"One-pot" synthesis of 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranose as intermediate for *N*-acetyl-α-D-lactosamine heptaacetate preparation

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Abstract

We have recently reported the synthesis of different acyl pyranoses, bearing only one free hydroxyl group, obtained by regioselective enzymatic hydrolysis of the peracetylated precursors and subsequent acyl migration of the hydrolyzed products.[1] As a part of this ongoing research, we have focused our attention onto the pure regioisomers of 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (**1**) as key intermediate for the preparation of a large number of oligosaccharides. Particularly, we have developed an efficient process to obtain a deprotected derivative of **1** having only one free hydroxyl group at the C-4 position (**3**) as a valuable synthon for the preparation of *N*-acetyl-α-D-lactosamine heptaacetate (**4**), building-block in a new synthetic pathway for oligosaccharides like Sialyl Lewis^x. Our chemo-enzymatic strategy allows to obtain the disaccharide (**4**) in good overall yield, starting from a commercially available cheap compound and without any intermediate purification. Our approach is, indeed, an interesting alternative to the classical multi-step chemical procedures that are often plagued by low yields and formation of undesired by-products.

Keywords: Carbohydrates, regioselective enzymatic hydrolysis, acyl group migration, *N*-acetyl-α-D-lactosamine heptaacetate, *Candida Rugosa* lipase

Introduction

Oligosaccharides have recently received considerable attention because of their biological roles. In fact, carbohydrates actively control a whole range of biological and pathological processes e.g. immune-defense, viral replication, parasitic infections, cell-cell adhesion, cell-cell recognition, etc.[2] This evidence provides further opportunities for the therapeutic treatment of diseases, such as diabetes, AIDS and cancer, with new oligosaccharides. Particularly, the challenge of developing carbohydrate-based anticancer vaccines is being actively sought. Tumor

immunotherapy is based on the theory that tumors possess specific antigens that can be recognized by a properly trained immune system. Those antigens are represented by carbohydrate motifs (glycoproteins or glycolipids) that are hyper-expressed by transformed cells. Indeed, the rationale underlying this approach is that the immunization by synthetic carbohydrate vaccines would produce antibodies against cancer cells preventing also from their spreading and invasiveness.[3-5] According to recent reports, some glycoderivatives have been clinically evaluated and are in ongoing human trials;[3-6] this might lead to a growing demand for carbohydrate derivatives as raw materials for the preparation of anticancer vaccines. However, one of the main problems of this promising therapy is to obtain significant amounts of the desired oligosaccharides (Figure 1) as their supply is extremely difficult both from isolation methods and total chemical synthesis. Moreover, due to their inherently low immunogenicity, carbohydrate antigens require an appropriate immunogenic carrier (generally a protein) to achieve the optimal response;[7] thus, the synthesis of these antigens is further complicated by the need to conjugate carbohydrates and peptides.

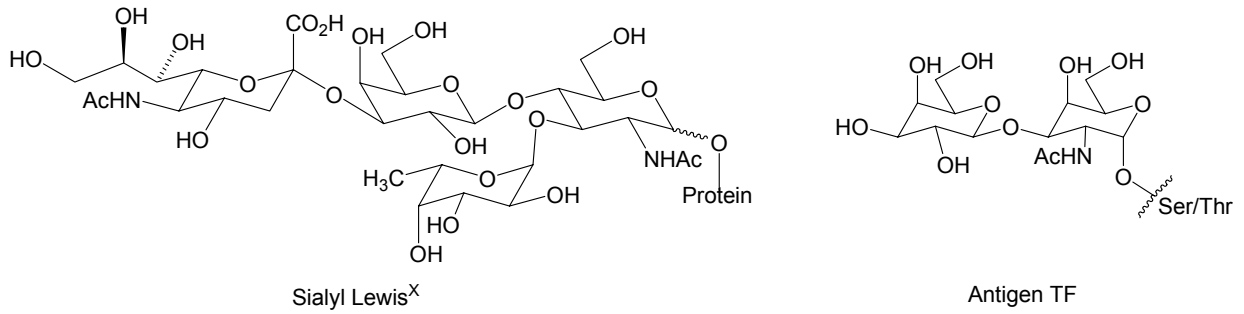
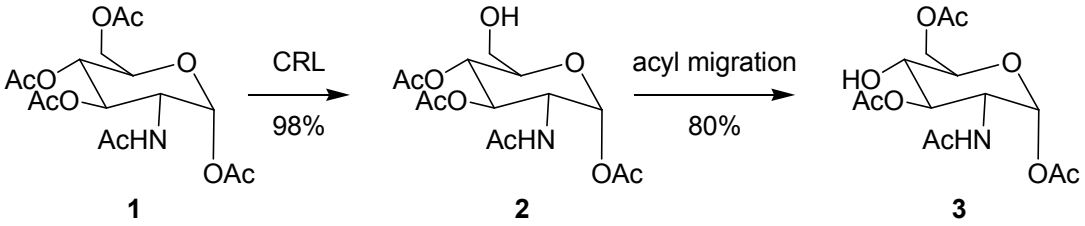


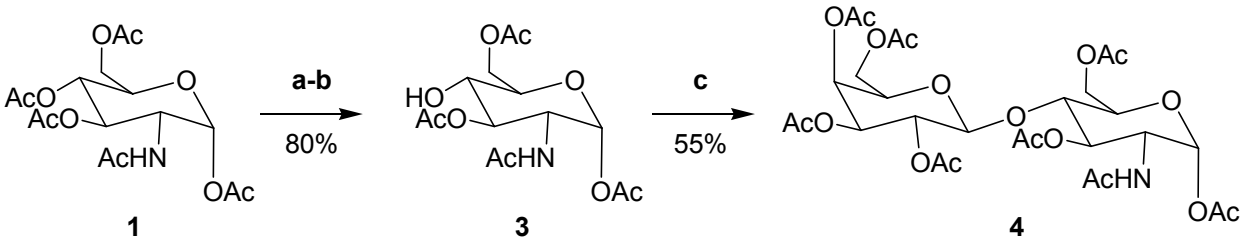
Figure 1. Structures of tumor associated antigens described throughout the text: Sialyl Lewis^x and TF antigen.

So far, several efforts have been made to pursue new efficient and economic synthetic processes of general applicability. Especially in the case of carbohydrates and other glycoderivatives (sugar esters, glycopeptides, etc.), pure regioisomers of *O*-acylated pyranosides bearing only one free hydroxyl group (AP-OH) are very useful building-blocks; nevertheless, their preparation usually requires multi-step chemical procedures involving regioselective protection/deprotection reactions. Due to the complexity of this classical chemical approach, we have considered the use of enzyme catalysts such as lipases for the regioselective deprotection of *O*-acylated pyranosides. Recently,[1] we have successfully developed a new process for the production, in high yield, of different APs-OH regioisomers using a chemo-enzymatic approach. On the basis of the previous studies, in the present work, we have investigated the regioselective enzymatic hydrolysis of 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (**1**) catalyzed by immobilized *Candida Rugosa* lipase (CRL),[1] and the subsequent acyl migration (pH and temperature dependent) of the hydrolyzed product (**2**, Scheme 1). The intermediate resulting from this "one-pot" chemo-enzymatic approach, namely 2-acetamido-2-deoxy-1,3,6-tri-

O-acetyl-α-D-glucopyranose (**3**), bearing only one free hydroxyl group at the C-4 position (AP-4OH), was then successfully used for the synthesis of *N*-acetyl-α-D-lactosamine heptaacetate (**4**) (Scheme 2). Although acetyl pyranoses with only one free hydroxyl group (APs-OH) can be used as precursors in the glycosylation reactions, they are not generally considered as valuable synthons because their chemical synthesis requires a multi-step process involving specific protection/deprotection reactions[8] and, besides, because the acetoxy moieties deactivate the free hydroxyl group. A novel procedure affording the target APs-OH in few steps, high yield and purity, avoiding intermediates purification, might enhance the use of these compounds in the glycosylations in spite of their poor reactivity. *N*-Acetyl-D-lactosamine, that is routinely prepared in several chemical steps,[9] is reported as an example of the successful application of the approach above described.



Scheme 1. Chemo-enzymatic synthesis of 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranose (AP-4OH, **3**).



a-b: "one-pot" enzymatic hydrolysis and acyl migration (4→6); **c**: chemical glycosylation

Experimental conditions:

a: acetone 20% (v/v) in 50 mM KH_2PO_4 buffer pH 4, 25 °C, [**1**] 50 mM, catalyst: CRL on octyl agarose

b: acetone 20% (v/v) in 50 mM KH_2PO_4 buffer pH 8.5, 0-4 °C

c: CH_2Cl_2, BF_3 $O(Et)_2$, tetra-*O*-acetyl-α-D-galactopyranosyl trichloroacetimidate, -20 °C

Scheme 2. Chemo-enzymatic synthesis of *N*-acetyl-α-D-lactosamine heptaacetate (**4**).

Results and Discussion

The process consists of three reactions (Scheme 2): the enzymatic hydrolysis of the peracylated 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (**1**) to form the AP-6OH (2-acetamido-2-deoxy-1,3,4-tri-*O*-acetyl-α-D-glucopyranose, **2**), followed by controlled acyl migration from the 4 to 6 position to afford the target AP-4OH (2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranose, **3**) and, finally, the synthesis of the disaccharide (**4**).

Chemo-enzymatic synthesis of 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranose (AP-4OH, 3)

The hydrolysis of **1**, catalyzed by CRL immobilized on octyl agarose, has been performed following the general procedure previously reported.[1] Selective removal of the C-6 acetoxy group in 50 mM phosphate buffer, containing acetonitrile as cosolvent (20%) at acid pH, gave the corresponding AP-6OH (**2**, Scheme 1).

In order to optimize the hydrolysis of **1** and, particularly, to increase the reaction rate, we considered the influence of different cosolvents and the substrate concentration. The change of the cosolvent did not display any sensitive improvement at low substrate concentration (5 mM) (Table 1) showing similar results when acetonitrile was replaced with acetone or DMSO as solvents.

Table 1. Enzymatic hydrolysis of **1**: effect of the cosolvent

Cosolvent[a]	V_h (UI/g)[b]	Conversion (%)[c]
Acetonitrile	4.3	45
Acetone	4.0	43
DMSO	4.0	38
DMF	3.0	33
Dioxane	1.9	27
EtOH	1.0	15
THF	n.d.	<5
None	2.7	27

Experimental conditions: pH 4, [**1**] 5 mM, 25 °C, enzyme: 1 g (activity: 200 UI/g)

[a] 20% (v/v) in 50 mM KH_2PO_4 buffer

[b] V_h values are expressed as 10^{-2}; UI=μmol of substrate hydrolyzed/min/g of enzyme derivative

[c] After 24 h

When the hydrolysis was performed with a higher concentration of substrate (20-50 mM), using acetonitrile or acetone as cosolvent, the yield was almost quantitative; whilst DMSO appeared to decrease the reaction rate and, indeed, the final conversion (Table 2).

Table 2. Enzymatic hydrolysis of **1**: effect of the substrate concentration

Cosolvent[a]	[1] (mM)	UI/mL[b]	V_h (UI/g)[c]	Conversion (%)[d]
Acetonitrile	20	25	20	92
Acetone	20	25	17	82
DMSO	20	25	12	61
Acetone	50	30	52	98

Experimental conditions: pH 4, 25 °C

[a] 20% (v/v) in 50 mM KH_2PO_4 buffer

[b] UI added per mL of reaction solution

[c] V_h values are expressed as 10^{-2}; UI=μmol of substrate hydrolyzed/min/g of enzyme derivative

[d] After 40 h

The study of the solvent effect was necessarily enlarged to the evaluation of the biocatalyst stability. Indeed, the immobilized CRL was suspended in phosphate buffer solutions containing acetonitrile, acetone or DMSO in a 20% ratio. These studies confirmed that acetonitrile was the best cosolvent in the enzymatic hydrolysis both in terms of the yield and for the reaction rate. However, acetonitrile resulted in reducing the stability of CRL, whereas DMSO and acetone did not sensitively affect the enzyme after the initial 35% drop in activity (data not reported). When the stability of the biocatalyst was tested in a buffer solution containing 50% of cosolvent, the enzymatic activity was dramatically repressed by acetonitrile, whereas DMSO and acetone, after the initial 50% drop, did not further influence the stability (Figure 2). These experiments were undertaken with the aim to test the biocatalyst in "drastic" reaction conditions necessary for the solubilization of the substrate at very high concentration for the scale-up development.

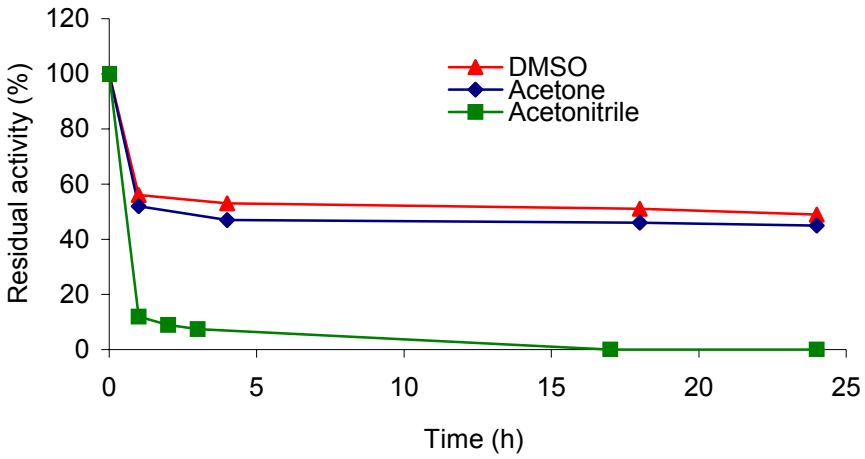


Figure 2. Stability of CRL in presence of different cosolvents (50% v/v).

Thus, acetone was selected as cosolvent for the successive experiments in order to evaluate the influence of the substrate concentration on the hydrolysis rate (V_h) and the conversion. This correlation may play a critical role in developing large scale processes and, indeed, cannot be understimated. A linear correlation between V_h and the substrate concentration (up to 50 mM) was found. Considering the rate of hydrolysis, the yield and the regioselectivity, the optimal reaction conditions for the enzymatic hydrolysis of 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (**1**, Scheme 2) were acetone 20% as cosolvent in phosphate buffer (50 mM) at pH 4 and r.t., using a 50 mM concentration of substrate (20 g/L). After 40 h, the conversion was 98% (Table 2).

The second step of the process concerned the optimization of the chemical acyl migration from the 4- to the deprotected 6-position. As previously reported for similar substrates,[1] this migration had been observed working in neutral or basic medium. As the influence of pH was found to play a crucial role in affecting the acyl migration, it was carefully considered as the pivotal parameter in order to control the exclusive formation of the 4-hydroxy derivative **3** (AP-4OH). The influence of the temperature and the type of cosolvent was also evaluated; the optimal reaction conditions, being acetone 20% as cosolvent in phosphate buffer (50 mM) pH 8.5 at 0-4 °C, afforded **3** (Scheme 1) in 80% conversion after 2 h. The extensive study performed for the acyl migration optimization will be reported in a forthcoming full paper.

Synthesis of *N*-acetyl-α-D-lactosamine heptaacetate (4)

According to the results obtained in the enzymatic hydrolysis of 2-deoxy-2-acetamido-1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (**1**) and in the acyl migration performed on the intermediate AP-6OH (**2**), we developed a "one-pot" chemo-enzymatic preparative synthesis of the AP-4OH (**3**). In fact, **1** was successfully hydrolyzed by immobilized CRL at pH 4 and 25 °C affording the 6-mono deprotected product (AP-6OH, **2**) in 98% yield. After removing the biocatalyst by filtration, the temperature was set to 4 °C and the pH brought to 8.5. Under these conditions, the

controlled acetyl group migration from the 4- to the 6-position gave AP-4OH (**3**) in 75% overall yield after the final purification step.

Once purified, AP-4OH (**3**) was used as starting material in a standard glycosylation reaction for the preparation of the disaccharide *N*-acetyl-α-D-lactosamine heptaacetate (**4**, Scheme 2). The synthesis proceeds through the condensation between tetra-*O*-acetyl-α-D-galactopyranosyltrichloroacetimidate and AP-4OH (**3**) catalyzed by boron trifluoride diethyl etherate at -20 °C. The peracetylated disaccharide **4** was obtained in 55% yield (not optimized). The global process is reported in Scheme 2.

Conclusions

The procedure here described, involving the "one-pot" enzymatic hydrolysis of a peracetylated pyranose followed by the controlled acyl migration, afforded the target AP-OH in high yield and purity.

To sum up, the main advantages of this strategy are:

1. the preparation of the enzyme substrates (peracetylated pyranoses) requires an easy and cheap chemical acetylation;
2. the synthesis of AP-OH can be performed by a two-step "one-pot" reaction performed in almost fully aqueous medium;
3. due to the regioselectivity of the catalyst (CRL), the target AP-OH is obtained in high yield and purity.

Thus, we have successfully demonstrated that using a chemo-enzymatic approach, a novel preparation of *N*-acetyl-α-D-lactosamine heptaacetate can be developed. This method allows an easy preparation of a high-value disaccharide that can serve as unit for some carbohydrate-based tumor antigens such as Sialyl Lewis^x and TF or, alternatively, for the obtainment of *N*-acetyl-D-lactosamine itself. The facile preparation of the acetylated precursors and the complete regioselectivity of the enzyme make this approach of general interest for the preparation of APs-OH, overcoming the limitations of these compounds in the glycosylation due to their poor reactivity.

The study of the biocatalyst and the reaction conditions may strongly influence the outcome of this strategy. In this context, new substrates are under investigation as well as the optimization of the chemical glycosylation. Those results are forthcoming and will be disclosed in due course.

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