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ARTICLE

Cholic acid dimers as invertible amphiphilic pockets: synthesis, molecular modeling, and inclusion studies

Meng Zhang, Nicolas Levaray, Josée R. Daniel, Karen C. Waldron, and X.X. Zhu

Abstract: Two dimers of cholic acid were synthesized through simple covalent linkers. The dimers form invertible molecular pockets in media of different polarity; hydrophobic pockets are formed in water and hydrophilic pockets are formed in organic media. Fluorescence studies show that pockets formed by these dimers can serve as invertible hosts for the hydrophobic guest pyrene and the hydrophilic guest coumarin 343. The molecular pocket also enhances dissolution of the weakly soluble cresol red sodium salt in organic media. Molecular modeling was performed to better understand the host–guest complexation process of the invertible amphiphilic pockets. The calculated free energy changes indicate that the two dimers form the most stable complexes with coumarin 343 at a host to guest ratio of 2:2, whereas the host to guest ratio differs in the formation of complexes with pyrene for the two dimers. The dimer with the shorter, less flexible linker seems to form host–guest complexes that are more stable in both water and organic solvents.

Key words: cholic acid dimers, molecular pockets, host–guest, molecular modeling.

Résumé : Nous avons synthétisé deux dimères de l'acide cholique à l'aide de coupleurs covalents simples. Les dimères forment des pochettes moléculaires réversibles dans des milieux de différents degrés de polarité; les pochettes hydrophobes se forment dans l'eau, et les pochettes hydrophiles, en milieu organique. Des études de fluorescence montrent que les pochettes formées par ces dimères peuvent servir d'hôtes réversibles au pyrène comme invité hydrophobe, et à la coumarine 343 comme invité hydrophile. La pochette moléculaire permet aussi de dissoudre plus facilement le sel de sodium du rouge de crésol, un composé peu soluble en milieu organique. Nous avons réalisé une étude de modélisation moléculaire afin de mieux comprendre le processus de complexation hôte–invité des pochettes amphiphiles réversibles. Les calculs des variations de l'énergie libre indiquent que les deux dimères forment les complexes les plus stables avec la coumarine 343, selon un rapport hôte–invité de 2:2. Cependant, dans le cas de la formation des complexes avec le pyrène, les calculs indiquent un rapport hôte-invité différent pour les deux dimères. Le dimère possédant le coupleur le plus court et le moins flexible semble former des complexes hôte–invité qui sont plus stables tant dans l'eau que dans les solvants organiques. [Traduit par la Rédaction]

Mots-clés : dimères de l'acide cholique, alvéoles moléculaires, hôte–invité, modélisation moléculaire.

Introduction

Bile acids are natural amphiphilic compounds that exist in the digestive tract of humans and animals.[1,2] They possess a convex hydrophobic face bearing three methyl groups and a concave hydrophilic face with hydroxyl and carboxylic acid groups.[3] Bile acids have been used to prepare molecular devices for use as drug delivery agents,[4–6] sensors for metal ions,[7,8] and ion transporters.[9–11] Bile acids also serve as attractive building blocks in the construction of star-shaped derivatives known as molecular pockets or umbrellas.[12–17] Due to their amphiphilicity, bile acid based materials are capable of responding to changes in solvent polarity by exposing either their hydrophobic or hydrophilic faces.[18,19]

Our group has previously synthesized dimers, trimers, and tetramers based on cholic acid,[7,20–23] the most abundant bile acid. These oligomers, possessing different linkages between cholic acid units, can form invertible pockets for binding and encapsulating metal ions and hydrophobic or hydrophilic species. The effect of the linker length on the stability of such complexes remains to be investigated. To this end, we have designed and synthesized two new dimers based on cholic acid through a shorter linker (dimer **1**, with an alkyl spacer of nine atoms comprising a tertiary amine and a butylene spacer) and a longer linker (dimer **2**, with a spacer of 15 atoms comprising two amide groups and a secondary amine).

Molecular modeling, often used in pharmacokinetics and in the study of biological receptor–ligand geometry, has been shown to be useful for investigating the dynamics and geometry of host–guest complexes[24] such as cyclodextrin[25] and calixarene,[26] and was thus applied to the cholic acid dimers. The combination of experimental and computational studies has been recognized as a powerful tool[27] to elucidate the effect of solvation energy,[28] ionic interactions[29] and insertion dynamics.[30,31] The two dimers used in this work were designed to have uncomplicated linear linkers to simplify the molecular modeling and to obtain a better understanding of the host–guest interaction in relation to the inclusion properties of the molecular pockets and solvation effects. For the guest molecules, we have selected polarity-sensitive probes such as pyrene, coumarin 343, and cresol red sodium salt.

Experimental

Materials and methods

All chemicals were purchased from Sigma-Aldrich and used without further purification, unless otherwise specified.

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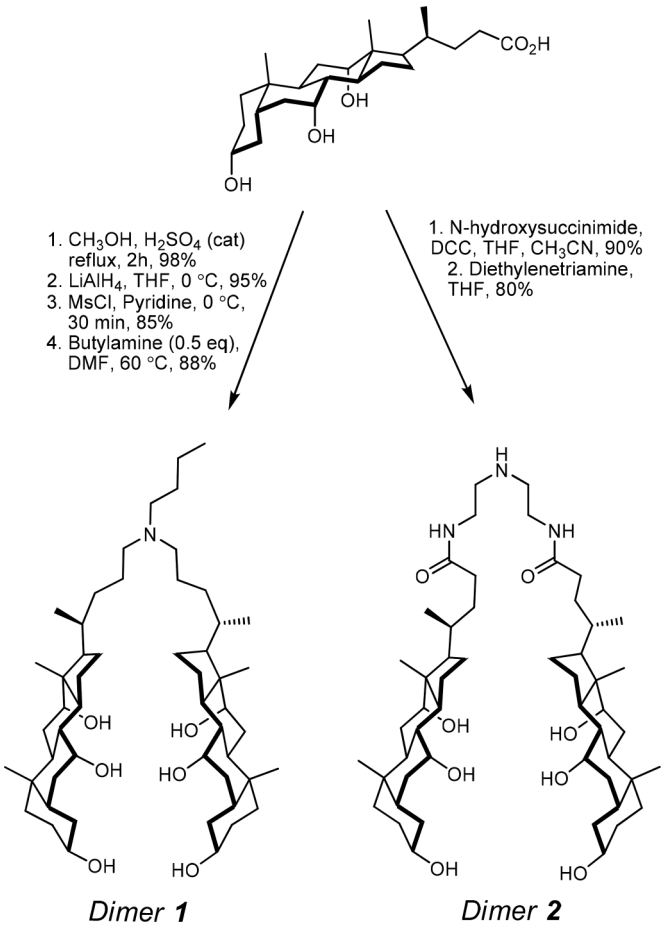
Scheme 1. Synthesis of dimers **1** and **2**.



Cholic acid methyl ester,[32] the alcohol derivative from the reduction of cholic acid, mesyl cholate,[33] and cholic acid succinimide ester[34] were synthesized according to the literature with minor modifications for dimer **2**.[35] The synthetic pathway for dimer **1** started with esterification, followed by reduction and mesylation of cholic acid. After these three steps, two equivalents of mesylated cholic acid were reacted with butylamine to form dimer **1** with a global yield of 70%. The synthetic pathway for dimer **2** was first published by Salunke et al.[35] and slightly modified in this work. Cholic acid was first activated by *N*-hydroxysuccinimide[34] and then reacted with diethylenetriamine to afford dimer **2** with a global yield of 67% (Scheme 1).

Dimer 1

To a solution of mesyl cholate (2.5 g, 5.28 mmol) in anhydrous dimethylformamide (DMF, 25 mL), butylamine (250 μL, 2.52 mmol) was added. The reaction mixture was stirred at 60 °C for 12 h. After cooling, water (100 mL) and ethyl acetate (100 mL) were added and the mixture was separated. The aqueous layer was extracted twice with ethyl acetate (2 × 50 mL). The organic layer was washed with brine, dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Flash chromatography on silica gel (80/20 hexane – ethyl acetate) afforded dimer **1** as a solid (1.85 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 4.06–4.00 (m, 2H, CHOH), 3.96–3.91 (m, 2H, CHOH), 3.90–3.86 (m, 2H, CHOH), 3.32–3.21 (m, 4H, CH_2N), 2.61–2.52 (m, 2H, CH_2N), 2.18–2.11 (m, 2H), 2.08–1.10 (m, 71H), 1.02 (d, *J* = 6.60 Hz, 6H, CH_3), 0.96 (s, 6H, CH_3), 0.73 (s, 6H, CH_3) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, δ): 77.20, 72.90, 68.16, 58.43, 51.56, 47.18, 46.30, 41.83, 39.43, 36.53, 35.13, 34.96, 33.86, 32.96, 32.80, 32.71, 30.92, 30.43, 29.53, 28.50, 27.42, 26.30, 25.52, 24.53, 23.08, 22.92, 22.59, 17.64 ppm. MS *m*/*z*, 826.689 $[M + H]^+$, 848.561 $[M + Na]^+$.

Dimer 2

To a solution of diethylenetriamine (440 μL, 4 mmol) in tetrahydrofuran (THF, 200 mL), cholic acid succinimide ester (4.04 g, 8 mmol) in THF (40 mL) was added dropwise. The reaction mixture was stirred at room temperature for 10 h and then filtered. The crude product was obtained by evaporation of the filtrate under reduced pressure and purified by flash chromatography on neutral aluminium oxide (80/20 chloroform–methanol). Dimer **2** was obtained as a white solid (2.58 g, 73%). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 4.32 (d, J = 4.18 Hz, 2H, OH), 4.10 (d, J = 3.33, 2H, OH), 4.01 (d, J = 3.35, 2H, OH), 3.78 (m, 2H, CHOH), 3.62 (m, 2H, CHOH), 3.17 (m, 2H, CHOH), 3.10 (q, J1 = J2 = 6.24, J3 = 6.16, 4H, $CONHCH_2$), 2.58 (t, J1 = J2 = 6.44, 4H, CH_2NH), 0.92 (d, J = 6.36, 6H, CH_3), 0.81 (s, 6H, CH_3), 0.58 (s, 6H, CH3) ppm. ^{13}C NMR (100 MHz, DMSO-d_6, δ),: 172.81, 70.98, 70.40, 66.21, 48.12, 46.10, 45.69, 41.47, 41.32, 38.14, 35.26, 35.13, 34.84, 34.33, 32.48, 31.62, 30.34, 28.51, 27.26, 26.17, 22.77, 22.57, 17.07, 12.29 ppm. MS *m*/*z*, 884.673 $[M + H]^+$, 906.656 $[M + Na]^+$.

Fluorescence spectroscopy

A stock solution of pyrene was prepared in methanol (2 mmol/L) then diluted in Milli-Q water (30 μL in 200 mL, 0.3 μmol/L). A stock solution of coumarin 343 was prepared in anhydrous THF (2 mmol/L) and then diluted with THF (30 μL in 200 mL, 0.3 μmol/L). Stock solutions of dimers **1** and **2** (25 μmol/L) were made separately in the solutions of either probe (0.3 μmol/L) and diluted to obtain desired concentrations for the experiments, in which the two probes were studied separately or as a mixture in water. The water solutions were purged with N_2 for 60 min to remove the trace of methanol used initially to dissolve pyrene. The fluorescence spectra of pyrene and coumarin 343 were recorded at room temperature on a Varian fluorescence spectrophotometer equipped with a Xe-900 lamp using excitation wavelengths of 336 nm and 410 nm for pyrene and coumarin 343, respectively. The bandwidths for excitation and emission were both 2.5 nm for pyrene, whereas the excitation and emission bandwidths for coumarin 343 were 5 and 4 nm, respectively.

Absorbance spectroscopy

A stock solution of dimer **1** (2 mmol/L) in chloroform and a solution of dimer **2** in chloroform–methanol (49/1, v/v, where the small quantity of methanol was added to help dissolve the dimer) were prepared and diluted to obtain a series of concentrations ranging from 0 to 1.0 mmol/L for dimer **1** and from 0 to 2.0 mmol/L for dimer **2**. A fixed amount (10 mg) of cresol red sodium salt was added to each of the solutions, which were then shaken at room temperature for 24 h to reach equilibrium and filtered through a 0.2 μm PTFE filter. The filtrates were diluted with methanol, and the absorbance spectra were recorded on a Cary Series UV–vis – NIR spectrophotometer (Agilent Technologies). The dissolved concentration of cresol red probe was calculated based on the maximum absorption (426 nm) for each solution with ε_{max} = 17 500 $(mol/L)^{-1}$ cm^{-1}.[36]

Molecular modeling

A 150 ps molecular dynamics study was performed using HyperChem (Hypercube Inc.) for the formation of the inclusion complexes; dynamics were conducted at 273 K. A docking approach for molecular mechanics optimization was used so that the free energy of binding could be estimated. Calculations were performed using the AMBER Force Field method and using dielectric constants (THF, 7.6; water, 80) to mimic the solvents. Initial geometric optimization was performed with the optimization algorithm Polak–Ribière with a convergence of 0.01 for the energy minimization.

Results and discussion

The formation of invertible molecular pockets was confirmed using the guest compounds pyrene and coumarin 343. To demonstrate the existence of the hydrophobic pocket in aqueous media,

Fig. 1. Fluorescence spectra of each probe (0.3 μmol/L) in the absence and presence of the dimers added at various concentrations (solid lines, 0 μmol/L; dashed lines, 5 μmol/L; dotted lines, 25 μmol/L). (A) Dimer **1** with pyrene in water; (B) dimer **1** with coumarin 343 in THF; (C) dimer **2** with pyrene in water; (D) dimer **2** with coumarin 343 in THF. [Colour online.]

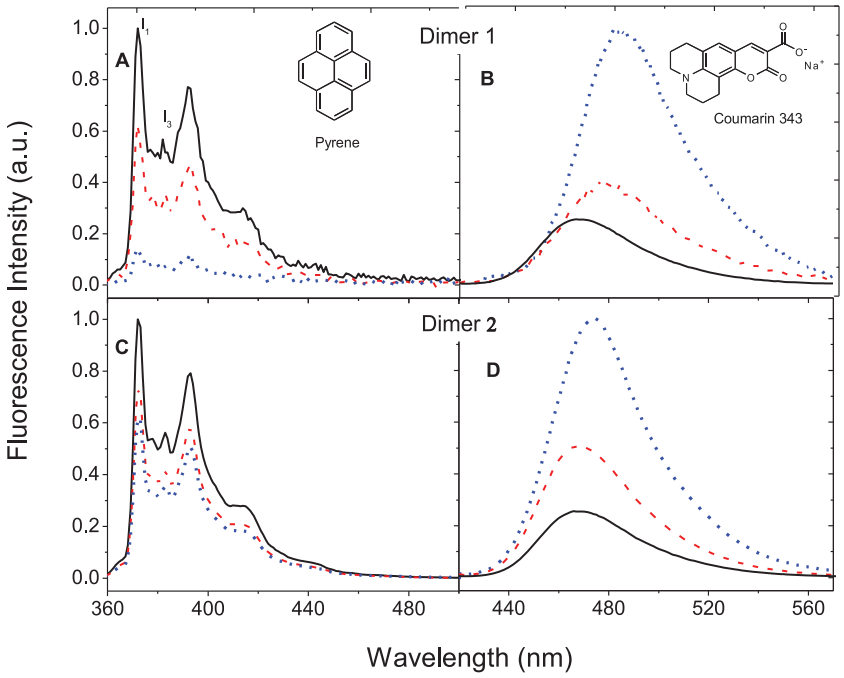


Fig. 2. The concentration of a hydrophilic probe, cresol red sodium salt, in pure chloroform and a mixture of chloroform and methanol (49/1, v/v) in the presence of either dimer **1** or **2** at varying concentrations. [Colour online.]

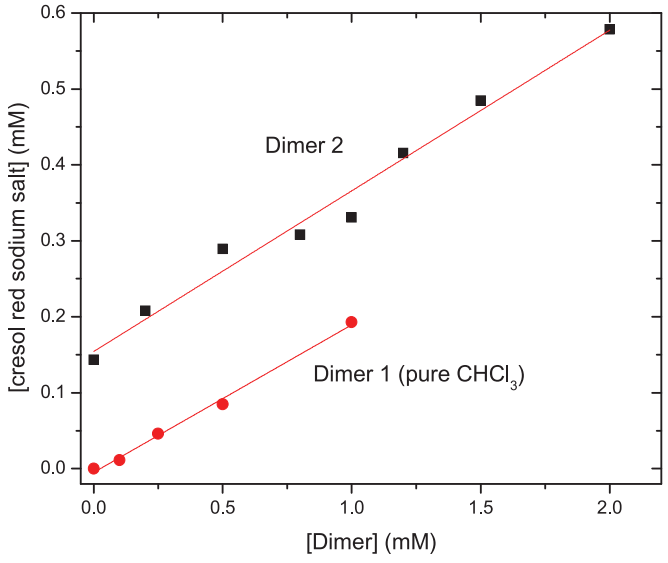
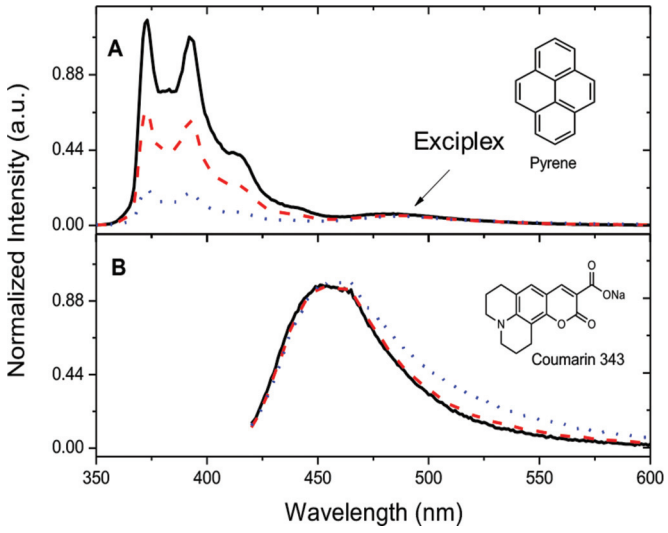


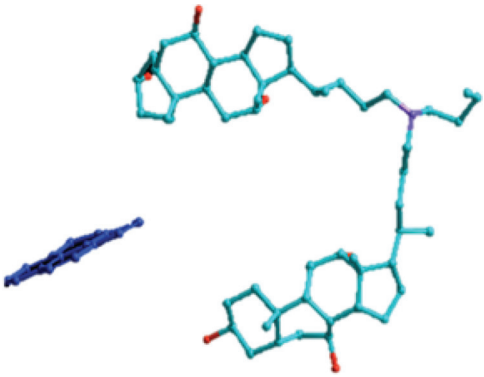
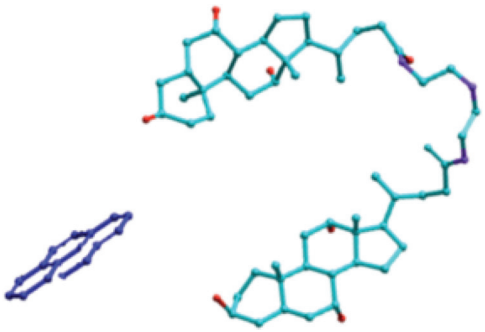
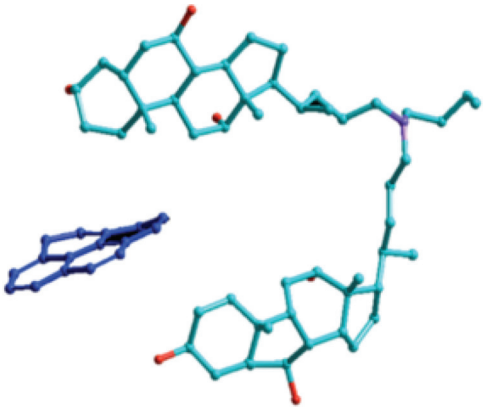
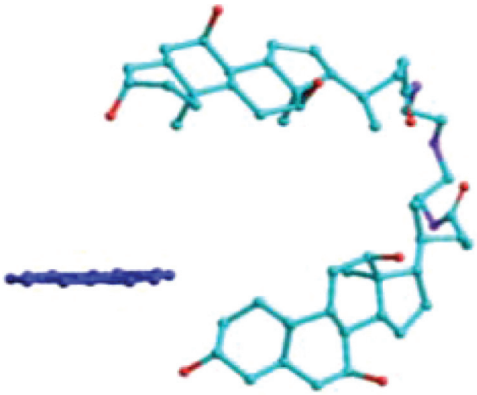
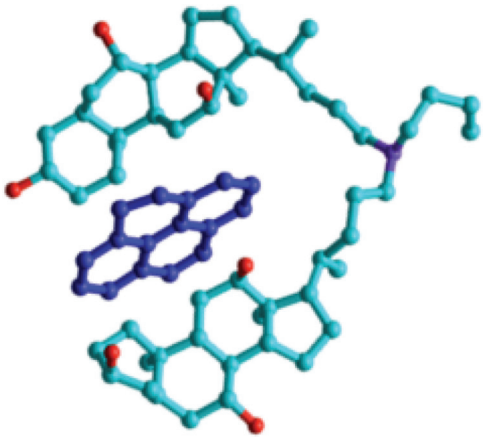
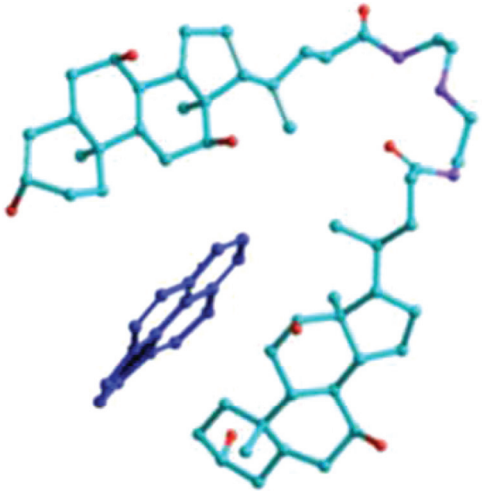
Fig. 3. Fluorescence spectra of (A) pyrene and (B) coumarin 343, mixed together in water (both concentrations at 0.3 μmol/L) for increasing concentrations of dimer **1** (solid lines, 0 μmol/L; dashed lines, 5 μmol/L; dotted lines, 25 μmol/L). [Colour online.]



pyrene was used as the probe. The intensity ratio of the third emission peak (I_3 at 383 nm) to the first emission peak (I_1 at 372 nm) in the pyrene fluorescence spectrum is known to increase when pyrene moves from a hydrophilic environment to a more hydrophobic one.[37,38] A slight increase in the ratio of I_3 to I_1 was indeed observed for pyrene in the presence of both dimers (Figs. 1A and 1C). The same figures also show an overall decrease of fluorescence intensity when dimer **1** or **2** was added, which may be caused by the precipitation that was observed during mixing. This suggests that the host–guest complex of pyrene with each dimer has very low solubility in the aqueous media. The less polar dimer **1**, which bears a butyl group, showed a greater reduction of intensity in the fluorescence spectra. It was reported previously that the amine groups on the dimers did not quench the fluorescence intensity of pyrene.[20,23] The decreased intensity of pyrene fluorescence spectra in the presence of either of the dimers may be caused by the phase separation of the dimer–pyrene complex in the aqueous solutions, which will be discussed later.

To demonstrate the existence of the hydrophilic pocket in a relatively nonpolar medium, coumarin 343 was added as it is a typical probe used to identify polar environments.[39,40] Both dimers and the coumarin 343 are quite soluble in THF, and there was no precipitate observed throughout the fluorescence study in

Table 1. Molecular dynamics of host–guest approach for dimer **1** and dimer **2** (host) with pyrene (guest) in water at different stages of complexation.

Time (ps)	Dimer **1**	Dimer **2**
0		
0.5		
20		

THF, in contrast to the fluorescence tests using pyrene. A red shift, accompanied by a significant increase in fluorescence intensity, is expected when the environment surrounding coumarin 343 becomes more polar or hydrophilic. The fluorescence spectrum of coumarin 343 in THF showed weak fluorescence intensity, with a maximum around 470 nm in the absence of the dimers (solid line in Figs. 1B and 1D). The addition of cholic acid dimer caused an increase in the fluorescence intensity of coumarin 343, and the maxima of the spectra shifted to 485 and 474 nm when in the presence of dimers **1** and **2**, respectively. These results indicate that coumarin 343 enters the hydrophilic pocket of the dimer to form a host–guest complex. Dimer **1** may form a more polar pocket in THF for the accommodation of coumarin 343 than dimer **2**, as evidenced by the larger maximum emission shift on the fluorescence spectra, as shown in Figs. 1B and 1D.

Cresol red sodium salt, another hydrophilic probe investigated, presents limited solubility in organic solvents such as chloroform, and thus its apparent dissolution in such nonpolar media can indicate the presence of a localized hydrophilic environment.[41,42] Therefore, the formation of host–guest complexes with the cholic acid dimers providing a hydrophilic cavity that helps dissolve a polar probe in organic solvents was investigated. The absorbance of homogeneous solutions filtered from a mixture containing 10 mg of the solid probe cresol red sodium salt and increasing concentrations of dimer were measured at 426 nm, then the absolute absorbance was converted to the concentration of solubi-

lized probe. Figure 2 shows that the apparent solubility of the cresol red probe in either pure chloroform or a mixture of chloroform and methanol (49/1, v/v) increased significantly with increasing concentration of each dimer. The linear relationship between the concentration of dimer and that of the dissolved probe confirms the formation of a hydrophilic pocket by both dimers in this relatively nonpolar solvent.

We also investigated the behavior of a mixture of both pyrene and coumarin 343 in water as a function of dimer **1** concentration. This experiment was designed to test whether dimer **1** interacts preferentially with one of the probes, with both probes simultaneously, or if the dissolved probe will interact with the pocket. Different excitation wavelengths were used to follow the fluorescence spectra of the two probes separately in the same solution. The fluorescence spectra of pyrene (Fig. 3A) remained almost identical to those of pyrene alone in aqueous solution (Fig. 1A) except for a red-shifted fluorescence emission band of pyrene at about 470 nm that was not detected for pyrene alone. The existence of such a red-shifted band in the presence of coumarin 343 may indicate the formation of a pyrene–coumarin exciplex via π–π stacking, which has been previously reported to appear at a similar shift of about 100 nm.[43] Precipitation occurred, as with pyrene probe alone, with increasing concentration of dimer **1**. The fluorescence spectrum of coumarin 343 (Fig. 3B) showed no significant change with increasing concentrations of dimer **1**, indicating that there was no observable interaction between coumarin 343 and dimer **1** in water.

The binding stoichiometry with a specific guest probe is an important characteristic for the evaluation of host molecular pocket size. If a molecular pocket can encapsulate the probe molecule at a binding ratio of 1:*n* (host:guest), the value *n* can be calculated by fitting the fluorescence signal of the guest versus the concentration of the host according to the Benesi–Hildebrand equation.[20] However, the fluorescence studies in this work were not able to calculate valid values of host:guest binding ratios with either probe for different reasons. Firstly, due to the low solubility in water of both dimers and pyrene, the dimer:pyrene complexes were observed to form suspensions in aqueous solutions, resulting in only a decrease of overall fluorescence intensities, instead of an obvious change in the vibronic band intensity ratio. Secondly, although both dimers and coumarin 343 formed homogeneous solutions in THF, no effective binding ratio could be obtained from the Benesi–Hildebrand equation by fitting either the fluorescence intensities or the emission shift. This indicates that both dimers may interact with coumarin 343 to form complexes other than a simple 1:*n* (host:guest) binding. As an alternative method for the evaluation of binding ratios, we used molecular modeling to speculate the structure of host–guest complexes of these two dimers with the probes pyrene and coumarin 343.

Molecular modeling of the host–guest complexes with a 150 ps molecular dynamics was carried out. Table 1 shows the simulation results of the molecular dynamics of dimers **1** and **2** with pyrene in water up to 20 ps equilibration time, beyond which no significant changes were observed. The guest molecule was placed along a vector orthogonal to the cavity's opening. As the simulation progresses, the guest molecule moves into the cholic acid dimer pocket, mainly due to hydrophobic forces.[44] In the last stage of the approach, pyrene seems to penetrate further inside the cavity of dimer **1** than that of dimer **2**.

To estimate the free energy of binding, a docking approach for molecular mechanics optimization was used. Molecular docking is typically used to predict the structure of an intermolecular complex of two molecules. The preferred orientation of one molecule is calculated in the presence of the other molecule when they interact to form a stable complex. The complexation energies of the hydrophobic guest (pyrene) and hydrophilic guest (coumarin 343) with dimers **1** and **2** were calculated by energy minimization in vacuum and in water. The experiments were conducted

Table 2. Computed free energy changes (in kJ/mol) at 0 K for the complexation of guests (pyrene or coumarin 343) with host dimers **1** and **2** in vacuum and in a solvent.

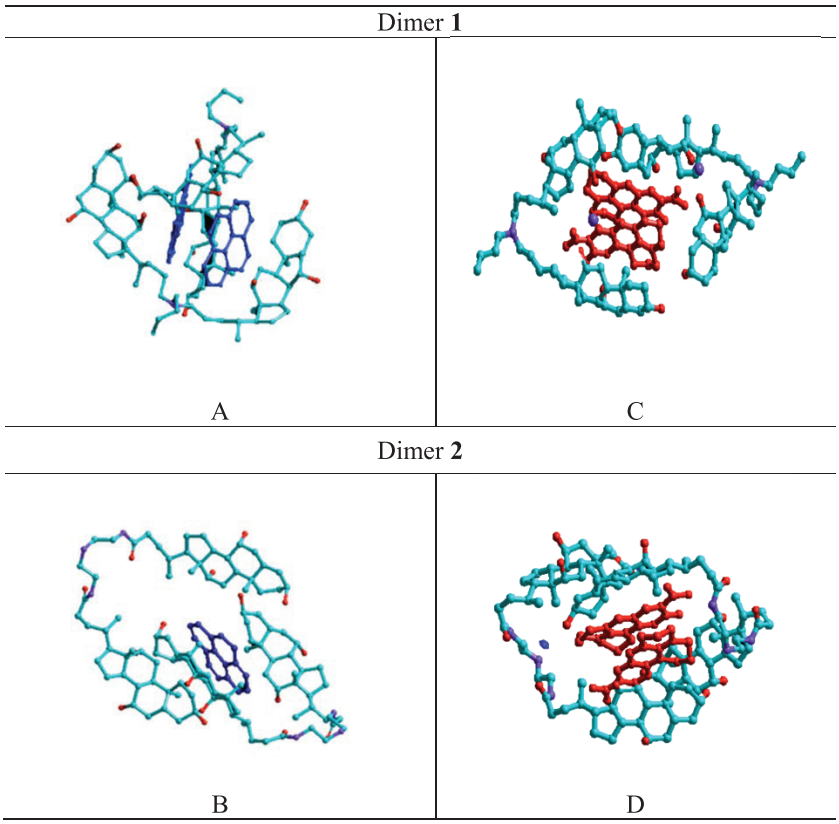
		Guest			
		Pyrene		Coumarin 343	
Host	Host:guest	In vacuum	In water	In vacuum	In THF
Dimer **1**	1:1	–91.5	–82.4	–98.1	–70.5
	1:2	–85.2	–66.7	–109.5	–65.6
	2:1	–112.9	–112.0	–160.4	–117.7
	2:2	**–193.7**	**–174.7**	**–192.0**	**–162.2**
Dimer **2**	1:1	–91.2	–81.1	–68.1	–61.9
	1:2	**–111.6**	–60.8	–97.0	–65.6
	2:1	–104.7	**–119.4**	–119.8	–108.7
	2:2	–103.5	–108.0	**–161.7**	**–141.0**

Note: The bold numbers illustrate the most stable complexes in each case.

in 1:1, 1:2, 2:1, and 2:2 molar ratios of dimer to guest probe to compare stabilities (Table 2). For example, the 1:1 ratio represents one host molecule (dimer) encapsulating one guest molecule, whereas the 2:2 ratio represents a situation where two dimers are needed to have inclusion of two stacked guest molecules. Table 2 shows that for dimer **1**, complexation with more than one pyrene molecule is favored (ratio of 2:2). A different behavior was predicted for dimer **2**, for which the 2:1 ratio yields a more stable complex (–119 kJ/mol). Such binding ratios may favor the formation of dimer–pyrene complexes of a large size, which may be phase separated from aqueous solutions due to the low solubility of both dimers in water. The pyrene encapsulated in dimers cannot be detected by fluorescence spectroscopy and only a fraction of unencapsulated pyrene remains in the solution to account for the reduced intensity across the whole fluorescence spectrum. This is further confirmed by the more reduced fluorescence intensity for the more hydrophobic dimer **1** versus dimer **2**. For the interaction of the inverted pockets with coumarin 343 in THF, a 2:2 host–guest ratio seems to provide the most stable inclusion complex for both dimers (–162 and –141 kJ/mol, respectively). This explains why no effective binding ratio could be obtained for 1:*n* complexes using the Benesi–Hildebrand equation. A comparison of the computed free energy changes for the complexes with the same guests listed in Table 2 seems to indicate that dimer **1**, with a shorter and less flexible linker, forms more stable complexes with pyrene in water (–175 vs –119 kJ/mol) and with coumarin 343 in THF (–162 vs –141 kJ/mol) than dimer **2**.

Figure 4 shows pyrene and coumarin 343 as guests inside the pockets of dimers **1** and **2** for the most stable conformations in solvent based on the computed Δ*G* changes (Table 1). The 2:2 mixture of the complex of dimer **1** with pyrene, which showed better stability (–174.7 kJ/mol), reveals the two molecules of pyrene are facing each other surrounded by four cholic acid moieties (Fig. 4A). In the dimer **2** : pyrene complex, two molecules of dimer **2** are needed to encapsulate one hydrophobic probe (Fig. 4B). The π–π interaction in the stacked pyrene molecules in the 2:2 complexes is likely responsible for the better stability of the dimer **1** complex compared with a 1:1 ratio. Simulation of the host:guest complexes with coumarin 343 show two guest molecules facing each other in pockets formed by two molecules of either dimer **1** or dimer **2** (Figs. 4C and 4D). It is interesting to note that the improvement in stability (calculated Δ*G* values, Table 2) of the dimer:coumarin 343 complexes was identical (i.e., 2.4 fold) for the two dimers when going from a ratio of 1:1 to 2:2. This is in contrast to the dimer: pyrene complexes for which the stability (Δ*G* values) improved over 2 fold for dimer **1** and only 1.3 fold for dimer **2** for the same change from a ratio of 1:1 to 2:2. In addition, the stabilities differ by only 10% between the 2:1 and 2:2 complexes of dimer **2** with pyrene in water and by only 1% in vacuum (Table 2) compared with differences of 56% and 71% in water and vacuum, respectively, for

Fig. 4. Molecular modeling of the host–guest systems. (A) dimer **1** : pyrene = 2:2, (B) dimer **2** : pyrene = 2:1, (C) dimer **1** : coumarin 343 = 2:2, (D) dimer **2** : coumarin 343 = 2:2. [Colour online.]



dimer **1**. The longer linker in dimer **2** may lead to some uncertainty during energy minimization over the 150 ps dynamics such that the probability of forming a 2:2 complex is similar to that of the 2:1 complex for dimer **2** : pyrene.

Conclusion

Two dimers of cholic acid with simple linkers were synthesized and used in the study of host–guest inclusion complexation with both hydrophobic and hydrophilic guest molecules in water and organic media. The pockets may be induced to invert themselves by switching the polarity of the media from water to organic solvents. This was confirmed by the behavior of three guest molecules of different polarity. Molecular modeling helped to gain insight into the behavior of the dimers with the hydrophobic (pyrene) and hydrophilic (coumarin 343) guests. Dimer **1** formed the most energetically stable complex with pyrene with a host–guest ratio of 2:2, whereas dimer **2** formed a less stable 2:2 complex with pyrene, as indicated by a 38% difference in calculated free energy. The modeling showed that dimer **2** favored the formation of a 2:1 complex with pyrene but only by a slight margin compared with the 2:2 complex. With coumarin 343, both dimers favored the formation of host–guest complexes with a ratio of 2:2, with dimer **1** showing 15% better stability. The results suggest that the host–guest complex stability may depend on the length and the functionality of the linker between the two cholic acid moieties in the dimer. Therefore, changes in the linker may allow fine-tuning of the host–guest complexation behavior for specific applications of these biomaterials such as selective extraction and release of environmentally or biologically relevant molecules.

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