Conference paper

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Chemo-enzymatic synthesis of 3-*O*-(β-D-glycopyranosyl)-sn-glycerols and their evaluation as preservative in cosmetics

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Abstract: D-Glycopyranosyl glycerols are common natural products and exhibit strong biological properties, notably as moisturizing agents in cosmetics. Their chemical synthesis remains tedious thus decreasing their potential industrial and economic development, as well as the study of their structure-function relationships. In this work, the chemo-enzymatic synthesis of three enantiopure 3-*O*-(β-D-glycopyranosyl)-*sn*-glycerols was efficiently performed using an original glycosidase from *Dictyoglomus thermophilum* and their preservatives properties were assessed using a challenge test method. Amongst them, the 3-*O*-(β-D-glucopyranosyl)-*sn*-glycerol exhibited a specific anti-fungus activity.

Keywords: antifungal activity; antimicrobial activity; carbohydrates; enzyme catalysis; ICS-28.

Introduction

D-Glycopyranosyl glycerols (GGs) are widespread natural products in archea, bacteria and plants [1]. For example, under the α-configuration, 2-*O*-(D-glucosyl)-*sn*-glycerol is the main compatible solute in photosynthetic bacteria and has recently attracted special attention for its application as moisturising agent in cosmetics [2]. Under the β-configuration, mono- and digalactosyldiacylglycerols constitute the major component of the membrane of the chloroplasts from plants and cyanobacteria, and thus, are known to be the most abundant lipids on Earth [3]. After removal of the lipidic part, they have already demonstrated efficient applications in foodstuffs, cosmetic and healthcare products, in antitumor medicines and in the inhibition of Epstein-Barr virus activation and skin cancer promotion [4–7].

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The chemical synthesis of GGs is not straightforward and requires multiple protection/deprotection steps, careful control of the anomeric configuration during the glycosylation reaction and tedious chromatographic purifications [8, 9]. Alternatively, direct enzymatic transglycosylation procedures using glycerol as acceptor have been recently developed and demonstrated promising results [8]. Amongst them, sucrose phosphorylase proved to be an extremely efficient biocatalyst and is now used to produce industrial scale of 2-*O*-(α-D-glucosyl)-*sn*-glycerol named Glycoin@.

For many years, glycosidases (EC 3.2.1.-) have been considered as an alternative choice to the classical processes of chemistry insofar as they allow the one-pot synthesis of glycosylated derivatives [10]. Among the latter, access to the alkyl glycosides raised a strong interest. These biodegradable surfactants possess remarkable properties including good emulsifying and hydrating agents in the agri-food, pharmaceutical or cosmetic industries. These compounds are also known for their dermatological properties, which make them compatible in the formulation of product [11].

In the course of our on-going research program devoted to rare carbohydrates [12], we are largely involved in the discovery of original enzymatic activities for the development of biocatalytic synthesis of glycoconjugates [13–15]. Herein, we wish to report the cloning, overexpression and purification of Dtgly, a versatile glycosidase from *Dictyoglomus thermophilum*, and its use as an efficient biocatalyst for the chemo-enzymatic preparation of a series of alkyl glycosides and glycosyl glycerols.

Experimental section

Cloning, expression and purification

The open reading frame containing *Dtgly* gene was amplified from a *D. thermophilum* strain H-6-12 (DSM 3960) genomic DNA template by PCR using primers Dt359-F (5′-AATTGCTAGCATGCTTAAATACAGGTTTC-CTG-3′) and Dt359-R (5′-TTTGCGGCCGCCTATTTAAGAAAC-3′). PCR product was then ligated into the pET-28a vector using restriction enzymes *Nhe I/Not I* (sequences underlined in primers). The correct insertion *Dtgly* gene into vector was confirmed by DNA sequencing (Eurofins Genomics) to be identical with the known genomic sequence of *D. thermophilum dicth_359* gene. *Escherichia coli* Rosetta (DE3) transformed with pET28a-*Dtgly* were cultivated in LB medium containing kanamycin (34 mg/mL) and chloramphenicol (30 mg/mL) at 37 °C until OD_{600} reach 0.6. The culture was then induced by adding IPTG 1 mM and incubated for 4 h at 37 °C and 25 °C (overnight) and 220 rpm. Cells were harvested by centrifugation, resuspended in 100 mL (1/10 culture volume) cocktail solution (Tris-HCl 50 mM pH 8.0, NaCl 200 mM, lysozyme and protease inhibitor), and lysed by thermic shock and sonication. Clarification of protein was carried out by heat treatment (15 min at 70 °C). Dtgly protein (uniprot B5YCI2) was further purified through IMAC (1 mL column equilibrated with tris-HCl (50 mM) –NaCl (200 mM) buffer (pH 8.0), the bound proteins were eluted using an imidazole gradient [10–500 mM)], and SEC [tris-HCl (50 mM)-NaCl (200 mM) buffer (pH 8.0)] purifications.

Enzyme activity assays

Dtgly activity was assayed at 37 °C in 200 µL reaction mixtures containing substrate (0.01–10 mM), 0.425 µg enzyme and Tris buffer (200 mM, pH 6.0). Residual spontaneous hydrolysis of the substrate was determined on sample containing H_2O instead of enzyme. For paranitrophenol (pNP) containing substrates, after 30 min reaction, 100 µL of sodium carbonate 1 M were added, and produced pNP was quantified by absorbance measurement at 405 nm. All kinetics parameters were calculated by fitting of saturation curves (as mean of triplicate measurements) with standard Michaelis-Menten and inhibition equations, using Prism 6 (GraphPad).

Effect of pH and temperature on Dtgly activity

The optimum pH for Dtgly was determined by measuring pNP-β-D-Glc hydrolysis under several pH values ranging from pH 4.0 to pH 10.0. The buffers used were: acid citric/sodium phosphate buffer (200 mM) from pH 4.0–6.0, imidazole/HCl buffer (200 mM) for pH 7.0, Tris/HCl buffer (200 mM) for pH 8.0, tris base buffer (200 mM) for pH 9.0 and sodium carbonate buffer (200 mM) for pH 10.0. One hundred microliter samples containing 1 mM pNP-β-D-Glc, 10 µL buffer and 5 µL of diluted Dtgly were incubated 5 min at 37 °C. Then reactions were quenched by adding 50 µL Na_2CO_3 (1 M) and absorbance was measured at 405 nm. The dependence of the enzyme activity on temperature was determined by measuring the hydrolysis of pNP-β-D-Glc for several temperatures ranging from 40 °C to 100 °C. Samples were prepared following the same protocol than pH-dependence study but only in 20 mM Imidazole-HCl buffer at pH 6.0. After 5 min incubation, reaction was quenched by adding 50 µL Na_2CO_3 (1 M) and relative activity was calculated according to absorbance measured for each temperature at 405 nm.

General procedure 1 – alkyl-*O*-glycoside synthesis

To a solution of pNP-β-D-glycopyranoside (1.0 equiv.) in citric acid/sodium phosphate buffer (20 mM, pH 6.0, 2 mL) were added acceptors (50 equiv.). The reaction mixture was stirred and warmed up to 37 °C, the enzyme Dtgly (0.35 mg · mL^{-1}, 20 nmol, 3 mL) was added and the reaction was monitored by TLC (ACN/H_2O, 6:1) until full disappearance of the glycosyl donor (typically 8 h). Then the mixture was concentrated under reduced pressure and purified.

Methyl 1-*O*-β-D-glucopyranoside 1 [16]

The title compound **1** was prepared from pNP-β-D-glucopyranoside (20.7 mg, 69 µmol) according the general procedure 1, with MeOH as acceptor. The expected product was obtained after a flash chromatography (DCM/MeOH, 9:1) as uncoloured oil (m = 8.3 mg; R = 54 %), R_f = 0.43 (ACN/H_2O, 6:1), $[\alpha]_D^{20} = -24$ (c = 1, H_2O). ^{1}H NMR (400 MHz, D_2O), δ = 4.4 (d, $J_{1/2}$ = 8 Hz, 1H, H-1); 3.95 (dd, $J_{5/6'}$ = 1.2 Hz, $J_{6'/6''}$ = 12.4 Hz, 1H, H-6′); 3.75 (dd, $J_{5/6''}$ = 6 Hz, $J_{6'/6''}$ = 11.6 Hz, 1H, H-6″); 3.6 (s, 3H, OCH_3); 3.5 (m, 2H, H-3, H-5); 3.4 (*b*t, $J_{3/4}$ = 9.6 Hz, 1H, H-4); 3.29 (*b*t, $J_{1/2}$ = 8 Hz, $J_{2/3}$ = 9 Hz, 1H, H-2), assignments were confirmed by gCOSY. ^{13}C NMR (100 MHz, D_2O), δ = 103.2 (C-1), 75.9 (C-5), 75.8 (C-3), 73.1 (C-2), 69.7 (C-4), 60.8 (C-6), 57.2 (OCH_3), assignments were confirmed by gHSQC. HR-MS (ESI): calcd. for $C_7H_{15}O_6$ $[M+H]^+$ 195.0863; found 195.0859.

Ethyl 1-*O*-β-D-glucopyranoside 2 [16]

Title compound **2** was obtained from pNP-β-D-glucopyranoside (20.0 mg, 66 µmol) following the general procedure 1, with EtOH as acceptor. A flash chromatography (DCM/MeOH, 9:1) afforded the expected compound as colourless oil (m = 7.9 mg; R = 57 %), R_f = 0.51 (ACN/H_2O, 6:1), $[\alpha]_D^{20} = -34.3$ (c = 1, H_2O). ^{1}H NMR (400 MHz, D_2O), δ = 4.5 (d, $J_{1/2}$ = 8 Hz, 1H, H-1); 4.0 (m, 1H, −OC$_a$*H*′−); 3.94 (dd, $J_{5/6'}$ = 4 Hz, $J_{6'/6''}$ = 12.4 Hz, 1H, H-6′); 3.75 (m, 2H, −OC$_a$*H*″−, H-6″); 3.5 (m, 2H, H-3, H-5); 3.4 (*b*t, $J_{3/4}$ = 9.2 Hz, 1H, H-4); 3.29 (*b*t, $J_{2/3}$ = 9.2 Hz, 1H, H-2); 1.26 (*b*t, $J_{a/b}$ = 8 Hz, 3H, −OCH_2−C$_b$*H*$_3$), assignments were confirmed by gCOSY. ^{13}C NMR (100 MHz, D_2O), δ = 101.9 (C-1); 75.9 (C-5), 75.8 (C-3); 73.1 (C-2); 69.7 (C-4); 66.2 (O*C*$_a$H$_2$CH$_3$); 60.8 (C-6); 14.3 ($OCH_2C_bH_3$), assignments were confirmed by gHSQC. HR-MS (ESI): calcd. for $C_8H_{17}O_6$ $[M+H]^+$ 209.1019; found 209.1021.

Isopropyl 1-*O*-β-D-glucopyranoside 3 [17]

Title compound **3** was synthesized from pNP-β-D-glucopyranoside (24.0 mg, 80 µmol) following the general procedure 1, with iPrOH as acceptor. The desired product was obtained after a flash chromatography

(DCM/MeOH, 9:1) as colourless oil (m = 5.9 mg; R = 33 %). R_f = 0.64 (ACN/H_2O, 6:1), $[\alpha]_D^{20}$ = –26 (c = 1, H_2O). ^{1}H NMR (400 MHz, D_2O), δ (ppm) = 4.56 (d, $J_{1/2}$ = 8 Hz, 1H, H-1); 4.14 (m, 1H, –OC_aH–); 3.93 (*b*dd, $J_{6'/6''}$ = 12.3 Hz, 1H, H-6′); 3.73 (dd, $J_{5/6''}$ = 2 Hz, $J_{6'/6''}$ = 12.3 Hz, 1H, H-6″); 3.5 (m, 2H, H-3, H-5); 3.4 (*b*t, $J_{3/4}$ = 9.6 Hz, 1H, H-4); 3.24 (*b*t, $J_{1/2}$ = 8 Hz, 1H, H-2); 1.27 (d, $J_{a/b}$ = 6 Hz, 3H, –OCH–C_bH_3); 1.24 (d, $J_{a/b'}$ = 6 Hz, 3H, –OCH–C_bH_3), assignments were confirmed by gCOSY. ^{13}C NMR (100 MHz, D_2O), δ = 100 (C-1); 76 (C-3, C-5); 73.2 (C-2); 73 (–OC_aHCH_3–); 69.7 (C-4); 60.8 (C-6); 22 (–OCH_2–C_bH_3); 21 (–OCH_2–$C_{b'}H_3$), assignments were confirmed by gHSQC. HR-MS (ESI): calcd. for $C_9H_{19}O_6$ $[M+H]^+$ 223.1176; found 223.1177.

Butyl 1-*O*-β-D-glucopyranoside 4 [16]

The title compound **4** was prepared from pNP-β-D-glucopyranoside (20.0 mg, 66 μmol) according the general procedure 1, with nButOH as acceptor. The expected product was obtained after a flash chromatography (DCM/MeOH, 9:1) as a colourless oil (m = 10 mg; R = 64 %). R_f = 0.63 (ACN/H_2O, 6:1), $[\alpha]_D^{20}$ = –35.6 (c = 1, H_2O). ^{1}H NMR (400 MHz, D_2O), δ = 4.5 (d, $J_{1/2}$ = 8 Hz, H-1); 3.95 (m, 2H, –$OC_aH'CH_2$–, H-6′); 3.73 (m, 2H, –$OC_aH''CH_2$–, H-6″); 3.5 (m, 2H, H-3, H-5); 3.4 (*b*t, $J_{3/4}$ = 9.2 Hz, 1H, H-4); 3.28 (*b*t, $J_{1/2}$ = 8 Hz, $J_{2/3}$ = 9.2 Hz, 1H, H-2); 1.63 (q, $J_{a/b}$ = 6.8 Hz, $J_{b/c}$ = 7.6 Hz, 2H, –$CH_2C_bH_2CH_2$–); 1.4 (m, 2H, –$CH_2C_cH_2CH_3$); 0.93 (*b*t, $J_{c/d}$ = 7.6 Hz, 3H, –$CH_2C_dH_3$), assignments were confirmed by gCOSY. ^{13}C NMR (100 MHz, D_2O), δ = 102.2 (C-1); 75.9–75.8 (C-3, C-5); 73.2 (C-2); 70.4 (–OC_aH_2–); 69.7 (C-4); 60.8 (C-6); 30.9 (–$C_bH_2CH_2$–); 18.45 (–$C_cH_2CH_3$); 13.0 (–$CH_2C_dH_3$), assignments were confirmed by gHSQC. HR-MS (ESI): calcd. for $C_{10}H_{21}O_6$ $[M+H]^+$ 237.1332; found 237.1332.

(Hexan-6-ol)-1-*O*-β-D-glucopyranoside 5 [18]

The title compound **5** was synthesized from pNP-β-D-glucopyranoside (20.0 mg, 66 μmol) according the general procedure 1, with 1,6-Hexanediol as acceptor. The expected compound was obtained after a flash chromatography (DCM/MeOH, 9:1) as colorless oil (m = 15.9 mg; R = 86 %). R_f = 0.49 (ACN/H_2O, 6:1), $[\alpha]_D^{20}$ = –32.1 (c = 1, H_2O). ^{1}H NMR (400 MHz, D_2O), δ = 4.48 (d, $J_{1/2}$ = 8 Hz, 1H, H-1); 3.94 (m, 2H, H-6′, –OC_aH'–CH_2); 3.74 (m, 2H, H-6″, OC_aH''–CH_2–); 3.63 (t, $J_{e/f}$ = 6.8 Hz, 3H, –C_fH_2OH); 3.49 (m, 2H, H-3, H-5); 3.42 (*b*t, $J_{3/4}$ = 9.6 Hz, 1H, H-4); 3.28 (d $J_{1/2}$ = 8 Hz, 1H, H-2); 1.65 (m, 2H, –CH_2–C_bH_2–); 1.59 (m, 2H, –C_dCH_2–C_eCH_2–); 1.40 (m, 4H, –C_cH_2–C_dCH_2–), assignments were confirmed by gCOSY. ^{13}C NMR (100 MHz, D_2O), δ = 102.3 (C-1); 76 (C-5); 75.9 (C-3); 73.2 (C-2); 70.6 (–OC_aH_2–); 69.8 (C-4); 61.8 (–C_fH_2OH); 60.9 (C-6); 31.3 (–C_eH_2–CH_2OH); 28.8 (–CH_2–C_bH_2–); 24.8 (–C_cH_2–C_dH_2–), assignments were confirmed by gHSQC. HR-MS (ESI): calcd. for $C_{12}H_{25}O_7$ $[M+H]^+$ 281.1595; found 281.1592.

1,2-Isopropylidene-3-*O*-(β-D-glucopyranosyl)-*sn*-glycerol 6

The title compound **6** was synthesized from pNP-β-D-glucopyranoside (33.2 mg, 110 μmol) according the general procedure 1, with (*S*)-solketal as acceptor. The expected compound was obtained after a flash chromatography (DCM/MeOH, 9:1) as colorless oil (m = 18.2 mg; R = 56 %). R_f = 0.50 (ACN/H_2O, 6:1), $[\alpha]_D^{20}$ = –31 (c = 1, H_2O). ^{1}H NMR (400 MHz, D_2O), δ = 4.52 (m, 2H, H-1) –OCH_2C_bH–); 4.21 (*b*t, $J_{c'/b}$ = 8.4 Hz, 1H, –$C_cH_2'O$–), 3.98 (m, 2H, H-6′, –OC_aH_2'–CH–); 3.88 (*b*t, $J_{c''/b}$ = 8 Hz, 1H, –CH–$C_cH_{2''}O$–); 3.83 (dd, $J_{a''/b}$ = 3.2 Hz, $J_{a'/a''}$ = 10.8 Hz, 1H, –OC_aH_2''–CH–); 3.77 (dd, $J_{5/6''}$ = 5.6 Hz, $J_{6'/6''}$ = 12 Hz, 1H, H-6″); 3.48 (*m*, 3H, H-3, H-5, H-4); 3.35 (*b*t, $J_{1/2}$ = 8 Hz, 1H, H-2); 1.52 (s, 3H, –$C_{e'}H_3$); 1.45 (s, 3H, –$C_{e''}H_3$), assignments were confirmed by gCOSY. ^{13}C NMR (100 MHz, D_2O), δ = 110 (C_d); 102.4 (C-1); 76 (C-5); 75.6 (C-3); 74 (OCH_2–C_bH); 73 (C-2); 70 (O–C_aH_2); 69.6 (C-4); 65 (C_cH_2–O); 60.7 (C-6); 25.5 ($C_{e'}H_3$); 24 ($C_{e''}H_3$), assignments were confirmed by gHSQC and HMBC. HR-MS (ESI): calcd. for $C_{12}H_{23}O_8$ $[M+H]^+$ 295.1387; found 295.1396.

1,2-Isopropylidene-3-*O*-(β-D-galactopyranosyl)-*sn*-glycerol 7

The title compound **7** was synthesized from pNP-β-D-galactopyranoside (27.4 mg, 91 µmol) according the general procedure 1, with (*S*)-solketal as acceptor. The expected compound was obtained after a flash chromatography (DCM/MeOH, 9:1) as colourless oil (m = 19.7 mg; R = 73 %). R_f = 0.46 (ACN/H_2O, 6:1), $[\alpha]_D^{20}$ = +5 (c = 1, H_2O). ^{1}H NMR (400 MHz, D_2O), δ = 4.50 (m, 1H, $-OCH_2-C_bH-$); 4.45 (d, $J_{1/2}$ = 8 Hz, 1H, H-1); 4.2 (t, $J_{b/c'}$ = 8 Hz, 1H, $-CH-C_cH_{2'}-O-$); 3.98 (m, 2H, H-4, $-O-C_aH_{2'}CH-$); 3.87 (*b*t, $J_{b/c''}$ = 8 Hz, 1H, $-CH-C_cH_2''-O$); 3.8 (m, 3H, H-6, $-O-C_aH_{2''}CH-$); 3.7 (m, 2H, H-3, H-5); 3.56 (*b*t, $J_{2/3}$ = 6.4 Hz, 1H, H-2); 1.5 (s, 3H, $-C_eH_3$); 1.43 (s, 3H, $-C_{e''}H_3$), assignments were confirmed by gCOSY. ^{13}C NMR (100 MHz, D_2O), δ = 110 ($CH_3-C_d-CH_3$); 103 (C-1); 75 (C-5); 74 ($OCH_2-C_bH-CH_2-$); 73 (C-3); 71 (C-2); 70 ($-OC_aH_2-CH-$); 69 (C-4); 65 ($CH-C_cH_2O$); 61 (C-6); 26 ($C_{e'}H_3$); 24 ($C_{e''}H_3$), assignments were confirmed by gHSQC and gHMBC. HR-MS (ESI): calcd. for $C_{12}H_{23}O_8$ $[M+H]^+$ 295.1387; found 295.1396.

1,2-Isopropylidene-3-*O*-(β-D-xylopyranosyl)-*sn*-glycerol 8

The title compound **8** was synthesized from pNP-β-D-xylopyranoside (21.3 mg, 114 µmol) according the general procedure 1, with (*S*)-solketal as acceptor. The expected compound was obtained after a flash chromatography (DCM/MeOH, 9:1) as colourless oil (m = 10.6 mg; R = 51 %). R_f = 0.68 (ACN/H_2O, 6:1), $[\alpha]_D^{20}$ = –32 (c = 1, MeOH). ^{1}H NMR (400 MHz, D_2O), δ = 4.48 (m, 1H, $-OCH_2-C_bH-$); 4.45 (d, $J_{1/2}$ = 8 Hz, 1H, H-1); 4.19 (t, $J_{b/c'}$ = 8 Hz, 1H, $-CH-C_cH_{2'}O-$); 3.98 (dd, $J_{4/5'}$ = 5.2 Hz; $J_{5'/5''}$ = 11.6 Hz, 1H,H-5′); 3.92 (*b*dd, $J_{a'/b}$ = 6.4 Hz; $J_{a'/a''}$ = 10.8 Hz, 1H, $-OC_aH_{2'}-CH-$); 3.89 (*b*t, $J_{c''/d}$ = 6.8 Hz, 1H, $-CH-C_cH_{2''}O-$); 3.79 (*b*dd, $J_{a''/b}$ = 3.6 Hz; $J_{a'/a''}$ = 10.8 Hz, 1H, $-OC_aH_{2''}-CH-$); 3.65 (m, 1H, H-4); 3.46 (t, $J_{2/3}$ = 9.2 Hz, 1H, H-3); 3.34 (m, 2H, H-5″, H-2), 1.49 (s, 3H, $-C_eH_3$); 1;42 (s, 3H, $-C_{e''}H_3$), assignments were confirmed by gCOSY. ^{13}C NMR (100 MHz, D_2O), δ = 110 ($CH_3-C_d-CH_3$); 103 (C-1); 76 (C-3); 74 (OCH_2-C_bH-); 73 (C-2); 70 (OC_aH_2-CH-); 69 (C-4); 65.4 ($-CH-C_cH_2O$); 65 (C-5); 26 ($-C_{e'}H_3$); 24 ($-C_{e''}H_3$), assignments were confirmed by gHSQC. HR-MS (ESI): calcd. for $C_{11}H_{21}O_7$ $[M+H]^+$ 265.1282; found 265.1282.

General procedure 2 – glyceride-*O*-glycoside synthesis

1,2-Isopropylidene-3-*O*-(β-D-glycopyranosyl)-*sn*-glycerol was dissolved in a solution of acetic acid (70 %) in water. The reaction was warmed up to 60 °C and left to stir until full deprotection was evident by TLC (~3 h). Then the mixture was concentrated, co-evaporated with toluene (three times) and purified on silica gel.

3-*O*-(β-D-Glucopyranosyl)-*sn*-glycerol 9 [19]

The title compound **9** was prepared from 1,2-isopropylidene-3-*O*-(β-D-glucopyranosyl)-*sn*-glycerol **6** (15.5 mg, 53 µmol) according the general procedure 2. The expected product was obtained after a flash chromatography (DCM/MeOH, 9:1) as uncoloured oil (m = 8.3 mg, R = 62 %), R_f = 0.21 (ACN/H_2O, 6:1), $[\alpha]_D^{20}$ = –25 (c = 1, H_2O). ^{1}H NMR (400 MHz, D_2O), δ = 4.49 (d, $J_{1/2}$ = 8 Hz, 1H, H-1); 3.95 (m, 3H, H-6', $-OCH_2-C_bH-$, $-CH-C_cH'OH$); 3.73 (m, 3H, H-6″, $-CH-C_cH''OH$, $-OC_aH'-CHOH$); 3.64 (*b*dd, $J_{a''/b}$ = 5.6 Hz, $J_{a'/a''}$ = 11.6 Hz, 1H, $-OC_aH''-CHOH$); 3.52 (*b*t, $J_{2/3}$ = 9.2 Hz, 1H, H-3); 3.47 (dd, $J_{5/6'}$ = 2 Hz, $J_{5/6''}$ = 6.4 Hz, 1H, H-5); 3.42 (*b*d, $J_{3/4}$ = 9.6 Hz, 1H, H-4); 3.33 (*b*t, $J_{2/3}$ = 8.8 Hz, 1H, H-2), assignments were confirmed by gCOSY. ^{13}C NMR (100 MHz, D_2O), δ = 102 (C-1); 76 (C-3); 75.6 (C-5); 73 (C-2); 71 ($-C_bHOH$); 70 ($-C_cH_2OH$); 69.6 (C-4); 62 ($-OC_aH_2-$); 61 (C-6), assignments were confirmed by gHSQC. HR-MS (ESI): calcd. for $C_9H_{19}O_8$ $[M+H]^+$ 255.1074; found 255.1073.

3-*O*-(β-D-Galactopyranosyl)-*sn*-glycerol 10 [19]

The title compound **10** was prepared from 1,2-isopropylidene-3-*O*-(β-D-galactopyranosyl)-*sn*-glycerol ***7*** (20.6 mg, 70 µmol) according the general procedure 2. The expected product was obtained after a flash chromatography (DCM/MeOH, 9:1) as colourless oil (m = 8.9 mg, R = 50 %), R_f = 0.18 (ACN/H_2O; 6:1), $[\alpha]_D^{20}$ = +7

(c = 1, H_2O). ^{1}H NMR (400 MHz, D_2O), δ = 4.44 (d, $J_{1/2}$ = 8 Hz, 1H, H-1); 3.95 (m, 3H, H-4, –OCH$_2$–C$_b$*H*–, –CH–C$_c$*H*′OH); 3.86–3.77 (m, 3H, H-6, –CH–C$_c$*H*″OH); 3.75-3.62 (m, 4H, H-3, H-5, –OC$_a$*H*$_2$–); 3.58 (*b*t, $J_{1/2}$ = 8 Hz, 1H, H-2), assignments were confirmed by gCOSY. ^{13}C NMR (100 MHz, D_2O), δ = 103 (C-1); 75 (C-5); 72.6 (C-3); 70.8 (–*C*$_b$HOH); 70.7 (–*C*$_c$H$_2$OH); 70.4 (C-2); 68.6 (C-4); 62.3 (C-6); 61 (–O*C*$_a$H$_2$), assignments were confirmed by gHSQC. HR-MS (ESI): calcd. for $C_9H_{19}O_8$ $[M+H]^+$ 255.1074; found 255.1075.

3-*O*-(β-D-xylopyranosyl)-*sn*-glycerol 11

The title compound **11** was prepared from 1,2-isopropylidene-3-*O*-(β-D-xylopyranosyl)-*sn*-glycerol **8** (15.2 mg, 57.6 μmol) according the general procedure 2. The expected product was obtained after a flash chromatography (DCM/MeOH, 9:1) as uncoloured oil (m = 8 mg; R = 62 %), R_f = 0.39 (ACN/H_2O; 6:1), $[\alpha]_D^{20}$ = –38.2 (c = 1, MeOH). ^{1}H NMR (400 MHz, D_2O), δ = 4.44 (d, $J_{1/2}$ = 8 Hz, 1H, H-1); 3.93 (m, 3H, H-5′, –OCH$_2$–C$_b$*H*–, –CH–C$_c$*H*′OH); 3.76 (*b*dd, $J_{b/c''}$ = 3.6 Hz, $J_{c'/c''}$ = 10.4 Hz, 1H, –CH–C$_c$*H*″OH); 3.64 (m, 3H, H-4, –OC$_a$*H*$_2$–); 3.47 (*b*t, $J_{2/3}$ = 9.2 Hz, 1H, H-3); 3.33 (m, 2H, H-2, H-5″), assignments were confirmed by gCOSY. ^{13}C NMR (100 MHz, D_2O), δ = 103.2 (C-1); 75.6 (C-3); 72.9 (C-2); 70.7 (–*C*$_c$HOH); 70.3 (–*C*$_b$H$_2$OH); 69.2 (C-4); 65 (C-5); 62.3 (–O*C*$_a$H$_2$), assignments were confirmed by gHSQC. HR-MS (ESI): calcd. for $C_8H_{17}O_7$ $[M+H]^+$ 225.0969; found 225.0967.

Antimicrobial assay

The potential antimicrobial activity of the products was assessed by the micro-challenge test method originally described in El Abdellaoui et al. [20] The microorganisms used include all the strains recommended in the current method for cosmetic preservative efficacy testing (EN ISO11930): *Pseudomonas aeruginosa* (*Pa*) ATCC 9027, *Staphylococcus aureus* (*Sa*) ATCC 6538, *Escherichia coli* (*Ec*) ATCC 8739, *Candida albicans* (*Ca*) ATCC 10231 and *Aspergillus brasiliensis* (*Ab*) ATCC 16404. These microorganisms were grown 24–48 h at 35 °C on tryptone soja broth (Oxoid, ref CM0129B) for the bacteria, YM broth (Difco, ref 268110) for *Candida albicans* or Sabouraud broth (Difco, ref A3314D) for *Aspergillus brasiliensis*, before preparation of a pure saline inoculum containing between 10^6 and 10^7 (fungus and yeast) or between 10^7 and 10^8 (bacteria) cfu/mL. Prior to the contamination, each sample (extracts and fractions) was first solubilised in DMSO, then in 0.85 % saline to obtain different concentrations from 12.5 $\mu g \cdot mL^{-1}$ to 200 or 400 or 500 $\mu g \cdot mL^{-1}$ for the extracts and from 10 $\mu g \cdot mL^{-1}$ to 160 $\mu g \cdot mL^{-1}$ for the fractions. Samples were deposed in duplicate, in five deep-wells plates (one for each tested microorganism) and contaminated with 10 μL of inoculum of each microorganism. The plates were covered with adhesive breathable film and incubated at 22 °C. Microbial counts were done at day 1 (24 h), days 7 and 14 for each sample. Counting was performed with a TTC micromethod for the yeast and bacteria and with the conventionnal agar plating method on Sabouraud gelose (Oxoid, ref BO0408M) for *Aspergillus brasiliensis*. Triphenyltetrazolium chloride (TTC) micromethod was performed as follow: in sterile 96 wells (250 μL) microplates, 20 μL of each sample were ten fold serialy diluted in 180 μL of Letheen broth (LB) (Difco, ref 268110) containing 1.5 % Tween 80 (Sigma, ref P1754) and TTC (Sigma, ref T8877) For the strain *Candida albicans*, Yeast Mold (YM) broth (Difco, ref 271120) was used instead of LB. The microplates were incubated 48 h at 32.5 °C and the microoganisms' growth was monitored as color change from colorless to pink/red. The reciprocal highest dilution indicating growth allows the determination of the log number of each microorganism at each time.

Results and discussion

Biochemical characterization of Dtgly

Dtgly gene (*dicth_0359*) was amplified from *D. thermophilum* genomic DNA and cloned into expression vector pET28a-(+) for heterologous production in *Escherichia coli*. This vector adds a *N*-terminal His-Tag that was

used for protein purification after cell lysis. Purification on Ni-NTA resin yielded good amounts of protein (5–10 mg/culture liter). This purified enzyme was further used to assess its catalytic activity. The kinetic parameters were evaluated by monitoring the hydrolytic activity of the enzyme towards 20 different para-nitrophenyl-sugars (pNP-sugars, Figure S1). Potentially cleaved pNP could be easily quantified at 405 nm. Amongst them, five pNP-pyranosides demonstrated to be substrates, all of them belonging to the D series and sharing a common β-linkage: Gal, Glc, Fuc, Man and Xyl. Only the two latter were too weak substrates to allow us to determine their kinetic parameters (Table 1, Figure S2). When comparing their specificity constant, Dtgly demonstrated formally to be more specific to D-Fuc and therefore is a β-D-fucosidase. However, Dtgly proved to be very versatile as it tolerates well the addition of an hydroxyl group at the C-6 position (D-Gal) and even better the additional inversion of configuration at the C-4 position (D-Glc). The values of optimum pH and temperature were determined to be 6.0 and 80 °C, respectively (see Figure S2). All these parameters are compatible with biocatalyzed synthesis and encouraged us to probe the transglycosylation properties of Dtgly.

Transglycosylation properties of Dtgly

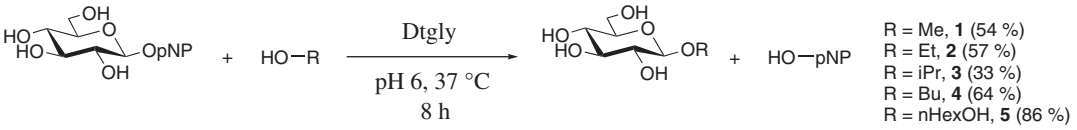
All the experiments were performed with the inexpensive pNP-Glc as a model substrate, at 37 °C and in a phosphate buffer pH 6.0, so (i) to limit the spontaneous hydrolysis of the donor, and (ii) more importantly, to insert this process in an eco-compatible approach. The transglycosylation is a kinetically-controlled reaction and in order to favour the formation of the product, i.e. the glycosides, enzymatic reactions were performed at high concentration of substrates: pNP-β-D-Glc as the donor (15 mM) and the alcohol as the acceptor (50 equiv., 750 mM). A series of alkyl chain lengths were tested in order to probe the synthetic potency of this biocatalyst (Scheme 1). The apparition of the alkyl glucosides **1–5** was followed by thin-layer chromatography and reactions were stopped after complete disappearance of the donor, generally after 8 h.

In an overall manner, all alcohols constitute moderate to good acceptors, yielding the alkyl glucosides from 33 % (**3**) to 86 % (**5**). As expected, primary alcohols (**1–2** and **4–5**) were more efficient than secondary ones (**3**). In principle, short chain alcohols are better nucleophiles for transglycosylation reactions than those having a long chain [11]. In total agreement with these previous observations, similar results were obtained when looking at methanol, ethanol and butanol (despite a biphasic mixture for this latter) as acceptors. The case of 1,6-hexanediol seems quite contradictory but can be easily explained by the increase polarity and thus solubility of the 1,6-hexanediol in the reaction mixture thus favouring the accessibility of the acceptor to the active site of Dtgly. The mono-glucosylated compound **5** was obtained due to the use of large excess of the alcohol acceptor.

Table 1: Kinetic parameters for the recombinant Dtgly toward the pNP sugars.

pNP sugar	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1} \cdot mM^{-1}$)
pNP β-D-Glc	0.46 ± 0.04	30.9 ± 0.7	66
pNP β-D-Gal	1.55 ± 0.13	65.0 ± 1.5	42
pNP β-D-Fuc	0.16 ± 0.03	28.3 ± 1.2	175
pNP β-D-Man	nd	nd	nd
pNP β-D-Xyl	nd	nd	nd

nd, Not determined.



Scheme 1: Biocatalyzed synthesis of alkyl glucosides **1–5**.

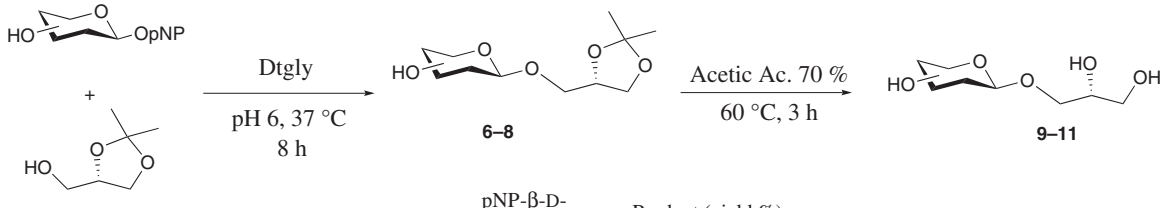
Chemo-enzymatic synthesis of 3-*O*-(β-D-glucopyranosyl)-*sn*-glycerols 9–11

As depicted in the introduction, GGs aroused an increasing interest due to their numerous biological properties, and especially in cosmetics, as moisturizing and anti-microbial agents for examples. However, their studies are still hampered by their poor availability both because of (i) their complex isolation from natural sources and (ii) their tedious chemical synthesis. In this study, we were thus interested in the chemo-enzymatic synthesis of a short series of 3-*O*-(β-D-glucopyranosyl)-*sn*-glycerol using our enzyme Dtgly following the easy and fast procedure developed for the transglycosylation reaction (Scheme 2). Thanks to the versatility of the enzyme at the donor site, we were expecting to be able to introduce a variety of different carbohydrate moieties.

Briefly, the different pNP-sugars were coupled with the commercially available (*S*)-solketal ((*S*)-1,2-*O*-isopropylidene-*sn*-glycerol) at 37 °C in a phosphate buffer pH 6.0 for 8 h. Reactions were firstly performed with the *rac*-solketal giving similar results but diastereoisomeric mixtures that were not separable in our hands (unpublished data). Surprisingly, pNP β-D-Fuc, the best substrate as far as the hydrolysis is concerned (Table 1), did not afford any product of glycosylation. On the contrary, Glc, Gal and even Xyl donors were able to afford us the corresponding 3-*O*-(β-D-glucopyranosyl)-*sn*-glycerols **6–8**, with 56 %, 73 % and 51 % of yields, respectively. The isopropylidene protecting groups were then easily removed by a treatment with 70 % acetic acid in aqueous solution at 60 °C for 3 h. The reaction was quantitative as visualized by thin-layer chromatography but the difficulty to purify such polar molecule decreased the isolated yields between 50 and 62 %. However, this straightforward biocatalyzed two-steps sequence provided us with 3-*O*-(β-D-glucosyl-, galactosyl- and xylosyl)-*sn*-glycerol **9–11** with an average 35 % overall yield. This chemo-enzymatic procedure represents a competitive and complementary alternative as previously reported multi-steps chemical or enzymatic synthesis [21–23]. In addition, the 3-*O*-(β-D-xylosyl)-*sn*-glycerol **11** is reported here for the first time to our knowledge.

Challenge test

Preservative agents are compounds that are able to kill or stop the growth of microorganisms over a long period of time. Therefore, they are largely utilized in the formulation of daily products for cosmetic to prevent microbial contaminations and guaranty the security of the consumer. The preservative potency of a molecule is assessed through the micro-challenge test method in accordance with the regulated challenge test defined by the European rules (EN ISO11930). In conformity, the anti-microbial effect was measured on five different species: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus brasiliensis* (Fig. 1). Surprisingly, among the four glycosides studied, only one (**9**) displayed a specific anti-fungus activity as shown in Fig. 1. Indeed, in comparison with the others products



pNP-β-D-sugars	Product (yield %)	
pNP β-D-Glc	**6** (56)	**9** (62)
pNP β-D-Gal	**7** (73)	**10** (50)
pNP β-D-Xyl	**8** (51)	**11** (62)

Scheme 2: Biocatalyzed synthesis of 3-*O*-(β-D-glucopyranosyl)-*sn*-glycerol **9–11**.

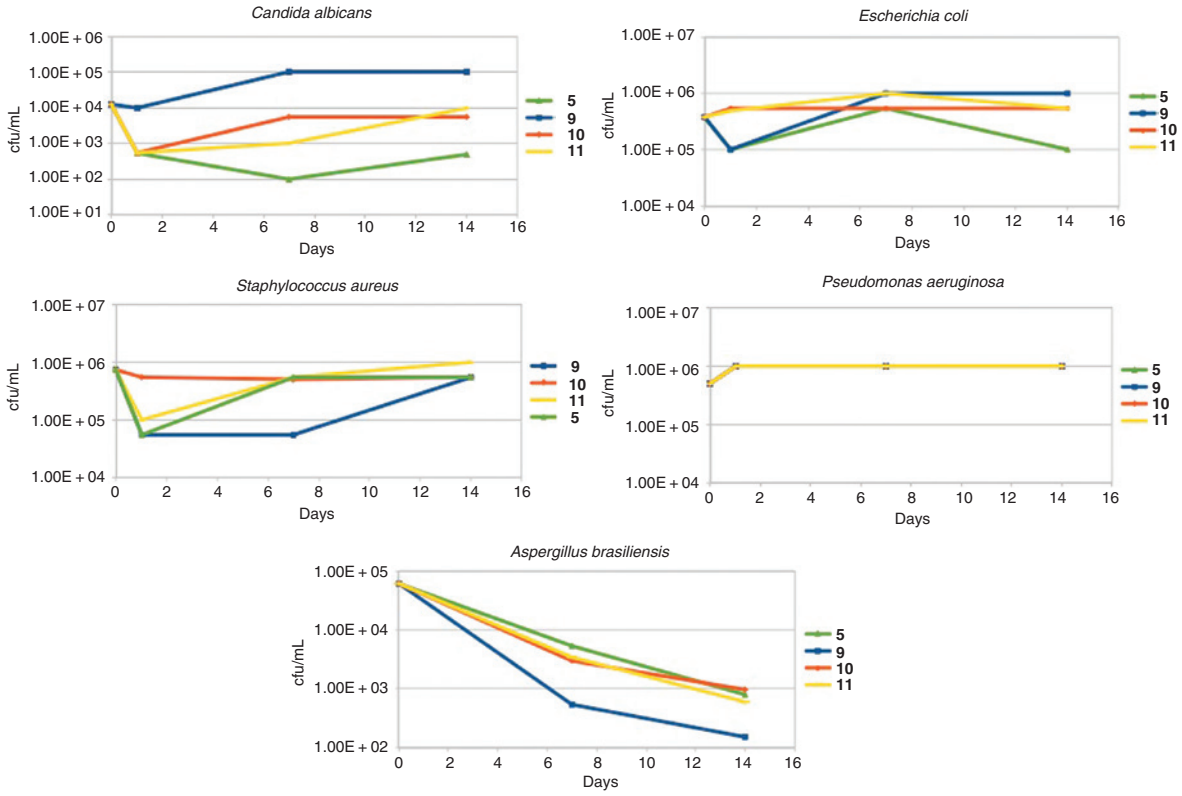


Fig. 1: Anti-microbial assays (compounds **5**, **9–11** were tested at 12.5 μg/mL).

tested, the 3-*O*-(β-D-glucosyl)-*sn*-glycerol **9** shown an interesting activity decreasing the fungus population of *A. brasiliensis* more than 2 log of magnitude (99 %) in 14 days and maintain its antimicrobial activity from the day 14–28 (data not shown), whereas the others products did not show this activity in the same time and display less anti-fungus activity at 7 days. Moreover, it is noteworthy that the simple replacement of the carbohydrate moiety (Glc) by another one (Gal or Xyl) can lead to a complete lost of the biological property. This might not be due to the physico-chemical properties of the three glycosyl (*S*)-glycerols **9–11** most likely similar, but to specific carbohydrate-lectin interactions at the surface of the microorganisms. To conclude, the compound **9** is of great interest because few compounds were identified exhibiting such a narrow anti-fungus activity. Works are currently under progress to increase this activity through the addition of fatty chains on the glycerol moiety as described previously [3, 9].

Conclusion

An original glycosidase Dtgly from *D. thermophilum* was cloned, overexpressed, purified and characterized towards its substrate specificity. It demonstrated to own efficient biocatalytic properties in term of transglycosylation and was ultimately used for the chemo-enzymatic synthesis of three enantiopure 3-*O*-(β-D-glycosyl)-*sn*-glycerols **9–11** with an overall yield of 35 %. Interestingly, in addition to their well-known moisturizing properties, the 3-*O*-(β-D-glucosyl)-*sn*-glycerol **9** proved to have also a specific anti-fungus activity. Due to the ample availability and low cost of pNP-sugars and solketal, and due the great versatility of this enzyme, this chemo-enzymatic procedure can be easily scaled up to allow the synthesis of numerous derivatives of GGs of potential biological interest.

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