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Characterization of chemo- and regioselectivity in enzyme-catalyzed consecutive hydrolytic deprotection of methyl acetyl derivatives of 1-β-*O*-acyl glucuronides

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ABSTRACT

Methyl acetyl derivatives of 1-β-*O*-(*o*-, *m*-, or *p*-phenyl)benzoyl glucuronides **2a**–**c** are fully deprotected by a one-pot consecutive enzyme-catalyzed hydrolytic reaction to afford **4a**–**c**, without isolation of the *O*-deacetylated derivatives **3a**–**c**. A lipase AS Amano from *Aspergillus niger* (LAS) and a carboxylesterase from *Streptomyces rochei* (CSR) showed high chemoselectivity toward the *O*-deacetylation of the *o*- and *m*-isomers, respectively. Chemoselective *O*-deacetylation of the *p*-isomer was promoted only in the presence of both enzymes. A lipase type B from *Candida antarctica* (CALB) was effective for the subsequent enzymatic hydrolysis of the methyl esters of **3a**–**c**. LAS exhibited also regioselective 3-*O*-deacetylation activity to afford the corresponding 2,4-di-*O*-acetyl intermediates **5a**–**c**, for which CSR showed higher *O*-deacetylation activity than that for **2a**–**c**. In kinetic studies using *p*-nitrophenyl ester substrates, LAS exhibited a broader acyl preference, the octanoyl ester being most effectively hydrolysed, whereas CSR exhibited the highest hydrolytic activity toward the acetyl ester. LAS and CSR play complementary as well as synergistic roles in the *O*-deacetylation of 2 bearing R groups of different steric bulkiness.

1. Introduction

It is important to understand pathways for the metabolic activation of drugs to reactive metabolites and subsequent irreversible covalent binding to target tissue macromolecules, as these processes are implicated in adverse drug reactions. Therefore, in discovery and development of safer drugs, efficient and widely applicable synthetic methodologies are required for preparing these reactive metabolites as standard samples and materials for toxicological and pharmacokinetic studies. 1-β-*O*-Acyl glucuronides (βGAs), a group of the above-mentioned reactive metabolites, are in general electrophilic species capable of binding to tissue proteins [1–4], possibly leading to adverse reaction of the parent carboxylic acid drugs [5–7]. For example, some non-steroidal anti-inflammatory drugs have been withdrawn due to their toxicities [8–10]. However, specific target macromolecules to which βGAs could covalently bind, toxicological consequences of the covalent binding, and relationships between the electrophilicity of βGAs and the toxicity of the parent carboxylic acid drugs remain largely unknown.

We have recently established a methodology for chemoenzymatic synthesis of βGAs with exclusive β-selectivity (Scheme 1) [11–13], and derived quantitative structure–activity relationships for the electrophilicity of their 1-β-*O*-acyl linkages to predict the electrophilic reactivity [13–15].

Among the enzymes screened under the conditions shown in Scheme 1, a lipase from *A. niger* (LAS) and a carboxylesterase from *S. rochei* (CSR) showed hydrolytic activity for the deprotection of the *O*-acetyl groups of the methyl acetyl precursor **2** to the corresponding methyl ester derivative **3**, whereas a lipase type B from *C. antarctica* (CALB) and an esterase from porcine liver (PLE) showed hydrolytic activity toward the methyl ester group of **3** to afford the desired βGA **4**. So far the chemoenzymatic method has been successfully applied to synthesis of 15 kinds of βGAs derived from six aromatic and nine aralkyl carboxylic acids [11–15]. LAS and CSR showed a good chemoselectivity toward the *O*-acetyl group among the three types of the ester functions of the compound **2**: the *O*-acetyl group, the 1-β-*O*-acyl linkage, and the carboxyl methyl ester. However, the structure of R group of 1-β-*O*-acyl linkage in a given compound **2** has been proved to considerably affect the chemoselectivity as well as the catalytic activity of these enzymes. For example, toward compounds **2** with phenyl and benzyl groups as the R group, LAS did not show good chemoselectivity toward the *O*-acetyl groups and unfavorably cleaved the 1-β-*O*-acyl linkage to liberate the parent benzoic and phenylacetic acids, respectively, whereas CSR showed much higher chemoselectivity toward these substrates to afford the corresponding fully *O*-deacetylated compounds **3** in good yields [12]. Furthermore, LAS exhibited high chemoselective *O*-deacetylation activity toward **2** with a bulky *o*-(anilino)phenyl group as the R group,

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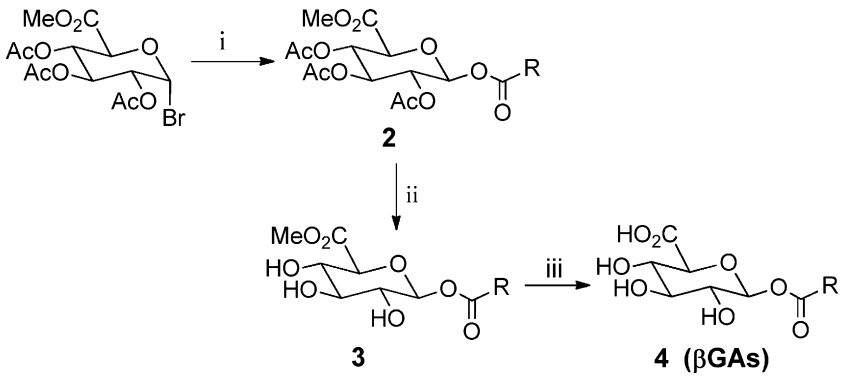
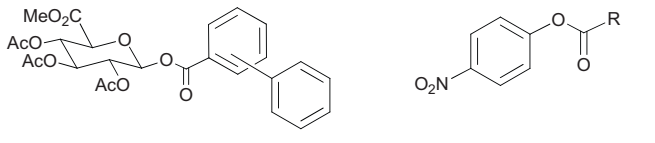


Scheme 1. Chemoenzymatic synthetic route to βGAs **4**. (i) RCO_2Cs, DMSO; (ii) LAS and/or CSR, 20% (v/v) DMSO in citrate buffer (pH 5.0); (iii) CALB, 20% (v/v) DMSO in citrate buffer (pH 5.0).

whereas CSR did not exhibit good hydrolytic activity toward this substrate [12]. In addition, only concurrent use of LAS and CSR synergistically showed good chemoselective *O*-deacetylation activities toward **2** with *p*-biphenyl and α,α-diethyl- and α,α-dimethyl-(*p*-phenyl)benzyl groups as the R group [12,13]. For these reasons, LAS and CSR are likely to play complementary and also synergistic roles in the hydrolytic deprotection of the *O*-acetyl group of **2** but the relationship between their hydrolytic activity and the structure of the R group has yet to be determined. Furthermore, LAS is different from CSR in that LAS is prone to afford some partially *O*-deacetylated intermediates in the reaction course.

There are several reviews for applications of hydrolytic enzymes to the regioselective *O*-deacylation of peracylated carbohydrates [16–19]. Among the enzymes, *A. niger* lipase has been reported to catalyze regioselective hydrolyses of anomeric *O*-acetyl esters of per-*O*-acetylated β-D-glucopyranose [20,21], β-D-ribofuranose [22] and oligosaccharides [23,24]. LAS-catalyzed chemoselective *O*-deacetylation of **2** to afford **3**, leaving the 1-β-*O*-acyl linkage intact, might strongly depend on the bulkiness of the R group of **2**. On the other hand, regioselective *O*-deacetylation activity has not been reported for CSR-catalyzed ester hydrolysis to the best of our knowledge.

It is, therefore, important and of interest to study the acyl preference and the influence of the steric effect of the R groups on LAS- and CSR-catalyzed chemo- and regioselective *O*-deacetylation of **2**, and thereby to gain insight into the enzymatic characteristics in the chemo- and regioselectivity. It has to be confirmed also the acyl preference with respect to the steric effect of R groups not yet available in the literature. Furthermore, a consecutive one-pot deprotection procedure from **2** to **4**, without isolation of **3**, seems to be an intriguing synthetic approach. Among the abovementioned 15 kinds of βGAs, the methyl acetyl derivatives of regioisomeric βGAs **2a**–**c** derived from *o*-, *m*- and *p*-phenylbenzoic acids **1a**–**c** (Scheme 2) have been selected as model substrates, because the compounds **2a**–**c** were *O*-deacetylated under different conditions using LAS, CSR, and concurrent use of LAS and CSR, respectively [12,13]. In this study, we report (1) the chemo- and regioselectivity of LAS and CSR toward **2a**–**c**, to characterize the effects of the R group, (2) acyl preferences in LAS- and CSR-catalyzed hydrolysis using several *p*-nitrophenyl esters **7a**–**e** (Scheme 2) by analysing the reaction kinetics, and (3) a one-pot deprotection procedure from **2a**–**c** to the corresponding 1-β-*O*-acyl glucuronides **4a**–**c**.



2a; *o*-isomer
2b; *m*-isomer
2c; *p*-isomer

7a; R = CH_3 **7d**; R = $(CH_2)_6CH_3$
7b; R = CH_2CH_3 **7e**; R = C_6H_5
7c; R = $(CH_2)_2CH_3$

Scheme 2. Substrates used for LAS- and CSR-catalyzed hydrolysis.

2. Experimental

2.1. General methods and materials

Lipases AS Amano from *A. niger* (LAS) and from *C. antarctica type B* (CALB), a carboxylesterase from *S. rochei* (CSR), and *o*-, *m*-, and *p*-phenylbenzoic acids were obtained from Wako Pure Chemical Industries (Osaka, Japan). Novozym 435 (an immobilized form of CALB) was purchased from MIK Pharmaceuticals Co., Ltd. (Tokyo, Japan) and Lipozyme® CALBL was kindly gifted from Novozymes Japan Ltd. (Chiba, Japan). The methyl acetyl derivatives **2a**–**c** were synthesized according to our previous papers [11–13]. *p*-Nitrophenyl esters **7a**, **7c**, and **7d** were obtained from Nacalai tesque Inc. (Kyoto, Japan); the propionyl [25] and benzoyl [26] esters **7b** and **7e** were synthesized according to reported procedures. Amberlite XAD-4 was obtained from the Organo Corporation (Tokyo, Japan) and used after grinding (80–200 mesh). All other chemicals were commercial products. ^1H and ^{13}C NMR were recorded using a JNM-AL400 (JEOL) and the chemical shifts are presented as δ-values in ppm with reference to the residual solvent signals [27] of d_6-DMSO (2.49 ppm for ^1H NMR and 39.50 ppm for ^{13}C NMR) or CD_3OD (3.30 ppm for ^1H NMR and 49.00 ppm for ^{13}C NMR). MS and HRMS were measured by electron impact (EI) ionization using a Hitachi M-2000 spectrometer.

2.1.1. General incubation conditions for enzymatic hydrolyses

The reaction mixtures were incubated at pH 5.0 to minimize a non-enzymatic intramolecular acyl migration of partially and fully *O*-deacetylated intermediates formed from **2a**–**c**. The progress of enzyme-catalyzed hydrolyses were monitored using HPLC. Unless otherwise indicated, the incubation was performed in 20 mM sodium citrate buffer (pH 5.0) containing 20% (v/v) DMSO as a co-solvent and at 40 ± 0.1 °C for LAS and CALB (including Lipozyme® CALBL and Novozym 435) and at 50 ± 0.1 °C for CSR, respectively. Among several cosolvents tested, DMSO was the best cosolvent for LAS and CSR using **2a** and **2b** as substrates, respectively. The concentration of LAS and CSR was 10 mg/mL of the incubation mixture. The incubation with both enzymes was performed at 40 ± 0.1 °C; the optimal temperature of LAS toward **2a** was 40 °C and the activity was gradually deactivated at 50 °C, whereas the catalytic efficiency of CSR was optimal at around 50 °C [12]. Incubation was started by the addition of substrate in DMSO solution to the incubation mixture. Hydrolytic activity of LAS and CSR toward **5a**–**c** was presented

as an initial rate of decrease in the substrate concentration. For the kinetic experiments on the hydrolysis of **7a**–**e**, kinetic parameters (K_m and V_{max}) were obtained from double-reciprocal plots of the initial rate data for LAS with **7a** and **7b** and for CSR with **7a**–**c**. For other *p*-nitrophenyl ester substrates, pseudo first-order rate constants were determined at low substrate concentrations. The rate of enzymatic hydrolysis of **7a**–**e** was corrected to account for non-enzymatic hydrolysis. Kinetic measurement of the hydrolysis of **3c**, with initial concentration of 0.1 mM, was performed using Lipozyme® CALBL, Novozyme 435, or CALB, at 100 μl of the enzyme solution, 25 mg of the immobilized enzyme, or 0.8 mg of the enzyme per mL of incubation mixture, respectively. The hydrolytic reaction obeys a pseudo-first order reaction and the rate constants obtained for Lipozyme® CALBL, Novozym 435, and CALB were 2.38 ± 0.02, 1.18 ± 0.10, and $3.81 \pm 0.01\ h^{-1}$, respectively. For preparative purposes, the initial concentrations of **2a**–**c** were in the range of 0.4–0.6 mM.

2.1.2. HPLC analysis of enzymatic hydrolyses

To analyse the time courses of the enzymatic hydrolyses of **2a**–**c**, **3a**–**c**, **5a**–**c**, and **7a**–**e**, at appropriate intervals, aliquots of the reaction mixture were diluted with HPLC mobile phase, to stop the enzymatic reaction. The HPLC samples were injected onto a Shimadzu HPLC that was equipped with a reversed-phase Symmetry C_{18} column (5 μm, 4.6 mm × 150 mm, Waters), a C-R8A Chromatopac data processor, a Shimadzu SPD-10A *VP* UV detector and a Shimadzu CTO-10AS *VP* column oven at 30 °C. The column was eluted with HPLC mobile phase at a flow rate of 0.7 mL/min with detection at 260 nm for **2a**–**c**, **3a**–**c**, and **5a**–**c** and 310 nm for **7a**–**e**, respectively. The mobile phase was aqueous CH_3CN containing 10 mM tetra-*n*-butylammonium bromide and 50 mM ammonium acetate buffer (pH 4.5) with variable concentrations (%, v/v) of CH_3CN; 35% for **3a**–**c**, 45% for **5a**–**c** and **7a**, 50% for **2a**, **7b**,**c**, and **7e**, 55% for **2b**,**c** and 70% for **7d**.

2.1.3. Computational chemistry

To obtain the optimized structures of **2a**–**c**, calculations were performed with a molecular mechanics model with MMFF, a semiempirical AM1 and PM3 models, and Hartree-Fock 3-21G and 6-31+G* models using Spartan '08 (Wave Function, Inc.).

2.2. Compound characterization

2.2.1. Methyl 1-O-(o-phenyl)benzoyl-β-D-glucopyranuronate (3a)

A solution of **2a** (14.1 mg, 27.4 μmol) in 68 mL of sodium citrate buffer (20 mM, pH 5.0) containing 20% (v/v) DMSO was incubated under stirring at 40 °C using LAS (680 mg). After 5 h, the conversion yield was 98%, as determined by HPLC analysis. The product was quantitatively extracted with EtOAc (50 mL × 3), and then EtOAc was evaporated in vacuo. The residue was dissolved in 4 mL of 20% (v/v) CH_3CN containing 0.01% (v/v) AcOH and applied to an Amberlite XAD-4 column (2.5 g, height 6 cm). Product **3a** was eluted with 30% (v/v) CH_3CN containing 0.01% AcOH. Yield (10.3 mg, 97%). ^1H NMR (400 MHz, CD_3OD) δ: 3.30 (dd, *J* 8.1 and 9.5 Hz, 1H, C_2-*H*), 3.44 (t, *J* 9.5 Hz, 1H, C_3-*H* or C_4-*H*), 3.53 (t, *J* 9.5 Hz, 1H, C_3-*H* or C_4-*H*), 3.78 (s, 3H, CO_2CH_3), 3.93 (d, *J* 9.5 Hz, 1H, C_5-*H*), 5.54 (d, *J* 8.1 Hz, 1H, C_1-*H*), 7.30–7.41 (m, 6H, aromatic *H*), 7.45 (dt, *J* 1.2 and 7.8 Hz, 1H, aromatic *H*), 7.60 (dt, *J* 1.2 and 7.8 Hz, 1H, aromatic *H*), 7.93 (dd, *J* 1.2 and 7.8 Hz, 1H, aromatic *H*). ^{13}C NMR (100 MHz, CD_3OD) δ: 52.9, 72.8 (C_4), 73.6 (C_2), 77.2 (C_5), 77.3 (C_3), 96.1 (C_1), 128.2, 128.3, 129.2, 129.8, 129.9, 131.1, 132.0, 133.0, 142.1, 144.5, 168.0, 170.7. HR MS (EI): calcd. for $C_{20}H_{20}O_8$ [M^+]: 388.1156; found 388.1140. MS (EI): *m*/*z* 388 (1%), 198 (100), 181 (98), 152 (46).

2.2.2. Methyl 1-O-(m-phenyl)benzoyl-β-D-glucopyranuronate (3b)

A solution of **2b** (15.7 mg, 30.5 μmol) in 51 mL of sodium citrate buffer (20 mM, pH 5.0) containing 20% (v/v) DMSO was incubated under stirring at 50 °C using CSR (510 mg). After 3 h, the conversion yield was 98%, as determined by HPLC analysis. The product was quantitatively extracted with EtOAc (50 mL × 3), and then EtOAc was evaporated in vacuo. The residue was dissolved in 4 mL of 20% (v/v) CH_3CN containing 0.01% (v/v) AcOH and applied to an Amberlite XAD-4 column (2.5 g, height 6 cm). Product **3b** was eluted with 30% (v/v) CH_3CN containing 0.01% AcOH. Yield (11.5 mg, 97%). ^1H NMR (400 MHz, CD_3OD) δ: 3.51–3.65 (m, 3H, C_2-*H*, C_3-*H*, and C_4-*H*), 3.76 (s, 3H, CO_2CH_3), 4.06 (d, *J* 9.5 Hz, 1H, C_5-*H*), 5.77 (d, *J* 7.8 Hz, 1H, C_1-*H*), 7.37 (t, *J* 7.6 Hz, 1H, aromatic *H*), 7.46 (t, *J* 7.1 Hz, 2H, aromatic *H*), 7.58 (t, *J* 7.8 Hz, 1H, aromatic *H*), 7.65–7.67 (m, 2H, aromatic *H*), 7.89–7.91 (m, 1H, aromatic *H*), 8.05–8.08 (m, 1H, aromatic *H*), 8.32 (t, *J* 1.5 Hz, 1H, aromatic *H*). ^{13}C NMR (100 MHz, CD_3OD) δ: 52.9, 73.0 (C_4), 73.7 (C_2), 77.3 (C_5), 77.4 (C_3), 96.3 (C_1), 128.1, 129.0, 129.3, 129.8, 130.1, 130.3, 131.1, 133.3, 141.2, 143.1, 166.5, 170.8. HR MS (EI): calcd. for $C_{20}H_{20}O_8$ [M^+]: 388.1156; found 388.1162. MS (EI): *m*/*z* 388 (0.2%), 198 (25), 181 (8), 152 (9), 105 (100).

2.2.3. Methyl 1-O-(p-phenyl)benzoyl-β-D-glucopyranuronate (3c)

A solution of **2c** (16.4 mg, 31.9 μmol) in 80 mL of sodium citrate buffer (20 mM, pH 5.0) containing 20% (v/v) DMSO was incubated under stirring at 40 °C using LAS (800 mg) and CSR (800 mg). After 2 h, the conversion yield was 95%, as determined by HPLC analysis. The product was quantitatively extracted with EtOAc (50 mL × 3), and then EtOAc was evaporated in vacuo. The residue was dissolved in 4 mL of 20% (v/v) CH_3CN containing 0.01% (v/v) AcOH and applied to an Amberlite XAD-4 column (2.5 g, height 6 cm). Product **3c** was eluted with 30% (v/v) CH_3CN containing 0.01% AcOH. Yield (11.7 mg, 95%). ^1H NMR (400 MHz, CD_3OD) δ: 3.51–3.65 (m, 3H, C_2-*H*, C_3-*H*, and C_4-*H*), 3.76 (s, 3H, CO_2CH_3), 4.06 (d, *J* 9.5 Hz, 1H, C_5-*H*), 5.77 (d, *J* 7.8 Hz, 1H, C_1-*H*), 7.38–7.42 (m, 1H, aromatic *H*), 7.45–7.49 (m, 2H, aromatic *H*), 7.67–7.70 (m, 2H, aromatic *H*), 7.76 (d, *J* 8.5 Hz, 2H, aromatic *H*), 8.17 (d, *J* 8.5 Hz, 2H, aromatic *H*). ^{13}C NMR (100 MHz, CD_3OD) δ: 52.9, 73.0 (C_4), 73.7 (C_2), 77.3 (C_5), 77.4 (C_3), 96.2 (C_1), 128.2, 128.3, 129.2, 129.4, 130.1, 131.6, 166.4, 170.8. HR MS (EI): calcd. for $C_{20}H_{20}O_8$ [M^+]: 388.1156; found 388.1128. MS (EI): *m*/*z* 388 (2%), 198 (100), 181 (82), 152 (40).

2.2.4. Methyl 1-O-(o-phenyl)benzoyl-2,4-di-O-acetyl-β-D-glucopyranuronate (5a)

A solution of **2a** (17.5 mg, 34.0 μmol) in 85 mL of sodium citrate buffer (20 mM, pH 5.0) containing 20% (v/v) DMSO was incubated under stirring at 40 °C using LAS (170 mg, 2.0 mg/mL of incubation mixture). After 0.5 h, the product **5a** was quantitatively extracted with EtOAc (50 mL × 2), and then EtOAc was evaporated in vacuo. The residue was dissolved in 4 mL of 40% (v/v) CH_3CN containing 0.01% (v/v) AcOH and applied to an Amberlite XAD-4 column (2.5 g, height 6 cm). Product **5a** was eluted with 50% (v/v) CH_3CN containing 0.01% AcOH. Yield (13.0 mg, 81%). ^1H NMR (400 MHz, d_6-DMSO) δ: 2.04 (s, 3H, $OCOCH_3$), 2.05 (s, 3H, $OCOCH_3$), 3.62 (s, 3H, CO_2CH_3), 3.96 (dt, *J* 6.1 and 9.5 Hz, 1H, C_3-*H*), 4.45 (d, *J* 9.5 Hz, 1H, C_5-*H*), 4.79 (t, *J* 9.5 Hz, 1H, C_4-*H*), 4.86 (dd, *J* 8.1 and 9.5 Hz, 1H, C_2-*H*), 5.86 (d, *J* 6.1 Hz, 1H, OH), 5.93 (d, *J* 8.1 Hz, 1H, C_1-*H*), 7.26 (dd, *J* 2.0 and 6.8 Hz, 2H, aromatic *H*), 7.36–7.41 (m, 3H, aromatic *H*), 7.44 (dd, *J* 1.2 and 7.8 Hz, 1H, aromatic *H*), 7.54 (dt, *J* 1.2 and 7.8 Hz, 1H, aromatic *H*), 7.68 (dt, *J* 1.2 and 7.8 Hz, 1H, aromatic *H*), 7.72 (dd, *J* 1.2 and 7.8 Hz, 1H, aromatic *H*). ^{13}C NMR (100 MHz, d_6-DMSO) δ: 20.5, 20.6, 52.4, 70.0 (C_3), 71.3 (C_4), 71.7 (C_5), 72.1 (C_2), 91.5 (C_1), 127.3, 127.6, 128.1, 128.3, 128.5, 129.3, 131.1, 132.4, 140.0, 142.4, 165.0, 167.4, 169.2, 169.5. HR MS (EI): calcd. for $C_{24}H_{24}O_{10}$ [M^+]:

472.1367; found 472.1348. MS (EI): *m*/*z* 472 (1%), 440 (3), 198 (28), 181 (100), 152 (18).

2.2.5. Methyl 1-O-(m-phenyl)benzoyl-2,4-di-O-acetyl-β-D-glucopyranuronate (**5b**)

A solution of **2b** (17.9 mg, 34.8 μmol) in 87 mL of sodium citrate buffer (20 mM, pH 5.0) containing 20% (v/v) DMSO was incubated under stirring at 40 °C using LAS (174 mg, 2.0 mg/mL of incubation mixture). After 17 min, the product **5b** was quantitatively extracted with EtOAc (50 mL × 2), and then EtOAc was evaporated in vacuo. The residue was dissolved in 4 mL of 40% (v/v) CH_3CN containing 0.01% (v/v) AcOH and applied to an Amberlite XAD-4 column (2.5 g, height 6 cm). Product **5b** was eluted with 50% (v/v) CH_3CN containing 0.01% AcOH. Yield (13.6 mg, 83%). ^{1}H NMR (400 MHz, d_6-DMSO) δ: 2.03 (s, 3H, $OCOCH_3$), 2.07 (s, 3H, $OCOCH_3$), 3.61 (s, 3H, CO_2CH_3), 4.06 (dt, *J* 5.9 and 9.5 Hz, 1H, C_3-*H*), 4.57 (d, *J* 9.5 Hz, 1H, C_5-*H*), 4.86 (t, *J* 9.5 Hz, 1H, C_4-*H*), 5.02 (dd, *J* 8.1 and 9.5 Hz, 1H, C_2-*H*), 5.91 (d, *J* 5.9 Hz, 1H, OH), 6.08 (d, *J* 8.1 Hz, 1H, C_1-*H*), 7.44 (t, *J* 7.1 Hz, 1H, aromatic *H*), 7.53 (t, *J* 7.1 Hz, 2H, aromatic *H*), 7.66–7.73 (m, 3H, aromatic *H*), 7.93 (d, *J* 7.8 Hz, 1H, aromatic *H*), 8.03 (d, *J* 7.8 Hz, 1H, aromatic *H*), 8.18 (t, *J* 1.5 Hz, 1H, aromatic *H*). ^{13}C NMR (100 MHz, d_6-DMSO) δ: 20.5, 20.6, 52.4, 69.7 (C_3), 71.4 (C_4), 71.8 (C_5), 72.2 (C_2), 91.9 (C_1), 126.8, 127.5, 128.1, 128.4, 128.8, 129.1, 129.8, 132.5, 138.8, 140.9, 163.8, 167.4, 169.5, 169.6. HR MS (EI): calcd. for $C_{24}H_{24}O_{10}$ [M^+]: 472.1367; found 472.1364. MS (EI): *m*/*z* 472 (7%), 440 (8), 198 (60), 181 (100), 152 (29).

2.2.6. Methyl 1-O-(p-phenyl)benzoyl-2,4-di-O-acetyl-β-D-glucopyranuronate (**5c**)

A solution of **2c** (19.8 mg, 38.5 μmol) in 96 mL of sodium citrate buffer (20 mM, pH 5.0) containing 20% (v/v) DMSO was incubated under stirring at 40 °C using LAS and CSR (each 960 mg). After 10 min, the product **5c** was quantitatively extracted with EtOAc (50 mL × 2), and then EtOAc was evaporated in vacuo. The residue was dispersed in 20 mL of 20% (v/v) CH_3CN containing 0.01% (v/v) AcOH and applied to an Amberlite XAD-4 column (2.5 g, height 6 cm). Elution was performed stepwise from 20, 30, 40 to 50% (v/v) CH_3CN containing 0.01% (v/v) AcOH. Product **5c** was eluted with 50% (v/v) CH_3CN containing 0.01% AcOH. Yield (9.5 mg, 52%). ^{1}H NMR (400 MHz, d_6-DMSO) δ: 2.03 (s, 3H, $OCOCH_3$), 2.07 (s, 3H, $OCOCH_3$), 3.51 (s, 3H, CO_2CH_3), 4.06 (t, *J* 9.3 Hz, 1H, C_3-*H*), 4.56 (d, *J* 9.3 Hz, 1H, C_5-*H*), 4.85 (t, *J* 9.3 Hz, 1H, C_4-*H*), 5.01 (t, *J* 8.5 Hz, 1H, C_2-*H*), 5.92 (brs, 1H, OH), 6.08 (d, *J* 8.1 Hz, 1H, C_1-*H*), 7.45 (t, *J* 6.8 Hz, 1H, aromatic *H*), 7.52 (t, *J* 7.8 Hz, 2H, aromatic *H*), 7.77 (d, *J* 8.1 Hz, 2H, aromatic *H*), 7.89 (d, *J* 8.3 Hz, 2H, aromatic *H*), 8.01 (d, *J* 8.1 Hz, 2H, aromatic *H*). ^{13}C NMR (100 MHz, d_6-DMSO) δ: 20.5, 20.6, 52.4, 69.8 (C_3), 71.4 (C_4), 71.8 (C_5), 72.2 (C_2), 91.7 (C_1), 126.8, 127.0, 127.2, 128.6, 129.1, 130.1, 138.5, 145.6, 163.8, 167.4, 169.4, 169.6. HR MS (EI): calcd. for $C_{24}H_{24}O_{10}$ [M^+]: 472.1367; found 472.1355. MS (EI): *m*/*z* 472 (4%), 440 (12), 198 (35), 181 (100), 152 (21)

2.2.7. Methyl 1-O-(p-phenyl)benzoyl-4-mono-O-acetyl-β-D-glucopyranuronate (**6c**)

In the fraction eluted with 40% (v/v) CH_3CN containing 0.01% (v/v) AcOH from the XAD-4 column applied the above-mentioned reaction mixture, another product **6c** was found. Yield (2.5 mg, 15%). ^{1}H NMR (400 MHz, CD_3OD) δ: 2.09 (s, 3H, $OCOCH_3$), 3.63–3.69 (m, 1H, C_2-*H*), 3.68 (s, 3H, CO_2CH_3), 3.79 (t, *J* 9.3 Hz, 1H, C_3-*H*), 4.27 (d, *J* 10.0 Hz, 1H, C_5-*H*), 4.92 (t, *J* 9.5 Hz, 1H, C_4-*H*), 5.81 (d, *J* 8.1 Hz, 1H, C_1-*H*), 7.38–7.41 (m, 1H, aromatic *H*), 7.47 (t, *J* 7.5 Hz, 2H, aromatic *H*), 7.66–7.69 (m, 2H, aromatic *H*), 7.77 (d, *J* 8.5 Hz, 2H, aromatic *H*), 8.17 (d, *J* 8.5 Hz, 2H, aromatic *H*). ^{13}C NMR (100 MHz, CD_3OD) δ: 20.7, 53.2, 73.1 (C_4), 73.7 (C_2), 74.1 (C_5), 74.9 (C_3), 95.7 (C_1), 128.2, 128.3, 129.1, 129.5, 130.1, 131.7, 141.0, 147.9, 166.3, 169.8, 172.0. HR MS (EI): calcd. for $C_{22}H_{22}O_9$ [M^+]: 430.1263; found 430.1273. MS (EI): *m*/*z* 430 (7%), 198 (98), 181 (100), 152 (54).

2.2.8. One-pot synthesis of 1-O-(o-phenyl)benzoyl-β-D-glucopyranuronate (**4a**)

A solution of **2a** (16.3 mg, 31.6 μmol) in 60 mL of sodium citrate buffer (20 mM, pH 5.0) containing 20% (v/v) DMSO was incubated under stirring at 40 °C using LAS (600 mg). After 5 h, CALB (60 mg in 1 mL of water) was added at one portion and incubated for further 1.0 h. The reaction mixture was then acidified with 2 M HCl to adjust pH to about 2.0. After filtration, the filtrate was applied to an Amberlite XAD-4 column (2.5 g, height 6 cm). After washing with 50 mL of 0.01% aqueous AcOH and then 10%(v/v) CH_3CN containing 0.01% AcOH, product **4a** was eluted with 20% (v/v) CH_3CN containing 0.01% AcOH. Yield (11.3 mg, overall yield of 95%). The HPLC retention time and the NMR data were identical with those of previously synthesised **4a** with a two-step procedure [13].

2.2.9. One-pot synthesis of 1-O-(m-phenyl)benzoyl-β-D-glucopyranuronate (**4b**)

A solution of **2a** (15.7 mg, 30.5 μmol) in 60 mL of sodium citrate buffer (20 mM, pH 5.0) containing 20% (v/v) DMSO was incubated under stirring at 50 °C using CSR (600 mg). After 3.5 h, CALB (60 mg in 1 mL of water) was added at one portion and incubated for further 1.0 h. The reaction mixture was then acidified with 2 M HCl to adjust pH to about 2.0. After filtration, the filtrate was applied to an Amberlite XAD-4 column (2.5 g, height 6 cm). After washing with 50 mL of 0.01% aqueous AcOH and then 10% (v/v) CH_3CN containing 0.01% AcOH, product **4b** was eluted with 25% (v/v) CH_3CN containing 0.01% AcOH. Yield (10.6 mg, overall yield of 93%). The HPLC retention time and the NMR data were identical with those of previously synthesised **4b** with a two-step procedure [13].

2.2.10. One-pot synthesis of 1-O-(p-phenyl)benzoyl-β-D-glucopyranuronate (**4c**)

A solution of **2c** (26.0 mg, 50.5 μmol) in 100 mL of sodium citrate buffer (20 mM, pH 5.0) containing 20% (v/v) DMSO was incubated under stirring at 40 °C using CSR (1.00 g) and LAS (1.00 g). After 2.5 h, the reaction mixture was heated in a boiling water to keep temperature of the reaction mixture at least more than 70 °C for 30 s to deactivate LAS. CALB (100 mg in 2 mL of water) was then added at one portion and incubated for further 1.5 h. The reaction mixture was then acidified with 2 M HCl to adjust pH to about 2.0. After filtration, the filtrate was applied to an Amberlite XAD-4 column (2.5 g, height 6 cm). After washing with 50 mL of 0.01% aqueous AcOH and then 10%(v/v) CH_3CN containing 0.01% AcOH, product **4c** was eluted with 25% (v/v) CH_3CN containing 0.01% AcOH. Yield (17.4 mg, overall yield of 92%). The HPLC retention time and the NMR data were identical with those of previously synthesised **4c** with a two-step procedure [12].

3. Results and discussion

3.1. Chemoselectivity of LAS and CSR in hydrolysis of **2a–c**

The compounds **2a–c**, from which the corresponding 1-β-*O*-acyl glucuronides **4a–c** had been synthesized previously with exclusive β-selectivity [12,13], were selected as the model compounds to examine in detail the chemo- and regioselectivity of LAS and CSR in the enzymatic *O*-deacetylation.

As shown in Table 1, both the enzymes exhibited different hydrolytic activities toward substrates **2a–c**; the chemoselective *O*-deacetylation of **2a–c** depended significantly on the position of the phenyl substituent. Fig. 1 shows the typical time courses of the

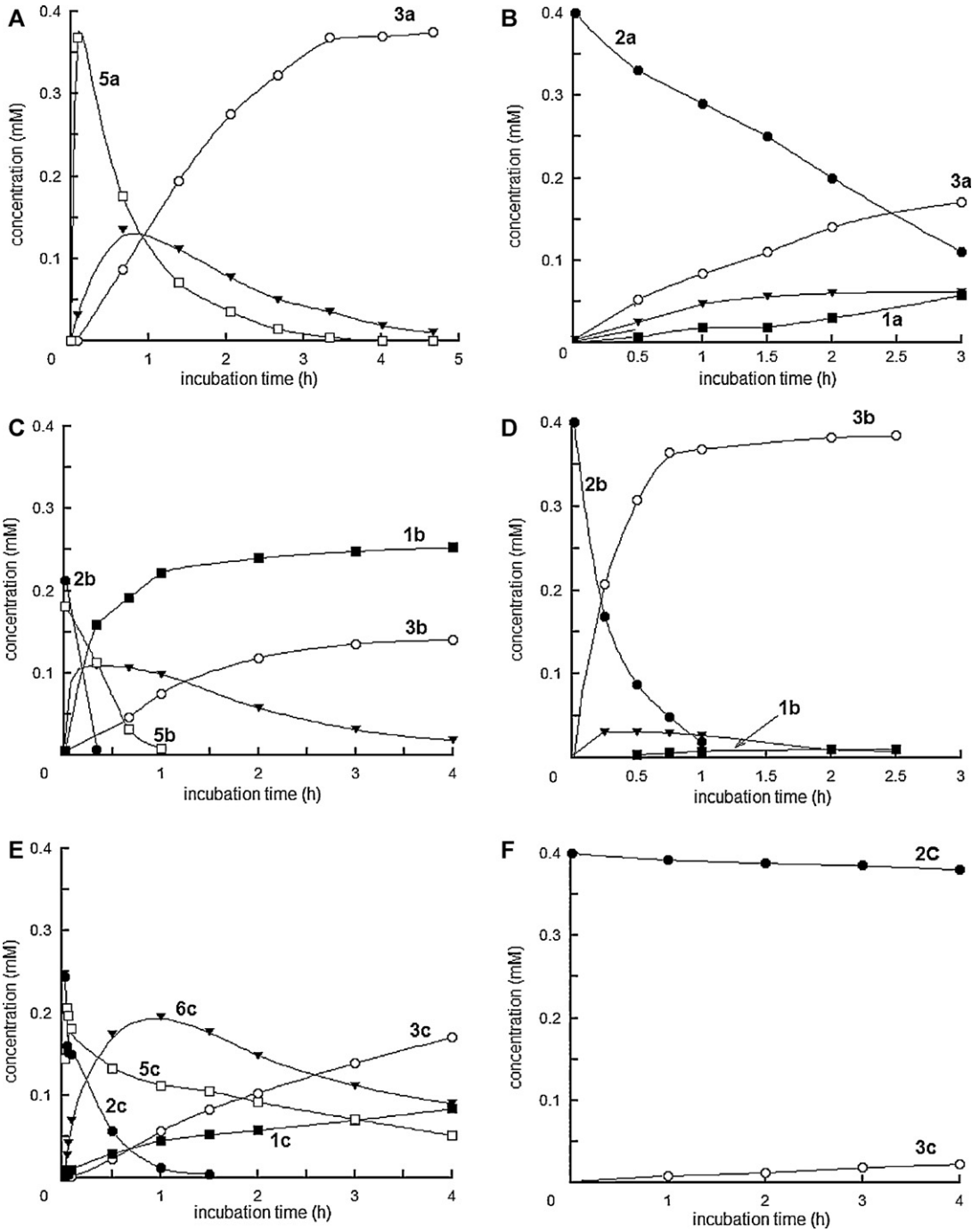


Fig. 1. Time courses of LAS- and CSR-catalyzed hydrolytic reactions of **2a**–**c**. (A) LAS toward **2a**; (B) CSR toward **2a**; (C) LAS toward **2b**; (D) CSR toward **2b**; (E) LAS toward **2c**; (F) CSR toward **2c**. Symbols are the concentrations of liberated parent carboxylic acids **1a**–**c** (■), substrates **2a**–**c** (●), fully *O*-deacetylated derivatives **3a**–**c** (○), 2,4-di-*O*-acetyl derivatives **5a**–**c** (□), and mono-*O*-acetyl derivatives (▼). Initial concentration of substrates was 0.4 mM. Enzyme concentration was 10 mg/mL of incubation mixture.

enzymatic *O*-deacetylation of **2a**–**c**. LAS and CSR exhibited the highest chemoselective *O*-deacetylation activity toward **2a** (Fig. 1A) and **2b** (Fig. 1D) to yield **3a** and **3b** in 97% yield in 5 h and in 97% yield in 3 h, respectively. The chemoselectivity worsened in the opposite combination (Fig. 1B and C). With LAS, **2b** liberated the parent carboxylic acid **1b**, other than the desired product **3b**, as a major product in 55% yield in 4 h without time-lag (Fig. 1C), indicating that LAS hydrolysed not only the *O*-acetyl groups but also 1-β-*O*-acyl ester linkage of **2b**. As shown in Fig. 1E with LAS, **2c** also liberated the parent carboxylic acid **1c** with accumulation of partially *O*-deacetylated intermediates (2,4-di-*O*-acetylated and 4-mono-*O*-acetylated compounds **5c** and **6c**, respectively); the structural assignment of **5c** and **6c** is described later in this paper. With CSR, **2a** was hydrolysed to **3a** (45% yield in 3 h) but with concomitant formation of a measurable amount of **1a** (Fig. 1B), whereas **2c** was hardly hydrolysed to afford only **3c** in 4% yield in 4 h (Fig. 1F).

The chemoselective *O*-deacetylation of **2c** was satisfactory, in a synergistic manner, only by concurrent use of LAS and CSR; **3c** was obtained in 95% yield in 2 h (Table 1 and Fig. 2A). Fig. 2B clearly shows the inhibition of CSR-catalyzed *O*-deacetylation of **2b** by the presence of **2c**, albeit a poor substrate for CSR as shown in Fig. 1F. This result indicates that **2c** could occupy the binding site(s) of CSR but the *p*-phenyl substituent might force **2c** into an unfavorable orientation for the enzymatic hydrolysis in the binding site.

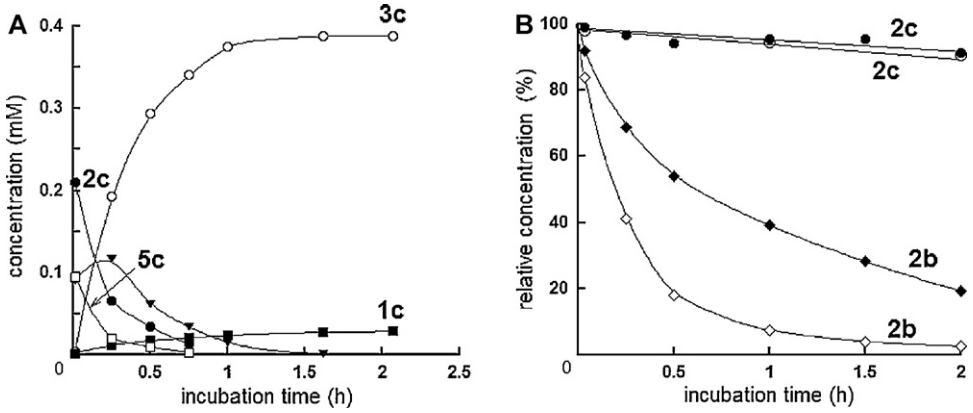


Fig. 2. Time courses of (A) chemoenzymatic *O*-deacetylation of **2c** (C_0 0.4 mM) with concurrent use of LAS and CSR and (B) CSR-catalyzed hydrolysis of **2b** (C_0 0.2 mM) in the absence and presence of **2c** (C_0 0.2 mM). Symbols in (A) were the concentrations of **1c** (■), **2c** (●), **3c** (○), and **5c** (□). Symbols in (B) were the concentrations of **2b** in the absence (◊) and presence (♦) of **2c** and **2c** in the absence (○) and presence (●) of **2b**. Enzyme concentration was 10 mg/mL of incubation mixture.

Table 1
Yields of the major products obtained from LAS- and CSR-catalyzed hydrolytic reactions of **2a–c**.

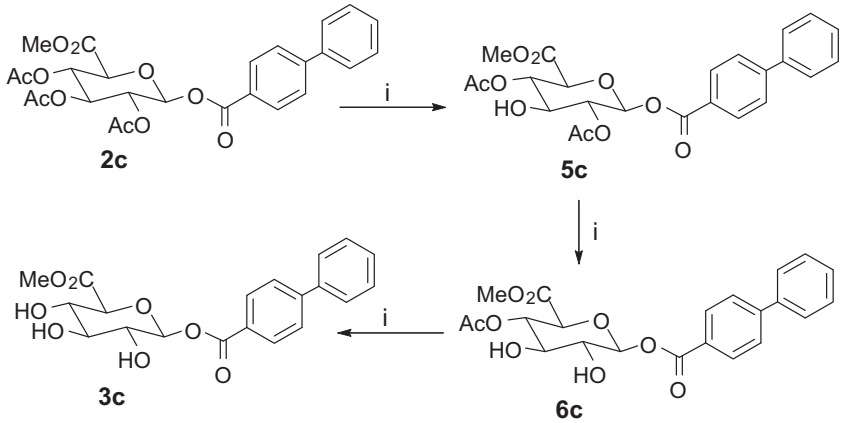
Substrate	Enzyme	Time (h)	Products and yield (%)
2a	LAS[a]	5	**3a**[c] (97%)
	CSR[a]	3	**3a**[c] (45%) and **1a**[d] (15%)
	LAS[a] + CSR[a]	3	**3a**[c] (81%) and **1a**[d] (16%)
	LAS[b]	0.5	**5a**[e] (81%)
2b	LAS[a]	4	**3b**[c] (35%) and **1b**[d] (55%)
	CSR[a]	3	**3b**[c] (97%)
	LAS[a] + CSR[a]	2	**3b**[c] (79%) and **1b**[d] (19%)
	LAS[b]	0.3	**5b**[e] (83%)
2c	LAS[a]	4	**3c**[c] (50%), **1c**[d] (23%) and **6c**[f] (23%)
	CSR[a]	4	**3c**[c] (4%)
	LAS[a] + CSR[a]	2	**3c**[c] (95%)
	LAS[a] + CSR[a]	0.2	**5c**[e] (52%) and **6c**[f] (15%)

[a] Enzymes was used at concentration of 10 mg/mL of incubation mixture.
[b] LAS was used at concentration of 2.0 mg/mL of incubation mixture.
[c] **3a–c** are the corresponding fully *O*-deacetylated compounds derived from **2a–c**.
[d] **1a–c** are the corresponding carboxylic acids, namely, *o*-, *m*-, and *p*-phenylbenzoic acids, respectively.
[e] **5a–c** are the 2,4-di-*O*-acetyl compounds derived from **2a–c**.
[f] **6c** is the 4-mono-*O*-acetyl compound derived from **2c**.

3.2. Regioselectivity of LAS in O-deacetylation of *2a–c*

In LAS-catalyzed *O*-deacetylation of **2a–c**, as mentioned above for **2c**, partially *O*-deacetylated intermediates **5a–c** and **6c** were as well detected. To investigate the regioselectivity of LAS-catalyzed *O*-deacetylation of **2a–c**, the major intermediates **5a–c** that accumulated in the early stage of the hydrolytic reactions were isolated and their structures were characterized. For the isolation of maximum amounts of **5a** and **5b** by retarding the reaction velocities, the concentrations of LAS and CSR, respectively, were reduced to 2 mg/mL of incubation mixture. ^{1}H and ^{13}C NMR spectra of **5a–c** indicate four carbonyl carbons and two acetyl methyl carbons with attached hydrogens. These results, together with MS data, indicate that the compounds **5a–c** are di-*O*-acetyl derivatives of **3a–c**. In the ^{1}H NMR spectra of **5a–c**, signals at δ ca. 5.9, exchangeable with D_2O, were assigned to the proton of the OH group at the 3-position, as determined from the ^{1}H-^{1}H COSY spectra (data not shown), in which the protons of C_1-H through C_5-H on the pyranose ring could be easily assigned. Comparing the C_1-H through C_5-H of **5a–c** with those of **2a–c**, the upfield shift observed for C_3-H of **5a–c** could be due to 3-*O*-deacetylation. In addition, large downfield shifts (1.3–1.5 ppm) were observed for both C_2-H and C_4-H of **5a–c**, compared with those of **4a–c**; this downfield shifts [28,29] also support the 2,4-di-*O*-acetyl structures. The structures are also supported by the downfield shifts (2.5–2.7 ppm) of C_2 and C_4 observed for **5a–c**, a typical deacetylation shift [30] at 3-*O*-acetyl group of **2a–c**. The isolation yields of **5a**, **5b** and **5c** were 81, 83 and 52%, respectively. In the isolation of **5c**, a more polar product **6c** (15%) was also obtained. **6c** was confirmed to be 4-mono-*O*-acetyl derivative of **4c** by comparing its spectral data with those of **2c** and **5c**, and by analysing the ^{1}H-^{1}H COSY spectrum of **6c** (data not shown). A major pathway for the enzymatic *O*-deacetylation of **2c** to **4c**, through major intermediates **5c** and **6c**, is shown in Scheme 3.

All the enzymatic hydrolyses in this study were performed at pH 5.0 to minimize a non-enzymatic intramolecular acyl migration [31,32] of **3a–c** and **5a–c** as well as other interme-



Scheme 3. Chemo- and regioselectivity in LAS-catalyzed *O*-deacetylation of **2c** to **3c** through intermediates **5c** and **6c**. (i) LAS, 20% (v/v) DMSO in citrate buffer (pH 5.0).

diates formed. The half-life times of 2,4-di-*O*-acetyl derivatives **5a**–**c** under the conditions without enzyme at 40 °C were over 13 h (data not shown), strongly indicating that these compounds are directly formed through LAS-catalyzed regioselective 3-*O*-deacetylation of **2a**–**c**. In literature, regioselective 2-*O*-deacetylation has been reported for methyl and benzyl 2,3,4-tri-*O*-acetyl-β-D-galactopyranosiduronates catalyzed by acylase I [33]. In addition to enzymatic regioselective *O*-deacetylation of primary ester of fully *O*-acetylated sugars [34–36] and glycosides [37–41], regioselective *O*-deacetylation has been reported for 3-*O*-deacetylation of secondary esters of methyl and 1-thiomethyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosides by a lipase from *A. niger* [42,43] and a cellulose acetate esterase from *Neisseria sicca* SB [44], 2-, 3- or 5-*O*-deacetylation of methyl 2,3,5-tri-*O*-acetyl-D-arabinofuranoside by several enzymes [45], and 2- or 3-*O*-deacetylation of fully *O*-acetylated adenosine derivative by an esterase from porcine liver [46]. In these reports, regioselective hydrolysis of the secondary *O*-acetyl ester has been observed for substrates without anomeric ester group. To our knowledge, our finding would be the first report for LAS-catalyzed regioselective 3-*O*-deacetylation toward the methyl acetyl derivatives of 1-β-*O*-acyl glucuronides **2a**–**c**.

3.3. Hydrolytic activity of LAS and CSR toward *2a–c* and *5a–c*

As described above, no significant accumulation of partially *O*-deacetylated intermediates was observed in CSR-catalyzed *O*-deacetylation of **2a**–**c** to **3a**–**c**. This result indicates that CSR could hydrolyse partially *O*-deacetylated intermediates such as **5a**–**c** much more effectively than the initial substrates **2a**–**c**. To examine the possibility, initial velocities of hydrolytic reactions catalyzed by CSR as well as LAS were determined using **2a**–**c** and **5a**–**c** as substrates. Table 2 summarizes the initial velocities of LAS- and CSR-catalyzed decrease in the concentration of the substrates **2a**–**c** and **5a**–**c**.

LAS exhibited higher hydrolytic activity toward **2a**–**c** than the 2,4-di-*O*-acetyl intermediates **5a**–**c**. Conversely, CSR exhibited much higher catalytic activity toward **5a**–**c** than **2a**–**c**, as expected. This result explains the reason because accumulation of **5a**–**c** was hardly observed during CSR-catalyzed *O*-deacetylation of **2a**–**c**. In the case of **2c**, the rate of LAS-catalyzed *O*-deacetylation of **2c** was shown to dramatically decrease due probably to increasing concentrations of the intermediates **5c** and **6c** (see Fig. 1E), indicating a competitive inhibition of enzyme activity by poorer substrates **5c** and **6c**. The synergistic acceleration of the *O*-deacetylation of **2c** to **3c** with concurrent use of LAS and CSR was, therefore, reasonably explained by thinking that the intermediates **5c** (and probably **6c**) could be effectively hydrolysed to the product **3c** by CSR, whose initial rate toward **5c** was approximately 15-fold higher than that of LAS (Table 2).

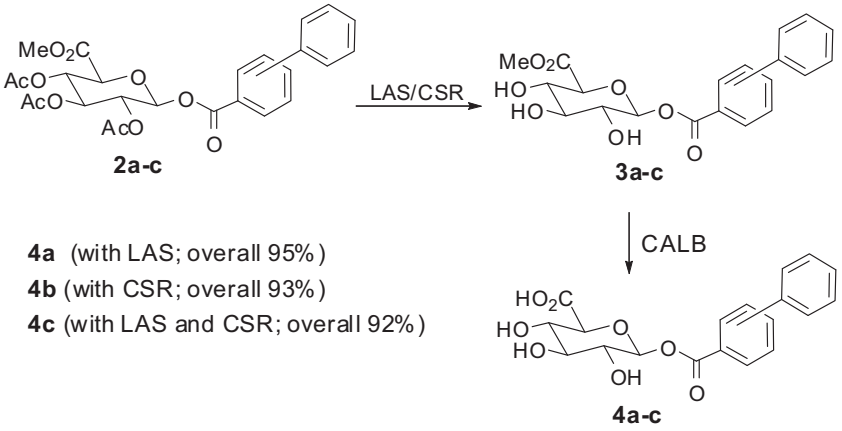
Table 2
Comparison of hydrolytic activities of LAS and CSR toward **2a**–**c** and **5a**–**c**.

Substrate	Initial velocity[a]	
	With LAS[b]	With CSR[d]
2a	11.7 ± 0.3	0.62 ± 0.02
2b	25.0 ± 0.2	1.9 ± 0.1
2c	22.4 ± 0.2	0.016 ± 0.002
5a	0.40 ± 0.01	205 ± 3
5b	1.5 ± 0.1	365 ± 3
5c	0.28 ± 0.05	4.3 ± 0.1

[a] Values were represented as mM/h at the initial concentrations of substrates (0.4 mM).
[b] The concentration of the enzyme (LAS or CSR) was 10 mg/mL of incubation mixture.

3.4. One-pot procedure for enzymatic deprotection of *2a–c* to *4a–c*

To reduce the time and costs, a one-pot procedure for the enzyme-catalyzed consecutive hydrolytic deprotection of **2a**–**c** to **4a**–**c**, without isolation of **3a**–**c**, was next examined. Because the hydrolysis of the methyl ester of **2** prior to the *O*-deaceylation did not give a good result [11] and CALB showed higher chemoselective hydrolytic activity than PLE toward the methyl ester [12], we tried a one-pot procedure using LAS and/or CSR as the enzyme(s) for the first step of *O*-deacetylation followed by addition of CALB as the enzyme for the second step of methyl ester hydrolysis. Therefore, a prerequisite for this one-pot procedure is that the enzyme(s) used for the first *O*-deacetylation step shows no hydrolytic activity toward the final products **4a**–**c**. As shown in Scheme 4, **2a** and **2b** were successfully converted to **4a** and **4b** in good yields (95 and 93%, respectively) using LAS and CSR as the first enzyme, respectively. LAS and CSR did not show significant hydrolytic activity toward **4a** and **4b**, respectively. In the case of **2c**, because LAS, but not CSR, showed hydrolytic activity toward **4c** with a half-life of ca. 1.2 h under the incubation conditions (data not shown), the reaction mixture of **2c** with both LAS and CSR was heated at least more than 70 °C for 30 sec to deactivate LAS before the addition of CALB; in consequence **4c** was obtained in overall yield of 92%. Besides CALB, the hydrolytic activity of commercially available lipases of Lipozyme® CALBL and Novozym 435 was also examined using **3c** as a model substrate. The hydrolysis of the methyl ester of **3c** smoothly the proceeded according to first-order reaction kinetics



Scheme 4. One-pot synthetic procedure from **2a**–**c** to **4a**–**c**.

Table 3
Kinetic parameters obtained for LAS- and CSR-catalyzed hydrolysis of **7a**–**e**.

Ester	With LAS			With CSR		
	V_{max}[a]	K_m[b]	V_{max}/K_m	V_{max}[a]	K_m[b]	V_{max}/K_m
7a	1.2	4.2	0.29	0.76	0.12	6.3
7b	2.0	11.5	0.17	0.19	0.62	0.31
7c	–[c]	–[c]	0.27	0.021	3.45	0.0061
7d	–[c]	–[c]	4.2[d]	–[c]	–[c]	0.0003[d]
7e	–[c]	–[c]	0.0093[d]	–[c]	–[c]	<0.0001[d]

[a] Expressed as μmol of liberated *p*-nitrophenol/min/mg of enzyme.
[b] mM.
[c] Values not determined.
[d] Values from the apparent first-order rate constants.

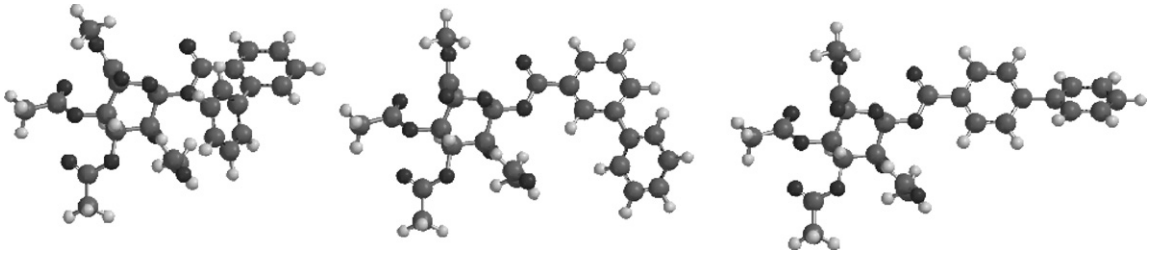


Fig. 3. Optimized structures of **2a**–**c** calculated by the Hartree-Fock 6-31 + G* models.

and the desired product **4c** was obtained in good yields (more than 98%) with chemoselectivity comparable with that of CALB. On the basis of the rate constants, the hydrolytic activities of 1 mL of the enzyme solution of Lipozyme® CALBL and 1 g of the Novozym 435 were estimated to be equivalent to that of 6.2 and 12.3 mg of CALB, respectively.

3.5. Acyl preference of LAS- and CSR-catalyzed hydrolysis of *7a*–*e*

Although *A. niger* lipase generally cleaved anomeric acetyl esters of fully *O*-acetylated sugars regioselectively [20–24], to our knowledge there have been no reports on acyl preference of LAS and CSR-catalyzed ester hydrolytic reaction. Since acyl preferences of LAS and CSR are thought to be an important factor [13] determining the chemoselectivity toward **2**, acyl preferences were investigated using *p*-nitrophenyl esters **7a**–**e** (shown in Scheme 2).

The enzymatic hydrolysis of **7a**–**e** was performed under the same reaction conditions used for the substrates **2a**–**c**, and the liberated *p*-nitrophenol was determined by HPLC. K_m and V_{max} values of LAS and CSR were obtained for **7a**–**b** and **7a**–**c**, respectively. Since the solubility of substrates (**7c**–**e** with LAS and **7d**–**e** with CSR) in the incubation mixture was too low to obtain the K_m and V_{max} values, apparent first-order rate constants (alternatives to V_{max}/K_m values) were determined at a low substrate concentration. The obtained kinetic parameters are listed in Table 3.

LAS exhibited hydrolytic activity toward all substrates **7a**–**e** with the medium-chain fatty acid ester **7d** being the most efficiently hydrolysed, as derived from the V_{max}/K_m values. The finding that LAS has a broad acyl preference including the benzoyl group (Table 3) might in part explain the low chemoselective *O*-deacetylation of **2** bearing phenyl and benzyl moieties as R groups [12] as well as the *m*- and *p*-isomers **2b** and **2c** in this study. Conversely, the finding that LAS showed high chemoselective *O*-deacetylation only toward the *o*-phenyl isomer **2a**, together with the findings [11,12] that LAS also showed high chemoselective *O*-deacetylation toward **2** with R groups such as *o*-(anilino)phenyl and *o*-(2,6-dichloroanilino)benzyl groups, indicates that sterically bulky R groups, especially as *ortho* substituents, might prevent the 1-β-*O*-acyl linkage from LAS-catalyzed hydrolysis. The poor chemoselectivity for **2b** and **2c** might be rationalized by considering that LAS has a binding site large enough to be occupied by **2b** and **2c** and a catalytic activity toward the 1-β-*O*- acyl linkages. In comparison with LAS, CSR showed a narrower acyl preference (Table 3), the acetyl ester **7a** being most efficiently catalyzed. The K_m value of CSR for **7a** was much lower than the value of LAS and the V_{max}/K_m value of CSR for **7a** was much higher than that of LAS. Furthermore, the V_{max}/K_m values of CSR for other substrates decreased with increasing numbers of methylene chains of **7b**–**d**, probably due to the increasing steric bulkiness of the acyl groups. The finding that CSR hardly hydrolysed benzoyl ester **7e** suggests that the binding site of CSR should be narrower than that of LAS. This might in part explain the high chemoselectivity of CSR in the *O*-deacetylation of **2** bearing, as R substituents, phenyl and benzyl groups in the previous paper [17], or *m*-(phenyl)benzoyl group **2b** in this study; however, the reasons for the low chemoselective *O*-deacetylation activity toward **2a** and the extremely low *O*-deacetylation activity toward **2c** remain unexplained.

Fig. 3 shows the optimized structures of **2a**–**c** calculated with Hartree-Fock 6-31 + G* model. Similar structures [15] were also obtained with other models used (data not shown). As expected, the *o*-phenyl substituent of **2a**, in contrast with **2b** and **2c**, covered the 1-β-*O*-acyl linkage; the *o*-phenyl substituent might consequently prevent access to the catalytic sites and protect the linkage from LAS- and CSR-catalyzed hydrolysis. Because LAS showed hydrolytic activity toward a medium-chain fatty acid ester (**7d**), the 1-β-*O*-acyl linkages of **2b** and **2c** also might be oriented to the catalytic site of LAS and be subsequently hydrolysed, resulting in a lowered chemoselectivity of LAS toward **2b** and **2c**. On the other hand, due to the hypothesized binding site of CSR, the 1-β-*O*-acyl linkages of **2b** and **2c** could neither be oriented to the catalytic site of CSR nor be hydrolysed by CSR.

4. Conclusion

We investigated LAS- and CSR-catalyzed chemoselective and regioselective *O*-deacetylation of **2a**–**c**. The position of the phenyl substituent of **2a**–**c** strongly affected the chemoselectivity; high chemoselectivity of LAS and CSR was observed toward *ortho*-isomer **2a** and *meta*-isomer **2b**, respectively. The chemoselectivity worsened in the opposite combination. These data indicate that LAS and

CSR play complementary roles in chemoselective *O*-deacetylation of **2** bearing R groups of different steric bulkiness. Based on the catalytic efficiency of the enzymes toward *p*-nitrophenyl esters **7a**–**e**, LAS was shown to have a broad acyl preference that was sufficient to hydrolyse the benzoyl ester **7e**. In contrast, CSR was shown to have a smaller binding site that was almost completely limited to the acetyl ester **7a**. These characteristics, together with the computationally optimized structures, could largely explain the differences observed in the chemoselective *O*-deacetylation of **2a**–**c**. LAS, but not CSR, exhibited regioselective 3-*O*-deacetylation activity toward **2a**–**c** to yield the corresponding 2,4-di-*O*-acetyl derivatives **5a**–**c**, among which **5a** and **5b** were obtained in good yields. CSR, differing from LAS, hydrolysed **5a**–**c** much more effectively than **2a**–**c**. A one-pot procedure for the synthesis of βGAs **4a**–**c** from **2a**–**c** were performed with LAS and/or CSR for the deprotection of the *O*-acetyl group followed by CALB for the deprotection of the carboxyl methyl ester. Application of these enzymes to hydrolytic deprotection for the synthesis of other glycosides with an alkaline-labile glycosidic bonding is currently under investigation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.12.014.

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