Articles

The Synthesis and Characterization of a Pyridine-Linked Cyclodextrin Dimer

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A cyclodextrin dimer has been synthesized in an overall 29% yield from β-cyclodextrin. This compound contains a pyridine bridge that links the secondary faces of two cyclodextrin units. A complete ^{1}H and ^{13}C NMR analysis was performed using a combination of one- and two-dimensional NMR pulse sequences. The results are consistent with the notion that each β-cyclodextrin moiety contains seven spectrally unique sugar residues. In addition, an analysis of key coupling constants suggest that the modified sugar moiety in the individual cyclodextrin units has undergone a chair inversion. This analysis represents the first complete NMR characterization of a cyclodextrin dimer. Finally, the binding properties of the cyclodextrin dimer for several guest molecules are reported. To the best of our knowledge, the association constant obtained with ethyl orange ($1.6 \pm 0.2 \times 10^7$) represents the largest formation constant ever reported for a dye molecule with a cyclodextrin-based host.

The relatively recent emergence of cyclodextrin dimers as water-soluble encapsulating agents offers new opportunities in the fields of molecular recognition and drug delivery.[1–12] These compounds possess the structural characteristics to completely encapsulate a guest molecule, shielding the bound species from the surrounding aqueous environment. As such, they serve as the unimolecular equivalent of a micelle, yet one in which the physical and chemical properties can be fine-tuned through the judicious incorporation of appropriate functionality. In conjunction with our work on self-assembling enzyme mimics,[13–15] we have recently synthesized several cyclodextrin dimer variants. However, the characterization of supramolecular complexes in which cyclodextrin dimers play a key role is critically dependent upon a complete analysis of the structural attributes associated with the cyclodextrin component itself. Perhaps no technique is more important in this regard than NMR, since chemical shift patterns serve as a direct assessment of the mode of cyclodextrin-guest interactions. Unfortunately, an analysis of the one-dimensional ^{1}H NMR spectrum of the parent cyclodextrins (i.e. α, β, γ) or their symmetrically modified derivatives can be problematical due to the extensive overlap of chemical shifts. This impediment is rendered even more formidable in cyclodextrin dimers, since the symmetry which embraces the parent cyclodextrins has been destroyed and the individual saccharide units are now spectrally unique. Fortunately, with the advent of two-dimensional pulse sequences, these complex oligosaccharide-containing systems can be analyzed in an unambiguous fashion. We describe herein the synthesis, subsequent characterization (^{1}H and ^{13}C NMR), and binding properties of a pyridine-linked β-cyclodextrin dimer. To the best of our knowledge, this report represents the first complete NMR characterization of a cyclodextrin dimer, a member of a family of compounds that has attracted increasing attention in recent years.

Experimental Section

All chemicals were obtained from commercial sources. ^{1}H NMR experiments were performed at 500 MHz for the cyclodextrin dimer **1**. Chemical shifts are reported with respect to TSP [the sodium salt of 3-(trimethylsilyl)proprionic-2,2,3,3-d_4 acid]. For the 3,5-bis(mercaptomethyl)pyridine (**7**), ^{1}H NMR and ^{13}C NMR experiments were performed at 400 and 75.5 MHz, respectively, and are reported relative to TMS (tetramethylsilane).

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Table 1. ^{1}H NMR Chemical Shifts (δ) for Compound 1. Ring and Atom Designations are Given in Figure 4[a]

ring	H-1	H-2	H-3	H-4	H-5	H-6
A	4.82	3.81	3.05	3.79	4.17	3.72
B	5.02	3.53	3.82	3.45	3.85	3.79
C	5.02	3.58	3.91	3.55	3.85	3.78
D	5.10	3.66	3.81	3.61	3.73	3.74
E	5.09	3.54	3.90	3.55	3.71	3.71
F	5.05	3.58	3.91	3.53	3.85	3.79
G	4.96	3.58	3.89	3.54	3.92	3.85

[a] Pyridine bridge: CH_2 4.01, pyridine (H-2) 8.42, pyridine (H-4) 7.93.

Table 2. ^{13}C NMR Chemical Shifts (δ) for Compound 1. Ring and Atom Designations are Given in Figure 4[a]

ring	C-1	C-2	C-3	C-4	C-5	C-6
A	103.7	73.5	50.3	78.3	76.5	59.2
B	101.4	71.3	73.3	80.9	70.8	60.4
C	101.8	71.3	73.3	80.9	70.8	60.4
D	102.1	71.3	73.3	80.9	70.8	60.4
E	102.1	71.3	73.3	80.9	70.8	60.4
F	101.6	71.3	73.3	80.9	70.8	60.4
G	101.2	71.3	73.3	80.9	70.8	60.4

[a] Pyridine bridge: CH_2 33.5; pyridine ring 134.5 (C-3), 138.2 (C-2), 147.6 (C-3).

Synthesis of 3,5-Bis(mercaptomethyl)pyridine (7). Thiourea (180 mg, 2.4 mmol) was dissolved in 95% ethanol (6 mL). 3,5-Bis(chloromethyl)pyridine (**5**) (200 mg, 1.2 mmol) was added to this solution and the reaction mixture was subsequently heated to reflux under an Ar atmosphere. After 3 h, a 5 N NaOH solution (2 mL) was added and reflux was continued for an additional 2 h. The reaction mixture was allowed to cool to room temperature, neutralized (1 N HCl), and then extracted with CH_2Cl_2. The organic extracts were combined and dried, (Na_2SO_4), and the solvent was removed *in vacuo* to provide an orange colored oil (100 mg, 48.8% yield). This material was immediately reacted with the cyclodextrin epoxide **4** (see below). ^{1}H NMR (CD_3OD): 8.43 (d, 2H, aromatic H at C2 and C6, $J = 2.1$ Hz), 7.91 (d, 1H, aromatic H at C4, $J = 2.1$ Hz), 3.84 (s, 4H, methylene protons). ^{13}C NMR (CD_3OD): 147.8, 139.4, 137.3, 25.3. Chemical ionization-MS *m/e* calculated for $C_7H_9NS_2$ = 171.284; found: 171.273 (M^+).

Synthesis of Cyclodextrin Dimer 1. The following protocol is based on a procedure described by Breslow and his colleagues:[16] In a 25 mL three-neck round-bottomed flask fitted with a condenser and magnetic stirring bar, the cyclodextrin epoxide **4** (200 mg, 0.18 mmol) was dissolved in aqueous $NaHCO_3$ (10 mL, 0.1 M). To the latter was added a solution of 3,5-bis(mercaptomethyl)pyridine[17] (15 mg, 0.09 mmol, 1 of mL of CH_3OH). The reaction mixture was stirred, under Ar, for 24 h at 60 °C. The solution was allowed to cool, and the desired product was isolated by sequential chromatography on G-25 sephadex and G-15 sephadex gel filtration columns. In each case the fractions containing the cyclodextrin dimer were identified by thin-layer chromatography (5:4:3 *n*-butanol:ethanol:water as eluent, silica gel plates; using a carbohydrate spray for visualization: anisaldehyde/ethanol/H_2SO_4/HOAc and subsequent heating on a hot plate) and combined, and the water was removed via lyophilization. The final chromatography furnished 170 mg (78.7% yield) of a white solid that exhibited a single peak by HPLC (Waters μBondapak C_{18} column; a 55 min linear gradient of 100% H_2O to 45% H_2O/55% CH_3CN; UV detector set at 273 nm). NMR data: see Tables 1 and 2. Fast atom bombardment-MS *m/e* calculated for $C_{91}H_{146}N_1O_{68}S_2$ = 2406.3; found: 2405.5 (M^+).

NMR Methods. All NMR spectra were recorded at 25 °C. A sample of **1** (8 mM in D_2O) was contained within a 5 mm NMR tube and was degassed before each experiment. The HDO peak was suppressed by single frequency irradiation. All two-dimensional (2D) spectra were symmetrized after 2D-Fourier transformation (FT). Total correlation spectroscopy (TOCSY) experiments were performed employing a previously described pulse sequence[18] at three mixing times (20, 45, and 75 ms); 256 experiments of 64 scans each with a relaxation delay of 1.5 s were recorded. The data were zero-filled to a 2K × 2K matrix and processed with a shifted sine-bell function. 1D TOCSY spectra were recorded by exciting the anomeric protons of the cyclodextrin units with a selective pulse and recording the magnetization transfer at three or four different mixing times. 2D nuclear Overhauser enhancement spectra (NOESY)[19] were collected at two mixing times (100 and 150 ms). For each mixing time, 256 experiments of 128 scans each were performed. The data was then processed as described for the TOCSY experiments. The 2D H−H correlated spectroscopy (COSY) data was obtained in the absolute intensity mode. Data sets consisting of 256 t1 increments (64 scans/t1) were collected and zero filled to a 1K × 1K matrix. The heteronuclear multiple quantum coherence (HMQC) spectrum was obtained with an inverse probe. The data set consisted of 256 t1 increments (64 scans/increment) with a delay of 2 s. The data was zero filled to a 4K × 2K matrix before FT. 1D NOE difference spectra were obtained by subtracting the spectra obtained with the decoupler placed on each of the proton resonances from the spectra acquired with the decoupler set on the base line. The power was adjusted to perturb a narrow band of the selected proton resonance.

Determination of Formation Constants for Compounds 8−10 with the Cyclodextrin Dimer 1. The fluorescence of the complexes of **1** with **8** (measured at 442−447 nm; excited at 318 nm), **9** (measured at 447−454 nm; excited at 320 nm), and **10** (measured at 447−454 nm; excited at 320 nm) was monitored via a fluorescence spectrophotometer equipped with a thermostated cell compartment maintained at 25 °C. All measurements were performed with quartz cuvettes. The stock solutions of **8−10** contained 500 μM guest in 200 mM pH 7.0 phosphate buffer. Two stock solutions of the β-cyclodextrin dimer **1** (1.0 mM and 2.0 mM in 200 mM phosphate buffer at pH 7.0) were employed. The order of addition of the component solutions were buffer (200 mM phosphate, pH 7.0), cyclodextrin dimer stock, and guest stock. The final concentration of guests was 1 μM. The cyclodextrin dimer concentration was varied from 1 μM up to saturation (i.e. no further change in fluorescence intensity). The resultant mixtures were preincubated for 5 min at 25 °C prior to fluorescence measurements. All measurements were performed in quadruplicate. Formation constants were computed by the scatchard treatment.[20]

Determination of the Formation Constants for Compounds 11 and 12 with the Cyclodextrin Dimer 1. The formation constants of **1** with **11** and **12** were obtained by monitoring the decrease in the fluorescence (measured at 447−454 nm; excited at 320 nm) of the **1•10** complex with increasing concentrations of either **11** or **12**. The data was treated as described in Connors (*vide infra*).[21] All measurements were obtained at 25 °C and were performed with quartz cuvettes. The stock solutions of **11** and **12** contained 200 μM guest in 200 mM pH 7.0 phosphate buffer. The stock solutions for the cyclodextrin dimer (200 μM) and **10** (200 μM) contained a 200 mM pH 7.0 phosphate buffer. The order of addition of the component solutions were buffer (200 mM phosphate, pH 7.0), **10** stock, cyclodextrin dimer stock, and guest stock. The final concentration of guests **11** and **12** was varied from 5 to 15 μM. For guest **11**, the final concentrations of cyclodextrin dimer and **10** were 1 and 100 μM, respectively. For guest **12**, the final concentrations of cyclodextrin dimer and **10** were 1 and 200 μM, respectively. The resultant mixtures were preincu-

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bated for 5 min at 25 °C prior to fluorescence measurements. All measurements were performed in quadruplicate. The formation constants for compounds **11** and **12** with the cyclodextrin dimer were extracted from the data obtained in this experiment using the following analysis:

The indicator I (**10**) as well as the substrates S (**11** and **12**) form complexes with the cyclodextrin dimer (CD) according to the following equilibria:

$$I + CD \rightleftharpoons I{-}CD \quad (1)$$

$$S + CD \rightleftharpoons S{-}CD \quad (2)$$

The binding constants for these equilibria are given by

$$K_{I-CD} = \frac{[I{-}CD]}{[I][CD]} \quad (3)$$

$$K_{S-CD} = \frac{[S{-}CD]}{[S][CD]} \quad (4)$$

Although K_{I-CD} (i.e. the association constant for the formation of **10**−CD) can be determined by standard analysis, K_{S-CD} (i.e. the association constants for the formation of **11**−CD and **12**−CD) is so large that the low concentrations required for S (in order to obtain an accurate measure of K_{S-CD}) preclude direct spectrophotometric analysis. However, the competitive equilibrium given by eq 5 generates the formation constant K_{com}. From the latter, the desired binding constant K_{S-CD} can be extracted.

$$I{-}CD + S \rightleftharpoons S{-}CD + I \quad (5)$$

$$K_{com} = \frac{[S{-}CD][I]}{[I{-}CD][S]} = \frac{K_{S-CD}}{K_{I-CD}} \quad (6)$$

The mass balance expressions are

$$S_t = [S] + [S{-}CD] \quad (7)$$

$$CD_t = [CD] + [S{-}CD] + [I{-}CD] \quad (8)$$

$$I_t = [I] + [I{-}CD] \quad (9)$$

[I], [S−CD], [I−CD], and [S] can be expressed in easily measurable quantities. K_{com} is obtained under conditions in which $I_t \gg CD_t$. Therefore $[I] = I_t$ and $[I{-}CD] = CD_t$. When the cyclodextrin is saturated with indicator the maximum fluorescence is observed and is given by

$$(F_{I-CD})_{max} = k_{I-CD} Q_{I-CD} CD_t \quad (10)$$

where F_{I-CD} is the observed fluorescence, Q_{I-CD} is the quantum yield, and k_{I-CD} is a constant. The saturation fraction (f) is provided in eq 11.

$$f = \frac{F_{I-CD}}{(F_{I-CD})_{max}} = \frac{[I{-}CD]}{CD_t} \quad (11)$$

Consequently, $[I{-}CD] = fCD_t$. In addition, [CD], [I−CD], and [S−CD] can be expressed in terms of readily obtained values from eqs 3, 7, and 8, respectively.

$$[CD] = \frac{[I{-}CD]}{[K_{I-CD}][I]} = \frac{fCD_t}{K_{I-CD} I_t} \quad (12)$$

$$[I{-}CD] = CD_t - [CD] - [I{-}CD] = CD_t - \frac{fCD_t}{K_{I-CD} I_t} - fCD_t \quad (13)$$

$$[S{-}CD] = S_t - [S{-}CD] \quad (14)$$

Substituting for all of the terms, except [S−CD], in eq 6, we obtain

$$K_{com} = \frac{I_t [S{-}CD]}{fCD_t(S_t - [S{-}CD])} \quad (15)$$

which can be rearranged into an equation for a straight line.

$$[S{-}CD] = K_{com} \frac{CD_t}{I_t} f(S_t - [S{-}CD]) \quad (16)$$

A plot of [S−CD] (eq 16) versus $f(S_t - [S{-}CD])$ yields a straight line with a slope of $K_{com}(CD_t/I_t)$. K_{S-CD} can be calculated from K_{11} (eq 6).

Results and Discussion

In the last few years, there has been a dramatic increase in the number of papers reporting the synthesis and analyzing the behavior of novel cyclodextrin dimers.[1−12] These species have been described in a variety of contexts, including their unusually high affinity for hydrophobic molecules[6] as well as their apparent ability to serve as membrane-bound receptors.[9] Nevertheless, as might be expected for such a relatively recent innovation, the potential applications of cyclodextrin dimers to biomimetic chemistry, drug delivery, and other areas has yet to be fully realized. Recently, we described the self-assembly of heme-containing protein mimics.[13−15] These species contain a heme moiety noncovalently associated with two β-cyclodextrin derivatives. In this context, it is apparent that cyclodextrin dimers may be of potential utility in the construction of metalloprotein models as well. We now report the synthesis of a pyridine-linked cyclodextrin dimer **1**. Unlike the majority of cyclodextrin dimers that have been reported to date, this species contains individual cyclodextrin units that are covalently attached to one another via their secondary faces. We have previously noted that this type of arrangement provides a hydrophobic groove that circumscribes the metal binding site of an appropriately ensconced metal-containing ligand.[13−15] In addition to the synthesis of **1**, we also describe the first complete NMR characterization of a cyclodextrin dimer. Finally, we report the results of an initial survey of the affinity of **1** for various guest compounds.

The synthesis of the pyridine-linked cyclodextrin dimer **1** is outlined in Scheme 1. The cyclodextrin epoxide **4** was synthesized from β-cyclodextrin **2** by monotosylation of a single C2 alcohol via D'Souza's protocol.[22] The resultant tosylated β-cyclodextrin was subsequently heated in aqueous base to furnish the desired epoxide using the procedure described by Breslow.[16] The dimercapto pyridine **7** was obtained from the corresponding dichloro pyridine **6**[17] by treatment of the latter with thiourea and subsequent hydrolysis (Scheme 2). Epoxide ring opening with **7** produced the cyclodextrin dimer in 78.7% yield (an overall 29% yield from β-cyclodextrin).

As noted above, the cyclodextrin dimer has been designed to serve as a key component in the self-assembly synthesis of various metalloprotein mimics. The solution characterization of such molecular assemblies is often conducted via NMR. Indeed, the structural elucidation

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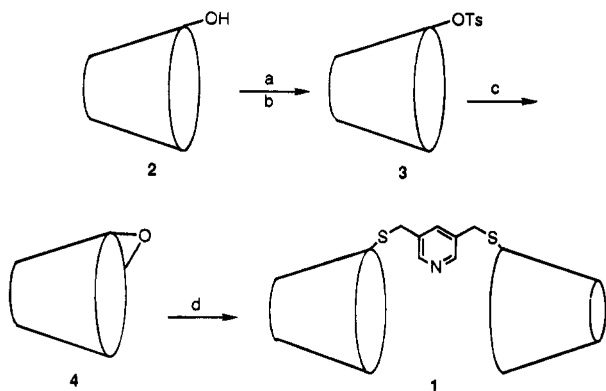
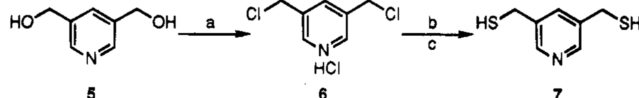
Scheme 1.[a] Synthesis of the Cyclodextrin Dimer 1 from β-Cyclodextrin



[a] (a) NaH in DMF at room temperature with stirring overnight; (b) p-toluenesulfonyl chloride at room temperature for 2 h (38% yield);[22] (c) 0.2 M $NaHCO_3$ at 60 °C for 5 h (96% yield);[16] (d) 3,5-bis(mercaptomethyl)pyridine (7) in 0.1 M $NaHCO_3$ at 60 °C for 24 h (78.7% yield).

Scheme 2.[a] Synthesis of 3,5-Bis(mercaptomethyl)pyridine (7)



[a] $SOCl_2$ heated to reflux for 30 min (100%);[17] (b) thiourea in 95% ethanol heated to reflux for 3 h; (c) 5 N NaOH heated to reflux for 2 h (48.8% yield).

of supramolecular entities containing several noncovalently-bound components can be typically achieved by observing changes in the chemical shifts of key nuclei. While 1 offers several structural features that should prove to be particularly useful in the construction of metalloprotein mimetics, it also presents an intricately complex ^1H NMR spectrum. This is not too surprising, given the fact that each cyclodextrin component in 1 bears seven spectrally unique sugar rings. Indeed, with the exception of Stoddart's nearly complete structural analysis of methylated β-cyclodextrin derivatives,[23] we are unaware of any unabridged NMR characterization of a chemically modified cyclodextrin. With these features in mind, and given the potential importance of chemical shift data in the analysis of structurally elaborate cyclodextrin-based inclusion complexes, we report the first complete NMR characterization of a cyclodextrin dimer.

The ^1H NMR spectrum of 1 is shown in Figure 1. The integrated intensities from the spectrum are consistent with the number and type of protons in the cyclodextrin dimer. The spectrum exhibits the expected patterns for the various groups of protons contained within 1: (1) the aromatic pyridine protons (7.5–8.0 ppm), (2) the CH_2 protons (as a broad singlet at 3.95 ppm), (3) the anomeric protons (i.e. H1 between 4.5–5.2 ppm), and (4) a group of resonances spread between 3–4 ppm for the H2, H3, H4, H5, and H6 protons in the sugar units of the cyclodextrin. A careful analysis of this 1D spectrum reveals that there are seven resolved H1 resonances. This indicates that the seven individual saccharide units associated with each cyclodextrin are spectrally unique

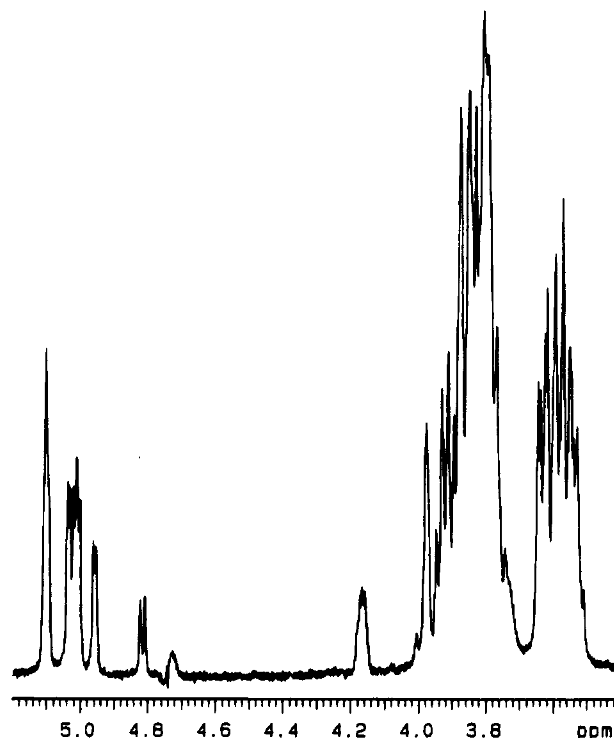


Figure 1. The 1D ^1H NMR spectrum (3.4–5.2 ppm) of the cyclodextrin dimer 1 at 500 MHz. The resonances for H3 of ring A and the aromatic protons are not shown.

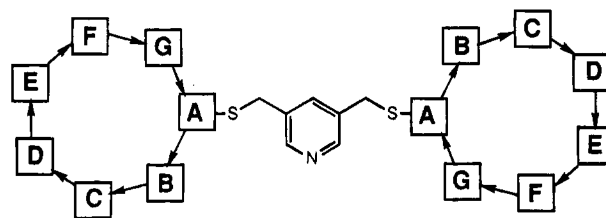


Figure 2. A schematic representation of the spectrally unique saccharide residues contained within 1. The residues are linked from C1 to C4 in a clockwise fashion. The cyclodextrin moiety is viewed from the secondary face.

(Figure 2). In addition, this result, along with the single peak for the CH_2 group, suggests that the two β-cyclodextrin halves of the dimer are spectrally indistinguishable.

Proton assignments were carried out in two steps: (1) intraresidue assignments were derived from TOCSY, COSY, and 1D-TOCSY experiments and (2) sequential residue connectivity information was obtained from NOE-difference spectra. Intraresidue assignments for H1, H2, and H3 were readily acquired from TOCSY/COSY spectra. In addition, all of the protons (i.e. H1, H2, H3, H4, H5, and H6) associated with the modified residue were easily identified as well (see TOCSY spectrum in Figure 3). In contrast, the chemical shift assignments for the H4, H5, and H6 protons of the six other unmodified residues in the cyclodextrin moiety were more difficult to extract. The resonances for these protons were eventually assigned from 1D slices taken from the TOCSY spectrum and were confirmed by data acquired from the 1D TOCSY spectra. The latter demonstrated successive magnetization transfer from H1 through H6 on the individual saccharide units, with increasing mixing time. Although this provides a complete assignment of the chemical shifts of the protons within a sugar

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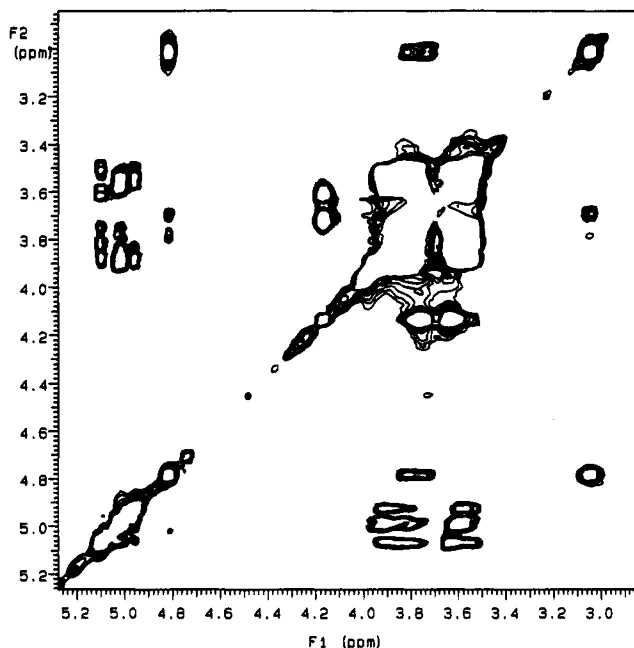


Figure 3. Total correlation (TOCSY) spectrum of compound **1**. The cross peaks from 3.0 to 4.0 ppm show the magnetization transfer from H1 → H2 → H3 → H4.

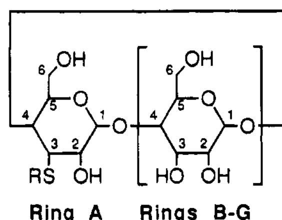


Figure 4. Ring and atom designations for Tables 1 and 2.

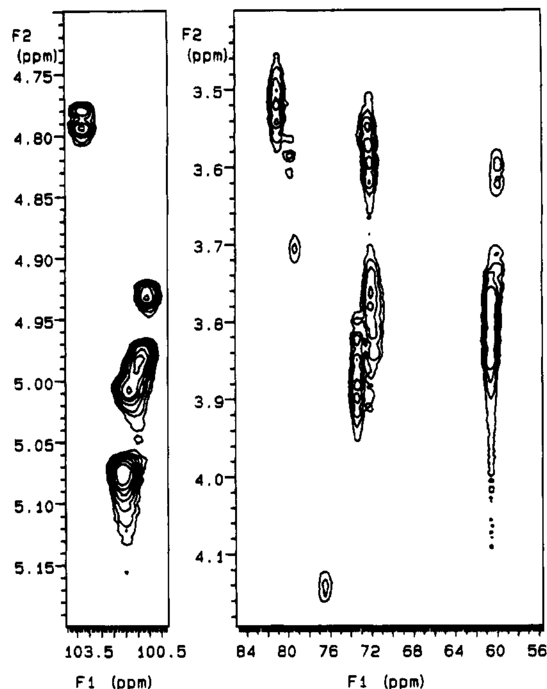
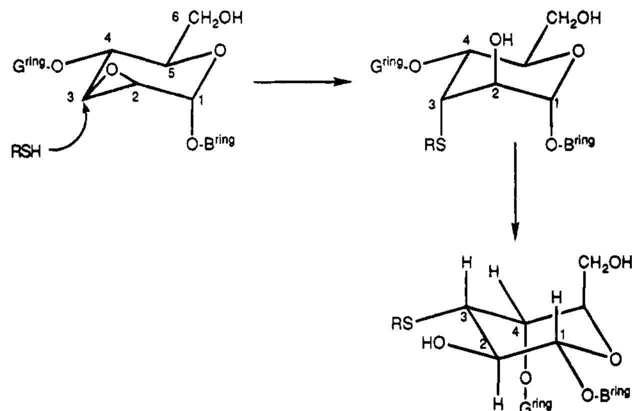


Figure 5. Heteronuclear multiple quantum coherence (HMQC) spectrum of **1**. The left panel shows the H1–C1 correlation, whereas the right panel provides all other H–C correlations (except for the aromatic and methylene nuclei).

Scheme 3. Conformational Analysis of the A Ring in Compound 1



residue, it does not furnish the assignments for a specific residue (i.e. residues A–G). The latter were established via 1D NOE experiments. Irradiation of the H1 proton, in addition to providing NOE enhancements of the H2 and H3 protons within the individual residue, also leads to an enhancement at H4 on the adjacent residue.[23] Consequently, we were able to assign the connectivity pattern among the seven sugar rings in the β-cyclodextrin. All assignments are provided in Table 1. Ring and atom designations are indicated in Figure 4.

With the proton chemical shifts in hand, we then derived the carbon chemical shifts from the HMQC spectra (Figure 5). These assignments are summarized in Table 2. Clearly, the resonances associated with the modified residue (i.e. residue A) exhibit a distinct shift relative to their counterparts in the unmodified sugar residues. Furthermore, with the exception of the anomeric carbon (i.e. C1) chemical shifts, all of the resonances in the B–G residues are invariant. In contrast, the proton chemical shifts are more sensitive to the subtle chemical differences among the individual residues. For example, the resonances associated with the anomeric protons are increasingly shifted downfield as one moves further away from the modified residue.

Breslow and Czarnick previously suggested that nucleophilic attack on the cyclodextrin epoxide at C3 proceeds from the axial direction and from inside the cyclodextrin cavity.[24] Under such circumstances, one might expect the C3 proton to assume an equatorial arrangement in the final product (see Scheme 3). However, these investigators reported a coupling constant of 11.2 Hz involving the proton at C3, implying that this hydrogen atom occupies an axial position. The interpretation offered by Breslow and Czarnick is that the modified ring has undergone either a chair inversion or a distortion to a nonchair conformation.[24] This appears to be the case in compound **1** as well. The J_{12} values for the seven H1 peaks fall into two groups: six residues have a J_{12} of 3.6 Hz, while the modified residue exhibits a J_{12} of 8.6 Hz. This suggests that both H1 and H2 in the modified residue lie in the axial orientation, which is consistent with the notion that the modified residue has assumed an inverted chair form. Indeed, H3 appears as a doublet of doublets in the 1D ^{1}H NMR spectrum. The two coupling constants are 8.2 and 3.2 Hz. The

(24) Breslow, R.; Czarnik, A. W. *J. Am. Chem. Soc.* **1983**, *105*, 1390–1391.

Table 3. Association Constants (± standard deviation; M^{-1}) for Cyclodextrin Dimer 1 and β-Cyclodextrin with Various Guest Molecules

guest	β-cyclodextrin[a] K_1	β-cyclodextrin[a] K_2	cyclodextrin dimer **1** K
8	2080		$1.6 \pm 0.1 \times 10^4$
9	1980	600	$2.0 \pm 0.1 \times 10^4$
10			$3.5 \pm 0.1 \times 10^5$
11	2970	606	$6.1 \pm 0.6 \times 10^6$
12	9030	388	$1.6 \pm 0.2 \times 10^7$

[a] Association constants for **8** and **9** are from ref 26 and those for **11** and **12** are from ref 4. The association constants for guests **8**, **9**, **11**, and **12** with β-cyclodextrin were obtained at 25 °C and pH 7.0.

former is due to H3–H2 coupling, which is compatible with an arrangement in which H3 is axial. Finally the 3.2 Hz coupling constant, which arises from H3–H4 coupling, is consistent with a geometry that contains one axial hydrogen and one equatorial hydrogen. Since we have established that H3 is axial, this implies that H4 must occupy the equatorial arrangement (see Scheme 3). These results are in agreement with the premise that the conformation of the two modified residues in **1** are inverted relative to their unmodified counterparts.

We have performed a preliminary survey of the affinity of **1** for several common guest molecules (**8**–**12**). Compounds **8**–**10** all exhibit a sharp enhancement in fluorescence upon interaction with hydrophobic entities in aqueous solution. As expected, these compounds exhibit analogous behavior in the presence of **1** as well. We found, using Job's method,[25] that all three guest molecules form 1:1 inclusion complexes with the cyclodextrin dimer (data not shown). From a Scatchard analysis, we obtained the association constants provided in Table 3. The binding constants affiliated with the cyclodextrin dimer are larger than those previously reported for β-cyclodextrin alone.[4,26] For compounds **8** and **9**, the binding enhancement is approximately 1 order of magnitude. In contrast, the binding enhancements for compounds **11** and **12** are significantly larger (more than 3 orders of magnitude greater than the 1:1 complex formed with β-cyclodextrin). Indeed, to the best of our knowledge, the binding constants obtained with the methyl and ethyl orange derivatives are the largest ever reported for any dye molecule with a cyclodextrin-based host. This observation is particularly intriguing since, in **11** and **12**, one of the two rings bears a hydrophilic substituent. Consequently, it seems likely that, through the rational design of guest molecules, even larger association constants can be achieved (for example, see ref 6). In addition, the binding constants reported in Table 3 are of potential utility in determining formation constants for species that exhibit even greater affinities for **1** than compounds **8**–**12**. For example, competition experiments involving various guest molecules in the presence of the fluorescent species **10** have been employed by Breslow and his colleagues to measure association constants as large as 10^8.[6]

We examined the interaction between methyl orange (**12**) and the host **1** by NOE difference spectroscopy in order to confirm that inclusion complex formation with the cyclodextrin dimer does occur. This experiment was performed with both host and guest present at a concentration of 6.3 mM. On the basis of the experimentally-

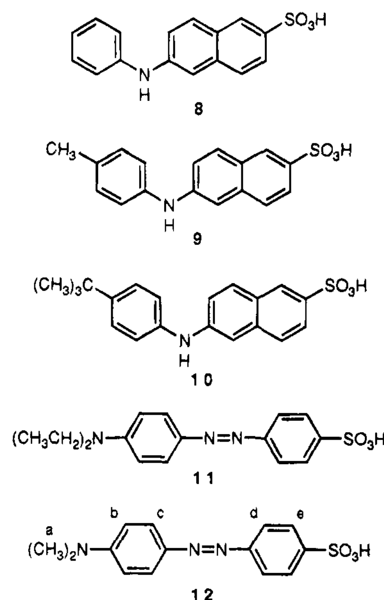
(25) Job, P. *Ann. Chim. Phys.* **1928**, *9*, 113–203.
(26) Catena, G. C.; Bright, F. V. *Anal. Chem.* **1989**, *61*, 905–909.

Table 4. Nuclear Overhauser Enhancements for the Methyl Orange (12)–Cyclodextrin Dimer (1) Inclusion Complex[a]

observed guest protons	irradiated host protons H-2	H-3	H-4	H-5
a	0	5.2	0	7.4
b	0	1.9	0	5.5
c	0	6.2	0	5.5
d	0	4.7	0	7.8
e	0	3.8	0	8.5

[a] The experiments were performed at 25 °C with [**12**] = 6.3 mM and [**1**] = 6.3 mM. The NOEs for the methyl orange protons were obtained by irradiating the resonances corresponding to the H-2, H-3, H-4, and H-5 of the cyclodextrin component. The values (±1%) are the percent enhancement in the integration associated with the methyl orange protons. The atom assignments for **12** are provided in Chart 1.

Chart 1. Guest Compounds 8–12



derived binding constant, under these conditions essentially all of the host and guest in solution are present as the host–guest complex. As is apparent from Table 4, irradiation of the H-2 and H-4 resonances of the cyclodextrin dimer failed to produce any discernible effect on the integration of the proton resonances for methyl orange. This is expected since the H-2 and H-4 nuclei are located on the exterior portion of the cyclodextrin cone. In contrast, the H-3 and H-5 protons are positioned within the interior of the cyclodextrin moiety and should therefore be situated near the bound guest molecule. Indeed, irradiation of H-3 and H-5 did produce the desired nuclear Overhauser enhancements. Furthermore, we observed upfield chemical shifts for each of the protons of methyl orange in the presence of the cyclodextrin dimer (Δδ ppm: $a = 0.026$; $b = 0.109$; $c = 0.002$; $d = 0.019$; $e = 0.037$; see Chart 1 for atom designations). These results are consistent with the notion that an inclusion complex between **1** and **12** does form. What is the structural nature of this inclusion complex? Specifically, do both of the cyclodextrin components in **1** simultaneously participate in guest coordination? Certainly, the large binding constants reported in this study suggest that they do. In addition, the NOE results provided in Table 4 (as well as the chemical shift data) indicates both rings of the guest are encapsulated within a cyclodextrin moiety. Although this does not categorically confirm that both halves of the cyclodextrin dimer

simultaneously interact with both aromatic halves of the methyl orange guest, it does indirectly suggest that simultaneous interaction is the likely scenario. An alternative interpretation of the NMR data is that at any given time only one of the two aromatic rings of methyl orange is ensconced within a cavity of the cyclodextrin dimer. On the basis of the strong NOE data, this would require that a mixture be present in solution, namely, a complex in which only the dimethylamino aromatic ring is encapsulated and a complex in which only the sulfonated aromatic ring is incorporated into the cyclodextrin. If this is the case, then this implies that the cyclodextrin moiety does not significantly discriminate between the two aromatic halves of methyl orange, an unlikely premise since the sulfonated ring is negatively charged, whereas the aromatic amine component is neutral. In short, the available evidence is most consistent with the notion that the cyclodextrin dimer **1** and the methyl orange guest **12** form an inclusion complex, one in which each aromatic component of **12** is simultaneously incorporated within each cyclodextrin component of the dimer host.

In summary, we have conducted the first full ^{1}H and ^{13}C NMR analysis of a cyclodextrin dimer. In addition, we have described the affinity of this cyclodextrin dimer for various guest molecules, including impressive binding constants for the methyl and ethyl orange species. These results represent an important first step in the characterization of cyclodextrin dimer-based metalloprotein mimics. The latter studies will be reported in due course.

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