ON THE SPECIFICITY OF NEURAMINIDASE
Synthesis of benzyl 5-acetamido-3,5-dideoxy-β-L-*arabino*-heptulosidaric acid, an α-ketosidic dicarboxylic derivative of *N*-acetyl-D-neuraminic acid resistant to *Vibrio cholerae* neuraminidase

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1. Introduction

In connection with our studies on the specificity of neuraminidase, the carboxymethyl α-ketoside of *N*-acetyl-D-neuraminic acid [1] and the benzyl α-ketoside of *N*-3-carboxypropionyl-D-neuraminic acid [2], both having two anionic sites have been synthesized. The transformation [3] of the carboxyl group in neuraminidase substrates to an alcohol and on amido group and the introduction of a cationic group in the aglyconic part of the substrate [4], have previously revealed that the carboxyl group in the glycone seems essential for the enzymatic cleavage of the α-ketosidic linkage.

In contrast to the carboxymethyl α-ketoside, being readily cleaved, the *N*-3-carboxypropionyl derivative was found not to be cleaved by *Vibrio cholerae* neuraminidase, nor did the compound inhibit the enzyme activity.

It has been demonstrated, however, that an exchange of the *N*-acetyl group in the substrate molecule to a *N*-propionyl, reduces the rate of the enzymatic hydrolysis of the α-ketosidic bond. An *N*-butyryl group instead of the *N*-acetyl abolishes the property of the ketoside to serve as a substrate for neuraminidase [5]. It was therefore suggested that the additional carboxyl group in the *N*-3-carboxypropionyl derivative sterically hindered the α-ketoside to reach the active center in the enzyme [2].

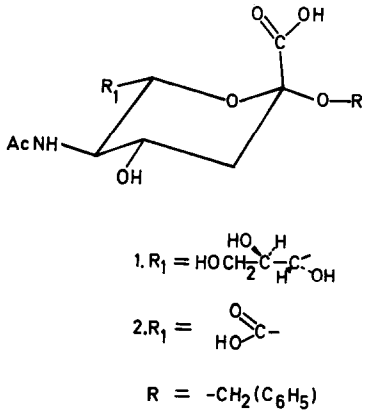

Fig. 1. Structure of the benzyl α-ketoside of *N*-acetyl-D-neuraminic acid, R_1 = 1 and the benzyl 5-acetamido-3,5-dideoxy-β-L-*arabino*-heptulosidaric acid, R_1 = 2.

Recently, however, it has been shown that the benzyl α-ketosides of 5-acetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*-nonulosaric acid [6] and 5-acetamido-3,5,9-trideoxy-D-*glycero*-β-D-*galacto*-deculosaric acid [7] also are resistant to the hydrolytic action of neuraminidase. These compounds were obtained from *N*-acetylneuraminic acid by oxidation of the primary alcohol group and by chain elongation, respectively. The present paper describes the synthesis and some properties of 5-acetamido-3,5-dideoxy-L-*arabino*-heptulosaric acid and its benzyl β-ketoside.

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The latter compound is related to the benzyl α-ketoside of *N*-acetyl-D-neuraminic acid in having a carboxyl group instead of a polyhydroxy side chain equatorially attached to the pyranosidic ring (fig. 1).

Being a L-sugar acid derivative, the β-anomer of the benzyl ketosides of the heptulosaric acid has the same absolute anomeric configuration as the benzyl α-ketoside of *N*-acetyl-D-neuraminic acid.

2. Materials and methods

2.1. *General methods*

Concentrations were carried out under reduced pressure at a bath temperature not exceeding 40°C.

NMR-analyses were performed with Varian A-60 A and 100 MHZ instruments and IR-analyses with a Perkin–Elmer 225 Grating Infrared Spectrometer. Optical rotations were determined with a Perkin–Elmer 141 polarimeter.

TLC was carried out on silica gel (Merck PF_{254}) with the solvents: (a) propanol: butanol: 0.1 M hydrochloric acid 2:1:1; (b) ethyl acetate, and paper electrophoresis in 0.2 M Veronal buffer pH 10, 10.6 V/cm. Spots were detected as previously described [3], R_{NA}-values represent the R_F-values of substances relative to *N*-acetylneuraminic acid (R_F 1.00).

Incubations with neuraminidase (25 units) were carried out as previously reported [3]. Neuraminidase from *Vibrio cholerae* (glycoprotein-*N*-acetylneuraminyl hydrolase, EC 3.2.1.18) was purchased from Behring–Werke, Marburg; 1ml containing 500 units (producer's specification).

2.2. *Syntheses*

2.2.1 Benzyl 5-acetamido-3,5-dideoxy-β-L-*arabino*-heptulosidaric acid

A solution of periodic acid (150 mg) in water (10 ml) was dropwise added to the methyl ester of the benzyl α-ketoside of *N*-acetylneuraminic acid (110 mg) dissolved in water (10 ml) at room temperature. The consumption of periodic acid was very fast and when the reaction mixture produced a constant blue colour with benzidine, the rest of the periodic acid solution was added. The time for the reaction was about 5 min. The solution was passed through a column with Dowex 1 × 4 (acetate form) ion exchanger. After washing the column with water the combined effluent and washings were freeze dried giving a product (100 mg) which was dissolved in water (10 ml) and shaken with bromine (50 mg) and barium carbonate (600 mg) for 15–60 min at room temperature.

The reaction mixture was filtered, the filtrate diluted with water and concentrated to remove excess bromine. After treatment with Dowex 50 W × 8 (H^+), silver carbonate and Dowex 50 W × 8 (H^+) again the solution was put on a column with Dowex 1 × 4 (1 × 5 cm, acetate form) ion exchanger. The column was washed with water and 2 M acetic acid and the product eluted with 4 M acetic acid.

When the substance was eluted completely, as controlled with Ehrlich's reagent, the eluate was concentrated to dryness, dissolved in some water and freeze dried. The resulting substance (50 mg) was dissolved in 1 M sodium hydroxide (5 ml). After 15 min at room temperature the solution was treated with Dowex 50 W × 8 (H^+) to remove sodium ions and freeze dried giving a chromatographically and electrophoretically homogenous dicarboxylic acid. The yield was 45 mg (45%). TLC: R_{NA} 1.87, solvent (a)

$[\alpha]_D^{25} = -23°C$ (*c* 1.0 in water)

Found: C 51.73; H 5.35; N 3.73

Calc. for $C_{16}H_{19}NO_8 \times H_2O$ C 51.77; H 5.70; N 3.77.

2.2.2. Dimethyl (benzyl 5-acetamido-4-*O*-acetyl-3,5-dideoxy-β-L-*arabino* heptulosid)arate

The benzyl α-ketoside of the dicarboxylic acid (25 mg) was transformed to its dimethylester with diazomethan. To a cooled solution of the ester in dry pyridine (0.5 ml) was dropwise added acetic anhydride (0.5 ml). After stirring overnight the reaction mixture was poured in ice–water and stirred for 2 hr. The solution was extracted with chloroform, the combined fractions being dried with sodium sulphate and concentrated to dryness. The residue was dissolved in ethanol and deionized

with Dowex 50 W X 8 (H^+). Evaporation of solvent yielded a syrup containing minor amounts of starting material as an impurity. The product was analyzed spectrometrically without further purifications.

TLC: R_{NA} 3.60, solvent (a)
R_F 0.21, solvent (b)

2.2.3. 5-Acetamido-3,5-dideoxy-α-L-*arabino*-heptulosaric acid

A solution of pure benzyl α-ketoside of the heptulosaric acid (25 mg) in ethanol–water (1:1, 10 ml) was added to palladium on charcoal (2 mg, chloride free) in ethanol–water (1:1, 10 ml). Hydrogenation for 10 hr at room temperature, removal of catalyst and evaporation of solvent yielded the acid as a syrup. The product was dissolved in water and freeze dried giving an electrophoretically and chromatographically homogeneous substance. The yield was 17 mg (93%). TLC: R_{NA} 0.80, solvent (a).
$[\alpha]_D^{25} = -28°C$ (c = 0.5 in water)
Found: C 39.87; H 5.04; N 5.36
Calc. for $C_9H_{13}NO_8 \times 1/2\ H_2O$ C 39.71; H 5.15; N 5.15.

3. Results and discussion

The new dicarboxylic derivatives of *N*-acetyl-D-neuraminic acid were prepared by periodate oxidation of the methyl ester of the benzyl α-ketoside of *N*-acetylneuraminic acid and oxidation of the formed formyl derivative with bromine–water in the presence of barium carbonate. The product was purified by ion exchange chromatography on Dowex 1 (acetate form), giving after saponification an electrophoretically and chromatographically homogeneous benzyl 5-acetamido-3,5-dideoxy-β-L-*arabino*-heptulosidaric acid. Methylation with diazomethane produced a dimethylester and acetylation with acetic anhydride in the presence of pyridine resulted in a mono-*O*-acetyl derivative. Hydrogenolysis of the ketosidic benzyl group yielded the free dicarboxylic acid. The proposed structures were confirmed by IR- and NMR-analyses. The dicarboxylic acids moved about 20% faster than the parent substances by electrophoresis in Veronal buffer, pH 10 and by titration correct equivalent weights were obtained. The heptulosaric acids formed humines faster than *N*-acetylneuraminic acid and did not develop, in contrast to the latter, a blue-violet colour with Bial's orcinol reagent.

In contrast to α-ketosides of the 7-carbon analogues of *N*-acetylneuraminic acid [8], the ketosidic linkage in the benzyl ketoside of the dicarboxylic acid was not cleaved by *Vibrio cholerae* neuraminidase, nor did the free or ketosidically bound heptulosaric acid inhibit the enzyme, using the benzyl α-ketoside of *N*-acetylneuraminic acid as substrate. These results are in agreement with the findings [6] from the oxidation of the primary hydroxyl group in the side chain of the benzyl α-ketoside of *N*-acetylneuraminic acid.

Attractant or repulsive forces between charged groups in the active center in neuraminidase and the new anionic site in the dicarboxylic acid may explain the failure of the enzyme to catalyze the hydrolysis of the α-ketosidic linkage in the *N*-acetylneuraminic acid derivative.

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References

[1] Holmquist, L. and Brossmer, R. (1972) FEBS Letters 22, 46.
[2] Brossmer, R. and Nebelin, E. (1969) FEBS Letters 4, 335.
[3] Brossmer, R. and Holmquist, L. (1971) Hoppe Seyler's Z. Physiol. Chem. 352, 1715.
[4] Holmquist, L. and Brossmer, R. (1972) Hoppe Seyler's Z. Physiol. Chem. 353, 1346.
[5] Meindl, P. and Tuppy, H. (1966) Mh. Chem. 97, 1628.
[6] Brossmer, R. and Neumann, B., to be published.
[7] Brossmer, R. and Bürk, G., to be published.
[8] McLean, R. L., Suttajit, M., Beidler, J. and Winzler, R. (1971) J. Biol. Chem. 246, 803.