

Accounts

Green Process in Glycotechnology

Shin-ichiro Shoda,* Ryukou Izumi, and Masaya Fujita

Department of Materials Chemistry, Graduate School of Engineering, Tohoku University, Aoba 07, Aoba-ku, Sendai 980-8579

(Received May 22, 2002)

Oligo- and poly-saccharides are important macromolecules in living systems. They show their multifunctional characteristics in the construction of cell walls, energy storage, and various biological phenomena like cell recognition. The development of a new glycosylation reaction is demanded in order to supply a sufficient amount of oligo- and poly-saccharides for basic research. Enzymes have several remarkable catalytic properties compared with other types of catalyst in terms of their selectivity, high catalytic activity, lack of undesirable side-reactions, and ability to operate under mild conditions. This account presents an overview of recent advances in the glycosylation reactions for synthesis of oligo- and poly-saccharides catalyzed by glycosyl hydrolases. A new carbon cyclic system of "Glyco-Chemistry Cycles" has been proposed based on the renewable polysaccharide biomass.

Carbohydrates are photosynthesized by using carbon dioxide and water and are the most widely distributed organic compounds as biomass on earth. Almost all naturally occurring carbohydrates exist as glycosidic compounds; they include oligo- or poly-saccharides, glycolipids, glycoproteins, nucleosides, and various alkyl glycosides. It is very rare for carbohydrates to be found in nature in the form of free sugar. Many important discoveries have been made to elucidate the roles of these glycosidic compounds from the viewpoint of basic science.[1] On the other hand, from technical points of view, these carbohydrates have long been utilized to improve human life.[2] Since carbohydrates show various chemical or physical properties derived from their complicated structures, the industrial fields where carbohydrates are employed have become extremely wide. Especially, oligo- or poly-saccharides which are composed of monosaccharide units connected through a glycosidic bond have a huge number of isomers, indicating that these compounds can act as informative molecules.

The formation of a glycosidic bond is realized by the reaction of a glycosyl donor and a glycosyl acceptor (Fig. 1). In glycosylating reactions, there are two possible geometric isomers related to the geometry of the anomeric carbon atom of monosaccharide, namely α-isomer and β-isomer. The control of the formation of these glycosidic bonds is called "stereoselectivity of glycosylation reaction". In addition to such stereoselectivity, there is one more prerequisite that is important when a glycosidic bond is formed. Monosaccharides such as glucose have four hydroxy groups that can participate in glycosyl bond formation: i.e., 2-, 3-, 4- and 6-hydroxy groups. Since oligo- and poly-saccharides can be formed by connecting the 1-hydroxy group of a monosaccharide unit and one of the four hydroxy groups of the adjacent monosaccharide unit, there are four possibilities: 1,2-bond, 1,3-bond, 1,4-bond, and 1,6-bond. Considering these possibilities, the numbers of possible isomers have been calculated: eleven isomers can be produced when a disaccharide is formed, 120 isomers can be pro-

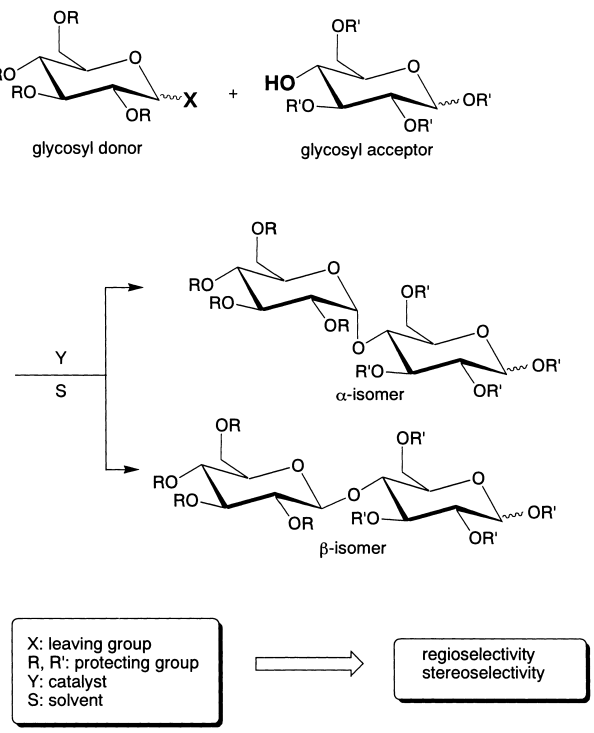


Fig. 1. Principle of glycosidic bond formation.

duced for a trisaccharide, 1424 isomers can be produced for a tetrasaccharide and 17872 isomers can be produced for a pentasaccharide.[3] Among these bonds, only one kind of bond must be formed in order to construct oligosaccharides of well-defined structures, which is called "regioselectivity of glycosylation reaction".

In order to generate regio- and stereo-selectivity, an appropriate leaving group X, protecting groups, an activator (catalyst), and a solvent should be selected. The classical Koenigs–Knorr reaction utilizes the combination of peracylated glycosyl halides as glycosyl donor and heavy metal salts like silver oxide or mercury(II) cyanide as activator in organic solvents.[4] Although many chemical glycosylation reactions with various combination of glycosyl donors and activators have appeared, the perfection of the glycosylating process still remains a challenging problem, especially in the synthesis of oligo- or polysaccharides where perfect control of regio- and stereo-chemistry is strictly required.

Oligo- and poly-saccharides can be divided into two classes: oligo- and poly-saccharides consisting of two or more types of monosaccharides that are connected in a diverse manner and oligo- or poly-saccharides having a repeating monosaccharide unit or a disaccharide unit. The former oligo- and poly-saccharides that show no repeating pattern play an important role in living systems. For example, some oligosaccharides on eucaryotic cell surfaces participate in cell recognition and immune responses.[5] The structure of the common unit of these oligosaccharides is shown in Fig. 2. It consists of a chitobiose moiety attached to the amide nitrogen of L-asparagine by a β-linkage. D-Mannose is attached to the chitobiose through a β-1,4 linkage, and two mannose residues are attached to this mannose by α-1,6 and α-1,3 glycosidic linkages. The development of a new methodology for regio- and stereo-selective synthesis of such oligo- and poly-saccharides has been an active topic in the synthetic field of carbohydrate chemistry. So far, various kinds of biologically important oligosaccharides have been prepared by connecting a monosaccharide unit through the use of chemical or enzymatic techniques.[6]

Typical examples of the another significant class of oligo- and poly-saccharides are cellooligosaccharide (cellulose) **1**, maltooligosaccharide (amylose) **2**, and chitooligosaccharide (chitin) **3** (Fig. 3). These compounds are important for construction of plant cell walls, energy storage, and as a skeletal component of invertebrates, respectively.[7] Nitrocellulose and acetylcellulose are classical examples of modified celluloses utilized as laquers, films, textiles, and tobacco filters. Recently, various functionalized celluloses like cellulose esters and

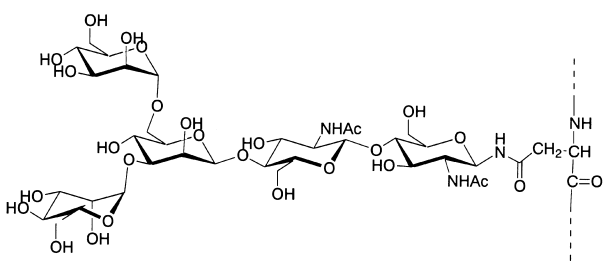


Fig. 2. Structure of pentasaccharide connected to asparagine of glycoprotein on cell surface.

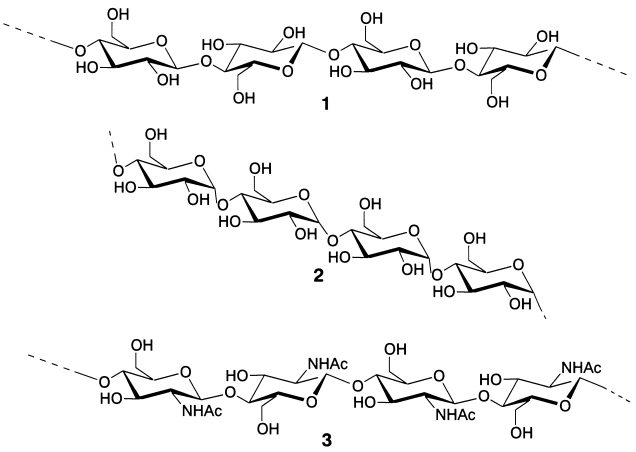


Fig. 3. Structure of cellooligosaccharide **1**, maltooligosaccharide **2**, and chitooligosaccharide **3**.

cellulose ethers have been developed as high performance polymers such as anion or cation exchangers, chromatographic materials, and electronic materials.[8] The construction of these oligosaccharide backbones is very difficult because complete regio- and stereo-control is required at every step of a continuous glycosylation. Whether a target molecule has a repeating unit or not, the regio- and stereo-selective construction of a glycosidic bond is one of the most challenging problems facing synthetic chemists.

Enzymes have several remarkable catalytic properties compared with other types of catalyst in terms of their regio- and stereo-selectivity.[9] In addition, from the viewpoint of environmental friendliness, enzymatic reaction is one of the most promising basic technologies with a simple operation under mild conditions, eliminating undesirable side-reactions. Enzymes that have been utilized so far for glycosidic bond formation are glycosyl hydrolases,[10] phosphorylases,[11] and glycosyltransferases.[12] This article presents an overview of recent advances in the synthesis of oligo- and poly-saccharides catalyzed by a hydrolase of glycosides, namely glycosidases and glycanases from a viewpoint of Green Chemistry. Glycosidases are enzymes that hydrolyze a glycosyl compound of small size. Glycanases refer to enzymes that hydrolyze polysaccharides. A new green process for synthesis of a typical glycosyl donor, glycosyl fluorides, and sugar oxazolines, will also be mentioned.

1. Green Chemistry in Synthesis of Glycosyl Donors

Glycosyl fluoride derivatives are one of the most useful glycosyl donors employed in chemical glycosylation and enzymatic glycosylation.[13] Because of the high C–F bond energy, glycosyl fluorides with appropriate protecting groups are stable in comparison with other glycosyl halides such as glycosyl chloride and glycosyl bromide which are utilized for the classical glycosylation reaction (Koenigs–Knorr reaction).[4] Since chemical glycosylations using protected glycosyl fluorides were developed in 1981,[14] the glycosyl fluoride method has been widely used and improved in organic synthesis.[15] Isomers of both α-glycosyl fluoride and β-glycosyl fluoride in their protected forms are prepared by the following two methods (Fig. 4(A)). β-Glycosyl fluorides are prepared by dis-

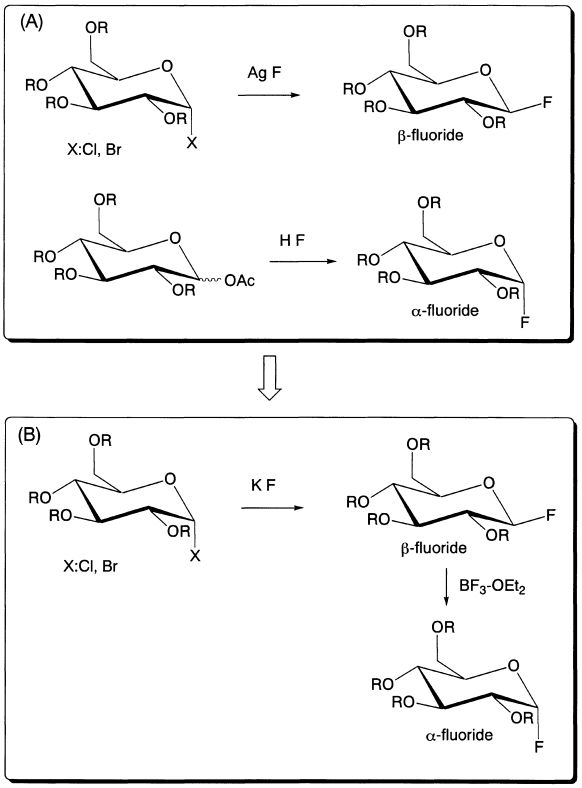


Fig. 4. Synthesis of glycosyl fluoride derivatives by classical method of using silver fluoride and hydrogen fluoride (A) and novel method based on Green Chemistry (B).

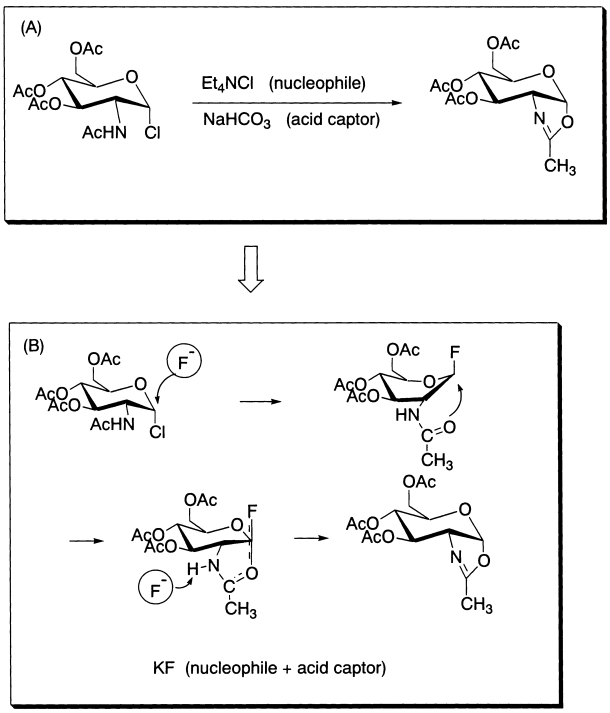


Fig. 5. Synthesis of 1,2-oxazoline derivatives of 2-acetamido-2-deoxy sugars by classical method of using tetraalkylammonium chloride as nucleophile and sodium hydrogencarbonate as acid captor (A), and novel method of using potassium fluoride as nucleophile and acid captor (B).

placement of the corresponding per-*O*-acy1ated glycosyl chlorides or bromides with silver fluoride via a S_N2 type reaction. The other anomer of α-glycosyl fluorides can be prepared by treatment of per-*O*-acylated sugars with hydrogen fluoride.

The synthetic route of β-fluorides can be replaced by another route that involves a nucleophilic substitution of α-glycosyl bromide derivatives by potassium fluoride (KF) (Fig. 4(B)).[16] The opposite anomer of α-glycosyl fluoride derivatives can be prepared via the anomerization of the resulting β-fluoride by the action of diethyl ether–boron trifluoride (1/1) (BF_3–OEt_2).[17] For laboratory scale experiments, the usage of KF and BF_3–OEt_2 is recommendable because the experimental procedures of these reactions without using a silver salt or hydrogen fluoride are much simpler than those of the conventional methods. When a glycosyl fluoride is employed for an enzymatic glycosylation, all the acyl protecting groups are removed with use of a base such as sodium methoxide.

The 1,2-oxazoline derivatives of 2-acetamido-2-deoxy sugars are the most useful key intermediates for preparation of various 2-acetamido-2-deoxy glycosides. Since Micheel et al. first reported the "oxazoline method" in 1958,[18] this methodology has been extensively employed in stereoselective synthesis of 1,2-*trans*-2-aminoglycosides.[19] Recently, sugar oxazolines having no protecting groups were found to be efficient glycosyl donors for enzymatic glycosylations.[20] Actually, several *N*-acetylglucosamination reactions have been reported by using an endo-*N*-acetylglucosaminidase as catalyst.

One of the classical methods for synthesis of sugar oxazoline derivative includes treatment of 2-acetamido-2-deoxy-glycosyl chlorides with sodium hydrogencarbonate in the presence of tetraalkylammonium chloride (Fig. 5(A)).[21] This reaction consists of the following two processes: 1) the anomerization of α-glycosyl chloride to β-glycosyl chloride by a nucleophilic attack of the chloride ion to the anomeric carbon atom and 2) the intramolecular attack of the carbonyl oxygen of the 2-acetamido group to the anomeric center as a result of proton abstraction on the nitrogen by sodium hydrogencarbonate. It is, therefore, necessary to utilize two kinds of reagents, quaternary ammonium salt and sodium hydrogencarbonate, for the reaction to occur. However, the yields of these reactions are normally low. In addition, these reactions require an ammonium compound that is very difficult to remove from the reaction mixture and consequently much organic solvent is necessary to purify the product by column chromatography. Such usage of tetraalkylammonium salt and organic solvent should be minimized from the viewpoint of Green Chemistry.

In the course of our investigation for synthesis of glycosyl fluoride donors by using KF, we found that a KF-promoted intramolecular cyclization reaction of 2-acetamido-2-deoxy-α-D-glucopyranosyl chloride takes place smoothly, giving rise to the corresponding 1,2-oxazoline derivative in good yield (Fig. 5(B)).[22] The intramolecular cyclization proceeds through a reactive intermediate of 2-acetamido-2-deoxy-β-D-glucopyranosyl fluoride as a result of a nucleophilic attack of the fluoride ion against the starting α-chloride. In this step, the fluoride ion behaves as a nucleophile toward the anomeric carbon atom to enhance the S_N2 type reaction with the inversion of configuration. The second step involves a participation of the carbonyl

oxygen of the 2-acetamido group from the α-side of the pyranose ring. The resulting cyclic intermediate suffers a proton abstraction on the nitrogen due to the action of another fluoride ion. According to the present method of using potassium fluoride, it is not necessary to utilize the quaternary ammonium chloride that is very difficult to be removed from the reaction mixture. This fact makes the reaction procedure extremely simple; filtrating the complex of potassium fluoride–hydrogen fluoride (KF–HF) and KCl can easily isolate the product. This methodology of using KF as nucleophile and acid captor can successfully be applied to an efficient synthesis of sugar orthoesters from the corresponding α-glycosyl bromide derivative and an alcohol.[23]

2. Endoglycosidase-Catalyzed Synthesis of Oligo- and Polysaccharides Using Glycosyl Fluorides as Glycosyl Donors

Glycosyl fluorides, sugar derivatives whose anomeric hydroxy group is replaced by a fluorine atom, have been extensively investigated as substrates for glycosidases, mainly in the fields of biochemistry or enzymology. Recently, glycosyl fluorides were found to be effective as glycosyl donors for enzymatic glycosylation catalyzed by endo-glycosidases. Endoglycosidases are defined as hydrolytic enzymes that cleave a glycosidic bond of the inner unit of polysaccharides. The merit of using glycosyl fluorides as glycosyl donors originates from the unique properties of the fluorine atom itself. First, the size of a fluorine atom is comparable in size to a hydroxy group, so that it can be accepted by an active site of an enzyme. Second, of the glycosyl halides, only glycosyl fluoride is stable as an unprotected form, which is necessary for most enzymatic reactions carried out in the presence of water. Ever since Barnett et al. demonstrated that a glycosyl fluoride could be recognized by a glycosidase,[24] numerous studies on the interaction of glycosyl fluorides and enzymes have been reported.[25] Nishizawa et al. reported that β-cellobiosyl fluoride is converted to cellobiose as main product together with a small amount of cellooligosaccharides as transglycosylated products catalyzed by endo-1,4-β-glucanase in a buffer solution (Fig. 6(A)).[26] When β-cellobiosyl fluoride was treated with the same enzyme in a mixed solvent of acetonitrile and acetate buffer, the transglycosylation predominates and the yield of cellooligosaccharides has significantly increased, providing a very efficient method for synthesis of cellooligosaccharide with perfect regio- and stereo-selectivity (Fig. 6(B)).[27] These reactions proceed via an oxocarbenium ion intermediate stabilized by a carboxylate of an acidic amino acid in the active site of the enzyme. If water attacks this intermediate, hydrolysis occurs to give cellobiose. If one of the hydroxy groups of the glycosyl acceptor attacks instead of water, the transglycosylation takes place.

The combination of a glycosyl fluoride and an endo-type glycosidase in an aqueous organic solvent has become an efficient general methodology of glycotechnology for preparation of various complicated oligosaccharides.[28] Actually, the methodology has successfully been applied to highly regio- and stereo-selective preparation of various oligosaccharides that are impossible to be isolated from nature. Many plant polysaccharides have a heterogeneous sequence composed of different monosaccharide units. For example, naturally occurring xylan, a xylose polymer having a β-1,4 glycosidic linkage in the main chain, normally contains L-arabinose and 4-*O*-methylglucuronic acid as side chains, its hydroxy group often being acetylated, and occasionally contains the β-1,3 glycosidic linkage as a minor unit (Fig. 7). This polysaccharide plays an important role in plant cell walls by interacting with cellulose microfibril. Therefore, a pure xylan, which is composed exclusively of xylopyranose units, can not be prepared from natural xylan (Fig. 7(A)).

A xylanase-catalyzed polycondensation of β-xylobiosyl fluoride took place in an aqueous organic solvent to give a mixture of linear oligosaccharides that contain only β-1,4 glyco-

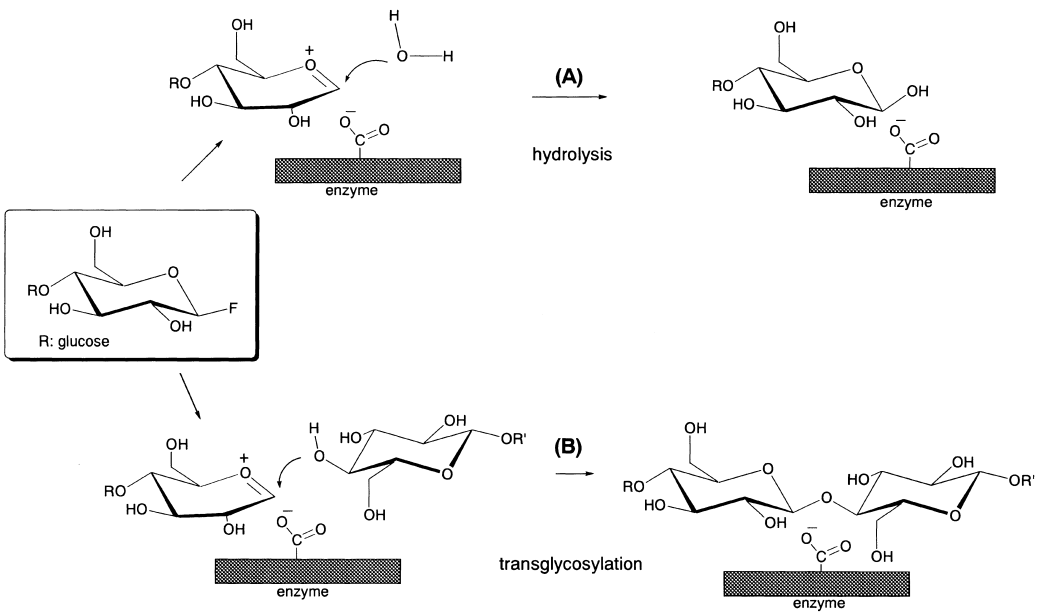


Fig. 6. The formation of oxocarbenium ion intermediate in the catalytic site of glycosidase as a result of C–F bond cleavage of β-cellobiosyl fluoride (R = glucose) followed by hydrolysis (A) and transglycosylation (B).

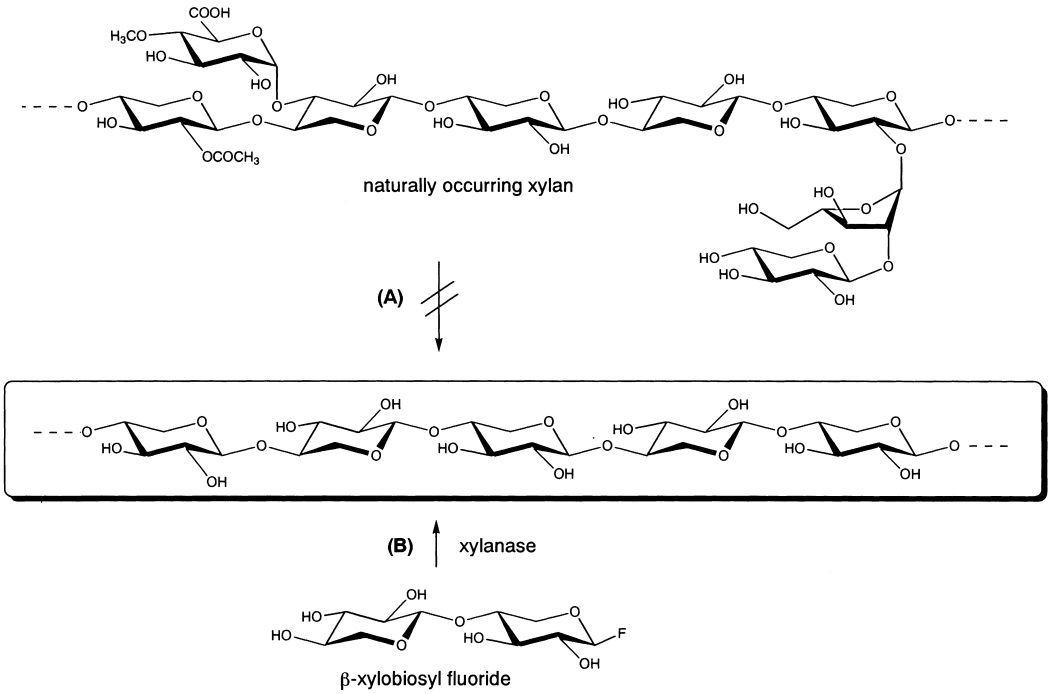


Fig. 7. Enzymatic polycondensation of β-xylobiosyl fluoride affording artificial xylooligomers that is impossible to prepare from naturally occurring xylan.

sidic bonds (Fig. 7(B)).[29] Although chemical synthesis of xylooligosaccharides by utilizing the classical Koenig–Knorr reaction was reported, the synthesis requires a repeating process that involves the regioselective deprotection of a hydroxy group.[30] Different from naturally occurring xylan, the oligosaccharides prepared enzymatically consists exclusively of D-xylopyranose residue with neither L-arabinose nor D-glucuronic acid as side chains.

The above reaction is to be interestingly compared with a biosynthetic pathway of naturally occurring xylan where the saccharide monomer is uridine diphosphate xylose formed as a result of a dehydrating process followed by a decarboxylating process starting from uridine 5′-diphosphoglucose (Fig. 8). Generally, the enzymes responsible for synthesis of polysaccharides are glycosyl transferases that have also become important catalysts in glycotechnology. In fact, glycosyltransferases have been extensively utilized for preparation of various biologically important oligosaccharides. However, the use of glycosyl transferases for the synthesis of a wide array of natural and non-natural oligosaccharides is restricted due to their higher substrate specificity. In contrast, glycosidases show much lower substrate specificity than transferases, which enables us to develop a new synthetic tool for functionalized oligosaccharides of non-natural types.

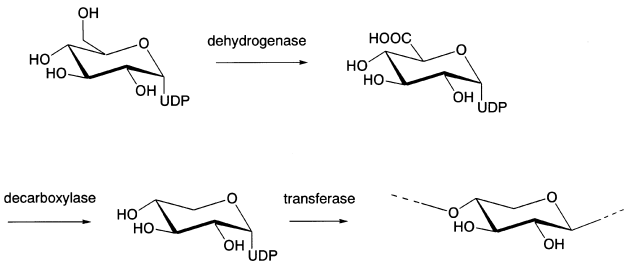


Fig. 8. Biosynthetic pathway of naturally occurring xylan.

The glycosyl fluoride method by the combination of a glycosyl fluoride substrate and a glycanase can be further applied to preparation of oligosaccharides of α-1,4 type, as well as that of oligosaccharides containing a β-1,3 bond and a thioglycosidic bond. Maltooligosaccharides have been prepared by polycondensation of α-D-maltosyl fluoride using α-amylase (from *Aspergillus oryzae*) in a mixed solvent of methanol–phosphate buffer.[31] The use of methanol as cosolvent enhances the polycondensation to afford higher maltooligomers. A 1,3-1,4-β-D-glucan 4-glucanohydrolase (from *Bacillus licheniformis*) has been shown to catalyze effective polycondensation of β-laminaribiosyl fluoride, and to lead to alternate 1,3-1,4-β-D-glucotetraose and -glucohexaose products.[32] The transglycosylation using the same glycosyl donor and methyl β-laminaribioside as acceptor gives the methyl 4-*O*-β-laminaribiosyl-β-laminaribioside in 40% overall yield. An enzymatic polycondensation of 4-thio-β-cellobiosyl fluoride in a buffer/organic solvent afforded a mixture of water-soluble hemithiocellodextrins with DP 4-14.[33] It is well known that cellooctaose is almost completely water-insoluble. The significant increase of the solubility of the hemithiocellodextrins is explained by the interruption of H-bonds that may introduce a distortion in the packing of the chains of oligo- and poly-saccharides.

3. Synthetic Strategy of Non-Natural Oligosaccharides

In the previous section, the advantage of using enzymes has been mentioned from the viewpoints of regio- and stereo-control in the construction of oligosaccharide structure of natural types. Another merit of enzymatic glycosylations can be seen in the formation of modified polysaccharides with well-defined

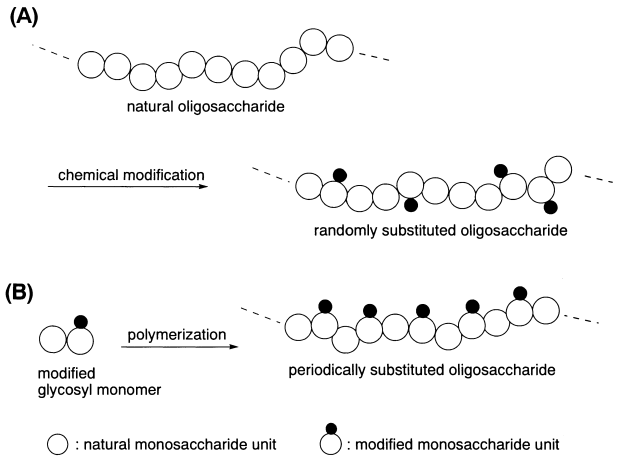


Fig. 9. Synthesis of substituted oligosaccharides by chemical modification of naturally occurring oligosaccharides (A), and polymerization of modified glycosyl monomer (B).

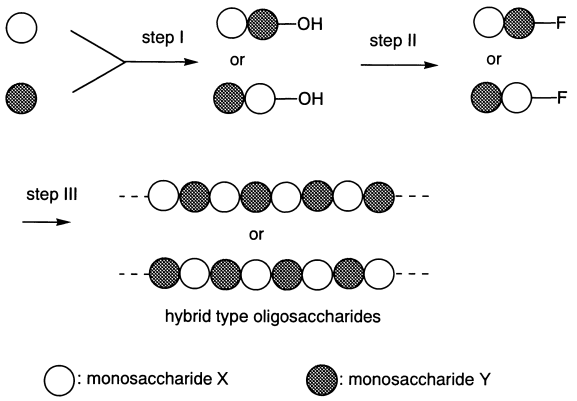


Fig. 10. Chemo-enzymatic procedures for synthesis of hybrid-type oligosaccharides having an alternating structure composed on a monosaccharide X and monosaccharide Y.

structures. The conventional synthetic strategies for functionalized polysaccharides have been based on the modification of hydroxy groups of naturally occurring polysaccharides by chemical reactions (Fig. 9(A)). For example, the derivatization of cellulose has extensively been studied for the development of high-performance cellulose materials. It is, however, obvious that these methodologies are not suitable for construction of a novel oligosaccharide having a well-defined structure because polymer reactions give a mixture of randomly substituted oligosaccharides.

In the present section, a new methodology for synthesis of non-natural oligosaccharides will be mentioned; an enzymatic polycondensation of a modified glycosyl monomer composed of a natural monosaccharide unit and an artificial monosaccharide unit will be demonstrated (Fig. 9(B)). According to this methodology, a novel functionalized oligosaccharide having a modified unit periodically in the main chain can be produced, provided that the regio- and stereo-chemistry of each glycosylating process is perfectly controlled. The perfection of glycosylation can be ensured by the usage of an endo-glycosidase as catalyst for successive glycosylating processes.

The cellulase-catalyzed polycondensation of β-cellbiosyl fluoride has successfully been applied to the synthesis of various modified cellooligosaccharides. Novel cellobiosyl fluoride derivatives, 6-*O*-methyl-β-cellobiosyl fluoride and 6′-*O*-methyl-β-cellobiosyl fluoride have been prepared as new monomers for enzymatic polycondensation, aiming at the synthesis of regioseletively *O*-methylated cellulose derivatives.[34] The 6-*O*-methylated monomer polymerized smoothly in a regio- and stereo-selective manner, giving rise to cellooligosaccharide derivatives having a methyl group alternatingly at the 6 position, whereas the 6′-*O*-methylated monomer showed a low polymerizability.

A new synthetic strategy for hybrid-type oligosaccharides having an alternating structure composed of a monosaccharide X and monosaccharide Y has been developed by chemoenzymatic procedures (Fig. 10). According to this principle, it is possible to design two kinds of disaccharide monomers, X-Y and Y-X. The skeleton of the disaccharide units (X-Y and Y-X) can be constructed by a chemical coupling of different kinds of monosaccharides (X and Y) (step I). The resulting disaccharide derivatives are converted to the corresponding fluorides (step II). In the final step III, the fluorides are polycondensed enzymatically.

Based on the principle mentioned above, a hybrid type oligosaccharide having a xylose unit and a glucose unit alternatingly in the main chain was synthesized by the enzymatic polycondensation of β-xylopyranosyl-glucopyranosyl fluoride, catalyzed by a xylanase, a hydrolase of xylan.[35] The regio- and stereo-selectivity of the glycosylating process between each disaccharide monomer unit is controlled perfectly, affording a β-1,4 glycosidic bond. The enzymatic polycondensation proceeds by the disaccharide unit. The transglycosylation at the glycosidic bond between the xylose moiety and the glucose moiety did not take place; no cleavage of the *O*-glycosidic bond of the disaccharide monomer was observed. An endo-xylanase was found to be a very efficient catalyst for the polycondensation, where both of the glycosyl donor site and the glycosyl acceptor site of the endo-xylanase could recognize the xylose-glucose moiety, enabling the polycondensation to proceed in a completely regio- and stereoselective manner.

Similarly, a new disaccharide monomer, mannopyranosyl-glucopyranosyl fluoride, was designed for synthesis of a novel glucomannan oligosaccharide derivative (Fig. 11).[36] The disaccharide monomer having an axial 2′-hydroxy group has successfully been polycondensed by cellulase mixture to give an oligosaccharide having a mannose unit and a glucose unit alternatingly in the main chain.

Some glycosyl fluorides were found to be suitable substrates for cyclodextrin-α-1,4-glucosyltransferase (CGTase). Cyclodextrin and its analogues were successfully synthesized by the polycondensation of α-glucosyl fluoride or α-maltosyl fluoride derivatives.[37] By use of immobilized CGTase (silica gel support functionalized with glutaraldehyde), α-glucosyl fluoride is transformed in high yield predominantly into cyclodextrins and maltooligomers as side products. The polymerization is carried out in the presence of sodium hydroxide, which neutralizes the liberated hydrogen fluoride as the reaction proceeds. New substrates of α-maltosyl fluorides substituted at the 6- or 6′-position with H, F, Br, OMe, and OAc were tested

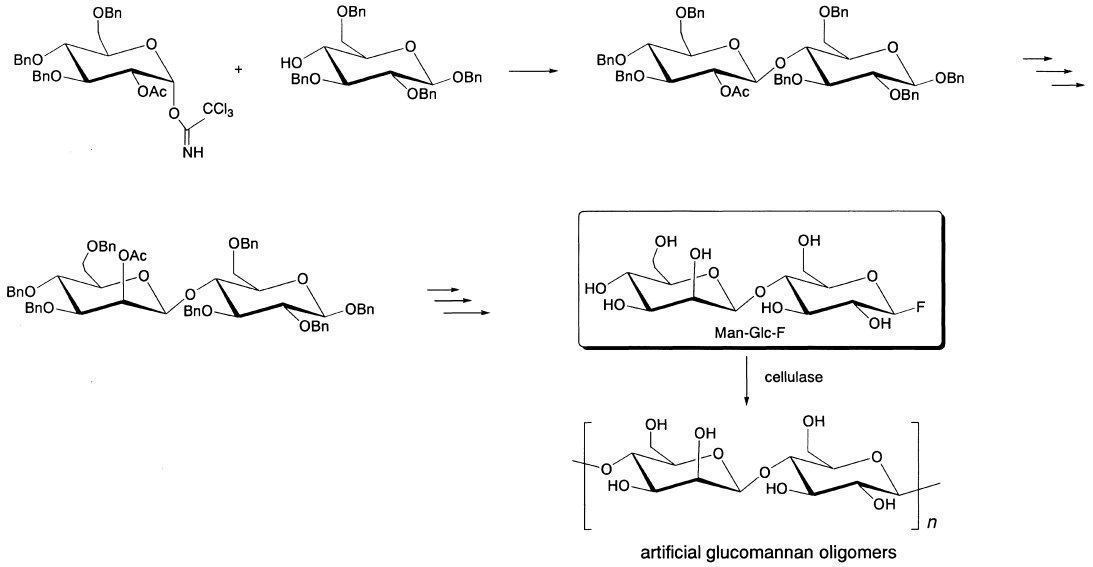


Fig. 11. Chemo-enzymatic synthesis of artificial glucomannan oligomers by enzymatic polycondensation of Man-Glc-F catalyzed by cellulase.

as substrates for the CGTase from *Bacillus macerans*.[38] All the modifications made at the 6-position of the maltose residue prevent binding in the donor part of the catalytic site, and only 6′-*O*-methyl and 6′-*O*-acetyl monomers were transformed into cyclic compounds.

4. Enzymatic Glycosylation Based on Reactive Intermediate or Transition State

4.1. Glycosynthase. In the previous section, the utility of endoglycosidases as catalysts for selective synthesis of oligosaccharides has been mentioned. However, the limitation of these reactions should be pointed out because the yield of the reaction is normally low due to the reversibility of the glycosylation reaction catalyzed by hydrolytic enzymes. In principle, glycosidases catalyze not only transglycosylation but also hydrolysis of the resulting glycosyl compounds unless the products are removed from the reaction mixture. Recently, Withers and his co-workers have solved this inherent problem of product hydrolysis (Fig. 12).[39] They prepared specific mutants to remove the catalytic nucleophile of acidic amino acid (glycosynthase). These mutants are hydrolytically inactive towards oligosaccharide substrates because the reactive glycosyl-enzyme intermediate can not be formed. In these reactions, a α-glycosyl fluoride having the opposite anomeric configuration is utilized as glycosyl donor for these mutants. The glycosyl fluoride having α-configuration can be recognized by the catalytic site of the mutant enzyme as an analogue of the covalent glycosyl-enzyme intermediate. The transglycosylation reactions proceed in excellent yields because the mutant enzymes are hydrolytically inactive towards the glycosides formed. The first glycosynthase was prepared from *Agrobacterium* β-glucosidase (Abg) E358A. Glycosynthase-mediated catalysis of glycoside formation is a promising technology for the future synthesis of oligosaccharides.

4.2. Enzymatic Addition of Sugar Oxazolines. In the previous sections, enzymatic glycosylation reactions using glycosyl fluorides as glycosyl donors were reviewed. The con-

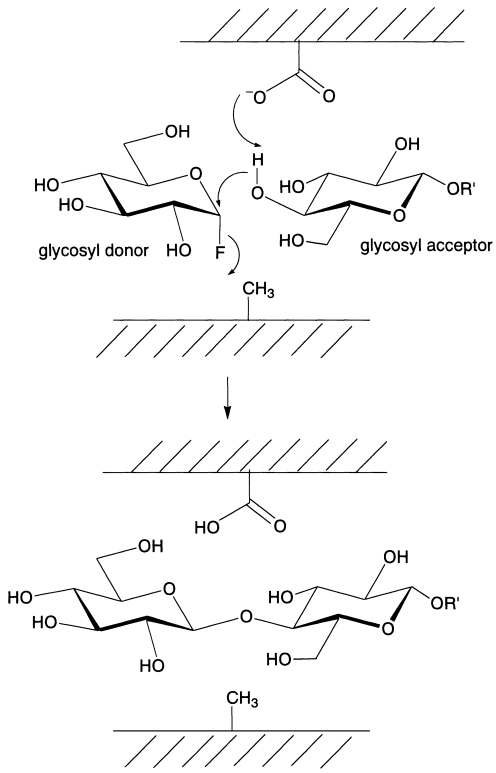


Fig. 12. Glycosynthase-mediated glycosylation.

formation of these glycosyl donors had to be very close to that of the product glycosides or to that of the glycosyl-enzyme intermediates for the reaction to occur. Smooth acceptance of the glycosyl donor into the active site of enzyme is ensured by the similarity of conformations between glycosyl fluoride donors and product glycosides or glycosyl-enzyme intermediates. In the present section, a new methodology for glycosylation by using a glycosyl donor having a distorted pyranose ring will be described. The sugar oxazoline are designed as a gly-

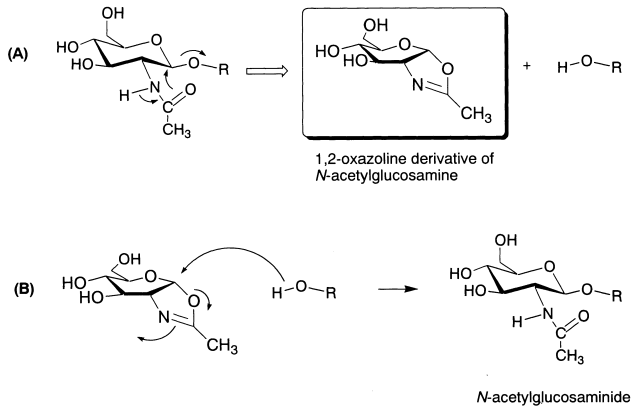


Fig. 13. Retrosynthesis of *N*-acetylglucosaminide unit leading to a distorted 1,2-oxazoline derivative (A) and addition of alcohol to the 1,2-oxazoline derivative leading to *N*-acetylglucosaminide.

cosyl donor on the basis of a retrosynthetic analysis of the product of *N*-acetylglucosaminide, leading to a transition state analogue of the enzymatic hydrolysis (Fig. 13(A)). When the *N*-acetylglucosaminide unit is retrosynthetically disconnected by the arrows shown in Fig. 13(A), a sugar oxazoline derivative is formed as a reasonable synthon. As a result of the retrosynthetic analysis, the real synthetic reaction of *N*-acetylglucosaminide would be the addition of an alcohol to the anomeric center of the sugar oxazoline derivative (Fig. 13(B)). Actually, the 1,2-oxazoline derivative of *N*,*N*′-diacetylchitobiose was found to be recognized by chitinase, a hydrolase of chitin, and the ring-opening polyaddition of proceeded smoothly, giving rise to the artificial chitin in quantitative yield.[20]

This novel methodology based on the design of the transition state analogue substrate for endoglycosidases can be applied to synthesis of various oligo- and poly-saccharides.[40] For example, a novel substrate of *N*-acetylcellobiosamine-oxazoline derivative has been synthesized as a monomer for the enzymatic polyaddition reaction catalyzed by the chitinase. The enzymatic polyaddition of this monomer took place smoothly to give a non-natural oligosaccharide having a glucose unit and an *N*-acetylglucosamine unit alternatingly in the main chain. These addition reactions by the combined use of a transition analogue substrate and a chitinase proceed only in the direction of addition at a high pH of 10–11 because the chitinase retains the transglycosylating activity without showing any hydrolytic activity at pH 10–11. These results have a very close relationship with the recent study concerning the hydrolysis mechanism of chitooligomers by a chitinase where a new concept of substrate-assisted mechanism involving an oxazolinium ion as intermediate is proposed (Fig. 14).[41]

The addition reaction of 1,2-oxazoline derivative of *N*-acetylglucosamine can be used not only for construction of an *N*-acetylglucosaminide structure but also for elucidation of the hydrolytic mechanism of endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M) and that of endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) (Fig. 15).[42] A disaccharide substrate of Man-GlcNAc-oxazoline was designed and synthesized as a novel probe for detection of the transglycosylating activity of endoglycosidases. A regio- and stereo-selective transglycosylation reaction of the substrate to *p*-nitrophenyl β-D-*N*-acetylglucosaminide (GlcNAcβ1-OpNP) catalyzed by Endo-M or Endo-A has been demonstrated, resulting in the core trisaccharide derivative Manβ1–4GlcNAcβ1–4GlcNAcβ1-OpNP. The transglycosylation proceeds irreversibly; the resulting trisaccharide 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. According to this mechanism the glycosidic bond is cleaved as fol-

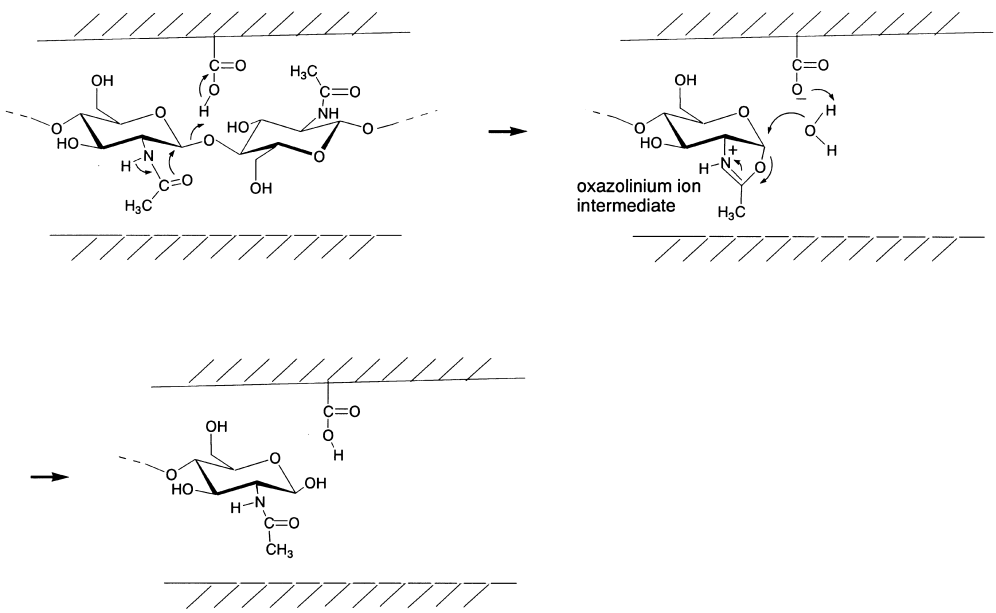


Fig. 14. Substrate assisted catalysis involving the neighboring group participation of *N*-acetyl group at the catalytic site of enzyme, leading to the formation of an oxazolinium ion intermediate.

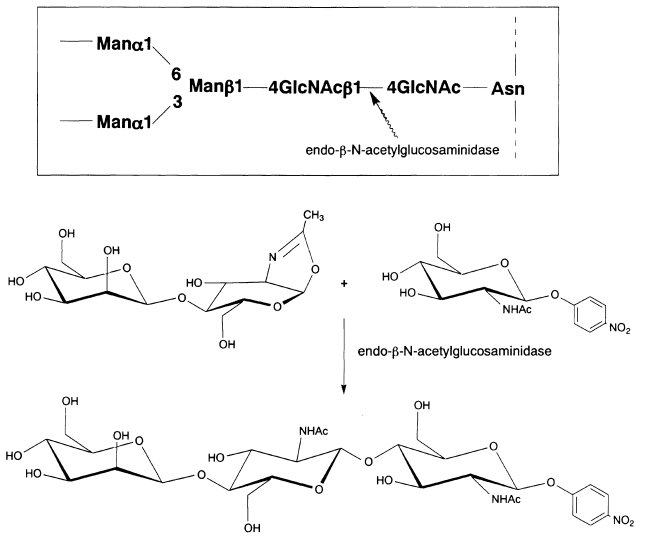


Fig. 15. Synthesis of trisaccharide core of glycoprotein by enzymatic addition reaction of a sugar oxazoline donor.

lows. 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. The resulting oxazolinium ion intermediate is then attacked by water or a glycosyl acceptor (R-OH) to give the hydolyzate or a transglycosylated product.

5. Galactose as Protecting Group in Enzymatic Synthesis of Oligosaccharides

Most of enzymatic glycosylations so far mentioned have been restricted to homo-coupling of activated substrates such as glycosyl fluorides or sugar oxazolines, where the substrates behave as glycosyl donor as well as glycosyl acceptor. The enzymatic hetero coupling was achieved by introducing a galactose unit at the non-reducing end of a glycosyl donor (Fig. 16). β-Lactosyl fluoride, which is accepted by cellulase but cannot be polycondensed owing to the axial hydroxy group at the 4′-position, has been used as a glycosyl donor for the preparation of oligosaccharides having a galactose unit at the non-reducing end. In this reaction, the galactose moiety acts as a protecting group of 4-hydroxy group on the glucose unit. As glycosyl acceptors, a variety of natural or non-natural monosaccharides and disaccharides in the form of their alkyl glycosides and thioglycosides have been employed.[43] The yields are very much dependent on the nature of the glycosyl acceptor and are in the range of 0 to 60%. The lactosylation takes place at the 4-position of the non-reducing end of the glycosyl acceptor forming the β-1,4 glycosidic bond exclusively.

High yields of the lactosylation were obtained using methyl β-glucoside derivatives, methyl β-mannoside, and methyl β-xyloside. Methyl α-cellobioside, methyl β-cellobioside, methyl β-laminaribioside, and methyl β-gentiobioside were also lactosylated effectively. Other derivatives, like methyl β-alloside and methyl α-glucoside carrying an axial hydroxy group do not seem to be accepted by cellulase and no lactosylation of these substrates occurs. These results can be used for the estimation of the steric repulsion of the methyl group and the active site (acceptor site) of cellulase. The following statements summarize the nature of the active site of cellulase. 1) The lactosylation reaction yields exclusively a β-1,4 glycosidic bond and therefore the glycosyl donor and acceptor must be oriented in a linear way. The retention of the configuration of the anomeric carbon can be explained by a double displacement mechanism involving the formation of a reactive intermediate, e.g. an oxocarbenium ion stabilized by the enzyme, and the nucleophilic attack of the 4-hydroxy group of the glycosyl acceptor. 2) The monosaccharide-acceptors having an axial group at the 1- or 3-position cannot be lactosylated, therefore, this group is directed towards the enzyme, inhibiting the binding owing to steric repulsion between the hydroxy group and the enzyme (Fig. 17). 3) An axial group at the 2-position of the monosaccharide or at the 1-position of the disaccharide does not inhibit binding, indicating that these groups are directed away from the enzyme.

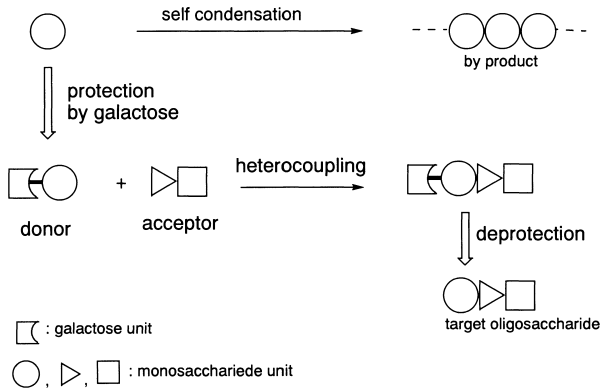


Fig. 16. Synthetic route to oligosaccharides by using galactose as protecting group for saccharide moiety.

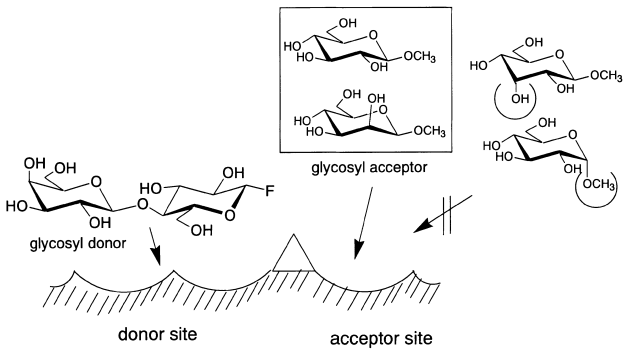


Fig. 17. Steric repulsion of axial group on glycosyl acceptor in enzymatic lactosylation catalyzed by cellulase.

As an application of this lactosylation reaction, a new route for regio- and stereo-selective synthesis of cellooligosaccharide derivatives has been developed by utilizing the following two enzymatic reactions (Fig. 18).[44] The first step involves the highly stereoselective β-lactosylation of methyl cellobioside ($n = 1$) catalyzed by cellulase using β-lactosyl fluoride as glycosyl donor, giving rise to a new oligosaccharide having a galactose unit at the non-reducing end (step I). The second step is a regioselective deprotection of the terminal galactose unit by the action of β-galactosidase, affording the corresponding

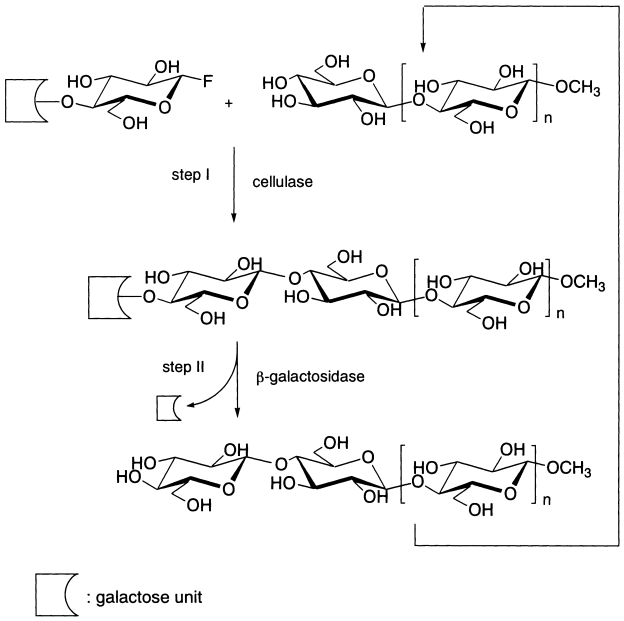


Fig. 18. Synthesis of cellooligosaccharide derivatives by combined use of endo-1,4-β-glucanase and β-galactosidase.

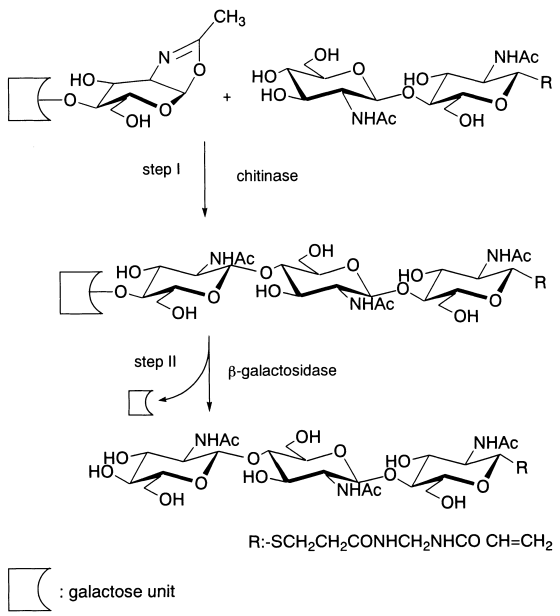


Fig. 19. Synthesis of chitotriose derivative having a polymerizable group the reducing end by combined use of chitinase and β-galacosidsase.

cellotrioside (n = 1) (step II). The resulting cellotrioside derivative was further subjected to the condensation with β-lactosyl fluoride, followed by the enzymatic degalactosylation process leading to the formation of methyl β-cellotetraoside (n = 2).

A chitotriose derivative having a polymerizable group has been synthesized via a process similar to the cellooligosaccharide synthesis via a chitinase-catalyzed addition reaction of the oxazoline derivative of *N*-acetyllactosamine, followed by β-galactosidase-catalyzed deprotection (Fig. 19).[45]

6. Glyco-Chemistry Cycle System

In biosyntheses of oligo- and poly-saccharides, the glycosidic bonds between each monosaccharide unit cannot be formed by the direct dehydration reaction of the corresponding monosaccharides. It is necessary to activate the anomeric carbon atom of the monosaccharide by introducing an appropriate leaving group so that the anomeric carbon atom is attacked by a hydroxy group of another monosaccharide unit. For example, in the biosynthesis of naturally occurring polysaccharides such as cellulose and chitin, the monomers of glucose and *N*-acetylglucosamine are activated as uridine 5′-diphosphoglucose and uridine 5′-diphospho-*N*-acetylglucosamine, respectively. Catalysts responsible for the polymerization of these activated monosaccharides are cellulose synthase and chitin synthase, respectively, which are classified as glycosyl transferases. These polysaccharides or their derivatives utilized are finally converted to carbon dioxide and water via combustion or degradation catalyzed by hydrolytic enzymes from bacteria. It is obvious that two kinds of enzymes, glycosyl transferases and glycosidases, are involved in the processes of polymerization and depolymerization in nature, constructing a large carbon cyclic system (Fig. 20).

In the present section, a new process for production of functionalized oligosaccharides based on the concept of "Glyco-Chemistry Cycle" will be proposed. In this cyclic system, a glycosidase plays two roles: glycosidic bond formation and glycosidic bond cleavage. Naturally occurring xyloglucan has a structure which consists of β-1,4 glucan chain with single xylopyranosyl residue linked α-1,6 to the main chain (Fig. 21). Some xyloglucan extracted from *Tamarindus* seeds contains a galactopyranosyl residue β-1,2 linked to the xylopyranosyl residue. The procedure for preparation of a novel trisaccharide from naturally occurring xyloglucan is as follows.[46] First, naturally occurring xyloglucan was treated with endo-1,4-β-glucanase to cleave the glycosidic bond between the xylosylated glucose unit and the unsubstituted glucose unit regioselectively (Fig. 21(A)). The resulting mixture of oligosaccharides was then treated with β-galactosidase to remove the galactopyranosyl residue, affording a heptasaccharide (Fig. 21(B)). The resulting heptasaccharide was further treated with isoprimeverose producing enzyme, giving rise to a mixture of smaller oligosaccharides (Fig. 21(C)). After purification by gel permeation chromatography, a trisaccharide, xylopyranosylcellobiose, was obtained effectively. The introduction of fluorine

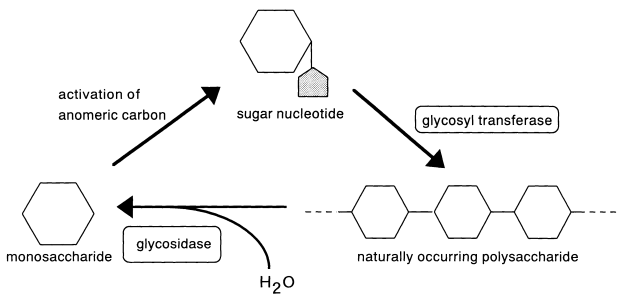


Fig. 20. Carbon cycle based on biosynthesis and biodegradation of polysaccharides.

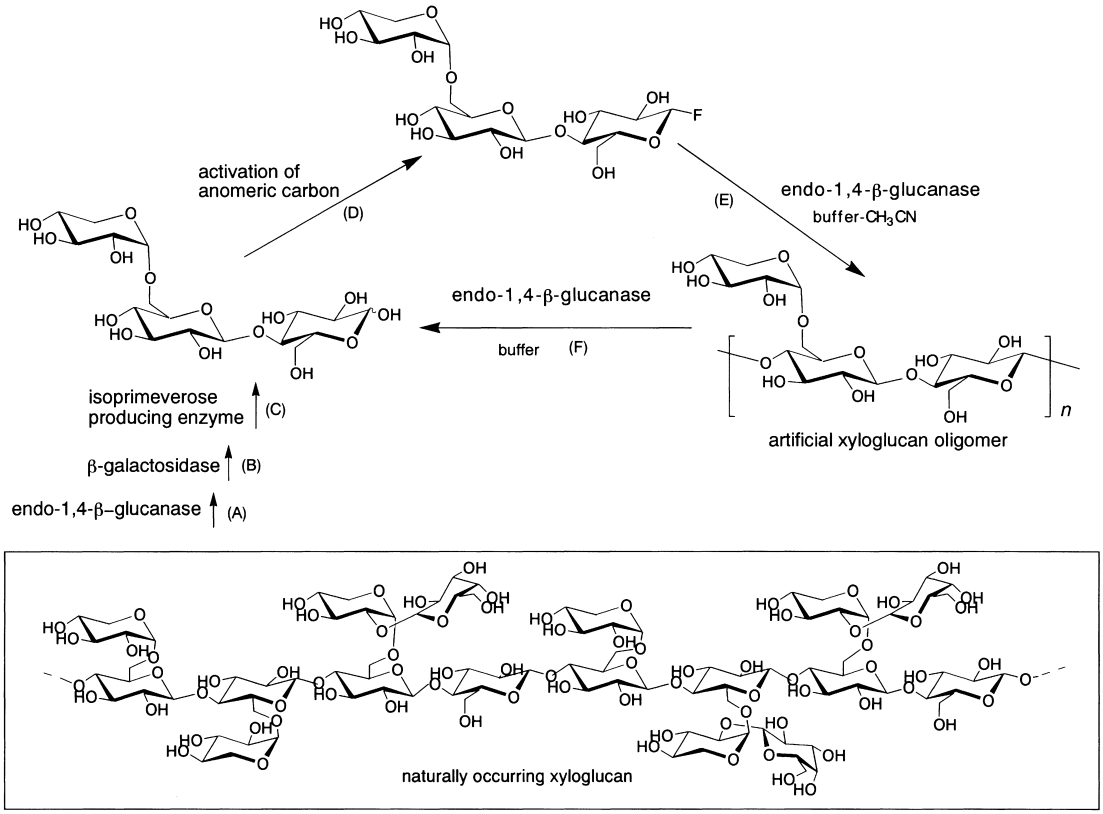


Fig. 21. Chemo-enzymatic synthesis of xyloglucan oligosaccharides.

atom to the anomeric center of this trisaccharide was achieved by 1) the protection of all hydroxy groups by acetyl group, 2) the bromination of the anomeric carbon, 3) the introduction of fluorine atom via inversion of configuration, and 4) deacetylation (Fig. 21(D)). When the trisaccharide monomer was treated with endo-1,4-β-glucanase (from *Trichoderma reesei*) under a preferable condition for transglycosylation, that is, in the presence of acetonitrile, a polycondensation reaction took place, giving rise to artificial xyloglucan oligomers (Fig. 21(E)).[47]

The ^{13}C NMR spectrum of the resulting product showed signals derived from the anomeric carbon atoms of β-1,4 main chain and α-1,6 side chain at 103 and 100 ppm, respectively. The appearance of a signal at 80 ppm clearly shows that β-1,4 glycosidic bond was formed during the polycondensation. The molecular weights of the resulting oligosaccharides were measured by MALDI-TOF mass spectroscopy. The molecular weight difference between each peak exactly corresponds to the molecular weight of the trisaccharide moiety that consists of two glucoses and one xylose, indicating that the polycondensation proceeds by trisacchride unit. The transglycosylation between the xylosylated glucose and unsubstituted glucose did not take place. Such results show that the products have an alternatingly xylosylated structure. It is impossible to construct such a structure by the conventional methodology of modifying a naturally occurring polysaccharide by chemical reactions. The resulting oligosaccharides can be degraded to the starting trisaccharide catalyzed by the endo-1,4-β-glucanase in different reaction conditions preferable to depolymerization (Fig. 21(F)). These results clearly show that a new carbon cyclic system has been constructed by using glycosidases.

Based on the conventional method of using modifications of naturally occurring oligosaccharides by chemical reactions, the construction of such a cyclic system would be impossible. The first reason is that it is difficult to convert the chemically modified products to the corresponding monomer unit enzymatically because the chemical modification occurs in a nonregioselective manner. The second reason is the fact that the glycosylating process from a monomer unit to a higher oligosaccharide structure without using protecting groups has long been believed to be impossible due to the lack of regio- and stereo-selectivity. By using the newly developed methodology of using glycosidases as catalysts based on naturally occurring polysaccharides, the construction of a novel carbon cyclic system becomes possible; so various kinds of functionalized oligo- and poly-saccharides can be designed and prepared (Glyco-Chemistry Cycles System). This concept consists of the transformation of a naturally occurring polysaccharide to a refined raw material (Fig. 22). The resulting raw material is then functionalized and further polymerized enzymatically to give a functionalized oligosaccharide derivative. This is a low environment-loading process because the characteristic feature of glycosidases is to catalyze both glycosylation reaction and deglycosylation reaction (complementarity of glycosidases) and, therefore, the resulting products can be converted to the starting materials enzymatically without using any drastic reaction conditions or strong acid catalyst.

7. Conclusions

It is noteworthy that all carbohydrate materials prepared by

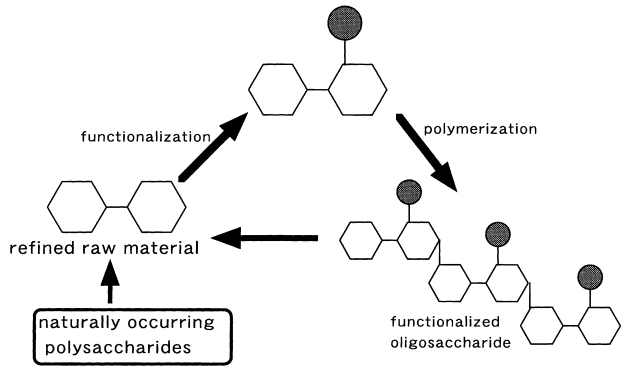


Fig. 22. Glyco-Chemistry Cycle System.

enzymes from natural origins can have a biodegradability that is tailored by the designer even if they have an artificial structure. The enzymatic glycosylation as well as the conventional chemical glycosylation will synergistically contribute not only to the creation of new glycotechnology, but also to the progress of the Green Chemistry of the future.

References

1 a) "Carbohydrates in Chemistry and Biology," ed by B. Ernst, G. W. Hart, and P. Sinay, WILEY-VCH, Weinheim (2000). b) "Essentials of Glycobiology," ed by A. Varki, R. Cummings, J. Esko, H. Freeze, G. Hart, and J. Mark, Cold Spring Harbor Labaratory Press (1999).

2 "Glycoscience," ed by B. O. Fraser-Reid, K. Tatsuta, and J. Thiem, Springer, Heidelberg (2001).

3 R. R. Schmidt, *Angew. Chem., Int. Ed. Engl.*, **25**, 212 (1986).

4 a) K. Igarashi, *Adv. Carbohydr. Chem. Biochem.*, **34**, 243 (1977). b) "Methods in Carbohydrate Chemistry," ed by R. L. Whistler, M. L. Wolfrom, and J. N. BeMiller, Academic Press, London, (1963), Vol. I, II.

5 A. D. Elbein, *Trends Biotechnol.*, **9**, 346 (1991).

6 a) "Chemistry of the *O*-Glycosidic Bond," ed by A. F. Bochkov, G. E. Zaikov, and C. Schuerch, Pergamon Press, Oxford (1979). b) "Modern Methods in Carbohydrate Synthesis," ed by S. H. Khan and R. A. O'Neill, Harwood Academic Publishers, Amsterdam (1996).

7 a) "Cellulose, Structure, Modification and Hydrolysis," ed by R. A. Young, R. M. Rowell, John Wiley & Sons, New York (1986). b) "Chitin and Chitinase," ed by P. Jolles and R. A. A. Muzzarelli, Birkhauser, Basel (1999).

8 a) "Methods in Carbohydrate Chemistry," ed by R. L. Whistler, J. W. Green, J. N. BeMiller, and M. L. Wolfrom, Academic Press, London (1963), Vol. III. b) "Essentials of Carbohydrate Chemistry," ed by J. F. Robyt, Springer, New York (1998).

9 "Enzymes in Synthetic Organic Chemistry," ed by C. H. Wong and G. M. Whitesides, Elsevier Science Ltd., New York (1994).

10 a) K. G. I. Nilsson, *Trends Biotechnol.*, **6**, 256 (1988). b) T. Murata and T. Usui, *Trends Glycosci. Glycotechnol.*, **12**, 161 (2000).

11 M. Kitaoka and K. Hayashi, *Trends Glycosci. Glycotechnol.*, **14**, 35 (2002).

12 a) C. H. Wong, R. L. Halcomb, Y. Ichikawa, and T. Kajimoto, *Angew. Chem., Int. Ed. Engl.*, **34**, 521 (1995). b) M. M. Palcic and O. Hindsgaul, *Trends Glycosci. Glycotechnol.*, **8**, 37 (1996).

13 T. Tsuchiya, *Adv. Carbohydr. Chem. Biochem.*, **48**, 91 (1990).

14 T. Mukaiyama, Y. Murai, and S. Shoda, *Chem. Lett.*, **1981**, 431.

15 a) M. Hayashi, S. Hashimoto, and R. Noyori, *Chem. Lett.*, **1984**, 1747. b) T. Matsumoto, H. Maeta, and K. Suzuki, *Tetrahedron Lett.*, **29**, 3567 (1988).

16 J. Thiem, M. Kreuzer, W. Fritsche-Lang, and H. M. Deger, German Patent 3626028 A1 (1987); Chem. Abstr., **107**, 176407e (1987).

17 T. Mukaiyama, Y. Hashimoto, and S. Shoda, *Chem. Lett.*, **1983**, 935.

18 F. Micheel and H. Koechling, *Chem. Ber.*, **91**, 673 (1958).

19 J. Kadokawa, M. Sato, M. Karasu, H. Tagaya, and K. Chiba, *Angew. Chem., Int. Ed.*, **37**, 2373 (1998).

20 S. Kobayashi, T. Kiyosada, and S. Shoda, *J. Am. Chem. Soc.*, **118**, 13113 (1996).

21 R. U. Lemieux and H. Driguez, *J. Am. Chem. Soc.*, **97**, 4063 (1975).

22 S. Shoda, R. Izumi, M. Suenaga, K. Saito, and M. Fujita, *Chem. Lett.*, **2002**, 150.

23 M. Motegi, R. Izumi, and S. Shoda, 81st Annu. Meet. Chem. Soc. Jpn. Prepr., II, 720 (2002).

24 J. E. G. Barnett, W. T. S. Jarvis, and K. A. Munday, *J. Biochem.*, **105**, 669 (1967).

25 a) E. J. Hehre, C. F. Brewer, and D. S. Genghof, *J. Biol. Chem.*, **254**, 5942 (1979). b) G. Okada, D. S. Genghof, and E. J. Hehre, *Carbohydr. Res.*, **71**, 287 (1979).

26 K. Kubo and K. Nishizawa, *Bull. Coll. Agric. Vet. Med. Nihon Univ.*, **41**, 9 (1984).

27 S. Kobayashi, K. Kashiwa, T. Kawasaki, and S. Shoda, *J. Am. Chem. Soc.*, **113**, 3079 (1991).

28 S. Shoda, M. Fujita, and S. Kobayashi, *Trends Glycosci. Glycotechnol.*, **54**, 279 (1998).

29 S. Kobayashi, X. Wen, and S. Shoda, *Macromolecules*, **29**, 2698 (1996).

30 a) P. Kovac, *Carbohydr. Res.*, **106**, 203 (1982). b) K. Takeo, Y. Murata, and S. Kitamura, *Carbohydr. Res.*, **224**, 311 (1992).

31 S. Kobayashi, J. Shimada, K. Kashiwa, and S. Shoda, *Macromolecules*, **25**, 3237 (1992).

32 J. L. Viladot, V. Moreau, A. Planas, and H. Driguez, *J. Chem. Soc., Perkin Trans. 1*, **1997**, 2383.

33 V. Moreau and H. Driguez, *J. Chem. Soc., Perkin Trans. 1*, **1996**, 525.

34 E. Okamoto, T. Kiyosada, S. Shoda, and S. Kobayashi, *Cellulose*, **4**, 161 (1997).

35 M. Fujita, S. Shoda, and S. Kobayashi, *J. Am. Chem. Soc.*, **120**, 6411 (1998).

36 R. Izumi and S. Shoda, 81st Annu. Meet. Chem. Soc. Jpn. Prepr., II, 724 (2002).

37 W. Treder, J. Thiem, and M. Schlingmann, *Tetrahedron Lett.*, **27**, 5605 (1986).

38 C. Cottaz, C. Apparu, and H. Driguez, *J. Chem. Soc., Perkin Trans. 1*, **1991**, 2235.

39 D. L. Jakeman and S. G. Withers, *Trends Glycosci. Glycotechnol.*, **14**, 13 (2002).

40 S. Kobayashi, H. Morii, R. Itoh, S. Kimura, and M. Ohmae, *J. Am. Chem. Soc.*, **123**, 11825 (2001).

41 a) I. Tews, A. C. Terwisscha van Scheltinga, A. Perrakis, K.

S. Wilson, and B. W. Dijkstra *J. Am. Chem. Soc.*, **119**, 7954 (1997). b) K. A. Brameld and W. A. Goddard III, *J. Am. Chem, Soc.*, **120**, 3571 (1998).

42 M. Fujita, S. Shoda, K. Haneda, T. Inazu, K. Takegawa, and K. Yamamoto, *Biochim. Biophys. Acta*, **1528**, 9 (2001).

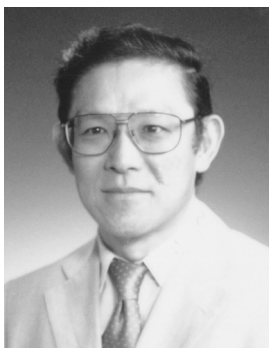
43 a) S. Shoda, K. Obata, O. Karthaus, and S. Kobayashi, *J. Chem. Soc., Chem. Commun.*, **1993**, 1402. b) O. Karthaus, S. Shoda, H. Takano, K. Obata, and S. Kobayashi, *J. Chem. Soc., Perkin Trans. 1*, **1994**, 1851.

44 a) S. Kobayashi, T. Kawasaki, K. Obata, and S. Shoda, *Chem. Lett.*, **1993**, 685. b) S. Shoda, T. Kawasaki, K. Obata, and S. Kobayashi, *Carbohydr. Res.*, **249**, 127 (1993).

45 Y. Misawa, C. Lohavisavapanichi, and S. Shoda, *Glycoconjugate J.*, **16**, S122 (1999).

46 Y. Mitsuishi and S. Shoda, *Cellu. Commun.*, **9**, 2 (2002).

47 S. Shoda, Y. Chigira, and Y. Mitsuishi, *Glycoconjugate J.*, **16**, S18 (1999).



Shin-ichiro Shoda received his Ph. D. in chemistry from the University of Tokyo in 1981, and then started his academic career as an research associate. In 1986, he moved to the Department of Materials Chemistry, Tohoku University where he became professor of functional polymer chemistry in 1999. His research interests include synthesis of carbohydrates, the development of novel glycosylation reactions, and macromolecular archtecture of high-performance oligo- and polysaccharides.

Ryukou Izumi received his M. Sc. Degree in 2000 from Ishinomaki Senshu University. He is currently a Ph.D. student of the Department of Materials Chemistry, Tohoku University, focusing on enzymatic synthesis of complex oligosaccharides having a β-mannoside linkage.

Masaya Fujita received his M. Sc. Degree in 1995 from Kochi University. He received his Ph. D. at Tohoku University in 1999, and then worked as a postdoctoral fellow at the Noguchi Institute. In 2000, he moved to Tohoku University to work as a research associate at the Department of Materials Chemistry. Since 2002 he has been working at the Noguchi Institute as a researcher. His research interests involve screening and modification of glycosyl hydrolases and investigation of their biological roles.