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Haemolytic activity of formyl- and acetyl-derivatives of bile acids and their gramine salts

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ABSTRACT

Bile acids (lithocholic: LCA, deoxycholic: DCA and cholic: CA) and their formyl- and acetyl-derivatives can be used as starting material in chemical synthesis of compounds with different biological activity strongly depended on their chemical structures. Our previous studies showed that biological activity of bile acids salts with gramine toward human erythrocytes was significantly different from the activity of bile acids alone. Moreover, gramine effectively modified the membrane perturbing activity of other steroids. As a continuation of our work, the haemolytic activity of formyl- and acetyl-substituet bile acids as well as their gramine salts was studied *in vitro*. The structures of new compounds were confirmed by spectral (NMR, FT-IR) analysis, mass spectrometry (ESI-MS) as well as PM5 semiempirical methods. The results shown that the haemolytic activity of formyl- and acetyl-LCA and DCA was significantly higher in comparison with their native forms at the whole concentration range. At high concentration, formyl derivative of CA was as effective as LCA and DCA derivatives whereas at lower concentration its haemolytic activity was at the level of original acid. The acetyl-CA was not active as membrane perturbing agents. Furthermore, gramine significantly decreased the membrane-perturbing activity of hydrophobic bile acids derivatives. The results obtained with the cellular system are in line with physicochemical calculation.

1. Introduction

Bile acids are present in the human bile and blood and some of them have cytotoxic activity [1,2]. Their biological effects strongly depend on the nature of the chemical structures e.g. hydrophilic ursodeoxycholic acid (UDCA) and its taurine and glycine conjugates protect cells against apoptosis induced by hydrophobic bile acids [3,4]. Bile acids and their derivatives have been used for treatment of bile acid deficiency and liver diseases [5] and some of them are TGR5 agonists [6] or P-glycoprotein (Pgp, ABCB1) inhibitors [7]. Bile acids and their derivatives are also attractive compounds for synthetic chemists because they have a large, rigid skeleton and possess chemically different polar hydroxyl groups. It is often necessary to protect this hydroxyl groups using, for example, acetate or formate as protecting moieties [8–10]. According to Tsemg at al. [11], the formyl groups on these compounds are quite stable to various reaction conditions. The stability and ready availability of these compounds make them suitable candidates for use as starting material in various synthetic schemes. The literature describes pharmacological applications for bile acids derivatives such as: antimicrobial [12–14], antifungal [15,16], antitumor [17] or as drug carriers [18,19]. The results show that acetyl-derivatives of litocholic acids exhibit significant antibacterial activity and some of them potentiate the effect of antibiotics such as amikacin, gentamicin and neomycin [20]. Moreover, lithocholic acid and LCA acetate has been shown to inhibit the proliferation and promote differentiation of human leukaemia THP-1 cells [21] and co-operation between lithocholic acid acetate and cotylenin A, shown promising pro-differentiating effects upon primary human myeloid leukaemia cells *in vitro* [22]. Considering all above, we decided to investigate the influence of formyl- and acetyl-substituents on the cytotoxicity of bile acids (lithocholic: LCA, deoxycholic: DCA and cholic: CA) on human red blood cells (RBC). It is known that RBC are very convenient systems in the study of the interactions of chemical compounds with the cell membrane. The amphiphilic molecules easily incorporated into the membrane of discoid RBC and can induce their cells shape transformation into echinocytes or stomatocytes. Depending on the membrane-perturbing activity of compounds, the membrane-structure alternation may undergo and cell damage, namely haemolysis, may occur. The number and position of

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hydroxyl group on the rigid steroidal backbone of bile acids are important with regard to their cytotoxicity. Depending on the value of the hydrophobicity index, bile acids can induce stomatocytogenic or echinocytogenic transformation of RBC shape, in the dose-and incubation time-dependent manner [23,24]. Our previous studies showed that alkaloids eg. gramine or nicotine, significantly decrease the capacity of bile acids to alter the lipid bilayer structure of RBC membrane and increase the membrane intercalating potency of sterols [25]. Therefore, the impact of gramine molecule on the cytotoxicity of substituted bile acids is also discussed. The structures of all new products were confirmed by spectral (NMR, FT-IR) analysis, mass spectrometry as well as PM5 semiempirical calculations. Moreover nicotine salts with formyl-bile acid were synthesized. The haemolytic activity of all compounds obtained was studied under physiological conditions *in vitro* (phosphate buffer, pH 7.4 at 37 °C).

2. Experimental

2.1. Instrumentation and chemicals

All melting points (mp) were obtained with a Büchi SMP-20 apparatus. ^{1}H NMR spectra were recorded on a Ultrashield spectrometer at 300 MHz with $CDCl_3$ or DMSO-d_6 as the solvent and TMS as the internal standard. Chemical shifts are reported in δ (parts per million) values. ESI mass spectra were measured on a ZQ Waters Mass Spectrometer. FT-IR spectra were recorded on Bruker FT-IR IFS 66v/S Spectrometer (KBr pellets). Analytical thin-layer chromatography (TLC) was carried out on silica gel plates 60 F254. Detection on TLC was made by the use of UV light and 10% aqueous H_2SO_4 (then the plates were heated at ~120 °C for approximately one minute and allow to cool). All chemicals or reagents used for syntheses were commercially available. PM5 semiempirical calculations were performed using the CAChe Fujitsu program.

2.2. General synthetic procedure for formyloxy- and acetoxy-bile acids

The starting formyl- (**2–4**) and acetyl- (**5–7**) esters of bile acids were prepared following the standard procedures. Compounds **2–4** were obtained according to Nascimento and Li et al. [20,26]. Lithocholic, deoxycholic or cholic acids were dissolved in formic acid. Next, some drops of perchloric acid was added. After stirring to 55–60 °C for 24 h, acetic anhydride was added carefully (to moment when bubbles gas were observed). The mixture was extracted with diethyl ether. The extract was washed with 10% $NaHCO_3$, water, brine and dried over anhydrous Na_2SO_4. The crude products were obtained from evaporation of solvent under reduced pressure and purification of the residue over silica gel ($CHCl_3$/MeOH, 100:1). Compounds **5–7** were synthesized according to Brycki et al. [27,28]. Lithocholic, deoxycholic or cholic acids were dissolved in anhydrous pyridine. Next, acetic anhydride and catalytic DMAP was added. After stirring at room temperature for 72 h, the mixture was extracted with chloroform. The extract was washed with 0.5 M HCl, 10% $NaHCO_3$, water, brine and dried over anhydrous Na_2SO_4. The crude products were obtained from evaporation of solvent under reduced pressure and purification of the residue over silica gel ($PhCH_3$/EtOAc, 50:1 for compounds **5** and **6**; $CHCl_3$/MeOH, 100:1 for compound **7**).

2.2.1. 3α-formyloxy-5β-cholan-24-oic acid (2)

Yield 84%, powder, mp 128–130 °C. ^{1}H NMR (500 MHz, $CDCl_3$, TMS, ppm): δ = 8.01 (s, 1H, 3α-OCHO), 4.82 (m, 1H, 3β-H), 0.90 (s, 3H, CH_3-19), 0.89 (br, 3H, CH_3-21), 0.62 (s, 3H, CH_3-18).

2.2.2. 3α,12α-Diformyloxy-5β-cholan-24-oic acid (3)

Yield 40%, powder, mp 170–171 °C. ^{1}H NMR (500 MHz, $CDCl_3$,TMS, ppm): δ = 8.12 (s, 1H, 12α-OCHO), 8.02 (s, 1H, 3α-OCHO), 5.23 (s, 1H, 12β-H), 4.83 (m, 1H, 3β-H), 0.91 (s, 3H, CH_3-19), 0.82 (d, 3H, CH_3-21), 0.73 (s, 3H, CH_3-18).

2.2.3. 3α,7α,12α-Triformyloxy-5β-cholan-24-oic acid (4)

Yield 81%, powder, mp 209–211 °C. ^{1}H NMR (500 MHz, $CDCl_3$,TMS, ppm): δ = 8.15 (s, 1H, 12α-OCHO), 8.10 (s, 1H, 7α-OCHO), 8.02 (s, 1H, 3α-OCHO), 5.26 (s, 1H, 12β-CH), 5.06 (s, 1H, 7β-H), 4.71 (m, 1H, 3β-H), 0.93 (s, 3H, CH_3-19), 0.83 (d, 3H, CH_3-21), 0.75 (s, 3H, CH_3-18).

2.2.4. 3α-acetoxy-5β-cholan-24-oic acid (5)

Yield 86%, powder, mp 156–158 °C. ^{1}H NMR (400 MHz, $CDCl_3$, TMS, ppm): δ = 4.72 (m, 1H, 3β-H), 2.03 (s, 3H, 3α-$OCOCH_3$), 0.93 (s, 3H, 19-CH_3), 0.83 (d, 3H, 21-CH_3), 0.65 (s, 3H, 18-CH_3).

2.2.5. 3α,12α-diacetoxy-5β-cholan-24-oic acid (6)

Yield 30%, powder, mp 125–126 °C. ^{1}H NMR (400 MHz, $CDCl_3$,TMS, ppm): δ = 4.71 (m, 1H, 12β-H), 3.99 (m, 1H, 3β-H), 2.02 (s, 6H, 3α-$OCOCH_3$), 0.98 (d, 3H, 21-CH_3), 0.92 (s, 3H, 19-CH_3), 0.69 (s, 3H, 18-CH_3).

2.2.6. 3α,7α,12α-Triacetoxy-5β-cholan-24-oic (7)

Yield 60%, powder, mp 69–70 °C. ^{1}H NMR (400 MHz, $CDCl_3$, TMS, ppm): δ = 4.89 (s, 1H, 12β-H), 4.58 (s, 1H, 7β-H), 3.86 (m, 1H, 3β-H), 2.22 (s, 3H, 12α-CH_3COO), 2.07 (s, 3H, 7α-CH_3COO), 2.03 (s, 3H, 3α-CH_3COO), 0.99 (d, 3H, CH_3-21), 0.93 (s, 3H, CH_3-19), 0.69 (s, 3H, CH_3-18).

2.3. General synthetic procedure for gramine salts 8–13

The typical and optimum process for preparation of gramine salts is shown as following: 3α-formyloxy-5β-cholan-24-oic acid (81 mg, 0.2 mmol), 3α,12α-diformyloxy-5β-cholan-24-oic acid (90 mg, 0.2 mmol), 3α,7α,12α-triformyloxy-5β-cholan-24-oic acid (98 mg, 0.2 mmol), 3α-acetoxy-5β-cholan-24-oic acid (84 mg, 0.2 mmol), 3α,12α-diacetoxy-5β-cholan-24-oic acid (95 mg, 0.2 mmol), 3α,7α,12α-triacetoxy-5β-cholan-24-oic acid (107 mg, 0.2 mmol) were dissolved separately in methanol (in the least volume of solvent). Then gramine in quality molar ratio was added. The reaction mixture was mixed at room temperature for 24 h, then the solvent was removed under reduced pressure. The crude products were crystallized from methanol.

2.3.1. Gramine-3α-formyloxy-5β-cholan-24-oic acid salt (8)

Yield 97%, powder, mp 32 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm): δ = 10.43 (s, 1H, $\equiv N^+$-H), 8.74 (s, 1H, NH), 8.04 (s, 1H, 3α-OCHO), 7.52 (d, 1H, 7′-H), 7.40 (d, 1H, 4′-H), 7.08–7.18 (m, 3H, 2′-H, 6′-H, 5′-H), 4.88–4.80 (m, 1H, 3β-H), 4.14 (s, 2H, 10′-H), 2.53 (s, 6H, $N^+(CH_3)_2$), 0.92 (bs, 3H, CH_3-19), 0.89 (bs, 3H, CH_3-21), 0.62 (bs, 3H, CH_3-18). ESI-MS: *m/z* 787 $[C_{48}H_{80}O_6+Cl]^-$, 751 $[C_{48}H_{80}O_6\text{-}H]^-$, 579 $[M+H]^+$, 551 $[C_{35}H_{54}N_2O_3+H]^+$, 304 $[C_{20}H_{22}N_3]^+$, 175 $[C_{11}H_{15}N_2]^+$, 130 $[C_9H_8N]^+$. FT-IR (KBr) ν_{max}: 3214, 2933, 2865, 1720, 1631, 1565, 1448, 1377, 1341.

2.3.2. Gramine-3α,12α-diformyloxy-5β-cholan-24-oic acid salt (9)

Yield 99%, powder, mp 135 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm): δ = 10.37 (s, 1H, $\equiv N^+$-H), 8.73 (s, 1H, NH), 8.10 (s, 1H, 12α-OCHO), 8.03 (bs, 1H, 3α-OCHO), 7.52 (d, 1H, 7′-H), 7.39 (d, 1H, 4′-H), 7.09–7.19 (m, 3H, 2′-H, 6′-H, 5′-H), 5.23 (bs, 1H, 12β-H), 4.86–4.79 (m, 1H, 3β-H), 4.15 (s, 2H, 10′-H), 2.53 (s, 6H, $N^+(CH_3)_2$), 0.91 (s, 3H, CH_3-19), 0.82 (bs, 3H, CH_3-21), 0.70 (bs, 3H, CH_3-18). ESI-MS: *m/z* 895 $[C_{52}H_{80}O_{12}\text{-}H]^-$, 867 $[C_{51}H_{80}O_{11}\text{-}H]^-$, 839 $[C_{50}H_{80}O_{10}\text{-}H]^-$, 447 $[C_{26}H_{40}O_6\text{-}H]^-$, 419 $[C_{25}H_{40}O_5\text{-}H]^-$, 175 $[C_{11}H_{15}N_2]^+$, 130 $[C_9H_8N]^+$. FT-IR (KBr) ν_{max}: 3187, 2935, 2867, 1719, 1631, 1568, 1449, 1377, 1180.

2.3.3. Gramine-3α,7α,12α-triformyloxy-5β-cholan-24-oic acid salt (10)

Yield 98%, powder, mp 70–73 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS,

ppm): δ = 9.69 (s, 1H, $\equiv N^+$-H), 8.69 (s, 1H, NH), 8.14 (s, 1H, 7α-OCHO), 8.10 (s, 1H, 12α-OCHO), 8.04 (bs, 1H, 3α-OCHO), 7.57 (d, 1H, 7′-H), 7.40 (d, 1H, 4′-H), 7.36 (s, 1H, 2′-H), 7.11–7.20 (m, 2H, 6′-H, 5′-H), 5.26 (bs, 1H, 12β-H), 5.06 (bs, 1H, 7β-H), 4.88–4.80 (m, 1H, 3β-H), 4.17 (s, 2H, 10′-H), 2.57 (s, 6H, $N^+(CH_3)_2$), 0.92 (s, 3H, CH_3-19), 0.84 (bs, 3H, CH_3-21), 0.73 (s, 3H, CH_3-18). ESI-MS: *m/z* 491 $[C_{27}H_{40}O_8\text{-}H]^-$, 304 $[C_{20}H_{22}N_3]^+$, 175 $[C_{11}H_{15}N_2]^+$, 130 $[C_9H_8N]^+$. FT-IR (KBr) ν_{max}: 3402, 2932, 2868, 1717, 1585, 1458, 1382, 1181.

*2.3.4. Gramine-3α-acetoxy-5β-cholan-24-oic acid salt (**11**)*

Yield 98%, powder, mp 50-55 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm*):* δ = 9.14 (s, 1H, $\equiv N^+$-H), 8.60 (s, 1H, NH), 7.59 (d, 1H, 7′-H), 7.41 (d, 1H, 4′-H), 7.28 (s, 1H, 2′-H), 7.11–7.21 (m, 2H, 6′-H, 5′-H), 4.76–4.68 (m, 1H, 3β-H), 4.02 (s, 2H, 10′-H), 2.47 (s, 6H, $N^+(CH_3)_2$), 2.03 (s, 3H, 3α-CH_3COO), 0.92 (bs, 6H, CH_3-21, CH_3-19), 0.63 (s, 3H, CH_3-18). ESI-MS: *m/z* 835 $[C_{52}H_{84}O_8\text{-}H]^-$, 453 $[C_{26}H_{42}O_4 + Cl]^-$, 417 $[C_{26}H_{42}O_4\text{-}H]^-$, 304 $[C_{20}H_{22}N_3]^+$, 175 $[C_{11}H_{15}N_2]^+$, 130 $[C_9H_8N]^+$. FT-IR (KBr) ν_{max}: 3215, 2936, 2866, 1735, 1562, 1448, 1379, 1361, 1244.

*2.3.5. Gramine-3α,12α-diacetoxy-5β-cholan-24-oic acid salt (**12**)*

Yield 80%, powder, mp 50–55 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm): δ = 12.36 (s, 1H, $\equiv N^+$-H), 9.56 (s, 1H, NH), 7.60 (d, 1H, 7′-H), 7.37 (d, 1H, 4′-H), 7.23 (s,1H, 2′-H), 7.02–7.16 (m, 2H, 6′-H, 5′-H), 4.81–4.75 (m, 1H, 3β-H), 4.15 (s, 1H, 12β-H), 3.93 (s, 2H, 10′-H), 2.41 (s, 6H, $N^+(CH_3)_2$), 2.03 (s, 6H, 3α-CH_3COO, 12α-CH_3COO), 0.90 (bs, 3H, CH_3-21), 0.89 (bs, 3H, CH_3-19), 0.66 (s, 3H, CH_3-18). ESI-MS: *m/z* 951 $[C_{56}H_{88}O_{12}\text{-}H]^-$, 475 $[C_{28}H_{44}O_6\text{-}H]^-$, 304 $[C_{20}H_{22}N_3]^+$, 175 $[C_{11}H_{15}N_2]^+$, 130 $[C_9H_8N]^+$. FT-IR (KBr) ν_{max}: 3183, 2944, 2869, 1734, 1631, 1563, 1449, 1378, 1378.

*2.3.6. Gramine-3α,7α,12α-triacetoxy-5β-cholan-24-oic acid salt (**13**)*

Yield 92%, powder, mp 90 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm): δ = 12,01 (s, 1H, $\equiv N^+$-H), 10.06 (s, 1H, NH), 7.55 (d, 1H, 7′-H), 7.37 (d, 1H, 4′-H), 7.29 (s,1H, 2′-H), 7.07–7.17 (m, 2H, 6′-H, 5′-H), 5.08 (bs, 1H, 12β-H), 4.90 (bs, 1H, 7β-H), 4.61–4.53 (m, 1H, 3β-H),4.00 (s, 2H, 10′-H), 2.46 (s, 6H, $N^+(CH_3)_2$), 2.10 (s, 3H, 12α-CH_3COO), 2.08 (s, 3H, 7α-CH_3COO), 2.05 (s, 3H, 3α-CH_3COO), 0.90 (s, 3H, CH_3-19), 0.83 (d, 3H, CH_3-21), 0.69 (s, 3H, CH_3-18). ESI-MS: *m/z* 569 $[C_{30}H_{46}O_8 + Cl]^-$, 533$[C_{30}H_{46}O_8\text{-}H]^-$, 175 $[C_{11}H_{15}N_2]^+$, 130 $[C_9H_8N]^+$. FT-IR (KBr) ν_{max}: 3386, 2945, 2871, 1732, 1732, 1562, 1439, 1377, 1249.

*2.4. General synthetic procedure for nicotine salts **14–16***

A mixture of (−)-(*S*)-nicotine (81 mg, 0.5 mmol) and 3α-formyloxy-5β-cholan-24-oic acid (203 mg, 0.5 mmol), 3α,12α-diformyloxy-5β-cholan-24-oic acid (225 mg, 0.5 mmol), 3α,7α,12α-triformyloxy-5β-cholan-24-oic acid (245 mg, 0.5 mmol) was stirred in methanol (10 mL) at room temperature for 20 h. After completion of the reaction as indicated by TLC the solvent was removed under reduced pressure.

*2.4.1. Nicotine-3α-formyloxy-5β-cholan-24-oic acid salt (**14**)*

Yield 95%, brown oil. ^{1}H NMR (300 MHz, DMSO-d_6, TMS, ppm): δ 8.55 (1H, ArH), 8.46 (1H, ArH); 7.71 (1H, ArH); 7.35 (1H, ArH); 2.06 (*N*-CH_3, s), 8.05 (s, 1H, 3α-OCHO), 0.91 (bs, 3H, CH_3-19), 0.88 (bs, 3H, CH_3-21), 0.61 (bs, 3H, CH_3-18). ESI-MS: *m/z* 539 $[C_{34}H_{55}O_3N_2]^+$, 403 $[C_{25}H_{39}O_4]^-$, 375 $[C_{24}H_{39}O_3]^-$, 163 $[C_{10}H_{15}N]^+$.

*2.4.2. Nicotine-3α,12α-diformyloxy-5β-cholan-24-oic acid salt (**15**)*

Yield 86%, brown oil. ^{1}H NMR (300 MHz, DMSO-d_6, TMS, ppm)*:* δ 8.54 (1H, ArH), 8.45 (1H, ArH); 7.71 (1H, ArH); 7.35 (1H, ArH); 2.07 (*N*-CH_3, s), 8.10 (s, 1H, 12α-OCHO), 8.03 (bs, 1H, 3α-OCHO), 0.90 (s, 3H, CH_3-19), 0.82 (bs, 3H, CH_3-21), 0.72 (bs, 3H, CH_3-18). ESI-MS: *m/z* 447 $[C_{26}H_{40}O_6\text{-}H]^-$, 419 $[C_{25}H_{40}O_5\text{-}H]^-$, 175 $[C_{11}H_{15}N_2]^+$, 163 $[C_{10}H_{15}N]^+$.

*2.4.3. Nicotine-3α,7α,12α-triformyloxy-5β-cholan-24-oic acid salt (**16**)*

Yield 89%, brown oil. ^{1}H NMR (300 MHz, DMSO-d_6, TMS, ppm): δ 8.59 (1H, ArH), 8.50 (1H, ArH); 7.73 (1H, ArH); 7.42 (1H, ArH); 2.09 (*N*-CH_3, s), 8.14 (s, 1H, 7α-OCHO), 8.10 (s, 1H, 12α-OCHO), 8.04 (bs, 1H, 3α-OCHO), 0.91 (s, 3H, CH_3-19), 0.86 (bs, 3H, CH_3-21), 0.72 (s, 3H, CH_3-18). ESI-MS: *m/z* 627 $[C_{36}H_{55}O_7N_2]^+$, 491 $[C_{27}H_{40}O_8\text{-}H]^-$, 163 $[C_{10}H_{15}N]^+$.

2.5. Human erythrocyte membrane perturbing activity of compounds studied

2.5.1. Erythrocyte preparation

Freshly human erythrocytes (RBC) suspensions were obtained from the blood bank. RBC were washed three times (3000 rpm/10 min/ + 4 °C) in phosphate buffered saline (PBS, pH 7.4) supplemented with 10 mM glucose. After washing, RBC were suspended in the buffer at 1.65x10^9 cells/mL. Cells were stored at + 4 °C and used within 5 h.

2.5.2. In vitro haemolytic assay

RBC (1.65×10^8 cells/mL, ~1.5% haematocrit) were incubated in phosphate-buffered saline (PBS, pH 7.4) supplemented with 10 mM glucose and containing tested compounds (in the concentration range from 0 mg/mL to 0.1 mg/mL) for 60 min at 37 °C in a shaking water bath. RBC incubated in PBS only were taken as the control. Controls and sample tests were run in triplicate and the experiments were repeated four times with RBC from different donors. After incubation, the RBC suspensions were centrifuged (3000 rpm/10 min/ + 4 °C) and the degree of haemolysis was estimated by measuring the absorbance of the supernatant at λ = 540 nm. The results were expressed as percentage (%) of haemolysis. Haemolysis 0% was taken as the absorbance of the supernatant of erythrocyte suspensions in PBS in the absence of tested compounds (control sample) and the total haemolysis (100%) was determined when PBS was replaced by cold distilled water. The values are the mean ± SD of four independent experiments.

2.5.3. Light microscope studies of erythrocytes shape transformation

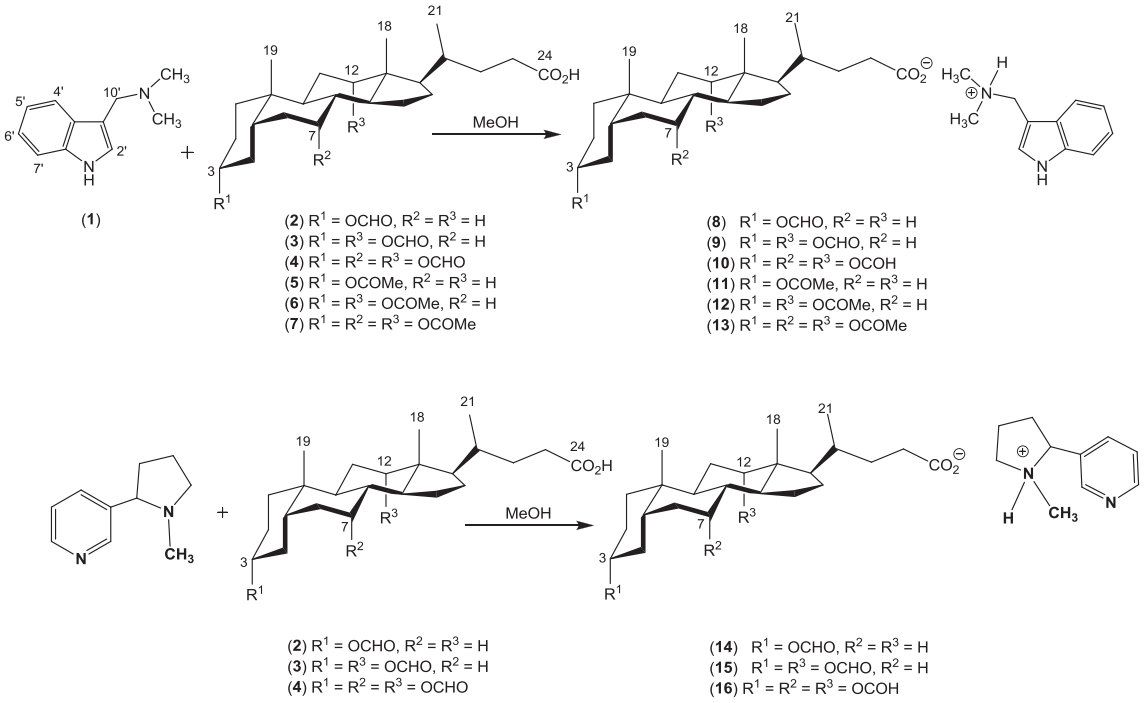
The cells were fixed with 5% paraformaldehyde plus 0.01% glutaraldehyde for 30 min at RT in the dark. After washing in PBS, RBC were settled on poly-L-lysine-treated (0.1 mg/mL, 10 min, RT) cover glasses and mounted on 80% glycerol. The cover slips were sealed with nail polish. A large number of RBC in several separate experimental samples were studied.

2.5.4. Scanning electron microscope studies of erythrocytes shape transformation

Erythrocytes fixed with 5% PFA plus 0.01% glutaraldehyde (as in Section 2.5.3) were post-fixed with 0.1% glutaraldehyde for 1 h at room temperature (RT). Cells were washed by exchanging of supernatant with PBS. The samples were gently vortexed and cells were fixed again with 2% glutaraldehyde for another hour at RT. After washing as above, cells were post-fixed with 1% OsO_4 for 30 min at RT. The supernatant was exchange with PBS and samples were very gently vortexed. Fixed erythrocytes were dehydrated in a series of ethanol solution (50%, 60%, 70%, 80%, 90%, 95%, and 100%), gold-sputtered, and examined in a *EVO 40* (ZEISS, Germany) scanning electron microscope.

2.5.5. Statistical analysis

The results were presented as mean value ± standard deviation (SD) for four independent experiments. A paired *t*-test was used to compare the every two suitable compounds. Statistical significance was defined as $p < 0.05$.



Scheme 1. Synthesis of gramine salts **8–13** and nicotine salts **14–16**.

3. Results and discussion

3.1. Chemistry

Bile acids were converted to their corresponding formyl derivatives by heating with formic acid [10,20]. The acetyl derivatives of bile acids were prepared following the standard Ac_2O/pyr/DMAP procedure [29]. A mixture of the cholic acids and gramine (or nicotine) in methanol gave salts (**8–16**) with the total yields 80–99%. The general synthetic method for the preparation of newly obtained salts is outlined in Scheme 1.

Structures of all salts were confirmed by their ^{1}H NMR and ESI MS spectra analyses. The ^{1}H NMR spectra of **8–13** besides characteristic signals in the range of 0.62–0.73 ppm (CH_3-18), 0.89–0.92 ppm (CH_3-19) and at 0.82–0.92 ppm (CH_3-21) shows signals attributed to $HCOO^-$ and CH_3COO^- groups at about 8 ppm and 2 ppm, respectively. In the spectra of these compounds characteristic singlets in the range of 2.41–2.57 ppm were assigned to the *N*-methyl protons and singlets at 3.93–4.17 ppm and 9.14–12.36 ppm to C10′-H and $\equiv N^+$-H protons, respectively. In the ^{1}H NMR spectra of nicotine salts (**14–16**) the two protons of pyridine ring being at α position to the nitrogen atom are largely deshielded and appear at about 8.5 ppm. The signal of *N*-methylene protons appear at about 2 ppm. The salts obtained gave mass spectra with the *m/z* values corresponding to fragmentation of acid molecule and gramine or nicotine. In ESI MS spectra of gramine salts, in positive ion mode the signal of indole fragment (*m/z* = 130) is observed, whereas in nicotine salts the signal of nicotinium cation at *m/z* = 163 is present. The absorption in the 3150–3250 cm^{-1} region (for **8–13**) corresponds to the $\nu(\equiv N^+H\ldots O)$ band which is a typical absorbance for hydrogen bond with an N…O distance about 3 Å.

3.2. Theoretical calculations

The final heats of formation (HOF) for the bile acids (LCA, DCA, CA) and their formyl- (**2–4**) and acetyl- (**5–7**) derivatives as well as formyl- (**8–10**) and acetyl- (**11–13**) salts are presented in Table 1. The esters derivatives of bile acids: 3α-formyloxy-5β-cholan-24-oic acid (**2**), 3α,12α-diformyloxy-5β-cholan-24-oic acid (**3**), 3α,7 α,12 α-triformyloxy-5β-cholan-24-oic acid (**4**) as well as 3α-acetoxy-5β-cholan-24-oic acid (**5**), 3α,12 α-diacetoxy-5β-cholan-24-oic acid (**6**), 3α,7α,12α-triacetoxy-5β-cholan-24-oic acid (**7**) have lower values of HOF than the acids with unsubstituted hydroxyl groups [25]. This fact can be explained by the greater stability by ester groups in isolated molecules and also caused by difficulty to form intramolecular hydrogen bonds, which can be formed in the case of unsubstituted hydroxyl groups. Furthermore, the increase of the number of free hydroxyl groups causes the increase of the HOF values [25]. The presence of ester group at 3α, 7α as well as 12 α position increases the stability of the compounds (**2–7**), and as a result of their conjugates with gramine (**8–13**). In addition, values of HOF decreases with increasing size of substituents in the reactive parts of steroid skeleton. The salts of formyl and acetyl-derivatives of lithocholic, deoxycholic and cholic acids are shown in Fig. 1. The lowest value of HOF for all salts was observed for

Table 1
Heat of formation (HOF) [kcal/mol] of bile acids (LCA, DCA, CA), its formyl (**2–4**) and acetyl (**5–7**) derivatives as well as their salts (**8–1 3**) and the length [Å] of intramolecular bonds between O···H···N.

Compound	HOF [kcal/mol]	ΔHOF [kcal/mol]	Length [Å] O···H···N
LCA	−235.0878[a]	–	–
DCA	−277.2635[a]	–	–
CA	−316.9693[a]	–	–
2	−271.7707	−36.6829	–
3	−348.9356	−71.6721	–
4	−424.7801	−107.8108	–
5	−276.4870	−41.3992	–
6	−367.4560	−90.1925	–
7	−448.4233	−131.4540	–
8	−229.4106	42.3601	1.72/1.45
9	−306.7766	42.1590	1.76/1.44
10	−382.7269	42.0532	1.77/1.43
11	−238.4242	38.0628	1.61/1.46
12	−325.3092	42.1468	1.68/1.46
13	−410.1348	38.2885	1.63/1.47

$\Delta HOF = HOF_{formyl/acetyl\ derivatives(2–7)} - HOF_{bile\ acids(LCA,\ DCA,\ CA)}$.
$\Delta HOF = HOF_{salts(8–10)} - HOF_{formyl\ derivatives(2–4)}$.
$\Delta HOF = HOF_{salts(11–13)} - HOF_{acetyl\ derivatives(5–7)}$.
[a] See [25].

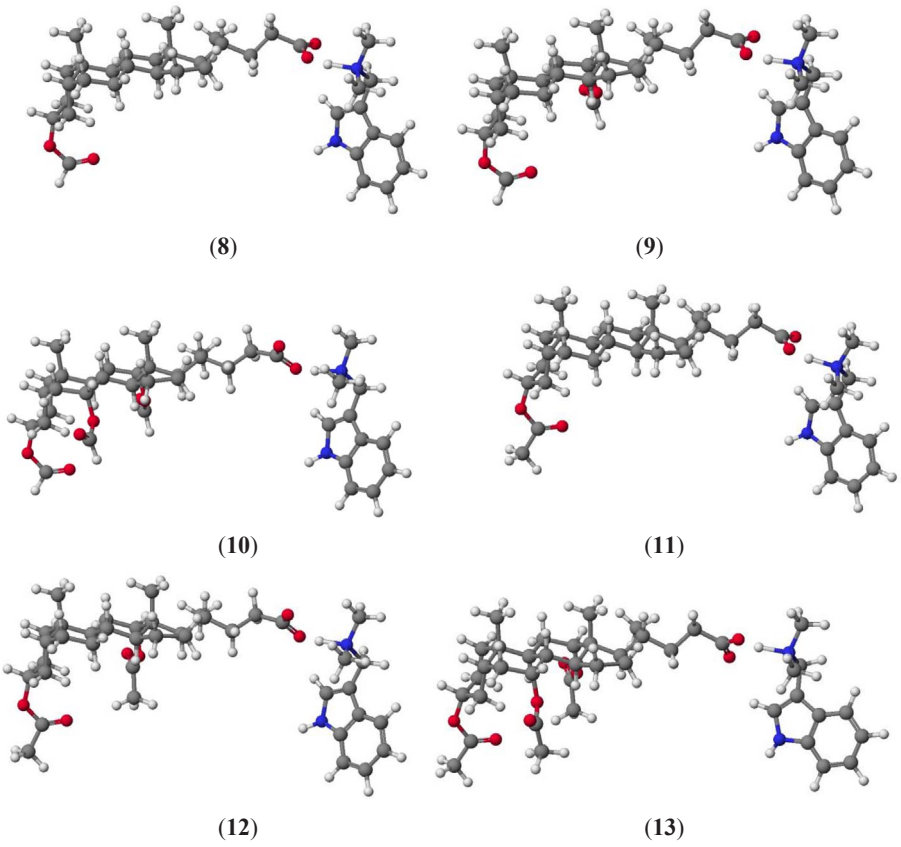


Fig. 1. Molecular models of gramine salts with formyl (**8–10**) and acetyl (**11–13**) derivatives of bile acids calculated by the PM5 method.

salts derived from cholic acid: **10** and **13**. In these salts, the number of formyl or acetyl groups in the steroid skeleton lowers the value of HOF. This effect is the same as in the case of bile acids containing a substituted hydroxyl group. Simultaneously bile acids having a substituted hydroxyl groups in positions 3α,7α and/or 12α (compounds **2–7**) have lower values HOF respect to the corresponding salts (compounds **8–13**). This follows from the fact that in the salts proton is delocalized between the oxygen atoms of carboxyl group and angular nitrogen atom of gramine. Furthermore the rings of gramine molecule are always oriented in perpendicular to the bile acid skeleton. The distances between the proton and quaternary nitrogen atom as well as the proton and oxygen atom of carboxylate anion are 1.43–1.45 Å and 1.72–1.77 Å for formyl salts: **8–10** and 1.46–1.47 Å and 1.61–1.68 Å for acetyl salts: **11–13**, respectively. This result is in a very good agreement with other findings as well as with our previous calculations [25].

3.3. Haemolytic activity of formyl- and acetyl-cholic acids and their gramine salts

The haemolytic activity of bile acids and their gramine salts was studied with a concentration in the range of 0.001 to 0.1 mg/mL (Fig. 2). Lithocholic acid, a monohydroxy bile acid, characterized the highest haemolytic activity as the most hydrophobic compound studied. Dihydroxydeoxycholic acid and trihydroxycholic acid, as more hydrophilic acids, were almost no membrane-perturbing agents at the used concentration rage. As shown in Fig. 2 the haemolytic activity of formyl- and acetyl-lithocholic (**2, 5**) and deoxycholic (**3, 6**) acids were significantly higher ($p < 0.05$) in comparison with their native forms at the whole concentration range. However, the differences were the most pronounced at their highest concentrations (0.05 and 0.1 mg/mL). Interestingly, at concentration equal to 0.1 mg/mL, formyl derivative of cholic acid (**4**) was as effective as lithocholic (**2, 5**) and deoxcholic (**3, 6**) acids derivatives. At lower concentration (0.05–0.001 mg/mL), the membrane perturbing activity of formyl derivative of cholic acid was similar to the original cholic acid. The acetyl-derivative of cholic acid (**7**) was not active as membrane perturbing agents as the original cholic acid at the all concentrations studied.

As shown in Fig. 2, gramine significantly decreased the membrane-perturbing activity of hydrophobic bile acids derivatives. However, both formyl- and acetyl-derivatives of lithocholic acid (**8, 11**) were an exception at the highest concentration (0.1 mg/mL). The possible explanation could be the extremely high haemolytic activity of this most hydrophobic bile acid. Therefore, the addition of gramine to formyl- and acetyl-derivatives of lithocholic acid was not efficient factor to reduce their cytotoxicity activity. These results are in line with our previous findings regarding the haemolytic activity of LCA and DCA salts with gramine [25]. Similarly to the natural bile acids, the formyl- and acetyl-derivatives of lithocholic and deoxycholic acids were echino- and/or stomatocytogenic agents, depending on their hydrophobicity and concentration. Gramine did not influence the efficiency of bile acids esters for their typical structure- and dose-dependent echinocytic or stomatocytogenic RBC shape transformation. Representative results of erythrocyte stomatocytic shape transformation observed for acetyl-lithocholic acid (**5**) and its gramine derivative (**11**) (at concentration equal 0.001 mg/mL) are presented in Fig. 3.

To study the influence of other alkaloids on the membrane perturbing activity of bile acids we synthesized formyl-derivatives of lithocholic, deoxycholic and cholic acids with nicotine salts, namely compound **14**, **15** and **16**. Our previous results showed that nicotine decreased the membrane interacting potential of bile acids depending on their hydrophobicity [26]. In the present study, nicotine decreased haemolytic activity of formyl-derivatives of bile acids, depending on their hydrophobicity and the concentration used. Namely, there was a more than 92% decrease in haemolysis, from 98,5% to 5,76% at the concentration 0.1 mg/mL of nicotine salt with formyl-cholic acid salt (**16**), with respect to that achieved for formyl-cholic acid alone (**4**). Similarly, there was a 95% decrease in haemolysis, namely from 97.5%

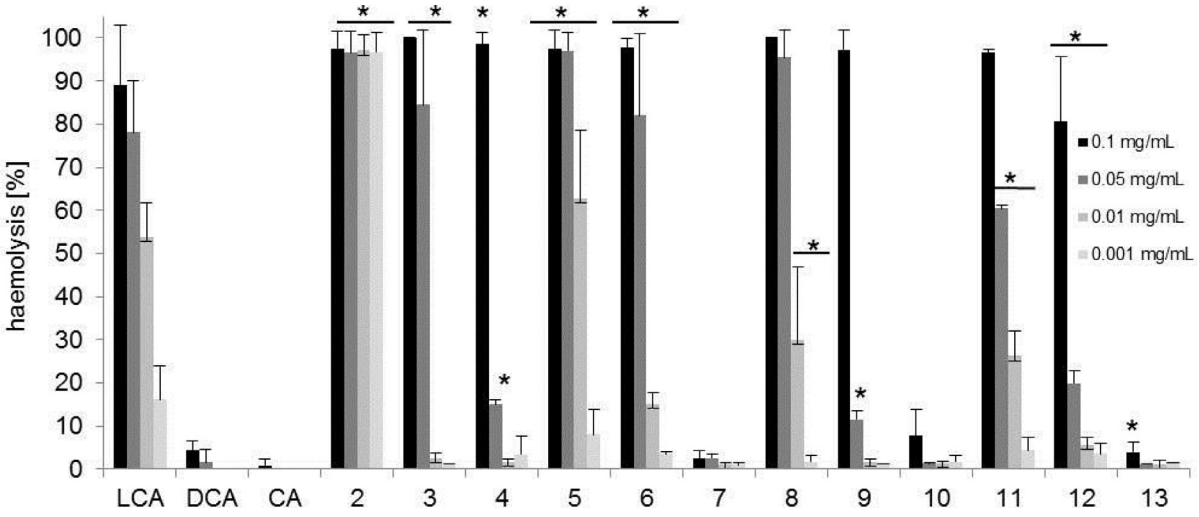


Fig. 2. The haemolytic activity of: bile acids (LCA, DCA, CA), their formyl-(**2–4**) and acetyl-(**5–6**) forms, their gramine salts with formyl-(**8–10**) and acetyl-(**11–13**) bile acids. The results are presented as mean ± SD (n = 4). *$p < 0.05$.

to 2.34% at the concentration 0.01 mg/mL of nicotine salt with formyl-lithocholic acid (**14**), with respect to that achieved for formyl-lithocholic acid alone (**2**). All new synthesized nicotine salts with formyl-bile acids induced RBC shape alterations, similar to those observed for bile acids alone (results not shown). However, at the concentration 0.01 mg/mL of nicotine salts with formyl-bile acids, RBC did not undergo any shape transformation. Therefore, it can be concluded, that different alkaloids, namely gramine and nicotine, decrease significantly the membrane interacting activity of formyl-bile acids.

To explain quantitative structure-haemolytic activity relationships of the bile acids and their derivatives (**2–7**), physicochemical calculations were conducted using Molinspiration Property Calculator, v2016.10. The physicochemical properties of molecules, such as, their lipophilicities and polar surface areas (PSAs) play important roles in determining biological responses, and are commonly used to study the structure activity relationships of bioactive molecules. This physiochemical parameters of bile acids and their derivatives **2–7** are listed in Table 2.

Low TPSA (< 75) has been associated with an increased risk of adverse events due to non-specific toxicity, particularly when combined with high lipophilicity. Therefore, the high haemolytic activity of lithocholic acid can be explain by its high lipophilicity (LogP > 5) and low TPSA. The introduction of formyl- and acetyl-substituents to bile acids molecule increases the values of TPSA and number of hydrogen-bond acceptors (up 3 relative to the parent acids). A high PSA and a large number of hydrogen bond acceptors has been associated with the poor membrane permeability due to the additional desolvation energy required to break hydrogen bonds when moving from an aqueous environment to the lipid membrane. Modification of deoxycholic and cholic acids reduces the number of hydrogen-bond donors particularly in the case of the compounds **4** and **7** which may affect their biological activity.

4. Conclusions

PM5 calculations performed for formyl and acetyl derivatives of bile acids indicate that the number of hydroxyl groups in the steroid skeleton influences the value of HOF. The higher number of unsubstituted or substituted hydroxyl groups reduced the HOF and consequently stability of the salt. The consequence of both formyl- and acetyl-modifications of hydrophobic bile acids, namely lithocholic and deoxycholic, was the increase of their membrane-perturbing activity. On the other hand, the haemolytic activity of acetyl-derivatives of hydrophilic cholic acid was no different from its original form; however, the formyl-derivative of cholic acid was as effective as the formyl-derivatives of lithocholic and deoxycholic acids. Although the presence of formyl- and

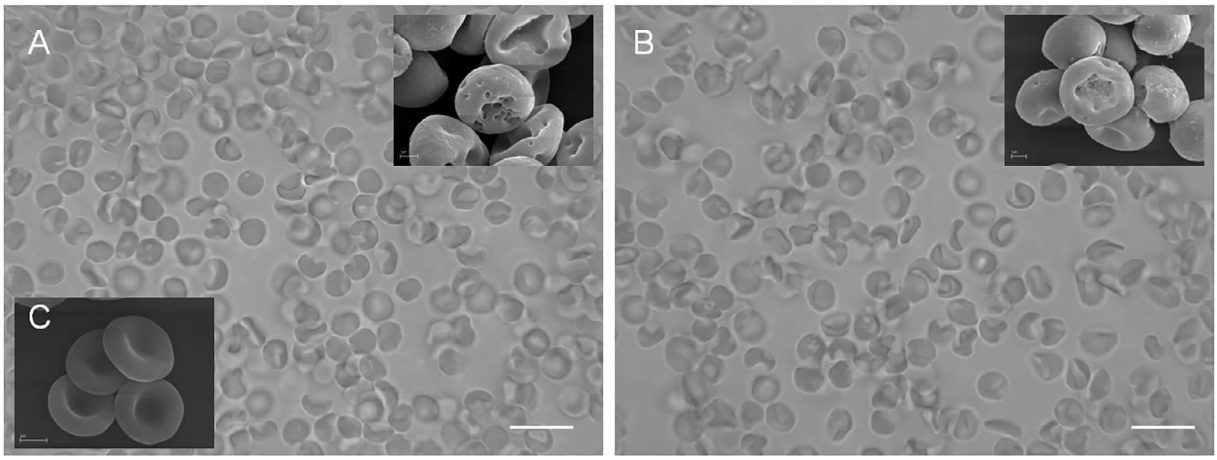


Fig. 3. Human erythrocytes incubated (A) with acetyl-lithocholic acid (**5**) and (B) with its gramine derivative (**11**) at 0.001 mg/mL (60 min, 37 °C). (C) discocytes (control cells), Stomatocytes (cupe cells) are dominated cell type in A and B. Insets in A and B: stomatocytes as observed under a scanning electron microscope. Scal bars represent 10 μm in A and B, 1 μm in C and 2 μm in both insets.

Table 2
Molinspiration calculations of the molecular properties of bile acids and their derivatives **2–7**.

Compound	MW (g/mol)[1]	Natoms[2]	LogP[3]	TPSA[4]	OH-NH interact[5]	O—N interact[6]	Nrotb[7]	Volume[8]
LCA	376.58	27	5.16	57.53	2	3	4	389.59
DCA	392.58	28	4.25	77.75	3	4	4	397.63
CA	408.58	29	3.33	97.98	4	5	4	405.68
2	436.59	31	3.75	104.06	3	6	6	425.63
3	464.60	33	4.18	110.14	2	7	8	445.58
4	492.61	35	4.60	116.21	1	8	10	465.53
5	450.62	32	4.04	104.06	3	6	6	442.19
6	492.65	35	4.74	110.14	2	7	8	478.70
7	534.60	38	5.44	116.21	1	8	10	515.21

[1] Molecular weight.
[2] Number of non hydrogen atoms.
[3] Calculated octanol/water partition coefficients.
[4] Topological polar surface areas.
[5] Number of hydrogen-bond donors (OH and NH groups).
[6] Number of hydrogen-bond acceptors (O and N atoms).
[7] Number of rotatable bonds.
[8] Molecular volume.

acetyl-substituents in bile acids increases their membrane-perturbing activity, this feature can be reduced or eliminated in the presence of different alkaloids e.g. gramine or nicotine. Because the cell membrane serves as a barrier in every type of cell, therefore, it can be concluded that alkaloids may reduce the cytotoxicity of membrane-active agents.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2017.07.003.

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