Tetrahedron 68 (2012) 2141–2145

Contents lists available at SciVerse ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Bio-solvents change regioselectivity in the synthesis of disaccharides using Biolacta β-galactosidase

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ARTICLE INFO

Article history:
Received 3 November 2011
Received in revised form 29 December 2011
Accepted 11 January 2012
Available online 16 January 2012

Keywords:
Biocatalysis
Solvent effects
Bio-solvents
Transglycosylation
β-Galactosidase

ABSTRACT

Bio-solvents are good alternative solvents that avoid the use of classical organic solvents when performing enzymatic reactions. A noticeably change in regioselectivity was observed in the synthetic behaviour of Biolacta β-galactosidase using bio-solvents derived from dimethylamide and glycerol as co-solvents. Under these conditions, the enzyme changes its well known tendency to produce β-(1→4) to β-(1→6) disaccharides. An evaluation of the bio-solvent concentration and the effects of the non proteic additives in commercially available Biolacta β-galactosidase was undertaken in order to optimize the reaction conditions to improve the yield of the β-(1→6) product.

1. Introduction

Oligosaccharides are involved in a wide range of biological processes including bacterial and viral infection, cancer metastasis, the blood-clotting cascade and many other crucial intercellular recognition events.[1–4] As the understanding of these biological functions increases, the need for practical synthetic procedures of oligosaccharides in large quantities has become a major issue. Organic chemical methods for obtaining them have been developed,[5–7] but they involve several elaborate protection and deprotection procedures. Glycosyl hydrolases (glycosidases) can be used to synthesize oligosaccharides in a kinetically controlled reaction, where a glycosyl donor is used to transfer its glycosyl residue to a sugar acceptor present in the reaction medium. In spite of the increased amount of work carried out with glycosyl hydrolases, their main drawback is the lack of regioselectivity, which limits their use for synthetic purposes. In an important contribution to glycosidase-catalyzed oligosaccharide synthesis, Usui et al.[8] reported for the first time the use of Biolacta β-galactosidase to synthesize some β-D-(1→4) galactosyl disaccharides bearing a GlcNAc residue at the reducing end, although some β-D-(1→6) linkages were also formed. This was one of the first examples of a preparative scale β-D-galactosyltransfer where the linkage occurs preferentially at the *O*-4 position using a galactosyl hydrolase.

It is also well documented that many enzymes can maintain their activity in a variety of organic solvents. This improves the solubility of the reagents, decreases unfavourable side reactions and shifts the equilibrium towards the product side, therefore minimising hydrolysis.[9] On the other hand, the presence of organic solvents in enzymatic reactions can alter the activity and specificity of the enzyme. There are numerous examples in the literature where enzyme specificity and enantioselectivity is closely related to the solvent's physical properties, such as, density, dipole moment and hydrophobicity.[10–13] Among the different families of the so- called bio-solvents, organic solvents derived from renewable sources are receiving an increasing attention.[14,15] For instance, glycosidases can catalyze transglycosylation reactions in aqueous-organic solvents mixtures.[16–18] However, the use of glycosidases in bio-solvents is still limited and a few studies about their use in transglycosylation reactions are available.[19–21] Recently, we have shown that in the presence of co-solvents derived from glycerol in transglycosylation reactions catalyzed by Biolacta β-galactosidase the regioselectivity is shifted towards the obtention of the β(1→6) regioisomer.[21] In this work, we report the influence of a series of bio-solvents derived from biomass on the activity and selectivity of the enzymatic synthesis of disaccharides catalyzed by Biolacta β-galactosidases.

2. Results and discussions

2.1. Transglycosylation reactions in bio-solvents

The transglycosylation reaction of *N*-acetylglucosamine (GlcNAc) with *p*-nitrophenyl-β-D-galactopyranoside (*p*NP-β-Gal),

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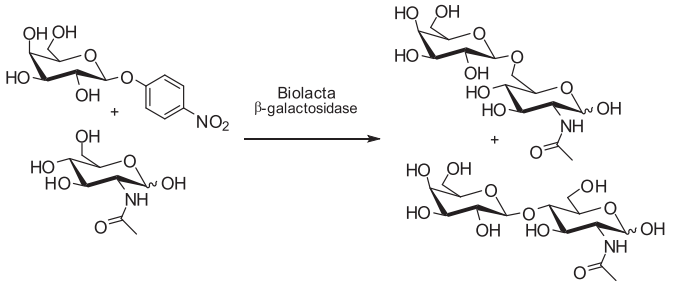
doi:10.1016/j.tet.2012.01.020

catalyzed by Biolacta No 5 β-galactosidase (Scheme 1) was used throughout this work. This reaction can afford Gal-β-(1→4)-GlcNAc as a major product and Gal-β-(1→6)-GlcNAc as a minor product.



Scheme 1. General scheme of transglycosylation reaction catalyzed by Biolacta β-galactosidase.

Transglycosylation reaction catalyzed by a commercial preparation of Biolacta β-galactosidase was carried out following the general procedure described in the Experimental section and monitored by HPLC (Scheme 1).

The concentration of bio-solvent was fixed to 2 M in the mixture with the 50 mM citrate/phosphate buffer at pH 5.0. The complete listing of the solvents used in this work is given in Fig. 1. Results obtained for the transglycosylation reaction in the presence of different solvents derived from glycerol are summarized in Table 1.

When the reaction is carried out only in a buffered medium, in the absence of organic solvents, good conversions of the desired Gal β-(1→4)GlcNAc (83%) product are achieved although a 17% of the Gal β-(1→6)GlcNAc isomer is also found. The use of either diethylamine or glycerol based solvents results in a change of the reaction regioselectivity towards the formation of the β(1→6) product except with solvent **S5**, that is, a solid under the experimental conditions. Solvents **S1** and **S4** led to the best yields for the synthesis of Gal-β-(1→6)-GlcNac (91% in both cases), with no traces of the hydrolysis product. This means an increase of activity compared to the natural behaviour of this enzyme in a 50 mM citrate/phosphate buffer at pH 5.0, along with an important change in the reaction regioselectivity, giving rise to the β-(1→6) isomer as a major reaction product. Therefore, these solvents were selected for further assays.

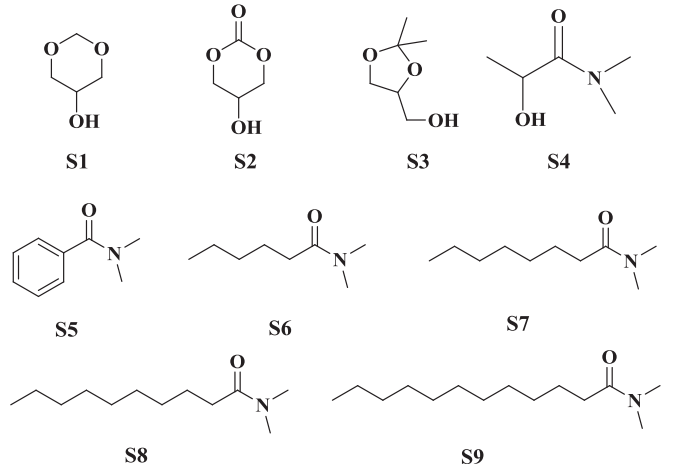


Fig. 1. Structures of bio-solvents derived from glycerol (**S1**–**S3**) and dimethylamide (**S4**–**S9**) employed in transglycosylation reactions with Biolacta β-galactosidase.

2.2. Effect of solvent molarity

In order to study the influence of co-solvents in enzymatic activity, different concentrations of **S2**, **S4** and **S6** (0.5, 2 and 5 M) in the reaction medium were assayed. Results obtained in these experiments are shown in Table 2.

Table 2
Transglycosylation yields (%) obtained with Biolacta β-galactosidase using 50 mM citrate/phosphate buffer at pH 5.0 at different bio-solvent concentrations

Solvent	Galactose (%)	Gal β-(1→4)GlcNAc (%)	Gal β-(1→6)GlcNAc (%)
S2 (0.5 M)	—	75	18
S2 (1 M)	—	36	45
S2 (2 M)	81	13	7
S4 (0.5 M)	—	86	14
S4 (2 M)	—	9	91
S4 (5 M)	—	—	25
S6 (0.5 M)	14	—	86
S6 (2 M)	—	25	75
S6 (5 M)	—	51	13

When a 0.5 M concentration of solvent **S2** was used a 75% yield β(1→4) product was obtained. However, at higher concentrations (1 M) the regioselectivity changes towards formation of the β(1→6) product (45%). The increase of the bio-solvent concentration in the reaction media (5 M) seems to favour the donor (*p*NP-β-Gal) hydrolysis giving galactose as the main product. In the case of solvent **S4**, we observed similar behaviour, but the best yield of β(1→6) product was obtained with a 2 M concentration (91%). In the case of solvent **S6**, the maximum yield in the transglycosylation reaction (β(1→6) product) is obtained with a 5 M concentration of green solvent (86%), and this yield systematically decreases with solvent concentration. For solvents, **S7**–**S9** no transglycosidation reaction was observed, only hydrolysis. These results indicate the need of

Table 1
Transglycosylation yields (%) obtained with Biolacta β-galactosidase using 50 mM citrate/phosphate buffer at pH 5.0 and 2 M of glycerol or diethylamine based solvent in the same buffer

Solvent	Solvent density (g mL^{-1})	log *P*	System composition	Galactose (%)	Gal β-(1→4)GlcNAc) (%)	Gal β-(1→6)GlcNAc (%)
Buffer	—	—	Monophasic	—	83	17
S1	1.235	−0.57	Monophasic	—	9	91
S2	1.405	−0.24	Monophasic	81	13	7
S3	1.063	0.03	Monophasic	—	29	71
S4	1.063	−0.69	Monophasic	—	9	91
S5[a]	1.147	1.41	Monophasic	—	83	12
S6	0.905	1.42	Monophasic	—	24	75
S7	0.885	2.25	Byphasic	100	—	—
S8	0.872	3.09	Byphasic	100	—	—
S9	0.892	3.92	Byphasic	100	—	—

[a] Solvent **S5** is solid under room temperature conditions (1 atm and 25 °C).

carefully choosing co-solvent concentrations in order to obtain the desired products.

The solubility in water of the co-solvents is probably a factor to be taken into account to explain these results (see Table 1). Solvents with long chains (**S7**–**S9**) tend to have low solubility in water and the presence of two liquid phases (buffer and bio-solvent) would explain the different behaviour obtained.

In fact, the change of regioselectivity cannot be easily related to solvent physical parameters (density, system composition, log *P* etc.). In principle, it could be due to the presence of impurities in the enzyme.

2.3. Enzymatic synthesis in presence of bio-solvents with semipurified Biolacta β-galactosidase

In order to explain the observed changes in regioselectivity, most of non-proteic substances present in Biolacta were removed by precipitation with $(NH_4)_2SO_4$. Powder of Biolacta contains about 11% of proteins in its composition. After the concentration process we achieved a 71% of proteins in the solid sample, which means that we considerably reduced the non-proteic components from the crude preparation. Commercial (C) and semipurified (S) enzyme showed a similar specific activity (4.0–4.2 U mg^{-1}, respectively).

The semipurified Biolacta β-galactosidase was used with the best bio-solvents (**S1** and **S4**), selected in view of previous results obtained with the commercially available enzyme. Table 3 shows the yields obtained in different media for these reactions.

Table 3
Transglycosylation yields (%) obtained with semipurified Biolacta β-galactosidase using 50 mM citrate/phosphate buffer at pH 5.0 and 2 M bio-solvent (**S1** and **S4**)

Solvent	Galactose (%)	Gal β-(1→4)GlcNAc (%)	Gal β-(1→6)GlcNAc (%)
Buffer	21	47	32
S1	9	6	85
S4	5	7	88

Using semipurified enzyme we observed that solvents **S1** and **S4** gave the best yields for Galβ-(1→6)GlcNAc product at 2 M bio-solvent concentration. Using commercial enzyme as catalyst in the same medium afforded similar yields and regioselectivity (91% of yield and β(1→6) as main product). Therefore, we concluded that the non-proteic substances present in Biolacta are not responsible for the change in regioselectivity observed.

As an added improvement to this process, the reaction time was reduced from 3 h to 30 min using this methodology. On the other hand, hydrolysis reaction is highly competitive and after 30 min the synthesized product starts to be consumed.

2.4. Enzymatic synthesis of glycoconjugate synthesis in the presence of bio-solvents

Transglycosidation was studied using different acceptors and *p*-nitrophenyl-β-D-galactopyranoside as galactosyl donor in the presence of Biolacta β-galactosidase with the aim of preparing new glycoconjugates. We carried out the synthesis of two functionalized monosaccharides using *N*-acetylglucosamine as starting material and tetraethylene glycol and 1-pentenol as linkers (Fig. 2).

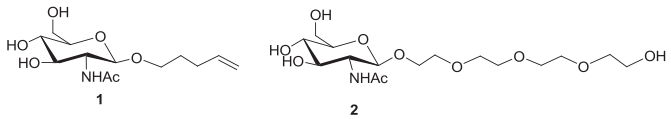


Fig. 2. Structure of alkyl-*N*-acetylglucosamine.

The preparation and characterization of these molecules has been reported by our group previously.[22]

Transglycosylation reaction catalyzed by a commercial preparation of Biolacta β-galactosidase was carried out following the general procedure described in the Experimental section and monitored by HPLC. The bio-solvent concentration was kept constant (2 M) in the mixture and a 50 mM citrate/phosphate buffer at pH 5.0 was used. The results obtained in the presence of solvents **S1** and **S4** are summarized in Table 4.

Table 4
Products obtained by transglycosylation catalyzed by β-galactosidase from *B. circulans* using **1** and **2** as acceptors using 50 mM citrate/phosphate buffer at pH 5.0 and 2 M of bio-solvent in the same buffer

Media	Acceptor	Galactose (%)	Gal β-(1→4)GlcNAc (%)	Gal β-(1→6)GlcNAc (%)
Buffer	1	100	—	—
Buffer	2	20	50	27
S1	1	100	—	—
S1	2	38	35	23
S4	1	100	—	—
S4	2	36	19	34
S6	1	100	—	—
S6	2	72	17	11

In none of these cases, compound **1** was recognized as acceptor by this enzyme. However, with compound **2** a mixture of two glycoconjugates were synthesized (β-(1→4) and β-(1→6)) in similar percentage.

3. Conclusions

A change in classical regioselectivity of Biolacta β-D-galactosidase when reaction is performed in the presence of bio-solvents is reported here. These solvents promote the β(1→6) linkages between the donor and acceptor (glycosides or alkyl-glycosides). This reactivity change is maintained even after removal of Biolacta non-proteic components. Yields of up to 90% of Gal-β-(1→6)-GlcNac were obtained and in the case of the semipurified enzyme the reaction times were considerably reduced from 3 h to 30 min. These reactions proceed with excellent regioselectivity, adding a considerable improvement over the use of aqueous buffer or conventional organic solvents. These results reinforce the concept of driving enzymatic syntheses to the desired product simply by adjusting the reaction medium with small amounts of bio-solvents.

4. Experimental section

4.1. General

Commercially available β-galactosidase from *Bacillus circulans* (Biolacta N5) was a gift from Daiwa Kasei. UV–vis spectra were recorded on a UV-2401 PC Shimadzu. HPLC Agilent 1100 with UV–vis detector using Mediterraneasea18 15 cm×0.46 5 mm column (Teknokroma) with water/acetonitrile (75:25) as a mobile phase at a flow of 0.7 mL/min. HPLC Jasco with light scattering detector using Lichrosorb NH2 5 mm 25×0.47 column (Teknokroma) with acetonitrile/water (80:20) as a mobile phase at a flow of 1.0 mL/min. NMR spectra were performed using Bruker AV. 250 MHz and AV III. 700 MHz. All solvents used in this work were a gift from COGNIS IP Management GmbH.

4.2. Enzyme activity assay

Protein concentration was determined by the Bradford method[23] using bovine serum albumin (BSA) as standard. The

activity of the β-galactosidases was assayed spectrophotometrically adding a sample of enzyme solution (20 μL) with 80 μL of sodium citrate/phosphate buffer 50 mM, pH 5.0 containing *p*-nitrophenyl-β-D-galactopyranoside (*p*NP-β-Gal) in order to obtain a final 5 mM concentration (substrate). The reaction mixture was incubated for 3 min at 37 °C. Absorbance was measured at 410 nm and the *p*-nitrophenol (*p*NP) released was quantified against a standard curve performed under the same experimental conditions. One unit of enzyme activity was defined as the quantity of enzyme hydrolyzing 1 mmol of *p*NP-β-Gal per minute, under the conditions stated above.

4.3. Semi-purification of enzymes

All operations were carried out at 4 °C. A 5% m/v solution of commercial β-galactosidase from *B. circulans* in sodium citrate/phosphate buffer 50 mM, pH 5.0 was precipitated by addition of ammonium sulfate, (55% saturation). Then the enzyme was centrifuged at 10,000 rpm, 20 min, 4 °C with the aim to separate the solid. The pellet was re-suspended in the same buffer and dialyzed during 36 h (three changes of buffers) with 10 mM buffer solution. Enzyme was lyophilized for 24 h. Proteic composition of final sample was observed by sodium docedyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli's method,[24] non denaturing PAGE was performed under the same method without SDS, β-mercaptoethanol and pre-heating the sample, in both cases, gels were running using 7.5% of acrylamide.

4.4. Synthesis of the functionalized monosaccharides

*4.4.1. Synthesis of 1-pentenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (**1**).* This compound was synthesized as previously described by de Paz et al.[25] To a solution of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucopyranoside (1 g, 2.5 mmol) in dry CH_2Cl_2 (15 mL), TMSOTf (3.63 mmol) was added and the mixture stirred at 30 °C for 72 h. Then, 4-pentenol (7.5 mmol) was added and the reaction stirred at that temperature for an additional 3 h. The suspension was neutralized with Et_3N, filtered and the solvent was evaporated to dryness. The residue was purified by flash chromatography (CH_2Cl_2/acetone 9:3) to yield 60%. TLC 0.75 (CH_2Cl_2/acetone 3:1). ^{1}H NMR:[25] (250 MHz, $CDCl_3$) 5.75 (m, 1H, −CH=CH_2); 5.61 (d, 1H, *J*=8.6 Hz, −NH−); 5.27 (t, 1H, *J*=9.9 Hz, H-3); 5.03 (t, 1H, *J*=9.6 Hz, H-4); 4.98−4.92 (m, 2H, −CH=CH_2); 4.64 (d, 1H, *J*=8.3 Hz, H-1); 4.22 (dd, 1H, *J*=4.8, 12.2 Hz, H-6a); 4.09 (dd, 1H, *J*=2.3, 12.2 Hz, H-6b); 3.86−3.77 (m, 2H, H-2, −CH_2−O−); 3.67 (m, 1H, −CH_2−O−); 2.17 (m, 4H, *J*=7.5 Hz, −$(CH_2)_2$−); 2.04, 1.99, 1.98, 1.91 (4s, 12H, −$COCH_3$); 2.05−1.59 (m, 4H, −$(CH_2)_2$). ^{13}C NMR (63 MHz, $CDCl_3$): 171.3, 171.2, 170.6, 169.9 (−CO−); 138.7 (−CH−); 115.4 (−CH_2−); 101.1 (H-1); 72.7, 72.1 (−CH−); 69.5, 69.0, 62.6 (−CH_2−); 55.3 (−CH−); 30.3, 28.9 (−CH_2−); 23.8, 21.2, 21.1, 21.0 (−CH_3−).

This compound (2 g) was dissolved in dry MeOH (2 mL) and NaOMe (2 g) was added. After stirring over the night at room temperature the reaction was neutralized with Amberlite IR-120H$^+$. The mixture was filtered and the solvent was evaporated in vacuo to obtain compound **5**. TLC 0.62 (isopropanol/nitromethane/water 10:9:2) ^{1}H NMR:[25] (250 MHz, D_2O) 84−5.68 (m, 1H, −CH=CH_2); 5.00−4.80 (m, 2H, −CH=CH_2); 4.43 (d, 1H, *J*=8.4 Hz, H-1); 3.67−3.32 (m, 2H, H6a, −CH_2−O−); 3.63−3.25 (m, 6H, H-4, −CH_2−O−, H-3, H-5, H6b); 2.01−1.95 (m, 4H, −$(CH_2)_2$−); 1.58−1.50 (m, 3H, −$COCH_3$−). ^{13}C NMR (63 MHz, D_2O): 174.8 (−CO−); 131.9 (−CH_2−); 115.2 (−CH=); 101.4 (H-1); 76.1, 74.1, 70.2 (−CH_2−); 69.9, 61.0, 55.9, 29.5 (−CH−); 28.7 (−CH_3−); 22.5 (−CH_2−).

*4.4.2. Synthesis of 1-tetraethylenglycol-2-acetamido-2-deoxy-β-D-glucopyranoside (**2**).* This compound was synthesized as previously described by Pérez et al.[22] Starting with *N*-acetyl-tri-*O*-acetyl-2-amino-2-deoxy-galactopyranosyl chloride (8.2 mmol) was dissolved in a 1:1 mixture of toluene and nitromethane (12 mL) and mercury cyanide (8.2 mmol), $MgSO_4$ and tetraethylene glycol (321 mmol) were added. The mixture was stirred at room temperature overnight. After addition of 20 mL CH_2Cl_2 the reaction mixture was filtered over Celite and the solvent was evaporated under reduced pressure. To the resulting syrup, 8.2 mmol NaOMe in 30 mL MeOH was added and the mixture was allowed to react for 3 h at room temperature. The reaction was neutralized with Amberlite, the mixture was filtered and the solvent evaporated in vacuo. The final product **6** (29%) was purified by flash chromatography (CH_2Cl_2/MeOH 3:1). TLC 0.32 (CH_2Cl_2/MeOH 3:1). ^{1}H NMR (250 MHz, D_2O) 5.32 (d, 1H, *J*=5.3 Hz, H-4); 5.15 (dd, J_1=10.5 Hz, J_2=7.8 Hz, H-2); 4.95 (dd, 1H, J_1=10.3 Hz, J_2=3.3 Hz, H-3); 4.52 (d, 1H, *J*=7.8 Hz, H-1); 4.14 (m, 2H, H-6a, H-6b); 3.86 (m, 2H, H-5, −$(CH_2)_2$−), 3.50−3.80 (m, 15H, −$(CH_2)_2$−); 2.17, 2.08, 2.07, 2.00 (s, 12H, CH_3). ^{13}C NMR (63 MHz, D_2O): 174.3 (−CO−); 101.3 (H-1); 76.2, 74.2 (−CH−); 71.9, 70.1, 70.1, 69.9, 69.8, 68.7, 69.7 (−CH_2−), 69.6 (−CH−), 60.9 (−CH_2−), 60.6 (−CH−), 55.7 (−CH_2−); 22.4 (−CH_3).

4.5. General procedure for transglycosylation reactions using glycoconjugates

A solution of 51.2 mg (0.17 M) *p*-nitrophenyl-β-D-galactopyranoside (donor) and 188 mg (0.85 M) of *N*-acetylglucosamine (acceptor) in 1 mL of green solvent (2 M)-buffer mixture was pre-equilibrated to 30 °C. Afterwards, 155 μmol/min (U) of β-galactosidase were added to the reaction mixture. Reaction was monitored by HPLC UV−vis and final products analysed by HPLC with an evaporative light scattering detector (ELSD). The reaction was stopped by heating the sample at 100 °C for 5 min.

Isolation of disaccharides was done by carbon/Celite (50% m/m), chromatography, the column was eluted with milliQ water and ethanol gradient (from 5% to 15% v/v).[21,26] The structures of the disaccharides (Gal-β(1→4)GlcNAc and Gal-β(1→6)GlcNAc) were assigned by ^{1}H and ^{13}C NMR (D_2O, 700 MHz), spectra were identical to previous references.[21,26,27]

In order to determine the effect of non-proteic substances present in Biolacta preparation in the reaction yield, transglycosylation reactions were carried out using semipurified (lyophilized) enzyme. The best performing solvents were chosen in view of the previous results, using crude extracts and setting the reactions with semipurified enzymes. The transglycosylation conditions were the same mentioned above and the reaction was monitored using HPLC-ELSD.

For functionalized disaccharides the same protocol previously described was used, and different glycoconjugates as acceptors were added. A solution of 51.2 mg (0.17 M) *p*NP-β-Gal (donor) and the different monosaccharides functionalized **1** and **2** (0.51 M).

Acknowledgements

This work has been supported by the MICINN ('Ministerio de Ciencia e Innovación'), CTQ2009-11801. M.S. is grateful for a Doctoral fellowship granted by Universidad Nacional de Costa Rica. We also thank the kind donation of Bio-solvents from Cognis IP Management GmbH, now part of BASF.

References and notes

1. Caines, M. E. C.; Zhu, H.; Vuckovic, M.; Willis, L. M.; Withers, S. G.; Wakarchuk, W. W.; Strynadka, N. C. J. *J. Biol. Chem.* **2008**, *283*, 31279−31283.
2. Shirato, H.; Ogawa, S.; Ito, H.; Sato, T.; Kameyama, A.; Narimatsu, H.; Xiaofan, Z.; Miyamura, T.; Wakita, T.; Ishii, K.; Takeda, N. *J. Virol.* **2008**, *82*, 10756−10767.
3. Springer, G. F. *Science* **1984**, *224*, 1198−1206.
4. Springer, G. F.; Desai, P. R.; Wise, W.; Carlstedt, S. C.; Tegtmeyer, H.; Stein, R.; Scanlon, E. F. *Immunol. Ser.* **1990**, *53*, 587−612.

5. Schmidt, R. R.; Rücker, E. *Tetrahedron Lett.* **1980**, *21*, 1421–1424.
6. Schmidt, R. R. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 212–235.
7. Paulsen, H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 155–173.
8. Usui, T.; Kubota, S.; Ohi, H. *Carbohydr. Res.* **1983**, *244*, 315–323.
9. Jongejan, J. A. In *Organic Synthesis with Enzymes in Non-Aqueous Media*; Wiley-VCH GmbH & KGaA: Weinheim, Germany, 2008; pp 25–46.
10. Bridiau, N.; Issaoui, N.; Maugard, T. *Biotechnol. Prog.* **2010**, *26*, 1278–1289.
11. Mantarosie, L.; Coman, S.; Parvulescu, V. I. *J. Mol. Catal. A: Chem.* **2008**, *279*, 223–229.
12. Yoon, J. H.; McKenzie, D. *Enzyme Microb. Technol.* **2005**, *36*, 439–446.
13. Gupta, M. N.; Roy, I. *Eur. J. Biochem.* **2004**, *271*, 2575–2583.
14. Sheldon, R. A. *Green Chem.* **2005**, *7*, 267–278.
15. Hernaiz, M. J.; Alcántara, A. R.; García, J. I.; Sinisterra, J. V. *Chem.—Eur. J.* **2010**, *16*, 9422–9437.
16. Giacomini, C.; Irazoqui, G.; Gonzalez, P.; Batista-Viera, F.; Brena, B. M. *J. Mol. Catal. B: Enzym.* **2002**, *19*, 159–165.
17. Finch, P.; Yoon, J. H. *Carbohydr. Res.* **1997**, *303*, 339–345.
18. Priya, K.; Loganathan, D. *Tetrahedron* **1999**, *55*, 1119–1128.
19. Pérez, M. S.; Sinisterra, J. V.; Hernáiz, M. J. *Curr. Org. Chem.* **2010**, *14*, 2366–2383.
20. Pérez-Sánchez, M.; Cortés-Cabrera, Á; García-Martín, H.; Sinisterra, J. V.; García, J. I.; Hernaíz, M. J. *Tetrahedron* **2011**, *67*, 7708–7712.
21. Pérez-Sánchez, M.; Sandoval, M.; Cortés-Cabrera, A.; García-Marín, H.; Sinisterra, J. V.; García, J. I.; Hernaiz, M. J. *Green Chem.* **2011**, *13*, 2810–2817.
22. Perez, M.; Munoz, F. J.; Munoz, E.; Fernandez, M.; Sinisterra, J. V.; Hernaiz, M. J. *J. Mol. Catal. B: Enzym.* **2008**, *52-3*, 153–157.
23. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
24. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
25. de Paz, J. L.; Ojeda, R.; Barrientos, A. G.; Penades, S.; Martin-Lomas, M. *Tetrahedron: Asymmetry* **2005**, *16*, 149–158.
26. Sandoval, M.; Ferreras, E.; Pérez-Sánchez, M.; Berenguer, J.; Sinisterra, J. V.; Hernaiz, M. J. *J. Mol. Catal. B: Enzym.* **2012**, *74*, 162–169.
27. Bridiau, N.; Maugard, T. *Biotechnol. Prog.* **2011**, *27*, 386–394.