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Investigation of inclusion complexation of paclitaxel by cyclohenicosakis-(1→2)-(β-D-glucopyranosyl), by cyclic-(1→2)-β-D-glucans (cyclosophoraoses), and by cyclomaltoheptaoses (β-cyclodextrins)

Sanghoo Lee,[a] Dong-hyuk Seo,[a] Hyun-Won Kim,[b] Seunho Jung[a,*]

[a]*Department of Microbial Engineering, Konkuk University, Hwayang-dong 1, Gwangjin-gu, Seoul 143-701, South Korea*

[b]*Department of Biochemistry and Institute of Basic Medical Sciences and Medical Engineering Institute, Yonsei University, Wonju College of Medicine, Wonju 220-701, South Korea*

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Abstract

Inclusion complexation of the poorly soluble drug, paclitaxel, was investigated with various host cyclooligosaccharides such as a family of isolated neutral cyclohenicosakis-(1→2)-(β-D-glucopyranosyl) (cyclic-(1→2)-β-D-glucans, cyclosophoraoses), dimethyl cyclomaltoheptaose (cyclodextrins, DM-β-CD) and hydroxypropyl cyclomaltoheptaose (cyclodextrins, HP-β-CD). Quantitative analysis with high-performance liquid chromatography (HPLC) indicated that all three cyclic oligosaccharides could increase the solubility of paclitaxel, where DM-β-CD gave the best results and a family of cyclosophoraoses and HP-β-CD, both gave similar results. Complexation of host molecules with paclitaxel was studied by NMR and fluorescence spectroscopic analyses. NMR spectroscopic analysis showed that the aromatic regions of paclitaxel experienced noticeable changes of the chemical shifts or peak shapes upon interaction with host molecules. The relatively bulky cyclosophoraoses allowed favorable accessibility to either the B-ring or A-ring of paclitaxel, while DM-β-CD and HP-β-CD allowed accessibility to all the aromatic rings including the C ring. The interaction of DM-β-CD with paclitaxel greatly increased the fluorescence intensity compared with other host molecules, suggesting the more effective partitioning of a moderate fluorophore into a hydrophobic cluster adjacent to the C-ring of paclitaxel.

Keywords: Paclitaxel; Cyclosophoraose; Cyclodextrin; Inclusion complex; Cyclo-oligosaccharides; *Rhizobium meliloti* 2011

1. Introduction

Cyclosophoraoses are unique molecules that are found almost exclusively in bacteria of the Rhizobiaceae family. They were originally found in fast-growing soil bacteria, in *Agrobacterium* and *Rhizobium* species as intra- or extra-oligosaccharides.[1,2] In *Agrobacterium* and *Rhizobium* species, these molecules contain glucose residues linked solely by β-(1→2) glycosidic bonds,[15] and contain D-glucose as the only hexose monomer. Cyclosophoraoses are a class of unbranched cyclic oligosaccharides composed of β-(1→2)-D-glucans varying in size from 17 to 40 as neutral or anionic forms. Cyclosophoraoses are synthesized in the cytosol and transported to the periplasmic

* Corresponding author. Tel./fax: +82-2-4503520.
E-mail address: shjung@konkuk.ac.kr (S. Jung).

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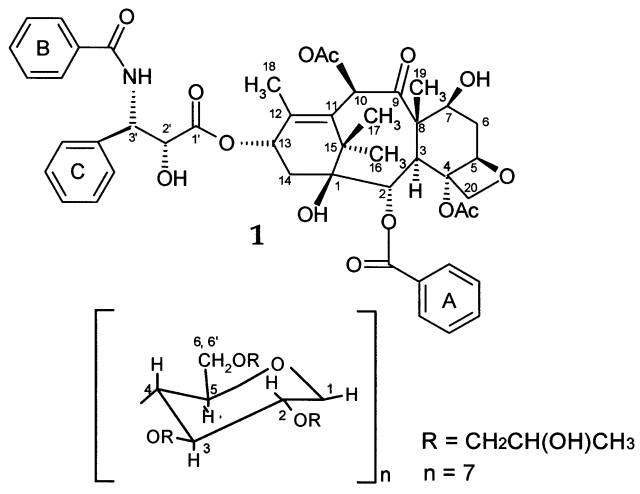
space where they play an important role in regulating the osmolarity in response to external osmotic shock.[3,16] Cyclosophoraoses are also known to be involved in the initial stage of root-nodule formation of *Rhizobium* species during nitrogen fixation.[4,17] Throughout this process, cyclosophoraoses are suspected to be involved in complexation with various plant flavonoids.[5] Thus, much attention has been focused, not only on their biological functions, but also on their potential ability to form inclusion complexes with other molecules. Additional studies have indicated that the cyclosophoraoses form inclusion complexes with hydrophobic plant metabolites such as naringenin, a legume-derived flavonoid.[5] Cyclosophoraoses have a greater solubility in water than that of CDs (cyclodextrins).[10]

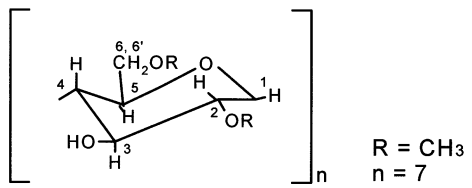
Currently, CDs and chemically modified CDs have been extensively used and studied as host molecules for inclusion complex formation in fields such as those of the pharmaceutical and the food industries.[6,11,23] CDs belong to a family of cyclic oligosaccharides with a hydrophilic surface and a hydrophobic cavity in the center. The most commonly used CDs are α-CD, β-CD, γ-CD, consisting of six, seven and eight glucopyranose rings, respectively. Chemically modified CDs have been used to increase the low solubility of natural CDs or modulate their toxicity,[9] and these can act as hosts for a wide variety of lipophilic drugs. These derivatized CDs have been used to resolve formulation difficulties encountered with poorly soluble drugs.

As for the application of inclusion complex formation, a single cyclosophoraose (degree of polymerization (DP) 17, Cys-A) was extensively used as a host molecule for complexation with guest molecules because of its easy availability. However, the isolated Cys-A showed limited possibilities on industrial applications related to inclusion complex formation over various hydrophobic guest molecules.[10] In the present investigation, we used a family of isolated neutral cyclosophoraoses with various DPs ranging from 17 to 27 along with HP-β-CD, DM-β-CD as a host molecule for inclusion complexation with the poorly soluble drug, paclitaxel (**1**). Paclitaxel is a complex diterpenoid natural product used clinically to treat advanced ovarian, breast and non-small cell lung cancers. It binds to the β-tubulin binding site on microtubles and interferes with their depolymerization.[8] Despite its potential in cancer therapy, its poor solubility in water makes a serious hindrance for its usage. Several attempts have been made to enhance its poor water solubility using derivatized CDs, such as HP-β-CD, HE-β-CD and DM-β-CD.[9,21,22] However, previous reports have not proposed any molecular mechanism for the solubility enhancement of **1** in the presence of cyclooligosaccharides. Here, we propose that the solubility enhancement of **1** with cyclooligosaccharides was due to the partial breakage of a hydrophobic cluster around the C-ring of **1**. Inclusion complex formation was



2-Hydroxypropyl-β-CD

(HP-β-CD)



2,6-Di-*O*-methyl-β-CD

(DM-β-CD)

investigated with NMR and fluorescence spectroscopy and then was quantitatively analyzed by HPLC.

2. Experimental

Preparation of cyclosophoraoses.—*Rhizobium meliloti* 2011 was generously provided by Dr R.I. Hollingsworth, MSU, E. Lansing, MI, USA. Cells were cultured in 500 mL of GMS medium to late logarithmic phase and incubated at 30 °C, at 150 rpm on a rotary shaker. Cells were harvested by centrifugation (8000 rpm, at 4 °C), washed once with a saline solution, and subjected to the hot EtOH extraction method. Cells were then extracted with 40 mL of 75% (v/v) EtOH at 70 °C for 30 min. After centrifugation, the supernatant was removed and concentrated on a vacuum rotary evaporator. The concentrated sample was chromatographed on a Sephadex G-50 column (1.5 × 110 cm) at a rate of 20 mL/h, and eluant fractions (7 mL) were assayed for carbohydrate by the phenol–H_2SO_4 method. The fractions containing cyclosophoraoses were pooled, concentrated, and desalted by a Sephadex G-15 column (2 × 27 cm) under conditions described above. The desalted sample was then applied to a column (2 × 35 cm) of DEAE-cellulose to separate neutral and anionic cyclosophoraoses. The column was first washed with distilled water containing 1 mM KCl, and a gradient was applied beginning with 1 mM KCl and ending with 100 mM KCl.

After the cyclosophoraoses were desalted by dialysis (Spectra/PorCE (cellulose ester membrane; MWCO: 1000)), their structures were confirmed by nuclear magnetic resonance (NMR) spectroscopy. In order to confirm the structure, various NMR spectroscopic analyses were performed on a Bruker AMX spectrometer (500 MHz for ^1H, 125 MHz for ^{13}C) with deuterated water (D_2O, 99.96%) or deuterated dimethyl sulfoxide (DMSO-d_6) as solvent at 27 °C.

The neutral cyclosophoraoses were further analyzed for the determination of molecular-weight distribution with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS, Voyager, PerSeptive Biosystems) using 2,5-dihydroxybenzoic acid (DHB) as the matrix under positive ionization. Mass spectra were recorded in DHB at a molar ratio of 10^{-3} with a total loading of around 1 μg of sample. Ions were formed by laser desorption at 337 nm.

Inclusion complex formation of HP-β-CD, DM-β-CD and neutral cyclosophoraoses with paclitaxel.—A family of isolated neutral cyclosophoraoses (DP 17–27) or HP-β-CD (Aldrich), DM-β-CD (Sigma) was used as host molecules for the inclusion complexation with **1** as a guest molecule. Based on the MALDI-MS analysis, number average-molecular weight ($M_n = \Sigma N_i M_i / \Sigma N_i$, where N_i is the measured peak intensities (peak area) of a molecular ion with degree of polymerization i and M_i is the mass of the ith cyclosophoraose) of a family of cyclosophoraoses was calculated[18] and further used for the determination of concentration. First, **1** was dissolved in EtOH to obtain 2.0 mM solution. Stock solution of **1** (1 mL) in a vial was added to each 1 mL of aq HP-β-CD, DM-β-CD and neutral cyclosophoraoses with various concentrations, and the mixtures were shaken for 24 h at 30 °C to equilibration in the dark. After equilibrium was reached, the mixture was evaporated, lyophilized and dissolved in 1 mL of water to remove insoluble **1** by filtration using a 0.2 μm membrane filter (Whatman). The concentration of **1** in the filtrate was determined by high-performance liquid chromatography (HPLC 10A, Shimadzu, Kyoto, Japan) with either an ultraviolet (UV) or a refractive index (RI) detector. Compound **1** was assayed at 230 nm, using a 60:40 MeCN–water mobile phase, 35 °C oven temperature and 0.7 mL/min flow rate. Lyophilization was carried out with a FD-3 freeze dryer (Heto Holten A/S, Denmark). An Econosphere C_{18} 5U column (25 cm × 4.6 mm, Alltech) was used for HPLC.

Inclusion complexation of HP-β-CD, DM-β-CD and a family of neutral cyclosophoraoses with **1** was also investigated by NMR and fluorescence (Hitachi F-2000) spectroscopic analyses.

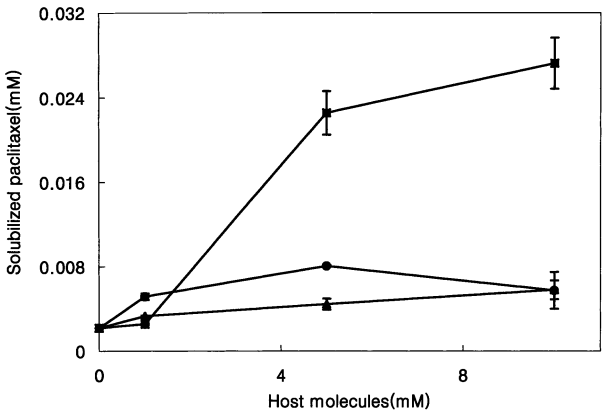


Fig. 1. Phase solubility diagram of **1** in solutions of host molecules. A 2 mM solution of **1** was mixed with 1, 5 and 10 mM solutions of the host molecule. The solubility of **1** at each stage was analyzed by isocratic HPLC (60:40 acetonitrile–water) at 230 nm. Each point represents the mean ± S.D. of three experiments. ■, **1** complexed with DM-β-CD; ●, **1** complexed with cyclosophoraoses; ▲, **1** complexed with HP-β-CD.

3. Results

Identification of neutral cyclosophoraoses.—Neutral cyclosophoraoses were isolated and purified from *R. meliloti* 2011 using several chromatographic methods, and their structures were identified with NMR spectra. The ^{13}C NMR chemical shifts of neutral cyclosophoraoses in DMSO-d_6 were assigned as C-1, δ 101.8; C-2, δ 82.6; C-3, δ 75.6; C-4, δ 68.8; C-5, δ 76.7; C-6, δ 60.6, respectively. MALDI-MS analysis showed the Gaussian distributions of ring size ranging from 17 to 27, of which the major forms were ranged from the 21-mer to the 23-mer (data not shown). The number-average molecular weight of cyclosophoraoses was determined at about 3568.6. The relatively large size of cyclosoporaoses may be advantageously compared to other CDs and its derivatized CDs in inclusion complex formation.

Phase solubility measurement of the inclusion complexes.—Fig. 1 shows the phase solubility diagrams for **1** over the entire concentration of the host molecules. The solubility of **1** was greatly enhanced according to the added concentrations of DM-β-CD compared to that enhanced by cyclosophoraoses and HP-β-CD. Cyclosophoraoses gave moderate solubility enhancement of **1** up to a concentration of 2–5 mM, but with concentrations more than 5 mM, solubility of **1** rather gradually decreased. Compared to cyclosophoraoses, HP-β-CD seemed to show a tendency to solubilize greater amounts of **1** in the higher concentrations than 5 mM. Out of three host molecules tested, DM-β-CD was the most effective inclusion agent of **1**.

^1H NMR chemical shift data.—The ^1H NMR spectra (DMSO-d_6, 5 mM, 500 MHz) were obtained for 5 mM of **1** with a twofold molar excess of host molecules. The chemical shift data for the complexed form of **1** were somewhat different from those for the free state **1** (Table 1). Other than chemical shift changes of NH, 1-OH, and 2′-OH observed for all the host molecules, noticeable changes were observed on non-aromatic protons (5-H, 14a-H, 14b-H) of **1** for the complexation with DM-β-CD and HP-β-CD, probably due to the interaction with the hydrophilic external part of these CDs. For the complexation with cyclosophoraoses, noticeable chemical shift change was observed only in the resonances of the aromatic regions of **1** (*o*-B). Fig. 2 shows proton NMR spectra of the ring moieties when **1** is complexed with host molecules. For the complexation with cyclosophoraoses (Fig. 2(c)), other than the chemical shift change noticed for *o*-B, a new resonance (7.677 ppm) appeared near the *m*-proton (7.633 ppm, *m*′-A) of A-ring of **1**, which was not induced by either of the derivatized CDs. A H–H COSY experiment confirmed the clear connectivity between the new *m*-proton (*m*′-A) and the *o*-, *p*-protons of the A-ring (Fig. 3). In case of the interaction of both of the derivatized CDs with **1** (Fig. 2(b,d)), however, distinctive changes in their peaks shapes were also observed in protons of the C-ring of **1**, whereas no changes were observed in the spectrum of **1** complexed with cyclosophoraoses (Fig. 2(c)), suggesting that DM-β-CD and HP-β-CD induced a change of coupling environments of those of the C-ring of **1**. This was confirmed by the peak shape change of the *p*-protons of the C-ring after the complexation, where the triplet of the *p*-proton of the C-ring became clear after the complexation (Table 2).

Fluorescence spectroscopic analysis of the inclusion complexes.—To investigate the envi-

Table 1
Chemical shifts (^{1}H NMR) of host molecules before and after inclusion complex with paclitaxel

Protons	DM-β-CD			Protons	Cyclosophoraoses			Protons	HP-β-CD		
	δ_1 [a]	δ_2 [b]	$\Delta\delta$ [c]		δ_1	δ_2	$\Delta\delta$		δ_1	δ_2	$\Delta\delta$
1-H	4.970	4.962	0.008	1-H	4.775	4.774	−0.001	1-H	4.932	4.922	−0.010
2-H	3.194	3.195	0.001	2-H	3.264	3.270	0.006	2-H	3.318	3.803	−0.015
3-H	3.699	3.707	0.008	3-H	3.520	3.531	0.011	3-H	3.771	3.785	0.014
4-H	3.344	3.346	0.002	4-H	3.174	3.173	−0.001	4-H	3.218	3.224	0.006
5-H	3.681	3.693	0.012	5-H	3.227	3.226	−0.001	5-H	3.560	3.575	0.015
6,6′-H	3.560	3.564	0.004	6-H	3.746	3.745	−0.001	6,6′-H	3.612	3.620	0.008
2-OCH_3	3.249	3.252	0.003	6′-H	3.510	3.522	0.012	CH_3 [d]	1.023	1.030	0.007
6-OCH_3	3.490	3.498	0.008	3,4-OH	5.123	5.137	0.014	CH_2 [d]	3.613	3.620	0.007
				6-OH	4.364	4.392	0.028				

[a] Free state.
[b] Complexed state.
[c] $\Delta\delta = \delta_2 - \delta_1$.
[d] Hydroxypropyl substituents.

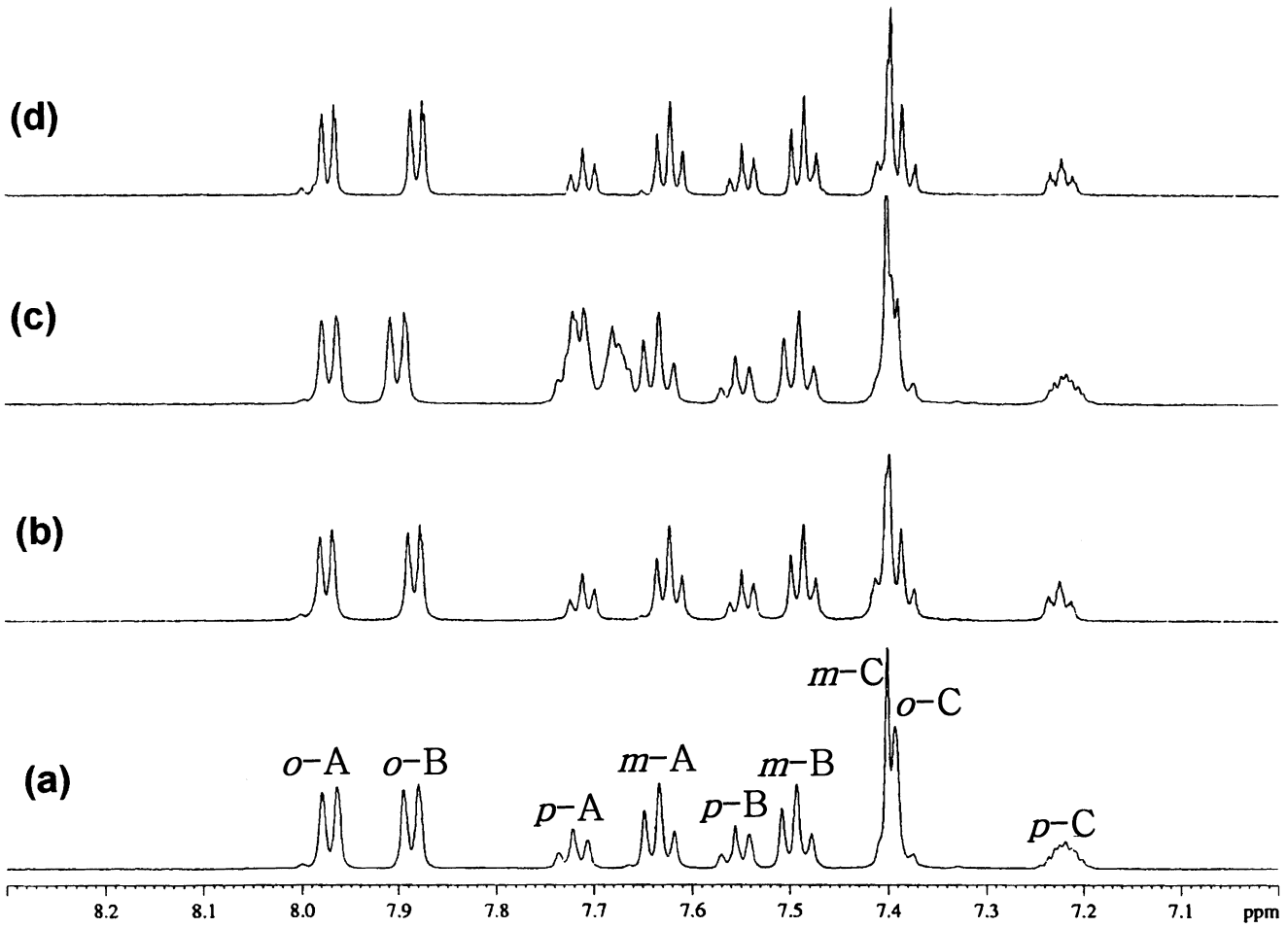


Fig. 2. ^{1}H NMR spectra (500 MHz, 25 °C, DMSO-d_6, internal standard Me_4Si) of (a) **1** (5 mM); (b) DM-β-CD–**1** mixture (1:2 molar ratio); (c) cyclosophoraoses–**1** mixture (1:2 molar ratio); and (d) HP-β-CD–**1** mixture (1:2 molar ratio).

ronment-dependent fluorescence changes of the host molecules–**1** mixtures in aqueous solution, fluorescence emission spectra were obtained on a finer time scale than that of NMR (Fig. 4). The fluorescence of **1** was observed to be environment dependent.[19] The stereospecific side chain of **1** is fluorescent due to the two hydrophobic aromatic rings (B- and C-ring) at position-13. The broad pattern of the emission spectrum suggested that the fluorophore of **1** showed a population-averaged spectrum in multiple environments of differing polarity. The interaction of DM-β-CD with **1** greatly increased the fluorescence intensity compared to other host molecules, suggesting the more effective partitioning of a moderate fluorophore into a hydrophobic environment.

4. Discussion

When inclusion complexation of a family of neutral cyclosophoraoses with **1** was compared with that of DM-β-CD and HP-β-CD, DM-β-CD gave the best result, and a family of cyclosophoraoses and HP-β-CD, both gave similar results. NMR and fluorescence spectra suggest a different mechanism of complexation between the derivatized β-CDs and cyclosophoraoses. NMR data for the ring moieties of **1** upon interaction with the host molecules show that cyclosophoraoses have favorable accessibility to the A- and B- rings, but not to the C-ring of **1**, while DM-β-CD and HP-β-CD have accessibility to the C-ring. The C-ring was known to be hydrophobically clustered among the 4-acetoxy (CH_3) moieties

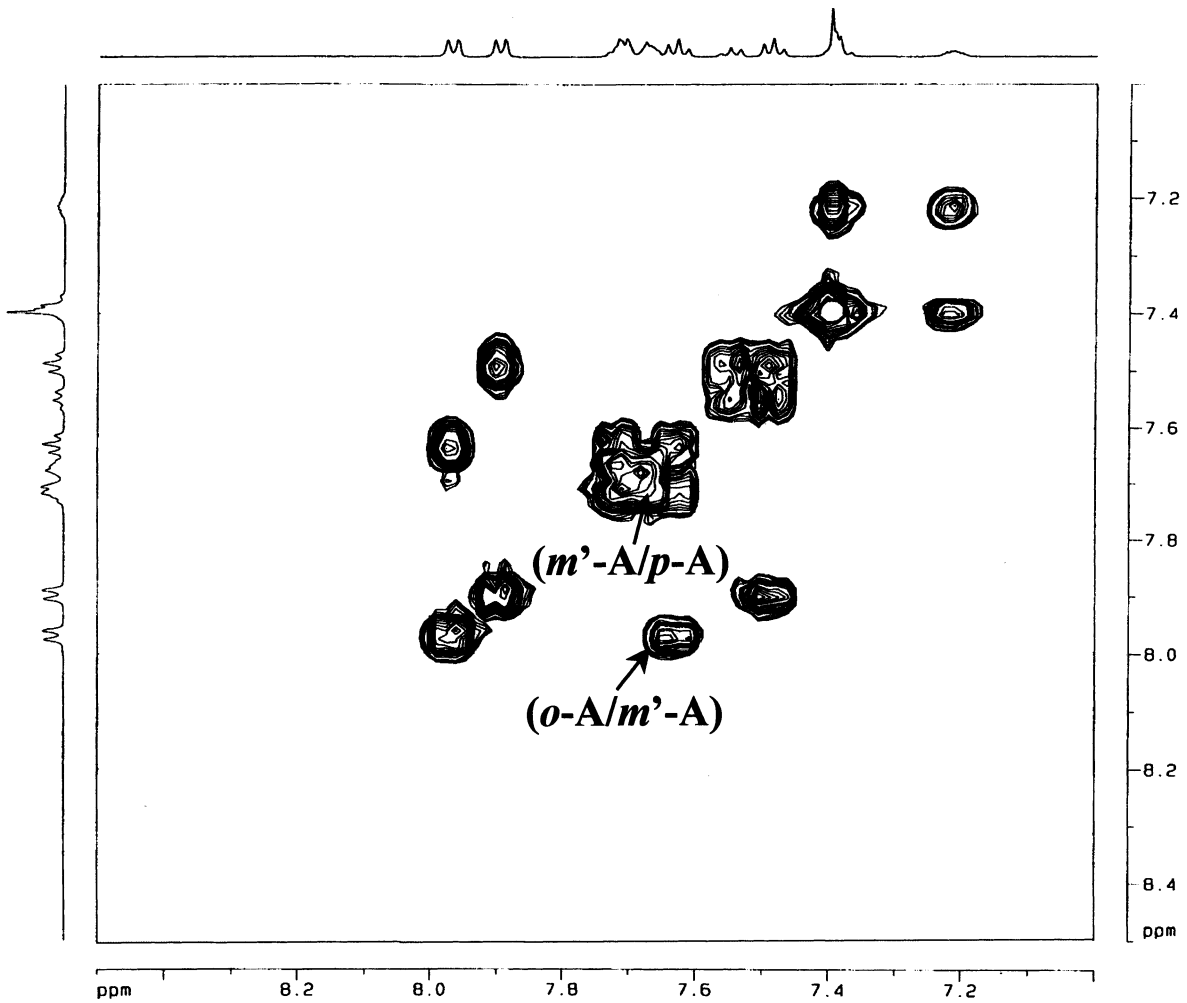


Fig. 3. H–H COSY spectrum (500 MHz) of the ring moieties of **1** in the presence of cyclosophoraoses. The connectivity between the new *m*-proton (7.633 ppm) and the *p*-proton of the A-ring is clearly seen along with that between the *o*-proton and new *m*-proton.

Table 2
^{1}H NMR chemical shifts (ppm) of paclitaxel before and after inclusion complexation

Protons	$\delta_{(\text{free taxol})}$ [a]	$\delta_{(\text{DM-β-CD})}$ [b]	$\Delta\delta$ [c]	$\delta_{(\text{Cys})}$ [d]	$\Delta\delta$ [e]	$\delta_{(\text{HP-β-CD})}$ [f]	$\Delta\delta$ [g]
NH	8.939	8.876	−0.063	8.975	0.036	8.873	−0.066
1-OH	4.715	4.682	−0.033	4.718	0.003	4.683	−0.032
2′-OH	6.216	6.162	−0.054	6.226	0.010	6.150	−0.066
2′-H	4.588	4.596	0.008	4.596	0.008	4.592	0.004
3′-H	5.397	5.410	0.013	5.404	0.007	5.409	0.012
2-H	5.412	5.421	0.009	5.418	0.006	5.420	0.008
3-H	3.613	3.622	0.009	3.613	0.000	3.620	0.007
5-H	4.925	4.881	−0.044	4.931	0.006	4.901	−0.024
6a-H	2.324	2.323	−0.001	2.320	−0.004	2.324	0.000
6b-H	1.639	1.646	0.007	1.643	0.004	1.642	0.003
7-H	4.104	4.110	0.006	4.105	0.001	4.108	0.004
10-H	6.292	6.294	0.002	6.292	0.000	6.292	0.000
13-H	5.889	5.900	0.011	5.887	−0.002	5.896	0.007
14a-H	1.889	1.917	0.028	1.887	−0.002	1.915	0.026
14b-H	1.727	1.751	0.024	1.719	−0.008	1.748	0.021
20a-H	4.022	4.027	0.005	4.021	−0.001	4.025	0.003
20b-H	4.006	4.014	0.008	4.005	−0.001	4.012	0.006
4-OAc	2.230	2.232	0.002	2.227	−0.003	2.230	0.000
10-OAc	2.115	2.113	−0.002	2.116	0.001	2.112	−0.003
16-Me	1.029	1.032	0.003	1.026	−0.003	1.030	0.001
17-Me	1.018	1.023	0.005	1.015	−0.003	1.022	0.004
18-Me	1.79	1.795	0.005	1.792	0.002	1.793	0.003
19-Me	1.507	1.510	0.003	1.504	−0.003	1.508	0.001
o-A [h]	7.972	7.974	0.002	7.971	−0.001	7.972	0.000
m-A [i]	7.630	7.622	−0.008	7.633	0.003	7.622	−0.008
m′-A [j]	7.630			7.677	0.047		
p-A [k]	7.721	7.711	−0.010	7.728	0.007	7.711	−0.010
o-B [l]	7.882	7.883	0.001	7.900	0.018	7.878	−0.004
m-B [m]	7.493	7.486	−0.007	7.490	−0.003	7.485	−0.008
p-B [n]	7.555	7.548	−0.007	7.556	0.001	7.548	−0.007
o-C [o]	7.388	7.386	−0.002	7.387	−0.001	7.384	−0.004
m-C [p]	7.397	7.399	0.002	7.399	−0.002	7.398	0.001
p-C [q]	7.220	7.223	0.003	7.219	−0.001	7.222	0.002

[a] Free taxol.
[b] Chemical shifts with DM-β-CD.
[c] $\Delta\delta = \delta_{\text{DM-β-CD}} - \delta_{\text{free taxol}}$.
[d] Chemical shifts with cyclosophoraoses.
[e] $\Delta\delta = \delta_{\text{cyclosophoraoses}} - \delta_{\text{free taxol}}$.
[f] Chemical shifts with HP-β-CD.
[g] $\Delta\delta = \delta_{\text{HP-β-CD}} - \delta_{\text{free taxol.}}$
[h] *o*-Protons in the A, B and C rings.
[i] *m*-Protons in the A, B and C rings.
[j] New *m*-proton induced when complexed with cyclosophoraoses.
[k] *p*-Protons in the A, B and C rings.
[l] *o*-Protons in the A, B and C rings.
[m] *m*-Protons in the A, B and C rings.
[n] *p*-Protons in the A, B and C rings.
[o] *o*-Protons in the A, B and C rings. In case of DM-β-CD and HP-β-CD, peak shapes were altered.
[p] *m*-Protons in the A, B and C rings. In case of DM-β-CD and HP-β-CD, peak shapes were altered.
[q] *p*-Protons in the A, B and C rings. In case of DM-β-CD and HP-β-CD, peak shapes were altered.

and the A-ring according to the proposed aqueous conformation of **1**.[20] NMR data suggests that the enhanced solubility of **1** by DM-β-CD and HP-β-CD would be due to the effective partitioning by the derivatizations. The fluoresence spectrum showed that parti-

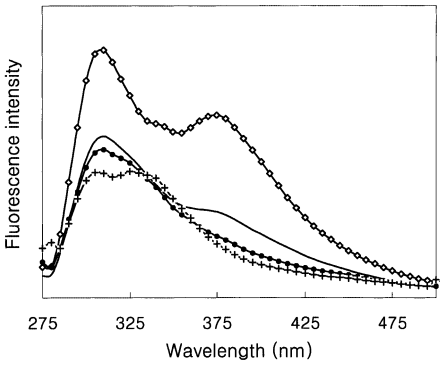


Fig. 4. Fluorescence emission spectra upon interaction of host molecules (10^{-3} M) with **1** (10^{-5} M) in aqueous solution. Samples were excited at 260 nm, with 5-nm excitation and emission slits. ◇, **1** complexed DM-β-CD; ●, **1** complexed with cyclosophoraoses, +, **1** complexed with HP-β-CD; and —, paclitaxel alone.

tioning by DM-β-CD would be more effective in breaking of hydrophobic clustering of the C-ring. Although cyclosophoraose showed favorable accessibility to both the A- and B-rings, its relatively large size[12,13] might prevent the accessibility to the C-ring. Thus, the solubility enhancement by cyclosophoraoses must rely on an entirely different mechanism, which might be due to thermodynamic stability of binding state based on its flexible nature.[7,14] Although DM-β-CD showed the best results on the solubility enhancement of **1**, it should be also considered that a family of cyclosophoraoses of DPs ranging from 17 to 27 with Gaussian distribution was used for the experiment. If specific cyclosphoraoses with effective ring size would be used, the solubility might be more enhanced. Further experiments are needed in this regard.

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