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Synthesis, Modeling and Binding Affinity of an Ester Analogue of the Terminal Trisaccharide of the Tumor-Associated Antigen Globo-H[1]

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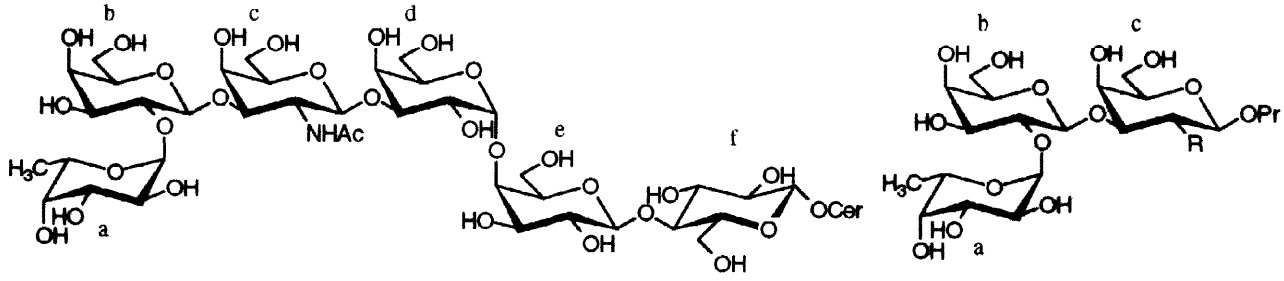
Abstract: The synthesis of the trisaccharide α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Gal*p*2Ac*O*Pr (**3**), mimicking the globo-H terminal trisaccharide unit (**2**), is described. The conformational properties of **3** were investigated with the aid of molecular mechanics energy minimizations, molecular dynamics simulations, and ^{1}H-NMR spectroscopic analysis and resulted in strict analogy with those of **2**. Nevertheless, analysis of MBr1 binding to these compounds indicate that substitution of the acetamido group at C-2 with the ester group lowers the affinity, thus suggesting that the amide hydrogen atom of **2** is involved in intermolecular interactions with the MBr1 antibody.

INTRODUCTION

The hexasaccharidic mojety of the glycosphingolipid globo-H antigen (**1**), recognised by the antibody MBr1, is overexpressed by a high percentage of human breast, ovary and lung carcinomas.[2-4]



1

2 R= NHAc
3 R= OAc

In order to obtain information on the MBr1-defined epitope, some overlapping di-, tri-, and tetrasaccharide sequences of **1** were synthesized and submitted to modeling and affinity studies.[5-9] In particular, the terminal *a*-

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b-c fragment, the trisaccharide α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc*O*Pr (**2**), which is the shortest sequence still retaining activity, was shown to be able to inhibit MBr1 binding to the target tumor cells (line MCF7) in a specific and dose dependent manner.[7]

Due to the importance of these kinds of tumor associated antigens, which could be clinically useful in generating active immunity against cancer, it would be of interest to ascertain whether modified analogues of **2** could present comparable or even enhanced biological activity. So, we planned to synthesize compounds mimicking the antigenic determinant, but structurally simpler, and in particular we chose as the first target the trisaccharide **3**, in which a 2-*O*-acetyl-β-D-galactose moiety replaces the 2-deoxy-2-acetamido-β-D-galactose unit *c*. In fact compound **3** should maintain the biological properties of **2** owing to the strict similarity of their structures and, in principle, of their conformational properties. Thus, in this paper we report on the synthesis of compound **3** and on its modeling and affinity studies made in comparison with trisaccharide **2**.

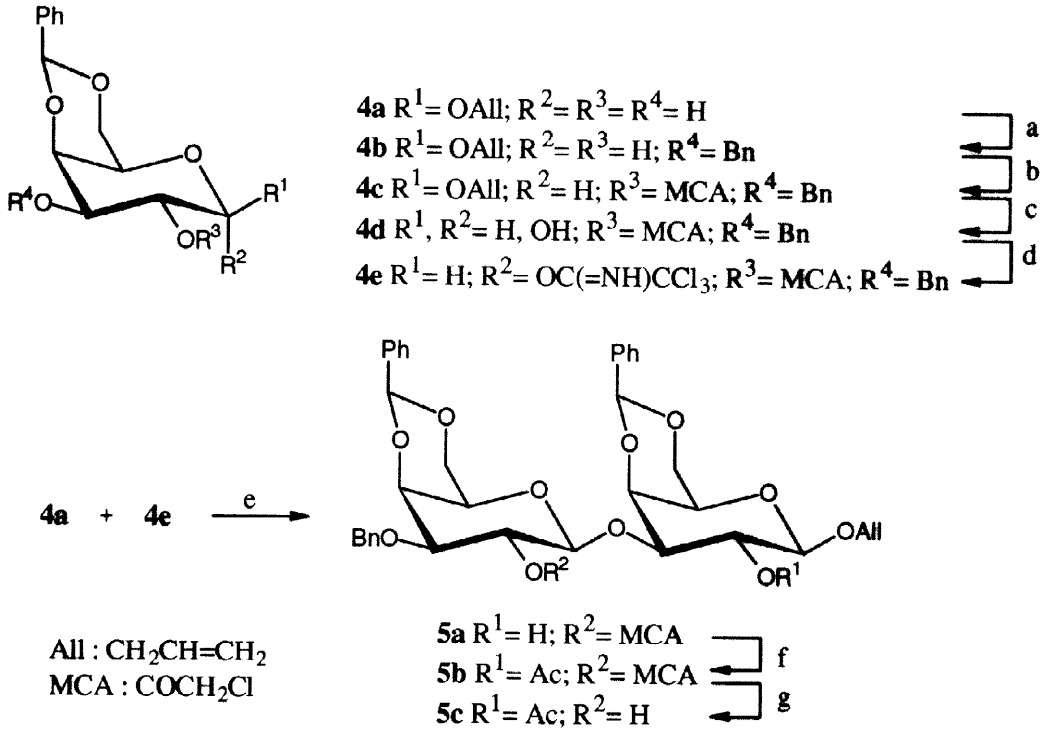
RESULTS AND DISCUSSION

The key intermediate in the synthesis of trisaccharide **3** was disaccharide **5c** which was constructed from properly protected monomers both deriving from the same sugar moiety, allyl 4,6-*O*-benzylidene-β-D-galactopyranoside **4a**,[10] a versatile building block for this synthetic approach. Compound **4a** furnished both the donor and the acceptor for the first coupling reaction: as acceptor it was directly employed without any other transformation, thus exploiting the generally observed higher reactivity of the 3-hydroxy group in the glycosidation step,[10,11] while as donor it was transformed into the trichloroacetimidate **4e**. The choice of the donor **4e** was made on the basis of the following requirements: i) a sterically demanding β-stereocontrolling group should be placed at the 2-position to ensure the formation of the β-anomer only; ii) after disaccharide construction, this β-orienting group should be selectively removed with respect to the *O*-acetyl group in the 2-position to allow the subsequent α-fucosylation. These two requirements were satisfied by means of the labile monochloroacetyl (MCA) group.

Thus, known allyl 4,6-*O*-benzylidene-β-D-galactopyranoside **4a** (scheme 1) was first selectively benzylated at the 3-position by refluxing in benzene in the presence of dibutyltin oxide, followed by treatment with benzyl bromide to yield **4b** which was protected at the 2-position with the MCA-group through the use of 3 eq. of $(ClCH_2CO)_2O$ in pyridine/dichloromethane (1:5, v/v)[12] to give the derivative **4c**.

Then, **4c** was converted into 1-*O*-unprotected **4d** by smooth isomerization of the allyloxy group with the bis (methyldiphenylphosphine)cyclooctadiene-iridium(I) hexafluorophosphate/hydrogen catalyst system,[13] followed by hydrolysis of the enol ether accomplished in neutral conditions by the use of N-bromosuccinimide and water in THF.[14] The overall mild system of anomeric deprotection did not seem to affect the sensitive monochloroacetyl group. Finally, by treatment of **4d** with trichloroacetonitrile and 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU), the desired α-trichloroacetimidate donor **4e** was obtained.

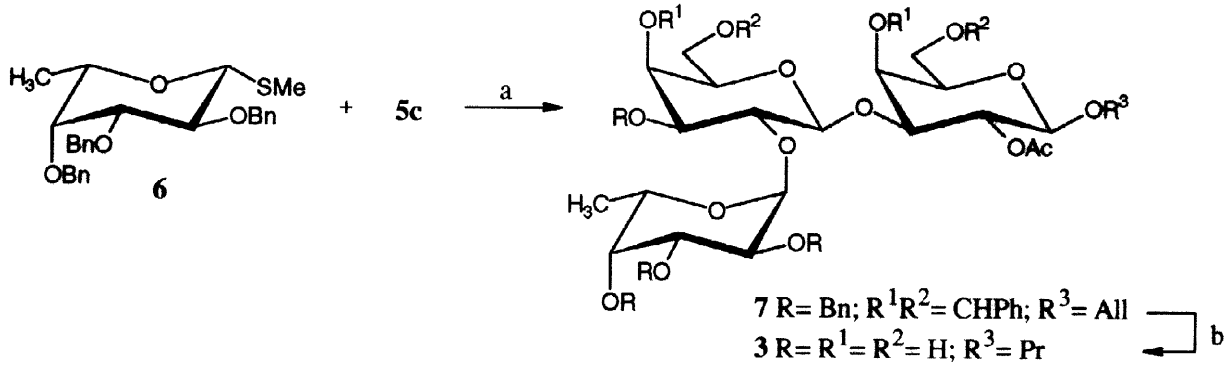
As a first step in the construction of the target trisaccharide, imidate **4e** (1.2 eq.) was condensed with acceptor **4a** (1 eq.) in dichloromethane at room temperature using trimethylsilyltriflate. These glycosylation conditions gave the β-(1→3) connected disaccharide **5a** in acceptable yield (50%), despite the lability of the glycosyl donor. The β-configuration of the glycosidic bond was demonstrated by the characteristic 8.0 Hz value of the trans-diaxal $J_{1',2'}$ coupling constant; at the same time the downfield shift of H-2, after **5a** acetylation to **5b**, confirmed the expected 1→3 linkage between the two monomers.



a) Bu_2SnO, benzene, reflux; then Bu_4NI, BnBr, reflux (62%); b) $(ClCH_2CO)_2O$, CH_2Cl_2/Py, 0°C (90%); c) $[Ir(cod)(PMePh_2)_2]PF_6$ cat., H_2, THF; then NBS, H_2O, THF (77%); d) Cl_3CCN, DBU, CH_2Cl_2 (54%); e) TMSOTf, CH_2Cl_2 (50%); f) AcCl, DMAP, Py, CH_2Cl_2 (98%); g) NH_2NH_2-CH_3COOH, AcOEt/MeOH (85%).

Scheme 1

After quantitative acetylation of **5a** with acetyl chloride in pyridine-dichloromethane, selective removal of the 2'-*O*-monochloroacetyl group to obtain **5c** was accomplished by means of hydrazine acetate (3 eq.) in methanol-ethyl acetate (1:1, v/v).[15]



a) NIS, TfOH, CH_2Cl_2 (76%); b) $Pd(OH)_2$ 20%, MeOH (quant.).

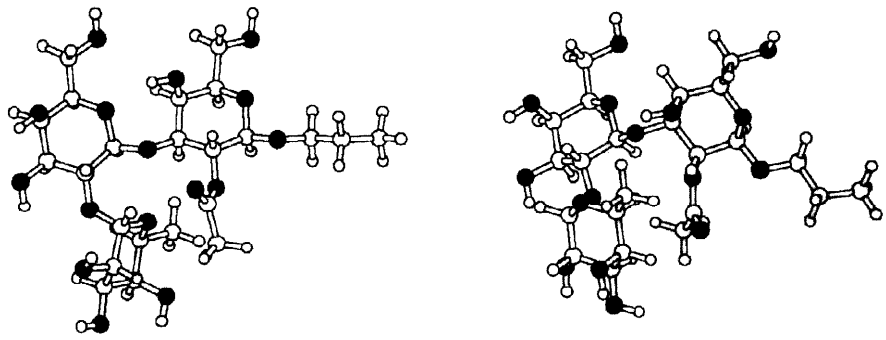
Scheme 2

Fucosylation of **5c** (scheme 2), carried out using 3 eq. of methyl 2,3,4-tri-*O*-benzyl-1-thio-β-L-fucopyranoside **6**,[16] N-iodosuccinimide (NIS) and catalytic triflic acid as promoter,[17] showed a good diastereoselectivity and gave, after chromatography, pure trisaccharide **7** in quite good yield (76%). The α-anomeric configuration of the newly formed glycosidic bond was confirmed by the value of the coupling constant of the H-1'' anomeric proton of **7** ($J_{1'',2''}$ = 4.0 Hz).

Compound **7** was finally deprotected by catalytic hydrogenolysis using Pearlman's catalyst in MeOH to afford the desired trisaccharide **3**. The coupling constants between the anomeric protons and the corresponding vicinal H-atom ($J_{1,2}$ = 8.0 Hz; $J_{1',2'}$ = 8.0 Hz; $J_{1'',2''}$ = 4.0 Hz) once more confirmed the anomeric configurations.

Table 1. Data on the Minimum Energy Conformations of Trisaccharide **3** as Calculated by Molecular Mechanics.

Conformation	φ_1, ψ_1	φ_2, ψ_2	E_{rel} (kJ/mol)	Equilibrium percentage
3A	23, -55	29, -54	0.00	73.5
3B	36, 28	38, 28	2.59	25.8
3C	22, -54	65, 69	13.60	0.3
3D	25, 176	49, 46	14.77	0.2
3E	32, 30	65, 64	15.36	0.1
3F	22, 174	23, -53	16.95	0.1



3A **3B**

Fig. 1. Three-dimensional plots of the most populated conformers of trisaccharide **3**.

The conformational behaviour of trisaccharide **3** was investigated with a molecular mechanics approach followed by molecular dynamics simulations. Through a random search, that considered the four single bonds at the glycosidic linkages (φ_1, ψ_1, φ_2, ψ_2) as degrees of freedom, several minima of the conformational space of **3** were located. For each conformer the orientation of the CH_2OH and OH groups was optimized to have, for each combination of φ and ψ, the lowest energy structure. Table 1 reports the data of conformers in a range of 5

kJ/mol above the global minimum and fig. 1 the three-dimensional plots of the two significantly populated conformers. From a comparison of the data in table 1 with the corresponding data already reported for **2**,[6] the strict analogy of **3** with **2** is evident.

Molecular dynamics (MD) simulations of compound **3** were performed starting from the two minima **3A** and **3B**, consisting in a pre-equilibration period (20 ps) followed by 80 ps data collection periods. The energy barrier between **3A** and **3B** was high enough that no transition between the two conformers could be observed in the course of the simulations. For each MD simulation the averaged values of the short inter-residue H-H distances were calculated and are reported in table 2. Though these distances were obtained *in vacuum*, a comparison of them with the corresponding distances obtained from proper experiments relying on ^{1}H-NMR spectroscopy should allow the true balance between the two conformers **3A** and **3B** in solution to be determined.

Complete assignment of the ^{1}H-NMR spectrum is a prerequisite to gain insight into the solution conformation. The ^{1}H-NMR signals were assigned by standard methods that rely on correlation through chemical bonds or through space and are reported in the experimental section while table 2 reports the inter-residue contacts obtained from phase-sensitive NOESY experiments together with the derived H-H distances referenced to an intra-residue distance for calibration. The NOESY results for **3** are in close agreement with the distances calculated in the simulation from **3B** and in contrast with those calculated from **3A**. In fact, the strong H5''/H2 cross peak accompanied by the weaker H6''/H2 and H3''/Ac cross peaks are diagnostic of a precise orientation of the fucose residue with respect to the 2-*O*-acetylgalactose residue, the same orientation already found for **2**.[6] A ROESY experiment was also performed, which confirmed the couplings detected by the NOESY technique and evidenced a further weak peak due to H1'/Ac cross coupling.

Thus, in all respects, trisaccharide **3** shows the same conformational behaviour already shown for its reference compound **2**.

Table 2. Inter-Residue Contacts of Compound **3** Derived from Molecular Dynamics Calculations and Phase Sensitive NOESY Experiments.

	Calcd distance (Å) in the MD simulations		NOESY cross peak volume	Calcd distance (Å) from NOESY
	3A	**3B**		
H1'/H3''[a]	2.74	2.67	1	2.70
H1''/H2'	2.28	2.43	2.475	2.32
H5''/H1'	3.04	3.69	[b]	
H5''/H2	6.17	2.65	1.965	2.41
$H_3$6''/H2	5.82	3.73	0.529	3.21
H1'/H3	2.29	2.43	1.889	2.43
H3''/Ac	6.12	3.52	0.606	3.14
H1'/Ac	6.20	3.63	[c]	

[a] Intra-residue contact used for calibration

[b] Not detectable due to signal overlapping

[c] Weak cross peak detected by ROESY

Biological characterisation indicated that trisaccharide **3** is able to inhibit MBr1 binding to the relevant target cell MCF7 in a dose-dependent manner with an IC_{50} 5-fold higher than that of the globo-H terminal trisaccharide unit **2**. These data suggest that, besides the *d* galactose residue,[8] also the 2-acetamido group present in the *c* galactose moiety should be included in the MBr1 recognized epitope and is critical in determining the binding affinity.

As modeling of compound **2** indicated[6] that the amide hydrogen atom is not involved in any intramolecular hydrogen bond and protrudes from the molecule surface, it probably forms an intermolecular hydrogen bond in the [**2**-MBr1] complex. Thus, the lower affinity of **3** towards the MBr1 antibody could be ascribed to the absence of this stabilizing interaction in the [**3**-MBr1] complex.

EXPERIMENTAL SECTION

General: Uncorrected melting points were determined on a Büchi apparatus. Optical rotations were determined on a Perkin-Elmer 241 polarimeter in a 1 dm cell at 20°C. Solvents were purified and dried in the usual way. All reactions were monitored by TLC on Silica Gel 60 F-254 plates (Merck) with detection by spraying with 50% H_2SO_4 solution and heating at 110°C. Flash column chromatography was performed on Silica Gel 60 (230-400 mesh, Merck). All evaporations were carried out under reduced pressure at 40°C. Mass experiments were performed as described by Colombo et al.[18]

NMR Experiments: All NMR spectra were recorded at 303 K with a Bruker AM-500 spectrometer equipped with an Aspect-3000 computer, a process controller, and an array processor in $CDCl_3$ solutions unless otherwise noted; chemical shifts of ^{1}H-NMR spectra are reported as δ (ppm) relative to tetramethylsilane as internal standard except those of compound **3** which were referenced to HDO (δ = 4.55). Compound **3** was lyophilized in 99.8% D_2O (Merck), and dried under vacuum before dissolving in 99.96% D_2O (Aldrich) under N_2 (0.05M soln); assignments of **3** are given by a combination of 1D and 2D COSY,[19] NOESY,[20] and ROESY[21] experiments. NOESY spectra: TPPI mode using the pulse sequence described in ref. 20; 512 t_1 increments (32 scans for each), spectral size in time domain 2K and spectral width 2824 Hz; zero filling to 1K in the t_1 dimension before Fourier transformation; the linearity in the build up of the NOE intensity was tested by performing the experiments at different mixing times (200-1000 ms), and no significant differences were found in the calculated distance obtained from the different data set. ROESY spectra: phase sensitive mode, pulse sequence 90-t_1-SL-FID where SL stands for a continuous spin-lock pulse of 200 ms at a field strength corresponding to a 90-deg-pulse width between 80 and 90 μs; carrier frequency placed at the left side of the spectrum at 6.5 ppm to minimize Hartmann-Hahn effects;[21] spectral width 6756 Hz; 512 t_1 increments (32 scans each), spectral size in the time domain 2K; zero filling to 1K in the t_1 dimension before Fourier transformation. All 2D spectra were weighted with the square sine-bell function shifted by π/2 in both dimensions. H-H distances were calculated from equation $r_i = r_j\ (\ V_i\ /V_j)^{-1/6}$ where V_i and V_j are the cross-peak volumes from unknown and calibration distances (r_i , r_j), respectively, measured by standard Bruker 2D integration routine from 2D-NOESY spectra. The cross-peak volumes for the interaction H/Me was divided by 1.5 before the application of the above equation according to Macura and Ernst[22] who showed that the NOESY cross-peak intensity is proportional to $N_i\,N_j\,/\,(N_i + N_j\,)$ where N_i and N_j are the number of i and j protons.

MM and MD Calculations: Molecular-mechanics energy minimizations and molecular-dynamics simulations were performed with the HyperChem™ software from Hypercube, Inc according to Toma et al.[9] The

glycosidic angles of compound **3** were defined as ϕ_1 H-1''– C-1''– O – C-2', ψ_1 C-1''– O – C-2'– H-2', ϕ_2 H-1'– C-1'– O – C-3, ψ_2 C-1'– O – C-3 – H-3.

Allyl 3-O-benzyl-4,6-O-benzylidene-β-D-galactopyranoside (**4b**): A mixture of allyl 4,6-benzylidene-β-D-galactopyranoside[10] (**4a**) (2.30 g, 7.46 mmol) and dibutyltin oxide (2.79 g, 11.19 mmol) in dry benzene (250 ml) was heated at reflux temperature with azeotropic removal of the generated water for 17 h. After concentration of the mixture to 50 ml, tetrabutylammonium iodide (2.76 g, 7.46 mmol) and benzyl bromide (2.66 ml, 22.38 mmol) were added, and the mixture was refluxed for additional 4.5 h. After removal of the solvent under reduced pressure, the residue was twice crystallized from diisopropyl ether/dichloromethane (9:1) to remove by-products, then purified by flash chromatography (dichloromethane/ethyl acetate 9:1) to give 1.84 g (62%) of **4b**. White solid. mp. 182°C dec. $[\alpha]_D$= +31 (*c* 1, $CHCl_3$). ^{1}H-NMR: 7.52-7.22 (*m*, 10H, arom. H); 5.95 (*m*, 1H, OCH_2CH=CH_2); 5.44 (*s*, 1H, C*H*Ph); 5.30, 5.19 (2 *m*, 2H, OCH_2CH=CH_2); 4.76, 4.72 (2 *d*, 2H, J 10.5Hz, OCH_2Ph); 4.41 (*m*, 1H, OCH_aH-); 4.34 (*d*, 1H, $J_{1,2}$ 8Hz, H-1); 4.30 (*dd*, 1H, $J_{5,6a}$ 1.5Hz, $J_{6a,6b}$ 12Hz, H-6a); 4.12 (*m*, 1H, OCH_bH-); 4.11 (br *d*, 1H, $J_{3,4}$ 3.5Hz, H-4); 4.04-3.98 (*m*, 2H, H-6b and H-2); 3.48 (*dd*, 1H, $J_{2,3}$ 10Hz, H-3); 3.33 (br *s*, 1H, H-5); 2.40 (*d*, 1H, $J_{OH,2}$ 2Hz, OH). Anal. Calcd for $C_{23}H_{26}O_6$: C, 69.33; H,6.58. Found: C,69.61; H, 6.63.

Allyl 3-O-benzyl-4,6-O-benzylidene-2-O-chloroacetyl-β-D-galactopyranoside (**4c**): Chloroacetic anhydride (2.02 g, 11.82 mmol) was added at 0°C to a solution of **4b** (1.57 g, 3.94 mmol) in CH_2Cl_2 (15 ml) and pyridine (3 ml). After stirring at 0°C for 1 h ice-cold water (2 ml) was added. After dilution with CH_2Cl_2 (60 ml), the mixture was washed successively with water (1 x 80 ml), sat. $NaHCO_3$ soln. (1 x 80ml), and water (2 x 80 ml). The organic layer was dried (Na_2SO_4), concentrated *in vacuo*, and the resulting residue was purified by flash chromatography (petroleum ether/ethyl acetate 6:4) to give **4c** (1.70 g; 90%). White solid. mp. 137°C. $[\alpha]_D$= +38 (*c* 1, $CHCl_3$). ^{1}H-NMR: 7.52-7.24 (*m*, 10H, arom); 5.84 (*m*, 1H, OCH_2CH=CH_2); 5.47 (*s*, 1H, C*H*Ph); 5.40 (*dd*, 1H, $J_{1,2}$ 8Hz, $J_{2,3}$ 10Hz, H-2); 5.23, 5.14 (2 *m*, 2H, OCH_2CH=CH_2); 4.69, 4.60 (2 *d*, 2H, J 12Hz, OCH_2Ph); 4.49 (*d*, 1H, H-1); 4.34 (*m*, 1H, OCH_aH-); 4.31 (*dd*, 1H, $J_{5,6a}$ 1.5Hz, $J_{6a,6b}$ 12.5Hz, H-6a); 4.16 (br *d*, 1H, $J_{3,4}$ 3.5Hz, H-4); 4.07 (*m*, 1H, OCH_bH); 4.02 (*dd*, 1H, $J_{5,6b}$ 1.5Hz, H-6b); 4.00, 3.96 (2 *d*, 2H, J 15Hz, $OCOCH_2Cl$); 3.63 (*dd*, 1H, H-3); 3.35 (br *s*, 1H, H-5). Anal. Calcd for $C_{25}H_{27}ClO_7$: C, 63.22; H, 5.73. Found: C, 63.40; H, 5.99.

3-O-Benzyl-4,6-O-benzylidene-2-O-chloroacetyl-α,β-D-galactopyranose (**4d**): Bis(methyldiphenyl phosphine)cyclooctadiene-iridium(I) hexafluorophosphate (0.090 g) was suspended in dry tetrahydrofuran (20 ml) under Ar, and hydrogen was bubbled for 15 min through the mixture. The resulting clear solution was then added dropwise to a stirred solution of **4c** (1.70 g, 3.58 mmol) in dry tetrahydrofuran (40 ml) under Ar. After 1h, ^{1}H-NMR spectroscopy showed total consumption of the starting material. Additional tetrahydrofuran (180 ml), water (12 ml), and N-bromosuccinimide (0.96 g) were then added. After 10 min, the mixture was concentrated *in vacuo*. Dichloromethane was added (150 ml), and the solution was washed with saturated $NaHCO_3$ solution (2 x 100 ml). The organic layer was dried (Na_2SO_4), evaporated under reduced pressure, and the resulting residue purified by flash chromatography (petroleum ether/ethyl acetate 1:1) to yield **4d** (1.19 g, 77%) as 85:15 α/β mixture. Foam. ^{1}H-NMR: 7.60-7.20 (*m*, 10H, arom); 5.59 (*dd*, 0.85H, $J_{1,2}$ 3.5Hz, $J_{1,OH}$ 3Hz, H-1α); 5.47 (*s*, 0.85H, C*H*Ph); 5.36 (*dd*, 0.85H, $J_{2,3}$ 10.5Hz, H-2α); 5.25 (*dd*, 0.15H, $J_{1,2}$ 8Hz, $J_{2,3}$ 10Hz, H-2β); 4.72-4.66 (*m*, 2H, OCH_2Ph); 4.24-4.18 (*m*, 1.7H, H-4α and H-6aα); 4.17 (br *d*, 0.15H, $J_{3,4}$ 3.5Hz, H-4β); 4.06 (*dd*, 0.85H, $J_{3,4}$ 3.5Hz, H-3α); 4.08-3.98 (*m*, 2.85H, $OCOCH_2Cl$ and H-6bα); 3.86 (br *s*, 0.85H, H-5α); 3.65 (*dd*, 0.15H, H-3β); 2.93 (*m*, 1H, OH). Anal. Calcd for $C_{22}H_{23}ClO_7$: C, 60.76; H, 5.33. Found: C, 60.39; H, 5.02.

3-O-Benzyl-4,6-O-benzylidene-2-O-chloroacetyl-α-D-galactopyranosyl trichloroacetimidate (**4e**): To a solution of **4d** (0.72 g, 1.64 mmol) in CH_2Cl_2 (8 ml) trichloroacetonitrile (1.65 ml) and 1,8-diazabicyclo[5,4,0]undec-7-ene (0.049 ml) were added and the mixture was stirred for 1 h at room temperature. The solvent was then removed *in vacuo*, and the residue purified by flash-chromatography (petroleum ether/ethyl acetate 8:2) to yield **4e** (0.51 g, 54%). Foam. $[\alpha]_D$ = +92 (*c* 1, $CHCl_3$). ^{1}H-NMR: 8.56 (*s*, 1H, NH); 7.58-7.24 (*m*, 10H, arom); 6.67 (*d*, 1H, $J_{1,2}$ 3.5Hz, H-1); 5.57 (*dd*, 1H, $J_{2,3}$ 10.5Hz, H-2); 5.51 (*s*, 1H, C*H*Ph); 4.75, 4.69 (2 *d*, 2H, J 12Hz, OCH_2Ph); 4.33 (br *d*, 1H, $J_{3,4}$ 3.5Hz, H-4); 4.29 (*dd*, $J_{5,6a}$ 1.2Hz, $J_{6a,6b}$ 12.5Hz, H-6a); 4.13 (*dd*, 1H, H-3); 4.02 (*dd*, 1H, $J_{5,6b}$ 1.5Hz, H-6b); 3.92 (*s*, 2H, OCOCH_2Cl); 3.86 (br *s*, 1H, H-5). Anal. Calcd for $C_{24}H_{23}Cl_4NO_7$: C, 49.76; H, 4.00; N, 2.42. Found: C,49.55; H, 3.92; N, 2.38.

Allyl (3-O-benzyl-4,6-O-benzylidene-2-O-chloroacetyl-β-D-galactopyranosyl)-(1→3)-4,6-O-benzylidene-β-D-galactopyranoside (**5a**): A mixture of **4e** (0.50 g, 0.86 mmol), **4a** (0.22 g, 0.72 mmol) and powdered 4Å molecular sieves (0.58 g) in dry CH_2Cl_2 (10 ml) was stirred for 30 min at room temperature under Ar. Trimethylsilyl triflate in toluene (1.0 M, 0.13 ml) was then added, and the mixture was stirred for 40 min at the same temperature. After quenching with triethylamine (0.11 ml), the residue, obtained by filtering and evaporation, was purified by flash-chromatography (toluene-ethyl acetate 1:1) to give **5a** (0.26 g, 50%). White solid. mp. 260-263°C dec. $[\alpha]_D$ = +34 (*c* 1, CH_2Cl_2). ^{1}H-NMR (500 MHz, $CDCl_3$): 7.60-7.20 (*m*, 15H, arom); 5.93 (*m*, 1H, OCH$_2$C*H*=CH$_2$); 5.52, 5.46 (2 *s*, 2H, 2 C*H*Ph); 5.40 (*dd*, 1H, $J_{1',2'}$ 8Hz, $J_{2',3'}$ 10Hz, H-2'); 5.30, 5.19 (2 *m*, 2H, OCH_2CH=CH$_2$); 5.06 (*d*, 1H, H-1'); 4.68, 4.60 (2 *d*, 2H, J 12Hz, OCH_2Ph); 4.40 (*m*, 1H, OCH_aH-); 4.33 (*d*, 1H, $J_{1,2}$ 7.5Hz, H-1); 4.31-4.27 (*m*, 2H, H-6a and H-6a'); 4.27 (br *d*, 1H, $J_{3,4}$ 3.5Hz, H-4); 4.15 (br *d*, 1H, $J_{3',4'}$ 3.5Hz, H-4'); 4.12 (*m*, 1H, OCH_bH-); 4.05-3.99 (*m*, 2H, H-6b and H-6b'); 3.96 (*m*, 1H, H-2); 3.90 (*s*, 2H, OCOCH_2Cl); 3.84 (*dd*, 1H, $J_{2,3}$ 10Hz, H-3); 3.62 (*dd*, 1H, H-3'); 3.39, 3.36 (2 br *s*, 2H, H-5 and H-5'). MS : *m/z* 742 $[M+NH_4]^+$. Anal. Calcd for $C_{38}H_{41}ClO_{12}$: C, 62.94; H, 5.70. Found: C, 63.15; H, 5.92.

Allyl (3-O-benzyl-4,6-O-benzylidene-2-O-chloroacetyl-β-D-galactopyranosyl)-(1→3)-2-O-acetyl-4,6-O-benzylidene-β-D-galactopyranoside (**5b**): To a solution of **5a** (0.26 g, 0.36 mmol) in CH_2Cl_2 (15 ml), pyridine (0.29 ml) and 4-dimethylaminopyridine (0.006 g) were added. Acetyl chloride in CH_2Cl_2 (0.67 M, 2.15 ml) was then added at 0°C. The mixture was allowed to warm to room temperature, and stirred for 17h. Dichloromethane was added (70 ml), and the solution washed with water (1 x 150 ml), 10% $NaHCO_3$ sol. (1 x 150 ml), 0.1N HCl soln. (1 x 150 ml) and water (1 x 150 ml). The organic layer was dried (Na_2SO_4), concentrated *in vacuo*, and the residue purified by flash-chromatography (ethyl acetate/petroleum ether 7:3) to afford **5b** (0.27 g, 98%). White solid. mp. 204°C dec. $[\alpha]_D$ = +43 (*c* 1, $CHCl_3$). ^{1}H-NMR: 7.60-7.20 (*m*, 15H, arom); 5.82 (*m*, 1H, OCH$_2$C*H*=CH$_2$); 5.55, 5.45 (2 *s*, 2H, 2 C*H*Ph); 5.35 (*dd*, 1H, $J_{1',2'}$ 8.5Hz, $J_{2',3'}$ 10Hz, H-2'); 5.34 (*dd*, 1H, , $J_{1,2}$ 8Hz, $J_{2,3}$ 10.5Hz, H-2); 5.23, 5.13 (2 *m*, 2H, OCH_2CH=CH$_2$); 4.74 (*d*, 1H, H-1'); 4.67, 4.59 (2 *d*, 2H, J 12Hz, OCH_2Ph); 4.43 (*d*, 1H, H-1); 4.33 (br *d*, 1H, $J_{3,4}$ 3.5Hz, H-4); 4.35-4.25 (*m*, 3H, H-6a, H-6a'and OCH_aH-); 4.14 (br *d*, 1H, $J_{3',4'}$ 3.5Hz, H-4'); 4.07 (*m*, 1H, OCH_bH); 4.04-3.98 (*m*, 4H, H-6b, H-6b'and OCOCH_2Cl); 3.90 (*dd*, 1H, H-3); 3.57 (*dd*, 1H, H-3'); 3.38, 3.33 (2 br *s*, 2H, H-5 and H-5'); 2.06 (*s*, 3H, OCOCH_3). MS: *m/z* 784 $[M+NH_4]^+$. Anal. Calcd for $C_{40}H_{43}ClO_{13}$: C, 62.62; H, 5.65. Found: C, 62.90; H, 5.85.

Allyl (3-O-benzyl-4,6-O-benzylidene-β-D-galactopyranosyl)-(1→3)-2-O-acetyl-4,6-O-benzylidene-β-D-galactopyranoside (**5c**): Hydrazine acetate (0.094 g, 1.02 mmol) was added to a solution of **5b** (0.26 g, 0.34 mmol) in ethyl acetate-methanol 1:1 (6.5 ml). The mixture was stirred at room temperature for 1.25 h, and then evaporated. Purification by flash-chromatography (toluene/acetone 8:2) yield **5c** (0.20 g, 85%). White solid. mp. 243-246°C dec. $[\alpha]_D$ = +45 (*c* 1, $CHCl_3$). ^{1}H-NMR: 7.60-7.20 (*m*, 15H, arom); 5.85 (*m*, 1H, OCH$_2$C*H*=CH$_2$); 5.57, 5.43 (2*s*, 2H, 2 C*H*Ph); 5.41 (*dd*, 1H, $J_{1,2}$ 8Hz, $J_{2,3}$ 10.5Hz, H-2); 5.25, 5.15 (2 *m*, 2H, OCH_2CH=CH$_2$); 4.79, 4.73 (2 *d*, 2H, J 12Hz, OCH_2Ph); 4.51 (*d*, 1H, H-1); 4.38 (br *d*, 1H, $J_{3,4}$ 3.5Hz, H-4); 4.37 (*d*, 1H, $J_{1',2'}$

8Hz, H-1'); 4.38-4.21 (*m*, 3H, OC*H*$_a$H-, H-6a and H-6a'); 4.12 (*m*, 1H, OC*H*$_b$H-); 4.07 (br *d*, 1H, $J_{3',4'}$ 3.5Hz, H-4'); 4.05-3.97 (*m*, 3H, H-2', H-6b and H-6b'); 3.82 (*dd*, 1H, H-3); 3.41 (br *s*, 1H, H-5 or H-5'); 3.40 (*dd*, 1H, $J_{2',3'}$ 10Hz, H-3'); 3.30 (br *s*, 1H, H-5 or H-5'); 2.47 (br *d*, 1H, $J_{OH,2'}$ 2Hz, OH); 2.07 (*s*, 3H, OCOC*H*$_3$). MS: *m/z* 708 $[M+NH_4]^+$. Anal. Calcd for $C_{38}H_{42}O_{12}$: C, 66.08; H, 6.13. Found: C, 65.89; H, 6.01.

Allyl (2,3,4-tri-O-benzyl-α-L-fucopyranosyl)-(1→2)-(3-O-benzyl-4,6-O-benzylidene-β-D-galactopyranosyl)-(1→3)-2-O-acetyl-4,6-O-benzylidene-β-D-galactopyranoside (**7**): To a mixture of **5c** (0.20 g, 0.29 mmol), methyl 2,3,4-tri-*O*-benzyl-1-thio-β-L-fucopyranoside **6**[13] (0.40 g, 0.87 mmol) and powdered 4Å molecular sieves (0.40 g) in dichloromethane (4 ml) at 0°C under Ar, a solution of N-iodosuccinimide (0.20 g) and triflic acid (0.024 ml) in dichloromethane (12 ml) was added. After 1h, the mixture was neutralized with 5% aq. $NaHCO_3$ soln., and filtered over Celite. The filtrate was washed with 20% $Na_2S_2O_3$ soln. (1 x 20 ml), 5% $NaHCO_3$ soln. (1 x 20 ml), and water (1 x 20 ml), dried (Na_2SO_4), and evaporated. Purification by flash-chromatography (toluene/acetone 85:15) furnished the trisaccharide as a 9:1 α/β mixture. A further flash-chromatography (ethyl acetate/petroleum ether 1:1) allowed to obtain pure **7** (0.24 g, 76%). Amorphous solid. $[\alpha]_D$= -26 (*c* 1, CH_2Cl_2). ^{1}H-NMR: 7.60-7.00 (*m*, 30H, arom); 5.86 (*m*, 1H, $OCH_2CH{=}CH_2$); 5.54 (*d*, 1H, $J_{1'',2''}$ 4Hz, H-1''); 5.53, 5.39 (2 *s*, 2H, 2 C*H*Ph); 5.34 (*dd*, 1H, $J_{1,2}$ 7.5Hz, $J_{2,3}$ 10.5Hz, H-2); 5.25, 5.16 (2 *m*, 2H, $OCH_2CH{=}CH_2$); 4.82-4.43 (*m*, 8H, OCH_2Ph); 4.61 (*d*, 1H, $J_{1',2'}$ 7.5Hz, H-1'); 4.45 (*d*, 1H, H-1); 4.37 (*m*, 1H, OC*H*$_a$H-); 4.35 (br *d*, 1H, $J_{3,4}$ 3.5Hz, H-4); 4.27 (*m*, 2H, H-6a and H-6a'); 4.16 (*dd*, 1H, $J_{2',3'}$ 10Hz, H-2'); 4.14-4.07 (*m*, 3H, H-4', H-5'' and OC*H*$_b$H-); 4.02 (*m*, 2H, H-6b and H-6'b); 3.93 (*dd*, 1H, H-3); 3.89 (*dd*, 1H, $J_{2'',3''}$ 10Hz; H-2''); 3.82 (*dd*, 1H, $J_{3'',4''}$ 2.75Hz, H-3''); 3.67 (*dd*, 1H, $J_{3',4'}$ 3.5Hz, H-3'); 3.64 (br *d*, 1H, H-4''); 3.39, 3.34 (2 br *s*, 2H, H-5 and H-5'); 2.02 (*s*, 3H, OCOC*H*$_3$); 0.60 (*d*, 3H, $J_{5'',6''}$ 6.5Hz, H-6''). Anal. Calcd for $C_{65}H_{70}O_{16}$: C, 70.51; H, 6.37. Found: C, 70.30; H, 6.31.

Propyl (α-L-fucopyranosyl)-(1→2)-(β-D-galactopyranosyl)-(1→3)-2-O-acetyl-β-D-galactopyranoside (**3**): To a solution of **7** (0.24 g, 0.22 mmol) in MeOH (20 ml), 20% $Pd(OH)_2$ (0.14 g) was added, and the mixture stirred for 5h under H_2 (TLC monitoring: ethyl acetate/isopropanol/water 3:3:1). The mixture was filtered over Celite, the solvent evaporated, the residue dissolved in D_2O, and the solution lyophilized to give **3** (0.12 g, quant.). White solid. mp. 200°-210°C dec. $[\alpha]_D$= -53 (*c* 1, MeOH). ^{1}H-NMR (D_2O): 5.05 (*d*, 1H, $J_{1'',2''}$ 4Hz, H-1''); 4.78 (*dd*, 1H, $J_{1,2}$ 8Hz, $J_{2,3}$ 10Hz, H-2); 4.47 (*d*, 1H, $J_{1',2'}$ 8Hz, H-1'); 4.36 (*d*, 1H, H-1); 4.01 (*dd*, 1H, $J_{3,4}$ 3.5Hz, H-3); 4.01 (*q*, 1H, $J_{5'',6''}$ 6.5Hz, H-5''); 3.95 (br *d*, 1H, H-4); 3.72 (br *d*, 1H, $J_{3',4'}$ 3.5Hz, H-4'); 3.68 (*m*, 1H, OC*H*$_a$H-); 3.66 (*dd*, 1H, $J_{2',3'}$ 10Hz, H-3'); 3.64-3.55 (*m*, 5H, H-5, H-6a, H-6b, H-6'a and H-6'b); 3.59 (*dd*, 1H, $J_{2'',3''}$ 10.5Hz, H-2''); 3.54 (br *d*, 1H, $J_{3'',4''}$ 3.5Hz, H-4''); 3.49 (*dd*, 1H, $J_{5',6'a}$ 4.5Hz, $J_{5',6'b}$ 8Hz, H-5'); 3.47 (*dd*, 1H, H-3''); 3.44 (*dd*, 1H, H-2'); 3.36 (*m*, 1H, OC*H*$_b$H-); 2.02 (*s*, 3H, OCOC*H*$_3$); 1.36 (*m*, 2H, $OCH_2CH_2CH_3$); 1.04 (*d*, 3H, H-6''); 0.68 (*t*, 3H, $OCH_2CH_2CH_3$). ^{13}C-NMR (125.76 MHz, D_2O, dioxane as internal standard-68.9 ppm): 175.6; 104.1; 103.7; 101.7; 78.7; 78.6; 77.4; 76.0; 74.7; 74.6; 74.1; 71.8; 71.6; 71.5; 70.4;0 63.3; 63.1; 32.6; 24.5; 22.9; 17.5; 12.0. MS: *m/z* 590 $[M+NH_4]^+$. Anal. Calcd for $C_{23}H_{40}O_{16}$: C, 48.25; H, 7.04. Found: C, 48.51; H, 7.41.

Evaluation of the Biological Activity: The effect of **3** and **2** on MBr1 binding to the relevant target cells MCF7 was tested by indirect immunofluorescence assays on live cell suspensions. Cells were incubated with a mixture of 1nM MBr1 and serial dilutions of each oligosaccharide (from 800 to 0.1 μM) in phosphate-buffered saline (PBS) containing 0.03% bovine serum albumin (BSA) for 90 min on ice. After 3 washings in PBS/BSA MBr1 binding was detected by incubating cells with fluorescein-conjugated goat anti-mouse IgM (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) for 30 min on ice. The bound fluorescence was detected by cytofluorimetric analysis using FacScan (Beckton Dickinson). The amount of **3** required to induce 50%

inhibition of MBr1 binding to the target cells (IC_{50}) was 250 μM (mean of 3 experiments); the trisaccharide **2** confirmed its previously published competition ability[7] with an IC_{50} of 50 μM (mean of 3 experiments).

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