Transferase Activity of a β-Glycosidase from *Thermus thermophilus*: Specificities and Limits – Application to the Synthesis of β-[1→3]-Disaccharides

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The aim of this paper is to test the ability of a β-glycosidase from *Thermus thermophilus* to catalyse transglycosylation reactions in the presence of nitrophenyl glycosides as donors and other monosaccharides as acceptors. Our results show that this enzyme is able to induce such reactions either with nitrophenylgalactosides, -glucosides and -fucosides. With the two former donors, the autocondensation of the donor, which thus acts also as an acceptor, is faster than the transglycosylation with other acceptors. Furthermore, as the regioselectivity of the reactions is mainly of the β-[1→3] type, good yields are obtained for the synthesis of 2-nitrophenyl-β-D-galactopyranosyl-[1→3]-β-D-galactopyranoside and 2-nitrophenyl-β-D-glucopyranosyl-[1→3]-β-D-glucopyranoside. Conversely, in the presence of *p*-nitrophenylfucoside, the autocondensation is very limited, and with methyl-α-D-galactoside as an acceptor, the regioselectivity is mainly of the β-[1→6]-type resulting in the synthesis of methyl-β-D-fucopyranosyl-[1→6]-α-D-galactopyranoside.

Introduction

The regioselective synthesis of the glycosidic bond may be achieved by means of the standard chemical approach[1] which usually requires cumbersome and sophisticated protection-deprotection multisteps. The enzymatic synthesis of the saccharides has become, over the last ten years, a very powerful alternative.[2] Two kinds of enzymes are able to catalyse the formation of the glycosidic bond: the glycosyltransferases and the glycosylhydrolases. The former generally lead to high yields and total regio- and stereoselectivities but they need very expensive sugar nucleotides as activated donors.[3−5] Furthermore, their high cost and low stability have greatly limited their use. The latter can also catalyse the formation of the glycosidic linkage not only through the reverse reaction but also through their transferase activity. Due to their stability and low cost, these enzymes are very attractive and they also induce a high stereoselectivity.[6−9] Their main disadvantages come from their rather low regioselectivity and the low yields usually obtained since the transferase activity remains in competition with the hydrolysis of the substrate and of the glycosides synthesized. In addition, other products may be synthesized, because the condensation of the activated glycoside donor can occur. Scheme 1 describes this rather complex situation encountered with the use of "retention" glycosidases. Fortunately, this complexity is very often apparent as the rates of transglycosylation reactions are much higher than the rates of hydrolysis. Furthermore, the rates of synthesis of the disaccharides are faster than those of the trisaccharides. To improve such reactions, suitable experimental conditions and/or new enzymatic activities must be found which can enhance the concentration of the transglycosylation products at the expense of the hydrolysis and provide a higher regioselectivity.

Considering the first proposition, high yields are usually obtained when using a donor bearing a good leaving group at the anomeric position. Thus, *p*- and *o*-nitrophenyl glycosides are most widely employed for this purpose leading, for instance, to the synthesis of blood determinant di- and trisaccharides.[10−15] We have also shown that vinyl β-galactoside is a good substrate for such reactions, leading in ice to very large amounts of disaccharide.[16] In a very elegant strategy, Withers et al.[17] have obtained very high yields of transglycosylation products when using a fluoro-α-glycoside as a donor and glycosidase mutants devoid of one of the carboxylates involved in the catalytic activity. These new enzymes catalyse efficiently the synthesis of a disaccharide by one nucleophilic attack but they are unable to induce the hydrolysis of this product. Considering the problem of regioselectivity, some natural enzymatic activities are also known to be highly regioselective. Good examples are the β-galactosidases from *Bacillus circulans*[13] or from *Bacillus singularis*[15] which allow a quasi-unique synthesis of β-[1→4]-disaccharides. The aim of this paper is to present the transglycosylation potential of a β-glycosidase from the thermophilic microorganism *Thermus thermophilus*. This enzyme (Tt-β-Gly), which has been cloned and overexpressed[18] into *E. coli*, is able to catalyse mainly the synthesis of β-(1→3)-linked disaccharides.

Results and Discussion

The study of a thermophilic glycosidase is of great interest since a very efficient catalyst can be obtained without carrying out a tedious purification procedure. Thus, the

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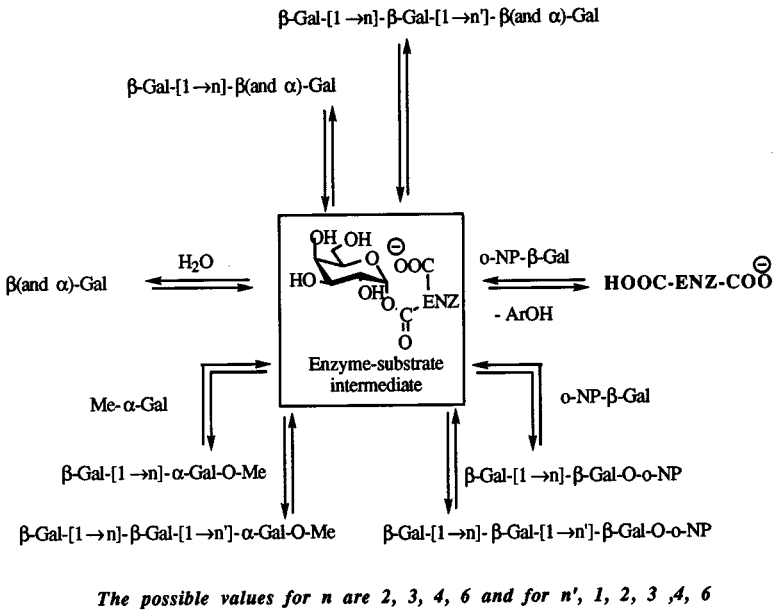


Scheme 1. Hydrolysis and possible transglycosylation reactions induced by a β-galactosidase; HOOC-ENZ-COO$^-$ represents the enzyme and the two carboxylate groups involved in the catalysis

gene tt-β-gly encoding a β-glycosidase has been cloned from *Thermus thermophilus* and overexpressed into *E. Coli*.[18] The amount of this enzyme can reach up to 50% of the total protein fraction in the cells of the host microorganism. After lysis of the cell membranes, simple heating at 65°C destroys all the glycosidasic activities normally present in the non-thermophilic species, apart from that of Tt-β-Glyc. In this work, the soluble fraction of the heated cells were used as a biocatalyst without any further purification.

Preliminary kinetic studies[18] have shown that the hydrolytic activity using *p*-nitrophenyl glycosides as substrates is higher for β-D-fucoside than for β-D-glucoside and β-D-galactoside (the respective values for k_{cat}/K_M are 713, 522 and 39 $mM^{-1}s^{-1}$). Furthermore, the regioselectivity exerted by this enzyme seems to be mainly of the β-[1→3]-type since the initial rate of hydrolysis of the β-[1→3]-disaccharides is higher than the rate of hydrolysis of all the other disaccharides tested.[18]

Since only a few glycosidases exhibit such a regioselectivity, an enzyme which could catalyse the synthesis of the β-[1→3]-glucosidic or β-[1→3]-galactosidic bonds would be of great interest considering the biological importance of the saccharides involving such structures. For instance, the β-D-Gal-[1→3]-D-Gal connection constitutes a part of the linkage region between the glycosaminoglycan chains and the protein parts in serine-linked connective tissue proteoglycans[19]. The β-Gal-[1→3]-α-GalNAc-O-Ser, known as the Thomsen-Friedenreich antigen determinant, has also been synthesized using the β-[1→3]-galactosidase from bovine testes.[20][21]

Thus, we have undertaken the study of the ability of Tt-β-Glyc to catalyse the synthesis of the β-[1→3]-glycosidic linkage using *o*-(or *p*-)nitrophenyl β-galactoside, glucoside and fucoside as donors in the presence of different acceptors. Table 1 indicates all the reactions studied in this work.

Table 1. Enzymatic transglycosylation reactions studied in this work; *o*-NP-β-Gal and *o*-NP-β-Glc: *o*-nitrophenyl β-D-galactopyranoside and β-D-glucopyranoside; *p*-NP-β-Fuc: *p*-nitrophenyl β-D-fucopyranoside; Me-α-Gal and Me-α-Glc: methyl α-D-galactopyranoside and glucopyranoside

Entry	Donor	Acceptor
1	*o*-NP-β-Gal	*o*-NP-β-Gal
2	*o*-NP-β-Gal	Me-α-Gal
3	*o*-NP-β-Gal	Me-α-Glc
4	*o*-NP-β-Glc	*o*-NP-β-Glc
5	*o*-NP-β-Glc	Me-α-Glc
6	*p*-NP-β-Fuc	*p*-NP-β-Fuc
7	*p*-NP-β-Fuc	Me-α-Gal

The kinetics of the reactions were monitored by means of ^{1}H-NMR spectroscopy at 500 MHz considering the anomeric proton resonances region (between δ = 4.2 and 5.2). To give an example, Figure 1 represents a mixture obtained with *o*-NP-β-Gal as a donor and Me-α-Gal as an acceptor (Table 1, Entry 2). The disaccharides were isolated by means of liquid chromatography (see Experimental Section). From the analysis of the ^{1}H-NMR spectra, the stereospecificity of the enzyme is clearly β since the 1-H signal always exhibits a high coupling constant. The structure of the regioisomers was elucidated by means of combined one- and two-dimensional ^{1}H- and ^{13}C-NMR experiments resulting in a complete analysis of the ^{13}C-NMR resonances. For instance, the ^{13}C-NMR spectrum of the disaccharide shown in Figure 2 clearly indicates that the linkage is not β-[1→6] since signals of two methylene groups are shifted to higher fields (δ = 61). Conversely, the resonance at lower field (δ = 82, C-3) strongly suggests a β-[1→3] linkage.[22] Figures 3,

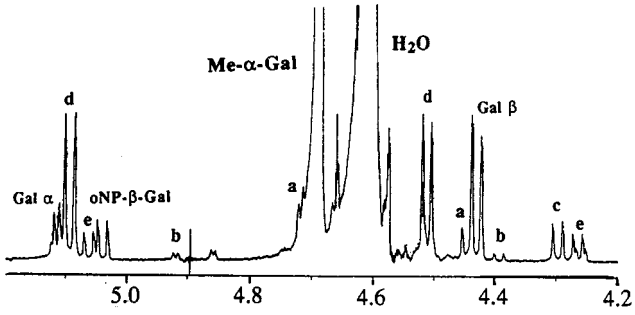


Figure 1. ^{1}H-NMR spectrum (500 MHz, solvent: D_2O) of a typical mixture obtained in the reaction of *o*-NP-β-Gal with Me-α-Gal using Tt-β-Gly as a catalyst; only the anomeric resonances are shown; a = β-Gal-[1→3]-β-Gal-O-Me; b = β-Gal-[1→2]-β-Gal-O-Me; c = β-Gal-[1→6]-β-Gal-O-Me); d = β-Gal-[1→3]-β-Gal-O-*o*-NP; e = β-Gal-[1→6]-β-Gal-O-*o*-NP

4, 5, 6 and 7 show the kinetic curves for the reactions (Table 1, entries 1, 2, 4, 5 and 7, respectively).

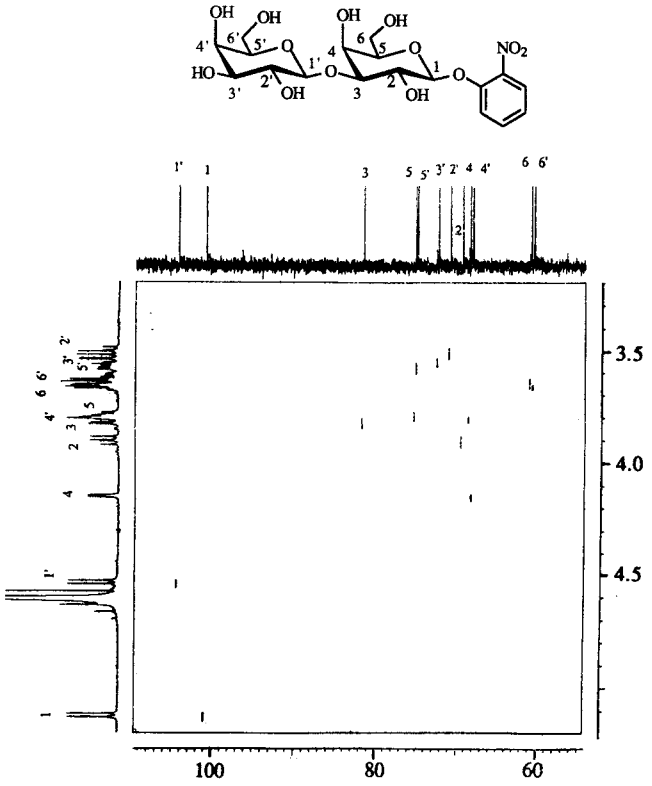


Figure 2. Two-dimensional proton-carbon correlation spectra of β-Gal-[1→3]-β-Gal-O-*o*-NP (solvent D_2O)

Although this glycosidase seems to exhibit mainly a β-fucosidase selectivity, the first reaction studied was a galactosidase transferase activity (Table 1, Entry 2). Considering this reaction, we were surprised to note that, even in the presence of a large excess (threefold) of the acceptor, the faster reaction was the autocondensation of the donor producing large amounts of *o*-nitrophenyl β-D-galactopyranosyl-[1→3]-β-D-galactopyranoside (β-Gal-[1→3]-β-Gal-O-*o*-NP, see Scheme 2, Figure 4). At the maximum concentration of this disaccharide, less than 10% of the other disaccharides were present in the reaction medium. After only

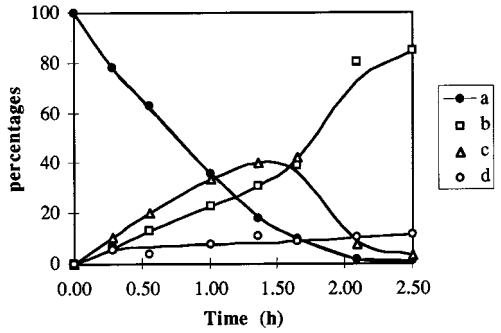


Figure 3. Kinetic study at 20°C of the autocondensation of *o*-NP-β-D-Gal catalysed by Tt-β-Gly; a = *o*-NP-β-Gal; b = Gal; c = β-Gal-[1→3]-β-Gal-O-*o*-NP; d = β-Gal-[1→6]-β-Gal-O-*o*-NP; the percentages are molar percentages of the initial *o*-NP-β-Gal

30 min of incubation at 55°C (2.5 h at 20°C), β-Gal-[1→3]-β-Gal-O-*o*-NP was completely hydrolysed, thus liberating *o*-NP-β-Gal which participated in the synthesis of other disaccharides by condensation with Me-α-Gal. This reaction has obviously no synthetic interest since a complex mixture of several disaccharides was obtained. Next, we have decided to study the same system but without the acceptor Me-α-Gal (Table 1, Entry 1). The results of the autocondensation given in Scheme 2 and Figure 3 show the ability of Tt-β-Glyc to catalyse the regioselective synthesis of β-Gal-[1→3]-β-Gal-O-*o*-NP with good yields. It should be pointed out that it is more convenient to perform this reaction at 20°C rather than at 55°C because, with the latter conditions, the maximum concentration of β-Gal-[1→3]-β-Gal-O-*o*-NP and the hydrolysis of this disaccharide occur in a very short time.

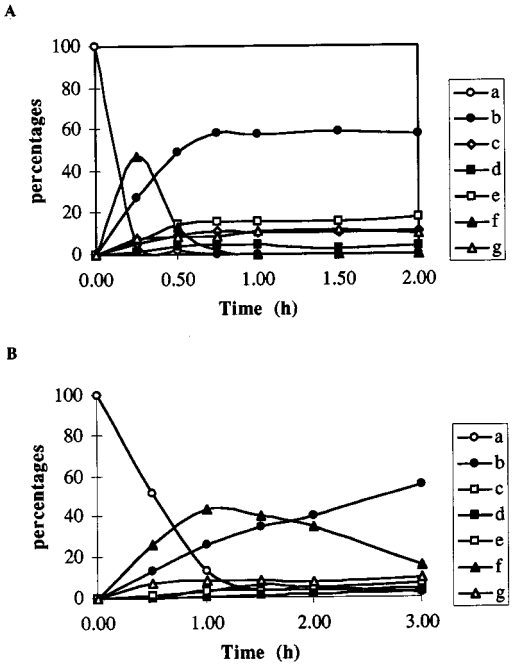


Figure 4. Kinetic study of the reaction of *o*-NP-β-Gal with Me-α-D-Gal catalysed by Tt-β-Gly; a = *o*-NP-β-Gal; b = Gal; c = β-Gal-[1→3]-β-Gal-O-Me; d = β-Gal-[1→2]-β-Gal-O-Me; e = β-Gal-[1→6]-β-Gal-O-Me); f = β-Gal-[1→3]-β-Gal-O-*o*-NP; g = β-Gal-[1→6]-β-Gal-O-*o*-NP; **A** = at 50°C and **B** = at 20°C; the percentages are molar percentages of the initial *o*-NP-β-Gal

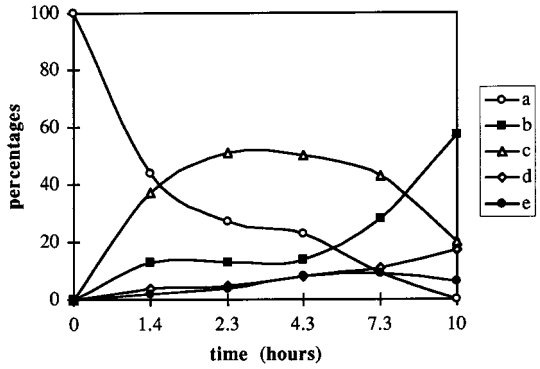


Figure 5. Kinetic study at 55°C of the autocondensation of *o*-NP-β-Glc catalysed by Tt-β-Gly; a = *o*-NP-β-Glc; b = Glc; c = β-Glc-[1→3]-β-Glc-O-*o*-NP; d = β-Glc-[1→6]-β-Glc-O-*o*-NP; e = represents the sum of the other disaccharides β-Glc-[1→*n*]-β-Glc-O-*o*-NP); the percentages are molar percentages of the initial *o*-NP-β-Glc

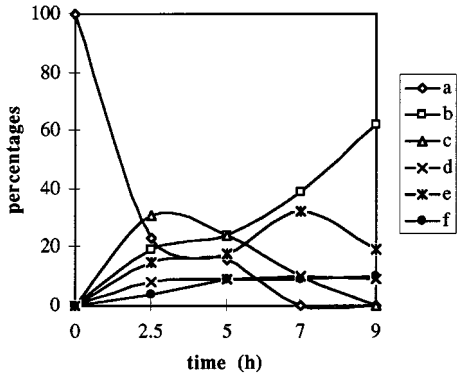
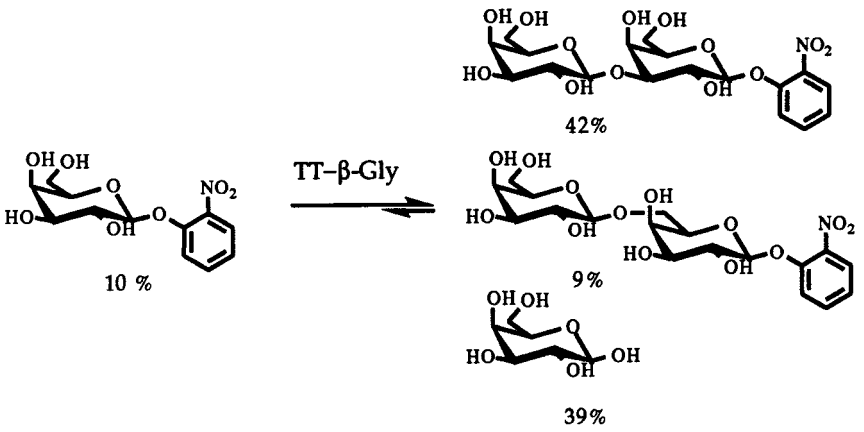


Figure 6. Kinetic study at 55°C of the reaction of *o*-NP-β-D-Glc with Me-α-D-Glc catalysed by Tt-β-Gly; a = *o*-NP-β-Glc; b = Glc; c = β-Glc-[1→3]-β-Glc-O-*o*-NP; d represents the sum of the other disaccharides β-Glc-[1→*n*]-β-Glc-O-*o*-NP; e = β-Glc-[1→3]-β-Glc-O-Me; f represents the sum of the other disaccharides β-Glc-[1→*n*]-β-Glc-O-Me; the percentages are molar percentages of the initial *o*-NP-β-Glc

Similar results were observed when the nature of the acceptor was changed. For instance, the use of Me-α-Glc as an acceptor did not dramatically affect the course of the reaction. The major product was the β-Gal-[1→3]-β-Gal-O-*o*-NP while minor amounts of β-Gal-[1→*n*]-α-Glc-OMe were synthesized (mainly β-Gal-[1→3]-α-Glc-O-Me). Another interesting feature of this enzyme lies in its capacity to discriminate between donors. While a rather broad range of nitrophenyl glycoside donors are recognized by Tt-β-Glyc, the nature of the aglycon part seems to play an important role. For instance, vinyl galactoside[16] which is accepted by several β-galactosidases (*E. coli*, *Aspergillus oryzae*) does not react at all in the presence of Tt-β-Gly.

The experiments in the presence of *o*-NP-β-Glc as a donor are performed at 55°C because the rate of the reactions was much too slow at room temperature. This rather curious behaviour, since the k_{cat}/K_M value is higher for *o*-NP-β-Glc than for *o*-NP-β-Gal, can be explained by considering the competitive inhibitor action of the donor used.[18] Furthermore and for similar reasons, lower concentrations were used in that case. Here again, we studied the autocondensation of *o*-NP-β-Glc (see Figure 6). The results are consistent with the previous reaction concerning the autocondensation of *o*-NP-β-Gal: The major product was β-Glc-[1→3]-β-Glc-O-*o*-NP with a 50% yield while only small amounts of the other disaccharides were formed (less than 10% as an overall yield). Meanwhile, in the case of *o*-NP-β-Glc, the rates of both reactions were reduced compared to that of *o*-NP-β-Gal. For instance, the maximum percentage of β-Glc-[1→3]-β-Glc-O-*o*-NP was reached within 2.5 h at 55°C, while the maximum percentage of β-Gal-[1→3]-β-Gal-O-*o*-NP was obtained in the same conditions within 0.25 h. Conversely, Tt-β-Gly induced a relatively fast transglycosylation reaction with the acceptor Me-α-Glc (see Figure 6). After 7 h of incubation, the reaction mixture was composed of 32% β-Glc-[1→3]-α-Glc-O-Me, about 10% of the other β-Glc-[1→*n*]-α-Glc-O-Me and 20% of all the β-Glc-[1→*n*]-β-Glc-O-*o*-NP. Despite the complex mixture obtained in this reaction, it was possible to purify the β-Glc-[1→3]-α-Glc-O-Me since the β-Glc-[1→*n*]-β-Glc-O-*o*-NP are retained on charcoal/Celite (see Experimental Section).

While transglycosylation using *o*-NP-β-Gal and *o*-NP-β-Glc as donors was markedly characterized by fast autocondensation reactions, such a behaviour was not observed with *p*-NP-β-Fuc. In that case, the study of the autocondensation (Table 1, Entry 6) showed that only very small amounts of disaccharide (less than 10%) were synthesized



Scheme 2. Autocondensation reaction of *o*-NP-β-Gal catalysed by Tt-β-Gly

while all the donor was hydrolysed. Due to the high cost of *p*-NP-β-D-Fuc, we investigated the transglycosylation with only one acceptor: Me-α-D-Gal (Table 1, Entry 7). Mainly, two disaccharides were present in the reaction mixture: methyl β-D-fucopyranosyl-[1→3]-β-D-galactopyranoside and its corresponding β-fuc-[1→6] regioisomer, the latter being largely predominant (Figure 7).

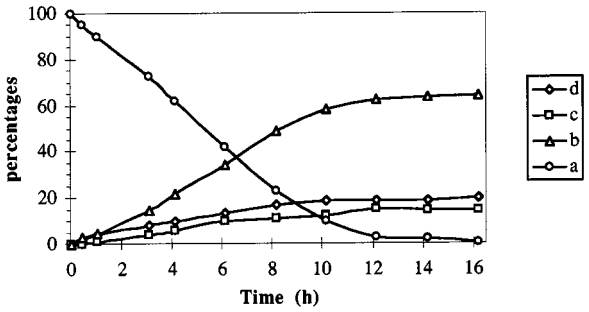


Figure 7. Kinetic study at 20°C of the reaction of *p*-NP-β-Fuc with Me-α-Gal catalysed by Tt-β-Gly; a = *p*-NP-β-Fuc; b = Fuc; c = β-Fuc-[1→6]-β-Gal-O-Me; d represents the sum of the other disaccharides β-Fuc-[1→*n*]-β-Gal-O-Me; the percentages are molar percentages of the initial *p*-NP-β-Fuc

In conclusion, we have shown the potential of the thermophilic Tt-β-Gly to catalyse the efficient synthesis of β-[1→3] disaccharides in the presence of nitrophenyl glycosides as donors in the following cases:

- autocondensation of *o*-NP-β-Gal and of *o*-NP-β-Glc providing good yields of the corresponding β-[1→3] disaccharides,
- synthesis of β-Glc-[1→3]-α-Glc-O-Me with moderate yield,
- synthesis of methyl β-D-fucopyranosyl-(1→6)-β-D-galactopyranoside (17% yield).

Experimental Section

General: The crude extract of *E. coli* cells (BL21pETbg8) in which the β-glycosidase from *Thermus thermophilus* had been overexpressed were used as a biocatalyst and prepared according to ref.[18] The chemicals supplied by Aldrich were used without further purification. The course of the reactions was monitored by means of TLC (precoated silica gel 60 sheets Merck F254) and ^{1}H-NMR spectroscopy. The components of the reaction mixtures were separated on charcoal (Darco G-60, 100 mesh, Aldrich)/Celite (Fluka 535) (1:1) columns (Seymour eluent: MeOH/$CHCl_3$/AcOH/H_2O, 60:30:6:4) and on silica gel columns. Complete analysis of the ^{1}H- and ^{13}C-NMR resonances and subsequent structure assignment were made using standard 2D sequences (COSY HH and HCOR correlations). The spectra were recorded with a Bruker AX500 spectrometer operating at 500 MHz for ^{1}H (solvent D_2O, chemical shifts in ppm quoted from H_2O resonance at δ = 4.6) and 126 MHz for ^{13}C (solvent D_2O, chemical shifts in ppm quoted from the methyl resonance of acetone at δ = 29.8).

Kinetic Study of the Autocondensation Reaction of *o*-NP-β-Gal Catalysed by Tt-β-Glyc: 100 mg (0.33 mmol) of *o*-NP-β-Gal was dissolved in 1.85 mL of phosphate buffer (0.05 M, pH = 7.0). 23 units (calculated from the hydrolysis of *o*-NP-β-Gal at 20 °C) were added and the solution was stirred at 20 °C or at 55 °C. Aliquots of 180 μL of the reaction mixture were taken off at different times. For each sample, the reaction was quenched by addition of 15 μL of 1 M NaOH. The water was then removed under reduced pressure. The powder obtained was redissolved in D_2O and the solution was submitted to quantitative ^{1}H-NMR analysis. A known amount of tetramethylurea was added as an internal standard.

Synthesis of β-Gal-[1→3]-β-Gal-O-*o*-NP and β-Gal-[1→6]-β-Gal-O-*o*-NP: 500 mg (1.66 mmol) of *o*-NP-β-Gal was dissolved in 9 mL of phosphate buffer (0.05 M, pH = 7.0). The enzymatic preparation (7.4 units, calculated from the hydrolysis of *o*-NP-β−Gal at 20°C) was added and the solution was stirred at room temperature. After 90 min of incubation, the reaction was quenched by the addition of 200 μL of 1 M NaOH. 500 mg of silica gel 60 (Merk F254) was added under stirring to the resulting solution and the water was removed under reduced pressure. The powder obtained was deposited on top of a silica gel column (3.5 cm diameter, 100 g of silica gel) and the products were allowed to elute (eluent: dichloromethane/methanol, 3:2). The course of the purification was monitored by TLC (precoated silica gel 60 plates, Merk F254). *o*-NP-β-Gal (44 mg) was eluted first, then β-Gal-[1→3]-β-Gal-O-*o*-NP (R_f = 0.67 with the eluent as above) was obtained (35 mg). Further elution gave a 71:29% mixture of both β-[1→3] and β-[1→6] regioisomers (109 mg). Overall yield for β-Gal-[1→3)]-β-Gal-O-*o*-NP: 30%.

β-Gal-[1→3)]-β-Gal-O-*o*-NP: ^{1}H NMR (D_2O): δ = 3.50 (dd, $^3J_{2'\text{-H},1'\text{-H}}$ = 7.7 Hz, $^3J_{2'\text{-H},3'\text{-H}}$ = 10.5 Hz, 2′-H), 3.54 (dd, $^3J_{3'\text{-H},2'\text{-H}}$ = 10.5 Hz, $^3J_{3'\text{-H},4'\text{-H}}$ = 3.0 Hz, 3′-H), 3.57 (m, 5′-H), 3.68−3.60 (m, 4 H, 6-H and 6′-H), 3.78 (m, 5-H), 3.80 (d, 4′-H), 3.81 (dd, $^3J_{3\text{-H},4\text{-H}}$ = 2.8 Hz, $^3J_{3\text{-H},2\text{-H}}$ = 9.6 Hz, 3-H), 3.90 (dd, $^3J_{2\text{-H},1\text{-H}}$ = 7.7 Hz, $^3J_{2\text{-H},3\text{-H}}$ = 9.6 Hz, 2-H), 4.14 (d, $^3J_{4\text{-H},3\text{-H}}$ = 2.8 Hz, 4-H), 4.43 (d, $^3J_{1'\text{-H},2'\text{-H}}$ = 7.7 Hz, 1′-H), 5.12 (d, $^3J_{1\text{-H},2\text{-H}}$ = 7.8 Hz, 1-H), 7.15, (t, 1 H, aromatic H), 7.32, (d, 1 H, aromatic H), 7.58, (t, 1 H, aromatic H), 7.81, (d, 1 H, aromatic H). − ^{13}C NMR (D_2O): δ = 60.6 (C-6 or C-6′), 60.9 (C-6 or C-6′), 68.2 (C-4), 68.6 (C-4′), 69.5 (C-2), 71.0 (C-2′), 72.5 (C-3′), 75.1 (C-5′), 75.3 (C-5), 81.7 (C-3), 100.9 (C-1), 104.3 (C-1′), 117.7 (aromatic C), 123.1 (aromatic C), 125.6 (aromatic C), 135.1 (aromatic C). − $C_{18}H_{25}NO_{13}$ (463): calcd. C 46.65, H 5.40, n 3.02; found C 46.59, H 5.36, n 3.12.

β-Gal-(1→6)-β-Gal-O-*o*-NP: ^{1}H NMR (D_2O): δ = 3.38 (dd, $^3J_{2'\text{-H},1'\text{-H}}$ = 7.6 Hz, $^3J_{2'\text{-H},3'\text{-H}}$ = 9.9 Hz, 2′-H), 3.44 (dd, $^3J_{3'\text{-H},2'\text{-H}}$ = 9.9 Hz, $^3J_{3'\text{-H},4'\text{-H}}$ = 3.5 Hz, 3′-H), 3.48 (m, 5′-H), 3.62 (m, 2 H, 6′-H), 3.74 (dd, $^3J_{2\text{-H},3\text{-H}}$ = 9.7 Hz, $^3J_{2\text{-H},1\text{-H}}$ = 7.8 Hz, 2-H), 3.77 (d, $^3J_{4'\text{-H},3'\text{-H}}$ = 3.3 Hz, 4′-H), 3.89 (d, $^3J_{4\text{-H},3\text{-H}}$ = 3.9 Hz, 4-H), 3.98 (m, 5-H), 4.28 (d, $^3J_{1'\text{-H},2\text{-H}'}$ = 7.7 Hz, 1′-H), 5.08 (d, $^3J_{1\text{-H},2\text{-H}}$ = 7.8 Hz, 1-H), 7.15 (t, 1 H, aromatic H), 7.41(d, 1 H, aromatic H), 7.58 (t, 1 H, aromatic H), 7.82 (d, 1 H, aromatic H). − ^{13}C NMR (D_2O): δ = 60.9 (C-6′), 68.5 (C-4), 68.6 (C-4′), 68.8 (C-6), 70.2 (C-2), 70.7 (C-2′), 71.9 (C-3), 72.6 (C-3′), 74.6 (C-5), 75.1 (C-5′), 101.0 (C-1), 103.2 (C-1′), 118 (aromatic C), 123 (aromatic C), 126.5 (aromatic C), 135.2 (aromatic C). − $C_{18}H_{25}NO_{13}$ (463): calcd. C 46.65, H 5.40, n 3.02; found C 46.61, H 5.35, n 3.09 (the analysis was performed using the mixture of the two regioisomers).

Kinetic Study of the Transglycosylation Reaction of *o*-NP-β-Gal with Me-α-Gal (or with Me-α−Glc) Catalysed by Tt-β-Gly: 70 mg (0.23 mM) of *o*-NP-β-Gal and 135 mg (0.70 mM) of Me-α-Gal (or Me-α-Glc) were dissolved in 1.3 mL of phosphate buffer (0.05 M, pH = 7.0). The mixture was stirred at room temperature or at 55°C and 16 units of the enzyme were added. Samples of 180 μL of the reaction mixture were taken off at different times. For each sample, the reaction was quenched by addition of 15 μL of 1 M NaOH. The water was then removed under reduced pressure. The powder thus obtained was redissolved in D_2O and the solution was submitted

to quantitative ^{1}H-NMR analysis. A known amount of tetramethylurea was added as an internal standard.

Kinetic Study of the Autocondensation Reaction of the *o*-NP-β-Glc Catalysed by Tt-β-Gly: This experiment was conducted directly in the NMR tube. In order to reduce as far as possible the amount of H_2O (and of hydroxy protons), the 0.1 M phosphate buffer (pH = 7) was prepared in distilled water. This solution was lyophilised and the powder was dissolved in D_2O and lyophilised once more. Then, an exact volume of D_2O was added in order to obtain the 0.1 M concentration. Similarly, the enzymatic preparation (1 unit for 8 μL) of Tt-β-Gly was lyophilised, washed with D_2O, lyophilised again and dissolved in a known volume of D_2O. In order to exchange the hydroxy protons, a similar treatment was also applied to *o*-NP-β-Glc. Thus, 556 μL of the buffer containing known amounts of tetramethylurea as an internal standard, 50 μmol (15 mg) of *o*-NP-β-Glc and 21 μL (2.6 units) of the enzymatic preparation were introduced into a 5-mm NMR tube. This mixture was incubated at 55°C for 7 h. From time to time, the solution was submitted to ^{1}H-NMR spectroscopy for quantitative analysis. After a reaction time of 5 h, some *o*-nitrophenol precipitated. The latter was redissolved by adding 40 μL of [D$_7$]DMF. At the end of the study (t = 7 h), the *o*-nitrophenol which precipitated once more was filtered before recording the last NMR spectrum. At that time, the *o*-NP-β-Glc was totally consumed.

Kinetic Study of the Transglycosylation Reaction of *o*-NP-β-Glc with Me-α-Glc: A procedure and quantities similar to those above were used apart from the supplementary addition of 150 μmol (29 mg) of Me-α-Glc (the hydroxy protons were pre-exchanged with D_2O).

Synthesis of β-Glc-[1→3]-α-Glc-O-Me: 602 mg (2 mmol) of *o*-NP-β-Glc and 1156 mg (6 mmol) of Me-α-Glc were dissolved in 22 mL of phosphate buffer (0.1 M, pH = 7.0). The enzymatic preparation (105 units, calculated from the hydrolysis of *o*-NP-β−Glc at 20°C) was added and the solution was stirred at 55°C. After 7 h of incubation, the reaction was quenched by addition of 330 μL of 1 M NaOH. The water was removed under reduced pressure and the compounds were separated on a charcoal/Celite column. The eluent was a mixture of ethanol and water (an ethanol gradient was used). Under such conditions all of the *o*-nitrophenyl saccharides were retained. The first fractions, eluted with a 5% ethanol/water mixture contained the glucose (135 mg) while the second ones were composed of Me-α-Glc (1040 mg).The disaccharides were allowed to elute with a 10% ethanol/water solution. β-Glc-[1→3]-α-Glc-O-Me came first (R_f = 0.37, Seymour eluent), but it was difficult to obtain this disaccharide as a pure compound. Several of the first fractions were mixed giving 150 mg of β-Glc-[1→3]-α-Glc-O-Me contaminated with less than 10% of other disaccharides. Further elution provided 61 mg of a mixture of β-Glc-(1→*n*)-α-Glc-OMe regioisomers. − ^{1}H NMR (400 MHz, D_2O): δ = 3.20 (t, $^3J_{2\text{-H},1\text{-H}} = {}^3J_{2\text{-H},3\text{-H}}$ = 10.6 Hz, 2-H), 3.25 (t, 1H), 3.28 (s, 3 H, CH_3), 3.3−3.8 (m, 10 H), 4.55 (d, $^3J_{1'\text{-H},2'\text{-H}}$ = 9.8 Hz, 1 H, 1′-H), 4.67 (d, $^3J_{1\text{-H},2\text{-H}}$ = 4.9 Hz, 1 H, 1-H). − ^{13}C NMR (250 MHz, D_2O): δ = 54.9 (OCH_3), 60.6 and 60.7 (C-6 and C-6′), 68.1 (C-4), 69.6 (C-2), 70.6 (C-2′), 71.4 (C-3′), 75.4 (C-5), 75.6 (C-5′), 75.9 (C-4′), 82.7 (C-3), 99.0 (C-1), 102.6 (C-1′). − $C_{13}H_{24}O_{11}$ (356): calcd. C 43.82, H 6.74; found C 43.72, H 6.69 (the analysis was performed using the mixture of the two regioisomers).

Kinetic Study of the Transglycosylation Reaction of *p*-NP-β−Fuc with Me-α-Gal: This experiment was performed at 20°C in an NMR tube under the same conditions as those used for the study of the reaction of *o*-NP-β-Glc with Me-α-Glc. The concentrations were lower because *p*-NP-β-Fuc is less soluble than *o*-NP-β-Glc. Thus, 14 mg (50 μmol) of *p*-NP-β-Fuc, 29 mg (150 μmol) of Me-α-Gal and 583 μL of the phosphate buffer were mixed with 1.8 units of the enzyme.

Synthesis of β-Fuc-[1→6]-β-Gal-O-Me: 85 mg (300 μmol) of *p*-NP-β-Fuc and 174 mg (900 μmol) of Me-α-Gal were dissolved in 2.9 mL of phosphate buffer (0.1 M; pH = 7.0). The enzymatic preparation (10.5 units, calculated from the hydrolysis of *o*-NP-β-Fuc) was added and the solution was incubated at 50°C for 5 h. Then the reaction was quenched by addition of 60 μL of 1 M NaOH. The water was removed under reduced pressure and the compounds were separeted on a charcoal/Celite column. The eluent was a mixture of ethanol and water. Under such conditions, all of the *o*-nitrophenyl saccharides were retained. The first fractions, eluted with a 5% ethanol/water mixture contained fucose and Me-α-Gal. The disaccharides were then eluted with a 25% ethanol/water solution. The fractions collected with 102 mL of the last eluent afforded 14 mg of a mixture composed of 60% β-Fuc-[1→6]-α-Gal-O-Me and 40% of the β-Fuc-[1→3]-α-Gal-O-Me. 10 mg more (92% and 8% of β-[1→6]- and the β-[1→3] disaccharides respectively) was collected with 80 mL of the eluent. Overall yield of β-Fuc-[1→6]-α-Gal-OMe: 17%. − $C_{13}H_{24}O_{10}$ (340): calcd. C 45.88, H 7.06; found C 43.72, H 6.69 (the analysis was performed using the 92:8% mixture of the two regioisomers).

β-Fuc-[1→6]-β-Gal-OMe: ^{1}H NMR (500 MHz, D_2O): δ = 1.23 ($^3J_{5'\text{-H},CH3}$ = 6.8 Hz, 3 H, CH_3), 3.28 (s, 3 H, OCH_3), 3.43 (dd, $^3J_{2'\text{-H},1'\text{-H}}$ = 7.9 Hz, $^3J_{2'\text{-H},3'\text{-H}}$ = 10.1 Hz, 1 H, 2′-H), 3.58 (dd, $^3J_{3'\text{-H},2'\text{-H}}$ = 10.1 Hz, $^3J_{3'\text{-H},4'\text{-H}}$ = 3.4 Hz, 1 H, 3′-H), 3.69 (d, $^3J_{4'\text{-H},3'\text{-H}}$ = 3.4 Hz, 1 H, 4′-H), 3.73 (dd, $^3J_{5'\text{-H},CH3}$ = 6.8 Hz, 1 H, 5′-H), 3.77 (m, 2 H, H-2, 3-H), 3.77 (dd, $^3J_{6b\text{-H},5\text{-H}}$ = 8.33 Hz, $^2J_{6b\text{-H},6a\text{-H}}$ = 11.3 Hz, 1 H, 6b-H), 3.96 (dd, $^3J_{6a\text{-H},5\text{-H}}$ = 4.3 Hz, $^2J_{6a\text{-H},6b\text{-H}}$ = 11.3 Hz, 1 H, 6a-H), 4.04 (dd, $^3J_{5\text{-H},6a\text{-H}}$ = 4.3 Hz, $^3J_{5\text{-H},6b\text{-H}}$ = 8.3 Hz, 1 H, 5-H), 4.38 (d, 1 H, 1′-H), 4.8 (d, 1 H, 1-H). − ^{13}C NMR (500 MHz, D_2O): δ = 15.3 (CH_3), 55.4 (OCH_3), 68.0 (C-6), 69.2, 69.2, 69.0 (C-2, C-3, C-4), 69.4 (C-5), 70.5 (C-2′), 70.9 (C-5′), 71.2 (C-4′), 72.8 (C-3′), 99.5 (C-1), 103.1 (C-1′).

β-Fuc-[1→3]-β-Gal-OMe: ^{1}H NMR (500 MHz, D_2O): δ = 1.08 (3 H, CH_3), 3.27 (s, 3 H, OCH_3), 3.40 (dd, $^3J_{2'\text{-H},1'\text{-H}}$ = 7.2 Hz, $^3J_{2'\text{-H},3'\text{-H}}$ = 9.9 Hz, 1 H, 2′-H), 3.50 (dd, $^3J_{3'\text{-H},2'\text{-H}}$ = 9.9 Hz, $^3J_{3'\text{-H},4'\text{-H}}$ = 2.9 Hz, 1 H, 3′-H), 3.58 (d, $^3J_{4'\text{-H},3'\text{-H}}$ = 2.9 Hz, 1 H, 4′-H), 3.61 (dd, 1 H, 5′-H), 3.77 (dd, $^3J_{3\text{-H},2\text{-H}}$ = 6.5 Hz, $^3J_{3\text{-H},4\text{-H}}$ = 2.70 Hz, 1 H, 3-H), 3.86 (dd, $^3J_{2\text{-H},3\text{-H}}$ = 6.53 Hz, 1 H, 2-H), 4.06 (d, $^3J_{4\text{-H},3\text{-H}}$ = 2.7 Hz, 1 H, 4-H), 4.43 (d, $^3J_{1'\text{-H},2'\text{-H}}$ = 7.2 Hz, 1 H, 1′-H), 4.72 (d, 1 H, 1-H). − ^{13}C NMR (500 MHz, D_2O): δ = 55.4 (CH_3), 61.1 (OCH_3), 67.3 (C-6), 69.0 (C-4), 69.4 (C-2), 70.4 (C-3′), 70.7 (C-5′), 70.9 (C-2′), 71.3 (C-4′), 79.3 (C-3).

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