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Synthesis of linkage-specific sialoside substrates for colorimetric assay of neuraminidases*

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ABSTRACT

Neuraminidase substrates suitable for analysis of linkage specificity were enzymically synthesized in good yield by linking *N*-acetylneuraminic acid (Neu*p*5Ac) to O-6 and O-3 of 4-nitrophenyl β-D-galactopyranoside with β-D-galactoside-α-(2→6)-sialyltransferase and β-D-galactoside-α-(2→3)-sialyltransferase, respectively. By use of these substrates, a convenient colorimetric assay method was developed for the determination of linkage specificity of bacterial and viral neuraminidases. The substrates are incubated with viral or bacterial neuraminidase and subsequently treated with β-D-galactosidase to convert the liberated 4-nitrophenyl β-D-galactopyranoside to 4-nitrophenol. The amount of liberated 4-nitrophenol is equivalent to the amount of Neu*p*5Ac released from the substrate, thus allowing measurement of neuraminidase activity. The results showed that bacterial and viral neuraminidases can discriminate between these two compounds, making them useful substrates for the rapid determination of neuraminidase linkage specificity.

INTRODUCTION

Neuraminidases have been found in viruses, bacteria, and animals. These enzymes catalyze the hydrolysis of α-linked *N*-acetylneuraminic acid (Neu*p*5Ac) units from glycoconjugates, oligo- and poly-saccharides, and synthetic substrates[1]. Neu*p*5Ac, which occupies a terminal position in the oligosaccharide chains of glycoproteins and glycolipids, is frequently attached in α-(2→3)- or α-(2→6)-linkage to Gal*p*, Glc*p*NAc, or Gal*p*NAc (D-galactopyranosyl, 2-acetamido-2-deoxy-D-glucopyranosyl, and 2-acetamido-2-deoxy-D-galactopyranosyl) residues and in α-(2→8)-linkage to another Neu*p*5Ac residue[2,3]. However, neuraminidases from different sources vary in their ability to recognize these different linkages, and the linkage specificity of neuraminidases has been the subject of considerable interest[4,5]. To date, a variety of natural substrates have been used to determine the relative specificities of neuraminidases for the α-Neu*p*5Ac-(2→6)-D-Gal*p* or α-Neu*p*5Ac-(2→3)-D-Gal*p* linkages. These include

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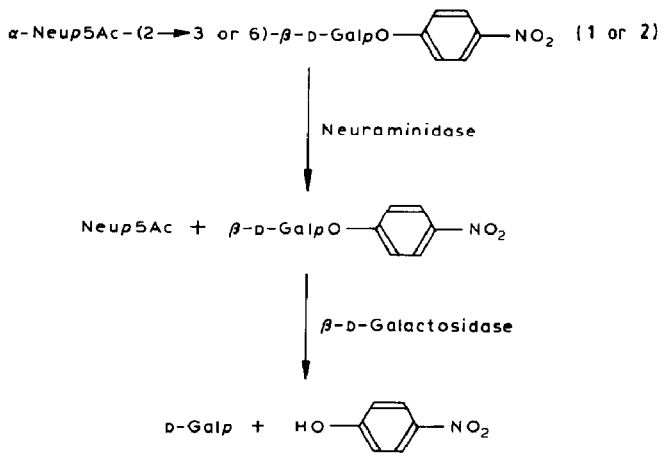
natural glycoproteins, purified milk oligosaccharides, resialylated ^{14}C- and ^{3}H-labeled glycoproteins, and ^{3}H-labeled sialyllactitol[4–9]. Although these substrates have provided useful information, they are not readily available, and can involve handling of radioactive materials.

This report describes the synthesis of nonradioactive substrates for the determination of neuraminidase linkage specificity in a rapid and convenient colorimetric assay. For the synthesis of the substrates, Neu*p*5Ac was specifically linked to O-3 or O-6 of a chromogenic β-D-galactoside, 4-nitrophenyl β-D-galactopyranoside, by use of a highly purified β-D-galactoside α-(2→6)- or β-D-galactoside α-(2→3)-sialyltransferase, respectively. In the assay, as shown in Scheme 1, each substrate is hydrolyzed by neuraminidase to yield 4-nitrophenyl β-D-galactopyranoside. Subsequent treatment with *Escherichia coli* β-D-galactosidase, which cannot hydrolyze the internal galactosyl bond in the sialylated 4-nitrophenyl β-D-galactoside, liberates the colored product 4-nitrophenol. The total amount of released 4-nitrophenol is equal to the amount of released Neu*p*5Ac. Neuraminidase linkage specificity can be determined by comparing the relative rates of hydrolysis of Neu*p*5Ac from the two substrates containing the sialoside in either the α-Neu*p*5Ac-(2→6)-D-Gal or α-Neu*p*5Ac-(2→3)-D-Gal linkage.



Scheme 1.

EXPERIMENTAL

General. — The β-D-galactoside α-(2→6)-sialyltransferase (EC 2.4.99.1) from rat liver and the β-D-galactoside α-(2→3)-sialyltransferase (EC 2.4.99.4) from porcine submaxillary glands used in this studies were purified as previously described[10–12]. β-D-Galactosidase (EC 3.2.1.23) from *Escherichia coli*, alkaline phosphatase, and *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DDN) were purchased from Boehringer Mannheim Corp. (Holtsville, NY). 4-Nitrophenyl β-D-galactopyranoside was pur-

chased from Sigma Chemical Co. (St. Louis, MO). Cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-Neu*p*5Ac) was prepared according to the published procedure[13]. Neuraminidases (EC 3.2.1.18) from *Vibrio cholerae* and *Arthrobacter ureafaciens* were purchased from Calbiochem Corp. (La Jolla, CA).

The ^{1}H-n.m.r. spectra were recorded with a Bruker AM 360 spectrometer at 296 K. The chemical shifts are expressed relative to the HOD signal (δ 4.81 at 296 K). The spectrophotometer used in this study was Carl Zeiss M4QIII.

Viruses. — A seed stock of A/Rhode Island/5$^+$/57 was the gift of Dr. Purnell Choppin, The Rockefeller University. A seed stock of A/Japan/305/57 was obtained from the American Type Culture Collection, Rockville, MD. The strains A/Victoria/3/75 and A/Memphis/102/72 were generously provided by Dr. Robert G. Webster, St. Jude Children's Research Hospital, Memphis, TN. All viruses were grown in the allantoic sack of 10- to 11-day-old embryonated eggs. Allantoic fluid was clarified by centrifugation at 10,000*g* for 20 mins, and the virus was collected as a pellet by centrifugation at 10,000*g* for 12 h. The virus was resuspended in 10mM Na_3PO_4, 0.15M NaCl, pH 7.0 (0.01 vol.), and aliquots were prepared and stored frozen at −80°.

Carbohydrate analysis. — Free Neu*p*5Ac and CMP-Neu*p*5Ac were analyzed by the method of Warren without and with $NaBH_4$ reduction, respectively[14], and glycosidically bound Neu*p*5Ac by the periodate–resorcinol method[15]. T.l.c. was performed on precoated silica gel plates (60-F254, E. Merck. Darmstadt) in 20:1 ethanol–M ammonium acetate, and the spots were detected by heating at 110° after spraying with orcinol–H_2SO_4.

Sialyltransferase assay. — Kinetic constants of β-D-galactoside α-(2→6)-sialyltransferase for 4-nitrophenyl β-D-galactopyranoside were determined as follows. The assay mixture (125 μL final volume) contained β-D-galactoside α-(2→6)-sialyltransferase (9 milli-units), bovine serum albumin (200 μg), and CMP-Neu*p*5Ac (200 μmol) in 0.1M sodium cacodylate, pH 6.7, with 1.0% Triton X-100. To this, 10–100mM of 4-nitrophenyl β-D-galactopyranoside (125 μL) was added and the mixture was incubated at 37°. The time of incubation was varied to limit CMP-Neu*p*5Ac consumption to 15%. The product was quantitatively determined by the periodate–resorcinol method.

Synthesis of 4-nitrophenyl O-*(5-acetamido-3,5-dideoxy-*α-D-glycero-D-galacto-*2-nonulopyranosylonic acid)-(2→6)-*β-D-*galactopyranoside* (**1**) *as substrate.* — 4-Nitrophenyl β-D-galactopyranoside (135 mg, 446 μmol) and CMP-Neu*p*5Ac (37.6 mg, 59.1 μmol) were dissolved in 50mM sodium cacodylate buffer (pH 6.5, 2.8 mL) containing bovine serum albumin (0.7 mg) and alkaline phosphatase (112 units)[16]. To this was added β-D-galactoside α-(2→6)-sialyltransferase (320 milli-units), and the solution was incubated at 37° for 24 h. After completion of the reaction, the mixture was diluted with distilled water (25 mL) and applied to a column (1.5 × 17.5 cm) of DEAE-Sephadex A25. The column was washed with distilled water (60 mL) and then eluted with 50mM NaCl; the fractions (3 mL) were monitored by t.l.c. The product was pooled, lyophilized, dissolved in water (2 mL), and applied to a column (1.6 × 77 cm) of Sephadex G-15 equilibrated and eluted with water. The fractions (3 mL) containing the product were

monitored by t.l.c., measured for conductivity to exclude the contamination by salts, pooled, and lyophilized. The yield determined by the periodate–resorcinol method was 19.6 μmol (33% based on CMP-Neu*p*5Ac); ^{1}H-n.m.r. (D_2O): δ 8.30 (d, 1 H, *J* 9.3Hz, Ph), 7.28 (d, 1H, *J* 9.3 Hz, Ph), 5.20 (d, 1 H, *J* 7.5Hz, H-1), 2.76 (dd, 1 H, $J_{3'e,4'}$ 4.4, $J_{3'a,3'e}$ 12.3Hz, H-3′*e*), 2.01 (s, 3 H, NAc), 1.65 (t, $J_{3'a,3c} = J_{3'a,4}$ 12.3 Hz, H-3′*a*).

Synthesis of 4-nitrophenyl O-*(5-acetamido-3,5-dideoxy-*α-D-glycero-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-*β-D-*galactopyranoside* (**2**) *as substrate.* — 4-Nitrophenyl β-D-galactopyranoside (29.5 mg, 98 μmol) and CMP-Neu*p*5Ac (5.2 mg, 8.2 μmol) were dissolved in 100mM sodium cacodylate buffer (pH 7.5, 0.6 mL) containing bovine serum albumin (78 μg). To this was added β-D-galactoside α-(2→3)-sialyltransferase (23 milli-units) from porcine submaxillary gland, and the solution was incubated at 37° for 52 h; alkaline phosphatase (10 units) was then added to this solution. After an additional 2-h incubation, the reaction mixture was diluted with distilled water (14 mL) and applied to a column (0.8 × 9.0 cm) of DEAE-Sephadex A25. The column was washed with distilled water (9 mL) and then eluted with 20 mL each of 10, 15, and 20mM Na_3PO_4 buffer (pH 6.7). The fractions (3 mL), monitored by t.l.c., which contained the product were lyophilized. The lyophilized product was dissolved in water (1 mL) and applied onto a column (1.6 × 77 cm) of Sephadex G-15 equilibrated and eluted with water. The fractions (3 mL) containing the product were monitored by t.l.c., measured for conductivity to exclude the contamination by salts, pooled, and lyophilized. The yield, determined by the periodate–resorcinol method, was 3.4 μmol (42% based on CMP-Neu*p*5Ac): ^{1}H-n.m.r. (D_2O):δ 8.30 (d, 1 H, *J* 9.3 Hz, Ph), 7.28 (d, 1 H, *J* 9.3 Hz, Ph), 5.33 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.27 (dd, 1 H, $J_{2,3}$ 9.9, $J_{3,4}$ 3.1 Hz, H-3), 4.07 (d, 1 H, $J_{3,4}$ 3.1 Hz, H-4), 3.93 (dd, 1 H, $J_{1,2}$ 7.8, $J_{2,3}$ 7.8 Hz, H-2), 2.81 (dd, 1 H, $J_{3'a,3'e}$ 12.4, $J_{3'e,4'}$ 4.6 Hz, H-3′*e*), 2.05 (s, 3 H, NAc), 1.85 (t, 1 H, $J_{3'e,3'a} = J_{3'a,4'}$ 12.4 Hz, H-3′*a*).

Neuraminidase assay. — Neuraminidase activity was assayed by incubating an appropriate amount of bacterial neuraminidase or virus (10 μL) with an assay mixture (100 μL) containing substrate (25 nmol) **1** or **2** and bovine serum albumin (1.25 mg) in a suitable buffer, as described in the figure legends. After incubation at 37° for 30 min, the reaction was stopped by addition of 50mM DDN (10 μL), an inhibitor of neuraminidase. To this reaction mixture was added 200mM Na_3PO_4 buffer (380 μL) (pH 7.9) containing β-D-galactosidase (0.8 unit). This mixture was incubated at 37° for 5 min, and liberated 4-nitrophenol was measured at A_{405}. The neuraminidase activity was expressed as per cent of Neu*p*5Ac released, which is equivalent to the amount of 4-nitrophenol produced. For determination of kinetic constants, the substrate concentrations were varied about K_m. The V_{max} was expressed as the relative rate of hydrolysis of the two substrates. The incubation times were varied to limit substrate consumption to 15%.

RESULTS

Synthesis of the (2→6)-sialoside substrate **1**. — For synthesis of linkage-specific neuraminidase substrates, several commercially available chromogenic β-D-galactosides were considered. Preliminary experiments using β-D-galactoside α-(2→6)-sia-

lyltransferase showed that 4-nitrophenyl and 2-nitrophenyl β-D-galactopyranoside were suitable acceptor substrates, whereas chlorophenyl red β-D-galactoside and resorcinyl β-D-galactoside were not. We chose 4-nitrophenyl β-D-galactopyranoside as the acceptor substrate for the linkage-specific introduction of Neu*p*5Ac, because it yields a chromophore having a higher extinction coefficient than 2-nitrophenol. In order to introduce the Neu*p*5Ac group at O-6 of 4-nitrophenyl β-D-galactopyranoside, CMP-Neu*p*5Ac and 4-nitrophenyl β-D-galactopyranoside were incubated with β-D-galactoside α-(2→6)-sialyltransferase isolated from rat liver. The reaction and purification procedures are identical to those described for the synthesis of related sialosides[17], except that DEAE-Sephadex A25 was used instead of Dowex 1 to avoid the unfavorable absorption of the product on ion-exchange resin, due to the aromaticity of the 4-nitrophenyl group. Although 4-nitrophenyl β-D-galactopyranoside is a poor acceptor substrate for this enzyme (K_m is 53.5mM), a good yield was obtained (33% based on CMP-Neu*p*5Ac) by use of this acceptor substrate at a high concentration (150mM). Linkage of the Neu*p*5Ac group at O-6 of the Gal*p* residue was confirmed by the chemical shift values of the H-3′ protons of the Neu*p*5Ac residue, which are similar to those reported for other (2→6)-sialosides[18]. There was no other product detected.

Synthesis of the (2→3)-sialoside substrate **2**. — Similar reaction of 4-nitrophenyl β-D-galactopyranoside with CMP-NeuAc and the highly purified β-D-galactoside α-(2→3)-sialyltransferase yielded a single sialylated product, as shown by t.l.c. During the purification procedure, phosphate buffer was used for the elution from the anion-exchange absorbent, because NaCl, which was used for **1**, could not be separated from this product by gel-filtration chromatography. The product was separated from phosphate by gel filtration on a column of Sephadex G-15. The α-Neu*p*5Ac-(2→3)-D-Gal*p* linkage was confirmed by ^{1}H-n.m.r. spectroscopy as follows. The chemical shift values of the H-3′ protons of the Neu*p*5Ac group are similar to those previously reported for (2→3)-sialosides[18]. In addition, the H-3 signal of the Gal*p* residue was deshielded relative to that of 4-nitrophenyl β-D-galactopyranoside, showing that the Neu*p*5Ac group is linked to O-3 of the Gal*p* residue[17]. Use of a high concentration of the β-D-galactoside acceptor substrate in the reaction mixture gave an excellent yield (42%, based on CMP-Neu*p*5Ac). There was no other product detected.

Determination of optimal assay conditions. — The assay procedure using the linkage-specific neuraminidase substrates is shown in Scheme 1, and is similar to that reported for linkage-specific fucosidase substrates reported earlier[19]. Conditions for neuraminidase digestion and subsequent release of 4-nitrophenol with *E. coli* β-D-galactosidase were optimized. To prevent further action of the neuraminidase during reaction with the β-D-galactosidase, the neuraminidase inhibitor DDN was used. The amount of DDN necessary for neuraminidase inhibition was determined for the neuraminidases of two strains of human influenza virus (A/Memphis102/72 and A/Rhode Island/5+/57), by use of α_1-acid glycoprotein as a substrate. At pH 6.5, 0.1mM DDN inhibited 90% of both neuraminidase reactions. Therefore, a final concentration of mM DDN was used for routine assays. To assess liberation of 4-nitrophenol by *E. coli* β-galactosidase, 4-nitrophenyl β-D-galactopyranoside (10 nmol) was incubated with

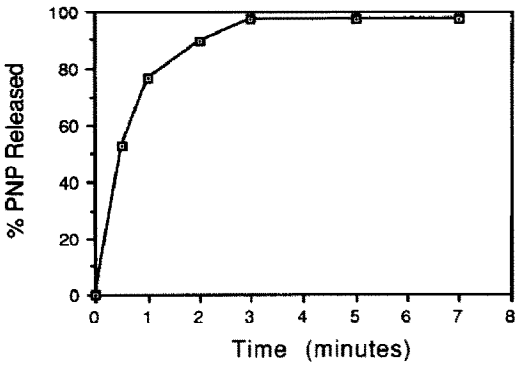


Fig. 1. Release of 4-nitrophenol from 4-nitrophenyl β-D-galactopyranoside by β-D-galactosidase. Assay medium contained 4-nitrophenyl β-D-galactopyranoside (10 nmol), β-D-galactosidase (0.8 unit), and bovine serum albumin (0.5 mg), made up to 500 μL with 200mM sodium phosphate buffer, pH 7.9, and were incubated at 37° for up to 7 min. The percent of 4-nitrophenol released was calculated from the extinction coefficient A_{405} (18.5 $mmol^{-1} \times 1 \times cm^{-1}$).

β-D-galactosidase (0.8 unit) under the conditions described in the legend of Fig. 1. As shown in Fig. 1, 98% of 4-nitrophenyl β-D-galactopyranoside was hydrolyzed within 3 min. The incubation time and amount of enzyme selected for routine assays were therefore set at 5 min and 0.8 unit of β-D-galactosidase, respectively. There was an excellent linear relationship between the amount of 4-nitrophenyl β-D-galactopyranoside released and the amount of 4-nitrophenol released, in the range between 1 and 10 nmol.

Substrate specificity of bacterial neuraminidases. — The two substrates **1** and **2** were evaluated for hydrolysis by two bacterial neuraminidases, (from *V. cholerae* and *A. ureafaciens*) which have been widely studied and are commercially available. The results shown in Fig. 2 showed that *V. cholerae* and *A. ureafaciens* neuraminidases exhibit

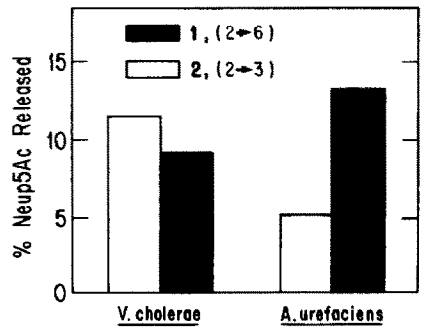


Fig. 2. Linkage specificity of bacterial neuraminidases. Incubations containing *V. cholerae* or *A. ureafaciens* neuraminidases and **1** [α-Neup5Ac-(2→6)-D-Galp-$OC_6H_4NO_2$(4), dark bars] or **2** [α-Neup5Ac-(2→3)-D-Galp-$OC_6H_4NO_2$(4), open bars] were done under optimal conditions for each enzyme. Assays with *V. cholerae* neuraminidase (1.12 mU) contained 50mM sodium acetate–9mM $CaCl_2$–0.15M NaCl, pH 5.5. Before measuring the absorbancy, the precipitated $Ca_3(PO_4)_2$ was removed by centrifugation. Assays with *A. ureafaciens* neuraminidase (5 μU) contained 200mM sodium acetate, pH 4.8.

preferential cleavage of the (2→3) and (2→6) substrates, respectively. The release of Neu*p*5Ac from **1** by *V. cholerae* neuraminidase was 79% of that released from **2**. In contrast, hydrolysis of **1** by *A. ureafaciens* neuraminidase was 255% of that of **2**. The differences observed for preferential hydrolysis of the two linkages by the bacterial neuraminidases are consistent with results reporting the use of resialylated α_1-acid glycoprotein[4] and sialyllactose[20] substrates.

Substrate specificity of viral neuraminidases. – The substrates **1** and **2** were also used to compare the linkage specificity of neuraminidases from four human influenza virus strains, A/Rhode Island/5$^+$/57 (RI/57), A/Japan/305/57 (Japn/57), A/Memphis/102/72 (Mem/72), and A/Victoria/3/75 (Vict/75), as shown in Fig. 3. These viral neuraminidases showed contrasting specificities. RI/57 and Japn/57 neuraminidases had strict specificity for the α-(2→3) linkage, whereas Mem/72 and Vict/75 neuraminidases exhibited substantial hydrolysis of both linkages.

Kinetics of viral neuraminidases. — The difference in activity of the two types of viral neuraminidases for hydrolysis of **1** was reflected in their kinetic constants. Table I shows the K_m and relative V_{max} values for the representative Vict/75 and RI/57 neuraminidases. Surprisingly, both viral neuraminidases exhibited similar relative V_{max} values for the α-(2→6) (**1**) and α-(2→3) (**2**) substrates. However, for Vict/75 neraminidase, the K_m values for the α-(2→6) (**1**) and α-(2→3) (**2**) substrates differed only two-fold, whereas for RI/57 neuraminidase they differed 50-fold. Thus, the differences in K_m values fully account for the substrate specificities of these viral neuraminidases illustrated in Fig. 3.

DISCUSSION

The results presented herein indicate that the chromogenic substrates, 4-nitrophenyl *O*-(5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic

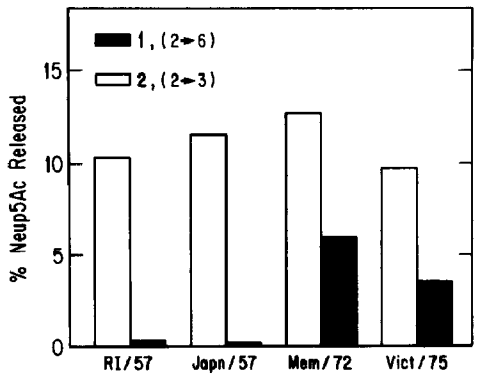


Fig. 3. Neuraminidase specificity of four human influenza virus strains. The viruses, A/Rhode Island/5$^+$/57 (RI/57), A/Japan/305/57 (Japn/57), A/Memphis/102/72 (Mem/72), and A/Victoria/3/75 (Vict/75), were incubated with **1** [α-Neu*p*5Ac-(2→6)-D-Gal*p*-$OC_6H_4NO_2$(4), dark bars] or **2** [α-Neu*p*5Ac-(2→3)-D-Gal*p*-$OC_6H_4NO_2$(4), open bars]. Assays contained 0.05-0.5 mU of viral neuraminidase to limit consumption of **2** to 15%, in 200mM sodium cacodylate, pH 6.6. The incubation time for RI/57 and Japn/57 neuraminidases with **1** was extended to 150 min, and the percent of Neu*p*5Ac released was corrected to the standard 30-min incubation.

TABLE I

Kinetic constants for the neuraminidases of A/Victoria/3/75 and A/Rhode Island/5$^+$/57 strains of human influenza virus

Substrate	*Vict/75*		*RI/57*	
	K_m (*m*M)	*Relative* V_{max} (%)[a]	K_m (*m*M)	*Relative* V_{max} (%)[a]
α-Neu*p*5Ac-(2→6)-D-Gal-$OC_6H_4NO_2$(4) (**1**)	0.8	61	18.0	52
α-Neu*p*5Ac-(2→3)-D-Gal-$OC_6H_4NO_2$(4) (**2**)	0.40	100	0.36	100

[a]100% V_{max} of Vict/75 and RI/57 neuraminidases were 71 and 142 pmol/min of Neu*p*5Ac released, respectively.

acid)-(2→6)-β-D-galactopyranoside (**1**) and 4-nitrophenyl *O*-(5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranoside (**2**), can be used to determine the linkage specificity of neuraminidases. In a previous report[6], it was suggested that the core oligosaccharide structure or the protein structure in glycoproteins (or both), as well as the linkage of the Neu*p*5Ac group, might also influence the rate of hydrolysis of different glycosidic linkages of the Neu*p*5Ac group. Since the substrates **1** and **2** have neither core oligosaccharides nor protein components, the different rates of hydrolysis of Neu*p*5Ac from these substrates are due to differential recognition of the α-Neu*p*5Ac-(2→6)-D-Gal and α-Neu*p*5Ac-(2→3)-D-Gal linkages.

The colorimetric assay described in this report is simple, and can be used to assay a large number of samples, since neither chromatography nor special precautions for handling are necessary. These substrates are also suitable for the assay of mammalian neuraminidases, such as lysosomal neuraminidases, which have low pH optimum. Indeed, no significant increase of the background release of Neu*p*5Ac was observed at the pH 4.8 used for the assay for *A. ureafaciens* neuraminidase.

Enzymic synthesis of such sialosides as the substrates reported here offers several advantages[17]. The most important of these is that sialyltransferases catalyze the stereo- and regio-specific formation of glycosidic linkages. Although 4-nitrophenyl β-D-galactopyranoside was a poor acceptor substrate for the highly purified sialyltransferases, good yields (based on CMP-Neu*p*5Ac) were obtained by use of a high acceptor-substrate concentration. In both cases, the sialyltransferases yielded a single product. Compounds **1** and **2** could be separated by gel-filtration chromatography, where **2** was eluted faster than **1**. The difference in elution position does not appear to be due to molecular size, since **1** and **2** are coeluted with NaCl and phosphate ion, respectively. Some interaction may exist between these compounds and Sephadex. Knowledge of the differential gel-filtration behavior of **1** and **2** was essential to develop a purification procedure to give a product free of salts.

The preparation of **1** and **2** using human platelets, in analytical scale, has been reported; in this case, the products were separated by paper chromatography[21]. Howev-

er, the reaction conditions and workup procedure were not optimized for milligram-scale preparative reactions. The synthetic method reported here can be scaled-up as required.

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