Bioorganic & Medicinal Chemistry Letters 20 (2010) 3588–3591

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

4,6-Dimethoxy-1,3,5-triazine oligoxyloglucans: Novel one-step preparable substrates for studying action of endo-β-1,4-glucanase III from *Trichoderma reesei*

Atsushi Kobayashi [a], Tomonari Tanaka [a], Kazuhito Watanabe [a], Masaki Ishihara [a], Masato Noguchi [a], Hirofumi Okada [b], Yasushi Morikawa [b], Shin-ichiro Shoda [a,*]

[a] *Graduate School of Engineering, Tohoku University, 6-6-11-514 Aoba, Sendai 980-8579, Japan*
[b] *Department of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan*

ARTICLE INFO

Article history:
Received 10 February 2010
Revised 23 April 2010
Accepted 27 April 2010
Available online 22 May 2010

Keywords:
Oligosaccharide
Glycosidase
Substrate
DMT-MM

ABSTRACT

Two kinds of 4,6-dimethoxy-1,3,5-triazine (DMT) oligoxyloglucans, DMT-β-XXXG and DMT-β-XLLG, have been synthesized via one-step procedure starting from the corresponding unprotected oligoxyloglucans in water. The resulting DMT derivatives were found to be hydrolyzed by endo-β-1,4-D-glucanase III from *Trichoderma reesei* (EGIII) and utilized as substrates for determination of the kinetic parameters of EGIII. The present DMT-method would be a convenient analytical tool for studying the action of glycosyl hydrolases due to the extremely simple synthetic process of DMT-glycosides without using protecting groups.

The determination of kinetic parameters for endo-glycanases, a hydrolytic enzyme of polysaccharides, is extremely important for evaluating their hydrolysis patterns in the field of food science and woody biomass utilization. There is a drawback to employ naturally occurring polysaccharides as substrates for studying the action of endo-type glycanases that the structure and the molecular weight of these substrates change during an enzymatic hydrolysis reaction. Therefore, the development of an artificial sugar substrate with a definite leaving group at the reducing end has long been a hot topic in polysaccharide chemistry.

Nitrophenyl glycosides are known to be useful compounds for kinetic studies of glycosidases, because the liberated nitrophenol derivatives show yellowish colors in aqueous solutions (pH >7.5).[1] However, the synthesis of nitrophenyl glycosides normally requires multi-step reactions including the usage of strong acids or bases as well as the protection and deprotection of the hydroxyl groups (Scheme 1(A)).[2] For example, in order to activate the anomeric center, severe acidic reaction conditions by using hydrogen bromide/acetic acid is necessary, and protecting groups like acetyl groups must be removed under a basic conditions by using sodium methoxide at the final stage of the synthesis.[3,4]

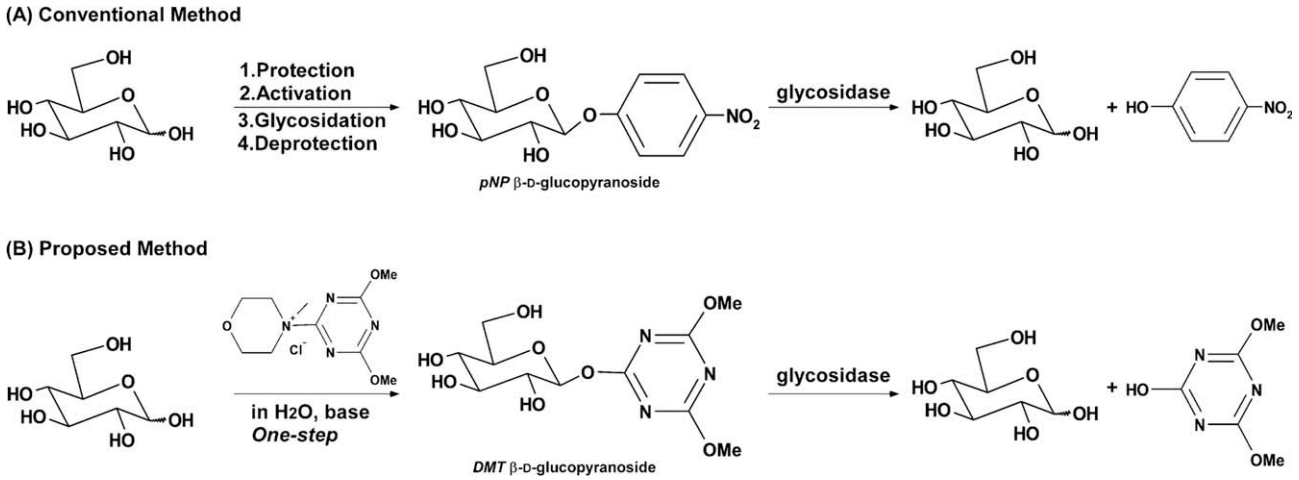
An enzymatic approach to the production of *p*-nitrophenyl (*p*NP) oligosaccharides has been reported. A substrate for human amylase in serum, *p*NP maltopentaoside, can be prepared by connecting a maltotetraose moiety to *p*NP glucoside by using an amylase as catalyst under mild reaction conditions.[5] However, these methods are highly restricted to the synthesis of specific oligosaccharides, its applicability in principle being dominated by the class of the enzyme catalyst employed. A novel substrate having other leaving group than nitrophenyl has, therefore, strongly been demanded in order to carry out kinetic analyses of glycosidases, particularly in case of using substrates whose glycon parts possess acid or base-labile functions.

Recently, we have found that 4,6-dimethoxy-1,3,5-triazin-2-yl β-lactoside (DMT-β-Lac), prepared by the reaction of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMT-MM)[6] and lactose, was hydrolyzed by a cellulase, giving rise to lactose and 2-hydroxy-4,6-dimethoxy-1,3,5-triazine.[7] The synthesis of DMT-β-Lac could be achieved in water without requiring any protection–deprotection process as well as any severe reaction conditions (Scheme 1(B)). These results prompted us to investigate a kinetic study on the enzymatic hydrolysis of DMT-oligosaccharides derived from naturally occurring xyloglucan (*Tamarindus* seeds).

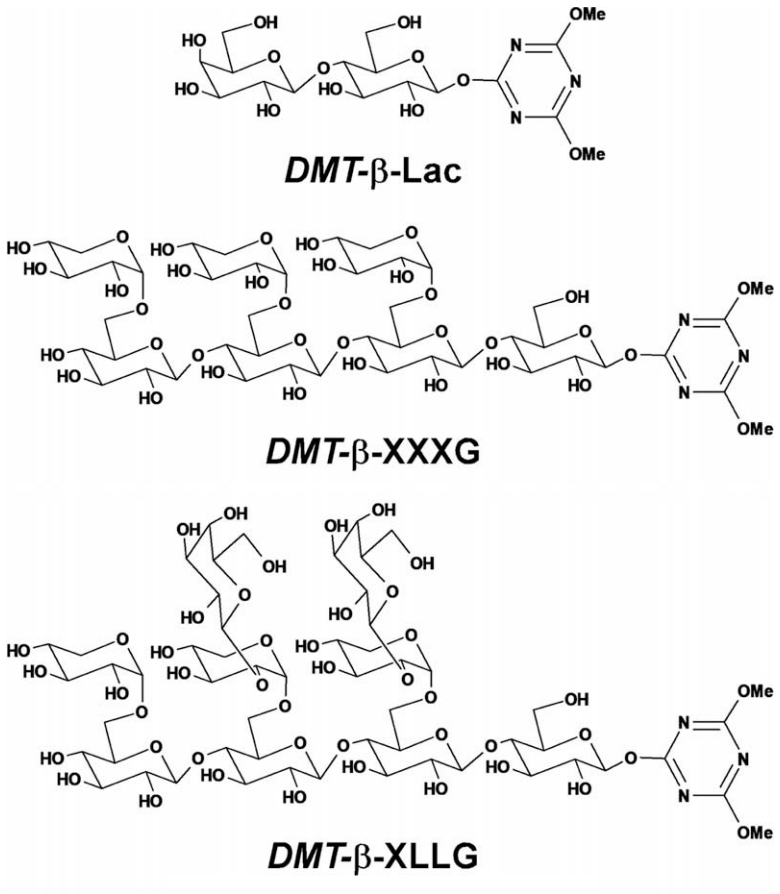
It is well known that endo-β-1,4-glucanase III from *Trichoderma reesei* (EGIII) is able to hydrolyze naturally occurring xyloglucan into the oligoxyloglucan (XXXG or XLLG).[8] In order to know substrate recognition of EGIII in more detail, kinetic analysis using oligoxyloglucans carrying a chromophore as their aglycon moiety is ideal. In this paper, we demonstrated a kinetic analysis of EGIII

* Corresponding author.
E-mail address: shoda@poly.che.tohoku.ac.jp (S. Shoda).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.04.122



Scheme 1. Comparison of chromogenic substrates: conventional *p*NP-glycoside versus one-step preparable substrate DMT-glycoside.

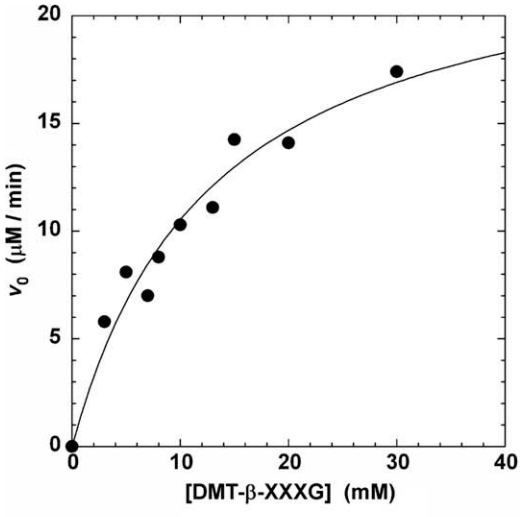


Scheme 2. DMT-glycosides used in this study. The backbone structure of naturally occurring xyloglucans from *Tamarindus* seeds is a mixture of at least two oligosaccharide units denoted by XXXG and XLLG, where a capital G represents an unsubstituted glucopyranose residue, a capital X represents a glucopyranose residue substituted with a xylopyranose through an α-1,6-glycosidic bond, and a capital L represents a glucopyranose residue substituted with a galactopyranose β(1–2)xylopyranose through an α-1,6-glycosidic bond.

by using two kinds of DMT-oligoxyloglucans, DMT-β-XXXG and DMT-β-XLLG, as novel sugar substrates (Scheme 2). The results of enzyme kinetics employing DMT-β-Lac have also been discussed.

The substrates, DMT-β-Lac, DMT-β-XXXG, and DMT-β-XLLG, were directly prepared starting from lactose,[7] XXXG,[9] and XLLG,[9] respectively, by the action of DMT-MM in the presence of 2,6-lutidine in water (yields: 61%, 44%, 36%, respectively). The enzyme EGIII was expressed and purified according to the previous report.[10] The reagent DMT-MM was synthesized from 2-chloro-4,6-dimethoxy 1,3,5-triazine and *N*-methylmorpholine.[6] Deuterium oxide was obtained by Acros.

NMR spectra (^{1}H, ^{13}C) were recorded on a Bruker DRX500 spectrometer. The samples were dissolved in D_2O, and acetone was used as an internal standard. HPLC analysis was carried out using



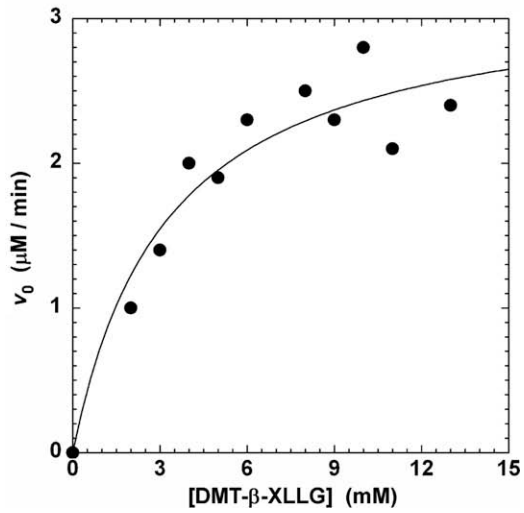


Figure 1. Michaelis–Menten analysis of EGIII-catalyzed reaction using DMT-β-XXXG and DMT-β-XLLG.

two type of columns (4.6 × 250 mm, eluent: acetonitrile aqueous solution); Inertsil® ODS-3 (GL Science) was used for DMT-β-Lac and TSK-gel Amide80 (Tosoh) was used for DMT-β-XXXG and DMT-β-XLLG, respectively. The UV absorbance of the eluate was monitored at 214 nm on a Shimadzu SPD-10AV detector. Molecular weight data were obtained using a Shimadzu AXIMA CFR plus mass spectrometer (2,5-dihydroxybenzoic acid matrix).

EGIII-catalyzed hydrolysis of DMT-glycosides was performed in 50 mM acetate buffer (pH 5.5) at 30 °C and all the reactions were monitored by quantitating the starting DMT-glycosides (UV at 214 nm) by HPLC. The kinetic parameters and their standard errors were calculated using the nonlinear regression analysis program '*KaleidaGraph* 4.1 J'.

The Michaelis constants (K_m) for DMT-β-XXXG and DMT-β-XLLG by EGIII were 13 and 3.3 mM, and the first-order rate constants (k_{cat}) for the substrate were 170 and 23 s^{-1}, respectively (Fig. 1.)

In order to clarify the merits of DMT-glycosides as substrates, we compared the kinetic parameters of DMT-β-Lac with that of *p*-nitrophenyl β-lactoside (*p*NP-β-Lac) for EGIII enzyme. It has been found that DMT-β-Lac was smoothly hydrolyzed by EGIII, affording lactose and 4,6-dimethoxy-1,3,5-triazine derivative. On the other hand, it is well known that *p*NP-β-Lac cannot be recognized by EGIII.[11] The Michaelis constant (K_m) for DMT-β-Lac by EGIII was

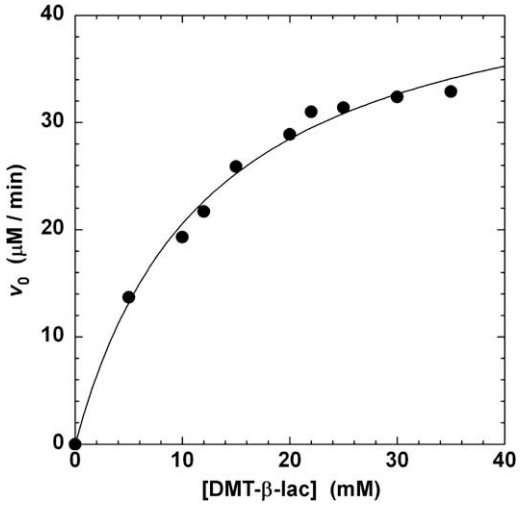


Figure 2. Michaelis–Menten analysis of EGIII-catalyzed reaction using DMT-β-Lac.

13 mM and the first-order rate constants (k_{cat}) for the substrate was 5.4 s^{-1} (Fig. 2).

These results clearly indicate that the existence of the 4,6-dimethoxy-1,3,5-triazine moiety is essential for the terminal glycosidic bond at the reducing end to be cleaved in the catalytic site of the enzyme. It is well understood from the viewpoint of stereoelectronic effect theory that the conformation of the pyranose ring, located in the −1 subsite, with a $^4C_1$ conformation must change to a half boat conformation in order that the orientation of the cleaving β-glycosidic bond becomes antiperiplanar to one of the lone pairs on the ring oxygen atom of the pyranose. This conformation change may be caused by a strong interaction between the dimethoxytriazine ring and specific amino acids in the vicinity of the +1 subsite.

On the other hand, *p*NP-β-Lac cannot be hydrolyzed by the same enzyme, clearly indicating that the conformation change of the pyranose ring in −1 subsite does not take place. These results shows that DMT-glycosides possess an advantageous character as enzyme substrates for kinetic studies of glycosidases over conventionally used *p*NP-glycosides.

In the present Letter, we demonstrated that two kinds of DMT-oligoxyloglucans, DMT-β-XXXG and DMT-β-XLLG, could be recognized as efficient substrates for an endo-β-glucanase. Taking the fact that preparation of DMT-glycosides is much easier than that of *p*NP-glycosides into consideration, the feasibility of DMT-glycosides as 'general substrates' for enzyme studies would emerge soon. In spite of the drawback that the hydrolytic product of DMT-glycosides, 2-hydroxy-4,6-dimethoxy-1,3,5-triazine (4,6-dimethoxy-1,3,5-triazine-2-one), shows no colors like *p*NP-glycoside, DMT-glycosides are attractive substrates for kinetic studies of glycosidases because of their easy-to-prepare and tailor-made characters as well as their higher activities from the viewpoints of synthetic organic chemistry and biomolecular engineering. Further studies for elucidating the detailed hydrolysis mechanism of the DMT-glycosidic bond in the catalytic site of a glycosidase is now in progress.

Acknowledgments

This work was supported by a Grant-in Aid for Scientific Research from the Ministry of Education, Sports, Science and Technology, and Industrial Technology Research Grant Program in 2006 from NEDO of Japan.

References and notes

1. Rauscher, E.. In *Methods of Enzymatic Analysis*; Bergmeyer, E., Ed.; VHC, 1984; Vol. 4, p 157.
2. Tokutake, S.; Yamaji, N.; Kato, M. *Chem. Pharm. Bull.* **1990**, *38*, 13.
3. Ibatullin, F. M.; Baumann, M. J.; Greffe, L.; Brumer, H. *Biochemistry* **2008**, *47*, 7762.
4. Ibatullin, F. M.; Banasiak, A.; Baumann, M. J.; Greffe, L.; Takahashi, J.; Mellerowicz, E. J.; Brumer, H. *Plant Physiol.* **2009**, *151*, 1741.
5. Usui, T.; Murata, T. *J. Biochem.* **1988**, *103*, 969.
6. Kunishima, M.; Kawachi, C.; Morita, J.; Terao, K.; Iwasaki, F.; Tani, S. *Tetrahedron* **1999**, *55*, 13159.
7. Tanaka, T.; Noguchi, M.; Kobayashi, A.; Shoda, S. *Chem. Commun.* **2008**, 2016.
8. Yuan, S.; Wu, Y.; Cosgrove, D. J. *Plant Physiol.* **2001**, *127*, 324.
9. Tanaka, T.; Noguchi, M.; Ishihara, M.; Kobayashi, A.; Shoda, S. *Macromol. Symp.*, in press.
10. Okada, H.; Tada, K.; Sekiya, T.; Yokoyama, K.; Takahashi, A.; Tohda, H.; Kumagai, H.; Morikawa, Y. *Appl. Environ. Microbiol.* **1998**, *64*, 555.
11. Claeyssens, M.; Henrissat, B. *Protein Sci.* **1992**, *1*, 1293.