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In situ ^{19}F NMR spectroscopy study of enzymatic transglycosylation reactions using α-D-aldohexopyranosyl fluorides as donors and acceptors

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Abstract—The use of ^{19}F NMR spectroscopy to study the kinetics of the self-condensation reaction of α-D-aldohexopyranosyl fluorides catalysed by α-glycosidases is described. The corresponding fluorinated disaccharides thus synthesised present separate individual fluorine resonances allowing the integration of each species. This method looks particularly useful to help in the choice of donor in enzymatic transglycosylation reactions since the self-condensation reaction always remains in competition with the condensation reaction (reaction of the donor with acceptors other than itself).

1. Introduction

The enzymatic synthesis of saccharides using the transferase activity of glycosidases has become a very powerful alternative to the regioselective chemical synthesis of the glycosidic bond.[1] These enzymes are attractive due to their stability and low cost and additionally most of them also induce highly stereoselective reactions.[2–5] The main drawbacks involved in the use of glycosidases arises from their partial regioselectivity and the moderate yields usually obtained since the transferase activity remains in competition with hydrolysis of the substrate and of the synthesised glycosides. In order to improve the yields of the transglycosylation reactions, suitable experimental conditions and/or new enzymatic activities must be found. High 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 are also very well recognised as acceptors by the glycosidases. Thus, in the presence of an acceptor, two transglycosylation reactions are in competition: the self-condensation and the condensation[12,13] leading to complex mixtures. The use of suitable disaccharides (melibiose, maltose etc.) avoids this problem but the activation is usually too low to afford high transglycosylation yields. Among the activated donors accepted by the glycosidases, the readily available α-D-aldohexopyranosyl fluorides have also been used successfully.[14] The self-condensation reaction also proceeds in this case, but we have shown that this undesirable reaction is generally reduced compared to the reaction with nitrophenylglycosides.[15] In our efforts to improve the yield of disaccharides obtained in these reactions, we found that monitoring the course of the reactions was absolutely determinant. In situ proton NMR has been used for this purpose.[16] Unfortunately, when applied to the self-condensation of α-D-aldohexopyranosyl fluorides, the limitations of the method became apparent. The anomeric H-1 protons of the fluorinated substrates and of the disaccharide products resonate at the same frequency making interpretation of the spectra difficult. However, the fluorine chemical shifts as published recently are more sensitive and amenable to this type of analysis.[17] Herein, we report that using in situ ^{19}F NMR spectroscopy discrimination between all the components could be achieved and thus allows the study of the kinetics of these self-condensation transglycosylations.

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2. Results and discussion

Three self-condensation reactions were studied by means of in situ ^{19}F NMR spectroscopy: α-D-galactopyranosyl fluoride **1** was reacted in the presence of α-galactosidases from green coffee beans (reaction **a**), Aga B from *Bacillus stearothermophilus* (reaction **b**)[13] and α-D-glucopyranosyl fluoride **2** with α-glycosidase from *Saccharomyces cerevisiae* (reaction **c**) (Scheme 1). The structure of the fluorinated disaccharide products was then determined by means of well known bidimensional NMR proton–proton and proton–carbon correlations and by comparison with the corresponding *p*-nitrophenyl analogues (see Section 3).

In the first case, two disaccharides, α-D-galactopyranosyl-[1→3]-α-D-galactopyranosyl fluoride **1**(**1**,**3**) and α-D-galactopyranosyl-[1→6]-α-D-galactopyranosyl fluoride **1**(**1**,**6**), were synthesised in low and nearly equal yields, while traces of α-D-galactopyranosyl-[1→2]-α-D-galactopyranosyl fluoride **1**(**1**,**2**) were also detected in the NMR spectra.

The α-galactosidase Aga B induced the synthesis of disaccharide **1**(**1**,**6**) as the major product with minor quantities of **1**(**1**,**3**) and of the **1**(**1**,**2**) regioisomers. Under the same conditions, the α-glucosidase from *S. cerevisiae* catalysed the synthesis of α-D-glucopyranosyl-[1→3]-α-D-glucopyranosyl fluoride **2**(**1**,**3**) (greatly predominant) with α-D-glucopyranosyl-[1→6]-α-D-glucopyranosyl fluoride **2**(**1**,**6**). Under the conditions employed, the formation of trisaccharides or higher oligosaccharides was not observed. These results are completely in accordance with the regioselectivity exhibited by these enzymes in the presence of other donor substrates.[4–13]

The ^{19}F NMR spectrum exhibits separate resonances for each fluorinated sugar, as shown for example in Fig. 1. The ^{19}F chemical shifts and H-F coupling constants of the fluorinated disaccharides are given in Table 1. From the integration of the ^{19}F resonances, it was possible to monitor the kinetics of the reactions. The results are shown in Fig. 2.

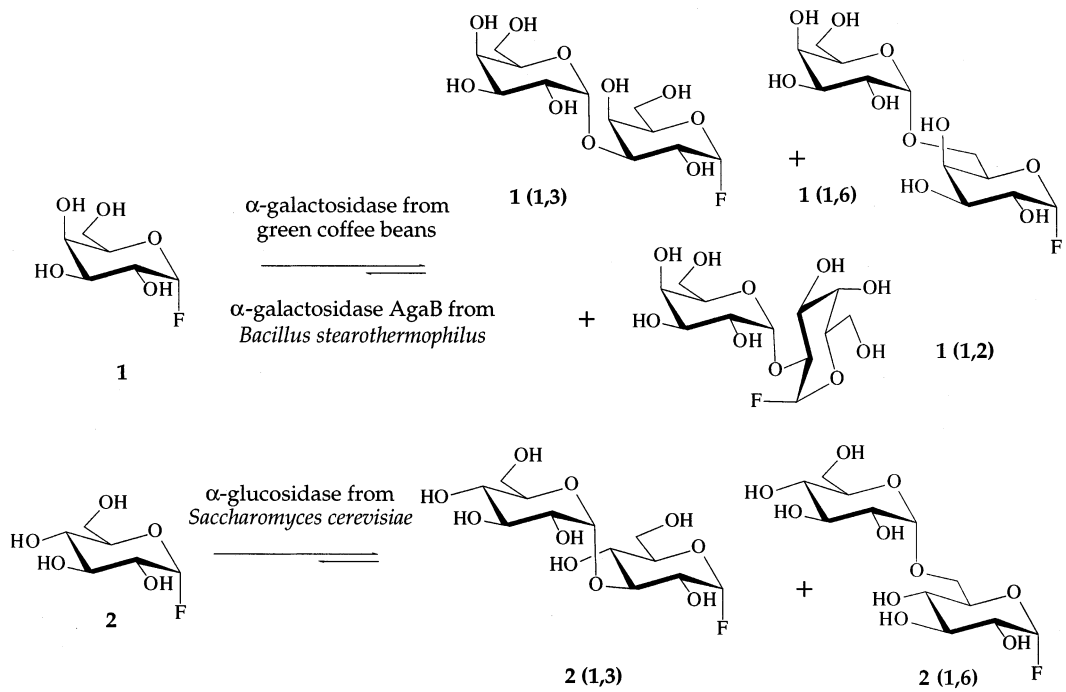
The molar percentages calculated from the integrations of the ^{19}F NMR signals represent the proportions of the α-D-aldohexopyranosyl fluoride which reacted to produce a given species. Since the synthesis of one equivalent of disaccharide needs two equivalents of donor, the concentrations of such compounds are half of the molar percentage values (or yields). The molar percentages of glucose or galactose resulting from the hydrolytic activity were easily deduced from the integration of the signal of the fluoride anion produced (the solvent used was 0.3 M sodium phosphate buffer in D_2O, pD 7.0) with the relationship:

$$[\text{glc or gal}] = [F^-] - \Sigma[\text{disaccharides}]$$

In this medium, the resonance located at −122 ppm was attributed to $NaHF_2$ since NaF should absorb at −133 ppm. The expressions used to determine the molar percentages were the following:

$$[C_{\text{mono}}]_i = \frac{[I_{\text{mono}}]_i}{\sum[I_{\text{mono}}]_i + 2\sum[I_{\text{di}}]_j} \qquad [C_{\text{di}}]_j = \frac{2[I_{\text{di}}]_j}{\sum[I_{\text{mono}}]_i + 2\sum[I_{\text{di}}]_j}$$

where $[C_{\text{mono}}]_i$ and $[C_{\text{di}}]_j$ are the molar percentages of the monosaccharide i and of the disaccharide j present in the reaction mixture; $[I_{\text{mono}}]_i$ and $[I_{\text{di}}]_j$ are the integration values for each fluorine resonance of a monosaccharide i and of a disaccharide j.



Scheme 1. Self-condensation reactions of α-D-aldohexopyranosyl fluorides catalysed by α-glycosidases.

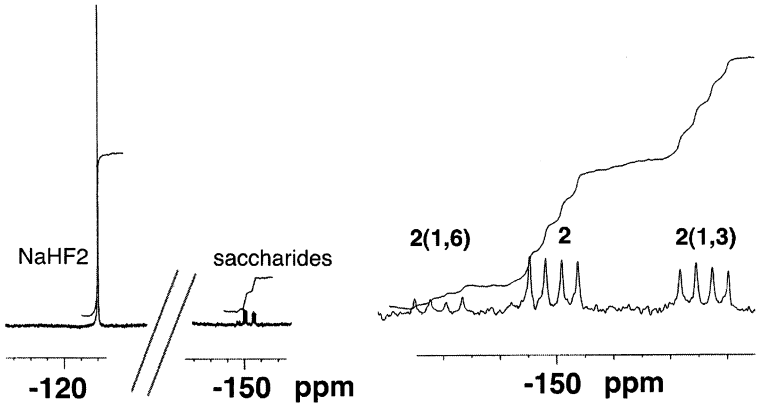


Figure 1. Non-decoupled $\{^1H\}^{19}F$ spectrum of the mixture obtained from the self-condensation of **2** catalysed by the α-glucosidase from *S. cerevisiae* (reaction **c**) after 2.5 h of incubation.

Table 1. ^{19}F chemical shifts and proton-fluor coupling constants of the fluorinated saccharides (the solvent is 0.3 M phosphate buffer, pD 7.0, chemical shifts calculated from sodium trifluoroacetate at −75.3 ppm)

Saccharide	δ_F (ppm)	$^1J_{F\text{-}H1}$ (Hz)	$^2J_{F\text{-}H2}$ (Hz)
1	−152.1	53.6	25.5
1(1,3)	−151.8	53.1	25.6
1(1,6)	−150.5	53.3	25.2
1(1,2)	−151.2	53.2	25.8
2	−150.0	53.5	26.4
2(1,3)	−151.5	53.2	26.1
2(1,6)	−149.6	54.0	26.6

It was assumed that the spontaneous hydrolysis of the carbon–fluorine bond of the substrate and of the products did not take place during the reactions. This hypothesis looks valid since we have verified that either the hydrolysis of the α-D-aldohexopyranosyl fluorides or the hydrolysis of several fluorinated disaccharides (cellobiosyl, maltosyl, melibiosyl fluorides) is very slow under our reaction conditions. For instance, only 4% of the α-D-galactopyranosyl fluoride was hydrolysed after 4 h at 25°C and pD 7.0. This result is in accordance with the work of Jencks et al.[18]

The self-condensation transglycosylation ability of each enzyme can easily be compared as shown in Fig. 2. Thus, it is obvious that the α-glucosidase and the α-galactosidase Aga B both recognise the α-D-aldohexopyranosyl fluorides as acceptors and lead to relatively high amounts of self-condensation disaccharides. For instance, the enzyme Aga B produced a 33% yield of fluorinated disaccharides. A similar potential was observed with α-glucosidase which synthesised an overall yield of 20% of disaccharides. Conversely, α-galactosidase from green coffee beans led to lower amounts of disaccharides (about 15% yield). However, all these enzymes are well known for their high transferase activity.[4–11] This is particularly the case with the latter

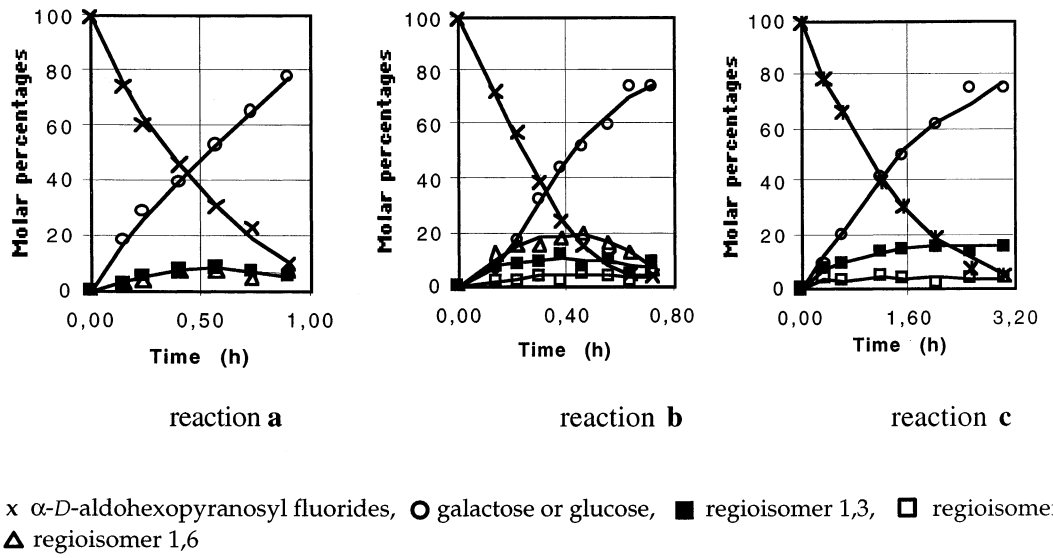


Figure 2. Kinetics of the self-condensation–hydrolysis reactions of α-D-aldohexopyranosyl fluorides. Reactions **a** and **b** are, respectively, the self-condensation reactions of α-D-galactopyranosyl fluoride catalysed by α-galactosidases from green coffee beans and Aga B from *Bacillus stearothermophilus*. Reaction **c** is the self-condensation of α-D-glucopyranosyl fluoride in the presence of α-glucosidase from *S. cerevisiae*.

enzyme which is able to produce high yields of transglycosylation products when using α-D-galactopyranosyl fluoride as a donor and other acceptors such as α-methylgalactoside in frozen media.[15] These results have to be compared to those observed with the titled galactosidases in the presence of 4-nitrophenyl-α-galactoside as a donor under the same conditions.[13,16] For instance, the overall yields of 35 and 55%, respectively, obtained for the self-condensation disaccharides in the presence of the α-galactosidases from green coffee beans and Aga B[13,16] indicate that the α-D-galactopyranosyl fluoride is a better candidate for the condensation reaction than the corresponding *O*-4-nitrophenyl analogue.

These observations clearly demonstrate that the choice of donor and of acceptor in transglycosylation reactions is of prime importance. Obviously the donor must be activated and bear a good leaving group. However if self-condensation is to be avoided, the enzyme must not recognise this compound as a good acceptor. From this point of view, we have shown the utility of ^{19}F NMR spectroscopy when using α-D-aldohexopyranosyl fluorides as donors.

3. Experimental

3.1. General procedures

The α-galactosidase from green coffee beans and the α-glucosidase from *S. cerevisiae* (recombinant) were purchased from Sigma. The enzymatic preparation of Aga B (*B. stearothermophilus*) was prepared according to References 19–22. The enzymatic activities are determined by monitoring the hydrolysis of the 4-nitrophenyl-α-D-glycopyranosides (10 mM solution at room temperature, pH 7.0, 0.1 M phosphate buffer) studied by the release of 4-nitrophenol measured spectroscopically at 420 nm. The unit is the number of μmol of 4-nitrophenol formed per minute. The chemicals supplied by Aldrich were used without further purification. The D_2O was purchased from Eurisotop (isotopic purity 99.9%). Structural assignments of the fluorinated disaccharides were made by comparison of their NMR spectra with the corresponding *O*-4-nitrophenyl analogues. Thus, we have shown[12,13] that two NMR parameters are highly characteristic of the type of glycosidic bond: $\delta(^1H_{1'})$ and $\delta(^{13}C_n)$ ($n=2$, 3, 4 or 6 is the position of the glycosidic bond on the reducing moiety). For instance, the comparison of the following data determined for 4-nitrophenyl α-D-galactopyranosyl-[1→*n*]-α-D-galactopyranosides [Gal-α-(1→*n*)-Gal-α-*O*-*p*NP, see below][12,13,15] with those obtained for the fluorinated analogues allows the structural elucidation of the later. Furthermore these assumptions were confirmed by the hydrolysis of the fluorine atom of disaccharides **1**(**1**,**6**) and **2**(**1**,**3**) which produced disaccharides whose NMR spectra were the same as those obtained by the hydrolysis of the corresponding *O*-4-nitrophenyl analogues. The spectra were recorded with a Bruker DRX500 spectrometer operating at 500.21 MHz for ^1H and 470.52 MHz for ^{19}F. The α-D-aldohexopyranosyl fluorides were prepared according to the known procedures starting from the corresponding β-peracetylglycosides treated with HF-pyridine followed by subsequent deprotection in the presence of sodium methoxide in methanol.[23]

3.2. Kinetic study of the self-condensation reaction of α-D-aldohexopyranosyl fluorides catalysed by α-glycosidases

The amount of salts necessary for the preparation of 0.78 mL of the sodium phosphate buffer (0.3 M, pH 7.0) were lyophilised, dissolved in D_2O and lyophilised once more. α-D-Aldohexopyranosyl fluoride (21 mg 70 μmol) was prepared by the same procedure. The α-glycosidase preparation was dried in a desiccator containing P_2O_5. The lyophilised buffer mixture was redissolved in D_2O (0.78 mL) and adjusted to pD 7.0. A portion of this solution (0.6 mL) was used to dissolve the substrate. The resulting solution was immediately filtered and transferred to an NMR tube. The dried α-glycosidase preparations (corresponding to 1.75 units) were redissolved in the remaining buffer mixture (0.18 mL) prior to their addition to the reactants in the NMR tube. The reaction was allowed to proceed in the magnet of the spectrometer pre-adjusted to the temperature of the reaction (25°C for our experiments).

Using this procedure, it was possible to perform the first measurements about 4 min after introduction of the enzyme into the NMR tube. Concerning the kinetics measurements, standard conditions were used (PW = 30, NS = 96, acquisition time = 0.344 s) regarding the rate of the reactions in order to obtain a sufficient signal to noise ratio (>20 for the major products) during a short time.

The T_1 relaxation time of the fluorines (which are usually of the same order of magnitude as those of protons) were assumed to be short enough to avoid the introduction of a delay between each pulse. The integrals were measured using the Bruker standard program. The accuracy of the concentration measurements is estimated at approx. ±5% for the major products.

δ (ppm)	Gal-α-(1→3)-Gal-α-*O*-*p*NP	Gal-α-(1→3)-Gal-α-F **1**(**1**,**3**)	Gal-α-(1→6)-Gal-α-*O*-*p*NP	Gal-α-(1→6)-Gal-α-F **1**(**1**,**6**)	Gal-α-(1→2)-Gal-α-*O*-*p*NP	Gal-α-(1→2)-Gal-α-F **1**(**1**,**2**)
δ ($^1H_{1'}$)	5.24	5.18	4.80	4.91	5.13	5.05
δ ($^{13}C_n$)	74.1	74.0	69.0	68.8	71.4	71.5

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