NMR Spectroscopic Studies on Complex Formation between Dimeric (*R*)-3-Hydroxybutanoic Acid and β-Cyclodextrin

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Dimeric (*R*)-3-hydroxybutanoic acid (3HB) was found to form an inclusion complex selectively with β-cyclodextrin (β-CD) in aqueous solution. The complex formation was confirmed by the changes of ^{1}H and ^{13}C NMR spectra of both dimeric 3HB and β-CD in D_2O solution. The continuous variation experiments showed the formation of a 1 : 1 inclusion complex. The dissociation constants at various temperatures between 15 and 47 °C were determined and the thermodynamic analysis of the complex formation was performed. The results indicated that the complex formation is enthalpically favorable but entropically unfavorable. We propose that the 3HB unit with carboxy end-group is located in the narrow side of the β-CD cavity.

Poly[(*R*)-hydroxybutanoate] (P(3HB)) is an optically active biopolyester synthesized as a storage material of carbon and energy in many prokaryotic microorganisms.[1,2] Since its discovery, P(3HB) and related poly[(*R*)-3-hydroxyalkanoates] have attracted growing interest both in basic research and in industry, because of their biodegradability and biocompatibility which allow them to be used as biodegradable substitutes for conventional plastics.[3—5]

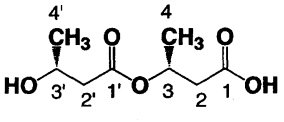
Besides storage properties, a trace of narrow-disperse low-molecular-weight P(3HB) of physiological significance was found in a variety of prokaryotic and eukaryotic organisms.[6—11] The low-molecular-weight P(3HB) was also detected in human blood plasma.[12] In addition, even monomeric and dimeric (*R*)-3-hydroxybutanoic acid (3HB) with physiological functions were found in some organisms.[13] The low-molecular-weight P(3HB) always appears to be complexed with other specific large molecules.[14] A typical example is P(3HB) complexes with calcium polyphosphate (Ca•PPi). It was proposed that the complexes may be located in the inner cell membrane, acting as a nonproteinogenic transmembrane ion channel, which is responsible for Ca, PPi, or even DNA uptake of the cell.[8] Although the mechanism is still not clear, there is no doubt that the molecular recognition plays an important role in the physiological processes with P(3HB).

Cyclodextrins (α-CD, β-CD, and γ-CD) are a series of cyclic oligosaccharides having doughnut-shaped hydrophobic cavities, which are able to bind various molecules to form inclusion complexes. They have been studied as models to mimic enzyme activity and to understand the mechanism of molecular recognition.[15—17] In order to understand the molecular recognition of the low-molecular-weight P(3HB) in the biological system, we have prepared dimeric 3HB (H-(3HB)$_2$A) as a model compound of low-molecular-weight P(3HB), and investigated its complex formation with cyclodextrins (Chart 1). We have found that dimeric 3HB selectively forms inclusion complexes with β-CD. In this paper, we report the NMR spectroscopic studies on the complex formation of dimeric 3HB with β-CD.

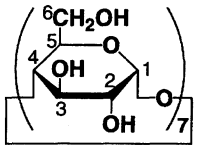
Results and Discussion

Complex Formation and Selectively. On addition of β-CD to a D_2O solution of dimeric 3HB, ^{1}H and ^{13}C NMR spectra of dimeric 3HB were found to show changes in chemical shift and/or in resonance pattern. Figure 1 shows the H2, H2′, and H3 resonances of ^{1}H NMR spectra of dimeric 3HB in the absence and the presence of β-CD in D_2O. With an increase in ratio of β-CD to dimeric 3HB, resonances of H2 and H3 shift to higher magnetic fields, and resonance patterns of H2 and H2′ also show remarkable changes. The results indicate that dimeric 3HB forms a complex with β-CD in aqueous solution.

Figure 2 shows the ^{1}H NMR spectra of β-CD in the absence and the presence of dimeric 3HB in D_2O. On addition of dimeric 3HB to a D_2O solution of β-CD, the spectra show remarkable changes in chemical shift of proton resonances of H-3 and H-5 of β-CD, which are located in the interior of



H(3HB)$_2$A



β-CD

Chart 1.

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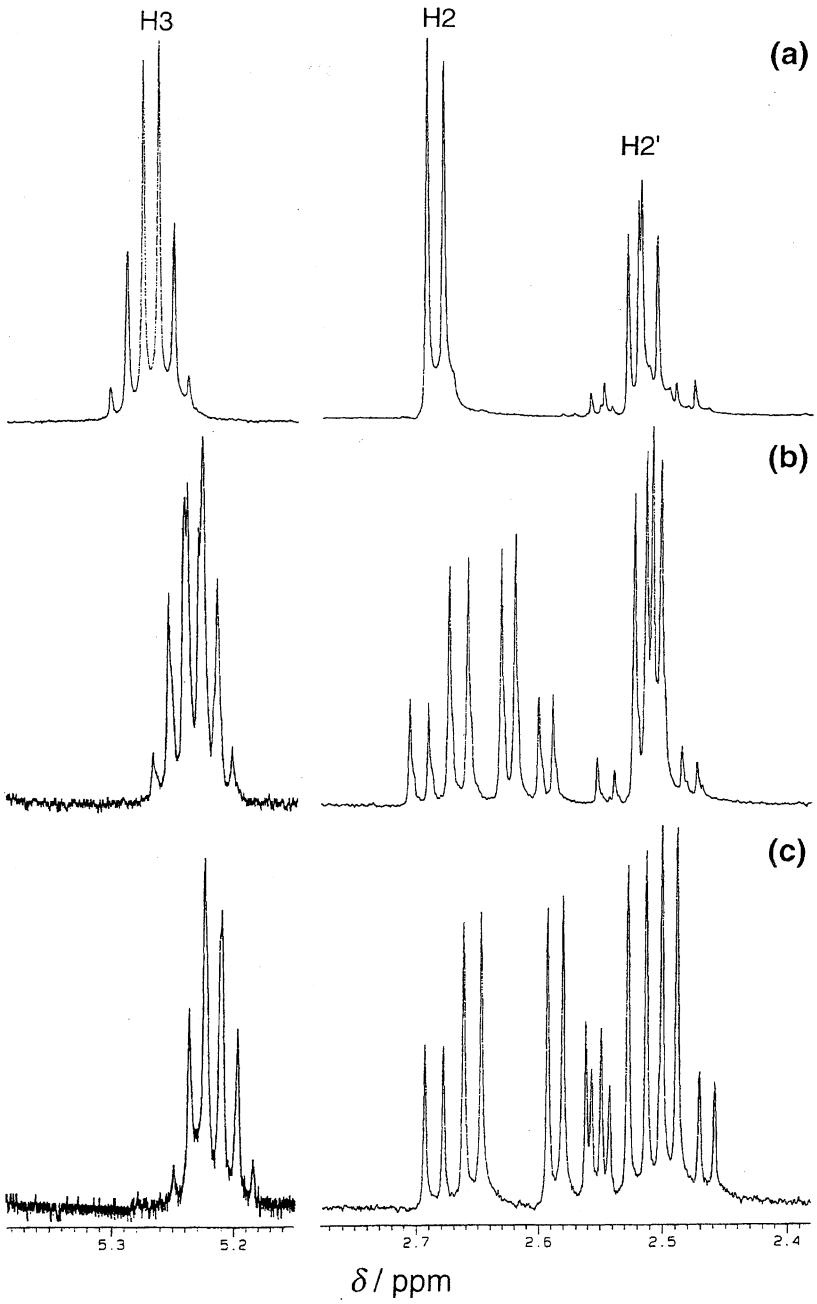

Fig. 1. 500-MHz ^{1}H NMR spectra for resonances of H2, H2′, and H3 of H(3HB)$_2$A in the absence and presence of β-CD in D_2O at 27 °C. (a) Pure H(3HB)$_2$A, (b) [β-CD]/[H(3HB)$_2$A] = 1.0, and (c) [β-CD]/[H(3HB)$_2$A] = 3.0.

the cavity,[15] while the resonances of H-1, H-2, H-4, and H-6, which are located in the exterior of the cavity,[15] remain unchanged. The results reveal that dimeric 3HB is included in the interior of the cyclodextrin cavity to form an inclusion complex.

In contract to β-CD, on addition of α-CD or γ-CD to a D_2O solution of dimeric 3HB. both ^{1}H and ^{13}C NMR spectra of dimeric 3HB were found to show no remarkable changes in chemical shift or in resonance pattern. The ^{1}H NMR spectra of α-CD and γ-CD in the absence and the presence of dimeric 3HB were recorded too. For all protons of α-CD and γ-CD, no remarkable changes in chemical shift were observed. The results indicate that dimeric 3HB does not form inclusion complexes with α-CD or γ-CD. We also found that monomeric 3HB does not form inclusion complexes with cyclodextrins at all. Therefore, the complex formation between dimeric 3HB and β-Cd is of high selectivity.

Stoichiometry. The stoichiometry of the inclusion complex formation between dimeric 3HB and β-CD may be obtained by continuous variation experiments, i.e., recording ^{1}H NMR spectra for solutions where the molar ratio of dimeric 3HB and β-CD is varied but the overall molar concentration of the two components is kept constant. Figure 3 shows the continuous variation plots (Job plots[18]) for chemical shifts for protons of dimeric 3HB (a) and β-CD (b). Plots for all protons in Fig. 3 show a maximum at molar ratio

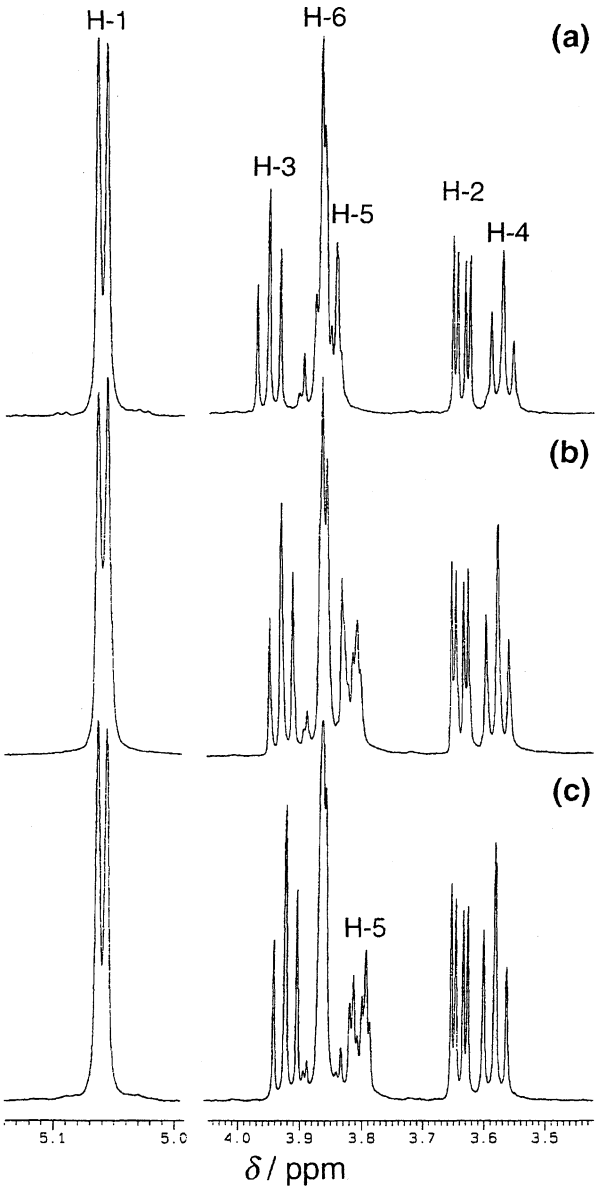

Fig. 2. 500-MHz ^{1}H NMR spectra of β-CD in the absence and the presence of H(3HB)$_2$A in D_2O at 27 °C. (a) Pure β-CD, (b) [H(3HB)$_2$A]/[β-CD] = 1.0, and (c) [H(3HB)$_2$A]/[β-CD] = 3.0.

between dimeric 3HB and β-CD equal to 0.5, indicating the formation of a 1 : 1 complex.

Dissociation Constant and Thermodynamic Analysis. Since dimeric 3HB (D) and β-CD (CD) form a 1 : 1 inclusion complex, the equilibrium of the complex may be expressed by:

$$D + CD \underset{K_d}{\overset{K_a}{\rightleftharpoons}} D{\cdot}CD. \quad (1)$$

Then, the dissociation constant K_d for the complex formation is given by

$$K_d = [D][CD]/[D{\cdot}CD], \quad (2)$$

where [D] and [CD] are the equilibrium concentrations of dimeric 3HB and β-CD, respectively, and [D·CD] is the equilibrium concentration of the complex. For a proton of dimeric 3HB, the observed chemical shift δ is:

$$\delta = \delta_D + ([D{\cdot}CD]/[D]_0)(\delta_{D{\cdot}CD} - \delta_D),$$

where $[D]_0$ is the initial concentration of dimeric 3HB, and δ_D and $\delta_{D{\cdot}CD}$ are the chemical shifts of the proton in pure dimeric 3HB and in pure complex, respectively. Therefore, the observed induced chemical shifts $\Delta\delta$ is:

$$\Delta\delta = \delta - \delta_D = ([D{\cdot}CD]/[D]_0)\Delta\delta_{D{\cdot}CD}, \quad (3)$$

where $\Delta\delta_{D{\cdot}CD} = \delta_{D{\cdot}CD} - \delta_D$. The following equation then is applicable from Eqs. 2 and 3:

$$K_d = (\Delta\delta_{D{\cdot}CD}/\Delta\delta - 1)[CD].$$

The above equation may be rearranged in the form:

$$1/\Delta\delta = \Delta\delta_{D{\cdot}CD} + (K_d/\Delta\delta_{D{\cdot}CD})(1/[CD]). \quad (4)$$

If one is working under a condition of initial β-CD concentration $[CD]_0$ in large excess over $[D]_0$, then Eq. 4 becomes:

$$1/\Delta\delta = 1/\Delta\delta_{D{\cdot}CD} + (K_d/\Delta\delta_{D{\cdot}CD})(1/[CD]_0). \quad (5)$$

This is an analog of the Benesi–Hildebrand equation.[19] For a series of solutions, a plot of $1/\Delta\delta$ against $1/[CD]_0$ should be linear. The intercept with the ordinate yields $1/\Delta\delta_{D{\cdot}CD}$ and from the gradient $K_d/\Delta\delta_{D{\cdot}CD}$ may be obtained.

However, our conditions were only $[CD]_0/[D]_0 > 5$. So we first obtained approximate values of $\Delta\delta_{D{\cdot}CD}$ and K_d by using Eq. 5. The values were taken to calculate [CD] in the given solutions. Then we estimated new values of $\Delta\delta_{D{\cdot}CD}$ and K_d by using Eq. 4. This procedure was repeated until new values of $\Delta\delta_{D{\cdot}CD}$ and K_d were identical to previous ones. This method is similar to that described by Lang.[20] For our data, the identical values were reached after three repetitions. The dissociation constants K_d at various temperatures between 15 and 47 °C were determined. The temperature dependence of the dissociation constants yields an enthalpy $\Delta H°$ of −10.4 kcal mol^{-1} for the complex formation. The free energy $\Delta G°$ and the entropy $\Delta S°$ were also calculated. Table 1 summarizes all of the thermodynamic parameters for the complex

Table 1. Thermodynamic Parameters for Complex Formation between H(3HB)$_2$A and β-CD

Temp/°C	K_d/mol dm^{-3}	$\Delta G°$/kcal mol^{-1}	$\Delta H°$/kcal mol^{-1}	$\Delta S°$/cal mol^{-1} K^{-1}
15.0	6.59×10^{-3}	−2.87		
27.0	1.06×10^{-2}	−2.71	−10.4	−25.6
37.0	2.56×10^{-2}	−2.21		
47.0	3.69×10^{-2}	−2.10		

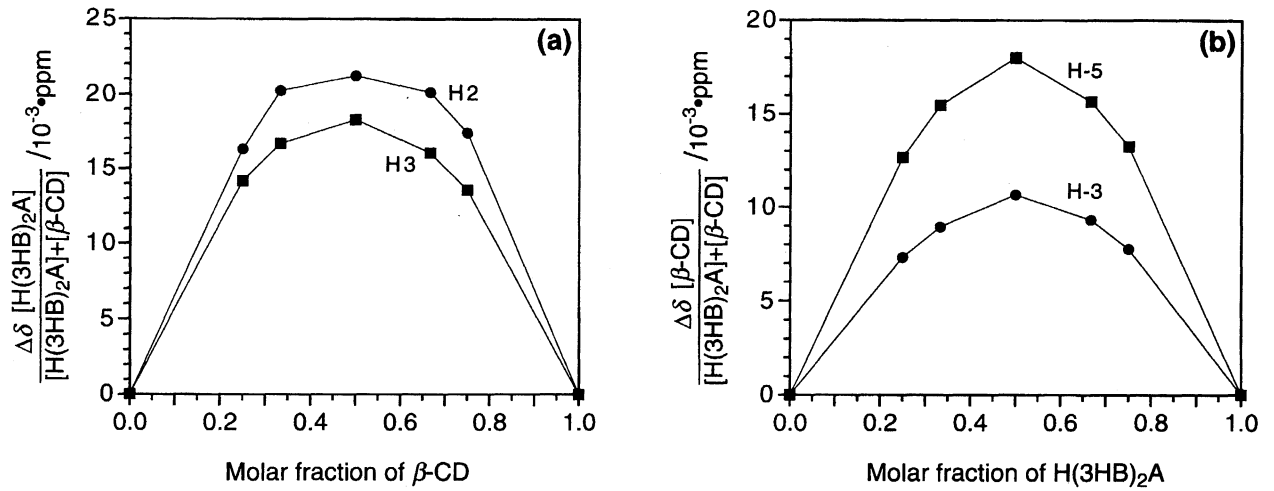

Fig. 3. Continuous variation plots for β-CD and H(3HB)$_2$A system. (a) Plots for chemical shifts of protons of H(3HB)$_2$A, and (b) Plots for chemical shifts of protons of β-CD.

formation between dimeric 3HB and β-CD. It seems that the large favorable enthalpy is due to the contribution of Van der Waals–London dispersion force and/or hydrogen bonding between the host and the guest molecules, while the large unfavorable entropy is due to the loss of conformational and rotational freedom of dimeric 3HB and/or conformational freedom of β-CD.[21,22] The changes both in enthalpy and in entropy reveal a tight inclusion and a deep penetration of dimeric 3HB within the cavity of β-CD. A key factor which affects the complex formation is the size correspondence between dimeric 3HB and β-CD cavity. Therefore, we can understand why dimeric 3HB does not form complexes with α-CD and γ-CD, whose cavities are too small or too large for 3HB dimer.

Inclusion Mode and Structure of Complex. Figure 4 shows the plots of the ^{1}H NMR induced chemical shifts for protons of dimeric 3HB against molar ratios of β-CD to dimeric 3HB. All protons of dimeric 3HB show changes in chemical shift with an increase in β-CD ratio. However, the changes for H2 and H3 are much more remarkable than those for other protons. Table 2 gives the data of the ^{13}C NMR chemical shifts of dimeric 3HB in the absence and the presence of β-CD and the induced chemical shifts of dimeric 3HB by β-CD. The values of $\Delta\delta$ ratios of C1 to C1′, of C2 to C2′, of C3 to C3′, and of C4 to C4′ are 5.9, 3.0, 4.7, and 1.9, respectively. It is apparent that the carbons of 3HB unit with carboxy end-group show relatively more

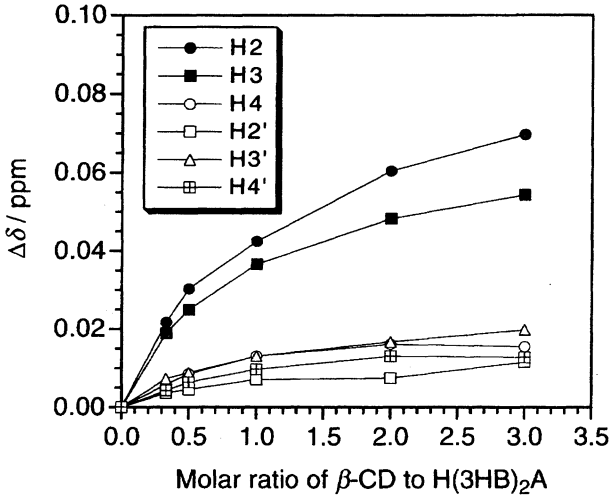

Fig. 4. Plots of the ^{1}H NMR induced chemical shifts by β-CD for protons of H(3HB)$_2$A against molar ratios of β-CD to H(3HB)$_2$A. Spectra determined in D$_2$O at 27 °C.

remarkable changes than those of another 3HB unit with hydroxy end-group. The results from both Fig. 4 and Table 2 indicate that the 3HB unit with carboxy end-group is more subject to the effect of the inner wall of the cavity of β-CD. A probable explanation of the results is that the 3HB unit with carboxy end-group is included in the narrow side of the cavity of β-CD, as shown in Fig. 5. Such geometry makes the 3HB unit with carboxy end-group closer to the inner wall

Table 2. ^{13}C NMR Chemical Shifts of Dimeric 3HB in the Absence and the Presence of β-CD and the Induced Chemical Shifts of Dimeric 3HB by β-CD in D$_2$O at 27 °C

	Carbon of H(3HB)$_2$A/ppm							
	C1	C1′	C2	C2′	C3	C3′	C4	C4′
H(3HB)$_2$A[a)]	178.03	178.03	43.10	46.16	71.60	67.21	21.60	24.50
β-CD–H(3BH)$_2$A[b)]	175.53	177.61	43.40	46.26	71.46	67.24	21.81	24.61
$\Delta\delta$	−2.49	−0.42	0.30	0.10	0.14	0.03	0.21	0.11

a) 20.0 mmol dm^{-3} H(3HB)$_2$A in D$_2$O. b) 10.0 mmol dm^{-3} H(3HB)$_2$A and 10.0 mmol dm^{-3} β-CD in D$_2$O.

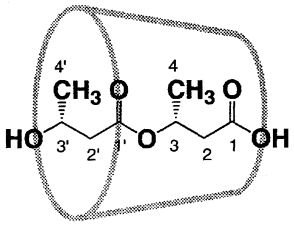

Fig. 5. Probable structure of β-CD–H(3HB)$_2$A complex.

of the cavity of β-CD; therefore the chemical shifts of both ^{1}H and ^{13}C of the 3HB unit with carboxy end-group are more subject to the effect of β-CD.

Conclusions

Molecular recognition between dimeric 3HB and cyclodextrins was studies by using ^{1}H and ^{13}C NMR spectroscopy. It was found that dimeric 3HB forms 1 : 1 inclusion complexes selectively with β-CD, while monomeric 3HB does not form any complex with cyclodextrins. It is proposed that the 3HB unit with carboxy end-group is located in the narrow side of the β-CD cavity in β-CD–dimeric 3HB complex. The dissociation constants of the complex at various temperatures between 15 and 47 °C are estimated to be between 6.59×10^{-3} to 3.69×10^{-2} mol dm^{-3}. The thermodynamic analysis reveals that the complex formation is enthalpically favorable but entropically unfavorable. The results of thermodynamic analysis indicate that the driving forces for the complex formation are Van der Waals–London dispersion force and hydrogen bonding, and the complex formation is accompanied by conformational variations of both components. Both the high selectivity and the thermodynamic analysis imply that the size correspondence plays an important role in the complex formation. It is assumed from the results that the size correspondence is also of great importance when low-molecular-weight P(3HB) forms a complex in a biological system. So, we can understand why P(3HB) in biological system forms complexes always with other specific large molecules rather than with small ones.

Experimental

Measurements. The ^{1}H and ^{13}C NMR spectra were recorded on a JEOL GX-500 NMR spectrometer at 500 and 125 MHz, respectively. The ^{1}H NMR measurements were carried out with 5.3-s pulse repetition, 5000-Hz spectral width, and 32768 data points. Chemical shifts were referred to DSS (δ=0.00 ppm) as external reference in a D_2O solution. Mass spectra were obtained on a JEOL JMS-HX 110 mass spectrometer by the fast atom bombardment (FAB) method in positive ion mode with glycerol matrix, 5.0—10.0 kV acceleration voltage, and 1000 resolution. Mass calibration was carried out with CsI spectrum.

Continuous Variation Experiments. The total concentrations of 3HB dimer and β-CD were kept to be 20.0 mmol dm^{-3} in D_2O, while the molar ratios of 3HB dimer and β-CD were varied to be 1 : 0, 3 : 1, 2 : 1, 1 : 1, 1 : 2, 1 : 3, and 0 : 1. The ^{1}H NMR spectra of the samples were recorded at 27 °C. The Job plots were drawn based on the induced chemical shifts of protons of 3HB dimer and β-CD, respectively.

Determination of Dissociation Constants. A series of D_2O solutions which include 1.5 mmol dm^{-3} of 3HB dimer 0.0, 8.0, 10.0, 12.0, 14.0, and 16.0 mmol dm^{-3} of β-CD were prepared. The ^{1}H NMR spectra of the solutions were recorded at 15, 27, 37, and 47 °C. The dissociation constants and other thermodynamic parameters were estimated from the induced chemical shifts of H3 of 3HB dimer. The average chemical shift values for the multiple peaks of H3 were used.

Materials. α-Cyclodextrin, β-cyclodextrin, and γ-cyclodextrin were supplied by Wako Pure Chemical Ind. Ltd. Other chemicals for synthesis were purchased from Kanto Chemical Co., Inc. Deuterium oxide (D_2O, 99.95%) was obtained from Merck.

Dimeric 3HB, (3*R*)-3{[(3*R*)-3-hydroxybutanoyl]oxy}butanoic acid (H(3HB)$_3$A), was prepared according to the procedures described in our previous papers.[23,24] ^{1}H NMR ($CDCl_3$, 500 MHz), δ=5.34 (m, 1H, C(3)H), 4.21 (m, 1H, C(3′)H), 2.55—2.69 (m, 2H, C(2)H$_2$), 2.39—2.48 (m, 2H, C(2′)H$_2$), 1.33 (d, J=6.4 Hz, 3H, C(4)H$_3$), 1.23 (d, J=6.4 Hz, 3H, C(4′)H$_3$). FAB-MS *m/z* (rel intensity) 191.3 (M^++1; 100), 105.6 (57). Anal. Calcd for $C_8H_{14}O_5$: C, 50.52; H, 7.42%. Found: C, 49.80; H, 7.44%.

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