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Regioselectivity in β-Galactosidase-catalyzed Transglycosylation for the Enzymatic Assembly of D-Galactosyl-D-mannose

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The regioselectivity of β-galactosidase derived from *Bacillus circulans* ATCC 31382 (β-1,3-galactosidase) in transgalactosylation reactions using D-mannose as an acceptor was investigated. This D-mannose associated regioselectivity was found to be different from reactions using either GlcNAc or GalNAc as acceptors, not only for β-1,3-galactosidase but also for β-galactosidases of different origins. The relative hydrolysis rate of Galβ-*p*NP and D-galactosyl-D-mannoses, of various linkages, was also measured in the presence of β-1,3-galactosidase and was found to correlate well with the ratio of disaccharides formed by transglycosylation. The unexpected regioselectivity using D-mannose can therefore be explained by an anomalous specificity in the hydrolysis reaction. By utilizing the identified characteristics of both regioselectivity and hydrolysis specificity using D-mannose, an efficient method for enzymatic synthesis of β-1,3-, β-1,4- and β-1,6-linked D-galactosyl-D-mannose was subsequently established.

Key words: β-galactosidase; *Bacillus circulans*; regioselectivity; transglycosylation; D-galactosyl-D-mannose

Oligosaccharides are known to be synthesized regioselectively by transglycosylation using glycosidases, and this holds true also for galactosyl oligosaccharides. Many biologically important galactosyl oligosaccharides have been synthesized enzymatically.[1,2] A recombinant β-galactosidase from *Bacillus circulans* ATCC 31382 (β-1,3-galactosidase)[3] has been reported to have a high specificity for both Galβ-(1→3)-GlcNAc and Galβ(1→3)-GalNAc derivatives. When this enzyme was used in transgalactosylation reactions using GlcNAc and GalNAc as acceptors, it showed high regioselectivity in the formation of Galβ-(1→3)-GlcNAc and Galβ-(1→3)-GalNAc respectively.[4] In contrast, this transglycosylation regioselectivity was found to change from a β-(1→3)-linkage to a β-(1→6)-linkage by a change of acceptor to D-galactose.[5] Hence, in transglycosylation reactions using D-galactose derivatives as acceptors, Galβ-(1→6)-Gal derivatives were obtained exclusively.

In the present study, we investigated the transglycosylation regioselectivity of β-1,3-galactosidase from *B. circulans* when the acceptor was changed from D-galactose to D-mannose. Moreover, we examined similar transglycosylation reactions using β-galactosidases from various origins and compared the regioselectively of these different reactions, using either GalNAc or D-mannose acceptors. We discuss the reasons for the altered regioselectivites due to the change of acceptor from either GlcNAc or GalNAc to D-mannose. To our knowledge, the synthesis of D-galactosyl-D-mannose using glycosidases has not been reported previously. We also explain an efficient method for the enzymatic synthesis of β-1,3-, β-1,4- and β-1,6-linked D-galactosyl-D-mannoses.

Materials and Methods

Materials. β-Galactosidases from *E. coli* and from bovine testes were purchased from Sigma. β-1,4-Galactosidase from *B. circulans* was purchased from Wako Pure Chemicals. (Osaka, Japan). β-Galactosidases from *Aspergillus oryzae* and from *Penicillium multicolor* were purchased from Toyobo (Osaka, Japan) and K.I. Chemicals (Shizuoka, Japan) respectively. β-Galactosidase from *Bifidobacterium bifidum* and β-1,3-galactosidase from *B. circulans* (recombinant) were used without further purification of the culture broth.

Analytical methods. HPLC was carried out with an HPAE-PAD Dionex PeakNet system using Carbopac PA1 (4.0 × 250 mm) column eluted by either 40 or 64 mM sodium hydroxide solutions. ^{1}H and ^{13}C NMR spectra were obtained using a Varian Inova 500 spectrometer.

Synthesis of Galβ-(1→3)-Man by β-1,3-galactosidase. A reaction mixture consisting of Galβ-*p*NP (150 mg), D-mannose (800 mg), and β-1,3-galactosidase from *B. circulans* (2.0 U) in 2.1 ml of sodium phosphate buffer (pH 6.0) containing 20% (v/v) DMF was incubated at 37 °C and the reaction was monitored by HPLC.

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When the Galβ-*p*NP peak disappeared from the HPLC spectrum, the reaction was stopped by heating the solution for 5 min in boiling water. The reaction produced a mixture of Galβ-(1→3)-Man and Galβ-(1→6)-Man in an approximate ratio of 2:1. In our typical experiment, the reactions were stopped at 2 h. The reaction mixture was then diluted into 1 liter with 0.1 M sodium phosphate buffer (pH 6.0), and incubated at 37 °C in the presence of β-galactosidase from *E. coli* (10 U). After overnight incubation, the solution was applied onto an activated carbon column (2.7 × 50 cm). The column was washed with 1 liter of water and subsequently eluted with a gradient of water (1 liter)-30% (v/v)-ethanol (1 liter). The sugar was detected with the PAD-HPLC system using a sodium hydroxide solution, and fractions containing disaccharides were collected and concentrated *in vacuo* and freeze-dried to give 15.4 mg of highly purified and 9.4 mg of moderately pure Galβ-(1→3)-Man. NMR (D_2O): δ 3.61 (t, 1H, $J_{2,3} = 7.62$ Hz, H-2(Gal)), δ 3.94 (bs, 1H, H-4(Gal)), δ 3.98 (dd, 1H, $J_{3,4} = 5.57$ Hz, $J_{3,4} = 3.23$ Hz, H-3(Manβ)), δ 4.06 (dd, 1H, $J_{2,3} = 2.93$ Hz, $J_{3,4} = 6.46$ Hz, H-3(Manα)), δ 4.13 (bs, 1H, H-2(Manα)), δ 4,15 (d, $J_{2,3} = 3.23$ Hz, 1H, H-2(Manβ)), δ 4.54 (d, 1H, $J_{1,2} = 7.62$ Hz, 1H, H-1(Galα)), δ 4.55 (d, 1H, $J_{1,2} = 7.92$ Hz, H-1(Galβ)), δ 4.91 (s, 1H, H-1(Manβ)), δ 5.23 (d, 1H, $J_{1,2} = 0.88$ Hz, H-1(Manα)).

Synthesis of Galβ-(1→4)-Man by β-1,4-galactosidase from B. circulans. A reaction mixture consisting of Galβ-*p*NP (150 mg), D-mannose (800 mg), and β-1,4-galactosidase from *B. circulans* (2.0 U) in sodium phosphate buffer (2.1 ml, pH 6.0) containing 20% (v/v) DMF was incubated at 37 °C. The reaction produced a mixture of Galβ-(1→3)-Man, Galβ-(1→4)-Man, and Galβ-(1→6)-Man in an approximately 1:3:1 ratio. In order to remove unwanted disaccharides, the hydrolysis reaction was performed using β-1,3-galactosidase from *B. circulans* (10 U), which was predicted to hydrolyze Galβ-(1→3)-Man and Galβ-(1→6)-Man. The solution containing Galβ-(1→4)-Man as a unique disaccharide was applied onto an activated carbon column, and the elution procedure was the same as that for the purification of Galβ-(1→3)-Man. A small amount (1.5 mg) of highly purified Galβ-(1→4)-Man was obtained together with 28.7 mg of Galβ-(1→4)-Man with lower purity. NMR (D_2O): δ 3.45 (dd, 1H, $J_{2,3} = 8.06$ Hz, H-2(Gal)), δ 3.79 (dd, 1H, $J_{2,3} = 3.43$ Hz, $J_{3,4} = 9.81$ Hz, H-3(Manα)), δ 4.00 (bs, 1H, H-2(Manα)), δ 4.34 (d, 1H, $J_{1,2} = 7.71$ Hz, H-1(Gal)), δ 4.47 (d, 1H, $J_{2,3} = 2.94$ Hz, H-2(Manβ)), δ 4.87 (s, 1H, H-1(Manβ)), δ 5.18 (s, 1H, H-1(Manα)).

Synthesis of Galβ-(1→6)-Man by β-galactosidase from E. coli. A reaction mixture consisting of Galβ-*p*NP (600 mg), D-mannose (3.2 g), and β-galactosidase from *E. coli* (8.0 U) in 0.1 M sodium phosphate buffer (8.0 ml, pH 6.0) containing 20% (v/v) DMF was incubated at 37 °C. The reaction mixture contained Galβ-(1→3)-Man, Galβ-(1→4)-Man, and Galβ-(1→6)-Man in an approximately 10:4:86 ratio, detected by HPLC. The unwanted Galβ-(1→3)-Man was hydrolyzed using β-galactosidase from bovine testes (40 U). Galβ-(1→6)-Man was purified using an activated carbon column by a procedure similar to that described in the previous sections. Highly purified (25.6 mg), moderately purified (41.9 mg), and crude preparations (more than 30% contaminants) (45.9 mg) of Galβ-(1→6)-Man were obtained. NMR (D_2O): δ 3.44 (dd, 1H, $J_{2,3} = 9.91$ Hz, H-2(Galβ)), δ 4.08 (dd, 1H, $J_{5,6} = 1.97$ Hz, $J_{6,6} = 11.29$ Hz, H-6(Gal)), δ 4.32 (d, 1H, $J_{1,2} = 7.86$ Hz, H-1(Galβ)), δ 4.33 (d, 1H, $J_{1,2} = 7.85$ Hz, H-1(Galα)), δ 4.79 (d, 1H, $J_{1,2} = 0.74$ Hz, H-1(Manβ)), δ 5.05 (d, 1H, $J_{1,2} = 1.97$ Hz, H-1(Manα)).

Synthesis of D-galactosyl-D-mannose using β-galactosidase from various origins. A reaction mixture consisting of Galβ-*p*NP (30 mg), D-mannose (160 mg), and β-galactosidase (0.8 U) in 400 μl of sodium phosphate buffer (pH 6.0) containing 20% (v/v) DMF was incubated at 37 °C, and the reaction was monitored by HPLC. When the peak of Galβ-*p*NP was no longer detectable by HPLC, the reaction was stopped by heating the solution for 5 min in boiling water. The reaction yield of each disaccharide was calculated from the peak areas in HPLC chromatograms, and is summarized in Table 1.

Table 1. Synthesis of Transfer Products Mediated by Various β-Galactosidases

Origin of enzyme	Linkage	Acceptor	
		GalNAc	Man
E. coli	β-(1→3)	trace	10
	β-(1→4)	trace	4
	β-(1→6)	95	86
A. Oryzae	β-(1→3)	5	17
	β-(1→4)	3	12
	β-(1→6)	92	71
P. multicolor	β-(1→3)	trace	25
	β-(1→4)	6	30
	β-(1→6)	94	45
β-1,4-galactosidase from *B. circulans*	β-(1→3)	trace	20
	β-(1→4)	63	62
	β-(1→6)	37	18
B. bifidum	β-(1→3)	trace	23
	β-(1→4)	64	39
	β-(1→6)	36	38
β-1,3-galactosidase from *B. circulans*	β-(1→3)	95	67
	β-(1→4)	trace	trace
	β-(1→6)	trace	33
Streptococcus 6646 K	β-(1→3)	trace	trace
	β-(1→4)	100	100
	β-(1→6)	trace	trace

Measurement of relative hydrolysis rate of Galβ-pNP and D-galactosyl-D-mannose disaccharides. 1.5 mM of each of the disaccharides or of a Galβ-*p*NP solution in 240 μl of 0.1 M sodium phosphate buffer (pH 6.0) and 20 μl of 24 mM L-fucose solution were mixed with 10 μl of enzyme solution (5.0 units/ml) and incubated at 37 °C. The ratio of either the remaining disaccharides or Galβ-*p*NP was calculated from the HPLC peak areas. L-Fucose was used as an internal standard.

Results and Discussion

Transgalactosylation to D-mannose using β-1,3-galactosidase

Zeng *et al.* reported that transgalactosylation with a D-galactose acceptor yielded Galβ-(1→6)-Gal as the main product.[5] In the present study, we used D-mannose as an acceptor to examine how β-galactosidase would discriminate among the acceptor substrates in the transglycosylation reactions. D-mannose has a stereochemistry largely different from those of D-galactose or D-GalNAc, which are characterized by axial and equatorial OH groups at the positions of C-2 and C-4 respectively. Moreover, the anomeric OH group is fixed predominantly at the α-configuration, which simplifies the identification of β-galactosylation products. The HPLC chromatogram of the reaction mixture of Galβ-*p*NP and D-mannose in the presence of β-1,3-galactosidase from *B. circulans* is shown in Fig. 1. The chart shows two disaccharide peaks representing Galβ-(1→3)-Man and Galβ-(1→6)-Man with a ratio of approximately 2:1, but peaks indicating the presence of Galβ-(1→2)-Man or Galβ-(1→4)-Man were not detected.

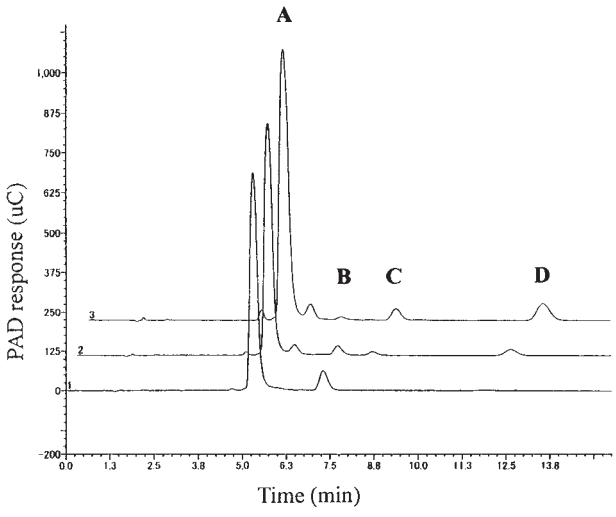

Fig. 1. HPAE-PAD Chromatography of the Reaction Mixture (3: 1.5 h, 2: 0.5 h, 1: 0 h) Using Galβ-*p*NP as a Donor and Man as an Acceptor in the Presence of β-1,3-Galactosidase from *B. circulans*.

Column: Carbopac PA1, Elution: 40 mM sodium hydroxide solution. Flow rate: 1 ml/min, peak A: D-galactose and D-mannose, peak B: Galβ-*p*NP, peak C: Galβ-(1→6)-Man, peak D: Galβ-(1→3)-Man.

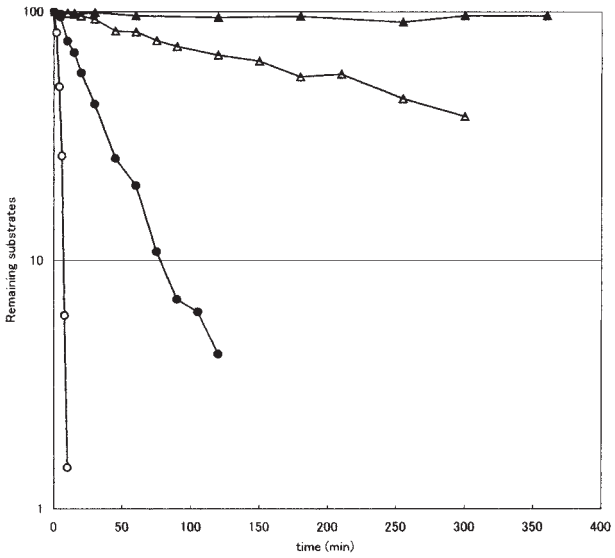

Fig. 2. Time Courses of the Peak Areas of Galβ-*p*NP and D-Galactosyl-D-mannose of Various Linkages in the Presence of β-1,3-Galactosidase from *B. circulans.*

Galβ-pNP (○), Galβ-(1→3)-Man (●), Galβ-(1→6)-Man (△), Galβ-(1→4)-Man (▲).

Hydrolysis of D-galactosyl-D-mannoses containing various different linkages by β-1,3-galactosidase

Generally, regioselectivity strongly correlates with hydrolysis specificity,[6,7] and hence we measured the relative hydrolysis rates of D-galactosyl-D-mannose and Galβ-*p*NP. The results, shown in Fig. 2, indicate that the relative hydrolysis rate is in the following order: Galβ-*p*NP > Galβ-(1→3)-Man > Galβ-(1→6)-Man > Galβ-(1→4)-Man. This finding is consistent with the results for the relative yield of the respective disaccharides by transglycosylation. Galβ-(1→3)-Man was hydrolyzed faster than Galβ-(1→6)-Man and was produced more readily by transgalactosylation. In addition, Galβ-(1→4)-Man was hydrolyzed slowly and was not obtained by transglycosylation, this result is also consistent with the hydrolysis specificity. Hence it may be concluded that the regioselectivity in these transglycosylation reactions is not anomalous, but due to the hydrolysis specificity of D-galactosyl-D-mannose, which is less restrictive than that of either Gal-GlcNAc or Gal-GalNAc.

Regioselectivity in transgalactosylation by β-galactosidases from various origins

We next examined reactions using other β-galactosidases of various origins.[8,9] The results are summarized in Table 1. β-1,4-Galactosidase from *B. circulans* is known to yield β-(1→4)-linked disaccharide predominantly by transgalactosylation when either GlcNAc or GalNAc is used as the acceptor.[10–12] In our experiments, however, we obtained yields of Galβ-(1→3)-Man, Galβ-(1→4)-Man, and Galβ-(1→6)-Man in a ratio of approximately 1:3:1 in reactions with this enzyme when D-mannose was used as an acceptor. The regioselectivity

changed due to the structural change in the acceptor molecule. In reactions using β-galactosidase from *E. coli*, the disaccharides that we obtained were Galβ-(1→3)-Man, Galβ-(1→4)-Man, and Galβ-(1→6)-Man, in a ratio of 10:4:86. This enzyme is known to yield Galβ-(1→6)-GalNAc exclusively in reactions using Galβ-*p*NP and GalNAc.[13] In this case also the regioselectivity of the reactions weakened slightly. It is noteworthy that β-galactosidase from *Streptococcus* 6646 K showed high regioselectivity for β-1,4-linkages, similarly to reactions using GlcNAc or GalNAc as an acceptor.[14] As can be seen in Table 1, the additional enzyme types showed different regioselectivities to the reactions using GlcNAc or GalNAc as an acceptor. Hence, the so-called "regioselectivity" is a regioselectivity in transglycosylation to either GlcNAc or GalNAc. Consequently, in transgalactosylation to D-galactose or D-mannose, one should bear in mind that there are alterations to or even a complete loss of established regioselectivities.

Preparative study of D-galactosyl-D-mannoses of differing linkages

Since regioselectivity in transgalactosylation to D-mannose is not as rigid as in the case of transgalactosylation to GlcNAc or GalNAc, the enzymatic preparation of D-galactosyl-D-mannose is somewhat more cumbersome. Galβ-(1→3)-Man was obtained as follows: First, by transglycosylation using Galβ-*p*NP and D-mannose in the presence of β-1,3-galactosidase from *B. circulans*, Galβ-(1→3)-Man was mainly produced with Galβ-(1→6)-Man as a minor component. In this reaction, Galβ-(1→4)-Man levels were negligibly small. Galβ-(1→6)-Man was then hydrolyzed using β-galactosidase from *E. coli* to generate a mixture of monosaccharides and Galβ-(1→3)-Man, which subsequently could easily be isolated using activated carbon column chromatography. Similarly, Galβ-(1→4)-Man was obtained as follows: A mixture of Galβ-(1→3)-Man, Galβ-(1→4)-Man, and Galβ-(1→6)-Man generated by β-1,4-galactosidase from *B. circulans* was treated by β-1,3-galactosidase from *B. circulans*, which has hydrolysis specificity for β-(1→3)- and β-(1→6)-linkages. Galβ-(1→3)-Man and Galβ-(1→6)-Man were therefore depleted by hydrolysis, leaving Galβ-(1→4)-Man as a remainder. The procedure for obtaining Galβ-(1→6)-Man is very simple, since β-galactosidase from *E. coli* generates a mixture of Galβ-(1→3)-Man and Galβ-(1→6)-Man in a ratio of approximately 1:9, together with small amounts of Galβ-(1→4)-Man. Galβ-(1→3)-Man is then easily removed by hydrolysis using β-galactosidase from bovine testes.[15,16] The remaining small amount of Galβ-(1→4)-Man can then be removed during the activated carbon column chromatography step. The results are summarized in Fig. 3. The ^{13}C NMR data of the purified D-galactosyl-D-mannoses are summarized in Table 2.

By combining transglycosylation with specific hydrolysis reactions, then, each disaccharide can be isolated and purified. Thus synthesized D-galactosyl-D-

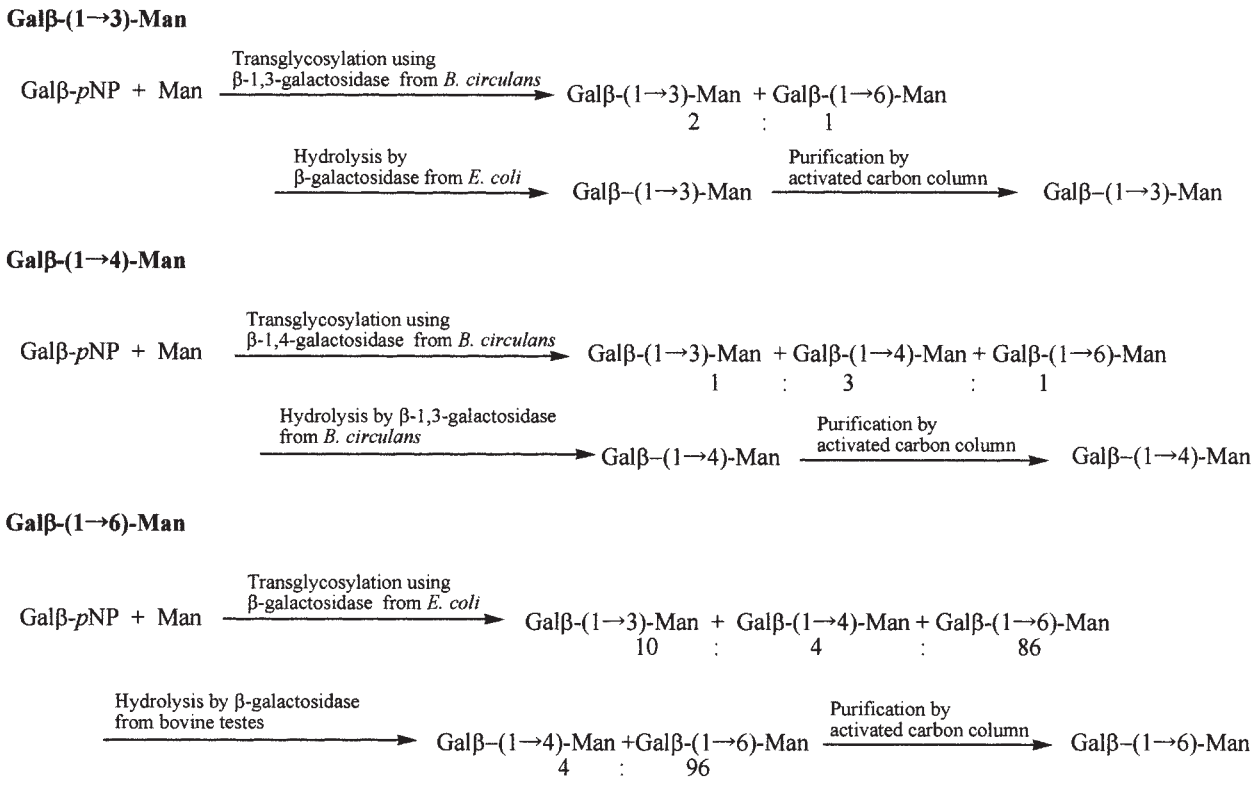

Fig. 3. Synthesis and Purification of D-Galactosyl-D-mannose of Various Linkages.

Table 2. ^{13}C NMR Data for D-Galactosyl-D-mannose

		Compound		
		Gal-β-(1→3)Man	Gal-β-(1→4)Man	Gal-β-(1→6)Man
Gal	C1	101.826	102.432	103.531
	C2	71.672	69.703	70.808
	C3	73.457	70.749	70.970
	C4	69.632	67.223	68.830
	C5	76.147	75.407	75.348
	C6	61.946	61.384	61.204
Man	C1	94.581	92.410	94.311
	C2	69.214	68.830	70.324
	C3	78.915	72.538	71.545
	C4	66.103	78.238	66.788
	C5	73.457	72.640	72.797
	C6	61.779	60.678	69.016

mannoses of different linkages may be utilized for various biological studies. For example, a repeating unit of D-galactosyl-D-mannose has been found to be a component unit of *Leishmania* lipophosphoglycan, and the disaccharide unit is supposed to be related to endothelial adhesion to monocyte.[17,18] Therefore these disaccharides are expected to be utilized to clarify the biological roles and functions of D-galactosyl-D-mannose sequence in *Leishmania*.

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