Efficient Method for the Elongation of the *N*-Acetylglucosamine Unit by Combined Use of Chitinase and β-Galactosidase

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Dedicated to Professor *Dieter Seebach* on the occasion of his 65th birthday

A novel strategy for the regio- and stereoselective synthesis by two enzymatic steps of oligosaccharides having an *N*-acetylglucosamine unit at the nonreducing end was developed. The first step involves a chitinase-catalyzed highly selective β-*N*-acetyllactosamination of an oligosaccharide acceptor with a 4,5-dihydrooxazole derivative of *N*-acetyllactosamine as the glycosyl donor. The usage of a transition-state-analogue substrate for the chitinase under basic conditions allows the reaction to proceed only in the synthetic direction while suppressing hydrolysis of the product in aqueous media. Several chitinase mutants also catalyzed the glycosylation efficiently under neutral conditions. The second step is a regioselective cleavage of the glycosidic bond between the terminal galactose unit and the adjacent *N*-acetylglucosamine unit by the action of a β-galactosidase. This constitutes a very useful method to add an *N*-acetylglucosamine unit to the nonreducing end of chito- and cello-oligosaccharide derivatives in a regio- and stereoselective manner.

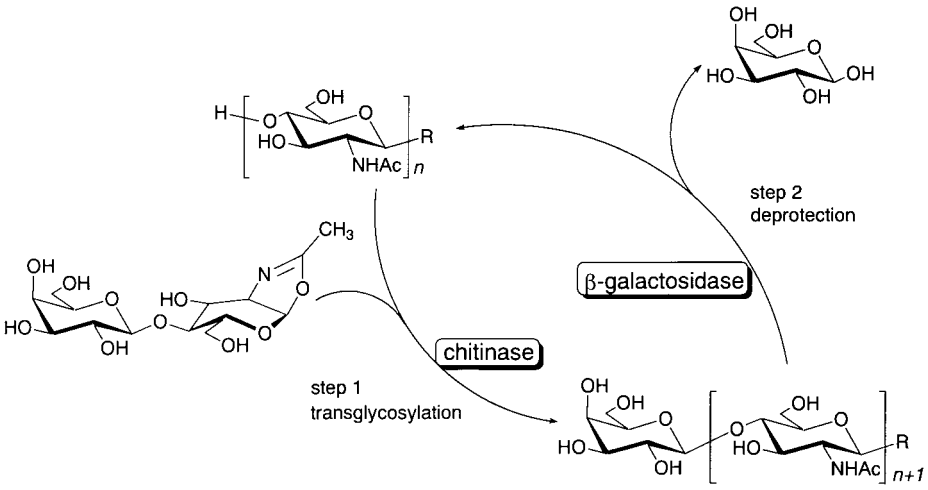
1. Introduction. – Invention of new synthetic tools for a highly selective glycosidic-bond formation is one of the most lively topics in glycotechnology [1]. To develop a highly regio- and stereoselective glycosylation, several requirements concerning the chemical properties of the leaving group on the glycosyl donor, the protecting groups, as well as the nature of the catalysts must be fulfilled. However, chemical approaches thus far reported for the regio- and stereoselective construction of a glycosidic bond have always required blocking and deblocking of at least some OH groups in the glycosyl donor and glycosyl acceptor. In addition, despite the numerous efforts toward development of new glycosylation reactions, complete stereocontrol of the glycosidic-bond formation has not been achieved [2].

Enzymatic glycosylation is a useful tool for the regio- and stereoselective construction of a glycosidic bond under mild reaction conditions where glycosyl donors and glycosyl acceptors can be employed in their unprotected forms, leading to the direct formation of unprotected oligosaccharides in aqueous media [3]. There are many possibilities concerning the combination of glycosyl donor, glycosyl acceptor, and enzyme catalyst. Since it was found that a dihydrooxazole (= 'oxazoline') derivative of

N-acetylglucosamine was recognized by a glycosidase, several studies on the enzymatic polyaddition reaction of sugar dihydrooxazole derivatives have been demonstrated. For example, the enzymatic glycosylation by the dihydrooxazole of *N,N′*-diacetylchitobiose or of *N*-acetylglucosamine catalyzed by a chitinase was reported [4]. However, in these reactions, it was impossible to achieve the exclusive elongation by a single *N*-acetylglucosamine unit. This is because the sugar dihydrooxazole derivative behaved as glycosyl donor and as glycosyl acceptor at the same time, leading to the formation of a mixture of self-coupling products; a selective cross-coupling between the *N*-acetylglucosamine moiety with another saccharide moiety could, therefore, not be achieved.

We postulated that, if the 4-OH group of the *N*-acetylglucosamine could be protected by an appropriate H_2O-soluble group, an enzymatic cross-coupling would be possible provided the protected substrate was recognized by the chitinase effectively, resulting in the formation of glycosyl-enzyme intermediate. The present paper describes a novel synthetic process for the preparation of oligosaccharides having an *N*-acetylglucosamine unit at the nonreducing end, with D-galactose as the protecting group for the glycosyl donor (*Scheme 1*). The reaction involves a chitinase-catalyzed, highly selective transglycosylation of the 4,5-dihydrooxazole derivative of *N*-acetyllactosamine to an oligosaccharide (step 1), followed by the enzymatic cleavage of the D-galactose unit from the nonreducing end of the product by the action of *β*-galactosidase (step 2). As a result of these enzymatic processes, an *N*-acetylglucosamine unit can be added in a defined way to the nonreducing end of the starting oligosaccharide without accompanying self-coupling reactions.

Scheme 1. *Strategy for Elongation of an* N-*Acetylglucosamine Unit by the Combined Use of Chitinase and β-Galactosidase.* The 4-OH group of the *N*-acetylglucosamine unit is protected by introducing a *β*-D-galactose moiety, avoiding self-coupling of the substrate.



2. Results and Discussion. – 2.1. *Design and Synthesis of the Glycosyl Donor, the Dihydrooxazole Derivative of* N-*Acetyllactosamine.* – First, a new glycosyl donor for the transglycosylation to transfer an *N*-acetylglucosamine unit was designed on the

basis of 1) the *retro*-synthetic analysis of an (*N*-acetylglucosamin)ide, and 2) a new mechanism for a chitinase-catalyzed hydrolysis of *N*-acetylglucosamine oligomers. When the (*N*-acetylglucosamin)ide unit is *retro*-synthetically disconnected, a sugar dihydrooxazole derivative is formed as a reasonable synthon. Dihydrooxazole derivatives of *N*-acetylglucosamine whose OH groups are protected have indeed been utilized as glycosyl donors for the preparation of various (*N*-acetylglucosamin)ides. The 'oxazoline method' found by *Micheel et al.* in 1958 [5] has been employed extensively in the stereoselective synthesis of 1,2-*trans*-2-amino-glycosides promoted by various *Broensted* or *Lewis* acids [6].

In addition, a new mechanism for hydrolysis of chitin by chitinase has recently been proposed [7]. According to this mechanism, a dihydrooxazolium ion intermediate is formed in the catalytic site of chitinase as a result of the neighboring participation of the 2-acetamido group. These reports prompted us to investigate the chemical behavior of a sugar dihydrooxazole derivative, the neutral form of the dihydrooxazolium ion intermediate, as a potential glycosyl-donor substrate for chitinases.

A novel irreversible chitinase-catalyzed glycosylation reaction can be designed based on the hypothesis that the usage of a distorted glycosyl-donor substrate lowers the free energy of activation significantly. By choosing basic conditions where the chitinase shows almost no hydrolytic activity, an irreversible glycosylation would be possible. The combined use of a transition-state-analogue substrate [8] and a deactivated hydrolytic enzyme would allow the reaction to proceed only in the direction of the transglycosylation because under basic conditions, the chitinase will not hydrolyze the product (irreversible glycosylation catalyzed by a glycosyl hydrolase).

The substrate, the dihydrooxazole derivative **1** of *N*-acetyllactosamine, was prepared from *N*-acetyllactosamine by acetylation followed by treating the acetate with trimethylsilyl trifluoromethanesulfonate [9]. Finally, the acetyl groups were removed by NaOMe in MeOH.

The ^{1}H-NMR spectrum of **1** shows a signal at δ 6.10 due to the anomeric proton at the reducing end (*Fig. 1,a*). The $J(1,2) = 7.32$ Hz and a signal at δ 2.07 for the Me group clearly indicate that the dihydrooxazole ring was formed. The ^{13}C-NMR spectrum shows signals at δ 104.8 and 78.3 due to C(1′) and C(4), respectively, indicating that the β-glycosidic bond of the Gal-GlcNAc moiety was retained during the synthesis (*Fig. 1,b*).

2.2. *Hydrolysis of* **1** *Catalyzed by Chitinase A1 from* Bacillus circulans *WL-12*. Chitinases (EC 3.2.1.14) cleave the β-1,4-glycosidic linkage in chitin, a homopolymer of *N*-acetylglucosamine. Based on the amino acid sequence similarities, chitinases are classified mostly into two families, *Families 18* and *19* in the classification scheme of *Henrissat* [10]. Recently, the hydrolysis mechanism of *Family 18* chitinases was found to be different from that of *Family 19* chitinases [7]. The mechanism involves a dihydrooxazolium ion as intermediate, as supported by several studies including an inhibition experiment [11] and by structural studies of a chitinase in complex with allosamidin, a natural product that mimics the dihydrooxazole moiety [7a].

On the basis of these reports, we decided to start our investigation by testing whether the dihydrooxazole derivative **1** is recognized by the active site of a *Family 18* chitinase. We used a cloned enzyme, chitinase A1 (from *Bacillus circulans* WL-12) for hydrolytic experiments [12]. The consumption of the dihydrooxazole substrate was monitored by means of high-performance liquid chromatography (HPLC). When the

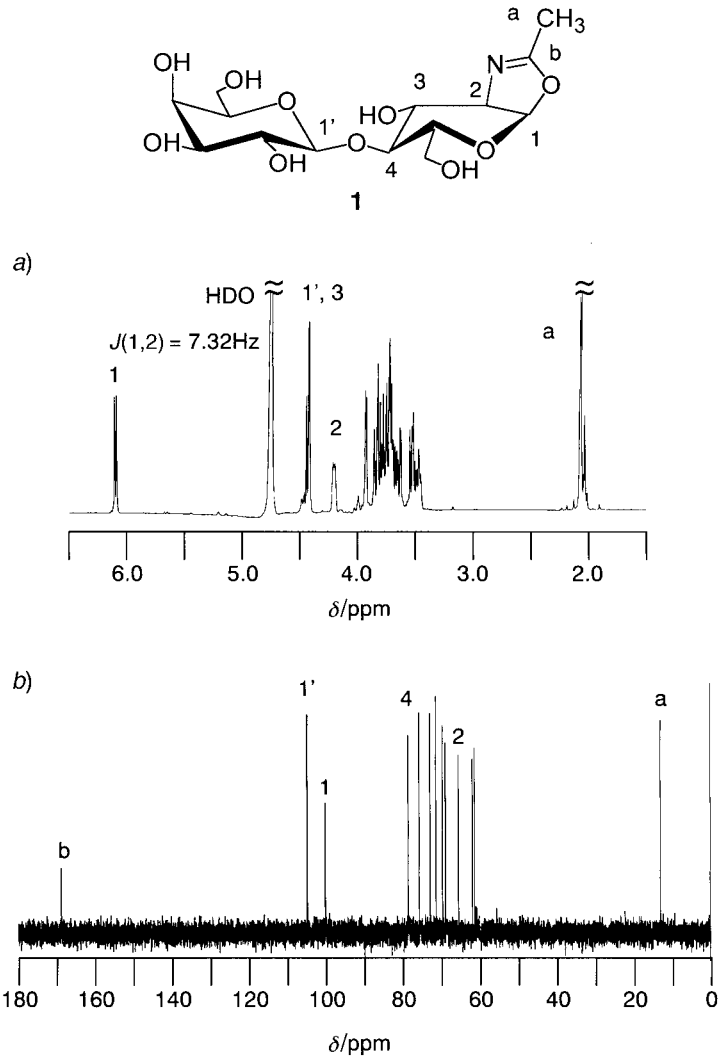


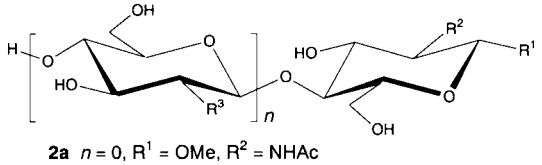
Fig. 1. a) *^{1}H-NMR* and b) *^{13}C-NMR spectra* (D_2O) *of glycosyl donor* **1** (LacNAc-oxa)

substrate **1** was kept at 37° in solution in a phosphate buffer (pH 6.0), only very slow hydrolysis (ring opening of the dihydrooxazole moiety) was observed. Addition of the chitinase A1 caused rapid cleavage of the anomeric C–O bond, yielding the corresponding *N*-acetyllactosamine. The commercially available crude chitinase from *Bacillus* sp., chitinase C1 (*Bacillus circulans* WL-12), and chitinase D1 (*Bacillus circulans* WL-12) also hydrolyzed **1** to *N*-acetyllactosamine. Neither lysozyme nor cellulase catalyzed the hydrolysis of **1**.

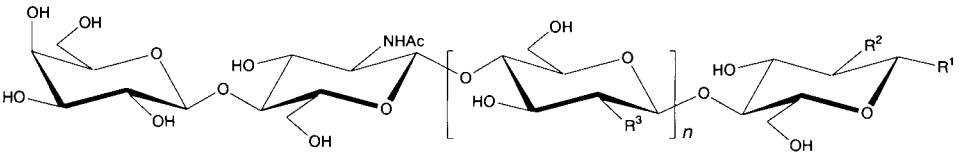
As a reference, hydrolysis of 4-nitrophenyl (*N*-acetyllactosamin)ide (Ga1(β1–4)GlcNAcβ-O4NP) was carried out with chitinase A1. The hydrolysis rate of

Gal(β1–4)GlcNAcβ-O4NP (liberation of 4-nitrophenol(HO4NP)) was much slower than that of **1**. These results indicate that compound **1**, having a distorted conformation close to the dihydrooxazolium-ion intermediate and an axial 4′-OH group, is accepted by the catalytic site of the chitinase A1 to form an enzyme-substrate complex whose anomeric center is attacked by H_2O. The use of such an artificial substrate having a higher free energy as a glycosyl donor promised to be very suitable for the irreversible transglycosylation catalyzed by a chitinase.

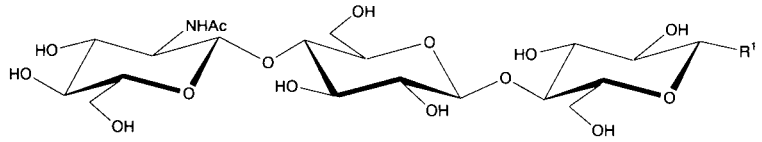
2.3. *Transglycosylation*. The glycosyl donor **1** was successfully transglycosylated to methyl (*N*-acetyl-β-D-glucosamin)ide **2a** (GlcNAcβ-OMe) (*Fig. 2*) catalyzed by chitinase (*Bacillus* sp.) to afford trisaccharide **3a** (Gal(β1–4)GlcNAc(β1–4)GlcNAcβ-OMe). The reaction was performed under basic conditions in citrate buffer (0.04M, pH 9.0) to favor transglycosylation over hydrolysis of the product **3a**. HPLC Monitoring of the mixture showed rapid consumption of the starting material **1** and the appearance of a new peak corresponding to trisaccharide **3a**. The mixture was



2a $n = 0$, R^1 = OMe, R^2 = NHAc
2b $n = 0$, R^1 = SMe, R^2 = NHAc
2c $n = 0$, R^1 = $SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$, R^2 = NHAc
4b $n = 1$, R^1 = SMe, R^2 = R^3 = NHAc
4c $n = 1$, R^1 = $SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$, R^2 = R^3 = NHAc
6c $n = 2$, R^1 = $SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$, R^2 = R^3 = NHAc
7 $n = 1$, R^1 = $SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$, R^2 = R^3 = OH



3a $n = 0$, R^1 = OMe, R^2 = NHAc
3b $n = 0$, R^1 = SMe, R^2 = NHAc
3c $n = 0$, R^1 = $SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$, R^2 = NHAc
5c $n = 1$, R^1 = $SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$, R^2 = R^3 = NHAc
8 $n = 1$, R^1 = $SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$, R^2 = R^3 = OH



9 R^1 = $SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$

Fig. 2. *Structure of glycosyl acceptors, transglycosylated products, and degalactosylated products*

heated for 20 min at 90° to deactivate the enzyme at the point of maximum formation of **3a**. The yield of the product was determined to be 55% after 30 min by comparing the peak area of the product with that of the other sugar units. The crude product was purified by prep. HPLC, and the structure of the resulting trisaccharide determined by means of ^{1}H- and ^{13}C-NMR spectroscopy. All anomeric protons were assigned on the basis of the reported chemical shift of methyl (*N*-acetyl-β-D-glucosamin)ide **2a** and *N*-acetyllactosamine (Gal(β-1–4)GlcNAc). The ^{13}C-NMR spectrum also supported the structure of trisaccharide **3a**.

It may be noted that, with methyl (*N*-acetyl-α-D-glucosamin)ide, the anomer of **2a**, as the glycosyl acceptor, no addition product was obtained, and hydrolysis of **1** to *N*-acetyllactosamine occurred quantitatively. These results indicate that the configuration at C(1) of the glycosyl acceptor is an important factor that controls the course of transglycosylation. These results may be explained by a steric repulsion between the anomeric MeO group of (*N*-acetyl-α-D-glucosamin)ide and an amino acid residue at the catalytic site of chitinase.

This transglycosylation was successfully applied to the 1-thioglycoside derivative **2b** (GlcNAcβ-SMe) as well as to the 1-thioglycoside **2c** having a polymerizable group at the reducing end (GlcNAcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$) [19] as glycosyl acceptors (*Fig. 2*). A chitinase-catalyzed transglycosylation between **1** and **2b** was performed in a carbonate buffer (0.05M, pH 9.0) at 40°, giving rise to the trisaccharide **3b** (Gal(β1–4)GlcNAc(β1–4)GlcNAcβ-SMe). HPLC Monitoring of the mixture showed after 3 h a decrease of the starting glycosyl donor **1**, and a new peak of the product **3b** appeared. The product yields as a function of reaction time at pH 9.0 as monitored by HPLC is shown in *Fig. 3*. During the course of the reaction, the yield of the product did not decrease at longer reaction times establishing that the newly formed glycosidic bonds of the obtained trisaccharide are not cleaved by the enzyme. These results clearly show that the addition reaction proceeded irreversibly under basic reaction conditions (pH 9.0). The structure of product **3b** was determined by ^{1}H- and ^{13}C-NMR spectroscopy (*Fig. 4*).

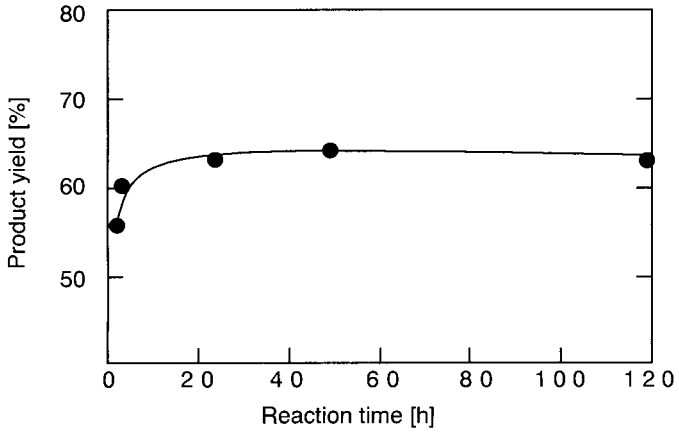


Fig. 3. *Product yields* vs. *reaction time for the chitinase-catalyzed addition reaction of GlcNAcβ-SMe* **2b** *to LacNAc-oxa* **1** (40° in carbonate buffer (pH 9.0))

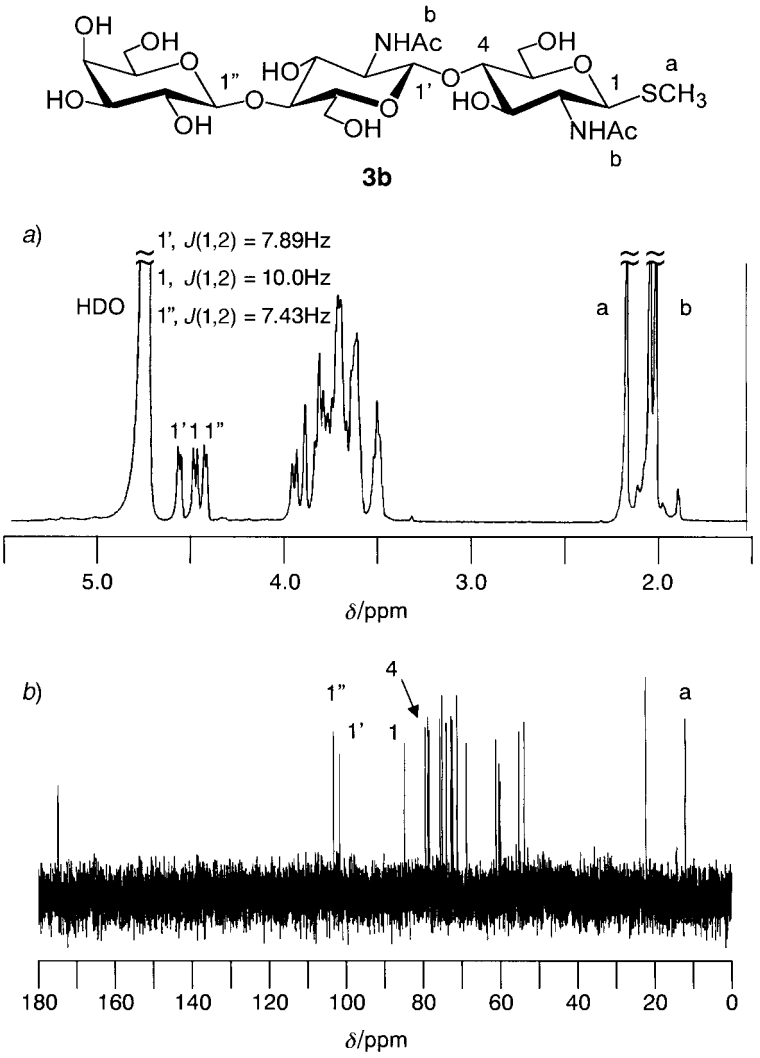


Fig. 4. a) *^{1}H-NMR and* b) *^{13}C-NMR spectra* (D_2O) *of addition product* **3b** (Gal(β1–4)GlcNAc(β1–4)GlcNAcβ-SMe)

The ^{1}H-NMR spectrum of **3b** showed a *d* with $J = 7.89$ Hz at δ 4.57 for H–C(1′), indicating that the newly formed glycosidic bond in **3b** is of the β-D-type (*Fig. 4,a*). In the ^{13}C-NMR, two peaks at δ 101.5 due to the C(1′) and at δ 79.3 due to the C(4) clearly indicate the regio- and stereoselective formation of the β-D-1,4 glycosidic linkage (*Fig. 4,b*).

The thioglycoside derivative **2c** with a polymerizable group at the reducing end was similarly glycosylated under basic conditions, giving rise to the trisaccharide derivative **3c** with a polymerizable group at the reducing end (Gal(β1–4)GlcNAc(β1–4)GlcNAcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$) whose structure was confirmed by ^{1}H- and ^{13}C-NMR spectroscopy.

The ^{1}H-NMR spectrum of **3c** showed the olefinic protons of the acrylamide group (δ 5.74–6.19 (3 H)) and three anomeric protons (δ 4.56 (H–C(1)), 4.53 (H–C(1′)), and 4.41 (H–C(1″))). The configuration of the resulting glycosidic bond was determined to be β-D, judging from the coupling constant of the signal assigned to the anomeric proton of the internal GlcNAc unit ($J(1',2') = 7.14$ Hz). These data show that the transglycosylation reaction of **1** to **2c** proceeds in a regio- and stereoselective manner, giving rise to the trisaccharide derivative **3c**.

The transglycosylation was monitored by HPLC. After the addition of chitinase, a peak corresponding to the glycosyl acceptor **2c** became smaller and a new peak attributed to **3c** appeared. Product **3c** was purified by prep. HPLC and freeze-dried. To optimize reaction conditions, the effect of pH on the transglycosylation yield was investigated at pH 9.0, 9.8, 10.4, and 11.0 (*Fig. 5*). When the reaction was carried out at pH 9.0 and 9.8, the yield of the product increased to a maximum after 2 h and then slowly decreased as the reaction proceeded. This behavior may be explained by the hydrolysis of product **3c** by the action of chitinase. When the reaction was performed at pH 10.4, the yield gradually increased as the reaction proceeded and became constant after 20 h, indicating that the addition reaction was irreversible; the newly produced glycosidic bond of **3c** was not cleaved by the catalyst chitinase. The reaction did not take place at pH 11.0.

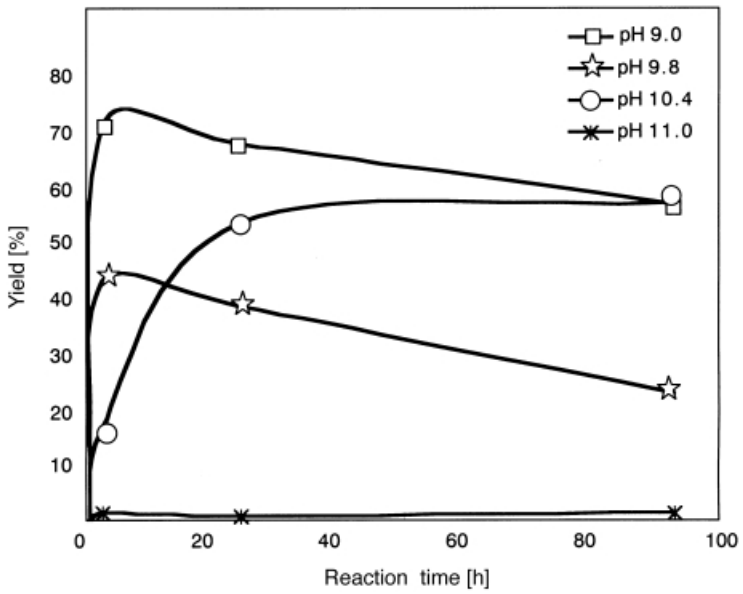
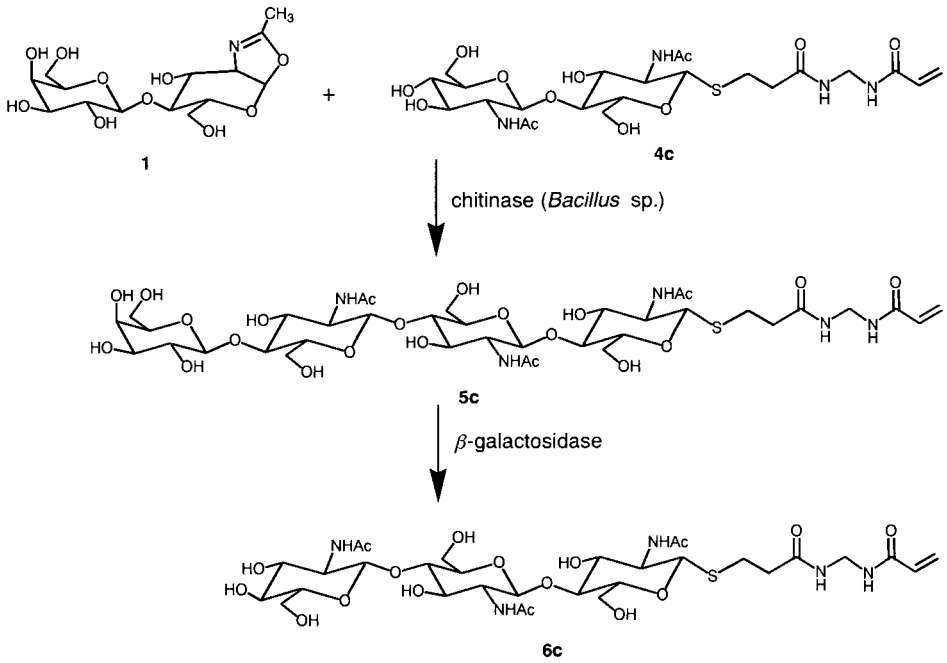


Fig. 5. *The effect of pH on the yields of* **3c** *in the chitinase-catalyzed addition reaction of* **2c** (GlcNAcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$) *to LacNAc-oxa* **1**

A chitobiose derivative **4c** having a polymerizable group at the reducing end (GlcNAc(β1–4)GlcNAcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$) was also subjected to the addition reaction with **1** in a buffer solution to give the corresponding tetrasaccharide derivative **5c** (Gal(β1–4)GlcNAc(β1–4)GlcNAc(β1–4)GlcNAcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$) (*Scheme 2*). The HPLC trace of the reaction

mixture showed that the peak of the glycosyl acceptor **4c** had completely disappeared after 2 h, and a new peak of the resulting tetrasaccharide **5c** was detected as a main product, indicating that the desired addition took place almost quantitatively (*Fig. 6,a*). However, in addition to the desired product **5c**, a small peak of **2c** (GlcNAcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$) appeared as a by-product. Since the chitinase is known to be inactive towards the glycosidic bond between two GlcNAc units of the glycosyl acceptor **4c**, the formation of this side product is attributed to the fact that the chitinase cleaved the glycosidic bond between the GlcNAc unit at the reducing end and the adjacent GlcNAc unit of the resulting tetrasaccharide derivative **5c**. A prolonged reaction time led to a considerable amount of by-product **2c**. To minimize this side reaction and to improve the yield, we stopped the reaction after 2 h.

Scheme 2



Interestingly, the chitinase from *Bacillus* sp. can also catalyze the *N*-acetyllactosamination of glycosyl acceptors that differ in structure from the natural substrate of chitinases, viz. cellooligosaccharides with a degree of polymerization (DP) 2–6. The formation of the glycosylation products was confirmed by measuring their molecular masses by means of MALDI-TOF mass spectroscopy. Cellobiose derivative **7** having a polymerizable group at the reducing end (Glc(β1–4)Glcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$) (*Fig. 2*) can also be *N*-acetyllactosaminated to give the corresponding tetrasaccharide derivative **8** (Gal(β1–4)GlcNAc(β1–4)Glc(β1–4)Glcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$). These results indicate that cellooligomers are recognized efficiently by the catalytic center as glycosyl acceptors.

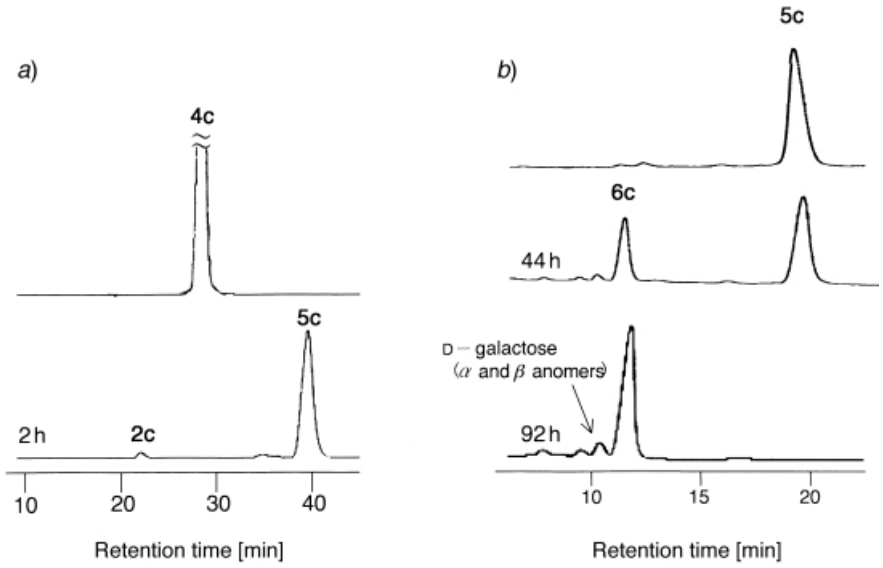


Fig. 6. a) *HPLC Trace of chitinase-catalyzed addition reaction of* **4c** (GlcNAc(β1–4)GlcNAcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$) *to LacNAc-oxa* **1**, *after 2 h*. b) *HPLC Trace of β-galactosidase-catalyzed degalactosylation reaction of* **5c** (Gal(β1–4)GlcNAc(β1–4)GlcNAc(β1–4)GlcNAcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$).

The ^{1}H-NMR spectrum of **8** shows a *d* for the anomeric proton (H–C(1″), J(1″,2″) = 7.82 Hz) at δ *ca*. 4.4, indicating the stereoselective formation of a β-D glycosidic linkage. The ^{13}C-NMR spectrum shows signals at δ 101.5 and 79.1 attributed to C(1″) and C(4′), respectively. No signals typical for β-D-1,3 and β-D-1,6 glycosidic bonds were detected. These data show that the enzymatic addition of **1** to **7** took place regio- and stereoselectively, leading to the exclusive formation of the β-D-1,4 linkage.

In the reaction presented involving **1** as a transition-state-analogue substrate, the N-atom of the dihydrooxazole ring may be protonated by an acidic amino acid located in the -1 subsite. The nucleophilic attack of the 4-OH group of the glycosyl acceptor occurs by the assistance of the carboxylate ion of the acidic amino acid that behaves as a general base. The α-D-glycosidic bond of **1** is then cleaved and the C=O bond is formed simultaneously, leading to the formation of the 2-acetamido group in the product oligosaccharides.

Chemical modification of the reducing end of oligosaccharides is an important topic in the field of glycotechnology, because these functionalized, end-reactive oligosaccharides can be used as precursors for the production of various glycoconjugate polymers. For example, oligosaccharides having a polymerizable group at the reducing end can be copolymerized with various vinyl monomers by a radical initiator to give copolymers having oligosaccharide graft chains [13]. However, it is extremely difficult to prepare such modified oligosaccharides by the conventional chemical approach, because it requires the introduction of several kinds of protecting groups into the oligosaccharide moiety. As the DP of an oligosaccharide increases, the chemical modification of the oligosaccharide becomes more difficult due to its low solubility in

organic solvents. The present enzymatic method of using an unprotected glycosyl donor and an unprotected glycosyl acceptor makes it possible to synthesize a functional oligosaccharide without employing laborious blocking and deblocking procedures.

2.4. *Degalactosylation*. In the above described reactions, the galactosyl group protects the *N*-acetylglucosamine unit and prevents self-coupling. The transglycosylation products possess a galactose unit at the nonreducing end (*Fig. 2*). We now discuss the β-galactosidase-catalyzed deprotection of these products. The trisaccharides **3b** and **3c** and the tetrasaccharide **8** were converted to the disaccharides **4b** (GlcNAc(β1–4)GlcNAcβ-SMe) and **4c** (GlcNAc(β1–4)GlcNAcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$) and the trisaccharide **9** (GlcNAc(β1–4)Glc(β1–4)Glcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$), respectively, by selective cleavage of the β-D glycosidic bond between the terminal D-galactose unit and the adjacent *N*-acetylglucosamine unit. The tetrasaccharide **5c** prepared by the enzymatic addition of **1** and **4c** was also converted to the corresponding trisaccharide **6c** (GlcNAc(β1–4)GlcNAc(β1–4)GlcNAcβ-$SCH_2CH_2CONHCH_2NHCOCH{=}CH_2$) by enzymatic removal of the D-galactose unit by the same procedure (*Scheme 2*). When **5c** was treated with β-galactosidase (*Streptococcus pneumoniae*) in phosphate buffer solution (0.02M, pH 6.0), the peak of **5c** disappeared completely after 92 h. A new peak attributed to product **6c** and two peaks due to the α-D and β-D isomers of the liberated D-galactose appeared (*Fig. 6,b*). The product **6c** was purified by HPLC and freeze-dried. The structures of all degalactosylated products were confirmed by comparing their ^{1}H- and ^{13}C-NMR spectra with those of GlcNAc-SR, chitobiose (GlcNAc(β1–4)GlcNAc), and chitotriose (GlcNAc(β1–4)GlcNAc(β1–4)GlcNAc). Thus, a new route for the preparation of chitooligosaccharides or cellooligosaccharides having an *N*-acetylglucosamine unit at the nonreducing end has been developed by the combination of a chitinase-catalyzed addition reaction and a β-galactosidase-catalyzed selective degalactosylation reaction.

The present method including the elongation of a single *N*-acetylglucosamine unit will become a convenient tool for the synthesis of various oligosaccharides useful in glycobiology. This method also makes it possible to prepare specifically labeled oligosaccharide derivatives that can be utilized for the elucidation of the roles of oligosaccharides *in vivo*, for example, the elicitor activity of oligosaccharides for higher plants [14]. The use of a thioglycoside moiety at the reducing end enables us to convert the products to free chitooligosaccharides or free hybrid-type oligosaccharides using an appropriate reagent without damaging the inner glycosidic bonds. Furthermore, thioglycosides can be converted to the corresponding sulfoxides that can be used as a glycosyl donor in acid-catalyzed glycosylations [15].

2.5. *Transglycosylation Catalyzed by Mutants of Chitinase A1*. So far, we had to carry out the chitinase-catalyzed addition reactions under basic conditions to ensure the irreversibility of the glycosylating process. To improve the presented glycosylation reaction so that it proceeds at a different pH, especially under neutral conditions, it is necessary to modify the catalyst enzyme. In *Sect. 2.5*, we wish to report that the transglycosylation can also be carried out under neutral conditions with several mutants of chitinase A1 from *Bacillus circulans* WL-12.

Recently, the three-dimensional structure of the catalytic domain of chitinase A1 from *Bacillus circulans* WL-12 was determined by X-ray analysis [16]. The study exhibited the amino acid residues that participate in hydrolysis of chitin as well as their peripheral residues. On the basis of this analysis, we investigated the addition reaction using several mutant chitinases as catalysts, the dihydrooxazole derivative **1** as glycosyl donor, and chitobiose (GlcNAc(β1–4)GlcNAc) or methyl (*N*-acetyl-β-D-glucosamin)ide (**2a**; GlcNAcβ-OMe) as glycosyl acceptor. The effect of several amino acids on the transglycosylating ability of these mutant chitinases was examined. The enzymes employed were mutant chitinases from *Bacillus circulans* WL-12, where the glutamic acid residue 204 was changed to a glutamine residue (E204Q), the aspartic acid residue 202 to an asparagine residue (D202N), the aspartic acid residue 200 to an asparagine residue (D200N), the tyrosine residue 279 to a phenylalanine residue (Y279F), the aspartic acid residue 280 to a asparagine residue (D280N), and the tryptophan residue 433 a to phenylalanine residue (W433F).

The enzymatic addition reaction was carried out under neutral conditions (pH 7.0) as well as under basic conditions (pH 10.4), and the transglycosylating activity was evaluated by tracing the formed tetrasaccharide (Gal(β1–4)GlcNAc(β1–4)GlcNAc(β1–4)GlcNAc) or **3a** (Gal(β1–4)GlcNAc(β1–4)GlcNAcβ-OMe) by means of HPLC. Interestingly, when the reaction was carried out with D202N, Y279F, D280N, and W433F as catalysts at pH 7.0, the yields were better than those obtained by wild-type chitinase. *Fig. 7* shows the trace of the addition product from chitobiose as glycosyl acceptor catalyzed by the wild-type chitinase A1 and D202N at

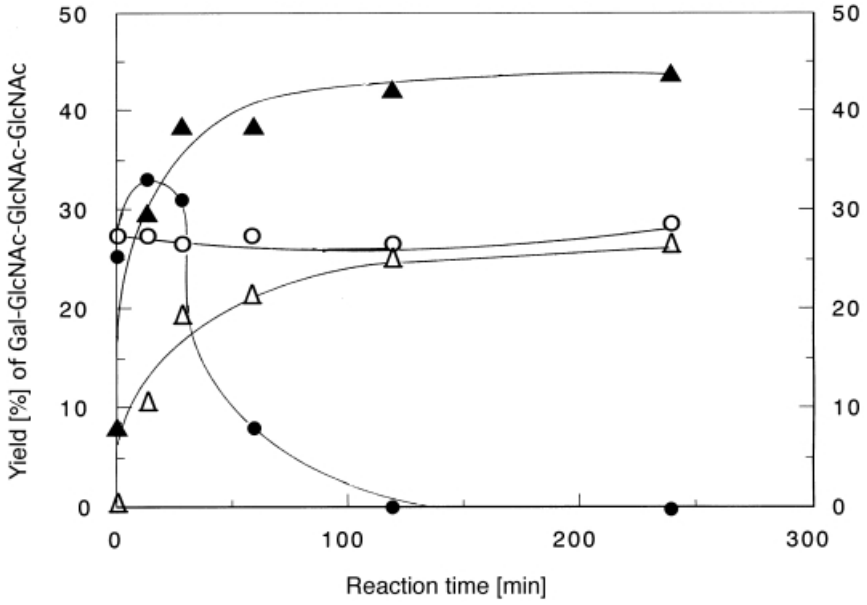


Fig. 7. *Product yields* vs. *reaction time for chitinase-catalyzed addition reaction of chitobiose* (GlcNAc(β1–4)GlcNAc) *to LacNAc-oxa* **1**. ▲: catalyst, mutant enzyme D202N; pH 7.0. ●: catalyst, wild-type enzyme; pH 7.0. △: catalyst, mutant enzyme D202N; pH 10.4. ○: catalyst, wild-type enzyme; pH 10.4.

pH 7.0 and 10.4. The addition reaction of chitobiose to the sugar dihydrooxazole **1** was smoothly catalyzed by the mutant D202N, giving rise to the corresponding tetrasaccharide (Gal(β1–4)GlcNAc(β1–4)GlcNAc(β1–4)GlcNAc). The yield of the formed tetrasaccharide was found to exceed 40% and did not decrease at a prolonged reaction time even at pH 7.0 (▲ in *Fig. 7*). When the reaction was performed under basic conditions (pH 10.4), the yields obtained with wild-type chitinase and mutant chitinase were almost the same at a prolonged reaction time (○ and △ in *Fig. 7*). The wild-type chitinase gave the addition product in quite good yield at the initial stage of the reaction; however, the resulting tetrasaccharide was immediately hydrolyzed at pH 7.0 (● in *Fig. 7*). These results indicate that the addition reaction catalyzed by the mutant enzyme D202N proceeded irreversibly at pH 7.0 where the wild-type enzyme shows strong hydrolysis activity. Thus, the hydrolyzing activity toward the resulting oligosaccharides was reduced by changing an amino acid of chitinase A1, while retaining their transglycosylating activities even under neutral conditions.

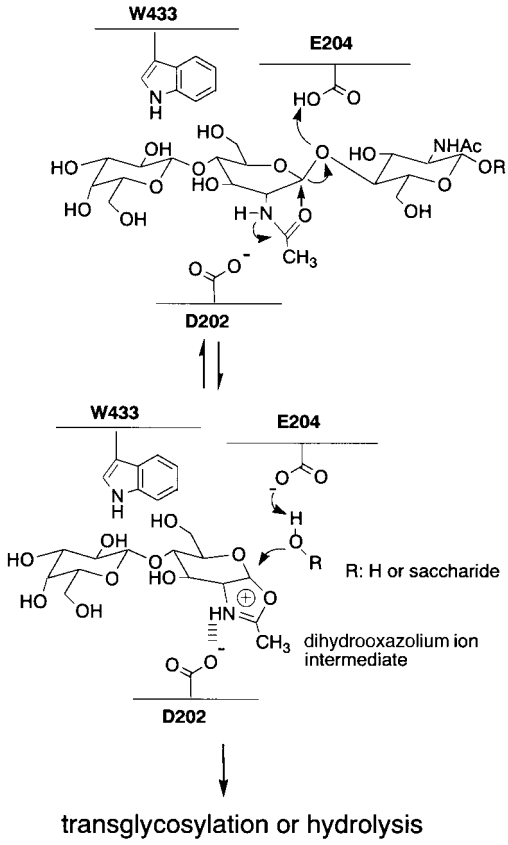
That the reaction catalyzed by these mutant enzymes is irreversible can be explained by their effective recognition of the transition-state analogue **1**, whereas the mutants show very low affinity toward the (*N*-acetylglucosamin)ide moiety of the product. It was assumed that the carboxy group of the aspartic acid residue 202 of the wild-type chitinase A1 interacts with the C(2) amide carbonyl of the substrate in the -1 subsite and serves to direct the carbonyl group toward the anomeric C-atom to assist the formation of the dihydrooxazole ring (*Scheme 3*). The replacement of the aspartic acid residue 202 by asparagine may cause a significant decrease of hydrolysis activity toward an (*N*-acetylglucosamin)ide unit of the formed tetrasaccharide.

The irreversible formation of the product with W433F as catalyst may also be explained by the decrease of hydrolytic activity of the mutant at pH 7.0. The specific activity of W433F toward 4-nitrophenyl chitobioside decreased to 0.02 U/mg whereas that of the wild type was 0.11 U/mg, indicating that the W433F has only 20% of hydrolytic activity compared to that of wild-type chitinase. These results are consistent with the decrease of hydrolytic activity observed in the enzymatic addition reaction catalyzed by W433F. It is well known that the tryptophane residue 433 of chitinase A1 assists the incorporation of the substrate into the -2 subsite of the catalytic site through a hydrophobic interaction. The replacement of the tryptophane residue 433 by the phenylalanine residue may cause the decrease of the interaction described above, leading to the significant deactivation of hydrolysis activity.

In contrast, the mutant E204Q where the essential glutamic acid residue is changed to glutamine could not promote the transglycosylation. The glutamic acid residue 204 plays a very important role for hydrolysis and transglycosylation of a (*N*-acetylglucosamin)ide unit [17]. It has been established that the carboxylate derived from the glutamic acid residue 204 acts as a general base to enhance the nucleophilic attack of the H_2O molecule or one of the OH groups of the glycosyl acceptor in the hydrolysis or transglycosylation, respectively (*Scheme 3*). Consequently, the lack of the glutamic acid residue 204 caused almost complete disappearance of hydrolytic activity as well as transglycosylating activity.

Although the detailed mechanism of the transglycosylation reaction catalyzed by these mutant enzymes has not been made clear, the usage of such modified catalysts will widen the scope of the enzymatic glycosylation based on the site-directed

Scheme 3. *Roles of aspartic acid residue 202* (D202), *glutamic acid residue 204* (E204), *and tryptophane residue 433* (W433) *in the catalytic site of wild-type chitinase A1 from* Bacillus circulans *WL-12*



mutagenesis technique. It is to be noted that some experimental evidence has been obtained that suggests that the transglycosylation yield can be optimized, depending upon various reaction conditions, by changing the nature of catalyst enzyme. Such optimizations will be extremely important for the development of a new multi-enzyme reaction system in the synthesis of complex oligosaccharides in the future.

Experimental Part

General. Commercially available trimethylsilyl trifluoromethanesulfonate was used without purification. The 3-[(acrylamidomethyl)amino]-3-oxopropyl 2-acetamido-2-deoxy-1-thio-β-D-glucopyranoside (**2c**) was prepared according to [18]. MeCN was distilled from CaH_2. Chitinase (*Bacillus* sp.) (0.04 U/mg) was purchased from *Wako Chemicals* (Osaka). β-Galactosidase (*Streptococcus pneumoniae*) (1.27 U/mg) was obtained from *Calbiochem*. The purity of wild-type chitinase A1, the corresponding mutants, chitinase C1, and chitinase D1 was confirmed by 10% SDS polyacrylamide-gel electrophoresis (SDS-PAGE). Sequence determination was carried out with *ALF express*™ DNA sequencer (*Amercham Pharmacia Biotech*). HPLC: *Inertsil ODS-3* and *TSK-gel-Amide-80* columns. UV Spectra: *Beckman DU-70* and *Shimadzu UV-160* spectrophotometers. NMR Spectra: *Bruker AC-250T*, *Bruker DPX-400*, and *Bruker ORX-500* spectrometers; δ in ppm, J in Hz.

*4,5-Dihydro-2-methyl{1,2-dideoxy-4-*O*-(β-*D*-galactopyranosyl)-α-*D*-glucopyranoso}[2,1-*d*]oxazole* (**1**). A mixture of *N*-acetyllactosamine (500 mg, 1.32 mmol), Ac_2O (2.7 ml), and pyridine (1.3 ml) was stirred at r.t. for 22 h. After diluting with $CHCl_3$, the resulting soln. was washed with H_2O, and sat. aq. $NaHCO_3$ soln., dried (Na_2SO_4), and evaporated: crude lactosamine peracetate that was used for the following experiment without further purification. To a soln. of lactosamine peracetate (948 mg, 1.40 mmol) in 1,2-dichloroethane (17 ml), trimethylsilyl trifluoromethanesulfonate (0.3 ml, 1.66 mmol) was added dropwise, and the mixture was heated at 50° for 5 h. Me_3N (0.6 ml) was added and the mixture stirred for 10 min. After diluting with $CHCl_3$, the mixture was washed with cold H_2O, the org. layer dried (Na_2SO_4) and evaporated, and the residue submitted to column chromatography (*Merck* silica gel *60* (0.040–0.063 mm), AcOEt/hexane 4:1) 520 mg of 4,5-dihydro-2-methyl{1,2-dideoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-glucopyranoso}[2,1-*d*]oxazole (61%). To a soln. of this oxazole derivative (498 mg, 0.81 mmol) in MeOH (50 ml) was added a solution of 0.5M NaOMe in MeOH (1.0 ml) at 0°, and the mixture was stirred at r.t. for 1 h. After neutralizing the mixture with *Amberlite IR-120* H^+, the solvent was evaporated: **1** (quant.). White solid. ^{1}H-NMR (D_2O, 400 MHz): 2.07 (*s*, N=C(Me)O); 6.10 (*d*, *J*(1,2) = 7.31, H–C(1)). ^{13}C-NMR (D_2O, 100 MHz): 13.1 (N=C(*Me*)O); 61.3 (C(6)); 61.8 (C(6′)); 65.3 (C(2)); 68.7 (C(4′)); 69.4 (C(5)); 71.0 (C(3)); 71.1 (C(2′)); 72.6 (C(3′)); 75.4 (C(5′)); 78.3 (C(4)); 100.0 (C(1)); 104.8 (C(1′)); 168.4 (N=*C*(Me)O).

Methyl O-β-D-*Galactopyranosyl-(1→4)*-O-*2-acetamido-2-deoxy-β-D-glucopyranosyl(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside* (Gal(β1–4)GlcNAc(β1–4)GlcNAcβ-OMe; **3a**). Glycosyl donor **1** (48 mg, 0.13 mmol) and methyl (*N*-acetyl-β-D-glucosamin)ide (**2a**; GlcNAcβ-OMe; 92 mg, 0.39 mmol) were dissolved in 150 µl of 0.05M citric buffer (pH 9.0). To this mixture, chitinase (*Bacillus* sp., 10 wt.-% for **1**) dissolved in 80 µl of 0.01M citric buffer (pH 9.0) was added, and the mixture was stirred for 0.5 h at 30°. Yields were determined by taking aliquots of the sample and evaporation of the solvent prior to HPLC analysis. For final workup, the mixture was treated with THF to inactivate the enzyme. To obtain samples for NMR spectral analysis, semi-prep. HPLC was performed. The HPLC fraction containing the product was chromatographically homogeneous. ^{1}H-NMR (D_2O, 400 MHz): 3.49 (*s*, MeO); 4.41 (*d*, *J*(1,2) = 7.85, H–C(1)); 4.44 (*d*, *J*(1″,2″) = 8.14, H–C(1″)); 4.58 (*d*, *J*(1′,2′) = 7.48, H–C(1′)). ^{13}C-NMR (D_2O, 100 MHz): 58.0 (MeO); 80.2 (C(4)); 102.2 (C(1′)); 102.7 (C(1)); 103.7 (C(1″)). MALDI-TOF-MS: 601.3 ($[M+Na]^+$; calc. 600.6).

Methyl O-β-D-*Galactopyranosyl-(1→4)*-O-*2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-1-thio-β-D-glucopyranoside* (Gal(β1–4)GlcNAc(β1–4)GlcNAcβ-SMe; **3b**. To **1** (18.5 mg, 0.05 mmol) in a microtube was added a soln. of methyl 2-acetamido-2-deoxy-1-thio-β-D-glucopyranoside (**2b**) in 0.5 ml of 0.05M carbonate buffer (pH 9.0), and the resulting mixture was incubated at 40°. After adding an excess of THF, the mixture was heated at 90° for 20 min to deactivate the enzyme. The solvent was evaporated and the residue dissolved in H_2O and separated by prep. HPLC: **3b** (13.0 mg, 64%). ^{1}H-NMR (D_2O, 500 MHz): 2.01 (2 COMe); 2.15 (*s*, MeS); 4.43 (*d*, *J*(1″,2″) = 7.43, H–C(1″)); 4.49 (*d*, *J*(1,2) = 10.0, H–C(1)); 4.56 (*d*, *J*(1′,2′) = 7.89, H–C(1′)). ^{13}C-NMR (D_2O, 125 MHz): 12.1 (MeS); 22.3 (2 N=C(Me_3)O); 53.9 (C(2)); 55.3 (C(2′)); 60.0 (C(6)); 60.4 (C(6′)); 61.2 (C(6″)); 68.7 (C(4″)); 71.1 (C(2″)); 72.3 (C(3)); 72.6 (C(3″)); 73.9 (C(3′)); 75.0 (C(5′)); 75.5 (C(5″)); 78.3 (C(5)); 78.6 (C(4′)); 79.3 (C(4)); 84.7 (C(1)); 101.5 (C(1′)); 103.0 (C(1″)); 174.6, 174.7 (2 *C*OMe).

Trisaccharide Gal(β1–4)GlcNAc(β1–4)GlcNAcβ-SCH$_2$*CH*$_2$*CONHCH*$_2$*NHCOCH=CH*$_2$ (**3c**). To **1** (73 mg, 0.2 mmol) in a microtube was added a soln. of GlcNAc derivative **2c** (26 mg, 66.7 mmol) and chitinase (*Bacillus* sp.; 7.3 mg, 292 mU) in 2.0 ml of 0.05M *Tris* buffer (pH 9.0), and the resulting mixture was incubated in a dry thermo bath at 40°. After adding an excess of THF, the mixture was heated at 90° for 20 min to deactivate the enzyme, and the solvent was evaporated. The residue was dissolved in H_2O and separated by prep. HPLC (*Inertsil-ODS*, H_2O/MeOH, 3.0 ml/min): **3c** (35 mg, 69%). ^{1}H-NMR (D_2O, 500 MHz): 1.96, 2.02 (*s*, 2 COMe); 2.54, 2.9 (*m*, SCH_2CH_2); 4.41 (*d*, *J*(1″,2″) = 7.82, H–C(1″)); 4.53 (*d*, *J*(1′,2′) = 7.14, H–C(1′)); 4.56 (*d*, *J*(1,2) = 10.0, H–C(1)); 4.60 (*s*, NCH_2N); 5.79–6.19 (*m*, CH=CH_2). ^{13}C-NMR (D_2O, 125 MHz): 22.3 (2 N=C(*Me*)O); 26.4, 36.0 (SCH_2CH_2); 44.4 (NCH_2N); 54.2 (C(2)); 55.3 (C(2′)); 60.0 (C(6′)); 60.5 (C(6′)); 61.2 (C(6″)); 68.7 (C(4″)); 71.1 (C(2″)); 72.2 (C(3′)); 72.6 (C(3″)); 73.8 (C(3)); 74.9 (C(5′)); 75.5 (C(5″)); 78.3 (C(4′)); 78.5 (C(5)); 79.4 (C(4)); 84.6 (C(1)); 101.5 (C(1′)); 103.0 (C(1″)); 128.4 (=CH_2); 129.7 (CH=); 168.8 (NH*C*OCH=); 174.5–174.8 (2 *C*OMe). MALDI-TOF-MS: 778.8 ($[M+Na]^+$; calc. 779.9).

Tetrasaccharide Gal(β1–4)GlcNAc(β1–4)GlcNAc(β1–4)GlcNAcβ-SCH$_2$*CH*$_2$*CONHCH*$_2$*NHCOCH=CH*$_2$ (**5c**). To **1** (18 mg, 48 µmol) in a microtube was added a solution of GlcNAc derivative **4c** (19 mg, 32 µmol) and chitinase (*Bacillus* sp., 70.4 mU) in 2.0 ml of 0.05M carbonate buffer (pH 10.4), and the resulting mixture was incubated at 40° for 2 h. The mixture was heated at 90° for 20 min to deactivate the enzyme, and the solvent was evaporated. The residue was dissolved in H_2O and separated by prep. HPLC (*Inertsil-ODS*, H_2O/MeOH 900:7, 5.0 ml/min): **5c** (17 mg, 54%). ^{1}H-NMR (D_2O, 400 MHz): 1.90–1.96 (*s*, 3 COMe); 2.48, 2.83 (*m*, SCH_2CH_2); 4.35 (*d*, *J*(1‴,2‴) = 7.76); 4.45–4.51 (H–C(1), H–C(1′), H–C(1″)); 4.55 (*s*, NCH_2N); 5.68–6.15

(*m*, $CH{=}CH_2$). ^{13}C-NMR (D_2O, 100 MHz): 22.3 (3 N=CO(Me)); 26.4, 36.0 (SCH_2CH_2); 44.4 (NCH_2N); 54.3 (C(2)); 55.2, (C(2′)); 55.3 (C(2″)); 60.0 (C(6′), C(6″)); 60.5 (C(6)); 61.2 (C(6‴)); 68.7 (C(4‴)); 71.1 (C(2‴)); 72.2 (C(3′), C(3″)); 72.6 (C(3‴)); 73.8 (C(3)); 74.7 (C(5′)); 75.0 (C(5″)); 75.5 (C(5‴)); 78.2 (C(4″)); 78.6 (C(5)); 79.1 (C(4′)); 79.2 (C(4)); 84.7 (C(1)); 101.4 (C(1′)); 101.5 (C(1″)); 103.0 (C(1‴)); 128.5 ($=CH_2$); 129.7 (CH=); 168.8 (NH*C*OCH=); 174.7–174.8 (3 *C*OMe). MALDI-TOF-MS: 981.6 ($[M+Na]^+$; calc. 983.1).

Hybrid-Type Tetrasaccharide Gal(β1–4)GlcNAc(β1–4)Glc(β1–4)Glcβ-SCH$_2$CH$_2$CONHCH$_2$-NHCOCH=CH$_2$ (**8**). To **1** (37 mg, 0.1 mmol) in a microtube was added a soln. of cellobioside derivative **7** (17 mg, 33 μmol) and chitinase (*Bacillus* sp., 325 mU) in 1.0 ml of 0.05M *Tris* buffer (pH 9.0), and the resulting mixture was incubated at 40°. The reaction was monitored by HPLC (MeCN/H_2O 3:1). After **1** was completely consumed, the mixture was heated at 90° for 20 min to deactivate the enzyme, and the solvent was evaporated. The residue was dissolved in H_2O and separated by prep. HPLC (*Inertsil-ODS*, H_2O/MeOH 900:70, 3.0 ml/min): **8** (5 mg, 16%). ^{1}H-NMR (D_2O, 400 MHz): 1.98 (*s*, COMe); 2.55, 2.91 (*m*, SCH_2CH_2); 4.39 (*d*, *J*(1‴,2‴) = 7.8, H–C(1‴)); 4.43 (*d*, *J*(1′,2′) = 7.9, H–C(1′)); 4.47 (*d*, *J*(1,2) = 10.0, H–C(1)); 4.50 (*d*, *J*(1″,2″) = 7.8, H–C(1″)); 4.58 (*s*, NCH_2N); 5.70–6.16 (*m*, $CH{=}CH_2$). ^{13}C-NMR (D_2O, 100 MHz): 22.3 (N=CO(*Me*)); 26.1, 36.2 (SCH_2CH_2); 44.5 (NCH_2N); 60.1 (C(6′), C(6″)); 60.3 (C(6)); 61.2 (C(6‴)); 79.1 (C(4′)); 85.6 (C(1)); 101.5 (C(1″)); 102.4 (C(1′)); 103.1 (C(1‴)); 128.4 ($=CH_2$); 129.7 (CH=); 168.8 (NH*C*OCH=); 174.7, 174.9 (*C*OMe, *C*OCH$_2$). MALDI-TOF-MS: 901.0 ($[M+Na]^+$; calc. 900.9).

*Methyl 2-Acetamido-4-*O*-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-2-deoxy-1-thio-β-*D*-glucopyranoside* (GlcNAc(β1–4)GlcNAcβ-SMe; **4b**). A typical procedure for the degalactosylation of **3b** by β-galactosidase is as follows: to a soln. of **3b** (10 mg, 16.2 μmol) in 2.0 ml of 0.05M phosphate buffer (pH 6.0) was added 0.2 ml of 0.05M phosphate buffer (pH 6.0) soln. of β-galactosidase (*Streptococcus pneumoniae*) (5 wt.-% rel. to **3b**), and the mixture was shaken for 1 h at 40°. After adding an excess of THF, the mixture was heated for 10 min at 90° to deactivate the enzyme, and the solvent was evaporated. The residue was dissolved in a minimum amount of H_2O and the H_2O-insoluble material was filtered off. The filtrate was chromatographed by prep. HPLC: **4b** (7 mg). ^{1}H-NMR (D_2O, 500 MHz): 2.03, 2.06 (2 COMe); 2.19 (MeS); 4.52 (*d*, *J*(1,2) = 10.4, H–C(1)); 4.59 (*d*, *J*(1′,2′) = 8.45, H–C(1′)). ^{13}C-NMR (D_2O, 125 MHz): 12.0 (MeS), 22.2, 22.3 (2 N=C(*Me*)O); 53.9 (C(2)); 55.7 (C(2′)); 60.4 (C(6)); 60.7 (C(6′)); 69.8 (C(4′)); 73.6 (C(3′)); 73.9 (C(3)); 76.0 (C(5′)); 78.6 (C(5)); 79.4 (C(4)); 84.6 (C(1)); 101.6 (C(1′)); 174.6, 174.7 (2 *C*OMe).

GlcNAc(β1–4)GlcNAcβ-SCH$_2$CH$_2$CONHCH$_2$NHCOCH=CH$_2$ (**4c**). A mixture of **3c** (35 mg, 46 μmol) and β-galactosidase (*Streptococcus pneumoniae*) (39 mU) in 6.5 ml of 0.02M phosphate buffer (pH 6.0) was incubated for 12 h at 37°. The mixture was heated at 90° for 25 min to deactivate the enzyme and filtered. The filtrate was chromatographed by prep. HPLC (*Inertsil ODS-3*, H_2O/MeOH 900:70; flow rate 5.0 ml/min): **4c** (25 mg). ^{1}H-NMR (D_2O, 400 MHz): 1.91, 1.97 (2 COMe); 2.49–2.88 (SCH_2CH_2); 4.48 (*d*, *J*(1′,2′) = 8.4, H–C(1′)); 4.54 (*d*, *J*(1,2) = 10.2, H–C(1)); 4.56 (*s*, NCH_2N); 5.69–6.15 ($CH{=}CH_2$). ^{13}C-NMR (D_2O, 100 MHz): 22.3 (2 N=CO(*Me*)); 26.4, 36.0 (SCH_2CH_2); 44.4 (NCH_2N); 54.2 (C(2)); 55.7 (C(2′)); 60.5 (C(6)); 60.7 (C(6′)); 69.8 (C(4′)); 73.6 (C(3′)); 73.8 (C(3)); 76.1 (C(5′)); 78.5 (C(5)); 79.5 (C(4)); 84.7 (C(1)); 101.6 (C(1′)); 128.5 ($=CH_2$); 129.7 (CH=); 168.8 (N*C*OCH=); 174.5, 174.7, 174.8 (2 N*C*OMe, N*C*OCH$_2$). MALDI-TOF-MS: 617.3 ($[M+Na]^+$; calc. 617.7).

GlcNAc(β1–4)GlcNAc(β1–4)GlcNAcβ-SCH$_2$CH$_2$CONHCH$_2$NHCOCH=CH$_2$ (**6c**). As described for **3c**, with **5c** (16 mg, 16 μmol), phosphate buffer (2.0 ml), and β-galactosidase (21 mU): 11 mg (86%) of **6c**. ^{1}H-NMR (D_2O, 400 MHz): 1.93 (*s*, COMe); 1.99 (*s*, 2 COMe); 2.51–2.87 (SCH_2CH_2); 4.48–4.54 (H–C(1), H–C(1′), H–C(1″)); 4.58 (*s*, NCH_2N); 5.71–6.17 (*m*, $CH{=}CH_2$). ^{13}C-NMR (D_2O, 100 MHz): 22.3 (N=CO(*Me*)); 26.4, 36.0 (SCH_2CH_2); 44.5 (NCH_2N); 54.3 (C(2)); 55.2 (C(2′)); 55.7 (C(2″)); 60.1 (C(6′)); 60.5 (C(6)); 60.7 (C(6″)); 69.8 (C(4″)); 72.3 (C(3′)); 73.6 (C(3″)); 73.8 (C(3)); 74.7 (C(5′)); 76.1 (C(5″)); 78.6 (C(5)); 79.2 (C(4), C(4′)); 84.7 (C(1)); 101.4 (C(1′)); 101.6 (C(1′)); 128.4 ($=CH_2$); 129.7 (CH=); 168.8 (N*C*OCH=); 174.5–174.8 (3 N*C*OMe, N*C*OCH$_2$). MALDI-TOF-MS: 820.8 ($[M+Na]^+$; calc. 820.9).

GlcNAc(β1–4)Glc(β1–4)Glc-β-SCH$_2$CH$_2$CONHCH$_2$NHCOCH=CH$_2$ (**9**). As described for **3b**, with **8** (5 mg, 5 μmol), phosphate buffer (730 μl), and β-galactosidase (7 mU): 3 mg (63%) of **8**. ^{1}H-NMR (D_2O, 400 MHz): 1.86 (*s*, COMe); 2.58, 2.95 (*m*, SCH_2CH_2); 4.46–4.54 (H–C(1), H–C(1′), H–C(1″)); 4.62 (*s*, NCH_2N); 5.75–6.20 (*m*, $CH{=}CH_2$). ^{13}C-NMR (D_2O, 100 MHz): 22.2 (N=CO(*Me*)); 26.1, 36.2 (SCH_2CH_2); 44.5 (NCH_2N), 55.7 (C(2″)); 60.1–60.7 (C(6′) or C(6″)); 60.3 (C(6)); 79.2 (C(4′)); 85.6 (C(1)); 101.6 (C(1″)); 102.3 (C(1′)); 128.4 ($=CH_2$); 129.7 (CH=); 168.8 (NH*C*OCH=), 174.7, 174.9 (*C*OMe, *C*OCH$_2$). MALDI-TOF-MS: 739.1 ($[M+Na]^+$, calc. 738.7).

Production and Purification of Wild-Type Chitinases and Their Mutants. Wild-type chitinase A1 and mutant chitinases were produced in *E. coli* HB101 cells carrying pHT012 and their derivatives encoding corresponding mutant chitinases, respectively. *E. coli* HB101 cells carrying a plasmid were grown in 100 ml of LB medium

containing 100 μg/ml of ampicillin for 20–23 h at 30°. The cells were collected by centrifugation, and chitinases were extracted from the cells by a cold osmotic-shock procedure. $(NH_4)_2SO_4$ was added to the extract to achieve 40% saturation. The precipitate formed was dissolved in 5 mM phosphate buffer and lyophilized. Wild-type and mutant chitinases were purified by a chitin affinity column (2.5 × 40.0 cm) equilibrated with 20 mM phosphate buffer. After washing with 200–300 ml of 20 mM NaOAc buffer (pH 5.5), adsorbed proteins were eluted with 100 ml of 20 mM NaOAc buffer (pH 5.5). The fractions containing chitinase A1 or their mutants were collected and lyophilized to give 5–10 mg of proteins. Wild-type chitinases C1 and D1 were produced in a manner similar as described above with *E. coli* JM109 and HB101 carrying the recombinant plasmid that encodes wild-type chitinase C1 and D1, respectively. After precipitating with $(NH_4)_2SO_4$ at 60% saturation, the precipitated proteins were subjected to chromatography on a hydroxyapatite column (2.5 × 25.0 cm) equilibrated with 1 mM phosphate buffer (pH 6.0) as follows. Chitinase C1: after washing with 1 mM phosphate buffer, adsorbed protein containing chitinase C1 was eluted with a linear gradient (1–400 mM) of phosphate buffer (pH 6.0), giving rise to 1 mg of chitinase C1. Chitinase D1: proteins containing chitinase D1 were eluted with 1 mM phosphate buffer for separation from other proteins adsorbed on hydroxyapatite; each peak fraction containing chitinase activity was collected and lyophilized to give 0.5 mg of chitinase D1.

Site-Directed Mutagenesis for Construction of Plasmid Encoding Mutant Gene. The site-directed mutant D200N, D202N, E204Q, Y279F, D280N, or W433F was made according to the procedure described by *Kunkel et al.* [20]. Plasmids encoding D200N, D202N, E204Q, or D280N were constructed by previous methods [17][21]. The mutants, Y279F and W433F, were constructed by using the *Quick Change*™ site-directed mutagenesis kit (*Stratagene*, CA). The recombinant plasmid pHT012, which carries the open reading frame of chitinase A1, was used as a template DNA for PCR. The synthetic oligonucleotides used for site-directed mutagenesis were 5′-GGATTAACATTATGACATACGATTTTAACG-3′ (Y279 → F) and 5′-AGCGATGTTCTTCGAGCT-CAGCGGT-3′ (W433 → F). To ensure that the desired mutation was the only mutation in the sequence, the entire region of the inserted DNA was sequenced.

Determination of Chitinase Activity. The activity of chitinases was evaluated by means of the amount of 4-nitrophenol released as a result of enzymatic hydrolysis of 4-nitrophenyl *N,N′*-diacetylchitobioside. The relative activity of chitinase A1, C1, and D1 was estimated to be 0.11, 3.93, and 5.11 U/mg, respectively. One unit of activity is defined as the amount of enzyme liberating 1 μmol of 4-nitrophenol per min.

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REFERENCES

[1] 'Carbohydrates in Chemistry and Biology', Eds. B. Ernst, G. W. Hart, and P. Sinaÿ, Wiley-VCH, Weinheim, 2000.
[2] 'Modern Methods in Carbohydrate Synthesis', Eds. S. H. Khan and R. A. O'Neill, Harwood Academic Publishers, Amsterdam, 1996.
[3] S. Shoda, in 'Glycoscience', Eds. B. O. Fraser-Reid, K. Tatsuta, and J. Thiem, Springer, Heidelberg, 2001.
[4] S. Kobayashi, T. Kiyosada, S. Shoda, *J. Am. Chem. Soc.* **1996**, *118*, 13113; S. Kobayashi, T. Kiyosada, S. Shoda, *Tetrahedron Lett.* **1997**, *38*, 2111.
[5] F. Micheel, H. Köchling, *Chem. Ber.* **1958**, *91*, 673.
[6] A. F. Bochkov, G. E. Zaikov, in 'Chemistry of the *O*-Glycosidic Bond', Ed. C. Schuerch, Pergamon Press, 1979, Chap. 2,p. 48;J. Kadokawa, S. Kasai, Y. Watanabe, M. Karasu, H. Tagaya, K. Chiba, *Macromolecules* **1997**, *30*, 8212; J. Kadokawa, M. Sato, M. Karasu, H. Tagaya, K. Chiba, *Angew. Chem., Int. Ed.* **1998**, *37*, 2373.
[7] a) A. C. Terwisscha van Scheltinga, S. Armand, K. H. Kalk, A. Isogai, B. Henrissat, B. W. Dijkstra, *Biochemistry* **1995**, *34*, 15619; b) A. C. Terwisscha van Scheltinga, M. Hennig, B. W. Dijkstra, *J. Mol. Biol.* **1996**, *262*, 243; c) I. Tews, A. C. Terwisscha van Scheltinga, A. Perrakis, K. S. Wilson, B. W. Dijkstra, *J. Am. Chem. Soc.* **1997**, *119*, 7954; d) K. A. Brameld, W. D. Shrader, B. Imperiali, W. A. Goddard III, *J. Mol. Biol.* **1998**, *280*, 913; e) K. A. Brameld, W. A. Goddard III, *J. Am. Chem. Soc.* **1998**, *120*, 3571.
[8] N. C. Price, L. Stevens, 'Fundamentals of Enzymology', Oxford Science Publications, New York, 1989, p. 187.
[9] S. Nakabayashi, C. D. Warren, R. W. Jeanloz, *Carbohydr. Res.* **1986**, *150*, C7.
[10] B. Henrissat, *Biochem. J.* **1991**, *280*, 309; B. Henrissat, A. Bairoch, *Biochem. J.* **1993**, *293*, 781; G. Davies, B. Henrissat, *Structure* **1995**, *3*, 853.

[11] S. Knapp, D. Vocadlo, A. Gao, B. Kirk, J. Lou, S. G. Withers, *J. Am. Chem. Soc.* **1996**, *118*, 6804.
[12] T. Watanabe, K. Suzuki, W. Oyanagi, K. Ohnishi, H. Tanaka, *J. Biol. Chem*. **1990**, *265*, 15659.
[13] R. Roy, *Trends Glycosci. Glycotechnol*. **1996**, *8*, 79; S. Lei, *Trends Glycosci. Glycotechnol*. **2000**, *12*, 229.
[14] J. Ebel, A. Mithofer, *Planta* **1998**, *206*, 335; A. Yamada, N. Shibuya, O. Kodama, T. Akatsuka, *Biosci. Biotech. Biochem*. **1993**, *57*, 405; N. Shibuya, H. Kaku, K. Kuchitsu, M. J. Maliarik, *FEBS Lett*. **1993**, *329*, 75.
[15] S. Raghavan, D. Kahne, *J. Am. Chem. Soc*. **1993**, *115*, 1580.
[16] T. Matsumoto, T. Nonaka, M. Hashimoto, T. Watanabe, Y. Mitsui, *Proc. Japan Acad. Ser. B* **1999**, *75*, 269.
[17] T. Watanabe, K. Kobori, K. Miyashita, T. Fujii, H. Sakai, M. Uchida, H. Tanaka, *J. Biol. Chem*. **1993**, *268*, 18567.
[18] T. Usui, S. Kubota, H. Ohi, *Carbohydr. Res.* **1983**, *244*, 315.
[19] M. C. Davies, *Langmuir* **1993**, *9*, 1637; R. T. Lee, S. Cascio, Y. C. Lee, *Anal. Biochem*. **1979**, *95*, 260.
[20] T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **1987**, *154*, 367.
[21] T. Watanabe, M. Uchida, K. Kobori, H. Tanaka, *Biosci. Biotech. Biochem.* **1994**, *58*, 2283; M. Hashimoto, Y. Honda, N. Nikaidou, T. Fukamizo, T. Watanabe, *J. Biosci. Bioeng.* **2000**, *89*, 100.

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