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α-Galactosyl fluoride in transfer reactions mediated by the green coffee beans α-galactosidase in ice

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Abstract

We show that the yields in saccharide synthesis by tranglycosylation with α-galactosidase from green coffee beans can be greatly enhanced when working in ice. Thus, methyl α-D-galactopyranosyl-(1→3)-α-D-galactopyranoside (**3a**) produced by reaction of α-D-galactopyranosyl fluoride **1** with methyl α-D-galactopyranoside (**2**) is obtained with 51% yield in ice while only 29% is synthesized at 37 °C. This result, already previously found by others with proteases and by us with a β-galactosidase appears to be a general property of hydrolases. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: α-Galactosidases; Transglycosylation; Glycosidases in ice; Disaccharides; Trisaccharides

1. Introduction

The enzymatic synthesis of saccharides has become a powerful alternative compared to the chemical approach for the regioselective synthesis of the glycosidic bond.[1] Due to their stability and low cost, glycosidases are very attractive since these enzymes induce high stereoselective synthesis of the glycosidic bond.[2–5] Their main drawbacks arise from a partial regioselectivity and moderate yields since the transferase activity remains in competition with the hydrolysis of the substrate and of the synthesized glycosides. In order to improve the yields of the transglycosylation reactions, suitable experimental conditions and/or new enzymatic activities must be found. Thus, better yields are usually obtained when using a donor bearing a good leaving group at the anomeric position. Nitrophenylglycosides are the most widely employed for this purpose leading, for instance, to the synthesis of blood determinant di- and trisaccharides.[6–11] In this case, another drawback arises: the nitrophenylglycosides used as donors may also be recognized as acceptors by the glycosidases. Thus, in the presence of an acceptor, two transglycosylation reactions are in competition: autocondensation and condensation[12] leading to complex mixtures. The extent of the autocondensation reaction may be clearly reduced when operating with high acceptor/donor ratio (typically 10/1) but, in this case, the separation of reaction mixture components may be problematic due to the excess of donor. The use of suitable disaccharides (melibiose, maltose…) generally overcome this problem but the activation at the anomeric carbon is usually too low to afford high transglycosylation yields.

Considering the transferase activity of proteases, it has been already shown that the hydrolysis can be greatly reduced when operating in ice. Using this procedure, high yields in peptides synthesis were obtained.[13,14] When applied to β-galactosidase, we have shown similarly, in a previous paper, that the yield of transglycosylation was greatly improved in ice although reaction rates were low.[12] The aim of this paper is to evaluate the potential of α-D-galactopyranosyl fluoride **1** as a candidate for providing good yields in ice for the transglycosylation reaction.

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2. Results and discussion

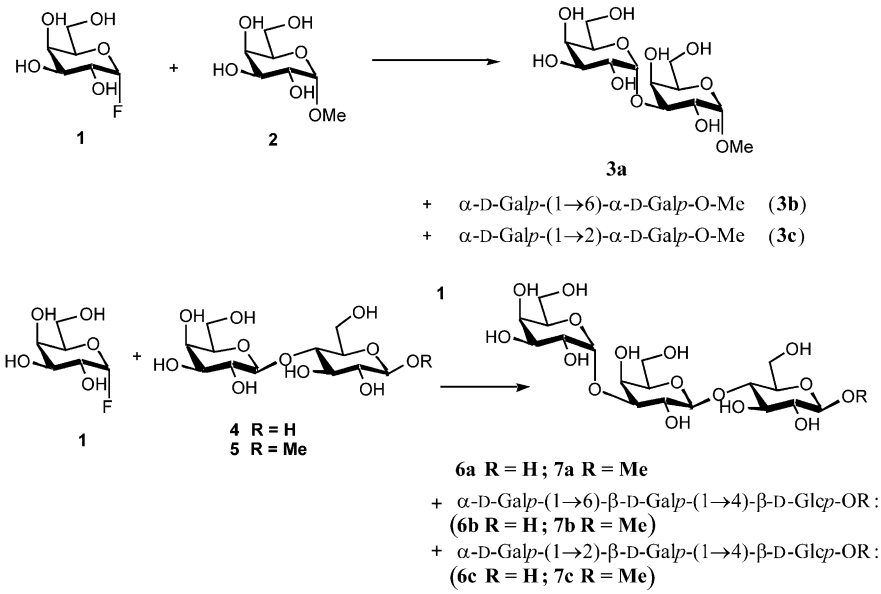
The ability of galactosylhydrolases to preserve their activity in ice seems to be dependent of at least two factors: (i) water solubility of the reactants must be as high as possible since the reaction is believed to occur in distinct lense-like liquid microinclusions containing high concentrations of the substrates and of the enzyme.[15] As the activity of water in such liquid phases is probably very low, the hydrolytic activity of the enzyme is therefore much more reduced than the transferring one; (ii) the class of galactosidase which also plays an important role.

The question of water solubility can be illustrated by the fact that *p*-nitrophenyl β-galactoside or its α anomer **8** and methyl α-galactoside **2** used, respectively as donor and acceptor in ice, have led to very low conversions. Conversely, in the presence of the same enzyme, a complete conversion was observed in ice when replacing **8** by the much more water soluble vinyl β-D-galactopyranoside.[12] Concerning the class of glycosidases, from our experience, all the thermophilic enzymes tested were unable to act in ice at the temperature of −15 °C. Such behavior has been observed with the β-glycosidase Tt-βgly from *Thermus thermophilus*[16] and with the α-galactosidase AgaB from *Bacillus stearothermophilus*.[17] Conversely, galactosidases from the mesophilic flora acted in these extreme conditions. It seems probable that psychrophilic glycosidases would be very good candidates for such reactions. Besides, related experiments are currently under investigation in our laboratory.

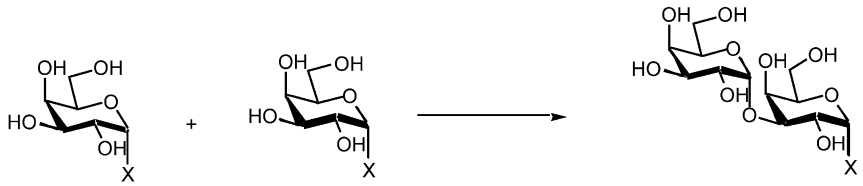
With the aim of extending our findings with β-galactosidases in ice, we have undertaken a similar study with the well known α-galactosidase from green coffee beans. As stated above, the donor substrate must be highly soluble in water and for this reason, we decided to use the vinyl α-D-galactopyranoside first. Unfortunately, the methods available for the synthesis of this compound are not very convenient for large-scale needs. So, we turned to the readily available fluoride **1**.[18,19] This compound is well accepted by α-galactosidases[20] as a donor and is also very soluble in water. Three acceptors have been tested in this work: **2**, lactose **4**, and methyl β-lactoside **5**. The last two compounds were chosen with the prospect to synthesize an important α-galactosyl epitope, α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose (**6a**) by using α-galactosidase from green coffee beans. The reactions studied are summarized in Scheme 1.

In order to get more information about the reactivity of **1**, we have undertaken the study of its autocondensation, i.e., the ability of this compound to act as a donor and an acceptor in transglycosylation reactions at room temperature (Scheme 2).

We were surprised to observe that unlike the behavior of donor **8**, very low amounts of autocondensation disaccharides were synthesized with donor **1** in our experimental conditions (donor/acceptor concentrations 90/270 mM). We have recently confirmed this property by means of ^{19}F NMR spectroscopy.[21] Fig. 1, which shows the kinetics of the self-condensations at 37 °C of **8** and **1** catalyzed by the α-galactosidase from green coffee bean, indicates that less than 10% of disaccharides were obtained with the latter versus 25% with the former at the same concentration. So, in addition to its high solubility in water, **1** is not a good acceptor for the enzyme studied. This property has an important appli-



Scheme 1. Transglycosylation reactions catalyzed by α-galactosidase from green coffee beans.



1 X = F
8 X= *O*-*p*-NO_2 Ph

9a X = F ; X = *O*-*p*-NO_2 Ph : **10a**
+ α-D-Gal*p*-(1→6)-α-D-Gal*p*-X :
X = F : **9b**; X = *O*-p-NO_2Ph : **10b**
+ α-D-Gal*p*-(1→2)-α-D-Gal*p*-X :
X = F : **9c**; X = p-NO_2Ph : **10c**

Scheme 2. Autocondensation transglycosylation reactions catalyzed by the α-galactosidase from green coffee beans using **1** and **8** as substrates.

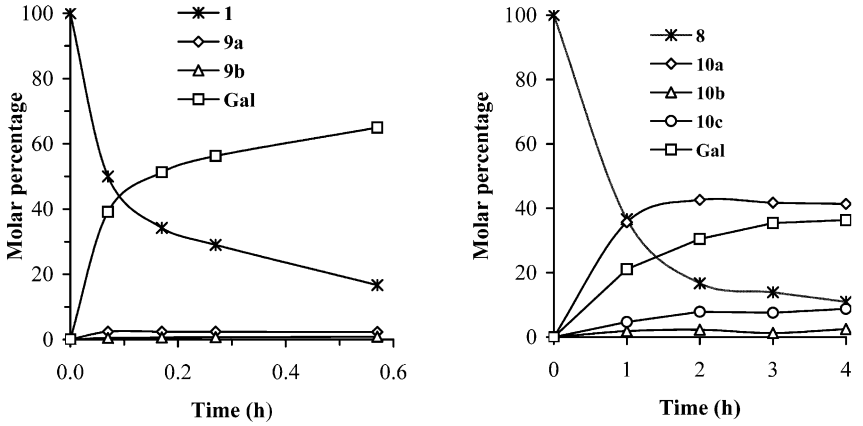


Fig. 1. Comparison of the ability of **1** and **8** as substrates to produce at 37 °C, the autocondensation reaction catalyzed by green coffee beans α-galactosidase. The relative proportions were determined from the proton NMR spectra using 10 mM trimethylsilyl–propansulfonate as a quantitative reference. Compounds **10a**, **10b** and **10c** are, respectively, 4-nitrophenyl (α-D-galactopyranosyl)-(1→3)-α-D-galactopyranoside, (1→6) and (1→2) regioisomers. **9a** and **9b** are the α-D-galactopyranosyl-(1→3)-α-D-galactopyranosyl fluoride and its 1→6 regioisomer.

cation in the condensation reactions since very low amounts (less than 5%) of the autocondensation disaccharides (at 37 °C) were present in the reaction media as shown by the ^{19}F NMR kinetic experiments performed for each reaction studied (data not shown).

Unfortunately, when the reactions were performed at 37 °C, this advantage became insignificant since the use of **1** instead of **8** as a donor in the transfer reaction in the presence of **2** as an acceptor did not provide higher yields in condensation disaccharides as seen in Fig. 2. This situation is understandable considering the rate of the hydrolysis of **1** which is faster than that of **8**. Thus, after 2 h reaction with **1**, 97% conversion, 53% hydrolysis and 29% synthesis of methyl (α-D-galactopyranosyl)-(1→3)-α-D-galactopyranoside (**3a**) were measured while in the case of **8**, after 6 h reaction, 80% conversion, 40% hydrolysis and 32% of **3a** were obtained.

The spontaneous hydrolysis of **1** was then suspected to be responsible for these results. Therefore, this spontaneous hydrolysis was quantified by ^1H NMR spectroscopy. Thus, we have shown, that at pH 7 in the buffer and at the concentrations used in our experiments (see Section 3), significant spontaneous hydrolysis occurred. For instance at 37 °C, after 2 h reaction, about 8% of **1** was hydrolyzed (see Fig. 3). Obviously, at lower temperatures, this drawback is minimized and even after several days in ice at −15 °C, less than 3% of **1** was hydrolyzed.

Considering the activity of glycosidases in ice, it was not necessary to study the kinetics of the reactions in order to optimize the yields of the transglycosylation reaction since we had already shown that the glycosidic bonds of the disaccharides synthesized were quite stable in such media.[12] Thus, we decided to quench the reactions in ice when most of **1** had disappeared. This was usually the case after 15 days reaction. In order to compare the results obtained in ice to the same reaction at 37 °C, we performed the latter in the same conditions except the reaction time which was chosen according to previous kinetic studies,[22] as that moment providing the maximum concentration of disaccharides. A comparison between each reaction can be made through Table

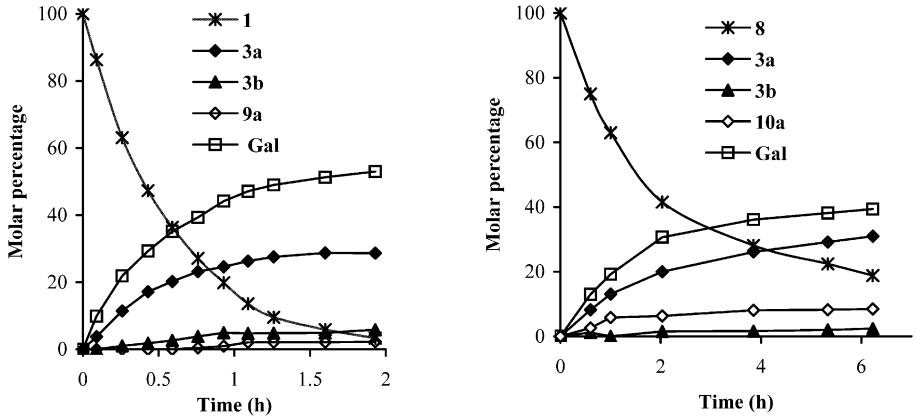


Fig. 2. Comparison of the ability of **1** and **8** to produce at 37 °C the transglycosylation reaction catalyzed by green coffee beans α-galactosidase using **2** as an acceptor. The relative proportion was determined from the proton NMR spectra using 10 mM trimethylsilyl–propansulfonate as a quantitative reference.

1 which shows that the reactions in ice provide, as expected, much higher transglycosylation yields than at 37 °C.

This is particularly true for the reaction using **2** as an acceptor as shown in Fig. 4 which represents a part of the proton NMR spectra of the reaction mixtures obtained in both conditions. Thus, a yield of 51% for **3a**, calculated from the integrations of the anomeric signals was obtained in ice while 10% of remaining donor was present. In the same conditions, at 37 °C, the calculated yield for **3a** was 29% while the donor was completely consumed.

The same was true for the reactions using **4** and **5** as acceptors, although the yields of transglycosylation products were lower than in the case of **2**, despite higher donor/acceptor ratio were used in the case of reactions with **4** and **5** (see Table 1 and Section 3). Other products synthesized from the condensation with galactose were also present (10–15%). Moreover, the regioselectivity of these two reactions was not as good as that observed with **2**. Thus besides the α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose (**6a**, α and β anomers) or methyl (α-D-galactopyranosyl)-(1→3)-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (**7a**) which were the main trisaccharides synthesized, noticeable amounts of the regioisomers (**6b** or **7b** and **6c** or **7c**) were also present (see Table 1). The structure of disaccharides **3a**, **3b** and **3c** has already been established elsewhere[4,6–12,16,17] while that of trisaccharides **6a**, **6b**, **6c**, **7a**, **7b** and **7c** was deduced by comparison of the ^{1}H and ^{13}C NMR parameters with available data[23] and with the corresponding NMR data of the disaccharide series. Thus, the ^{1}H and ^{13}C NMR chemical shifts of the anomeric protons are very characteristic of the type of glycosidic bond and their value is not affected to a large extent by the substituents of the glycoside at the reducing moiety. For instance, the chemical shift $\delta(^{II}H\text{-}1)$ for **3a** is 5.14 ppm, while $\delta(^{III}H\text{-}1)$ of **6a** and **7a** are, respectively, 5.15 and 5.14 ppm. The NMR spectra of the mixture of trisaccharides **7a**, **7b** and **7c** obtained after removing the mono and disaccharides are given in Fig. 5.

Nevertheless, whatever the conditions used, the relative percentages of the regioisomers remained the same showing that the transglycosidase activity of the enzyme was not affected in ice. These results indicate that α-galactosidases, as well as their β-analogues, are able to improve their transglycosylation activity in ice at the expense of the hydrolytic reaction. In such conditions, one can expect substantial enhancements of the transferase activity of all 'mesophilic' glycosidases. This could be of interest, for instance, in the case of the highly regiolective α-galactosidase from *Penicillium multicolor*, which catalyzes the synthesis of trisaccharide **6a** as a single transglycosylation product.[24] This property, which was first observed with proteases,[13] seems to be general for the hydrolases and it is likely that lipases or esterases would also enhance their transferase activ-

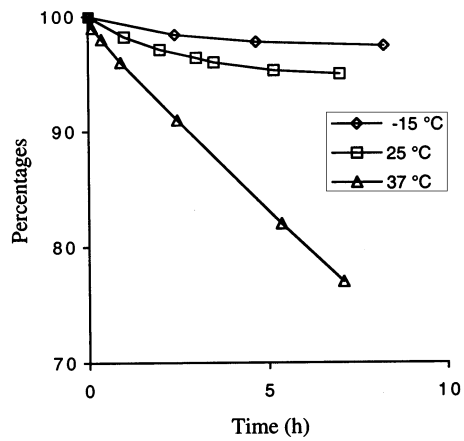


Fig. 3. Study of the spontaneous hydrolysis of fluoride **1** in deuterated phosphate buffer solutions, 0.3 M, pD 7. Relative proportions were determined from the proton NMR spectra.

Table 1
Composition of the mixtures obtained from the condensation reaction of fluoride **1** (concentration = 90 mM) used as a donor catalyzed by green coffee beans α-galactosidase

Conditions	Acceptor	Donor/acceptor	**1**	**1,3** (%) [a]	**1,6** (%) [a]	**1,2** (%) [a]	Other [b]	Gal
$t = 2$ h, $T = 37$ °C	**2**	1/3	3	29	6	3	9	50
$t = 15$ d, $T = -15$ °C	**2**	1/3	10	51	10	7	5	17
$t = 2$ h, $T = 37$ °C	**5**	1/5	0	14	10	6	15	55
$t = 15$ d, $T = -15$ °C	**5**	1/5	0	28	20	11	13	28
$t = 2$ h, $T = 37$ °C	**4**	1/8	0	15	10	6	15	54
$t = 15$ d, $T = -15$ °C	**4**	1/8	0	31	20	15	10	24

Relative proportions indicated in the table were calculated from the ^{1}H NMR spectra using a 10 mM trimethylsilyl–propansulfonate reference.

[a] Proportions of di- or trisaccharides with (1→3) or (1→6) or (1→2) α-glycosidic bonds.

[b] Other minor and non-identified compounds also present.

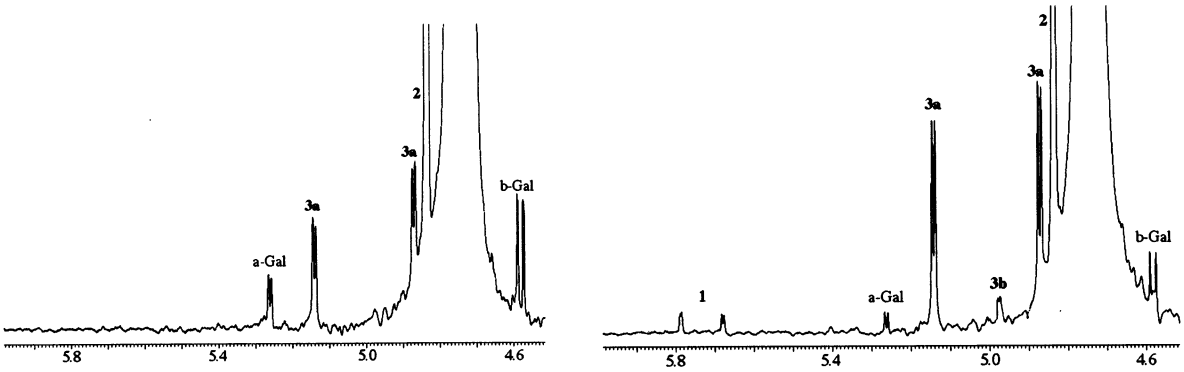


Fig. 4. ^{1}H NMR spectra 500 MHz, anomeric proton region, at 37 °C in D_2O of the crude reaction mixtures obtained at 37 °C (left) and at −15 °C (right) for the transglycosylation reaction catalyzed by the α-galactosidase from green coffee beans using fluoride **1** as a donor and **2** as an acceptor.

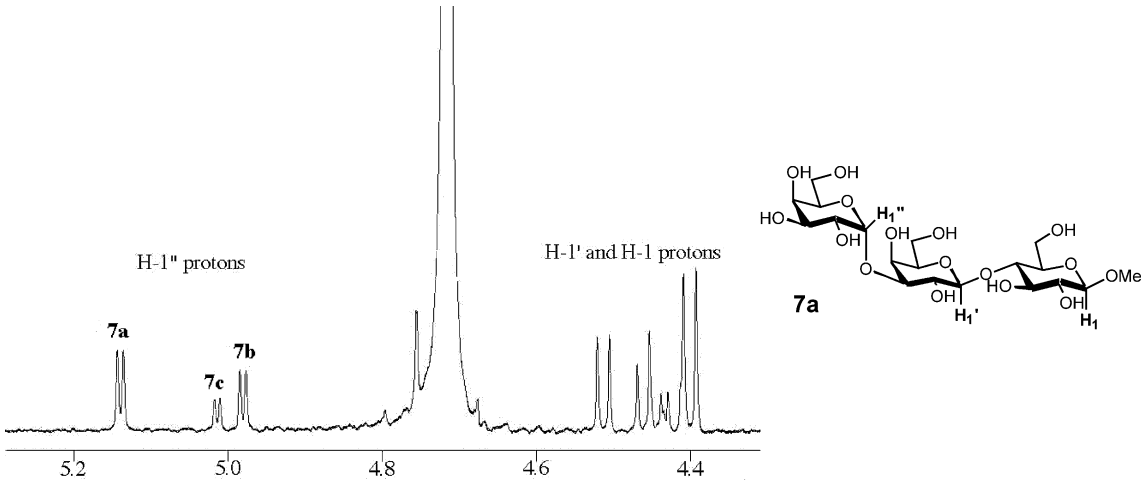


Fig. 5. ^{1}H NMR spectrum 500 MHz, at 25 °C in D_2O (only the anomeric region is shown) of the trisaccharides synthesized using the α-galactosidase from green coffee beans, **1** as a donor and **5** as acceptor at −15 °C.

ity in ice. Obviously, this is not so important for this class of enzymes since they generally show catalytic activity in organic media. However, some applications could be found with organic solvent-insoluble compounds such as carbohydrates. Thus, for instance, the regioselective acylation of saccharides, which is per-

formed in aggressive solvents like pyridine, could be replaced by reactions in ice. Further work is in progress in our laboratory along this line.

3. Experimental

General procedures.—α-Galactosidase from green coffee beans was purchased from Sigma. The chemicals supplied by Sigma were used without further purification. The enzymatic preparation of the α-galactosidase Aga B (from *B. stearothermophilus* was supplied by Professor R. Mattes (Institute for Industrial Genetics, University of Stuttgart, Germany). Deuterium oxide was purchased from Eurisotop (isotopic purity 99.9%). The course of the reactions was followed by means of TLC (precoated Silica Gel 60 sheets, E. Merck F254) and by ^{1}H NMR spectroscopy at 500 MHz (Bruker AX500 spectrometer). The components of the reaction mixtures of autocondensation reactions were separated on silica gel columns (Seymour eluent: 60:30:3:5 MeOH–$CHCl_3$–AcOH–water) and the components of condensation reactions on 1:1 charcoal (Darco G-60, 100 mesh, Aldrich)–Celite (Fluka 535) columns (1:5 EtOH–water). The complete analysis of the ^{1}H and ^{13}C NMR resonances and the subsequent structure assignment were made using standard 2D sequences (COSY HH and HCOOR correlations) and by comparison with previously published data.[4,6–12,16,17,23] 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 the resonance of methyl protons of (trimethylsilyl)-3-propansulfonic acid at −0.17 ppm) and 126 MHz for ^{13}C (solvent D_2O, chemical shifts in ppm quoted from the methyl acetone resonance at 29.8 ppm).

Study of the kinetics of the reactions at 37 °C.—All the experiments were conducted according to a procedure already reported in previous papers.[12,16,17,21]

Study of the kinetics of the spontaneous hydrolysis of fluoride **1** *by means of ^{1}H NMR spectroscopy.*—Fluoride **1** (9 mg, 50 μmol) was dissolved in 556 μL of deuterated phosphate buffer (0.3 M, pH 7). This mixture was filtered prior to transferring to an NMR tube. The latter was introduced into a thermostated bath at the desired temperature. At intervals, NMR spectra of this mixture were recorded at the temperature of the reaction, allowing the measurement of the concentrations by means of integrations of the anomeric proton signals. For the reactions at −15 °C, the solid mixture had to be melted before each NMR measurement and the spectra were recorded at 25 °C.

Methyl (α-D-galactopyranosyl)-(1→3)-α-D-galactopyranoside (**3a**)

At T = 37 °C. Fluoride **1** (46 mg; 250 μmol) and the acceptor **2** (146 mg; 750 μmol) were dissolved in 2.78 mL of the phosphate buffer (0.3 M, pH 7). The α-galactosidase from a green coffee bean (8.75 U; Sigma) was added to this solution at 37 °C. After 2 h of incubation at this temperature, the reaction was quenched by adding MeOH (6 mL). The reaction media was then concentrated under reduced pressure and the components were purified on a charcoal–Celite column by using an EtOH gradient in water. The first two fractions eluted with 5% EtOH solution contained, respectively, galactose (16 mg) and the remaining donor **1** (6 mg) plus **2**. The disaccharides were eluted with solutions of 10–15% of EtOH. Compound **3a** was obtained as a first condensation disaccharide with a yield of 23% (21 mg; R_f 0.39, TLC Silica Gel, E. Merck F254 and Seymour eluent). A second fraction yielded **3b** (2 mg; R_f 0.33 TLC Silica Gel plates, E. Merck F254 and Seymour eluent).

At T = −15 °C. Fluoride **1** (91 mg, 0.5 mmol) and **2** (291 mg, 1.5 mmol) were dissolved in 5.56 mL of the phosphate buffer (0.3 M, pH 7). This solution was cooled in an ice bath for 10 mn and 17.5 units of the α-galactosidase were added. Immediately after, the reactor was introduced into an EtOH bath, cooled at −25 °C with liquid nitrogen, until the solidification of the mixture occurred. Then, the reactor was transferred into a cryostat at $T = -15$ °C. After 15 days of incubation at this temperature, the reaction was quenched by adding 12 mL of MeOH. Then the procedure of purification used above was applied, leading to a first fraction containing galactose and the remaining acceptor **2** and a second fraction composed of galactose and the remaining **1** (elution with 5% EtOH). Elution with 10–15% EtOH yielded a third fraction containing **3b** (9 mg), **3c** (10 mg) and **3a** (57 mg) and a fourth fraction of pure **3a** (27 mg). The overall yield for **3a** was 47%.

α-D-*Gal*p-*(1→3)*-α-D-*Gal*p-*OMe* (**3a**). $[\alpha]_D$ +254° (*c* 1, water), lit.[4] +252° (*c* 1, water), ^{1}H NMR (D_2O): δ Gal*p*^{I} 4.87 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 4.11 (dd, 1 H, $J_{4,3}$ 2.3 Hz, $J_{4,5}$ n.m., H-4), 3.86 (dd, 1 H, $J_{2,3}$ 10.3 Hz, H-2), 3.82 (dd, 1 H, $J_{3,4}$ 3.0 Hz, H-3), 3.79 (dd, 1 H, $J_{5,6a}$ 6.2, $J_{5,6b}$ 6.2 Hz, H-5), 3.64 (m, 4 H, H-6a and H-6b), 3.31 (s, 3 H, OCH_3); Gal*p*^{II} 5.14 (d, 1 H, $J_{1,2}$ 3.9 Hz, H-1), 4.08 (dd, 1 H, $J_{5,6a}$ 6.3, $J_{5,6b}$ 6.5 Hz, H-5), 3.90 (d, 1 H, $J_{4,3}$ 3.9 Hz, H-4), 3.84 (dd, 1 H, $J_{3,2}$ 10.3 Hz, H-3), 3.75 (dd, 1 H, H-2), 3.64 (m, 4 H, H-6a and H-6b); ^{13}C NMR (D_2O): δ Gal*p*^{I} 99.1 (C-1), 74.0 (C-3), 70.6 (C-5), 68.9 (C-4), (C-2), 60.7 (C-6), 54.8 (OCH_3); Gal*p*^{II} 94.8 (C-1), 70.2 (C-5), 68.9 (C-3), 67.9 66.3 (C-2), 65.2 (C-4), 60.9 (C-6). Anal. Calcd for $C_{13}H_{24}O_{11}$ (356): C, 43.82; H, 6.74. Found: C, 43.77; H, 6.81.

α-D-*Gal*p-*(1→6)*-α-D-*Gal*p-*OMe* (**3b**). $[\alpha]_D$ +285° (*c* 0.4, water), lit. no data; ^{1}H NMR (D_2O): δ Gal*p*^{I} 4.83 (H-1), 4.08 (H-5), 4.03 (H-3), 3.87 (H-6a), 3.82 (H-2), 3.73 (H-4), 3.70 (H-6b), 3.41 (s, 3 H, OCH_3); Gal*p*^{II}

4.97 (H-1), 4.00 (H-5), 3.98 (H-3), 3.83 (H-2), 3.74 (H-4), 3.63 (H-6a and H-6b); ^{13}C NMR (D_2O): δ Gal*p*^{I} 99.1 (C-1), 69.0 (C-3), 68.8 (C-6), 68.2 (C-5), 67.9 (C-2), 66.1 (C-4), 54.7 (OCH_3); Gal*p*^{II} 97.9 (C-1′), 70.7 (C-5′), 69.1 (C-2′), 68.9 (C-3′), 60.8 (C-4′), 57.0 (C-6′). Anal. Calcd for $C_{13}H_{24}O_{11}$ (356): C, 43.82; H, 6.74. Found: C, 43.73, H, 6.82.

α-D-Galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose (**6a**), *in a mixture with* **6b** *and* **6c** *regioisomers in ice at −15 °C.*—Fluoride **1** (46 mg, 250 μmol) and lactose (721 mg, 2 mmol) were dissolved in 2.78 mL of phosphate buffer (0.3 M, pH 7). Then, 8.75 units of the galactosidase were added and the same procedure as above (at −15 °C) was applied. Elution of the mixture on a charcoal–Celite column with 5% EtOH afforded galactose in the first fraction, then **1** in the second fraction. Lactose **4** was eluted with solutions containing 10–15% EtOH while 70 mg of the mixture of trisaccharides (as α, β anomers) were obtained; R_f (Silica Gel, E. Merck F254, Seymour eluent) 0.1. The total amount of these trisaccharides represents a transglycosylation yield of 57% (the relative percentages determined by means of proton NMR spectroscopy were 47% for **6a**, 30% for **6c** and 23% for **6b**). Since we were unable to separate these compounds, we give the analysis for the mixture of regioisomers. The structures were elucidated by comparison of the anomeric H-1″ proton chemical shifts of the trisaccharides with the H-1′ anomeric proton chemical shifts of the corresponding preceding disaccharides.

*α-D-Gal*p-*(1→2)-β-D-Gal*p-*(1→4)-D-Glc*p (**6c**). β Anomer: ^{1}H NMR (D_2O): δ 5.08 (^{III}H-1), 4.66 (^{I}H-1), 4.43 (^{II}H-1); ^{13}C NMR (D_2O): δ 102.5 (^{II}C-1), 95.3 (^{I}C-1), 95.2(^{III}C-1).

α Anomer: ^{1}H NMR (D_2O): δ 5.08 (^{III}H-1), 4.96 (^{I}H-1), 4.45 (^{II}H-1); ^{13}C NMR (D_2O):δ 102.8 (^{II}C-1), 98.0 (^{I}C-1), 95.2 (^{III}C-1).

*α-D-Gal*p-*(1→3)-β-D-Gal*p-*(1→4)-D-Glc*p (**6a**). β Anomer: ^{1}H NMR (D_2O): δ 5.15 (^{III}H-1), 4.66 (^{I}H-1), 4.43 (^{II}H-1); ^{13}C NMR (D_2O): δ 102.7 (^{II}C-1′), 95.3 (^{I}C-1), 92.5 (^{III}C-1).

α Anomer: ^{1}H NMR (D_2O): δ 5.15 (^{III}H-1), 4.98 (^{I}H-1), 4.43 (^{II}H-1); ^{13}C NMR (D_2O): δ 102.5 (^{II}C-1), 98.4 (^{I}C-1), 92.4 (^{III}C-1).

*α-D-Gal*p-*(1→6)-β-D-Gal*p-*(1→4)-D-Glc*p (**6c**). β Anomer: ^{1}H NMR (D_2O): δ 4.94 (^{III}H-1), 4.64 (^{I}H-1), 4.50 (^{II}H-1); ^{13}C NMR (D_2O): δ 102.6 (^{II}C-1′), 95.4 (^{I}C-1), 98.5 (^{III}C-1).

α Anomer: ^{1}H NMR (D_2O): δ 4.97 (^{I}H-1), 4.94 (^{III}H-1), 4.50 (^{II}H-1); ^{13}C NMR (D_2O): δ 102.4 (^{II}C-1), 98.6 (^{I}C-1), 98.5 (^{III}C-1).

Anal. Calcd for $C_{18}H_{32}O_{16}$ (504): C, 42.86; H, 6.35. Found: C, 43.01; H, 6.29 (mixture of **6a**, **6b**, **6c**).

Synthesis of methyl (α-D-galactopyranosyl)-(1→3)-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (**7a**) *in a mixture with* **7b** *and* **7c** *regioisomer in ice (−15 °C).*—Fluoride **1** (26 mg, 140 μmol) and **5** (250 mg, 702 μmol) were dissolved in 1.56 mL of phosphate buffer (0.3 M, pH 7). Then, 2.45 units of the galactosidase were added and the same procedure as above (at −15 °C) was applied. Elution of the mixture on a charcoal–Celite column with 5% EtOH afforded galactose and **1** in the first fraction. Compound **2** was eluted with solutions containing 5–10% EtOH, then 38 mg of the mixture of trisaccharides were obtained by elution with 10–15% EtOH. The total amount of these trisaccharides represents a transglycosylation yield of 52% (the relative percentages determined by means of proton NMR spectroscopy were 47% for **7a**, 34% for **7b** and 19% for **7c**. Since we were unable to separate these compounds, we give the analysis for the mixture of regioisomers. The structures were elucidated by comparison of the anomeric H-1″ proton chemical shifts of the trisaccharides with the H-1′ anomeric proton chemical shifts of the corresponding preceding disaccharides.

*α-D-Gal*p-*(1→2)-β-D-Gal*p-*(1→4)-β-D-Glc*p-*OMe* (**7c**). ^{1}H NMR (D_2O): δ 5.02 (^{III}H-1), 4.42 (^{II}H-1), 4.41 (^{I}H-1); ^{13}C NMR (D_2O): δ 102.6 (^{I}C-1 and ^{II}C-1), 98.3 (^{III}C-1).

*α-D-Gal*p-*(1→3)-β-D-Gal*p-*(1→4)-β-D-Glc*p-*OMe* (**7a**). ^{1}H NMR (D_2O): δ 5.14 (^{III}H-1), 4.51 (^{II}H-1), 4.40 (^{I}H-1); ^{13}C NMR (D_2O): δ 102.6 (^{I}C-1 and ^{II}C-1), 95.1 (^{III}C-1).

*α-D-Gal*p-*(1→6)-β-D-Gal*p-*(1→4)-β-D-Glc*p-*OMe* (**7b**). ^{1}H NMR (D_2O): δ 4.98 (^{III}H-1), 4.43 (^{II}H-1), 4.40 (^{I}H-1); ^{13}C NMR (D_2O): δ 102.6 (^{I}C-1 and ^{II}C-1), 98.0 (^{III}C-1).

Anal. Calcd for $C_{19}H_{34}O_{16}$ (518): C, 44.02; H, 6.56. Found: C, 43.93; H, 6.59 (mixture of **7a**, **7b**, **7c**).

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