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# Investigation of new acyloxy derivatives of cholic acid and their esters as drug absorption modifiers

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ABSTRACT

Skin penetration enhancers are used in the formulation of transdermal delivery systems for drugs that are otherwise not sufficiently skin-permeable. Intestinal absorption promoters/enhancers are used as excipients in oral formulations of poorly oral-bioavailable drugs. Series of fourteen acyloxy derivatives of 5β-cholic acid as potential drug absorption modifiers was generated by multistep synthesis. The synthesis of all newly prepared compounds is presented here. Structure confirmation of all generated compounds was accomplished by $^{1}$H NMR, $^{13}$C NMR, IR and MS spectroscopy methods. All the prepared compounds were analyzed using RP-TLC, and their lipophilicity ($R_M$) was determined. The hydrophobicity ($\log P$) and solubility ($\log S$) of the studied compounds were also calculated using two commercially available programs. All the target compounds were tested for their *in vitro* transdermal penetration activity and as potential intestinal absorption enhancers. The anti-proliferative activity of all the final compounds was also assessed against the human cancer cell lines: T-lymphoblastic leukemia cell line and the breast adenocarcinoma cell line. Their cytotoxicity was also evaluated against the normal human skin fibroblast cells. Two compounds showed anti-proliferative effect on cancer cells without affecting the growth of normal cells, which should be promising in potential development of new drugs. Most of the target compounds showed minimal anti-proliferative activity ($IC_{50} > 37$ μM), indicating they would have low cytotoxicity when administered as chemical absorption modifiers. The relationships between the lipophilicity and the chemical structure of the studied compounds as well as the relationships between their chemical structure and enhancement effects are discussed in this article.



## 1. Introduction

Development in the field of pharmaceutical administration has resulted in the discovery of highly sophisticated drug delivery systems that allow for the maintenance of a constant drug level in an organism. Contrary to these revolution biopharmaceutical/galenical results, it is estimated that about 40% of marketed active pharmaceutical ingredients and 60% of active new chemical entities identified in R&D screening programs employed by many pharmaceutical companies are poorly water soluble. These poorly soluble "modern" drugs are distinguished by incomplete absorption and low, erratic bioavailability as a result of either mostly intestinal absorption after oral administration or less commonly transdermal absorption after local application [1]. One of possibilities to modify/optimize drug unfavourable physico-chemical properties is to use absorption modifiers, e.g., bile acid derivatives [2–5].

Cholic acid is one of the most important human bile acids. Bile acid derivatives/analogues are an important class of compounds with a range of pharmacological activities. Bile acids could be easily modified by derivatisation of the functional groups on the steroid nucleus. Nontoxic bile acid/salt derivatives (as amphiphilic compounds) are used widely in drug formulations as excipients (intestinal absorption enhancers, promoters) and can influence gastrointestinal solubility, absorption and chemical/enzymatic stability of drugs [6–12]. Cholic acid derivatives were studied also as transdermal penetration enhancers [13–21]. The reason for their activity may be their specific features in solvation and self-assembly [22–28].

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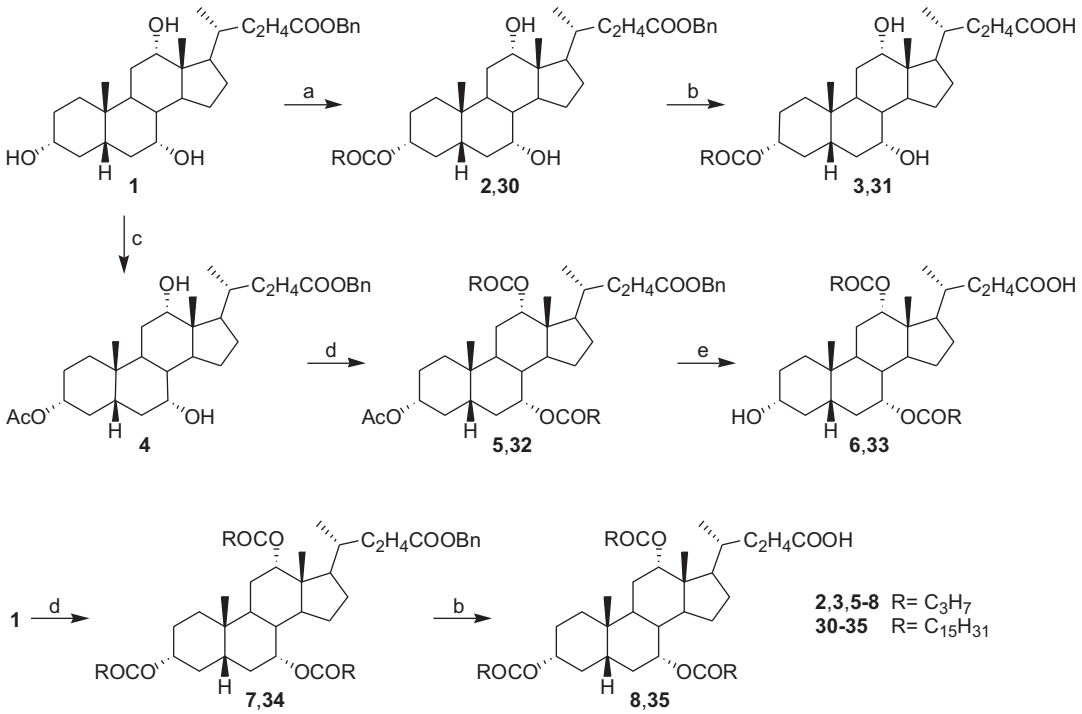



**Scheme 1.** Synthesis of target mono-, bis-, tris(butanoyl) **3**, **6**, **8** and mono-, bis-, tris(hexadecanoyl) **31**, **33**, **35** substituted compounds: (a) $C_3H_7COCl$ or $C_{15}H_{31}COCl$, DMAP, $CH_2Cl_2$; (b) $HCOONH_4$, Pd/C, MeOH; (c) $Ac_2O$, $KHCO_3$, toluene; (d) $C_3H_7COCl$ or $C_{15}H_{31}COCl$, BTEAC, $CaH_2$, toluene; (e) (i) NaOH, *i*-PrOH, (ii) AcOH, $CH_2Cl_2$.

Transdermal therapeutic systems are an excellent alternative to conventional pharmaceutical administration forms. However, the application of transdermal drug delivery faces the problem of insufficient or no penetration of active pharmaceutical substances through the skin, as the outermost layer of skin, namely the stratum corneum (SC), forms a strong barrier for most of exogenous substances including drugs. The barrier function of the SC is attributed to its multilayered wall-like structure, in which terminally differentiated keratin-rich epidermal cells (corneocytes) are embedded in an intercellular lipid-rich matrix [4,21].

Transdermal penetration enhancers are special pharmaceutical excipients that interact with skin components, to increase penetration of drugs to blood circulation after topical application. Numerous compounds of different chemical structures were evaluated as penetration enhancers and several possible mechanisms of action of enhancers have been hypothesized, but exact mechanisms have not been elucidated [21]. In spite of the extensive research in this field, chemical penetration enhancers have not reached their full potential in transdermal or topical systems so far. Transdermal chemical penetration enhancers are compounds which can partition into and interact with the SC constituents when incorporated into a transdermal formulation, thereby reducing the resistance of the skin to drug diffusion [21].

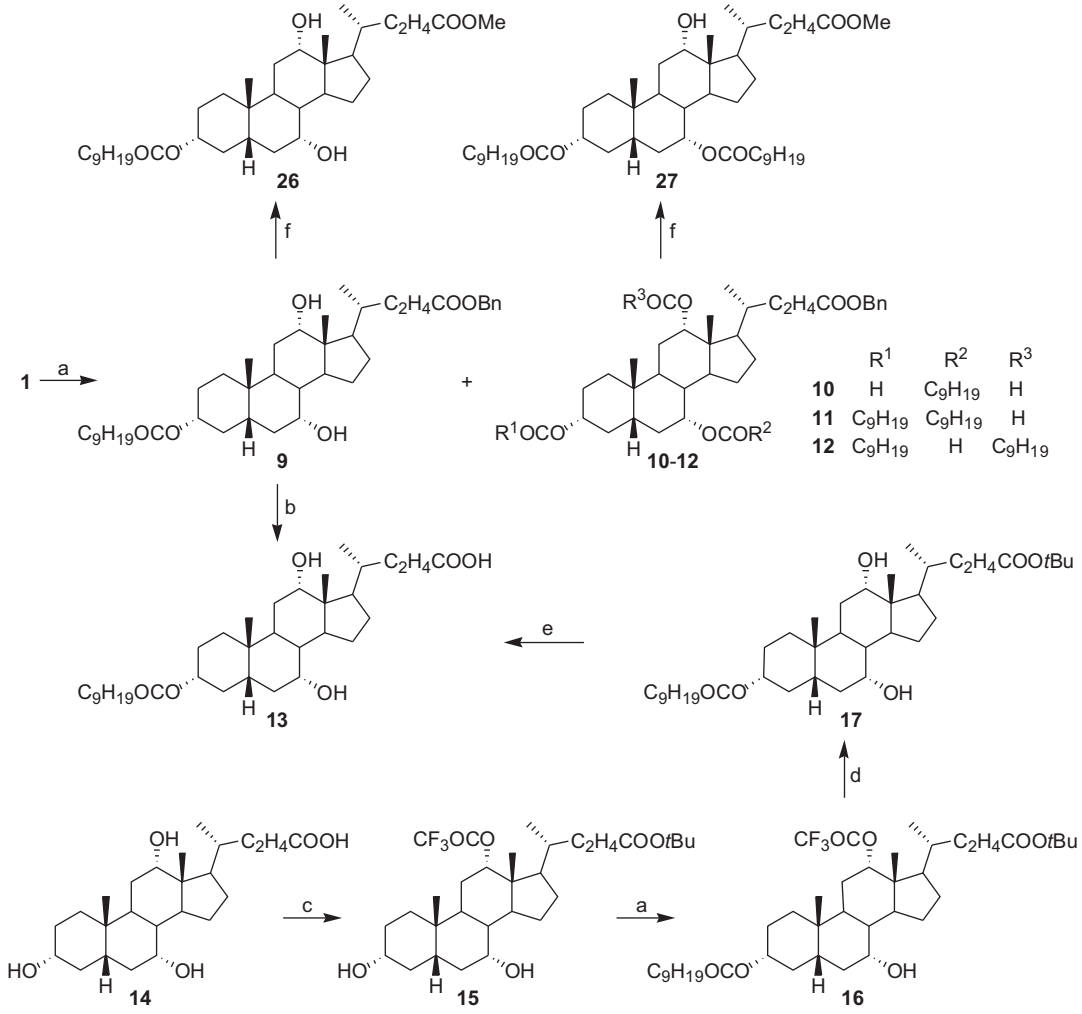
The traditional lipophilicity parameter, log *P*, is a well-known physico-chemical descriptor widely used in QSAR analysis. In some experimental studies of penetration enhancement, the lipophilicity (non-polarity) of enhancers was measured and the corresponding relationship between enhancer lipophilicity and penetration enhancement potency was investigated [21,29,30]. Therefore we have examined both the experimental lipophilicity $R_M$ (RP-TLC) data and calculated lipophilicity log *P* of all compounds in this article. The solubility (polarity) log *S* of the discussed compounds was calculated as well.

The multistep synthesis of a series of fourteen acyloxy derivatives of 5β-cholic acid with $C_4$, $C_{10}$ and $C_{16}$ linear acyl chains as novel transdermal chemical penetration enhancers and/or as intestinal drug absorption modifiers is described herein. Mono-, di- and tri-*O*-acylation of cholic acid were chosen for clarifying the correlations between the physico-chemical properties of the absorption/penetration enhancers of bile acid-type inducing the enhancement effect. Various acyl chain lengths as well as different degree and position of substitution of the compounds discussed in this paper impart specific solvation and surface features influencing structural modifications of the biological membranes. Primary *in vitro* screening of transdermal penetration activity of all the final synthesized compounds was performed using a Franz cell [31] and intestinal absorption enhancement activity was evaluated using PAMPA (parallel artificial membrane permeability assays) experiments [32,33]. All the discussed compounds were evaluated for their anti-proliferative activity against the T-lymphoblastic leukemia cell line and the breast adenocarcinoma cell line as well as also for their cytotoxicity against normal human skin fibroblast cells. The relationships between the lipophilicity/solubility and the chemical structure of the studied compounds as well as the relationships between their chemical structure and activity/enhancement effects (SAR) are discussed in this article.

## 2. Result and discussion

### 2.1. Chemistry

3α-Mono(acyloxy) derivatives **3**, **13**, **31** were prepared by the reaction of benzyl 3α,7α,12α-trihydroxy-5β-cholate (benzyl cholate, **1**) [34] and 4-dimethylaminopyridine (DMAP) with acyl chlorides under mild conditions followed by hydrogenolysis of protecting groups, see Schemes 1 and 2. 3α-Mono(decanoyloxy) derivative **13** was also prepared by modification of a synthetic pathway described by Bonar-Law et al., using 3α,7α,12α-trihydroxy-5β-cholic acid **14**, in which the 12α-hydroxyl was protected with a trifluoroacetyl group [35], see Scheme 2. Methyl 3α-decanoyloxy-7α,12α-dihydroxy-5β-cholate (**26**) was obtained by re-esterification of benzyl 3α-decanoyloxy-7α,12α-dihydroxy-5β-cholate (**9**) by methanol, see Scheme 2.




**Scheme 2.** Synthesis of target mono(decanoyl) substituted derivative **13** and mono- and 3α,7α-bis(decanoyl) substituted derivatives **26**, **27**: (a) $C_9H_{19}COCl$, DMAP, $CH_2Cl_2$;. (b) $HCOONH_4$, Pd/C, MeOH; (c) $(CF_3CO)_2O$, *t*-BuOH, THF; (d) MeOH, THF, $NH_3$(aq); (e) $CF_3COOH$, AcONa, $CH_2Cl_2$; (f) MeONa, MeOH, THF, AcOH.

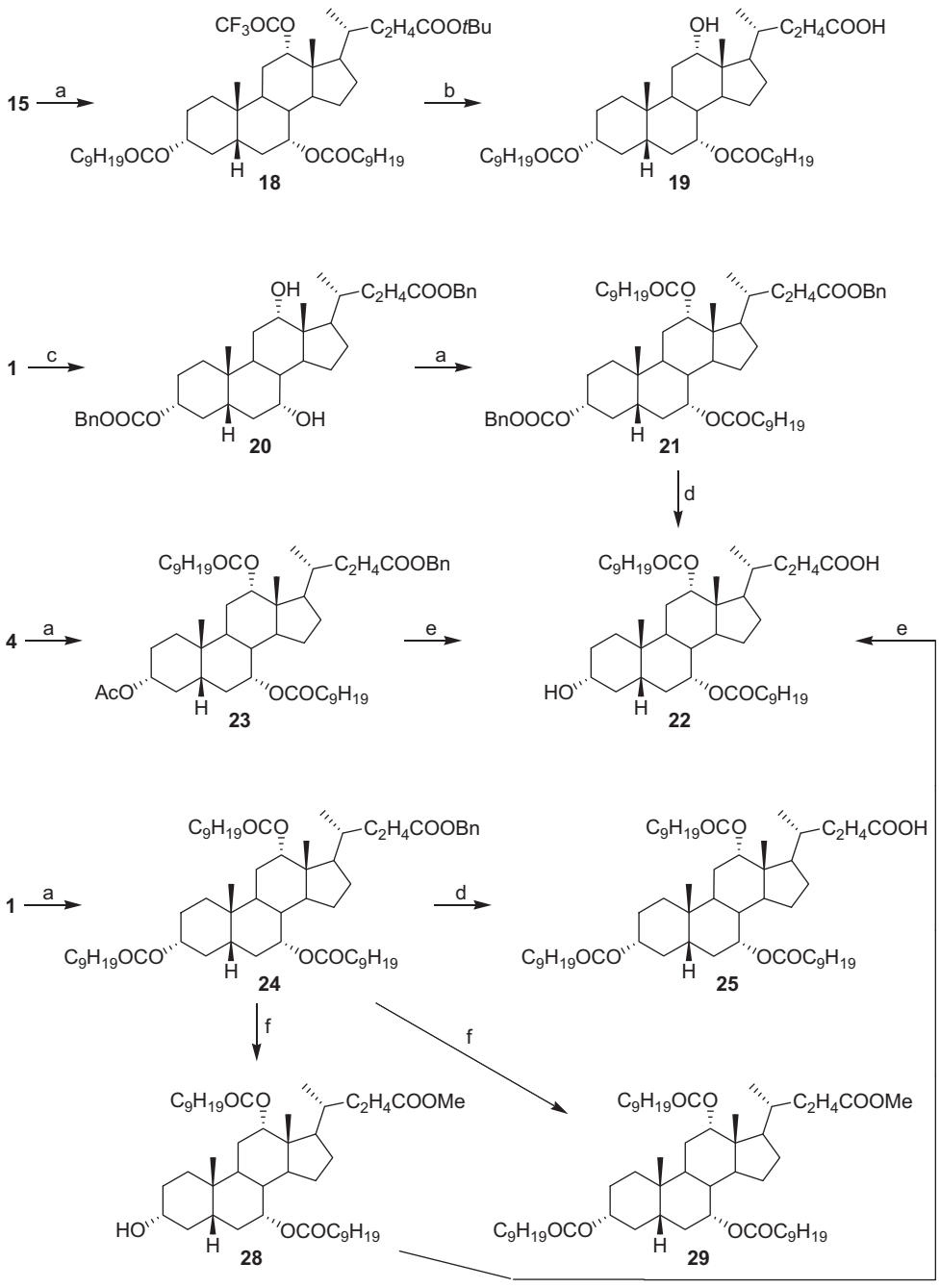
The preparation of 7α,12α-bis(acyloxy) derivatives **6**, **22**, **33** included the protection of 3α-hydroxy group of benzyl ester **1** by the acetyl group, according to Schwarz et al. [36], resulting in compound **4** and subsequent acylation of both free 7α,12α-dihydroxy moieties. Long-chain acyls were derived from fatty acids. Finally, the acetyl group was selectively cleaved by solvolysis in the presence of a base. Methyl 7α,12α-bis(decanoyloxy)-3α-hydroxy-5β-cholate (**28**) was prepared by mild hydrolysis and re-esterification of benzyl 3α,7α,12α-tris(decanoyloxy)-5β-cholate (**24**) by methanol, see Scheme 3. This method including the protection of 3α-hydroxy group in ester **1** by a benzyloxycarbonyl moiety was developed for the selective preparation of 7α,12α-bis(decanoyloxy)-3α-hydroxy-5β-cholic acid (**22**), see Scheme 3. The preparation of 7α,12α-bis(acyloxy) substituted derivatives was based on regioselective alcoholysis described by Wess et al. [37].

3α,7α-Bis(decanoyloxy) derivatives **19**, **27** were prepared by modification of the procedures used for preparation of 3α-mono(decanoyloxy) derivatives **13** and **26**, *i.e.* the protection of 12α-hydroxyl with trifluoroacetyl group (for compound **19**) or re-esterification of benzyl ester **12** (for compound **27**), see Schemes 2 and 3.

3α,7α,12α-Tris(acyloxy) derivatives **8**, **25**, **35** were prepared by the reaction of benzyl cholate **1** with acyl chlorides in the presence of benzyltriethylammonium chloride (BTEAC) as a phase transfer catalyst with subsequent hydrogenolysis of the protecting group. Methyl 3α,7α,12α-tris(decanoyloxy)-5β-cholate (**29**) was obtained by re-esterification of benzyl 3α,7α,12α-tris(decanoyloxy)-5β-cholate (**24**) by methanol, see Schemes 1 and 3.

### 2.2. Lipophilicity and solubility of the prepared compounds

Different lipophilicity descriptors such as $\log k_w$, $\log P$, $\log D$, etc. are used for structure–activity relations description and prediction. However, the algorithms used in their calculation do not take into account configuration specificity and sometimes even regiospecificity of steroidal derivatives. Hence, having in mind the importance of permeability and solubility (polarity) for biological activity [38], this study compares calculated $\log P$ and $\log S$ values with the related experimental parameter, the $R_M$ values of the final derivatives, as a measure of their lipophilicity. The $R_M$ values were determined by RP-TLC. Solubility was estimated using the software that is applied by many industrial companies, ACD/Solubility DB. This program calculates aqueous solubility values at any pH under the standard conditions (and zero ionic strength). The accuracy of calculations for simple structures is usually better than 0.2–0.5 logarithmic units. So, it is not derived from $\log P$ and takes into account not only the pH (solubility as a function of pH) but compares the fragmental estimations with experimental material from ca 6000 compounds databased. Nevertheless, it is important to note that all $\log P$ and $\log S$ values calculated using various programs should be




**Scheme 3.** Synthesis of target bis-, tris(decanoyl) substituted derivatives **19**, **22**, **25** and 7α,12α-bis-, tris(decanoyl) substituted derivatives **28**, **29**: (a) $C_9H_{19}COCl$, BTEAC, $CaH_2$, toluene; (b) *i)* MeOH, THF, $NH_3$(aq), *ii)* $CF_3COOH$, AcONa, $CH_2Cl_2$; (c) BnOCOCl, pyridine; (d) $HCOONH_4$, Pd/C, MeOH; (e) (i) NaOH, *i*-PrOH, (ii) AcOH, $CH_2Cl_2$; (f) MeONa, MeOH, THF, AcOH.

understood as approximate. The results are shown in Table 1 and illustrated in Fig. 1.

As expected, compound **35** possessed the highest lipophilicity. Contrary to all expectations and calculated log*P* and log*S* data, compound **6** showed the lowest lipophilicity. Generally, it can be stated that mono(acyloxy) substituted derivatives expressed the lowest lipophilicity, except for compound **6**, and tris(acyloxy) substituted compounds showed the highest lipophilicity within the individual series. The calculated log*P* data and the determined $R_M$ values correspond to the expected trend in lipophilicity, increasing within the series of the evaluated compounds (butanoyl < decanoyl < decanoyl-Me ester < hexadecanoyl derivatives). This dependence is approximately linear. The $R_M$ data corresponds to the lipophilicity within the series of the discussed compounds. For most of the discussed compounds a logical dependence can be found: polarity/solubility (log*S* data) decreases with a lipophilicity increase, see Fig. 1.

Interesting anomalies were observed for 3α,7α- and 7α,12α-bis(acyloxy) derivatives **19**/**22** and **27**/**28**: 7α,12α-bis(acyloxy) substituted compounds unexpectedly expressed much lower lipophilicity and much higher polarity than 3α,7α-bis(acyloxy) substituted derivatives. They could be probably explained by different solvation and other types of non-covalent interactions (especially van der Waals interactions, hydrogen bonds, dipole-dipole interactions) of hydroxyl moieties in the $C_{(3)}$ and $C_{(12)}$ positions of the steroidal skeleton. This fact is extremely important

**Table 1**
Comparison of determined $R_M$ with calculated lipophilicity (log *P*) and solubility (log *S*) values.

| Compound | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R_M$ | log *P* (ACD/log *P* DB) | log *S* (ACD/log *S* DB) |
|---|---|---|---|---|---|---|---|
| **3** | $C_3H_7$ | H | H | H | −0.51 | 4.87 | −2.96 |
| **6** | H | $C_3H_7$ | $C_3H_7$ | H | −0.53 | 6.85 | −3.42 |
| **8** | $C_3H_7$ | $C_3H_7$ | $C_3H_7$ | H | −0.34 | 8.84 | −4.71 |
| **13** | $C_9H_{19}$ | H | H | H | −0.20 | 7.92 | −4.21 |
| **19** | $C_9H_{19}$ | $C_9H_{19}$ | H | H | 0.08 | 12.97 | −6.42 |
| **22** | H | $C_9H_{19}$ | $C_9H_{19}$ | H | −0.12 | 12.95 | −6.42 |
| **25** | $C_9H_{19}$ | $C_9H_{19}$ | $C_9H_{19}$ | H | 0.44 | 18.01 | −8.55 |
| **26** | $C_9H_{19}$ | H | H | $CH_3$ | −0.04 | 8.52 | −7.05 |
| **27** | $C_9H_{19}$ | $C_9H_{19}$ | H | $CH_3$ | 0.29 | 13.56 | −9.18 |
| **28** | H | $C_9H_{19}$ | $C_9H_{19}$ | $CH_3$ | 0.10 | 13.56 | −9.18 |
| **29** | $C_9H_{19}$ | $C_9H_{19}$ | $C_9H_{19}$ | $CH_3$ | 0.76 | 18.61 | −11.22 |
| **31** | $C_{15}H_{31}$ | H | H | H | 0.15 | 10.98 | −5.76 |
| **33** | H | $C_{15}H_{31}$ | $C_{15}H_{31}$ | H | 0.52 | 19.08 | −8.70 |
| **35** | $C_{15}H_{31}$ | $C_{15}H_{31}$ | $C_{15}H_{31}$ | H | 1.67 | 27.18 | −10.74 |
| Cholic acid | – | – | – | – | −0.55 | 2.88 | −1.39 |

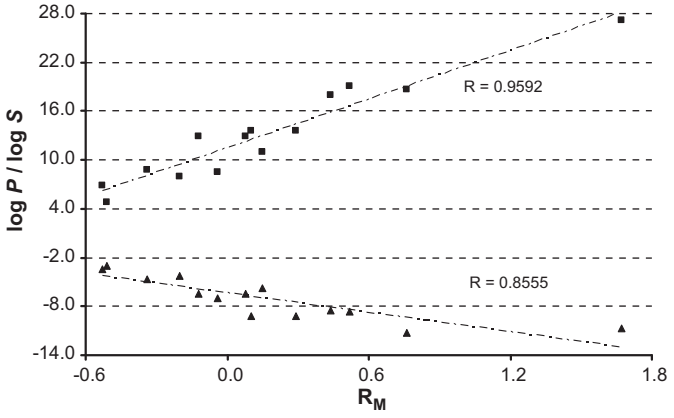



**Fig. 1.** Comparison of log *P* and log *S* values (lipo/hydrophilicity properties) computed using two programs with the determined $R_M$ values.

for the absolutely different enhancer activity of these bis(acyloxy) substituted derivatives, see below. Some differences between the determined lipophilicity and predicted lipophilicity/solubility data were also observed for compounds **26**, **31** and **29**. All these unexpected differences influence penetration activity.

Generally, it could be concluded that the prediction power of using either experimental $R_M$ or calculated log *S* or log *P* for extrapolation of transport modifications may be a good tool for searching potential transdermal penetration modifiers.

### *2.3. In vitro screening of transdermal penetration-enhancing activity*

The penetration enhancement activity of the prepared compounds was evaluated using theophylline as a model penetrant and propylene glycol/water 1:1 (v/v) as a donor vehicle. Theophylline was used as a model drug of medium polarity (log *P* −0.06; log $D_8$ −0.05) [39,40], as it has been extensively studied in transdermal penetration experiments [41,42]. Most of the studies involved the use of propylene glycol (PG) or its mixture with water or ethanol as a donor vehicle. Previous studies have indicated that PG by itself (or a PG/water co-solvent system) does not interfere with membranes, but rather exhibits a synergistic effect in combination with other penetration enhancers [43–45]. Porcine ear skin was selected for initial evaluation of enhancement activity of prepared compounds as this tissue is a suitable *in vitro* model of human skin [46,47]. Porcine skin has been shown to be histologically and biochemically similar to human skin, therefore full-thickness pig ear skin has been used in numerous percutaneous absorption studies [48]. Nevertheless, for testing of hydrophobic penetrants, dermatomed skin has been recommended [49]. The skin permeation experiments were performed using static Franz diffusion cells [31].

As it could be expected, the presented transdermal penetration enhancement results of the target compounds and the data obtained using PAMPA method (see below) are different (see Table 2, where the enhancement ratios (ERs) are presented). The principal difference is probably due to different properties of the transport "membrane"; the PAMPA diaphragm is an artificial model of cell lipid bilayer "simulating" intestine wall, whereas the

**Table 2**
ERs of the prepared target compounds and *in vitro* anti-proliferative activity/cytotoxicity of tested compounds on normal and cancer cell lines. $IC_{50}$ (μmol/L) assessed by Calcein AM assay of surviving cells. ERs data for skin and anti-proliferative activity of the compounds are expressed as mean ± SD ($n$ = 3 experiments), ERs data for PAMPA are expressed as mean ± SD ($n$ = 4 experiments). Means followed by different letters are significantly different at $P$ = 0.05.

| Compound | ERs | | Cell lines $IC_{50}$ (μmol/L) | | |
|---|---|---|---|---|---|
| | Skin | PAMPA | CEM | MCF7 | BJ |
| **3** | $1.67 \pm 0.10^{fg}$ | $1.08 \pm 0.04^{g}$ | >37 | >37 | >37 |
| **6** | $1.57 \pm 0.11^{ef}$ | $1.18 \pm 0.04^{h}$ | >37 | >37 | >37 |
| **8** | $1.67 \pm 0.08^{fg}$ | $1.16 \pm 0.04^{h}$ | $33.0 \pm 0.8^{c}$ | >37 | >37 |
| **13** | 2.13 ± 0.13i | $0.35 \pm 0.04^{a}$ | $27.3 \pm 1.9^{b}$ | $34.6 \pm 0.9^{b}$ | >37 |
| **19** | $1.30 \pm 0.13^{cd}$ | $0.60 \pm 0.05^{c}$ | >37 | >37 | >37 |
| **22** | $1.24 \pm 0.14^{bc}$ | $1.03 \pm 0.05^{g}$ | $16.4 \pm 4.3^{a}$ | $21.8 \pm 0.5^{a}$ | 14.8 ± 2.1 |
| **25** | $2.09 \pm 0.14^{i}$ | $0.73 \pm 0.05^{d}$ | >37 | >37 | >37 |
| **26** | $1.83 \pm 0.12^{gh}$ | $0.73 \pm 0.05^{d}$ | >37 | >37 | >37 |
| **27** | $1.08 \pm 0.12^{ab}$ | $0.95 \pm 0.04^{f}$ | >37 | >37 | >37 |
| **28** | $0.92 \pm 0.14^{a}$ | $1.21 \pm 0.04^{h}$ | >37 | >37 | >37 |
| **29** | $1.45 \pm 0.12^{de}$ | $0.93 \pm 0.05^{f}$ | >37 | >37 | >37 |
| **31** | $2.01 \pm 0.11^{hi}$ | $0.52 \pm 0.04^{b}$ | $25.7 \pm 3.8^{b}$ | $30.4 \pm 2.5^{b}$ | >37 |
| **33** | $1.96 \pm 0.11^{hi}$ | $0.86 \pm 0.04^{e}$ | >37 | >37 | >37 |
| **35** | $2.01 \pm 0.11^{hi}$ | $0.80 \pm 0.04^{e}$ | >37 | >37 | >37 |

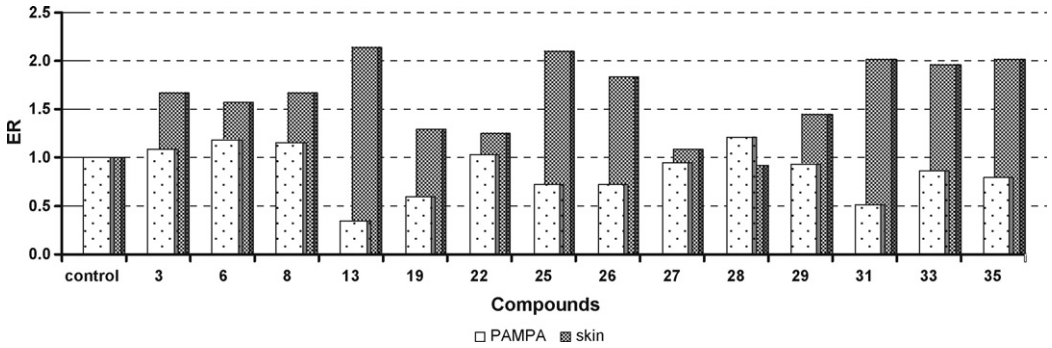



**Fig. 2.** ERs through porcine ear skin and PAMPA of the prepared target compounds. Control experiments used theophylline in the donor vehicle without any enhancer. ERs data for skin were calculated from 3 experiments, ERs data for PAMPA were calculated from 4 experiments.

ear porcine skin is a much more complex naturally constructed barrier.

The effect of target cholic acid derivatives on penetration of theophylline through the porcine skin is presented in Fig. 2 (hatched columns). Control experiments were run with only theophylline in the donor vehicle in the absence of any enhancer. The used system PG/water ensured entire solubility of theophylline, which is crucial for evaluation of penetration of theophylline through membranes. This solvent system also provides stable emulsion or microsuspension of the investigated enhancer [21,29,30,39–45]. A tested enhancer cannot be completely dissolved, similarly as in topical formulations, because by penetration of enhancer to the skin the dynamic balance (dissolved/undissolved part) is continuously changing. The investigated enhancer should be understood as a pharmaceutical excipient influencing only drug transport across the skin barrier [3,4].

The highest ERs were obtained with **13** (mono(decanoyl)) and **25** (tris(decanoyl)) derivatives with ERs of 2.13 and 2.09, respectively. According to the above presented data (Table 2, Fig. 2 – hatched columns), it can be concluded that all the discussed compounds showed only moderate penetration activity. Although acylation of cholic acid did not lead to potentially better enhancers of transdermal absorption, some interesting structure–activity relationships can be observed.

The results of one-way analysis of the variance (ANOVA) test complemented by the Bonferroni's multicomparison test are presented in Table 2 where differences were considered significant at $P = 0.05$. Considerable differences between determined ER were found. If comparison was made related to ER in the skin test of the most active compound **13** (2.13), the ER values of compounds **27** and **28** were significantly different from it at the probability level $P = 0.001$, the ER values of compounds **3**, **6**, **8**, **19**, **22** and **29** at $P = 0.01$ and the ER value of compound **26** at $P = 0.05$.

Generally, it can be postulated that the highest activity within the individual series was shown by mono- and tris(acyloxy) substituted derivatives, whereas bis(acyloxy) substituted derivatives, especially 7α,12α-bis(acyloxy) substituted compounds, possessed the lowest enhancing activity. However, comparable ER values were obtained within the series of the least lipophilic butanoyl derivatives (**3**, **6**, **8**) and the most lipophilic hexadodecanoyl derivatives (**31**, **33**, **35**), whereas hexadodecanoyl series expressed higher penetration-enhancing activity compared with butanoyl series.

Great differences can be observed in both decanoyl series, whereas methyl ester series **26**–**29** showed to be less active than acids **13**, **19**, **22**, **25**. From the results (Table 2, Fig. 2 – hatched columns) it is evident that penetration–enhancement activity is strongly dependent on balanced lipo/hydrophilic properties (lipophilicity/solubility–polarity) of the individual discussed enhancers due to their optimal interaction with skin components. The dependences of transdermal penetration–enhancement activity (ER) on the lipophilicity ($R_M$ values) and solubility (log $S$ values) of the studied compounds are shown in Fig. 3.

When compounds with the lowest penetration–enhancement activity are eliminated, dependences illustrated in Fig. 3 can be presented. The eliminated compounds are mostly 3α,7α- and 7α,12α-bis(acyloxy) derivatives and generally compounds with great differences between determined and predicted lipophilicity and/or solubility, *i.e.* compounds with strong inter- and intramolecular interactions. The parabolic dependence of activity on lipophilicity can be observed in Fig. 3a, and almost a mirror image can be seen in Fig. 3b, where the dependence of activity on solubility is demonstrated. It can be concluded that the higher

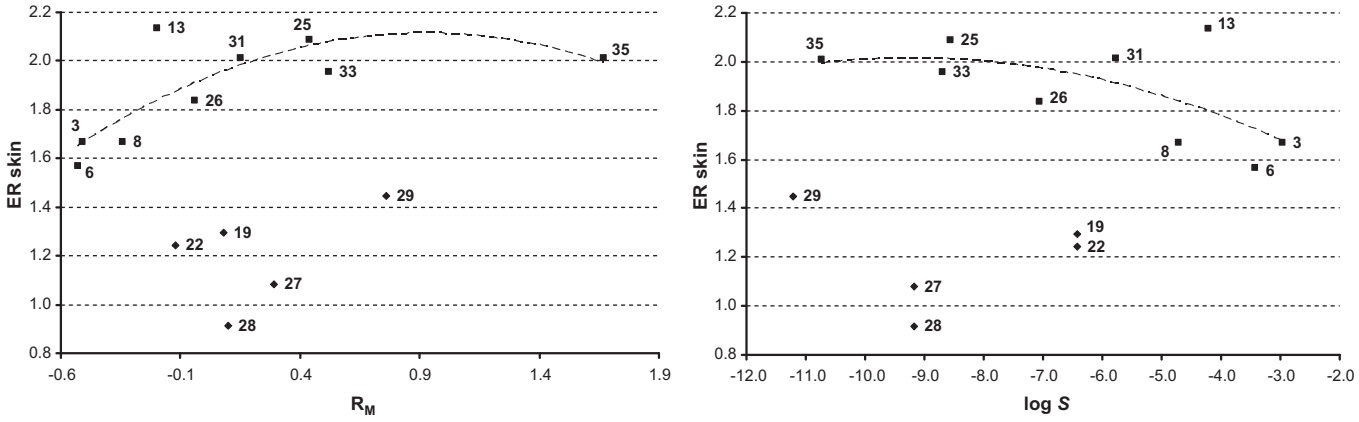



**Fig. 3.** The dependences of transdermal penetration–enhancement activity ER on the lipophilicity $R_M$ (a) and solubility log $S$ (b) of the studied target compounds.

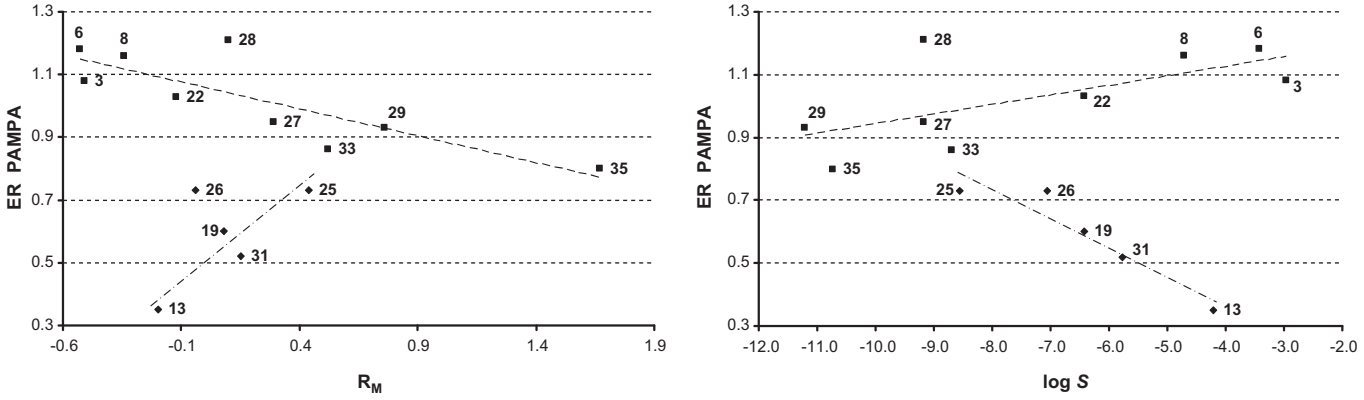



**Fig. 4.** The dependences of intestinal absorption-enhancement activity ER on the lipophilicity $R_M$ (a) and solubility log $S$ (b) of the studied target compounds.

transdermal penetration–enhancement activity is connected with higher solubility (Fig. 3b) and an optimum range of lipophilicity (Fig. 3a). When lipophilicity decreases dramatically with higher solubility, a decrease in enhancement activity can also be observed (butanoyl derivatives **3**, **6**, **8**). This confirms the fact that alkyl chains $C_{10}$–$C_{16}$ are preferred, because transdermal chemical penetration enhancers are compounds that intercalate between ceramides in the SC, disrupt ceramide–ceramide bonds and, by doing this, form a "channel" in the SC [21].

### 2.4. *In vitro screening of intestinal absorption-enhancing activity (PAMPA experiments)*

PAMPA have become a very useful and quite cheap tool for predicting *in vivo* drug permeability and are well-suited as a ranking tool for the assessment of compounds with passive transport mechanisms. An absorption study of binary mixtures or final formulations is also possible on PAMPA plates.

The effect of target cholic acid derivatives on the penetration of theophylline through the artificial polyvinylidene fluoride (PVDF) PAMPA membrane is presented in Fig. 2, dotted columns. Control experiments were run with only theophylline in the donor vehicle in the absence of any enhancer. The used solvents ensure solubility of theophylline as a model penetrating compound, which is important for facilitation of penetration through a membrane. This system also supports the stability of the microsuspension of the tested enhancer. The same as for the skin, the ratio between theophylline penetration with and without an enhancer was determined for the selected concentration based on the previous experience [29,30]. Only if some of evaluated potential enhancers expressed significant enhancer activity, the influence of the enhancer concentration on enhancement effect would be investigated.

Penetration in Franz cells is different from that in PAMPA tests, because the real skin is used as a barrier in Franz cells. Another parameter that may influence the measurements is solubility (solvation) supramolecular superassembly properties of cholic acid derivatives (donors), but explanation of this parameter is not the aim of this study. The difference between both experiments can also be seen in the geometrical arrangement of the experiment: in the PAMPA method a donor is below the diaphragm, whereas in the Franz cell the donor is above it. The greatest differences were noticeable in the results of mono(decanoyl) derivative **13** and mono(hexadecanoyl) derivative **31**.

The highest ERs were obtained for compounds **28** (7α,12α-bis(decanoyl)-Me ester), **6** (7α,12α-bis(butanoyl)), **8** (tris(butanoyl)), **3** (mono(butanoyl)) and **22** (7α,12α-bis(decanoyl)): 1.21, 1.18 1.16, 1.08 and 1.03, respectively. According to the above presented data (Table 2, Fig. 2 – dotted columns), it can be concluded that all the discussed compounds showed only moderate enhancement activity; the resting non-mentioned compounds are decelerators. Although it can be concluded that prepared acylcholic acid derivatives showed only low intestinal absorption enhancement, some interesting structure–activity relationships can be observed.

The results of one-way ANOVA test complemented by the Bonferroni's multicomparison test are presented in Table 2 where differences were considered significant at $P = 0.01$. Considerable differences between determined ER were found. If comparison was made related to ER of the less active compound in PAMPA test, *i.e.* **28**, the ER values of compounds **13**, **19**, **25**, **26**, **27**, **29**, **31**, **33** and **35** significantly differed from it at the probability level $P = 0.001$ and the ER values of compounds **3** and **22** at $P = 0.01$.

Significant changes even with small differences in the chemical structure can be observed in PAMPA experiments. Generally, it can be stated that trends in enhancement efficacy are absolutely opposite compared to transdermal penetration–enhancement activity ER. While transdermal enhancement activity is the highest for mono- a tris(acyloxy) substituted derivatives and the lowest for 7α,12α-bis(acyloxy) substituted compounds, in PAMPA experiments namely 7α,12α-bis(acyloxy) substituted compounds showed the highest activities within individual series, see Table 2, Fig. 2 – dotted columns. This fact can be probably connected with the above discussed specific non-covalent interactions of hydroxyl moieties, *i.e.* specific lipo/hydrophilic interactions and properties, see Section 2.2.

The compounds shown in Fig. 4 can be divided into intestinal absorption promoters/enhancers (accelerators) and compounds with the lowest enhancer activity, *i.e.* the highest "decelerator activity" (decelerators). Practically the whole series of decanoyl derivatives **13**, **19**, **25** and some other mono(acyloxy) substituted compounds, generally with relatively low lipophilicity can be classified as decelerators. Fig. 4a illustrates the linear dependence of activity on lipophilicity; intestinal penetration–enhancement activity dramatically decreases with lipophilicity increase (dashed line). Fig. 4b shows linear dependence of activity on solubility (dashed line), and it is almost a mirror image of the dependence demonstrated in Fig. 4a. Absolutely different dependences can be observed in the series of "decelerators". Fig. 4a shows decelerator activity decrease (intestinal enhancement activity increase) with lipophilicity ($R_M$) increase (dashed and dotted line), whereas Fig. 4b illustrates decelerator activity increase (decrease of enhancement activity) with solubility (log $S$) increase (dashed and dotted line). Based on these facts, it can be concluded that in the case of accelerants/enhancers (not decelerators) intestinal penetration–enhancement activity is also connected with higher

solubility (Fig. 4b) and relatively low lipophilicity (Fig. 4a). 7α,12α-Bis(decanoyl) derivative **28** showed the highest activity again, probably due to specific strong inter- and intramolecular interactions.

As all the butanoyl derivatives (**3**, **6**, **8**) expressed the balanced transdermal and intestinal penetration–enhancement activity, contrary to the rest of the discussed compounds, it can be suggested that these butanoyl derivatives as amphiphilic compounds show high hydrotropic effect and can reduce the resistance of membranes to drug diffusion [50,51].

The highest activity of **28** and the total loss of the intestinal penetration–enhancement activity of the majority of decanoyl and all hexadecanoyl derivatives compared to transdermal absorption can be explained also by a "cut-off" effect [52,53]. This "cut-off" effect is observed for *n*-alkyl substituted series of amphiphilic compounds, when the dependence of biological activity upon the alkyl chain length shows quasi-parabolic or bilinear dependence. The total inversion of activity is connected with the structural diversity of skin and PAMPA membrane. The hydrophobic parts of surfactants interact with lipidic parts of biological membranes. However, the water solubility of surfactants with longer alkyl chains is limited, and too large values of surfactant partition coefficient do not enable the penetration of such molecules through hydrophilic (aqueous) regions of biological membranes. Consequently, the final concentration of long-chain surfactants in the membrane will be lower than that of surfactants with shorter alkyl chains, which results in the loss of activity. It is suggested that the lateral expansion of the phospholipid bilayer of biological membranes caused by the intercalation of long-chain amphiphilic molecules between the phospholipid molecules and the mismatch between their hydrocarbon chains lengths results in the creation of free volume in the bilayer hydrophobic region [53]. According to the free volume theory the extent of membrane disturbance due to surfactant incorporation depends on the size of free volume created under its alkyl chain which can be then filled up with chains of neighbouring lipids as well as on the partition coefficient of the surfactants [52,54]. Therefore the most effective disturbance of the membrane and thus the highest inhibitory effect will be exhibited by surfactants with middle alkyl chain length ensuring not only sufficiently high free volume under an alkyl chain but also high concentration of the surfactant in the membrane due to the suitable value of the surfactant partition coefficient [55].

### 2.5. In vitro anti-proliferative/cytotoxicity assays

To evaluate the cytotoxic properties of tested compounds cells of different histopathological origin were used: a T-lymphoblastic leukemia cell line (CEM), a breast adenocarcinoma cell line (MCF7), and, as controls, normal human skin fibroblast cells (BJ). Treatment with compounds **8** (tris(butanoyl)), **13** (mono(decanoyl)), **22** (7α,12α-bis(decanoyl)), and **31** (mono(hexadecanoyl)) resulted in dose-dependent inhibition of cancer cells viability. Compound **22** showed the highest cytotoxicity but compounds **8**, **13** and **31** had cytotoxic effect on cancer cells without affecting the growth of normal BJ cells, which should be promising in potential development of new antiproliferative drugs.

Other discussed final compounds demonstrated poor anti-proliferative effect (or insignificant cytotoxicity effect) against all the cell lines, with $IC_{50}$ values greater than 37 μmol/L, see Table 2. These results suggest that the poor anti-proliferative activity of the target compounds will lead to limited cytotoxicity if they are used *in vivo* as absorption modifiers.

The results of one-way ANOVA test complemented by the Bonferroni's multicomparison test are presented in Table 2 where differences were considered significant at $P = 0.05$. Considerable differences between determined anti-proliferative $IC_{50}$ values were found. If comparison was made related to $IC_{50}$ values of the less anti-proliferatively active (insignificantly toxic) compound against CEM cells, *i.e.* **8** (33.0 μmol/L) the $IC_{50}$ values of compounds **13** and **22** were significantly different from it at the probability level $P = 0.01$ and the $IC_{50}$ value of compound **31** at $P = 0.05$. Similarly, in the cytotoxicity test against MCF7 if related to the $IC_{50}$ value of the less anti-proliferatively active (insignificantly toxic) compound **13** (34.6 μmol/L), the $IC_{50}$ value of compound **22** significantly differed from it at the probability level $P = 0.001$.

## 3. Conclusions

In this work a series of fourteen final acyloxy derivatives of 5β-cholic acid with $C_4$, $C_{10}$ and $C_{16}$ linear acyl chains were prepared as novel potential transdermal penetration enhancers and intestinal drug absorption modifiers. Experimental (relative) lipophilicity $R_M$, calculated from RP-TLC measurements was compared with predicted log *P* and log *S* values. The determined $R_M$ values as well as the calculated log *P* and log *S* values were compared with the membrane permeability influence studied by the PAMPA method and transdermal absorption *in vitro*. The comparison of the values influencing theophylline transport through the artificial and natural membranes ($R_M$, log *P*, log *S*) with experimental ERs of the cholic acid ester derivatives confirmed the expected correlation. The ability of the final compounds to enhance the penetration of theophylline through porcine skin was examined using a Franz cell, and the intestinal drug absorption enhancing effect was evaluated by means of PAMPA experiments. The highest transdermal penetration–enhancement activity in this study was exhibited by compounds **13** (3α-mono(decanoyl)) and **25** (3α,7α,12α-tris(decanoyl)), while the highest intestinal absorption-enhancement activity was exhibited by compounds **28** (7α,12α-bis(decanoyl)-Me ester) and **6** (7α,12α-bis(butanoyl)). All the compounds were additionally evaluated for their anti-proliferative/cytotoxic activity against two human cancer cell lines and against normal human skin fibroblast cells. Two compounds, **13** and **31**, showed anti-proliferative effect on cancer cells without affecting the growth of normal cells, which suggests that these compounds would have low cytotoxic side-effects when administered as enhancers/excipients. Ten other target compounds exhibited limited cytotoxicity, so they could be used as absorption modifiers. Although all the discussed compounds expressed only moderate intestinal absorption/transdermal penetration enhancement effects, nevertheless the obtained data provided important parameters for correlations between solubility/lipophilicity and enhancement activity, and noteworthy structure–activity relationships were found for subsequent structure optimization and rationalization of the design of novel potential cholic acid-type enhancers.

## 4. Experimental

### 4.1. Chemistry

All reagents were purchased from Sigma–Aldrich (Schnelldorf, Germany) and Merck (Darmstadt, Germany). Kieselgel 60, 0.063–0.200 mm (Merck, Darmstadt, Germany) was used for column chromatography. Thin layer chromatography (TLC) experiments were performed on aluminium foil-backed silica gel 40 $F_{254}$ plates (Merck, Darmstadt, Germany). Detection was performed by spraying with a solution of 20 g of $Ce(SO_4)_2$ in 200 ml 10% $H_2SO_4$ and subsequent heating. The melting points were determined on a Boetius apparatus (Nagema, Germany) and are uncorrected. Infrared (IR) spectra were recorded on a Smart MIRacle™ ATR ZnSe for Nicolet™ 6700 FT-IR Spectrometer (Thermo Scientific,

USA). The spectra were obtained by accumulation of 256 scans with 2 $cm^{-1}$ resolution in the 4000–600 $cm^{-1}$ region. Parameter "zero-filling" was 0. Happ–Gensel apodisation function was used. Elemental analyses were performed, using a Vario EL III Universal CHNOS Elemental Analyzer (Elementar Analysensysteme, Germany). All $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker Avance-250 FT-NMR spectrometer (250 MHz for $^1H$ and 62.9 MHz for $^{13}C$, Bruker Comp., Karlsruhe, Germany). Chemicals shifts are reported in ppm ($\delta$) using internal $Si(CH_3)_4$ as the reference, with diffuse, easily exchangeable signals being omitted. Mass spectra were measured using a LTQ Orbitrap Hybrid Mass Spectrometer (Thermo Electron Corporation, USA) with direct injection into an APCI source (400 °C) in the negative mode.

Benzyl 3α-butanoyloxy-7α,12α-dihydroxy-5β-cholate (**2**): Butanoyl chloride (1.50 ml, 14.33 mmol) dissolved in $CH_2Cl_2$ (20 ml) was slowly dropwise added to the solution of benzyl cholate (**1**, 6.03 g, 12.09 mmol) and DMAP (1.79 g, 14.65 mmol) in $CH_2Cl_2$ (40 ml) at 0 °C (ice bath) under argon. Then the mixture was stirred and warmed to a room temperature. After 2 h it was poured into aqueous AcOH (100 ml, 6%), and the aqueous layer was washed with $CH_2Cl_2$. Combined organic layers were washed with saturated aqueous $NaHCO_3$ and water and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/MeOH/AcOH 300:5:1 to 300:30:2). This provided a colourless oil. Yield: 2.79 g (40%). $^1H$ NMR (250 MHz, $CDCl_3$), $\delta$: 0.67 (s, 3H, C$H_3$), 0.90 (s, 3H, C$H_3$), 0.93 (m, 3H, C$H_3$), 0.97 (d, 3H, C$H_3$, $J$ = 6.87 Hz), 1.05–2.50 (m, 30H), 3.85 (m, 1H, $C_{(7)}H$), 3.97 (m, 1H, $C_{(12)}H$), 4.59 (m, 1H, $C_{(3)}H$), 5.11 (2H, PhC$H_2$), 7.30–7.40 (m, 5H, *Ph*). $^{13}C$ NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.69, 13.81, 17.48, 18.69, 22.69, 23.29, 26.88, 26.91, 27.56, 28.54, 31.02, 31.45, 34.55, 34.84, 35.05, 35.24, 35.42, 36.81, 39.72, 41.39, 42.22, 46.71, 47.41, 66.26, 68.41, 73.05, 74.14, 128.32, 128.37, 128.68, 136.28, 173.48 (*C*OOR), 174.14 (*C*OOR). HR-MS: for $C_{35}H_{51}O_6$ $[M-H]^-$ calculated: 567.3764 $m/z$; found: 567.3772 $m/z$.

3α-Butanoyloxy-7α,12α-dihydroxy-5β-cholic acid (**3**): Benzyl ester **2** (2.79 g, 4.91 mmol) and $HCOONH_4$ (1.05 g, 16.7 mmol) were dissolved in MeOH (70 ml), and 10% Pd/C (0.29 g) was added. The mixture was stirred under argon for 1.5 h. Then the second portion of 10% Pd/C (0.29 g) was added to the mixture and it was stirred for next 2 h. Then the suspension was filtered through celite and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/AcOEt/AcOH 400:100:4 to 250:250:4). This provided a white crystalline compound. Yield: 1.37 g (58%). Mp. 127–130 °C. $^1H$ NMR (250 MHz, $CDCl_3$), $\delta$: 0.70 (s, 3H, C$H_3$), 0.91 (s, 3H, C$H_3$), 0.93 (m, 3H, C$H_3$), 1.00 (d, 3H, C$H_3$, $J$ = 5.84 Hz), 1.05–2.50 (m, 30H), 3.87 (m, 1H, $C_{(7)}H$), 4.00 (m, 1H, $C_{(12)}H$), 4.59 (m, 1H, $C_{(3)}H$). $^{13}C$ NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.68, 13.81, 17.45, 18.69, 22.65, 23.31, 26.81, 26.88, 27.62, 28.43, 30.86, 31.11, 34.58, 34.86, 35.05, 35.37, 35.40, 36.81, 39.64, 41.36, 42.15, 46.70, 47.28, 68.53, 73.21, 74.19, 173.53 (*C*OOR), 179.32 (*C*OOH). IR ($cm^{-1}$): $\nu$(OH) 3331, $\nu$(CH) 2936, 2863, $\nu$(C=O) 1719, $\delta$(CH) 1465, $\nu$(CO) 1250, 1185, 1075, 1020. Anal. Calc. for $C_{28}H_{46}O_6$ (478.66): 70.26% C, 9.69% H; found: 70.02% C, 10.16% H. HR-MS: for $C_{28}H_{45}O_6$ $[M-H]^-$ calculated: 477.3216 $m/z$; found: 477.3227 $m/z$.

Benzyl 3α-acetoxy-7α,12α-dihydroxy-5β-cholate (**4**): The mixture of ester 1 (10.04 g, 20.14 mmol) and $KHCO_3$ (8.81 g, 88 mmol) in toluene (80 ml) and $Ac_2O$ (6.6 ml; 69.8 mmol) was refluxed under argon for 20 min. Then the mixture was poured on ice, the aqueous layer was separated and washed with toluene (50 ml). Combined organic extracts were washed with brine and evaporated under reduced pressure. The crude product was purified by crystallization from toluene, and a white crystalline compound was obtained. Yield: 4.94 g, (45%). $^1H$ NMR (250 MHz, $CDCl_3$), $\delta$: 0.67 (s, 3H, C$H_3$), 0.90 (s, 3H, C$H_3$), 0.97 (d, 3H, C$H_3$, $J$ = 5.97 Hz), 1.00–2.50 (m, 26H), 1.99 (s, 3H, C$H_3$CO), 3.84 (m, 1H, $C_{(7)}H$), 3.97 (m, 1H, $C_{(12)}H$), 4.57 (m, 1H, $C_{(3)}H$), 5.11 (2H, PhC$H_2$), 7.30–7.40 (m, 5H, *Ph*). $^{13}C$ NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.63, 17.45, 21.55, 22.61, 23.28, 26.73, 26.78, 27.54, 28.42, 30.98, 31.43, 34.56, 34.81, 35.02, 35.25, 35.30, 39.64, 41.32, 41.61, 42.06, 46.66, 47.36, 66.22, 68.39, 73.03, 74.44, 128.27, 128.33, 128.64, 136.23, 170.85 (*C*OOR), 174.13 (*C*OOR). HR-MS: for $C_{33}H_{47}O_6$ $[M-H]^-$ calculated: 539.3451 $m/z$; found: 539.3463 $m/z$.

Benzyl 3α-acetoxy-7α,12α-bis(butanoyloxy)-5β-cholate (**5**): Ester **4** (2.50 g, 4.63 mmol), BTEAC (0.41 g, 1.80 mmol), $CaH_2$ (0.82 g, 19.48 mmol) and butanoyl chloride (2 ml, 19.11 mmol) dissolved in toluene (40 ml) were refluxed under argon for 1 h. Then the mixture was poured into aqueous AcOH (70 ml, 4%). The aqueous layer was washed with toluene (3 × 50 ml), and combined organic extracts were washed with saturated aqueous $NaHCO_3$ (200 ml) and brine and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (petroleum ether/acetone 640:240). This provided a colourless oil. Yield: 2.96 g (94%). $^1H$ NMR (250 MHz, $CDCl_3$), $\delta$: 0.70 (s, 3H, C$H_3$), 0.82 (d, 3H, C$H_3$, $J$ = 6.14 Hz), 0.92 (s, 3H, C$H_3$), 0.98 (m, 3H, C$H_3$), 1.03 (t, 3H, C$H_3$, $J$ = 7.42 Hz), 1.07–2.50 (m, 32H), 2.01 (s, 3H, C$H_3$CO), 4.56 (m, 1H, $C_{(3)}H$), 4.94 (m, 1H, $C_{(7)}H$), 5.10 (m, 1H, $C_{(12)}H$), 5.10 (2H, PhC$H_2$), 7.30–7.40 (m, 5H, *Ph*). $^{13}C$ NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.20, 13.87, 14.05, 17.62, 18.51, 18.88, 21.46, 22.53, 23.06, 25.39, 26.82, 27.31, 28.74, 30.94, 31.32, 31.47, 34.43, 34.74, 34.79, 34.90, 36.98, 37.03, 38.03, 40.94, 43.34, 45.16, 47.55, 66.24, 70.61, 73.96, 75.17, 128.31, 128.36, 128.64, 136.16, 170.58 (*C*OOR), 172.73 (*C*OOR), 172.81 (*C*OOR), 173.90 (*C*OOR). HR-MS: for $C_{41}H_{59}O_8$ $[M-H]^-$ calculated: 679.4288 $m/z$; found: 679.4295 $m/z$.

7α,12α-Bis(butanoyloxy)-3α-hydroxy-5β-cholic acid (**6**): The mixture of ester **5** (2.81 g, 4.13 mmol), *i*-PrOH (37 ml) and aqueous NaOH (9 ml; 4%) was stirred at 80 °C for 30 min. It was poured into diluted aqueous AcOH (100 ml; 2.9%). The aqueous layer was washed with $CH_2Cl_2$ (3 × 50 ml) and combined organic extracts were washed with water (100 ml) and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (hexane/$Et_2O$/AcOH 250:250:10 to 60:300:8). This provided a white crystalline compound. Yield: 1.90 g (84%). Mp. 133–136 °C. $^1H$ NMR (250 MHz, $CDCl_3$), $\delta$: 0.73 (s, 3H, C$H_3$), 0.83 (d, 3H, C$H_3$, $J$ = 6.21 Hz), 0.91 (s, 3H, C$H_3$), 0.99 (m, 3H, C$H_3$), 1.02 (t, 3H, C$H_3$, $J$ = 7.47 Hz), 1.05–2.50 (m, 33H), 3.49 (m, 1H, $C_{(3)}H$), 4.94 (m, 1H, $C_{(7)}H$), 5.11 (m, 1H, $C_{(12)}H$), 5.60 (s, 1H). $^{13}C$ NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.25, 13.85, 14.07, 17.62, 18.57, 18.79, 22.61, 23.06, 25.49, 27.32, 28.84, 30.57, 30.74, 31.04, 31.60, 34.42, 34.84, 35.00, 36.95, 37.03, 38.05, 38.97, 41.13, 43.39, 45.20, 47.52, 70.71, 71.79, 75.19, 173.03 (*C*OOR), 173.10 (*C*OOR), 179.40 (*C*OOH). IR ($cm^{-1}$): $\nu$(OH) 3438, $\nu$(CH) 2932, 2870, $\nu$(C=O) 1724, $\delta$(CH) 1448, $\nu$(CO) 1253, 1181, 1072, 1039. Anal. Calc. for $C_{32}H_{52}O_7$ (548.75): 70.04% C, 9.55% H; found: 69.50% C, 9.97% H. HR-MS: for $C_{32}H_{51}O_6$ $[M-H]^-$ calculated: 547.3635 $m/z$; found: 547.3646 $m/z$.

Benzyl 3α,7α,12α-tris(butanoyloxy)-5β-cholate (**7**): Benzyl cholate **1** (4.00 g, 8.02 mmol), BTEAC (0.71 g, 3.12 mmol) $CaH_2$ (2.02 g, 47.98 mmol) and butanoyl chloride (5.00 ml, 47.77 mmol) dissolved in toluene (100 ml) were refluxed under argon for 3 h. Then the mixture was poured into aqueous AcOH (100 ml, 6%). The aqueous layer was washed with toluene (3 × 50 ml) and combined organic extracts were washed with saturated aqueous $NaHCO_3$ (200 ml) and brine and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/MeOH/AcOH 300:5:1). This provided a colourless oil. Yield: 4.4 g (77%). $^1H$ NMR (250 MHz, $CDCl_3$), $\delta$: 0.70 (s, 3H, C$H_3$), 0.82 (d, 3H, C$H_3$, $J$ = 6.13 Hz), 0.92 (s, 3H, C$H_3$), 0.94 (m, 3H, C$H_3$), 0.98 (m, 3H, C$H_3$), 1.03 (m, 3H, C$H_3$), 1.10–2.50 (m, 36H), 4.58 (m, 1H, $C_{(3)}H$), 4.93 (m, 1H, $C_{(7)}H$), 5.09 (m, 1H, $C_{(12)}H$), 5.10 (2H, PhC$H_2$), 7.30–7.38 (m, 5H, *Ph*). $^{13}C$ NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.23, 13.71, 13.90, 14.10, 17.66, 18.53, 18.70, 18.93, 22.54, 23.09, 25.37, 26.86, 27.34, 28.75, 30.98, 31.35, 31.49, 34.46, 34.78, 34.82, 34.97, 36.74, 37.05, 37.09, 38.06, 40.94, 43.36, 45.19, 47.60, 66.28, 70.69, 73.63, 75.24, 128.35, 128.40, 128.68, 136.19, 172.72 (*C*OOR), 172.82 (*C*OOR),

173.19 (*C*OOR), 173.95 (*C*OOR). HR-MS: for $C_{43}H_{65}O_8$ $[M-H]^-$ calculated: 707.4601 *m*/*z*; found: 707.4610 *m*/*z*.

3α,7α,12α-Tris(butanoyloxy)-5β-cholic acid (**8**): Benzyl ester **7** (4.4 g, 6.21 mmol) and $HCOONH_4$ (1.60 g, 25.37 mmol) were dissolved in MeOH (70 ml), and 10% Pd/C (0.29 g) was added. The mixture was stirred under argon for 1.5 h. Then the second portion of 10% Pd/C (0.29 g) was added to the mixture, and it was stirred for next 2 h. After that the suspension was filtered through celite and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/MeOH/AcOH 300:5:1 to 300:15:1.5). This provided a colourless oil. Yield: 3.0 g (78%). $^1$H NMR (250 MHz, $CDCl_3$), *δ*: 0.74 (s, 3H, C*H*$_3$), 0.84 (d, 3H, C*H*$_3$, *J* = 6.20 Hz), 0.92 (s, 3H, C*H*$_3$), 0.94 (m, 3H, C*H*$_3$), 0.98 (t, 3H, C*H*$_3$, *J* = 7.46 Hz), 1.04 (t, 3H, C*H*$_3$, *J* = 7.29 Hz), 1.10–2.50 (m, 37H), 4.59 (m, 1H, $C_{(3)}H$), 4.95 (m, 1H, $C_{(7)}H$), 5.12 (m, 1H, $C_{(12)}H$), 9.50 (s, 1H, COO*H*). $^{13}$C NMR (62.5 MHz, $CDCl_3$), *δ*: 12.23, 13.68, 13.87, 14.07, 17.63, 18.52, 18.68, 18.91, 22.51, 23.06, 25.35, 26.83, 27.32, 28.73, 30.72, 31.03, 31.46, 34.44, 34.75, 34.81, 34.94, 36.72, 37.03, 37.08, 38.04, 40.90, 43.34, 45.19, 47.53, 70.72, 73.65, 75.25, 172.79 (*C*OOR), 172.87 (*C*OOR), 173.27 (*C*OOR), 179.85 (*C*OOH). IR ($cm^{-1}$): *ν*(CH) 2961, 2873, *ν*(C=O) 1726, *δ*(CH) 1456, *ν*(CO) 1252, 1180, 1089. Anal. Calc. for $C_{36}H_{58}O_8$ (616.84): 69.87% C, 9.45% H; found: 68.05% C, 9.48% H. HR-MS: for $C_{36}H_{57}O_6$ $[M-H]^-$ calculated: 617.4053 *m*/*z*; found: 617.4049 *m*/*z*.

Benzyl decanoyloxy-hydroxy-cholates **9**–**12**: Decanoyl chloride (3.75 ml, 18.07 mmol) dissolved in $CH_2Cl_2$ (40 ml) was slowly dropwise added to the solution of benzyl cholate (**1**, 10.03 g, 20.12 mmol), and DMAP (3.08 g; 25.21 mmol) in $CH_2Cl_2$ (60 ml) at 0 °C (ice bath) under argon. Then the mixture was stirred and warmed to a room temperature. After 2 h it was poured into aqueous AcOH (100 ml, 6%), and the aqueous layer was washed with $CH_2Cl_2$. Combined organic layers were washed with saturated aqueous $NaHCO_3$ and water and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (toluene/AcOEt/AcOH 100:5:0.5 to 0:100:1). This provided four products:

Benzyl 3α-decanoyloxy-7α,12α-dihydroxy-5β-cholate (**9**): A colourless oil. Yield: 2.40 g (37%). $^1$H NMR (250 MHz, $CDCl_3$), *δ*: 0.66 (s, 3H, C*H*$_3$), 0.88 (m, 3H, C*H*$_3$), 0.90 (s, 3H, C*H*$_3$), 0.97 (d, 3H, C*H*$_3$, *J* = 5.88 Hz), 1.00–2.5 (m, 42H), 3.84 (m, 1H, $C_{(7)}H$), 3.97 (m, 1H, $C_{(12)}H$), 4.58 (m, 1H, $C_{(3)}H$), 5.11 (2H, PhC*H*$_2$), 7.3–7.4 (m, 5H, *Ph*). $^{13}$C NMR (62.5 MHz, $CDCl_3$), *δ*: 12.60, 14.20, 17.43, 22.58, 22.77, 23.28, 25.19, 26.73, 26.87, 27.56, 28.40, 29.30, 29.37, 29.40, 29.53, 30.96, 31.39, 31.96, 34.62, 34.81, 34.94, 35.04, 35.28, 35.32, 39.58, 41.35, 42.09, 46.66, 47.34, 66.21, 68.42, 73.11, 74.14, 128.26, 128.31, 128.62, 136.22, 173.59 (*C*OOR), 174.14 (*C*OOR). HR-MS: for $C_{41}H_{63}O_6$ $[M-H]^-$ calculated: 651.4703 *m*/*z*; found: 651.4714 *m*/*z*.

Benzyl 7α-decanoyloxy-3α,12α-dihydroxy-5β-cholate (**10**): A colourless oil. Yield: 1.51 g (11%). $^1$H NMR (250 MHz, $CDCl_3$), *δ*: 0.64 (s, 3H, C*H*$_3$), 0.88 (m, 3H, C*H*$_3$), 0.90 (s, 3H, C*H*$_3$), 0.96 (d, 3H, C*H*$_3$, *J* = 5.93 Hz), 1.0–2.5 (m, 42H), 3.47 (m, 1H, $C_{(3)}H$), 3.97 (m, 1H, $C_{(12)}H$), 4.89 (m, 1H, $C_{(7)}H$), 5.10 (2H, PhC*H*$_2$), 7.30–7.40 (m, 5H, *Ph*). $^{13}$C NMR (62.5 MHz, $CDCl_3$), *δ*: 12.57, 14.18, 17.37, 27.33, 28.20, 28.65, 29.29, 29.37, 29.47, 29.57, 30.57, 30.94, 31.43, 31.58, 31.95, 34.39, 34.99, 35.10, 35.22, 38.26, 39.06, 41.22, 42.16, 46.60, 47.29, 66.18, 70.73, 71.78, 72.72, 128.25, 128.30, 128.60, 136.17, 173.44 (*C*OOR), 173.96 (*C*OOR). HR-MS: for $C_{41}H_{63}O_6$ $[M-H]^-$ calculated: 651.4703 *m*/*z*; found: 651.4716 *m*/*z*.

Benzyl 3α,7α-bis(decanoyloxy)-12α-hydroxy-5β-cholate (**11**) yield about 21%, was isolated together with benzyl 3α,12α-bis(decanoyloxy)-7α-hydroxy-5β-cholate (**12**), yield about 12%, and was not further purified (about 12%).

*tert*-Butyl 3α,7α-dihydroxy-12α-trifluoroacetoxy-5β-cholate (**15**): The solution of 3α,7α,12α-trihydroxy-5β-cholic acid (**14**, 10.08 g, 24.67 mmol) in THF (250 ml) cooled to −45 °C under argon was mixed with $(TFAc)_2O$ (62 ml, 446 mmol) and stirred at this temperature for 1.5 h. The temperature was allowed to rise and maintained at −30 to −40 °C during 2 h. Then it was cooled down to −57 °C, and *tert*-BuOH (100 ml, 1.046 mol) was added slowly. After 16 h the mixture was poured on ice at the laboratory temperature. The aqueous layer was washed with $Et_2O$ (250 ml). $Et_2O$ layer was washed with ice-cooled aqueous NaOH (150 ml; 2 M) and saturated aqueous $NaHCO_3$ (150 ml) and evaporated under reduced pressure. The residue was mixed with MeOH (150 ml) and THF (150 ml) at 0 °C; the saturated aqueous solution of $NaHCO_3$ (75 ml) was added, and mixture was stirred for 1 h. The second portion of the saturated aqueous solution of $NaHCO_3$ (20 ml) was added, and the mixture was stirred at room temperature for 3 h and poured into the mixture of $Et_2O$ (500 ml) and ice-cooled water (300 ml). After shaking, the $Et_2O$ layer was washed with water (300 ml) and phosphate buffer (300 ml; 1 M) and evaporated under reduced pressure. The crude product was purified by crystallization form hexane, and a white crystalline compound was obtained. Yield: 9.42 g (68%). Mp. 123–125 °C. $^1$H NMR (250 MHz, $CDCl_3$), *δ*: 0.79 (s, 3H, C*H*$_3$), 0.81 (d, 3H, C*H*$_3$, *J* = 6.47 Hz), 0.90 (s, 3H, C*H*$_3$), 0.80–2.3 (m, 26H), 1.44 (s, 9H, (C*H*$_3$)$_3$C–OCO), 3.43 (m, 1H, $C_{(3)}H$), 3.88 (m, 1H, $C_{(7)}H$), 5.31 (m, 1H, $C_{(12)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), *δ*: 12.32, 17.62, 22.64, 22.77, 22.90, 25.51, 27.29, 27.77, 28.24, 30.71, 30.85, 32.35, 34.60, 34.69, 35.15, 39.33, 39.85, 41.49, 43.49, 45.24, 47.65, 67.91, 71.92, 80.17 (*C*(CH$_3$)$_3$), 81.01, 114.79 (q, *C*F$_3$CO, $^1J_{CF}$ = 286.20 Hz), 157.06 (q, CF$_3$*C*O, $^2J_{CF}$ = 42.14 Hz), 173.53 (*C*OOR). HR-MS: for $C_{30}H_{46}F_3O_6$ $[M-H]^-$ calculated: 559.3325 *m*/*z*; found: 559.3337 *m*/*z*.

*tert*-Butyl 3α-decanoyloxy-7α-hydroxy-12α-trifluoroacetoxy-5β-cholate (**16**): The solution of ester **15** (1.99 g, 3.55 mmol), DMAP (0.13 g, 1.06 mmol) and TEA (1.1 ml, 7.9 mmol) in $CH_2Cl_2$ (55 ml) was mixed with decanoyl chloride (1.6 ml, 7.7 mmol), and the mixture was stirred for 50 min at room temperature. Then it was evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (toluene/AcOEt/AcOH 100:5:0.5). This provided a colourless oil. Yield: 1.43 g (56%).$^1$H NMR (250 MHz, $CDCl_3$), *δ*: 0.75–0.94 (m, 12H, 4 × C*H*$_3$), 0.95–2.38 (m, 42H), 1.44 (s, 9H, (C*H*$_3$)$_3$C–OCO), 3.88 (m, 1H, $C_{(7)}H$), 4.55 (m, 1H, $C_{(3)}H$), 5.32 (m, 1H, $C_{(12)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), *δ*: 12.32, 14.19, 17.62, 22.59, 22.78, 22.90, 25.17, 25.49, 26.66, 27.30, 27.75, 28.23, 29.38 (t, $J_{CF}$ = 9.1 Hz), 30.86, 31.98, 32.36, 34.56, 34.60, 34.62, 34.85, 35.36, 39.29, 41.24, 43.50, 45.25, 47.66, 67.84, 73.77, 80.16, 80.95, 114.80 (q, *C*F$_3$CO, $^1J_{CF}$ = 286 Hz), 157.02 (q, CF$_3$*C*O, $^2J_{CF}$ = 42.14 Hz), 173.50 (*C*OOR), 173.72 (*C*OOR). HR-MS: for $C_{40}H_{64}F_3O_7$ $[M-H]^-$ calculated: 713.4682 *m*/*z*; found: 713.4691 *m*/*z*.

*tert*-Butyl 3α-decanoyloxy-7α,12α-dihydroxy-5β-cholate (**17**): The solution of ester **16** (1.43 g, 2.0 mmol) in THF (40 ml) and MeOH (40 ml) was mixed with concentrated $NH_3$(aq) (30.5 ml) and the mixture was stirred at room temperature for 1 h, poured into phosphate buffer (200 ml, pH 7) and washed with $Et_2O$ (2 × 160 ml). Combined $Et_2O$ extracts were washed with brine and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (toluene/AcOEt/AcOH 100:5:0.5). This provided a yellowish oil. Yield: 1.33 g (90%). $^1$H NMR (250 MHz, $CDCl_3$), *δ*: 0.68 (s, 3H, C*H*$_3$), 0.86 (m, 3H, C*H*$_3$), 0.89 (s, 3H, C*H*$_3$), 0.96 (d, 3H, C*H*$_3$, *J* = 6.24 Hz), 1.00–2.00 (m, 37H), 1.43 (s, 9H, (C*H*$_3$)$_3$C–OCO), 2.03–2.40 (m, 7H), 3.84 (m, 1H, $C_{(7)}H$), 3.98 (m, 1H, $C_{(12)}H$), 4.56 (m, 1H, $C_{(3)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), *δ*: 12.62, 14.21, 17.48, 22.59, 22.78, 23.27, 25.19, 26.76, 26.84, 27.58, 28.23, 28.36, 29.30, 29.38, 29.40, 29.54, 31.04, 31.97, 32.65, 34.59, 34.82, 34.94, 35.02, 35.23, 35.33, 39.60, 41.33, 42.11, 46.65, 47.41, 68.46, 73.16, 74.16, 80.13, 173.73 (*C*OOR), 173.88 (*C*OOR). HR-MS: for $C_{38}H_{65}O_6$ $[M-H]^-$ calculated: 617.4859 *m*/*z*; found: 617.4870 *m*/*z*.

3α-Decanoyloxy-7α,12α-dihydroxy-5β-cholic acid (**13**): *Method A*: Benzyl ester **9** (2.33 g, 3.57 mmol) and $HCOONH_4$ (1.05 g, 16.7 mmol) were dissolved in MeOH (50 ml), and 10% Pd/C

(0.27 g) was added. The mixture was stirred under argon for 1 h. Then the second portion of 10% Pd/C (0.2 g) was added to the mixture, and it was stirred for next 2 h. Then the suspension was filtered through celite and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/MeOH/AcOH 30:1.5:0.3). This provided a colourless oil. Yield: 1.43 g (71%).

*Method B*: *tert*-Butyl ester **17** (1.30 g, 2.20 mmol) was dissolved in $CH_2Cl_2$ (38 ml), the solution was cooled to 0 °C and mixed with TFAcOH (7.5 ml) under argon. The reaction mixture was warmed to the room temperature and after 2 h poured into aqueous AcONa (100 ml; 8%). The aqueous mixture was washed with $CH_2Cl_2$ (2 × 100 ml) and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/$Et_2O$/AcOH 100:8:0.5 to 100:64:0.4). This provided a colourless oil. Yield: 0.60 g (53%). $^1$H NMR (250 MHz, $CDCl_3$), $\delta$: 0.70 (s, 3H, C$H_3$), 0.87 (m, 3H, C$H_3$), 0.90 (s, 3H, C$H_3$), 0.99 (d, 3H, C$H_3$, $J$ = 5.76 Hz), 1.03–2.50 (m, 46H), 3.86 (m, 1H, $C_{(7)}H$), 3.99 (m, 1H, $C_{(12)}H$), 4.58 (m, 1H, $C_{(3)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.68, 14.24, 17.47, 22.65, 22.81, 23.32, 25.23, 26.82, 26.88, 27.63, 28.45, 29.34, 29.41, 29.44, 29.58, 30.91, 31.16, 32.01, 34.59, 34.87, 34.98, 35.07, 35.37, 35.41, 39.66, 41.37, 42.17, 46.70, 47.26, 68.52, 73.19, 74.18, 173.71 (*C*OOR), 179.15 (*C*OOH). IR (cm$^{-1}$): $\nu$(OH) 3431, $\nu$(CH) 2922, 2855, $\nu$(C=O) 1706, $\delta$(CH) 1464, $\nu$(CO) 1247, 1186, 1072, 1023. Anal. Calc. for $C_{34}H_{58}O_6$ (562.82): 72.56% C, 10.39% H; found 71.84% C, 10.84% H. HR-MS: for $C_{34}H_{57}O_6$ [M−H]$^-$ calculated: 561.4155 $m/z$; found: 561.4168 $m/z$.

*tert*-Butyl 3α,7α-bis(decanoyloxy)-12α-trifluoroacetoxy-5β-cholate (**18**): *tert*-Butyl ester **15** (4 g, 7.13 mmol), BTEAC (0.61 g, 2.7 mmol) $CaH_2$ (1.2 g, 28.5 mmol) and decanoyl chloride (5.6 g, 29.4 mmol) dissolved in toluene (100 ml) were refluxed under argon for 2 h. Then the mixture was poured into aqueous AcOH (250 ml, 2%). The aqueous layer was washed with toluene (3 × 50 ml) and combined organic extracts were washed with saturated aqueous $NaHCO_3$ (200 ml) and brine and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/$Et_2O$/AcOH 100:2:0.4). This provided a colourless oil. Yield: 2.29 g (37%). $^1$H NMR (250 MHz, $CDCl_3$), $\delta$: 0.76 (s, 3H, C$H_3$), 0.80 (d, 3H, C$H_3$, $J$ = 6.39 Hz), 0.86 (m, 6H, 2 × C$H_3$), 0.91 (s, 3H, C$H_3$), 0.95–2.50 (m, 56H), 1.41 (s, 9H, ($CH_3$)$_3$C–OCO), 4.54 (m, 1H, $C_{(3)}H$), 4.92 (m, 1H, $C_{(7)}H$), 5.31 (m, 1H, $C_{(12)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.17, 14.17, 17.66, 21.95, 22.48, 22.77, 22.90, 25.09, 25.19, 25.28, 26.67, 27.15, 28.20, 28.63, 29.23, 29.40, 29.52, 29.61, 30.86, 31.37, 31.97, 32.44, 34.37, 34.61, 34.75, 34.81, 34.95, 35.23, 37.94, 40.87, 43.20, 45.19, 47.76, 70.21, 73.39, 80.15, 80.74, 114.82 (q, *C*$F_3$CO, $^1J_{CF}$ = 286.41 Hz), 156.79 (q, $CF_3$*C*O, $^2J_{CF}$ = 41.93 Hz), 172.86 (*C*OOR), 173.36 (*C*OOR), 173.52 (*C*OOR). HR-MS: for $C_{50}H_{82}F_3O_8$ [M−H]$^-$ calculated: 867.6040 $m/z$; found: 867.6052 $m/z$.

3α,7α-Bis(decanoyloxy)-12α-hydroxy-5β-cholic acid (**19**): The solution of ester **18** (2.28 g, 2.62 mmol) in THF (55 ml) and MeOH (55 ml) was mixed with concentrated $NH_3$(aq) (38 ml, 23%), and the mixture was stirred at room temperature for 1 h, poured into phosphate buffer (500 ml; 1 M; pH 7) and washed with AcOEt (3 × 250 ml). Combined AcOEt extracts were evaporated under reduced pressure and then dissolved in $CH_2Cl_2$ (35 ml). TFAcOH (10 ml) was added at 12 °C and the mixture was stirred at room temperature for 2 h. Then it was poured into aqueous AcONa (200 ml; 10%). The aqueous layer was washed with $CH_2Cl_2$ (3 × 100 ml). Combined organic extracts were washed with brine (100 ml) and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/$Et_2O$/AcOH 800:32:3.2). This provided a white crystalline compound. Yield: 0.98 g (52%). Mp. 129–131 °C. $^1$H NMR (250 MHz, $CDCl_3$), $\delta$: 0.68 (s, 3H, C$H_3$), 0.87 (m, 6H, 2 × C$H_3$), 0.91 (s, 3H, C$H_3$), 0.98 (d, 3H, C$H_3$, $J$ = 6.04 Hz), 1.00–2.50 (m, 58 H), 4.00 (m, 1H, $C_{(12)}H$), 4.58 (m, 1H, $C_{(3)}H$), 4.90 (m, 1H, $C_{(7)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.67, 14.23, 17.46, 22.68, 22.81, 23.16, 25.21, 25.25, 26.84, 27.39, 28.27, 28.72, 29.31, 29.42, 29.47, 29.55, 29.57, 29.67, 30.76, 31.13, 31.49, 32.00, 32.03, 34.50, 34.91, 35.11, 35.14, 38.29, 41.08, 42.28, 46.69, 47.40, 70.71, 72.91, 73.91, 173.35 (*C*OOR), 173.58 (*C*OOR), 179.61 (*C*OOH). IR (cm$^{-1}$): $\nu$(OH) 3529, $\nu$(CH) 2919, 2852, $\nu$(C=O) 1732, 1705, $\delta$(CH) 1378, $\nu$(CO) 1169. Anal. Calc. for $C_{44}H_{76}O_7$ (717.07): 73.70% C, 10.68% H; found 73.54% C, 11.27% H. HR-MS: for $C_{44}H_{75}O_7$ [M−H]$^-$ calculated: 715.5513 $m/z$; found: 715.5474 $m/z$.

Benzyl 3α-benzyloxycarboxy-7α,12α-dihydroxy-5β-cholate (**20**). The solution of benzyl cholate **1** (5.03 g, 10.1 mmol) in dry pyridine (75 ml) was cooled to 0 °C and mixed under argon with BnOCOCl (4.37 g, 25.6 mmol). Then the mixture was stirred for 4 h, and another portion of BnOCOCl (4.38 g, 25.7 mmol) was added. After 20 h the solvents were evaporated under reduced pressure. The residue was dissolved in toluene (50 ml) and washed with aqueous AcOH (60 ml, 17%). The aqueous layer was washed with toluene (2 × 50 ml). Combined organic extracts were washed with brine and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/MeOH/AcOH 300:5:1). This provided a colourless oil. Yield: 2.97 g (46%). $^1$H NMR (250 MHz, $CDCl_3$), $\delta$: 0.65 (s, 3H, C$H_3$), 0.89 (s, 3H, C$H_3$), 0.96 (d, 3H, C$H_3$, $J$ = 5.95 Hz), 1.00–2.50 (m, 27H), 3.83 (m, 1H, $C_{(7)}H$), 3.95 (m, 1H, $C_{(12)}H$), 4.45 (m, 1H, $C_{(3)}H$), 5.10 (2H, PhC$H_2$), 5.12 (s, 2H, PhC$H_2$), 7.30–7.40 (m, 10H, 2 × *Ph*). $^{13}$C NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.64, 17.43, 22.54, 23.26, 26.67, 26.70, 27.52, 28.41, 30.96, 31.41, 34.43, 34.76, 34.89, 35.16, 35.22, 39.65, 41.29, 42.00, 46.64, 47.30, 66.23, 68.30, 69.27, 72.94, 78.59, 128.29, 128.32, 128.34, 128.46, 128.64, 135.63, 136.23, 154.81 (*O*COO), 174.16 (*C*OOR). HR-MS: for $C_{39}H_{51}O_7$ [M−H]$^-$ calculated: 631.3713 $m/z$; found: 631.3726 $m/z$.

Benzyl 3α-benzyloxycarboxy-7α,12α-bis(decanoyloxy)-5β-cholate (**21**): Ester **20** (0.20 g, 0.32 mmol), BTEAC (0.03 g, 0.13 mmol) $CaH_2$ (0.05 g, 1.19 mmol) and decanoyl chloride (0.25 g, 1.31 mmol) dissolved in toluene (5 ml) were refluxed for 2 h under argon. Then the mixture was poured into aqueous AcOH (20 ml, 2.5%). The aqueous layer was washed with $CH_2Cl_2$ (3 × 20 ml), and combined organic extracts were washed with brine and evaporated under reduced pressure. The residue (0.26 g, 87%) was used in the subsequent step without further purification. $^1$H NMR (250 MHz, $CDCl_3$), $\delta$: 0.69 (s, 3H, C$H_3$), 0.80 (d, 3H, C$H_3$, $J$ = 6.13 Hz), 0.83–0.90 (m, 6H, 2 × C$H_3$), 0.92 (s, 3H, C$H_3$), 0.95–2.45 (m, 57H), 4.46 (m, 1H, $C_{(3)}H$), 4.92 (m, 1H, $C_{(7)}H$), 5.09 (m, 1H, $C_{(12)}H$), 5.10 (2H, PhC$H_2$), 5.13 (2H, PhC$H_2$), 7.30–7.40 (m, 10H, 2 × *Ph*). $^{13}$C NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.29, 14.22, 17.66, 22.56, 22.80, 23.10, 25.27, 25.45, 25.57, 26.89, 27.32, 28.85, 29.35, 29.39, 29.45, 29.47, 29.58, 29.60, 29.65, 30.97, 31.39, 31.51, 32.01, 32.02, 34.47, 34.71, 34.80, 35.04, 35.16, 38.04, 41.05, 43.41, 45.23, 47.63, 66.26, 69.51, 70.43, 75.03, 78.28, 128.33, 128.38, 128.45, 128.63, 128.67, 128.71, 135.46, 136.20, 154.75 (*O*COO), 173.14 (*C*OOR), 173.22 (*C*OOR), 173.86 (*C*OOR). HR-MS: for $C_{59}H_{87}O_9$ [M−H]$^-$ calculated: 939.6428 $m/z$; found: 939.6439 $m/z$.

Benzyl 3α-acetoxy-7α,12α-bis(decanoyloxy)-5β-cholate (**23**): Ester **4** (3.00 g, 5.55 mmol), BTEAC (0.53 g, 2.33 mmol) $CaH_2$ (0.97 g, 23.0 mmol) and decanoyl chloride (4.6 ml; 22.0 mmol) dissolved in toluene (50 ml) were refluxed for 2 h under argon. Then the mixture was poured into aqueous AcOH (50 ml, 6%). The aqueous layer was washed with toluene (3 × 50 ml) and combined organic extracts were washed with saturated aqueous $NaHCO_3$ (2 × 50 ml) and water (100 ml) and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (toluene/AcOEt 100:2 to 100:10). This provided a colourless oil. Yield: 3.4 g (72%). $^1$H NMR (250 MHz, $CDCl_3$), $\delta$: 0.70 (s, 3H, C$H_3$), 0.81 (d, 3H, C$H_3$, $J$ = 6.08 Hz), 0.88 (m, 6H, 2 × C$H_3$), 0.92 (s, 3H, C$H_3$), 0.95–2.45 (m, 57 H), 2.01 (s, 3H, C$H_3$CO), 4.57 (m, 1H, $C_{(3)}H$), 4.93 (m, 1H, $C_{(7)}H$), 5.1 (m, 1H, $C_{(12)}H$), 5.10 (2H, PhC$H_2$), 7.32–7.37

(m, 5H, *Ph*). $^{13}$C NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.25, 14.19, 17.65, 21.48, 22.61, 22.78, 23.08, 25.21, 25.47, 25.61, 26.98, 27.31, 28.79, 29.43, 29.45, 29.47, 29.53, 29.59, 29.65, 30.96, 31.36, 31.50, 32.00, 34.48, 34.78, 34.80, 35.04, 35.14, 35.19, 38.05, 41.04, 43.39, 45.19, 47.64, 66.25, 70.58, 74.09, 75.14, 128.32, 128.38, 128.66, 136.19, 170.55 (*C*OOR), 172.91 (*C*OOR), 173.01 (*C*OOR), 173.85 (*C*OOR). HR-MS: for $C_{53}H_{83}O_8$ $[M-H]^-$ calculated: 847.6166 *m*/*z*; found: 847.6175 *m*/*z*.

7α,12α-Bis(decanoyloxy)-3α-hydroxy-5β-cholic acid (**22**). *Method A*: Benzyl ester **21** (0.10 g, 0.106 mmol) and $HCOONH_4$ (0.06 g, 0.95 mmol) were dissolved in MeOH (5 ml), and 10% Pd/C (0.01 g) was added. The mixture was stirred under argon for 1 h. Then the suspension was filtered through celite and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/MeOH/AcOH 300:15:3). This provided a colourless oil. Yield: 70 mg (92%).

*Method B*: The mixture of ester **23** (1.66 g, 1.92 mmol), *i*-PrOH (17 ml) and aqueous NaOH (4.2 ml, 4%) was stirred at 80 °C for 30 min. It was poured into diluted aqueous AcOH (80 ml, 2.5%). The aqueous layer was washed with $CH_2Cl_2$ (3 × 50 ml), and combined organic extracts were washed with water (100 ml) and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (toluene/AcOEt/AcOH 100:10:1 to 100:15:1). This provided a colourless oil. Yield: 0.72 g (51%).

*Method C*: The mixture of methyl ester **28** (3.47 g, 4.75 mmol), THF (27 ml), *i*-PrOH (26 ml) and aqueous NaOH (26 ml, 0.8%, 5.25 mmol NaOH) was stirred for 20 h under room temperature. Then it was poured into diluted aqueous AcOH (250 ml, 1%). The aqueous layer was washed with $CH_2Cl_2$ (3 × 100 ml), and combined organic extracts were washed with water (100 ml) and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/MeOH/AcOH 300:5:1). This provided a colourless oil. Yield: 2.55 g (75%). $^1$H NMR (250 MHz, $CDCl_3$), $\delta$: 0.73 (s, 3H, C*H*$_3$), 0.83 (d, 3H, C*H*$_3$, *J* = 6.27 Hz), 0.88 (m, 6H, 2 × C*H*$_3$), 0.91 (s, 3H, C*H*$_3$), 0.95–2.45 (m, 58H), 3.48 (m, 1H, $C_{(3)}H$), 4.93 (m, 1H, $C_{(7)}H$), 5.11 (m, 1H, $C_{(12)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.31, 14.21, 17.66, 22.66, 22.80, 23.11, 25.27, 25.48, 25.56, 27.35, 28.88, 29.40, 29.44, 29.48, 29.53, 29.64, 29.70, 30.75, 31.09, 31.62, 32.01, 32.03, 34.45, 34.85, 35.08, 35.21, 38.09, 39.11, 41.20, 43.44, 45.24, 47.63, 70.69, 71.86, 75.17, 173.26 (*C*OOR), 179.50 (*C*OOH). IR ($cm^{-1}$): $\nu$(OH) 3429, $\nu$(CH) 2923, 2854, $\nu$(C=O) 1727, $\delta$(CH) 1466, $\nu$(CO) 1247, 1176, 1073, 1015. Anal. Calc. for $C_{44}H_{76}O_7$ (717.07): 73.70% C, 10.68% H; found: 72.81% C, 11.27% H. HR-MS: for $C_{44}H_{75}O_7$ $[M-H]^-$ calculated: 715.5513 *m*/*z*; found: 715.5528 *m*/*z*.

Benzyl 3α,7α,12α-tris(decanoyloxy)-5β-cholate (24): Benzyl cholate **1** (8.17 g, 16.4 mmol), BTEAC (1.44 g, 6.3 mmol) $CaH_2$ (4.55 g, 108 mmol) and decanoyl chloride (20 ml, 96.4 mmol) dissolved in toluene (200 ml) were refluxed for 3 h under argon. Then the mixture was poured into aqueous AcOH (200 ml, 6%). The aqueous layer was washed with toluene (3 × 100 ml), and combined organic extracts were washed with saturated aqueous $NaHCO_3$ (200 ml) and brine and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (toluene/$Et_2O$/TEA 180:18:2). This provided a yellowish oil. Yield: 15.6 g (99%). $^1$H NMR (250 MHz, $CDCl_3$), $\delta$: 0.63 (s, 3H, C*H*$_3$), 0.81 (d, 3H, C*H*$_3$, *J* = 6.10 Hz), 0.87 (m, 9H, 3 × C*H*$_3$), 0.91 (s, 3H, C*H*$_3$), 1.00–2.50 (m, 73 H), 4.58 (m, 1H, $C_{(3)}H$), 4.93 (m, 1H, $C_{(7)}H$), 5.10 (m, 1H, $C_{(12)}H$), 5.1 (2H, PhC*H*$_2$), 7.30–7.37 (m, 5H, *Ph*). $^{13}$C NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.23, 14.18, 17.63, 22.57, 22.76, 22.78, 23.06, 24.82, 25.19, 25.44, 25.58, 26.99, 27.30, 28.77, 29.17, 29.28, 29.34, 29.39, 29.43, 29.45, 29.51, 29.56, 29.60, 29.67, 30.94, 31.34, 31.46, 31.97, 32.02, 34.04, 34.45, 34.76, 34.80, 35.11, 35.18, 38.03, 41.00, 43.37, 45.17, 47.63, 66.24, 70.62, 73.73, 75.16, 128.30, 128.36, 128.63, 136.17, 172.88 (COOR), 173.01 (*C*OOR), 173.37 (*C*OOR), 173.86 (*C*OOR). HR-MS: for $C_{61}H_{99}O_8$ $[M-H]^-$ calculated: 959.7418 *m*/*z*; found: 959.7429 *m*/*z*.

3α,7α,12α-Tris(decanoyloxy)-5β-cholic acid (**25**): Benzyl ester **24** (4.04 g, 4.2 mmol) and $HCOONH_4$ (1.09 g, 17.3 mmol) were dissolved in MeOH (40 ml), and 10% Pd/C (0.39 g) was added. The mixture was stirred under argon for 3 h. Then the suspension was filtered through celite and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/MeOH/AcOH 300:5:1). This provided a colourless oil. Yield: 3.62 g (99%). $^1$H NMR (250 MHz, $CDCl_3$), $\delta$: 0.73 (s, 3H, C*H*$_3$), 0.83 (d, 3H, C*H*$_3$, *J* = 6.00 Hz), 0.88 (m, 9H, 3 × C*H*$_3$), 0.92 (s, 3H, C*H*$_3$), 0.95–2.50 (m, 73H), 4.58 (m, 1H, $C_{(3)}H$), 4.94 (m, 1H, $C_{(7)}H$), 5.11 (m, 1H, $C_{(12)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.29, 14.20, 17.66, 22.61, 22.81, 23.10, 25.23, 25.48, 25.62, 27.02, 27.35, 28.80, 29.32, 29.42, 29.47, 29.48, 29.56, 29.59, 29.65, 29.73, 30.74, 31.08, 31.49, 32.00, 32.05, 34.49, 34.81, 34.84, 35.10, 35.16, 35.24, 38.07, 41.03, 43.41, 45.22, 47.63, 70.66, 73.77, 75.19, 172.95 (*C*OOR), 173.07 (*C*OOR), 173.45 (*C*OOR), 179.75 (*C*OOH). IR ($cm^{-1}$): $\nu$(CH) 2923, 2854, $\nu$(C=O) 1729, $\delta$(CH) 1466, $\nu$(CO) 1247, 1174, 1103, 1008. Anal. Calc. for $C_{54}H_{94}O_8$ (871.61): 74.44% C, 10.87% H; found: 73.71% C, 11.83% H. HR-MS: for $C_{54}H_{93}O_8$ $[M-H]^-$ calculated: 869.6870 *m*/*z*; found: 869.6876 *m*/*z*.

Methyl 3α-decanoyloxy-7α,12α-dihydroxy-5β-cholate (**26**): To benzyl ester **9** (2.51 g, 3.84 mmol) dissolved in THF (6 ml) and MeOH (34 ml), MeONa (0.11 g, 2.04 mmol) was added stepwise, and the solution was stirred at room temperature for 45 min. Then it was poured into aqueous AcOH (100 ml, 0.5%). The aqueous layer was washed with $CH_2Cl_2$ (3 × 50 ml), and combined organic extracts were washed with brine and evaporated under reduced pressure. The crude product was purified by crystallisation from hexane. This provided a white crystalline compound. Yield: 0.70 g (32%). Mp. 94–97 °C. $^1$H NMR (250 MHz, $CDCl_3$), $\delta$: 0.69 (s, 3H, C*H*$_3$), 0.87 (m, 3H, C*H*$_3$), 0.90 (s, 3H, C*H*$_3$), 0.98 (d, 3H, C*H*$_3$, *J* = 6.13 Hz), 1.00–2.50 (m, 43H), 3.66 (s, 3H, COOC*H*$_3$), 3.85 (m, 1H, $C_{(7)}H$), 3.97 (m, 1H, $C_{(12)}H$), 4.57 (m, 1H, $C_{(3)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.68, 14.24, 17.48, 22.66, 22.80, 23.29, 25.21, 26.84, 26.87, 27.57, 28.50, 29.31, 29.40, 29.42, 29.56, 31.01, 31.18, 31.99, 34.56, 34.83, 34.96, 35.04, 35.30, 35.37, 39.67, 41.35, 42.19, 46.69, 47.36, 51.64, 68.41, 73.07, 74.12, 173.67 (*C*OOR), 174.84 (*C*OOR). IR ($cm^{-1}$): $\nu$(OH) 3598, 3534, $\nu$(CH) 2928, 2863, $\nu$(C=O) 1738, $\delta$(CH) 1366, $\nu$(CO) 1167. Anal. Calc. for $C_{35}H_{60}O_6$ (576.84): 72.87% C, 10.48% H; found: 72.65% C, 10.95% H. HR-MS: for $C_{35}H_{59}O_6$ $[M-H]^-$ calculated: 576.4077 *m*/*z*; found: 576.4081 *m*/*z*.

Methyl 3α,7α-bis(decanoyloxy)-12α-hydroxy-5β-cholate (**27**): The mixture of benzyl esters **11** and **12** (1.33 g, 1.65 mmol) was mixed with THF (5 ml), MeOH (20 ml) and MeONa (0.09 g, 1.67 mmol) and stirred at room temperature for 30 min. Then it was poured into aqueous AcOH (250 ml, 0.4%). The aqueous layer was washed with $CH_2Cl_2$ (3 × 100 ml), and combined organic extracts were washed with brine and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (petroleum ether/acetone/AcOH 800:100:10). This yielded a crystalline material 0.95 g (79%) that was a mixture of methyl 3α,7α-bis(decanoyloxy)-12α-hydroxy-5β-cholate (**27**, 90% of mixture) and methyl 3α,12α-bis(decanoyloxy)-7α-hydroxy-5β-cholate (10% of mixture). Crystallisation from toluene/hexane mixture provided 0.6 g (50%) of **27** as a white crystalline compound. Mp. 100–102 °C. $^1$H NMR (250 MHz, $CDCl_3$), $\delta$: 0.68 (s, 3H, C*H*$_3$), 0.87 (m, 6H, 2 × C*H*$_3$), 0.92 (s, 3H, C*H*$_3$), 0.97 (d, 3H, C*H*$_3$, *J* = 6.12 Hz), 1.00–2.50 (m, 57H), 3.65 (s, 3H, COOC*H*$_3$), 3.99 (m, 1H, $C_{(12)}H$), 4.58 (m, 1H, $C_{(3)}H$), 4.90 (m, 1H, $C_{(7)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), $\delta$: 12.67, 14.23, 17.51, 22.68, 22.81, 23.16, 25.22, 25.26, 26.85, 27.40, 28.29, 28.72, 29.31, 29.43, 29.55, 29.57, 29.67, 31.00, 31.22, 31.50, 32.01, 32.03, 34.50, 34.91, 35.11, 35.17, 38.29, 41.09, 42.29, 46.70, 47.43, 51.64, 70.69, 72.87, 73.88, 173.31 (*C*OOR), 173.53 (*C*OOR), 174.70 (*C*OOR). IR ($cm^{-1}$): $\nu$(OH) 3529, $\nu$(CH) 2921, 2851, $\nu$(C=O) 1735, 1705, $\nu$(CO) 1167. Anal. Calc. for $C_{45}H_{78}O_7$ (731.10): 73.93% C,

10.75% H; found: 73.73% C, 11.43% H. HR-MS: for $C_{45}H_{77}O_7$ $[M-H]^-$ calculated: 729.5669 *m/z*; found: 729.56468 *m/z*.

Methyl 7α,12α-bis(decanoyloxy)-3α-hydroxy-5β-cholate (**28**): Benzyl ester **24** (6.01 g, 6.25 mmol), THF (30 ml), MeOH (60 ml) and MeONa (0.36 g, 6.67 mmol) were mixed and stirred at room temperature for 16 h. Then the mixture was poured into aqueous AcOH (250 ml, 1%). The aqueous layer was washed with $CH_2Cl_2$ (3 × 100 ml), and combined organic extracts were washed with brine and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (toluene/$Et_2O$/TEA 160:40:2). This provided a colourless oil. Yield: 4.30 g (94%). $^1$H NMR (250 MHz, $CDCl_3$), δ: 0.71 (s, 3H, C$H_3$), 0.80 (d, 3H, C$H_3$, *J* = 6.26 Hz), 0.87 (m, 6H, 2 × C$H_3$), 0.89 (s, 3H, C$H_3$), 0.95–2.50 (m, 57H), 3.46 (m, 1H, $C_{(3)}H$), 3.64 (s, 3H, COOC$H_3$), 4.91 (m, 1H, $C_{(7)}H$), 5.08 (m, 1H, $C_{(12)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), δ: 12.29, 14.21, 17.66, 22.67, 22.80, 23.10, 25.26, 25.47, 25.56, 27.35, 28.88, 29.39, 29.44, 29.47, 29.52, 29.54, 29.63, 29.68, 30.73, 30.96, 31.11, 31.61, 32.01, 34.44, 34.87, 35.07, 35.20, 38.06, 39.13, 41.18, 43.43, 45.21, 47.56, 51.60, 70.65, 71.82, 75.15, 173.23 (*C*OOR), 174.57 (*C*OOR). IR ($cm^{-1}$): ν(OH) 3507, ν(CH) 1727, ν(CO) 1247, 1171, 1075. Anal. Calc. for $C_{45}H_{78}O_7$ (731.10): 73.93% C, 10.75% H; found: 72.52% C, 11.38% H. HR-MS: for $C_{45}H_{77}O_7$ $[M-H]^-$ calculated: 729.5669 *m/z*; found: 729.56476 *m/z*.

Methyl 3α,7α,12α-tris(decanoyloxy)-5β-cholate (**29**): Benzyl ester **24** (2.52 g, 2.62 mmol), THF (6 ml), MeOH (25 ml) and MeONa (0.15 g, 2.78 mmol) were mixed and stirred at room temperature for 30 min. Then the mixture was poured into aqueous AcOH (250 ml, 2%). The aqueous layer was washed with $CH_2Cl_2$ (3 × 100 ml), and combined organic extracts were washed with brine and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (toluene/$Et_2O$/TEA 180:18:2). This provided a colourless oil. Yield: 1.9 g (82%). $^1$H NMR (250 MHz, $CDCl_3$), δ: 0.66 (s, 3H, C$H_3$), 0.75 (d, 3H, C$H_3$, *J* = 6.2 Hz), 0.81 (m, 9H, 3 × C$H_3$), 0.85 (s, 3H, C$H_3$), 0.90–2.40 (m, 73H), 3.58 (s, 3H, COOC$H_3$), 4.51 (m, 1H, $C_{(3)}H$), 4.87 (m, 1H, $C_{(7)}H$), 5.03 (m, 1H, $C_{(12)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), δ: 12.31, 14.22, 17.71, 22.64, 22.81, 22.83, 23.13, 25.25, 25.51, 25.64, 27.05, 27.38, 28.83, 29.34, 29.44, 29.49, 29.52, 29.58, 29.61, 29.66, 29.74, 31.01, 31.16, 31.52, 32.02, 32.07, 34.52, 34.86, 34.90, 35.12, 35.18, 35.25, 38.09, 41.06, 43.44, 45.24, 47.64, 51.62, 70.66, 73.77, 75.21, 172.92 (*C*OOR), 173.06 (*C*OOR), 173.42 (*C*OOR), 174.58 (*C*OOR). IR ($cm^{-1}$): ν(CH) 2922, 2854, ν(C=O) 1729, δ(CH) 1378, ν(CO) 1171. Anal. Calc. for $C_{55}H_{96}O_8$ (885.35): 74.61% C, 10.93% H; found: 73.45% C, 11.88% H. HR-MS: for $C_{55}H_{95}O_8$ $[M-H]^-$ calculated: 883.7027 *m/z*; found: 883.69904 *m/z*.

Benzyl 3α-hexadecanoyloxy-7α,12α-dihydroxy-5β-cholate (**30**): Hexadecanoyl chloride (4.4 ml, 14.52 mmol) dissolved in $CH_2Cl_2$ (20 ml) was slowly dropwise added to the solution of benzyl cholate **1** (6.02 g, 12.07 mmol) and DMAP (1.80 g, 14.73 mmol) in $CH_2Cl_2$ (40 ml) at 0 °C (ice bath) under argon. Then the mixture was stirred and warmed to a room temperature. After 2 h it was poured into aqueous AcOH (100 ml, 6%), and the aqueous layer was washed with $CH_2Cl_2$. Combined organic layers were washed with saturated aqueous $NaHCO_3$ and water and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (toluene/AcOEt/AcOH 500:25:2.5 to 500:70:2.5). This provided a colourless oil. Yield: 5.20 g (58%). $^1$H NMR (250 MHz, $CDCl_3$), δ: 0.66 (s, 3H, C$H_3$), 0.88 (m, 3H, C$H_3$), 0.89 (s, 3H, C$H_3$), 0.97 (d, 3H, C$H_3$, *J* = 5.9 Hz), 1.00–2.50 (m, 54 H), 3.84 (m, 1H, $C_{(7)}H$), 3.97 (m, 1H, $C_{(12)}H$), 4.58 (m, 1H, $C_{(3)}H$), 5.11 (2H, PhC$H_2$), 7.30–7.37 (m, 5H, *Ph*). $^{13}$C NMR (62.5 MHz, $CDCl_3$), δ: 12.61, 14.22, 17.44, 22.59, 22.79, 23.29, 25.20, 26.74, 26.88, 27.57, 28.40, 29.32, 29.42, 29.47, 29.60, 29.74, 29.77, 29.81, 30.97, 31.39, 32.03, 34.62, 34.82, 34.95, 35.05, 35.28, 35.33, 39.59, 41.36, 42.09, 46.66, 47.35, 66.22, 68.44, 73.13, 74.15, 128.26, 128.32, 128.63, 136.23, 173.60 (*C*OOR), 174.15 (*C*OOR). HR-MS: for $C_{47}H_{75}O_6$ $[M-H]^-$ calculated: 735.5642 *m/z*; found: 735.5651 *m/z*.

3α-Hexadecanoyloxy-7α,12α-dihydroxy-5β-cholic acid (**31**): Benzyl ester **30** (4.57 g, 6.20 mmol) and $HCOONH_4$ (3.07 g; 48.68 mmol) were dissolved in MeOH (150 ml), and 10% Pd/C (0.11 g) was added. The mixture was stirred under argon for 1.5 h. Then the second portion of 10% Pd/C (0.54 g) was added to the mixture and it was stirred for next 2 h. After that the suspension was filtered through celite and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/MeOH/AcOH 600:30:6). This provided a yellowish waxy compound. Yield: 3.51 g (88%). $^1$H NMR (250 MHz, $CDCl_3$), δ: 0.69 (s, 3H, C$H_3$), 0.86 (m, 3H, C$H_3$), 0.89 (s, 3H, C$H_3$), 0.98 (d, 3H, C$H_3$, *J* = 5.73 Hz), 1.02–2.05 (m, 47H), 2.10–2.50 (m, 6H), 3.85 (m, 1H, $C_{(7)}H$), 3.99 (m, 1H, $C_{(12)}H$), 4.56 (m, 1H, $C_{(3)}H$), 5.30 (s, O*H*). $^{13}$C NMR (62.5 MHz, $CDCl_3$), δ: 12.62, 14.22, 17.43, 22.57, 22.80, 23.31, 24.85, 25.20, 26.70, 26.87, 27.63, 28.32, 29.22, 29.34, 29.44, 29.47, 29.61, 29.78, 29.82, 30.84, 31.17, 32.04, 34.16, 34.60, 34.85, 34.96, 35.05, 35.33, 35.42, 39.56, 41.35, 42.05, 46.66, 47.23, 68.56, 73.27, 74.20, 173.69 (*C*OOR), 179.58 (*C*OOH). IR ($cm^{-1}$): ν(OH) 3485, 3333, ν(CH) 2918, 2851, ν(C=O) 1723, 1709, δ(CH) 1464, ν(CO) 1258, 1176, 1037. Anal. Calc. for $C_{40}H_{70}O_6$ (646.98): 74.26% C, 10.91% H; found: 73.94% C, 11.62% H. HR-MS: for $C_{40}H_{69}O_6$ $[M-H]^-$ calculated: 645.5094 *m/z*; found: 645.5101 *m/z*.

Benzyl 3α-acetoxy-7α,12α-bis(hexadecanoyloxy)-5β-cholate (**32**): Ester **4** (2.51 g, 4.64 mmol), BTEAC (0.42 g, 1.84 mmol), $CaH_2$ (0.79 g, 18.76 mmol) and hexadecanoyl chloride (5.5 ml, 18.15 mmol) dissolved in toluene (40 ml) were refluxed under argon for 1 h. Then the mixture was poured into aqueous AcOH (50 ml; 6%). The aqueous layer was washed with toluene (3 × 50 ml), and combined organic extracts were washed with saturated aqueous $NaHCO_3$ (200 ml) and brine and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (petroleum ether/acetone 800:165). This provided a colourless oil. Yield: 3.18 g (67%). $^1$H NMR (250 MHz, $CDCl_3$), δ: 0.70 (s, 3H, C$H_3$), 0.81 (d, 3H, C$H_3$, *J* = 6.10 Hz), 0.88 (m, 6H, 2 × C$H_3$), 0.92 (s, 3H, C$H_3$), 0.95–2.50 (m, 80H), 2.00 (s, 3H, C$H_3$CO), 4.57 (m, 1H, $C_{(3)}H$), 4.93 (m, 1H, $C_{(7)}H$), 5.10 (m, 1H, $C_{(12)}H$), 5.10 (2H, PhC$H_2$), 7.30–7.40 (m, 5H, *Ph*). $^{13}$C NMR (62.5 MHz, $CDCl_3$), δ: 12.25, 14.22, 17.65, 21.49, 22.60, 22.80, 23.09, 25.22, 25.47, 25.63, 26.98, 27.31, 28.78, 29.44, 29.48, 29.51, 29.55, 29.61, 29.67, 29.73, 29.78, 29.84, 30.95, 31.34, 31.50, 32.04, 34.48, 34.76, 34.80, 35.04, 35.16, 35.20, 38.05, 41.04, 43.38, 45.19, 47.63, 66.25, 70.58, 74.09, 75.14, 128.32, 128.37, 128.65, 136.19, 170.54 (*C*OOR), 172.90 (*C*OOR), 173.00 (*C*OOR), 173.84 (*C*OOR). HR-MS: for $C_{65}H_{107}O_8$ $[M-H]^-$ calculated: 1015.8044 *m/z*; found: 1015.8056 *m/z*.

7α,12α-Bis(hexadecanoyloxy)-3α-hydroxy-5β-cholic acid (**33**): The mixture of ester **32** (2.94 g, 2.89 mmol), *i*-PrOH (25 ml) and aqueous NaOH (6 ml; 4.3%) was stirred at 80 °C for 30 min. Then it was poured into diluted aqueous AcOH (110 ml; 2.7%). The aqueous layer was washed with $CH_2Cl_2$ (3 × 50 ml), and combined organic extracts were washed with water (100 ml) and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (petroleum ether/$Et_2O$/AcOH 375:125:10 to 50:150:4). This provided a colourless oil. Yield: 2.03 g (79%). $^1$H NMR (250 MHz, $CDCl_3$), δ: 0.73 (s, 3H, C$H_3$), 0.83 (d, 3H, C$H_3$, *J* = 6.26 Hz), 0.88 (m, 6H, 2 × C$H_3$), 0.91 (s, 3H, C$H_3$), 0.95–2.50 (m, 81H), 3.48 (m, 1H, $C_{(3)}H$), 4.93 (m, 1H, $C_{(7)}H$), 5.10 (m, 1H, $C_{(12)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), δ: 12.32, 14.23, 17.67, 22.66, 22.82, 23.11, 25.26, 25.49, 25.56, 27.33, 28.89, 29.41, 29.50, 29.54, 29.56, 29.65, 29.72, 29.76, 29.80, 29.85, 30.72, 31.09, 31.63, 32.06, 34.45, 34.82, 35.08, 35.20, 38.10, 39.13, 41.20, 43.43, 45.25, 47.64, 70.68, 71.86, 75.16, 173.24 (*C*OOR), 179.49 (*C*OOH). IR ($cm^{-1}$): ν(OH) 3435, ν(CH) 2921, 2852, ν(C=O) 1729, 1709, δ(CH) 1466, ν(CO) 1250, 1176, 1073, 1005. Anal. Calc. for $C_{56}H_{100}O_7$ (885.39): 75.97% C, 11.38% H; found: 75.31% C, 12.00% H. HR-MS: for $C_{56}H_{99}O_6$ $[M-H]^-$ calculated: 883.7391 *m/z*; found: 883.7403 *m/z*.

Benzyl 3α,7α,12α-tris(hexadecanoyloxy)-5β-cholate (**34**): Benzyl cholate **1** (2.00 g, 4.01 mmol), BTEAC (0.36 g, 1.58 mmol), $CaH_2$ (1.01 g, 24.00 mmol) and hexadecanoyl chloride (7.5 ml, 24.70 mmol) dissolved in toluene (50 ml) were refluxed under argon for 5 h. Then the mixture was poured into aqueous AcOH (50 ml; 6%). The aqueous layer was washed with toluene (3 × 50 ml), and combined organic extracts were washed with saturated aqueous $NaHCO_3$ (200 ml) and brine and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (toluene/AcOEt, 0–8%). This provided a colourless oil. Yield: 2.95 g (60%). $^1$H NMR (250 MHz, $CDCl_3$), δ: 0.70 (s, 3H, C$H_3$), 0.81 (d, 3H, C$H_3$, $J$ = 6.08 Hz), 0.88 (m, 9H, 3 × C$H_3$), 0.92 (s, 3H, C$H_3$), 0.95–2.45 (m, 108H), 4.57 (m, 1H, $C_{(3)}H$), 4.93 (m, 1H, $C_{(7)}H$), 5.09 (m, 1H, $C_{(12)}H$), 5.1 (2H, PhC$H_2$), 7.30–7.37 (m, 5H, *Ph*). $^{13}$C NMR (62.5 MHz, $CDCl_3$), δ: 12.27, 14.23, 17.68, 22.62, 22.82, 23.11, 25.25, 25.47, 25.64, 27.03, 27.34, 28.80, 29.36, 29.51, 29.58, 29.68, 29.73, 29.82, 29.85, 29.89, 30.97, 31.37, 31.51, 32.07, 34.50, 34.79, 34.85, 35.11, 35.17, 35.23, 38.08, 41.05, 43.40, 45.21, 47.67, 66.27, 70.63, 73.74, 75.18, 128.34, 128.39, 128.67, 136.21, 172.87 (*C*OOR), 173.00 (*C*OOR), 173.36 (*C*OOR), 173.86 (*C*OOR). HR-MS: for $C_{79}H_{135}O_8$ $[M-H]^-$ calculated: 1212.0235 $m/z$; found: 1212.0247 $m/z$.

3α,7α,12α-Tris(hexadecanoyloxy)-5β-cholic acid (**35**): Ester **34** (2.96 g, 2.44 mmol) and $HCOONH_4$ (0.62 g, 9.83 mmol) were dissolved in MeOH (70 ml), and 10% Pd/C (0.30 g) was added. The mixture was stirred under argon for 3 h. Then the suspension was filtered through celite and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel ($CH_2Cl_2$/MeOH/AcOH 300:5:1). This provided a white crystalline compound. Yield: 2.56 g (93%). Mp. 53–55 °C. $^1$H NMR (250 MHz, $CDCl_3$), δ: 0.73 (s, 3H, C$H_3$), 0.83 (d, 3H, C$H_3$, $J$ = 6.00 Hz), 0.88 (m, 9H, 3 × C$H_3$), 0.92 (s, 3H, C$H_3$), 1.00–2.50 (m, 109), 4.58 (m, 1H, $C_{(3)}H$), 4.93 (m, 1H, $C_{(7)}H$), 5.10 (m, 1H, $C_{(12)}H$). $^{13}$C NMR (62.5 MHz, $CDCl_3$), δ: 12.31, 14.24, 17.69, 22.62, 22.83, 23.12, 25.26, 25.49, 25.65, 27.04, 27.35, 28.81, 29.36, 29.52, 29.59, 29.69, 29.74, 29.83, 29.86, 29.89, 30.73, 31.06, 31.51, 32.08, 34.51, 34.81, 34.86, 35.13, 35.18, 35.25, 38.10, 41.05, 43.41, 45.24, 47.67, 70.66, 73.77, 75.19, 172.93 (*C*OOR), 173.05 (*C*OOR), 173.43 (*C*OOR), 179.61 (*C*OOH). IR ($cm^{-1}$): ν(CH) 2916, 2849, ν(C=O) 1727, 1706, δ(CH) 1468, ν(CO) 1248, 1171, 1071, 1008. Anal. Calc. for $C_{72}H_{130}O_8$ (1123.80): 76.95% C, 11.66% H; found: 76.52% C, 12.35% H. HR-MS: for $C_{72}H_{129}O_8$ $[M-H]^-$ calculated: 1121.9687 $m/z$; found: 1121.9700 $m/z$.

### 4.2. Lipophilicity and solubility determination/calculations

$R_M$ values were determined from the RP-18 TLC measurements. The solution of a compound in $CH_2Cl_2$ was spotted on a TLC plate (TLC silica gel 60 RP-18 $F_{254s}$, 20 cm × 20 cm, Merck), 1.5 cm from the edge. The volatiles were carefully evaporated, and the plate was developed by MeOH/AcOH 100:1 solvent mixture for 2.5 h. After drying, the plate was sprayed for the detection by solution of $Ce(SO_4)_2$ in $H_2SO_4$ and heated. $R_M$ data were obtained from equation: $R_M = \log(1/R_f - 1)$. $R_M$ values as well as $\log k$, $\log k_w$ or Kovats retention indexes can be used as the lipophilicity index converted to $\log P$ scale [1,56].

$\log P$ values (*i.e.*, the logarithm of the partition coefficient for *n*-octanol/water) were predicted using ACD/Log *P* DB software (ACD/Labs, ver. 12.01, Advanced Chemistry Development, Inc., Toronto, ON, Canada, 2010). $\log S$ values (as ACD/Labs aqueous $\log S$ at pH 7.4) were calculated by ACD/Log S DB software (ver. 12.01, Advanced Chemistry Development, Inc., Toronto, ON, Canada, 2010). The results are shown in Table 1.

ACD/Solubility DB (http://www.acdlabs.com/products/pc_admet/physchem/physchemsuite/) is a program that calculates aqueous solubility values at any pH under the standard conditions (and zero ionic strength). The accuracy of calculations (according to the vendor) for simple structures is usually better than 0.2–0.5 logarithmic units (for complex structures it is better than 0.5–1.0 logarithmic units). Solubility is not derived from $\log P$ and takes into account not only the pH (solubility as a function of pH) but compares the fragmental estimations with experimental material from ca 6000 compounds databased.

### 4.3. In vitro screening of penetration enhancing activity and sample analysis

Skin samples were obtained from porcine ear. Full thickness dorsal skin was cut in fragments and stored at −20 °C until utilized. Skin samples were slowly thawed (at 4 °C overnight and then at ambient temperature) before each experiment. The penetration enhancing activity of newly synthesized target compounds was evaluated *in vitro*, using a vertical Franz diffusion cell (SES – Analytical Systems, GmbH, Germany), with a donor surface area of 0.635 $cm^2$ and receptor volume of 5.2 ml. The skin was mounted between the donor and receptor compartments of the Franz diffusion cell with the epidermal side up. The receptor compartment was filled with phosphate buffered saline (pH 7.4) and was maintained at 37 ± 0.5 °C, while using circulating water bath. The receptor compartment content was continuously stirred using a magnetic stirring bar. The skin was kept in contact with the receptor phase for 0.5 h prior to the experiment. The donor samples were prepared by dissolving the tested enhancer (1 mg) in propylene glycol (0.5 ml), and the solution of theophylline (5 mg) in water (0.5 ml) was added. This mixture was shaken vigorously and then sonicated for 10 min at 40 °C, then this stable system (dissolved theophylline in enhancer emulsion) was applied to the skin surface and the donor compartment of the cell was covered by Parafilm®. The control samples were prepared in the same manner without enhancers. Samples (0.5 ml) of the receptor phase were withdrawn at seven pre-determined time intervals over 24 h (1, 2, 4, 6, 8, 12 and 24 h) and the cell was refilled with an equivalent amount of fresh buffer solution. A minimum of three determinations was performed using skin fragments from a minimum of two animals for each compound. The samples were stored at −18 °C until analyzed by HPLC.

Analysis of samples for theophylline content was performed using an Agilent 1200 series HPLC system, equipped with a diode array detection (DAD) system, a quaternary model pump and an automatic injector (Agilent Technologies, Germany). Data acquisition was performed using ChemStation chromatography software. A Waters Symmetry® $C_8$ 5 μm, 4.6 mm × 250 mm (Waters Corp., Milford, MA, USA) chromatographic column was used. A mixture of MeCN (HPLC grade, 50.0%) and $H_2O$ (HPLC – Mili-Q Grade, 50.0%) was used as a mobile phase. The total flow of the column was 0.5 ml/min, injection 10 μl, column temperature 25 °C and sample temperature 10 °C. The detection wavelength of 280 nm was chosen. The retention time ($t_R$) of theophylline was 5.07 ± 0.05 min.

The cumulative amounts of theophylline that penetrated through the skin into the receptor compartment (μg/$cm^2$), were corrected for sample removal, and plotted against time (h). An approximately linear dependence was found ($R^2 \geq 0.98$), and steady state fluxes (μg/$cm^2$/h) were calculated using the linear region of the plots. ERs were calculated as ratios of theophylline flux with and without the enhancer. The results are summarized in Table 2.

### 4.4. PAMPA experiments

The penetration enhancing activity of newly synthesized final compounds was evaluated *in vitro*, using a vertical PAMPA system (BD GentestTMPre-Coated PAMPA Plate System, 96 wells,

http://www.bdbeurope.com). The donor samples were prepared by dissolving the tested enhancer (16.9 μmol) and theophylline (83.3 μmol) in ethanol (0.3 ml) and water (2.7 ml). This mixture was shaken vigorously for 30 s and then sonicated for 10 min at 40 °C. The control samples were prepared in the same manner without enhancers. As a receptor phase carbonate buffer saline (pH 7.4) was used. About 0.5 h before the experiment the PAMPA system was taken out from the freezer and warm up to the room temperature. The receptor phase (200 μl/well) was pipetted into the upper wells. The donor phase (stable system of dissolved theophylline in enhancer emulsion/microsuspension) was pipetted into the lower ones (300 μl/well). After 5 h 10 μl of the receptor phase was taken from each well and mixed with physiological solution (990 μl). A minimum of four determinations was performed. The samples were stored at −18 °C until analyzed by HPLC.

Analysis of samples for theophylline content was performed, using a Waters Alliance 2695 XE HPLC separation module and a Waters Photodiode Array Detector 2996 (Waters Corp., Milford, MA, USA). A Waters Symmetry® $C_8$ 5 μm, 4.6 mm × 250 mm (Waters Corp., Milford, MA, USA) chromatographic column was also used. The HPLC separation process was monitored by Empower™ 2 Chromatography Data Software, Waters 2009 (Waters Corp., Milford, MA, USA). The mixture of MeCN (HPLC grade, 50.0%) and $H_2O$ (HPLC – Mili-Q Grade, 50.0%) was used as a mobile phase. The total flow of the column was 0.5 ml/min, injection 10 μl, column temperature 25 °C and sample temperature 10 °C. The detection wavelength of 280 nm was chosen. The retention time ($t_R$) of theophylline was 5.07 ± 0.05 min. ERs were calculated as ratios of theophylline flux with and without the enhancer. The results are summarized in Table 2.

### 4.5. In vitro anti-proliferative/cytotoxicity assay

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, trypsin, penicillin and streptomycin were purchased from Sigma (MO, USA); foetal bovine serum (FBS) and Calcein AM from Invitrogen (CA, USA). The cell lines used for screening, T-lymphoblastic leukemia CEM, breast adenocarcinoma MCF7, and human foreskin fibroblasts BJ, were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM medium (Sigma, MO, USA). All media used were supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 10,000 U penicillin and 10 mg/ml streptomycin. The cell lines were maintained under standard cell culture conditions at 37 °C and 5% $CO_2$ in a humid environment. Cells were subcultured twice or three times a week using the standard trypsinization procedure. Suspensions with approximately $1.0 \times 10^5$ cells/ml ($0.5 \times 10^5$ cells/ml for BJ) were distributed in 96-well microtiter plates, and after 12 h of stabilization the tested compounds were added at the desired concentrations in Lutrol F 127. Control cultures were treated with Lutrol F 127 alone, and the final concentration of Lutrol F 127 in the reaction mixture never exceeded 0.5%. In most cases six serial 3-fold dilutions of the test substances were added at time zero in 20 μl aliquots to the microtiter plate wells, and the highest final concentration in the wells was 37 μM. After incubation for 72 h, Calcein AM solution (2 μM, Molecular Probes, Invitrogen, CA, USA) was added, and the cells were incubated for a further hour. The fluorescence from viable cells was then quantified, using a Fluoroskan Ascent fluorometer (Labsystems, Finland). The percentage of surviving cells in each well was calculated from the equation $IC_{50}$ = (OD drug exposed well/mean OD control wells) × 100%. Each compound was tested in triplicate, and the entire test was repeated at least three times. The $IC_{50}$ value, the concentration lethal for 50% of tumor cells of each tested substance, was calculated from the obtained dose response curves [57].

### 4.6. Statistical analysis

All experiments were carried out in triplicate (ERs data for skin and anti-proliferative activity) or quadruplicate (PAMPA). Data were expressed as means ± SD. Differences were evaluated by one-way ANOVA test completed by the Bonferroni's multicomparison test (ORIGIN PRO7). The differences were considered significant at $P = 0.05$. Student's $t$-test was used to detect statistical differences ($P = 0.001$, $P = 0.01$ or $P = 0.05$) between the less active compound and other compounds from the series.

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