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Synthesis of *mono*-glucose-branched cyclodextrins with a high inclusion ability for doxorubicin and their efficient glycosylation using *Mucor hiemalis endo*-β-*N*-acetylglucosaminidase

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Abstract—The *mono*-glucose-branched cyclodextrins having an appropriate spacer between the β-cyclodextrin and a glucose moiety were synthesized from β-cyclodextrin and arbutin. They had the significantly high association constants for doxorubicin, the anticancer agent, in the range of 10^5–10^6 M^{-1}, and worked as highly reactive glycosyl acceptors for the transglycosylation reaction by *endo*-β-*N*-acetylglucosaminidase of *Mucor hiemalis* to produce sialo-complex type oligosaccharide-branched cyclodextrins in the high yields of 65–67%.

Saccharides are known to be involved in a number of significant biological recognition phenomena on the surfaces of cell membranes. Cyclodextrins (CDs) have the ability to carry drugs in their cavities. Therefore, the CD derivatives conjugated with saccharide(s) are expected to be drug carrier molecules capable of specific cell recognition, and might be useful as targeting drug-delivery systems.[1] However, no targeting drug carrier using the CDs has yet been reported. In order to obtain effective drug carrier molecules based on the CD–saccharide(s), efficient methods for binding saccharide(s) to the CDs and for increasing the drug inclusion ability of the CD cavities are required.

To synthesize the CD–saccharide(s) conjugates is a challenging objective. Enzymatic glycosylations have often been utilized for synthesizing them.[1p,q] The *endo*-β-*N*-acetylglucosaminidase of *Mucor hiemalis* (Endo-M) has proved to be a practical tool to synthesize glycoconjugates by transferring the natural oligosaccharide blocks from asparagine-linked glycopeptides to acceptors.[2] In our previous study,[3] we found that the enzyme had the transglycosylation ability of transferring the oligosaccharides to the CD derivative prepared from *N*-α-Fmoc-*N*-ω-(2-acetamide-2-deoxy-β-D-glucopyranosyl)-asparagine and 6-mono-amino-β-CD. Three kinds of natural (sialo-complex type, asialo-complex type, and high-mannose type)[4]oligosaccharide-branched CDs were successfully obtained although the transglycosylation yields were only 6–12%.

This letter describes an efficient method for preparing targeting drug carriers based on the CD–saccharide(s) molecules using Endo-M. We designed novel CD derivatives which were expected to work as highly reactive glycosyl acceptors of the Endo-M transglycosylation and to have a high drug inclusion ability. The molecular

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interaction between the CD derivatives and the anticancer agent, doxorubicin (DXR), was evaluated using the SPR optical biosensor, and the transglycosylation reaction by recombinant Endo-M to the CD derivatives was examined.

Our approach was to introduce the commercially available 4-hydroxyphenyl-β-D-glucopyranoside[5] (natural product called arbutin **1**) into the CDs in order to synthesize the *mono*-glucose-branched CDs, which may function as the glycosyl acceptors of the Endo-M transglycosylation. The reasons were as follows: (1) As Endo-M showed a high transglycosylation activity on the β-isomer of glucopyranose among the naturally occurring hexopyranoses, the transglycosylation reaction was expected to efficiently proceed on the β-glucopyranose of the arbutin. (2) We speculated that the phenyl group of the arbutin linked with CD through an appropriate spacer would exhibit a structure like the cap of the CD, and this 'pseudo-capping structure' and the hydrophobicity of the phenyl group would increase the hydrophobicity of the CD cavity. It was also speculated that when DXR was included into the CD cavities, the stacking complex by the π–π interaction between the phenyl group and DXR would be formed as shown in Figure 1. These effects were expected to enhance the inclusion association between these CD derivatives and DXR.

First, we synthesized the *mono*-glucose-branched CDs (**9** and **14**) as the glycosyl acceptors of the Endo-M transglycosylation. The reaction of allyl bromide (1.2 equiv) and the dry sodium salt of arbutin **1**, which was obtained by the treatment of **1** with NaOH (1 equiv) in H_2O, was carried out in DMF for 24 h and produced

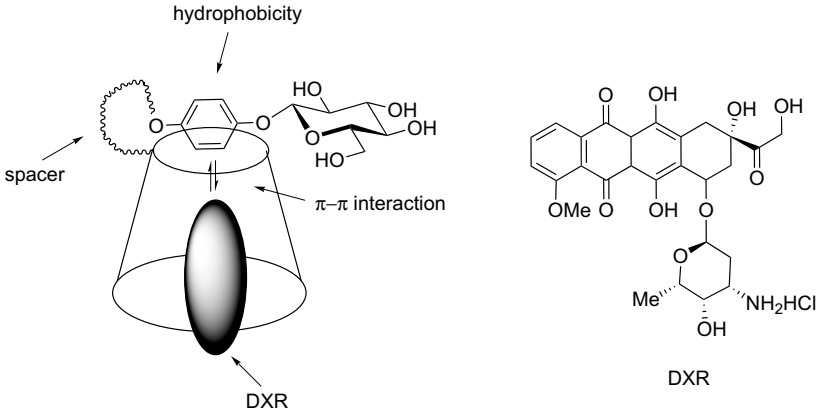
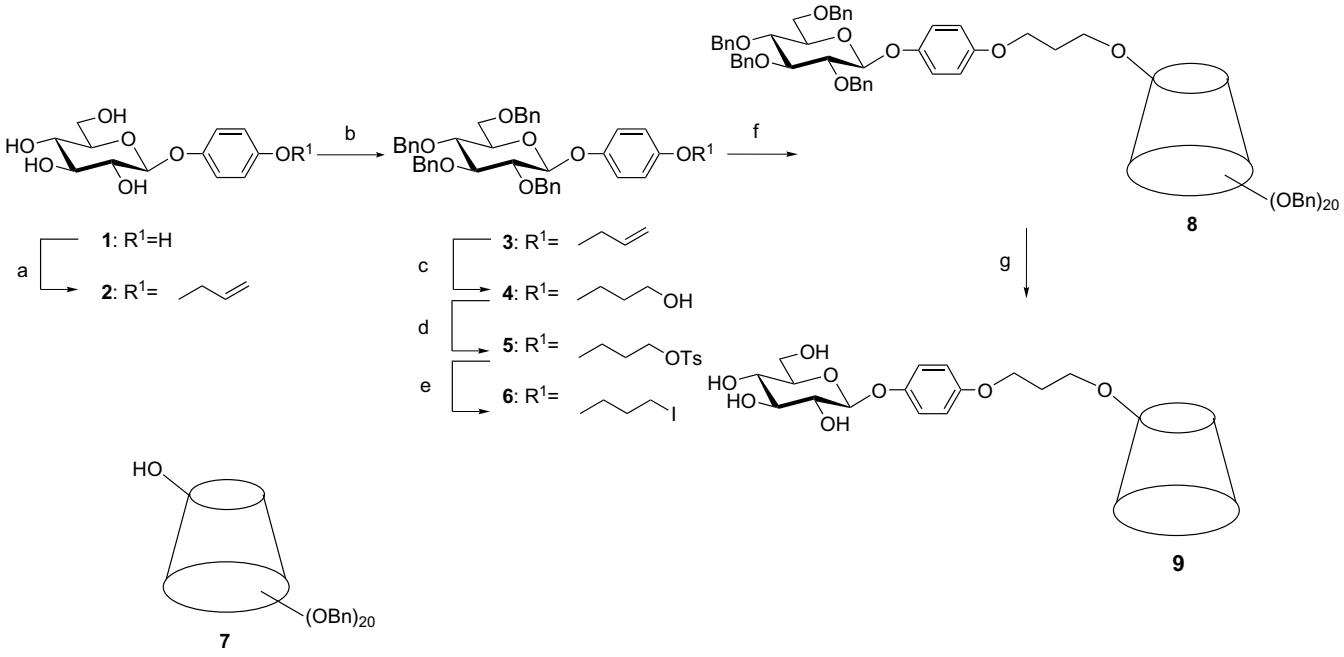
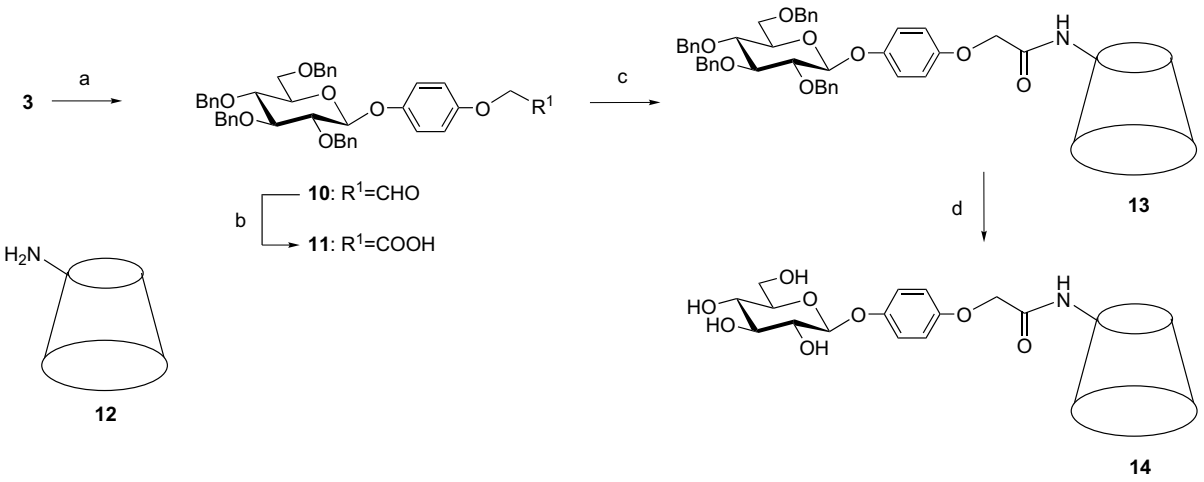


Figure 1.



Scheme 1. Reagents and conditions: (a) NaOH/H_2O, then dry, AllBr/DMF, 99%; (b) NaH, BnBr/DMF, 91%; (c) 9-BBN/THF, then NaOHaq, H_2O_2aq, 95%; (d) TsCl, Et_3N/CH_2Cl_2, quant; (e) NaI/DMF, 60 °C, 92%; (f) KOH, **7**/DMF, 51%; (g) H_2/Pd(OH)$_2$/ether–MeOH–H_2O, then gel-filtration, 73%.



Scheme 2. Reagents and conditions: (a) O_3, Ph_3P/CH_2Cl_2, 97%; (b) 2-methyl-2-butene, NaH_2PO_4, $NaClO_2$/*t*-BuOH–H_2O, 98%; (c) $Me_2P(S)Cl$, DIEA, DMF; (d) $H_2/Pd(OH)_2$/ether–MeOH–H_2O, then gel-filtration, 63% from **11**.

Table 1. Kinetic parameters of CD derivatives (**9** and **14**) for the association with the immobilized DXR

Entry	CD derivative	$K_a \times 10^3$ M^{-1}	$k_{ass} \times 10^3$ M^{-1} s^{-1}	$k_{diss} \times 10^{-2}$ s^{-1}
1	**9**	2.2×10^2	4.6 ± 0.6	2.1 ± 0.3
2	**14**	5.3×10^3	18 ± 2	0.34 ± 0.2
3	**15**	3.1	0.12 ± 0.005	4.1 ± 0.3

the allylated compound **2**, followed by separation using silica-gel column chromatography (chloroform/methanol = 8:1) in 99% from **1**. The benzylation of **2** using benzyl bromide (12 equiv) and NaH (16 equiv) in DMF was carried out for 3 d, and afforded the compound **3**, which was purified by silica-gel column chromatography (hexane/ethyl acetate = 4:1) in 91%. The hydroboration of **3** with 9-BBN (2 equiv) in THF at 0 °C for 24 h, followed by oxidation using aq H_2O_2 (10 equiv) and by hydrolysis with aq NaOH (3 equiv) for 24 h gave the compound **4**, which was purified by silica-gel column chromatography (hexane/ethyl acetate = 4:1) in 95%. The reaction of **4** with TsCl (4 equiv) in pyridine for 5 h quantitatively gave **5**, followed by separation on preparative silica-gel TLC (hexane/ethyl acetate = 2:1). The treatment of **5** with NaI (4 equiv) in DMF at 60 °C for 1.5 h afforded **6**, followed by separation on preparative TLC (hexane/ethyl acetate = 2:1) in 92%. The reaction of **6** (4.5 equiv) with the benzylated β-CD **7**[6] using KOH (120 equiv) in DMF for 24 h gave **8**, which was purified by preparative silica-gel TLC (hexane/ethyl acetate = 2:1) in 51%. The treatment of **8** with H_2–$Pd(OH)_2$ in ether–methanol–H_2O for 24 h provided the desired compound **9**, which was purified by gel-filtration using Sephadex G25 (5% EtOH aq) in 73% (Scheme 1).

The ozonization of **3** in the presence of Ph_3P (3 equiv) gave **10**, followed by separation on silica-gel preparative TLC (hexane/ethyl acetate = 1:1) in 97%. The oxidation of **10** using $NaClO_2$ (10 equiv)–NaH_2PO_4 (1.5 equiv) in the presence of 2-methyl-2-butene in *t*-BuOH–H_2O afforded the compound **11**, which was purified by silica-gel column chromatography (chloroform/methanol = 5:1) in 98%. The condensation of **11** (1 equiv) with 6-*mono*-amino-β-CD **12** using $Me_2P(S)Cl$ (1 equiv) was carried out in the presence of DIEA (1 equiv) in DMF for 4 d, and gave the crude **13**. The following debenzylation of **13** by H_2–$Pd(OH)_2$ afforded the desired product **14**, which was purified by gel-filtration using Sephadex G25 (5% EtOH aq) in 63% (Scheme 2).

Next, we estimated the inclusion associations of **9** and **14** with the immobilized DXR by the SPR biosensor according to the reported method.[1a] The association constants (K_a), association rate constants (k_{ass}) and dissociation rate constants (k_{diss}) of **9** and **14** are summarized in Table 1 and compared with the previously reported data of the CD derivative **15**, which has no phenyl group (Figure 2). The k_{ass} and k_{diss} values of **9** were 4.6×10^3 M^{-1} s^{-1} and 2.1×10^{-2} s^{-1}, and those of **14** were 1.8×10^4 M^{-1} s^{-1} and 3.4×10^{-3} s^{-1}. The K_a values of **9** and **14** were calculated to be 2.2×10^5 M^{-1}

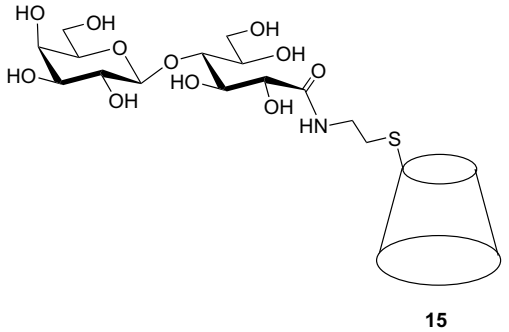


Figure 2.

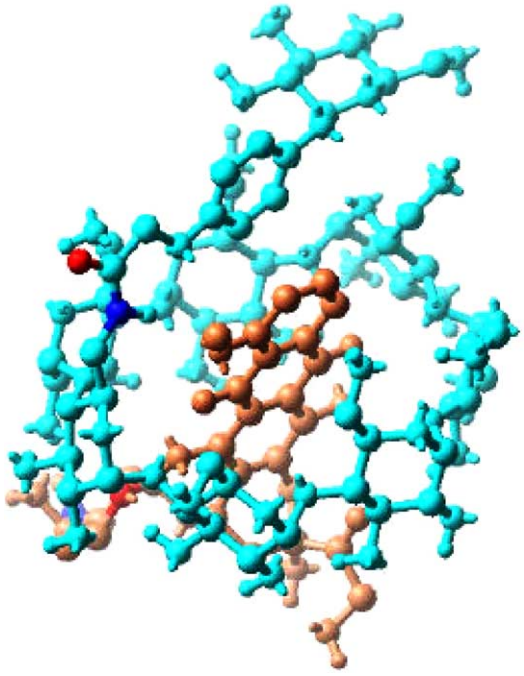


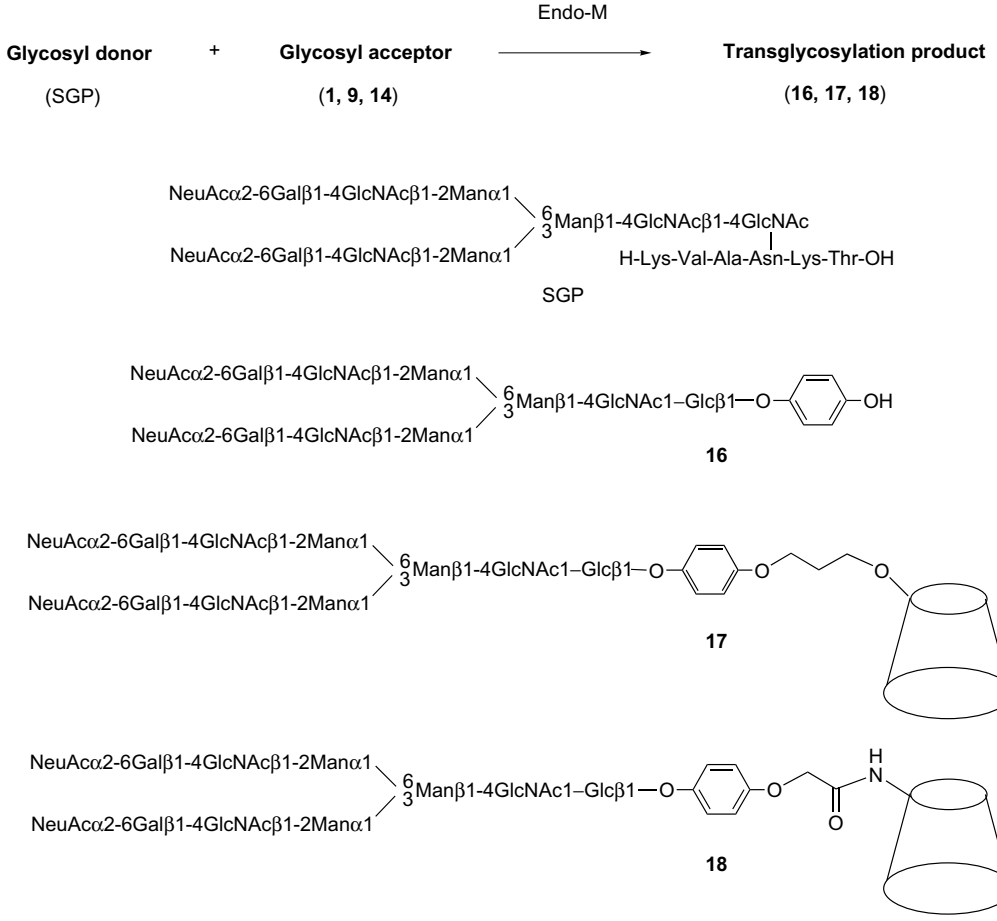
Figure 3. Molecular model of the inclusion association between **14** and DXR.

and $5.3 \times 10^6\ M^{-1}$, respectively. The CD derivatives **9** and **14** indicated the significantly high association constants for DXR in the range of 10^5–$10^6\ M^{-1}$. Particularly, the K_a value of **14** was 1700 times higher (the k_{ass} value was 150 times higher and the k_{diss} value was 12 times lower) than that of **15**. As expected, the phenyl groups of **9** and **14** had a remarkable effect of enhancing the inclusion interactions with the immobilized DXR. The molecular model of **14** including DXR also supported our speculation (Fig. 3). The comparison of the K_a values of **9** and **14** suggested that **14** would form a more rigid 'capping structure' than **9** due to the generation of the hydrogen bonds by the existence of the amide group of the spacer.

Finally, we investigated the synthesis of natural oligosaccharide-branched CDs from **9** and **14** by the transglycosylation activity of the recombinant Endo-M using

Table 2. Transglycosylations using recombinant Endo-M to **1**, **9**, **14**

Entry	Glycosyl acceptor	Product	Transglycosylation yield/%
1	**1**	**16**	56
2	**9**	**17**	67
3	**14**	**18**	65



Scheme 3. Reaction conditions: 0.1 μmol of glycosyl acceptor, 0.2 μmol of SGP, and 1.48 mU of Endo-M in a total volume of 10 μL of 60 mM potassium phosphate buffer (pH 6.25) for 2 h at 37 °C.

the hen egg yolk glycopeptide, H-Lys-Val-Ala-Asn-[(NeuAc-Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$]-Lys-Thr-OH (SGP), as the oligosaccharide donor. The transglycosylation reaction was carried out using 0.2 μmol of the oligosaccharide donor (SGP), 0.1 μmol of the glycosyl acceptors (**1**, **9** and **14**), and 1.48 mU of recombinant Endo-M according to the reported procedure.[7] First, arbutin **1** was used as the glycosyl acceptor in order to ascertain its reactivity in the Endo-M transglycosylation. After incubation for 2 h at 37 °C, HPLC analysis of the reaction mixture indicated that the transglycosylation reaction proceeded with the good yield of 56%. In general, after the yields of the Endo-M transglycosylation reached the maximum, the transglycosylation product was gradually degraded by the hydrolytic activity of Endo-M. In the case of **1** used as the glycosyl acceptor, the degradation of the transglycosylation product of **16** was hardly observed even after 5 h. When **9** and **14** were used as the glycosyl acceptors, the corresponding sialo complex-type oligosaccharide-branched CDs **17** and **18** were similarly obtained in the high yields of 67% and 65%, respectively.[8] Both yields using **9** and **14** were slightly higher than that of **1**. The CD cavities in these glycosyl acceptors might influence the transglycosylation activity of the enzyme. Thus we could successfully develop an efficient Endo-M transglycosylation system to produce oligosaccharide-branched CDs (Table 2, Scheme 3).

In summary, we could synthesize the *mono*-glucose-branched CDs, which had an appropriate spacer between the β-cyclodextrin and a glucose moiety, from β-CD and arbutin. The obtained *mono*-glucose-branched cyclodextrins indicated significantly high association constants for doxorubicin in the range of 10^5–10^6 M^{-1}. In addition, they worked as highly reactive glycosyl acceptors for the transglycosylation reaction by Endo-M to produce sialo-complex type oligosaccharide-branched CDs in the high yields of 65–67%. The arbutin derivatives can be widely used as the useful tags of various substrates for the Endo-M transglycosylation. Their introduction into CDs through an appropriate spacer can also enhance the CD's inclusion ability for drugs having an aromatic ring like DXR. These findings will contribute to the development of drug carriers for targeting drug-delivery systems.

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8. Analyses or isolation of the transglycosylation product were done using HPLC (Shimadzu LC-10AT chromatograph equipped with a SPD-10A ultraviolet spectrophotometer) on a reversed-phase column (4.6 × 250 mm, Inertsil ODS-3, GL Sciences, Inc.). Elution was carried out with a linear gradient of acetonitrile (5–35%) containing 0.1% aqueous trifluoromethanesulfonic acid (TFA) in 30 min at the flow rate of 1 mL/min. The reaction products were monitored by absorptions at 214 nm. MALDI-TOF MS; **17**: Found: *m/z* $[M-H]^-$ 3450.5: calcd for $C_{133}H_{213}N_5O_{98}[M-H]^-$ 3447.2; **18**: Found: *m/z* $[M+K]^+$ 3484.6: calcd for $C_{132}H_{210}N_6O_{98}$ $[M+K]^+$ 3486.1.