New amphiphilic derivatives of cyclodextrins for the purpose of insertion in biological membranes: the "Cup and Ball" molecules

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Structural investigations of β-cyclodextrin derivatives obtained in solution by nucleophilic substitution with diamino alkanes *N*-protected by the bulky Boc† group are presented. These new compounds are models for amphiphilic transporters to be included in organized phases such as liposomes. It is shown here that the terminal bulky hydrophobic Boc group precludes inclusion of the chain in the cavity of the cyclodextrin. Two-dimensional NMR experiments are used in conjunction with competition with external guests to assess the general concept of "Cup and Ball" molecules. The incorporation of these new amphiphilic cyclodextrins into preorganized molecular systems such as phospholipidic vesicles is investigated using ^{1}H, ^{13}C and ^{31}P NMR. The validation and quantification of this incorporation are further evidenced by differential scanning calorimetry and immunological methods using specific antibodies.

Introduction

The continuing challenge of using cyclooligosaccharides (cyclodextrins or CDs) for solubilization and drugs targeting has led to the preparation of a wide variety of chemically modified derivatives in order to improve the properties of these host molecules.[1] A possible approach as far as pharmaceutical applications are concerned would be to combine the size specificity of cyclodextrins with the use of the transport properties of organized structures such as vesicles, liposomes or micelles.

Many amphiphilic cyclodextrins have been described in the literature. Per-6-alkylamino-6-deoxy-β-CD derivatives[2] have been shown to generate highly stable Langmuir–Blodgett films in pure form or in conjunction with phospholipids.[3] Synthetic routes used to afford such molecules involved the synthesis of heptakis-6-*O*-*p*-tosyl-cyclomaltoheptaose‡ which can only be obtained in chemically pure form after tedious separation.[4] The formation of amphiphilic cyclodextrins through esterification or etherification at the secondary hydroxy face has also been described.[5] On the other hand, the "Bouquet" family based on a β-cyclodextrin core with per-heptakis-2,6 esterification[6] has been shown to be incorporated into lipid membranes where they are shown to act as artificial channels of the "chundle"-type structures.[7]

However, in all cases previously described, the β-cyclodextrin has been per-6, per-3, per-2,3 or per-2,6 substituted by relatively bulky groups leading to steric hindrance effects. Under these conditions the inclusion properties of the cavity of the β-cyclodextrin could be partially lost. These types of derivatives imply an extensive purification or a long synthesis leading often to very low yields of pure amphiphilic cyclodextrins. Moreover, these amphiphilic compounds are not soluble in aqueous media and lack biocompatibility, precluding their use in pharmaceutical applications.

These basic ideas have led to the synthesis and to the characterization of amphiphilic monosubstituted cyclodextrin derivatives. Petter *et al.*[8] have described a nucleophilic substitution of mono-6-*O*-*p*-tosyl-β-CD by an alkyl amine but the overall yield remains poor (5%). Molecular Lollipops[9] obtained in good yields were expected to combine the inclusion properties of cyclodextrins and the spontaneous formation of organized structures. However it has been demonstrated by proton NMR that self-inclusion processes are encountered in amphiphilic cyclodextrin derivatives such as lollipops. This unexpected behaviour strongly depends on the length of the aliphatic chain and can lead to inclusion complexes which are stable enough to preclude inclusion of external guests.

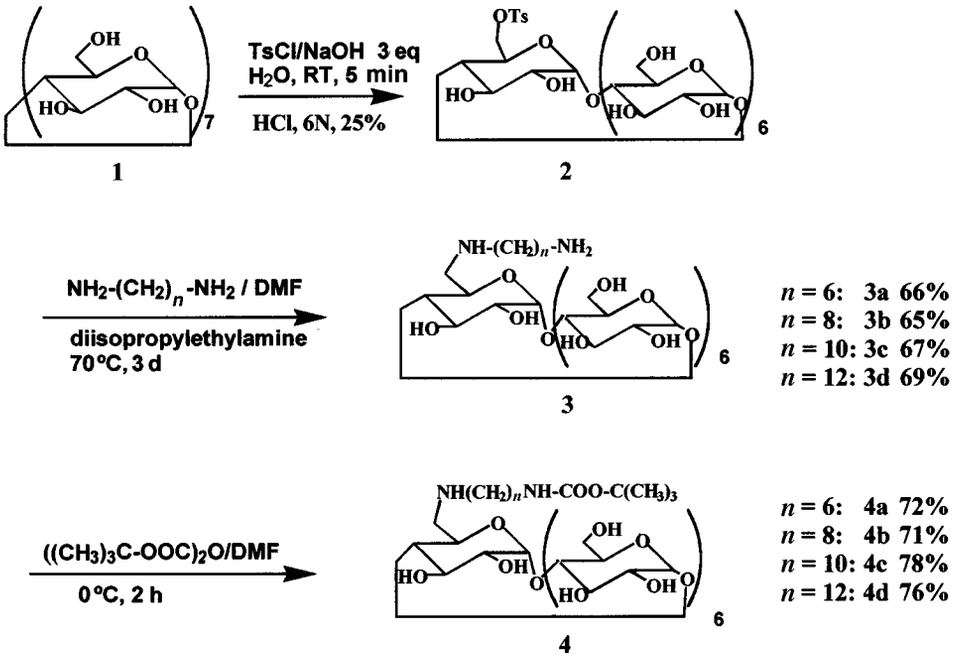
In order to prevent the amphiphilic cyclodextrins from undergoing the self-inclusion process, we now report on a general concept of "Cup and Ball" molecules. In these compounds, the grafted aliphatic chain of variable length is terminated by a bulky hydrophobic group hampering inclusion of the chain in the cavity.[10] These new compounds were prepared and fully characterized by NMR. This technique is used throughout to evidence the peculiar solution behaviour of these compounds in aqueous media. Their inclusion properties towards hydrophobic guests were evaluated by the same technique. We have investigated the incorporation of these molecules and their inclusion complexes into di-myristoyl-phosphatidylcholine (DMPC) vesicles and liposomes by the three following independent techniques: ^{31}P NMR in the liquid phase, immunological titration of β-CD derivatives and differential scanning calorimetry (DSC).

Synthesis

The described "Cup and Ball" derivatives were prepared by grafting a diamino alkane onto β-cyclodextrin. The primary amine was protected by a Boc group according to Scheme 1. Mono-6-*O*-*p*-tosyl-6-deoxy-cyclomaltoheptaose **2** was obtained in aqueous solution as already described[11] and converted into mono-*N*-aminoalkane-6-amino-6-deoxy-cyclomaltoheptaose **3** by nucleophilic substitution with the pertinent diamino alkane in dimethylformamide (DMF) and in the presence of 10 equivalents of diisopropylethylamine at 70 °C for 3 days.[10]

Protection of the *N*-terminal group of compounds **3** with di-*tert*-butyl dicarbonate was achieved in DMF at 0 °C for 2 h. The final material was purified by high pressure liquid chromatography (elution with MeOH–H_2O 40:60) and freeze-dried. The chemical structure of compounds **3a–d** and **4a–d** was confirmed by elemental analysis, NMR spectra and mass spectrometry (see Experimental section).

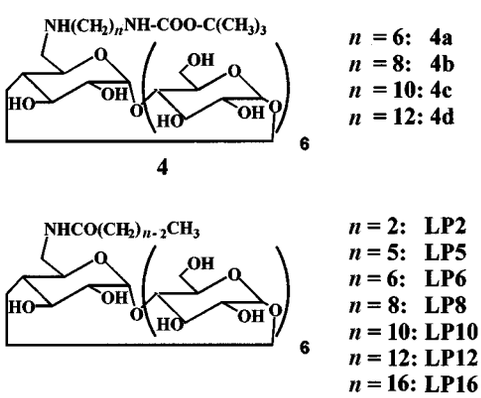
† Boc = *tert*-butyloxycarbonyl.
‡ *p*-Tosyl = toluene-*p*-sulfonyl.



Scheme 1

Solubility in water of the "Cup and Ball" cyclodextrin derivatives

The results of the solubility in water at 20 °C for **4a**–**d** are displayed in Table 1 and have to be compared with solubility data for β-cyclodextrin and Lollipop molecules (LP_n) (structures given in Scheme 2) under the same conditions.[9] The following



Scheme 2

major conclusions can be drawn. The "Cup and Ball" derivatives exhibit a much higher solubility in water than the parent β-cyclodextrin even for **4c** and **4d** which bear the largest hydrophobic moieties, although sonication was required to solubilize the latter compounds. Conversely, as described elsewhere,[9] compounds LP_n exhibit low solubility in water. For both families of β-cyclodextrin derivatives the solubility rapidly drops when the length of the aliphatic chain increases.

Considering the gain in solubility, *N*-Boc-aminoalkane-6-amino-6-deoxy-cyclomaltoheptaoses **4** appear to be more favourable than the 6-amido-alkyl-6-deoxy-cyclomaltoheptaoses LP_n even if the hydrophobic moiety seems to be larger.

Results and discussion

NMR investigations of the self-inclusion and inclusion properties of *N*-Boc-aminoalkane-6-amino-6-deoxy-cyclomaltoheptaoses 4

For the sake of clarity, the detailed NMR study will be presented for derivative **4a** only. All derivatives **4** exhibit the same properties. A large number of organic molecules are known to form inclusion complexes with cyclodextrins.[12] For the present purpose ASANa (sodium anthraquinone-2-sulfonate) has been selected since its inclusion in cyclodextrin has been extensively studied by NMR.[13] The molecular structure of this pertinent compound is displayed in Fig. 2. The association constant determined with β-CD is 850 M^{-1}. This compound was also selected on the basis of its aromatic character, which induces large shifts of proton NMR lines upon inclusion. These shifts, generally in the upfield direction, are due to the ring current effects and will mainly concern H_3 and H_5 protons of cyclodextrin if inclusion occurs. In this sense, it can be defined as an "organic shift reagent".[14] First, one-dimensional spectra of **4a** in the absence and in the presence of the potential guest will be compared. The results are presented in Fig. 1. It is observed that ASANa induces shifts consistent with inclusion.

Since the spectra are relatively complex owing to the lack of molecular symmetry, a more complete analysis was obtained from stepwise identification of protons of **4a** by successive two-dimensional RELAY experiments. Being located in a very specific spectral domain, anomeric protons were used as a starting point for stepwise assignments.[15] Fig. 2 shows contour plots obtained from a single RELAY experiment (taking magnetization from H_1 to H_3) on **4a** in the absence and in the presence of ASANa. Among the seven H_3 protons several have experienced very large upfield shifts upon addition of ASANa (up to 0.2

Table 1 Comparison of the solubilities in water of the native cyclodextrin, of Lollipops and of "Cup and Ball" under identical conditions

Compound	Solubility at 20 °C/mmol dm^{-3}
β-CD	15
LP2	17.2
LP5	7
LP6	1.8
LP8	1.1
LP10	1
LP12	0.7
LP16	0.6
4a ($n = 6$)	370
4b ($n = 8$)	180
4c ($n = 10$)	35
4d ($n = 12$)	20

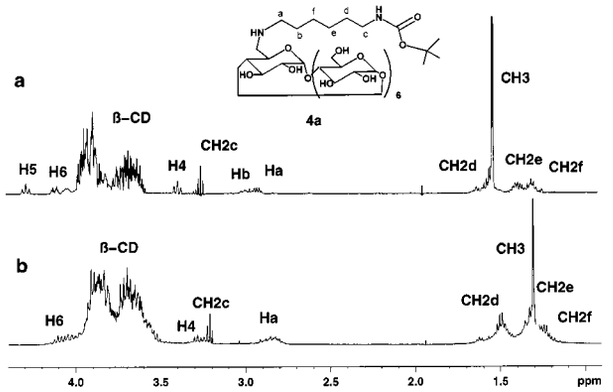
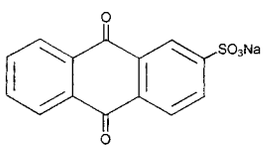


Fig. 1 Partial ^{1}H NMR spectra (298 K, 500 MHz, 10 mmol dm^{-3} in D_2O) of **4a** (a) alone and (b) in the presence of ASANa (10 mmol dm^{-3}).



ASANa

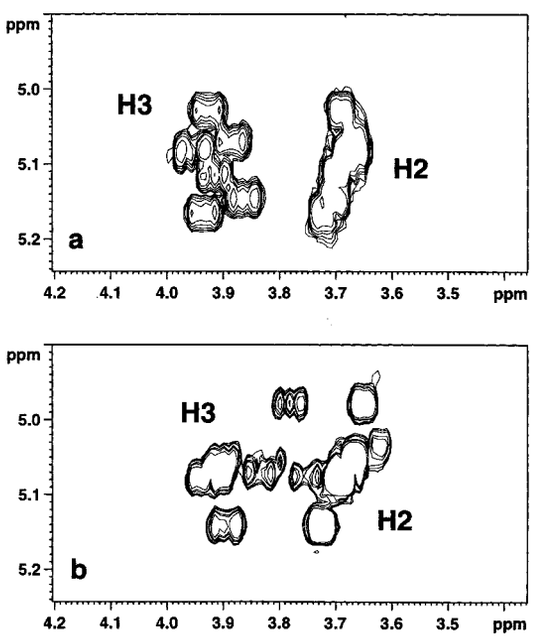


Fig. 2 Partial contour plots of RELAY experiments (D_2O, 298 K, 500 MHz) performed on **4a** (10 mmol dm^{-3}) (a) alone and (b) in the presence of ASANa (10 mmol dm^{-3}).

ppm). Since protons H_3 are located in the hydrophobic cavity, this observation fully proves that ASANa is included in the cavity with high efficiency. Furthermore, the large inequivalence of protons H_3 in terms of induced shifts implies a very specific binding of ASANa in the cavity.

Surprisingly, ASANa also induces shifts in the resonance of the methyl protons of the Boc group. This very large upfield shift upon addition of ASANa (up to 0.23 ppm) was quite unexpected since the *N*-terminal protected Boc group should not be implicated by the inclusion process.

More detailed indications concerning the geometry of this inclusion complex can be derived by evidencing spatial proximities between protons of the host and guest molecules. This can be achieved by investigation of dipolar interactions using two-dimensional T-ROESY experiments[16] since it was shown that this provides the most sensitive approach to the structural analysis of inclusion complexes with CD[17] in solution. As

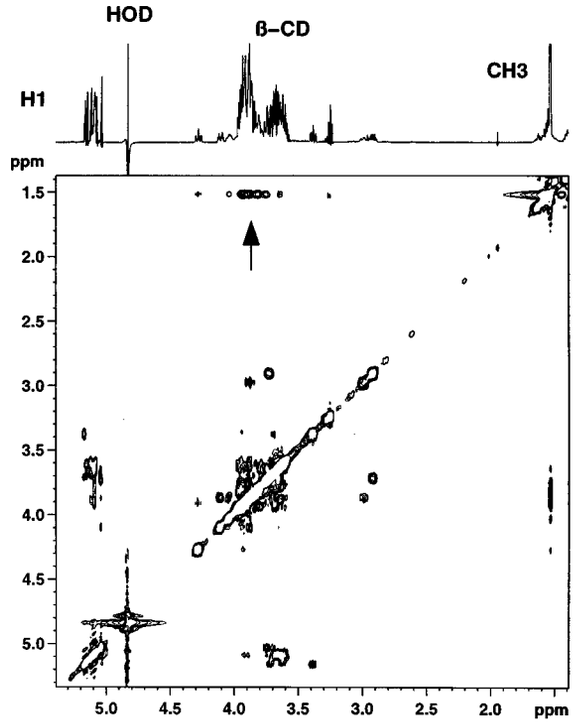


Fig. 3 Contour plot of T-ROESY experiment (D_2O, 298 K, 500 MHz, 300 ms spin-lock time at 18 dB attenuation) performed on a sample containing 10 mmol dm^{-3} of **4a**. Arrow indicates dipolar interactions between methyl group and protons H_5 and H_6 of β-CD moiety.

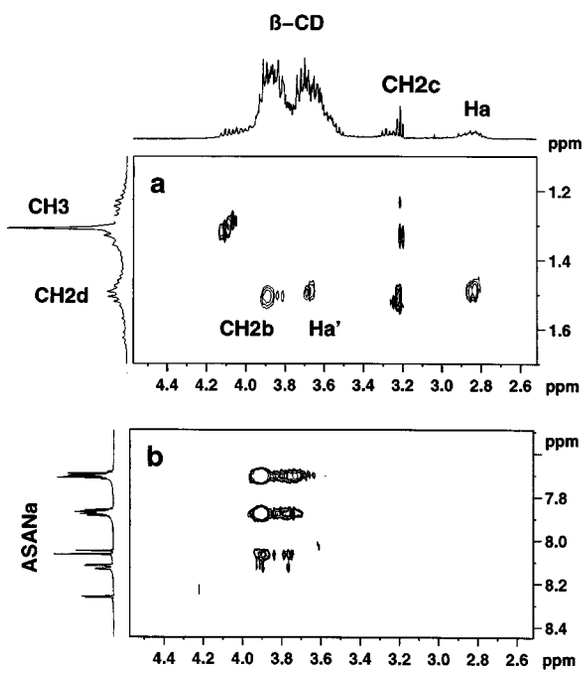


Fig. 4 Partial contour plots of T-ROESY experiment (D_2O, 298 K, 500 MHz, 300 ms spin-lock time at 18 dB attenuation) performed on a sample containing 10 mmol dm^{-3} of **4a** and 10 mmol dm^{-3} of ASANa. Areas of (a) aliphatic protons and (b) aromatic protons.

found in many other situations regarding inclusion complexes in water,[18] a 300 ms mixing time was selected to provide reliable dipolar cross-peaks with a minimal contribution of scalar transfer and spin diffusion. A typical example is displayed in Figs. 3 and 4 for **4a** alone and in the presence of ASANa. All non-diagonal peaks are indicative of spatial proximities between protons. Dipolar contacts are observed between protons H_3–H_5 of the cavity of **4a** and aromatic protons of ASANa indicating the formation of an inclusion complex. Moreover, in the absence of ASANa, dipolar interactions

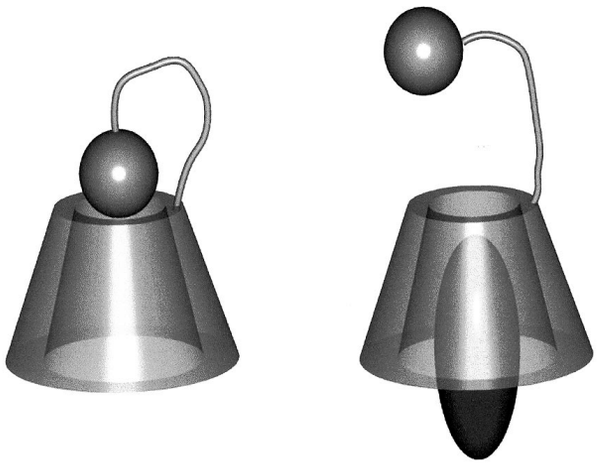


Fig. 5 Schematic representations of the "Cup and Ball" molecule alone and with a guest.

are observed between protons of the methyl of the Boc group and protons H_5 and H_6 of the cyclodextrin (Fig. 3). These interactions disappear upon addition of ASANa as observed in Fig. 4.

This suggests that the bulky Boc group comes close to the narrow primary hydroxy side in the absence of ASANa and that upon formation of an inclusion complex, the aliphatic chain is expelled from the cavity. This assumption is fully supported by the study of ^1H–^{13}C correlation experiments performed on **4a** alone and on the inclusion complex with ASANa under strictly identical experimental conditions. The difference of shifts experienced by the protons H_b of the guest molecule upon complex formation is very informative (data not shown). Indeed, protons H_b are isochronous for the ASANa–**4a** inclusion complex and strongly inequivalent for **4a** alone in aqueous solution. In the latter case, it can be concluded that the C_a–C_b–C_c bonds are locked in a single position. Conversely, in the ASANa–**4a** inclusion complex, conformational freedom is restored. As expected, the strong conformational strain observed in the **4a** derivative alone can be related to a self-inclusion process. Conversely, the conformational equilibrium observed in the inclusion complex is consistent with ejection of the aliphatic chain from the cavity.

At the present stage, it is reasonable to postulate that the amphiphilic derivative **4a** forms an intramolecular inclusion complex in aqueous solution. But attempts to perform a competition experiment with ASANa leads to the following conclusion: the cavity is not vacant but could be occupied by a competitive guest such as ASANa. From the Job Plot analysis[19] (data not shown), the 1:1 stoichiometry was ascertained. A numerical simulation was used to derive the corresponding association constant. It was observed that the association constant K drops from 850 M^{-1} for β-CD to 600 M^{-1} for **4a**. A lower value for the ASANa–**4a** complex can be explained by weak steric hindrance effects. However the capacity of compound **4a** to form a complex is retained even if the association constant is lower than that obtained with β-CD. This confirms that the Boc group of **4a** is self-included in the cavity of the β-CD carrier. However, interactions are clearly much weaker than for the lollipop β-CD analogues[9] suggesting a much looser fitting of the bulky Boc group in the cavity which does not inhibit the inclusion capacity of this derivative. Fig. 5 displays realistic models for **4a** alone and in the presence of ASANa, illustrating the name of "Cup and Ball" given to these molecules.

Incorporation of derivatives 4 into preorganized molecular system

Di-myristoyl-phosphatidyl-choline (DMPC) was selected as a convenient surfactant for our ternary system since it shows very weak interactions with the cavity of β-CD as described elsewhere.[20] We have investigated the incorporation of **4a–d** derivatives and of their inclusion complexes obtained with ASANa into DMPC small unilamellar vesicles (SUV) by three independent techniques, *i.e.* ^{31}P NMR, differential scanning calorimetry (DSC) and immunological titration of the β-CD derivatives.[21] The homogeneous solution of DMPC SUV was prepared[22] by suspension of DMPC in water and then sonication for 10 min. The solution became transparent and the presence of SUV alone was confirmed by the characteristic[23] and well resolved lines obtained by ^1H and ^{31}P NMR.

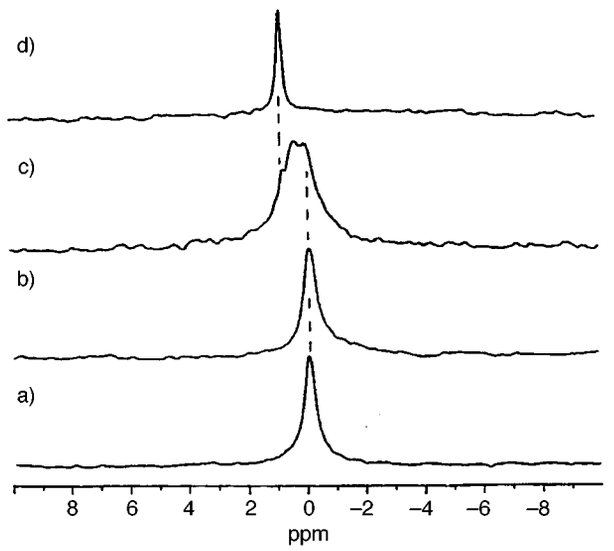


Fig. 6 ^{31}P NMR spectra (303 K, 81 MHz) of (a) DMPC (15 mmol dm^{-3}), (b) with ASANa (6 mmol dm^{-3}), (c) with **4a** (6 mmol dm^{-3}) and (d) with inclusion complex ASANa–**4a** (6 mmol dm^{-3}).

Preliminary ^{31}P NMR, DSC and immunological titration studies have shown that there is no incorporation or interaction between small unilamellar vesicles of DMPC and β-CD alone, ASANa alone or the ASANa–β-CD inclusion complex. These results support the proposition that any interaction observed between derivatives **4** and vesicles of DMPC is due to the specific amphiphilic character of these new molecules.

Investigation of the incorporation by ^{31}P NMR. A 15 mmol dm^{-3} solution of DMPC SUV was prepared in D_2O. Addition of defined amounts of derivatives **4** and/or ASANa led to a 6 mmol dm^{-3} concentration of ASANa or derivatives **4** alone or the inclusion complex. The ^{31}P NMR spectra were recorded after incubation of the mixtures at 313 K for 24 h. As shown by the following spectra (Figs. 6 and 7), it appears that the compounds **4a–d** and their inclusion complexes with ASANa can be incorporated into the DMPC small unilamellar vesicles (SUV). For the short chain derivatives (**4a** $n = 6$, **4b** $n = 8$), this incorporation has been evidenced by the significant shift (65 Hz) of the resonance peak as displayed in Fig. 6. One can also notice that the inclusion complexes incorporate much more efficiently (complete disappearance of the original peak) than the host molecules alone, probably owing to the ejection of the aliphatic chain from the cavity.

In the case of $n = 10$, **4c**, and $n = 12$, **4d**, the incorporation of these amphiphilic cyclodextrins induces the appearance of a broad signal indicating the existence of large multilamellar vesicles (MLV).[24] The equilibrium between the SUV and the MLV is a fast process since the respective proportion of the SUV and the MLV varies slowly with time as observed in Fig. 7. For the derivative with linger chains, it seems that the efficiency of the incorporation is identical for the hosts alone and for inclusion complexes.

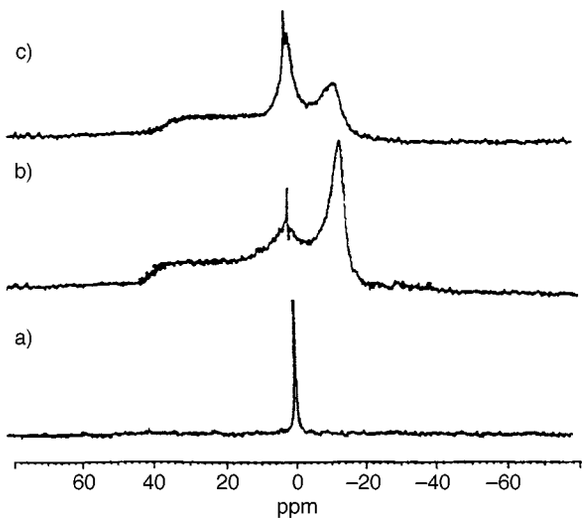


Fig. 7 ^{31}P NMR spectra (303 K, 81 MHz) of (a) DMPC (15 mmol dm^{-3}), (b) with **4c** (6 mmol dm^{-3}) after incubation of 24 h and (c) with **4c** (6 mmol dm^{-3}) after incubation of two weeks.

Table 2 DSC investigations of the influence of the insertion of the "Cup and Ball" **4a**–**d** on the transition temperature (T_c) and on the enthalpy (ΔH) of the DMPC matrix

Sample	T_c/°C	ΔH/kJ mol^{-1}	$\Delta H/\Delta H_{DMPC}$ (%)
DMPC alone	23.04	24.9	100
DMPC + **4a**	22.97	12.7	51
DMPC + **4b**	23.08	15.3	61
DMPC + **4c**	22.99	20.1	80
DMPC + **4d**	22.93	18.1	72

These first results show that the length of the hydrophobic chain directly influences the incorporation process of derivatives **4** into DMPC SUV. It seems that the parameters of the incorporation such as efficiency, rate or final structure obtained dramatically depend upon the length of the chain and also upon the formation of an inclusion complex. This last point leads to the ejection of the chain from the cavity of the cyclodextrin carrier and consequently increases possible affinities between the phospholipid and the aliphatic moiety of the CD derivative.

Investigation of the incorporation by DSC. Unilamellar vesicular solutions of DMPC (75 mmol dm^{-3}) containing the different derivatives **4** (7.5 mM) were ultracentrifuged at 303 K (30 000 *g*, 1 h) after incubation at 313 K for 4 days; supernatants and pellets were then separated. DSC[25] was used to derive the transition temperature of the lipid in the pellets in the absence and in the presence of derivatives **4**. In the latter cases, the disappearance of the pre-transition peak and the important broadening of the transition peak are characteristic of the incorporation phenomenon which represents a perturbation of the lipidic matrix and loss of cooperativity.[26] The temperatures of the pre-transition and transition and the value of the enthalpy were determined for each sample. The main results are displayed in Table 2. We can observe that the experimental data obtained with the pure DMPC are consistent with the literature.[27] Addition of amphiphilic cyclodextrins **4** did not modify significantly the temperature of the transition peak but decreased the enthalpy with a clear relation to the chain length. These observations confirm the incorporation of derivatives **4** alone in the lamellar phase of DMPC.

Immunological titration of derivatives 4. Unilamellar vesicular solutions of DMPC, at different concentrations, containing derivatives **4** or their inclusion complexes with ASANa (6 mmol dm^{-3}) were ultracentrifuged at 303 K (200 000 *g*, 20 h) after incubation at 313 K for 24 h; supernatants and pellets were then separated. The initial solution and supernatants were titrated for β-CD derivatives by an immunological method using antibodies raised against β-CD as described elsewhere.[21] Small variations of concentration were observed for different supernatants obtained from the samples containing only derivatives **4** as evidenced by ^{31}P NMR, the incorporation of derivatives **4** alone into DPMC vesicles being low. Conversely, samples containing inclusion complexes of ASANa showed a clear decrease of their supernatant concentrations as the DMPC concentration increased. The titration results are illustrated in Table 3 for the derivative **4c** and proved a much more efficient incorporation of the inclusion complexes as compared to the amphiphilic compounds alone.

Table 3 Immunological titration results for **4c**

[DMPC]/ mmol dm^{-3}	[**4c**] alone/ mmol dm^{-3}	[**4c**] in presence of ASANa/ mmol dm^{-3}
0	3.88	3.90
15	3.79	3.73
30	3.65	3.51
75	3.70	3.15

Table 4 Lipid–water partition coefficients (K_p) for **4a**–**d**

	4a $n = 6$	**4b** $n = 8$	**4c** $n = 10$	**4d** $n = 12$
K_p (mass)	3.7	4.0	4.1	4.3
K_p (mole)	139	150	155	162

An important parameter is the partition coefficient K_p reflecting the efficiency of the incorporation:

$$K_p = \frac{m_{BL}/m_L}{m_{BA}/m_A} = [B]_L/[B]_A$$

where m_{BL} = mass of cyclodextrin in the lipid phase (mg), m_{BA} = mass of cyclodextrin in the aqueous phase (mg), m_L = mass of the lipid phase (mg) and m_A = mass of the aqueous phase (mg).

The partition coefficients K_p (molar ratio) of each inclusion complex between lipid and water have been calculated from these experimental results and are displayed in Table 4. The values of K_p appear to be independent of the aliphatic chain length. Although K_p remains weak as compared to values obtained with more classical glycolipids,[28] it is increased by a ten fold factor upon formation of inclusion complexes.

The goal of this work was to prepare water-soluble amphiphilic cyclodextrin derivatives with the aim of vectorizing and/or targeting pharmaceutically active compounds. The incorporation of these compounds into preorganized molecular systems such as lipidic vesicles or liposomes implies that the grafted aliphatic chain has to be left outside of the hydrophobic cavity of the cyclodextrin. In order to preclude the self-inclusion process, the general concept of "Cup and Ball" molecules was developed. In these compounds, the grafted aliphatic chain is terminated by a bulky hydrophobic group hampering strong inclusion of the chain in the cavity. These compounds were prepared and fully characterized by NMR and their inclusion properties towards hydrophobic guests were evaluated by the same technique.

It has been further shown that these molecules and their inclusion complexes are incorporated into the phospholipidic vesicles by using ^{31}P NMR as well as more global methods such as differential scanning calorimetry. Furthermore, the final validation and quantification of this incorporation was evidenced by immunological methods using specific antibodies prepared against β-cyclodextrin. The inclusion complexes are incorporated much more efficiently into a phospholipidic

phase owing to the ejection of the aliphatic chain from the cavity.

Physico-chemical techniques such as ^2H and neutron scattering will be used further in order to better understand the mechanism of the incorporation and to optimize the mixed colloidal system.

Experimental

General procedures and instrumentation

The β-cyclodextrin obtained from Roquette Frères was freeze-dried before synthesis. Toluene-*p*-sulfonyl chloride (TsCl) was recrystallized from light petroleum. TLC was performed on silica gel 60 plates (E. Merck) followed by charring with 10% H_2SO_4. Preparative HPLC was carried out with a Waters Delta Prep 3000 Chromatograph equipped with an LSED detector and a μBondapak C_{18}-bonded silica column, by elution of the appropriate solvent at 10 cm^3 min^{-1}. Mass spectrometry was performed on a SCIEX spectrometer with electrospray infusion. Elemental analyses were obtained from samples previously freeze-dried and were performed at the Service Central de Microanalyses of CNRS, Lyon, France. IR spectra obtained on a Perkin-Elmer 1725X spectrometer are reported in cm^{-1}.

^1H NMR experiments were performed at 500 MHz using a Bruker DRX500 spectrometer. In all cases, the samples were prepared in deuterium oxide and d_6-DMSO (Euriso-TOP, Saclay, France) and measurements were performed at 298 K under careful temperature regulation. The length of the 90° pulse was *ca.* 7 μs. 1D NMR spectra were collected using 16 K data points. Chemical shifts (in ppm) are given relative to external tetramethylsilane (TMS = 0 ppm) and calibration was performed using the signal of the residual protons of the solvent as a secondary reference. T-ROESY experiments[16] were obtained using the pulse program available from the Bruker library using a 300 ms spin-lock time. These bidimensional experiments were acquired using 2 K data points and 256 time increments. The phase sensitive (TPPI) sequence was used and processing resulted in a 1K*1K (real–real) matrix. Details concerning experimental conditions are given in the figure captions. All NMR data were processed and plotted using the UX-NMR program (Bruker Analytische Messtechnik) on a Silicon graphic workstation. ^{31}P NMR experiments were performed at 81 MHz using a Bruker AC200 spectrometer. In all cases, the samples were prepared in D_2O and measurements were performed at 303 K under careful temperature regulation. DSC experiments were performed using a Perkin-Elmer DSC-7 instrument with a scan rate of 1° K min^{-1} in the 278–323 K range.

Immunological titration

Competitive enzyme immunoassay (EIA) was performed as described elsewhere.[21] 96-well microtiter plates (Immunoplate Maxisorb, Nunc, Denmark) were coated with mouse monoclonal antirabbit immunoglobulins in order to ensure separation between the free and the bound moieties of the enzymatic tracer during the immunological reaction. Before use, the plates were washed with 10 mmol phosphate buffer (pH 7.4) containing 0.05% Tween 20. The total volume of the immunological reaction was 0.15 cm^3, each component (enzymatic tracer, diluted rabbit polyclonal antisera and cyclodextrin standard) being added in a 0.05 cm^3 volume. β-CD–AChE enzyme conjugate was used at a concentration of 5 Ellman units cm^{-3}. The working dilution for the different rabbit bleedings was previously determined by performing serial dilution experiments.

After an 18 h incubation period at 277 K, the plates were washed and the enzyme activity of the bound immunological complex revealed by addition of 0.2 cm^3 of enzymatic substrate and chromogen (Ellman's reagent) in each well. After 2 h of gentle shaking in the dark at room temperature, the absorbance at 414 nm in each well was measured automatically, Results are given in terms of $B\ B_0^{-1}$ as a function of the dose, B and B_0 representing the bound enzyme activity in the presence or absence of competitor respectively. A linear log–lin transformation was used to fit the standard curve. Determination of concentrations was performed using the experimental data B and the standard enzyme immunoassay titration curves obtained for the β-CD derivatives **4**. All experiments were made in duplicate and quadruplicate for B_0.

Synthesis

***N*-Aminoalkyl-6-amino-6-deoxy-cyclomaltoheptaoses 3: general procedure.** To a solution of diamino alkane (11.6 mmol, 10 equivalents) in dimethylformamide (DMF, 35 cm^3), diisopropylethylamine (2 cm^3, 11.6 mmol) was added and the reaction mixture was stirred for 10 min at room temperature. 6-*O*-*p*-tolylsulfonyl-cyclomaltoheptaose **2**[11] (1.16 mmol) was added and the reaction mixture was stirred for 3 days at 348 K. The solvent was removed under reduced pressure and the crude material dissolved in 10 cm^3 of water. Addition of 200 cm^3 of acetone to the cooled aqueous solution resulted in a precipitate which was recovered by filtration, washed with acetone and dried. It was dissolved in 5 cm^3 of water and layered into a Lewatit SP1080H^+ column (3*25 cm prepared with water) and eluted with 300 cm^3 of water and then with 200 cm^3 of aq. ammonia (6%). The pure compound appeared in ammonia fractions. These fractions were evaporated almost to dryness and lyophilized to yield *N*-aminoalkane-6-amino-6-deoxy-cyclomaltoheptaoses **3**.

3a: N-Aminohexyl-6-amino-6-deoxy-cyclomaltoheptaose. m = 0.8 g (66%) from 6-*O*-*p*-tolylsulfonyl-cyclomaltoheptaose **2**[11] (1.49 g, 1.166 mmol) and diaminohexane (1.34 g, 11.6 mmol). **TLC**: R_f = 0.1 (BuOH–DMF–H_2O 2:1:1). **HPLC**: T_r = 7 min 36 s (MeOH–H_2O–CH_3COOH 15:85:0.2). Found: C, 46.47; H, 6.73; Calc. for $C_{48}N_2O_{34}H_{84}\cdot H_2O$: C, 46.08; H, 6.88%. **ES-MS**: *m*/*z* 1233, $[M + H]^+$. **NMR**: δ_C (125 MHz, d_6-DMSO) 106.0 (C-1), 88.1 and 85.5 (C-4), 77.0–74.5 (C-2, C-3 and C-5), 63.8 (C-6), 54.2 (NH–*CH*$_2$ and *CH*$_2$–NH), 33.6, 33.1, 30.8, 30.5 (4C, 4CH_2). δ_H (500.13 MHz, d_6-DMSO) 8.01 (2H, NH_2), 5.85 ($OH_{2,3}$), 4.84 (7H, H_1), 4.52 (OH_6), 3.75–3.33 ($H_{2,3,4,5,6}$), 3.09 (1H, H_a), 2.94 (1H, H_f), 2.75 (1H, N_H), 2.63 (1H, $H_{a'}$), 2.48 (1H, H_f), 1.41 (4H, $H_{b,e}$), 1.28 (4H, $H_{c,d}$).

3b: N-Aminooctyl-6-amino-6-deoxy-cyclomaltoheptaose. m = 0.95 g (65%) from 6-*O*-*p*-tolylsulfonyl-cyclomaltoheptaose **2**[11] (1.49 g, 1.166 mmol) and diaminooctane (1.67 g, 11.6 mmol). **TLC**: R_f = 0.14 (BuOH–DMF–H_2O 2:1:1). **HPLC**: T_r = 8 min (MeOH–H_2O–CH_3COOH 15:85:0.2). Found: C, 46.57; H, 6.93; Calc. for $C_{50}N_2O_{34}H_{88}\cdot 2H_2O$: C, 46.30; H, 7.10%. **ES-MS**: *m*/*z* 1261, $[M + H]^+$. **NMR**: δ_C (125 MHz, d_6-DMSO) 105.5 (C-1), 87.6 and 85.5 (C-4), 76.9–74.7 (C-2, C-3 and C-5), 63.8 (C-6), 53.8 (2C, NH–*CH*$_2$ and *CH*$_2$–NH), 40.9, 33.6, 32.9, 30.8, 30.2 (6C, 6CH_2). δ_H (500.13 MHz, d_6-DMSO) 8.00 (2H, NH_2), 5.87 ($OH_{2,3}$), 4.85 (7H, H_1), 4.52 (OH_6), 3.76–3.32 ($H_{2,3,4,5,6}$), 3.07 (1H, NH_2–*CH*), 2.94 (1H, β-CD–NH–*CH*), 2.77 (1H, N_H), 2.65 (1H, NH_2–*CH'*), 2.49 (1H, β-CD–NH–*CH'*), 1.4–1.28 (12H, 6CH_2).

3c: N-Aminodecyl-6-amino-6-deoxy-cyclomaltoheptaose. m = 1.0 g (67%) from 6-*O*-*p*-tolylsulfonyl-cyclomaltoheptaose **2**[11] (1.49 g, 1.166 mmol) and diaminodecane (1.99 g, 11.6 mmol). **TLC**: R_f = 0.18 (BuOH–DMF–H_2O 2:1:1). **HPLC**: T_r = 10 min 36 s (MeOH–H_2O–CH_3COOH 15:85:0.2). Found: C, 47.45; H, 7.43; Calc. for $C_{52}N_2O_{34}H_{92}\cdot H_2O$: C, 47.78; H, 7.20%. **ES-MS**: *m*/*z* 1289, $[M + H]^+$. **NMR**: δ_C (125 MHz, d_6-DMSO) 105.5 (C-1), 87.5 and 85.5 (C-4), 76.9–74.5 (C-2, C-3 and C-5), 63.8 (C-6), 53.7 (2C, NH–*CH*$_2$ and *CH*$_2$–NH), 41.0, 33.4, 32.9, 32.6, 30.7, 30.2, 29.7 (8C, 8CH_2). δ_H (500.13 MHz, d_6-DMSO) 8.02 (2H, NH_2), 5.75 ($OH_{2,3}$), 4.87 (7H, H_1), 4.48 (OH_6), 3.71–3.27 ($H_{2,3,4,5,6}$), 3.10 (1H, NH_2–*CH*), 2.91 (1H, β-CD–

NH–*CH*), 2.75 (1H, N_H), 2.66 (1H, NH_2–*CH′*), 2.49 (1H, β-CD–NH–*CH′*), 1.45–1.30 (16H, $8CH_2$).

3d: N-Aminododecyl-6-amino-6-deoxy-cyclomaltoheptaose. $m = 1.05$ g (69%) from 6-*O*-*p*-tolylsulfonyl-cyclomaltoheptaose **2**[11] (1.49 g, 1.166 mmol) and diaminododecane (2.32 g, 11.6 mmol). **TLC**: $R_f = 0.23$ (BuOH–DMF–H_2O 2:1:1). **HPLC**: $T = 13$ min 24 s (MeOH–H_2O–CH_3COOH 15:85:0.2). Found: C, 48.27; H, 7.26; Calc. for $C_{54}N_2O_{34}H_{96}\cdot H_2O$: C, 48.58; H, 7.35%. **ES-MS**: *m/z* 1317, $[M + H]^+$. **NMR**: δ_C (125 MHz, d_6-DMSO) 106.0 (C-1), 87.6 and 85.7 (C-4), 77.2–74.8 (C-2, C-3 and C-5), 63.8 (C-6), 53.7 (2C, NH–CH_2 and CH_2–NH), 41.4, 33.4, 32.8, 32.7, 32.6, 30.6, 30.2 (10C, $10CH_2$). δ_H (500.13 MHz, d_6-DMSO) 8.05 (2H, NH_2), 5.74 ($OH_{2,3}$), 4.89 (7H, H_1), 4.50 (OH_6), 3.74–3.33 ($H_{2,3,4,5,6}$), 3.10 (1H, NH_2–*CH*), 2.92 (1H, β-CD–NH–*CH*), 2.77 (1H, N_H), 2.67 (1H, NH_2–*CH′*), 2.48 (1H, β-CD–NH–*CH′*). 1.42–1.27 (20H, $10CH_2$).

***N*-Boc-aminoalkyl-6-amino-6-deoxy-cyclomaltoheptaoses 4: general procedure.** To a solution of *N*-aminoalkyl-6-amino-6-deoxy-cyclomaltoheptaose **3** (0.4 mmol) in 40 cm^3 of DMF, di-*tert*-butyl dicarbonate (0.38 mmol) was added at 273 K. The reaction mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure. Addition of 200 cm^3 of acetone to the cooled solution resulted in a precipitate which was recovered by filtration, washed with acetone and dried to give quantitatively a solid which contained almost pure **4**. Final purification was achieved by HPLC (MeOH–H_2O 40:60) to afford **4** as an amorphous powder after freeze-drying.

4a: N-Boc-aminohexyl-6-amino-6-deoxy-cyclomaltoheptaose. $m = 383$ mg (72%) from *N*-aminohexyl-6-amino-6-deoxy-cyclomaltoheptaose **3a** (492 mg, 0.4 mmol). **TLC**: $R_f = 0.44$ (NH_4OH 6%–EtOH–BuOH 5:5:3). **HPLC**: $T_r = 5$ min 3 s (MeOH–H_2O 40:60). Found: C, 46.36; H, 6.94; Calc. for $C_{53}N_2O_{36}H_{92}\cdot 2H_2O$: C, 46.49; H, 7.02%. **ES-MS**: *m/z* 1333, $[M + H]^+$. **IR**: $\nu = 1665$ cm^{-1} (NHCO); **NMR**: δ_C (125 MHz, d_6-DMSO) 104.0 and 102.1 (C-1), 86.0, 85.2–84.1 and 82.3 (C-4), 78.0–72.9 (C-2, C-3 and C-5), 63.3 (C-6), 52.3 and 51.1 (NH–CH_2 and CH_2–NH), 40.8, 30.1, 29.9, 29.8 (4C, $4CH_2$), 31.9 ($3CH_3$). δ_H (500.13 MHz, d_6-DMSO) 7.98 (1H, NHCO), 5.96–5.47 ($OH_{2,3}$), 5.04–4.80 (7H, H_1), 4.56–4.29 (OH_6), 4.01 (1H, $H_{5'}$), 3.86–3.25 ($H_{2,3,4,5,6}$), 3.06 (1H, $H_{4'}$), 2.71 (1H, $H_{6'}$), 3.81 (2H, β-CD–NH–CH_2), 3.68 (1H, N_H), 3.06 (2H, CH_2–NHCO), 1.38 (2H, $CH_{e'}$), 1.23 (2H, $CH_{2'}$), 1.20 (2H, CH_2), 1.17 (2H, CH_2), 1.30 (9H, $3CH_3$).

4b: N-Boc-aminooctyl-6-amino-6-deoxy-cyclomaltoheptaose. $m = 386$ mg (71%) from *N*-aminooctyl-6-amino-6-deoxy-cyclomaltoheptaose **3b** (504 mg, 0.4 mmol). **TLC**: $R_f = 0.57$ (NH_4OH 6%–EtOH–BuOH 5:5:3). **HPLC**: $T_r = 8$ min 33 s (MeOH–H_2O 40:60). Found: C, 47.79; H, 7.06; Calc. for $C_{55}N_2O_{36}H_{96}\cdot H_2O$: C, 47.97; H, 7.12%. **ES-MS**: *m/z* 1361, $[M + H]^+$. **IR**: $\nu = 1673$ cm^{-1} (NHCO); **NMR**: δ_C (125 MHz, d_6-DMSO) 104.0 and 102.1 (C-1), 85.3–81.2 (C-4), 76.4–70.6 (C-2, C-3 and C-5), 62.8 (C-6), 52.3 and 51.1 (NH–CH_2 and CH_2–NH), 39.6, 29.4–27.0 (6C, $6CH_2$), 29.8 ($3CH_3$). δ_H (500.13 MHz, d_6-DMSO) 7.99 (1H, NHCO), 5.97–5.43 ($OH_{2,3}$), 5.03–4.81 (7H, H_1), 4.55–4.25 (OH_6), 4.02–2.72 ($H_{2,3,4,5,6}$), 3.82 (2H, β-CD–NH–CH_2), 3.66 (1H, N_H), 3.07 (2H, CH_2–NHCO), 1.40–1.15 (12H, $6CH_2$), 1.33 (9H, $3CH_3$).

4c: N-Boc-aminodecyl-6-amino-6-deoxy-cyclomaltoheptaose. $m = 433$ mg (78%) from *N*-aminodecyl-6-amino-6-deoxy-cyclomaltoheptaose **3c** (515 mg, 0.4 mmol). **TLC**: $R_f = 0.68$ (NH_4OH 6%–EtOH–BuOH 5:5:3). **HPLC**: $T_r = 7$ min 42 s (MeOH–H_2O 50:50). Found: C, 48.58; H, 7.21; Calc. for $C_{57}N_2O_{36}H_{100}\cdot H_2O$: C, 48.65; H, 7.25%. **ES-MS**: *m/z* 1389, $[M + H]^+$. **IR**: $\nu = 1665$ cm^{-1} (NHCO); **NMR**: δ_C (125 MHz, d_6-DMSO) 103.1 (C-1), 82.5–80.1 (C-4), 74.3–73.1 (C-2, C-3 and C-5), 61.0 (C-6), 52.3 and 51.1 (NH–CH_2 and CH_2–NH), 38.9, 29.4–26.9 (8C, $8CH_2$), 29.0 ($3CH_3$). δ_H (500.13 MHz, d_6-DMSO) 7.98 (1H, NHCO), 5.99–5.45 ($OH_{2,3}$), 5.04–4.82 (7H, H_1), 4.57–4.29 (OH_6), 4.01–2.72 ($H_{2,3,4,5,6}$), 3.80 (2H, β-CD–NH–CH_2), 3.67 (1H, N_H), 3.08 (2H, CH_2–NHCO), 1.39–1.18 (16H, $8CH_2$), 1.30 (9H, $3CH_3$).

4d: N-Boc-aminododecyl-6-amino-6-deoxy-cyclomaltoheptaose. $m = 430$ mg (76%) from *N*-aminododecyl-6-amino-6-deoxy-cyclomaltoheptaose **3d** (526 mg, 0.4 mmol). **TLC**: $R_f = 0.78$ (NH_4OH 6%–EtOH–BuOH 5:5:3). **HPLC**: $T_r = 14$ min 45 s (MeOH–H_2O 50:50). Found: C, 48.58; H, 7.29; Calc. for $C_{59}N_2O_{36}H_{104}\cdot 2H_2O$: C, 48.76; H, 7.44%. **ES-MS**: *m/z* 1417, $[M + H]^+$. **IR**: $\nu = 1660$ cm^{-1} (NHCO); **NMR**: δ_C (125 MHz, d_6-DMSO) 102.8 (C-1), 84.1–82.0 (C-4), 75.1–72.5 (C-2, C-3 and C-5), 61.1–60.0 (C-6), 52.3 and 51.1 (NH–CH_2 and CH_2–NH), 39.1, 29.4–27.0 (10C, $10CH_2$), 28.9 ($3CH_3$). δ_H (500.13 MHz, d_6-DMSO) 7.99 (1H, NHCO), 5.98–5.44 ($OH_{2,3}$), 5.06–4.79 (7H, H_1), 4.58–4.28 (OH_6), 4.02–2.73 ($H_{2,3,4,5,6}$), 3.81 (2H, β-CD–NH–CH_2), 3.66 (1H, N_H), 3.08 (2H, CH_2–NHCO), 1.41–1.19 (20H, $10CH_2$), 1.32 (9H, $3CH_3$).

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