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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,