Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Molecular Catalysis B: Enzymatic 39 (2006) 128–134
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Unique transglycosylation potential of extracellular α-D-galactosidase from *Talaromyces flavus*

Pavla Simerská [a], Marek Kuzma [a], Daniela Monti [b], Sergio Riva [b], Martina Macková [c], Vladimír Křen [a,*]

[a] *Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ 142 20 Prague 4, Czech Republic*
[b] *Istituto di Chimica del Riconoscimento Molecolare, CNR, Via Mario Bianco 9, I 201 31 Milan, Italy*
[c] *Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, CZ 168 20 Prague 6, Czech Republic*

Available online 28 February 2006

Abstract

The transglycosylation potential of the extracellular α-D-galactosidase from the filamentous fungus *Talaromyces flavus* CCF 2686, chosen as the best enzyme from the screening, was investigated using a series of sterically hindered alcohols (primary, secondary and tertiary) as galactosyl acceptors. Nine alkyl α-D-galactopyranosides derived from the following alcohols – *tert*-butyl alcohol, 2-methyl-2-butyl alcohol, 2-methyl-1-propyl alcohol, 2,2,2-trifluoroethyl alcohol, 2-propyn-1-ol, *n*-pentyl alcohol, 3,5-dihydroxybenzyl alcohol, 1-phenylethyl alcohol and 1,4-dithio-DL-threitol – were prepared on a semi-preparative scale. This demonstrates a broad synthetic potential of the *T. flavus* α-D-galactosidase that has not been observed with another enzyme tested. Moreover, this enzyme exhibits good transglycosylation yields (6–34%). The enzymatic synthesis of *tert*-butyl α-D-galactopyranoside by transglycosylation was studied in detail.

Keywords: α-D-Galactosidase; *Talaromyces flavus*; Transglycosylation; *tert*-Butyl alcohol; 1,4-Dithio-DL-threitol

1. Introduction

α-D-Galactosidases (α-D-galactopyranoside galactohydrolase, E.C. 3.2.1.22) catalyse the cleavage of terminal galactosyl residues from a wide range of substrates, including linear and branched oligosaccharides, polysaccharides and synthetic substrates such as *p*-nitrophenyl α-D-galactopyranoside (*p*NPαGal). Many microorganisms, plants and animals produce α-D-galactosidases, often in multiple isoenzyme forms [1]. In the food industry they are used for different technological applications, e.g., for the hydrolysis of raffinose in beet sugar syrups [2] or galacto-oligosaccharides in soybean milk [3,4] and for the improving of the gelling properties of galactomannans used as food thickeners [5]. Moreover, since galactomannans and galactoglucomannans occur in softwoods [6], α-D-galactosidases may also be employed for the modification of wood-derived materials. Finally, α-D-galactosidases are of interest in medicine, e.g., for the treatment of Fabry's disease by enzyme replacement therapy [7] or for the blood type conversion (B type cell to O type cell) [8].

The enzymatic synthesis of glycosides is a complementary alternative to chemical synthesis, which is rather time-consuming due to the need of several protection/deprotection steps. Major advantages of the glycosidase-catalysed synthesis are an absolute anomeric stereoselectivity and, in some cases, regioselectivity [9].

The transglycosylation activity of α-D-galactosidases from different sources, e.g., *Coffea arabica*, *Thermus brockianus* and *Candida guilliermondii* [10–12], has already been described, also in the presence of various alcohols as acceptors [13].

We previously reported that a crude preparation of *T. flavus* α-D-galactosidase shows an unusual regioselectivity in transglycosylation of *p*NPαGal, with a selective preference for the formation of α(1→3) bonds [14], and tolerates *p*-nitrophenyl 6-*O*-acetyl-α-D-galactopyranoside as an acceptor yielding *p*-nitrophenyl α-D-galactopyranosyl-(1→3)-6-*O*-acetyl-α-D-galactopyranoside [15].

Besides its peculiar selectivity, this inducible enzyme was unexpectedly found to be active in the transfer of α-galactosyl residue onto sterically hindered acceptors, e.g., *tert*-butyl alcohol [15], which is generally quite inert to hydrolases and, consequently, used as a co-solvent in order to increase substrate solubility [16].

* Corresponding author. Tel.: +420 2 9644 2510; fax: +420 2 9644 2509.
E-mail address: kren@biomed.cas.cz (V. Křen).

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.01.006

Here we investigated the acceptor specificity of the *T. flavus* α-D-galactosidase and its transglycosylation activity using various alcohols (primary, secondary and tertiary) as acceptors. Moreover, examples of enzymatic glycosylation of thioalcohols catalysed by this biocatalyst are presented.

2. Experimental

2.1. Materials and methods

2-Methyl-2-butyl alcohol (**2a**), 2-methyl-1-propyl alcohol (**3a**), 2,2,2-trifluoroethyl alcohol (**4a**), 2-propyn-1-ol (**5a**), *n*-pentyl alcohol (**6a**), 3,5-dihydroxybenzyl alcohol (**7a**), 1-phenylethyl alcohol (**8a**) and 1,4-dithio-DL-threitol (DL-*threo*-1,4-dimercapto-2,3-butanediol) (**9a**), *p*-nitrophenol and *p*NPαGal were purchased from Sigma–Aldrich, D-galactose and *tert*-butyl alcohol (**1a**) were from Lachema Brno. All chemicals were of analytical grade.

The α-D-galactosidases from different strains were obtained from the Culture Collection of Fungi (CCF), Department of Botany, Charles University, Prague, Czech Republic. Flasks (500 ml) with 100 ml medium were inoculated with the suspension of spores in 0.1% Tween 80. The flasks were cultivated on a rotary shaker at 28 °C. Medium used (g/l): yeast extract 0.5, mycological peptone 5, KH_2PO_4 3, $NH_4H_2PO_4$ 5, pH 6.0. As the enzyme inducers the following compounds were used: raffinose (2 g/l) for *Aspergillus parasiticus* CCF 3058, *A. sojae* CCF 3060, *A. tamarii* CCF 3085, *Circinella muscae* CCF 1568, *Penicillium daleae* CCF 2365, *P. chrysogenum* CCF 1269, *P. melinii* CCF 2440 and *P. multicolor* CCF 2244; 2-deoxyglucose (1 g/l) for *Aspergillus flavipes* CCF 2026 and 6-deoxyglucose (1 g/l) for *Talaromyces flavus* CCF 2324 and *T. flavus* CCF 2686. After the sterilization each flask was supplemented with sterile $MgSO_4 \cdot 7H_2O$ (0.5 g/l). The α-D-galactosidase from *C. arabica* (green coffee beans) was purchased from Sigma. Crude enzymes were obtained by ammonium sulfate precipitation (80% saturation) of respective culture filtrates (4-day-old culture) and centrifugation (20 min at 13000 rpm and 4 °C).

α-D-Galactosidase activity was assayed using *p*NPαGal as a substrate. One unit of α-D-galactosidase activity is defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute at pH 5.0 and 35 °C with *p*NPαGal as a substrate. All incubations were performed in Thermomixer comfort at 850 rpm (Eppendorf, D). Products yields were calculated relative to *p*NPαGal.

Thin-layer chromatography (TLC) was carried out on precoated silica gel DC-Alufolien Kieselgel 60 F_{254} plates, Merck (SRN), detection was performed with 5% H_2SO_4 in ethanol. Flash column chromatography was performed on silica gel 60 (40–63 μm), Merck (SRN). ^1H and ^{13}C NMR spectra were measured on a Varian^{UNITY} *INOVA*-400 spectrometer (399.89 and 100.55 MHz, respectively) in D_2O, CD_3OD and $CDCl_3$ at 30 °C. Residual signal of solvents was used as an internal standard (D_2O: δ_H 4.508 ppm, CD_3OD: δ_H 3.33 ppm, δ_C 49.30 ppm, $CDCl_3$: δ_H 7.265, δ_C 77.00). ^{13}C NMR spectra in D_2O were referenced to acetone (δ_C 30.5 ppm).

2.2. Influence of tert-butyl alcohol on the activity of the T. flavus α-D-galactosidase

Activity of three selected α-D-galactosidases from *T. flavus* CCF 2686, *Penicillium multicolor* CCF 2244 and *P. chrysogenum* CCF 1269 was tested in the presence of high concentrations of *tert*-butyl alcohol. Each reaction mixture contained *p*NPαGal (2 mM), the respective α-D-galactosidase (0.015 U/ml), 0–60% (v/v) of *tert*-butyl alcohol and 50 mM citrate-phosphate buffer (pH 3.5 for *T. flavus* α-D-galactosidase and pH 5.0 for both *P. multicolor* and *P. chrysogenum* α-D-galactosidases). The reactions were incubated for 10 min at 37 °C and samples (50 μl) were stopped by adding to 0.1 M Na_2CO_3 (1 ml). The absorbance was measured at 420 nm against the corresponding blank. As a reference, a reaction without *tert*-butyl alcohol was performed.

The influence of different concentrations of *tert*-butyl alcohol on the transglycosylation yields was evaluated by adding the *T. flavus* α-D-galactosidase (2 U/ml) to reaction mixtures containing *p*NPαGal (0.03 M) and different amounts of *tert*-butyl alcohol (25, 50 and 75%, v/v, respectively), in 50 mM citrate-phosphate buffer, pH 3.5. The reaction mixtures were shaken at 37 °C and monitored by TLC (AcOEt/MeOH/H_2O = 8/1.5/0.3) after 3.5, 6.5 and 48 h.

2.3. Influence of alcohol concentration on the stability of the T. flavus α-D-galactosidase

The *T. flavus* α-D-galactosidase (0.12 U/ml) was incubated at 37 °C in 50 mM citrate-phosphate buffer, pH 3.5 and *tert*-butyl alcohol (25%, v/v). At scheduled times (1, 2, 4, 6, 23, 25 and 27 h), *p*NPαGal (2 mM) was added and the reactions were carried out for 10 min. A sample (50 μl) of each reaction mixture was added to 1 ml 0.1 M Na_2CO_3 to stop the reaction and the absorbances were measured as previously described.

2.4. Screening of α-D-galactosidases for galactosylation of tert-butyl alcohol and 2-propyn-1-ol

*p*NPαGal (0.03 M), *tert*-butyl alcohol (2.6 M, 25%, v/v) or 2-propyn-1-ol (0.02 M, 1%, v/v), 50 mM citrate-phosphate buffer, pH 5.0 and the α-D-galactosidase (2 U/ml) from *Aspergillus flavipes* CCF 2026, *A. parasiticus* CCF 3058, *A. sojae* CCF 3060, *A. tamarii* CCF 3085, *Circinella muscae* CCF 1568, *C. arabica* (Sigma), *Penicillium daleae* CCF 2365, *P. chrysogenum* CCF 1269, *P. melinii* CCF 2440, *P. multicolor* CCF 2244, *T. flavus* CCF 2324 and *T. flavus* CCF 2686, respectively, were shaken at 37 °C for 4 h. Production of *tert*-butyl and propyn-1-yl α-D-galactopyranosides was monitored by TLC (AcOEt/MeOH/H_2O = 8/1.5/0.3, v/v/v).

2.5. Galactosylation of tertiary alcohols

2.5.1. tert-Butyl α-D-galactopyranoside (*1*)

tert-Butyl α-D-galactopyranoside (**1**) was prepared by reaction of *p*NPαGal (100 mg, 0.3 mmol) and *tert*-butyl alcohol (5.2 ml, 0.05 mol) in 50 mM citrate-phosphate buffer,

pH 3.5 (5.6 ml). Crude *T. flavus* enzyme (16.4 U) was added and the reaction mixture was shaken at 37 °C for 25 h. The course of the reaction was monitored by TLC (AcOEt/MeOH/H_2O = 8/1.5/0.3). Crude product **1** (10.6 mg, 13.5% yield) was purified by flash chromatography (the same eluent as for TLC) and characterised by NMR spectroscopy. **1**. ^{1}H NMR (D_2O): 1.055 (9H, s, $(CH_3)_3C$), 3.472 (1H, dd, J = 7.2, 11.7 Hz, H-6a), 3.508 (1H, dd, J = 5.4, 11.7 Hz, H-6b), 3.523 (1H, dd, J = 4.1, 10.4 Hz, H-2), 3.631 (1H, dd, J = 3.4, 10.4 Hz, H-3), 3.743 (1H, dd, J = 1.3, 3.4 Hz, H-4), 3.890 (1H, ddd, J = 1.3, 5.4, 7.2 Hz, H-5), 5.001 (1H, d, J = 4.1 Hz, H-1). ^{13}C NMR (D_2O): 28.09 (q, $(CH_3)_3CO$), 61.52 (t, C-6), 68.81 (d, C-2), 69.75 (d, C-4), 69.95 (d, C-3), 70.61 (d, C-5), 76.29 (s, $(CH_3)_3CO$), 93.39 (d, C-1).

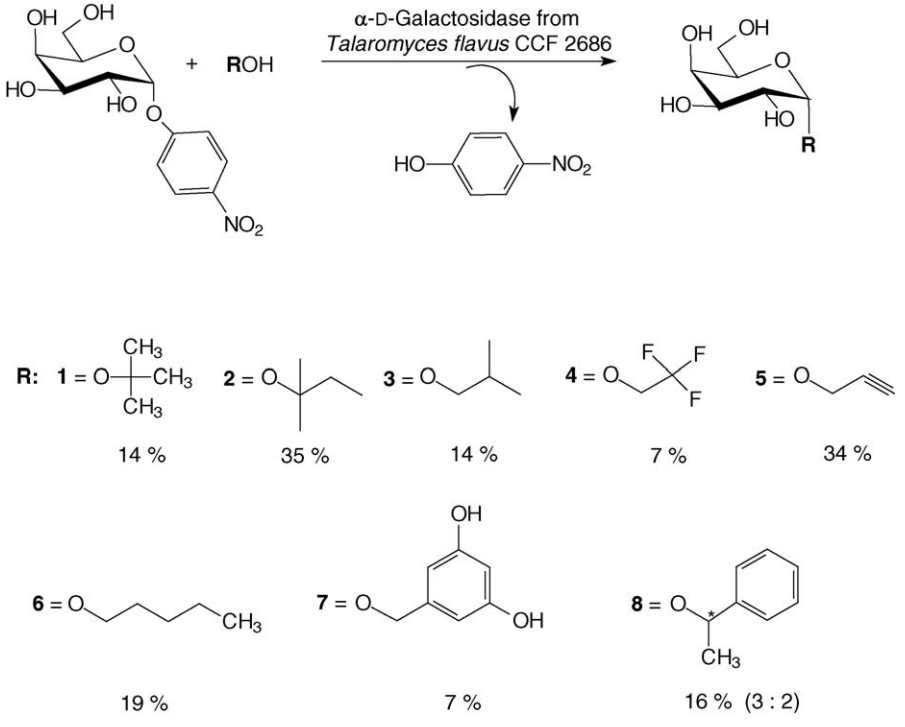
2.5.2. 2-Methyl-2-butyl α-D-galactopyranoside (2)

2-Methyl-2-butyl α-D-galactopyranoside (**2**) was prepared by reaction of *p*NPαGal (50 mg, 0.16 mmol) and 2-methyl-2-butyl alcohol (1.8 ml, 16 mmol) in 50 mM citrate-phosphate buffer, pH 3.5 (5.6 ml), catalysed by the *T. flavus* α-D-galactosidase (10 U). The reaction was shaken at 37 °C for 4 h. The crude residue was purified by flash chromatography (AcOEt/MeOH/H_2O = 8/1.5/0.3) to give **2** (14.5 mg, 35% yield) and characterised by NMR spectroscopy. **2**. ^{1}H NMR (D_2O) 0.672 (3 H, dd, J = 7.5, 7.5 Hz, H-3′), 0.996 (3 H, s, 1′-Me_b), 1.007 (3 H, s, 1′-Me_a), 1.328 (1 H, dq, J = 13.9, 7.5 Hz, H-2′b), 1.394 (1 H, dq, J = 13.9, 7.5 Hz, H-2′a), 3.462 (1 H, dd, J = 7.2, 11.6 Hz, H-6b), 3.501 (1 H, dd, J = 5.3, 11.6 Hz, H-6a), 3.519 (1 H, dd, J = 4.1, 10.4 Hz, H-2), 3.642 (1 H, dd, J = 10.4, 3.5 Hz, H-3), 3.747 (1 H, dd, J = 3.5, 1.3 Hz, H-4), 3.892 (1 H, ddd, J = 1.3, 7.2 Hz, 5.3 Hz, H-5), 4.996 (1 H, d, J = 4.1 Hz, H-1). ^{13}C NMR (D_2O, HMQC readouts) 8.2 (C-3′), 24.9 (1′-Me_a), 25.1 (1′-Me_b), 33.9 (C-2′), 61.3 (C-6), 68.8 (C-2), 69.5 (C-4), 69.7 (C-3), 70.5 (C-5), 93.0 (C-1). a, downfield; b, upfield.

2.6. Synthesis of alkyl α-D-galactopyranosides

Analytical reactions were performed by mixing *p*NPαGal (5 mg, 0.02 mmol), 50 mM citrate-phosphate buffer (pH 3.5, 1 ml), the *T. flavus* α-D-galactosidase (1 U) and the respective alcohol (0.2 mmol): 2-methyl-1-propyl alcohol (15.3 μl), 2,2,2-trifluoroethyl alcohol (11.9 μl), propyn-1-ol (9.7 μl), *n*-pentyl alcohol (18.3 μl), 3,5-dihydroxybenzyl alcohol (23.3 mg), 1-phenylethyl alcohol (20.1 μl), 1,4-dithio-DL-threitol (25.6 mg). Semipreparative procedures were accomplished using 10 times higher amount of the components of analytical reactions. Reaction mixtures were shaken at 37 °C for 4 h and monitored by TLC (propane-2-ol/H_2O/NH_3 = 7/2/1). Crude products were purified by flash chromatography (AcOEt/MeOH/H_2O = 8/1.5/0.3, or PE/AcOEt = 2.5/1 in the case of DL-*threo*-1,4-dimercapto-3-hydroxy-2-butyl α-D-galactopyranoside (**9**), which was peracetylated to yield compound **10**). The following products (Scheme 1) were isolated: 2-methyl-1-propyl (**3**, 5.6 mg, 14% yield), 2,2,2-trifluoroethyl (**4**, 2.9 mg, 7% yield), 2-propyn-1-yl (**5**, 12.3 mg, 34% yield), *n*-pentyl (**6**, 7.8 mg, 19% yield), 3,5-dihydroxybenzyl (**7**, 3.7 mg, 7% yield), 1-phenylethyl (**8**, 7.7 mg, 16% yield) and 2,3,3′,4,6-penta-*O*-acetyl-1′,4′-di-*S*-acetyl-DL-threo-1,4-dimercapto-3-hydroxy-2-butyl (**10**, 5.4 mg, 6% yield) α-D-galactopyranosides.

2-Methyl-1-propyl α-D-*galactopyranoside* (**3**). ^{1}H NMR (D_2O): 0.685 (3 H, d, J = 6.7 Hz, H-3′a), 0.697 (3 H, d, J = 6.7 Hz, H-3′a), 1.691 (1 H, m, H-2′), 3.096 (1 H, dd, J = 9.5, 6.2 Hz, H-1′b), 3.229 (1 H, dd, J = 9.5, 7.5 Hz, H-1′a), 3.509 (2 H, d, J = 6.1 Hz, H-6), 3.586 (1 H, dd, J = 10.3, 3.8 Hz, H-2), 3.645 (1



Scheme 1.

H, dd, J = 10.3, 3.3 Hz, H-3), 3.720 (1 H, dt, J = 1.2, 6.1 Hz, H-5), 3.756 (1 H, dd, J = 3.3, 1.2 Hz, H-4), 4.705 (1 H, d, J = 3.8 Hz, H-1). ^{13}C NMR (D_2O, HMQC readouts): 18.7 (C-3′b), 18.8 (C-3′a), 27.7 (C-2′), 61.4 (C-6), 68.7 (C-2), 69.5 (C-4), 69.8 (C-3), 71.0 (C-5), 75.1 (C-1′), 98.5 (C-1). a, downfield; b, upfield.

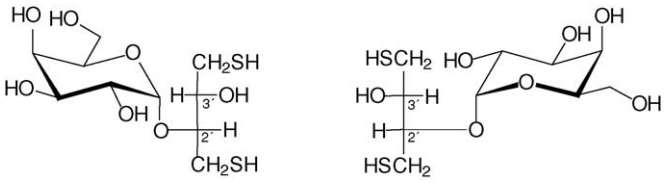
2,2,2-Trifluoroethyl α-D-galactopyranoside (**4**). ^{1}H NMR (CD_3OD) 3.708 (1 H, dd, J = 5.3, 11.3 Hz, H-6b), 3.758 (1 H, dd, J = 6.7, 11.3 Hz, H-6a), 3.772 (1 H, dd, J = 10.1, 3.3 Hz, H-3), 3.830 (1 H, ddd, J = 1.3 Hz, 5.3 Hz, 6.7 Hz, H-5), 3.847 (1 H, dd, J = 3.7, 10.1 Hz, H-2), 3.931 (1 H, dd, J = 3.3, 1.3 Hz, H-4), 4.043 (1 H, dq, J = 12.4, 9.0 Hz, H-1′b), 4.138 (1 H, dq, J = 12.4, 9.1 Hz, H-1′a), 4.952 (1 H, d, J = 3.7 Hz). ^{13}C NMR (CD_3OD, HMQC and HMBC readouts), 62.9 (C-6), 65.7 (C-1′), 70.0 (C-2), 71.1 (C-4), 71.3 (C-3), 73.3 (C-5), 101.3 (C-1), 125.9 (C-2′). a, downfield; b, upfield.

Propyn-1-yl α-D-galactopyranoside (**5**). ^{1}H NMR (D_2O): 2.671 (1H, t, J = 2.4 Hz, H-3′), 3.511 (2H, d, J = 6.3 Hz, H-6), 3.580–3.647 (2H, m, H-2, H-3), 3.726 (1H, td, J = 1.1, 6.3 Hz, H-5), 3.764 (1H, dd, J = 1.1, 2.4 Hz, H-4), 4.077 (1H, dd, J = 2.4, 16.0 Hz, H-1′b), 4.131 (1H, dd, J = 2.4, 16.0 Hz, H-1′a), 4.891 (1H, d, J = 3.0 Hz, H-1). ^{13}C NMR (D_2O, HMQC and HMBC readouts): 55.1 (t, C-1′), 61.2 (t, C-6), 68.2 (d, C-2 or C-3), 69.3 (d, C-4), 69.6 (d, C-2 or C-3), 71.5 (d, C-5), 76.1 (d, C-3′), 79.3 (s, C-2′), 97.7 (d, C-1). a, downfield; b, upfield.

n-Pentyl α-D-galactopyranoside (**6**). ^{1}H NMR (CD_3OD) 0.945 (3 H, m, H-5′), *1.39 (2 H, m, H-4′), *1.40 (2 H, m, H-3′), 1.663 (2 H, m, H-2′), 3.461 (1 H, dt, J = 9.7, 6.5 Hz, H-1′b), *3.72 (2 H, m, H-6), 3.742 (1 H, dd, J = 2.8, 9.7 Hz, H-1′a), 3.745 (1 H, dd, J = 3.1, 10.0 Hz, H-3), 3.782 (1 H, dd, J = 3.3, 10.0 Hz, H-2), 3.825 (1 H, dt, J = 1.3, 6.1 Hz, H-5), 3.908 (1 H, dd, J = 3.1, 1.3 Hz, H-4), 4.819 (1 H, d, J = 3.3 Hz, H-1). ^{13}C NMR (CD_3OD, HSQC readouts) 14.6 (C-5′), 24.0 (C-4′), 29.9 (C-3′), 30.6 (C-2′), 63.1 (C-6), 69.5 (C-1′), 70.5 (C-2), 71.3 (C-4), 71.8 (C-3), 72.6 (C-5), 100.6 (C-1). a, downfield; b, upfield, *HSQC readouts.

3,5-Dihydroxybenzyl α-D-galactopyranoside (**7**). ^{1}H NMR (D_2O) 3.434 (1 H, dd, J = 7.6, 11.6 Hz, H-6b), 3.528 (1 H, dd, J = 4.9 Hz, 11.6 Hz, H-6a), 3.604 (1 H, dd, J = 3.7, 10.3 Hz, H-2), 3.653 (1 H, dd, J = 10.3, 3.2 Hz, H-3), 3.715 (1 H, ddd, J = 1.2, 7.6 Hz, 4.9 Hz, H-5), 3.766 (1 H, J = 3.2, 1.2 Hz, H-4), 4.283 (1 H, d, J = 12.0 Hz, H-1′b), 4.403 (1 H, d, J = 12.0 Hz, H-1′a), 4.800 (1 H, d, J = 3.7 Hz, H-1), 6.139 (1 H, t, J = 2.3 Hz, H-5′), 6.285 (2 H, d, J = 2.3 Hz, H-3′, H-7′). ^{13}C NMR (D_2O, HMQC and HMBC readouts) 61.2 (C-6), 68.5 (C-2), 69.4 (C-1′, C-4), 69.6 (C-3), 71.2 (C-5), 98.0 (C-1), 102.6 (C-5′), 107.5 (C-3′, C-7′), 140.4 (C-2′). a, downfield; b, upfield.

1-Phenylethyl α-D-galactopyranoside (**8**). The sample contains two diastereomers in 3:2 ratio. ^{1}H NMR (D_2O), major component: 1.284 (3 H, d, J = 6.6 Hz, CH_3–), 3.006 (1 H, dd, J = 11.4, 6.9 Hz, H-6b), 3.240 (1 H, dd, J = 11.4, 6.0 Hz, H-6a), 3.417 (1 H, ddd, J = 6.9, 6.0, 1.2 Hz, H-5), 3.546 (1 H, dd, J = 3.1, 1.2 Hz, H-4), 3.580 (1 H, dd, J = 10.3, 3.5 Hz, H-2), 3.620 (1 H, dd, J = 10.3, 3.1 Hz, H-3), 4.574 (1 H, q, J = 6.6 Hz, OCH), 4.952 (1 H, d, J = 3.5 Hz, H-1), 7.164 (1 H, m, H-*para*), 7.212 (2 H, m, H-*meta*), 7.236 (2 H, m, H-*ortho*); minor component: 1.276 (3 H, d, J = 6.6 Hz, CH_3–), 3.456 (2 H, d, J = 6.2 Hz, H-6), 3.496 (1 H, dd, J = 10.3, 4.0 Hz, H-2), 3.731 (1 H, dd, J = 10.3, 3.4 Hz, H-3), 3.789 (1 H, dd, J = 3.5, 1.3 Hz, H-4), 3.865 (1 H, dt, J = 1.3, 6.2 Hz, H-5), 4.516 (1 H, d, J = 4.0 Hz, H-1), 4.672 (1 H, q, J = 6.6 Hz, OCH), 7.164 (1 H, m, H-*para*), 7.212 (2 H, m, H-*meta*), 7.236 (2 H, m, H-*ortho*). ^{13}C NMR (D_2O), major component: 21.35 (Me), 60.42 (C-6), 68.56 (C-2), 69.15 (C-4), 69.77 (C-3), 70.82 (C-5), 77.19 (OCH), 98.20 (C-1), 126.58 (2 × C-*ortho*), 128.10 (C-*para*), 128.86 (C-*meta*), 143.80 (C-*ipso*); minor component: 23.09 (Me), 61.49 (C-6), 68.48 (C-2), 69.58 (C-4), 69.72 (C-3), 71.23 (C-5), 73.95 (OCH), 95.50 (C-1), 127.09 (2 × C-*ortho*), 128.31 (C-*para*), 128.96 (C-*meta*), 142.37 (C-*ipso*). a, downfield; b, upfield.



Scheme 2. Structures of both diastereoisomers (A and B) of compound **9**.

2,3,3′,4,6-Penta-O-acetyl-1′,4′-di-S-acetyl-DL-threo-1,4-dimercapto-3-hydroxy-2-butyl α-D-galactopyranoside (**10**). The sample is a mixture of two diastereomers in a 62:38 ratio. The two structures could not be unequivocally assigned (Scheme 2). A: ^{1}H NMR ($CDCl_3$) 1.999, 2.044, 2.131, 2.134, 2.144 (15H, 5 × Ac), 2.338, 2.343 (6H, 2 × AcS), 2.939 (1 H, dd, J = 14.2, 7.2 Hz, H-1′b), 3.012 (1 H, dd, J = 9.1, 14.2 Hz, H-4′b), 3.185 (1 H, dd, J = 14.2, 4.9 Hz, H-1′a), 3.414 (1 H, dd, J = 3.7, 14.2 Hz, H-4′a), 3.845 (1 H, m, H-2′), 4.085 (1 H, dd, J = 6.7, 11.5 Hz, H-6b), 4.114 (1 H, dd, J = 6.7, 11.5 Hz, H-6a), 4.413 (1 H, ddd, J = 1.4, 6.3, 6.7 Hz, H-5), 5.12* (1 H, m, H-3′), 5.164 (1 H, dd, J = 3.8, 11.0 Hz, H-2), 5.319 (1 H, d, J = 3.8 Hz, H-1), 5.388 (1 H, dd, J = 11.0, 3.3 Hz, H-3), 5.500 (1 H, dd, J = 3.3 Hz, 1.4 Hz, H-4). ^{13}C NMR ($CDCl_3$, gHSQC readouts) 29.10 (C-4′), 29.36 (C-1′), 61.91 (C-6), 67.15 (C-5), 67.28 (C-3), [c]67.84 (C-2), [c]68.10 (C-4), 72.27 (C-3′), 78.49 (C-2′), 97.34 (C-1), 194.29 (4′-SCO), 194.59 (1′-SCO).

B: ^{1}H NMR ($CDCl_3$) 1.993, 2.030, 2.066, 2.129, 2.152 (15H, 5 × Ac), 2.335, 2.357 (6H, 2 × AcS), 3.098 (1 H, dd, J = 8.1, 13.9 Hz, H-4′b), 3.172 (2 H, d, J = 6.3 Hz, H-1′), 3.266 (1 H, dd, J = 4.8, 13.9 Hz, H-4′a), 3.842 (1 H, m, H-2′), 4.080 (1 H, dd, J = 6.3, 11.3 Hz, H-6b), 4.157 (1 H, dd, J = 6.8, 11.3 Hz, H-6a), 4.463 (1 H, ddd, J = 1.4, 6.3, 6.8 Hz, H-5), 5.10* (1 H, m, H-3′), 5.243 (1 H, dd, J = 3.7, 10.8 Hz, H-2), 5.280 (1 H, d, J = 3.7 Hz, H-1), 5.340 (1 H, dd, J = 10.8, 3.2 Hz, H-3), 5.491 (1 H, dd, J = 3.2, 1.4 Hz, H-4). ^{13}C NMR ($CDCl_3$, gHSQC readouts) 28.64 (C-4′), 29.15 (C-1′), 61.56 (C-6), 66.93 (C-5), 67.52 (C-3), 67.61 (C-2), 67.92 (C-4), 71.19 (C-3′), 78.18 (C-2′), 97.34 (C-1), 194.22 (4′-SCO), 194.59 (1′-SCO). a, downfield; b, upfield, [c]might be interchanged, *gHSQC readouts

3. Results and discussion

3.1. Transglycosylation of tert-butyl alcohol catalysed by the T. flavus α-D-galactosidase

Different water-miscible organic solvents are often employed in transglycosylation reactions to increase the solubility of reac-

tants and to lower water activity, similarly to the approach used with lipases and proteases [17]. Sterically hindered tertiary alcohols (like *tert*-butyl alcohol) are usually considered to be a good choice as, contrary to primary and secondary aliphatic alcohols [13], they are usually inert to hydrolases [16]. When *tert*-butyl alcohol was used as a cosolvent in a reaction catalysed by the *T. flavus* α-D-galactosidase [15], the unexpected formation of *tert*-butyl α-D-galactopyranoside (**1**) as a by-product was observed. To our best knowledge, this is the first case of an enzyme-catalysed α-galactosylation of a sterically hindered alcohol. Therefore, we studied this unique phenomenon in detail with a selection of alcohols as acceptors and various α-D-galactosidase sources.

Transglycosylation reaction with *tert*-butyl alcohol was scaled-up using 25% (v/v) of this alcohol and *tert*-butyl α-D-galactopyranoside **1** was isolated in 14% yield. Similarly, reaction with another tertiary alcohol – *tert*-amyl alcohol (2-methyl-2-butyl alcohol) allowed the isolation of the corresponding glycoside **2**.

3.2. Activity and stability of the T. flavus α-D-galactosidase in the presence of tert-butyl alcohol

High alcohol concentrations in reaction mixtures lead to appreciable transglycosylation yields and better solubility of substrates. Nevertheless, such reactions often fail due to considerable enzyme inactivation by the high concentration of organic cosolvent [18].

Higher *tert*-butyl alcohol concentrations prolonged total conversion of *p*NPαGal: 3.5 h in 25% alcohol, 6.5 h in 50% alcohol and 48 h in 75% alcohol. In the later cases, the enzyme was added in several portions to remain active throughout the whole reaction time.

The activities of 12 α-D-galactosidases from different sources were tested for the transglycosylation reactions with *tert*-butyl alcohol (25%, v/v) and 2-propyn-1-ol (1%, v/v) (Table 1). The α-D-galactosidases from *Aspergillus tamari* CCF 3085 and *Penicillium chrysogenum* CCF 1269 did not cleave *p*NPαGal due to the inhibition by *tert*-butyl alcohol. The α-D-galactosidases from *C. arabica* (Sigma) and *Penicillium melinii* CCF 2440 were able to cleave *p*NPαGal but did not catalyse transfer of α-galactosyl moiety onto *tert*-butyl alcohol. All the other tested enzymes showed a significantly lower activity than the *T. flavus* α-D-galactosidase (Fig. 1), therefore the transglycosylation reaction conditions were optimised with this enzyme. In the reactions with 2-propyn-1-ol, all the tested enzymes were active (Table 1).

We found that the *T. flavus* α-D-galactosidase retains most of its activity and stability in the presence of *tert*-butyl alcohol (25%, v/v). Enzyme activity decreased to 77% after 10 min incubation with *tert*-butyl alcohol and the enzyme kept its stability for at least 27 h (37% of the residual activity) (Figs. 2 and 3).

3.3. Transglycosylation reactions with other alcohols

We further investigated the ability of the *T. flavus* enzyme to catalyse galactosylation of alcohols different from *tert*-butyl

Table 1
Transglycosylation activity of the α-D-galactosidases from different strains (see Section 2) using *tert*-butyl alcohol (**1a**) or 2-propyn-1-ol (**5a**) as acceptors[a]

Source	Intensity of product spot[b] on TLC		Reaction time[c] (h)	
	1a	**5a**	**1a**	**5a**
Aspergillus flavipes CCF 2026	++	+++	1	1
A. parasiticus CCF 3058	+	++	4	1
A. sojae CCF 3060	+	++	5	1
A. tamarii CCF 3085	–	+	–	1
Circinella muscae CCF 1568	+	++	8	1
Coffea arabica (Sigma)	–	+	–	1
Penicillium daleae CCF 2365	+	+++	3	1
P. chrysogenum CCF 1269	–	+++	–	1
P. melinii CCF 2440	–	+++	–	1
P. multicolor CCF 2244	+	++	2	3
Talaromyces flavus CCF 2324	++	++	7	3
T. flavus CCF 2686	+++	++	3	1

[a] Final α-D-galactosidase activity in the reaction mixtures 2 U/ml; alcohol concentrations: *tert*-butyl alcohol (25%, v/v) (**1a**) or 2-propyn-1-ol (1%, v/v) (**5a**), 37 °C, pH 5.0–for more details see Section 2.
[b] –, no product; +, traces of the product; ++, 10–25% of the product; +++, 25–50% of the product (estimated by TLC).
[c] 100% conversion of *p*NPαGal.

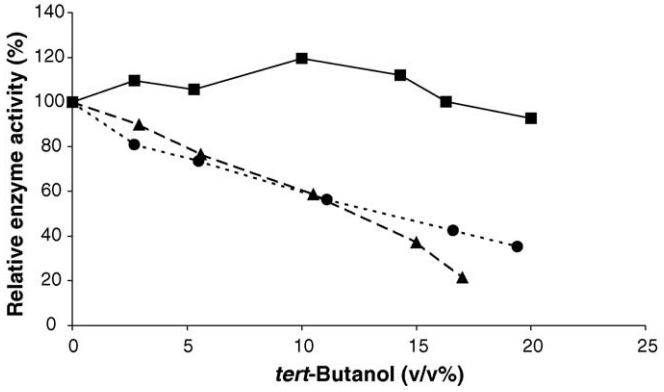


Fig. 1. Influence of *tert*-butyl alcohol on the initial activity of the α-D-galactosidases from *T. flavus* (■), compared to *Penicillium multicolor* (●) and *P. chrysogenum* (▲).

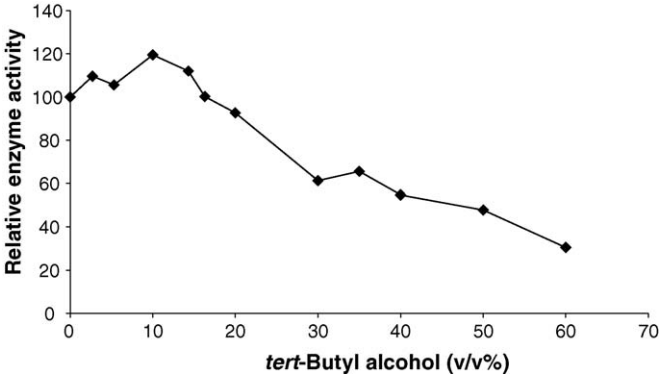


Fig. 2. Influence of *tert*-butyl alcohol on the activity of the *T. flavus* α-D-galactosidase after incubation of 10 min.

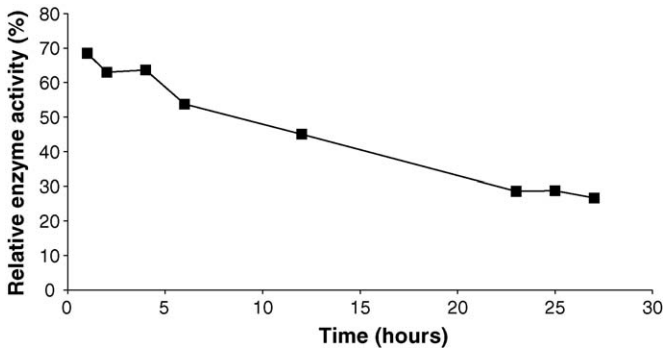


Fig. 3. Stability of the *T. flavus* α-D-galactosidase in *tert*-butyl alcohol (25%, v/v).

and *tert*-amyl alcohol (Scheme 1). The substrate specificity of the α-D-galactosidase was first screened on an analytical scale, then scaled-up in order to isolate respective galactosides. The following products were prepared: *tert*-butyl (**1**, 14% yield), 2-methyl-2-butyl (**2**, 35%), 2-methyl-1-propyl (**3**, 14%), 2,2,2-trifluoroethyl (**4**, 7%), propyn-1-yl (**5**, 34%), *n*-pentyl (**6**, 19%), 3,5-dihydroxybenzyl (**7**, 7%), 1-phenylethyl (**8**, 16%) and DL-*threo*-1,4-dimercapto-3-hydroxy-2-butyl (**9**, 6%) -α-D-galactopyranosides. Very weak enrichment of one diastereomer of the product **8** was observed that is in accordance of a weak capability of glycosidases to discriminate racemic alcohols [19].

3.4. Glycosylation of thioalcohols catalysed by the T. flavus α-D-galactosidase

Glycosylation of thioalcohols with wild-type enzyme has been reported using almond β-glucosidase with 1,3-dithiopropane as an acceptor and glucose as a donor [17]. Another reference to *O*- and/or *S*-glycosylation of thioalcohols comprised the reaction with mercaptoethanol catalysed by β-glucosidase (almonds) [20]. Condensation with free glucose under the catalysis of a β-glucosidase yielded both *O*- and *S*-mercaptoethyl glucosides, however, no spectral characterisation was provided. Meulenbeld et al. [21] studied the glycosylation of 1- and 2-propanethiol and showed again that only the β-glucosidase from almonds could glycosylate both these thioalcohols in transglycosylation mode with *p*NPβGlc as a sugar donor. α-Glucosidase, β-galactosidase, α-mannosidase and thioglucosidase (from *Sinapis alba*) did not glycosylate those thiols [21].

We found that the *T. flavus* α-D-galactosidase was not inhibited by thioalcohols and, moreover, it was able to galactosylate them. For instance, this enzyme catalysed the transglycosylation reaction with mercaptoethanol (1%, v/v). The fact that it was active under strong reducing conditions indicates the probable absence of disulfide bonds in its structure. The formed product (visible by TLC and HPLC) is supposed to be mercaptoethyl-*O*-galactopyranoside but, unfortunately, we did not succeed in its isolation due to its high lability during separations.

We performed transglycosylation reaction with a racemic 1,4-dithio-DL-threitol as an acceptor, which yielded the respective alkyl *O*-galactopyranosides. Introduction of the α-D-galactopyranosyl moiety with new chiral centres into the acceptor molecule creates a mixture of two diastereomers that could be distinguished in the NMR spectrum as a non-equimolar mixture of diastereomers, as a result of either an enzymatic discrimination or a separation procedure, but they could not be unequivocally assigned (see Scheme 2). Structural proof of product **9** was performed with respective peracetate (**10**) that excludes also possible oxidative oligomerization via respective thiol groups.

4. Conclusions

α-D-Galactosidase from the strain *T. flavus* CCF 2686 showed the highest transglycosylation activity towards *tert*-butyl alcohol from all the strains tested. Therefore, this enzyme was used as a catalyst in synthetic reactions presented here. We have demonstrated the broad acceptor specificity of the *T. flavus* α-D-galactosidase on the preparation of nine alkyl α-D-galactopyranosides by transglycosylation. Especially remarkable is the galactosylation of *tert*-butyl alcohol, *tert*-amyl alcohol and 1,4-dithio-DL-threitol.

Acknowledgements

This work was supported by GACR (grant 203/05/0172) and COST chemistry D25/0001/02, project MŠMT OC D25.002, NATO (Collaborative project No. LST.CLG.980125 to S.R. and V.K.) and Institutional research concept No. AV0Z50200510.

References

[1] P. Dey, J.B. Pridham, Adv. Enzymol. Relat. Areas Mol. Biol. 36 (1972) 91–130.
[2] H. Kobayashi, H. Suzuki, J. Ferment. Tech. 50 (1972) 625–632.
[3] V.M. Guimaraes, S.T. de Rezende, M.A. Moreira, E.G. de Barros, C.R. Felix, Phytochemistry 58 (2001) 67–73.
[4] K. Sakai, T. Tachiki, H. Kumagai, T. Tochikura, Agric. Biol. Chem. 51 (1987) 315–322.
[5] P.V. Bulpin, M.J. Gidley, R. Jeffcoat, D.J. Underwood, Carbohydr. Polym. 12 (1990) 155–168.
[6] J.H. Clarke, K. Davidson, J.H. Rixon, J.R. Halstead, M.P. Fransen, H.J. Gilbert, G.P. Hazlewood, Appl. Microbiol. Biotechnol. 53 (2000) 661–667.
[7] M. Fuller, M. Lovejoy, D.A. Brooks, M.L. Harkin, J.J. Hopwood, P.J. Meikle, Clin. Chem. 50 (2004) 1979–1985.
[8] L.D. Varbanets, V.M. Malanchuk, T.T. Buglova, R.A. Kuhlmann, Carbohydr. Polym. 44 (2001) 357–363.
[9] V. Křen, J. Thiem, Chem. Soc. Rev. 26 (1997) 463–473.
[10] P. Biely, V. Puchart, G.L. Cote, Carbohydr. Res. 332 (2001) 299–303.
[11] P. Spangenberg, C. André, M. Dion, C. Rabiller, R. Mattes, Carbohydr. Res. 329 (2000) 65–73.
[12] H. Hashimoto, Ch. Katayama, M. Goto, T. Okinaga, S. Kitahata, Biosci. Biotechnol. Biochem. 59 (1995) 619–623.
[13] E.V. Eneyskaya, A.M. Golubev, A.M. Kachurin, A.N. Savelév, K.N. Neustroev, Carbohydr. Res. 305 (1997) 83–91.
[14] L. Weignerová, Z. Huňková, M. Kuzma, V. Křen, J. Mol. Cat. B: Enzym. 11 (2001) 219–224.
[15] P. Simerská, M. Kuzma, A. Pišvejcová, L. Weignerová, M. Macková, S. Riva, V. Křen, Folia Microbiol. 48 (2003) 329–338.

[16] F. van Rantwijk, M. Woudenberg-van Oosterom, R.A. Sheldon, J. Mol. Cat. B: Enzym. 6 (1999) 511–532.
[17] G. Vic, D.H.G. Crout, Tetrahedron: Asymmetry 5 (1994) 2513–2516.
[18] M. Casali, L. Tarantini, S. Riva, Z. Huňková, L. Weignerová, V. Křen, Biotechnol. Bioeng. 77 (2002) 105–110.
[19] B. Werschkun, W.A. Konig, V. Křen, J. Thiem, J. Chem. Soc. Perkin Trans. 1 (1995) 2459–2466.
[20] T. Dintinger, D. Dutheil-Bouedec, M. Bouchonneau, C. Tellier, Biotechnol. Lett. 16 (1994) 689–692.
[21] G.H. Meulenbeld, B.M. de Roode, S. Hartmans, Biocatal. Biotransform. 20 (2002) 251–256.