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NMR structure elucidation of cyclic sialyl 6-sulfo Lewis x, a biologically dormant form of L-selectin ligand

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Abstract—The structure of cyclic sialyl 6-sulfo Lewis x, a new biologically dormant form of L-selectin ligand, was determined unambiguously by the accurate NMR analysis to have the lactamized ^{5,2}B conformation of its sialic acid. For the NMR structure elucidation were used various informations, such as chemical shifts values, appearance of amide proton, and NOE.

Selectins (E-, P-, and L-)[1] are cell adhesion molecules expressed on endothelial cells, platelets, and leukocytes. Those are implicated in extravasation of leukocytes, homing of lymphocytes, and infiltration or metastasis of malignant cells including leukemia cells and cancer cells. Each selectin has a calcium-dependent carbohydrate binding domain, and binds cell-specific oligosaccharide ligands, such as sialyl Lewis x (sialyl Le^x),[2] sialyl Lewis a,[3] and several other determinants. Recently, it has been reported that sialyl 6-sulfo Lewis x (sialyl 6-sulfo Le^x; **2**), a sialyl Le^x determinant sulfated at β-GlcNAc, serves as the major ligand for L-selectin on high endothelial venules (HEV) in human lymph nodes by generating a specific monoclonal antibody (mAb) G152.[4] However, in leukocytes, expression of the sialyl 6-sulfo Le^x determinant is very unstable upon manipulation of cells. Soon we noticed that **2** in leukocytes is rapidly metabolized through a distinct and previously-unknown pathway, containing modification of sialic acid part.[5,6] That is, the pathway consists of three metabolite members shown in Figure 1; genuine sialyl 6-sulfo Le^x (**2**), de-*N*-acetyl sialyl 6-sulfo Lewis x (de-*N*-Ac sialyl 6-sulfo Le^x; **3**), and cyclic sialyl 6-sulfo Lewis x (cyclic sialyl 6-sulfo Le^x; **1**). De-*N*-Ac sialyl 6-sulfo Le^x (**3**) is active in cell adhesion, but is an unstable metabolite presented in only small amount in cells and tissues. On the other hand, cyclic determinant **1** is a minor by-product obtained incidentally during the chemical synthesis of **2**. This compound **1** was recognized by a (mAb) G159, which was obtained from the same fusion as the G152, and is inactive in cell adhesion. This suggests that **1** is a stable and dormant intracellular pool of selectin ligand.

The chemical structure of cyclic sialyl 6-sulfo Le^x (**1**) has been investigated by the negative ion mode FABMS analysis, and the TLC mobilities of **1** under comparison with related compounds.[6,7] The molecular-related ion $[M–H]^-$ of **1** was clearly detected at m/z 1710, which is 18 mass units lower than that of **3** (m/z 1728). Moreover, as shown in Figure 2, **1** had a much higher TLC mobility than the other compounds, compatible with the formation of an intramolecular ring, such as lactone or lactam structure. The remarkable stability of **1** against alkaline treatment indicated that the presence of a lactam ring rather than a lactone ring in the sialic acid moiety. However, the lactam structure of **1** could not yet be fully confirmed because of the shortage and the complex chemical structure of **1**. In

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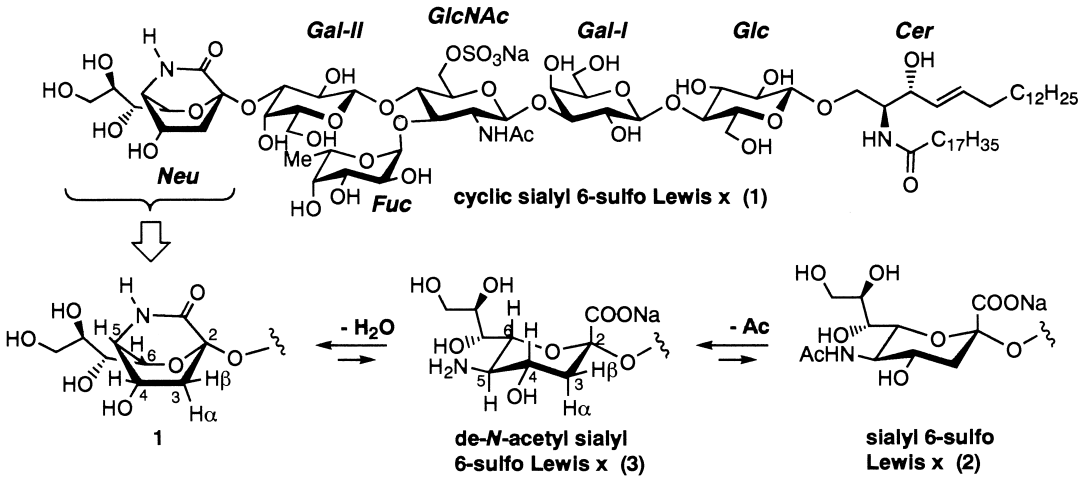

Figure 1. Chemical structure of cyclic sialyl 6-sulfo Lewis x (**1**) and hypothetical metabolic pathway of sialyl 6-sulfo Lewis x.[5,6] Neu = neuraminic acid (sialic acid), Gal = galactopyranose, GlcNAc = 2-acetamido-2-deoxyglucopyranose, Glc = glucopyranose, Fuc = fucopyranose, Cer = ceramide.

order to obtain an NMR-detectable amount of **1**, de-*N*-Ac sialyl 6-sulfo Le^x **3** was treated with water-soluble carbodiimide,1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (WSC) in dimethylsulfoxide (DMSO) at 60°C.[5] The reaction mixture was then purified by the preparative SiO_2 TLC impregnated in a solvent mixture of chloroform:methanol:0.5% calcium chloride (55:40:10), gave ca. 0.5 mg of the G159-reactive compound **1**, as indicated in Figure 2. Here, we describe the unambiguous structure elucidation of **1** by two-dimensional NMR techniques, and also with the aid of the comparison of the ^{1}H NMR chemical shifts with those of GM4 analogues assigned recently.[8,9]

1D and 2D NMR studies of **1** were carried out on an 800 MHz spectrometer. Except for some overlapped signals, most of the ^{1}H NMR resonances for **1** in 90% DMSO-d_6/10% D_2O solution were assigned on the basis of correlation transmitted through scalar coupling interactions in COSY and TOCSY spectra, and from analysis of through-space interactions observed in NOESY spectrum.

Figure 3B described a part of the TOCSY correlations of H-4 to H-9 protons of sialic acid (Neu) residue for **1**. In comparison with those for **3**, the ^{1}H NMR chemical shifts of Neu H-4 to H-7 for **1** were downfield-shifted largely, as shown in Table 1. The fact that the chemical shift value (δ 3.50) of Neu H-5 for **1** is much lower field than that (δ 2.51) for **3**, in which Neu C-5 attached with free amine, suggests the acylation of the C-5 amino group of **1**. Together with the molecular weight information, it is supposed that the carboxyl group of the sialic acid for **1** forms an amide linkage with the C-5 NH_2 within the residue, which composes a new six-membered lactam ring. In contrast with Neu residue, the ^{1}H NMR chemical shift differences of Gal-II residue between **1** and **3** were very small, indicating no appearance of the inter-residual lactone[10] formed between the carboxyl group of sialic acid and hydroxyl groups of galactose.

The formation of the intra-residual six-membered lactam structure involves a significant conformational change of the sugar backbone for sialic acid from $^2C_5$ chair to ^{5,2}B boat conformation. The ^{1}H NMR coupling pattern of H-6 in **1**, as shown in Figure 3B, is a broad singlet with very small coupling constants with both H-5 and H-7, which is consistent with those in a typical ^{5,2}B boat conformation.[8,9,11] In contract with that in **1**, the coupling pattern of Neu H-6 in **3**, which has a chair conformation, is a broad doublet, with a large coupling with Neu H-5 (J = 10 Hz).

The ^{1}H NMR coupling pattern of Neu H-3 protons in **1** must be changed during the conformational conversion of sialic acid with that of H-6. Although the

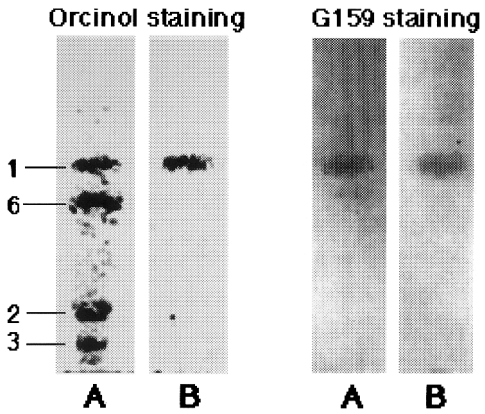

Figure 2. Purification of glycolipid defined by the G159 antibody. Left panel, Orciol/H_2SO_4 staining; right panel, immunostaining preparation of same TLC plate with the G159 antibody. Lane A, crude preparation obtained by the WSC-treatment of de-*N*-acetyl sialyl 6-sulfo Le^x; lane B, pure G159 antibody-reactive glycolipid obtained by preparative TLC from crude WSC-treated de-*N*-acetyl sialyl 6-sulfo Le^x. Spot 1, cyclic sialyl 6-sulfo Le^x; spot 2, sialyl 6-sulfo Le^x; spot 3, de-*N*-acetyl sialyl 6-sulfo Le^x; spot 6, sialyl 6-sulfo Le^x lactone (undefined).

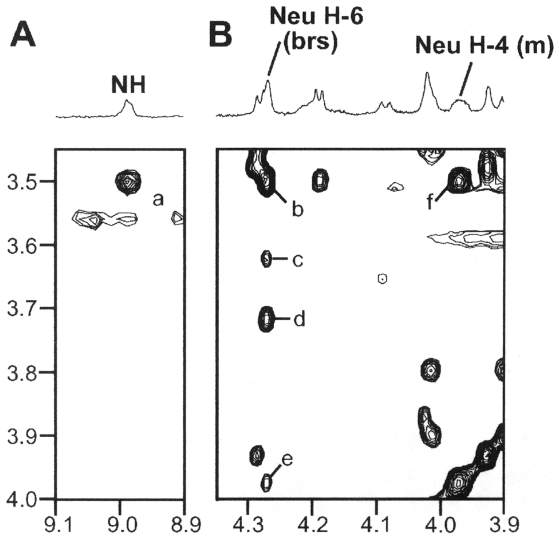

Figure 3. Parts of COSY in 90% DMSO-d_6/H_2O (A) and TOCSY in 90% DMSO-d_6/D_2O (B) of **1**. Cross peaks are assigned to (a) Neu NH/Neu H-5; (b) Neu H-6/Neu H-5; (c) Neu H-6/Neu H-9; (d) Neu H-6/Neu H-7; (e) Neu H-6/Neu H-4; (f) Neu H-4/Neu H-5.

chemical shifts of H-3 protons are assigned stereospecifically by NOE informations [i.e. the NOE between the H-3β and H-4 was stronger than that between the H-3α and H-4 (data not shown)], coupling pattern could not be observed due to the signal overlapping. This coupling information deficiency for **1** could be supplied from the previous NMR study of cyclic sialyl GM4 (**4**, Fig. 4),[9] a lactamized sialic acid derivative. This cyclic sialyl GM4 (**4**) has very similar chemical shift values of Neu H-3 protons with those of **1** and also shows the same NOE informations in the Neu part. In contrast with the ^{5,2}B boat conformation for **1** and **4**, Neu part of **3** has an ordinary $^2C_5$ chair conformation by deducing from a comparison between **3** and de-*N*-acetyl sialyl GM4 (**5**, Fig. 4).

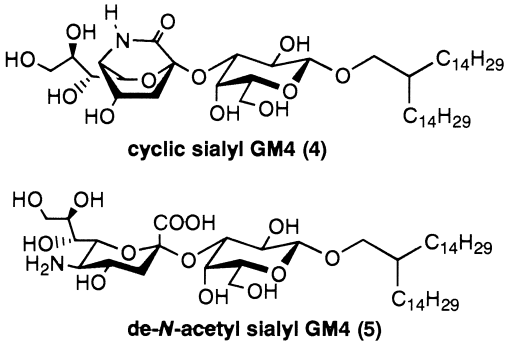

Figure 4. Chemical structures of cyclic sialyl GM4 (**4**) and de-*N*-acetyl sialyl GM4 (**5**).

Table 1 shows the comparison of the chemical shift values of Neu and Gal-II for **1**, **3**, **4**, and **5**. The Gal-II part's chemical shift differences between cyclic derivatives **1** and **4** and between de-*N*-acetyl derivatives **3** and **5** are very small except for H-1 of Gal-II, respectively. This indicates the Gal-II parts of these compounds have the similar structures.

The amide linkage is also supported from the presence of the amide proton in the lactamized sialic acid in **1**. The ^{1}H NMR spectrum of **1** in 90% DMSO-d_6/10% H_2O shows a broad doublet signal at δ 8.98 ppm ($J \leq 6.0$ Hz), which does not exhibit in the spectrum in 90% DMSO-d_6/10% D_2O solution. This exchangeable proton can be assigned to the amide proton. The COSY spectrum of **1** in 90% DMSO-d_6/10% H_2O shows a correlation between this amide proton and the H-5 proton at δ 3.50 ppm, confirming the linkage sites exist within the sugar core (Fig. 3A).

Table 1. ^{1}H chemical shift (δ) values of Neu and Gal-II residues for **1**, **3**, **4**, and **5** at 300 K in 90% DMSO-d_6/D_2O[a]

Residue No	**1**	**3**	**4**[b]	**5**[b]
Neu				
3α	1.91[c]	1.40[c]	1.84 (4.4, 13)	1.28[c]
3β	2.30[c]	2.65 (5.0, 12)	2.26 (13, 14)	2.65 (4.5, 12)
4	3.97 (m)	3.31[c]	3.93 (m)	3.14 (m)
5	3.50[c]	2.51[c]	3.48[c]	2.45[c]
6	4.28 (brs)	3.19[c]	4.19 (brs)	3.31 (brd, 10)
7	3.71[c]	3.32[c]	3.70 (4.0, 7.3)	3.29[c]
8	3.45[c]	3.51[c]	3.43[c]	3.49[c]
9a	3.61[c]	3.66[c]	3.58[c]	3.39[c]
9b	3.44[c]	3.66[c]	3.23[c]	3.60[c]
Gal-II				
1	4.42 (7.4)	4.33 (7.4)	4.08 (7.3)	3.99 (7.7)
2	3.40[c]	3.28[c]	3.35 (7.8, 9.0)	3.19[c]
3	3.87 (brd, 10)	3.86[c]	3.78 (2.5, 10)	3.85 (3.0, 10)
4	4.02[c]	3.47[c]	4.01 (1.5)	3.67 (3.0)
5	3.32[c]	3.65[c]	3.30 (5.9)	3.18[c]
6a	3.46[c]	3.77[c]	3.44[c]	3.41[c]
6b	3.46[c]	3.77[c]	3.50[c]	3.47[c]

[a] Spectra (*J* values in Hz) recorded at 800 MHz for **1** and **3**, and 600 MHz for **4** and **5**.
[b] Cited in Ref. 9.
[c] Signals are overlapped.

We have shown the structural determination of cyclic sialyl 6-sulfo Le^x (**1**) unambiguously by NMR experiments. ^1H NMR chemical shift values and the appearance of an amide proton signal prove the $^{5,2}B$ form lactam structure of **1**. The results described in this paper is compatible with the former investigations by MS and TLC analyses of **1**, and provide the occurrence of cyclic sialyl 6-sulfo Le^x as a new biologically dormant form of selectin ligands. Further investigation on the biological functions and structural information of selectin ligands will clarify the mechanism of the selectin-ligands interaction.

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