ヒドロキシ基の保護を必要としない新規糖供与体の一段階合成

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Dimethoxy Triazine Glycosides as New Glycosyl Donors for Chemo-enzymatic Synthesis of Oligosaccharides

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Abstract: Various 4-(4,6-dimethoxy-1,3,5-triazin-2-yl) glycosides (DMT-glycosides) have been synthesized from the corresponding free sugars in water by using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMT-MM) as a dehydrative condensing agent. The resulting DMT-glycosides were found to be recognized as substrates for glycosyl hydrolases and could be utilized as novel glycosyl donors for chemo-enzymatic glycosylations. DMT-glycosides will be efficient and general glycosyl donors for glycosidase-catalyzed transglycosylation reaction in the field of carbohydrate chemistry.

Key words: transglycosylation, glycosidase, activated glycosyl donor, one-step preparation, aqueous solution

Enzymatic glycosylation has become an attractive methodology in the field of synthetic carbohydrate chemistry.[1,2] In particular, glycosidase-catalyzed transglycosylation reactions play central roles for the synthesis of various glycosyl compounds such as oligosaccharides, polysaccharides, and glycoconjugates, although other proteinous catalysts like glycosyl transferases,[3-5] phosphorylases[6,7] and glycosynthases[8] are alternative to glycosidases. The main reason why glycosidases are frequently employed for glycoside synthesis is because they satisfy necessary and sufficient properties as catalysts for industrial scale. Glycosidases show high stability and availability, and the substrates for glycosidases are inexpensive.

A number of studies on glycosidase-catalyzed glycosylations have been reported where various compounds such as phenyl glycosides,[9] *p*-nitrophenyl glycosides,[10] glycosyl fluorides[11] and sugar oxazolines[12] are employed as glycosyl donors. However, the preparation of these glycosyl donors requires laborious task including the protection and deprotection of the hydroxy groups and the purification of products. Furthermore, cleavage of the glycosidic bonds by acidic reagents is often observed during the introduction of a leaving group to the anomeric position, especially in case of oligosaccharides with higher molecular weights.

For example, in order to replace the anomeric hydroxy group by a fluorine atom, a multi-step process including acetylation, bromination, fluorination and deacetylation is necessary (Fig. 1, path ①→②→③→④). Another example is the synthesis of phenyl glycoside derivatives where protected 1-hydroxy sugar derivatives must firstly be prepared via several steps from the corresponding free sugars, and the anomeric hydroxy group is substituted with a phenoxy group by using, for example, the Mitsunobu reagent (Fig. 1, path ①→⑤→⑥→④).[13,14] Therefore, the development of new glycosyl donors that can be prepared directly from free sugars has been strongly demanded (Fig. 1, path ⑦).

There are three kinds of hydroxy groups in a saccharide moiety: the primary hydroxy group, the secondary hydroxy groups, and the hemiacetal (Fig. 2). The pK_a values of the primary and secondary hydroxy groups are around 16 whereas that of the hemiacetal is 12.2, indicating that the hemiacetal is more acidic than other hydroxy groups.[15] On the other hand, the pK_a value of water is known to be 15.7. All of these data suggest that a selective nucleophilic attack of the hemiacetal to an electrophile would be possible in water without protecting other hydroxy groups provided that an appropriate electrophilic reagent is chosen.

We already reported that the anomeric hydroxy group of various monosaccharides can be activated by Mitsunobu reagent in polar organic solvent without protecting other hydroxy groups (Scheme 1).[16] However, when the reaction was applied to oligosaccharides of higher molecular weights, the reaction did not proceed due to the poor solubility of the oligosaccharides toward organic solvents. It was, therefore, necessary to carry out the reaction in an aqueous solution by using a water-soluble electrophilic reagent in order to activate the anomeric hydroxy group of higher oligosaccharides effectively.

Preparation of a DMT-glycoside and its characterization.

Recently, Kunishima *et al.* developed a water-soluble dehydrative condensing agent, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) for peptide synthesis.[17] The reaction consists of the initial formation of an activated ester intermediate, which is then attacked by an amine to give the corresponding amide. We postulated that a selective activation of sugar compounds would be possible if hemiacetals behave like carboxylic acids, affording glycosyl compounds having the

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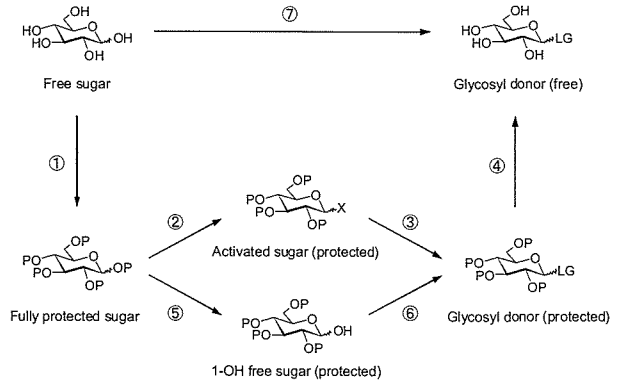


Fig. 1. Strategy of synthesizing activated glycosyl donor.

Path ①, protection of hydroxy group; ②, activation at anomeric position; ③, ⑥, introduction of leaving group; ④, deprotection; ⑤, selective deprotection at anomeric position; ⑦, direct activation by dehydrative condensing agent without any protection of hydroxy groups.

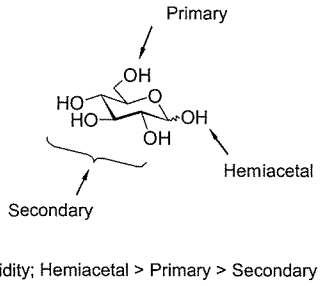
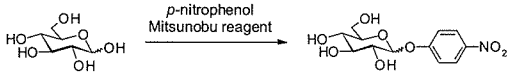
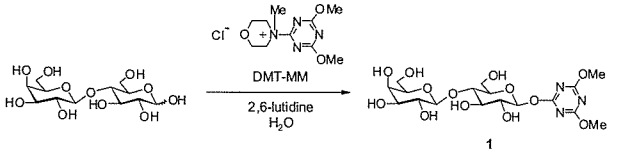


Fig. 2. Hydroxy groups of free sugar.



Scheme 1. Direct synthesis of *p*-nitrophenyl glucoside by using the Mitsunobu reagent.



Scheme 2. Preparation of DMT-β-Lac **1** by using DMT-MM.

DMT moiety at the anomeric position. After screening various bases such as 2,6-lutidine, pyridine, triethylamine, diisopropylethylamine, *N*-methylmorpholine, sodium hydrogen carbonate as acid scavengers, we found that the use of 2,6-lutidine gave the best results concerning the yield of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl glycosides (DMT-glycosides).

The following is a typical procedure for synthesis of DMT-β-lactoside (DMT-β-Lac) **1** (Scheme 2). An aqueous solution of lactose (α/β=26/74), DMT-MM (2 equiv.) and 2,6-lutidine (1 equiv.) was stirred at room temperature for 18 h. After confirming the disappearance of lactose, the solvent was evaporated and the residue was crystallized from ethanol, giving rise to **1** in 73% yield.

The ^{1}H-NMR spectrum of **1** showed a doublet peak at 5.8 ppm derived from the anomeric proton with the coupling constant of 8.06 Hz, indicating that the anomeric configuration of the product is β-type (Fig. 3). It is to be noted that the anomeric hydroxy group predominantly attacked the 2-position of DMT-MM because it has a higher

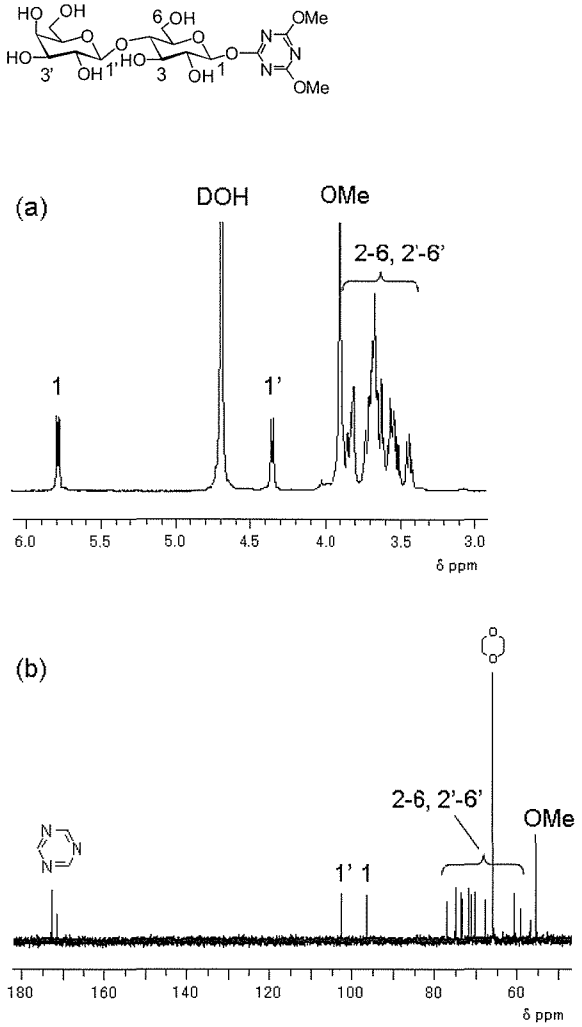


Fig. 3. NMR spectra of DMT-β-Lac **1** in D_2O.
(a) ^{1}H-NMR, (b) ^{13}C-NMR.

acidity compared with other hydroxy groups and water.

Next, we investigated the time-course of the DMT-β-Lac formation in deuterium oxide solution by ^{1}H-NMR spectroscopy (Fig. 4). An anomeric proton signal derived from DMT-β-Lac could be observed after 1 h and gradually increased. At the early stage of the reaction, the formation of mono-substituted DMT-α-lactoside (DMT-α-Lac) was observed, which is converted to the di-substituted product ((DMT)$_2$-α-Lac) (Fig. 5). The nucleophilicity of the 2-hydroxy group of the DMT-α-lactoside may be intramolecularly enhanced by the lone-pair on the nitrogen atom of the triazine ring, resulting in the formation of the di-substituted product (Scheme 3). It is noteworthy that the di-substituted by-product can easily be removed by crystallization or chromatography because there is large difference of physical properties between the mono-substituted product and di-substituted by-product. Other sugars like glucose, galactose, xylose, cellobiose, maltose, xyloglucan-oligosaccharide are converted to the corresponding DMT-derivatives in a similar manner.

Hydrolysis of DMT-glycosides catalyzed by glycosidases.

Prior to being utilized as a glycosyl donor for glycosidase-catalyzed transglycosylation, DMT-β-Lac **1** was subjected to hydrolysis experiment in order to know

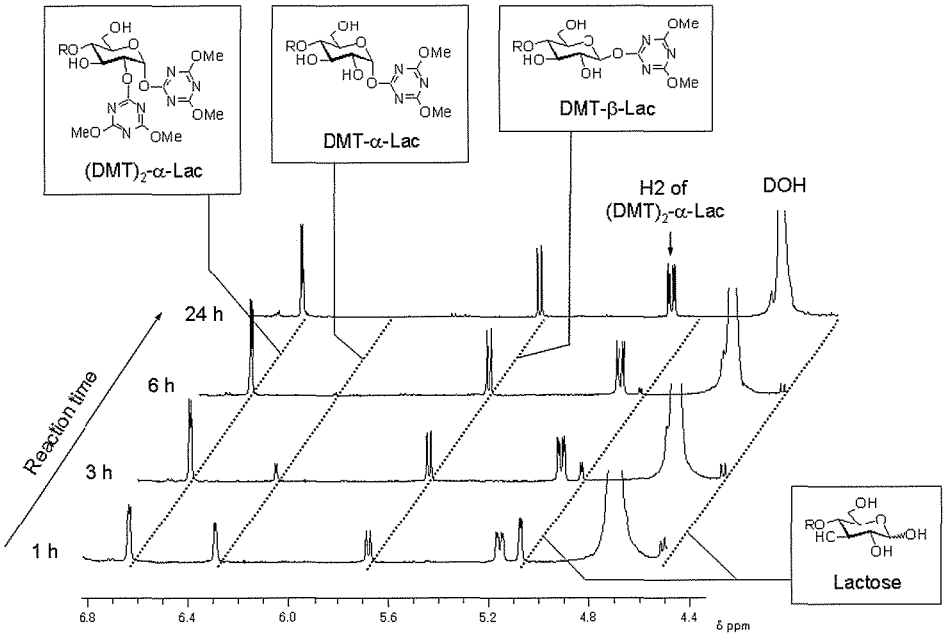


Fig. 4. Time-course of the formation of DMT-β-Lac **1**, DMT-α-Lac and $(DMT)_2$-α-Lac traced by ^{1}H-NMR spectroscopy.

A deuterium oxide solution (0.5 mL) of lactose monohydrate (0.1 mmol, α/β = 9/1) was treated with DMT-MM (0.2 mmol) and 2,6-lutidine (0.2 mmol) at room temperature.

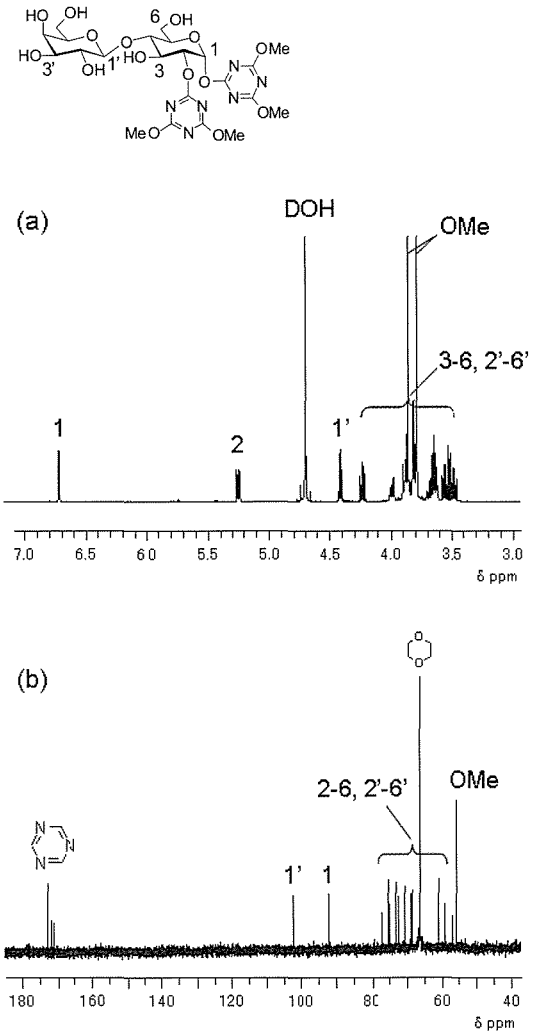
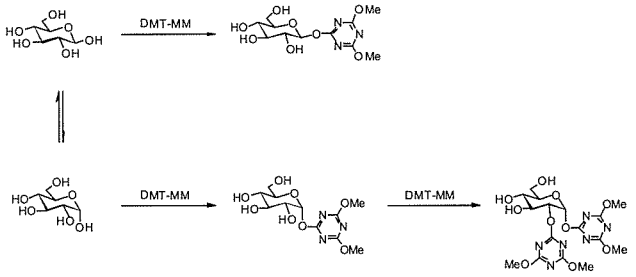


Fig. 5. NMR spectra of $(DMT)_2$-α-Lac in D_2O.

(a) ^{1}H-NMR, (b) ^{13}C-NMR.



Scheme 3. Plausible reaction mechanism of DMT-glycoside formation.

whether the DMT moiety can be recognized by enzyme to form an enzyme-substrate intermediate. We had already found that β-D-lactosyl fluoride can be recognized by endo-β1,4-glucanase III (EGIII) from *Trichoderma reesei*,[18] while the conventional chromogenic substrate of *p*-nitrophenyl β-D-lactoside can not be hydrolyzed by EGIII.[19] It was interesting for us to investigate the substrate specificity of the DMT moiety for EGIII.

Firstly, enzymatic hydrolysis experiment of **1** was performed in 200 mM acetate buffer (pH 5.5) at 30°C. Obviously, almost no glycosidic bond cleavage was observed in the absence of the enzyme, indicating that compound **1** is stable in acetate buffer. Interestingly, when EGIII was added, the compound **1** was found to be hydrolyzed smoothly, giving rise to lactose and 4,6-dimethoxy-1,3,5-triazine derivative (Fig. 6). These results clearly indicated that the compound **1** can be accepted by the −2 subsite as well as −1 subsite of the enzyme. The hydrolysis mechanism has not been made clear, however, it is assumed that the glycosidic oxygen atom or the nitrogen atom (1 or 3 position) of the triazine ring is protonated with an acidic amino acid located at the catalytic center of EGIII, affording an oxocarbenium ion intermediate. The resulting intermediate is then attached by water to give the hydrolyzates.

We also tested other glycosidases, β-D-glucosidase, β-D-galactosidase, by using DMT-β-glucoside and DMT-β-galactoside, respectively, as substrates, and found these DMT-derivatives can also be hydrolyzed by the corresponding glycosidases, giving rise to glucose and galactose, respectively.

Transglycosylation by using DMT-β-lactoside as glycosyl donor catalyzed by EGIII.

Having been encouraged by these new findings, we tried to synthesize a tetrasaccharide by the reaction of **1** as a glycosyl donor and α-thiophenyl cellobioside (Cel-α-SPh) **2** as a glycosyl acceptor (Scheme 4). When **1** and **2** were mixed in the presence of EGIII in 200 mM acetate buffer (pH 5.5) at 30°C (donor/acceptor ratio=1/1), the corresponding tetrasaccharide derivative (Lac-Cel-α-SPh) **3** was obtained in 66%. The yield of **3** on the basis of the acceptor increased to 95% when the donor/acceptor ratio was changed to 2.5/1. A new formed glycosyl bound was β-1,4 type between lactoside and cellobioside, which was confirmed by NMR spectroscopy.

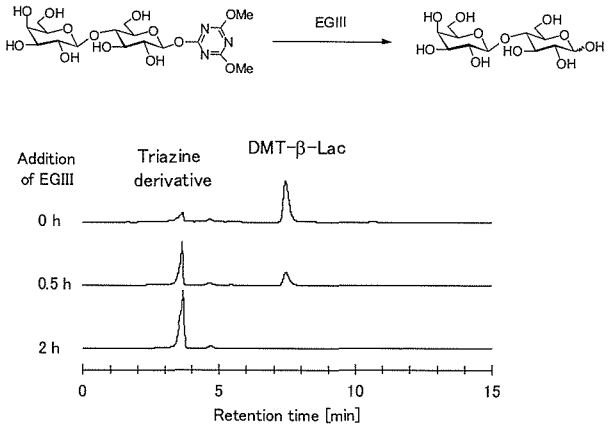


Fig. 6. Scheme and HPLC chart of enzymatic hydrolysis of DMT-β-Lac **1**.

Thirty millimolars DMT-β-Lac was reacted in 200 mM acetate buffer (pH 5.5) with EGIII at 30°C. The reaction mixture was analyzed by HPLC (column, Amide80; detector, UV (214 nm)).

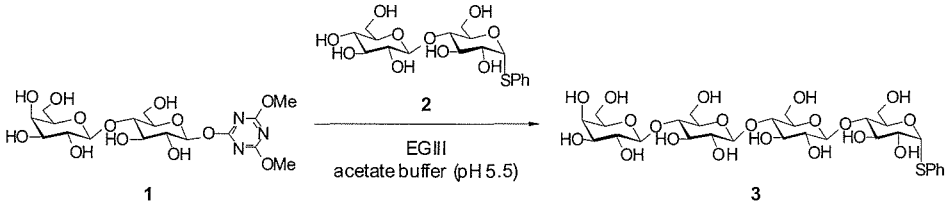
Polycondensation of DMT-derivative of xyloglucan oligomer catalyzed by cellulase.

Xyloglucans are one of the major polysaccharides emerged in plant cell walls, and have a cellulose back bone partially substituted by a xylopyranosyl moiety through an α-1,6 bond.[20-22] Much attention has been paid to artificial xyloglucans having a repeating oligosaccharide unit because these compounds would lead to a greater understanding of the mechanism of xyloglucan metabolism and the binding mechanism of xyloglucan to cellulose in growing plant cell wall. Naturally occurring xyloglucans, however, do not possess a complete regularity of saccharide sequences. Therefore, development of an efficient method for synthesis of artificial xyloglucan derivatives with definite structures has strongly been demanded.

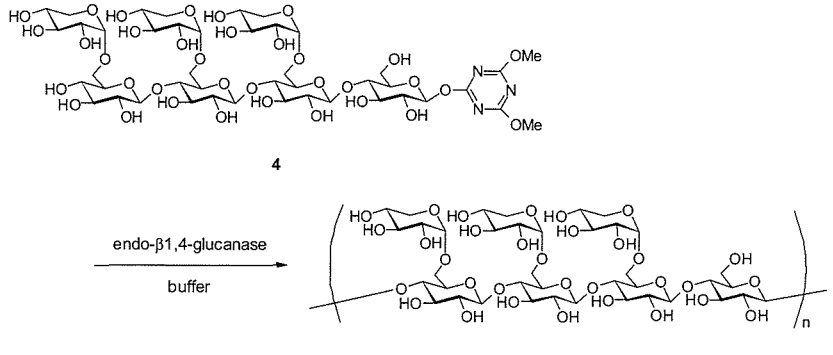
A xyloglucan heptasaccharide (XXXG) has directly been converted to the corresponding 4,6-dimethoxy-1,3,5-triazin-2-yl derivatives (DMT-β-XXXG) **4**. The resulting activated oligosaccharide derivatives **4** were found to polymerize catalyzed by an endo-β1,4-glucanase as catalyst. The polymerization took place in a complete regio- and stereo-selective manner, affording non-natural polysaccharides **5** having a XXXG-repeating unit in the main chain (Scheme 5).

One-pot chemo-enzymatic oligosaccharide synthesis.

One of the most significant characteristics of the present glycosylation reaction is that DMT-glycosides can be directly prepared in aqueous solution starting from free sugars. This fact prompted us to investigate a one-pot procedure without isolating the synthetic intermediate DMT-glycoside (Fig. 7). When lactose was treated with DMT-MM and 2,6-lutidine in aqueous solution followed by the addition of the glycosyl acceptor **2** and EGIII, the corresponding tetrasaccharide **3** was directly obtained in 32% yield (on the basis of lactose). As well as lactosylation reaction, other kind of one-pot glycosylation and polycon-



Scheme 4. EGIII-catalyzed transglycosylation using DMT-β-Lac **1** as a glycosyl donor.



Scheme 5. Glycosidase-catalyzed polycondensation using DMT-β-XXXG **4** as a substrate.

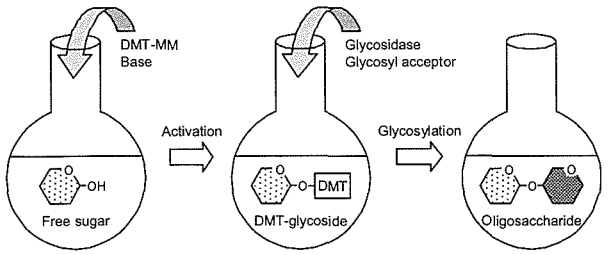


Fig. 7. One-pot chemo-enzymatic oligosaccharide synthesis through DMT-glycoside.

densation reactions can be performed through DMT-glycosides starting from free sugars.

Future application of DMT-glycosides.

Novel glycosyl compounds, DMT-glycosides, can be directly obtained by the reaction of free sugars and a dehydrative condensing agent, DMT-MM, in water without using any protecting groups. The resulting DMT-glycosides can be utilized as efficient glycosyl donors for oligosaccharide synthesis. It is possible to introduce various functional groups at 2 and/or 4 position of the triazine ring, indicating that various triazine-based glycosyl donors with different reactivities can be designed corresponding to enzyme catalysts in future.

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ヒドロキシ基の保護を必要としない新規糖供与体の一段階合成 —糖加水分解酵素を用いる糖転移反応の効率化—

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糖加水分解酵素の糖転移能を利用した糖鎖合成反応は，酵素の入手が容易，糖転移酵素では実現不可能なオリゴ糖単位での転移が可能という利点を有していることから広く用いられている．さらには，*p*NP糖やフッ化糖のような優れた脱離基を有する合成糖供与体を用いることにより，反応効率の向上が可能である．しかし，これら合成糖供与体の調製は，多段階の工程が必要である上，アノマー位への脱離基の導入の際，グリコシド結合の開裂も懸念されることから，分子量が大きく分岐を有するオリゴ糖を合成糖供与体化することは困難とされてきた．我々はこれらの問題点を解決するため，糖のアノマー位のヒドロキシ基の反応性の高さに着目し，無保護糖を原料とした簡便な糖供与体合成法として，光延試薬を用いた*p*NP糖の一段階合成，水溶性カルボジイミド塩酸塩を用いた糖オキサゾリン誘導体の一段階合成を報告した．さらには，DMT-MMを用いた場合には，糖オキサゾリン誘導体が得られる他に，副生成物としてアノマー位にジメトキシトリアジンを有する化合物 (DMT糖) が得られ，糖供与体として働くことが期待された．水溶液中での無保護糖とDMT-MMとの反応においては，グルコースなどの単糖，ラクトースなどの二糖，さらには分子量が大きく分岐を有するオリゴ糖にも適用可能であった．得られたDMT糖に糖加水分解酵素を作用させたところ，速やかに加水分解を受けたことから，DMT糖が有用な糖供与体となり得ることを見出した．合成したDMT-β-Lactosideを糖供与体とし，EGIIIによる糖受容体への酵素的ラクトシル化反応に成功した．また，合成したDMT糖を単離することなく無保護糖からのワンポットグリコシル化反応にも成功した．本DMT糖合成法は，従来の有機合成法では糖供与体の合成が非常に困難であったキシログルカンオリゴ糖にも適用可能であり，DMT糖を基質とした酵素的重縮合反応にも成功した．

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〔質問〕 北大院・農 木村

1）反応条件を検討することでα型の一置換体を得ることはできないか？例えばDMT-MMと糖を等モル反応させるなど．

2）マンノースの場合は，α型の一置換体が得られるか？

3）DMT糖は酵素の阻害剤にならないのか？

〔答〕

1）DMT-MMを等モル，あるいはそれ以下で反応した場合でも，反応開始直後からα型二置換体の生成がみられる．種々の検討を行っているところではあるが，現時点でα型二置換体を生成することなく，α型一置換体を得ることはできていない．

2）マンノースの場合にはα型一置換体が主生成物として得られ，副生成物としてβ型二置換体が得られる．2位へのDMT基の導入は，始めにアノマー位に導入されたDMT基のトリアジン環窒素原子が塩基として2位ヒドロキシ基のプロトンに作用していることが原因と考察しており，二置換体は1,2-*cis*型が得られる．

3）β型DMT糖は水中において自然加水分解されるため，阻害剤として用いるには基質の安定性に欠けると思われる．阻害剤としての利用を考える場合には，基質の安定性を向上させる誘導体化が必要と考えられる．

〔質問〕 京大院・生命科学 芦田

K_m値が大きいようだが，基質の水溶性に問題はないか．

〔答〕

DMT糖の水溶性は，速度論解析に汎用されている*p*-ニトロフェニル化糖よりも高く，全く問題ない．水溶性が高いために糖加水分解酵素を用いる糖転移反応では有利な条件である高基質濃度での反応が可能となる利点を有している．

〔質問〕 日大・生資化・農化 袴田

1）β型のDMT糖にジメトキシトリアジンアルコール (DMT-OH) を反応系に加えることによって，α型のDMT糖はできないのか？

2）レアシュガーに本方法を用いると保護基がついていないので，フラノースになるか，ピラノースになるのか，わからないのではないでしょうか？

〔答〕

1）DMT-OHは水中では1,3,5-triazin-2(1H)-one (ケト構造) となるため，α型のDMT糖は生成しない．また，系内にアルコールが存在しても求核性が低いために生成物は得られない．

2）フルクトースを基質に反応した場合には，DMT糖は得られない．また，キシロースの場合には，得られる生成物は全てピラノース型であり，フラノース型の生成物はこれまで確認されていない．