Enzymatic Synthesis of 3-*O*-Methylated Chitin Oligomers from New Derivatives of a Chitobiose Oxazoline

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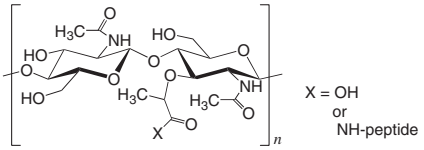
Regiospecifically 3-*O*- and/or 3′-*O*-methylated derivatives of a chitobiose oxazoline have been synthesized as new substrate monomers and subjected to a chitinase catalysis, leading to the first synthesis of 3-*O*-methylated chitin oligomers via enzymatic oligomerization.

Chitin, a $\beta(1 \rightarrow 4)$-linked polymer of *N*-acetyl-D-glucosaminopyranose, is utilized as structural materials in nature for animals such as invertebrates. Chitin usually exists in the form of a stable crystal lacking solubility in ordinary solvents,[1,2] while its oligomers are water-soluble and attracting a big interest in their biological activities.[3] To develop new functional materials of chitin, chemical modification to natural chitin has been extensively studied, normally under heterogeneous conditions. Although difficulties remain in perfect control of the regioselectivity as well as of the substitution degree,[4] various 6-*O*-substituted chitin derivatives were thus prepared.[2]

3-*O*-Substitution of chitin is of great interest in view of disturbing an intramolecular hydrogen-bonding for maintaining chain linearity of chitin,[1] which is responsible often for the physical, chemical, and biological properties. It is to be noted that a bacterial cell-wall peptidoglycan is well known as an alternatingly 3-*O*-modified derivative of chitin and also as a natural substrate of lysozyme enzymes (Scheme 1).[5] Regiospecific modification of 3-OH position of natural chitin is, however, very difficult because of the lower reactivity.

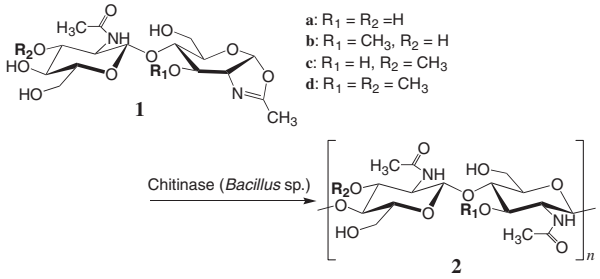


Scheme 1.

On the other hand, in vitro synthesis of chitin (**2a**) via enzymatic polymerization of a chitobiose oxazoline (**1a**) has been recently accomplished (Scheme 2).[6,7] This polymerization proceeds perfectly in a regio- and stereoselective manner to form $\beta(1 \rightarrow 4)$ glycosidic linkages of chitin, which opened a new possible way to prepare well-defined derivatives of chitin.

In the present study, regiospecifically 3-*O*-methylated (**1b**), 3′-*O*-methylated (**1c**), and 3,3′-di-*O*-methylated (**1d**) derivatives of the chitobiose oxazoline have been designed as new candidate monomers for the enzymatic polymerization, and their behaviors with a chitinase enzyme have been investigated, aiming at a novel synthesis of 3-*O*-substituted chitin derivatives.

Compounds **1b**–**1d** have been synthesized from D-glucosamine hydrochloride[8] and identified by NMR analysis.[9] These compounds were subjected to a chitinase catalysis as follows; the compound was dissolved in 40-mM carbonate buffer (pH 9.0) to adjust the concentration as 200 mM. The enzymatic reaction was started by adding chitinase (*Bacillus* sp., Wako, EC 3.2.1.14, 0.04 U/mg) to the solution with an amount of 10 wt % toward the compound. The reaction solution was incubated at 30 °C. The conversion and the products were analyzed by HPLC. As a result, compounds **1b**–**1d** have been disclosed as new substrates for the chitinase; they were recognized by the enzyme, thus undergoing both enzymatic oligomerization and hydrolysis at the oxazoline moieties to yield water-soluble oligosaccharides and the ring-opened hydrolysis products (G2). Water-insoluble products due to higher degree of polymerization (DP) were not produced. As shown in Table 1, significant difference was found among the reactions of **1b**–**1d** in distribution of the products and in reaction rate.



Scheme 2.

The oligosaccharide products were formed most efficiently from **1b**: HPLC analysis of the reaction solution indicated the yield of the oligomerization products over tetrasaccharide (DP > 2). The selective formation of $\beta(1 \rightarrow 4)$-glycosidic linkages was confirmed by NMR analysis. It is characteristic to **1b** that considerable amount of odd-numbered oligosaccharide products was also produced. This indicates that hydrolysis at the $\beta(1 \rightarrow 4)$-glycosidic linkages of the oligomerization products also occurred concurrently. To lower the hydrolysis activity, the enzymatic reaction of **1b** at pH 10.0 was attempted;[7b] however, the oligomerization was also decelerated pari passu with the hydrolysis.

With respects to the reaction rate, significant difference was found between **1c** and the others (**1b** and **1d**). Despite the much higher reactivity of **1c**, its oligomerizability was lower than that of **1b**: the hydrolysis at the oxazoline moiety of **1c** to give the ring-opened product of G2 was catalyzed more efficiently than the oligomerization. In the case of **1d**, the ring-opening hydrolysis proceeded predominantly.

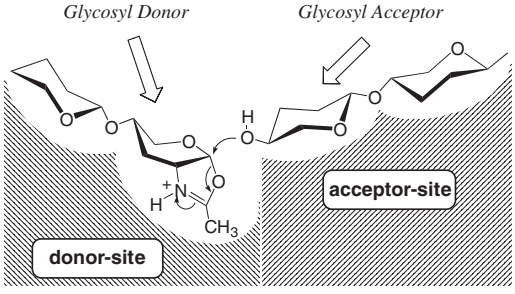
The enzymatic oligomerization as well as polymerization is brought about by repetition of the glycosyl coupling between a glycosyl donor (GD) and a glycosyl acceptor (GA) at the active center of enzyme. In the present study, the oxazoline moiety of GD is activated to open the ring by nucleophilic addition of

Table 1. Water-soluble products from the new oxazoline substrates[a]

Substrate	Time /h	Conv.[b] /%	Yield of **2**[c] /%						
			G2	G3	G4	G5	G6	G7	G8···
1b	14.0	100	67	10	12	4	2	2	1
1b (ctrl)	14.0	8	8	0	0	0	0	0	0
1c	2.5	100	79	tr	18	0	2	0	0
1c (ctrl)	2.5	2	2	0	0	0	0	0	0
1d	14.0	96	93	tr	2	0	0	0	0
1d (ctrl)	14.0	9	9	0	0	0	0	0	0

[a]"conv.", "ctrl", and "tr" are abbreviations of conversion, a control experiment without enzyme, and trace amount (<0.5), respectively. G2–G8 are symbols of the di- to the octa-saccharide products. [b]Determined by HPLC on Chemcobond ODS–W column with water–acetonitrile (100:3) eluent and RI detector. [c]Determined by HPLC on Shodex sugar KS-802 column with water eluent and RI detector. For each enzymatic reaction, the yield of monosaccharide was detected as tr.

4-OH group of GA, which forms the $\beta(1 \rightarrow 4)$-glycosidic linkage (Scheme 3).[7] The ring-opening hydrolysis is a case where water molecule reacts with GD in competition to GA.



Scheme 3.

As for the previous polymerization of **1a**, it is considered that the uptake of GD and GA to the corresponding binding sites of the enzyme are well balanced in order to induce the successive glycosylations to yield chitin efficiently.[10] In the present reactions, the 3-*O*-methylation and 3′-*O*-methylation of **1a** would have reduced the uptake of GD at the donor-site and that of GA at the acceptor-site, respectively. Namely, the slow consumption of **1b** and the competitively occurring hydrolysis of the oligomerization products can be explained by the reduced uptake of **1b** at the donor-site, while the enzymatic ring-opening hydrolysis of **1c** occurred owing to less uptake of GA at the acceptor-site. The reaction result of **1d** revealed a reflection of the both contributions. This suggests that easier uptake of the substituted substrates is required for both the donor- and acceptor-sites of the enzyme to realize more efficient glycosylation; for instance, an enzyme having higher lysozyme activity[5,11] is expected as a promising catalyst for the enzymatic polymerization to produce alternatingly 3-*O*-modified derivatives of chitin.

To conclude, regiospecifically 3-*O*- and/or 3′-*O*-methylated derivatives of a chitobiose oxazoline have been designed for the first synthesis of 3-*O*-methylated chitin oligomers via the enzymatic oligomerization. Selection of an appropriate enzyme, however, remains as the next important strategy to achieve the enzymatic polymerization efficiently. Further investigations are under progress.

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8 Brief description on the synthetic procedure for **1b** is as follows: 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-D-glucopyranosyl trichloroacetimidate and *p*-methoxyphenyl 6-*O*-acetyl-2-deoxy-3-*O*-methyl-2-phthalimido-β-D-glucopyranoside were coupled to the corresponding disaccharide, followed by dephthaloylation, *N*-acetylation, oxidative removal of *p*-methoxyphenyl group, 1-*O*-acetylation, oxazoline formation with trimethylsilyl trifluoromethanesulfonate, and *O*-deacetylation to **1b**.
9 ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of **1b–1d** in D_2O solution are as follows: **1b** δ_H 5.91 (d, 1H, H-1), 4.50 (d, 1H, H-1′), 4.22 (ddd, 1H, H-2), 4.00 (dd, 1H, H-3), 3.79 (dd, 1H, H-6′a), 3.69 (dd, 1H, H-6′b), 3.64–3.49 (m, 2H, H-4 and H-2′), 3.48–3.26 (m, 5H, H-6a, H-6b, H-3′, H-4′, and H-5′), 3.39 (s, 3H, OCH_3-3), 3.14 (ddd, 1H, H-5), 1.95 (s, 3H, N=C(C$\underline{H}_3$)O), 1.92 (s, 3H, NHC(=O)C$\underline{H}_3$); $J_{1,2}$ = 7.5 Hz, $J_{1',2'}$ = 8.5 Hz; δ_C 174.7 (NH$\underline{C}$(=O)CH_3), 168.9 (N=$\underline{C}$(CH_3)O), 103.6 (C-1′), 99.8 (C-1), 78.1 (C-3), 76.4 (C-4), 76.0 (C-5′), 73.8 (C-3′), 70.9 (C-5), 69.7 (C-4′), 63.1 (C-2), 62.1 (C-6), 60.5 (C-6′), 57.5 (OCH_3-3), 56.0 (C-2′), 22.3 (NHC(=O)$\underline{C}H_3$), 13.1 (N=C($\underline{C}H_3$)O). **1c** δ_H 5.95 (d, 1H, H-1), 4.45 (d, 1H, H-1′), 4.29 (dd, 1H, H-3), 4.07 (ddd, 1H, H-2), 3.80 (dd, 1H, H-6′a), 3.69–3.59 (m, 2H, H-6′b, and H-2′), 3.57 (dd, 1H, H-6a), 3.53–3.31 (m, 4H, H-4, H-6b, H-4′, and H-5′), 3.39 (s, 3H, OCH_3-3′), 3.24 (dd, 1H, H-3′), 3.17 (ddd, 1H, H-5), 1.93 (s, 3H, N=C(C$\underline{H}_3$)O), 1.92 (s, 3H, NHC(=O)C$\underline{H}_3$); $J_{1,2}$ = 7.5 Hz, $J_{1',2'}$ = 8.5 Hz; δ_C 174.5 (NH$\underline{C}$(=O)CH_3), 168.6 (N=$\underline{C}$(CH_3)O), 103.1 (C-1′), 100.0 (C-1), 83.0 (C-3′), 79.1 (C-4), 76.0 (C-5′), 71.1 (C-5), 68.9 (C-4′), 68.8 (C-3), 65.4 (C-2), 62.1 (C-6), 60.8 (C-6′), 59.3 (OCH_3-3′), 54.6 (C-2′), 22.3 (NHC(=O)$\underline{C}H_3$), 13.1 (N=C($\underline{C}H_3$)O). **1d** δ_H 5.91 (d, 1H, H-1), 4.51 (d, 1H, H-1′), 4.21 (ddd, 1H, H-2), 4.00 (dd, 1H, H-3), 3.78 (dd, 1H, H-6′a), 3.73–3.54 (m, 4H, H-6′b, H-2′, H-4, and H-6a), 3.53–3.31 (m, 3H, H-4′, H-6b, and H-5′), 3.40 (s, 3H, OCH_3-3′), 3.39 (s, 3H, OCH_3-3), 3.26 (dd, 1H, H-3′), 3.14 (ddd, 1H, H-5), 1.94 (s, 3H, N=C(C$\underline{H}_3$)O), 1.92 (s, 3H, NHC(=O)C$\underline{H}_3$); $J_{1,2}$ = 7.5 Hz, $J_{1',2'}$ = 8.5 Hz; δ_C 174.5 (NH$\underline{C}$(=O)CH_3), 168.9 (N=$\underline{C}$(CH_3)O), 103.5 (C-1′), 99.8 (C-1), 83.0 (C-3′), 78.1 (C-3), 76.4 (C-4), 75.8 (C-5′), 70.9 (C-5), 68.6 (C-4′), 63.1 (C-2), 62.1 (C-6) 60.4 (C-6′), 59.3 (OCH_3-3′), 57.5 (OCH_3-3), 54.6 (C-2′), 22.3 (NHC(=O)$\underline{C}H_3$), 13.1 (N=C($\underline{C}H_3$)O).
10 For comparison, enzymatic reaction of **1a** was examined under the similar condition with those of **1b–1d**, which completed in 2.5 h giving **2a** with the following distribution; 63% of water-insoluble products (synthetic chitin), 1% of chitohexaose, 3% of chitotetraose, 1% of chitotriose, and 32% of chitobiose.
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