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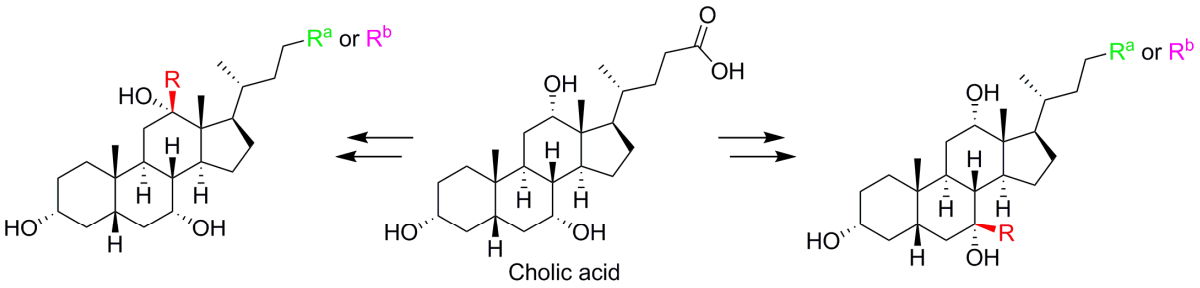
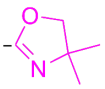


R^a: R= methyl
R^b: R= methyl, ethyl, butyl or octyl

R^a= -COOH

R^b= 

R^a: R= methyl, ethyl, butyl or octyl

IC_{50}<5 μM

R^b: R= methyl, ethyl, butyl or octyl

Synthesis and antitumor activity of alkylated bile acids and oxazolines

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Key words:

- Bile acid
- Stereoselective alkylation
- Grignard reaction
- Antitumor activity
- Oxazoline

Abstract

Bile acid derivatives with a free carboxylic group or an oxazoline ring in the side chain and with different lengths of alkyl chains on steroid skeleton were synthetized and their antitumor activity against six human cancer cell lines was investigated. Methyl, ethyl, butyl or octyl chains were introduced stereoselectively by Grignard reaction at C-7 of acid and oxazoline, and at C-12 of oxazoline. Carbonyl group at C-12 of acid compound gave addition product only with methyl Grignard reagent, and complex mixture of products with other used reagents. Due to enolization, the C-3 carbonyl group did not participate in the Grignard reaction. Steric reasons are a main cause of this chemical behavior. Compounds with a butyl chain at the C-7 position showed very good antitumor activity with IC_{50}<5 μM.

1. Introduction

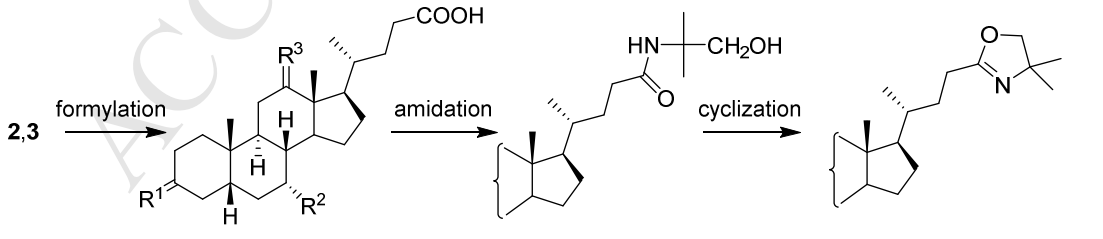
Perception of bile acids (BAs) as merely lipid emulsifying and solubilizing molecules was fundamentally changed after discovery of their hormonal roles. BAs are natural agonists for a number of nuclear and transmembrane receptors involved in a variety of metabolic functions, ranging from BA biosynthesis to energy homeostasis.[1] These findings considerably broadened therapeutic potential of BA and their derivatives.

Relation of hydrophobic/hydrophilic area of BA is important for their biological properties. Hydrophobic BAs, such as secondary BAs, deoxycholic acid (DCA) and lithocholic acid (LCA) which lack one hydroxyl group relative to corresponding primary BAs, can induce apoptosis and have been implicated as tumor promoters.[2, 3] BAs with oxo groups have lower membranolytic activity then their hydroxyl analogues.[4, 5] Also, obeticholic acid, semisynthetic BA, with ethyl group connected to steroid C-6 is a strong farnesoid X receptor (FXR) agonist, and in the final phases of clinical studies for treatment of some liver diseases.[6, 7] In our previous study we have showed that introduction of an ethylidene group at the C-7 position on the steroid skeleton greatly influenced antitumor activity of these compounds.[8] Perception of BA steroid skeleton as a pharmacophore, and findings that introduction of hydrophobic groups on BA steroid skeleton can significantly influence biological activity prompted us to investigate the most common way to change hydrophobicity-introduction of alkyl groups. This work should give insights into specific chemical behavior of bile acid steroid skeleton in alkylation reactions with Grignard reagents and in vitro antitumor activity of alkyl derivatives.

2. Results and discussion

2.1. Chemistry

In order to investigate the possibilities of bile acid alkylation we decided to employ Grignard reactions as they are simple, cheap and fast. Also we decided to try Grignard reactions on bile acid derivatives with free carboxylic groups, as well as with protected carboxylic groups in form of oxazoline. For the starting compound we used cheap and commercially available cholic acid (CA). By known procedures we oxidized CA in corresponding 7-oxo (**1**), 12-oxo (**2**) and 3-oxo (**3**) derivatives.[9-11] 7,12-Diformyloxy-3-oxo compound **4** was obtained by known method.[12]

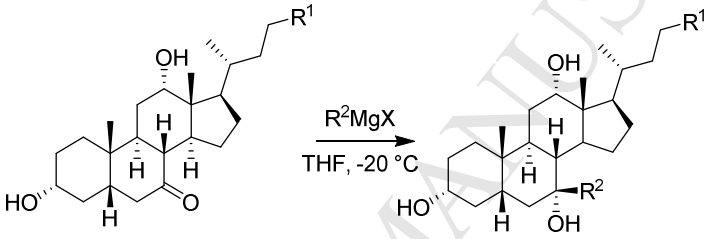


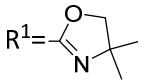
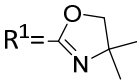
2, 6 R^1=αOH, βH; R^2= OH; R^3=O
3, **7** R^1=O; R^2=OH; R^3=αOH, βH
8, **9**, **11** R^1=αOOCH, βH; R^2= OOCH; R^3=O
4, **10**, **12** R^1=O; R^2=OOCH; R^3=αOOCH, βH

Scheme 1. Synthesis of oxazoline derivatives

Protection of carboxylic group in the form of oxazoline can resolve low solubility of bile salt in ether solvents and potential problems of carboxylic carbon participation in Grignard reaction. The synthesis of bile oxazoline **5** is described in our previous publication.[8] By similar manner we have synthesized 12-oxo **6** and 3-oxo **7** oxazoline in good yields (Scheme 1.). The sequence included formylation (**8**), condensation of carboxylic group with 2-amino-2-methyl-1-propanol (**9**, **10**) using EEDQ as coupling agent followed by cyclization to oxazoline (**11**, **12**) ring by treatment with thionyl chloride and deformylation (**6** and **7**).

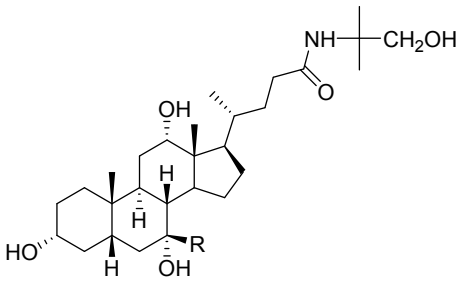
Bile acid derivatives, 7-oxo bile acid **1** and 7-oxo bile oxazoline **5**, were subjected to Grignard reaction with methyl, ethyl, butyl and octylmagnesium halides. Reaction condition, products and yields of reactions are shown in Scheme 2.



Substrate	Reagent	Eqvivalents of R^2MgX	Reaction time [min]	Product			Yield [%]
R^1= COOH **1**	MeMgI	34	30	R^1= COOH	**13**	R^2=Me	83
	EtMgBr	24	90		**14**	R^2=Et	88
	BuMgCl	25	30		**15**	R^2=Bu	77
	OctMgBr	30	135		**16**	R^2=Oct	83
R^1=  **5**	MeMgI	19	20	R^1= 	**17**	R^2=Me	63
	EtMgBr	45	15		**18**	R^2=Et	74
	BuMgCl	23	60		**19**	R^2=Bu	72
	OctMgBr	28	60		**20**	R^2=Oct	56

Scheme 2. 7-Oxo derivatives in Grignard reaction

Both oxo derivatives, **1** and **5,** gave corresponding alkyl adducts in good yields. However, adducts with free carboxyl group are formed in better yields then oxazolines with similar reaction conditions. Reaction times were slightly shorter for **5** then for **1** probably due to better solubility of oxazolines in THF. Reactions with Grignard reagent were carried out in dry THF on temperature of –20 °C. When higher temperature was applied oxazoline ring was partially hydrolyzed into amides **21**, **22** and **23** (Fig. 1.), though alkylation of C-7 carbonyl group still occurs.



21 R=Me
22 R=Bu
23 R=Oct

Fig. 1. Alkylated amides formed by partial oxazoline hydrolysis

Yields of alkylated amides at –20 °C were less than 8%, while at reflux yields of byproduct were increased up to 26%. Alkylation reactions were stereoselective, and only one epimer was detected. Determination of configuration in steroid skeleton is always challenging, especially in the case when newly introduced substituent is alkyl group. NOE NMR experiments usually give ambiguous results due to considerable overlap of signals in NMR spectra, also bile acid derivatives with long hydrocarbon chains are hard to crystallize. Fortunately, we succeeded to crystallize carboxylic derivate with methyl group **13**, and determined configuration at C-7 by X-ray crystallography experiment. Structure of **13** shown in Fig 2, shows that methyl group at C-7 is β orientated. This configuration was expected considering considerable steric hindrance of α side of steroid by A ring, and β methyl group has thermodynamically favorable equatorial orientation.

If a nucleophile approaches from the α side of the steroidal skeleton the maximal overlap of HOMO orbitals of nucleophile and LUMO orbitals of carbonyl group would result in 105° to 107°angle of attack (Bürgi-Dunitz angle α_{BD}, Nu-C(carbonyl)-O(carbonyl)). This would make nucleophilic carbon of Grignard reagent in syn-axial orientation with C-4, C-9 and C-12 hydrogens. That means that there is no variance in Flippin-Lodge angle (α_{FL}) in α-azimuth plane, and that nucleophile attack from α side is excluded. While in attack from β side at $\alpha_{BD} \approx 107°$ angle Flippin-Lodge angle (β-azimuth plane) shows maximal variance, i. e. attack is not sterically inhibited (**Appendix A – Supplementary data**).

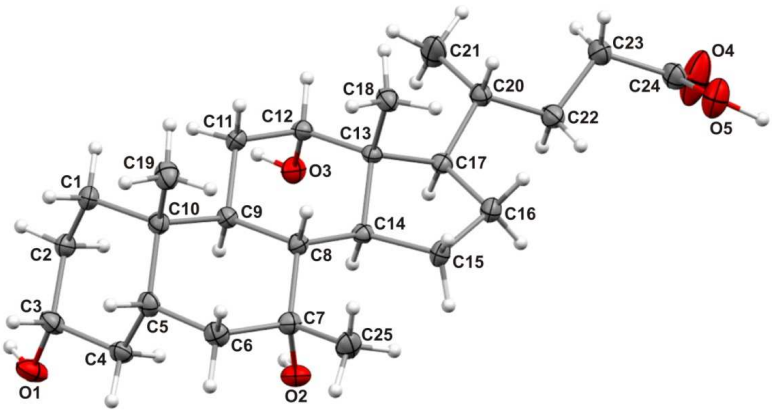
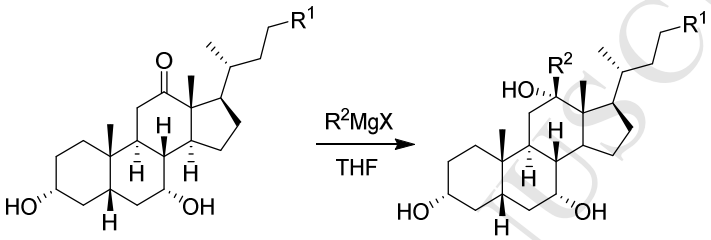
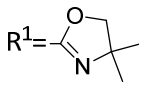
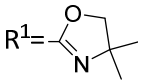


Fig. 2. ORTEP drawing of the molecular structure of compound **13** with labeled non-H atoms. Displacement ellipsoids are shown at 30% probability, and H atoms are drawn as spheres of arbitrary radii.

Example of **13** gave enough evidence that is safe to conclude, with considerable degree of certainty, that other 7-alkyl derivatives have also β-orientated alkyl groups.

Somewhat different results were obtained in the reaction of 12-oxo BA **2** or 12-oxo oxazoline **6** with Grignard reagents. Reaction condition, products and yields of these reactions are shown in Scheme 3.



Substrate	Reagent	Eqvivalents of R^2MgX	Reaction conditions	Product			Yield [%]
R^1= COOH **2**	MeMgI	20	90 min, -20 °C	R^1= COOH	**24**	R^2=Me	27
	EtMgBr	20	5 h, 0 °C or RT		**CA**	R^2=H	/
	BuMgCl	20	5 h, 0 °C or RT		**CA**	R^2=H	10
 **6**	MeMgI	21	30 min, 0 °C		**25**	R^2=Me	53
	EtMgBr	22	2 h -20 °C → 3 h 0 °C		**26**	R^2=Et	48
					27	R^2=H	17
	BuMgCl	51	2 h -20 °C → 3 h 0 °C		**28**	R^2=Bu	49
					27	R^2=H	19
	OctMgBr	52	3 h 0 °C		**29**	R^2=Oct	67

Scheme 3. 12-Oxo derivatives in Grignard reaction

Alkylation of 12-oxo BA was only successful with MeMgI, and **24** was isolated in moderate yield. Reactions of **2** with EtMgBr and BuMgCl were carried out at three temperatures: at –20 °C after 2 h the reaction did not occur; at 0 °C or room temperature (RT) after 5 h complex mixture of products was obtained with CA as main product (10% with BuMgCl). In reaction with EtMgBr reaction mixture was not purified, ad CA as main product was detected by TLC check.

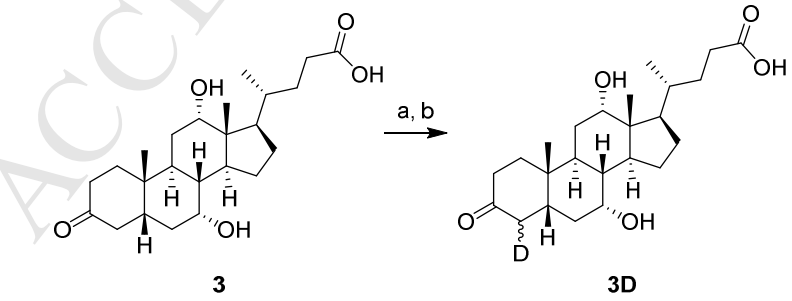
Oxazoline **6** with MeMgI at 0 °C after 30 minutes gave only adduct **25** but with EtMgBr and BuMgCl, at –20 °C and then at 0 °C, beside alkylated products **26** i.e **28**, gave also reduced product **27**. Reduced product **27**, was created as a result of the reduction of the keto group with Grignard reagent, and it is related to steric hindrance of C-12 carbonyl group.[12] Reaction of **6** with OctMgBr was carried out at 0 °C

for 3 h, and only octyl product **29** was isolated. These findings show that higher reaction temperatures (0 °C) disfavors reduction reaction, and only addition of alkyl group occurs.

Configuration at C-12 of **24** was determined by ROESY NMR experiment, and a strong cross-peak between methyl groups on C-12 and C-13 indicate β orientation of C-12 methyl group. Same stereochemistries at C-12 presumably have all C-12 alkyl derivatives, as Flippin-Lodge angle in α-azimuth plane tends to zero value (**Appendix B - Supplementary data**).

The difference in the chemical behavior between 7-oxo and 12-oxo BAs is attributed to a lower steric accessibility of C-12 carbonyl carbon. In this way it could be also explained different reactivity of these groups in Grignard reaction. The Grignard reagent reacted relatively quickly with the C-7 carbonyl group of **1**, to give the corresponding addition product, while in the case of **2** only methyl adducts were isolated in poor yield, and ethyl- and butyl- reagents gave complex mixture of products. Approach of the alkylmagnesium halide reagent to C-12 carbonyl of **2** is hampered by C-18 methyl group, therefore reaction occurs only at the higher temperatures (RT), and at higher temperatures side reactions also occur. Weakly reactive carboxylic carbon C-24 undergoes addition reactions on room temperature, which with the combination of C-12 adducts and reduction of C-12 carbonyl group leads to complex mixture of products. Smaller value of Flippin-Lodger angle in β-azimuth plane of C-12 carbonyl makes successful collision with Grignard reagent molecules only with molecules that approach carbonyl group in angle no greater than α_{FL}-2 angle. Therefore, increasing the temperature increases the number of collisions and this number tends to number of collisions seen at C-7 carbonyl group (attack from β side) (**Appendix B**).

In the next phase Grignard reactions of acid **3** with MeMgI (THF, 1 h RT; benzene, 3 h reflux), EtMgBr (THF, 100 min 0 °C) and BuMgCl (THF, 4 h RT) were investigated. The reactions have not occurred, and only a starting compound was isolated. This result was unexpected given that the carbonyl group at C-3 position is the least hindered in comparison to the C-7 and C-12 carbonyl groups. Explanation of this result is the formation of a nucleophilic enolate anion caused by abstraction of α-protons by Grignard reagent. This is confirmed by reaction of **3** with EtMgBr which is quenched by addition of D_2O, to afford compound **3D** (Scheme 4) (**Appendix C - Supplementary data**)



3 **3D**

Scheme 4. (a) EtMgBr, THF, −20 °C, 2.5 h; (b) i) D_2O; ii) AcOH.

Also, addition reaction did not occur in the case of oxazoline analogue **7** and EtMgBr or BuMgCl (–20 °C, RT or reflux), and only partial hydrolysis of oxazoline ring to amide **30** occurred in a 8% yield.

2.2. Antitumor activity

Antiproliferative activities of synthesized derivatives (**13**, **14**, **15**, **16**, **17**, **18**, **19**, **20**, **24**, **25**, **26**, **28**, **29**) were evaluated *in vitro* against six tumor cell lines (estrogen receptor positive breast adenocarcinoma MCF-7, estrogen receptor negative breast adenocarcinoma MDA-MB-231, human prostate cancer PC3, cervix carcinoma HeLa, colon adenocarcinoma HT-29, adenocarcinomic human alveolar basal epithelial cells A549) and one normal cell line (fetal fibroblasts MRC-5). Cytotoxicity was evaluated by using standard MTT assay,[14] after exposure of cells to the tested compounds for 72 h. The commercial nonselective antitumor agent doxorubicin (DOX) was used as a positive control in this assay.

Three compounds showed antiproliferative activity with the IC_{50} values lower than 10 μM. 7-Butyl-acid derivative **15** showed activity against MCF-7 with IC_{50} value of 9.00 μM, and against MDA-MB-231 with IC_{50} value of 3.07 μM. 7-Butyl-oxazoline **19** showed activity in low micromolar range (IC_{50} 4.43 μM) against HeLa cell line. Against MDA-MB-231 cell line compound **20** showed antiproliferative activity of IC_{50} 9.70 μM. It is important to highlight that none of the tested compounds were active against fetal fibroblast normal cell line MRC-5, unlike Doxorubicine which is very toxic towards this cell line. Data of *In vitro* antiproliferative activities of other synthetized compounds could be found in Table 1. in the supplementary data.

3. Conclusion

We have explored alkylation of bile acid derivatives at the positions C-3, C-7 and C-12 by using Grignard reaction. Alkylation was done on the derivatives with either free carboxylic group or oxazoline group in side chain Alkylation of substrates with free the carboxylic group gave slightly better yields, but considering the additional synthetic steps for the protection of the carboxylic group, direct alkylation is definitively method of choice. Oxazoline group was beneficial for the synthesis of C-12 alkyl derivatives since all alkyl products were obtained in the reactions with corresponding reagents, while only 12-methyl derivative was successfully synthesized from the derivative with free carboxylic group (alkylation with ethyl and butyl reagents gave a substantial amount of by-products due to side reactions such as alkylation of C-24 and reduction of C-12 carbonyl group). Alkylation at C-12 is also stereoselective with only β-alkyl products isolated. Substrates with the C-3 carbonyl group failed to react with Grignard reagents due to formation of unreactive enol anion. Selected synthesized derivatives were evaluated for their in vitro antiproliferative activity against tumor cell lines and one normal cell line. The most potent activities were recorded in the cultures of MDA-MB-231 cells (7-butyl compounds **15** and **19** with IC_{50} 3.07 μM and 4.43 μM, respectively) and MCF-7 cells (7-butyl compound **15** with IC_{50} 9.00 μM). None of the synthesized compounds were active toward normal MRC-5 cells.

4. Experimental section

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 250 apparatus (^{1}H 250 MHz and ^{13}C 62.5 MHz) Bruker Avance III HD 400 (400 MHz ^{1}H, 101 MHz ^{13}C) Avance III HD 500 (500 MHz ^{1}H, 125 MHz ^{13}C) apparatus using tetramethylsilane as the internal standard. IR spectra were recorded on a Nexus 670 FT-IR spectrometer. HRMS spectra (TOF) were recorded on a 6210 Time-of-Flight LC/MS Agilent Technologies (ESI+) instrument. Melting points were determined using a Nagema Boeitus melting point apparatus and the results are uncorrected. The colorimetric MTT assay was carried out following the reported procedure.[14] Flash chromatography was performed on silica gel 60 (0.04–0.063 mm, Merck). Grignard's reagents (EtMgBr, 2 M solution in Et_2O; BuMgCl, 2 M solution in Et_2O; OctMgBr, 2 M solution in Et_2O) were purchased from Sigma-Aldrich, solutions were dried with anhydrous Na_2SO_4. Tetrahydrofuran (THF) was dried and distilled from sodium metal using benzophenone as an indicator.

4.1 3α,7α-Diformyloxy-12-oxo-5β-cholanoic acid (8)

Oxo BA **2** (0.487 g, 1.20 mmol) was dissolved in formic acid (3.5 mL) with addition of $HClO_4$ (0.05 mL). Solution was heated at 50–55 °C, after 1 h solution was cooled to room temperature and acetic anhydride (1 mL) was added until appearance of large amount of bubbles (decomposition of acetic formic anhydride formed after acetic anhydride reacted with all water from formic acid), and then poured in cold water (100 mL). White precipitate was filtered, washed with water to neutral pH and dried on air. Compound **8** was obtained in yield of 0.530 g (96%). Rf ($CHCl_3$/acetone 7:1 with glacial acetic acid 0.05 mL/10 mL of eluent) 0.80.

IR (cm^{-1}): 2941, 2874, 1717, 1182.

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.76 (d, *J*=6 Hz, 3H, H-21), 0.99 (s, 3H, H-18) and 1.03 (s, 3H, H-19), 4.60 (m, 1H, H-3), 5.00 (s, 1H, H-7), 8.17 and 8.22 (2s, 2×1H, 2CHO), 11.94 (s, 1H, COOH).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 11.6 (C-18), 19.0 (C-21), 22.1 (C-19), 23.5, 26.9, 27.3, 30.7, 31.4, 31.5, 34.5, 34.7, 35.4, 35.5, 37.1, 37.6, 37.8, 40.1, 46.5, 53.1, 56.9, 70.6 (C-7), 73.4 (C-3), 162.1 (2CHO), 175.2 (C-24), 213.5 (C-12).

HRMS (TOF) *m/z*: calculated for $C_{26}H_{38}O_7$ $[M+Na]^+$ 485.2510, found 485.2514.

4.2 2-(3α,7α-Diformyloxy-12-oxo-5β-cholan-24-amido)-2-methyl-1-propanol (9)

To the suspension of compound **8** (0.530 g, 1.15 mmol) in EtOAc (13 mL) were added: triethylamine (0.3 mL), 2-amino-2-methyl-1-propanol (0.2 mL, 2.1 mmol), H_2O (1 mL) and EEDQ (0.42 g, 1.7 mmol). Reaction mixture was refluxed for 4 h. After cooling to room temperature, the reaction mixture was washed successively with 3 M HCl (1×4 mL), H_2O (1×4 mL), 10% $NaHCO_3$ (2×10 mL) and then with water to neutrality (3×5 mL). The organic layer was dried and evaporated in vacuum to give an oily residue which was purified by flash column chromatography (CH_2Cl_2/acetone 8:1). Pure **9** was obtained as colorless oil in yield of 0.366 g (60%). Rf ($CHCl_3$/acetone 7:1) 0.61.

IR (cm^{-1}): 3393, 2936, 2873, 2249, 1717, 1643, 1184.

^{1}H NMR (250 MHz, $CDCl_3$) δ = 0.86 (d, *J*=6 Hz, 3H, H-21), 1.04 and 1.06 (2s, 2×3H, H-18 i H-19), 1.29 (s, 6H, 2xCH_3 amide), 3.57 (s, 2H, CH_2 amide), 4.72 (m, 1H, H-3), 5.15 (s, 1H, H-7), 5.56 (s, 1H, NH), 8.01 (s, 1H, CHO), 8.04 (s, 1H, CHO).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ = 11.5, 18.7, 22.1, 23.7, 24.8, 25.9, 26.5, 27.3, 31.1, 33.5, 34.1, 34.4, 34.7, 35.4, 35.5, 37.5, 37.7, 37.7, 45.0, 49.00, 52.74, 56.2, 57.0, 70.4, 70.9, 72.5, 160.3 (CHO), 160.6 (CHO), 174.5 (C-24), 213.6 (C-12).

HRMS (TOF) *m/z*: calculated for $C_{30}H_{47}O_7$ $[M+H]^+$ 534.34253, found 534.34165.

4.3 2-(3α,7α-Diformyloxy-12-oxo-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (11)

Freshly distilled $SOCl_2$ (0.06 mL, 0.83 mmol), was added dropwise to the stirred ice-cooled solution of compound **9** (0.092 g, 0.17 mmol) in THF (0.5 mL) and the reaction mixture was stirred further for 1 h. A saturated $NaHCO_3$ solution (5 mL) was added with vigorous stirring. Reaction mixture was extracted with EtOAc (4×5 mL). The organic extracts were combined, dried and evaporated to dryness. Crude product was purified by flash chromatography (EtOAc /CH_2Cl_2 2:1). Pure **11** was obtained as oil in yield of 0.086 g (94%). Rf ($CHCl_3$/acetone 7:1) 0.80.

IR (cm^{-1}): 2964, 2940, 2873, 1665, 1464, 1448

^{1}H NMR (250 MHz, $CDCl_3$) δ = 0.74 (s, 3H, H-18), 0.86 (d, *J*=6.0 Hz, 3H, H-21), 1.20 (s, 3H, H-19), 1.25 (s, 6H, 2xCH_3 on C-4'), 3.88 (s, 2H, H-5'), 4.81 (m, 1H, H-3), 5.27 (s, 1H, H-12), 7.98 (s, 1H, CHO on C-3), 8.10 (s, 1H, CHO on C-12).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ = 12.4 (C-18), 17.6 (C-21), 22.6 (C-19), 24.0, 25.1, 25.9, 26.3, 27.5, 28.3 (CH3), 28.4 (CH3), 31.9, 32.9, 33.5, 34.6, 34.8, 36.5, 41.8, 44.9, 45.6, 46.3, 49.0, 66.8 (C-4'), 72.5 (C-3), 74.6 (C-12), 78.9 (C-5'), 160.3 and 160.4 (2xCHO), 166.2 (C-2'), 210.5 (C-7).

HRMS (TOF) *m/z*: calculated for $C_{30}H_{45}NO_6$ 516.33196, found 516.33113.

4.4 2-(3α,7α-Dihydroxy-12-oxo-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (6)

Solution of **11** (0.322 g, 0.63 mmol) and KOH (0.28 g, 4.99 mmol) in methanol (6 mL) was refluxed for 20 min., whereupon saturated solution of NH_4Cl in H_2O (40 mL) was added to neutrality. Solution was extracted with EtOAc (4×8 mL). The organic extracts were combined, dried and evaporated to dryness and purified by flash chromatography (CH_2Cl_2/acetone 1:1). Compound **6** was obtained as a small white needles crystal (mp 193 °C after recrystallization from acetone) in yield of 0.273 g (90%). Rf (EtOAc/acetone 1:1) 0.34.

IR (cm^{-1}): 3386, 2965, 2930, 2870, 1701, 1664.

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.78 (d, *J*=5.5 Hz, 3H, H-21), 0.94 (s, 3H, H-19), 0.96 (s, 3H, H-18), 1.15 (s, 6H, 2xCH_3 on C-4'), 3.18 (m, 1H, H-3), 3.72 (s, 1H, H-7), 3.84 (s, 2H, H-5'), 4.33–4.36 (more signals, 2H, OH on C-3 i C-7).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 11.6 (C-18), 19.1 (C-21), 22.5 (C-19), 23.7, 25.2, 27.6, 28.7 i 28.8 (2xCH_3 on C-4'), 30.8, 31.9, 35.1, 35.5, 35.6, 35.9, 37.1, 37.9, 41.4, 46.6 (C-17), 53.9, 56.8, 66.5 (C-7), 67.2 (C-4'), 70.4 (C-3), 78.4 (C-5'), 165.1 (C-2'), 214.42 (C-12).

HRMS (TOF) *m/z*: calculated for $C_{28}H_{45}NO_4$ $[M+H]^+$ 460.34214, found 460.34070.

4.5 2-(7α,12α-Diformyloxy-3-oxo-5β-cholan-24-amido)-2-methyl-1-propanol (10)

To the suspension of compound **4** (0.799 g, 1.73 mmol) in EtOAc (17 mL) were added: triethylamine (0.4 mL), 2-amino-2-methyl-1-propanol (0.3 mL, 3.1 mmol) and EEDQ (0.74 g, 2.99 mmol). Reaction mixture was refluxed for 3 h and stirred at room temperature for 12 h. The reaction mixture was washed successively with 3 M HCl (1×4 mL), H_2O (1×4 mL), 10% $NaHCO_3$ (2×10 mL) and then with water to neutrality (3×5 mL). The organic layer was dried and evaporated in vacuum to give an oily residue which was purified by flash column chromatography (CH_2Cl_2/acetone 5:1). Pure **10** was obtained as colorless oil in yield of 0.703 g (76%). Rf (CH_2Cl_2/acetone 5:1) 0.48.

IR (cm^{-1}): 3391, 2963, 2919, 1715, 1261, 1095, 1021.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.81 (s, 3H, CH_3), 0.87 (d, *J*=6.5 Hz, 3H, H-21), 1.05 (s, 3H, CH_3), 1.29 (s, 6H, 2xCH_3 amide), 3.58 (s, 2H amide), 4.84 (bs, 1H, OH amide), 5.17 (d, *J*=2.5 Hz, 1H, H-12), 5.51 (s, 1H, H-7), 8.10 (s, 1H, CHO), 8.17 (s, 1H, CHO).

^{13}C NMR (101 MHz, CDCl3) δ = 12.2, 17.6, 21.6, 22.8, 24.8, 24.8, 25.9, 27.2, 29.5, 31.1, 31.4, 33.9, 34.4, 34.7, 36.2, 36.5, 37.8, 42.2, 42.9, 44.6, 45.1, 47.3, 56.2, 70.6, 70.9, 75.2, 160.3, 160.4, 174.3 (C-24), 211.6 (C-3).

HRMS (TOF) *m/z*: calculated for $C_{30}H_{47}NO_7$ $[M+Na]^+$ 556.32447, found 556.32315.

4.6 2-(7α,12α-Diformyloxy-3-oxo-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (12)

Freshly distilled $SOCl_2$ (0.1 mL, 1.37 mmol), was added dropwise to the stirred ice-cooled solution of compound **10** (0.168 g, 0.31 mmol) in THF (3 mL) and the reaction mixture was stirred further for 1 h. A saturated $NaHCO_3$ solution (10 mL) was added with vigorous stirring. Reaction mixture was extracted with EtOAc (4×10 mL). The organic extracts were combined, dried and evaporated to dryness. Crude product was purified by flash chromatography (CH_2Cl_2/acetone 8:1). Pure **12** was obtained as colorless oil in yield of 0.112 g (69%). Rf (CH_2Cl_3/acetone 5:1) 0.53.

IR (cm^{-1}): 3416, 2961, 2927, 2872, 1722, 1415, 1667, 1176.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.79 (s, 3H, H-18), 0.88 (d, *J*=6 Hz, 3H, H-21), 1.04 (s, 3H, H-19), 1.25 (s, 6H, 2xCH_3 on C-4'), 3.01 (t, *J*=15 Hz, H-4α) 1H, 3.89 (s, 2H, H-5'), 5.15 (d, *J*=2 Hz, 1H, H-7), 5.31 (s, 1H, H-12), 8.09 (s, 1H, CHO), 8.15 (s, 1H, CHO).

^{13}C NMR (101 MHz, $CDCl_3$) δ = 12.1 (C-18), 17.6 (C-21), 21.6 (C-19), 22.8, 24.9, 25.9, 27.2, 28.4 (CH_3 on C-4'), 28.4 (CH3 on C-4'), 29.5, 31.1, 31.8, 34.4, 34.8, 36.2, 36.5, 37.7, 42.2, 42.9, 44.6 (C-4), 45.1, 47.0, 66.8 (C-4'), 70.6 (C-7), 75. (C-12), 78.9 (C-5'), 160.3 (CHO), 160.4 (CHO), 166.3 (C-2'), 211.6 (C-3).

HRMS (TOF) *m/z*: calculated for $C_{30}H_{45}NO_6$ $[M+H]^+$ 516.33196, found 516.33071.

4.7 2-(7α,12α-Dihydroxy-3-oxo-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (7)

Solution of **12** (0.602 g, 1.17 mmol) and KOH (0.60 g, 10.69 mmol) in methanol (15 mL) was refluxed for 20 min., whereupon saturated solution of NH_4Cl in H_2O (60 mL) was added to neutrality. Solution was extracted with EtOAc (5×10 mL). The organic extracts were combined, dried and evaporated to dryness and purified by flash chromatography (CH_2Cl_2/acetone 1:1). Compound **7** was obtained as a white needle crystal (mp 148 °C after recrystallization from acetone) in yield of 0.416 g (77%). Rf ($CHCl_3$/acetone 2:1) 0.54.

IR (cm^{-1}): 3428, 2941, 2868, 1708, 1663.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.73 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.01 (d, *J*=3 Hz, 3H, H-21), 1.26 (2s, 6H, 2xCH_3 on C-4'), 3.90 (d, *J*=3, 1H, H-7), 3.92 (s, 2H, H-5'), 4.00 (s, 1H, H-12).

^{13}C NMR (101 MHz, $CDCl_3$) δ = 12.4, 17.7, 21.5, 23.3, 24.4, 26.8, 27.8, 28.2, 28.4, 28.4, 31.3, 34.2, 34.9, 35.6, 36.8, 36.8, 39.3, 41.9, 43.4, 45.5, 46.2, 46.6, 66.7 (C-4'), 67.9 (C-7), 73.1 (C-12), 79.0 (C-5'), 167.4 (C-2'), 213.5 (C-3).

HRMS (TOF) *m/z*: calculated for $C_{28}H_{45}NO_4$ $[M+H]^+$ 460.34214, found 460.34053.

General procedure for Grignard reaction

Appropriate Grignard reagent was added dropwise over 3 min to a cooled (–20 °C) solution of corresponding compound in dry THF and stirred at same temperature (unless stated otherwise) for the reaction time. Reaction was quenched by addition of saturated aqueous solution of NH_4Cl, and then glacial acetic acid. After extraction with EtOAc, organic phases were combined, dried and evaporated in vacuum. The crude reaction mixture was purified by flash column chromatography.

4.8 3α,7α,12α-Trihydroxy-7β-methyl-5β-cholanic acid (13)

MeMgI in Et_2O (2 M, 7.4 mL, 14.8 mmol); **1** (0.173 g, 0.43 mmol) in THF (12.5 mL); reaction time 30 min; NH_4Cl (10 mL); CH_3COOH (3 mL); EtOAc (5x10 mL); flash chromatography: CH_2Cl_2/MeOH 9:1 with addition of glacial acetic acid 0.05 mL/10 mL of eluent. Compound **13** was obtained as transparent cube crystals (mp. 224 °C, after crystallization from acetone, lit.[15] mp. 221–223 °C) in yield of 0.154 g (83%). Rf (same solvent system as for flash chromatography) 0.28.

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.62 (s, 3H, H-18), 0.78 (s, 3H, H-19), 0.94 (d, *J*=6 Hz, 3H, H-21), 1.10 (s, 3H, CH_3 on C-7), 3.22 (m, 1H, H-3), 3.56 (s, 1H, OH on C-7), 3.76 (s, 1H, H-12), 4.09 (d, *J*=3.5 Hz, 1H, OH on C-12), 4.31 (d, *J*=4 Hz, 1H, OH on C-3), 11.92 (bs, 1H, COOH).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 13.2 (C-18), 17.5 (C-21), 23.2 (C-19), 27.4, 28.0, 28.9, 29.5, 30.7, 31.2, 31.3, 33.7 (CH_3 on C-7), 34.5, 35.4, 36.1, 38.6, 42.5, 42.7, 43.6, 45.0, 45.1, 47.5, 70.8 (C-3), 70.9 (C-12), 71.4 (C-7), 175.6 (C-24).

Structure Determination and Refinement for 13

A suitable single crystal of compound **13** was mounted on a glass fiber and crystallographic data were collected using an Oxford Diffraction Gemini S diffractometer with a CCD area detector ($\lambda_{MoK\alpha}$ = 71073 Å, monochromator: graphite) at 293 K. CrysAlisPro and CrysAlis RED software packages (Oxford Diffraction, 2009, Abingdon) were used for data collection and data integration. Analysis of the integrated data did not reveal any decay. Final cell parameters were determined by a global refinement of 1,942 reflections ($2.836 < \theta < 28.903°$). Collected data were corrected for absorption effects by using a Multi-scan absorption correction.[15] Structure solution and refinement were carried out with the programs SHELXT and SHELXL-2014/6 respectively.[16] ORTEP-3 for Windows[17] was employed for molecular graphics and WinGx software was used to prepare material for publication.[18]

Full-matrix least-squares refinement was carried out by minimizing ($F_o^2 - F_c^2$). All nonhydrogen atoms were refined anisotropically and refinement was carried out without geometric or ADP restraints. Hydrogen atoms attached to carbon atoms were placed in geometrically idealized positions and refined as riding on their parent atoms, with C–H = 0.98–1.00 Å with U_{iso}(H) = 1.2U_{eq}(C) for methylene and methyne groups, and U_{iso} (H) = 1.5 U_{eq}(C) for methyl groups. At the final stage of the refinement, H atoms attached to O atoms were identified on difference electron density maps and isotropically refined. Crystal data and experimental details of the structure determination are listed in Table 1.

Table 1 Crystal data and refinement parameters for compound **13**

Compound **13**	Parameters
Chemical formula	$C_{25}H_{42}O_5$
M_r	422.58
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Temperature	293 K
Unit cell dimensions	*a* = 9.0862 (6) Å
a, *b*, *c* (Å)	*b* = 15.8629 (12) Å
	c = 16.8304 (11) Å
Volume	2425.8 (3) Å^3
Z	4
Absorption coefficient	0.08 mm^{-1}
Crystal size	0.57 × 0.31 × 0.29 mm^3

Absorption correction	Multi-scan *CrysAlis PRO* 1.171.38.41 (Rigaku Oxford Diffraction, 2015) Empirical absorption correction using spherical harmonics, implemented in SCALE3 ABSPACK scaling algorithm.
T_{min}, T_{max}	0.975, 1.000
Reflections collected	5979
Independent reflection	3817 [R_{int} = 0.02]
Observed [I > 2s(I)] reflections	3425
Data/restrains/parameters	3817/0/291
Goodness-of-fit on F^2	1.061
Final R [F^2 > 2σ(F^2)]	*R1* = 0.0442, *wR2* = 0.0950
R indices (all data)	*R1* = 0.0502, *wR2* = 0.0984
ρ_{max}, ρ_{min} (e Å^{-3})	0.21, -0.22

Crystallographic data for compound **13** are deposited in the Cambridge Crystallographic Data Centre as supplementary material number CCDC 1561497. The CCDC files 1561497. contain the supplementary crystallographic data. Copies of this information may be obtained free of charge from the Director, CCDC, 12 Union Road, Cambridge, CB21FZ, UK (email: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk).

4.9 3α,7α,12α-Trihydroxy-7β-ethyl-5β-cholanic acid (14)

EtMgBr in Et_2O (3 M, 3 mL, 9 mmol); **1** (0.154 g, 0.38 mmol) in THF (6.4 mL); reaction time 90 min; NH_4Cl (10 mL); CH_3COOH (3 mL); EtOAc (5x10 mL); flash chromatography: CH_2Cl_2/MeOH 9:1 with addition of glacial acetic acid 0.05 mL/10 mL of eluent. Compound **14** (0.146 g; 88%) was obtained in the form of oil. Rf (same solvent system as for flash chromatography) 0.31.

IR (cm^{-1}): 3401, 2930, 1713, 1377, 1097, 1034.

^{1}H NMR (400 MHz, DMSO-*d6*) δ = 0.64 (s, 3H, H-18), 0.76 (s, 3H, H-19), 0.79 (t, *J*=7 Hz, 3H, CH_3 ethyl chain), 0.94 (d, *J*=6 Hz, 3H, H-21), 3.22 (m, 1H, H-3), 3.41 (s, 1H, OH on H-7), 3.75 (s, 1H, H-12), 4.10 (s, 1H, OH on C-12), 4.33 (s, 1H, OH on C-3),

^{13}C NMR (101 MHz, DMSO-*d6*) δ = 10.0 (CH_3 ethyl chain), 13.2 (C-18), 17.5 (C-21), 21.5, 23.0 (C-19), 26.2, 27.8, 28.8, 29.7, 30.6, 31.3, 31.5, 34.2, 35.5, 36.1, 37.0, 38.9, 39.0, 42.0, 43.0, 44.9, 47.7, 70.8, 70.9, 73.8 (C-7), 175.6 (C-24).

HRMS (TOF) *m/z*: calculated for $C_{26}H_{43}O_5$ $[M+K]^+$ 475.28203, found 475.28151.

4.10 3α,7α,12α-Trihydroxy-7β-butyl-5β-cholanic acid (15)

BuMgCl in Et_2O (2 M, 3.4 mL, 6.8 mmol); **1** (0.111 g, 0.27 mmol) in THF (4.25 mL); reaction time 30 min; NH_4Cl (10 mL); CH_3COOH (3 mL); EtOAc (5x10 mL); flash chromatography: CH_2Cl_2/MeOH 9:1 with addition of glacial acetic acid 0.05 mL/10 mL of eluent. Compound **15** was obtained in the form of oil in yield of 0.080 g (94% calculated in regard to reacted **1**), and regenerated **1**, 0.037 g. Rf (same solvent system as for flash chromatography) 0.33.

IR (cm^{-1}): 3401, 2932, 2871, 1709, 1379, 1078, 1033, 736.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.75 (s, 3H, H-18), 0.85 (s, 3H, H-19), 0.92 (t, *J*=7 Hz, 3H, CH_3 butyl chain), 1.03 (d, *J*=6 Hz, 3H, H-21), 3.54 (m, 1H, H-3), 3.95 (s, 1H, H-12).

^{13}C NMR (101 MHz, $CDCl_3$) δ = 13.0 (C-18), 14.2 (CH_3 butyl chain), 17.3 (C-21), 22.4, 23.3, 26.4, 27.4, 27.8, 28.8, 28.9, 29.7, 30.7, 31.1, 34.0, 35.5, 38.0, 39.3, 40.5, 41.6, 43.1, 44.4, 45.4, 48.0, 72.1 (C-3), 72.6 (C-12), 75.4, 77.2, 178.3 (C-24).

HRMS (TOF) *m/z*: calculated for $C_{28}H_{48}O_5$ $[M+K]^+$ 503.31333, found 503.31222.

4.11 3α,7α,12α-Trihydroxy-7β-octyl-5β-cholanic acid (16)

OctMgBr in Et_2O (2 M, 11.5 mL, 23 mmol); **1** (0.311 g, 0.76 mmol) in THF (15 mL); reaction time 135 min; NH_4Cl (10 mL); CH_3COOH (3 mL); EtOAc (5x10 mL); flash chromatography: CH_2Cl_2/MeOH 9:1 with addition of glacial acetic acid 0.05 mL/10 mL of eluent. Compound **16** was obtained in the form of oil in yield of 0.330 g (83%). Rf (same solvent system as for flash chromatography) 0.33.

IR (cm^{-1}): 3400, 2925, 2855, 1711, 1377, 1262, 1081, 1032.

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.62 (s, 3H, H-18), 0.75 (s, 3H, H-19), 0.85 (t, *J*=7 Hz, 3H, CH_3 octyl chain), 0.93 (d, *J*=6 Hz, 3H, H-21), 3.22 (m, 1H, H-3), 3.41 (s, 1H, OH on C-7), 3.74 (s, 1H, H-12), 4.11 (s, 1H, OH on C-12), 4.33 (s, 1H, OH on C-3).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 13.1 (C-18), 14.4 (CH_3 octyl chain), 17.5 (C-21), 22.6 (C-19), 23.0, 25.1, 26.2, 27.8, 28.9, 29.1, 29.4, 29.7, 30.3, 30.6, 31.2, 31.3, 31.7, 34.2, 35.5, 36.1, 38.9, 40.5, 42.1, 43.1, 44.9, 45.0, 47.6 (C-13), 70.9 (C-3), 70.9 (C-12), 73.6 (C-7), 175.4 (C-24).

HRMS (TOF) *m/z*: calculated for $C_{32}H_{56}O_5$ $[M+CHO]^-$ 565.41098, found 565.40997.

4.12 2-(3α,7α,12α-Trihydroxy-7β-methyl-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (17), 2-(3α,7α,12α-trihydroxy-7β-methyl-5β-cholano -24-amido)-2-methyl-1-propanol (21)

MeMgI in Et_2O (2 M, 6 mL, 12 mmol); **5** (0.289 g, 0.63 mmol) in THF (13 mL); reaction time 20 min; NH_4Cl (10 mL); EtOAc (5x10 mL); flash chromatography: CH_2Cl_2/MeOH 9:1. Compound **17** was obtained as a white needle crystal (mp. 105 °C crystalized from acetone, lit.[19] mp. 103–104.5 °C) in the yield of 0.193 g (63%), and **21** in the form of oil in yield of 0.019 g (6%). Rf (CH_2Cl_2/MeOH 92:8) for **17** 0.20, and for **21** 0.10.

Spectroscopic data for compound **17**:

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.61 (s, 3H, H-18), 0.78 (s, 3H, H-19), 0.95 (d, *J*=6 Hz, 3H, H-21), 1.09 (s, 3H, CH_3 on C-7), 1.14 (s, 6H, 2xCH_3 on C-4'), 3.21 (m, 1H, H-3), 3.57 (s, 1H, OH on C-7), 3.76 (s, 1H, H-12), 3.83 (s, 2H, H-5'), 4.10 (d, *J*=3.1 Hz, 1H, OH on C-12), 4.33 (d, *J*=3.9 Hz, 1H, OH on C-3).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 13.1 (C-18), 17.6 (C-21), 23.1 (C-19), 24.9, 27.4, 28.1, 28.7 and 28.8 (2xCH_3 on C-4'), 28.9, 29.5, 30.7, 32.3, 33.7 (CH_3 on C-7), 34.4, 35.3, 36.1, 38.6, 42.4, 42.7, 43.6, 45.0, 45.0, 47.5 (C-13), 67.1 (C-4'), 70.8, 70.9, 71.4 (C-7), 78.4 (C-5'), 165.3 (C-2').

Spectroscopic data for compound **21**:

IR (cm^{-1}): 3369, 2930, 1649, 1549.

^{1}H NMR (400 MHz, $CDCl_3$) δ= 0.70 (s, 3H, H-18), 0.86 (s, 3H, H-19), 1.01 (d, *J* = 6 Hz, 3H, H-21), 1.22 (s, 3H, CH_3 on C-7), 1.29 (s, 6H, 2xCH_3 on amide), 3.45 (m, 1H, H-3), 3.57 (s, 2H, CH_2 on amide), 3.94 (s, 1H, H-12), 6.15 (s, 1H, NH).

^{13}C NMR (101 MHz, $CDCl_3$) δ= 12.8 (C-18), 17.6 (C-21), 22.5 (C-19), 24.6 and 24.5 (2xCH_3 on amide), 27.5, 27.8, 28.6, 28.8, 30.2, 31.6, 33.1, 33.3 (CH_3 on C-7), 34.3, 35.0, 35.6, 38.3, 41.9, 42.5, 43.2, 44.5, 44.7, 47.6, 55.9, 70.7 (C-7), 71.8 (CH_2 on amide), 72.6 (C-3), 73.3 (C-12), 175.2 (C-24).

HRMS (TOF) *m/z*: calculated for $C_{29}H_{51}NO_5$ $[M+H]^+$ 494.3840, found 494.3843.

4.13 2-(3α,7α,12α-Trihydroxy-7β-ethyl-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (18)

EtMgBr in Et_2O (3 M, 3 mL, 9 mmol); **5** (0.091 g, 0.2 mmol) in THF (2 mL); reaction time 15 min; NH_4Cl (8 mL); EtOAc (5x10 mL); flash chromatography: acetone/EtOAc 1:3. Compound **18** was obtained as an oil in the yield of 0.072 g (74%). Rf (CH_2Cl_2/MeOH 9:1) 0.29.

IR (cm^{-1}): 3391, 2964, 2935, 2873, 1724, 1663, 1462, 1366, 1271, 1159, 1086, 1038, 996, 933, 912, 735.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.73 (s, 3H, H-18), 0.84 (s, 3H, H-19), 0.87 (t, *J*=7 Hz, 3H, CH_3 ethyl chain), 1.03 (d, *J*=6 Hz, 3H, H-21), 1.26 (s, 6H, 2xCH_3 on C-4'), 3.47 (m, 1H, H-3), 3.90 (s, 2H, H-5'), 3.94 (s, 1H, H-12).

^{13}C NMR (101 MHz, $CDCl_3$) δ = 9.5 (CH_3 ethyl chain), 12.9 (C-18), 17.5 (C-21), 22.4 (C-19), 25.0, 26.4, 27.7, 28.4 and 28.4 (2xCH_3 on C-4'), 28.8, 29.7, 30.2, 32.0, 34.1, 35.3, 35.7, 36.9, 38.6, 38.7, 39.9, 41.6, 42.7, 45.1, 47.9, 66.7 (C-4'), 71.7 (C-3), 72.4 (C-12), 75.5 (C-7), 78.9 (C-5'), 166.8 (C-2').

HRMS (TOF) *m/z*: calculated for $C_{30}H_{51}NO_4$ $[M+H]^+$ 490.38909, found 490.38929.

4.14 2-(3α,7α,12α-Trihydroxy-7β-butyl-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (19), 2-(3α,7α,12α-trihydroxy-7β-butyl-5β-cholano-24-amido)-2-methyl-1-propanol (22)

Procedure A: BuMgCl in Et_2O (2 M, 75 mL, 0.15 mol); **5** (3.0 g, 6.52 mmol) in THF (50 mL); reaction time 60 min; NH_4Cl (100 mL); EtOAc (5x30 mL); flash chromatography: acetone/EtOAc 3:1, then CH_2Cl_2/MeOH 9:1. Compound **19** was obtained as a colorless oil in the yield of 2.41 g (79% in regard to reacted **5**) and compound **22** (0.263 g, 8% in regard to reacted **5**), and regenerated **5**, 0.290 g. Rf (EtOAc/acetone 1:3) for **19** 0.41, for **22** 0.35.

Procedure B: BuMgCl in Et_2O (2 M, 0.2 mL, 0.4 mmol); **5** (0.064 g, 0.14 mmol) in THF (1.6 mL); heated to reflux 2 h; NH_4Cl (10 mL); EtOAc (5x10 mL); flash chromatography same as in procedure A. Compound **19** was obtained as an oil in the yield of 0.019 g (33% in regard to reacted **5**) and compound **22** (0.015 g, 20% in regard to reacted **5**), and regenerated **5**, 0.012 g.

Spectroscopic data for compound **19**:

IR (cm^{-1}): 3370, 2957, 2930, 2868, 1663, 1463, 1366, 1081, 908, 734.

^{1}H NMR (500 MHz, DMSO-*d*6) δ = 0.62 (s, 3H, H-19), 0.75 (s, 3H, H-18), 0.83 (t, *J*=7 Hz, 3H, CH_3 butyl chain), 0.94 (d, *J*=6 Hz, 3H, H-21), 1.14 (s, 6H, 2xCH_3 on C-4'), 3.21 (m, 1H, H-3), 3.40 (s, 1H, OH on C-7), 3.74 (d, *J*=3 Hz, 1H, C-12), 3.83 (s, 2H, H-5'), 4.09 (d, *J*=4 Hz, 1H, OH on C-12), 4.32 (d, *J*=4 Hz, 1H, OH on C-3).

^{13}C NMR (125 MHz, DMSO-*d*6) δ = 12.7 (C-18), 14.1 (CH_3 butyl chain), 17.1 (C-21), 22.6 (C-19), 23.0, 24.4, 25.7, 27.0, 27.4, 28.2 and 28.3 (2xCH_3 on C-4'), 28.4, 29.2, 30.2, 31.8, 33.7 (C-10), 34.8, 35.6, 38.4, 40.1, 41.6, 42.6, 44.3, 44.4, 47.2 (C-13), 66.6 (C-4'), 70.4 (C-3), 70.4 (C-12), 73.1 (C-7), 77.9 (C-5'), 164.8 (C-2').

HRMS (TOF) *m/z*: calculated for $C_{32}H_{55}NO_4$ $[M+H]^+$ 518.42039, found 518.41963.

Spectroscopic data for compound **22**:

IR (cm^{-1}): 3369, 2932, 2869, 1651, 1552, 1456, 1378, 1266, 1181, 1102, 1074, 1035, 994, 738, 703.

^{1}H NMR (250 MHz, $CDCl_3$) δ = 0.69 and 0.81 (2s, 2x3H, H-18 and H-19), 0.87 (t, *J*=6 Hz, 3H, CH_3 butyl chain), 0.99 (d, *J*=5 Hz, 3H, H-21), 1.27 (s, 6H, 2xCH_3 amide), 3.42 (bs, 1H, OH on C-7), 3.53 (s, 2H, CH_2 amide), 3.88 (s, 1H, H-12), 4.00 (bs, 1H, OH on C-12), 5.45 (bs, 1H, OH on C-3), 6.44 (s, 1H, NH).

^{13}C NMR (62.5 MHz, CDCl3) δ = 12.8 (CH_3), 14.1 (CH_3), 17.5 (CH_3), 22.4 (CH_3), 23.2, 24.3 (CH_3 amide), 24.4 (CH_3 amide), 26.2, 27.3, 27.6, 28.7, 29.6, 30.1, 31.5, 33.0, 34.0, 35.0, 35.6, 38.5, 39.5, 40.3, 41.6, 42.7, 44.4, 44.4, 47.7, 55.6 (qC amide), 70.5 (CH_2 amide), 71.6, 72.5, 75.3 (C-7), 175.1 (C-24).

HRMS (TOF) *m/z*: calculated for $C_{32}H_{57}NO_5$ $[M+Na]^+$ 558.41290, found 558.41193.

4.15 2-(3α,7α,12α-Trihydroxy-7β-octyl-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (20), 2-(3α,7α,12α-trihydroxy-7β-octyl-5β-cholano-24-amido)-2-methyl-1-propanol (23)

Procedure A: OctMgBr in Et_2O (2 M, 32 mL, 64 mmol); **5** (1.031 g, 2.24 mmol) in THF (17 mL); reaction time 1 h; NH_4Cl (50 mL); EtOAc (5x20 mL); flash chromatography: acetone/EtOAc 2:3, then CH_2Cl_2/MeOH 93:7. Compound **20** was obtained as an oil in the yield of 0.720 g (69% in regard to reacted **5**) and regenerated **5**, 0.196 g. Rf (CH_2Cl_2/MeOH 9:1) for **20** 0.28.

Procedure B: OctMgBr in Et_2O (2 M, 0.2 mL, 0.4 mmol); **5** (0.069 g, 0.15 mmol) in THF (1.7 mL); heated to reflux 2 h; NH_4Cl (20 mL); EtOAc (5x10 mL); flash chromatography same as in procedure A. Compound **20** was obtained as an colorless oil in the yield of 0.016 g (18% in regard to reacted **5**) and compound **23** (0.022 g, 26% in regard to reacted **5**), and regenerated **5**, 0.012 g. Rf (CH_2Cl_2/MeOH 9:1) for **23** 0.40.

Spectroscopic data for compound **20**:

IR (cm^{-1}): 3370, 2925, 2855, 1726, 1653, 1553, 1464, 1378, 1263, 1174, 1082, 1037, 941, 911, 731, 610.

^{1}H NMR (500 MHz, DMSO-*d*6) δ = 0.62 (s, 3H, H-18), 0.75 (s, 3H, H-19), 0.86 (t, *J*=7 Hz, 3H, CH_3 octyl chain), 0.95 (d, *J*=6 Hz, 3H, H-21), 1.14 (s, 6H, 2xCH_3 on C-4'), 3.21 (m, 1H, H-3), 3.41 (s, 1H, OH on C-7), 3.75 (s, 1H, H-12), 3.83 (s, 2H, H-5'), 4.10 (d, *J*=3.5 Hz, 1H, OH on C-12), 4.33 (d, *J*=3.3 Hz, 1H, OH on C-3).

^{13}C NMR (125 MHz, DMSO-*d*6) δ = 13.1 (C-18), 14.4 (CH_3 octyl chain), 17.6 (C-21), 22.6, 23.1 (C-19), 24.8, 25.1, 26.2, 27.1, 27.9, 28.7, 28.8, 28.9, 29.1, 29.4, 29.7, 30.2, 30.6, 31.7, 32.3, 34.2, 35.3, 36.1, 38.9, 40.5, 42.1, 43.0, 44.9, 45.0, 47.7 (C-13), 67.1 (C-4'), 70.8 (C-3), 70.9 (C-12), 73.6 (C-7), 78.4 (C-5'), 165.3 (C-2').

HRMS (TOF) *m/z*: calculated for $C_{36}H_{63}NO_4$ $[M+H]^+$ 574.48299, found 574.48370.

Spectroscopic data for compound **23**:

IR (cm^{-1}): 3369, 2927, 2857, 1648, 1550, 1465, 1378, 1073, 1035, 758.

^{1}H NMR (250 MHz, $CDCl_3$) δ = 0.62 (s, 3H, H-18), 0.75 (s, 3H, H-19), 0.86 (t, *J*=7 Hz, 3H, CH_3 octyl chain), 0.94 (d, *J*=6 Hz, 3H, H-21), 1.16 (s, 6H, 2xCH_3 amide), 3.22 (m, 1H, H-3), 3.37 (d, *J*=6 Hz, 2H, CH_2 amide), 3.40 (s, 1H, OH on C-7), 3.74 (s, 1H, H-12), 4.07 (d, *J*=4 Hz, 1H, OH on C-12), 4.33 (d, *J*=4 Hz, 1H, OH on C-3), 4.91 (t, *J*=6 Hz, 1H, OH amide), 7.23 (s, 1H, NH).

^{13}C NMR (62.5 MHz, $CDCl_3$) δ = 13.2 (C-18), 14.4 (CH_3 octyl chain), 17.8 (C-21), 22.6, 23.1 (C-19), 24.2 (2xCH_3 amide), 25.1, 26.2, 27.9, 28.9, 29.1, 29.4, 29.7, 30.2, 30.6, 31.7, 32.2, 33.7, 34.2, 35.5, 36.1, 38.9, 40.5, 42.1, 43.0, 45.0, 47.6, 54.6, 55.4, 68.2 (CH_2 amide), 70.8 (C-3), 70.9 (C-12), 73.6 (C-7), 173.4 (C-24).

HRMS (TOF) *m/z*: calculated for $C_{36}H_{65}NO_5$ $[M+H]^+$ 592.49355, found 592.49248.

4.16 3α,7α,12α-Trihydroxy-12β-methyl-5β-cholanic acid (24)

MeMgI in Et_2O (2 M, 5 mL, 10 mmol); **2** (0.197 g, 0.49 mmol) in THF (12.5 mL); reaction time 90 min; NH_4Cl (30 mL); EtOAc (5x10 mL); flash chromatography: CH_2Cl_2/MeOH 9:1 with addition of glacial acetic acid 0.05 mL/10 mL of eluent). Compound **24** was obtained as a colorless oil in yield of 0.056 g (27%). Rf (CH_2Cl_2/MeOH 9:1) 0.44.

IR (cm^{-1}): 3401, 2926, 1707, 1454.

^{1}HNMR (400 MHz, DMSO-*d*6) δ = 0.67 (s, 3H, H-18), 0.80 (s, 3H, H-19), 0.89 (d, *J*=7 Hz, 3H, H-21), 1.13 (s, 3H, CH_3 on C-12), 3.18 (m, 1H, H-3), 3.62 (s, 1H, H-7), 3.86 (s, 1H, OH on C-12), 4.00 (d, *J*=3 Hz, 1H, OH on C-7), 4.35 (s, 1H, OH on C-3).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 12.7 (C-18), 21.1, 21.8 (C-21), 23.1, 23.2, 27.5 (CH_3 on C-12), 28.3, 28.8, 30.9 (C-6), 32.3 (C-20), 33.3, 34.9, 35.4, 35.7, 36.8 (C-11), 39.0 (C-8), 40.0, 42.0 (C-5), 42.5 (C-14), 47.6 (C-17), 49.5 (C-13), 66.7 (C-7), 70.9 (C-3), 73.0 (C-12), 175.3 (C-24).

HRMS (TOF) *m/z*: calculated for $C_{25}H_{42}O_5$ $[M+Na]^+$ 445.2924, found 445.2921.

4.17 2-(3α,7α,12α-Trihydroxy-12β-methyl-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (25)

MeMgI in Et_2O (2 M, 7 mL, 14 mmol); **6** (0.303 g, 0.66 mmol) in THF (13 mL); reaction time 30 min; NH_4Cl (20 mL); EtOAc (5x7 mL); flash chromatography: CH_2Cl_2/MeOH 92:8. Compound **25** was obtained as a colorless oil in the yield of 0.166 g (53%). Rf (CH_2Cl_2/MeOH 92:8) 0.43.

IR (cm^{-1}): 3391, 2969, 2932, 1662, 1463.

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.65 (s, 3H, H-18), 0.80 (s, 3H, H-19), 0.91 (d, *J*=7 Hz, 3H, H-21), 1.14 (s, 9H, 2xCH_3 on C-4' and CH_3 on C-12), 3.18 (m, 1H, H-3), 3.62 (s, 1H, H-7), 3.82–3.86 (more signals, 3H, H-5' and OH on C-12), 3.96 (d, *J*=3 Hz, 1H, OH on C-7), 4.32 (d, *J*=4 Hz, 1H, OH on C-3).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 12.7 (C-18), 21.2, 21.8, 23.1, 23.3, 24.2, 26.4, 27.6, 28.3, 28.8 (2xCH_3 on C-4'), 29.8, 30.9, 31.8, 34.9, 35.4, 35.7, 36.7, 39.0, 42.0, 42.5, 47.5, 49.5, 66.7 (C-7), 67.1 (C-4'), 70.9 (C-3), 73.0 (C-12), 78.4 (C-5'), 164.9 (C-2').

HRMS (TOF) *m/z*: calculated for $C_{29}H_{49}NO_4$ $[M+Na]^+$ 498.3554, found 498.3550.

4.18 2-(3α,7α,12α-Trihydroxy-12β-ethyl-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (26), 2-(3α,7α,12α-trihydroxy-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (27)

EtMgBr in Et_2O (3 M, 2.2 mL, 6 mmol); **6** (0.122 g, 0.27 mmol) in THF (4 mL); reaction time 2 h; NH_4Cl (10 mL); EtOAc (5x5 mL); flash chromatography: EtOAc/acetone 3:2, EtOAc/acetone 3:1, CH_2Cl_2/MeOH

98:2). Compound **26** was obtained as an colorless oil in the yield of 0.062 g (48%), and compound **27** in the yield of 0.021 g (17%, mp 104 °C recrystallised from acetone as a white needle crystals, lit.[20] mp. 104 °C). Rf (CH_2Cl_2/MeOH 9:1) of **26** 0.30, and of **27** 0.21.

Spectroscopic data for compound **26**:

IR (cm^{-1}): 3392, 2961, 2931, 2871, 1663, 1463, 1366, 1275, 1115, 1079, 1004, 940, 910, 737, 611.

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.66 (s, 3H, H-18), 0.80 (s, 3H, H-19), 0.84 (t, *J*=7 Hz, 3H, CH_3 ethyl chain), 0.89 (d, *J*=7 Hz, 3H, H-21), 1.14 (s, 3H, CH_3 on C-4'), 1.15 (s, 3H, CH_3 on C-4'), 3.20 (m, 1H, H-3), 3.63 (s, 1H, H-7), 3.69 (s, 1H, OH on C-12), 3.80 (d, *J*=8 Hz, 1H, H-5'), 3.85 (d, *J*=8 Hz, 1H, H-5'), 3.96 (d, *J*=3 Hz, 1H, OH on C-7), 4.33 (d, *J*=4 Hz, 1H, OH on C-3).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 8.1 (CH_3 ethyl chain), 13.1 (C-18), 21.3, 21.6 (C-21), 23.1 (C-19), 23.4, 26.4, 27.9, 28.8 (2xCH_3 on C-4'), 29.6, 30.3, 30.7, 30.8, 32.3, 35.2, 35.4, 35.7, 39.1, 40.0, 42.0, 42.7, 47.1, 50.3, 66.9 (C-7), 67.1 (C-4'), 70.9 (C-3), 75.0 (C-12), 78.4 (C-5'), 164.9 (C-2').

HRMS (TOF) *m/z*: calculated for $C_{30}H_{51}NO_4$ $[M+H]^+$ 490.38909, found 490.38809.

Spectroscopic data for compound **27**:

IR (cm^{-1}): 3370, 2931, 2867, 1727, 1662, 1463, 1366, 1276, 1195, 1119, 1078, 1046, 981, 950, 914, 754.

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.58 (s, 3H, CH_3, H-18), 0.81 (s, 3H, CH_3, H-19), 0.94 (d, *J*=6 Hz, 3H, H-21), 1.14 (s, 6H, 2xCH_3 on C-4'), 3.18 (m, 1H, H-3), 3.61 (s, 1H, H-7), 3.78 (s, 1H, H-12), 3.83 (s, 2H, H-5'), 4.02 (d, *J*=3 Hz, 1H, OH on C-7), 4.12 (d, *J*=3 Hz, 1H, OH on C-12), 4.34 (d, *J*=4 Hz, 1H, OH on C-3).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 12.7 (C-18), 17.5 (C-21), 23.1 (C-19), 23.3, 24.9, 26.7, 27.8, 28.7 and 28.8 (2xCH_3 on C-4'), 29.0, 30.8, 32.3, 34.8, 35.3, 35.4, 35.8, 39.9, 40.0, 41.8 (C-17), 42.0, 46.3 (C-13), 46.5, 66.7 (C-7), 67.1 (C-4'), 70.9 (C-3), 71.5 (C-12), 78.4 (C-5'), 165.4 (C-2').

HRMS (TOF) *m/z*: calculated for $C_{28}H_{47}NO_4$ $[M+H]^+$ 462.35779, found 462.35760.

4.19 2-(3α,7α,12α-Trihydroxy-12β-butyl-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (28)

BuMgCl in Et_2O (2 M, 6.4 mL, 12.8 mmol); **6** (0.115 g, 0.25 mmol) in THF (4.25 mL); reaction time 2 h, –20 °C, 3 h 0 °C; NH_4Cl (20 mL); EtOAc (5x10 mL); flash chromatography: acetone/EtOAc 1:2. Compound **28** was obtained as an oil in the yield of 0.063 g (49%) and compound **27** (0.022 g, 19%). Rf (CH_2Cl_2/MeOH 9:1) 0.62.

Spectroscopic data for compound **28**:

IR (cm^{-1}): 3391, 2957, 2931, 2870, 1663, 1462, 1365, 1267.

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.65 (s, 3H, H-18), 0.80 (s, 3H, H-19), 0.87–0.91 (more signals, 6H, CH_3 butyl chain and H-21), 1.14 and 1.15 (2s, 6H, 2xCH_3 on C-4'), 3.18 (s, 1H, H-3), 3.62 (s, 1H, H-7), 3.69 (s,

1H, OH on C-12), 3.79–3.86 (more signals, 2H, H-5'), 3.95 (d, *J*=3 Hz, 1H, OH on C-7), 4.31 (d, *J*=4 Hz, 1H, OH on C-3).

^{13}C NMR (101 MHz, DMSO-*d*6) δ= 13.1 (C-18), 14.7 (CH_3 butyl chain), 21.7, 21.7 (C-19), 23.1, 23.5, 23.9, 25.5, 26.5, 28.0, 28.7 and 28.9 (2xCH_3 on C-4'), 29.8, 30.8, 31.2, 32.4, 35.2, 35.4, 35.7, 38.3, 39.1, 42.0, 42.6, 47.0, 50.4, 55.4 (C-13), 66.9 (C-4'), 67.1 (C-7), 70.9 (C-3), 75.1 (C-12), 78.4 (C-5'), 165.0 (C-2').

HRMS (TOF) *m/z*: calculated for $C_{32}H_{55}NO_4$ $[M+H]^+$ 518.42039, found 518.41949.

4.20 2-(3α,7α,12α-Trihydroxy-12β-octyl-24-nor-5β-cholano-23-yl)-4,4-dimethyloxazoline (29)

OctMgBr in Et_2O (2 M, 6 mL, 12 mmol); **6** (0.104 g, 0.23 mmol) in THF (6 mL); reaction time 3 h; NH_4Cl (20 mL); EtOAc (5x10 mL); flash chromatography: CH_2Cl_2/MeOH 92:8. Compound **29** was obtained as a small white crystals (mp. 96 °C after crystallization from acetone/MeOH) in the yield of 0.057 g (67%). Rf (CH_2Cl_2/MeOH 9:1) 0.42.

IR (cm^{-1}): 3391, 2927, 2856, 1652, 1549, 1463, 1377.

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.65 (s, 3H, H-18), 0.79 (s, 3H, H-19), 0.85–0.91 (more signals, 6H, CH_3 octyl chain and H-21), 1.14 and 1.15 (2s, 6H, 2xCH_3 on C-4'), 3.18 (m, 1H, H-3), 3.62 (s, 1H, H-7), 3.69 (s, 1H, OH on C-12), 3.80-3.85 (more signals, 2H, H-5'), 3.95 (d, *J*=3 Hz, 1H, OH on C-7), 4.32 (d, *J*=4 Hz, 1H, OH on C-3).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 13.1 (C-18), 14.4 (CH_3 octyl chain), 21.6, 21.7 (C-21), 22.5, 23.1 (C-19), 23.3, 23.5, 26.5, 28.0, 28.7 and 28.9 (2xCH_3 on C-4'), 29.3, 29.7, 29.8, 30.8, 31.2, 31.7, 32.4, 35.2, 35.4, 35.7, 38.6, 39.1, 40.2, 42.0, 42.6, 47.0, 50.4 (C-13), 66.9 (C-4'), 67.1 (C-7), 70.9 (C-3), 75.1 (C-12), 78.4 (C-5'), 164.9 (C-2').

HRMS (TOF) *m/z*: calculated for $C_{36}H_{63}NO_4$ $[M+H]^+$ 574.48299, found 574.48158.

4.21 7α,12α-Dihydroxy-3-oxo[4-$^2H_1$]-5β-cholanic acid (3D)

EtMgBr in Et_2O (3 M, 2 mL, 6 mmol); **3** (0.114 g, 0.28 mmol) in THF (3 mL); reaction time 90 min; reaction was quenched with addition of D_2O (0.5 mL), mixed for 10 min and then AcOH (3 mL) was added; EtOAc (5x10 mL); flash chromatography: CH_2Cl_2/MeOH 93:7 with addition of glacial acetic acid 0.05 mL/10 mL of eluent). Compound **3D** (0.108 g; 95%) was obtained as a white needle crystal (mp. 158 °C recrystallized from H_2O/MeOH). Rf (CH_2Cl_2/MeOH with addition of glacial acetic acid 0.05 mL/10 mL of eluent) 0.58.

IR (cm^{-1}): 3435, 2938, 2865, 1707, 1268.

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.63 (s, 3H, H-18), 0.92-0.94 (more signals, 6H, H-19 and H-21), 3.69 (s, 1H, H-7), 3.83 (s, 1H, H-12), 4.19 (s, 1H, OH), 4.32 (s, 1H, OH).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 12.8 (C-18), 17.4 (C-21), 21.9 (C-19), 23.2, 27.0, 27.7, 29.1, 31.3, 31.3, 34.3, 34.9, 35.5, 36.7, 36.9, 41.8, 43.2, 45.5 (m, C-D), 46.3, 46.6, 55.4, 66.8 (C-7), 71.4 (C-12), 175.4 (C-24), 212.4 (C-3).

HRMS (TOF) *m/z*: calculated for $C_{24}H_{37}DO_5$ $[M+K]^+$ 446.24136, found 446.24155.

4.22 2-(7α,12α-Trihydroxy-3-oxo-5β-cholano-24-amido)-2-methyl-1-propanol (30)

BuMgCl in Et_2O (2 M, 2 mL, 4 mmol); **7** (0.071 g, 0.15 mmol) in THF (2 mL); after 2 h one more portion of BuMgCl was added (2 M, 2 mL, 4 mmol) and mixed for 2 h, and then reaction temperature was heated to the room temperature and mixed for 18 h more; NH_4Cl (10 mL); EtOAc (4x10 mL); flash chromatography: EtOAc/toluene/MeOH 7:2:1). Compound **30** was obtained as an oil in the yield of 0.006 g (8%). Rf (same solvent system as for flash chromatography) 0.2.

IR (cm^{-1}): 3401, 2925, 2865, 1705, 1652, 1548.

^{1}H NMR (400 MHz, DMSO-*d*6) δ = 0.62 (s, 3H, H-18), 0.92-0.94 (more signals, 6H, H-19 i H-21), 1.16 (s, 6H, 2×CH_3 amide), 3.69 (s, 1H, H-7), 3.82 (s, 2H, H-12), 4.16 (d, *J*=9 Hz, 1H, OH), 4.33 (d, *J*=3 Hz, 1H, OH), 4.91 (s, 1H, OH amide), 7.24 (s, 1H, NH).

^{13}C NMR (101 MHz, DMSO-*d*6) δ = 12.8 (C-18), 17.7 (C-21), 21.9, 23.2, 24.2 (2×CH_3 amide), 27.0, 27.7, 29.2, 32.2, 33.6, 34.3, 34.9, 35.6, 36.6, 36.9, 41.8, 43.2, 45.8, 46.3, 46.7, 54.6, 66.8, 68.2, 71.4, 173.4 (C-24), 212.3 (C-3).

HRMS (TOF) *m/z*: calculated for $C_{28}H_{47}NO_5$ $[M+H]^+$ 478.3527, found 478.3536.

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