Enzymatic Synthesis of Specifically Modified Linear Oligosaccharides from γ-Cyclodextrin Derivatives. Study on Importance of Active Sites of Taka Amylase A[1)]

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(Received May 11, 1989)

Specifically modified linear oligosaccharides were prepared by enzymatic hydrolysis of 6- or 2-*O*-substituted γ-cyclodextrin by Taka amylase A. These results suggest that the importance of the interactions between hydroxyl groups and the subsites (P—V) of Taka amylase A decreases in the following order; for C-6-OH, S>R>T>Q,U and for C-2-OH, R,S>T>Q, U.

Substrate specificity of Taka amylase A[2-8)] has been successfully studied by use of partially *O*-methylated amylose and specifically modified phenyl maltosides in order to obtain information about interaction between the subsites (Fig. 1)[6-8)] of Taka amylase A and substrates.[9-14)] We have reported a novel synthetic method for the preparation of specifically modified linear oligosaccharides by Taka amylase A degradation of α-cyclodextrin (α-CD) or β-cyclodextrin (β-CD) derivatives.[15-21)] This also provided new information about interactions between substrates and the subsites of Taka amylase A. The Taka amylase A-catalyzed hydrolysis of 6-*O*-substituted cyclodextrins (CDs)[16,17,22)] and $6^A,6^X$-*O*-disubstituted (X=B, C, or D) CDs[15-17)] revealed that interaction between subsite S and the C-6-OH was important. Also, the Taka amylase A-catalyzed hydrolysis of 2-*O*-substituted CDs disclosed the importance of interaction between the C-2-OH and subsites R and S.[21)] However, these experiments using the α- or β-CD derivatives can not tell us about the importance of other subsites. The reason is that the slow rate of cleavage of the CD macrocyclic ring does not allow accumulation of intermediate linear oligosaccharides in enough amount to be isolated. However, since γ-cyclodextrin (γ-CD) is hydrolyzed much faster than α- or β-CD by Taka amylase A,[23)] the hydrolysis of γ-CD derivatives can reasonably be expected to give new specifically modified linear oligosaccharides (new intermediates in the enzymatic hydrolysis) which can not be isolated in the hydrolysis of α- or β-CD. Also, this result will provide novel information concerning the importance of the subsites. We describe here Taka amylase A-catalyzed hydrolysis of 6-*O*-tosyl-γ-cyclodextrin (**1**), 6-*O*-(1-naphthylsulfonyl)-γ-cyclodextrin (**2**), 6-*O*-(2-naphthylsulfonyl)-γ-cyclodextrin (**3**), 2-*O*-(1-naphthylsulfonyl)-γ-cyclodextrin (**4**), and 2-*O*-(2-naphthylsulfonyl)-γ-cyclodextrin (**5**).

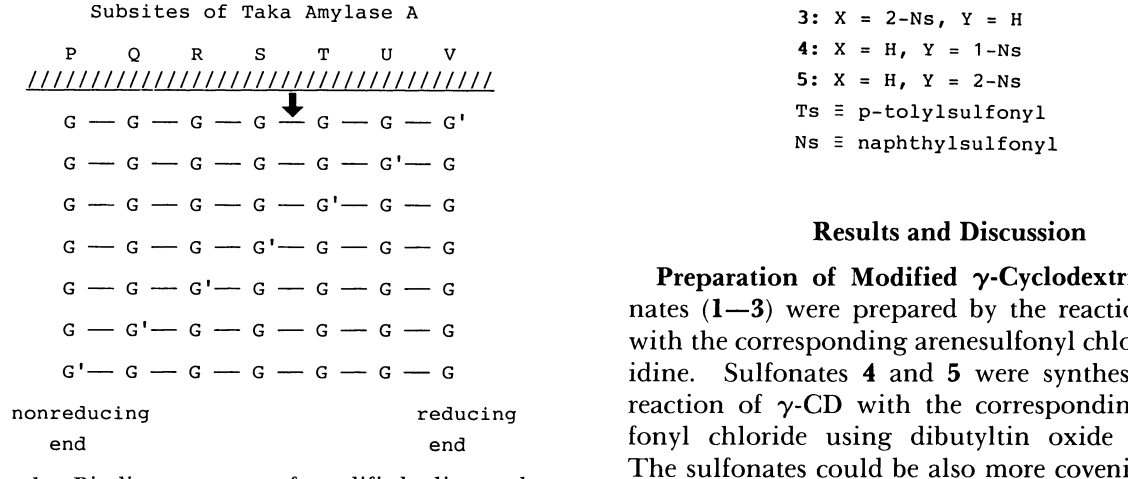
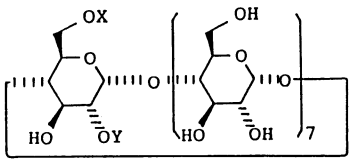


Fig. 1. Binding patterns of modified oligosaccharide to Taka amylase A.
P—V: subsite, G: glucose unit, G′: modified glucose unit, ↓: cleavage point of glucosidic bond.



1: X = Ts, Y = H
2: X = 1-Ns, Y = H
3: X = 2-Ns, Y = H
4: X = H, Y = 1-Ns
5: X = H, Y = 2-Ns
Ts ≡ p-tolylsulfonyl
Ns ≡ naphthylsulfonyl

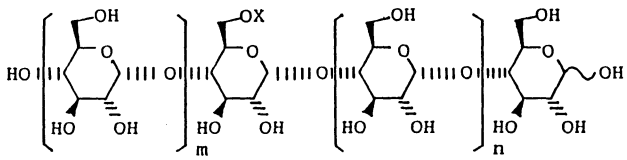
Results and Discussion

Preparation of Modified γ-Cyclodextrins. Sulfonates (**1—3**) were prepared by the reaction of γ-CD with the corresponding arenesulfonyl chloride in pyridine. Sulfonates **4** and **5** were synthesized by the reaction of γ-CD with the corresponding arenesulfonyl chloride using dibutyltin oxide in DMF.[24)] The sulfonates could be also more coveniently, however in lower yield, synthesized by the reaction of γ-CD with the corresponding arenesulfonyl chloride in water (see Experimental).

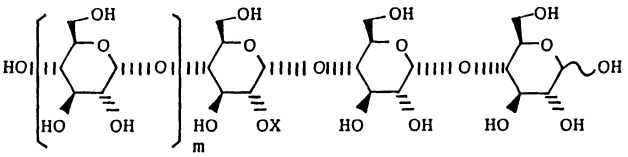
Taka Amylase A-Catalyzed Hydrolysis of the Modified γ-Cyclodextrins. The γ-CD derivatives were hydrolyzed by Taka amylase A to give specifically modified linear oligosaccharides. The reaction conditions, products, and yields are summarized in Table 1.

The modified maltose (**12**) or the modified maltotriose (**17** or **18**) was identified by comparing its high-performance liquid chromatography (HPLC) retention time and its fast atom bombardment mass (FAB MS) spectrum with those of the corresponding authentic compound.[21,22] The other 6′-*O*-(arylsulfonyl)maltoses (**13** and **14**) were converted to *S*-phenyl-6′-thiomaltose[16,17] and then identified similarly. Structures of other linear oligosaccharides were determined as follows. All compounds showed FAB MS spectra and ^{13}C NMR spectra consistent with their structures, whereas the position of sulfonylation in the chain of oligosaccharide was not determined by the spectral data. Sulfonates **6** and **9** were reduced with $NaBH_4$ to give **19** and **20**, respectively, followed by complete acetylation with acetic anhydride in pyridine. The resultant acetates (**21** and **22**) were analyzed by electron impact mass (EI MS) spectra in order to determine the sulfonylation position. The results are shown in Fig. 2. The sulfonate, **10**, or **11**, was converted to the 6″-phenylthio derivative (**23**) which was identified by comparing its HPLC retention time and its FAB MS spectrum to those of an authentic sample prepared from the reaction of **9** with thiophenol.

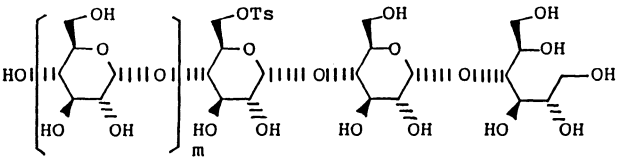
The sulfonate, **7** or **8**, was converted to the 3,6-anhydro derivative (**24**) by treatment with $Ba(OH)_2$ in water and identified by comparing its HPLC retention time and its FAB MS spectrum to those of the authentic compound which was prepared by the reaction of **6** with aqueous $Ba(OH)_2$. Authentic 3″,6″-anhydromaltotetraose (**24**) was also prepared by Taka-amylolysis of 3,6-anhydro-γ-cyclodextrin.[25,26]



6: m = 1, n = 1, X = Ts
7: m = 1, n = 1, X = 1-Ns
8: m = 1, n = 1, X = 2-Ns
9: m = 0, n = 1, X = Ts
10: m = 0, n = 1, X = 1-Ns
11: m = 0, n = 1, X = 2-Ns
12: m = 0, n = 0, X = Ts
13: m = 0, n = 0, X = 1-Ns
14: m = 0, n = 0, X = 2-Ns
23: m = 0, n = 1, OX = S-C_6H_5



15: m = 1, X = 1-Ns
16: m = 1, X = 2-Ns
17: m = 0, X = 1-Ns
18: m = 0, X = 2-Ns



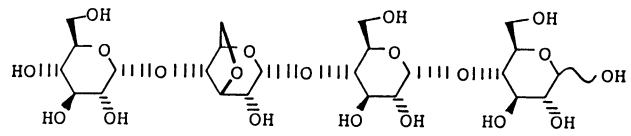
19: m = 1
20: m = 0

On reaction with aqueous $Ba(OH)_2$, the secondary sulfonate, **15** or **16**, gave an epoxide (**25**) which was identified by comparing its HPLC retention time and

Table 1. Taka Amylase A-Catalyzed Hydrolysis of Modified γ-Cyclodextrins[a,b]

CD derivative mg	Reaction temperature	Reaction period	Product
1 (50)	40 °C	3 h	**12** (13.6 mg, 79.5%)
(100)	10 °C	2 h	**6** (17.9 mg, 31.6%)+**9** (19.8 mg, 43.6%)
(100)	10 °C	2 days	**12** (34 mg, 99.4%)
2 (80)	40 °C	4 h	**13** (19.6 mg, 68.6%)
(63)	10 °C	1.5 h	**7** (12.9 mg, 35.5%)+**10** (16 mg, 54.4%)
3 (70)	40 °C	3 h	**14** (24.8 mg, 98.8%)
(200)	10 °C	2 h	**8** (33.2 mg, 28.8%)+**11** (27.2 mg, 29.1%)
(100)	10 °C	2 days	**14** (27.1 mg, 75.7%)
4 (20)	40 °C	3 h	**17** (6.3 mg, 67.7%)
(39.5)	10 °C	2.5 h	**15** (9.8 mg, 43%)+**17** (6.1 mg, 33.2%)
(50)	10 °C	1 day	**17** (15.8 mg, 67.5%)
5 (50)	40 °C	3 h	**18** (19 mg, 81.2%)
(80)	10 °C	1.5 h	**16** (13.2 mg, 28.6%)+**18** (8.8 mg, 23.5%)

a) Solvent, 0.1 mol dm^{-3} acetate buffer (pH 5.5) containing calcium chloride (0.01 mol dm^{-3}).
b) The concentrations of the γ-cyclodextrin derivative and Taka amylase A were each 10 mg/(1 cm^3 of solvent).



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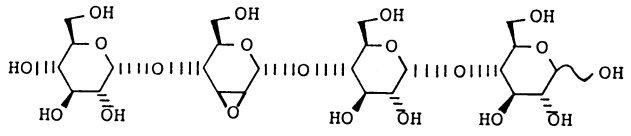

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its FAB MS spectrum to those of the authentic sample.[21]

α- or β-CD analogues of **1**, **2** or **3** and **4** or **5** gave only G′-G and G′-G-G, respectively, upon Taka amylase A-catalyzed hydrolysis at 40 °C[21] (Hereafter, the following abbreviations are used: G, glucose unit; G′, modified glucose unit.). In an attempt to obtain intermediate linear oligosaccharides, reactions at lower temperature (10 °C or 0 °C) were carried out. However, the reaction rates were too slow to allow accumulation of stable linear oligosaccharide intermediates. The derivatives of γ-CD (**1**—**5**) were enzymatically hydrolyzed much more rapidly than the α- and β-CD analogues at 40 °C, although **1**—**3**, and **4** or **5** gave G′-G and G′-G-G, respectively, at this temperature similarly to the α- and β-CD analogues (Table 1). This suggests the accumulation of intermediates at lower temperature in the case of γ-CD derivatives.

The enzymatic hydrolysis of **1**—**3** at 10 °C afforded a mixture of G′-G-G and G-G′-G-G. Amylolyses of **1**—**3** at 40 °C or at 10 °C for longer reaction period (2 days) gave G′-G exclusively, demonstrating that G′-G-G and G-G′-G-G were the intermediates and that G-G′-G-G was a precursor of G′-G-G which led to G′-G. The enzymatic hydrolysis of **4** and **5** at 10 °C afforded a mixture of G′-G-G and G-G′-G-G. The reactions of **4** and **5** at 40 °C or 10 °C for 1 day gave G′-G-G, showing that G-G′-G-G was an intermediate leading to G′-G-G. Taka amylase A has seven subsites (P—V) in the active site and an oligosaccharide is cleaved between S and T subsites as shown in Fig. 1. The results with **1**—**3** demonstrate that interaction of the C-6-OH with the subsites R and T is important, but that interaction with T is less important than that with R and much less important than that with S. The results with **4** and **5** demonstrate that interaction of the C-2-OH with T is less important than those with R and S. Thus, the order of importance of the subsites is as follows. For C-6-OH, S>R>T>Q,U and for C-2-OH, R, S>T>Q, U. Unfortunately, estimation of importance of the subsites Q, U, P, and V was not possible because the higher oligosaccharides did not accumulate in enough amount to be isolated in this reaction system. It is interesting to note that difference in kind of modification (Ts, 1-Ns or 2-Ns) on the hydroxyl group of saccharide did not influence the kind of product (G′-G, G-G′-G-G, etc.) and that only the position of the modification (2-O or 6-O) determined the kind of product.

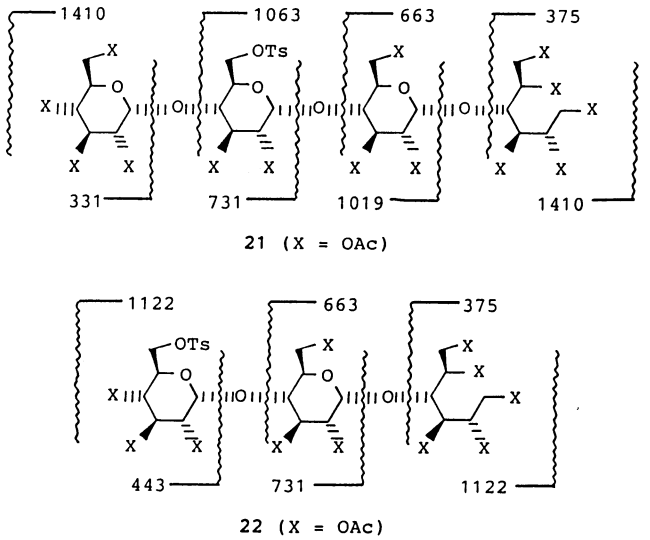

Fig. 2. EI MS spectral fragmentation patterns of **21** and **22**, which also showed correct molecular ions in FD MS spectra.

As mentioned before, the specifically activated (sulfonylated) oligosaccharides **6**—**11**, **15**, and **16** descibed in the present study can not be isolated from the enzymatic hydrolysis of 6- and 2-*O*-arylsulfonyl-α- or β-CDs, even when Taka amylase A was used in an amount equal to that of the CD derivative and/or the reaction was carried out at 5 °C or 10 °C. On the other hand, purely chemical methods for the preparation of these compounds from smaller sugars will need troublesome repeats of activation, protection, and deprotection of hydroxyl groups of the sugars. One-step chemical synthesis of specifically modified linear oligosaccharides from the corresponding linear oligosaccharides is also impossible. This method will require tedious separation of many regioisomeric monosulfonylated oligosaccharides.

Since there does not exist any regiochemical isomer, 2- and 6-*O*-arylsulfonyl-γ-CDs can be easily prepared and easily isolated as a pure material. As shown above, they can be smoothly hydrolyzed to give one or two modified oligosaccharide(s) at 10 °C. Furthermore, the products can be effectively separated by reversed-phase column chromatography. Therefore, the selective chemical modification of γ-CD followed by the Taka amylase A-catalyzed hydrolysis has several advantages for preparation of specifically modified oligosaccharides.

Experimental

General. ^{13}CNMR spectra were determined with a

JEOL FX-90Q spectrometer (22.5 MHz) and a JEOL FX-100 spectrometer (25 MHz). Fast atom bombardment mass spectra (FAB MS), field desorption mass spectra (FD MS), and electron impact mass spectra (EI MS) were recorded with a JEOL JMX DX-300 or DX-303/JMA 5000 data system. Thin-layer chromatography (TLC) was run with precoated silica-gel plates (Merck, Art 5554). Spot detection was carried out by UV light and/or staining with 0.1% 1,3-naphthalenediol in EtOH-H_2O-H_2SO_4 (200 : 157 : 43 v/v/v). The solvent for TLC development was n-C_3H_7OH-AcOH-H_2O(7 : 7 : 5 v/v/v). A Merck Lobar prepeaked column (LiChroprep RP18 column, 25 mm×310 mm) and/or a Kusano prepacked column ODS (CPO-HS-221-20, 22 mm×100 mm) was used for reversed-phase column chromatography. High-performance liquid chromatography (HPLC) was performed on a Shimadzu LC3A with a Zorbax ODS column (4.6 mm×150 mm, 5 μm, DuPont) or with a TSKgel ODS-120T (4.6 mm×250 mm, 5 μm, TOSOH).

6-*O*-Arylsulfonyl-γ-cyclodextrins (1—3). p-Toluenesulfonyl chloride (12 g) was added to a solution of γ-CD (10 g) in pyridine (800 cm^3) and the mixture was stirred for 1 h. After addition of water (10 cm^3), the mixture was concentrated in vacuo and chromatographed on a reversed-phase column with gradient elution from water (1 dm^3) to 50% aqueous ethanol (1 dm^3) to give the monotosylate **1** (3.5 g, 31%). The other arylsulfonates **2** and **3** were similarly prepared and isolated in similar yields.

2-*O*-Arylsulfonyl-γ-cyclodextrins (4 and 5). The titled compound was synthesized by the reaction of γ-CD with the corresponding arenesulfonyl chloride using dibutyltin oxide in DMF following the method of Murakami et al.[24] The compounds **4** and **5** can be more coveniently, however in lower yields, prepared as follows. Powdered arenesulfonyl chloride (3.0 g) was added in one portion to 10 cm^3 of aqueous solution (pH 12.0, adjusted by addition of aqueous NaOH) of γ-CD (2.0 g). The suspension was vigorously stirred at room temperature and the pH of the suspension was allowed to decrease. After the mixture became neutral, it was filtered and chromatographed on a reversed-phase column with gradient elution from water (1 dm^3) to 30% aqueous CH_3CN (1 dm^3) to give **4** (117.0 mg) or **5** (235.2 mg).

4: ^{13}C NMR (25 MHz, Me_2SO-d_6, characteristic nonaromatic absorptions) δ=59.9, 69.0, 72.1, 72.4, 77.2, 80.4, 80.9, 81.1, 96.7, 101.6; FAB MS m/z 1487 ($M+H^+$).

5: ^{13}C NMR (25 MHz, Me_2SO-d_6, characteristic nonaromatic absorptions) δ=59.9, 69.0, 72.2, 72.4, 72.6, 72.8, 77.9, 79.9, 80.0, 80.3, 80.6, 80.9, 97.1, 101.5, 101.7; FAB MS m/z 1487 ($M+H^+$).

Taka Amylase A-Catalyzed Hydrolysis. Commercially available Taka amylase A (α-amylase from Aspergillus oryzae, Type X-A, Sigma) was used. A solution of Taka amylase A and a modified γ-CD (**1—5**) in acetate buffer solution (pH 5.5, 0.2 mol dm^{-3}) containing calcium chloride (0.01 mol dm^{-3}) was kept at 40 °C or 10 °C to give a limited mixture of oligosaccharides as main products. The enzymatic reaction was monitored by silica-gel TLC. Although the reaction at 40 °C gave mainly a single product (**12**, **13**, **14**, **17**, or **18**), a similar reaction for a shorter reaction period at 10 °C afforded mainly two products. After 1% aqueous HCl was added to make the solution acid (pH 2—3) for denaturation of the enzyme, the supernatant obtained by centrifugation of the mixture was filtered, and applied on a reversed-phase column. After elution with water to remove inorganic salts, gradient elution, changing from water to methanol or acetonitrile, was used to give pure products. The reaction conditions and the yields are shown in Table 1.

6: ^{13}C NMR (25 MHz, D_2O, characteristic nonaromatic absorptions) δ=23.5, 62.7, 63.1, 94.8, 98.4, 102.2, 102.6; FAB MS m/z 843 ($M+Na^+$).

7: ^{13}C NMR (25 MHz, D_2O, characteristic nonaromatic absorptions) δ=62.7, 63.1, 98.4, 101.7, 102.0, 102.4; FAB MS m/z 857 ($M+H^+$), 879 ($M+Na^+$).

8: ^{13}C NMR (25 MHz, D_2O, characteristic nonaromatic absorptions) δ=62.7, 63.2, 94.5, 98.4, 102.2, 102.6; FAB MS m/z 857 ($M+H^+$).

9: ^{13}C NMR (25 MHz, D_2O, characteristic nonaromatic absorptions) δ=23.5, 63.2, 94.5, 98.4, 102.2, 102.5; FAB MS m/z 659 ($M+H^+$).

10: ^{13}C NMR (25 MHz, D_2O, characteristic nonaromatic absorptions) δ=63.1, 94.5, 98.4, 102.2, 102.5; FAB MS m/z 695 ($M+H^+$).

11: ^{13}C NMR (25 MHz, D_2O, characteristic nonaromatic absorptions) δ=63.2, 98.4, 102.2, 102.5; FAB MS m/z 695 ($M+H^+$), 717 ($M+Na^+$).

12: ^{13}C NMR (25 MHz, D_2O, characteristic nonaromatic absorptions) δ=23.5, 63.4, 71.6, 72.4, 72.5, 72.9, 73.9, 74.0, 75.3, 75.8, 76.6, 77.2, 78.8, 80.0, 80.2, 94.5, 98.4, 102.2; FAB MS m/z 519 ($M+Na^+$).

13: FAB MS m/z 533 ($M+H^+$).

14: FAB MS m/z 533 ($M+H^+$).

15: ^{13}C NMR (25 MHz, D_2O, characteristic nonaromatic absorptions) δ=62.9, 81.0, 81.3, 81.8, 82.7, 94.7, 96.3, 98.5, 103.0, 103.3; FAB MS m/z 857 ($M+H^+$).

16: ^{13}C NMR (22.5 MHz, D_2O, characteristic nonaromatic absorptions) δ=63.0, 80.6, 80.9, 82.1, 94.5, 97.7, 98.4, 102.9; FAB MS m/z 857 ($M+H^+$).

Treatment of 6″-*O*-(Arylsulfonyl)maltotrioses (9—11) and 6′-*O*-(Arylsulfonyl)maltoses (12—14) with Thiophenol. A mixture of 6″-O-(arylsulfonyl)maltotriose (**9**) (10 mg), thiophenol (0.1 cm^3), and $CsCO_3$ (33 mg) in dry DMF (1 cm^3) was heated at 60 °C for 17 h under nitrogen. After addition of water, the solution was neutralized by addition of dilute HCl, and washed with ether. The mixture was chromatographed on a reversed-phase column with gradient elution of water-CH_3CN to give 7.7 mg (85.0%) of S-phenyl-6″-thiomaltotriose (**23**). Similarly, **10** (20 mg) or **11** (20 mg) gave **23** (10.3 mg, 59.9% or 8.1 mg, 47.1%, respectively). Following the procedure described above, **12—14** were converted to the known compound, S-phenyl-6′-thiomaltose.

23: FAB MS m/z 597 ($M+H^+$).

Treatment of 6″-*O*-(Arylsulfonyl)maltotetraoses (6—8) with Aqueous Ba(OH)$_2$. A solution of **6** (11.8 mg) in 0.05 mol dm^{-3} $Ba(OH)_2$ (1.2 cm^3) was stirred at room temperature for 5 h, neutralized with 0.05 mol dm^{-3} H_2SO_4 and filtered. The filtrate was chromatographed on a reversed-phase column with eluting with water to give 8.7 mg (93.5%) of 3″,6″-anhydromaltotetraose (**24**) which was identified by comparing its HPLC retention time and its FAB MS spectrum with those of the authentic compound.[25,26] Similarly, **7** (9 mg) or **8** (45.0 mg) gave **24** (4.2 mg, 61.8% or 24.5 mg, 71.8%, respectively).

Treatment of 2″-*O*-(Arylsulfonyl)maltotetraoses (15—16) with Aqueous Ba(OH)$_2$. A solution of **16** (40.6 mg) in 0.05 mol dm^{-3} $Ba(OH)_2$ (4.06 cm^3) was stirred at room temperature for 1 h and neutralized with 0.05 mol dm^{-3} H_2SO_4. After filtration, the filtrate was chromatographed on a

reversed-phase column with elution of water to give 21.3 mg (69.4%) of (2″*S*,3″*S*)-2″,3″-anhydromaltotetraose (**25**) which was identified by comparing its HPLC retention time and its FAB MS spectra with those of the authentic compound.[21] Similarly, **15** (7.6 mg) gave **25** (3.3 mg, 56.9%).

Reduction of 6″-*O*-Tosylmaltotetraose (6) or 6″-*O*-Tosylmaltotriose (9) with $NaBH_4$. A solution of **9** (50 mg) in 1% aqueous $NaBH_4$ (5 cm^3) was kept at 2 °C for 1 h. After neutralization of the solution by addition of dilute HCl, the mixture was chromatographed on a reversed-phase column with gradient elution of aqueous methanol to give **20** (49.9 mg, 99.5%). Similarly, **6** (50 mg) give **19** (44.5 mg, 88.8%).

19: ^{13}CNMR (22.5 MHz, D_2O, characteristic nonaromatic absorptions) δ=23.5, 62.7, 63.1, 64.9, 65.4, 79.4, 80.1, 84.5, 102.2, 102.6, 102.9; FAB MS *m/z* 823 ($M+H^+$), 845 ($M+Na^+$).

20: ^{13}CNMR (22.5 MHz, D_2O, characteristic nonaromatic absorptions) δ=23.6, 63.2, 65.0, 65.5, 80.1, 84.6, 102.5, 103.0; FAB MS *m/z* 661 ($M+H^+$).

Acetylation of 19 or 20. A solution of **20** (10 mg) and acetic anhydride (1 cm^3) in pyridine (1 cm^3) was allowed to stand at room temperature for 48 h and concentrated by evaporation of all volatile materials with a stream of nitrogen. After dry chloroform (1 cm^3) was added to the residue, evaporation was repeated. This procedure was carried out two more times. The crude product was purified by reversed-phase HPLC to give **22** (10.5 mg, 61.8%). Similarly, **19** (10 mg) gave **21** (6.8 mg, 39.5%).

21: MS *m/z* 1433 ($M+Na^+$).

22: FD MS *m/z* 1145 ($M+Na^+$).

We are indebted to Japan Maize Products Co. Ltd. for a generous gift of γ-CD.

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