Pergamon

Tetrahedron Letters, Vol. 38, No. 11, pp. 1865-1868, 1997

0040-4039/97 $17.00 + 0.00

PII: S0040-4039(97)00237-2

Synthesis of ^{13}C Enriched Sialyllactones and Their Characterization Using Isotope Edited Inverse Detected NMR Spectroscopy[1]

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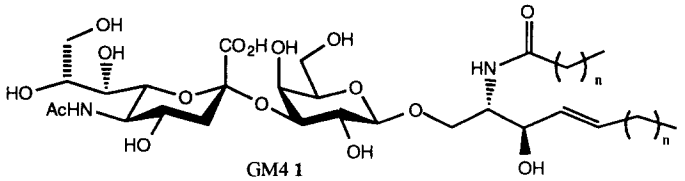
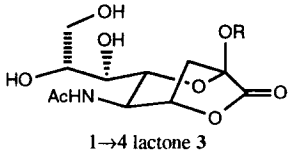
Abstract: *^{13}C enriched monosaccharide based sialyllactones were prepared in order to perform isotope edited NMR experiments for their characterization. In the inverse detected experiments only those protons long-range coupled to the enriched nuclei are detected, providing a potentially powerful tool for studying in vitro sialyllactone formation.*

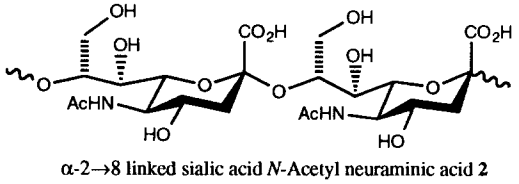
Sialyllactones have been implicated as important components of several biological processes.[2] For example, lactones of GM4 (**1**) may serve as recognition elements in tumor metastasis[3] and lactone formation in α-2→8 linked homooligomers of *N*-acetyl neuraminic acid (NeuAc) (**2**) may help bacteria encapsulated in this polysaccharide evade detection by the immune system.[4] Although there is strong indirect evidence for the involvement of sialyllactones in these events, they have not been directly detected *in vitro*. As part of our program aimed at studying sialyllactone formation in sialoglycoconjugate/protein binding events, we are exploring the possibility of using isotope edited NMR spectroscopy to directly detect the presence of lactones *in vitro* and to distinguish between possible lactone products. We began our studies using simple sialyllactones derived from intramolecular cyclization of the C-4 (**3**) and the C-7 (**4**) hydroxyls of NeuAc derivatives. These model compounds have not been implicated in any known biological processes, however modeling studies suggest that they could be used as scaffolds in the design and synthesis of neuraminidase inhibitors.[5] If the lactones are to be used as scaffolds, it is important to confirm that they maintain structural integrity in the binding site of the enzyme. Ultimately this requires that we develop a method for observing a small molecule in a virtual sea of protons (i.e. a protein) and our approach is to use isotope edited NMR spectroscopy. However, as a first step, it was important to establish that characterization of possible sialyllactone products could be achieved using these spectroscopic methods.



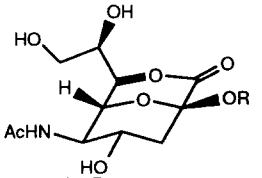
GM4 **1**



1→4 lactone **3**



α-2→8 linked sialic acid *N*-Acetyl neuraminic acid **2**



1→7 lactone **4**

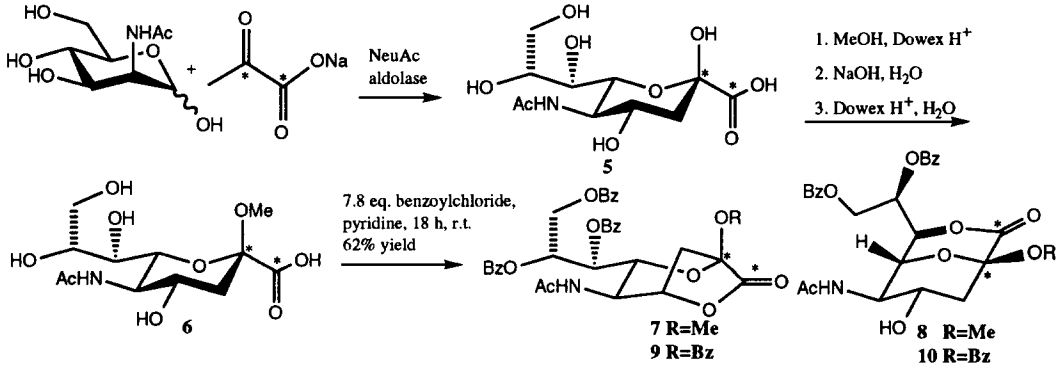
Inverse detected single quantum filtered long range spectroscopy (INSQLR1D),[6] is a specialized NMR experiment that provides isotope edited spectroscopic data. Selective irradiation of a ^{13}C enriched sample results in magnetization transfer to protons that are coupled to the ^{13}C enriched nucleus according to the pulse sequence shown in figure 1. Magnetization transfer is usually optimized for 2 or 3 bond coupling, but longer range data may also be acquired.[6] Only those protons coupled to the ^{13}C enriched nucleus appear in the proton spectrum, effectively editing out the background proton resonances. Herein we report the characterization of sialyllactones using INSQLRID spectroscopy.

$$\begin{array}{lccccccccccc} ^{1}H: & 90^{\circ} & - & 1/4J & - & 180^{\circ} & - & 1/4J & - & 90^{\circ} - t_1/2 & - & 180^{\circ} - t_1/2 - 90^{\circ} - \text{Acq} \\ ^{13}C: & & & & & 180^{\circ} & & & & 90^{\circ} & & 90^{\circ} \end{array}$$

Figure 1: Pulse sequence for INSQLRID experiment. J is chosen to optimize for either 2, 3, or 4 bond coupling. The t_1 delay is a remnant of the 2D experiment and has no effect, it is set to a minimum amount such as 3 msec.

Enriched *N*-acetyl neuraminic acid (**5**) was prepared according to the method reported by Prestegard[7] using a 50:50 mixture of 1-^{13}C and 2-^{13}C enriched sodium pyruvate (Scheme 1).[8] Glycosylation of **5** with methanol in the presence of an acid catalyst provided the methyl glycoside methyl ester in 70% yield after recrystalization.[9] Ester hydrolysis under basic conditions followed by acidification provided the carboxylic acid (**6**). Treatment of **6** with 7.8 equivalents of benzoyl chloride at room temperature for 18 hours gave a 2.4:1 mixture of lactones **7** and **8** in 62% yield. Interestingly, direct benzolyation of **5** results in a 1:3 mixture of lactones **9** and **10** suggesting that the glycosidic linkage may conformationally bias the regiochemical outcome of the lactonization.[10]

Scheme 1



The ^{13}C spectrum of **7** showed two peaks at δ169.0 ppm and δ97.0 ppm corresponding to C1 and C2, respectively, and the ^{13}C spectrum of **8** showed peaks at δ166.0 ppm and δ95.0 ppm. Full proton assignments for **7** and **8** were obtained using 2D techniques.[11] Finally, these compounds were investigated using INSQLR1D in order to characterize the lactones through long range coupling to the labeled carbon atoms.

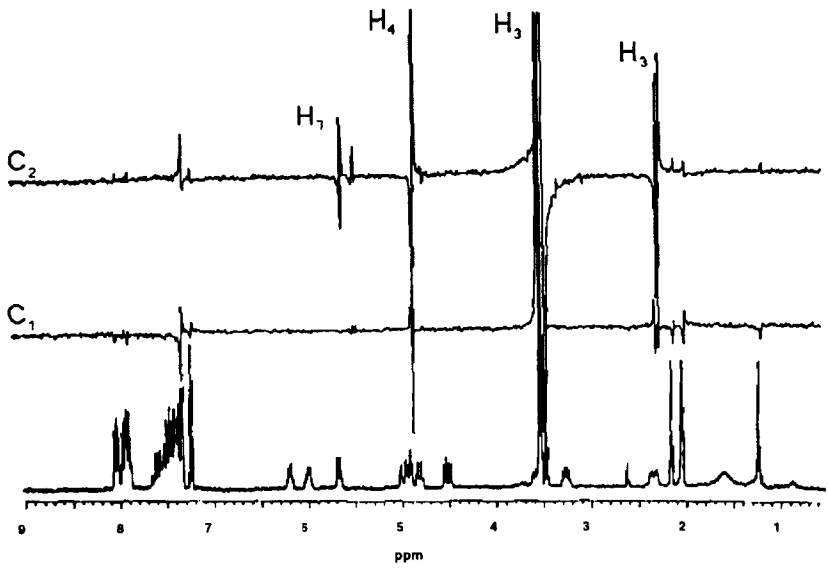


Figure 2: Lower spectrum is ^{1}H NMR of 7, middle spectrum is $^{13}CNMR$ from inverse spectrum after irradiation of C1, upper spectrum is $^{13}CNMR$ from inverse spectrum after C2 irradiation.

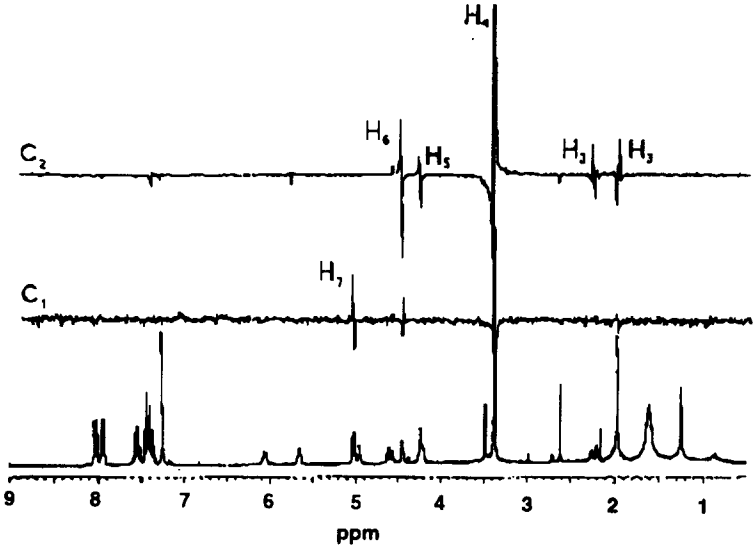


Figure 3: Lower spectrum is ^{1}H NMR of 8, middle spectrum is $^{13}CNMR$ from inverse spectrum after irradiation of C1, upper spectrum is $^{13}CNMR$ from inverse spectrum after C2 irradiation.

In the first inverse experiment of **7**, C1 was irradiated and a 1D proton spectrum was acquired.[12] Only those protons that are coupled through three or four bonds (H3ax, H3eq, H4, and OMe) appear in the proton spectrum. When C2 was irradiated, the inverse spectrum showed resonances corresponding to H3ax, H3eq (2-bond coupled to C2), H4 and OMe, both of which are 3-bond coupled to C2. Although H6 is also within three bonds of C2, no

coupling to this nucleus was observed regardless of the delay value. However 4-bond coupling to H7 was evidenced by the resonance peak at δ5.56 ppm (figure 2). In similar experiments C1 of the 1,7-lactone **8** was irradiated. The proton spectrum obtained from this experiment showed three peaks corresponding to H7, H6, OMe and/or H4.[13] The spectrum acquired after irradiation of C2 contained resonances for H3ax and H3eq (2-bond coupled to C2), H6 and H4 (3-bond coupled to C2), and H5 (4-bond coupled to C2), (figure 3).

Two important features are clearly demonstrated by these experiments. First, the spectra are greatly simplified by filtering out all protons that are not coupled to the ^{13}C enriched nuclei. Secondly, both **7** and **8** have characteristic spectra depending upon which ^{13}C enriched carbon is irradiated. These combined features suggest that this NMR technique may be a powerful tool for detecting sialyllactones *in vitro*. Further investigations are underway in our laboratories including preparation of the unprotected analogs **3** and **4** in order to establish that they are also distinguishable, and protein binding studies to probe the effects of slow molecular tumbling upon spectral resolution.

Acknowledgment. Acknowledgment is made to the Donors of The Petroleum Research Fund, administered by the American Chemical Society, and The State of Arizona Materials Characterization Program for partial support of this research. We are also grateful to the University of Arizona Department of Chemistry and the Vice President for Research for their generous support.

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8. A 50:50 mixture was used to avoid complications from ^{13}C-^{13}C coupling. In scheme 1 there is a 50:50 mixture of molecules having either 1-^{13}C or 2-^{13}C enrichment.
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11. Data for **6**: $^{13}C_1C_{11}H_{21}O_9N$ ^1H NMR (250MHz, D_2O) δ 1.69 (dd, *J* = 13.0, 11.2 Hz, H3), 1.99 (s, Ac), 2.32 (dd, *J* = 13.2, 4.8 Hz, H3), 3.21 (s, Me), 3.52 (d, *J* = 9.5 Hz, H7), 3.60 (dd, *J* = 11.9, 5.71 Hz, H9), 3.79 (dd, *J*= 12.2, 5.7 Hz, H9) 3.82-3.76 (m, H6, H8) 3.88 (d, *J* = 10.21 Hz, H5), 3.97 (dd, *J* = 11.25, 5.0 Hz, H4).
    Data for **7**: $[\alpha]^{25}_D$ -0.06 (c 1.2x10^3, $CHCl_3$). ^1H NMR (500MHz, $CDCl_3$) δ 2.33 (dd, *J* =12.4, 5.5Hz, H3), 3.25 (dd, *J* =10.7, 6.8Hz, H5), 3.50 (dd, *J*=12.4, 5.4Hz, H3), 4.51 (dd, *J* =12.6, 5.8Hz, H9), 4.81 (d, *J* =9.7Hz, H6), 4.91 (dd, *J* =5.4, 5.5Hz, H4), 4.98 (dd, *J* =13.6, 2.5Hz, H9), 5.56 (d, *J* =7.6Hz, H7), 5.99 (ddd, *J* =7.6, 5.8, 2.5Hz, H8), 6.2 (d, *J* =6.8Hz, NH). HRFAB calcd. for $^{13}C_1C_{32}H_{31}O_{11}N$: 618.1931; found 619.2054 ($M+H^+$).
    Data for **8**: $C_{26}H_{27}O_{10}N$ $[\alpha]^{25}_D$ +0.04 (c 1.6x10^3, $CHCl_3$). ^1H NMR (500MHz, $CDCl_3$) δ 2.00 (d, *J* =7.3Hz, H3), 2.15 (d, *J* = 7.0Hz, H3), 3.41 (s, OH), 4.2 (d, *J*= 4.0Hz, H4), 4.19 (dd, *J* =8.1, 4.0Hz, H5), 4.48 (br s, H6), 4.60 (dd, *J* =12.3, 5.6Hz, H9), 4.94 (dd, *J* =12.3, 3.5Hz, H9), 5.02 (d, *J* =5.9Hz, H7), 5.67 (ddd, *J* =5.9, 5.6, 3.5Hz, H8), 6.13 (d, *J* =8.3Hz, NH). HRFAB calcd. for $^{13}C_1C_{25}H_{27}O_{10}N$: 514.1669; found 515.1786 ($M+H^+$).
12. The following are the parameters we used for the NMR experiment described in reference 3: O2=8700; D1=1; P1=8.6 (^1H 90°); D2=0.08, 0.06; P2=17.2 (2xP1) (^1H 180°); P3=16.4 (^{13}C 90°); P4=32.8 (2xP3) (^{13}C 180°); S1=0H; SI=4K; NE=64; TD=4K, t_1=3msec.
13. Because the resonances for H4 and OMe overlap it is not possible to unambiguously distinguish between the 4-bond coupling to either of these nuclei.

(*Received in USA* 29 *July* 1996; *revised* 22 *January* 1997; *accepted* 23 *January* 1997)