SYNTHETIC INHIBITORS OF *VIBRIO CHOLERAE* NEURAMINIDASE AND NEURAMINIDASES OF SOME INFLUENZA VIRUS STRAINS

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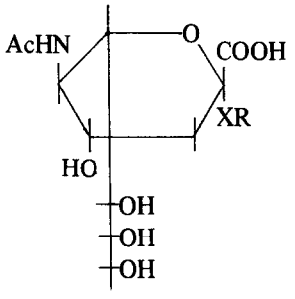
1. Introduction

Specific inhibitors of neuraminidases (E.C.3.2.1.18) have been found to be effective tools in mechanistic studies of these enzymes and in elucidation of their biological function, especially for the case of neuraminidases from pathogenic bacteria and viruses [1–4]. In this communication, the interaction of neuraminidases from *Vibrio cholerae* and from some strains of influenza viruses with 2-*O*-*p*-nitrophenyl-(1), 2-methylthio-(2), 2-*p*-nitrophenylthio-(3), 2-*p*-nitrophenylamino-2-deoxy-*N*-acetyl-α-D-neuraminic acid(4) and with a structural analog of *N*-acetyl-D-neuraminic acid (NANA), i.e. 3-aza-2,3,4-trideoxy-4-oxo-D-arabino-octonic acid δ-lactone(5) were investigated.

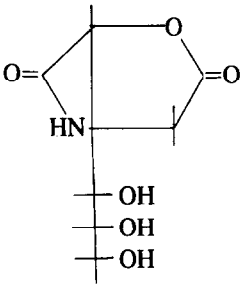
Synthetic ketoside (1) was suggested as a convenient substrate for kinetic studies of neuraminidases. Ketosides (2)–(4) were shown to be competitive inhibitors of the enzymes. The strongest inhibitor activity was exhibited by the δ-lactone (5). Compounds (2)–(5) differ in their inhibitory actions towards neuraminidases of different origin, and these differences, which appear to be closely associated with the specificity of the enzymes, could be used as an additional test for distinguishing infuenza virus strain.

2. Materials and methods

Vibrio cholerae neuraminidase (N.V.Philips-Duphar,



(1), X = O, R = *p*-$NO_2C_6H_4$
(2), X = S, R = CH_3
(3), X = S, R = *p*-$NO_2C_6H_4$
(4), X = NH, R = *p*-$NO_2C_6H_4$



(5)

Holland) was employed. Preparations of virus strains A2/Singapore/1157, A2/USSR/0395/69, A2/USSR/O46/69, B/Berkeley/3/69, A/Swine/15/31 and parainfluenza types 2 and 3 were also used as sources of the enzyme. Viruses were propagated in the allantoic cavity of 10–11-day-old chick embryos and purified by means of differential centrifugation or by adsorbtion on 50% formalinased chick erythrocytes, with subsequent elution with 3% NaCl solution [5].

Collocalia mucoid [6] and *p*-nitrophenyl *N*-acetyl-α-D-neuraminoside [7] were used as substrates. Inhibitors (2)–(4) were synthesized as described in [7]. Lactone (5) was obtained by condensation of D-arabinic acid γ-lactone with glycine methyl ester hydrochloride in pyridine in the presence of equimolecular amounts of dicyclohexylcarbodiimide and 1 N KOH; m.p. 213–215° (from ethanol, decomp.), $[\alpha]_D^{20}$-2.5 ± 2° (Found: C 40.27, H 5.32, N 6.30%. Calc. for $C_7H_{11}O_6N$ C 40.98, H 5.41, N 6.83%).

Hydrolysis of substrate (1) with *V. cholerae* neuraminidase was performed by incubation in 0.1 M acetate buffer, pH 5.6 containing 0.9% NaCl and 0.1% $CaCl_2$ at 37°, and 1.17 mM substrates. Liberated *p*-nitrophenol was determined spectrophotometrically at 400 nm. Hydrolyses with viral neuraminidases were carried out in 0.3 M phosphate buffer, pH 5.8 and 37° for 30 min. Virus eluate (0.1 ml) and 500 μg/ml of Collocalia mucoid were used for each test. NANA liberated in the cource of hydrolysis was determined by the thiobarbiturate technique [8]. In all the experiments, the concentration of the inhibitors (2)–(5) was 1.1 mM. In control tests substrates were incubated without enzymes.

3. Results and discussion

The rate of *V. cholerae* neuraminidase-catalysed hydrolysis of (1) measured by *p*-nitrophenol release is identical to that obtained by the thiobarbituric acid method, but the former procedure significantly simplifies kinetic studies of the reaction. At a substrate concentration of 1.7 mM, the rate of enzymatic hydrolysis is a linear function of enzyme concentration in the range 0.1–0.5 mg/ml. Results were plotted according to Lineweaver and Burk and values for K_m of 1.67 mM and V_{max} of 53 μmoles/l. min were obtained.

Vibro cholerae and viral neuraminidases do not split *S*-ketosides (2) and (3) and *N*-ketoside (4) during 3 hr incubation. Compounds (2)–(5) were proved to be competitive inhibitors of the enzyme hydrolysis of the *O*-ketone (1), K_i-values being given in table 1.

Table 1

K_i-Values for NANA and compounds (2)–(5) obtained from hydrolysis of *p*-nitrophenyl *N*-acetyl-α-D-neuraminoside with *V. cholerae* neuraminidase.

Compound	K_I (mM)
NANA	4.88
2-Deoxy-2-*p*-nitrophenylamino-*N*-acetyl-α-D-neuraminic acid (4)	2.34
2-Deoxy-2-methylthio-*N*-acetyl-α-D-neuraminic acid (2)	0.84
2-Deoxy-2-*p*-nitrophenylthio-*N*-acetyl-α-D-neuraminic acid (3)	0.23
3-Aza-2,3,4-trideoxy-4-oxo-D-arabino-octonic acid δ-lactone (5)	0.04

The highest inhibitory activity was exhibited by lactone (5). On simultaneous incubation of substrate (1) and δ-lactone (5) with *V. cholerae* neuraminidase, compound (5) behaves as a competitive inhibitor, not affecting V_{max} for hydrolysis of substrate (1) (see table 1). A 3 hr preincubation of the enzyme with lactone (5) at 37° leads, however, to complete inhibition of neuraminidase activity. A 12 hr dialysis of the preincubated mixture against 0.1 M acetate buffer (pH 5.6) at 0° results in partial (about 25%) reactivation of the enzyme. Thus the above data suggest that lactone (5) forms at least two types of enzyme-inhibitor complex with *V. cholerae* neuraminidase.

Inhibition of neuraminidase activity of some human and animal influenza viruses with compounds (2)–(5), measured by hydrolytic release of NANA from Collocalia mucoid after 30 min incubation at 37°, is summarized in table 2.

These findings show that compounds (2)–(5) differ in their inhibitory action towards the different influenza-strain neuraminidases, lactone (5) exhibiting the highest inhibitory activity. Compound (5) completely inhibits the neuraminidase activity of most of the viruses investigated, including the original pandemic variant A2/Sing 1/57 and Hong-Kong line strain A2/USSR 0395/69. Both these varieties are known to

Table 2
Inhibition of viral neuraminidase activity with compounds (2)–(5).

Virus strain	NANA Released (μmoles)				
	Control	In presence of inhibitors			
		(2)	(3)	(4)	(5)
A2/Sing 1/57	112	–	–	–	0
A2/USSR/0395/69	35.2	22.4	0	28.8	0
A2/USSR/046/69	64	–	–	–	0
B/Berkeley/3/69	35.2	16	0	28.8	0
A/Swine/15/31	48	41.6	0	38.4	0
parainfluenza, type 2	54.4	41.6	22.2	32	54.4
parainfluenza, type 3	35.2	25.6	25.2	32	25.6

possess neuraminidases belonging to different kinetic groups [9]. The neuraminidases of the parainfluenza viruses of types 2 and 3 are not affected by inhibitors (2)–(5), which is indicative of a somewhat abnormal specificity of the enzymes in question. It is noteworthy that compounds (2)–(5) did not inhibit the hemagglutinating action of the influenza and parainfluenza viruses studied.

The inhibitory activities of lactone (5) on *V. cholerae* neuraminidase under the conditions where the compound behaves as competitive inhibitor, and that of 2-deoxy-2,3-dehydro-*N*-acetyl-neuraminic acid (6) described by Meindl and Tuppy [10], are of the same order. The high affinity of inhibitors (5) and (6) for the enzyme seems to indicate that the conformations of these compounds closely imitate the strained conformation of NANA residues in the substrates bonded to the active site.

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