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In situ proton NMR study of acetyl and formyl group migration in mono-*O*-acyl D-glucose†

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Acetyl and formyl group migration, mutarotation, and hydrolysis of mono-*O*-acylated glucose are studied by *in situ* 1D and 2D ^{1}H NMR spectroscopy. α-D-Glucosyl-1-acetate and α-D-glucosyl-1-formate serve as sole starting materials. They are generated *in situ* by configuration retaining glucosyltransfer from α-D-glucosyl-1-phosphate to formate and acetate, which is catalyzed by the Glu-237 → Gln mutant of *Leuconostoc mesenteroides* sucrose phosphorylase. Temporary accumulated regio-isomeric mono-*O*-acyl D-glucoses are identified, characterized, and quantified directly from the reaction mixture. Time courses of the transformations give insight into pH dependence of acyl group migration and mutarotation as well as into the stability of various regioisomers. Copyright © 2009 John Wiley & Sons, Ltd.

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Introduction

Migration of small acyl groups in partially *O*-acylated carbohydrates or polyhydroxy compounds is well known since the 1920s[1,2] and has occasionally been used in synthetic strategies.[3] However, it makes the *O*-acetyl group an unusual protecting group when selective protection of a secondary hydroxyl group in a saccharide is required.[4] Further on it complicates structural investigations of partially *O*-acetylated oligosaccharides and polysaccharides, which naturally occur in plants and bacteria.[5–7] Migrating acyl groups also impede studies of lipases [EC 3.1.1.3] and acetylesterases [EC 3.1.1.6] acting on partially acylated saccharides.[8,9] Irreversible acyl group migration (AcM) from secondary to primary hydroxyl groups is well documented in some cases,[10,11] but reversible AcM in polyhydroxy compounds has only been occasionally studied with respect to kinetic, thermodynamic, and positional aspects.[12–14] This discrepancy is likely caused by difficulties to analyze intermediates, which are accumulated in small amounts for a short time.

During the last decades there is a growing interest to determine the details of AcM reaction mechanism and its kinetic.[12–19] NMR spectroscopy has frequently been applied as an excellent analytical tool for this purpose.[13,14,17–19] Acylated saccharides used as starting materials in some of these investigations are isomeric mixtures, which make AcM difficult to analyze.[13] For some other studies, laboriously synthesized selective *O*-acylated glycopyranosides serve as model substrates. These tailor-made compounds are not subjected to mutarotation and AcM is only possible over a constricted number of hydroxyl functions.[12,14]

We now use the recently described sucrose phosphorylase catalyzed glucosyl transfer for selective generation of α-D-glucosyl-1-acetate (**α-1a**) and α-D-glucosyl-1-formate (**α-2a**).[20] These compounds serve as excellent starting materials to study several details of acetyl and formyl group migration as well as of accompanied mutarotation in mono-*O*-acyl glucose. 1D and 2D *in situ* ^{1}H NMR spectroscopy is used as the most versatile technique to identify and quantify up to 14 intermediates and products *in situ* and in parallel during the reasonable short reaction times.

Results and Discussion

Wild-type sucrose phosphorylase from *Leuconostoc mesenteroides* [*Lm*SPase, EC 2.4.1.7] catalyses reversible glucosyl transfer from sucrose (**3**) to phosphate with retention of the α-configuration (Scheme 1(A)). The equilibrium is by far on the side of the products fructose (**4**) and α-D-glucosyl-1-phosphate (**5**).[21] The corresponding *Lm*SPase Glu-237 → Gln mutant (E237Q) also catalyses α-retaining glucosyl transfer from α-D-glucosyl-1-phosphate (**5**) to small acceptor molecules like acetate and formate (Scheme 1(B)).[20] The formed α-D-glucosyl-1-acetate (**α-1a**) and α-D-glucosyl-1-formate (**α-2a**) are subjected to spontaneous AcM, mutarotation, and partial hydrolysis in aqueous solution, which has made the use of *in situ* ^{1}H NMR necessary to identify and investigate the E237Q catalyzed transformations.[20]

We apply 1D and 2D *in situ* ^{1}H NMR to gain insight into the spontaneous consecutive reactions without influences during work up procedures. Successively recorded ^{1}H NMR spectra allow identification of known compounds and enable efficient analysis of the reaction time courses.[22–24] 2D COSY and TOCSY as well as 1D selTOCSY spectra are recorded directly from the reaction mixture for additional structural analysis of not yet described intermediates.[25,26]

An *in situ* recorded ^{1}H NMR spectrum is given in Fig. 1(A) and shows indicative signals of all sufficiently accumulated

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† *Dedicated to the memory of Dr. Marion F. Kögl. Her insights, enthusiasm, and unique humanity have been a scientific and personal enrichment to many, and her presence is greatly missed.*

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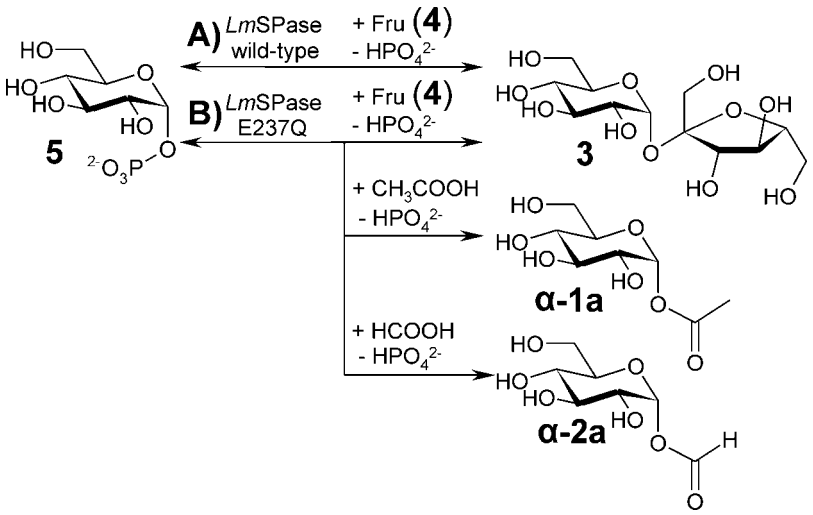
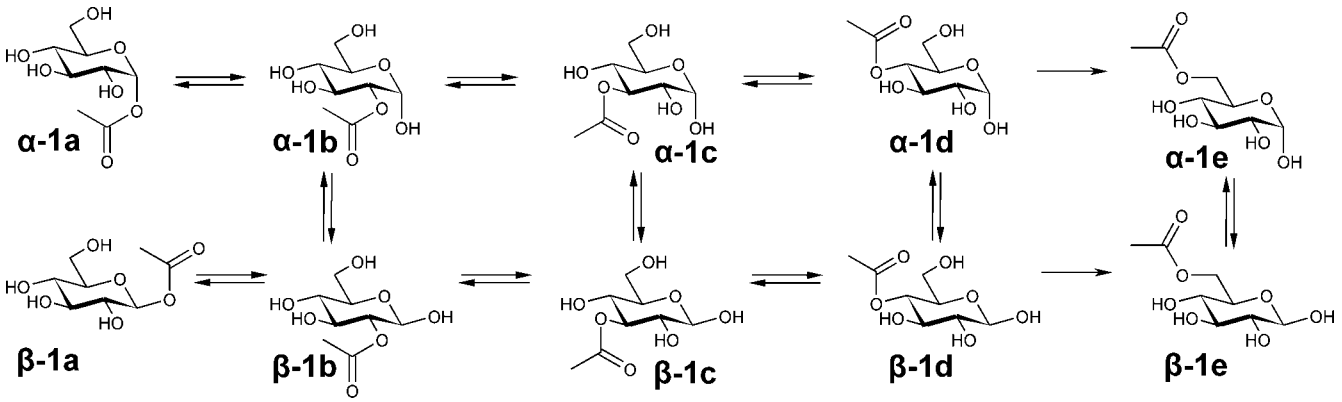


Scheme 1. (A) Wild-type *Lm*SPase catalyzed formation of sucrose (**3**) by glucosyl transfer from α-D-glucosyl-1-phosphate (**5**) to fructose (**4**). (B) E237Q catalyzed syntheses of sucrose (**3**), α-D-glucosyl-1-acetate (**α-1a**), and α-D-glucosyl-1-formate (**α-2a**) by glucosyl transfer from α-D-glucosyl-1-phosphate (**5**) to fructose (**4**), acetate, and formate, respectively.



Scheme 2. Reaction scheme of acetyl group migration and mutarotation between D-glucosyl-1-acetate (**1a**) and the *O*-acetyl D-glucoses (**1b-e**).

compounds during α-D-glucosyl-1-formate (**α-2a**) generation, formyl group migration, and hydrolysis. The corresponding time course (Fig. 1(B)) indicates the E237Q catalyzed glycosyl transfer to formate to be reasonably slow, which is caused by moderate catalytic efficiency ($k_{cat}/K_m = 3.7 \times 10^{-4}$ s^{-1} mM^{-1}).[20] Several steps of the formyl group migration, however, are fast and lead to only ~400 µM maximum concentrations of **2a**. Intermittently generated 2-*O*-formyl D-glucose (**2b**) and 3-*O*-formyl D-glucose (**2c**) are accumulated in detectable amounts only in a time frame of 6–8 h. Concentrations of both anomers of 4-*O*-formyl D-glucose (**2d**) are below the detection limit, because fast and irreversible AcM causes direct formation of the two 6-*O*-formyl-D-glucose anomers (**2e**).[10] A simultaneous and rapid formyl ester hydrolysis of all intermediates and products leads to fast formation of free glucose and formate. The quite low concentrations of accumulated *O*-formyl glucoses (**2**) allow only to determine a small number of ^{1}H NMR data (Table 1), which are in good accordance with sparse results published earlier.[20,27] Further analysis of the intermediates, however, is cumbered by fast hydrolysis of the formyl esters.

Using acetate as acceptor E237Q catalyses formation of α-D-glucosyl-1-acetate (**α-1a**) with moderate catalytic efficiency ($k_{cat}/K_m = 3.9 \times 10^{-4}$ s^{-1} mM^{-1}).[20] The consecutive AcM is strongly pH dependant and small pH changes lead to pronounced differences in concentrations of the intermittently generated mono-*O*-acetyl D-glucoses (**1**).[28] A transformation at pH 7.8 causes fast AcM only leading to accumulation of the terminally formed 6-*O*-acetyl D-glucose (**1e**). At pH 6.8 the biotransformation and AcM are very slow resulting in exclusive formation of small amounts 2-*O*-acetyl D-glucose (**1b**) after 10 h. At pH 7.2–7.3 the enzyme catalyzed transformation, AcM, and mutarotation are well balanced and allow *in situ* NMR monitoring of time courses between 12 h and 90 h. During the longer reaction time, additional

Table 1. Proton [δ_H (ppm)] chemical shifts of *O*-formyl D-glucoses

Compound	H-1	H-6a	H-6b	HCOO-
α-D-glucosyl-1-formate (**α-2a**)	5.36	ND	ND	8.21
2-*O*-formyl D-glucose (**2b**)	ND	ND	ND	8.27
3-*O*-formyl α-D-glucose (**α-2c**)	5.25	ND	ND	8.31[a]
3-*O*-formyl β-D-glucose (**β-2c**)	ND	ND	ND	8.30[a]
6-*O*-formyl α-D-glucose (**α-2e**)	5.18	4.42	4.38	8.15
6-*O*-formyl β-D-glucose (**β-2e**)	4.63	4.47	4.32	8.15

ND, not determined due to signal overlap.
[a] Exchangeable.

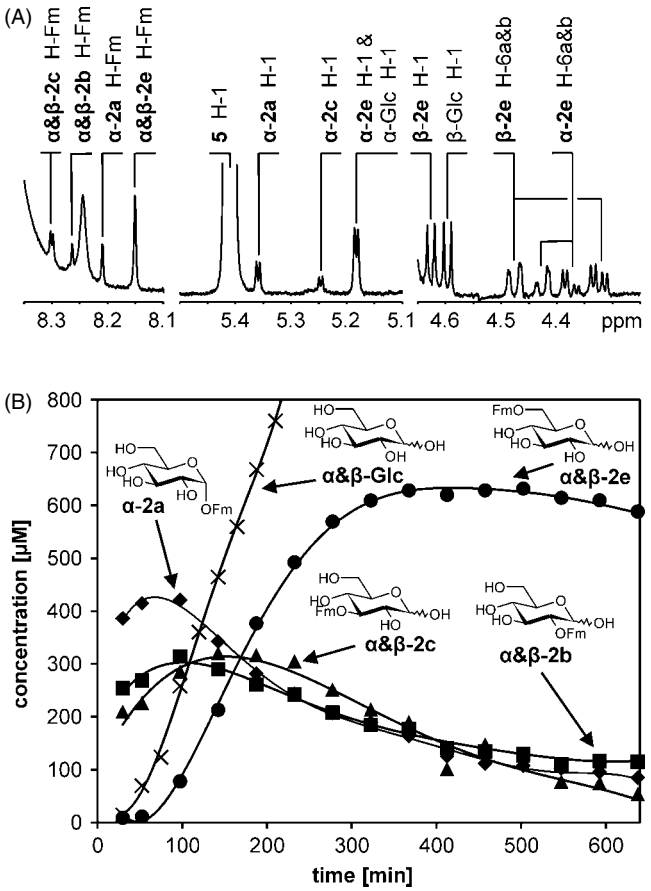


Figure 1. (A) ^{1}H NMR spectrum taken during E237Q catalyzed glucosyl transfer from α-D-glucosyl-1-phosphate (**5**) to formate and consecutive formyl group migration, mutarotation, and hydrolysis. Representative signals of the accumulated intermediates and terminally formed glucose are indicated (HCOO-: proton in formyl group). (B) *In situ* ^{1}H NMR monitored time course of the reaction cascade at pH 7.2. Shown are concentrations of the initially formed α-D-glucosyl-1-formate (**α-2a**) as well as the summed concentrations of the respective anomers of *O*-formyl D-glucoses (**2b**), (**2c**), and (**2e**), and of the terminal formed glucose (Fm: formyl group). The compounds are assigned by the indicative signals shown in (A) and by the order of their appearance in the reaction cascade. Starting concentration of α-D-glucosyl-1-phosphate (**5**) and of formate are 10 mM and 250 mM, respectively and glucose concentration after 630 min is 3.1 mM. 4-*O*-Formyl D-glucose (**2d**) does not accumulate in detectable amounts.

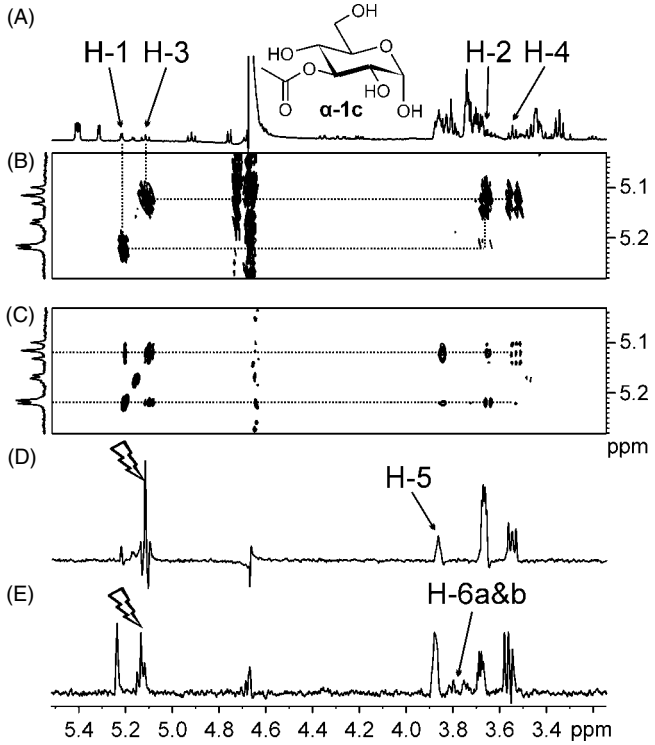


Figure 2. *In situ* ^{1}H NMR structural analysis of 3-*O*-acetyl α-D-glucose (**α-1c**). In the ^{1}H NMR (A) H-1 shows a doublet (5.22 ppm; $^{3}J_{H-H} = 4.1$ Hz), which indicates the α-pyranose form. DQF-COSY cutting (B) shows consecutive couplings to H-2 (3.67 ppm), H-3 (5.12 ppm), and H-4 (3.55 ppm). Reasonable low field shift of H-3 indicates the hydroxyl group in this position to be acetylated. The glucose configuration is confirmed by large coupling constants of H-3 and H-4, which are also detectable in ^{1}H NMR. They indicate no enzyme catalyzed modification of the saccharide skeleton. H-1 and H-3 traces in 2D TOCSY with 100 ms mixing time (C) indicate the shift of H-5 (3.87 ppm). 1D selTOCSY with 25 ms mixing time and irradiation at H-3 (D) confirms the shifts of H-2, H-4, and H-5. Corresponding 1D selTOCSY with 100 ms mixing time (E) allows additional determination of H-6a and H-6b (3.80 ppm and 3.74 ppm). Spectra correspond to the time course shown in Fig. 4(B) and are recorded between 30 h and 40 h after the start of the experiment.

in situ 1D selTOCSY as well as 2D COSY, TOCSY, and NOESY spectra can be recorded, when intermediates are accumulated in higher concentrations. These measurements enable for the first time a detailed structure elucidation of several mono-*O*-acetyl D-glucoses (**1**) in parallel. A comprehensive analysis is exemplarily shown in Fig. 2 for identification of 3-*O*-acetyl α-D-glucose (**α-1c**).

Apart from the initially generated α-D-glucosyl-1-acetate (**α-1a**), both anomers of 2-*O*-acetyl D-glucose (**1b**) and 3-*O*-acetyl D-glucose (**1c**) are accumulated in amounts suitable for detailed characterization. β-D-Glucosyl-1-acetate (**β-1a**), however, is only formed in small concentrations by AcM from position two to one in **β-1b**. Little signals of the two further acetyl groups indicate the two anomers of 4-*O*-acetyl D-glucose (**1d**) to be accumulated only in small amounts. Their low concentration is caused by the fast and irreversible AcM from position four to six in gluco-configured aldohexoses.[10] Hence, 6-*O*-Acetyl D-glucose (**1e**) is finally generated in larger amounts (Scheme 2). Signals of the acetate groups of all compounds are assigned to the mono-*O*-acetyl D-glucoses (**1**) by parallel time courses of corresponding H-1 and CH_3 group signal integrals (Fig. 3). All detectable ^{1}H NMR shifts of the accumulated intermediates are listed in Table 2. They are in good accordance with a few earlier reported shifts of these compounds.[20,29–33]

^{1}H NMR signals of the acetyl groups and of some H-1 are used to determine time courses of the reactions monitored at pH 7.3 and 7.2 over 13 and 90 h, respectively. This small pH variation leads to very similar transformation rates of the E237Q catalyzed reaction, but the AcM rates show reasonable differences (Fig. 4(A) and (B)). For example, maximum concentration of 2-*O*-acetyl D-glucose (**1b**) is reached after ~6 and ~20 h, respectively. However, several details of the consecutive AcMs are very similar in both time courses. At the beginning, 2-*O*-acetyl α-D-glucose (**α-1b**) is formed in excess above the corresponding β-anomer **β-1b** before mutarotation inverted their proportion towards the thermodynamically favored ratio after a couple of minutes. The corresponding equilibration of 3-*O*-acetyl D-glucose (**1c**) is reached considerably later, which is very likely caused by faster AcM from position two to three in the α-anomer compared to the β-anomer as well as by slower mutarotion of **1c** compared to those of

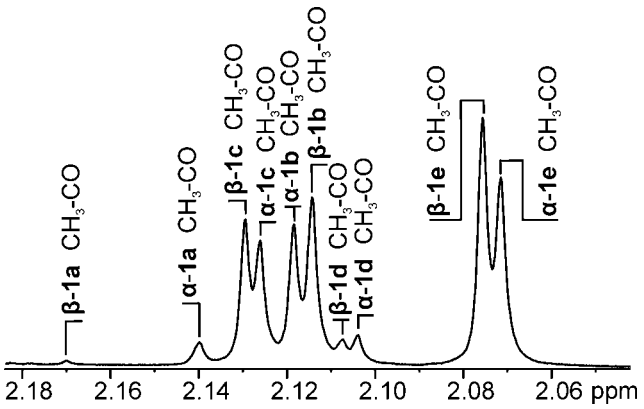


Figure 3. ^{1}H NMR signals of the acetate groups assigned to the anomers of D-glucosyl-1-acetate (**1a**) and of the *O*-acetyl D-glucoses (**1b-e**). Spectrum matches the last *in situ* ^{1}H NMR measurement of the time course shown in Fig. 4(B).

1b (Fig. 4(A)). The anomeric ratios of intermediate **1d** and of terminal product **1e** do not change during the reaction, as they are formed after the equilibrium conditions of the mutarotation are reached. Progress of the total product formation shows that the E237Q catalyzed transformation decelerated, caused by product inhibition and enzyme denaturation (Fig. 4(C)). Hydrolysis of the acetates is quite small as by far no free glucose is monitored. The pH hence drops only very slightly during the reaction (−0.03 pH) in spite of high buffer concentration. However, even this small decrease in pH leads to the slowing of AcM. Such changes during transformations impede a sensible determination of the large number of pH dependant rate constants (Scheme 2), which make the reported system more complex compared to recently studied AcM in partly protected α-D-galactose.[14]

Conclusions

*Lm*SPase E237Q mutant catalyzed reactions provide the specific synthesis of α-D-glucosyl-1-acetate (**α-1a**) and α-D-glucosyl-1-formate (**α-2a**) which are excellent starting materials for a first-time study of free formyl and acetyl group migration in unprotected mono-*O*-acyl D-glucose (**1**). 1D and 2D *in situ* ^{1}H NMR monitoring is an optimal analytical technique for parallel identification of accumulated intermediates and gives insight into time courses of acyl group migration as well as into accompanied mutarotation. Such investigations complement AcM studies using partly protected model substrates, which allow monitoring of only a restricted number of migration steps.[12,14] The resulting data are valuable for future identification of partially *O*-acetylated natural saccharides as well as for analysis of biocatalyzed regiospecific acylation/deacylation of carbohydrates.

Experimental

Chemicals

All chemicals are purchased from Sigma-Aldrich Chemical Co., St Louis, USA in the highest available purity and used without further purification.

Site-directed mutagenesis, protein production, and purification

Site-directed mutagenesis, protein production, and purification of E237Q have been described previously.[20]

NMR sample preparation

For *in situ* NMR measurements the reactions are performed directly in a NMR sample tube. Samples contain 10 mM α-D-glucosyl-1-phosphate (**5**), 9 μM E237Q, and 250 mM sodium formate or sodium acetate, respectively, in D_2O (0.70 mL, 99.9% D). The pH value is adjusted by addition of 1.0 M NaOD or DCl solutions in D_2O and measured at the start and at the end of the reaction. Small amounts of MES buffer (1 mM), which originate from protein purification, serve for *in situ* estimation of pH changes during the reaction.

In situ ^{1}H NMR measurements

Reactions taking 13–14 h are performed in the magnet and up to 64 ^{1}H NMR spectra are directly taken from the sample at regular intervals.[22] The reactions over 90 h are performed in the magnet and temporarily kept in a temperature-controlled water bath (303 ± 0.2 K). 1D and 2D ^{1}H NMR measurements of these reactions are measured in non-uniform intervals.[25,26]

Table 2. Proton [δ_H (ppm)] chemical shifts of *O*-acetyl D-glucoses

Compound	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	CH_3CO-
α-D-glucosyl-1-acetate (**α-1a**)	6.05	3.74	3.80	3.75	3.75	3.75	3.43	2.141
β-D-glucosyl-1-acetate (**β-1a**)	5.49				ND			2.170
2-*O*-acetyl α-D-glucose (**α-1b**)	5.32	4.66	3.85	3.46	3.81	3.91	3.82	2.118
2-*O*-acetyl β-D-glucose (**β-1b**)	4.76	4.61	3.62	3.45	3.80	3.84	3.67	2.113
3-*O*-acetyl α-D-glucose (**α-1c**)	5.22	3.67	5.12	3.55	3.87	3.80	3.74	2.124
3-*O*-acetyl β-D-glucose (**β-1c**)	4.69	3.37	4.93	3.55	3.51	3.88	3.69	2.130
4-*O*-acetyl α-D-glucose (**α-1d**)				ND				2.104[a]
4-*O*-acetyl β-D-glucose (**β-1d**)				ND				2.107[a]
6-*O*-acetyl α-D-glucose (**α-1e**)	5.18	3.69	3.50	3.42	3.99	4.32	4.25	2.070
6-*O*-acetyl β-D-glucose (**β-1e**)	4.62	3.23	3.46	3.64	4.37	4.37	4.21	2.074

ND, not determined due to low concentration.
[a] Exchangeable.

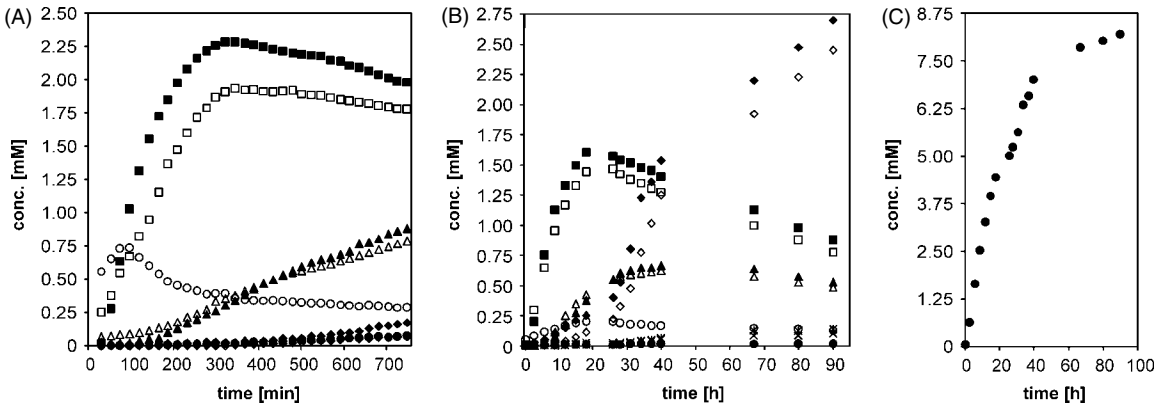


Figure 4. *In situ* ^{1}H NMR time course of α-D-glucosyl-1-acetate (**α-1a**) formation, AcM, and mutarotation at pH 7.3 (A) and 7.2 (B) over 13 h and 90 h, respectively. [Symbols: close circle (**β-1a**); open circle (**α-1a**); close square (**β-1b**); open square (**α-1b**); close triangle (**β-1c**); open triangle (**α-1c**); star (**β-1d**); cross (**α-1d**); close diamond (**β-1e**); open diamond (**α-1e**).] Concentration of summed products in the reaction performed at pH 7.2 over 90 h is shown in (C). Starting concentrations of α-D-glucosyl-1-phosphate (**5**) and of acetate are 10 mM and 250 mM, respectively. Signals of H-1 and of the acetate groups are used to obtain the progress curves of the intermediates and products.

NMR spectroscopic measurements

All spectra are recorded on a DRX-600 AVANCE spectrometer (Bruker, Rheinstetten, Germany), equipped with a triple resonance *xyz*-gradient inverse probe and processed using the Topspin 1.3 software. ^{1}H irradiation and measurement frequency is 600.13 MHz and the sample temperature is adjusted to 303.15 ± 0.01 K. The 1D ^{1}H and selTOCSY spectra are recorded with a 30° ^{1}H-pulse, acquisition of 32 768 data points, digital FID resolution of 0.18 Hz, a relaxation delay of 1.0 s, and accumulation of 16 to 512 scans.[26] The overwhelming HDO signal in ^{1}H NMR spectra is suppressed by presaturation. For selective excitation in selTOCSY Gaussian pulses with 50 ms duration are used and the mixing times varied between 25 and 100 ms. After zero filling to 65 536 data points the free induction decays are Fourier transformed to spectra of 6000 Hz range. 2D TOCSY and NOESY spectra are recorded with 1024 data points and 8 scans in the t_2-dimension, as well as with 256 experiments in t_1-dimension leading to measurement times between 75 and 100 min and to digital FID resolutions of 4.7 Hz (t_2-dimension) and 18.7 Hz (t_1-dimension). Zero filling to 512 data points in t_1-dimension, appropriate sinusoidal multiplication, and Fourier transformation lead to spectra with 6000 Hz range in both dimensions. Mixing time in 2D NOESY spectra is 800 ms. All spectra are referenced to external acetone (δ ^{1}H: 2.225 ppm).

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