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Synthesis, spectroscopy, theoretical and biological studies of new gramine-steroids salts and conjugates

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New gramine connections with bile acids (lithocholic, deoxycholic, cholic) and sterols (cholesterol, cholestanol) were synthesized. The structures of products were confirmed by spectral (NMR, FT-IR) analysis, mass spectrometry (ESI-MS) as well as PM5 semiempirical methods. Unexpectedly, the products of the reaction of gramine with cholesterol and cholestanol were symmetrical compounds consisting of two molecules of sterols connected by $N(CH_3)_2$ group. All new synthesized compounds interact *in vitro* with the human erythrocyte membrane and alter discoid erythrocyte shape inducing stomatocytosis or echinocytosis. Increase in the incorporation of the fluorescent dye merocyanine 540 (MC540) into the erythrocyte membrane indicates that new compounds at sublytic concentrations are capable of disturbing membrane phospholipids asymmetry and loosening the molecular packing of phospholipids in the bilayer. Gramine significantly decreases the membrane partitioning properties as well as haemolytic activity of lithocholic acid in its new salt. Moreover, both deoxycholic and cholic acids completely lost their membrane perturbing activities in the gramine salts. On the other hand, the capacity of new gramine-sterols connections to alter the erythrocyte membrane structure and its permeability is much higher in comparison with sterols alone. The dual effect of gramine on the bile acid and sterols cell membrane partitioning activity observed in our study should not be neglected *in vivo*.

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1. Introduction

Gramine (*N*-(1H-indol-3-ylmethyl)-*N*,*N*-dimethylamine) (**1**) is one of the best known indole derivatives. It is a widely used initial compound in the synthesis of a large variety of substituted indoles, including agents playing important roles in living organism. In particular, gramine is among the substances most frequently used in the synthesis of L-tryptophan and its derivatives and for the synthesis of various biologically active indole-containing compounds [1,2]. Gramine itself exhibits radioprotector activity and effectively inhibits the growth of *Microcystis aeruginosa* [3,4]. This indole compound has great synthetic potential and is capable of participating in a variety of transformations leading to interesting and important types of compounds showing high biological activity [5]. For example 1-benzylgramines containing various substituents in different positions are capable of blocking serotonin activity [6]. For this reason, several examples of syntheses of gramine derivatives have been reported, and gramine still remains a good starting material in the preparation of new drugs, e.g. those showing antifungal, antibacterial and anticancer activity [7].

On the other hand, steroids are a important class of natural products and play different roles in higher organisms and microorganisms. One of the best known sterols is cholesterol. It is an important component of mammalian cell membranes and it is present insignificant concentrations in the nervous tissue [8–11]. Furthermore cholesterol is the biosynthetic precursor of bile acids, steroid hormones, as well as vitamin D and lipoproteins [12–14]. Bile acids are metabolites of cholesterol, biosynthesized in the liver [15,16]. They are divided into two groups, namely the primary bile acids, e.g. chenodeoxycholic acid and cholic acid, and secondary bile acids such as ursodeoxycholic, deoxycholic and lithocholic acids [17–19]. Bile acids are present in the human bile and blood and some of them have strong cytotoxic activity [20,21]. They display a rigid, large and curved skeleton. Moreover, they comprise chemically different polar hydroxy groups (3α; 3α, 7α; 3α, 12α and 3α, 7α, 12α or 3α, 7β, 12α) and amphiphilic properties [22–26]. Literature gives very little information on the connection of steroids and alkaloids. Therefore, there is growing interest in the synthesis of conjugates of natural compounds to find new active substances

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[27]. Bioconjugation has emerged as a fast growing methodology in medicinal chemistry and aims at the binding of two or more active molecules to form new complex substances showing combined properties of their individual components. In the course of our recent investigation on synthetic modifications of natural compounds such as bile acids and alkaloids [28], we focused on the synthesis of the new compounds to study their biological activity. Natural compounds present in the environment may enter the living organisms and interact with the cellular membranes, especially with membrane of human red blood cells (RBC) [29]. Some of chemical compounds alter the cell membrane structure, which can bean initial step of cell lysis [5]. RBC, which have no nucleus and other organelles, are the most studied model of cell membrane and haemolysis is a good indication of membrane perturbing properties of both natural and synthetic compounds. According to the bilayer couple hypothesis amphiphilic molecules incorporate into RBC membrane, disturb the structure of lipid bilayer and induce cell shape transformation into echinocytes (crenation) or stomatocytes (invagination) [30]. Finally, amphiphilic compounds can increase the cell membrane permeability to ions and induce haemolysis [21]. In the course of our previous investigation we have shown that nicotine alkaloids decrease the RBC membrane perturbing activity of bile acids in alkaloid bile acid salts [28].

In this work, the impact of newly synthesized series of gramine-bile acids and sterols connections on RBC morphology and their lipid bilayer structure and permeability, was studied. The structures of all products were confirmed by spectral (NMR, FT-IR) analysis, as well as mass spectrometry (ESI-MS). Moreover, PM5 calculations were performed for all compounds [31–33].

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 Warian 300/400 spectrometer at 300 MHz with $CDCl_3$ 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). The elemental analysis was carried out on a Vario EL III Elemental Analyzer. Analytical thin-layer chromatography (TLC) was carried out on silica gel plates 60 F254. Detection on TLC was made by the use of Dragendorff's Reagent, 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, were of AR grade, and were used as received. All solvents and reagents were analytical reagent and used directly without purification. PM5 semiempirical calculations were performed using the CAChe Fujitsu program.

2.2. General synthetic procedure for gramine salts and conjugates

The typical and optimum process for preparation of gramine salts is shown as following: lithocholic acid (190 mg, 0.5 mmol), deoxycholic acid (196 mg, 0.5 mmol) or cholic acid (204 mg, 0.5 mmol), was dissolved in methanol (in the least volume of solvent). Then gramine (87 mg, 0.5 mmol) was added. The reaction mixture was mixed at room temperature, then the solvent was removed and the product was recrystallized from acetone.

2.2.1. Gramine-lithocholic acid (**7**)

Yield 98%. White powder, mp 137–139 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm): δ = 8.80 (s, 1H, ≡N$^+$–H), 7.65 (d, 1H, 7′-H), 7.40 (d, 1H, 4′-H), 7.30 (s, 1H, 2′-H), 7.23 (t, 1H, 6′-H), 7.15 (t, 1H, 5′-H), 4.00 (s, 2H, 10′-H), 3.58–3.67 (m, 1H, 3β-H), 2.47 (s, 6H, $N^+(CH_3)_2$), 0.95 (d, 3H, CH_3-21), 0.91 (s, 3H, CH_3-19), 0.64 (s, 3H, CH_3-18). ESI-MS: m/z 551 $[M+H]^+$, 304 $[C_{20}H_{22}N_3]^+$, 175 $[C_{11}H_{15}N_2]^+$, 130 $[C_9H_8N]^+$. Anal. calcd. for $C_{35}H_{54}N_2O_3$: C, 76.36; H, 9.82; N, 5.09. Found: C, 76.52; H, 9.72; N, 5.02.

2.2.2. Gramine-deoxycholic acid (**8**)

Yield 96%. Light yellow powder, mp 78–79 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm): δ = 9.07 (s, 1H, ≡N$^+$–H), 7.61 (d, 1H, 7′-H), 7.39 (d, 1H, 4′-H), 7.36 (s, 1H, 2′-H), 7.21 (t, 1H, 6′-H), 7.14 (t, 1H, 5′-H), 4.04 (s, 2H, 10′-H), 4.00 (s, 1H, 12β-H), 3.57–3.64 (m, 1H, 3β-H), 2.48 (s, 6H, $N^+(CH_3)_2$), 0.98 (d, 3H, CH_3-21), 0.89 (s, 3H, CH_3-19), 0.68 (s, 3H, CH_3-18). ESI-MS: m/z 567 $[M+H]^+$, 304 $[C_{20}H_{22}N_3]^+$, 175 $[C_{11}H_{15}N_2]^+$, 130 $[C_9H_8N]^+$. Anal. calcd. for $C_{35}H_{54}N_2O_4$: C, 74.20; H, 9.54; N, 4.95. Found: C, 74.18; H, 9.38; N, 4.84.

2.2.3. Gramine-cholic acid (**9**)

Yield 93%. Light yellow powder, mp 89–92 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm): δ = 10.89 (s, 1H, ≡N$^+$–H), 7.60 (d, 1H, 7′-H), 7.35 (d, 1H, 4′-H), 7.20 (s, 1H, 2′-H), 7.08 (t, 1H, 6′-H), 6.98 (t, 1H, 5′-H), 3.82 (s, 1H, 12β-H), 3.65 (s, 1H, 7β-H), 3.50 (s, 2H, 10′-H), 3.13–3.22 (m, 1H, 3β-H), 2.14 (s, 6H, $N^+(CH_3)_2$), 0.92 (d, 3H, CH_3-21), 0.81 (s, 3H, CH_3-19), 0.58 (s, 3H, CH_3-18). ESI-MS: m/z 583 $[M+H]^+$, 304 $[C_{20}H_{22}N_3]^+$, 175 $[C_{11}H_{15}N_2]^+$, 130 $[C_9H_8N]^+$. Anal. calcd. for $C_{35}H_{54}N_2O_5$: C, 72.16; H, 9.28; N, 4.81. Found: C, 72.08; H, 9.26; N, 4.65.

The typical and optimum process for preparation of gramine conjugates is shown as following: 3β-bromoacetoxycholesterol (100 mg, 0.2 mmol), or 3β-bromoacetoxy-dihydrocholesterol (100 mg, 0.2 mmol) was heated at reflux in acetonitrile (7 mL). The gramine (44 mg, 0.26 mmol) was added when steroid was solved completely. The reaction mixture was heated for 5 h. The precipitated residue was isolated by filtration, dried at room temperature, and recrystallized from acetonitrile.

2.2.4. Gramine-3β-bromoacetoxycholesterol (**10**)

Yield 22%. Light yellow powder, mp 179–181 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm): δ 5.39 (s, 2H, 6-H), 5.05 (4H, N^+–CH_2–), 4.74–4.65 (m, 2H, 3α-H), 3.82 (s, 6H, $N^+(CH_3)_2$), 1.01 (s, 6H, CH_3-19), 0.92–0.91 (d, 6H, CH_3-21), 0.88–0.85 (dd, 12H; CH_3-26, 27), 0.68 (s, 6H, CH_3-18). ESI-MS: m/z 899 $[M-Br]^+$, 544 $[C_{34}H_{58}NO_4]^+$, 190 $[C_{12}H_{17}N_2]^+$, 130 $[C_6H_{12}NO_2]^+$. FT-IR (KBr) ν_{max}: 3000, 2750, 2730, 1650, 1500, 1350, 1310, 1170. Anal. calcd. for $C_{60}H_{100}BrNO_4$: C, 73.62; H, 10.23; N, 1.43. Found: C, 73.61; H, 10.22; N, 1.47.

2.2.5. Gramine-3β-bromoacetoxydihydrocholesterol (**11**)

Yield 69%. Light yellow powder, mp 168–169 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS, ppm): δ 4.92 (s, 4H, N^+–CH_2–), 4.85–4.75 (m, 2H, 3α-H), 3.76 (s, 6H, $N^+(CH_3)_2$), 0.90–0.89 (d, 6H, CH_3-21), 0.87–0.85 (dd, 12H, CH_3-26, 27), 0.82 (s, 6H, CH_3-19), 0.65 (s, 6H, CH_3-18). ESI-MS: m/z 903 $[M-Br]^+$, 546 $[C_{34}H_{60}NO_4]^+$, 190 $[C_{12}H_{17}N_2]^+$, 130 $[C_6H_{12}NO_2]^+$. FT-IR (KBr) ν_{max}: 3000, 2750, 2730, 1500, 1350, 1310, 1170. Anal. calcd. for $C_{60}H_{104}BrNO_4$: C, 73.32; H, 10.59; N, 1.43. Found: C, 73.41; H, 10.32; N, 1.43.

2.3. Human erythrocyte membrane perturbing activity of compounds tested

2.3.1. 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

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