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Complexation of Microcystins and Nodularin by Cyclodextrins in Aqueous Solution, a Potential Removal Strategy

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Abstract

Cyanotoxins are potent toxic compounds produced by cyanobacteria during algal blooms, which threaten drinking water supplies. These compounds can poison and kill animals and humans. The host—guest interactions of α-, β-, and γ-cyclodextrins (CD) with problematic cyanotoxins, including microcystins (MCs) and nodularin (NOD), were investigated to demonstrate the potential application of CDs for the removal of these toxins from drinking water or applications related to their separation or purification. MCs and NOD have a hydrophobic Adda chain, which contains diene and benzene functional groups. The complexation of these cyanotoxins with CDs was monitored by nuclear magnetic resonance (NMR). The ^{1}H NMR spectra for MCs are unchanged upon addition of α-CD (smallest host). However, addition of larger hosts, β-CD and γ-CD, leads to significant changes in chemical shifts of the benzene and diene resonances on the 3-amino-9-methoxy-2,6,8-trimethal-10-phenyldeca-4,6-dienoic acid (Adda) chain of MCs and NOD. Solution pH, natural organic matter, and salinity do not appreciably influence the host—guest complexation under our experimental conditions. The experimental binding constants for MCs and NOD with γ-CD are relatively strong, ranging from 1155 to 507 M^{-1}. The observed changes in chemical shifts for specific protons and competitive binding experiments demonstrate a 1:1 inclusion complex between γ-CD and MCs or NOD, with the Adda chains threading through the CD ring, resulting in an inclusion complex. Our results suggest that CD-type substrates are useful hosts for the complexation of MCs and NOD. CDs can be readily attached to a number of polymeric or solid supports and their functionality tailored to strengthen specific host—guest interactions. With further development of such materials, CD host—guest chemistry may find direct application in the removal and/or separation science of these compounds.

INTRODUCTION

Cyanobacteria are a series of bacteria that obtain their energy through photosynthesis. Cyanobacteria produce potent toxic compounds during algal blooms, which can poison and even kill animals and humans.[1–3] The cyclic peptide toxins of the microcystin and nodularin families are the most widespread and threatening compounds produced by cyanobacteria in blooms from fresh and brackish waters.[4–6] They contain five (nodularin) and seven (microcystins) amino acids, with the two terminal amino acids of the peptide joined to form a cyclic compound (Figure 1). These toxins are hepatotoxins and tumor promoters.[7] The lethal doses for 50% of the population (LD_{50}) for approximately 70 different MCs range from 50 to 500 μg/kg.[8] The hydrophobic Adda moiety, which is common to all microcystins

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and nodularin, is considered to be the key component for biological activity and their ability to bind and inhibit protein phosphatase.[9]

Microcystins are extremely stable and resistant to chemical hydrolysis or oxidation. In natural waters and in the dark, microcystins can persist for months or years[1] and are recalcitrant to conventional water treatment processes. The high toxicity of MCs and their impact on environmental, social, and economic aspects propelled the scientific interest for developing methods for the detection, characterization, and treatment of these toxins. Activated carbon adsorption[10] and ozone and chlorine dioxide oxidation[11,12] can effectively remove MCs from drinking water. However, the presence of natural organic materials causes a reduction of the operational lifetime and reduces the effectiveness. TiO_2 photocatalysis and ultrasonic irradiation have been used to degrade MCs efficiently, and the associated toxicity is dramatically reduced.[13–15] While TiO_2 leads to degradation of MCs under bench-scale conditions, these methods are typically strongly influenced by water quality and are often not cost-effective.[16] Moreover, the potential health risks associated with TiO_2 nanoparticles in water have become a concern recently.[17] Biodegradation by different types of bacteria has been studied, and the probable pathway was elucidated.[18,19] Bioremediation of these toxins has been reported and has potential as a treatment method for these substrates, but it can also be highly sensitive to water quality and require extended periods of time (20—30 days).[20,21] The combination of bioremediation with advanced oxidation technologies is another possible treatment strategy to consider for the treatment of cyanotoxins. Although CDs have been used as agents (cages) to trap and remove a variety of organic pollutants by host—guest complexation,[22–24] the use of CDs for chemical remediation of cyanotoxins is unexplored.

CDs are hollow open-ended cone-shaped molecules composed of repetitive D-glucopyranose (cyclic sugar) units joined through α (1—4) linkages, as illustrated in Figure 2. Secondary hydroxyl groups at the second and third positions of the cyclic sugar units define the wider opening of the CD substrates, while primary hydroxyl groups at the sixth position of the individual sugar units define the perimeter of the narrower opening. The interiors of the CD rings are lined with ether oxygens and methane functional groups.[25] Thus cyclodextrins have hydrophilic openings and hydrophobic interiors. α-, β-, and γ-CDs have six, seven, and eight glucopyranose units, respectively. They are inexpensive, nontoxic, and readily available. It is well-known that cyclodextrins can act as hosts for hydrophobic guests and exhibit specific complexation with a number of organic compounds.[26] Formation of cyclodextrin complexes is an equilibrium process where free guest molecules are in equilibrium with incapsulated molecules. In general, the complexation is controlled by the size, structural shape, and hydrophobic properties of the guests. The detection and characterization of these complexes have been explored through calorimetric,[27,28] X-ray diffraction,[29,30] NMR,[31–33] computational studies,[34] and ESI-MS.[35] Among these analytical methods, NMR is one of the most useful methods due to its easy application and the detailed structural information on the nature of the complex that can be obtained. NMR is widely used in host—guest chemistry because it can readily distinguish encapsulation and surface or peripheral binding of the substrate to the host. Moreover, specific shifts of the proton signals are a direct indication of the specific nature/region of a substance that is encapsulated.[31–33]

The current study was conducted to demonstrate and characterize the complex of cyanotoxins with α, β, and γ-CDs as hosts (complexing agents) for the potential removal of MCs and/or NOD from drinking water sources. The strength and nature of the binding of these cyanotoxins with the different CDs and the influence of water quality on the complexation are reported here.

EXPERIMENTAL SECTION

Chemicals

Microcystin-LR (MCLR) was purified according to a method by Song.[36] All reagents were HPLC grade or analytical grade, including microcystin-RR (MCRR); nodularin; α-, β-, and γ-cyclodextrins (Sigma-Aldrich); methanol (Fisher Scientific); and adamantanecarboxylic acid (Sigma-Aldrich). The sample solutions for ^{1}H NMR measurement were prepared by dissolving MCs, NOD, and CDs in the D_2O solution.

Solution Preparation

MCLR was mixed with three types of CDs in different molar ratios from 1:1 to 1:64, respectively. MC variants and NOD were individually mixed with γ-CD at a specific molar ratio 1:12 at a high concentration. The sample solutions were subsequently diluted to several specific concentrations, and complexation was monitored by ^{1}H NMR to determine the equilibrium binding constant. The desired solution pH was obtained by addition of appropriate volumes of stock DCl or NaOD solutions. The effect of salinity and humic materials was determined by preparing solutions containing specific amounts of NaCl or humic acid. The salt and humic material were carefully weighed using an analytical balance and all solutions were prepared using volumetric glassware.

NMR Experiments

All NMR experiments were conducted in 99.9% D_2O with 0.1% DSS [sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (δ = 0.00 ppm)] as the internal standard. The NMR experiments were carried out on a Bruker DRX 400 spectrometer at 20 °C.

RESULTS AND DISCUSSION

Complex of MCLR with α-, β-, and γ-Cyclodextrins

MCLR ($C_{49}H_{74}O_{12}N_{10}$) is a relatively large compound with a molecular weight of 994 g/mol. The ^{1}H NMR spectrum for MCLR is complicated with extensive overlapping resonances in the aliphatic region (4.0—0.5 ppm). The host CD also possesses a multitude of overlapping peaks in the aliphatic region. Fortunately, upfield resonances of protons on the benzene ring and diene moieties of the Adda chain of the potential guest occur in a region of the ^{1}H NMR spectra that is unique and unobstructed by other peaks (8.0—5.0 ppm). The ^{1}H NMR peaks in this region correspond to the ortho, para, and meta protons on the benzene ring, H_o, H_m, H_p, and the diene protons, H_1, H_2, and H_3 (Figure 3).

^{1}H NMR spectra of pure MCLR and MCLR in the presence of three different CDs at a molar ratio of 1:2 are also shown in Figure 3. α-CD does not induce significant change in the chemical shift for protons on the Adda hydrophobic chain of MCLR, and the lack of a shift indicates that there is no measurable interaction between α-CD and the Adda chain. The ^{1}H NMR spectra of MCLR in the presence of β-CD and γ-CD show distinct changes in the chemical shift for the aromatic ring and diene moieties relative to free MCLR. Modest chemical shift changes of proton peaks are observed for the complex of MCLR and β-CD, but the complexation with γ-CD yields the largest changes of chemical shift for the proton associated with the Adda chain.

The formation of a distinct stable complex between MCLR and CD would yield two sets of MCLR peaks, one set for complexed MCLR and the other set for free MCLR. We did not observe two sets of peaks, but rather observed a shift of the aromatic and olefinic peaks for MCLR upon addition of β and γ-CD, indicating that the exchange of MCLR between the free and host—guest complexed environments is fast relative to the ^{1}H NMR time scale (0.5

ms). The resonances for protons on aromatic and diene moieties shift upfield (to lower chemical shift in ppm) upon complexation, indicating increased shielding of the protons from the magnetic field. This is consistent with the protons (Adda) being complexed within the CD ring, a hydrophobic environment.

For the benzene proton resonances for H_o, H_m, and H_p were assigned on the basis of their multiplicity and integration.[37] For free MCLR, H_o and H_p, the protons ortho and para relative to the alkyl chain, will be more shielded and thus have a lower chemical shift relative to the meta proton, H_m. The individual proton resonances of the benzene ring (H_o, H_m) shift only slightly upon complexation with β-CD (Figure 4). The H_m triplet on the benzene ring of MCLR shifts downfield, while olefinic proton peaks do not shift. We suggest this is the result of the smaller size of the β-CD cavity (i.d. 0.62 nm) that can only encapsulate the benzene ring (i.d. 0.56 nm) of the Adda chain. More pronounced changes in chemical shift are observed for the olefinic and benzene protons in the presence of γ-CD (i.d. 0.78 nm) compared to β-CD (Figure 4). The protons H_o (doublet), H_1 (triplet), H_2 (doublet), and H_3 (quartet) shift downfield upon addition of γ-CD. Moreover, chemical shift for the protons on the OCH_3 group of Adda chain also changes from 3.0 to 3.2 ppm. The significant changes in chemical shifts indicate MCLR may be encapsulated within γ-CD; thus, the benzene and olefinic protons are exposed to different regions of the γ-CD (top and bottom). These observations suggest γ-CD complexes the middle of the Adda chain, analogous to a bracelet fitting onto an arm or a bead threading a string. The olefinic protons (H_4 and H_5) within the cyclic structure of MCLR do not shift, implying that there is minimal or no measurable specific interaction between the cyclic region of MCLR and γ-CD.

Characterization Nature of Complex

Different complexing modes, for example, internal host—guest complex or external association not involving the cavity of CD, are possible. The pronounced chemical shift change for the protons on the Adda moiety suggests that the host—guest complex involves Adda. In an attempt to confirm that the nature of the interaction of MCLR and γ-CD occurs via an inclusion complex, a powerful competitive guest, adamantanecarboxylic acid (ADA), with a binding constant of 34 000 M^{-1}, known to form 1:1 inclusion complex,[38] was added into MCLR and γ-CD solution. ADA undergoes the strongest binding with γ-CD at neutural pH.[34] If the MCLR:γ-CD complex involves an inclusion complex, the resonances for several protons on benzene ring and diene group of MCLR shift upfield upon addition of γ-CD, as illustrated in Figure 5. When ADA is added into the mixture of MCLR and γ-CD, the NMR spectrum of the free MCLR is observed, indicating that ADA replaces MCLR in the cavity of γ-CD.

Effects of Solution pH, Salinity, and NOM

To probe the effects of water quality on the complexation of MCLR and γ-CD, the effects of solution pH and ionic strength were investigated. The affinity of the neutral/hydrophobic compounds for the hydrophobic cavity of CDs may be enhanced relative to ionized forms in solution of increased ionic strength.[39] The solution pH has a direct effect on the charge and hydrophobicity of MCLR, which in turn could influence the host—guest interaction with CDs. We investigated the effect of solution pH on the MC:CD complex under the acidic, basic, and the neutral conditions normally associated with typical natural waters. A series of MCLR:γ-CD solutions at various solution pH values (1, 3, 5, 7, 8, 9, and 10) was characterized by ^{1}H NMR. Since MCLR possesses acidic COOH groups, a stronger binding may be expected under acid conditions, where the nonionized MC is more hydrophobic. However, no appreciable change was observed in the complexation as a function of solution pH over the range from 1 to 7. The very small change for H_3 observed at pH 5 is assigned to a chemical environment change due to protonation of carboxylic acid in the vicinity of H_3 or

changes in conformation of MCLR. The other resonances were not influenced by solution pH. Under basic conditions, the binding becomes slightly weaker, likely the result of ionization of functional groups on the cyclic peptide region.

Microcystins are most widely distributed in freshwaters, but they have also been identified in marine environments 1; thus, the effect of salinity on the complexation was investigated. It is reported that CD solubilization of organic compounds can be enhanced by increasing the ionic strength of the solution due to formation of guest and cyclodextrin complex aggregates.[40] NaCl was added to the solution of MCLR and γ-CD to increase the ionic strength of the solution. Even at 5 mol/L NaCl no changes in the binding were observed on the basis of the NMR spectra. According to Loftsson et al., salts enhance the contribution of the noninclusion complexation to the overall guest solubility.[40] The absence of a salt effect is consistent with the proposal that MCLR and γ-CD form an inclusion complex. The presence of natural organic matter (NOD) has a negative effect on the removal of cyanotoxins by several common methods, such as activated carbon adsorption,[41] ozonation,[42] and TiO_2 photodegradation.[43] In our study, up to 5 g/L humic acid was added into the solution of MCLR and γ-CD, yet no changes were observed in the binding or complexation of the toxin, a distinct advantage over existing treatment methods, which are negatively impacted by the presence of humic materials.

Binding Constant for the Complex of γ-CD with Different MCs and NOD

Binding constant for MCLR, MCRR, and NOD with γ-CD were obtained according to the method described by Ramstad et al.[31] The binding isotherm for a 1:1 molecular stoichiometry can be obtained by measuring the chemical shift change as a function of host at a specific ratio of the host and γ-CD, 1:12. In accordance with the theory, Δ_{max}, the maximum of chemical shift difference, cannot be experimentally obtained in the rare instance that complete binding is achieved. However, the binding constant, K, and Δ_{max} may be calculated from eq 2 through application of nonlinear curve fitting to the empirical data.

$$\Delta = \frac{\Delta_{max} K [\mathrm{CD} - \gamma]}{1 + K [\mathrm{CD} - \gamma]} \quad (1)$$

$$\frac{1}{\Delta} = \frac{1}{\Delta_{max}} + \frac{1}{\Delta_{max} K} \frac{1}{[\mathrm{CD} - \gamma]} \quad (2)$$

where Δ is the chemical shift difference, K is the binding constant, [γ-CD] is the the concentration of γ-CD, and Δ_{max} is the the maximum of chemical shift difference.

The data for calculating binding constants were obtained by monitoring chemical shift change as a function of [γ-CD]. The solutions employed for these experiments were prepared using serial dilution. The change in chemical shift was monitored relative to protons of the internal standard dextran sulfate sodium (DSS) at 0 ppm. The proton resonances for H_1, H_2, H_3, and H_o shift upfield as the [CD] increases. Table 1 and Figure 6 show the measured data for chemical shift difference of H_1, H_2, H_3, and H_o for MCLR. H_1 exhibits the largest chemical shift change, which is consistent with a complexation made containing H_1 (the diene) in the middle of the CD ring, the most hydrophobic and shielded environment of the CD interior, as discussed earlier. Relatively small shifts for H_3 suggest that this proton may be at the edge of the CD ring or just outside, and thus the influence is less pronounced. To further probe the specific position of CD on the Adda chain, advanced NMR techniques (NOESY) were carried out in an attempt to determine the distances

between protons on the host and guest molecules. We were unable to detect specific interactions between the host and guest molecules from our NOESY experiments. This could be due to experimental limitations, dynamic properties of the host—guest complexation, or movement of the host within the guest, i.e., rotating and sliding within the complex.

The binding constant (K) and Δ_{max} were obtained using curve-fitting analyses. Ideally each proton on a particular host would yield the same K. Although the value of binding constant is expected to be independent of the proton measured, K values for each measured proton are different, due to slightly different chemical environment.[44] The average binding constant for the complex of MCLR and γ-CD is 1045 ± 144 M^{-1} and Δ_{max} varies from 0.12 to 0.45 ppm (Table 2). Excellent modeling of the experimental data by eq 1 indicates a 1:1 complex and not a higher order complex.

At least 70 different MC variants have been identified. All MC variants and NOD possess the Adda side chain and thus should be complexed by γ-CD.[8,9] To test the broader applicability of CD complexation, experiments were conducted with MCRR and NOD substrates. The differences between the structure of MCRR and MCLR are the two variable L-amino acids at the 2 and 4 positions. Titration of the chemical shift change as a function of [γ-CD] leads to an average binding constant of 835 ± 77 M^{-1} for the complex of MCRR and γ-CD, slightly lower than the value for MCLR (1045 ± 144 M^{-1}). The values of Δ_{max} for MCRR are from 0.13 to 0.45 ppm (Table 2). Although MCRR and MCLR have different amino acid residues with the cyclic peptide ring structure, the similar K values further indicate the complexation occurs at the Adda chain common to both compounds. MC's are composed of seven amino acid residues, while NOD consists of five residues (Figure 1B), but all possess the Adda chain. The average binding constant for the complex of NOD and γ-CD is 612 ± 145 M^{-1}, which is lower than that of MCLR. The values for Δ_{max} are from 0.08 to 0.44 ppm (Table 2). The different structures on the cyclic peptide of NOD and MCs affect their ability to complex with CDs.

The NMR studies demonstrate that α-CD does not exhibit a detectable host—guest interaction with MCLR. The slightly larger β-CD induces chemical shift changes in the benzene protons in the Adda side chain, but these shifts were too small to allow accurate determination of the K for β-CD with MCLR. Since the shifts are only observed for the benzene proton resonances, we propose the binding occurs at the end the Adda chain and the interaction between MCLR and β-CD is weak. However, γ-CD exhibits relatively strong binding with MC variants and NOD. ^{1}H NMR was used to obtain the binding isotherms, curves of the observed chemical shifts versus free ligand (γ-CD) concentrations, which yield binding constants for MCLR, MCRR, and NOD of 1045, 835, and 612 M^{-1}, respectively. To probe the nature of the MCLR:γ-CD complex, ADA, which forms a strong inclusion complex with γ-CD, was added to the MC:γ-CD complex. Upon addition of ADA, the MCLR:γ-CD complex disappears with the complementary formation of free MCLR. This observation indicates that the ADA replaces MCLR and the MCLR:γ-CD interactions occur via an inclusion complex. Our studies demonstrate that specific CDs form strong inclusion complexes with MCs and NOD, which are not influenced over a range of solution pH, by salinity, or in the presence of NOM.

Our results suggest that CDs are promising substrates for complexing MCs and NOD under a variety of different water qualities, thus forming the basis for potential treatment/removal methods. The applications of this process will require improvement in the binding properties of the host, but CDs can be readily tailored to enhance the binding properties through simple functional transformations of the peripheral hydroxyl groups.[45] The practical application of this process for the removal or trapping of MCs and NOD will also require immobilized CD

substrates, such as those employed for the removal of dyes, organic pollutants, and metal ions from wastewater.[22–24,46] While testing modified CD materials for removal of MCs and NOD is beyond the scope of this paper, we are planning future studies in this direction.

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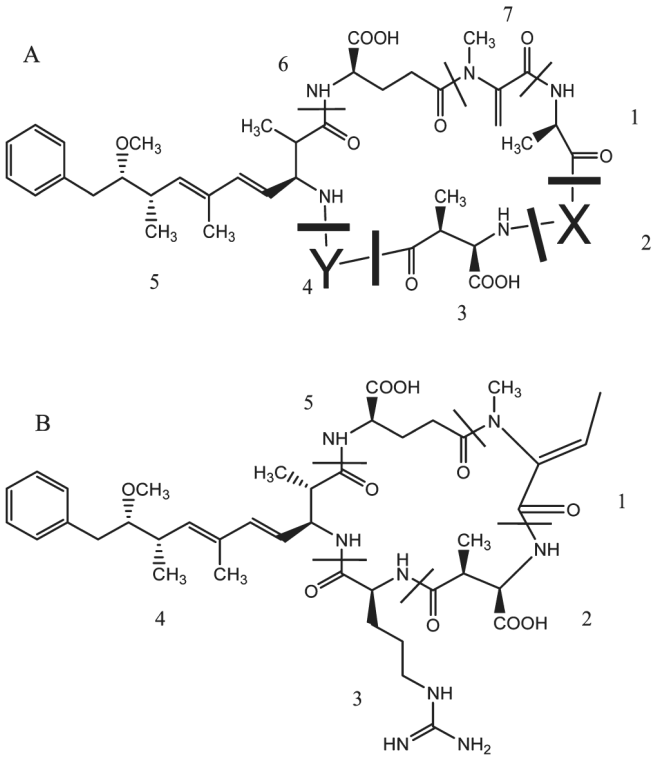
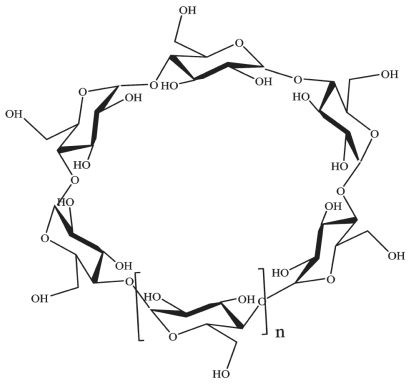
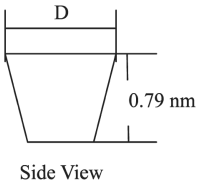


Figure 1.

(A) Microcystins [1, D-alanine; 2, X = variable D- and L-amino acid; 3, D-methylaspartic acid; 4,Y = variable D- and L-amino acid; 5, 3-amino-9-methoxy-2,6,8-trimethal-10-phenyldeca-4,6-dienoic acid (Adda); 6, D-glutamic acid; 7, *N*-methaldehydralanine] and (B) nodularin [1, 2-(methyl-amino)-2-dehydrobutyric acid; 2, methylaspartic acid; 3, arginine acid; 4, Adda; 5, D-glutamic acid].





n	Name	D (nm)
1	α-CD	0.49
2	β-CD	0.62
3	γ-CD	0.79

Figure 2.
The structure and dimension of α-, β-, and γ-cyclodextrins.

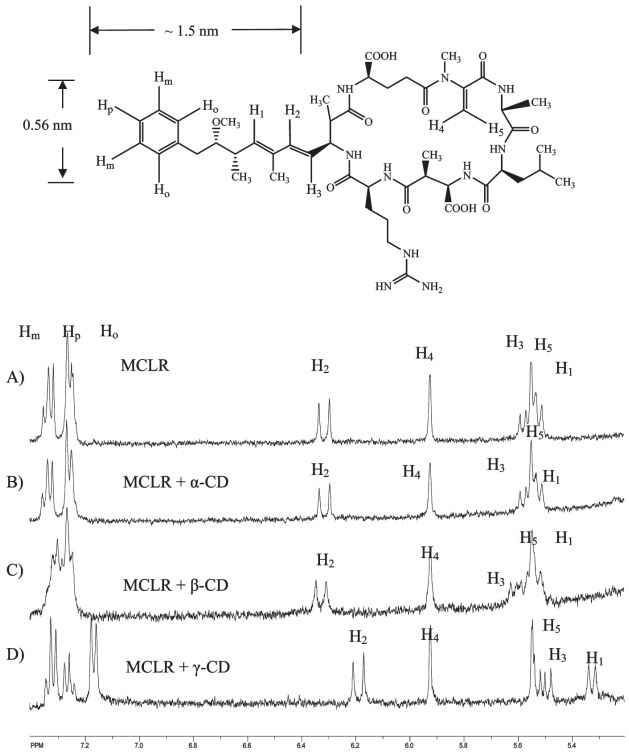


Figure 3.
^{1}HNMR spectra of D_2O solutions of MCLR (A) in the presence of (B) α-CD, (C) β-CD, and (D) γ-CD at a ratio of 1:2.

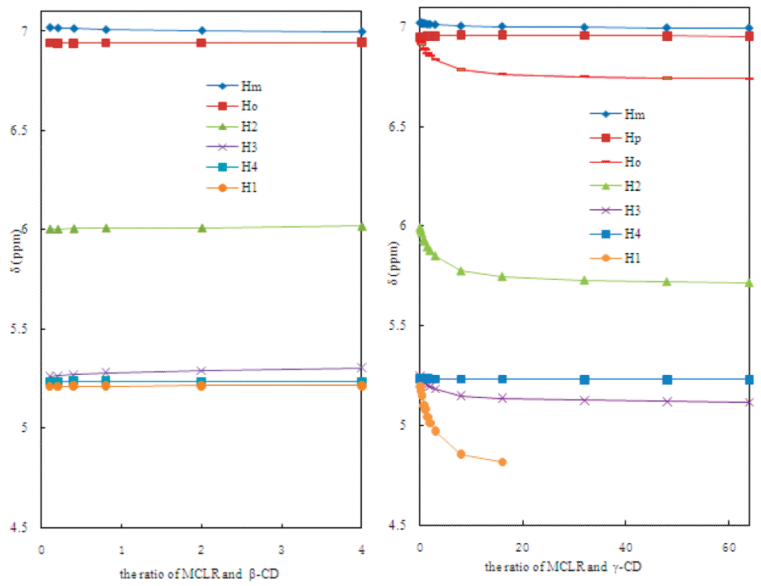


Figure 4.

Chemical shift changes on benzene ring and diene group on MCLR in the presence of (A) β-CD and (B) γ-CD by increasing the ratio of CDs and MCLR. Each curve represents the middle point of multiplets for each hydrogen. H_p in B is not included because of overlap by other H peaks.

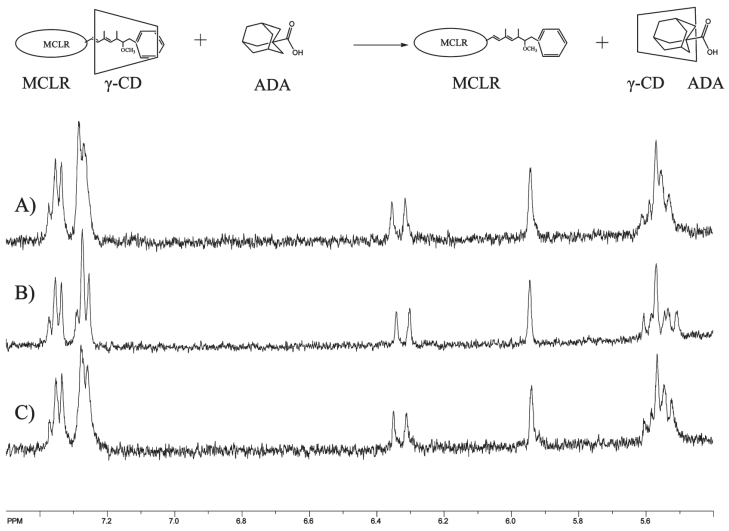


Figure 5.

^{1}HNMR spectra resulting from addition of adamantanecarboxylic acid (ADA) to MCLR—γ-CD solution for (A) MCLR, (B) MCLR:γ-CD, 1:2, (C) MCLR:γ-CD:ADA, 1:2:10.

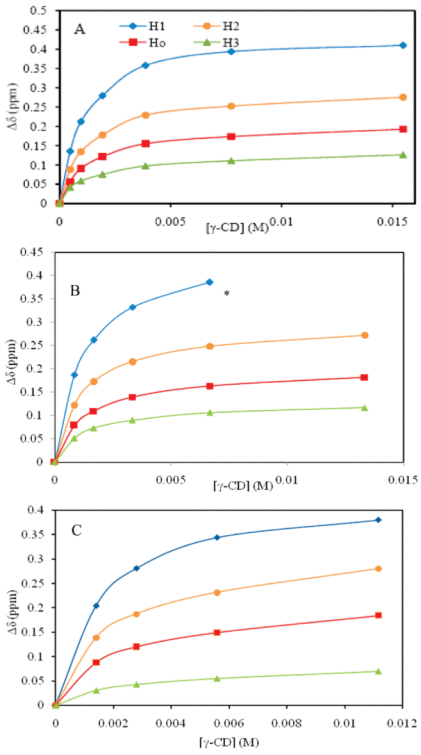


Figure 6.

Chemical shift change of hydrogens on MCLR as a function of [γ-CD] by complexation of MCLR (A), MCRR (B), NOD (C), and γ-CD at the molar ratio of 1:12 in D_2O solution. *The peak after this point was overlapped by other peaks and incalculable.

Table 1

Chemical Shift Difference Data in the Function of [γ-CD] for Binding Curve at the Ratio of [MCLR]:[γ-CD], 1:12

	Δδ (ppm)			
γ-CD (mM)	H_o	H_1	H_2	H_3
0.48	0.06	0.14	0.09	0.04
0.96	0.09	0.21	0.13	0.06
1.9	0.12	0.28	0.18	0.07
3.9	0.15	0.36	0.23	0.09
7.7	0.17	0.39	0.25	0.11
15.5	0.19	0.41	0.28	0.12

Table 2

Parameters for the Complexation of γ-CD and MCLR, MCRR, and NOD, respectively

	parameter	H_o	H_1	H_2	H_3
MCLR	Δ_{max} (ppm)	0.20	0.45	0.29	0.12
	K (M^{-1})	1014	1112	1155	902
MCRR	Δ_{max} (ppm)	0.19	0.45	0.29	0.13
	K (M^{-1})	858	739	856	884
NOD	Δ_{max} (ppm)	0.21	0.44	0.31	0.08
	K (M^{-1})	538	757	643	506