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Investigation of the Stability of Thiosialosides toward Hydrolysis by Sialidases Using NMR Spectroscopy

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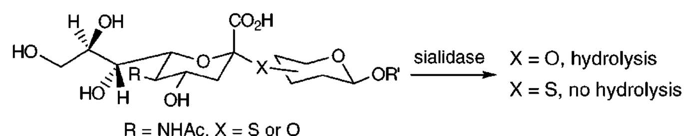
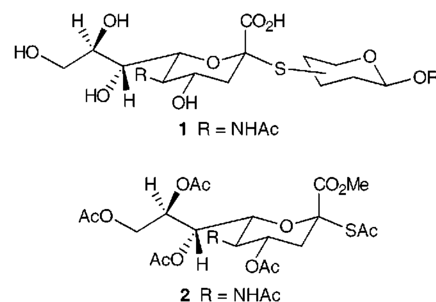
ABSTRACT



^{1}H NMR spectroscopy has been used to investigate whether the α(2→6)-linked thiosialoside 3 and the α(2→3)-linked thiosialoside 9 are hydrolyzed in the presence of *Vibrio cholerae* sialidase. Similarly, the hydrolysis of the *O*-ketosides Neu5Ac-2-*O*-α-(2→3)-Gal*β*Me (4) and the α-(2→6)-sialyllactoside 7, representing natural α(2→3)- and α(2→6)-linked sialosides, respectively, was investigated. The results of the ^{1}H NMR experiments clearly demonstrate that the thiosialosides are not hydrolyzed by *Vibrio cholerae* sialidase. As expected, the *O*-sialosides are hydrolyzed to give *N*-acetyl-α-D-neuraminic acid as the first product of substrate cleavage.

Sialidases are glycohydrolases that catalyze the cleavage of terminal sialic acids from glycoconjugates.[1] It is becoming increasingly evident that sialidases and *trans*-sialidases play important roles in several disease states.[1,2] One strategy for inhibition and structural studies of glycohydrolases is to design substrate-like compounds that are resistant to hydrolysis by the enzyme.[3,4] These compounds are purposely designed to mimic the key elements of the sialosides that are the natural substrates for sialidases. In general this approach has the potential to yield compounds with a high affinity for the sialidase-binding pocket. One such class of substrate-based compound that has been widely accepted as being metabolically stable to the action of sialidases are the sialic acid thioglycosides (e.g., **1**) or thiosialosides. Efficient synthetic routes for the production of a range of thiosialosides have been developed which generally involve coupling between a 2-thio-Neu5Ac derivative and an activated sialosyl acceptor.[4−6] Recent studies within the von Itzstein group have led to the development of a mild and facile route to the synthesis of thiosialosides, by coupling an activated acceptor with the 2-thioacetyl-Neu5Ac derivative **2** in the presence of diethylamine in *N,N*-dimethylformamide.[7,8] This method has provided ready access to a range of α-linked thiosialosides.[7−9]



Although the stability of thioglycosides toward hydrolysis by glycosidases is generally accepted, there are only a few reports which actually demonstrate the stability of the

(1) For reviews see: (a) Schauer, R.; Kamerling, J. P. *Glycoproteins II*; Montreuil, J., Vliegenthart, J. F. G., Schachter, H., Eds.; Elsevier: Amsterdam, 1997; pp 243−402. (b) Saito, M.; Yu, R. K. *Biology of the Sialic Acids*; Rosenberg, A., Ed.; Plenum Press: New York, 1995; pp 261−313. (c) Traving, C.; Schauer, R. *Cell. Mol. Life Sci.* **1998**, *54*, 1330−1349.
(2) Taylor, G. *Curr. Opin. Struct. Biol.* **1996**, *6*, 830−837.
(3) Driguez, H. *Top. Curr. Chem.* **1997**, *187*, 85−116.
(4) Hasegawa, A.; Kiso, M. *Carbohydrates: Synthetic Methods and Applications in Medicinal Chemistry*; Ogura, H., Hasegawa, A., Suami, T., Eds.; VCH: New York, 1992; pp 243−266.

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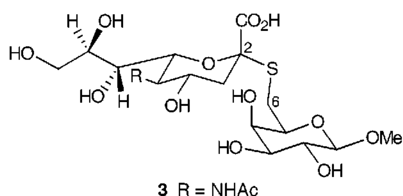
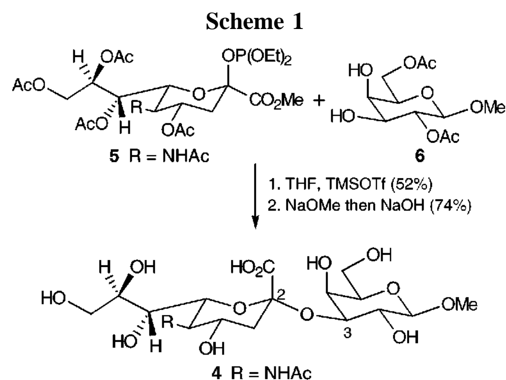
thioglycosidic linkage in thiosialosides toward the action of sialidases.[5,10−12] For example, it has been shown that thioglycosidic analogues of gangliosides are stable to the action of influenza virus sialidase.[5] Similarly, it has been reported that simple aryl thiosialosides are not hydrolyzed by *Vibrio cholerae* sialidase during incubation for 3 h,[10] although these same authors have described the "slow" hydrolysis of aryl thiosialosides by influenza virus sialidase.[11]

Our own interests in the stability of thiosialosides in the presence of sialidases has in part stemmed from some rather curious observations when attempting to perform X-ray structural analysis of a sialidase, using crystals soaked with Neu5Ac-2-*S*-α-(2→6)-Gal*β*Me (**3**). On several occasions (Graeme Laver, personal communication) no electron density was observed for the aglycon unit. There has therefore been some speculation that thiosialosides such as **3** are not metabolically stable under the conditions necessary to obtain soaked crystals for X-ray analysis. To address this controversy we decided to use NMR spectroscopy to investigate whether any hydrolysis of thiosialosides is detectable in the presence a sialidase. The sialidase chosen as a model for this study was *Vibrio cholerae* sialidase, primarily because *Vibrio cholerae* sialidase is readily available, its biology and 3D structure are well-characterized and it is a relatively promiscuous sialidase, accepting both α(2→6)- and α(2→3)-linked sialosides. This NMR spectroscopy method also has the potential to be utilized for mechanistic studies into other α(2→3)-recognizing enzymes such as *trans*-sialidases.

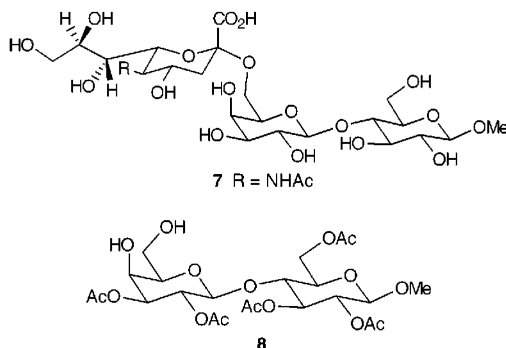


To establish appropriate ^{1}H NMR experimental conditions for our hydrolysis studies, we required representative examples of natural α(2→3)- and α(2→6)-*O*-linked sialosides. For the α(2→3)-linked sialoside, we opted for Neu5Ac-2-*O*-α(2→3)-Gal*β*Me (**4**),[13] which was readily prepared (Scheme 1) *via* a TMSOTf-mediated coupling between the sialosyl

Scheme 1



phosphite **5** and the sialosyl acceptor **6** using a modification[14] of the method reported by Schmidt and co-workers.[15] Similarly, the α(2→6)-linked sialyllactoside **7** was prepared in an analogous manner to that shown in Scheme 1 for **4**, employing the lactoside **8** as the sialosyl acceptor.



With the sialosides **4** and **7** representing natural α(2→3)- and α(2→6)-*O*-linked sialosides, respectively, our attention turned to the hydrolysis of these compounds using *Vibrio cholerae* sialidase. The progress of the sialidase hydrolysis reactions is conveniently studied by incubating *Vibrio cholerae* sialidase with substrate and observing the reaction *in vitro* using NMR spectroscopy. The H3a and H3e protons on Neu5Ac appear at characteristic chemical shifts, well-separated from other peaks in the ^{1}H NMR spectrum,

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(6) (a) Warner, T. G.; Lee, L. A. *Carbohydr. Res.* **1988**, *176*, 211−218. (b) Roy, R. *Carbohydrates in Drug Design*; Witczak, Z. J., Nieforth, K. A., Eds.; Marcel Dekker: New York, 1997; pp 83−135. (c) von Itzstein, M.; Kiefel, M. J. *Carbohydrates in Drug Design*; Witczak, Z. J., Nieforth, K. A., Eds.; Marcel Dekker: New York, 1997; pp 39−82. (d) Zanini, D.; Roy, R. *J. Org. Chem.* **1998**, *63*, 3486−3491. (e) Park, W. K. C.; Meunier, S. J.; Zanini, D.; Roy, R. *Carbohydr. Lett.* **1995**, *1*, 179−184. (f) Eisele, T.; Schmidt, R. R. *Liebigs Ann.* **1997**, 865−872. (g) Eisele, T.; Toepfer, A.; Kretzschmar, G.; Schmidt, R. R. *Tetrahedron Lett.* **1996**, *37*, 1389−1392.

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(10) Khorlin, A. Y.; Privalova, I. M.; Zakstelskaya, L. Y.; Molibog, E. V.; Evstigneeva, N. A. *FEBS Lett.* **1970**, *8*, 17−19.

(11) Privalova, I. M.; Khorlin, A. Y. *Izv. Akad. Nauk SSSR* **1969**, *12*, 2785−2792; *Chem. Abstr.* **1970**, *72*, 90785y.

(12) Sabesan, S.; Neira, S.; Davidson, F.; Duus, J. Ø.; Bock, K. *J. Am. Chem. Soc.* **1994**, *116*, 1616−1634.

(13) All new compounds gave satisfactory spectroscopic and microanalytical data. Compound **4**: ^{1}H NMR (500 MHz, D_2O) δ 1.66 (1H, dd, $J_{3a,3e}$ 12.5, $J_{3a,4}$ 12.0 Hz, NeuH-3a), 1.89 (3H, s, AcN), 2.62 (1H, dd, $J_{3e,4}$ 4.5 Hz, NeuH-3e), 3.40 (1H, dd, $J_{2,3}$ 9.5, $J_{2,1}$ 8.0 Hz, GalH-2), 4.32 (3H, s, OMe), 3.45−3.51 (3H, m, NeuH-6/H-9/GalH-5), 3.52−3.57 (2H, m, NeuH-4/NeuH-7), 3.59−3.63 (2H, m, GalH-6/H-6′), 3.68−3.75 (3H, m, NeuH-5/H-8/H-9′), 3.80 (1H, d, $J_{4,3}$ 3.0 Hz, GalH-4), 3.95 (1H, dd, GalH-3), 4.25 (1H, d, GalH-1).

(14) **General Procedure for the Synthesis of Sialosides.** To a solution of the phosphite **5** and sialosyl acceptor (2.5 equiv) in THF containing 4 Å sieves at −40 °C was added TMSOTf (0.3 equiv). After 1 h at −40 °C and 3 h at 0 °C the mixture was neutralized (Et_3N), filtered, concentrated, and then chromatographed.

(15) Martin, T. J.; Brescello, R.; Toepfer, A.; Schmidt, R. R. *Glycoconjugate J.* **1993**, *10*, 16−25.

depending on the aglycon unit of Neu5Ac and the anomeric configuration at C2.[16] Accordingly, these resonances can easily be tracked throughout the hydrolysis reaction, and in this way, all reaction mixture components are observable during the course of the reaction.

The hydrolyses of the sialosides **4** and **7** were performed in D_2O by incubating the compound (6 mM) with *Vibrio cholerae* sialidase (50 μL, 0.16U of activity) at 25 °C in an NMR tube, and periodically running a ^{1}H NMR spectrum.[17] As expected, complete hydrolysis of Neu5Ac-2-*O*-α(2→3)-GalβMe (**4**) (shown in Figure 1) and the α(2→6)-sialyllactoside **7** was observed in the presence of *Vibrio cholerae* sialidase.

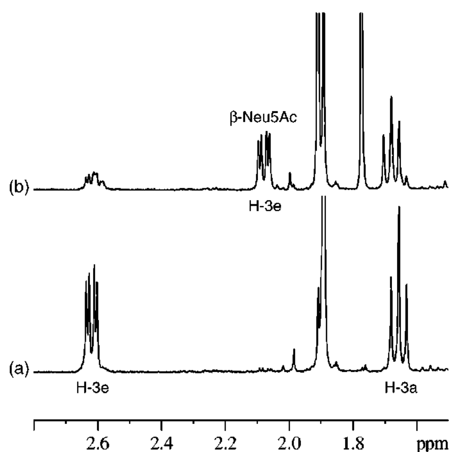
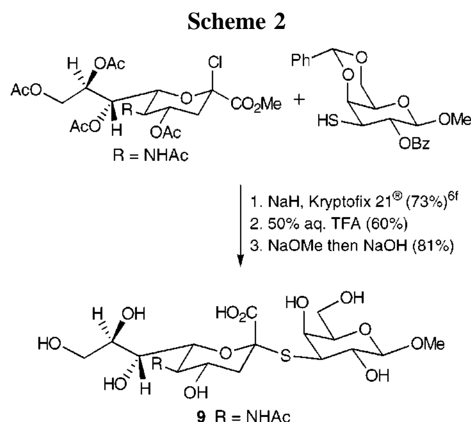


Figure 1. ^{1}H NMR analysis of the hydrolysis of *O*-linked sialosides by *Vibrio cholerae* sialidase: (a) Partial ^{1}H NMR spectrum of Neu5Ac-2-*O*-α(2→3)-GalβMe (**4**) at time = 0 min; and (b) the same ^{1}H NMR region after incubation of compound **4** with *Vibrio cholerae* sialidase for 120 min.

By monitoring the H3 protons of the sialosides in the ^{1}H NMR spectra of these reactions, it was revealed that in both cases the α-anomer of Neu5Ac was the initial product liberated from the reaction, which subsequently mutarotated to the β-anomer of Neu5Ac. These results are entirely consistent with previous studies[18] which have shown that *Vibrio cholerae* sialidase, like all other sialidases studied to date, is a retaining enzyme, i.e., the anomeric configuration of the substrate and initial product are the same. While it is possible to use ^{1}H NMR experiments such as these to obtain kinetic data,[19] unfortunately in this case the H3 resonances of the initial product (α-Neu5Ac) overlap with those of **4** and **7**.

Having established that we could successfully use ^{1}H NMR spectroscopy to monitor the hydrolysis of *O*-linked Neu5Ac glycosides, we turned our investigation to thiosialosides. To complement the hydrolysis of both α(2→6)- and α(2→3)-linked sialyl *O*-glycosides, we opted to employ the known[8] α(2→6)-linked thiosialoside Neu5Ac-2-*S*-α(2→6)-GalβMe (**3**), as well as the α(2→3)-linked thiosialoside Neu5Ac-2-*S*-α(2→3)-GalβMe (**9**),[20] which was prepared as shown in Scheme 2.[6f,21]

Scheme 2



The thiosialosides Neu5Ac-2-*S*-α(2→6)-GalβMe (**3**) and Neu5Ac-2-*S*-α(2→3)-GalβMe (**9**) were incubated in the presence of *Vibrio cholerae* sialidase under identical conditions[17] to those described for the sialosides **4** and **7**.[22] Periodically over 70 days, the ^{1}H NMR spectra of the thiosialosides **3** and **9** in the presence of sialidase were checked. No detectable changes in the ^{1}H NMR spectrum of either the α(2→6)-linked thiosialoside **3** or the α(2→3)-linked thiosialoside **9** (shown in Figure 2) were observed over this time period.

These results clearly demonstrate that both α(2→6)- and α(2→3)-thioglycosides of Neu5Ac are metabolically stable to the action of *Vibrio cholerae* sialidase, even at the high concentrations of enzyme and sialoside required for NMR analysis. The stability of thioglycosides toward the action of glycohydrolases is presumably partly due to the slightly longer C−S bond resulting in a minor alteration of glycoside conformation, as well as the reduced Lewis basicity of

(16) Vliegenthart, J. F. G.; Dorland, L.; van Halbeek, H.; Haverkamp, J. In *Sialic Acids: Chemistry Metabolism and Function*; Schauer, R., Ed.; *Cell Biology Monographs*; Springer-Verlag: Wein, 1982; Vol. 10, pp 127−171.

(17) The hydrolysis of substrates **4** and **7** in the presence of 0.16 U of *Vibrio cholerae* sialidase (1 U is the amount of enzyme required to catalyze the hydrolysis of 1 μmol of substrate per min) was monitored by ^{1}H NMR spectroscopy as a time course reaction on a 600 MHz Bruker DRX spectrometer at 25 °C, in 0.6 mL of D_2O. Spectra of sialosides **4** and **7** were acquired in D_2O prior to the addition of enzyme (representing time = 0 s). After addition of enzyme, spectra of 32 scans were acquired frequently over the first hour of incubation (e.g., every 5−10 min) and then less frequently for several hours longer (approximately every 15 min), with 16K data points over a spectral width of 6024 Hz and a relaxation delay of 2 s.

(18) (a) Wilson, J. C.; Angus, D. I.; von Itzstein, M. *J. Am. Chem. Soc.* **1995**, *117*, 4214−4217. (b) Wilson, J. C.; Kong, D. C. M.; Li, Y.-T.; von Itzstein, M. *Glycoconjugate J.* **1996**, *13*, 927−931.

(19) Chong, A. K. J.; Pegg, M. S.; Taylor, N. R.; von Itzstein, M. *Eur. J. Biochem.* **1992**, *207*, 335−343.

(20) Compound **9**: ^{1}H NMR (300 MHz, D_2O): δ 1.69 (1H, dd, $J_{3a,3e}$ = $J_{3a,4}$ =12.0 Hz, NeuH-3a), 1.87 (3H, s, AcN), 2.64 (1H, dd, $J_{3e,4}$ 4.2 Hz, NeuH-3e), 3.17−3.20 (2H, m, GalH-2/H-3), 3.25−3.28 (4H, m, OMe/NeuH-7), 3.45−3.49 (2H, m, NeuH-6/H-9), 3.53 (1H, ddd, $J_{4,5}$ 10 Hz, NeuH-4), 3.54−3.60 (3H, m, GalH-5/H-6/H-6′), 3.67 (1H, bs, GalH-4), 3.71−3.77 (3H, m, NeuH-5/H-8/H-9′), 4.27 (1H, d, $J_{1,2}$ 6.6 Hz, GalH-1).

(21) Full details of the synthesis of compound **9**, and related compounds, will be published in due course.

(22) For the thiosialosides **3** and **9**, ^{1}H NMR spectra were acquired after 8 h, 14 days, and 70 days of incubation with enzyme.

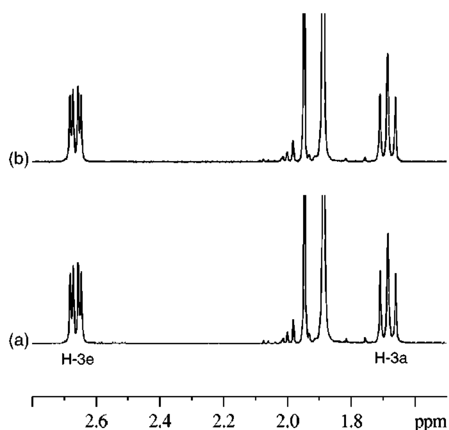


Figure 2. ^{1}H NMR analysis of the stability of thiosialosides toward *Vibrio cholerae* sialidase: (a) partial ^{1}H NMR spectrum of Neu5Ac-2-*S*-α(2→3)-Gal*β*Me (**9**) at time = 0 min; and (b) the same ^{1}H NMR region after incubation of thiosialoside **9** with *Vibrio cholerae* sialidase for 14 days.

sulfur.[3,12,23] In light of this stability, it is not unreasonable to suggest that thiosialosides are also stable during crystal-soaking experiments for X-ray analysis. The apparent inability to observe electron density for the aglycon unit of thiosialosides such as **3** during X-ray analysis of a sialidase soaked with these thiosialosides is therefore difficult to explain, although it is possible that the flexibility of the aglycon unit hinders its detection during X-ray analysis. Alternatively, we have speculated elsewhere[24] that this inability to observe the aglycon unit may have been due to the presence of minor (<5%) amounts of Neu5Ac2en contaminant in the thiosialoside **3**, since Neu5Ac2en has a much greater affinity for the binding site of sialidases than **3**.

It is evident however, from the results presented here, that thiosialosides have the potential to be useful as metabolically stable probes for a range of sialic acid-recognizing proteins including sialidases and *trans*-sialidases. The observations of metabolic stability presented above show that thiosialosides may be able to survive for extended periods *in vivo*, which suggests that their potential use as pharmaceuticals should not be underestimated.

Acknowledgment. We thank the NIH (Grant number PO1 A1 38084) and the ARC for financial support.

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