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Synthesis and Guest Binding Properties of Regioselectively Anthranilate-Tosyl-Labeled β-Cyclodextrins

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Flexible hosts, regioselectively hetero-labeled 6^A-anthranilate-6^X-*O*-*p*-tosyl-labeled β-cyclodextrins (X=B or G, C or F, and D or E for β-1, β-2, and β-3, respectively), have been synthesized to investigate their chemo-sensor potential as host compounds for biochemical substances such as bile acids and terpenoids as guest molecules. These hosts showed pure monomer fluorescence, exhibiting increase in fluorescence intensity on complexation of the all guests examined. The extent of the fluorescence variation with a guest was employed to evaluate the sensing abilities of these hosts, and the sensing parameter ($\Delta I/I^0$) was used to describe the sensing abilities of the hosts. Hosts β-2 and β-3 recognized bile acids with high sensitivity, whereas β-1 detected all guests examined with low sensitivity. On the whole, the sequence of the binding ability of these hosts was β-3 > β-2 > β-1 for bile acids. The behaviors of the appended units of these hosts during a formation of the host-guest complexation were studied by induced circular dichroism (ICD) and fluorescence spectra. The ICD spectra of these hosts showed same patterns. The guest-induced variations in ICD and fluorescence spectra suggest that anthranilate and tosyl units take movement with a changing their mutual relationship in the place to work as spacers. The parameter values of the titled hosts are much higher than those of homo-labeled anthranilate β-cyclodextrin analogs.

Key Words : Cyclodextrin, Anthranilate, Molecular sensing, Fluorescent sensor system

1. Introduction

Macrocyclic hosts molecules such as cyclodextrins, calixarenes and cavitands have been attracted interest as an enzyme model or supramolecular compounds[1], because these hosts can accommodate various compounds such as organic molecules or metals in their cavities.[1)-2)] We have reported the synthesis of fluorescent active cyclodextrins which were modified with hetero units, which are dansyl and tosyl[3)], pyrene and tosyl[4)-5)], or pyrene and cyanobenzene[6)]. These hosts molecules show the molecular recognition for steroids or endocrine disruptor[7)-9)]. It was proved that these analogs show much better sensory system than those of homo units-labeled cyclodextrins such as bis-anthranilate modified β- and γ-cyclodextrins, bis-dansyl modified γ-cyclodextrins 6^A, 6^B-, 6^A, 6^C-, and 6^A, 6^D-bis-dansylglysine-modified β-Cyclodextrins[10)-12)], because of their much more sensitivity and high selectivity. For further extension of our work, we synthesized regioselectively hetero-labeled β-cyclodextrins, which are 6^A-anthranilate-6^X-*O*-*p*-tosyl-labeled β-cyclodextrins (X=B or G, C or F, and D or E for β-1, β-2, and β-3, respectively) as shown in Scheme 1.

2. Experimental

^{1}H NMR spectra were recorded with BURUKER DPX 300 spectrometer using TMS as an internal standard, and mass spectra were obtained with a JMS-700 mass spectrometer. Elemental analyses were carried out by a Yanagimoto MT-5 CHN corder.

2.1 Preparations of 6^A, $6^{B\,or\,G}$-, 6^A, $6^{C\,or\,F}$-, 6^A, $6^{D\,or\,E}$-anthranilate-tosyl-labeled β-cyclodextrins (β-1, β-2 and β-3, respectively)

A mixture of 6^A, 6^B-di-*O*-(*p*-tosyl) β-cyclodextrin[10)] (500 mg, 0.35 mmol) and sodium anthranilate (73 mg, 0.46 mmol) in 10 mL of DMF was heated at 80℃ for 24 h. After cooling, the reaction mixture was poured into 500 mL of acetone. The precipitates were filtered off and dissolved in 1.5 mL of DMF. The DMF soluble fraction was applied to reverse-phase column (Lobar column Lichroprep RP-18, Merck Ltd., 240 mm x 10 mm). Stepwise elution using 250 mL of 10 vol.% and 250 mL of 20 vol.% aqueous CH_3CN, and 250 mL of 30 vol.% aqueous CH_3CN was applied to obtain β-1. Yield 1.9%. R_f 0.60 (1-butanol-ethanol-water 5:4:3 by volume, TLC; silica gel $60F_{254}$; Merck Ltd.) and 0.57 (methanol-water 2:1 by volume, TLC; silica gel RP-$18F_{254S}$; Merck Ltd.). ^{1}H-NMR (DMSO-D_6): δ 2.34 (3H, s, -CH_3 of tosyl), 3.2-3.8 (42H, m, C^2-C^6H of cyclodextrin), 3.9-5.0 (12H, m, O^6H and C^1H of cyclodextrin), 5.6-6.0 (14H, br, O^2H and O^3H of cyclodextrin), 6.55 (1H, t, J=7.5 Hz, aromatic-H of anthranilate), 6.63-6.80 (1H, br, -NH of anthranilate), 6.78 (1H, d, J=7.8 Hz, aromatic-H

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of anthranilate), 7.16-7.48 (3H, m, aromatic-H of tosyl and anthranilate), 7.61-7.78 (3H, m, aromatic-H of tosyl and anthranilate). Calcd. for $C_{56}H_{80}O_{38}NSNa \cdot 4H_2O$: C, 44.77; H, 5.91; N, 0.93%. Found: C, 44.66; H, 6.07; N, 1.07%. MS (FAB): 1430 ($[M+H]^+$).

Compound *β*-2 was prepared from 6^A, 6^C-di-*O*-(*p*-tosyl) *β*-cyclodextrin and sodium anthranilate by the same procedure as *β*-1. Yield 15.1%. R_f 0.60 (1-butanol-ethanol-water 5:4:3 by volume, TLC; silica gel $60F_{254}$; Merck Ltd.) and 0.59 (methanol-water 2:1 by volume, TLC; silica gel RP-$18F_{254S}$; Merck Ltd.). ^{1}H-NMR (DMSO-D_6): δ 2.41 (3H, s, -CH_3 of tosyl), 3.2-3.8 (42H, m, C^2-C^6H of cyclodextrin), 3.9-5.0 (12H, m, O^6H and C^1H of cyclodextrin), 5.6-6.0 (14H, br, O^2H and O^3H of cyclodextrin), 6.48-6.57 (1H, m, aromatic-H of anthranilate), 6.60-6.71 (1H, br, -NH of anthranilate), 6.76 (1H, d, J=8.4 Hz, aromatic-H of anthranilate), 7.08-7.32 (1H, m. aromatic-H of anthranilate), 7.42 (2H, t, J=8.9 Hz, aromatic-H of tosyl), 7.69-7.79 (3H, m, aromatic-H of tosyl and anthranilate). Calcd. for $C_{56}H_{80}O_{38}NSNa \cdot 3H_2O$: C, 45.31; H, 5.84; N, 0.94%. Found: C, 45.24; H, 5.98; N, 1.00%. MS (FAB): 1430 ($[M+H]^+$).

Compound *β*-3 was prepared from 6^A, 6^E-di-*O*-(*p*-tosyl) *β*-cyclodextrin and sodium anthranilate by the same procedure as *β*-1. Yield 6.5%. R_f 0.60 (1-butanol-ethanol-water 5:4:3 by volume, TLC; silica gel $60F_{254}$; Merck Ltd.) and 0.71 (methanol-water 2:1 by volume, TLC; silica gel RP-$18F_{254S}$; Merck Ltd.). ^{1}H-NMR (DMSO-D_6): δ 2.41 (3H, s, -CH_3 of tosyl), 3.2-3.8 (42H, m, C^2-C^6H of cyclodextrin), 3.9-4.9 (12H, m, O^6H and C^1H of cyclodextrin), 5.65-5.9 (14H, br, O^2H and O^3H of cyclodextrin), 6.51 (1H, t, J=6.8 Hz, aromatic-H of anthranilate), 6.57-6.65 (1H, br, -NH of anthranilate), 6.75 (1H, t, J=7.7 Hz, aromatic-H of anthranilate), 7.18-7.28 (1H, m. aromatic-H of anthranilate), 7.42 (2H, t, J=7.8 Hz, aromatic-H of tosyl), 7.61-7.77 (3H, m, aromatic-H of tosyl and anthranilate). Calcd. for $C_{56}H_{80}O_{38}NSNa \cdot 12H_2O$: C, 40.86; H, 6.37; N, 0.85%. Found: C, 40.80; H, 5.74; N, 1.05%. MS (FAB): 1430 ($[M+H]^+$).

2.2 Measurements

Fluorescence and circular dichroism spectra were measured at 25℃ using a Perkin-Elmer LS 40B fluorescence spectrophotometer and a JASCO J-700 spectropolarimeter, respectively. For the fluorescence measurements, the excitation wavelength of the fluorescence spectra was 330 nm, and the width of excitation and emission slits were 5 nm. Ethylene glycol aqueous solution (10 vol.%) was used as a solvent for the host, because the solubility of the host in pure water is poor.[10] Five μL of the guest species (0.5, 0.05 and 0.005 M) in dimethyl sulfoxide (DMSO) or MeOH were injected into a 10 vol.% ethylene glycol aqueous solution of the host (2.5 mL) to make a sample solution with a host concentration of 1.0×10^{-6} M and guest concentration of 1.0, 0.1 and 0.01 mM, respectively. For the circular dichroism measurements, five μL of the guest species (0.05 M) in dimethyl sulfoxide (DMSO) were injected into a 10 vol.% ethylene glycol aqueous solution of the host (2.5 mL) to give a sample solution with a host concentration of 1.0×10^{-4} M and guest concentration of 1.0×10^{-4} M.

2.3 Determination of binding constants

The binding constants of three hosts, *β*-1, *β*-2 and *β*-3, for several guests were obtained from the guest-induced fluorescence variations at 418 nm by employing a Benesi-Hildbrand-type equation, as reported previously.[4)-5)]

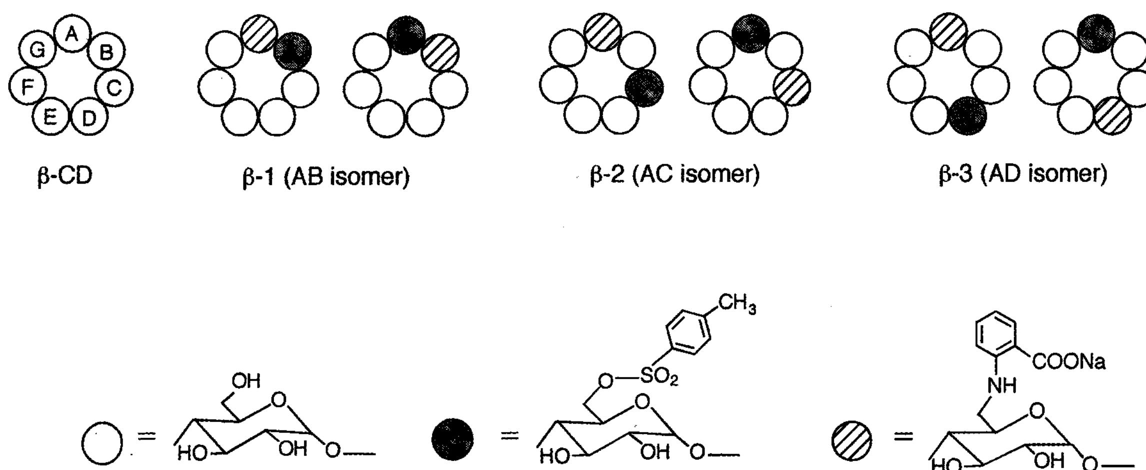
3. Results and discussion

3.1 The Preparations of 6^A, $6^{B\ or\ G}$-, 6^A, $6^{C\ or\ F}$-, 6^A, $6^{D\ or\ E}$-anthranilate-tosyl-labeled *β*-cyclodextrins (*β*-1, *β*-2 and *β*-3, respectively)

Hosts *β*-1, *β*-2 and *β*-3 were prepared from 6^A, 6^B-, 6^A, 6^C- and 6^A, 6^D-di-*O*-(*p*-tosyl) *β*-cyclodextrin, respectively, using sodium anthranilate at 80℃, as shown in Figure 1. These hosts were purified by reverse-phase column chromatography. Although, it is suspected that *β*-1, *β*-2 and *β*-3 are isolated as a mixture of diastereomers, including 6^A, 6^B- and 6^A, 6^G-, 6^A, 6^C- and 6^A, 6^F-, and 6^A, 6^D- and 6^A, 6^E-anthranilate-tosyl-labeled *β*-cyclodextrins, respectively. This is because each of these diastereomers is inseparable by reverse-column chromatography and the existing ratio of these diastereomers was unable to be determined by ^{1}H-NMR analysis.[3)-6)] In this paper, the hosts were assumed to exist as diastereomers and have been named *β*-1, *β*-2 and *β*-3 for 6^A,6^B- and 6^A,6^G-, 6^A, 6^C- and 6^A, 6^F-, and 6^A, 6^D- and 6^A, 6^E-anthranilate-tosyl-labeled *β*-cyclodextrins, respectively.

3.2 Induced circular dichroism (ICD) spectra

The ICD spectra of the three hosts, *β*-1, *β*-2 and *β*-3, alone



Scheme 1 Structures of *β*-1, *β*-2 and *β*-3.

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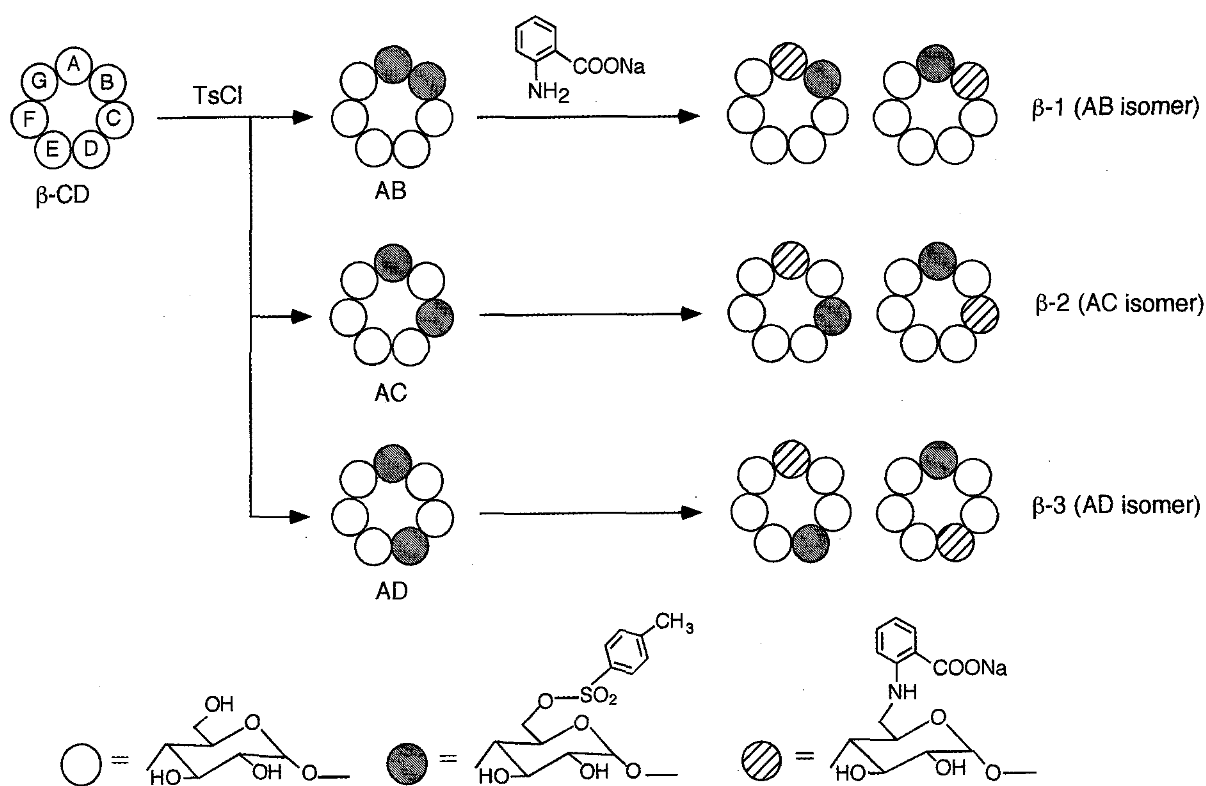

Figure 1 Preparations of β-1, β-2 and β-3.

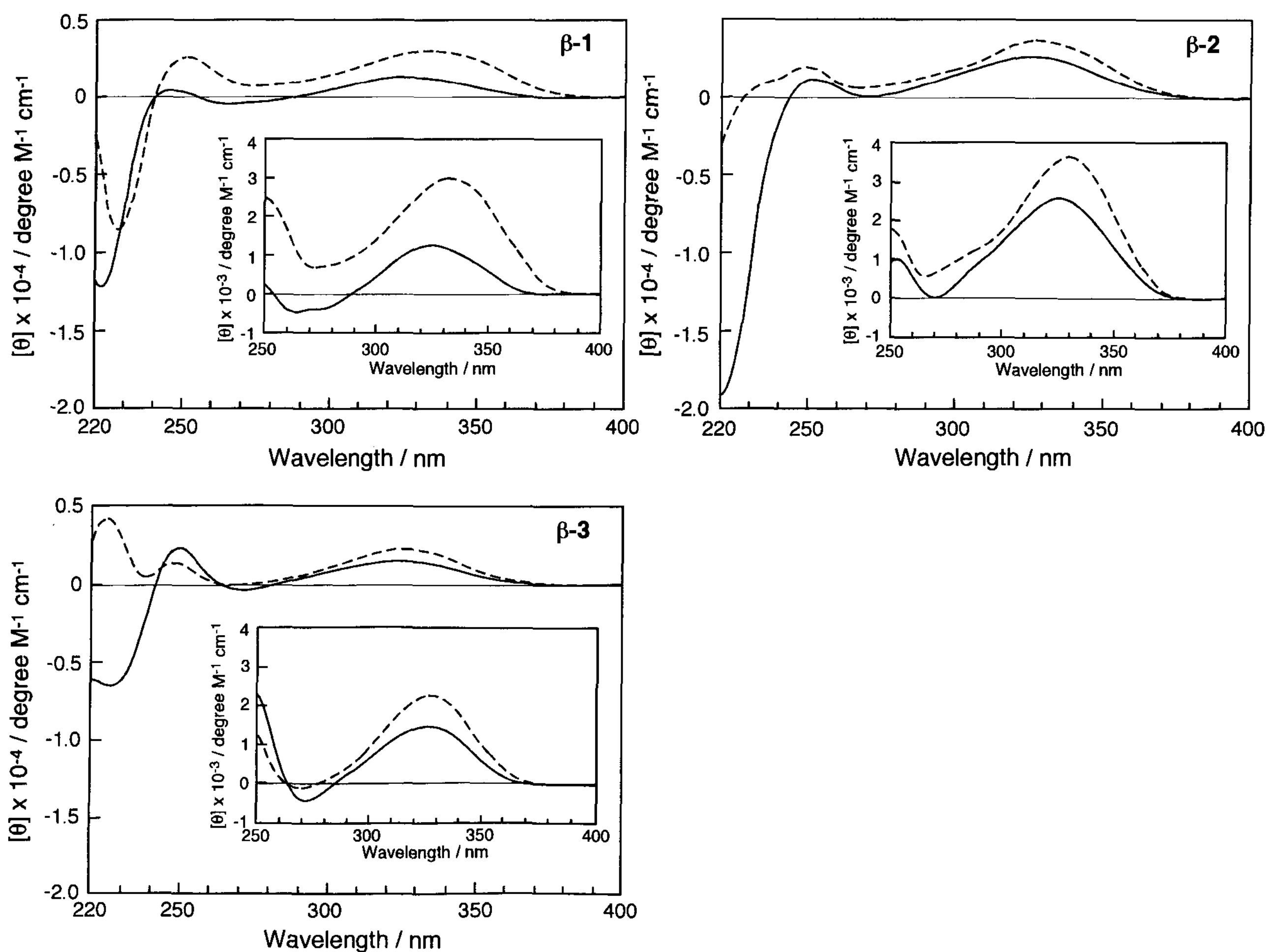

Figure 2 ICD spectra of β-1, β-2 and β-3 in a 10 vol.% ehtylene glycol aqueous solution (1.0×10^{-4} M:---, 25℃) and containing ursodeoxycholic acid (1.0×10^{-4} M:---).

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and in the presence of ursodeoxycholic acid in a 10 vol.% ethylene glycol aqueous solution are taken to investigate the movement of the appended units when a host-guest complexation occurs, as shown in Figure 2. The spectrum of β-1 exhibits a negative Cotton peak around 220-240 nm and positive Cotton peaks around at 245 and 325 nm, in which these peaks decrease upon addition of a guest. It indicates that the appended units penetrate into the chiral cyclodextrin cavity[10], as it is well known that an increase in the ICD intensity means the appended unit is located in the chiral environment of the cyclodextrin cavity. The ICD spectral patterns of β-2 and β-3 are basically similar to that of β-1, however, changes of [θ] value are not same. The [θ] values of β-1 around at 325 nm increase with much greater than those of β-2 and β-3. On the other hand, ICD intensity of β-2 of the negative band at 220-245 nm with a guest is decreased much more than those of β-1 and β-3, and the negative band at 220-255 nm of β-3 changes to positive one on accommodation of a guest. These results suggest that the movements of the anthranilate and tosyl units associated with a guest are not the same for the three hosts. It is reported that ICD signs of the spectroscopic active cyclodextrin derivatives indicated a type of inclusion which is equatorial or axial self-inclusion of the appended unit.[15)-19)] Therefore, opposite of ICD pattern at 220-255 nm of β-3 before and after guest addition suggests that the anthranilate and tosyl units of β-3 may replace each other, probably due to ease of movement of the appended units, because the positions of these appended units of β-3, which are A and D or E at C-6 of seven glucose units, are more distant than those of β-1 and β-2. These phenomena should be advantageous for molecular sensing by these hosts, because they might cause differences of sensitivity and selectivity for the guest molecules.

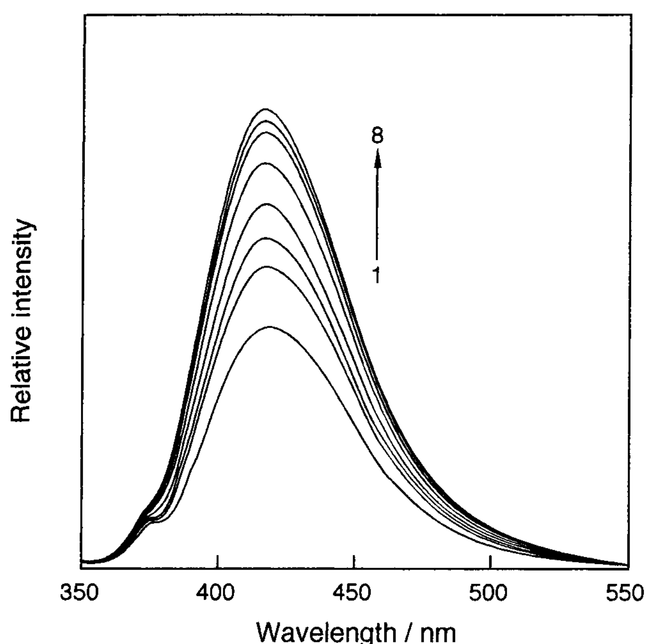
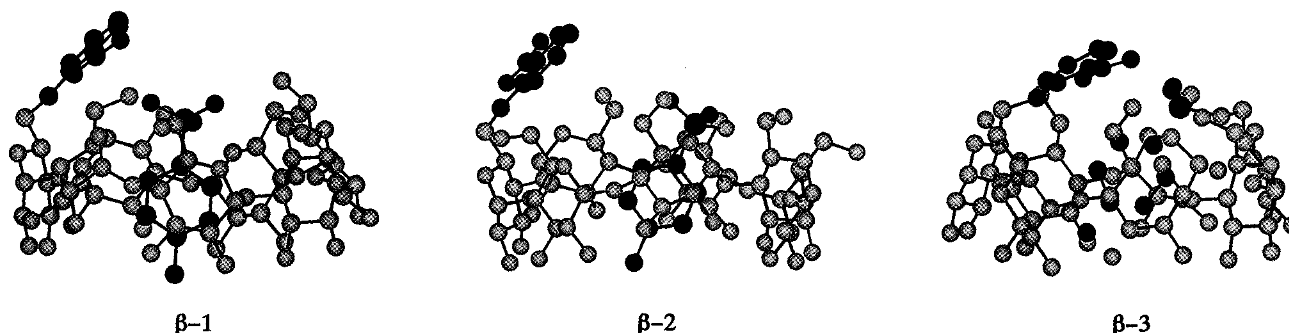


Figure 3 Fliorescence spectra of β-2 in a 10 vol.% ethylene glycol aqueous solution (1.0x10^{-6} M, 25℃) at various concentrations of ursodeoxycholic acid (1: 0, 2: 4.0x10^{-6}, 3: 8.0x10^{-6}, 4: 1.2x10^{-5}, 5: 2.4x10^{-5}, 6: 4.0x10^{-5}, 7: 6.1x10^{-5}, 8: 8.3x10^{-5} M).

3.3 Fluorescence spectra

Figure 3 shows fluorescence spectra of β-2 in the absence and presence of ursodeoxycholic acid in a 10 vol.% ethylene glycol aqueous solution. In the study of anthranilate modified cyclodextrin system, we found that most effective excitation wavelength is 330 nm.[10), 20)-21)] The fluorescence spectra of β-1, β-2 and β-3 are composed of pure monomer emission with a peak around 418 nm, and their intensity increases with increasing of ursodeoxycholic acid concentration. It is reported that a guest-induced fluorescence enhancement means that the labeled unit is moving into the cyclodextrin cavity deeply[10] and a decrease means that the labeled unit is moving out of the cavity.[3), 11)-12)] The ICD and fluorescence spectral changed of the three hosts suggest that the anthranilate unit is included into the cyclodextrin cavity upon guest binding and acts as a spacer. On the other hand, we attempted to examine the guest-induced behavior of the tosyl unit, which is fluorescent inert, by ROESY ^{1}H-NMR. Unfortunately, our attempt was unsuccessful, because the host in a 10 vol.% DMSO-d_6 D_2O solution was deposited through the NMR measurement. The host might cause aggregation. The energy-minimized structures of the three hosts obtained using molecular mechanics in CS Chem 3D (MM2)[13)-14)], as illustrated in Scheme 2. However, the energy-minimized structures obtained are estimated ones, so it is probably indicates that the tosyl unit of the three hosts are located into the cyclodextrin cavity deeply as self-inclusion formation. Therefore, as the ICD and fluorescence spectral changed of the hosts, it is speculated that the tosyl units of β-1 and β-2 move in the cyclodextrin cavity to play the roll of a spacer when host-guest complexation occurs. On the other hand, the tosyl unit of β-3 is estimated to be excluded from the cyclodextrin cavity and not play the role of a spacer when host-guest complexation is



Scheme 2 Energy-minimized structures of β-1, β-2 and β-3 obtained using molecular mechanics in CS Chem 3D (MM2)

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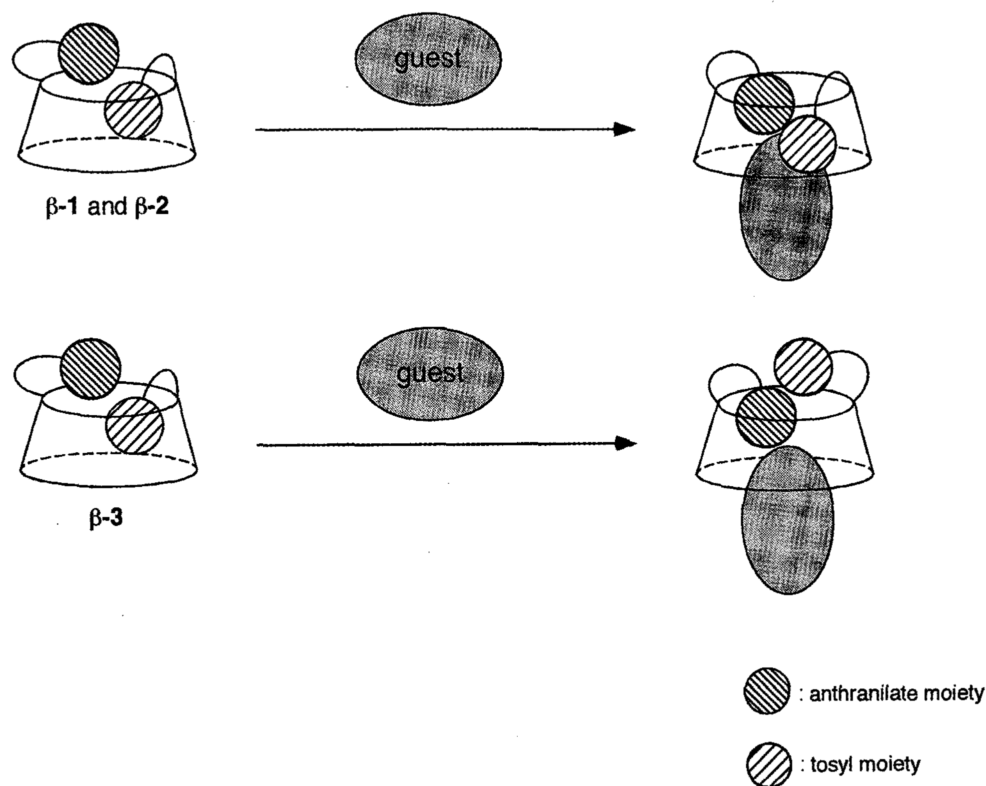

Scheme 3 Estimated host-guest complexation of the host.

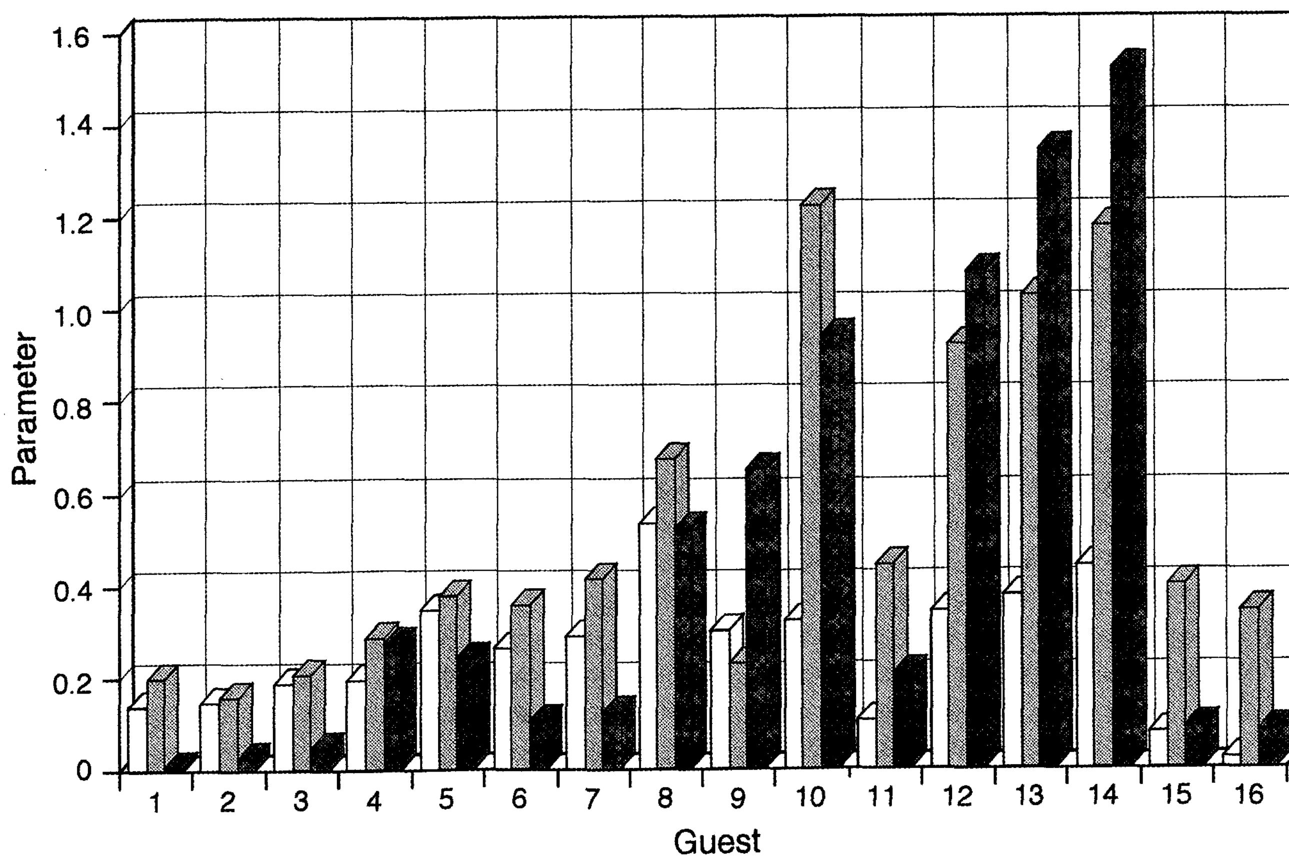

Figure 4 Sensing factors of β-1 (□), β-2 (▩) and β-3 (■) in a 10 vol.% ethylene glycol aqueous solution (1.0×10^{-6} M, 25℃) for all gueats examined (guest concentrations, 1-9: 1.0 mM, 10: 0.01 mM, 11-16: 0.1 mM).

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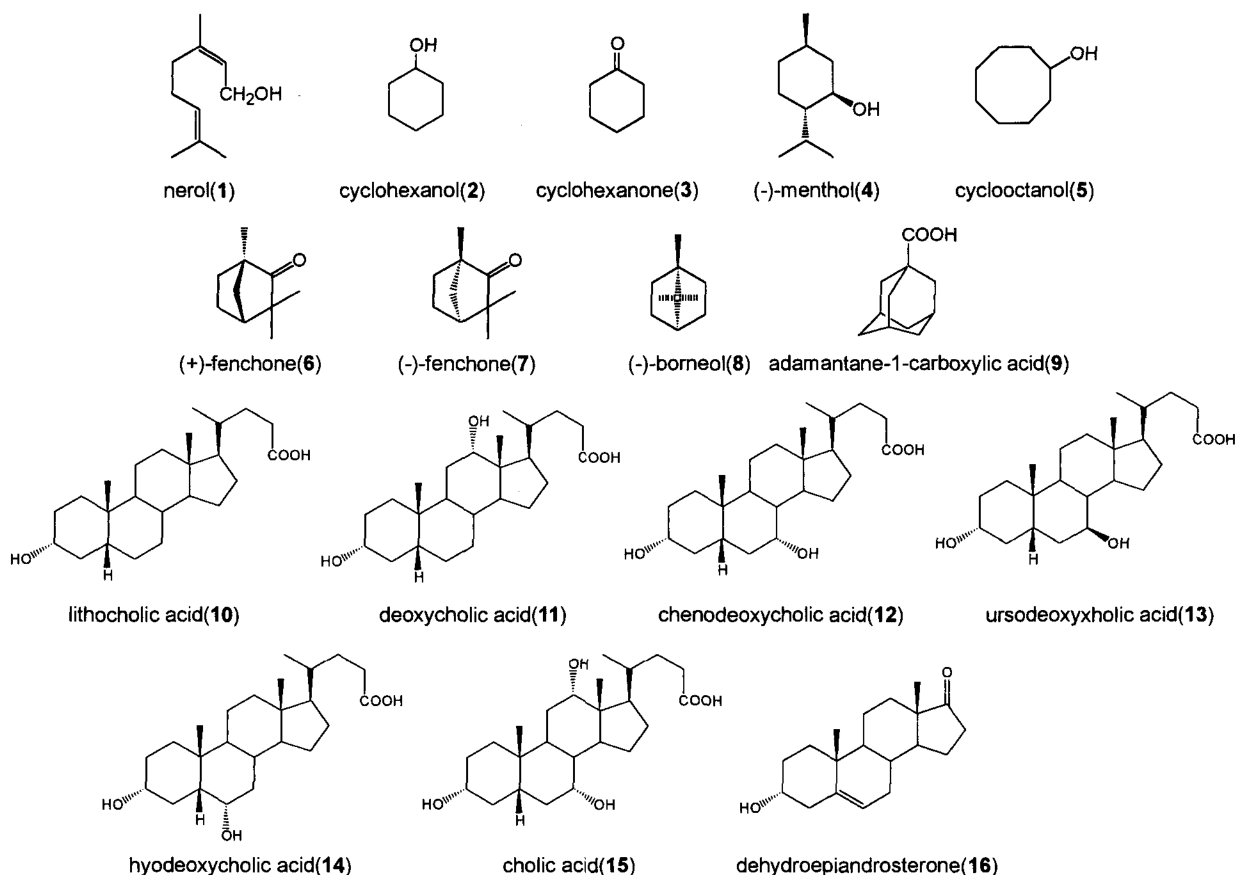
formed, and the anthranilate unit of β-3 is included into the cyclodextrin cavity, as illustrated in Scheme 3.

3.4 Sensing abilities of β-1, β-2 and β-3 for terpenoids and steroids

As reported previously, the extent of the variation of the fluorescence intensity of these hosts depended on the nature of a guest, even at low concentration; therefore, those hosts can be used as fluorescent molecular sensors, as seen in the case of anthranilate-labeled cyclodextrin analogs reported previously.[10] In order to evaluate the sensing ability of modified cyclodextrins, the $\Delta I/I^0$ value was used as a sensitivity parameter. Here, ΔI is I/I^0, where I^0 is the fluorescence intensity for the host alone and I is that of a complex. Figure 4 shows the parameter values of β-1, β-2 and



Scheme 4 Guest molecules.

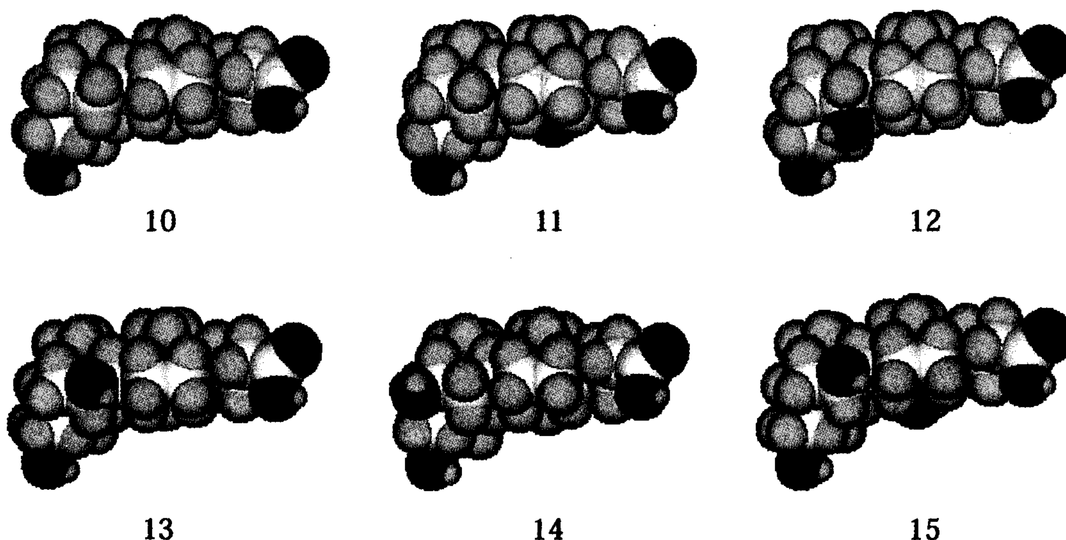


Figure 5 Space filling figures of guests 10-15 after carring out MM2 energy minimized calculation. Oxygen atoms are shown by dark color.

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β-3 with terpenoids at 1.0 mM and steroids at 0.1 mM except for lithocholic acid (10), which was examined at 0.01 mM because 0.1 mM of lithocholic acid is not soluble into a 10 vol.% ethylene glycol aqueous solution. The structures of 16 guest molecules are shown in Scheme 4. Hyodeoxycholic acid (14), which has two hydroxyl groups at C-3 and C-6 of the steroidal framework, ursodeoxycholic acid (13) and chenodeoxycholic acid (12), which bear two hydroxyl groups at C-3 and C-7 of the steroidal framework and are diastereoisomers each other, were detected by β-2 and β-3 with the highest sensitivities, exhibiting the sensing values of 1.178 and 1.520 for guest 14, 1.028 and 1.343 for guest 13 and 0.92 and 1.08 for guest 12, respectively. Lithocholic acid (10), which has one hydroxyl group at C-7 of the steroidal frame work at C-3 of the steroidal framework and its concentration is one tenth of other bile acid concentration, was detected by β-2 and β-3 with high sensitivities, exhibiting the sensing values of 1.22 and 0.94, respectively. It is achieved that the high sensitivity of these hosts for the guests such as 12, 13 and 14. Unfortunately, the selectivity for these guest molecules by β-2 and β-3 is not enough. On the other hand, host β-1 detected these guests 10, 12, 13, and 14 with low sensitivities and its sensing parameters were negligible. The sensing factors of the three hosts for deoxycholic acid (11) and cholic acid (15), which have hydroxyl group at C-12 of the steroidal framework, and dehydroepiandrosterone (16), which bears ketone at C-17 of the steroidal framework, were also negligible. The sensing factors of β-2 and β-3 for bile acids are more sensitive than those of 6^A, 6^C- and 6^A, 6^D-di-anthranilate-labeled β-cyclodextrins reported previously and these of β-1 for bile acids are lower than those of 6^A, 6^B-di-anthranilate-labeled β-cyclodextrin.[10] The sequence of the sensing factors of the three hosts for guests 12, 13 and 14, are β-3 > β-2 > β-1, on the other hand, the sequence of the sensing factors of these hosts for guest 10, is β-2 > β-3 > β-1. These facts mean that the positions of the appended units, which have influence on the flexibility of each ones, and the group at C-17 of the steroidal framework affect the sensing ability for bile acids. The sensing factors, which are ruled by conformational change, of the three hosts for guests 12, 13 and 14, which have a hydroxyl group on the B ring of themselves, are in inverse proportion to the distance from the C-17 to the hydroxyl group at the B ring of the steroidal framework as shown in Figure 5. Furthermore, the sensing factors are small by existence of hydroxyl group on the C ring of the steroidal framework. Thus, host β-1, β-2 and β-3 can recognize the presence of hydroxyl group

Table 1 Binding constants ($K/mol^{-1}\ dm^3$) of β-1, β-2 and β-3 in a 10 vol.% ethylene glycol aqueous solution (10×10^{-6} M, 25℃)[a].

Guest	β-1	β-2	β-3
Borneol (**8**)	17,600±520 [b]	12,800±310	4,280±150
Lithocholic acid (**10**)	88,500±8,690	295,000±13,000	615,000±21,800
Chenodeoxycholic acid (**12**)	51,400±1,110	44,100±870	33,600±1,050
Ursodeoxycholic acid (**13**)	*78,800±5,490*	*77,800±1,360*	*67,600±3,640*
Hyodeoxycholic acid (**14**)	148,000±5,000	317,000±3,360	101,000±1,680

a) The K values were obtained from guest-induced fluorescence variations.
b) The statistical errors were values of standerd deviation assessed by guest-induced fluorescence variations.

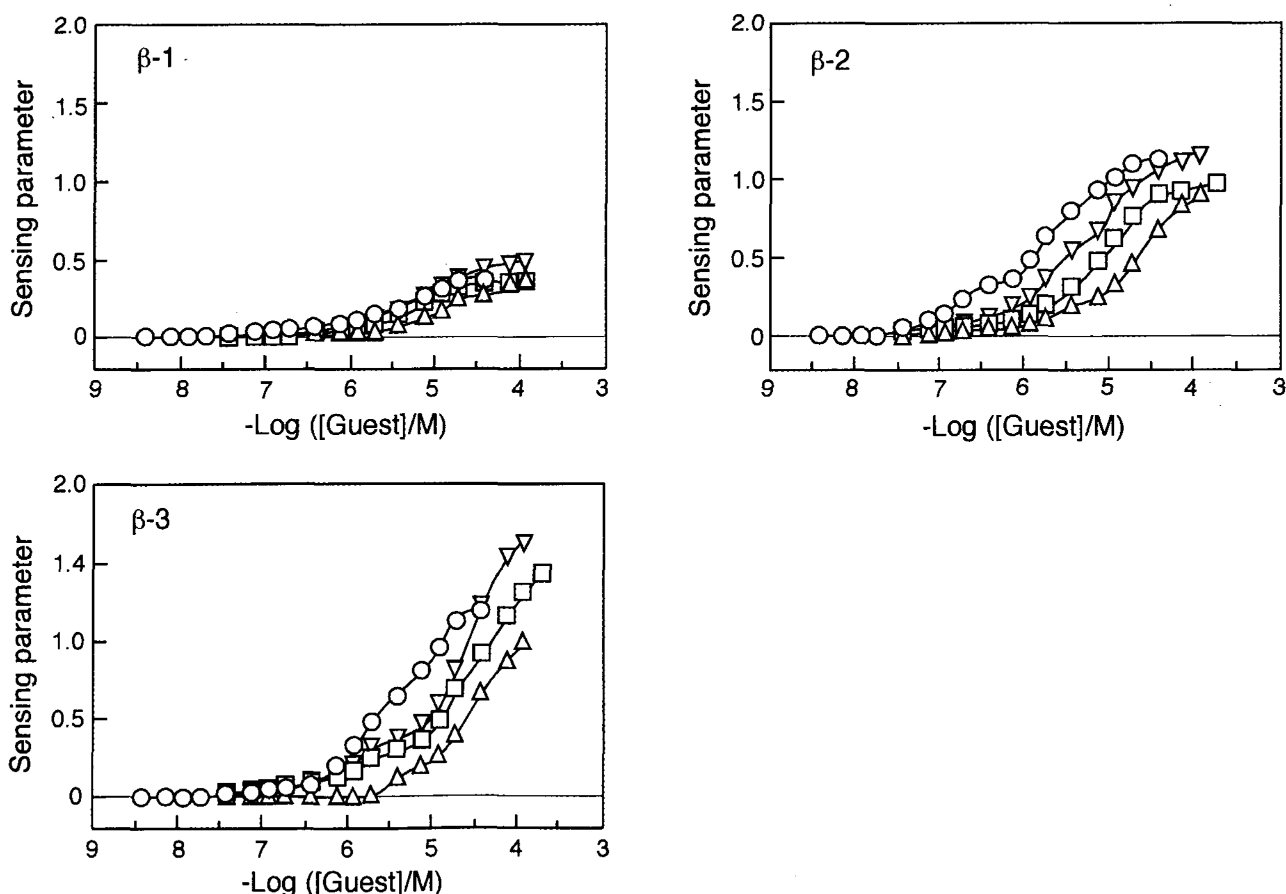

Figure 6 Fluorescence variations of β-1, β-2 and β-3 (1.0×10^{-6} M, 25℃) in a 10 vol.% ehtylene glycol aqueous solution for lithcholic acid (○), chenodeoxycholic acid (△), ursodeoxycholic acid (□), and hydeoxycgolic acid (▽) as a function of guest concentration.

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on the B and C ring of the steroidal framework on the cholic acid analogs, and the distance from the C-17 to the hydroxyl group on the B ring of the steroidal one. These suggest that guests 10 and 12-14 enter the cyclodextrin cavity from the site of carboxylic acid and form hydrogen bonding between the carboxylic acid of the guest and the amino group of the anthranilate unit of the host, moreover, A and B rings of the steroidal framework are located on out of the cyclodextrin one. For terpenoid guests, the three hosts exhibited low sensitivities for guests 1-7, exhibiting lower sensitivities than homo-anthranilate-labeled β-cyclodextrins.[10] On the other hand, for guests 8 and 9, which are bicyclic and tricyclic compounds, respectively, the three hosts detected guest 8 and β-3 detected guest 9 with higher sensitivities than other guests, however, these sensing factors are not recognized advantage over those of homo-anthranilate-labeled β-cyclodextrins.[10] These results obtained as sensing factors suggest that hetero units, which are anthranilate and tosyl, of β-2 and β-3 contribute to qualitative molecular recognitions.

3.5 Binding constants

The guest-induced fluorescence variation at 418 nm was employed to calculate the binding constants (K) of three hosts using Eq. 1.

$$1/(I_f - I_f^0) = 1/a[\mathrm{CD}] + 1/a[\mathrm{CD}]K \times 1/[\mathrm{G}] \qquad \text{Eq. 1}$$

Here, I is the fluorescence intensity at 418 nm (I_f for complex, I_f^0 for the host alone), [CD] the total host concentration, [G] the total guest concentration, and a is constant. The binding constants of the three hosts were obtained in order to examine the correlation between the fluorescence variations and the binding abilities of the hosts. The results are shown in Table 1. In every host, there is no simple correlation between the binding constants and the sensitivity factors. This means that the sensitivity values give a relative, but no absolute, measure of the binding abilities of the hosts.

3.6 Response ranges

Figure 6 shows response curves of β-1, β-2 and β-3 for guests such as lithocholic acid (10), chenodeoxycholic acid (12), ursodeoxycholic acid (13), and hyodeoxycholic acid (14). Host β-1 detected with response ranges $10^{-6.5}-10^{-4.5}$, $10^{-5.5}-10^{-4}$, and $10^{-6}-10^{-4}$ M to 10, 12 and 13, and 14, respectively. Host β-2 detected with response ranges $10^{-7}-10^{-4.5}$, $10^{-6}-10^{-4}$, and $10^{-6.5}-10^{-4}$ M to 10, 12 and 13, and 14, respectively. Host β-3 detected with response ranges $10^{-6.5}-10^{-4.5}$, $10^{-5.5}-10^{-4}$, $10^{-6.5}-10^{-4}$, and $10^{-6.5}-10^{-4}$ M to 10, 12, 13, and 14, respectively. These results mean that each host perceives on different guest concentration range, and especially host β-3 has unique response ranges to each guest. All hosts give almost clear concentration dependency for the guests, reflecting the sensitivities of the system for the guests. Although, it is true that response curves are not simply increased with increase of guest concentration, sometimes-different dependency. This result suggests that two ways of the host-guest binding pattern is taking place such as 1:1 and 1:2 complex patterns.

4. Conclusion

Three hetero-functionalized analogs of anthranilate- and tosyl-labeled β-cyclodextrins were investigated as new chemo-sensors for organic guests such as terpenoids and bile acids, which are biologically significant substances. These hosts show pure monomer fluorescence, the variation of which was used as a parameter to describe the sensing ability. The introduction of hetero functional groups such as antharanilate and tosyl, which are in different positions such as 6A and 6X in the cuclodextrin upper rim, gives new sensing factors that impart high sensitivity and selectivity to these hosts. In this system, it is obvious that the collaboration of the anthranilate and tosyl units, which substituted at 6A and 6X (X=C or F, and D or E) in the cyclodextrin upper rim, contributes to improve the selective molecular binding ability. The system shown here is a very convenient and useful method, because the chemical modification of a guest, even if it is spectroscopically inert, is not necessary, a guest can be examined directly.

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