Molecular Binding Behavior of Pyridine-2,6-dicarboxamide-Bridged Bis(*β*-cyclodextrin) with Oligopeptides: Switchable Molecular Binding Mode

Yu Liu,* Guo-Song Chen, Yong Chen, Fei Ding, Tao Liu, and Yan-Li Zhao

Department of Chemistry, State Key Laboratory of Elemento-Organic Chemistry, Nankai University, Tianjin 300071, P. R. China. Received December 20, 2003; Revised Manuscript Received January 30, 2004

Bridged bis(*β*-cyclodextrin) **1** with a pyridine-2,6-dicarboxamide linker was synthesized, and its inclusion complexation behavior with some aliphatic oligopeptides was investigated in aqueous buffer solution of pH 2.0 and 7.2 at 25 °C by means of circular dichroism, fluorescence, and 2D NMR techniques. The results show that the resulting inclusion complexes of **1** with oligopeptides adopt a cooperative "cyclodextrin−guest−cyclodextrin" sandwich binding mode in a neutral media, but a "guest−linker−cyclodextrin" coinclusion binding mode in an acidic media. These switchable binding modes consequently rationalize the binding ability of bis(*β*-cyclodextrin) **1** at different pH values; that is, **1** shows the stronger association with oligopeptides in a neutral media. Because of the simultaneous contributions of hydrophobic, hydrogen bond, and electrostatic interactions, bis(*β*-cyclodextrin) **1** affords length-selectivity up to 4.7 for the Gly-Gly/Gly-Gly-Gly pair at pH 2.0 and sequence-selectivity up to 4.2 for the Gly-Leu/Leu-Gly pair at pH 7.2. These phenomena are discussed from the viewpoint of the size−fit concept and the multipoint recognitions between host and guest.

INTRODUCTION

Cyclodextrins are a class of cyclic oligosaccharides consisting, for the most common representatives, of six to eight D-glucose units linked by α-1,4-glucose bonds. They are well-known to encapsulate various organic guests within their hydrophobic cavities to afford host−guest complexes or supramolecular species in aqueous solution (*1*). This fascinating property enables them to be successfully utilized as drug carriers (*2*−*4*), separation reagents (*5*), enzyme mimics (*6*), and photochemical sensors (*7*), etc. Bis-cyclodextrin derivatives can greatly enhance the original binding ability and molecular recognition of parent cyclodextrins through the cooperative binding of two adjacent hydrophobic cavities. In addition, the functional linker group introduced in bridged bis-cyclodextrins can not only adjust the distance and orientation of two cyclodextrin cavities in cyclodextrin dimers to affect the penetration depth of guest molecules but also supply additional binding ability toward accommodated guest upon inclusion complexation. Owing to these potential advantages, a variety of dimeric cyclodextrins with different functional linkers have been designed and synthesized to understand the multiple recognition mechanisms of cyclodextrin dimers and to mimic the "multimode, multipoint" binding often observed in the biological systems (*8*−*10*). Among the various families of organic and biological guests for these studies, oligopeptides have attracted considerable interest because they represent an intermediate step toward the recognition of protein surfaces, and the short peptide sequences are themselves worthwhile targets for recognition due to their potential applications in separation, diagnostic, or biological areas (*11*, *12*). Recently, a number of works on the recognition and separation of peptides by cyclodextrins have been published (*13*−*16*). Schneider reported a detailed analysis on the inclusion complexation of some di- and tripeptides with amino cyclodextrins, demonstrating the simultaneous presence of complexes with a peptide phenyl unit approaching from both the narrow and the wide side of the cyclodextrin cavity (*17*). Breslow et al. reported a series of elegant works on the molecular recognition of oligopeptides by cyclodextrin cavities, which showed that the cyclodextrin dimers could strongly associate with the oligopeptides through the cooperative binding of two hydrophobic cavities (*18*). Inoue et al. reported the chiral recognition thermodynamics of dipeptides with native *γ*-cyclodextrin and discussed the effects of the length, size, and flexibility of the tether connecting the two aromatic moieties in a peptide on the molecular recognition (*19*). More recently, synthetic receptors bearing a 2,6-disubstituted pyridine group were proven to be one type of effective host compound for the recognition of peptide guests with a carboxylic acid terminus, since the pyridine unit can specifically bind to the carboxylic acid fragment of the peptide (*20*, *21*). However, most of the studies on the molecular recognition of peptides by cyclodextrins are carried in the neutral media, and the corresponding investigations in the acidic media are less involved (*17*, *22*). Herein, we wish to report the synthesis of a bis(*β*-cyclodextrin) with a fluorescent pyridine-2,6-dicarboxamide linker (Chart 1) and its inclusion complexation behavior with six aliphatic oligopeptides at different pH values. These studies will help us to gain a deeper insight into the recognition process of peptides in different biological environments, such as serum (pH ca. 7.3) or gastric acid (pH ca. 1.5), and consequently meet their potential applications as drug carriers.

EXPERIMENTAL PROCEDURES

Materials. All guest oligopeptides, i.e., Glu-Glu, Gly-Gly, Gly-Leu, Leu-Gly, Met-Met, and Gly-Gly-Gly (Chart 2), were commercially available and used without further

* Author for correspondence. Tel: +86-022-23503625; fax: +86-022-23503625 (or 4853). E-mail: yuliu@public.tpt.tj.cn.

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Chart 1

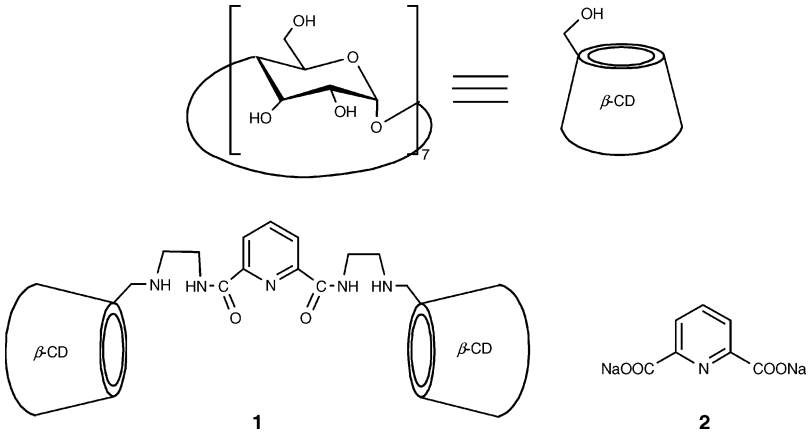
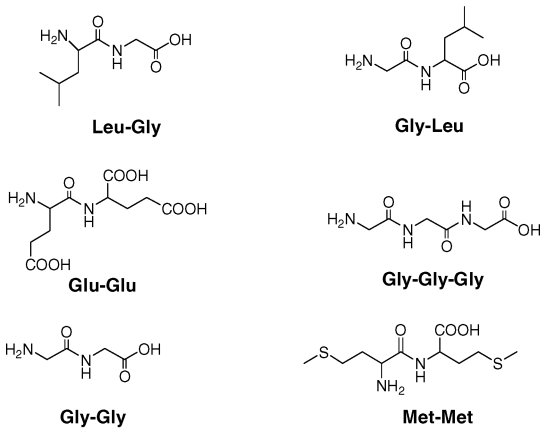


Chart 2



purification. Pyridine-2,6-dicarboxylic acid was purchased from Alfa Aesar. β-Cyclodextrin of reagent grade was recrystallized twice from water and dried in vacuo at 95 °C for 24 h prior to use. *N*,*N*-Dimethylformamide (DMF) was dried over calcium hydride for 2 days and then distilled under reduced pressure prior to use. Mono-[6-*O*-(*p*-toluenesulfonyl)]-β-cyclodextrin (6-OTs-β-cyclodextrin) was prepared by the reaction of tosyl chloride with β-cyclodextrin in an alkaline aqueous solution according to the literature reports (*23*). Then, 6-OTs-β-cyclodextrin was converted to mono[6-(2-aminoeythylamino)-6-deoxy]-β-cyclodextrin in 70% yield on heating in excess ethylenediamine at 70 °C for 7 h (*24*).

Measurements. Elemental analyses were performed on a Perkin-Elmer-2400C instrument. NMR spectra were recorded on a Varian Mercury VX300 instrument. UV and circular dichroism (CD) spectra were performed on a Shimadzu UV 2401 spectrophotometer and a JASCO J-715S spectropolarimeter, respectively. Fluorescence spectra were measured in a conventional rectangular quartz cell (10 × 10 × 45 mm) at 25 °C on a JASCO FP-750 spectrometer equipped with a constant-temperature water bath, with the excitation and emission slits width of 10 nm. In the spectral measurements, disodium hydrogen phosphate dodecahydrate (25.79 g) and sodium dihydrogen phosphate dihydrate (4.37 g) were dissolved in 1000 mL of deionized water to make a 0.10 M aqueous phosphate buffer solution of pH 7.2, whereas 20 mM potassium chloride was adjusted to pH 2.0 with 1 M hydrochloric acid to give an acidic buffer solution (*25*), which were used as solvent for all measurements.

Synthesis of Pyridine-2,6-dicarboxamide-Bridged Bis(β-cyclodextrin) (1). To a solution of DMF (50 mL) containing 2.6 g of mono[6-(2-aminoeythylamino)-6-deoxy]-β-cyclodextrin and 1.24 g of dicyclohexylcarbodiimide (DCC) was added 0.16 g of pyridine-2,6-dicarboxylic acid in the presence of a small amount of 4 Å molecular sieves. The reaction mixture was stirred for 3 d in an ice bath and another 4 d at room temperature. The precipitate was removed by filtration, and the filtrate was poured into 300 mL of acetone. The white precipitate was collected and subsequently purified on a Sephadex G-25 column with deionized water as eluent. After drying in vacuo, a pure sample was obtained in 4% yield. ^{1}H NMR (D_2O, TMS, ppm) δ 2.5−3.0 (m, 8H), 3.1−4.0 (m, 84H), 4.9 (m, 14H), 7.9 (m, 3H); FT-IR (KBr) ν/cm^{-1} 3330, 3064, 2928, 1702, 1615, 1573, 1434 1368, 1301, 1239, 1155, 1079, 1031, 945, 847, 757, 707, 580, 529, 504, 411. Anal. Calcd for $C_{95}H_{153}O_{70}N_5 \cdot 8H_2O$: C, 43.40; H, 6.48; N, 2.66. Found: C, 43.53; H, 6.27; N, 2.94.

RESULTS AND DISCUSSION

Conformational Analysis. Over the past decades, circular dichroism (CD) spectrometry has become a convenient and widely employed method for the elucidation of the absolute conformation of chiral compounds (*26*). Moreover, achiral compounds can also show the induced circular dichroism (ICD) signal in the corresponding transition band in cases where there is a chiral microenvironment. Possessing an inherent chiral cavity, cyclodextrins can certainly provide such a microenvironment for the attached achiral moiety. In this context, we have measured the CD spectra of **1** at 1.0 × 10^{-4} mol dm^{-3} concentration in aqueous buffer solutions (Figure 1) to investigate the conformation of this β-cyclodextrin dimer with a chromophoric pyridine linker.

As shown in Figure 1a, host **1** displays quite different CD spectra in the two buffer solutions of different pH values considered in this study, which indicates that there should exist significant but different degrees of interaction between the linker group and the cyclodextrin cavity. The CD spectrum of **1** shows a strong positive Cotton effect peak around 213 nm ($\Delta\epsilon$ = 3.91 dm^3 mol^{-1} cm^{-1}) in a pH 7.2 buffer but displays a moderate negative Cotton effect peak around 220 nm ($\Delta\epsilon$ = −1.54 dm^3 mol^{-1} cm^{-1}) and a weak negative Cotton effect peak around 270 nm ($\Delta\epsilon$ = −0.14 dm^3 mol^{-1} cm^{-1}) in a pH 2.0 buffer. In the control experiment, the UV spectrum of the reference compound **2**, disodium pyridine-2,6-dicarboxylate, shows a peak around 272 nm and a shoulder around 220 nm, while the CD spectrum of **2** only exhibits a weak positive Cotton effect peak around 219 nm ($\Delta\epsilon$ = 0.1 dm^3 mol^{-1} cm^{-1}) assigned to the ^1L_a transition of the pyridine chromophore. According to the empirical rule proposed

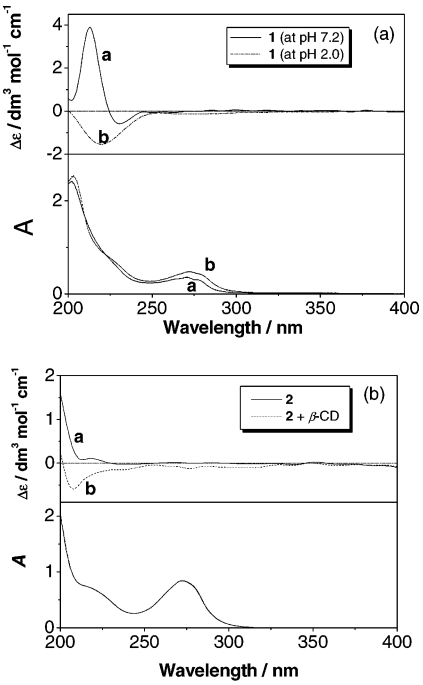


Figure 1. (a) Circular dichroism and UV spectra of host **1** (1.0×10^{-4}mol dm^{-3}) in the pH 7.2 and 2.0 buffers at 25 °C. (b) Circular dichroism and UV spectra of **2** (1×10^{-4}mol dm^{-3}) in the absence and presence of β-cyclodextrin (20 equiv.) in the pH 7.2 buffer at 25 °C.

by Kajtár (*27*), and Harata (*28*), 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 cyclodextrin. For the chromophore located inside the cyclodextrin cavity, its electronic transition parallel to the cyclodextrin axis gives a positive ICD signal, whereas the perpendicular transitions gives a negative signal, but this situation is reversed for the chromophore located outside the cyclodextrin cavity. So we can deduce that the pyridine group in the linker of host **1** may be located outside the cyclodextrin cavity in a pH 7.2 buffer, where the transition moment of ^1L_a band around 213 nm is nearly perpendicular to the cyclodextrin axis and thus induces the positive CD signal. On the other hand, in a pH 2.0 buffer, the pyridine chromophore may be shallowly included in the cyclodextrin cavity, where both the ^1L_a and ^1L_b transition moments around 220 and 270 nm are nearly perpendicular to the cyclodextrin axis, resulting in the two negative Cotton effect peaks. As a good reference system, the CD spectrum of **2** in the presence of excess amount of β-cyclodextrin is also measured. As can be seen in Figure 1b, compound **2** displays similar, but weak, CD signals to those of bis(β-cyclodextrin) **1** in a pH 7.2 buffer. However, in the presence of β-cyclodextrin, the CD spectrum of **2** shows two negative Cotton effect peaks around 208 and 277 nm, respectively, which is like the spectrum of **1** in a pH 2.0 buffer. In addition, the fluorescence spectra of **1** in the pH 7.2 and 2.0 buffers also provide some useful information. As can be seen in Figure 2, bis(β-cyclodextrin) **1** emits stronger fluorescence in a pH 2.0 buffer than in a pH 7.2 buffer, which indicates that the pyridine chromophore in **1** is certainly located in a more hydrophobic environment in the pH 2.0 buffer. The results of the CD and fluorescence experiments jointly indicate that there exist some interactions between the pyridine chromophore in the linker group and the cyclodextrin cavity, which consequently supports our hypothesis about the self-inclusion conformation of **1** in an acidic media.

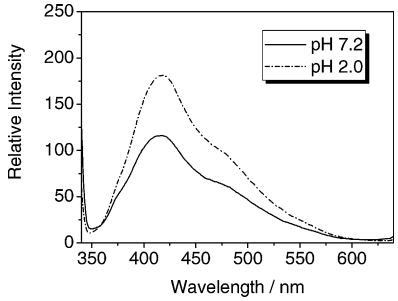


Figure 2. Fluorescence spectra of **1** (1.0×10^{-4} mol dm^{-3}) in the pH 7.2 and 2.0 buffers.

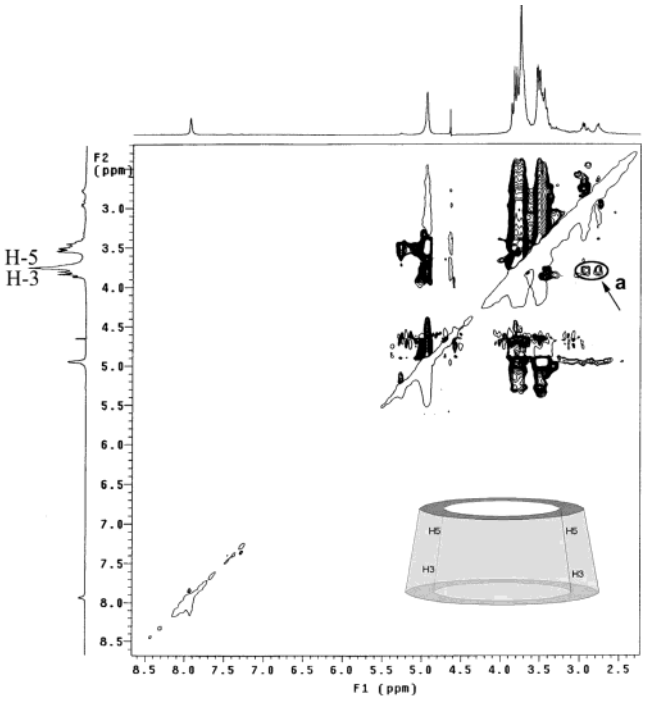


Figure 3. 2D ROESY spectra of host **1** (5 mM) in pD 7.2 buffer at 298 K with a mixing time of 400 ms.

2D NMR experiment provides reliable information about the conformation of bis(β-cyclodextrin) **1** in a pH 7.2 buffer. As illustrated in Figure 3, the ROSEY spectrum of **1** displays clear NOE cross-peaks between the H-5 protons of cyclodextrin and the ethylene protons of the linker group (peaks a). On the other hand, no NOE correlations between the inner protons (H-3 and H-5) of the cyclodextrin moieties and the pyridine protons can be observed. These phenomena indicate that only the ethylene unit of the linker group is shallowly embedded in the cyclodextrin cavity, but the pyridine group is located at the exterior. From the above CD, fluorescence, and NMR results, we can draw the conclusion that the conformation of **1** switches according to the pH value of the buffer solution as represented in Figure 4. Unfortunately, the ROSEY signals of **1** at pD 2.0 are too ambiguous to examine the NOE correlations between the pyridine unit and the H-3/H-5 protons of cyclodextrin, which may be due to the protonation of the pyridine unit, although the mechanism is still unclear. However, we can still get some useful information about the self-inclusion conformation of host **1** at pH 2.0 from the ^{1}H NMR spectra. Seen from the structure of host **1**, one can deduce that its self-inclusion conformation at pH 2.0 should result in the existence of a degenerated chemical-exchange process, i.e. the pyridinium ring included in one or the other cyclodextrin cavity, which would result in

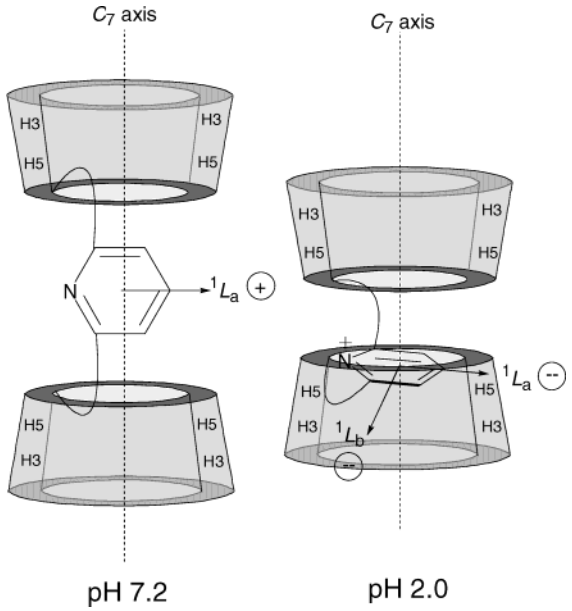


Figure 4. Possible conformation of bis(β-cyclodextrin) **1** in pH 7.2 and 2.0 buffers.

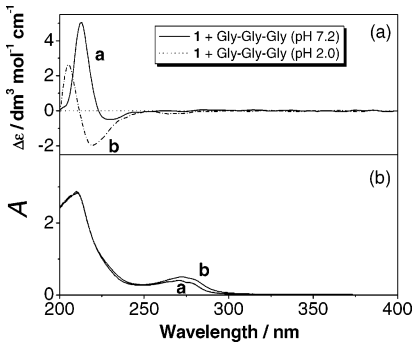


Figure 5. Circular dichroism (a) and UV (b) spectra of host **1** (1.0×10^{-4} mol dm^{-3}) in the presence of Gly-Gly-Gly (6.0×10^{-4} mol dm^{-3}) in the pH 7.2 and 2.0 buffers at 25 °C.

the significant line broadening in the ^{1}H NMR spectra unless this exchange is very rapid. Therefore, the obvious line broadening of the pyridinium protons in **1** at pD 2.0 clearly indicates the self-inclusion conformation of **1** in the acidic media.

Binding Mode. In our previous researches, we found that bridged bis(β-cyclodextrin) hosts could adopt different binding modes upon cooperative association with a given guest molecule, leading to either a sandwich or a coinclusion complex (*29*). In this work, we examine the CD spectra of bis(β-cyclodextrin) **1** in the presence of Gly-Gly-Gly to investigate its binding mode at different pH values. The reason for choosing Gly-Gly-Gly as guest molecule is that it displays no appreciable CD signals in the range of 200−400 nm. As seen from Figure 5, in either the pH 7.2 or the pH 2.0 buffer, the CD spectrum of **1** remains unchanged upon addition of Gly-Gly-Gly, but the Cotton effect intensities increase significantly. The enhanced Cotton effects ($\Delta\Delta\epsilon = 1.13$ dm^3 mol^{-1} cm^{-1} for pH 7.2 buffer and $\Delta\Delta\epsilon = 0.43$ dm^3 mol^{-1} cm^{-1} for pH 2.0 buffer) indicate that **1** has associated with Gly-Gly-Gly to form a host−guest complex. The fact that **1** exhibits similar pH dependent CD signals in the absence and in the presence of Gly-Gly-Gly supports that it adopts binding modes upon inclusion complexation that are compatible with the above-discussed host conformations. In a pH 7.2 buffer, bis(β-cyclodextrin) **1** may adopt a sandwich binding mode, where the guest molecule is cooperatively bound by two cyclodextrin cavities (Figure 6). However, in a pH 2.0 buffer, bis(β-cyclodextrin) **1** probably adopts a coinclusion binding mode; that is, the linker group is partly self-included in one of the hydrophobic cavities, while the guest molecule penetrates into the other cavity (Figure 6).

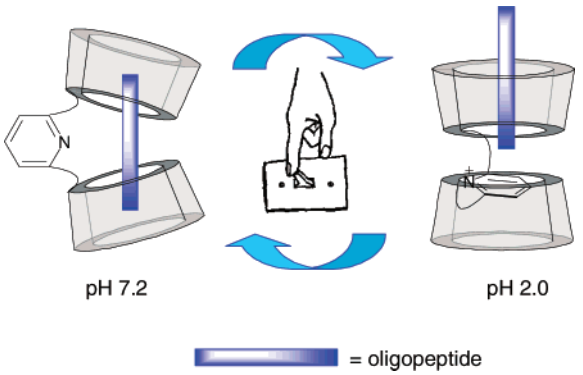


Figure 6. Possible binding mode of bis(β-cyclodextrin) **1** with oligopeptides.

The NMR data further reveal these binding modes in more detail. Since the chemical shifts of the isopropyl protons in Leu fragment are distant from those of the protons in bis(β-cyclodextrin) **1** and can be easily recognized in NMR spectra, we select Gly-Leu as guest to examine the binding geometry of the bridged bis(cyclodextrin) **1** with the guest oligopeptides. As illustrated in Figure 7, the ROESY spectrum of an equimolar mixture of **1** with Gly-Leu displays clear NOE cross-peaks between the H-3 protons of cyclodextrin and the methyl protons (H-5′) in Gly-Leu (peak a) as well as those between the H-5 protons of cyclodextrin and the methine proton (H-4′) of Gly-Leu (peak b). In addition, the initial strong correlation between the ethylene protons in the linker group of bis(cyclodextrin) **1** and the H-5 protons of cyclodextrin (peak a in Figure 3) disappears after the addition of guest oligopeptide. These results jointly indicate that the guest Gly-Leu is accommodated in the cyclodextrin cavity and the linker group is entirely driven out after the guest inclusion, which confirms the cooperative sandwich binding mode between host and guest. On the other hand, the appreciable line broadening of the pyridinium protons in **1** at pD 2.0 in the presence of Gly-Leu indicates that the pyridinium group is still self-included in the cyclodextrin cavity even after host−guest complexation, which consequently verifies the coinclusion binding mode as illustrated in Figure 6.

Spectral Titration. Quantitative investigation of the complexation behavior of **1** with the selected oligopeptides has been examined at pH 7.2 and 2.0 by means of titration fluorimetry. The fluorescence intensity of **1** gradually increases with the stepwise addition of oligopeptides (Figure 8). As expected, all of the continuous variation (Job) plots show the maximum at a molar fraction of 0.5, confirming the formation of 1:1 host−guest inclusion complexes for either the sandwich or the coinclusion binding mode. (Figure 9)

Using a nonlinear least squares curve-fitting method (*30*), we obtain the complexation stability constant for each host−guest combination. The excellent curve fits support the reliability of the stability constants obtained. The stability constant (K_s) and Gibbs free energy change ($-\Delta G°$) for the inclusion complexation of host bis(β-cyclodextrin) **1** with a series of oligopeptide guests are listed in Table 1.

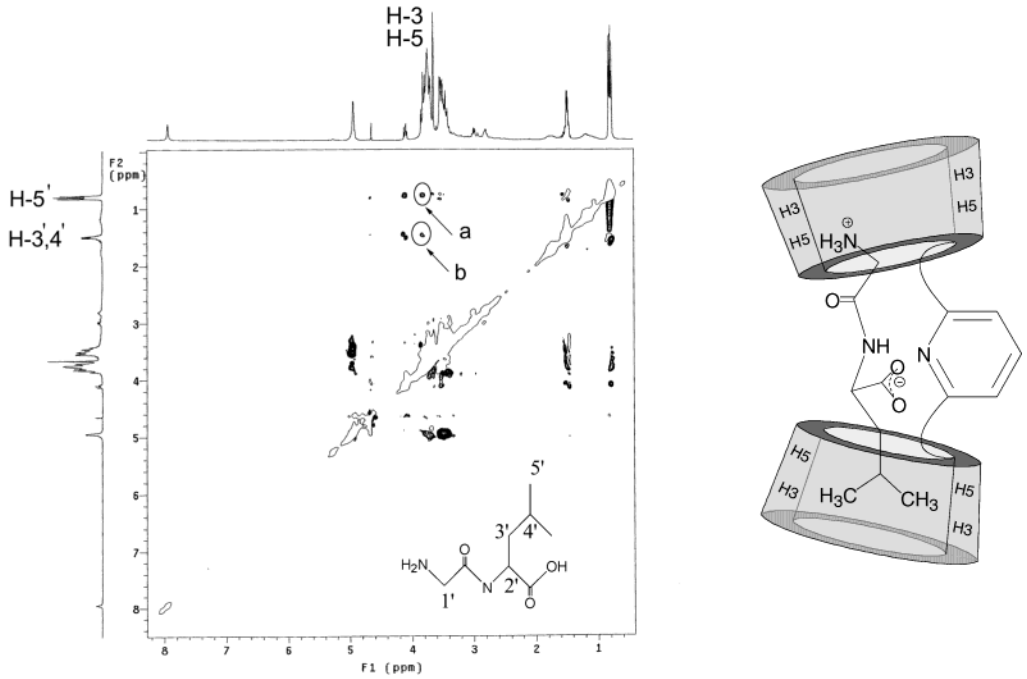


Figure 7. (left) 2D ROESY spectrum of mixture of host **1** (5 mM) and Gly-Leu (5 mM) in pD 7.2 buffer at 298 K with a mixing time of 400 ms, (right) possible binding mode of host **1** with Gly-Leu.

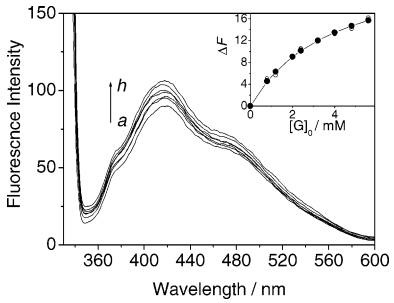


Figure 8. Fluorescence spectral changes bis(*β*-cyclodextrin) **1** (0.08 mM) upon addition of guest Gly-Gly-Gly (0−5.6 mM from *a* to *h*) at pH 7.2 and the nonlinear least-squares analysis (inset) of the differential intensity (Δ*F*) to calculate the complex stability constant (K_s). The excitation wavelength is 330 nm.

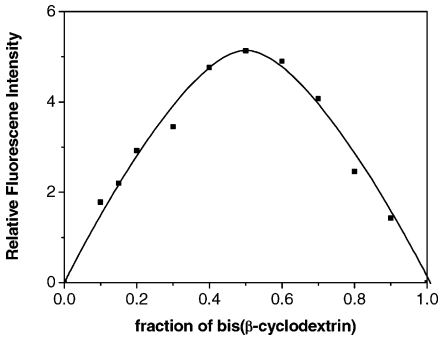


Figure 9. Continuous variation plot of the **1**/Gly-Gly system ([bis(*β*-cyclodextrin)] + [Gly-Gly] = 1.0×10^{-4} mol dm^{-3}) in a pH 2.0 buffer.

Molecular Binding Ability and Molecular Selectivity. It is well-known that several weak interactions, including van der Waals, hydrophobic, and hydrogen bond interactions, simultaneously contribute to the inclusion complexation of cyclodextrins with guest molecules. Additionally, other intermolecular interactions, such as electrostatic interaction and electron transfer, can also affect the binding behavior of cyclodextrins to some extent. In the present case, we find that the host−guest size/shape matching and induced fit dominate the stability of the complexes formed between host **1** and the assayed oligopeptides.

Table 1. Complex Stability Constant (K_s) and Gibbs Free Energy Change ($-\Delta G°$) for 1:1 Inclusion Complexation of Host 1 with Various Oligopeptide Guests in Aqueous Buffer Solution (pH 7.2 and 2.0) at 25 °C

pH	oligopeptide	Ks/M^{-1}	log K_s	$-\Delta G°$/kJ mol^{-1}
7.2	Glu-Glu	320 ± 10	2.50	14.3
2.0	Glu-Glu	153 ± 3	2.18	12.5
7.2	Gly-Gly	832 ± 30	2.92	16.7
2.0	Gly-Gly	339 ± 10	2.53	14.4
7.2	Gly-Leu	1208 ± 40	3.08	17.6
2.0	Gly-Leu	517 ± 20	2.71	15.5
7.2	Leu-Gly	356 ± 10	2.55	14.6
2.0	Leu-Gly	330 ± 10	2.52	14.4
7.2	Met-Met	700 ± 30	2.84	16.2
2.0	Met-Met	641 ± 20	2.81	16.0
7.2	Gly-Gly-Gly	263 ± 10	2.42	13.8
2.0	Gly-Gly-Gly	72 ± 3	1.86	10.6

From Table 1, we can see that the corresponding K_s value for a given oligopeptide guest at pH 7.2 is higher than that at pH 2.0. This observation is consistent with the cooperative contributions of two cyclodextrin cavities on binding with one guest. On the other hand, for the coinclusion mode, the guest molecule is only bound by one cyclodextrin, which will inevitably lead to weaker interactions between host and guest. Among the guest oligopeptides, bis(*β*-cyclodextrin) **1** displays the strongest binding ability toward Gly-Leu and Met-Met, both of which possess a carboxyl group located in the middle of the peptide chain. It is known that the diamidopyridine group can interact with the carboxyl group and thus effectively recognizes complementary peptide guests (*20*, *31*). In our case, this interaction rationalize not only the strong associations of bis(*β*-cyclodextrin) **1** with guest Gly-Leu and Met-Met but also the high sequence selectivity of **1** for Gly-Leu as compared with Leu-Gly ($K_s^{Gly-Leu}/K_s^{Leu-Gly} = 4.2$ at pH 7.2). On the other hand, although Glu-Glu has a similar structure to Met-Met, its poor hydrophobicity arising from the presence of three hydrophilic carboxyl groups in the molecule reduces the

hydrophobic interactions upon complexation with the cyclodextrin cavities and thus results in the moderate K_s value. Another interesting point is that, although possessing a relatively long linker group, bis(β-cyclodextrin) **1** exhibits a higher K_s value for the shorter Gly-Gly than for the longer Gly-Gly-Gly ($K_s^{Gly-Gly}/K_s^{Gly-Gly-Gly} = 4.7$ at pH 2.0), which may be attributed to the size−fit relationship between host and guest. That is, for a coinclusion mode in which the guest is only associated with one cyclodextrin cavity, the shorter guest Gly-Gly (skeleton length 7.246 Å, estimated by MM2) is fits better to the height of the cyclodextrin cavity (7.9 Å) than Gly-Gly-Gly (skeleton length 10.87 Å, estimated by MM2).

Protonation Effect. Because of the relatively low pK_a value of the pyridine group (pK_a ca. 5.4) and the ethylenediamino group (pK_a ca. 3.2), all of the N atoms in the linker group of **1** should be protonated in a pH 2.0 buffer. On the other hand, for the selected guest oligopeptides, the pK_a values of the carboxyl groups are around 3 (*22a*), while the corresponding values for the amino terminus are around 8 (*32*), which indicates that the C-terminals of the guests should exist as COOH the N-terminals as NH_3^+ at pH 2.0. That is to say, in an acidic media (pH 2.0), both host **1** and oligopeptide are positively charged, which will inevitably lead to unfavorable electrostatic repulsions between host and guest. However, this negative contribution will be partly compensated by the hydrogen bonds between the carboxyl, amino, and/or sulfydryl groups of guest peptides and the hydroxyl groups of the cyclodextrin. As a cumulative result of the hydrophobic, electrostatic and hydrogen bond interactions, the differential Gibbs energy changes for the complexation of bis(β-cyclodextrin) at different pH values ($\Delta\Delta G° = \Delta G°_{pH\ 7.2} - \Delta G°_{pH\ 2.0}$) vary in a narrow range (0.2−3.2 kJ/mol). This phenomenon indicates that the protonation effect can only affects the binding ability of bis(β-cyclodextrin) **1** with oligopeptides to a limited extent.

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