lidine ring could then be important subsequent to the enzymatic acylation reaction.

The penicillins and cephalosporins are effective bacteriocidal agents presumably because they disrupt bacterial cell wall synthesis.[23,48,49] This effect is exerted by the inhibition of enzymes that catalyze the cross-linking reaction of peptidoglycan strands.[48,49] The process of transpeptidation is thought to involve the cleavage of the terminal D-alanyl-D-alanine of the peptidoglycan to give an acyl enzyme intermediate with release of D-alanine. Inhibition may arise because penicillin is a structural or transition-state analogue of acyl D-ala-D-ala.[50,51] It has been proposed that penicillin acylates the relevant transpeptidase enzymes via a nucleophilic reaction at the β-lactam carbonyl.[50-52] Removal in this manner of the amide substituent should allow the thiazolidine ring to open with reasonable rapidity. Indeed ring opening does occur in the breakdown of the benzyl penicillin–DD-carboxypeptidase–transpeptidase enzyme complex of Streptomyces R61.[52] Such a process could increase the difficulty of enzyme reactivation by altering the alignment of the acyl enzyme carbonyl to functional groups in the active site.

It has generally been considered that the product of alkaline hydrolysis of penicillin derivatives will be the corresponding thiazolidine, e.g., benzylpenicillin undergoes opening of the β-lactam ring to give benzylpenicilloic acid.[23,53] This implies that thiazolidine ring opening is unfavorable at those pH values. As seen in the present work the equilibrium for thiazolidine ring opening is indeed unfavorable at high pH (>10), even when the forward reaction is rapid because of significant internal stabilization of the developing carbonium ion. This is because the reverse ring-closure reaction involving nucleophilic attack of the thiol anion is facile. As a consequence, ring opening is only favorable at pH <10 where the thiol group is un-ionized. However, both the ring-opening and hydrolysis reactions are rapid and pH independent at pH values considerably greater than 7. Thus, the present studies of the hydrolysis of thiazolidines, in which conclusive evidence for an iminium ion intermediate was obtained with the derivatives of *p*-(dimethylamino)cinnamaldehyde, and in which a detailed kinetic analysis of the hydrolysis reactions was possible, have provided insight into the reactivity of the thiazolidine ring and into the kinetic consequences of preequilibrium ring-opening reactions. These factors may be important in the reactions of penicillin and its derivatives.

Acknowledgment. This work was supported by research grants from the National Institutes of Health and the National Science Foundation.

Registry No. I, 130904-39-1; II, 130904-40-4; III, 130904-41-5; IV, 130904-42-6; V, 79661-90-8; *p*-(dimethylamino)cinnamaldehyde, 6203-18-5; cinnamaldehyde, 104-55-2; β-aminoethanethiol, 60-23-1; β-(butylamino)ethanethiol, 5842-00-2; β-(phenylamino)ethanethiol, 5977-99-1.

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Novel Method for Polysaccharide Synthesis Using an Enzyme: The First in Vitro Synthesis of Cellulose via a Nonbiosynthetic Path Utilizing Cellulase as Catalyst

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Abstract: The in vitro synthesis of cellulose via a nonbiosynthetic path has been achieved for the first time by condensation of β-D-cellobiosyl fluoride as substrate for cellulase, a hydrolysis enzyme of cellulose, in a mixed solvent of acetonitrile/acetate buffer (pH 5, 5:1). The water-insoluble part of the products is "synthetic cellulose", the structure of which was confirmed by comparison with an authentic natural cellulose sample with use of solid ^{13}C NMR and IR spectroscopies as well as with a hydrolysis experiment. The present synthetic cellulose was converted to the corresponding triacetate whose molecular weight was at least 6.3×10^3 (degree of polymerization (DP) ≥22). X-ray as well as ^{13}C NMR analyses showed that its crystal structure is of type II with high crystallinity. Under reaction conditions of a higher substrate concentration or higher acetonitrile concentration, water-soluble cellooligosaccharides (DP ≤ 8) were produced predominantly.

Cellulose is the most abundant organic substance occurring on the earth. Some 10^{15} kg of cellulose are photosynthesized and degraded each year.[1] For over a century, many researchers have been attracted by this natural substance, and enormous studies, structure determinations, biosyntheses, and chemical and physical properties determinations have been performed in view of both fundamental sciences and practical applications.[2] In vitro synthesis of cellulose, therefore, has long been one of the most difficult, yet important, challenging topics from the early stages of macromolecular science. Many efforts have been devoted to regio- and stereoselective preparation of cellulose, i.e., construction of stereoregular polysaccharides having β(1→4) glycosidic linkage. The chemical approaches so far attempted, however, have failed to solve the problem in spite of remarkable development of modern synthetic methods.[3,4] The condensation of 2,3,6-glucose tricar-

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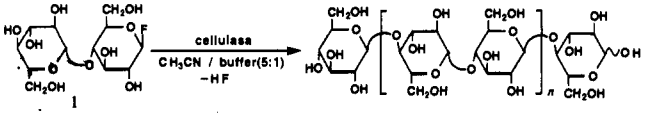
Scheme I



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banilate with phosphorus pentoxide in a mixture of chloroform/dimethyl sulfoxide gave branched products, and the molecular weight of the resulting polysaccharide after removing the protecting group was low.[3b] 1,4-Anhydro-2,3,6-tri-*O*-benzyl-α-D-glucose has been polymerized via a Lewis acid as catalyst, giving rise to polymers having mixed structures of β(1→4) (cellulose-type) and α(1→4) (amylose-type) linkages.[4b,c] Uryu and coworkers investigated the possibility of synthesizing polysaccharides having β(1→4) linkage (cellulose) by the cationic ring-opening polymerization of 1,4-anhydroglucose derivatives. However, stereoregular polysaccharides having the desired structure were not obtained due to the lack of regioselective ring opening.[4f] Concerning a stepwise synthesis of cellooligosaccharide derivatives, several oligomers up to an octamer have been synthesized starting from allyl 2,3,6-tri-*O*-benzyl-4-*O*-(*p*-methoxybenzyl)-β-D-glucoside; however, elimination of the protecting groups to an oligomer, e.g., cellooctaose, has not been achieved yet.[5] Some in vitro formations of cellulose according to a biosynthetic path with use of *Acetobacter xylinum*[6] or *Phaseolus aureus* extracts[7] with a nucleoside diphosphate sugar (ADP–, CDP–, or GDP–glucose) as substrate have been reported.

We report here a completely novel approach for the synthesis of cellulose by a transglycosylation reaction (condensation polymerization) catalyzed by cellulase, an extracellular hydrolysis enzyme of cellulose, with use of β-D-cellobiosyl fluoride (**1**)[8] as a glycosyl donor (substrate monomer) (Scheme I). It was postulated that a cellobiose (disaccharide) unit would be preferable as a substrate because it can be recognized by the binding site of the enzyme more readily than a glucose (monosaccharide) derivative; that is, the smallest substrate recognized by the enzyme may be a disaccharide. We have therefore chosen β-D-cellobiosyl fluoride (**1**) as an activated glycosyl donor. The configuration of the Cl fluorine atom of the starting material has been designed in order to form a reactive intermediate leading to a β(1→4) product (cellulose) via a "double displacement mechanism"[9] at the active site of the enzyme. The present reaction does not involve protection and deprotection of the hydroxyl group. It has been found necessary to carry out the reaction in a mixture of an organic solvent and acetate buffer solution to promote the transglycosylation.[10]

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Table I. Enzymatic Polymerization of **1** Catalyzed by Cellulase in Various Solvents[a]

entry	solvent	yield[c] (%)
1	CH_3CN/buffer[b] (5:1)	64 (54)
2	C_2H_5CN/buffer (5:1)	10 (3)
3	$(CH_3)_2CO$/buffer (5:1)	13 (7)
4	CH_3OH/buffer (5:1)	17 (6)
5	C_2H_5OH/buffer (5:1)	20 (7)
6	1,4-dioxane/buffer (5:1)	4 (~0)
7	CH_3NO_2/buffer (5:1)	15 (1)
8	DMF/buffer (5:1)	~0 (0)
9	DMSO/buffer (5:1)	~0 (0)

[a] Polymerized at 30 °C for 12 h: [**1**] = 2.5 × 10^{-2} mol/L, cellulase 5 wt % for **1**. [b] Acetate buffer (0.05 M, pH 5). [c] 5:1 methanol/water-insoluble part. In parentheses, the yield of the water-insoluble part is given.

Table II. Reaction of D-Cellobiose Catalyzed by Cellulase in Various Solvents[a]

			wt % of product[c]			
entry	solvent	time (h)	G_1	G_2	G_3	G_4
1	buffer[b]	24	76	22	2	0
2	CH_3CN/buffer (2:1)	24	53	38	18	1
3	CH_3CN/buffer (5:1)	48	19	60	18	3
4	$(CH_3)_2CO$/buffer (2:1)	24	26	62	10	2
5	$(CH_3)_2CO$/buffer (5:1)	48	10	84	5	1
6	DMF/buffer (2:1)	48	2	98	0	0
7	DMSO/buffer (2:1)	48	0	100	0	0

[a] The reaction was carried out at 30 °C: [cellobiose] = 2.5 × 10^{-2} mol/L, cellulase 7.5 wt % for cellobiose. [b] Acetate buffer (0.05 M, pH 5). [c] G_1, G_2, G_3, and G_4 denote glucose, cellobiose, cellotriose, and cellotetraose, respectively.

Table III. Effect of Acetonitrile Concentration on Enzymatic Polymerization of **1** Catalyzed by Cellulase[a]

entry	solvent	yield[c] (%)
1	buffer	0 (0)
2	CH_3CN/buffer[b] (2:1)	16 (3)
3	CH_3CN/buffer (5:1)	64 (54)
4	CH_3CN/buffer (7.5:1)	77 (31)
5	CH_3CN/buffer (10:1)	67 (14)
6	CH_3CN/buffer (15:1)	50 (5)

[a] Polymerized at 30 °C for 12h: [**1**] = 2.5 × 10^{-2} mol/L, cellulase 5 wt % for **1**. [b] Acetate buffer (0.05 M, pH 5). [c] 5:1 Methanol/water-insoluble part. In parentheses, the yield of the water-insoluble part is given.

Results and Discussion

Enzymatic Polymerization. To a solution of β-D-cellobiosyl fluoride (**1**) in a mixture of an organic solvent and 0.05 M acetate buffer (pH 5) was added a 0.05 M acetate buffer (1.0 mL) solution of cellulase, and the mixture was stirred at 30 °C. As the reaction proceeded, the initially homogeneous solution gradually became heterogeneous with a white precipitation of the product polysaccharide. After 12 h, the resulting suspension was heated at 100 °C for 10 min to inactivate the enzyme and was poured into an excess amount of methanol/water (5:1) to remove the catalyst cellulase, lower molecular weight products like D-glucose and D-cellobiose, and a trace amount of inorganic compounds derived from the buffer solution. The fluorine atom at the polymer end was converted into a hydroxy group during these workup procedures. The insoluble part was collected by filtration. Further purification was achieved by suspending the product in water and by successive filtration, giving rise to the water-insoluble part of the white powdery materials. On the other hand, the filtrate was concentrated in vacuo to give a white powdery water-soluble part.

Table I summarizes the results of solvent effect on the enzymatic polymerization of **1** catalyzed by cellulase. The values of the yield denote that of the 5:1 methanol/water-insoluble part, which consists of the water-insoluble part and the water-soluble part (cellulose and cellooligosaccharide, respectively, as shown below). The yield of the water-insoluble part is given in parenthesis. Of

Table IV. Enzymatic Polymerization of β-D-Cellobiosyl Fluoride (1) Catalyzed by Cellulase:[a] Effect of Substrate Concentration and Reaction Time[b]

entry	substrate concn (mol/L)	wt % of catal	time (h)	product yield[c]
1	2.5×10^{-2}	5.1	12	64 (54)
2	4.0×10^{-2}	5.0	6.5	68 (40)
3	8.0×10^{-2}	5.0	5	58 (12)
4	1.2×10^{-2}	5.1	2	69 (8)

[a] All reactions were carried out in 5:1 CH_3CN/acetate (0.05 M) buffer (pH 5) at 30 °C. [b] The reaction was stopped at the stage of maximum formation of the water-soluble oligosaccharide by monitoring the reaction mixture with use of GPC. [c] Isolated yields. In parentheses, the yield of the water-insoluble part is given.

the organic solvent screened, acetonitrile was found most effective for promotion of the reaction (entry 1). The use of propionitrile was inadequate because it was not miscible with the buffer solution (entry 2). The polymerization proceeded also when other organic solvents like acetone, methanol, ethanol, 1,4-dioxane, and nitromethane were used; however, the yields of the products decreased markedly (entries 3–7). No polymerization product was obtained when *N*,*N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were used as cosolvent, which indicates that the enzyme was completely inactivated in the presence of these highly dipolar aprotic solvents; **1** remained unchanged (entries 8 and 9).

When cellobiose was used instead of cellobiosyl fluoride as substrate in a mixture of an organic solvent and buffer, only a small amount of oligomers (cellooligosaccharides (G_3 and G_4)) were detected by high-performance liquid chromatography (HPLC); no polymerized product was obtained even under the optimized conditions for substrate **1** with use of acetonitrile as cosolvent (Table II). These results indicate that the replacement of the 1-hydroxy group of cellobiose by the fluorine atom is essential for the production of higher molecular weight polysaccharides.

Table III shows the effect of acetonitrile concentration on the polymerization of **1**. The best result in terms of the yield of the water-insoluble part (in parentheses) was obtained when the ratio of acetonitrile/buffer was 5:1 (entry 3). As the acetonitrile concentration increased, the yield of the water-insoluble part decreased, and hence the water-soluble part (oligosaccharides) was produced predominantly (entries 4–6). These results indicate that a chain extension reaction stopped at the early stage of the reaction, which may be explained by the poor solubility of the propagating water-soluble cellooligosaccharide, a precursor of cellulose, toward the solvent system of high acetonitrile concentration. This explanation is partly supported by a gel permeation chromatography (GPC) analysis of the resulting water-soluble oligosaccharides; that is, cellohexaose (G_6) was formed more preferentially than other oligomers of cellopentaose (G_5)[11] and cellotetraose (G_4) when the reaction was carried out in a mixture of acetonitrile:buffer = 7.5:1 and 10:1 (entries 4 and 5, respectively) (the ratio of these oligomers is approximately G_4:G_5:G_6 = 25:33:42). On the contrary, the reaction in acetonitrile:buffer = 15:1 (entry 6) afforded the tetramer (G_4) predominantly (G_4:G_5:G_6 = 40:33:27). In a solvent of low acetonitrile concentration (entry 2), the hydrolysis of **1** to D-cellobiose (the elimination of anomeric fluorine atom) occurred predominantly compared with the transglycosylation reaction because of the more preferable attack of a water molecule to a reactive intermediate than that of the 4′-hydroxy group of another monomer **1**.

The effect of substrate concentration was also investigated (Table IV). The yield of the water-insoluble part depends upon the substrate concentration; the water-insoluble part became the main product (entries 1 and 2) or the minor one (entries 3 and 4). This result may be explained by assuming the further hydrolysis of the once-formed water-insoluble cellulose with production of the water-soluble part (the main product of entries 3 and 4); the rate of hydrolysis of the resulting polysaccharide was enhanced, and consequently the enzymatic cleavage of the β(1→4) glycosidic bond between glucose units occurred more preferably than the desired transglycosylation reaction under a higher substrate concentration.[12]

Table V. Enzymatic Polymerization of **1** with Cellulase from Different Origin[a]

entry	cellulase	yield[b] (%)
1	*T. viride*	64 (54)
2	*A. niger*	30 (8)
3	*P. tulipiferae*	43 (25)
4	β-glucosidase (almonds)	0 (0)

[a] Polymerized in CH_3CN/acetate buffer (0.05 M, pH 5) at 30 °C for 12 h: [**1**] = 2.5×10^{-2} mol/L, cellulase 5 wt % for **1**. [b] 5:1 Methanol/water-insoluble part. In parentheses, the yield of the water-insoluble part is given.

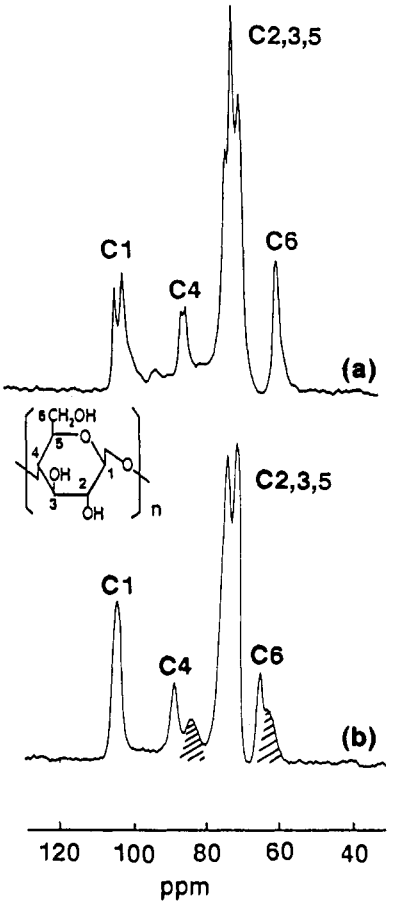


Figure 1. CP/MAS ^{13}C NMR spectra of (a) the water-insoluble part of the product and (b) natural cellulose.

Table V shows the results of enzymatic polymerization catalyzed by cellulase from various origins. Cellulase produced from *Trichoderma viride* appeared to be the most effective for the synthesis of cellulose. Other cellulase, from *Aspergillus niger* or *Polyporus tulipiferae*, was found less effective. β-Glucosidase from almonds did not catalyze the polymerization.

Structure Determination of the Products. The structure of the water-insoluble part has been determined by ^{13}C NMR and IR spectroscopies as well as by its hydrolysis product. The cross-

(11) There are several possible explanations for the formation of cellooligosaccharides having glucose units of odd number. A transglycosylation of cellobiose may occur not only at the C1 carbon atom (the carbon atom to which the fluorine atom attaches) but also at the C1′ anomeric center, and consequently a monosaccharide (G) unit was transferred, giving rise to the product of G_{2n+1}. Polysaccharides having glucose units of even number once formed may be hydrolyzed enzymatically to afford also the oligosaccharides of G_{2n+1}.

(12) The reaction was stopped at the stage of maximum formation of the water-soluble oligosaccharide by monitoring the reaction mixture with use of GPC.

Table VI. ^{13}C NMR Chemical Shifts of Cellooligosaccharide in D_2O Solution[a]

residue		C1	C2	C3	C4	C5	C6
reducing end unit	α	92.7	72.1	72.1	79.2	71.0	60.7
	β	96.6	75.0	74.8	79.2	75.6	60.7
internal unit		103.2	73.8	74.8	79.1	75.6	60.7
nonreducing end unit		103.4	74.0	76.3	70.3	76.8	61.4

[a] Chemical shifts (ppm) relative to TMS by setting the shift of 1,4-dioxane at 67.4 ppm.

polarization/magic angle spinning (CP/MAS) solid ^{13}C NMR spectrum of the water-insoluble part (Figure 1a) is very similar to that of natural cellulose (Figure 1b), and hence, each signal can be assigned to the corresponding carbon atom of the glucose unit (C1–C6).[13] The signals at 104.9–107.1 and 87.6–88.7 ppm are assigned to the C1 and C4 carbon atoms, respectively. The signals from 72.8 to 76.6 ppm are due to C2, C3, and C5 carbon atoms. The signal at 62.2 ppm is ascribed to the C6 carbon atom. No signal due to the methylene carbon atom (C6) adjacent to the β(1→6) glycosidic bond was observed around 69 ppm, which is normally detected when an isomeric β(1→6) linkage is formed.[14]

In order to obtain more detailed information concerning the stereoregularity and the molecular weight, the product was converted to the corresponding acetylated derivative with acetic anhydride/perchloric acid. The ^{13}C NMR spectrum of the acetylated product in $CDCl_3$ showed exactly the same pattern as that of an authentic cellulose triacetate.[15] Treatment of the water-insoluble part with cellulase in 0.05 M acetate buffer afforded hydrolysis products of D-glucose and D-cellobiose exclusively. These results clearly indicate that the glycosidic bond formation occurred in a regio and stereoselective manner between cellobiose units to afford a stereoregular polysaccharide having β(1→4) linkage. The gel permeation chromatographic (GPC) analysis of the acetylated product revealed that the average molecular weight is 6.3×10^3 (polystyrene standard), which corresponds to the degree of polymerization (DP) being 22.[16] Under the present acetylation conditions using perchloric acid as catalyst, the glycosidic bond cleavage may occur.[17] The real average DP of the water-insoluble part is, therefore, at least 22. All of the above data allow us to refer to the water-insoluble part as "cellulose".[18]

Table VI shows the ^{13}C NMR chemical shifts of the water-soluble part in D_2O solution. Each peak can be assigned to the corresponding carbon atom (C1–C6) of oligosaccharides having β(1→4) linkage.[19] The spectrum showed signals of carbon atoms from internal glucose units as well as those of the reducing and nonreducing glucose end units in the cellooligosaccharides. No signal derived from an isomeric C6 carbon atom, which is contained in the β(1→6) linkage, was detected around 69 ppm.[19] These results indicate that the water-soluble part consists exclu-

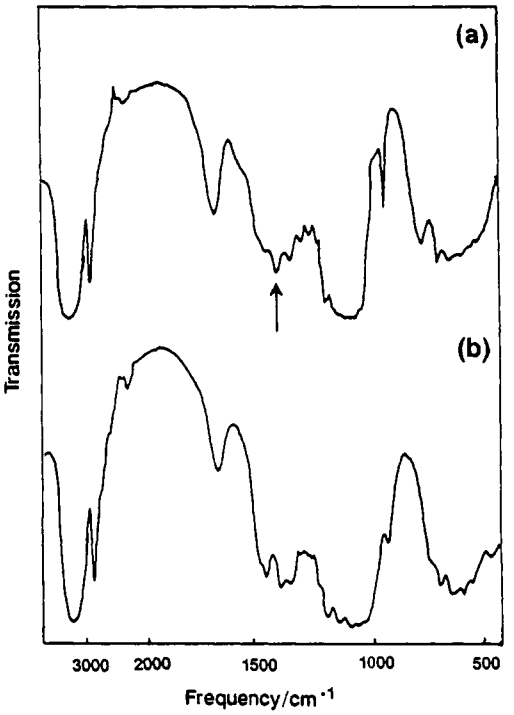


Figure 2. IR spectra (KBr) of (a) the water-insoluble part of the product and (b) natural cellulose (type I).

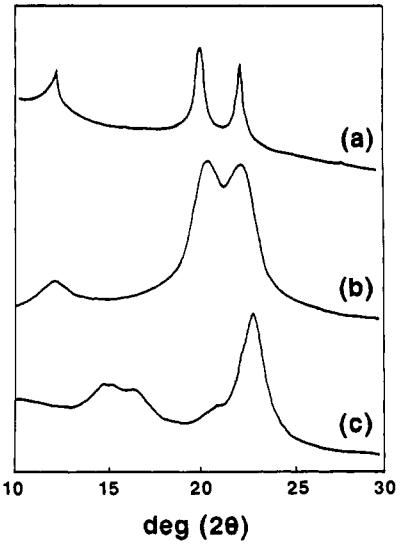
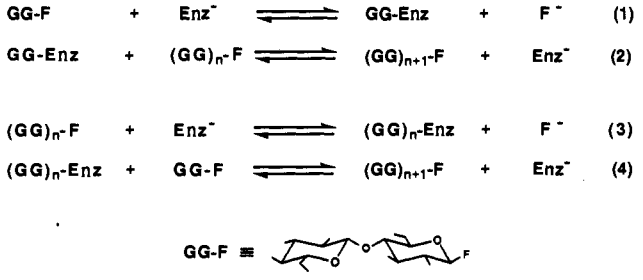


Figure 3. X-ray diffractograms of (a) the water-insoluble part of the product (type II), (b) mercerized cellulose (type II), and (c) natural cellulose (type I).

Scheme II



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(16) This value may be overestimated since cellulose derivatives have more extended chain conformations than those of polystyrene. Johnson, J. F. Size-exclusion (Gel-permeation) Chromatography. In *Encyclopedia of Polymer Science and Engineering*; Mark, H. F., Bikales, N. M., Overberger, C. G., Menger, G., Eds.; Wiley-Interscience: New York, 1985; Vol. 3, pp 501–523.

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(18) Generally in carbohydrate chemistry, a "polysaccharide" refers to a substance having more than ten monosaccharide units: Kennedy, J. F.; White, C. A. Polysaccharides. In *Comprehensive Organic Chemistry*; Barton, D., Ollis, W. D., Haslam, E., Eds.; Pergamon Press: Oxford, 1979; Vol. 5, pp 755–830. According to this terminology, the present product belongs to the cellulose, a typical polysaccharide, family.

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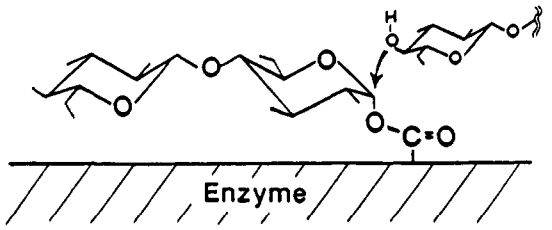
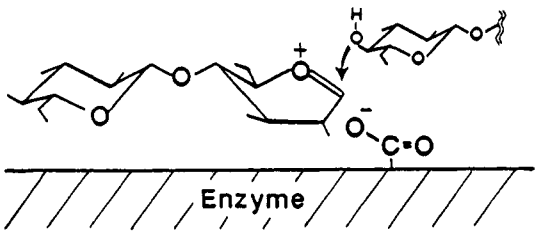


(a) Glycosyl enzyme intermediate



(b) Glycosyl oxocarbenium ion intermediate

Figure 4. Proposed models in the stereoselective formation of the $\beta(1\rightarrow4)$ glycosidic linkage via a substrate–enzyme complex involving double inversion of configuration at the C1 carbon. (a) and (b) show the case of reaction 2 of Scheme II.

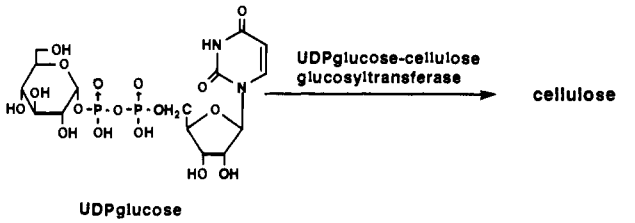
sively of stereoregular oligosaccharides having a $\beta(1\rightarrow4)$ unit (cellooligosaccharide). The average degree of polymerization (DP) of the product oligomers was found to be 6 determined by HPLC with authentic cellohexaose as a standard.

Crystal Structure of the Water-Insoluble Part. The ^{13}C NMR signal pattern at 62.6 ppm due to the C6 carbon in the present synthetic cellulose indicates that the cellulose has a crystal structure of type II (Figure 1a), different from that of natural cellulose of type I whose C6 carbon signal was observed at 65.5–66.0 ppm and had a shoulder peak (Figure 1b).[13] Furthermore, the clear splitting of the signal due to the C1 carbon atom in Figure 1a suggests that the synthetic cellulose has higher crystallinity. The IR spectrum of the water-insoluble part exhibited also a characteristic feature of type II cellulose; the definite absorption at 1370 cm^{-1} (upward arrow in Figure 2a) was more intense than absorptions at 1430 and 1333 cm^{-1}, whereas, in the spectrum of a type I cellulose (Figure 2b), these absorptions were observed with almost equal intensity.[20]

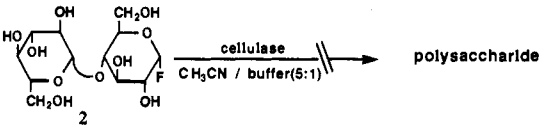
The X-ray diffraction measurement of the synthetic cellulose (the water-insoluble part) showed three peaks at 2θ = 22.0, 19.9, and 12.2 characteristic of the type II cellulose (Figure 3a), which clearly differentiates the product from type I natural cellulose (Figure 3c).[21] In addition, the sharp peaks of the synthetic cellulose compared with that of mercerized cellulose of type II (Figure 3b) indicate that the present product has higher crystallinity than the latter. This result is consistent with the CP/MAS ^{13}C NMR spectroscopic observations.

Reaction Mechanism. At present, there are two conceivable reaction mechanisms concerning the chain propagation for the present polymerization (Scheme II). The first one involves the formation of an active intermediate of the disaccharide unit (GG–Enz) by reaction of **1** (GG–F) and the cellulase (Enz^-) followed by an attack of the terminal 4′-hydroxy group of the propagating polymer ($(GG)_n$–F), giving rise to the product ($(GG)_{n+1}$–F) (activated monomer mechanism (eqs 1 and 2)). In the second mechanism, an active intermediate ($(GG)_n$–Enz) is formed on the chain end and the propagating process is realized by the attack of the 4′-hydroxy group of the disaccharide unit

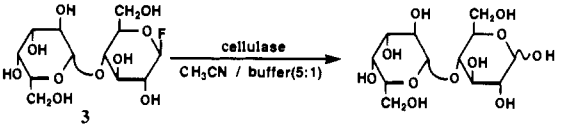
Scheme III



Scheme IV



Scheme V



(GG–F) on this intermediate (active chain-end mechanism (eqs 3 and 4)).

The formation of the stereoregular polysaccharides may be explained by assuming the following two processes. The first step involves the formation of a glycosyl–enzyme intermediate (Figure 4a) or a glycosyl oxocarbenium ion (Figure 4b) at an active site of cellulase with the elimination of fluoride anion.[22] This reactive intermediate is then attacked by the 4′-hydroxy group of another $(GG)_n$–F, which locates in a subsite of the enzyme, leading to the stereoselective formation of the $\beta(1\rightarrow4)$ linkage. Consequently, the stereochemistry of the product is retention of configuration via "double inversion" concerning the anomeric carbon atom of the β-D-cellobiosyl fluoride (**1**).

The present reaction mechanism is to be interestingly compared with a biosynthetic pathway of cellulose that involves the "inversion" of the configuration concerning the C1 carbon atom of the substrate of uridine diphosphate–glucose (UDP–glucose) (Scheme III).[23]

In relation to the first step of the proposed reaction mechanism (eq. 1), the effect of the stereochemistry of the anomeric carbon atom of **1** was investigated. The reaction of α-D-cellobiosyl fluoride (**2**) having the opposite configuration (the anomer of **1**) in the presence of a catalytic amount of cellulase (5 wt %) in acetonitrile/buffer (5:1) afforded no product of polysaccharide, **2** being recovered unchanged after the reaction (Scheme IV). This result clearly indicates that substrate **2** with the α configuration of the fluorine atom is not recognized by cellulase to give the reactive intermediate that is essential for the transglycosylation.

The cellulase-catalyzed reaction of β-D-lactosyl fluoride (**3**) having the same anomeric configuration as **1** and an axial hydroxy group at the C-4′ carbon atom in acetonitrile/buffer (5:1) afforded the corresponding hydrolysis product D-lactose, quantitatively giving no polymeric product (Scheme V). This result shows that β-D-lactosyl fluoride is recognizable to the active site of cellulase; however, the resulting glycosyl–enzyme complex cannot be attacked by the 4′-hydroxyl group of another lactosyl fluoride because it is impossible for the 4′-hydroxy group having axial configuration to be close to the anomeric center of the substrate–enzyme complex.

Experimental Section

Materials. The solvents CH_2Cl_2, CH_3CN, and pyridine were purified by distillation over CaH_2. A monomer of β-D-cellobiosyl fluoride was

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(21) Hayashi, J.; Yamada, T.; Kimura, K. *J. Appl. Polym. Sci.*: *Appl. Polym. Symp.* **1976**, *28*, 713.

(22) See, for example, Nishizawa, K.; Hashimoto, Y. In *The Carbohydrates, Chemistry and Biochemistry*, 2nd ed.; Pigman, W., Horton, D., Eds.; Academic Press: New York and London, Vol. 2A, pp 241–300.
(23) Delmer, D. Y. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 105.

prepared according to the literature.[8] α-D-Cellobiosyl fluoride was synthesized by anomerizing the fluorine atom of **1** by the action of AgF/BF_3OEt_2 in CH_2Cl_2.[24] Cellulases from *T. viride*, *A. niger*, and *P. tulipiferae* were purchased from Yakult Co. (Tokyo), Sigma Co. (U. S.A.), and Kyowa Hakko Co. (Tokyo), respectively. β-Glucosidase (almonds) was obtained from Sigma. The crude product of α-D-cellobiosyl fluoride was purified by silica gel chromatography (Merck 7734, Kieselgel 60). The buffer solution (0.05 M, pH 5) was prepared by mixing an acetic acid (0.05 M) solution and a sodium acetate (0.05 M) solution and monitoring the pH value with a pH meter (Toa Denki Kogyo HM-20E). Natural cellulose (DP ≃ 200) from pulp was obtained from Daicel Chemical Industries (Tokyo).

Measurements. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC250-T spectrometer with tetramethylsilane or 1,4-dioxane as standard. Solid-state ^{13}C NMR spectrum was measured by the cross-polarization/magic angle spinning technique on a Bruker MSL-400 spectrometer. IR spectra were taken on a Shimadzu IR-460 spectrophotometer. Molecular weight data were obtained by use of a Hitachi 655A liquid chromatograph with a Hitachi GL-616 column (water eluent, cellooligosaccharide standard) or a Hitachi GL-A130,150 column (chloroform eluent, polystyrene standard). The X-ray diffractograms were measured as powders by means of a powder X-ray diffractometer (Rigaku-Denki) under a Ni-filtered Cu Kα radiation (30 kV, 15 mA).

Polymerization of β-D-Cellobiosyl Fluoride. A typical procedure for the polymerization of β-D-cellobiosyl fluoride (**1**) is given as follows (entry 1, Table I). A substrate of β-D-cellobiosyl fluoride (334 mg, 0.97 mmol) was dissolved in a mixture of acetonitrile (33 mL) and 0.05 M acetate buffer (pH 5, 5.6 mL). To this solution was added a 0.05 M acetate buffer (1.0 mL) solution of cellulase (17 mg, 26 units, 5.1 wt % for the substrate) from *T. viride*, and the mixture was shaken at 30 °C for 12 h. The resulting suspension was then heated at 100 °C for 10 min to inactivate the enzyme. After the acetonitrile was evaporated, the residue was poured into an excess amount of methanol/water (5:1). The precipitate was collected by filtration, giving rise to 212 mg of the 5:1 methanol/water-insoluble part (64%) after drying in vacuo. The insoluble part was then suspended in distilled water, and the mixture was filtered. The white powder was dried in vacuo, affording 180 mg of the water-insoluble part (54%). On the other hand, the filtrate was concentrated in vacuo to give 32 mg of the white powdery water-soluble part (10%). The reactions with other organic solvents were carried out in a similar manner. Water-soluble part: ^{13}C NMR (D_2O) 60.7 (C6), 61.4 (C6 of nonreducing end unit), 70.3 (C4 of nonreducing end unit), 71.0 (C5 of reducing end unit, α form), 72.1 (C2 and C3 of reducing end unit, α form), 73.8 (C2), 74.0 (C2 of nonreducing end unit), 74.8 (C3), 75.6 (C5 of reducing end unit, β form), 76.3 (C3 of nonreducing end unit), 76.8 (C5 of nonreducing end unit), 79.2 (C4), 92.7 (C1 of reducing end unit, α form), 96.6 (C1 of reducing end unit, β form), 103.2 (C1), 103.4 ppm (C1 of nonreducing end unit). Water-insoluble part: ^{13}C NMR (solid) 62.2 (C6), 72.8–76.6 (C2,3,5), 87.6–88.7 (C4), 104.9–107.1 ppm (C1).

Acetylation of the Water-Insoluble Part. The water-insoluble part (255 mg) was immersed in acetic acid (1 mL) for 12 h. To this mixture were added acetic acid (2 mL), toluene (4 mL), and a catalytic amount (0.0013 mL) of 70% perchloric acid, and the reaction mixture was vigorously stirred at room temperature. Acetic anhydride (2.5 mL) was added, and the mixture was stirred for 24 days at room temperature. The resulting mixture was poured into an excess of ethanol, and the resulting white precipitate was collected by filtration. The precipitate was dried in vacuo to give 186 mg of white powdery peracetylated product: ^1H NMR ($CDCl_3$) 1.95–2.14 ($CH_3C{=}O$), 5.65 (d, J = 8.6 Hz, anomeric proton of reducing end unit, β form), 6.24 ppm (d, J = 3.6 Hz, anomeric proton of reducing end unit, α form); ^{13}C NMR (DMSO-d_6) 62.2 (C6), 71.4–75.8 (C2,3,5), 99.3 (C1), 168.0–170.4 ($CH_3C{=}O$).

Enzymatic Hydrolysis of the Water-Insoluble Part. To a suspension of the water-insoluble part (25 mg) in 0.05 M acetate buffer (3.0 mL) was added cellulase (5.0 mg; *T. viride*), and the mixture was stirred at room temperature for 24 h. The resulting homogeneous solution was directly analyzed by gel permeation chromatography, which showed the exclusive formations of D-glucose and D-cellobiose (16:1).

Hydrolysis of β-D-Lactosyl Fluoride. β-D-Lactosyl fluoride (**3**) (150 mg, 0.43 mmol) was dissolved in a mixture of acetonitrile (15 mL) and 0.05 M acetate buffer (pH 5, 2.0 mL). To this solution was added a 0.05 M acetate buffer (1.0 mL) solution of cellulase (9.5 mg, 14.5 units, 6.4 wt % for the substrate) from *T. viride*. The reaction mixture was shaken at 30 °C, and the formation of the hydrolysis product of D-lactose was monitored by means of HPLC. After 4 h, the β-D-lactosyl fluoride was converted to the corresponding D-lactose quantitatively.

Conclusion

The first in vitro synthesis of cellulose via a nonbiosynthetic path has successfully been achieved with use of β-D-cellobiosyl fluoride as a substrate for cellulase in an organic/water mixed solvent. With use of the present enzymatic polymerization, a sufficient amount of cellulose having a high crystallinity can be readily prepared in good yields. In addition, the change of the reaction conditions (the substrate concentration or organic solvent concentration) enables us to synthesize selectively the water-soluble cellooligosaccharides, which have recently become important substances due to their biological activities and to other utilizations.[25] It is also to be noted that the present method of the use of a glycosyl fluoride as substrate for a hydrolytic enzyme in an organic/water mixed solvent has opened a way to the future development of a new methodology for synthesis of various polysaccharides including cellulose and a variety of biologically important poly- and oligosaccharides. Further studies concerning the detailed reaction mechanism and inherent properties of the synthetic cellulose are now in progress.

Acknowledgment. We thank Professor J. Hayashi of Hokkaido University for sending the X-ray diffraction data of mercerized cellulose and Dr. F. Horii of Kyoto University for helpful discussions concerning the ^{13}C NMR spectra.

Registry No. 1, 7584-85-2; cellulose, 9004-34-6; cellulase, 9012-54-8; D-glucose, 50-99-7; cellobiose, 528-50-7; cellotriose, 33404-34-1; cellotetraose, 38819-01-1; acetonitrile, 75-05-8.

(24) Voznij, Y. V.; Koikov, L. N.; Galoyan, A. A. *Carbohydr. Res.* **1984**, *132*, 339.

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