Pergamon

Tetrahedron: *Asymmetry* 10 (1999) 2905–2912

TETRAHEDRON: *ASYMMETRY*

Probing the transferase activity of glycosidases by means of in situ NMR spectroscopy

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Received 11 June 1999; accepted 14 July 1999

Abstract

This paper describes the conditions in which in situ NMR spectroscopy is a suitable technique to use when following the course of enzymatic transglycosylation reactions. Using this methodology, the reactions must be carried out in D_2O. Our experiments indicate that the rate of the transglycosylation reaction is reduced in this solvent while the rate of the hydrolysis of the disaccharides produced is enhanced depending on the nature of the anomeric substituent. However, this undesirable effect is generally weak because the rates of the transglycosylation reactions are always faster than the rates of the hydrolysis whatever the solvent. The great advantage of NMR spectroscopy lies in its potential to detect, in a single experiment, all the components of the reaction without any disturbance of the reaction medium.

1. Introduction

The key role played by carbohydrates in biological mechanisms has led to the rapid development of sugar chemistry. On the one hand, 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. On the other hand, the enzymatic synthesis of the saccharides has become, over the last 10 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 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 via the reverse reaction but also via the transferase activity. Due to their stability and low cost, these enzymes are very attractive

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0957-4166/99/$ - see front matter
PII: S0957-4166(99)00297-9

especially since they also induce a high stereoselectivity.[6–9] Their main disadvantages come from their rather low regioselectivity and the moderate yields usually obtained since the transferase activity remains in competition with the hydrolysis of the substrate and of the glycosides synthesised. In addition, other products may be synthesised since the autocondensation of the activated glycoside donor can occur.[10] To improve the yields in 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 can provide a greater regioselectivity.[10,11] We have shown, for instance, the possibility of performing such enzymatic reactions in ice.[11] In order to avoid the hydrolysis of the glycosides produced, a very elegant method has also been developed by Withers et al. Their strategy, which includes the directed mutation of β-glycosidases, gives very powerful enzymes producing high yields of transglycosylation products.[12]

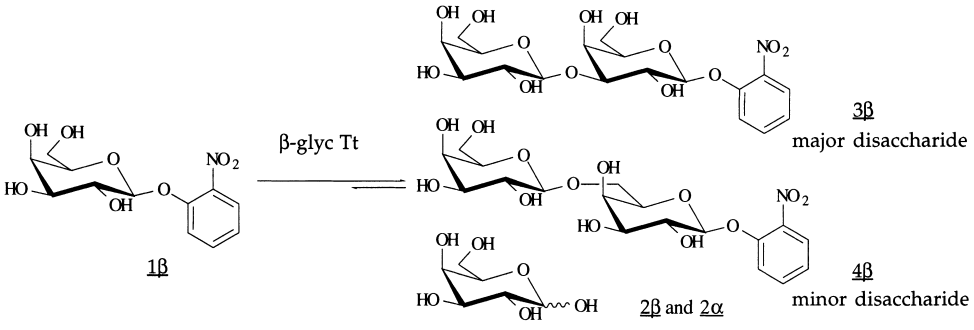
Since transglycosylation reactions are under kinetic control, it is of great interest to know the time when the highest concentration of a given disaccharide is present in the reaction medium in order to quench the conversion before any significant hydrolysis takes place. The aim of this paper is to present the conditions in which proton NMR spectroscopy allows the direct measurement of each component in such reactions.

2. Results and discussion

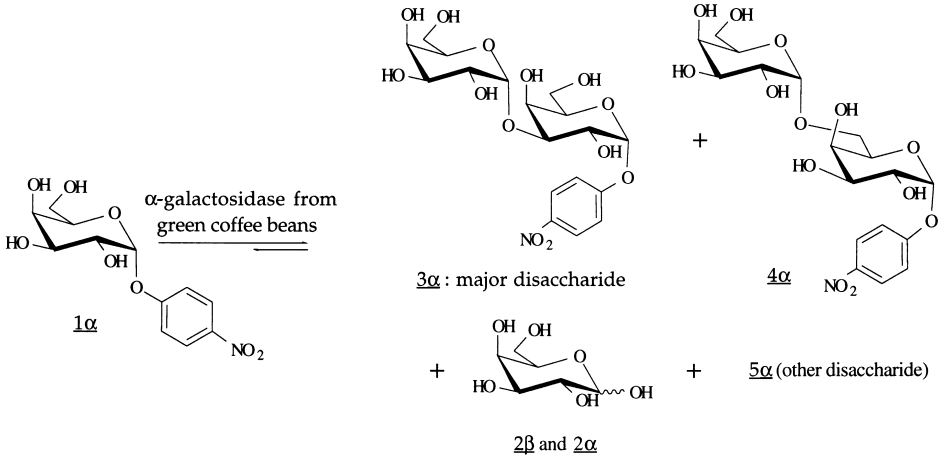
Several analytical methods are available for this purpose, particularly chromatographical techniques. For instance, the relative concentrations of all the compounds involved in the reaction described in Scheme 1 can be measured by means of HPLC. Capillary electrophoresis also seems to be a powerful method[13,14] for the kinetic study of such reactions. Another approach is proton NMR spectroscopy. This technique has the advantage of giving signals where intensities are directly proportional to the concentrations of the substances. Furthermore, this non-invasive spectroscopy enables the study of the enzymatic reactions thus suppressing the possible side-effects arising from the taking of aliquots. In addition, the resonance region between 4.0 and 6.0 ppm, despite being rather narrow, allows a clear separation (at 500 MHz) of all the anomeric protons of the saccharides present in the reaction medium (see Figs. 1 and 5) and the resolution is quite good although the enzyme is present in the solutions. However, when carried out in an NMR tube, the course of the enzymatic reaction has to be studied at a very high D_2O/H_2O ratio since the residual water protons present their resonance absorption around 4.6–4.8 ppm. Thus, the question is whether the substitution of H_2O by heavy water exerts an influence on the glycosidase activity and consequently on the rates of the reactions. In order to investigate the kinetics of two reactions were first studied:

– Reaction A: autocondensation of 2-nitrophenyl β-D-galactopyranoside (*o*-NP-β-Gal) in the presence of a thermostable β-glycosidase from *Thermus thermophilus* (Tt β-gly), (Scheme 1).
– Reaction B: autocondensation of 4-nitrophenyl α-D-galactopyranoside (*p*-NP-α-Gal) in the presence of the α-galactosidase from green coffee beans (Scheme 2).

Each reaction was performed in the same conditions (temperature, concentration, enzymatic activity, see experimental section) either in H_2O or in D_2O. In the first case, samples were taken of the reaction mixture and analysed by means of NMR spectroscopy (after quenching the enzymatic activity by adding 1 M NaOH) while in the second, the NMR tube was the reactor. The major kinetic compounds obtained in these two already known reactions are the 2-nitrophenyl β-D-galactopyranosyl-[1→3]-β-D-galactopyranoside **3β** and the 4-nitrophenyl α-D-galactopyranosyl-[1→3]-α-D-galactopyranoside **3α**[10,15–17] (Schemes 1 and 2). The resonances of all the anomeric α and β protons located between 4.2



Scheme 1. Reaction A: autocondensation of the 2-nitrophenyl β-D-galactopyranoside catalysed by a β-glycosidase (Tt β-gly) from the thermophilic species *Thermus thermophilus*



Scheme 2. Reaction B: autocondensation of the 4-nitrophenyl α-D-galactopyranoside catalysed by the α-galactosidase from green coffee beans

and 6 ppm are sufficiently separated to allow the integration of the resonances corresponding to each component as shown in Fig. 1.

The results of the kinetic studies are shown in Fig. 2 (reaction A) and Fig. 3 (reaction B). A comparison between the kinetic profiles obtained in H_2O and D_2O indicates some differences. The most important observation is that the hydrolytic activity of the glycosidases is enhanced in deuterium oxide while the transglycosylation rates are reduced in this solvent. For instance, in the case of reaction A, after 1.75 h of incubation, the galactose concentrations are 27 mM and 38 mM, respectively, in H_2O and D_2O while in both cases very low amounts of the donor **1β** remain. A similar effect is observed in the case of reaction B: an incubation of 3–4 h produces galactose concentrations of 32 mM (in H_2O) and 41 mM (in D_2O). In each reaction, the rate of hydrolysis of the major disaccharide is higher in deuterium oxide solutions. As a consequence, the time at which the maximum concentrations for **3β** or **3α** obtained in D_2O is higher than in H_2O and thus leads to lower concentrations of the disaccharides in deuterium oxide. This is particularly true with reaction B where the highest concentrations in H_2O and in D_2O for **3α** are 19.2 mM (within 2 h) and 13.4 mM (within 2.5 h), respectively. For reaction A, this effect is less important because the rate of the transglycosylation reaction is very fast whatever the solvent. Thus, the highest concentrations for compound **3β** in D_2O and in H_2O are, respectively, 10.3 mM (1 h of incubation) and 12.4 mM (0.5 h of incubation). These results show that proton NMR spectroscopy is a suitable

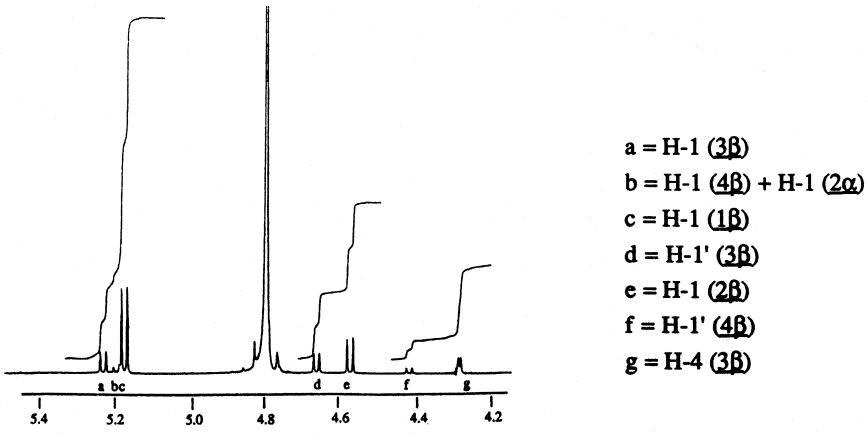


Figure 1. Typical ^{1}H NMR spectra (500 MHz) of the mixture of compounds in reaction A carried out in the NMR tube after 0.5 h of incubation (solvent D_2O, buffer pD 7). Only the anomeric region is shown

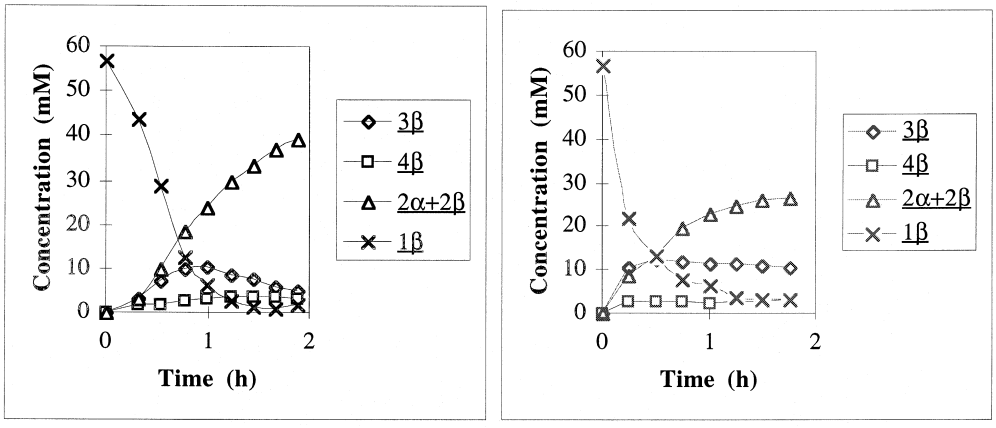


Figure 2. Kinetic study of reaction A at T=25°C. Left curves: in situ NMR experiment in D_2O, right curves: reaction in H_2O (the maximum concentrations for the disaccharide **3β** are, respectively, 10.3 mM and 12.4 mM corresponding to yields of 36 and 43%)

technique to use when following the course of the glycosidase transferase activity only if the rate of transglycosylation is much faster than the rate of hydrolysis of the products which seems to be enhanced in D_2O. This undesirable effect is highly reduced when the reactions are performed in the presence of an excess of acceptor. For instance, the transglycosylation C was performed using **1α** (1 equivalent) as a donor and α-methyl galactoside (3 equivalents) as an acceptor in the presence of the α-galactosidase from green coffee beans. Fig. 4 shows the kinetic profiles in H_2O and in D_2O. It is clear that the rate of the reaction is once again less rapid in the latter solvent. The difference from the previous reactions discussed in this work lies in the fact that the hydrolysis of the disaccharides bearing the methoxy group at the anomeric position is much slower than that of the disaccharides substituted with a nitrophenoxy group. As a result, after the same incubation time (5.3 h), the concentrations of the major disaccharide **3α**(**X**) are 33 mM in H_2O and 28 mM in D_2O while the concentrations of the remaining donor **1α** were, respectively, 8 mM and 18 mM (Fig. 4). In our laboratory, a systematic study of the reactions catalysed by glycosidases[18,19] also indicated similar trends thus showing that in situ NMR can be used to optimise such reactions, taking into account the fact that a small reduction in the rates of the reactions occurs in the presence of D_2O.

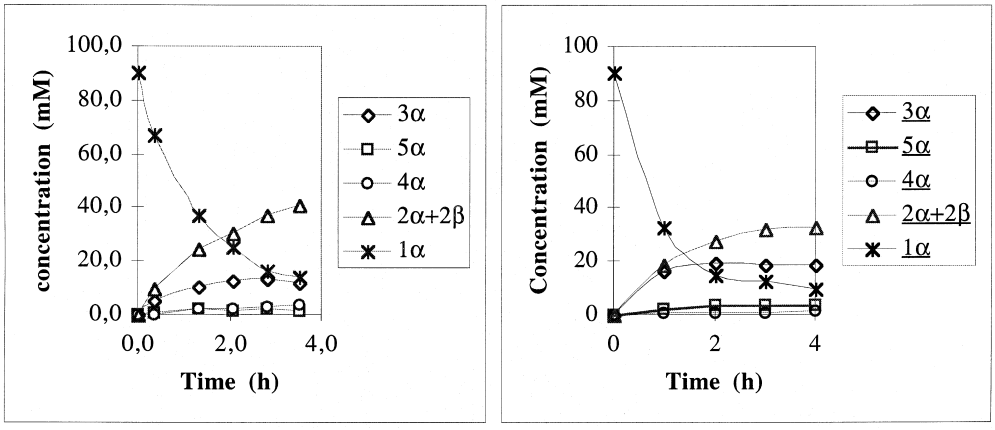


Figure 3. Kinetic study of reaction B at T=37°C. Left curves: in situ NMR experiment in D_2O, right curves: reaction in H_2O (the maximum concentrations for the disaccharide **3α** are, respectively, 13.4 mM and 19.2 mM corresponding to yields of 30 and 43%)

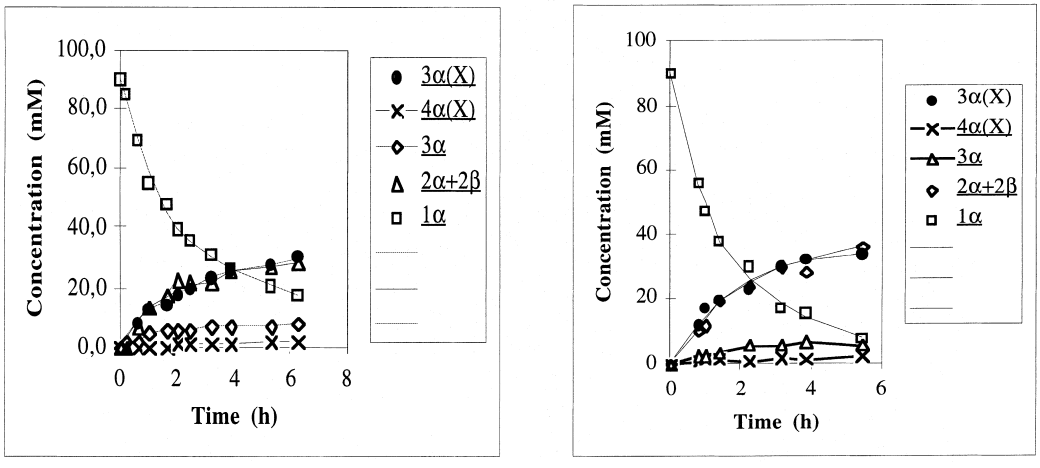


Figure 4. Kinetic study of reaction C at T=37°C. Left curves: in situ NMR experiment in D_2O, right curves: reaction in H_2O. **3α(X)** is the methyl α-D-galactopyranosyl-[1→3]-α-D-galactopyranoside and **4α(X)** the corresponding [1→4] regioisomer. Small amounts of other disaccharides were also present

In order to exemplify the power of in situ NMR for studying these very complex transformations, Fig. 5 shows the spectra of mixtures obtained with the above reaction catalysed by two different α-galactosidases at T=25 and T=37°C. At the higher temperature, the water resonance moves to high fields, thus allowing the analysis of a part of the spectrum completely masked by the water resonance at 25°C. Incidentally, an advantage of NMR spectroscopy over other techniques is clearly shown in the autocondensation experiments (reactions A and B) which can also be considered as the enzymatic hydrolysis of the activated donors **1β** and **1α**. The corresponding phenol thus liberated can be measured by means of visible spectroscopy. Usually, this technique is used to determine the glycosidase activity of an enzymatic preparation. Obviously, vis is not suitable for this purpose since significant autocondensation takes place and thus the concentration of phenol is not identical to the concentration of nitrophenylgalactoside which has really been transformed, particularly at the beginning of the transformation.

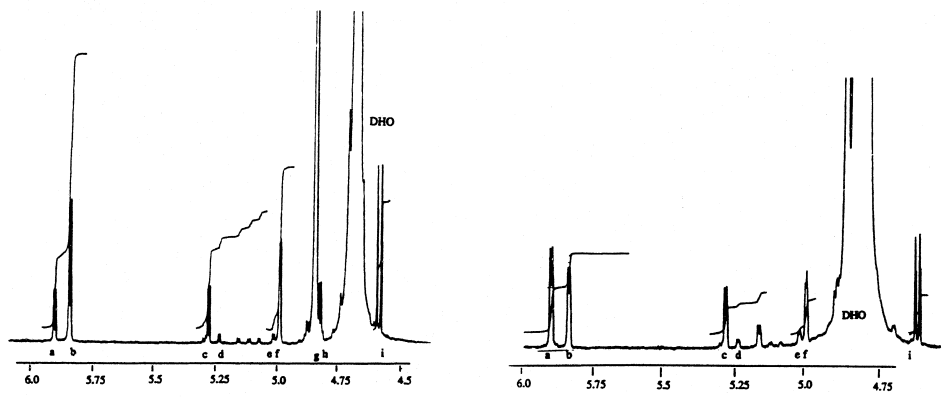


Figure 5. Proton NMR spectra at 500 MHz (only the anomeric protons are shown) of mixtures obtained with reaction C (condensation of 4-nitrophenyl α-D-galactopyranoside **1α** with methyl α-D-galactopyranoside) catalysed with the α-galactosidases Raf A[19] at 37°C (left spectrum) and Aga B[19] at 25°C (right spectrum). Lines: a: represents the resonances of H-1 (**3α** and **4α**), b: H-1 (**1α**), c: H-1 (**2α**), d: H-1′ (**3α**), f: H-1′ [**4α**(**X**)], g: H-1 (α-methyl galactopyranoside), h: H-1′ (**4α**), i: H-1 (**2β**). Other disaccharides are also present

3. Experimental

3.1. General

The crude extract cells 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 the literature.[14] The α-galactosidase from green coffee beans was purchased from Sigma. The chemicals supplied by Aldrich were used without further purification. The α-galactosidases Aga B (from *Bacillus stearothermophilus*) and Raf A (from *E. coli*) were supplied by Prof. R. Mattes (Institute of Industrial Genetics, University of Stuttgart). The D_2O was purchased from Eurisotop (isotopic purity 99.9%). The complete analysis of the NMR ^1H and ^{13}C resonances and subsequent structure assignment were made using standard 2D sequences (COSY HH and HCOOR correlations) and by comparisons with previous data.[8,10,11] The spectra were recorded with a Bruker AX500 spectrometer operating at 500 MHz for ^1H (solvent D_2O) and chemical shifts in ppm quoted from the resonance of the methyl groups of (trimethylsilyl)-3-propansulfonic acid was used as an internal reference.

3.2. Kinetic study of the autocondensation reaction of o-NP-β-Gal catalysed by Tt β-Gly (reaction A)

Solvent H_2O at 25°C: 6.1 mL of the phosphate buffer containing 12.1 mg (55.7 μM) of the reference was prepared and adjusted to pH 7. In a separate experiment, 57 μL of the crude extract of *E. coli* cells (BL21pETBg8) containing 1.8 units (calculated from the hydrolysis of *o*-NP-β-Gal at 25°C) of the *Thermus thermophilus* β-glycosidase were concentrated overnight in a desiccator (P_2O_5) and redissolved in 0.6 mL of the buffer solution. To this solution, 110 mg (0.365 mmol) of 2-nitrophenyl β-D-galactoside dissolved in 5.5 mL of the buffer and 0.35 mL of DMF (used as a co-solvent to dissolve all the components of the mixture, particularly the large amounts of 2-nitrophenol synthesised at the end of the experiment) was added. The solution was allowed to proceed at 25°C. Aliquots of 540 μL of the reaction mixture were taken 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 and the resulting mixture

was dried in a desiccator (P_2O_5). The powders obtained were redissolved in D_2O and the solution was submitted to ^1H NMR quantitative analysis.

Solvent D_2O at 25°C (kinetics in the NMR tube): 0.83 mL of 0.1 M, pH 7 phosphate buffer was lyophilised, dissolved in 0.5 mL of D_2O, lyophilised once more and redissolved in 0.83 mL of D_2O buffer. Then, to 0.5 mL of the D_2O buffer, 15 mg (50 μmol) of 2-nitrophenyl galactoside, 1.7 mg (7.6 μmol) of the reference (trimethylsilyl)-3-propansulfonic acid and 48 μL of DMF-d_6 were added. The presence of DMF was necessary to dissolve the phenol synthesised. In a separated experiment, 59.7 μL of the crude extract of *E. coli* cells (BL21pETbg8)[10] containing 0.25 units (calculated from the hydrolysis of *o*-NP-β-Gal at 25°C) were concentrated overnight in a desiccator (P_2O_5) and dissolved in the remaining 0.13 mL phosphate buffer at pD 7.0. This enzymatic solution was added to the reactant solution. The resulting mixture was immediately filtered in an NMR tube and the reaction was allowed to proceed in the probe of the magnet of the spectrometer at 25°C.

3.3. Kinetic study of the autocondensation reaction of p-*NP-α-Gal catalysed by α-galactosidase from green coffee beans (reaction B)*

Solvent H_2O at 37°C: 130 μL of the Sigma preparation of the α-galactosidase from green coffee beans containing 15.7 units were dried in a desiccator containing P_2O_5. The resulting powder was redissolved into 0.72 mL from the 8.72 mL of the 0.1 M phosphate buffer containing the silylated reference (9.16 mM). The pH of this solution was adjusted to 7 prior to the introduction of the enzyme. 4-Nitrophenyl-β-D-galactoside (236.6 mg, 0.785 mmol) was redissolved in 8 mL of the above phosphate buffer and the enzyme preparation was added. The reaction was allowed to proceed at 37°C. Aliquots of 360 μL of the reaction mixture were taken off at different times. For each sample, the reaction was quenched by addition of 10 μL of 1 M NaOH. The water was then removed under reduced pressure and the resulting mixture was dried in a desiccator (P_2O_5). The powders obtained were redissolved in D_2O and the solution was submitted to ^1H NMR quantitative analysis.

Solvent D_2O at 37°C (kinetic study in the NMR tube): 21 mg (70 μmol) of 4-nitrophenyl α-D-galactoside and the amount of salts necessary for 0.78 mL from the 0.1 M (pH 7) phosphate buffer were lyophilised, dissolved in D_2O and lyophilised once more. The buffer reference mixture containing the silylated reference (0.78 mL) was prepared as above and adjusted to pD 7. The α-galactosidase from green coffee beans (Sigma) was dried in a desiccator containing P_2O_5 and redissolved in 0.18 mL of the phosphate buffer reference mixture. The solutions were warmed to 37°C and the enzymatic preparation was added to the reactant solution. The resulting mixture was immediately filtered in an NMR tube and the reaction was allowed to proceed in the magnet of the spectrometer at 37°C.

3.4. Kinetic study of the condensation reaction of p-*NP-β-Gal with methyl* α-D-*galactopyranoside catalysed by α-galactosidase from green coffee beans (reaction C)*

The procedure and the amounts of all the components were identical to those used in the above reactions apart from the presence of the α-methylgalactoside (3 equivalents for 1 of *p*-NP-α-Gal).

Acknowledgements

Thanks are due to the French Ministry of Education and Research for a grant to one of us (V.C.G.) and for financially supporting this work. R. Mattes (Institute of Industrial Genetics, University of Stuttgart) is gratefully acknowledged for providing the α-galactosidases Raf A and Aga B.

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