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Interaction of *N*-Acetyl-4-, 7-, 8- or 9-deoxyneuraminic Acids and *N*-Acetyl-4-, 7- or 8-mono-epi- and -7,8-di-epineuraminic Acids with *N*-Acetylneuraminate Lyase

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Various deoxy- and epi-derivatives of *N*-acetylneuraminic acid were synthesized and tested for their substrate properties with *N*-acetylneuraminate lyase from *Clostridium perfringens*. *N*-Acetyl-9-deoxyneuraminic acid is a good substrate, *N*-acetylneuraminic acid derivatives with epimeric configuration at C-7, C-8 or both are cleaved slowly, while *N*-acetyl-4-epi-, *N*-acetyl-4-deoxy-, *N*-acetyl-7-deoxy- and *N*-acetyl-8-deoxyneuraminic acid are resistant to enzyme action. *N*-Acetyl-4-deoxyneuraminic acid and *N*-acetyl-4-epineuraminic acid competitively inhibit the enzyme. These studies give further insight into a mechanism proposed for the reversible cleavage of sialic acids by *N*-acetylneuraminate lyase.

N-Acetylneuraminate lyase (EC 4.1.3.3) reversibly cleaves sialic acids into acylmannosamines and pyruvate [1, 2] and thus may have a regulatory role in the metabolism of sialic acids. This enzyme occurs regularly in cells of higher animals and in some bacterial species [1, 2]. The lyase has been purified e.g. from *Clostridium perfringens* [3, 4] and *Escherichia coli* [5, 6], and has also been isolated from pig kidney [7]. The partial purification from various other microbial and mammalian sources has also been described, as summarized in [1, 2, 8]. The enzyme from *E. coli* has been cloned by two groups and its nucleotide sequence described [5, 9].

In the catalysis of the lyase from *C. perfringens* [10] and pig kidney (Schauer *et al.*, unpublished results) evidence for the participation of lysine residues forming intermediary Schiff bases between enzyme and substrate molecules, as well as of

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histidine residues, was obtained. Especially the interaction of sialic acids with the latter amino acid gives an explanation for the resistance of sialic acids with substituted hydroxyl groups at C-4 (*N*-acetyl-4-*O*-acetylneuraminic acid and *N*-acetyl-4-*O*-methylneuraminic acid) towards the lyase [11-13]. From this evidence for the requirement of a free hydroxyl group at C-4 for lyase action involving a proton transfer to the histidine residue during the realdolization process [10] it is to be expected that a sialic acid lacking a hydroxyl group at this C-atom (*N*-acetyl-4-deoxyneuraminic acid; 4-deoxy-Neu5Ac) is also resistant towards the action of the lyase. Further insight into the mechanism of the lyase action was expected from the study of a sialic acid having a hydroxy group at C-4 with inverse configuration (*N*-acetyl-4-epineuraminic acid; 4-epi-Neu5Ac) when compared with the natural compound. These investigations revealed that both synthetic sialic acids cannot be cleaved by the lyase, but appear to interact with the substrate binding site of the enzyme in a competitive manner.

Earlier studies have shown that the lyase requires an intact glycerol side chain of sialic acid for full activity [14, 15]. While the C-8 analogue of Neu5Ac obtained by periodate oxidation and borohydride reduction showed only 20% of the activity observed with Neu5Ac, the C-7 analogue was not cleaved at all by the enzyme. It was therefore of interest to study the influence of chemically modified side chains of Neu5Ac, i.e. the behaviour of various deoxy- and epi-derivatives towards lyase activity. Strong influences on enzyme activity were observed with these synthetic substances.

Materials and Methods

The following substances were tested, the synthesis of which has been described previously: 4-deoxy-Neu5Ac [16], *N*-acetyl-7-deoxyneuraminic acid (7-deoxy-Neu5Ac) [17], *N*-acetyl-8-deoxyneuraminic acid (8-deoxy-Neu5Ac) [17], *N*-acetyl-9-deoxyneuraminic acid (9-deoxy-Neu5Ac) [17], 4-epi-NeuAc [18, 19], *N*-acetyl-7-epineuraminic acid (7-epi-Neu5Ac) [20], *N*-acetyl-8-epineuraminic acid (8-epi-Neu5Ac) [20] and *N*-acetyl-7,8-di-epineuraminic acid (7,8-di-epi-Neu5Ac) [20]. Sodium salts of these sialic acids or of Neu5Ac (obtained as a gift from MECT, Tokyo) as reference compound were incubated at 1 mM concentration in 50 mM sodium phosphate buffer, pH 7.2, in the presence of 80 mU/ml lyase from *C. perfringens* (Sigma, Munich, W. Germany) for various times between 2 and 12 min at 37°C (total assay volume 600-800 µl). Blanks without the enzyme were run in parallel. Pyruvate which may have been formed during the enzyme reaction corresponds to the amount of sialic acid cleaved and was determined with the aid of lactate dehydrogenase (LDH) similar to the methods described [11, 21, 22]: 100 µl of the incubation mixtures described above were removed every 2 min and, after inactivation of the lyase by heating at 94°C for 2 min, mixed with 700 µl H_2O, 180 µl 50 mM sodium phosphate buffer, pH 7.2, and 100 µl 2 mM NADH solution (Boehringer Mannheim, Mannheim, W. Germany). The extinction of this solution was measured at 340 nm, and the enzyme reaction started by the addition of 10 µl (5 µg) of LDH (from rabbit muscle; Boehringer Mannheim). After 2 min at room temperature the reaction was terminated, the extinction read at 340 nm, and the amount of pyruvate originally present calculated on the basis of calibration curves from the amount of NADH consumed. From the time curves (2-12 min) of lyase reaction obtained in this way the µmol pyruvate formed per min could exactly be calculated as the basis of kinetic studies. The reaction rates were constant during this time of incubation.

Table 1. Kinetic data of *N*-acetylneuraminate lyase from *Clostridium perfringens* for various epi- and deoxy-derivatives of Neu5Ac[a].

Substance	Relative cleavage rates (%)	K_M Values (mM)	V_{max} Value (mU/mg protein)	Inhibitor constant K_i (mM)
Neu5Ac	100	2.7	3.13	
4-epi-Neu5Ac	0		0	0.16
7-epi-Neu5Ac	16	14.8	1.10	
8-epi-Neu5Ac	3	52.7	0.78	
7,8-di-epi-Neu5Ac	14	11.9	0.92	
4-deoxy-Neu5Ac	-[b]		0	0.93
7-deoxy-Neu5Ac	0		0	N.D.[c]
8-deoxy-Neu5Ac	0		0	N.D.
9-deoxy-Neu5Ac	73	3.1	1.45	

[a] For experimental details see the text. The values are means from 2-3 experiments.
[b] A retroaldol reaction is impossible, in contrast to the other sialic acids.
[c] N.D. = not determined.

The influence of those synthetic sialic acids, which were not substrates for the lyase (4-deoxy-Neu5Ac, 7-deoxy-Neu5Ac, 8-deoxy-Neu5Ac and 4-epi-Neu5Ac), on the rate of cleavage of Neu5Ac by this enzyme, was studied in the following way. Various concentrations of Neu5Ac (2.5, 5 and 10 mM) were incubated with the lyase (80 mU/ml incubation mixture) in the presence of 0, 0.77, 1.93 and 3.85 mM, respectively, of these sialic acids. The formation of pyruvate was followed as described above and plotted as reciprocal values against the reciprocal Neu5Ac concentrations. The K_i values were calculated using the formula [23]

$$K_i = \frac{K_M \times i}{K'_M - K_M}$$

where K'_M is the effective Michaelis constant in the presence of inhibitor at the concentration i. Furthermore, the inhibitory potency of the lyase-resistant synthetic sialic acids, i.e. concentrations leading to 50% inhibition of the cleavage rate of Neu5Ac, was estimated by incubating Neu5Ac at 6 or 10 mM concentrations with the synthetic sialic acids at concentrations varying between 1 and 6 mM.

Results

Among the synthetic neuraminic acid derivatives tested with *N*-acetylneuraminate lyase, 9-deoxy-Neu5Ac, 7-epi-Neu5Ac, 8-epi-Neu5Ac and 7,8-di-epi-Neu5Ac are cleaved by the lyase, as determined by the formation of pyruvic acid. In contrast, 4-epi-Neu5Ac, as well as 4-, 7- or 8-deoxy-Neu5Ac are resistant towards the action of the enzyme, as tested in the LDH assay, and in the case of 4-epi-Neu5Ac and 4-deoxy-Neu5Ac additionally by HPLC [12]. The cleavage rates of the sialic acids susceptible to enzyme action show great differences, but are in all cases lower than that of Neu5Ac. In the epi-series, 7-epi-Neu5Ac was cleaved at the highest rate (16% when compared with Neu5Ac), whereas

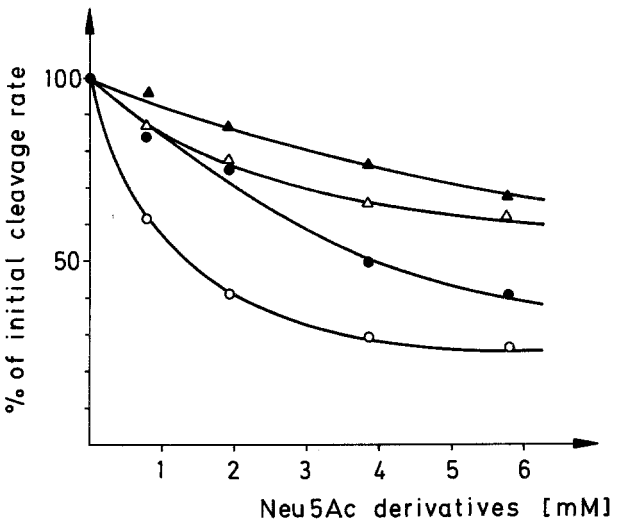

Figure 1. Inhibition of *N*-acetylneuraminate lyase reaction by epi- or deoxy-derivatives of Neu5Ac. The concentrations of the inhibitors were variable and those of Neu5Ac were kept constant (10 mM; ○, ●: or 6 mM; △, ▲) in each experiment. For further details see the text.
○, 4-epi-Neu5Ac; ●, 4-deoxy-Neu5Ac; △, 7-deoxy-Neu5Ac; ▲, 8-deoxy-Neu5Ac.

8-epi-Neu5Ac is a weak substrate (3%). The cleavage rate, however, increases after the additional epimerisation at C-7 yielding 7,8-epi-Neu5Ac (11% relative rate). In the deoxy-series, only 9-deoxy-Neu5Ac is a substrate. It leads to the formation of pyruvate at a rate 73% of that obtained with Neu5Ac. These relative degradation rates and some kinetic parameters are summarized in Table 1. The K_M values show that the affinity of the side chain epimers of Neu5Ac to the enzyme is much lower than that of Neu5Ac; also the V_{max} values are lower. In contrast, the K_M value of 9-deoxy-Neu5Ac is comparable to that of Neu5Ac.

When testing the lyase-resistant Neu5Ac derivatives for a possible inhibitory potency, 4-deoxy-Neu5Ac and 4-epi-Neu5Ac were found to inhibit the lyase action. The cleavage rate of Neu5Ac at 10 mM concentration was inhibited by 50% at 3.8 mM 4-deoxy-Neu5Ac and 1.4 mM 4-epi-Neu5Ac concentrations, respectively (Fig. 1). Applying constant inhibitor and variable Neu5Ac concentrations, plots were obtained which demonstrate the competitive type of this inhibition (Figs. 2 and 3). A K_i value of 0.93 mM (K_M of Neu5Ac 5 mM in this experiment; K'_M values ranging from 9-22 mM in the individual experiments) was calculated for 4-deoxy-Neu5Ac (Fig. 2). The average V_{max} value for Neu5Ac in the presence of various concentrations of 4-deoxy-Neu5Ac was 4 mU/mg enzyme protein; 3.5 mU/mg was found as the corresponding value for Neu5Ac in the absence of the inhibitor. The K_i value for 4-epi-Neu5Ac was estimated in a similar way and found to be 0.16 mM (K_M of Neu5Ac in this experiment 4.1 mM; K'_M values ranging from 18-42 mM in the individual experiments; Fig. 3). The average V_{max} value for Neu5Ac in the presence of 4-epi-Neu5Ac was 1.7 mU/mg lyase. This value for the non-inhibited cleavage reaction of Neu5Ac was 1.2 mU/mg protein.

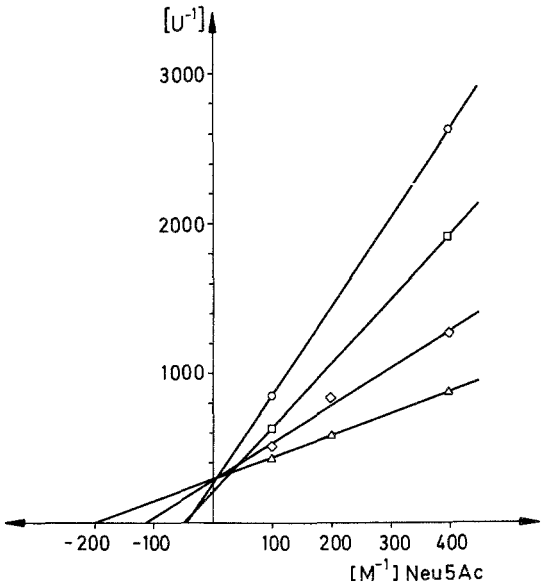

Figure 2. Inhibition of the action of *N*-acetylneuraminate lyase on Neu5Ac by various concentrations of 4-deoxy-Neu5Ac for determination of the inhibitor constant (0.93 mM): △, no inhibitor; ◇, 0.77 mM; □, 1.93 mM; ○, 3.85 mM 4-deoxy-Neu5Ac. For further experimental details see the Methods section.

Both 7-deoxy-Neu5Ac and 8-deoxy-Neu5Ac inhibit lyase activity, too, but this effect is much weaker than that of 4-deoxy-Neu5Ac and 4-epi-Neu5Ac. At the relatively high concentration of 6 mM 7-deoxy-Neu5Ac, the cleavage rate of Neu5Ac (6 mM) is inhibited by about 35%, and at the same concentration of 8-deoxy-Neu5Ac by only 30% (Fig. 1). A more detailed investigation, however, did not reveal that this inhibition follows a special type of kinetics. The sialic acids epimeric in the side chain (7-epi-Neu5Ac, 8-epi-Neu5Ac and 7,8-di-epi-Neu5Ac), which are relatively poor substrates, at millimolar concentration had no significant influence on the activity of the lyase with Neu5Ac, as estimated from the overall yield of pyruvate in the assay mixtures.

Discussion

These studies show that *N*-acetylneuraminate lyase, which is considered to be a specific enzyme for the degradation of non-glycosidically bound sialic acids into pyruvic acid and acylmannosamines [1, 2, 10], cannot tolerate the epimerization or removal of the hydroxyl residue at C-4 of the pyranose ring, but can tolerate similar modification at some of the C-atoms of the glycerol side chain. All side-chain epimers of Neu5Ac are cleaved by the lyase, although at much slower rates than Neu5Ac. These results show that the lyase is not absolutely dependent on the natural configuration of the C-7 to C-8 moiety of neuraminic acid, corresponding to C-3 to C-5 of mannose, for activity.

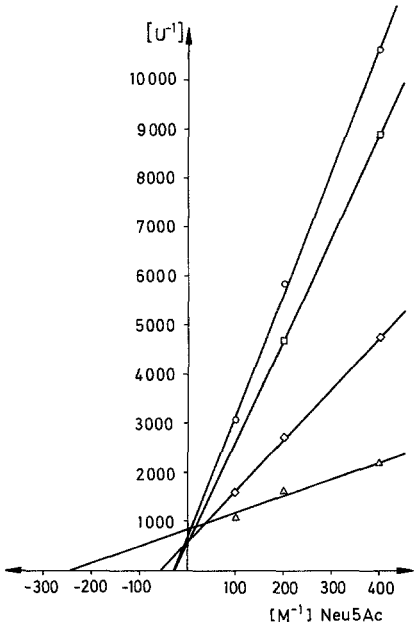

Figure 3. Inhibition of the cleavage of Neu5Ac by *N*-acetylneuraminate lyase in the presence of various concentrations of 4-epi-Neu5Ac to evaluate the inhibitor constant (0.16 mM): △, no inhibitor; ◇, 0.77 mM; □, 1.93 mM; ○, 3.85 mM 4-epi-Neu5Ac. For further experimental details see the Methods section.

Epimerizations at the side-chain have also been found to influence the activity of CMP-sialate synthase (EC 2.7.7.43) to a different degree [20]. Similar to the lyase, 8-epimerization of Neu5Ac has the strongest influence on enzyme reaction, as it can no longer be activated by CTP in contrast to the 7-mono- and 7,8-di-epimers. In contrast, the 8-epimer of 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en) is well recognized by sialidase, as evaluated by its competitive inhibitory effect on the activity of this enzyme, whereas the other epimers have an intermediary position and are weaker inhibitors [24].

Removal of one of the hydroxyl groups from this side chain has rather variable effects which strongly depend on the position of the deoxy centre: while both 7- and 8-deoxy-Neu5Ac are not susceptible to lyase action, 9-deoxy-Neu5Ac is readily cleaved. This shows a reduced sensitivity of the lyase towards chemical modification at C-9. Correspondingly, Neu5Ac *O*-acetylated at C-9 is also cleaved by the lyase, although at only one fourth of the speed of unsubstituted Neu5Ac [12]. An *O*-acetyl group at C-7 and thus closer to the pyranose ring, however, almost prevents the action of the lyase (3% cleavage rate when compared with Neu5Ac; [12]). All these findings are in agreement with earlier observations made with the C-7 and C-8 analogues of Neu5Ac, that the side-chain of neuraminic acid strongly influences lyase action [14, 15].

It is remarkable in this context that it was possible with the aid of Neu5Ac lyase from *Clostridium perfringens* to synthesize 9-fluoro-9-deoxy-Neu5Ac from 6-fluoro-6-deoxy-*N*-acetylmannosamine and pyruvate [25] as well as the C-8 analogue of Neu5Ac from

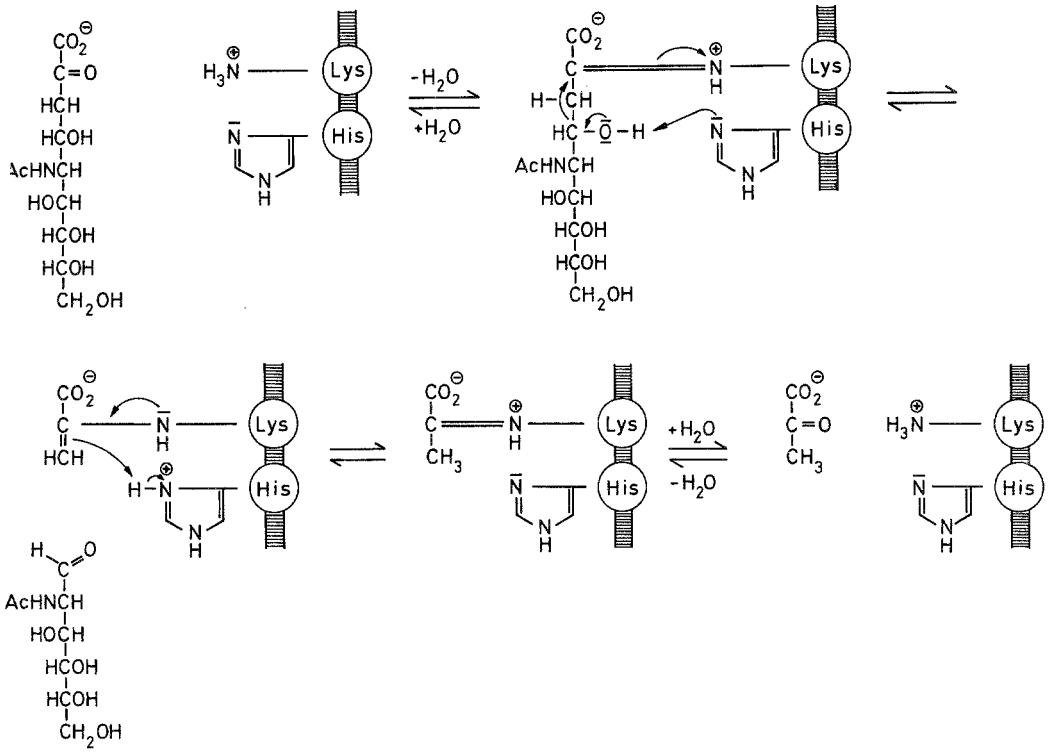

Figure 4. Proposed mechanism of the reversible cleavage of Neu5Ac by *N*-acetylneuraminate lyase [2, 10]. For explanation see the text.

2-acetamido-2-deoxy-D-lyxose and pyruvate [15], respectively. Furthermore, the enzymatic formation of 9-azido-9-deoxy-Neu5Ac from 6-azido-6-deoxy-*N*-acetylmannosamine and pyruvate has been reported [26].

The inability of *N*-acetylneuraminate lyase to cleave 4-deoxy-Neu5Ac as described here and reported at a conference [27], shows the requirement of a hydroxyl residue not only at C-7 and C-8 but also at C-4 of neuraminic acid for the lyase reaction and supports the mechanism of reversible cleavage of Neu5Ac by the lyase, as proposed [2, 4, 10]. Accordingly, after the formation of a Schiff's base between the substrate molecule (e.g. Neu5Ac) and a lysine residue of the enzyme, the unprotonated imidazole nitrogen atom of a histidine residue serves as acceptor of the proton from the hydroxyl group at C-4 of neuraminic acid. This deprotonation leads to cleavage of the bond between C-3 and C-4 and to the formation of *N*-acetylmannosamine and an enamine from pyruvate and a lysine residue. The enzymatic reaction is terminated by the subsequent transfer of the proton from the nucleophilic imidazole group to the enamine and hydrolysis of the (protonated) Schiff's base, leading to free pyruvate and the original enzyme molecule, similar to a mechanism proposed in [4] (Fig. 4). This mechanism has been elucidated by e.g. photo-oxidation of the lyase, reagents for histidine modification, and borohydride

reduction in the presence of substrate [4, 10]. In agreement with this mechanism, cleavage of Neu5Ac is principally not possible if the C-4 hydroxyl is missing as described here, or is substituted by e.g. an *O*-acetyl or *O*-methyl residue as described earlier [11-13]. The proton transfer from the C-4 hydroxyl to the catalytically active histidine residue of the enzyme seems to be possible only if this hydroxyl is in an equatorial position, as in the natural neuraminic acid derivatives. This is shown by the resistance of 4-epi-Neu5Ac, having an axial hydroxyl group at C-4, to lyase action, although this sialic acid seems to fulfil the requirements for a retroaldol reaction.

Although both synthetic sialic acids modified at C-4 are not substrates for the lyase, they appear to interact with the active site of the enzyme. Under the conditions applied, the cleavage rate of Neu5Ac is inhibited by about 50% in the presence of millimolar concentrations of both synthetic sialic acids. The type of inhibition seems to be competitive, as the K'_M values for Neu5Ac are enhanced depending on an increase of the inhibitor concentration. In addition, the V_{max} values for Neu5Ac do not change much under the influence of different inhibitor concentrations. This shows that binding of neuraminic acid derivatives to the lyase can take place in spite of altered structural features of the pyranose ring of Neu5Ac, as e.g. the removal or change of the epimeric configuration of the hydroxyl residue at C-4. Even a substituent at C-4, as in *N*-acetyl-4-*O*-acetylneuraminic acid, does not prevent interaction with the enzyme, as this sialic acid also inhibits the lyase action competitively [11, 12]. These observations point to the role that the natural configuration of the neuraminic acid side chain, especially at C-7 and C-8, plays in the interaction with the lyase. This is in agreement with the inability of 7-deoxy-Neu5Ac and 8-deoxy-Neu5Ac to be substrates of the lyase or to competitively inhibit the enzyme. Epimerization in the neuraminic acid side chain also seems to weaken the binding of the corresponding sialic acids to the active centre of the lyase, as these sialic acids are relatively poor substrates for this enzyme.

Other sialic acids have been reported to be competitive inhibitors of the lyase: *N*-monofluoroacetylneuraminic acid [11] and *N*-acetyl-3-fluoroneuraminic acid [28]. *N*-Acetyl-3-hydroxyneuraminic acid, however, has been described to inhibit the lyase in a non-competitive way [29, 30].

Retroaldolization of sialic acids by the lyase not only requires a free hydroxyl at C-4 in the correct configuration, but also the presence of a free glycosidic hydroxyl residue, which is assumed to interact with the lysine residue of the enzyme, most probably after opening of the pyranose ring of neuraminic acid [2, 4]. Accordingly, 2-deoxy-2,3-didehydro sialic acids as well as glycosidically bound sialic acids are not susceptible to the action of Neu5Ac lyase [1, 2, 12].

In conclusion, the synthesis of modified sialic acids is a valuable approach to obtain more information about the mechanism of action of enzymes regulating the metabolism of sialic acids and may lead to a way of influencing their pathways.

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