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Enzymatic α(1→2)-L-fucosylation: investigation of the specificity of the α(1→2)-L-galactosyltransferase from *Helix pomatia*

Angela Michelle Scheppokat,[a] Minoru Morita,[a,†] Joachim Thiem[a,*] and Hagen Bretting[b]

[a]*Institut für Organische Chemie, Universität Hamburg, Martin-Luther-King-Platz 6, 21073 Hamburg, Germany*
[b]*Zoologisches Institut, Universität Hamburg, Martin-Luther-King-Platz 3, 21073 Hamburg, Germany*

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Abstract—The α(1→2)-L-galactosyltransferase from *Helix pomatia* transfers an L-fucosyl residue from GDP-L-Fucose to a terminal, non-reducing D-galactopyranosyl moiety of an oligosaccharide. The extent of the enzyme's specificity towards the stereochemistry at the D-galactopyranosyl anomeric centre, the site of interglycosidic linkage and the nature of the subterminal oligosaccharide residue has been investigated using HPAEC-PAD and MALDI-TOF technology. This α(1→2)-L-galactosyltransferase is specific for D-galactopyranosyl β-linkages, independent of the site of the interglycosidic linkage and aglycone configuration and with limited specificity for the nature of the subterminal sugar residue.

1. Introduction

The L-fucose moiety is commonly found in mammalian glycoproteins, glycolipids and oligosaccharides. For instance, the H blood group determinant, precursor of the ABO blood antigens, is defined by the disaccharide sequence L-Fuc-α(1→2)-D-Gal.[1] To mention but a few of their myriad biological functions, L-fucosylated glycoconjugates are involved in the control of chain length during biosynthesis, in growth regulation and in cell–cell interactions.[2–4] Glycoconjugates containing L-fucose have also been implicated in malignancy.[5,6] It is widely acknowledged that the quickest and most efficient synthetic pathway to a complex oligosaccharide involves enzymatic steps, therefore the availability of a series of highly active glycosyltransferases is of utmost importance. Glycosyltransferases are noted for their excellent yields and high regio- and stereospecificity, but may require the employment of expensive nucleotide donors, which often leads to labour-intensive recycling processes.[7] However, the recent availability of large amounts of inexpensive GDP-L-Fuc[8] has made the use of glycosyltransferases for oligosaccharide synthesis more attractive than it was previously.

In search of enzymes with significantly higher α(1→2)-L-fucosyltransferase activity than those known to date, the albumen gland of the vineyard snail *Helix pomatia* was examined, where an α(1→2)-L-galactosyltransferase (L-GalT) is utilised in the synthesis of galactan. Galactan is a highly-branched polysaccharide used as the exclusive carbohydrate source for embryonic and newly-hatched snails,[9] and substituted at terminal non-reducing D-galactosyl moieties with α(1→2)-L-Gal residues.[10] It was hoped that the L-GalT would exhibit L-FucT activity, which indeed proved to be the case. Starting from the acceptor disaccharide D-Gal-β(1→3)-D-Gal-βOMe **1**, the trisaccharides L-Gal-α(1→2)-D-Gal-β(1→3)-D-Gal-βOMe **2** and L-Fuc-α(1→2)-D-Gal-β(1→3)-D-Gal-βOMe **4** (where either GDP-L-Gal or GDP-L-Fuc was used as donor, respectively) were synthesised on a milligram scale and characterised by permethylation studies and NMR of the peracetylated derivatives **3** and **5** (Scheme 1).[10]

Having seen that the L-GalT recognises GDP-L-Fuc, it was decided to optimise the transfer reactions and to explore the synthetic potential of this enzyme. HPAEC-PAD is an extremely sensitive and convenient method of monitoring small-scale glycosylation reactions, and was therefore employed as the method of choice.

* Corresponding author.
† Current address: Snowbrand Milk Products Co. Ltd., Technology and Research Institute, Minamidai 1-1-2, Kawagoe-shi, Saitama 351-0061, Japan.

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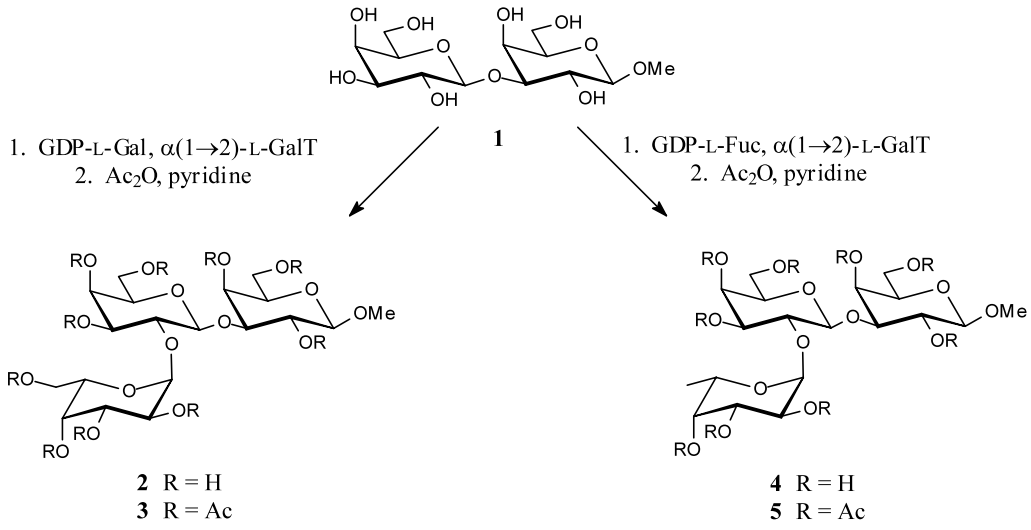


Scheme 1. α(1→2)-L-Galactosylation and α(1→2)-L-fucosylation of acceptor **1** using *H. pomatia* α(1→2)-L-GalT.

2. Results and discussion

2.1. Small-scale acceptor specificity testing

The L-galactosylation and L-fucosylation reactions were optimised with respect to temperature, incubation period, eventual inclusion of antimicrobial agents, and/or promoting agents such as cysteine, and donor:acceptor ratio. A suitable compromise between the accelerated donor degradation at higher temperatures and the slower fucosylation/galactosylation rates at lower temperatures proved to be 28°C, with an ideal incubation period of 18 h in Tris–HCl buffer (pH 7.6). Investigation of the optimal donor:acceptor ratio led to the values 5:2 for L-galactosylation and 3:2 for L-fucosylation. It has been reported that cysteine accelerates the transfer rate, however, it was observed that cysteine simultaneously accelerates the rate of donor degradation, and was henceforth excluded from reaction systems. The necessity of an antimicrobial agent arose, and sodium azide avoided consumption of the reaction components by bacteria, without detrimentally affecting the transfer. The presence of an alkaline bovine phosphatase from calf intestine (EC 3.1.3.1) in the system was seen to enhance significantly the L-fucosylation yield and to slightly enhance the L-galactosylation yield. Thus bovine phosphatase was employed to remove GDP, which is accumulated as a side-product of the transfer from the donor and which can lead to feedback inhibition of the transfer.[11]

Using the optimised L-fucosylation conditions and GDP-L-Fuc as donor, the L-GalT was tested with a series of acceptors (Table 1). The glycosylation yields were determined using Dionex HPAEC-PAD chromatography. Because the albumen gland sediment represents a crude enzyme mixture, all Dionex spectra were also examined for traces of side products, arising for example through acceptor hydrolysis. However, in all instances, except for the unsuccessful acceptor **23**, the carbohydrate components of the reaction mixtures consisted of only the acceptor, the expected product and L-fucose. The enzyme recognised a broad range of acceptors, showing only limited specificity towards the site of interglycosidic linkage. All four disaccharides of the homologous series D-Galβ(1→*n*)-D-GlcOMe **6–9**

Table 1. α(1→2)-L-Fucosylation of various acceptor substrates using *H. pomatia* α(1→2)-L-GalT

Acceptor	Transfer (%)[a]	Acceptor	Transfer (%)[a]
D-Galβ(1→3)-D-GalβOMe **1**	93	D-Galβ(1→4)-D-Man **16**	75
D-Galβ(1→2)-D-GlcβOMe **6**	75	D-Lactal **17**	47
D-Galβ(1→3)-D-GlcβOMe **7**	81	D-Galβ(1→4)-D-GlcNAc **18**	53
D-Galβ(1→4)-D-GlcβOMe **8**	87	D-Galβ(1→3)-D-Ara **19**	0
D-Galβ(1→6)-D-GlcβOMe **9**	74	D-Galβ(1→4)-D-1,2-deoxyglucose **20**	0
D-Galβ(1→4)-D-GlcβOPent **10**	0	D-Lactitol **21**	0
D-Galβ(1→4)-D-Glc **11**	87	D-Galα(1→6)-D-GlcβOMe **22**	0
[D-Galβ(1→3)]$_2$-D-Galβ(1→4)-D-Glc **12**	Good[b]	D-Galβ(1→4)-D-GalβO*p*NP **23**	0
[D-Galβ(1→3)]$_3$-D-Galβ(1→4)-D-Glc **13**	Good[b]	D-Galβ(1→4)-D-Gal **24**	0
[D-Galβ(1→3)]$_4$-D-Galβ(1→4)-D-Glc **14**	Good[b]	D-Galβ(1→6)-D-Gal **25**	69
D-Galβ(1→3)-[D-Galβ(1→6)]-D-GalβOMe **15**	Good[b]		

[a] Evaluated by Dionex HPAEC-PAD chromatography, with respect to reduction in acceptor peak area under identical dilution conditions. The presence of the product was confirmed in all cases by MALDI-TOF spectroscopy.

[b] Reaction observed only by MALDI-TOF spectroscopy–complete removal of acceptor observed.

($n=2$, 3, 4, 6) proved to be excellent acceptors, with transfer rates of 74–87%. In the case of D-Gal-β(1→4)-D-Glc-βOPent **10**, the aglycone had a detrimental effect on the solubility of the acceptor in aqueous solution and thus inhibited transfer fully. This was directly due to the length and hydrophobicity of the aglycone, as lactose **11** and methyl lactoside **8** were successful acceptors, each having L-fucosylation yields of 87%. Monosaccharides were not accepted by L-GalT, with disaccharides being the smallest oligosaccharides recognised. The tetra-, penta- and hexasaccharides [D-Gal-β(1→3)-]$_n$-D-Gal-β(1→4)-D-Glc **12–14** ($n=1$–4) were isolated from the milk of the tammar wallaby *Macropus eugenii*.[12,13] Each of the oligosaccharides **12–14** was recognised by L-GalT, which was not surprising considering that L-GalT was employed in vivo to cap side chain growth in the highly-branched galactan, which consists of 25000 sugar units.[14] The branched trisaccharide D-Galβ(1→3)-[D-Galβ(1→6)]-D-GalβOMe **15**, with two terminal, non-reducing D-galactopyranosyl residues, was also recognised, with observation of both the mono and difucosylated products by MALDI-TOF spectroscopy. The nature of the subterminal sugar units was also of relatively little importance, with many conventional D-series subterminal sugars being recognised—e.g. D-Gal-β(1→4)-D-Glc **11**, D-Gal-β(1→4)-D-Man **16**, D-lactal **17**, *N*-acetyl-D-lactosamine **18**. However, D-Gal-β(1→4)-D-Ara **19**, in which the subterminal sugar may be considered an L-series analogue, was not accepted. D-Gal-β(1→4)-1,2-dideoxy-D-Glc **20** and disaccharide alditols (e.g. D-lactitol **21**) were also unsuccessful.

An α-interglycosidic linkage between the terminal, non-reducing D-galactose and the subterminal sugar residue was not tolerated by the enzyme (e.g. D-Galα(1→6)-D-GlcOMe **22**). Similarly, the corresponding β-interglycosidic linkage may not be attached to an axial hydroxyl group in the non-terminal sugar residue, as occurs for example in a (1→4) linkage between two D-galactosyl units. Initial testing with D-Gal-β(1→4)-D-Gal-βO*p*NP **23** showed that this disaccharide was not accepted. This may have been due to either the -interglycosidic linkage to an axial hydroxyl group, or to cleavage of the pNP group, which was seen to occur immediately upon incubation of D-Gal-β(1→4)-D-Gal-βO*p*NP **23** with L-GalT. This *p*-nitrophenol thus formed may have inhibited glycosylation and to test this, the successful acceptor D-Gal-β(1→3)-D-Gal-βOMe **1** was treated with GDP-L-Fuc and L-GalT both in the presence and in the absence of *p*-nitrophenol. Apparently, *p*-nitrophenol had no effect on the L-fucosylation, indicating that the (1→4) linkage was the disturbing factor. This could be confirmed by incubating commercially available D-Gal-β(1→4)-D-Gal **24** with GDP-L-Fuc and L-GalT, whereby no transfer could be observed. D-Gal-βO*p*NP is not hydrolysed by the albumen gland sediment under identical conditions. In fact, hydrolysis of acceptor substrates was observed only in the case of D-Gal-β(1→4)-D-Gal-βO*p*NP **23**, which indicates that the albumen gland sediment contains no significant β-D-galactoside activity, which would interfere with the glycosation reactions described herein. As expected, D-Gal-β(1→6)-D-Gal **25** turned out to be a successful acceptor substrate.

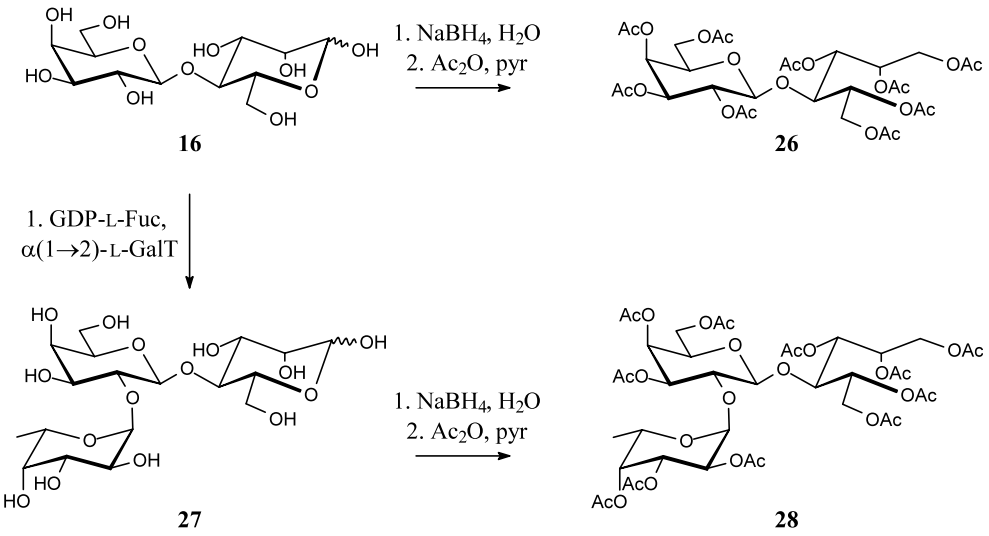
2.2. Larger scale synthesis of H-antigen-bearing trisaccharides

The acceptance or non-acceptance of an acceptor by L-GalT was observed by HPAEC-PAD, and the presence or absence of the desired L-fucosylated oligosaccharide product was verified by MALDI-TOF spectroscopy. It is presumed, by analogy, that the products formed by treating the above-mentioned oligosaccharides with the L-GalT are those resulting from α(1→2)-L-fucosylation at the terminal, non-reducing D-galactopyranosyl moiety. To confirm these conclusions, it would be desirable to carry out the respective L-fucosylations on a mg scale, which allows NMR studies of the product trisaccharides to be conducted. Unprotected D-Gal-β(1→3)-D-Gal-βOMe **1** was fucosylated on a mg scale to give the trisaccharide L-Fuc-α(1→2)-D-Gal-β(1→3)-D-Gal-βOMe **4**, which was purified using semi-preparative HPAEC-PAD and peracetylated giving compound **5** in almost double the previously reported yield (70%).[15] The disaccharide D-Gal-β(1→4)-D-Man **16** was reduced using $NaBH_4$, whereupon the sugar alditol was peracetylated to give compound **26** in 92% yield, for NMR comparison purposes. Disaccharide D-Gal-β(1→4)-D-Man **16** was reacted to give the novel trisaccharide L-Fuc-α(1→2)-D-Gal-β(1→4)-D-Man **27**. Trisaccharide **27** was reduced in the same manner as described for **16** and peracetylated to give compound **28** (67%, Scheme 2). Full NMR characterisation of all peracetylated compounds was conducted, with HMBC experiments being used to confirm the sites of fucosylation in the trisaccharides **5** and **28**.

3. Conclusion

Having recognised that the L-GalT transfers either an L-fucosyl residue or an L-galactosyl residue to a terminal, non-reducing D-galactosyl moiety, forming an α(1→2)-linkage, it was decided to optimise the fucosylation reaction and to explore the synthetic potential of this enzyme. To this end, a series of oligosaccharide acceptors were tested with GDP-L-Fuc as donor substance. The enzyme recognised a pleasingly broad range of acceptors, showing only limited specificity towards the site of interglycosidic linkage, nature of the non-terminal sugar residue and type of aglycone. Neither monosaccharides nor disaccharide alditols were successful acceptors. Oligosaccharides with an α-interglycosidic linkage between the terminal, non-reducing D-galactose and the subterminal sugar residue were not recognised by the enzyme, and the corresponding β-interglycosidic linkage may not be attached to an axial hydroxyl group in the non-terminal sugar residue.

The disaccharide D-Gal-β(1→3)-D-Gal-βOMe **1** was reacted with GDP-L-Fuc to give the trisaccharide L-Fuc-α(1→2)-D-Gal-β(1→3)-D-Gal-βOMe **2** in almost double the previously reported yields.[15] The disaccha-



Scheme 2. *H. pomatia* α(1→2)-L-GalT-mediated α(1→2)-L-fucosylation of D-Galβ(1→4)-D-Man.

ride D-Gal-β(1→4)-D-Man **16** was reacted with GDP-L-Fuc to give the trisaccharide L-Fuc-α(1→2)-D-Gal-β(1→4)-D-Man **27** in excellent yield, with 67% yield of derivative **28** with respect to the acceptor **16** being achieved after reduction and peracetylation.

4. Experimental

4.1. General

Unless otherwise stated, all reactions were monitored using Dionex HPAEC-PAD (Carbopac 100, 100 mM aqueous NaOH), Dionex GmbH, Idstein, Germany. Aqueous samples were freeze dried using a Lyovac GT2 from Leybold-Heraeus. MALDI-TOF mass spectra were recorded using a Bruker Biflex III spectrometer in positive reflector mode. DHB (2,5-dihydroxybenzoic acid) was used as matrix. ^{1}H and ^{13}C NMR spectra were recorded using Bruker AMX-400 (^{1}H: 400 MHz, ^{13}C: 100.62 MHz) and DRX-500 (^{1}H: 500 MHz, ^{13}C: 125.77 MHz) spectrometers. Chemical shifts are calibrated to $(Me)_4Si$ ($\delta = 0.00$ ppm) in $CDCl_3$.

Specimens of the snail *H. pomatia* were collected from their natural habitat near Hamburg or Schwandorf, Germany. The snails were collected in April/May, shortly before the main breeding season from May to July, which is when the highest enzyme activity may be expected. The glands were stored at −70°C until processing, whereby no substantial loss of activity could be observed after storage for 3 years.

The marsupial oligosaccharides [D-Galβ(1→3)]$_2$-D-Galβ(1→4)-D-Glc **12**, [D-Galβ(1→3)]$_3$-D-Galβ(1→4)-D-Glc **13** and [D-Galβ(1→3)]$_4$-D-Galβ(1→4)-D-Glc **14** are those isolated from the tammar wallaby *Macropus eugenii*.[12,13]

4.2. Preparation of the *H. pomatia* albumen gland sediment

The *H. pomatia* albumen glands (2.0 g) were homogenised in Tris–HCl buffer (50 mM, pH 7.6, 5 ml) in a Potter–Elvehjem homogeniser, and centrifuged for 45 min at 4000 rpm at 4°C. The supernatant was removed from the pellet and discarded, and this process was repeated five times with 30 min centrifugation at 4000 rpm. The resulting albumen gland sediment (approximately 2000 μl) was rehomogenised and either used immediately or stored at −70°C. Albumen gland sediment which has been frozen and stored must be rehomogenised before use. 300 μl of albumen gland sediment is required to react 2.81 μmol of acceptor. One snail provides one albumen gland, with an average weight of approximately 2.0 g. Therefore, roughly 18.7 μmol of acceptor can be α(1→2)-L-fucosylated per gland (this corresponds to 6.7 mg of disaccharide acceptor per snail). No further activity values can be given for the α(1→2)-L-GalT as it could not be purified to date. However, frequently repeated experiments have shown that these values are reproducible.

4.3. L-Fucosylation with *H. pomatia* α(1→2)-L-GalT and GDP-L-Fuc (small scale)

$MnCl_2$ (6 μl, 100 mM), NaN_3 (1.2 μl, 10 mg/ml), Tris–HCl buffer (pH 7.6, 3.3 μl), calf intestine alkaline phosphatase (185 mU/μl, 1 μl), the acceptor (0.281 μmol, 1 equiv., as 25 μg/μl aqueous solution) and GDP-L-Fuc (0.375 μmol, 1.33 equiv., as 4.5 μl of 50 μg/μl solution) were combined in a 1 ml Eppendorf tube, to which 40 μl of the albumen gland sediment was added. The reaction was stirred and incubated at 28°C for 18 h, then terminated by dilution with bidistilled water to a total volume of 1 ml and centrifugation at 14000 rpm for 10 min. The supernatant was removed,

and the pellet washed with 300 μl bidistilled water and centrifuged twice at 14000 rpm for 10 min. The combined aqueous fractions were shaken for 10 min with mixed bed anion exchange resin. After centrifugation at 10000 rpm for 10 min, the supernatant was removed and lyophilised.

4.4. Preparation of HPAEC-PAD samples

From a test reaction as described in Section 4.3, with 0.281 μmol acceptor in 60 μl reaction volume, 5 μl were removed and diluted with bidistilled water to 100 μl. The sample was shaken with mixed bed anion exchange resin for 10 min, and centrifuged at 10000 rpm for 10 min. The supernatant was removed and used directly for HPAEC-PAD analysis.

4.5. Methyl 2,4,6-tri-*O*-acetyl-3-*O*-[3,4,6-tri-*O*-acetyl-2-*O*-(2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl)-β-D-galactopyranosyl]-β-D-galactopyranoside 5

Methyl 3-*O*-(β-D-galactopyranosyl)-β-D-galactopyranoside **1** (1.20 mg, 25 μg/μl, 3.37 μmol) was added to a solution of GDP-L-Fuc (2.70 mg, 100 μg/μl, 4.49 μmol), $MnCl_2$ (100 mM, 72 μl), NaN_3 (10 mg/ml, 14.4 μl), calf intestine alkaline phosphatase (EC 3.1.3.1, Boehringer-Mannheim, 185 mU/μl, 12 μl), and *H. pomatia* albumen gland sediment (360 μl), and incubated at 28°C for 18 h with stirring. The reaction was terminated by dilution with bidistilled water to a volume of 2 ml and centrifugation at 14000 rpm for 10 min. The supernatant was removed, and the pellet washed with 500 μl bidistilled water and centrifuged at 14000 rpm for 10 min, twice. The combined aqueous fractions were lyophilised, diluted to 500 μl and purified by semi-preparative HPAEC-PAD. The relevant fractions were combined, lyophilised and acetylated by incubation with acetic anhydride (1 ml) and pyridine (500 μl) for 2 h at 80°C. The reaction mixture was evaporated to dryness by flushing with nitrogen gas, and the resulting residue was dissolved in dichloromethane (2 ml) and washed with bidistilled water (2×3 ml). The organic phase was evaporated to dryness, giving the title compound as a yellow syrup (70%, 2.1 mg); $[\alpha]_D^{20} = -33.0$ (*c* 0.10, $CHCl_3$); MALDI-TOF (DBH, positive mode) (*m*/*z*): 903.20 $[M+Na]^+$, 919.18 $[M+K]^+$; ^{1}H NMR ($CDCl_3$): δ 5.39 (vd, 1H, H-4, $J_{3,4} = 3.8$, $J_{4,5} \cong 1.0$ Hz), 5.24–5.20 (m, 3H, H-4′, H-4″, H-1″, $J_{4',5'} = 0.8$ Hz), 5.16 (dd, 1H, H-2″, $J_{1'',2''} = 3.6$, $J_{2'',3''} = 10.4$ Hz), 5.04 (dd, 1H, H-2, $J_{1,2} = 7.9$, $J_{2,3} = 9.9$ Hz), 4.90 (dd, 2H, H-3′, H-3″, $J_{2',3'} = 10.4$, $J_{3',4'} = 3.8$, $J_{2'',3''} = 10.4$, $J_{3'',4''} = 3.8$ Hz), 4.53 (d, 1H, H-1′, $J_{1',2'} = 7.6$ Hz), 4.27 (d, 1H, H-1, $J_{1,2} = 7.9$ Hz), 4.23 (m, 1H, H-5″), 4.15 (dd, 1H, H-6a′, $J_{5',6a'} = 6.1$, $J_{6a',6b'} = 11.2$ Hz), 4.10–3.99 (m, 3H, H-6a, H-6b, H-6b′, $J_{5,6a} = 5.6$, $J_{6a,6b} = 11.4$, $J_{5',6b'} = 6.9$ Hz), 3.86 (dd, 1H, H-3, $J_{2,3} = 9.9$, $J_{3,4} = 3.8$ Hz), 3.78–3.72 (m, 3H, H-2′, H-5′, H-5), 3.42 (s, 3H, OCH_3), 2.08, 2.07, 2.06, 2.02, 2.01, 2.00, 1.90, 1.90, 1.89 (9×s, each 3H, CH_3COO), 1.08 (d, 3H, H-6″, $J_{5'',6''} = 6.4$); ^{13}C NMR ($CDCl_3$): δ 102.47 (C-1), 101.93 (C-1′), 96.31 (C-1″), 76.12 (C-3), 74.14 (C-3′/C-3″), 72.92, 72.17, 70.99, 70.94 (C-2′, C-5, C-5′ or C-2, where C-2 is either 70.99 or 70.94), 71.69 (C-4′/C-4″), 70.56 (C-4), 69.06 (C-3′/C-3″), 67.65 (C-2″), 67.27 (C-4′/C-4″), 65.38 (C-5″), 61.98 (C-6), 61.25 (C-6′), 57.28 (OCH_3), 21.29, 21.12, 21.01 (each CH_3COO), 16.01 (C-6″).

4.6. 1,2,3,5,6-Penta-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl]-D-mannitol 26

4-*O*-(β-D-Galactopyranosyl]-D-mannose **16** (5.00 mg, 0.015 mmol) was dissolved in bidistilled water (100 μl) and treated with aqueous $NaBH_4$ (10 μg/μl, 0.439 mmol, 1.66 ml). The mixture was stirred for 2 h at room temperature, acidified with concentrated CH_3COOH (until pH ≈ 3) and 1 M HCl (300 μl), frozen and lyophilised, then codistilled with MeOH (5×5 ml). The subsequent residue was acetylated by incubation with acetic anhydride (1 ml) and pyridine (200 μl) for 2 h at 80°C. The reaction mixture was evaporated to dryness by flushing with nitrogen gas, and the resulting residue was dissolved in dichloromethane (2 ml) and washed with bidistilled water (2×3 ml). The organic phase was evaporated to dryness, giving the title compound as a yellow oil (7.3 mg, 69%); $[\alpha]_D^{20} = -11.4$ (*c* 0.36, $CHCl_3$); MALDI-TOF (DHB, positive mode) (*m*/*z*): 745.35 $[M+Na]^+$, 761.30 $[M+K]^+$; ^{1}H-NMR ($CDCl_3$): δ 5.31 (m, 1H, H-3), 5.29 (m, 1H, H-4′), 5.25 (m, 1H, H-2), 5.12 (dd, 1H, H-2′, $J_{1',2'} = 7.9$, $J_{2',3'} = 10.4$ Hz), 4.96 (m, 1H, H-5), 4.92 (dd, 1H, H-3′, $J_{2',3'} = 10.4$, $J_{3',4'} = 3.6$ Hz), 4.56 (d, 1H, H-1′, $J_{1',2'} = 7.9$ Hz), 4.50 (dd, 1H, H-6a, $J_{5,6a} = 2.6$, $J_{6a,6b} = 12.5$ Hz), 4.44 (dd, 1H, H-1a, $J_{1a,1b} = 12.2$, $J_{1a,2} = 2.3$ Hz), 4.13 (dd, 1H, H-1b, $J_{1a,1b} = 12.2$, $J_{1b,2} = 7.1$ Hz), 4.11 (m, 1H, H-4), 4.05 (m, 2H, H-6a′, H-6b′), 3.95 (dd, 1H, H-6b, $J_{5,6b} = 5.1$, $J_{6a,6b} = 12.5$ Hz), 3.86 (m, 1H, H-5′), 2.14, 2.09, 2.02, 2.01, 2.00, 1.99, 1.98, 1.96, 1.91 (9×s, each 3H, CH_3COO); ^{13}C-NMR ($CDCl_3$): δ 101.15 (C-1′), 76.00 (C-4), 72.70 (C-2), 71.63, 71.36 (C-3′, C-5′), 70.22, 70.02 (C-3, C-5), 69.22 (C-2′), 66.80 (C-4′), 63.20 (C-1), 62.19, 62.15 (C-6′, C-6), 22.25–20.65 (CH_3COO).

4.7. 1,2,3,5,6-Penta-*O*-acetyl-4-*O*-[3,4,6-tri-*O*-acetyl-2-*O*-(2,3,4-tri-*O*-acetyl-α-L-fuco-pyranosyl)-β-D-galactopyranosyl]-D-mannitol 28

Five batches of the following composition were simultaneously prepared: The acceptor 4-*O*-(β-D-galactopyranosyl]-D-mannose **16** (200 μg, 25μg/μl, 0.585 μmol) was added to a solution of GDP-L-Fuc (450 μg, 0.738 μmol), $MnCl_2$ (100 mM, 12 μl), NaN_3 (10 mg/ml, 2.4 μl), Tris–HCl buffer (pH 7.6, 6.6 μl), calf intestine alkaline phosphatase (EC 3.1.3.1, Boehringer-Mannheim, 185 mU/μl, 2 μl), and *H. pomatia* albumen gland sediment (80 μl), and stirred at 28°C for 18 h. The reaction was terminated by dilution with bidistilled water (800 μl) and centrifugation at 14000 rpm for 10 min. The supernatant was removed, and the pellet washed with 500 μl bidistilled water and centrifuged at 14000 rpm for 10 min, twice. The combined supernatants were treated with mixed bed anion exchange resin, which was removed and washed (3×300 μl). The combined aqueous phases were lyophilised, and treated with solutions of GDP-L-Fuc (225 μg, 0.369 μmol), 6 μl of $MnCl_2$ (100 mM), 1.2 μl of NaN_3 (10 mg/ml), 3.3 μl of Tris–HCl buffer (pH 7.6), 1 μl of calf intestine

alkaline phosphatase (185 mU/μl), and 40 μl of albumen gland sediment. The reaction mixture was incubated at 28°C for 18 h with stirring, before termination and work-up exactly as described above. The combined aqueous fractions were lyophilised, diluted to 500 μl and purified by semi-preparative HPAEC-PAD, then dissolved in bidistilled water (20 μl) and stirred with aqueous $NaBH_4$ (10 μg/μl, 0.088 mmol, 332 μl) for 2 h at room temperature, then acidified with concentrated CH_3COOH (until pH ≈ 3), frozen and lyophilised, then codistilled with MeOH (5×5 ml). Treatment of the product with acetic anhydride (1 ml) and pyridine (200 μl), with heating to 80°C for 2 h, resulted in a yellow oil which was dissolved in dichloromethane (2 ml) and washed with bidistilled water (2×3 ml). The organic phase was evaporated to dryness, giving the title compound as a pale yellow glass (66%, 1.8 mg); $[\alpha]_D^{20} = -36.7$ (*c* 0.09, $CHCl_3$); MALDI-TOF (DBH, positive mode) (*m*/*z*): 975.27 $[M+Na]^+$, 991.21 $[M+K]^+$; ^1H NMR ($CDCl_3$): δ 5.39 (dd, 1H, H-3, $J_{2,3}=5.1$, $J_{3,4}=6.6$ Hz), 5.37 (d, 1H, H-1″, $J_{1'',2''}=4.1$ Hz), 5.25 (dd, 1H, H-4″, $J_{3'',4''}=3.4$, $J_{4'',5''}=1.0$ Hz), 5.21–5.17 (m, 3H, H-2, H-4′, H-3″, $J_{4',5'}=1.0$ Hz), 5.08 (m, 1H, H-5), 4.94–4.91 (m, 2H, H-3′, H-2″, $J_{2',3'}=9.9$, $J_{1'',2''}=4.1$, $J_{2'',3''}=9.7$ Hz), 4.60 (dd, 1H, H-6a, $J_{5,6a}=6.6$, $J_{6a,6b}=12.5$ Hz), 4.58 (d, 1H, H-1′, $J_{1',2'}=7.6$ Hz), 4.44 (m, 1H, H-5″), 4.36 (dd, 1H, H-1a, $J_{1a,1b}=12.2$, $J_{1a,2}=2.3$ Hz), 4.28 (dd, 1H, H-4, $J_{3,4}=6.6$, $J_{4,5}=2.3$ Hz), 4.10 (dd, 1H, H-6b, $J_{5,6b}=6.1$, $J_{6a,6b}=12.5$ Hz), 4.08 (dd, 1H, H-1b, $J_{1b,2}=6.1$, $J_{1a,1b}=12.2$ Hz), 4.02 (m, 2H, 2×H-6′), 3.89 (dd, 1H, H-2′, $J_{1',2'}=7.6$, $J_{2',3'}=9.9$ Hz), 3.82 (m, 1H, H-5′, $J_{5',6'}=6.9$ Hz), 2.09, 2.07, 2.03, 2.02, 2.01, 1.99, 1.98, 1.97, 1.93, 1.92, 1.89 (11×s, each 3H, CH_3COO), 1.18 (d, 3H, 3×H-6″, $J_{5'',6''}=6.4$ Hz); ^{13}C NMR ($CDCl_3$): δ 171.02, 170.79, 169.97 (CH_3COO), 100.06 (C-1′), 95.97 (C-1″), 74.28 (C-4), 73.94 (C-2″), 71.60, 71.60, 71.55(C-2′, C-3″, C-4″), 71.32 (C-5′), 70.15, 70.15 (C-3, C-5), 68.50 (C-3′), 67.44 (C-2), 67.33 (C-4′), 65.40 (C-5″), 62.46 (C-1), 62.30 (C-6), 61.56 (C-6′), 21.28, 21.11, 21.06, 21.02, 21.01, 20.96, 20.94, 20.94, 20.93, 20.87, 20.75 (CH_3COO), 16.28 (C-6″).

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