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Sialyltransferase-catalyzed transfer of KDN onto oligosaccharides [1]

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Abstract

Sialyltransferases catalyze transfer of *N*-acetylneuraminic, the most common sialic acid, from cytidine 5-monophospho-*N*-acetylneuraminic acid, onto oligosaccharide chains. 3-Deoxy-β-D-glycero-D-galacto-2-nonulopyranosonic acid (KDN), the deaminated analogue of *N*-acetylneuraminic acid, was converted into CMP-KDN by a chemical procedure involving CMP phosphoramidite. KDN was then successfully transferred, from CMP-KDN, onto Galβ1-3(2*O*Ac)Galβ*O*CH$_3$, in porcine liver α(2-3) sialyltransferase-catalyzed reaction, allowing preparation of KDNα2-3Galβ1-3(2*O*Ac)Galβ*O*CH$_3$ in 88% yield. KDNα2-6Galβ1-4GlcNAc could be also prepared using rat liver sialyltransferase.

Keywords: *N*-Acetylneuraminic; Oligosaccharide synthesis; Sugar-nucleotide synthesis

1. Introduction

Sialic acids, present on animal cell surface, are located at the non-reducing end of oligosaccharides of glycoproteins and glycolipids; they are known to mask galactose residues involved in recognition of Ashwell lectin, responsible for removal of desialylated glycoconjugates from blood circulation. Sialyltransferases are the enzymes catalyzing in vivo transfer of *N*-acetylneuraminic acid, the most common sialic acid, from cytidine 5-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc), the sugar-nucleotide donor, onto oligosaccharide chains, according to the following scheme:

CMP-NeuAc + oligosaccharide

→ NeuAc-oligosaccharide + CMP

These enzymes have been shown to efficiently act in vitro on a variety of oligosaccharide acceptors [1–3]. Of great interest would be the replacement of *N*-acetylneuraminic acid by a sialic acid resistant to sialidases in order to increase the bio-availability of oligosaccharides as potential drugs, or the plasmatic lifetime of recombinant glycoproteins. KDN (**1**), the deaminated neuraminic acid, first isolated from the polysialoglycoprotein of rainbow trout [4] has been found to resist viral and bacterial sialidases [5]. Thus, it was interesting to test the activity of

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[1] Dedicated to Roger W. Jeanloz on the occasion of his 80th birthday.

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mammalian sialyltransferases towards CMP-KDN (**2**), which has not yet been investigated.

We previously reported that calf brain sialate synthetase, unlike the *E. coli* enzyme, was able to accept KDN as a substrate, catalyzing the following reaction:

$$KDN + CTP \rightarrow CMP\text{-}KDN + PP_i$$

But because of the poor kinetic constants of the enzyme towards **1** and the inherent instability of the sugar-nucleotide, **2** was only obtained in a low yield [6]. On the other hand, a KDN specific synthetase occurring in rainbow trout testis has been reported in the literature [7], but was not at our disposal. In order to make this sugar-nucleotide available on a large scale, we decided to turn to chemical synthesis of **2**.

Thus, we report herein, at first, the chemical synthesis of CMP-KDN (**2**) and then KDN transfer onto oligosaccharides catalyzed by natural mammalian sialyltransferases.

2. Experimental

All general procedures were as described previously [8].

*2.1. Methyl-4,5,7,8,9-penta-O-acetyl-3-deoxy-β-D-glycero-D-galacto-2-nonulo-pyranosonate (**5**)*

To a solution of **1** (1.54 g, 5.74 mmol) in anhydrous MeOH (100 ml) was added Dowex 50 (H^+) resin (5 g) and the mixture was stirred at room temperature under N_2 for 15 h. After filtration of the resin, the filtrate was evaporated to dryness giving **3** (1.6 g). A CH_3COCl:CH_3COOH 2:1 mixture (100 ml) was added to **3** and the solution was saturated at 0°C with anhydrous HCl. After 20 h at room temperature, the reaction mixture was concentrated and co-evaporated several times with toluene, affording the chloride **4** as the residual syrup. To a solution of **4** (2.8 g, 5.7 mmol) in a THF:water 9:1 mixture (62 ml) were added HgO (920 mg, 4.3 mmol) and $HgCl_2$ (770 mg, 2.84 mmol). The mixture was stirred at room temperature overnight, then filtered on Celite and evaporated to dryness. The residue was chromatographed (hexane:ethyl acetate, 2:1) to give compound **5** (1.5 g, 54% from **1**); $[\alpha]_D^{30}$ 3 (*c* 1.1, CH_2Cl_2); ^{1}H NMR (DMSO): δ 1.87 (t, 1H, *J* 12.5 Hz, H-3a), 1.90–2.05 (15H, 5 OAc), 2.22 (dd, 1H, *J* 5 Hz, H-3e), 3.70 (s, 3H, CH_3), 4.07 (dd, 1H, *J* 11.5, *J* 6.5 Hz, H-9a), 4.21 (dd, 1H, *J* 2, *J* 9 Hz, H-6), 4.40 (dd, 1H, *J* 3 Hz, H-9b), 4.75 (t, 1H, *J* = *J* 9 Hz, H-5), 5.05 (m, 1H, H-8), 5.15 (m, 1H, H-4), 5.26 (dd, 1H, *J* 5 Hz, H-7), 7.55 (s, 1H, OH); ^{13}C NMR ($CDCl_3$) δ 20.57, 20.67, 20.69, 20.84, 20.89 (5 OAc), 35.62 (C-3), 53.57 (CH_3), 62.37 (C-9), 66.76 (C-4), 67.95 (C-7), 68.93, 69.19, 69.59 (C-8, C-6, C-5), 94.65 (C-2), 168.87 (COOMe), 169.88, 169.90, 170.01, 170.07, 170.64 (5 CO). Anal. Calcd. for $C_{20}H_{28}O_{14}$: C, 48.78; H, 5.73; O, 45.82. Found: C, 48.33; H, 5.71; O, 45.82.

*2.2. 2-Cyanoethyl 2′,3′-O,N⁴-triacetylcytidin-5′-yl-N,N-di-sopropylphosphoramidite (**7**)*

To a solution of triacetylcytidine **6** (300 mg, 0.81 mmol), in anhydrous THF (3 ml) were added diisopropylethylamine (0.565 ml, 3.24 mmol) and 2-cyanoethyl-*N*,*N*′-di-isopropylchlorophosphoramidite (0.362 ml, 1.62 mmol) and the mixture was stirred for 1 h at room temperature. The mixture was then concentrated to dryness and the residue purified by flash chromatography (ethyl acetate:hexane 4:1, containing 1.2% pyridine), affording **7** (393 mg, 85%) as a 1:1 diastereomeric mixture, ^{1}H and ^{31}P NMR in agreement with literature [5].

*2.3. 2-Cyanoethyl 2′,3′-O,N⁴-triacetylcytidin-5′-yl-methyl-4,5,7,8,9-penta-O-acetyl-3-deoxy-β-D-glycero-D-galacto-2-nonulopyranosid-2″-yl phosphite (**8**)*

To a cooled (−40°C) solution of phosphoramidite **7** (391 mg, 0.686 mmol) in anhydrous CH_3CN (1 ml) were added derivative **5** (65 mg, 0.137 mmol) dissolved in CH_3CN (0.7 ml) and 1*H*-tetrazole (48 mg, 0.686 mmol). The mixture was stirred at −40°C under nitrogen for 5 min,

then the temperature was allowed to warm up to 20°C and the mixture was further stirred for 1 h. After evaporation to dryness, the residue was purified on Sephadex LH-20 (1.5 × 90 cm) with MeOH as the eluent, affording phosphite **8** (85 mg, 60%, 1.3:1 diastereomeric mixture); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 = 0 ppm): δ 137.13, 135.90; selected ^{1}H NMR data ($CDCl_3$): δ 2.15–2.0 (21H, 7 OAc), 2.40 (s, 3H, NHAc), 2.75 (m, 4H, $(CH_2)_2$), 3,82 (2s, CH_3), 6.10, 6.20 (2d, *J* 5 Hz, 2 H-1′), 7.57, 7.60 (2d, 2 H-5″), 8.02, 8.07 (2d, *J* 7.5 Hz, 2 H-6′).

*2.4. 2-Cyanoethyl 2′,3′-O,N^4-triacetylcytidin-5′-yl-methyl-4,5,7,8,9-penta -O- acetyl -3- deoxy-β-*D - glycero - D - galacto-*2-nonulopyranosid-2′-yl phosphate (**9**)*

To a cooled (0°C) solution of phosphite **8** (85 mg, 0.089 mmol) in CH_3CN (0.415 ml) was added *t*-BuOOH 3 M in isooctane (0.297 ml, 0.897 mmol); the mixture was stirred at 0°C for 30 min, then diluted with EtOAc (5 ml), washed with saturated $NaHCO_3$ solution and water. After drying ($MgSO_4$), the organic phase was evaporated to dryness (80 mg, 93%, 1:1 diastereomeric mixture); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 = 0 ppm): δ −6.43, −6.63.

*2.5. Cytidine 5′-(3-deoxy-β-*D - glycero - D - galacto-*2-nonulopyranosylonic acid monophosphate (CMP-KDN) (**2**)*

A solution of phosphate **9** (80 mg, 0.083 mmol) in THF (1.1 ml) was stirred with DBU (15 mg, 0.099 mmol) for 5 min at room temperature; to this mixture a 4 M NaOMe solution (0.110 ml, 0.880 mmol) was then added. After 3 h, water (1.4 ml) was added and the reaction mixture was further stirred at room temperature for 5 h. Deacetylation was monitored on TLC (*n*-propanol:water 7:3, *Rf* **2** 0.32). Then the mixture was diluted with MeOH (10 ml), neutralized to pH 7.9 by careful addition of BioRad 50W-X-8 (H^+ form) resin. After filtration of the resin, the filtrate was purified on a DEAE-Sephadex A-25 column (2 × 30 cm^2) (HCO_3^- form). Elution with a gradient of 0.1–0.8 M $HCO_3^-NEt_3^+$ (pH 7.9) gave **2** as its bis(triethylammonium) salt (40 mg, 60%); ^{1}H NMR data in agreement with literature [3,4]; ^{31}P NMR ($CDCl_3$, 85% H_3PO_4: 0 ppm) δ: −4.25; $[\alpha]_D^{30}$ −9 (*c* 1.1, water).

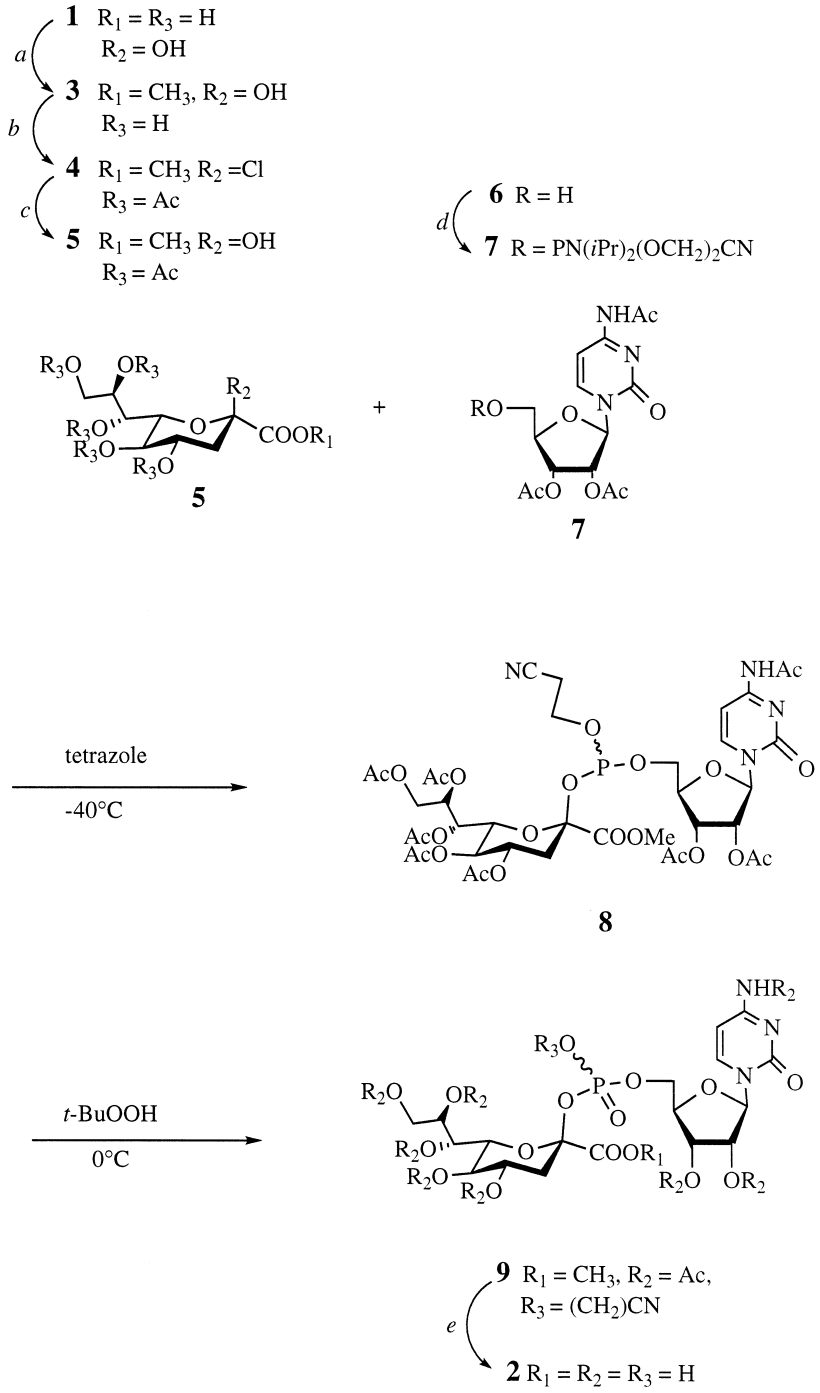
*2.6. Methyl -O- (3- deoxy -α-*D - glycero - D - galacto-*2-nonulopyranosylonic acid-(2 → 3)-O-β-D-galactopyranosyl-(1 → 3)-O-(2-O-acetyl) -β-D-galactopyranoside (**11**)*

A solution of disaccharide **10** (5 mg, 12.75 μmol) in 50 mM Na cacodylate buffer (pH 7.5, 1.7 ml) containing 100 mM NaCl, 0.1% Triton X-100 and 20 mM $MnCl_2$ was incubated at 37°C with ST3Gal-1 (26 mU), CMP-KDN (**2**) (11.25 mg, 13 μmol) added in three portions (at 6 h interval time) and alkaline phosphatase (7 U) for 36 h. Reaction mixture was first applied to a DEAE-Sephadex A-25 (HCO_3^- form) column (1.5 × 15 cm), eluted with a 0 to 0.2 M $HCO_3^-HNEt_3^+$ gradient (pH 7.8); fractions containing trisaccharide **11** were freeze-dried and then applied to a column of Sephadex G-15 (2 × 60 cm), affording pure trisaccharide **11** which was converted to its sodium salt by passage down a column of BioRad 50W-X-8 resin (Na^+ form) (7.3 mg, 88%); *Rf*: 0.41 (*n*-propanol:water 4:1); ^{1}H NMR (250 MHz, D_2O): δ 5.0 (dd, 1H, *J* 8, *J* 9 Hz, H-2), 4.55 (d, 1H, *J* 8 Hz, H-1′), 4.50 (d, 1H, *J* 8 Hz, H-1), 4.15 (d, 1H, *J* 3 Hz, H-4), 4.0 (dd, 1H, *J* 3.5 Hz, H-3′), 3.95 (dd, 1H, *J* 10 Hz, H-3), 3.84 (d, 1H, H-4′), 3.45 (s, 3H, OCH_3), 2.65 (dd, 1H, *J* 12 *J* 4.5 Hz, H-3″e), 2.1 (s, 3H, OAc), 1.70 (t, 1H, *J* 12 Hz, H-3″a); LRMS (negative mode): *m/z* 647.1 $[M - Na]^-$.

*2.7. 3-Deoxy-α-*D - glycero - D - galacto-*2-nonulopyranosylonic acid-(2 → 6)-O-β-D-galactopyranosyl-(1 → 4)-O-2-acetamido-2-deoxy-β-D-glucopyranose (**13**)*

A solution of disaccharide **12** (16 mg, 5 μmol) in 50 mM Na cacodylate buffer (pH 7.5,

1 ml) containing 100 mM NaCl, 0.4% Triton CF-54 and bovin serum albumin (1 mg) was incubated at 37°C with ST6Gal (10 mU), CMP-KDN (**2**) (8.5 mg, 10 μmol) added in three portions (at 12 h interval time) and alkaline phosphatase (1 U) for 36 h. The sialylation



Scheme 1. Chemical synthesis of CMP-KDN **2**; (a) H+, MeOH; (b) CH_3COCl; CH_3COOH; HCl; (c) HgO–$HgCl_2$, THF–water; (d) (iPr_2)NP(Cl)O(CH_2)$_2$CN, THF; (e) (1) DBU, (2) NaOMe, (3) H_2O.

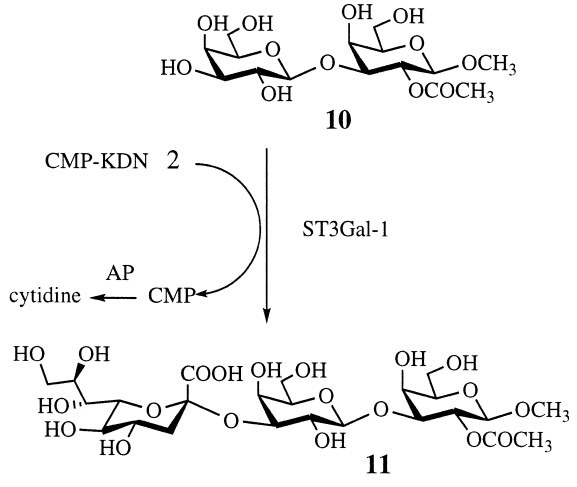
product was purified in the same way as **11** affording pure trisaccharide **13** as α, β anomeric mixture (1 mg, 35%); ^{1}H NMR (250 MHz, D_2O): δ 5.13 (d, 0.6H, *J* 2.5, H-1α), 4.65 (d, 0.4H, *J* 8 Hz, H-1β), 4.37 (d, 1H, *J* 7.5 Hz, H-1′), 2.57 (dd, 1H, *J* 12.5, *J* 4.5 Hz, H-3″e), 2.1 (s, 3H, NAc), 1.64 (t, 1H, *J* 12.5 Hz, H-3″a).

3. Results and discussion

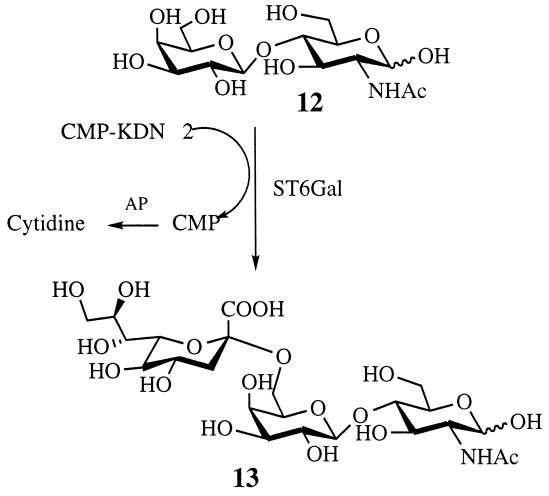
The CMP-KDN donor **2** was synthesized according to the phosphoramidite procedure, described for CMP-NeuAC [9] (Scheme 1). To this end KDN (**1**) readily available by condensation of D-mannose with pyruvate, catalyzed by sialyl aldolase [10], was first converted by treatment with anhydrous methanol in the presence of Dowex 50 (H^+), into its methyl ester **3**, which was treated with a mixture of AcCl–AcOH (1:1) saturated with HCl at 0°C to give the peracetylated chloro derivative **4**. Without any purification step, compound **4** was hydrolyzed with mercury salts to give compound **5** having free hydroxyl group on the anomeric position, in 54% overall yield (from **1**).

To prepare the cytidine 5′-*O*-amidite **7**, introduction of the amidite group to the 5′ position of the cytidine derivative **6** [11] was achieved with 2-cyanoethyl-*N*-*N*′-diisopropylchlorophosphoramidite in the presence of diisopropyl ethylamine, affording **7** as a 1:1 diastereomeric mixture in 85% yield. This procedure was preferred to the published one [9], in view of the lower cost of this reagent compared with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetra-isopropylphosphorodiamidite. Coupling of pentaacetyl KDN **5** with phosphoramidite **7** in the presence of 1*H*-tetrazole provided the phosphite derivative **8** in 60% isolated yield, after purification on Sephadex LH-20. The 1:1.6 diastereomeric mixture was evidenced from ^{31}P and ^{1}H NMR spectroscopy. Oxidation of **8** to the phosphate derivative **9** was performed with *tert*-butylhydroperoxide in 93% yield, but the instable compound **9** could not be purified. Complete deprotection of **9** could be achieved in the following way: first de-*O*-cyano-ethylation with DBU, and subsequent de-*O*- and de-*N*-acetylation with excess NaOMe followed by hydrolysis of the methyl ester with NaOH, afforded after purification on anionic exchanger, CMP-KDN (**2**) in 60% yield.

Having in hands this sugar-nucleotide, we examined the enzymatic transfer of KDN catalyzed by sialyltransferases. The porcine liver α(2-3)-sialyltransferase (ST3Gal-1) that we easily prepared in the laboratory, was selected, and the disaccharide, Galβ1-3(2-*O*Ac)Galβ*O*CH$_3$ **10**, a mimic of the natural T antigen but excellent substrate [8] was chosen as the oligosaccharide acceptor (Scheme 2). Incubation of partially purified ST3Gal-1 [12] with disaccharide **10** and stoichiometric amount of **2** in cacodylate buffer pH 7.5, in the presence of alkaline phosphatase (AP) [2] which hydrolyzed inhibitory CMP released in the course of the enzymatic reaction, into cytidine, allowed the synthesis of trisaccharide **11** isolated, in 88% yield, after purification by anion exchange and size exclusion chromatography. By using commercial rat liver α(2-6)sialyltransferase (ST6Gal), we also demonstrated KDN transfer onto *N*-acetyllac-



Scheme 2. Sialyltransferase-catalyzed transfer of KDN onto Galβ1-3(2*O*Ac)Galβ*O*CH$_3$ **10**.



Scheme 3. Sialyltransferase-catalyzed transfer of KDN onto *N*-acetyllactosamine **12**.

tosamine (Scheme 3). However, KDNα2-6Galβ1-4GlcNAc was obtained in a much lower yield (35%), probably because of the greater instability of ST6Gal compared with ST3Gal-1.

4. Conclusion

The new availability in CMP-KDN allowed to successfully transfer KDN onto oligosaccharide sequences, which could be achieved in high yield. The chemical access to activated KDN will be extended to other neuraminic acid analogues, with the hope to enlarge the use of sialyltransferase-catalyzed reaction in vitro and in vivo as well.

After submitting the abstract of this paper to Biotrans 1997, a similar work from R. Halcomb et al. appeared [13].

Acknowledgements

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