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Cholesteryl-cyclodextrins: synthesis and insertion into phospholipid membranes

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Abstract

6^{I}-(Cholest-5-en-3β-yloxycarbonyl)amino-6^{I}-deoxycyclomaltoheptaose and 6^{I}-(cholest-5-en-3α-ylamido)succinylamido-6^{I}-deoxycyclomaltoheptaose were synthesized and fully characterized by NMR spectroscopy experiments and mass spectrometry analysis. Incorporation of these monosubstituted amphiphilic cyclodextrins into phospholipid bilayers was investigated using small-angle X-ray scattering, differential scanning calorimetry and ^{31}P nuclear magnetic resonance. Different modes of incorporation depending on the spacer length between the cyclodextrin and cholesterol moieties were observed.

Keywords: Amphiphilic cyclodextrins; Phospholipid bilayers; NMR spectroscopy; X-ray scattering

1. Introduction

Literature data dealing with the synthesis of amphiphilic derivatives of cyclodextrins point out outstanding properties for such compounds. Amphiphilic cyclodextrins bearing multiple hydrophobic chains on the primary face have been extensively investigated. Per(6-dodecylamino-6-deoxy)-β-cyclodextrin [1] and per(6-alkylsulfonyl-6-deoxy)-β-cyclodextrins [2] have been shown to form stable Langmuir–Blodgett layers and the corresponding per(6-*S*-alkyl-6-thio) derivatives [3] to give rise to thermotropic liquid crystals. Cyclodextrin derivatives obtained by attaching long chains on the secondary face or both the primary and the secondary faces have also been investigated. Modified cyclodextrins named 'bouquets', obtained by grafting poly(oxyethylene) or polymethylene chains have been synthesized in order to act as transmembrane ion channels since they can be incorporated into lipidic vesicles [4,5]. It was recently shown that 'skirt-shaped cyclodextrins' bearing fatty acyl chains linked to their secondary hydroxyl groups can form stable nanospheres [6]. These supramolecular assemblies show promising properties for drug encapsulation and delivery owing to the cumulative effects of size specificity and transport properties of cyclodextrins and organized materials, respectively [7].

The design and synthesis of new amphiphilic cyclodextrins with specific properties appear, therefore, as a real challenge. However, the synthesis of chemically pure polysubstituted amphiphilic cyclodextrins in good overall yields remains a major limitation ow-

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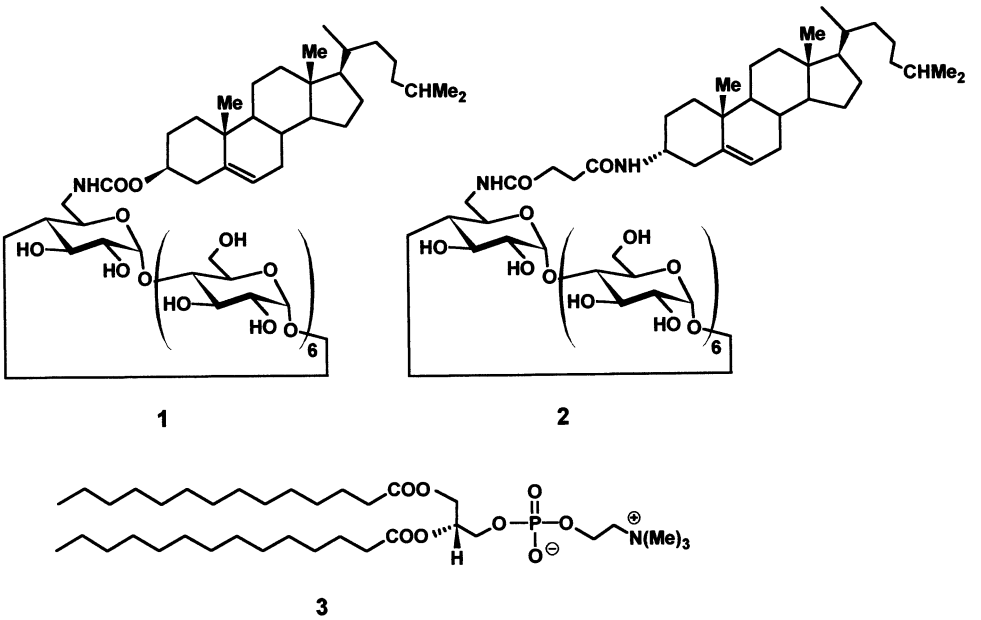


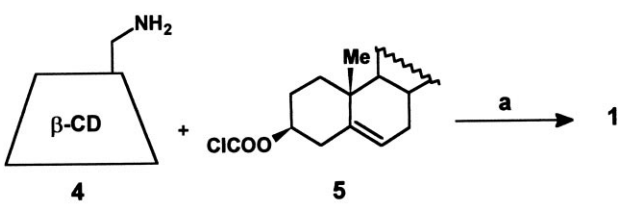
Scheme 1.

ing to the requirements of tedious purification steps. Conversely, previous results from our laboratory have shown that amphiphilic monosubstituted β-cyclodextrins, the 'cup and ball' molecules [8], exhibit attractive properties. These derivatives, which possess a linear chain of variable length with a bulky *tert*-butyloxycarbonyl end-group, can indeed be inserted into phospholipidic vesicles and still retain their inclusion properties [9]. This general approach provides clues for the transport of bioactive molecules under different administration pathways.

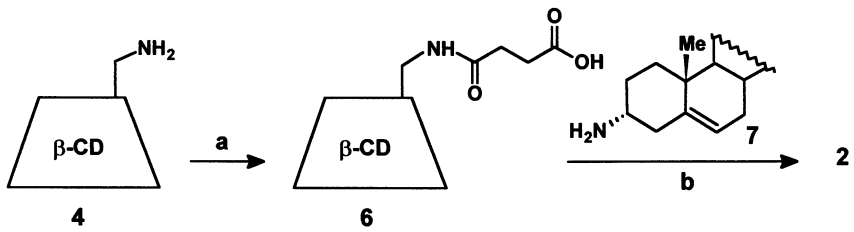
We report here on the synthesis of 6^I-(cholest-5-en-3β-yloxycarbonyl)amino-6^I-deoxycyclomaltoheptaose (**1**) and 6^I-(cholest-5-en-3α-ylamido)succinylamido-6^I-deoxycyclomaltoheptaose (**2**), and on the investigation of their incorporation into lipid model membranes such as dimyristoyl-phosphatidylcholine (DMPC) (**3**) using several complementary techniques, i.e., small-angle X-ray scattering (SAXS), differential scanning calorimetry (DSC) and ^{31}P nuclear magnetic resonance. Comparison of the properties of **1** and **2** was expected to provide information concerning the influence of the distance between the hydrophilic and hydrophobic moieties in terms of their incorporation into phospholipid bilayers.

2. Results and discussion

Synthesis of cholesteryl-β-cyclodextrins.— The synthetic pathway to cholesteryl-β-cyclodextrins **1** and **2** (Scheme 1) involved the preparation of 6^I-amino-6^I-deoxycyclomaltoheptaose (**4**). This β-cyclodextrin derivative was synthesized in three steps from the parent cyclodextrin, as described in Ref. [10]. Reaction of cholesteryl chloroformate (**5**) with **4** in *N*,*N*-dimethylformamide and in the presence of triethylamine afforded **1** in 67% yield (Scheme 2) . Nucleophilic addition of **4** on succinic anhydride in *N*,*N*-dimethylformamide afforded **6** (89% yield), as described in Ref. [11]. Compound **6** was further reacted with 3-α-aminocholesterol (**7**) under standard coupling conditions (*N*,*N*′-diisopropylcarbodiimide (DIC), hydroxybenzotriazole (HOBT)) resulting in a 67% yield of **2** (Scheme 3) . The final material was purified by high-performance liquid chromatography (water–MeOH,



Scheme 2. (a) NEt_3, DMF, 17 h, rt.



Scheme 3. (a) Succinic anhydride, DMF, 3 h, rt; (b) DIC, HOBT, DMF–THF, 48 h, rt.

gradient elution). 3-α-Aminocholesterol (**7**) was prepared according to the following sequence: 3-β-cholesterol was converted into 3-α-azidocholesterol under Mitsunobu conditions using diphenylphosphoryl azide as the nucleophilic partner [12]. 3-α-Azidocholesterol was then reduced by treatment with triphenylphosphine in the presence of water [13]. Complete configuration inversion at the 3 carbon atom of cholesterol was evidenced by the different coupling constants of the H-3 protons of 3-β-cholesterol in axial position ($J_{3,2ax}$ 12, $J_{3,2eq}$ 2.5, $J_{3,4eq}$ 5 Hz) and 3-α-azidocholesterol in equatorial position ($J_{3,4ax}$ 5.2, $J_{3,4eq}$ 7 Hz). Moreover, the optical rotation value obtained for 3-α-azidocholesterol, $[\alpha]_D^{20}$ −4.7° (*c* 0.85, $CHCl_3$), is in very good agreement with literature data [14], $[\alpha]_D^{20}$ −4.7° (*c* 1.38, $CHCl_3$).

The chemical structures of **1** and **2** were assessed using high-resolution ^1H NMR and mass spectrometry with electrospray infusion mode (high and low resolution). The accurate mass data obtained by ESI-HRMS have confirmed the molecular formulae of **1** and **2**. The NMR analysis was performed in Me_2SO-d_6, since both compounds are insoluble in water, and demonstrated that the purified final samples were free of any included by-product or reagent. The monosubstitution of the cyclodextrin derivatives has been shown by digital integration of the NMR signals arising from cholesterol and cyclodextrin moieties. Because the spectra are relatively complex owing to the lack of molecular symmetry of the cyclodextrin moiety, a complete analysis was obtained from stepwise identification of protons of **1** and **2** by COSY and successive RELAY experiments. Being located in a very specific spectral domain, anomeric protons were used as a starting point for stepwise assignment of the cyclodextrin moiety. The cholesterol and cyclodextrin moieties were then sequenced using NOESY [15] experiments. As an example, Fig. 1 displays a partial contour plot of a NOESY experiment performed on **2** in Me_2SO-d_6. This experiment confirms the attachment of 3-α-aminocholesterol to the 6^I-amino-β-CD derivative through the succinic acid spacer by evidencing dipolar interactions (indicating spatial proximities). Indeed, in the partial NOESY contour plot, each amide proton yields two dipolar cross peaks, one arising from protons of its own moiety (i.e., NH Chol with H-3 Chol or NH β-CD with H-6 β-CD and H-6′ β-CD) and one from the neighboring methylene group of the succinic acid part.

Insertion of cholesteryl-cyclodextrins into phospholipid membranes.—Cholesteryl-β-cyclodextrins (Chol-β-CD) **1** and **2** were incorporated at different concentrations into lamellar phases of dimyristoyl-phosphatidylcholine (DMPC) (**3**), according to a procedure which leads to perfect molecular dispersion for all types of host molecules, as described previously in Ref. [16] (the DMPC/Chol-β-CD ratios were 83:17, 7:3, and 2:3 (w/w), whereas the total lipid/water ratio was kept constant at 1:4 (w/w)). The procedure used for molecular mixing involves preparation of a mixed lipidic film by evaporation of an organic solution of DMPC/**1** or **2**, hydration of the dried lipidic film with a large excess of water, ultrasonication of the resulting suspension to obtain mixed vesicles, lyophilization and rehydration at fixed water contents. DMPC was selected since it forms the L_α lamellar structure beyond 23 °C in water [17,18], corresponding to the liquid-crystal phase of biological membranes at physiological temperatures. The incorporation was investigated by three physico–chemical techniques, i.e., SAXS, DSC and ^{31}P NMR. All experiments were performed on the

same samples and, in the case of the SAXS and ^{31}P NMR studies, at a temperature greater than 23 °C.

Obtaining a single phase upon adding a large molecule into a lamellar phase derived from a zwitterionic molecule such as DMPC requires the following conditions [19]: (i) the existence of a molar fraction of the DMPC/host molecule for which the curvature is large enough to avoid frustration leading to cubic or vesicle phases [20]; (ii) no segregation between molecules, i.e., low enthalpy of mixing of head-groups and chains as usually revealed through the additivity of area per molecule [21]; and (iii) weak modification of the molecular forces stabilizing the lamellar phase [22]. For DMPC, a delicate force balance between dispersive and hydration forces ensures a zero osmotic pressure for a 62 Å period, i.e., in the case of the coexistence of a single lamellar phase with excess water.

We demonstrate here using SAXS that the three previously defined conditions are fulfilled for mixtures of DMPC and **2**. Insertion of **2** in DMPC at a weight ratio of 17:83 respectively results, after homogeneization, in four Bragg peaks on the SAXS spectrum (see Fig. 2(B)). The presence of four Bragg peaks indicates the coexistence of two lamellar phases. The peaks at 0.1005 and 0.2001 $Å^{-1}$ correspond to the first- and second-order lines of the pure L_α phase of DMPC [16], as observed in Fig. 2(A). The peaks at 0.0845 and 0.1672 $Å^{-1}$ can be assigned to an additional lamellar phase (L_{CD}) with a larger swelling. The repetition distance of the L_{CD} phase is increased by 12 Å with respect to that of pure DMPC (62 Å). The increased swelling of this second lamellar phase likely results from the modification of the force balance between the van der Waals attraction and short-range repulsive hydration forces due to the incorporation of compound **2** into the lamellar phase of DMPC. The restoration of the coexisting L_α and L_{CD} lamellar phases, after heating up to 75 °C (resulting in a single phase as indicated by the presence of only one set of Bragg peaks) and cooling down to ambient temperature, confirmed the thermodynamic stability of the two phases. Moreover, after 1 month of equilibration, spectra obtained under identical conditions as in Fig. 2(A, B) showed no significant alterations. Upon increasing the molar proportion of Chol-β-CD **2** with respect to DMPC (DMPC/Chol-β-CD **2** 7:3 (w/w)), the L_α and L_{CD} phases still coexist and the respective weight of the L_{CD} phase increases. Since no monotonic swelling of the lamellar phase of DMPC is observed, it can be concluded that all added **2** is included in the L_{CD} phase, supporting the idea of fully separated phases designated **I** and **II** in Fig. 2(D). This unusual behavior might result from strong attractive interactions between Chol-β-CD **2** molecules. The presence of Bragg peaks at a fixed position, independent of the initial ratio, is the

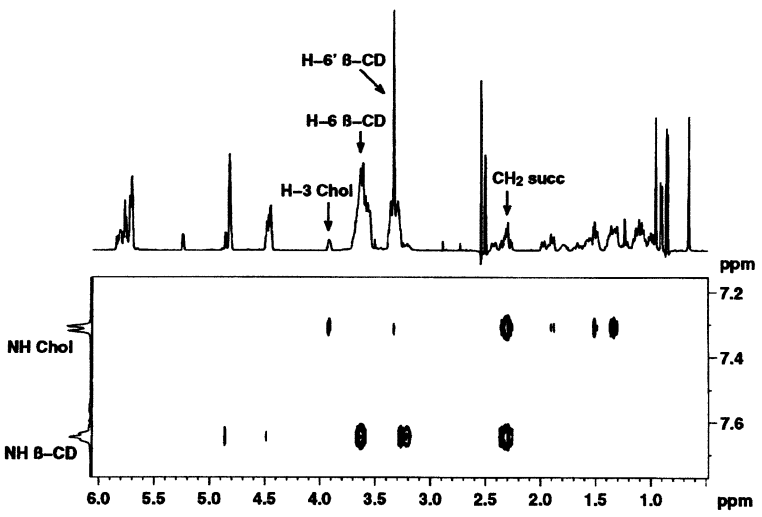


Fig. 1. Partial contour of a NOESY experiment (500 MHz, Me_2SO-d_6, 25 °C, 300 ms mixing time) performed on **2** (4.8×10^{-3} M).

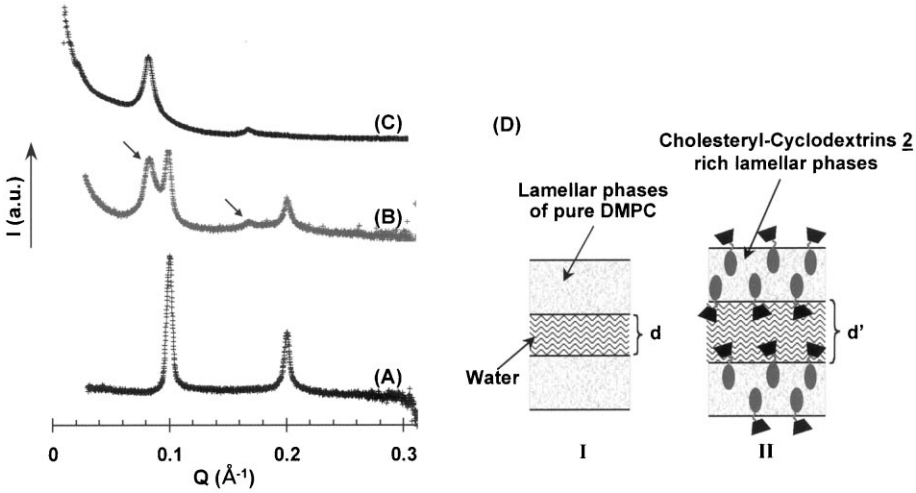


Fig. 2. SAXS spectra obtained at 30 °C and schematic representation of the mixed DMPC/Chol-β-CD **2** phases. (A) Pure DMPC in excess water (80% w/w); (B) DMPC/Chol-β-CD **2** system (83:17 w/w); the peaks corresponding to the first- and second-order lines of the L_{CD} lamellar phase are indicated by an arrow; (C) DMPC/Chol-β-CD **2** system (2:3 w/w): pure L_{CD} lamellar phase; (D) schematic representation of coexisting L_α and L_{CD} lamellar phases.

signature of the existence of a pure phase of the mixed lipids in the phase diagram. Increasing the DMPC/Chol-β-CD **2** to 2:3 (w/w) resulted in the complete disappearance of the pure DMPC lines (see Fig. 2(C)). From these data, it can hence be concluded that the pure L_{CD} phase corresponds to the DMPC/Chol-β-CD **2** 2:3 (w/w) mixture.

The behavior of **1** strongly differs from that of **2**. The addition of **1** to DMPC in water does not result in the appearance of additional Bragg peaks as observed above, but induces a broadening of the Bragg peaks corresponding to the L_α phase of DMPC (see Fig. 3(A, B)). This is indicative of a short-range disorder in the mixed bilayers although the mechanism of this membrane perturbation cannot be elucidated by SAXS only.

DSC studies fully support the SAXS data. In Fig. 4(B), the thermogram of the DMPC/Chol-β-CD **2** system exhibits three endothermic transition peaks, two of which correspond to the pretransition and to the main gel-to-fluid transition of pure DMPC in water, as shown by the reference thermogram (see Fig. 4(A)). Even in the presence of **2**, the pretransition for DMPC lamellar phase is retained, indicating that the lamellar phase of DMPC remains pure. Moreover, the transition peak that appears between those of the pure DMPC phase can be attributed to the Chol-β-CD **2** rich lamellar phase observed by SAXS. The small enthalpy of this phase transition is due to the presence of the cholesterol moiety of **2** in the hydrophobic core of the DMPC membrane. It is indeed well documented that the addition of cholesterol to phospholipid membranes strongly reduces or even abolishes the gel-to-fluid phase transition [23]. These results confirm the coexistence of two different lamellar phases, corresponding to the pure lamellar phase of DMPC and to L_{CD}. In the case of the DMPC/Chol-β-CD **1** system, the thermogram presents several peaks (see Fig. 4(C)) representative of a strong perturbation of the DMPC

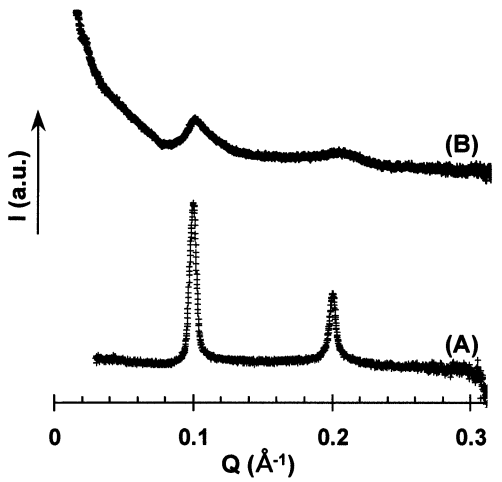


Fig. 3. SAXS spectra of fully hydrated DMPC alone and in the presence of Chol-β-CD **1** at 30 °C. (A) Pure DMPC in excess water (80% w/w); (B) DMPC/Chol-β-CD **1** system (83:17 w/w).

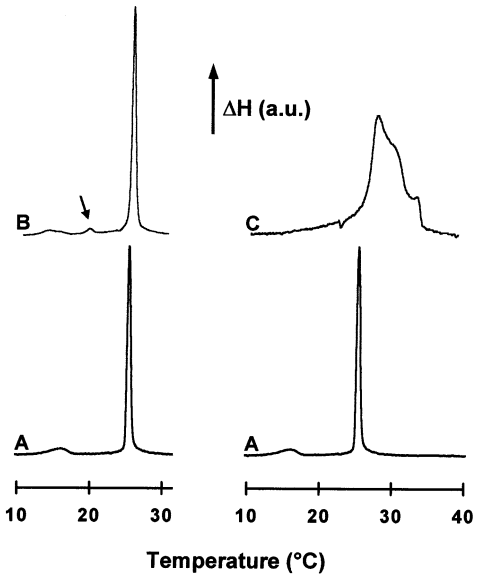


Fig. 4. DSC thermograms (first heating runs) of the mixed DMPC/Chol-β-CD phases. (A) Pure DMPC in excess water (80% w/w); (B) DMPC/Chol-β-CD **2** system (83:17 w/w); the transition peak of the Chol-β-CD **2** rich lamellar phase is indicated by an arrow; (C) DMPC/Chol-β-CD **1** system (83:17 w/w).

chains. The more rigid nature of **1** compared with **2** appears to induce a higher disorder into the phospholipid bilayer. This emphasizes the influence of the length of the spacer on the overall behavior of the mixed phase.

^{31}P NMR gave further important information about the incorporation of cholesteryl-β-cyclodextrins. Fig. 5 displays the ^{31}P NMR spectra of fully hydrated DMPC samples alone and in the presence of **1** and **2**. The reference spectrum is typical of the L_α phase with a chemical shift anisotropy (CSA) of ca. 50 ppm [24]. Upon addition of **2**, the bilayer structure is retained with similar molecular order as shown by the conservation of the CSA value. Conversely, in the presence of **1**, a broad isotropic peak appears, indicating the formation of smaller systems (LUV or SUV).

To conclude, new amphiphilic derivatives of cyclodextrins were prepared by grafting the cholesterol moiety on the primary hydroxyl face of the CD molecule through spacers of various lengths. These compounds can be incorporated into bilayer phospholipidic systems at high molar ratio and give rise to separated bilayer systems. Further investigations will concern the full characterization of these peculiar systems using diffusion techniques and deuterium NMR spectroscopy of specifically labeled samples on both the polar head and the hydrophobic cores with a special attention to the effect of the spacer length. Experiments dealing with the inclusion of guest molecules and the potential applications of these systems for transdermal formulations and drug delivery are currently under investigation.

3. Experimental

Synthesis of materials.—The β-cyclodextrin obtained from Roquette Frères was freeze-dried before synthesis. 6^{I}-Amino-6^{I}-deoxycyclomaltoheptaose was obtained in three steps from the parent cyclodextrin, as described [10]. Cholest-5-en-3α-ylamine (3-α-aminocholesterol) was prepared in two steps from cholest-5-en-3β-ol (3β-cholesterol) according to literature methods [12,13]. Other chemicals were purchased from Sigma or

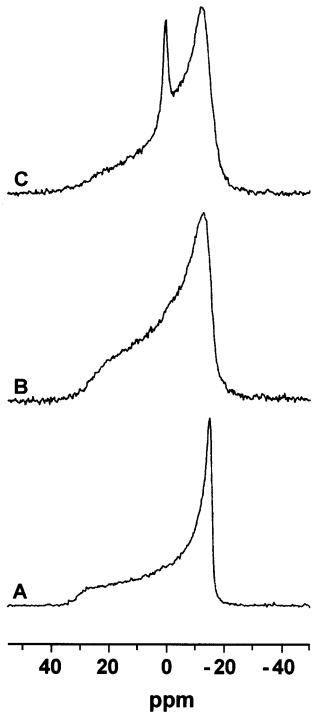


Fig. 5. ^{31}P NMR spectra (81 MHz, 25 °C) of the mixed DMPC/Chol-β-CD phases. (A) Pure DMPC in excess water (80% w/w); (B) DMPC/Chol-β-CD **2** system (83:17 w/w); (C) DMPC/Chol-β-CD **1** system (83:17 w/w).

Fluka. 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 fitted with an ELS detector and a μBondapak C_{18}-bonded silica column, by elution of the appropriate solvent at 10 mL/min. Electrospray mass spectrometry analysis was performed in positive mode on a ZabSpec TOF (Micromass, UK) mass spectrometer. The compounds were individually dissolved in a 1:1 water–MeCN mixture at a concentration of 0.01 mg/mL and infused into the electrospray ion source. The mass spectrometer was operated at 4 kV. Accurate mass measurement has been achieved using polyethylene glycol as the internal reference masses. Melting points were determined with an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured using a Perkin–Elmer 341 polarimeter. Elemental analysis were performed at the Service Central de Microanalyses of CNRS, Lyon, France. The samples (cyclomaltoheptaose derivatives) were previously dried under vacuum for 48 h in presence of P_2O_5.

^{1}H NMR experiments were performed at 500 MHz using a Bruker DRX500 spectrometer. In all cases, measurements were performed at 25 °C under careful temperature regulation. Deuterium oxide and Me_2SO-d_6 were obtained from Euriso-Top (France). The length of the 90° pulse was ca. 7 μs. 1D NMR spectra were collected using 16 K data points. Chemical shifts are given relative to external Me_4Si (0 ppm) and calibration was performed using the signal of the residual protons of the solvent as a secondary reference. The NOESY experiment [15] was obtained using the PULSE program available from the Bruker library using a 300 ms mixing time. This bidimensional experiment was acquired using 2 K data points and 300 time increments. The phase sensitive (TPPI) sequence was used and processing resulted in a 1 K × 1 K (real–real) matrix. Details concerning experimental conditions are given in the figure captions. All NMR data were processed and plotted using the UXNMR program (Bruker Analytische Messtechnik) on a Silicon graphic workstation.

6^I-(Cholest-5-en-3β-yloxycarbonyl)amino-6^I-deoxycyclomaltoheptaose (**1**).—Cholesteryl chloroformate (0.19 g, 0.42 mmol) was added in one portion to a solution of freeze-dried 6^I-amino-6^I-deoxycyclomaltoheptaose (0.40 g, 0.35 mmol) and NEt_3 (0.06 mL, 0.42 mmol) in dry DMF (4 mL). The reaction mixture was stirred at room temperature (rt) under nitrogen overnight, the solvent was removed under reduced pressure and the residual syrup was poured into ether (50 mL). The white precipitate was filtered, washed with ether, water and dried. Pure **1** (0.36 g, 67%) was obtained as a white amorphous powder; mp 235 °C (dec); R_f 0.71 (5:5:1 NH_4OH–EtOH–BuOH); ^{1}H NMR (Me_2SO-d_6): δ 6.75 (t, 1 H, ^3J 5.3 Hz, NH), 5.72 (m, 14 H, OH-2, OH-3 β-CD), 5.33 (m, 1 H, H-6 Chol), 4.83 (m, 7 H, H-1 β-CD), 4.45 (m, 6 H, OH-6 β-CD), 4.39 (m, 1 H, H-3 Chol), 3.66–3.08 (m, 42 H, H-2, H-3, H-4, H-5, H-6, H-6′ β-CD), 2.24 (m, 2 H, H-4, H-4′ Chol), 1.98–0.66 (m, 41 H, H Chol); ESI-HRMS: $[M + Na]^+$ m/z 1568.7097 Calcd for $C_{70}H_{115}NNaO_{36}$ Found: 1568.7094. Anal. Calcd for $C_{70}H_{115}NO_{36}\cdot 2H_2O$: C, 53.12; H, 7.58. Found: C, 53.32; H, 7.67.

6^I-(Cholest-5-en-3α-ylamido)succinylamido-6^I-deoxycyclomaltoheptaose (**2**).—Succinic anhydride (0.045 g, 0.45 mmol) dissolved in dry DMF (1.5 mL) was added to a solution of freeze-dried 6^I-amino-6^I-deoxycyclomaltoheptaose (0.410 g, 0.36 mmol) in dry DMF (12 mL). The reaction mixture was stirred for 3 h at rt under nitrogen. After evaporation of most of the solvent, the residual syrup was poured into acetone (150 mL). The crude product was isolated by filtration, washed with acetone and dried. Pure 6^I-succinylamido-6^I-deoxycyclomaltoheptaose (**6**) was obtained (0.400 g, 0.32 mmol). To a solution of this compound in dry DMF (14 mL), DIC (0.2 mL, 1.29 mmol) and hydroxybenzotriazole (0.20 g, 1.29 mmol) dissolved in DMF (3 mL) were added successively. The mixture was stirred under nitrogen for 1 h at rt and cholest-5-en-3α-ylamine (0.15 g, 0.39 mmol) dissolved in dry THF (14 mL) was added. The mixture was stirred at rt for 48 h. The reaction was stopped by addition of water (0.5 mL) at 0 °C. After evaporation of most of the solvents, the residual oil was poured into acetone

(150 mL). The white precipitate was filtered, washed with acetone and dried to give a solid which contained almost pure **2** (0.355 g, 67%). Final purification by HPLC (water–MeOH, gradient elution) afforded **2** as an amorphous powder; mp 225 °C (dec); R_f 0.63 (5:5:4 NH_4OH–EtOH–BuOH); ^1H NMR (Me_2SO-d_6): δ 7.65 (t, 1 H, ^3J 5.2 Hz, NH β-CD), 7.32 (d, 1 H, $^3J = 7.6$ Hz, NH Chol), 5.77 (m, 14 H, OH-2, OH-3 β-CD), 5.24 (m, 1 H, H-6 Chol), 4.85 (m, 7 H, H-1 β-CD), 4.47 (m, 6 H, OH-6 β-CD), 3.92 (m, 1 H, H-3 Chol), 3.69–3.18 (m, 42 H, H-2, H-3, H-4, H-5, H-6, H-6′ β-CD), 2.42 (m, 1 H, H-4 Chol), 2.31 (m, 4 H, CH_2 succ), 1.99–0.65 (m, 42 H, H Chol); ESI-HRMS: $[M + Na]^+$ m/z 1623.7518 Calcd for $C_{73}H_{120}N_2NaO_{36}$ Found 1623.7513. Anal. Calcd for $C_{73}H_{120}N_2O_{36}\cdot 2H_2O$: C, 53.54; H, 7.63. Found: C, 53.24; H, 7.84.

Measurements of physical properties

Preparation of the samples. DMPC (99%) was purchased from Sigma. Water was produced from the Milli-Q water purification from Millipore. Samples were prepared according to a procedure leading to perfect molecular dispersion as described previously [16].

As an example, cholesteryl-β-cyclodextrin **2** (20.7 mg, 0.013 mmol) was dissolved in a solution of DMPC (100 mg, 0.15 mmol) in a mixture of 4:1 $CHCl_3$–MeOH (60 mL). The solvents were removed under reduced pressure. After elimination of the residual traces of $CHCl_3$–MeOH under high vacuum overnight, the obtained lipidic film was hydrated by a large excess of water (1 mg DMPC–**2** mixture/mL). The suspension was ultrasonicated, using an ultrasonic disintegrator (Vibracell 72412), and then freeze-dried. The resulting powder was weighed and rehydrated at fixed water contents. The sample was stored at 30 °C and all steps were performed at a temperature much higher than the gel–liquid crystal phase transition temperature of DMPC (23 °C). The same procedure was used to prepare all the other samples according to the appropriate weight ratios.

Instrumentation.—X-ray scattering experiments were performed at 30 °C on a pinhole collimation Huxley–Holmes type SAXS camera [25]. The X-rays are generated by a rotating anode (RU 400, Rigaku) with 15 kW electrical power and a 1×8 mm actual size of X-ray source. The camera is equipped with a 2D 256×256 channels detector operating under vacuum. The sample to detector distance is 2100 mm. In our experiments the accessible Q range was 0.015–0.35 $Å^{-1}$.

DSC experiments were performed using a Perkin–Elmer DSC-7 instrument with a scan rate of 2 °C/min in the 5–50 °C range.

^{31}P NMR experiments were performed at 81 MHz under broad-band proton decoupling using a Bruker AC 200 spectrometer. In all cases, measurements were performed at 25 °C under careful temperature regulation.

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