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Chemo- and Enzymatic Synthesis of Partially and Fully *N*-Deacetylated 4-Methylumbelliferyl Chitobiosides: Fluorogenic Substrates for Chitinase

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Abstract—Partially and fully *N*-deacetylated 4-methylumberlliferyl chitobioside (**1**) derivatives, such as GlcN-GlcNAc-UMB (**2**), GlcNAc-GlcN-UMB (**3**), and $(GlcN)_2$-UMB (**4**), were synthesized using chemo- and enzymatic procedure. Fluorescent aglycon was released from the chitobiosides **1**, **2** and **3** by the action of chitinase. These UMB glycosides of heterochitobiose were versatile probes for the investigation of substrate binding chitinase from various sources.

Chitin is the most abundant linear polymer of GlcNAc (2-acetamido-2-deoxy-D-glucopyranose), and the polymer is found in invertebrates such as crustaceans, insects, spiders, and the cell walls of most fungi and many algae.[1] For degradation of the biopolymer, chitinases [EC 3.2.1.14] are produced from many bacteria. In nature, a partially *N*-deacetylated chitosan (DAC), which is made of GlcNAc residues randomly localized in the chitosan chain, is produced from chitin by chitin deacetylase.[2] Although the DAC is a soluble substrate for chitosanases, the bacterial chitinases can also hydrolyze the β-1,4-glycosidic linkage between GlcNAc and GlcN (2-amino-2-deoxy-D-glucopyranose) of the DAC.[3–5] In this situation, we have been interested in the recognition of DAC on GlcNAc by the chitinase. The substrate specificity of chitinase against DAC has been investigated by the determination of enzymatic products using exo-type glycosidases (β-*N*-acetylhexosaminidase and exo-β-D-glucosaminidase) or NMR spectroscopy.[3–5] These methods, however, are found to be complicated procedure, because it is difficult to separate and analyze the hetero-DAC oligomers from the enzymatic product mixture. In addition, the quantitative chitinase activity using DAC as a substrate has been evaluated by the reducing sugar method.[6] The reducing method, however, cannot differentiate the cleavage bond between GlcN-GlcNAc and GlcNAc-GlcN of DAC. Therefore, it is highly desired to establish well-characterized DAC substrates for kinetic analysis of the chitinases.

On the other hand, 4-methylumberlliferyl (UMB) glycosides have been widely used for the kinetic analysis of carbohydrolases, because the released 4-methylumbelliferone strongly fluoresced in ultraviolet light at pH 9—10.[7] These results prompted us to design and synthesize the UMB-glycosides of partially and fully deacetylated DAC oligomers, such as GlcN-GlcNAc-UMB (**2**), GlcNAc-GlcN-UMB (**3**), and $(GlcN)_2$-UMB (**4**), as versatile substrates for the kinetic analysis of the chitinases. In this report, we describe the synthesis of the UMB glycosides of chitobiose by the selective *N*-deacetylation using chemical and enzymatic procedures, and also the effect of the *N*-deacetylation of **1** on the activity of *Bacillus circulans* WL12 chitinase A1.

The starting compound, hexa-*O*-acetylchitobiose (**5**), which was prepared from chitin, was converted to $(GlcNAc)_2$-UMB (**1**) by the method described in the literature.[8,9] However, it was quite difficult to selectively eliminate the C2′ *N*-acetyl groups of **1** by a chemical method without affecting the UMB glycosidic bond. Thus, we have employed chitin deacetylase from *Colletotrichum lindemuthianum* to produce GlcN-GlcNAc-UMB (**2**) as shown in Scheme 1.[10]

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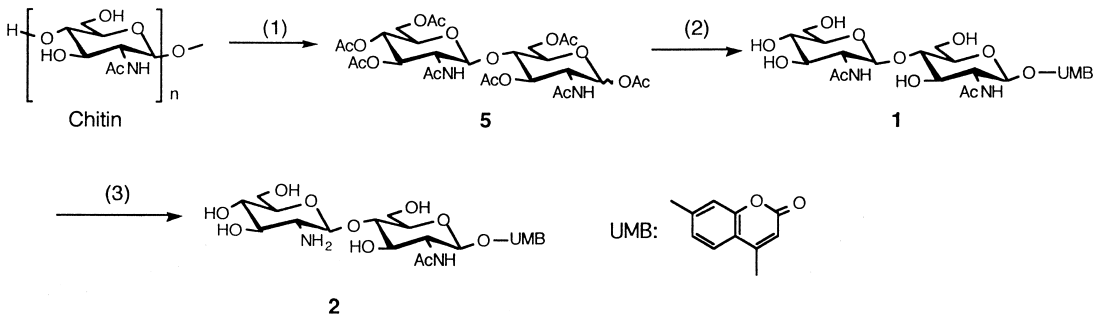
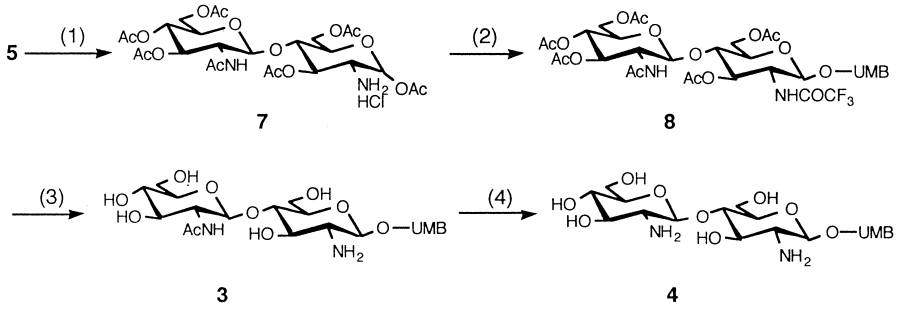


Scheme 1. (1) Concd. H_2SO_4/Ac_2O; (2) refs 8 and 9; (3) chitin deacetylase (ref 10).



Scheme 2. (1) (a) HCl/AcOH, quant., (b) H_2O/acetone, 73%; (2) (a) $(CF_3CO)_2O$/acetone, 70%, (b) HCl/AcOH, quant.; (c) UMBONa/DMF, 44%; (3) NH_3/MeOH, 80 h, 61%; (4) chitin deacetylase (ref 10).

The second stage of our synthesis was the transformation of **5** into GlcNAc-GlcN-UMB (**3**), and $(GlcN)_2$-UMB (**4**) as shown in Scheme 2. The treatment of **5** with hydrogen chloride in AcOH gave the chloride, which then underwent an acyl transfer reaction to give **7** in acetone containing 1 equiv water by gently refluxing according to the reported method.[11] The amine hydrochloride **7** was converted to UMB glycoside (**8**) by the following sequence: protection of **7** with the trifluoroacetyl group which could be removed under mild conditions, and subsequent chlorination and condensation afforded **8** using common methods.[8,9] The deprotection of the *O*-acetyl and *N*-trifluoroacetyl groups in **8** was effected by ammonolysis in NH_3/MeOH at rt to furnish GlcNAc-GlcN-UMB (**3**).[12] Finally, the enzymatic deacetylation of **3** was carried out using the chitin deacetylase described for **2**, yielding $(GlcN)_2$-UMB (**4**).[10] The ^{1}H NMR and MALDI-TOF MS analyses for each target compound, **2**, **3** and **4** were in full accord with assigned structures.[10,11]

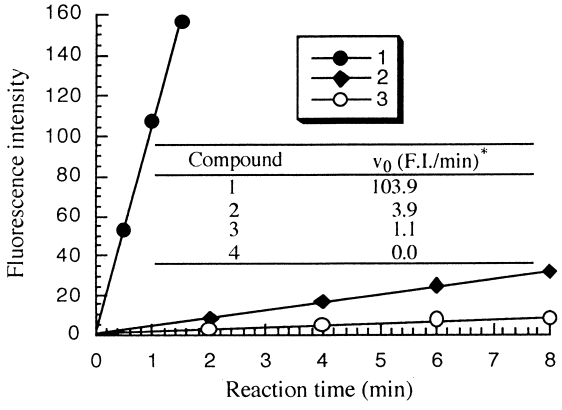


Figure 1. Time courses of the increase in fluorescence intensity from $(GlcNAc)_2$-UMB derivatives hydrolyzed by chitinase A1 from *Bacillus circulans* WL 12. *F.I. indicated fluorescence intensity at 450 nm with excitation at 360 nm. Substrate concentrations are 36.0 μM. Enzyme concentration is 3.1 nM. The other experimental conditions were adjusted as previously reported.[14]

Each of **1**–**4** was mixed with chitinase A1 from *Bacillus circulans* WL 12, family 18, and free UMB was measured by the increased fluorescence intensity at 450 nm with excitation at 360 nm. In the case of **4**, no UMB release was observed despite a long incubation time. As shown in Figure 1, the release of free UMB from partially *N*-deacetylated glycosides (**2** and **3**) decreased by 3.8 and 1.0 % of **1**, respectively. Although Mitsutomi et al.[5] reported that the chitinase hydrolyzes the glycosidic bond between GlcNAc and GlcN in the DAC, the chitinase hydrolyze the glycosidic linkage between GlcNAc-GlcN and the UMB moiety of **3**. Furthermore, a 'substrate assisted catalysis' mechanism for the bacterial chitinase has been postulated, that is, the hydrolytic reaction takes place by the acetamido group participation at the −1 subsite because the chitinase has only one catalytic residue as a proton donor.[6,13] Thus, the significant increase in fluorescence intensity from compound **3** is worth noting from the viewpoint of the catalytic mechanism. In order to obtain further information on the reaction mechanism, a kinetic analysis of the chitinase using these UMB derivatives is now in progress in our laboratory.

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