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Synthesis and Vaccine Evaluation of the Tumor Associated Carbohydrate Antigen RM2 from Prostate Cancer

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Hong-Yang Chuang

Synthesis and Vaccine Evaluation of the Tumor Associated Carbohydrate Antigen RM2 from Prostate Cancer

Doctoral Thesis accepted by
National Taiwan University, Taipei, Taiwan

Springer

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Supervisor's Foreword

This thesis is focused on the synthesis and evaluation of the RM2 antigen as a prostate cancer vaccine. This study is the first to successfully synthesize this complex glycan molecule; the introduction of two sialic acid units to the compact and rigid 3,4 dibranched galactoside unit is very challenging and the development of a method for the β-selective and efficient glycosylation of the galactosamine moiety at the 4-position of dibranched galactose is problematic owing to the steric hindrance of the adjacent sialyl moiety. Here, the author has successfully synthesized the carbohydrate RM2 antigen and analogues, using the [1+2+3] one-pot sequential strategy as single stereoisomers in every glycosylation step in good yields and stereoselectivity. He then conjugated the synthetic RM2 antigen to the carrier protein CRM197 in an average number of 1–10 epitopes per carrier to create the prostate cancer vaccine candidate, which was combined with α-galactosylceramide C1, its analogue C34, or Alu as adjuvant. After vaccination studies in mice, glycan arrays were used to monitor the titers of the induced antibody and specificity. The results indicated that, when one molecule of DT was conjugated with 4.7 molecules of RM2 antigen in average (DT-RM4.7) and adjuvanted with the glycolipid C34, the strongest anti-RM2 antibody titer was exhibited. More importantly, the induced mouse antibodies mediated effective complement-dependent cytotoxicity (CDC) against the prostate cancer cell line LNCap.

Taipei, Taiwan
February 2015

Chi-Huey Wong

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February 2015

Hong-Yang Chuang

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Chapter 1
Chemical Synthesis of Proposed RM2 and Derivatives

1.1 Introduction

1.1.1 Glycosphingolipids

About several hundred different glycosphingolipid structures have been identified, and the nature of both the hydrophobic chains in the ceramide moiety and, in particular, the carbohydrate content varies considerably. An oligosaccharide chain linked to the primary hydroxyl group of ceramide or amino alcohol of sphingoid long-chain aliphatic constitutes the polar headgroup of GSLs. Depending on the carbohydrate species, GSLs can be classified as neutral GSLs containing only uncharged sugar molecules and as acidic GSLs, which include sulfatides and gangliosides, containing a negative charged sulfate group or at least one sialic acid.

Glycosphingolipids (GSLs) were initiated through isolation and structural characterization of lacto-series-type and neo-lacto-series-type GSLs, globo-series, iso-globo-series GSLs, muco-series GSLs, and ganglio-series GSLs. All of them have the common precursor, β-lactosylceramides (LacCer, Galβ4Glcβ1Cer), which can be further modified to produce a wide variety of glycosylated structures (Fig. 1.1). Besides, GSLs are named using a series of abbreviations. The letter following "G" indicates the number of sialic acid (M for one, D for two, T for three, Q for four, and P for five) and the index number represents the result of [5 minus (the number of neutral monosaccharide units)] (Fig. 1.2) [1].

GSLs are involved in cell adhesion, cell growth regulation, modulating functional membrane proteins, regulating transmembrane signaling, and mediating cellular interactions [2]. Altered glycosylations of glycoshingolipids are observed in various types of cancer [3]. Some studies have reported that structures and functions of GSLs are altered greatly in various types of cancer, providing a basis for invasive

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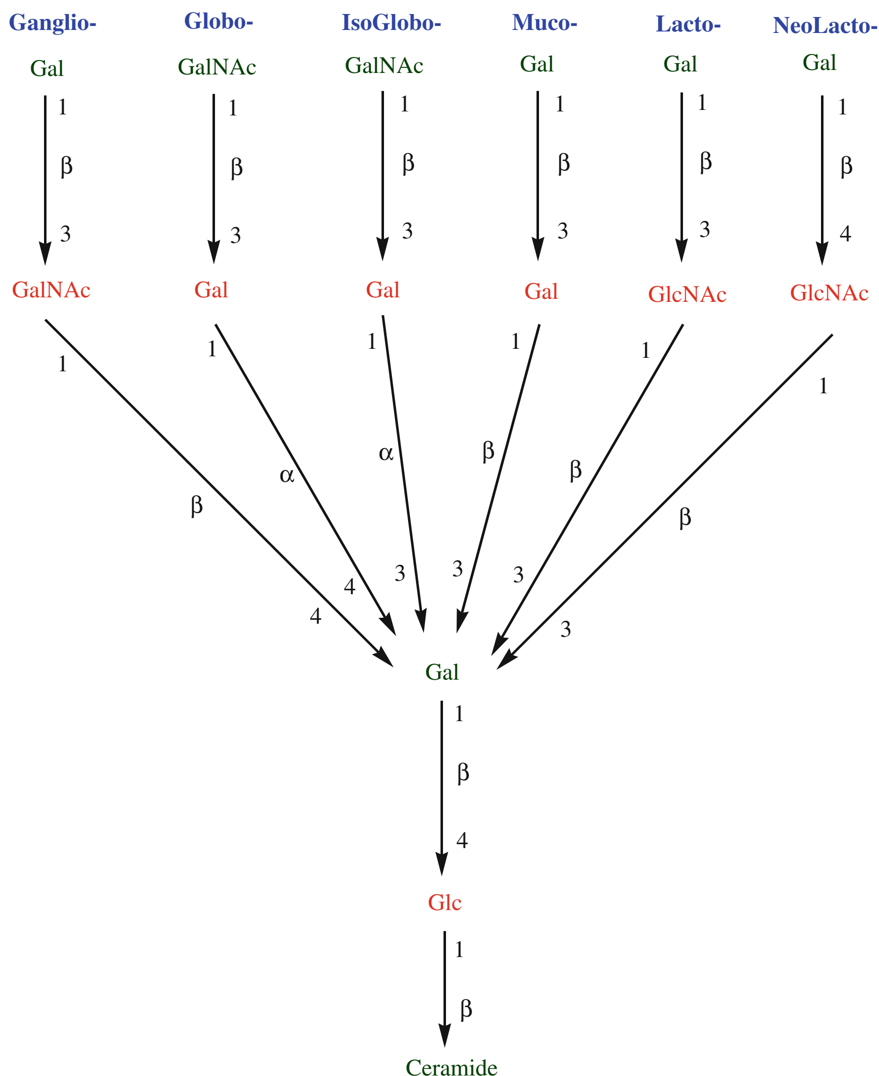


Fig. 1.1 Structure of the different families of GSLs, compared to *β*-lactosyl moiety in the ceramide

and metastatic properties of tumor cells [3]. Expression of tumor-associated GSLs or glycoepitopes in primary tumor is strongly correlated with the outcome of tumor progression, metastasis, and invasion [4].

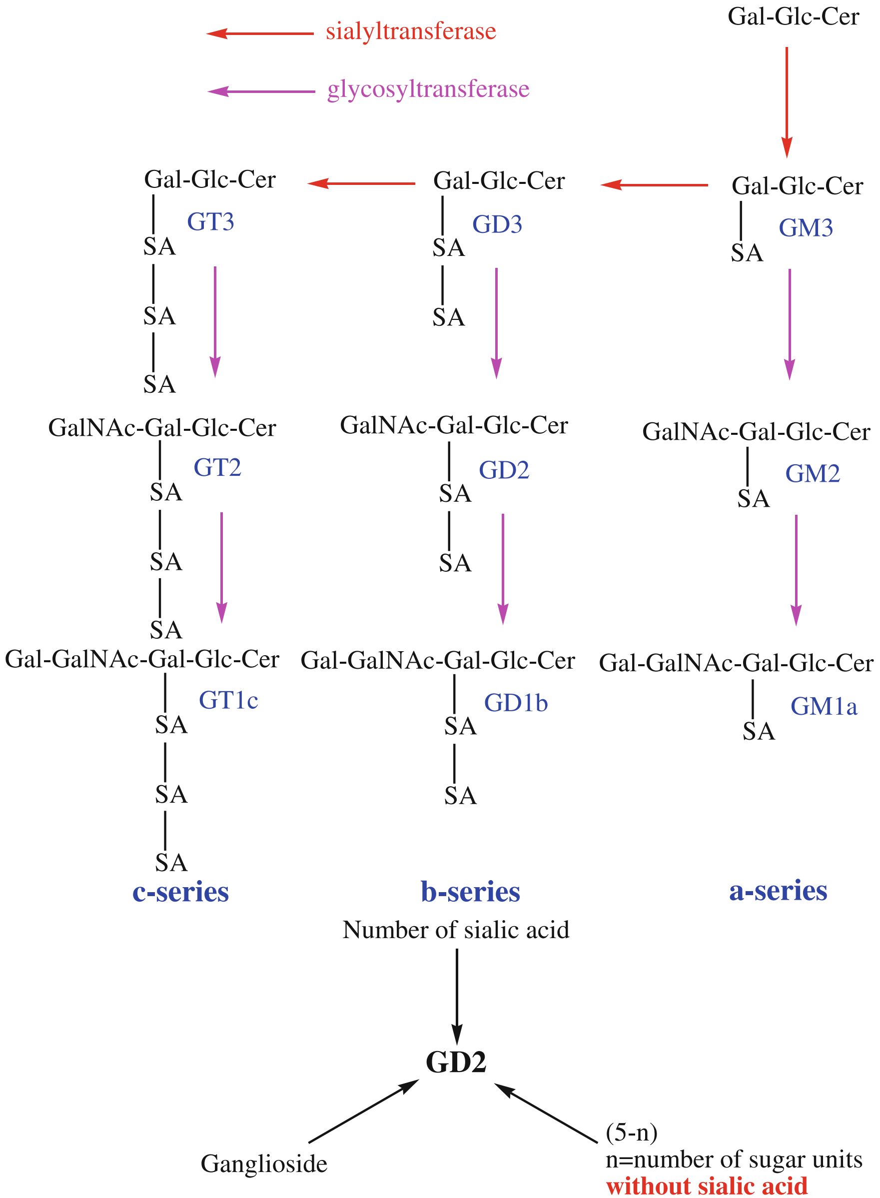


Fig. 1.2 Glycospingolipids are named using a series of abbreviations and major structures of gangliosides

1.1.2 Tumor-Associated Carbohydrate Antigens

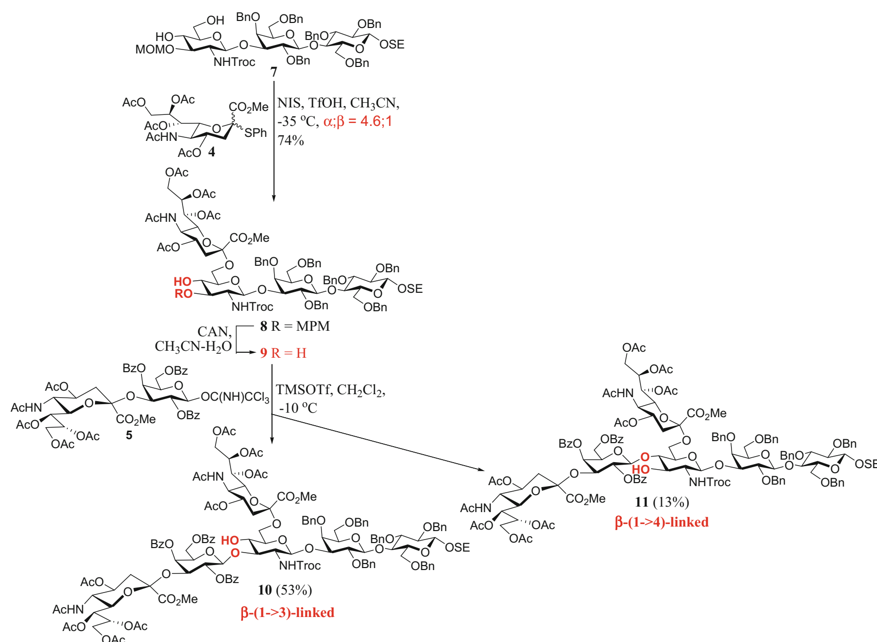
It is well established that carbohydrate antigens are the most abundantly expressed antigens on the surface of most cancer cells. The aberrant glycosylations include loss or overexpression of certain glycan structures, the persistence of truncated structures, and the emergence of novel structures. The association of aberrant glycosylation with tumor progression was first described by Meezan et al. in 1969 with the demonstration that cancer glycans differ from healthy cells [5]. This finding was later supported by much histological evidence that lectins show differential binding to healthy tissue [6].

More recently, a group of tumor-associated antigens was identified and characterized. They are called tumor-associated carbohydrate antigens (TACAs) because they are carbohydrates that react with lectins or monoclonal antibodies [7] (Fig. 1.3 and Table 1.1) [8]. TACAs are not only overexpressed on the surface of cancer cells but are also absent or rarely expressed on normal cells [9]. Besides, they are related to tumor cell adhesion and metastasis so tumor-associated carbohydrate antigens provide a specific target for the development of tumor-specific carbohydrate vaccine to stimulate antibody production.

1.1.3 RM2 Antigen (β1,4-GalNAc-Disialyl-Lc4) as a New Marker for Prostate Cancer

In 2001, Hakomari and colleagues focused on a novel structure, RM2, a glycosphingolipid (GSL) isolated and identified from a renal cell carcinoma cell line TOS-1 and prostate cancer cell lines LNCap and PC-3 [11]. It was immunocharacterized by monoclonal antibody RM2 and, therefore, termed “RM2 antigen”. The RM2 antigen, β-1.4-GalNAc-disialyl-Lc4, is highly expressed on the prostate cancer cell surface as a glycolipid and possibly a glycoprotein (Fig. 1.5) [12]. In addition, the RM2 antigen expression level has been found to be closely associated with the prostate cancer staging Gleason grading system (Table 1.2). The RM2 antigen expression is upregulated as the Gleason grade increases [13]; therefore, RM2 antigen can be developed into a new and novel marker for prostate cancer. The proposed structure of the RM2 antigen consists of a novel hybrid core with the “ganglio-series” and the “disialyl lacto-series type 1 chain” groups (Fig. 1.4).

Recently, Kiso and co-workers reported a systematic synthesis of DSLc4 via α-stereoselective sialylation of O-6 and introduction of the sialyl-α-(2 → 3)-D-galactose unit to the O-3 position of GlcNAc [15]. The low selectivity of the reported DSLc4 synthesis [15] is resulted from the low α-selectivity of sialylation (synthesis of compound **8**) and low regioselectivity for the synthesis of compound **10** as shown in the scheme below:



1.2 Chemical Synthesis of Hexasaccharide RM2 and Its Derivatives

1.2.1 Design of Sugar Building Blocks

The synthesis of both α(2,3) and α(2,6) sialic acid units and the compact and rigid 3,4 dibranched galactoside unit is challenging because (1) the presence of the C1 carboxyl group of sialic acids reduces the reactivity of the anomeric position; and (2) the absence of C-3 participating group fails to direct the stereoselectivity and, at the same time, readily promotes the undesired 2,3-elimination product. Many approaches have been developed to address these problems: (1) the conversion of different leaving groups on the anomeric center [1, 16, 17]; (2) the installation of TFA [18] and Troc [15] at *N*-5 position; (3) the 1,5-intramolecular lactamization [19]; (4) the application of nitrile solvent in glycosylation [20]; and (5) installation of a 5-*N*,4,*O*-carbonly group (oxazolidinone ring) onto the sialic acid [21]. Recently, our group incorporated dibutyl phosphate as a leaving group into *N*-acetyl-5-*N*,4-*O*-carbonyl-protected sialoside as a new sialyl donor to increase α-selectivity and reaction yield [22]. Besides the difficulty of sialylation, β-selective and efficient glycosylation of galactosamine moiety into the 4-position of

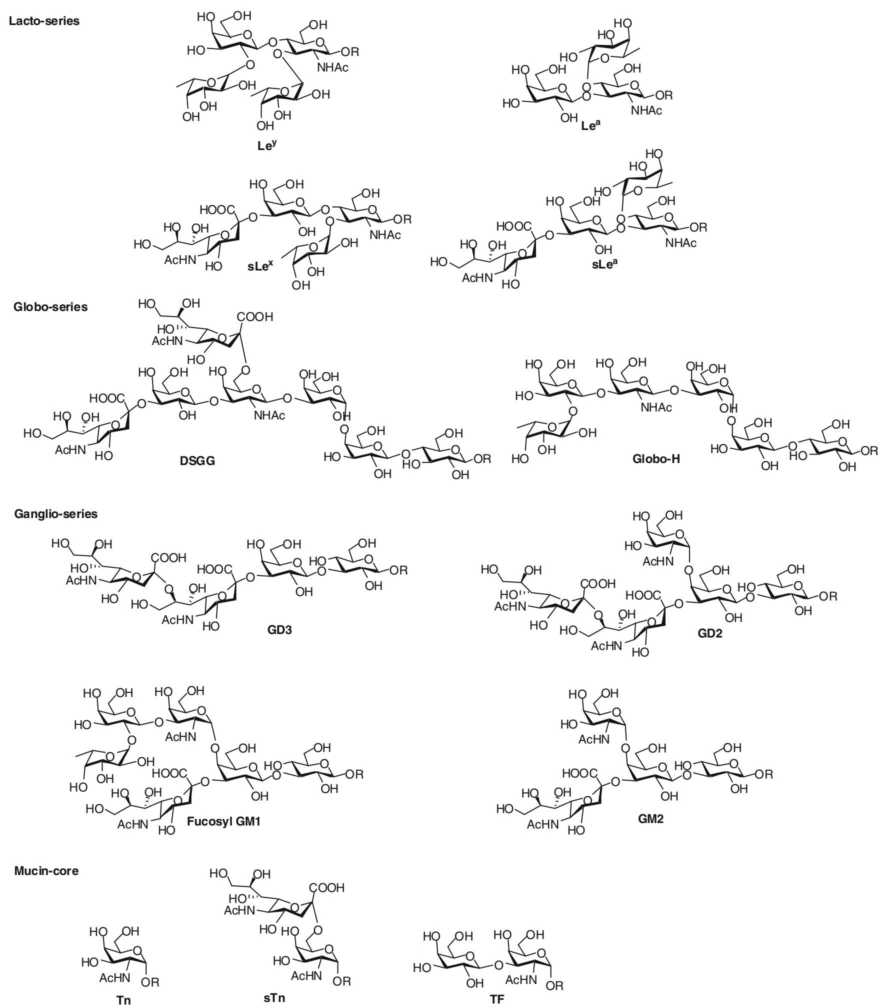


Fig. 1.3 Cancer-associated carbohydrate antigens. A small subset of tumor-associated antigens and can be simply divided into related lacto-series, globo-series, ganglio-series, and O-linked mucin core. Adapted from [10], copyright 2015, with permission from Macmillan Publishers Ltd

dibranched galactose is problematic owing to steric hinderance of the adjacent sialyl moiety.

To overcome these problems, the strategy is to divide the target hexasaccharide **1-1** into three parts: monosaccharide **1-B**, disaccharide **1-C**, and sialyated trisaccharide **1-D** (Fig. 1.5). Considering the difficulty of fashioning a branch from galactose residue, we decided to perform this step at the beginning. Because the reactivities of these three building blocks decrease progressively, the building blocks can react sequentially in a single flask to construct the entire hexasaccharide in a single one-pot reaction performed using a [1 + 2 + 3] approach (Fig. 1.7). Note that

Table 1.1 Common expression patterns of cancer glycans on malignant tissues

Cancer glycan	Malignant tissue								
	Ovary	Pancreas	Blood	Breast	Colon	Brain	Prostate	Shin	Lung
sLe^x	–	X	–	X	X	–	–	–	X
sLe^a	–	X	–	X	X	–	–	–	X
sTn	X	X	–	X	X	–	X	–	X
TF	X	–	–	X	X	–	X	–	–
Le^y	X	X	–	X	X	–	X	–	X
Globo H	X	X	–	X	X	–	X	–	X
PSA	–	X	X	X	–	X	–	–	X
GD2	–	–	X	–	–	X	–	X	–
GD3	–	–	–	–	–	X	–	X	–
Fucosyl GM1	–	–	–	–	–	–	–	–	X
GM2	X	X	X	X	X	X	X	X	X

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Table 1.2 Relationship between RM2 immunoreactivity and Gleason score of radical prostatectomy specimen

Gleason score	RM2 immunoreactivity	
	Lower expression	Higher expression
3 + 3	4	0
3 + 4	13	7
4 + 3	3	19
4 + 4	0	7
4 + 5	0	17
5 + 4	0	3
5 + 5	0	2
	20	55

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Lower expression, negative to weak immunoreactivity
Higher expression, moderate to strong immunoreactivity

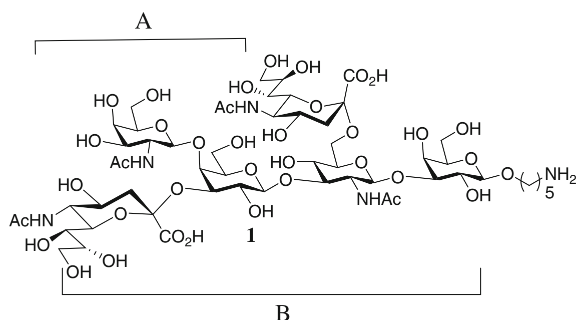


Fig. 1.4 The structure of an RM2 carbohydrate hapten *1*. *A* ganglio-series structure. *B* disialyl lacto-series type 1 structure. Adapted from [14], copyright 2015, with permission from American Chemical Society

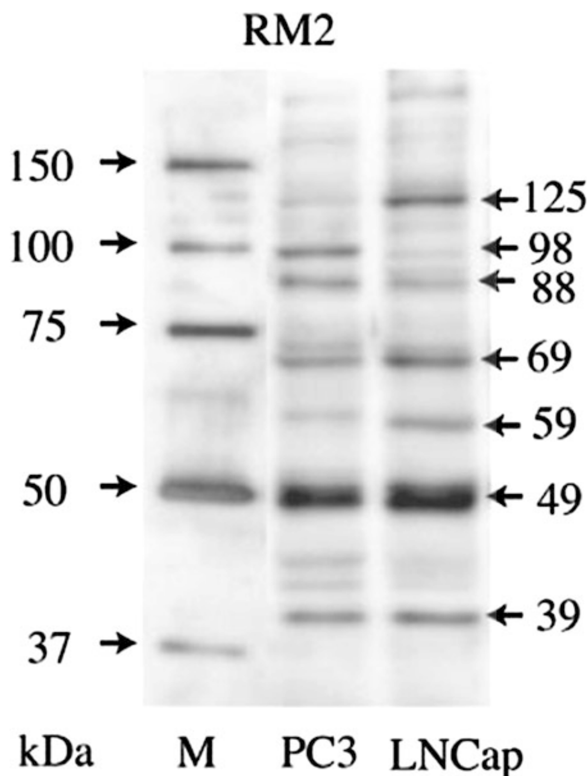


Fig. 1.5 Western blot analysis of prostate cancer cell lines by RM2. A total of 10 mg of cancer cells was applied in each lane. *Right panel* immunostaining by RM2. *M* size marker. RM2 detected a 49-kDa glycoprotein as the major band in LNCap and PC3, in addition to several minor bands found in one or both cell lines. Adapted from [13], copyright 2015, with permission from John Wiley & Sons Ltd

this is the first application of using sialic acid $\alpha(2 \rightarrow 3)$ galactose as a building block to perform one-pot synthesis. Moreover, in order to perform the one-pot synthesis, we prepared the hexasaccharide RM2 using the step-by-step protocol for oligosaccharide synthesis, and orthogonal protection groups were required to provide potential acceptor sites for later glycosylation. Besides, the hydroxylpentamine linker was initially installed into the reducing end of the galactose, for the purpose of immobilizing hexasaccharide **1-1** onto NHS-coated slides or conjugating with carrier protein to form vaccine candidate through amide formation. Truncated RM2 derivatives can also be attached to the glycan microarray by the same strategy (Fig. 1.6).

1.2.2 Syntheses of Sialyated Trisaccharide Building Block 1-D

The sialyated trisaccharide **1-D** was further designed into building block **1-2**, which was retrosynthetically disconnected to obtain three functional building block units: sialoside **1-3**, glucosamine **1-4**, and galactose **1-5** (Fig. 1.8).

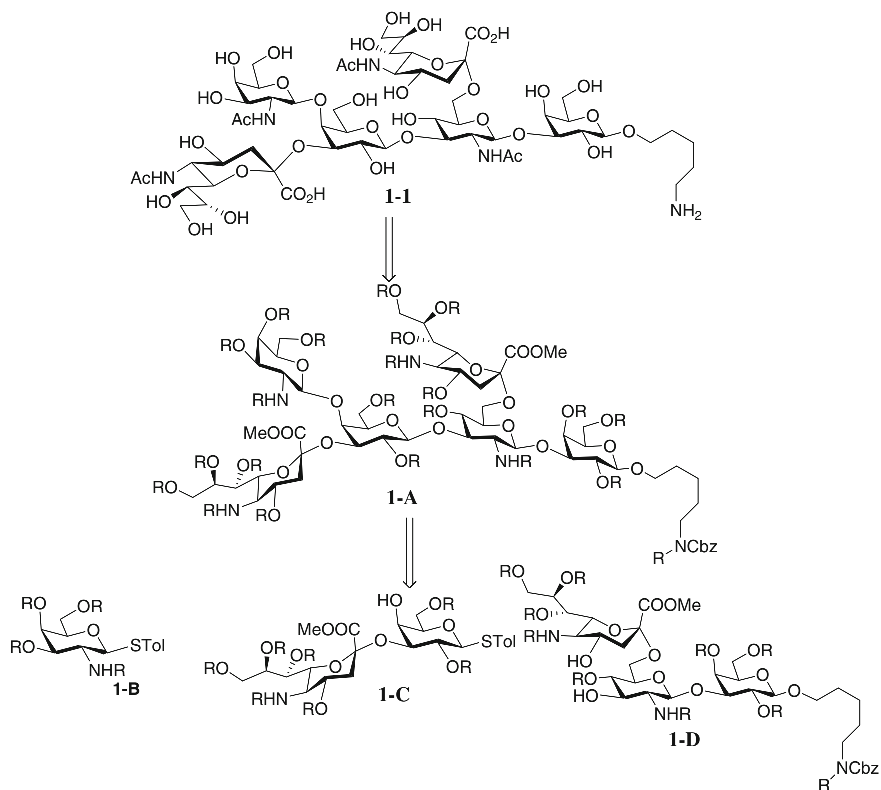


Fig. 1.6 Retrosynthetic analysis of the RM2 hapten

The known compound **1-6** [23] was converted into compound **1-4** by selective ring-opening of the 4,6-*O*-benzylidine to the 4-*O*-benzyl ether with the unsubstituted 6-hydroxy. Without purification, the glucosamine was acetylated by the standard acetylation procedure to obtain the desired building block **1-4** in 50 % yield by flash column chromatography [24] (Fig. 1.9).

1.2.3 Synthesis of Galactose Building Block 1-5

The known compound **1-7** [25] was acetylated by the standard acetylation procedure (Fig. 1.10), and the acetylated compound was glycosylated with the benzyl-5-hydroxypentylcarbamate in dichloromethane using NIS/TfOH as a promoter to afford **1-8** in 60 % yield. Compound **1-8** was de-acetylated using the Zemplen condition; then benzylation of the triol intermediate afforded **1-9** in 85 % yield over two steps. The acetyl group was replaced by the benzyl group to increase the

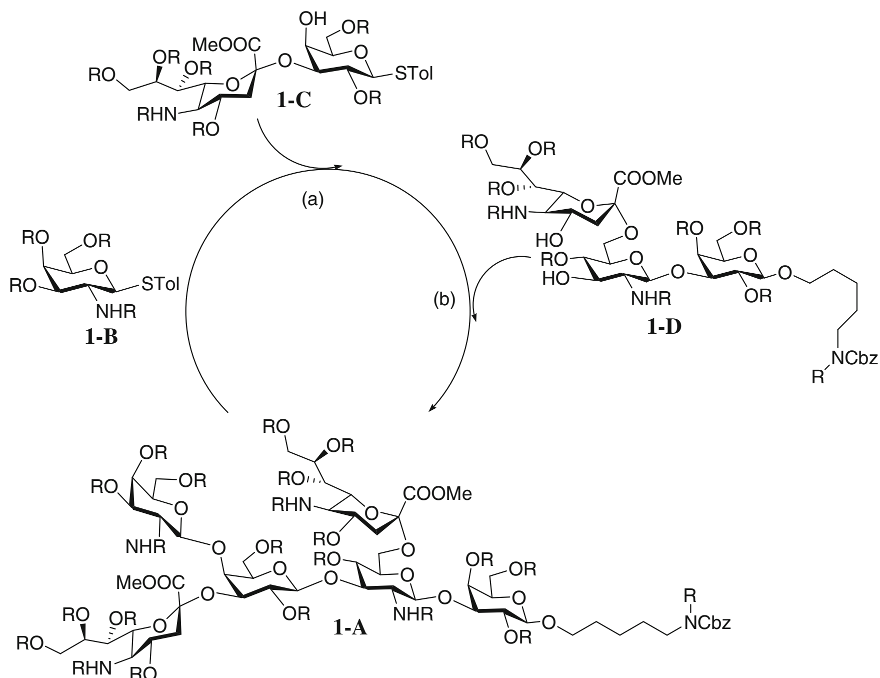


Fig. 1.7 The one-pot programmable protocol for hexasaccharide synthesis

reactivity of the galactose building block, followed by selective removal of the PMB protecting group with DDQ to give **1-5** in 77 % yield over two steps

1.2.4 Synthesis of Sialylated Trisaccharide Building Block 1-2

To synthesize the sialylated trisaccharide building block **1-2**, glycosylation of donor **1-4** and acceptor **1-5** in dichloromethane using NIS/TfOH as a promoter afforded protected disaccharide. Disaccharide was de-acetylated using the Zemplen condition to obtain desired compound **1-10** in nearly 40–50 % at 0 °C. Notably, acetyl group of the disaccharide was incompletely hydrolyzed, and the Troc group was highly susceptible to the basic condition resulting in the formation of side products **1-10a** and **1-10b**. It is difficult to separate the desired compound **1-10** from the side products **1-10a** and **1-10b** because they were compatible in the TLC plates. Thus, we decided to remove all acetyl and Troc protecting groups at the same time by using sodium hydroxide, then protected free amino group by TrocCl to afford **1-10**. We further tested whether glycosylation of 3,6-diol disaccharide **1-10** with sialylated donor **1-3** [26] could obtain regioselective α(2,6) sialyated trisaccharide

Fig. 1.8 Retrosynthesis analysis of sialyated trisaccharide **1-D**

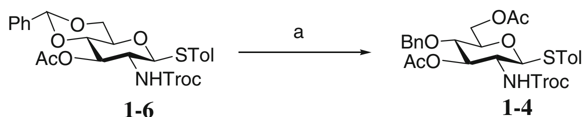


Fig. 1.9 Synthesis of GluNAc building block **1-4**. *a* (i) BH_3-THF, Bu_2BOTf, 0 °C, 6 h; (ii) Ac_2O, pyridine, CH_2Cl_2, rt, 50 % for two steps

building block **1-2** in good yield. The result showed that such coupling gave relatively large amount of side products α(2,3) sialylated trisaccharide **1-2a** (27 %) and sialylated tetrasaccharide **1-2b** (10 %) and only yielded the desired α(2,6) sialylated trisaccharide building block **1-2** in 43 % ($\alpha : \beta = 3 : 1$) (Fig. 1.11). Hence, we designed a building block **1-12** to modify this synthetic scheme to improve the

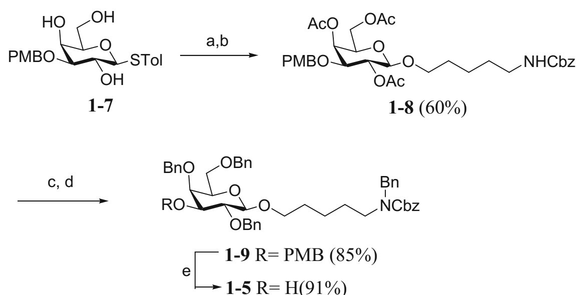


Fig. 1.10 Synthesis of Galactose building block **1-5**. *a* Ac_2O, pyridine, rt, 12 h; *b* NIS, cat. TfOH, benzyl 5-hydroxypentylcarbamate, CH_2Cl_2, −30 °C, 3 h, 60 % (over two steps); *c* NaOMe, MeOH, rt, 10 h; *d* BnBr, NaH, rt, 2 h, 85 % (over two steps); *e* DDQ, CH_2Cl_2/H_2O, rt, 2 h, 91 %

yield and enlargement of the α(2,6) sialylated trisaccharide building block **1-2** to the gram scale.

1.2.5 Synthesis of GlcNAc Building Block 1-12

The synthesis of monosaccharide **1-12** can be achieved from the known 4,6-*O*-benzylidene acetal **1-11** [27] (Fig. 1.12). The *p*-methoxybenzylation of **1-11** was carried out by *p*-methoxylbenzyl chloride (PMBCl) and sodium hydride (NaH) in DMF, followed by selective ring-opening of the 4,6-*O*-benzylidene group with dibutylboranetriflate (Bu_2BOTf) and borane-tetrahydrofuran complex (BH_3.THF). After sequential removal of the phthalimido group and Troc formation, the desired product **1-12** was produced in four steps in 24 % yield.

1.2.6 Synthesis of Trisaccharide 1-2

Compound **1-13** can be obtained after acetylation by pyridine and acetic anhydride overnight. The preparation of the target trisaccharide building block **1-2** started from the NIS/TfOH-promoted coupling of the GlcNAc donor **1-13** with the 3′-OH **1-5**; then, acetyl and Troc protecting group was removed using sodium hydroxide, and the free amino group was protected by TrocCl to afford **1-14a**. Trisaccharide can be synthesized by reacting sialyl donor **1-3** with disaccharide **1-14a** using in dichloromethane using NIS/TfOH as a promoter at −40 °C. Finally, selective removal of the PMB protecting group by DDQ produced sialylated trisaccharide **1-2** as only α product in 52 % yield over two steps (Fig. 1.13). Compared to the method mentioned before, this modified method could easily isolate the fully

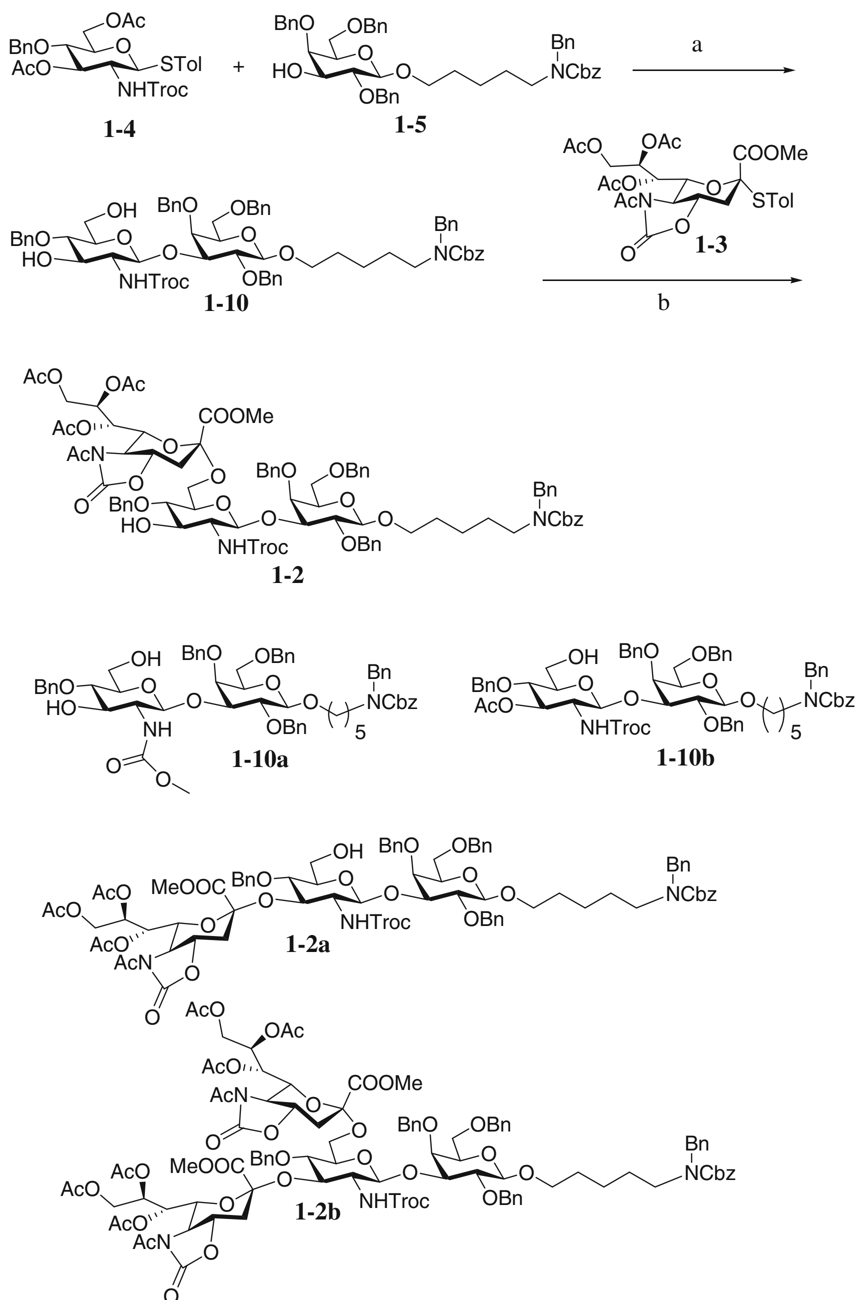


Fig. 1.11 Synthesis of sialyated trisaccharide building block **1-2**. *a* (i) NIS, cat. TfOH, CH_2Cl_2, −30 °C; (ii) NaOH, 1,4-Dioxane/MeOH/H_2O, 60 °C; (iii) TrocCl, $NaHCO_3$, H_2O/1,4-Dioxane, 68 % for three steps; *b* NIS, cat. TfOH, CH_2Cl_2, −40 °C, 2 h, 43 %

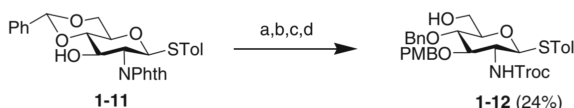


Fig. 1.12 Synthesis of GlcNAc building block **1-12**. *a* NaH, PMBCl, DMF, rt, 1 h; *b* BH_3.THF, Bu_2BOTf, 0 °C, 2 h; *c* Ethylenediamine, ethanol, 80 °C, 16 h; *d* TrocCl, $NaHCO_3$, THF, rt, 16 h, overall yield 24 %. Adapted from [14], copyright 2015, with permission from American Chemical Society

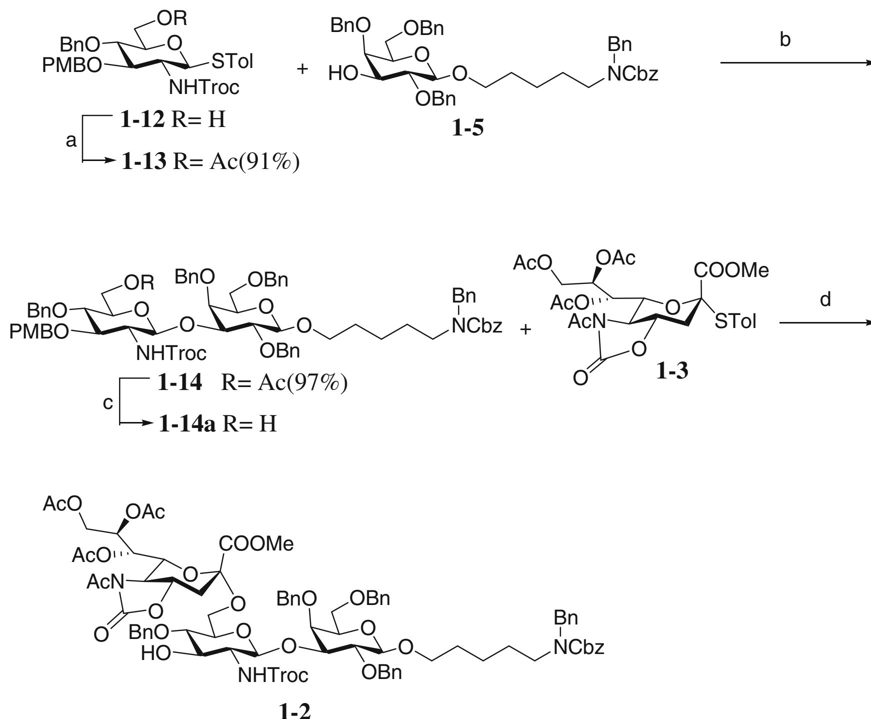


Fig. 1.13 Synthesis of trisaccharide building block **1-2**. *a* Ac_2O, pyridine, DMAP, rt, 12 h, 91 %; *b* NIS, cat. TfOH, CH_2Cl_2, −30 °C, 97 %; *c* (i) NaOH, 1,4-Dioxane/MeOH/H_2O, 60 °C; (ii) TrocCl, $NaHCO_3$, H_2O/1,4-Dioxane, 69 % for two steps; *d* (i) NIS, cat. TfOH, CH_2Cl_2, −40 °C, 2 h; (ii) DDQ, H_2O/CH_2Cl_2, rt, 2 h, 52 % for two steps

protected trisaccharide compound **1-2** in high overall yield (35 %) and large scale (10 g)

We further conducted an orthogonal one-pot [1 + 2 + 3] glycosylation and successfully synthesized the target trisaccharide **1-2** (Fig. 1.14). The one-pot synthetic operation was performed by sialyl phosphate **1-15** (1.5 equiv) and GlcNAc acceptor **1-12** (1.0 equiv) in the presence of TMSOTf at −60 °C. The second glycosylation between **1-12** and **1-5** was carried out by adding NIS (2.0 equiv) to

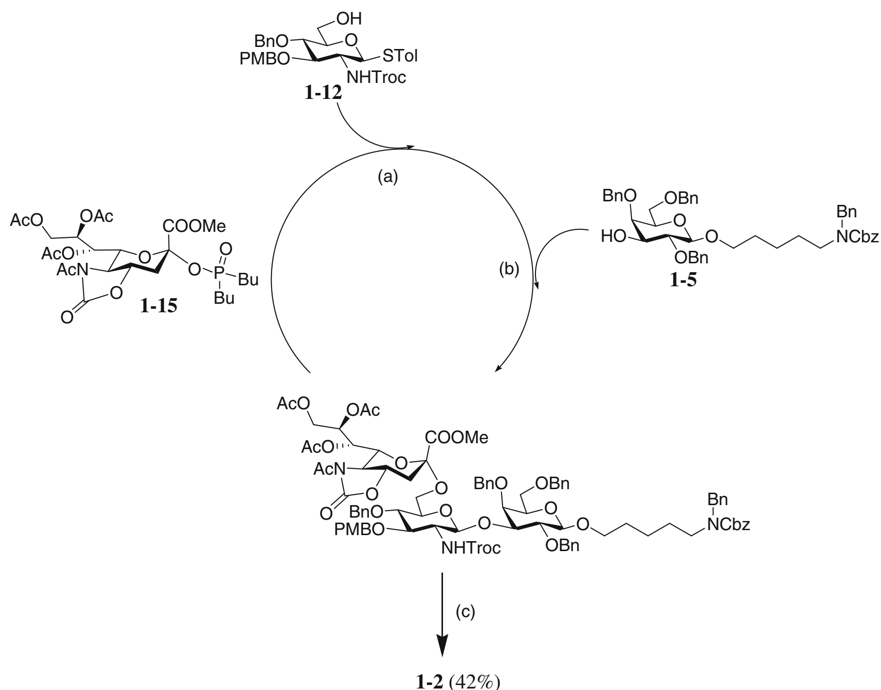


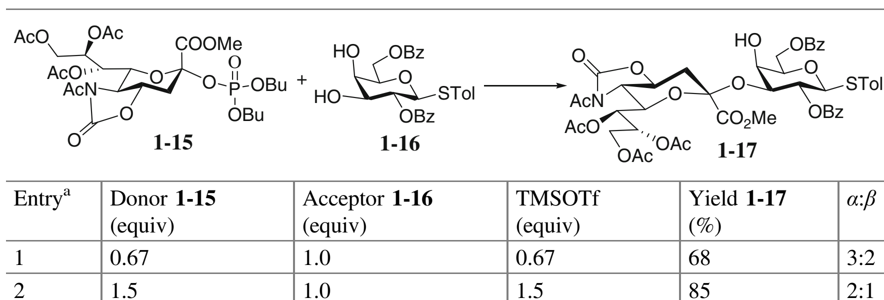
Fig. 1.14 Orthogonal one-pot synthesis of trisaccharide **1-2**. *a* **1-15**, **12**, TMSOTf, MS4 Å, CH_2Cl_2 : CH_3CN = 1 : 2, −60 °C, 1 h; *b* **1-5**, NIS, −20 °C, 2 h; *c* DDQ, CH_2Cl_2, H_2O, rt, 12 h, overall yield 42 %. Adapted from [14], copyright 2015, with permission from American Chemical Society

the reaction solution at higher temperature (−20 °C). Finally, removal of the PMB group by DDQ produced trisaccharide **1-2** in 42 % yield. The configuration of the trisaccharide **1-2** was examined by NMR spectrometry, and the new formed *α*-glycosidic bond was confirmed by coupling constant $^3J(C_1\text{-}H_{3ax})$ = 6.0 Hz.

1.2.7 Synthesis of Disaccharide 1-17

To synthesize the Sialα2 → 3Gal disaccharide, the galactose acceptor **1-16** with the sialyl donor **1-15** [22] was coupled to afford *α*(2,3)-sialyl galactoside **1-17.** Interestingly, the yield of coupling reaction using **1-15** was improved when the equivalent of donor **1-15** was increased from 0.67 to 1.5, and the *α*/*β* ratio was also greatly improved from 3:2 to 2:1 (Table 1.3).

Table 1.3 Glycosylation reaction between **1-15** and **1-16**



Entry[a]	Donor **1-15** (equiv)	Acceptor **1-16** (equiv)	TMSOTf (equiv)	Yield **1-17** (%)	α:β
1	0.67	1.0	0.67	68	3:2
2	1.5	1.0	1.5	85	2:1

[a]In a typical glycosylation procedure, the reaction time was stirred for 4 h and the reaction temperature was −40 °C in the presence of MS3 Å

1.2.8 Examine Synthesis of Hexasaccharide

To synthesize the β-selective trisaccharide building block, GlaNAc **1-18** [28] and **1-19** [29] were examined by coupling with the disaccharide acceptor **1-17** in CH_2Cl_2 with different promoters because the GalNAc donor **1-18** was usually coupled with disaccharide in the presence of NIS and TfOH to synthesize the GM2-core trisaccharide. Besides, the triacetyl group was replaced by the tribenzyl group to increase the reactivity of the galactosamine building block to synthesize GlaNAc **1-19**. Glycosylation of **1-18** or **1-19** as the donor with the acceptor **1-17** by using the NIS and TfOH promoting system in dichloromethane cannot afford the desired trisaccharide. Thus, we changed the promoter from the NIS/TfOH to the BSP/Tf_2O system using the pre-activation method. Therefore, donor **1-18** or **1-19** (1.2 equiv) and BSP (0.65 equiv) were mixed together with molecular sieves and stirred in anhydrous dichloromethane for 1 h at room temperature. After cooling down to −60 °C, Tf_2O (0.65 equiv) and acceptor **1-17** were transferred to the solution sequentially, and then the temperature was raised up to 0 °C over 2 h. The result of the TLC plate was a mess and no desired trisaccharide **1-20** or **1-21** was obtained after purification (Table 1.4). Therefore, we tried another strategy to prepare the hexasaccharide by conjugating the pentasaccharide with the GlaNAc **1-18** or **1-19.**

To prepare the target hexasaccharide **1-23** or **1-24**, we first prepared pentasaccharide **1-22** by convergent synthesis using glycosylation of trisaccharide acceptor **1-2** with disaccharide donor **1-17** and the NIS/TfOH promoting system in anhydrous CH_2Cl_2 at –20 °C in good yield (55 %) (Fig. 1.15). However, the coupling of GlaNAc donors **1-18** or **1-19** and the acceptor **1-22** cannot provide hexasaccharide **1-23** or **1-24** (Table 1.5). We speculated that the major reason might be the hydrogen bond between the 4′-alcohol of galactose and the benzoyl protecting group on the 6-position of galactose resulting in the low reactivity and poor yield. To increase yield and β-selective glycosylation of galactosamine moiety, we planned to replace the 6-position protecting group of galactose from benzoyl protecting group to benzyl protecting group to reduce the hydrogen bond interaction.

Table 1.4 Glycosylation reaction between **1-17** and **1-18/19**

1-18, R=Ac
1-19, R=Bn
1-17
Promoter
MS4A, CH_2Cl_2
1-20, R=Ac
1-21, R=Bn

Entry	Donor	Promoter[c]	Temp (°C)	Yield (%)
1	**1-18**[a]	NIS/TfOH	−60 to 0	–
2	**1-18**[a]	NIS/TfOH	−60 to 0	–
3	**1-18**[b]	BSP/Tf_2O	−70 to 0	–
3	**1-19**[b]	BSP/Tf_2O	−70 to 0	–

[a]Donor (2.0 equiv), acceptor (1.0 equiv)
[b]Donor (1.2 equiv), acceptor (1.0 equiv)
[c]NIS (3.0 equiv), TfOH (1.0 equiv), BSP (0.65 equiv), and Tf_2O (0.65 equiv)

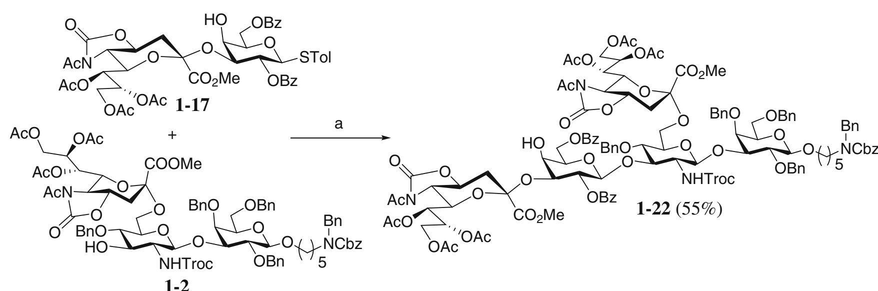
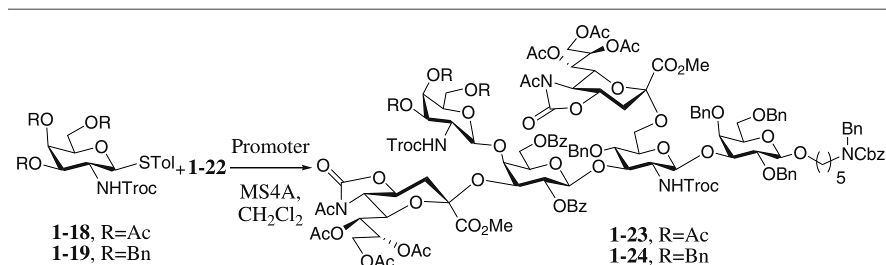


Fig. 1.15 Synthesis of pentasaccharide **1-22**. *a* **1-17** (2 eq), **1-2** (1 eq), NIS (3 eq), TfOH (1 eq), MS4 Å, CH_2Cl_2, −20 °C, 16 h, 55 %

1.2.9 Hexasaccharide RM2 Antigen: Investigate Sialα2 → 3Gal Disaccharide with High α-Stereoselectivity and Yield

In order to synthesize the Sialα2 → 3Gal disaccharide with high *α*-stereoselectivity and yield, the compound **1-25** [22] was efficiently converted into the corresponding 6-*O*-benzyl-4-hydroxy derivative **1-26** using triethylsilane (Et_3SiH) and trifluorosulfonic acid (TfOH) in a high yield with excellent regioselectivity (Fig. 1.16). During the course of our investigation, Takahashi and co-workers reported a related study on the use of 5-*N*,4-*O*-oxazolidinones as sialic acid donors **1-28** [30] and acceptor 3,4-diol galactoside to achieve an elegant synthesis of GP1c ganglioside

Table 1.5 Glycosylation reaction between **1-22** and **1-18/19**



Entry	Donor[a]	Promoter[b]	Temp (°C)	Yield (%)
1	**1-18**	NIS/TfOH	−40 to 0	–
2	**1-19**	NIS/TfOH	−40 to 0	–

[a]Donor (2.0 equiv), acceptor (1.0 equiv)
[b]NIS (3.0 equiv), TfOH (1.0 equiv)

epitope. In addition, because phosphate-based leaving groups can be selectively activated under thioglycoside, the sialic acid donors of **1-15**, **1-29**, **1-31**, and **1-33** were tested by coupling with thiogalactose acceptor **1-26** to compare the effect of the 7′ and 8′ modification. The 7′ and 8′ hydroxyl group of known compound **1-27** [30] was protected using 2,2-dimethoxypropane with a catalytic amount of camphorsulfonic acid to obtain product **1-28**. Compound **1-30** can be obtained after acetylation by pyridine and acetic anhydride overnight. Furthermore, to make thioglycoside **1-33** for investigation of the effect of the *N*-Acetyl-5-*N*,4-*O*-Carbonyl protection group for α-selectivity, acetyl chloride and DIPEA were added to the reaction at 0 °C in dichloromethane. To our surprise, the thiophenyl group in the anomeric center of known 5-*N*,4-*O*-carbonyl-protected thiosialoside **1-28** was replaced by dibutyl phosphate group at 0 °C under N-iodosuccinimide (NIS) and trifluoromethansulfonic acid (TfOH) activation for 10 h to obtain **1-29** in 89 % yield ($\alpha:\beta = 13:1$). Under the same reaction condition, donors **1-30** or **1-32** were coupled to dibutyl phosphate to give dibutyl sialyl phosphate **1-31** or **1-33**, respectively.

With the α-sialyl phosphate donor **1-29** and 3,4-dihydroxyl galactose acceptor **26** in hand, glycosylation of **1-26** and **1-29** under the activation of TMSOTf in CH_2Cl_2 at −78 °C for 2 h gave Neu5Acα2 → 3Gal disaccharide **1-35** as a single isomer in 87 % yield (Table 1.6, entry 2). The configuration of the disaccharide **1-35** was examined by NMR spectrometer, and the new formed α-glycosidic bond was confirmed by coupling constant $^3J(C_1\text{-}H_{3ax}) = 6.1$ Hz. For comparison, the glycosylations of sialyl phosphate **1-29**, **1-30**, or **1-31** were also evaluated under the same reaction condition. The result showed that using phosphate donor **1-29** produced higher α-selectivity and yield (Table 1.6). Next, to synthesize the β-selective trisaccharide building block **1-39**, **1-40**, or **1-41**, GlaNAc **1-18**, **1-19**, and **1-38** were examined by coupling with the disaccharide acceptor **1-35** in CH_2Cl_2 at different temperatures because Kiso and co-workers employed a related study on the use of GlaNAc **1-18** or **1-38** to couple with disaccharide to achieve an elegant synthesis of GM2-core trisaccharide. Besides, the

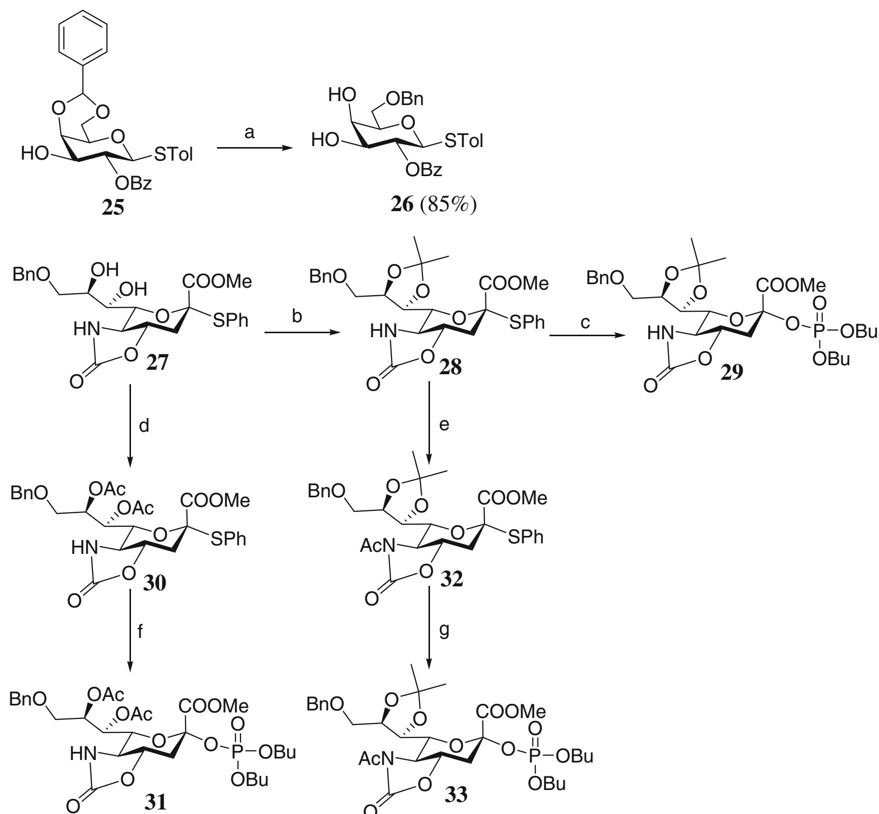


Fig. 1.16 Synthesis of sialyl donor **1-27**, **1-29** and **1-31**. *a* Et_3SiH, TfOH, MS4 Å, CH_2Cl_2, −78 °C, 1 h, 85 %; *b* 2,2-dimethoxypropane, CSA, rt, 12 h, 93 %; *c* NIS, TfOH, Dibutylphosphate, MS4 Å, CH_2Cl_2, 0 °C, 10 h, 89 %; *d* Ac_2O, pyridine, rt, 12 h; *e* AcCl, DIPEA, CH_2Cl_2, 0 °C, 2 h, 98 %; *f* NIS, TfOH, Dibutylphosphate, MS4 Å, CH_2Cl_2, 0 °C, 10 h; *g* NIS, TfOH, Dibutylphosphate, MS4 Å, CH_2Cl_2, 0 °C, 10 h, 60 %. Adapted from [14], copyright 2015, with permission from American Chemical Society

traditional promoter could not be used in this reaction, so we changed from the traditional NIS/TfOH promoter to the BSP/Tf_2O promoter for the pre-activation method. The triacetyl group was replaced by the tribenzyl group to increase reactivity to obtain GlaNAc **1-19**. Following pre-activation of the donor **1-38** with BSP/Tf_2O at −50 °C and subsequent addition of the acceptor **1-35**, the reaction mixture was allowed to warm to −40 °C to obtain trisaccharide **1-41** in 43 % yield as a 13–1 mixture of rotamers (determined by ^{1}H NMR spectroscopic analysis of the crude reaction) (Table 1.7, entry 3). For comparison, the glycosylations of GlaNAc **1-18** and **1-19** were also evaluated under the similar reaction condition to afford trisaccharide in low yield of 10 and 23 %, respectively. Convergent synthesis of hexasaccharide **1-42** was achieved in a good yield (62 %) using glycosylation of

Table 1.6 Synthesis the Sialα2 → 3Gal Disaccharide

1-15,1-29,1-31,1-33 + 1-26 → (TMSOTf, −78 °C / CH_2Cl_2, MS4A) 1-34,1-35,1-36,1-37

Entry	Donor[a]	Product	Yield[%][b]	α:β[c]	δ_{C1} [ppm]	^3J(C-1,H-3_{eq})[Hz]
1	**1-15**	**1-34**	65	7.4:1	168.5	5.9
2	**1-29**	**1-35**	87	α only	168.7	6.1
3	**1-31**	**1-36**	52	1:2	168.4	5.9
4	**1-33**	**1-37**	80	5:1	169.2	6.0

[a]Donor (1.25 equiv), acceptor (1.0 equiv)
[b]Isolated yield
[c]Determined by ^{1}H NMR spectroscopic analysis of the crude reaction mixture

trisaccharide acceptor **1-2** with trisaccharide donor **1-41** and the NIS/TfOH promoting system in anhydrous CH_2Cl_2 at 0 °C (Fig. 1.17).

Alternatively, an orthogonal one-pot [1 + 2 + 3] glycosylation was investigated (Fig. 1.18). The one-pot synthetic operation was performed by pre-activation of the donor **1-38** with BSP/Tf_2O at −50 °C and subsequent addition of the acceptor **1-35.** The trisaccharide **1-41** was formed when the reaction mixture was warmed to −40 °C. The second glycosylation between **1-35** and **1-2** by adding NIS and TfOH to the reaction solution required a higher temperature (0 °C). Finally, the results showed that such coupling gave tetrasaccharide **1-43** (43 %) as the major product, while the reaction only yielded 5 % of hexasaccharide **1-42** (Fig. 1.19).

Because phosphate-based leaving groups can be selectively activated over thioglycoside, the GlaNAc donor **1-39** [31] was tested by coupling with disaccharide **35** to reduce the equivalent of the GlaNAc donor to improve the yield of the one-pot reaction. Glycosylation of **1-39** (1.3 eq) and **1-35** (1.0 eq) under the activation of TBDMSOTf in CH_2Cl_2 at −50 °C for 2 h gave trisaccharide **1-41** in 63 % yield as 6–1 mixture of rotamers (determined by ^{1}H NMR spectroscopic analysis of the crude reaction) (Table 1.8). Thus, we further conducted an orthogonal one-pot [1 + 2 + 3]

Table 1.7 Glycosylation reaction between **1-35** and **1-18**, **1-19**, or **1-38**

1-18, R_1, R_2, R_3=Ac
1-19, R_1, R_2, R_3=Bn
1-38, R_1=Ac, R_2=Bn, R_3=TBS

1-35

promoter
MS4A, CH_2Cl_2

1-39, R_1, R_2, R_3=Ac
1-40, R_1, R_2, R_3=Bn
1-41, R_1=Ac, R_2=Bn, R_3=TBS

Entry	Donor[a]	Promoter[b]	Temp (°C)	Yield (%)
1	**1-18**	BSP/Tf_20	−60 to −40	10
2	**1-19**	BSP/Tf_20	−60 to −40	23
3	**1-38**	BSP/Tf_20	−50 to −40	43

[a]Donor (2.0 equiv), acceptor (1.0 equiv)
[b]BSP (1.01 equiv), and Tf_2O (1.08 equiv)

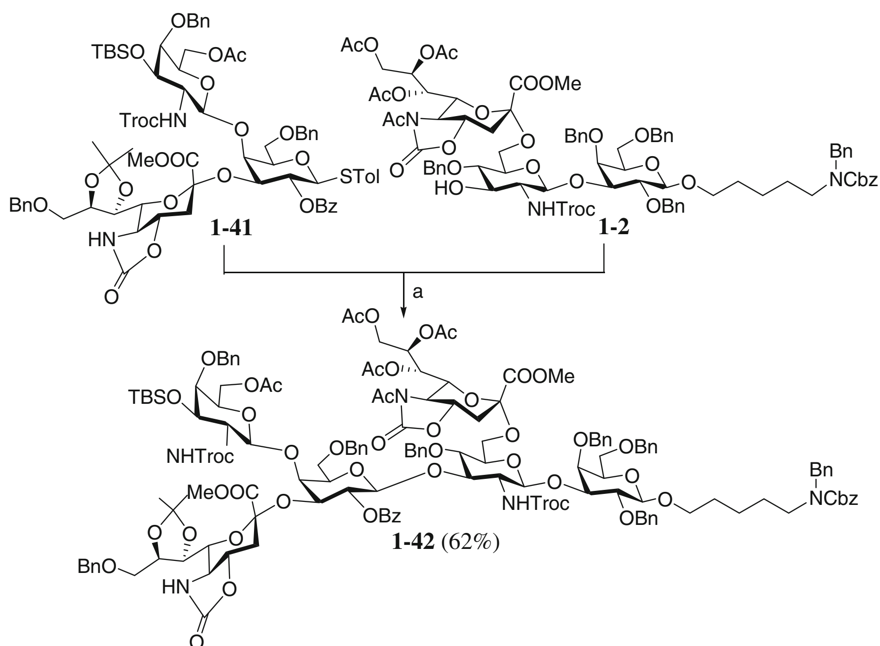


Fig. 1.17 Synthesis of hexasaccharide **1-42**. *a* NIS, TfOH, CH_2Cl_2, 0 °C, MS4 Å, 22 h, 62 %. Adapted from [14], copyright 2015, with permission from American Chemical Society

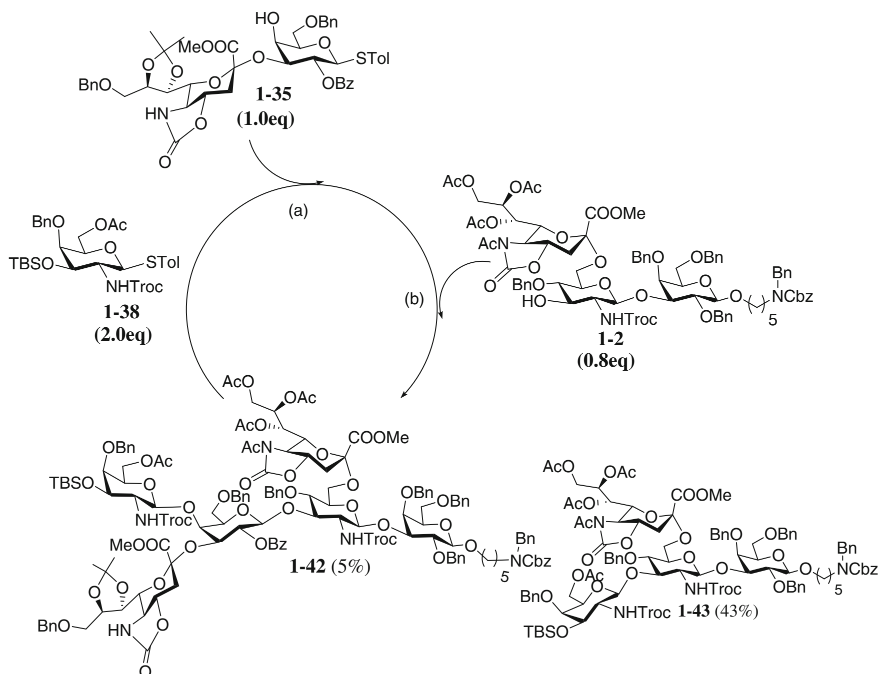
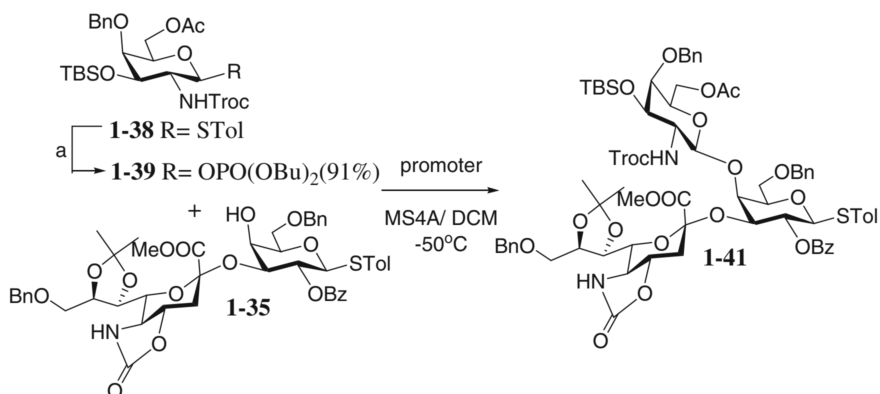


Fig. 1.18 One-pot synthesis of hexasaccharide **1-42**. *a* **1-35**, **1-38**, BSP, Tf_2O, MS4 Å, CH_2Cl_2, −50 °C → −40 °C, 2 h; *b* **2**, NIS, TfOH, 0 °C, 22 h

Table 1.8 Glycosylation reaction between **1-39** and **1-35**



Entry	Donor **1-39** (equiv)	Acceptor **1-35** (equiv)	Promoter[b]	Yield (%)
1	2.0	1.0	TBDMSOTf	73
2	1.8	1.0	TBDMSOTf	69
3	1.5	1.0	TBDMSOTf	68
4	1.3	1.0	TBDMSOTf	63

[a]Dibutylphosphate (3.0 equiv), NIS (2.0 equiv), TfOH (0.25 equiv), CH_2Cl_2, MS4 Å, 0 °C
[b]TMSOTf (the same equiv of donor)

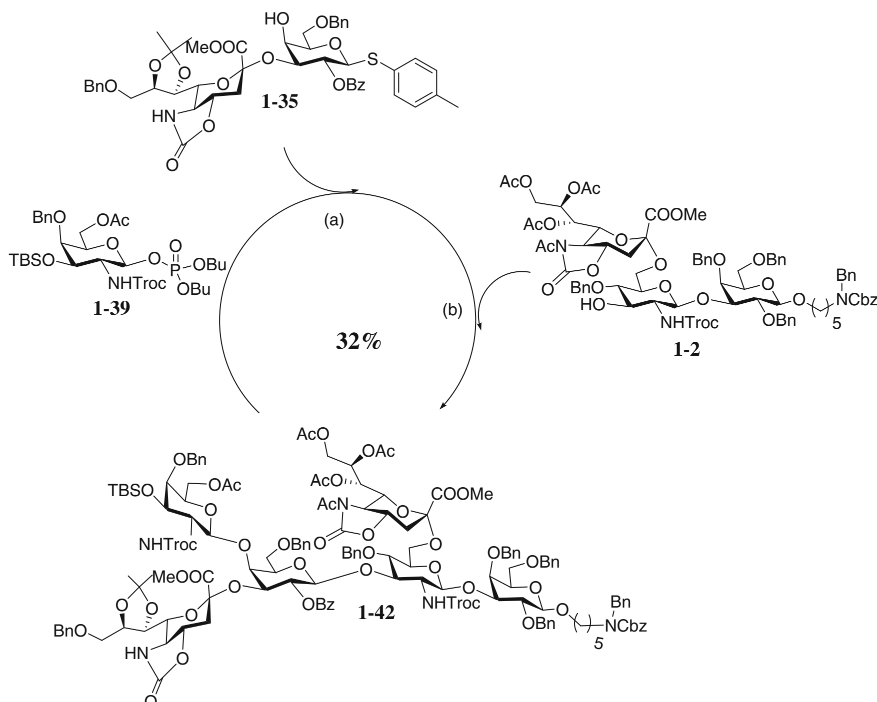


Fig. 1.19 One-pot synthesis of hexasaccharide **1-42**. *a* **1-39**, **1-35**, TBDMSOTf, MS4 Å, CH_2Cl_2, −50 °C, 2 h; *b* **1-2**, NIS, 0 °C, 22 h, 32 % (over two steps) as a single stereoisomer. Adapted from [14], copyright 2015, with permission from American Chemical Society

glycosylation and successfully synthesized the target hexasaccharide **1-42** (Fig. 1.19). Chemoselective glycosylation of the phosphate donor **1-39** with the thioglycoside **1-35** under the TBDMSOTf activation in CH_2Cl_2 at −50 °C provided trisaccharide **1-41**. Without isolation, the acceptor **1-2** and NIS were subsequently added to the reaction vessel at 0 °C, and the reaction was finished in 23 h to afford the protected hexasaccharide **1-42** as a single stereoisomer in an overall yield of 32 % based on **1-2**.

1.2.10 Syntheses of Truncated RM2 Derivatives 1-44, 1-46, 1-48, and 1-50

Although RM2 antibody has been reported to recognize RM2 antigen, its specificity for related carbohydrate is not well studied. To understand the specificity of RM2 antibody, we synthesized the RM2 fragments **1-44**, **1-46**, **1-48**, and **1-50** to determine the specificity of monoclonal anti-RM2 antibody. The syntheses of the

RM2 fragments **1-44**, **1-46**, **1-48**, and **1-50** followed the above method (Fig. 1.20). Donors **1-43** [32], **1-45** [32], or **1-47** [22] were coupled to acceptor **1-2** to give tetrasaccharide **1-44**, pentasaccharide **1-46**, or pentasaccharide **1-48,** respectively. In addition, treatment of the trisaccharide donor **1-41** and disaccharide acceptor **1-49** with NIS and a catalytic amount of TBDMSOTf at 0 °C gave the protected pentasaccharide **1-50** in 67 % yield.

Global deprotection of the protected hexasaccharide **1-42** and pentasaccharide **1-50** was achieved using a four-step procedure: (i) hydrolysis of acyl protecting groups; (ii) acetylation of the amino groups; (iii) removal of the isopropyl groups; and (iv) hydrogenolysis of the resulting benzyl ethers to provide the fully deprotected hexasaccharide **1-1** and pentasaccharide **1-55**. In addition, with the similar strategy, deprotection of the **1-2**, **1-44**, **1-46**, and **1-50** gave the corresponding deprotected oligosaccharides **1-51-1-54**, respectively, in good yields (Fig. 1.21). These materials can be used for preparation of a glycan array for antibody binding study and for the preparation of glycoconjugates for vaccine development.

1.3 Summary

We successfully developed a [1 + 2 + 3] one-pot strategy to synthesize the RM2 antigen hexasaccharide that was proposed to be a prostate tumor antigen. We also synthesized some of the truncated analogs of the proposed RM2 antigen in good yields and stereoselectivity. The structure of the synthetic products was verified by 1D and 2D NMR analysis and evaluated this proposed hexasaccharide recognition by monoclonal antibody RM2.

1.3.1 Experimental Section

Materials Commercial solvents and reagents were purchased from Sigma-Aldrich and Acros and used as received without further purification. Monoclonal antibody RM2 was supplied by Prof. Seiichi Saito (Department of Urology, Graduate School of Medicine, University of Ryukyus, Nishihara 903-0215, Japan) and Cy3-conjugated anti-mouse IgG (IgG, IgG1, IgG2a, IgG2b, IgG2c, and IgG3) and IgM antibodies were obtained from Jackson IMMUNO Research. Diphtheria toxoid (CRM 197) was purchased from *PF*enex Incorporation; aluminum phosphate ($AlPO_4$) from Brenntag Biosector; and glycolipid derivatives (C1 and C34) were from Dr. Chi-Huey Wong's lab.

General Molecular sieves 4 Å (Reidel-deHaen No.31812) for glycosylations were crushed and activated by heating at 350 °C for 10 h before use. Reactions were monitored with analytical TLC plates (PLC silica gel-60, F_{254}, 2 mm, Merck) and visualized under UV (254 nm) or by staining with acidic ceric ammonium molybdate or *p*-anisaldehyde. Flash column chromatography was performed on

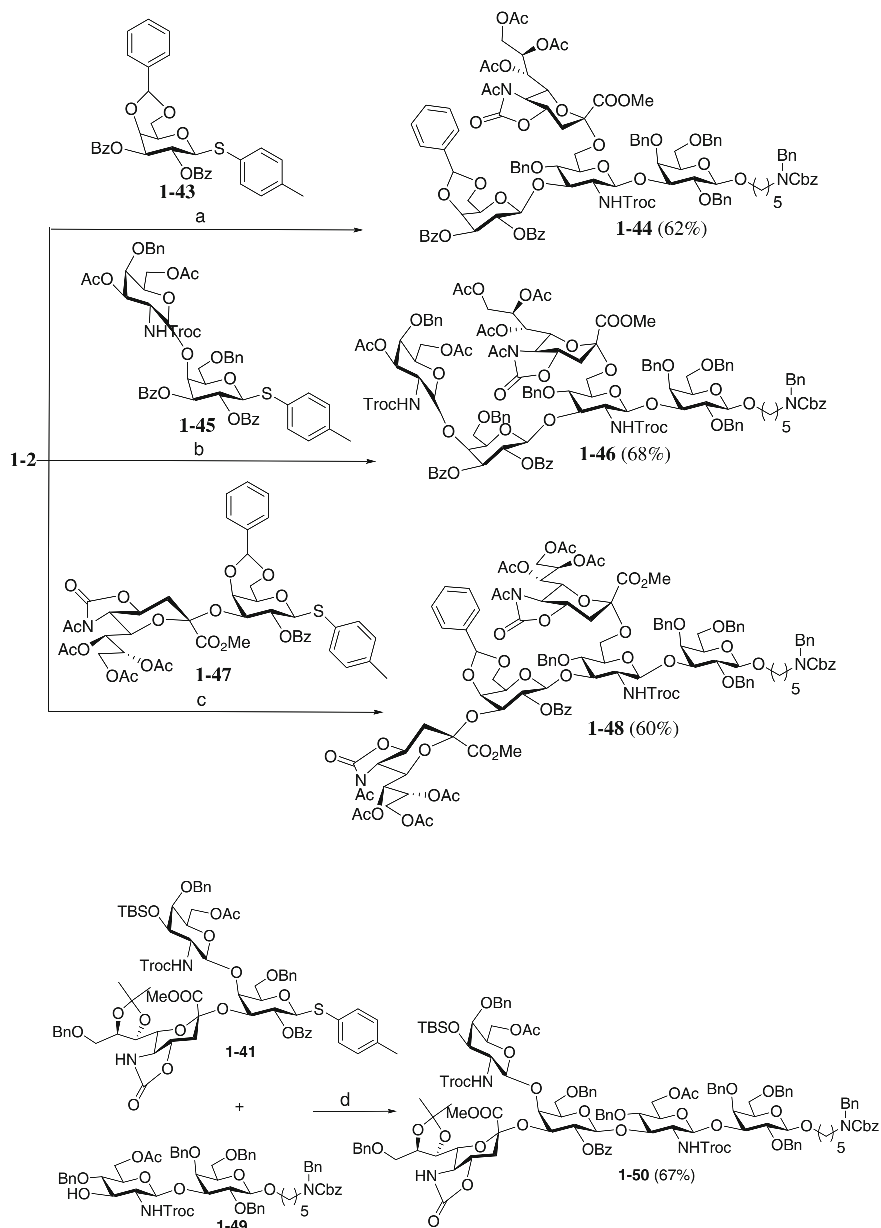


Fig. 1.20 Synthesis of **1-44**, **1-46**, **1-48**, and **1-50**. *a* NIS, TfOH, MS4 Å, CH_2Cl_2, 0 °C, 9 h; *b* NIS, TfOH, MS4 Å, CH_2Cl_2, 0 °C, 11 h; *c* NIS, TfOH, MS4 Å, CH_2Cl_2, −20 °C, 3 h. *d* NIS, TBDMSOTf, MS4 Å, CH_2Cl_2, 0 °C, 8 h. Adapted from [14], copyright 2015, with permission from American Chemical Society

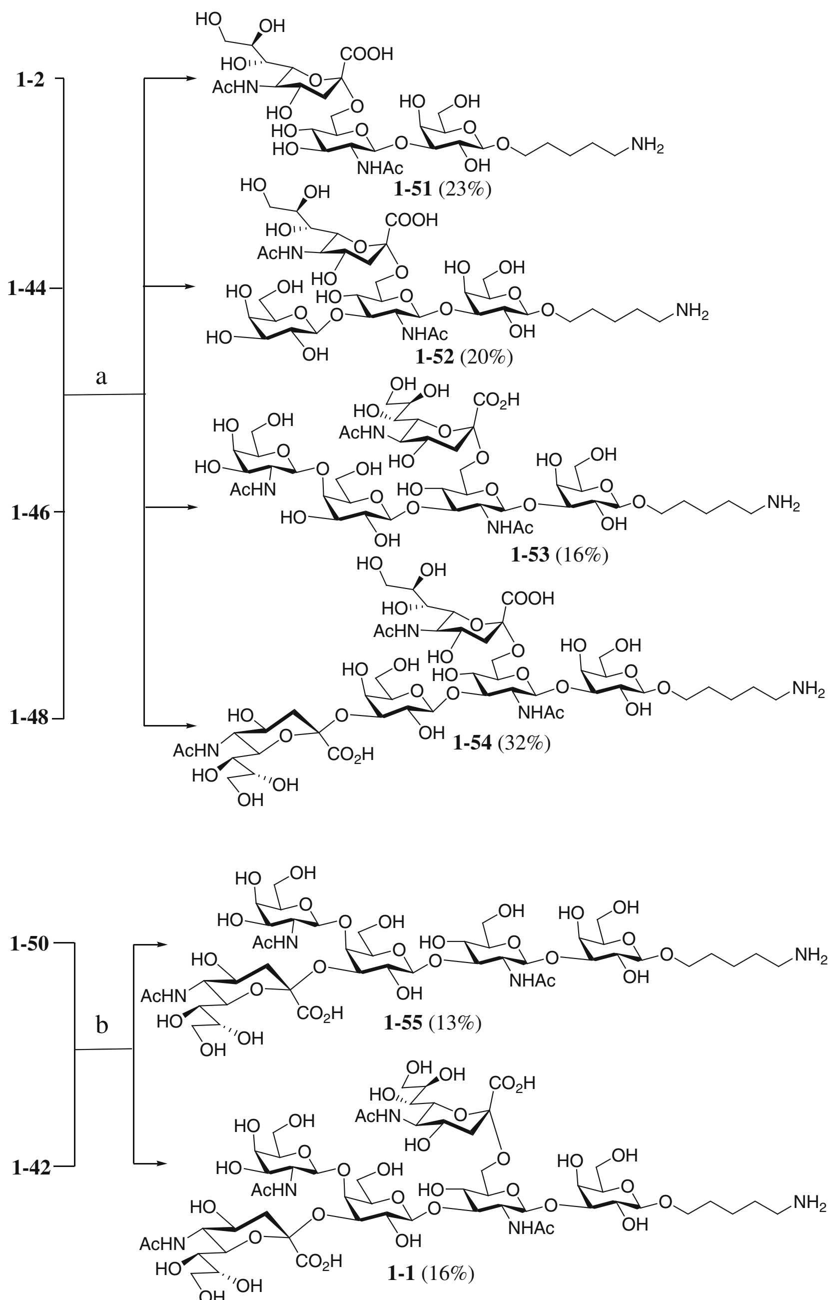


Fig. 1.21 Global deprotection of **1-2**, **1-42**, **1-44**, **1-46**, **1-48**, and **1-50**. *a* (i) LiOH · H_2O, Dioxane, H_2O, 90–95 °C, 36 h; (ii) Ac_2O, $NaHCO_3$, H_2O then LiOH, H_2O, rt, 12 h; (iii) $Pd(OH)_2$, H_2, CH_3OH, H_2O, rt, 12 h. *b* (i) LiOH · H_2O, Dioxane, H_2O, 90−95 °C, 36 h; (ii) Ac_2O, pyridine, DMAP, 12 h; (iii) $BF_3.OEt_2$, CH_3CN, 0 °C → rt, 18 h; (iv) LiOH, H_2O, rt, 12 h (v) $Pd(OH)_2$, H_2, CH_3OH, H_2O, rt, 12 h. Adapted from [14], copyright 2015, with permission from American Chemical Society

silica gel (40–63 μm, Merck), LiChroprep RP8 (40–63 μm), and LiChroprep RP18 (40–63 μm).

Instrumentation Proton nuclear magnetic resonance (^1H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker Advance 600 (600/150 MHz) NMR spectrometers. Chemical shifts of protons were reported in ppm (δ scale) and referenced to tetramethylsilane ($\delta = 0$). Chemical shifts of carbon were also reported in parts per million (ppm, δ scale) and calibrated with tetramethylsilane ($\delta = 0$). DEPT 135 (distortionless enhancement by polarization transfer) was employed for determination of multiplicity. Data were represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant (J) in Hz, and integration. High resolution mass spectra were obtained using BioTOF III, and MALDI-TOF MS were obtained using Ultraflex II TOF/TOF.

((2R,3S,4R,5R,6S)-4-acetoxy-3-(benzyloxy)-6-(p-tolylthio)-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yl)methyl acetate (1-4)

Compound 1-6 (7.0 g, 11.85 mmole) was cooled to −20 °C, dissolved in BH_3-THF complex (36 mL, 1 M solution in THF), and treated with Bu_2BOTf (13 mL, 1 M solution in CH_2Cl_2). The reaction mixture was stirred from −20 to 0 °C over 1.5 h under argon. After stirring was continued for 6 h at 0 °C, the reaction mixture was quenched with Et_3N (10 mL), followed by dropwise addition of MeOH (30 mL). The reaction mixture was warmed to room temperature and evaporated to dryness. The residual syrup was coevaporated with MeOH (2 × 50 mL) and then toluene (50 mL). A solution of C-6 alcohol in anhydrous CH_2Cl_2 (75 mL) was treated with acetic anhydride (5 mL, 53 mmole) and pyridine (10 mL, 123.6 mmole). The reaction mixture was stirred at room temperature for 8 h under argon. The solution was concentrated to syrup which was extracted with dichloromethane. The extract was successively washed with 2 N HCl, water, $NaHCO_{3(aq)}$ and water, dried with Na_2SO_4 and concentrated. Purification by flash chromatography on silica gel (1 : 10 to 1 : 3 ethyl acetate/hexane) gave **1-4** as white solid (6.85 g, 91 % yield). **1-4**: $R_f = 0.43$ (Hexane : EtOAc = 3 : 1); ^1H NMR (600 MHz, $CDCl_3$) δ 7.39 (d, J = 7.2 Hz, 2H; Ar–H), 7.28 (m 3H; Ar–H), 7.18 (d, J = 5.9 Hz, 2H; Ar–H), 7.08 (d, J = 7.2 Hz, 2H; Ar–H), 5.6 (d, J = 9.7 Hz, 1H), 5.19 (m, 1H), 4.82 (d, J = 12.0 Hz, 1H; PhCH), 4.71 (d, J = 12.0 Hz, 1H; PhCH), 4.59 (d, J = 10.2 Hz, 1H; H-1 anomeric), 4.52 (m, 2H), 4.31 (d, J = 11.5 Hz, 1H; CH NHTroc), 4.12 (d, J = 11.5 Hz, 1H; CH NHTroc), 3.73 (m, 1H), 3.56 (m, 2H), 2.31 (s, 3H; CH_3 STol), 2.04 (s, 3H; CH_3 Ac), 1.95 (s, 3H; CH_3 Ac); ^{13}C NMR (150 MHz $CDCl_3$) δ170.9, 170.6, 154.4, 138.4, 137.2, 133.4, 129.7, 128.7, 128.3, 128.1, 95.6, 87.1, 76.8, 76.1, 75.8, 74.8, 74.6, 62.9, 55.2, 21.2, 21.0, 20.9.

HRMS(ESI) calculated for $C_{27}H_{30}Cl_3NO_8SNa$ $[M + Na]^+$: 658.0625, found: 658.0631.

(2R,3S,4S,5R,6R)-2-(acetoxymethyl)-6-(5-(benzyloxycarbonylamino)pentyloxy)-4-(4-methoxybenzyloxy)tetrahydro-2H-pyran-3,5-diyl diacetate (1-8)

Acetic anhydride (15 mL) was added to a solution of compound **1-7** (4.0 g, 9.96 mmole) in pyridine (15 mL) at 0 °C. The mixture was stirred for 12 h at 40 °C, and methanol (5 mL) was added. The solution was concentrated into syrup and

extracted with dichloromethane. The extract was successively washed with 2 N HCl, water, $NaHCO_{3(aq)}$, and water, then dried with Na_2SO_4, and concentrated. Column chromatography (1 : 2 ethyl acetate/hexane) of the residue on silica gel gave the per-acetylated thioglycoside as a white solid (5.17 g, 99 %). To a solution of purified thioglycoside (5.17 g, 9.95 mmole) and benzyl 5-hydroxy pentylcarbamate (3.52 g, 15.92 mmole) in dichloromethane (30 mL), molecular sieves 4 Å (10.0 g) were added, and the mixture was stirred for 1 h at room temperature and then cooled to −30 °C. To the stirred mixture, NIS (3.58 g, 15.92 mmole) and TfOH (0.42 mL, 4.78 mmole) were added, and the solution was stirred continuously for 3 h at −30 °C. The precipitates were filtered off and washed with dichloromethane. The filtrates were combined, and the solution was successively washed with saturated $NaHCO_{3(aq)}$ and saturated $Na_2S_2O_{3(aq)}$, then dried with Na_2SO_4, and concentrated. Purification by flash silica gel column chromatography (1 : 2 to 1 : 1 ethyl acetate/hexane) gave **1-8** as colorless oil. (3.86 g, 60 %). **1-8**: R_f = 0.65 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.34–7.29 (m, 5H; Cbz-H), 7.18 (d, *J* = 8.4 Hz, 2H; Ar–H), 6.85 (d, *J* = 8.4 Hz, 2H; Ar–H), 5.47 (d, *J* = 3.2 Hz, 1H), 5.09–5.05 (m, 3H), 4.61 (d, *J* = 11.9 Hz, 1H; H-1 anomeric), 4.33 (m, 2H), 4.16 (d, *J* = 6.6 Hz, 2H), 3.87–3.82 (m, 1H), 3.79–3.75 (m, 4H), 3.50 (dd, *J* = 10.0, 3.4 Hz, 1H), 3.44–3.39 (m, 1H), 3.16 (m, 2H), 2.13 (s, 3H; CH_3 Ac), 2.06 (s, 3H; CH_3 Ac), 2.00 (s, 3H; CH_3 Ac), 1.61–1.45 (m, 4H; aliphatic), 1.37–1.30 (m, 2H; aliphatic); ^{13}C NMR (150 MHz $CDCl_3$) δ 170.3, 170.3, 169.2, 159.2, 156.3, 136.6, 129.4, 129.3, 128.3, 127.9, 113.6, 101.1, 76.0, 70.8, 70.7, 70.4, 69.4, 66.2, 65.9, 61.8, 55.1, 40.7, 29.4, 28.8, 22.9, 20.7, 20.6, 20.6; HRMS (ESI-TOF, MNa^+) calculated for $C_{33}H_{43}NO_{12}Na$ 668.2677, found 668.2674.

Benzylbenzyl(5-((2R,3R,4S,5S,6R)-3,5-bis(benzyloxy)-6-(benzyloxymethyl)-4-(4-methoxybenzyloxy)tetrahydro-2H-pyran-2-yloxy)pentyl)carbamate (1-9)

NaOMe (0.65 g, 12 mmole) was added to a solution of compound **8** (3.86 g, 5.98 mmole) in MeOH (120 mL), and the mixture was stirred at room temperature under N_2 atmosphere. After stirring for 10 h, the reaction mixture was neutralized with Amberlite IR-120 resin. The resin was removed by filtration, and the filtrate was concentrated and dried under reduced pressure. The residue was dried under high vacuum for 5 h. Then NaH (60 % in mineral oil; 1.12 g, 28.0 mmole) was added to a solution of residue in DMF (100 mL) at 0 °C, and the resulting mixture was stirred for 10 min. Benzylbromide (3.0 mL, 25.08 mmole) was added, and the resulting mixture was warmed to 25 °C and stirred for 2 h. MeOH (4.0 mL) was then added to remove any remaining benzyl bromide. After further 30 min of stirring, the mixture was poured into iced water. The aqueous phase was extracted with EtOAc, and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated. Purification by flash silica gel column chromatography (1 : 4 to 1 : 3 ethyl acetate/hexane) gave **1-9** as colorless oil (4.47 g, 85 %). **1-9**: R_f = 0.28 (Hexane : EtOAc = 3 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.33–7.24 (m, 27H; Ar–H), 6.84 (d, *J* = 8.6 Hz, 2H; Ar–H), 5.15 (d, *J* = 13.8 Hz, 2H; $PhCH_2$), 4.92 (d, *J* = 10.9 Hz, 1H; PhCH), 4.88–4.84 (m, 1H), 4.73 (d, *J* = 10.9 Hz, 1H; PhCH), 4.68–4.59 (m, 3H), 4.46–4.38 (m, 4H), 4.29 (m, 1H), 3.91–3.84 (m, 2H), 3.79 (s, 3H; CH_3 PMB), 3.77–3.74 (m, 1H), 3.56 (m, 2H),

3.49–3.46 (m, 3H), 3.18 (m, 2H), 1.60–1.47 (m, 4H; aliphatic), 1.29–1.24 (m, 2H; aliphatic); ^{13}C NMR (150 MHz $CDCl_3$) δ 159.1, 156.7, 156.1, 138.9, 138.7, 138.0, 136.8, 130.7, 129.2, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.8, 127.5, 127.5, 127.2, 113.7, 103.9, 81.9, 79.6, 75.1, 74.5, 73.5, 73.4, 72.7, 69.7, 69.6, 68.9, 67.1, 55.2, 50.5, 50.2, 47.2, 46.2, 29.4, 28.0; 27.5, 23.4, HRMS (ESI-TOF, MNa^+) calculated for $C_{55}H_{61}NO_9Na$ 902.4239, found 902.4223.

Benzylbenzyl(5-((2R,3R,4S,5R,6R)-3,5-bis(benzyloxy)-6-(benzyloxymethyl)-4-hydroxytetrahydro-2H-pyran-2-yloxy)pentyl)carbamate (1-5)

DDQ (1.16 g, 5.1 mmole) was added to a solution of compound **1-9** (4.47 g, 5.08 mmole) in CH_2Cl_2/H_2O (10 : 1, 33 mL) at 0 °C, and the resulting mixture was warmed to 25 °C and stirred for 2 h. The reaction mixture was diluted with CH_2Cl_2 (250 mL) and washed with saturated aqueous $NaHCO_3$ (50 mL) and brine (20 mL). The organic layer was dried with Na_2SO_4, and the solvents were removed under reduced pressure. The residue was purified by flash silica gel column chromatography (1 : 3 to 1 : 2.5 ethylacetate/hexane) to give **1-5** as colorless oil (3.47 g, 90 %). **1-5**: R_f = 0.58 (Hexane : EtOAc = 2 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.39–7.21 (m, 25H; Ar–H), 5.2 (m, 2H; $PhCH_2$), 4.99 (m, 1H), 4.85 (d, J = 11.7 Hz, 1H; H-1 anomeric), 4.7 (dd, J = 12.2, 12.2 Hz, 2H; $PhCH_2$), 4.53 (m, 4H), 4.36 (m, 1H), 3.96–3.91 (m, 2H), 3.70–3.68 (m, 4H), 3.62 (dd, J = 7.9, 7.9 Hz, 1H), 3.49 (m, 1H), 3.30–3.22 (m, 2H), 2.40 (s, 1H), 1.69–1.55 (m, 4H; aliphatic), 1.41–1.33 (m, 2H; aliphatic); ^{13}C NMR (150 MHz $CDCl_3$) δ 156.7, 156.2, 138.5, 137.9, 136.9, 136.8, 128.6, 128.5, 128.5, 128.3, 128.2, 128.1, 127.9, 127.8, 127.7, 127.3, 127.3, 127.2, 103.8, 79.6, 75.6, 75.0. 74.6, 74.1, 73.6, 73.5, 69.7, 69.6, 68.8, 67.2, 50.5, 50.2, 47.2, 46.2, 29.7, 29.4, 28.0, 27.5, 23.4; HRMS (ESI-TOF, MNa^+) calculated for $C_{47}H_{53}NO_8Na$ 782.3663, found 782.3664.

GlcNAc donor **1-4** (1.0 g, 1.57 mmole), acceptor **1-5** (0.87 g, 1.14 mmole), and molecule sieve (MS) (AW-300, 2.5 g) were suspended in dry CH_2Cl_2 (30 mL) under argon at room temperature stirring for 1 h. The reaction mixture was then cooled to −30 °C, followed by addition of NIS (0.34 g, 1.49 mmole) and TfOH (0.55 mL, 0.56 mmole). The reaction mixture was stirred at −20 °C for 15 h and quenched with Et_3N. MS was filtered off and the filtrate was washed with sat. $Na_2S_2O_3$, sat. $NaHCO_3$, H_2O, and brine, dried over $MgSO_4$, and concentrated. Purification by flash silica gel column chromatography (1 : 5 to 1 : 3 ethyl acetate/hexane) gave a white solid. To a solution of residue in $MeOH/H_2O$/1,4-Dioxane (1 : 1 : 1, 90 mL) was added NaOH (0.21 g, 5.48 mmole) at 25 °C and the resulting mixture was warmed to 90 °C and stirred for 12 h. The reaction mixture was quenched by aqueous 1 N HCl. The solvents were removed by reduced pressure. Trichloroethoxycarbonyl chloride (0.25 mL, 1.83 mmole) was added dropwise over a period of 1 h at room temperature to a vigorously stirred solution of residue and $NaHCO_3$ (0.46 g, 5.47 mmole) in water/1,4-dioxane (1 : 1, 80 mL) was added to solution. The mixture was stirred for another 1 h. The solvents were removed by reduced pressure. The residue was purified by flash silica gel column chromatography (1 : 3 to 1 : 2 ethyl acetate.hexane) to give **1-10** as white solid (0.92 g, 68 %).

1-10: $R_f = 0.58$ (Hexane : EtOAc = 3 : 2); ^{1}H NMR (600 MHz $CDCl_3$) δ 7.35–7.24 (m, 30H; Ar–H), 5.13 (m, 2H; $PhCH_2$), 5.04 (m, 1H), 4.85 (dd, *J* = 11.1, 11.1 Hz, 2H; $PhCH_2$), 4.72–4.69 (m, 2H), 4.62–4.54 (m, 4H), 4.46–4.40 (m, 4H), 4.29 (m, 1H), 3.86–3.82 (m, 3H), 3.77–3.74 (m, 3H), 3.59–3.57 (m, 3H), 3.48–3.43 (m, 2H), 3.40–3.37 (m, 2H), 3.27–3.24 (m, 1H), 3.15 (m, 2H), 2.94 (s, 1H), 1.60–1.47 (m, 4H; aliphatic), 1.29–1.24 (m, 2H; aliphatic); ^{13}C NMR (150 MHz $CDCl_3$) δ 156.8, 156.3, 155.6, 138.9, 138.7, 138.1, 137.9, 137.0, 136.9, 128.9, 128.8, 128.7, 128.6, 128.4, 128.2, 128.1, 128.0, 127.9, 127.7, 127.4, 127.3, 104.0, 101.7, 95.4, 80.3, 80.0, 77.9, 75.9, 75.6, 75.2, 75.0, 74.9, 74.7, 74.6, 73.7, 73.6, 69.9, 68.8, 67.3, 62.0, 58.6, 50.6, 50.4, 47.2, 46.3, 29.5, 28.0, 27.6, 23.5; HRMS (ESI-TOF, MNa^+) calculated for $C_{63}H_{71}Cl_3N_2O_{14}N$ 1209.3854, found 1209.3870.

2,2,2-trichloroethyl(2S,3R,4R,5S,6R)-5-(benzyloxy)-6-(hydroxymethyl)-4-(4-methoxybenzyloxy)-2-(p-tolylthio)tetrahydro-2H-pyran-3-ylcarbamate (1-12)

Compound **1-11** (2.66 g, 5.286 mmole) was dissolved in dry DMF under argon, followed by the addition of *p*-methoxybenzyl chloride (1.5 mL, 10.8 mmole). The resulting mixture was treated with NaH (60 %) (0.46 g, 10.75 mmole) at 0 °C and slowly warmed to room temperature. After 1 h, the reaction mixture was quenched with methanol, and extracted with ethyl acetate (50 mL), and then washed with brine. The resulting organic phase was evaporated at low pressure to give a crude product, which was used without purification. This residue was dissolved in CH_2Cl_2 (30 mL) and BH_3THF in THF (1.0 M) (28 mL, 28 mmole) was added. The reaction mixture was stirred for 10 min, followed by dropwise addition of Bu_2BOTf (5.4 mL, 5.4 mmole) at 0 °C. TLC indicated that the starting material had disappeared after 2 h. The resulting mixture was neutralized with triethylamine and excess borane was quenched with methanol. The solution was concentrated and the residue was purified by chromatography (1 : 4 to 1 : 2 ethyl acetate/hexane). Then, the material residue and ethylenediamine (20 mL, 298 mmole) were dissolved in dry ethanol (50 mL). The mixture was heated at reflux for 16 h. The reaction was evaporated at low pressure, and the residue was dissolved in a mixture of THF and saturated $NaHCO_3$ (35 mL), followed by the addition of TrocCl (0.9 mL, 6.53 mmole). The mixture was stirred for 16 h, and then extracted with EtOAc (60 mL) and saturated aqueous $NaHCO_3$ (40 mL). The organic layer was dried over Na_2SO_4 and purified by chromatography (1 : 5 to 1 : 3 ethyl acetate/hexane) to give **1-12** as the white solid (0.85 g, 1.268 mmole). **1-12**: $R_f = 0.68$ (Hexane : EtOAc = 3 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.36 (m, 7H; Ar–H), 7.20 (d, *J* = 8.6 Hz, 2H; Ar–H), 7.10 (d, *J* = 8.0 Hz, 2H; Ar–H), 6.84 (d, *J* = 8.6 Hz, 2H; Ar–H), 5.08 (d, *J* = 8.3 Hz, 1H), 4.88 (d, *J* = 10.2 Hz, 1H; H-1), 4.83 (d, *J* = 11.0 Hz, 1H), 4.78–4.72 (m, 3H), 4.65–4.62 (m, 2H), 3.88–3.82 (m, 2H), 3.78 (s, 3H; CH_3 PMB), 3.72–3.66 (m, 1H), 3.53 (t, *J* = 9.3 Hz, 1H), 3.42–3.34 (m, 2H), 2.32 (s, 3H; CH_3 STol), 1.96 (t, *J* = 6.6 Hz, 1H); ^{13}C NMR (150 MHz $CDCl_3$) δ 159.6, 154.0, 138.6, 137.9, 133.3, 130.0, 128.7, 128.2, 128.1, 114.1, 95.6, 86.2, 81.6, 79.6, 78.5, 75.1, 75.0, 74.6, 62.2, 56.8, 55.4, 21.3; HRMS (ESI-TOF, MNa^+) calculated for $C_{31}H_{34}Cl_3NO_7SNa$ 694.0990, found 694.1135.

((2R,3S,4R,5R,6S)-6-((2R,3R,4S,5S,6R)-2-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-3,5-bis(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-(benzyloxy)-4-(4-methoxybenzyloxy)-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yl)methyl acetate (1-14)

To a solution of compound **1-12** (4.12 g, 6.14 mmole) in pyridine (7 mL) was added acetic anhydride (7 mL) at room temperature. The mixture was stirred for 12 h at the same temperature, and methanol (5 mL) was added. The solution was concentrated into syrup and extracted with dichloromethane. The extract was successively washed with 2 N HCl, water, $NaHCO_{3(aq)}$, and water, dried with Na_2SO_4 and concentrated. Column chromatography (1 : 3 ethyl acetate/hexane) of the residue on silica gel gave the per-acetylated thioglycoside as a white solid **1-13** (3.8 g, 87 %). GlcNAc donor **1-13** (3.6 g, 5.05 mmole), acceptor **1-5** (2.32 g, 3.05 mmole), and molecule sieve (MS) (AW-300, 2.5 g) were suspended in dry CH_2Cl_2 (30 mL) under argon at room temperature stirring for 1 h. The reaction mixture was then cooled to −30 °C, followed by addition of NIS (1.44 g, 6.40 mmole) and TfOH (0.06 mL, 0.675 mmole). The reaction mixture was stirred at −20 °C for 2 h and quenched with Et_3N. MS was filtered off and the filtrate was washed with sat. $Na_2S_2O_3$, sat. $NaHCO_3$, H_2O, and brine, dried over $MgSO_4$, and concentrated. Purification by flash silica gel column chromatography (1 : 5 to 1 : 2 ethyl acetate/hexane) gave white solid **1-14** (5.25 g, 3.89 mmole). **1-14**: R_f = 0.33 (Hexane : EtOAc = 2 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.42–7.15 (m, 32H; Ar–H), 6.86 (d, *J* = 8.6 Hz, 2H; Ar–H), 5.16 (m, 2H; $PhCH_2$), 5.03 (m, 1H), 4.93 (d, *J* = 11.6 Hz, 1H), 4.83 (d, *J* = 10.9 Hz, 1H), 4.71 (d, *J* = 8.2 Hz, 1H), 4.63 (m, 3H), 4.57–4.32 (m, 11H), 4.23 (dd, *J* = 11.9, 4.6 Hz, 1H), 3.91–3.89 (m, 2H), 3.79–3.76 (m, 5H), 3.65–3.53 (m, 5H), 3.46–3.41 (m, 2H), 3.33 (s, 1H), 3.18 (m, 2H), 2.00 (s, 3H; CH_3 Ac), 1.61–1.47 (m, 4H; aliphatic), 1.32–1.25 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.7, 159.4, 156.7, 156.2, 154.3, 139.2, 138.9, 138.0, 137.6, 130.1, 129.5, 128.7, 128.6, 128.6, 128.6, 128.5, 128.2, 128.1, 128.1, 127.9, 127.9, 127.8, 127.6, 127.5, 127.5, 127.3, 127.3, 127.2, 113.9, 103.9, 101.6, 95.6, 81.9, 80.5, 80.0, 77.9, 75.7, 75.0, 74.8, 74.6, 74.4, 73.7, 73.6, 72.9, 69.7, 69.7, 69.0, 67.2, 63.0, 57.7, 55.3, 50.6, 50.3, 47.2, 46.2, 29.4, 27.9, 27.5, 23.4, 23.4, 20.9.

(2R)-1-((3aR,6S,7aR)-3-acetyl-6-(dibutoxyphosphoryloxy)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2,3-triyl triacetate (1-15)

A solution of **1-3** (2.0 g, 3.44 mmole, 1.0 equiv), dibutyl phosphate (1.92 mL, 10.3 mmole, 3.0 equiv), and activated 3 Å powdered molecular sieves (4.0 g) in anhydrous dichloromethane (60 mL) was stirred 1 h under an argon atmosphere, and then cooled to 0 °C followed by addition of NIS (1.55 g, 6.88 mmole, 2.0 equiv) and TfOH (76.0 μL, 0.86 mmole, 0.25 equiv). The reaction mixture was stirred at 4 °C for 5 h until the disappearance of the donor on TLC, then quenched with 20 % aqueous $Na_2S_2O_3$ solution and saturated $NaHCO_3$. The mixture was diluted with dichloromethane, filtered through Celite. The organic layer was washed with 20 % aqueous $Na_2S_2O_3$ solution, saturated $NaHCO_3$, and brine. The organics were dried over Na_2SO_4, filtered, and concentrated under reduced

pressure. Purification by flash silica gel column chromatography (1 : 2 to 1 : 1 ethyl acetate/hexane) gave **1-15** (1.83 g, 80 %).

1-15 (*α* form) R_f = 0.36 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz $CDCl_3$) δ 5.65 (dd, *J* = 7.5, 1.3 Hz, 1H), 5.34 (dt, *J* = 2.7, 6.4 Hz, 1H), 4.73 (dd, *J* = 9.5, 1.3 Hz, 1H), 4.38 (dd, *J* = 12.3, 2.8 Hz, 1H), 4.22–4.08 (m, 6H), 3.89–3.86 (m, 4H), 3.03 (dd, *J* = 12.2, 4.0 Hz, 1H; H-3 $_{eq}$), 2.71 (t, *J* = 12.7 Hz, 1H; H-3$_{ax}$), 2.53 (s, 3H; CH_3 Ac), 2.17 (s, 3H; CH_3 Ac), 2.13 (s, 3H; CH_3 Ac), 2.06 (s, 3H; CH_3 Ac), 1.72–1.66 (m, 4H; Bu), 1.47–1.40 (m, 4H; Bu), 0.96 (m, 6H; Bu); ^{13}C NMR (150 MHz $CDCl_3$) δ 171.9, 170.6, 170.0, 169.9, 167.3, 167.2, 153.5, 98.2, 98.1, 74.2, 71.5, 69.8, 68.1, 68.0, 68.0, 62.5, 58.3, 53.5, 35.9, 35.9, 32.1, 32.1, 32.1, 32.0, 29.7, 24.6, 21.0, 20.8, 20.7, 18.6, 18.6, 13.6.

1-15 (*β* form) R_f = 0.35 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz $CDCl_3$) δ 5.65 (dd, *J* = 2.1, 2.0 Hz, 1H), 5.26 (td, *J* = 4.3, 2.8 Hz, 1H), 4.72 (dd, *J* = 9.5, 1.7 Hz, 1H), 4.57 (ddd, *J* = 12.2, 11.2, 3.6 Hz, 1H), 4.53 (dd, *J* = 12.2, 7.2 Hz, 1H), 4.15–4.05 (m, 4H), 3.85 (s,3H), 3.75 (dd, *J* = 11.2, 9.7 Hz, 1H; H-4), 2.89 (dd, *J* = 12.8, 3.6 Hz, 1H; H-3 $_{eq}$), 2.49 (s, 3H; CH_3 Ac), 2.29 (dt, *J* = 12.7, 2.6 Hz, 1H; H-3$_{ax}$), 2.11 (s, 3H; CH_3 Ac), 2.08 (s. 3H; CH_3 Ac), 2.02 (s, 3H; CH_3 Ac), 1.68–1.63 (m, 4H; Bu), 1.43–1.37 (m, 4H; Bu), 0.96–0.93 (m, 6H; Bu); ^{13}C NMR (150 MHz $CDCl_3$) δ 172.2, 170.6, 170.6, 169.8, 165.5, 153.4, 108.6, 102.7, 98.8, 98.8, 76.6, 74.0, 72.5, 71.8, 68.5, 68.5, 68.4, 68.3, 62.8, 58.8, 53.5, 36.6, 36.0, 36.0, 32.1, 32.0, 29.7, 28.4, 24.6, 23.3, 23.2, 21.0, 20.8, 18.6, 18.5, 13.5.

HRMS (ESI) calculated for $C_{27}H_{42}NO_{16}PNa$ $[M + Na]^+$: 690.2133, Found: 690.2136.

(1S,2R)-1-((3aR,4R,6R,7aS)-3-acetyl-6-(((2R,3S,4R,5R,6S)-6-((2R,3R,4S,5S,6R)-2-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-3,5-bis (benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-(benzyloxy)-4-hydroxy-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yl)methoxy)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2,3-triyl triacetate (1-2)

Method A

A solution of donor **1-3** (0.30 g, 0.52 mmole, 1.0 equiv), acceptor **1-10** (0.92 g, 0.78 mmole, 1.5 equiv), and activated 4 Å powdered molecular sieves (2.0 g) in anhydrous dichloromethane (40 mL) was stirred overnight under an argon atmosphere, and then cooled to −40 °C followed by addition of NIS (0.29 g, 1.30 mmole, 2.5 equiv) and TfOH (46.0 μL, 0.52 mmole, 1.0 equiv). The reaction mixture was stirred at −40 °C for 5 h until the disappearance of the donor on TLC, then quenched with triethylamine and warmed to room temperature. The mixture was diluted with dichloromethane, filtered through Celite, washed with 20 % aqueous $Na_2S_2O_3$ solution, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash silica gel column chromatography (1 : 2 to 1 : 1.5 ethyl acetate/hexane) gave **1-2** (0.4 g, 47 %) (*α* : *β* = 6 : 1).

Method B

A solution of donor **1-3** (1.06 g, 1.82 mmole, 1.74 equiv), acceptor **1-14a** (1.37 g, 1.05 mmole, 1.0 equiv), and activated 4 Å powdered molecular sieves (3.0 g) in anhydrous dichloromethane (28 mL) was stirred overnight under an argon

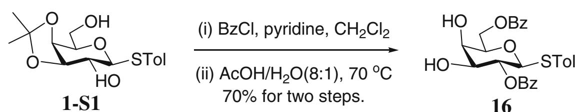
atmosphere, and then cooled to −40 °C followed by addition of NIS (0.52 g, 2.32 mmole, 2.21 equiv) and TfOH (18.0 μL, 0.2 mmole, 0.19 equiv). The reaction mixture was stirred at −40 °C for 2 h until the disappearance of the donor on TLC, then quenched with triethylamine and warmed to room temperature. The mixture was diluted with dichloromethane, filtered through Celite, washed with 20 % aqueous $Na_2S_2O_3$ solution, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash silica gel column chromatography (1 : 3 to 1 : 1.25 ethyl acetate/hexane) gave trisaccharide (1.3 g, 70 %) (*α* only). DDQ (0.26 g, 1.15 mmole) was added to a solution of trisaccharide in CH_2Cl_2/H_2O (10 : 1, 22 mL) at room temperature and stirred overnight. The reaction mixture was diluted with CH_2Cl_2 (30 mL) and washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried with Na_2SO_4, and the solvents were removed under reduced pressure. The residue was purified by flash silica gel column chromatography (1 : 3 to 1 : 1.25 ethylacetate/hexane) to give **1-2** as white solid (0.762 g, 63 %).

One-Pot

A solution of dibutyl sialyl phosphate **1-15** (0.1775 g, 0.27 mmole), acceptor **1-12** (0.1364 g, 0.20 mmole), and pulverized activated 4 Å MS (0.4 g) in dry CH_2Cl_2 (3.5 mL), and acetonitrile (7 mL) was stirred under argon at room temperature for 3 h. The mixture was then cooled to −60 °C, followed by addition of TMSOTf (50 μL, 0.28 mmole) via micro-syringe and stirring for 30 min. Then a solution of the acceptor **1-5** (0.0842 g, 0.11 mmole) in dry CH_2Cl_2 (1.0 mL) was slowly added to the reaction mixture. The mixture was warmed to −20 °C and stirred for 30 min, followed by addition of NIS (0.324 mmole). After 2 h, when TLC indicated that acceptor **1-5** was fully consumed, the reaction was neutralized by triethylamine, diluted with dichloromethane, and filtered with a pad of Celite. The filtrate was poured into a mixture of saturated aq. $NaHCO_3$ and saturated aq. $Na_2S_2O_3$. The aqueous layer was extracted with two portions of ethyl acetate. The combined extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 3 to 1 : 1.5 ethylacetate/hexane) to give trisaccharide as white solid.

DDQ (0.0187 g, 0.082 mmole) was added to a solution of trisaccharide in CH_2Cl_2/H_2O (10 : 1, 5.5 mL) at room temperature and stirred overnight. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried with Na_2SO_4, and the solvents were removed under reduced pressure. The residue was purified by flash silica gel column chromatography (1 : 3 to 1 : 1.5 ethylacetate/hexane) to give **1-2** as white solid (0.077 g, 42 %). **1-2**: R_f = 0.23 (Hexane : EtOAc = 2 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.38–7.11 (m, 30H; Ar–H), 5.58–5.57 (m, 1H), 5.48–5.45 (m, 1H), 5.13 (m, 2H; $PhCH_2$), 5.00–4.93 (m, 2H), 4.82–4.74 (m, 3H), 4.69 (d, *J* = 12.0 Hz, 1H; H^{gal}-1 anomeric), 4.64 (d, *J* = 10.5 Hz, 2H), 4.59–4.55 (m, 2H), 4.50–4.31 (m, 7H), 4.19 (dd, *J* = 11.0, 4.1 Hz, 1H), 3.99–3.93 (m, 2H), 3.85–3.81 (m, 2H), 3.76–3.74 (m, 2H), 3.66 (dd, *J* = 10.6, 9.8 Hz, 1H), 3.62–3.42 (m, 9H), 3.38–3.35 (m, 2H), 3.18–3.10 (m, 3H), 2.87 (dd, *J* = 11.8, 2.9 Hz, 1H; H^{sial}-3 $_{eq}$), 2.45 (s, 3H; CH_3 Ac), 2.12–2.07 (m, 4H; CH_3 Ac, H^{sial}-3_{ax}), 2.00 (s, 3H; CH_3 Ac), 1.74 (s, 3H; CH_3 Ac), 1.56–1.43 (m, 4H; aliphatic), 1.28–1.19 (m, 2H; aliphatic).

The α-form was confirmed by coupling constant $^{3}J(C_1\text{-}H_{3ax})$ = 6.0 Hz; ^{13}C NMR (150 MHz $CDCl_3$) δ 171.9, 170.6, 169.9, 169.9, 168.3, 156.6, 156.0, 155.1, 153.5, 138.8, 138.8, 138.1, 137.8, 137.7, 136.7, 136.6, 128.6, 128.9, 128.5, 128.4, 128.3, 128.3, 128.1, 128.0, 127.8, 127.7, 127.6, 127.3, 127.1, 103.8, 101.8, 99.2, 95.3, 80.2, 79.8, 77.4, 75.7, 75.2, 75.2, 74.8, 74.5, 74.3, 73.7, 73.5, 73.4, 71.4, 69.6, 68.5, 68.7, 68.3, 67.0, 67.0, 64.5, 63.0, 58.9, 58.1, 52.7, 50.4, 50.1, 47.0, 46.0, 36.4, 29.5, 29.2, 27.8, 27.4, 24.6, 23.2, 23.2, 21.1, 20.7, 20.4; HRMS (ESI-TOF, MNa^+) calculated for $C_{82}H_{94}Cl_3N_3O_{26}Na$ 1666.5085, found 1666.5017.



((3R,4S,5S,6R)-5-(benzoyloxy)-3,4-dihydroxy-6-(p-tolylthio)tetrahydro-2H-pyran-2-yl)methyl benzoate (1-16)

To a stirred solution of compound **1-S1** (1.6 g, 4.9 mmole) in pyridine (15 mL) was added benzoyl chloride (15 mL) at 0 °C. The mixture was stirred for 12 h at 40 °C, and methanol (10 mL) was added. The solution was concentrated to syrup which was extracted with dichloromethane. The extract was successively washed with 2 N HCl, water, $NaHCO_{3(aq)}$, water, and dried over Na_2SO_4 and concentrated. Column chromatography (1 : 4 to 1 : 3 ethyl acetate/hexane) of the residue on silica gel gave white solid. To a solution of residue in MeOH/H_2O (1 : 1, 50 mL) was added acetic acid (50 mL) at 25 °C and the resulting mixture was warmed to 80 °C and stirred for 12 h. The solvents were removed by reduced pressure. The residue was purified by flash silica gel column chromatography (1 : 2.5 to 1 : 1 ethyl acetate/hexane) to give **1-16** as white solid (1.69 g, 70 %). **1-16**: R_f = 0.48 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz $CDCl_3$) δ 8.09–8.05 (m, 4H; Ar–H), 7.61–7.59 (m, 2H; Ar–H), 7.48–7.45 (m, 4H; ArH), 7.35 (d, J = 8.00 Hz, 2H; Ar–H), 6.95 (d, J = 8.00 Hz, 2H), 5.19 (dd, J = 9.6, 9.6 Hz, 1H), 4.77 (d, J = 10.0 Hz, 1H; H-1 anomeric), 4.70 (dd, J = 11.6, 5.5 Hz, 1H), 4.60 (dd, J = 11.5, 7.2 Hz, 1H), 4.07 (m, 1H), 3.92 (dd, J = 6.4, 6.4 Hz, 1H), 3.86 (dd, J = 9.0, 2.7 Hz, 1H), 3.50 (s, 1H), 3.03 (s, 1H), 2.27 (s, 3H; CH_3 STol); ^{13}C NMR (150 MHz $CDCl_3$) δ 167.3, 166.7, 138.4, 133.7, 133.5, 133.2, 130.3, 130.0, 129.8, 129.8, 129.5, 128.9, 128.6, 128.6, 86.6, 76.3, 73.9, 72.5, 69.1, 63.6, 21.3.

HRMS (ESI) calculated for $C_{27}H_{26}O_7SNa$ $[M + Na]^+$: 517.1291, Found: 517.1274.

(1S,2R)-1-((3aS,6R,7aS)-3-acetyl-6-((2S,3S,4S,5S)-3-(benzoyloxy)-6-(benzoyloxymethyl)-5-hydroxy-2-(p-tolylthio)tetrahydro-2H-pyran-4-yloxy)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2,3-triyl triacetate (1-17) A solution of donor **1-15** (1.83 g, 2.74 mmole, 1.5 equiv), acceptor **1-16** (0.90 g, 1.83 mmole, 1.0 equiv), and activated 3 Å powdered molecular sieves (3.0 g) in anhydrous dichloromethane (40 mL) was stirred 2–4 h under an argon atmosphere, and then cooled to −40 °C followed by addition of TMSOTf (0.25 mL, 1.35 mmole, 0.75 equiv). After 1 h, an extra amount of

TMSOTf (0.25 mL, 1.35 mmole, 0.75 equiv) was added to the mixture, and the solution was kept stirring at the −40 °C for 4 h until disappearance of the donor on TLC, then quenched with Et_3N (1 mL). The mixture was diluted with dichloromethane and filtered through Celite. The organic layer was washed with 20 % aqueous $Na_2S_2O_3$ solution, saturated $NaHCO_3$, and brine. The organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash silica gel column chromatography (1 : 3 to 1 : 2 to 2 : 3 ethyl acetate/hexane) gave **1-17** (1.47 g, 85 %)($\alpha : \beta = 2 : 1$). **1-17** :$R_f = 0.58$ (Hexane : EtOAc = 2 : 3); ^{1}H NMR (600 MHz $CDCl_3$) δ 8.15 (d, $J = 8.0$ Hz, 2H; Ar–H), 8.05 (d, $J = 7.2$ Hz, 1H; Ar–H), 7.59–7.58 (m, 2H; Ar–H), 7.49–7.44 (m, 4H; Ar–H), 7.35 (m, 2H; Ar–H), 6.95 (d, $J = 7.7$ Hz, 2H; Ar–H), 5.54–5.52 (m, 1H), 5.48 (d, $J = 9.1$ Hz, 1H), 5.41 (dd, $J = 9.7, 9.7$ Hz, 1H), 4.91 (d, $J = 10.1$ Hz, 1H), 4.57–4.55 (m, 2H), 4.56 (dd, $J = 2.8, 9.4$ Hz, 1H), 4.50 (d, $J = 9.4$ Hz, 1H), 4.39 (d, $J = 12.2$ Hz, 1H), 4.00 (dd, $J = 5.9, 5.9$ Hz, 1H), 3.93 (dd, $J = 7.4, 12.1$ Hz, 1H), 3.83 (m, 1H), 3.80 (d, $J = 1.3$ Hz, 1H), 3.72 (s, 3H; CH_3 OMe), 3.53 (dd, $J = 11.0, 11.0$ Hz, 1H), 2.83 (dd, $J = 2.9, 12.1$ Hz, 1H; H^{sial}-3 $_{eq}$), 2.73 (s, 3H; CH_3 STol), 2.41 (s, 3H; CH_3 Ac), 2.26 (s, 3H; CH_3 Ac), 2.06 (s, 3H; CH_3 Ac), 2.06–2.01 (m, 1H; H^{sial}-3_{ax}), 1.98 (s, 3H; CH_3 Ac); ^{13}C NMR (150 MHz $CDCl_3$) δ 178.0, 171.9, 171.3, 170.9, 170.5, 170.2, 168.7, 166.3, 165.5, 153.5, 138.0, 133.4, 133.3, 130.2, 130.1, 130.0, 129.3, 128.6, 97.4, 87.2, 75.6, 75.3, 75.2, 74.9, 71.5, 68.9, 68.3, 67.3, 63.7, 63.6, 60.5, 58.8, 53.4, 36.0, 24.6, 21.2, 21.2, 21.1, 20.8, 20.3, 14.3.

HRMS (ESI) calculated for $C_{46}H_{49}NO_{19}SNa$ $[M + Na]^+$: 974.2512, Found: 974.2510.

(2R)-1-(3-acetyl-6-(((3S,4R,5S,6R)-4-((3S,4S,5S)-4-((3aS,6R,7aS)-3-acetyl-6-(methoxycarbonyl)-2-oxo-4-((1R,2S)-1,2,3-triacetoxypropyl)hexahydro-2H-pyrano[3,4-d]oxazol-6-yloxy)-3-(benzoyloxy)-6-(benzoyloxymethyl)-5-hydroxytetrahydro-2H-pyran-2-yloxy)-6-((2S,3S,4S,5S)-2-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-3,5-bis(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-(benzyloxy)-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yl)methoxy)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2,3-triyl triacetate (1-22).

A solution of donor **1-17** (0.189 g, 0.20 mmole, 2.0 equiv), acceptor **1-2** (0.164 g, 0.10 mmole, 1.0 equiv), and activated 4 Å powdered molecular sieves (2.0 g) in anhydrous dichloromethane (2 mL) was stirred overnight under an argon atmosphere, and then cooled to −40 °C followed by addition of NIS (67 mg, 0.3 mmole, 3.0 equiv) and TfOH (0.009 mL, 0.1 mmole, 1.0 equiv). The reaction mixture was stirred at −20 °C for 5 h, and then quenched with triethylamine and warmed to room temperature. The mixture was diluted with dichloromethane, filtered through Celite, washed with 20 % aqueous $Na_2S_2O_3$ solution, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash silica gel column chromatography (1 : 3 to 1 : 1 ethyl acetate/hexane) gave **1-22** (0.246 g, 55 %). **1-22**: $R_f = 0.31$ (Hexane : EtOAc = 1:1); ^{1}H NMR (400 MHz $CDCl_3$) δ 8.12 (d, $J = 7.6$ Hz, 2H; Ar–H), 7.98 (d, $J = 7.6$ Hz, 2H; Ar–H), 7.59–7.53 (m, 2H; Ar–H), 7.46–7.39 (m, 6H; Ar–H), 7.32–7.21 (m, 28H; Ar–H), 5.55 (m, 2H), 5.45 (dd, $J = 8.2, 8.2$ Hz, 1H), 5.41–5.34 (m, 2H), 5.14 (m, 2H), 4.98 (d,

J = 9.3 Hz, 1H), 4.88–4.85 (m, 1H), 4.79–4.75 (m, 2H), 4.69–4.65 (m, 2H), 4.62–4.56 (m, 2H), 4.51–4.32 (m, 10H), 4.30–3.99 (m, 8H), 3.94–3.85 (m, 5H), 3.80 (m, 2H), 3.74–3.71 (m, 5H), 3.64–3.52 (m, 8H), 3.43 (m, 5H), 3.09 (m, 3H), 2.80 (dd, J = 3.3, 12.1 Hz, 1H; $H^{sial(2\rightarrow6)}$-3 $_{eq}$), 2.60 (dd, J = 3.5, 12.3 Hz, 1H; $H^{sial(2\rightarrow3)}$-3 $_{eq}$), 2.47 (s, 3H; CH_3 Ac), 2.45 (s, 3H; CH_3 Ac), 2.17–2.7 (m, 5H; CH_3 Ac, H^{sial} $^{(2\rightarrow6)}$-3_{ax}, and $H^{sial(2\rightarrow3)}$-3_{ax}), 2.04 (s, 3H; CH_3 Ac), 2.01 (s, 3H; CH_3 Ac), 1.82 (s, 3H; CH_3 Ac), 1.78 (s, 3H; CH_3 Ac), 1.52–1.45 (m, 4H; aliphatic), 1.30–1.23 (m, 2H; aliphatic); ^{13}C NMR (150 MHz $CDCl_3$) δ 171.9, 171.8, 170.7, 170.6, 170.1, 170.0, 169.9, 169.7, 168.4, 168.2, 166.2, 165.0, 153.6, 153.6, 153.4, 139.3, 139.0, 138.5, 137.9, 136.9, 136.7, 133.4, 133.2, 129.9, 129.8, 129.6, 128.7, 128.5, 128.4, 128.3, 128.2, 128.2, 128.2, 127.9, 127.9, 127.8, 127.4, 127.3, 127.1, 126.8, 103.9, 100.7, 99.3, 98.1, 96.1, 80.6, 79.6, 78.6, 76.1, 75.5, 75.1, 74.8, 74.6, 74.6, 74.4, 74.2, 73.5, 71.6, 71.3, 71.3, 71.0, 69.7, 68.9, 68.7, 68.4, 67.1, 64.5, 63.0, 62.7, 62.4, 60.4, 59.1, 58.8, 58.3, 53.3, 52.7, 50.4, 50.1, 47.1, 46.1, 36.4, 35.2, 31.9, 31.6, 29.7, 29.3, 27.8, 27.4, 24.7, 24.6, 23.2, 22.7, 21.1, 21.0, 20.9, 20.7, 20.6, 14.2, 14.1.

HRMS (MALDI) calculated for $C_{121}H_{135}Cl_3N_4O_{45}Na$ $[M + Na]^+$: 2491.7356, Found: 2491.8092.

(2S,3R,4S,5R,6R)-6-(benzyloxymethyl)-4,5-dihydroxy-2-(p-tolylthio)tetrahydro-2H-pyran-3-yl benzoate (1-26)

A solution of compound **1-25** (0.7 g, 1.464 mmole) and pulverized activated 4 Å MS (1.7 g) in CH_2Cl_2 was stirred at room temperature. After stirring for 1 h, the mixture was cooled to −78 °C. Then, to the stirred solution, Et_3SiH (0.71 mL, 4.39 mmole) and TfOH (0.4 mL, 4.5 mmole) were added successively. After stirring for 1 h at −78 °C, Et_3N (5 mL) and MeOH (5 mL) were added successively, and the mixture was diluted with $CHCl_3$, washed with aqueous $NaHCO_3$, dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by flash silica gel column chromatography (1 :2 to 1 :1 ethylacetate/hexane) to give **1-26** as white solid (0.6 g, 85 %). **1-26**: R_f = 0.38 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.06 (dd, J = 8.0, 0.95 Hz, 2H; Ar–H), 7.58–7.55 (m, 1H; Ar–H), 7.43 (dd, J = 7.7, 7.7 Hz, 2H; Ar–H), 7.37–7.28 (m, 7H; Ar–H), 7.02 (d, J = 7.9 Hz, 2H; Ar–H), 5.20 (t, J = 9.6 Hz, 1H), 4.72 (d, J = 9.75 Hz, 1H; H-1 anomeric), 4.59–4.55 (m, 2H; $PhCH_2$), 4.06 (t, J = 3.5 Hz, 1H), 3.82 (d, J = 5.3 Hz, 2H), 3.78–3.74 (m, 1H), 3.70–3.68 (m, 1H), 3.41 (d, J = 7.8 Hz, 1H), 3.17 (d, J = 4.0 Hz, 1H), 2.28 (s, 3H; CH_3 STol); ^{13}C NMR (150 MHz $CDCl_3$) δ 166.8, 138.1, 137.7, 133.3, 133.1, 130.0, 129.6, 129.6, 128.7, 128.4, 128.4, 127.8, 127.8, 86.3, 77.3, 73.9, 73.7, 72.1, 69.7, 69.7, 21.1; HRMS (ESI-TOF, MNa^+) Calculated for $C_{27}H_{28}O_6SNa$ 503.1499, found 503.1494.

(3aR,4R,6S,7aS)-methyl-4-((4S,5R)-5-(benzyloxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)-2-oxo-6-(phenylthio)hexahydro-2H-pyrano[3,4-d]oxazole-6-carboxylate (1-28)

To a solution compound **1-27** (5.00 g, 10.2 mmole, 1.00 eq.) in acetone dimethyl acetal (40.0 ml, 4 mL/mmole) was added 10-camphorsulfonic acid (2.40 g, 10.2 mmole, 1.00 eq.) at room temperature under argon. After being stirred at room temperature for 4 h, the reaction mixture was neutralized with Et_3N (4 ml) and

evaporated in vacuo. The crude product was purified by flash silica gel column chromatography (1 : 2 to 1 : 1 ethylacetate/hexane) to give **1-28** as white solid (4.60 g, 8.69 mmole, 85 %). **1-28**: R_f = 0.50 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.66 (d, *J* = 7.9 Hz, 2H; Ar–H), 7.38–7.21 (m, 8H; Ar–H), 6.40 (s, 1H), 4.48–4.42 (m, 2H), 4.30 (d, *J* = 11.9 Hz, 1H), 4.08–4.05 (m, 1H), 3.86–3.82 (m, 1H), 3.78–3.75 (m, 1H), 3.71–3.68 (m, 1H), 3.62–3.56 (m, 2H), 3.46 (s, 3H; CH_3 OMe), 3.16 (dd, *J* = 11.6, 2.9 Hz, 1H; H-3 $_{eq}$), 2.14 (t, *J* = 12.2 Hz, 1H; H-3_{ax}), 1.72 (s, 3H; CH_3), 1.40 (s, 3H; CH_3); ^{13}C NMR (150 MHz $CDCl_3$) δ 168.4, 160.0, 137.8, 135.7, 129.9, 129.8, 129.3, 128.5, 128.1, 127.8, 109.9, 88.9, 77.5, 75.9, 75.6, 73.9, 73.6, 68.9, 58.7, 52.8, 35.8, 26.4, 25.6; HRMS (ESI-TOF, MNa^+) calculated for $C_{27}H_{31}NO_8SNa$ 552.1663, found 552.1625.

(3aR,4R,6S,7aS)-methyl 4-((4S,5R)-5-(benzyloxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)-6-(dibutoxyphosphoryloxy)-2-oxohexahydro-2H-pyrano[3,4-d] oxazole-6-carboxylate (1-29)

A solution of thiosialoside donor **1-28** (6.2 g, 11.71 mmole), dibutyl phosphate (6.8 mL, 35.23 mmole) and pulverized activated 4 Å MS (8 g) in dry CH_2Cl_2 (140 mL) was stirred under argon at room temperature for 3 h. The reaction mixture was then cooled to 0 °C, followed by the addition of NIS (5.55 g, 24.67 mmole) and 0.5 M TfOH solution in dry Et_2O (6.8 mL, 3.4 mmole). After stirring for 10 h, the reaction mixture was diluted with dichloromethane and filtered through a pad of Celite. The filtrate was then poured into a mixture of saturated aq. $Na_2S_2O_3$ and $NaHCO_3$. The aqueous layer was extracted with two portions of dichloromethane. The collected organic phases were then washed with brine, dried over Mg_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 2 to 1 : 1.25 ethylacetate/hexane) to give **1-29** as oil (5.36 g, 72 %). **1-29**: R_f = 0.40 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.31–7.25 (m, 4H; Ar–H), 7.22–7.19 (m, 1H; Ar–H), 5.56 (s, 1H; NH), 4.54 (m, 2H; $PhCH_2$), 4.43 (q, *J* = 6.3 Hz, 1H), 4.27 (dd, *J* = 9.7, 1.5 Hz, 1H), 4.08–3.94 (m, 7H), 3.75 (s, 3H; CH_3 OMe), 3.59 (t, *J* = 10.5 Hz, 1H; H-4), 2.90 (dd, *J* = 11.8, 3.4 Hz, 1H; H-3 $_{eq}$), 2.26 (t, *J* = 12.3 Hz, 1H; H-3_{ax}), 1.61–1.55 (m, 4H; Bu), 1.42 (s, 3H; CH_3), 1.37–1.29 (m, 4H; Bu), 1.27 (s, 3H; CH_3), 0.88–0.83 (m, 6H; Bu); ^{13}C NMR (150 MHz $CDCl_3$) δ 167.8, 167.8, 159.7, 159.6, 138.0, 128.2, 128.0, 127.5, 109.3, 99.2, 99.2, 76.1, 76.0, 76.0, 75.2, 73.2, 68.4, 68.2, 68.1, 67.7, 67.7, 57.6, 53.1, 38.1, 38.0, 32.0, 31.9, 31.9, 31.9, 26.2, 18.5, 13.4, 13.4; HRMS (ESI-TOF, MNa^+) Calculated for $C_{29}H_{44}N_1O_{12}P_1Na$ 652.2493, found 652.2495.

(1S,2R)-3-(benzyloxy)-1-((3aR,4R,6S,7aS)-6-(methoxycarbonyl)-2-oxo-6-(phenylthio)hexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2-diyl diacetate (1-30)

A solution of compound **1-27** (5.0 g, 10.21 mmole) in pyridine (10 mL) was treated with Ac_2O (12 mL) and stirred at room temperature for 2 h, then concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (1 : 2 to 1 : 1.25 ethylacetate/hexane) to give **1-30** as a white solid (4.8 g, 81 %). **1-30**: R_f = 0.55 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.48–7.46 (m, 2H; Ar–H), 7.39–7.29 (m, 5H; Ar–H), 7.27 (dd, *J* = 6.9, 1.3 Hz, 2H; Ar–H), 5.42 (s, 1H), 5.31–5.26 (m, 2H), 4.60 (d, *J* = 12.1 Hz, 1H;

PhCH), 4.36 (d, J = 12.1 Hz, 1H; PhCH), 4.06 (dd, J = 9.9, 1.3 Hz, 1H), 3.92 (ddd, J = 12.6, 11.0, 3.7 Hz, 1H), 3.74 (dd, J = 11.2, 1.6 Hz, 1H), 3.61 (dd, J = 11.2, 2.3 Hz, 1H), 3.52 (s, 3H; CH_3 OMe), 3.12 (dd, J = 12.1, 3.6 Hz, 1H; H-3 $_{eq}$), 2.98–2.94 (m, 1H; H-4), 2.12 (t, J = 12.4 Hz, 1H; H-3$_{ax}$), 2.06 (s, 3H; CH_3 Ac), 2.00 (s, 3H; CH_3 Ac); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1, 169.8, 167.6, 159.0, 137.4, 135.9, 129.8, 128.8, 128.6, 128.5, 128.1, 127.9, 88.2, 75.3, 73.4, 69.1, 68.9, 67.0, 57.9, 52.8, 37.7, 21.0, 20.7; HRMS (ESI-TOF, M^+) calculated for $C_{28}H_{31}NO_{10}S$ 596.1561, found 596.1575.

(1S,2R)-3-(benzyloxy)-1-((3aR,4R,6S,7aS)-6-(dibutoxyphosphoryloxy)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2-diyl diacetate (1-31)

A solution of thiosialoside donor **1-30** (1.3 g, 2.27 mmole), dibutyl phosphate (1.3 mL, 6.99 mmole), and pulverized activated 4 Å MS (2 g) in dry CH_2Cl_2 (25 mL) was stirred under argon at room temperature for 3 h. The reaction mixture was then cooled to 0 °C, followed by the addition of NIS (1.03 g, 4.58 mmole) and 0.5 M TfOH solution in dry Et_2O (0.9 mL, 0.45 mmole). After stirring for 10 h, the reaction mixture was diluted with dichloromethane and filtered through a pad of Celite. The filtrate was then poured into a mixture of saturated aq. $Na_2S_2O_3$ and $NaHCO_3$. The aqueous layer was extracted with two portions of dichloromethane. The collected organic phases were then washed with brine, dried over Mg_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 2 to 1 : 1.25 ethylacetate/hexane) to give **1-31** as oil (1.05 g, 69 %). **1-31**: R_f = 0.40 (Hexane : EtOAc = 1 : 1); ^1H NMR (600 MHz, $CDCl_3$) δ 7.33–7.22 (m, 5H; Ar–H), 5.45 (s, 1H), 5.30 (dd, J = 9.8, 1.5 Hz, 1H), 5.22 (dt, J = 9.8, 2.5 Hz, 1H), 4.58 (d, J = 12.2 Hz, 1H; PhCH), 4.42 (dd, J = 10.0, 1.6 Hz, 1H), 4.33 (d, J = 12.2 Hz, 1H; PhCH), 4.09–3.96 (m, 5H), 3.78 (s, 3H; CH_3 OMe), 3.65 (dd, J = 11.2, 2.0 Hz, 1H), 3.50 (dd, J = 11.1, 2.9 Hz, 1H), 3.20–3.16 (m, 1H; H-4), 2.87 (dd, J = 12.0, 3.8 Hz, 1H; H-3 $_{eq}$), 2.60 (t, J = 12.7 Hz, 1H; H-3$_{ax}$), 2.12 (s, 3H; CH_3 Ac), 1.96 (s, 3H; CH_3 Ac), 1.63–1.58 (m, 4H; Bu), 1.39–1.32 (m, 4H; Bu), 0.90–0.87 (m, 6H; Bu); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.4, 171.4, 169.9, 167.5, 167.5, 159.4, 137.5, 128.6, 128.2, 128.1, 99.1, 99.0, 76.2, 75.5, 73.5, 68.9, 68.8, 68.1, 68.1, 68.0, 68.0, 67.2, 57.5, 53.4, 37.4, 32.3, 32.2, 32.2, 32.2, 29.7, 21.1, 20.7, 18.8, 18.7, 13.7, 13.7; HRMS (ESI-TOF, M^+) calculated for $C_{30}H_{44}NO_{14}P$ 674.2572, found 674.2585.

(3aR,4R,6S,7aS)-methyl-3-acetyl-4-((4S,5R)-5-(benzyloxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)-2-oxo-6-(phenylthio)hexahydro-2H-pyrano[3,4-d]oxazole-6-carboxylate (1-32)

The compound **1-28** (1.22 g, 2.30 mmole) was dissolved in anhydrous CH_2Cl_2 (10 mL), treated with $EtN(i\text{-}Pr)_2$ (3.8 mL, 23 mmole, 10 equiv), and cooled to 0 °C before acetyl chloride (1.3 mL, 18.2 mmole, 8 equiv) was added dropwise, then the solution stirred at 0 °C for 1 h. After warming to room temperature, the reaction mixture was diluted with CH_2Cl_2 (10 mL) and washed with saturated aqueous $NaHCO_3$. The aqueous layer was extracted with two portions of ethyl acetate. The combined extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash silica gel column

chromatography (1 : 2 to 1 : 1.5 ethylacetate/hexane) to give **1-32** as a white solid (4.8 g, 81 %). **1-32**: R_f = 0.69 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.74 (d, *J* = 6.9 Hz, 2H; Ar–H), 7.40 (t, *J* = 4.3 Hz, 1H; Ar–H), 7.36 (t, *J* = 7.5 Hz, 2H; Ar–H), 7.30 (t, *J* = 5.6 Hz, 2H; Ar–H), 7.29–7.25 (m, 3H; Ar–H), 4.97 (d, *J* = 7.3 Hz, 1H), 4.53–4.47 (m, 2H), 4.30 (d, *J* = 12.0 Hz, 1H; PhCH), 4.03–3.95 (m, 2H), 3.88–3.79 (m, 2H), 3.75–3.73 (m, 1H; H-4), 3.51 (s, 3H; CH_3 OMe), 3.18 (dd, *J* = 11.8, 3.5 Hz, 1H; H-3 $_{eq}$), 2.46 (s, 3H; CH_3 Ac), 2.17 (t, *J* = 12.2 Hz, 1H; H-3_{ax}), 1.79 (s, 3H; CH_3), 1.47 (s, 3H; CH_3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.4, 168.2, 153.7, 140.5, 138.3, 137.0, 136.9, 130.2, 129.6, 128.8, 128.2, 127.8, 127.7, 127.5, 125.0, 109.8, 88.6, 78.1, 76.6, 76.2, 75.5, 72.8, 67.8, 59.9, 52.6, 36.6, 26.5, 25.8, 25.2, 21.3; HRMS (ESI-TOF, M^+) calculated for $C_{29}H_{33}NO_9S$ 597.1768, found 594.1782.

(3aR,4R,6S,7aS)-methyl-3-acetyl-4-((4S,5R)-5-(benzyloxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)-6-(dibutoxyphosphoryloxy)-2-oxohexahydro-2H-pyrano[3,4-d]oxazole-6-carboxylate (1-33)

A solution of thiosialoside donor **1-32** (1.28 g, 2.24 mmole), dibutyl phosphate (1.3 mL, 6.99 mmole), and pulverized activated 4 Å MS (2 g) in dry CH_2Cl_2 (29 mL) was stirred under argon at room temperature for 3 h. The reaction mixture was then cooled to 0 °C, followed by the addition of NIS (1.03 g, 4.58 mmole) and 0.5 M TfOH solution in dry Et_2O (0.9 mL, 0.45 mmole). After stirring for 10 h, the reaction mixture was diluted with dichloromethane and filtered through a pad of Celite. The filtrate was then poured into a mixture of saturated aq. $Na_2S_2O_3$ and $NaHCO_3$. The aqueous layer was extracted with two portions of dichloromethane. The collected organic phases were then washed with brine, dried over Mg_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 2 to 1 : 1.5 ethylacetate/hexane) to give **1-33** as oil (0.48 g, 32 %). **1-33**: R_f = 0.63 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.38–7.28 (m, 5H; Ar–H), 4.95 (dd, *J* = 7.1, 1.7 Hz, 1H), 4.56 (m, 2H), 4.52–4.46 (m, 2H), 4.36 (dd, *J* = 9.4, 1.7 Hz, 1H), 4.18–4.04 (m, 4H), 3.96–3.84 (m, 3H), 3.83 (s, 3H; CH_3 OMe), 2.97 (dd, *J* = 13.0, 3.7 Hz, 1H; H-3 $_{eq}$), 2.53 (t, *J* = 12.9 Hz, 1H; H-3_{ax}), 2.50 (s, 3H; CH_3 Ac), 1.70–1.63 (m, 4H; Bu), 1.44–1.36 (m, 10H; Bu and 2 ^{X} CH_3), 0.95–0.92 (m, 4H; Bu); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.8, 128.6, 127.9, 109.5, 100.1, 100.0, 76.4, 76.3, 75.8, 74.5, 73.5, 69.0, 68.5, 68.5, 59.8, 53.5, 35.6, 32.4, 32.3, 32.3, 26.4, 25.8, 25.2, 18.8, 18.8, 13.7, 13.7; HRMS (ESI-TOF, M^+) calculated for $C_{31}H_{46}NO_{13}P$ 694.2599, found 694.2611.

(1S,2R)-1-((3aR,4R,6S,7aS)-3-acetyl-6-((2R,3S,4R,5R,6S)-3-(benzoyloxy)-6-(benzyloxymethyl)-5-hydroxy-2-(p-tolylthio)tetrahydro-2H-pyran-4-yloxy)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2,3-triyl triacetate (1-34)

A solution of sialyl phosphate donor **1-15** (1.2 g, 1.80 mmole), acceptor **1-26** (0.69 g, 1.43 mmole), and pulverized activated 4 Å MS (2.2 g) in CH_2Cl_2 was stirred under argon at room temperature for 2 h. The reaction mixture was then cooled to −78 °C, followed by addition of TMSOTf (0.46 mL, 2.54 mmole) via syringe. After stirring at the same temperature for 2 h, the reaction mixture was neutralized with triethylamine, diluted with dichloromethane, and filtered through a

pad of Celite. The filtrate was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash silica gel column chromatography (1 : 2 to 1 : 1.25 ethylacetate/hexane) to give **1-34** as white solid (0.87 g, 65 %).

1-34 (*α* form) :R_f = 0.35 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.16 (dd, *J* = 8.1, 1.1 Hz, 2H; Ar–H), 7.59–7.56 (m, 1H; Ar–H), 7.47 (t, *J* = 7.8 Hz, 2H; Ar–H), 7.36–7.27 (m, 7H; Ar–H), 7.03 (d, *J* = 8.1 Hz, 3H; Ar–H), 5.53–5.50 (m, 1H), 5.47 (dd, *J* = 8.7, 1.8 Hz, 1H), 5.37 (t, *J* = 9.7 Hz, 1H), 4.87 (d, *J* = 10.0 Hz, 1H), 4.59–4.55 (m, 2H; $PhCH_2$), 4.47 (td, *J* = 9.5, 2.4 Hz, 2H), 4.41 (dd, *J* = 12.3, 2.5 Hz, 1H), 3.93 (dd, *J* = 12.3, 7.3 Hz, 1H), 3.85–3.78 (m, 5H), 3.70 (s, 3H; CH_3 OMe), 3.52 (dd, *J* = 11.2, 9.4 Hz, 1H), 2.83 (dd, *J* = 12.0, 3.4 Hz, 1H; H^{sial}-3 $_{eq}$), 2.73 (d, *J* = 3.0 Hz, 1H), 2.42 (s, 3H; CH_3 STol), 2.29 (s, 3H; CH_3 Ac), 2.11 (s, 3H; CH_3 Ac), 2.03 (t, *J* = 12.8 Hz, 1H; H^{sial}-3_{ax}), 2.00 (s, 3H; CH_3 Ac), 1.46 (s, 3H; CH_3 Ac). The *α*-form was confirmed by coupling constant $^3J(C_1$-$H_{3ax})$ = 5.9 Hz; ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.8, 170.7, 170.4, 169.9, 168.4, 165.2, 153.4, 138.1, 138.04, 133.2, 130.1, 129.5, 128.8, 128.5, 128.3, 127.7, 127.6, 97.5, 86.9, 76.5, 75.5, 75.1, 74.9, 73.5, 71.5, 69.0, 69.0, 68.5, 67.5, 63.3, 58.7, 53.2, 35.8, 24.5, 21.2, 21.1, 20.7, 20.1.

1-34 (*β* form) R_f = 0.50 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.03 (dd, *J* = 8.3, 1.2 Hz, 2H; Ar–H), 7.64–7.60 (m, 1H; Ar–H), 7.49 (t, *J* = 7.9 Hz, 2H; Ar–H), 7.39–7.29 (m, 7H; Ar–H), 7.03 (d, *J* = 7.9 Hz, 2H; Ar–H), 5.49 (dt, *J* = 10.6, 5.5 Hz, 2H), 5.36 (t, *J* = 9.7 Hz, 1H), 4.74 (d, *J* = 10.0 Hz, 1H), 4.61–4.56 (m, 3H), 4.50 (dd, *J* = 9.3, 2.3 Hz, 1H), 4.43–4.36 (m, 1H), 4.08 (t, *J* = 3.0 Hz, 1H), 4.00 (dd, *J* = 12.1, 7.6 Hz, 1H), 3.93 (dd, *J* = 9.4, 3.1 Hz, 1H), 3.85 (d, *J* = 5.1 Hz, 2H), 3.71 (t, *J* = 5.2 Hz, 1H), 3.61 (dd, *J* = 11.1, 9.6 Hz, 1H), 3.53–3.48 (m, 4H), 2.62 (dd, *J* = 12.3, 3.7 Hz, 1H; H^{sial}-3 $_{eq}$), 2.40 (s, 3H; CH_3 STol), 2.30 (s, 3H; CH_3 Ac), 2.08 (s, 3H; CH_3 Ac), 2.05 (s, 3H; CH_3 Ac), 1.99 (s, 3H; CH_3 Ac), 1.98–1.93 (m, 1H; H^{sial}-3_{ax}). The $^3J(C_1$-$H_{3ax})$ coupling constant of the S6 (*β*-form) was too small to be detected; ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.5, 171.0, 170.7, 169.7, 166.4, 165.2, 153.4, 138.3, 137.8, 133.6, 133.4, 129.7, 129.5, 128.6, 128.4, 128.0, 127.7, 127.7, 98.8, 86.2, 77.7, 74.9, 74.1, 73.7, 72.4, 70.2, 69.8, 68.6, 68.5, 63.3, 59.0, 52.9, 35.3, 24.5, 21.1, 21.0, 20.8, 20.7.HRMS (ESI-TOF, M^+) calculated for $C_{46}H_{51}NO_{18}S$ 960.2719, found 960.2756.

(3aR,4R,6S,7aS)-methyl 6-((2S,3R,4S,5S,6R)-3-(benzoyloxy)-6-(benzyloxymethyl)-5-hydroxy-2-(p-tolylthio)tetrahydro-2H-pyran-4-yloxy)-4-((4S,5R)-5-(benzyloxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazole-6-carboxylate (1-35)

A solution of sialyl phosphate donor **1-29** (3.28 g, 5.21 mmole), acceptor **1-26** (2 g, 4.17 mmole), and pulverized activated 4 Å MS (6.2 g) in CH_2Cl_2 was stirred under argon at room temperature for 2 h. The reaction mixture was then cooled to −78 °C, followed by the addition of TMSOTf (1.51 mL, 8.32 mmole) via syringe. After stirring at the same temperature for 2 h the reaction mixture was neutralized with triethylamine (3 mL), diluted with dichloromethane, and filtered through a pad of Celite. The filtrate was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash silica gel column

chromatography (1 : 2 to 1 : 1.25 ethylacetate/hexane) to give **1-35** as white solid (3.75 g, 87 %). **1-35**: R_f = 0.34 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.01 (dd, J = 8.5, 1.2 Hz, 2H; Ar–H), 7.59–7.56 (m, 1H; Ar–H), 7.44 (dd, J = 7.7, 7.7 Hz, 2H; Ar–H), 7.34 (d, J = 8.0 Hz, 2H; Ar–H), 7.31–7.24 (m, 10H; Ar–H), 7.00 (d, J = 8.0 Hz, 2H; Ar–H), 5.48 (s, 1H), 5.42 (t, J = 9.8 Hz, 1H), 4.70 (d, J = 10.0 Hz, 1H), 4.54 (dd, J = 9.5, 6.4 Hz, 2H), 4.51 (dd, J = 10.0, 7.2 Hz, 2H), 4.45–4.42 (m, 1H), 4.30 (dd, J = 9.6, 3.0 Hz, 1H), 4.13 (s, 1H), 4.04 (dd, J = 7.0, 1.7 Hz, 1H), 3.93–3.85 (m, 3H), 3.81–3.74 (m, 3H), 3.68–3.66 (m, 4H), 3.42 (t, J = 10.6 Hz, 1H; H^{sial}-4), 2.61–2.58 (m, 2H; NH and H^{sial}-3 $_{eq}$), 2.27 (s, 3H; CH_3 STol), 1.94 (t, J = 12.5 Hz, 1H; H^{sial}-3_{ax}), 1.73(s, 1H), 1.41 (s, 3H; CH_3), 1.30 (s, 3H; CH_3). The α-form was confirmed by coupling constant $^3J(C_1\text{-}H_{3ax})$ = 6.1 Hz; ^{13}C NMR (150 MHz $CDCl_3$) δ 168.7, 165.3, 159.8, 138.2, 137.9, 133.6, 133.1, 129.9, 129.7, 129.2, 128.7, 128.6, 128.5, 128.0, 127.9, 127.8, 127.8, 109.1, 99.9, 87.2, 77.5, 76.9, 76.2, 75.6, 75.3, 75.1, 73.7, 73.6, 69.6, 68.9, 68.7, 68.5, 58.2, 53.4, 36.5, 27.1, 24.7, 21.3; HRMS (ESI-TOF, M^+) calculated for $C_{48}H_{53}NO_{14}S$ 900.3260, found 900.3283.

(1S,2R)-1-((3aR,4R,6S,7aS)-6-((2R,3S,4R,5R,6S)-3-(benzoyloxy)-6-(benzyl-oxymethyl)-5-hydroxy-2-(p-tolylthio)tetrahydro-2H-pyran-4-yloxy)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)-3-(benzyl-oxy)propane-1,2-diyl diacetate (1-36)

A solution of sialyl phosphate donor **1-31** (1.05 g, 1.56 mmole), acceptor **1-26** (0.61 g, 1.27 mmole), and pulverized activated 4 Å MS (2.5 g) in CH_2Cl_2 was stirred under argon at room temperature for 2 h. The reaction mixture was then cooled to −78 °C, followed by addition of TMSOTf (0.36 mL, 1.98 mmole) via syringe. After stirring at the same temperature for 2 h, the reaction mixture was neutralized with triethylamine, diluted with dichloromethane, and filtered through a pad of Celite. The filtrate was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash silica gel column chromatography (1 : 2 to 1 : 1.25 ethylacetate/hexane) to give **1-36** as white solid (0.62 g, 52 %).

1-36 (α form) : R_f = 0.35 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.03 (dd, J = 8.2, 1.1 Hz, 3H; Ar–H), 7.50–7.46 (m, 1H; Ar–H), 7.38–7.27 (m, 12H; Ar–H), 7.25–7.22 (m, 2H; Ar–H), 7.01 (d, J = 7.9 Hz, 2H; Ar–H), 5.45 (dt, J = 4.8, 2.6 Hz, 1H), 5.32–5.26 (m, 2H), 5.21 (dd, J = 10.2, 2.2 Hz, 1H), 4.84 (d, J = 9.9 Hz, 1H), 4.63 (d, J = 12.1 Hz, 1H), 4.60–4.54 (m, 2H; $PhCH_2$), 4.37 (dd, J = 9.4, 3.0 Hz, 1H), 4.31 (d, J = 12.1 Hz, 1H), 4.07 (dd, J = 9.9, 2.2 Hz, 1H), 3.84–3.74 (m, 4H), 3.69 (s, 3H; CH_3 OMe), 3.47 (dd, J = 11.0, 1.8 Hz, 1H), 3.36 (dd, J = 11.0, 3.0 Hz, 1H), 2.86 (dd, J = 12.0, 3.3 Hz, 1H; H^{sial}-3 $_{eq}$), 2.81–2.75 (m, 1H; H^{sial}-4), 2.57 (d, J = 2.3 Hz, 1H), 2.29 (s, 3H; CH_3 STol), 2.15 (s, 3H; CH_3 Ac), 1.93 (t, J = 12.2 Hz, 1H; H^{sial}-3_{ax}), 1.31 (s, 3H; CH_3 Ac). The α-form was confirmed by coupling constant $^3J(C_1\text{-}H_{3ax})$ = 5.9 Hz; ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.0, 170.2, 168.4, 164.9, 159.0, 138.1, 138.0, 137.2, 133.0, 133.0, 130.1, 130.0, 129.5, 128.7, 128.5, 128.3, 128.3, 128.2, 128.0, 127.7, 127.5, 98.3, 86.5, 76.5, 76.4, 75.4, 73.7, 73.5, 73.3, 69.0, 68.9, 68.4, 67.4, 67.3, 57.6, 53.1, 36.9, 21.4, 21.1, 19.9.

1-36 (*β* form) $R_f = 0.34$ (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.03 (d, $J = 7.4$ Hz, 2H; Ar–H), 7.62 (t, $J = 7.4$ Hz, 1H; Ar–H), 7.48 (t, $J = 7.7$ Hz, 2H; Ar–H), 7.37–7.26 (m, 12H; Ar–H), 7.03 (d, $J = 8.0$ Hz, 2H; Ar–H), 5.35–5.30 (m, 2H), 5.28 (s, 1H), 5.15 (dd, $J = 5.9$, 3.3 Hz, 1H), 4.70 (d, $J = 10.0$ Hz, 1H), 4.57–4.52 (m, 3H), 4.42 (d, $J = 12.0$ Hz, 1H), 4.27 (qd, $J = 7.1$, 3.9 Hz, 2H), 3.97 (t, $J = 3.4$ Hz, 1H), 3.82–3.73 (m, 4H), 3.60–3.55 (m, 2H), 3.54 (s, 3H; CH_3 OMe), 3.30 (d, $J = 3.9$ Hz, 1H), 2.95 (t, $J = 10.5$ Hz, 1H; H^{sial}-4), 2.56 (dd, $J = 12.5$, 3.8 Hz, 1H; H^{sial}-3 $_{eq}$), 2.30 (s, 3H; CH_3 STol), 2.10 (s, 3H; CH_3 Ac), 1.97 (s, 3H; CH_3 Ac), 1.90 (t, $J = 12.5$ Hz, 1H; H^{sial}-3_{ax}). The *β*-form was confirmed by coupling constant $^3J(C_1\text{-}H_{3ax}) = 1.2$ Hz; ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.8, 170.7, 166.8, 165.2, 158.9, 138.3, 137.8, 137.5, 133.6, 133.5, 129.6, 129.5, 128.6, 128.5, 128.4, 128.1, 127.9, 127.7, 99.6, 86.3, 78.1, 76.0, 73.7, 73.3, 72.7, 70.7, 70.2, 69.6, 68.4, 68.1, 68.1, 58.2, 52.9, 35.5, 21.1, 21.0, 20.6. HRMS (ESI-TOF, M^+) calculated for $C_{49}H_{53}NO_{16}S$ 944.3158, found 944.3194.

(3aR,4R,6S,7aS)-methyl-3-acetyl-6-((2R,3S,4R,5R,6S)-3-(benzoyloxy)-6-(benzyloxymethyl)-5-hydroxy-2-(p-tolylthio)tetrahydro-2H-pyran-4-yloxy)-4-((4S,5R)-5-(benzyloxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)-2-oxohexahy-dro-2H-pyrano[3,4-d]oxazole-6-carboxylate (1-37)

A solution of sialyl phosphate donor **1-33** (0.52 g, 0.78 mmole), acceptor **1-26** (0.30 g, 0.63 mmole), and pulverized activated 4Å MS (2.5 g) in CH_2Cl_2 was stirred under argon at room temperature for 2 h. The reaction mixture was then cooled to −78 °C, followed by addition of TMSOTf (0.17 mL, 0.94 mmole) via syringe. After stirring at the same temperature for 2 h, the reaction mixture was neutralized with triethylamine, diluted with dichloromethane, and filtered through a pad of Celite. The filtrate was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash silica gel column chromatography (1 : 3 to 1 : 1.5 ethylacetate/hexane) to give **1-37** as white solid (0.51 g, 80 %). **1-37**: $R_f = 0.57$ (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.03 (dd, $J = 8.3$, 1.2 Hz, 2H; Ar–H), 7.60–7.57 (m, 1H; Ar–H), 7.46 (t, $J = 7.8$ Hz, 2H; Ar–H), 7.36 (d, $J = 8.0$ Hz, 2H; Ar–H), 7.32–7.24 (m, 10H; Ar–H), 7.02 (d, $J = 8.0$ Hz, 2H; Ar–H), 5.46 (t, $J = 9.9$ Hz, 1H), 4.84 (dd, $J = 7.3$, 2.1 Hz, 1H), 4.72 (d, $J = 10.1$ Hz, 1H), 4.56 (s, 2H), 4.53 (d, $J = 12.0$ Hz, 1H), 4.49 (dd, $J = 9.7$, 3.1 Hz, 1H), 4.45–4.41 (m, 2H), 4.20 (dd, $J = 9.1$, 2.1 Hz, 1H), 4.16 (t, $J = 2.5$ Hz, 1H), 3.90 (ddd, $J = 13.3$, 11.4, 3.5 Hz, 1H), 3.84 (dd, $J = 10.3$, 4.9 Hz, 1H), 3.81–3.75 (m, 2H), 3.73–3.70 (m, 5H), 2.65 (dd, $J = 12.1$, 3.5 Hz, 1H; H^{sial}-3 $_{eq}$), 2.63 (d, $J = 2.2$ Hz, 1H), 2.42 (s, 3H; CH_3 STol), 2.29 (s, 3H; CH_3 Ac), 1.89 (t, $J = 13.0$ Hz, 1H; H^{sial}-3_{ax}), 1.50 (s, 3H; CH_3 Ac), 1.40 (s, 3H; CH_3 Ac). The *α*-form was confirmed by coupling constant $^3J(C_1\text{-}H_{3ax}) = 6.0$ Hz; ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.8, 169.1, 165.3, 153.9, 138.2, 138.1, 138.1, 133.5, 133.2, 129.9, 129.8, 129.7, 129.1, 128.6, 128.5, 128.0, 127.9, 127.8, 108.5, 98.2, 87.1, 76.8, 76.3, 75.9, 75.4, 74.5, 73.7, 73.5, 69.5, 69.3, 68.6, 68.1, 60.1, 53.4, 36.3, 27.3, 25.2, 24.7, 21.3; HRMS (ESI-TOF, M^+) calculated for $C_{50}H_{55}NO_{15}S$ 964.3185, found 964.3219.

(3aR,4R,6S,7aS)-methyl 6-((2R,3S,4S,5R,6S)-3-((2S,3R,4R,5S,6R)-6-(acetoxy methyl)-5-(benzyloxy)-4-(tert-butyldimethylsilyloxy)-3-((2,2,2-trichloroethoxy) carbonylamino)tetrahydro-2H-pyran-2-yloxy)-5-(benzoyloxy)-2-(benzyloxymethyl)-6-(p-tolylthio)tetrahydro-2H-pyran-4-yloxy)-4-((4S,5R)-5-(benzyloxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazole-6-carboxylate (1-41)

A solution of donor **1-39** (2.34 g, 2.95 mmole), acceptor **1-35** (1.85 g, 2.06 mmole), and pulverized activated 4 Å MS (6.2 g) in CH_2Cl_2 was stirred under argon at room temperature for 2 h. The reaction mixture was then cooled to −50 °C, followed by the addition of TMSOTf (0.6 mL, 3.307 mmole) via micro-syringe. After stirring at the same temperature for 2 h, the reaction mixture was neutralized with triethylamine (3 mL), diluted with dichloromethane, and filtered through a pad of Celite. The filtrate was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 2.5 to 1 : 1.25 ethylacetate/hexane) to give **1-41** as white solid (2.23 g, 73 %). **1-41**: $R_f = 0.37$ (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00 (d, J = 7.4 Hz, 2H; Ar–H), 7.59 (t, J = 7.4 Hz, 1H; Ar–H), 7.47 (t, J = 7.8 Hz, 2H; Ar–H), 7.38–7.36 (m, 4H; Ar–H), 7.32–7.25 (m, 13H; Ar–H), 6.99 (d, J = 8.0 Hz, 2H; Ar–H), 5.50 (s, 1H), 5.36 (s, 1H), 5.07 (m, 2H), 4.90 (d, J = 11.5 Hz, 1H), 4.72–4.54 (m, 6H), 4.49–4.46 (m, 2H), 4.43 (dd, J = 13.0, 7.2 Hz, 1H), 4.10–4.02 (m, 7H), 3.98 (td, J = 12.0, 3.6 Hz, 1H), 3.86–3.78 (m, 2H), 3.75–3.72 (m, 5H), 3.66–3.56 (m, 4H), 3.49 (t, J = 10.6 Hz, 1H; H^{sial}-4), 2.34 (dd, J = 12.2, 3.6 Hz, 1H; H^{sial}-3 $_{eq}$), 2.29–2.22 (m, 4H; H^{sial}-3_{ax} and CH_3 STol), 1.89 (s, 3H; CH_3 Ac), 1.33 (s, 3H; CH_3), 1.28 (s, 3H; CH_3), 0.92 (s, 9H; t-Bu TBS), 0.17 (s, 3H; CH_3 TBS), 0.16 (s, 3H; CH_3 TBS); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.6, 168.9, 159.6, 154.2, 138.5, 138.5, 137.7, 133.5, 132.6, 129.8, 129.6, 129.2, 128.6, 128.5, 128.3, 128.3, 128.2, 127.9, 127.9, 127.8, 127.7, 127.6, 127.5, 127.3, 108.8, 101.8, 96.1, 78.5, 76.4, 75.4, 75.1, 75.0, 74.7, 74.6, 74.4, 73.6, 73.0, 72.2, 70.5, 68.1, 63.6, 58.1, 54.6, 54.0, 34.4, 27.0, 25.8, 25.8, 24.3, 21.1, 20.7, 18.0, −4.1, −4.7; HRMS (ESI-TOF, MNa^+) calculated for $C_{72}H_{87}Cl_3N_2O_{21}SSiNa$ 1505.4244, found 1505.4245.

(1S,2R)-1-((3aR,4R,6R,7aS)-6-(((2R,3R,4R,5S,6S)-4-((2R,3R,4S,5S,6R)-5-((2S,3R,4S,6R)-6-(acetoxymethyl)-5-(benzyloxy)-4-(tert-butyldimethylsilyloxy)-3-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yloxy)-3-(benzoyloxy)-6-(benzyloxymethyl)-4-((3aR,4R,6S,7aS)-4-((4S,5R)-5-(benzyloxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-6-yloxy)tetrahydro-2H-pyran-2-yloxy)-6-((2R,3S,4S,5S,6R)-2-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-3,5-bis (benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-(benzyloxy)-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yl)methoxy)-3-acetyl-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl) propane-1,2,3-triyl triacetate (1-42)

A solution of donor **1-41** (0.47 g, 0.317 mmole), acceptor **2** (0.26 g, 0.158 mmole), and pulverized activated 4 Å MS (1.3 g) in CH_2Cl_2 was stirred under argon at room temperature for 2 h. The reaction mixture was then cooled to −30 °C followed by addition of NIS (0.107 g, 0.476 mmole) and 0.5 M TfOH solution in dry

Et_2O (0.3 mL, 0.15 mmole). After stirring for 23 h at 0 °C, the reaction mixture was diluted with dichloromethane and filtered through a pad of Celite. The filtrate was then poured into a mixture of saturated aq. $Na_2S_2O_3$ and $NaHCO_3$. The aqueous layer was extracted with two portions of dichloromethane. The collected organic phases were then washed with brine, dried over Mg_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 :2.5 to 1 :1.25 ethylacetate/hexane) to give **1-42** as white solid (0.296 g, 62 %).

One-Pot:

A solution of GlaNAc donor **1-39** (0.302 g, 0.38 mmole), acceptor **1-35** (0.262 g, 0.291 mmole) and pulverized activated 4 Å MS (0.9 g) in dry CH_2Cl_2 (3.5 mL) was stirred under argon at room temperature for 3 h. The mixture was then cooled to −50 °C, followed by addition of TBDMSOTf (88 μL, 0.382 mmole) via micro-syringe and stirring for 2 h. Then, a solution of the acceptor **1-2** (0.382 g, 0.232 mmole) in dry CH_2Cl_2 (1.0 mL) was slowly added to the reaction mixture. The mixture was warmed to 0 °C and stirred for 30 min, followed by the addition of NIS (0.085 g, 0.377 mmole). After 24 h, when TLC indicated that acceptor **1-2** was fully consumed, the reaction was neutralized by triethylamine, diluted with dichloromethane, and filtered with a pad of Celite. The filtrate was poured into a mixture of saturated aq. $NaHCO_3$ and saturated aq. $Na_2S_2O_3$. The aqueous layer was extracted with two portions of ethyl acetate. The combined extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 2.5 to 1 : 1.25 ethylacetate/hexane) to give **1-42** as white solid (0.223 g, 32 %). **1-42**: R_f = 0.32 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.08 (d, J = 7.4 Hz, 2H; Ar–H), 7.62 (t, J = 7.4 Hz, 1H; Ar–H), 7.49 (t, J = 7.9 Hz, 4H; Ar–H), 7.40 (d, J = 7.2 Hz, 2H; Ar–H), 7.35–7.10 (m, 42H; Ar–H), 6.71 (t, J = 7.4 Hz, 1H), 5.57 (dd, J = 9.1, 1.3 Hz, 1H), 5.46 (ddd, J = 9.0, 6.3, 2.6 Hz, 1H), 5.32 (s, 1H), 5.17–5.09 (m, 5H), 4.95–4.80 (m, 2H), 4.75–4.68 (m, 3H), 4.63 (dd, J = 14.1, 6.4 Hz, 2H), 4.57 (d, J = 10.9 Hz, 1H), 4.52–4.22 (m, 17H), 4.13 (t, J = 8.6 Hz, 1H), 4.09–4.03 (m, 5H), 3.96–3.88 (m, 3H), 3.83–3.71 (m, 10H), 3.64–3.50 (m, 11H), 3.44–3.27 (m, 9H), 3.12–2.93 (m, 3H), 2.82 (dd, J = 11.9, 3.2 Hz, 1H; $H^{sial(2\rightarrow6)}$-3 $_{eq}$), 2.48 (s, 3H; CH_3 Ac), 2.32–2.25 (m, 2H; $H^{sial(2\rightarrow6)}$-3_{ax} and $H^{sial(2\rightarrow3)}$-3 $_{eq}$), 2.15–2.10 (m, 4H; CH_3 Ac and $H^{sial(2\rightarrow3)}$-$3_{ax)}$, 2.03 (s, 3H; CH_3 Ac), 1.90 (s, 3H; CH_3 Ac), 1.69 (s, 3H; CH_3 Ac), 1.50–1.14 (m, 12H; aliphatic and 2 ^{X} CH_3), 0.94 (s, 9H; t-Bu TBS), 0.22–0.20 (m, 6H; 2 ^{X} CH_3 TBS); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.8, 170.7, 170.6, 170.4, 169.8, 169.3, 168.5, 164.3, 159.4, 156.6, 156.1, 154.3, 153.7, 153.2, 139.3, 139.1, 139.0, 138.3, 138.2, 137.8, 137.7, 136.8, 136.7, 133.6, 129.9, 129.7, 129.3, 129.2, 128.8, 128.7, 128.5, 128.4, 128.4, 128.3, 128.3, 128.1, 128.0, 127.9, 127.8, 127.8, 127.6, 127.3, 127.2, 127.1, 127.0, 126.6, 109.0, 103.8, 101.8, 101.0, 100.9, 99.1, 96.3, 96.0, 81.1, 79.3, 78.5, 78.0, 76.2, 75.7, 75.3, 75.3, 75.1, 75.1, 75.0, 74.8, 74.7, 74.6, 74.4, 74.1, 73.5, 73.4, 73.1, 72.9, 72.2, 71.1, 69.7, 69.7, 69.2, 69.0, 68.3, 67.9, 67.1, 64.3, 63.6, 62.9, 59.1, 58.3, 58.1, 54.5, 54.0, 53.7, 52.6, 50.4, 50.1, 47.1, 46.1, 36.6, 34.6, 31.7, 29.2, 27.8, 27.4, 27.1, 25.8, 25.7, 24.7, 24.2, 23.3, 21.1, 20.8, 20.6, 20.5, 18.0, −4.1, −4.8; HRMS (ESI-TOF, MNa^+) calculated for $C_{147}H_{173}Cl_6N_5O_{47}SiNa$ 3024.9106, found 3024.9080.

(1S,2R)-1-((3aR,4R,6R,7aS)-3-acetyl-6-(((2R,3S,4R,5R,6S)-6-((2R,3R,4S,5S,6R)-2-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-3,5-bis(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-(benzyloxy)-4-((4aR,6R,7R,8S)-7,8-bis(benzoyloxy)-2-phenylhexahydropyrano[3,2-d] [1, 3] dioxin-6-yloxy)-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yl)methoxy)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d] oxazol-4-yl)propane-1,2,3-triyl triacetate (1-44)

A solution of donor **1-43** (0.239 g, 0.410 mmole), acceptor **1-2** (0.151 g, 0.092 mmole), and pulverized activated 4 Å MS (0.8 g) in CH_2Cl_2 was stirred under argon at room temperature for 2 h. The reaction mixture was then cooled to −30 °C, followed by addition of NIS (0.104 g, 0.462 mmole) and TfOH (9 μL, 0.1 mmole). After stirring at 0 °C for 9 h, the reaction mixture was diluted with dichloromethane and filtered through a pad of Celite. The filtrate was then poured into a mixture of saturated aq. $Na_2S_2O_3$ and $NaHCO_3$. The aqueous layer was extracted with two portions of dichloromethane. The collected organic phases were then washed with brine, dried over Mg_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 3 to 1 : 1.25 ethylacetate/hexane) to give **1-44** as oil (0.12 g, 62 %). **1-44**: R_f = 0.23 (Hexane : EtOAc = 2 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.02 (d, *J* = 7.3 Hz, 2H; Ar–H), 7.95 (d, *J* = 7.3 Hz, 2H; Ar–H), 7.50–7.44 (m, 6H; Ar–H), 7.36–7.12 (m, 35H; Ar–H), 5.84 (dd, *J* = 10.3, 8.1 Hz, 1H), 5.58 (d, *J* = 7.7 Hz, 1H), 5.53 (s, 1H), 5.41–5.38 (m, 1H), 5.23 (dd, *J* = 10.3, 3.5 Hz, 1H), 5.13 (d, *J* = 9.7 Hz, 3H), 4.87–4.77 (m, 4H), 4.63 (mz, 2H), 4.57–4.33 (m, 9H), 4.26–4.24 (m, 2H), 4.18–4.16 (m, 2H), 4.03–4.01 (m, 2H), 3.92–3.80 (m, 5H), 3.67–3.33 (m, 14H), 3.13–3.06 (m, 3H), 2.83 (dd, *J* = 11.8, 2.9 Hz, 1H; $H^{sial(2\rightarrow6)}$-3$_{eq}$), 2.47 (s, 3H; CH_3 Ac), 2.15–2.05 (m, 4H; CH_3 Ac and $H^{sial(2\rightarrow6)}$-3$_{ax}$), 2.01 (s, 3H; CH_3 Ac), 1.82 (s, 3H; CH_3 Ac), 1.57–1.34 (m, 4H; aliphatic), 1.28–1.09 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.2, 171.8, 170.7, 170.0, 169.9, 168.4, 166.0, 165.3, 156.7, 156.1, 153.6, 139.4, 139.0, 138.2, 137.9, 137.7, 136.9, 136.7, 133.3, 133.2, 129.9, 129.8, 129.6, 129.1, 128.8, 128.5, 128.4, 128.4, 128.2, 128.2, 128.0, 127.9, 127.9, 127.8, 127.6, 127.3, 127.2, 127.1, 126.9, 126.2, 103.8, 101.3, 100.7, 99.4, 95.7, 80.5, 79.8, 79.6, 76.2, 75.7, 75.2, 75.0, 74.9, 74.6, 74.1, 73.9, 73.6, 73.5, 73.5, 72.7, 71.4, 69.7, 68.9, 68.5, 67.1, 66.6, 64.7, 63.5, 62.8, 59.1, 58.4, 52.8, 50.5, 50.2, 47.1, 46.1, 36.4, 29.3, 27.9, 27.4, 24.7, 23.3, 23.2, 21.1, 20.8, 20.7; HRMS (ESI-TOF, MNa^+) calculated for $C_{109}H_{116}Cl_3N_3O_{33}Na$ 2124.6462, found 2124.6302.

(1S,2R)-1-((3aR,4R,6R,7aS)-6-(((2R,3S,5R,6S)-4-((2R,3S,5S,6R)-5-((3R,4S,5S,6R)-4-acetoxy-6-(acetoxymethyl)-5-(benzyloxy)-3-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yloxy)-3,4-bis(benzoyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-2-yloxy)-6-((2R,3R,4S,5S,6R)-2-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-3,5-bis(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-(benzyloxy)-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yl)methoxy)-3-acetyl-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2,3-triyl triacetate (1-46)

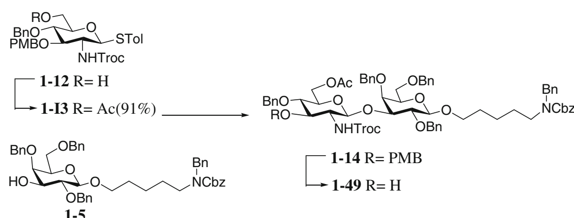
A solution of donor **1-45** (0.365 g, 0.333 mmole), acceptor **1-2** (0.2 g, 0.122 mmole), and pulverized activated 4 Å MS (0.54 g) in CH_2Cl_2 was stirred

under argon at room temperature for 2 h. The reaction mixture was then cooled to −30 °C, followed by addition of NIS (0.093 g, 0.413 mmole) and TfOH (0.012 mL, 0.135 mmole). After stirring for 11 h at 0 °C, the reaction mixture was diluted with dichloromethane and filtered through a pad of Celite. The filtrate was then poured into a mixture of saturated aq. $Na_2S_2O_3$ and $NaHCO_3$. The aqueous layer was extracted with two portions of dichloromethane. The collected organic phases were then washed with brine, dried over Mg_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 4 to 1 : 1.75 ethylacetate/hexane) to give **1-46** as oil (0.217 g, 68 %). **1-46**: R_f = 0.23 (Hexane : EtOAc = 2 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.04 (d, *J* = 7.5 Hz, 2H; Ar–H), 7.91 (d, *J* = 7.5 Hz, 2H; Ar–H), 7.62 (d, *J* = 7.2 Hz, 2H; Ar–H), 7.55 (t, *J* = 7.4 Hz, 1H; Ar–H), 7.45 (m, 3H; Ar–H), 7.38–7.10 (m, 44H; Ar–H), 6.85 (t, *J* = 7.2 Hz, 1H), 5.64–5.61 (m, 2H), 5.48–5.47 (m, 1H), 5.28 (d, *J* = 12.3 Hz, 1H), 5.18–5.11 (m, 3H), 5.03–4.92 (m, 3H), 4.84 (d, *J* = 11.1 Hz, 1H), 4.76–4.65 (m, 5H), 4.56–4.28 (m, 16H), 4.20–4.17 (m, 3H), 4.13–4.10 (m, 1H), 4.01–3.93 (m, 3H), 3.85–3.80 (m, 2H), 3.75–3.52 (m, 13H), 3.47–3.24 (m, 7H), 3.14 (m, 2H), 2.91–2.89 (m, 1H; $H^{sial(2\rightarrow6)}$-3 $_{eq}$), 2.49 (s, 3H; CH_3 Ac), 2.20–2.13 (m, 4H; CH_3 Ac and $H^{sial(2\rightarrow6)}$-3_{ax}), 2.04–2.02 (m, 6H; 2 ^{X} CH_3 Ac), 1.95 (s, 3H; CH_3 Ac), 1.79 (s, 3H; CH_3 Ac), 1.55–1.42 (m, 4H; aliphatic), 1.25–1.20 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.8, 171.0, 170.6, 170.3, 170.2, 169.9, 168.6, 166.1, 165.3, 156.6, 156.1, 154.2, 153.8, 153.7, 139.3, 138.8, 138.7, 138.4, 137.9, 137.4, 136.8, 136.8, 134.0, 133.0, 129.8, 129.7, 129.7, 129.5, 128.9, 128.7, 128.6, 128.6, 128.5, 128.4, 128.4, 128.4, 128.3, 128.2, 128.2, 127.9, 127.8, 127.8, 127.6, 127.6, 127.3, 127.3, 127.2, 127.1, 127.1, 103.8, 103.2, 101.1, 100.9, 99.3, 96.3, 95.6, 80.6, 79.7, 79.3, 75.6, 75.4, 75.1, 75.0, 75.0, 74.8, 74.7, 74.5, 74.5, 74.3, 74.0, 73.6, 73.5, 73.4, 73.3, 73.0, 71.9, 71.3, 70.3, 69.7, 69.6, 69.0, 68.8, 68.4, 64.5, 62.9, 62.3, 59.1, 57.5, 50.5, 50.2, 47.1, 46.1, 36.6, 29.3, 27.8, 27.4, 24.7, 23.3, 23.2, 21.2, 20.8, 20.8, 20.7; HRMS (ESI-TOF, MNa^+) calculated for $C_{129}H_{140}Cl_6N_4O_{41}Na$ 2637.7021, found 2637.6947.

(1S,2R)-1-((3aR,4R,6R,7aS)-3-acetyl-6-(((2R,3S,4R,5R,6S)-4-((4aR,6R,7S,8S,8aS)-8-((3aS,4S,6R,7aR)-3-acetyl-6-(methoxycarbonyl)-2-oxo-4-((1S,2R)-1,2,3-triacetoxypropyl)hexahydro-2H-pyrano[3,4-d]oxazol-6-yloxy)-7-(benzoyloxy)-2-phenylhexahydropyrano[3,2-d] [1, 3] dioxin-6-yloxy)-6-((2R,3R,4S,5S,6R)-2-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-3,5-bis(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-(benzyloxy)-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yl)methoxy)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2,3-triyl triacetate (1-48)

A solution of donor **1-47** (0.28 g, 0.299 mmole), acceptor **1-2** (0.32 g, 0.195 mmole), and pulverized activated 4 Å MS (0.6 g) in CH_2Cl_2 was stirred under argon at room temperature for 2 h. The reaction mixture was then cooled to −20 °C, followed by addition of NIS (0.102 g, 0.455 mmole) and TfOH (0.009 mL, 0.101 mmole). After stirring at −20 °C for 3 h, the reaction mixture was diluted with dichloromethane and filtered through a pad of Celite. The filtrate was then poured into a mixture of saturated aq. $Na_2S_2O_3$ and $NaHCO_3$. The aqueous layer

was extracted with two portions of dichloromethane. The collected organic phases were then washed with brine, dried over Mg_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 2.5 to 1 : 1 ethylacetate/hexane) to give **1-48** as oil (0.2884 g, 60 %). **1-48**: R_f = 0.19 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.15 (d, *J* = 7.3 Hz, 2H; Ar–H), 7.58–7.52 (m, 3H; Ar–H), 7.45 (t, *J* = 7.8 Hz, 2H; Ar–H), 7.39 (d, *J* = 7.0 Hz, 2H; Ar–H), 7.32–7.19 (m, 27H; Ar–H), 7.14–7.08 (m, 4H; Ar–H), 5.62–5.55 (m, 3H), 5.47 (ddd, *J* = 9.5, 7.3, 2.4 Hz, 1H), 5.43 (s, 1H), 5.35 (ddd, *J* = 8.6, 5.9, 2.7 Hz, 1H), 5.18–5.09 (m, 3H), 4.87–4.74 (m, 4H), 4.65–4.35 (m, 13H), 4.27–4.26 (m, 3H), 4.20–4.04 (m, 6H), 3.91–3.74 (m, 5H), 3.66–3.43 (m, 16H), 3.28 (s, 3H; CH_3 OMe), 3.09 (d, *J* = 45.3 Hz, 2H), 2.81–2.77 (m, 2H; $H^{sial(2\rightarrow6)}$-3 $_{eq}$ and $H^{sial(2\rightarrow3)}$-3 $_{eq}$), 2.47 (s, 3H; CH_3 Ac), 2.47 (s, 3H; CH_3 Ac), 2.19 (s, 3H; CH_3 Ac), 2.15 (s, 3H; CH_3 Ac), 2.09–2.06 (m, 5H; CH_3 Ac, $H^{sial(2\rightarrow6)}$-3_{ax}, and $H^{sial(2\rightarrow3)}$-3_{ax}), 2.00 (s, 3H; CH_3 Ac), 2.00 (s, 3H; CH_3 Ac), 1.79–1.77 (s, 3H; CH_3 Ac), 1.50–1.38 (m, 4H; aliphatic), 1.25–1.09 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.0, 171.8, 170.8, 170.7, 170.1, 170.0, 169.9, 169.8, 168.3, 168.0, 165.1, 156.6, 156.1, 153.6, 153.4, 139.3, 139.0, 138.2, 138.1, 137.8, 136.8, 136.7, 133.3, 130.1, 129.7, 128.8, 128.7, 128.6, 128.4, 128.4, 128.4, 128.1, 128.1, 127.9, 127.9, 127.8, 127.7, 127.7, 127.4, 127.3, 127.2, 127.1, 126.8, 126.5, 103.8, 100.8, 100.6, 100.3, 99.3, 97.5, 96.2, 80.7, 79.4, 79.1, 76.3, 75.9, 75.1, 75.0, 74.9, 74.9, 74.8, 74.5, 74.1, 73.6, 73.5, 72.9, 72.8, 71.3, 70.8, 69.7, 69.7, 69.0, 68.5, 68.3, 68.0, 67.0, 66.2, 64.6, 63.5, 62.6, 59.0, 58.9, 58.5, 52.8, 52.7, 50.4, 50.1, 47.1, 46.1, 36.8, 36.3, 29.6, 29.3, 27.8, 27.4, 24.6, 23.3, 21.3, 21.1, 21.1, 20.8, 20.7, 20.6; HRMS (ESI-TOF, MNa^+) calculated for $C_{121}H_{135}Cl_3N_4O_{44}Na$ 2477.7425, found 2477.7390.



((2R,3S,4R,5R,6S)-6-((2R,3R,4S,5S,6R)-2-(5-(benzyl(benzyloxycarbonyl) amino)pentyloxy)-3,5-bis(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-(benzyloxy)-4-hydroxy-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yl)methyl acetate (1-49)

Acetic anhydride (7 mL) was added to a solution of compound **1-12** (4.12 g, 6.14 mmole) in pyridine (7 mL) at room temperature. The mixture was stirred for 12 h at the same temperature, and methanol (5 mL) was added. The solution was concentrated into a syrup and extracted with dichloromethane. The extract was successively washed with 2 N HCl, water, $NaHCO_{3(aq)}$, and water, then dried with Na_2SO_4, and concentrated. Column chromatography (1 : 3 ethyl acetate/hexane) of the residue on silica gel gave the per-acetylated thioglycoside as white solid **1-13** (3.8 g, 87 %). A solution of donor **1-13** (1.42 g, 1.99 mmole), acceptor **1-5** (1.00 g,

1.316 mmole), and pulverized activated 4 Å MS (2.0 g) in CH_2Cl_2 was stirred under argon at room temperature for 2 h. The reaction mixture was then cooled to −20 °C, followed by addition of NIS (0.60 g, 2.67 mmole) and TfOH (0.016 mL, 0.18 mmole). After stirring at the same temperature for 2 h, the reaction mixture was diluted with dichloromethane and filtered through a pad of Celite. The filtrate was then poured into a mixture of saturated aq. $Na_2S_2O_3$ and $NaHCO_3$. The aqueous layer was extracted with two portions of dichloromethane. The collected organic phases were then washed with brine, dried over Mg_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 3 to 1 : 2 ethylacetate/hexane) to give **1-14** as white solid (1.14 g, 64 %). DDQ (0.40 g, 1.76 mmole) was added to a solution of compound **1-14** (1.14 g, 0.845 mmole) in CH_2Cl_2/H_2O (11 : 1, 36 mL) at rt and the resulting mixture was stirred for 2 h. The reaction mixture was diluted with CH_2Cl_2 (250 mL) and washed with saturated aqueous $NaHCO_3$ (200 mL) and brine (35 mL). The organic layer was dried with Na_2SO_4, and the solvents were removed under reduced pressure. The residue was purified by flash silica gel column chromatography (1 : 3 to 1 : 2 ethylacetate/hexane) to give **1-49** as colorless oil (0.635 g, 66 %). **1-49**: R_f = 0.22 (Hexane : EtOAc = 2 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.35–7.11 (m, 30H; Ar–H), 5.13 (d, *J* = 14.6 Hz, 2H; $PhCH_2$), 5.01 (t, *J* = 12.8 Hz, 1H), 4.89–4.84 (m, 3H), 4.67–4.64 (m, 3H), 4.58–4.53 (m, 3H), 4.44–4.27 (m, 6H), 4.22 (dd, *J* = 11.4, 3.7 Hz, 1H), 3.86–3.81 (m, 2H), 3.76–3.68 (m, 2H), 3.55–3.46 (m, 5H), 3.45–3.33 (m, 4H), 3.14 (m, 2H), 1.96 (s, 3H; CH_3 Ac), 1.55–1.42 (m, 4H; aliphatic), 1.27–1.18 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.6, 156.6, 156.0, 155.5, 138.7, 138.6, 137.7, 137.6, 136.7, 136.6, 128.5, 128.4, 128.4, 128.3, 128.3, 128.0, 127.7, 127.7, 127.4, 127.3, 127.2, 127.1, 127.0, 103.7, 101.7, 95.1, 80.7, 79.5, 77.4, 76.1, 75.5, 74.7, 74.6, 74.5, 74.3, 73.4, 72.7, 69.6, 69.5, 68.7, 67.0, 67.0, 62.9, 58.5, 50.4, 50.1, 47.0 46.0, 29.2, 27.8, 27.3, 23.2, 23.2, 20.7; HRMS (ESI-TOF, MNa^+) calculated for $C_{65}H_{73}Cl_3N_2O_{15}Na$ 1251.3961, found 1251.3963.

(3aR,4R,6S,7aS)-methyl 6-((2R,3S,4S,5R,6R)-3-((2S,3R,4R,5S,6R)-6-(acetoxy methyl)-5-(benzyloxy)-4-(tert-butyldimethylsilyloxy)-3-((2,2,2-trichloroethoxy) carbonylamino)tetrahydro-2H-pyran-2-yloxy)-6-((2R,3S,4R,5R,6S)-2-(acetoxy-methyl)-6-((2R,3R,4S,5S,6R)-2-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-3,5-bis(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-(benzyl-oxy)-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-4-yloxy)-5-(benzoyloxy)-2-(benzyloxymethyl)tetrahydro-2H-pyran-4-yloxy)-4-((4S,5R)-5-(benzyloxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)-2-oxohexahydro-2H-pyrano [3,4-d]oxazole-6-carboxylate (1-50)

A solution of donor **1-41** (0.209 g, 0.141 mmole), acceptor **1-49** (0.124 g, 0.101 mmole) and pulverized activated 4 Å MS (0.65 g) in dry CH_2Cl_2 (7 mL) was stirred under argon at room temperature for 2 h. The mixture was then cooled to −30 °C, followed by addition of NIS (0.045 g, 0.2 mmole) and TBDMSOTf (23

μL, 0.10 mmole) via micro-syringe. After stirring at 0 °C for 8 h, the reaction mixture was diluted with dichloromethane and filtered through a pad of Celite. The filtrate was then poured into a mixture of saturated aq. $Na_2S_2O_3$ and $NaHCO_3$. The aqueous layer was extracted with two portions of dichloromethane. The collected organic phases were then washed with brine, dried over Mg_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 4 to 1 : 2 ethylacetate/hexane) to give **1-50** as oil (0.173 g, 67 %). **1-50**: R_f = 0.41 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.17 (d, *J* = 7.6 Hz, 2H; Ar–H), 7.66 (t, *J* = 7.6 Hz, 1H; Ar–H), 7.53 (t, *J* = 7.6 Hz, 2H; Ar–H), 7.41–7.10 (m, 45H; Ar–H), 6.00 (d, *J* = 10.0 Hz, 1H), 5.67 (s, 1H), 5.50–5.47 (m, 1H), 5.18–5.17 (m, 3H), 5.03 (m, 2H), 4.87–4.84 (m, 3H), 4.77 (d, *J* = 11.9 Hz, 1H), 4.69–4.35 (m, 19H), 4.29–4.20 (m, 5H), 4.15–4.06 (m, 4H), 4.00 (m, 2H), 3.90–3.80 (m, 3H), 3.70–3.47 (m, 14H), 3.43–3.37 (m, 3H), 3.12 (m, 2H), 2.89 (s, 1H), 2.46–2.44 (m, 1H; $H^{sial(2\rightarrow3)}$-3 $_{eq}$), 2.20–2.16 (m, 1H; $H^{sial(2\rightarrow3)}$-3$_{ax}$), 2.01 (s, 3H; CH_3 Ac), 1.80 (s, 3H; CH_3 Ac), 1.53–1.18 (m, 12H; aliphatic and 2 ^{X} CH_3), 0.90 (s, 9H; t-Bu TBS), 0.04 (s, 3H; CH_3 TBS), 0.02 (s, 3H; CH_3 TBS); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.4, 170.4, 166.8, 164.4, 159.5, 156.6, 156.0, 154.2, 153.4, 139.3, 138.8, 138.6, 138.0, 137.8, 137.8, 137.0, 136.8, 136.7, 133.7, 129.7, 129.2, 128.9, 128.5, 128.5, 128.4, 128.3, 128.3, 128.2, 128.0, 127.9, 127.9, 127.8, 127.8, 127.7, 127.7, 127.7, 127.6, 127.5, 127.3, 127.2, 127.1, 126.6, 108.4, 103.7, 101.3, 100.3, 96.1, 95.3, 81.2, 79.3, 79.2, 76.7, 76.0, 75.3, 75.0, 74.9, 74.7, 74.4, 74.3, 74.1, 73.7, 73.4, 73.3, 73.2, 72.9, 72.4, 72.3, 71.1, 70.2, 69.7, 69.6, 69.4, 68.7, 68.3, 67.0, 65.9, 62.7, 62.3, 58.5, 58.1, 53.3, 53.2, 50.4, 50.1, 47.0, 46.0, 35.2, 29.2, 27.7, 27.3, 25.6, 24.5, 23.2, 20.7, 20.6, 17.8, –4.1, –4.9. HRMS (ESI-TOF, MNa^+) calculated for $C_{130}H_{152}Cl_6N_4O_{36}SiNa$ 2609.7983, found 2609.8115.

General procedures for deprotection of oligosaccharide 1-2, 1-44, 1-46, and 1-48 to give 1-51, 1-52, 1-53, and 1-54, respectively.

LiOH (5.0 mmole, 50.0 eq) was added to a stirred solution of protected oligosaccharide (0.1 mmole, 1.00 eq) in 1,4-dioxane (5.00 mL) and H_2O (5.00 mL) at room temperature. After stirring at 80 °C for 36 h, the reaction mixture was evaporated in vacuo. The residue was purified by reverse–phase column chromatography (Bond Elut-C18) to give the product residue. $NaHCO_3$ (5.0 mmole, 50.0 eq) and acetic anhydride (5.0 mmole, 50.0 eq) were added to a stirred solution of the above residue in H_2O (3.00 mL) at room temperature. After stirring at the same temperature for 1 h, $NaHCO_3$ (5.0 mmole, 50.0 eq) and acetic anhydride (5.0 mmole, 50.0 eq) were added into the reaction mixture at room temperature. After stirring at the same temperature for 1 h, LiOH (5.0 mmole, 50.0 eq) was added into the reaction mixture. After stirring at the same temperature for 12 h, the reaction mixture was evaporated in vacuo. The residue was purified by reverse–phase column chromatography (Bond Elut-C18). $Pd(OH)_2$ (1 mmole) was added to

a stirred solution of the above residue in methanol (2.00 mL) and H_2O (2.00 mL). The reaction mixture was hydrogenolyzed for 12 h under H_2 gas atmosphere. The reaction mixture was filtered, and the filtrate was evaporated in vacuo. The residue was purified by reverse–phase column chromatography (Bond Elut-C18) to give deprotected oligosaccharide.

(2R,4S,5R,6R)-5-acetamido-2-(((2R,3S,4R,5R,6S)-5-acetamido-6-((2R,3R,4S,5S,6R)-2-(5-aminopentyloxy)-3,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-4-yloxy)-3,4-dihydroxytetrahydro-2H-pyran-2-yl)methoxy)-4-hydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-carboxylic acid (1-51)

Compound **1-51** (19 mg, 0.025 mmole, 23 %) was prepared according to the general procedure for the deprotection of oligosaccharide from trisaccharide **1-2** (0.18 g, 0.109 mmole).

^{1}H NMR (600 MHz, D_2O) δ 4.55 (d, *J* = 8.4 Hz, 1H; H^{Gla}-1), 4.26 (d, *J* = 7.9 Hz, 1H; H^{GlcNAc}-1), 4.04 (d, *J* = 3.2 Hz, 1H), 3.86–3.39 (m, 20H), 2.64 (dd, *J* = 12.4, 4.7 Hz, 1H; $H^{sial(2\rightarrow6)}$-3 $_{eq}$), 2.60 (t, *J* = 7.1 Hz, 2H; aliphatic),1.92 (s, 3H; CH_3 Ac), 1.91 (s, 3H; CH_3 Ac), 1.59 (t, *J* = 12.2 Hz, 1H; $H^{sial(2\rightarrow6)}$-3_{ax}), 1.55–1.50 (m, 2H; aliphatic), 1.43–1.38 (m, 2H; aliphatic), 1.31–1.26 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, D_2O) δ 175.0, 174.9, 173.3, 102.7, 102.7, 100.0, 82.5, 74.7, 74.0, 73.4, 72.4, 71.6, 70.2, 69.6, 69.5, 68.2, 68.1, 68.1, 62.7, 62.5, 60.9, 55.5, 51.8, 40.0, 29.8, 28.3, 22.2, 22.1, 21.9; HRMS (ESI-TOF, MH^+) calculated for $C_{30}H_{53}N_3O_{19}H$ 760.3346, found 760.3363.

(2R,4S,5R,6R)-5-acetamido-2-(((2R,3S,4R,5R,6S)-5-acetamido-6-((2R,3R,4S,5S,6R)-2-(5-aminopentyloxy)-3,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-hydroxy-4-((2R,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)tetrahydro-2H-pyran-2-yl) methoxy)-4-hydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-carboxylic acid (1-52)

Compound **1-52** (46 mg, 0.050 mmole, 20 %) was prepared according to the general procedure for the deprotection of oligosaccharide from tetrasaccharide **1-44** (0.46 g, 0.249 mmole).

^{1}H NMR (600 MHz, D_2O) δ 4.70 (d, *J* = 8.4 Hz, 1H; $H^{Gla(I)}$-1), 4.43 (d, *J* = 7.5 Hz, 1H; $H^{Gla(II)}$-1), 4.37 (d, *J* = 8.3 Hz, 1H; H^{GlcNAc}-1), 4.16 (d, *J* = 3.3 Hz, 1H), 3.98–3.50 (m, 26H), 3.00 (t, *J* = 7.5 Hz, 2H; aliphatic), 2.73 (dd, *J* = 12.3, 4.5 Hz, 1H; $H^{sial(2\rightarrow6)}$-3 $_{eq}$), 2.02 (s, 3H; CH_3 Ac), 2.01 (s, 3H; CH_3 Ac), 1.71–1.63 (m, 5H; aliphatic and $H^{sial(2\rightarrow6)}$-3_{ax}), 1.48–1.43 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, D_2O) δ 174.9, 174.8, 173.3, 103.3, 102.7, 102.4, 100.1, 82.5, 81.6, 75.2, 74.7, 73.6, 72.4, 71.6, 70.6, 69.8, 69.6, 68.5, 68.3, 68.2, 68.1, 68.0, 62.8, 62.5, 60.9, 54.7, 51.7, 40.0, 39.3, 28.1, 26.3, 22.1, 22.0, 21.9; HRMS (ESI-TOF, MH^+) calculated for $C_{36}H_{63}N_3O_{24}H$ 922.3874, found 922.3988.

(2R,4S,5R,6R)-5-acetamido-2-(((2R,3S,4R,5R,6S)-5-acetamido-4-((2R,3R,4R,5R,6R)-5-((2S,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)-3,4-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)-6-((2R,3R,4S,5S,6R)-2-(5-aminopentyloxy)-3,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-hydroxytetrahydro-2H-pyran-2-yl)methoxy)-4-hydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-carboxylic acid (1-53)

Compound **1-53** (23 mg, 0.020 mmole, 16 %) was prepared according to the general procedure for the deprotection of oligosaccharide from pentasaccharide **1-46** (0.33 g, 0.126 mmole).

^{1}H NMR (600 MHz, D_2O) δ 4.69 (d, *J* = 8.4 Hz, 1H; $H^{Gla(I)}$-1), 4.62 (d, *J* = 8.1 Hz, 1H; H^{GlaNAc}-1), 4.43 (d, *J* = 7.6 Hz, 1H; $H^{Gla(II)}$-1), 4.37 (d, *J* = 7.7 Hz, 1H; H^{GlcNAc}-1), 4.16 (d, *J* = 3.4 Hz, 1H), 4.07 (d, *J* = 2.9 Hz, 1H), 3.97–3.52 (m, 30H), 3.40–3.37 (m, 1H), 2.99 (t, *J* = 7.5 Hz, 2H; aliphatic), 2.73 (dd, *J* = 12.4, 4.6 Hz, 1H; $H^{sial(2\rightarrow6)}$-3 $_{eq}$), 2.04 (s, 3H; CH_3 Ac), 2.02 (s, 3H; CH_3 Ac), 2.00 (s, 3H; CH_3 Ac), 1.71–1.63 (m, 5H; aliphatic and $H^{sial(2\rightarrow6)}$-3_{ax}), 1.48–1.43 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, D_2O) δ 174.9 174.8 173.3 103.0 102.7 102.5 102.4 100.1 82.5 81.1 75.9 74.7 74.7 74.2 73.6 72.4 72.3 71.6 70.8 70.6 69.8 69.6 68.2 68.1 68.1 68.0 67.7 62.8 62.5 61.0 60.5 54.7 52.6 51.7 40.0 39.3 28.1 26.3 22.3 22.2 22.0 21.9; HRMS (ESI-TOF, MH^+) calculated for $C_{44}H_{76}N_4O_{29}H$ 1125.4668, found 1125.4872.

(2R,4S,5R,6R)-5-acetamido-2-(((2R,3S,4R,5R,6S)-5-acetamido-4-((2R,3R,4S,5S,6R)-4-((2S,4R,5R,6S)-5-acetamido-2-carboxy-4-hydroxy-6-((1S,2S)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-yloxy)-3,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)-6-((2R,3R,4S,5S,6R)-2-(5-aminopentyloxy)-3,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-hydroxytetrahydro-2H-pyran-2-yl)methoxy)-4-hydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-carboxylic acid (1-54)

Compound **1-54** (28 mg, 0.023 mmole, 32 %) was prepared according to the general procedure for the deprotection of oligosaccharide from pentasaccharide **1-48** (0.18 g, 0.073 mmole).

^{1}H NMR (600 MHz, D_2O) δ 4.70 (d, *J* = 8.6 Hz, 1H; $H^{Gla(I)}$-1), 4.48 (d, *J* = 7.8 Hz, 1H; $H^{Gla(II)}$-1), 4.37 (d, *J* = 7.9 Hz, 1H; H^{GlcNAc}-1), 4.15 (d, *J* = 3.2 Hz, 1H), 4.06 (dd, *J* = 9.8, 3.0 Hz, 1H), 3.96–3.50 (m, 32H), 2.99 (t, *J* = 7.5 Hz, 2H; aliphatic), 2.75–2.71 (m, 2H; $H^{sial(2\rightarrow6)}$-3 $_{eq}$ and $H^{sial(2\rightarrow3)}$-3 $_{eq}$), 2.01 (s, 3H; CH_3 Ac), 2.01 (s, 3H; CH_3 Ac), 2.00 (s, 3H; CH_3 Ac), 1.76 (t, *J* = 12.1 Hz, 1H; H^{sial} $^{(2\rightarrow3)}$-3_{ax}), 1.70–1.63 (m, 5H; aliphatic and $H^{sial(2\rightarrow6)}$-3_{ax}), 1.47–1.42 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, D_2O) δ 174.9, 174.9, 174.8, 173.8, 173.3, 103.3, 102.6, 102.3, 100.1, 99.5, 82.4, 81.8, 75.5, 75.0, 74.7, 73.6, 72.7, 72.4, 71.7, 71.6, 69.8, 69.6, 69.0, 68.3, 68.2, 68.1, 68.0, 67.9, 67.1, 62.8, 62.5, 62.3, 60.9, 54.6,

51.7, 51.5, 40.0, 39.7, 37.2, 30.1, 28.0, 26.3, 22.2, 22.0, 21.9; HRMS (ESI-TOF, MNa^+) calculated for $C_{47}H_{80}N_4O_{32}Na$ 1235.4648, found 1235.4697.

General procedures for deprotection of oligosaccharide 1-50, and 1-42 to give 1-55, and 1-1, respectively.

LiOH (5.0 mmole, 50.0 eq) was added to a stirred solution of protected oligosaccharide (0.1 mmole, 1.00 eq) in 1,4-dioxane (5.00 mL) and H_2O (5.00 mL) at room temperature. After stirring at 95 °C for 36 h, the reaction mixture was evaporated in vacuo. The residue was purified by reverse–phase column chromatography (Bond Elut-C18) to give the product residue. A solution of the above residue in pyridine (10.0 mmole, 100.0 eq) and acetic anhydride (10.0 mmole, 100.0 eq) was stirred at room temperature for 14 h. The resulting solution was concentrated and coevaporated with toluene twice. Purification of the residue by silica gel chromatography (1 : 11 to 1 : 2 MeOH:DCM) provided the product residue. A resulting residue was dissolved in acetonitrile (1 mL), and BF_3-OEt_2 (2.5 mmole, 25.00 eq) and one drop of water was added at 0 °C. After stirring at the same temperature for 4 h, the reaction mixture was allowed to warm gradually to room temperature and stirred for 2 h continually at room temperature. The $NaHCO_3$(aq) was poured into the solution and the solution was extracted by DCM four times and concentrated under reduced vacuum. The residue was purified by flash column chromatography (1 : 9 to 1 : 2 MeOH:DCM) to give the product residue. A solution of resulting residue and LiOH (5.0 mmole, 50.0 eq) in 1,4-Dioxane (1.00 mL) and H_2O (1.00 mL)was stirred for 12 h at room temperature. The reaction mixture was evaporated in vacuo and the residue was purified by reverse–phase column chromatography (Bond Elut-C18). $Pd(OH)_2$ (1 mmole) was added to a stirred solution of the above residue in methanol (2.00 mL) and H_2O (2.00 mL). The reaction mixture was hydrogenolyzed for 18 h under H_2 gas atmosphere. The reaction mixture was filtered, and the filtrate was evaporated in vacuo. The residue was purified by reverse–phase column chromatography (Bond Elut-C18) to give deprotected oligosaccharide.

(2R,4R,5S,6S)-5-acetamido-2-((2R,3R,4R,5S,6R)-2-((2S,3R,4R,5S,6R)-3-acetamido-2-((2R,3R,4S,5S,6R)-2-(5-aminopentyloxy)-3,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-4-yloxy)-5-hydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-4-yloxy)-5-((2S,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)-3-hydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-4-yloxy)-4-hydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-carboxylic acid (1-55)

Compound **1-55** (12 mg, 0.011 mmole, 13 %) was prepared according to the general procedure for the deprotection of oligosaccharide from pentasaccharide **1-50** (0.22 g, 0.085 mmole).

^{1}H NMR (600 MHz, D_2O) δ 4.73 (d, J = 11.0 Hz, 1H; $H^{Gla(I)}$-1), 4.72 (d, J = 11.0 Hz, 1H; H^{GlaNAc}-1), 4.51 (d, J = 8.0 Hz, 1H; $H^{Gla(II)}$-1), 4.37 (d, J = 8.0 Hz, 1H; H^{GlcNAc}-1), 4.13–4.08 (m, 3H), 3.94–3.44 (m, 29H), 3.36–3.33 (m, 1H), 2.98 (t, J = 7.4 Hz, 2H; aliphatic), 2.66 (dd, J = 12.7, 4.7 Hz, 1H; $H^{sial(2\rightarrow3)}$-3$_{eq}$), 2.02 (s, 3H; CH_3 Ac), 2.01 (s, 3H; CH_3 Ac), 2.01 (s, 3H; CH_3 Ac), 1.90 (t, J = 12.0 Hz, 1H; $H^{sial(2\rightarrow3)}$-3$_{ax}$), 1.70–1.63 (m, 4H; aliphatic), 1.47–1.42 (m, 2H;

aliphatic); ^{13}C NMR (150 MHz, D_2O) δ 174.9, 174.8, 174.8, 174.0, 102.8, 102.7, 102.6, 102.3, 101.3, 82.2, 81.9, 76.7, 75.1, 74.6, 74.4, 73.9, 72.9, 72.2, 71.1, 69.9, 69.6, 69.5, 68.6, 68.3, 68.2, 67.9, 67.6, 62.7, 61.0, 60.8, 60.4, 54.5, 52.2, 51.5, 39.3, 37.1, 28.1, 26.4, 26.4, 26.4, 22.5, 22.2, 22.0, 21.9; HRMS (ESI-TOF, MH^+) calculated for $C_{44}H_{77}N_4O_{29}7$ 1125.4668, found 1125.4471.

(2R,4R,5S,6S)-5-acetamido-2-((2R,3S,4R,5R,6R)-3-((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)-6-((2S,3R,4R,5S,6R)-3-acetamido-6-(((2R,4S,5R,6R)-5-acetamido-2-carboxy-4-hydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-yloxy) methyl)-2-((2R,3R,4S,5S,6R)-2-(5-aminopentyloxy)-3,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-4-yloxy)-5-hydroxytetrahydro-2H-pyran-4-yloxy)-5-hydroxy-2-(hydroxymethyl)tetrahydro-2H-pyran-4-yloxy)-4-hydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-carboxylic acid (1-1)

Compound **1-1** (53 mg, 0.037 mmole, 16 %) was prepared according to the general procedure for the deprotection of oligosaccharide from hexasaccharide **1-42** (0.70 g, 0.234 mmole).

^{1}H NMR (600 MHz, D_2O) δ 4.75 (d, J = 8.5 Hz, 1H; $H^{Gla(I)}$-1), 4.74 (d, J = 8.4 Hz, 1H; H^{GlaNAc}-1), 4.54 (d, J = 7.9 Hz, 1H; $H^{Gla(II)}$-1), 4.40 (d, J = 7.98 Hz, 1H; H^{GlcNAc}-1), 4.18–4.11 (m, 3H), 3.99–3.55 (m, 37H), 3.52–3.50 (m, 1H), 3.37 (t, J = 9 Hz, 1H), 3.03 (t, J = 7.5 Hz, 2H; aliphatic), 2.76 (dd, J = 12.4, 4.5 Hz, 1H; $H^{sial(2\rightarrow6)}$-3 $_{eq}$), 2.69 (dd, J = 12.8, 4.5 Hz, 1H; $H^{sial(2\rightarrow3)}$-3 $_{eq}$), 2.05 (s, 3H; CH_3 Ac), 2.05 (s, 3H; CH_3 Ac), 2.04 (s, 3H; CH_3 Ac), 2.04 (s, 3H; CH_3 Ac), 1.93 (t, J = 12 Hz, 1H; $H^{sial(2\rightarrow3)}$-3_{ax}), 1.74–1.66 (m, 5H; aliphatic and $H^{sial(2\rightarrow6)}$-3_{ax}), 1.51–1.46 (m, 2H; aliphatic). The α-form of Sial(2 → 6) was confirmed by coupling constant $^3J(C_1\text{-}H_{3ax})$ = 10.60 Hz and The α-form of Sial (2 → 3) was confirmed by coupling constant $^3J(C_1\text{-}H_{3ax})$ = 5.60 Hz; ^{13}C NMR (150 MHz, D_2O) δ 174.9, 174.8, 174.0, 173.4, 102.9, 102.6, 102.4, 101.3, 100.1, 82.5, 81.7, 76.7, 74.7, 74.6, 74.4, 74.0, 73.6, 72.9, 72.4, 72.2, 71.6, 71.1, 69.8, 69.6, 69.5, 68.6, 68.3, 68.3, 68.2, 68.1, 67.9, 67.7, 62.9, 62.7, 62.5, 61.1, 61.0, 60.4, 54.5, 52.3, 51.8, 51.5, 40.0, 39.3, 37.1, 28.1, 26.3, 22.5, 22.3, 22.0, 21.9; HRMS (ESI-TOF, MH^+) calculated for $C_{55}H_{94}N_5O_{37}$ 1416.5622, found 1416.6175.

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Chapter 2
RM2 Antigen: Structural Characterization and Determination of $K_{D,Surf}$ for Multivalent Carbohydrate–Protein Interaction

2.1 Introduction

2.1.1 A Novel Ganglioside (RM2) Isolated from Renal Cell Carcinoma

Gangliosides are glycosphingolipids (GSL) containing sialic acids and are present in the outer leaflet of plasma membranes. GSL expression is a complex phenomenon, and pathological or abnormal expression at the tumor cell surface must be defined as the ability of the tumor cell to interact with specific target cell. This interaction may thus promote metastasis through the lymphatic or blood circulatory system.

Renal cell carcinoma (RCC) has been characterized by high expression of disialogangliosides when enhanced expression of higher gangliosides is correlated with the metastatic potential. Two RCC cell lines, TOS-1 and ACNH, derived from its metastatic deposits are characterized by high expression of disialogliosides. Initially, disialosylgalactosylgloboside (DSGG) was identified previously as one of the three types of major disialogangliosides from RCC tissues. However, further studies indicated that DSGG is the major disialoganglioside of ACHN cell lines, but absent from TOS-1 cell lines [1].

The proposed structure of RM2 can be easily confused with DSGG antigen because (1) RM2 and DSGG are indistinguishable on high performance thin-layer chromatography (TLC) with similar and overlapping relative retention factor (Rf); and (2) the RCC expresses many types of disialogangliosides and other globo-series monosialogangliosides, leading to varying proportions of globo-series and ganglio/lacto-series antigens (Fig. 2.1) [2]. Above all, mAb RM2 was observed to react strongly only with the novel TOS-1 heptaglycosylceramide RM2 but not significantly with DSGG. Besides, the DSGG was purified from metastatic renal carcinoma cell line, ACNH. ACNH is the immunogen used to generate mAb 5F3, which was specific for DSGG and not cross-reactive with the RM2 antigen.

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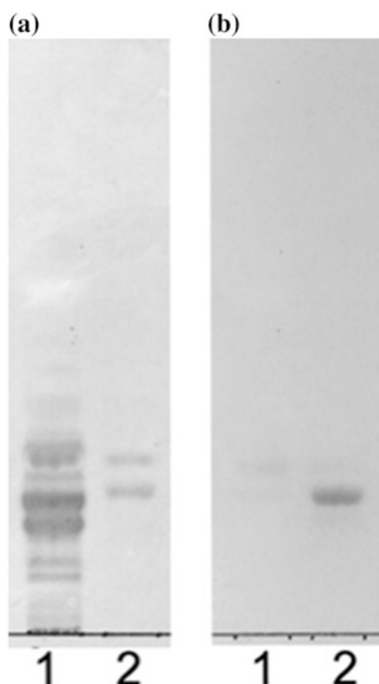

Fig. 2.1 TLC immunostaining of TOS-1 and ACHN disialogangliosides. Lane 1, disialosyl fractions of TOS-1 gangliosides. Lane 2, disialosyl fractions of ACHN gangliosides. *Panel A* mAb RM2 immunostaining. *Panel B* mAb 5F3 immunostaining. Adapted from [2], copyright 2015, with permission from Springer Science+Business Media

On the other hand, this internal hexasaccharide RM2 without the GlaNAc residue is identical to α(2,3)/α(2,6)-disialyl lactotetraose (DSLc4), which was observed as a colon cancer-associated antigen by using a monoclonal antibody (FH9) [3] for siglec-7, which is expressed in natural killer (NK) cells and is able to affect NK activity through binding [4]. To further verify the proposed structure of the RM2 antigen, we chemically synthesized the proposed hexasaccharide **1-1** and evaluated its recognition by monoclonal antibody RM2.

2.2 Characterize the Structure of RM2 Antigen by Monoclonal RM2 Antibody and Further Determine the $K_{D,Surf}$ by Carbohydrate–Protein Interaction

2.2.1 Structural Characterization of RM2 Antigen

To confirm that the synthesized hexasaccharide **1-1** was indeed the RM2 antigen and to further understand the antibody binding specificity, we prepared an expanded glycan array containing the synthesized hexasaccharide **1-1** and its analogs along with the other 90 glycans prepared in our laboratory to form a glycan array for testing (Fig. 2.6). All glycans were directly immobilized onto NHS-coated glass

slides by taking an aliquot from a stock solution of sugar at a fixed concentration (100 μM).

The assay involved an initial treatment with RM2 (a mouse IgM anti-RM2 monoclonal antibody, a kind gift from Prof. Saito), followed by incubation with a fluorescein-tagged anti-mouse IgM secondary antibody against its primary antibody. After 1 h incubation, the slides were washed with ddH_2O twice and scanned to show the binding specificity of the antibody to printed oligosaccharides spots. The resulted images showed that monoclonal RM2 antibody recognize hexasaccharide **1-1** specifically, indicating that the synthetic hexasaccharide **1-1** contains the same antigenic epitope with which RM2 antibody reacts on prostate cancer cells (Fig. 2.2); therefore, we concluded that the synthesized hexasaccharide **1-1** was indeed the RM2 antigen. Moreover, the structure assignment of the synthesized hexasaccharide **1-1** is confirmed by both 1D and 2D-NMR (Fig. 2.3 and Table 2.1).

2.2.2 Determination of $K_{D,Surf}$ for Multivalent Carbohydrate–Protein Interaction on the Surface

To determine the dissociation constants of RM2 and truncated analogs on the surface interacting with antibodies in a multivalent manner, we utilized the direct measurement method developed in our lab [6], adopting the different concentrations of antibodies and printed sugars. The binding curves were analyzed as Langmuir isotherms, assuming that the system reached equilibrium during incubation,

$$F_{\text{obs}} = F_{\max}[P]/\left(K_{\text{D,surf}} + [P]\right)$$

where $F_{\max}$ is the maximum fluorescence intensity, which is a measure of the amount of active carbohydrate on the surface, $[P]$ is the total antibody concentration, and $K_{\text{D,surf}}$ is the equilibrium dissociation constant for surface carbohydrate and antibody. Each array was printed at five different concentrations with sugars from 100 to 6.25 μM (100, 50, 25, 12.5, and 6.25 μM). The arrays were incubated with 12 concentrations of diluted anti-RM2 antibody, ranging from 25 to 0.06 nM, in combination with the Cy3-labeled goat anti-mouse IgM secondary antibody. After washing, the fluorescence intensity generated the saturated curve for dissociation constant ($K_{\text{D,surf}}$) determination. The results showed that when the printing concentration was below 6.25 μM, the average distance between printed sugars was too far to have enough multivalent effect with antibody RM2. At printing concentrations from 100 to 6.25 μM, however, we observed that the $K_{\text{D,surf}}$ values measured were narrowly distributed from the individual curves (Fig. 2.4 and Table 2.2). Using the same method, the $K_{\text{D,surf}}$ values were determined for the truncated RM2 analog **1-53** interacting with antibodies (Fig. 2.5 and Table 2.3). Overall, the relative binding specificity of RM2 for the epitopes was RM2 > pentasaccharide **1-53** (Table 2.4).

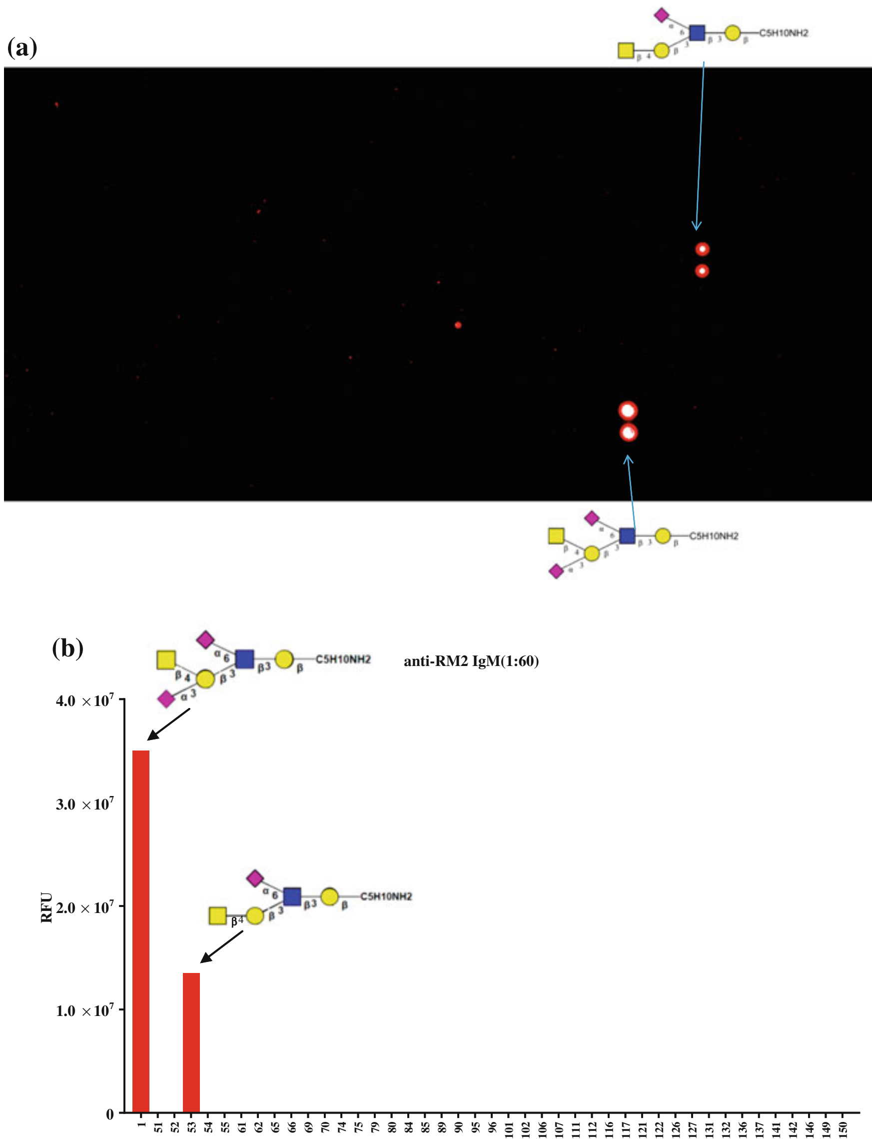

Fig. 2.2 Binding of monoclonal antibodies to RM2 and truncated sequences. **a** Slide image obtained from fluorescence scan after antibody incubation assay with RM2. **b** Binding specificity of monoclonal antibodies RM2 to RM2 antigen and its fragments. Adapted from [5], copyright 2015, with permission from American Chemical Society

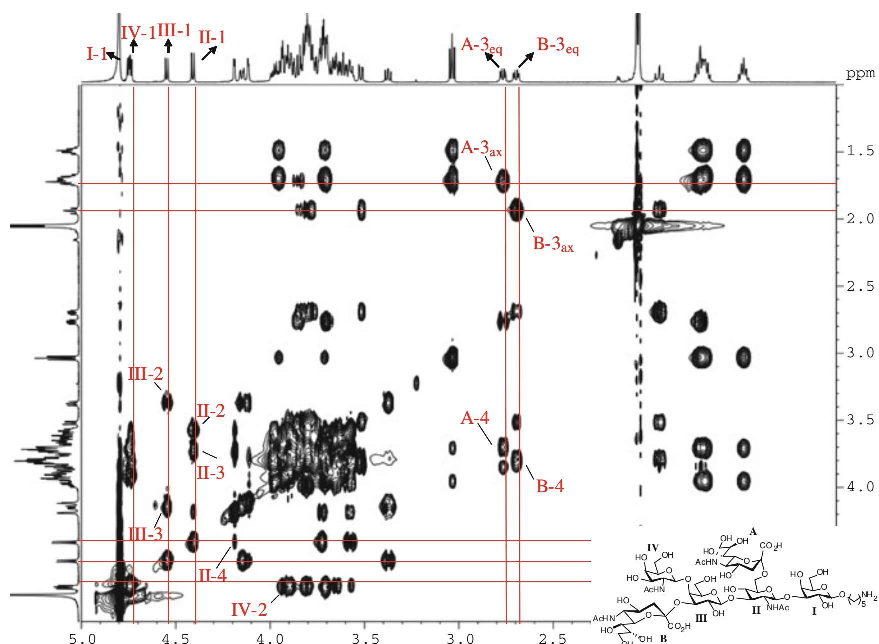

Fig. 2.3 Two-dimensional TOCSY NMR spectra analysis of synthetic RM2 antigen in D_2O at 298 K. Adapted from [5], copyright 2015, with permission from American Chemical Society

Table 2.1 Proton chemical shift, $^3J_{1,2}$ coupling constant (Hz), and ^3J(C-1,H-3_{ax}) (Hz) for synthetic RM2 in D_2O at 298 K. Adapted from [5], copyright 2015, with permission from American Chemical Society

Synthetic RM2 NeuAcα2 ⟶ 6
GalNAcβ1 ⟶ 4 GlcNAcβ1 ⟶ 3Galβ1 ⟶ linker
NeuAcα2 ⟶ 3Galβ1 ⟶ 3

	NeuAcα2 (→3Galβ1)	NeuAcα2 (→6)	GalNAcβ1	3Galβ1	GlcNAcβ1	3Galβ1
H-1 ($^3J_{1,2}$)			4.74 (8.4)	4.54 (7.9)	4.40 (8.0)	4.75 (8.5)
H-2			3.909	3.375	3.576	4.119
H-3	2.69(eq) 1.93(ax)	2.76(eq) 1.72(ax)	3.826	4.150	3.726	
H-4	3.799	3.699			4.191	
H-5						
H-6						
NAc	2.058	2.057	2.045		2.045	
^3J(C-1,H-3_{ax}) [Hz]	**5.60**	**10.60**				

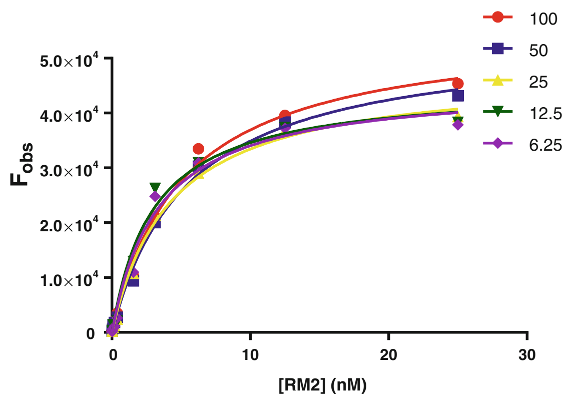

Fig. 2.4 Binding curves for RM2 printed at different concentrations (100, 50, 25, 12.5, and 6.25 µM). The *curves* were obtained using Cy3-labeled goat anti-mouse IgM secondary antibody. Adapted from [5], copyright 2015, with permission from American Chemical Society

Table 2.2 Surface dissociation constants ($K_{D,surf}$) of antibody RM2 and RM2 on microarray

Printing concentration RM2, µM	F_{max}	$K_{D,surf}$, nM
100	55,756	5.104
50	53,982	5.479
25	47,714	4.277
12.5	45,028	3.140
6.25	45,483	4.724

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$K_{D,surf}$ values (nM) for RM2 concentrations against fluorescent intensity at different concentrations of printed sugar

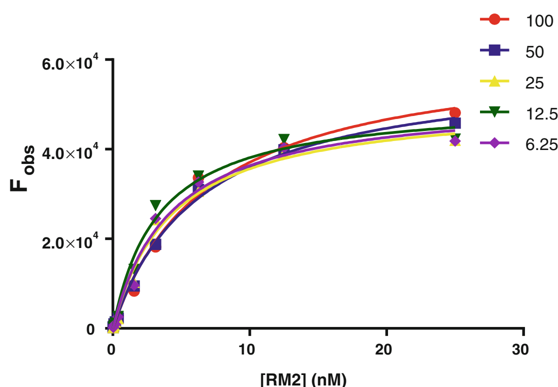

Fig. 2.5 Binding curve for RM2 antibody for **1-53** printed at different concentrations. Adapted from [5], copyright 2015, with permission from American Chemical Society

Table 2.3 Surface dissociation constants ($K_{D,surf}$) of antibody RM2 and **1-53** on microarray

Printing concentration RM2, μM	F_{max}	$K_{D,surf}$, nM
100	62,859	6.962
50	59,045	6.440
25	50,970	4.496
12.5	50,900	4.047
6.25	51,597	5.127

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$K_{D,surf}$ values (nM) for RM2 concentrations against fluorescent intensity at different concentrations of printed sugar

Table 2.4 $K_{D,surf}$ (nM) values of different antibodies and different RM2 analogs

	$K_{D,surf}$ (nM) ± SD (nM)	
Antibody	RM2 (**1**)	Pentasaccharide, **1-53**
RM2	4.545 ± 0.903	5.756 ± 1.140

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2.3 Summary

With these synthetic carbohydrates in hand, we used these glycans combined with other related glycans to form a 96-member glycan microarray to study the specificity of monoclonal RM2 antibody and confirmed the structure of our synthetic RM2 hexasaccharide antigen. Moreover, we also used the glycan array to determine the surface dissociation constant with monoclonal antibody RM2. The $K_{D,surf}$ values determined for the RM2 antigen interacting with antibodies was 4.5 nM. Overall, the relative binding specificity of RM2 for the epitopes was RM2 > pentasaccharide **1-53**.

2.4 Experimental Section

Glycan Microarray Fabrication Microarrays were printed (BioDot; Cartesian Technologies) by robotic pin (SMP3; TeleChem International Inc.) deposition of ~0.7 nL 100 μM amine-containing glycans in printing buffer (300 mM phosphate buffer, pH 8.5, containing 0.01 % TritonX-100) from a 14-well microtiter plate onto N−Hydroxysuccinimide (NHS)-coated glass slides. Subsequently, a 96-glycan microarray (Fig. 2.6) was used to determine the polyclonal Ab spectrum of DT-RM series–induced antiserum. Printed slides were allowed to react in an atmosphere of 80 % humidity for 1 h followed by desiccation overnight. These slides were stored at room temperature in a desiccator prior to use.

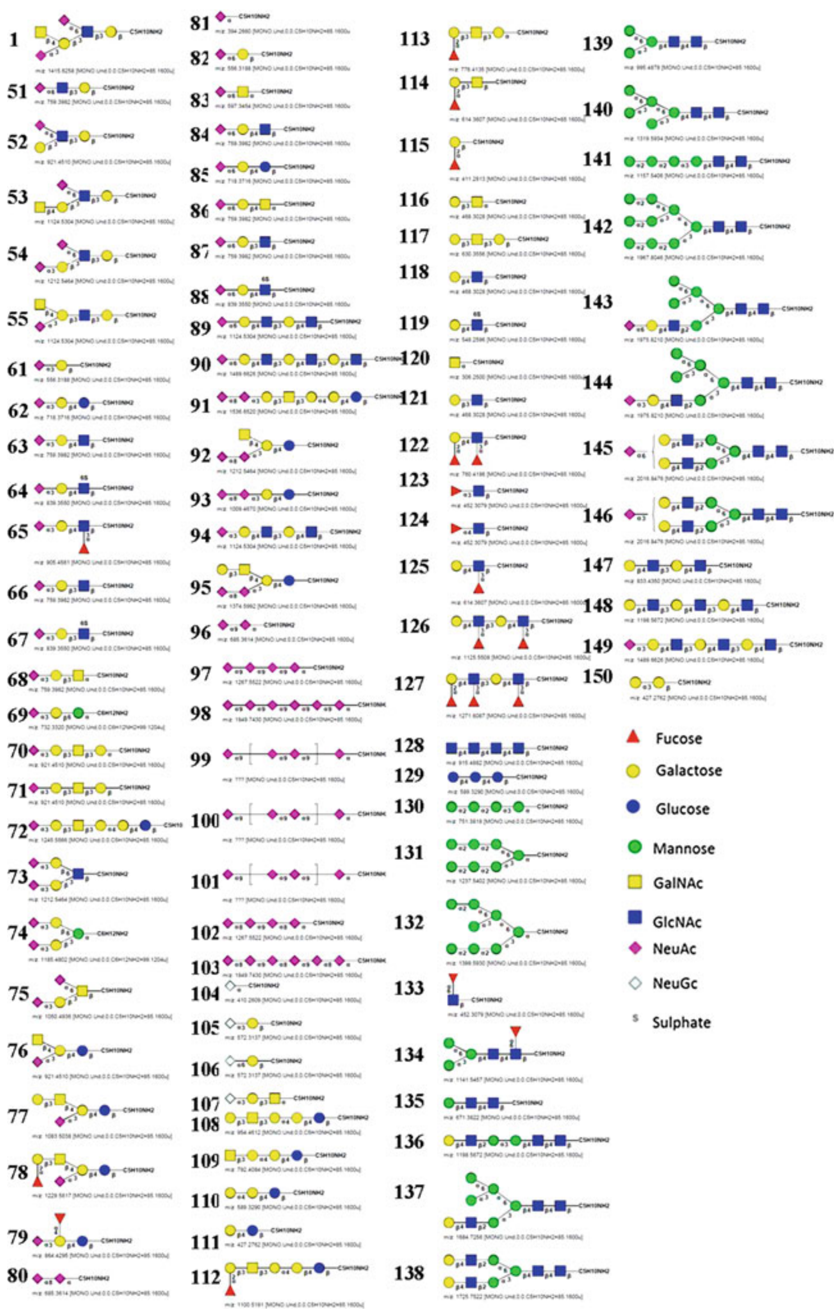

Fig. 2.6 Chemical structures of 96 various oligosaccharides for glycan array chip

Determination of Surface Dissociation Constant The slides were spotted with solutions of RM2 **1-1** and **1-53** with concentrations 100, 50, 25, 12.5, and 6.25 μM two rows from top to bottom with five replicates horizontally placed in each grid. The glycan microarray was blocked with 50 mM ethanolamine for 1 h at 0 °C and washed thrice with PBST buffer before use. The monoclonal RM2 antibody was diluted with 3 % BSA/PBST buffer (PBST buffer: PBS and 0.05 % Tween-20, pH 7.4). Next, Cy3-conjugated goat anti-mouse IgM antibody was added into the diluted solution to precomplexed with primary antibody. The precomplexed solution was applied to each well and incubated at 0 °C for 1 h in the dark. Finally, the slides were washed by PBST washing buffer and ddH_2O in sequence. The slides were spin-dried for 5 min before scanning at 635 nm with a microarray fluorescence chip reader (GenePix 4300A; Molecular Devices Corporation). Scanned images were analyzed with GenePix Pro-6.0 analysis software (Axon Instruments, Union City, CA, USA).

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Chapter 3
RM2 Antigen: Synthesis of Glycoconjugates

3.1 Introduction

3.1.1 Carbohydrate-Based Vaccines

Attractive carbohydrate-based vaccine targets include the aberrant glycosylation on malignant cells and the unique glycan structures on the surface of diverse pathogens. Francis and Tillett first reported carbohydrate-based vaccines in 1930 [1]. They found that injection of type-specific polysaccharides could induce antibodies for heterologous types of pneumococci. In the late 1940s, Macleod et al. [2] were the first to attempt to use polysaccharide as immunogens against a wide range of bacteria pathogens. Bacterial pathogens possess a cell surface capsular polysaccharide or a liposaccharide shell, which in turn helps to evade the human immune system. The immense polysaccharides not only hide the cell surface component of the bacteria from attacks by the host immune system but also prevent antibody-triggered complement activation and phagocytosis. Thus, antibodies specifically targeted at the bacterial surface polysaccharide may prevent infection from pathogens.

The first licensed synthetic oligosaccharide vaccine was the Hib vaccine produced in Cuba by Bencomo et al. They synthesized an eight-repeating unit of oligosaccharide with an amine group coupled to a maleimide-activated ester, which

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allowed the saccharide to be conjugated to thiolated proteins by Michaelic addition (Fig. 3.1) [3]. After the success of antibacterial glycoconjugate vaccines, researchers shifted their attention to the development of antiprotozoan, antiviral, and anticancer vaccines.

An ideal target antigen for development of cancer vaccine should be specifically expressed on cancer cells and accessible to the immune system. Tumor-associated carbohydrate antigens (TACAs) are not only most abundantly and sometimes aberrantly expressed on the surface of cancer cells but are also absent or rarely expressed on normal cells [4]. Many TACAs have been characterized for specific types of cancer [5] and suggested for the development of tumor-specific vaccines

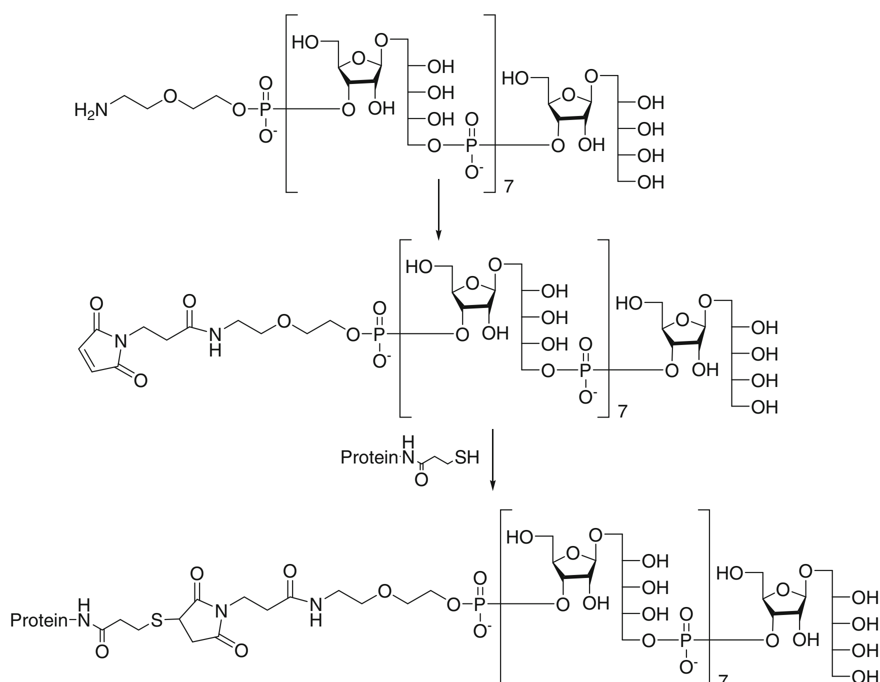


Fig. 3.1 The first commercial vaccine made from a synthetic carbohydrate. Adapted from [3], copyright 2015, with permission from John Wiley & Sons Ltd

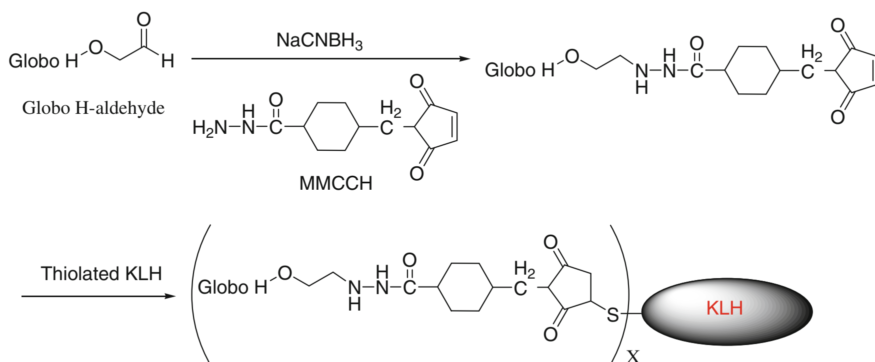


Fig. 3.2 Synthesis of Globo H–KLH conjugates by reductive amination and MMCCH crosslinker. Modified from [6, 7]

[6, 7]. However, because carbohydrates generally display poor immunogenicity, a glycan vaccine conjugated with a carrier protein can stimulate the immune response. One example is the Globo H–KLH vaccine, which was first synthesized by Danishefsky and co-workers via the convergent glycal assembly strategy to Keyhole limpet hemocyanin (KLH) conjugate (Fig. 3.2) [8, 9]. In the phase I clinical trial of Globo H–KLH [10, 11], ~700 (Globo H) GH units conjugated to one KLH protein was administered together with QS21 adjuvant. The treatment appeared to be fairly well-tolerated and was capable of inducing a high titer of IgM antibodies that participate in complement-mediated tumor cell lysis. Currently, this vaccine is in phase III clinical trials in Taiwan and in phase II clinical trials in USA, Korea, Hong-Kong, and India. Moreover, similar synthetic approaches have been utilized to synthesize other vaccines (e.g., fucosyl-GM1-KLH [12, 13] and Le^y-KLH [14]) against other types of cancer. Compared to a mixture of the corresponding monovalent vaccines, the unimolecular polyvalent vaccine used less carrier protein leading to less epitopic immune suppression. By sufficient coupling the mentioned tumor-associated carbohydrate antigens with immunogenic carrier protein, vaccines are currently undergoing clinical evaluation with encouraging results (Table 3.1) [15].

Table 3.1 Carbohydrate-based anticancer vaccines under development

Target antigen	Spacer	Carrier	Cancer type	References
Monomeric vaccine				
Globo H	CH_2CH_2	BSA, KLH	Breast	[8, 9]
	p-nitrophenyl	CRM197, TT	Breast	[16]
	MMCCH	KLH	Breast, prostate	[10]
Fuc-GM1	Ceramide-reductive amination	KLH	Small-cell lung	[17]
GD3	Ceramide-reductive amination	KLH	Melanoma	[18, 19]
GM2	Ceramide-reductive amination	KLH	Melanoma	[20, 21]
GD2	Ceramide-lactone	KLH	Melanoma	[22]
GM3	Proteoliposomes	OMPC	Melanoma	[23]
Le^y	CH_2CH_2	KLH	Ovarian	[24]
STn	Crotyl linker	KLH	Breast	[25, 26]
PSA, NP-PSA	Reductive amination	KLH	Small-cell lung	[27]
Monomeric cluster vaccine				
Tn(c)	MBS	KLH, PAM	Prostate	[28]
TF(c)	MBS	KLH	Prostate	
STn(c)	STn© crotyl linker-MMCCH	KLH	Breast	[29]
Polyvalent vaccine (pooled monomeric vaccines)				
GD3, Le^y, MUC1 and MUC2	(GD3)-reductive amination (Le^y)-CH_2CH_2 (MUC1 and MUC2)-MBS	KLH	Melanoma Ovarian Breast	[30]
GM2, Globo H, Ley, Tn(c) and TF(c) MUC1 (32mer)	(GM2)-reductive amination (Le^y, Globo H)-MMCCH (MUC1, Tn, TF, and STn)-MBS	KLH	Prostate	[31]
Unimolecular polyvalent vaccine (consists of multiantigens on unimolecule)				
Globo H, Tn, STn, TF, Le^y, and (GM2)	Diaminopropyl-MBS	KLH	Breast, prostate	[32–34]
Globo H, Tn, STn, TF, and GM2	Diaminopropyl-MUC1-alanine-MBS	KLH	Breast	[35]
Multicomponent vaccine				
Fucosyl GM1	FucGM1-norleucine-MHC II binding peptide-MBS	KLH	Small-cell lung	[12]
Tn, TF or STF	Pam3CySK4-ethylene glycol-MUC1	KLH	Breast	[36]
Tn and TF (three-component branched)	Pan-DR epitope-Lys-MUC1-Tn-Ala-MUC1-TF	KLH	Breast	[37]

Modified from [15]

3.1.2 Applications of Glycolipids as Immunological Adjuvants

Among the variety of ligands that bind to CD1d, the most well-studied CD1d antigen, a glycolipid termed agelashins, is a structurally optimized compound of the natural product from *Agelas mauritianus* found by Koezuka and co-workers at Kirin Brewery in 1993 (Fig. 3.3) [38]. The α-galactosylceramide not only exhibited antitumor responses but also mediated adjuvant activity. A series of studies showed that the NKT cell activation is induced rapidly to produce large quantities of cytokines after interaction between the invariant TCR on the NKT cells and α-GalCer presenting CD1d molecule on the cell surface of APCs [39].

Several natural and synthetic analogs of α-GalCer have been investigated for their effects on NKT cell activation. Recently, Wong and co-workers evaluated the cytokine secretion profiles of 22 α-GalCer analogs, the same glycan head as α-GalCer but presence of double bond on either of the two lipids or with different chain length, and summarized in (Fig. 3.4) [40] that acyl modifications on the phenyl ring could promote Th1-biased polarization. The C34, C23, and 7DW8-5 induced higher ratio of IFN-γ to IL-4 in mice and in humans, exhibiting greater anticancer effects in mice (Figs. 3.5 and 3.6) [40].

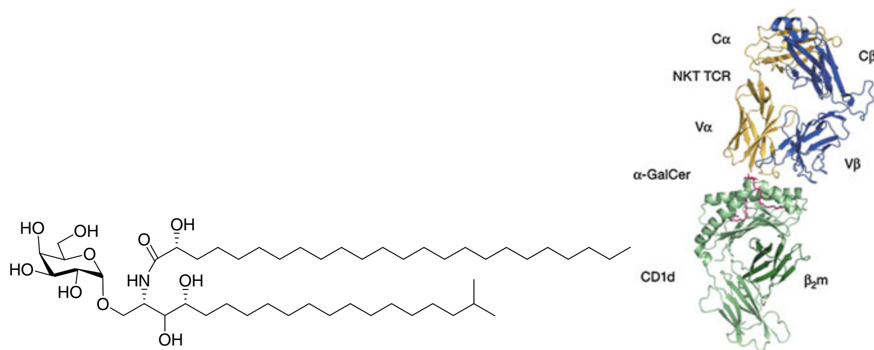
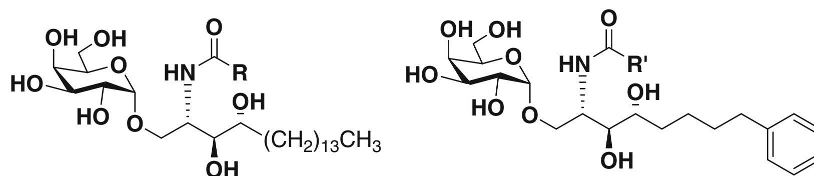


Fig. 3.3 Structure of agelashin and the *Agelas mauritianus*. Overview of the NKT TCR-human CD1d-α-GalCer complex



glycolipid	structure
α GalCer (C1)	$R=(CH_2)_{24}CH_3$
C10	$R=(CH_2)_5Ph$
C18	$R=(CH_2)_5Ph(p\text{-}OMe)$
C19	$R=(CH_2)_5Ph(p\text{-}F)$
C20	$R=(CH_2)_5Ph(p\text{-}CF_3)$
C21	$R=(CH_2)_5Ph(p\text{-}Ph)$
C11	$R=(CH_2)_7Ph$
C22	$R=(CH_2)_7Ph(p\text{-}OMe)$
C23	$R=(CH_2)_7Ph(p\text{-}F)$
C24	$R=(CH_2)_7Ph(p\text{-}CF_3)$
C25	$R=(CH_2)_7Ph(p\text{-}Ph)$
C16	$R=(CH_2)_{10}Ph$
7DW8-5	$R=(CH_2)_{10}Ph(p\text{-}F)$
7DW8-6	$R=(CH_2)_{10}Ph(p\text{-}CF_3)$
C26	$R=(CH_2)_{10}Ph(p\text{-}Ph)$
C30	$R=(CH_2)_{10}Ph(p\text{-}Ph)(p\text{-}F)$
C34	$R=(CH_2)_{10}Ph(p\text{-}O\text{-}Ph)(p\text{-}F)$
C27	$R=(CH_2)_{14}Ph$
C28	$R=(CH_2)_{20}Ph$
C29	$R=(CH_2)_{24}Ph$
C35	$R=(CH_2)_{10}Ph(p\text{-}F)$
C36	$R=(CH_2)_7Ph$
C37	$R=(CH_2)_7Ph(p\text{-}F)$

Fig. 3.4 The structure of α-GalCer analogs. The analogs of α-GalCer (C1) are separated into two categories: those with one or two phenyl ring(s) on the acyl chain only and those with one phenyl ring on each lipid tail (C35, C36, and C37). The glycolipids with one acyl phenyl ring without further modifications were arranged in the order of their chain length: C10, C11, C16 and then C27–C29. Adapted from [40], copyright 2015, with permission from National Academy of Sciences

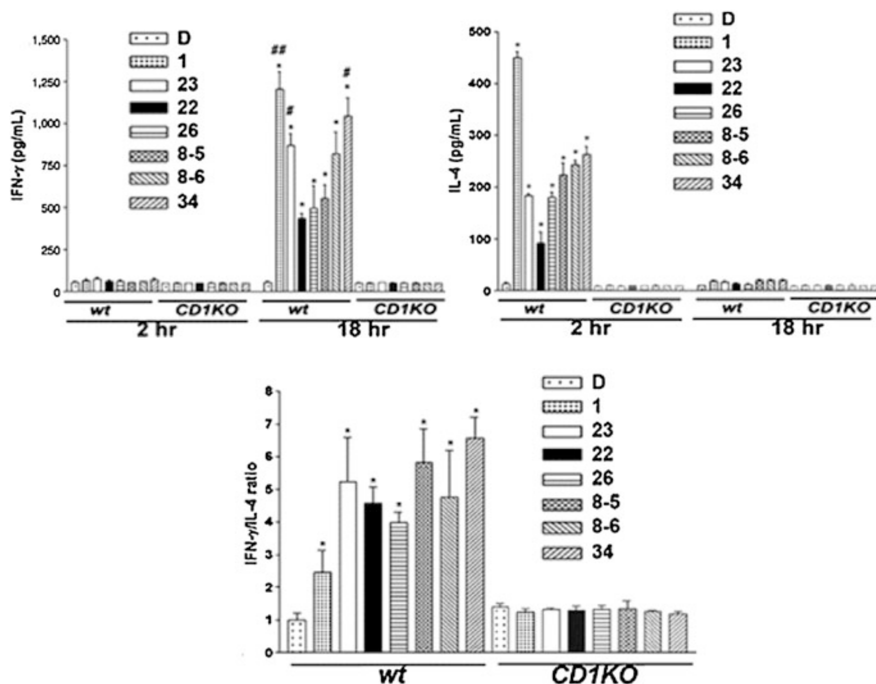


Fig. 3.5 Phenyl glycolipids-induced cytokines secretion was CD1d dependent in mice. Secretion of IFN-γ and IL-4 in mice after i.v. administration of glycolipids. BALB/c mice were injected i.v. with 0.1 μg of indicated glycolipids. Sera were collected at 2 and 18 h were analyzed for cytokines production. Adapted from [40], copyright 2015, with permission from National Academy of Sciences

3.2 Generation and Characterization of RM2 Glycoconjugate

3.2.1 *Synthesis of DT-RM$_{4.7}$ as a Vaccine Candidate with Glycolipid C34 as a Potent Adjuvant*

To develop a general protocol [16] for carbohydrate-carrier protein conjugation (Fig. 3.7; Table 3.2), we adopted the thiol-maleimide coupling method for sialic acid-rich compounds. The amine group of the hexasaccharide **1-1** was reacted with 2 equiv 3,3′-Dithiobis(sulfo-succinimidylpropionate) (DTSSP) and an amine-reactive *N*-hydroxysulfosuccinimide (sulfo-NHS) ester at each end of an 8-carbon spacer arm in pH 7.4 phosphate buffer at room temperature for 8 h to afford the corresponding half ester. Next, the disulfide bond was cleaved in the presence of dithiothreitol (DTT) at 40 °C for 2 h to obtain the free thio product **3-1** as Michael donors in 78 % yield after purification on a size exclusion column LH-20.

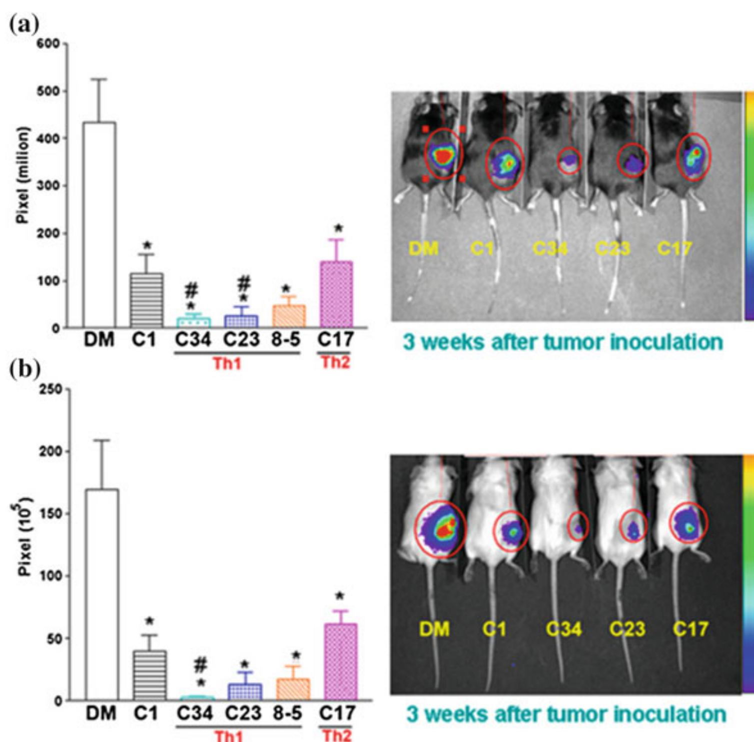


Fig. 3.6 Anticancer efficacy of phenyl glycolipids. **a** C57BL/Six mice (n ¼ 5) were injected intravenously with lung cancer cells (TC1) and the indicated glycolipids (0.1 μg/mouse) or 0.1 % DMSO once a week for 4 weeks, and BALB/c mice (n ¼ 5) were s.c. inoculated with breast cancer cells (**b**). Three days later, mice were i.v. injected with indicated glycolipids (0.1 μg/mouse) a week for 4 week. Adapted from [40], copyright 2015, with permission from National Academy of Sciences

Furthermore, in order to generate thio active maleimide group on the protein, CRM197 was reacted with excess *N*-(ε-maleimidocaproyloxy) sulfosuccinimide ester (Sulfo-EMCS) in pH 7.0 phosphate buffer for 2 h. The number of maleimide-linkers on the protein was determined by MALDI-TOF mass spectrometer. In average, 12.85 molecules of maleimide linkers were coupled on each molecule of diphtheria toxin mutant CRM197. Finally, for protein conjugation, the purified thiolated hexasaccharide **3-1** were incubated with the derivatized protein in pH 7.2 phosphate buffer for 2 h at room temperature to obtain the RM2 antigen-CRM197 glycoconjugate which was shown to contain 4.7 molecules of RM2 antigen per molecule of CRM197 (DT-$RM_{4.7}$ (Table 3.2). Our previous report showed that the glycoconjugate vaccine exhibited better immunization result to induce higher IgG titer antibodies when combined with the α-galactosylceramide analog C34 as an adjuvant [41]. To study the effect of adjuvant on Ab response, groups of BALB/c mice were immunized intramuscularly with 2 μg of DT-$RM_{4.7}$ in combination with

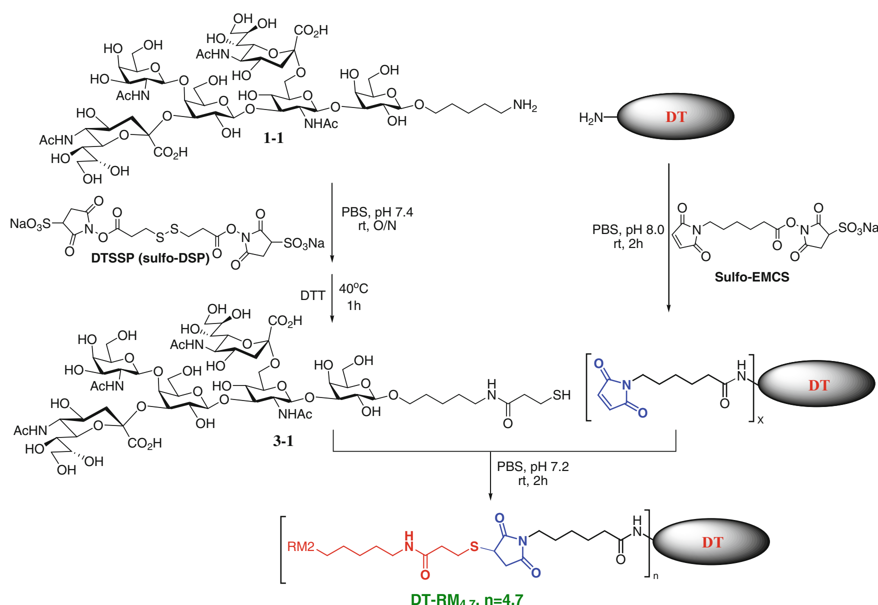


Fig. 3.7 Scheme for producing synthetic candidate carbohydrate-based vaccine. Adapted from [42], copyright 2015, with permission from American Chemical Society

Table 3.2 The preparation of RM2-DT conjugates

Entry	DT (mg)	Number of maleimide	RM2-SH (5 mg/mL)	Hapten incorporation	Product
1	1	26.6 (4.1×10^{-7} mole)	0.33 mL (11.1×10^{-7} mole)	10	**DT-RM$_{10}$**
2	1	12.85 (2.14×10^{-7} mole)	0.19 mL (6.42×10^{-7} mole)	4.7	**DT-RM$_{4.7}$**
3	2	12.85 (4.28×10^{-7} mole)	0.14 mL (4.63×10^{-7} mole)	3.0	**DT-RM$_{3.0}$**
4	6	12.85 (12.84×10^{-7} mole)	0.15 mL (4.96×10^{-7} mole)	1.0	**DT-RM$_{3.0}$**

(1) MALDI-TOF found 78822 → DT-RM$_{10}$; (2) MALDI-TOF found 67975 → DT-RM$_{4.7}$; (3) MALDI-TOF found 65316 → DT-RM$_{3.0}$; (4) MALDI-TOF found 62025 → DT-RM$_{3.0}$

2 μg of α-galactosylceramide C1, its analog C34 [16], or Alu. Three vaccinations were given at 2-week intervals. Two weeks after the third injection, sera were collected and subsequently tested with the previously mentioned glycan microarray (96 glycans) (Fig. 2.6) to estimate the level and diversity of anti-RM2-related antibody. The results showed that mouse anti-RM2 IgG titers increased as vaccination proceeded and peaked after the third vaccination. Among the DT-RM$_{4.7}$ vaccinated groups, we found that DT-RM$_{4.7}$/C34 induced higher levels of anti-RM2 IgG titers than DT-RM$_{4.7}$/C1 and DT-RM$_{4.7}$/Alu after dilution to 12,000-fold (Fig. 3.8).

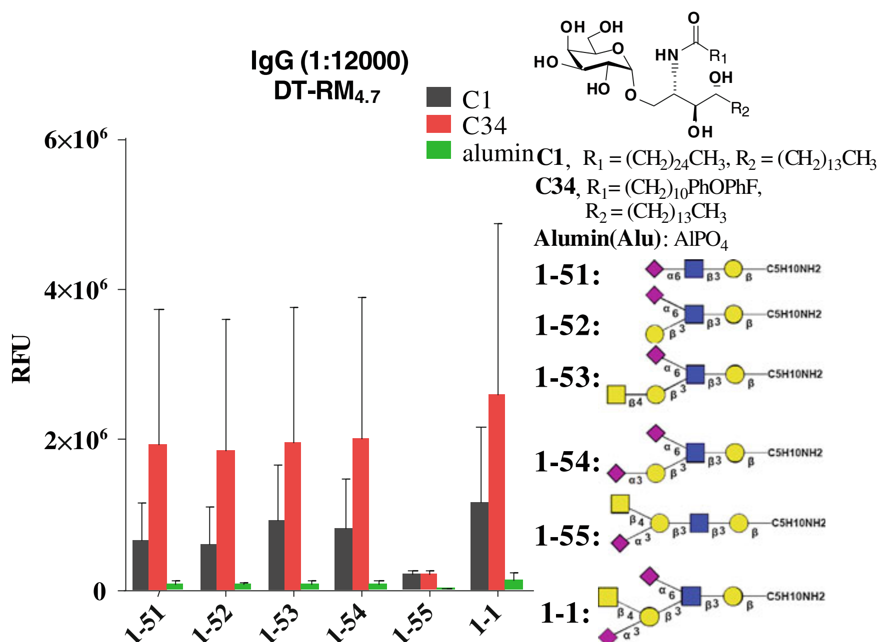
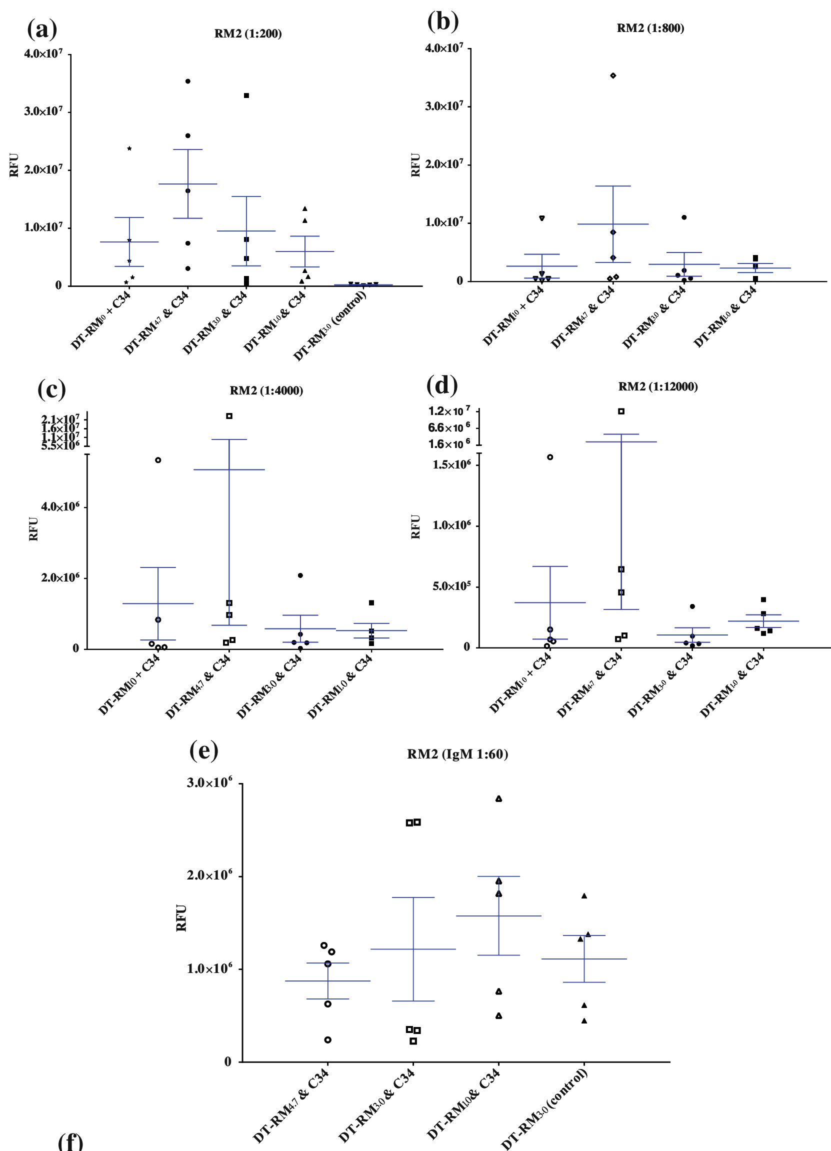


Fig. 3.8 Female BALB/c mice were immunized with 2 μg of RM2 antigen of DT-$RM_{4.7}$ in combination with 2 μg of C1, C34, or Alu. Mouse serum was collected 2 weeks after final immunization, and the production of IgG against RM2 antigen and its truncated analogs after dilution to 12,000-fold. Data represent as total fluorescence intensity of five mice ± the SEM. Adapted from [42], copyright 2015, with permission from American Chemical Society

3.2.2 Search for the Best Epitope Ratio of DT-RM Vaccine Adjuvanted with C34

After identifying C34 as the most effective adjuvant for DT-$RM_{4.7}$, we determined the best epitope ratio of vaccine by changing the amount of RM2 antigen attached to each carrier protein DT (Fig. 3.8). Various equivalents of thiolated hexasaccharide **3-1** and different protein concentrations generate diverse carbohydrate-protein ratios. The number of RM2 antigens on the protein was determined by MALDI-TOF mass spectrometer. After dialysis and analysis, we were able to conjugate, on average, 1.0, 3.0, 4.7, and 10 molecules of RM2 antigen to one molecule DT to give DT-$RM_{1.0}$, DT-$RM_{3.0}$, DT-$RM_{4.7}$, and DT-$RM_{10.0}$ (Table 3.2). Using the same vaccination protocol mentioned previously, sera were collected 2 weeks after the third vaccination, and the elicited Abs were subsequently profiled by an RM2-coated glycan microarray. In general, we observed that when immunized with DT-$RM_{3.0}$ alone without adjuvant, mice generated only low titers of anti-RM2 IgG (Fig. 3.9a). The glycan microarray results showed that, on average, 4.7 RM2 antigens conjugated to one DT induced the most abundant IgG titers against RM2 (Figs. 3.9 and 3.10).



(f)

	DT-RM$_{10}$					DT-RM$_{4.7}$					DT-RM$_{3.0}$					DT-RM$_{1.0}$				
12000	2	63	0.6	6	3	3	4	26	467	18	4	0.7	2	14	1	6	6	5	16	11
4000	2	178	5	28	2	6	9	44	753	32	14	1	7	70	6	11	11	5	44	17
800	3	271	11	32	12	12	20	212	884	103	48	5	14	275	27	65	14	11	103	97
200	17	595	110	199	38	76	185	650	885	412	202	12	33	823	119	21	67	41	335	284

Fig. 3.9 Serum IgG antibodies and IgM antibodies response against RM2 in immunized mice with different epitope ratios of DT-RM/C34. **a** 200-fold dilution of IgG antibodies. **b** 800-fold dilution of IgG antibodies. **c** 4,000-fold dilution of IgG antibodies. **d** 12,000-fold dilution of IgG antibodies. **e** 60-fold dilution of IgM antibodies. **f** Signal-to-noise (S/N) ratio of serum IgG antibodies against RM2 in different immunized mice with various fold dilution. Adapted from [42], copyright 2015, with permission from American Chemical Society

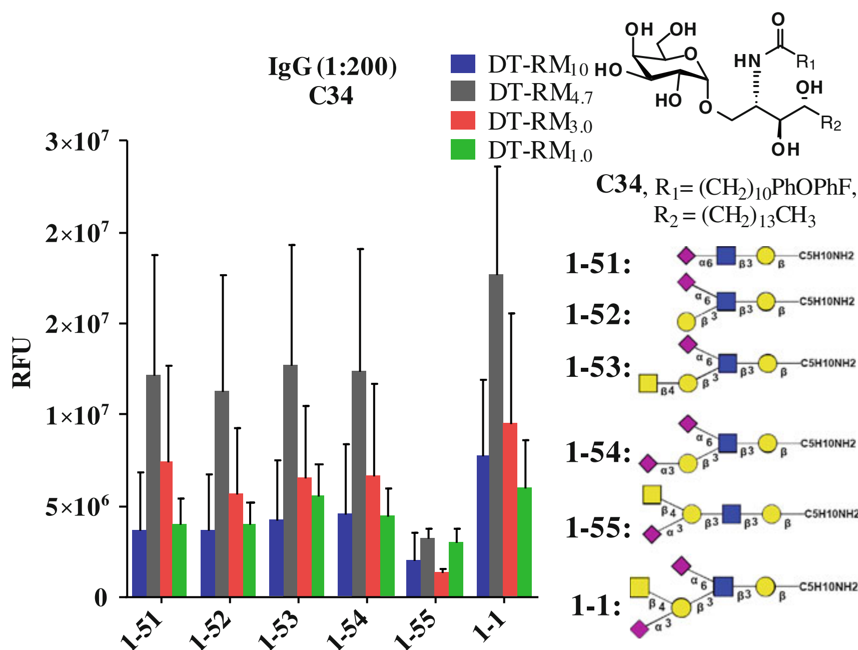


Fig. 3.10 The induced IgG against RM2 titers and glycan binding profile of IgG collected from different epitope ratios of DT-RM/C34-immunized mice. Adapted from [42], copyright 2015, with permission from American Chemical Society

Alternatively, when immunized with DT-$RM_{3.0}$ alone without adjuvant, mice generated only low titers of anti-RM2 IgG (Fig. 3.9a). When we fixed the signal-to-noise (S/N) ratio >3, the induced IgG could be diluted to 12,000-fold before the signal disappeared (Fig. 3.9f). However, the induced IgM titer could only be diluted to 60-fold (Fig. 3.9e), and the IgM signals reached to the background after dilution to 200-fold.

Interestingly, the specificity analysis of the induced IgG antibodies by DT-$RM_{4.7}$/C34 vaccine showed that the induced antibodies had a strong binding to the RM2 antigen and weaker binding to its trisaccharide **1-51**, tetrasaccharide **1-52**, pentasacharide **1-53**, and pentasacharide **1-54** (Fig. 3.10). We observed that these oligosaccharides all contain the same epitope trisaccharide **1-51** (NeuAcα2 → 6GlcNAcβ1 → 3Galβ1 → R). To further evaluate the vaccine specificity, we constructed a glycan microarray created by STn, DSGG, RM2 antigen, RM2 antigen analogs, and RM2 antigen fragments (Fig. 3.11). In general, specificity analysis of the induced antibodies by DT-$RM_{4.7}$/C34 vaccine showed that the induced antibodies mainly bound to the RM2 antigen and to its trisaccharide epitope **1-1** to **1-51**, a lesser extent to disaccharide **3-2** (NeuAcα2 → 6GlcNAcβ1 → R) and trisaccharide **3-3** (NeuAcα2 → 6GlcNAcβ1 → 3Gluβ1 → R). Besides, there is no detectable signal of binding to NeuAcα2 → 6Gal, DSGG, or STn.

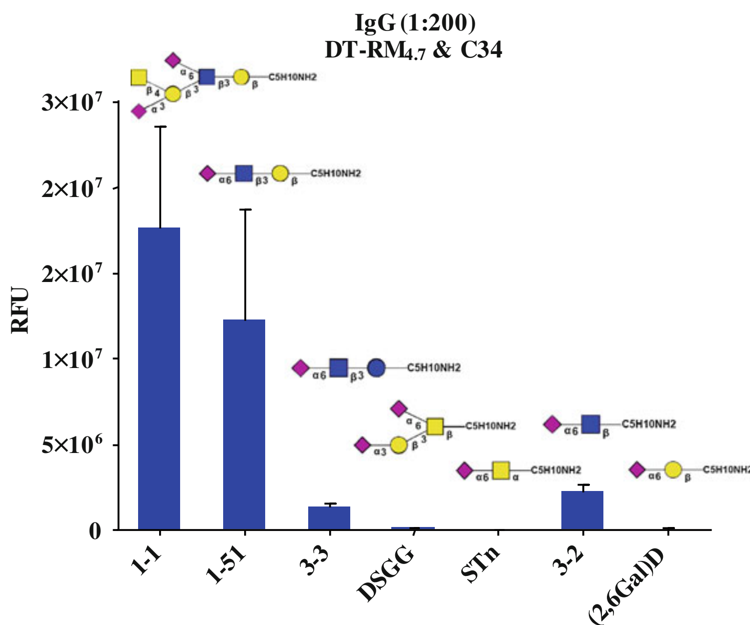


Fig. 3.11 Specificity analysis of the induced antibodies by DT-$RM_{4.7}$/C34 vaccine. Adapted from [42], copyright 2015, with permission from American Chemical Society

It was reported that DT could induce antigen-specific T cell proliferation and elevate splenocytes production of IL-2, IFN-γ, and IL-6, suggesting their role in the Th1-driven pathway [43–45]. Furthermore, the glycolipid C34 was able to induce higher production of IFN-γ and IL-4, indicating a more Th1-skewed antigen [40]. But, the induced subtypes anti-DT-$RM_{4.7}$/C34 antibodies were mainly IgG1 antibodies with a trace amount of IgG2b and IgG2c antibodies and no detectable IgG2a (Fig. 3.12).

Complement-dependent cytotoxicity (CDC) is one of the most potent cell killing mechanisms mediating the immune response in which IgG or IgM antibodies bind to antigens on the tumor or cancer cell surface. Complement activation, initiated through complement protein C1q binding to the Fc region of the IgM or IgG isotype antibodies, represents the important activity of antibodies against circulating tumor cells and micrometastases. To evaluate the therapeutic potential of anti-RM2 antibodies, we tested the complement-dependent cytotoxicity with prostate cancer cell line LNCap in the presence of new born rabbit complement. The ability of antibodies to induce complement activation is strongly dependent on the antibody isotype and epitope recognized. Thus, isotype IgG1 and IgG3 are able to activate the complement cascade particularly well through C1q, in contrast to IgG2 and IgG4 [46]. As shown above, our vaccine formulation resulted in a higher titer of subclass IgG1 antibodies than other subclasses, and the immune serum showed strong complement-mediated cytotoxicity activity on the RM2-positive human

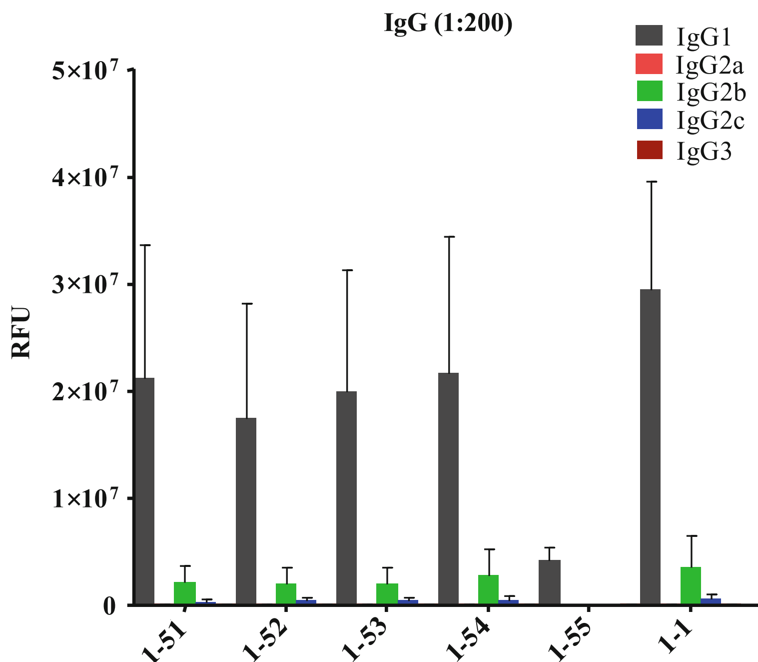


Fig. 3.12 The glycan binding profiles of induced IgG subtypes titers collected from DT-$RM_{4.7}$/C34-immunized mice 2 weeks after the third injection. Adapted from [42], copyright 2015, with permission from American Chemical Society

prostate cancer cell line LNCap (Fig. 3.13). These data suggest that our carbohydrate-based vaccine DT-RM, based on the chemically synthetic hexasaccharide **1-1** and a mutated diphtheria toxin (DT) with adjuvant C34 may create an efficient immune stimulation in humans.

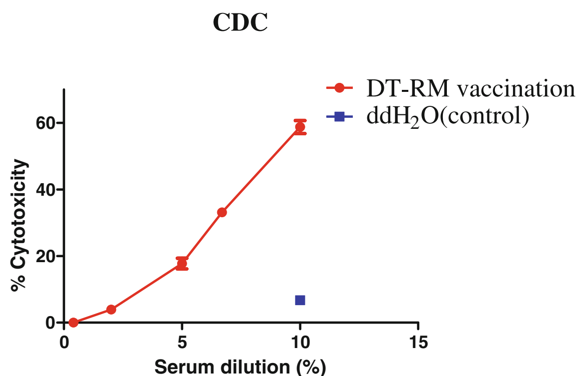


Fig. 3.13 CDC activity of the induced antibodies by serum from vaccination mice in the presence of rabbit complement. Lysis of human prostate cancer cell line LNCap at different concentrations. Adapted from [42], copyright 2015, with permission from American Chemical Society

3.2.3 Synthesis of RM2 Conjugated with Different Carrier Proteins

Because the DT-$RM_{4.7}$/C34 showed better vaccination results, it was of our interest to find the best composition of vaccine by changing the carrier proteins. To increase conjugation efficiency, we modified the aminopentanyl group of RM2 to the thiopentanyl group and also modified the lysine NH_2 group of protein to the maleimine functional group. Followed by a Michaelic addition in pH 7.2 phosphate buffer for 2 h at room temperature, we were able to generate RM2-protein conjugates, including DT-$RM_{5.0}$, BSA-$RM_{8.0}$, TT-$RM_{16.0}$, and KLH-RM (Fig. 3.14). The molecular weights of RM2-protein conjugates were determined by MALDI-TOF to calculate the average number of RM2 epitopes on each carrier protein (Table 3.3).

Using the same vaccination protocol mentioned previously, sera were collected 2 weeks after the third vaccination, and the elicited Abs were subsequently profiled by an RM2-coated glycan microarray to evaluate the level and diversity of anti-RM2 related Ab. In the group employed C34 as an adjuvant, we found that DT-$RM_{5.0}$ and TT-$RM_{16.0}$ could induce higher levels of anti-RM2 IgG than BSA-$RM_{8.0}$ and KLH-RM (Fig. 3.15). The levels of anti-RM2 IgG were significantly enhanced when co-administered with C34.

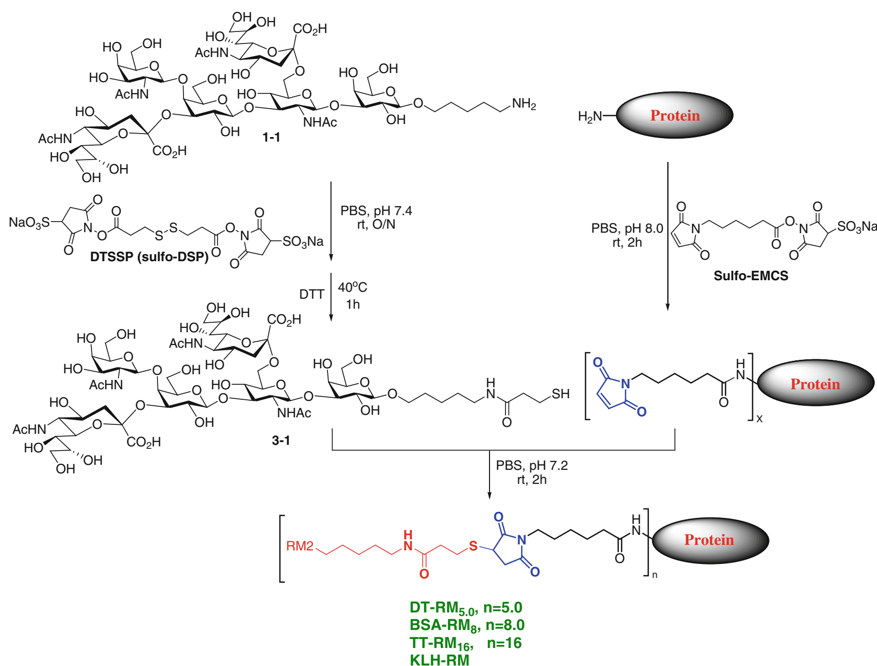


Fig. 3.14 Scheme for producing synthetic glycoconjugates

Table 3.3 The preparation of glycoconjugates

Entry	Protein (mg)	RM2-SH (5 mg/mL)	Hapten incorporation	Product
1	DT	0.068 mL (2.29 × 10^{-7} mole)	5	**DT-RM$_{5.0}$**
2	BSA	0.15 mL (5.07 × 10^{-7} mole)	8	**BSA-RM$_{8.0}$**
3	TT	0.12 mL (3.97 × 10^{-7} mole)	16	**TT-RM$_{16}$**
4	KLH	0.12 mL (3.97 × 10^{-7} mole)		**KLH-RM**
Entry	Protein (mg)	TR-SH (**3-4**) (5 mg/mL)	Hapten incorporation	Product
1	DT	1.0 mL	8.1	**DT-TR$_{8.1}$**

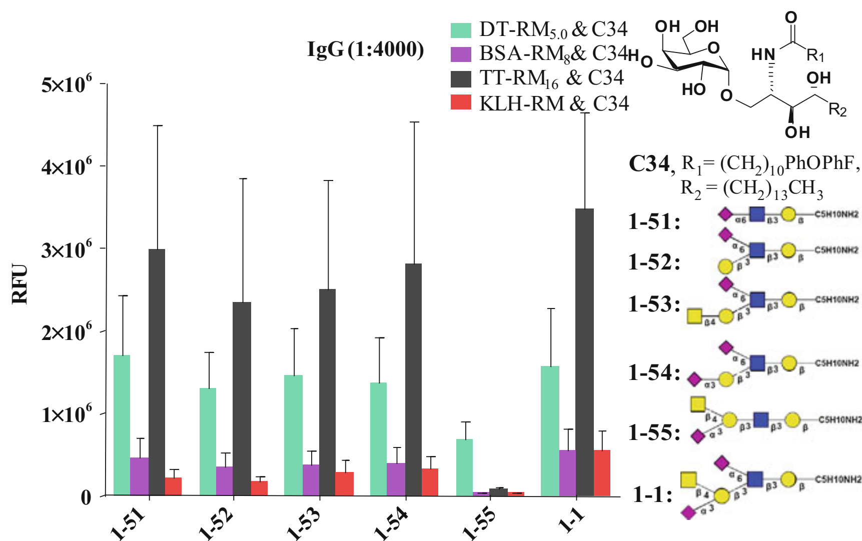


Fig. 3.15 Anti-RM2 Ab elicited by different RM2-carrier protein conjugates

In order to evaluate whether the vaccine provides long-term protective levels of anti-RM2 antibodies, groups of BALB/c mice were immunized intramuscularly with 2 µg of glycoconjugates in combination with or without 2 µg of C34. Three vaccinations were given at 2-week intervals. Two weeks after the third injection, sera were collected and subsequently tested with the previously mentioned glycan microarray (96 glycans) (Fig. S1) to estimate the level of anti-RM2-related antibody. Analysis of the kinetics of the antibody response revealed that anti-RM2 antibody titers peaked after the third vaccination in the four groups (DT-RM$_{5.0}$, BSA-RM$_{8.0}$, TT-RM$_{16.0}$, and KLH-RM) treated with adjuvant C34 and was markedly higher than DT-RM$_{5.0}$ alone (Fig. 3.16). In contrast, the titers gradually dropped to lower concentration over time. Besides, the specificity analysis of the induced IgG antibodies by glycoconjugates showed that the induced antibodies had

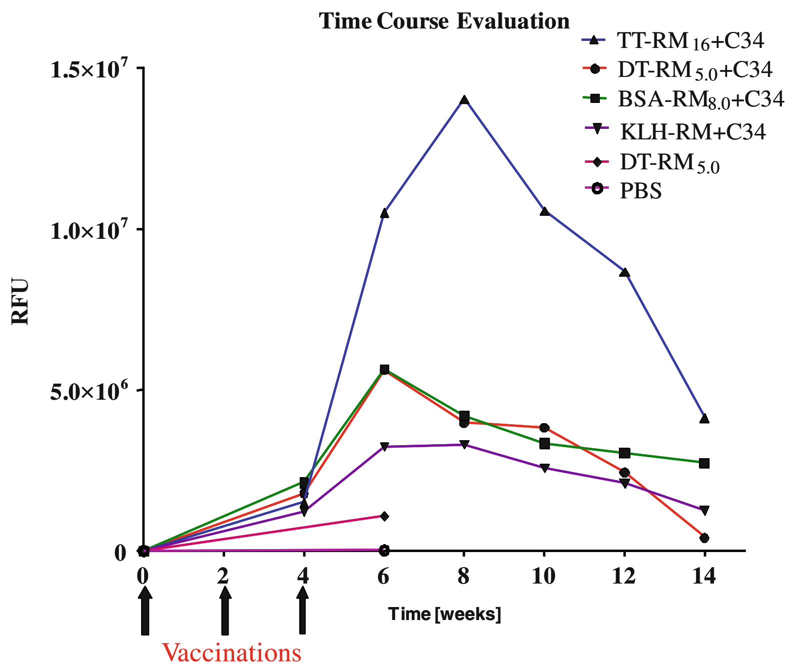


Fig. 3.16 Time course evaluation of mouse anti-RM2 IgG antibody

strong binding to these oligosaccharides containing the same epitope trisaccharide **1-51** (NeuAcα2 → 6GlcNAcβ1 → 3Galβ1 → R) (Fig. 3.15). Consequently, we decided to conjugate carrier protein DT to trisaccharide **1-51** to evaluate the specificity of the induced antibody. Followed the same conjugation method mentioned previously, we were able to conjugate, on average, 8.1 molecules of trisaccharide **1-51** to 1 molecule of DT (DT-$TR_{8.1}$) (Fig. 3.17; Table 3.3). Groups of BALB/c mice were immunized i.m. with DT-$TR_{8.1}$ (2 μg) with 2 μg glycolipid (C1 or C34) at 2-week intervals for three injections. Sera were obtained 10 days after the third vaccination, and the elicited Abs were examined by RM2-coated glycan microarray.

Among the DT-$TR_{8.1}$ vaccinated groups, we found that DT-$TR_{8.1}$/C34 induced higher levels of anti-RM2 IgG titers than DT-$TR_{8.1}$/C1 after dilution to 4,000-fold (Fig. 3.18). For IgG response, strong binding to trisaccharide **1-51** and a lesser extent to tetrasaccharide **1-52**, pentasaccharide **1-53**, pentasaccharide **1-54**, and hexasaccharide **1-1** epitopes were observed in both C1- and C34-adjuvanted vaccines. Above all, the specificity analysis of the induced IgG antibodies by vaccines (DT-$RM_{5.0}$/C34, BSA-$RM_{8.0}$/C34, TT-$RM_{16.0}$/C34, KLH-RM/C34, and DT-$TR_{8.1}$/C34) showed that the induced antibodies had strong binding to these oligosaccharides containing the same epitope trisaccharide **1-51** (NeuAcα2 → 6GlcNAcβ1 → 3Galβ1 → R). We wondered if this trisaccharide **1-51** (inner core of the RM2

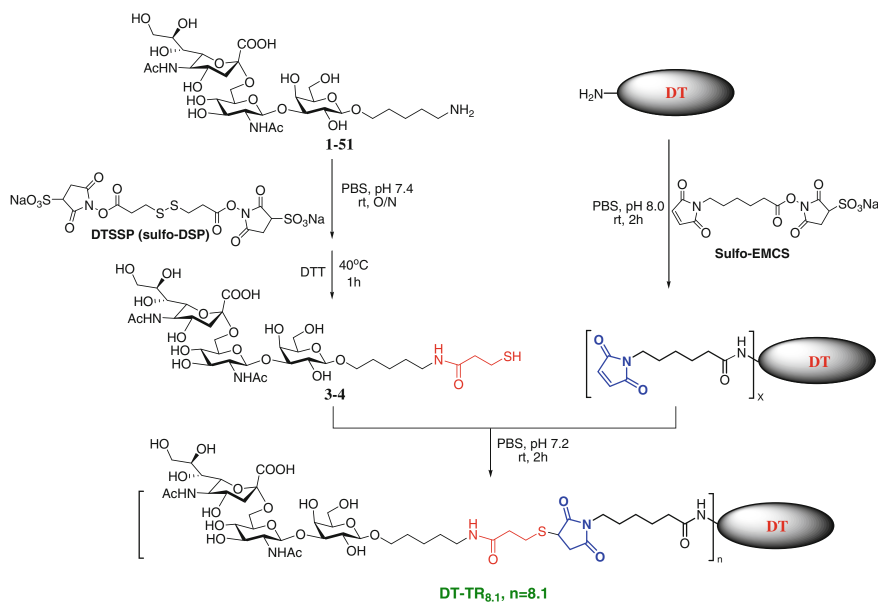


Fig. 3.17 Scheme for producing synthetic candidate trisaccharide-based vaccine

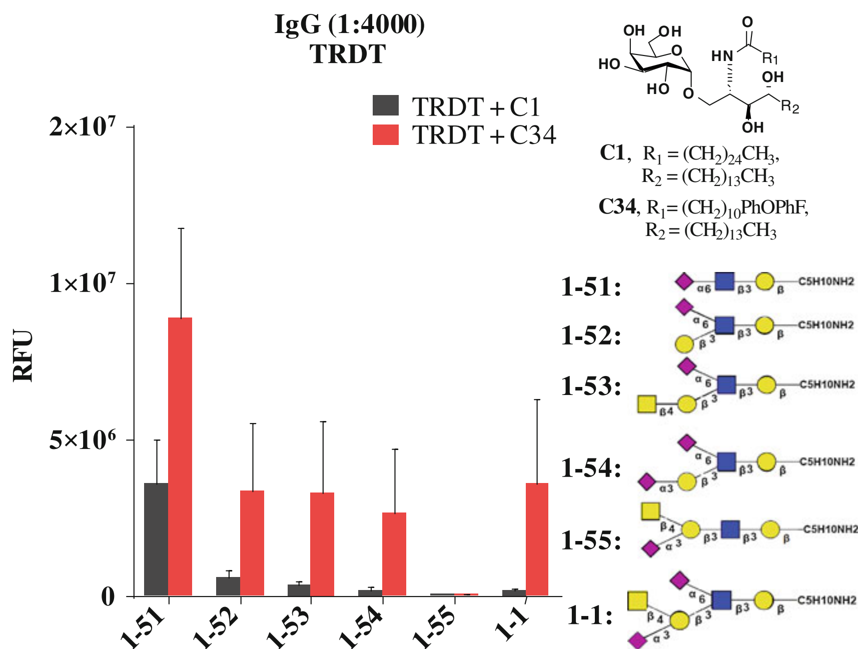


Fig. 3.18 Female BALB/c mice were immunized with 2 μg of trisaccharide **1-51** of DT-$TR_{8.1}$ in combination with 2 μg of C1 or C34

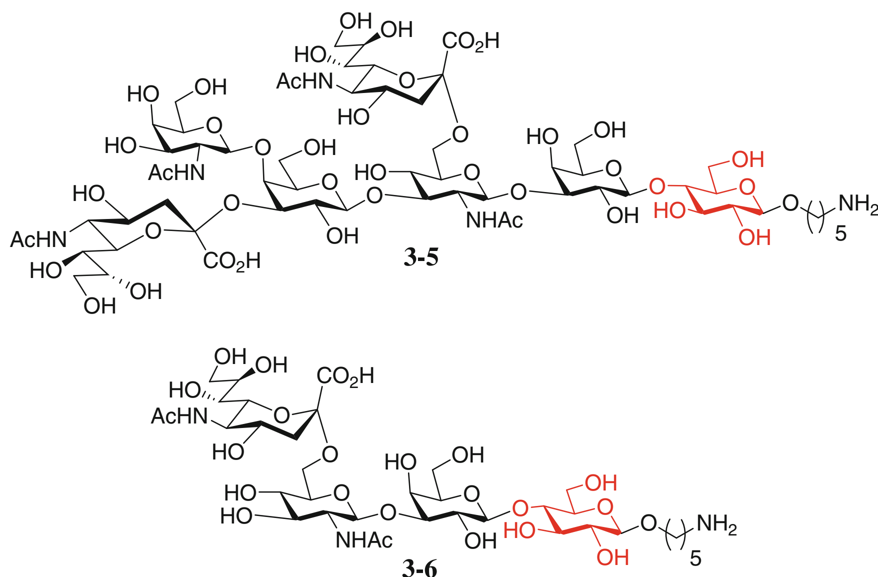


Fig. 3.19 The structure of an RM2 carbohydrate heptasaccharide **3-5** and truncated derivative tetrasaccharide **3-6**

antigen) played an important role on the immune response. Furthermore, RM2 is identified as a novel hybrid structure between "ganglio-series" and the "disialyl lacto-series type 1 chain" groups. For these reasons, we wanted to chemically synthesize the proposed heptasaccharide **3-5** and tetrasaccharide **3-6** to further evaluate the importance of the inner core of RM2 antigen (Fig. 3.19).

3.3 Summary

The synthetic antigen was conjugated to a mutated diphtheria toxin (DT, CRM197) with different copy numbers and adjuvant combinations to form the vaccine candidates. After vaccination in mice, we used glycan microarrays to monitor their immune response, and the results indicated that, when one molecule of DT was incorporated with 4.7 molecules of RM2 on average (DT-RM 4.7) and adjuvanted with the glycolipid C34, the combination exhibited the strongest anti-RM2 IgG titer. In addition, to identify the optimal carrier, we chemically synthesized and linked RM2 to a carrier protein, including keyhole limpet hemocyanion (KLH),

tetanus toxoid (TT), BSA, and mutated diphtheria toxin (DT, CRM197) and it was administered to mice for the study of immune response. In the group employed C34 as an adjuvant, we found that DT and TT could induce higher levels of anti-RM2 IgG than BSA and KLH-RM. Moreover, the induced mouse antibodies mediated effective complement-dependent cytotoxicity (CDC) against the prostate cancer cell line LNCap.

3.4 Experimental Section

Materials Commercial solvents and reagents were purchased from Sigma-Aldrich and Acros and used as received without further purification. Monoclonal antibody RM2 was provided by Prof. Seiichi Saito (Department of Urology, Graduate School of Medicine, University of Ryukyus, Nishihara 903-0215, Japan) and Cy3-conjugated anti-mouse IgG (IgG, IgG1, IgG2a, IgG2b, IgG2c, and IgG3) and IgM antibodies were from Jackson IMMUNO Research. Diphtheria toxoid (CRM 197) was purchased from *PF*enex Incorporation. Aluminum phosphate ($AlPO_4$) was from Brenntag Biosector and Glycolipid derivatives (C1 and C34) were from Dr. Chi-Huey Wong's lab.

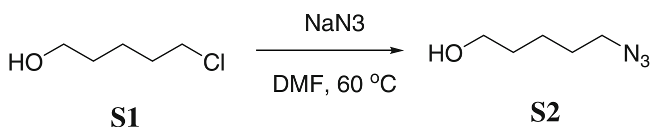
General Molecular sieves 4 Å (Reidel-deHaen No. 31812) for glycosylations were crushed and activated by heating at 350 °C for 10 h before use. Reactions were monitored with analytical TLC plates (PLC silica gel-60, F_{254}, 2 mm, Merck) and visualized under UV (254 nm) or by staining with acidic ceric ammonium molybdate or *p*-anisaldehyde. Flash column chromatography was performed on silica gel (40–63 µm, Merck), LiChroprep RP8 (40–63 µm), and LiChroprep RP18 (40–63 µm).

Instrumentation Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker Advance 600 (600 MHz/150 MHz) NMR spectrometers. Chemical shifts of protons were reported in ppm (δ scale) and referenced to tetramethylsilane ($\delta = 0$). Chemical shifts of carbon were also reported in parts per million (ppm, δ scale) and were calibrated with tetramethylsilane ($\delta = 0$). DEPT 135 (distortion less enhancement by polarization transfer) was employed for determination of multiplicity. Data were represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant (*J*) in Hz, and integration. High resolution mass spectra were obtained using BioTOF III, and MALDI-TOF MS were obtained using Ultraflex II TOF/TOF.

(2R,4R,5S,6S)-5-acetamido-2-((2R,3S,4R,5R,6R)-3-((2S,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)-6-((2S,3R,4R,5S,6R)-3-acetamido-6-(((2R,4S,5R,6R)-5-acetamido-2-carboxy-4-hydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-yloxy)methyl)-2-((2R,3S,4S,5R,6R)-3,5-dihydroxy-2-(hydroxymethyl)-6-(5-(3-mercaptopropanamido)pentyloxy)tetrahydro-2H-pyran-4-yloxy)-5-hydroxytetrahydro-2H-pyran-4-yloxy)-5-hydroxy-2-(hydroxymethyl)tetrahydro-2H-pyran-4-

yloxy)-4-hydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-carboxylic acid (3)

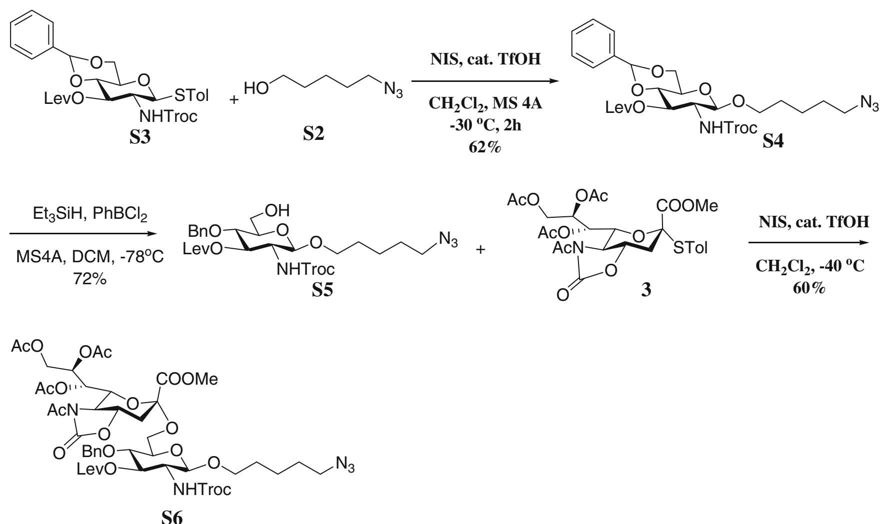
DTSSP (8.6 mg, 0.0141 mmole) was added to a solution of free amine **1-1** (10 mg, 0.007 mole) in 0.1 M phosphate buffer (3 mL), pH 7.4. Then the solution was stirred overnight. The reaction mixture was warmed to 40 °C, followed by addition of DTT (9.5 mg, 0.0615 mmole). After stirring at the same temperature for 1.5 h, the reaction mixture was concentrated in vacuo, and the residue was purified by LH-20 column to afford a white solid **3-1** (8.2 mg, 78 %). ^{1}H NMR (600 MHz, D_2O) δ 4.75–4.73 (m, 2H; $H^{Gla(I)}$-1 and H^{GlaNAc}-1), 4.55 (d, J = 8.1 Hz, 1H; $H^{Gla(II)}$-1), 4.40 (d, J = 8.0 Hz, 1H; H^{GlcNAc}-1), 4.17–4.11 (m, 3H), 4.01 (dd, J = 10.9, 5.4 Hz, 1H), 3.95–3.52 (m, 36H), 3.38 (dd, J = 9.6, 8.0 Hz, 1H), 3.24 (t, J = 6.8 Hz, 2H; aliphatic), 2.80 (t, J = 6.7 Hz, 2H; aliphatic), 2.76 (dd, J = 12.5, 4.6 Hz, 1H; $H^{sial(2\rightarrow6)}$-3 $_{eq}$), 2.70 (dd, J = 12.6, 4.6 Hz, 1H; $H^{sial(2\rightarrow3)}$-3 $_{eq}$), 2.56 (t, J = 6.7 Hz, 2H; aliphatic), 2.06 (s, 3H; CH_3 Ac), 2.06 (s, 3H; CH_3 Ac), 2.05 (s, 3H; CH_3 Ac), 2.04 (s, 3H; CH_3 Ac), 1.95 (t, J = 12.1 Hz, 1H; $H^{sial(2\rightarrow3)}$-3_{ax}), 1.77 (t, J = 12.2 Hz, 1H; $H^{sial(2\rightarrow6)}$-3_{ax}), 1.68–1.64 (m, 2H; aliphatic), 1.59–1.55 (m, 2H; aliphatic), 1.47–1.36 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, D_2O) δ 174.9, 174.8, 174.1, 174.0, 173.4, 102.9, 102.7, 102.6, 102.5, 101.4, 100.2, 82.5, 81.7, 76.8, 74.7, 74.6, 74.4, 74.0, 73.7, 72.9, 72.4, 72.2, 71.6, 71.2, 70.2, 69.7, 69.6, 68.6, 68.3, 68.2, 68.1, 67.9, 67.7, 64.6, 62.9, 62.7, 62.5, 61.1, 61.0, 60.4, 54.6, 52.3, 51.8, 51.5, 40.0, 39.3, 39.2, 37.1, 28.3, 27.9, 22.5, 22.4, 22.3, 22.0, 19.9; HRMS (ESI-TOF, MNa^+) calculated for $C_{58}H_{97}N_5O_{38}SNa$ 1526.5424, found 1526.5442.



5-azidopentan-1-ol (S2)

To a solution of 5-Chloro-1-pentanol (10.00 g, 80.16 mmole) in DMF (100 mL) was added NaN_3 (10.6 g, 163.2 mmole). After being stirred at 60 °C for 3 days, the reaction mixture was concentrated in vacuo. The residue was purified by silica gel column chromatography (1 : 4 to 1 : 3 ethylacetate/hexane) to give **S2** as oil (10.00 g, 95 %).

^{1}H NMR (600 MHz, $CDCl_3$) δ 3.62 (m, 2H), 3.25 (t, J = 6.9 Hz, 2H), 1.63–1.53 (m, 4H), 1.45–1.40 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 62.5, 51.3, 32.0, 28.5, 22.9; HRMS (ESI-TOF, MNa^+) calculated for $C_5H_{11}N_3ONa$ 152.0800, found 152.0804.



(1S,2R)-1-((3aR,4R,6R,7aS)-3-acetyl-6-(((2R,3S,4R,5R,6R)-6-(5-azidopentyloxy)-3-(benzyloxy)-4-(4-oxopentanoyloxy)-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yl)methoxy)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2,3-triyl triacetate (S6)

To a solution of purified thioglycoside (4.4 g, 6.82 mmole) and 5-azidopentan-1-ol (1.5 g, 11.62 mmole) in dichloromethane (80 mL), molecular sieves 4 Å (4 g) were added, and the mixture was stirred for 1 h at room temperature and then cooled to −20 °C. To the stirred mixture, NIS (2.63 g, 11.68 mmole) and TfOH (0.25 mL, 2.81 mmole) were added, and the solution was stirred continuously for 2 h at −20 °C. The precipitates were filtered off and washed with dichloromethane. The filtrates were combined, and the solution was successively washed with saturated $NaHCO_{3(aq)}$ and saturated $Na_2S_2O_{3(aq)}$, then dried with Na_2SO_4, and concentrated. Purification by flash silica gel column chromatography (1 : 4 to 1 : 3 ethyl acetate/hexane) gave **S4** as colorless oil. (2.75 g, 62 %).

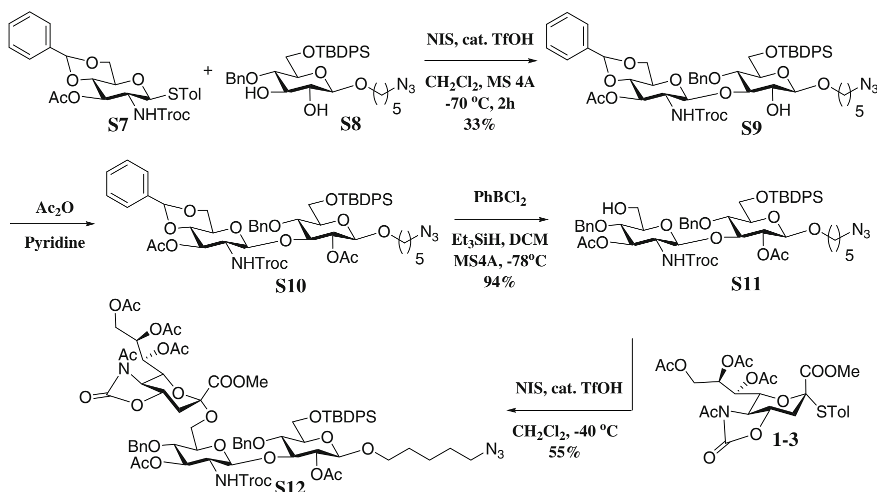
A solution of donor **S4** (2.8 g, 4.3 mmole) and activated 4 Å powdered molecular sieves (6.0 g) in anhydrous dichloromethane (80 mL) was stirred for 1 h under an argon atmosphere at room temperature, and then cooled to −78 °C followed by addition of Et_3SiH (2.7 mL, 16.71 mmole, 3.89 equiv) and $PhBCl_2$ (2.4 mL, 18.43 mmole, 4.28 equiv). The reaction mixture was stirred at −78 °C for 2 h until the disappearance of the donor on TLC. The resulting mixture was neutralized with triethylamine and excess borane was quenched with methanol. The mixture was diluted with dichloromethane, filtered through Celite, washed with saturated aqueous $NaHCO_3$ solution, and concentrated under reduced pressure. Purification by flash silica gel column chromatography (1 : 3 to 1 : 1.75 ethyl acetate/hexane) gave **S5** (2.02 g, 72 %).

A solution of donor **3** (0.85 g, 1.46 mmole, 1.49 equiv), acceptor **S5** (0.64 g, 0.98 mmole, 1.0 equiv), and activated 4 Å powdered molecular sieves (2.0 g) in anhydrous dichloromethane (35 mL) was stirred overnight under an argon atmosphere, and then cooled to −40 °C followed by addition of NIS (0.45 g, 2.01 mmole, 2.0 equiv) and TfOH (27.0 μL, 0.3 mmole, 0.3 equiv). The reaction mixture was stirred at −40 °C for 1 h until the disappearance of the donor on TLC, then quenched with triethylamine and warmed to room temperature. The mixture was diluted with dichloromethane, filtered through Celite, washed with 20 % aqueous $Na_2S_2O_3$ solution, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash silica gel column chromatography (1 : 2 to 1 : 1.25 ethyl acetate/hexane) gave **S6** (0.65 g, 60 %) (α only).

(2R,4S,5R,6R)-5-acetamido-2-(((2R,3S,4R,5R,6R)-5-acetamido-6-(5-aminopentyloxy)-3,4-dihydroxytetrahydro-2H-pyran-2-yl)methoxy)-4-hydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-carboxylic acid (3-2)

Compound **3-2** (200 mg, 0.334 mmole, 57 %) was prepared according to the general procedure for deprotection of oligosaccharide from disaccharide **S6** (0.65 g, 0.585 mmole).

^{1}H NMR (600 MHz, D_2O) δ 4.496 (d, J = 8.5 Hz, 1H; H^{GlcNAc}-1), 3.96 (dd, J = 10.7, 5.3 Hz, 1H), 3.90–3.86 (m, 3H), 3.83 (t, J = 10.1 Hz, 1H), 3.75 (d, J = 9.7 Hz, 1H), 3.72–3.59 (m, 6H), 3.54–3.48 (m, 3H), 3.00 (t, J = 7.6 Hz, 2H; aliphatic), 2.75 (dd, J = 12.5, 4.7 Hz, 1H; $H^{sial(2\rightarrow6)}$-3 $_{eq}$), 2.04 (s, 3H; CH_3 Ac), 2.04 (s, 3H; CH_3 Ac), 1.74–1.66 (m, 3H; aliphatic and $H^{sial(2\rightarrow6)}$-3$_{ax}$), 1.63–1.58 (m, 2H; aliphatic), 1.45–1.37 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, D_2O) δ 175.0, 174.4, 173.4, 101.3, 100.1, 74.2, 73.6, 72.5, 71.7, 70.2, 69.7, 68.2, 68.1, 62.8, 62.6, 55.5, 51.8, 40.1, 39.3, 28.1, 26.3, 22.1, 22.0; HRMS (ESI-TOF, MH^+) calculated for $C_{24}H_{43}N_3O_{14}$ 598.2818, found 598.2805.



(1S,2R)-1-((3aR,4R,6S,7aS)-6-(((2R,3S,4R,5R,6S)-4-acetoxy-6-((2R,3R,4S,5R,6R)-3-acetoxy-2-(5-azidopentyloxy)-5-(benzyloxy)-6-((tert-butyldiphenylsilyloxy)methyl)tetrahydro-2H-pyran-4-yloxy)-3-(benzyloxy)-5-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yl)methoxy)-3-acetyl-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2,3-triyl triacetate (S12)

A solution of donor **S7** (2.46 g, 4.16 mmole, 1.56 equiv), acceptor **S8** (1.65 g, 2.66 mmole, 1.0 equiv), and activated 4 Å powdered molecular sieves (5.0 g) in anhydrous dichloromethane (65 mL) was stirred overnight under an argon atmosphere, and then cooled to −70 °C followed by addition of NIS (1.19 g, 5.29 mmole, 2.0 equiv) and TfOH (95.0 μL, 1.07 mmole, 0.4 equiv). The reaction mixture was stirred at −70 °C for 2 h until the disappearance of the donor on TLC, then quenched with triethylamine and warmed to room temperature. The mixture was diluted with dichloromethane, filtered through Celite, washed with 20 % aqueous $Na_2S_2O_3$ solution, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash silica gel column chromatography (1 : 5 to 1 : 4 ethyl acetate/hexane) gave **S9** (1.75 g, 33 %).

A solution of **S9** in anhydrous CH_2Cl_2 (25 mL) was treated with acetic anhydride (10 mL) and pyridine (15 mL). The reaction mixture was stirred at room temperature for 8 h under argon. The solution was concentrated to syrup which was extracted with dichloromethane. The extract was successively washed with 2 N HCl, water, $NaHCO_{3(aq)}$ and water, dried with Na_2SO_4 and concentrated. Purification by flash chromatography on silica gel (1:10 to 1:5 ethyl acetate/hexane) gave **S10** as white solid (1.75 g, 1.55 mmole, 95 %).

A solution of donor **S10** (1.75 g, 1.55 mmole) and activated 4 Å powdered moleecular sieves (4.0 g) in anhydrous dichloromethane (50 mL) was stirred for 1 h under an argon atmosphere at room temperature, and then cooled to −78 °C followed by addition of Et_3SiH (0.8 mL, 4.95 mmole, 3.19 equiv) and $PhBCl_2$ (0.63 mL, 4.69 mmole, 3.02 equiv). The reaction mixture was stirred at −78 °C for 2 h until the disappearance of the donor on TLC. The resulting mixture was neutralized with triethylamine and excess borane was quenched with methanol. The mixture was diluted with dichloromethane, filtered through Celite, washed with saturated aqueous $NaHCO_3$ solution, and concentrated under reduced pressure. Purification by flash silica gel column chromatography (1:4 to 1:3 ethyl acetate/hexane) gave **S11** (1.65 g, 94 %)

A solution of donor **1-3** (1.41 g, 2.42 mmole, 1.66 equiv), acceptor **S11** (1.65 g, 1.46 mmole, 1.0 equiv), and activated 4 Å powdered molecular sieves (4.0 g) in anhydrous dichloromethane (65 mL) was stirred overnight under an argon atmosphere, and then cooled to −40 °C followed by addition of NIS (0.72 g, 3.2 mmole, 2.2 equiv) and TfOH (74.0 μL, 0.83 mmole, 0.6 equiv). The reaction mixture was stirred at −40 °C for 1 h until the disappearance of the donor on TLC, then quenched with triethylamine and warmed to room temperature. The mixture was diluted with dichloromethane, filtered through Celite, washed with 20 % aqueous $Na_2S_2O_3$ solution, dried over Na_2SO_4, filtered, and concentrated under reduced

pressure. Purification by flash silica gel column chromatography (1:3 to 1:1.75 ethyl acetate/hexane) gave **S12** (1.27 g, 55 %) (α only).

(2R,4S,5R,6R)-5-acetamido-2-(((2R,3S,4R,5R,6S)-5-acetamido-6-((2R,3R,4S,5R,6R)-2-(5-aminopentyloxy)-3,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-4-yloxy)-3,4-dihydroxytetrahydro-2H-pyran-2-yl)methoxy)-4-hydroxy-6-((1R,2R)-1,2,3-trihydroxypropyl)tetrahydro-2H-pyran-2-carboxylic acid (3-3)

Compound **3-3** (120 mg, 0.334 mmole, 20 %) was prepared according to the general procedure for the deprotection of oligosaccharide from disaccharide **S12** (1.27 g, 0.802 mmole).

^{1}H NMR (600 MHz, D_2O) δ 4.66 (d, J = 8.5 Hz, 1H; H^{Glc}-1), 4.43 (d, J = 8.1 Hz, 1H; H^{GlcNAc}-1), 3.96–3.85 (m, 5H), 3.83 (t, J = 10.1 Hz, 1H), 3.77–3.72 (m, 3H), 3.71–3.62 (m, 4H), 3.60–3.54 (m, 4H), 3.50–3.44 (m, 3H), 3.31 (t, J = 8.6 Hz, 1H), 3.00 (t, J = 7.5 Hz, 2H; aliphatic), 2.72 (dd, J = 12.4, 4.7 Hz, 1H; $H^{sial(2\rightarrow6)}$-3$_{eq}$), 2.04 (s, 3H; CH_3 Ac), 2.03 (s, 3H; CH_3 Ac), 1.75–1.64 (m, 5H; aliphatic and $H^{sial(2\rightarrow6)}$-3$_{ax}$), 1.49–1.42 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, D_2O) δ 174.9, 174.7, 173.4, 102.2, 102.1, 100.0, 86.1, 75.3, 74.2, 73.4, 72.4, 72.3, 71.7, 70.0, 69.9, 68.5, 68.2, 68.2, 63.1, 62.5, 60.8, 55.5, 51.8, 40.0, 39.3, 28.1, 28.4, 22.1, 22.0; HRMS (ESI-TOF, MNa^+) calculated for $C_{30}H_{53}N_3O_{19}Na$ 782.3165, found 782.3174.

General procedure for generating maleimide activation DT

DT (Diphtheria toxin)(1 mg) was dissolved in 0.1 M phosphate buffer pH 7.0 or 8.0 (~1 mg/ml), and Sulfo-EMCS (1 mg) was added to the solution. The solution was stirred gently for 2 h at room temperature. The mixture was then diluted with deionized water and centrifuge against 5 changes of deionized water by Amicon Ultra-0.5 (10 kDa, 2X). The solution was lyphophilized to white solid. The obtained maleimide DT can be characterized by MALDI-TOF (positive mode, matrix sinapinic acid, H_2O) analysis to determine the maleimide incorporation rate. MALDI-TOF found 60806 (60,806 − 58,326/193 = 12.85); MALDI-TOF found 63464 (63,464 − 58,326/193 = 26.6)

General procedure for generating protein carbohydrate conjugates

Modified DT was dissolved in 0.1 M phosphate buffer pH 7.2 (~1 mg/ml), and a different ratio of thiolated hexasaccharide **3-1** was added to the solution. The mixture was stirred for 2 h at room temperature. The mixture was then diluted with deionized water and centrifuged against 5 changes of deionized water by Amicon Ultra-0.5 (10 kDa, 2X). The solution was lyphophilized to white solid. The obtained **DT-RM** series can be characterized by MALDI-TOF (positive mode, matrix sinapinic acid, H_2O) analysis to determine the carbohydrate incorporation rate.

General procedure for generating maleimide activation protein (BSA, TT)

Protein (BSA, TT)(1 mg, 2 mg, 1.1 mg) was dissolved in 0.1 M phosphate buffer pH 7.2 (~1 mg/ml), and Sulfo-EMCS (1.4 mg) was added to the solution. The solution was stirred gently for 2 h at room temperature. The mixture was then diluted with deionized water and centrifuged against 5 changes of deionized water by Amicon Ultra-0.5 (10 kDa, 2X). The solution was lyphophilized to white solid.

The obtained maleimide BSA or DT can be characterized by MALDI-TOF (positive mode, matrix sinapinic acid, H_2O) analysis to determine the maleimide incorporation rate.

General procedure for generating protein carbohydrate conjugates

Modified protein was dissolved in 0.1 M phosphate buffer pH 7.2 ($\sim$1 mg/ml), and a different ratio of thiolated hexasaccharide **3-1** or thiolated trisaccharide **3-4** was added to the solution. The mixture was stirred for 2 h at room temperature. The mixture was then diluted with deionized water and centrifuged against 5 changes of deionized water by Amicon Ultra-0.5 (10 kDa, 2X). The solution was lyphophilized to white solid. The obtained **glycoconjugates** series can be characterized by MALDI-TOF (positive mode, matrix sinapinic acid, H_2O) analysis to determine the carbohydrate incorporation rate.

Mice Dosage and Immunization Schedule For comparing immunogenicity of DT-RM vaccine with different amounts of RM2 attached to each carrier protein DT, groups of three–five mice (8-week-old female Balb/c mice, BioLASCO, Taiwan) were immunized intramuscularly with **DT-RM$_{1.0}$**, **DT-RM$_{3.0}$**, **DT-RM$_{4.7}$**, or **DT-RM$_{10.0}$** with or without adjuvant C1, C34, or $AlPO_3$, respectively. Each vaccination contained 2 μg of RM2 (**DT-RM$_{1.0}$**, **DT-RM$_{3.0}$**, **DT-RM$_{4.7}$**, or **DT-RM$_{10.0}$**) and with or without 2 μg of C1, C34, or Alu, respectively. Control mice were injected with phosphate buffer saline (PBS). Three vaccinations were given at 2-week intervals. Two weeks after the third injection, sera were obtained and subsequent tested with an RM2-coated glycan microarray to estimate the level and diversity of anti-RM2 related antibody.

Glycan Microarray Fabrication Microarrays were printed (BioDot; Cartesian Technologies) by robotic pin (SMP3; TeleChem International Inc.) deposition of $\sim$0.7 nL 100 μM amine-containing glycans in printing buffer (300 mM phosphate buffer, pH 8.5, containing 0.01 % TritonX-100) from a 14-well microtiter plate onto N-Hydroxysuccinimide (NHS)-coated glass slides. Subsequently, a 96-glycan microarray (Fig. S1) was used to determine the polyclonal Ab spectrum of DT-RM series-induced antiserum. Printed slides were allowed to react in an atmosphere of 80 % humidity for 1 h followed by desiccation overnight. These slides were stored at room temperature in a desiccator prior to use.

Serologic Assay with Glycan Microarray Mouse sera were diluted with 1 % BSA/PBST buffer (PBST buffer: PBS and 0.05 % Tween-20, pH 7.4). The glycan microarray was blocked with 50 mM ethanolamine for 1 h at 0 °C and washed thrice with PBST buffer before use. The serum dilutions were then introduced to the glycan microarray and incubated at 0 °C for 1 h. Next, Cy3-conjugated goat anti-mouse IgG (H + L), IgG1, IgG2a, IgG2b, IgG2c, IgG3, or anti-mouse IgM was added to the microarray slide and then sealed for 1 h incubation at 0 °C in the dark. Finally, the slides were washed thrice with PBST, PBST washing buffer, and ddH_2O in sequence. The slides were spin-dried for 5 min before scanned at 635 nm with a microarray fluorescence chip reader (GenePix 4300A; Molecular Devices Corporation). Scanned images were analyzed with GenePix Pro-6.0 analysis software (Axon Instruments, Union City, CA, USA).

Complement-dependent cytotoxicity (CDC) test Complement-dependent cytotoxicity was tested by nonradioactive cytotoxicity assay. LNCap cells were added to wells of U-bottomed tissue culture microtiter plates at a concentration of 1.25×10^4 cells/25 μL and incubated with different dilutions of PBS or post-vaccination sera or with medium (RPMI-1640) alone. Next, 50 μL of rabbit complement diluted 1:25 in complete medium (RPMI-1640) was added to triplicate samples and incubated for 90 min at 37 °C. Thus, the final complement dilution in the assay was 1:50. Following incubation, 150 μL medium (RPMI-1640) were added for every well and supernatants (50 μL) were collected by centrifugation. Reconstituted LDH substrate mix (50 μL) was added to each well of the assay plate containing sample transferred from the cytotoxicity assay plate. Then, the plate was sealed for 30 min for incubation at room temperature in the dark. Finally, stop solution (50 μL) was added to each well, and the absorbance at 490 nm was recorded immediately. All assays were performed in triplicate, and the spontaneous release was evaluated in the presence of complement. The percentage specific lysis was calculated as follows: Cytotoxicity (%) = 100 × [experimental release − spontaneous release]/[maximum release − spontaneous release].

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Chapter 4
Synthesis of Heptasaccharide RM2 Prostate Tumor Antigen: Chemical Synthesis of Heptasaccharide and Tetrasaccharide (Inner Core of the RM2 Antigen)

4.1 Introduction

4.1.1 Heptasaccharide Form and Hexasaccharide Form of RM2 Antigen

Previous studies have shown that inducing IgG antibodies by vaccines (DT-$RM_{5.0}$/C34, BSA-$RM_{8.0}$/C34, TT-$RM_{16.0}$/C34, KLH-RM/C34, and DT-$TR_{8.1}$/C34) had a strong binding to oligosaccharides containing the same epitope trisaccharide **1-51** (NeuAcα2 → 6GlcNAcβ1 → 3Galβ1 → R). Besides, the heptasaccharide form is highly expressed on the prostate cancer cell surface as a glycolipid, while the hexasaccharide form is expressed on the surface possibly as a glycoprotein. Furthermore, RM2 is identified as a novel hybrid structure between the “ganglio-series” and “disialyl lacto-series type 1 chain” groups. Above all, we suspect that this trisaccharide (inner core of the RM2 antigen) plays an important role in immune response. For these reasons, we were interested to chemically synthesize the proposed heptasaccharide **3-5** and tetrasaccharide **3-6** to further evaluate the importance of the inner core of RM2 antigen (Fig. 4.1). Our group will develop a vaccine using DT as carrier protein and a glycolipid C34 as adjuvant to induce a class switch with IgG antibody response to study the different impacts on these oligosaccharides.

4.2 Chemical Synthesis of Proposed Heptasaccharide

4.2.1 Chemical Synthesis of Heptasaccharide 3-5 and Tetrasaccharide 3-6

After careful analysis of the structure of heptasaccharide **3-5**, we decided to approach the synthesis from a suitably protected heptasaccharide **4-1** (Fig. 4.2). To use the step-by-step protocol for oligosaccharide synthesis, orthogonal protecting

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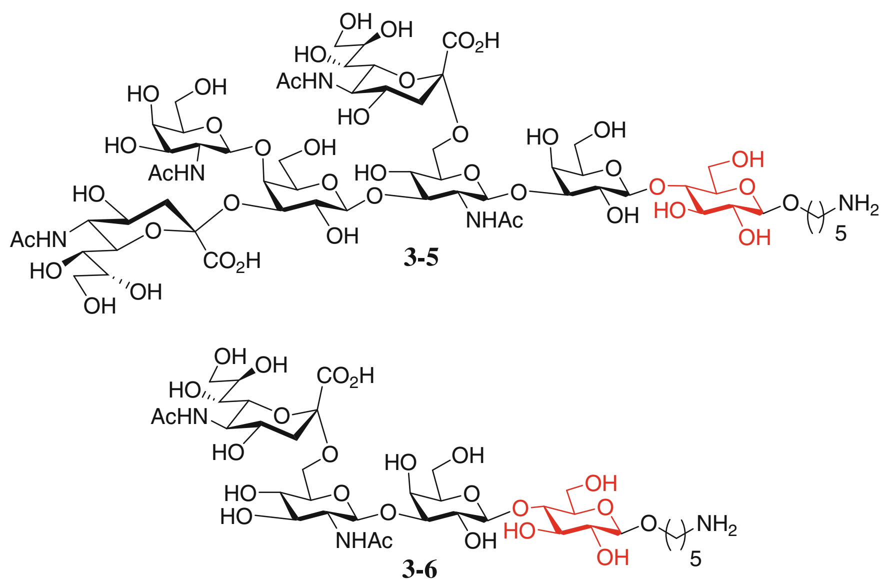

Fig. 4.1 The structure of an RM2 carbohydrate heptasaccharide **3-5** and truncated derivative tetrasaccharide **3-6**

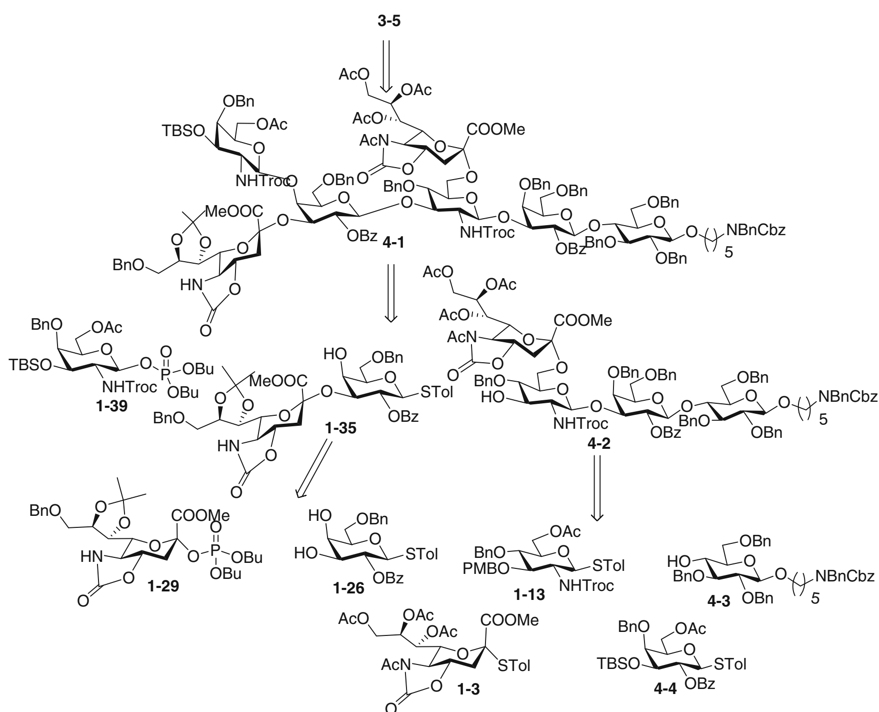

Fig. 4.2 Retrosynthetic analysis of RM2 Heptasaccharide **3-5**

groups were required to provide potential acceptor sites for later glycosylation. Overall, the target heptasaccharide can be divided into three parts, monosaccharide **1-39**, disaccharide **1-35**, and tetrasaccharide **4-2**, which are further divided into seven monosaccharide building blocks **1-3** [1], **1-13**, **1-26**, **1-29**, **1-39**, **4-3,** and **4-4**.

4.2.2 Synthesis of Galactose Building Block 4-4

The known compound **4-5** [2] was converted into compound **4-6** by regioselective acetal ring-opening of the 4,6-*O*-benzylidene group using BH_3·THF and TMSOTf (97 %). After sequential standard acetylation, the desired product **4-4** was produced in 98 % yield (Fig. 4.3).

4.2.3 Synthesis of Disaccharide Building Block 4-9

The preparation of the target disaccharide building block **4-9** started from the NIS/TfOH-promoted coupling of the Gal donor **4-4** with the 4′-OH **4-3** to afford **4-7** (94 %). Compound **4-7** was deacetylated under the Zemplen condition; and then underwent Williamson etherification to generate the disaccharide **4-8** in 92 % yield. For the TBS group cleavage, the 3′-OH **4-9** was efficiently afforded after treatment of **4-8** with BF_3·OEt_2 (Fig. 4.4).

4.2.4 Synthesis of Sialylated Tetrasaccharide Building Block 4-2

To synthesize the sialylated tetrasaccharide building block **4-2**, glycosylation of GlcNAc donor **1-13** and acceptor **4-9** in dichloromethane using NIS/TfOH as a promoter afforded trisaccharide **4-10** in 87 % yield. Trisaccharide **4-10** was

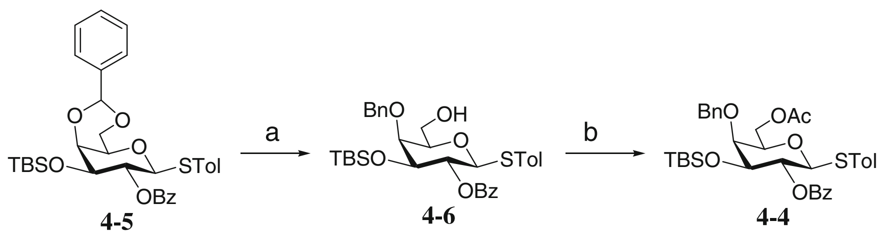

Fig. 4.3 Synthesis of Galactose building block **4-4**. *a* TMSOTf, BH_3-THF, CH_2Cl_2, 0 °C, 5 h, 97 %; *b* Ac_2O, pyridine, CH_2Cl_2, O/N, rt, 98 %

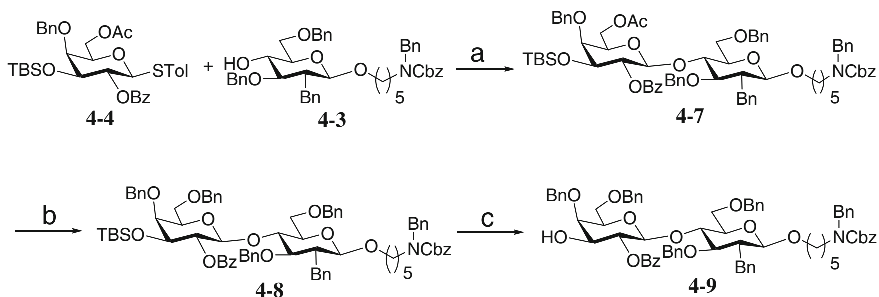

Fig. 4.4 Synthesis of disaccharide building block **4-9**. *a* NIS, TfOH, –20 °C, MS4Å, CH_2Cl_2, 94 %; *b* (i) NaOMe, MeOH, 60–65 °C, 4 h; (ii) BnBr, NaH, DMF, rt, 12 h, two steps 92 %; *c* BF_3-OEt_2, CH_3CN, 0 °C, 30 min, 96 %

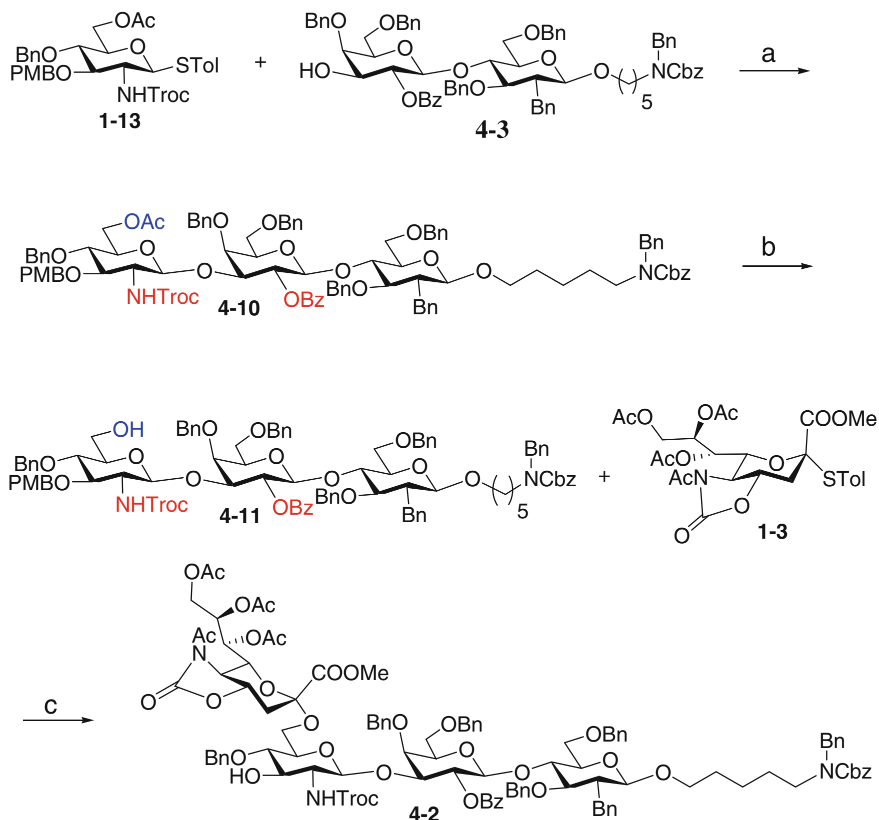

Fig. 4.5 Synthesis of sialylated tetrasaccharide building block **4-2**. *a* NIS, TfOH, –20 °C, MS4 Å, CH_2Cl_2, 87 %; *b* NaOMe, MeOH:CH_2Cl_2 = 5:1, rt, 24 h, 84 %; *c* (i) NIS, TfOH, −40 °C, MS4 Å, CH_2Cl_2, 1.5 h; (ii) DDQ, H_2O, CH_2Cl_2, rt, 2 h, 40 % for two steps

deacetylated using the Zemplen condition in diluted concentration to obtain **4-11** (84 %). Tetrasaccharide can be synthesized by reacting sialyl donor **1-3** with trisaccharide **4-11** in dichloromethane using NIS/TfOH as a promoter at −40 °C. Finally, selective removal of the PMB protecting group by DDQ produced sialylated tetrasaccharide **4-2** in 40 % yield over two steps ($\alpha : \beta = 17 : 1$ determined by ^{1}H NMR spectroscopic analysis of the crude reaction) (Fig. 4.5).

4.2.5 Synthesis of Compound 4-2

Convergent synthesis of heptasaccharide **4-12** was achieved as a single stereoisomer in 20 % yield by glycosylation of tetrasaccharide acceptor **4-2** with trisaccharide donor **1-41** in the NIS/TfOH promoting system at 0 °C for 23 h (Fig. 4.6).

Global deprotection of the protected tetrasaccharide **4-2** was achieved using a four-step procedure: (i) hydrolysis of acyl protecting groups; (ii) acetylation of the amine groups; (iii) removal of the acetonide groups; and (iv) hydrogenolysis of the resulting benzyl ethers to provide the fully deprotected tetrasaccharide **4-13** (Fig. 4.7).

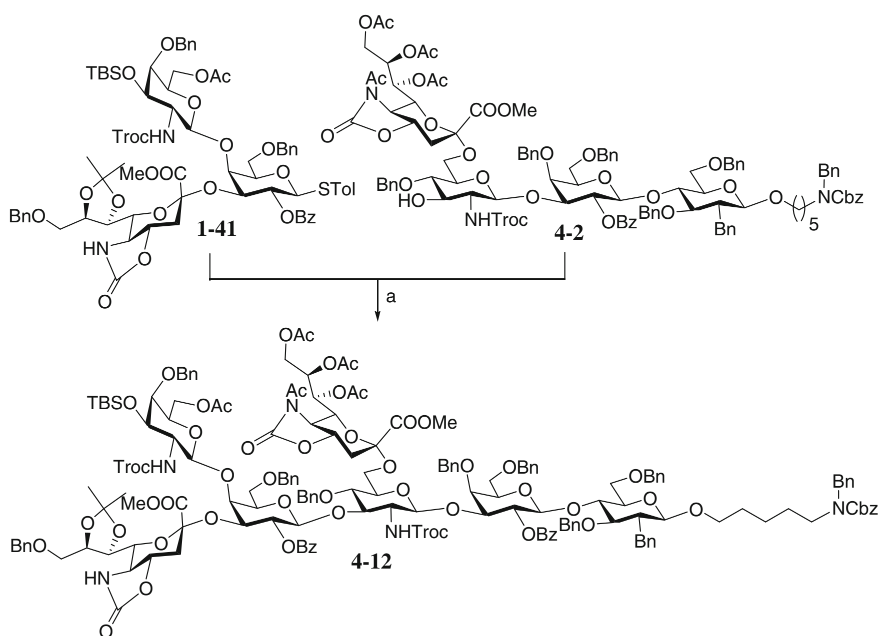

Fig. 4.6 Synthesis of heptasaccharide **4-12**. *a* NIS, TfOH, MS 4 Å, CH_2Cl_2, 0 °C, 23 h, 20 %

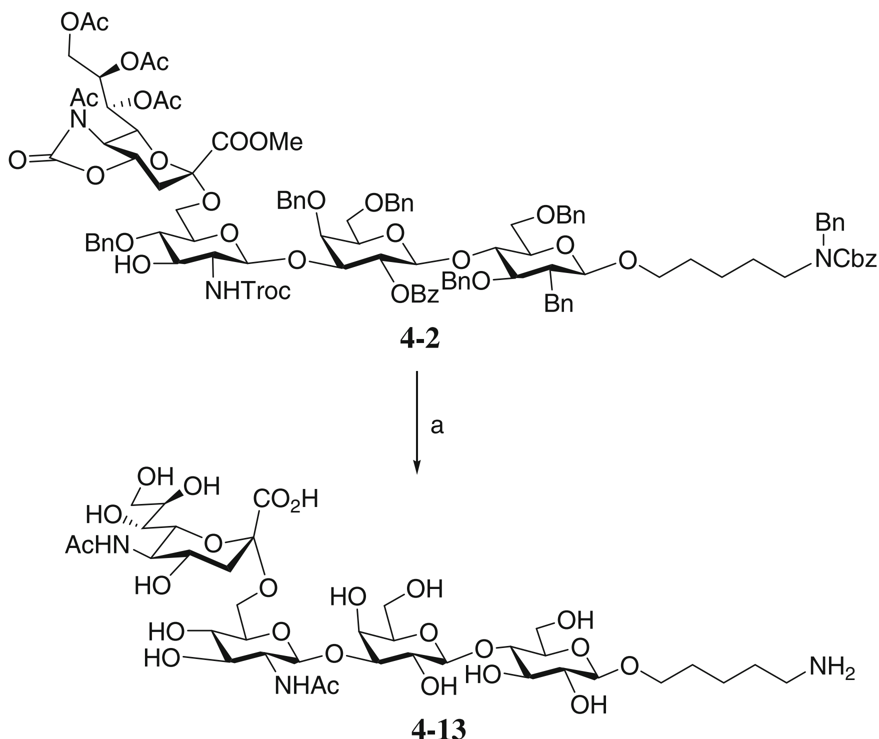

Fig. 4.7 Global deprotection of **4-2**. *a* (i) LiOH H_2O, Dioxane, H_2O, 90–95 °C, 36 h; (ii) Ac_2O, $NaHCO_3$, H_2O then LiOH, H_2O, rt, 12 h; (iii) $Pd(OH)_2$, H_2, CH_3OH, H_2O, rt, 12 h

4.3 Summary

To verify the proposed idea, we chemically synthesized the proposed heptasaccharide and tetrasaccharide in the similar methods mentioned before with good stereoselectivity. These carbohydrates moieties will be conjugated with DT to form vaccines for immunogenicity studies in the mouse model in the presence of C34 as the adjuvants.

4.4 Experimental Section

Materials Commercial solvents and reagents were purchased from Sigma-Aldrich and Acros and used as received without further purification. Monoclonal antibody RM2 was provided by Prof. Seiichi Saito (Department of Urology, Graduate School of Medicine, University of Ryukyus, Nishihara 903-0215, Japan), and Cy3-conjugated anti-mouse IgG (IgG, IgG1, IgG2a, IgG2b, IgG2c, and IgG3) and

IgM antibodies were from Jackson IMMUNO Research. Diphtheria toxoid (CRM 197) was purchased from *PF*enex Incorporation. Aluminum phosphate ($AlPO_4$) was from Brenntag Biosector and Glycolipid derivatives (C1 and C34) were from Dr. Chi-Huey Wong's lab.

General Molecular sieves 4 Å (Reidel-deHaen No.31812) for glycosylations were crushed and activated by heating at 350 °C for 10 h before use. Reactions were monitored with analytical TLC plates (PLC silica gel-60, F_{254}, 2 mm, Merck) and visualized under UV (254 nm) or by staining with acidic ceric ammonium molybdate or *p*-anisaldehyde. Flash column chromatography was performed on silica gel (40–63 μm, Merck), LiChroprep RP8 (40–63 μm), and LiChroprep RP18 (40–63 μm).

Instrumentation Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker Advance 600 (600 MHz/150 MHz) NMR spectrometers. Chemical shifts of protons were reported in ppm (δ scale) and referenced to tetramethylsilane (δ = 0). Chemical shifts of carbon were also reported in parts per million (ppm, δ scale) and were calibrated with tetramethylsilane (δ = 0). DEPT 135 (distortionless enhancement by polarization transfer) was employed for determination of multiplicity. Data were represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant (*J*) in Hz, and integration. High resolution mass spectra were obtained using BioTOF III, and MALDI-TOF MS were obtained using Ultraflex II TOF/TOF.

(2S,3R,4S,5S,6R)-5-(benzyloxy)-4-(tert-butyldimethylsilyloxy)-6-(hydroxymethyl)-2-(p-tolylthio)tetrahydro-2H-pyran-3-yl benzoate (4-6)

Compound **4-5** (11.40 g, 19.23 mmol) was cooled to 0 ºC, dissolved in anhydrous dichloromethane (120 mL), and treated with a 1 M solution of borane-tetrahydrofuran complex in THF (70 mL, 70 mmol) and TMSOTf (1.76 mL, 9.50 mmol). After stirring was continued for 5 h at 0 °C, the reaction was quenched by sequential additions of Et_3N (2 mL) and methanol (20 mL). The reaction mixture was warmed to room temperature and evaporated to dryness. The resulting mixture was concentrated under reduced pressure followed by coevaporation with methanol. The residue was purified by flash column chromatography (1 : 5 to 1 : 3 ethyl acetate/hexane) on silica gel to give **4-6** as white solid (11.08 g, 97 %); **4-6**: $R_f = 0.31$ (Hexane : EtOAc = 3 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05 (dd, *J* = 8.3, 1.2 Hz, 2H; Ar–H), 7.60–7.55 (m, 1H; Ar–H), 7.45 (dd, *J* = 11.0, 4.6 Hz, 2H; Ar–H), 7.38–7.28 (m, 7H; Ar–H), 7.02 (d, *J* = 7.9 Hz, 2H; Ar–H), 5.63 (s, 1H), 5.08 (d, *J* = 11.6 Hz, 1H; PhCH), 4.72 (d, *J* = 8.7 Hz, 1H; H-1 anomeric), 4.57 (d, *J* = 11.6 Hz, 1H; PhCH), 3.97 (d, *J* = 7.9 Hz, 1H), 3.91–3.85 (m, 1H), 3.77 (d, *J* = 2.6 Hz, 1H), 3.63–3.56 (m, 2H), 2.29 (s, 3H; CH_3 STol), 1.72 (s, 1H; OH), 0.78 (s, 9H; t-Bu TBS), 0.10 (s, 3H; CH_3 TBS), −0.10 (s, 3H; CH_3 TBS); ^{13}C NMR (150 MHz, $CDCl_3$) δ 165.1, 138.4, 137.7, 132.9, 132.5, 130.3, 129.8, 129.6, 129.5, 128.3, 128.3, 128.0, 127.7, 87.3, 78.9, 75.8, 74.7, 62.2, 25.5, 21.1, 17.7, −4.0, −5.0; HRMS (ESI, MNa+) calculated for $C_{33}H_{42}O_6NaSSi$ 617.2369, found 617.2367.

(2S,3R,4S,5S,6R)-6-(acetoxymethyl)-5-(benzyloxy)-4-(tert-butyldimethylsilyloxy)-2-(p-tolylthio)tetrahydro-2H-pyran-3-yl benzoate (4-4)

A solution of C-6 alcohol **4-6** (11.08 g, 18.65 mmol) in anhydrous CH_2Cl_2 (100 mL) was treated with acetic anhydride (10 mL, 106 mmol) and pyridine (10 mL, 123.6 mmol). The reaction mixture was stirred at room temperature for 12 h under argon. The solution was concentrated to syrup which was extracted with dichloromethane. The extract was successively washed with 1 N HCl, water, $NaHCO_{3(aq)}$, and water, dried with Na_2SO_4, and concentrated. Purification by flash chromatography on silica gel (1 : 6 to 1 : 4 ethyl acetate/hexane) gave **4-4** as white solid (11.64 g, 98 % yield). **4-4**: R_f = 0.58 (Hexane : EtOAc = 3 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05 (dd, *J* = 8.3, 1.2 Hz, 2H; Ar–H), 7.60–7.54 (m, 1H; Ar–H), 7.45 (t, *J* = 7.8 Hz, 2H; Ar–H), 7.38–7.32 (m, 5H; Ar–H), 7.01 (d, *J* = 8.0 Hz, 2H; Ar–H), 5.62 (s, 1H), 5.10 (d, *J* = 11.4 Hz, 1H; PhCH), 4.70 (d, *J* = 7.5 Hz, 1H; H-1 anomeric), 4.57 (d, *J* = 11.4 Hz, 1H; PhCH), 4.31 (dd, *J* = 11.3, 7.1 Hz, 1H), 4.17 (dd, *J* = 11.3, 5.2 Hz, 1H), 3.97 (d, *J* = 7.5 Hz, 1H), 3.79–3.74 (m, 2H), 2.29 (s, 3H; CH_3 STol), 2.03 (s, 3H; CH_3 Ac), 0.78 (s, 9H; t-Bu TBS), 0.10 (s, 3H; CH_3 TBS), −0.10 (s, 3H; CH_3 TBS); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.6, 165.1, 138.3, 137.6, 133.0, 132.6, 130.2, 129.8, 129.4, 128.3, 128.2, 127.8, 127.5, 76.1, 75.7, 74.8, 63.5, 25.5, 21.1, 20.8, 17.7, −4.0, −5.1; HRMS (ESI, MNa+) calculated for $C_{35}H_{44}O_7NaSSi$, 659.2469, found 659.2481.

(2S,3R,4S,5S,6R)-6-(acetoxymethyl)-2-((2R,3S,4R,5R,6R)-5-benzyl-6-(5-(benzyl (benzyloxycarbonyl)amino)pentyloxy)-4-(benzyloxy)-2-(benzyloxymethyl) tetrahydro-2H-pyran-3-yloxy)-5-(benzyloxy)-4-(tert-butyldimethylsilyloxy) tetrahydro-2H-pyran-3-yl benzoate (4-7)

Galactose donor **4-4** (8.13 g, 12.77 mmol), acceptor **4-3** (6.50 g, 8.73 mmol), and molecule sieve (MS) (AW-300, 20 g) were suspended in dry CH_2Cl_2 (250 mL) under argon at room temperature stirring for 1 h. The reaction mixture was then cooled to −30 °C, followed by addition of NIS (3.42 g, 15.19 mmol) and TfOH (0.076 mL, 0.85 mmol). The reaction mixture was stirred at −20 °C for 2 h and quenched with Et_3N. MS was filtered off and the filtrate was washed with sat. $Na_2S_2O_3$, sat. $NaHCO_3$, H_2O, and brine, dried over $MgSO_4$, and concentrated. Purification by flash silica gel column chromatography (1 : 6 to 1 : 3.5 ethyl acetate/hexane) gave white solid **4-7** (10.31 g, 94 %). **4-7**: R_f = 0.25 (Hexane : EtOAc = 3 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.98 (dd, *J* = 8.2, 1.1 Hz, 2H; Ar–H), 7.58–7.53 (m, 1H; Ar–H), 7.42 (t, *J* = 7.8 Hz, 2H; Ar–H), 7.38–7.09 (m, 30H; Ar–H), 5.56 (t, *J* = 8.8 Hz, 1H), 5.12 (m, 3H; PhCH), 5.00 (d, *J* = 10.8 Hz, 1H; PhCH), 4.82 (t, *J* = 11.2 Hz, 1H; PhCH), 4.75 (d, *J* = 10.8 Hz, 1H; PhCH), 4.71 (d, *J* = 7.9 Hz, 1H; Glucose H-1 anomeric), 4.66 (d, *J* = 11.2 Hz, 1H; PhCH), 4.59 (d, *J* = 12.2 Hz, 1H; PhCH), 4.52 (d, *J* = 11.3 Hz, 1H; PhCH), 4.44 (m, 2H; PhCH), 4.30 (d, *J* = 12.2 Hz, 1H; PhCH), 4.21 (m, 1H), 4.08 (dd, *J* = 11.1, 6.7 Hz, 1H), 4.02 (dd, *J* = 11.1, 6.0 Hz, 1H), 3.94–3.87 (m, 1H), 3.77 (dd, *J* = 9.8, 2.5 Hz, 2H), 3.63 (d, *J* = 2.2 Hz, 1H), 3.59–3.50 (m, 3H), 3.48 (t, *J* = 6.7 Hz, 1H), 3.35 (m, 2H), 3.16 (m, 3H), 1.94 (s, 3H; CH_3 Ac), 1.62 –1.40 (m, 4H; aliphatic), 1.36–1.17 (m, 2H; aliphatic), 0.77 (s, 9H; t-Bu TBS), 0.11 (s, 3H; CH_3 TBS), −0.10 (s, 3H; CH_3 TBS); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.3, 164.8, 156.6, 156.1, 139.1, 138.6, 138.4, 137.9, 136.8, 136.7, 133.0, 130.0,

129.7, 128.4, 128.4, 128.3, 128.2, 128.1, 128.0, 127.8, 127.7, 127.7, 127.6, 127.5, 127.4, 127.2, 127.2, 127.1, 127.0, 103.4, 100.3, 82.8, 81.7, 76.2, 75.1, 75.1, 74.6, 74.3, 74.3, 73.4, 73.3, 72.1, 69.6, 69.5, 68.3, 67.1, 62.8, 60.3, 50.5, 50.1, 47.1, 46.1, 29.6, 29.3, 29.3, 27.8, 27.4, 25.5, 23.3, 23.2, 20.7, 17.7, 14.1, −3.9, −5.0; HRMS (ESI, MNa+) calculated for $C_{75}H_{89}NO_{15}NaSi$, 1294.5899, found 1294.5945.

(2S,3R,4S,5S,6R)-2-((2R,3S,4R,5R,6R)-5-benzyl-6-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-4-(benzyloxy)-2-(benzyloxymethyl)tetrahydro-2H-pyran-3-yloxy)-5-(benzyloxy)-6-(benzyloxymethyl)-4-(tert-butyldimethylsilyloxy)tetrahydro-2H-pyran-3-yl benzoate (4-8)

NaOMe (0.3 g, 5.55 mmol) was added to a solution of compound **4-7** (11.0 g, 8.75 mmol) in MeOH (250 mL), and the mixture was stirred at 60–65 °C under N_2 atmosphere. After stirring for 4 h, the reaction mixture was neutralized with Amberlite IR-120 resin. The resin was removed by filtration, and the filtrate was concentrated and dried under reduced pressure. The residue was dried under high vacuum for 9 h. Then, NaH (60 % in mineral oil; 1.50 g, 37.5 mmol) was added to a solution of residue in DMF (300 mL) at 0 °C, and the resulting mixture was stirred for 10 min. Benzylbromide (5.0 mL, 41.80 mmol) was added, and the resulting mixture was warmed to 25 °C and stirred for 12 h. MeOH (8.0 mL) was then added to remove any remaining benzyl bromide. After further 30 min of stirring, the mixture was poured into iced water. The aqueous phase was extracted with EtOAc, and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated. Purification by flash silica gel column chromatography (1 : 5 to 1 : 3.5 ethyl acetate/hexane) gave **4-8** as colorless oil (10.50 g, 92 %). **4-8**: R_f = 0.67 (Hexane : EtOAc = 2 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.99–7.95 (m, 2H; Ar–H), 7.56–7.52 (m, 1H; Ar–H), 7.41 (t, *J* = 7.8 Hz, 2H; Ar–H), 7.37–7.07 (m, 35H; Ar–H), 5.56 (t, *J* = 8.8 Hz, 1H), 5.12 (m, 3H; PhCH), 5.01 (d, *J* = 10.7 Hz, 1H; PhCH), 4.82 (t, *J* = 11.1 Hz, 1H), 4.75–4.65 (m, 3H; PhCH and Glucose H-1 anomeric), 4.59 (d, *J* = 12.2 Hz, 1H; PhCH), 4.48 (d, *J* = 11.1 Hz, 1H; PhCH), 4.43 (m, 2H; PhCH), 4.31 (m, 2H; PhCH), 4.26–4.16 (m, 2H), 3.89 (t, *J* = 9.3 Hz, 1H), 3.83–3.71 (m, 3H), 3.60–3.47 (m, 5H), 3.41–3.28 (m, 3H), 3.24–3.08 (m, 3H), 1.63–1.39 (m, 4H; aliphatic), 1.35–1.17 (m, 2H; aliphatic), 0.76 (s, 9H; t-Bu TBS), 0.10 (s, 3H; CH_3 TBS), −0.11 (s, 3H; CH_3 TBS); ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.8, 156.6, 156.1, 139.1, 138.9, 138.6, 138.4, 138.0, 137.8, 136.8, 136.7, 132.9, 130.1, 129.7, 128.4, 128.3, 128.3, 128.1, 128.1, 127.9, 127.8, 127.7, 127.7, 127.6, 127.6, 127.3, 127.3, 127.2, 127.1, 127.1, 126.9, 103.4, 100.5, 82.8, 81.7, 76.3, 75.4, 75.3, 74.7, 74.4, 74.3, 73.7, 73.4, 73.2, 73.2, 69.6, 69.5, 68.2, 68.1, 67.0, 50.5, 50.1, 47.1, 46.1, 29.6, 29.3, 29.2, 27.8, 27.4, 25.5, 23.3, 23.2, 17.7, 14.1, 14.1, −3.9, −5.0; HRMS (ESI, MNa+) calculated for $C_{80}H_{93}NO_{14}NaSi$, 1342.6263, found 1342.6304.

(2S,3R,4S,5R,6R)-2-((2R,3S,5R,6R)-5-benzyl-6-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-4-(benzyloxy)-2-(benzyloxymethyl)tetrahydro-2H-pyran-3-yloxy)-5-(benzyloxy)-6-(benzyloxymethyl)-4-hydroxytetrahydro-2H-pyran-3-yl benzoate (4-9)

To a solution of compound **4-8** (2.48 g, 1.90 mmol) in dry CH_3CN (30 mL) was added $BF_3 \cdot Et_2O$ (0.6 mL, 2.27 mmol) at 0 °C. After stirring for 30 min, the reaction

was quenched with saturated $NaHCO_3$ (12 mL), and extracted with ethyl acetate (3 × 30 mL), and the combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. Purification by flash silica gel column chromatography (1 : 5 to 1 : 2.5 ethyl acetate/hexane) gave **4-9** as colorless oil (2.17 g, 96 %). **4-9**: R_f = 0.42 (Hexane : EtOAc = 2 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.95 (dd, *J* = 8.3, 1.2 Hz, 2H; Ar–H), 7.56–7.51 (m, 1H; Ar–H), 7.40 (q, *J* = 7.7 Hz, 2H; Ar–H), 7.36–7.10 (m, 35H; Ar–H), 5.22 (dd, *J* = 10.0, 8.0 Hz, 1H), 5.14 (d, *J* = 12.5 Hz, 2H; PhCH), 4.99 (d, *J* = 10.9 Hz, 1H; PhCH), 4.83 (t, *J* = 11.4 Hz, 1H), 4.78 (d, *J* = 10.9 Hz, 1H; PhCH), 4.74–4.65 (m, 4H; PhCH and Glucose H-1 anomeric), 4.56 (d, *J* = 12.2 Hz, 1H; PhCH), 4.44 (m, 2H; PhCH), 4.37 (d, *J* = 11.7 Hz, 1H; PhCH), 4.29 (t, *J* = 12.1 Hz, 2H), 4.23 (m, 1H), 3.92–3.85 (m, 2H), 3.85–3.73 (m, 1H), 3.65–3.51 (m, 5H), 3.49 (dd, *J* = 8.7, 5.7 Hz, 1H), 3.44–3.31 (m, 3H), 3.18 (m, 3H), 2.30 (d, *J* = 10.3 Hz, 1H), 1.64–1.41 (m, 4H; aliphatic), 1.37–1.18 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.3, 156.6, 156.1, 139.2, 138.5, 138.2, 138.1, 137.8, 137.7, 136.8, 136.7, 133.2, 129.7, 129.6, 128.5, 128.4, 128.4, 128.3, 128.2, 128.0, 127.8, 127.8, 127.7, 127.7, 127.5, 127.4, 127.2, 127.2, 127.0, 103.4, 100.3, 82.8, 81.7, 76.4, 75.4, 75.2, 74.7, 74.7, 74.3, 73.4, 73.3, 73.1, 73.0, 69.7, 69.6, 68.2, 67.6, 67.1, 50.5, 50.1, 47.1, 46.1, 29.6, 29.3, 27.8, 27.4, 23.3; HRMS (ESI, MH+) calculated for $C_{74}H_{79}NO_{14}Na$, 1206.5579, found 1206.5621.

(2S,3R,4S,5S,6R)-4-((2S,3R,4R,5S,6R)-6-(acetoxymethyl)-5-(benzyloxy)-4-(4-methoxybenzyloxy)-3-((2,2,2-trichloroethoxy)carbonylamino)tetrahydro-2H-pyran-2-yloxy)-2-((2R,3S,4R,5R,6R)-5-benzyl-6-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-4-(benzyloxy)-2-(benzyloxymethyl)tetrahydro-2H-pyran-3-yloxy)-5-(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-3-yl benzoate (4-10)

GlcNAc donor **1-13** (1.96 g, 2.75 mmol), acceptor **4-9** (2.17 g, 1.82 mmol), and molecule sieve (MS) (AW-300, 5.0 g) were suspended in dry CH_2Cl_2 (48 mL) under argon at room temperature stirring for 8 h. The reaction mixture was then cooled to −30 °C, followed by addition of NIS (0.82 g, 3.64 mmol) and TfOH (0.034 mL, 0.38 mmol). The reaction mixture was stirred at −20 °C for 2 h and quenched with Et_3N. MS was filtered off and the filtrate was washed with sat. $Na_2S_2O_3$, sat. $NaHCO_3$, H_2O, and brine, dried over $MgSO_4$, and concentrated. Purification by flash silica gel column chromatography (1 : 5 to 1 : 2.5 ethyl acetate/hexane) gave white solid **4-10** (2.82 g, 87 %). **4-10**: R_f = 0.63 (Hexane : EtOAc = 3 : 2); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.95 (d, *J* = 7.8 Hz, 2H; Ar–H), 7.58 (t, *J* = 7.4 Hz, 1H; Ar–H), 7.46 (t, *J* = 7.8 Hz, 2H; Ar–H), 7.39–7.07 (m, 42H; Ar–H), 6.78 (d, *J* = 8.7 Hz, 2H; Ar–H), 5.52 (dd, *J* = 9.8, 8.3 Hz, 1H), 5.13 (d, *J* = 9.6 Hz, 2H), 5.01–4.94 (m, 2H), 4.90 (d, *J* = 7.2 Hz, 1H), 4.81 (m, 3H), 4.74–4.59 (m, 5H), 4.59–4.49 (m, 3H), 4.43 (dd, *J* = 10.4, 8.6 Hz, 3H), 4.36 (d, *J* = 12.2 Hz, 1H), 4.32 (d, *J* = 11.8 Hz, 1H), 4.27 (d, *J* = 12.2 Hz, 1H), 4.24–4.15 (m, 3H), 4.05 (d, *J* = 2.9 Hz, 1H), 3.94 (t, *J* = 9.1 Hz, 1H), 3.87 (t, *J* = 9.1 Hz, 1H), 3.83–3.67 (m, 5H), 3.62 (d, *J* = 10.6 Hz, 2H), 3.55–3.27 (m, 8H), 3.15 (m, 3H), 1.90 (s, 3H; CH_3 Ac), 1.61–1.40 (m, 4H; aliphatic), 1.26 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.4, 164.6, 159.2, 156.6, 156.0, 153.4, 139.0, 138.6, 138.3, 138.0, 137.8, 137.4, 136.7, 136.7, 133.2, 129.7, 129.4,

128.6, 128.5, 128.5, 128.4, 128.3, 128.2, 128.1, 128.1, 128.0, 127.9, 127.9, 127.9, 127.8, 127.7, 127.5, 127.4, 127.4, 127.3, 127.2, 127.1, 127.1, 127.0, 126.9, 113.8, 103.3, 101.1, 100.3, 95.5, 82.6, 81.6, 80.7, 79.5, 78.3, 76.2, 76.2, 75.3, 74.8, 74.7, 74.6, 74.3, 73.5, 73.4, 73.3, 72.7, 72.5, 69.6, 69.5, 68.0, 67.8, 67.0, 62.9, 60.3, 58.5, 55.1, 50.4, 50.1, 47.0, 46.1, 29.6, 29.3, 27.8, 27.4, 23.2, 20.6; HRMS (ESI, MNa+) calculated for $C_{100}H_{107}Cl_3N_2O_{22}Na$, 1815.6279, found 1815.6336.

(2S,3R,4S,5S,6R)-2-((2R,3S,4R,5R,6R)-5-benzyl-6-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-4-(benzyloxy)-2-(benzyloxymethyl)tetrahydro-2H-pyran-3-yloxy)-5-(benzyloxy)-4-((2S,3R,4R,5S,6R)-5-(benzyloxy)-6-(hydroxymethyl)-4-(4-methoxybenzyloxy)-3-((2,2,2-trichloroethoxy)carbonylamino) tetrahydro-2H-pyran-2-yloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-3-yl benzoate (4-11)

NaOMe (0.032 g, 0.59 mmol) was added to a solution of compound **4-10** (8.25 g, 4.63 mmol) in a mixed MeOH (390 mL) and CH_2Cl_2 (60 mL), and the mixture was stirred at room temperature under N_2 atmosphere. After stirring for 24 h, the reaction mixture was neutralized with Amberlite IR-120 resin. The resin was removed by filtration, and the filtrate was concentrated and dried under reduced pressure. Purification by flash silica gel column chromatography (1 : 5 to 1 : 2.5 ethyl acetate/hexane) gave white solid **4-11** (6.76 g, 84 %). **4-11**: $R_f = 0.44$ (Hexane : EtOAc = 3 : 2); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.93 (d, *J* = 7.7 Hz, 2H; Ar–H), 7.58 (t, *J* = 7.4 Hz, 1H; Ar–H), 7.44 (t, *J* = 7.7 Hz, 2H; Ar–H), 7.39–7.09 (m, 42H; Ar–H), 6.77 (d, *J* = 8.7 Hz, 2H; Ar–H), 5.54 (t, *J* = 9.0 Hz, 1H), 5.13 (d, *J* = 10.0 Hz, 2H), 4.98 (m, 2H), 4.81 (t, *J* = 9.6 Hz, 2H), 4.72 (d, *J* = 10.6 Hz, 1H), 4.71–4.56 (m, 7H), 4.50 (d, *J* = 10.7 Hz, 1H), 4.47–4.31 (m, 4H), 4.29–4.14 (m, 3H), 4.02 (d, *J* = 11.9 Hz, 1H), 3.96 (d, *J* = 2.4 Hz, 1H), 3.87 (t, *J* = 9.3 Hz, 2H), 3.82–3.68 (m, 7H), 3.52 (m, 5H), 3.45–3.28 (m, 4H), 3.28–3.07 (m, 4H), 1.87–1.77 (m, 1H; OH), 1.61–1.40 (m, 4H; aliphatic), 1.33–1.18 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.6, 159.2, 156.6, 156.0, 153.5, 139.1, 138.8, 138.6, 138.3, 137.9, 137.8, 137.7, 136.7, 136.7, 133.3, 129.7, 129.6, 129.5, 128.6, 128.5, 128.4, 128.3, 128.3, 128.1, 128.1, 128.0, 128.0, 127.9, 127.9, 127.9, 127.8, 127.7, 127.7, 127.4, 127.2, 127.1, 127.1, 127.0, 113.8, 103.3, 101.3, 100.4, 95.6, 82.7, 81.6, 79.8, 79.5, 78.0, 76.3, 75.7, 75.3, 75.2, 74.8, 74.7, 74.5, 74.3, 74.3, 73.7, 73.3, 73.3, 72.7, 69.6, 69.5, 67.8, 67.1, 61.7, 58.1, 55.1, 50.4, 50.1, 47.0, 46.1, 29.6, 29.3, 27.8, 27.4, 23.2; HRMS (ESI, MNa+) calculated for $C_{98}H_{105}Cl_3N_2O_{21}Na$, 1773.6173, found 1773.6237.

(1S,2R)-1-((4R,6S,7aS)-3-acetyl-6-(((2R,3S,4R,5R,6S)-6-((2S,3R,4S,5S,6R)-3-(benzoyloxy)-2-((2R,3S,4R,5R,6R)-5-benzyl-6-(5-(benzyl(benzyloxycarbonyl)amino)pentyloxy)-4-(benzyloxy)-2-(benzyloxymethyl)tetrahydro-2H-pyran-3-yloxy)-5-(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-4-yloxy)-3-(benzyloxy)-4-hydroxy-5-((2,2,2-trichloroethoxy)carbonylamino) tetrahydro-2H-pyran-2-yl)methoxy)-6-(methoxycarbonyl)-2-oxohexahydro-2H-pyrano[3,4-d]oxazol-4-yl)propane-1,2,3-triyl triacetate (4-2)

A solution of donor **1-3** (3.65 g, 6.28 mmol, 1.56 equiv), acceptor **4-11** (7 g, 4.02 mmol, 1.0 equiv), and activated 4 Å powdered molecular sieves (15 g) in anhydrous dichloromethane (128 mL) was stirred overnight under an argon

atmosphere, and then cooled to –40 °C followed by addition of NIS (1.92 g, 8.53 mmol, 2.12 equiv) and TfOH (66.0 μL, 0.74 mmol, 0.19 equiv). The reaction mixture was stirred at −40 °C for 2 h until the disappearance of the donor on TLC, then quenched with triethylamine and warmed to room temperature. The mixture was diluted with dichloromethane, filtered through Celite, washed with 20 % aqueous $Na_2S_2O_3$ solution, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash silica gel column chromatography (1 : 3 to 1 : 1.25 ethyl acetate/hexane) gave tetrasaccharide (8 g, 91 %) (α:β = 17:1). DDQ (1.44 g, 6.35 mmol) was added to a solution of trisaccharide in CH_2Cl_2/H_2O (10 : 1, 88 mL) at room temperature and stirred overnight. The reaction mixture was diluted with CH_2Cl_2 (150 mL) and washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried with Na_2SO_4, and the solvents were removed under reduced pressure. The residue was purified by flash silica gel column chromatography (1 : 3 to 1 : 1.25 ethylacetate/hexane) to give **4-2** as white solid (3.33 g, 44 %). **4-2**: R_f = 0.42 (Hexane : EtOAc = 1 : 1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.93 (d, J = 7.8 Hz, 2H; Ar–H), 7.58 (t, J = 7.4 Hz, 1H; Ar–H), 7.43 (t, J = 7.8 Hz, 2H; Ar–H), 7.41–7.10 (m, 40H; Ar–H), 5.59–5.51 (m, 2H), 5.50–5.44 (m, 1H), 5.13 (d, J = 10.2 Hz, 2H), 5.04 (d, J = 11.7 Hz, 1H), 4.97 (d, J = 10.7 Hz, 1H), 4.91 (s, 1H), 4.85–4.77 (m, 2H), 4.77–4.64 (m, 5H), 4.62 (d, J = 12.3 Hz, 1H), 4.57 (d, J = 11.8 Hz, 1H), 4.48–4.40 (m, 3H), 4.34 (m, 5H), 4.25–4.15 (m, 3H), 4.00–3.86 (m, 4H), 3.82–3.72 (m, 2H), 3.65 (dd, J = 11.1, 9.6 Hz, 1H), 3.62–3.43 (m, 12H), 3.40–3.29 (m, 3H), 3.27–3.08 (m, 5H), 2.83 (dd, J = 12.0, 3.4 Hz, 1H; H^{sial}-3 $_{eq}$), 2.47 (s, 3H; CH_3 Ac), 2.15–2.08 (m, 4H; CH_3 Ac, H^{sial}-3_{ax}), 2.00 (s, 3H; CH_3 Ac), 1.76–1.66 (m, 3H; CH_3 Ac), 1.49 (m, 4H; aliphatic), 1.35–1.16 (m, 2H; aliphatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.9, 170.6, 170.0, 169.9, 168.5, 165.1, 156.6, 156.1, 154.9, 153.5, 139.1, 139.0, 138.6, 138.4, 138.1, 137.9, 137.9, 136.8, 136.7, 133.5, 129.7, 129.4, 128.7, 128.5, 128.5, 128.4, 128.3, 128.3, 128.2, 128.1, 127.9, 127.9, 127.8, 127.8, 127.7, 127.7, 127.7, 127.4, 127.4, 127.2, 127.2, 127.1, 127.0, 103.4, 101.2, 100.2, 99.2, 95.4, 82.7, 81.6, 79.8, 77.4, 76.4, 76.4, 75.3, 75.1, 74.8, 74.7, 74.6, 74.3, 74.2, 74.2, 73.9, 73.5, 73.3, 73.1, 71.4, 69.7, 69.6, 68.3, 68.2, 67.9, 67.1, 64.5, 63.0, 59.0, 58.4, 52.8, 50.5, 50.1, 47.1, 46.1, 29.6, 29.3, 27.8, 27.4, 24.6, 23.3, 21.1, 20.7, 20.5; HRMS (ESI, MNa+) calculated for $C_{109}H_{120}Cl_3N_3O_{32}Na$, 2110.6818, found 2110.6903.

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