ELSEVIER

Carbohydrate Research 300 (1997) 161–167

CARBOHYDRATE RESEARCH

Porcine liver (2 → 3)-α-sialyltransferase: substrate specificity studies and application of the immobilized enzyme to the synthesis of various sialylated oligosaccharide sequences [1]

André Lubineau, Karine Basset-Carpentier, Claudine Augé *

Institut de Chimie Moléculaire d'Orsay, U.R.A. C.N.R.S. 462, Université de Paris-Sud, Bt 420, F-91405 Orsay, France

Received 9 December 1996; accepted 28 January 1997

Abstract

In search of substrate analogues for the porcine liver β-D-Gal*p*-(1 → 3)-D-Gal*p*-NAc: CMP-Neu5Ac-(2 → 3′)-α-sialyltransferase, three disaccharides β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-*O*-CH_3 (**5**), β-D-Gal*p*-(1 → 3)-β-D-(2-*O*Ac)-Gal*p*-*O*-CH_3 (**7**) and β-D-Gal*p*-(1 → 3)-β-D-(2-*O*Ac)-Gal*p*-*O*-Bn (**11**) were synthesized and tested with the enzyme. Disaccharide **7** turned out to be a very good substrate allowing a rapid access to the trisaccharide α-Neu5Ac-(2 → 3)-β-D-Gal*p*-(1 → 3)-β-D-(2-*O*Ac)-Gal*p*-*O*-CH_3 (**13**) on a preparative scale using the crude enzyme immobilized on cationic exchanger. Trisaccharide **13** was further exploited, first as a sialyl donor in *Trypanosoma cruzi trans*-sialidase catalyzed reaction and second through acetolysis reaction as a source for the synthon α-Neu5Ac-(2 → 3)-D-Gal (**16**). © 1997 Elsevier Science Ltd.

Keywords: Sialylation; Enzyme specificity; Sialyltransferase; *Trans*-sialidase; Acetolysis

1. Introduction

Glycosyltransferases, and especially sialyltransferases, are becoming widely used in the synthesis of oligosaccharides of interest in cellular adhesion processes involving recognition by selectins [1]. However, a major drawback of the enzymatic approach is the strict specificity of these enzymes. Thus for example, in the sialyltransferase family, eight different enzymes all transferring a sialic acid residue to the 3-hydroxyl group of a galactose residue, but with different substrate specificities sometimes very close, are known [2]. Great advances have been made recently in the cloning of sialyltransferases, but only a few reports have been concerned with structural modifications of the acceptor substrate [3–5].

In view of the fact that cloned sialyltransferases will become available in large amount in the near future, it is important to look in more detail at the in vitro specificity of these enzymes in order to further extend their synthetic potential. With this aim, we were interested in studying the specificity of a natural

* Corresponding author.

[1] Presented at the XVIIIth International Carbohydrate Symposium, Milan, Italy, July 21–26, 1996.

PII S0008-6215(97)00043-8

sialyltransferase available from porcine liver, β-D-Gal *p*-(1 → 3)-D-Gal *p*-NAc: CMP-Neu5Ac-(2 → 3′)-α-sialyltransferase (EC 2.4.99.4). This enzyme catalyzes the in vivo incorporation of sialic acid into glycoproteins and glycolipids which possess a terminal β-D-Gal *p*-(1 → 3)-D-Gal *p*-NAc unit. The enzyme has been successfully used in vitro in the synthesis of α-Neu5Ac-(2 → 3)-β-D-Gal *p*-(1 → 3)-D-Gal *p*NAc, α-Neu5Gc-(2 → 3)-β-D-Gal *p*-(1 → 3)-D-Gal *p*NAc and α-Neu5Ac-(2 → 3)-β-D-Gal *p*-(1 → 3)-D-Glc *p*NAc [6,7].

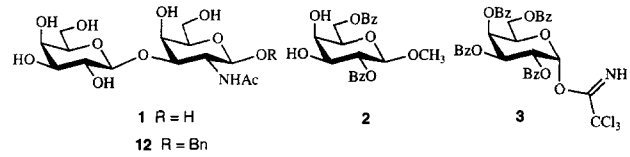
In search for natural substrate analog more readily available than β-D-Gal *p*-(1 → 3)-D-Gal *p*NAc (**1**) which is prepared by inversion of configuration of carbon-4 of the disaccharide β-D-Gal *p*-(1 → 3)-D-Glc *p*NAc [8], we synthesized the disaccharides β-D-Gal *p*-(1 → 3)-β-D-Gal *p*-O-R with methyl or benzyl as the aglycon and substituted at C-2 by an acetate (**5**, **7** and **11**). Evaluation with the enzyme showed that disaccharide **7** was an excellent substrate, allowing the preparative synthesis of the sialylated trisaccharide **13**. This trisaccharide was further exploited in two ways, first as a sialyl donor in *Trypanosoma cruzi trans*-sialidase catalyzed reaction and second through acetolysis reaction as a source for the synthon α-Neu5Ac-(2 → 3)-D-Gal (**16**).

2. Results and discussion

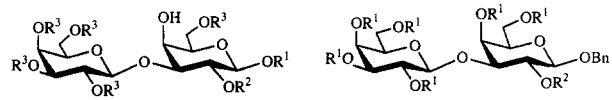
The synthesis of disaccharide **4** was achieved through the trichloroacetimidate procedure [9]. Condensation of diol **2**, readily obtained via methyl 3,4-*O*-isopropylidene-β-D-galactopyranoside [10] with trichloroacetimidate **3** [11] in dichloromethane at 0 °C in the presence of trimethylsilyl triflate, afforded after chromatography the crystalline disaccharide **4** in 75% yield. Debenzoylation of **4**, with sodium methoxide in methanol, gave in quantitative yield the methyl glycoside **5** which was tested as an acceptor substrate towards porcine liver β-D-Gal *p*-(1 → 3)-D-Gal *p*-NAc: CMP-Neu5Ac-(2 → 3′)-α-sialyltransferase. Compound **5** turned out to be a poor substrate with an affinity constant (K_m) ten times higher and a maximum velocity (V_{max}) three times lower than those for the natural substrate **1** (Table 1). Conventional acetylation of **5** afforded the crystalline peracetylated derivative **6** in 84% yield. Selective deacetylation at 0 °C with a mixture of triethylamine, methanol and water led to the C-2 acetylated disaccharide **7** in 76% isolated yield. The downfield shift for H-2 clearly confirmed the structure of **7**. It is worth noting that similar sterically hindered deacetylations on 3-O-substituted-D-galactosides have been previously reported [12]. Disaccharide **7** proved to be a much better substrate than **5**, comparable to **1** with a slightly increased V_{max}, but a two fold greater K_m (Table 1).

Table 1
Kinetic parameters, K_m (Michaelis constant) and V_{max} (maximum velocity) for disaccharide analogues as acceptor substrates of β-D-Gal *p*-(1 → 3)-D-Gal *p*-NAc: CMP-Neu5Ac-(2 → 3′)-α-sialyltransferase from porcine liver

Compound	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})	V_{max}/K_m (%)
1	0.41 ± 0.02	16.8 ± 0.5	100
5	5.9 ± 1.6	6.8 ± 0.4	2.8
7	0.94 ± 0.16	25.7 ± 3.4	68
11	–	–	–



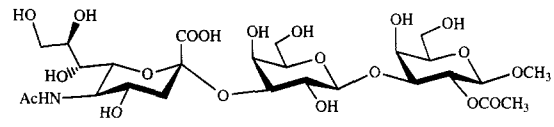
The perbenzoylated disaccharide **8**, prepared according to the published procedure [13], was debenzoylated and peracetylated in the same way, yielding disaccharide **10** in 83% yield. Again, selective deacetylation to the monoacetate **11** could be achieved at 0 °C in 65% yield. Surprisingly disaccharide **11**, with a β benzyl aglycon, was not an acceptor substrate for (2 → 3′)-α-sialyltransferase, whereas a relative activity of 50% compared with the free disaccharide **1** was observed for the benzyl β-glycoside **12**. The kinetic parameters for substrate **1** and the substrate analogues **5**, **7** and **11** are reported in Table 1. The relatively high V_{max}/K_m ratio measured for disaccharide **7** allowed the enzymic sialylation of this disaccharide on a preparative scale.



Preparation of porcine liver β-D-Galp-(1 → 3)-D-GalpNAc: CMP-Neup5Ac-(2 → 3')-α-sialyltransferase adsorbed on SP-C50 Sephadex.—The porcine liver enzyme has been purified to homogeneity and cloned [14]. In our case we used, for simplicity, a crude enzyme preparation. The 1.4% Triton X-100 enzymatic extract was adsorbed onto a Cibacron Blue F36A-agarose column and was eluted with a stepwise gradient of NaCl [7]. After dialysis, the elution fractions having at this stage a very low specific activity $\cong 2$ mU · mg^{-1} were then directly applied onto SP-C50 Sephadex at pH 6. After extensive washing with the equilibration buffer the gel, which retained 42% of the initial enzymic activity, was used for enzymic synthesis. The main advantages of ionic adsorption of the enzyme were: (i) concentration of the enzymic activity; (ii) elimination of glycerol; (iii) decrease in the Triton X-100 concentration, which greatly facilitated the purification of the reaction product.

For kinetic studies, the enzyme eluted from the Cibacron Blue F3GA-agarose column was further purified using a CDP-hexanolamine agarose column which was eluted with a linear gradient of CTP [6]. This preparation could be lyophilized in the presence of BSA. The freeze-dried material, taken up in water, was used after dialysis for kinetic studies.

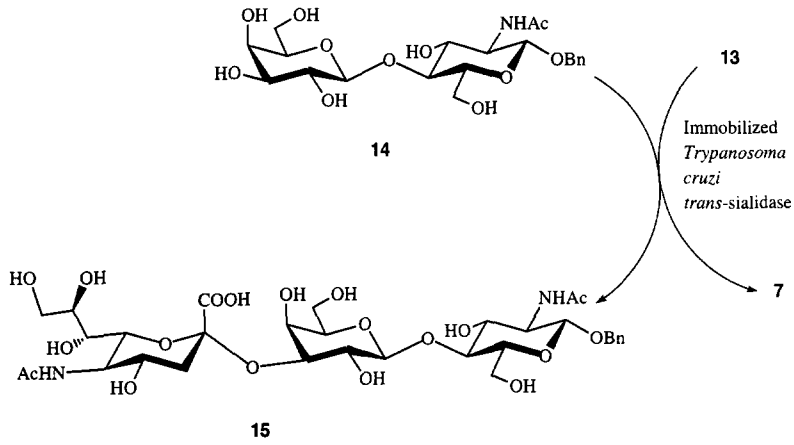
Preparative-scale synthesis of trisaccharide 13.—The disaccharide acceptor **7** (3 mM) was incubated with a stoichiometric amount of CMP-Neu5Ac (added in three portions), in the presence of sialyltransferase adsorbed on SP-C50 Sephadex in 50 mM sodium cacodylate buffer (pH 7.5) containing 100 mM NaCl and 0.1% Triton X-100. Calf intestine alkaline phosphatase was added in the course of the incubation, in order to hydrolyse CMP released in the reaction into cytidine, according to the published procedure [15]. By TLC analysis, the reaction had proceeded to completion after 36 h. Because the sialyltransferase was still active after this time period, additional portion of both substrates **7** and CMP-Neu5Ac were added and the reaction was allowed to proceed for one day more. The trisaccharide was purified by anion exchange chromatography; elution with volatile triethylammonium hydrogen carbonate afforded pure trisaccharide **13** on 100 mg-scale in an excellent yield (85%). It is worthwhile to note that this synthesis was achieved with 0.16 U of insolubilized enzyme isolated from 50 g of fresh porcine liver.



13

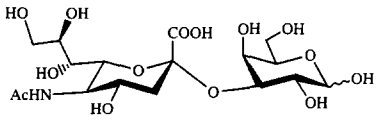
Sialylation through trans-sialidase.—The *trans*-sialidase from *Trypanosoma cruzi* has the unique property of catalyzing the reversible transfer of Neu5Ac, from an α-(2 → 3)-sialylated sugar and not from CMP-Neu5Ac, to virtually any linear oligosaccharides with a β-galactose at the non reducing end to yield a new α-(2 → 3) sialylated oligosaccharide [16,17]. The practical limitation of using this enzyme for synthetic purposes is that the desired sialylated oligosaccharide is produced at the expense of another sialoside used as the donor substrate. Trisaccharide **13** proved to be as good substrate as α-(2 → 3)-sialyllactose, commonly used as donor substrate in *trans*-sialidase catalyzed sialylation, and much better than *p*-nitrophenyl-α-sialoside [16]. As an illustration of the possible use of trisaccharide **13** as a sialic donor, sialylation of β-benzyl-*N*-acetyllactosamine **14** was achieved with *Trypanosoma cruzi trans*-sialidase immobilized on concanavalin A-sepharose according to the procedure of Scudder et al. [16] (Scheme 1). A three-fold excess of **14** was used in order to drive the equilibrium of the reaction. The sialyllactosamine **15** was isolated in 50% yield after purification by anion-exchange chromatography, affording a mixture of **13** and **15**. A subsequent reverse phase chromatography, allowed separation of **15** from **13**. The ^{1}H NMR spectrum of **15** was in good agreement with that reported for the corresponding methyl glycoside [18].

Acetolysis of trisaccharide 13.—In the acetolysis of polysaccharides, it is well known that (1 → 6) linkages are split preferentially, presumably because of the greater ease of approach of the acetylium cation compared to that on (1 → 2), (1 → 3) and (1 → 4) linkages [19]. The high stability of the sialic acid linkage during acetolysis, which might be explained by steric hindrance to acetylium cation attack, has been previously observed in gangliosides [20] and in a hexasaccharide from human milk [21]. All these observations prompted us to submit trisaccharide **13** to acetolysis reaction in order to selectively cleave the (1 → 3)-β-Gal linkage and prepare the synthon α-Neu5Ac-(2 → 3)-D-Gal. Acetolysis conditions were first checked for disaccharide **5**, and 5% sulfuric acid



Scheme 1. Sialylation of β-D-Gal*p*-(1 → 4)-β-D-Glc*p*-NAc-O-Bn (**14**) using concanavalin A-immobilized *Trypanosoma cruzi trans*-sialidase and trisaccharide **13** as the sialyl donor.

in a mixture of acetic anhydride-acetic acid for 5 days was selected and applied for acetolysis of trisaccharide **13**. Then, carefully controlled neutralization of the solution was followed by extraction with dichloromethane and deacetylation affording a crude mixture which, after purification by anion exchange chromatography, allowed to isolate the expected disaccharide **16** as a mixture of α and β anomers in 60% yield. ^{1}H NMR data and optical rotation of **16** were in good agreement with the values previously reported [22]. We are presently optimizing the reaction in order to make this chemo-enzymatic strategy a practical access to α-D-Neu5Ac-(2 → 3)-D-Gal, a very useful synthon in chemical oligosaccharide synthesis.



16

3. Experimental

General.—^{1}H NMR spectra were recorded at 250 MHz with a Bruker AM-250 spectrometer. The chemical shifts are given relative to the signal of tetramethylsilane as internal standard (0.2% soln in $CDCl_3$) for soln in D_2O. ^{13}C NMR spectra were recorded at 50 MHz with a Bruker AM-200 spectrometer; 1,4-dioxane was used as the internal standard (δ 66.64 ppm from the signal of tetramethylsilane). Optical rotations were measured with a Jasco digital micropolarimeter. CMP-Neu5Ac was synthesized according to a published procedure [23]. β-D-Gal*p*-(1 → 3)-D-Gal*p*NAc was prepared according to the procedure of Lubineau et al. [8]. *trans*-Sialidase was a gift from Dr. Miercio E.A. Pereira, New England Medical Center Hospitals, Boston MA 02111. One *trans*-sialidase unit is the amount of enzyme which sialylates 1 μmole of lactose min^{-1} at 37 °C and pH 7. *trans*-Sialidase assay was performed according to Scudder et al. [16], except that [^{14}C] lactose was used instead of [^{14}C] *N*-acetyllactosamine. CMP-[9-^{3}H] Neu5Ac (specific activity, 555 GBq/mmol) was from Isotopchim and [D-glucose-1-^{14}C] lactose (specific activity, 200 GBq/mmol) from Amersham.

Measurements of enzyme kinetics.—Radiochemical assays were performed as previously described [6]. For kinetic studies, the following concentrations of acceptor were used: 0.16, 0.62, 0.83, 1.66, 4.16, 8.33 and 16.6 mM for **1** and **7**, and 10.3, 20.4, 35.7, 71.4, 142.8 and 250 mM for **5**.

Immobilization of crude porcine liver β-D-Galp-(1 → 3)-D-GalpNAc: CMP-Neup5Ac-(2 → 3′)-α-sialyltransferase.—The membrane bound enzyme was first extracted from porcine liver homogenate with 1.4% Triton X-100, according to the published procedure [7]. Then, the Triton extract (100 mL, 0.9 U, specific activity: 0.4 mU · mg^{-1} of proteins) was applied to a column (3 × 6 cm) of Cibacron Blue F3GA-agarose (4 μmol mL^{-1}) previously equilibrated with 10 mM sodium cacodylate buffer (pH 6) containing 0.1 M NaCl, 1% Triton X-100 and 25% glycerol. After washing with this buffer, the enzyme was eluted with a stepwise gradient of NaCl (0.5 M, 1 M, 2 M) in the same buffer. The 1 M NaCl eluate (157 mL, 0.39 U, specific activity: 2 mU mg^{-1} of proteins), dialyzed against 10 mM sodium cacodylate

buffer (pH 6) containing 1% Triton X-100 and 25% glycerol (2 × 2 L), was then applied at a flow rate of 30 mL h^{-1} to a small column (2 × 5 cm) of SP-C50 Sephadex equilibrated in the same buffer. After washing with the equilibration buffer (200 mL), the gel was stored in 50 mM sodium cacodylate buffer (pH 7.5), containing 0.1 M NaCl, 0.1% Triton X-100 and 0.02% NaN_3 (0.16 U of sialyltransferase adsorbed on SP-C50 Sephadex, 41% of loaded enzyme activity).

Methyl 2,6-di-O-benzoyl-3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-β-D-galactopyranoside (**4**).—A mixture of **2** (658 mg, 1.637 mmol) and 2,3,4,6-tetra-*O*-benzoyl-α-D-galactopyranosyl trichloroacetimidate (**3**, 1.45 g, 1.964 mmol) in dry dichloromethane (10 mL) was stirred at room temperature under N_2, then cooled to 0 °C. Trimethylsilyltriflate (0.5 M) in CH_2Cl_2 (0.59 mL, 0.295 mmol) was added and the mixture was stirred for 40 min at 0 °C. *N,N*-Diisopropylethylamine (0.5 mL) was added; the mixture was then concd and the residue was chromatographed on silica gel with 15:1 toluene–EtOAc as the eluent, affording **4** (1.2 g, 75%) which crystallized from EtOH; mp 219–220 °C, $[\alpha]_D^{26}$ +105° (*c* 0.93, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): δ 8.20–7.10 (m, 30 H, 5 Ph), 5.95 (d, 1 H, $J_{3,4'}$ 3 Hz, H-4'), 5.75 (dd, 1 H, $J_{2,3}$ 10, $J_{1,2}$ 8 Hz, H-2), 5.48 (m, 2 H, H-2', H-3'), 5.00 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 4.42 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.27 (d, 1 H, H-4), 3.99 (dd, 1 H, $J_{3,4}$ 3, $J_{2,3}$ 10 Hz, H-3), 3.41 (s, 3 H, OCH_3) 3.10 (s, 1 H, OH); ^{13}C NMR ($CDCl_3$): δ 166.21, 165.97, 165.56, 165.39, 164.70 (6 CO), 133.72, 133.47, 133.27, 133.11, 132.74, 132.66 (6 C–Ph), 129.99 129.83, 129.68, 129.59, 129.38, 129.12, 128.66, 128.55, 128.39, 128.19, 128.09, 128.02 (30 C–Ph), 101.69, 101.72 (C-1, C-1'), 81.09 (C-3), 72.04, 71.78, 71.37 (C-5, C-5', C-3'), 70.32 (C-2), 69.26 (C-2'), 68.36 (C-4), 67.82 (C-4'), 62.11, 62.66 (C-6, C-6') and 56.29 (OCH_3). Anal. Calcd for $C_{55}H_{48}O_{17}$: C, 67.34; H, 4.93; O, 27.73. Found: C, 67.57; H, 4.92; O, 27.66.

Methyl 2-O-acetyl-3-O-β-D-galactopyranosyl-β-D-galactopyranoside (**7**).—A soln of NaOMe in MeOH (0.2 M, 10 mL) was added to a soln of **4** (1 g, 1.02 mmol) in MeOH (5 mL). After 15 h at room temperature, the soln was neutralized with Dowex 50W-(H^+) resin, filtered and concd to dryness to give disaccharide **5** (360 mg, quantitative yield), which crystallized from MeOH; mp 202–203 °C [lit. 201–202 °C [24]. Disaccharide **5** (400 mg, 1.1 mmol) was treated with Ac_2O (10 mL) and pyridine (10 mL) at room temperature for 24 h. The mixture was then evaporated to dryness, coevaporated several times with toluene, then crystallized from EtOH, affording disaccharide **6** (600 mg, 84%). ^{1}H NMR ($CDCl_3$) δ: 5.39 (d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.35 (d, 1 H, $J_{3',4'}$ 3 Hz, H-4'), 5.18 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 10 Hz, H-2), 5.09 (dd, 1 H, $J_{1',2'}$ 8, $J_{2',3'}$ 10 Hz, H-2'), 4.93 (dd, 1 H, H-3') 4.57 (dd, 1 H, H-1'), 4.30 (dd, 1 H, H-1), 3.50 (s, 3 H, OCH_3) and 2.17, 2.14, 2.11, 2.08, 2.06, 2.02, 1.97 (7s, 21 H, 7OAc).

A soln of disaccharide **6** (600 mg, 0.92 mmol) in 0.16:8:1 Et_3N–MeOH–water (27.5 mL) was stirred at 0 °C for 48 h. The TLC (6:6:1 2-propanol–EtOAc–water) showed a major spot (R_f 0.24) just above the disaccharide **5** (R_f 0.18). Solvents were evaporated and the residue was purified by flash column chromatography (6:8:1 2-propanol–EtOAc–water) to give **7** (280 mg, 76%); $[\alpha]_D^{26}$ +9° (*c* 1.12, H_2O); ^{1}H NMR (D_2O): δ 4.99 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 10 Hz, H-2), 4.52 (d,1 H, H-1), 4.44 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1'), 4.17 (d, 1 H, $J_{3,4}$ 3 Hz, H-4), 3.98 (dd, 1 H, H-3), 3.85 (d, 1 H, $J_{3',4'}$ 3 Hz, H-4'), 3.47 (s, 3 H, CH_3) and 2.09 (s, 3 H, $COCH_3$). ^{13}C NMR (D_2O): δ 173.37 (CO), 104.78 (C-1'), 101.53 (C-1), 80.17 (C-3), 74.94 (2 C-5), 72.59 (C-3'), 70.98 (C-2), 70.46 (C-2'), 68.78, 68.58 (C-4, C-4'), 60.96, 60.81 (2 C-6), 56.89 (OCH_3) and 20.52 ($COCH_3$). Anal. Calcd for $C_{15}H_{26}O_{12}$, H_2O: C, 43.27; H, 6.78; O, 49.95. Found: C, 43.39; H, 6.83; O, 49.57.

Benzyl 2-O-acetyl-3-O-β-D-galactopyranosyl-β-D-galactopyranoside (**11**).—A soln of NaOMe in MeOH (0.2M, 3 mL) was added to a soln of **8** [12] (360 mg, 0.31 mmol) in MeOH (1.5 mL). After 15 h at room temperature, the soln was neutralized with Dowex 50W-(H^+) resin, filtered and concd to dryness to give crude disaccharide **9** (125 mg, 93%). The residual sirup was treated with Ac_2O (5 mL) and pyridine (5 mL) at room temperature for 24 h. The mixture was then evaporated to dryness and coevaporated several times with toluene. Flash chromatography (1:4 AcOEt:Hexane) of the residue gave disaccharide **10** (187 mg, 83%); ^{1}H NMR ($CDCl_3$): δ 7.35 (m, 5 H, Ph) 5.38 (d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.34 (d, 1 H, $J_{3',4}$ 3.2, $J_{4',5'}$, 1 Hz, H-4'), 5.26 (dd, 1 H, $J_{1,2}$, 7,5, $J_{2',3'}$ 10.5 Hz, H-2), 4.91 (dd, 1 H, H-3'), 4.89 (d, 1 H, J_{gem} 12.5 Hz, CHPh), 4.62 (dd, 1 H, CH'Ph), 4.54 (d, 1 H, H-1), 4.39 (dd, 1 H, H-1') and 2.17, 2.15, 2.11, 2.05, 2.01, 1.97 (6s, 21 H, 7 OAc).

A soln of disaccharide **10** (500 mg, 0.688 mmol), in 0.32:8:1 Et_3N–MeOH–water (29 mL) was stirred at 0 °C for 7 h. TLC (6:6:1 2-propanol–EtOAc–water) showed a major spot (R_f 0.43). Solvents were evaporated and the residue was purified by flash column chromatography (4:1 EtOAc:MeOH) to give com-

pound **11** (210 mg, 65%): $[\alpha]_D^{25}$ −7° (*c*, 0.87, H_2O); ^1H NMR (D_2O): 7.45 (m, 5 H, Ph), 5.07 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 10 Hz, H-2), 4.90 (d, 1 H, J_{gem} 12 Hz, CHPh), 4.70 (d, 1 H, CH'Ph), 4.65 (d, 1 H, H-1), 4.44 (d, 1 H, H-1'), 4,22 (d, 1 H, $J_{3,4}$ 3 Hz, H-4), 3.95 (dd, 1 H, H-3), 3.90 (d, 1 H, $J_{3',4'}$ 3.2 Hz, H-4'), 3.49 (dd, 1 H, $J_{2',3'}$ 10 Hz, H-2') and 2.05 (s, 3 H, $COCH_3$). ^{13}C NMR (D_2O): δ 173.26 (CO), 136.74, 128.85, 128.61 (C–Ph), 104.84 (C-1'), 99.72 (C-1), 80.06 (C-3), 75.09, 75.00 (C-5, C-5'), 72.58 (C-3'), 71.44 (CH_2Ph), 71.21 (C-2) 70.48 (C-2'), 68.77, 68.62 (C-4, C-4'), 61.01, 60.84 (C-6, C-6') 56.89 (OCH_3) and 20.52 ($COCH_3$). Anal. calcd for $C_{21}H_{30}O_{12}$, H_2O: C, 51.22; H, 6.55. Found: C, 51.19; H 6.49.

Methyl-O-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic acid)-(2 → 3)-O-β-D-galactopyranosyl-(1 → 3)-O-(2-O-acetyl)-β-D-galactopyranoside (**13**).—Disaccharide glycoside **7** (32 mg, 0.08 mmol) and CMP-Neu5Ac bis(triethylammonium) salt (19 mg, 0.02 mmol) were dissolved in 50 mM Na cacodylate buffer (pH 7.5, 15 mL) containing 100 mM NaCl, 0.1% Triton X-100. Sialyltransferase (0.16 U) adsorbed on SP-C50 Sephadex (10 mL) was added and the mixture was incubated at 37 °C. The reaction was monitored by TLC on silica gel (4:1 *n*-propanol–water). After 5 h more CMP-Neu5Ac (0.02 mmol) was added, and again after 10 h (0.04 mmol) together with alkaline phosphatase (7.5 U). After 36 h, a major spot (R_f 0.49) corresponding to trisaccharide **13** was observed on TLC. Because the enzymatic assay of an aliquot of the immobilized sialyltransferase, withdrawn from the reaction mixture, showed that 53% of the enzymatic activity was still retained on the gel, more substrates (disaccharide **7**, 36 mg, 0.09 mmol and CMP-Neu5Ac, 0.09 mmol in two portions at 10 h interval time) were added. The incubation was stopped after 60 h. The reaction mixture was filtered and the sephadex resin was washed with 50 mM Na cacodylate buffer (pH 7.5). The filtrate and washings were combined and purified by chromatography on a DEAE-Sephadex A-25 (HCO_3^- form) column (2 × 20 cm). Elution with a gradient of 0–0.4 M triethylammonium hydrogen carbonate (pH 8.0) gave **13** as its triethylammonium salt (114 mg, 85%), $[\alpha]_D^{25}$ +3° (*c* 1, H_2O); ^1H NMR (D_2O): δ 4.98 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 10 Hz, H-2), 4.52 (d, 1 H, $J_{1'2'}$ 8 Hz, H-1'), 4.50 (d, 1 H, H-1), 4.16 (d, 1 H, $J_{3,4}$ 3 Hz, H-4), 4,02 (dd, 1 H, H-3'), 3.96 (dd, 1 H, $J_{2',3'}$ 10, $J_{3',4'}$, 3 Hz, H-3), 3.88 (d, 1 H, H-4'), 3.47 (s, 3 H, OCH_3), 3.15 (q, 6 H, $(CH_2–CH_{3)3}$), 2.69 (dd, 1 H, $J_{3''a,3''e}$ 12.5, $J_{3''e,4}$ 4.5 Hz, H-3''e), 2.09 (s, 3 H, OAc), 1.97 (s, 3H, NHAc), 1.73 (t, 1 H, $J_{3''a,4}$ 12.5 Hz, H-3''a) and 1.23 (t, 9 H, $(CH_2–CH_3)_3$). ^{13}C NMR (D_2O): 175.24 ($NHCOCH_3$), 174.22 (C-1''), 173.69 ($COCH_3$), 104.81 (C-1'), 101.73 (C-1), 100.00 (C-2''), 80.69 (C-3), 75.93, 75.20, 74.99 (C-5, C-5', C-3'), 73.05 (C-6''), 72.04 (C-8''), 71.11 (C-2), 69.22 (C-2'), 68.93, 68.67, 68.27, 67.66 (C-4, C-4', C-4'', C-7''), 62.69 (C-9''), 61.24, 61.09 (C-6, C-6'), 57.14 (OCH_3), 51.92 (C-5''), 39.95 (C-3''), 22.30 (NAc) and 20.88 (OAc).

Benzyl-O-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic acid)-(2 → 3)-O-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**15**).—Crude *trans*-sialidase (2.8 mL, 7 mU) was stirred for 4 h in the presence of 1 mM $CaCl_2$ with concanavalin A-sepharose 4B (Sigma, 14 mg of lectin/mL of gel), previously equilibrated with 10 mM Na cacodylate buffer (pH 6.9) containing 1 mM $CaCl_2$ and 0.5 M NaCl. The gel was then recovered on a sintered glass funnel, washed with the above buffer containing 1 mM $CaCl_2$ and 0.02% NaN_3 (20 mL) and stored in suspension in 50 mM Na cacodylate buffer pH 6.9 containing 2 mM $CaCl_2$ (6 mU were adsorbed on concanavalin A-sepharose).

A soln of trisaccharide **13** (7.3 mg, 9.24 μmol) and disaccharide **14** (15.7 mg, 33.2 μmol) was incubated with *trans*-sialidase adsorbed on concanavalin A-sepharose (0.6 mL, 6 mU) in 50 mM Na cacodylate buffer (pH 6.9) containing 2 mM $CaCl_2$ for 4 d at 37 °C with gentle shaking. The gel was filtered and washed with the same buffer. The filtrate and washings were applied to a DEAE-Sephadex A-25 (HCO_3^-) column. Elution with a linear gradient of 0 to 0.2 M triethylammonium hydrogen carbonate buffer (pH 7.8) afforded fractions containing a mixture of trisaccharides **15** and **13** which were collected and freeze-dried (15.5 mg). The lyophilizate was dissolved in a small amount of water and purified on a column of Bio-Beads SM2 (BioRad). Washing with water afforded trisaccharide **13**. Pure trisaccharide **15** was then eluted with 4:1 *n*-propanol–water (4 mg, 50%); $[\alpha]_D^{29}$ −12° (*c* 0.5, H_2O); ^1H NMR (D_2O): δ 7.35 (m, 5 H, Ph), 4.60 (d, 1 H, J_{gem} 12 Hz, CHPh), 4.47 (d, 1 H $J_{1'2'}$ 8 Hz, H-1'), 4.46 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 2.70 (dd, 1 H, $J_{3e,4}$ 4.5 Hz, $J_{3a,3e}$ 12.5 Hz, H-3e''), 1.97 (s, 3 H, NAc), 1.86 (s, 3 H, NAc) and 1.74 (t, 1 H, $J_{3a,4}$ 12.5 Hz, H-3a'').

Acetolysis of 13.—Trisaccharide **13** (42.7 mg, 54 μmol) was dissolved in a mixture of 1:1 Ac_2O–AcOH containing 5% sulfuric acid (5 mL) and stirred

at room temperature for 4 d. Then, the mixture was slowly poured into 3 M ice-cold $KHCO_3$ solution (30 mL). The soln was adjusted to pH 2.5 by adding 6 N HCl and then extracted with CH_2Cl_2. The organic phase was evaporated to dryness and coevaporated twice with toluene. The residue was then dissolved in MeOH (1 mL), a soln of MeONa (0.2 M, 1 mL) was added and the mixture was stirred for 12 h at room temperature. The soln was neutralized with Dowex 50W-(H^+) resin, filtered and concd to dryness. The residue was purified by chromatography on DEAE-Sephadex A-25 (HCO_3^- form). Elution with a gradient of 0 to 0.15 M triethylammonium hydrogen carbonate buffer (pH 7.8) afforded disaccharide **16** (18.1 mg, 60%), as a mixture of α and β anomers; $[\alpha]_D^{27}$ +19° (*c* 1.18, H_2O); [lit. [22] +23.1° (*c* 1, H_2O)]; ^1H NMR (D_2O): δ 5.29 (d, 0.3 H, $J_{1,2}$ 3.7 Hz, H-1α), 4.64 (d, 0.7 H, $J_{1,2}$ 8 Hz, H-1β), 4.34 (dd, 0.3 H, $J_{3,2}$ 10.5 Hz, $J_{3,4}$ 3 Hz, H-3α), 4.09 (dd, 0.7 H, $J_{3,2}$ 10, $J_{3,4}$ 3 Hz, H-3β), 4.02 (d, 0.3 H, H-4α), 3.95 (d, 0.7 H, H-4β), 3.53 (dd, 0.7 H, H-2β), 2.77 (dd, 0.7 H, $J_{3'e,3'a}$ 12.5, $J_{3'e,4'}$ 4.5 Hz, H-3'eβ), 2.73 (dd, 0.3 H, $J_{3'e,3'a}$ 12.5, $J_{3'e,4'}$ 5 Hz, H-3'eα), 2.04 (s, 3 H, NAc), 1.83 (t, 0.7 H, $J_{3'a,4'}$ 12.5 Hz, H-3'aβ) and 1.80 (t, 0.3 H, $J_{3'a,4'}$ 12.5 Hz, H-3'aα).

Acknowledgements

We are very grateful to Pr. Miercio E.A. Pereira, New England Medical Center Hospitals, Tufts University School of Medicine, Division of Geographic and Infectious Diseases, Boston, Massachusetts, USA, who provided us with *Trypanosoma cruzi trans*-sialidase.

This work has been supported in part by the M.E.N.E.S.R.: Actions Concertées des Sciences du Vivant, ACCSV14 No. 9514111, Réseau GTrec.

References

[1] H.J.M. Gijsen, L. Qiao, W. Fitz, and C.-H. Wong, *Chem. Rev.*, 96 (1996) 443–473.
[2] A. Harduin-Lepers, M.-A. Recchi, and P. Delannoy, *Glycobiology*, 5 (1995) 741–758.
[3] Y. Ito and J.C. Paulson, *J. Am. Chem. Soc.*, 115 (1993) 1603–1605.
[4] K.B. Wlasichuk, M.A. Kashem, P.V. Nikrad, P. Bird, C. Jiang, and A.P. Venot, *J. Biol. Chem.*, 268 (1993) 13971–13977.
[5] G. Baisch, R. Öhrlein, M. Streiff, and B. Ernst, *Bioorg. Med. Chem. Lett.*, 6 (1996) 755–758.
[6] A. Lubineau, C. Augé, and P. François, *Carbohydr. Res.*, 228 (1992) 137–144.
[7] A. Lubineau, C. Augé, C. Gautheron-Le Narvor, and J.-C. Ginet, *Bioorg. Med. Chem.*, 2 (1994) 669–674.
[8] A. Lubineau and H. Bienaymé, *Carbohydr. Res.*, 212 (1991) 267–271.
[9] R.R. Schmidt, *Angew. Chem. Int. Ed. Engl.*, 25 (1986) 212–235.
[10] G. Catelani, F. Colonna, and A. Marra, *Carbohydr. Res.*, 182 (1988) 297–300.
[11] S. Rio, J.-M. Beau, and J.-C. Jacquinet, *Carbohydr. Res.*, 219 (1991) 71–90.
[12] Z. Szurmai, A. Liptàk, and G. Snatzke, *Carbohydr. Res.*, 200 (1990) 201–208.
[13] S. Rio, J.-M. Beau, and J.-C. Jacquinet, *Carbohydr. Res.*, 244 (1993) 295–313.
[14] W. Gillespie, S. Kelm, and J.-C. Paulson, *J. Biol. Chem.*, 267 (1992) 21004–21010.
[15] C. Unverzagt, H. Kunz, and J.C. Paulson, *J. Am. Chem. Soc.*, 112 (1990) 9308–9309.
[16] P. Scudder, J. Doom, M. Chuenkova, I.D. Manger, and M.E.A. Pereira, *J. Biol. Chem.*, 268 (1993) 9886–9891.
[17] Y. Ito and J.C. Paulson, *J. Am. Chem. Soc.*, 115 (1993) 7862–7863.
[18] S. Sabesan and J.C. Paulson, *J. Am. Chem. Soc.*, 108 (1986) 2068–2080.
[19] R.D. Guthrie and J.F. Mc Carthy, *Adv. Carbohydr. Chem. Biochem.*, 22 (1967) 11–23.
[20] R. Kuhn and H. Wiegandt, *Chem. Ber.*, 96 (1963) 866–880.
[21] L. Grimmonprez, S. Bouquelet, B. Bayard, G. Spik, M. Monsigny, and J. Montreuil, *Eur. J. Biochem.*, 13 (1970) 484–492.
[22] T. Ogawa and M. Sugimoto, *Carbohydr. Res.*, 135 (1985) C5–C9.
[23] S. David, C. Augé, and C. Gautheron, *Adv. Carbohydr. Chem. Biochem.*, 49 (1991) 175–237.
[24] P. Kovàc, C.P.J. Glaudemans, and R.B. Taylor, *Carbohydr. Res.*, 142 (1985) 158–164.