.p. 230° (decomp.); $[\alpha]_D$ + 29.7° (water)] and 0.354 ml of triethylamine in dry tetrahydrofuran. The mixture was kept for 10-15 minutes at 0° and then shaken in a shaker for 3-4 hours at 20°. Triethylamine hydrochloride was filtered off and washed with tetrahydrofuran. The washings were added to the filtrate, and the whole was vacuum-evaporated. The white substance was washed carefully with ether; weight 1.424 g. The crystallization of (I) from ethanol gave fine long needles; yield 1.338 g (75.2%); m.p. 205° (evaporated out of alcohol); 206-206.7° (evaporated out of acetone); $[\alpha]_D^{23}$ + 11.0 ± 0.4° (N,N-dimethylformamide); $[\alpha]_D^{19}$ + 7.2°; (c 1; acetone). Found: C 58.00; H 5.54; N 4.08%. $C_{33}H_{38}N_2O_{14}$. Calculated: C 57.73; H 5.53; N 4.08%.

In the recently published paper by Marks and Neuberger [18], a description is given of the synthesis of (I) from the same starting substances, but by the carbodiimide method. The constants of their product: m.p. 209.5-210.5° (from alcohol); $[\alpha]_D^{22}$ + 6.3° (c 1.27; acetone).

N-(N-BOC-O-methyl-L-β-aspartyl)-D-glucosamine (II). 6.34 ml of 0.92 N CH_3ONa was added to a solution of 1 g of (I) (m.p. 200.5-200.8°) in 10 ml of dry chloroform cooled to −10°. The mixture was left for ten minutes at −10° and for 20 minutes at 20°, and it was then vacuum-evaporated at not above 20°. The residue was washed with water (0°) and dried over P_2O_5 in a vacuum desiccator. The weight of the substance was 0.343 g; m.p. 160-161° (decomp.). From the washings we isolated also 0.169 g of a substance of m.p. 156-159°. The total yield was 0.512 g (67.7%). For analysis the substance was purified by reprecipitation with ether from alcoholic solution and was vacuum-dried at 74°; $[\alpha]_D^{22}$ + 25.3° (c 0.55 CH_3OH) R_f 0.53-0.55 (in system n-C_4H_9OH: CH_3COOH : H_2O 4 : 1 : 5). Found: C 48.85, H 5.59; N 5.92%. $C_{19}H_{26}N_2O_{11} \cdot 1/2\ H_2O$. Calculated: C 48.82; H 5.78; N 5.99%.

N-(O-Benzyl-N-BOC-L-β-aspartyl)-D-glucosamine (III). At 0° a solution of D-glucosamine in a mixture of tetrahydrofuran and methanol, prepared from 1.11 g of D-glucosamine hydrochloride and an equimolecular amount of CH_3ONa, was added to a tetrahydrofuran solution of a mixed anhydride prepared at 0° from 2.24 g of the 1-benzyl ester of N-BOC-L-aspartic acid and ethyl chloroformate. The mixture was kept for 30 minutes at −10°, then for one hour at 0°, and finally left overnight at 20°. On evaporation of the reaction mixture we obtained a white gel-like precipitate, which was shaken successively with water at 0°, ether, and chloroform. The precipitate was centrifuged off and dried in a vacuum desiccator. The substance was found to be chromatographically pure; weight 1.33 g (50%); m.p. 167-168°; $[\alpha]_D^{23}$ + 20.0° (c 0.3; CH_3OH). Found: C 57.02; H 5.76; N 5.31%. $C_{25}H_{30}N_2O_{10} \cdot 1/2\ H_2O$. Calculated: C 56.92; H 5.88; N 5.31%.

N-(L-β-Aspartyl)-D-glucosamine (IV). 0.44 g of the substance (III) was hydrogenated in 15 ml of aqueous methanol with a small addition of acetone over palladium black in presence of an equimolecular amount of HCl (0.8 ml of 1 N HCl) for 24 hours. In the course of the hydrogenation a white precipitate formed, and this dissolved on the addition of water. Catalyst was separated, and the filtrate was evaporated. The residue (0.332 g) consisted of the substance (IV) (the predominating component) with the starting substance, N-(O-benzyl-L-β-aspartyl)-D-glucosamine, aspartic acid, and glucosamine as impurities (electrophoresis; pH 2.7; 1000 V; 1 h; development with ninhydrin and an alkaline solution of $AgNO_3$). The substance was crystallized from the least possible amount of hot water. A white precipitate formed in the cold; weight 0.088 g (36%); m.p. > 320° (starts to blacken at 210°); $[\alpha]_D^{19}$ + 30.2° → + 23.3° (c 0.28; water). A little more (IV) was isolated from the mother solution.

Synthesis of (IV) without the Isolation of (III). A solution of 1.35 g of (I) (m.p. 201-202°) in absolute methanol was mixed with 10.25 ml of 0.78 N $Ba(OCH_3)_2$ solution (see [19]), and the mixture was left for 20 hours at 0°. After the addition of 10 ml of water, carbon dioxide was passed; barium carbonate was separated by centrifugation. The clear solution was vacuum-concentrated to half bulk and again saturated with carbon dioxide. The filtrate was vacuum-evaporated; weight of residue 1.022 g. Without further purification 1 g of this substance was hydrogenated over palladium black in presence of 1 M HCl. In the course of the hydrogenation water was added to the reaction mixture. Catalyst was separated, and the solution was vacuum-evaporated. The oil, which did not crystallize on standing, solidifed when rubbed out with absolute methanol. The substance was separated (698 mg), dissolved in a little hot water, and mixed with methanol. The precipitate formed was separated and dried in a vacuum desiccator; weight 341 mg (59%). For analysis 200 mg of the substance was recrystallized from hot water; we obtained 53 mg. The substance was chromatographically pure; m.p. > 320° (starts to blacken at 210°); $[\alpha]_D^{23}$ + 31.9° → + 22.7° ± 0.3 (c 0.28, water). Found: C 38.48; H 6.32; N 5.93%. $C_{10}H_{18}N_2O_8 \cdot H_2O$. Calculated: C 38.46; H 6.41; N 5.95%.

In Neuberger's paper the following data are given for the sesquihydrate of (IV); m.p. > 360° (starts to blacken at 200°); $[\alpha]_D$ + 29° → + 22° (c 0.28; water).

Methyl N-(N-BOC-O-methyl-L-β-aspartyl)-α-D-glucosaminide (V). 1.5 g of (I) (m. p. 203-204°) was refluxed for two hours with 30 ml of a 2% solution of HCl in methanol. 4 g of $PbCO_3$ was added to the solution while it had not yet cooled, and the mixture was shaken in a shaker. This operation was repeated, and the filtrate was checked for the absence of chloride ions. The filtrate was then vacuum-evaporated (< 30°), and the residue was dried in a vacuum desiccator; the oil amounted to 1.02 g; it was dissolved in the least possible amount of methanol. On addition of a large excess of ether in the cold, a white precipitate of (V) formed; weight 0.65 g. On chromatography of this substance in the system butyl alcohol– acetic acid – water (4 : 8 : 5) we detected (V) (R_f 0.26-0.27) together with methyl N-(O-benzyl-N-BOC-L-β-aspartyl)-α-D-glucosaminide and some unknown substance (R_f 0.06 and 0.13 respectively) as impurities. The substance (V) and the impurities present did not react with an alkaline silver solution. For analysis the compound (V) was repeatedly purified by precipitating the impurities with ether from methanolic solution. The substance (V) was amorphous; $[\alpha]_D^{23}$ + 63.0 (c 0.6; CH_3OH). Found: C 52.91; H 6.30; N 5.97%. $C_{20}H_{28}N_2O_{10}$. Calculated: C 52.63; H 6.13; N 6.13%.

Methyl N-(O-Methyl-L-β-aspartyl)-α-D-glucosaminide (VI). 100 mg of (V) was hydrogenated in 5 ml of methanol with the addition of two drops of glacial acetic acid over palladium black. Catalyst was filtered off, and the filtrate was vacuum-evaporated (< 30°). The oil solidified when rubbed out with ether; the yield of the hemihydrate of the acetate (VI) was 54 mg (81%); $[\alpha]_D^{23}$ + 80.6° (c 0.65; water). Found: C 43.31; H 6.85; H 6.85; N 7.49%. $C_{12}H_{22}N_2O_8 \cdot CH_3COOH$ 1/2 H_2O. Calculated: C 42.97; H 6.90; N 7.16%.

SUMMARY

A description is given of the synthesis and some properties of N-(L-β-aspartyl)-D-glucosamine, methyl N-(O-methyl-L-β-aspartyl)-α-D-glucosaminide, and some of their derivatives.

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