GLYCOPEPTIDES

XVI. Synthesis of the Methyl Ester of N-Glycyl-Methoxyneuraminic Acid

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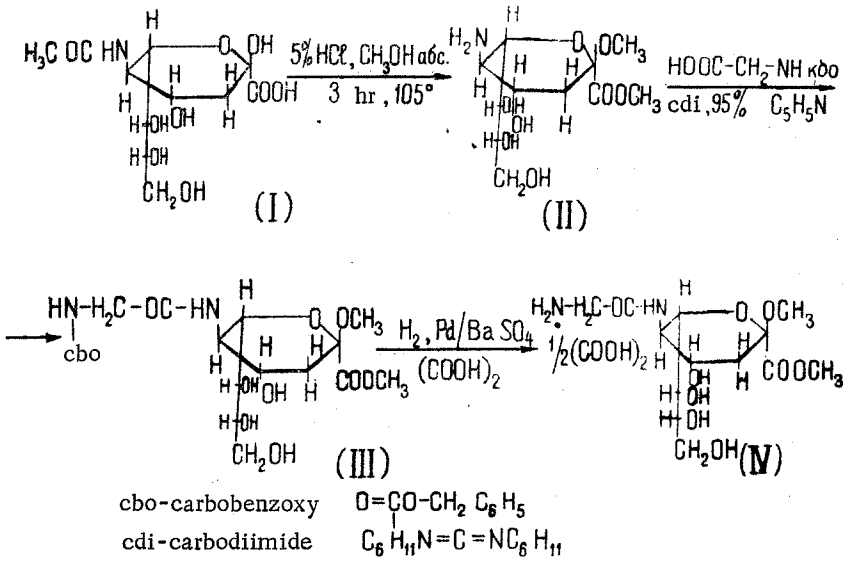
In spite of the exceptional importance of neuraminic acid, which is contained in many essential biopolymers, there is almost no information on the synthesis of its derivatives [1]. Compounds modelling fragments of natural glycopeptides containing neuraminic acid and necessary for the elucidation of the linkage of the latter with the skeleton of the polymer are of particular interest. We have previously obtained the methyl ester of 9-O-glycyl-N-acetylneuraminic acid [2].

Continuing this work, we have synthesized by the carbodiimide method [3] the methyl ester of N-glycylmethoxyneuraminic acid, which contains a N-aminoacyl bond. The synthesis was carried out from the methyl ester of methoxyneuraminic acid by the following route:



The first stage of the synthesis presented certain difficulties, since no satisfactory method of obtaining the methyl ester of methoxyneuraminic acid (II) was known.

The methyl ester of methoxyneuraminic acid was obtained by heating acetylneuraminic acid with 5% hydrochloric acid in absolute methanol at 105°C for 3 hr. Although partial resinification of substance (I) took place under these conditions, compound (II) was obtained in high yield (≈ 80%). The hydrochloride of (II) was freed from Cl^- ions on a column of the anion-exchanger "Dowex" 2 × 8 (CO_3^{--} form). The methyl ester of methoxyneuraminic acid (II) was mobile on electrophoresis, was shown up by ninhydrin (presence of an amino group), by potassium periodatecuprate (presence of a glycol grouping), ferrous chloride and hydroxylamine (presence of an ester linkage) and C_2 and benzidine (test for an NH_2 group). On thin-layer chromatography, substance (II) was shown up by resorcinol. It was impossible to obtain compound (II) by methylation with diazomethane of the methoxyneuraminic acid isolated by methanolysis from the submaxillary mucin [4], since in this process almost equal amounts of compound (II) and a substance apparently consisting of a methylation product of the methoxyneuraminic acid at the amino group were formed. Substance (II) readily undergoes condensation with carbobenzoxyglycine in the presence of N, N'-dicyclohexylcarbodiimide in 95% aqueous pyridine in the cold [5]. With equimolecular amounts of the substances, the reaction takes place exclusively at the amino group, checked by electrophoresis and thin-layer chromatography. Only traces of another reaction product running above the main substance was found in the reaction mixture.

The structure of the condensation product obtained (III) was confirmed by elementary analysis, tests on the chromatogram with potassium periodatecuprate, with Cl_2 and benzidine, with ferrous chloride and hydroxylamine, and with resorcinol. Compound (III) is immobile on electrophoresis and is not shown up by ninhydrin.

The structure of the compound obtained was confirmed definitively by periodate oxidation. The absorption of

2 moles of periodic acid unambiguously showed that the product obtained (III) is the methyl ester of N-(N'-carbobenoxyglycyl)-methoxyneuraminic acid. To exclude over-oxidation, the periodate oxidation was carried out in an acetate buffer at pH 3.72 in the cold (+4°C) in the dark.

An attempt to synthesize a N-aminoacyl derivative of methoxyneuraminic acid with a free carboxyl group has not yet been successful. The condensation of methoxyneuraminic acid with carbobenzoxyglycine by the carbodiimide method could not take place, this apparently being explainable by the formation of a Zwitter ion.

The hydrogenation of substance (III) on $Pd/BaSO_4$ in 75% aqueous CH_3OH in the presence of $(COOH)_2 \cdot 2H_2O$ gave an aminoacyl derivative with a free amino group (IV), which was isolated in the form of the oxalate. On electrophoresis, it migrated towards the anode, was shown up by ninhydrin, by potassium periodatecuprate, and by Cl_2 and benzidine.

An investigation of the stability of the aminoacyl bond in the compounds obtained and of other features of the chemical behavior of this new model of the natural glycopeptides is continuing.

Experimental

The chromatograms were obtained by the descending and ascending methods. Mobile phase: butan-1-ol – acetic acid – water (4 : 1 : 1), upper layer (system 1). Thin-layer chromatography was carried out on silica gel plates in the systems: n-propanol – 1 N NH_4OH – water (6 : 2 : 1) (system 2) and chloroform – methanol – acetic acid (20 : 30 : 0.1) (system 3). Electrophoresis was carried out in a buffer consisting of pyridine (2 ml) – acetic acid (4 ml) – water (to 1 liter), pH 4.2-4.5, and at a voltage of 900-1000 V. The spots were revealed by means of ninhydrin, potassium periodatecuprate, chlorine and benzidine, ferrous chloride and hydroxylamine, and resorcinol.

Methyl ester of methoxyneuraminic acid (II). A mixture of 500 mg (0.6 mmole) of N-acetylneuraminic acid and 200 ml of freshly-prepared 5% hydrochloric acid in absolute methanol was heated in a tube at 105-110°C for 3 hr. The excess of hydrochloric acid in the cooled solution was neutralized with $PbCO_3$ to pH 6-6.5, and the precipitate was carefully washed with hot methanol. The methanolic solution was evaporated to dryness in vacuum, and the residue was left overnight over caustic potash in a desiccator to eliminate traces of Cl^- ions; it was then dissolved in 100 ml of distilled water, the solution was filtered to remove humic substances, and the aqueous solution was extracted with butanol (3 × 15 ml) to eliminate colored substances. The colorless aqueous solution was lyophilized.

The yield of crude product was 373 mg (80%). The lyophilized product contained Cl^- ions (Beilstein test). To eliminate the Cl^- ions, the substance was dissolved in 25 ml of water and the solution was passed through a column containing "Dowex" 2 × 8 (CO_3^{--} form). Then the column was washed with 100 ml of water, the aqueous eluate was evaporated in vacuum, and was lyophilized. The yield of compound (II) was 305 mg. The substance was electrophoretically and chromatographically homogeneous. On electrophoresis it migrated towards the anode. It was shown up by ninhydrin, periodate, $FeCl_3$ and hydroxylamine, Cl_2 and benzidine, and resorcinol. R_f in system 1 – 0.43; in system 2 – 0.64; in system 3 – 0.32.

Found, %: C 45.45; H 7.48. Calculated for $C_{11}H_{21}O_8N$, %: C 44.73; H 7.16.

The substance was hygroscopic, readily soluble in water and methanol, and insoluble in ether.

Methyl ester of N-(N'-carbobenzoxyglycyl)-methoxyneuraminic acid (III). A solution of 305 mg (0.001 mole) of the methyl ester of methoxyneuraminic acid in 5 ml of 95% aqueous pyridine was cooled to +4°C, 209 mg (0.001 mole) of N-carbobenzoxyglycine and 300 mg (0.015 mole) of N, N'-dicyclohexylcarbodiimide were added, and the mixture was left at +4° for a day. The pyridine was distilled off to dryness, residue was washed with absolute toluene, and the latter was also distilled off to dryness.

The dry residue was dissolved in a mixture of water and ether (25 + 25 ml) and filtered from the insoluble dicyclohexylurea; the aqueous solution was extracted with ether (10 × 10 ml) to eliminate the unchanged N-carbobenzoxyglycine and carbodiimide and the N, N'-dicyclohexylurea. The aqueous solution was extracted with butan-1-ol (5 × 15 ml), the butanolic extracts were washed with water (3 × 15 ml), the the butanolic solution was evaporated to small bulk and left overnight in the refrigerator. In the course of 2-3 days, crystals of condensation product precipitated; they were filtered off and washed with anhydrous n-butanol. The yield of crystalline substance (III) was 130 mg. Mp 189-190°C (on a Kofler block); $[\alpha]_D^{18}$ –25.28° (c 2.2; water).

The substance was chromatographically and electrophoretically homogeneous and was immobile on electrophoresis. It was readily soluble in pyridine, water, and methanol, and insoluble in ether. R_f in systems 1 and 3 – 0.7.

Found, %: C 52.03, 52.23; H 6.21, 6.20; N 6.05, 6.18. Calculated for $C_{21}H_{30}N_2O_{11}$, %: C 51.84; H 6.21; N 5.75.

By evaporating the mother liquor and lyophilizing the aqueous solution, 87 mg of a substance containing, together with the condensation product, another substance with a R_f value in system 2 of 0.9 was obtained. The combined

aqueous extracts were re-extracted with butan-1-ol (5 × 15 ml), the butanolic extract was washed with water (3 × 20 ml), and evaporated to dryness, and the residue was dissolved in water and lyophilized, giving an additional 67 mg of chromatographically and electrophoretically homogeneous condensation product.

The total yield of compound (III) was 58%. The combined aqueous extracts were lyophilized, giving 106.3 mg of unchanged initial methyl ester of methoxyneuraminic acid.

Methyl ester of N-glycylmethoxyneuraminic acid (IV). A solution of 47.4 mg (0.1 mole) of the methyl ester of N-(N'-carbobenzoxyglycyl)-methoxyneuraminic acid in 1 ml of 75% aqueous methanol was treated with 25 ml of Pd/$BaSO_4$ and 75 mg (0.55 mole) of oxalic acid and was hydrogenated at 20° for 45 min. The solid matter was centrifuged off, the solution was filtered through a dense filter, and the hydrogenation product was precipitated with a ten-fold volume of dry acetone. The flocculent precipitate was rapidly centrifuged off, dissolved in the minimum amount of water (2-3 drops), and precipitated with a large volume of dry acetone. The oil which separated was triturated to a powder with absolute acetone, giving 21 mg (60%) of substance (IV) in the form of the oxalate, mp 153-155°C (decomp., on the Kofler block), $[\alpha]_D^{18}$ −17.16° (c 1.9; water).

Found, %: C 42.83, 42.93; H 6.35, 6.53. Calculated for $C_{28}H_{50}N_4O_{22}$, %: C 42.31; H 6.34.

The substance was electrophoretically homogeneous and migrated towards the anode.

Oxidation of the methyl ester of N-(N'-carbobenzoxyglycyl)-methoxyneuraminic acid with periodate. A solution of 20.4 mg of the substance in 35 ml of an acetate buffer with pH 3.72 was treated with 5 ml of a 0.05 M solution of sodium metaperiodate (c 10.7 g/*l*) and was made up to 50 ml with distilled water. Oxidation was carried out at +5°C in the dark. After predetermined intervals of time (30 min, 1 hr, 2 hr, 3 hr, 5 hr), aliquots (5 ml) were taken and neutralized with 0.2 mg of $NaHCO_3$, and 1.5 ml of 4% $NaHCO_3$ solution (buffer) and 0.5 g of KI were added. The samples were left in the dark for 5 min and were titrated with sodium arsenite (to starch). After 1 hour, 1.955 mole of periodate had been absorbed. No over-oxidation was observed.

The paper chromatography was carried out on grade "B" paper of the Leningrad "Goznak" mill.

Summary

1. The methyl ester of N-(N'-carbobenzoxyglycyl)-methoxyneuraminic acid has been synthesized by the carbodiimide method.

2. Hydrogenation of the methyl ester of N-(N'-carbobenzoxyglycyl)-methoxyneuraminic acid over Pb/$BaSO_4$ has given the methyl ester of N-glycyl-methoxyneuraminic acid.

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