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Structure of a β-Cyclodextrin–Vanillin Inclusion Complex

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Vanillin was found to form a stable complex with β-cyclodextrin. A binding constant value of 1.11×10^4 M^{-1} was determined for the 1:1 complex. ^1H NMR chemical shift differences and circular dichroism spectra indicated that both phenolic end and aldehyde end were affected by complexation. 2D nuclear Overhauser enhancement spectroscopy indicated proximity of OCH_3 in vanillin to the anomeric protons of β-cyclodextrin. 1D difference NOE experiments have revealed that vanillin exists with the phenolic end nearer to the narrower end and the aldehyde end to the wider end of the torus-shaped β-cyclodextrin molecule.

Extensive literature is available since Freudenberg and Cramer (1948) discovered that cyclodextrin can form inclusion compounds (Saenger, 1980). β-Cyclodextrin (BCD) consists of seven glucose units in a ring with α(1→4) links

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enclosing a cavity of size 7.0 Å (Bender and Komiyama, 1978). The glucose units are in an undistorted C1 conformation. While the secondary hydroxyl groups (namely 2-OH and 3-OH) are located on the inner side facing the center, the primary hydroxyl is on the outside. Since the interior of the torus consists of CH groups and glucosidic oxygens, it is relatively apolar compared to the outside. X-ray and NMR studies were carried out to arrive at the structure of the complexes between BCD and some guest molecules (Demarco and Thakkar, 1970; Manor and Saenger, 1974).

The use of cyclodextrins in the food industry covers a range of applications. Controlling flavor release, masking odors and tastes, stabilizing emulsions, controlling or masking color, protecting ingredients from oxidation, light-induced reactions, thermal decomposition, and evaporation loss are some of the areas where cyclodextrins have found wide applications (Pszczola, 1988). In this connection several useful technological processes were developed in food industries based upon cyclodextrins. One such is the debittering of fruit juices where a polymer from cyclodextrin and epichlorohydrin is used to reduce about 45% of the naringen (bittering principle) level (Wilson et al., 1989; Spalding, 1987). Microencapsulation of flavor compounds by BCD has resulted in trapping of volatile compounds, protecting thermally unstable or reactive compounds and thereby increasing their shelf life (Reinacaus and Risch, 1986; Pagington, 1986, 1987). In order to derive information about the structure of BCD complexes with flavor molecules, vanillin was chosen as a model compound to investigate the structure by a detailed NMR study.

MATERIALS AND METHODS

BCD was purchased from Sigma and vanillin from Fluka. BCD–vanillin complex was prepared by adding equimolar amounts of vanillin to an aqueous solution of BCD and lyophilizing the solution.

Straight ^{1}H NMR spectra were recorded on a Bruker WH 270-MHz instrument fitted with a Spectrospin magnet operating at 20 ± 1 °C and an Aspect 3000 computer. About 20 mg/mL of the complex dissolved in 0.5 mL of D_2O or DMSO-d_6 was used for obtaining the spectra. Typically 20 scans were accumulated per spectrum. All other details are described elsewhere (Divakar, 1985). 1D difference NOE (nuclear Overhauser enhancement) experiments were performed by irradiating the sample first at a point far removed from the signals (off-resonance) and then at a specified frequency. The duration of irradiation was 3 s to allow sufficient NOE to build. About 64 scans were accumulated for both off-resonance and on-resonance irradiations, and the difference between these two was obtained by subtracting one spectrum from the other. NOE effects were then measured from this difference spectrum.

Binding constant values were determined from ^{1}H NMR measurements of vanillin H-5′ (ortho to phenolic OH) signal based on the method of Reuban (1973). The shifts of H-5′ were monitored when increasing amounts of BCD were added to vanillin. A Scatchard plot was constructed in which r/C_f (ratio of fraction of ligand molecules bound (r) to concentration of free ligand molecules) plotted against r, gives a curve with a slope equal to $-1/K_D$ (K_D = dissociation constant of the complex), which is the binding constant value for the complex. In such an instance $r = \Delta\nu/\rho\Delta\nu_f$, where $\Delta\nu$ = chemical shift difference (Hz) between free and complexed vanillin at a particular concentration of BCD, ρ = ratio of BCD to vanillin, and $\Delta\nu_f$ = difference in chemical shift value of H-5′ (Hz) for the highest concentration of BCD, and $r/C_f = \Delta\nu/(\Delta\nu_f - \Delta\nu)R_t$, where R_t = concentration of BCD. A binding constant value of $1.11 \times 10^4 \pm 1800$ M^{-1} was determined for the vanillin–BCD complex. Also, a plot of $\Delta\nu$ versus ρ indicated a 1:1 stoichiometry for the complex formed between BCD and vanillin.

^{1}H COSY (correlated spectroscopy) and NOESY (nuclear Overhauser enhancement spectroscopy) (Wider et al., 1984) were obtained on a Bruker AM 500 NMR instrument fitted with a Spectrospin magnet and an Aspect 3000 computer. The pulse sequences used were COSY (D_1–90°–t_1–90°–FID) and NOESY (D_1–90°–t_1–90°–t_m–90°–FID). Delays were D_1 = 1 s, t_1 = variable from 3 μs in 256 increments of 144 μs (D_2O) and 106 μs (DMSO-d_6), and mixing time t_m = 500 ms. Typically 16 scans were accumulated for each trace. Quadrature detection was employed. Free induction decays were acquired over 1024 data points and 9433.9 Hz (6944.4 Hz for D_2O) for each of 256 traces. The second dimension (f_2) sweep width was 4716.9 Hz (3472.2 Hz for D_2O). The raw data were zero-filled from 256 to 512 data points in the second dimension before double Fourier transformation.

Circular dichroism (CD) spectra were recorded on a Jasco J-20 instrument at 20 °C for solutions of vanillin containing BCD in DMSO. Molar ellipticity values $[\theta]_\lambda$ were calculated from the relation $[\theta]_\lambda = 100 \cdot \theta_{deg}/c \cdot l$, where θ_{deg} = rotation (deg) at the specified wavelength, c = concentration (mol/L), and l = path length (dm).

RESULTS AND DISCUSSION

^{1}H NMR spectra of the BCD–vanillin complex in both DMSO-d_6 and D_2O are shown in Figure 1. The difference in chemical shift values on complexation in DMSO-d_6 is shown in Table I. The most affected protons are the vanillin protons. All of them show upfield shifts on complexation invariably: H-5′ exhibits a maximum shift of −0.11 ppm, H-6′ (ortho to CHO and para to OCH_3) −0.07 ppm, H-2′ (ortho to CHO and OCH_3) −0.04 ppm, CHO −0.03 ppm, and OCH_3 −0.03 ppm. Phenolic OH, though not seen in free vanillin, was observed as a broad peak in the complex.

A circular dichroism spectrum of the complex showed a positive band at 313 nm and a negative band at 279 nm (Figure 2). Neither BCD nor vanillin exhibits CD spectra in the wavelength region 350–250 nm. Due to strong absorption from DMSO, the region below 250 nm could not be examined satisfactorily. However, large molar ellipticity values were observed for the complex: $[\theta]_{313}$ = 36 000 and $[\theta]_{279}$ = 19 200 deg·cm^2·dmol^{-1}. The ultraviolet spectrum of vanillin exhibits bands at 309 nm (molar extinction coefficient, ϵ = 11 673), 270 nm (ϵ = 12 881), and 252 nm (ϵ = 7487). While the CD band at $[\theta]_{313}$ arises from an n–π* transition of the aldehyde group, the one at $[\theta]_{279}$ arises from a π–π* transition of the phenolic OH group. Observation of positive and negative bands indicated an induced CD for the complex and that complexation probably affects both the aldehyde and phenolic OH groups in terms of their relative disposition within the BCD cavity.

^{1}H COSY and NOESY spectra in both D_2O and DMSO-d_6 are given in Figure 3. The COSY spectrum of the sample in DMSO-d_6 was more informative than that in D_2O. The signals at 5.74, 5.68, and 4.47 ppm were from sugar hydroxyl protons 2-OH, 3-OH, and 6-OH (Casu et al., 1966), respectively, as confirmed by deuterium exchange experiment and observation of cross-peaks between the hydroxyl protons and the protons attached to the respective carbons (H-2, H-3, H-6a,b). The H-1 signal resonates upfield to its position in D_2O at 4.83 ppm. The hydroxyl 2-OH exhibits a small splitting (6.2 Hz; Figure 1). The peak at 3.62 ppm was found to arise from H-6a,b, H-4, and H-3 proton signals. The H-5 signal resonates at 3.55 ppm; H-2 was found to be most upfield in DMSO-d_6, resonanting at 3.31 ppm.

The NOESY spectrum of the complex in D_2O (Figure 3) did not reveal much. However, in DMSO-d_6, the NOESY spectrum (Figure 3) indicated proximity of vanillin to groups in BCD. Proximity of various groups in BCD among themselves is revealed very clearly by prom-

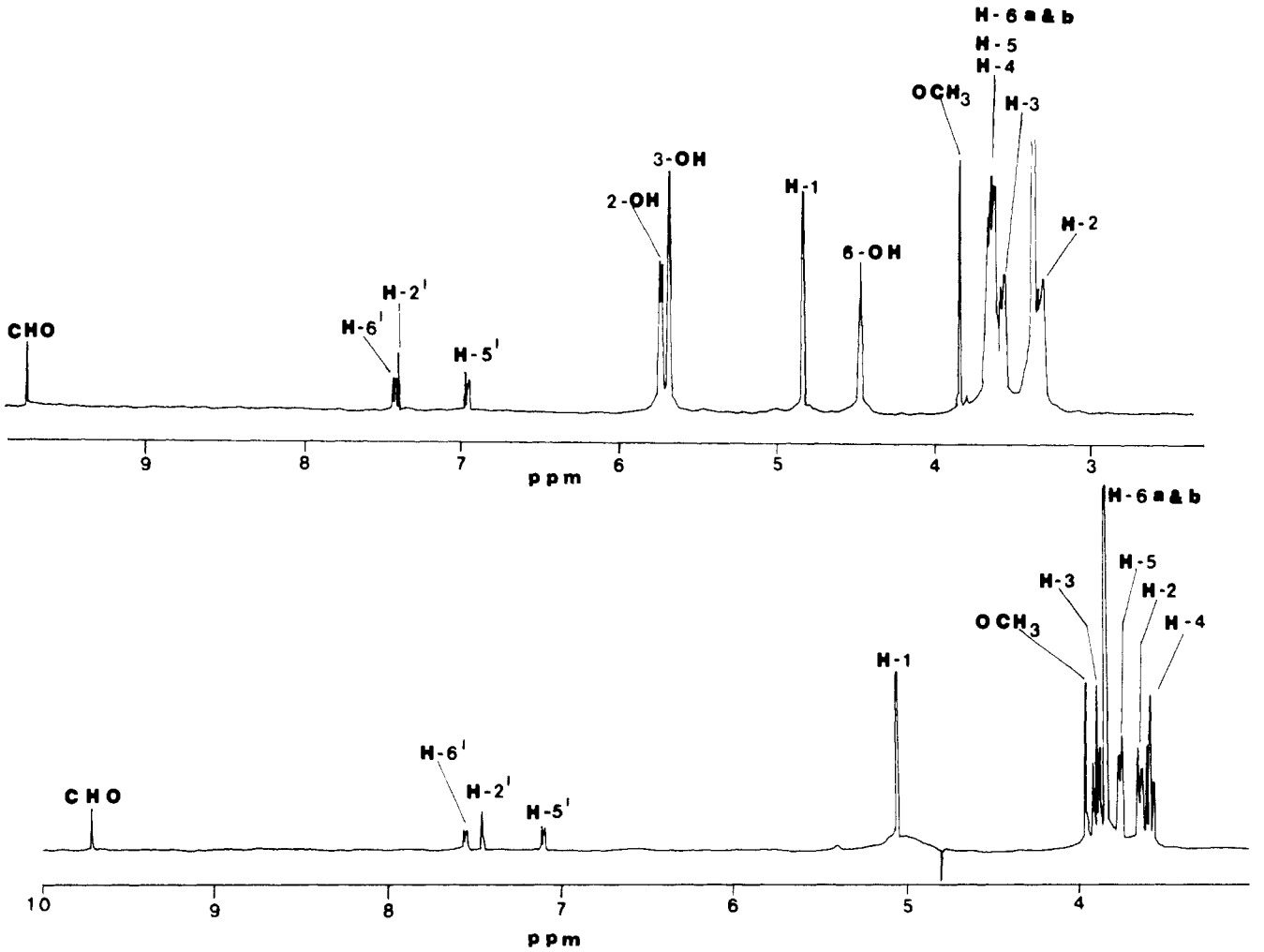

Figure 1. 270-MHz ^{1}H NMR spectra of the BCD–vanillin complex in DMSO-d_6 (upper trace) and D_2O (lower trace).

Table I. ^{1}H NMR Studies of the BCD–Vanillin Complex[a]

signal	diff in chem shift values: DMSO-d_6	diff in chem shift values: D_2O
	Vanillin	
CHO	−0.03[b]	*c*, *e*
H-6′	−0.07	*e*
H-2′	−0.04	*e*
H-5′	−0.11	*e*
OCH_3	−0.03	*e*
phenolic OH	*c*	*e*
	BCD	
2-OH	0	*c*
3-OH	−0.01	*c*
H-1	0	0.01
6-OH	−0.01	*c*
H-4	*d*	0.03
H-5	*d*	0.05
H-6a,b	*d*	−0.01
H-3	0.05[d]	−0.06
H-2	−0.04	−0.06

[a] Values were measured at 270 MHz. [b] Negative sign indicates upfield shift. [c] Could not be observed. [d] Overlapping of signals. [e] At room temperature the solubility of vanillin in D_2O is very small; hence, signals could not be observed.

inent cross-peaks. Observation of a cross-peak between the primary hydroxyl on the exterior of the BCD torus to the water indicates proximity to water molecules. The methoxy group in vanillin exhibits a faint cross-peak with H-1 from BCD, indicating that OCH_3 group is nearer to the anomeric protons in BCD cavity. Similarly, a faint cross-peak was also observed between the aromatic proton H-1′ and H-1.

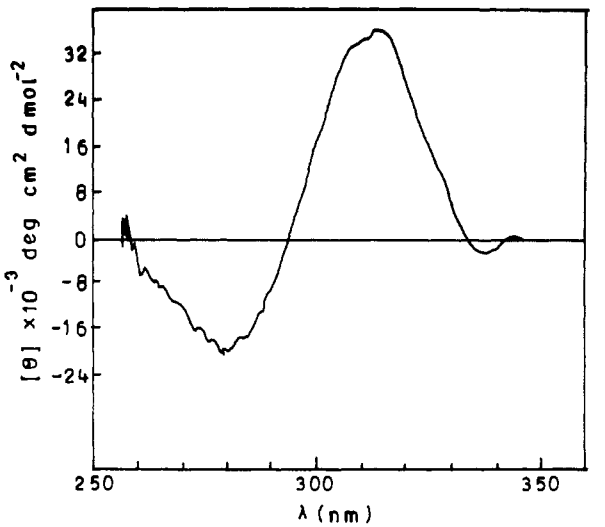

Figure 2. Circular dichroism spectra of BCD–vanillin in DMSO. Concentrations used: vanillin, 40.0×10^{-6} M; BCD, 2.5×10^{-4} M.

2D NOESY spectra revealed some details but were not very informative. This is probably because small positive NOE's found in molecules of this size are difficult to detect reliably because of sensitivity problems plagued by long relaxation delays required between the pulses (Morris, 1986). Also the relationship between NOE and τ_c (correlation time) is extremely important in determining the increase in intensity or observation of prominent NOESY cross-peaks (Wuthrich, 1971; Roberts and Jar-

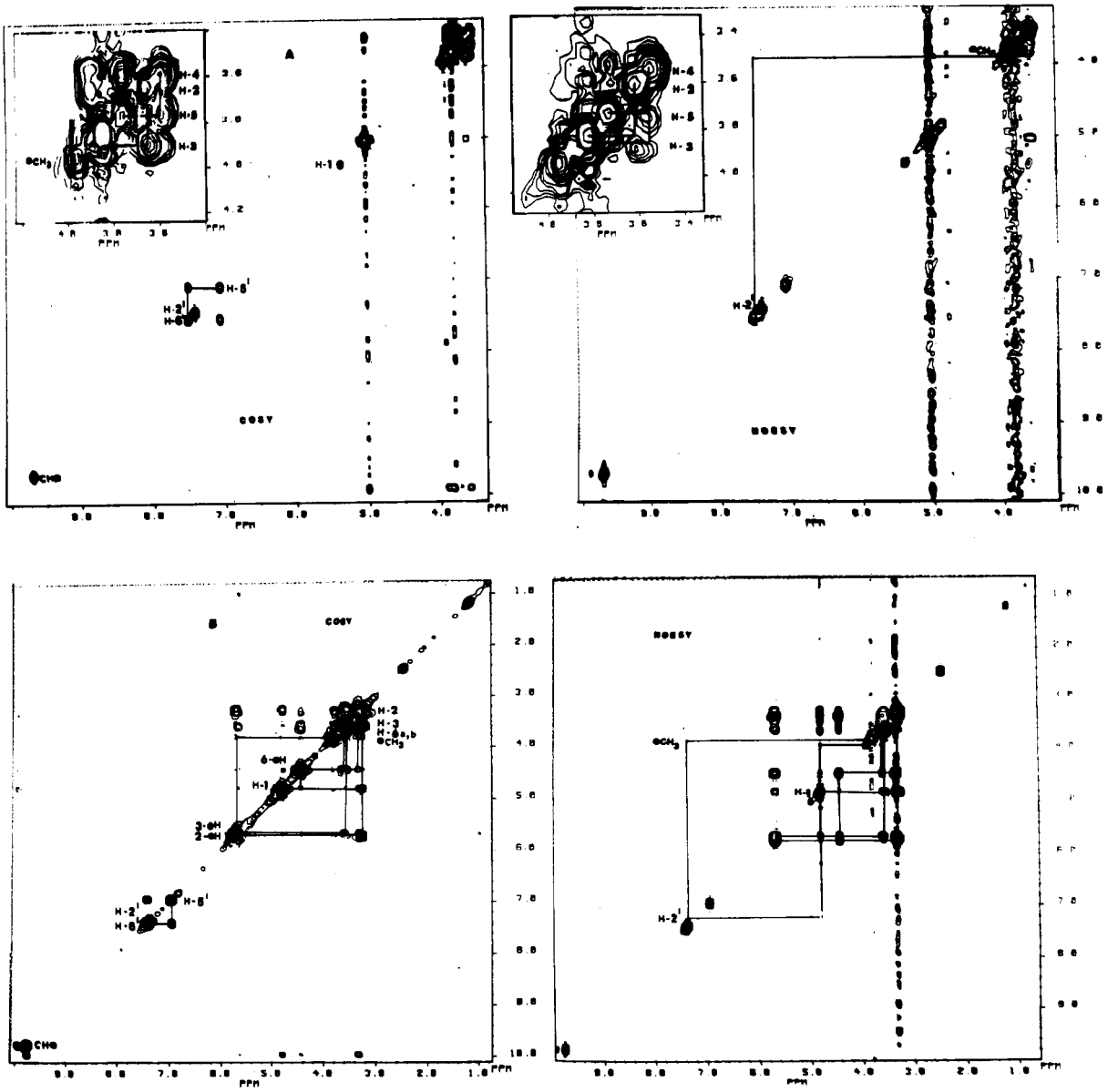

Figure 3. (A) 500-MHz 2D COSY and NOESY spectra of the BCD–vanillin complex in D_2O. The connectivities between the protons are indicated. (B) COSY and NOESY spectra in DMSO-d_6. The conditions for obtaining the spectra are given in Materials and Methods.

detzky, 1981). For $^1H\{^1H\}$ NOE a value of 0.5 can be obtained for the extreme motional narrowing situation when $\omega^2\tau_c^2 \ll 1$ (ω = precessional frequency), and it can vary up to −1 for $\omega^2\tau_c^2 \gg 1$. At 500 MHz, $\omega^{-1} = 3.1 \times 10^{-10}$ s. The BCD–vanillin complex can have a τ_c value of that order, and hence the observed NOE may be practically low. In order to overcome the problems, 1D NOE experiments were carried out at 270 MHz. Since at 270 MHz $\omega = 1.78 \times 10^9$ rad·s^{-1}, NOE's of small magnitudes can be expected.

The results from experiments carried out in DMSO-d_6 are illustrated in Table II. No attempts were made to carry out distance measurements from NOE. Only qualitative inferences were drawn. It was assumed that dipolar relaxation is the principal contributor to NOE (Noggle and Schirmer, 1971). On the 270-MHz instrument, decoupling and observing frequencies were used simultaneously during spectral accumulation. This will lead to problems in quantification if spin diffusion and other such processes contribute to relaxation (Roberts and Jardetzky, 1981).

Close examination of Table II reveals significant changes in intensities when both vanillin and BCD protons were irradiated. BCD possess a V-shaped cavity with the secondary hydroxyl side more open than the primary hydroxyl

Table II

signal irradiated	signals affected with % increase in intensity					
vanillin	2-OH	3-OH	H-2	H-1	H-4, H-5, H-6a,b	6-OH
phenolic OH[a,b]	c			5.2	3.33	
H-5′	3.17	3.33		5.1	5.3	2.83
OCH_3					3.41	
CHO			1.38	1.25		
BCD	phenolic OH	H-5′	OCH_3	H-6′	H-2′	CHO
2-OH		2.81	2.25			2.25
3-OH	1.34		2.06			2.23
H-2	2.11	2.0				1.4
H-1	2.35	2.75	1.82			
H-4, H-5,H-6a,b	2.1	2.48				1.55
6-OH		2.88	1.67			

[a] The intensity changes for all the signals are not given. When vanillin protons are irradiated, the increases in intensity observed for BCD protons are given and vice versa. [b] Negative values are observed for the BCD protons when vanillin protons are irradiated. [c] Could not be measured.

(Bender and Komiyama, 1978). BCD protons can be classified into two broad groups: H-2, H-3, 2-OH, and 3-OH protons (group 1) present at the wider part of the molecule, and H-1, H-4, H-5, and H-6a,b (group 2) present at the narrower end. Intensity changes were observed for protons within their own molecular frame when BCD or vanillin protons were irradiated where negative NOE's were observed for BCD protons and positive NOE's were observed for vanillin protons. Also, when the effect of irradiation of protons in one molecular frame (either host or guest) on the other was examined, negative NOE's were observed for BCD protons when vanillin protons were irradiated and positive NOE's were observed for vanillin protons when BCD protons were irradiated. This is probably because of two different correlation times operating for the host and guest molecules, vanillin protons exhibiting higher τ_c than BCD protons. Irradiation of vanillin protons causes an intensity increase in BCD protons from both ends. However, depending on the specific vanillin proton irradiated, protons from one of the ends of BCD exhibit more effect than the other. For example, irradiation of the phenolic OH proton affects group 2 (H-1, H-6a,b) more than group 1 (3-OH); CHO affects 2-OH, 3-OH, and H-2 more than H-1; H-5′ and methoxyl affect both groups of protons almost equally. Similarly, irradiation of BCD protons shows slightly selective enhancement in certain vanillin protons. Irradiation of H-1, H-6a,b (H-4, H-5), and 6-OH invariably affect H-5′ and phenolic OH more than H-2′, H-6′, and CHO. Hence, from these qualitative data it can be deduced that phenolic OH, OCH_3, and H-5′ are in the vicinity of group 2 protons, which constitute the narrower end of BCD, while the aldehyde is nearer to the open end. If processes like spin diffusion were operative, then the difference in distribution of intensities may not be observable.

From the foregoing studies, it is evident that the disposition of vanillin in BCD is such that the phenolic end within the BCD cavity is nearer to the narrower end and the aldehyde end and to the 2-OH and 3-OH groups at the wider end. A large number of apolar compounds were reported to form stable complexes with BCD (Bender and Komiyama, 1978). In vanillin, the phenolic end is hydrophobic and BCD possesses a slight apolar cavity and hence can accommodate the hydrophobic end with ease. Apolar binding can be favored by van der Waal's interaction and thermodynamics if accompanied by a large enthalpy change and a small unfavorable entropy change, as in the 1-adamantanecarboxylate complex with BCD where the bulky substrate is accommodated within the BCD cavity (Bender and Komiyama, 1978).

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