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CHITINASE-CATALYZED SYNTHESIS OF OLIGOSACCHARIDES BY USING A SUGAR OXAZOLINE AS GLYCOSYL DONOR

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Abstract - The regio- and stereoselective addition reaction of various methyl glycosides having an *N*-acetylglucosamine unit to the oxazoline derivative of *N*-acetyllactosamine has been achieved by using a chitinase as catalyst, giving rise to the corresponding oligosaccharides.

The enzymatic glycosylation is a topic of current research.[1] So far there have been several successful approaches to enzymatic synthesis of poly- and oligosaccharides *in vitro*. Glycosyltransferases, enzymes which catalyze glycosylations in natural systems,[2] or glycosidases which are responsible for the cleavage of glycosidic bonds[3] have been used for the synthesis of poly- and oligosaccharides.

Recently, the *in vitro* synthesis of chitin has been achieved by an enzymatic polyaddition of a chitobiose oxazoline monomer.[4] Nucleophilic attack of the 4'-OH group of the glycosyl acceptor molecules at the anomeric center of the glycosyl donor molecule leads to the selective formation of a β-glycosidic bond. It was also demonstrated that an oxazoline derivative of *N*-acetylglucosamine can be used as a substrate of chitinase for the preparation of chitobiose.[5]

In this paper, the chitinase-catalyzed addition reaction was successfully applied to the synthesis of oligosaccharides (**3**) by using an oxazoline derivative of *N*-acetyllactosamine (**1**) as glycosyl donor and various methyl glycosides (ROH) (**2**) as glycosyl acceptors. The axial OH group at the 4'-position of **1** cannot attack the anomeric center, thus polyaddition is prevented. Yet, *N*-acetyllactosamine moiety is recognized by chitinase, therefore the sugar oxazoline derivative (**1**) can act as a glycosyl donor.

First, the specificity of the substrate (**1**) for chitinase (*Bacillus* sp.) was tested. The concentration of **1** was followed by means of ^{1}H NMR spectroscopy in deuterium oxide. When the solution was kept at 20 °C, a slight decrease of the substrate concentration was observed, indicating that a very slow hydrolysis of **1** (carbon-oxygen bond cleavage to produce *N*-acetyllactosamine) occurred even in the absence of chitinase.

Dedicated to Professor Teruaki Mukaiyama on the occasion of his 73rd birthday.

The addition of chitinase caused a rapid hydrolysis within 20 hours, giving rise to *N*-acetyllactosamine. These results clearly indicate that **1** can be recognized by the catalytic site of chitinase and the oxazoline ring is activated for hydrolysis. Other hydrolases such as lysozyme and cellulase were found inactive to **1**.

When the oxazoline (**1**) was reacted with a glycosyl donor (ROH) (**2**) in the presence of a catalytic amount of chitinase in citric buffer (pH 9.0), the corresponding *N*-acetyllactosaminated product (**3**) was obtained in good yields (Table 1)[6].

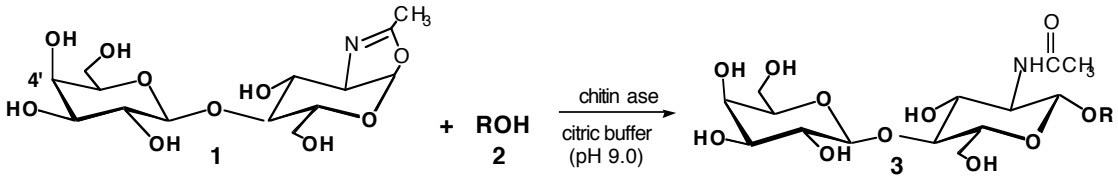

Table 1 Enzymatic addition of various glycosyl acceptors to **1**[a]

entry	acceptor (**2**)	enzyme (wt%)	pH	time (h)	yield[b] (%) of **3**
1	GlcNAc-β-OMe	10	9.0	0.5	55
2	GlcNAc-α-OMe	10	9.0	—	0
3	GlcNAc-β-OEt	10	8.8	0.5	64
4	GlcNAc-β-OPr	5	9.0	2.0	47
5	GlcNAc-β-O*i*-Pr	5	9.0	2.0	41
6	Glc-β-OMe	5	9.0	—	0
7	Glc-GlcNAc- β-OMe	5	9.0	2.0	78

[a][**1**] = 0.1M, [acceptor] = 0.3 M, temp = 30 °C. [b]Determined by HPLC.

Fairly good yields are achieved with methyl *N*-acetyl-β-D-glucosaminide (GlcNAc-β-OMe), ethyl *N*-acetyl-β-D-glucosaminide (GlcNAc-β-OEt), propyl *N*-acetyl-β-D-glucosaminide (GlcNAc-β-OPr), and *i*-propyl *N*-acetyl-β-D-glucosaminide (GlcNAc-β-O*i*-Pr) (entries 1,3-5). A disaccharide acceptor, methyl 2-acetamido-2-deoxy- 4-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside, was also glycosylated in good yield (entry 7). Methyl β-D-glucopyranoside could not be glycosylated (entry 6). Interestingly, the orientation of the anomeric methoxy group of the acceptor was found crucial for the reaction to proceed. Methyl *N*-acetyl-β-D-glucosaminide (GlcNAc-β-OMe) gives the product (**3**) whereas the corresponding α-anomer did not react at all (entry 2). This fact gives us some information about the orientation of the substrates as well as the interaction with the amino acid residues in the active site. The axial group at the 1-position of the glycosyl acceptor may point toward the enzyme and thus prevents binding due to steric repulsion. The result for Glc-β-OMe (entry 6) indicates that the glucoside moiety has almost no affinity to the acceptor site of the enzyme compared with GlcNAc-OR moiety or Glc-GlcNAc-OMe moiety.

The stereochemistry of the newly formed glycosidic bonds was determined as β(1→4) by ^{1}H and ^{13}C NMR spectroscopy. In all cases the ^{1}H NMR analysis showed signals for the anomeric proton of *N*-

acetyllactosamine unit at around 4.5 ppm with J values of 6-8 Hz. The ^{13}C NMR spectra of the products indicated signals due to the anomeric carbon atom of *N*-acetyllactosamine unit ($C_{1'}$) and C_4 of the reducing unit of **3** at around 102 and 80 ppm, respectively. These values are characteristic of β(1→4) glycosidic bond.[7] No signal from the methylene carbon C_6 (δ 69 ppm) or from the methyne carbon C_3 (δ 85 ppm) adjacent to a β(1→6)[8] or a β(1→3)[9] glycosidic bond were observed in the product, indicating that the β(1→4) glycosidic linkage was formed in a regio- and stereoselective manner.

The yield of the resulting oligosaccharides did not decrease at longer reaction times, showing the irreversible behavior of chitinase under a high pH value of *ca*. 9.0. The addition reaction of **1** by chitinase was efficiently catalyzed at this pH whereas the hydrolysis of the product oligosaccharide could not be observed, since chitinase shows an optimal hydrolysis at pH 7.8.[10] Actually, when the reaction was carried out under more acidic conditions like pH 7.5 and pH 8.3, the hydrolysis of the glycosidic bond between the glycosyl donor and the glycosyl acceptor occurred, which caused the decrease of the product yields. It is to be noted that the usage of the transition state analogue substrate (**1**) allows the reaction to proceed only in the direction of addition reaction while suppressing hydrolysis of the product in aqueous media.[4]

Chitinase, the enzyme responsible for the hydrolysis of β(1→4) *N*-acetylglucosaminide moiety in chitin, proved to be useful for the enzymatic addition reaction of an oxazoline derivative of *N*-acetyllactosamine. The present methodology which utilizes a sugar oxazoline derivative as a glycosyl donor paves a way for development of enzymatic glycosylation of 2-amino-2-deoxysugars.

EXTERIMENTAL

General notes:

All organic solvents were distilled from the appropriate drying reagents before use. NMR spectral data were obtained from a Bruker AC-250T spectrometer. For analytical and semipreparative HPLC a Hitachi 655A liquid chromatograph, equipped with a Merck Lichrosorb RP-18 (7 μm) column, a Hitachi 655A-30 RI detector was used (water eluent). MALDI-TOF mass spectra were obtained on a Bruker Protein TOF spectrometer (2,5-dihydroxybenzoic acid matrix). The starting material for the glycosyl donor (**1**), *N*-acetyllactosamine heptaacetate, was prepared from lactose *via* seven steps according to the literature.[11] The resulting heptaacetate was converted to **1** by using trimethylsilyl trifluoromethanesulfonate[12] followed by methanolysis by sodium methoxide in methanol.[13] Chitinase (*Bacillus* sp., EC3.2.1.14, 0.04 U/mg) was obtained from Wako Chemicals (Japan).

Enzymatic lactosamination of glycosyl acceptors

In a typical run, the glycosyl donor (**1**) (48 mg, 0.13 mmol) and methyl *N*-acetyl-β-D-glucosaminide (GlcNAc-β-OMe) (92 mg, 0.39 mmol) were dissolved in 150 μL of citric buffer (0.05 M, pH 9.0). To

this mixture chitinase (*Bacillus* sp., 10 wt% for **1**) dissolved in 80 μL citric buffer (0.01 M, pH 9.0) was added and the reaction mixture was stirred for 0.5 h at 30 °C. Yields were determined by taking aliquots of the sample and evaporation of the solvent prior to HPLC analysis. For final work-up the reaction mixture was treated with tetrahydrofuran in order to inactivate the enzyme, prior to semipreparative HPLC to obtain samples for NMR spectral analysis. The HPLC fraction containing the product was found to be chromatographically homogeneous.[14]

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6. MALDI-TOF mass spectra of the products: entry 1, 601.3 (calcd 600.6); entry 3, 615.9 (calcd 614.6; entry 4, 628.3 (calcd 628.6); entry 5, 627.9 (calcd 628.6); entry 7, 763.8 (calcd 762.7).

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13. NMR spectral data of **1** are as follows. ^{1}H NMR (D_2O): 6.09 (d, 1H, $J_{1,2}$ = 7.35 Hz, H_1), 4.42 (d, 1H, $J_{1',2'}$ = 8.00 Hz, $H_{1'}$), 2.06 (s, 3H, methyl). ^{13}C NMR (D_2O): 169.0 (N=*C*(Me)O), 105.1 ($C_{1'}$), 100.3 (C_1), 13.9 (N=C(*Me*)O).

14. NMR spectra of the product trisaccharide (entry 1) are as follows. ^{1}H NMR (D_2O): 4.58 (d, 1H, $J_{1',2'}$ = 7.48 Hz, $H_{1'}$), 4.44 (d, 1H, $J_{1'',2''}$ = 8.14 Hz, $H_{1''}$), 4.41 (d, 1H, $J_{1,2}$ = 7.85 Hz, H_1), 3.49 (s, 3H, OCH_3). ^{13}C NMR (D_2O): 103.7 ($C_{1''}$), 102.7 (C_1), 102.2 ($C_{1'}$), 80.2 (C_4), 58.0 (O*C*H$_3$).