Molecular Complexation: β-Cyclodextrin and Benzaldehyde Inclusion Complex

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INTRODUCTION

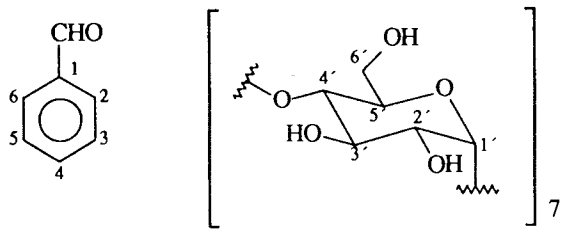
In the search for carcinostatic constituents in the steam distillate of fig fruit (*Ficus carcia*), Takeuchi *et al.* isolated benzaldehyde as the active component on the basis of its *in vivo* antitumor activities against adenocarcinoma 755 and Ehrlich carcinoma (1). However, benzaldehyde is a highly volatile substance and can be readily oxidized in the air, which presents a serious problem in administering it during clinical trials. An inclusion complex with β-cyclodextrin was thus prepared and shown to be effective in the prevention of 4-nitroquinoline-1-oxide induced papilloma in ICR mice (Scheme I) (1). The overall response was better than 50% in a clinical trial with 102 patients (2). This inclusion complex was also highly active in the inhibition of mouse pulmonary metastasis (3,4). These interesting bioactivities prompted us to study further its structural specificity and stability in solution. The *in vitro* antitumor cytotoxicity of this complex against human tumor cell lines was evaluated as well.

MATERIALS AND METHODS

Materials. β-Cyclodextrin was obtained from Chemical Dynamic Corp., South Plainfield, NJ. Benzaldehyde was obtained from Sigma Chemical Co., St. Louis, MO.

High-Performance Liquid Chromatography. A liquid chromatograph equipped with a Waters Model 440 solvent delivery system and a Waters Model ALC/GPC 202 UV absorption detector which operates at 254 nm was used. As the mobile phase system, 28% CH_3CN (acetonitrile) and 72% acetic acid (1%) solution were used. The pH of the system was adjusted to 5.5 with 2 *N* NaOH.

NMR Spectroscopy. All structural studies of complexes by ^1H NMR were recorded on a Nicolet NT-470 spectrometer with 16K computer memory operating at 469.5 MHz. The spectra were measured with a 0.5-μsec (90°) pulse width and 10-sec repetition time. DMSO-d_6 (Aldrich Chemical Co.) was used as an external reference with a signal at 3.03 ppm relative to TMS (at 0.0 ppm) for ^1H NMR spectra. A 5-mm sample tube was used. All 470-MHz ^1H NMR coupling constants and chemical shifts were calculated by the Raccoon spin simulation program.



Scheme I

Mass Spectrometry. Fast atom bombardment (FAB) mass spectra were obtained with a Kratos MS-50 sector mass spectrometer utilizing 3:1 dithiothreitol/dithioerythritol as the matrix. An accelerating voltage of 8 kV and a scanning rate of 100 sec/dec were used for the experiments.

Preparation of β-Cyclodextrin–Benzaldehyde Inclusion Complex. β-Cyclodextrin (2.702 g, 2 mmol) was dissolved in 25 ml of double-distilled water at 80°C. Nitrogen was then passed in the solution, and benzaldehyde (0.3 ml, 2.7 mmol) was added to it. This mixture was stirred and kept at room temperature overnight. The precipitated white crystals were filtered and washed with small amounts of water and ether. This complex was redissolved in double-distilled water at 50°C under nitrogen for 24 hr. This recrystallized complex was filtered and dried in low vacuum at room temperature for 24 hr to yield 2.162 g, 74.2%; mp 284°C.

The 470-MHz ^1H NMR spectral integration showed that this was a 1:1 ratio complex of β-cyclodextrin and benzaldehyde. Detailed discussion of these spectral data is given under Results and Discussion.

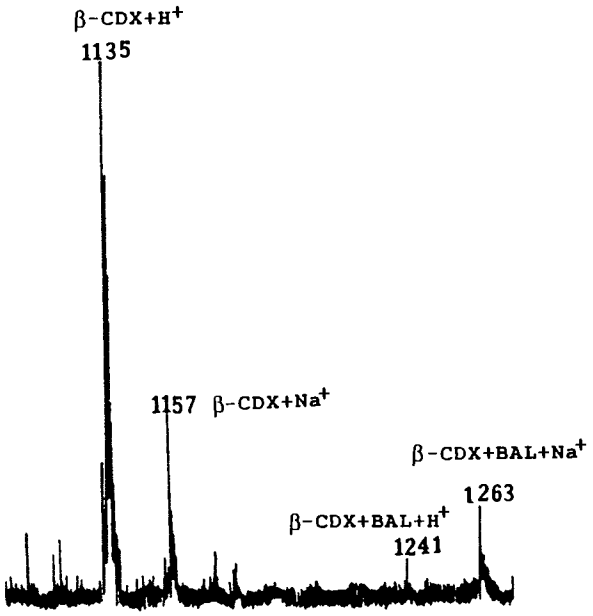


Fig. 1. Fast atom bombardment mass spectrum of β-cyclodextrin–benzaldehyde complex.

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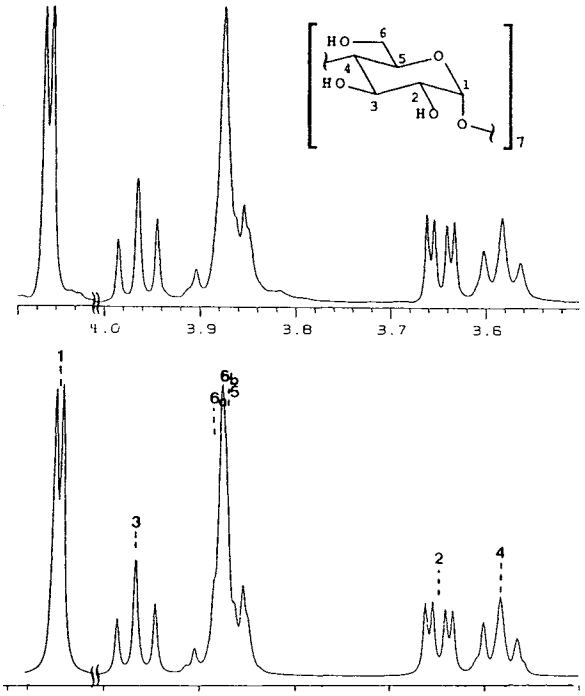

Fig. 2. ^{1}H NMR (470 MHz) spectrum of β-cyclodextrin (top) and its computer-simulated spectrum (bottom).

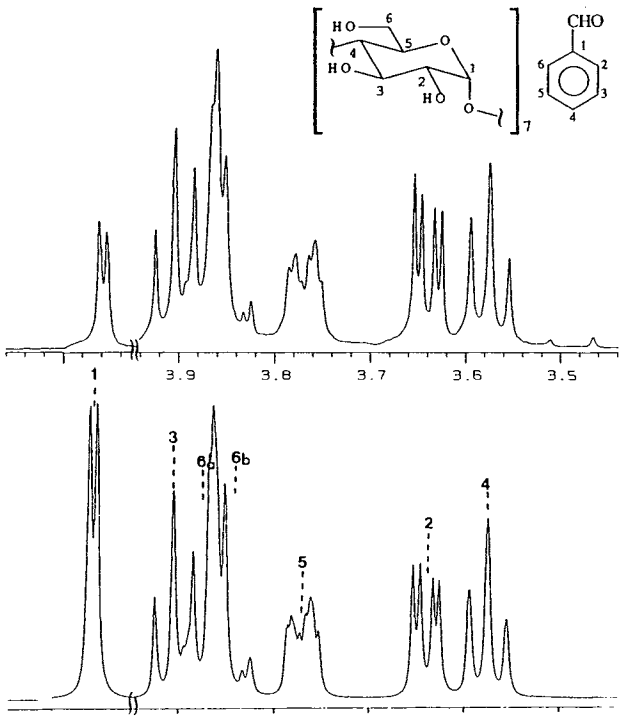

Fig. 3. ^{1}H NMR (470 MHz) spectrum of β-cyclodextrin–benzaldehyde (top) and its computer-simulated spectrum (bottom).

Autooxidation Rate Studies for Benzaldehyde and Benzaldehyde–Cyclodextrin Complexes. Benzaldehyde (10 m*M*) with an equal molar amount of β-cyclodextrins was dissolved in phosphate buffer (0.1 *M*, pH 7.0) at 70°C. The rate of autooxidation of samples was followed by HPLC. The peak area of remaining benzaldehyde from the HPLC chromatograms was used to calculate the degree of oxidation. The half-life ($t_{1/2}$) of benzaldehyde oxidation was obtained by plotting the percentage of remaining benzaldehyde versus time.

In Vitro *Antitumor Cytotoxicity Assay.* Evaluation of the *in vitro* antitumor activity was performed at the Purdue Cancer Center Cell Culture Laboratories. Compounds were examined in HT-29 (human colon adenocarcinoma) and MCF-7 (human breast adenocarcinoma) cells in 96-well microtiter plates and incubated for 6 days. The cytotoxicity was measured as ED_{50}, the effective dose at which cell growth is retarded to 50% of controlled culture using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] dye (5,6).

RESULTS AND DISCUSSION

Inclusion Complexation. Solid-state ^{13}C NMR was previously used to study the structure of β-cyclodextrin–benzaldehyde (β-CDX-BAL) complex in the solid state (7). The carbon resonances of benzaldehyde in the solid-state complex were almost identical to those in solution without β-cyclodextrin. It appears that solid-state ^{13}C NMR is un-

Table I. ^{1}H NMR (470 MHz) Chemical Shifts of Benzaldehyde Before and After Complexation with β-Cyclodextrin in pH 7.4 0.1 *M* Phosphate D_2O Buffer

Proton(s)	β-Cyclodextrin–benzaldehyde 1:1 complex (ppm)	β-CDX (ppm)	Benzaldehyde (ppm)	Difference (Hz)
CHO	9.9500		9.9350	7.05
2, 6	7.9580		7.9590	−0.47
3, 5	7.6460		7.6250	9.87
4	7.7880		7.7600	13.16
1′	5.0565	5.0690		−5.88
2′	3.6395	3.6480		−4.00
3′	3.9017	3.9640		−29.28
4′	3.5755	3.5840		−4.00
5′	3.7685	3.8637		−44.74
6a′	3.8713	3.8870		−7.38
6b′	3.8460	3.8637		−8.32

Table II. ^{1}H NMR (470-MHz) Computer-Simulated Spin Coupling Constants for β-Cyclodextrin and β-Cyclodextrin–Benzaldehyde Complex

Protons	Coupling constant (Hz) β-CDX	β-CDX-BAL
J_{12}	3.5	3.5
J_{15}	−0.6	−0.7
J_{23}	9.8	9.6
J_{34}	9.2	9.0
J_{45}	9.4	9.4
J_{46a}	−0.7	−0.5
J_{46b}	−0.7	−1.1
J_{56a}	1.9	1.9
J_{56b}	4.7	4.5
J_{6a6b}	−12.4	−12.4

able to reveal the characteristics of this inclusion complex. Recently, we have demonstrated that fast atom bombardment mass spectrometry (FAB-MS) may be utilized to determine the molecular species of the noncovalent inclusion complexes of β-cyclodextrin–tolbutamide (8) and α-cyclodextrin–penicillin V (9) using dithiothreitol/dithioerythritol (3:1) as a matrix. The FAB-MS of the benzaldehyde complex is shown in Fig. 1. The protonated molecular ion at *m/z* 1241 (β-CD-BAL + H^+) and the sodium ion adduct at *m/z* 1263 (β-CD-BAL + Na^+) are clearly observed, which provide strong evidence for the formation of a stable complex between β-cyclodextrin and benzaldehyde.

Integration of the 470-MHz ^{1}H NMR spectrum of the β-cyclodextrin–benzaldehyde complex in neutral solution showed a 1:1 molar ratio. It also disclosed the second-order spectral characteristics of the cyclodextrin proton resonances. The chemical shifts and coupling constants of the β-cyclodextrin and its benzaldehyde complex were therefore calculated using a computer spin simulation program. The experimental and simulated spectra are shown in Figs. 2 and 3, and the simulated results are summarized in Tables I and II. The downfield shifts of the *para* and *meta* phenyl protons were initially interpreted as being due to the absence of aromatic ring stacking upon complexation. However, this could not account for the slight upfield shift of the *ortho* phenyl protons. The shift may also result from the hydrophobic medium effect or induced dipole–dipole interactions. Most importantly, the H-3′ and H-5′ of β-cyclodextrin were significantly shifted upfield by the anisotropic effect of an aromatic ring current, strongly indicating that the aromatic moiety was included in the cavity.

Prevention of Autoxidation by β-Cyclodextrin. Autoxidation of benzaldehyde in the solid state could be inhibited by the formation of a 3:2 (benzaldehyde:β-cyclodextrin) complex as reported previously by Uekama *et al.* (10). We monitored the autoxidation rate of the β-cyclodextrin–benzaldehyde (1:1) inclusion complex in neutral solution at 70°C by HPLC. The half-life for this complex is sevenfold longer than that of free benzaldehyde (Table III). The stabilization of the aldehyde group indicates that the aldehyde group is included in the cavity of β-cyclodextrin and thus prevents its direct contact with oxygen. This protection effect makes the preparation of aqueous benzaldehyde solution for antitumor cytotoxicity evaluations much easier.

Table III. Autoxidation Rates of Benzaldehyde and Its β-Cyclodextrin Complex in pH 7.0 Phosphate Buffer Solution at 70°C

Compound	Half-life (hr)
Benzaldehyde	1.7
β-Cyclodextrin–benzaldehyde	11.6

Table IV. Antitumor Cytotoxicity Against Human Tumor Cell Lines

Compound	ED_{50} (*M*) MCF-7 (breast)	HT-29 (colon)
Benzaldehyde	7×10^{-4}	5×10^{-4}
β-Cyclodextrin–benzaldehyde	6×10^{-4}	7×10^{-4}

Antitumor Cytotoxicity of Benzaldehyde. The main objective for preparing β-cyclodextrin–benzaldehyde inclusion complex is to evaluate the antitumor efficacy of benzaldehyde. We have tested the *in vitro* cytotoxicity against human tumor cell lines (HT-29, colon adenocarcinoma, and MCF-7, breast adenocarcinoma). These results clearly indicate that the inclusion complex still retained the antitumor cytotoxicity of benzaldehyde (Table IV).

In summary, both physical and chemical data show the formation of a stable inclusion complex between benzaldehyde and β-cyclodextrin in aqueous solution and in the solid state. The ^{1}H NMR and FAB-MS results indicate the formation of a 1:1 (β benzaldehyde:β-cyclodextrin) complex in the solid state instead of a 3:2 (benzaldehyde:β-cyclodextrin) as previously reported by Uekama *et al.* (10). The antioxidation effect and ^{1}H NMR chemical shift changes suggest that the aldehyde group is the leading group inserted into the hydrophobic cavity of β-cyclodextrin, similar to the inclusion structure of α-cyclodextrin–benzaldehyde complex previously determined by the X-ray crystallographic method (11).

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