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Amino Acid-Bile Acid Based Molecules: Extremely Narrow Surfactant Nanotubes Formed by a Phenylalanine-Substituted Cholic Acid

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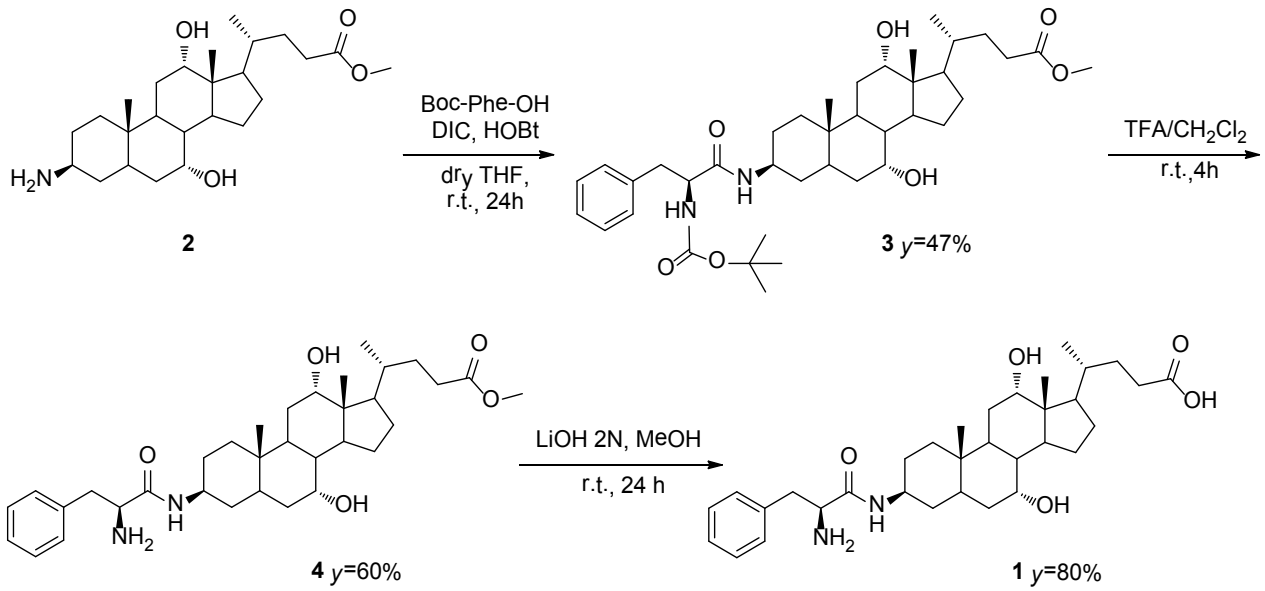
Contents

Materials and Methods

Synthesis of β-L-PheC

The novel derivative β-L-PheC **1** was prepared by the synthetic route shown in scheme 1. The 3β-aminoderivative **2** of cholic acid used as starting material, was obtained by the procedures previously described in the literature.[1-3]

Scheme 1



All the reagents were purchased from Sigma-Aldrich and used without further purification. Dry solvents, namely dichloromethane, tetrahydrofuran (THF) and methanol, were distilled following standard procedures before use. Reactions and chromatographic separations were monitored by thin layer chromatography (TLC) on 0.25 mm silica gel plate (Merck Kieselgel 60 F_{254}). Phosphomolybdic acid 12% solution in EtOH, I_2 vapor, and UV light (254 nm) were used as revealing agents. Column chromatography was carried out on silica-gel (Merck. Kieselgel 60, 70-230 mesh, 0.063-0.20 mm). ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL 300 Mercury (300 MHz) spectrometer using 5 mm tubes and chloroform-*d* ($CDCl_3$), methanol-d_4 (CD_3OD), dimethyl sulfoxide-d_6 ($(CD_3)_2SO$) as internal standards. IR spectra of products ($CHCl_3$ solution, 10 mg/ml) were recorded with a Shimadzu IR 470 spectrophotometer. High resolution ESI mass spectra were carried out on a Q-TOF Micro

spectrometer operating in a positive ion mode. Melting points were determined using a Mettler FP 80 apparatus, interfaced with a Microstar IV microscope.

Synthesis of N-*tert*-butyloxycarbonyl-L-Phenylalanine. In a 100 ml round bottom flask, di-*tert*-butyl dicarbonate (Boc_2O) (2.84 g, 13.0 mmol) and NaOH (800 mg, 20.0 mmol) were added to a solution of L-Phenylalanine (1.65 g, 10 mmol) in THF/H_2O (1:1, 40 mL). The resulting mixture was stirred at room temperature for 16 hour, then concentrated to half volume under reduced pressure. CH_2Cl_2 (40 mL) was added, and the biphasic mixture cautiously acidified by HCl 10% dropping, until no formation of a white precipitate was observed. The organic layer was thereafter separated, dried over Na_2SO_4, and finally concentrated to give an oily residue. The product was recrystallized from an ethyl acetate-petroleum ether 1:9 mixture as a white solid (1.86 g, y=70%). m.p. 84-85 °C; ^{1}H NMR (300 MHz, $CDCl_3$, δ): (2:1 rotameric mixture) 11.43 (bs, 1H, COOH), 7.27 (m, 5H, aromatic), 6.68 (m, 1H, -NHCO-), 5.08 (m, 1H, CH major rotamer), 4.65 (m, 1H, CH, minor rotamer), 3.21 (m, 2H, CH_B of CH_2, both rotamers), 3.09 (m, $1H_A$, CH_A of CH_2, major rotamer), 2.93 (m, 1H, CH_A of CH_2, minor rotamer), 1.44 (s, 9H, CH_3 of *t*-Bu, major rotamer), 1.31 (s, 9 H, CH_3 of *t*-Bu, minor rotamer); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 176.5, 176.0, 156.4, 155.3, 135.8, 129.4, 128.5, 127.0, 81.5, 80.2, 56.0, 54.2, 39.1, 37.8, 28.2, 27.9; IR [$CHCl_3$] 3316, 2979, 1712, 1649, 1366, 1159 cm^{-1}.

Synthesis of compound 3. *N,N'*-diisopropylcarbodiimide (DIC) (0.11 mL, 0.7 mmol) was added to an ice-bath cooled solution of **2** (421 mg, 1.0 mmol), N-*tert*-butoxycarbonyl-(*S*)-phenylalanine (318 mg, 1.2 mmol), and 1-hydroxybenzotriazole (HOBt) (121 mg, 0.9 mmol) in 10 mL of dry THF under inert atmosphere. The reaction mixture was kept under stirring at 0°C for 30 min, then at r.t. for 48 h. After reaction completion the white suspension was filtered, and the filtrate concentrated *in vacuo*. The resulting oily residue was dissolved in ethyl acetate (20 mL), and the organic layer washed with 2N citric acid (2 x 20 mL), with satd. $NaHCO_3$ (2 x 20 mL) and with brine (2 x 20 mL). After drying over Na_2SO_4, the solvent was removed under reduced pressure, affording a crude residue that was purified by silica-gel column (EtOAc-petroleum ether 3:7), giving methyl 3β-(2'-(S)-(*tert*-butoxycarbonyl)amino-3'-phenylpropanamido)-7α,12α-dihydroxy-5β-cholan-24-oate **3** (314 mg, y=47%). mp 98-99 °C; $[\alpha]_D$ = 43.65 deg cm^3 g^{-1} dm^{-1} (c=0.90 in MeOH); ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.26 (m, 6H, aromatic, -NHCO-), 5.83 (s, 1H, -NHCOO-), 5.38 (m, 1H, Phe CH), 4.24 (1H, m, CH-3), 3.93 (m, 3H, CH-12 and 2 OH), 3.80 (bs, 1H, CH-7), 3.64 (s, 3H, OCH_3), 3.09 (m,

H_B of Phe CH_2), 2.90 (m, H_A of Phe CH_2), 1.42 (s, 9H, *t*-Bu), 1.00-2.50 (m, 21H, CH_2 and CH of steroid skeleton and side chain), 0.95 (d, *J*=4.95, 3H, CH_3 -21), 0.78 (s, 3H, CH_3 -19), 0.65 (s, 3H, CH_3 -18); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 174.7, 170.2, 155.4, 136.9, 129.1, 128.6, 126.7, 79.9, 72.8, 68.2, 56.2, 51.4, 47.1, 46.4, 45.3, 41.7, 39.3, 38.9, 36.8, 35.1, 34.9, 34.3, 33.1, 31.0, 30.9, 30.8, 28.4, 28.2, 27.4, 25.8, 24.4, 23.1, 22.8, 17.2, 12.4. IR [$CHCl_3$] 3445, 2985, 1720, 1699, 1668, 1160 cm^{-1}; HRMS (ESI, m/z): calcd for $[C_{39}H_{60}N_2O_7 + Na]^+$ 691.4293; found 691.4301.

Synthesis of compound 4. Trifluoroacetic acid (1 mL) was added dropwise at 0°C to a stirred solution of compound **3** (668 mg, 1.0 mmol) in dry CH_2Cl_2 (19 mL). The mixture was stirred at r.t. for 4 h and then washed with satd. $NaHCO_3$ (3 x 25 mL) water and brine (2 x 20 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. Purification by silica-gel column chromatography (eluting first with ethyl acetate, then with MeOH-Et_3N 99.5:0.5) afforded the methyl 3β-(2'-(*S*)-amino-3'-phenylpropanamido)-7α,12α-dihydroxy-5β-cholan-24-oate **4** (341 mg, y=60%) as white solid. M.p. 205-207 °C, $[\alpha]_D$ = 37.72 deg cm^3 g^{-1} dm^{-1} (c=0.91 in MeOH); ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.45 (m, 1H, -NHCO-), 7.26 (m, 5H, aromatic), 4.06 (m, 1H, Phe CH), 3.94 (bs, 1H, CH-12), 3.83 (bs, 1H, CH-7), 3.64 (s, 4H, OCH_3, CH-3), 3.19 (m, 1H, Phe CH_2), 1.10-2.70 (m, 28H, CH_2 and CH of steroid skeleton and side chain, 2OH, NH_2), 0.96 (d, 3H, *J*=5.0 Hz, CH_3-21), 0.88 (s, 3H, CH_3-19), 0.66 (s, 3H, CH_3-18); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 174.6, 172.8, 137.5, 129.2, 128.6, 126.7, 72.9, 68.2, 56.3, 51.3, 47.1, 46.4, 44.9, 41.7, 40.6, 39.3, 37.2, 35.2, 35.1, 34.3, 33.4, 31.1, 31.0, 30.8, 28.4, 27.4, 25.8, 24.5, 23.2, 22.9, 17.2, 12.4; IR [$CHCl_3$] 3445, 2985, 1720, 1680, 1160 cm^{-1}; HRMS (ESI, m/z): calcd for $[C_{34}H_{52}N_2O_5 + H]^+$ 569.3949; found 569.3953.

Synthesis of compound 1. The compound **4** (568 mg, 1mmol) was dissolved in MeOH (5 mL) and 2N LiOH solution (5 mL) was added. The mixture was stirred for 24 h at room temperature. The mixture was concentrated to half volume under reduced pressure, and HCl 1N was added dropwise until the precipitation of product was complete (pH 6.7). The white solid was collected by vacuum filtration and then dried in desiccator for 24 hrs. This procedure afforded the pure product **1** (444 mg, y = 80%). m.p. 203-205 °C; $[\alpha]_D$ = 1.27 deg cm^3 g^{-1} dm^{-1} (c=0.75 in MeOH); ^{1}H NMR (400 MHz, CD_3OD, δ): 7.26 (m, 5H, aromatic), 3.92 (m, 3H, CH-12, CH-7, Phe CH), 3.75 (bs, 1H, CH-3), 2.97 (m, 2H, Phe CH_2), 1.00-2.50 (m, 24 H, CH_2 and CH of steroid skeleton and side chain), 1.00 (d, 3H, J = 6.05 Hz, CH_3 -21), 0.84 (s, 3H, CH_3 -19); ^{13}C NMR (400 MHz, DMSO-d_6, δ): 174.9, 172.5, 129.1, 127.8, 125.9,

70.9, 66.1, 55.6, 46.0, 45.7, 44.1, 41.3, 41.1, 39.3, 36.5, 34.9, 34.6, 34.3, 33.2, 30.9, 30.8, 30.5, 28.6, 27.1, 25.5, 24.3, 22.8, 22.7, 16.9, 12.2; HRMS (ESI, m/z) calcd for $[C_{33}H_{50}N_2O_5 + H]^+$ 555.3792; found 555.3788.

Cryo-TEM measurements.

The freezing of the cryo-TEM sample was carried out using a controlled environment vitrification system,[4] keeping the relative humidiy close to saturation at around 26 °C. A thin film of the gel on a lacey carbon-coated copper grid was rapidly vitrified by plunging into liquid ethane (-180 °C) and stored in liquid nitrogen before the examination. The micrographs were recorded using a Philips CM120 Bio TWIN electron microscope equipped with a Gatan MSC791 cooled-CCD camera detection system, operating at 120 kV, under low electron dose conditions.

AFM measurements.

AFM experiments were carried out with a DIMENSION ICON controlled by a Nanoscope V Controller (Brucker AXS, Germany) equipped with a closed loop scanner The images were obtained in AFM intermittent contact (Tapping) mode in air. The sample was prepared by smearing the gel through a gentle rotation by spin coating at around 600 rpm for several minutes, until the surface appear dried. No rinsing was employed in order to avoid modification of network structure.

SAXS measurements.

SAXS measurements were performed at the MAX II SAXS beamline I911-4 at MAXIV Laboratory in Lund, Sweden.[5] Scattering curves, recorded within the range of $0.01<q<0.4$ $Å^{-1}$ ($q=4\pi \sin(\theta)/\lambda$, where 2θ is the scattering angle and λ is the X-ray wavelength), were corrected for solvent and capillary contributions. The Indirect Fourier Transform method was used for interpreting the spectra of the micellar solutions. The pair distance distribution function $p(r)$ of the single particle was obtained, which provides the maximum dimension and the gyration radius.[6]

The SAXS spectra of the gel were analyzed using the expression of the intensity of a hollow cylinder, obtained starting from the general equation of the form factor for cylinder with shells as[7]

$$I(q) \propto \left[D_o^2 \frac{J_1(q D_o/2)}{q D_o/2} - D_i^2 \frac{J_1(q D_i/2)}{q D_i/2} \right]^2$$

where D_o and D_i are the outer and inner diameters of the tube, respectively, q is the scattering vector and $J_n(x)$ is the n order Bessel function of the first kind. A theoretical equation for the intensity of a collection of N tubules can be estimated by extending the expression proposed by Oster and Riley for bundles of cylinders,[14] in analogy with the Debye formula, as

$$I_N(q) \propto \frac{1}{N^2} P(q) \sum_{i=1}^{N} \sum_{j=1}^{N} J_o(q d_{ij})$$

where d_{ij} is the distance between the centers of the ith and jth tubule. The fits of the experimental curves of Figure 3 were performed assuming

$$I(q) \propto \sum_{N=1}^{K} w_N N \langle I_N(q) \rangle \qquad (1)$$

where w_N is the weight fraction of the bundle of N tubules. The average in Equation 1 is considered with respect to a distribution of diameters, in our calculations a Gaussian distribution with a 10% standard deviation was considered.

CD measurements.

CD spectra were recorded on a JASCO model 715 and reported in molar ellipticity [θ]. The UV spectra were reported in molar extinction coefficient ε, after correction for the solvent contribution. The spectra were recorded in the range of wavelength (λ) 200-300 nm.

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Supporting Figures

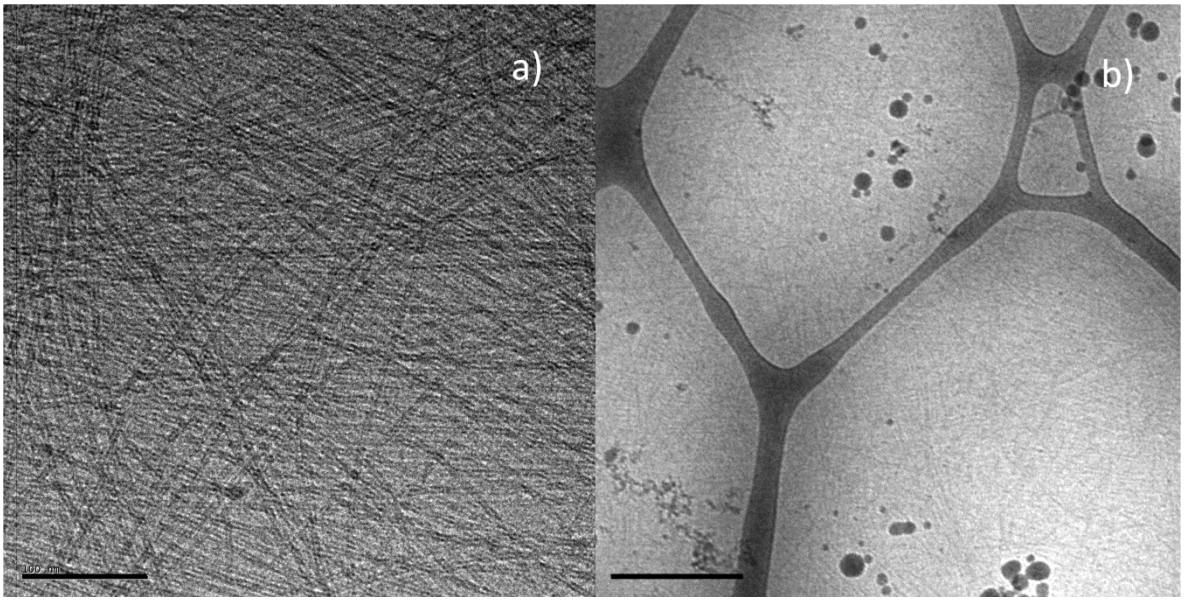

Figure S1. Cryo-TEM images of the 36.0 mM β-L-PheC gel, at pH=1.1 and NaCl concentration of 0.150 M. The bars are: a) 100 nm and b) 500 nm.

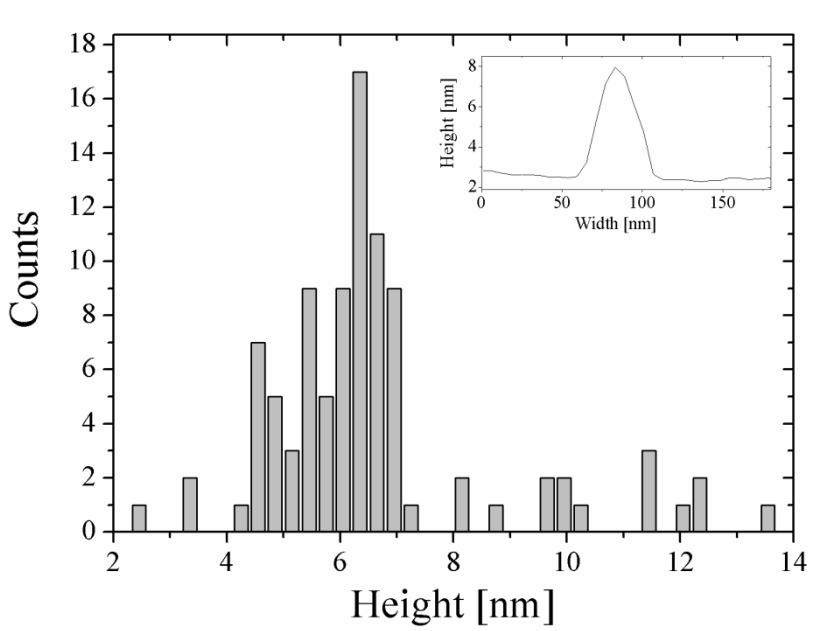

Figure S2. Distribution of the heights from AFM images. An example of tubule profile is reported in the inset.

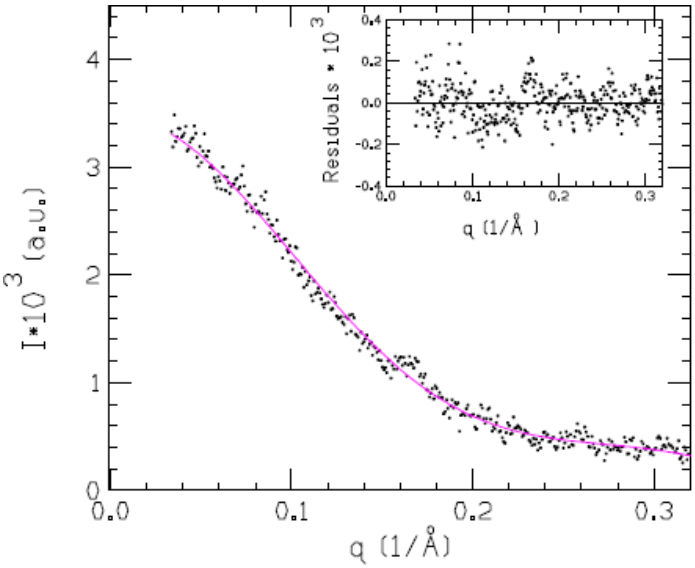

Figure S3. Experimental SAXS data (dots) of β-L-PheC 36.0 mM at pH=1.1 and T=55°C, and best fitting curve obtained by ITP (line). The fitting curve is consistent with globular micelles with gyration radius and maximum dimension of 1.0±0.1 and 2.7±0.1 nm, respectively. The residuals are shown in the inset.

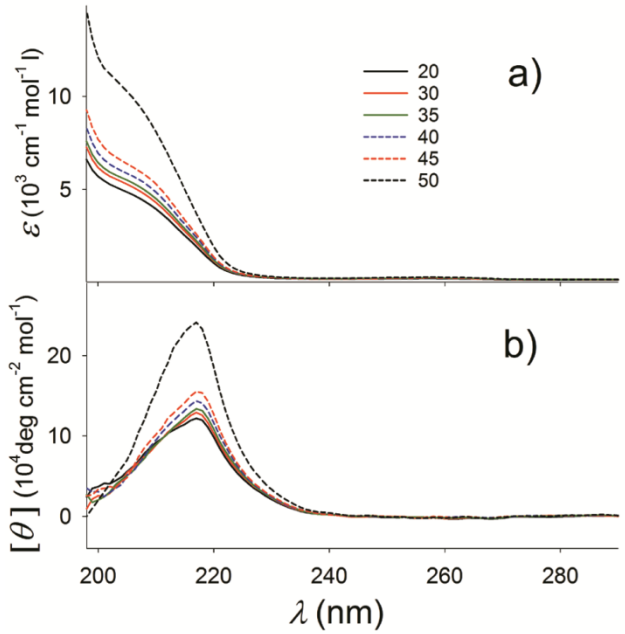

Figure S4. a) UV and b) CD spectra of β-L-PheC at concentration of 18.0 mM and pH=1.1 as a function of temperature. The legend reports the temperature (°C).