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Studies on the inhibition of sialyl- and galactosyltransferase‡

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The inhibition of the α-2,6-sialyltransferase from rat liver, the α-2,3-sialyltransferase from porcine submandibular gland and of the galactosyltransferase from human milk were studied using monosaccharide-, nucleoside- and nucleotide-derivatives of their naturally occurring donor substrates cytidine 5′-monophosphate-*N*-acetylneuraminic acid and uridine 5′-diphosphate-galactose, respectively. Only the corresponding nucleosides/nucleotides showed inhibitory activity. Periodate oxidation of CMP or CMP-Neu5Ac and of UMP or UDP-Gal led to reduced inhibitory efficiency with the respective transferase. The type and reversibility of the inhibition of some of these compounds, as well as the corresponding K_i values were determined.

***Keywords*: sialyltransferase, galactosyltransferase, inhibitor, nucleoside monosaccharide conjugates**

Introduction

Sialyltransferases are a family of enzymes which catalyse the last step in the biosynthesis of complex oligosaccharides by transferring sialic acids onto terminal positions of carbohydrate groups from glycoproteins and glycolipids [1–3]. The reaction proceeds according to the following equation:

$$\text{CMP-sialic acid} + \text{acceptor} \rightarrow \text{sialyl-acceptor} + \text{CMP}$$

They are widely distributed among some microorganisms and higher animals and are localized in the Golgi apparatus of animal cells [4, 5], but they can also be found in soluble form as in the colostrum of goat, cow and man [6]. Sialyltransferases differ by their acceptor specificity or the type of bond they form between sialic acid and the sugars galactose, *N*-acetylgalactosamine or sialic acid to which it is attached. Only sialic acids which are β-glycosidically linked to CMP are recognized as donor substrates [1]. The transfer catalysed by sialyltransferases can only take place if suitable acceptor residues are available. The acceptor galactoses are attached to the growing glycan chain by galactosyltransferases, also localized in the Golgi apparatus, by the following reaction:

$$\text{UDP-Gal} + \text{acceptor} \rightarrow \text{Gal-acceptor} + \text{UDP}$$

Many of these enzymes have already been purified from natural sources and characterized and about 15 animal and bacterial sialyltransferases have already been cloned [3, 7].

Alterations of the carbohydrate structure and composition of cell surface glycoproteins and glycolipids have an essential influence on the metastatic potential of tumour cells [8, 9]. In this context sialic acids play a very important ole. Investigations revealed a direct correlation between the number of sialic acids on the cell surface and the metastatic potential of tumour cells [8, 10, 11]. The hypersialylation, which has been observed on malign transformed cells, goes along with an enhanced sialyltransferase activity [12]. Therefore, inhibition of the transfer of sialic acids onto terminal positions of oligosaccharide chains could lead to a decrease of metastases. Corresponding observations have been described by Kijima-Suda *et al.* [13, 14] and by Wagner *et al.* [15] in different systems. They applied the CMP-sialic acid analogue 5-fluoro-2′,3′-*O*-isopropylidene-5′-*O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-uridine (Kl-8110) to metastatic cell lines and noticed a significant inhibition of the sialic acid transfer and a reduced formation of metastases when the cells were applied to the nude mice model. Recent investigations by Harvey and Thomas [16] and Schmelter and Ivanow (unpublished) showed that Kl-8110 does not achieve its effects via a modulation of sialyltransferase activity but by inhibiting the transport of CMP-sialic acid into Golgi vesicles thereby depleting the donor substrate pool of sialyltransferases.

‡ Dedicated to Professor Dr Edward Kean on the occasion of his 70th birthday.

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A systematic search for inhibitors of sialyltransferases has not been published up to now. In the 1970s Bernacki [17], Shah and Raghupathy [18] and Klohs *et al.* [19] investigated the influence of nucleotides on the sialyltransferases activities of rat liver microsomes and on the sialyltransferases of rat and human sera. The inhibition of purified α-2,6-sialyltransferase from bovine colostrum by CDP [20] and for the microsomal lactosylceramide α-2,3-sialyltransferase from embryonic chicken brain by CMP, periodate-oxidized CMP, UDP and periodate-oxidized UDP [21] has also been analysed. The experiments reported below were carried out to investigate the possibilities of inhibition of glycoprotein sialylation in more detail by directly inhibiting the commercially available purified enzymes α-2,3-sialyltransferase from porcine submandibular gland, α-2,6-sialyltransferase from rat liver and human milk galactosyltransferase *in vitro*. A series of different sialic acids, nucleosides, nucleotides and nucleoside conjugates with sialic acid or galactose were tested in order to identify the structural elements of effective inhibitors.

Materials and methods

Materials

All chemicals were of highest purity available and purchased from Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany), unless stated otherwise. The following compounds were kindly made available by other groups: deoxy [22, 23] and epi analogues [24] of *N*-acetylneuraminic acid (Neu5Ac), compounds **1** [25] and **2** by Professor Zbiral (Vienna); compound **3** [26] and 6-C-methyl-Neu5Ac [27] by Professor Vasella (Zurich); 2,7-anhydro-Neu5Ac [28] and compounds **4–15** [29–32] by Professor Ogura (Tokyo) and the natural inhibitors by Professor Caputto (Córdoba, Argentina) and Professor van den Eijnden (Amsterdam) [33, 34]. (The numbered compounds are shown in Fig. 1.) CMP-[^{14}C]Neu5Ac and UDP[^{14}C]Gal were obtained from Amersham-Buchler (Braunschweig, Germany). Fetuin was obtained from Sigma and desialylated according to Schauer *et al.* [35].

CMP-*N*-Acetylneuraminate: D-galactosyl-β(1-4)-*N*-acetyl-β-D-gluosamine α-2,6-sialyltransferase (E.C. 2.4.99.1) and UDP-galactose: *N*-acetyl-β-D-glucosamine β-1,4-galactosyltransferase (E.C. 2.4.1.90) were purchased from Boehringer Mannheim (Germany)and CMP-*N*-acetylneuraminate: D-galactosyl-β(1-3)-*N*-acetyl-β-D-galactosamine α-2,3-sialyltransferase (E.C. 2.4.99.4) from Genzyme (Cambridge, USA).

Sephadex gels for chromatography were purchased from Pharmacia (Freiburg, Germany), ion exchange resins from Fluka (Neu-Ulm, Germany).

Analytical methods

IR spectroscopy was carried out on a Perkin-Elmer 1310 spectrometer. Mass spectra were recorded with Varian MAT-CH-5 and MAT-731 spectrometers. The electron energy was 70 eV. Four hundred MHz ^{1}H-NMR spectroscopy was carried out on a Bruker AM-800 spectrometer. The samples were dissolved in 0.4 ml $CDCl_3$ at ambient temperature. Chemical shifts (δ) are expressed in ppm downfield from the signal for external TMS.

The determination of the total amount of bound and free sialic acid was carried out by the method of Böhm *et al.* [36] according to Schauer [37]. Free sialic acid was determined using the microadaptation of the periodate/thiobarbituric acid assay of Warren [38] as described by Schauer [37]. The protein content was determined with the BioRad protein reagent (Bio-Rad Laboratories, Munich) by using bovine serum albumin as standard. The method of Avigad [39] was used for quantification of aldehyde groups.

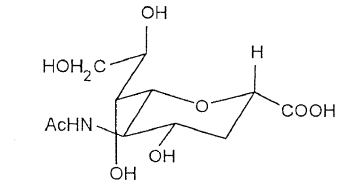
Chemical syntheses

Moisture-sensitive reactions were performed in oven-dried glassware under a positive pressure of argon. Liquids and solutions were transferred by syringe. Usual work-up means partitioning the reaction mixture between water and an organic solvent (given in parentheses) and removal of organic solvents in vacuo at 40 °C using a rotary evaporator.

N-Acetylneuraminic acid methyl ester (Neu5Ac1Me) and *N*-acetylneuraminic acid β-methyl glycoside (βNeu5Ac2Me) were prepared according to Yu and Ledeen [40] in 84% and 56% yield. The glycosyldonor methyl-5-acetamido-4,7,8,9-tetra-*O*-acetyl-chloro-2,3,5-trideoxy-D-*glycero* β-D-*galacto*-2-nonulopyranosonate (βNeu4,5,7,8,9Ac$_5$2Cl1Me) was synthesized by modification of the method of Ogura *et al.* [31]. Three hundred mg (0.92 mmol) Neu5Ac1Me were stirred in 6 ml acetylchloride for 5 h at roomtemperature. Then the mixture was cooled to −45 °C and dry, freshly prepared HCl gas was bubbled for 15 min through the solution. It was warmed to room temperature within 3 h and stirred another 6 h. The acetylchloride was removed and the residue codistilled twice with absolute ether. After solvent evaporation the peracetylated glycosylchloride βNeu4,5,7,8,9Ac$_5$2Cl1Me (yield: 88%) was used immediately for glycosidation.

Synthesis of 2′,3′-*O*-isopropylidene-5′-*O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α/β-D-*galacto*-2-nonulopyranosylonate)-inosine (**16/17**) and 2′,3′-*O*-isopropylidene-5′-*O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonate)-uridine (**18**)

To a solution of silver trifluoromethanesulfonate (56 mg, 0.22 mmol), 443 mg molecular sieve 4A and 56 mg (0.18 mmol, 2′,3′-*O*-isopropylideneinosine (Sigma, Germany) in *N*,*N*-dimethylformamide (DMF; 10 ml) were added 112 mg (0.22 mmol) of βNeu4,5,7,8,9Ac$_5$2Cl1Me and stirred at 20 °C for 18 h in the dark. Usual work-up (CH_2Cl_2), removal of DMF by lyophilization and column



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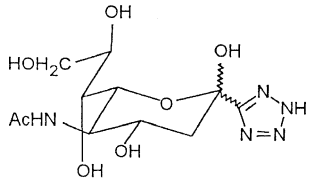

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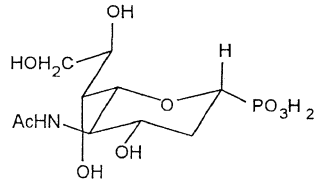

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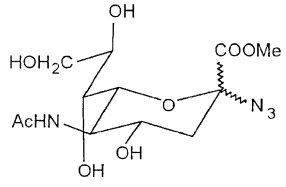
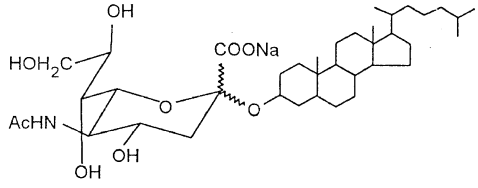
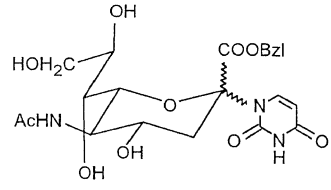


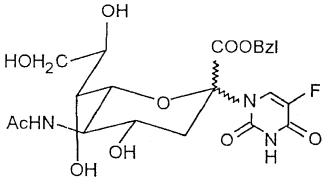
4 = α - anomer
5 = β - anomer



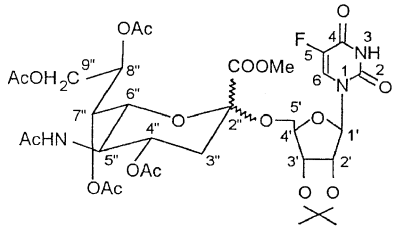
6 = α - anomer
7 = β – anomer



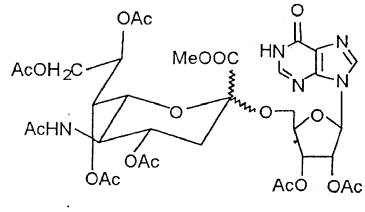
8 = α – anomer
9 = β – anomer



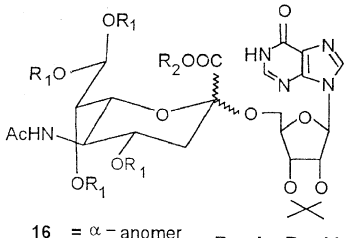
10 = α – anomer
11 = β – anomer



12 = α - anomer (KI - 8110)
13 = β – anomer



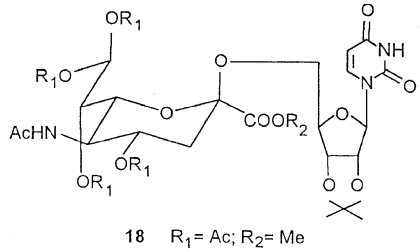
14 = α – anomer (KI - 8115)
15 = β – anomer



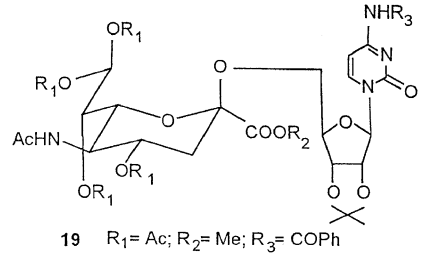
16 = α – anomer
17 = β – anomer
16A = α – anomer
17A = β – anomer

R_1= Ac; R_2= Me

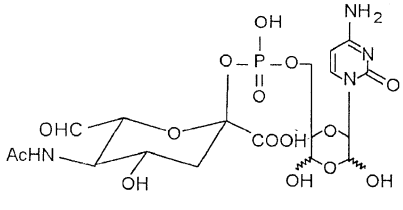
R_1= R_2= H



18 R_1= Ac; R_2= Me
18A R_1= R_2= H



19 R_1= Ac; R_2= Me; R_3= COPh
19A R_1= R_2= R_3= H



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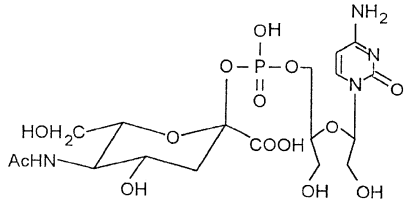

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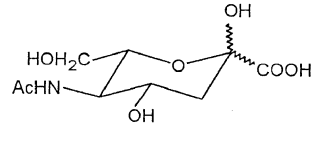

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Figure 1. Structures of the numbered compounds described in the text.

chromatography (25 g of silica gel; $CHCl_3$–CH_3OH 30/1–10/1) furnished **16/17** (31 mg, 18%, $\alpha/\beta \sim 1/3$). The spectroscopic data are in accordance with those of Ogura *et al.* [41].

The corresponding uridine analogue **18** was synthesized by the method of Ogura *et al.* [31]; yield 11.5%.

Synthesis of N^4-benzoyl-2′,3′-*O*-isopropylidene-5′-*O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonate)-cytidine (**19**)

Four hundred and twelve mg (1.29 mmol) 2′,3′-*O*-isopropylidenecytidine (Sigma, Germany) were dissolved in 20 ml pyridine and 400 µl (3.20 mmol) trimethylchlorosilane was added. After stirring for 15 min at 20 °C, 370 µl (3.20 mmol) benzoylchloride was added and the reaction mixture stirred for another 90 min. Then the solution was cooled to 0 °C, before 2 ml H_2O and after 5 min at 20 °C. Solvent evaporation and work-up (ethyl acetate) of the resulting residue, followed by column chromatography (50 g of silica gel; $CHCl_3$–CH_3OH 60/1) gave N^4-benzoyl-2′,3′-*O*-isopropylidenecytidine (439 mg, 88%). M.p. 181.5 °C (from petroletherethanol) (lit. 181–182 °C, [42]). ^{1}H-NMR (400 MHz, $CDCl_3$): $\delta = 1.32$ (isopropylidene-CH_3), 1.58 (isopropylidene-CH_3), 3.81 (5′-H_a), 3.91 (5′-H_b), 4.39 (4′-H), 5.05 (3′-H), 5.20 (2′-H), 5.58 (1′-H), 7.50 (5-H), 7.81 (6-H), 7.41–7.92 (Ar-H).

To a solution of 658 mg (1.70 mmol) N^4-benzoyl-2′,3′-*O*-isopropylidenecytidine, 1 g molecular sieve 4A, 240 mg (0.95 mmol) $Hg(CN)_2$ and 480 mg (1.33 mmol) $Hg(Br)_2$ in 50 mol CH_2Cl_2 was added 763 mg (1.50 mmol) βNeu4,5,7,8,9Ac$_5$2Cl1Me . The reaction mixture was stirred for 1 d at 20 °C in the dark. Usual work-up (CH_2Cl_2) and column chromatography (100 g of silica gel; $CHCl_3$–CH_3OH 70/1–40/1) provided **19** (128 mg, 10%). M.p. 201.6–203 °C (from ethyl acetate). ^{1}H-NMR (400 MHz, $CDCl_3$): $\delta = 1.31$ (isopropylidene-CH_3), 1.52 (isopropylidene-CH_3), 1.86 (NAc), 1.98, 2.00, 2.06 and 2.10 (OAc × 4), 2.52 (3″-H_{eq}), 3.73 (COOMe), 3.81 (5′-H_a), 4.09 (9″-H_a), 4.36 (4′-H), 4.46 (5′-H_b), 4.98 (3′-H), 5.05 (8″-H), 5.11 (2′-H), 5.11 (2′-H), 5.44 (7″-H), 5.61 (1′-H), 7.47 (5-H), 7.90 (6-H), 7.39–7.91 (Ar-H), ^{13}C-NMR (100.6 MHz, $CDCl_3$), DEPT: 20.97, 21.02, 21.09, 21.16 and 23.38 ($CH_3CO \times 5$), 25.49 and 27.46 ($C(CH_3)_2$), 36.18 (C-3″), 49.18 (C-5″), 53.41 (CH_3O), 62.49 and 62.84 (C-9″, C-5′), 77.51 ($C(CH_3)_2$), 84.50 (C-4′) 88.41 (C-1′), 97.25 (C-5), 98.18 (C-2″), 127.60, 128.13 128.50, 132.22 132.99 and 133.51 (Ar-C), 147.75 (C-6), 155.45 (C-2), 163.48, 166.68, 168.65, 170.40, 170.64, 170.78, 170.88 and 171.20 (C-4, CO × 7). IR ($CHCl_3$): 3335 (NH), 1730 (ester), 1660 (amide I), 1550 (amide II) cm^{-1}. MS: m/z = 491 (0.4), 387 (0.7), 329 (1.0).

Solutions of **16/17**, **18** or **19** in 1 M NaOH were stirred at 20 °C for 2 h. After cooling down to − 20 °C and adjusting the pH to 3 with Dowex 50W × 8 (H^+-form), the resulting filtrates were lyophilized. Yield of **16A/17A**, **18A** and **19A**: 78–85%.

Synthesis of ox-CMP-C7-Neu5Ac (**20**) and ox/red-CMP-C7-Neu5Ac (**21**)

To a solution of 10 mg CMP-Neu5Ac (16.2 µmol) in 1 ml 40 mM phosphate buffer, pH 6.8, 3.5 mg (16.2 µmol) $NaIO_4$ was added. After 60 min at 0 °C in the dark, the reaction was stopped by addition of 30 µl ethylene glycol. The reaction mixtures were applied to a column of Sephadex G-25 (1.2 × 35 cm) and eluted with 1 mM ammonia at 4 °C. Fractions containing sialic acids were pooled. Further purification of these fractions was achieved by means of a Sephadex G-10 column (1.2 × 80 cm) using the same eluant. Fractions containing the same compounds according to comparison by TLC were pooled and lyophilized. For further analysis by TLC [37] and HPLC [43] the products were reduced with $NaBH_4$. The identification was carried out with reference substances for the CMP and sialic acid moiety of CMP-Neu5Ac, separately. Therefore, the CMP-glycosides had to be hydrolyzed (1 M formic acid, 1 h, 80 °C) and the resulting compounds were purified by means of a Sephadex G-25 (0.8 × 30 cm) and a Dowex 50W × 8 (20–50 mesh, H^+-form, 0.8 × 20 cm) column [35].

Periodate oxidation of galactose-, Neu5Ac- and ribose-derivatives

To 7.2 mg (12.4 µmol) UDP-galactose in 1 ml 40 mM phosphate buffer, pH 6.8, 26.4 mg (123.6 µmol) $NaIO_4$ was added. The reaction, purification and analysis of the products were performed as described for CMP-Neu5Ac. The oxidation of 5 mg (15.4 µmol) βNeu5Ac2Me, 5 mg (17.6 µmol) cytidine and 4 mg (26.6 µmol) D-ribose in 1 ml of 40 mM phosphate buffer, pH 6.8, was carried out with a five-fold molar excess of $NaIO_4$ (16.5, 18.9 and 28.5 mg, corresponding to 77.1, 88.2 and 133.2 µmol) under the usual conditions. Purification of the reaction products was achieved by chromatography on a Sephadex G-10 column (1.2 × 35 cm) with water as eluant.

Activity of glycosyltransferases, kinetics

The sialyltransferase assays were performed in a total volume of 50 µl containing 14 mM sodium cacodylate buffer, pH 6.8, with 0.2% Triton CF-54 (in case of the α-2,3-sialyltransferase 40 mM sodium cacodylate buffer, pH 6.5, with 0.2% Triton X-100 was used), 0.1 mU enzyme as determined by the manufacturers, 50 µg bovine serum albumin 0.2 mg asialofetuin (30 nmol Galβ(1-4)GlcNAc acceptors sites for the α-2,6-sialyltransferase and 15 nmol Galβ(1-3)GalNAc termini for the α-2,3-sialyltransferases), 4 pmol CMP-[^{14}C]Neu5Ac (262 mCi mmol^{-1}) and compounds to be tested for inhibitory effects in the concentration range between 0.25 and 4.2 mM. With the low substrate concentrations used for screening the potential inhibitors the activity is reduced to about 0.1 µU. After preincubation of the enzymes with the potential inhibitors for 10 min at 37 °C

Table 1. Inhibition of the α-2,6-sialyltransferase (α-2,6 ST) from rat liver and the α-2,3-sialyltransferase (α-2,3 ST) from porcine submandibular gland.

Substance	α-2,6 ST		α-2,3 ST	
	Inhibition by (%)	K_i *(mM)*	*Inhibition by (%)*	K_i *(mM)*
Cytidine	70	0.13*	30 (0.65 mM)	22*
2′-fluoro-2′-deoxycytidine	74	–	0	–
5-methyl-2′-deoxycytidine	13	–	–	–
Ox-cytidine	30	–	0	–
2-thiocytidine	76	0.15*	3	–
28A	22	–	0	–
5′-CDP	85	0.05*	89	0.05*
5′-CDP-ethanolamine	39	–	–	–
5′-CDP-glucose	42	–	–	–
5′-CDP-glycerol	48	–	–	–
Ox-5′-CDP	47	–	15	–
2′-CMP	40	–	–	–
2′,3′-cCMP	33	–	–	–
3′-CMP	39	–	–	–
3′,5′-cCMP	24	–	0	–
5′-CMP	71	0.09*	84	0.064*
Ox-5′-CMP	38	3.3‡	12	5.7‡
Ox/red-5′-CMP	4	–	–	–
Ox-CMP-C7-Neu5Ac (**20**)	44	2.8‡	19	4.9‡
Ox/red-CMP-C7-Neu5Ac (**21**)	9	–	6	–
5′-CTP	90	0.046*	80	0.06*
2′-deoxy-CTP	84	–	72	–
5-iodo-CTP	86	–	–	–
Ox-5′-CTP	58	–	21	–
5′-α-thio-CTP	87	–	76	–
5′-GMP	27	–	8	–
2′-deoxythymidine	16	–	–	–
5′-UMP	24	–	14	–

The relative inhibition data were determined at 0.25 mM inhibitor if no other concentration is indicated; -, not determined; *, competitive inhibition; ‡, non-competitive inhibition.

enzyme reactions were started by the addition of CMP-[^{14}C]Neu5Ac. After 20 min, the reaction mixtures were frozen at − 80 °C. The radioactivity bound to glycoproteins was quantified as described by Paulson *et al.* [44]. The results are the means of triplicate determinations.

The transfer of UDP-[^{14}C]Gal onto ovalbumin by UDP-galactose: *N*-acetyl-β-D-glucosamine β-1,4-galactosyltransferase (EC 2.4.1.90) from human milk [47] was tested in the presence of the potential inhibitors listed in Table 2. The galactosyltransferase assay was performed in a total volume of 50 µl containing 40 mM sodium cacodylate buffer, pH 7.0, 12 mM $MgCl_2$, 0.1 mU enzyme as determined by the manufacturers, 50 µg bovine serum albumin, 0.2 mg ovalbumin (9 nmol acceptor sites), 3 pmol UDP-[^{14}C]Gal (329 mCi mmol^{-1}) and potential inhibitors. After preincubation of the enzyme with the potential inhibitors for 10 min at 37 °C, enzyme reactions were started by addition of UDP-[^{14}C]Gal. After 25 min, the reaction was stopped and worked up as described for sialyltransferases.

For the determination of inhibitor constants, the donor substrates were used at 0.18 and 0.30 mM concentration and the acceptor substrates at acceptor site concentrations of 2 mM for the α-2,6-sialyltransferase, 1 mM for the α-2,3-sialyltransferase and 0.26 mM for the β-1,4-galactosyltrasferase in a total volume of 60 µl. The K_i values and the type of inhibition were determined by the graphical methods of Dixon [45] and Cornish-Bowden [46] using the Enzfitter program (Elsevier-Biosoft, Cambridge, UK).

Reversibility of inhibition

The components of an α-2,6-sialyltransferase assay were preincubated for 15 min at 37 °C in the presence of 0.25 mM inhibitor, but in the absence of donor substrate. Subsequently, the

Table 2. Inhibition of the galactosyltransferase from human milk.

Substance	*Inhibition by (%)*	K_i *[mM]*
Uracil	0	–
Uridine	40	0.39 mM, competitive
5′-UMP	73	0.19 mM, competitive
Ox-5′-UMP	22	0.55 mM, non-competitive
Ox-UDP-Gal	29	0.50 mM, non-competitive

The relative inhibition data were determined at 0.38 mM inhibitor concentration; –, not determined.

assay mixture was transferred to a 100 µl dialysis vial and dialysed for 3 h at 4 °C against 1 l of 14 mM sodium cacodylate buffer, pH 6.8. Afterwards the content of the dialysis vial was transferred back to the incubation vial, the donor substrate was added, and the whole mixture was incubated as usual. For control, the same procedure was repeated in the absence of any inhibitors.

Effect of $NaBH_4$ on the α-2,6-sialyltransferase and its inhibition

Studies on the influence of $NaBH_4$ on the inhibition of the enzyme with ox-CMP and ox-CMP-C7-Neu5Ac (**20**) were made using the following methods:

1. The enzyme was preincubated for 15 min at 37 °C in a volume of 20 µl with 1 mM ox-5′-CMP or 1 mM ox-CMP-C7-Neu5Ac, respectively, reduced subsequently with 2.3 equivalents of $NaBH_4$ at room temperature and finally incubated under standard conditions.

2. In a reversed sequence the enzyme was first incubated for 45 min at room temperature with 2.3 m $NaBH_4$ and then 1 mM ox-5′-CMP or 1 mM ox-CMP-C7-Neu5Ac were added, respectively. After preincubation for 15 min at 37 °C, the assay was performed as usual. In control experiments the assays were treated in the same way but without inhibitors.

Results and discussion

Periodate oxidations

Periodate oxidation of CMP-Neu5Ac with four- and ten-fold molar excess of $NaIO_4$, respectively, led to the formation of identical products. Starting with 10 mg CMP-Neu5Ac, 5.3 mg (60%) ox-CMP-C7 Neu5Ac (**20**) were obtained. Oxidation of CMP-Neu5Ac (10 mg) with an excess of oxidizing agent, followed by treatment with $NaBH_4$, gave 4.6 mg of the reduced compound ox/red-CMP-C7-Neu5Ac (**21**) in 51% yield. Analysis of the hydrolysis products of **21** by TLC with reference substances revealed, that CMP was oxidized at the 2′ and 3′-positions of ribose. The Neu5Ac derivative was identified as ox/red-C7-Neu5Ac (**22**), which means that the glycerol side chain was shortened by two carbon atoms. The structure was identified on the basis of TLC, HPLC and the absorption spectrum of the Bial-complex [48] of the reduced compound. No contaminating substances were detected by HPLC. Quantification of the aldehyde functions before reduction confirmed the proposed structure. A preferred oxidation either in the ribose or the Neu5Ac moiety of CMP-Neu5Ac could not be established. The oxidation of 5 mg βNeu5Ac2Me or cytidine and 4 mg D-ribose furnished the following results: 2.8 mg ox-βNeu5Ac2Me (69%), 3.1 mg oxcytidine (61%) and 2.6 mg oxyribose (64%).

Treatment of 7.2 mg UDP-Gal with a ten-fold molar excess of $NaIO_4$ gave 3.8 mg (54%) ox-UDP-Gal. Comparison by TLC with reference substances showed, that the vicinal hydroxy groups of the ribose moiety from UDP were oxidized and that the galactose part had reacted, too. For the latter molecule different structures are possible [49]. A selective oxidation site in one of the two parts of this disacharide nucleotide could not be observed.

Glycosidation of sialic acids

The glycosyl donor βNeu4,5,7,8,9$Ac_5$2Cl1Me was synthesized by simultaneous peracetylation and chlorination of Neu5Ac1Me. The formation of the intramolecular elimination product 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu2en5Ac) could be minimized by the reaction conditions used. The glycosyl acceptor N^4-benzoyl-2′,3′-*O*-isopropylidenecytidine was prepared by an alternative procedure, which gave a better yield (88%) than the two original syntheses (57% and 50%) described by Holy and Pischel [42]. Starting from βNeu4,5,7,8,9$Ac_5$2Cl1Me and N^4-benzoyl-2′,3′-*O*-isopropylidenecytidine, the disaccharide nucleoside **19** was obtained in 10% yield. The β-configuration was established according to Sato *et al.* [32]. The synthesis of the disaccharide nucleosides **16/17**, **18** and **19** was performed under Koenigs-Knorr-like reaction conditions. Together with the glycosidation products the side product Neu2en5Ac was formed in 20–30% yield, The protecting groups with the exception of the isopropylidene group could easily be removed under alkaline conditions giving **16A/17A**, **18A** and **19A**.

Inhibition of sialyltransferases

Since CMP-Neu5Ac is the naturally occurring donor substrate of these enzymes, we tried to develop an inhibitor on the basis of derivatives of CMP and Neu5Ac. The Neu5Ac derivatives were chosen in such a way that each of the nine positions of this sugar was modified at least once. *N*-Acetylneuraminic acid and its monosccharide derivatives 2,7-anhydro-Neu5Ac, ox-C7-Neu5Ac, ox/red-C7-Neu5Ac (**22**), the phosphonic acid analogue of βNeu5Ac **3**, the 2-azido-αNeu5AclMe **4** and its β-isomer **5** and the cholesteryl glycoside of αNeu5Ac **6** and its β-isomer **7**, were tested for their inhibitory effect on both sialyltransferases in at least

1 mM concentration. The α-2,6-sialyltransferase was additionally tested with 6-C-methyl-Neu5Ac, the 2-deoxy derivative 2d-2H_{ax}-Neu5Ac 1, 4-deoxy-Neu5Ac, 7-deoxy-Neu5Ac, 8-deoxy-Neu5Ac, 9-deoxy-Neu5Ac, 7-epi-Neu5Ac, 7,8-diepi-Neu5Ac, 8-epi-Neu5Ac, Neu2en5Ac, βNeu5Ac2Me, ox-C7-βNeu5Ac2Me, ox/red-C7-Neu5Ac2Me and N-glycolylneuraminic acid. No inhibition of the sialyltransferases was observed with these sialic acid derivatives investigated at 1 or 2 mM concentration. Klohs *et al.* [19] demonstrated that the human serum sialyltransferase is only inhibited by sialic acid at concentrations of 5 mM and higher. This minor inhibition could be explained by assuming that the transferases tested do not interact significantly with Neu5Ac and its derivatives.

In contrast to the sialic acid derivatives tested, some nuleosides and nucleotides have interesting inhibitory properties. A list of those compounds exhibiting significant inhibitory effects, their concentrations used and the K_i-values determined for some of the inhibitors are shown in Table 1. The most effective inhibitors were found amount cytosine nucleosides and nucleotides. The inhibitory potency increased from cytidine via 5′-CMP and 5′-CDP to 5′-CTP for the α-2,6-sialyltransferase. The α-2,3-sialyltransferase's best inhibitor, however, was 5′-CDP, which inhibited both transferases comparably. For the homologous α-2,6-sialyltransferase from bovine colostrum Paulson *et al.* [20] determined an inhibition constant of 0.025 mM for the interaction of CDP with the enzyme acceptor substrate complex, which is comparable to the K_i of 0.05 mM obtained under conditions of acceptor saturation (Table 1). Surprisingly, cytidine inhibited the α-2,6-transferase by a similar extent as 5′-CMP, whereas a 2.6-fold higher concentration of this nucleoside inhibited the α-2,3-transferase only by 30%. Tests with different CMP isomers revealed the importance of the position of the phosphate group. The inhibitory efficiencies of 2′-CMP, 3′-CMP, 2′,3′-cCMP and 3′,5′-cCMP for the α-2,6-sialyltransferase were comparable, but significantly below the efficiency of 5′-CMP. Although not being essential, one or more phosphate groups in 5′-position enhance the inhibition possibly by providing a negative charge equivalent to the carboxylate group of CMPNeu5Ac. Substitution of the 5′-hydroxyl group of cytidine by chlorine completely abolished binding by both transferases, whereas the exchange of oxygen by sulfur at the α-phosphate as in 5′-α-thio-CTP was without noticeable effect. Cytosine and ribose alone had no inhibitory effect indicating that the nucleoside is the minimal unit to be recognized by the transferases. A comparable lack of inhibition by cytosine was found by Ortiz *et al.* [50] for the membrane bound α-2,8-sialyltransferase from *Escherichia coli* K-235. That enzyme, however, was also not influenced by cytidine. In line with the observed importance of the ribose part was the complete lack of inhibition of both sialyltransferases by the uracil-and 5-fluorouracil-α/β-glycosides **8–11.**

The investigation of cytidine derivatives revealed that some modifications in the cytosine part, e.g. methylation at position 5, a hydroxyl group at position 4 as in 5′-UMP, or an additional 3-methyl group as in 2′-deoxythymidine, resulted in a strong reduction of inhibition of the α-2,6-sialyltransferase. However, 2-thiocytidine and 5-iodo-CTP exhibited inhibitory potencies comparable to their unsubstituted analogues. An activation by 5′-UMP, that had been described for 5′-UMP, 5′-UDP and 5′-UTP with the rat liver microsomal enzyme [17, 18], was not observed with the purified sialyltransferases used here. The 2′-deoxy modification slightly reduced the inhibition of both transferases. However, 2′-fluoro-2′-deoxycytidine inhibited only the α-2,6-sialyltransferase by a comparable degree as cytidine, no inhibition of the α-2,3-transferase was observed. The periodate oxidation of 5′-CMP, 5′-CDP and 5′-CTP lead to significant losses of inhibitory potential. These effects were more pronounced with the α-2,3-sialyltransferase, illustrating the increased sensitivity of this enzyme towards modifications at C-2′ or ribose. Ox-5′-CMP has been shown to inhibit the human serum sialyltransferase by 13.6% at 0.25 mM concentration [19]. Periodateoxidized and subsequently reduced 5′-CMP exhibited nearly no inhibition of the α-2,6-sialyltransferase.

In an extension of the periodate oxidation studies, the natural substrate CMP-Neu5Ac was modified, furnishing ox-CMP-C7-Neu5Ac (**20**). A comparable approach has been successfully applied to hyaluronate synthase which is inhibited irreversibly by periodate-oxidized UDP-*N*-acetylglucosamine and UDP-glucuronic acid [51]. However, 0.25 mM of ox-CMP-C-7-Neu5Ac led only to 44% inhibition of the α-2,6-sialyltransferase, and the enzyme with α-2,3-specificity was inhibited by 19%. The observation that this compound exhibited an only slightly higher inhibitory efficiency than ox-5′-CMP shows, that the Neu5Ac moiety of the molecule is not very important for recognition. The results with the periodate-modified nucleotides suggest that the two sialyltransferases do not well interact with oxidized or oxidized and reduced ribose structures.

Another series of CMP-Neu5Ac analogues included substances composed of a nucleoside linked to Neu5Ac. The inefficiency of the 5-fluorouridine derivative **12** (Kl-8110) to inhibit at 0.45 mM concentration the two transferases parallels the results of Harvey and Thomas [16] obtained with other sialyltransferases. The β-glycosidic isomer **13** also failed to exhibit any inhibition as did the α- and β-glycosidic forms (**14**, **15**) of the inosine sialic acid conjugate Kl-8115. In order to expand the series of nucleoside derivatives and to exclude a negative influence of the Neu5Ac-4,7,8,9-tetra-O-acetylation, the inosine glyosides **16A/17A**, the uridine glycoside **18A** and the cytidine glycoside **19A** were synthesized and tested. The only effect observable was produced by **19A** that inhibited the α-2,6-sialyltransferase by 22%.

The two natural inhibitor proteins, isolated by Albarracin *et al.* [33] from rat brain and by van den Eijnden and Schiphorst [34] from calf brain, exhibited the highest inhibitory capacities of all substances tested when applied to the

α-2,6-sialyltransferase. At 1 μM concentration (1 μg in an assay volume of 50 μl) the calf brain inhibitor reduced the transferase activity by 80%, whereas 1.1 μg of the rat brain protein inhibited by 50% . The calf inhibitor was reported to be specific for the CMP-Neu5Ac:GalNAcα-protein α-2,6-sialyltransferase of bovine submaxilary gland. The very good inhibition of the rat liver transferase found by us is an interesting extension of the applicability of this inhibitor. Brefeldin A, that is known for blocking the intracellular transport and processing of different glycoproteins [52, 53], had no effect on both sialyltransferases.

Further information about the mechanism of the glycosyltransferase inhibition with some of the substances of Tables 1 and 2 is given by the type of inhibition. Calculation and plotting of the kinetic data according to Dixon [45] showed, that the inhibition observed was competitive, non-competitive or mixed. These forms could be distinguished unequivocally by Cornish-Bowden plots. The K_i values of the most efficient nucleotide inhibitors of the sialyltransferases were 50 μM for 5′-CDP (α-2,3) and 46 μM for 5′-CTP (α-2,6), respectively. Comparable inhibition constants have been published for an *E. coli* α-2,8-sialyltransferase (30 μM [50]) and for the inhibition of a human serum sialyltransferase by CTP (16 μM [19]).

The inhibition of these enzymes by cytidine and the cytidine nucleotides was of the competitive type, indicating that these substances share the essential structural elements with the natural donor substrate CMP-Neu5Ac. However, the periodate-oxidized compounds ox-CMP and ox-CMP-C7-Neu5Ac (**20**) exhibited non-competitive inhibition. An explanation could be that the oxidative cleavage of the C2′-C3′ bond alters the structure of the ribose in such a way that the substances are not recognized as CMP-Neu5Ac analogues by both sialyltransferases, but inhibits by being bound at other areas of the enzymes. Ox-CMP has recently been described as an inhibitor of the CMP-Neu5Ac: lactosylceramide α-2,3-sialyltransferase from chicken embryo brain [21]. In that system ox-CMP and surprisingly also ox-UDP inhibited competitively to the acceptor substrate lactosylceramide. The authors observed a time-dependent increase of sialyltransferase inhibition by ox-UDP, suggesting that this dialdehyde is an irreversible competitive inhibitor. This mechanism might also apply to the α-2,6-sialyltransferase studied here, especially because an apparent non-competitive inhibition has been observed with several irreversibly inhibited enzymes [54].

Reversibility of α-2,6-sialyltransferase inhibition and influence of $NaBH_4$

The reversibility of inhibition by 5′-CMP, ox-5′-CMP and ox-CMP-C-7-Neu5Ac was tested by two methods. In the case of reversible inhibition, the inhibitor should be removable by dialysis resulting in a decrease in inhibition. As shown in Table 3 the reduction of inhibition by the potentially irreversible inhibitors ox-5′-CMP and ox-CMP-C-7-Neu5Ac was comparable to that measured with the reversible inhibitor 5′-CMP, indicating that the oxidized nucleotides were probably not reacting irreversibly.

Table 3. Influence of dialysis on the inhibitory efficiency of inhibitors of the α-2,6-sialyltransferase from rat liver.

Inhibitor	*Inhibition (%)*	
	Without dialysis	After dialysis
5′-CMP	68	19
Ox-5′-CMP	39	12
Ox-CMP-C7-Neu5Ac	41	16

If the aldehyde groups of ox-CMP and ox-CMP-C7-Neu5Ac react with amino groups in the active site of the transferase under formation of an unstable imine, the latter should be stabilized by reduction thereby leading to irreversible inhibition. In order to test the inhibitory properties of ox-CMP or ox-CMP-C7-Neu5Ac in the presence of $NaBH_4$ the enzyme was preincubated with one of the two compounds and $NaBH_4$ was added subsequently or vice versa. For both substances the results are comparable. In the first case the inhibitory efficiency was reduced by about 20%. In the second case the inhibition was negligible. The addition of $NaBH_4$ had no positive effect on the inhibitory capacity. Therefore, it can be concluded that the inhibitory effect of ox-CMP and ox-CMP-C7-Neu5Ac is reversible and that it is not likely that the inhibitor bind covalently to the enzyme.

Inhibition of galctosyltransferase

Among the different purines, pyrimidines, nucleosides and nucleotides tested on their inhibitory properties towards the human galactosyltransferase adenine, adenosine, 5′-AMP, 3′,5′-cAMP, cytosine, cytidine, 5′-CMP, 3′,5′-cCMP, 5′-CDP, 5′-CTP, guanine, guanosine, 5′-GMP, hypoxanthine, inosine and ribose showed no or only negligible inhibition at 0.38 mM concentration. Only 2′-deoxythymidine and D-galactose inhibited the enzyme by about 10%. Uracil had no effect, whereas uridine and 5′-UMP inhibited the enzyme to 40% and 73%, respectively, the latter being the most effective inhibitor of the compounds tested (Table 2). As in the case of the sialyltransferases, the nucleotide had better inhibitory properties than the nucleoside. The inhibitor constant of 0.19 mM for 5′-UMP is comparable to that of 5′-UTP (0.128 mM) determined for the galactosyltransferase of cell-free L1210 leukaemia ascites fluid [55]. Periodate oxidation of 5′-UMP and UDP-Gal led to significant losses of inhibitory potency, ox-5′-CDP and ox-5′-CMP were completely ineffective. The present study shows many parallels between the inhibition of sialyltransferases and galactosyltransferase.

The inhibitory capacity of all compounds tested seems not to be sufficient for biological application. Further studies will have to show, if alternative strategies might be more successful. Vaghefi *et al.* [55, 56] synthesized phosphonate analogues of nucleoside-disphosphate-sugars which inhibited galactosyltransferase activity *in vitro*. A similar modification of CMP-Neu5Ac or the introduction of a stabilizing methylene group between the pyranose ring and a phosphate oxygen (see [57] for the corresponding CMP-KDO derivative) may provide applicable inhibitors. Moreover, CMP-Neu5Ac derivatives, in which the phosphate group is substituted by a sulfur atom or by sulfur-containing groups [58] could have interesting properties, too. However, the crystallization of the glycosyltransferases and the availability of X-ray crystallographic data would be the best prerequisite for the design of most effective inhibitors.

Besides the development of inhibitors specific for galactosyltransferases or sialyltransferases, an indirect reduction of sialylation by inhibition of the donor substrate transport into the Golgi vesicles is also possible. Further studies will have to elucidate whether the direct or the indirect variant of inhibition is more effective.

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