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Stereoselective phosphorylation of branched cyclodextrins with inorganic *cyclo*-triphosphate

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Abstract

The phosphorylation by inorganic sodium *cyclo*-triphosphate (P_{3m}) having a six-membered ring was examined for cyclomaltohexaose (α-cyclodextrin) and branched cyclodextrins (mono-6-*O*-α-D-glucopyranosylcyclomaltohexaose, mono-6-*O*-α-D-maltosylcyclomaltohexaose, mono-6-*O*-α-D-glucopyranosylcyclomaltoheptaose, and mono-6-*O*-α-D-maltosylcyclomaltoheptaose) in aqueous solution. For all cyclomaltooligosaccharides (cyclodextrins) studied, the 2-OH group was stereoselectively phosphorylated. In the reaction of branched cyclodextrins and P_{3m}, only the 2-OH on the α-D-glucopyranosyl group of the cyclodextrin rings was phosphorylated with maximum yields of more than 27%. The phosphorylation mechanism of branched cyclodextrins with P_{3m} is also discussed.

Keywords: Phosphorylation; Branched cyclodextrin; *cyclo*-Triphosphate; Triphosphate ester

1. Introduction

Sodium *cyclo*-triphosphate, $Na_3P_3O_9$ (P_{3m}), was first prepared by Fleitmann and Henneberg in 1848.[1] P_{3m} undergoes acidic and alkaline hydrolysis to produce a linear triphosphate. We have demonstrated that the reaction of D-glucose with P_{3m} in aqueous solution afforded β-D-glucopyranosyl 1-triphosphate in good yield (47%) by a one-step process without protection of hydroxyl groups.[2,3] Although D-glucose exists as the equilibrium mixture of α and β anomers under reaction conditions, only β-D-glucopyranosyl 1-triphosphate was formed stereoselectively.[3] This method could be applicable to the phosphorylation of other monosaccharides.[4,5] The phosphorylated products of oligo- and polysaccharides are expected to be useful as chiral selectors,[6] masking agents for metal ions,[7] and materials for drug delivery system (DDS).[8] Although this stereoselective phosphorylation has been shown to be effective with monosaccharides, where there is a 1-OH group, it is more significant to apply this phosphorylation reaction to oligosaccharides having no available 1-OH group.

We have already shown that cyclomaltohexaose and cyclomaltooctaose (α- and γ-cyclodextrin, α- and γ-CD) react with P_{3m} in aqueous solution to form phosphate esters of α- and γ-CD in maximum yields of 22.6 and 19.0%, respectively.[9] In the phosphorylation reaction of α- and γ-CD with P_{3m}, the resultant products were their monophosphate and triphosphate esters. However, it was impossible to identify and assign structures to the phosphorylated products at that time.

The phosphorylation of α- and β-cyclodextirn with inorganic metaphosphate as a solid-phase reaction was reported in 1997.[10] The products were mixtures of three different kinds of α- and β-cyclodextrin monophosphates.

More recently, we demonstrated that 2- or 3-OH on the nonreducing unit of disaccharides could be readily phosphorylated by P_{3m} in aqueous solution.[11,12] For example, in the reaction of sucrose with P_{3m}, two phosphorylated products, β-D-fructofranosyl-(2→1)-2-*O*-triphospho-α-D-glucopyranoside and 3-*O*-triphospho-β-D-fructofranosyl-(2→1)-α-D-glucopyranoside, were obtained. In previous studies,[9] β-cyclodextrin (β-

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CD), which has no reducing glucopyranosyl group, could not be phosphorylated by P_{3m} because of its low solubility in water. Because β-CD is available at reasonable cost and can include a variety of guest compounds, various applications of the compound are expected in many fields. However, its application has been limited due to its low solubility in water. Branched cyclodextrins synthesized by Kobayashi and co-workers[13] are valuable because of their high solubility in water. Labile compounds such as fragrance constituents and volatile components have been maintained, protected, and stabilized by the formation of inclusion compounds with branched CDs.

In the present study, first, the reaction of α-cyclodextirn (**1**) with P_{3m} was reinvestigated, and the product was definitively identified. Then, the reaction of mono-6-*O*-α-D-glucopyranosylcyclomaltohexaose (mono-G_1-α-CD; **2**), mono-6-*O*-α-D-maltosylcyclomaltohexaose (mono-G_2-α-CD; **3**), mono-6-*O*-α-D-glucopyranosylcyclomaltoheptaose (mono-G_1-β-CD; **4**), or mono-6-*O*-α-D-maltosylcyclomaltoheptaose (mono-G_2-β-CD; **5**), with P_{3m} was studied to develop a selective phosphorylation in aqueous solution for aiming at the synthesis of a new DDS host.

2. Results and discussion

The syntheses of cyclodextrin triphosphates (**6**–**10**) were carried out essentially according to the previous method.[3] Table 1 summarizes the yields of the product of cyclomaltohexaose (α-cyclodextrin, **1**) and P_{3m} under various reaction conditions. On decreasing the reaction temperature, the yield of **6** gradually increased, and the yield increased with the increase in the initial concentration of P_{3m}. Therefore, the maximum yield and its time suggested that the optimum conditions for the phosphorylation of **1** with P_{3m} is a molar ratio of **1**:P_{3m} = 1:3, and 10 °C. In the case of mono- and disaccharides, the maximum yields were attained in cases where there was an excess of saccharides. However, the maximum yield of **6** was obtained under the condition of excess P_{3m} for α-CD, due to the low solubility of α-CD.

Table 1
Yields of the product **6** in the reaction of α-CD with P_{3m}

Conc (M)		T (°C)	Time (h)	Yield (%)
1	P_{3m}			
0.15	0.15	40	8	4
0.15	0.15	25	53	9
0.15	0.15	10	220	13
0.15	0.30	10	78	20
0.15	0.45	10	74	22

^1H and ^{31}P NMR spectra were measured to confirm the reaction product of **1** with P_{3m}. The ^{31}P NMR spectrum for the reaction product in Fig. 1 shows the characteristic spectrum for the presumed triphosphate derivative of **1**. The ^1H-nondecoupled spectrum showed one doublet of doublets at −10.8 ppm, which became a doublet in the ^1H-decoupled spectrum, indicating the characteristic peak of P_α similar to triphosphate derivatives of D-aldoses.[4] The other doublet at −5.87 ppm was assigned to Pγ. The doublet of doublets at −20.9 ppm did not change in a ^1H-decoupling experiment, suggesting P_β of the presumed triphosphate derivative. Also, Fig. 1 shows the ^{31}P–^1H heteronuclear multiple bond correlation (HMBC) 2D NMR spectrum. A correlation between P_α at −10.8 ppm and the ^1H signal at 4.05 ppm was observed. The ^1H COSY spectrum showed a correlation between H-2 (4.05 ppm) and H-1 (5.13 ppm). The doublet of doublets of doublets at 4.05 ppm could be assigned to H-2. As shown in Table 2, a downfield shift was observed both for the H-1 and H-2 signals of the phosphorylated glucopyranosyl residue (named as residue D). Therefore, the 2-OH of one glucopyranosyl residue in α-CD was stereoselectively phosphorylated. ^1H NMR chemical shifts and coupling constants determined by ^1H, COSY, TOCSY, and ROESY NMR spectra are shown in Table 2. For maltose and sucrose triphosphates,[12] the chemical shifts of H-2 on a phosphorylated glucopyranosyl group were 4.14 and 4.08 ppm, respectively, supporting the above assignment. The $^3J_{P\alpha,H\text{-}2}$ value was also consistent with the data of oligosaccharide triphosphates.[12] As shown in the H-1 region of the ^1H NMR spectrum, there are three types of glucopyranosyl residues. The doublets at 4.96 and 5.13 ppm were assigned to H-1 of the residues C″ and D, respectively. The doublet at 4.90 ppm was assigned to H-1 of residues C and C′.[14] The ROESY spectrum showed that residues C′ and C″ are linked to the 1-OH and the 4-OH of residue D, respectively. From the result of the integration of the H-1 signals, only one of the six glucose units of α-CD was verified to be phosphorylated by P_{3m}. Therefore, the phosphorylated product in the reaction of **1** with P_{3m} was confirmed to be 2-*O*-triphosphocyclomaltohexaose (**6**) as shown in Scheme 1.

Branched cyclodextrins have higher solubility in water due to the presence of the branched glucopyranosyl residue bonded to cyclodextrin through a (1→6)-α-glucopyranosidic linkage. Therefore, the phosphorylation of mono-G_1-α-CD (**2**), mono-G_2-α-CD (**3**), mono-G_1-β-CD (**4**), or mono-G_2-β-CD (**5**) with P_{3m} was carried out under various molar ratios and temperatures.

Table 3 shows the yields of the triphosphate esters of **2**–**5**. The maximum yields for **2**–**5** were 29, 35, 27 and 28%, respectively. The higher yields were attained compared with **1** due to the higher solubility of the

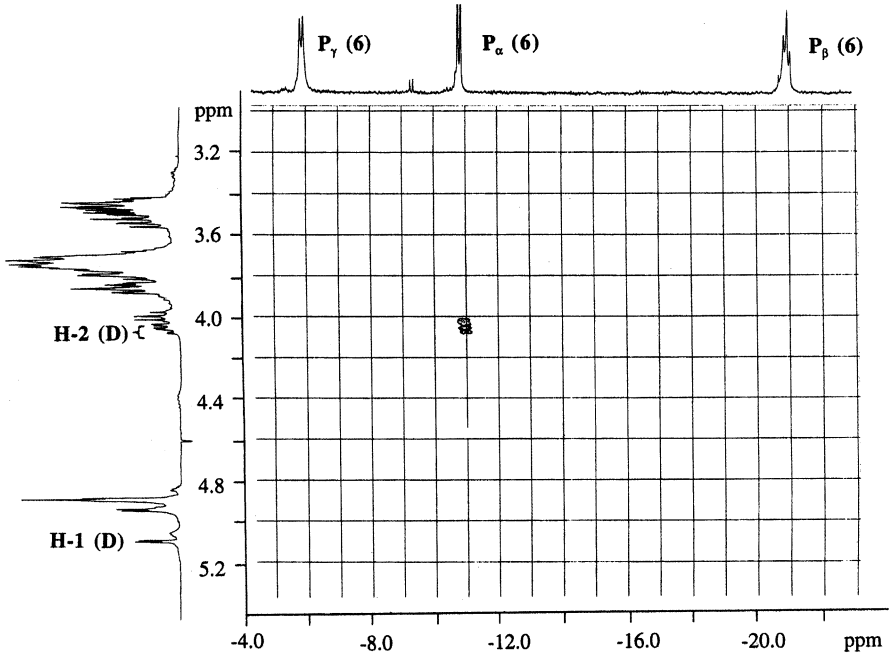


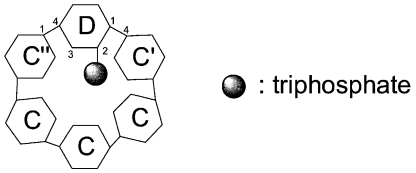
Fig. 1. $^{31}P-^{1}H$ 2D HMBC NMR spectrum of **6**.

branched CDs in water. The yields increased with the increase in the initial concentration of **2**–**5**. The optimum conditions for the phosphorylation of **2** and **3** with P_{3m} was a molar ratio of P_{3m}:**2** or **3** = 1:3 and 25 °C. On the other hand, the optimum conditions for the phosphorylation of **4** and **5** with P_{3m} was a molar ratio of P_{3m}:**4** or **5** = 1:3 and 10 °C. The stability of the phosphorylated products of **2** and **3** might be different from that of α-CD.

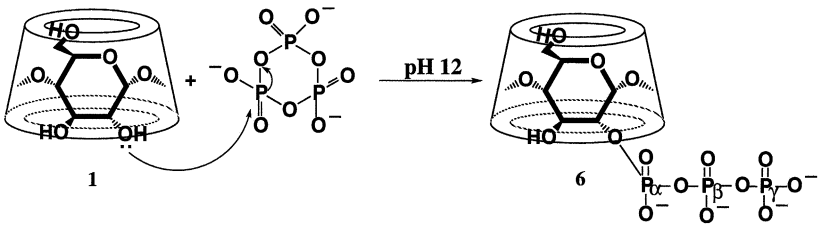
The identification of reaction products **7**–**10** was performed in a similar way as **6**. Fig. 2 shows the $^{31}P-^{1}H$ HMBC 2D NMR spectrum of the phosphorylation product **9** of **4**. The ^{31}P NMR spectrum (Table 4) showed three peaks at −11.0, −21.1, and −5.4 ppm, which are characteristic for triphosphate esters of adenosine.[15] A correlation between P_α at −11.0 ppm and ^{1}H signal at 4.09 ppm was observed. The doublet of doublets of doublets at 4.09 ppm could be assigned to H-2 by the ^{1}H COSY spectrum.

In the case of α-CD, all the glucopyranosyl residues are equivalent. However, all the glucopyranosyl residues are nonequivalent in the branched CDs. Therefore, it is necessary to assign the phosphorylated glucopyranosyl residue. The ROESY and TOCSY NMR spectra were measured, and the data are summarized in Table 5. As shown in the ^{1}H NMR spectrum of the product **6** (Fig.

Table 2
^{1}H NMR chemical shifts and coupling constants of **6** and α-CD



	Residue	δ (ppm)				J (Hz)			
		H-1	H-2	H-3	H-4	P_α, H-2	H-1, H-2	H-2, H-3	H-3, H-4
6	C	4.90	3.46	3.85	3.43		3.8	9.6	9.1
	C′	4.90	3.46	3.85	3.53		3.8	9.6	9.3
	C″	4.96	3.41	3.85	3.43		3.5	9.6	9.3
	D	5.13	4.05	3.98	3.53	8.8	3.5	9.6	9.4
α-CD		5.05	3.62	3.99	3.57		3.5	10.0	8.7



Scheme 1.

Table 3
Yields of the triphosphate esters of **2**–**5**

Conc (M)	T (°C)	Time (h)	Yield (%)
Mono-G_1-α-CD (**2**):P_{3m}			
0.15:0.45	10	118	20
0.15:0.30	10	99	15
0.15:0.15	10	73	9
0.30:0.15	10	169	23
0.45:0.15	10	98	25
0.45:0.45	10	52	26
0.45:0.15	25	29	29
0.45:0.15	40	8	18
Mono-G_2-α-CD (**3**):P_{3m}			
0.15:0.45	10	50	19
0.15:0.30	10	49	18
0.15:0.15	10	75	14
0.30:0.15	10	126	20
0.45:0.15	10	71	27
0.45:0.45	10	50	25
0.45:0.15	25	22	35
0.45:0.15	40	7	25
Mono-G_1-β-CD (**4**):P_{3m}			
0.15:0.45	10	146	20
0.15:0.30	10	52	14
0.15:0.15	10	120	9
0.30:0.15	10	102	18
0.45:0.15	10	98	27
0.45:0.45	10	25	20
0.45:0.15	25	21	24
Mono-G_2-β-CD (**5**):P_{3m}			
0.15:0.45	10	34	18
0.15:0.30	10	54	15
0.15:0.15	10	101	14
0.30:0.15	10	46	19
0.45:0.15	10	69	28
0.45:0.45	10	48	18
0.45:0.15	25	23	28

2), there are four different H-1 signals. The doublet at 5.19 ppm could be assigned to H-1 of the phosphorylated residue D as mentioned above. The doublets at 4.82 and 4.99 ppm were assigned to H-1 of the residues A and C″, respectively. The H-1 signals of the residues B, C, and C′ were overlapping at 4.94 ppm. The correlations between H-1 of the residue D and H-4 of the residue C′, and between H-4 of the residue D and H-1 of the residue C″ were observed in the ROESY spectra. If the phosphorylation occurred in residue A, three different H-1 signals would have been observed in the ^{1}H NMR spectrum, and the H-1 signal at 4.82 ppm would have disappeared. The H-1 signal of residue A showed no change by phosphorylation.[14] Therefore, it was found that the phosphorylation had not occurred in the branched glucopyranosyl residue but was at one of the residues of the β-CD ring. On the basis of the integration of the H-1 signals, only one of the eight glucose units of **4** was verified to be phosphorylated by P_{3m}, and H-1 at 4.99 ppm was made up of five signals (the residues B, C, C, C, and C′ in Fig. 3). The three sets of H-2 and H-4 signals due to the residues B, C, and C′ could be distinguished by the TOCSY spectra. In the ROESY spectra, the correlations between H-1 of residue B and H-4 of residue D, and H-4 of residue B and H-1 of residue D could not be observed. Therefore, it was found that residue B and its neighbors were not phosphorylated as shown in Fig. 3. Fig. 3 shows four possible structures of **9**. Unfortunately, it is impossible to confirm **9** from these candidates at the present stage. Anyway, the 2-OH in one of the glucopyranosyl residues that constitutes of a past β-CD ring was stereoselectively phosphorylated. Product **9** was verified to be 6-*O*-(α-D-glucopyranosyl)-2-*O*-triphosphocyclomaltoheptaose (Scheme 2), comparable with the phosphoryaltion reaction of **1**. The other NMR data in Tables 4 and 5 were consistent with this assignment.

The other products **7**, **8**, and **10** were similarily identified. The phosphorylation products of **2**, **3**, and **5** with P_{3m} were verified to be 6-*O*-(α-D-glucopyranosyl)-2-*O*-triphosphocyclomaltohexaose (**7**), 6-*O*-(α-maltosyl)-2-*O*-triphosphocyclomaltohexaose (**8**), and 6-*O*-(α-maltosyl)-2-*O*-triphosphocyclomaltoheptaose (**10**). Therefore, **2**–**5** are stereoselectively phosphorylated with P_{3m} in aqueous solution to form **7**–**10**, respectively (Scheme 2).

The phosphorylation mechanism of **1**–**5** with P_{3m} is formulated as follows. At pH 12, P_{3m} is easily attacked by nucleophilic reagents such as ammonia,[16] alcohol,[17,18] nucleoside,[19] and amino acid.[20] In the present study, the lone-electron pair on 2-OH of the α-D-glucopyranosyl group nucleophilically attacks a phosphorus atom on P_{3m} to open its six-membered ring. The

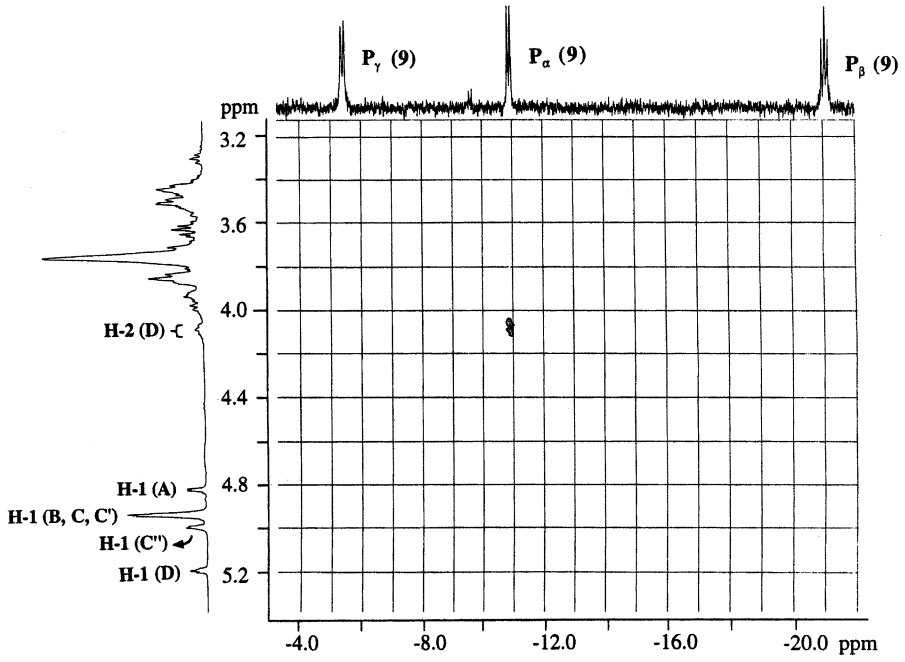


Fig. 2. $^{31}P-^{1}H$ 2D HMBC NMR spectrum of **9**. Mono-G_1-β-CD (**4**):P_{3m} = 0.45:0.15 M, pH 12, and 10 °C, after 24 h.

reactivity of cyclodextrins strongly depend on the acidity of the hydroxyl groups at C-2, C-3, and C-6. The secondary hydroxyl group 2-OH has the highest acidity (pK_a 12.2) in the α-D-glucopyranosyl group.[21,22] Under anhydrous conditions, the 2-OH of β-CD can be selectively deprotonated and allowed to react with electrophilic reagents.[23] In the reaction of **1**–**5** with P_{3m}, the 2-OH group of the glucopyranosyl group constituting CD ring was selectively phosphorylated. The 2-OH on the branched glucopyranosyl or maltosyl groups was not phosphorylated, probably due to steric hindrance.[24]

The reaction of α- and β-CD with metaphosphate gave three isomers of monophosphate ester.[10] The yields of the 2-, 3-, and 6-isomers were in an approximate ratio of 1:1:3. In the reaction of β-CD and phosphoryl chloride, a primary hydroxyl group was stereoselectively phosphorylated.[8] Compared with the phosphorylation of CDs by metaphosphate, in the present study, stereoselective phosphorylation was attained by P_{3m}.

In conclusion, the phosphorylation of branched cyclodextrins was found to be possibly similar to that for α-CD. These results will open a new path for the phosphorylation of CDs and polysaccharides in a one-step process in aqueous solution.

3. Experimental

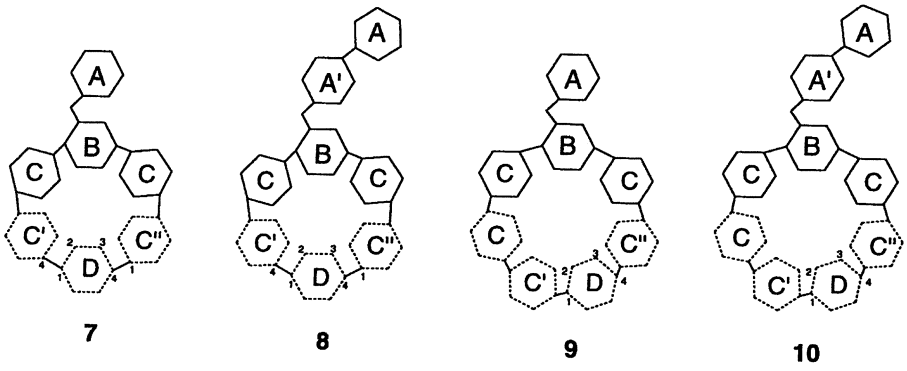
3.1. Chemicals

Sodium *cyclo*-triphosphate (P_{3m}), $Na_3P_3O_9 6H_2O$, was prepared by the procedure described in previous papers.[1,25] Branched CDs (**2**–**5**) were purchased from Bio Research Corporation of Yokohama (Yokohama, Japan). Sodium 2,2-dimethyl-2-silapentane-5-sulfonate

Table 4
^{31}P NMR chemical shifts and coupling constants of the products **6**–**10**

Product	δ (ppm)			J (Hz)		
	P_α	P_β	P_γ	P_α, P_β	P_β, P_γ	P_α, H-2
6	−10.8	−20.9	−5.87	18.2	20.2	8.8
7	−10.7	−20.4	−4.85	17.8	20.0	9.5
8	−10.8	−20.4	−4.87	17.8	20.0	9.0
9	−11.0	−21.1	−5.41	18.4	19.6	9.7
10	−11.0	−20.9	−5.16	18.0	20.8	9.0

Table 5
^{1}H NMR chemical shifts and coupling constants of **7**—**10**



Compound	Residue	δ (ppm)				J (Hz)			
		H-1	H-2	H-3	H-4	P_α, H-2	H-1, H-2	H-2, H-3	H-3, H-4
7	A	4.95	3.54	3.75	3.44		3.3	9.6	9.6
	B	5.06	3.69	4.01	3.58		3.5	9.8	9.8
	C	5.06	3.61	4.01	3.58		3.5	9.8	9.8
	C′	5.06	3.61	4.01	3.61		3.5	9.8	9.8
	C″	5.10	3.42	4.01	*		3.5	9.8	9.8
	D	5.28	4.21	4.14	3.68	9.5	3.0	9.8	9.8
8	A	5.36	3.56	3.71	3.40		3.5	9.5	9.3
	A′	4.93	3.56	3.99	3.64		3.5	9.5	9.5
	B	5.04	3.62	3.98	3.57		3.5	9.8	9.5
	C	5.04	3.60	3.98	3.57		3.5	9.8	9.5
	C′	5.04	3.60	3.98	3.65		3.5	9.8	9.5
	C″	5.09	3.55	3.98	*		3.5	9.8	9.5
	D	5.27	4.19	4.12	3.66	9.0	3.5	9.8	9.8
9	A	4.82	3.41	3.62	3.30		3.5	10.0	9.3
	B	4.94	3.57	3.85	3.47		3.3	9.5	9.5
	C	4.94	3.51	3.85	3.44		3.1	9.4	10.0
	C′	4.94	3.54	3.85	3.54		3.3	9.5	9.5
	C″	4.99	3.44	3.86	*		3.0	10.0	9.5
	D	5.19	4.09	3.97	3.56	9.7	4.0	9.3	9.5
10	A	5.33	3.53	3.71	3.39		3.5	9.0	9.0
	A′	4.91	3.55	3.98	3.62		4.0	9.0	9.0
	B	5.04	3.63	3.95	3.57		3.5	10.1	9.9
	C	5.04	3.58	3.95	3.54		3.5	9.9	9.8
	C′	5.04	3.57	3.95	3.54		3.5	9.9	9.8
	C″	5.09	3.53	3.95	*		3.5	9.8	9.8
	D	5.29	4.19	4.08	3.69	9.0	3.8	9.8	9.8

*Could not be determined. ⓟ: Possible residue of phosphorylation.

(DSS) was purchased from Sigma Chemical Co. (St. Louis, USA). Other reagents were purchased from Wako Chemicals (Osaka, Japan).

3.2. ESI mass spectrometry

The apparatus used a API3000 (Applied Biosystems) using electrospray ionization (ESI) for ion production. The mass spectrometer was operated in the negative-ion mode.

3.3. NMR measurements

^{1}H NMR spectra were measured with Varian Gemini 300 spectrometer. Samples were dissolved in D_2O (99.9%). DSS was used as an external reference for ^{1}H NMR spectra. ^{31}P NMR spectra with and without

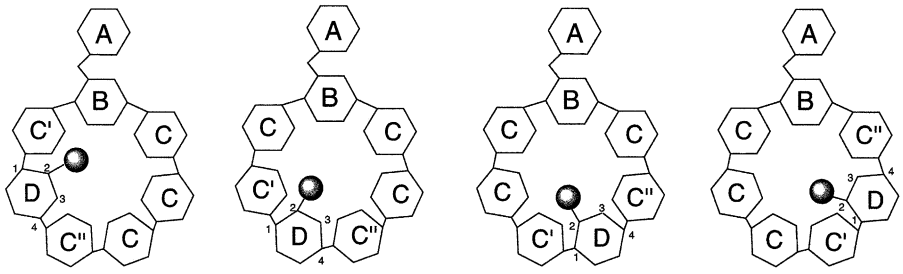


Fig. 3. Possible structures of **9**.

broad-band decoupling, and ^{31}P–^{1}H 2D HMBC spectra were obtained with a Varian Unity INOVA 500 spectrometer. An external standard of 85% H_3PO_4 was used.

3.4. HPLC measurements

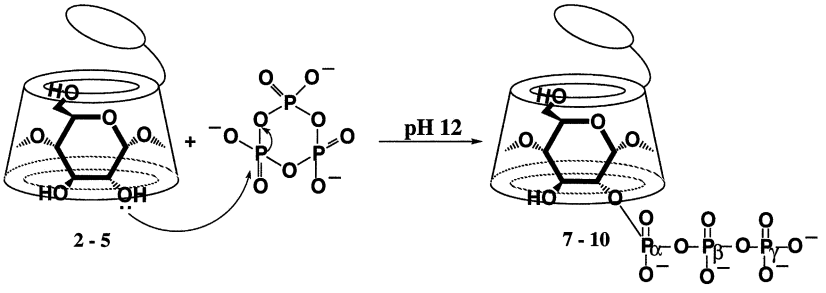
High performance liquid chromatography (HPLC) analyses were carried out with a JASCO GULLIVER HPLC system (Tokyo, Japan) coupled with a JASCO DU-4F flow injection system to detect phosphate by a post-column reaction. A column (150 × 6.0 mm i.d.) packed with a polystyrene-based anion-exchanger (TSK gel, SAX, 5 μm, TOSOH, Japan) was used for the analysis of phosphate. The flow rate was 1.0 mL min^{-1}, and the column temperature was maintained at 40 °C. A convex gradient elution technique using 0.2 and 0.45 M of aqueous potassium chloride was employed for the analysis of phosphate. Sugar phosphate esters, diphosphate (P_2), triphosphate (P_3), and *cyclo*-triphosphate (P_{3m}) were hydrolyzed to monophosphate (P_1) by 6 M sulfuric acid at 140 °C, and the resulting monophosphate was allowed to react with the chromogenic reagent (molybdenum(V)–molybdenum(VI) reagent) to form a phosphorus–molybdenum heteropoly blue complex. The absorbance of the complex was measured at 830 nm.

3.5. Synthesis of 6

Cyclomaltohexaose (**1**) (1.4593 g) and P_{3m} (1.8629 g) were dissolved in H_2O (20 mL), and then the solution was adjusted to pH 12 at 10 °C. After 3 days, the solution was adjusted to pH 7 by 1 M HCl. The yield of the phosphorylated product (**6**) was 15.6% by HPLC. The separation of **6** from the reaction solution was accomplished by anion-exchange chromatography with a 2 × 80 cm column filled with Dowex 1-X2 resin (100–200 mesh, Cl^- form). Elution was carried out with distilled water until no further cyclodextrin appears. Then elution was carried out with aqueous 0.3 M KCl, and each 50-mL fraction was measured by HPLC. The fractionated solution containing only **6** was concentrated at −113 °C in vacuo (freeze-drying). For the purpose of desalting, an aqueous solution of the concentrate was passed over a PD-10 column (Amersham Biosciences, NJ, USA). Each 0.5-mL fraction was measured by HPLC, and the fractionated solution containing only **6** was freeze-dried. The final yield and purity of **6** was 82.5 mg and 93%, respectively. The ESI mass spectrum of **6** showed *m*/*z* 1211.4 corresponding to molecular ion of 2-*O*-triphosphocyclomaltohexaose (Calcd 1210.8 for the protonated dipotassium salt, $[C_{36}H_{61}O_{39}P_3]^{2-}$).

3.6. Syntheses of 7−10

The reactions of branched cyclodextrins (**2**–**5**) (0.45 M, 5 mL) with P_{3m} (0.15 M, 5 mL) were carried out at pH 12 and 10 °C. The isolation procedure of **7**–**10** was the same as **6**. The total yields and purity of **7**–**10** were **7** (74.5 mg, 98%), **8** (71.5 mg, 99%), **9** (90.3 mg, 92%), and **10** (116.7 mg, 94%), respectively. The ESI mass spectrum of **7**–**10** showed their molecular ions for the protonated dipotassium salts; **7**: ESIMS (*m*/*z* 1373.3; Calcd 1373.9 for $[C_{42}H_{72}O_{44}P_3]^{2-}$), **8**: ESIMS (*m*/*z* 1535.7; Calcd 1536.0 for $[C_{48}H_{82}O_{49}P_3]^{2-}$), **9**: ESIMS



Scheme 2.

(m/z 1535.8; Calcd 1536.0 for $[C_{48}H_{82}O_{49}P_3]^{2-}$), **10**: ESIMS ($m/z$ 1697.9; Calcd 1698.2 for $[C_{54}H_{92}O_{54}P_3]^{2-}$).

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