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Biological Properties of *N*-Acyl and *N*-Haloacetyl Neuraminic Acids: Processing by Enzymes of Sialic Acid Metabolism, and Interaction with Influenza Virus

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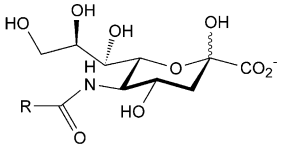
Abstract—Several unnatural *N*-acyl neuraminic acids (*N*-propionyl, *N*-hexanoyl, *N*-benzoyl, *N*-trifluoroacetyl, *N*-chloroacetyl, *N*-difluoroacetyl) were prepared enzymatically using immobilised sialic acid aldolase. *N*-Trifluoroacetyl-, *N*-chloroacetyl- and *N*-difluoroacetyl neuraminic acids were shown to enhance up to 10-fold the rate of association of influenza virus A to a sialoglycolipid neomembrane by surface plasmon resonance, and were found to act as weak inhibitors (K_{iapp} 0.45–2.0 mM) of influenza virus neuraminidase. The *N*-propionyl, *N*-chloroacetyl- and *N*-difluoroacetyl neuraminic acids were found to be substrates for recombinant *Escherichia coli* CMP sialate synthase, to give the corresponding CMP-*N*-acyl-neuraminic acids. CMP-*N*-propionyl neuraminic acid was found not to be a substrate for CMP-*N*-acetyl neuraminic acid hydroxylase from pig submandibular gland.

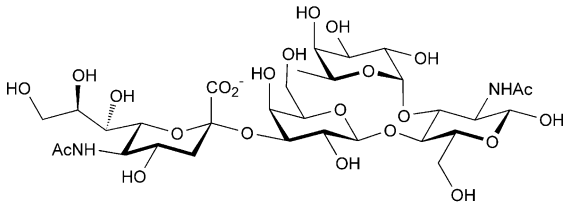
Introduction

The sialic acids are a widely distributed family of sugars present in viruses, bacteria and higher organisms. The 9-carbon sugar D-neuraminic acid is most commonly found as the *N*-acetyl derivative [**1**, *N*-acetyl-D-neuraminic acid (Neu5Ac)], which is often found as the terminal position of cell surface glycoproteins and glycolipids.[1] Notably, Neu5Ac is a component of sialyl Lewis^x (**2**), the leukocyte antigen responsible for E-selectin binding, implicated in chronic inflammation.[2] Adherence of influenza virus particles to their target cells also involves the interaction of sialic acid-containing glycoproteins and glycolipids with viral haemagglutinnin, which presents opportunities for anti-viral intervention using sialic acid analogues.[3–5]

N-Glycolyl-D neuraminic acid (**3**, Neu5Gc) is also abundant in many mammals, but is not normally present in humans,[1] although it has been detected in certain human cancer cells.[6] The biosynthesis of Neu5Gc occurs at the level of the nucleotide sugar CMP-*N*-acetyl-neuraminic acid, via a non-heme iron-dependent oxygenase enzyme cytidine 5′-monophosphate (CMP)-



R = CH_3 Neu5Ac (**1**)
R = CH_2OH Neu5Gc (**3**)



Sialyl Lewis^x (**2**)

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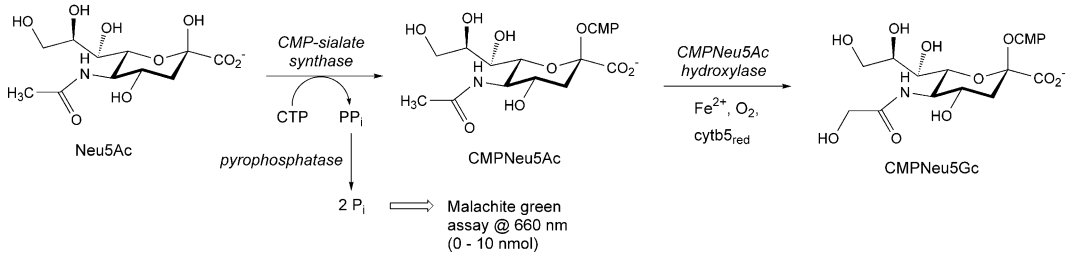


Scheme 1. Biosynthesis of CMP-*N*-acetyl-neuraminic acid via CMP-sialate synthase and CMPNeu5Ac hydroxylase. The coupled assay for CMP-sialate synthase is illustrated.

N-acetyl-neuraminic acid hydroxylase (see Scheme 1), which has been purified from mouse liver and pig submandibular gland.[7,8] Cloning of the cDNA encoding the mammalian hydroxylase has been reported.[9–11]

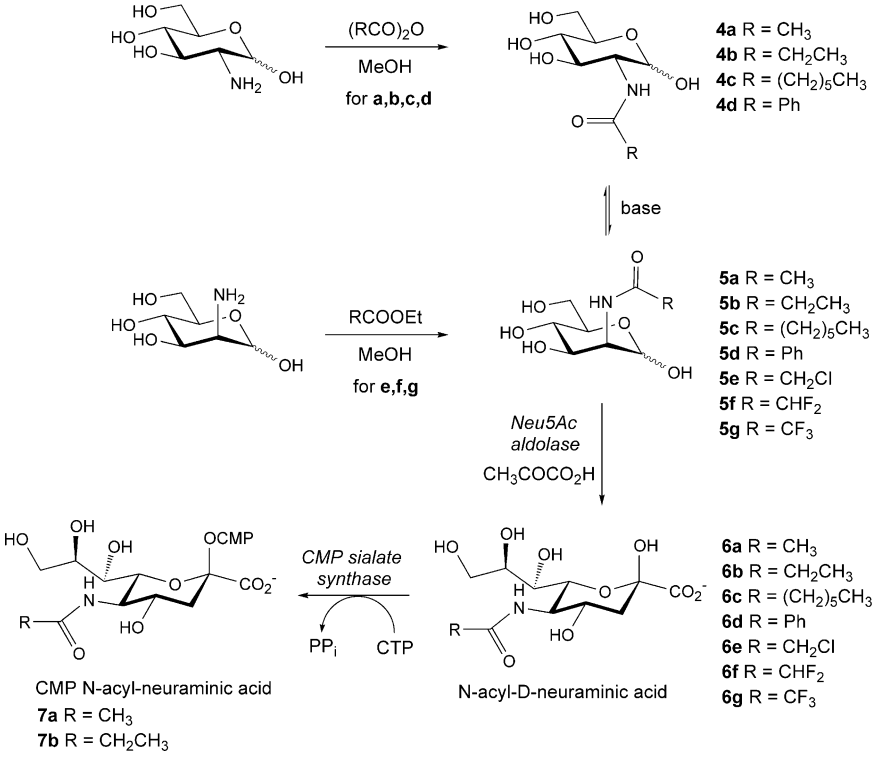
The preparation of *N*-acetyl neuraminic acid in large quantities using immobilised sialic acid aldolase has previously been reported.[12–14] The substrates for this biotransformation are *N*-acetyl-D-mannosamine, which can be prepared by base-catalysed epimerisation of *N*-acetyl-D-glucosamine,[15] and pyruvic acid. Sialic acid aldolase has been used to prepare *N*-Cbz-D-neuraminic acid[13] and several *N*-acylated neuraminic acids.[16] The activation of *N*-acetyl neuraminic acid to CMP-*N*-acetyl neuraminic acid has been reported using CMP sialate synthase from *Escherichia coli*[17–20] and from *Neisseria meningitidis*.[21,22] The *N. meningitidis* CMP sialate synthase has a higher specific activity than the *E. coli* enzyme, and has been used to prepare several unnatural CMP-*N*-acyl neuraminic acids in high yield.[22]

There is considerable interest in the biological properties of sialic acids containing unnatural substituents. Several *n*-alkyl chain *N*-acyl-D-mannosamines have been incorporated in whole cells into cell-surface sialoglycoconjugates[23,24] and the unnatural modifications have recently been shown to affect cell adhesion properties.[25] In this paper we report the enzymatic processing of several *N*-acyl and *N*-haloacetyl neuraminic acids by CMP sialate synthase and CMP-Neu5Ac hydroxylase, and their interaction with influenza virus particles and with influenza virus neuraminidase.

Results

Preparation of *N*-acyl-neuraminic acids

The *N*-acyl-neuraminic acids were prepared from D-glucosamine or D-mannosamine, using the immobilised sialic acid aldolase, as illustrated in Scheme 2.



Scheme 2. Chemo-enzymatic route for preparation of *N*-acyl-D-glucosamines **4a**–**d**, *N*-acyl-D-mannosamines **5a**–**g**, *N*-acyl-D-neuraminic acids **6a**–**g**, and CMP-*N*-acyl-D-neuraminic acids **7a**,**b**.

D-glucosamine was acylated by treatment of a supersaturated solution of the free base with the appropriate anhydride in 70–95% yields, using a modification of the method of Inouye et al., [26] to give a range of *N*-acyl-glucosamines (**4a**–**d**). The *N*-acyl-glucosamines were epimerised under alkaline conditions (pH 11.0) to give equilibrium mixtures of *N*-acyl-glucosamine and *N*-acyl-mannosamine. As reported by Spivak and Roseman,[15] the equilibrium favours the *N*-acyl-D-glucosamine, by approximately 4:1. The mixtures were analysed by HPLC, using an Organic Acids Aminex HPX-87H column: it was found that the *N*-acetyl-pyranoses (**4a**,**5a**) and *N*-propionyl-pyranoses (**4b**,**5b**) could not be resolved at 20 °C, but could be satisfactorily resolved by heating the HPLC column to 65 °C. The *N*-hexanoyl-pyranoses (**4c**,**5c**) and *N*-benzoyl-pyranoses (**4d**,**5d**) could be resolved at 20 °C.

By optimisation of the method of Mahmoudian et al., [14] a procedure for enrichment of this mixture in favour of *N*-acyl-mannosamine was developed, involving selective methanol extraction of *N*-acyl-glucosamine. Via this method samples of enriched *N*-acyl-mannosamines were prepared which contained 40–70% *N*-acyl-mannosamine.

The following halogenated sidechain analogues were also prepared: the trifluoroacetyl analogue, the chloroacetyl analogue, and the difluoroacetyl analogue (**5e**–**5g**). Using the above route, D-glucosamine could be successfully acylated with the trifluoroacetyl, difluoroacetyl or chloroacetyl groups. However, problems were experienced with the base-catalysed epimerisation, in the former case due to de-acylation, and in the latter cases due to intramolecular cyclisation. Therefore, acylation of the more expensive D-mannosamine was carried out in these cases, using the corresponding ethyl or methyl ester (possible due to the higher reactivity of the acyl donor), which proceeded in 70–100% yield.

Samples of *N*-acyl-D-mannosamine were then converted, using immobilised *N*-acetyl-neuraminic acid aldolase and co-substrate pyruvic acid (in 1.2–1.5-fold excess), to give samples of *N*-acyl-neuraminic acids. Enzymatic conversions were carried out on a 100 mg scale, and were monitored analytically by Organic Acids HPLC for a 48–72 h incubation. Attempts to carry out aldolase biotransformations on unenriched *N*-acyl-glucosamine/*N*-acyl-mannosamine mixtures were unsuccessful, since the *N*-acyl-glucosamine is a potent inhibitor for Neu5Ac aldolase. The *N*-acyl-D-neuraminic acid products (**6a**–**g**) were then purified by Dowex 1 anion exchange chromatography, and characterised by ^{1}H and ^{13}C NMR spectroscopy and electrospray MS. Overall isolated yields were in the range 10–43%. After ion exchange purification, the products were >95% pure by ^{1}H NMR spectroscopy.

Interaction of *N*-acyl-neuraminic acids with influenza virus A particles

The technique of surface plasmon resonance (SPR) has been used extensively to follow specific molecular association, in real time, of a number of antigen and antibody reactions,[27] and lectin binding to carbohydrate ligands,[28] on the surface of a sensor chip. Treatment of a BiaCore HPA sensor chip, containing an alkanethiol monolayer, with bovine brain lipid gave a stable neomembrane containing surface sialogangliosides, to which the association and dissociation of influenza virus A particles could be measured (see Fig. 1). We have found previously that the presence of free *N*-acetyl-neuraminic acid *increases* the rate of association of virions to such a neomembrane by approximately 10-fold.[29] Therefore it was of interest to compare the effect of free *N*-acyl neuraminic acids on virion binding.

Few SPR studies have been published using whole virions,[30] because there is often more than one binding

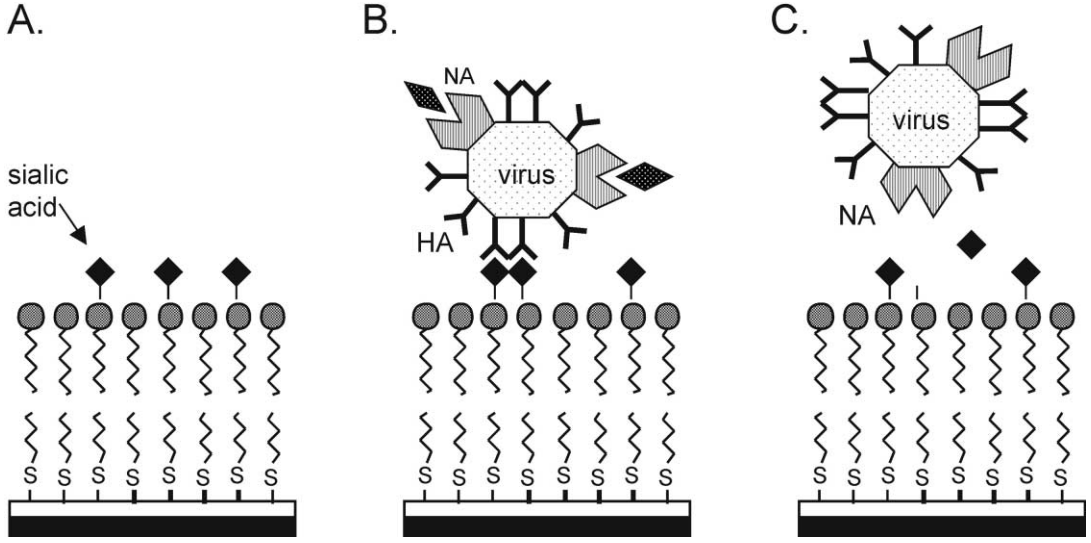


Figure 1. Illustration of influenza virion binding to sialoglycolipid neomembrane. (A) Neomembrane, consisting of monolayer of brain lipid (mixture of brain phospholipids and glycolipids) applied to HPA sensor chip (Au surface coated with alkanethiol monolayer). (B) Binding of virion to neomembrane via multi-valent interaction of heamagglutinin with sialoglycolipids. Inhibition of neuraminidase by *N*-acyl neuraminic acid is also indicated. (C) Competing hydrolysis of sialoglycolipid ligands by neuraminidase.

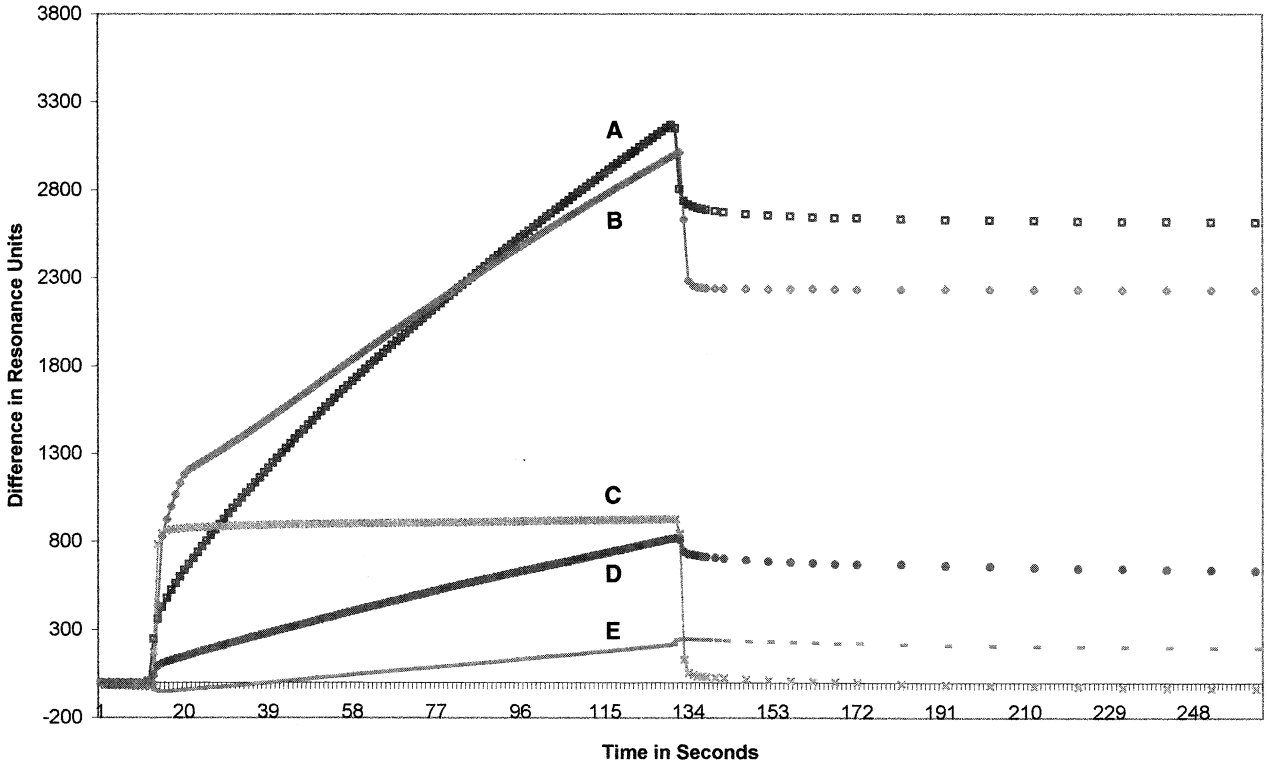


Figure 2. Binding of influenza virus A particles to bovine brain sialoglycolipid neomembrane, in the presence of **6e–g**, assessed by surface plasmon resonance. A, virus + **6g** (R = CF_3CO; 3.88×10^7 virus particles/γmole inhibitor/μL); B, virus + **6a** (R = CH_3CO; 2.27×10^7 virus particles/μmole inhibitor/μL); C, virus + methyl *N*-acetyl neuraminate (2.62×10^8 virus particles/μmole inhibitor/μL); D, virus + **6b** (R = CH_3CH_2CO; 1.37×10^8 virus particles/μmole inhibitor/μL); E, virus alone (1.78×10^8 virus particles/μmole inhibitor/μL).

protein on the surface of the virus and many copies of the protein on each particle. The strain of influenza virus A used in this study [strain A/PR/8/34(PR8;HIN1)] has between 600 and 1000 haemagglutinin (HA) spikes extending from the surface of each virus particle and up to 200 spikes of neuraminidase (NA). The multiple copies of HA produce a cooperative multivalent binding to any suitable sialoglycoside (in our case brain sialogangliosides). The first reaction of neuraminidase is also to bind to the sialogangliosides followed by hydrolysis of the sialic acid residues. Thus, repeat injections of virions onto the chip showed a lower binding, and surfaces were regenerated frequently to ensure that reproducible data was obtained.

A typical set of binding curves is shown in Fig. 2. The lowest binding curve is due to the virus alone, where the highest concentration of virus was required to detect a response. Ten-fold higher rates of association were observed in the presence of *N*-acetyl neuraminic acid **6a**, and in the presence of *N*-trifluoroacetyl neuraminic acid **6g** (that reaches the highest binding shown in Fig. 2). Similarly high rates of association were measured using *N*-chloroacetyl neuraminic acid **6e** and *N*-difluoroacetyl neuraminic acid **6**f. In the presence of *N*-propionyl neuraminic acid **6b** only a slightly enhanced rate of association was observed. No association was observed in the presence of methyl N-acetyl neuraminate, the only compound to reduce the binding of the virus, suggesting that its action was mainly blocking the HA binding to the sialoganglioside.

The relative association rate constants for three experiments on each of the test solutions are shown in Table 1, with the number of virus particles bound per micromole of inhibitor present. One possible explanation for the enhanced binding of influenza virus A to the sialoganglioside layer by **6a** and **6e–g** is that these compounds are acting as inhibitors of the viral neuraminidase, therefore allowing increased binding of viral HA to the surface

Table 1. Rates of association of influenza virus A particles with sialoglycolipid neomembrane, in the presence of *N*-acyl neuraminic acids **6a–6g**

Test solution	Concentration range of virus (particles per μmole inhibitor per μL)	Rate of association k_a (M^{-1} s^{-1})
Virus alone	$1.8–1.9\times10^8$	2.7 ± 0.5
Virus + methyl ester	$2.6–2.8\times10^7$	0.57 ± 0.2
Virus + *N*-acetyl **6a**	$2.1–2.3\times10^7$	25.1 ± 5.0
Virus + *N*-propionyl **6b**	$1.4–1.5\times10^8$	7.3 ± 2.0
Virus + chloroacetyl **6e**	$3.9–4.2\times10^7$	35.5 ± 5.0
Virus + difluoroacetyl **6f**	$4.1–4.4\times10^7$	22.5 ± 1.0
Virus + trifluoroacetyl **6g**	$3.8–3.9\times10^7$	28.5 ± 5.0

Rates of association were determined by surface plasmon resonance, as described in the Experimental.

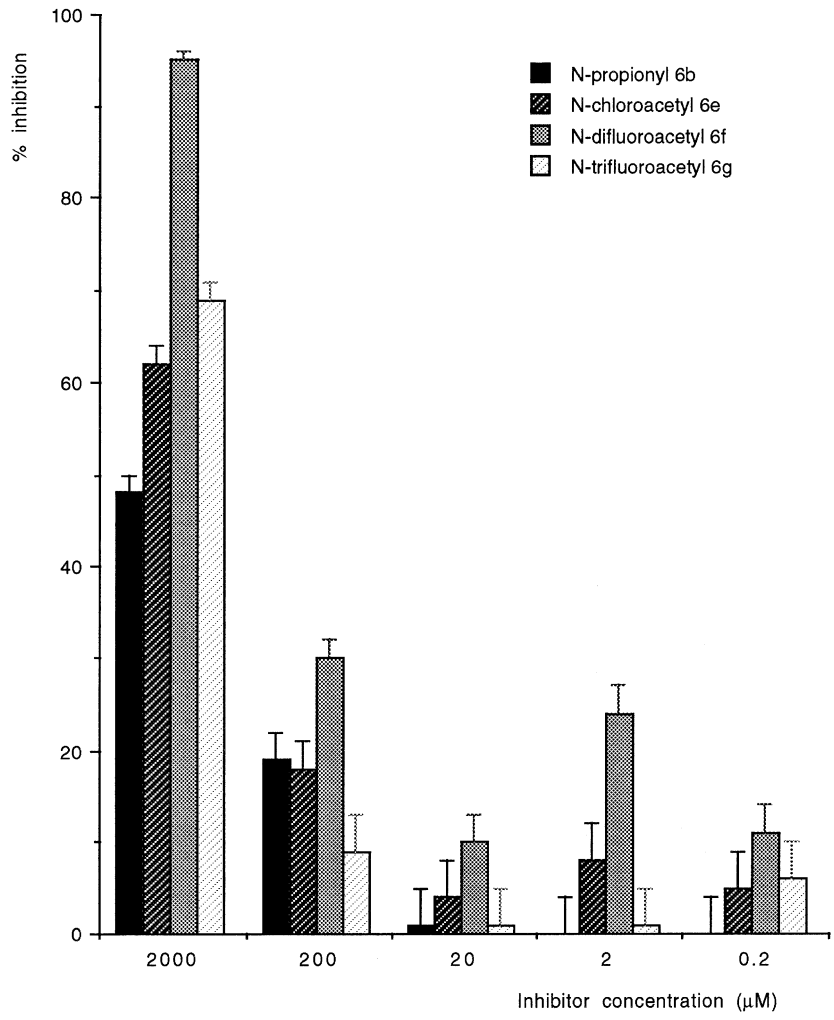


Figure 3. Inhibition of B/Beijing/1/87 influenza virus neuraminidase by *N*-acyl-neuraminic acids **6b** (*N*-propionyl), **6e** (*N*-chloroacetyl), **6f** (*N*-difluoroacetyl), and **6g** (*N*-trifluoroacetyl) at 2 mM, 200 μM, 20 μM, 2 μM, and 0.2 μM inhibitor concentrations. Percentage inhibition (vs control) is plotted for each inhibitor at each concentration.

(see Fig. 2A). The results show that as the electronegativity of the 5-*N*-substituent increased, the rate of association of the virus particles increased, at a lower concentration of virus, suggesting a favourable binding interaction of the more electronegative sidechain with the NA active site.

Assay of *N*-acyl-neuraminic acids as inhibitors of influenza virus Neuraminidase

In order to examine the above hypothesis, samples of the halogenated *N*-acyl neuraminic acids **6b** and **6e–g** were tested as inhibitors of influenza virus neuraminidase, using a chemiluminescent substrate NA-Star. The results are shown in Fig. 3. At 2 mM inhibitor concentration 50–95% inhibition was observed in a 25 min assay, the most potent inhibitor being the *N*-difluoroacetyl neuraminic acid **6f**. At 200 μM inhibitor concentration 10–30% inhibition was observed, again the most active inhibitor being the *N*-difluoroacetyl neuraminic acid **6f**.

Plots of rate versus inhibitor concentration (data not shown) gave the following apparent K_i values: 2.0 mM for *N*-propionyl analogue **6b**; 1.3 mM for *N*-chloroacetyl analogue **6e**; 0.45 mM for *N*-difluoroacetyl analogue **6f**; 1.0 mM for *N*-trifluoroacetyl analogue **6g**. These results confirm that **6e–g** do function as inhibitors of influenza virus neuraminidase, although comparison with the K_m for the *N*-acetyl-neuraminic acid substrate (60 μM) indicates that the inhibition is modest, and that the enzyme shows some selectivity for the *N*-acetyl side chain.

Preparation of CMP-*N*-acyl-neuraminic acids

Activation of neuraminic acids to their CMP glycosides is carried out biologically by the enzyme CMP sialate synthase. The CMP sialate synthase gene from *E. coli* has previously been cloned onto plasmid pWG123.[18] Recombinant enzyme was expressed from *E. coli* W3110/pWG123. A coupled enzyme assay was developed, in which the release of pyrophosphate was coupled with inorganic pyrophosphatase, and the phosphate product detected using a colourimetric phosphate release assay, as shown in Scheme 2. The enzyme was then purified from cell-free extracts by orange A dye chromatography and ammonium sulphate precipitation. Assays carried out at a range of Neu5Ac concentrations gave a K_m value of 7 mM for Neu5Ac

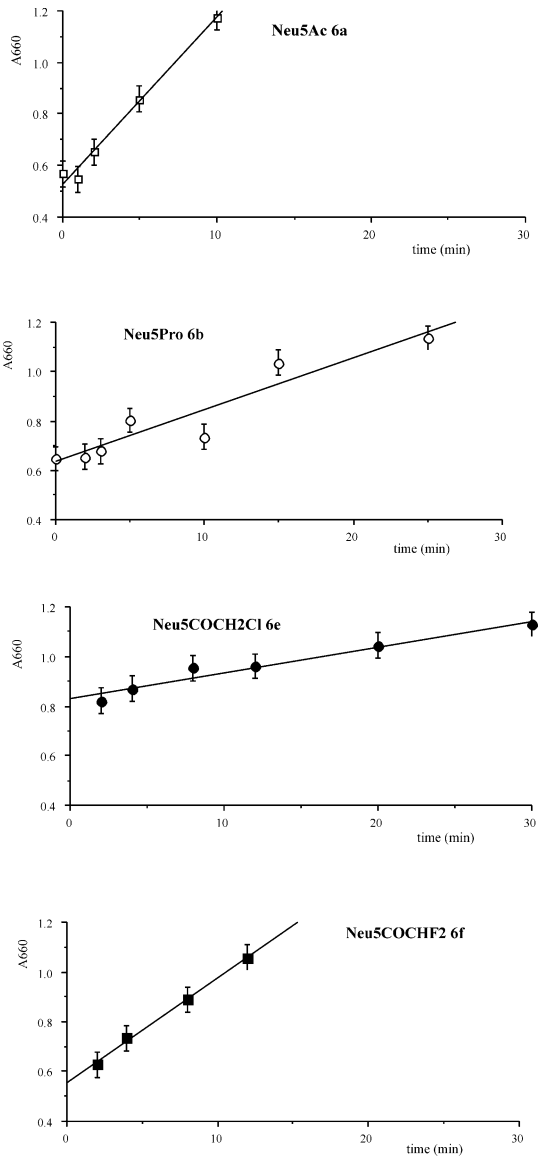


Figure 4. Processing of *N*-acyl-neuraminic acids **6a**, **6b**, **6e**, and **6f** at 10 mM concentration by recombinant *E. coli* CMP-sialate synthase, using coupled Malachite green stopped assay (described in the Experimental). Absorbance at 660 nm is plotted against assay time.

(data not shown), comparable with the reported value of 4 mM.[18]

Assays were carried out with several *N*-acyl neuraminic acids, using the coupled phosphate release assay, in order to determine their relative activity as substrates for CMP sialate synthase. Each substrate was tested at 10 mM concentration, over a 30-min time course, and the results are shown in Fig. 4. The following relative velocities were measured (1.00 for Neu5Ac): 0.32 for *N*-propionyl analogue **6b**; 0.16 for *N*-chloroacetyl analogue **6e**; and 0.65 for *N*-difluoroacetyl analogue **6f**. Assays with the *N*-trifluoroacetyl substrate **6g** revealed > 10 nmol levels of background phosphate present in the synthetic substrate, which precluded a rate determination.

Preparative CMP activation of *N*-acetyl- and *N*-propionyl-neuraminic acids was carried out on a 10 mg scale by treatment with CMP sialate synthase (0.1 units) and CTP in 50 mM Tris buffer (pH 8.5) for 36 h, in the presence of inorganic pyrophosphatase (0.5 units). After centrifugation, the CMP-sialic acid products were purified by Dowex 1 anion exchange chromatography, eluting with a gradient of 50 mM-1 M ammonium bicarbonate, followed by lyophilisation. ^{1}H NMR spectroscopic data of the CMP sialic acid products agreed with published data.[22]

Interaction of CMP-*N*-propionyl-neuraminic acid with CMPNeu5Ac hydroxylase

CMP-*N*-propionyl-neuraminic acid was tested as a substrate at 50 μM and 0.5 mM concentrations for CMPNeu5Ac hydroxylase, purified from pig submandibular gland.[31] Hydroxylation of the *N*-propionyl sidechain to a lactyl sidechain would allow an investigation of the stereochemical course of the reaction. However, no product was detected by HPLC analysis, with fluorometric detection. Although the sample of CMP-*N*-propionyl-neuraminic acid contained ammonium bicarbonate salts, control assays revealed that the enzyme had > 50% activity at 5 mM ammonium bicarbonate (the maximum concentration which might be present in the assay), thus the presence of buffer salts would not prevent substrate turnover under the assay conditions. It is therefore apparent that the pig hydroxylase enzyme does not accept an *N*-propionyl sidechain in place of an *N*-acetyl sidechain.

Conclusion

The chemo-enzymatic route involving sialic acid aldolase was used to prepare a number of unnatural *N*-acyl-D-neuraminic acids. The CMP activation of several *N*-acyl-D-neuraminic acids by recombinant *E. coli* CMP-sialate synthase has been demonstrated on an analytical scale for *N*-acetyl, *N*-propionyl, *N*-chloroacetyl and *N*-difluoroacetyl analogues, and on a preparative scale for *N*-acetyl and *N*-propionyl analogues. The reduced activity of the *E. coli* enzyme with *N*-chloroacetyl and *N*-propionyl analogues is in contrast to the *N. meningitidis* enzyme, which is reported to show high activity with substrates bearing larger side chains such as N-Cbz.[22]

Synthetic CMP-*N*-propionyl-D-neuraminic acid was found not to be a substrate for pig submandibular gland CMPNeu5Ac hydroxylase. The high specificity of this enzyme is in contrast to the relaxed specificity shown by other mammalian enzymes of sialic acid glycoconjugate biosynthesis, as shown by in vivo incorporation of unnatural N-acyl mannosamines.[23–25]

We have shown using surface plasmon resonance that the rate of association of influenza virus A particles to a sialoglycolipid neomembrane is increased 10-fold in the presence of the *N*-acetyl, *N*-chloroacetyl, *N*-difluoroacetyl, or *N*-trifluoroacetyl neuraminic acids, but only 2–3-fold by the *N*-propionyl analogue, and is decreased in the presence of the methyl ester. Therefore, the observed effect is influenced by the nature of the *N*-acyl

and carboxyl substituents. Since the experiment is carried out using whole virus particles and a sialoglycolipid monolayer, the cause of the effect is not certain, but one possible explanation is via inhibition of the viral neuraminidase. We have shown that these compounds are modest inhibitors of influenza virus neuraminidase. It is known that neuraminidase from viral strains has a larger substrate binding cleft than mammalian strains, and the binding site is conserved between different strains of influenza virus.[32] The binding site contains several arginine residues, for example B/Lee/40 has five arginines in the binding site, two of which are in close proximity to the 5-*N*-acyl position.[32] An increase in electronegativity at the 5-*N*-position of neuraminic acid would allow stronger interaction with the arginine groups and may explain why the difluoro, trifluoro and monochloro derivatives showed the best inhibition of NA, thereby allowing more virus particles to bind to the neomembrane. These compounds may be useful tools for further molecular studies of sialic acid enzymology.

Experimental

General

Immobilised neuraminic acid aldolase, *N*-acetyl neuraminic acid and methyl *N*-acetyl neuraminate were gifts of GlaxoSmithKline Pharmaceuticals. Plasmid pWG123 was a gift of Prof. C. H. Wong (Scripps, CA, USA). Other chemicals and biochemicals were obtained from Sigma-Aldrich Ltd. High performance liquid chromatography (HPLC) was carried out on a Waters LKB chromatograph, using an Aminex HPX-87H Organic Acids column, eluting with 6 mM H_2SO_4 at a flow rate of 0.5 mL/min, detecting at 254 nm. Retention times under these conditions are quoted in the text below. Unless otherwise stated, chromatography was carried out at 20 °C.

Purification of *E. coli* CMP sialate synthase

Recombinant *E. coli* CMP sialate synthase was expressed from plasmid pWG123, containing the gene encoding *E. coli* CMP sialate synthase, and purified using a modification of the procedure of Liu et al. [18] A 1.5-L culture of W3110/pWG123 was grown with aeration at 37 °C in Luria broth containing 100 μg/mL ampicillin. Cultures were induced by addition of 0.5 mM isopropylthio-D-galactoside (IPTG) at A_{595} 0.6–1.0, and were grown for a further 5 h at 37 °C. Cells were harvested by centrifugation (10,000*g*, 20 min), and the pellets stored overnight at 4 °C.

Cells were re-suspended in 50 mL of purification buffer [200 mM Tris buffer pH 7.5 containing 20 mM $MgCl_2$ and 0.2 mM dithiothreitol (DTT)]. Cells were broken by sonication, and cell debris removed by centrifugation (40,000*g*, 25 min). The extract was applied to a Cibacron reactive orange dye column (18×2.2 cm), and the column eluted with 100-mL purification buffer, followed by a gradient (300 mL) of buffer containing 0–1 M KCl, at a flow rate of 1 mL/min. Fractions were assayed for CMP sialate synthase activity (see below). Activity was detected in fractions 28–48 (out of 72), which were pooled. Enzyme was precipitated by treatment with 60% ammonium sulfate, followed by centrifugation (15,000*g*, 25 min). The purified enzyme was found to have a total activity of 1.08 units, and a specific activity of 0.126 units/mg.

Coupled assay for CMP sialate synthase

Assay mixtures (total volume 500 μL) contained 100 mM glycine buffer pH 9.5, 5 mM *N*-acyl-neuraminic acid, 2.7 mM cytidine 5′-triphosphate, 10 mM $MgCl_2$, and 0.5 units inorganic pyrophosphatase, to which was added CMP sialate synthase (0.005–0.01 units) to start the assay. Assays were incubated at 25 °C in microcentrifuge tubes for 30 min. 50-μL aliquots were withdrawn at time intervals between 0 and 30 min, and were treated immediately with 800 μL malachite green reagent (as described by Lanzetta et al.[33]), then after 4 min treated with 100 μL 34% sodium citrate solution. The absorbance at 660 nm was measured, and phosphate release calibrated against a set of sodium phosphate standards (0–10 nmol range). Control assays lacking CMP sialate synthase, and lacking Neu5Ac (background CTPase activity), were also carried out. Activity of CMP sialate synthase was calculated as half the rate of phosphate release (since 1 μmol of pyrophosphate yields 2 μmol of phosphate).

Assays at a range of Neu5Ac concentrations (2.5–10 mM) were used to determine a K_m value for Neu5Ac. *N*-Acyl-D-neuraminic acid substrates were tested at 10 mM concentration, using the above conditions.

Procedure for *N*-acylation of D-glucosamine

D-Glucosamine hydrochloride (5.52 g, 25.6 mmol) was treated with sodium methoxide, freshly prepared by dissolving sodium metal (0.64 g, 27.8 mmol) in dry methanol (25 mL), under nitrogen at 0 °C. After stirring for 2 min to liberate free glucosamine, the solution was filtered using a Buchner funnel. The super-saturated solution was treated immediately with propionic anhydride (4.0 mL, 31.2 mmol), and stirred for 16 h at room temperature. The precipitated product was filtered, and washed with cold methanol, giving 2.93 g of a white solid. The filtrate was left for 2 h at room temperature, and a further precipitate filtered, and washed with EtOAc, giving a further 1.73 g product. The combined product (4.66 g, 77%) was found to be pure *N*-propionyl-D-glucosamine (**4b**) by ^1H NMR spectroscopy.

The same procedure was used to prepare *N*-acyl-D-glucosamines **4a**–**d**, using the appropriate anhydride. Yields and data as follows.

***N*-Acetyl-D-glucosamine (4a).** 67% yield; HPLC retention time 13.3 min; δ_H (300 MHz, D_2O) 5.11 (1H, d, $J=3.7$ Hz, H-1 α anomer), 4.62 (1H, d, $J=8.1$ Hz, H-1 β anomer), 3.8–3.3 (6H, m), 1.96 (2×3H, s, CH_3CO–) ppm; m/z (ES^+) 222.1 (MH^+, 100%).

***N*-Propionyl-D-glucosamine (4b).** 70% yield; HPLC retention time 13.1 min; δ_H (300 MHz, D_2O) 5.10 (1H, d, $J=3.7$ Hz, H-1 α anomer), 4.62 (1H, d, $J=8.8$ Hz, H-1 β anomer), 3.8–3.3 (6H, m), 2.22 and 2.21 (2×2H, q, $J=7.4$ Hz, CH_2CO-), 1.03 & 1.02 (2×3H, t, $J=7.4$ Hz, $C\underline{H}_3CH_2$–) ppm; *m*/*z* (ES^+) 236.0 (MH^+, 100%).

***N*-hexanoyl-D-glucosamine (4c).** 93% yield; HPLC retention time 26.6 min; δ_H (300 MHz, D_2O) 5.12 (1H, d, $J=3.7$ Hz, H-1 α anomer), 4.64 (1H, d, $J=8.1$ Hz, H-1 β anomer), 3.9–3.3 (6H, m), 2.23 and 2.22 (2×2H, t, $J=7.4$ Hz, CH_2CO–), 1.54 (2×2H, qui, $J=7$ Hz, $C\underline{H}_2CH_2CO$), 1.3–1.15 (4H, m, $CH_3C\underline{H}_2C\underline{H}_2$–), 0.80 (2×3H, t, $J=6.6$ Hz, $C\underline{H}_3CH_2$–) ppm; *m*/*z* (ES^+) 278.2 (MH^+, 50%).

***N*-Benzoyl-D-glucosamine (4d).** 99% yield; HPLC retention time 25.7 min; δ_H (300 MHz, D_2O) 7.8–7.3 (5H, m, Ar), 5.24 (1H, d, $J=3.7$ Hz, H-1 α anomer), 4.78 (1H, d, $J=8.8$ Hz, H-1 β anomer), 4.1–3.4 (6H, m) ppm; *m*/*z* (ES^+) 284.1 (MH^+, 28%).

^{13}C NMR data (75 MHz, D_2O) for **4a–d** (ND = not detected):

		4a		4b		4c		4d	
		α	β	α	β	α	β	α	β
C-1		93.5	97.6	93.5	97.6	93.5	97.6	93.6	97.6
C-2		56.7	59.3	56.6	59.2	56.6	59.1	57.3	59.9
C-3		73.3	76.5	73.3	76.5	73.2	76.4	73.3	76.5
C-4		72.7	72.5	72.7	72.5	72.8	72.6	72.8	72.6
C-5		74.2	78.6	74.2	78.6	74.2	78.5	74.2	78.6
C-6		63.2	63.4	63.2	63.4	63.2	63.4	63.3	63.4
C=O		177.4	177.4	181.1	181.3	180.4	180.4	ND	ND
Acyl	C-2	24.5	24.8	31.7	32.0	38.7	38.3	129.7–135.0	
	C-3			12.2	12.1	33.2	33.0		
	C-4					27.7	27.7		
	C-5					24.3	24.3		
	C-6					15.8	15.8		

The same procedure was used to acylate D-mannosamine to synthesise *N*-acyl-D-mannosamines **5e–g**. For the halogenated sidechains, it was possible to use the appropriate ester as acylating agent: methyl chloroacetate (for **5e**); ethyl difluoroacetate (for **5f**); and ethyl trifluoroacetate (for **5g**). Yields and data as follows.

***N*-Chloroacetyl-D-mannosamine (5e).** 73% yield; HPLC retention time 14.4 min; δ_H (300 MHz, D_2O) 5.06 (1H, d, $J=1.5$ Hz, H-1 α anomer), 4.97 (1H, d, $J=1.5$ Hz, H-1 β anomer), 4.40 and 4.27 (2×1H, dd, $J=4.5$, 1.5 Hz, H-2), 4.15 & 4.10 (2×2H, s, CH_2Cl–), 4.0–3.4 (5H, m) ppm.

***N*-Difluoroacetyl-D-mannosamine (5f).** 100% yield; HPLC retention time 14.5 min; δ_H (300 MHz, D_2O)) 6.14 and 6.10 (2×1H, t, $J_{H\text{-}F}=54$ Hz, CHF_2–), 5.09 (1H, d, $J=1.5$ Hz, H-1 α anomer), 5.00 (1H, d, $J=1.5$ Hz, H-1 β anomer), 4.45 and 4.33 (2×1H, dd, $J=4.5$, 1.5 Hz, H-2), 4.0–3.4 (5H, m) ppm.

***N*-Trifluoroacetyl-D-mannosamine (5g).** 99% yield; HPLC retention time 15.8 min; δ_H (300 MHz, D_2O) 5.13 (1H, d, $J=1.5$ Hz, H-1 α anomer), 5.03 (1H, d, $J=1.5$ Hz, H-1 β anomer), 4.50 and 4.37 (2×1H, dd, $J=4.5$, 1.5 Hz, H-2), 4.0–3.4 (5H, m) ppm.

Procedure for epimerisation of *N*-acyl-D-glucosamine to *N*-acyl-D-mannosamine

N-Propionyl-D-glucosamine (**4b**, 1.03 g, 4.38 mmol) was dissolved in water (20 mL), the pH adjusted to 11.0 by addition of 2 M sodium hydroxide, and the reaction stirred at room temperature for 4 days. The reaction was neutralised to pH 7.0 by addition of acetic acid, then lyophilised to give the initial mixture of GlcPro/ManPro.

The residue was treated with a small volume of cold methanol, and the resulting suspension was filtered through cotton wool. The filtrate was kept at 4 °C overnight, filtered once more, then the solvent evaporated at reduced pressure, to give the enriched ManPro/GlcPro mixture (0.494 g) as a 2:1 ratio by ^1H NMR spectroscopy. The same procedure was used to prepare enriched samples of **5a–d**. In the case of *N*-hexanoyl-D-mannosamine **5c**, it was found that extraction with cold water was more effective, giving a 1:1 ratio of **5c**/**4c**, rather than 1:2.5 obtained by methanol extraction.

^1H NMR spectroscopic data for *N*-acyl-D-mannosamines **5a–c** identical to published data.[24]

***N*-Benzoyl-D-mannosamine (5d).** δ_H (300 MHz, D_2O) 7.8–7.35 (2×5H, m, Ar), 5.18 (1H, d, $J=1.5$ Hz, H-1 α anomer), 5.06 (1H, d, $J=1.5$ Hz, H-1 β anomer), 4.8–3.9 (6H, m) ppm. HPLC retention times: **5a** 13.0 min (65 °C): **5b** 12.8 min (65 °C): **5c** 23.7 min (20 °C); **5d** 20.8 min (20 °C).

Procedure for sialic acid aldolase biotransformation of *N*-acyl-D-mannosamine to *N*-acyl-D-neuraminic acid

Enriched *N*-acetyl-D-mannosamine (206 mg of 1:1 GlcNAc/ManNAc, 0.46 mmol) and sodium pyruvate (58 mg, 0.53 mmol) were dissolved in 50 mM sodium phosphate buffer pH 7.5 (10 mL), and stirred for 140 h at room temperature in the presence of immobilised sialic acid aldolase (1 mL, approx. 70 units). At intervals, 0.5 mL aliquots were withdrawn, centrifuged (13,000 rpm, 15 min), and analysed by HPLC. To 5 μL supernatant was added 995 μL 6 mM H_2SO_4, and an aliquot injected onto a Bio-Rad Aminex HPX-87X Organic Acids column, eluting isocratically with 6 mM H_2SO_4 at 0.5 mL/min. Retention times: pyruvic acid 13.8 min, GlcNAc/ManNAc 16.0 min, Neu5Ac 12.2 min.

Upon completion, the reaction mixture was centrifuged (13,000 rpm, 15 min), and the pellet washed with water (5 mL) and re-centrifuged. The combined supernatants were loaded onto a Dowex-1 (formate form) anion exchange column, and eluted with a gradient of 0–1 M

formic acid (200 mL), collecting 6-mL fractions. Fractions were analysed by silica thin-layer chromatography [eluent ethyl acetate/acetic acid/water (5:2:2)], staining with ninhydrin. Fractions 1–4 contained GlcNAc, ManNAc and phosphate salts. Neu5Ac (R_f 0.10) was present in fractions 17–24, which were lyophilised to give the product *N*-acetyl-D-neuraminic acid as a brown powder (48 mg, 33%), which was found to be >95% pure by ^{1}H NMR spectroscopy.

The same procedure was used to prepare *N*-propionyl, *N*-hexanoyl, and *N*-benzoyl D-neuraminic acids, from enriched *N*-acyl-D-mannosamine/*N*-acyl-D-glucosamine mixtures. *N*-Chloroacetyl, *N*-difluoroacetyl and *N*-trifluoroacetyl D-neuraminic acids were prepared using the same procedure from samples of pure *N*-acyl-D-mannosamines. Isolated yields and data as follows.

***N*-Acetyl-D-neuraminic acid (6a).** Yield 33%; HPLC retention time 12.2 min; δ_H (300 MHz, D_2O) 3.91 (1H, d, J=8.1 Hz, H-6), 3.86 (1H, m, H-4), 3.83 (1H, t, J=9.4 Hz, H-5), 3.76 (1H, dd, J=11.2, 2.3 Hz, H-9), 3.68 (1H, m, H-8), 3.53 (1H, dd, J=11.2, 5.9 Hz, H-9′), 3.43 (1H, d, J=8.8 Hz, H-7), 2.13 (1H, dd, J=12.7, 5.4 Hz, H-3$_{eq}$), 1.97 (3H, s, CH_3CO–), 1.74 (1H, dd, J=12.7, 10.9 Hz, H-3$_{ax}$) ppm, m/z (ES^+) 310 (MH^+ 69%).

***N*-Propionyl-D-neuraminic acid (6b).** Yield 38%; HPLC retention time 12.0 min; δ_H (300 MHz, D_2O) 4.00 (1H, m, H-4), 3.88 (1H, d, J=11.1 Hz, H-6), 3.84 (1H, t, J=9.6 Hz, H-5), 3.75 (1H, dd, J=11.8, 2.2 Hz, H-9), 3.66 (1H, ddd, J=9.6, 5.9, 2.2 Hz, H-8), 3.52 (1H, dd, J=11.8, 5.9 Hz, H-9′), 3.44 (1H, d, J=9.6 Hz, H-7), 2.27–2.17 (1H, m, H-3$_{eq}$), 2.23 (2H, q, J=7.4 Hz, CH_2CO–), 1.79 (1H, dd, J=13.2, 11.8 Hz, H-3$_{ax}$), 1.04 (3H, t, J=7.4 Hz, $C\underline{H}_3CH_2CO$–) ppm, m/z (ES^+) 324.2 (MH^+ 100%).

***N*-Hexanoyl-D-neuraminic acid (6c).** Yield 5%; HPLC retention time 15.2 min; δ_H (300 MHz, D_2O) 4.00 (1H, m, H-4), 3.94 (1H, d, J=10.3 Hz, H-6), 3.82 (1H, t, J=10.3 Hz, H-5), 3.73 (1H, dd, J=11.8, 2.2 Hz, H-9), 3.64 (1H, ddd, J=8.8, 5.9, 2.2 Hz, H-8), 3.49 (1H, dd, J=11.8, 5.9 Hz, H-9′), 3.43 (1H, d, J=8.8 Hz, H-7), 2.23–2.16 (1H, m, H-3$_{eq}$), 2.19 (2H, t, J=6.6 Hz, CH_2CO–), 1.76 (1H, t, J=12.5 Hz, H-3$_{ax}$), 1.50 (2H, qui, J=6.6 Hz, $C\underline{H}_2CH_2CO$–), 1.19 (4H, m), 0.76 (3H, t, J=6.6 Hz, CH_3) ppm, m/z (ES^+) 366.3 (MH^+ 43%).

***N*-Benzoyl-D-neuraminic acid (6d).** Yield 11%; HPLC retention time 14.3 min; δ_H (300 MHz, D_2O) 7.7–7.35 (5H, m, Ar), 4.2–3.8 (3H, m, H-4, H-5, H-6), 3.72 (1H, dd, J=11.7, 2.2 Hz, H-9), 3.67 (1H, ddd, J=8.8, 5.9, 2.2 Hz, H-8), 3.51 (1H, d, J=8.8 Hz, H-7), 3.48 (1H, dd, J=11.1, 5.9 Hz, H-9′), 2.24 (1H, dd, J=13.2, 3.7 Hz, H-3$_{eq}$), 1.82 (1H, dd, J=12.9, 11.8 Hz, H-3$_{ax}$) ppm, m/z (ES^+) 372.3 (MH^+ 16%).

***N*-Chloroacetyl-D-neuraminic acid 6e.** 10% yield; HPLC retention time 11.6 min; δ_H (300 MHz, D_2O) 4.08 (2H, s, CH_2Cl-), 4.04 (1H, d, J=9.0 Hz, H-6), 4.00 (1H, m, H-4, 3.89 (1H, t, J=10.1 Hz, H-5), 3.73 (1H, dd, J=11.9, 2.6 Hz, H-9), 3.66 (1H, m, H-8), 3.50 (1H, dd, J=11.5, 6.4 Hz, H-9′), 3.46 (1H, d, J=9.2 Hz, H-7), 2.21 (1H, dd, J=12.7, 4.6 Hz, H-3$_{eq}$), 1.78 (1H, dd, J=13.0, 11.4 Hz, H-3$_{ax}$) ppm.

***N*-Difluoroacetyl-D-neuraminic acid 6f.** 39% yield; HPLC retention time 11.0 min; δ_H (300 MHz, D_2O) 6.09 (1H, t, $J_{H\text{-}F}$=54 Hz, CHF_2–), 4.10 (1H, d, J=10.3 Hz, H-6), 4.06 (1H, m, H-4), 3.96 (1H, t, J=10.1 Hz, H-5), 3.75 (1H, dd, J=11.8, 2.6 Hz, H-9), 3.65 (1H, m, H-8), 3.51 (1H, dd, J=12.4, 6.3 Hz, H-9′), 3.44 (1H, d, J=9.2 Hz, H-7), 2.23 (1H, dd, J=13.2, 4.8 Hz, H-3$_{eq}$), 1.80 (1H, dd, J=12.8, 11.2 Hz, H-3$_{ax}$) ppm.

***N*-trifluoroacetyl-D-neuraminic acid 6g.** 43% yield; HPLC retention time 11.9 min; δ_H (300 MHz, D_2O) 4.13 (1H, d, J=10.5 Hz, H-6), 4.06 (1H, m, H-4), 3.98 (1H, t, J=9.8 Hz, H-5), 3.76 (1H, dd, J=11.4, 2.7 Hz, H-9), 3.66 (1H, m, H-8), 3.51 (1H, dd, J=11.8, 6.4 Hz, H-9′), 3.43 (1H, d, J=9.2 Hz, H-7), 2.24 (1H, dd, J=13.1, 4.8 Hz, H-3$_{eq}$), 1.81 (1H, dd, J=13.1, 11.0 Hz, H-3$_{ax}$) ppm.

^{13}C NMR data (75 MHz, D_2O) for **6a-g** (ND = not detected).

		6a	6b	6c	6d	6e	6f	6g
C-1		177.4	181.5	180.8	203.4	173.0	188.2	200.6
C-2		97.9	98.0	98.0	98.0	98.0	98.0	98.0
C-3		41.4	41.5	41.6	41.7	41.5	41.5	41.5
C-4		69.3	69.2	69.2	69.3	69.1	69.0	68.8
C-5		54.6	54.5	54.5	55.3	55.1	54.7	55.2
C-6		72.7	72.7	72.8	72.8	72.7	72.4	72.2
C-7		70.8	70.8	71.0	71.1	70.8	70.8	70.8
C-8		73.0	73.0	73.0	73.0	72.8	72.8	72.7
C-9		65.7	65.7	65.8	65.8	65.7	65.7	65.7
Acyl	C=O	176.0	176.0	176.1	ND	176.3	176.2	176.0
	C-2	24.7	31.9	38.6	ND	44.9	110.9	138.4
	C-3		12.2	33.2	129.8			
	C-4			27.7	131.4			
	C-5			24.3	134.9			
	C-6			15.8				

Preparation of the sialoglycolipid neomembrane

One track at a time of an HPA sensor chip was impregnated with bovine brain lipid (Type VII brain extract from Sigma, containing bovine brain phospholipids and glycolipids) in a BIAcore 2000 instrument at 30 °C. A concentration of lipid (16.6 mg/10 mL) was suspended in 0.01 M HEPES, 0.15 M NaCl pH 7.4 and homogenized thoroughly at 30 °C before injection (2×325 μL) onto a sensor chip at a flow rate of 5 μL/min. The tracks were washed between injections with a fast flow of buffer to remove excess layers of lipid and with 50 mM NaOH (2×15 μL) to produce a stable neomembrane.

Interaction of influenza virus particles to the neomembrane by surface plasmon resonance

Human influenza A virus strain A/PR/8/34 (PR8; H1N1) (harvested from the allantoic fluid of 10-day-old

fertilized chicken eggs that had been inoculated with virus) was purified by centrifugation on successive velocity and density gradients of sucrose. Virus was assayed by haemagglutination titration,[30] and the resulting titre converted to virus particles by multiplying by a factor of 10^9 (unpublished data). Virus was diluted in buffer to 2.56×10^{10} particles/μL (approximately 10^{13} haemagglutination spikes/μL).

Test solutions were prepared and aliquots (20 μL), containing between 1.78×10^8 and 1.89×10^8 virus particles/μL and between 1.30 and 8.35 μmol/μL of putative inhibitor, were injected at a flow rate of 10 μL/min onto the relevant tracks of the sensor chip coated with brain lipid at 30 °C, and the binding followed in real time. Due to the gradual decay of the surface layer, new neomembrane was deposited after three or four injections, and the order of injection of the putative inhibitors was varied. Experiments were carried out in triplicate.

Assay of *N*-acyl-D-neuraminic acids as inhibitors of influenza virus neuraminidase

N-propionyl-D-neuraminic acid (**6b**), *N*-chloroacetyl-D-neuraminic acid (**6e**), *N*-difluoroacetyl-D-neuraminic acid (**6f**) and *N*-trifluoroacetyl-D-neuraminic acid (**6g**) were assayed as inhibitors of neuraminidase from influenza virus B/Beijing/1/87. Assays were carried out as described by Buxton et al., [34] using a chemilumescent substrate NA-Star (at 60 μM concentration), over a 25-min assay period, at the following inhibitor concentrations: 2.0 mM; 200 μM; 20 μM; 2 μM. Results are shown in Figure 3.

Preparation of CMP-*N*-propionyl-D-neuraminic acid using CMP sialate synthase

N-Propionyl-D-neuraminic acid (**6b**, 10.0 mg, 30 μmol) and CTP (17 mg, 30 μmol) were dissolved in 50 mM Tris buffer pH 8.5 (10 mL) containing 20 mM $MgCl_2$. Inorganic pyrophosphatase (0.5 units) and *E. coli* CMP sialate synthase (0.5 units) were added, and the mixture was stirred at room temperature for 24 h. The mixture was centrifuged (13,000 rpm, 20 min), and the supernatant applied to a Dowex-1 (formate form) anion exchange column (13×1 cm), which had been pre-equilibrated with 50 mM ammonium bicarbonate solution. The column was eluted with 50 mM ammonium bicarbonate (50 mL), followed by a gradient of 50 mM to 1 M ammonium bicarbonate (200 mL). The second of three fractions showing UV absorbance at 273 nm, containing the CMP-*N*-propionyl neuraminic acid product, was pooled, and solid Dowex-50W resin (H^+ form) carefully added until the pH of the solution reached 7.4. The resin was then removed by filtration, and the filtrate lyophilised to give the product as a white powder (329 mg), containing excess ammonium bicarbonate. Repeated lyophilisation gave a sample (200 mg) of CMP-*N*-propionyl-D-neuraminic acid (**7b**) containing ammonium bicarbonate buffer salts, which showed identical ^{1}H NMR spectroscopic data to published data.[22]

Assay of CMP-*N*-propionyl-D-neuraminic acid as substrate for CMP-Neu5Ac hydroxylase

CMP-Neu5Ac hydroxylase was purified from pig submandibular gland, as previously described.[31] CMP-Neu5Ac hydroxylase (4 μU) was incubated with 1 mM NADH, 0.5 mM $FeSO_4$, pig liver microsomes (as a source of cytochrome b_5 and cytochrome b_5 reductase, 30 μg protein solubilised in a final concentration of 0.5% w/v Triton X-100), and CMP-N-propionyl-neuraminic acid (50 μM and 5 mM) in 50 mM HEPES buffer pH 7.4, in a total volume of 25 μL. The mixture was incubated at 37 °C for 2 h, then treated with 1 M trichloroacetic acid (5 μL), and centrifuged (14,000 rpm, 2 min) to remove protein. The supernatant was lyophilised, and sialic acids derivatised by treatment with 1,2-diamino-4,5-methylenedioxybenzene, as previously described.[31] HPLC analysis was carried out with fluorometric detection on a TSK gel ODS-120T column, eluting with MeCN/MeOH/H_2O (9:7:84), as previously described.[31] In each case N-propionyl-neuraminic acid substrates were observed, but in no case was any hydroxylated product detected. Assays of CMP-Neu5Ac hydroxylase with ^{14}C-CMP-*N*-acetyl-neuraminic acid were carried out as previously described,[31] in the presence and absence of 5 mM ammonium bicarbonate.

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