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Enzymatic synthesis of a novel cyclic pentasaccharide consisting of α-D-glucopyranose with 6-α-glucosyltransferase and 3-α-isomaltosyltransferase

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Abstract—A novel cyclic pentasaccharide (CPS) and a branched cyclic pentasaccharide (6G-CPS) consisting of D-glucopyranose were synthesized with 6-α-glucosyltransferase (6GT) and 3-α-isomaltosyltransferase (IMT) from *Bacillus globisporus* N75. The structure of CPS was *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→}. The other, 6G-CPS, had the structure *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-[α-D-Glc*p*-(1→6)]-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→}. The formation of CPS was presumed to occur after the following four successive reactions: a 6-glucosyltransfer reaction with 6GT, a 4-glucosyltransfer reaction with 6GT, a 3-isomaltosyltransfer reaction with IMT, and a cyclization reaction with IMT.

Keywords: Cyclic pentasaccharide; Branched cyclic pentasaccharide; 6-α-Glucosyltransferase; 3-α-Isomaltosyltransferase

1. Introduction

The cyclic tetrasaccharide, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}, (abbreviated CTS) has a unique structure consisting of four glucose residues joined by alternate α-(1→3)- and α-(1→6)-linkages. Côté and co-workers first reported that CTS was produced from a dextran-like polysaccharide, alternan, by its degradation enzyme.[1,2] Recently, we have found a new enzymatic system to synthesize this saccharide from starch by transglycosylation using two glycosyltransferases, 6-α-glucosyltransferase (abbreviated 6GT) and 3-α-isomaltosyltransferase (abbreviated IMT).[3,4] Two enzymes work jointly and convert maltodextrins into CTS by the following three successive reactions. (1) 6GT catalyzes an intermolecular transglucosylation in which a glucose residue at the nonreducing end of α-(1→4)-glucan is transferred to the 6-OH of another nonreducing glucose to produce isomaltosyl-α-(1→4)-glucan. (2) The isomaltosyl part of the intermediate product is transferred to another isomaltosyl-α-(1→4)-glucan by IMT. The second intermediate product is isomaltosyl-α-(1→3)-isomaltosyl-α-(1→4)-glucan. (3) Another activity of IMT, an intramolecular transglycosylation, cuts off and cyclizes the isomaltosyl-α-(1→3)-isomaltosyl part of the second intermediate, to produce CTS. Cyclomaltodextrin glucanotransferase (abbreviated CGTase: EC 2.4.1.19), one of the most well-known cyclic glucan-synthesizing enzymes, is produced cyclomaltooligosaccharides (cyclodextrins, abbreviated CDs) from linear α-(1→4)-glucans.[5] CGTase produce CDs that have various degrees of polymerization. Therefore, IMT, one of the cyclic glucan-synthesizing enzymes, is expected to synthesize a novel cyclic glucan that would have different degrees of polymerization from CTS. In this study, we report the enzymatic synthesis of a novel cyclic pentasaccharide (abbreviated CPS) consisting of α-D-glucopyranose with 6GT and IMT.

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2. Results

2.1. Reaction to starch with 6GT and IMT

A reaction mixture (1.0 g) containing 0.3 g of partially hydrolyzed starch (Pinedex no. 100, Matsutani Chemical Industry, Hyogo, Japan), 6GT and IMT (4.0 U/g-dry solid [abbreviated DS] of CTS-forming activity), and 50 μL of 1 M NaOAc buffer (pH 6.0) was incubated at 45 °C for 72 h. After heating at 100 °C for 10 min, the mixture was diluted with 5 mM NaOAc buffer (pH 5.0) to 60 mL. To degrade oligosaccharides other than the cyclic saccharide including CTS into glucose, α-glucosidase (8000 U/g-DS), glucoamylase (1000 U/g-DS), and α-amylase (15 U/g-DS) were added to the mixture and held at 50 °C for 24 h. After the hydrolysis reaction, the solution was heated to 100 °C for 10 min to inactivate the enzymes. Furthermore, an alkaline treatment (to pH 12 with NaOH, 100 °C, 60 min) was performed to remove the reducing sugars after concentration by evaporation. After neutralization by passing the mixture through 2.0 mL of Diaion SK1B (Mitsubishi Chem. Co., Tokyo, Japan), the resultant solution was desalted by passing it through ion-exchange resins, 0.5 mL of Diaion SK1B, 0.5 mL of Diaion WA30 (Mitsubishi Chem. Co.), and 1.0 mL of Amberlite IRA411S (Japan Organo, Tokyo, Japan). As shown in Figure 1, when the saccharides in the resultant solution were analyzed by HPLC, two peaks other than CTS were mainly detected around the retention time of 44 min (saccharide **1**) and 41 min (saccharide **2**). The analytical yields of saccharides **1**, **2**, and CTS were 0.4%, 0.3%, and 99.0%, respectively. In the LC–MS analysis, the adduct ions with sodium ion $[M+Na]^+$ at *m*/*z* 833 (saccharide **1**) and 995 (**2**) were monitored in the positive-ion electrospray ionization mode. These results show that saccharides **1** and **2** are novel cyclic oligosaccharides having a degree of polymerization of 5 and 6.

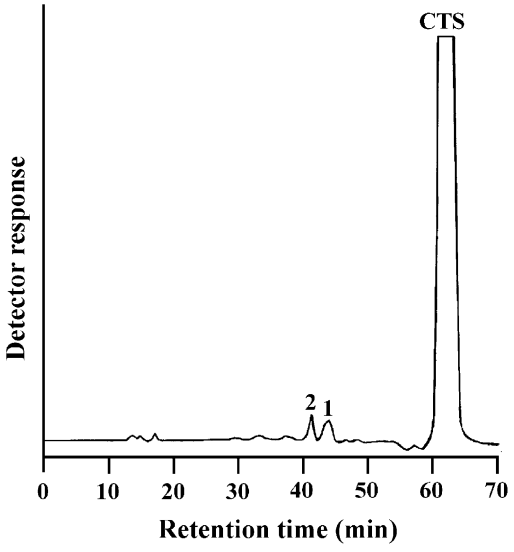

Figure 1. HPLC profile of reaction products after an alkaline treatment. CTS: cyclic tetrasaccharide, **1**: saccharide **1**, **2**: saccharide **2**.

2.2. Preparation of saccharides 1 and 2

A reaction mixture (333 g) containing 100 g of Pinedex no. 100, 6GT and IMT (4.0 U/g-DS of CTS-forming activity), and 16.7 mL of 1 M NaOAc buffer (pH 6.0) was incubated at 45 °C for 72 h. After heating at 100 °C for 10 min, the mixture was diluted with 5 mM NaOAc buffer (pH 5.0) to 20 L. To degrade non-cyclic oligosaccharides, α-glucosidase (8000 U/g-DS), glucoamylase (1000 U/g-DS), and α-amylase (15 U/g-DS) were added to the mixture and held at 50 °C for 24 h. After the hydrolysis reaction, the solution was heated to 100 °C for 30 min to inactivate those enzymes. Furthermore, an alkaline treatment was performed to remove the reducing sugars after concentration by evaporation. After neutralization by passing the mixture through 500 mL of Diaion SK1B, the resultant solution was desalted by passing it through ion-exchange resins, 150 mL of Diaion SK1B, 150 mL of Diaion WA30, and 300 mL of Amberlite IRA411S. The eluent was concentrated to 260 mL (45.8 g-DS) by evaporation. The resulting saccharide mixture contained 0.4% of saccharide **1**, 0.3% of saccharide **2**, and 99.0% of CTS.

To separate saccharides **1** and **2** from the saccharide mixture, preparative ODS column chromatography on a YMC-pack ODS-AQ R-355-15-AQ column (50 mm i.d. × 500 mm: YMC Co., Ltd, Kyoto, Japan) was performed, eluting with water as a solvent at a flow rate of 30 mL/min at 25 °C. The fractions containing saccharides **1** and **2** were separately collected, and then evaporated. After 5 cycles of chromatography while injecting 50 mL (8.8 g-DS) of samples per cycle, two fractions, saccharide **1** (8 mL: 103 mg-DS) and **2** (14 mL: 168 mg-DS), were obtained.

To purify saccharide **1**, a repeated ion-exchange column chromatography on an MCI GEL CK04SS column (10 mm i.d. × 200 mm × 2; Mitsubishi Chem. Co.) was carried out, eluting with water as a solvent at a flow rate of 0.4 mL/min at 80 °C. The fraction containing saccharide **1** was collected and then evaporated. After 100 cycles of chromatography while injecting 50 μL (0.6 mg-DS) of samples per cycle, the purified saccharide **1**, in 99.1% purity, was obtained in a yield of 30.7 mg. Saccharide **2** was also purified by the same ion-exchange column chromatography. Finally, saccharide **2** (94.0% purity) was obtained in a yield of 14.5 mg.

2.3. Characterization of saccharide 1

The molecular mass of saccharide **1** was found to be 810 by measuring the $[M+Na]^+$ ion (*m*/*z* 833) by ESIMS. The molecular mass was determined to be $M = 162 \times n$

($n = 5$), indicating that the pentasaccharide contained a cyclic structure. Methylation analysis gave no 2,3,4,6-tetra-*O*-methyl product derived from a nonreducing-end glucose, as shown in Table 1. Compared with CTS, 1 mol of the 2,3,6-tri-*O*-methyl product was newly generated. To confirm this structure, NMR spectroscopy measurements were done. The ^{13}C NMR spectrum of saccharide **1** contained 30 signals (Table 2), indicating that it should be a pentamer of hexose. To clarify the connectivity of the glucose residues, heteronuclear multiple bond correlation (HMBC) measurement was carried out. Each glucose residue is represented as I, II, III, IV, and V as indicated in Figure 2a. The proton and carbon positions in a particular residue are represented by, for example, H-1–I and C-1–I, respectively. The connectivity of I (1→3) V and IV (1→3) III were deduced from HMBC correlations between H-1–I (δ_H, 5.11 ppm) and C-3–V (δ_c, 87.9 ppm) and between H-1–IV (δ_H, 5.58 ppm) and C-3-III (δ_c, 78.4 ppm). Furthermore, the connectivity of II (1→6) I or IV, III (1→4) I

Table 1. Methylation analysis of saccharides **1** and **2**

Saccharide	2,3,4,6-Tetra-*O*-methyl-Glc	2,3,6-Tri-*O*-methyl-Glc	2,4,6-Tri-*O*-methyl-Glc	2,3,4-Tri-*O*-methyl-Glc	2,4-Di-*O*-methyl-Glc
1	—	1.0	1.9	2.1	—
2	0.8	1.0	1.0	2.1	1.0
CTS	—	—	2.0	2.0	—

Table 2. ^{13}C NMR chemical shift data for saccharides **1** and **2**[a]

Residue[b]	Carbon atom	Saccharide **1**	Saccharide **2**
I	C-1	103.1	103.2
	C-2	74.7	74.7
	C-3	75.6	75.6
	C-4	73.1	73.1
	C-5	74.6	74.5
	C-6	70.2	70.2
II	C-1	100.3	100.3
	C-2	73.3	73.3
	C-3	76.2	76.2
	C-4	79.7	80.3
	C-5	73.1	73.1
	C-6	63.2	63.3
III	C-1	102.3	102.5
	C-2	71.4	71.4
	C-3	78.4	78.4
	C-4	73.3	73.5
	C-5	75.0	73.8
	C-6	62.9	68.5
IV	C-1	99.9	99.8
	C-2	74.0	74.1
	C-3	74.8	74.8
	C-4	73.8	73.8
	C-5	71.5	71.6
	C-6	68.4	68.6
V	C-1	99.1	99.1
	C-2	72.8	72.9
	C-3	87.9	87.9
	C-4	73.5	73.5
	C-5	73.9	74.0
	C-6	63.2	63.3
VI	C-1		100.9
	C-2		74.2
	C-3		75.8
	C-4		72.3
	C-5		74.5
	C-6		63.3

[a] NMR spectra data were recorded for solutions in D_2O at 40 °C. The chemical shifts were expressed in parts per million downfield from the signal of 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (TPS), which was used as an internal standard.

[b] Roman numerals indicate the positions of the hexose residues in the saccharides **1** and **2** shown in Figure 2.

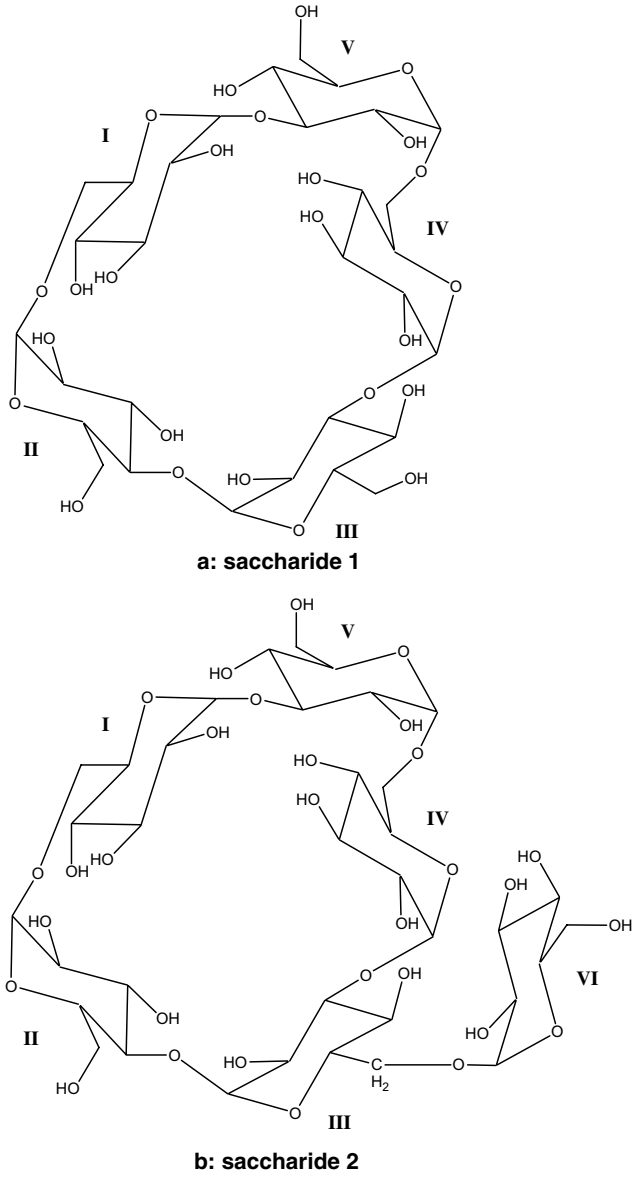

Figure 2. Structure of saccharides **1** (a) and **2** (b).

or II, and V (1→6) II or IV were also deduced from HMBC correlations between H-1–II (δ_H, 4.91 ppm) and C-6–I or IV (δ_c, 70.2 ppm), between H-1–III (δ_H, 5.42 ppm) and C-4–I or II (δ_c, 79.7 ppm) and between H-1–V (δ_H, 5.02 ppm) and C-6–II or IV (δ_c, 68.4 ppm). Based on the results of HMBC measurement, the structure of saccharide **1** was limited to *cyclo*-{→6) I (1→3) V (1→6) IV (1→3) III (1→4) II (1→}(candidate A) or *cyclo*-{→4) I (1→3) V (1→6) II (1→6) IV (1→3) III (1→}(candidate B). To determine the structure from two candidates, saccharide **1** was partially hydrolyzed by 0.2 N HCl at 95 °C for 4 h. The main products after hydrolysis were isomaltose and isopanose. Isopanose was included only in candidate A. Isomaltotriose, which was the partial structure of candidate B, was not detected. From this result, saccharide **1**, named CPS, was deduced to be *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→} (Fig. 2a).

2.4. Characterization of saccharide 2

The mass spectrum of saccharide **2** showed its molecular weight to be 972 by measuring the $[M+Na]^+$ ion (*m*/*z* 995). The molecular mass was determined to be $M = 162 \times n$ ($n = 6$), indicating that the hexasaccharide contained a cyclic structure. Methylation analysis gave 1 mol of the 2,3,4,6-tetra-*O*-methyl product derived from a nonreducing-end glucose, as shown in Table 1. Compared with saccharide **1**, 1 mol of the 2,4,6-tri-*O*-methyl product was lost, and a corresponding amount of the 2,4-tetra-*O*-methyl product was generated. Therefore, saccharide **2** would be expected to have a structure containing one glucose residue attached to saccharide **1** by a (1→6)-linkage. The ^{13}C NMR spectrum of saccharide **2** contained 36 signals (Table 2), indicating that it should be a hexamer of hexose. ^1H–^{13}C COSY showed that the α-configuration of the glucose residue in saccharide **2** was confirmed by the C-1 signal of this residue at 100.9 ppm {δ^1H, 4.94 ppm (d, $J_{1,2}$ 3.7 Hz)}. A large downfield shift (5.6 ppm) of the C-6 signal and an upfield shift (1.2 ppm) of the C-5 signal of the 3-*O*-glycosylated ring residue (residue III in Fig. 2b) were observed in the spectrum. Based on these results, saccharide **2**, named 6G-CPS, was deduced to be *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-[α-D-Glc*p*-(1→6)]-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→} (Fig. 2b).

3. Discussion

It has been known that bacterial enzymes produce cyclic glucans. Cyclomaltooligosaccharides (cyclodextrin, cyclic α-(1→4)-glucans), consisting of six or more glucose units, are produced from starch by CGTase.[5] A cyclic α-(1→6)-glucan with seven to nine glucose units is produced from dextran by an extracellular enzyme, called cycloisomaltooligosaccharide glucanotransferase, from *Bacillus circulans* T-3040.[6] All of these cyclic glucans have homogeneous linkages in their structure. Moreover, these cyclic glucans are synthesized from the substrate in one step. Recently, we found a new enzymatic system to synthesize CTS from starch in three steps with two glycosyltransferases.[3] CTS is smaller than the other cyclic glucans mentioned above and has heterogeneous linkages in its structure.

In this study, we found that 6GT and IMT work jointly and convert starch into not only CTS but also CPS. CPS contained a α-(1→4)-linkage in its structure. When transglucosylation to CTS by 6GT was examined, 4-*O*-α-glucosyl-CTS was generated.[7] Therefore, the α-(1→4)-linkage in CPS was thought to be the action of 6GT. Based on this phenomenon, the formation mechanism of CPS was presumed by the following four successive reactions. (1) 6GT catalyzes an intermolecular transglucosylation in which a glucose residue at the nonreducing end of α-(1→4)-glucan is transferred to the 6-OH of another nonreducing glucose, to produce isomaltosyl-α-(1→4)-glucan. (2) 6GT catalyzes an intermolecular transglucosylation in which a glucose residue at the nonreducing end of α-(1→4)-glucan is transferred to the 4-OH of a nonreducing-end glucose of isomaltosyl-α-(1→4)-glucan, to produce glucosyl-α-(1→4)-isomaltosyl-α-(1→4)-glucan. (3) The isomaltosyl part of the isomaltosyl-α-(1→4)-glucan, the first intermediate product, is transferred to the 3-OH of a nonreducing-end glucose of glucosyl-α-(1→4)-isomaltosyl-α-(1→4)-glucan by IMT. The third intermediate product is

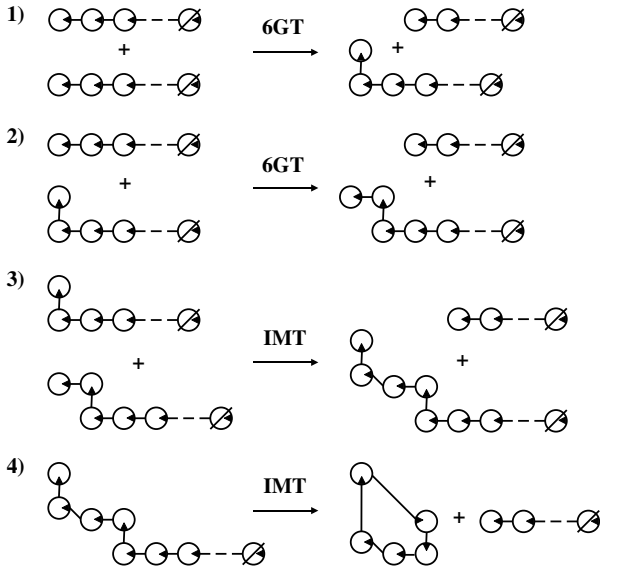

Figure 3. Presumptive mechanism for the formation of CPS. ○: glucose residue, Ø: reducing end glucose residue, —: α-(1→4)-linkage, |: α-(1→6)-linkage, \: α-(1→3)-linkage, ◀: position of anomeric carbon.

isomaltosyl-α-(1→3)-glucosyl-α-(1→4)-isomaltosyl-α-(1→4)-glucan. (4) Another activity of IMT, an intramolecular transglycosylation, cuts off and cyclizes the isomaltosyl-α-(1→3)-glucosyl-α-(1→4)-isomaltosyl part of the third intermediate, to produce CPS (Fig. 3). On the other hand, it was expected that 6G-CPS was synthesized by the transglucosylation to CPS with 6GT. In this study, 6G-CPS has remained after hydrolysis with the α-glucosidase. It seems that the branched part of 6G-CPS is inaccessible to the active center of α-glucosidase because of steric hindrance.

The crystalline structure of CPS is not clarified yet; therefore, we examined the three-dimensional structure by computer-modeling studies using MOPAC and AM1.[8] This result showed that CPS has a depression considerably deeper than CTS, so that various low molecular compounds may be included in CPS (data not shown).

CPS generated by enzyme reactions is a novel cyclic oligosaccharide that consists of five glucose molecules, and it might have functions and physical properties different from already-known cyclic oligosaccharides.

4. Experimental

4.1. Carbohydrate and enzymes

Partially hydrolyzed starch, Pinedex no. 100 (hydrolysis 1.5%) was obtained from Matsutani Chemical Industry. Glucoamylase from *Rizopus delemar* (Glucozyme) and α-amylase from *B. subtilis* (Neospitase PK2) were purchased from Nagase ChemteX Corporation (Hyogo, Japan). α-Glucosidase from *Aspergillus niger* (Transglucosidase L) was obtained from Amano Enzyme, Inc. (Aichi, Japan). 6GT and IMT from *Bacillus globisporus* N75 were purified as described previously.[9] The activities of glucoamylase, α-amylase, and α-glucosidase were assayed according to the methods recommended by their respective suppliers. CTS-forming activity was measured as reported in a previous publication.[4]

4.2. High-performance liquid chromatography (HPLC)

The amounts of neutral saccharides in samples were determined by HPLC. Samples were first treated by filtration using a filter kit, KC prep dura (0.45 μm, Katayama Chemical Co., Osaka, Japan) and by deionization using a microacilyzer G0 (Asahi Chemical Co., Tokyo, Japan). HPLC analysis was performed with an LC-10AD pump, an RID-10A refractive index monitor, and a C-R7A data processor (Shimadzu Corporation, Kyoto, Japan) equipped with an MCI GEL CK04SS column (10 mm i.d. × 200 mm × 2; Mitsubishi Chem. Co., Tokyo, Japan) at a flow rate of 0.4 mL/min, using water as a solvent at 80 °C.

4.3. Methylation analysis

Methylation analysis was performed according to the method of Hakomori.[10] The saccharide samples (100 μg each) were methylated and after extracting with $CHCl_3$, the methylated sample was hydrolyzed with 90% HOAc containing 0.5 N H_2SO_4 at 80 °C for 6 h. The methylated monosaccharides were reduced with sodium borohydride and then acetylated with Ac_2O at 100 °C for 4 h. The resulting partially methylated alditol acetates were analyzed by GLC (GC-14B, Shimadzu Corporation) in a DB-5 capillary column (J&W Scientific, Folsom, CA) at 130–250 °C (5 °C/min).

4.4. LC–MS analysis

The molecular masses of the products were determined by LC–MS. The LC–MS was carried out using the same HPLC system as the above interfaced to an LCQ advantage ion-trap mass analyzer (Thermo Electron Corporation, Kanagawa, Japan) fitted with an electrospray ionization (ESI) interface. The mass spectrometer was operated in the positive-ion mode; the ESI voltage was set at 5 kV, and the capillary temperature was 350 °C.

4.5. NMR measurement

NMR spectral data were recorded for 2–5% solutions in D_2O at 40 °C with a JNM-AL300 spectrometer (^1H 300.4 MHz, ^{13}C 75.45 MHz: JEOL, Tokyo, Japan). The chemical shifts were expressed in parts per million downfield from the signal of 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (TPS), which was used as an internal standard.

4.6. Computer modeling

The MOPAC2000 program was used for the semiempirical level of molecular orbital calculations.[8] Molecular structures were displayed using the Mercury graphic software. In the semiempirical calculation, the AM1 hamiltonian was used.[8]

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