Research Article

Aglycone Specificity of Endo-ß-1,3-Glucanase

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Abstract

Four β-glucan trisaccharides with different aglycones were synthesized as endo-β-1,3-glucanase substrates from β-1,3-trisaccharide, where glycosylation with methanol or *p*-methoxyphenol yielded a glycoside lacking a linker. The introduction of an aminopentyl group provided the glycoside with a linker, after which an acetyl group and fluorescein were introduced on the amino group on the pentyl linker. The synthesized substrates were used to investigate the effects of hydrophobicity, length, and size of the aglycone on hydrolysis, specifically on hydrolysis by endo-β-1,3-glucanase. Hydrolysis was evaluated using TLC and HPLC, which demonstrated that the substrates with hydrophobic aglycone were hydrolyzed completely and that the *p*-methoxyphenyl glycoside was hydrolyzed in 2 hours. The aglycones of glycosides were thus shown to affect the activity of endo-β-1,3-glucanase, and it was furthermore found that the catalytic domain of the enzyme recognition specificity for both the carbohydrate and the aglycone moiety.

ABBREVIATIONS

TLC: Thin-Layer Chromatography; HPLC: High Pressure Liquid Chromatography

INTRODUCTION

The glycoside hydrolases include various enzymes that hydrolyze glycosidic bonds in carbohydrates and include over 100 enzyme families grouped based on 3D structure and amino acid sequence [1-5]. Glycoside hydrolases exist in bacteria, plants, and animals and the specific enzyme types present differ between various tissues and organs [6-8]. Substrate specificity for carbohydrate hydrolysis has been investigated to clarify the currently ambiguous substrate specificity, and methods for the detection of these substrates in target samples have also been investigated for the development of a diagnosis method for infection and disease [9-13]. The substrate specificity of carbohydrate-hydrolyzing enzymes can be quantitatively evaluated using a synthesized glycan with an identified structure, such that the part of the structure required for substrate recognition of enzymes can be determined. Such synthetic glycans, which are chemically synthesized to contain easily detectable functional groups, can moreover be applied as indicators of glycoside hydrolases associated with infection and disease.

Endo-β-1,3-glucanases in the glycoside hydrolase family hydrolyze cell walls of bacteria and plants [15,16]. In higher plants, these glucanases are produced to protect against bacterial and fungal infection as well as to contribute to morphogenetic processes and energy supply [17,19]. Unlike bacteria, humans do not express β-1,3-glucanasesand clarifying the substrate specificity of these enzymes is thus an important research drive toward bacterial detection in the diagnosis of infection. The substrate specificity of β-1,3-glucanaseshavenot been investigated using synthetic substrates. Instead, the β-glucans laminar in and curdlan, whose structures are not known, have been used to investigate the specificity and hydrolysis rates of β-1,3-glucanases [20-24], since the biological preparation of carbohydrates with homogeneous structures and the chemical synthesis of polysaccharides are challenging. The detection of carbohydrates is also limited in that they do not contain functional groups that are detectable by conventional methods such as UV absorbance. For more sensitive detection of carbohydrates, therefore, the aglycone groups of glycans are usually modified with UV-absorbing and fluorescent groups. It must be noted, however, that these introduced functional groups may affect the hydrolysis by the enzyme [25-27]. The probes conjugated fluorescent group have been developed in many fields to detect an enzyme and ion with high sensitivity. The detection techniques utilize structural changes of the probes which bound to them. By binding to them, the probes exhibit a fluorescent or a fluorescent wavelength change. Therefore, the high sensitive detection are achieved by overcoming background of unbound probes [28-32].

An endo-β-1,3-glucanase from *Cellulosimicrobium cellulans*

DK-1 was previously purified for sequence and crystal structure determination [15]. While X-ray analysis of the co-crystal of endo-β-1,3-glucanase with its substrate is important for substrate recognition analysis, it is difficult to achieve this due to substrate hydrolysis by the enzyme. For this reason, hydrolases which are genetically modified to have no hydrolytic activity are often used to investigate substrate recognition using thermodynamic analysis and X-ray analysis of co-crystals. In such cases, however, the modified enzymes must be shown to have the same structure as its native counterpart.

In this study, the effect of the aglycone portion of glycan on hydrolysis by endo-β-1,3-glucanase was investigated. The aglycones may interact with the amino acid residues near the catalytic domain of endo-β-1,3-glucanase and thus not only the carbohydrate units but also the aglycone units are important for recognition by endo-β-1,3-glucanase. To investigate the substrate specificity of endo-β-1,3-glucanase, glycol conjugates with various functional groups on the aglycone of β-glucan trisaccharide were synthesized and used to evaluate the hydrolysis activity of the enzyme.

MATERIALS AND METHODS

^{1}H NMR spectra were recorded at 400 or 700 MHz using a Bruker AVANCE 400 Plus Nanobay, or a JEOL RESONANCE JNM-ECZ700R spectrometer in chloroform-*d* and deuterium oxide. ^{13}C NMR spectra were recorded at 101 or 176 MHz with the same instruments. Chemical shifts are given in ppm (δ) and referenced to Me$_4$Si or to the internal solvent signal used as an internal standard. Assignments in the NMR spectra were made by first-order analysis of spectra, and supported by correlation spectroscopy and heteronuclear chemical shift correlation. Matrix-assisted laser desorption ionization time-of-flight high-resolution mass spectra (MALDI-TOF HRMS) were recorded on a JEOL JMS-S3000 using 2,5-dihydroxylbenzoic acid as matrix.

Unless otherwise stated, all commercially available solvents and reagents were used without further purification. Dry solvents were prepared by storage over molecular sieves which were activated in vacuum at 200 °C. Silica gel column chromatography was performed with silica gel 60N, spherical neutral, particle size 40-50 μm or 63-210 μm (Kanto Chemical Co. Inc.). Reverse phase column chromatography was carried out using C18 Sep-pak VacCartridge (particle size 55-105 μm, 10g; Waters). Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel 60 F$_{254}$ (layer thickness, 0.25 mm; Merk). For detection of intermediates, TLC plates were dipped in a solution of 85:10:5 (v/v/v) methanol:resorcinol:concentrated sulfuric acid and heated for a few minutes or irradiated with UV lamp.

HPLC analysis was performed on a Shimadzu HPLC system equipped with a TSKgel Amide-80 column (Tosoh Bioscience, 4.6 mm × 250 mm, 5 μm). The HPLC system was consisted of a system controller (CBM-20A), a diode array detector (SPD-M20A), a pumps (LC-20AD), an auto sampler (SIL-20AC), a column oven (CTO-20AC), and a degasser (DGU-20As).

2,4,6-Tri-*O*-acetyl-3-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl)-α-D-glucopyranosyl 2,2,2-trichloroacetimidate (2)

Compound **1** (20 mg, 39.6 μmol) was dissolved in pyridine (2.0 mL) and acetic anhydride (1.5 mL), and the mixture was stirred at room temperature for 6 h. The mixture was then co-evaporated with toluene to give peracetate (38 mg), of which 15 mg (15.5 μmol) was dissolved in *N*, *N*-dimethylformamide (100 μL). Ammonium carbonate (20.6 mg) was added to the peracetate solution and the mixture was stirred vigorously at room temperature for 21 h. Next, ethyl acetate was added to the suspension which was then washed with water, dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was dissolved in dichloromethane (206 μL) trichloroacetonitrile (10.3μL, 103μmol) was added to the resulting solution. After the solution was cooled in an ice bath, 1, 8-diazabicyclo [5.4.0]-7-undecene (0.2μL, 1.3μmol) was added and the mixture was then stirred at room temperature for 7 h. The solution was concentrated and the residue was purified by silica gel chromatography with 3:1 to 1:1 (v/v) hexane: ethyl acetate to give **2** (13mg, 79%): ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.71 (s, 1H), 6.47 (d, $J_{1,2}$= 3.7 Hz, 1H, H-1), 5.16-5.03 (m, 3H), 4.96-4.88 (m, 3H), 4.56 (d, $J_{1'',2''}$ = 8.4 Hz, 1H, H-1''), 4.52 (d, $J_{1',2'}$ = 8.0 Hz, 1H, H-1'), 4.44-4.37 (m, 2H), 4.26-4.10 (m, 7H), 4.07-4.02 (m, 1H), 3.86 (t, J = 9.4 Hz, 1H), 3.80-3.76 (m, 1H), 3.71-3.67 (m, 1H), 2.15-1.99 (m, 27H).

Methyl 2,4,6-tri-*O*-acetyl-3-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl)-β-D-glucopyranoside (3)

To a solution of **2** (50 mg, 46.7 μmol) and methanol (9.47 μL, 234 μmol) in dichloromethane (233 μL), molecular sieves 3Å powder (40.7 mg) was added and the mixture was then stirred at room temperature for 15 min before being cooled at -40°C for 30 min. Trimethylsilyl trifluoromethansulfonate (1.7 μL, 9.4 μmol) diluted in dichloromethane (33 μL) was added to the mixture which was then stirred at -10°C for 30 min. Next, the mixture was cooled at -40°C and methanol (9.47 μL, 234 μmol) and trimethylsilyl trifluoromethansulfonate (1.7 μL, 9.4 μmol) diluted in dichloromethane (20 μL) were added. The resulting mixture was then stirred at -40°C for 80 min. The mixture was diluted with dichloromethane and filtered through celite. The filtrate was then washed successively with aqueous saturated sodium hydrogen carbonate and brine, dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was purified by silica gel chromatography with 3:1 (v/v) toluene:acetone to give mixture (18 mg). The mixture was finally purified by C18 Sep-pak cartridge (10g) with 3–10% methanol solution to give **3** (18 mg, 41%). ^{1}H NMR (700 MHz, CDCl$_3$)δ 5.11-5.09 (dd, 1 H, H-2, $J_{1,2}$ = 8.4 Hz, $J_{2,3}$ = 9.8 Hz), 5.06-5.03 (t, 1 H, H-3'', $J_{2'',3''}$ = $J_{3'',4''}$ = 9.8 Hz), 4.95-4.89 (m, 4 H, H-2', H-3, H-3', H-4''), 4.49-4.48 (d, 1 H, H-1, $J_{1,2}$ = 8.4 Hz), 4.44-4.43 (d, 1 H, H-1', $J_{1',2'}$ = 8.4 Hz), 4.37-4.36 (m, 1 H, H-6a'), 4.31-4.28 (m, 2 H, H-1'', H-6b', $J_{1'',2''}$ = 8.4 Hz), 4.19-4.14 (m, 2 H, H-6a, H-6b), 4.05-4.00 (m, 2 H, H-6a'', H-6b''), 3.86-3.84 (br-dd, 1 H, H-4), 3.80-3.78 (br-dd, 1 H, H-4'), 3.67-3.63 (m, 3 H, H-5, H-5', H-5''), 3.45 (s, 3 H, CH$_3$O-), 2.34-2.01 (m, 30 H, Ac×30); ^{13}C NMR (176 MHz, D$_2$O) δ 170.9, 170.7, 170.6, 170.4, 169.6, 169.4, 169.2, 168.9, 129.1, 128.3, 125.4, 101.5, 101.1, 100.8, 79.1, 78.3,

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77.3, 77.1, 76.9, 72.9, 72.9, 72.7, 71.9, 71.7, 71.7, 70.9, 68.5, 68.1, 62.3, 62.1, 61.7, 56.7, 53.5, 31.0, 29.8, 29.4, 21.2, 20.9, 20.8, 20.7, 20.6, 20.5, 20.4, MALDI-TOF-HRMS (positive ion): (m/z) calcd for ($C_{45}H_{58}O_{27}$+Na⁺): 968.2801; found m/z: 968.2805.

p-Methoxyphenyl 2,4,6-tri-*O*-acetyl-3-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl)-β-D-glucopyranoside (4)

To a solution of **2** (19 mg, 17.8 µmol) and *p*-methoxyphenol (11 mg, 89.0 µmol) in dichloromethane (340 µL), molecular seives 4Å powder (50 mg) was added and the mixture was then stirred at room temperature for 30 min before being cooled at -40 °C for 30 min. Trimethylsilyl trifluoromethansulfonate (1.6 µL, 8.85 µmol) diluted in dichloromethane (16 µL) was added to the mixture which was then stirred at -40 °C for 45 min. The mixture was diluted with dichloromethane and filtered through celite. The filtrate was then washed successively with aqueous sodium hydrogen carbonate and brine, dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was purified by silica gel chromatography with 8:1 to 5:1 (v/v) toluene:acetone to give **4** (15 mg, 83%): ¹H NMR (400 MHz, CDCl₃) δ 6.93-6.89 (m, 2 H, Ph), 6.83-6.79 (m, 2 H, Ph), 5.23-5.18 (dd, 1 H, H-2, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 9.2 Hz) 5.15-5.04 (m, 2 H, H-2″ H-4′), 5.02-4.96 (br-dd, 1 H, H-4), 4.93-4.88 (m, 3 H, H-1″, H-2′, H-4″), 4.83-4.81 (d, 1 H, H-1), 4.52-4.48 (m, 2 H, H-1′, H-3″, $J_{1',2'}$= 8.0 Hz), 4.42-4.37 (dd, 1 H, H-6a′,$J_{5',6a'}$=4.0 Hz, $J_{6a',6b'}$= 12.8 Hz), 4.34-4.30 (dd, 1 H, H-6b′, $J_{5',6b'}$ =4.4 Hz), 4.24-4.16 (m, 2 H, H-6a, H-6b), 4.08-4.01 (m, 2 H, H-6a″, H-6b″), 3.97-3.92 (t, 1 H, H-3, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz), 3.85-3.77 (m, 5 H, CH₃O-, H-3′, H-5), 3.70-3.66 (m, 2 H, H-5′, H-5″), 2.35-1.98 (m, 30 H, Ac-×10); ¹³C NMR (101 MHz, CDCl₃) δ 170.7, 170.6, 170.5, 170.3, 169.5, 169.3, 169.2, 168.9, 168.8, 155.7, 151.0, 137.9, 129.0, 128.2, 125.3, 118.5, 114.5, 101.1, 100.7, 100.1, 79.0, 78.1, 77.4, 77.0, 76.7, 72.9, 72.7, 72.0, 71.7, 71.6, 70.9, 68.4, 68.2, 68.1, 62.2, 62.0, 61.7, 55.7, 21.5, 21.1, 21.0, 20.8, 20.8, 20.7, 20.6, 20.5, 20.4, 20.4; MALDI-TOF-HRMS (positive ion): (m/z) calcd for ($C_{45}H_{58}O_{27}$+Na⁺): 1053.3063; found m/z:1053.3006.

5-*N*-(9-Fluorenylmethoxycarbonyl)aminopentyl 2,4,6-tri-*O*-acetyl-3-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl)-β-D-glucopyranoside (5)

To a solution of **2** (200 mg, 187 µmol) and 5-*N*-(9-fluorenylmethoxycarbonyl) aminopentan-1-ol (304 mg, 934 µmol) in dichloromethane (17.2 mL), molecular seives 4Å powder (1.33 g) was added and the mixture was then stirred at room temperature for 1 h before being cooled at -40 °C for 30 min. Trimethylsilyl trifluoromethansulfonate (6.8 µL, 37.6 µmol) diluted in dichloromethane (1.2 mL) was added to the mixture which was then stirred at -40 °C for 1 h. Next, the mixture was cooled at -40 °C and trimethylsilyl trifluoromethansulfonate (6.8 µL, 37.6 µmol) diluted in dichloromethane (1.2 mL) was added. The resulting mixture was then stirred at -40 °C for 1 h. The mixture was diluted with dichloromethane and filtered through celite, and the filtrate was washed successively with aqueous sodium hydrogen carbonate and brine, dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was purified by silica gel chromatography with 4:1 to 3:1 (v/v)

toluene:acetone to give **5** (176 mg, 77%): ¹H NMR (400 MHz, CDCl₃) δ 7.78- 7.76 (m, 2H, Ph), 7.61-7.59 (m, 2H, Ph), 7.43-7.39 (m, 2H, Ph), 7.34-7.30 (m, 2H, Ph), 5.14-5.04 (m, 2 H, CH₂-), 4.97-4.91 (m, 5 H, H-2, H-2′, H-2″, H-5′, NH-), 4.51-4.49 (d, 1 H, H-1, $J_{1,2}$ = 8.0 Hz), 4.46-4.44 (d, 1 H, H-1′, $J_{1,2}$ = 8.0 Hz), 4.41-4.29 (m, 5 H, H-1″, H-3″, H-4′, H-5, H-5″, $J_{1'',2''}$ = 8.0 Hz), 4.24-4.20 (m, 2 H, H-4, H-4″), 4.07-4.01 (m, 2 H, H-6a, H-6b), 4.07-4.01 (m, 2 H, H-6a″, H-6b″), 3.88-3.78 (m, 3 H, CH₂-, H-6a′), 3.69-3.64 (m, 3 H, H-3, H-3′, H-6b′), 3.46-3.40 (m, 1 H, -CH), 3.22-3.14 (m, 2 H, CH₂-), 2.18-1.97 (m, 30 H, Ac×10), 1.69-1.48 (m, 6 H, CH₂-×3);¹³C NMR (101 MHz, CDCl₃) δ 170.8, 170.6, 170.5, 170.3, 169.4, 169.3, 169.1, 169.1, 168.8, 168.8, 156.4, 144.0, 141.3, 127.7, 127.0, 125.0, 120.0, 101.0, 100.7, 100.6, 79.0, 78.3, 77.4, 77.0, 76.7, 73.0, 72.9, 72.6, 71.8, 71.7, 71.6, 70.8, 69.3, 68.4, 68.2, 68.0, 66.5, 62.2, 62.0, 61.7, 53.4, 47.3, 40.9, 31.6, 29.6, 29.0, 23.0, 22.7, 21.0, 20.8, 20.7, 20.6, 20.5, 20.4, 20.4, 14.1; MALDI-TOF-HRMS (positive ion): (m/z) calcd for ($C_{58}H_{73}N_{1}O_{28}$+Na⁺): 1254.4217; found m/z:1254.4214.

Methyl 3-*O*-(3-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl)-β-D-glucopyranoside (6)

Compound**5** (18 mg, 19.2 µmol) was dissolved in 1.3% NaOMe—MeOH (1.05 mL), and the mixture was stirred at room temperature for 23 h. The mixture was then added SK1B (H⁺) resin to neutralize the solution. Next, the suspension was filtered and the filtrate was evaporated. The residue was purified by C18 Sep-pak cartridge (10g) with 3% methanol solution to give **6** (10 mg, quant.); ¹H NMR (700 MHz, D₂O) δ 4.74-4.70 (m, 1 H, H-3), 4.67-4.66 (d, 1 H, H-1′, $J_{1',2'}$= 7.7 Hz), 4.66-4.65 (d, 1 H, H-1″, $J_{1'',2''}$= 8.4 Hz), 4.32-4.31 (d, 1 H, H-1, $J_{1,2}$= 8.4 Hz), 3.84-3.81 (m, 3 H, H-6a, H-6a′, H-6a″), 3.68-3.62 (m, 5 H, H-5, H-5′, H-6b, H-6b′, H-6b″), 3.48 (s, 3 H, CH₃O-), 3.47-3.35 (m, 7 H, H-2″, H-3′, H-3″, H-4, H-4′, H-4″, H-5″), 3.32-3.31 (dd, 1 H, H-2′), 3.27-3.26 (dd, 1 H, H-2); ¹³C NMR (176 MHz, D₂O) δ 103.0, 102.9, 102.6, 84.5, 84.3, 76.0, 75.7, 75.6, 73.5, 73.3, 72.9, 69.6, 68.2, 68.2, 60.7, 57.3; MALDI-TOF-HRMS (positive ion): (m/z) calcd for ($C_{19}H_{34}O_{16}$+Na⁺): 541.1745; found m/z: 541.1731.

p-Methoxyphenyl 3-*O*-(3-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl)-β-D-glucopyranoside (7)

Compound 4 (35 mg, 33.9 µmol) was dissolved in 2.8% NaOMe—MeOH (1.1 mL) and the mixture was then stirred at room temperature for 15.8 h. The mixture was added SK1B (H⁺) resin to neutralize the solution. Next, the mixture was filtered and the filtrate was evaporated to give **7** (20 mg, 95%): ¹H NMR (700 MHz, D₂O) δ 7.03-7.01 (m, 2 H, Ph), 6.90-6.88 (m, 2 H, Ph), 4.94-4.93 (d, 1 H, H-1, $J_{1,2}$ = 8.4 Hz), 4.74-4.70 (m, 2 H, H-1′, H-3′), 4.66-4.65 (d, 1 H, H-1″, $J_{1'',2''}$ = 7.7 Hz), 3.84-3.81 (m, 3 H, H-6b, H-6a″, H-6b″), 3.76-3.74 (br-dd, 1 H, H-2), 3.71 (s, 3 H, CH₃O-), 3.70-3.61 (m, 4 H, H-5, H-5″, H-6a, H-6b′), 3.51-3.37 (m, 7 H, H-3, H-3″, H-4, H-4′, H-4″, H-5′, H-6a′), 3.32-3.29 (dd, 1 H, H-2″, $J_{2'',3''}$ = 9.8 Hz), 3.27-3.25 (dd, 1 H, H-2′, $J_{2',3'}$ = 9.8 Hz);¹³C NMR (176 MHz, D₂O) δ 154.8, 150.9, 118.3, 115.1, 102.9, 102.6, 101.1, 84.3, 84.0, 76.0, 75.8, 75.7, 75.6, 73.5, 73.3, 72.8, 69.6, 68.2, 68.0, 60.7, 60.5, 55.8; MALDI-TOF-HRMS (positive ion): (m/z) calcd for ($C_{25}H_{38}O_{17}$+Na⁺): 633.2007; found m/z:633.2048.

5-Acetamidopentyl 3-*O*-(3-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl)-β-D-glucopyranoside (8)

Compound **2** (39 mg, 31.7 μmol) was dissolved in dichloromethane (2.0mL) and the solution was added piperidine (939 μL) in dichloromethane (1.76 mL) at room temperature for 75 min.The solution was then evaporated and the residue which was cooled on ice bath was added acetic anhydride (1.5 mL) at room temperature for 30 min. Next, the mixture was diluted with dichloromethane and washed successively with ice water, 1M HCl aqueous, saturated sodium hydrogen carbonate and brine, dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was purified by silica gel chromatography with 13:10 to 1:1 (v/v) toluene:acetone to give acetamide derivative (27 mg), which was dissolved in 0.28% NaOMe—MeOH (2.02 mL) and stirred at room temperature for 16 h. The mixture was added SK1B (H⁺) resin to neutralize the solution, and the mixture was filtered and the filtrate was evaporated to give **8** (10 mg, 50%): ^{1}H NMR (700 MHz, D$_2$O) δ 4.74-4.70 (br-dd, 1 H, H-3″), 4.68-4.67 (d, 1 H, H-1″, $J_{1″,2″}$ = 9.1 Hz), 4.65-4.64 (d, 1 H, H-1′, $J_{1′,2′}$ = 7.7 Hz), 4.38-4.37 (d, 1 H, H-1, $J_{1,2}$ = 8.4 Hz), 3.84-3.81 (m, 4 H, -NH, H-3, H-4′, H-6b), 3.69-3.56 (m, 6 H, CH$_2$-, H-3′, H-4, H-5″, H-6a), 3.46-3.34 (m, 7 H, H-2′, H-4″, H-5, H-5′, H-6a, H-6b, H-6a′, H-6b′), 3.32-3.29 (dd, 1 H, H-2, $J_{2,3}$ = 9.8 Hz), 3.27-3.24 (dd, 1 H, H-2″ $J_{2″,3″}$= 8.4 Hz), 3.27-3.06 (dd, 2 H, CH$_2$-, *J*= 6.3 Hz, 7.0 Hz), 1.87 (s, 3 H, Ac-), 1.57-1.52 (m, 2 H, CH$_2$-), 1.45-1.41 (m, 2 H, CH$_2$-), 1.31-1.26 (m, 2 H, CH$_2$-); ^{13}C NMR (176 MHz, D$_2$O) δ 174.0, 102.9, 102.6, 102.0, 84.4, 84.2, 76.0, 75.7, 75.6, 73.5, 73.3, 73.0, 70.5, 69.6, 68.2, 68.2, 60.7, 39.4, 28.4, 28.0, 22.5, 21.9; MALDI-TOF-HRMS (positive ion): (m/z) calcd for (C$_{25}$H$_{45}$N$_1$O$_{17}$+Na⁺): 654.2585; found m/z:654.2556.

5-(*N*-(Fluorescein-5-yl)-thioureido)aminopentyl 3-*O*-(3-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl)-β-D-glucopyranoside (9)

Compound **2** (41 mg, 33.3 μmol) was dissolved in 14 w% aqueous NH$_3$ solution (10.0 mL), and the mixture was stirred at room temperature for 18.0 h. The mixture was then evaporated and the residue was dissolved in water. The solution was washed with dichloromethane and the aqueous layer was lyophilized to give the amine derivative (20 mg), which was dissolved in methanol (1.0 mL). The solution was added 5-isothiocyanatofluorescein (20.6 mg) in methanol (1.0 mL) and the mixture was stirred at room temperature for 4 h. The solution was then evaporated and the residue was purified by C18 Seppak cartridge (10g) with 60% methanol solution. Subsequently, the mixture was finally purified by silica gel chromatography with 2:1 to 1:2 (v/v) dichloromethane:methanol to give **9** (8 mg, 24%): ^{1}H NMR (400 MHz, D$_2$O) δ 7.64-6.59 (m, 9 H, Ph), 4.60-4.58 (m, 2 H, H-1′, H-1″), 4.40-4.34 (d, 1 H, H-1, $J_{1,2}$ = 8.0 Hz), 3.83-3.77 (m, 4 H, H-4, H-4′, CH$_2$-), 3.68-3.58 (m, 8 H, H-3, H-3′, H-3″, H-4″, H-6a, H-6b′, H-6a″, H-6b″), 3.43-3.22 (m, 10 H, H-2, H-2′, H-2″, H-5, H-5′, H-5″, H-6b, H-6b′, CH$_2$-), 1.57-1.33 (m, 6 H, CH$_2$-×3);^{13}C NMR (101 MHz, CDCl$_3$) δ 218.3, 214.8, 191.2, 103.1, 102.8, 102.8, 102.5, 102.1, 101.8, 84.3, 84.3, 75.9, 75.9, 75.6, 75.4, 73.7, 73.5, 73.4, 73.4, 73.2, 73.1, 72.9, 72.8, 70.5, 70.4, 70.2, 70.2, 69.5, 69.4, 69.3, 68.4, 68.1, 68.0, 67.8, 61.0, 60.7, 60.5, 60.3, 54.7, 54.5, 54.4, 48.8, 48.8, 48.7, 31.6, 31.6, 28.4, 28.3, 28.1, 28.1, 27.8,

27.6, 22.6, 20.0; MALDI-TOF-HRMS (positive ion): (m/z) calcd for (C$_{44}$H$_{54}$N$_2$O$_{21}$S$_1$+Na⁺): 1001.2837; found m/z:1001.2816.

Hydrolysis activity assay

Enzyme reactions (total volume: 1.0 mL) containing substrates (50 μM) and endo-β-1,3-glucanase (25 μM) purified as previously reported [14] were carried out in 40 mM potassium phosphate (pH 7.0) at 40°C. Between 10 min and 4 h after the reactions were initiated, 100 μL samples of the reaction volume were removed and diluted in 100 μL acetonitrile and 300 μL water, after which the resulting solutions were lyophilized (for **6**, **7** and **8**). The residues were dissolved in 15 μL acetonitrile and 15 μL water before being subjected to TLC analysis along with 5 nmol of each substrate. The TLC developing solvents used were 3:3:2 (v/v/v) ethyl acetate:2-propanol:water for compounds **6** and **8** and 3:3:1 (v/v/v) ethyl acetate:2-propanol:water for compound **7**. Only the reactions carried out with compound **9** were analyzed by HPLC (TSK-gel Amide-80 [3.0 μm; 4.6 mm × 15 cm] column with water: CH$_3$CN solvent mixture [5:95 to 45:55, linear gradient for 20 min] at 40°C, 1 mL/min). After 10 min, 30 min, 1 h, 2 h and 4 h, 15 μL of the reaction mixture containing **9** (50 μM) were collected and diluted with 75 μL of CH$_3$CN and 60 μL of H$_2$O. The diluted solution (1 μL) was then further diluted with 500 μL of CH$_3$CN and 500 μL of H$_2$O. A 10 μL aliquot of this diluted solution was then injected into the HPLC system to analyze the cleavage rate of **9**. The compounds were detected using fluorescent detector (Ex. 490 nm, Em. 525 nm).

RESULTS AND DISCUSSION

In Scheme 1, β-glucantrisaccharide **1**, which is commercially available, was used as the starting material. **1** was per acetylated with acetic anhydride in pyridine, after which the peracetate was deprotected selectively at the 1-position to give hemiacetal. The hemiacetal was then reacted with trichloroacetonitrile by the addition of DBU, yielding trichloriacetimidate **2** (79% yield) in three steps [32,33]. Treatment of **2** and methanol with TMSOTf in CH$_2$Cl$_2$ yielded in methyl glycoside **3** (41% yield) [34]. Similarly, **2** was reacted with *p*-methoxyphenol to give *p*-methoxyphenyl glycoside **4** (83% yield). *N*-F moc aminopentyl glycoside **5** was synthesized by glycosidation of **2** with *N*-Fmoc amino pentanol to introduce functional groups on the aglycone.

The acetyl groups on **3** and **4** were deprotected under Zemplén conditions to yield **6** and **7** as substrates for hydrolysis by endo-β-1,3-glucanase (Scheme 2) [35-37]. Onto the glycoside **5** with an alkyl chain as a spacer on the aglycone, an acetyl group or fluorescent label was introduced as a small or bulky functional group at the ω-position. First, **5** was deprotected selectively at the Fmoc group, after which the exposed amino group was converted to an acetoamido group. The compound was then deacetylated (except at the acetamido group) to give **8** at a 50% yield. Next, **5** was soaked in a NaOMe-MeOH solution to deprotect all protecting groups, after which FITC was introduced to yield **9** at 24% yield (2 steps) [39-42]. The fluorescently labeled glycoside **9** is a useful compound that can be used for the sensitive detection of endo-β-1,3-glucanase.

Finally, the synthesized compounds (**6**,**7**,**8**,**9**) were treated with endo-β-1,3-glucanase to evaluate the resulting hydrolysis activity. While compounds **9** can be detected by fluorescence

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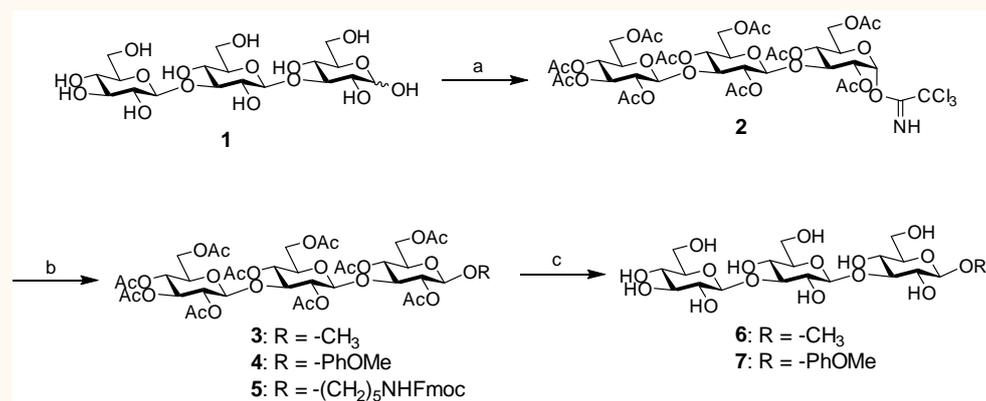
Scheme 1 Reagents and conditions: (a) (i) Ac_2O, Pyridine, rt; (ii) $(NH_3)_2CO_3$, DMF, rt; (iii) Cl_3CCN, DBU, CH_2Cl_2, rt, (b) methanol, TMSOTf, CH_2Cl_2, -40 °C for **3**; *p*-methoxyphenol, TMSOTf, CH_2Cl_2, -40 °C for **4**; FmocNH$(CH_2)_5$OH, TMSOTf, CH_2Cl_2, -40 °C for **5**; (c) NaOMe, MeOH, rt.
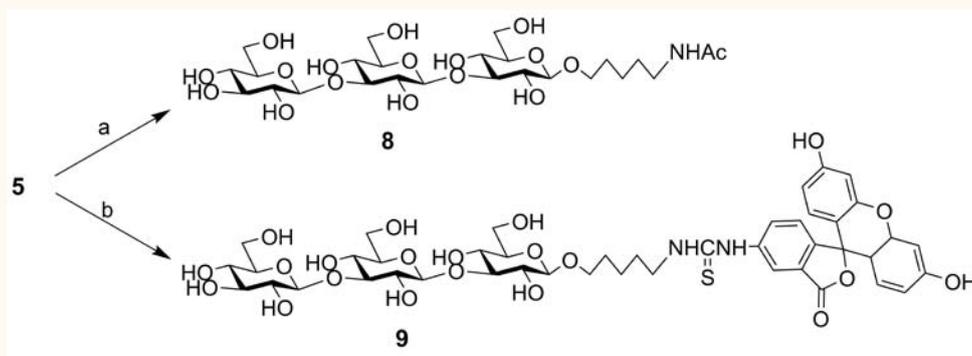
Scheme 2 Reagents and conditions: (a) (i) piperidine, CH_2Cl_2, rt; (ii) Ac_2O, rt; (iii) NaOMe, MeOH, rt, (b) (i) aqueous NH_3, rt; (ii) FITC, methanol, rt.
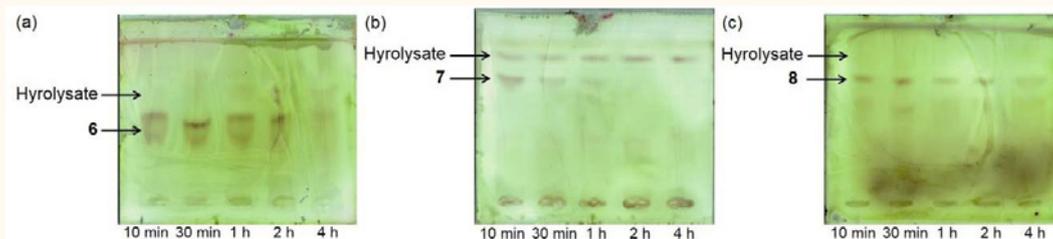

Figure 1 Thin-layer chromatography analysis of hydrolytic reactions by endo-β-1,3-glucanase with (a) Methyl glycoside **6**, (b) *p*-methoxyphenyl glycoside **7**, and (c) acetamidopentyl glycoside **8** substrates.
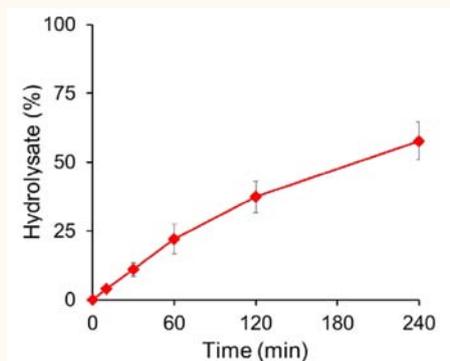

Figure 2 Time course study of hydrolytic reactions carried out with the fluorescent glycoside **9** as a substrate for endo-β-1,3-glucanase assessed by HPLC. Error bars represent one standard deviation from the mean (n = 4 experimental replicates).

analysis, compounds **6**, **7,** and **8** were shown to be inadequate for highly sensitive detection. The hydrolysis byendo-β-1,3-glucanase for compounds **6,7**, and **8** was therefore assessed using TLC. As shown in Figure 1, **7** were hydrolyzed faster than **6** and **8** and had disappeared completely in 2 hours. Half of **6** were hydrolyzed after 4 hours and **8** were not observed a hydrolysate. **9** were evaluated in terms of hydrolysis activity using HPLC, which revealed thatca. 60% of **9** had been hydrolyzed after 4 hours. The results reported here demonstrate that hydrophobic functional groups on the aglycone are required for a strong interaction with the catalytic domain of endo-β-1,3-glucanase. Moreover, the hydrophobic groups which are close to the carbohydrate unit were found to have an effect on the hydrolysis activity of the enzyme. The slightly bulkier FITC group on**9,** compared with the *p*-methoxypheny group of **7** may have caused steric hindrance with the amino acid residues close to the catalytic site of endo-β-1,3-glucanase; however, hydrolytic activity was detected at a few femtomole of **9,** demonstrating the potency of the fluorescent probe **9** as an indicator of the enzyme activity. Overall, these findings demonstrate that the aglycone structure is also important for high activity in endo-type enzymes, and that the carbohydrate unit of a trisaccharide was indeed hydrolyzed by endo-β-1,3-glucanase. This study also demonstrates that substrates for endo-β-1,3-glucanase can be simply synthesized from commercially available reagents.

CONCLUSION

The β-glucan trisaccharides with methyl group, *p*-methoxyphenyl group, acetamido group and fluorescein on the aglycones were synthesized. The hydrolysis activity of endo-β-1,3-glucanase was evaluated using the synthesized substrates on TLC and HPLC. The result showed that the substrates with the hydrophobic aglycones such as *p*-methoxyphenol and fluorescein were hydrolyzed efficient and *p*-methoxyphenyl glycoside was hydrolyzed completely in 2 hours. Therefore, the aglycones of glycosides affect the hydrolysis of endo-β-1,3-glucanase and the catalytic domain may also occur the interaction with aglycone moiety. Consequently, the aglycones should be selected a suitable functional group to give highly hydrolytic activity. The substrate with high sensitivity for endo-β-1,3-glucanase will be utilized as the indicator for infection by bacteria and fungi. Further, synthesis of β-glucan oligosaccharides with branches bound in β-1,6 linkage are currently ongoing. They clarify the substrate specificity of endo-β-1,3-glucanase for carbohydrate unit and a more sensitive substrate will be developed.

REFERENCES

1. Henrissat B, Bairoch, A. Updating the sequence-based classification of glycosyl hydrolases. Biochem J. 1996; 316: 695-696.

2. Davies GJ, Wilson KS, Henrissat B. Nomenclature for sugar-binding subsites in glycosyl hydrolases. Biochem J. 1997; 321: 557-559.

3. McCarter JD, Withers GS. Mechanisms of enzymatic glycoside hydrolysis. Curr Opin Struct Biol. 1994; 4: 885-892.

4. Davies G, Henrissat B. Structures and mechanisms of glycosyl hydrolases. Structure.1995; 3: 853-859.

5. Lammens W, Le Roy K, Schroeven, L, Van Laere A, Rabijns A, Van den Ende W. Structural insights into glycoside hydrolase family 32 and 68 enzymes: functional implications. J Exp Bot. 2009; 60: 727-740.

6. Henrissat B, Davies G. Structural and sequence-based classification of glycoside hydrolases. Curr Opin Struct Biol. 1996; 7: 637-644.

7. Adams DJ. Fungal cell wall chitinases and glucanases. Microbiology. 2004; 150: 2029-2035.

8. Yamamoto K, Li SC, Li YT, Ernst B, Hart, GW, Sinaý P. Microbial glycosidases. Carbohydr Chem Biol. 2000; 497-511.

9. Kumagai Y, Yamashita K, Tagami T, Uraji M, Wan K, Okuyama M, et al. The loop structure of Actinomycete glycoside hydrolase family 5 mannanases governs substrate recognition. FEBS J. 2015; 282: 4001-4014.

10. Hakki Z, Thompson AJ, Bellmaine S, Speciale G, Davies GJ, Williams SJ. Structural and Kinetic Dissection of the *endo*-α-1,2-Mannanase Activity of Bacterial GH99 Glycoside Hydrolases from *Bacteroides spp.* Chemistry. 2015; 21: 1966-1977.

11. Stubbs KA, Scaffidi A, Debowski AW, Mark BL, Stick RV, Vocadlo DJ. Synthesis and use of mechanism-based protein-profiling probes for retaining β-D-glucosaminidases facilitate identification of *Pseudomonas aeruginosa* NagZ. J Am Chem Soc. 2008; 130: 327-335.

12. Walvoort MT, Kallemeijn WW, Willems LI, Witte MD, Aerts JM, van der Marel GA, et al. Tuning the leaving group in 2-deoxy-2-fluoroglucoside results in improved activity-based retaining β-glucosidase probes. Chem Commun (Camb). 2012; 48: 10386-10388.

13. Saikia R, Singh BP, Kumar R, Arora DK. Detection of pathogenesisrelated proteins-chitinase and β-1, 3-glucanase in induced chickpea. Curr Sci. 2005; 9: 659-663.

14. Glunde K, Foss CA, Takagi T, Wildes F, Bhujwalla ZM. Synthesis of 6'-O-lissamine-rhodamine B-glucosamine as a novel probe for fluorescence imaging of lysosomes in breast tumors. Bioconjugate chem. 2005; 16: 843-851.

15. Tanabe Y, Oda M. Molecular characterization of endo-1,3-β-glucanase from *Cellulosimicrobium cellulans*: effects of carbohydrate-binding module on enzymatic function and stability. Biochim Biophys Acta. 2011; 1814: 1713-1719.

16. Akiyama T, Pillai MA, Sentoku N. Cloning, characterization and expression of *OsGLN2*, a rice endo-1,3-β-glucanase gene regulated developmentally in flowers and hormonally in germinating seeds. Planta. 2004; 220: 129-139.

17. Martin K, McDougall BM, McIlroy S, Chen J, Seviour RJ. Biochemistry and molecular biology of exocellular fungal β-(1, 3)-and β-(1, 6)-glucanases. FEMS Microbiol Rev. 2007; 31: 168-192.

18. Patrick WM, Nakatani Y, Cutfield SM, Sharpe ML, Ramsay RJ, Cutfield JF. Carbohydrate binding sites in Candida albicans exo-β-1,3-glucanase and the role of the Phe-Phe ′clamp′ at the active site entrance. FEBS J. 2007; 277: 4549-4561.

19. Kawai R, Igarashi K, Yoshida M, Kitaoka M, Samejima M. Hydrolysis of β-1,3/1,6-glucan by glycoside hydrolase family 16 endo-1, 3 (4)-β-glucanase from the basidiomycete *Phanerochaete chrysosporium*. Appl Microbiol Biotechnol. 2006; 71: 898-906.

20. Wong YS, Maclachlan GA. 1,3-β-D-glucanases from *Pisum Sativum* seedlingsII. Substrate specificities and enzymic action patterns. Biochim Biophys Acta. 1979; 571: 256-269.

21. Hong TY, Cheng CW, Huang JW, Meng M. Isolation and biochemical characterization of an endo-1,3-β-glucanase from *Streptomyces sioyaensis* containing a C-terminal family 6 carbohydrate-binding module that binds to 1,3-β-glucan. Microbiology. 2002; 148: 1151-1159.

22. Zverlov VV, Volkov IY, Velikodvorskaya GA, Schwarz WH. The binding pattern of two carbohydrate-binding modules of laminarinase

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Lam16A from *Thermotoga neapolitana*: differences in β-glucan binding within family CBM4. Microbiology. 2001; 147: 621-629.

23. Krah M, Misselwitz R, Politz O, Thomsen KK, Welfle H, Borriss R. The laminarinase from thermophilic eubacterium *Rhodothermus marinus*. Eur J Biochem. 1998; 257: 101-111.

24. Lafond M, Navarro D, Haon M, Couturier M, Berrin JG. Characterization of a broad-specificity β-glucanase acting on β-(1,3)-, β-(1,4)-, and β-(1,6)-glucans that defines a new glycoside hydrolase family. Appl Environ Microb. 2012; 78: 8540-8546.

25. Hösel W, Conn EE. The aglycone specificity of plant β-glycosidases. Trends Biochem Sci. 1982; 7: 219-221.

26. Berrin JG, Czjzek M, Kroon PA, McLauchlan WR, Puigserver A, Williamson G, et al. Substrate (aglycone) specificity of human cytosolic β-glucosidase. Biochem J. 2003; 373: 41-48.

27. Verdoucq L, Czjzek M, Moriniere J, Bevan DR, Esen A. Mutational and structural analysis of aglycone specificity in maize and sorghum β-glucosidases. J Biol Chem. 2003; 278: 25055-25062.

28. Mao Z, Wang M, Liu J, Liu LJ, Lee SMY, Leung CH, Ma DL. A long lifetime switch-on iridium (iii) chemosensor for the visualization of cysteine in live zebrafish. Chem Commun. 2016; 52: 4450-4453.

29. Wong CY, Chung LH, Lin S, Chan DSH, Leung CH, Ma DL. A Ruthenium (II) Complex Supported by Trithiacyclononane and Aromatic Diimine Ligand as Luminescent Switch-On Probe for Biomolecule Detection and Protein Staining. Sci Rep. 2014; 4: 7136.

30. van Es HH, Veldwijk M, Havenga M, Valerio D. A flow cytometric assay for lysosomal glucocerebrosidase. Anal Biochem. 1997; 247: 268-271.

31. Komatsu T, Kikuchi K, Takakusa H, Hanaoka K, Ueno T, Kamiya M, et al. Design and synthesis of an enzyme activity-based labeling molecule with fluorescence spectral change. J Am Chem Soc. 2006; 128: 15946-15947.

32. Kurishita Y, Kohira T, Ojida A, Hamachi I. Rational design of FRET-based ratiometric chemosensors for *in vitro* and in cell fluorescence analyses of nucleoside polyphosphates. J Am Chem Soc. 2010; 132: 13290-13299.

33. Matsuo I, Isomura M, Miyazaki T, Sakakibara T, Ajisaka K. Chemoenzymatic synthesis of the branched oligosaccharides which correspond to the core structures of N-linked sugar chains. Carbohydr Res. 1997; 305: 401-413.

34. van der Peet P, Gannon CT, Walker I, Dinev Z, Angelin M, Tam S, et al. Use of Click Chemistry to Define the Substrate Specificity of Leishmania β-1, 2-Mannosyltransferases. Chem Bio Chem. 2006; 7: 1384-1391.

35. Schmidt RR, Grundler G. α-Linked disaccharides from *O*-(β-D-glycopyranosyl) trichloroacetimidates using trimethylsilyl trifluoromethanesulfonate as catalyst. Angew Chem Int Ed Engl. 1982; 21: 781-782.

36. Miyagawa A, Matsuda T, Yamamura H. Synthesis of Branched Tetrasaccharide Derivatives of Schizophyllan-like β-Glucan. J Carbohydr Chem. 2015; 34: 215-246.

37. Cheng H, Cao X, Xian M, Fang L, Cai TB, Ji JJ, et al. Synthesis and enzyme-specific activation of carbohydrate-geldanamycin conjugates with potent anticancer activity. J Med Chem. 2005; 48: 645-652.

38. Miyagawa A, Kurosawa H, Watanabe T, Koyama T, Terunuma D, Matsuoka K. Synthesis of glycoconjugate polymer carrying globotriaose as artificial multivalent ligand for Shiga toxin-producing *Escherichia coli* O157: H7. Carbohydr Polym. 2004; 57: 441-450.

39. Ziegler T, Röseling D, Subramanian LR. Preparation of glycosyl amino acids as building blocks for the combinatorial synthesis of neoglycoconjugates. Tetrahedron Asymmetry. 2002; 13: 911-914.

40. Neilson T, Werstiuk ES. Oligoribonucleotide Synthesis. II. Preparation of 2'-*O*-tetrahydropyranyl Derivatives of Adenosine and Cytidine Necessary for Insertion in Stepwise Synthesis. Can J Chem. 1971; 49: 493-499.

41. Gentsch R, Pippig F, Nilles K, Theato P, Kikkeri R, Maglinao M, et al. Modular approach toward bioactive fiber meshes carrying oligosaccharides. Macromolecules. 2010; 43: 9239-9247.

42. Dowlut M, Hall DG, Hindsgaul O. Investigation of nonspecific effects of different dyes in the screening of labeled carbohydrates against immobilized proteins. J Org Chem. 2005; 70: 9809-9813.