Synthesis of substrates for the characterisation of sialate-*O*-acetyltransferases

Inês Eleutério and Hansjörg Streicher*

Department of Chemistry and Biochemistry, University of Sussex, Brighton BN1 9QG, UK

The synthesis of a series of α- and β-configured sialosides using a Koenigs–Knorr methodology is described. The target compounds can serve as substrates for the investigation of biologically relevant sialate-*O*-acetyltransferase activity.

Keywords: sialic acid, *N*-acetylneuraminic acid, sialate-*O*-acetyltransferase, CMP-*N*-acetylneuraminic acid, skin tumor, leukaemia

O-Acetylation is one of the most common modifications of sialic acids in mammals and microorganisms. It occurs either at C-4 or at any position on the glycerol side chain of sialic acid. Multiple acetylations are possible.[1–4]

Biological sialate-*O*-acetylation has attracted increased interest in recent years due its abundance and involvement in many, including pathological, biological processes. For instance, acetylation may promote or hinder recognition of sialic acid by proteins, cells or pathogens or slow down the activity of degradative enzymes such as sialate lyases or sialidases. Related biological events include cell differentiation, tumour growth, immunity, apoptosis, microbial infections and in particular cancer where they are considered markers for certain skin tumours and a form of leukaemia.[1–3,5–7]

In bacteria, *O*-acetyltransferases responsible for this modification are common and specific sialate-*O*-acetyltransferases (SOATs) have been identified in the bacterial pathogens *Escherichia coli K1*, *type III group B streptococci*, *Campylobacter jejuni* and *Neisseria meningitis* where they act on sialic acid components of their capsular polysaccharides or lipo-oligosaccharides.[8–11]

In mammals, two systems transferring acetyl groups from the common donor acetyl-coenzyme A have been studied in detail. The AcCoA:sialate-7(9)-*O*-acetyltransferase, obtained from the human colon, rat liver and bovine submandibular gland and the AcCoA:sialate-4-*O*-acetyltransferase from guinea pig liver[12–16] (Fig. 1).

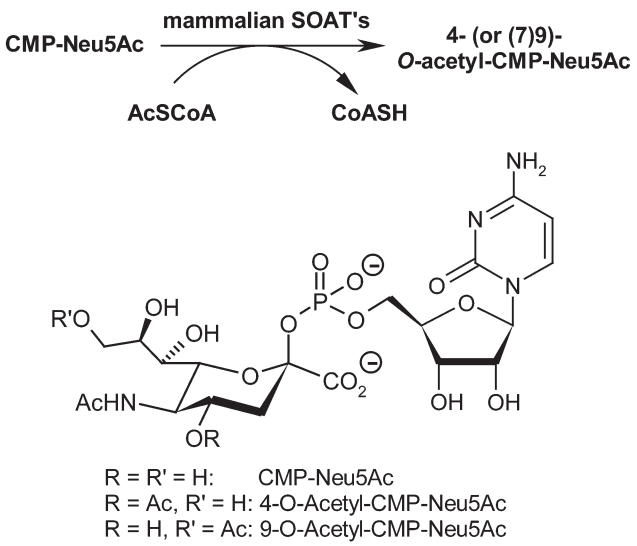


Fig. 1 Sialate-O-acetylation of the natural substrate CMP-*N*-acetylneuraminic acid.

* Correspondent. E-mail: h.streicher@sussex.ac.uk

Whilst several of the bacterial SOAT genes have been identified[8–11] the structures of the systems exhibiting SOAT activity in eukaryotic cells have remained elusive. The mammalian enzymes studied are localised in the Golgi membrane and utilise CMP-*N*-acetylneuraminic acid as a substrate which, in turn, is then the substrate for the respective sialyltransferases responsible for attachment of the acetylated sialic acid to a glyconconjugate[8,9] (Fig. 1).

CMP-Neu5Ac is special amongst the sialoconjugates as it possesses the sialic acid in its less common β-configuration. We now report the synthesis of potential substrates designed to aid the analysis of mammalian SOAT-specificity and -activity. Dicarboxylic acids **1α** and **1β** aim to mimic the two negative charges (under physiological conditions) of CMP-Neu5Ac and the fluorescent conjugates **2α** and **2β** should yield more readily detectable products, if SOAT-activity is present (Fig. 2).

Syntheses

Commercially available *N*-acetylneuraminic acid was converted into its methyl ester and subsequently per-acetylation and anomeric chlorination in one step yielded the Koenigs–Knorr donor **3** in high yield, as described in the literature.[17,18] Glycosidation with glycolic acid benzyl ester as acceptor (Method A) gave the mixture of diastereoisomers **4(α,β)** in a combined yield of 92% with a ratio of α:β = 9:1. The diasteroisomers **4α** and **4β** were readily separated by flash chromatography using a petrol ether/ethyl acetate gradient. Pure **4α** and **4β** were then quantitatively deprotected in identical fashion by hydrogenation over a Pd/C catalyst followed by treatment with sodium methoxide in methanol and then aqueous ammonium hydroxide (Method B). Gel permeation chromatography (Biogel P2, 0.1 M NH_4HCO_3) was used to remove residual salt from target compounds **1α** and **1β** (Scheme 1).

In order to prepare the dansyl conjugates **5α** and **5β**, compounds **4α** and **4β** were hydrogenated and the corresponding free acids were then reacted with *N*-dansyl ethylenediamine, using PyBOP (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate) as condensating agent (Method C). Following purification, dansyl conjugates **5α** and **5β** were deprotected (Method D) to yield the fluorescent SOAT substrates **2α** and **2β** (Scheme 1).

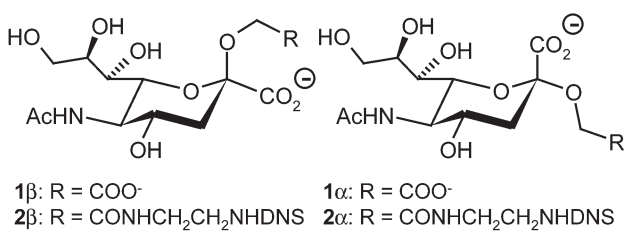
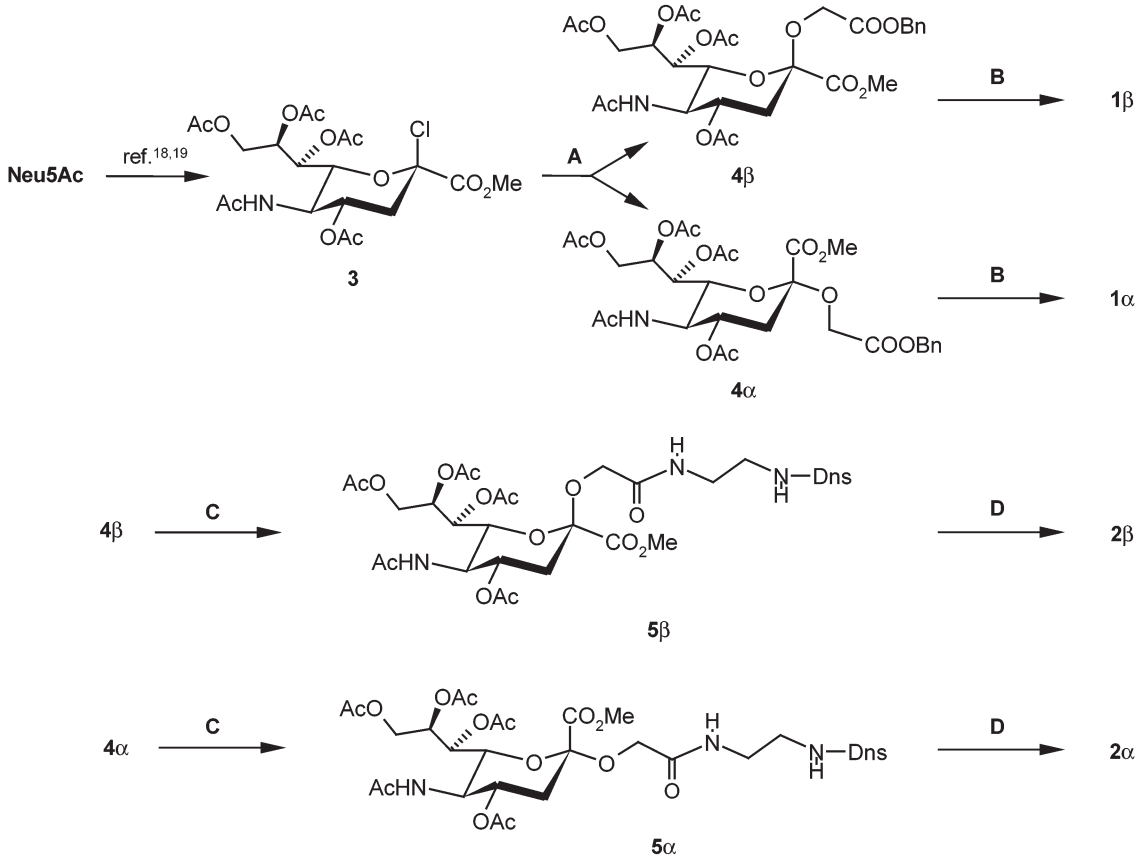


Fig. 2 Target α- and β-sialosides.



Scheme 1 Syntheses of target sialosides.

In conclusion, we have presented the synthesis of a set of sialosides as potential substrates for SOATs. Acetylations of the target compounds with selected enzyme preparations will be carried out in due course.

Experimental

Reaction solvents were purchased anhydrous and used as received. Solvents for chromatography were distilled before use. Reactions were monitored by TLC using precoated silica gel 60 F_{254} plates. Compounds were detected by UV absorption and/or by staining with a molybdenum phosphate reagent (20 g ammonium molybdate and 0.4 g cerium(IV) sulfate in 400 mL of 10% aq. sulfuric acid) or a basic $KMnO_4$-solution and subsequent heating at 120 °C for 5 min. Silica gel 60 A 'Davisil' (particle size 35–70 μm) from Fisher Scientific, UK was used for flash chromatography. ^{1}H NMR, ^{13}C NMR, and all multidimensional NMR spectra were recorded on a Bruker DPX 300 MHz spectrometer. Chemical shifts in ^{1}H NMR and ^{13}C NMR spectra were referenced to the residual proton resonance of the respective deuterated solvent, $CDCl_3$ (7.24 ppm), D_2O (4.80 ppm), D_2O in CD_3OD (4.88 ppm). ESI MS spectra were recorded on a Bruker Daltonics Apex III in positive mode with MeOH/H_2O as solvent. Molecular mass data were analysed with the Bruker Daltronics DataAnalysis 3.4 package. In the case of the deprotected and thus highly polar target molecules, various charged species were observed. The signal with the highest intensity was repectively analysed. Gel permeation chromatography was carried out in the 1–10 mg scale on a XK 16/70 column (bed volume 130 mL), from Amersham packed with Sephadex G-10 (particle size 40–120 μm) and 0.1 M NH_4HCO_3 as buffer. Detection was achieved with a differential refractometer from Knauer, Berlin, Germany. Fine chemicals were purchased from Aldrich-, Sigma- or Acros-Chemicals and were of the highest purity available.

Method A: Under an atmosphere of dry nitrogen, Ag_2CO_3 (5.17 g, 37.6 mmol) and $AgClO_4$ (396 mg, 1.91 mmol) were suspended in a solution of glycolic acid benzyl ester (5.66 g, 37.7 mmol) in dichloromethane (98 mL). A solution of donor **3** (1.92 g, 3.76 mmol) in dichloromethane (98 mL) was added and the mixture was stirred for 24h at room temperature in the dark. The mixture was filtered, washed with dichloromethane and the combined filtrates were concentrated *in vacuo*. Flash chromatography (petrol ether/ethyl acetate 5:1 → ethyl acetate only) gave **4β** (0.22 g, 0.34 mmol) and **4α** (1.99 g, 3.11 mmol) in a total yield of 92%.

Method B: **4α** (or **4β**) (308 mg, 0.48 mmol) was dissolved in a mixture of dichloromethane/methanol (1:2, 10 mL) and 50 mg of Pd/C was added. The mixture was stirred vigorously under an atmosphere of hydrogen until tlc indicated the absence of starting material (~ 30 min). Following neutralisation with Amberlite IR-120 (H^+-form), the mixture was filtered through Celite and evaporated. The resulting oil was dissolved in a mixture of dioxane/aq. ammonia (25%) (1:1, 20 mL), stirred overnight and lyophilised. Gel permeation chromatography (as described above) gave **1α** (or **1β**) (164 mg, 0.44 mmol) in 93% yield.

Method C: **4α** (or **4β**) were converted into the free acid as described in procedure B. The acid (55 mg, 0.1 mmol), Py BOP (52 mg, 0.11 mmol), *N*-dansylethylenediamine (33 mg, 0.11 mmol) and triethylamine (68 μL) were dissolved in this order in 0.2 mL DMF and the mixture was stirred for 2 h. After quenching with saturated NH_4Cl-solution (10 mL) the mixture was extracted three times with methylene chloride (10 mL each time), dried over $MgSO_4$, filtered and evaporated. The crude product was purified by flash chromatography (ethyl acetate/methanol, 95:5) to give **5α** (or **5β**) (55 mg, 0.066 mmol) in 67% yield.

Method D: **5α** (or **5β**) (20 mg, 24 μmol) was dissolved in a mixture of dioxane/aq. ammonia (25%) (1:1, 4 mL), stirred overnight and lyophilised. Gel permeation chromatography (as described above) gave **2α** (or **2β**) (10 mg, 15 μmol) in 64% yield.

Glycolic acid benzyl ester: Glycolic acid (3 g, 39.45 mmol), benzyl bromide (5.53 mL, 47.16 mmol) and potassium carbonate (5 g) were suspended in DMF (150 mL) and the mixture was stirred overnight at room temperature. The mixture was filtered, diluted with dichloromethane (300 mL) and extracted three times with saturated $NaHCO_3$-solution. The organic phase was dried (Mg_2SO_4), filtered and evaporated. The ester was purified by flash-chromatography

(petrol ether/ethyl acetate, 5:10). R_f = 0.55 (ethyl acetate). The physical properties were identical to a commercially obtained sample (Sigma-Aldrich).

N-Dansyl-ethylenediamine: Dansyl chloride (1 g, 3.7 mmol) and triethylamine (5 mL) were dissolved in dichloromethane (10 mL) and 1,2-diaminoethane (2.47 mL, 37 mmol) were added. After stirring overnight at room temperature, the mixture was evaporated and the crude product was purified by flash chromatography (dichloromethane). The physical properties corresponded to those published.[17]

2-Chlorosialyldonor (**3**): Synthesised as described in the literature[18,19] and used without further purification.

Protected α-carboxymethyl sialoside (**4α**): Obtained from **3** following Method A. Colourless oil. R_f = 0.30 (DCM/MeOH, 95:5). ^{1}H NMR (300 MHz, $CDCl_3$). δ 7.35–7.15 (m, 5H, C_6H_5), 5.30–4.95 (m, 5H, H-7, H-8, N*H*, CH_2Ph), 4.85 (ddd, 1H, *J* = 4.4, 10.0, 12.3 Hz, H-4), 4.30–3.80 (m, 5H, H-5, H-9, H-9, CH_2CO), 3.62 (s, 3H, OCH_3), 2.65 (dd, 1H, *J* = 12.5, 4.4 Hz, H-3$_{eq}$), 2.05–1.78 (5s, 15H, 5 COCH_3), 1.78 (dd, 1H, *J* = 12.5, 12.3 Hz, H-3$_{ax}$). HR-ESI-MS ($C_{29}H_{37}NO_{15}$): Calcd: 662.2060 (M+Na)$^+$. Found: 662.2041.

Protected β-carboxymethysialoside (**4β**): Obtained from **3** following Method A. Colourless oil. R_f = 0.37 (DCM/MeOH, 95:5). ^{1}H NMR (300 MHz, $CDCl_3$). δ 7.35–7.15 (m, 5H, C_6H_5), 5.30–5.00 (m, 6H, H-4, H-7, H-8, N*H*, CH_2Ph), 4.65, 4.25–3.80 (m, 5H, H-5, H-9, H-9, CH_2CO), 3.65 (s, 3H, OCH_3), 2.42 (dd, 1H, *J* = 12.2, 4.3 Hz, H-3$_{eq}$), 2.05–1.78 (m, 16H, H-3$_{ax}$, 5COCH_3). HR-ESI-MS ($C_{29}H_{37}NO_{15}$): Calcd: 662.2060 (M+Na)$^+$. Found: 662.2044.

α-Carboxymethylsialoside (**1α**): Synthesised from **4α** following Method B. Colourless solid. NMR and MS data conformed to those published.[18]

β-Carboxymethylsialoside (**1β**): Synthesised from **4β** following Method B. Colourless solid. ^{1}H NMR (300 MHz, D_2O). δ 4.50–3.30 (m, 9H), 2.25 (dd, 1H, *J* = 12.5, 4.4 Hz, H-3$_{eq}$), 1.78 (s, 3H, COCH_3), 1.55 (dd, 1H, *J* = 12.5, 11.0 Hz, H-3$_{ax}$). HR-ESI-MS ($C_{13}H_{21}NO_{11}$): Calcd: 389.0934 (M-H+Na)$^+$. Found: 389.1172.

Protected α-configured dansyl conjugate (**5α**): Obtained from **4α** following Method C. Light yellow oil. R_f = 0.32 (DCM/MeOH, 95:5). ^{1}H NMR (300 MHz, $CDCl_3$). δ 8.65, 8.35, 8.20, 7.50, 7.20 (6H, $C_{10}H_6$), 6.90, 6.20 (2H, N*H*), 5.45, 5.21 (2H, H-7, H-8), 4.95 (ddd, 1H, H-4), 4.72 (dd, 1H, *J* = 12.5 Hz, H-9), 4.31–3.65 (m, 5H, H-5, H-6, H-9, CH_2CO), 3.73 (s, 3H, OCH_3), 3.45–2.75 (4m, 4H, NHCH_2 CH_2NH), 2.90 (bs, 6H, N(CH_3)$_2$), 2.55 (dd, 1H, *J* = 12.2, 4.6 Hz, H-3$_{eq}$), 2.05–1.65 (5s, 1dd, 5COCH_3, H-3$_{ax}$). HR-ESI-MS ($C_{36}H_{48}N_4O_{16}S$): Calcd: 847.2683 (M+Na)$^+$. Found: 847.2875.

Protected β-configured dansyl conjugate (**5β**): Obtained from **4β** following Method C. Light yellow oil. R_f = 0.68 (DCM/MeOH, 7:1). ^{1}H NMR (300 MHz, $CDCl_3$). δ 8.65–7.20 (4d, 2dd, 6H, $C_{10}H_6$), 6.78, 6.50 (2H, N*H*), 5.50–5.32 (m, 2H, H-4, H-7), 5.05 (bd, 1H, H-8), 4.72 (dd, 1H, *J* = 12.5 Hz, H-9), 4.35–3.90 (m, 5H, H-5, H-6, H-9, CH_2CO), 3.60–2.70 (4m, 4H, NHCH_2CH_2NH), 3.75 (s, 3H, OCH_3), 2.90 (s, 6H, N(CH_3)$_2$), 2.58 (dd, 1H, *J* = 12.3, 4.5 Hz, H-3$_{eq}$), 2.05–1.65 (5s, 1dd, 5COCH_3, H-3$_{ax}$). HR-ESI-MS ($C_{36}H_{48}N_4O_{16}S$): Calcd: 847.2683 (M+Na)$^+$. Found: 847.2680.

α-Configured dansyl conjugate (**2α**):Synthesised from **5α** following Method D. Light yellow solid. ^{1}H NMR (300 MHz, D_2O). δ 8.35–7.30 (2d, 2m, 6H, $C_{10}H_6$), 4.80–2.70 (m, 13H, H-4, H-5, H-6, H-7, H-8, H-9, H-9, CH_2CO, HNCH_2CH_2NH), 2.73 (s, 6H, N(CH_3)$_2$), 2.56 (dd, 1H, *J* = 12.0, 4.3 Hz, H-3$_{eq}$), 1.95 (s, 3H, COCH_3), 1.75 (dd, 1H, *J* = 12.3 Hz, H-3$_{ax}$). ESI-MS ($C_{27}H_{38}N_4O_{12}S$): Calcd: 664.2026 (M-H+Na)$^+$. Found: 664.2269.

β-Configured dansyl conjugate (**2β**): Synthesised from **5β** following Method D. Light yellow solid. The ^{1}H NMR of the purified compound shows clearly separated signal sets for the H-3 protons and the acetyl group with an intensity of approximately 0.5:0.5. This indicates the existence of two populations of either amide bond rotamers or ring conformers. Attempts to unequivocally correlate the signal sets with distinct isomers have so far not been successful. ^{1}H NMR (500 MHz, D_2O). δ 8.40–7.28 (2d, 2m, 6H, $C_{10}H_6$), 4.20–3.00 (m, 13H, H-4, H-5, H-6, H-7, H-8, H-9, H-9, CH_2CO, HNCH_2CH_2NH), 2.80 (s, 6H, N(CH_3)$_2$), 2.41, 2.24 (2bdd, 1H, *J* = ~12, ~4 Hz, H-3$_{eq}$), 1.98, 1.92 (2s, 3H, COCH_3), 1.65, 1.45 (2bdd, 1H, *J* = ~12 Hz, H-3$_{ax}$). HR-ESI-MS ($C_{27}H_{38}N_4O_{12}S$): Calcd: 664.2026 (M-H+Na)$^+$. Found: 664.2223.

Received 27 July 2010; accepted 7 August 2010
Paper 1000274 *doi: 10.3184/030823410X12862035050403*
Published online: 22 October 2010

References

1 T. Angata and A. Varki, *Chem. Rev.*, 2002, **102**, 439.
2 J. Tiralongo and R. Schauer, *Trends Glycosci. Glycotechnol.*, 2004, **16**, 1.
3 R. Schauer, *Zoology*, 2004, **107**, 49.
4 R. Schauer and J.P. Kamerling, *Glycoproteins II*, eds J. Montreuil, J.F.G. Vliegenthart and H. Schachter, Elsevier, Amsterdam, 1997, p. 243.
5 C. Fahr and R. Schauer, *J. Invest. Dermatol.*, 2001, **116**, 254.
6 U. Hubl, H. Ishida, M. Kiso, A. Hasegawa and R. Schauer, *J. Biochem.*, 2000, **127**, 1021.
7 D. Sinha, C. Mandal and D.K. Bhattacharya, *Leukemia*, 1999, **13**, 119.
8 E.L. Deszo, S.M. Steenbergen, D.I. Freedberg and E.R. Vimr, *Proc. Natl. Acad. Sci. USA*, 2005, **102**, 5564.
9 A.K. Bergfeld, H. Claus, U. Vogel and M. Mühlenhoff, *J. Biol. Chem.*, 2007, **282**, 22217.
10 R.S. Houliston, H.P. Endtz, N. Yuki, J. Li, H.C. Jarrell, M. Koga, A. van Belkum, M.F. Karwaski, W.W. Wakarchuk and M. Gilbert, *J. Biol. Chem.*, 2006, **281**, 11480.
11 A.L. Lewis, M.E. Hensler, A. Varki and V. Nizet, *J. Biol. Chem.*, 2006, **281**, 11186.
12 M. Iwersen, V. Vandamme-Feldhaus and R. Schauer, *Glycoconj. J.*, 1998, **15**, 895.
13 M. Iwersen, H. Dora, G. Kohla, S. Gasa and R. Schauer, *Biol. Chem.*, 2003, **384**, 1035.
14 H.H. Higa, C. Butor, S. Diaz and A. Varki, *J. Biol. Chem.*, 1989, **264**, 19427.
15 Y. Shen, G. Kohla, A.L. Lrhofi, B. Sipos, H. Kalthoff, G.J. Gerwig, J.P. Kamerling, R. Schauer and J. Tiralongo, *Eur. J. Biochem.*, 2004, **271**, 281.
16 L.A. Lrhofi, G.V. Srinivasan and R. Schauer, *Biol. Chem.*, 2007, **388**, 297.
17 M.M. Abdel-Monem and K. Ohno, *J. Pharmaceut. Sci.*, 1977, **66**, 1089.
18 J.C. McAuliffe, D. Rabuka and O. Hindsgaul, *Org. Lett.*, 2002, **4**, 3067.
19 R. Roy and C. Laferreire, *Can. J. Chem.*, 1990, **68**, 2045.