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Enzymatic β-glycosidation of primary alcohols

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Abstract

For the purpose of finding the best reaction conditions of β-glucosidation of primary alcohols, screening experiments in respect of the enzymes, glycosyl donors and solvents were carried out. The effective enzyme and glycosyl donor for the synthesis of benzyl β-D-glucopyranoside (**4**) appeared to be β-glucosidase (EC 3.2.1.21) from almonds and 4-nitrophenyl β-D-glucopyranoside (**7**), respectively. Enzymatic glycosidation of 10 kinds of primary alcohols (methanol, ethanol, *n*-propanol, *n*-butanol, *n*-pentanol, *n*-hexanol, 2-methoxyethanol, 2-ethoxyethanol, benzyl alcohol, (*S*)-(−)-perillyl alcohol) gave stereoselectively the corresponding β-D-glucopyranosides (**14**–**21**, **4** and **22**) in moderate yield, respectively.

Keywords: β-Glucosidase; β-Glucosidation; 4-Nitrophenyl β-D-glucopyranoside; Benzyl β-D-glucopyranoside

1. Introduction

There are many β-D-glucopyranosides possessing primary alcohol moiety as an aglycone part in nature. The development of stereoselective methods for the synthesis of glycosidic linkages presents a considerable challenge to synthetic chemists [1,2]. Although well-developed chemical synthesis of the glycosidic structure is increasingly being established, several steps of selective protection, activation and coupling using metal catalyst are necessary. This problem in chemical synthesis has promoted the development of enzymatic approaches. Lipase-catalyzed synthesis of acyl sugar is reported [3], whereas much less is known about glycosidase-catalyzed synthesis of alkyl glycosides [4]. Glycosidases are responsible for catalytic hydrolysis of the glycosidic linkage and are increasingly being used in carbohydrate synthesis. For example, β-glucosidase catalyzes the stereospecific hydrolytic cleavage of the β-glucosidic bond in the substrate (**1**) to give glucose (**2**) (Fig. 1, path a). Meanwhile, the reaction of β-D-glucopyranoside (**1**) and a nucleophile such as an alcohol is reported to afford a new β-D-glucopyranoside (**3**) exclusively with the β-configuration (Fig. 1, path b) [5]. In case of the latter case, serine congeners were used as acceptor alcohol. The success of the glycosidic bond formation depends on the reactive intermediate (enzyme-bound glycosyl cation) being trapped faster by the glycosyl acceptor than by water. We are attracted to this transglycosylation reaction since alcohols as the glucosyl acceptor are better bound at the active site than water. This can be achieved by using predominantly organic reaction media. Research concerning enzymatic

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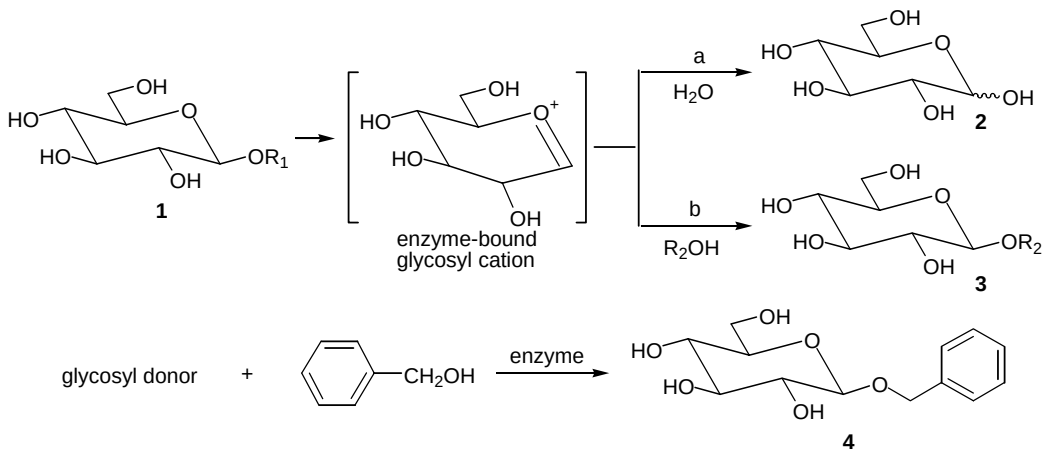


Fig. 1. Formation of glucose (**2**) or β-D-glucopyranoside (**3**) from β-D-glucopyranside (**1**) via glycosy cation.

reactions in organic media has been extremely active during the last few years. There are two approaches to optimizing the product yield from a given glycosidase in enzymatic glycoside synthesis, i.e. the use of either a high donor or high acceptor concentration [6]. High concentration of both is usually impractical due to the solubility limitation. High donor concentration is only practical if the donor is cheap such as glucose. High acceptor concentration is practical if the acceptor is cheap or can be recovered from the reaction mixture. For the purpose of the synthesis of naturally occurring β-D-glucopyranosides, the use of equal portions of both glycosy donor and acceptor alcohol from a point of synthetic view are desirable. Our current work has been directed towards establishing which reaction conditions in respect of the glycosyl donor and the enzyme including an immobilized form in aqueous or organic media are better in the synthesis of β-D-glucopyranosides. In order to determine the effective reaction conditions, the synthesis of benzyl β-D-glucopyranoside (**4**) was selected as a model transglycosylation reaction.

2. Material and methods

2.1. Analytical methods

^{1}H and ^{13}C NMR spectra were recorded by a JEOL EX 400 spectrometer (Tokyo, Japan). Spectra were taken with 5–10% (w/v) solution in DMSO-d_6 with Me_4Si as an internal reference. Melting points were determined on a Yanaco MP-3S micro melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. The FAB mass spectra were obtained with a JEOL JMS-AX 500 (matrix; glycerol) spectrometer. IR spectra were recorded on a JASCO FT/IR-300 spectrometer. The HPLC system was composed of a detector (Shodex RI SE-61), pump (Shodex DS-3), integrator (Sic chromatocorder 12), column oven (Shodex OVEN AO-50) and column (Shodex KS-801, solvent: H_2O, temperature; 80 °C, flow rate; 1 ml/min). All evaporations were performed under reduced pressure. For column chromatography, silica gel (Kieselgel 60) was employed.

2.2. Materials

2.2.1. Enzymes

The following enzymes possessing β-glycosidation activity were obtained. β-D-Glucosidase (EC 3.2.1.21) from almonds was purchased from Sigma (G-0395, 2.5–3.6 U/mg). Both GODO-TCL 4002 (0.076 U/mg) and GODO-TCL B15E (0.075 U/mg) were obtained from Godo Shusei Co. Ltd. (Tokyo, Japan). ENZYMA SANTEX CM-100 (1.547 U/mg) was obtained from Yamato Kasei Co. Ltd. (Tokyo, Japan). CELLULASE (1.252 U/mg) was obtained from Rakuto Kasei Co. Ltd. (Ohtsu, Japan).

2.2.2. Glycosyl donors

(1) The following glycosyl donors were commercially available. 2-Nitrophenyl β-D-glucopyraniside (**5**), 3-nitrophenyl β-D-glucopyranoside (**6**) were purchased from Funakoshi Yakuhin (Tokyo, Japan). Phenyl β-D-glycopyranoside (**8**), arbutin (**9**) and cellobiose (**10**) were purchased from Sigma.

(2) By applying the reported procedure [7], 4-nitrophenyl β-D-glycopyranoside (**7**) was synthesized. A mixture of penta *O*-acetyl α-glycopyranose (12 g) and *p*-nitrophenol (12 g) was melted at 120 °C and a suspension of *p*-toluenesulfonic acid (170 mg) in Ac_2O/AcOH (5/95) solution (10 ml) was added to the above mixture. The whole mixture was stirred at 120 °C for 2 h under reduced pressure by means of a vacuum pump and the reaction mixture was diluted with benzene (300 ml). The organic layer was washed with water and 1 M aqueous NaOH and dried over anhydrous Na_2SO_4. Evaporation of the organic solvent gave a residue which was chromatographed on silicagel with *n*-hexane–AcOEt (4/1) to afford a less polar fraction I (4.11 g, 28.5%) and a more polar fraction II (1.69 g, 11.7%). Both fractions were individually crystallized from EtOH to provide colorless prisms I mp 110–112 °C (from the less polar fraction) and colorless prisms II mp 171–173 °C (from the more polar fraction), respectively. A mixture of crystal I (500 mg) and NaOMe (60 mg) in MeOH (20 ml) was stirred for 20 min at room temperature and the reaction mixture was acidified with the aid of ion exchange resin (SK-1B) and filtered. The filtrate was evaporated to give an oily compound which was crystallized from EtOH to afford colorless needles (4-nitrophenyl α-D-glycopyranoside, 288 mg, 90%, mp 212–217 °C), whose physical data (mp) were consistent with those of the commercially available product (mp 214–217 °C). A mixture of crystal II (500 mg) and NaOMe (60 mg) in MeOH (20 ml) was stirred for 20 min at room temperature and the reaction mixture was worked up in the same way for the preparation of 4-nitrophenyl α-D-glycopyranoside to afford colorless needles (4-nitrophenyl β-D-glycopyranoside (**7**), 298 mg, 93%, mp 165–167 °C, FAB-MS *m*/*z*: 302 $(M + 1)^+$), whose physical data (mp) were consistent with those of the commercially available product (mp 162–165 °C). Mass production of **7** (5.75 g) was achieved in 92% yield from the reaction of crystal II (9.73 g) and NaOMe (450 mg) in MeOH (150 ml) (Fig. 2).

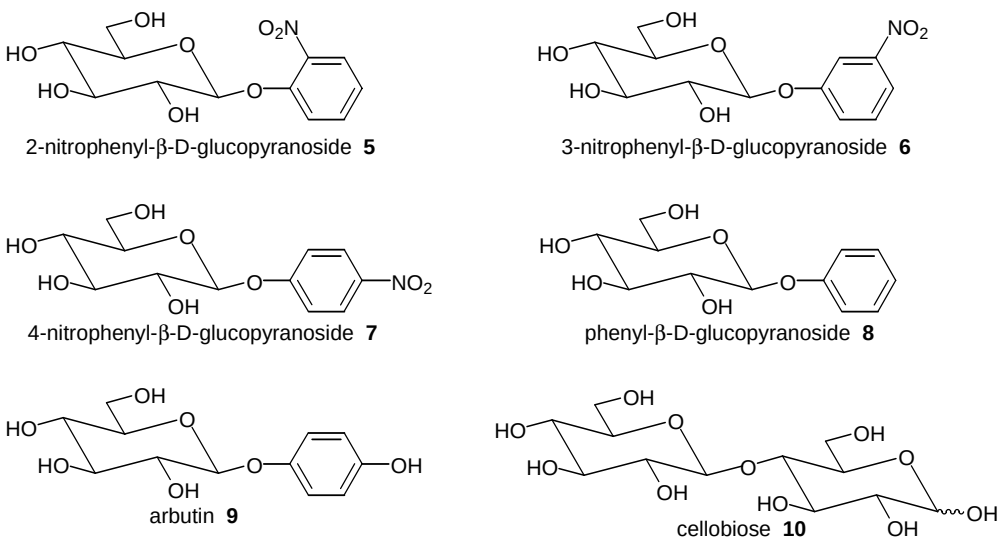


Fig. 2. Structure of six kinds of glycosyl donors.

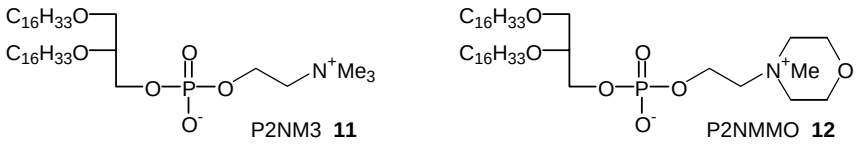


Fig. 3. Structure of two kinds of phospholipid analogues.

2.2.3. Preparation of the lipid-β-glucosidase aggregates

A mixture of 50 mg of β-D-glucosidase from almonds (3.4 U/mg) in water (5 ml) and 50 mg of 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonocholine (P2NM3) (**11**) [8] in benzene (40 ml) was sonicated for 30 min at 0 °C. The resulting precipitate was centrifugated at 3120 × *g* and the solvent was lyophilized to obtain an amorphous powder (aggregate I: 14.6 mg). The aggregate II of β-D-glucosidase from almonds and 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy ethyl *N*-methyl morphonium (inner salt; P2NMMO) (**12**) [8] was prepared in the same way as for the preparation of aggregate I to give an amorphous powder (aggregate II: 10.8 mg) (Fig. 3).

2.2.4. Immobilization of β-D-glucosidase using prepolymer

Immobilization of β-D-glucosidase from almonds on photocross-linkable resin prepolymer (ENTP-4000) was carried out by the following procedure. One gram of ENTP-4000 (**13**) [9] was mixed with 10 mg of a photosensitizer, benzoin ethyl ether and 110 mg of β-D-glucosidase from almonds (3.4 U/mg). The mixture was layered on a sheet of transparent polyester film (thickness, ca. 0.5 mm). The layer was covered with transparent thin film and then illuminated with chemical lamps (wavelength range, 300–400 nm) for 3 min. The gel film III thus obtained is cut into small pieces (0.5 mm × 5 mm × 5 mm) and used for bioconversion reaction (Fig. 4).

2.3. Enzymatic transglycosylation

2.3.1. Screening experiment

General procedure of the synthesis of benzyl β-D-glucopyranoside (**4**) using benzyl alcohol was carried out by the following procedure. A mixture of 4-nitrophenyl β-D-glycopyranoside (**7**, 30 mg), enzyme and benzyl alcohol (11 mg in phosphate buffer (pH 5, 1 ml) or reaction solvent (1 ml) was incubated for 24 h at 30 °C. After the reaction mixture was boiled for 5 min, it was filtered with the aid of Millipore Samprep LCR13-LH. The filtrate was directly subjected to analysis by means of HPLC. The standard benzyl β-D-glucopyranoside (**4**) was purchased from Sigma.

2.3.2. Preparative scale-synthesis of β-D-glucopyranosides

*2.3.2.1. Methyl β-D-glucopyranoside (**14**).* A mixture of **7** (500 mg, 1.66 mmol), methanol (10.63 g, 332 mmol), and β-glucosidase 23 mg (83 U) in phosphate buffer (pH 5, 17 ml) was incubated for 24 h at 30 °C. After the reaction mixture was boiled for 5 min and evaporated under reduced pressure, the residue was chromatographed on silica gel (20 g) with $CHCl_3$/MeOH (20/1) to afford **14** (262 mg, 81%) as colorless crystals. **14**: mp 112–113 °C; $[\alpha]_D^{22}$ − 33.5 (c = 0.5, H_2O); IR (KBr): 3398, 2922, 1075, 1038 cm^{-1}, ^1H NMR (DMSO-d_6): δ 3.38 (3H, s), 3.67 (1H, dq, J = 1.5, 5.9 Hz), 4.02 (1H, d, J = 7.8 Hz), 4.50 (1H, t, J = 5.9 Hz), 4.90 (2H, dd,

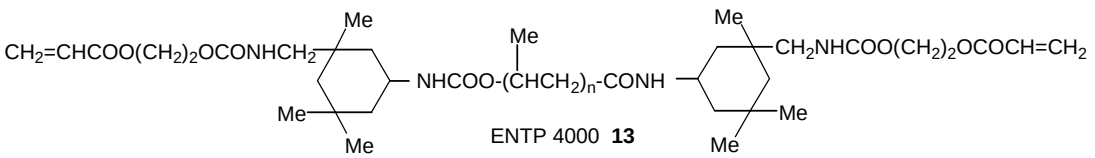


Fig. 4. Structure of photocross-linkable resin prepolymer.

J = 7.3, 10.2 Hz), 5.03 (1H, d, J = 4.9 Hz); ^{13}C NMR (pyridine-d_5): δ 105.7, 78.6, 78.5, 75.1, 71.7, 62.8, 56.8; FAB MS m/z: 195 $(M+1)^+$; Anal. Found: C, 41.28; H, 7.59. Calcd. for $C_7H_{14}O_6 \cdot 1/2H_2O$: C, 41.38; H, 7.44%.

*2.3.2.2. Ethyl β-D-glucopyranoside (**15**).* A mixture of **7** (500 mg, 1.66 mmol), ethanol (11.46 g, 249 mmol), and β-glucosidase 20 mg (72 U) in phosphate buffer (pH 5, 17 ml) was incubated for 24 h at 30 °C. After the reaction mixture was boiled for 5 min and evaporated under reduced pressure, the residue was chromatographed on silica gel (20 g) with $CHCl_3$/MeOH (20/1) to afford **15** (224 mg, 64%) as colorless crystals. **15**: mp 93–98 °C; $[\alpha]_D^{23}$ −37.9 (c = 0.5, H_2O); IR (KBr): 3420, 2935, 1075, 1034 cm^{-1}, ^{1}H NMR (DMSO-d_6): δ 1.13 (3H, t, J = 6.8 Hz), 3.66 (1H, dq, J = 1.5, 5.9 Hz), 3.81 (1H, dq, J = 2.4, 7.1 Hz), 4.11 (1H, d, J = 7.8 Hz), 4.89 (2H, dd, J = 4.9, 12.7 Hz), 4.96 (1H, d, J = 4.9 Hz); ^{13}C NMR (pyridine-d_5): δ 104.3, 78.5, 78.4, 75.2, 71.7, 65.0, 62.8, 15.7; FAB MS m/z: 209 $(M+1)^+$; Anal. Found: C, 45.85; H, 7.75. Calcd. for $C_8H_{16}O_6$: C, 46.15; H, 7.75%.

*2.3.2.3. n-Propyl β-D-glucopyranoside (**16**).* A mixture of **7** (500 mg, 1.66 mmol), *n*-propanol (1.000 g, 16.7 mmol), and β-glucosidase 20 mg (72 U) in phosphate buffer (pH 5, 17 ml) was incubated for 24 h at 30 °C. After the reaction mixture was boiled for 5 min and evaporated under reduced pressure, the residue was chromatographed on silica gel (20 g) with $CHCl_3$/MeOH (30/1) to afford **16** (178 mg, 48%) as colorless crystals. **16**: mp 97–99 °C; $[\alpha]_D^{26}$ − 40.1 (c = 0.5, H_2O); IR (KBr): 3400, 2925, 1080, 1040 cm^{-1}, ^{1}H NMR (DMSO-d_6): δ 0.87 (3H, t, J = 7.6 Hz), 1.53 (2H, sixtet, J = 7.1 Hz), 4.10 (1H, d, J = 7.8 Hz), 4.46 (1H, t, J = 5.9 Hz), 4.88 (2H, dd, J = 4.6, 11.9 Hz), 4.94 (1H, d, J = 4.9 Hz); ^{13}C NMR (pyridine-d_5): δ 104.4, 78.3, 77.2, 73.0, 71.5, 70.2, 62.7, 23.4, 10.9; FAB MS m/z: 223 $(M+1)^+$; Anal. Found: C, 48.26; H, 8.06. Calcd. for $C_9H_{18}O_6$: C, 48.64; H, 8.16%.

*2.3.2.4. n-Butyl β-D-glucopyranoside (**17**).* A mixture of **7** (1.000 g, 3.32 mmol), *n*-butanol (250 mg, 3.37 mmol), and β-glucosidase 67 mg (240 U) in phosphate buffer (pH 5, 34 ml) was incubated for 24 h at 30 °C. After the reaction mixture was boiled for 5 min and evaporated under reduced pressure, the residue was chromatographed on silica gel (40 g) with $CHCl_3$/MeOH (30/1) to afford **17** (199 mg, 25%) as colorless crystals. **17**: mp 84–85 °C; $[\alpha]_D^{26}$ −35.8 (c = 0.5, H_2O); IR (KBr): 3446, 2920, 1081, 1031 cm^{-1}, ^{1}H NMR (DMSO-d_6): δ 0.88 (3H, t, J = 7.3 Hz), 1.33 (2H, sixtet, J = 7.3 Hz), 1.50 (2H, sixtet, J = 6.8 Hz), 3.66 (1H, dt, J = 1.7, 5.9 Hz), 3.76 (1H, dt, J = 5.8, 6.8 Hz), 4.10 (1H, d, J = 7.8 Hz), 4.47 (1H, t, J = 5.9 Hz), 4.89 (2H, dd, J = 4.9, 12.2 Hz), 4.93 (1H, d, J = 4.9 Hz); ^{13}C NMR (pyridine-d_5): δ 104.6, 78.6, 78.5, 75.2, 71.7, 62.9, 62.9, 32.3, 19.7, 14.2; FAB MS m/z: 237 $(M+1)^+$; Anal. Found: C, 50.58; H, 8.59. Calcd. for $C_{10}H_{20}O_6$: C, 50.83; H, 8.53%.

*2.3.2.5. n-Pentyl β-D-glucopyranoside (**18**).* A mixture of **7** (500 mg, 1.66 mmol), *n*-pentanol (147 mg, 1.66 mmol), and β-glucosidase 60 mg (205 U) in phosphate buffer (pH 5, 17 ml) was incubated for 24 h at 30 °C. After the reaction mixture was boiled for 5 min and evaporated under reduced pressure, the residue was chromatographed on silica gel (20 g) with $CHCl_3$/MeOH (30/1) to afford **18** (93 mg, 22%) as colorless crystals. **18**: mp 89–91 °C; $[\alpha]_D^{27}$ −35.5 (c = 0.5, H_2O); IR (KBr): 3380, 2918, 1079, 1036 cm^{-1}, ^{1}H NMR (DMSO-d_6): δ 0.87 (3H, t, J = 6.8 Hz), 3.66 (1H, dd, J = 5.9, 11 Hz), 3.75 (1H, dt, J = 6.8 Hz), 4.09 (1H, d, J = 7.8 Hz), 4.47 (1H, t, J = 5.9 Hz), 4.90 (2H, dd, J = 4.4, 13.2 Hz), 4.94 (1H, d, J = 4.9 Hz); ^{13}C NMR (pyridine-d_5): δ 104.7, 78.6, 78.5, 75.3, 71.8, 69.8, 62.9, 30.0, 28.6, 22.9, 14.3; FAB MS m/z: 251 $(M+1)^+$; Anal. Found: C, 52.49; H, 8.90. Calcd. for $C_{11}H_{22}O_6$: C, 52.78; H, 8.66%.

*2.3.2.6. n-Hexyl β-D-glucopyranoside (**19**).* A mixture of **7** (500 mg, 1.66 mmol), *n*-hexanol (172 mg, 1.68 mmol), and β-glucosidase 50 mg (169 U) in phosphate buffer (pH 5, 17 ml) was incubated for 24 h at 30 °C. After the reaction mixture was boiled for 5 min and evaporated under reduced pressure, the residue was chromatographed on silica gel (20 g) with $CHCl_3$/MeOH (30/1) to afford **19** (79 mg, 18%) as colorless crystals. **17**: mp 88–89 °C; $[\alpha]_D^{27}$ −34.9 (c = 0.5, H_2O); IR (KBr): 3410, 2927, 1075, 1030 cm^{-1}, ^{1}H NMR (DMSO-d_6): δ 0.86 (3H, t, J = 6.8 Hz), 3.66 (1H, dd, J = 5.9, 11.7 Hz), 3.75 (1H, sixtet, J = 6.8 Hz), 4.09 (1H, d, J = 7.8 Hz), 4.46 (1H, t,

$J = 5.9$ Hz), 4.90 (2H, dd, $J = 4.9$, 12.7 Hz), 4.93 (1H, d, $J = 4.9$ Hz); ^{13}C NMR (MeOH-d_4): δ 104.4, 78.1, 77.9, 75.1, 71.7, 70.9, 62.8, 32.9, 30.8, 26.8, 23.7, 14.4; FAB MS *m/z*: 265 $(M+1)^+$; Anal. Found: C, 54.28; H, 9.25. Calcd. for $C_{12}H_{24}O_6$: C, 54.53; H, 9.15%.

*2.3.2.7. Methoxyethyl β-D-glucopyranoside (**20**).* A mixture of **7** (1.000 g, 3.32 mmol), 2-methoxyethanol (2.526 g, 33.2 mmol), and β-glucosidase 42 mg (150 U) in phosphate buffer (pH 5, 34 ml) was incubated for 24 h at 30 °C. After the reaction mixture was boiled for 5 min and evaporated under reduced pressure, the residue was chromatographed on silica gel (40 g) with $CHCl_3$/MeOH (30/1) to afford **20** (156 mg, 20%) as colorless crystals. **20**: mp 107–109 °C; $[\alpha]_D^{27} - 29.4$ ($c = 0.32$, H_2O); IR (KBr): 3448, 2932, 1073, 1040 cm^{-1}, ^{1}H NMR (DMSO-d_6): δ 4.14 (1H, d, $J = 7.8$ Hz), 4.49 (1H, t, $J = 5.6$ Hz), 4.89 (2H, dd, $J = 4.9$, 14.6 Hz), 4.99 (1H, d, $J = 4.9$ Hz); ^{13}C NMR (pyridine-d_5): δ 104.8, 78.6, 78.6, 75.2, 72.2, 71.7, 68.9, 62.8, 58.7; FAB MS *m/z*: 239 $(M+1)^+$; Anal. Found: C, 45.09; H, 7.78. Calcd. for $C_9H_{18}O_7$: C, 45.37; H, 7.62%.

*2.3.2.8. Ethoxyethyl β-D-glucopyranoside (**21**).* A mixture of **7** (1.000 g, 3.32 mmol), 2-ethoxyethanol (2.991 g, 33.2 mmol), and β-glucosidase 41 mg (147 U) in phosphate buffer (pH 5, 34 ml) was incubated for 24 h at 30 °C. After the reaction mixture was boiled for 5 min and evaporated under reduced pressure, the residue was chromatographed on silica gel (40 g) with $CHCl_3$/MeOH (30/1) to afford **21** (198 mg, 24%) as colorless crystals. **21**: mp 137–139 °C; $[\alpha]_D^{24} - 23.4$ ($c = 0.49$, H_2O); IR (KBr): 3362, 2943, 1073, 1040 cm^{-1}, ^{1}H NMR (DMSO-d_6): δ 4.14 (1H, d, $J = 7.8$ Hz), 4.49 (1H, t, $J = 5.8$ Hz), 4.91 (2H, dd, $J = 4.9$, 15.6 Hz), 4.99 (1H, d, $J = 4.9$ Hz); ^{13}C NMR (pyridine-d_5): δ 104.7, 78.3, 78.3, 75.0, 71.5, 70.1, 69.0, 66.5, 62.7, 15.6; FAB MS *m/z*: 253 $(M+1)^+$; Anal. Found: C, 47.42; H, 8.11. Calcd. for $C_{10}H_{20}O_7$: C, 47.61; H, 7.99%.

*2.3.2.9. Benzyl β-D-glucopyranoside (**4**).* A mixture of **7** (1.000 g, 3.32 mmol), benzyl alcohol (363 mg, 3.36 mmol), and β-glucosidase 120 mg (480 U) in phosphate buffer (pH 5, 34 ml) was incubated for 24 h at 30 °C. After the reaction mixture was boiled for 5 min and evaporated under reduced pressure, the residue was chromatographed on silica gel (40 g) with $CHCl_3$/MeOH (30/1) to afford **4** (187 mg, 21%) as colorless crystals. **4**: mp 114–116 °C; $[\alpha]_D^{26} - 59.3$ ($c = 0.3$, MeOH); IR (KBr): 3422, 2886, 1108, 1081, 1052, 1033 cm^{-1}, ^{1}H NMR (D_2O, acetone): δ 3.18 (1H, t, $J = 8.6$ Hz), 3.35–3.22 (3H, m), 3.60 (1H, d, $J = 5.9$, 12.5 Hz), 3.79 (1H, d, $J = 1.5$, 12.5 Hz), 4.39 (1H, d, $J = 8.3$ Hz), 4.62 (1H, d, $J = 11.5$ Hz), 4.80 (1H, d, $J = 11.5$ Hz), 7.35–7.26 (5H, m); ^{13}C NMR (D_2O): δ 137.5, 129.6, 129.6, 129.5, 129.5, 129.4, 102.1, 76.8, 76.7, 74.0, 72.3, 70.6, 61.7; FAB MS *m/z*: 271 $(M+1)^+$; Anal. Found: C, 57.34; H, 6.77. Calcd. for $C_{13}H_{18}O_6$: C, 57.77; H, 6.71%.

*2.3.2.10. Perilloside A (**22**).* A mixture of **7** (2.000 g, 6.64 mmol), (*S*)-(−)-perillyl alcohol (1.030 g, 6.67 mmol), and β-glucosidase 10 mg (34 U) in phosphate buffer (pH 5, 68 ml) was incubated for 24 h at 30 °C. After the reaction mixture was boiled for 5 min and concentrated under reduced pressure, the residue was chromatographed on silica gel (80 g) with $CHCl_3$/MeOH (30/1) to afford **22** (159 mg, 8%) as colorless crystals. **22**: mp 113–114.5 °C; $[\alpha]_D^{26} - 90.3$ ($c = 0.48$, MeOH); IR (KBr): 3398, 2922, 1100, 1075, 1038 cm^{-1}, ^{1}H NMR (CD_3OD): δ 1.48 (1H, m), 1.73 (3H, s), 1.81 (1H, m), 3.67 (1H, dd, $J = 5.2$, 11.9 Hz), 3.86 (1H, dd, $J = 1.8$, 11.9 Hz), 4.02 (1H, d, $J = 11.9$ Hz), 4.22 (1H, d, $J = 11.9$ Hz), 4.27 (1H, d, $J = 7.9$ Hz), 4.71 (2H, s), 5.76 (1H, br, s); ^{13}C NMR (pyridine-d_5): δ 151.1, 136.8, 126.1, 109.3, 103.1, 78.3, 78.0, 75.2, 74.6, 71.8, 62.9, 42.6, 31.8, 28.9, 27.6, 21.1; HRMS (FAB MS) *m/z*: 315.1802; Calcd. for $C_{16}H_{27}O_6$ *m/z*: 315.1808 $(M+1)^+$; FAB MS *m/z*: 315 $(M+1)^+$; Anal. Found: C, 60.57; H, 8.41. Calcd. for $C_{16}H_{26}O_6 \cdot 1/6H_2O$: C, 60.55; H, 8.36%.

3. Results and discussion

3.1. Screening for suitable enzyme

Five kinds of commercially available enzymes possessing β-D-glucosidase activity were applied for a screening experiment in phosphate buffer (pH 5) solution as shown in Table 1, and β-D-glucosidase (EC 3.2.1.21) from almonds was found to be a suitable enzyme for transglycosylation (Table 1, entry 1).

Table 1
Effect on transglucosylation using five kinds of enzymes

$$\underset{\mathbf{7}\,(30\,\mathrm{mg})}{\text{glu-O-Ph-NO}_2} + \underset{(11\,\mathrm{mg})}{\text{benzyl alcohol}} \xrightarrow[\text{in phosphate buffer (pH 5.0, 1 ml) 30 °C, 24 h}]{\text{enzyme (3 U)}} \underset{\mathbf{4}}{\text{glu-OCH}_2\text{Ph}}$$

Entry	Enzyme	glu-OCH_2Ph (**4**; %)	Glucose	SM (**7**; %)	Other (%)
1	β-Glucosidase (EC 3.2.1.21)	28.6	50.6	20.8	–
2	GODO-TCI 4002	18.0	73.3	–	8.7
3	GODO-TCI B15E	21.0	70.9	–	8.1
4	ENZYMA SANTEX CM-100	15.9	74.9		9.2
5	CELLULASE	13.9	77.4	–	8.7

3.2. Selection of glycosyl donor

Six kinds of glycosyl donors (**5**–**10**) as shown in Fig. 2 were used for transglycosylation in phosphate buffer solution, and the results are shown in Table 2. Three kinds of nitrophenyl β-D-glycopyranosides (**5**–**7**) were found to be suitable glycosyl donors. The unit numbers of β-D-glucosidase activity to give a high yield of beznyl β-D-glucopyranoside (**4**) were different among the three kinds of glycosyl donors (**5**–**7**). When the proper units of β-D-glucosidase were applied for transglycosylation using three kinds of nitrophenyl β-D-glycopyranosides (**5**–**7**), no effect on the yield of benzyl β-D-glucopyranoside (**4**) was observed and this phenomenon was a common feature for the three kinds of nitrophenyl β-D-glycopyranosides (**5**–**7**) (Table 2, entries 2, 3, 5–10). In the case of using glycosyl donor (**5**), it was difficult to determine the best amount of β-D-glucosidase because the yield of **4** varied considerably by a very small amount of

Table 2
Effect on transglucosylation using six kinds of glucosyl donors

$$\underset{(30\,\mathrm{mg})}{\text{glucosyl donor}} + \underset{(11\,\mathrm{mg})}{\text{benzyl alcohol}} \xrightarrow[\text{in phosphate buffer (pH 5.0, 1 ml) 30 °C, 24 h}]{\text{β-glucosidase (EC 3.2.1.21)}} \underset{\mathbf{4}}{\text{glu-OCH}_2\text{Ph}}$$

Entry	Glucosy donor	β-Glucosidase (U/ml)	glu-OCH_2Ph (**4**; %)	Glucose	SM (**7**; %)	Other (%)
1	**5**	0.01	13.1	17.1	61.4	8.4
2	**5**	0.03	24.8	41.2	19.2	14.8
3	**5**	0.06	22.3	61.0	1.2	15.5
4	**5**	0.1	17.0	70.6	–	12.4
5	**6**	1.0	25.0	56.2	8.9	9.9
6	**6**	5.0	24.1	63.7	2.1	10.1
7	**6**	10.0	20.3	70.8	–	8.9
8	**7**	5.0	28.0	56.5	15.5	–
9	**7**	10.0	27.0	64.9	8.1	–
10	**7**	15.0	26.4	66.9	6.7	–
11	**7**	20.0	17.3	79.5	3.2	–
12	**8**	0.1	2.6	20.5	76.9	–
13	**8**	0.5	1.1	84.5	14.4	–
14	**8**	1.0	–	97.7	2.3	–
15	**9**	0.1	1.6	5.8	92.2	–
16	**9**	1.0	1.4	60.2	38.4	–
17	**9**	5.0	–	100	Trace	–
18	**10**	1.0	1>	89.6	10.4	–
19	**10**	3.0	1>	98.7	1.3	–

enzyme (Table 2, entries 1–4). When other glycosyl donors (**8**–**10**) were used for transglycosylation, the yield of benzyl β-D-glucopyranoside (**4**) was found to be extremely low. The prices of 2- and 3-nitrophenyl β-D-glycopyranoside (**5**, **6**) were too high to use as the starting material. Therefore, 4-nitrophenyl β-D-glycopyranoside (**7**) was selected as a suitable glycosyl donor from the results (Table 1, entry 1 and Table 2, entry 8) and for the above-mentioned reason.

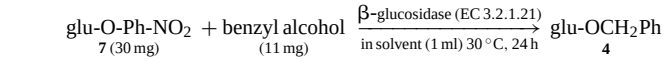
3.3. Solvent effect on transglycosylation

Transglycosylation can be achieved by using predominantly organic media because hydrophobic alcohols as the glycosyl acceptor are better bound at the active site (enzyme-bound glycosyl cation) than water. One approach to glycosidic bond formation is to use a water-miscible solvent, which decreases the thermodynamic water activity and thereby favors transglycosylation. An alternative approach is to add a water-immiscible solvent. In this case, the hydrophobic alcohol is extracted to an organic phase so that its concentration in the aqueous phase is kept low and the reaction should be shifted to the transglycosylation. This approach should be applied in a two-phase system using the hydrophobic alcohol as both the substrate and the organic phase. At first, the reaction of **7** (30 mg) and benzyl alcohol (11 mg) in the presence of 10 U of β-glycosidase in a water-miscible solvent (1 ml) such as DMSO, DMF, acetone and CH_3CN was carried out, but the reaction did not occur (Table 3, entries 1–4). The presence of water-miscible solvents has a strong denaturing effect on the β-glycosidase. When the same scale reaction as the above case was carried out in a homogeneous solvent system consisted of water and a water-miscible solvent, transglycosylation reaction proceeded to give benzyl β-D-glucopyranoside (**4**) in poor yield (Table 3, entries 5–8). The same scale reaction in a two-phase system (*n*-hexane/H_2O = 1:1 or *n*-hexane/buffer (pH 5) = 1:1) afforded benzyl β-D-glucopyranoside (**4**) in moderate yield (Table 3, entries 9, 10).

3.4. Effect on transglycosylation using immobilized β-glycosidase

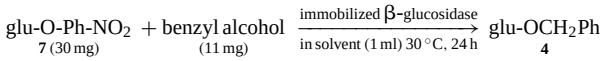
For the purpose of carrying out smoothly enzymatic reaction in organic solvents, asymmetric hydrolysis of water-insoluble α-acyloxy ester was carried out using lipases immobilized with synthetic prepolymer ENTP-4000 in water-saturated organic solvents such as isopropyl ether or benzene to prepare a chiral intermediate for the synthesis of diltiazem hydrochloride [10], while a new type of immobilized enzyme, a lipid-lipase aggregate was also found to catalyze the enantioselective hydrolysis of the above-mentioned substrate in water-saturated organic solvent [11]. In the present case, both aggregates I and II were found

Table 3
Effect on transglucosylation by solvents



Entry	Solvent	β-Glucosidase (U/ml)	glu-OCH_2Ph (**4**; %)	Glucose	SM (**7**; %)
1	DMSO	10	Not detected		
2	DMF	10	Not detected		
3	Acetone	10	Not detected		
4	CH_3CN	10	Not detected		
5	DMSO/H_2O = 6:4	10	7.5	92.5	–
6	DMF/H_2O = 6:4	10	3.9	96.1	–
7	Acetone/H_2O = 8:2	10	2.4	97.6	–
8	CH_3CN/H_2O = 8:2	10	2.2	97.8	–
9	Hexane/H_2O = 1:1	3	19.4	66.5	14.2
10	Hexane/buffer = 1:1	3	20.6	68.5	10.9
11	Benzene/H_2O = 1:1	3	–	100	–
12	Benzene/buffer = 1:1	0.5	7.0	93.0	–

Table 4
Effect on transglucosylation using three kinds of immobilized enzymes



Entry	Immobilized β-glucosidase	Solvent	glu-OCH_2Ph (**4**; %)	Glucose	SM (**7**; %)
1	Aggregate I (3 mg)	Phosphate buffer (pH 5)	22.1	56.1	21.8
2	Aggregate I (3 mg)	Hexane/H_2O = 1:1	17.9	76.7	5.4
3	Aggregate I (3 mg)	DMSO/H_2O = 100:1	Not detected		
4	Aggregate I (3 mg)	Hexane/DMSO/H_2O = 50:50:1	Not detected		
5	Aggregate II (2 mg)	Phosphate buffer (pH 5)	22.0	62.1	15.9
6	Aggregate II (2 mg)	Hexane/H_2O = 1:1	18.6	71.2	10.2
7	Aggregate II (2 mg)	DMSO/H_2O = 6:4	4.6	31.8	63.6
8	Aggregate II (2 mg)	DMSO/H_2O = 8:2	Not detected		
9	Gel film III (16.4 mg)	Phosphate buffer (pH 5)	14.5	85.5	–
10	Gel film III (19.3 mg)	Hexane/H_2O = 1:1	17.0	83.0	–
11	Gel film III (15.3 mg)	$CHCl_3$/H_2O = 1:1	2.8	97.2	–
12	Gel film III (17.8 mg)	DMSO/H_2O = 6:4	0.7	7.0	92.3

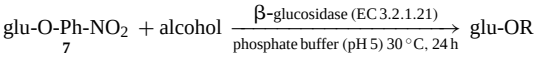
to prepare the β-D-glucopyranoside (**4**) in phosphate buffer (Table 4, entries 1, 5) or a two-phase solvent system (*n*-hexane/H_2O = 1:1) (Table 4, entries 2, 6). The gel film III prepared with β-glycosidase and synthetic prepolymer ENTP-4000 catalyzed the β-glycosidation to give β-D-glycopyranoside (**4**) in moderate yield in phosphate buffer (Table 4, entry 9) or a two-phase solvent system (*n*-hexane/H_2O = 1:1) (Table 4, entry 10).

3.5. Preparative scale-synthesis of β-D-glucopyranosides

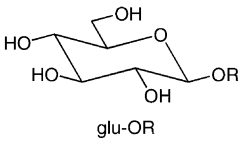
From a screening experiment including the use of immobilized β-glucosidase, an effective enzyme and glycosyl donor for the synthesis of benzyl β-D-glucopyranoside (**4**) appeared to be β-glucosidase (EC 3.2.1.21) from almonds and 4-nitrophenyl-β-D-glucopyranoside (**7**), respectively. The following primary alcohols such as methanol, ethanol, *n*-propanol, *n*-butanol, *n*-pentanol, *n*-hextanol, 2-methoxyethanol, 2-ethoxyethanol, benzyl alcohol and (*S*)-(−)-perillyl alcohol were selected as a nucleophile. Addition of the glycosyl donor (**7**) to a solution of the acceptor substrate dissolved in phosphate buffer (pH 5) containing β-glucosidase was carried out over a period of 24 h. The reaction can be easily monitored by reverse-phase HPLC and is terminated when the formation of the desired product is at a maximum. The results are summarized in Table 5.

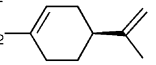
The structures of all products were determined by direct comparison with the corresponding β-D-glucopyranosides. Identification of the β-configuration of the anomeric center was easily achieved via analysis of the C–H/C–H coupling constant (d, J = 7.8 Hz). The spectral data of the synthetic β-D-glucopyranosides (**14**; mp 112–113 °C, $[\alpha]_D$ −33.5 (c = 0.5, H_2O), 81% yield, 15; mp 93–98 °C, $[\alpha]_D$ − 37.9 (c = 0.5, H_2O), 65% yield, **16**; mp 97–99 °C, $[\alpha]_D$ − 40.1 (c = 0.5, H_2O), 48% yield, **17**; mp 84–85 °C, $[\alpha]_D$ − 35.8 (c = 0.5, H_2O), 25% yield, **18**; mp 89–91 °C, $[\alpha]_D$ − 35.5 (c = 0.5, H_2O), 22% yield, **19**; mp 88–89 °C, $[\alpha]_D$ − 34.9 (c = 0.5, H_2O), 18% yield, **20**; mp 107–109 °C, $[\alpha]_D$ − 29.4 (c = 0.32, H_2O), 20% yield, **21**; mp 137–139 °C, $[\alpha]_D$ − 23.4 (c = 0.49, H_2O), 24% yield, **4**; mp 114–116 °C, $[\alpha]_D$ − 59.3 (c = 0.3, MeOH), 21% yield, **22**; mp 113–114.5 °C, $[\alpha]_D$ − 90.3 (c = 0.48, MeOH), 8% yield) were identical with those of the reported β-D-glucopyranosides (methyl β-D-glucopyranoside **14** [12]; mp 115–116 °C, $[\alpha]_D$ − 34.2 (H_2O), ethyl β-D-glucopyranoside **15** [13]; mp 98–100 °C, $[\alpha]_D$ − 37.9 (H_2O), *n*-propyl β-D-glucopyranoside **16** [14]; mp 102–103 °C, $[\alpha]_D$ − 39.1 (H_2O), *n*-butyl β-D-glucopyranoside **17** [15]; mp 86–87 °C, $[\alpha]_D$ − 38.4 (MeOH), *n*-pentyl β-D-glucopyranoside **18**

Table 5
Preparative scale-synthesis of β-D-glucopyranoside using 10 kinds of alcohols



Entry	ROH (eq)	β-Glucosidase/buffer (U/ml)	glu-OR (yield; %)	
1	CH_3OH (200)	4.9	glu-OCH_3	**14** (81)
2	CH_3CH_2OH (150)	4.2	glu-OCH_2CH_3	**15** (64)
3	$CH_3CH_2CH_2OH$ (10)	4.2	glu-$O(CH_2)_2CH_3$	**16** (48)
4	$CH_3(CH_2)_2CH_2OH$ (1)	7.0	glu-$O(CH_2)_3CH_3$	**17** (25)
5	$CH_3(CH_2)_3CH_2OH$ (1.6)	12.0	glu-$O(CH_2)_4CH_3$	**18** (22)
6	$CH_3(CH_2)_4CH_2OH$ (1.7)	10.0	glu-$O(CH_2)_5CH_3$	**19** (18)
7	$CH_3OCH_2CH_2OH$ (10)	4.4	glu-$O(OH_2)_2OCH_3$	**20** (20)
8	$CH_3CH_2OCH_2CH_2OH$ (10)	4.3	glu-$O(CH_2)_2OCH_2CH_3$	**21** (24)
9	$PhCH_2OH$ (1)	12.0	glu-OCH_2Ph	**4** (21)
10	(*S*)-(−)-Perillyl alcohol (1)	3.4	glu-$OC_{10}H_{15}$	**22** (8)



R = CH_3 **14**
R = CH_2CH_3 **15**
R = $CH_2CH_2CH_3$ **16**
R = $CH_2(CH_2)_2CH_3$ **17**
R = $CH_2(CH_2)_3CH_3$ **18**
R = $CH_2(CH_2)_4CH_3$ **19**
R = $CH_2CH_2OCH_3$ **20**
R = $CH_2CH_2OCH_2CH_3$ **21**
R = CH_2Ph **4**
R = CH_2  **22**

[16]; mp 91–93 °C, $[\alpha]_D$ − 36.3 (H_2O), *n*-hexyl β-D-glucopyranoside **19** [16]; mp 90–92 °C, $[\alpha]_D$ − 34.5 (H_2O), benzyl β-D-glucopyranoside **4** [17]; mp 123–124 °C, $[\alpha]_D$ − 59.2 (*c* = 0.67, MeOH), perilloside A **22** [18]; mp 114.5–115 °C, $[\alpha]_D$ − 92.7 (*c* = 0.77, MeOH)), respectively. In spite of the moderate chemical yield, in all cases only the β-D-glucopyranoside was obtained. Prolonged reaction times (>24 h) generally resulted in decreased yields of the glucosides presumably due to competing hydrolysis of the product by β-glucosidase.

3.6. Discussion

When the enzymatic β-glycosidation by the present method was carried out under high acceptor concentration, chemical yield of β-D-glucopyranosides (**14**–**16**) appeared to be relatively high (Table 5, entries 1–3). In case of the use of equal portions of glycosyl donor **7** and acceptor alcohol bearing monoterpene alcohol, the yield of β-D-glucopyranosides (**22**) (Table 5, entry 10) was found to be unsatisfied but could look promising in the β-glycosidation of linear type primary alcohol. In case of the transglucosylation of serine congeners with 2-nitrophenyl-β-D-glucopyranoside (**5**) or 4-nitrophenyl-β-D-glucopyranoside (**7**) in the presence of β-glucosidase in phosphate buffer, chemical yield of the β-D-glucopyranoside was reported to be low and less than 30% [5]. In order to avoid the cleavage of the glycosidic bond of the produced β-D-glucopyranoside, transglucosylation of 5-phenyl-1-pentanol with **7** using lipid-coated β-glucosidase in dry isopropyl ether was reported to give the corresponding β-D-glucopyranoside in 23% yield [19]. Enzymatic formation of a glycosidic bond is thought to be mechanistically similar to the acid-catalyzed formation of glycosides [20]. The active site of β-glucosidase was constructed with two carboxylic acid parts which play the important role of catalyzing the hydrolysis of glycosidic linkages. One is the carboxylate ion which acts as a

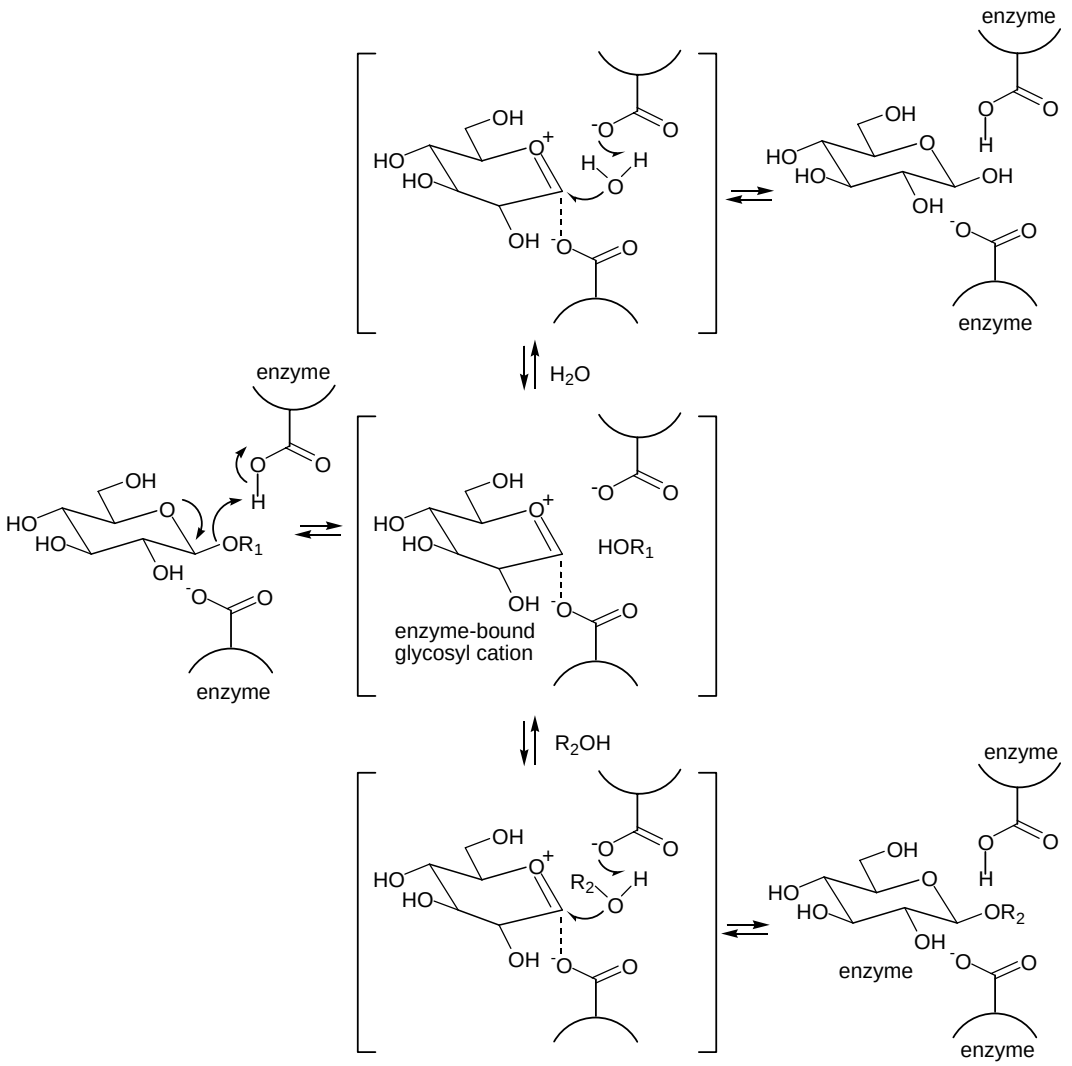


Fig. 5. Plausible mechanism of hydrolysis or transglycosylation via glycosyl cation.

general base and the other is carboxylic acid which acts as a general acid. When the substrate is brought close to the active site of the enzyme, the oxocarbenium ion with α-configuration at the anomeric carbon as shown in Fig. 5 was formed. This oxonium ion or the enzyme-bound glycosy cation was stabilized by an ion-pair intermediate or covalent bonding and can be captured by an alcohol to yield a glycoside. Nucleophilic alcohol presumably attacks at the anomeric carbon from the β-side to afford exclusively β-glucopyranoside.

4. Conclusion

In order to investigate the best reaction conditions of β-glucosidation of primary alcohols, screening experiments in respect of the enzymes, glycosyl donors and solvents were carried out. From a screening experiment including the use of immobilized β-glucosidase, the effective enzyme and glycosyl donor for the synthesis of benzyl β-D-glucopyranoside (**4**) appeared to be β-glucosidase (EC 3.2.1.21) from almonds and 4-nitrophenyl β-D-glucopyranoside (**7**),

respectively. Enzymatic glycosidation of 10 kinds of primary alcohols (methanol, ethanol, *n*-propanol, *n*-butanol, *n*-pentanol, *n*-hexanol, 2-methoxyethanol, 2-ethoxyethanol, benzyl alcohol, (*S*)-(−)-perillyl alcohol) and 4-nitrophenyl β-D-glucopyranoside (**7**) using β-glucosidase from almonds gave stereoselectively the corresponding β-D-glucopyranosides (**14**–**21**, **4** and **22**) in moderate yield, respectively.

References

[1] C.-H. Wong, G.M. Whitesides, Enzymes in Synthetic Organic Chemistry, Tetrahedron Organic Chemistry Series, vol. 12, Pergamon Press, Oxford, 1994, p. 252.
[2] K. Faber, Biotransformations in Organic Chemistry: A Text Book, fourth ed., Springer-Verlag, Berlin, 2000, p. 307.
[3] X. Zhang, T. Kamiya, N. Ohtsubo, H. Ishida, M. Kiso, J. Carbohydrate Chem. 18 (1999) 225.
[4] A. Basso, A. Ducret, L. Gardossi, R. Lortie, Tetrahedron Lett. 43 (2002) 2005.
[5] N.J. Turner, M.C. Webberley, J. Chem. Soc. Chem. Commun. (1991) 1349.
[6] D.E. Stevenson, R.A. Stanley, R.H. Furneaux, Biotechnol. Bioeng. 42 (1993) 657.
[7] Beilsteins Handbuch der Organischen Chemie 17 (3) 2950.
[8] H. Akita, I. Umezawa, H. Matsukura, T. Oishi, Chem. Pharm. Bull. 40 (1992) 318.
[9] S. Fukui, A. Tanaka, Adv. Biochem. Eng./Biotechnol. 29 (1984) 1.
[10] H. Akita, Y. Enoki, H. Yamada, T. Oishi, Chem. Pharm. Bull. 37 (1989) 2876.
[11] H. Akita, I. Umezawa, H. Matsukura, T. Oishi, Chem. Pharm. Bull. 39 (1991) 1632.
[12] P.M. Collins (Ed.), Dictionary of Carbohydrates, Chapman & Hall, London, 1998, p. 541.
[13] Y. Matsubara, T. Mizuno, A. Sawabe, Y. Iizuka, K. Okamoto, Nippon Nogeikagaku Kaishi 63 (1989) 1373.
[14] L.R. Schroeder, J.W. Green, J. Chem. Soc. C (1966) 530.
[15] T. Matsumoto, W. Trueb, R. Gwinner, G.H. Eugster, Helv. Chim. Acta 52 (1969) 716.
[16] W.W. Pigman, N.K. Richtmyer, J. Am. Chem. Soc. 64 (1942) 369.
[17] J. Kitajima, T. Ishikawa, Y. Tanaka, M. Ono, Y. Ito, T. Nohara, Chem. Pharm. Bull. 46 (1998) 1587.
[18] T. Fujita, M. Nakayama, Phytochemistry 31 (1992) 3265.
[19] T. Mori, Y. Okahata, Tetrahedron Lett. 38 (1997) 1971.
[20] D.G. Drueckhammer, W.J. Hennen, R.L. Pederson, C.F. Barbas, C.M. Gautheron, T. Krach, C.-H. Wong, Synthesis (1991) 499.