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Chemical trans-glycosylation of bioactive glycolinkage: synthesis of an α-lycotetraosyl cholesterol

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Abstract—The aim of this study was to verify the antitumor role of the β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-D-galactopyranosyl (lycotetraosyl) moiety present in steroidal glycosides from Solanaceous plants. We explored a new chemical trans-glycosylation method using an endoglycosidase called tomatinase that is produced by the tomato pathogen, *Fusarium oxysporum* f. sp. *lycopersici*. The lycotetraose, which was prepared by enzymatic hydrolysis of α-tomatine with tomatinase, was converted to glycosyl donors such as trichloroacetimidate, fluoride, and thioglycoside. All obtained glycosyl donors were glycosylated with cholesterol to form α-lycotetraosyl cholesterols in a stereoselective manner. The obtained lycotetraosyl derivatives together with typical natural lycotetraosyl glycosides were examined for their antiproliferative activity.

Solanaceous plants are widely distributed the world over and are used as traditional drugs against cancer and herpes as well as food for the prevention of cancer.[1,2] We have studied the steroidal oligosaccharides in 40 types of solanaceous plants.[3,4] Our detailed studies on the relationship between the structure and bioactivity of the glycosides from *Solanum* plants showed that spirostanol type steroidal glycosides having β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-D-galactopyranosyl glycone moiety have potent antitumor activity.[5,6] This tetrasaccharide, known as lycotetraose,[7] is one of the major oligosaccharide moieties in steroidal glycosides. Lycotetraose is found in a very wide variety of plant species including members of the *Solanaceae* and *Liliaceae* families such as tomatoes, potatoes, garlic, and other plants that are important in human diet. Recently, Morrow et al. reported that α-tomatine (lycotetraosyl tomatidine), a major steroidal alkaloid from tomatoes, triggered cell death by apoptosis.[8] Although the lycotetraosyl moiety along with an aglycone part is a crucial structure in antiproliferative and apoptotic activities in human cancer cell lines, the function of lycotetraose is unclear. In continuing our efforts[6] to study the structure–activity relationship of aglycone parts having the lycotetraosyl moiety and in order to obtain an insight into the mechanism of action of a lycotetraose-binding biomolecule, we planned to carry out the chemical trans-glycosylation of lycotetraose to appropriate aglycones in order to obtain neoglycoconjugates.[9] Here, we describe our attempts to synthesize an unnatural α-lycotetraosyl cholesterol and the results of the antiproliferative activity of the synthesized lycotetraosyl derivatives. Since this method is flexible, it may be used for constructing a lycotetraose-containing chemical library.

Syntheses of lycotetraose,[10] methyl lycotetraoside,[11] and lycotetraose-containing saponin desgalactotigonin[12,13] have been reported in the literature. On the other hand, in an earlier paper, we have reported the trans-glycosylation of bioactive glycolinkages from natural glycosides to some aglycones.[14–17] This method is useful in the direct conversion of an oligosaccharide moiety to various aglycones when constructing an oligosaccharide-based library. Endoglycosidases are powerful tools for determining oligosaccharide sequences. Fortunately, an endogalactosidase called tomatinase,[18] which is produced by the vascular wilt pathogen of tomato *Fusarium*

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oxysporum f. sp. *lycopersici* cleaves α-tomatine into tomatidine and lycotetraose.[19] To enzymatically synthesize lycotetraose from α-tomatine, the endogalactosidase was prepared in the form of crude tomatinase. The crude tomatinase was obtained from culture filtrates of *F. oxysporum* f. sp. *lycopersici*, induced by α-tomatine.[20] As described previously,[20] proteins in the filtrate were precipitated by the addition of ammonium sulfate to 80% saturation. The precipitated proteins were collected on the filter by suction and then desalted. The obtained crude tomatinase included a protein with a molecular mass of 64 kDa that accumulated in the α-tomatine-induced culture filtrates that had tomatinase activity. The extract was used without further purification since undesired hydrolysis of lycotetraose was not observed. Recently, Woods and co-workers reported preparative enzymatic synthesis of lycotetraose from α-tomatine.[21] In this synthesis, lycotetraose was liberated by a recombinant endoglycosidase from the same plant pathogen. Although, this method involving the use of an *Escherichia coli* transformant containing recombinant tomatinase was elegant, our procedure for the production of tomatinase is easy to perform because it does not require special instruments or a special environment for manipulating microbes containing products of genetic recombination. Also, our method is sufficient to prepare the starting material for organic synthesis. For example, lycotetraose (ca. 200 mg) was prepared from a 1 L culture medium of *F. oxysporum* f. sp. *lycopersici*.

The steroid glycoalkaloid α-tomatine was isolated from fresh leaves of tomato (*Lycopersicon esculentum*), after harvesting the fruit. A 0.01% yield (w/w wet plant body) was obtained. Lycotetraose was prepared from α-tomatine by enzymatic hydrolysis with crude tomatinase. Enzymatic hydrolysis was followed by gel filtration with Sephadex LH20 and silica gel chromatography resulting in a 75% yield (Fig. 1).

The obtained lycotetraose[22] was protected with acetic anhydride in pyridine to give a peracetylated lycotetraoside **1**[23] (Scheme 1). In this procedure, the conformation of the xylose moiety was changed from $^4C_1$ to $^1C_4$ (δ 5.16, d, $J_{H1,2} = 1.8$ Hz, xyl H-1) due to steric hindrance.[11,24] The acetyl group of the reducing end of **1** was deacetylated with hydrazine acetate[25] to afford a tetrasaccharide **2**[26] in 70% yield after separation by silica gel. The tetrasaccharide **2** was converted to an α-trichloroacetimidate **3**[26] in the presence of trichloroacetonitrile and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in 98% yield.[27]

The tetrasaccharide donor **3** was transferred to cholesterol by Schmidt's method[27] using boron trifluoride etherate ($BF_3{\cdot}Et_2O$) as the promoter, and the fully protected glycoside **4**[28] was selectively obtained as an α anomer (δ 5.11, d, $J_{H1,2} = 3.7$ Hz, gal H-1), in 37% yield (Scheme 2). Following deprotection by sodium methoxide in MeOH, α-lycotetraosyl cholesterol **5**,[28] an unnatural glycoconjugate, was obtained in 97% yield. After deacetylation of **4**, the conformation of a xylose unit of **5** was observed in the $^4C_1$ form (δ 5.27, d, $J_{H1,2} = 7.9$ Hz, xyl H-1), which is the same as that in naturally occurring lycotetraosides.

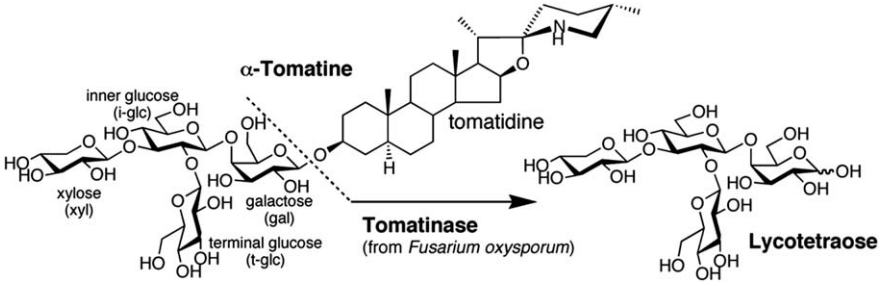
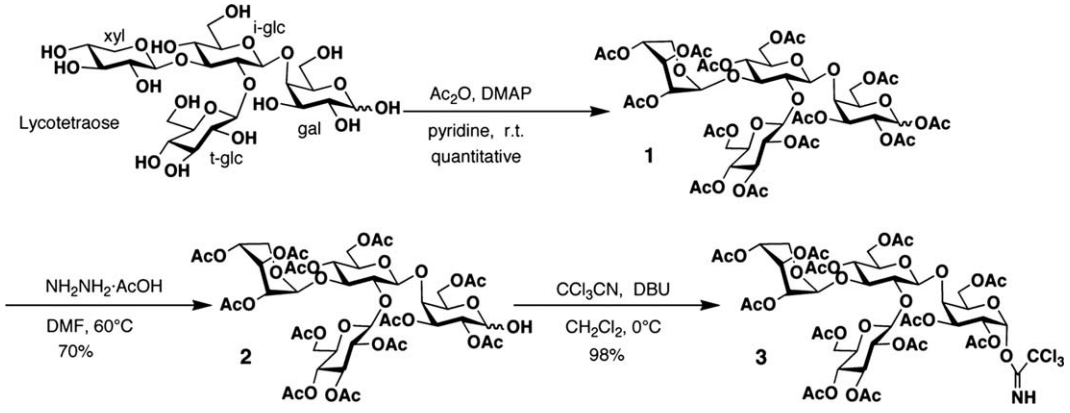
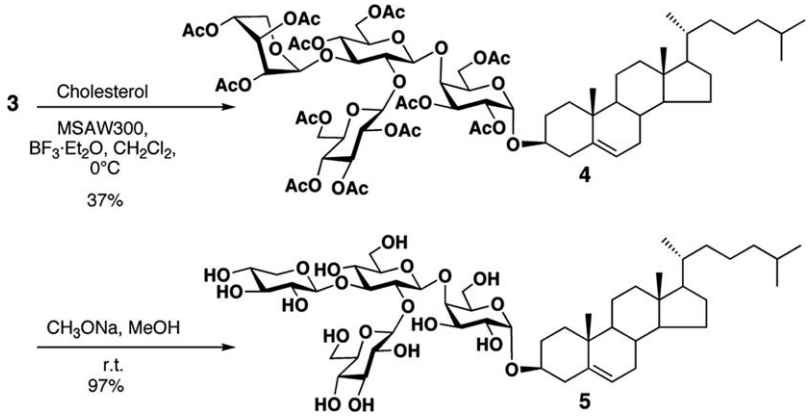


Figure 1. Preparation of lycotetraose using the pathogen induced endoglycosidase, tomatinase.



Scheme 1. Synthesis of lycotetraosyl trichloroacetimidate **3**.



Scheme 2. Trans-glycosylation using lycotetraosyl trichloroacetimidate **3**.

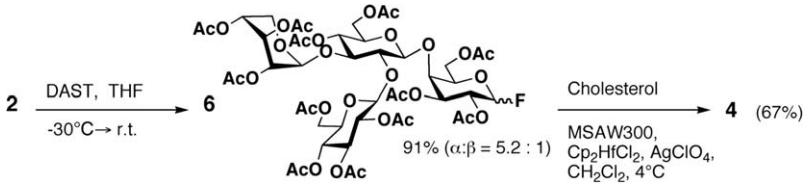
Since the stereoselectivity of the glycosylation reaction did not yield a satisfactory naturally occurring β anomer, we tried converting the glycosyl donor **3** to the fluoride **6** (Scheme 3) and the thioglycoside **7** (Scheme 4). The 1-OH galactosyl derivative **2** and diethylaminosulfur trifluoride (DAST)[29] were reacted to give the fluoride **6** in 91% yield in the form of an anomeric mixture (α:β = 5.2:1).[30] The α-anomer fluoride donor **6** was activated by Cp_2HfCl_2 and $AgClO_4$[31] and then glycosylated with cholesterol to afford the α-glycoside **4** in 67% yield.

The lycotetraosyl thioglycoside **7** was prepared with peracetylated lycotetraoside **1** and thiocresol in the presence of $BF_3{\cdot}Et_2O$ to afford an anomeric mixture (α:β = 1:1) in 85% yield.[32] The lycotetrasyl donor **7**[33] was glycosylated with cholesterol in the presence of *N*-iodosuccinimide/trimethylsilyl trifluoromethanesulfonate (NIS/TMSOTf)[34,35] to afford the α-glycoside **4** in 52% yield.

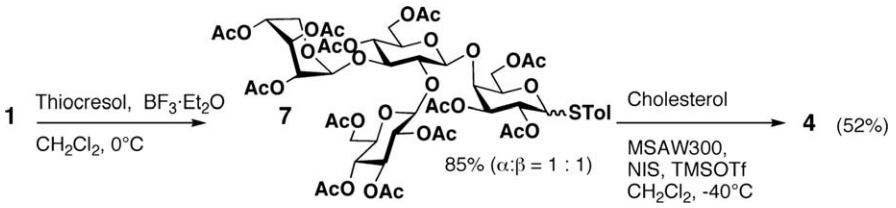
During these experiments, we found that using either **6** or **7** as glycosyl donors significantly increased the reaction yield, which was calculated from acetate **1** to the cholesterol derivative **4**. That is, if **6** was the glycosyl donor, the yield was 41% when three steps were used. If **7** was used, it was 43% in two steps; while in the case of **3**, it was 25% when three steps were used. Focusing on the newly generated glycosidic bond, the stereoselectivity of the glycosylation was not influenced by these glycosyl donors to give the α-glycosidic linkage of **4**.

The cytotoxicity of the obtained neosaponins **5** and related compounds **4**, lycotetraose, α-tomatine, and desgalactotigonin toward NCI-H460 (non small-cell lung cancer) and MDA-MB-231 (breast cancer) cell lines was tested (Table 1). Only compound **5** showed some cytotoxicity [IC_{50}: 10.1 μM (NCI-H460); 2.54 μM (MDA-MB-231)], while lycotetraose and acetylated glycoside **5** did not show cytotoxicity (>50 μM).

In conclusion, a simple preparation of β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-D-galactopyranose (lycotetraose) was obtained by the enzymatic hydrolysis of α-tomatine by tomatinase present in culture filtrates of *F. oxysporum* f. sp. *lycopersici*. Despite having a C-2 acetyl protecting group, which participates in neighboring reactions to



Scheme 3. Trans-glycosylation using lycotetraosyl fluoride **6**.



Scheme 4. Trans-glycosylation using lycotetraosyl thioglycoside **7**.

Table 1. Inhibitory effects on the growth of two human cancer cell lines [IC_{50} (μM)]

	NCI-H460	MDA-MB-231
CDDP	0.48	11.6
Desgalactotigonin	2.82	3.35
α-Tomatine	1.79	1.88
Lycotetraose	>50	>50
4	>50	>50
5	10.1	2.54

form a predominantly 1,2-*trans*-β-glycosyl bond, only the unnatural α-glycoside was synthesized, irrespective of the glycosyl donor (**3**, **6**, or **7**) used. It is noteworthy that our experiments show that neighboring group participation in these examples is far from sufficient to ensure glycosylation in complex oligosaccharide transglycosylation. Since the unnatural lycotetraosyl derivative **5** showed inhibitory effects in two human cancer cell lines that were comparable with those of the natural glycosides desgalactotigonin and α-tomatine, it might be possible to alter both the stereochemistry of the anomeric center of the reducing end of the lycotetraosyl glycosides and the steroidal aglycone moieties in order to produce bioactivity. To investigate the structure–activity relationship in detail, a number of lycotetraosyl derivatives will be synthesized in due course. Thus, the transglycosylation method developed here could be applied in the synthesis of novel bioactive glycosides.

Acknowledgements

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23. Selected spectroscopic data: compound **1**: $[\alpha]_D^{25}$ −13.2 (*c* 0.13, $CHCl_3$); HR positive FAB-MS (*m*/*z*): 1205.3412 $[M+Na]^+$ (calcd for $C_{49}H_{66}O_{33}Na$; 1205.3384); ^1H NMR (in $CDCl_3$) δ: (α anomer) 4.26 (1H, d, $J = 7.3$ Hz, i-glc H-1), 4.87 (1H, d, $J = 7.3$ Hz, t-glc H-1), 5.16 (1H, d, $J = 1.8$ Hz, xyl H-1), 6.31 (1H, d, $J = 3.7$ Hz, gal H-1); (β anomer) 4.29 (1H, d, $J = 7.9$ Hz, i-glc H-1), 4.86 (1H, d, $J = 7.9$ Hz, t-glc H-1), 5.18 (1H, d, $J = 1.8$ Hz, xyl H-1), 5.73 (1H, d, $J = 8.5$ Hz, gal H-1).
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26. Selected spectroscopic data: compound **2**: $[\alpha]_D^{25}$ −12.5 (*c* 0.31, $CHCl_3$); HR positive FAB-MS (*m*/*z*): 1163.3296 $[M+Na]^+$ (calcd for $C_{47}H_{64}O_{32}Na$; 1163.3278); ^1H NMR (in $CDCl_3$) δ: (α anomer) 4.29 (1H, d, $J = 7.3$ Hz, i-glc H-1), 4.88 (1H, d, $J = 7.9$ Hz, t-glc H-1α), 5.16 (1H, d, $J = 2.4$ Hz, xyl H-1), 5.37 (1H, d, $J = 3.7$ Hz, gal H-1); (β anomer) 4.23 (1H, d, $J = 7.3$ Hz, i-glc H-1), 4.54 (1H, d, $J = 7.9$ Hz, gal H-1), 4.86 (1H, d, $J = 7.9$ Hz, t-glc H-1), 5.13 (1H, d, $J = 3.1$ Hz, xyl H-1). Compound **3**: Positive FAB-MS (*m*/*z*): 1308 $[M+Na]^+$; ^1H NMR (in $CDCl_3$) δ: 4.27 (1H, d, $J = 7.9$ Hz, i-glc H-1), 4.87 (1H, d, $J = 7.9$ Hz, t-glc H-1), 5.18 (1H, d, $J = 2.4$ Hz, xyl H-1α), 6.53 (1H, d, $J = 3.7$ Hz, gal H-1α), 8.62 (1H, s, N*H*).
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28. Selected spectroscopic data: compound **4**: $[\alpha]_D^{25}$ +17.2 (*c* 0.15, $CHCl_3$); HR positive FAB-MS (*m*/*z*): 1531.6755 $[M+Na]^+$ (calcd for $C_{74}H_{108}O_{32}Na$; 1531.6721); ^1H NMR (in $CDCl_3$) δ: 0.67 (3H, s, H_3-18), 0.862 (3H, d, $J = 6.1$ Hz, H_3-26), 0.863 (3H, d, $J = 6.7$ Hz, H_3-27), 0.91 (3H, d, $J = 6.7$ Hz, H_3-21), 0.99 (3H, s, H_3-19), 4.22 (1H, d, $J = 7.9$ Hz, i-glc H-1), 4.86 (1H, d, $J = 7.9$ Hz, t-glc H-1), 5.11 (1H, d, $J = 3.7$ Hz, gal H-1), 5.17 (1H, d, $J = 2.4$ Hz, xyl H-1). Compound **5**: $[\alpha]_D^{25}$ +16.0 (*c* 0.10, MeOH); HR ESI-MS (*m*/*z*): 1027.54542 $[M+Na]^+$ (calcd

for $C_{50}H_{84}O_{20}Na$; 1027.54536); ^1H NMR (in pyridine-d_5) δ: 0.67 (3H, s, H_3-18), 0.902 (3H, d, $J = 6.7$ Hz, H_3-26), 0.904 (3H, d, $J = 6.7$ Hz, H_3-27), 0.95 (3H, s, H_3-19), 0.98 (3H, d, $J = 6.7$ Hz, H_3-21), 5.16 (1H, d, $J = 7.9$ Hz, i-glc H-1), 5.27 (1H, d, $J = 7.9$ Hz, xyl H-1), 5.47 (1H, d, $J = 3.1$ Hz, gal H-1), 5.67 (1H, d, $J = 7.9$ Hz, t-glc H-1).

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30. Selected spectroscopic data: compound **6**: (α-form) $[\alpha]_D^{25}$ −21.6 (*c* 0.26, $CHCl_3$); HR positive FAB-MS (*m*/*z*): 1165.3208 $[M+Na]^+$ (calcd for $C_{47}H_{63}FO_{31}Na$; 1165.3235); ^1H NMR (in $CDCl_3$) δ: 4.27 (1H, d, $J = 7.9$ Hz, i-glc H-1), 4.86 (1H, d, $J = 7.9$ Hz, t-glc H-1), 5.17 (1H, d, $J = 2.4$ Hz, xyl H-1), 5.68 (1H, dd, $J_{1,F} = 53.7$ Hz, $J_{1,2} = 3.1$ Hz, gal H-1); ^{13}C NMR (in $CDCl_3$) δ: 96.8 (xyl C-1), 99.4 (t-glc C-1), 101.0 (i-glc C-1), 104.7 (d, $J_{1,F} = 226.2$ Hz, gal C-1).
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33. Selected spectroscopic data: compound **7**: (α-form) $[\alpha]_D^{25}$ +69.7 (*c* 0.1, $CHCl_3$); Positive ESI-MS (*m*/*z*): 1269 $[M+Na]^+$; ^1H NMR (in $CDCl_3$) δ: 4.24 (1H, d, $J = 7.3$ Hz, i-glc H-1), 4.86 (1H, d, $J = 7.9$ Hz, t-glc H-1), 5.19 (1H, d, $J = 2.4$ Hz, xyl H-1), 5.83 (1H, d, $J = 5.5$ Hz, gal H-1); ^{13}C NMR (in $CDCl_3$) δ: 85.2 (gal C-1), 96.9 (xyl C-1), 99.4 (t-glc C-1), 101.0 (i-glc C-1); (β-form) $[\alpha]_D^{25}$ −16.2 (*c* 0.1, $CHCl_3$); Positive ESI-MS (*m*/*z*): 1269 $[M+Na]^+$; ^1H NMR (in $CDCl_3$) δ: 4.31 (1H, d, $J = 7.3$ Hz, i-glc H-1), 4.60 (1H, d, $J = 10.4$ Hz, gal H-1), 4.88 (1H, d, $J = 7.8$ Hz, t-glc H-1), 5.14 (1H, d, $J = 3.1$ Hz, xyl H-1); ^{13}C NMR (in $CDCl_3$) δ: 87.8 (gal C-1), 97.5 (xyl C-1), 99.4 (t-glc C-1), 101.1 (i-glc C-1).
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