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Molecular Recognition Thermodynamics and Structural Elucidation of Interactions between Steroids and Bridged Bis(*β*-cyclodextrin)s

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A series of bridged bis(*β*-cyclodextrin(CD))s (**2**−**7**) were synthesized, i.e., bridged bis(*β*-CD)s **2** and **3** bearing binaphthyl or biquinoline tethers and bridged bis(*β*-CD)s **4**−**7** possessing dithiobis(benzoyl) tether, and their complex stability constants (K_S), enthalpy ($\Delta H°$), and entropy changes ($\Delta S°$) for the 1:2 inclusion complexation with representative steroids, deoxycholate, cholate, glycocholate, and taurocholate, have been determined in an aqueous phosphate buffer solution of pH 7.20 at 298.15 K by means of titration microcalorimetry. The original conformations of bridged bis(*β*-cyclodextrin)s were investigated by circular dichroism and ^{1}H ROESY spectroscopy. Structures of the inclusion complexes between steroids and bridged bis(*β*-CD)s in solution were elucidated by 2D NMR experiments, indicating that anionic groups of two steroid molecules penetrate, respectively, into the two hydrophobic CD cavities in one 6,6′-bridged bis(*β*-CD) molecule from the secondary rim to give a 1:2 binding mode upon inclusion complexation. The results obtained from titration microcalorimetry and 2D NMR experiments jointly demonstrate that bridged bis(*β*-CD)s **2**, **3** and **5**−**7** tethered by protonated amino group possessing different substituted groups can enhance not only the molecular binding ability toward steroids by electrostatic interaction but also molecular selectivity. Thermodynamically, the resulting 1:2 bis(*β*-CD)−steroid complexes are formed by an enthalpy-driven process, accompanied by smaller entropy loss. The increased complex stability mainly results from enthalpy gain, accompanied by large conformational change and extensive desolvation effects for the 1:2 inclusion complexation between bis(*β*-CD)s and steroids.

Introduction

Bridged bis(*β*-cyclodextrin(CD))s, as a very important family of CD derivatives, have been known to alter significantly the molecular binding ability and selectivity toward a variety of guests in comparison with parent *β*-CDs through the cooperative binding of a single model substrate by two hydrophobic cavities located in a closely vicinity[1] and therefore provide an excellent model system mimicking the substrate-specific interaction of enzymes.[2] Consequently, a wide variety of bridged bis(*β*-CD)s have been synthesized in order to examine and compare the molecular binding abilities of native *β*-CD and bridged bis(*β*-CD)s and also to gain insights into factors governing the inclusion complexation phenomena between the host bis(*β*-CD)s and guest molecules.[1−8] On the other hand, the inclusion complexation of bridged bis(*β*-CD)s toward guest molecules does not always give the cooperative 1:1 binding of a single guest molecule by the two hydrophobic cavities located in close vicinity. Our recent investigation results demonstrated that a paclitaxel and a bridged bis(*β*-CD) containing an appropriate spacer could form a 1:2 inclusion complex through the binding of each cavity of the bis(*β*-CD) with one paclitaxel molecule, and this noncooperative binding mode had been successfully applied onto the solubilization increase of paclitaxel.[9] In

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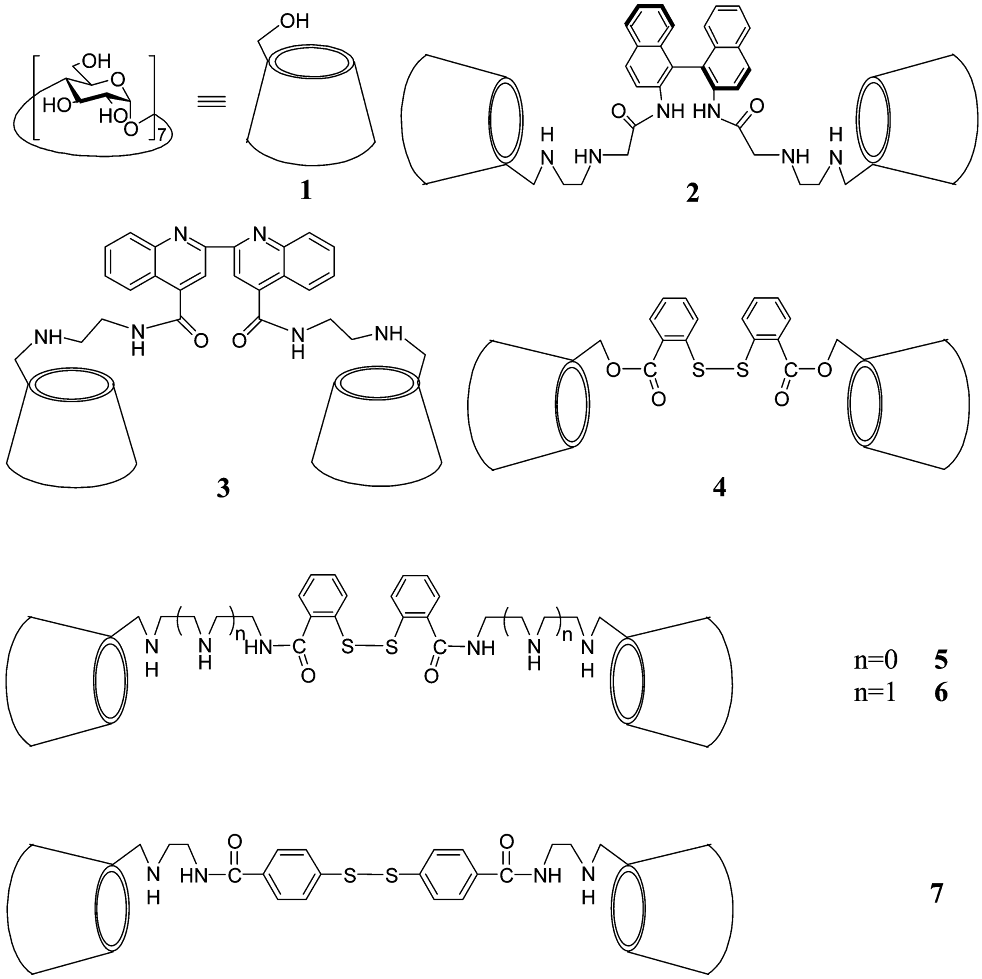
CHART 1



particluar, in our present studies on the inclusion complexation of 6,6′-bridged bis(β-CD)s with anionic biological amphiphilic surfactant-like compounds, steroids, we observed a particular complexation mode. Anionic groups of two steroid molecules penetrated respectively into the two hydrophobic CD cavities in one 6,6′-bridged bis(β-CD) molecule from the secondary rim to give a 1:2 bis(β-CD)−guest binding mode, instead of the cooperative binding of a guest molecule by the two hydrophobic cavities located in a close vicinity. According to recent reports, this inclusion complexation is significant for the understanding of the multiple-recognition mechanism of CDs. Recently, Kano and Kuroda et al. reported the inclusion complexation of CDs and charged porphyrins, demonstrating that the anionic porphyrins penetrated into the hydrophobic CD cavity from the secondary side, and thermodynamically, strong van der Waals interactions and dehydration from CD played an important role in those systems.[10] Tato and co-workers[11] studied the complex geometry of β-CD and its derivatives in D_2O by ROESY experiments, which exhibit different binding modes for the inclusion complexation with steroids and showing that the steroid molecule can enter into the hydrophobic CD cavity from the secondary side with the carboxylate group (tail). More recently, we investigated the 1:1 inclusion complexation of aminated mono-β-CDs with representative steroid molecules, demonstrating that steroid molecules can enter into aminated mono-modified β-CDs that possess different substituted groups from the secondary side by the cooperation of electrostatic interaction and van der Waals interaction and, therefore, enhance the molecular binding ability toward steroids.[12] However, to the best of our knowledge, the mechanism and thermodynamic origin of this 1:2 bis(β-CD)−guest inclusion complexation between bridged bis(β-CD)s and guest molecules have rarely been thoroughly investigated, despite its importance on the understanding of the multiple-recognition mechanism of CDs.

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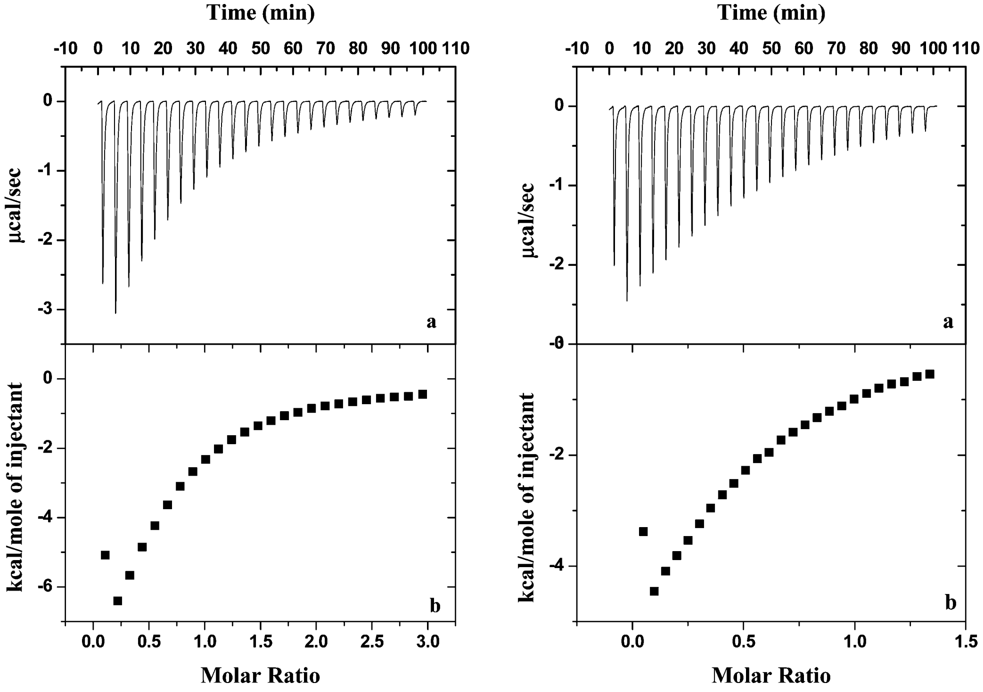
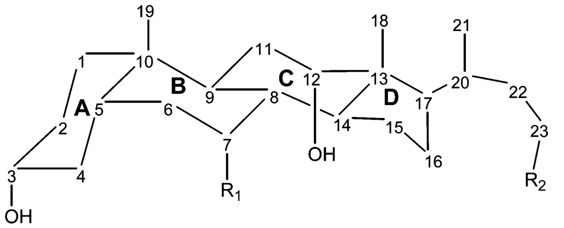


FIGURE 1. Calorimetric titrations of host **2** with **CA** (left) and **GCA** (right) in a phosphate buffer solution of pH 7.2 at 25 °C. (a) Raw data for sequential 10 μL injections of CD solution (2.01 mM) into steroid solution (0.13 and 0.287 mM). (b) Heats of reaction as obtained from the integration of the calorimetric traces.

CHART 2



In this paper, we report the syntheses of bridged bis(β-CD)s **2**−**7** with different spacers (Chart 1) and their molecular recognition thermodynamics and solution structures upon inclusion complexation with representative steroids (Chart 2). It is our special interest to examine the main factors governing the above 1:2 bis(β-CD)−guest inclusion complexation, which will serve our further understanding of this recently developing, but thermodynamically less investigated, area of bridged bis(β-CD).

Experimental Section

Materials. β-CD of reagent grade was recrystallized twice from water and dried in vacuo at 100 °C for 24 h prior to use. *N*,*N*-Dimethylformamide (DMF) and pyridine were dried over calcium hydride for 2 days and then distilled under a reduced pressure prior to use. All chemicals, i.e., dicyclohexylcarbodiimide (DCC) and triethylamine, were commercially available and used without further purification. All steroid guests, i.e., deoxycholate (DCA), cholate (CA), glycocholate (GCA), and taurocholate (TCA), were purchased from Sigma and used as received. Mono[6-*O*-(*p*-toluenesulfonyl)]-β-CD (6-OTs-β-CD) was prepared by the reaction of β-CD with *p*-toluenesulfonyl chloride in aqueous alkaline solution.[13] Bridged bis(β-CD)s **3**, **5**, **6**, and **7** were prepared according to our reported procedures.[3c,14] (*R*)-(+)-2,2′-Diamino-1,1′-binaphthyl was synthesized by the literature method.[15] Disodium hydrogen phosphate and sodium dihydrogen phosphate were dissolved in distilled, deionized water to make a 0.1 M phosphate buffer solution of pH 7.20 for spectral measurements and microcalorimetric titrations.

NMR Measurements. All of the ^{1}H NMR experiments were carried out in deuterium oxide at 298.2 ±0.1 K. Two-dimensional rotating frame nuclear Overhauser effect spectroscopy (ROESY) was performed on a Bruker AV600 spectrometer at 600.13 MHz. A Bruker standard sequence with water suppression (roesyphpr) was necessary to remove the signal of residual HOD and to be able to observe weak intermolecular NOE interactions. The data consisted of eight scans collected over 2048 complex points and for a spectral width of 6127 Hz. A mixing time of 200 ms, a repetition delay of 1.5 s, an acquisition time of 0.167 s, and a 90° pulse width of 7.95 μs at −3 dB power attenuation were used. The data were zero-filled to 1024 × 1024 points and processed by applying a $\pi/2$ shifted Q-sine window in both dimensions. Small cross-peaks were neglected when their magnitude was close to that of noise.

Synthesis of Bis(β-CD) 2. (*R*)-(+)-2,2′-Diaminoacetylmethylene chloride−1,1′-binaphthyl was prepared by the reaction of acetyl chloride with (*R*)-(+)-2,2′-diamino-1,1′-binaphthyl in acetonitrile in 86% yield. Then (*R*)-(+)-2,2′-diaminoacetyl chloride−1,1′-binaphthyl (0.2 g) was added to a solution of 6-ethylenediamine−β-CD (1.2 g) in 40 mL of dry DMF containing triethylamine (5 mL) with magnetic stirring under N_2 protection. The solution was stirred at room temperature for 24 h and then was allowed to warm and react at 80 °C for 3 days. The reaction mixture was poured into acetone to give a brown precipitate. The crude solid product was collected by filtration and then purified by Sephadex G-25 column chromatography with deionizated water as eluent to give brown product (400 mg) in 29% yield. ^{1}H NMR (D_2O, TMS, ppm): δ

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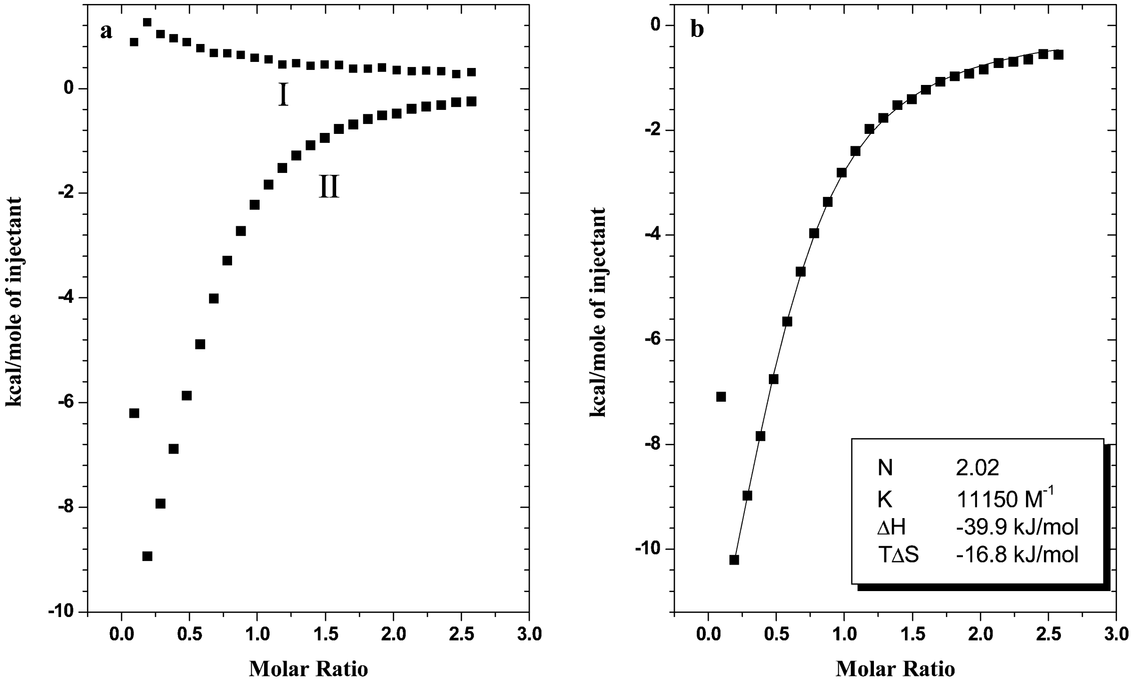

FIGURE 2. (a) Heat effects of dilution (I) and of complexation (II) of **6** with **DCA** for each injection during titration microcalorimetric experiment. (b) "Net" heat effect obtained by subtracting the heat of dilution from the heat of reaction, which was analyzed by computer simulation using the "one set of binding sites" model.

2.5−2.9 (m, 8H); 2.9−3.8 (m, 88H); 4.9 (d, 14H); 6.8−8.1 (m, 12H). ^{13}C NMR (D_2O, ppm): δ 164.9, 132.1, 128.1, 124.6, 101.9, 83.4, 81.2, 72.5, 69.6, 60.3, 47.5, 48.9, 47.8, 30.3. Anal. Calcd for $C_{112}H_{170}O_{70}N_6 \cdot 14H_2O$: C, 45.25; H, 6.71; N, 2.83. Found: C, 45.26; H, 6.70; N, 2.67.

Synthesis of Bis(β-CD) 4. Native β-CD (3.0 g) and 25 mL of dry pyridine was added to a solution of DMF (100 mL) containing 2,2′-dithiobis(benzoic acid) (0.4 g) and dicyclohexylcarbodiimide (DCC) (0.44 g) in the presence of molecular sieves 4 Å. The resultant mixture was stirred for 12 h in an ice bath and another 48 h at room temperature. The precipitate was removed by filtration, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 25 mL of hot water and then poured into 400 mL of acetone. The precipitate formed was collected by filtration to afford a yellow powder, which was purified on a column of Sephadex G-25 to give 0.33 g (10% yield) of 6,6′-[2,2′-dithiobis[2-(benzoylamino)ethylamino]]-bridged bis(β-CD) **4** as a light yellow solid. ^{1}H NMR (D_2O, TMS, ppm): δ 2.64−3.01 (m, 8H); 3.27−3.79 (m, 84H); 4.87 (d, 14H); 6.72−7.8 (m, Ar 8H). ^{13}C NMR (D_2O, ppm): δ 177.0, 163.1, 135.8, 120.6, 118.7, 104.5, 83.8, 75.7, 74.7, 74.4, 62.9. Anal. Calcd for $C_{98}H_{146}O_{72}S_2 \cdot 22H_2O$: C, 40.08; H, 6.52; S, 2.18. Found: C, 39.87; H, 6.98; S, 2.15. IR (KBr): ν_{max}/cm^{-1} 3335, 2927, 2063, 1658, 1422, 1333, 1230, 1249, 1203, 1156, 1079, 1029, 945, 856, 757, 707, 577, 529.

Microcalorimetric Titration. The microcalorimetric titrations were performed at 25 °C under atmospheric pressure in an aqueous phosphate buffer solution of pH 7.20 by using a Microcal VP-ITC titration microcalorimeter, which allows us to simultaneously determine the enthalpy and equilibrium constant from a single titration curve. The instrument was calibrated chemically by performing the complexation reaction of β-CD with cyclohexanol, which gave thermodynamic parameters in good agreement with the literature data.[16] All solutions were degassed and thermostated using a ThermoVac accessory before the titration experiment and titrations were performed below the critical micelle concentration of the steroid molecules.

In each run, a phosphate buffer solution of the host molecule in a 0.250 mL syringe was sequentially injected into the calorimeter sample cell containing a buffer solution of steroid guests with stirring at 300 rpm. The sample cell volume was 1.4227 mL in all experiments. Each titration experiment was composed of 25 successive injections (10 μL per injection). Steroid solutions were applied at a concentration of the range between 0.10 and 0.52 mM, which is below their critical micelle concentration (CMC). (The CMC of the steroids employed here are >1 mM[17]) Typical titration curves are shown in Figure 1. Each addition of CD into the sample cell gave rise to a heat of reaction, caused by the formation of inclusion complexes between steroid molecules and CDs. The heats of reaction decrease after each injection of host CD because less and less steroid molecules are available to form inclusion complexes.

A control experiment was performed to determine the heat of dilution by injecting a host buffer solution into a pure buffer solution containing no steroid molecules. The dilution enthalpy was subtracted from the apparent enthalpy obtained in each titration run, and the net reaction enthalpy was analyzed by using the "one set of binding sites" model for bridged bis(β-CD)s **2**−**7**, as exemplified in Figure 2 for the complexation of **DCA** with bridged bis(β-CD) **6**. This model will work for any number of sites *N* if all sites have the same K_S and $\Delta H°$. In this case, the total heat *Q* was fitted via a nolinear least-squares minimization method to the total CD concentration in cell (M_t) using the following equation:

$$Q = \frac{NX_t\Delta HVo}{2} \left[1 + \frac{M_t}{NX_t} + \frac{1}{NK_SX_t} - \sqrt{\left(1 + \frac{M_t}{NX_t} + \frac{1}{NK_SX_t}\right)^2 - \frac{4M_t}{NX_t}}\right] \quad (1)$$

Where *N* is the number of binding sites of CD, X_t is the total concentration of steroids in cell and V_o is the cell volume. The

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TABLE 1. Complex Stability Constants (K_S) and Standard Enthalpies ($\Delta H°$) and Entropy Changes ($T\Delta S°$) for the Inclusion Complexation of Steroid Guests with Native β-CD 1 and Bridged Bis(β-CD)s 2−7 in a Phosphate Buffer Solution of pH 7.20 at T = 298.1 K

host[a]	guest[b]	N[c]	K_S/M^{-1}	$-\Delta H°$/kJ·mol^{-1}	$T\Delta S°$/kJ·mol^{-1}
1	DCA	1	4844 ± 16	25.8 ± 0.0	−4.76 ± 0.0
	CA	1	4068 ± 84	23.0 ± 0.5	−2.38 ± 0.5
	GCA	1	2394 ± 69	23.0 ± 0.1	−3.7 ± 0.1
	TCA	1	2293 ± 13	23.8 ± 0.1	−4.59 ± 0.1
2	DCA	2	5504 ± 50	42.7 ± 0.2	−21.4 ± 0.2
	CA	2.02	7351 ± 48	33.0 ± 0.1	−10.9 ± 0.1
	GCA	2.05	5936 ± 58	15.1 ± 0.2	6.4 ± 0.2
	TCA	2	3058 ± 38	24.5 ± 0.2	−4.6 ± 0.2
3	DCA	2.01	8372 ± 71	48.1 ± 0.2	−25.7 ± 0.2
	CA	2	5559 ± 38	49.3 ± 0.4	−27.9 ± 0.4
	GCA	2	2979 ± 15	18.1 ± 0.1	4.2 ± 0.1
	TCA	2	4441 ± 31	19.7 ± 0.1	1.1 ± 0.1
4	DCA	2	7900 ± 32	31.6 ± 0.1	−9.4 ± 0.1
	CA	2.09	5039 ± 32	28.2 ± 0.1	−7.1 ± 0.1
	GCA	2	4262 ± 27	21.5 ± 0.1	−0.8 ± 0.1
	TCA	2	1975 ± 10	22.0 ± 0.1	−3.2 ± 0.1
5	DCA	2	8912 ± 37	38.1 ± 0.1	−15.6 ± 0.1
	CA	2.13	10700 ± 98	30.6 ± 0.2	−7.6 ± 0.2
	GCA	2	5689 ± 38	22.7 ± 0.1	−1.3 ± 0.1
	TCA	2	2762 ± 36	37.3 ± 0.1	−17.6 ± 0.1
6	DCA	2.02	11150 ± 130	39.9 ± 0.2	−16.8 ± 0.2
	CA	2	9899 ± 36	37.5 ± 0.1	−14.7 ± 0.1
	GCA	2	4061 ± 22	23.5 ± 0.2	−2.9 ± 0.2
	TCA	2	2502 ± 15	20.2 ± 0.2	−0.8 ± 0.2
7	DCA	2	10325 ± 245	39.4 ± 0.6	−16.5 ± 0.7
	CA	2.14	6196 ± 134	39.3 ± 0.5	−17.6 ± 0.5
	GCA	2	2891 ± 25	23.3 ± 0.1	−3.5 ± 0.1
	TCA	2	2189 ± 18	20.0 ± 0.1	−0.9 ± 0.1

[a] [Host] = 1.97−2.06 mM. [b] [Guest] = 0.12−0.30 mM. [c] Stoichiometry given by fitting program or fixed.

value of Q above can be calculated (for any designated values of N, K, and ΔH) at the end of the ith injection and designated $Q(i)$. Then the correct expression for the heat released, $\Delta Q(i)$, from the ith injection is

$$\Delta Q(i) = Q(i) + \frac{dV_i}{\text{<ital>}V_o}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1) \quad (2)$$

where dV_i is the volume of titrant added to the solution. Along with obtaining of K_S and $\Delta H°$ in this fitting program, the N value in eq 1 can also be obtained, which represents the numbers of steroids bound to one CD molecule.

If a CD molecule has sites with two different values of K_S and/or $\Delta H°$, then the model with "two sets of binding sites" must be used. The calculations of the present system were also performed by using the two sets of binding site models as well as the sequential binding model in addition to the one set of binding site model. However, the parameters calculated by the latter two binding models gave large uncertainties, and the quality of fit was not improved. Therefore, two independent titration experiments were performed to afford self-consistent parameters by fitting with one set of binding sites model, and the averaged values were reported in Table 1.

The ORIGIN software (Microcal), used for the calculation of the binding constant (K_S) and standard molar reaction enthalpy ($\Delta H°$) from the titration curve, gave the relevant standard derivation on the basis of the scatter of data points in a single titration experiment. The binding stoichiometry was also given as a parameter when fitting the binding isotherm (panel b in Figure 2). Knowledge of the binding constant (K_S) and molar reaction enthalpy ($\Delta H°$) enabled the calculation of the standard free energy of binding ($\Delta G°$) and entropy change ($\Delta S°$), according to

$$\Delta G° = -RT \ln K_S = \Delta H° - T\Delta S° \quad (1)$$

where R is the gas constant and T is the absolute temperature.

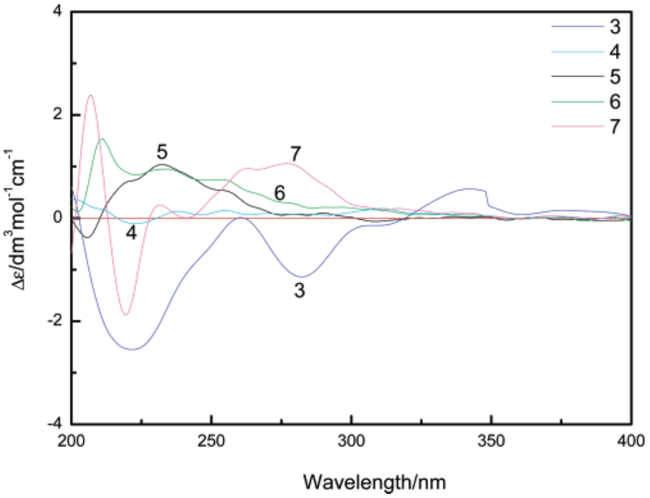


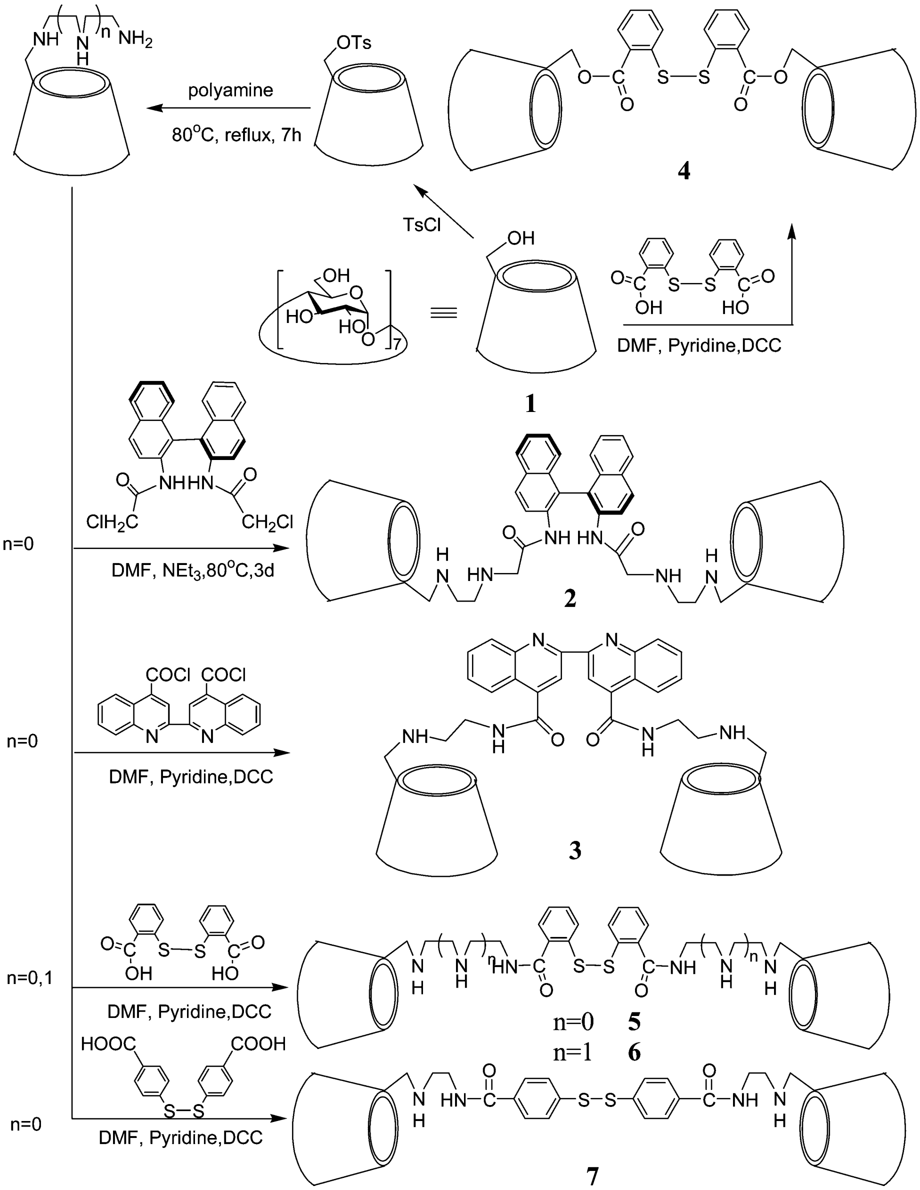
FIGURE 3. Circular dichroism spectra of bis(β-CD)s **3**−**7** in an aqueous phosphate buffer solution of pH = 7.20 at 25 °C.

Multiple independent titration runs (N = 2−4) were performed to afford self-consistent thermodynamic parameters, and the averaged values are reported in Table 1. The reported uncertainties in the thermodynamic parameters reported for host−guest complexation are the standard deviations of the mean values unless stated otherwise.

Results and Discussion

Synthesis. As illustrated in Scheme 1, the (R)-(+)-2,2′-diamino-1,1′-binaphthyl, which was synthesized according to the reported procedure, was converted to (R)-(+)-2,2′-diaminoacetyl chloride−1,1′-binaphthyl and then

SCHEME 1



reacted with 6-ethylenediamine−β-CD to give the final product **2** with a chiral tether moiety in a moderate yield. The bridged bis(β-CD) **4** was synthesized in moderate yield by the reaction of 2,2′-dithiobis(benzoic acid) with native β-CD, while bis(β-CD)s **5**−**7** were synthesized from the corresponding mono[6-oligo(ethylenediamino)-6-deoxy]-β-CD. For the syntheses of bis(β-CD)s **4**−**7**, caution should be exercised to keep the mixture anhydrous and at low temperature during the reaction, particularly at the initial stage, for a smooth and clean reaction without undesirable products.

Original Conformation of Bridged Bis(β-CD)s. It has been amply demonstrated that the inclusion of a chromophoric achiral guest/moiety in a chiral host such as CDs produces induced circular dichroism (ICD) signals at the wavelengths at which the guest chromophore has absorbance.[18] To confirm the original conformation of the bridged bis(β-CD)s possessing chromophoric achiral tethers in an aqueous solution, their ICD spectra were measured at 25 °C. As can be seen from Figure 3, hosts **3**−**7** display different or similar ICD spectra in the absence of guest, which indicates that there exist different degrees of interaction between the aromatic tether and two chiral cavities of the bridged bis-CDs.

The circular dichroism spectrum of bridged bis(β-CD) **3** exhibits two negative Cotton effect peaks with moderate intensities at 225 nm ($\Delta\epsilon = -3.83$ M^{-1} cm^{-1}) and 283 nm ($\Delta\epsilon = -2.22$ M^{-1} cm^{-1}), which may be assigned to the ^1L_a and ^1L_b bands, respectively. The sign of the induced Cotton effect enables us to elucidate the conformation of biquinoline unit in bridged bis(β-CD) **3**. The negative Cotton effects observed for the ^1L_a and ^1L_b bands indicate that the biquinoline unit is shallowly included into the two CD cavities of bridged bis(β-CD) **3** in the longitudinal direction, which is in good agreement with the 2D NMR spectral study.[3c]

The circular dichroism spectrum of bridged bis(β-CD) **4** possessing a relatively rigid tether does not show any

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[18] (a) Connors, K. A. *Chem. Rev.* **1997**, *97*, 1325. (b) Rekharsky, M. V.; Inoue, Y. *Chem. Rev.* **1998**, *98*, 1875. (c) Zhdanov, Y. A.; Alekseev, Y. E.; Kompantseva, E. V.; Vergeichik, E. N. *Russ. Chem. Rev.* **1992**, *61*, 563.

significant ICD signal, indicating that the chromophoric achiral tether is far away from the CD cavities. Meanwhile, bridged bis(β-CD) **5** possessing the same chromophoric group but a longer tether as compared with bis(β-CD) **4** displays a very weak positive Cotton effect peaks at 290 nm ($\Delta\epsilon$ = 0.10 M^{-1} cm^{-1}) for the ^1L_b transition and a relatively strong positive Cotton effect at 232 nm ($\Delta\epsilon$ = 1.04 M^{-1} cm^{-1}) for the ^1L_a transition of phenyl group in 2,2′-dithiobisbenzoic group. As a higher homologue of host **5**, host **6** gives a similar circular dichroism sign; that is, two positive Cotton effect peaks with moderate intensities at 234 nm ($\Delta\epsilon$ = 0.95 M^{-1} cm^{-1}) and 296 nm ($\Delta\epsilon$ = 0.21 M^{-1} cm^{-1}), which may be assigned to the ^1L_a and ^1L_b bands, respectively. Host **7** also displays two weak positive Cotton effect peaks for the ^1L_a and ^1L_b bands at 278 nm ($\Delta\epsilon$ = 1.05 M^{-1} cm^{-1}) and 232 ($\Delta\epsilon$ = 0.25 M^{-1} cm^{-1}). According to the generally accepted empirical rule,[19] the sign of ICD signal depends on the orientation of the transition dipole moment of the chromophore with respect to the dipole moment of the CD. The electronic transition parallel to the CD axis gives a positive ICD signal, whereas the perpendicular transition gives a negative signal. However, the situation is reversed for a guest located just outside the CD cavity. Therefore, we can deduce that the phenyl chromophores in 2,2′-dithiobisbenzoic group of **5** and **6** and 4,4′-dithiobisbenzoic group of **7** penetrate deeply or shallowly into the CD cavities.

2D NMR spectroscopy has recently become an essential method in the study of the conformation of the CD derivatives and the interaction between host CDs and guest molecules, since one can conclude that two protons are closely located in space if an NOE cross-peak is detected between the relevant protons in the NOESY or ROESY spectrum. Therefore, it is possible to estimate the orientation of the tether moiety in the CD cavity using the assigned NOE correlations. If the tether moiety is self-included in the CD cavity, the NOE correlations between the protons of the tether moiety and the H3/H5 protons of the CD should be observed. To this end, to obtain further evidence about the initial geometry of the self-included model of the bridged bis(β-CD)s with different tether groups, 2D NMR spectroscopy experiments of bridged bis(β-CD)s **2**−**7** have been performed in neutral D_2O solution on a Bruker AV600 spectrometer following the circular dichroism experiments. It is worthy to note that the concentration of the hosts in the ROESY experiments is closer to that in the calorimetric titration experiments as compared with the low concentration used in the above CD experiments.

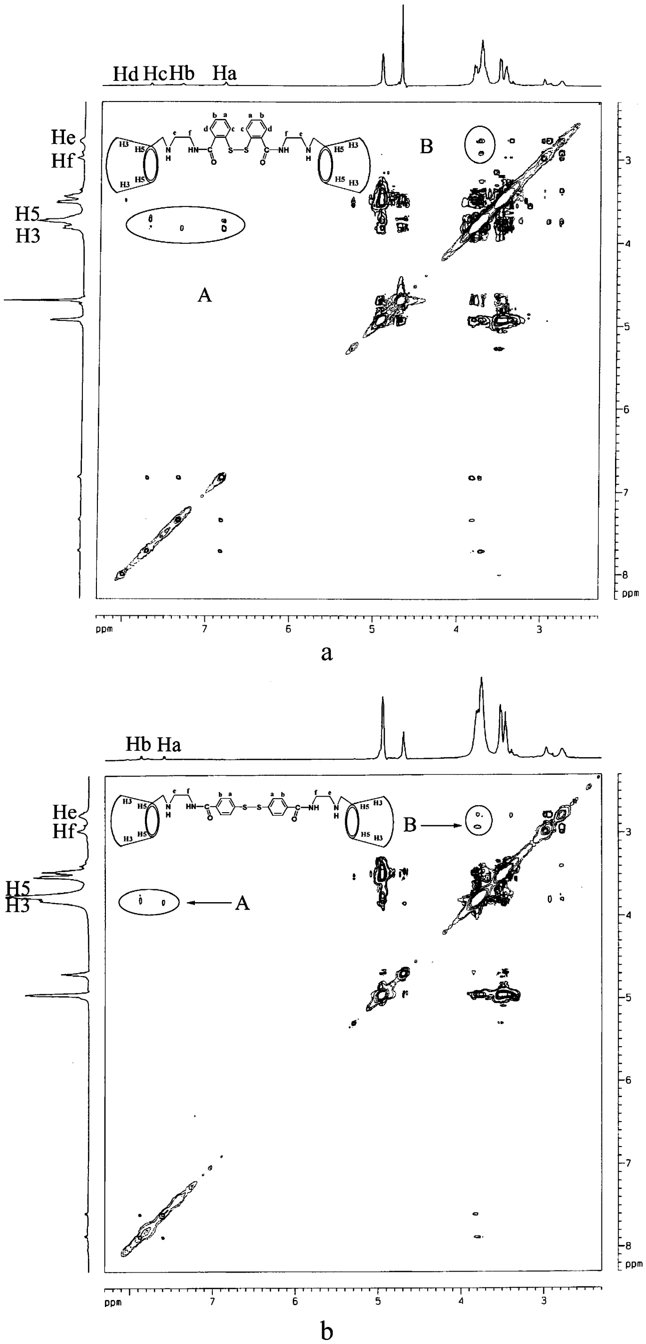

FIGURE 4. ROESY spectra of (a) bridged bis(β-CD) **5** and (b) bridged bis(β-CD) **7** with a mixing time of 200 ms at 298.1 K.

The representative spectra obtained for bridged bis(β-CD)s **5** and **7** are shown in parts a and b, respectively, of Figure 4. Both of them give appreciable NOE correlations between the protons of the tether moiety and the H3/H5 protons of the CD cavity. As can be seen from Figure 4a, the NOE cross-peaks (peaks A) between H3 and H5 of CD cavity and Ha/Hc of the aromatic protons and that between H3 and Hb are observed, suggesting that the Ha−Hb−Hc side of the tether phenyl group is included into the CD cavity. Meantime, NOE cross-peaks between Hd of the tether phenyl group and H3/H5 of the CD cavity are not observed, indicating that the aromatic moiety of the CD tether is not entirely but partially self-included into the hydrophobic cavity from the secondary side of the CD. Furthermore, those cross-peaks (peaks B) between the ethylamino protons He and Hf of the CD tether and H5 of CD cavity are also clearly observed. We thus conclude that both the ethylamino tether of bridged CD and the tether phenyl group are included into the CD cavity. On the other hand, in the ROESY spectrum of bridged bis(β-CD) **7**, not only the cross-peaks (peaks A and B) between Hb of the phenyl moiety and He of the tether ethylamino group with both H3 and H5 of CD

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[19] (a) Kajtar, M.; Horvath, T. C.; Kuthi, E.; Szejtli, J. *Acta Chim. Acad. Sci. Hung.* **1982**, *110*, 327. (b) M. Kodaka, *J. Am. Chem. Soc.* **1993**, *115*, 3702. (c) Bright, F. V. Catena, G. C. *Anal. Chem.* **1989**, *61*, 905. (d) Harata, K.; Uedaira, H. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 375.

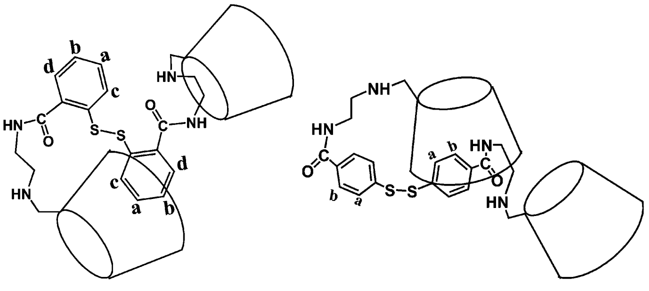

FIGURE 5. Plausible molecular structures of bridged bis(β-CD)s **5** and **7** deduced from the ROESY spectra.

cavity, but also those between Ha/Hf of the tether and CDs H3 are found, as can be seen from Figure 4b. Noting that the interactions of Ha/He and H3 are stronger than those with H5, we can further conclude that the tether phenyl group and ethylamino group are cooperatively self-included into the CD cavity from the secondary rim of CD. The self-included binding modes of hosts **5** and **7** obtained from the ROESY experiments are in excellent agreement with the results obtained from the previous circular dichroism experiments. Based on the above facts, we propose the following two self-inclusion binding modes of bridged bis(β-CD)s **5** and **7** (Figure 5)

Binding Stoichiometry. Several stoichiometric ratios between CDs and guests, such as 1:2, 2:1, and 2:2, have been reported,[1−8,11] but the most common ratio has hitherto shown to be 1:1. The stoichiometric ratios (N value) that we observed from the binding patterns for the titrations of steroids with bridged bis(β-CD)s **2**−**7** fell within the range of ~1.8−2.1:1. This clearly indicates that the majority of the inclusion complexes have a 1:2 stoichiometry of steroids and bridged bis(β-CD)s. The 1:2 binding modes for the inclusion complexation of bridged bis(β-CD)s with steroids have also been investigated by ROESY experiments, which further verifies the above microcalorimetric experiment results.

Binding Modes. Previous studies on the complexation of steroids with CDs demonstrated that the steroid body could enter into the inner cavity of β-CD or monomodified β-CDs by the side of the secondary hydroxyl groups, with the side chain folded or unfolded toward the steroid body, to give different binding stabilities upon complexation with CDs.[11,12] In the present study on the inclusion complexation of steroids and bridged bis(β-CD)s **2**−**7**, aminated bridged bis(β-CD)s **2**, **3**, and **5**−**7** showed larger stability constants than the native β-CyD **1** and the neutral bridged bis(β-CyD) **4**, which does not have amino groups in the tether. To further investigate the mechanism of the enhanced binding ability and the binding mode of interactions between bridged bis(β-CD)s **2**−**7** and steroids as well as to establish the correlation between the conformation of the resulting complexes and the thermodynamics obtained for bridged bis(β-CD)s **2**−**7** tethered with different groups, the ROESY experiments of bridged bis(β-CD)s **2**−**7** were performed in the presence of CA/DCA/GCA in D_2O to elucidate the complex structure in an aqueous solution. The spectra obtained are shown in Figure 6 and Figures S3−S11 (seeing the Supporting Information), respectively.

The notations used are Hn for CD protons and Pn for steroids protons, where n is the carbon number indicated in Chart 2. In the control experiments, the ROESY spectra of the steroids show the intramolecular NOE correlations among the steroid protons, which will help us easily recognize the NOE correlations between host and guest from the ROESY spectra of bis(β-CD)−steroid complexes (see the Supporting Information). As shown in Figure 6, the spectrum for the resulting complex of CA-**5** exhibits clear NOE cross-peaks (peaks A) between the side-chain protons (P-21) and H3, H5 of CD. Meanwhile, the cross-peaks B, C, and D correspond to the interactions of the protons at D-ring of CA with H3 and/or H5 of CD cavity, showing that the D-ring of CA is accommodated in the cavity. It is also clear that P18 of CA (peaks B) only interacts with H3 of CD and the interactions (peaks C) of P15, 16 of CA with H3 of CD are slightly stronger than those with H5. From the above information, it is deduced that CA enters the CD cavity of **5** from the second side of CD with the side chain and D-ring. The ROESY experiments suggest that the side chain with the negative carboxylate group of CA moves toward the positive protonated amino group of **5**. The close examination of the ROESY spectra (see the Supporting Information) of the resulting complexes CA-**7**, CA-**2**, DCA-**5**, DCA-**7**, DCA-**2**, and GCA-**2** consistently demonstrate the similar binding mode with the complex CA-**5**, with only a slight degree of difference in the depth of guest insertion. Since the above-mentioned microcalorimetric titration results indicate that two molecules of steroids are included ino one bis-CD molecule, we propose the possible 1:2 bis(β-CD)-steroid binding modes (Figure 7).

Complexation of Steroids by β-CD 1 and Bridged Bis(β-CD) 4. As can be seen from Table 1, native β-CD **1** and bridged bis(β-CD) **4** show relatively lower binding ability upon inclusion complexation with steroids as compared with aminated bridged bis(β-CD)s **2**, **3**, and **5**−**7**. Even so, bridged bis(β-CD) **4**, possessing a relatively short and rigid tether without amino groups, still gives an enhanced binding ability upon complexation with steroids, except TCA, when compared its one single unit of cavity with that of native β-CD **1**. One possible reason is that although the spacers of bridged bis(β-CD) **4** cannot interact directly with the steroid molecules during the independent identical 1:2 binding process due to the relatively shallow inclusion of steroid body in the CD cavity, they can still change the host−guest binding abilities to some extent by affecting the conformation of bis(β-CD) and/or the penetration depth of the guest into the CD cavity. Thermodynamically, the enthalpy changes for the inclusion complexation of bridged bis(β-CD) **4** with DCA and CA are more negative than that of native β-CD **1** ($-\Delta\Delta H°$ ca. 5 kJ·mol^{-1}), resulting in the relatively stronger binding. On the other hand, the enthalpy change for the complexation of **4** with DCA (31.6 ± 0.1 kJ mol^{-1}) is higher than that with CA (28.2 ± 0.1 kJ mol^{-1}), which directly contributes to the increased complex stability. Indeed, the binding ability ($K_S = 7900 \pm 32$) of **4** with DCA is higher than that with CA ($K_S = 5039 \pm 32$), which is similar to that of **1**. It is reasonable that, possessing the more hydrophobic structure due to the absence of C-7 hydroxyl group as compared with CA, DCA is easier to bind into the β-CD cavity than CA, which should lead to the more favorable van der Waals interactions. Therefore, the stronger interaction between **4** and DCA gives the larger negative enthalpic change and directly leads to the

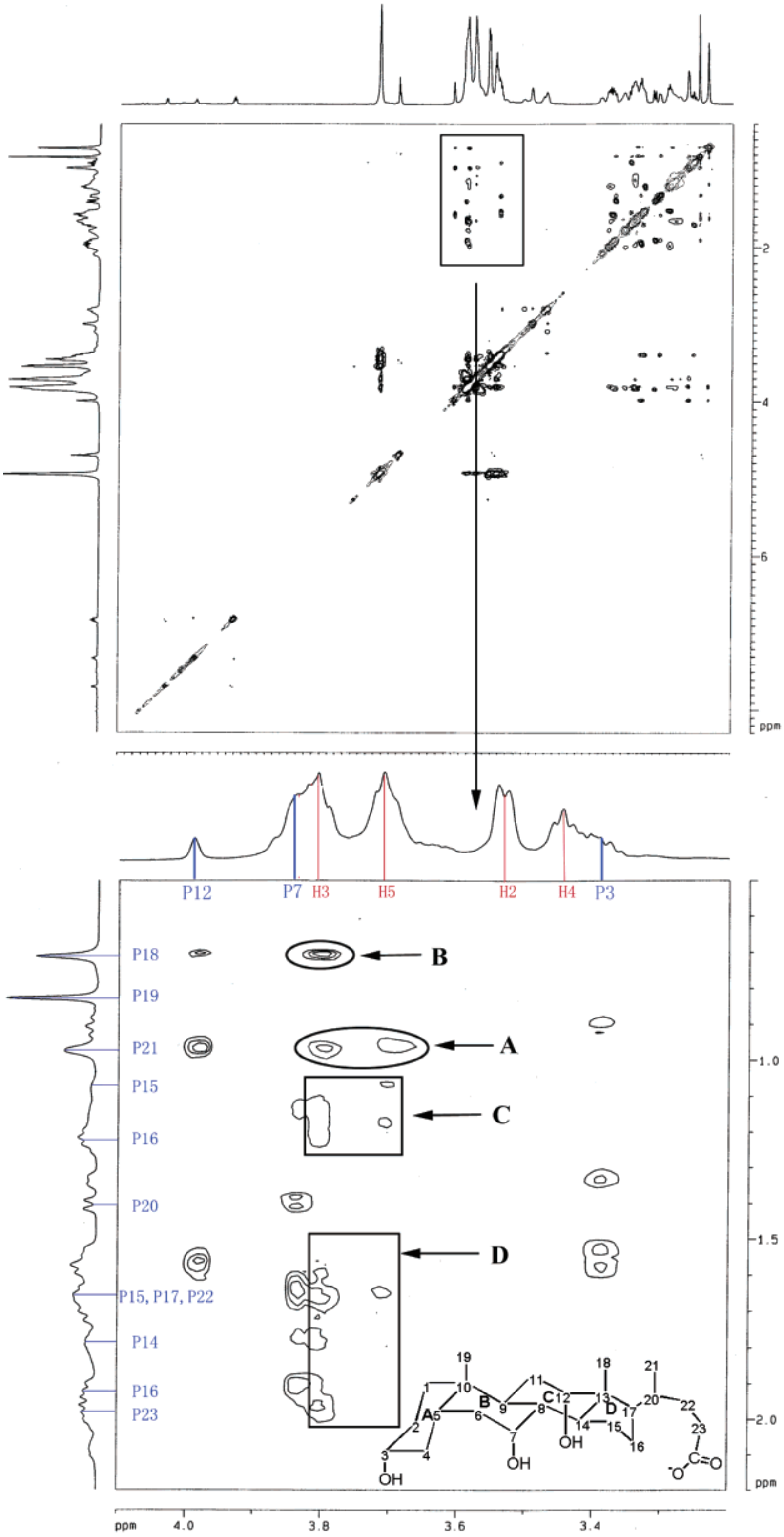

FIGURE 6. ROESY spectrum of CA and host **5** with a mixing time of 200 ms at 298 K.

relatively larger complex stability constant. It is worthy to note that bridged bis(β-CD) **4** enhances the guest selectivity for GCA/TCA pair up to 2.16, as compared with the nearly nonselectivity toward GCA/TCA by native β-CD.

Complexation of CA and DCA by Bridged Bis(β-CD)s 2, 3, and 5−7 Possessing Aminated Tethers. As indicated above from the circular dichroism spectra and ROESY experiments, the spacer of hosts **2**, **3**, and **5**−**7** self-includes, more or less, into the CD cavity. Upon complexation with steroid, these bridged bis(CD)s exhibit outspread conformation, with their tether out of the CD cavity. These ICD and ROESY results indicate that steroid molecules penetrate into the CD cavity of ami-

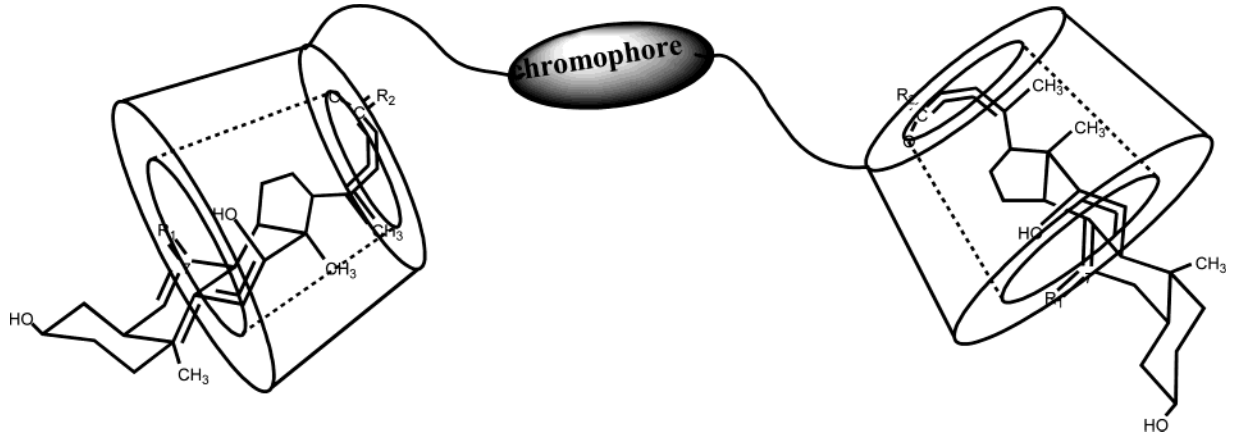

FIGURE 7. Possible binding modes of bridged bis(β-CD)−steroid complexes.

nated bridged bis(β-CD)s from the secondary side with the steroid side chain, which possesses the negative carboxylate group, moving toward the positive protonated amino group of CD tethers. It is also demonstrated that large conformation changes of aminated bridged bis(β-CD)s occur in the complexation process. The electrostatic interaction between protonated amino group of hosts **2**, **3**, and **5**−**7** and the negative charged carboxyl or sulfonic acid group of the steroids must play an important role in the binding behavior and should be responsible for the different thermodynamic behavior. Herein, two families of bridged bis(β-CD)s possessing spacers with different structure and flexibility, i.e., bridged bis(β-CD)s **2** and **3** bearing binaphthyl or biquinoline tethers and bridged bis(β-CD)s **4**−**7** possessing dithiobis(benzoyl) tether, are taken as excellent hosts binding with steroids in order to quantitatively investigate the aminated spacer effects of bridged bis(β-CD)s and host−guest electrostatic interactions upon complexation with steroids. We had demonstrated that monoaminated β-CDs, possessing positive charge at pH 7.2, could enhance the binding ability toward negatively charged steroid molecules through the additional electrostatic interactions in the opposite charged host−guest complexation.[12] In the present study, the bridged bis(β-CD)s possess positive charged 6,6′-amino groups at pH 7.2 and, therefore, should also enhance the binding ability toward negatively charged steroid molecules, as compared with neutral bridged bis(β-CD) **4**, through the additional electrostatic interactions upon inclusion complexation with steroid molecules. Anionic groups of the two steroid molecules penetrated, respectively, into the two hydrophobic CD cavities in one 6,6′-bridged bis(β-CD) molecule from the secondary rim to give a 1:2 binding mode. Therefore, the positive charged spacer of bridged bis(β-CD)s can act as a charged cap covering the CD cavity and further participate in the inclusion complexation toward guest steroids.

As can be seen from Table 1, thermodynamically, the binding behavior of DCA and CA by CDs **1**−**7** is entirely driven by favorable enthalpy changes with accompanying unfavorable entropy changes ($\Delta H° < 0$; $T\Delta S° < 0$), which are attributed to the predominant contribution of the van der Waals interaction arising from the size/shape-fit and geometrical complement between host and guest and the accompanying decreases in translational and structural freedoms upon complexation.[20] Meanwhile, all the complexation of aminated bridged bis(β-CD)s toward DCA and CA give more negative enthalpy changes ($-\Delta\Delta H° = 6.5-16.5$ kJ·mol^{-1} and $2.4-21.1$ kJ·mol^{-1}) as compared with that of neutral bridged bis(β-CD)s **4**, validating the contribution of the attractive electrostatic interactions between positively charged protonated amino group of β-CD tethers and negatively charged carboxylate group of DCA and CA. Our previous study[21] also indicated that the effective electrostatic interaction between host and guest usually led to a more exothermic reaction enthalpy. In deed, the disturbance and rearrangement of the originally well-optimized host−guest van der Waals interaction in the β-CD cavity caused by the modification of CD and the complexation of guest is also another main factor governing the degree of enthalpy changes upon the host−guest complexation. Accompanied with the more exothermic reaction enthalpies, the inclusion complexation of DCA and CA by aminated bridged bis(β-CD)s exhibits more unfavorable $\Delta S°$ compared to that for neutral bridged bis(β-CD) **4** and native β-CD **1**, which possibly originates from the conformation fixation of host and guest and the rigid complex formation upon complexation. As a result, the additional attractive electrostatic interactions evidently enhance the molecular binding ability of bridged bis(β-CD)s except for **2**-DCA pair and alter the molecular selectivity toward CA and DCA. Thermodynamically, the enhanced binding ability is mainly driven by the experimentally observed enthalpic increases despite of the more unfavorable entropy changes. In another word, the additional electrostatic interactions between positively charged protonated amino group of CD tethers and negatively charged carboxylate group of DCA and CA influence and control the binding ability of the host bis-CDs.

Complexation of GCA and TCA by Bridged Bis(β-CD)s 2, 3, and 5−7 Possessing Aminated Tether. Bridged bis(β-CD)s **2**, **3**, and **5**−**7** give the lower binding ability upon complexation with GCA and TCA as compared with the complexation with CA and DCA, which is similar with the complexation of β-CD **1** and bridged

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(20) (a) Rekharsky, M. V.; Inoue, Y. *J. Am. Chem. Soc.* **2002**, *124*, 813. (b) Rekharsky, M.; Inoue, Y. *J. Am. Chem. Soc.* **2000**, *122*, 10949.

(21) (a) Inoue, Y.; Liu, Y.; Tong, L.-H.; Shen, B.-J.; Jin, D.-S. *J. Am. Chem. Soc.* **199**3, *115*, 10637. (b) Inoue, Y.; Hakushi, T.; Liu, Y.; Tong, L.-H.; Shen, B.-J.; Jin. D.-S. *J. Am. Chem. Soc.* **1993**, *115*, 475.

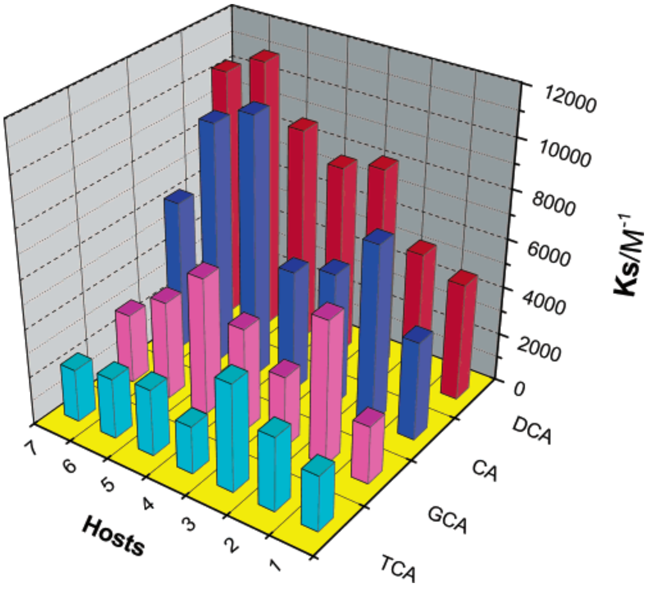

FIGURE 8. Complex stability constants (K_S) upon inclusion complexation of steroids with β-CD **1** and bridged bis(β-CD)s **2**−**7** in phosphate aqueous buffer solution at 298 K.

bis(β-CD) **4**. The universal decreased binding ability toward GCA and TCA must relate to the structure differences from CA and DCA. GCA and TCA possess the same steroid skeleton with CA, but GCA and TCA are the resulting compounds of the conjugation of chololic acid with glycine and taurine, respectively. Thus, the more polar side chains at C23 for GCA and TCA remarkably affect their binding thermodynamics. The results from the ROESY experiments indicate that the representative **2**−GCA system (Figure S3 in the Supporting Information) exhibits the main binding mode: GCA enters the interior of CD with their "tail" first and the D-ring is shallowly included in the CD cavity. The NOE cross-peak of the interactions for P19 of GCA and H3 of CD is also observed despite the very weak intensity, suggesting that another binding mode should also exist in the **2**−GCA system, that is, GCA enters the interior of CD with their A-ring (OH). Significantly, due to the mostly existed former binding mode caused by the attracted electrostatic interactions, bridged bis(β-CD) **2** gives strong binding ability (examining one cavity's binding ability) toward GCA up to 5936 ± 58 and enhances the binding ability toward GCA as compared with those of bridged bis(β-CD) **4** (4262 ± 27 M^{-1}) and native β-CD **1** (2394 ± 69 M^{-1}). This mainly contributes to the favorable entropy changes (6.4 ± 0.2 kJ·mol^{-1}), showing that there must exist relative larger conformation changes and more extensive solvent-desolvating effect during the complexation process.

Binding Ability and Molecular Selectivity Toward Steroids. To visualize and easily discuss the molecular binding behavior and selectivity between CDs and steroid molecules, the changing profiles of the complex stability constants (K_S) as well as the standard enthalpy ($\Delta H°$) and entropy changes ($T\Delta S°$) are plotted in Figures 8 and 9, respectively.

Native β-CD **1** and bridged bis(β-CD) **4** afford relatively small binding constants probably due to the weak van der Waals, the hydrophobic interactions, and the host structure features, as discussed previously. However, possessing positively charged amino tethers, the aminated bridged bis(β-CD)s **2**, **3**, and **5**−**7** exhibit the enhanced molecular binding ability through the cooperative electrostatic, van der Waals interactions, etc. Experimental data indicate that the additional attractive electrostatic interactions play an important role in their complexation.

It is also interesting to compare the obtained "host selectivity" sequence for each steroid. The binding constant for the complexation of each steroid by neutral bridged bis(β-CD) **4** and aminated bridged bis(β-CD)s **2**−**3** and **5**−**7** increases in the following order:

DCA: **2** < **4** < **3** < **5** < **7** < **6**

CA: **4** < **3** < **7** < **2** < **6** < **5**

GCA: **7** < **3** < **6** < **4** < **5** < **2**

TCA: **4** < **7** < **6** < **5** < **2** < **3**

As can be seen from Table 1 and Figure 8, aminated bridged bis(β-CD)s **2**, **3**, and **5**−**7** show mostly enhanced

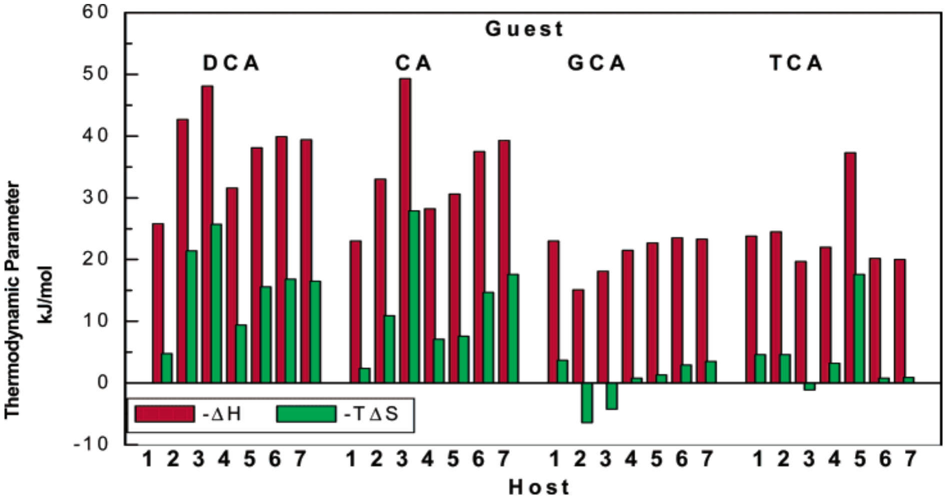

FIGURE 9. Standard enthalpy ($\Delta H°$) and entropy changes ($T\Delta S°$) for the 1:1 inclusion complexation of β-CD **1** and the 2:1 inclusion complexation of bridged bis(β-CD)s **2**−**7** in phosphate aqueous buffer solution at 298 K.

binding ability toward steroid molecules as compared with neutral bridged bis(β-CD) **4**, except for **2**-DCA, **7**-GCA, **3**-GCA, and **6**-GCA pairs. For instance, the K_S values for the inclusion complexation of aminated bridged bis(β-CD)s **2**, **3**, and **5**−**7** with CA are enhanced by factors of 1.46 for **2**, 1.10 for **3**, 2.12 for **5**, 1.96 for **6**, and 1.23 for **7**, respectively, as compared with that for bridged bis(β-CD) **4**. The differential reaction enthalpy and entropy changes calculated by using the data presented in Table 1 reveal that nearly all the enthalpy changes (-2.4 kJ/mol < $\Delta H°_{\text{hosts } \mathbf{2,3,5,6,7}} - \Delta H°_{\text{host } \mathbf{4}} < -21.1$ kJ/mol for CA) are more remarkable than the entropy changes (-0.5 kJ·mol^{-1} < $T\Delta S°_{\text{hosts } \mathbf{2,3,5,6,7}} - T\Delta S°_{\text{host } \mathbf{4}} < -20.8$ kJ·mol^{-1} for CA). Therefore, the enthalpy changes apparently control the enhanced binding ability of aminated bridged bis(β-CD)s as compared to neutral bridged bis(β-CD).

On the other hand, hosts **3**, **6**, and **7** give stronger binding ability toward DCA versus CA, which is the same as that for host **4**. Hosts **2** and **5** reverse this binding selectivity, showing larger binding constants for CA than DCA. Bridged bis(β-CD) **2** gives the largest guest selectivity for CA/DCA up to 1.33. Meanwhile, bridged bis(β-CD) **7** gives the largest guest selectivity for DCA/TCA up to 4.7 ($K_S^{(7-\text{DCA})}/K_S^{(7-\text{TCA})}$) and 1.7 times guest selectivity for DCA/CA. Aminated bridged bis(β-CD) **6** gives 2.44 times selectivity for CA versus GCA, enhancing the selectivity (1.18 for CA/GCA) of neutral bridged bis(β-CD) **4**. Thermodynamically, the complexation of GCA with **6** gives $\Delta H°$ and $T\Delta S°$ values comparable to those for neutral bridged bis(β-CD) **4**, and the enthalpic gain ($\Delta H°_{\mathbf{6}} - \Delta H°_{\mathbf{4}} = -2.0$ kJ·mol^{-1}) is well compensated by the equally increasing entropic loss ($T\Delta S°_{\mathbf{6}} - T\Delta S°_{\mathbf{4}} = -2.1$ kJ·mol^{-1}). In contrast, the complexation of CA with **6** gives a larger enthalpic gain than that for neutral bridged bis(β-CD) **4** ($\Delta H°_{\mathbf{6}} - \Delta H°_{\mathbf{4}} = -9.3$ kJ·mol^{-1}), which is not compensated by a relatively small entropic loss ($T\Delta S°_{\mathbf{6}} - T\Delta S°_{\mathbf{4}} = -7.6$ kJ·mol^{-1}). As a consequence of such opposite behavior of $\Delta H°$ and $T\Delta S°$, the negligible molecular selectivity of β-CD ($\Delta\Delta G° = \Delta G°_{\text{TCA}} - \Delta G°_{\text{CA}} = 1.2$ kJ·mol^{-1}) is substantially enhanced to give a $\Delta\Delta G°$ value of 3.8 kJ·mol^{-1} for host **6**.

Enthalpy−Entropy Compensation. Enthalpy−entropy compensation has often been observed empirically in the kinetic and thermodynamic parameters determined for a wide variety of reactions and equilibria and has long been an active topic in the chemical field. Numerous experimental data in the original and review articles indicate that the compensation enthalpy−entropy relationship is a powerful tool to understand and even to predict thermodynamic behavior.[21,22]

The linear ΔH-ΔS relationship observed experimentally leads to eq 3 and gives eq 4 when integrated.

$$T\Delta\Delta S = \alpha\Delta\Delta H \tag{3}$$

$$T\Delta S = \alpha\Delta H + T\Delta S_0 \tag{4}$$

Thus, the slope (α) of the $T\Delta S$ versus ΔH plot (eq 4) indicates to what extent the enthalpic gain ($\Delta\Delta H$) is canceled by entropic loss. The positive intercept indicates that the complex is stabilized even in the absence of enthalpic contributions, as far as the $T\Delta S$ term is positive. In the present study, the $T\Delta S$ values are plotted against the ΔH values to give an excellent straight line with a correlation coefficient of 0.99, as can be seen from Figure 10, and the reporting limited bridged bis(CD)-steroid systems presently examined has an intercept $T\Delta S_0 = 19.3$ kJ·mol^{-1} and slope = 0.94, respectively. Previous reports on enthalpy−entropy compensation for the complexation of 1070 guest molecules with natural CDs gave a slope (α = 0.88) and intercept ($T\Delta S_0 = 12$ kJ·mol^{-1}). And our previous studies on enthalpy−entropy compensation for the complexation of steroids with mono aminated CDs gave a slope (α = 0.93) and intercept ($T\Delta S_0 = 18.7$ kJ·mol^{-1}). From the above results, we can see that the values we obtained are much larger than those relative to the natural CDs and slightly larger than those relative to the aminated mono-CDs, and in agreement with those reported in the literature for complexes formed by bis(CD)s with flexible tethers. This indicates that the inclusion complexation of aminated bridged bis(β-CD)s with steroid occurs the larger conformational change and extensive desolvation effect. Although the analysis was performed with a limited number of data, we have gained clear and sensible insights into the factors and mechanism governing the inclusion complexation of bis(β-CD)s in the present case. Work concerned with extending the reliability of these thermodynamic data is currently in progress.

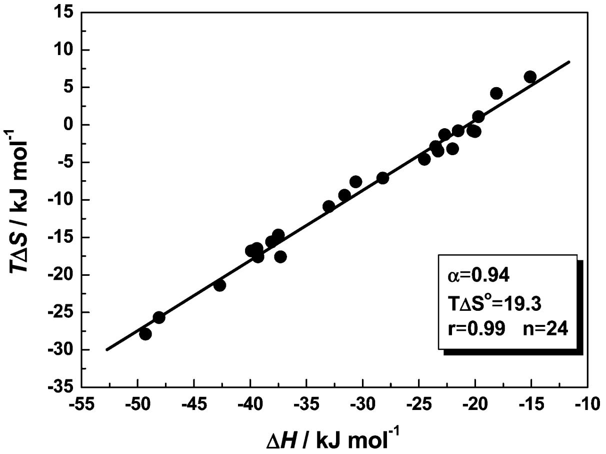

FIGURE 10. Enthalpy−entropy compensation plot for the inclusion complexation of various steroids with bridged bis(β-CD)s **2**−**7** obtained in the present work in an aqueous buffer solution at 298.15 K.

Conclusions

In summary, solution structural investigations on the inclusion complex of bridged bis(β-CD)s and steroids showed that anionic groups of two steroid molecules penetrated respectively into the two hydrophobic CD cavities in one bis(β-CD) molecule from the secondary rim to exhibit 1:2 host−guest binding mode. From the structures of bis(β-CD)s and their complexes, it is noted that the relative large conformation changes and self-inclusion of the spacer must occur in the inclusion complexation with steroid molecules, corresponding to the more negative entropy changes. Thermodynamically, the enhanced

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[22] (a) Rekharsky, M. V.; Inoue, Y. *Chem. Rev.* **1998**, *98*, 1875. (b) Grunwald, E. *Thermodynamics of Molecular Species*; Wiley-Inerscience: New York, 1996. (c) Chen, R.-T. *Correlation Analysis in Coordination Chemistry*; Anhui Educational Publishing: Hefei, 1995 (in Chinese). (d) Exner, O. *Correlation Analysis of Chemical Data*; Plenum: New York, 1988. (e) Rekharsky, M. V.; Inoue, Y. *J. Am. Chem. Soc.* **2000**, *122*, 4418.

van der Waals interactions and additional attractive electrostatic interactions between bis(β-CD) and steroid led to a more exothermic reaction enthalpy, which directly contributed to the increased inclusion complex stability. Therefore, the current studies not only revealed the enhanced binding ability and molecular selectivity of steroid by bis(β-CD) but also established the correlation between thermodynamic parameters and solution structure in the complexation of bridged bis(β-CD) and steroid, which will further serve the understanding of the molecular multiple-recognition mechanism in supramolecular systems.

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Supporting Information Available: ROESY spectra of CA, DCA, CA-**2**, CA-**7**, DCA-**2**, DCA-**5**, DCA-**7**, and GCA-**2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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