Residue- and Sequence-Selective Binding of Nonaromatic Dipeptides by Bis(β-cyclodextrin) with a Functional Tether

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Peptide recognition by synthetic receptors is known to mediate versatile protein–protein interactions and give specific biochemical functions.[1–6] Among them, the development of cyclodextrin (CD)-based receptors for residue- and sequence-selective recognition of peptides is one of the most challenging tasks, which may also lead us to deeper understanding and smarter mimicking of vital biological functions.[7–12] Recent investigations have demonstrated that bis(β-CD)s linked with a short tether possess binding abilities and selectivities for specific guests much higher than native β-CD. This was made possible through the cooperative two-point recognition[11a,13–15] that mimics the highly substrate-specific binding of enzymes.[16] Thus, a variety of bis(β-CD)s with considerable structural diversity have been prepared in order to elucidate their complexation behavior as well as the factors and mechanisms governing the multipoint recognition upon inclusion complexation by bis-CDs.

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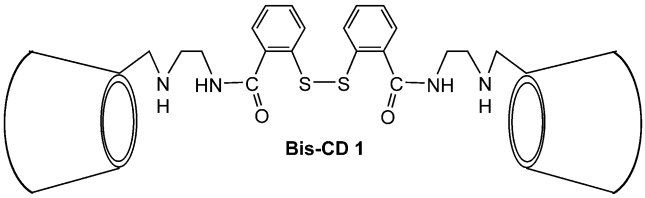
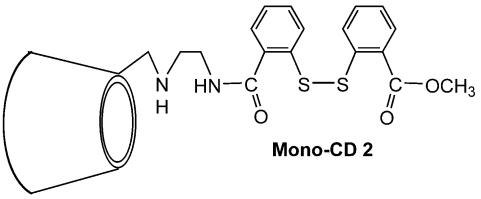
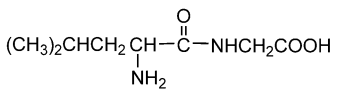
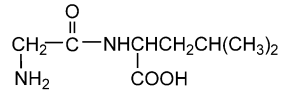
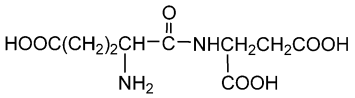
However, the work on molecular recognition by bis(β-CD)s has been concentrated mostly on the complexation of rather simple organic guests and amino acids, and practically no attempt has been made on the recognition of nonaromatic oligopeptides by bis(β-CD)s, outside the elegant work on oligopeptides carrying two aromatic amino acid residues for a simultaneous complexation by CD cavities[11] and on testing a library of aromatic and nonaromatic tripeptides for binding to a bis- as well as a mono-CD receptor.[12] Here, we report the unique molecular-recognition behavior of a newly synthesized dithiobenzoylamino-bridged bis(β-CD) (**1**) with representative nonaromatic dipeptides and the remarkable fluorescence enhancement by coinclusion of a dipeptide guest with the tether moiety into the cavity.

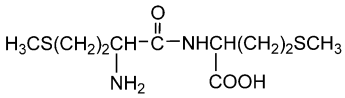


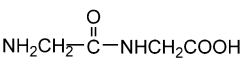


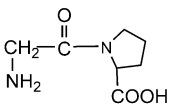












6,6′-(2,2′-Dithiobis(2-(benzoylamino)ethylamino))-bridged bis(β-CD) (**1**) was synthesized by the reaction of mono(6-(2-aminoethyleneamino)-6-deoxy)-β-CD with 2,2′-dithiobis-(benzoic acid) in the presence of dicyclohexylcarbodiimide (Scheme 1). Chromatographic purification over Sephadex G-25 gave a pure sample in 28% yield as a bright brown solid.

The original conformation of bis(β-CD) **1** and mono(β-CD) **2** in the absence of a guest was investigated in aqueous solution by circular dichroism and/or 2D NMR spectroscopy (see Supporting Information) to reveal that the tether moiety is shallowly self-included in the CD cavity. However, upon addition of Gly-Leu as a guest, the tether's aromatic protons and the CD's

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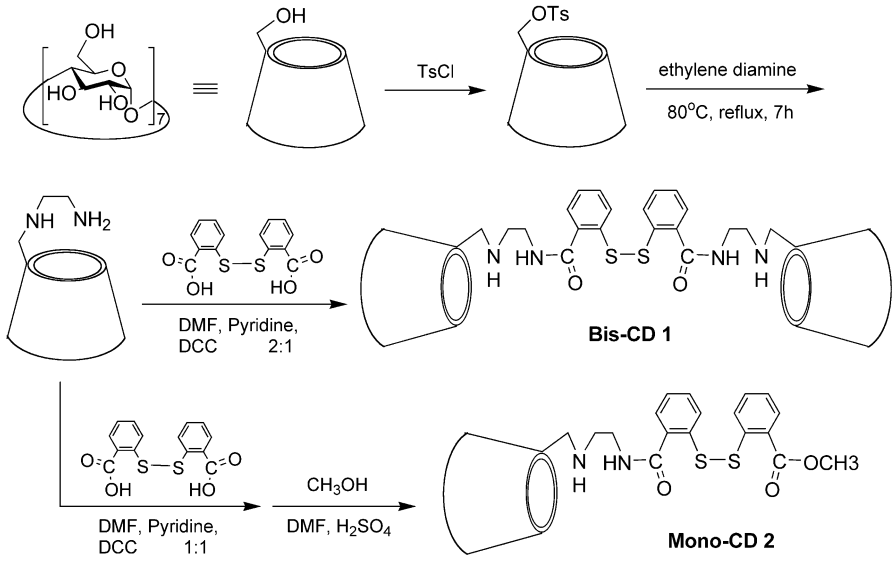

Scheme 1. *Synthetic routes of host CDs.*

H-5 showed a clear crosspeak in the ROESY spectrum (A in Figure 1); this demonstrated that the aromatic group is perching on the primary side of the cavity. Furthermore, not only the protons of isobutyl group of Gly-Leu but also the ethyl protons of the CD tether gave crosspeaks (B and C) with the protons of H-3 and/or H-5 of **1**; this indicated that both the isobutyl group of the Leu residue and the ethylamino group of the CD tether are simultaneously included in the same CD cavity. This was further validated by the observation of weak NOE crosspeaks (D) between the isobutyl protons of Gly-Leu and the ethylamino protons of the CD tether. On the other hand, we did not find any NOE signals between the aromatic protons of phenyl group of bis-CD and the protons of the isobutyl group of Gly-Leu. Therefore, we consider that the isobutyl of this Leu residue and the ethylamino moiety of the CD tether are cooperatively included in one CD cavity and the tether phenyl group is shallowly included in the other CD cavity.

To further examine the cooperative binding behavior by two CD cavities, the complexation stoichiometry was investigated. The Job plot obtained for the inclusion complexation of bis(β-CD) **1** with Gly-Leu (Figure 2) confirmed the formation of 1:1 sandwich complex. The complex stability constants (K_s) and Gibbs free energy changes

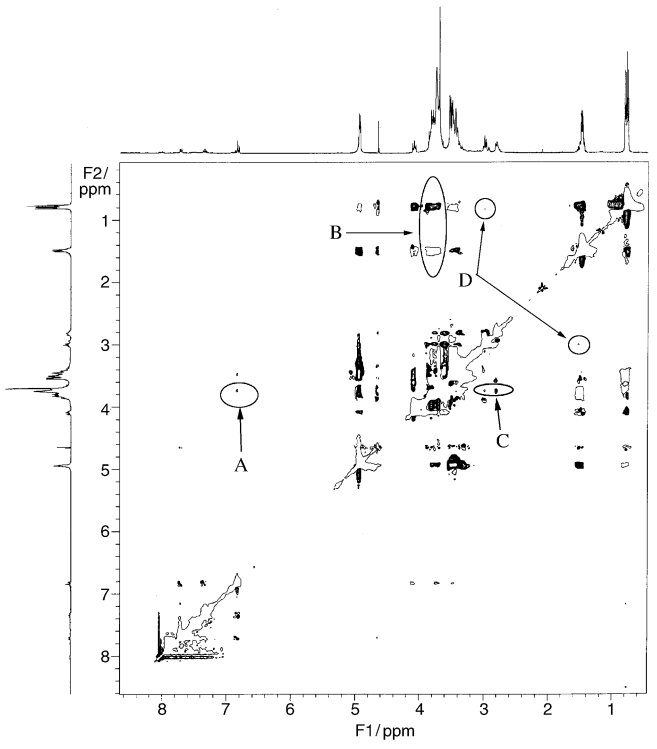

Figure 1. *2D ROESY spectrum of Gly-Leu-**1** ([**1**] = [Gly-Leu] = 2.0 × 10^{-3} M) with a mixing time of 400 0 ms in D_2O.*

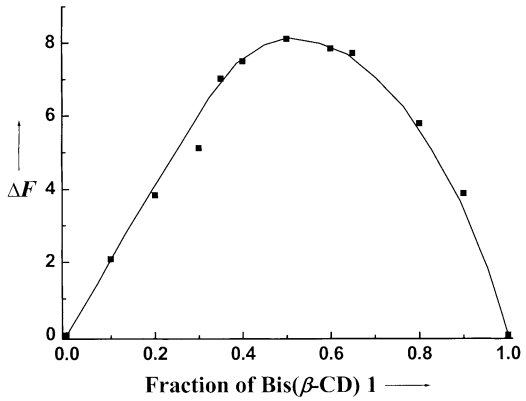

Figure 2. *Continuous variation plot of the bis(β-CD) **1**/Gly-Leu system ([bis(β-CD) unit] + [Gly-Leu]) 1.0 × 10^{-6} mol dm^{-3}) ΔF = relative fluorescent intensity.*

(−Δ*G*°) for the 1:1 complexation of dipeptides Leu-Gly, Gly-Leu, Gly-Pro, Glu-Glu, Gly-Gly, and Met-Met with bis(β-CD) **1** were determined at 25 °C by fluorometric titration (JASCO FP-750), since practically no significant changes for reliable spectrometry were observed in the ultraviolet and circular dichroism spectra upon addition of the guests.

Fluorometric titrations of bis(β-CD) **1** (8 × 10^{-6} M) with dipeptides (up to 4–6 × 10^{-4} M) were performed at 25 °C in aqueous phosphate buffer solution at pH 7.20. As shown in Figure 3, the stepwise addition of dipeptides caused significant successive enhancement in fluorescence intensity (I_F). The dramatic enhancement of fluorescence intensity may indicate the increased hydrophobicity around the fluorophore as a result of the hydrogen-bonding interaction between the tether carboxamide and guest peptide groups. A conventional nonlinear

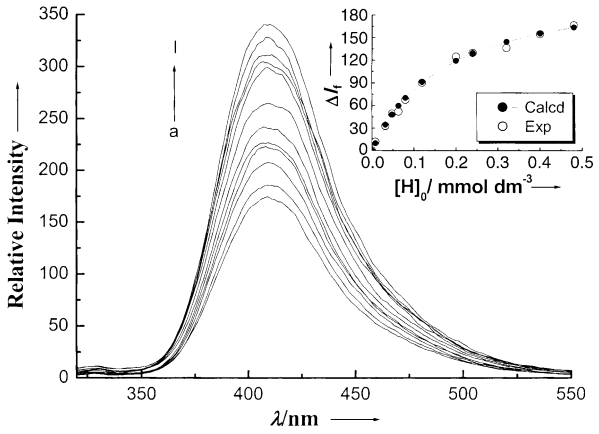

Figure 3. *Fluorescence spectral changes of bis(β-CD) **1** (8 μM) and the nonlinear least-squares analysis (inset) of the differential intensity (ΔI_F) used to calculate the complex stability constant (K_S) upon addition of Gly-Pro (0–480 μM; traces a to l) in aqueous buffer solution at 25 °C and pH 7.20; excitation at 295 nm.*

least-squares fit of the I_F data to the theoretical curve for the 1:1 complexation gave the complex stability constants (K_S), listed in Table 1, along with the relative K_S's (K_{rel}) and the Gibbs free energy changes ($-\Delta G°$) calculated therefrom. On the other hand, the stepwise addition of all the employed dipeptides caused no appreciable fluorescence changes of mono(β-CD) **2**, which lacks the second CD cavity. These observations validated not only the importance of the second cavity of bis-CD **1** upon inclusion complexation with dipeptide, but also the coinclusion binding mode.

Table 1. *Complex stability constants (K_S), relative stability constant (K_{rel}), and Gibbs free-energy changes ($-\Delta G$) for 1:1 inclusion complexation of dipeptides with bis(β-CD) **1** in aqueous buffer solution at 25 °C and pH 7.20.*

Guest	K_S [M^{-1}]	K_{rel}	$-\Delta G°$ [kJ mol^{-1}]
Glu-Glu	590 ± 50	≡1	15.8
Gly-Gly	1680 ± 80	2.9	18.4
Leu-Gly	3300 ± 200	5.6	20.1
Met-Met	5600 ± 250	9.5	21.4
Gly-Pro	6200 ± 300	10.5	21.7
Gly-Leu	16600 ± 800	28.1	24.1

As can be seen from Table 1, the complex stability increases in the order: Glu-Glu < Gly-Gly < Leu-Gly < Met-Met < Gly-Pro < Gly-Leu. Although the obtained K_S's are moderate, bis(β-CD) **1** can discriminate the nonaromatic dipeptides with good to excellent selectivities, affording the largest K_{rel} of 28.1 (or a stability difference of 8.3 kJ mol^{-1}) for Gly-Leu versus Glu-Glu. The best affinity of bis(β-CD) **1** to Gly-Leu is reasonably accounted for in terms of the inclusion of the hydrophobic isobutyl group of the Leu residue into the CD cavity and the favorable electrostatic attraction between the closely located tether NH_2^+ and Leu CO_2^- groups, both of which greatly promote the cooperative inclusion by the two CDs in **1**. In the Gly-containing dipeptide series, the affinity order follows in general the hydrophobicity order of the partner amino acid residue: Gly-Gly < Leu-Gly < Gly-Pro, excepting Gly-Leu, for which we have no reasonable rationalization at present. Similarly, the smallest K_S for Glu-Glu is attributable to its high hydrophilicity. In addition, it seems reasonable that bis(β-CD) **1** shows relatively higher affinity for Met-Met, since Met-Met possesses a hydrophobic side chain at each end of the molecule, which greatly promote inclusion by the two hydrophobic cavities of bridged bis(β-CD).

A more intriguing finding is the particularly high sequence selectivity. Upon complexation of Leu-Gly and Gly-Leu, bis(β-CD) **1** can differentiate the dipeptide sequences with a selectivity as high as 5.0 ($\Delta\Delta G° = 4.0$ kJ mol^{-1}). This remarkable difference in K_S between Leu-Gly and Gly-Leu can be accounted for in terms of the attractive/repulsive interaction between the protonated amino group in the tether ($-NH_2^+-$) and the relevant charged group (CO_2^- or NH_3^+) remaining in the amino acid residues. For a Gly-Leu guest, the inclusion of the hydrophobic isobutyl group of the Leu residue into the CD cavity induces the electrostatic attraction between the closely located tether NH_2^+ and Leu CO_2^- groups. In contrast, the inclusion of Leu-Gly inevitably causes electrostatic repulsion between the tether NH_2^+ and Leu NH_3^+ groups.

Although this is a preliminary study dealing with a limited number of dipeptide guests, the present results are of particular interest and importance in designing peptide receptors with high binding ability and/or sequence selectivity and also in discussing their binding behavior. We also believe that the present host design, which brings about cooperative, multipoint/multimode recognition by introducing a functional tether to bis-CDs, is potentially applicable in general to the complexation of homologous oligopeptides. Studies to compare the differences of the binding affinities between aromatic and nonaromatic dipeptides upon inclusion complexation with fluorescent bis-CD and to elucidate the scope and limitations of this host design and the fundamental strategy as well as the detailed recognition mechanism are currently in progress.

Experimental Section

Synthesis of 6,6′-(2,2′-dithiobis(2-(benzoylamino)ethylamino))-bridged bis (β-CD) (1): Mono(6-(2-aminoethyleneamino)-6-deoxy)-β-CD (3.0 g) and dry pyridine (25 mL) were added to a solution of 2,2′-dithiobis(benzoic acid) (0.3 g) and dicyclohexyl-carbodiimide (DCC) (0.33 g) in DMF (100 mL) in the presence of 4 Å molecular sieves. The resultant mixture was stirred for 12 h in an ice bath and another 18 h at room temperature and then allowed to stand for 3 d until no more precipitation occurred. The precipitate was removed by filtration, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in a minimum amount of hot water and then poured into acetone (300 mL). The precipitate formed was collected by filtration to obtain a brown powder, which was purified on a Sephadex G-25 column to give 0.8 g (28%) of **1** as a light brown solid. ^{1}H NMR (D_2O, TMS): $\delta =$ 2.63–2.91 (m, 8H), 3.25–3.78 (m, 84H), 4.86 (d, 14H), 6.71–7.65 ppm (m, 8H; Ar); ^{13}C NMR (D_2O): $\delta = 177.2$, 163.0, 135.9, 120.6, 118.5, 104.1, 85.5, 83.4, 75.5, 74.2, 72.5, 62.5, 51.1, 48.2, 40.7 ppm; IR (KBr): $\tilde{\nu}_{max}$: 3333, 2927, 1631, 1592, 1485, 1458, 1334, 1302, 1253, 1203, 1155, 1079, 1031, 945, 859, 757, 705, 607, 580, 532, 439, 410 cm^{-1} UV/Vis (water) λ_{max} (ε) = 296.8 nm^{-1} (2850 dm^3 mol^{-1} cm^{-1}); elemental analysis calcd (%) for

$C_{102}H_{158}O_{70}N_4S_2 \cdot 20H_2O$: C 41.05, H 6.69, N 1.88, S 2.15; found: C 40.86, H 6.74, N 2.00, S 2.19;.

Synthesis of mono[6-(2,2′-dithio-1′-benzoylmethylester-1-benzoylaminoethylamino)-6-deoxy]-β-CD (2): Dry pyridine (25 mL) was added to a solution of 2,2′-dithiobis(benzoic acid) (0.3 g) and dicyclohexyl-carbodiimide (DCC; 0.33 g) in DMF (80 mL) in the presence of 4 Å molecular sieves, and then a solution of mono(6-(2-aminoethyleneamino)-6-deoxy)-β-CD (1.2 g) in DMF (20 mL) was added dropwise. The resultant mixture was stirred for 24 h in an ice bath and for another 48 h at room temperature and then allowed to stand for 2 d until no more precipitation occurred. The precipitate was removed by filtration, and the filtrate was evaporated to dryness under reduced pressure. The above product was then dissolved in DMF (30 mL), and then methanol (2 mL) and DCC (0.2 g) were added. The resultant mixture was stirred and heated to 80 °C to react for about 24 h under the catalysis of several drops of sulfuric acid. Then the precipitate was removed by filtration, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in a minimum amount of hot water and then poured into acetone (300 mL). The precipitate formed was collected by filtration to obtain a brown powder, which was purified twice on a Sephadex G-25 column to give 0.48 g (32 %) of **2** as a light brown solid. ^{1}H NMR (D_2O, TMS): $\delta = 2.8$–3.2 (m, 4H), 3.2–3.8 (m, 42H), 3.9 (s, 3H), 4.9 (m, 7H), 6.8–8.0 ppm (m, 8H; Ar); ^{13}C NMR (D_2O): $\delta = 177.7$, 163.5, 135.8, 133.0, 120.8, 120.0, 118.2, 109.9, 101.9, 81.1, 73.0, 71.9, 67.3, 60.2, 50.9, 48.5, 44.8 ppm; IR (KBr): $\tilde{\nu}_{max} = 3298$, 2943, 1649, 1540, 1455, 1336, 1155, 1080, 1053, 944, 754, 609, 578, 437, 407 cm^{-1}; elemental analysis calcd (%) for $C_{59}H_{86}O_{37}N_2S_2 \cdot 5H_2O$: C 45.15, H 6.17, N 1.79, S 4.09; found: C 45.26, H 6.05, N 1.66, S 3.90.

Spectral titrations: Fluorescence spectra were measured in a conventional quartz cell (10×10×45 mm) at 25 °C on a JASCO FP-750 spectrometer equipped with a constant-temperature water bath and with excitation and emission slits of 5 nm width for all the dipeptides. Hosts and dipeptides were resolved in aqueous buffer solution (pH 7.20) at 25 °C, and allowed to stand for 30 min before use in the following fluorescence experiments. The stepwise addition of a known amount of the peptides to a solution of **1** (8 μM) caused significant enhancement in the fluorescence intensity of **1**. According to the fluorescence-spectral changes of **1**, the complex stability constant (K_S) upon addition of the buffered dipeptides could be calculated by nonlinear least-squares analysis. For mono-CD **2**, the fluorescence-spectral changes were too small to allow the calculation of the binding constants of **2** and dipeptides.

Acknowledgements

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Keywords: binding · cyclodextrins · peptides · recognition

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