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Detailed acceptor specificities of human α1,3-fucosyltransferases, Fuc-TVII and Fuc-TVI

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To clarify the acceptor specificity of Fuc-TVII, its activity toward various analogs of a 2-(trimethylsilyl)ethyl α2,3-sialyl lacto-*N*-neotetraose, an acceptor for both Fuc-TVII and Fuc -TVI, was examined in comparison with that of Fuc-TVI. Fuc-TVII required three portions of α2,3-sialylated type-2 oligosaccharide structures (*i.e.,* the hydroxyl group at C-4 of Gal, the hydroxyl group at C-3 of GlcNAc, and the carbonylamino group at C-2 of GlcNAc) for its acceptor recognition. Fuc-TVI required the carbonylamino group at C-2 of GlcNAc for its acceptor recognition. Fuc-TVI I showed higher affinity toward two analogs, in which the hydroxyl group at C-6 of GlcNAc has been deoxygenated and the acetamide group of *N*-acetylneuraminic acid has been replaced with a glycolylamino group, respectively, than that toward the original compound. On the other hand, Fuc-TVI showed higher affinity toward an analog, in which the acetamide group of GlcNAc has been modified with a lauroylamino group, than that toward the original compound. Analysis involving mass spectrometry confirmed that both Fuc-TVII and Fuc-TVI could fucosylate these three analogs to yield sialyl Lewis x derivatives.

Keywords: **Acceptor specificity, α1,3-Fucosyltransferase, Key functional group, Selectin, Sialyl Lewis x**

Introduction

Fuc-TVII [1, 2], a member of the α1,3-fucosyltransferase (Fuc-T) family, has evoked considerable interest, since it is a key enzyme in the biosynthesis of selectin ligands, sialyl Lewis x (sLe^x; NeuAcα2-3Galβ1-4(Fucα1-3) GlcNAc), and related oligosaccharides [3, 4]. Recent studies have demonstrated the involvement of Fuc-TVII in the biosynthesis of E-, P-, and L- selectin ligands [5, 6]. We previously reported the enzymatic properties of Fuc-TVII, as determined with its soluble form produced by a human Burkitt lymphoma cell line [7]. Five cloned human α1,3-Fuc-Ts (Fuc-TIII, Fuc-TIV, Fuc-TV, Fuc-TVI, and Fuc-TVII) [1, 2, 8-14] have been classified as to their acceptor specificities as well as their tissue distributions and *N*-ethylmaleimide sensitivities [1, 2, 7, 15-20]. Fuc-TIII, Fuc-TV, and Fuc-TVI showed activity toward both α2,3-sialylated and nonsialylated type-2 oligosaccharides [12-14, 19], whereas Fuc-TVII showed activity only toward α2,3-sialylated ones [1, 2]. Fuc-TIV showed little activity toward the α2,3-sialylated oligosaccharides *in vitro* [12]. In addition, the hydroxyl groups on acceptors required for the recognition by the protein (*i.e.,* key polar groups) have been partly determined for Fuc-TIII, Fuc-TIV, Fuc-TV, and Fuc-TVI [21, 22], whereas those for Fuc-TVII have never been determined. In the present study, we determined the acceptor specificity of Fuc-TVII in comparison with that of Fuc-TVI toward a broad panel of 2-(trimethylsilyl)ethyl α2,3-sialyl lacto-*N*-neotetraose **(1)** to extend our previous studies, which revealed the enzymatic basis of Fuc-TVII.

Recently, Fuc-TVII-deficient mice were shown to exhibit a leukocyte adhesion deficiency that is characterized by both the absence of leukocyte E- and P-selectin ligand activity and a deficiency of L- selectin ligand activity in high endothelial venules [6]. Therefore, selective inhibitors for Fuc-TVII are expected to be therapeutics for the treatment of inflammatory diseases. Detailed knowledge about the acceptor specificity of Fuc-TVII may also allow us to design selective inhibitors for it. In addition, these α1,3-Fuc-Ts are expected to be valuable tools for the *in vitro* synthesis of sLe^x and related oligosaccharides [23], which were demonstrated to be capable of blunting tissue injury in several *in vivo* models [24-26]. Elucidation of the detailed acceptor specificities of these Fuc-Ts may also facilitate their efficient use for the synthesis of various derivatives of sLe^x.

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Materials and methods

α1,3-Fuc-T and Fuc-T assays.

Soluble recombinant Fuc-TVII and Fuc-TVI were prepared as described previously [7]. 22 analogs of 2-(trimethylsilyl)ethyl α2,3-sialyl lacto-*N*-neotetraose **(1-22)** were rationally synthesized for this purpose. The synthesis of these compounds will be described elsewhere. Standard Fuc-T assays were performed, in a total volume of 30 μl of 100 mM cacodylate buffer (pH 7.5), 25 mM $MnCl_2$, 0.05 mM GDP-fucose, 0.025 mM pyridylaminated α2,3-sialyl lacto-*N*-neotetraose (NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc), and one of the recombinant enzymes (1.0 μg each). When the effects of the above analogs on Fuc-TVII or Fuc-TVI were examined, the analogs were added to the reaction mixture at 100 μM. After incubation at 37°C for 2 h, the reaction was stopped by boiling for 5 min. After centrifugation, each reaction mixture was subjected to HPLC analysis on an ODS column. The reaction product was eluted with 20 mM NH_4OAc (pH 4.0) at the flow rate of 1.0 ml/min and monitored with a fluorescence spectrometer.

Measurement of K_i values.

The enzyme activity was measured in a reaction mixture containing different concentrations of pyridylaminated α2,3-sialyl lacto-*N*-neotetraose (12.5, 25, and 50 μM), that was mixed with the test compounds at final concentrations of 12.5-100 μM. The K_i value for each compound was estimated from Dixon plots.

MS analysis of reaction mixtures.

15 nmol of each compound was incubated with either Fuc-TVII or Fuc-TVI in a total volume of 30 μl of 100 mM cacodylate buffer (pH 7.5), 25 mM $MnCl_2$, and 0.5 mM GDP-fucose. After incubation at 27°C for 24 h, the reaction mixture was boiled for 5 min, followed by centrifugation. Mass spectra of the supernatant were recorded with a JOEL JMX HX/HX 110A mass spectrometer.

Results

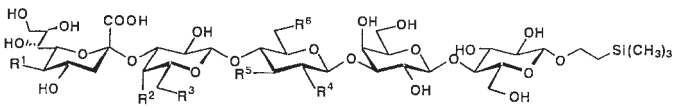
Competitive enzyme assay

In our assay system, the addition of analogs which compete with a labeled-acceptor (pyridylaminated α2,3-sialyl lacto-*N*-neotetraose) leads to a reduction in the generation of pyridylaminated sLe^x hexasaccharide. As expected, 2-(trimethylsilyl)ethyl α2,3-sialyl lacto-*N*-neotetraose **(1)**, another acceptor for both Fuc-TVII and Fuc-TVI, reduced the generation of pyridylaminated sLe^x hexasaccharide in the assay involving either Fuc-TVII or Fuc-TVI (63.0 and 52.7%, respectively, compared to without compound **1**). Therefore, the competition of compound 1 with pyridylaminated α2,3-sialyl lacto-*N*-neotetraose was 37.0 and 47.3% for Fuc-TVII and Fuc-TVI, respectively. Using this assay system, the competition of 21 analogs **(2-22)** [27] was measured and compared to that of compound 1 to determine the detailed acceptor specificities of Fuc-TVII and Fuc-TVI (Table 1).

At first, the competition of deoxygenated analogs **(2-5)** with pyridylaminated α2,3-sialyl lacto-*N*-neotetraose was measured to determine the role of each hydroxyl group for Fuc-TVII or Fuc-TVI. Two analogs deoxygenated at C-4 of Gal **(2)** and C-3 of GlcNAc **(3)**, respectively, did not exhibit any detectable competition in the Fuc-TVII assay, although they exhibited weak but detectable competition in Fuc-TVI assay. On the other hand, an analog deoxygenated at C-6 of Gal **(4)** exhibited weak but detectable competition in both assays. In contrast, an analog deoxygenated at C-6 of GlcNAc **(5)** competed more potently than compound **1** for Fuc-TVII. Second, the role of the acetamide group at C-2 of GlcNAc was examined. Compound **6**, in which the acetamide group at C-2 of GlcNAc has been replaced with a free amino group, did not exhibit detectable competition for Fuc-TVII or Fuc-TVI.

To examine the effects of replacement of functional groups on the competition with pyridylaminated α2,3-sialyl lacto-*N*-neotetraose for Fuc-TVII or Fuc-TVI, sixteen analogs **(7-22)** were also subjected to the assay with Fuc-TVII or Fuc-TVI. Modification of the acetamide group at position 5 of *N*-acetylneuraminic acid with a glycolylamino group **(7)** increased the competition for Fuc-TVII, although it did not cause a drastic effect in that for Fuc-TVI. In contrast, substitution at this position with a hydroxyl group **(8)** was not effective for the competition for either Fuc-TVII or Fuc-TVI. Compound **9**, in which the acetamide group at C-2 of GlcNAc has been replaced with a hydroxyl group, did not exhibit any detectable competition for either enzyme. On the other hand, replacement of this acetamide group with a butylylamino group **(10)** decreased the degree of competition for both enzymes, while compound **11**, in which this acetamide group has been replaced with an octanoylamino group, showed competition as potent as compound **1** for Fuc-TVI. Moreover, replacement of this acetamide group with a lauroylamino group **(12)** increased the competition for Fuc-TVI compared to in the case of compound **1**, while replacement with a palmitoylamino group **(13)** decreased the competition for Fuc-TVI. On the other hand, modification of this acetamide group with a benzoylamino **(14)** or a phathaloylamino **(15)** group decreased the competition for Fuc-TVI, respectively. These analogs **(10-15)** exhibited similar patterns of competition for Fuc-TVII, although they were all less potent than compound **1** for Fuc-TVII. Neither compound **16** nor **17**, which are stereo isomers of compounds **1** and **9** at C-2 of GlcNAc, respectively, competed with pyridylaminated α2,3-sialyl lacto-*N*-neotetraose for either enzyme. Replacement of the hydroxyl group at C-6 of Gal with a free amino- **(18)** or an

Table 1. The structures and relative activities of 2-(trimethylsilyl)ethyl α2,3-sialyl lacto-*N*-neotetraose **(1)** and its analogs **(2-22).**



	Compound	R^1	R^2	R^3	R^4	R^5	R^6	Relative competition (%) Fuc-TVII	Fuc-TVI
1	GSC253	NHAc	OH	OH	NHAc	OH	OH	100	100
2	GSC255	NHAc	OH	OH	NHAc	OH	OH	ND**	30.2
3	GSC298	NHAc	OH	OH	NHAc	H	OH	ND	24.2
4	GSC256	NHAc	OH	H	NHAc	OH	OH	30.8	32.8
5	GSC322	NHAc	OH	OH	NHAc	OH	H	122.2	88.0
6	GSC290	NHAc	OH	OH	NH_2	OH	OH	ND	ND
7	GSC306	$NHC(O)CH_2OH$	OH	OH	NHAc	OH	OH	145.1	88.7
8	GSC304	OH	OH	OH	NHAc	OH	OH	78.9	90.8
9	GSC323	NHAc	OH	OH	OH	OH	OH	ND	ND
10	GSC291	NHAc	OH	OH	$NHC(O)(CH_2)_2CH_3$	OH	OH	28.4	53.1
11	GSC292	NHAc	OH	OH	$NHC(O)(CH_2)_6CH_3$	OH	OH	44.6	104.6
12	GSC302	NHAc	OH	OH	$NHC(O)(CH_2)_{10}CH_3$	OH	OH	73.8	141.8
13	GSC303	NHAc	OH	OH	$NHC(O)(CH_2)_{14}CH_3$	OH	OH	9.2	25.6
14	GSC293	NHAc	OH	OH	NHBz	OH	OH	20.5	40.9
15	GSC294	NHAc	OH	OH	NPhth	OH	OH	19.5	35.3
16	GSC347	NHAc	OH	OH	NHAc***	OH	OH	ND	ND
17	GSC348	NHAc	OH	OH	OH***	OH	OH	ND	ND
18	GSC305	NHAc	OH	NH_2	NHAc	OH	OH	14.1	5.5
19	GSC307	NHAc	OH	NHAc	OH	OH	19.5	14.5	
20	GSC254	NHAc	OH	OH	NHAc	OMe	OH	ND	19.9
21	GSC295	NHAc	OH	OH	NHAc	OH***	OH	ND	29.3
22	GSC351	$NHC(O)CH_2OH$	OH	OH	$NHC(O)(CH_2)_{10}CH_3$	OH	OH	102.4	103.5

*Relative competition was determined with the competition of compound **1** as a standard, in the presence of 25 μM pyridylaminated acceptor.
**ND, Not detected (< 5.0%).
***Epimer.

acetamide **(19)** group caused decreased competition for either enzyme. Neither a methoxyganated analog **(20)** nor a stereo isomer **(21)** at C-3 of GlcNAc did not exhibit detectable competition for Fuc-TVII. In contrast, they showed weak but detectable competition for Fuc-TVI compared to in the case of compound **1**. In addition to the above 20 analogs, we also examined an analog **(22)** in which the acetamide groups of *N*-acetylneuraminic acid and GlcNAc have been replaced with *N*-glycolyl and lauroylamino groups, respectively. Each modification was demonstrated to enhance the competition for Fuc-TVII or Fuc-TVI, respectively. Contrary to our expectation, compound **22** showed less potent competition than compounds **7** and **12** for Fuc-TVII and Fuc-TVI, respectively.

Measurement of K_i values

Compounds **5**,**7**, and **12** exhibited more potent competition with pyridylaminated α2,3-sialyl lacto-*N*-neotetraose than compound **1** for Fuc-TVII or Fuc-TVI. The K_i values of these compounds for Fuc-TVII and Fuc-TVI were determined (Table 2). The K_i values of **5** and **7** for Fuc-TVII were lower than that of compound **1**, indicating that Fuc-TVII had higher affinity toward compounds **5** and **7** than compound **1**. The K_i value of compound **12** for Fuc-TVI was lower than that of compound **1**, also indicating that Fuc-TVI had higher affinity toward compound **12** than compound **1**.

Analysis by mass spectrometry

Fuc-TVII or Fuc-TVI exhibited higher affinity toward one of the above analogs (**1**,**5**,**7**, or **12**) than compound **1**. To determine whether these analogs are acceptors or inhibitors for either enzyme, they were subjected to reactions with each enzyme. The reaction mixtures were analyzed by mass spectrometry to detect the fucosylation of these compounds. After incubation of compound **1** with either

Table 2. The K_i values of 2-(trimethylsilyl)ethyl α2,3-sialyl lacto-*N*-neotetraose **(1)** and its three analogs (**5**, **7**, and **12**) for Fuc-TVII and Fuc-TVI.

	K_i (μM)	
Compound	*Fuc-TVII*	*Fuc-TVI*
1 GSC253	71.9	33.8
5 GSC322	57.1	60.3
7 GSC306	40.2	59.5
12 GSC302	276.3	9.1

Fuc-TVII or Fuc-TVI, an 146 increased mass ion peak indicating fucose transfer to compound **1** was observed at *m/z* 1243 ([M-H], Figure 1 A-D). Fucosylation of compounds **5** and **7** by Fuc-TVII was also confirmed by an 146 increased mass ion peak at *m/z* 1227 and 1259, respectively ([M-H],Figure 1 E-H). After incubation of compound **12** with Fuc-TVI, 146 increased mass ion peak was also observed at *m/z* 1383 ([M-H], Figure 1 I and J). These results demonstrated that compounds **5** and **7** were acceptors for Fuc-TVII, and that compound **12** was one for Fuc-TVI.

Discussion

In this study, we examined the acceptor specificities of Fuc-TVII and Fuc-TVI in detail, using various analogs of 2-(trimethylsilyl)ethyl α2,3-sialyl lacto-*N*-neotetraose **(1-22)**. The competition of these compounds with pyridylaminated α2,3-sialyl lacto-*N*-neotetraose for Fuc-TVII or Fuc-TVI indicates the acceptor recognition for each enzyme. Therefore, compounds that do not show any competition cannot be recognized by these enzymes. Compounds **2** and **3** did not exhibit any detectable competition for Fuc-TVII, and compound **6** did not show competition for either Fuc-TVII or Fuc-TVI. These results indicate that the hydroxyl groups at C-4 of Gal and C-3 of GlcNAc are essential for acceptor recognition for Fuc-TVII, and that the acetamide group at C-2 of GlcNAc is essential for acceptor recognition for both Fuc-TVII and Fuc-TVI. In this point, these functional groups can be defined as "key functional groups" for Fuc-TVII or both Fuc-TVII and Fuc-TVI. Compounds in which the acetamide group at C-2 of GlcNAc has been replaced with several functional groups containing an carbonylamino portion **(10-15)** showed competition for both Fuc-TVII and Fuc-TVI. Therefore, the carbonylamino portion at C-2 of GlcNAc is essential for acceptor recognition and can be designated as a "key functional group" for both enzymes. These results indicate that Fuc-TVII and Fuc-TVI exhibit distinct specificities toward α2,3-sialylated type-2 oligosaccharide acceptors. On the other hand, an analog deoxyganated at C-6 of Gal **(4)** showed competition for Fuc-TVI, while a recent report by de Vries et al. demonstrated that Fuc-TVI had an absolute requirement for this hydroxyl group as to nonsialylated type-2 oligosaccharide acceptors [22]. Fuc-TVI may recognize α2,3-sialylated and nonsialylated type-2 oligosaccharide acceptors in different ways. Interestingly, an analog deoxygenated at C-6 of GlcNAc **(5)** was more potent than compound **1** in the competition for Fuc-TVII. This hydroxyl group may limit the acceptor binding to Fuc-TVII.

To date, several inhibitors for α1,3-Fuc-Ts, most of which were synthesized as unreactive analogs of GDP-fucose, have been prepared, but selective ones have never been reported. The presently described acceptor specificity of Fuc-TVII, in comparison with that of Fuc-TVI, will be useful for designing selective and effective inhibitors for Fuc-TVII. In particular, our results for compounds **5**,**7** and **12** suggest that the design of selective inhibitors for Fuc-TVII or Fuc-TVI is possible. The K_i values indicated that compounds **5** and **7** were recognized more favorably than compound **1** by Fuc -TVII, and that compound **12** was recognized more favorably than compound **1** by Fuc-TVI. Therefore, these analogs could be good candidates for designing selective inhibitors for Fuc-TVII or Fuc-TVI. Moreover, these compounds have potential as selective inhibitors for Fuc-TVII, which compete with endogenous acceptors in cells. On the other hand, the hydrophilic nature of sugars prevents these acceptor-based inhibitors from permeating the cell membrane. To solve this problem, the acetylation of hydroxyl groups will be effective, as described in the report by Sarkar et al., indicating that the acetylated disaccharide, Galβ1-4GlcNAcβ-*O*-naphthalenemethanol, reduced sLe^x expression on HL-60 cells through competitive inhibition of glycosyltransferases involved in its biosynthesis [27].

The results obtained in this study summarized in Figure 2 may be due to direct interaction of the substituted groups of acceptors with each enzyme, or the conformational changes caused by the modifications. Further investigation may provide more selective and effective inhibitors for Fuc-TVII.

The acceptor analogs used in this study are also valuable as precursors of sLe^x derivatives, which will facilitate the development of novel selectin blockers. At present, we are attempting to obtain sLe^x derivatives on a preparative scale from these acceptor analogs.

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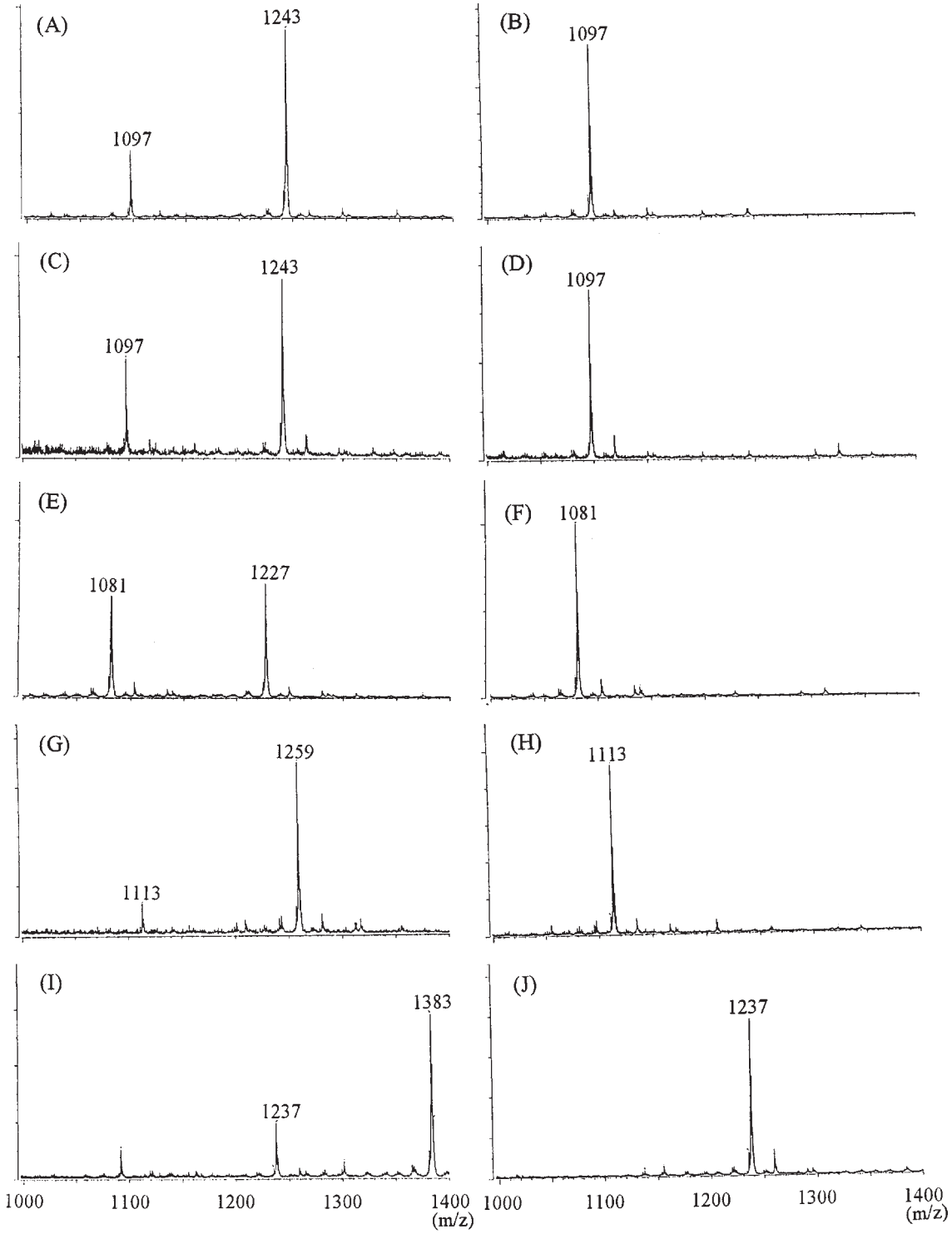

Figure 1. Mass spectra of reaction mixtures of 2-(trimethylsilyl)ethyl α2,3-sialyl lacto-*N*-neotetraose **(1)** and its three analogs (**5**, **7**, and **12**) after incubation with or without either Fuc-TVI or Fuc-TVII.

A and *B;* **1** with or without Fuc-TVII. *C* and *D;* **1** with or without Fuc-TVI. *E* and *F;* **5** with or without Fuc-TVII. *G* and *H;* **7** with or without Fuc-TVII. *I* and *J;* **12** with or without Fuc-TVI.

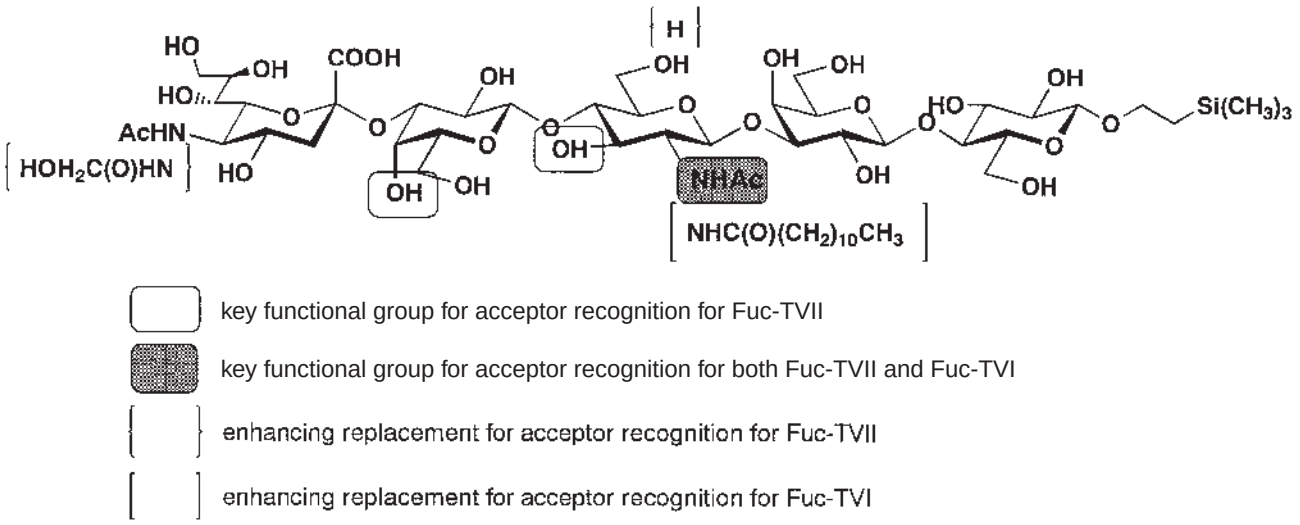

Figure 2. Summary of the acceptor specificities of Fuc-TVII and Fuc-TVI, and the effects of the replacement of functional groups in acceptors on the recognition by Fuc-TVII or Fuc-TVI.

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