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# pH-Sensitive liposomes with embedded ampholytic derivatives of cholan-24-oic acid

**Anton S. Popov, Anna A. Efimova, Alexey V. Kazantsev , Dmitry A. Erzunov, Nikolay V. Lukashev, Irina D. Grozdova, Nikolay S. Melik-Nubarov and Alexander A. Yaroslavov**

## *Methods*

Mean hydrodynamic diameter of liposomes was measured by dynamic light scattering (DLS) at a fixed scattering angle (90°) in a thermostatic cell with a Brookhaven Zeta Plus instrument (USA). Software provided by the manufacturer was employed to calculate diameter values (error ±7%). Electrophoretic mobility (EPM) of liposomes was determined in a thermostatic cell by laser microelectrophoresis by using Brookhaven Zeta Plus instrument with the corresponding software (error ±3-5%).

The pH values of solutions were measured with a Radiometer pHM 83 pH meter (Denmark) equipped with a measuring P1041 glass electrode and a K4041 calomel reference electrode (error ±0.02 units).

The conductivities of solutions were determined with a Radiometer CDM 83 conductometer (Denmark) equipped with a PP1042 platinum electrode (error ±0.01 units).

NMR spectra were recorded with a Bruker Avance 400 (1H 400 MHz, 13C 100.6 MHz) spectrometer at ambient temperature. Chemical shifts are presented in ppm (δ scale) and referenced to tetramethylsilane (δ = 0 ppm) in the 1H NMR spectra and to the solvent signal in the 13C NMR spectra. MALDI-TOF spectra were recorded with a Bruker Daltonics UltraFlex instrument in a dithranol matrix using PEG 400 or PEG 600 as the internal standard.

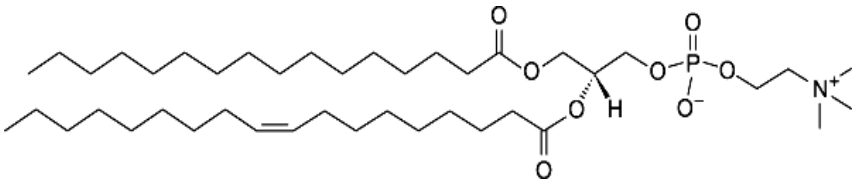
All experiments were carried out in quadruplicates. Double-distilled water was used for making solutions after additionally treating it with a Milli-Q Millipore system.

## *Experimental procedures*

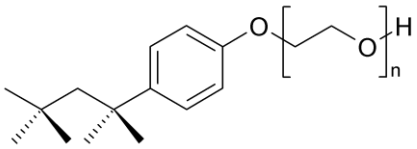
Liposomes were prepared via mixing of methanol-chloroform (1/1 wt/wt) solutions of 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and AMS, evaporation of the organic solvent under vacuum at 30°C, dispersion of the lipid film a $10^{-2}$ M buffer solution with appropriate pH followed by final sonication of the resulting lipid/water mixture with a Cole-Parmer 4710

ultrasonic homogenizer for 400 s (2 × 200 s) at 20 °C. The resulting liposomes were separated from titanium dust via centrifugation in a J-11 centrifuge (Beckman) for 5 min at 10000 rpm.

Liposomes loaded with 1 M NaCl were prepared via dispersion of the lipid film in a $10^{-3}$ M buffer solution containing 1 M NaCl and dialysis of the suspension against the $10^{-3}$ M buffer that was renewed every 30 minutes.



DOPC

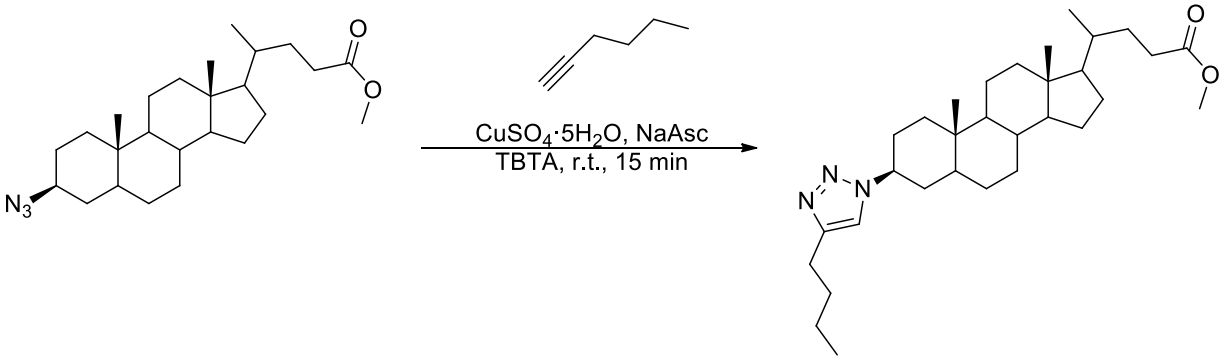


n=9-10

Triton X-100

**Figure S1** The structure of the compounds: zwitter-ionic 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti polar lipids), Triton X-100 (Sigma).

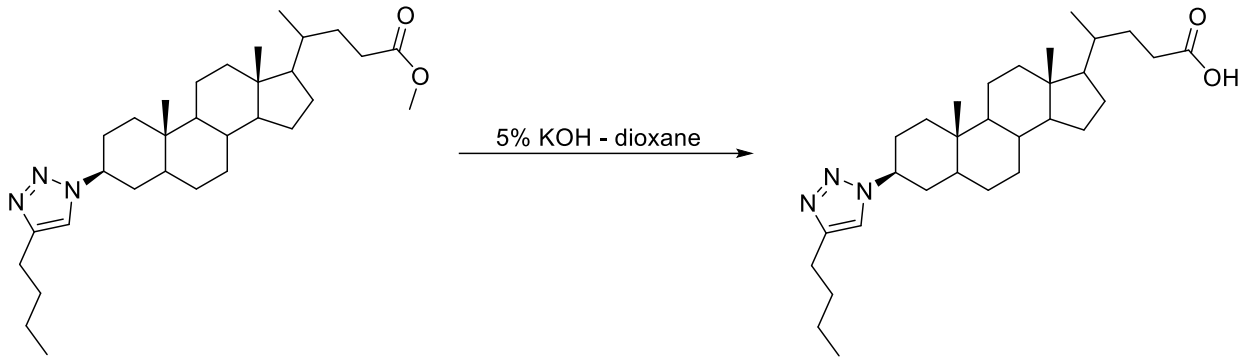
**Procedure S1. Methyl 3-(4-Butyl-1H-1,2,3-triazolyl)-5β-cholan-24-oate** was synthesized according:




In a vial with a screw cap 83,1 mg (0,2 mmol) methyl 3β-azido-5β-cholan-24-ate was mixed with 5 mg (0,02 mmol) $CuSO_4 \cdot 5H_2O$, 16 mg (0,08 mmol) sodium ascorbate and 11 mg (0,02 mmol) TBTA (Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine) under Ar; 1 ml of mixture THF-$H_2O$ (4:1), 26 μl (0,22 mmol) hexyne-1 were added and stirred for 15 min at r.t. The obtained solution was diluted with $CH_2Cl_2$ (100 ml), washed with sat. aqueous solution of EDTA (50 ml) and water (2x50 ml). Organic layer was separated, dried with $Na_2SO_4$ and solvents

evaporated. The product was purified by column chromatography ($CH_2Cl_2$/MeOH 50:1). Yield – 77%, white powder. $^1$H NMR (400 MHz, $CDCl_3$): δ = 7.33 (s, 1H, CH(triaz)), 4.63 (bs, 1H, 3-CH), 3.65 (s, 3H, COO$\underline{CH_3}$), 2.70 (t, *J* = 7.6, 2H, $CH_2$(triaz)), 0.96-0.87 (s+d+t, 9H, 21-$CH_3$+19-$CH_3$+$\underline{CH_3}CH_2$), 0.64 (s, 3H, 18-$CH_3$). $^{13}$C NMR (100 MHz, $CDCl_3$): 174.7 (COOMe), 147.8 (C(triaz)), 119.6 (CH(triaz)), 56.6, 56.4, 56.0, 51.4, 42.8, 40.5, 40.2, 37.3, 35.6, 35.4, 34.8, 31.6, 31.1, 31.0, 30.8, 30.0, 28.2, 26.5, 26.1, 25.4, 25.0, 24.2, 23.8, 22.4, 21.0, 18.3, 13.8, 12.0.

**3-(4-Butyl-1H-1,2,3-triazolyl)-5β-cholan-24-oic acid (AMS 1)** was synthesized according:




In a vial with a screw cap 53 mg (0,106 mmol) of methyl 3-(4-Butyl-1H-1,2,3-triazolyl)-5β-cholan-24-oate was placed and 3 ml of 1,4-dioxane with 5% aq. KOH (1:1) added. Obtained mixture was stirred for 3 h at 80°C, evaporated to the half of volume and acidified by HCl to pH 5-6. Product was extracted by EtOAc (3x30 ml), organic layer separated and dried with $Na_2SO_4$ and solvents were evaporated. Yield – 95%, white powder. $^1$H NMR (400 MHz, $CDCl_3$): δ = 7.33 (s, 1H, CH(triaz)), 4.63 (bs, 1H, 3-CH), 2.71 (t, *J* = 7.6, 2H, $CH_2$(triaz)), 0.96-0.87 (s+d+t, 9H, 21-$CH_3$+19-$CH_3$+$\underline{CH_3}CH_2$), 0.64 (s, 3H, 18-$CH_3$). $^{13}$C NMR (100 MHz, $CDCl_3$): 179.3 (COOH), 147.8 (C(triaz)), 119.7 (CH(triaz)), 56.6, 56.4, 56.0, 42.8, 40.4, 40.1, 37.3, 35.6. 35.3, 34.8, 31.6, 31.0, 30.8, 30.77, 29.9, 28.2, 26.5, 26.1, 25.4, 24.9, 24.2, 23.8, 22.4, 21.0, 18.3, 13.8, 12.1. MALDI-TOF: 484.3927 $[M+H]^+$. Calculated for $C_{30}H_{50}N_3O_2$: 484.3898.

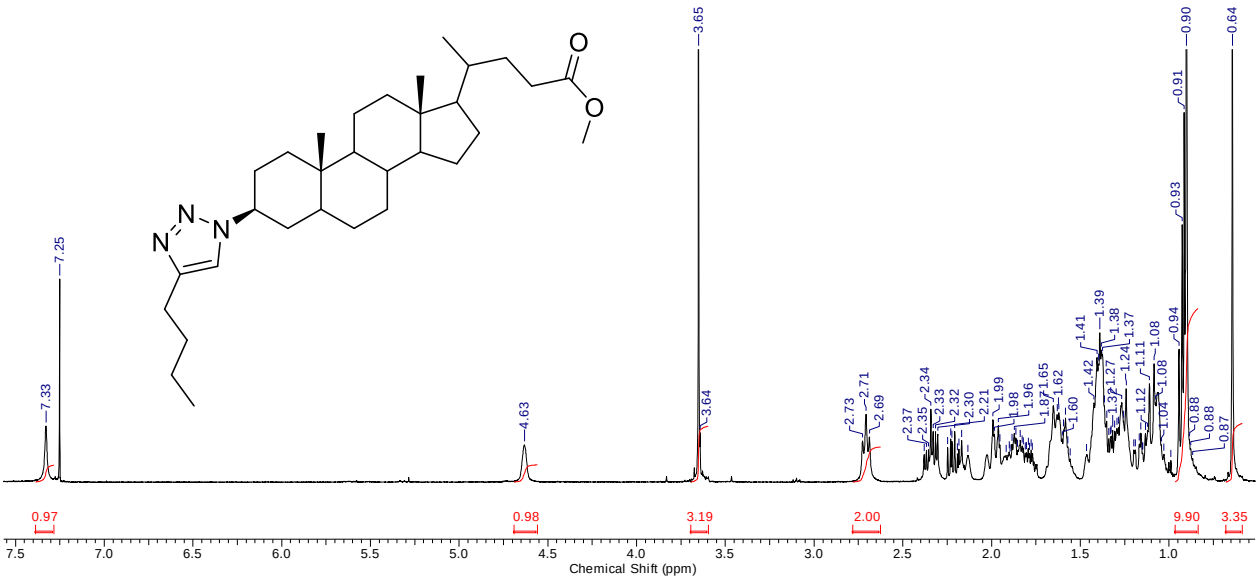



**Figure S2** Methyl 3-(4-Butyl-1H-1,2,3-triazolyl)-5β-cholan-24-oate ($^{1}$H NMR $CDCl_3$)

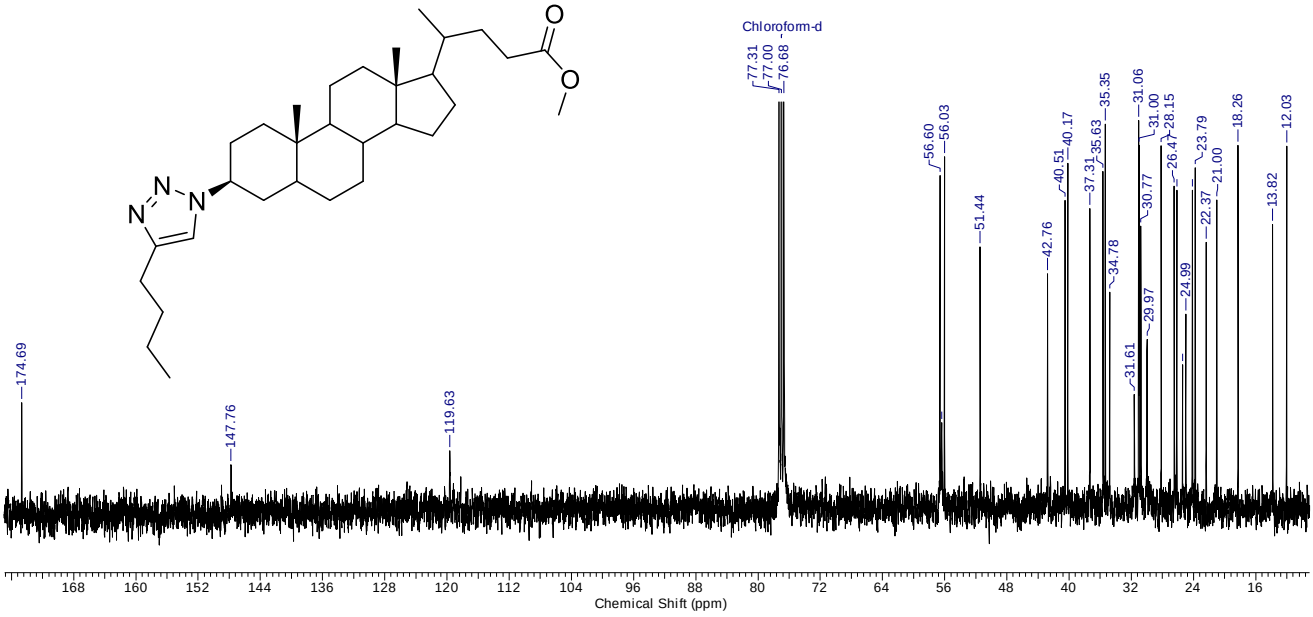



**Figure S3 Methyl** 3-(4-butyl-1H-1,2,3-triazolyl)-5β-cholan-24-oate ($^{13}$C NMR $CDCl_3$)

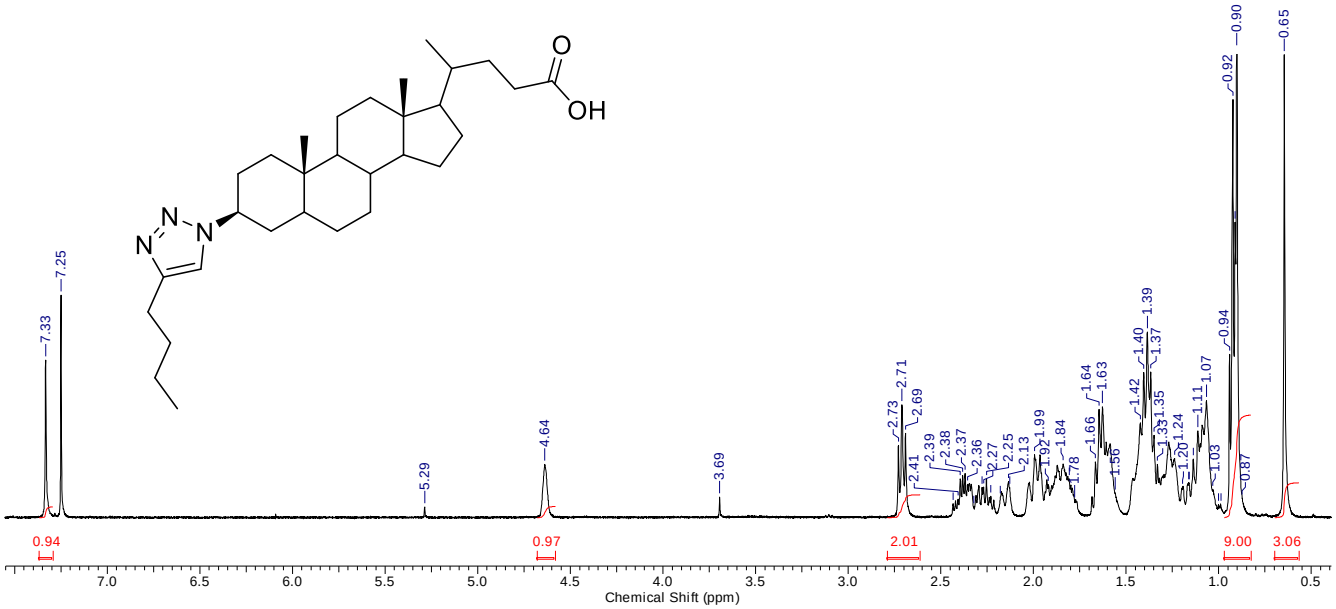



**Figure S4** 3-(4-Butyl-1H-1,2,3-triazolyl)-5β-cholan-24-oic acid ($^{1}$H NMR $CDCl_3$) (**AMS 1**)

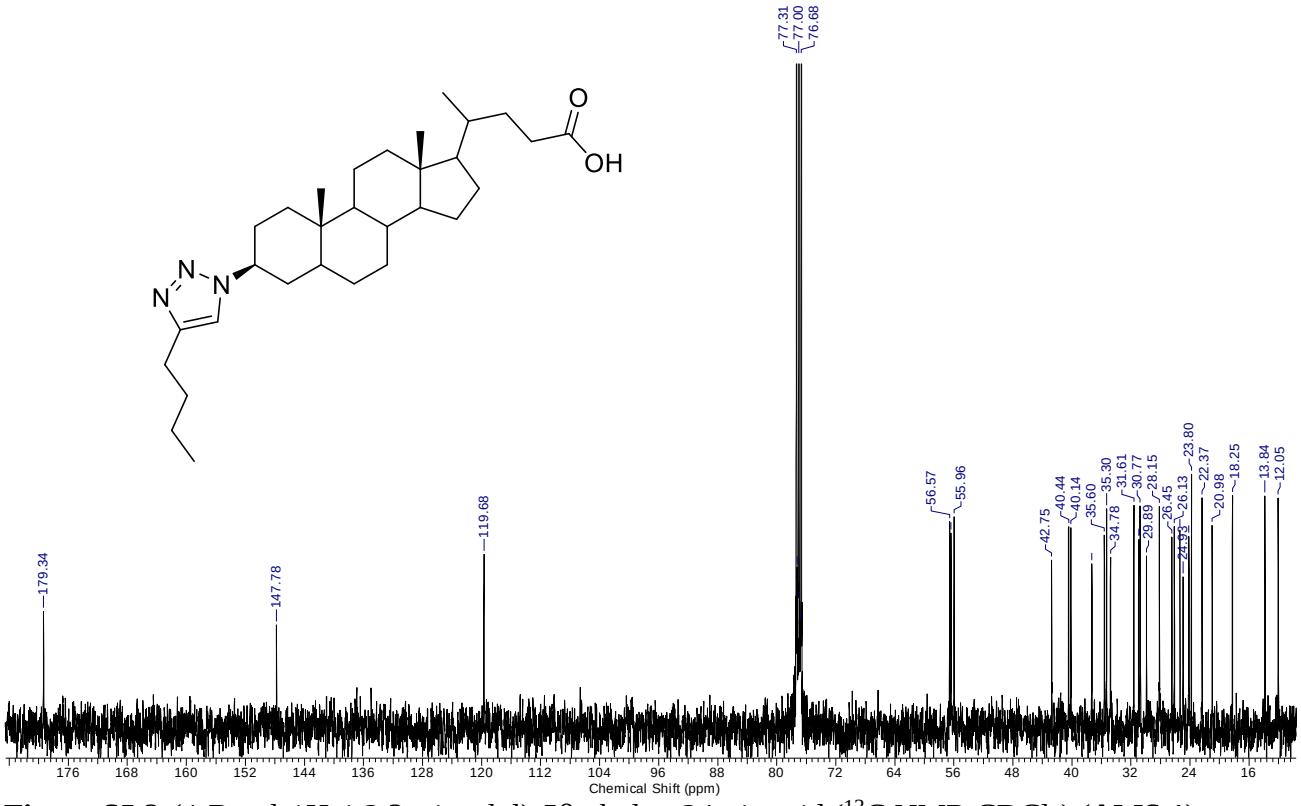
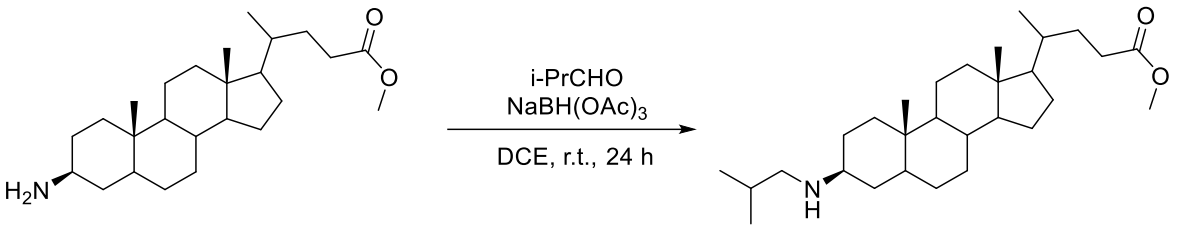



**Figure S5** 3-(4-Butyl-1H-1,2,3-triazolyl)-5β-cholan-24-oic acid ($^{13}$C NMR $CDCl_3$) (**AMS 1**)

**Procedure S2**

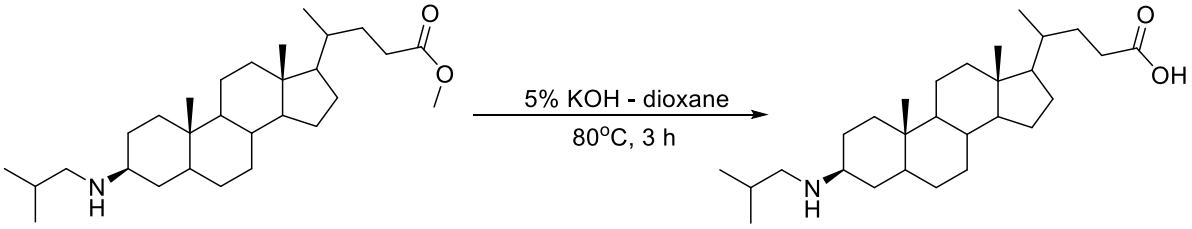
Methyl 3β-amino-5β-cholan-24-oate was synthesized from methyl 3β-azido-5β-cholan-24-oate according [Y. Zhao, Zh. Zhong, *J. Am. Chem. Soc.*, 2005, **127**, 17894].

**Methyl 3β-(isobutylamino)-5β-cholan-24-oate** was synthesized according:




To a mixture of 97 mg (0.25 mmol) methyl 3β-amino-5β-cholan-24-oate and 25 μl (19.8 mg, 0.275 mmol) isobutyraldehyde in 6 ml of dry 1,2-dichloroethane 80 mg (0.375 mmol) of sodium triacetoxyborohydride was added at ambient temperature and obtained solution was stirred for 24 h at ambient temperature. The reaction mixture was diluted with $CH_2Cl_2$ (30 ml), washed twice with sat. aqueous solution of $Na_2CO_3$. Organic layer was separated, dried with $Na_2SO_4$ and solvents evaporated. The product was purified by column chromatography ($CH_2Cl_2$/MeOH 10:1). Yield – 91%, white powder. $^1$H NMR (400 MHz, $CDCl_3$), δ: 3.66 (s, 3H, $COOCH_3$), 2.95 (bs, 1H, 3α-CH), 2.43 (d, *J* = 6.7 Hz, 2H, $NCH_2$), 0.96 (s, 3H, 19-$CH_3$), 0.93 (d, *J* = 6.7 Hz, 6H, ($\underline{CH_3}$)$_2$CH), 0.91 (d, *J* = 6.5 Hz, 3H, 21-$CH_3$), 0.64 (s, 3H, 18-$CH_3$). $^{13}$C NMR (100 MHz, $CDCl_3$): 174.7, 56.6, 55.9, 54.8, 52.9, 51.4, 42.7, 40.2, 40.0, 36.6, 35.6, 35.3, 35.1, 31.0, 30.3, 30.3, 28.2, 27.7, 26.9, 26.2, 24.1, 24.0, 23.8, 21.0, 20.9, 20.8, 18.2, 12.0.

**3β-(Isobutylamino)-5β-cholan-24-oic acid (AMS 2)** was synthesized according:




In a vial with a screw cap 59 mg (0,132 mmol) of methyl 3β-(isobutylamino)-5β-cholan-24-oate was placed and 5 ml of 1,4-dioxane with 5% aq. KOH (1:1) added. Obtained mixture was stirred for 3 h at 80°C, evaporated to the half of volume and acidified by HCl to pH 5-6. Product was extracted by EtOAc (3x30 ml), organic layer separated and dried with $Na_2SO_4$ and solvents were evaporated. Yield – 97%, white powder. $^1$H NMR (400 MHz, $CD_3OD$), δ: 3.46 (bs, 1H, 3α-CH), 2.86 (d, *J* = 6.9 Hz, 2H, N$\underline{CH_2}$), 1.05 (d, *J* = 6.5 Hz, 6H, ($\underline{CH_3}$)$_2$CH), 1.04 (s, 3H, 19-$CH_3$), 0.94 (d, *J* = 6.3 Hz, 3H, 21-$CH_3$), 0.70 (s, 3H, 18-$CH_3$). $^{13}$C NMR (100 MHz, $CD_3OD$): 176.2, 57.7, 57.4, 57.0, 54.2, 43.9, 41.4, 41.3, 37.5, 36.9, 36.7, 36.0, 32.3, 32.1, 30.7, 29.2, 28.4, 27.4, 27.0, 26.7, 25.2, 23.6, 22.5, 22.1, 20.5, 18.8, 12.0. MALDI-TOF: 432.3966 $[M+H]^+$. Calculated for $C_{28}H_{50}NO_2$: 432.3836.

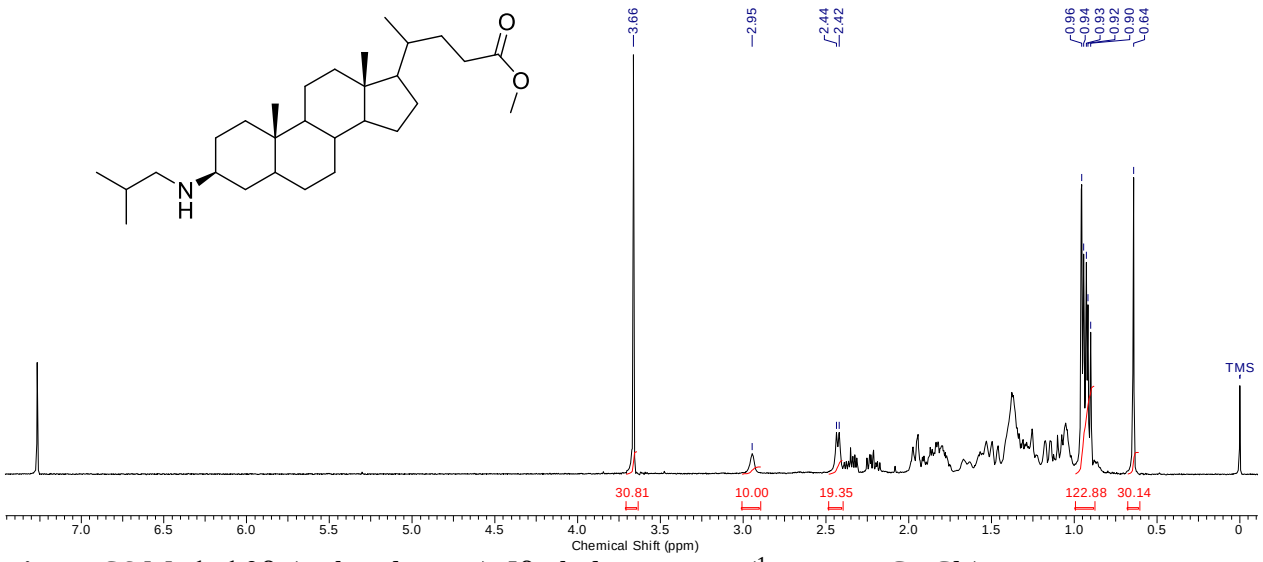



**Figure S6** Methyl 3β-(isobutylamino)-5β-cholan-24-oate ($^{1}$H NMR, $CDCl_3$)

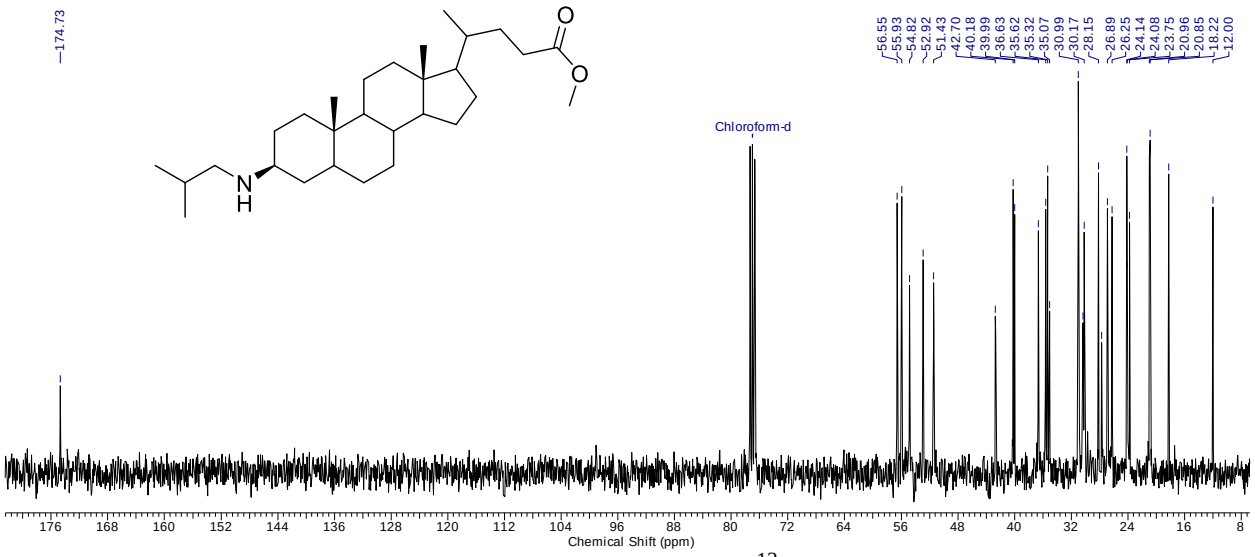



**Figure S7** Methyl 3β-(isobutylamino)-5β-cholan-24-oate ($^{13}$C NMR, $CDCl_3$)

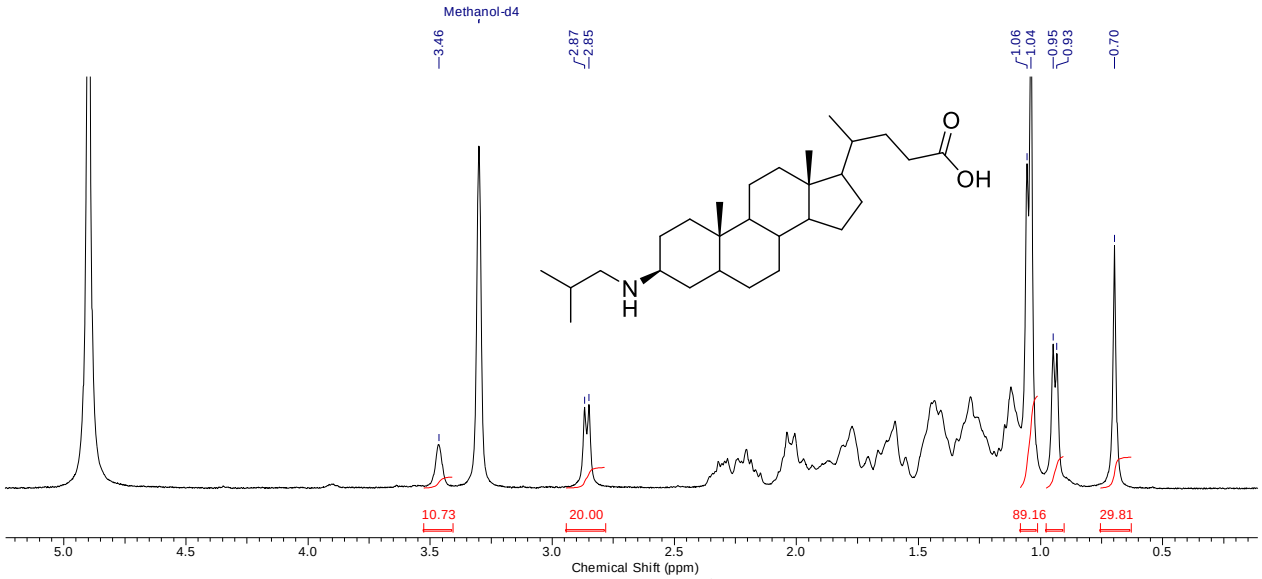



**Figure S8** 3β-(Isobutylamino)-5β-cholan-24-oic acid ($^{1}$H NMR, $CD_3OD$) (**AMS 2**)

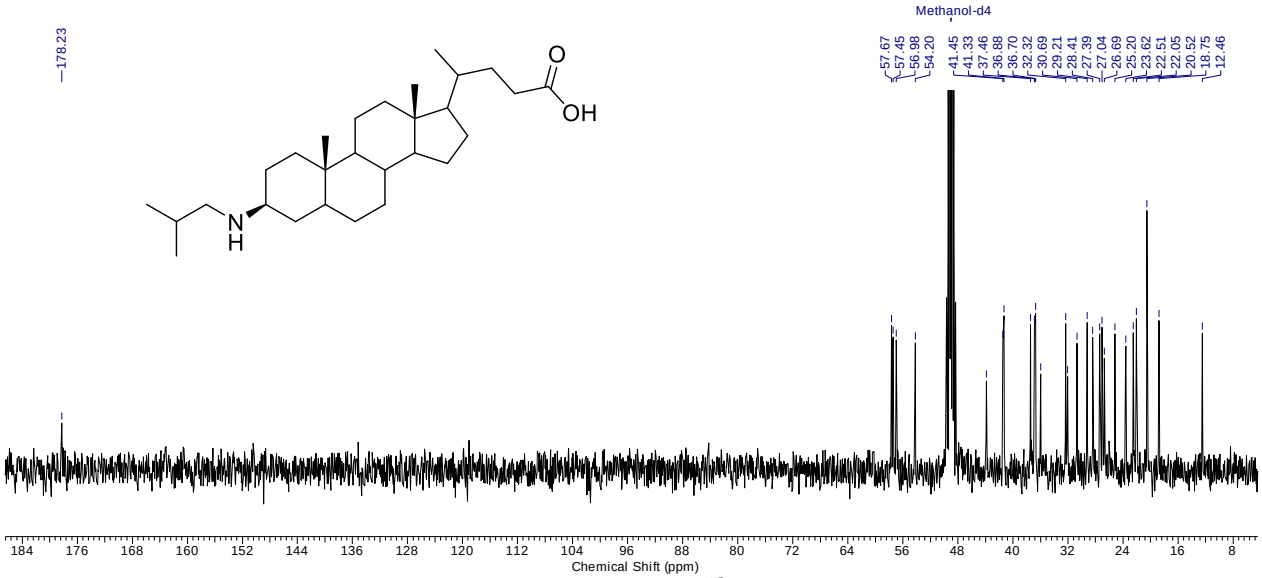



**Figure S9** 3β-(Isobutylamino)-5β-cholan-24-oic acid ($^{13}$C NMR, $CD_3OD$) (**AMS 2**)

Cytotoxicity of the liposomes towards human breast adenocarcinoma MCF-7/R cells was evaluated with a methyl-tetrazolium blue assay. The dye, penetrated into the living cells and attacked by redox enzymes, is oxidized to formazan and precipitates as dark-blue crystals whereas in dead cells such transformation does not occur. The standard procedure involves incubation of the test suspension (solution) with the cells, addition of the dye solution, dissolution of the cells in dimethylsulfoxide (DMSO), measurement of the optical density of the resulting solution and comparison of the result with the calibration curve in the absence of the tested objects. Briefly, the day before the experiment, MCF-7/R cells were seeded on a 96-well

plate (Costar, USA) at a density 3000–4000 cells per well. The next day, the culturing medium was removed and 0.2 mL of the solutions of liposomes at varying concentrations in the serum-free medium were placed in the wells for 1 h. In control wells (100% of surviving cells), the assayed compounds were replaced with equal volume of the appropriate medium. Then, the solutions were removed and the cells were cultured in 0.2 mL of fresh complete medium containing 10% of serum for 3 days. The amount of remaining living cells was assayed by addition of 0.05 mL of MTT solution (1 mg/mL) in the culturing medium for 3–4 h. Then, the medium was removed, violet crystals of formazan were dissolved in 0.1 mL of DMSO, and the optical density at 550 nm was measured on a Multiscan photometer (Titertek, USA). The portion of survived cells was calculated as a ratio of optical density in well with certain concentration to that in control well. All runs were carried out in quadruplicate.