BIOORGANIC CHEMISTRY 11, 72–84 (1982)

Guest-Induced Conformational Change of β-Cyclodextrin Capped with an Environmentally Sensitive Chromophore

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Received June 19, 1981

A capped cyclodextrin, 6-deoxy-6-(*p*-hydroxy-*m*-nitrophenacylthio)-β-cyclodextrin, was prepared in order to detect any conformational change of the host upon the guest binding. The association constant between the cyclodextrin and 1-adamantanecarboxylate, cyclohexanecarboxylate, *p*-methylbenzoate, 3,3-dimethylbutyrate, or 2,2-dimethylpropionate was enhanced 20, 5.3, 3.7, 2.3, or 2.0 times, respectively, by chromophore capping. The changes in the electronic, NMR, and circular dichroism spectra as well as pK_a of this cyclodextrin upon binding of the guest strongly indicate a conformational change around the chromophoric moiety of the cyclodextrin.

INTRODUCTION

Modeling of enzyme function by use of cyclodextrins has been extensively and successfully studied in the past decade (*1*). Molecular recognition of cyclodextrins originally found by Cramer (*2*) and Bender (*3*) was much enhanced by flexible (*4*) or rigid (*5*) cappings with hydrophobic groups on the primary hydroxyl side of cyclodextrins. Also, multiple recognition was attained with duplex cyclodextrin (*6*) and metal complex-capped cyclodextrin (*7*). The catalytic ability of cyclodextrins, again originally found by Cramer (*2*) and Bender (*3*), was much improved by the introduction of an appropriate group, such as imidazole (*8*) or oximate (*9*). Thus, by the use of cyclodextrins, models of ribonuclease (*10*), transaminase (*11*), ligase (*12*), carbonic anhydrase (*13*), and rhodopsin (*14*) have been constructed by Breslow and Tabushi.

Although conformational changes upon substrate binding are an important aspect of enzyme catalysis (*15*), no modeling of this phenomenon has been studied by the use of a modified cyclodextrin (*16*). In the present paper, we describe a substrate-induced conformational change for β-cyclodextrin modified with an appropriate flexible chromophore moiety (**3a**).

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0045-2068/82/010072-13$02.00/0

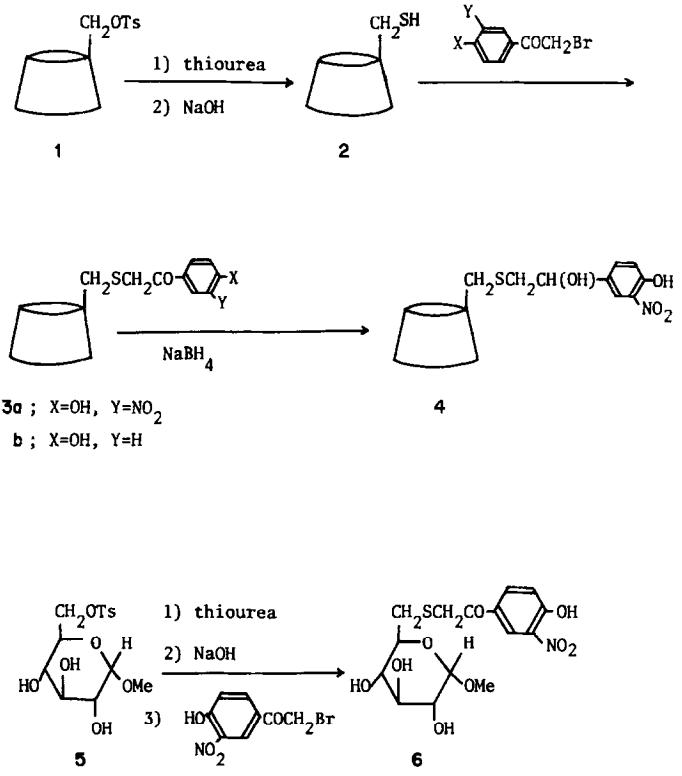
RESULTS AND DISCUSSION

Preparation of Chromophore-Modified Cyclodextrins and Chromophore-Modified Glucoside

The starting materials, β-cyclodextrin-6-monotosylate (**1**) (*17*) and α-D-methyl-glucoside-6-tosylate (**5**) (*18*), were prepared according to reported procedures. The synthetic reactions for compounds **3a, 3b, 4,** and **6** are shown in Scheme 1. These substances were purified through silica gel, DEAE-cellulose, and Sephadex columns, followed by recrystallization. Characterization of these compounds were carried out by NMR, ir, and electronic spectra as well as elemental analysis or field desorption mass spectrum.

Electronic Spectral Change of Chromophore-Modified Cyclodextrin on Guest Binding

The electronic spectrum of **3a** in a borate buffer (pH 9.18) is depicted in Fig. 1 with λ_{max} at 396 and 323 nm. Addition of a guest compound such as 1-adamantane-carboxylate, 1-adamantylammonium, cyclohexanecarboxylate, *p*-methylben-zoate, 3,3-dimethylbutyrate, and 2,2-dimethylpropionate caused a blue shift of the



SCHEME 1

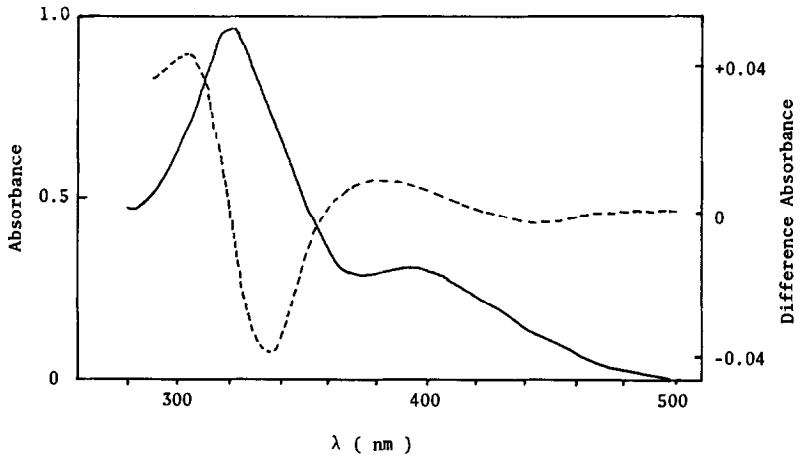


FIG. 1. Absorption (——) and difference (– – –) spectra of chromophore-modified β-cyclodextrin, **3a** (5×10^{-5} *M*, pH 9.18). For difference spectrum, sample compartment also contained sodium 1 adamantanecarboxylate (5.8×10^{-4} *M*) where the cyclodextrin was bound 82%.

absorption of **3a** as shown in the difference spectrum depicted in Fig. 1. On treatment of the spectral data by the Hildebrand–Benesi method (*19*) and/or the Scatchard method (*20*), the formation of 1 : 1, host–guest association was indicated, and the association constant was estimated (Table 1).

The guest compounds were more strongly bound by the chromophore-modified cyclodextrin, **3a**, than by native β-cyclodextrin, even though the chromophore moiety could have been included in the cavity to inhibit competitively the binding of the guest. Table 1 showed that **3a** bound 1-adamantanecarboxylate, cyclohexanecarboxylate, *p*-methylbenzoate, 3,3-dimethylbutyrate, and 2,2-dimethylpro-

TABLE 1

ASSOCIATION CONSTANTS BETWEEN CYCLODEXTRINS AND GUEST MOLECULES[a]

Guest	K_a of host (M^{-1})	
	β-Cyclodextrin	Modified β-cyclodextrin (**3a**)
Sodium 1-adamantanecarboxylate	625[b]	12,700[c] (10,000)[c,d]
1-Adamantylammonium chloride		2,100[e] (2054)[d,e]
Sodium cyclohexanecarboxylate	136[c]	714[c]
Sodium *p*-methylbenzoate	109[c]	407[c]
Sodium 3,3-dimethylbutyrate	215[f]	500[f]
Sodium 2,2-dimethylpropionate	29[c]	59[c]

[a] Unless otherwise noted, the constants were estimated on the basis of the electronic absorption change.

[b] Reported value, pH 9.00 carbonate buffer (see Ref. (5)).

[c] In borate buffer (pH 9.18), 25°C.

[d] The constants were estimated on the basis of the circular dichroism spectral change, 25°C.

[e] In phosphate buffer (pH 6.86), 25°C.

[f] In phosphate buffer (pH 11.0), 25°C.

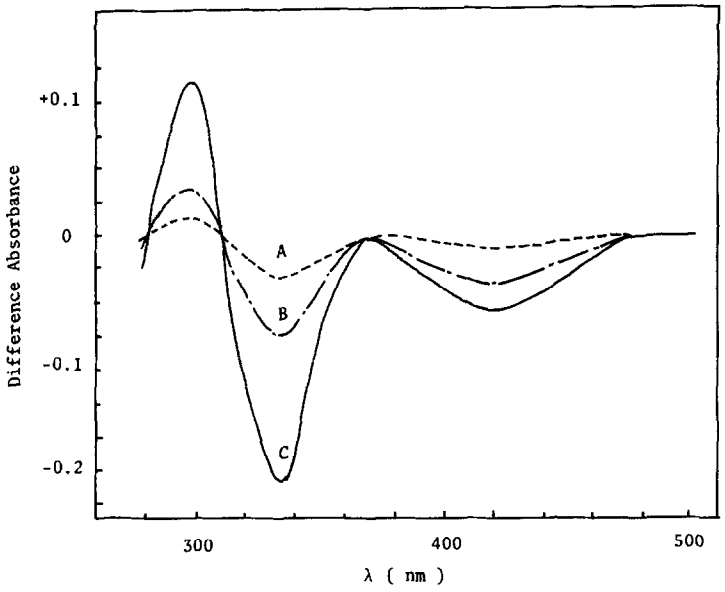
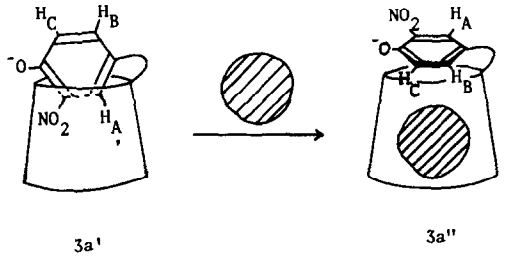


FIG. 2. Difference spectra of **7**. Reference compartment contained **7** (4.94×10^{-5} *M*) in 50/50 dioxane-borate buffer (pH 9.18). Sample compartment contained **7** (4.94×10^{-5} *M*) in dioxane-borate buffer (pH 9.18): (A) 40/60; (B) 20/80; (C) 0/100.

pionate, 20, 5.3, 3.7, 2.3, and 2.0 times more strongly, respectively, than the parent β-cyclodextrin. Thus, this chromophore substituent not only enhanced the guest binding but also enhanced the binding selectivity. Such selective recognition was also operative in the case of flexibly or rigidly capped β-cyclodextrin (*4*, *5*), suggesting significant interaction between the chromophore substituent and the guest molecule in the present case also.

The observed blue shifts of the absorptions of **3a** upon guest binding suggest the movement of the chromophore moiety to a less hydrophobic environment because the model compound, **7**, showed a similar behavior when perturbed with a hydrophilic solvent. The absorption at 404 and 319 nm of **7** in aqueous dioxane (pH 9.18, borate buffer + dioxane) showed blue shift as water content was increased (Fig. 2). Red shift of the absorptions of *p*-nitrophenolate by cyclodextrin inclusion (*21*) also support the above suggestion. Thus, the chromophore moiety may be partially included in the hydrophobic cavity in the absence of a guest and,



3a' 3a''

SCHEME 2

on guest binding, it may move out of the cavity, presumably, to accomodate the guest (Scheme 2).

^{1}H-NMR Spectral Change of Chromophore-Modified Cyclodextrin

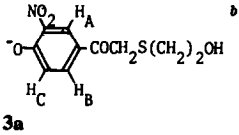
The ^{1}H-NMR of **3a**, **7**, and a mixture of **3a** and excess of 1-adamantanecarboxylate were measured in D_2O. In the last case, 84.6% of the host was estimated to form the inclusion complex with 1-adamantanecarboxylate based on the association constant. The aromatic ^{1}H-NMR chemical shifts of **3a** and **7** are summarized in Table 2. When comparing the chemical shifts of **3a** with those of **7**, it can be seen that H_A in **3a** is the most shielded ($\Delta\delta = 0.29$) by the cyclodextrin moiety. In the present case, a shielding effect by the β-cyclodextrin moiety was observed. However, deshielding effects are also reported in the inclusion of *p*-nitrophenolate, benzoic acid, or benzoate by α-cyclodextrin (*22*). This difference may be due to the difference in the depth and the mode of the guest inclusion. On the other hand, the inclusion of 1-adamantanecarboxylate in the cavity of **3a** diminished the shielding effect. These NMR observations as well as the electronic spectral data of **3a** suggest that a conformational change occurs around the chromophore moiety upon guest binding as shown in Scheme 2, where H_A is included in the cavity (**3a'**) in the absence of a guest and the chromophore moiety is pushed out of the cavity by the guest molecule upon complex formation (**3a''**).

pK_a Change of Chromophore-Modified Cyclodextrin

The pK_a of the phenolic moiety of **7** and **3a** were titrated photomoetrically at 25°C. The pK_a of **7** in aqueous solution was 4.60. The pK_a of **7** included (96%) by β-cyclodextrin was smaller by 0.47 pH unit while the pK_a of **3a** was smaller by 0.20 pH unit. This finding strongly supports the mechanism shown in Scheme 2 featuring partial inclusion of the chromophore moiety of **3a** in the cyclodextrin cavity in aqueous solution. Interestingly, inclusion (97%) of 1-adamantylammo-

TABLE 2

^{1}H-NMR CHEMICAL SHIFT OF PHENOLIC MOIETY[a]

Phenolate	Guest	Chemical shift (δ)		
		H_A	H_B	H_C
 [b]	—	8.49	7.79	6.69
3a	—	8.20	7.61	6.70
3a[c,d]	Sodium 1-adamantanecarboxylate	8.44	7.71	6.61

[a] D_2O solvent.
[b] The internal standard: sodium acetate and sodium 3-(trimethylsilyl)propionate.
[c] The internal standard: sodium acetate.
[d] 84.6% of **3a** included the guest molecule.

nium shifted the pK_a value of **3a** up to that of **7** in aqueous solution. This result is in accordance with the proposed mechanism.

Circular Dichroism Spectral Change of Chromophore-Modified Cyclodextrin

The compounds, **3a** and **6**, gave rise to the circular dichroism (CD) spectra depicted in Fig. 3. Cramer and his co-workers (*23*) reported that peracetyl-β-cyclodextrin xanthate, **8**, showed very similar ORD spectrum to that of peracetyl-α-D-methylglucoside xanthate, **9**, strongly suggesting no specific interaction between the xanthate moiety and the cyclodextrin cavity of **8**. In marked contrast, the spectrum of **3a** was quite different from that of **6**. Also, **3a** exhibited a much more intense CD spectrum than **6**. These results suggest that significant and specific interaction exists in **3a** between the chromophore and the asymmetric moiety, cyclodextrin.

Dependency of the CD spectrum of **3a** on temperature and the solvent were elucidated (Figs. 4 and 5). As the temperature decreased, the ellipticities increased, suggesting that the conformation of **3a** becomes more rigid. The temperature dependency seems to be similar to a transition between native and denatured forms of an enzyme. The increase of the content of ethanol in the solvent brought marked changes of the spectrum. The spectrum in the 80% ethanol was broad, suggesting a much greater degree of mobility of the chromophore moiety than that

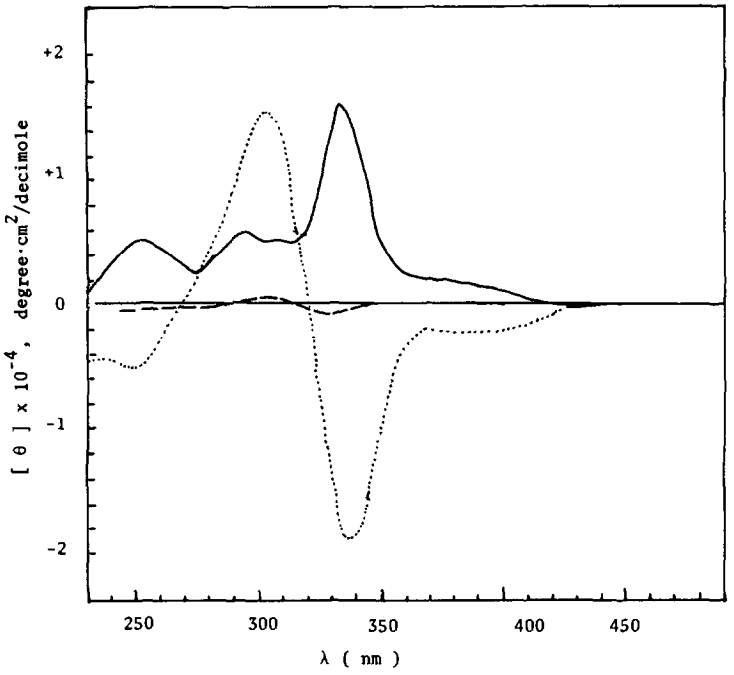
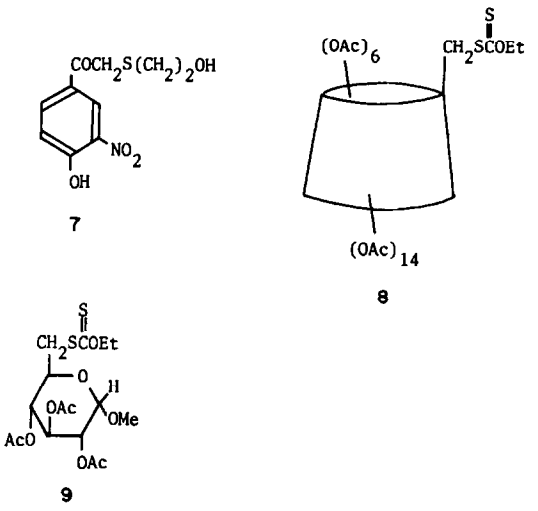


FIG. 3. Circular dichroism spectra of **3a** (——). **6** (– – –) and inclusion complex of **3a** with sodium 1-adamantanecarboxylate (· · ·) in phosphate buffer (pH 11.0) at 25°C. The spectrum of **6** (6×10^{-4} *M*) in the presence of sodium 1-adamantanecarboxylate (1×10^{-2} *M*) was same as that of **6** alone.



SCHEME 3

in the aqueous solution. These dependencies also supported the specific interaction (**3a'**) of the chromophore moiety with the cyclodextrin cavity in aqueous solution.

Although the presence of 1-adamantanecarboxylate did not affect the CD spectrum of **6**, the inclusion of 1-adamantanecarboxylate caused a dramatic change of

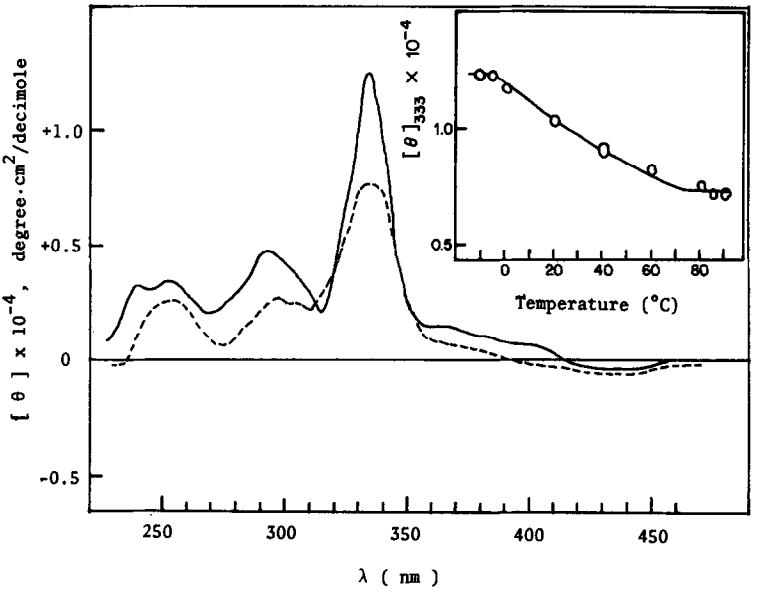


FIG. 4. Temperature dependency of circular dichroism spectrum of **3a** in phosphate buffer (pH 11.0) containing ethanol (2%). Addition of ethanol was necessary for low temperature measurements: −10°C (——), 90°C (---). Insert: Temperature dependency of the ellipticity at 333 nm.

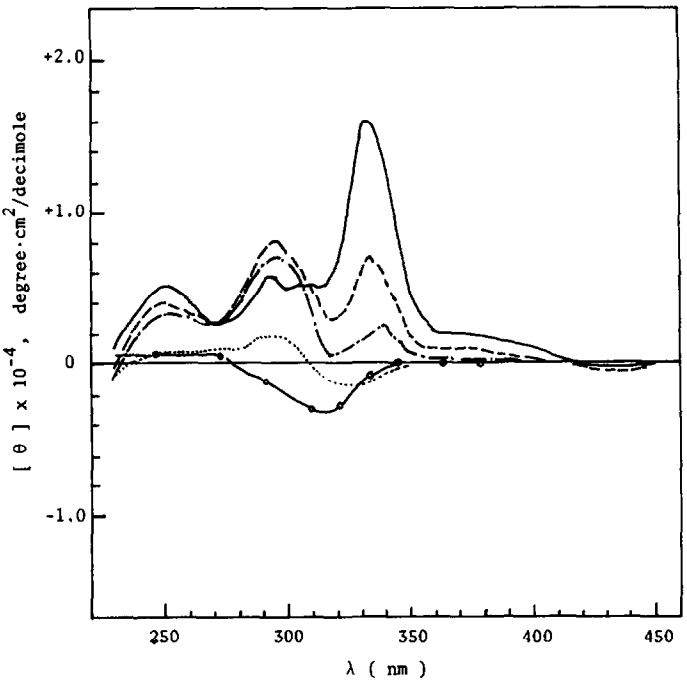


FIG. 5. Solvent effect of circular dichroism spectrum of **3a** in phosphate buffer (pH 11.0) at 25°C. Ethanol/phosphate buffer; 0/100 (——), 10/90 (– – –), 20/80 (– · –), 40/60 (· · ·), 80/20 (–o–).

the CD spectrum of **3a**, in particular reversions of the Cotton effects at 400, 333, and 250 nm (Figure 3). These observations also support the idea of a conformational change upon guest inclusion in the vicinity of the chromophore moiety. The

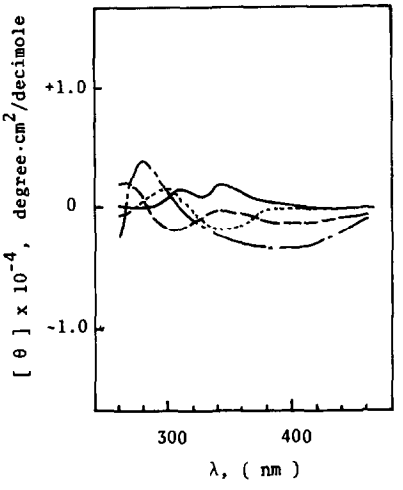


FIG. 6. Circular dichroism spectra (pH 11.0) of the chromophore-modified cyclodextrin, **3b** (6.8×10^{-4} *M*), and **4** (2×10^{-4} *M*) alone (—— and – – –, respectively) and in the presence of sodium 1-adamantanecarboxylate (1×10^{-2} *M*) (· · · and – · –, respectively) at 25°C.

CD spectral change by the Scatchard method (Table 1) shows good agreement with the corresponding values obtained from the electronic spectral changes.

From the comparison of CD spectrum of **3a** with those of **3b** and **4** shown in Fig. 6, it is suggested that the presence of carbonyl and nitro groups is necessary for the appearance of the strongest ellipticity at 333 nm in opposite sign.

SUMMARY AND CONCLUSION

All of the spectral and pK_a changes that occurred upon guest binding by the chromophore-modified cyclodextrin (**3a**) are consistent with a guest-induced conformational change around the chromophore moiety. This chromophore modification has not only strengthened the binding by the cyclodextrin but also enhanced the binding selectivity.

EXPERIMENTAL

General Notes

Electronic spectra, ir spectra, ^{1}H-NMR spectra, field desorption mass spectra, and circular dichroism spectra were obtained on a Hitachi Model 200-10 spectrophotometer, a Hitachi 215 grating infrared spectrophotometer, a JOEL FX FT-NMR (or a JOEL C 60H spectrometer), a JOEL D 300 spectrometer, and a Jasco 20C spectrometer, respectively.

6-Deoxy-6-mercapto-β-cyclodextrin (**2**)

A mixture of 2 g of 6-deoxy-6-tosyloxy-β-cyclodextrin (**1**) (*17*) and 2 g of thiourea in 100 ml of 80% aqueous methanol was refluxed for 2 days and was evaporated *in vacuo*. Methanol (30 ml) was added to the residue followed by stirring for 1 hr. The solid was filtered (1.54 g) and dissolved in 69 ml of 10% NaOH and kept at 50° for 5 hr. After the pH of the solution was adjusted to 2 by addition of 10% HCl, 5 ml of trichloroethylene was added to the solution. After stirring overnight, the precipitate was filtered and washed with water. Evaporation of trichloroethylene *in vacuo* followed by repeated recrystallization from water gave **2** (1.13 g, 58.4%). R_f of TLC (silica gel, CH_3CO_2Et/n-PrOH/H_2O, 7/7/5), 0.23; ^{1}H-NMR and ir are very similar to those of β-cyclodextrin.

6-Deoxy-6-(p-hydroxy-m-nitrophenacylthio)-β-cyclodextrin (**3a**)

A solution of 230 mg of **2** and 104 mg of 2-nitro-4-bromoacetylphenol in 60 ml of degassed aqueous sodium carbonate (pH 10.0) containing 30% of ethanol was stirred under nitrogen at room temperature. After 2 days, the pH of the solution was adjusted to 3 by addition of 1 *N* HCl. Trichloroethylene (1 ml) was added to the solution and the precipitate formed was collected. After evaporation of trichloroethylene *in vacuo*, the solid was dissolved in a small amount of water and

lyophylized (223 mg). A part of the lyophylized material (100 mg) was dissolved in 50 ml of water and the pH of the solution was adjusted to 7.5 by addition of 0.01 *N* NaOH. This solution was applied on DEAE-cellulose column chromatography (1.95 × 25 cm). After 50 ml of water was eluted, a gradient elution with 500 ml of water–500 ml of 0.5 *N* NH_4HCO_3 was applied. The product was easily detectable as a yellow band. The yellow fraction was lyophylized to give **3a**–sodium salt (66 mg). Overall yield was 54%. R_f of TLC (silica gel, $CH_3CO_2Et/n\text{-}PrOH/H_2O$, 7/7/5), 0.29; ir (KBr), 1682, 1618, 1576, 1325, 1155, 1030, 839 cm^{-1}; ^{1}H-NMR (δ, DMSO), 8.33 (d, 1H; J = 1.8 Hz), 7.48 (dd, 1H; J = 1.8 Hz, 9.0 Hz), 6.27 (d, 1H, J = 9.0 Hz), 5.76 (14H, secondary OH of cyclodextrin), 4.83 (7H, C_1H of cyclodextrin), 4.46 (6H, primary OH of cyclodextrin), 4.0–3.2 (42H, C_2–C_6H of cyclodextrin and $-COCH_2S-$), 2.90 (2H, cyclodextrin–CH_2S-), electronic absorption (pH 9.18 borate buffer) λ_{max}, 396 (ε 6200), 323 (19,700), 260 (11,600), 227 (13,000). *Anal.* Calcd for $C_{50}H_{74}O_{38}NSNa \cdot H_2O$: C, 43.64; H, 5.59; N, 1.04. Found: C, 43.64; H, 5.71; N, 1.32. Other spectral data and pK_a values are shown in Table 2, the text, and Figs. 1 and 3–5.

6-Deoxy-6-(p-hydroxyphenacylthio)-β-cyclodextrin (**3b**)

A solution of 60 mg of 6-deoxy-6-mercapto-β-cyclodextrin (**2**) and 200 mg of 4-bromoacetylphenol in 70 ml of aqueous Na_2CO_3 (pH 10) containing 20 ml of ethanol was stirred under nitrogen for 2 days. After acidification of the solution to pH 3 and concentration *in vacuo* to 40 ml, 5 ml of trichloroethylene was added. After stirring for 1 day, the precipitate was filtered. After evaporation of trichloroethylene *in vacuo*, chromatographic separation of the precipitate with Sephadex G-10 (1.5 × 15 cm, water) gave 28 mg of **3b**: ^{1}H-NMR (δ, DMSO), 7.81 (d, 2H, J = 9.0 Hz), 6.82 (d, 2H, J = 9.0 Hz), 5.70 and 4.42 (OH), 4.48 (7H, C_1H of cyclodextrin), 4.0–2.8 (others).

Reduction of the Ketone (**3a**) *to* **4** *with Sodium Borohydride*

To a solution of 30 mg of the chromophore-modified cyclodextrin, **3a**, in 500 ml of aqueous K_2CO_3 (pH 11), a solution of sodium borohydride (5×10^{-3} *M*) in aqueous K_2CO_3 (pH 11) was added in small portions. The reduction was monitored by the disappearance of the absorption at 323 nm and the shift of the absorption from 396 to 414 nm. The final spectrum was practically identical to that of *o*-nitrophenolate. This solution was used for measurements of CD spectra. The ^{1}H-NMR spectrum was taken for the solution of 3 mg of **4** in K_2CO_3/D_2O (0.5 ml, pH 11); ^{1}H-NMR (δ), 7.74 (d, 1H, J = 1.4 Hz), 7.16 (dd, 1H, J = 1.4 Hz, 8.3 Hz), 6.93 (d, 1H, J = 8.3 Hz), 5.00 (7H, C_1H of cyclodextrin), 4.5–3.0 (others).

*6-Deoxy-6-(p-hydroxy-m-nitrophenacylthio)-α-*D*-methyl glucoside* (**6**)

6-Deoxy-6-tosyloxy-α-D-methylglucoside (**5**) was prepared according to the procedure described by Cramer *et al.* (*18*). A solution of 1.5 g of the tosylate (**5**) and 1.5 g of thiourea in 150 ml of 75% methanol was refluxed for 6 hr and the solvent was evaporated *in vacuo*. The residue was a mixture of two compounds

(silica gel TLC, CH_3CO_2Et/n-PrOH/H_2O, 7/7/5) and easily purified by CMC column chromatography (water and then acetic acid) to give 50 mg of the thiuronium salt. Another component obtained in water fraction was the unreacted tosylate (**5**) (ca. 1 g). A solution of 50 mg of the thiuronium salt and 40 ml of 10% NaOH was stirred at 50°C under nitrogen. After addition of 20 mg of sodium borohydride, the solution was acidified to pH 4 with 5% HCl and then alkalified to pH 10 with K_2CO_3. To the solution, 80 mg of 2-nitro-4-bromoacetylphenol dissolved in 2 ml of ethanol was added. The mixture was stirred at room temperature for 2 days under nitrogen and neutralized with 5% HCl. After evaporation of the solvent, 7 mg of the desired glucoside (**6**) was separated by DEAE-cellulose column chromatography (water and then acetic acid): field desorption mass spectrum, *m/z* 428 ($M + K^+$), 412 ($M + Na^+$); ^{1}H-NMR (δ, D_2O), 8.60 (d, 1H, J = 2 Hz), 8.02 (dd, 1H, J = 2 Hz, 8 Hz), 7.10 (d, 1H, J = 8 Hz), 4.60 (d, 1H, J = 2 Hz, C_1H of glucoside), 4.02 (2H, $-SCH_2CO$), 3.30 (s, 3H, CH_3), 3.80–2.60 (others); ir (KBr), 1682, 1575, 1325 cm^{-1}.

2-Nitro-4-bromoacetylphenol

To a hot (80°C) solution of 2 g of 2-nitro-4-acetylphenol (*24*) in 500 ml of acetic acid was added dropwise 80 ml of a solution of bromine (0.25 *M*) in acetic acid. After the addition, the solution was stirred for 30 min and evaporated *in vacuo* to give a solid. This was recrystallized from chloroform to give 1.5 g of the title compound, mp 85–87°C; ir (KBr), 1692, 1615, 1570, 1530, 1325, 848 cm^{-1}; ^{1}H-NMR (δ, $CDCl_3$), 8.70 (d, 1H, J = 1.8 Hz), 8.17 (dd, 1H, J = 1.8 Hz, 9.0 Hz), 7.20 (d, 1H, J = 9.0 Hz), 4.37 (s, 2H).

2-Hydroxyethyl (p-hydroxy-m-nitrophenacyl) Sulfide (**7**)

A solution of 400 mg of mercaptoethanol and 130 mg of 2-nitro-4-bromoacetylphenol in 20 ml of degassed aqueous Na_2CO_3 (pH 10) was stirred at room temperature. After the pH of the solution was adjusted to 2 by addition of 1 *N* HCl, the solution was extracted with chloroform. The extract was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was applied on silica gel column ($CHCl_3$) to give 82 mg of pure **7**, mp 89–90°C; ir (KBr), 3300, 2960, 1670, 1575, 1535, 1325, 798 cm^{-1}; ^{1}H-NMR (δ, DMSO), 8.40 (d, 1H, J = 1.8 Hz), 8.05 (dd, 1H, J = 1.8 Hz, 9 Hz), 7.16 (d, 1H, J = 9 Hz), 3.97 (s, 2H), 3.52 (t, 2H, J = 7 Hz), 2.56 (t, 2H, J = 7 Hz).

Association Constants between **3a** *and Guest Molecules*

The difference electronic spectrum was taken between **3a** (5×10^{-5} *M*) alone and **3a** (5×10^{-5} *M*) in the presence of a guest in borate (pH 9.18) or phosphate (pH 11.0) buffer solution at 25°C. The reciprocal of the difference absorbance around 305 or 380 nm was plotted against the reciprocal of the guest concentration. From the slope and the intercept, the association constant was obtained. The guest concentration ranges from 3.9×10^{-4} to 2.14×10^{-3} *M* for sodium 1-adamantanecarboxylate, from 3.96×10^{-4} to 5.22×10^{-3} *M* for sodium cyclohexanecarboxy-

late, from 7.8×10^{-4} to 3.30×10^{-3} *M* for sodium 2,2-dimethylpropionate, from 3.92×10^{-4} to 2.98×10^{-3} *M* for sodium *p*-methylbenzoate, or from 2.68×10^{-4} to 1.31×10^{-3} *M* for 3,3-dimethylbutyrate, respectively. The association constants between β-cyclodextrin and sodium *p*-methylbenzoate (5×10^{-5} *M*) or 2-hydroxyethyl (*p*-hydroxy-*m*-nitrophenacyl) sulfide (**7**) (5×10^{-5} *M*) were also estimated by the difference spectra at 25°C and pH 9.18 (borate buffer), where β-cyclodextrin concentration ranges from 2.6×10^{-3} to 4.9×10^{-4} *M* for the benzoate or from 2.4×10^{-3} to 4.9×10^{-4} *M* for the sulfide (**7**). The association constant between β-cyclodextrin and sodium cyclohexanecarboxylate was measured by the competitive association between cyclohexanecarboxylate and *p*-nitrophenolate toward β-cyclodextrin at 25°C and pH 9.18, where the association constant of *p*-nitrophenolate was estimated as 660 M^{-1}. The association constant between **3a** and sodium 1-adamantanecaboxylate or 1-adamantylammonium chloride was also estimated by means of circular dichroism spectra, where the concentration of **3a**, the carboxylate, and the ammonium were 2.0×10^{-4}, $3.0 \times 10^{-4} - 1.0 \times 10^{-2}$, and $5.66 \times 10^{-4} - 3.10 \times 10^{-3}$ *M*, respectively. These spectral data were treated by the Scatchard method.

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