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Engineering of glucoside acceptors for the regioselective synthesis of β-(1→3)-disaccharides with glycosynthases

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ABSTRACT

Glycosynthase mutants obtained from *Thermotoga maritima* were able to catalyze the regioselective synthesis of aryl β-D-Gal*p*-(1→3)-β-D-Glc*p* and aryl β-D-Glc*p*-(1→3)-β-D-Glc*p* in high yields (up to 90 %) using aryl β-D-glucosides as acceptors. The need for an aglyconic aryl group was rationalized by molecular modeling calculations, which have emphasized a high stabilizing interaction of this group by stacking with W312 of the enzyme. Unfortunately, the deprotection of the aromatic group of the disaccharides was not possible without partial hydrolysis of the glycosidic bond. The replacement of aryl groups by benzyl ones could offer the opportunity to deprotect the anomeric position under very mild conditions. Assuming that benzyl acceptors could preserve the stabilizing stacking, benzyl β-D-glucoside firstly assayed as acceptor resulted in both poor yields and poor regioselectivity. Thus, we decided to undertake molecular modeling calculations in order to design which suitable substituted benzyl acceptors could be used. This study resulted in the choice of 2-biphenylmethyl β-D-glucopyranoside. This choice was validated experimentally, since the corresponding β-(1→3) disaccharide was obtained in good yields and with a high regioselectivity. At the same time, we have shown that phenyl 1-thio-β-D-glucopyranoside was also an excellent substrate leading to similar results as those obtained with the *O*-phenyl analogue. The NBS deprotection of the *S*-phenyl group afforded the corresponding disaccharide quantitatively.

1. Introduction

Carbohydrate ligands play an important role in a number of biological mechanisms. The understanding of their action and, consequently, their use as drugs depend on the availability of the oligosaccharides involved in the processes. The difficulties encountered in building a glycosidic bond regioselectively and stereoselectively are well known. Despite the considerable development of efficient methods in this field, the synthesis by means of conventional chemical methods suffers from the need for cumbersome protection–deprotection sequences. In order to avoid this disadvantage, enzymatic methods have been proposed in the last decade considering the regio- and stereo-selectivities usually induced by biocatalysts. This is the case for glycosyl transferases, which catalyze such a synthesis with both high yield and high selectivity. Unfortunately, the need for costly nucleotide donors greatly limits their application for large-scale production of saccharides. In another approach, the transfer activity exerted by 'retaining' glycosidases also provided an efficient alternative, since these enzymes accommodate much simpler and readily available donors. Problems encountered in this case were incomplete regioselectivity, which resulted in mixtures of regioisomers that were difficult to separate and in competition between the transglycosylation reaction and the hydrolysis of the substrate and of the product. In order to improve the properties of the glycosidases, two strategies were undertaken.

The first one, using directed mutagenesis was developed by Withers and co-workers with exoglycosidases[1–7] and by Planas and co-workers with endoglycosidases.[8–12] The mutant enzymes thus produced were called glycosynthases because they were devoid of hydrolytic activity but maintained a high transglycosylation ability. Thus, chemo-enzymatic synthesis of β-(1→3)-oligosaccharides was achieved using the 'glycosynthase methodology'.[13] We have also recently validated this approach with a β-D-glycosidase (TtβGly) from *Thermus thermophilus*,[14] an enzyme cloned and overexpressed in our laboratory.[15] Thus, E338G or E338S mutants allowed the synthesis of phenyl β-D-Glc*p*-(1→3)-β-D-Glc*p* and phenyl β-D-Gal*p*-(1→3)-β-D-Glc*p* in yields of up to 85% starting from α-glycosyl fluorides as donors and phenyl β-D-glucopyranoside ($\beta GlcC_6H_5$) as an acceptor (see Scheme 1).

The second strategy developed recently by us was based on directed evolution.[16,17] Our results indicated that some mutant enzymes had completely lost their hydrolytic activity while keeping the transglycosidase one. Such enzymes were called transglycosidases.

A disadvantage of the Ttβgly glycosidases is the need for a phenyl ring at the (+2) sub-site to ensure the correct positioning of the

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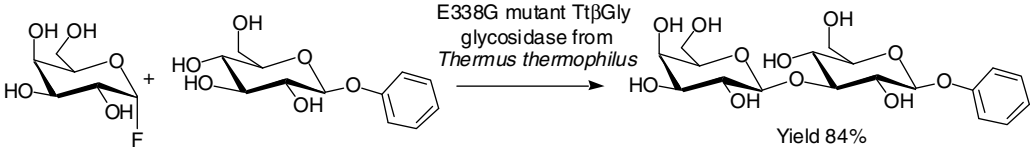


Scheme 1. Regioselective synthesis of phenyl β-D-Gal*p*-(1→3)-β-D-Glc*p* catalyzed by the glycosynthase E338G obtained by mutagenesis of TtβGly glycosidase from *Thermus thermophilus*.

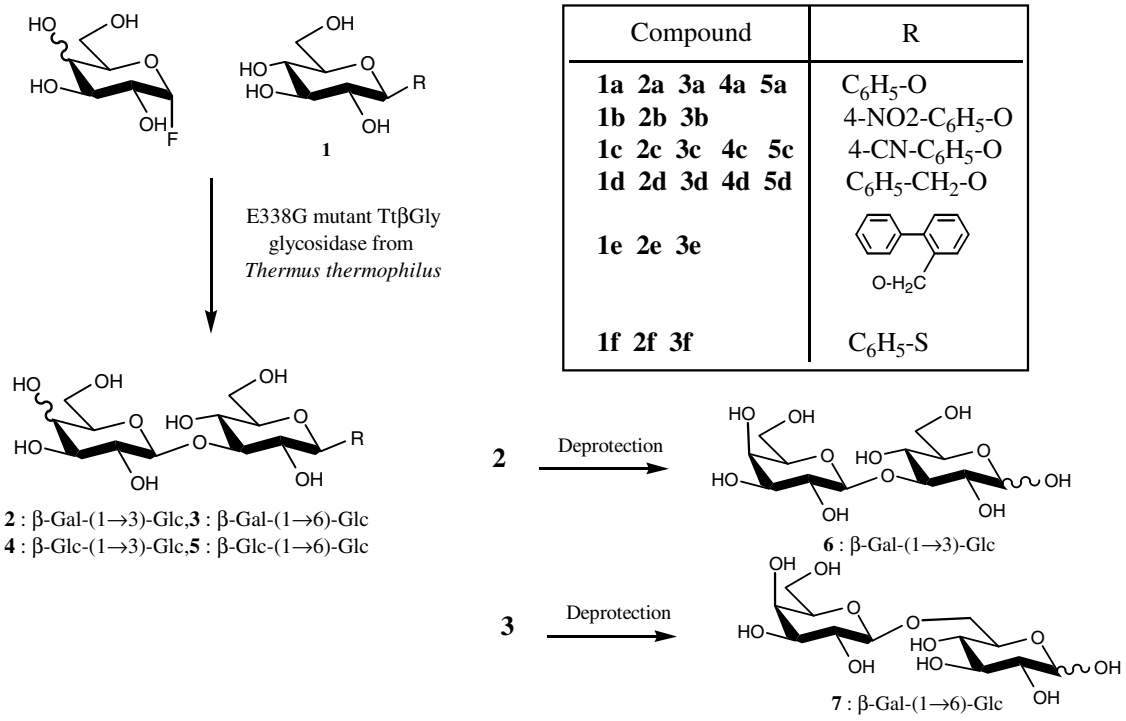
acceptor in the active site.[14] Furthermore, this substituent has to be eliminated in order to subsequently functionalize the anomeric position. Previous attempts to achieve this step were unsuccessful, since the chemical deprotection of the aglycon by hydrolysis was in competition with the breakdown of the glycosidic bond. This paper deals with the design of suitable acceptors bearing readily removable anomeric substituents and able to induce the same regioselectivity. For this purpose, several acceptor subtrates like aryl and benzyl β-D-glucopyranosides, were tested regarding the ability of their aglycon part to be further eliminated. Selection of suitable β-D-glucopyranosides was previously based on molecular modeling calculations on Ttβgly enzyme/substrates complexes.

2. Results and discussion

Therapeutic applications of oligosaccharides are determined by their multi-grafting on proteins. For this purpose, the functionalization of the reactive free anomeric carbon is often used. Meanwhile, the glycosynthase E338G (Ttβgly from *Thermus thermophilus*) acted as an efficient catalyst for the regioselective synthesis of the β-(1→3)-disaccharides when the acceptor beared a β-*O*-aryl group. The phenyl group used in our previous studies was shown to be non-easily removable. Thus, two strategies could be considered in order to produce useful oligosaccharides: either to find an aromatic group bearing chemical functions further usable for the grafting with proteins or to design aglycon aromatic groups easier to remove than the phenyl group. According to these requirements, we have tested several glucosides acting as acceptors in the glycosylation reaction mediated by the glycosynthase E338G using α-galactopyranosyl fluoride and α-glucopyranosyl fluoride as donors. The list of the acceptors used in this work is shown in Scheme 2, and the results obtained are summarized in Table 1.

Our primary strategy was to replace the phenyl group by another substituted aromatic ring that is able to allow the grafting of a saccharide on a polymeric framework. For example, 4-nitrophenyl or 4-cyanophenyl groups could be hydrogenated to provide amines that could be used for this purpose according to well-known methodologies. Moreover, *O*-4-nitrophenyl group, a better leaving group than *O*-phenyl one, could be hydrolyzed without cleavage of the β-(1→3) glycosidic linkage.

As indicated by our previous results, the replacement of the β-*O*-phenyl group by a β-4-nitrophenyl analogue had no significant effect on either the regioselectivity or the glycosylation yields (see Table 1, entries 1–3). Unfortunately, although the reduction of the nitro group was quite easy to achieve, the further use of the amino group resulted in bad grafting yields of the disaccharide on proteins. In other respects, the removal of *O*-4-nitrophenyl group via hydrolysis could not be performed without partial breakdown of the β-(1→3) disaccharide bond. The use of 4-cyanophenyl β-D-glucopyranoside **1c** as an acceptor led to similar results although the glycosylation reaction rate was lower in that case. Consequently, a higher ratio of [donor]/[**1c**] or [**1c**]/[[donor] had to be used since the rate of spontaneous hydrolysis of galactosyl



Compound	R
1a 2a 3a 4a 5a	C_6H_5-O
1b 2b 3b	4-NO2-C_6H_5-O
1c 2c 3c 4c 5c	4-CN-C_6H_5-O
1d 2d 3d 4d 5d	C_6H_5-CH_2-O
1e 2e 3e	O-H_2C
1f 2f 3f	C_6H_5-S

Scheme 2. Reactions, substrates and compounds.

Table 1
Experimental conditions and β-(1→3) disaccharide yields using various acceptors **1** and a glycosynthase (E338G) obtained from *Thermus thermophilus* glycoside hydrolase as a catalyst

Entry	Donor	Acceptor	[Donor] (mmol /L)	[Acceptor] (mmol/L)	β-(1→3)-Disaccharide	β-(1→6)-Disaccharide	Transglycosylation yields (%)
1	Gal-F	**1a**	50	50	100	0	86*
2	Glc-F	**1a**	50	50	100	0	96*
3	Gal-F	**1b**	50	50	100	0	84
4	Gal-F	**1c**	50	50	100	0	45
5	Gal-F	**1c**	100	50	100	0	85
6	Glc-F	**1c**	50	50	100	0	45
7	Glc-F	**1c**	100	50	100	0	95
8	Gal-F	**1d**	100	50	55	45	67
9	Glc-F	**1d**	100	50	56	44	82
10	Gal-F	**1e**	50	25	84	16	70
11	Gal-F	**1f**	25	25	96	4	81
12	Gal-F	**1f**	30	15	94	6	94

* See Ref. 14.

fluoride became competitive (see Table 1, entries 4–6). The non-commercially available monosaccharide **1c** was obtained with the standard sugar chemistry procedures: coupling of 4-hydroxybenzonitrile with 2,3,4,6-tetraacetyl-α-D-glucopyranosyl bromide in the presence of silver carbonate, followed by Zemplén deacetylation. Unfortunately, the reduction of the nitrile group of saccharides **1c**, **2c** or **4c** proved to be very difficult to achieve in good conditions. Moreover, this first strategy suffered from the presence of an aromatically substituted cycle that may induce undesirable immunogenic responses, when these molecules are used as therapeutic agents. So, the need for saccharides deprotected at the anomeric position remained very important since the functionalization at this position is quite easy via the synthesis of anomeric amino derivatives.

According to these requirements, the use of 4-methoxyphenyl β-D-glucopyranoside as an acceptor was assayed, since the resulting disaccharide could be deprotected using ceric ammonium nitrate.[18] Unfortunately, this acceptor was a poor substrate for our mutant enzyme, thus resulting in very low transglycosylation yields. Similarly, benzyl derivatives looked like very attractive candidates due to an easy deprotection of the β-*O*-benzyl aglycon by catalytic reduction under very mild conditions. Moreover, the presence of the aromatic group separated by a single methylene group from the anomeric carbon could preserve the stabilizing stacking with W312 of Ttβgly glycosynthases. So, we synthesized benzyl β-D-glucopyranoside **1d** using the transglycosylation reaction catalyzed by the wild type Ttβgly glycosidase in the presence of *p*-nitrophenyl β-D-glucopyranoside (pNPβGlc) as a donor and benzyl alcohol as an acceptor. The kinetic study of the corresponding reaction indicated that theoretical yields of about 40% could be reached. This very easy one-step enzymatic reaction was preferred in that case to the conventional chemical approach, since benzyl alcohol was a good substrate for the enzyme. The ability of **1d** to act efficiently in the glycosylation reactions is tested as described in Scheme 2. Unfortunately, **1d** was also shown to be a poor substrate, since the yields were lower than those previously obtained and the regioselectivity was also poor (see Table 1, entry 8). This was particularly the case for the synthesis of the β-(1→3)-disaccharide **2d**, which was obtained in similar amounts to its β-(1→6)-regioisomer **3d**. The same was true when using Glc-F as a donor (see Table 1, entry 9). Despite this disappointing result, we have undertaken the deprotection of β-*O*-benzyl aglycon by catalytic hydrogenation of the mixture of regioisomers **2d** and **3d**. This reaction afforded quantitatively the corresponding mixture of the free disaccharides, β-D-Gal*p*-(1→3)-D-Glc*p* **6** and β-D-Gal*p*-(1→6)-D-Glc*p* **7**. Correspondingly, molecular modeling calculations revealed that the benzylic acceptor **1d** was unable to fit properly in the active site in order to synthesize the desired β-(1→3)-disaccharide. It is likely that the introduction of a methylene group between the anomeric position and the phenyl group promoted an increased conformational mobility that induced a relaxed regioselectivity. This disappointing result prompted us to use molecular modeling techniques in order to choose a suitable benzylic acceptor. For this purpose, we screened in silico a set of acceptors like 2-, 3-, or 4-hydroxybenzyl , 2-, 3- or 4-methylbenzyl β-D-glucopyranosides and 4- or 2-biphenylmethyl β-D-glucopyranosides. Calculations showed that hydroxybenzyl derivatives fit more or less correctly in the active site, while the introduction of methylbenzyl ones resulted in higher energy interactions. The same was true for 4-biphenylmethyl β-D-glucopyranoside, while an unexpected high stabilization was obtained with the 2-biphenylmethyl analogue **1e** in which the second phenyl group produced a stabilizing stacking with W312 of the mutant glycosynthase (see Table 2, Fig. 1 and Section 3).

Table 2
Enzyme–acceptor docking energy (kcal/mol)

Acceptor	Energy	Variation
1a	−46.6	Ref.
1f	−44.4	+2.2
1d	−39.7	+6.9
1e	−62.7	−16.1

The complex with **1a** was chosen as a reference. Its replacement by **1f** had no significant effect on energy docking, while **1d** induced much more significant rise in docking energy. Conversely, **1e** led to a severe docking energy decrease. In this case, the increase in ligand–enzyme contact surface only partly explains this strong affinity between the two partners.

The preparation of **1e** by means of the enzymatic approach used for **1d** was not possible due to the very poor solubility of 2-biphenylmethanol in water. Thus, the synthesis of **1e** was achieved using conventional sugar chemistry. Coupling of 2-biphenylmethanol with 2,3,4,6-tetra-*O*-acetyl-1-*O*-trichloroacetimidoyl-α-D-glucopyranose was catalyzed by means of trifluoroboride etherate. Subsequent deacetylation in the presence of ammoniac in methanol afforded **1e**. The glycosylation reaction then performed in the presence of Gal-F as a donor in the same conditions as those previously used, resulted in dramatic improvement of the β-(1→3) regioselectivity of the E338G [relative percentages of β-(1→3) **2e** regio-isomer versus β-(1→6) **3e**: 84/16, see Table 1, entry 10]. This result constituted an additional indication of the need for the stabilization of the acceptor by stacking a phenyl group of the latter with W312. The deprotection of the biphenylmethyl at O-2 group, achieved via Pd/C catalyzed hydrogenolysis, afforded quantitatively the corresponding β-D-Gal*p*-(1→3)-D-Glc*p* **6**. The attachment of a readily removable functional group to a glycosidase substrate facilitated a rational control of the regioselectivity. The same

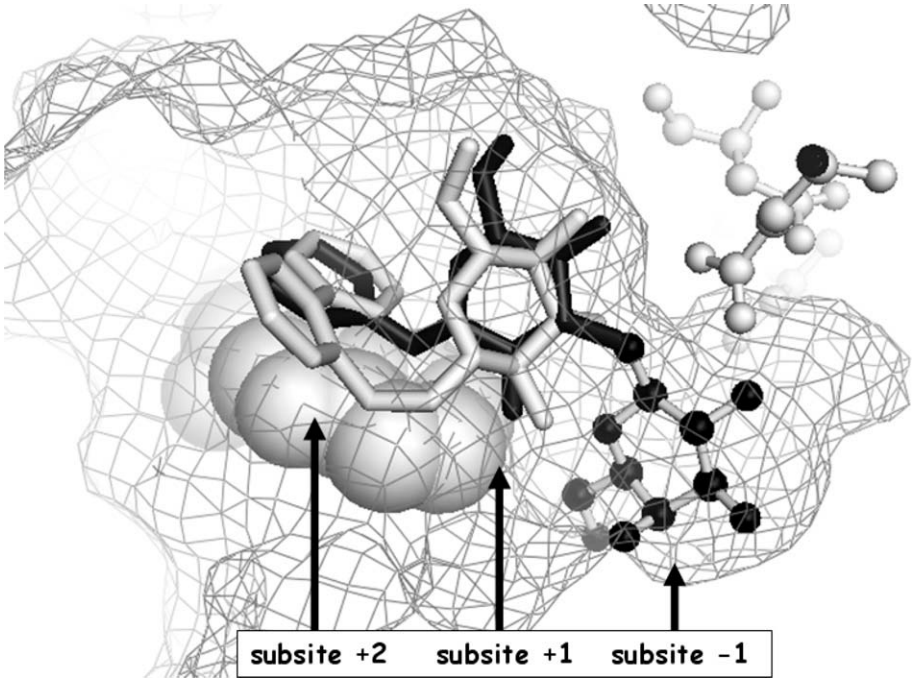


Figure 1. Rational design of an acceptor in the transglycosylation reaction catalyzed by TtβGly glycosynthase. Phenyl β-D-glucopyranoside (*__1a__, *black sticks) and 2-biphenylmethyl β-D-glucopyranoside (*__1e__, *white sticks) are acting as acceptors in the glycosylation reaction. They both present one aromatic ring strongly stacked with W312 (spheres) for the stabilization of the ligand fragment at sub-site (+2) with respect to β-(1→3) linkage formation (glycosyl enzyme inside the cleft in black ball and stick). The catalytic site is visualized in mesh representation. Important catalytic residues (E164, N282) are also shown (ball and stick, medium, and light, respectively).

approach, previously reported by Lairson et al. in the case of glycosyltransferases resulted in similar results.[19]

As an alternative, we also tested phenyl 1-thio-β-D-glucopyranoside **1f**, which is known to be easily deprotected via hydrolysis of the thioglycosidic bond under very mild conditions. In this way, we verified, by means of molecular modeling calculations that acceptor **1f** is fitted into approximately the same position as its oxygenated analogue. As expected, the replacement of the oxygen atom by a sulfur did not induce any important topological modifications of the position of the reactants in the active site, thus indicating that the synthesis of the corresponding β-(1→3)-regioisomer should be preferred. Once again, this prediction was quite satisfactory in accordance with experimental data, since the use of **1f** as an acceptor led to similar results to those obtained with phenyl glucoside **1a** (Table 1, entries 1 and 11) although very minor amounts of the β-(1→6) regioisomer were present. It should be noted that phenyl 1-thio-β-D-glucopyranoside **1f** was already successfully used as an acceptor in transglycosylation reactions catalyzed by exoglycosidases.[20] Substantial improvement of the yield (81–94%) was obtained when working with a ratio of [donor]/[acceptor] = 2 (see Table 1, entries 11 and 12). The deprotection of the thiophenyl group by the action of *N*-bromosuccinimide in water afforded quantitatively β-D-Gal*p*-(1→3)-Glc*p* **6**.

In conclusion, we have shown that substrate engineering can be a complementary approach to enzyme engineering to afford high yields and regioselectivity in glucosidation reactions catalyzed by glycosynthases. Thus, two acceptors, 2-biphenylmethyl β-D-glucopyranoside **1e** and phenyl 1-thio-β-D-glucopyranoside **1f** were chosen for their ability to produce regioselectively the corresponding β-(1→3)-disaccharides in good yields. In both cases, the removal of the aglycon afforded quantitatively β-D-Gal*p*-(1→3)-D-Glc*p* **6**. Moreover, this choice was supported by molecular modeling calculation, which proved to constitute a rational approach for the design of suitable reactants.

3. Experimental

3.1. General procedures

Chemicals supplied by Aldrich were used without further purification. The courses of the reactions were followed by means of TLC (precoated Silica Gel 60 sheets Merck F254) and proton NMR spectroscopy. The components of the reaction mixtures were separated by means of silica gel chromatography. Analysis of the NMR ^{1}H and ^{13}C resonances and subsequent structure assignments were made by using standard 2D sequences (COSY HH HOHAHA and HC correlations). The spectra were recorded with a Bruker DRX500 spectrometer operating at 500 MHz for ^{1}H and at 126 MHz for ^{13}C or with a Bruker AX400 spectrometer operating at 400 MHz for ^{1}H and at 100.6 MHz for ^{13}C. Due to their solubility in water, the spectra of the saccharides were recorded in D_2O, and the chemical shifts (in ppm) were quoted from the resonance of methyl protons of sodium 3-(trimethylsilyl)-propansulfonate (DSS) used as an internal reference. For each compound, the proton chemical shifts and coupling constants are given in Table 3, and the carbon chemical shifts are given in Table 4.

3.2. Molecular modeling calculations

All molecular modeling visualizations and calculations were performed on an SGI workstation with Accelrys tools (InsightII software and CFF91 force field). For the enzyme, the crystal structure of the native TtβGly (pdb:1ug6) and the starting modeled complex with a DP3 oligomer β-D-Gal*p*-(1→3)-β-D-Glc*p*-(1→4)-D-Glc*p* has already been described.[16]

Molecular modeling calculations were performed in two steps. The first consisted in generating all probable conformations of substrates mentioned above, regardless of the catalytic space con-

Table 3
^{1}H NMR data of compounds described (500 MHz), solvent D_2O, T = 35 °C

Comp.		H-1	H-2	H-3	H-4	H-5	H-6	H-6′	Ph-CH_2	Others
1c	—	5.22	3.51	3.61	3.63	3.67	3.75	3.93	—	7.76, 7.22 (aromatics)
1d	—	4.55	3.37	3.53	3.45	3.45	3.97	3.78	4.97, 4.78	7.40–7.55 (aromatics)
Tetraacetyl **1e**	—	4.45	5.05	5.08	5.16	3.57	4.02	4.23	4.56, 4.80	2.00, 2.01, 2.02, 2.05 (CH_3) 7.29–7.50 (aromatics)
1e	—	4.29	3.38[a]	3.23	3.34[a]	3.25	3.78	3.65	4.71, 4.87	7.3–7.7 (aromatics)
1f	—	4.78	3.35	3.52	3.41	3.47	3.71	3.88	—	7.40, 7.57 (aromatics)
2b	I	5.28	3.87	3.90	3.65	3.67	3.78	3.95	—	7.40–7.55 (aromatics)
	II	4.70	3.63	3.69	3.93	3.73	n.d.	3.80	—	—
2c	I	5.24	3.81	3.88	3.63	3.69	3.77	3.93	—	7.23, 7.76, 7.76, 7.77 (aromatics)
	II	4.70	3.63	3.69	3.94	3.73	3.80	3.77	—	—
4c	I	5.24	3.81	3.87	3.62	3.70	3.77	3.93	—	7.26, 7.26, 7.26, 7.77 (aromatics)
	II	4.78	3.38	3.53	3.41	3.49	3.72	3.93	—	—
2d	I	4.35	3.27	3.55	n.d.	3.34	3.81	n.d.	4.70, 4.86	7.40–7.55 (aromatics)
	II	4.40	3.50	3.67	3.90	3.40	n.d.	n.d	—	—
2e	I	4.31	3.28	3.54	n.d.	3.36	3.82	n.d.	4.71, 4.87	7.35–7.65 (aromatics)
	II	4.42	3.52	3.65	3.91	3.39	n.d	n.d.	—	—
2f	I	4.81	3.53	3.78	n.d.	n.d.	3.89	3.72	—	7.35–7.60 (aromatics)
	II	4.67	3.57	3.66	3.91	n.d.	n.d	n.d.	—	—
6 (α,β)	I α	5.23	3.71	3.90	3.51	3.86	3.75	3.83	—	—
	I β	4.66	3.43	3.73	3.52	3.48	3.72	3.89	—	—
	II	4.64	3.60	3.67	3.92	n.d.	n.d.	n.d.	—	—
		$^3J_{1,2}$	$^3J_{2,3}$	$^3J_{3,4}$	$^3J_{4,5}$	$^3J_{5,6}$	$^3J_{5,6}$	$^2J_{6,6'}$	$^2J_{CH_2\text{-Ph}}$	
1c	—	7.4	9.4	9.4	9.9	5.7	2.2	−12.3	—	8.9 (aromatics)
1d	—	7.9	9.1	7.9	1.1	5.6	1.8	−12.3	−12.3	n.d.
Tetraacetyl **1e**	—	7.8	9.3	9.3	9.3	2.5	4.9	−12.2	−11.7	n.d.
1e	—	7.8	n.d	n.d.	n.d	2.1	5.4	−12.2	−11.7	n.d.
1f	—	9.8	8.3	8.8	9.8	5.6	2.4	−12.7	–	n.d.
2b	I	7.9	8.4	9.0	9.9	5.3	2.2	−12.4	—	n.d
	II	7.7	9.9	3.4	0.8	3.8	8.1	−12.1	—	—
2c	I	7.9	9.3	8.6	9.8	5.4	2.3	−12.4	—	8.9 (aromatics)
	II	7.7	9.9	3.4	0.9	4.1	8.0	−12.4	—	—
4c	I	7.8	9.3	8.6	9.9	5.1	2.3	−12.4	—	8.9 (aromatics)
	II	7.9	9.5	9.1	9.8	5.1	2.3	−12.4	—	—
2d	I	7.8	8.8	8.3	9.6	2.3	4.4	−12.2	−11.7	n.d
	II	7.8	n.d.	n.d.	3.4	n.d.	n.d.	n.d	—	—
2e	I	7.8	8.8	8.3	9.6	2.3	4.4	−12.2	−11.7	n.d.
	II	7.8	n.d.	n.d.	3.4	n.d	n.d	n.d	—	—
2f	I	10.0	8.8	8.8	9.6	1.1.	n.d.	n.d.	−12.2	n.d.
	II	7.9	9.9	3.4	n.d.	n.d.	n.d.	n.d	n.d.	—
6 (α,β)	I α	3.8	9.7	9.0	9.9	5.0	2.5	−12.3	—	—
	Iβ	8.0	9.2	8.3	9.9	5.3	2.3	−12.3	—	—
	II	7.6	9.8	3.4	n.d.	n.d	n.d	n.d	—	—

Chemical shifts in ppm, ref: sodium 3-(trimethylsilyl)-propansulfonate, coupling constants J in Hz. Tetraacetyl **1e**, solvent $CDCl_3$, ref: TMS.
[a] May be reversed.

Table 4
^{13}C NMR data of the compounds described (125 MHz), solvent D_2O, T = 35 °C)

Comp.		C-1	C-2	C-3	C-4	C-5	C-6	Ph-CH_2	Others
1c	—	102.1	72.0	78.2	75.5	78.9	63.2	—	122.5 (CN) ; 119.7, 137.7, 107.7, 162.8 (aromatics)
1d	—	100.3	72.2	74.9	68.7	75.0	59.9	70.4	
Tetraacetyl **1e**	—	89.4	71.5	68.8	73.1	71.9	62.0	68.6	20.8, 20.9 (CH_3) ; 170.9,170.5,169.6, 169.5 (CO); 127.5 − 142.3 (aromatics)
1e	—	100.8	72.1[a]	75.0	68.9[a]	75.2	60.3	71.1	127.6, 127.9, 128.4, 128.8, 129.3, 130.1, 130.4, 133.6, 140.4, 142.4 (aromatics)
1f	—	90.0	74.4	80.0	72.2	82.6	63.6	—	134.7, 134.3,132.0, 130.8 (aromatics)
2b	I	104.7	76.9	87.9	72.2	79.0	63.7	—	
	II	106.0	74.0	75.3	71.3	78.0	63.8	—	—
2c	I	102.0	75.4	86.0	70.8	78.6	63.3	—	107.8, 119.8, 137.4, 162.8 (aromatics), 122.6 (CN)
	II	106.0	74.0	75.2	71.4	78.1	63.8	—	
4c	I	101.9	75.2	86.7	70.5	78.5	63.1	—	119.7, 137.3, 107.7, 162.7 (aromatics); 122.4 (CN)
	II	105.5	76.1	78.2	72.3	78.7	63.4		
2d	I	102.6	72.4	78.1	n.d.	74.3	60.2	70.4	(Aromatics)
	II	100.4	71.0	72.4	68.6	n.d.	61.1	—	—
2e	I	102.9	72.8	78.3	n.d.	74.6	60.0	68.8	127.6, 127.8, 128.5, 128.8, 129.4, 130.1, 130.6, 133.5, 140.4, 142.5 (aromatics)
	II	100.8	71.0	72.4	68.6	n.d.	61.1	—	—
2f	I	89.7	70.8	88.3	73.9	82.3	63.5	—	130.8, 132.0, 134.3, 134.7 (aromatics)
	II	105.9	74.0	75.3	71.3	78.0	63.8	—	—
6 (α,β)	I α	94.7	78.0	85.3	71.0	74.0	63.4	—	—
	I β	98.4	76.4	87.6	71.0	78.2	63.6	—	—
	II	106.1	74.0	75.4	71.3	73.6	63.6	—	—

Chemical shifts in ppm, ref : sodium 3-(trimethylsilyl)-propansulfonate. Tetraacetyl **1e** solvent $CDCl_3$, ref : TMS.
[a] May be reversed.

straints: all linkage bonds yielding substrate flexibility were systematically scanned at regular intervals (10°) according to the protocol already described.[21]

The second step consisted in locating these substrates in their right place in the catalytic site for subsequent complex energy minimization. From previous analysis of the TtβGly/DP3 substrate complex[16], it was possible to deduce structural information for specific docking at each sub-site. Sub-site (−1), deep in the catalytic site, was characterized by a very strong docking energy involving all possible stabilizing factors. Contrary to this first case, sub-site (+1) presented a weak docking mainly due to Van der Waals terms. Finally, sub-site (+2) also had a rather weak docking partly due to W312 stacking (with a sugar or aglycon aromatic ring). Thus, it could be deduced that the sugar ring initially located at subsite (+1) had a significant degree of freedom without notable consequence in the total enzyme/substrate docking energy. Conversely, W312 should have a pivotal influence on the stabilization of the acceptor moiety. Accordingly, all conformations of acceptors were tested by first superimposing the sugar ring atoms on those of the corresponding modeled ring at sub-site (+1) with β-(1→3) disposition with sub-site (−1). All complexes presenting high steric hindrance between enzyme and substrate were discarded. The few remaining solutions were minimized with all heavy atoms of the enzyme fixed. The same energy minimization protocol (steepest descent algorithm, 10,000 iterations) was applied to all calculations to evaluate the capability of each of these conformations to adapt to severe catalytic cavity constraints preserving the β-(1→3) selectivity, while using (when possible) the stacking opportunity between the aglycon moiety and W312. Due to these drastic energy minimization conditions, these results should be used qualitatively, and it is generally admitted that this interaction energy is a relevant criterion of comparison when the total enzyme/substrate contact surface is quite comparable. In practice, this condition is only fulfilled between some compounds. For example (see Table 2), the replacement of phenyl glucoside (**1a**) by phenyl thioglucoside (**1f**) led to an increase of interaction energy of only 2.2 kcal/mol (not significant within the protocol used here). When 2-biphenylmethyl β-D-glucopyranoside (**1e**) was tested, the interaction energy was much more favorable (−16.1 kcal/mol). Although a part of the gain was probably due to a greater contact surface, it seems reasonable to consider that the stacking of one phenyl ring with W312 significantly contributed to stabilize this acceptor in the catalytic site.

3.3. Kinetic study of the synthesis of benzyl β-D-glucopyranoside 1d using 4-nitrophenyl β-D-glucopyranoside and benzylic alcohol as substrates for Ttβgly

Sodium phosphate buffer (0.74 mL, 0.05 M, pH 7.0) was lyophilized, dissolved in D_2O (2 mL), and lyophilized once more. The resulting salts were re-dissolved into 0.74 mL D_2O. Then, 0.2 mL of DMF containing 9 mg (30 μmol) of pNPβGlc and 32 mg (300 μmol) of benzylic alcohol were successively added. The resulting soln was immediately filtered, transferred to an NMR tube, and a ^1H spectrum of the mixture was recorded at 30 °C. Then, 6 μL of the enzymatic Ttβgly sol.[15] was added, and the reaction was allowed to proceed in the magnet of the spectrometer at 30 °C. Using this procedure, it was possible to perform the first measurements at about 4 min after the introduction of the enzyme in the NMR tube. Concerning the kinetics measurements, standard conditions were used (PW = 30°, NS = 32, acquisition time = 0.344 s), and the integrals were measured using the Brucker standard program. The accuracy of the measurements of the concentrations was estimated at about 5% for the major products. This study showed that a 40 % maximum concentration was obtained for benzyl β-D-glucopyranoside **1d** after 2 h.

3.4. Enzymatic synthesis of benzyl β-D-glucopyranoside 1d

To a soln of pNPβGlc (500 mg, 1.66 mmol) in a solvent composed of 20% DMF and 80% sodium phosphate buffer (55 mL, 0.05 M, pH 7), were successively introduced benzylic alcohol (1.6 mL, 1.79 g, 16.6 mmol) and Ttβgly glycosidase sol. (333 μL) prepared as previously described.[15] The mixture was stirred at 30 °C for 2.0 h (increased reaction time resulted in a fast hydrolysis of **1d**). The reaction mixture was then quenched by warming at 100 °C for 5 min. Thus, water was distilled under diminished pressure, and the resulting mixture was washed with $CHCl_3$ in order to remove DMF and excess benzylic alcohol. Next, saccharide **1d** was purified on a silica gel column (5:2:2 BuOH–EtOH–water, R_f 0.68). After collection of the fractions containing **1d**, and elimination of the solvent, pure **1d** was obtained (135 mg, yield 30%, ^1H and ^{13}C NMR spectra of **1d**, see Tables 3 and 4). The present spectra were identical to those already published for authentic samples.[22]

3.5. Synthesis of 4-cyanophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside

To 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (5.2 g, 12.7 mmol), prepared according to a standard procedure[23] and dissolved in 1:1 MeCN–CH_2Cl_2 (55 mL), molecular sieves (1 g, 3 Å) were added. The mixture was stirred in the dark for 30 min at rt and silver carbonate (6.46 g, 23.4 mmol) and 4-cyanophenol (2.28 g, 19.2 mmol) were successively added. The reaction mixture was stirred in the dark at rt for 12 h (completion of the reaction followed by TLC of the remaining bromosugar). The reaction mixture was then filtered over Celite, and the filtrate was concentrated over diminished pressure. Dissolution of the oily product in CH_2Cl_2 resulted in the precipitation of AgBr as a white solid, which was filtered over Celite. Solvent evaporation resulted in an oily residue, which was purified by silica gel chromatography (3:2 petroleum ether–EtOAc) resulting in 4-cyanophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside as a syrup (3.1 g, 7.0 mmol).The present ^1H and ^{13}C NMR spectra (Tables 3 and 4) were identical to those values previously published for 4-cyanophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside.[24]

3.6. Synthesis of 4-cyanophenyl β-D-glucopyranoside 1c

Zemplén deacetylation[23] of 4-cyanophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (3.1 g, 7.0 mmol) afforded **1c**, which was obtained as white crystals after recrystallization in ethanol (1.8 g, 6.4 mmol). The NMR parameters (see Tables 3 and 4) and physical constants were consistent with the structure of 4-cyanophenyl β-D-glucopyranoside **1c**, mp 185–187 °C, $[\alpha]_D^{20}$ −92.3, (*c* 1.5, water lit.[25] mp 188–189 °C, $[\alpha]_D^{20}$ −91.9, (*c* 1.5, water).

3.7. 2-Biphenylmethyl β-D-glucopyranoside 1e

3.7.1. 2-Biphenylmethyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside

2,3,4,6-Tetra-*O*-acetyl-1-*O*-trichloroacetimidoyl-α-D-glucopyranose (prepared according to the standard procedure,[23] 724 mg, 1 .47 mmol), 2-biphenylmethanol (271 mg, 1.47 mmol), dry CH_2Cl_2 (7.5 mL), and $BF_3(Et)_2O$ (19 drops) were successively introduced in a reactor under a dry nitrogen flow. The reaction was allowed to proceed for 2 h and the reaction mixture was quenched by adding EtOAC (37 mL). Then, this soln was washed three times with a saturated sodium hydrogencarbonate sol. (3 × 15 mL) and with distilled water (15 mL). The organic layer was dried over sodium sulfate, and the solvent was removed under diminished pressure. Purification of the product thus obtained via silica gel chromatography (2:3 AcOMe–petroleum ether, R_f 0.48) afforded

peracetylated **1e** as an oily product (236 mg, 0.46 mmol, yield 31%). Anal. Calcd for $C_{25}H_{28}O_{10}$: C, 61.48; H, 5.74. Found: C, 61.36; H, 5.82. NMR data for 2-biphenylmethyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside, see Tables 3 and 4.

3.7.2. 2-Biphenylmethyl β-D-glucopyranoside 1e

To a soln of peracetylated **1e** (236 mg, 0.46 mmol) in MeOH (5 mL) was added concentrated ammonia (36%, 0.11 mL). The reaction was allowed to proceed at room temperature for 6 h. Then, the solvent was removed by distillation under diminished pressure, and ether was added in order to precipitate **1e**, which was obtained as white crystals (105 mg, 0.33 mmol). mp 173–175 °C, $[\alpha]_D^{20}$ −7.8 (*c* 3.0, water), Anal. Calcd for $C_{17}H_{20}O_6$: C, 63.75; H, 6.25. Found: C, 63.69; H, 6.35. The NMR data of **1e** are given in Tables 3 and 4.

3.8. Synthesis of phenyl 1-thio-β-D-glucopyranoside 1f

The synthesis of **1f** was achieved via deacetylation of commercial (Aldrich) phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside in the presence of catalytic amounts of NaOMe in MeOH.[23]

3.9. Mixture of benzyl β-D-galactopyranosyl-(1→3 and 1→6)-β-D-glucopyranosides 2d and 3d

α-D-Galactopyranosyl fluoride (36 mg, 0.2 mmol, prepared according to Malet and Planas[12]) and benzyl β-D-glucopyranoside **1d** (27 mg, 0.1 mmol) were dissolved in hydrogencarbonate buffer (150 mmol/L, pH 7.8). Then, glycosynthase E338G sol. (0.9 mL), prepared as previously described,[15] was added and the reaction was allowed to proceed at 55 °C until the complete consumption of the fluoride (12 h). After removal of the solvent under diminished pressure, purification by silica gel chromatography (5:2:2 BuOH–EtOH–water, R_f 0.52) afforded a mixture of disaccharides **2d** and **3d**, 55% and 45%, respectively, 29 mg, overall yield 67%. Anal. (mixture of regioisomers) Calcd for $C_{19}H_{28}O_{11}$: C, 52.78; H, 6.48. Found: C, 52.69; H, 6.59). Some fractions enriched in disaccharide **2d** allowed the NMR structural identification of this compound (see Tables 3 and 4). The structure of benzyl β-D-Gal*p*-(1→6)-β-D-Glc*p* **3d** was determined on the basis of the ^{13}C NMR spectrum of the mixture of regioisomers: in the typical 60–65 ppm zone for the CH_2OH resonances, three peaks were present, two of them with equal intensity were easily attributed to **2d** while the single third one belonged to **3d**, thus confirming the 1→6 linkage.

3.10. Mixture of benzyl β-D-glucopyranosyl-(1→3 and 1→6)-β-D-glucopyranosides 4d and 5d

α-D-Glucopyranosyl fluoride (36 mg, 0.2 mmol) and benzyl β-D-glucopyranoside **1d** (27 mg, 0.1 mmol) were dissolved in hydrogencarbonate buffer (150 mmol/L, pH 7.8). Then, 0.9 mL of glycosynthase E338G sol., prepared as previously described,[15] was added and the reaction was allowed to proceed at 55 °C until complete consumption of the fluoride (12 h). After removal of the solvent under diminished pressure, purification by silica gel chromatography (5:2:2 BuOH–EtOH–water, R_f 0.50) afforded a mixture of disaccharides **4d** and **5d** (56% and 44%, respectively, 37 mg overall yield 82%, Anal. (mixture of regioisomers) Calcd for $C_{19}H_{28}O_{11}$: C, 52.78; H, 6.48. Found: C, 52.61; H, 6.61. Some fractions enriched in disaccharide **4d** allowed the NMR structural identification of this compound. The ^1H and ^{13}C NMR spectra were consistent with the Glc(1→3)-Glc structure of **4d** (see Tables 3 and 4).The structure of benzyl β-D-Glc*p*(1→6)-β-D-Glc*p* **5d** was established on the same basis as already described for the structure determination of **3d** (see above).

3.11. Synthesis of 4-nitrophenyl β-D-galactopyranosyl-(1→3)-β-D-glucopyranoside 2b

α-D-Galactopyranosyl fluoride (18.2 mg, 0.1 mmol) and 4-nitrophenyl β-D-glucopyranoside **1b** (0.1 mmol) were dissolved into 2 mL of 150 mmol/L of ammonium carbonate buffer (pH 7.8). Then, 0.9 mL of Tt-β-Gly E338G sol., prepared as already described,[15] was added and the mixture was stirred overnight at 55 °C. After solvent elimination under diminished pressure, the residue purified by silica gel chromatography (7:2:1 AcOEt–MeOH–water) gave **2b** (39 mg, 84%) as white solid. R_f 0.49 (eluent above). Calcd for $C_{18}H_{25}NO_{13}$: C, 46.65; H, 5.40. Found: C, 46.80; H, 5.26. ^1H and ^{13}C NMR parameters (see Tables 3 and 4) were identical to those previously reported.[26]

3.12. 4-Cyanophenyl β-D-galactopyranosyl-(1→3)-β-D-glucopyranoside 2c and 4-cyanophenyl β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside 4c

α-D-Galactopyranosyl or α-D-glucopyranosyl fluoride (34 mg, 0.2 mmol) and 4-cyanophenyl β-D-glucopyranoside **1c** (28 mg, 0.1 mmol) were dissolved in hydrogencarbonate buffer (150 mmol/L, pH 7.8). Then, glycosynthase E338G sol. (0.9 mL), prepared as previously described,[15] was added and the reaction was allowed to proceed at 55 °C until complete consumption of the fluoride (12 h). After removal of the solvent under diminished pressure, purification by silica gel chromatography (5:2:2 BuOH–EtOH–water, R_f of **2c** 0.55 and R_f of **4c** 0.64) afforded 38 mg of pure **2c** (yield 85 %, mp 162–163 °C, $[\alpha]_D^{20}$ −34.3 (*c* 2.8, water). Calcd for $C_{19}H_{25}NO_{11}$: C, 51.47; H, 5.64. Found: C, 51.67; H, 5.49) and 42 mg of pure **4c** (yield 95%, mp 166–168 °C, $[\alpha]_D^{20}$ −24.8, *c* 2.4, water. Calcd for $C_{19}H_{25}NO_{11}$: C, 51.47; H, 5.64. Found: C, 51.39; H, 5.71).

3.13. 2-Biphenylmethyl β-D-galactopyranosyl-(1→3)-β-D-glucopyranoside 2e

α-D-Galactosyl fluoride (83 mg, 0.46 mmol) and 2-biphenylmethyl β-D-glucopyranoside **1e** (91 mg, 0.27 mmol) were dissolved in ammonium hydrogencarbonate buffer (50 mmol/L, pH 7.8, 8 mL). Then, E338G glycosynthase sol. (3 mL), prepared as previously described,[15] was added. The reaction was allowed to proceed at 55 °C for 6.5 h. At this time, the Gal-F had completely disappeared. After elimination of the solvent under diminished pressure, flash silica gel chromatography of the mixture (28.3:10:1:1.7:4.1 $CHCl_3$–MeOH–AcOH–water–petroleum ether) gave fractions containing a mixture of two regioisomers as revealed by the ^1H NMR spectrum (80 mg, 86% and 14%, respectively, overall yield 70%). A further purification by silica gel chromatography (same eluent as above) afforded first, fractions containing the minor disaccharide (R_f 0.75) as a major compound, which was identified as the β-(1→6) regioisomer **3e** on the basis of the ^{13}C NMR spectrum of the mixture. Further fractions contained only the β-(1→3) regioisomer **2e** (R_f 0.62, identified on the basis of the ^1H and ^{13}C NMR spectra, see Tables 3 and 4). After distillation of the solvents under diminished pressure and washing the residue with CH_2Cl_2 (3 × 10 mL) in order to remove traces of acetic acid, **2e** (38 mg) was obtained as a syrup ($[\alpha]_D^{20}$ −34.3 (*c* 2.8, water). Anal. Calcd for $C_{23}H_{30}O_{11}$: C, 57.26; H, 6.22. Found: C, 57.17; H, 6.31).

3.14. Synthesis of phenyl β-D-galactopyranosyl-(1→3)-1-thio-β-D-glucopyranoside 2f

α-D-Galactosyl fluoride (150 mg, 0.8 mmol) and phenyl 1-thio-β-D-glucopyranoside **1f** (220 mg, 0.8 mmol) were dissolved in 20 mL of ammonium hydrogencarbonate buffer (150 mmol/L, pH 7.8). Then 9 mL of the E338G glycosynthase sol., prepared as

previously described,[15] was added. The reaction was allowed to proceed at 55 °C for 7 h while stirring. At this time, the Gal-F had completely disappeared. Then, the solvent was removed under diminished pressure, and the products were purified by silica gel flash chromatography (9:1 BuOH–water). The disaccharide fractions were collected and after removal of the solvent, 281 mg of a mixture of the two regioisomers were obtained (96% and 4%, respectively, as determined on the basis of the ^{1}H NMR spectra, overall yield 81%). A further purification performed with silica gel chromatography using the same eluent as above yielded 205 mg of pure **2f** (R_f 0.46) and 58 mg of a mixture of **2f** and **3f** (R_f of **3f** 0.60). Anal. Calcd for $C_{18}H_{26}O_{10}S$ (**2f**): C, 49.77; H, 5.99. Found: C, 49.91; H, 5.81. The ^{1}H and ^{13}C NMR spectra of **2f** were identical as those previously described for phenyl β-D-Gal*p*-(1→3)-1-thio-β-D-Glc*p*.[27] The structure of **3f** was determined on the basis of the ^{13}C NMR spectrum of the mixture of **2f** and **3f** (CH_2OH resonances).

3.15. Hydrogenolysis of benzyl and of 2-biphenylmethyl disaccharides

(a) Synthesis of mixtures of β-D-galactopyranosyl-(1→3 and 1→6)-D-glucopyranoside **6** and **7** by hydrogenolysis of benzyl β-D-Gal*p*-(1→3 and 1→6)-β-D-Glc*p* **2d** and **3d**.
The mixture of disaccharides **2d** and **3d** (43.2 mg, 0.1 mmol, 55% and 45%, respectively) was dissolved in 10 mL of MeOH. The catalyst (Pd/C, 50 mg) and H_2 were introduced in the flask. The mixture was stirred at rt for 2 h. After filtration of the catalyst and elimination of the solvent under diminished pressure, a white solid was obtained (33 mg, 0.096 mmol, yield 96%). The ^{1}H NMR spectrum revealed the presence of the two β-(1→3) β-(1→6) regioisomers **6** and **7** (relative percentages 55:45). Comparison with the literature data for β-D-Gal*p*-(1→3)-D-Glc*p*[28] and with the main product obtained from the hydrolysis of **2f** (see below) allowed the structure elucidation. The attribution of the NMR resonances of **6** is given in Tables 3 and 4.

(b) Synthesis of β-D-galactopyranosyl-(1→3)-D-glucopyranoside **6** by hydrogenolysis of 2-biphenylmethyl β-D-Gal*p*-(1→3)-β-D-Glc*p* **2e**. The same procedure as above was used, but with a 8 h reaction time. Thus, hydrogenation of **2e** (24 mg, 0.50 mmol) produced the free disaccharide **6** (16 mg, 0.47 mmol, yield 94%). The NMR spectra were identical to those described below (see Section 3.16).

3.16. Synthesis of β-D-galactopyranosyl-(1→3)-D-glucopyranoside 6 by hydrolysis of phenyl β-D-galactopyranosyl-(1→3)-1-thio-β-D-glucopyranoside 2f in the presence of NBS.[29]

The β-(1→3) disaccharide **2f** (43.4 mg, 0.1 mmol) was dissolved in water (8 mL). After addition of 2 equiv of NBS (71.2 mg, 0.4 mmol), a white precipitate appeared. After redissolving of the later, the reaction was allowed to proceed for 30 min at rt. Then, water was eliminated under diminished pressure, and the disaccharide purified using silica gel chromatography (7:2:1 EtOAc–MeOH–water) was obtained nearly quantitatively (yield 94 %, 32 mg, Anal. Calcd for $C_{12}H_{22}O_{11}$: C, 42.11; H, 6.43. Found: C, 42.02; H, 6.32). Comparison with the literature data for β-D-Gal*p*-(1→3)-D-Glc*p*[28] allowed the structure elucidation of **6**. The assignments of the NMR resonances of **6** are given in Tables 3 and 4.

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