Synthesis of the Disaccharides of Phenolic Glycolipid I of *Mycobacterium leprae* with Some Kinds of Aglycone and Their Serological Activities

Tsuyoshi FUJIWARA* and Shinzo IZUMI**

* Laboratory of Chemistry, Institute for Natural Science, Nara University, Nara
** Leprosy Research Laboratory, Department of Dermatology, Faculty of Medicine, Kyoto University, Kyoto

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ABSTRACT

The bromide of the peracetate of the non-reducing-end disaccharide of phenolic glycolipid I of *Mycobacterium leprae* was coupled with lauryl alcohol or 3-phenylpropanol in acetonitrile in the presence of mercury cyanide. The product was the 1 : 1 mixture of α- and β-linked disaccharides. The coupling of the bromide with stearyl alcohol in nitromethane-toluene gave mainly α-linked stearyl disaccharide. The complete loss of the stereoselectivity in acetonitrile was thought to be due to the lack of neibouring group participation.

The activities of these disaccharides and some other synthetic disaccharides were tested in ELISA inhibition assay and direct ELISA. No compound tested in this study showed lower activity than free disaccharide in ELISA inhibition assay. Among the tested disaccharides, the compounds with long lipophilic chain as lauryl and stearyl disaccharides showed high activity in both ELISA inhibition assay and direct ELISA. On the other hand the compounds with short chain showed only the same level of the activity as that of free disaccharide in ELISA inhibition assay. And these compounds showed no activity in direct ELISA. These results suggest that long lipophilic compounds like lauryl or stearyl alcohol should be chosen for the development of the synthetic antigen convenient for direct ELISA.

I. INTRODUCTION

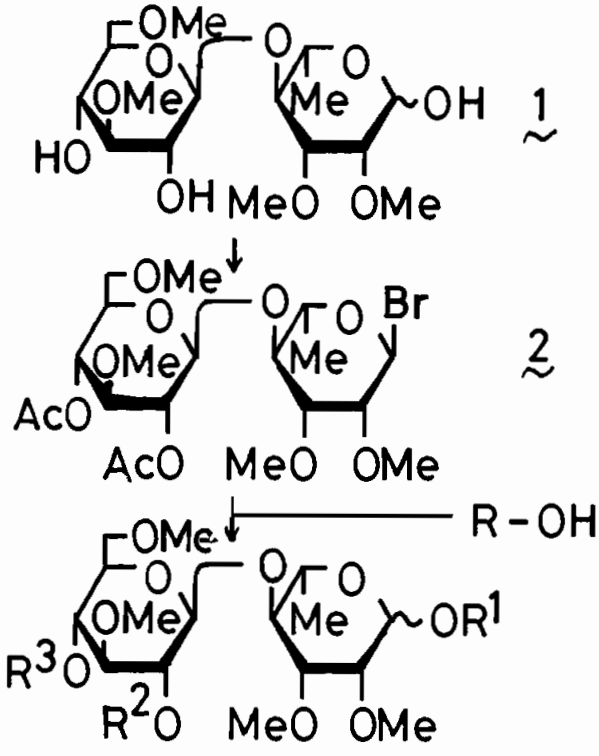
The trisaccharide *O*-(3, 6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-*O*-(2, 3-di-*O*-methyl-α-L-rhamnopyranosyl)-(1→2)-3-*O*-methyl-α-L-rhamnopyranose is the species-specific segment of phenolic glycolipid I (PGL I) which is the antigen very specific to *Mycobacterium laprae*.[1-3] The synthetic study of the trisaccharide and related saccharides have revealed that nonreducing end disaccharide has the same serological activity as the trisaccharide.[3-7] This result shows the possibility of developing new method of serodiagnosis of leprosy infection by using this synthetic disaccharide. We used ELISA inhibition assay to measure the activities of the synthesized saccharides in the previous study.[3] But, this method is rather complicated and is not suitable for the use in clinical or preventive works of leprosy. In order to overcome this, the synthesis of the disaccharides carring the linker arm to access to protein carrier has been reported.[8-13]

And also the serological activity of the protein-disaccharide conjugate which was prepared by reductive amination has been reported.[3,14] During the course of this study, we have found that some disaccharides with aglycone attached to microplate. This suggests that direct ELISA is possible if proper aglycone is employed. In this paper, we describe the synthesis of such disacchaies and their serological activities in ELISA inhibition assay and direct ELISA.

II. RESULTS AND DISCUSSION

A. Synthesis

Fig. 1 shows the synthetic pathway to the disaccharides with several kinds of aglycone. The disaccharide *O*-(3, 6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-2, 3-di-*O*-methyl-α-L-rhamnose (**1**) was acetylated and brominated by titanium tetrabromide.[15] Bromide (**2**) was unstable and its preparation contained substantial amount (*ca.* 30%) of degraded product. But it was used for the next reaction without further purification. Lauryl alcohol was stirred with **2** in acetonitrile in the presence of mercury cyanide. The reaction was almost complete after 3 hr. Laulyl disaccharide fraction was separated by silica gel column chromatography from the reaction products. T.L.C. of the lauryl disaccharide fraction with solvent system of benzene-acetone (4 : 1) showed only one spot and N.M.R. supported that this fraction was the disaccharide with lauryl group as an aglycone. But the lauryl disaccharide fraction showed relatively small specific rotation value ($[\alpha]_D^{20}$ −20.6°) and also showed two spots on T.L.C after deacetylation with sodium methoxide. Therefore, they were sepearted each other by a preparative T.L.C. to give **3** and **4** in a ratio of about 1 : 1. N.M.R. of less polar cpmpound, **3**, showed two anomeric proton



	R^1	1-C	R^2	R^3
3	$CH_3(CH_2)_{11}$	α	H	H
4	$CH_3(CH_2)_{11}$	β	H	H
5	$C_6H_5(CH_2)_3$	α	H	H
6	$C_6H_5(CH_2)_3$	β	H	H
7	$CH_3(CH_2)_{17}$	α	Ac	H
8	$CH_3(CH_2)_{17}$	α	H	H
9	$C_6H_5CH_2$	α	H	H
10	$CH_3CO_2(CH_2)_2C_6H_5$	α	H	H
11	$CH_3CO_2(CH_2)_2C_6H_5$	α	Ac	H
12	$CH_3CO_2(CH_2)_2C_6H_5$	α	H	Ac

Fig. 1. Synthesis of the disaccharides having various kinds of aglycone.

signals at 4.81 ppm (J= 0 Hz) and 4.42 ppm (doulet, J=8.3 Hz) due to the α-linked rhamnose and β-linked glucose residues, respectively. The large negative specific rotation value also supported the structure of **4**.

Compound **2** was also stirred with 3-phenylpropanol under the same condition for lauryl alcohol to give the anomeric mixture of 3-phenylpropyl disaccharide as the case of lauryl alcohol. They were deacetylated and then separetd each other by silica gel column chromatogaphy to give the anomeric mixture of α-linked disaccharide (**5**) and β-linked disaccharide (**6**) in a ratio of about 1 : 1, which were supported by N.M.R. and specific ritation value.

The complet loss of the stereoselectivity and the unstability of the bromide was thought to be due to the presence of C-2-OMe group on the rhamnose residue, because neibouring group assistance is not present in 2-*O*-methylated saccharides. Therefore, in order to improve the selectivity and stability, nitromethane-toluene system was employed.[16] Stearyl alcohol was stirred with **2** in nitromethane-toluene (5 : 3) in the presence of mercury cyanide to give mainly α-linked stearyl disaccharide in contrast to the above two cases. The yield of the disaccharide derivatives was almost same (*ca.* 25%) as those of lauryl disaccharide and 3-phenylpropyl disaccharide. Deacetylation of the stearyl disaccharide fraction under mild condition gave partially deacetylated disaccharide (**7**) and the repeated treatment of **7** with sodium methoxide gave completely deacetylated stearyl disaccharide (**8**). In order to determine the position of *O*-acetyl group, **7** was methylated with nitromethane-boron trifluoride and then hydrolyzed with trifluoroacetic acid and converted into alditol acetates. They were analyzed by G.C. with a column of OV225 and by G.C.-M.S. with a OV-1 capillary column. G.C. of the alditol acetaets gave two peaks with relative retention time of Rt=0.92 (m/z 43, 101, 117, 143, 161, 203) and Rt= 1.92 (m/z 45, 129, 161, 189), showing **8** was the disaccharide with *O*-acetyl group on 2-C-OH of the 3, 6-di-*O*-methylglucose residue.

B. Serology

In order to test the effect of the aglycone to the serological reactivity of the disaccharide, the synthesized disaccharides discussed in this paper and several other synthetic compounds which had natural disaccharide parts were tested by ELISA inhibition assay. Namely, the disaccharide was incubated with standard LL serum, and then the remaining activity was measured by standard ELISA protocol. The results of ELISA inhibition assay is given in Fig. 2. Control sugars like rhamnose, maltose or glucose did not show an inhibition activity. Free natural disaccharide (**1**, ND) showed about 40% inhibition over the sample sugar concentration of 250 nmole/ml, while PGL I showed very high inhibition activity of about 75% inhibition at 25 nmole/ml, which was the highest activity among the tested compounds. One of the reasones for the lower activi-

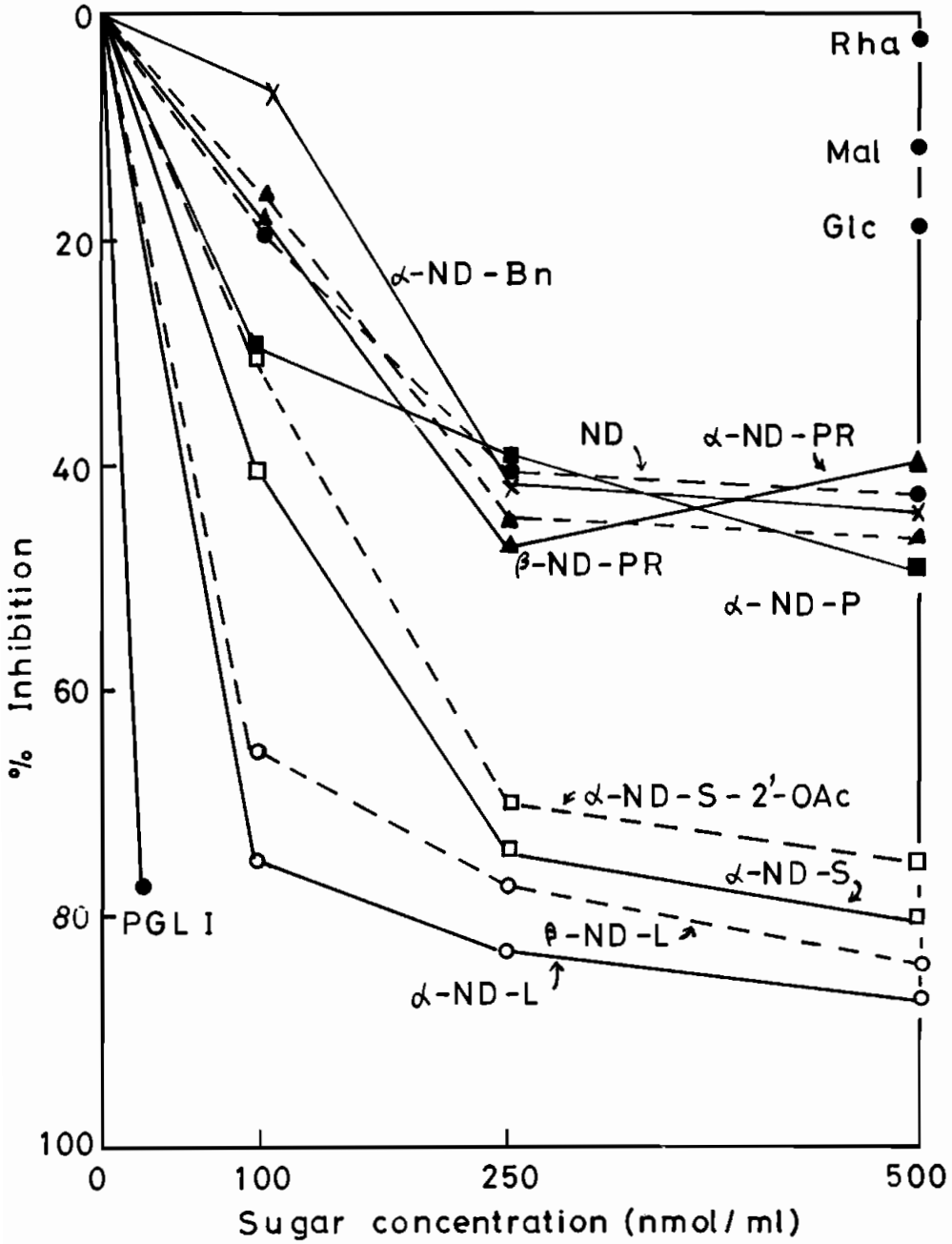


Fig. 2. Results of ELISA inhibition assay. Rha = rhamnose, Glc = glucose, Mal = maltose. For other abbreviations, see text.

ties of the synthesized disaccharides than that of PGL I is thought to be the contributions of the lipid part of PGL I molecule to the binding of PGL I to the antibody in the standard serum, and another is the contributions of the reducing-end monosaccharide.

The tested disaccharides were divided into two groups according to the degree of the inhibition activity. One is the group which showed almost same activity as ND. This group included bnzyl disaccharide (**9** , α－ND－Bn), *p*-(methoxycarbonylethyl)phenyl disaccharide (**10**, α－ND－P) and 3-phenylpropyl disacchaccharides (**5** , α－ND－PR and **6** , β－ND－PR), which all had aglycones with relatively short carbon-chains. The other is the group which showed much higher inhibition activities than that of ND. This group included lauryl disaccharides (**3** , α－ND－L and **4** , β－ND－L), stearyl disaccharide (**8** , α－ND－S) and partially-acetylated stearyl disaccharide (**7** , α－ND－S－2'－OAc), which all had the aglycones with long carbon-chains. The fact that no disaccharide tested in this experiment showed lower activity than ND suggested that we can use any aglycone without the loss of the activity, which is very important for the development of the best compound for the practical use. However, the fact that α－ND－L and β－ND－L had higher activity than that α－ND－S suggested that the length of the chain of the aglycone was not only one factor.

It was found that α－ and β－anomer showed almost same activity in all compounds. This showed that the aglycone attached to the natural disaccharide did not decrease the inhibition activity of the natural disaccharide. These results agree with the fact that natural trisaccharide, its epimer in rhamnose-rhamnose linkage and ND showed the same activity in ELISA inhibitivn assay.[3]

Table 1. The results of direct ELISA *

Compound		Exp. I	Exp. II	Exp. III
1	ND	−71± 12		
3	α－ND－L	567± 67	407±22	319± 34
4	β－ND－L	128±126	85±10	110± 10
8	α－ND－S			111± 7
7	α－ND－S－2'－OAc			128± 5
9	α－ND－Bn	102± 20	104±18	
5	α－ND－PR	−132± 4		
6	β－ND－PR	−5± 6		
10	α－ND－P	202± 16		
11	α－ND－P－2'－OAc	398± 35		
12	α－ND－P－4'－OAc	505± 32		
	PGL I	3096± 16	1331±69	1636±116

* All values is given in 1,000 times magnification.

In order to see the activities of the synthesized disaccharides in direct ELISA, ELISA plate was coated with synthetic disaccharide and the serological activity was measured by standard ELISA protocol. The results given in Table I showed that ND, α − ND − PR and β − ND − PR did not show the activity. α − ND − Bn, α − ND − P, α − ND − S and α − ND − S − 2' − OAc showed the activities but their levels were low, while PGL I was highly active. α − ND − L showed relatively high activity, but β − ND − L had only low activity in contrast to the results of ELISA inhibition assay. This means that the difference of the reactivities of the synthetic disaccharide to antibody in direct ELISA is not due to the anomeric configuration in free solution but is due to how closely the anibody can be present to the saccharide attached to the plate. As the disaccharide is thought to attach to the plate at the aglycone parts and α − ND − L has an angled form, the possibility that the sugar part of the disaccharide is present in apart from the surface of the plate is higher in α − ND − L than in β − ND − L. This is probably one of the reasone why the α − ND − L was more active than β − ND − L in direct ELISA. acetylated disaccharides (**11**, α − ND − P − 2' − OAc and **12**, α − ND − P − 4' − OAc) showed relatively high activities in direct ELISA, suggesting the possibility to develop mere serologically active disaccharide than ND by introducing proper groups to the free hydroxy groups on the sugar residues.

Ⅲ. EXPERIMENTAL

Optical rotations were measured with a Horiba SEPA 200 polarimeter, using 10-cm microtube (2 ml). T.L.C. was conducted on plates of Merck silica gel 60 F-254 (0.25 mm); compounds were detected by charring with 10% sulfuric acid. I.R. spectra were recorded with a Hitachi 210 infrared spectrometer. ^{1}H-N.M.R. spectra were recorded with Hitachi R24B nuclear magnetic reasonance spectrometer. G.C.-M.S. was conducted with Hitachi M70 mass spectrometer with a OV-1 capillary column (25 m).

*O-(2, 4-Di-O-acetyl-3, 6-di-O-methyl-β-*D*-glucopyransyl)-(1→4)-2, 3-di-O-methyl-α-*L*-rhamnopyranosyl bromide* (**2**). - - - Compound **1** (330 mg) was acetylated and brominated with titanium tetrabromide by the method of Paulsen,[15] to give 502 mg of bromide **2**. This preparation contained about 30% of degradation product, maybe 1-OH disaccharide, R_f 0.58 (benzene-acetone, 4 : 1). N.M.R. ($CDCl_3$, 60 MHz) δ : 6.50 (s, H-1).

*Lauryl O-(2, 4-di-O-acetyl-3, 6-di-O-methyl-β-*D*-glucopyranosyl)-(1→4)-2, 3-di-O-methyl-α-and β-*L*-rhamnopyranoside* (**3** *and* **4**). - - - Compound **2** (125 mg) and lauryl alcohol (35 mg) were stirred in 1 ml of acetonitrile at room temperature overnigt in the presence of mercury cyanide (35 mg). The reaction mix-

ture was proccessed as previously reported and then evaporated. The residue was charged on a silica gel column in benzene. The factions eluted by 2% *t*-butanol in benzene were collected, giving 31.5 mg of the acetylated lauryl disaccharide fraction, which was anomeric mixture, $[\alpha]_D^{20}$ −20.6° (c 0.28, chloroform), R_f 0.51 (benzene-acetone, 2 : 1).

The lauryl disaccharide fraction (31.5 mg) was dissolved in 3 ml of dry methanol. Sodium methoxide (1.3 M, 0.3 ml) was added and the mixture was boiled for 1 min, then stood for 30 min at room temperature. The mixture was neutralized with Amberlite IR-120 (H^+) and evaporate. Densitometry of the residual syrup on T.L.C. plate showed two spots (α-and β-anomers) in a ratio of about 1 : 1. They were separated by preparative T.L.C., to give **3** (5.13 mg) and 4 (8.88 mg). For **3** $[\alpha]_D^{20}$ −46.2° (c 0.17, chloroform), R_f 0.52 (benzene-acetone, 2 : 1). I.R. ν_{max} (liquid film) cm^{-1} : 3700−3200 (s, OH), 2950, 2860 (vs, C-H), 1460, 1445, 1380, 1370, 1285 (m), 1120, 1070 (vs, broad, C-O-C). N.M.R. ($CDCl_3$, 60 MHz) δ : 4.81 (s, 1H, H-1), 4.42 (d, 1H, $J_{1',2'}$ 8.3 Hz, H-1'), 4.0−3.0 (14H), 3.68, 3.47, 3.46, 3.40 (4s, 12H, OMeX 4), 1.8−1.1 (23H, methylene of lauryl group and Rha-Me), 0.95 (m, 3H, Me of lauryl group). For **4** : $[\alpha]_D^{20}$ 9.9° (c 0.28, chloroform), R_f 0.45 (benzene-acetone, I.R. ν_{max} (liquid film) cm^{-1} : 3700−3200 (s, OH), 2950, 2860 (vs, C-H), 1445, 1460, 1370, 1310, 1290 (m), 1120, 1070 (vs, broad, C-O-C), 980, 770 (m). N.M.R. ($CDCl_3$, 60 MHz) δ : 4.43 (d, 1H, $J_{1',2'}$ 8.3 Hz, H-1'), 4.38 (s, 1H, H-1), 4.0−3.0 (14H), 3.70, 3.66, 3.50, 3.40 (4s, 12H, OMeX 4), 1.8−1.1 (23H, methylene of lauryl group and Rha-Me), 0.95 (m, 3H, Me of lauryl group).

***3-Phenylpropyl O-(3, 6-di-O-methyl-β-D-glucopyranosyl)-(1→4)-2, 3-di-O-methyl-α-and β-L-rhamnopyranoside* (5 *and* 6).**— Compound **2** (125 mg) and 3-phenylpropanol (26 mg) were coupled and processed as for **3** and **4**, to give 26.5 mg of the 3-phenylpropyl disaccharide fraction (anomeric mixture), $[\alpha]_D^{20}$ −23.2° (c 1.47, chloroform), R_f 0.44 (benzene-acetone, 4 : 1).

The 3-phenylpropyl disaccharide fraction (49 mg) was deacetylated and separated by preparative T.L.C., to give **5** (10.2 mg) and **6** (12.3 mg). For **5** : $[\alpha]_D^{20}$ −39.8° (c 0.37, chloroform), R_f 0.39 (benzene-acetone, 2 : 1). I.R. ν_{max} (liquid film) cm^{-1} : 3600−3100 (s, OH), 1440 (m), 1110, 1060 (vs, broad, C-O-C), 700 (w). N.M.R. ($CDCl_3$, 60 MHz) δ : 7.27 (s, 5H, ph), 4.45 (d, 1H, $J_{1',2'}$ 8.2Hz, H-1'), 3.9−3.0 (14H), 3.70, 3.50, 3.50, 3.41 (4s, 12H, OMeX 4), 2.9−2.5 (m, 2H, ph-CH_2-CH_2-), 2.1−1.6 (m, 2H, ph-CH_2-CH_2-), 1.35 (d, 3H, $J_{5,6}$ 6 Hz, Rha-Me). For **6** : $[\alpha]_D^{20}$ 3.46° (c 0.60, chloroform), R_f 0.33 (benzene-acetone, 2 : 1). I.R. ν_{max} (liquid film) cm^{-1} : 3600−3100 (s, OH), 1440 (m), 1110, 1060 (vs, C-O-C), 980, 700 (m). N.M.R. ($CDCl_3$, 60 MHz) δ : 7.24 (s, 5H, ph), 4.45 (d, 1H, $J_{1',2'}$ 8.3 Hz, H-1'), 4.43 (s, 1H, H-1), 3.9−3.0 (14H), 3.70, 3.65, 3.49, 3.40 (4s, 12H, OMeX 4), 3.0−2.5 (m, 2H, ph-CH_2-), 2.2−1.7 (m, 2H, ph-

CH_2-CH_2-), 1.40 (d, 3H, *J* 6 Hz, Rha-Me).

Stearyl O-(2-O-acetyl-3, 6-di-O-methyl-β-D-glucopyranosyl)-(1→4)-2, 3-di-O-methyl-α-L-rhamnopyranoside (**7**) *and stearyl O-(3, 6-di-O-methyl-β-D-glucopyranosyl)-(1→4)-2, 3-di-O-methyl-α-L-rhamnopyranoside* (**8**).- - - Compound **2** (125 mg) and stearyl alcohol (42 mg) were stirred at 35 C for 3 hr in nitromethane-toluene (5 : 3) in the presence of mercury cyanide, and proccessed as usual. The product was purified by silicagel column chromatography to give stearyl disaccharide fraction : $[\alpha]_D^{20}$ −20.8° (c 1.21, chloroform), R_f 0.56 (benzene-acetone, 4 : 1).

The steary disaccharide fraction (39 mg) was deacetylated with sodium methoxide (0.01 M) and purified by preparative T.L.C., to give **7** (27.5 mg), $[\alpha]_D^{20}$ −34.5° (c 0.43, chlorofvrm), R_f 0.62 (benzene-acetone, 2 : 1). I.R. ν_{max} (liquid film) cm-1 : 3700−3100 (s, OH), 3050−2800 (vs, CH), 1750 (vs, C=O), 1460, 1450 (s), 1370 (s), 1230 (s, O-C=O), 1170−1000 (vs, broad, C-O-C). N.M.R. ($CDCl_3$, 60 MHz) δ : 4.80 (s, 1 H, H-1), 4.77 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 3.7−3.3 (13H), 3.51, 3.46, 3.41, 3.38 (4 s, 12H, 4 XOMe), 2.09 (s, 3 H, 2'-OAc), 1.26 (35H, methylene of stearyl group and Rha-Me), 0.95 (m, 3H, Me of steearyl group).

Compound 2 (20 mg) was deacetylated again with sodium methoxide (0.1 M) and purifed by preparative T.L.C., to give **8** (9.8 mg), $[\alpha]_D^{20}$ −39.6° (c 0.34, chloroform), R_f 0.33 (benzene-acetone, 2 : 1). I.R. ν_{max}. (liquid film) cm^{-1} : 3700 − 3100 (s, OH), 1110, 1070 (s, broad, C-O-C). N.M.R. ($CDCl_3$, 60 MHz) δ : 4.80 (s, 1 H, H-1), 4.41 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1'), 3.8−3.0 (14H), 3.68, 3.48, 3.48, 3.39 (4 s, 12H, OMeX 4), 1.25 (35H, methylene of stearyl group and Rha-Me), 0.90 (m, Me of stearyl group).

ELISA inhibition assay.- - - Disaccharide derivative dissolved in ethanol was added to the serum (50 μl, 1000 times diluted with 20% fetal calf serum-phosphate buffered saline) of active LL patient (standard serum) to the final concentrations of 100, 250 or 500 nmole/ml. It was incubated at 37 C for 3 hr and then remaining activity was measured by standard ELISA protocol reported previously.[18]

Direct ELISA.- - - A methanol solution of the synthetic disaccharide (5 μg/well) was added to the wells of polystylene microplate (Immuno Plate II, Nunk, Kamstrup, Denmark), and the solvent was evaporated by heating at 60 C. The dried wells were blocked and the activities of the disaccharides were measured by standard ELISA protocol reported previously.[18]

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