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A novel disaccharide substrate having 1,2-oxazoline moiety for detection of transglycosylating activity of endoglycosidases

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Abstract

A disaccharide substrate of Manβ1-4GlcNAc-oxazoline **2** was designed and synthesized as a novel probe for detection of the transglycosylating activity of endoglycosidases. A regio- and stereoselective transglycosylation reaction of **2** to GlcNAcβ1-*O*-*p*NP or Dns-Asn(GlcNAc)-OH catalyzed by endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M) and endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) has been demonstrated for the first time, resulting in the core trisaccharide derivative Manβ1-4GlcNAcβ1-4GlcNAcβ1-*O*-*p*NP **8** (or -(Dns)Asn-OH). Interestingly, the transglycosylation proceeds irreversibly; the resulting trisaccharide **8** was not hydrolyzed by Endo-M and Endo-A. Based on these results, a new mechanism including an oxazolinium ion intermediate has been proposed for the endoglycosidase-catalyzed hydrolysis or transglycosylation.

Keywords: Sugar oxazoline; Transition state analogue substrate; Endo-β-*N*-acetylglucosaminidase; Transglycosylation; Regioselective glycosylation; Stereoselective glycosylation; Oxazolinium ion intermediate

1. Introduction

N-Linked glycoproteins have a relatively fixed pentasaccharide core **1** which comprises the proximal trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc (Fig. 1). It is apparent that a wide variety of oligosaccharides including this pentasaccharide core result in recognition by many receptors such as antibodies and lectins. Introduction of these oligosaccharides into proteins or peptides brings about the possibility that achieves the synthesis of novel functional glycoproteins or glycopeptides [1–3].

It is well known that some endoglycosidases such as endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M) and endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) can transglycosylate a large oligosaccharide donor onto various glycosyl acceptors [4,5]. For example, glycopeptides having N-linked oligosaccharide have been successfully prepared by forming a GlcNAcβ1-4GlcNAc bond under mild reaction conditions [6–8]. Accordingly, screening of such enzymes with high transglycosylating activity would provide more industrial benefits. However, the preparation of these oligosaccharide donors having a complex structure requires many chemical or enzymatic procedures. In addition, as the structure of the oligosaccharide donor becomes more complicated, the differentiation of the glycosidic bond formed as a result of transglycosylation becomes more difficult [9,10]. Therefore, development of a new probe substrate having a simple chemical structure for efficient search of endoglycosidases has strongly been required.

Here we report the synthesis of a novel disaccharide substrate **2** having 1,2-oxazoline moiety for detection of the transglycosylating activity of Endo-M and Endo-A

Abbreviations: GlcNAcβ1-*O*-*p*NP, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside; Manβ1-4GlcNAc-oxazoline, 2-methyl{4-*O*-(β-D-mannopyranosyl)-1,2-dideoxy-α-D-glucopyrano}-[2,1-*d*]-2-oxazoline; Dns-Asn(GlcNAc)-OH, N^{α}-dansyl-N^{β}-2-acetamido-2-deoxy-β-D-glucopyranosyl-L-asparagine; Galβ1-4GlcNAc-oxazoline, 2-methyl{4-*O*-(β-D-galactopyranosyl)-1,2-dideoxy-α-D-glucopyrano}-[2,1-*d*]-2-oxazoline

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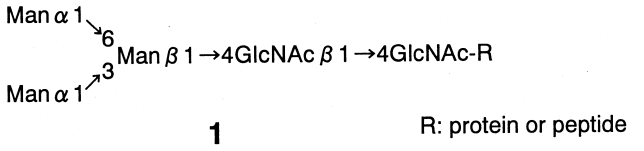

Fig. 1. Structure of pentasaccharide core **1** of N-linked glycoproteins or glycopeptides.

(Fig. 2). The transglycosylation of **2** to GlcNAcβ1-*O*-*p*NP proceeded in a regio- and stereoselective manner. A fluorescence energy transfer substrate having a dansyl group [11] can also be transglycosylated effectively. Surprisingly, the transglycosylated product of trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc-R was not hydrolyzed by endoglycosidases. Thus this novel oxazoline substrate is useful for screening of endoglycosidases which catalyze the GlcNAcβ1-4GlcNAc bond formation in the synthesis of glycoprotein derivatives as well as for elucidation of the mechanism of endoglycosidase-catalyzed reactions. In fact, the reaction catalyzed by Endo-M and Endo-A appeared to proceed via the anchimeric assisted mechanism which involves an oxazolinium ion intermediate stabilized by the neighboring acetamido group [12].

2. Materials and methods

2.1. Synthesis of Man-GlcNAc-oxazoline 2

The synthetic route of Manβ1-4GlcNAc-oxazoline **2** consists of the construction of the disaccharide backbone (Manβ1-4GlcNAc) and the introduction of the oxazoline moiety to the reducing end unit (Fig. 3). As a glycosyl donor for stereoselective β1-4 mannosidation, we prepared phenyl 4,6-*O*-benzylidene-2,3-di-*O*-benzyl-1-thio-α-D-mannopyranoside *S*-oxide **3** from D-mannose via six steps (overall yield 60%) [13]. As a glycosyl acceptor, 1,6-anhydro-2-azido-3-*O*-benzyl-2-deoxy-β-D-glucopyranose **4** was synthesized from D-glucose via six steps (overall yield 45%) [14]. The mannosidation was carried out according to Kahne's method [15]. To a solution of **3** and 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP) in dichloromethane was added trifluoromethanesulfonic anhydride at −78°C and the resulting solution was stirred for 2–5 min. A solution of **4** in dichloromethane was added dropwise and the reaction mixture was quenched by triethylamine. After evaporation of the solvent, the residue was chromatographed on silica gel, giving rise to a disaccharide derivative **5** (yield 60% for **3**) [15,16].

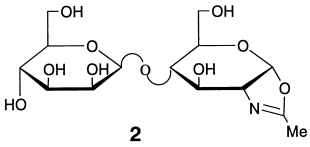

Fig. 2. Structure of novel disaccharide substrate **2**.

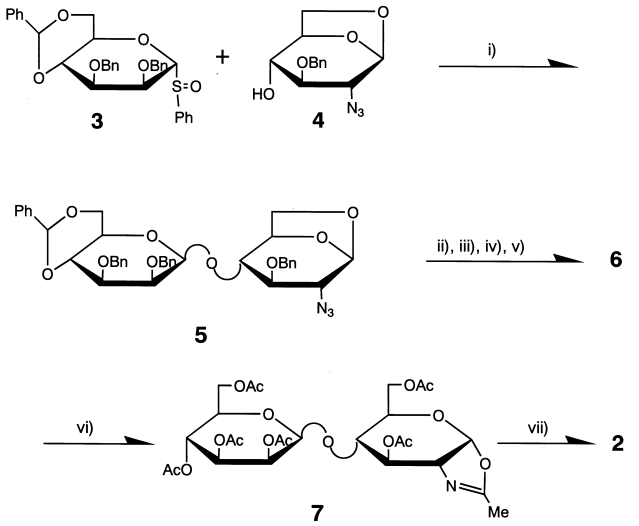

Fig. 3. Synthetic route of **2**. (i) Tf_2O, DTBMP/CH_2Cl_2, −78°C; (ii) TFA, Ac_2O, 24 h; (iii) CH_3COSH, 48 h; (iv) Pd-C, H_2/MeOH; (v) Ac_2O/Py; (vi) TMSOTf/$C_2H_4Cl_2$; (vii) MeONa/MeOH.

After cleavage of the 1,6-anhydro ring and removal of the benzyl groups, all hydroxyl groups were acetylated, affording peracetylated disaccharide derivatives **6**. The formation of the oxazoline ring was performed as follows [17]. To a solution of **6** in 1,2-dichloroethane was added dropwise a 1,2-dichloroethane solution of trimethylsilyl trifluoromethanesulfonate at 0°C. The reaction mixture was stirred at 50°C for 24 h and then neutralized with triethylamine. Purification by chromatography on silica gel resulted in peracetylated oxazoline derivative **7** (yield 60%). All acetyl groups of **7** were removed by the action of diethylamine in methanol to give Manβ1-4GlcNAc-oxazoline **2**.

2: ^{1}H NMR (^2H_2O): δ = 5.84 (d, 1H, $J_{1,2}$ = 6.8 Hz, H-1), 4.45 (d, 1H, $J_{1',2'}$ < 1.0 Hz, H-1′), 3.11–4.08 (m, 12H, sugar ring protons), 1.85 (s, 3H, methyl protons of oxazoline). ^{13}C NMR (^2H_2O): δ = 168.8 ($\underline{C}OCH_3$), 102.5 (J_{C-H} = 158.4 Hz, C-1′), 101.9 (C-1), 13.9 ($CO\underline{C}H_3$).

The glycosyl acceptor of Dns-Asn(GlcNAc)-OH was prepared from Fmoc-Asn(GlcNAc(OAc)$_3$)-*O*-tBu [18] by the following procedure [19]: (1) deprotection of the Fmoc group by piperidine, (2) introduction of the Dns group by Dns-Cl and triethylamine, (3) deprotection of the *O*-acetyl group by NaOMe in methanol, and (4) deprotection of the tBu ester by trifluoroacetic acid. GlcNAcβ1-*O*-*p*NP was purchased from Seikagaku Kogyo.

2.2. HPLC analysis and structure determination of transglycosylated product

The enzymatic reaction was monitored by high performance liquid chromatography (HPLC: Hitachi 6000 series) using a Mightysil RP-18 (Cica-Merck) column. The glycosyl acceptor GlcNAcβ1-*O*-*p*NP and the product (Manβ1-4GlcNAcβ1-4GlcNAcβ1-*O*-*p*NP) were detected

at 260 nm on a UV detector. Dns-Asn(GlcNAc)-OH and the product (Manβ1-4GlcNAcβ1-4GlcNAc-(Dns)Asn-OH) were detected at 520 nm (emission wave length) on a fluorescence detector. The yield of the resulting trisaccharide derivatives could be estimated by comparing the spectral area of the product with that of a sum of the product and the unreacted acceptor, based on the assumption that the absorptivity of the acceptor and that of the product are approximately the same. The ^{1}H and ^{13}C NMR spectra of the substrate **2** and the product **8** were measured in $^{2}H_2O$ by means of Jeol EX-400 spectrometer.

2.3. Preparation of enzymes

Endo-M was partially purified from the culture broth of *M. hiemalis* [20]. Endo-A was purified from the culture fluid of *A. protophormiae* [21]. These enzyme preparations were free from other glycosidase activities.

2.4. Enzymatic addition of Man-GlcNAc-oxazoline **2**

A typical procedure for enzymatic addition of Manβ1-4-GlcNAc-oxazoline **2** to GlcNAcβ1-*O*-*p*NP catalyzed by Endo-M is as follows: to substrate **2** (175 μg, 0.44 μmol) and GlcNAcβ1-*O*-*p*NP (75.3 μg, 0.22 μmol) dissolved in 20 mM potassium phosphate buffer (pH 6.25) was added Endo-M (1.37 mU) and the reaction mixture was incubated at 30°C for 120 min with gentle shaking. The resulting product was separated by HPLC, concentrated and lyophilized to give Manβ1-4GlcNAcβ1-4GlcNAcβ1-*O*-*p*NP **8** in 54% yield.

8: ^{1}H NMR ($^{2}H_2O$): δ = 8.09 (d, 2H, J = 9.2 Hz, *m*-Ph), 7.02 (d, 2H, J = 9.2 Hz, *o*-Ph), 5.16 (d, 1H, $J_{1,2}$ = 8.4 Hz, H-1), 4.62 (d, 1H, $J_{1',2'}$ < 1.0 Hz, H-1′), 4.48 (d, 1H, $J_{1'',2''}$ = 7.6 Hz, H-1″), 3.27–3.94 (m, 18H, sugar ring protons), 1.93 (s, 3H, methyl protons of NAc in GlcNAcβ1-*O*-*p*NP), 1.85 (s, 3H, methyl protons of NAc in the internal GlcNAc unit). ^{13}C NMR ($^{2}H_2O$): δ = 176.2 and 175.9 ($\underline{C}OCH_3$ of C-1 and C-1′), 162.8 (phenyl carbon attached to the phenolic oxygen), 143.8 (*p*-Ph), 127.2 (*o*-Ph), 117.5 (*m*-Ph), 102.5 (C-1′), 101.1 (C-1), 99.5 (C-1″), 23.0 and 22.9 ($CO\underline{C}H_3$).

3. Results and discussion

3.1. Transglycosylation by endo-type glycosidases

The transglycosylating ability of the novel substrate **2** toward GlcNAcβ1-*O*-*p*NP has been investigated using various endo-type glycosidases (Table 1). Among the enzymes tested, Endo-M and Endo-A were found to catalyze the transglycosylation of the Man-GlcNAc moiety of **2** to GlcNAcβ1-*O*-*p*NP or Dns-Asn(GlcNAc)-OH effectively (entries 1 and 2). When other endo-type glycosidases such as chitinase, Endo-H, Endo-F, and Endo-Fsp were used, the transglycosylated product could not be obtained (entries 3–8). We have already reported that the optimum pH for the transglycosylation of Galβ1-4GlcNAc-oxazoline catalyzed by chitinase (*Bacillus* sp.) is higher than that for hydrolysis (entry 3 in parentheses) [22]. We used this chitinase for **2** under a basic condition of pH 8.0; however, no transglycosylated product was produced. These results clearly indicate the difference in recognition by endo-β-*N*-acetylglucosaminidase and chitinase toward the Manβ1-4GlcNAc moiety.

3.2. Selectivity of transglycosylation using Endo-M and Endo-A

The transglycosylation reaction was monitored by HPLC. After the addition of Endo-M or Endo-A, the peak corresponding to the GlcNAcβ1-*O*-*p*NP became

Table 1
Transglycosylation activity of endo-type glycosidases for Man-GlcNAc-oxazoline **2**[a]

Entry	Enzyme	Time (min)	pH	Activity (mU)	Transglycosylation[e] yield (%)
1	endo-β-*N*-acetylglucosaminidase M (*M. hiemalis*)	120 (138)[c]	6.25 (6.25)[c]	1.4 (1.4)[c]	54 (77)[b] (49)[c]
2	endo-β-*N*-acetylglucosaminidase A (*A. protophormiae*)	120	6.0	13.8	44
3	chitinase (*Bacillus* sp.)	300 (30)[d]	8.0 (8.0)[d]	1.4 (1.4)[d]	0 (62)[d]
4	chitinase (*Serratia marcescens*)	120	6.0	1.4	0
5	chitinase (*Streptomyces griseus*)	120	6.0	13.8	0
6	endo-β-*N*-acetylglucosaminidase H (*Streptomyces plicatus*)	120	5.5	1.4	0
7	endo-β-*N*-acetylglucosaminidase F_1 (*Flavobacterium meningosepticum*)	120	5.0	1.4	0
8	endo-β-*N*-acetylglucosaminidase (Endo-Flavo; *Flavobacterium* sp.)	300	6.0	5.5	1

[a]Total volume: 20 μl; temperature: 30°C; donor (Man-GlcNAc-oxazoline)/acceptor (GlcNAc-PNP) = 21.54 mM/10.77 mM = 2:1.
[b]Donor/acceptor = 32.31 mM/10.77 mM = 3:1.
[c]Donor (Man-GlcNAc-oxazoline)/acceptor (Dns-Asn(GlcNAc)-OH = 44 mM/10 mM = 4.4:1.0.
[d]Donor (Gal-GlcNAc-oxazoline)/acceptor (GlcNAc-PNP) = 21.54 mM/10.77 mM = 2:1.
[e]Determined by HPLC. HPLC column: Mightysil RP-18 (φ 4.6–250 (Cica-Merck)); flow conditions: 8–16% (CH_3CN 0–40 min) gradient; 1.0 ml/min; detector: UV 260 nm (c: HPLC column; Inertsil ODS-3 (φ 4.6–250 (Cica-Merck)); solvent A: 25 mM borate buffer (pH 7.5); solvent B: CH_3CN/25 mM borate buffer (4:1); gradient: 20–50% of B (0-40 min).

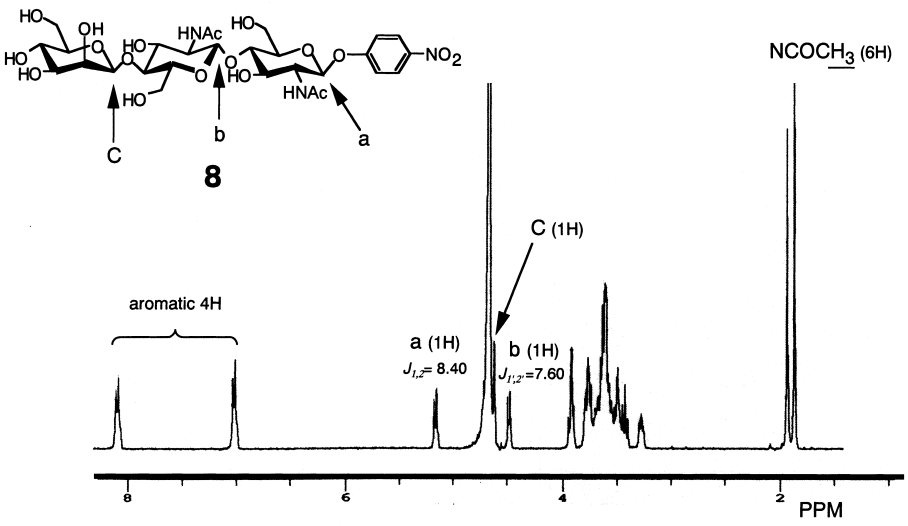

Fig. 4. ^{1}H NMR spectrum of trisaccharide product **8**.

smaller and a new peak appeared. The resulting product was purified using a preparative HPLC and freeze-dried.

The ^{1}H NMR spectrum of the product indicated the presence of a *p*-nitrophenyl group ($\delta = 8.09$ ppm (2H) and 7.02 ppm (2H)), methyl protons of two *N*-acetyl groups ($\delta = 1.93$ and 1.85 ppm (6H)) and three anomeric protons (a, $\delta = 5.16$ ppm; c, $\delta = 4.62$ ppm; and b, $\delta = 4.48$ ppm) (Fig. 4). The stereochemistry of the resulting glycosidic bond was determined to be β type according to the coupling constant of peak b ascribable to the anomeric proton of the internal GlcNAc unit ($J_{1',2'} = 7.60$ Hz). These data show that the transglycosylation reaction of **2** to GlcNAcβ1-*O*-*p*NP proceeds in a regio- and stereoselective manner, giving rise to a trisaccharide derivative **8** (Manβ1-4GlcNAcβ1-4GlcNAcβ1-*O*-*p*NP).

The structure of **8** was also supported by ^{13}C NMR spectroscopy and ^{13}C-^{1}H COSY NMR analysis. All of these spectral data indicated that the product is a trisaccharide derivative containing two GlcNAc units and one *p*NP group which were connected through a β-glycosidic bond.

In order to confirm the structure, an enzymatic cleavage of the β-mannosidic bond was performed by treating **8** with a β1-4-mannosidase. When a buffer solution of **8** treated with β1-4-mannosidase (from *Xanthomonas holicola*: Calbiochem), the peak derived from **8** disappeared and a new peak appeared (Fig. 5). The hydrolyzate was purified by HPLC and freeze-dried. The ^{13}C NMR spectrum of the hydrolyzate was found to be identical to that of commercially available *p*-nitrophenyl β-D-*N*-acetylchitobioside ((GlcNAc)$_2$β1-*O*-*p*NP). Thus, the transglycosylated product was confirmed to be Manβ1-4GlcNAcβ1-4-GlcNAcβ1-*O*-*p*NP **8**.

3.3. Evidence of irreversible transglycosylation of 2

Fig. 6 shows the relationship between the product yield

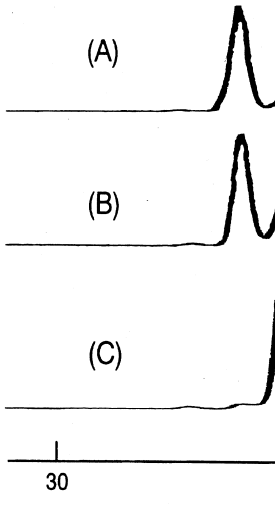

Fig. 5. High performance liquid chromatograms after addition of mannosidase after 0.5 h (A), after 2 h (B), and after 17 h (C).

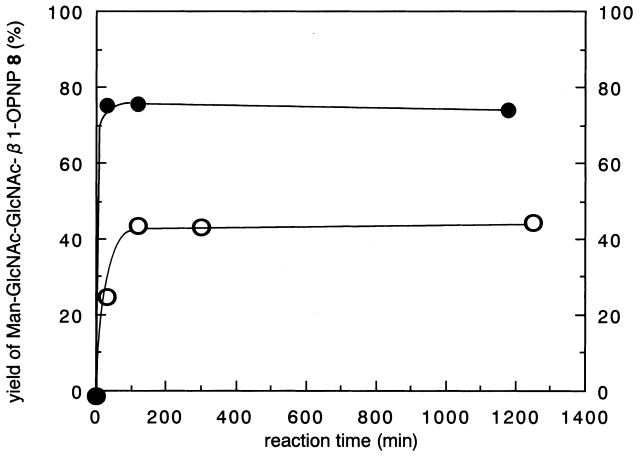

Fig. 6. Effect of incubation time on yield. ●, Endo-M; ○, Endo-A. Ratios of donor to acceptor are 3:1 (Endo-M) and 2:1 (Endo-A), respectively.

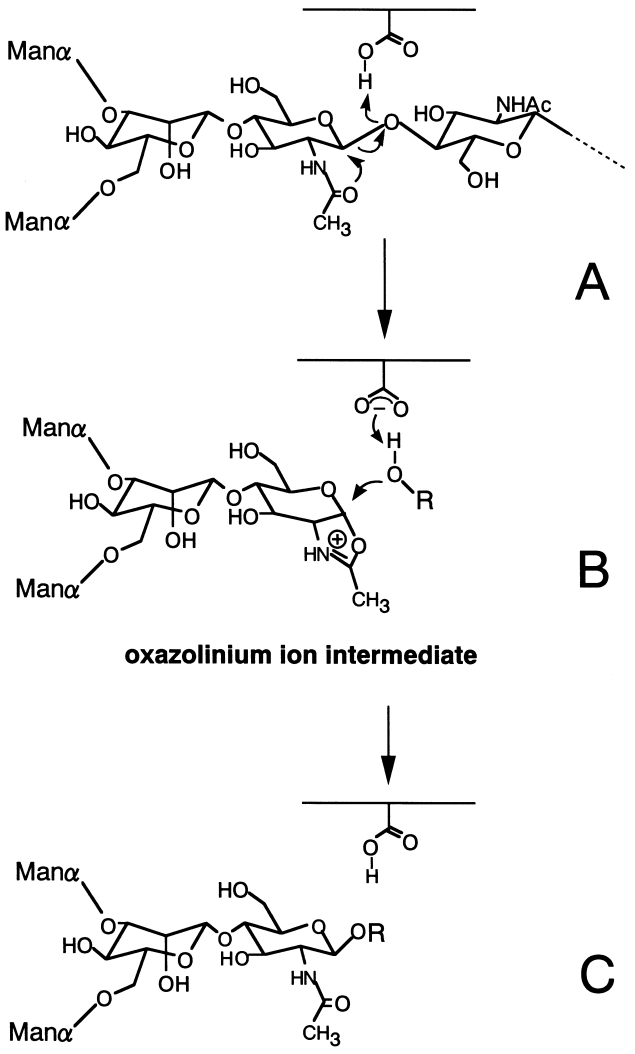

Fig. 7. Proposed reaction mechanism of Endo-M and Endo-A via oxazolinium ion intermediate.

and the incubation time. The formation of the trisaccharide product **8** has been completed within 0.5–1.0 h. The yields of **8** did not decrease on prolonged incubation; the transglycosylating process is irreversible. These observations clearly indicate that the resulting trisaccharide derivative **8** was not recognized as substrate by the catalytic site of Endo-M and Endo-A. It is well known that the recognizing ability of endoglycosidases strongly depends on the size of the oligosaccharide substrates. The branching part of the oligosaccharides is essential for cleavage of the GlcNAc-GlcNAc bond; the hydrolysis rate for oligosaccharides without branching becomes small [23]. This fact is consistent with the present results that the resulting trisaccharide derivative cannot be hydrolyzed by Endo-M or Endo-A.

In the present transglycosylation reaction, even a small molecule of Manβ1-4GlcNAc-oxazoline **2** could be recognized by endoglycosidases, indicating that the oxazoline moiety has a very strong affinity toward the catalytic site. This may be explained by assuming that compound **2** with a distorted conformation behaves as a transition state analogue substrate. The transglycosylation occurred very smoothly because the activation energy between the starting material **2** and the product **8** was significantly lowered [24].

These findings enable us to presume a new mechanism that involves an oxazolinium ion intermediate for the hydrolysis or transglycosylation reaction catalyzed by Endo-M and Endo-A (Fig. 7) [25,26]. According to this mechanism the glycosidic bond is cleaved as follows. First, the oxygen of the glycosidic bond between two GlcNAc units is protonated by the carboxylic acid of an acidic amino acid followed by the formation of the oxazolinium ion intermediate by the nucleophilic attack of the amide carbonyl group to the anomeric center (Fig. 7A). The resulting oxazolinium ion intermediate is then attacked by water or a glycosyl acceptor (R-OH) to give the hydrolyzate or a transglycosylated product (Fig. 7B,C).

In the present reaction of utilizing **2** as transition state analogue substrate, the nitrogen atom of the oxazoline ring may be protonated by the proton of an acidic amino acid located in the −1 subsite. The nucleophilic attack of the 4-hydroxyl group of the glycosyl acceptor occurs by the assistance of the carboxylate ion of the other acidic amino acid that behaves as a general base. The α-glycosidic bond of **2** is then cleaved and the carbon–oxygen double bond is formed simultaneously, leading to the formation of the 2-acetamide group in the product trisaccharide.

This is the first example of a transglycosylation reaction catalyzed by endo-β-*N*-acetylglucosaminidases (Endo-M and -A) using a transition state analogue substrate. It is noteworthy that based on the design for a transition state analogue substrate, the combined use of a small molecule of trisaccharide derivative and an endo-type glycosidase brings about substantial advantage of detecting the transglycosylating activity of an endoglycosidase in the synthetic field of glycotechnology. Moreover, this new substrate will be useful for the elucidation of the mechanism of hydrolysis or transglycosylation catalyzed by endo-type glycosidases. Further studies on the precise mechanism of hydrolysis as well as the application of the present transglycosylation to larger oligosaccharide donors are now in progress.

Acknowledgements

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