Synthesis of Potential Inhibitors of Hemagglutination by Influenza Virus: Chemoenzymic Preparation of N-5 Analogs of *N*-Acetylneuraminic Acid.[1]

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Abstract: *A chemoenzymic route to neuraminic acid 2 is described. This method is based on conversion of N-carbobenzoxymannosamine (ManCBz) 3 to N-carbobenzoxyneuraminic acid (Neu5CBz) 4, catalyzed by Neu5Ac aldolase. The Neu5CBz 4 was converted to the α-methyl glycoside 8 and deprotected to afford the free amine 2. This procedure has been scaled up to generate 10-gram quantities of 2. N-Acylation of 2 produced several new N-acyl neuraminic acid analogs; these have been evaluated as inhibitors of adhesion of influenza virus to chicken erythrocytes in a hemagglutination inhibition assay (HAI). This preparation of neuraminic acid 2 is compared with other literature procedures.*

N-Acetylneuraminic acid (**1**, R = H, Neu5Ac) is present as the terminal sugar of many oligosaccharides on the surfaces of mammalian cells[6] and is the ligand recognized by the influenza A virus.[7] Two proteins on the surface of this virus bind to carbohydrates terminated by Neu5Ac: hemagglutinin (HA), the carbohydrate binding protein that specifically recognizes Neu5Ac and is responsible for adhesion of virus to cell,[6] and neuraminidase (NA, EC 3.2.1.18), a glycosidase that cleaves the O-glycoside bond of terminal neuraminic acids.[8] The detailed mechanisms of adhesion of virus to cell and escape of newly formed virion from an infected cell surface are not well understood, but both the adhesion and detachment events are essential steps in the viral infection process.

We and others have developed syntheses of monomeric Neu5Ac analogs in order to evaluate these compounds as inhibitors of the influenza virus-cell adhesion process mediated by HA.[9,10,11] The X-ray crystal structure of the complex between influenza HA and *N*-acetylneuraminic acid α-methyl glycoside (**1**, R = CH3,

Neu5Acα2Me) shows a relatively spacious hydrophobic region that interacts with the N-acetyl group of Neu5Acα2Me 1.[12] The presence of this hydrophobic region suggests that acylation of the N-5 nitrogen of neuraminic acid with lipophilic residues might lead to derivatives that bind more tightly than Neu5Acα2Me itself. To explore the influence of different N-acyl groups on the binding of HA to derivatives of Neu5Acα2Me 1, we required a practical synthetic route to the neuraminic acid α-methyl glycoside (Neuα2Me) 2, a key intermediate in preparing N-acylated analogs. We have recently reported a procedure for the Neu5Ac aldolase (EC 4.1.3.3) catalyzed preparation of 2 from 2-deoxy-2-azidomannopyranose and sodium pyruvate.[9] The preparation of the 2-deoxy-2-azidomannopyranose requires 4 steps; this synthetic approach is, as a result, inefficient. This paper describes a chemoenzymic preparation of 2 from the readily available 2-*N*-carbobenzoxy-2-deoxy-D-mannosamine (ManCBz) 3a (see Scheme I). In addition, several new N-acyl neuraminic acid analogs have been prepared and their ability to inhibit the adhesion of influenza virus (X-31)[13] to chicken erythrocytes has been evaluated using a standard hemagglutination assay (HAI).[14] Finally, we evaluate and compare this procedure for the synthesis of 2 to several previously reported synthetic routes.[9,15-22]
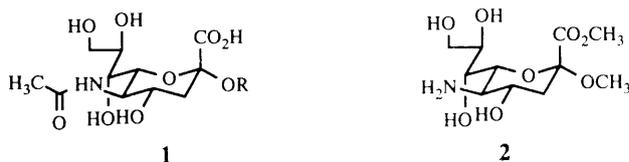
Preparation of Mannose Derivatives. We chose ManCBz 3a as the starting material based on three criteria: that it be 1) commercially available or *easily* prepared; 2) accepted by Neu5Ac aldolase as a substrate for condensation with sodium pyruvate, and 3) protected on nitrogen by a group that was removable under mild conditions. While it was not clear at the start of this investigation that Neu5Ac aldolase would tolerate the steric and electronic characteristics of a CBz protecting group, the other two criteria were fulfilled.[23]

The ManCBz 3a was not commercially available and acylation of 2-mannosamine under Schotten-Bauman[24] conditions with benzyl chlorocarbonate had been reported to be difficult.[25] Our attempts to produce 3a using standard acylation conditions[26] gave either recovered starting material, or, under more forcing conditions,[27] a complex mixture of carbohydrate derived products (including products isomerized to the glucosamine skeleton) that we did not characterize further. We have found that a slight modification of the Schotten-Bauman procedure[24]--the addition of 2-mannosamine hydrochloride to a stirred biphasic solution of aqueous sodium bicarbonate and benzyl chlorocarbonate at ambient temperature--produced the desired ManCBz 3a in 65% yield on a 15-gram scale.

Neu5Ac Aldolase Catalyzed Conversion of ManCBz 3a to Neu5CBz 4a. The Neu5Ac aldolase catalyzed condensation of ManCBz 3a with sodium pyruvate in tris buffer (pH 7.5) proceeded in good yield to produce Neu5CBz 4a in 75% isolated yield (see Scheme I). Due to the limited solubility of 3a in water, excess Neu5Ac aldolase and extended reaction times were required for satisfactory conversion. Neu5Ac aldolase is commercially available and relatively inexpensive, and we generally used non-immobilized enzyme in solution for small or medium scale preparation (< 2 g). In large scale reactions (5-10g), membrane enclosed enzyme

3a, R=NHCBz
3b, R=N$_3$

Na pyruvate / NeuAc aldolase

4a, 75%
4b, 70%

methanol cat. TFA

5a, >90%
5b, 91%

Ac$_2$O pyridine

6a, 99%
6b, 92%

HCl Et$_2$O cat AcCl

7a, >90%
7b, >90%

methanol Ag salicylate

8a, 83%
8b, 83%

NaOCH$_3$ methanol

9a, >99%
9b, >90%

10% Pd/C methanol

2 from 9a, 51%
2 from 9b, 94%
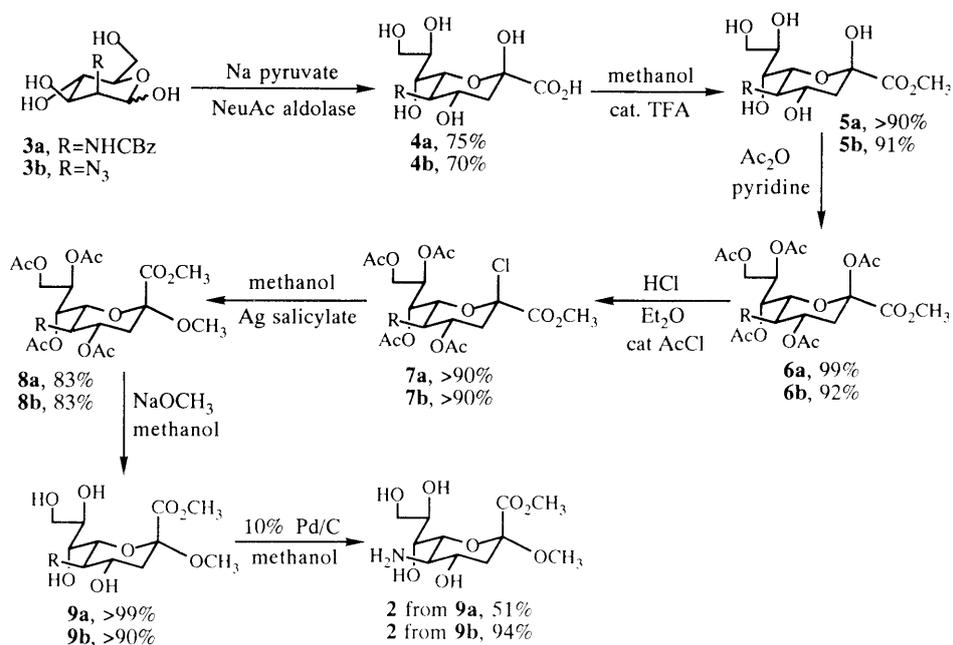
Scheme I. Synthesis of Neuraminic Acid Methyl Ester (2)

preparations were used.[28] The workup and subsequent isolation of **4a** consisted of a simple ion exchange chromatography on Dowex AG-1-X8 (formate form) with a formic acid gradient (0 - 2 M).

Preparation of α-Methylgycoside 9a. Conversion of the Neu5CBz **4a** to the α-methylglycoside methyl ester **9a** followed established procedures with only minor modifications (see Scheme I). Methyl esterification of **4a** with Dowex W50 (H$^+$ form) in methanol[29] or catalytic TFA in methanol,[30] peracylation with acetic anhydride/DMAP/pyridine and treatment with HCl in ether[31] gave the glycosyl chloride **7a**. Silver salicylate in methanol was by far the most selective, efficient and reliable method for the methyl glycosidation reaction.[32] We routinely obtained >70% isolated yield of α-methylglycoside **8a** with no detectable formation of the β-anomer. Deacylation with NaOMe in methanol afforded the tetraol **9a**. In order to compare the results obtained from this series of reactions with those observed by converting 2-deoxy-2-azidomannopyranose **3b** to **2**,[9] the results from the azide series are also summarized in Scheme I.

Deprotection of the C5 Amino Functionality. Removal of the amino protecting group had to be accomplished under non-acidic conditions because of the configurational instability of the α-methylglycosides. The Cbz protecting group was removed easily by catalytic hydrogenation (methanol with 10% Pd-C catalyst) at ambient temperature and atmospheric pressure to afford **2** in 51% purified yield.[33]

Acylation of Free Amine 2. Standard acylation of the free amino intermediate **2** proved problematic. Reaction with stoichiometric amounts of the corresponding acyl chlorides often afforded mixtures of mono- and bisacylated materials, in which the second acyl group could be removed only by prolonged treatment with 1N NaOH. Bisacylation was circumvented by treatment of the free amine with the preformed *N*-hydroxysuccinimide ester of the corresponding acyl compound. This procedure afforded the desired N-acyl derivatives of neuraminic acid in good yields. The methyl ester of each N-acyl neuraminic acid (**1, 9a, 10, 11, 12** and **13**) was hydrolyzed to the corresponding acid before testing these compounds in an HAI assay. Several of these N-acylated neuraminic acids and their values of $K_i^{(HAI)}$[34] are listed in Table I.

2
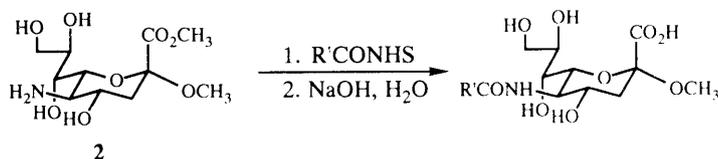
Table I. $K_i^{(HAI)}$ **Values of N-Acylated Neuraminic Acids**

Compound	R'	$K_i^{(HAI)}$ (mM)[a]
1	CH_3-	2.5 [b]
9a	$PhCH_2O$-	10.0
10	CH_3O-	1.5
11	H-	12.0
12		3.5
13	$CH_3(CH_2)_2$-	5.0

[a] $K_i^{(HAI)}$ is an apparent inhibition constant and represents the lowest concentration of inhibitor that prevents agglutination of chicken erythrocytes by virus; see Experimental.
[b] Neu5Acα2Me **1** is used as a control and the value $K_i^{(HAI)}$ = 2.8 mM is comparable to that obtained by an alternative assay.[36]

Inhibition Constants of N-Acyl Neuraminic Acid Analogs from the Hemagglutination Inhibition (HAI) Assay. We tested several N-acyl analogs of Neu5Ac for their ability to inhibit the binding of influenza A virus to chicken erythrocytes using the HAI assay.[14] The HAI assay consists of serial dilutions of inhibitors in 96-well microtitre plates followed by the addition of influenza virus X-31 and incubation. A buffered solution of chicken erythrocytes is added and after further incubation, the wells are checked for agglutination of erythrocytes. We define the hemagglutination inhibition constant $K_i^{(HAI)}$ as the lowest concentration of inhibitor

that prevents agglutination of erythrocytes by influenza at 4 °C under these assay conditions (see Experimental).[35]

The N-acetyl group of Neu5Ac is thought to interact with a nearby tryptophan group on HA; it therefore seemed possible that a N-cyclopropanoyl or a N-Cbz group would lead to tighter binding due to π-overlap. The HAI assay of these N-acyl derivatives of neuraminic acid (Table I) indicates that these derivatives inhibit hemagglutination only as well as the α-methyl glycoside of Neu5Ac itself (Neu5Acα2Me **1**, $K_i^{(HAI)}$= 2.8 x 10^-3 **M**, see Table I).[36]

Other Methods for the Preparation of Neu5Ac Analogs. At the onset of this investigation no useful procedures were available for the preparation of any neuraminic acid analogs carrying a free amino function on C-5.[9] The parent keto-amino acid is believed to be too unstable to be isolated, presumably due to decomposition involving cyclic imine formation. There are of course other methods of obtaining derivatives of neuraminic acid, the most common of which is to obtain Neu5Ac by extraction from natural sources such as meconium[15] and the nest of the chinese swiftlet bird.[16] The latter method is the most popular: as much as 12% by weight can be collected by simple acidic extraction of the commercially available nest substance.[16,17] With the exception of *N*-glycoylneuraminic acid[18] and Neu5Ac,[15,16] other derivatives of neuraminic acid are difficult to obtain from natural sources. Although β-glycosides of the naturally occurring *N*-glycolyl neuraminic acid have been successfully N-deacylated using strong mineral acids,[19] the α-glycosides of glycolyl neuraminic acids are not available in useful quantities. The difficulties associated with the removal of N-acetyl or N-glycoyl groups from naturally occurring neuraminic acids is such that we do not, at present, consider them practical starting materials for the preparation of N-acyl neuraminic acid analogs. Our general experience has been that the synthesis of neuraminic acid and its derivatives is often most conveniently accomplished using enzymes.

We note, however, the recent publication of a procedure in which the free amine **2** was obtained from Neu5Acα2Me **1** by N-deacylation with a methanolic solution of tetramethylammonium hydroxide.[20] We have evaluated this method and found it to be a feasible alternative approach to the preparation of **2** provided a suitable source of Neu5Acα2Me **1** is available.[15,16]

A number of synthetic routes to neuraminic acid analogs have been developed. Early examples focused mainly on the chemical condensation of pyruvate equivalents with *N*-acetylmannosamine.[21] These methods often suffer from low yields (<30%) and difficult purifications. An elegant total chemical synthesis of Neu5Ac has been reported.[22] Unfortunately the large number of steps in synthesis make it impractical for preparing large quantities of Neu5Ac. It is conceivable that these chemical methods could be useful in preparing derivatives of neuraminic acid other than Neu5Ac. We believe, however, that either the method described here using a chemoenzymic protocol, or that involving deacylation of Neu5Acα2Me **1**,[20] offer the most efficient methods now available for the preparation of N-substituted analogs of neuraminic acid.

CONCLUSION

We have developed a route for the synthesis of the key intermediate α-methylneuraminic acid methyl ester **2** via an enzyme-catalyzed condensation of sodium pyruvate with CBz-mannosamine **3a**. This procedure takes advantage of Neu5Ac aldolase and its ability to accept a range of N-substituted mannosamines. The Cbz

route and the previously reported azido route[9] fulfill the requirements of enzyme compatibility and nitrogen protection group removability. The azide route is, however, less convenient than the Cbz route.[9] The Cbz route provides **2** efficiently and on multigram scales.

The simple N-substituted derivatives of Neu5Ac, prepared by N-acylation of **2**, inhibited hemagglutination of chicken erythrocytes, but no better than Neu5Acα2Me **1**.[36] We are currently preparing new analogs of Neu5Ac that incorporate long N-5 lipophilic side chains (C4-C14) with the anticipation that these compounds will show enhanced HA binding and therefore enhanced hemagglutination inhibition.[37]

EXPERIMENTAL SECTION

General Methods and Materials. Reagents and solvents were reagent grade and used as received unless otherwise noted. Neu5Ac aldolase was obtained from Sigma. Mannosamine·HCl was purchased from Pfhanstiehl Laboratories, Inc. (Waukegan, Il.) Tlc was preformed on glass plates with UV fluorescent indicator (Merck, silica gel 60 F_{254}). Plates were stained using cerium molybdate stain (2 g of ceric sulfate, 48 g of ammonium molybdate and 10% aq H_2SO_4, 1L). Flash chromatography[38] employed silica gel from Merck. Ion exchange resins AG1-X8 (formate form, 100-200 mesh) and Dowex 50W-X8 were purchased from Biorad.

N-acetylneuraminic acid was obtained from extraction of the edible chinese swiftlet's nest.[16,17] Erythrocytes from two week old chickens were from Spafas Inc. Influenza virus (X-31) was from obtained the laboratory of Professor John Skehel. Phosphate buffered saline used in the HAI assays was prepared from 80 g of NaCl, 2 g of KCl, 11 g of Na_2HPO_4 and 2 g of KH_2PO_4 in 1L of distilled H_2O. This stock solution was diluted 1 part in 10 in distilled H_2O and then adjusted to pH 7.2 with 1N NaOH.

Proton and carbon NMR spectra were measured on Bruker AM-300, -400 and -500 MHz spectrophometers. Chemical shifts are reported in ppm relative to the solvent; $CHCl_3$ in $CDCl_3$ at 7.24 ppm, HOD in D_2O at 4.80 ppm, CH_3CN in CD_3CN at 1.97 and CDH_2OH in CD_3OD at 3.30 ppm for the proton spectra. For carbon spectra the references are 77.0 ppm for $CDCl_3$, 1.70 ppm for CD_3CN, 39.8 for DMSO-d_6 and 49.9 ppm for CD_3OD.

Hemagglutination inhibition assay. The HA titer of a PBS stock solution of X-31 influenza virus was determined by serial dilution of 50 μL of the virus solution in 12 microtiter plate wells (96 well microtiter plate) each containing 50 μL of PBS. Chicken erythrocytes in PBS (5% suspension, 100 μl) was added to each well, mixed and incubated for 1h at 4 °C. The HA endpoint is defined as the last dilution well before erythrocyte pellets begin to form and is expressed as a reciprocal of the endpoint dilution.

The monomeric inhibitors were diluted in PBS (pH 7.2). The inhibitor (50 μL) was serially diluted in 12 microtiter plate wells containing 50 μL PBS. To each well was added 50 μL of an X-31 virus solution that had been diluted to the titer endpoint concentration determined as described above. After a 30 min incubation period (4 °C), 100 μL of the suspension of chicken erythrocytes was added to each well and the plate was gently agitated and then incubated at 4 °C for an additional 2 h. The endpoint ($K_i^{(HAI)}$) is defined as the lowest concentration of Neu5Ac residues that inhibits 50% agglutination of erythrocytes by influenza virus (X-31).

The inhibition of hemagglutination by N-acyl neuraminic acids was measured relative to Neu5Acα2Me **1** ($K_i^{(HAI)}$ = 2.8 x 10^{-3} M).[36]

2-*N*-Carbobenzoxyl-2-deoxy-D-mannosamine (ManCbz) 3a.

Mannosamine hydrochloride (15 g, 69 mmol) was dissolved in saturated aqueous sodium bicarbonate (250 mL) and benzylchlorocarbonate (11.7 mL, 83 mmol, 1.2 equiv) was added. The two-phase system was stirred for 4 h at room temperature. The yellow solution was concentrated by evaporation, redissolved in ethanol, filtered and concentrated to a viscous oil. The oil was filtered through silica gel and concentrated by evaporation to produce a white solid (14 g, 45 mmol, 65%); ^{1}H NMR (500 MHz, D$_2$O, TSP) δ 7.50-7.45 (m, 5H), 5.17 (d, 1H, J = 12.6 Hz), 5.10 (d, 1H, J = 12.6 Hz), 4.20 (m, 1H), 4.07 (m, 1H), 3.91-3.75 (m, 2H), 3.60 (m, 1H), 3.51 (m, 1H), 3.42 (m, 1H); tlc (15% methanol/ethyl acetate) R$_f$=0.75.

5-*N*-Carbobenzoxylamino-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosidonic Acid (Neu5Cbz) 4a.

Cbz-Mannosamine **3a** (622 mg, 2 mmol) dissolved in tris-buffer (3.8 mL) was added to a solution of sodium pyruvate (2.20 g, 20 mmol) in the same buffer solution (7 mL). To this mixture was added several hundred units (μmol min^{-1} mg^{-1}) of Neu5Ac aldolase. The reaction mixture was stirred at 37 °C, pH 7.6, for one day under air. The reaction mixture was followed by tlc and ^{1}H NMR spectroscopy. Using ion-exchange chromatography, the product was eluted from an AG-1-X8 column (formate form) with a gradient of 0-2 M aqueous formic acid. The product was obtained as white flaky solid by evaporation in vacuo and subsequent lyophilized (520 mg, 65%). The entire procedure was later run with 5.0 g (161 mmol) of **3a** to yield 4.76 g (11.9 mmol, 75%); ^{1}H NMR (500 MHz, D$_2$O, pH 7, TSP) δ 7.43 (m, 5H), 5.17 (d, 1H, J = 12.6 Hz), 5.10 (d, 1H, J = 12.6 Hz), 4.02 (m, 1H), 3.98 (d, 1H, J = 10.7), 3.82 (d, 1H, J= 11.9), 3.74 (t, 1H, J = 6.9 Hz), 3.65 (t, 1H, J = 10.3 Hz), 3.57 (dd, 1H, J = 6.8, 11.9 Hz), 3.53 (d, 1H, J = 9.1 Hz), 2.20 (dd, 1H, J = 4.8, 12.8 Hz), 1.82 (dd, 1H, J = 12.4, 12.8 Hz); tlc (6:2:3 1-propanol/ammonium hydroxide/water) R$_f$=0.59.

5-*N*-Carbobenzoxylamino-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosidonic Acid Methyl Ester 5a.

The carboxylic acid **4a** (200 mg, 0.50 mmol) was dissolved in anhydrous methanol (10 mL) with catalytic trifluoroacetic acid (38 μL, 0.050 mmol) and stirred under nitrogen for 40 h until tlc no longer showed any starting material. Excess MeOH and CF$_3$CO$_2$H were removed in vacuo. The product was a white solid, which was pure by ^{1}H NMR spectroscopy. It was taken on to the next step without further purification; ^{1}H NMR (500 MHz, CD$_3$OD) δ 7.34 (m, 5H), 5.12 (d, 1H, J = 12.4 Hz), 5.07 (d, 1H, J = 12.4 Hz), 4.03 (d, 1H, J = 10.5 Hz), 3.99 (dd, 1H, J = 5.6, 15.8 Hz), 3.79 (dd, 1H, J = 2.6, 11.5 Hz), 3.77 (s, 3H), 3.67 (m, 1H), 3.63-3.55 (m, 3H), 2.19 (dd, 1H, J = 4.9, 12.8 Hz), 1.87 (dd, 1H, J = 11.6, 12.8 Hz); ^{13}C NMR (125 MHz, DMSO-d$_6$) δ 170.56, 157.51, 137.33, 128.64(2C), 128.10, 128.04(2C), 95.18, 70.71, 70.36, 69.27, 65.98, 65.80, 63.92, 54.53, 52.53, 40.33; tlc (3:1 dichloromethane/methanol) R$_f$=0.63. FAB-HRMS calcd for C$_{18}$H$_{25}$O$_{10}$N=415.1478, Found [M+H]$^+$= 416.1475. Anal. calcd for C$_{18}$H$_{25}$O$_{10}$N C 52.05, H 6.07, N 3.37, Found C 51.85, H 5.98, N 3.39.

2,4,7,8,9-Penta-O-acetyl-5-*N*-carbobenzoxylamino-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulo-pyranosidonic Acid Methyl Ester 6a.

The compound **5a** (207 mg, 0.50 mmol)

was dissolved in pyridine (3 mL) and treated with acetic anhydride (2 mL) and cat DMAP (ca 5 mg). The solution was stirred under nitrogen at 0 °C for 30 min, and then overnight at room temperature. Flash chromatography (4:1 ethyl acetate:ethyl ether) followed by evaporation gave a white foam (**6a**, 261 mg, 0.42 mmol, 83%). This compound could be obtained in higher purity when the reaction was run on recrystallized (methanol:ethyl acetate) starting material. In this event, subsequent chromatography was not necessary. Instead, the brown solid was redissolved in methylene chloride, washed with saturated bicarbonate, 1N HCl, and saturated NaCl, then dried over $MgSO_4$, filtered and evaporated to give a white foam (**6a**, 99% yield); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (m, 5H), 5.45 (m, 1H), 5.22 (dd, 1H, J = 6.0, 12.0 Hz), 5.13 (d, 1H, J = 12.0 Hz), 5.11 (m, 2H), 4.95 (d, 1H, J = 9.0 Hz), 4.62 (dd, 1H, J = 6.0, 12.0 Hz), 4.13-4.04 (m, 2H), 3.75 (s, 3H), 2.54 (dd, 1H, J = 4.9, 13.3 Hz), 2.15 (s, 3H), 2.13 (s, 3H), 2.06 (s, 3H), 1.99 (m, 1H), 1.76 (s, 3H); tlc (1:1 hexane/ethyl acetate) R_f=0.34.

4,7,8,9-Tetra-O-acetyl-5-*N*-carbobenzoxylamino-2-β-chloro-2,3,5-trideoxy-β-D-glycero-D-galacto-2-nonulopyranosidonic Acid Methyl Ester 7a.

A portion of **6a** (261 mg, 0.42 mmol) was added to a 50-mL three-necked round-bottomed flask fitted with a gas inlet valve, glass stopper and $CaCl_2$ drying tube. The flask was purged with dry, freshly distilled ethyl ether (~30 mL). The solution was chilled to -45 °C (CH_3CN/dry ice bath) and treated with 0.1 mL of acetyl chloride. Using a gas inlet valve, HCl gas (dried through a sulfuric acid bubbler) was added over 3-4 h, at which time the starting material was consumed (tlc). The HCl was allowed to bubble an additional 15 min and then stopped. The solution warmed to room temperature and was concentrated to dryness on a rotary evaporator (*Caution! care was taken to avoid heating the water bath to over ~45 °C to avoid elimination*). The chloride **7a** (240 mg, 100%) was collected as a white foam; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35-7.30 (m, 5H), 5.55 (dd, 1H, J = 2.3, 7.6 Hz), 5.35 (ddd, 1H, J = 4.8, 10.6, 10.6 Hz), 5.20 (m, 1H), 5.06 (ABq, 2H, J = 12.2, 54.1 Hz), 4.61 (d, 1H, J = 10.6 Hz), 4.38 (dd, 1H, J = 2.4, 12.5 Hz), 4.31 (dd, 1H, J = 2.4, 10.6 Hz), 4.07 (dd, 1H, J = 5.2, 12.5 Hz), 3.87 (s, 3H), 2.80 (dd, 1H, J = 4.8, 13.8 Hz), 2.22 (dd, 1H, J = 11.6, 13.8 Hz), 2.14 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 1.86 (s, 3H); tlc (1:1 hexane/ ethyl acetate) R_f=0.53.

α-Methyl-4,7,8,9-tetra-O-acetyl-5-*N*-carbobenzoxylamino-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosidonic Acid Methyl Ester 8a.

The chloride **7a** (252 mg, 0.42 mmol) was dissolved in dry methanol and 1.5 equiv of freshly precipitated silver carbonate[39] was added. The solution was stirred under N_2 in the dark for 2h and then filtered over Celite (545), washed generously with methanol, and concentrated by evaporation to a purple foam. The compound was purified by flash chromatography (1:1 hexane/ethyl acetate). The α-methyl glycoside **8a** (83%) was collected as a white solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30-7.15 (m, 5H), 5.38 (s, 1H), 5.04 (m, 1H), 4.85 (m, 2H), 4.80 (s, 1H), 4.74 (m, 1H), 4.23 (m, 1H), 4.08 (m, 2H), 3.72 (s, 3H), 3.25 (s, 3H), 2.51 (dd, 1H, J = 4.6, 13.6 Hz), 2.14 (s, 3H), 2.10 (s, 3H), 1.98 (m, 1H), 1.95 (s, 3H), 1.75 (s, 3H); tlc (1:1 hexane:ethyl acetate) R_f=0.47.

α-2-methyl-5-*N*-Carbobenzoxylamino-2,3,5-trideoxy-β-D-glycero-D-galacto-2-nonulopyranosidonic Acid Methyl Ester 9a.

A catalytic amount of sodium (<1 mg) was added to a solution of **8a** (240 mg) in anhydrous methanol (5 mL). The solution was stirred under Ar for 12 h. The

solution was acidified to pH 4 with Dowex 50W-X8 (H^+ form), filtered and concentrated by evaporation to afford a yellow oil. The oil crystallized from chloroform to yield white flakes (**9a**, 120 mg, 99%); ^{1}H NMR (300 MHz, CD$_3$OD) δ 7.38-7.25 (m, 5H), 5.10 (m, 2H), 3.89 (m, 2H), 3.85 (s, 3H), 3.65-3.54 (m, 5H), 3.37 (s, 3H), 2.62 (dd, 1H, J = 4.8, 13.0 Hz), 1.71 (dd, 1H, J = 12.1, 13.0 Hz); ^{13}C NMR (125 MHz, CD$_3$CN) 174.0, 170.1, 138.7, 131.0(2C), 130.5, 129.7(2C), 101.5, 74.0, 72.3, 69.9, 68.0, 67.9, 65.7, 55.1(2C), 52.6, 40.9; tlc (9:1 ethyl acetate/ methanol) R$_f$=0.64; FAB-HRMS calcd for C$_{19}$H$_{27}$O$_{10}$N=429.1634, Found [M+H]$^+$ = 430.1714; Anal. calcd for C$_{19}$H$_{27}$O$_{10}$N C 53.13, H 6.34, N 3.26, found C 52.98, H 6.28, N 3.20.

α-Methyl-5-*N*-carbomethoxyamino-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosidonic Acid Methyl Ester 10.

^{1}H NMR (300 MHz, CD$_3$OD) δ 3.90-3.75 (m, 5H), 3.69-3.47 (m, 6H), 3.40-3.25 (m, 5H), 2.62 (m, 1H), 1.70 (t, 1H, J = 12.0 Hz); tlc (9:1 ethyl acetate/ methanol) R$_f$=0.27.

Methyl 5-amino-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosidonic Acid Methyl Ester 2.

The Cbz precursor **9a** (120 mg, 0.28 mmol) was dissolved in methanol (8 mL). To the reaction solution was added 10% Pd/C (10 mg) and then kept under an atmosphere of H$_2$. After stirring for 24 h, the compound was purified by flash chromatography (9:1 methanol/ ethyl acetate). The amine **2** (0.42 mg, 0.142 mmol, 51%)[33] was obtained by evaporation followed by lyophilization; ^{1}H NMR (500 MHz, D$_2$O) δ 3.91 (m, 2H), 3.91 (s, 3H), 3.86 (dd, 1H, J = 4.0, 10.0 Hz), 3.73 (t, 1H, J =10.0 Hz), 3.70 (dd, 1H, J = 4.0, 12.0 Hz), 3.55 (m, 1H), 3.39 (s, 3H), 2.81 (t, 1H, J =12.0 Hz), 2.64 (dd, 1H, J = 4.0, 12.0 Hz), 1.75 (t, 1H, J =12 Hz); ^{13}C NMR (125 MHz) δ 170.2, 99.8, 73.5, 71.0, 69.0, 68.2, 63.9, 53.0, 52.5, 51.0, 38.0; tlc (1:1 methanol/ethyl acetate) R$_f$=0.28; FAB-HRMS calcd for C$_{11}$H$_{21}$O$_8$N=295.1267, Found [M+H]$^+$= 296.1246; Anal. calcd for C$_{11}$H$_{21}$O$_8$N; C 44.74, H 7.16, N 4.74, Found C 44.25, H 7.16, N 4.90.

General procedure for the acetylation of the 5-amino-α-methyl ketoside **2**. To a mixture of α-methyl ketoside **2** (17 mg, 57.8 μmol) in pyridine (1 mL) was added 1.1 equiv of the appropiate acid chloride or acyl succinimide at 10 °C under a nitrogen atmosphere. The solution was stirred at room temperature until tlc (ethyl acetate/ methanol 9:1) showed the complete conversion of the starting material. The mixture was concentrated by evaporation and resulting oil was chromatographed on a column of silica gel.

α-Methyl-5-*N*-formylamino-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosidonic Acid Methyl Ester 11.

Formic acid (50 mL) was added slowly to acetic anhydride (100 mL) at 0 °C. The solution was allowed to warm to room temperature. After 2 h, *N*-hydroxylsuccinnimide was added to the solution and stirred overnight. The solution was evaporated to a white solid which was suspended in ethyl acetate (100 mL) and then filtered to remove excess acetic anhydride. The addition of hexanes caused the ester to crystallize as a white solid which was collected by filtration to afford the required formyl-NHS ester (6.2 g, 43 mmol, 50 %); ^{1}H NMR (300 MHz, CD$_3$CN) δ 8.30 (s, 1H, formyl H), 2.78 (s, 4H). The free amine **2** (40 mg, 0.135 mmol) was dissolved in pyridine (2.5 mL), and the preformed formyl-*N*-hydroxylsuccinnimide (29 mg, 0.203 mmol) was added. The solution was stirred overnight, purified by chromatography (3:2 ethyl

acetate/methanol), and passed through an ion exchange column (Dowex AG-1-X8, formate form). The final product was obtained as a white solid (32 mg, 0.1 mmol, 74 %); ^{1}H NMR (300 MHz, CD_3CN) δ 8.0 (s, 1H), 3.90-3.40 (m, 7H), 3.88 (s, 3H), 3.39 (s, 3H), 3.35 (s, 3H), 2.67 (dd, 1H, J = 6.8, 13.0 Hz), 1.80 (m, 1H); ^{13}C NMR (125 MHz, CD_3CN) δ 170.7, 168.7, 101.5, 75.0, 73.8, 71.0, 69.4, 65.6, 56.0, 53.7, 52.8, 39.5; tlc (4:5:1 butanol/acetone/water) R_f=0.62.

α-Methyl-5-*N*-cyclopropanoyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosidonic Acid Methyl Ester 12.[9]

Eluant (ethyl acetate/methanol 12:1, R_f=0.21 in 9:1 ethyl acetate/methanol). Yield: 12.5 mg (34.7 μmol, 60 %) as a white powder; ^{1}H NMR (D_2O, 400 MHz) δ 3.81 (m, 5H), 3.81 (s, 3H), 3.62 (dd, 1H, J = 5.5, 11.5 Hz), 3.53 (d, 1H, J = 9.0 Hz), 3.35 (s, 3H), 2.66 (dd, 1H, J = 4.0, 12.0 Hz), 1.77 (t, 1H, J = 12 Hz, 1H), 1.59 (m, 1H), 0.85 (m, 4H); ^{13}C NMR (125 MHz) δ 170.5, 168.9, 99.0, 73.6, 73.2, 72.4, 68.9, 68.6, 67.6, 63.9, 54.8, 54.1, 52.5, 51.0, 39.1.

α-Methyl-5-*N*-butrylcarbonylamino-3,5-dideoxy-β-D-glycero-D-galacto-2-nonulopyranosidonic Acid Methyl Ester 13.

The free amine 2 (44.2 mg, 0.15 mmol) was dissolved in dry pyridine (1 mL) and cooled to 0 °C under nitrogen. Butyryl chloride (23 μL, 0.23 mmol) in dry CH_2Cl_2 (1 mL) was added dropwise over 5 min to the solution of 2. After 24 h the reaction was incomplete, so over the next 2 days butyric anhydride (24.5 μL, 0.15 mmol) was added three more times. After a total of 5 days, three drops of water was added. After 1 h, the solution was pumped down to 1 mL, dissolved in methanol and run through a column (1:1 methanol/ethyl acetate) of silica gel. The solution was then run through a 5 mm anion exchange column (ammonium formate form) and concentrated by evaporation providing a white solid. This solid was taken up in methanol (1 mL) and KO^tBu (several mgs) was added. This solution was left overnight, neutralized with Dowex (H^+), and filtered. A column (1:1 methanol/ethyl acetate) of silica gel afforded product 14 (30 mg, 55 %) with a trace of bis-acetylated material. Half of this material was purified further by preparative tlc yielding 10 mg of the desired product. The other half was combined with H_2O (1 mL) and triethylamine (0.5 mL), stirred overnight, concentrated by evaporation, then passed through a pipette of Dowex Na^+ form, producing a clear oil (13 mg). The two products were combined and purified by passing the N-acetate through a column of silica gel (1:1 methanol/ ethyl acetate) leaving a white solid (6 mg); ^{1}H NMR (400 MHz, D_2O) δ 3.90-3.55 (m, 7H), 3.80 (s, 3H), 3.31 (s, 3H), 2.71 (dd, 1H, J = 2.0, 10.0 Hz), 2.25 (t, 2H, J = 10.0 Hz), 1.60 (m, 3H), 0.90 (t, 3H, J = 8.0 Hz); tlc (1:1 methanol/ethyl acetate) R_f=0.5.

General procedure for the hydrolysis of the neuraminic acid methyl ester as described for 9a.   Methyl ester 9a (120 mg, 0.28 mmol) was added to a 25 mL round bottom flask equipped with a stir bar. To the flask was added 5 mL 1.0 N NaOH and the reaction was stirred at room temperature until tlc (chloroform/methanol 9:1) showed the complete consumption of starting material (~ 1h). The reaction mixture was passed through a Dowex 50W-X8 column (hydrogen form) to remove the sodium salts. The water was removed by lyophilization to afford a white powder (110 mg, 0.28 mmol, 100%). The acid was dissolved in PBS (pH 7.2) and used in the HAI assay without further purification.

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     i   **10**
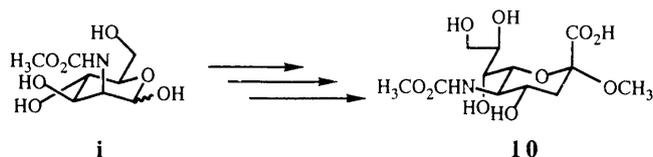
     This 2-mannosamine derivative **i** was carried through each reaction sequence in Scheme I to produce **10** in 29% overall yield. The methyl carbamate could be only be removed in low and variable yields using 40% KOH/nBuOH (see Corey, E. J.; Bock, M. G.; Kozikowski, A. P.; Rama Rao, A.V.; Floyd, D.; Lipshutz, B. *Tetrahedron Lett.* **1978**, 12, 1051). This route was abandoned as a synthesis of **2**.

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