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Abstract

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Synthesis of the Methyl Ester of 9-O-Glycyl-N-acetylneuraminic Acid

Neuraminic acid (NANA), in the form of its N-acyl derivatives, is a constituent of the most important biopolymers that perform essential functions in the human and animal organism ([1]), and also of polysaccharides of various species of microorganisms ([2]).

Although the significance of NANA is still not clear in detail, the exceptional role of this monomer follows from the fact that many biopolymers completely lose their biological activity after cleavage of NANA residues occupying a terminal position in the chain (see, for example ([3])). There is every reason to suppose that NANA, for example, takes a direct part in phenomena of immunity.

Since most biopolymers containing NANA belong to mixed biopolymers, especially glycopeptides and glycolipids, the polyfunctional residues of NANA may be linked by various types of bonds with other units of the polymer chain, above all with monosaccharides and amino acids. It is known that in those compounds in which NANA occupies a terminal position, it is linked with neighboring carbohydrate units by an O-glycosidic bond and is readily cleaved during acid hydrolysis and under the action of neuraminidase ([4]). However, certain biologically important substances also contain NANA (from 30 to 50%) that is resistant to the action of neuraminidase ([5–7]). Nothing is known about the nature of such bonds of NANA with neighboring units. The great difficulties encountered in attempts to determine the character of these bonds in natural biopolymers make the study of model compounds, in particular aminoacyl derivatives of NANA, highly important. The preparation of such hitherto unknown compounds is hindered by the practically complete absence of data on the chemistry of NANA and on the preparation of even its simplest derivatives.

In the present article we report the first synthesis of the simplest O-aminoacyl derivative of NANA—methyl 9-O-glycyl-NANA—by the carbodiimide method ([8]); this is at the same time the first example of selective acylation. NANA was obtained by hydrolysis of serum protein from pig, horse, and human blood by the method developed by Svennerholm ([9]); the methyl ester of NANA (I) was obtained by methylation in methanol with diazomethane under strong cooling. Condensation of I with N-carbobenzoxyglycine was carried out by the previously developed method (8) in dry pyridine in the presence of N,N'-dicyclohexylcarbodiimide (I–III).

The sufficiently universal carbodiimide method for the synthesis of aminoacyl derivatives of sugars also proved very convenient in the case of I. On interaction of equimolecular amounts of the reagents in the cold, the condensation proceeded almost exclusively at the primary hydroxyl, with formation of methyl 9-O-carbobenzoxyglycyl-NANA (II). In the reaction mixture, chromatography revealed only traces of another reaction product, migrating above the main substance and apparently an acyl derivative at a secondary hydroxyl or a diacyl-

derivative of I. These data show that in I the primary hydroxyl group possesses a significantly higher activity toward acylation and, as in the case of hexoses, differs sharply from the other hydroxyls, despite the fact that in I the hydroxyl groups are in an open chain.

The structure of the compound II obtained was confirmed by positive tests on chromatograms with resorcinol reagent(10), potassium periodate cuprate, hydroxylamine with $FeCl_3$, and fluorescein.

$$\text{NANA} \xrightarrow{CH_2N_2} \text{methyl ester of NANA} \xrightarrow[\text{KDI, Py}]{K_{63}-NH-CH_2-COOH} \text{(II)}$$

$$\text{(II)} \xrightarrow[(COOH)_2]{Pd/BaSO_4} [(\text{III})]_2\,(COO^-)_2$$

$$K_{63} = C(=O)OCH_2C_6H_5; \qquad \text{KDI} \;\; C_6H_{11}N=C=N-C_6H_{11}$$

The final structure of the compound obtained was confirmed by periodate oxidation. The uptake of 2 moles of periodic acid unequivocally indicates that the compound obtained is a 9-*O*-aminoacyl derivative of NANA. To avoid overoxidation, periodate oxidation was carried out in acetate buffer at pH 3.72, in the cold and in the dark. To refine the oxidation conditions and compare the results, oxidation of NANA and I was carried out simultaneously.

The final product of the synthesis—the aminoacyl derivative with a free amino group (III)—was obtained by hydrogenolysis of II over $Pb/BaSO_4$ in aqueous methanol in the presence of oxalic acid. The oxalate III obtained is chromatographically and electrophoretically homogeneous and gives a positive reaction with ninhydrin.

As indicated, this compound is the first example of an *O*-aminoacyl derivative of NANA; the study of its properties is of undoubted interest for comparison with the properties of certain natural biopolymers containing NANA. Data on the stability of the compound obtained will be presented somewhat later.

Experimental part

Chromatography was carried out on Whatman No. 3 paper; the chromatograms were descending. Mobile phase: *n*-butanol—acetic acid—water, 4:1:1, upper layer (system 1); *n*-butanol—pyridine—water, 9:5:8, upper layer (system 2); *n*-propanol —0.1 N HCl—*n*-butanol, 2:1:1, upper layer (system 3); isobutanol saturated with water (system 4). Thin-layer chromatography on plates with silica gel was carried out in the system: chloroform—methanol, 7:3 (system 5). Electrophoresis was carried out in buffer: pyridine (2 ml)—acetic acid (4 ml)—water

(up to 1 l), pH 4.2-4.5 and a voltage of 900-1000 V. Spots on paper were detected with Svennerholm' s resorcinol reagent, Ehrlich' s reagent, potassium periodate-cuprate, ninhydrin, fluorescein, and hydroxylamine-$FeCl_3$. Spots on the plate were detected with Svennerholm' s resorcinol reagent, potassium periodate-cuprate, and hydroxylamine-$FeCl_3$.

Isolation of NANA was carried out by hydrolysis of pig, horse, or human blood-serum protein by the Svennerholm method. The blood serum was freed from erythrocytes by centrifugation, and the protein was precipitated with a fourfold volume of ethanol. The protein obtained from 4 liters of serum was washed at +4° with stirring with several portions of 0.01 N H_2SO_4 (total volume 20 l) to remove low-molecular impurities to pH 2.5-3, and was hydrolyzed with 5 l of 0.03 N H_2SO_4 at 80° for 1 hour. The unhydrolyzed protein was separated by centrifugation and again hydrolyzed with a fresh portion of acid. Triple hydrolysis is sufficient for practically complete cleavage of NANA from the protein. The combined hydrolysates were neutralized with saturated $Ba(OH)_2$ solution to pH 5-6 (according to methyl red). The $BaSO_4$ precipitate was separated by centrifugation; the clear solution was passed through a column with Dowex 50 × 8 cation exchanger (50-100 mesh) in the H^+ form, and then through a column (30 × 250) with Dowex 1 × 8 anion exchanger (100-200 mesh) in the $HCOO^-$ form. The second column was washed with 2 l of distilled water, and NANA was eluted with 0.3 N formic acid, collecting 150-ml fractions. Fractions 4-8, containing NANA, were diluted twice with distilled water and evaporated in vacuo at 40° with constant dilution with water to $^1/_3$ of the initial volume. The concentrated eluate was lyophilized, giving NANA in a yield of 0.5 g per liter of serum. The preparation was purified by crystallization; for this purpose 1 g of NANA was dissolved in 3 ml of distilled water, 10 volumes of dry methanol were added with careful heating to +40°, and then 15 volumes of dry ether. From 1.8 g of crude NANA, 1.3 g of pure NANA was obtained, m.p. 182-184° (with decomposition in a Kofler block), $[\alpha]_D^{18} - 32.98°$ (C 0.496, water). The IR spectrum of NANA is identical with that reported in the literature (^{11}). R_f of NANA in system 2 is 0.19; in system 3, 0.35.

Methyl ester of NANA (I). To a solution of 1 g (0.0032 M) of NANA in 150 ml of absolute methanol, with caution and cooling with dry ice in acetone, an ethereal solution of diazomethane was added dropwise until the yellow diazomethane solution ceased to become decolorized and nitrogen was evolved.

After distillation of the solvent in vacuo to dryness, a chromatographically pure ester was obtained in quantitative yield. After recrystallization from a mixture of absolute methanol with dry ether, I was obtained with m.p. 185-186° (with decomposition in a Kofler block), $[\alpha]_D^{20} 32.3°$ (C 0.511, water), R_f of I in systems 1, 2, 4, 5 respectively 0.4; 0.51; 0.26; 0.32.

Found, %: C 44.78; H 6.60; N 4.50; 4.38
$C_{12}H_{21}NO_9$. Calculated, %: C 44.58; H 6.54; N 4.64

Methyl ester of 9-O-(N-carbobenzoxyglycyl)-N-acetylneuraminic acid (II).
To a cooled solution of 625 mg (0.0019 M) of I in 20 ml of absolute pyridine and 403 mg (0.0019 M) of N-carbobenzoxyglycine, 492 mg (0.0027 M) of N—N′-dicyclohexylcarbodiimide was added, and the mixture was left for 49 hours at +5°. The pyridine was distilled off in vacuo to dryness; the residue was treated with a mixture of 50 ml of water and 25 ml of ether, the insoluble N,N′-dicyclohexylurea was filtered off, the aqueous layer was separated and extracted with ether (25 ml × 10), and the ethereal extracts were washed with water (25 ml × 5), which was combined with the main aqueous solution. The aqueous solution, slightly acidified with acetic acid, was extracted with *n*-butanol (25 ml × 15); the butanol extracts, washed with water (15 ml × 5), were evaporated in vacuo to dryness. Yield of II 469.2 mg (47%), m.p. 100-102° (with decomposition in a···

Kofler block), $[\alpha]_D^{28}$ 18.68° (C 0.493, water), R_f II in systems 1, 2, 4, 5 respectively 0.74; 0.83; 0.69; 0.62.

Found, %: C 51.52; 51.31; H 6.22; 6.29
$C_{22}H_{30}N_2O_{12}$. Calculated, %: C 51.36; H 5.87

Methyl ester of 9-O-glycyl-N-acetylneuraminic acid (oxalate) III. 103 mg (0.2 mM) of II was dissolved in 2 ml of 75% methanol; 51.5 mg of $Pd/BaSO_4$, 15.1 mg (0.12 mM) of oxalic acid were added, and the mixture was hydrogenated for 2 hours. The catalyst was separated by centrifugation; the filtrate was diluted with a fivefold volume of absolute acetone; the flocculent precipitate that separated was collected by centrifugation, dissolved in a minimal amount of water (2-3 drops), and the substance was precipitated with absolute acetone. The oil was triturated 3 times in absolute acetone until it was converted into a powder; the residual acetone was removed in vacuo. 45 mg of chromatographically and electrophoretically homogeneous substance III was obtained, $[\alpha]_D^{27} - 15.27°$ (C 0.511, water).

Found, %: C 42.81; H 5.93
$C_{30}H_{50}N_4O_{24}$. Calculated, %: C 42.35, 42.26; H 6.06, 6.23

Periodate oxidation of NANA, I, II. 20 mg of the substance was dissolved in 35 ml of acetate buffer with pH 3.72; 10 ml of 0.05 N aqueous $NaJO_4$ solution was added; the volume was brought to 50 ml with distilled water and the mixture was left in the dark at +4°. Periodically, 5-ml samples were taken, neutralized with 0.2–0.25 g of $NaHCO_3$ and 1.5 ml of cooled 4% sodium bicarbonate solution; 0.5 g of KJ was added, and titration was carried out with sodium arsenite. In the oxidation of NANA, 2.48 moles were consumed in the first hour, 3.08 moles in 3 hours, and 3.12 moles of HJO_4 in 17 hours; in the oxidation of I, 2.83 moles in the first hour, 3.09 moles in 6 hours, and 3.22 moles of HJO_4 in 24 hours; in the oxidation of II, 1.9 moles were consumed in the first hour, 2.08 moles in 4 hours, and 2.11 moles of HJO_4 in 22 hours.

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