ON THE SPECIFICITY OF NEURAMINIDASE

The carboxymethyl α-ketoside of N-acetyl-D-neuraminic acid, a *Vibrio cholerae* neuraminidase substrate having two anionic sites

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1. Introduction

In a previous study on the specificity of *Vibrio cholerae* neuraminidase [1] the carboxyl group of the benzyl α-ketoside of *N*-acetyl-D-neuraminic acid was transformed into a primary alcohol and an amido group giving derivatives of 5-*N*-acetyl-D-nonulosamine and 5-*N*-acetyl-D-neuraminamide, respectively. The resulting compounds were not cleaved by neuraminidase, nor did they inhibit the enzyme activity.

These data strongly support the assumption that the anionic site of the *N*-acetylneuraminic acid molecule in neuraminidase labile ketosides is necessary for the enzymatic hydrolysis of the α-ketosidic bond.

In the present work the synthesis of the carboxymethyl α-ketoside of *N*-acetylneuraminic acid was performed in order to obtain a ketoside with all the structural requirements for being cleaved by neuraminidase but having a second anionic site close to the carboxyl group of the nonulosaminic acid (fig. 1). This additional anionic site was hoped to compete with the original one at its binding to the enzyme, or to bind to any basic function near the active center of the neuraminidase. The possibility was envisaged that such an extra binding could cause a disturbance in the catalytic function of the enzyme or promote the formation of a strong enzyme–substrate complex.

In addition the carboxymethyl β-ketoside was prepared. Also having an axially orientated carboxyl group (fig. 1), this ketoside is a potential inhibitor of the neuraminidase.

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2. Material and methods

2.1. *General methods*

The same general methods were used as previously described [1], In addition TLC was performed in propanol:butanol:0.1 M hydrochloric acid, 2:1:1, and paper electrophoresis in sodium borate:sodium chloride buffer, pH 7.8, 6 V/cm. Spots were detected with Ehrlich's p-dimethyl-aminobenzaldehyde reagent.

Incubations with neuraminidase (12.5 units) were carried out in 0.05 M Tris-maleate buffer pH 6.40, 0.005 M with respect to calcium chloride at 37° in a total volume of 0.5 ml. Samples were taken from the incubation mixtures at different intervals of time and immediately frozen. Released *N*-acetylneuraminic acid was determined according to Warren [2].

Neuraminidase from *Vibrio cholerae* (glycoprotein-*N*-acetylneuraminyl-hydrolase, EC 3.2.1.18) was purchased from Behring-Werke, Marburg; 1 ml containing 500 units (producer's specification).

2.2. *Syntheses*

2.2.1. Carboxymethyl α-ketoside of *N*-acetyl-D-neuraminic acid

Acetochloroneuraminic acid [3] prepared from peracetylated neuraminic acid (1 g) was shaken with

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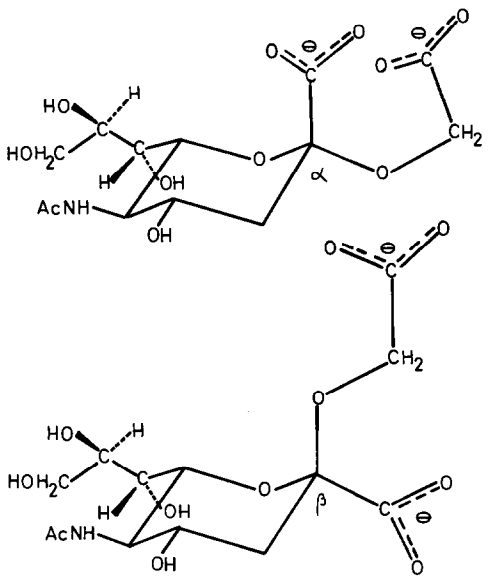

Fig. 1. Carboxymethyl α- and β-ketosides of *N*-acetylneuraminic acid.

silver carbonate (500 mg), pulverized Drierite (2 g) and redistilled butyl glycollate (10 g) for 48 hr.

The mixture was filtered and solids washed with acetone–water (1:1, v/v). The combined filtrate and washings were concentrated to a sirup which was dissolved in a small volume of acetone–water (1:1, v/v). The solution was passed through a Dowex 1 X 4 column (acetate form, 2 X 15 cm).

After washing the column the *N*-acetylneuraminic acid derivatives were eluted according to [4]. The sirup obtained after evaporation of solvent was dissolved in chloroform (20 ml). This solution was shaken with water (3 X 20 ml). After being dried with magnesium sulphate the organic layer was concentrated to dryness giving the peracetylated butoxycarbonylmethyl α-ketoside as a hard glass containing only traces of impurities (370 mg).

Crystallization from water yielded the pure compound. The peracetylated compound was hydrolyzed according to [1] giving a chromatographically homogeneous carboxymethyl α-ketoside. Attempts to crystallize the product proved to be unsuccessful so far. Physical constants and analyses are given in table 1.

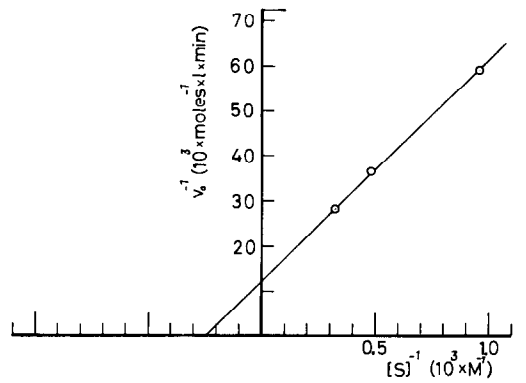

Fig. 2. Dependence of the initial rate of enzymatic hydrolysis at pH 6.4 of the carboxymethyl α-ketoside of *N*-acetylneuraminic acid on its concentration.

2.2.2. Carboxymethyl β-ketoside of *N*-acetyl-D-neuraminic acid

This compound was prepared as above using methylglycollate (1 g). The product obtained from the ion exchange column was hydrolyzed with 1 M sodium hydroxide for 15 min at room temp. After deionization with Dowex 50W X8 (H^+-form) the solution was freeze dried. The resulting complex mixture was chromatographed on a DEAE-Sephadex A-25 column (bicarbonate form, 2 X 50 cm) usign a linear 0.01–0.5 M ammonium bicarbonate gradient, giving two main fractions containing mono- and dicarboxylic acids, respectively. The fractions containing the anomeric dicarboxylic acids were collected and freeze dried, dissolved in water, deionized with Dowex 50W X8 (H^+-form) and freeze dried again. The product (260 mg) was dissolved in hot methanol (10 ml) and ethyl ether (10 ml) and petroleum ether (10 ml) was added. On standing at room temp the β-ketoside crystallized as well formed needles (50 mg).

Physical constants and analyses are given in table 1.

The mother liquid gave after concentration to dryness an impure α-ketoside.

3. Results

The carboxymethyl α-ketoside of *N*-acetylneuraminic acid was prepared by the condensation of acetochloroneuraminic acid with butylglycollate using the Königs-Knorr technique according to [3].

Table 1
Properties of carboxymethyl α- and β-ketosides of *N*-acetyl-D-neuraminic acid.

Derivative of *N*-acetylneuraminic acid	Mp. (dec) (°C)	$(a)_D^{25°}$ (*C* 0.5)	Formula	Calculated			Found		
				C (%)	H (%)	N (%)	C (%)	H (%)	N (%)
Tetra-*O*-acetyl-butoxy-carbonyl-methyl α-ketoside	184–186	−10° (methanol)	$C_{25}H_{37}NO_{15}$	50.76	6.31	2.37	50.59	6.24	2.18
Carboxymethyl α-ketoside	not crystalline	− 9° (water)	$C_{13}H_{21}NO_{11}\cdot H_2O$	40.52	6.01	3.60	40.0	5.7	3.8*
Carboxymethyl β-ketoside	193–195	−24° (water)	$C_{13}H_{21}NO_{11}$	42.51	5.76	3.81	42.3	5.9	3.8*

* Ultramicro analysis.

Water extraction of impurities from a solution of the crude reaction product in chloroform conveniently yielded the pure peracetylated butoxycarbonyl α-ketoside which crystallized from water. Mild alkaline hydrolysis of ester groups resulted in the carboxymethyl α-ketoside. Using methylglycollate instead of butylglycollate, saponification of the crude reaction product and ion exchange chromatography on DEAE-Sephadex A-25 (bicarbonate-form) the carboxymethyl β-anomer of *N*-acetylneuraminic acid could be isolated in crystalline form.

The structures of the *N*-acetylneuraminic acid derivatives were confirmed by IR-analysis. By titration of the acids a correct equivalent weight was obtained but only one inflexion point was observed. The dicarboxylic acids moved faster than *N*-acetylneuraminic acid by electrophoresis in borate buffer.

The carboxymethyl α-ketoside of *N*-acetylneuraminic acid was found to be completely cleaved by *Vibrio cholerae* neuraminidase, the enzymatic reaction having a pH optimum between 6 and 6.5.

By means of the graphical method of Lineweaver and Burk a K_m-value of 4×10^{-3} M at pH 6.4 was obtained for the enzymatic hydrolysis of the carboxymethyl α-ketoside (fig. 2).

It has been demonstrated [5] that the K_m-values obtained with different *Vibrio cholerae* neuraminidase substrates containing neutral aglycones is in the range $1-7 \times 10^{-3}$ M at pH 6.4, having pH optima for the cleavage between 6 and 6.5.

Thus the additional anionic site in the aglycone of the carboxymethyl α-ketoside of *N*-acetylneuraminic acid has no drastic effect on the neuraminidase activity. The carboxymethyl β-ketoside was not cleaved by the enzyme, nor was its activity inhibited by this substance.

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