The One-step Preparation of Sugar Oxazoline Enables the Synthesis of Glycoprotein Having a Definite Structure

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Abstract
Sugar oxazoline derivatives are useful donor substrates for N-acetylg glucosaminidase-catalyzed glycosylation reactions. In this review, we describe the direct conversion of free sugars to their oxazoline derivatives by using water-soluble dehydrating agents and its application to enzymatic synthesis of complex glycosidic compounds including glycoproteins.

A. Introduction
Over half of all proteins typically expressed in mammalian cells are estimated to be glycosylated. Recent research has revealed that the stability and activity of glycoproteins greatly depend on their carbohydrate structures. For example, the quality of glycoproteins is strictly controlled by the structure of the oligosaccharides attached. The details were reviewed in the previous TIGG (1–3).

The biosynthesis of glycoproteins is achieved via multi-step reactions catalyzed by various glycosyl transferases and glycosyl hydrolases. Therefore, the synthesis of glycoproteins having a definite oligosaccharide structure is extremely difficult even if a highly controlled biosynthetic pathway is available. To date, the synthesis of glycoproteins having a single glycoform has only been achieved through a chemical or chemo–enzymatic path. The chemo–enzymatic method is more advantageous than chemical methods because glycoproteins having a large oligosaccharide moiety can be prepared as long as the enzyme can recognize the protein moiety as glycosyl acceptor.

Sugar oxazoline derivatives are used as efficient glycosyl donors for the synthesis of various glycoproteins and glycopolypeptides catalyzed by endo-N-acetylglucosaminidases. However, these sugar oxazoline derivatives must be prepared via extremely laborious process including protection and deprotection of the hydroxy groups. Simplification of the method for sugar oxazolines synthesis has, therefore, been strongly required. This review describes the recent progress in chemo–enzymatic synthesis of glycoproteins where the donor substrates of sugar oxazolines can be directly prepared from free sugars and utilized for the transglycosylation catalyzed by endo-N-acetylglucosaminidases with a lower hydrolytic activity.

B. Direct Synthesis of Sugar Oxazolines Using Water-soluble Dehydrating Agent
Sugar oxazoline derivatives are regarded as dehydrated products of the corresponding 2-acetamido sugars (Fig. 1). We postulated that the oxazoline ring formation might be achieved by using a dehydrating agent in aqueous media. To form the oxazoline derivative via dehydration reaction, the anomeric hydroxy group must selectively react with a dehydrating agent. The reported pK_{a} value of the anomeric hydroxy group in glucose is 12.4 (4). This fact indicates that the acidity of the hemiacetal is much larger than that of water, and the hemiacetal hydroxy group possesses sufficient nucleophilicity towards an electrophile in water. In 1995, Penadés reported a synthesis of 2,4-dinitrophenyl glycosides by using 2,4-dinitrofluorobenzene as electrophile in an aqueous ethanol solution (5). Recently, we have reported the first direct synthesis of the oxazoline derivative of N-acetylg glucosamine by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as dehydrating agent in aqueous media. However, the reaction yields were low under various conditions using EDC (Scheme 1) (6).
We assumed that the low yield of oxazoline formation was attributed to the lower electrophilicity of EDC and the lower leaving ability of the isourea moiety on the intermediate. After screening various dehydrating agents, we found that 2-chloro-1,3-dimethylimidazolium chloride (DMC), a formamidine type dehydrating agent, is the most suitable agent for the reaction. The strong dehydrating ability of DMC was also supported by the fact that an amide can be converted to the corresponding nitrile (7).

The synthesis of oxazoline derivatives was carried out in the presence of excess amount of DMC and triethylamine (Table 1) (8). This method was able to be applied not only to monosaccharides like GlcNAc but also oligosaccharides such as N-acetyllactosamine and chitooligosaccharides. Moreover, the oxazoline derivative of N-linked disialooligosaccharide could be prepared for the first time without lactonization and cleavage of the sialic acid moieties. The purification of oxazoline derivatives of chitooligosaccharides was achieved by using reversed phase HPLC. The oxazoline derivative having a carboxylic acid like disialooligosaccharide could be separated by preparative gel filtration chromatography (Sephadex G-15) (9). The present DMC method is a general synthetic methodology for preparation of complex oligosaccharide oxazolines, which has required the multi-step chemical routes including protection, and deprotection (10–13).

The DMC-mediated direct anomeric activation has also been found to be useful for preparation of other sugar derivatives in aqueous media. For example, various kinds of 1,6-anhydro sugars could be prepared directly from the corresponding unprotected sugars in good yields (14). Intermolecular dehydration reaction smoothly occurs by using sodium azide and arylthiols as nucleophiles, affording glycosyl azides and arylthioglycosides, respectively in excellent yields (Fig. 2) (15–17).

Although the efficient synthesis of oxazoline derivatives was achieved by using DMC, the method contains some problems. Excess amount of DMC was required under diluted conditions due to spontaneous hydrolysis of DMC. In addition, the manipulation of DMC was very difficult because of its hygroscopic nature. Moreover, the removal of 1,3-dimethylimidazolidinone (DMI), the decomposed product of DMC, was difficult due to the higher boiling temperature of DMI. We then continued to find a novel dehydrating agent which is much easier to handle. We postulated that the

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate (mM)</th>
<th>DMC eq.</th>
<th>Base (eq.)</th>
<th>Yield (%)</th>
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<td>Et₃N (9)</td>
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<td>Et₃N (45)</td>
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<td>Et₃N (45)</td>
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</table>

Fig. 2. DMC mediated derivatization of free saccharides.
stability would be increased by introducing an electron donating group into the formamidine skeleton.

We synthesized 2-chloro-1,3-dimethylbenzimidazolinium chloride (CDMBI) having a phenylene moiety instead of ethylene moiety in DMC (18). CDMBI was found to be considerably stable against moisture and showed no hygroscopicity. Moreover, the removal of the decomposed product of CDMBI, 1,3-dimethylbenzimidazolone, was easy due to its poor solubility toward water. CDMBI was soluble in water (1.2 g CDMBI/1 mL water at room temperature), and the intramolecular dehydration of 2-acetamido sugars took place smoothly in water, giving rise to the corresponding oxazoline derivatives. Although CDMBI and DMC showed almost the same efficiency for oxazoline formation at 150 mM substrate concentration, the difference was observed below 50 mM substrate concentration (Table 2). Sodium phosphate or sodium carbonate could also be employed as a base.

CDMBI is a useful reagent for preparation of oligosaccharide oxazolines having larger molecular weights and low solubilities. Chitobiose oxazoline derivative was obtained in 30% yield using five equivalents of CDMBI at 1 mM conditions (unpublished data). Although the reactivity of CDMBI is lower than that of DMC, the dehydrating ability of CDMBI is strong enough to promote the intramolecular dehydration between the 2-acetamido group and the hemiacetal. Based on the fact that the decomposed product can be removed as precipitates, we have successfully demonstrated a two-step enzymatic glycosylation without isolating oxazoline intermediate (Scheme 2).

<table>
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<tr>
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<th>Base (eq.)</th>
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<td>Na3PO4 (7.5)</td>
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<td>Na3PO4 (12.5)</td>
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</tr>
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<td>CDMBI (5)</td>
<td>K2CO3 (30)</td>
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<tr>
<td>9</td>
<td>disialooligo saccharide (50)</td>
<td>CDMBI (5)</td>
<td>K3PO4 3H2O (15)</td>
<td>91</td>
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</table>

Scheme 2. One-solution glycosylation reaction using CDMBI mediated oxazoline synthesis and Endo-M mutant.
C. Combined Use of Sugar Oxazolines and Mutant $N$-Acetylglucosaminidase Enables Efficient Transglycosylations

In general, the efficiency of glycosidase-catalyzed transglycosylations is low since the resulting glycosylated products are hydrolyzed by the enzyme catalysts. Glycosynthase developed by Withers et al. is one of solutions of this problem (19). Glycosynthases are designed by mutating a retaining glycosidase by replacing a nucleophilic amino acid to a non-nucleophilic amino acid of the enzyme. The glycosylation reaction proceeds without hydrolysis of the product in sufficient yields. As glycosyl donors, glycosyl fluorides having the opposite anomeric configuration to the products are employed. The details of glycosynthases were reviewed previously in TIGG (20).

Glycosidases are classified into glycoside hydrolase family (GH) 18, 20 and 85, such as chitinase, hexosaminidase, and endo-$N$-acetylglucosaminidase, lack nucleophilic amino acids in their active sites. The hydrolysis of glycosides by these enzymes proceeds via neighboring group participation, which is called “substrate assisted catalysis” (Fig. 3) (21). Recently, various sugar oxazolines have been extensively utilized as transition state analogue substrates for glycosidases (22, 23). Because of the lack of nucleophilic amino acids, these enzymes cannot be converted to the corresponding glycosynthases. In order to obtain glycosidases with high transglycosylating abilities, another strategy was required for preparation of mutants.

Chitinase A1 from Bacillus circulans WL-12 classified into GH 18 recognized the oxazoline derivative of N-acetyllactosamine (24). We have developed a chitinase A1 having a low hydrolytic activity by replacing the tryptophan 433 in the $-2$ subsite to alanine (ChiA1 W433A) (25, 26). ChiA1 W433A does not hydrolyze $p$-nitrophenyl $\beta$-chitobioside that is frequently utilized for measuring chitinase activity. However, the ChiA1 W433A was found to catalyze the transglycosylation reaction using the oxazoline derivative as donor substrate. The glycosylation reaction between oxazoline derivative of N-acetyllactosamine and chitobiose occurs by using ChiA1 W433A, giving rise to a transglycosylated tetrasaccharide in 96% yield. This is the first example of enzymatic transglycosylation by the combined use of an activated substrate and a deactivated glycosidase. The synthesis of chitoheptaose has been achieved by using chitopentaose oxazoline as glycosyl donor and chitobiose as glycosyl acceptor catalyzed by ChiA1 W433A (Scheme 3) (26). A combined use of one-step preparable oxazoline derivatives and an enzyme with a low hydrolytic activity enables

Fig. 3. Reaction mechanism for substrate assisted catalysis by hexosaminidase.


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D. Application to Glycoprotein Synthesis

Endo-N-acetylgalactosaminidase from *Mucor hiemalis* (Endo-M) classified into GH 85 hydrolyzes the GlcNAc–GlcNAc bond in N-linked oligosaccharides (27). Endo-M shows a transglycosylation activity in the presence of an appropriate acceptor substrate. Various glycopeptides were synthesized by using disialoglycopeptide (SGP) as glycosyl donor catalyzed by Endo-M (28–33).

Recently, Endo-M has been found to recognize the oxazoline derivative of Man–GlcNAc disaccharide as a substrate for transglycosylation reaction in the presence of a GlcNAc derivative acceptor (34). Interestingly, a trisaccharide, Man–GlcNAc–GlcNAc, was not recognized by Endo-M, suggesting that the transglycosylated product would not be hydrolyzed by Endo-M.

Yamamoto et al. has developed a mutant Endo-M having a higher transglycosylation activity and a lower hydrolytic activity by site-directed mutagenesis. When the Tyr217 is replaced by Phe (Endo-M Y217F), the hydrolytic activity was decreased to 72% and the transglycosylation activity increased to 147% (35). Endo-M Y217F was a suitable mutant for the transglycosylation reaction using SGP as a donor substrate. However, the resulting transglycosylation products were hydrolyzed during the reaction.

On the other hand, when the Asn175 was replaced by Ala (Endo-M N175A), the resulting mutant was able to recognize oxazoline derivatives as donor substrates and catalyze the transglycosylation reaction effectively in spite of the fact that the mutant showed almost no hydrolytic activity with SGP substrate. It is assumed that the Asn175 residue can stabilize the oxazolinium ion intermediate (Fig. 4). After screening other mutants of Asn175 residue, N175Q mutant (Endo-M N175Q) was found to be the best enzyme for the transglycosylation of N-linked oligosaccharide moiety (36). Endo-M N175Q enables the quantitative transglycosylation reaction using sugar oxazoline derivatives as donor substrates. Various glycopeptides and glycoproteins having a definite carbohydrate structure have been synthesized catalyzed by the mutant Endo-M (37–42). By using Endo-M N175Q as a catalyst, the one-solution glycosylation of a GlcNAc derivative has successfully been demonstrated without purifying the unstable oxazoline intermediate (Scheme 2) (18). Endo-M N175Q is now commercially available.

Endo-M recognizes various N-linked oligosaccharides such as high mannose type and hybrid type. However, N-linked oligosaccharides having core fucose are not recognized. Consequently, the remodeling of these fucosylated glycoproteins has been considered to be difficult. Endo-N-acetylgalactosaminidase from *Streptococcus pyogenes* (Endo-S) is able to recognize these fucosylated glycoproteins (43). B. G. Davis et al. achieved the preparation of human IgG having a single glycoform from fucosylated IgG by remodel-
ing using Endo-S (Fig. 5) (44). The inherent N-linked oligosaccharide containing core fucose in IgG was removed by Endo-S, giving rise to Fuc-GlcNAc-Asn structure. The resulting IgG was then glycosylated using Man₅GlcNAc tetrasaccharide oxazoline as a donor catalyzed by Endo-S, affording single glycoform IgG.

L. X. Wang et al. developed an endoglycosidase having a lower hydrolysis activity from Endo-S by using the same strategy used for the creation of Endo-M mutant. Endo-S whose Asp233 is replaced by Gln or Ala could catalyze transglycosylation reaction efficiently (45). Endo-S is a suitable enzyme for remodeling of IgG, since Endo-S recognizes IgG with high affinity (46).

Endo-N-acetylglucosaminidase from *Streptococcus pneumoniae* (Endo-D) also recognized fucosylated N-linked oligosaccharides. The preparation of Endo-D having a decreased hydrolytic activity was achieved by the same strategy as Endo-M (47). Endo-M, Endo-S and Endo-D are classified GH 85. The one-step preparation of sugar oxazolines is a general methodology that can be applied to various endo-N-acetylglucosaminidase-catalyzed transglycosylation in glycolotechnology field. The use of mutants endo-N-acetylglucosaminidase for the transglycosylation of sugar oxazolines was reviewed in TIGG (48).

**E. Future Prospects**

The direct synthesis of sugar oxazoline derivatives is an essential basic technology to produce various complex glycosidic compounds including N-linked glycoproteins. However, there are still some problems concerning the availability of the starting oligosaccharides in large scale. Although it has become much easier to obtain the disialooligosaccharide derivatives (SGP) from hen egg yolk (49), the availability of inexpensive oligosaccharide in a large-scale will be expected in order to promote basic researches in glycoscience.

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**References**

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Shin-ichiro Shoda received his Ph.D. degree from the University of Tokyo in 1981 under the supervision of Professor Teruaki Mukaiyama in the field of synthetic organic chemistry, where he developed the glycosyl fluoride method as a novel glycosylating technology. After spending three years working as an Assistant Professor at the University of Tokyo, he conducted his postdoctoral fellowship at ETH-Zurich (from 1984 till 1986) with Professor Dieter Seebach. In 1986, he moved to Tohoku University and joined the labs of Professor Shiro Kobayashi in the field of polymer synthesis, where he developed new chemo-enzymatic glycosylations by using glycosyl fluorides and sugar oxazolines as glycosyl donors. In 1999, he was promoted to a Full Professor at Tohoku University (Functional Macromolecular Chemistry Laboratory). His research interests include synthesis of carbohydrates, the development of novel glycosylations, and macromolecular architecture and precision synthesis of well-designed functional oligo- and polysaccharides.