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How pure is your thiosialoside? A reinvestigation into the HPLC purification of thioglycosides of *N*-acetylneuraminic acid

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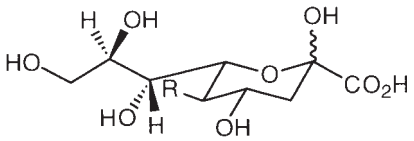
The synthesis of thiosialosides as potential biological probes for investigations involving the use of sialic acid-recognising proteins has been reinvestigated. It has been found that the most efficient method for the preparation of thiosialosides free from any 2,3-didehydro sialic acid contaminants involves an intermediate HPLC purification of thiosialosides as their methyl esters. Subsequent methyl ester hydrolysis provides thiosialosides (eg. 6 and 14) which are suitable for studies involving the use of sialic acid-recognising proteins.

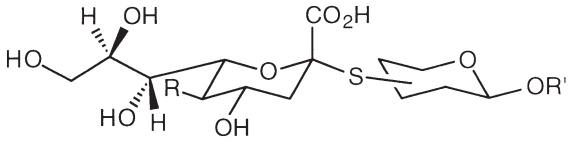
Keywords: **thiosialosides, sialic acids, thioglycosides, HPLC purification, biological probes**

Introduction

Sialic acids, and the proteins and enzymes which utilise them, are intimately involved in a variety of important biological processes, including cell recognition phenomena, the pathogenesis of some microorganisms, and various disease states [1–8]. As a consequence of their involvement in such biological processes, there has been a continual increase over recent years in the synthesis of novel sialic acid derivatives as probes for studies into sialic acid-recognising proteins [9–11]. In particular, the synthesis of metabolically stable glycosides of *N*-acetylneuraminic acid [Neu5Ac (**1**)] has attracted attention [10,12–14]. Thiosialosides (eg. **2**), which have been shown to be resistant to enzyme hydrolysis [14,15], are ideally suited to studies aimed at elucidating information regarding structure-activity relationships between sialic acid-recognising proteins and Neu5Ac derivatives.



(**1**) R = NHAc



(**2**) R = NHAc

Our own efforts in this area have led us to develop new methods for the synthesis of thiosialosides [16–20], and has resulted in the synthesis of thiosialosides as potential inhibitors of rotaviral infection [17] as well as the preparation of an alkylmercury containing thiosialoside for use in X-ray crystallographic studies with sialic acid-recognising proteins [20]. In many of these investigations we have prepared the requisite thiosialosides by performing a diethylamine mediated coupling between the 2-thioacetyl-Neu5Ac derivative **3** and an activated sialosyl acceptor (eg. **4**, X = Br or OTf) [16,17], to give the corresponding (2,6)-linked thiosialosides **5** after deprotection (Figure 1).

Interestingly, the Neu5Ac-2-*S*-α-(2,6)-Gal derivative **6**, prepared in this way was found to be unsuitable for co-crystallisation with influenza virus sialidase, since X-ray crystallographic analysis of sialidase crystals grown in the presence of an excess of **6** showed apparantly only Neu5Ac2en (**7**) in the catalytic site [Laver WG, personal communication]. Whilst influenza virus sialic acid-recognising proteins generally have a low affinity for monomeric sialosides [12], Neu5Ac2en (**7**), which is recognised as a transition state mimic for sialidases [21], has a much

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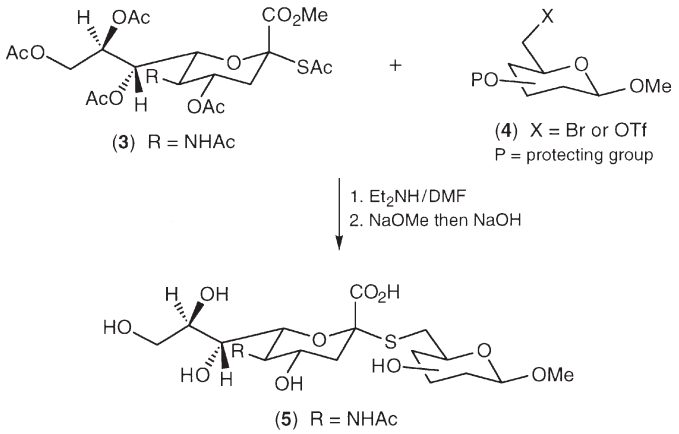
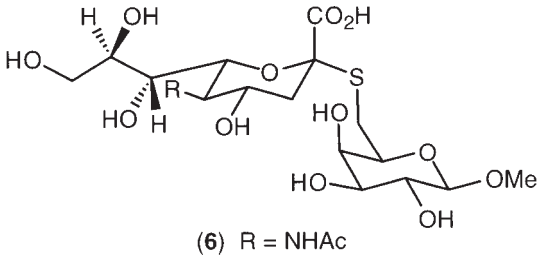
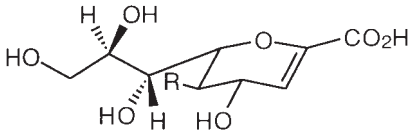


Figure 1. Synthesis of thiosialosides [16,17].

greater affinity (K_i ~ 10^{-5} – 10^{-6} M [22]) for sialidases. Given these differences in affinity, it is conceivable that even a small amount (<5%) of Neu5Ac2en (**7**) in a sample of the thiosialoside **6** would result in virtual saturation of the sialic acid binding domain of the sialidase with **7** rather than **6.**





The source of Neu5Ac2en in our sample of **6** was somewhat mystifying, since compound **6** had been HPLC purified, and satisfactory spectroscopic and analytical data obtained. This paper describes our reinvestigation into the synthesis of thiosialosides such as **6** and reports the use of a simple protocol, based on the HPLC purification of thiosialoside methyl esters, as a way of ensuring biological probes based on thiosialosides are not contaminated with Neu5Ac2en.

Materials and methods

General methods

Melting points were determined on a Mettler FP21 hot-stage melting point apparatus and are uncorrected. Infrared spectra were recorded on a Hitachi 270-30 infrared spectrophotometer. ^{1}H and ^{13}C (JMOD) NMR spectra were recorded using a Brüker AM 300WB multinuclear magnetic resonance spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to an external reference for D_2O. Mass spectra were obtained using a Micromass Platform II spectrometer (LRESIMS & HRESIMS). HPLC purification was performed on a Waters 600E system controller, using reverse phase C_{18} Waters μBondapak™ columns (analytical: 8×100mm, part no. WAT085721; preparative: 25×100mm, part no. WAT038505), detecting at 220nm with a Waters 484 tunable absorbance detector. All solvents were distilled prior to use or were of analytical grade.

Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (**3**) [23], methyl *S*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic)-(2,6)-6-thio-β-D-galactopyranoside (**6**) [17], methyl *S*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulo-pyranosylonate)-(2,6)-2,3,4-tri-*O*-acetyl-6-thio-β-D-galactopyranoside (**12**) [17], methyl *S*-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2,6)-2,3,4-tri-*O*-acety-6 - thio-β-D-glucopyranoside (**13**) [16], methyl *S*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopy-ranosyl onic)-2,6)-6-thio-β-D-glucopyranoside (**14**) [17], methyl 2,3,4-tri-*O*-acetyl-6-*O*-triflyl-β-D-galactopyranoside (**10**) [17], methyl 2,3,4-tri-*O*-acetyl-6-bromo-6-deoxy-β-D-glucopyranoside (**11**) [16], 5-acetamido-2,6-anhydro-3, 5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid (**7**) [24], and methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonate (**9**) [24], were all prepared as previously described.

Methyl S-*(methyl 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosylonate)-(2,6)-6-thio-β-D-galacto-pyranoside* (**15**)

To a solution of methyl *S*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2,6)-2,3,4-tri-*O*-acetyl-6-thio-β-D-galactopyranoside (**12**) (380 mg, 0.47 mmol) in dry MeOH (10 ml) at 0° C under N_2 was added sodium (*ca.* 50 mg). After 1h at 0° C the solution was allowed to warm to room temperature and stirred for a further 2h before being neutralised (to pH 5) with Amberlyte IR120 (H^+) resin. The resin was removed by filtration, washed with MeOH and the methanolic washes concentrated under reduced pressure to give a pale yellow foam. HPLC purification (see Table 1 for conditions) gave

the title compound (**15**) (196 mg, 81%) as an amorphous mass: mp 130–135°C; ν_{max} (KBr) 3412, 1728, 1641, 1554, 1122, 1056 cm^{-1}; ^1H NMR (300 MHz; D_2O): δ 1.87 (1H, dd, $J_{3a,3e}$ 12.9, $J_{3a,4}$ 11.5 Hz, NeuH-3a), 2.01 (3H, s, AcN), 2.81 (1H, dd, $J_{3e,3a}$ 12.9, $J_{3e,4}$ 4.5 Hz, NeuH-3e), 2.90 (1 H, dd, $J_{6,6'}$ 13.8, $J_{6,5}$ 6.9 Hz, GalH-6), 3.02 (1H, dd, $J_{6',6}$ 13.8, $J_{6',5}$ 7.2 Hz, GalH-6′), 3.45 (1H, dd, $J_{2,3}$ 9.9, $J_{2,1}$ 7.8 Hz, GalH-2), 3.50–3.54 (1H, m, NeuH-6), 3.54 (3 H, s, OMe), 3.56 (1H, dd, $J_{3,2}$ 9.9, $J_{3,4}$ 3.3 Hz, GalH-3), 3.61–3.64 (1H, m, NeuH-7), 3.63–3.70 (3H, m, NeuH-8/NeuH-9/GalH-5), 3.70 (1H, ddd, $J_{4,3a}$ 11.5, $J_{4,5}$ 10.5, $J_{4,3e}$ 4.5 Hz, NeuH-4), 3.78–3.85 (2H, m, NeuH-5/ NeuH-9′), 3.87 (3H, s, CO_2Me), 3.96 (1H, d, $J_{4,3}$ 3.3 Hz, GalH-4), 4.24 (1H, d, $J_{1,2}$ 7.8 Hz, GalH-1) assignments confirmed by ^1H-^1H COSY; ^{13}C NMR (75.5 MHz; D_2O): δ 22.0 (NC(O)*Me*), 28.9 (NeuC-3), 39.9 (GalC-6), 51.6 (NeuC-5), 53.6 (CO_2Me), 57.2 (OMe), 63.0 (NeuC-9), 67.5, 68.1, 68.9. 70.4, 70.9, 72.8, 73.7, 74.9 (all CH, NeuC-4/NeuC-6/NeuC-7/NeuC-8/GalC-2/GalC-3/GalC-4/GalC-5), 83.3 (NeuC-2), 103.8 (GalC-1), 171.0 (NeuC-1), 174.9 (N*C*(O)Me); LRESIMS: m/z 538 [(M + Na)^{N}1, 100%], 516 [(M + 1)^{N}1, 75], 484 (60), 306 (74), 288 (43).

Analytical data. Calculated for $C_{19}H_{33}NO_{13}S{\cdot}4H_2O$: C, 38.8; H, 7.0; N, 2.4. Found: C, 38.3; H, 6.2; N, 2.3%. HRESIMS: $C_{19}H_{34}NO_{13}S$ requires 516.17509; found 516.17620.

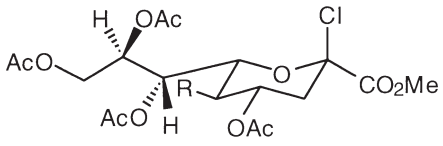
Methyl S-*(methyl 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosylonate)-(2,6)-6-thio-β-D-glucopyranoside (***16***)*

Methyl *S*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2,6)-2,3,4-tri-*O*-acetyl-6-thio-β-D-glucopyranoside (**13**) (196 mg, 0.24 mmol) was treated in the same way as described for **12** above, and purified using HPLC (see Table 1 for conditions) to give **16** (103 mg, 83%) as an amorphous mass: mp 108-112 °C; ν_{max} (KBr) 3450, 1726, 1644, 1446, 1394, 1288, 1058 cm^{-1}; ^1H NMR (300 MHz; D_2O): δ 1.90 (1H, dd, $J_{3a,3e}$ 12.3, $J_{3a,4}$ 11.7 Hz, NeuH-3a), 2.03 (3H, s, AcN), 2.82 (1H, dd, $J_{3e,3a}$ 12.3, $J_{3e,4}$ 4.6 Hz, NeuH-3e), 2.97 (1 H, dd, $J_{6,6'}$ 12.9, $J_{6,5}$ 7.8 Hz, GlcH-6), 3.24 (1H, dd, $J_{2,3}$ 9.3, $J_{2,1}$ 7.8 Hz, GlcH-2), 3.29–3.36 (2H, m, GlcH-4/GlcH-6′), 3.41–3.48 (2H, m, GlcH-3/GlcH-5), 3.52 (3 H, s, OMe), 3.52–3.55 (1H, m, NeuH-6), 3.59–3.67 (2H, m NeuH-7/NeuH-9), 3.71 (1H, ddd, $J_{4,3a}$ 11.7, $J_{4,5}$ 10.3, $J_{4,3e}$ 4.6 Hz, NeuH-4), 3.81–3.90 (3H, m, NeuH-5/NeuH-8/NeuH-9′), 3.88 (3H, s, CO_2Me), 4.31 (1H, d, $J_{1,2}$ 7.8 Hz, GlcH-1) assignments confirmed by ^1H-^1H COSY; ^{13}C NMR (75.5 MHz; D_2O): δ 22.0 (NC(O)*Me*), 29.9 (NeuC-3), 39.9 (GlcC-6), 51.6 (NeuC-5), 53.6 (CO_2Me), 57.2 (OMe), 63.0 (NeuC-9), 67.6, 68.3, 70.7. 72.3, 73.0, 74.4, 74.8, 75.4 (all CH, NeuC-4/NeuC-6/NeuC-7/NeuC-8/GlcC-2/GlcC-3/GlcC-4/GlcC-5), 82.3 (NeuC-2), 103.3 (GalC-1), 171.1 (NeuC-1), 174.9 (N*C*(O)Me); LRESIMS: m/z 538 [(M + Na)$^+$, 100%], 516 [(M + 1)$^+$, 15], 414 (32).

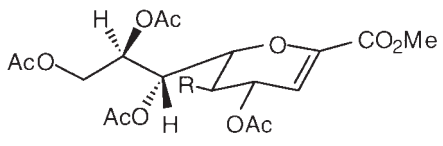
Analytical data. Calculated for $C_{19}H_{33}NO_{13}S{\cdot}3H_2O$: C, 40.1; H, 6.9; N, 2.5. Found: C, 39.9; H, 6.4; N, 2.4%.

Results and discussion

We have previously reported [16–20] a method for the synthesis of thiosialosides, involving the selective *in situ* thiodeacetylation of the 2-α-thioacetyl-Neu5Ac derivative **3** using diethylamine, and coupling of the resultant thiolate with activated acceptors (Figure 1). The source of the Neu5Ac2en contaminant in thiosialosides (eg. **6**) prepared in this way presumeably stems from the preparation of the 2-thioacetyl-Neu5Ac derivative **3**, which is formed by reaction of the 2-β-chloro-Neu5Ac derivative **8** with potassium thioacetate [23]. In our hands, the peracetylated Neu5Ac2en1Me derivative **9** is typically formed in *ca.* 10–15% (by ^1H n.m.r. analysis of the reaction product) during the synthesis of **3**. Separation of the Neu5Ac2en derivative **9** from the thioacetyl derivative **3** is extremely difficult, since compounds **3** and **9** have almost identical chromatographic properties and also co-crystallise. Whilst exhaustive chromatographic purification of the thioacetyl derivative **3** can result in a sample which is spectroscopically and analytically pure this does not necessarily result in **3** being totally free of **9**. For example, combustion analysis of **3** ($C_{22}H_{31}NO_{13}S$) should give C, 48.08; H, 5.69; N, 2.55%. However, if **3** is contaminated with 5% of **9**, the calculated analytical data are almost identical (C, 48.20; H, 5.69; N, 2.57%). Since contaminants of 5% or less are extremely difficult to detect spectroscopically, it is conceivable that such quantities of the Neu5Ac2en derivative **9** can be carried through into the coupling of **3** with sialosyl acceptors.

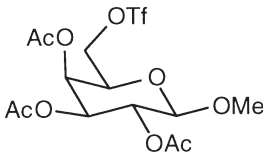


(**8**) R = NHAc

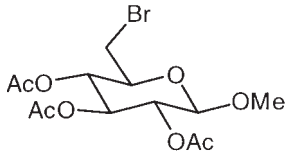


(**9**) R = NHAc

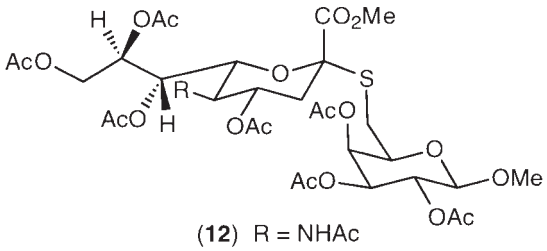
Unfortunately, after coupling of **3** with the activated acceptors **10** and **11,** to give the thiosialosides **12** and **13**, respectively [16,17], the problem of separating the Neu5Ac2en derivative **9** still remains, since the thiosialosides **12** and **13** have extremely similar chromatographic behaviour to the Neu5Ac2en derivative **9**.



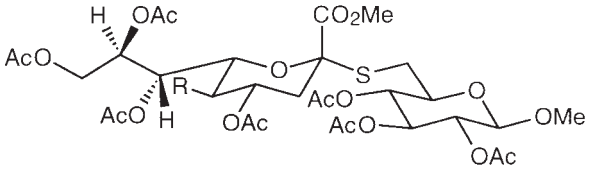
(**10**)



(**11**)

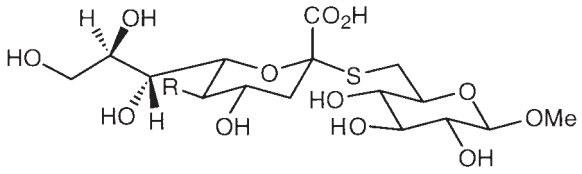


(**12**) R = NHAc



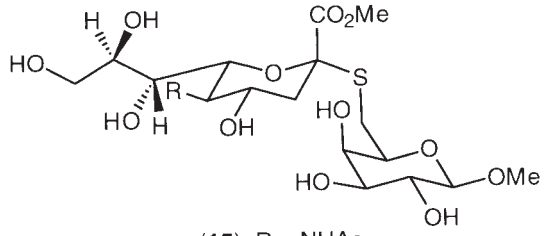
(**13**) R = NHAc

Our previous work towards the synthesis of thiosialosides as biological probes involved the total deprotection of compounds such as **12** and **13** (NaOMe followed by NaOH) to give the corresponding Neu5Ac-2-*S*-α-(2,6)-Gal and Neu5Ac-2-*S*-α-(2,6)-Glc derivatives **6** and **14**, respectively [17]. However, we have subsequently found (*vide infra*) that HPLC purification of the thiosialosides **6** and **14** would not result in separation of any Neu5Ac2en (**7**) contaminant, since compounds **6**, **14**, and **7** all have similar chromatographic properties.

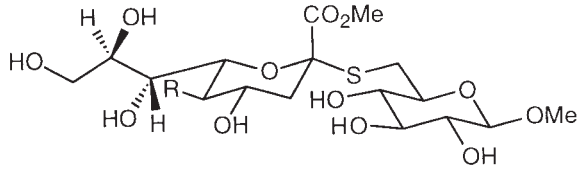


(**14**) R = NHAc

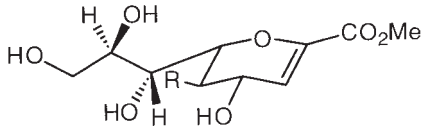
In an attempt to overcome this problem, it was decided to investigate a two-step deprotection strategy for the preparation of thiosialosides for use in studies with sialic acid-recognising proteins. Accordingly, the thiosialosides **12** and **13** were treated with sodium methoxide in methanol to give the corresponding methyl ester derivatives **15** and **16**, respectively. In a similar manner the peracetylated Neu5Ac2en1Me derivative **9** was deprotected to give Neu5Ac2en1Me (**17**) [25].



(**15**) R = NHAc



(**16**) R = NHAc



(**17**) R = NHAc

To our delight, the thiosialoside methyl esters **15** and **16** have very different chromatographic behaviour to Neu5Ac2en1Me (**17**). The results from the HPLC purification of thiosialosides **15** and **16**, together with the data obtained for Neu5Ac2en1Me (**17**), Neu5Ac2en (**7**) and the fully deprotected thiosialosides **6** and **14** are presented in Table 1. As can be seen, the thiosialoside methyl esters **15** and **16** have significantly longer retention on a C_{18} reverse phase HPLC column (Waters μBondapak™) than the methyl ester derivative **17** of Neu5Ac2en. This is in stark contrast to the fully deprotected compounds **6**, **14**, and **7** (Table 1), which could not be separated under a

Table 1. HPLC purification of thiosialosides[a]

Compound	*Retention time (min)*
Neu5Ac1Me-2-*S*-α-(2,6)-Gal-β-OMe (**15**)	28.8
Neu5Ac1Me-2-*S*-α-(2,6)-Glc-β-Ome (**16**)	33.3
Neu5Ac2en1Me (**17**)	11.2
Neu5Ac-2-*S*-α-(2,6)-Gal-β-OMe (**6**)	6.8[b]
Neu5Ac-2-*S*-α-(2,6)-Glc-β-OMe (**14**)	7.1[b]
Neu5Ac2en (**7**)	6.7[b]

a. All purification was performed using a Waters μBondapak™ C_{18} column with 1% CH_3CN in H_2O as the mobile phase and a flow rate of 6.1 ml/min.

variety of conditions involving different mobile phases and flow rates.

In order to confirm that the Neu5Ac2en derivative **17** can indeed be separated from the thiosialosides **15** and **16**, an HPLC purified sample of **15** (20 mg) was deliberately contaminated with **17** (2 mg) and the mixture resubjected to HPLC purification. Under the conditions specified above (Table 1), the thiosialoside methyl ester **15** and Neu5Ac2en1Me (**17**) were easily separated, and each recovered quantitatively.

Conclusion

The HPLC purification of thiosialosides as their methyl esters represents a reliable method for ensuring thiosialosides used in studies with sialic acid-recognising proteins are free from Neu5Ac2en contaminants. Furthermore, the two step deprotection strategy does not alter the overall efficiency of the synthetic sequence.

Acknowledgements

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References

1 Schauer R (1982) *Adv Carbohydr Chem Biochem* **40**: 131–234.
2 Corfield AP, Schauer R (1982) In *Sialic Acids—Chemistry, Metabolism and Function,* vol. 10 in *Cell Biology Monographs* (Schauer R, ed) pp 195–261. Wein: Springer Verlag.
3 Horowitz MJ, Pigman W (1978) *The Glycoconjugates,* vol I and II. New York: Academic Press.
4 Schauer R, Kamerling JP (1997) In *Glycoproteins II* (Montreuil J, Vliengenthart JFG, Schachter H, eds) pp 243–402. Amsterdam: Elsevier.
5 Corfield T (1992) *Glycobiology* **2**: 509–21.
6 Reutter W, Köttgen E, Bauer C, Gerok W (1982) In *Sialic Acids—Chemistry, Metabolism and Function,* vol. 10 in *Cell Biology Monographs* (Schauer R, ed) pp 263–305. Wein: Springer Verlag.
7 Varki A (1992) *Glycobiology* **2**: 25–40.
8 Schauer R, Kelm S, Reuter G, Roggentin P, Shaw L (1995) In *Biology of the Sialic Acids* (Rosenberg A, ed) pp 7–67. New York: Plenum Press.
9 Zbiral E (1992) In *Carbohydrates: Synthetic Methods and Applications in Medicinal Chemistry* (Ogura H, Hasegawa A, Suami T, eds) pp 304–39. New York: VCH.
10 von Itzstein M, Kiefel MJ (1997) In *Carbohydrates in Drug Design* (Witczak ZJ, Nieforth KA, eds) pp 39–82. New York: MDI.
11 DeNinno MP (1991) *Synthesis* 583–93.
12 Roy R (1997) In *Carbohydrates in Drug Design* (Witczak ZJ, Nieforth KA, eds) pp 83–135. New York: MDI.
13 Hasegawa A, Kiso M (1992) In *Carbohydrates: Synthetic Methods and Applications in Medicinal Chemistry* (Ogura H, Hasegawa A, Suami T, eds) pp 243–66. New York: VCH.
14 Suzuki Y, Sato K, Kiso M, Hasegawa A (1990) *Glycoconjugate J* **7**: 349–56.
15 Sabesan S, Neira S, Davidson F, Duus JØ, Bock K (1994) *J Am Chem Soc* **116**: 1616–34.
16 Bennett S, von Itzstein M, Kiefel MJ (1994) *Carbohydr Res* **259**: 293–99.
17 Kiefel MJ, Beisner B, Bennett S, Holmes ID, von Itzstein M (1996) *J Med Chem* **39**: 1314–20.
18 Angus DI, von Itzstein M (1995) *Carbohydr Res* **274**: 279–83.
19 Smalec B, von Itzstein M (1995) *Carbohydr Res* **266**: 269–72.
20 Kiefel MJ, von Itzstein M (1996) *Tetrahedron Lett* **37**: 7307–10.
21 Chong AKJ, Pegg MS, Taylor NR, von Itzstein M (1992) *Eur J Biochem* **207**: 335–43.
22 Holzer CT, von Itzstein M, Jin B, Pegg MS, Stewart WP, Wu W-Y (1993) *Glycoconjugate J* **10**: 40–44.
23 Hasegawa A, Nakamura J, Kiso M (1986) *J Carbohydr Chem* **5**: 11–19.
24 Meindl P, Tuppy H (1969) *Monatshefte Chem* **100**: 1295–306.
25 Kumar V, Kessler J, Scott ME, Patwardhan BH, Tanenbaum SW, Flashner M (1981) *Carbohydr Res* **94**: 123–30.

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