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Inclusion Complexation of a Family of Cyclosophoraoses with Indomethacin

LEE, SANGHOO[1], CHANHO KWON[1], YOUNGJIN CHOI[1], DONG-HYUK SEO[1], HYUN-WON KIM[2], AND SEUNHO JUNG[1]*

[1]*Department of Microbial Engineering, Konkuk University, Hwayang-dong 1, Gwangjin-ku, Seoul 143-701, Korea*
[2]*Department of Biochemistry and Institute of Basic Medical Sciences and Medical Engineering Institute, Yonsei University, Wonju College of Medicine, Wonju 220-701, Korea*

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Abstract Cyclosophoraoses are a class of unbranched cyclic-(1→2)-β-D-glucans found in the *Rhizobium* species. Their unique cyclic structures and high solubility make them potent for inclusion complexation as a host for an insoluble guest molecule. A family of neutral cyclosophoraoses (DP 17-27) isolated from *Rhizobium meliloti* 2011 was used as a host for inclusion complexation with an insoluble guest drug, indomethacin. A high performance liquid chromatographic analysis indicated that the inclusion complexation of cyclosophoraoses greatly enhanced the solubility of indomethacin compared with β-cyclodextrin. The estimated value of the association constant of the complex in water for β-cyclodextrin and cyclosophoraoses was 523 M^{-1} and 17,570 M^{-1}, respectively. NMR spectroscopy showed that the inclusion complex was characterized by the interaction of the indole ring moiety of indomethacin with the cavity of cyclosophoraoses.

Key words: Cyclosophoraoses, inclusion complex, indomethacin, β-cyclodextrin

Inclusion complexation technology using host cyclic-oligosaccharides such as α, β, and γ-cyclodextrin has been applied to several bioindustrial fields, including the solubilization of insoluble drugs, odor control, biosensor, and enhancement of guest stability [19]. Cyclodextrins are cyclic oligosaccharides with six (α-CD), seven (β-CD), and eight (γ-CD) α-1, 4-glycosidically linked glucopyranose units synthesized by cyclodextrin glucanotransferase [8, 15, 16]. They are in truncated cone-shaped structures with the hydrophobic inner cavities whose diameters range from approximately 5 to 9 Å, depending on the number of glucose moieties. In particular, β-CD has been widely utilized due to its easy availability, even though it has limited solubility with a small inner hydrophobic cavity. As a possible substitute for cyclodextrins in inclusion complexation, a family of neutral cyclosophoraoses was used, which has a better solubility and more variable molecular sizes. Cyclosophoraoses are a class of unbranched cyclic oligosaccharides composed of β-1,2-D-glucans varying in size from 17 to 40 in a neutral or anionic form. They were originally found in fast growing soil bacteria, *Agrobacterium* and *Rhizobium* species, as *intra-* or *extra*-oligosaccharides [6, 13]. Cyclosophoraoses are synthesized in the cytosol and transported to the periplasmic space where they play an important role in regulating the osmolarity in response to external osmotic shock [12]. Cyclosophoraoses are also known to be involved in the initial stage of the root-nodule formation of the *Rhizobium* species during nitrogen fixation [18]. Throughout this process, cyclosophoraoses are suspected to be involved in complexation with various plant flavonoids. 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. As for the application of inclusion complex formation, a single cyclosophoraose (Degree of Polymerization (DP) 17, Cys-A) was used as a host for complexation with guest molecules because of its easy availability. However, the isolated Cys-A showed limited possibilities for industrial applications related to inclusion complex formation over various hydrophobic guest molecules [9]. Both Cys-A and β-CD showed similar ability for inclusion complexation [9]. In the present investigation, we used a family of isolated neutral cyclosophoraoses with various DPs ranging from 17 to 27 as a host, instead of a single form of Cys-A previously used, for inclusion complexation with the hardly soluble indomethacin sodium salt [1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindoleacetic acid sodium salt], a prostaglandin synthetase inhibitor, which inhibits the action of cyclooxygenase, a kind of master enzyme involved in inflammation or blood clotting [1, 4, 10]. The complexation of indomethacin with cyclosophoraoses

*Corresponding author
Phone: 82-2-450-3520; Fax: 82-2-450-3520;
E-mail: shjung@konkuk.ac.kr

was investigated with NMR and FTIR spectroscopy, then the binding constant of cyclosophoraoses to indomethacin was estimated from HPLC analysis.

MATERIALS AND METHODS

Bacterial Strain and Growth Medium

Rhizobium meliloti 2011 was generously provided by Dr. R. I. Hollingsworth, MSU, E. Lansing, Michigan, U.S.A. The cells were cultured in 500 ml of a GMS medium to late logarithmic phase, and then incubated at 30°C, at 150 rpm on a rotary shaker. The culture medium (GMS) contained the following components per liter of distilled water: 1 g of glutamic acid, 5 g of mannitol, 1 g of potassium phosphate dibasic, 0.2 g of magnesium sulfate, 0.04 g of calcium chloride, and vitamins [12].

Preparation of Cyclosophoraoses

The *Rhizobium meliloti* 2011 was cultured in 500 ml of the GMS medium to late logarithmic phase, and then incubated at 30°C, at 150 rpm on a rotary shaker. The cells were harvested by centrifugation (8,000 rpm, at 4°C), washed once with a saline solution, and subjected to the hot-ethanol extraction method. The cells were then extracted with 40 ml of 75% (vol/vol) ethanol at 70°C for 30 min. After centrifugation, the supernatant was removed and concentrated using a vacuum rotary evaporator. The concentrated sample was chromatographed on a Sephadex G50 column (1.5×110 cm) at a rate of 20 ml/h, and the eluant fractions (7 ml) were assayed for carbohydrate using the phenol-sulfuric acid method. The fractions containing cyclosophoraoses were pooled, concentrated, and desalted through a Sephadex G15 column (2×27 cm) under the previous conditions. The desalted sample was then applied to a column (2×35 cm) of DEAE-cellulose to separate the neutral and anionic cyclosophoraoses. The column was first washed with distilled water containing 1 mM KCl, and then a gradient was applied, beginning with 1 mM KCl and ending with 100 mM KCl.

Structural Analysis

After the cyclosophoraoses were desalted by dialysis [Spectra/Por CE (cellulose ester membrane; MWCO: 1000), Spectrum Laboratories, Inc., CA, U.S.A.], their structures were confirmed by NMR spectroscopy. Various NMR spectroscopic analyses were performed on a Bruker (AMX, Germany) spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C) with deuterated water (D_2O, 99.96%) or deuterated dimethyl sulfoxide (DMSO-d_6) as the solvent at 27°C. The neutral cyclosophoraoses were further analyzed to determine the molecular weight distribution using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS, Voyager, PerSeptive Biosystems, Framingham, U.S.A.) with 2, 5-dihydroxybenzoic acid (DHB) as the matrix at a positive ionization. The mass spectra were recorded in DHB at a mole ratio of 10^{-3} with a total loading of around 1 μg of sample. Ions were formed by laser desorption at 337 nm. The inclusion complexation of a family of cyclosophoraoses with indomethacin was also investigated with NMR and FTIR (JASCO FTIR-300E, REV, U.K.) spectroscopic analyses.

Inclusion Complex Formation of Cyclosophoraoses and β-Cyclodextrin with Indomethacin

A family of isolated cyclosophoraoses or commercial β-cyclodextrin (Sigma, St. Louis, U.S.A.) was used as the host for the inclusion complex forming ability, with indomethacin (Sigma, St. Louis, U.S.A.) as the guest molecule. First, indomethacin was dissolved in acetone to obtain a 15×10^{-3} M solution. One milliliter of the indomethacin stock solution in a vial was added to 1 ml of aqueous neutral cyclosophoraoses or a β-cyclodextrin solution with various concentrations, and the mixture was shaken for 24 h at 30°C to accomplish an equilibration in the dark. After equilibrium was reached, the mixture was evaporated, lyophilized, and dissolved in 1 ml of water to remove insoluble indomethacin by filtration using a 0.2 μm membrane filter (Whatman BioScience, U.K.). The complexed indomethacin structures with host molecules were confirmed by NMR and FTIR spectroscopic analyses. The indomethacin concentration in the filtrate was determined by high performance liquid chromatography [HPLC 10A (Shimadzu, Kyoto, Japan)] with an ultraviolet (UV) or refractive index (RI) detector. The indomethacin was assayed by reading at 320 nm, at 35°C, with 90% methanol and 10% water as the mobile phase, and a constant flow rate of 1.0 ml/min. Econosphere C18 5U column (25 cm×4.6 mm, Alltech Associates, Inc., IL, U.S.A.) was used for the HPLC. For a more exact quantitative analysis, the filtrate was added to 2.0 M trifluoroacetic acid (TFA) and hydrolyzed for 3 h at 120°C, which then degraded only host cyclic oligosaccharides. After lyophilization [FD-3 freeze dryer (Heto Holten A/S, Denmark)] of the filtrate, the exact concentration of the complexed indomethacin was confirmed by high performance liquid chromatography. The concentration of a family of cyclosophoraoses was calculated using the number average molecular weight based on the MALDI/MS analysis [5, 20], which gave a quantitative distribution of the cyclosophoraoses [11]. The association constant (K) of the inclusion complex was also estimated using HPLC analysis.

RESULTS AND DISCUSSION

Identification of Neutral Cyclosophoraoses

Neutral cyclosophoraoses were isolated and purified from the *Rhizobium meliloti* 2011 using several chromatographic

techniques, and the exact structure was identified with various NMR spectra. The ^{1}H NMR spectral data for the neutral cyclosophoraoses in DMSO-d_6 are shown in Table 1. The ^{13}C NMR chemical shifts of the neutral cyclosophoraoses in DMSO-d_6 were also 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. Although the exact three-dimensional structures of cyclosophoraoses still remain unknown, several conformational studies have been performed. Figure 1 shows three molecular models of cyclosophoraoses as proposed structures [14, 21, 2]. All three structures appear to be equilibrated in aqueous solutions. The MALDI-MS analysis also showed the Gaussian distributions of the ring size ranging from 17 to 27, of which the major forms ranged from 21 mer to 23 mer (data not shown). Based on the MALDI-MS analysis, the number average molecular weight ($Mn=\Sigma N_iM_i/\Sigma N_i$, where N_i is the measured peak intensities (peak area) of a molecular ion with the degree of polymerization i and M_i is the mass of the ith cyclosophoraose) of a family of cyclosophoraoses was calculated and then used for the determination of concentration. The calculated number average molecular weight of the cyclosophoraoses was determined at about 3568.6, which was then used for the HPLC analysis.

Table 1. Chemical shifts (ppm) of the protons of indomethacin and cyclosophoraoses in the free and complex states.

Proton	δ_1[a]	δ_2[b]	$\Delta\delta$[c]
Indomethacin			
Me	2.215	2.270	0.055
CH_2	3.655	3.685	0.030
OMe	3.760	3.717	-0.043
COOH	nd[d]	10.294	
H4	7.035	6.939	-0.096
H6	6.925	7.308	0.383
H7	6.713	6.545	-0.168
H14,18	7.679	variable	
H15,17	7.650	variable	
Cyclosophoraoses			
H1	4.772	4.780	0.008
H2	3.267	3.216	-0.051
H3	3.516	3.528	0.012
H4	3.174	3.150	-0.024
H5	3.225	3.206	-0.019
H6	3.758	3.747	-0.011
H6	3.499	3.517	0.018
OH3,4[e]	5.123	5.172	0.049
OH6	4.363	4.392	0.029

[a]Free state, [b]Inclusion complex, [c]$\Delta\delta=\delta_2-\delta_1$, [d]not detected, [e]OH group attached to the C3,4 in cyclosophoraoses.

Phase Solubility Measurement of the Inclusion Complexes

The complex-forming ability of cyclosophoraoses was estimated from the enhancement of the solubility of indomethacin as an insoluble guest molecule in comparison with β-cyclodextrin. Figure 2 shows the solubility diagrams of indomethacin corresponding to the added concentration of cyclosophoraoses or β-cyclodextrin. The slope of each isotherm was quite different, as the solubilization of

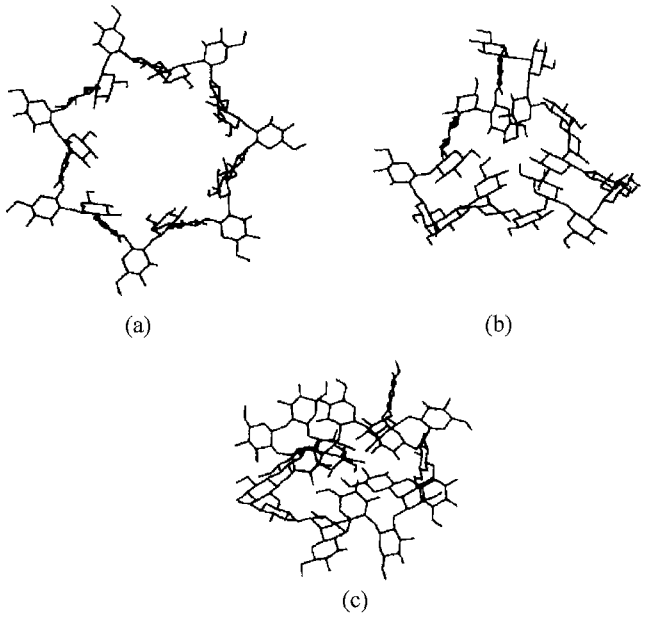

Fig. 1. Molecular models of cyclosophoraoses.
Proposed by (a) Palleschi *et al.* [14], (b) York *et al.* [21], and (c) Jung *et al.* [2].

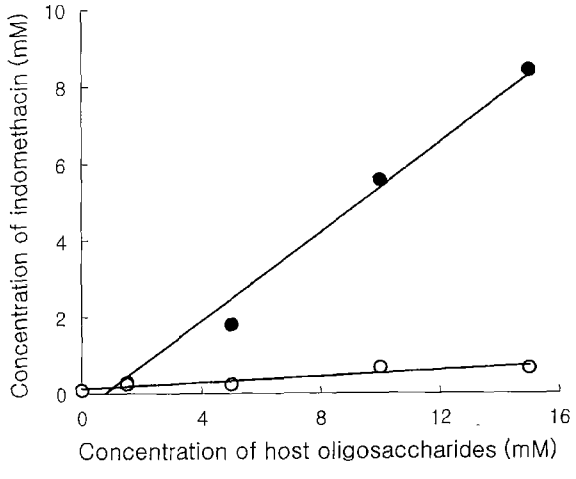

Fig. 2. Solubility increase of indomethacin in cyclosophoraoses and β-cyclodextrin solutions.
A 15 mM indomethacin solution was mixed with 1.5, 5, 10, and 15 mM of cyclicoligosaccharides solutions. The solubility of indomethacin at each stage was analyzed by an isocratic HPLC (methanol:water=90:10) at 320 nm. The calculated number average molecular weights of cyclosophoraoses for the HPLC analysis were determined to be about 3568.6. (● Indomethacin complexed with cyclosophoraoses, ○ indomethacin complexed with β-cyclodextrin).

indomethacin was more greatly enhanced by cyclosophoraoses than by β-cyclodextrin. It has been reported that Cys-A (DP 17) and β-cyclodextrin exhibit similar results in increasing the solubility of indomethacin [9]. However, the present data showed that the solubility of indomethacin was greatly increased by a family of cyclosophoraoses (DP 17-27), when compared with that by β-cyclodextrin (Fig. 2). This could be explained by the enhanced capability of inclusion complex formation of cyclosophoraoses with the guest indomethacin. The association constant (K) of the inclusion complex based on the number average molecular weights was also estimated with HPLC analysis. The solubility measurement is a general method for determining the K value. In phase-solubility diagrams, in the case of a straight solubility curve, the K value can be calculated based on the assumption of 1:1 complex stoichiometry [7]. The association constant K for this 1:1 complex is given by K=S/I(1−S), where S is the slope of the solubility curve, and I is the solubility of the indomethacin in the absence of host molecules. In the previous study by Duchene *et al.* [17], the association constant K for β-cyclodextrin was 570–680 M^{-1} at pH 6.8, whereas, in this study, the values of the association constant in water for β-cyclodextrin and cyclosophoraoses were 523 M^{-1} and 17,570 M^{-1}, respectively. The cyclosophoraoses produced by *Rhizobium meliloti* 2011 showed a definite superiority in their solublizing effect and inclusion complex forming ability for indomethacin when compared with *R. phaseoli*, producing a single form of Cys A with similar solubility enhancement to that of cyclodextrin (DP 17) [9]. This might be mainly due to the presence of wide and various hydrophobic cavities within a family of cyclosophoraoses.

Spectroscopic Analyses of Inclusion Complexes of Indomethacin with Host Molecules

The inclusion complexes were investigated by NMR spectroscopy. Figure 3 shows the proton NMR spectra before and after the complexation of indomethacin with cyclosophoraoses. Chemical shift variations in all the aromatic protons of indomethacin in the presence of cyclosophoraoses clearly indicated the formation of the complex (Fig. 3). Table 1 presents a summary of the variations in the chemical shifts of indomethacin and cyclosophoraoses due to the complexation. The H4, H5, and H6 protons for the indole ring of indomethacin experienced significant peak shifts before and after the inclusion complexation with cyclosophoraoses. As shown in Fig. 3, the H6 proton of indomethacin shifted more downfield than the H4 proton. The chemical shifts of the *p*-chlorobenzoyl moiety were also widely separated and

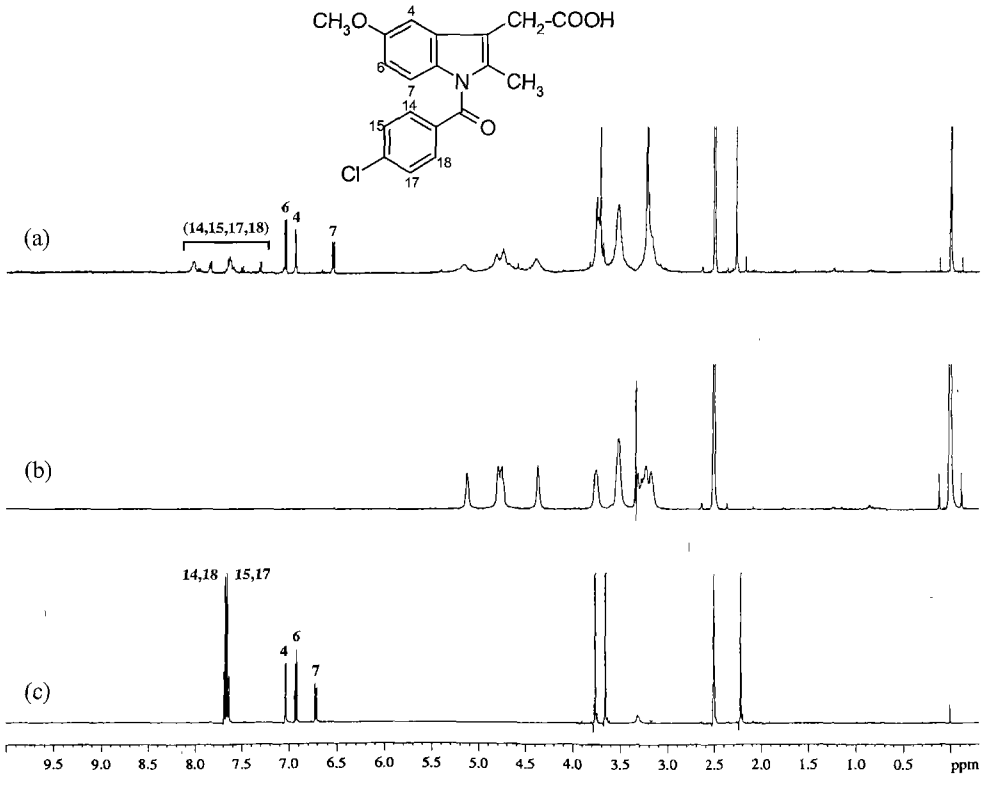

Fig. 3. Proton NMR spectrum before and after complexation of indomethacin with cyclosophoraoses (DMSO-d_6).
(a) Indomethacin (10 mM); (b) cyclosophoraoses (10 mM); (c) inclusion complex (1:1 molar ratio). Peaks for DMSO-d_6 appeared at δ 2.49. All chemical shifts were given relative to internal TMS at 0 ppm (top; indomethacin structure).

broadened upon inclusion complexation. The broad range of chemical shift changes and the peak shapes of the complexation with a family of cyclosophoraoses are in contrast to those of previous NMR data on the complexation of indomethacin with β-cyclodextrin [4], where the major variations concentrated on the aromatic protons H4, H6, H7 of the indole ring, and a broader range of variations of chemical shifts were clearly observed upon complexation with cyclosophoraoses. This phenomenon indicated that the complexation induced a change in the coupling environment for the protons in the *p*-chlorobenzoyl moiety of indomethacin. As such, the chemical environment of indomethacin was also dramatically changed upon complexation with cyclosophoraoses. In addition, the H2, H4, H5, H6 protons of cyclosophoraoses experienced significant upfield shifts, while the other protons were shifted downfield (Table 1). This effect was clearly observed on the H2 and H4 protons, probably due to the anisotropic effects of the induced magnetic field of the ring moieties of indomethacin. FTIR spectroscopy was also used to investigate the interaction of indomethacin with cyclosophoraoses in the solid state (Fig. 4). Spectra were obtained in the range of 4,000–400 cm^{-1}. The sharp peaks shown at 1,693 and 1,597 cm^{-1} are corresponding to the (N-)C=O and (O-)C=O stretch of indomethacin, respectively [Fig. 4(a)]. In this region,

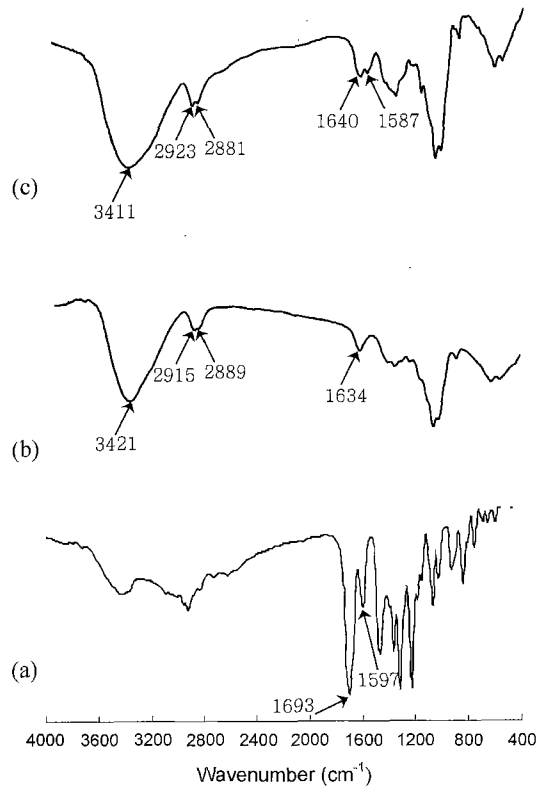

Fig. 4. FTIR spectra (4,000–400 cm^{-1}) in KBr matrix.
(a) Indomethacin alone, (b) cyclosophoraoses alone, and (c) inclusion complex.

the spectrum of cyclosophoraoses showed a broad band due to the bending of (C-)O-H (1,634 cm^{-1}) [Fig. 4(b)]. However, the spectrum observed upon complexation of indomethacin with cyclosophoraoses showed a restricted peak intensity in the carbonyl ((N-) C=O) stretching region at 1,640 cm^{-1} [Fig. 4(c)], where there is a lower frequency shift. This shift might be explained by the breakdown of the intramolecular interaction of the (N-)C=O---H14 or H18 due to the inclusion complexation between *p*-chlorobenzoyl moieties and cyclosophoroases. This was also confirmed by NMR spectroscopy.

In the present investigation, we attempted to demonstrate that cyclosophoraoses showed a much greater accessibility for complexation with indomethacin compared with β-cyclodextrin, and thus made a better complex with indomethacin. This phenomenon could be due to the preferential thermodynamic stability of the complexed cyclosophoraoses, which provided a stronger intermolecular energy for both the electrostatic and the van der Waals interactions compared with β-cyclodextrin, as was recently proposed using Monte Carlo docking simulation [3]. It should also be 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 sizes are used, the solubility might be more enhanced. Further experiments are needed in this regard.

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