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New cholic acid analogs: synthesis and 17β-hydroxydehydrogenase (17β-HSD) inhibition activity

https://doi.org/10.1515/znb-2018-0192
Received November 16, 2017; accepted December 7, 2017

Abstract: The 17β-hydroxysteroid dehydrogenase (17β-HSD) enzyme family is involved in the biosynthesis of active steroids and its inhibition constitutes an interesting approach for treating estrogen-, androgen-dependent cancers and osteoporosis. In this study, a new series of cholic acid analogs was designed with the goal of improving the biological activity as 17β-HSD1 and 17β-HSD2 inhibitors. To this end, 23-cholyl amides **4–7**, 3-*O*-*p*-toluenesulfonyl-23-cholyl amides **10–12**, 23-cholyl-carbohydrazide **14**, carbothioamide analog **15**, and 23-cholyl-acylhydrazone derivatives **18–22** were synthesized from cholic acid (**3**) *via* coupling, sulfonation and substitution reactions. Basic treatment of keto group of **5** with *p*-bromoaniline afforded **8**, meanwhile acidic treatment of **3** with thiosemicarbazide furnished the 23-cholyl-thiadiazole derivative **16**. The synthesized compounds were evaluated for their inhibition activity against 17β-HSD1 and 17β-HSD2, and were found inactive at 1.0 μM concentration (inhibition <10%). However, the steroids **12**, **21** and **22** showed inhibition of 21.1, 23.9 and 21.3%, respectively, against 17β-HSD2 at the same concentration. Therefore, these steroidal analogs can be further structurally modified to optimize their inhibition activity against 17β-HSD2 for the development of potential therapeutics.

Keywords: breast cancer; cholic acid; coupling reaction; 17β-hydroxydehydrogenase (17β-HSD); osteoporosis.

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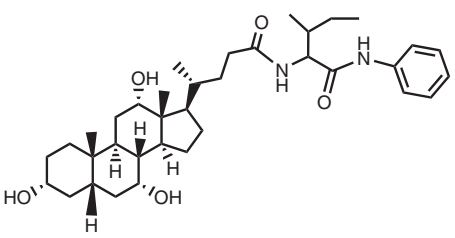
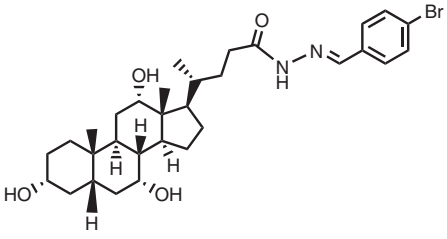
1 Introduction

Cholic acid is a major primary bile acid produced in the liver and found in the bile of mammals and other vertebrates and usually conjugated with glycine or taurine. It facilitates fat absorption and cholesterol excretion [1]. A number of bile acid derivatives possess diverse pharmacological activities, notably antimicrobial [2–4], antifungal [4–6], carbonic anhydrase inhibition [7, 8] and potential antioxidant [9]. One of the most promising applications of bile acid derivatives is as drug carriers, a direct consequence of their amphiphilic character [5]. They have already been reported to improve the permeability of cell membranes, including the bacterial wall [2, 10]. Therefore, conjugation of active substances with bile acid derivatives through chemical modifications at the 3- and 24-positions of the sterol nucleus led to various potential active analogs [11–21]. Brossard et al. [22] have synthesized several bile acid derivatives like *N*-(4-*N*-cinnamylpiperazin-1-yl)-3α,7α-dihydroxy-5β-cholan-24-amide and its 7β-analog showed significant activity against three human cancer cells [multiple myeloma (KMS-11), glioblastoma multiforme (GBM) and colonic carcinoma (HCT-116) human cell lines], within IC_{50} = 8.5–31.4 μM. Additionally, Zhang et al. [23] reported that cholic acid *n*-butyl ester had lower IC_{50} against MCF-7 human breast cancer (BC) but it did not have cytotoxicity to normal cell at low concentration. Diastereoisomeric-cholic-acid-derived 1,2,4,5-tetraoxanes were also tested and found to have high anticancer activity against human melanoma (Fem X), and cervix cancer (HeLa) [24] *cis* stereoisomers were twofold more active. Recently, Sakhuja et al. [25] have synthesized a series of cholanamide derivatives linked *via* α-amino acid, and some of these analogs exhibited fairly good activity against the BC cell line (IC_{50} = 1.35–4.52 μM) (e.g.: **1**, Fig. 1). Furthermore, cholic acid derivatives displayed significant activity against some viral infections. Salunke et al. [26] have reported the first examples of C-11 azido/amino functionalized cholic acid derivatives induce HIV replication and syncytia formation in T cells. Moreover, bile acids have been suggested as useful moieties to liver organotropic drugs, since cisplatin-bile acid derivatives



1. Cytotoxicity (GI_{50} = 1.35 μM) against human breast adenocarcinoma cell lines



2. Cytotoxicity (GI_{50} = 1.96 μM) against Gram positive bacteria *E. faecalis*

Fig. 1: Some potentially active steroid analogs.

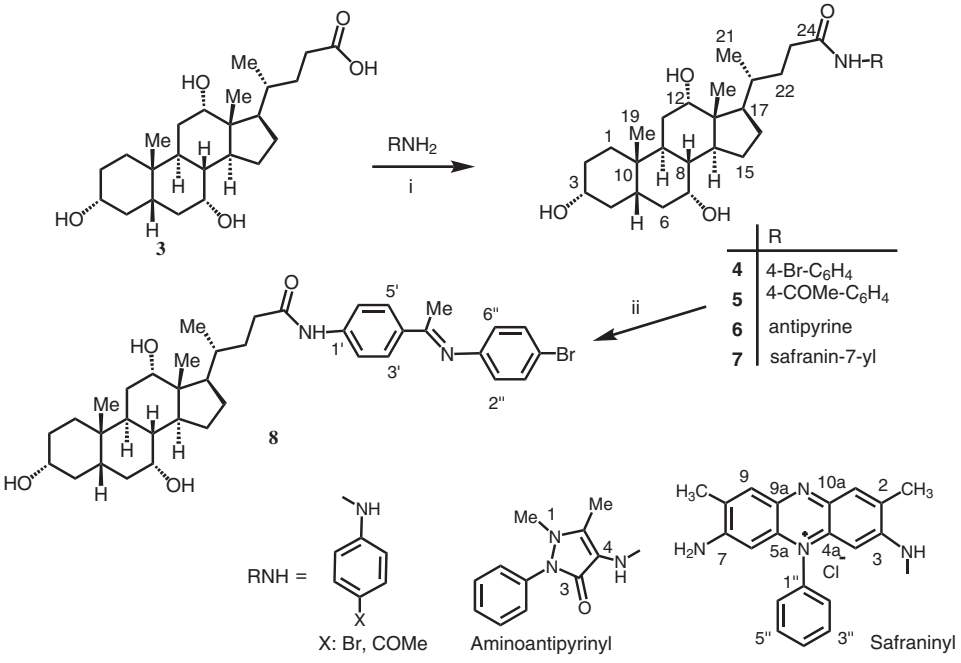
(Bamets) have been used *in vitro* to determine the production of virions by HBV-transfected hepatoblastoma cells (HepG2) [27] and effects of DNA-reactive bile acid derivatives (Bamets) on hepatitis B virus life cycle [28]. However, number of cholic acid analogs revealed significant antimicrobial activity [4, 29–33]. Recently, Al-Qawasmeh et al. [34] have reported the synthesis and antimicrobial activity of cholic acid hydrazone analogs, as some of the derivatives showed stronger antimicrobial activity against Gram-positive bacteria than Cefaclor and Cefixime, for example analog **2** (IC_{50} = 1.96 μM) (Fig. 1) against Gram positive bacteria *Enterococcus faecalis*.

In respect with the biological significance of bile acid derivatives and in continuation of our work on the 17β-hydroxydehydrogenase inhibition activity [35], we report here the synthesis and the biological activity of new cholic acid analogs as inhibitors of 17β-HSD1, 17β-HSD2 enzymes or antimicrobial candidates (Fig. 1).

2 Result and discussion

2.1 Chemistry

Treatment of cholic acid **3** with the appropriate amines (e.g.: 4-bromoaniline-, *N*′-aminoacetophenoe, 4-aminoantipyrine and safranine) in the presence of DCC as coupling reagent and DMAP as catalyst in CH_2Cl_2-DMF gave *N*-(substituted-aryl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide derivatives **4–7** in 68%–88% yield. The amide derivative **4** has been selected as a key intermediate for



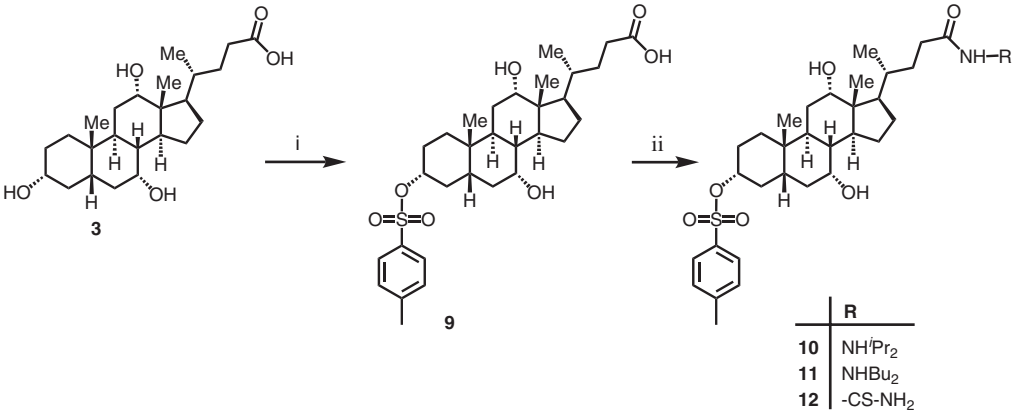
Scheme 1: Reagents and conditions: (i) DCC, DMAP, CH_2Cl_2-DMF, r.t., 16–20 h; (ii) 4-Br-C_6H_4-NH_2, AcOH, EtOH, reflux, 12 h.

the synthesis of new imine analog **8** in 74% yield, by treatment with 4-bromoaniline (Scheme 1).

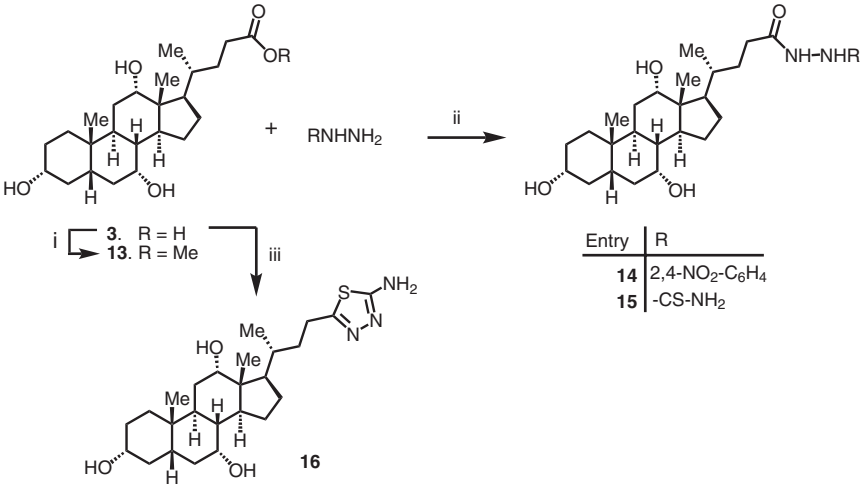
A common feature of bile acid-derived antimicrobials is their potential to exhibit a facially amphiphilic nature due to both a hydrophobic and a hydrophilic face [36]. Therefore, our work was modified by selective tosylation of **9** at C-3 [37] followed by amidation of carboxylic group leading to a facial amphilphile analog with two oriented hydroxy groups at C-7 and C-12 that may be exploited in podant-type receptors. Thus, the tosylate **9** was subjected to an amide forming reaction using DCC and amines (e.g.: *i*-propyl-, *n*-butylamines and thiourea), following the procedure described previously in preparation of **4–7**, furnished the 3α-*p*-toluenesulfonyl cholan-24-amide derivatives **10–12** in 75, 73 and 71% yield, respectively (Scheme 2).

Our work was modified by selecting methyl cholate **13**, prepared previously by Shen et al. [38], as a precursor for the synthesis of new carbohydrazide derivatives aiming to examine their anti-HSD and antimicrobial activities in comparison to those of the analogs **4–7**. Thus, treatment of **13** with 2,4-dinitrophenylhydrazine or thiosemicarbazide afforded the carbohydrazide analogs **14** and **15** in 73 and 81% yield, respectively. Further, the broad and potent activity of thiadiazole and their derivatives has established them as pharmacologically significant scaffolds [39]. In this study, the carboxylic acid moiety has been converted into a 1,3,4-thiazdiazole group by treatment of cholic acid (**3**) with thiosemicarbazide in alcoholic H_2SO_4 furnishing **16** in 83% yield (Scheme 3).

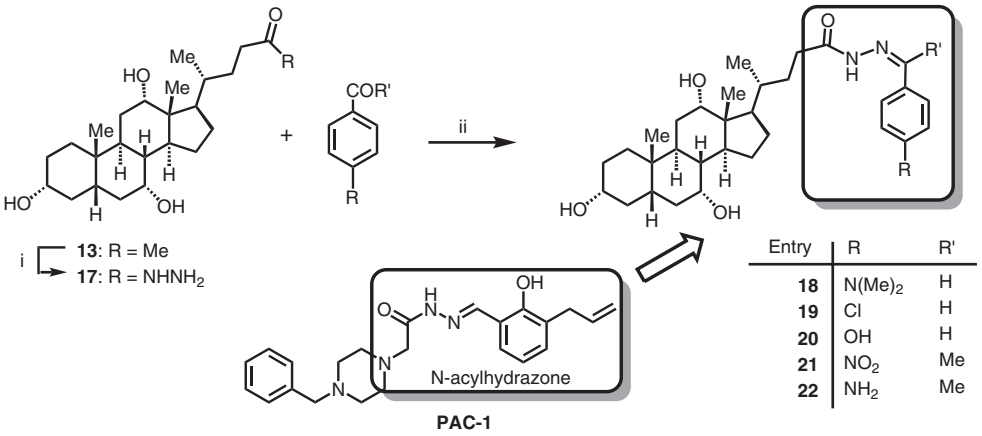
PAC-1 (2-(4-benzylpiperazin-1-yl)-*N*′-[(*Z*)-(6-oxo-5-prop-2-enylcyclohexa-2,4-dien-1-ylidene) methyl]acetohydrazide) is the first procaspase activating compound that selectively induces apoptosis in cancerous cells having the potential *N*-acylhydrazone pharmacophore [40, 41]. In an attempt to discover new antitumor agents *via* the inhibition of



Scheme 2: Reagents and conditions: (i) TsCl, pyridine, 0°C, (30 min), 55°C, 5 h; (ii) RNH_2, DCC, DMAP, CH_2Cl_2-DMF, r.t., 16–20 h.



Scheme 3: Reagents and conditions: (i) MeOH, HCl, reflux, 5 h; (ii) EtOH, DMF, reflux, 12 h; (iii) $NH_2NHCSNH_2$, EtOH, conc. H_2SO_4, reflux, 5 h.



Scheme 4: Reagents and conditions: (i) NH_2NH_2. H_2O, EtOH, reflux, 12 h; (ii) EtOH, AcOH, 10–12 h.

17β-HSD enzymes or for the treatment of osteoporosis, we combined the cholic acid moiety and *N*-acylhydrazone based in a hybrid pharmacophore design [42]. The carbohydrazide derivative **17** was prepared according to the procedure of Al-Qawasmeh et al. [34] from methyl cholate **13** and hydrazine hydrate. Condensation of **17** with the carbonyl compounds (e.g.: 4-*N*,*N*-dimethyl-, 4-choloro-, and 4-hydroxybenzaldehydes as with as well 4-nitro- and 4-aminoacetophenones) in refluxing acidic EtOH gave the desired benzylidene and ethylidene analogs **18–22** in 71%–90% yield (Scheme 4).

The structures of **4–16** and **18–22** were assigned on the basis of their IR and NMR (^{1}H, ^{13}C and 2D) spectra, which showed rather similar patterns of the aliphatic proton and carbon atoms. The ^{1}H NMR spectra of **4–8**, **14–16** (ok?) and **18–22** were characterized by the presence of aromatic protons and carbon atoms, indicative for amidation of the cholic acid backbone. The aromatic protons 3′-H and 5′-H of all analogs, except **6** and **14**, appeared as doublets or at the regions $\delta = 8.49–6.58$ ppm ($J = 8.8–7.5$ Hz), while 2′-H and 6′-H were resonated at the regions $\delta = 8.17–6.55$ ppm. The multiplet at $\delta = 7.35–7.49$ ppm assigned for the five aromatic protons of **6**, whereas the singlet at δ 8.60 ppm and two doublets at $\delta = 8.37$ and 7.65 ppm ($J = 7.9$ Hz) attributed to aromatic protons 3′-H, 5′-H and 6′-H of **14**, respectively. The multiplets at $\delta = 1.36–1.29$ ppm were assigned to 20-H, while the doublets at the regions at $\delta = 0.94–0.91$ ppm ($J_{Me21,H20} = 6.6–6.1$ Hz) were attributed to the methylene protons (21-Me). The N = CH protons of **18–20** resonated as singlets at $\delta = 8.08$, 8.42 and 8.52 ppm, respectively, while methylene protons of N = C*Me* group of **21** and **22** appeared as singlets at $\delta = 2.32$ and 2.23 ppm, respectively. The other aliphatic protons were fully analyzed (*c.f.* Experimental section). In the ^{13}C NMR, the resonances at the regions $\delta = 173.7–167.8$ ppm were assigned for the carbonyl carbon atoms of the amide group (CONH), whereas the aromatic carbon atoms appeared at the regions $\delta = 160.2–106.0$ ppm. The signals at the lower fields $\delta = 180.0$ and 183.8 ppm were assigned to C = S carbon atoms of **12** and **15**, whereas the resonances at $\delta = 173.3$ and 170.2 ppm were attributed to C-2′ and C-5′ of the thiadiazole moiety of **16**. The resonances at the region $\delta = 150.2–142.8$ and 169.2 ppm were assigned for C = N carbon atoms of **18–22** and **8**, respectively. C-22 and C-23 and C-24 appeared at the regions $\delta = 33.3–31.0$ and 32.7–26.7 ppm, respectively. The other aliphatic carbon atoms and the substituents have been fully identified (*c.f.* Experimental section).

Compound **6** was selected for further NMR studies. From a gradient heteronuclear multiple bond correlation (HMBC) [43] NMR spectrum, $^3J_{C,H}$ couplings between 23a-H, 23b-H protons at $\delta = 2.09$, 2.02 ppm and carbonyl carbon atoms of the amide group at $\delta = 171.5$ ppm, was observed. Further, 22a-H and 22b-H at $\delta = 1.67$, 1.23 ppm showed $^2J_{C,H}$ couplings with the same carbonyl carbon atom of the amide group. Additionally, C-4′ and C-5′ of the pyrazolone ring at $\delta = 107.8$ and 135.0 ppm showed two $^2J_{C,H}$ couplings with methylene protons at $\delta = 2.73$ and 3.02 ppm, respectively (Fig. 2). All the compounds have been identified by their ^{1}H,^{13}C HSQC NMR spectra [44].

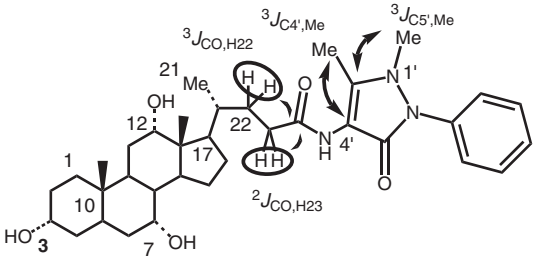


Fig. 2: $J_{C,H}$ correlations in the NMR HMBC spectrum of **6**.

2.2 *In vitro* inhibition activity of 17β-HSD1 and 17β-HSD2

17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) catalyzes the conversion of the weakly active estrone (E1) to the highly active estradiol (E2), meanwhile 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) catalyzes the conversion of E2 and testosterone (T) into the E1 and δ4-androstene-3,17-dione (δ4-AD), respectively, [45]. The enzyme 17β-HSD1 came into the focus of interest as a novel therapeutic target for the treatment of estrogen dependent diseases like BC and endometriosis [46–48]. 17β-HSD2 is expressed in human osteoblastic (OB) cells [49], therefore its inhibition can lead to the desired increase of E2, formed by reduction of E1, and T levels in the bone tissue and may thus be a novel approach for the treatment of osteoporosis [50]. As 17β-HSD2 catalyzes the inactivation of E2 into E1, inhibitory activity toward this enzyme must be avoided. However, 17β-HSD1 inhibitors should not inhibit 17β-HSD2 and, of course, should not be estrogenic. Our new synthesized compounds were tested for their ability to inhibit 17β-HSD1 and 17β-HSD2. The inhibition values of the tested compounds are shown in Table 1. All these derivatives showed less than 10% inhibition against 17β-HSD1 at 1.0 μM and were considered to be inactive. On the other hand, compounds **12**, **20** and **21** exhibited inhibition activities against 17β-HSD2 of 21.1%, 23.9% and 21.3%, respectively, at 1.0 μM concentration, and thus turned out to be promising analogs for treatment of osteoporosis. In general, the introduction of arylated acylhydrazone group *via* derivatization of the carboxylic group of cholic acid, had a positive impact on inhibitory activity, in comparison for those of other cholic acid derivatives.

Table 1: 17β-HSD1 and 17β-HSD2 inhibitory activity of some cholic acid analogs[a].

Comp	17β-HSD1[a] (% inhib.) (1.0 μM)	17β-HSD2[b] (% inhib.) (1.0 μM)
4	3.0	2.4
5	7.5	14.4
6	ni	3.6
7	−8.6	0.9
8	−0.3	8.5
9	7.1	10.2
10	−3.3	4.9
11	0.2	2.0
12	2.2	21.1
13	6.2	15.2
14	8.5	11.5
15	−0.8	4.9
16	3.1	8.1
17	ni	ni
18	−4.6	12.5
19	−8.5	4.2
20	−2.2	23.9
21	−7.3	21.3
22	ni	5.7

[a]ni: no inhibition. Human placenta, cytosolic fraction, substrate [^{3}H]E1 + E1 [500 nM], cofactor NADH [500 μM]; [b]human placenta, microsomal fraction, substrate [^{3}H]E2 + E2 [500 nM], cofactor NAD + [1500 μM]; [c]mean values of two determinations, standard deviation less than 10%.

3 Conclusion

A new series of amides, tosylates, carbohydrazide, carbothioamide analog and acylhydrazone analogs derived cholic acid were synthesized. The synthesized compounds were screened for their inhibitory activity against 17β-HSD1 and 17β-HSD2 at 1.0 μM concentration. Only compounds **12**, **20** and **21** showed inhibition against 17β-HSD2 (21.1, 23.9 and 21.3%, respectively). These analogs can be considered as promising agents for treatment of osteoporosis waiting for further structural modification.

4 Experimental

4.1 General

Melting points are uncorrected and were measured on a Büchi melting point apparatus B-545 (Büchi Labortechnik AG, Switzerland). Microanalytical data were obtained with a Vario Elemental Analyzer (Shimadzu, Japan). NMR spectra were obtained at 400 and 600 MHz (^{1}H) as well as 100 MHz and 150:91 MHz (^{13}C) spectrometers (Avance III, Bruker, Germany) with TMS as internal standard and on δ scale in ppm. Signal assignments for protons were performed by selective proton decoupling or by COSY spectra. Heteronuclear assignments were verified by HSQC and HMBC experiments. TLC plates 60 F_{254} were purchased from Merck.

4.2 General procedure for the synthesis of amides analogs of cholic acid (4–7)

To a cold solution of cholic acid **3** (409 mg, 1.00 mmol) in CH_2Cl_2-DMF (20 mL, 1:1 *v*/*v*) were added 4-(dimethylamino)pyridine (DMAP) (16 mg, 0.13 mmol) and amines (1.00 mmol) with stirring for 10 min, followed by addition of

N,*N*′-dicyclohexylcarbodiimid (DCC) (206 mg, 1.00 mmol). The reaction mixture was stirred at room temperature for 16–20 h and completion of reaction was monitored by TLC. A white precipitate was filtered and the solvent was evaporated to dryness and the residue was dissolved in ethyl acetate (50 mL). The organic layer was washed with aqueous 4% HCl and saturated $NaHCO_3$ (3×30 mL) then dried (Na_2SO_4) and evaporated to dryness. The residue was purified on a SiO_2 column chromatography using, in gradient, MeOH (0%–10%) and $CHCl_3$ as eluent to give the desired product.

4.2.1 *N*-(4-bromophenyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (4)

From 4-bromoaniline (172 mg). Yield: 438 mg (78%) as colorless solid, m.p.: 85–87°C; R_f = 0.32. – IR (KBr): ν = 3373(OH), 2931, 2854 (CH_2), 1697 (C=O)$_{amid}$, 1635 (C=C)$_{arom.}$ cm^{-1}. – ^{1}H NMR ([D$_6$]DMSO): δ = 9.95 (s, 1H, NH_{amide}), 7.58 (d, 2H, J = 8.7 Hz, 3′-$H_{arom.}$ + 5′-$H_{arom.}$), 6.55 (d, 2H, J = 8.7 Hz, 2′-$H_{arom.}$ + 6′-$H_{arom.}$), 6.00 (bs, 1H, OH), 5.58 (d, 1H, J = 8.0 Hz, OH), 4.34 (bs, 1H, OH), 3.98 (m, 1H, 12-H), 3.78 (m, 1H, 7-H), 3.19 (m, 1H, 3-H), 2.27 (m, 2H, CH_2-4), 2.22 (m, 1H, 9-H), 2.15 (m, 1H, 23a-H), 2.13 (m, 1H, 23b-H), 1.94 (m, 1H, 14-H), 1.82 (m, 1H, 6-H), 1.79 (m, 1H, 7-H), 1.77 (m, 1H, 16a-H), 1.67 (m, 2H, 1a-H + 22-H), 1.64 (m, 2H, 2a-H + 15a-H), 1.47 (m, 1H, 4a-H), 1.43 (m, 1H, 11a-H), 1.39 (m, 1H, 6b-H), 1.35 (m, 1H, 11b-H), 1.33 (m, 2H, 8-H + 20-H), 1.29 (m, 1H, 2b-H), 1.27 (m, 1H, 5-H), 1.22 (m, 1H, 22-H), 1.18 (m, 1H, 16b-H), 0.93 (d, 3H, $J_{Me21,H20}$ = 6.3 Hz, 21-Me), 0.91 (m, 1H, 15b-H), 0.83 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.57 (s, 3H, 18-Me). – ^{13}C NMR ([D$_6$]DMSO): δ = 171.5 (C=O), 135.2 ($C_{arom.}$-1′), 130.4 ($C_{arom.}$-3′ + $C_{arom.}$-5′), 124.8 (C-4′), 120.8 ($C_{arom.}$-2′ + $C_{arom.}$-6′), 73.9 (C-12), 70.9 (C-3), 66.2 (C-7), 47.4 (C-17), 46.1 (C-13), 45.2 (C-5), 41.4 (C-14), 40.1 (C-8), 39.8 (C-4), 37.7 (C-20), 36.6 (C-1 + C-6), 35.2 (C-10), 31.8 (C-2 + C-22), 31.7 (C-23), 28.5 (C-11), 27.2 (C-16), 26.6 (C-9), 22.5 (Me-19 + C-15), 18.4 (Me-21), 12.2 (Me-18). – $C_{30}H_{44}BrNO_4$ (562.59): calcd. C 64.05, H 7.88, N 2.49; found C 63.81, H 7.75, N 2.22.

4.2.2 *N*-(4-acetylphenyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (5)

From 4-aminoacetophenone (135 mg). Yield: 357 mg (68%) as dark yellow solid, m.p.: 128–130°C, R_f = 0.83. – IR (KBr): ν = 3394 (OH), 2931, 2854 (CH_2), 1712 (C=O)$_{acetyl}$, 1697 (C=O)$_{amid}$, 1650 (C=C)$_{arom.}$ cm^{-1}. – ^{1}H NMR ([D$_6$]DMSO): δ = 9.95 (s, 1H, NH_{amide}), 7.57 (d, 2H, J = 7.8 Hz, 3′-$H_{arom.}$ + 5′-$H_{arom.}$), 7.44 (d, 2H, J = 7.8 Hz, 32′-$H_{arom.}$ + 56′-$H_{arom.}$), 6.61 (d, 1H, J = 7.9 Hz, OH), 5.58 (d, 1H, J = 7.6 Hz, OH), 4.30 (bs, 1H, OH), 4.06 (m, 1H, 12-H), 3.78 (m, 1H, 7-H), 3.18 (m, 1H, 3-H), 2.58 (s, 3H, COMe), 2.24 (m, 1H, 4a-H), 2.15 (m, 2H, 9-H), 2.12 (m, 1H, 23a-H), 2.06 (m, 1H, 23b-H), 1.94 (m, H, 14-H), 1.80 (m, 1H, 6a-H), 1.78 (m, 1H, 17-H), 1.75 (m, 1H, 16a-H), 1.67 (m, 2H, 1a-H + 22a-H), 1.64 (m, 2H, 2a-H + 15a-H), 1.48 (m, 1H, 4b-H), 1.44 (m, 1H, 11a-H), 1.39 (m, 1H, 6b-H), 1.35 (m, 1H, 11b-H), 1.33 (m, 2H, 8-H + 20-H), 1.28 (m, 1H, 2b-H), 1.25 (m, 1H, 5-H), 1.23 (m, 1H, 22b-H), 1.21 (m, 1H, 5-H), 1.17 (m, 1H, 16b-H), 0.93 (d, 3H, $J_{Me21,H20}$ = 6.3 Hz, 21-Me), 0.91 (m, 1H, 15b-H), 0.83 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.57 (s, 3H, 18-Me). – ^{13}C NMR ([D$_6$]DMSO): δ = 191.2 (C=O), 171.5 (CONH), 139.7 (C-1′), 138.7 (C-4′), 131.3 (C-3′ + C-5′), 120.8 (C-2′ + C-6′), 73.1 (C-12), 70.9 (C-3), 66.2 (C-7), 47.1 (C-17), 45.6 (C-13), 41.5 (C-5), 41.3 (C-14), 40.1 (C-4 + C-8), 37.7 (C-20), 36.7 (C-1), 35.4 (C-6), 35.2 (C-10), 31.7 (C-2 + C-22), 30.9 (C-23), 28.4 (C-11), 27.2 (C-16), 26.1 (C-9), 24.5 (COMe), 18.4 (Me-21), 12.2 (Me-18). – ($C_{32}H_{47}NO_5$) (525.73): calcd. C 73.11, H 9.01, N 2.66; found C 72.90, H 8.91, N 2.43.

4.2.3 *N*-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (6)

From 4-aminoantipyrine (203 mg). Yield: 523 mg (88%) as orange solid, m.p.: 142–145°C, R_f = 0.64. – IR (KBr): ν = 3404 (OH), 2931, 2854 (CH_2), 1705 (C=O)$_{antipy.}$, 1681 (C=O)$_{amid}$, 1666 (C=C)$_{arom.}$ cm^{-1}. – ^{1}H NMR ([D$_6$]DMSO): δ = 8.98 (s, 1H, NH_{amide}), 7.35–7.49 (m, 5H, H_{arom}), 5.58 (d, 1H, J = 7.8 Hz, OH), 4.32 (m, 1H, OH), 4.07 (m, 1H, 17-H), 3.79 (m, 1H, 7-H), 3.19 (m, 1H, 3-H), 3.02 (s, 3H, $C^1_{pyrazolon}$-N*Me*), 2.73 (s, 3H, $C^5_{pyrazolon}$-*Me*), 2.24 (m, 1H, 4a-H), 2.14 (m, 1H, 9-H), 2.09 (m, 1H, 23a-H), 2.02 (m, 1H, 23b-H), 1.97 (m, 1H, 14-H), 1.81 (m, 1H, 17-H), 1.76 (m, 1H, 16a-H), 1.75 (m, 1H, 16b-H), 1.67 (m, 2H, 1a-H + 22a-H), 1.64 (m, 2H, 2a-H + 15a-H), 1.48 (m, 1H, 4a-H), 1.42 (m, 1H, 11a-H), 1.39 (m, 1H, 6a-H), 1.36 (m, 1H, 11b-H), 1.34 (m, 2H, 8-H + 20-H), 1.29 (m, 1H, 2b-H), 1.26 (m, 1H, 5-H), 1.23 (m, 1H, 22b-H), 1.18 (m, 1H, 16b-H), 0.93 (d, 3H, $J_{Me21,H20}$ = 6.3 Hz, 21-Me), 0.91 (m, 1H, 15b-H), 0.83 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.57 (s, 3H, 18-Me). – ^{13}C NMR ([D$_6$]DMSO): δ = 171.5 (CONH), 162.2 (C=O$_{pyrazolon}$), 135.0 ($C_{pyrazolon}$-5′ + $C_{arom.}$-1′), 128.9, 123.2 ($C_{arom.}$), 107.8 ($C_{pyrazolon}$-4′), 73.1 (C-12), 70.9 (C-3), 66.2 (C-7), 49.5 (C-17), 47.4 (C-13), 45.7 (C-5), 43.4 (C-14), 41.3 (C-4 + C-8), 36.2 (C-20), 35.7 (C-1), 35.2 (C-6), 34.3 (C-10), 33.2 (NMe), 32.4 (C-22), 31.7 (C-2), 30.9 (C-23), 28.4 (C-11), 27.2 (C-16), 26.1 (C-9), 24.5 (C-15), 22.5 (Me-19), 18.4 (Me-21), 12.2 (Me-18), 11.1 ($C^5_{pyrazolon}$-*Me*). – r $C_{35}H_{51}N_3O_5$ (593.81): calcd. C 70.79, H 8.66, N 7.08; found C 70.54, H 8.59, N 6.88.

4.2.4 3-Amino-2,8-dimethyl-5-phenyl-7-yl-(*N*-(3α,7α,12α-trihydroxy-5β-cholan-24-amido))-phenazin-5-ium chloride (7)

From safranine (351 mg). Yield: 615 mg (83%) as brown crystals, m.p.: 161–163°C, R_f = 0.42. – IR (KBr): ν = 3420 (OH), 2931, 2854 (CH_2), 1690 (C=O)$_{amid}$, 1650 (C=C)$_{arom.}$ cm^{-1}. – ^{1}H NMR ([D$_6$]DMSO): δ = 8.49 (d, 1H, J = 7.8 Hz, 9′-H$_{arom.}$), 8.09 (d, 2H, J = 7.9 Hz, 2″-H$_{arom.}$ + 6″-H$_{arom.}$), 7.95 (s, 1H, H-4′), 7.46 (bs, 2H, 1′-H$_{arom.}$ + 6′-H$_{arom.}$), 7.32 (m, 3H, 3″-H$_{arom.}$ + 4″-H$_{arom.}$ + 5″-H), 5.69 (d, 1H, J = 8.0 Hz, OH), 4.41 (d, 1H, J = 7.8 Hz, OH), 4.00 (m, 1H, 12-H), 3.77 (m, 1H, 7-H), 3.20 (m, 1H, 3-H), 2.26 (m, 1H, 4a-H), 2.14 (m, 7H, H-9 + (2 × Me-safranin)), 2.11 (m, 1H, 23a-H), 2.08 (m, 1H, 23b-H), 1.96 (m, 1H, 14-H), 1.80 (m, 1H, 6a-H), 1.77 (m, 1H, 17-H), 1.73 (m, 1H, 16a-H), 1.65 (m, 2H, 1a-H + 22a-H), 1.63 (m, 2H, 2a-H + 15a-H), 1.48 (m, 1H, 4b-H), 1.42 (m, 2H, 11a-H), 1.37 (m, 1H, 6b-H), 1.35 (m, 1H, 11b-H), 1.30 (m, 1H, 8-H + 20-H), 1.26 (m, 2H, 2b-H + 5-H), 1.24 (m, 1H, 22b-H), 1.17 (m, 1H, 16b-H), 0.92 (d, 3H, $J_{Me21,H20}$ = 6.2 Hz, 21-Me), 0.91 (m, 1H, 15b-H), 0.83 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.58 (s, 3H, 18-Me). – ^{13}C NMR ([D$_6$]DMSO): δ = 173.3 (C=O), 150.1 (C-4a′), 141.4 (C7′-NH$_2$), 138.8 (C$_{arom.}$-1″), 136.9, 134.5, 131.2, 131.1, 129.4, 128.0, 127.7, 126.6, 125.6 (C$_{arom.}$), 121.6 (C-10a′ + C-9a′), 106.6 (C-4′), 71.8 (C-12), 70.3 (C-3), 66.1 (C-7), 47.4 (C-17), 46.2 (C-13), 45.6 (C-5), 41.4 (C-14), 40.9 (C-8), 40.4 (C-4), 36.2 (C-20), 35.6 (C-1), 35.4 (C-6), 35.1 (C-10), 31.8 (C-2 + C-22), 31.0 (C-23), 28.0 (C-11), 27.1 (C-16), 26.1 (C-9), 22.6 (C-15), 22.5 (Me-19), 19.3 (C2′-Me), 18.4 (Me-21), 16.9 (C8′-Me), 12.2 (Me-18). – $C_{44}H_{57}ClN_4O_4$ (741.41): calcd. C 71.28, H 7.75, N 7.56; found C 71.01, H 7.65, N 7.26.

4.3 *N*-(4-(1-((4-bromophenyl)imino)ethyl) phenyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (8)

To a solution of **5** (310 mg, 0.60 mmol) in EtOH (20 mL) and glacial acetic acid (1 mL) was added 4-bromoaniline (100 mg, 0.60 mmol) and the mixture was heated under reflux for 12 h. The reaction progress was monitored by TLC. After cooling, the mixture was poured into cold water, and the solid product was collected by filtration followed by recrystallization from EtOH to give **8** (230 mg, 74%) as red solid, m.p.: 180–182°C, R_f = 0.7. – IR (KBr): ν = 3409 (OH), 2931, 2854 (CH_2), 1649 (C=N), 1595 (C=C)$_{arom.}$ cm^{-1}. – ^{1}H NMR ([D$_6$]DMSO): δ = 10.09 (s, 1H, NH), 7.88 (d, 2H, J = 7.9 Hz, 3′-H$_{arom.}$ + 5′-H$_{arom.}$), 7.65 (d, 2H, J = 7.9 Hz, 3″-H$_{arom.}$ + 5″-H$_{arom.}$), 7.57 (d, 2H, J = 7.9 Hz, 2′-H$_{arom.}$ + 6′-H$_{arom.}$), 7.11 (d, 2H, J = 7.9 Hz, 2″-H$_{arom.}$ + 6″-H$_{arom.}$), 5.61 (bs, 1H, OH), 5.21 (bs, 1H, OH), 4.44 (bs, 1H, OH), 4.04 (m, 1H, 12-H), 3.78 (m, 1H, 7-H), 3.19 (m, 1H, 3-H), 2.22 (m, 1H, 4a-H), 2.14 (m, 1H, 9-H), 2.11 (s, 3H, N=CMe), 2.09 (m, 1H, 23a-H), 2.02 (m, 1H, 23b-H), 1.95 (m, 1H, 14-H), 1.80 (m, 1H, 6a-H), 1.77 (m, 1H, 17-H), 1.67 (m, 2H, 1a-H + 22a-H), 1.64 (m, 2H, 2a-H + 15a-H), 1.46 (m, 1H, 4b-H), 1.44 (m, 1H, 11a-H), 1.39 (m, 1H, 6a-H), 1.35 (m, 1H, 11b-H), 1.33 (m, 1H, 8-H), 1.30 (m, 1H, 20-H), 1.27 (m, 1H, 2b-H), 1.25 (m, 1H, 5-H), 1.23 (m, 1H, 12-22b-H), 1.19 (m, 1H, 16b-H), 0.93 (d, 3H, $J_{Me21,H20}$ = 6.5 Hz, 21-Me), 0.91 (m, 1H, 15b-H), 0.84 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.57 (s, 3H, 18-Me). – ^{13}C NMR ([D$_6$]DMSO): δ = 172.1 (CONH) 169.2 (N=*C*Me), 148.5 (C$_{arom.}$-1″), 139.2 (C$_{arom.}$-1′), 135.1 (C$_{arom.}$-4′), 131.8, 125.4, 122.4, 121.4, 119.8 (C$_{arom.}$), 71.6 (C-12), 70.2 (C-3), 66.8 (C-7), 47.7 (C-17), 46.2 (C-13), 45.4 (C-5), 41.3 (C-14), 40.2 (C-4 + C-8), 36.1 (C-20), 35.8 (C-1), 35.6 (C-6), 34.9 (C-10), 32.3 (C-2 + C-22), 30.9 (C-23), 29.0 (C-11), 27.8 (C-16), 25.8 (C-9), 23.1 (C-15), 22.7 (Me-19), 19.0 (Me-21), 19.0 (Me-21), 17.6 (N=C*Me*). – $C_{38}H_{51}BrN_2O_4$ (679.74): calcd. C 67.15, H 7.56, N 4.12; found C 66.91, H 7.42, N 4.22.

4.4 3 α-*p*-Toluenesulfonyl-7α,12α-dihydroxy-5β-cholan-24-oic acid (9)

This compound was prepared according to the procedure reported by Barnett and Reichstein [37]. To a solution of cholic acid **3** (613 mg, 1.50 mmol) in dry pyridine (15 mL) was added a solution of *p*-toluenesulfonyl chloride (300 mg, 1.58 mmol) in dry pyridine (5 mL) with stirring at 0°C for 30 min. The solution was allowed to warm up, stirred at room temperature for 5 h, and the progress of the reaction was monitored by TLC. The reaction mixture was slowly added to 1.5 M HCl (100 mL). The white precipitate was collected by filtration and dried *in vacuo*. The crude product was purified on a SiO_2 column using in gradient, MeOH (0%–20%) and $CHCl_3$ as eluent to give pure **13** (642 mg, 76%), m.p.: 130–133°C (Lit. [37], m.p.: 131°C).

4.5 General procedure for synthesis of 3α-tosyloxy-7α,12α-dihydroxy-5β-cholan-24-amide derivatives (10–12)

The amide analogs were prepared according to the procedure for preparation of the analogs **4–7** from the tosylate derivative **9** (281 mg, 0.50 mmol) and amines (0.50 mmol) in CH_2Cl_2-DMF (10 mL), DMAP (0.07 mmol) and DCC (0.50 mmol). The products were purified from cyclohexylurea by SiO_2 column using, in gradient, MeOH (0%–10%) and $CHCl_3$ as eluent.

4.5.1 (*N,N*-diisopropyl)- 7α,12α-dihydroxy-3α-tosyloxy-5β-cholan-24-amide (10)

From *N,N*-diisopropylamine (51 mg). Yield: 242 mg (75%) as yellow solid, m.p.: 155–156°C, $R_f = 0.28$. – IR (KBr): $\nu = 3410$ (OH), 2931, 2854 (CH_2), 1695 $(C=O)_{amid}$, 1650 $(C=C)_{arom}$, 1314, 1338 (S=O) cm^{-1}. – ^1H NMR ([D_6]DMSO): $\delta = 7.60$ (d, 2H, $J = 7.8$ Hz, 2′-$H_{arom.}$ + 6′-$H_{arom.}$), 7.38 (d, 2H, $J = 7.8$ Hz, 3′-$H_{arom.}$ + 5′-$H_{arom.}$), 5.66 (m, 1H, OH), 3.99, 4.00 (d, 1H, $J = 3.2$ Hz, OH), 3.78 (m, 1H, 12-H), 3.61 (m, 1H, 7-H), 3.49 (m, 2H, 2×*CH*Me_2), 3.18 (m, 1H, 3-H), 2.39 (s, 3H, *Me*-Ar), 2.23 (m, 1H, 4a-H), 2.14 (m, 1H, 9-H), 2.12 (m, 1H, 23a-H), 2.04 (m, 1H, 23b-H), 1.96 (m, 1H, 14-H), 1.78 (m, 1H, 6a-H), 1.73 (m, 1H, 17-H), 1.70 (m, 1H, 16a-H), 1.67 (m, 2H, 1a-H + 22a-H), 1.63 (m, 2H, 2a-H + 15a-H), 1.46 (m, 1H, 4b-H), 1.43 (m, 1H, 11a-H), 1.41 (m, 2H, 6b-H + 11b-H), 1.38 (m, 2H, 8-H + 11b-H), 1.36 (m, 1H, 20-H), 1.33 (m, 1H, 2b-H), 1.28 (m, 1H, 5-H), 1.24 (m, 12H, 2 × CH*Me_2*), 1.22 (m, 1H, 22b-H), 1.20 (m, 1H, 16b-H), 0.93 (d, 3H, $J_{Me21,H20} = 6.1$ Hz, 21-Me), 0.91 (m, 1H, H-15b), 0.83 (m, 1H, 1b-Hb), 0.80 (s, 3H, 19-Me), 0.57 (s, 3H, 18-Me). – ^{13}C NMR ([D_6]DMSO): $\delta = 171.5$ (C=O), 148.0 (C-1′), 138.7 (C-4′), 130.2 (C-2′ + C-6′), 128.3 (C-3′ + C-5′), 71.8 (C-12), 70.3 (C-3), 66.1 (C-7), 53.8 (*CH*Me_2), 47.5 (C-17), 45.6 (C-13), 45.6 (C-5), 41.4 (C-14), 40.5 (C-8), 40.2 (C-4), 36.0 (C-20), 35.7 (C-1), 35.5 (C-6), 35.2 (C-10), 31.7 (C-2 + C-22), 30.9 (C-23), 28.4 (C-11), 27.2 (C-16), 26.1 (C-9), 24.5 (2 × CH*Me_2*), 22.7 (C-15), 22.5 (Me-19), 21.9 (*Me*-Ar), 17.0 (Me-21), 12.2 (Me-18). – $C_{37}H_{59}NO_6S$ (645.94): calcd. C 68.80, H 9.21, N 2.17; found C 68.58, H 9.10, N 1.92.

4.5.2 (*N,N*-dibutyl)-7α,12α-dihydroxy-3α-tosyloxy-5β-cholan-24-amide (11)

From *N,N*-dibutylamine (65 mg). Yield: 246 mg (73%) as light brown solid, m.p.: 133–132°C, $R_f = 0.41$. – IR (KBr): $\nu = 3407$ (OH), 2931, 2854 (CH_2),, 1699 $(C=O)_{amid}$, 1649 $(C=C)_{arom.}$, 1314, 1338 (S=O) cm^{-1}. – ^1H NMR ([D_6]DMSO): $\delta = 7.59$ (d, 2H, $J = 7.9$ Hz, 2′-$H_{arom.}$ + 6′-$H_{arom.}$), 7.48 (d, 2H, $J = 7.9$ Hz, 3′-$H_{arom.}$ + 5′-$H_{arom.}$), 5.54 (m, 1H, OH), 4.07 (m, 1H, OH), 3.78 (m, 1H, 12-H), 3.61 (m, 1H, 7-H), 3.34 (m, 4H, 2 × N*CH_2*CH_2CH_2Me), 3.20 (m, 1H, 3-H), 2.39 (s, 3H, *Me*-Ar), 2.22 (m, 1H, 4a-H), 2.15 (m, 1H, 9-H), 2.13 (m, 1H, 23a-H), 2.05 (m, 1H, 23b-H), 1.94 (m, 1H, 14-H), 1.80 (m, 1H, 6a-H), 1.78 (m, 1H, 17-H), 1.75 (m, 1H, 16a-H), 1.67 (m, 2H, 1a-H + 22a-H), 1.64 (m, 2H, 2a-H + 15a-H), 1.53 (m, 4H, 2 × NCH_2*CH_2*CH_2Me), 1.46 (m, 1H, 4a-H), 1.44 (m, 1H, 11a-H), 1.38 (m, 1H, 6b-H), 1.36 (m, 1H, 11b-H), 1.33 (m, 1H, 8-H) 1.30 (m, 4H, 2 × $N(CH_2)_2$*CH_2*Me), 1.29 (m, 1H, 20-H), 1.28 (m, 1H, 2b-H), 1.24 (m, 1H, 5-H), 1.22 (m, 1H, 22b-H), 1.18 (m, 1H, 16b-H), 0.92 (d, 3H, $J_{Me21,H20} = 6.5$ Hz, Me-21), 0.91 (m, 1H, 15b-H), 0.89 (m, 6H, 2 × $N(CH_2)_3$*Me*), 0.83 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.57 (s, 3H, 18-Me). – ^{13}C NMR ([D_6]DMSO): $\delta = 171.4$ (C=O), 145.6 (C-1′), 137.5 (C-4′), 130.1 (C-2′ + C-6′), 127.9 (C-3′ + C-5′), 71.8 (C-12), 70.9 (C-3), 66.1 (C-7), 50.3 (2 × NCH_2), 47.1 (C-17), 46.1 (C-13), 45.6 (C-5), 41.4 (C-14), 40.5 (C-8), 40.0 (C-4), 36.1 (C-20), 35.6 (C-1), 35.5 (C-6), 35.2 (C-10), 31.7 (C-2 + C-22), 30.9 (C-23), 30.3 (NCH_2*CH_2*CH_2CH_3), 28.4 (C-11), 27.2 (C-16), 26.0 (C-9), 22.7 (C-15), 22.5 (Me-19), 22.0 (Me-Ar), 20.7 (NCH_2CH_2*CH_2*CH_3), 17.0 (Me-21), 13.6 ($N(CH_2)_3$*Me*), 12.2 (Me-18). – $C_{39}H_{63}NO_6S$ (673.99): calcd. C 69.50, H 9.42, N 2.08, found C 69.28, H 9.30, N 1.82.

4.5.3 *N*-carbamothioyl-7α,12α-dihydroxy-3α-tosyloxy-5β-cholanamide (12)

From thiourea (38 mg). Yield: 220 mg (71%) as yellow solid, m.p.: 137–139°C; $R_f = 0.23$. – IR (KBr): $\nu = 3420$ (OH), 2931, 2854 (CH_2), 1728 $(C=O)_{amid}$, 1629 $(C=C)_{arom.}$, 1321, 1342 (S=O) cm^{-1}. ^1H NMR ([D_6]DMSO): $\delta = 9.66$ (bs, 2H, NH_2), 8.48 (s, 1H, NH), 7.64 (d, 2H, $J = 8.4$ Hz, 2′-$H_{arom.}$ + 6′-$H_{arom.}$), 7.47 (d, 2H, $J = 8.4$ Hz, 3′-$H_{arom.}$ + 5′-$H_{arom.}$), 5.70 (bs, 1H, OH), 4.03 (bs, 1H, OH), 3.78 (m, 1H, 12-H), 3.61 (m, 1H, 7-H), 3.18 (m, 1H, 3-H), 2.39 (s, 3H, *Me*-Ar), 2.22 (m, 1H, 4a-H), 2.14 (m, 1H, 9-H), 2.12 (m, 1H, 23a-H), 2.04 (m, 1H, 23b-H), 1.98 (m, 1H, 14-H), 1.82 (m, 1H, 6a-H), 1.77 (m, 1H, 17-H), 1.71 (m, 1H, 16a-H), 1.65 (m, 2H, 1a-H + 22a-H), 1.63 (m, 2H, 2a-H + 15a-H), 1.47 (m, 1H, 4b-H), 1.43 (m, 1H, 11a-H), 1.37 (m, 1H, 6b-H), 1.35 (m, 1H, 11b-H), 1.33 (m, 1H, 8-H) 1.30 (m, 1H, 20-H), 1.27 (m, 1H, 2b-H), 1.24 (m, 1H, 5-H), 1.21 (m, 1H, 22b-H), 1.17 (m, 1H, 16b-H), 0.93 (d, 3H, $J_{Me21,H20} = 6.6$ Hz, 21-Me), 0.92 (m, 1H, 15b-H), 0.83 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.58 (s, 3H, 18-Me). – ^{13}C NMR ([D_6]DMSO): $\delta = 180.0$ (C=S), 172.4 (C=O), 146.9 (C-1′), 138.6 (C-4′), 129.4 (C-2′ + C-6′), 128.1 (C-3′ + C-5′), 72.0 (C-12), 70.4 (C-3), 66.2 (C-7), 46.0 (C-17), 45.7 (C-13), 41.7 (C-5), 41.4 (C-14), 40.5 (C-8), 40.0 (C-4), 36.2 (C-20), 35.8 (C-1), 35.5 (C-6), 35.2 (C-10), 31.4 (C-22), 31.0 (C-2), 30.3 (C-23), 28.5 (C-11), 27.2 (C-16), 26.1 (C-9), 23.7 (C-15), 22.5 (Me-19), 21.9 (*Me*-Ar), 18.5 (Me-21), 12.3 (Me-18). – $C_{32}H_{48}N_2O_6S$ (620.86): calcd. C 61.91, H 7.79, N 4.51; found: C 61.70, H 7.67, N 4.29.

4.6 Methyl cholate (13)

This analog was prepared by typical procedure described Shen et al. [38] (m.p.: 155–156°C).

4.7 General procedure for synthesis of carbohydrazide analogs of cholic acid (14 and 15)

A mixture of methyl cholate (**9**) (211 mg, 0.50 mmol) and hydrazide (0.50 mmol) in abs. EtOH (15 mL) was heated under reflux for 12 h and completion of reaction was monitored by TLC. The precipitate formed was filtered off, dried and recrystallized from EtOH to give the desired product.

4.7.1 *N′*-(2,4-dinitrophenyl)-3α,7α,12α-trihydroxy-5β-cholan-24-carbohydrazide (14)

From 2,4-dinitrophenylhydrazide (100 mg). Yield: 215 mg (73%) as orange solid, m.p.: 165–167°C, R_f = 0.33. – IR (KBr): ν = 3412 (OH), 2931, 2854 (CH_2), 1726 (C=O)$_{amid}$, 1641 (C=C)$_{arom.}$, 1301 (NO_2) cm^{-1}. – ^{1}H NMR ([D$_6$]DMSO): δ = 8.60 (s, 1H, 3′-H$_{arom}$), 8.37 (d, 1H, J = 7.9 Hz, 5′-H$_{arom.}$), 7.64 (d, 1H, J = 7.9 Hz, 6′-H$_{arom.}$), 4.02 (m, 1H, 12-H), 3.77 (m, 1H, 7-H), 3.17 (m, 1H, 3-H), 2.25 (m, 1H, 4a-H), 2.15 (m, 1H, 9-H), 2.10 (m, 1H, 23a-H), 2.02 (m, 1H, 23b-H), 1.97 (m, 1H, 14-H), 1.79 (m, 1H, 6a-H), 1.76 (m, 1H, 17-H), 1.70 (m, 1H, 16a-H), 1.65 (m, 2H, 1a-H + 22a-H), 1.62 (m, 2H, 2a-H + 15a-H), 1.48 (m, 1H, 4b-H), 1.42 (m, 1H, H-11a), 1.39 (m, 1H, 6b-H), 1.36 (m, 1H, 11b-H), 1.34 (m, 1H, 8-H) 1.31 (m, 1H, 20-H), 1.29 (m, 1H, 2b-H), 1.25 (m, 1H, 5-H), 1.21 (m, 1H, 22b-H), 1.16 (m, 1H, 16b-H), 0.92 (d, 3H, $J_{Me21,H20}$ = 6.4 Hz, 21-Me), 0.90 (m, 1H, 15b-H), 0.82 (m, 1H, 1b-H), 0.79 (s, 3H, 19-Me), 0.57 (s, 3H, 18-Me). – ^{13}C NMR ([D$_6$]DMSO): δ = 173.7 (CONH), 153.4 (C$_{arom.}$-1′), 140.0 (C4′-NO_2), 135.4 (C2′-NO_2), 128.7 (C$_{arom.}$-5′), 121.9 (C$_{arom.}$-3′), 116.6 (C$_{arom.}$-6′), 71.5 (C-12), 70.4 (C-3), 66.2 (C-7), 47.5 (C-17), 45.7 (C-13), 41.5 (C-5), 41.3 (C-14), 40.4 (C-4 + C-8), 38.1 (C-20), 36.1 (C-1), 34.3 (C-6), 33.3 (C-22), 32.7 (C-23) 30.4 (C-2), 28.4 (C-11 + C-16), 27.2 (C-9), 23.7 (C-15), 22.5 (Me-19), 18.5 (Me-21), 12.2 (Me-18). – $C_{30}H_{44}N_4O_8$ (588.70): calcd. C 61.21, H 7.53, N 9.52; found C 60.90, H 7.41, N 9.32.

4.7.2 2-(3α,7α,12α-Trihydroxy-5β-cholanoyl)-hydrazine-1-carbothioamide (15)

From thiosemicarbazide (50 mg). Yield: 195 mg (81%) as brown crystals, m.p.: 135–137°C, R_f = 0.46. – IR (KBr): ν = 3406 (OH), 2931, 2854 (CH_2), 1726 (C = O)$_{amid}$, 1641 (C=C)$_{arom.}$ cm^{-1}. – ^{1}H NMR ([D$_6$]DMSO): δ = 5.01 (br s., 1H, OH), 4.30 (bs, 1H, J = 7.8 Hz, OH), 4.09 (m, 1H, 12-H), 3.77 (m, 1H, 7-H), 3.17 (m, 1H, 3-H), 2.19 (m, 1H, 4-H), 2.15 (m, 1H, 9-H), 2.10 (m, 2H, 23a-H), 2.03 (m, 1H, 23b-H), 1.97 (m, 1H, 14-H), 1.80 (m, 1H, 6a-H), 1.76 (m, 1H, 17-H), 1.72 (m, 1H, 16a-H), 1.67 (m, 2H, 1a-H + 22a-H), 1.65 (m, 2H, 2a-H + 15a-H), 1.47 (m, 1H, 4a-H), 1.43 (m, 1H, 11a-H), 1.38 (m, 2H, 6b-H), 1.35 (m, 1H, 11b-H), 1.33 (m, 1H, 8-H), 1.31 (m, 1H, 20-H), 1.28 (m, 1H, 2b-H), 1.25 (m, 1H, 5-H), 1.23 (m, 1H, 22b-H), 1.18 (m, 1H, 16b-H), 0.92 (d, 3H, $J_{Me21,H20}$ = 6.4 Hz, 21-Me), 0.91 (m, 1H, 15b-H), 0.84 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me). – ^{13}C NMR ([D$_6$]DMSO): δ = 183.8 (C=S), 173.7 (CONH), 71.8 (C-12), 70.3 (C-3), 66.2 (C-7), 47.5 (C-17), 45.9 (C-13), 45.2 (C-5), 41.4 (C-14), 40.1 (C-4 + C-8), 36.0 (C-20), 35.8 (C-1), 35.2 (C-6), 34.9 (C-10), 32.7 (C-2 + C-22), 30.6 (C-23), 28.4 (C-11), 27.2 (C-16), 26.1 (C-9), 22.7 (C-15 + Me-19), 17.0 (Me-21), 12.2 (Me-18). – $C_{25}H_{43}N_3O_4S$ (481.70): calcd. C 62.34, H 9.00, N 8.72; found C 62.05, H 8.78, N 8.50.

4.8 5-Amino-2-(3α,7α,12α-trihydroxy-5β-cholyl-23-yl)-1,3,4-thiadiazole (16)

To a stirred mixture of cholic acid **3** (409 mg, 1.0 mmol) and thiosemicarbazide (91 mg, 1.0 mmol) in EtOH (25 mL) was added conc. H_2SO_4 (1.0 mL) dropwise and heated under reflux for 5 h and the reaction progress was monitored by TLC. The mixture was poured into a crushed ice and the solid was filtered, washed with cold water and recrystallized from EtOH to give **12** as brown solid (385 mg, 83%), m.p.: 210–212°C, R_f = 0.36. – IR (KBr): ν = 3413 (OH), 2931, 2866 (CH_2), 1594 (C=N), 690 (C-S-C) cm^{-1}. – ^{1}H NMR ([D$_6$]DMSO): δ = 9.01 (s, 1H, NH_2), 5.55 (bs, 1H, OH), 4.00 (bs, 1H, OH), 3.75 (m, 1H, 12-H), 3.62 (m, 1H, 7-H), 3.45 (bs, 1H, OH), 3.16 (m, 1H, 3-H), 3.00 (m, 2H, 23a,b-H), 2.23 (m, 1H, 4a-H), 2.16 (m, 1H, 9-H), 1.90 (m, 1H, 14-H), 1.80 (m, 1H, 6a-H), 1.73 (m, 1H, 16a-H), 1.71 (m, 1H, 17-H), 1.68 (m, 2H, 1a-H + 22a-H), 1.63 (m, 2H, 2a-H + 15a-H), 1.46 (m, 1H, 4b-H), 1.44 (m, 1H, 11a-H), 1.35 (m, 3H, H-6b + H-8 + H-11b), 1.31 (m, 1H, 20-H), 1.28 (m, 1H, 2b-H), 1.25 (m, 1H, 5-H), 1.23 (m, 1H, 22b-H), 1.17 (m, 1H, 16b-H), 1.11 (m, 1H, 17-H), 0.96 (d, 3H, $J_{Me21,H20}$ = 6.2 Hz, 21-Me), 0.94 (m, 1H, 15b-H), 0.84 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.56 (s, 3H, 18-Me). – ^{13}C NMR ([D$_6$]DMSO): δ = 173.3 (C$_{thiadiazole}$-2′), 170.2 (C$_{thiadiazole}$-5′), 71.7 (C-12), 70.4 (C-7), 66.2 (C-3), 47.4 (C-17), 45.3 (C-13), 41.9 (C-5), 40.6 (C-8 + C-4), 38.7 (C-20), 35.7 (C-1), 35.4 (C-6), 35.1 (C-10), 31.7 (C-2 + C-22), 29.0 (C-11), 27.8 (C-16), 26.7 (C-23), 26.0 (C-9), 22.9 (C-15), 22.7 (Me-19), 19.0 (Me-21); 12.8 (Me-18). – $C_{25}H_{41}N_3O_3S$ (463.68): calcd. C 64.76, H 8.91, N 9.06; found C 64.53, H 8.80, N 8.78.

4.9 Cholylhydrazide (17)

This compound was prepared by typical procedure described by Al-Qawasmeh et al. [34] from methyl cholate

13 and hydrazine hydrate, and characterized by melting point comparison with literature value (m.p.: 160–162°C)

4.10 General procedure for the synthesis of *N′*-[(1*E*)-4-substituted-benzylidene or ethylidene]-3α,7α,12α-trihydroxy-5β-cholan-24-ohydrazide derivatives (18–22)

To a solution of **17** (211 mg, 0.50 mmol) in abs. EtOH (15 mL) and glacial acetic acid (1 mL) was added the corresponding aromatic aldehyde or ketone (0.55 mmol) and the mixture was heated under reflux for 10–12 h and the reaction progress was monitored by TLC. After cooling, the mixture poured into cold water and left standing for 10 h in order to complete precipitation. The solid product was collected by filtration, washed by petroleum ether and recrystallized from EtOH.

4.10.1 *N′*-[(1*E*)-4-Dimethylaminobenzylidene]-3α,7α,12α-trihydroxy-5β-cholan-24-ohydrazide (18)

From 4-(dimethylamino)benzaldehyde (82 mg). Yield: 249 mg (90%) as yellow solid, m.p.: 190–192°C; R_f = 0.53. – IR (KBr): ν = 3049 (OH), 2931, 2862 (CH_2), 1698 (C=O)$_{amid}$, 1650 (C=N), 1604 (C=C)$_{arom.}$ cm^{-1}. – ^{1}H NMR ([D_6]DMSO): δ = 8.08 (s, 1H, N=CH), 7.30 (d, 2H, J = 7.9 Hz, 2′-H$_{arom.}$ + 6′-H$_{arom.}$'), 6.58 (d, 2H, J = 7.9 Hz, 3′-H$_{arom.}$ + 5′-H$_{arom.}$), 5.64 (bs, 1H, OH), 3.96 (bs, 1H, OH), 3.78 (m, 1H, 12-H), 3.57 (m, 1H, H-7), 3.19 (m, 1H, 3-H), 2.94 (s, 6H, NMe_2), 2.29 (m, 1H, 4a-H), 2.15 (m, 1H, 9-H), 2.12 (m, 1H, 23a-H), 2.06 (m, 1H, 23b-H), 1.97 (m, 1H, 14-H), 1.83 (m, 1H, 6a-H), 1.76 (m, 1H, 17-H), 1.72 (m, 1H, 16a-H), 1.63 (m, 2H, 1a-H + 22a-H), 1.65 (m, 2H, 2a-H + 15a-H), 1.54 (m, 1H, 4b-H), 1.52 (m, 1H, 11a-H), 1.39 (m, 1H, 6b-H), 1.37 (m, 2H, 6a-H + 11b-H), 1.35 (m, 1H, 8-H), 1.30 (m, 1H, 20-H), 1.29 (m, 1H, 2b-H), 1.22 (m, 1H, 5-H), 1.18 (m, 1H, 22b-H), 0.92 (d, 3H, $J_{Me21,H20}$ = 6.5 Hz, 21-Me), 0.91 (m, 1H, 15b-H), 0.83 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.58 (s, 3H, 18-Me). – ^{13}C NMR ([D_6]DMSO): δ = 171.2 (CONH), 156.5 (C-4′), 149.2 (N=CH), 127.9 (C$_{arom.}$-2′ + C$_{arom.}$-6′), 122.2 (C$_{arom.}$-1′), 106.6 (C$_{arom.}$-3′ + C$_{arom.}$-5′), 71.4 (C-12), 70.8 (C-3), 66.7 (C-7), 46.4 (C-17), 45.4 (C-13), 42.0 (NMe_2), 41.3 (C-5 + C-14), 40.3 (C-4 + C-8), 36.1 (C-20), 35.6 (C-1), 35.2 (C-6), 35.0 (C-10), 31.3 (C-22), 31.1 (C-2), 30.3 (C-23), 28.4 (C-11), 27.2 (C-16), 26.2 (C-9), 22.7 (C-15), 22.5 (Me-19), 17.1 (Me-21), 12.2 (Me-18). – $C_{33}H_{51}N_3O_4$ (553.79): calcd. C 71.57, H 9.28, N 7.59; found C 71.29, H 9.11, N 7.38.

4.10.2 *N′*-[(1*E*)-4-Cholorobenzylidene]-3α,7α,12α-trihydroxy-5β-cholan-24-ohydrazide (19)

From 4-chlorobenzaldehyde (77 mg). Yield: 234 mg (86%) as white solid, m.p.: 259–261°C (Lit. [36] 260–261°C), R_f = 0.50. – IR (KBr): ν = 3040 (OH), 2931, 2862 (CH_2), 1694 (C=O)$_{amid}$, 1650 (C=N), 1604 (C=C)$_{arom.}$ cm^{-1}. – ^{1}H NMR ([D_6]DMSO): δ = 9.61 (s, 1H, NH), 8.42 (s, 1H, N=CH), 7.94 (d, 2H, J = 7.5 Hz, 2′-H$_{arom.}$ + 6′-H$_{arom.}$), 7.56 (d, 2H, J = 7.5 Hz, 3′-H$_{arom.}$ + 5′-H$_{arom.}$), 6.21 (bs, 1H, OH), 4.09 (bs, 1H, OH), 3.85 (m, 1H, 12-H), 3.61 (m, 1H, 7-H), 3.18 (m, 1H, 3-H), 2.22 (m, 1H, 4a-H), 2.14 (m, 1H, 9-H), 2.12 (m, 1H, 23a-H), 2.03 (m, 1H, 23b-H), 1.98 (m, 1H, 14-H), 1.83 (m, 1H, 6a-H), 1.79 (m, 1H, 17-H), 1.72 (m, 1H, 16a-H), 1.65 (m, 2H, 1a-H + 22a-H), 1.62 (m, 2H, 2-H + 15a-H), 1.46 (m, 1H, 4b-H), 1.44 (m, 1H, 11a-H), 1.37 (m, 1H, 6b-H), 1.35 (m, 1H, 11b-H), 1.33 (m, 1H, 8-H), 1.30 (m, 1H, 20-H), 1.29 (m, 1H, 2b-H), 1.24 (m, 1H, 5-H), 1.22 (m, 1H, 22b-H), 1.16 (m, 1H, 16b-H), 0.93 (d, 3H, $J_{Me21,H20}$ = 6.4 Hz, 21-Me), 0.92 (m, 1H, 15b-H), 0.83 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.58 (s, 3H, 18-Me). – ^{13}C NMR ([D_6]DMSO): δ = 167.8 (CONH), 144.5 (N=CH), 137.7 (C4′-Cl), 131.8 (C-1′), 130.8 (C$_{arom.}$-2′ + C$_{arom.}$-6′), 128.7 (C$_{arom.}$-3′ + C$_{arom.}$-5′), 71.9 (C-12), 70.3 (C-3), 66.1 (C-7), 46.3 (C-17), 45.6 (C-13), 41.4 (C-5 + C-14), 40.4 (C-4 + C-8), 36.3 (C-20), 35.8 (C-1), 35.3 (C-6), 35.2 (C-10), 31.3 (C-22), 31.1 (C-2), 30.3 (C-23), 28.4 (C-11), 27.2 (C-16), 26.2 (C-9), 22.7 (C-15), 22.5 (Me-19), 17.1 (Me-21), 12.2 (Me-18). – $C_{31}H_{45}ClN_2O_4$ (545.16): calcd. C 68.30, H 8.32, N 5.14; found C 60.12, H 8.23, N 4.89.

4.10.3 *N′*-[(1*E*)-4-hydroxybenzylidene]-3α,7α,12α-trihydroxy-5β-cholan-24-ohydrazide (20)

From 4-hydroxyaldehyde (67 mg). Yield: 200 mg (76%) as light brown solid, m.p.: 206–208°C, R_f = 0.61. – IR (KBr): ν = 3046 (OH), 2935, 2868 (CH_2), 1696 (C=O)$_{amid}$, 1658 (C=N), 1558 (C=C)$_{arom.}$ cm^{-1}. – ^{1}H NMR ([D_6]DMSO): δ = 9.63 (bs, 1H, NH), 8.53 (s, 1H, N=CH), 7.67 (d, 2H, J = 8.4 Hz, 2′-H$_{arom.}$ + 6′-H$_{arom.}$), 6.87 (d, 2H, J = 8.4 Hz, 3′-H$_{arom.}$ + 5′-H$_{arom.}$), 4.00 (bs, 1H, OH), 3.78 (m, 1H, 12-H), 3.61 (m, 1H, 7-H), 3.43 (s, 1H, Ar-*OH*), 3.19 (m, 1H, 3-H), 2.21 (m, 1H, 4a-H), 2.15 (m, 1H, 9-H), 2.13 (m, 1H, 23a-H), 2.01 (m, 1H, 23b-H), 1.91 (m, 1H, 14-H), 1.83 (m, 1H, 6a-H), 1.78 (m, 1H, 17-H), 1.71 (m, 1H, 16a-H), 1.65 (m, 2H, 1a-H + 22a-H), 1.63 (m, 2H, 2a-H + 15a-H) 1.44 (m, 1H, 4b-H), 1.42 (m, 1H, 11a-H), 1.38 (m, 1H, 6b-H), 1.35 (m, 1H, 11b-H), 1.33 (m, 1H, 8-H), 1.31 (m, 1H, 20-H), 1.28 (m, 1H, 2b-H), 1.24 (m, 1H, 5-H), 1.22 (m, 1H, 22b-H), 1.17 (m, 1H, 16b-H), 0.94 (d, 3H, $J_{Me21,H20}$ = 6.4 Hz, 21-Me), 0.93 (m, 1H, 15b-H), 0.83 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.58 (s, 3H, 18-Me). – ^{13}C NMR ([D_6]DMSO): δ = 171.5 (CONH), 160.2 (C4′-OH), 142.8 (N=CH), 129.9

($C_{arom.}$-2′ + $C_{arom.}$-6′), 128.2 (C-1′), 115.7 ($C_{arom.}$-3′ + $C_{arom.}$-5′), 71.8 (C-12), 70.4 (C-3), 66.2 (C-7), 46.5 (C-17), 45.7 (C-13), 41.5 (C-5 + C-14), 40.3 (C-4 + C-8), 36.2 (C-20), 35.9 (C-1), 35.3 (C-6), 35.1 (C-10), 31.4 (C-22), 31.2 (C-2), 30.3 (C-23), 28.5 (C-11), 27.2 (C-16), 26.1 (C-9), 23.1 (C-15), 22.7 (Me-19), 18.5 (Me-21), 12.3 (Me-18). – $C_{31}H_{46}N_2O_5$ (526.72): calcd. C 70.69, H 8.80, N 5.32; found C 70.38, H 8.67, N 5.03.

4.10.4 *N′*-(1-(4-nitrophenyl)ethylidene)-3*α*,7*α*,12*α*-trihydroxy-5*β*-cholanhydrazide (21)

From 4-nitroacetophenone (108 mg). Yield: 202 mg (71%) as red solid, m.p.: 188–190°C, R_f = 0.71. – IR (KBr): ν = 3051 (OH), 2933, 2866 (CH_2), 1672 $(C=O)_{amid}$, 1595 (C=N), 1558 $(C=C)_{arom.}$ cm^{-1}. – ^{1}H NMR ([D_6]DMSO): δ = 10.60 (bs, 1H, NH), 8.31 (d, 2H, J = 8.8 Hz, 3′-$H_{arom.}$ + 5′-$H_{arom.}$), 8.17 (d, 2H, J = 8.8 Hz, 2′-$H_{arom.}$ + 6′-$H_{arom.}$), 6.61 (bs, 1H, OH), 5.62 (bs, 1H, OH), 4.00 (bs, 1H, OH), 3.79 (m, 1H, 12-H), 3.60 (m, 1H, 7-H), 3.18 (m, 1H, 3-H), 2.32 (s, 3H, N=C*Me*), 2.23 (m, 1H, 4a-H), 2.15 (m, 1H, 9-H), 2.13 (m, 1H, 23a-H), 2.07 (m, 1H, 14-H), 1.96 (m, 1H, 23b-H), 1.83 (m, 1H, 6a-H), 1.79 (m, 1H, 17-H), 1.69 (m, 1H, 16a-H), 1.65 (m, 2H, 1a-H + 22a-H), 163 (m, 2H, 2a-H + 15a-H), 1.46 (m, 1H, 4b-H), 1.44 (m, 1H, 11a-H), 1.38 (m, 1H, 6b-H), 1.35 (m, 1H, 11b-H), 1.33 (m, 1H, 8-H), 1.30 (m, 1H, 20-H), 1.28 (m, 1H, 2b-H), 1.24 (m, 1H, 5-H), 1.21 (m, 1H, 22b-H), 1.18 (m, 1H, 16b-H), 0.94 (d, 3H, $J_{Me21,H20}$ = 6.6 Hz, 21-Me), 0.93 (m, 1H, 15b-H), 0.83 (m, 1H, 1b-H), 0.80 (s, 3H, 19-Me), 0.59 (s, 3H, 18-Me). – ^{13}C NMR ([D_6]DMSO): δ = 170.9 (CONH), 156.0 (C4′-NO_2), 148.0 (N=*C*Me), 143.2 (C-1′), 127.8 ($C_{arom.}$-3′ + $C_{arom.}$5′), 123.5 ($C_{arom.}$-2′ + $C_{arom.}$6′), 71.5 (C-12), 70.3 (C-3), 66.1 (C-7), 47.5 (C-17), 45.7 (C-13), 41.9 (C-5), 41.6 (C-14), 40.4 (C-8), 39.8 (C-4), 36.5 (C-20), 35.8 (C-1), 35.4 (C-6), 35.2 (C-10), 31.7 (C-2 + C-22), 31.0 (C-23), 28.5 (C-11), 27.2 (C-16), 26.1 (C-9), 22.8 (C-15 + N=C*Me*), 22.5 (Me-19), 18.4 (Me-21), 12.2 (Me-18). – $C_{32}H_{47}N_3O_6$ (569.74): calcd. C 67.46, H 8.32, N 7.38; found C 67.19, H 8.15, N 7.12.

4.10.5 *N′*-(1-(4-aminophenyl)ethylidene)-3*α*,7*α*,12*α*-trihydroxy-5*β*-cholanhydrazide (22)

From 4-aminoacetophenone (74 mg). Yield: 232 mg (86%) as dark red solid, m.p.: 198–200°C, R_f = 0.66. – IR (KBr): ν = 3049 (OH), 2931, 2866 (CH_2), 1896 $(C=O)_{amid}$, 1631 (C=N), 1564 $(C=C)_{arom.}$ cm^{-1}. – ^{1}H NMR ([D_6]DMSO): δ = 10.04 (d, 2H, J = 8.6 Hz, NH_2), 8.92 (s, 1H, NH), 7.62 (d, 2H, J = 8.7 Hz, 2′-$H_{arom.}$ + 6′-$H_{arom.}$), 6.58 (d, 2H, J = 8.7 Hz, 3′-$H_{arom.}$ + 5′-$H_{arom.}$), 5.37 (bs, 1H, OH), 4.00 (bs, 1H, OH), 3.80 (m, 1H, 12-H), 3.61 (m, 1H, 7-H), 3.44 (bs, 1H, OH), 3.19 (m, 1H, 3-H), 2.23 (s, 3H, N=C*Me*), 2.21 (m, 1H, 4-H), 2.14 (m, 1H, 9-H), 2.11 (m, 1H, 23a-H), 2.05 (m, 1H, 23b-H), 1.98 (m, 1H, 14-H), 1.83 (m, 1H, 6a-H), 1.75 (m, 1H, 17-H), 1.72 (m, 1H, 16a-H), 1.66 (m, 2H, 1a-H + 22a-H), 1.63 (m, 2H, 2a-H + 15a-H), 1.47 (m, 1H, 4b-H), 1.43 (m, 1H, 11a-H), 1.38 (m, 1H, 6b-H), 1.36 (m, 1H, 11b-H), 1.33 (m, 1H, 8-H), 1.31 (m, 1H, 20-H), 1.28 (m, 1H, 2b-H), 1.25 (m, 1H, 5-H), 1.23 (m, 1H, 22b-H), 1.18 (m, 1H, 16b-H), 0.94 (d, 3H, $J_{Me21,H20}$ = 6.2 Hz, 21-Me), 0.92 (m, 1H, 15b-H), 0.84 (m, 1H, 1b-H), 0.81 (s, 3H, 19-Me), 0.60 (s, 3H, 18-Me). – ^{13}C NMR ([D_6]DMSO): δ = 171.5 (CONH), 158.0 (C4′-NH_2), 150.2 (N=*C*Me), 123.5 ($C_{arom.}$-2′ + $C_{arom.}$-6′), 127.5 (C-1′), 113.0 ($C_{arom.}$-3′ + $C_{arom.}$-5′), 71.5 (C-12), 70.3 (C-3), 66.2 (C-7), 47.5 (C-17), 45.7 (C-13), 41.7 (C-5), 41.6 (C-14), 40.8 (C-4 + C-8), 36.2 (C-20), 35.9 (C-1), 35.2 (C-6), 34.8 (C-10), 31.9 (C-22), 31.4 (C-2), 30.9 (C-23), 28.5 (C-11), 27.3 (C-16), 26.2 (C-9), 23.0 (C-15), 22.7 (N=C*Me*), 22.5 (Me-19), 18.5 (Me-21), 13.9 (Me-18). – $C_{32}H_{49}N_3O_4$ (539.76): calcd. C 71.21, H 9.15, N 7.79; found C 70.89, H 9.01, N 7.55.

4.11 Bioactivity assays

4.11.1 Inhibition assay of human 17*β*-HSD1

Inhibitory activities were evaluated by an established method with minor modifications [51]. Briefly, the enzyme preparation was incubated with NADPH (500 µM) in the presence of potential inhibitors at 37°C in a phosphate buffer (50 mM) supplemented with 20% of glycerol and EDTA (1 mM). Synthesized compounds stock solutions were prepared in DMSO. The final concentration of DMSO was adjusted to 1% in all samples. The enzymatic reaction was started by addition of a mixture of unlabeled- and [2,4,6,7-^{3}H]-E1 (from Perkin Elmer, Boston and Quickszint Flow 302 scintillator fluid was from Zinsser Analytic, Frankfurt) (final concentration: 500 nM, 0.15 µCi). After 10 min, the incubation was stopped with $HgCl_2$ and the mixture was extracted with diethylether. After evaporation, the steroids were dissolved in acetonitrile. E1 and E2 were separated using acetonitrile-water (45:55) as mobile phase in a C18 reverse phase chromatography column (Nucleodur C18 125/3 100-5, Macherey-Nagel) connected to a HPLC-system (Agilent 1200 Series, Agilent Technologies). Detection and quantification of the steroids were performed using a radioflow detector (Ramona, raytest). The conversion rate was calculated after analysis of the resulting chromatograms according to the following equation:

$$\% \text{ conversion} = \%E2 / (\%E2 + \%E1) \times 100$$

Each value was calculated from at least three independent experiments in triplicate.

4.11.2 Inhibition of human 17β-HSD2

The 17β-HSD2 inhibition assay was performed similarly to the 17β-HSD1 procedure. The microsomal fraction was incubated with NAD+ [1500 μM], test compound and a mixture of unlabeled- and [2,4,6,7-^{3}H]-E2 (final concentration: 500 nM, 0.11 μCi) for 20 min at 37°C. Further treatment of the samples and HPLC separation was carried out as mentioned above. The conversion rate was calculated after analysis of the resulting chromatograms according to the following equation:

$$\% \text{ conversion} = (\% \text{ E1}) / (\%\text{E1} + \%\text{E2}) \times 100.$$

Acknowledgment: We thank Miss A. Friemel of the Department of Chemistry, University of Konstanz, Germany, for the 2D-NMR experiments.

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