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Note

α-Mannosidase-catalyzed synthesis of a (1 → 2)-α-D-rhamnodisaccharide derivative

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Abstract—Enzymatic transglycosylation using *p*-nitrophenyl α-D-rhamnopyranoside as the glycosyl donor and 6 equiv of ethyl 1-thio-α-D-rhamnopyranoside as the glycosyl acceptor yielded a D-rhamnooligosaccharide derivative. The reaction was catalyzed by jack bean α-mannosidase in a 1:1 (v/v) mixture of 0.1 M sodium citrate buffer (pH 4.5)–MeCN at 25 °C. The enzyme exhibited high catalytic activity for the reaction, to afford in 32.1% isolated yield (based on donor substrate) ethyl α-D-rhamnopyranosyl-(1 → 2)-1-thio-α-D-rhamnopyranoside, which is a derivative of the common oligosaccharide unit of the antigenic lipopolysaccharides from *Pseudomonas*.

Keywords: Jack bean α-mannosidase; α-D-Rhamnosidase activity; Transglycosylation reaction; (1 → 2)-α-D-Rhamnodisaccharide derivative; *Pseudomonas* lipopolysaccharide

1. Introduction

D-Rhamnopyranose (Rha*p*), a rare sugar in nature, has been found in the O-specific polysaccharide fractions from the antigenic lipopolysaccharides of *Pseudomonas cepacia*, *P. syringae*, and *P. aeruginosa*. The repeating units in these polysaccharides have the following structures: → 3)-α-Rha*p*-(1 → 3)-α-Rha*p*-(1 → 2)-β-Rha*p*-(1 → and → 3)-α-Rha*p*-(1 → 3)-α-Rha*p*-(1 → 2)-α-Rha*p*-(1 →.[1–5] The chemical synthesis of these trisaccharide units has been previously undertaken.[6] Synthetic methodologies for the oligosaccharides, however, often require complicated processes, such as regioselective protection and deprotection of the sugar hydroxyl groups, and separation of the α and β anomers from the glycosylation products. In recent years, considerable attention has been given to the use of enzymes for oligosaccharide construction—specifically, *exo*-type carbohydrate hydrolases, such as glycosidases. In contrast to chemical synthesis, enzymatic reactions involve mild conditions, are simple, and possess precise positional and anomeric selectivity in the formation of glycosidic linkages. Several glycosidases have demonstrated efficient catalytic activities in the synthesis of oligosaccharides from donor glycosides and acceptor sugars via transglycosylation. Glycosidases are classified into different types based on their strict specificities for the glycon structure of the substrate glycosides. In the case of α-D-rhamnooligosaccharides, it seemed appropriate to use α-D-rhamnosidase for the enzymatic transglycosylation reaction; however, α-D-rhamnosidase is rarely found in nature. We have previously reported that α-mannosidases (EC 3.2.1.24) from jack bean and almond, which are commercially available, show high hydrolytic activity, not only toward α-D-mannopyranosides, but also toward their 6-deoxy analogues, α-D-rhamnopyranosides.[7] Accordingly, the specificity of these α-mannosidases toward α-D-rhamnopyranosides

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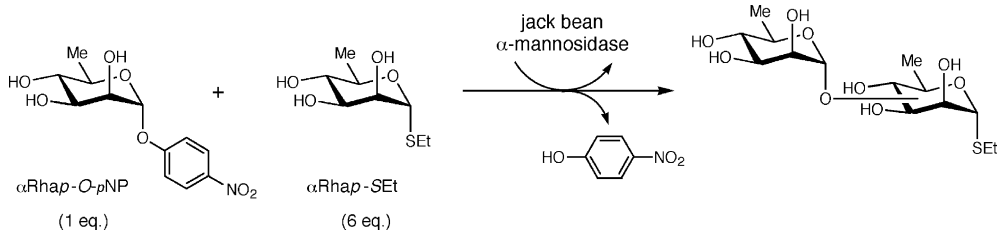

Figure 1. Schematic representation of the transglycosylation reaction between α-Rha*p*-*O*-*p*NP and α-Rha*p*-*S*Et.

can be employed in the transglycosylation reaction for the synthesis of the α-D-rhamnooligosaccharide component in the O-specific polysaccharides of antigenic lipopolysaccharides from *Pseudomonas*.

Herein, we report the jack bean α-mannosidase-catalyzed synthesis of D-rhamnodisaccharide derivatives, using *p*-nitrophenyl α-D-rhamnopyranoside (α-Rha*p*-*O*-*p*NP) as the glycosyl donor and ethyl 1-thio-α-D-rhamnopyranoside (α-Rha*p*-*S*Et) as the glycosyl acceptor (Fig. 1). Since the anomeric alkylthio group of the sugar can be readily converted into a hydroxyl group, and furthermore, since alkyl 1-thio sugars can be useful as a glycosyl donor for the chemical glycosylation in the subsequent elongation of the oligosaccharide chain, α-Rha*p*-*S*Et was chosen as the glycosyl acceptor for the enzymatic transglycosylation. Although there have been numerous reports on glycosidase-catalyzed oligosaccharide synthesis, to the best of our knowledge, there are no reports that describe the synthesis of D-rhamnooligosaccharides using the glycosidase-catalyzed transglycosylation reaction.

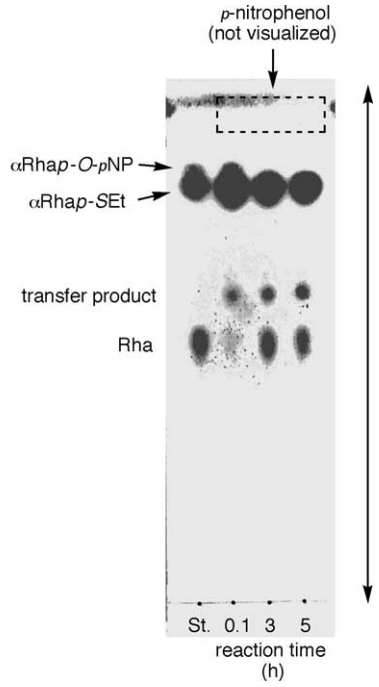

Figure 2. TLC of the compounds in the transglycosylation mixture. The positions of α-Rha*p*-*O*-*p*NP, α-Rha*p*-*S*Et, and Rha are identified based on standards are marked on the margin.

2. Results and discussion

To effectively dissolve the donor (α-Rha*p*-*O*-*p*NP) and 6 equiv of the acceptor (α-Rha*p*-*S*Et) for transglycosylation using jack bean α-mannosidase, a mixture of MeCN and 0.1 M sodium citrate buffer (1:1, v/v) was used as the reaction solvent. Using this mixture, the transglycosylation reaction was shown to proceed efficiently at 25 °C. TLC analysis of the reaction mixture showed a single transglycosylation product (Fig. 2). As shown in the HPLC analysis of the mixture (Fig. 3), most of the donor substrate was consumed within 5 h. In contrast to the TLC results (Fig. 2), HPLC analysis (Fig. 3) showed two new peaks (peaks **c** and **d**), corresponding to minor and major compounds, respectively. Attempts to further purify the product on the TLC plate and to fully separate from hydrolyzate D-rhamnose (Rha) using normal or reversed-phase column chromatography were unsuccessful. Consequently, all sugar derivatives in the mixture were isolated by silica gel column chromatography as their acetylated derivatives. Acetylation was performed on the reaction mixture after 5 h of reaction and after the solvent had been removed by evaporation. In addition to the spots corresponding to the peracetates of the remaining substrates (α-Rha*p*-*O*-*p*NP and α-Rha*p*-*S*Et) and the hydrolyzate (Rha), only one additional spot was identified on the TLC plate (data not shown). The compound corresponding to this spot was purified by silica gel column chromatography, deacetylated using 1:2:7 (v/v) Et_3N–H_2O–MeOH, and then further purified by silica gel flash column chromatography to remove contaminants produced by the deacetylation. The fractions were combined, the solvent removed by evaporation, and the resulting sample dried in vacuo to give a pure compound, identified as compound **d** by HPLC. The minor compound corresponding to peak **c** was not identified; it is possible that it is not a sugar derivative.

The structure of purified compound **d** was identified by MS and NMR spectrometry. The FABMS spectra corresponded to a $[M+H]^+$ species at m/z of 355, con-

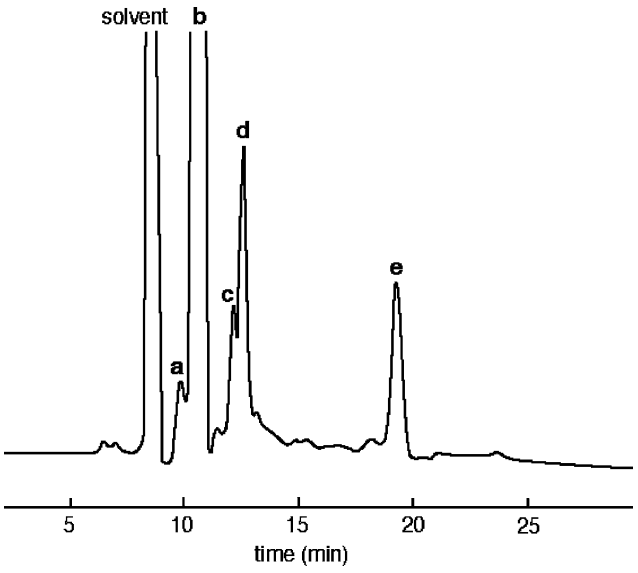

Figure 3. HPLC chromatograms of the compounds in the transglycosylation mixture after 5 h reaction. Peaks **a**, **b**, and **e** correspond to α-Rha*p*-*O*-*p*NP, α-Rha*p*-*S*Et, and *p*-nitrophenol, and Rha, respectively. The structure of compound corresponding to peak **d** is shown in Figure 4.

firming that compound **d** consisted of ethyl 1-thio-α-rhamnobioside. ^{1}H and ^{13}C NMR assignments of the disaccharide derivative are presented in Figure 4. The position of the glycosidic linkage of the aglycon moiety (α-Rha*p*-*S*Et) was detected by ^{1}H-detected multiple-bond connectivity (HMBC) NMR experiments. The

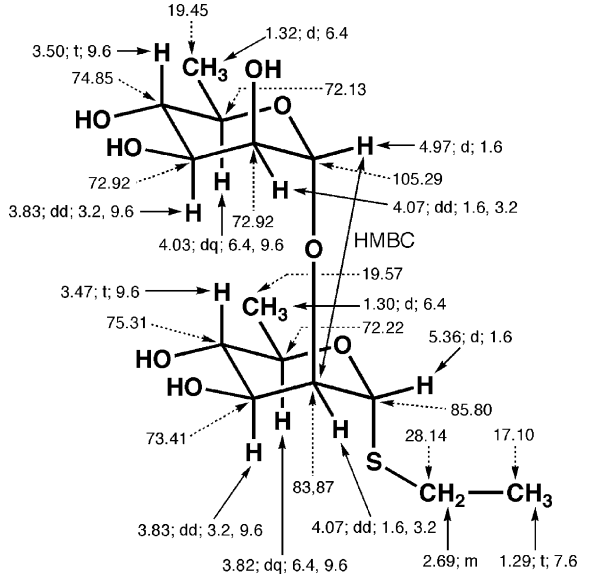

Figure 4. ^{1}H and ^{13}C NMR shifts (in D_2O) and the structure of the transglycosylation product (compound **d** in Fig. 3). ^{1}H NMR data (chemical shift in ppm; multiplicity; coupling constant in Hz) and ^{13}C NMR chemical shift (ppm) are shown, using arrows and dashed line arrows, respectively. The bent double-headed arrow indicates the HMBC crosspeak.

anomeric hydrogen of the glycon (H-1) exhibited cross-peaks with C-2, C-3, and C-5 of the glycon and C-2 of the aglycon (C-2′) (Fig. 5). A coupling constant ($J_{1,2}$) of 1.6 Hz was observed for the glycon anomeric hydrogen. An identical coupling constant is observed in the glycon anomeric hydrogen of the D-rhamnodisaccharide moiety having an α-(1→2)-glycosidic linkage in the repeating unit of the O-specific polysaccharide from the antigenic lipopolysaccharides of *P. cepacia*.[2] Generally, α-glycosidases are known to act with retention of anomeric configuration in hydrolysis and transglycosylation reactions. These results allowed us to identify this disaccharide derivative as ethyl α-D-rhamnopyranosyl-(1→2)-1-thio-α-D-rhamnopyranoside, α-Rha*p*-(1→2)-α-Rha*p*-*S*Et. The optical rotation ($[\alpha]_D$) and melting point of this disaccharide derivative were 119.2° (*c* 0.1, H_2O) and 90.5–93.0 °C, respectively. The isolated yield of this disaccharide derivative was 32.1% (based on donor substrate, after 5 h of reaction time). This fact demonstrates the feasible and practical application of the jack bean α-mannosidase-catalyzed transglycosylation reaction to produce D-rhamnodisaccharide derivatives having an α-(1→2)-glycosidic linkage. For the construction of repeating D-rhamnotrisaccharide units in O-specific polysaccharides of antigenic bacterial lipopolysaccharides from *Pseudomonas*, this D-rhamnodisaccharide derivative may be useful as a common building block.

3. Materials and methods

Materials. *p*-Nitrophenyl α-D-mannopyranoside (α-Man*p*-*O*-*p*NP) was purchased from Wako Pure Chemical Industries (Tokyo, Japan), and recrystallized from EtOH prior to use. Rha and α-Rha*p*-*O*-*p*NP were synthesized following procedures described in our previous paper.[7,8] α-Rha*p*-*S*Et was synthesized from Rha following a previously reported method.[9] α-Mannosidase from jack bean was purchased from Sigma–Aldrich (St. Louis, USA). Silica Gel 60 N (230–400 mesh) for column chromatography and Silica Gel 60 plates (0.25 mm) for TLC were purchased from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Assay of the enzymatic hydrolytic activity. The hydrolytic activity of jack bean α-mannosidase was assayed by using α-Man*p*-*O*-*p*NP as a substrate. To the incubation mixture of the substrate (1.25 mM) in 0.1 M sodium citrate buffer (0.4 mL, pH 4.5) at 25 °C, was added the enzyme solution (0.1 mL). The enzyme activity was monitored by measuring the amount of *p*-nitrophenol that was released from α-Man*p*-*O*-*p*NP. The reaction was stopped by the addition of 0.3 M Na_2CO_3 (0.5 mL). The amount of liberated *p*-nitrophenol in the mixture was determined by measuring the

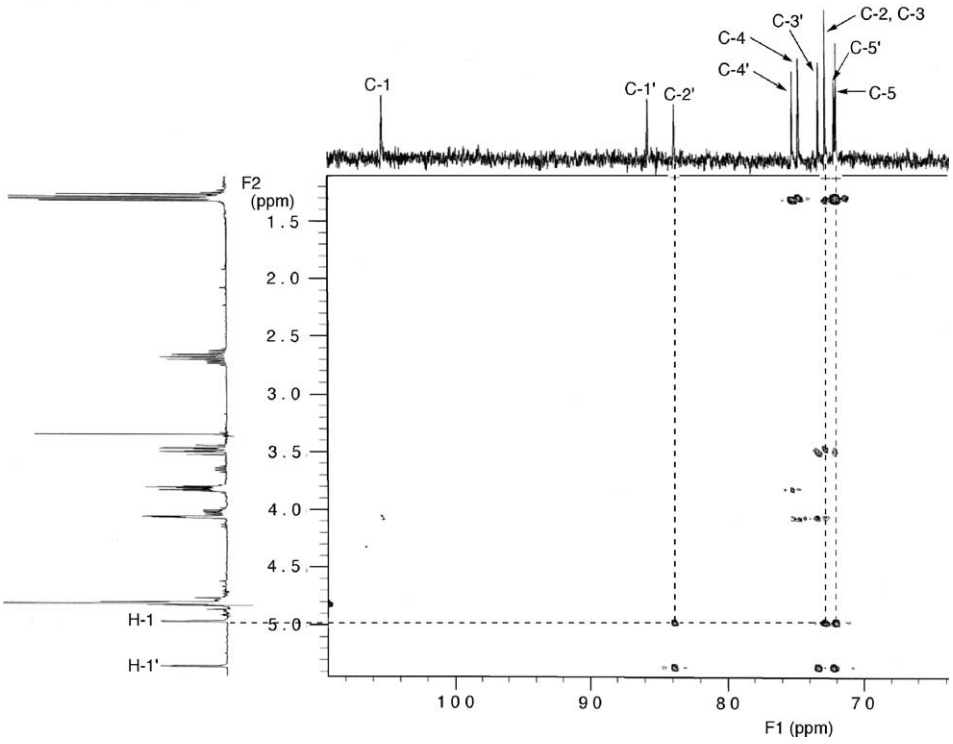

Figure 5. HMBC NMR crosspeaks (in D_2O) with glycon anomeric hydrogen of transglycosylation product (compound **d** in Fig. 3). Peaks H-1 and C-1–5 are anomeric hydrogen and ring carbons of glycon moiety of the rhamnodisaccharide derivative. Peaks H-1′ and C-1′–5′ are anomeric hydrogen and ring carbons of the aglycon moiety of the rhamnodisaccharide derivative. Dashed lines indicate crossing between the peak of H-1 and those of C-2, C-3, C-5, and C-2′.

absorbance of the solution at 405 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol/min under the assay conditions.

Transglycosylation reaction. To a mixture of α-Rha*p*-*O*-*p*NP (228.2 mg, 0.80 mmol) and α-Rha*p*-*S*Et (999.7 mg, 4.8 mmol) in 3:4 (v/v) 0.1 M sodium citrate buffer (pH 4.5)–MeCN (3.5 mL) was added 9.6 units of jack bean α-mannosidase solution (0.5 mL). The mixture was stirred magnetically at 25 °C; at specific intervals, the progress of the reaction was monitored by TLC and HPLC analyses using 100-μL portions of the reaction mixture, which were combined with EtOH (400 μL) in order to inactivate the enzyme. The compounds were analyzed by TLC with 100:35:10:8 CH_2Cl_2–MeOH–EtOH–H_2O as the mobile phase, and visualized by spraying an aqueous solution containing 2.4% (w/v) phosphomolybdic acid, 5% (v/v) H_2SO_4, and 1.5% (v/v) H_3PO_4, followed by heating. Upon completion of the reaction, the solvent was removed by evaporation, and the resulting residue redissolved in 76:24 (v/v) MeCN–H_2O prior to HPLC analysis. The HPLC analysis was performed using a Shimadzu LC-10AS pump equipped with a Shodex RI-71 differential refractometer and a Shodex NH2P-50 4E column (250×4.6 mm) under isocratic conditions using 76:24 (v/v) MeCN–H_2O as the mobile phase at a flow rate of 0.2 mL/min.

Purification of oligosaccharides. After stirring the foregoing reaction mixture for 5 h at 25 °C, the solvent was removed by evaporation. To acetylate all the sugar derivatives, pyridine (15 mL) and Ac_2O (15 mL) were added to the residue, which was stirred for 24 h at 25 °C. The resulting peracetates were identified by TLC with 3:2 (v/v) hexane–AcOEt as the mobile phase. Upon identification, the compounds were extracted using EtOAc (80 mL), which was subsequently washed with HCl (3×80 mL), saturated aqueous $NaHCO_3$, and brine. After removing the solvent by evaporation, the sample was dried in vacuo, and the peracetated transglycosylation product was purified by silica gel column chromatography with 3:2 (v/v) hexane–AcOEt as the mobile phase, and monitored using TLC with 3:2 (v/v) hexane–AcOEt. The fractions containing the pure compound were combined and the solvent removed by evaporation to afford 191 mg of the product.

For deacetylation, this compound was dissolved in 1:2:7 (v/v) Et_3N–H_2O–MeOH and stirred for 24 h at 25 °C. After removal of the solvent by evaporation, the product was purified by silica gel flash column chromatography with 100:35:10:8 (v/v) CH_2Cl_2–MeOH–EtOH–H_2O as the mobile phase, and monitored by TLC with 100:35:10:8 (v/v) CH_2Cl_2–MeOH–EtOH–H_2O. After combining the fractions containing the pure compound and removing the solvent by evaporation,

the sample was dried in vacuo to afford 91.1 mg of the product.

Structural identification of the oligosaccharides. The disaccharide derivative, synthesized by the described enzymatic reaction, was characterized by ^{1}H and ^{13}C NMR spectrometry and positive-ion FABMS. The ^{1}H and ^{13}C NMR spectra of the sample were recorded in D_2O at 25 °C using a Varian VXR-400 spectrometer. Chemical shifts are expressed as downfield shifts (ppm) from Me_4Si. The mass spectra were recorded in glycerol (as the matrix) using a JMX SX-102A instrument under positive-ion FAB conditions. The optical rotation of the sample was measured in H_2O at 20 °C using a Jasco P-1030 polarimeter. The melting point was determined with a Yamato Model MP-21 capillary apparatus and is uncorrected.

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