Carbohydrate Research 345 (2010) 2458–2463

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Acceptor specificity in the transglycosylation reaction using Endo-M

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ARTICLE INFO

Article history:
Received 3 July 2010
Received in revised form 21 August 2010
Accepted 26 August 2010
Available online 17 September 2010

Keywords:
Endo-β-*N*-acetylglucosaminidase
Endo-M
Transglycosylation reaction
Recognition site

ABSTRACT

To determine the structural specificity of the glycosyl acceptor of the transglycosylation reaction using endo-β-*N*-acetylglucosaminidase (ENGase) (EC 3.2.1.96) from *Mucor hiemalis* (Endo-M), several acceptor derivatives were designed and synthesized. The narrow regions of the 1,3-diol structure from the 4- to 6-hydroxy functions of GlcNAc were found to be essential for the transglycosylation reaction using Endo-M. Furthermore, it was determined that Endo-M strictly recognizes a 1,3-diol structure consisting of primary and secondary hydroxyl groups.

1. Introduction

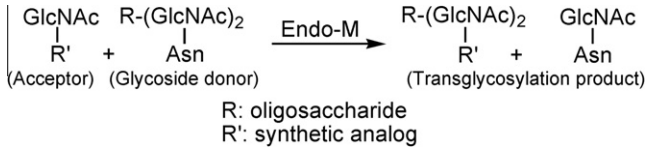
Endo-β-*N*-acetylglucosaminidase (ENGase, EC 3.2.1.96) catalyzes the hydrolysis of the β-(1→4)-glycosidic linkage between the *N,N′*-diacetylchitobiose moiety of N-glycans.[1] This enzyme from *Mucor hiemalis* (Endo-M), is unique in that it can act on all three types of N-glycans, that is, high-mannose-type, complex-type, and hybrid-type oligosaccharides, for the hydrolysis and transglycosylation of the appropriate acceptors containing the *N*-acetylglucosamine (GlcNAc) residue (Scheme 1).[2–5] Endo-M is an effective tool for the reconstruction and remodeling of glycopeptides and glycoproteins.[6–10]

However, rapid hydrolysis of the product with N-glycan by the wild-type endoglycosidases is difficult to avoid, thus limiting its wide application. Development of unique ENGase-based glycosynthases that cannot hydrolyze the product but are still able to take highly activated species as a donor substrate is a solution to this problem.[11,12] Umekawa et al. achieved site-directed mutagenesis of residues in the putative catalytic region of Endo-M mutants with superior transglycosylation activity.[5,13–15]

On the other hand, a summary of acceptor specificity of the transglycosylation activity of Endo-M has been previously reported.[1] Endo-M transfers a sugar chain to glucose, mannose, and other sugars as well as GlcNAc. It has been revealed that the enzyme requires the C-4 equatorial hydroxy group of the carbohydrate, and the C-1 and C-2 of GlcNAc are not important for the transglycosylation activity. The importance of the C-5 hydroxymethyl group of the sugar was suggested from reported data[1] indicating that a transglycosylation reaction does not occur with glucuronic acid and xylose. The acceptor specificity of Endo-M was examined in this study, with focus on the C-5 hydroxymethyl group of the glycosyl acceptor and oxygen atom of the pyranose ring.

2. Results and discussion

First, cyclohexyl asparagines **2** and **3** were synthesized and designed based on the structure of Fmoc-Asn(GlcNAc)-OH (**1**, Fig. 1). Fmoc-Asn(c-Hex(OH))-OH (**2**) possessing only a mono-



Scheme 1. Transglycosylation reaction using Endo-M.

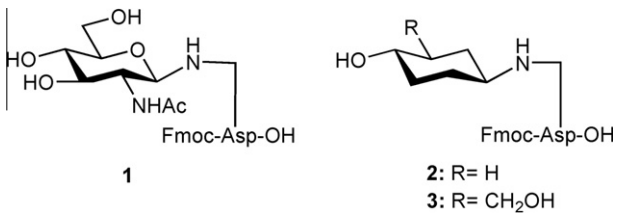


Figure 1. Structure of glycosyl acceptors.

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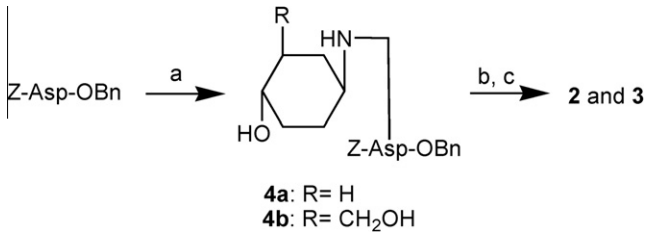
doi:10.1016/j.carres.2010.08.022



Scheme 2. Reagents and conditions: (a) MptCl, Et_3N, 1-amino-4-hydroxycyclohexane or 3-hydroxymethyl-4-hydroxycyclohexylamine, CH_2Cl_2; (b) H_2 gas, AcOH, Pd/C, MeOH; (c) Fmoc-OSu, Na_2CO_3, dioxane–H_2O (1:1).

hydroxyl functionality, a mimic of the equatorial hydroxyl group at C-4 of GlcNAc, was synthesized as follows: Benzyloxycarbonyl aspartic acid α-benzyl ester and *trans*-4-aminocyclohexanol were synthesized using the dimethylphosphinothioic mixed anhydride (Mpt-MA) method[16] producing the amide derivative **4a** in 75% yield (Scheme 2).

The benzyl and benzyloxycarbonyl groups of **4a** were removed by hydrogenation in the presence of 10% Pd–C, the Fmoc group was reacted with the amino group by the reaction of the *N*-(9-fluorenylmethoxycarbonyl) succinimide reagent, to obtain the desired product **2** in 85% yield. Next, a transglycosylation of the complex-type oligosaccharide block of **2** was attempted using Endo-M. Using **2** as the glycosyl acceptor, the transglycosylation of a complex-type oligosaccharide block [$(NeuAc\text{-}Gal\text{-}GlcNAc\text{-}Man)_2$-Man-GlcNAc-] with sialylglycopeptide (SGP)[17] as the glycosyl donor was examined in the presence of Endo-M under previously described reaction conditions.[18] Under these conditions, the transglycosylation reaction did not proceed at all. This result showed that Endo-M does not recognize only 4-OH of GlcNAc.

Next, Fmoc-Asn(c-Hex(OH)(CH_2OH))-OH (**3**), which is a mimic of the C-4 equatorial hydroxyl group and C-5 hydroxymethyl group of GlcNAc, was synthesized. Using a method similar to that described for the synthesis of compound **2**, compound **3** was obtained from 3-hydroxymethyl-4-hydroxycyclohexylamine, (which has been previously described)[19] and benzyloxycarbonyl aspartic acid α-benzyl ester. Although this product was a mixture of diastereomers, each isomer was isolated using reversed-phase high-performance liquid chromatography (RP-HPLC). It was assumed that one isomer was in the same conformation as D-GlcNAc, and the other was an L-isomer.

The transglycosylation reaction of one of the two isomers did not proceed at all. It was assumed that the L-GlcNAc mimetic did not react. However, the transglycosylation of the other isomer using Endo-M yielded the desired transglycosylated product. The RP-HPLC profile of the transglycosylation of SGP to **3** after a reaction time of 4 h is shown in Figure 2. Characterization of the new product was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The *m/z* of the product

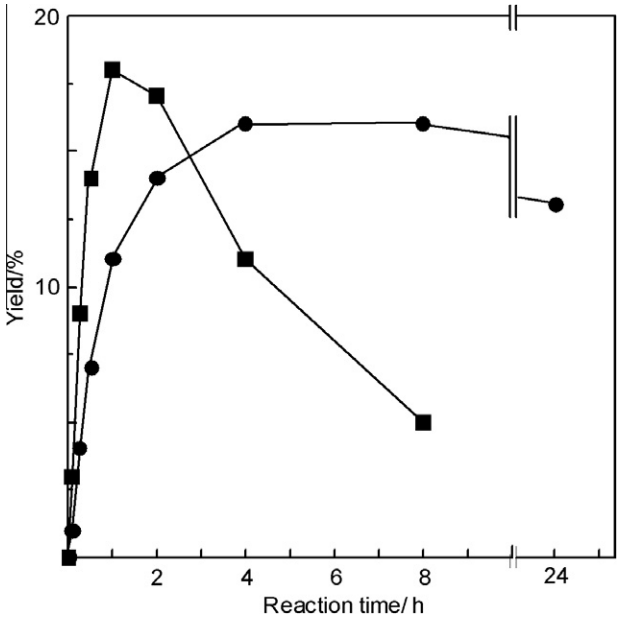


Figure 3. Time course of the transglycosylation reaction using Endo-M. ■: Fmoc-Asn(GlcNAc)-OH (**1**), ●: Fmoc-Asn(c-Hex(OH)(CH_2OH))-OH (**3**).

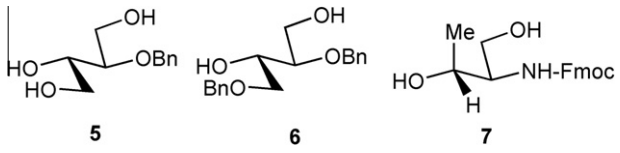


Figure 4. Structure of new glycosyl acceptors.

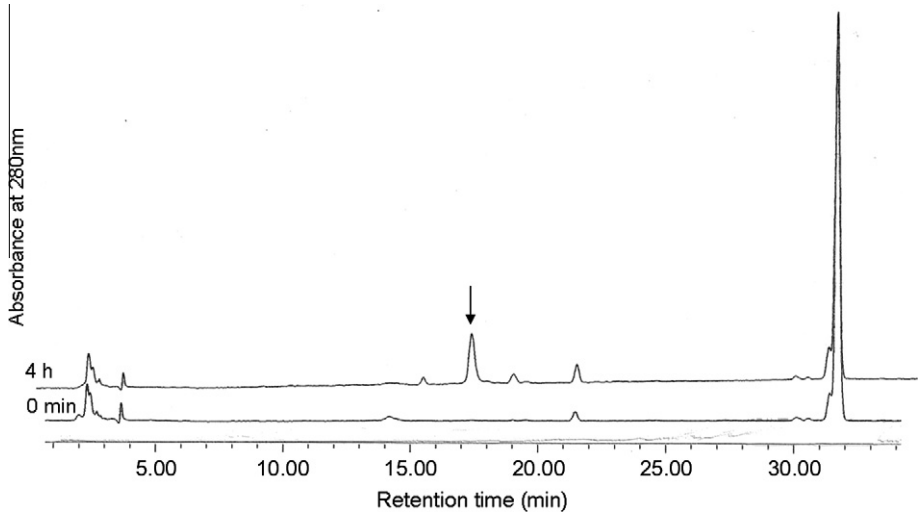
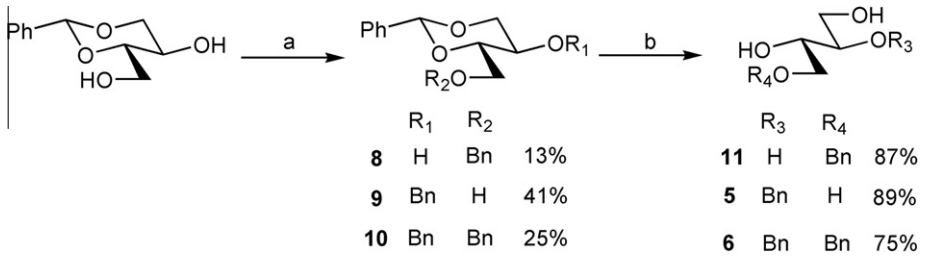


Figure 2. RP-HPLC profile of the reaction mixture incubated with cyclohexanediol derivative **3** and SGP in the presence of Endo-M after a reaction time of 4 h, as detected by absorbance at 280 nm. The large peak at the retention time of 31–32 min corresponds to remaining amounts of acceptor **3**.



Scheme 3. Reagents and conditions: (a) BnBr, NaH, DMF, rt; (b) 80%-AcOH, 40 °C.

was 2484.5 whereas the calculated *m/z* was 2482.9. The time course of the transglycosylation reaction of **3** is shown in Figure 3.

When the GlcNAc derivative **1** was used as an acceptor, the yield of the transglycosylated product reached 18% after 1 h. Thereafter, the product was promptly hydrolyzed. On the other hand, the highest yield resulting from using cyclohexanediol derivative **3** as an acceptor was 16% after 4 h. Cyclohexanediol derivative **3** was found to have transglycosylation activity similar to that of GlcNAc derivatives **1**. However, the hydrolysis rate of cyclohexanediol derivative **3** was considerably slower compared to the hydrolysis rate of GlcNAc derivative **1**. Therefore, it was determined that the oxygen atom on the pyranose ring was not essential; however, a narrow region of the acceptor—the area from 4-OH to 6-OH of GlcNAc—was essential for the transglycosylation reaction using Endo-M. From these results it was assumed that 6-OH of GlcNAc would be important for the transglycosylation reaction, which was similar to the reported study.[15,20,21]

To narrow down the structural specificity areas of the glycosyl acceptor in this reaction, several acceptor derivatives with a 1,3-diol structure were designed and synthesized (Fig. 4). Furthermore, linear 1,3-diol structures, such as erythritol derivatives **5** and **6**, and threoninol derivative **7**[22–24] were also designed.

For the preparation of erythritol derivatives **5** and **6**, 1,3-*O*-benzylidene-L-erythritol[25,26] was benzylated using 1.2 equiv benzyl bromide to yield 4-*O*-benzyl erythritol derivative **8** (13%), 2-*O*-benzyl erythritol derivative **9** (41%), and 2,4-di-*O*-benzyl erythritol derivative **10** (25%) (Scheme 3). After deprotection of the benzylidene group using 80% acetic acid, erythritol derivatives **11**, **5**, and **6** were obtained in 87%, 89%, and 75% yields, respectively.

Next, the transglycosylation reaction was carried out using 1,3-diol derivatives **5**–**7** as acceptors, SGP as a glycosyl donor, and Endo-M. Surprisingly, Endo-M recognized linear 1,3-diol derivatives, such as erythritol derivatives **5** and **6**, and threoninol derivative **7** (Fig. 5). In the case of monobenzylated erythritol **5**, which was the most superior acceptor, the yield of the transglycosylated product reached 26% after 1 h (Fig. 6). Dibenzylated erythritol **6** and threoninol derivative **7** also demonstrated transglycosylation activity (15% and 7%, respectively). The hydrolysis rate of these compounds and the cyclohexanediol derivatives **3** were slow compared to that of GlcNAc derivative **1**. These data suggest that the C-3 hydroxyl group of GlcNAc may be related to hydrolysis activity. To determine the transglycosylated position of these non-natural compounds using NMR, transglycosylated dibenzylated erythritol **6** was treated with sialidase from *Arthrobacter ureafaciens* and b-galactosidase from *Jack bean* and β-*N*-acetylhexosaminidase from *Jack bean*. Long-range correlations were detected between the NMR signals of the anomer protons of GlcNAc and the C-2 methine carbon of erythritol in HMBC experiments. As a result, the transglycosylated position of the secondary hydroxyl group was observed, similar to the transglycosylation reaction of GlcNAc.[†]

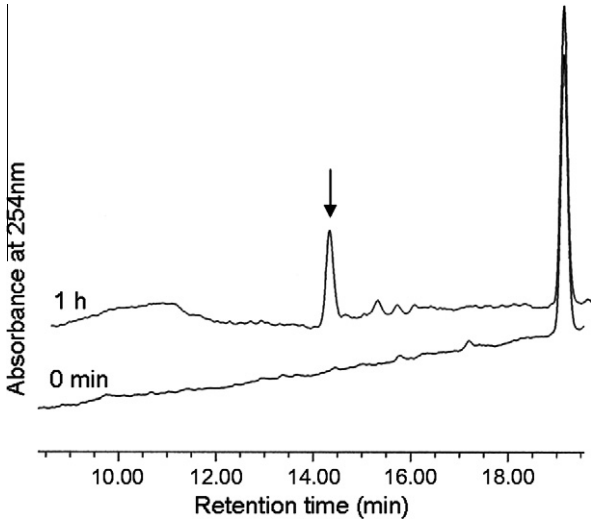


Figure 5. RP-HPLC profile of the reaction mixture incubated with 2-*O*-benzyl erythritol (**5**) and SGP in the presence of Endo-M after a reaction time of 1 h as detected by absorbance at 254 nm. The large peak at the retention time of 18–19 min corresponds to remaining amounts of acceptor **5**.

On the other hand, erythritol derivative **11** and 2-*O*-benzyl glycerol[‡] yielded no transglycosylated products. From erythritol derivative **11**, it was hypothesized that the free hydroxyl group, which exists in the position corresponding to C-5 of GlcNAc, would interrupt the transglycosylation reaction. For confirmation, the transglycosylation reaction was attempted with several 1,2,3-triol derivatives, but the reaction did not proceed (data is not shown). In spite of having an essential 1,3-diol structure, 2-*O*-benzyl glycerol, which consists of two primary hydroxyl groups, did not function as an acceptor. Based on these results, the two hydroxyl groups need to act as primary and secondary hydroxyl groups.

3. Conclusions

To determine the structural specificity of the glycosyl acceptor in the transglycosylation reaction using Endo-M, several acceptor derivatives were synthesized. It was determined that the oxygen atom on the pyranose ring was not essential; however, the narrow regions of the 1,3-diol structure from the 4-OH to 6-OH of GlcNAc were considered essential for the transglycosylation reaction using Endo-M. The enzyme recognizes not only cyclic 1,3-diol structures such as GlcNAc but also linear 1,3-diol structures such as erythritol and threoninol. The reaction did not proceed to glycerol using two primary hydroxyl groups: Endo-M strictly recognizes the 1,3-diol structures consisting of primary and a secondary hydroxyl groups. Based on these data, new effective acceptors for Endo-M are currently being designed and synthesized.

[†] These results will be described elsewhere.

[‡] 2-Benzyloxy glycerol was available from Tokyo Chemical Industry Co., Ltd.

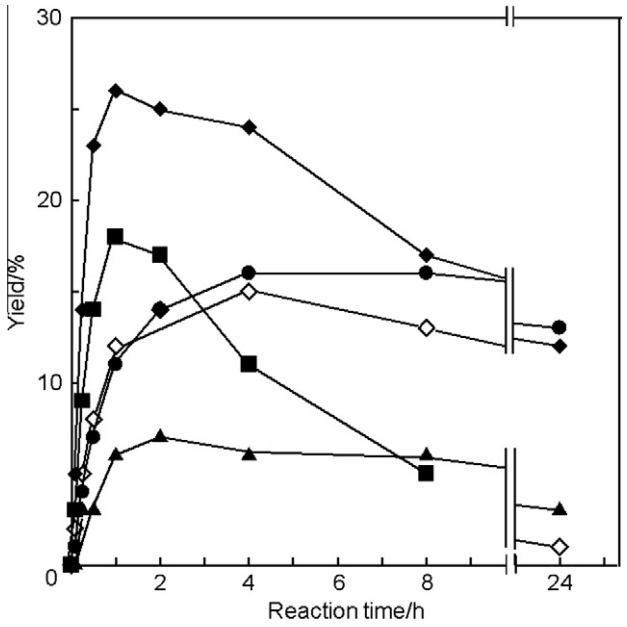


Figure 6. Time course of the transglycosylation reaction using Endo-M. ■: Fmoc-Asn(GlcNAc)-OH (**1**), ●: Fmoc-Asn(c-Hex(OH)(CH_2OH))-OH (**3**), ♦: 2-*O*-Benzyl erythritol (**5**), ◇: 1,3-*O*-dibenzyl-erythritol (**6**), ▲: Fmoc-Thr-ol (**7**).

4. Experimental methods

4.1. General methods

^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz on a JEOL JNM-400. MALDI-TOF mass spectra were obtained using a Daltonix-Bruker AUTOFLEX. The structure of the acceptors was confirmed by MALDI-TOF MS in linear and negative ion modes using 2,5-dihydroxy-benzoic acid as a matrix, unless otherwise described. HRMS data were obtained on an LC–MS-IT-TOF (Shimadzu Corporation). Reactions were monitored using thin-layer chromatography on a pre-coated plate of Silica Gel 60F_{254} (Merck) and/or RP-HPLC (HPLC system equipped with a Delta 600 series interface). Flash chromatography was performed on Silica Gel (Silica Gel 60N, 40–50 μm; Kanto Chemical Co., Inc.). The biantennary complex-type sialylglycopeptide (SGP), Endo-M, and Lys-Val-Ala-Asn[(NeuAc-Gal-GlcNAc-Man)$_2$ Man-GlcNAc$_2$]-Lys-Thr were obtained from Tokyo Chemical Industry Co., Ltd.

4.2. N^{α}-Benzyloxycarbonyl-N^{β}-4-hydroxycyclohexyl-L-asparagine α-benzyl ester, Z-Asn(c-Hex(OH))-OBn (4a)

Triethylamine (610 mg, 6.03 mmol) and dimethylphosphinothioyl chloride (390 mg, 3.03 mmol) were added to a solution of N^{α}-benzyloxycarbonyl-L-aspartic acid α-benzyl ester (Z-Asp-OBn) (1.07 g, 2.99 mmol) in CH_2Cl_2 (15 mL), and the mixture was stirred at 0 °C to prepare the corresponding Mpt-MA. After stirring at room temperature for 30 min, the reaction mixture was added to a stirred solution of *trans*-4-aminocyclohexanol (350 mg, 3.04 mmol) in CH_2Cl_2 (30 mL). After stirring at room temperature for 1 h, the reaction mixture was washed with 1 N HCl, water, 5% aq $NaHCO_3$, water, and brine. The organic layer was dried over anhydrous Na_2SO_4, and concentrated in vacuo. Purification by flash column chromatography (CH_2Cl_2–MeOH 50:1) yielded **4a** (1.03 g, 75%) as colorless amorphous crystals. ^{1}H NMR ($CDCl_3$): δ 7.36–7.30 (10H, m, Ph × 2), 6.06 (1H, br d, J = 8.4 Hz, NH), 5.40 (1H, br d, J = 6.8 Hz, NH), 5.18 (2H, dd, J = 12.4, 12.4 Hz, OCH_2), 5.11 (2H, br s, OCH_2), 4.59 (1H, t, J = 4.0 Hz, Asn-α-CH), 3.65 (1H, m, c-Hex-CH), 3.55 (1H, m, c-Hex-CH), 2.78 (2H, dd, J = 4.4, 4.0 Hz, Asn-β-CH_2), 1.94–1.85 (4H, m, c-Hex), 1.52 (1H, br s, OH), 1.36–1.33 (2H, m, c-Hex), 1.11–1.05 (2H, m, c-Hex). ^{13}C NMR (DMSO-d_6): δ 171.5, 167.8, 155.8, 136.9, 135.9, 128.3–127.6 (aromatic carbon), 68.1, 66.0, 65.5, 50.9, 47.3, 37.1, 33.9, 30.1. HRMS calcd for $C_{25}H_{30}N_2O_6{\cdot}K^+$ $[M+K]^+$ 493.1741; found *m/z* 493.1741.

4.3. N^{α}-9-Fluorenylmethoxycarbonyl-N^{β}-4-hydroxycyclohexyl-L-asparagine, Fmoc-Asn(c-Hex(OH))-OH (2)

Compound **4a** (1.97 g, 4.34 mmol) and acetic acid (261 mg, 4.34 mmol) in MeOH (80 mL) were stirred under an H_2 atmosphere in the presence of 10% Pd–C (394 mg) at room temperature for 2 h. The mixture was filtered through filter paper and concentrated in vacuo. A solution of Fmoc succinimide (1.36 g, 4.02 mmol) in dioxane (20 mL) was added to a stirred solution of the compound in 10% sodium carbonate aq (20 mL) at room temperature. The reaction mixture was stirred for 24 h. EtOAc was added to the reaction mixture and washed with water. The water layer was adjusted to pH 2–3 using 1 N HCl; white solids were separated out. The solids were washed with water, EtOAc, and Et_2O. The solid when dried in vacuo yielded **2** (1.41 g, 72% in two steps). ^{1}H NMR (CD_3OD): δ 7.80–7.29 (8H, m, Fmoc), 4.52–4.45 (1H, m, Asn-α-CH), 4.34−4.32 (2H, m, OCH_2), 4.23 (1H, t, J = 6.8 Hz, CH), 3.63−3.53 (1H, m, c-Hex-CH), 3.52−3.43 (1H, m, c-Hex-CH), 2.66 (2H, m, Asn-β-CH2), 1.95–1.82 (4H, m, c-Hex), 1.33−1.23 (4H, m, c-Hex). ^{13}C NMR (DMSO-d_6): δ 173.1, 168.2, 155.8, 143.8, 140.7, 127.6–120.1 (aromatic carbon), 68.1, 65.7, 50.8, 47.3, 46.6, 37.2, 33.9, 30.2. HRMS calcd for $C_{25}H_{27}N_2O_6$ $[M-H^+]^-$ 451.1869; found *m/z* 451.1856.

4.4. N^{α}-Benzyloxycarbonyl-N^{β}-3-hydroxymethyl-4-hydroxycyclohexyl-L-asparagine α-benzyl ester, Z-Asn(c-Hex(OH)(CH_2OH))-OBn (4b)

Triethylamine (198 mg, 1.93 mmol) and dimethylphosphinothioyl chloride (150 mg, 1.17 mmol) were added to a solution of N^{α}-benzyloxycarbonyl-L-aspartic acid α-benzyl ester (Z-Asp-OBn) (353 mg, 0.988 mmol) in CH_2Cl_2 (3 mL) and the mixture was stirred at 0 °C to prepare the corresponding Mpt-MA. After stirring at room temperature for 1 h, the reaction mixture was added to a stirred solution of 1-amino-3-hydroxymethyl-4-hydroxycyclohexane (110 mg, 0.758 mmol) in MeOH (2 mL). After stirring at room temperature for 3 h, the reaction mixture was washed with 1 N HCl, water, saturated $NaHCO_3$, water, and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo. Purification by flash column chromatography ($CHCl_3$–MeOH–AcOH 85:25:20) yielded **4b** (291 mg, 79%) as colorless amorphous crystals. ^{1}H NMR ($CDCl_3$): δ 7.32–7.26 (10H, m, Ph × 2), 6.20 (1H, br d, J = 7.2 Hz, NH), 6.11 (1H, br d, J = 7.2 Hz, NH), 5.18 (2H, dd, J = 13.2, 12.8 Hz, OCH_2), 5.10 (2H, br s, OCH_2), 4.62 (1H, t, J = 4.4 Hz, Asn-α-CH), 4.08 (1H, br d, J = 4.0 Hz, c-Hex-CH), 3.63–3.60 (2H, m, CH_2–OH), 3.56–3.51 (1H, m, c-Hex-CH), 2.93–2.72 (2H, m, Asn-β-CH_2), 2.04 (1H, br s, OH), 1.25 (1H, br s, OH), 1.81–1.11 (7H, m, c-Hex). ^{13}C NMR ($CDCl_3$): δ 174.1, 170.5, 163.0, 136.0, 135.1, 128.4–128.0 (aromatic carbon), 67.5, 67.1, 50.4, 38.7, 36.8, 36.4, 31.7, 28.9, 23.0, 14.1. HRMS calcd for $C_{26}H_{33}N_2O_7$ $[M+H^+]^+$ 485.2288; found *m/z* 485.2289.

4.5. N^{α}-9-Fluorenylmethoxycarbonyl-N^{β}-3-hydroxymetyl-4-hydroxy-cyclohexyl-L-asparagine, Fmoc-Asn(c-Hex(OH)(CH_2OH))-OH (3)

Compound **4b** (195 mg, 0.403 mmol) and acetic acid (48.0 mg, 0.799 mmol) in MeOH (5 mL) were stirred overnight under a H_2 atmosphere in the presence of 10% Pd–C (28 mg) at room temper-

ature. The mixture was filtered through filter paper and concentrated in vacuo. A solution of Fmoc succinimide (140 mg, 0.415 mmol) in dioxane (5 mL) was added to a stirred solution of the compound in 10% sodium carbonate aq (5 mL) at room temperature. The reaction mixture was stirred for 18 h, to which Et_2O was added and it was extracted with water. The water layer was washed with Et_2O, adjusted to pH 1 using 1 N HCl, and was extracted with EtOAc. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. The organic solution was concentrated in vacuo. Purification by flash column chromatography ($CHCl_3$–MeOH 5:1) yielded **3** (127 mg, 65% in two steps) as a colorless amorphous crystal. ^{1}H NMR (CD_3OD): δ 7.79–7.31 (8H, m, Fmoc), 4.58–4.55 (1H, m, AsnHα), 4.37–4.29 (2H, m, Fmoc-CH_2CONH), 4.22 (1H, t, J = 6.8 Hz, Fmoc-CH), 3.95 (1H, br, CHNH), 3.61 (2H, d, J = 5.4 Hz, CH_2OH), 3.52 (1H, m, CHOH), 2.82–2.69 (2H, m, AsnHβ), 1.82–1.40 (7H, m, c-Hex). ^{13}C NMR (DMSO-d_6): δ 169.6, 155.6, 143.9, 140.7, 127.6–120.1 (aromatic carbon), 69.1, 65.6, 63.1, 52.5, 46.7, 43.5, 41.5, 38.8, 31.0, 29.2, 28.0. HRMS calcd for $C_{26}H_{29}N_2O_7$ $[M-H^+]^-$481.1975; found *m/z* 481.1976.

4.6. Preparation of benzylidene erythritol derivatives 8–10

Sodium hydride (140 mg, 5.83 mmol) was added to a stirred solution of 1,3-*O*-benzylidene-L-erythritol (500 mg, 2.38 mmol) in DMF (20 mL) at 0 °C. After the suspension was stirred for 40 min, benzyl bromide (485 mg, 2.84 mmol) was added dropwise over 5 min period and stirred for 30 min. The reaction temperature was then allowed to increase to room temperature for 1.5 h, MeOH was then slowly added to react with the excess sodium hydride, and water was added to the reaction. The reaction mixture was extracted with EtOAc, and the organic layer was washed with water and brine, and then dried over anhydrous Na_2SO_4. The organic solution was concentrated in vacuo. Purification by flash column chromatography (hexane–EtOAc gradient elution 9:1→2:1) yielded **8** (95.0 mg, 13%), **9** (295 mg, 41%), and **10** (232 mg, 25%) as colorless amorphous crystals.

4.6.1. 1,3-*O*-Benzylidene-4-*O*-benzyl-L-erythritol (8)

^{1}H NMR (CD_3OD): δ 7.49–7.23 (10H, m, Ph × 2), 5.52 (1H, s, Ph-CH), 4.60 (2H, s, Ph-CH_2), 4.20 (1H, dd, J = 10.4, 4.8 Hz, H-1_b), 3.88–3.56 (5H, m, H-1_a, H-2, H-3, H-4). ^{13}C NMR (CD_3OD): δ 139.6, 139.4, 129.8–127.4 (aromatic carbon), 102.3, 102.3, 83.1, 74.5, 72.3, 70.8, 62.8. HRMS calcd for $C_{18}H_{21}O_4$ $[M+H^+]^+$ 301.1440; found *m/z* 301.1398.

4.6.2. 1,3-*O*-Benzylidene-2-*O*-benzyl-L-erythritol (9)

^{1}H NMR (CD_3OD): δ 7.49–7.28 (10H, m, Ph × 2), 5.53 (1H, s, Ph-CH), 4.61 (2H, s, Ph-CH_2), 4.34–4.32 (1H, m, H-1_a), 3.91–3.86 (1H, m, H-4_a), 3.77–3.70 (2H, m, H-2, H-4_b), 3.67–3.60 (2H, m, H-1_b, H-3). ^{13}C NMR (CD_3OD): δ 139.6, 139.4, 129.8-127.5 (aromatic carbon), 102.4, 82.9, 73.4, 70.3, 69.9, 62.5. HRMS calcd for $C_{18}H_{20}O_4$·Na $[M+Na^+]^+$ 323.1259; found *m/z* 323.1249.

4.6.3. 1,3-*O*-Benzylidene-2,4-di-*O*-benzyl-L-erythritol (10)

^{1}H NMR (CD_3OD): δ 7.47–7.24 (15H, m, Ph × 3), 5.51 (1H, s, Ph-CH), 4.61–4.49 (4H, m, Ph-CH_2 × 2), 4.32 (1H, dd, J = 9.6, 3.6 Hz, H-1_{eq}), and 3.93–3.59 (5H, m, H-1_{ax}, H-2, H-3, H-4). ^{13}C NMR (CD_3OD): δ 139.5, 139.5, 139.4, 129.8–127.5 (aromatic carbon), 102.4, 102.3, 81.7, 74.3, 73.3, 70.4, 70.3, 69.8. HRMS calcd for $C_{25}H_{26}O_4$·Na $[M+Na^+]^+$ 413.1729; found *m/z* 413.1714.

4.7. General procedure for the preparation of erythritol derivatives 5, 6, and 11

Compound **9** (92.0 mg, 0.307 mmol) in 80% acetic acid (2 mL) was heated at 40 °C for 4 h. The reaction mixture was allowed to cool, washed with saturated $NaHCO_3$, water, and brine, and then dried over anhydrous Na_2SO_4. The organic solution was concentrated in vacuo. Purification by flash column chromatography (hexane–EtOAc gradient elution 1:2→100% EtOAc) yielded **5** (55.0 mg, 87%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.38–7.30 (5H, m, Ph), 4.66 (1H, d, J = 11.6 Hz, Ph-CH_{2a}), 4.59 (1H, d, J = 11.6 Hz, Ph-CH_{2b}), 3.92–3.41 (6H, m, H-1, H-2, H-3, H-4), 2.50-2.10 (3H, br, OH × 3). ^{13}C NMR (CD_3OD) δ 139.8, 129.2–128.5 (aromatic carbon), 81.4, 73.2, 72.6, 64.3, 61.8. HRMS calcd for $C_{11}H_{16}O_4$·Na $[M+Na^+]^+$ 235.0946; found *m/z* 235.0989.

4.7.1. 1,3-Di-*O*-benzyl-D-erythritol (6)

75% yield as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.38–7.28 (10H, m, Ph × 2), 4.64–4.50 (4H, m, CH_2 × 2), 3.96 (1H, dt, J = 6.3, 3.9 Hz, CH–OH), 3.84 (1H, dd, J = 12.2, 4.4 Hz, CH_{2a}–OH), 3.79 (1H, dd, J = 12.2, 4.4 Hz, CH_{2b}–OH), 3.66 (1H, dd, J = 9.3, 3.9 Hz, CH_{2a}–OBn), 3.58 (1H, dd, J = 9.3, 6.3 Hz, CH_{2b}–OBn), and 3.54 (1H, td, J = 6.3, 4.4, 4.4 Hz, CH–OBn). ^{13}C NMR ($CDCl_3$): δ 137.9, 128.5–127.9 (aromatic carbon), 78.8, 73.5, 72.3, 70.9, 61.5. HRMS calcd for $C_{18}H_{22}O_4$·Na $[M+Na^+]^+$ 325.1416; found *m/z* 325.1399.

4.7.2. 1-*O*-Benzyl-D-erythritol (11)

87% yield as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.38–7.24 (5H, m, Ph), 4.88 (2H, s, $PhCH_2$), 3.75–3.67 (3H, m, H-1_a, H-3, H-4_a), 3.62–3.54 (3H, m, H-1_b, H-2, H-4_b). ^{13}C NMR ($CDCl_3$): δ 139.7, 129.3–128.6 (aromatic carbon), 74.3, 73.7, 73.0, 72.4, 64.6. HRMS calcd for $C_{11}H_{16}O_4$·Na $[M+Na^+]^+$ 235.0946; found *m/z* 235.0921.

4.8. Transglycosylation reaction with Endo-M

The transglycosylation reaction was performed with a reaction mixture composed of 10 mM **3**, 20 mM SGP, and 0.2 U/mL Endo-M in a total volume of 20 mL of 0.4 M phosphate buffer (pH 6.25) containing 30% DMSO (v/v). After incubation for 0–24 h at 25 °C, aliquots (2 μL) were added to 98 μL of 0.2% trifluoroacetic acid (TFA) solution to finish the reaction. Analyses of the transglycosylation products were performed using RP-HPLC. The elution was carried out using a linear gradient of acetonitrile (20–45%) containing 0.1% aq TFA for 35 min at a flow rate of 1 mL/min. The reaction products were monitored at an absorption of 280 nm. The yields of the transglycosylation products were calculated from the ratio of the peaks of the transglycosylation products to the glycosyl acceptors. This was based on the assumption that the absorption wavelength of the transglycosylation products and those of the acceptors would be approximately the same. The purification of the transglycosylation products was performed using RP-HPLC, using the same procedure described in the methods of analysis section.

4.8.1. Glycosylated Fmoc-Asn(c-Hex(OH)(CH_2OH))-OH

MALDI-TOF MS *m/z* ratio for $C_{102}H_{152}N_7O_{63}$ $[M-H^+]^-$ calcd for 2482.9; found 2484.5.

4.8.2. Glycosylated Fmoc-Thr-ol

MALDI-TOF MS *m/z* ratio for $C_{95}H_{143}N_6O_{60}$ $[M-H^+]^-$ calcd for 2327.8; found 2329.9.

4.8.3. Glycosylated 2-*O*-benzyl-D-erythritol

MALDI-TOF MS *m/z* ratio for $C_{87}H_{138}N_5O_{60}$ $[M-H^+]^-$ calcd for 2212.8; found 2214.1.

4.8.4. Glycosylated 1,3-di-*O*-benzyl-D-erythritol

MALDI-TOF MS *m/z* ratio for $C_{94}H_{144}N_5O_{60}$ $[M-H^+]^-$ calcd for 2304.2; found 2303.7.

Acknowledgments

The authors gratefully acknowledge Professor Kenji Yamamoto from Ishikawa Prefectural University for valuable discussions of this work. The authors also thank the Tokyo Chemical Industry Co., Ltd for providing Endo-M and SGP, and Dr. Takashi Yamanoi and Mr. Yoshiki Oda from the Noguchi Institute for participating in structural NMR analyses of the transglycosylated products, and Professor Akemi Suzuki from Institute of Glycoscience, Tokai University for participating in HRMS analyses. Part of this work was supported by a Grant for Hi-Tech Research from Tokai University, a Sasakawa Scientific Research Grant from The Japan Science Society, and a Research and Study Project funding from the Tokai University Educational System General Research Organization.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2010.08.022.

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