Steroids 78 (2013) 435–453

Contents lists available at SciVerse ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

New propanoyloxy derivatives of 5β-cholan-24-oic acid as drug absorption modifiers

Lenka Coufalová [a,*], Lech Mrózek [b], Lucie Rárová [c], Lukáš Plaček [d], Radka Opatřilová [a], Jiří Dohnal [a,e], Katarína Král'ová [f], Oldřich Paleta [g], Vladimír Král [g], Pavel Drašar [g], Josef Jampílek [a,e,*]

[a] Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho 1/3, 612 42 Brno, Czech Republic
[b] BorsodChem MCHZ, s.r.o., Chemicka 1/2039, 709 03 Ostrava-Marianske Hory, Czech Republic
[c] Centre of the Region Hana for Biotechnological and Agricultural Research, Department of Growth Regulators, Faculty of Science, Palacky University, Slechtitelu 11, 783 71 Olomouc, Czech Republic
[d] Pragolab s.r.o., Nad Krocinkou 55/285, 190 00 Prague 9, Czech Republic
[e] Research Institute for Pharmacy and Biochemistry, Lidicka 1879/48, 602 00 Brno, Czech Republic
[f] Institute of Chemistry, Faculty of Natural Sciences, Comenius University, Mlynska dolina Ch-2, 842 15 Bratislava, Slovakia
[g] Institute of Chemical Technology, Technicka 5, 166 28 Prague 6, Czech Republic

ARTICLE INFO

Article history:
Received 17 September 2012
Received in revised form 18 January 2013
Accepted 9 February 2013
Available online 19 February 2013

Keywords:
Cholic acid derivatives
Lipophilicity
Solubility
Transdermal penetration enhancers
Intestinal absorption promoters
In vitro cytotoxicity

ABSTRACT

A series of final twelve propanoyloxy derivatives of 5β-cholan-24-oic acid (*O*-propanoyl derivatives of cholic acid) as potential drug absorption modifiers (skin penetration enhancers, intestinal absorption promoters) was generated by multistep synthesis. 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*), solubility (log *S*), polar surface area (PSA) and molar volume (MV) of the studied compounds were also calculated. All the target compounds were tested for their *in vitro* transdermal penetration effect and as potential intestinal absorption enhancers. The cytotoxicity of all the evaluated compounds was evaluated against normal human skin fibroblast cells. Their anti-proliferative activity was also assessed against human cancer cell lines: T-lymphoblastic leukemia cell line and breast adenocarcinoma cell line. One compound showed selective cytotoxicity against human skin fibroblast cells and another compound possessed the highest cytotoxicity against all the tested cell lines. Only one compound expressed anti-proliferative effect on leukemia 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 moderate cytotoxicity when administered as chemical absorption modifiers. The relationships between the lipophilicity/polarity and the chemical structure of the studied compounds as well as the relationships between their chemical structure and enhancement effect 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 results, over the last ten years, the number of poorly soluble drugs has steadily increased. Literature states that about 60% of all drugs coming directly from synthesis are nowadays poorly soluble. Compounds with insufficient solubility carry a higher risk of failure during discovery and development, since insufficient solubility may compromise other property assays, mask additional undesirable properties, influence both pharmacokinetic and pharmacodynamic properties of the compound and finally may affect the developability of the compound. Poor solubility in water correlates with poor bioavailability. If there is no way to improve drug solubility, it will not be able to be absorbed from the gastrointestinal tract into the bloodstream and reach the site of action [1,2].

Modification/optimization of unfavorable physico-chemical properties of these drugs is possible through increasing their water solubility or improving permeability. Generally, these strategies are based on a few fundamental concepts: change of ionizability, lipophilicity, polarity or change of hydrogen bond donors or acceptors. Pre-formulation/formulation can be another and mostly successful strategy for improving aqueous solubility and/or permeability and

* Corresponding authors. Tel.: +420 72125 6271 (J. Jampílek), +420 54156 2923 (L. Coufalová).
E-mail addresses: coufaloval@vfu.cz (L. Coufalová), josef.jampilek@gmail.com (J. Jampílek).

0039-128X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.steroids.2013.02.001

subsequently bioavailability. For example, selection of a suitable salt, particle size reduction (till nano size) connected with an increase of the surface area, change of polymorphic forms, selection of appropriate excipients to function as solubilizers/transporters (surfactants or pharmaceutical complexing agents, permeability enhancers) can be used for the oral dosage form [3]. One of possibilities to modify/optimize drug unfavorable physico-chemical properties is to use absorption modifiers, e.g. bile acid derivatives [4–8].

Cholic acid is one of the most important human bile acids. Bile acid derivatives/analogs are an important class of compounds with a range of pharmacological activities. 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 [8–17]. Cholic acid derivatives were studied also as transdermal penetration enhancers [8,18–26]. The reason for their effect may be their specific features in solvation and self-assembly [27–35].

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 [6,26]. Transdermal penetration enhancers are special pharmaceutical excipients that interact with skin components, to increase absorption 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 [26]. 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 [26].

The multistep synthesis of a series of long chain propanoyloxy derivatives of 5β-cholan-24-oic acid prepared as new potential transdermal chemical penetration enhancers and/or as intestinal drug absorption modifiers is described herein. This study is based on recently published results of the enhancement effect of cholic acid derivatives *O*-substituted by mono-, di- and tri-acyl C_4, C_{10} and C_{16} linear saturated chains [8]. The design of all new long-chain substituents of hydroxyl moieties of cholic acid is based on isosteric replacement of oxygen for carbon with the aim to increase the polar surface area and thus influence lipophilicity, polarity and interactions with components in the SC as well as in the phospholipid membrane.

Various acyl chain lengths (diethyleneglycol, decanoyl-diethyleneglycol, bis(decanoyl)-glycerol, tocopherol) as well as different degrees (mono-, di- and tri-substituted derivatives) and positions (3 and/or 7 and/or 12 positions) of substitution of the compounds discussed in this paper impart specific solvation and surface features influencing structural modifications of biological membranes. 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 [8,26,36–38]. Therefore both the experimentally determined lipophilicity (logarithm of the retention factor, R_M values) and calculated lipophilicity (log *P*) as well as other molecular descriptors of all the final compounds were investigated in this article.

Primary *in vitro* screening of transdermal penetration effect of all the final synthesized compounds was performed using a Franz cell [26,39], and intestinal absorption enhancement effect was evaluated using PAMPA (parallel artificial membrane permeability assays) experiments [40–42]. 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 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 effect) (SAR) are discussed in this article.

2. Result and discussion

2.1. Chemistry

Various synthetic pathways leading to preparation of acyl chlorides **4**, **8**, **13** and **16** are described in Schemes 1–4. The hydroxyl moieties of benzyl 3α,7α,12α-trihydroxy-5β-cholanoate (benzyl cholate, **17**) were subsequently acylated by those acyl chlorides [8,43].

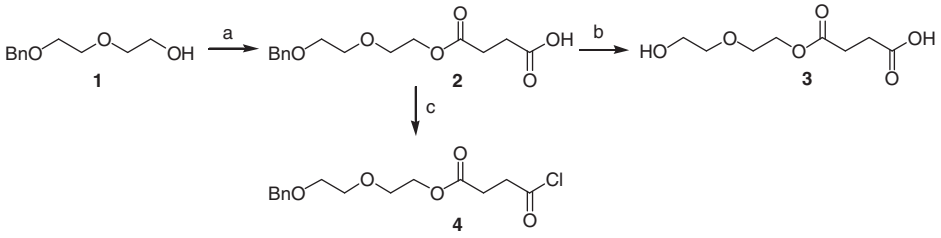
The reaction of mono benzyl alcohol **1** with succinic acid anhydride provided benzylated acid **2** [44] and subsequent hydrogenolysis of the benzyl ether group in the presence of palladium catalyst yielded hydroxy acid **3**. The reaction of benzylated acid **2** with oxalyl chloride gave acyl chloride **4** that was used for acylation of benzyl cholate (**17**) and preparation of *O*-substituted cholic acid derivatives, see Scheme 1.

The free hydroxyl group of monobenzyl alcohol **1** was acylated with decanoyl chloride providing benzyl derivative **5**. Hydrogenolysis of this compound afforded hydroxy derivative **6**, which by reaction with succinic acid anhydride led to **7**. This acid was treated with oxalyl chloride to give acyl chloride **8**, see Scheme 2.

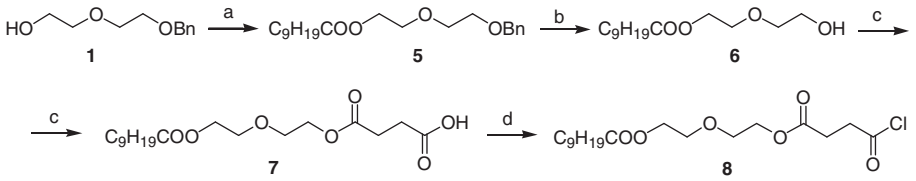
Acylation of benzylated glycerol **9** [45] with decanoyl chloride gave ester **10**. Cleavage of the benzyl group in **10** led to alcohol **11**, which by treatment with succinic acid anhydride gave **12**. The latter by reaction with oxalyl chloride afforded acid chloride **13**, see Scheme 3.

(+/−)-α-Tocopherol hemisuccinate **15** was prepared by a reaction of (+/−)-α-tocopherol (**14**) with succinic acid anhydride [46] and then converted to acyl chloride **16** [47] by the standard procedure with oxalyl chloride, see Scheme 4.

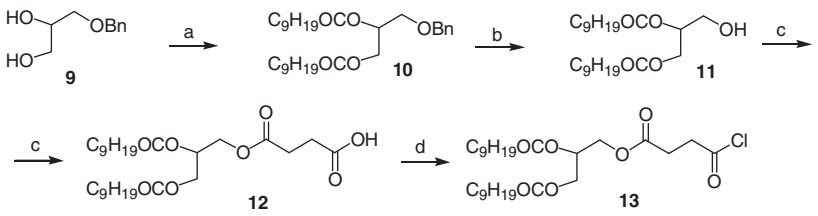
The reaction of benzyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate (benzyl cholate, **17**) [43] with acyl chlorides in the presence



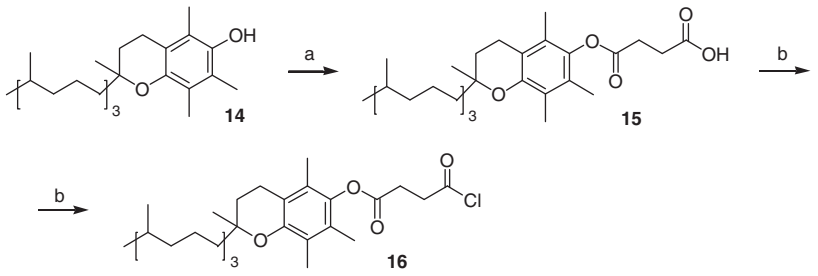
Scheme 1. Synthesis of 3-[2-(2-hydroxyethoxy)ethoxycarbonyl]propanoic acid (**3**) and 3-[2-(2-benzyloxyethoxy)ethoxycarbonyl]propanoyl chloride (**4**): (a) succinic acid anhydride, DMAP, CH_2Cl_2, 22 °C; (b) H_2, Pd/C, MeOH, 22 °C; (c) $(COCl)_2$, toluene, 22 °C.



Scheme 2. Synthesis of 3-[2-(2-decanoyloxyethoxy)ethoxycarbonyl]propanoic acid (**7**) and 3-[2-(2-decanoyloxyethoxy)ethoxycarbonyl]propanoyl chloride (**8**): (a) $C_9H_{19}COCl$, pyridine, DMAP, CH_2Cl_2, 22 °C; (b) H_2, Pd/C, AcOEt, 40 °C; (c) succinic acid anhydride, DMAP, CH_2Cl_2, 22 °C; (d) $(COCl)_2$, toluene, DMF, 22 °C.



Scheme 3. Synthesis of 3-[2,3-bis(decanoyloxy)propyloxycarbonyl]propanoic acid (**12**) and 3-[2,3-bis(decanoyloxy)propyloxycarbonyl]propanoyl chloride (**13**): (a) $C_9H_{19}COCl$, pyridine, DMAP, CH_2Cl_2, 22 °C; (b) H_2, Pd/C, 22 °C; (c) succinic acid anhydride, DMAP, CH_2Cl_2, 22 °C; (d) $(COCl)_2$, toluene, 22 °C.



Scheme 4. Synthesis of (+/−)-α-tocopherol hemisuccinate (**15**) and 3-{[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl]oxycarbonyl}propanoyl chloride (**16**): (a) succinic acid anhydride, DMAP, CH_2Cl_2, 22 °C; (b) $(COCl)_2$, toluene, 22 °C.

of 4-dimethylaminopyridine (DMAP) under mild conditions followed by hydrogenolysis of protecting groups provided 3α-mono(acyloxy) derivatives **19**, **26**, **32** and **38**, see Scheme 5.

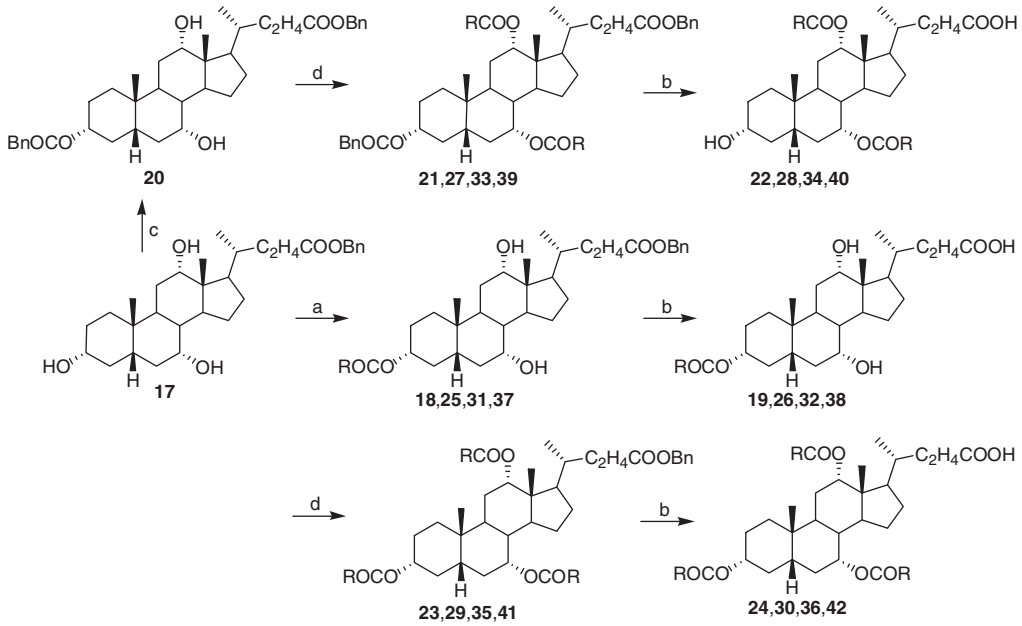
The preparation of 7α,12α-bis(acyloxy) derivatives **22**, **28**, **34** and **40** included the protection of 3α-hydroxy group of benzyl ester **17** by the benzyloxycarbonyl group, resulting in compound **20** [8] and subsequent acylation of both free 7α,12α-dihydroxy moieties. Finally, the benzyloxycarbonyl group was selectively cleaved by hydrogenolysis on palladium catalyst with simultaneous deprotection of the carboxy group. This method, including the protection of 3α-hydroxy group in ester **17** by the benzyloxycarbonyl moiety, was developed for the selective preparation of 7α,12α-bis(decanoyloxy)-3α-hydroxy-5β-cholan-24-oic acid [8]. Hydrogenolysis of the benzylether group in diethyleneglycol series was successfully performed only in hydrogen atmosphere on palladium catalyst, see Scheme 5.

3α,7α,12α-Tris(acyloxy) derivatives **24**, **30**, **36** and **42** were prepared by a reaction of benzyl cholate **17** with acyl chlorides in the presence of benzyltriethylammonium chloride (BTEAC) as a phase transfer catalyst with subsequent hydrogenolysis of the protecting group, see Scheme 5.

2.2. Lipophilicity and solubility of the prepared compounds

The well-known physico-chemical descriptors such as lipophilicity, solubility, polar surface area and molar volume are largely employed during quantitative structure–activity relationship analysis. In a number of studies examining penetration enhancement, the relationship between lipophilicity and other descriptors and their potency of penetration enhancement was investigated [26]. In the current investigation we examined both the calculated lipophilicity (log *P*) and the experimental lipophilicity R_M data [48] as well as calculated solubility (log *S*), polar surface area (PSA) and molar volume (MV) of all compounds to determine if these factors play a role in their penetration enhancement potency.

However, the algorithms used in calculations of descriptors, especially log *P* and log *S*, in principle do not take into account the aspects of configuration and conformation. The positions of substituents are implemented in the calculation protocol, however, the prediction depends on the number of similar compounds in the database. Hence, having in mind the importance of permeability and solubility (polarity) for biological activity [49], this study compares calculated log *P* and log *S* values with the related experimental parameter, the R_M (linear physico-chemical descriptor in TLC) values of the final derivatives, as a measure of their lipophilicity. The R_M values were determined by reverse phase thin layer chromatography (RP-TLC). R_M represents the logarithmic ratio of distances between solute spot and solvent front on the one hand and the migration distance of the solute on the other hand [48]. 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 [50] with experimental material from ca. 6000 compounds databased. Nevertheless, it is important to note that all



18,**21**,**23**	R= $Bn(OCH_2CH_2)_2OCOCH_2CH_2$
19,**22**,**24**	R= $H(OCH_2CH_2)_2OCOCH_2CH_2$
25,**26**,**27**,**28**,**29**,**30**	R= $C_9H_{19}CO(OCH_2CH_2)_2OCOCH_2CH_2$
31,**32**,**33**,**34**,**35**,**36**	R= $C_9H_{19}OCOCH_2CH(C_9H_{19}OCO)CH_2OCOCH_2CH_2$
37,**38**,**39**,**40**,**41**,**42**	R= tocopherylOCOCH$_2$CH$_2$

Scheme 5. Synthesis of target mono-, bis- and tris(acyl) derivatives (a) RCOCl, DMAP, CH_2Cl_2, 22 °C; (b) $HCOONH_4$, Pd/C or H_2, Pd/C, 40 °C; (c) BnOCOCl, pyridine, 0 °C; (d) RCOCl, BTEAC, CaH_2, toluene, 112 °C.

log *P* and log *S* values calculated using various programmes should be understood as approximate. The results are shown in Table 1 and illustrated in Fig. 1.

The compounds under investigation could be divided into four groups based on the substituents on the steroidal scaffold: Group 1 includes diethyleneglycols **19**, **22** and **24** and starting acid **3**; Group 2 contains decanoyl-diethyleneglycols **26**, **28** and **30** and starting acid **7**; Group 3 is composed of bis(decanoyl)-glycerols **32**, **34** and **36** and starting acid **12**; Group 4 includes tocopherol substituted compounds **38**, **40** and **42** and starting acid **15**.

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 as follows: Group 1 (diethyleneglycols) < Group 2 (decanoyl-diethyleneglycols) < Group 3 [bis(decanoyl)-glycerols] < Group 4 (tocopherol derivatives). This dependence is approximately linear. As expected, compound **42** possessed the highest lipophilicity. Contrary to all expectations and calculated log *P* and log *S* data, compound **24** showed the lowest lipophilicity (R_M). According to ACD, the lowest lipophilicity was computed for compound **3** (log $P = -0.42$), and the lowest polarity/solubility was computed for compound **40** (log $S = -11.0$). Noteworthy dissimilarity between Group 1 and other groups can be found. Starting acid **3** in Group 1 possessed higher lipophilicity than substituted 5β-cholan-24-oic acid derivatives **19**, **22** and **24** within this group, and it can be stated that within this series lipophilicity decreased with substitution, *i.e.* that lipophilicity increased subsequently: **24** (trisubstituted) < **22** (disubstituted) < **19** (monosubstituted) < **3** (starting acid). In general, it can be stated for Groups 2–4 that the starting acid showed the lowest lipophilicity (according to the R_M values) together with the highest solubility (according to the log *S* values), *i.e.* that lipophilicity increased subsequently: starting acid < mono(substituted) compound < bis(substituted) compound < tris(substituted) compound.

As expected, the highest solubility/polarity (log *S*) was shown by Group 1, and polarity decreased with the chain length: Group 1 > Group 2 > Group 3 > Group 4, nevertheless interesting anomalies were observed. Within Group 1 solubility decreased with the decreasing number of substituents, *i.e.* **3** (acid) > **24** (tris) > **22** (bis) > **19** (mono), while within Groups 2–4 bis(substituted) derivatives **28**, **34** and **40** unexpectedly expressed much lower polarity/solubility than the mono(substituted) (Group 2 and Group 3) or tris(substituted) (Group 4) compounds. This trend is also reflected in poor correlation of polarity/solubility (log *S* data) with the experimentally determined lipophilicity (R_M), see Fig. 1.

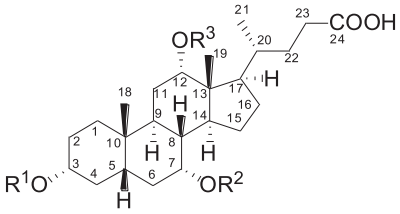
All these differences could be probably explained by different solvation and other types of non-covalent interactions (especially van der Waals interactions, hydrogen bonds, dipole–dipole interactions) of hydroxyl moieties in the $C_{(3)}$, $C_{(7)}$ and $C_{(12)}$ positions of the steroidal skeleton as well as free hydroxyl moieties in aliphatic chains in the case of diethyleneglycols (Group 1). These interactions are extremely important for the significantly different enhancer effect of these bis(substituted) derivatives, see below. All these unexpected differences influence enhancement effect. It is not possible to explain these specific properties/behavior of bis(substituted) derivatives using PSA that logically increases the number of polar atoms in the molecule.

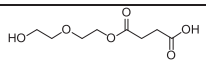
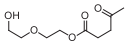
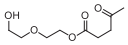
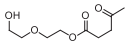
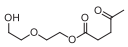
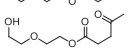
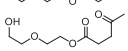
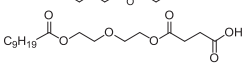
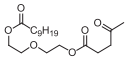
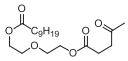
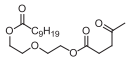
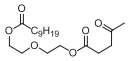
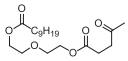
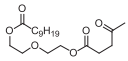
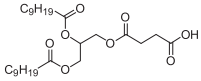
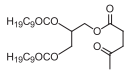
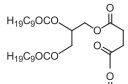
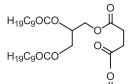
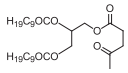
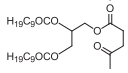
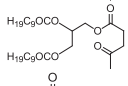
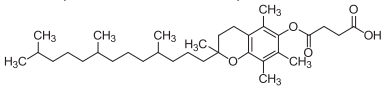
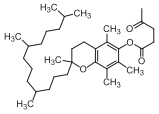
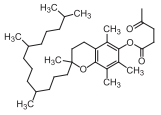
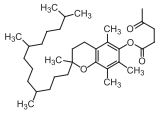
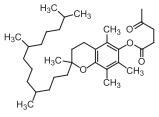
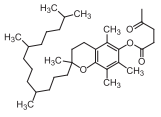
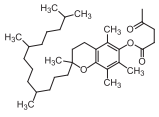
Generally, it could be concluded that the prediction power of used experimental R_M or calculated values for extrapolation of transport modifications may be a good tool for searching potential absorption modifiers.

2.3. In vitro screening of transdermal penetration-enhancing effect

The penetration enhancement effect of the prepared compounds was evaluated using theophylline as a model penetrant and propylene glycol/water 1:1 (v/v) as a donor vehicle by means of a Franz cell [26,39]. Theophylline was used as a model drug of medium polarity (log $P = -0.06$) [51,52], as it has been extensively studied in transdermal penetration experiments [53,54]. 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

Table 1
Comparison of determined R_m with calculated lipophilicity (log *P*) and solubility (log *S*) values; calculated values of polar surface area (PSA [Å^2]) and molar volume (MV [cm^3]). (Log *P*, log *S* and MV were calculated using ACD ver. 12, PSA was calculated using CambrigeSoftware ChemBio3D, ver. 12.).



Comp.	R^1	R^2	R^3	R_M	log *P*	log *S*	PSA [Å^2]	MV [cm^3]
3				-0.44	-0.42	0.69	93.06	163
19		H	H	-0.56	2.97	-1.75	159.82	479
22	H			-0.61	3.05	-1.59	221.65	619
24				-0.69	3.13	-1.04	283.48	757
7				-0.61	4.47	-0.28	99.13	334
26		H	H	-0.35	7.86	-3.90	165.89	645
28	H			-0.15	12.85	-4.52	233.79	949
30				0.21	17.83	-3.76	301.69	1251
12				-0.26	8.53	-2.92	116.20	480
32		H	H	0.01	11.92	-5.74	182.96	786
34	H			0.49	20.96	-6.10	267.93	1229
36				1.25	30.01	-3.03	352.90	1670
15				0.25	10.16	-5.84	72.83	530
38		H	H	0.57	14.12	-8.55	139.59	825
40	H			1.51	25.35	-11.00	181.19	1307
42				2.42	36.58	-9.31	222.79	1786
cholic acid	–	–	–	-0.55	2.88	-1.39	97.99	345

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 [55–57]. Porcine ear skin was selected for initial evaluation of enhancement effect of prepared compounds as this tissue is a suitable *in vitro* model of human skin [58,59]. 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 [60]. Nevertheless, for testing of hydrophobic penetrants, dermatomed skin has been recommended [61]. The skin penetration experiments were performed using static Franz diffusion cells [39].

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

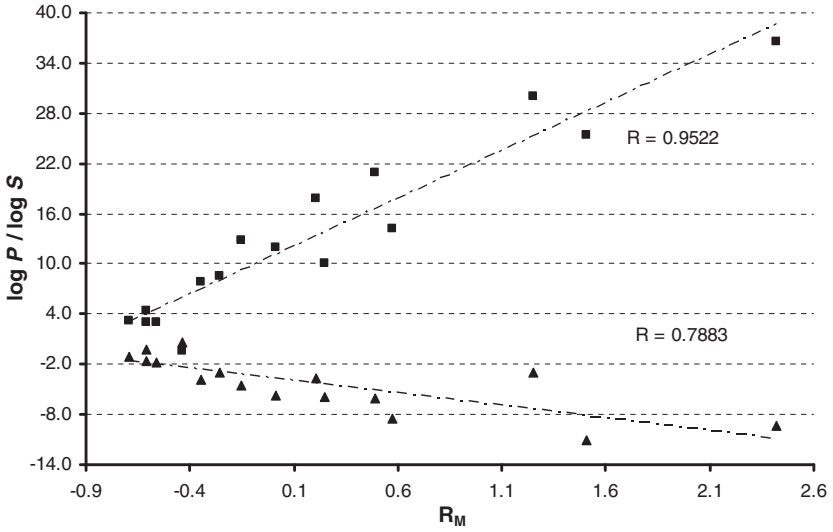


Fig. 1. Comparison of log *P* (squares, *y*-axis, positive values) and/or log *S* (triangles, *y*-axis, negative values) values (lipo/hydrophilicity properties) computed using two programs (ACD/LogP DB, ACD/LogS DB) with the determined R_M values.

lipid bilayer "simulating" intestine wall, whereas the ear porcine skin is a much more complex naturally constructed barrier.

The effect of target 5β-cholan-24-oic acid derivatives on penetration of theophylline through the porcine skin 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 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 [8,26,36–38,51–57]. 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 [5,6].

The highest enhancement ratios (ERs) were obtained with **19** [mono(diethyleneglycol)] and **24** [tris(diethyleneglycol)] derivatives with ERs of 2.63 and 2.50, respectively, see Table 2, Fig. 2 (dotted columns).

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 $P = 0.05$. Whereas no significant differences between transdermal ER of some tested acids were found, namely between **7** and **15**, considerable differences between determined ER were estimated for studied steroid-like compounds. Due to structural difference the starting acids cannot be compared with derivatives of 5β-cholan-24-oic acid.

Generally, it can be postulated that the highest enhancement effect within the individual series was shown by mono(substituted) and tris(substituted) derivatives, whereas bis(substituted) derivatives possessed the lowest enhancing effect, *i.e.* penetration-enhancement effect decreased as follows: mono(substituted) > tris(substituted) > bis(substituted) > starting acid, except Group 3, where starting acid **12** showed higher ER value than bis(substituted) derivative **34**. While the most active compounds were from Group 1, it seems that decanoyl-diethyleneglycol (Group 2, compounds **26**, **28**, **30**) is the most advantageous substituent, because this series expressed the highest well-balanced enhancement ratios without any evident dependence of enhancement effect on the position of the substituent in the steroid skeleton. The penetration-enhancement effect of both Group 3 [bis(decanoyl)-glycerols] and Group 4 (tocopherols) is low in comparison with Group 1 and Group 2. This observation confirms the fact that highly lipophilic branched long-chains or chains containing cycles (aromatic or saturated ring) are not preferred substituents of transdermal chemical penetration enhancers [26].

From the results presented in Table 2 and Fig. 2 (dotted columns) it is evident that transdermal penetration-enhancement effect is strongly dependent on balanced lipo/hydrophilic properties (lipophilicity/solubility-polarity) of individual discussed enhancers due to their optimal interaction with skin components. The dependences of the transdermal penetration-enhancement effect (ER) on the lipophilicity expressed by experimentally determined R_M values or by log *P* as well as on the molar volume (MV [cm^3]) for the studied steroid-like compounds are shown in Figs. 3A–C.

From the above mentioned Figs. 3A–C, where starting acids (**3**, **7**, **12**, **15**) are not shown, it is evident that for 3α-mono(substituted) and 3α,7α,12α-tris(substituted) compounds the transdermal ER enhancement effect linearly decreases with the increasing lipophilicity or with the increasing molar volume. After the elimination of compound **32** (that showed lower ER than expected) the correlation coefficient for linear regression including the rest seven compounds was −0.967 for R_M, −0.970 for log *P* and −0.923 for MV. On the other hand, no definite dependence of ER of 7α,12α-bis(substituted) compounds on the above mentioned descriptors was found. The compounds **22**, **34** and **40** showed significantly lower enhancement effect than mono(substituted) and tris(substituted) derivatives, but it is problematic to explain the reason of the too high effectiveness of compound **28**.

In our previous paper [8] we found that the dependence of ER enhancing effect on log *S* was a mirror image to the dependence of transdermal ER on lipophilicity. This was also confirmed in present study (figure not shown), only with lower correlation coefficients. The definite dependence of transdermal ER on PSA (see Fig. 3D) cannot be determined, however from Table 1 it can be concluded that PSA value approx. 160 $Å^2$ [compound **19**, mono(diethyleneglycol)] is the most advantageous for the highest enhancement effect within the discussed types of substituents, nevertheless within tris(substituted) derivatives secondary maximum can be found [PSA about 380 $Å^2$ for compound **24**, tris(diethyleneglycol)].

The above described molecular descriptors characterize properties of the compounds, nevertheless a mechanism of the higher

Table 2
Enhancement ratios (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 (T-lymphoblastic leukemia CEM, breast adenocarcinoma MCF7, human foreskin fibroblasts BJ). ERs data for skin are expressed as mean ± SD (n = 5 experiments), ERs data for PAMPA are expressed as mean ± SD (n = 4 experiments) and anti-proliferative activity of the compounds are expressed as mean ± SD (n = 3 experiments). The means followed by different letters are significantly different at P = 0.05. The starting acids were compared separately from the final steroid-like compounds.

Comp.	ERs		Cell lines IC_{50} [μmol/l]		
	Skin	PAMPA	CEM	MCF7	BJ
3	1.88 ± 0.11^{y}	2.27 ± 0.19^{x}	>37	>37	>37
19	2.63 ± 0.47^{e}	2.45 ± 0.20^{bc}	>37	>37	>37
22	1.54 ± 0.25^{ab}	2.66 ± 0.05^{cde}	>37	>37	>37
24	2.50 ± 0.19^{e}	2.75 ± 0.24^{e}	>37	>37	>37
7	1.47 ± 0.26^{x}	3.03 ± 0.10^{y}	>37	>37	>37
26	2.41 ± 0.07^{e}	2.72 ± 0.09^{de}	>37	>37	>37
28	2.28 ± 0.14^{de}	2.86 ± 0.03^{e}	26.6 ± 2.9^{a}	>37	>37
30	2.36 ± 0.24^{de}	2.68 ± 0.15^{cde}	>37	>37	18.5 ± 1.4^{a}
12	1.60 ± 0.18^{xy}	2.43 ± 0.19^{x}	>37	>37	>37
32	2.01 ± 0.20^{cd}	2.23 ± 0.08^{a}	>37	>37	>37
34	1.20 ± 0.08^{a}	2.67 ± 0.17^{cde}	>37	>37	>37
36	1.99 ± 0.41^{cd}	2.53 ± 0.10^{bcd}	>37	>37	>37
15	1.50 ± 0.20^{x}	2.33 ± 0.09^{x}	26.9 ± 7.7^{a}	35.8 ± 3.6	31.8 ± 3.8^{b}
38	2.26 ± 0.12^{de}	2.23 ± 0.22^{a}	>37	>37	>37
40	1.61 ± 0.20^{b}	$2.52 \pm 0.18b^{cd}$	>37	>37	>37
42	1.85 ± 0.25^{bc}	2.36 ± 0.10^{ab}	>37	>37	>37

enhancement effect of 3α-mono(substituted) and 3α,7α,12α-tris(substituted) derivatives would be also explained by substitution of $C_{(3)}$ position of bile acid by an aliphatic chain, which can simulate 3-*O*-cholesterol-esters that belong among major skin components and thus, due to structural similarity, the discussed compounds can easier intercalate between skin components in the SC and disrupt ceramide–ceramide, ceramide–cholesterol or cholesterol–cholesterol bonds and, by doing this, form a "channel" in the SC. Conversely, the free hydroxyl moiety in $C_{(3)}$ position of bile acid (7α,12α-bis(substituted) derivatives) can interact with skin components, which can result in more impermeable SC [26,62].

2.4. In vitro screening of intestinal absorption-enhancing effect (PAMPA experiments)

Parallel artificial membrane permeability assays (PAMPA) have become a very useful and quite cheap tool for predicting intestinal drug permeability and are well-suited as a ranking tool for the assessment of compounds with passive intestinal transport mechanisms. An absorption study of binary mixtures or final formulations is also possible on PAMPA plates [40–42].

The effect of target 5β-cholan-24-oic acid derivatives on the penetration of theophylline through the artificial polyvinylidene fluoride (PVDF) PAMPA membrane 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 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 [36–38]. Only if some of evaluated potential enhancers expressed significant enhancer effect, 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 the starting acids and especially bis(diethyleneglycol) derivative **22** and bis[bis(decanoyl)-glycerol] derivative **34**.

The highest enhancement ratios (ERs) were obtained for compounds **28** [bis(decanoyl-diethyleneglycol)], **24** [tris(diethyleneglycol)] and **26** [mono(decanoyl-diethyleneglycol)]: 2.86, 2.75 and 2.72, respectively. 3-[2-(2-Decanoyloxyethoxy)ethoxycarbonyl]propanoic acid (**7**) expressed the highest intestinal absorption-promoting effect (ER = 3.03). According to the above presented data, see Table 2, Fig. 2 (hatched columns), it can be concluded that most discussed compounds showed noteworthy intestinal absorption-enhancing effect. It can be stated that trends of intestinal permeability enhancement effect in this study are contrary to those of transdermal enhancement effect, as also discussed in [8]. While transdermal enhancement effect was the highest for mono(substituted) and tris(substituted) derivatives and the lowest for bis(substituted) compounds, in PAMPA experiments bis(substituted) compounds showed high activities within individual series, see Table 2, Fig. 2 (hatched 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 results of the 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

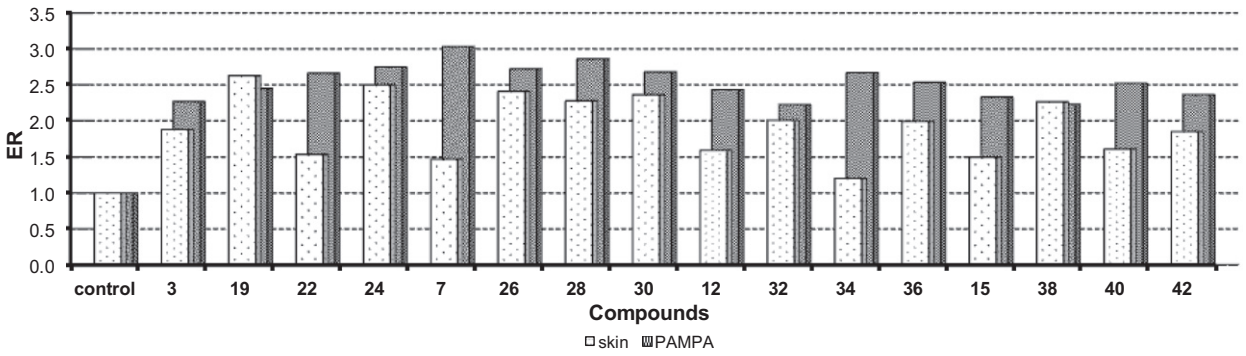


Fig. 2. Enhancement ratios (ERs) through porcine ear skin and PAMPA of the prepared target compounds. Control experiments used theophylline in the donor vehicle without any enhancer. ERs data for skin were calculated from 5 experiments, ERs data for PAMPA were calculated from 4 experiments.

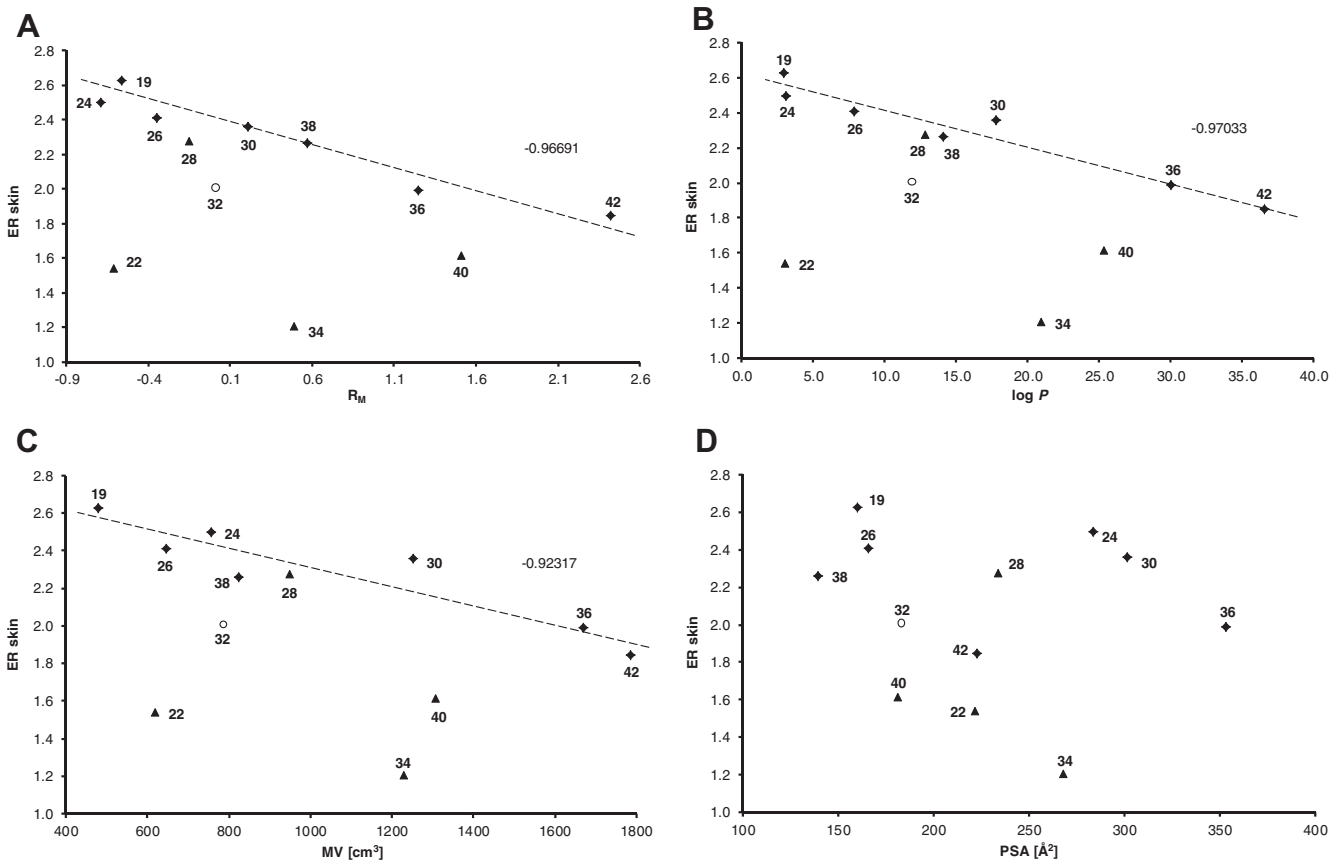


Fig. 3. The dependences of transdermal penetration-enhancement effect ER on the lipophilicity R_M (Fig. 3A), log P (Fig. 3B), molar volume (MV [cm^3]) (Fig. 3C) and polar surface area (PSA [Å2]) (Fig. 3D) of the studied steroid-like target compounds.

at $P = 0.05$. Whereas no significant differences between intestinal ER of some tested acids were found, namely between **3**, **12** and **15**, considerable differences between determined ER were estimated for studied steroid-like compounds. As mentioned above, the starting acids cannot be compared with derivatives of 5β-cholan-24-oic acid due to structural difference.

Based on the results, decanoyl-diethyleneglycol (Group 2) seems to be the most advantageous substituent of hydroxyl moieties of cholic acid. The intestinal absorption-enhancing effect depending on substituents decreased subsequently: Group 2 (decanoyl-diethyleneglycols) > Group 1 (diethyleneglycols) > Group 3 [bis(decanoyl)-glycerols] > Group 4 (tocopherols). While all compounds within Group 2 showed effect, within Group 3 and Group 4 only di- and tri-substituted derivatives can be considered to have effect, *i.e.* compounds with high lipophilicity and low polarity within individual series. Starting acids **12** and **15** were more active than mono(substituted) derivatives **32** and **38**. A totally opposite trend can be observed within Group 1. Tris(substituted) derivative **24** showed the highest intestinal absorption-enhancing effect, but this compound possessed the highest hydrophilicity as well as polarity/solubility. Similarly to the results obtained for ER-skin penetration enhancing effect, also intestinal penetration enhancing effect significantly depended on the lipophilicity and the molar volume of tested steroid-like compounds (Fig. 4A–C).

From the mentioned above Figs. 4A–C, where starting acids (**3**, **7**, **12**, **15**) are not shown, it is evident that for di- and tri-substituted compounds the dependence of intestinal ER enhancement effect on the lipophilicity or molar volume shows bilinear course. The highest ER penetration enhancing effect was observed for compound **28** ($R_M = -0.15$, log $P = 12.85$, MV = 949 cm^3), and the correlation coefficients evaluated for descending part of the dependence were −0.973 (R_M), −0.970 (log P) and −0.920 (MV). The mono-substituted compounds **19**, **32** and **38** showed significantly lower enhancement effect than bis(substituted) and tris(substituted) compounds. Considerably higher efficiency was shown by compound **26**. However, from four ER values that are available for mono-substituted compounds it is not possible to determine clearly whether in this case the dependence would also be biphasic.

The definite dependence of ER on PSA (see Fig. 4D) the same as the definite dependence of transdermal penetration-enhancement effect ER on PSA (Fig. 3D) cannot be determined; however from Table 1 it can be concluded that the optimum of PSA value was approx. 234 Å^2 [compound **28**, bis(decanoyl-diethyleneglycol)].

As all the decanoyl-diethyleneglycol (Group 2) and diethyleneglycol derivatives (Group 1) expressed balanced transdermal and intestinal permeability enhancement effect, contrary to the rest of the discussed compounds, it can be suggested that these compounds as amphiphilic compounds showed high hydrotropic effect, and they can reduce the resistance of membranes to drug diffusion [63,64].

The higher intestinal absorption enhancement effect of the discussed compounds compared with the transdermal penetration-enhancement effect, especially low transdermal enhancement effect of bis(substituted) derivatives, can be also explained by a "cut-off" effect [8,65,66]. This "cut-off" effect can be observed for *n*-alkyl substituted series of amphiphilic compounds. The inversion of effect is connected with the structural diversity of the skin and the 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 effect. It is suggested that the lateral expansion of the phospholipid bilayer of biological membranes caused by the intercalation of long-chain amphiphilic molecules between phospholipid molecules and the mismatch between their hydrocarbon chains lengths result in the creation of free volume in the bilayer hydrophobic region [66]. 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 neighboring lipids, as well as on the partition coefficient of the surfactants [65,67]. 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 the alkyl chain but also a high concentration of the surfactant in the membrane due to the suitable value of the surfactant partition coefficient [68].

2.5. In vitro cytotoxicity/anti-proliferative assays

To evaluate the cytotoxic properties of the 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 **28** [bis(decanoyl-diethyleneglycol)] and **15** (3-{[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl]oxycarbonyl}propanoic acid) resulted in dose-dependent inhibition of cancer cells viability. Compound **15** showed the highest cytotoxicity against all the tested cell lines and compound **30** [tris(decanoyl-diethyleneglycol)] showed selective cytotoxicity against human skin fibroblast cells. Based on these observations both compounds cannot be used as penetration modifiers. But compound **28** expressed cytotoxicity effect only against leukemia cells without affecting the growth of normal BJ cells, which should be promising in potential development of new anti-proliferative drugs.

Other discussed tested 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 target compounds will lead to limited cytotoxicity if they are used *in vivo* as absorption modifiers.

In the cytotoxicity test against BJ, the IC_{50} value of compound **30** (18.5 μmol/l) significantly differed from the IC_{50} value of compound **15** (31.8 μmol/l) at the probability level $P = 0.05$. On the other hand, no significant difference was found between IC_{50} values against CEM cells of compound **28** (26.6 μmol/l) and compound **15** (26.9 μmol/l) (see Table 2).

3. Conclusions

In this paper a series of twelve final propanoyloxy derivatives of 5β-cholan-24-oic acid with diethyleneglycol, decanoyl-diethyleneglycol, bis(decanoyl)-glycerol and tocopherol chains were prepared by multistep synthesis as new 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 as well as polar surface areas and molar volumes of the target compounds were compared with the membrane permeability influence

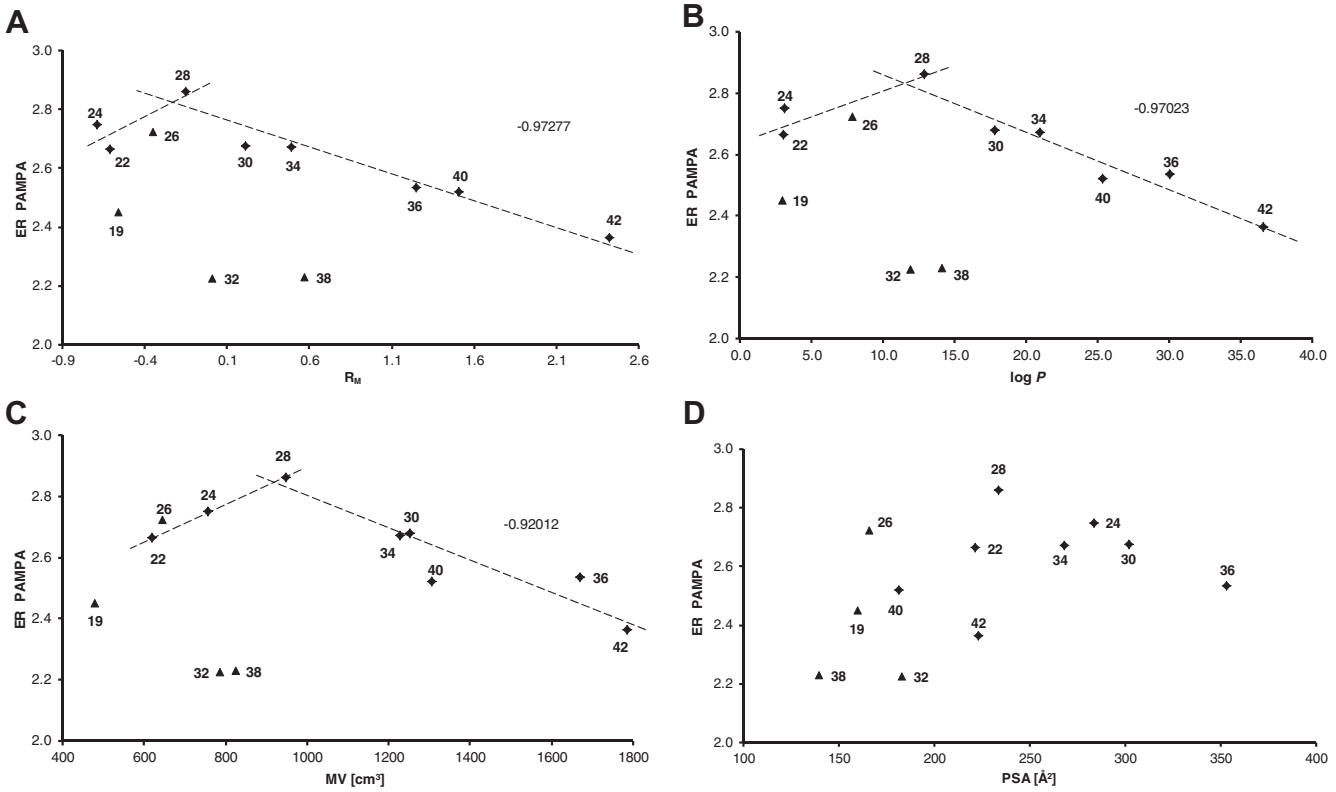


Fig. 4. The dependences of intestinal absorption enhancement effect ER on the lipophilicity R_M (Fig. 4A), log *P* (Fig. 4B), molar volume (MV [cm^3]) (Fig. 4C) and polar surface area (PSA [Å2]) (Fig. 4D) of the studied steroid-like target compounds.

studied by the PAMPA method and transdermal absorption *in vitro*. 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. All these derivatives, based on isosteric replacement of oxygen for carbon in the alkyl chain, expressed much higher enhancement effect than the primary C_4, C_{10} and C_{16} linear acyloxy derivatives of 5β-cholic acid. The highest transdermal penetration-enhancement effect in this study was exhibited by compounds **19** [mono(diethyleneglycol)] and **24** [tris(diethyleneglycol)], while the highest intestinal absorption enhancement effect was expressed by compounds **28** [bis(decanoyl-diethyleneglycol)], **24** [tris(diethyleneglycol)] and **26** [mono(decanoyl-diethyleneglycol)]. Generally, it can be postulated that the highest transdermal enhancement effect within the individual series was shown by mono-substituted derivatives, whereas bis(substituted) derivatives demonstrated the highest intestinal permeability enhancement effect. Decanoyl-diethyleneglycol and/or diethyleneglycol are the most preferred substituents of cholic acid hydroxyl moieties. All the compounds were additionally evaluated their cytotoxicity against two human cancer cell lines and against normal human skin fibroblasts. Compound **28** showed anti-proliferative effect on cancer cells without affecting the growth of normal cells, which suggests that this compound would have low cytotoxic side-effects when administered as an enhancer/excipient. Ten other target compounds exhibited limited cytotoxicity, so they could be used as absorption modifiers. The obtained data provided important parameters for correlations between solubility/lipophilicity and enhancement effect, 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 flash chromatography (F_c). 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 ^{1}H- and ^{13}C NMR spectra were recorded on a Bruker Avance-250 FT-NMR spectrometer (250 MHz for ^{1}H-NMR and 62.9 MHz for ^{13}C NMR, Bruker Comp., Karlsruhe, Germany). Chemicals shifts are reported in ppm (δ) 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.

4.1.1. 3-[2-(2-Benzyloxyethoxy)ethoxycarbonyl]propanoic acid (**2**)

2-(2-Benzyloxyethoxy)ethanol [44] (**1**, 12.96 g, 66 mmol) and succinic acid anhydride (8.12 g, 81.1 mmol) were dissolved in CH_2Cl_2 (200 ml), and DMAP (9.95 g, 81.4 mmol) was added. The solution was stirred at ambient temperature for 1 h. Then AcOH (1 ml) was added and the mixture was evaporated under reduced pressure. The crude product was purified by gradient F_c (toluene/AcOEt/AcOH 150:50:2 to 25:75:1). This provided an oily liquid. Yield: 10 g (51%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 2.64 (s, 4H, $COCH_2$-CH_2CO), 3.60–3.72 (m, 6H, $PhCH_2OCH_2CH_2OCH_2CH_2$), 4.22–4.30 (m, 2H, CH_2CH_2OCO), 4.57 (s, 2H, $PhCH_2O$), 7.30–7.37 (m, 5H, *Ph*), 10.53 (s, 1H, COO*H*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 29.0, 29.0, 63.9, 69.1, 69.4, 70.6, 73.3, 127.7, 127.9, 128.4, 138.1, 172.2 (*C*OOR), 177.3 (*C*OOH).

4.1.2. 3-[2-(2-Hydroxyethoxy)ethoxycarbonyl]propanoic acid (**3**)

To the solution of acid **2** (2.56 g, 8.64 mmol) in MeOH (100 ml) 10% Pd/C (0.27 g) was added, and the mixture was stirred under hydrogen for 1 h. Then the mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by gradient F_c (toluene/AcOEt/AcOH 150:50:2 to 25:75:1). This provided an oily liquid. Yield: 1.2 g (67%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 2.62–2.69 (m, 4H, $COCH_2CH_2CO$), 3.55–3.68 (m, 2H, CH_2OH), 3.65–3.78 (m, 4H, CH_2OCH_2), 4.24–4.32 (m, 2H, CH_2OCO), 6.37 (s, 2H, O*H*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 29.1, 29.3, 61.7, 63.7, 69.1, 72.3, 172.3 (*C*OOR), 176.5 (*C*OOH). IR (cm^{-1}): ν(CH) 2930, ν(C=O) 1725, ν(CO) 1162, 1126, 1061. Anal. Calc. for $C_8H_{14}O_6$ (206.19): 46.60% C, 6.84% H; found: 45.33% C, 7.19% H. HR-MS: for $C_8H_{15}O_6$ $[M+H]^+$ calculated: 207.0869 m/z; found: 207.08655.

4.1.3. 3-[2-(2-Benzyloxyethoxy)ethoxycarbonyl]propanoyl chloride (**4**)

To the solution of acid **2** (5.33 g, 17.99 mmol) in toluene (7.5 ml) oxalyl chloride (7.5 ml, 88.63 mmol) was added at ambient temperature, after 30 min DMF (2 drops) was added, and then the mixture was stirred for 5 min. After that the mixture was evaporated under reduced pressure. Crude acyl chloride **4** (6.0 g) was used in the subsequent acylation reaction without further purification.

4.1.4. 2-(2-Benzyloxyethoxy)ethyl decanoate (**5**)

The solution of decanoyl chloride (9.5 ml, 45.78 mmol) in CH_2Cl_2 (20 ml) was slowly added dropwise at 0 °C (ice bath) under argon to the solution of 2-(2-benzyloxyethoxy)ethanol [44] (**1**, 8.01 g, 40.82 mmol), pyridine (3.6 ml, 44.6 mmol) and DMAP (0.50 g, 4.09 mmol) in CH_2Cl_2 (30 ml). Then the mixture was stirred and warmed to ambient temperature. After 2 h it was poured into aqueous AcOH (6%), and the aqueous layer was washed with CH_2Cl_2. The combined organic layers were washed with water, brine, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by gradient F_c (CH_2Cl_2/MeOH/AcOH 900:7.5:3 to 150:5:1). This provided an oily liquid. Yield: 14.0 g (97%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.88 (m, 3H, CH_3), 1.18–1.40 (m, 12H, 6 × CH_2), 1.62 (m, 2H, $CH_2CH_2CH_2COO$), 2.32 (m, 2H, CH_2COO), 3.60–3.74 (m, 6H, 3 × CH_2OCH_2), 4.20–4.27 (m, 2H, CH_2OCO), 4.57 (s, 2H, $PhCH_2OCH_2$), 7.25–7.37 (m, 5H, *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 14.2, 22.8, 25.0, 29.3, 29.4, 29.5, 31.9, 34.3, 63.5, 69.4, 69.5, 70.8, 73.4, 127.8, 127.9, 128.5, 138.3, 174.0 (COOR).

4.1.5. 2-(2-Hydroxyethoxy)ethyl decanoate (**6**)

Decanoate **5** (14.04 g, 40.06 mmol) was dissolved in AcOEt (300 ml), 10% Pd/C (1.33 g) was added, and the mixture was stirred under hydrogen at 40 °C for 24 h. Then additional 10% Pd/C was added (1.01 g), and the mixture was stirred under hydrogen at 40 °C for 24 h. Then the mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by gradient F_c (toluene/AcOEt/AcOH 90:15:1 to 50:50:1). This

provided an oily liquid. Yield: 7.9 g (76%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.88 (m, 3H, CH_3), 1.20–1.40 (m, 12H, 6 × CH_2), 1.62 (m, 2H, $CH_2CH_2CH_2COO$), 2.33 (m, 2H, CH_2COO), 3.57–3.63 (m, 2H, CH_2OH), 3.67–3.78 (m, 4H, CH_2OCH_2), 4.20–4.28 (m, 2H, CH_2-OCO). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 14.2, 22.7, 25.0, 29.2, 29.3, 29.5, 31.9, 34.3, 61.8, 63.3, 69.3, 72.5, 173.9 (*C*OOR).

*4.1.6. 3-[2-(2-Decanoyloxyethoxy)ethoxycarbonyl]propanoic acid (**7**)*

Decanoate 6 (7.92 g, 30.42 mmol), succinic acid anhydride (3.79 g, 37.9 mmol) and DMAP (4.50 g, 36.8 mmol) were dissolved in CH_2Cl_2 (120 ml) and stirred at ambient temperature for 2 h. After addition of concentrated AcOH (3 ml, 52.4 mmol) the mixture was poured into water. The mixture was extracted with CH_2Cl_2. The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by F_c (AcOEt/AcOH 100:1) and crystallized from toluene/petroleum ether (6:1). This provided a white crystalline compound. Yield: 9.3 g (84%). Mp. 36 °C. ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.88 (m, 3H, CH_3), 1.2–1.4 (m, 12H, 6 × CH_2), 1.62 (m, 2H, $CH_2CH_2CH_2COO$), 2.33 (m, 2H, CH_2COO), 2.60–2.75 (m, 4H, $COCH_2CH_2CO$), 3.66–3.74 (m, 4H, CH_2OCH_2), 4.20–4.30 (m, 4H, 2 × CH_2OCO), 10.84 (s, 1H, COO*H*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 14.2, 22.7, 25.0, 28.9, 29.0, 29.2, 29.3, 29.5, 31.9, 34.3, 63.3, 63.9, 69.1, 69.2, 172.2 (*C*OOR), 174.0 (*C*OOR), 177.8 (*C*OOH). IR (cm^{-1}): ν(CH) 2953, 2915, 2848, ν(C=O) 1724, 1694, ν(CH) 1325, ν(CO) 1176, 1132. Anal. Calc. for $C_{18}H_{32}O_7$ (360.44): 59.98% C, 8.95% H; found: 59.45% C, 9.15% H. HR-MS: for $C_{18}H_{31}O_7$ $[M-H]^-$ calculated: 359.2070 m/z; found: 359.20435 m/z.

*4.1.7. 3-[2-(2-Decanoyloxyethoxy)ethoxycarbonyl]propanoyl chloride (**8**)*

To the solution of acid **7** (6.07 g, 16.84 mmol) in toluene (25 ml) oxalyl chloride (7.0 ml, 82.72 mmol) was added at ambient temperature and after 15 min DMF (2 drops). Then the mixture was stirred for 20 min and evaporated under reduced pressure. Crude acyl chloride **8** (6.9 g) was used in the subsequent acylation reaction without further purification.

*4.1.8. 1,2-O,O′-Bisdecanoyl-3-O′′-benzyl-rac-glycerol (**10**)*

Decanoyl chloride (35 ml, 168.7 mmol) dissolved in CH_2Cl_2 (50 ml) was slowly (during 30 min) added dropwise to the cooled (15 °C) solution of 1-*O*-benzyl-*rac*-glycerol [45] (**9**, 15.05 g, 82.59 mmol), DMAP (0.16 g, 1.31 mmol) and pyridine (15 ml, 185.8 mmol) in CH_2Cl_2 (100 ml). The mixture was stirred at ambient temperature for 2.5 h and then heated at 40 °C for 8.5 h with elimination of air humidity. Then glacial AcOH (4 ml) was added to the mixture. The water layer was separated and washed with CH_2Cl_2. The organic layer was washed with water and brine, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by gradient F_c (CH_2Cl_2/AcOEt 100:0 to 100:6). This provided an oily liquid. Yield: 35.2 g (87%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.88 (m, 6H, 2 × CH_3), 1.15–1.40 (m, 24H, 2×(CH_2)$_6$), 1.60 (m, 4H, 2 × CH_2CH_2COO), 2.23–2.37 (m, 4H, 2 × CH_2COO), 3.59 (d, 2H, CH_2OCH_2Ph, J = 5.18 Hz), 4.19 (dd, 1H, ^2J = 11.89 Hz, ^3J = 6.42 Hz), 4.35 (dd, 1H, ^2J = 11.89 Hz, ^3J = 3.84 Hz), 4.54 (2H, PhCH_2), 5.19–5.30 (m, 1H), 7.25–7.40 (m, 5H, *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 14.3, 22.8, 25.0, 25.1, 29.2, 29.2, 29.4, 29.6, 32.0, 34.24, 34.5, 62.8, 68.4, 70.1, 73.4, 127.8, 127.9, 128.6, 137.8, 173.3 (*C*OOR), 173.6 (*C*OOR).

*4.1.9. 1,2-O,O′-Bisdecanoyl-rac-glycerol (**11**)*

The solution of compound **10** (30.76 g, 62.68 mmol) in AcOEt (300 ml) was stirred with 10% Pd/C (3.07 g) under hydrogen at ambient temperature for 22 h. Then a further portion of 10% Pd/C (1 g) was added, and the mixture was stirred under hydrogen for 24 h, filtered and evaporated under reduced pressure. The crude product was purified by F_c (CH_2Cl_2/MeOH/AcOH 300:10:3). This provided an oily liquid. Yield: 12.37 g (49%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.88 (m, 6H, 2 × CH_3), 1.20–1.40 (m, 24H, 2×(CH_2)$_6$), 1.54–1.71 (m, 4H, 2 × CH_2CH_2COO), 2.20–2.40 (m, 5H, 2 × CH_2-COO, O*H*), 3.72 (d, 2H, CH_2OH, J = 5.05 Hz), 4.22 (dd, 1H, ^2J = 11.93 Hz, ^3J = 5.77 Hz), 4.33 (dd, 1H, ^2J = 11.93 Hz, ^3J = 4.43 Hz), 5.02–5.15 (m, 1H). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 14.2, 22.8, 25.0, 25.1, 29.2, 29.2, 29.4, 29.5, 31.9, 34.2, 34.4, 61.6, 62.2, 72.3, 173.6 (*C*OOR), 173.9 (*C*OOR).

*4.1.10. 3-[2,3-Bis(decanoyloxy)propyloxycarbonyl]propanoic acid (**12**)*

Succinic acid anhydride (3.75 g, 37.37 mmol) was added to the solution of compound 11 (12.37 g, 30.88 mmol) and DMAP (4.52 g, 36.99 mmol) in CH_2Cl_2 (120 ml), and the mixture was stirred for 2.5 h. Then AcOH (1 ml) was added, and the mixture was evaporated under reduced pressure. The crude product was purified by gradient F_c (CH_2Cl_2/AcOEt/AcOH 800:65:8.5 and 800:150:9). This provided an oily liquid. Yield: 12.2 g (79%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.88 (m, 6H, 2 × CH_3), 1.20–1.40 (m, 24H, 2×(CH_2)$_6$), 1.54–1.69 (m, 4H, 2 × CH_2CH_2COO), 2.26–2.37 (m, 4H, 2 × CH_2-COO), 2.60–2.72 (m, 4H, $COCH_2CH_2CO$), 4.00–4.38 (m, 4H, 2 × CH_2-OCO), 5.21–5.32 (m, 1H, *C*HOCO), 9.79 (s, COO*H*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 14.2, 22.8, 24.9, 25.0, 28.8, 28.9, 29.2, 29.2, 29.4, 29.5, 32.0, 34.2, 34.3, 62.14, 62.8, 68.9, 171.7 (*C*OOR), 173.1 (*C*OOR), 173.5 (*C*OOR), 177.7 (*C*OOH). IR (cm^{-1}): ν(CH) 2953, 2917, 2851, ν(C=O) 1730, 1697, ν(CH) 1386, ν(CO) 1172. Anal. Calc. for $C_{27}H_{48}O_8$ (500.67): 64.77% C, 9.66% H; found: 66.08% C, 10.09% H. HR-MS: for $C_{27}H_{47}O_8$ $[M-H]^-$ calculated: 499.3271 m/z; found: 499.32578 m/z.

*4.1.11. 3-[2,3-Bis(decanoyloxy)propyloxycarbonyl]propanoyl chloride (**13**)*

To the solution of acid **12** (6.99 g, 13.96 mmol) in toluene (12 ml) oxalyl chloride (6 ml, 70.90 mmol) was added at ambient temperature, after 15 min DMF (2 drops) was added, and the mixture was stirred for 20 min. Then the mixture was evaporated under reduced pressure. Crude acyl chloride **13** (7.5 g) was used in the subsequent acylation reaction without further purification.

*4.1.12. (+/−)-α-Tocopherol hemisuccinate (**15**)*

Succinic acid anhydride (3.64 g; 36.38 mmol) was added to the solution of α-tocopherol (**14**, 12.62 g; 29.3 mmol) and DMAP (4.76 g, 38.96 mmol) in CH_2Cl_2 (200 ml), and the mixture was stirred at ambient temperature for 2 h [46]. Then the mixture was evaporated under reduced pressure. The crude product was purified by F_c (CH_2Cl_2/MeOH/AcOH 30:1.5:0.3). This provided an oily liquid. Yield: 15.8 g (99%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.81–0.90 (m, 12H, 4 × CH_3), 0.95–1.65 (m, 25H), 1.60–1.90 (m, 2H), 1.96 (s, 3H, ArCH_3), 2.00 (s, 3H, ArCH_3), 2,08 (s, 3H, ArCH_3), 2.58 (m, 2H), 2.75–2.97 (m, 4H, $COCH_2CH_2CO$). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 11.9, 12.2, 13.0, 19.7, 19.8, 19.8, 19.8, 19.9, 19.7, 19.8, 19.8, 19.8, 19.9, 20.7, 21.2, 22.8, 22.9, 24.0, 24.6, 24.9, 28.1, 28.7, 29.1, 31.2, 32.9, 32.9, 37.4, 37.5, 37.6, 37.7, 39.5, 75.2, 117.6, 123.2, 125.0, 126.8, 140.6, 149.6, 170.9 (*C*OOR), 178.2 (*C*OOH). IR (cm^{-1}): ν(CH) 2952, 2922, 2866, ν(C=O) 1745, 1692, ν(CH) 1460, ν(CO) 1152, 1105, 1087. Anal. Calc. for $C_{33}H_{54}O_5$ (530.78): 74.67% C, 10.25% H; found: 74.52% C, 10.56% H.

*4.1.13. 3-{[2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl]oxycarbonyl} propanoyl chloride (**16**)*

To the solution of compound **15** (2.72 g, 5.13 mmol) in toluene (4 ml) oxalyl chloride (2 ml, 23.64 mmol) was added, and the mixture was stirred for 2 h [47]. Then the mixture was evaporated under reduced pressure. The crude acyl chloride **16** (2.9 g) was used in the subsequent acylation reaction without further purification.

*4.1.14. Benzyl 3α-{3-[2-(2-benzyloxyethoxy)ethoxycarbonyl]propanoyloxy}-7α,12α-dihydroxy-5β-cholan-24-oate (**18**)*

Acyl chloride **4** (4.58 g, 14.55 mmol) dissolved in CH_2Cl_2 (20 ml) was slowly added dropwise to the solution of benzyl cholate (**17**, 6.00 g, 12.03 mmol) and DMAP (1.77 g, 14.49 mmol) in CH_2Cl_2 (40 ml) at 0 °C under argon. Then the mixture was stirred and warmed to an ambient temperature. After 3 h it was poured into aqueous AcOH (250 ml, 1.2%), and the aqueous layer was washed with CH_2Cl_2. The combined organic layers were dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by F_c (CH_2Cl_2/MeOH/AcOH 300:10:1). This provided a colorless oil. Yield: 1.9 g (20%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.66 (s, 3H, C*H*$_3$), 0.89 (s, 3H, C*H*$_3$), 0.96 (d, 3H, C*H*$_3$, J = 5.71 Hz), 1.00–2.50 (m, 26H), 2.52–2.67 (m, 4H, COC*H*$_2$C*H*$_2$CO), 3.60–3.75 (m, 6H, 3 × C*H*$_2$OCH$_2$), 3.82 (m, 1H, C$_{(7)}$*H*), 3.96 (m, 1H, C$_{(12)}$*H*), 4.20–4.30 (m, 2H, C*H*$_2$OCO), 4.57 (s, 2H, PhC*H*$_2$OCH$_2$), 4.58 (m, 1H, C$_{(3)}$*H*), 5.11 (2H, PhC*H*$_2$OCO), 7.32–7.38 (m, 10H, 2 × *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.6, 17.4, 22.6, 23.2, 26.7, 26.7, 27.5, 28.4, 29.2, 29.4, 29.6, 30.9, 31.4, 34.5, 34.8, 34.9, 35.2, 35.2, 39.6, 41.3, 42.0, 46.6, 47.3, 63.9, 66.2, 68.3, 69.2, 69.4, 70.7, 73.0, 73.4, 74.8, 127.7, 127.8, 128.3, 128.3, 128.5, 128.6, 136.2, 138.2, 171.9 (*C*OOR), 172.5 (*C*OOR), 174.2 (*C*OOR).

*4.1.15. 7α,12α-Dihydroxy-3α-{3-[2-(2-hydroxyethoxy)ethoxycarbonyl]propanoyloxy}-5β-cholan-24-oic acid (**19**)*

Benzyl ester **18** (1.49 g, 1.92 mmol) was dissolved in MeOH (60 ml), and 10% Pd/C (0.12 g) was added. The mixture was stirred under hydrogen at 40 °C for 1.5 h. Then the suspension was filtered through celite and evaporated under reduced pressure. The crude product was purified by F_c (CH_2Cl_2/MeOH/AcOH 150:15:1). This provided a colorless oil. Yield: 0.8 g (69%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.69 (s, 3H, C*H*$_3$), 0.90 (s, 3H, C*H*$_3$), 0.99 (d, 3H, C*H*$_3$, J = 5.56 Hz), 1.00–2.47 (m, 27H), 2.53–2.70 (m, 4H, COC*H*$_2$C*H*$_2$CO), 3.55–3.80 (m, 6H, 3 × C*H*$_2$OCH$_2$), 3.85 (m, 1H, C$_{(7)}$*H*), 3.99 (m, 1H, C$_{(12)}$*H*), 4.20–4.30 (m, 2H, C*H*$_2$OCO), 4.58 (m, 1H, C$_{(3)}$*H*), 4.85 (s, O*H*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.6, 17.3, 22.6, 23.2, 26.6, 26.7, 27.6, 28.3, 29.4, 29.7, 30.9, 31.1, 34.5, 34.8, 34.9, 35.2, 35.4, 39.6, 41.3, 41.9, 46.6, 47.0, 61.6, 63.8, 68.4, 69.1, 72.5, 73.1, 75.0, 171.9 (*C*OOR), 172.5 (*C*OOR), 178.5 (*C*OOH). IR (cm^{-1}): ν(OH) 3441, 3260, ν(CH) 2936, 2871, ν(C=O) 1713, ν(CH) 1382, ν(CO) 1163. Anal. Calc. for $C_{32}H_{52}O_{10}$ (596.74): 64.41% C, 8.78% H; found: 62.78% C, 8.93% H. HR-MS: for $C_{32}H_{52}O_{10}$ $[M-H]^-$ calculated: 595.3482 m/z; found: 595.3441 m/z.

*4.1.16. Benzyl 3α-benzyloxycarboxy-7α,12α-dihydroxy-5β-cholan-24-oate (**20**)*

The solution of benzyl cholate **17** (5.03 g, 10.1 mmol) in dry pyridine (75 ml) was cooled to 0 °C and mixed with BnOCOCl (4.37 g, 25.6 mmol) under argon. Then the mixture was stirred for 4 h, and a further 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 and washed with aqueous AcOH (17%). The aqueous layer was washed with toluene. Combined organic extracts were washed with brine and evaporated under reduced pressure. The crude product was purified by F_c (CH_2Cl_2/MeOH/AcOH 300:5:1). This provided a colorless oil. Yield: 3.0 g (46%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 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, $CDCl_3$), δ: 12.6, 17.4, 22.5, 23.3, 26.7, 26.7, 27.5, 28.4, 31.0, 31.4, 34.4, 34.8, 34.9, 35.2, 35.2, 39.7, 41.3, 42.0, 46.6, 47.3, 66.2, 68.3, 69.3, 72.9, 78.6, 128.3, 128.3, 128.3, 128.5, 128.6, 135.6, 136.2, 154.8 (OCOO), 174.2 (*C*OOR). HR-MS: for $C_{39}H_{51}O_7$ $[M-H]^-$ calculated: 631.3713 m/z; found: 631.3726 m/z [8].

*4.1.17. Benzyl 3α-benzyloxycarboxy-7α,12α-bis{3-[2-(2-benzyloxyethoxy)ethoxycarbonyl] propanoyloxy}-5β-cholan-24-oate (**21**)*

The mixture of ester **20** (2.52 g, 3.98 mmol), BTEAC (0.36 g, 1.58 mmol) and CaH_2 (0.67 g, 15.90 mmol) in toluene (40 ml) was mixed with the solution of crude acyl chloride **4** (4.98 g, 15.83 mmol) in toluene (20 ml), and the resulting mixture was refluxed under argon for 4 h. Then the reaction mixture was poured into the mixture of aqueous AcOH (6%) and toluene. After shaking the aqueous layer was washed with toluene. The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by F_c (toluene/Et_2O/TEA 80:20:1 and toluene/AcOEt/TEA 60:30:1). This provided a colorless oil. Yield: 3.3 g (69%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.68 (s, 3H, C*H*$_3$), 0.79 (d, 3H, C*H*$_3$, J = 5.71 Hz), 0.91 (s, 3H, C*H*$_3$), 0.95–2.50 (m, 24H), 2.50–2.75 (m, 8H, 2 × COC*H*$_2$C*H*$_2$CO), 3.56–3.72 (m, 12H, 6 × C*H*$_2$OCH$_2$), 4.20–4.30 (m, 4H, 2 × C*H*$_2$OCO), 4.45 (m, 1H, C$_{(3)}$*H*), 4.55 (s, 2H, PhC*H*$_2$-OCH$_2$), 4.56 (s, 2H, PhC*H*$_2$OCH$_2$), 4.92 (m, 1H, C$_{(7)}$*H*), 5.09 (2H, PhC*H*$_2$OCO), 5.10 (m, 1H, C$_{(12)}$*H*), 5.14 (s, 2H, PhC*H*$_2$OCOO),7.30–7.40 (m, 20H, 4 × *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.3, 17.5, 22.6, 22.9, 25.6, 26.8, 27.4, 28.9, 29.0, 29.1, 29.5, 29.6, 30.8, 31.3, 31.4, 34.4, 34.6, 34.7, 34.7, 37.9, 41.0, 43.4, 45.3, 47.4, 63.9, 64.0, 66.2, 69.2, 69.4, 69.5, 69.6, 70.6, 70.7, 71.1, 73.4, 75.7, 78.3, 127.7, 127.9, 128.3, 128.4, 128.5, 128.6, 128.7, 128.8, 135.4, 136.2, 138.3, 154.7 (OCOO), 171.8 (*C*OOR), 171.9 (*C*OOR), 172.3 (*C*OOR), 172.4 (*C*OOR), 174.0 (*C*OOR).

*4.1.18. 3α-Hydroxy-7α,12α-bis{3-[2-(2-hydroxyethoxy)ethoxycarbonyl]propanoyloxy}-5β-cholan-24-oic acid (**22**)*

The mixture of ester **21** (3.19 g, 2.68 mmol), MeOH (160 ml), AcOEt (40 ml) and 10% Pd/C (0.62 g) was stirred under hydrogen at 40 °C for 5.5 h. Then the mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by F_c (CH_2Cl_2/MeOH/AcOH 150:15:1). This provided a colorless oil. Yield: 1.6 g (75%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.72 (s, 3H, C*H*$_3$), 0.81 (d, 3H, C*H*$_3$, J = 5.86 Hz), 0.89 (s, 3H, C*H*$_3$), 0.95–2.50 (m, 26H), 2.60–2.75 (m, 8H, 2 × COC*H*$_2$C*H*$_2$CO), 3.44 (m, 1H, C$_{(3)}$*H*), 3.55–3.62 (m, 4H, 2 × C*H*$_2$OH), 3.65–3.75 (m, 8H, 2 × C*H*$_2$-OC*H*$_2$), 4.00–4.45 (s, O*H*), 4.18–4.37 (m, 4H, 2 × C*H*$_2$OCO), 4.94 (m, 1H, C$_{(7)}$*H*), 5.10 (m, 1H, C$_{(12)}$*H*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.2, 17.6, 22.6, 23.0, 25.4, 27.4, 28.8, 29.3, 29.4, 29.74, 29.8, 30.3, 30.7, 31.5, 34.4, 34.8, 35.1, 38.0, 39.1, 41.0, 43.4, 45.2, 47.1, 61.6, 61.7, 63.9, 63.9, 69.1, 71.6, 72.6, 72.7, 76.0, 171.7 (*C*OOR), 172.5 (*C*OOR), 178.3 (*C*OOH). IR (cm^{-1}): ν(OH) 3412, ν(CH) 2927, 2867, ν(C=O) 1724, ν(CO) 1161, 1125, 1067. Anal. Calc. for $C_{40}H_{64}O_{15}$ (784.93): 61.21% C, 8.22% H; found: 59.19% C, 8.49% H. HR-MS: for $C_{40}H_{63}O_{15}$ $[M-H]^-$ calculated: 783.4167 m/z; found: 783.4159 m/z.

*4.1.19. Benzyl 3α,7α,12α-tris{3-[2-(2-benzyloxyethoxy)ethoxycarbonyl]propanoyloxy}-5β-cholan-24-oate (**23**)*

The mixture of benzyl ester **17** (1.50 g, 3.01 mmol), BTEAC (0.26 g, 1.14 mmol), CaH_2 (0.77 g, 18.29 mmol) and toluene (25 ml) was mixed with the solution of crude acyl chloride **4** (5.66 g, 17.99 mmol) in toluene (25 ml), and the mixture was refluxed for 3 h. Then the reaction mixture was poured into the mixture of aqueous AcOH (6%) and toluene. After shaking the aqueous layer was washed with toluene. The combined organic layers were washed with saturated aqueous $NaHCO_3$ and brine, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The

crude product was purified by gradient F_c (hexane/Et_2O/AcOH 25:25:1 to 0:50:1 and then Et_2O/MeOH/AcOH 100:5:2). This provided a colorless oil. Yield: 2.8 g (69%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.69 (s, 3H, CH_3), 0.80 (d, 3H, CH_3, J = 5.69 Hz), 0.90 (s, 3H, CH_3), 0.95–2.50 (m, 24H), 2.53–2.78 (m, 12H, 3 × COCH_2CH_2CO), 3.55–3.75 (m, 18H, 9 × CH_2OCH$_2$), 4.20–4.30 (m, 6H, 3 × CH_2OCO), 4.56 (s, 6H, 3 × PhCH_2OCH$_2$), 4.57 (m, 1H, C$_{(3)}H$), 4.92 (m, 1H, C$_{(7)}H$), 5.09 (2H, PhCH_2OCO), 5.10 (m, 1H, C$_{(12)}H$), 7.30–7.36 (m, 20H, 4 × *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.3, 17.5, 22.6, 22.9, 25.7, 26.9, 27.3, 28.9, 29.0, 29.1, 29.2, 29.5, 29.6, 30.9, 31.3, 31.4, 34.4, 34.7, 34.8, 37.9, 41.0, 43.4, 45.3, 47.4, 63.9, 63.9, 66.2, 69.2, 69.5, 70.7, 71.2, 73.4, 74.5, 75.7, 127.7, 127.8, 128.3, 128.3, 128.5, 128.6, 136.2, 138.3, 171.7 (*C*OOR), 171.8 (*C*OOR), 171.9 (*C*OOR), 172.2 (*C*OOR), 172.3 (*C*OOR), 172.4 (*C*OOR), 173.9 (*C*OOR).

4.1.20. 3α,7α,12α-Tris{3-[2-(2-hydroxyethoxy)ethoxycarbonyl] propanoyloxy}-5β-cholan-24-oic acid (**24**)

The solution of benzyl ester **23** (2.45 g, 1.84 mmol) in MeOH (110 ml) and AcOEt (28 ml) was stirred with 10% Pd/C (0.43 g) under hydrogen at 40 °C for 5.5 h. Then the mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by F_c (CH_2Cl_2/MeOH/AcOH 150:15:1). This provided an oily liquid. Yield: 1.1 g (61%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.71 (s, 3H, CH_3), 0.80 (d, 3H, CH_3, J = 5.41 Hz), 0.89 (s, 3H, CH_3), 0.95–2.45 (m, 24H), 2.55–2.80 (m, 12H, 3 × COCH_2CH_2CO), 3.53–3.62 (m, 6H, 3 × CH_2OH), 3.64–3.74 (m, 12H, 6 × CH_2OCH$_2$), 4.0–4.3 (O*H*), 4.2–4.35 (m, 6H, 3 × CH_2OCO), 4.55 (m, 1H, C$_{(3)}H$), 4.91 (m, 1H, C$_{(7)}H$), 5.10 (m, 1H, C$_{(12)}H$). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.2, 17.5, 22.6, 22.9, 25.6, 26.8, 27.4, 28.9, 29.1, 29.2, 29.3, 29.6, 29.7, 30.7, 31.3, 34.4, 34.7, 34.7, 37.9, 40.9, 43.4, 45.2, 47.0, 61.6, 61.6, 61.7, 63.7, 63.7, 63.8, 69.1, 71.4, 72.6, 74.6, 75.8, 171.8 (*C*OOR), 172.0 (*C*OOR), 172.4 (*C*OOR), 172.5 (*C*OOR), 178.2 (*C*OOH). IR (cm^{-1}): ν(OH) 3445, ν(CH) 2939, 2870, ν(C=O) 1725, ν(CH) 1381, ν(CO) 1160, 1127. Anal. Calc. for $C_{48}H_{76}O_{20}$ (973.11): 59.24% C, 7.87% H; found: 56.62% C, 8.03% H. HR-MS: for $C_{48}H_{75}O_{20}$ [M−H]$^-$ calculated: 971.4852 m/z; found: 971.4811 m/z.

4.1.21. Benzyl 3α-{3-[2-(2-decanoyloxyethoxy)ethoxycarbonyl] propanoyloxy}-7α,12α-dihydroxy-5β-cholan-24-oate (**25**)

The solution of acyl chloride **8** (1.84 g, 4.85 mmol) in CH_2Cl_2 (20 ml) was slowly added to the solution of benzyl cholate (**17**, 2.01 g, 4.03 mmol) and DMAP (0.58 g, 4.75 mmol) in CH_2Cl_2 (30 ml) at 0 °C. The mixture was stirred at this temperature for 45 min. Then the mixture was warmed to an ambient temperature and poured into aqueous AcOH (6%). The aqueous layer was washed with CH_2Cl_2. The combined organic layers were washed with saturated aqueous $NaHCO_3$, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by F_c (CH_2Cl_2/MeOH/AcOH 300:10:1). This provided an oily liquid. Yield: 0.7 g (22%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.67 (s, 3H, CH_3), 0.88 (m, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.98 (d, 3H, CH_3, J = 5.87 Hz), 1.00–2.00 (m, 36H), 2.13–2.50 (m, 6H), 2.53–2.69 (m, 4H, COCH_2-CH_2CO), 3.65–3.73 (m, 4H, CH_2OCH_2), 3.84 (m, 1H, C$_{(7)}H$), 3.97 (m, 1H, C$_{(12)}H$), 4.18–4.29 (m, 4H, 2 × OCH$_2$CH_2OCO), 4.59 (m, 1H, C$_{(3)}H$), 5.11 (2H, PhCH_2), 7.32–7.38 (m, 5H, *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.7, 14.2, 17.5, 22.7, 22.8, 23.3, 25.0, 26.8, 26.9, 27.5, 28.6, 29.3, 29.3, 29.4, 29.5, 29.6, 31.0, 31.4, 32.0, 34.4, 34.5, 34.8, 35.0, 35.2, 35.3 39.7, 41.4, 42.2, 46.7, 47.4, 63.4, 63.8, 66.2, 68.3, 69.1, 69.3, 73.0, 74.9, 128.3, 128.4, 128.7, 136.3, 171.9, 172.4, 174.0, 174.1.

4.1.22. 3α-{3-[2-(2-Decanoyloxyethoxy)ethoxycarbonyl] propanoyloxy}-7α,12α-dihydroxy-5β-cholan-24-oic acid (**26**)

The solution of benzyl ester **25** (0.66 g, 0.79 mmol) in AcOEt (15 ml) was stirred with 10% Pd/C (0.13 g) under hydrogen at 30 °C for 30 min. After that the suspension was filtered through celite and evaporated under reduced pressure. The crude product was purified by gradient F_c (CH_2Cl_2/MeOH/AcOH 30:0.5:0.1 to 30:3:0.2). This provided an oily liquid. Yield: 0.4 g (68%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.70 (s, 3H, CH_3), 0.88 (m, 3H, CH_3), 0.91 (s, 3H, CH_3), 1.00 (d, 3H, CH_3, J = 5.93 Hz), 1.00–2.05 (m, 36H), 2.13–2.50 (m, 6H), 2.53–2.70 (m, 4H, COCH_2CH_2CO), 3.66–3.73 (m, 4H, CH_2OCH_2), 3.86 (m, 1H, C$_{(7)}H$), 3.99 (m, 1H, C$_{(12)}H$), 4.19–4.28 (m, 4H, 2 × OCH$_2$CH_2OCO), 4.59 (m, 1H, C$_{(3)}H$). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.7, 14.2, 17.5, 22.7, 22.8, 23.3, 25.1, 26.8, 26.9, 27.6, 28.5, 29.3, 29.3, 29.4, 29.6, 29.7, 30.9, 31.0, 32.0, 34.4, 34.5, 34.9, 35.0, 35.3, 35.4, 39.7, 41.4, 42.2, 46.7, 47.3, 63.4, 63.9, 68.4, 69.2, 69.3, 73.1, 74.9, 171.9 (*C*OOR), 172.5 (*C*OOR), 174.1 (*C*OOR), 178.8 (*C*OOH). IR (cm^{-1}): ν(OH) 3487, ν(CH) 2923, 2855, ν(C=O) 1729, ν(CH) 1378, ν(CO) 1162. Anal. Calc. for $C_{42}H_{70}O_{11}$ (751.00): 67.17% C, 9.39% H; found: 66.50% C, 9.72% H. HR-MS: for $C_{42}H_{69}O_{11}$ [M−H]$^-$ calculated: 749.4840 m/z; found: 749.4801 m/z.

4.1.23. Benzyl 3α-benzyloxycarboxy-7α,12α-bis{3-[2-(2-decanoyloxyethoxy)ethoxycarbonyl]propanoyloxy}-5β-cholan-24-oate (**27**)

Ester **20** (2.09 g, 3.30 mmol), BTEAC (0.27 g, 1.19 mmol) and CaH_2 (0.53 g, 12.59 mmol) were dissolved in toluene (40 ml) and mixed with the solution of crude acyl chloride **8** (4.84 g, 12.76 mmol) in toluene (20 ml). The mixture was refluxed under argon for 3 h. The reaction mixture was poured into the mixture of aqueous AcOH (3%) and toluene. After shaking the aqueous layer was washed with toluene. The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by F_c (toluene/AcOEt/TEA 80:20:1). This provided an oily liquid. Yield: 3.0 g (68%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.69 (s, 3H, CH_3), 0.80 (d, 3H, CH_3, J = 5.78 Hz), 0.88 (m, 6H, 2 × CH_3), 0.91 (s, 3H, CH_3), 0.95–2.20 (m, 50H), 2.20–2.50 (m, 6H), 2.50–2.80 (m, 8H, 2 × COCH_2CH_2-CO), 3.60–3.72 (m, 8H, 2 × CH_2OCH_2), 4.16–4.29 (m, 8H, 4 × CH_2-OCO), 4.46 (m, 1H, C$_{(3)}H$), 4.94 (m, 1H, C$_{(7)}H$), 5.09 (2H, PhCH_2O), 5.10 (m, 1H, C$_{(12)}H$), 5.13 (s, 2H, PhCH_2OCOO), 7.30–7.40 (m, 10H, 2 × *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.3, 14.2, 17.5, 22.5, 22.7, 22.9, 25.0, 25.6, 26.8, 27.3, 28.9, 29.0, 29.1, 29.2, 29.4, 29.5, 30.8, 31.2, 31.4, 31.9, 34.3, 34.4, 34.6, 34.6, 34.7, 37.9, 41.0, 43.4, 45.2, 47.4, 63.3, 63.7, 63.8, 66.2, 69.1, 69.2, 69.3, 69.5, 71.1, 75.7, 78.3, 128.3, 128.4, 128.5, 128.6, 128.7, 128.8, 135.4, 136.2, 154.6 (OCOO), 171.7 (*C*OOR), 171.8 (*C*OOR), 172.2 (*C*OOR), 172.3 (*C*OOR), 173.8 (*C*OOR), 173.9 (*C*OOR).

4.1.24. 7α,12α-Bis{3-[2-(2-decanoyloxyethoxy)ethoxycarbonyl] propanoyloxy}-3α-hydroxy-5β-cholan-24-oic acid (**28**)

The solution of $HCOONH_4$ (0.54 g, 8.56 mmol) in MeOH (50 ml) and 10% Pd/C (0.58 g) were added to the solution of ester **27** (2.90 g, 2.20 mmol) in AcOEt (50 ml). The mixture was stirred under argon for 1.5 h. Then the mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by gradient F_c (CH_2Cl_2/MeOH/AcOH 100:5:1). This provided an oily liquid. Yield: 1.8 g (74%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.70 (s, 3H, CH_3), 0.80 (d, 3H, CH_3, J = 6.05 Hz), 0.85 (m, 6H, 2 × CH_3), 0.88 (s, 3H, CH_3), 0.95–2.15 (m, 51H), 2.15–2.45 (m, 6H), 2.55–2.75 (m, 8H, 2 × COCH_2CH_2CO), 3.43 (m, 1H, C$_{(3)}H$), 3.60–3.73 (m, 8H, 2 × CH_2OCH_2), 4.14–4.28 (m, 8H, 4 × CH_2OCO), 4.91 (m, 1H, C$_{(7)}H$), 5.08 (m, 1H, C$_{(12)}H$). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.2, 14.2, 17.5, 22.5, 22.7, 22.9, 25.0, 25.4, 27.3, 28.8, 29.1, 29.2, 29.3, 29.5, 29.6, 30.4, 30.6, 30.8, 31.4, 31.9, 34.3, 34.4, 34.7, 35.1, 38.0, 39.0, 41.1, 43.3, 45.2, 47.2, 63.3, 63.8, 63.9, 69.0, 69.1, 69.2, 71.5, 71.6, 75.9, 171.7 (*C*OOR), 171.7 (*C*OOR), 172.3 (*C*OOR), 172.4 (*C*OOR), 174.0 (*C*OOR), 179.0 (*C*OOH). IR (cm^{-1}): ν(OH) 3445, ν(CH) 2924, 2854, ν(C=O) 1728, ν(CH) 1378, ν(CO) 1161. Anal. Calc. for $C_{60}H_{100}O_{17}$ (1093.43): 65.91% C, 9.22% H;

found: 64.13% C, 9.20% H. HR-MS: for $C_{60}H_{99}O_{17}$ $[M-H]^-$ calculated: 1091.6882 m/z; found: 1091.6843 m/z.

4.1.25. Benzyl 3α,7α,12α-tris{3-[2-(2-decanoyloxyethoxy)ethoxycarbonyl] propanoyloxy}-5β-cholan-24-oate (**29**)

The mixture of benzyl cholate (**17**, 1.01 g, 2.03 mmol), BTEAC (0.18 g, 0.79 mmol) and CaH_2 (0.55 g, 13.06 mmol) in toluene (15 ml) was mixed with the solution of crude acyl chloride **8** (4.53 g, 11.96 mmol) in toluene (15 ml). The mixture was refluxed under argon for 3 h. The reaction mixture was poured into aqueous AcOH (6%) and the aqueous layer was washed with toluene. The combined organic layers were washed with saturated aqueous $NaHCO_3$, water, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by F_c (hexane/acetone/AcOH 80:20:1). This provided an oily liquid. Yield: 1.8 g (59%). The product without characterization was used in the subsequent reaction.

4.1.26. 3α,7α,12α-Tris{3-[2-(2-decanoyloxyethoxy)ethoxycarbonyl] propanoyloxy}-5β-cholan-24-oic acid (**30**)

The solution of benzyl ester **29** (1.81 g, 1.19 mmol) in AcOEt (25 ml) was stirred with 10% Pd/C (0.19 g) at ambient temperature under hydrogen for 1.5 h. Then the mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by F_c (hexane/acetone/AcOH 80:20:1). This provided an oily liquid. Yield: 1.1 g (66%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.73 (s, 3H, CH_3), 0.83 (d, 3H, CH_3, J = 6.02 Hz), 0.88 (m, 9H, 3 × CH_3), 0.91 (s, 3H, CH_3), 1.00–2.10 (m, 64H), 2.20–2.40 (m, 8H), 2.55–2.80 (m, 12H, 3 × COCH_2CH_2CO), 3.65–3.75 (m, 12H, 3 × CH_2OCH_2), 4.19–4.30 (m, 12H, 6 × CH_2OCO), 4.58 (m, 1H, $C_{(3)}H$), 4.94 (m, 1H, $C_{(7)}H$), 5.13 (m, 1H, $C_{(12)}H$). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.3, 14.2, 17.5, 22.6, 22.6, 22.9, 25.0, 25.7, 26.9, 27.4, 28.9, 29.0, 29.1, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 30.6, 30.7, 31.4, 32.0, 34.3, 34.5, 34.7, 34.8, 38.0, 41.1, 43.4, 45.3, 47.2, 63.3, 63.7, 63.8, 63.9, 69.1, 69.3, 71.3, 74.6, 75.7, 171.7 (COOR), 171.8 (COOR), 172.0 (COOR), 172.3 (COOR), 172.4 (COOR), 172.5 (COOR), 173.8 (COOR), 173.9 (COOR), 178.8 (COOH). IR (cm^{-1}): ν(CH) 2924, 2855, ν(C=O) 1729, ν(CH) 1380, ν(CO) 1160. Anal. Calc. for $C_{78}H_{130}O_{23}$ (1435.85): 65.25% C, 9.13% H; found: 65.20% C, 9.21% H. HR-MS: for $C_{78}H_{129}O_{23}$ $[M-H]^-$ calculated: 1433.8925 m/z; found: 1433.8870 m/z.

4.1.27. Benzyl 3α-{3-[2,3-bis(decanoyloxy)propyloxycarbonyl] propanoyloxy}-7α,12α-dihydroxy-5β-cholan-24-oate (**31**)

Acyl chloride **13** (3.84 g, 7.39 mmol) dissolved in CH_2Cl_2 (40 ml) was slowly added to the solution of benzyl cholate (**17**, 6.32 g, 12.68 mmol) and DMAP (0.92 g, 7.53 mmol) in CH_2Cl_2 (60 ml) at 0 °C (ice bath). Then the mixture was warmed to ambient temperature and stirred for 1.5 h. The mixture was poured into the mixture of aqueous AcOH (3%) and CH_2Cl_2. After shaking aqueous layer was separated and washed with CH_2Cl_2. The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by gradient F_c (hexane/acetone 10:1 to 4:1). This provided an oily liquid. Yield: 2.2 g (30%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.59 (s, 3H, CH_3), 0.81 (m, 6H, 2 × CH_3), 0.83 (s, 3H, CH_3), 0.90 (d, 3H, CH_3, J = 5.54 Hz), 0.95–2.00 (m, 49H), 2.05–2.43 (m, 9H), 2.43–2.60 (m, 4H, COCH_2CH_2CO), 3.77 (m, 1H, $C_{(7)}H$), 3.90 (m, 1H, $C_{(12)}H$), 4.02–4.16 (m, 2H, CH_2OCO), 4.18–4.30 (m, 2H, CH_2OCO), 4.51 (m, 1H, $C_{(3)}H$), 5.04 (2H, PhCH_2), 5.18 (m, 1H, CHOCO), 7.22–7.32 (m, *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.6, 14.2, 17.4, 22.6, 22.8, 23.2, 24.9, 25.0, 26.7, 26.8, 27.5, 28.5, 29.1, 29.2, 29.4, 29.5, 31.0, 31.4, 32.0, 34.2, 34.3, 34.5, 34.8, 35.0, 35.2, 39.7, 41.3, 42.1, 46.6, 47.3, 62.3, 62.5, 66.2, 68.2, 69.0, 72.9, 74.9, 128.2, 128.3, 128.6, 136.2, 171.6 (COOR), 172.0 (COOR), 173.1 (COOR), 173.5 (COOR), 174.1 (COOR).

4.1.28. 3α-{3-[2,3-Bis(decanoyloxy)propyloxycarbonyl] propanoyloxy}-7α,12α-dihydroxy-5β-cholan-24-oic acid (**32**)

The solution of $HCOONH_4$ (0.57 g, 9.04 mmol) in MeOH (20 ml) and 10% Pd/C (0.26 g) were added to the solution of ester **31** (2.13 g, 2.17 mmol) in AcOEt (20 ml). The mixture was stirred under argon for 1.5 h. Then the mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by F_c (CH_2Cl_2/MeOH/AcOH 300:5:1). This provided an oily liquid. Yield: 1.3 g (68%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.69 (s, 3H, CH_3), 0.87 (m, 6H, 2 × CH_3), 0.89 (s, 3H, CH_3), 0.99 (d, 3H, CH_3, J = 5.91 Hz), 1.00–2.01 (m, 49H), 2.13–2.48 (m, 9H), 2.50–2.65 (m, 4H, COCH_2CH_2CO), 3.85 (m, 1H, $C_{(7)}H$), 3.98 (m, 1H, $C_{(12)}H$), 4.08–4.22 (m, 2H, CH_2OCO), 4.23–4.36 (m, 2H, CH_2OCO), 4.57 (m, 1H, $C_{(3)}H$), 5.24 (m, 1H, CHOCO). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.7, 14.2, 17.4, 22.6, 22.8, 23.3, 24.9, 25.0, 26.7, 26.8, 27.6, 28.5, 29.2, 29.3, 29.4, 29.5, 29.6, 30.9, 31.1, 32.0, 34.2, 34.3, 34.5, 34.8, 35.0, 35.2, 35.3, 39.7, 41.4, 42.1, 46.7, 47.2, 62.3, 62.6, 68.4, 69.0, 73.1, 75.0, 171.7 (COOR), 172.0 (COOR), 173.2 (COOR), 173.6 (COOR), 179.1 (COOH). IR (cm^{-1}): ν(OH) 3479, ν(CH) 2921, 2853, ν(C=O) 1732, ν(CH) 1377, ν(CO) 1151. Anal. Calc. for $C_{51}H_{86}O_{12}$ (891.22): 68.73% C, 9.73% H; found: 67.51% C, 9.88% H. HR-MS: for $C_{51}H_{85}O_{12}$ $[M-H]^-$ calculated: 889.6041 m/z; found: 889.6025 m/z.

4.1.29. Benzyl 3α-benzyloxycarboxy-7α,12α-bis{3-[2,3-bis(decanoyloxy)propyloxycarbonyl]propanoyloxy}-5β-cholan-24-oate (**33**)

The mixture of benzyl ester **20** (1.09 g, 1.72 mmol), BTEAC (0.14 g, 0.61 mmol), CaH_2 (0.28 g, 6.65 mmol) and toluene (20 ml) was mixed with the solution of acyl chloride **13** (3.66 g, 7.05 mmol) in toluene (10 ml), and the resulting mixture was refluxed under argon for 4 h. The mixture was poured into the mixture of aqueous AcOH (3%) and toluene. After shaking the aqueous layer was washed with toluene. The combined organic layers were washed with brine (100 ml), dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by F_c (toluene/AcOEt 15:1 and 25:2). This provided an oily liquid. Yield: 1.4 g (50%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.69 (s, 3H, CH_3), 0.80 (d, 3H, CH_3, J = 5.59 Hz), 0.88 (m, 12H, 4 × CH_3), 0.91 (s, 3H, CH_3), 0.95–2.20 (m, 78H), 2.23–2.45 (m, 10H), 2.50–2.75 (m, 8H, 2 × COCH_2CH_2CO), 4.05–4.23 (m, 4H, 2 × CH_2OCO), 4.23–4.36 (m, 4H, 2 × CH_2OCO), 4.46 (m, 1H, $C_{(3)}H$), 4.93 (m, 1H, $C_{(7)}H$), 5.10 (m, 3H, PhCH_2, $C_{(12)}H$), 5.14 (s, 2H, PhCH_2), 5.25 (m, 2H, 2 × CHOCO), 7.30–7.40 (m, 10H, 2 × *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.3, 14.2, 17.5, 22.6, 22.8, 22.9, 24.9, 25.0, 25.7, 26.8, 27.4, 28.8, 28.8, 29.0, 29.2, 29.3, 29.4, 29.4, 29.6, 30.9, 31.3, 31.4, 32.0, 34.2, 34.3, 34.5, 34.6, 34.7, 34.8, 38.0, 41.0, 43.5, 45.3, 47.5, 62.3, 62.8, 66.2, 69.0, 69.6, 71.3, 75.8, 78.3, 128.3, 128.4, 128.5, 128.6, 128.7, 128.7, 135.5, 136.3, 154.7 (OCOO), 171.7 (COOR), 171.8 (COOR), 171.9 (COOR), 172.0 (COOR), 172.1 (COOR), 173.0 (COOR), 173.1 (COOR), 173.3 (COOR), 173.4 (COOR), 174.0 (COOR).

4.1.30. 7α,12α-Bis{3-[2,3-bis(decanoyloxy)propyloxycarbonyl] propanoyloxy}-3α-hydroxy-5β-cholan-24-oic acid (**34**)

The solution of $HCOONH_4$ (0.44 g, 6.98 mmol) in MeOH (30 ml) and 10% Pd/C (0.14 g) were added to the solution of ester **33** (1.35 g, 0.84 mmol) in AcOEt (40 ml), and the mixture was stirred under argon for 3.5 h. Then the mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by F_c (hexane/acetone/AcOH 80:30:1). This provided an oily liquid. Yield: 1.0 g (86%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.70 (s, 3H, CH_3), 0.80 (d, 3H, CH_3, J = 5.95 Hz), 0.85 (m, 12H, 4 × CH_3), 0.88 (s, 3H, CH_3), 0.90–2.20 (m, 79H), 2.23–2.37 (m, 10H), 2.55–2.75 (m, 8H, 2 × COCH_2CH_2CO), 3.45 (m, 1H, $C_{(3)}H$), 4.05–4.22 (m, 4H, 2 × CH_2OCO), 4.23–4.35 (m, 4H, 2 × CH_2OCO), 4.90 (m, 1H, $C_{(7)}H$), 5.09 (m, 1H, $C_{(12)}H$), 5.18–5.31 (m, 2H, 2 × CHOCO). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.3, 14.2, 17.5, 22.6, 22.7, 22.9, 24.9,

25.0, 25.5, 27.3, 28.9, 29.1, 29.2, 29.4, 29.5, 29.6, 30.4, 30.6, 30.8, 31.4, 32.0, 34.1, 34.3, 34.3, 34.7, 35.0, 37.9, 39.0, 41.0, 43.4, 45.2, 47.3, 62.2, 62.3, 62.6, 62.7, 62.8, 68.9, 71.5, 71.6, 75.9, 76.0, 171.7 (*C*OOR), 172.0 (*C*OOR), 173.1 (*C*OOR), 173.4 (*C*OOR), 173.5 (*C*OOR), 173.6 (*C*OOR), 179.2 (*C*OOH). IR (cm^{-1}): *ν*(OH) 3476, *ν*(CH) 2923, 2854, *ν*(C = O) 1732, *ν*(CH) 1378, *ν*(CO) 1148. Anal. Calc. for $C_{78}H_{132}O_{19}$ (1373.87): 68.19% C, 9.68% H; found: 66.89% C, 9.75% H. HR-MS: for $C_{78}H_{131}O_{19}$ $[M-H]^-$ calculated: 1371.9285 m/z; found: 1371.9222 m/z.

4.1.31. Benzyl 3α,7α,12α-tris{3-[2,3-bis(decanoyloxy)propyloxycarbonyl] propanoyloxy}-5β-cholan-24-oate (**35**)

The mixture of benzyl ester **17** (0.86 g, 1.72 mmol), BTEAC (0.22 g, 0.97 mmol), CaH_2 (0.45 g, 10.69 mmol) and toluene (15 ml) was mixed with the solution of acyl chloride **13** (5.17 g, 9.96 mmol) in toluene (10 ml), and the resulting mixture was refluxed under argon for 3 h. The reaction mixture was poured into the mixture of aqueous AcOH (6%) and toluene. After shaking the aqueous layer was washed with toluene. The combined organic layers were washed with saturated aqueous $NaHCO_3$ and water, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by gradient F_c (hexane/acetone 20:1 to 5:1). This provided an oily liquid. Yield: 1.6 g (48%). ^{1}H NMR (250 MHz, $CDCl_3$), *δ*: 0.70 (s, 3H, C*H*$_3$), 0.80 (d, 3H, C*H*$_3$, *J* = 5.53 Hz), 0.88 (m, 21H, 7 × C*H*$_3$), 0.95–2.15 (m, 106H), 2.20–2.45 (m, 14H), 2.50–2.80 (m, 12H, 3 × COC*H*$_2$C*H*$_2$CO), 4.08–4.24 (m, 6H, 3 × C*H*$_2$OCO), 4.24–4.37 (m, 6H, 3 × C*H*$_2$OCO), 4.58 (m, 1H, $C_{(3)}H$), 4.93 (m, 1H, $C_{(7)}H$), 5.07–5.15 (m, 3H, PhC*H*$_2$, $C_{(12)}H$), 5.20–5.33 (m, 3H, 3 × C*H*OCO), 7.31–7.38 (m, 5H, *Ph*).

4.1.32. 3α,7α,12α-Tris{3-[2,3-bis(decanoyloxy)propyloxycarbonyl] propanoyloxy}-5β-cholan-24-oic acid (**36**)

To the solution of benzyl ester **35** (1.54 g, 0.79 mmol) in AcOEt (20 ml) 10% Pd/C (0.13 g) was added and the mixture was stirred under hydrogen for 30 min. The mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by gradient F_c (hexane/acetone/AcOH 80:5:1 and 80:10:1). This provided an oily liquid. Yield: 1.0 g (70%). ^{1}H NMR (250 MHz, $CDCl_3$), *δ*: 0.72 (s, 3H, C*H*$_3$), 0.78–0.94 (m, 24H, 8 × C*H*$_3$), 0.95–2.15 (m, 106H), 2.20–2.45 (m, 14H), 2.50–2.80 (m, 12H, 3 × COC*H*$_2$C*H*$_2$CO), 4.05–4.23 (m, 6H, 3 × C*H*$_2$OCO), 4.23–4.36 (m, 6H, 3 × C*H*$_2$OCO), 4.57 (m, 1H, $C_{(3)}H$), 4.92 (m, 1H, $C_{(7)}H$), 5.12 (m, 1H, $C_{(12)}H$), 5.18–5.33 (m, 3H, 3 × C*H*OCO). ^{13}C NMR (62.5 MHz, $CDCl_3$), *δ*: 12.3, 14.2, 17.5, 22.7, 22.8, 22.9, 24.9, 25.0, 28.8, 28.9, 29.0, 29.1, 29.2, 29.3, 29.4, 29.6, 30.6, 30.7, 31.4, 32.0, 34.2, 34.3, 34.5, 34.8, 34.9, 38.0, 41.1, 43.5, 45.3, 47.3, 62.2, 62.3, 62.7, 62.8, 68.9, 71.3, 74.6, 75.8, 171.6 (*C*OOR), 171.7 (*C*OOR), 171.9 (*C*OOR), 172.0 (*C*OOR), 172.1 (*C*OOR), 173.0 (*C*OOR), 173.1 (*C*OOR), 173.2 (*C*OOR), 173.3 (*C*OOR), 173.4 (*C*OOR), 173.5 (*C*OOR), 178.6 (*C*OOH). IR (cm^{-1}): *ν*(CH) 2923, 2854, *ν*(C=O) 1733, *ν*(CH) 1378, *ν*(CO) 1148. Anal. Calc. for $C_{105}H_{178}O_{26}$ (1856.52): 67.93% C, 9.66% H; found: 67.07% C, 10.05% H. HR-MS: for $C_{105}H_{177}O_{26}$ $[M-H]^-$ calculated: 1854.2528 m/z; found: 1854.2402 m/z.

4.1.33. Benzyl (+/−)-7α,12α-dihydroxy-3α-(3-{[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl]oxycarbonyl}propanoyloxy)-5β-cholan-24-oate (**37**)

Acyl chloride **16** (7.96 g, 14.49 mmol) dissolved in CH_2Cl_2 (20 ml) was slowly added to the solution of benzyl cholate (**17**, 6.03 g, 12.1 mmol) and DMAP (1.80 g, 14.73 mmol) in CH_2Cl_2 (40 ml) at 0 °C (ice bath). The mixture was stirred at this temperature for 20 min. Then the mixture was warmed to ambient temperature and poured into aqueous AcOH (6%). After shaking the aqueous layer was washed with CH_2Cl_2. The combined organic layers were washed with saturated aqueous $NaHCO_3$, water, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by gradient F_c (toluene/AcOEt 100:0 to 0:100). Yield: 4.5 g (37%). ^{1}H NMR (250 MHz, $CDCl_3$), *δ*: 0.66 (s, 3H, C*H*$_3$), 0.80–0.92 (m, 15H, 5 × C*H*$_3$), 0.95–2.50 (m, 64H), 2.58 (m, 2H), 2.70 (m, 2H), 2.90 (m, 2H), 3.83 (m, 1H, $C_{(7)}H$), 3.96 (m, 1H, $C_{(12)}H$), 4.62 (m, 1H, $C_{(3)}H$), 5.11 (2H, PhC*H*$_2$), 7.30–7.38 (m, 5H, *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), *δ*: 11.9, 12.3, 12.7, 12.9, 13.1, 17.5, 19.6, 19.7, 19.8, 19.9, 20.0, 20.7, 21.2, 22.6, 22.7, 22.9, 23.3, 24.6, 24.9, 26.7, 26.8, 27.5, 28.1, 28.5, 29.1, 29.7, 31.0, 31.2, 31.4, 32.8, 32.9, 34.6, 34.8, 35.0, 35.2, 35.3, 37.4, 37.5, 37.6, 37.7, 39.5, 39.7, 41.3, 42.2, 46.7, 47.7, 66.2, 68.3, 73.0, 74.9, 75.2, 117.5, 123.1, 125.1, 126.9, 128.3, 128.4, 128.7, 136.3, 140.6, 149.5, 171.1 (*C*OOR), 171.8 (*C*OOR), 174.1 (*C*OOR).

4.1.34. (+/−)-7α,12α-Dihydroxy-3α-(3-{[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl]oxycarbonyl}propanoyloxy)-5β-cholan-24-oic acid (**38**)

To the solution of $HCOONH_4$ (1.20 g, 19.03 mmol) and benzyl ester **37** (4.50 g, 4.45 mmol) in AcOEt (50 ml) and MeOH (150 ml) 10% Pd/C (0.54 g) was added and the mixture was stirred under argon for 5.5 h. The mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by gradient F_c (CH_2Cl_2/MeOH/AcOH 100:5:1 and 100:10:1). This provided an oily liquid. Yield: 3.3 g (81%). ^{1}H NMR (250 MHz, $CDCl_3$), *δ*: 0.69 (s, 3H, CH_3), 0.80–0.92 (m, 15H, 5 × CH_3), 0.95–2.50 (m, 64H), 2.58 (m, 2H), 2.71 (m, 2H), 2.91 (m, 2H), 3.85 (m, 1H, $C_{(7)}$H), 3.99 (m, 1H, $C_{(12)}$H), 4.62 (m, 1H, $C_{(3)}$H). ^{13}C NMR (62.5 MHz, $CDCl_3$), *δ*: 11.9, 12.3, 12.7, 13.1, 17.5, 19.7, 19.8, 19.9, 20.7, 21.2, 22.6, 22.7, 22.8, 23.3, 24.0, 24.6, 24.9, 26.8, 26.8, 27.6, 28.1, 28.5, 29.1, 29.7, 30.9, 31.1, 31.2, 32.8, 32.9, 34.6, 34.8, 35.0, 35.3, 37.4, 37.5, 37.6, 37.7, 39.5, 39.7, 41.3, 42.2, 46.7, 47.3, 68.4, 73.1, 74.9, 75.2, 117.5, 123.1, 125.1, 126.9, 140.6, 149.5, 171.2 (COOR), 171.9 (COOR), 179.1 (COOH). IR (cm^{-1}): *ν*(OH) 3476, *ν*(CH) 2923, 2866, *ν*(C=O) 1732, 1709, *ν*(CH) 1456, *ν*(CO) 1144, 1072, 1022. Anal. Calc. for $C_{57}H_{92}O_9$ (921.33): 74.31% C, 10.06% H; found: 73.69% C, 10.58% H. HR-MS: for $C_{57}H_{91}O_9$ $[M-H]^-$ calculated: 919.6663 m/z; found: 919.6680 m/z.

4.1.35. Benzyl 3α-benzyloxycarboxy-7α,12α-bis(3-{[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl]oxycarbonyl}propanoyloxy)-5β-cholan-24-oate (**39**)

The mixture of ester **20** (1.00 g, 1.58 mmol), BTEAC (0.16 g, 0.70 mmol), CaH_2 (0.26 g, 6.18 mmol) and toluene (20 ml) was mixed with the solution of acyl chloride **16** (2.81 g, 5.12 mmol) in toluene (12 ml) and refluxed under argon for 3 h. The reaction mixture was poured into the mixture of aqueous AcOH (3%) and toluene. After shaking the aqueous layer was washed with toluene. The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by F_c (toluene/AcOEt/AcOH 200:5:1). This provided an oily liquid. Yield: 1.5 g (57%). ^{1}H NMR (250 MHz, $CDCl_3$), *δ*: 0.68 (s, 3H, C*H*$_3$), 0.75 (d, 3H, C*H*$_3$, *J* = 5.95 Hz), 0.81–0.93 (m, 27H, 9 × C*H*$_3$), 0.95–2.40 (m, 94H), 2.49–2.62 (m, 4H, 2 × C*H*$_2$Ar), 2.65–3.10 (m, 8H, 2 × COC*H*$_2$C*H*$_2$-CO), 4.43 (m, 1H, $C_{(3)}H$), 4.95 (m, 1H, $C_{(7)}H$), 5.06 (4H, 2 × PhC*H*$_2$-O), 5.14 (m, 1H, $C_{(12)}H$), 7.29–7.37 (m, 10H, 2 × *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), *δ*: 11.9, 12.0, 12.2, 12.3, 13.1, 17.5, 19.6, 19.7, 19.8, 19.9, 20.7, 21.2, 22.6, 22.8, 22.9, 24.6, 25.0, 25.8, 26.8, 27.2, 28.1, 28.8, 28.9, 29.1, 29.4, 29.5, 30.7, 31.2, 32.8, 32.9, 34.4, 34.6, 34.7, 37.4, 37.5, 37.6, 37.7, 37.9, 39.5, 41.0, 43.5, 45.3, 47.6, 66.2, 69.5, 71.3, 75.2, 75.8, 78.2, 117.4, 117.5, 123.0, 123.1, 125.2, 126.9, 128.3, 128.4, 128.5, 128.6, 128.6, 128.7, 135.4, 136.2, 140.6, 140.7, 149.5, 149.6, 154.6, 170.8 (*C*OOR), 171.0 (*C*OOR), 171.9 (*C*OOR), 172.0 (*C*OOR), 173.9 (*C*OOR).

*4.1.36. 3α-Hydroxy-7α,12α-bis(3-{[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl]oxycarbonyl}propanoyloxy)-5β-cholan-24-oic acid (**40**)*

The mixture of ester **39** (1.34 g, 0.808 mmol), $HCOONH_4$ (0.42 g, 6.66 mmol), AcOEt (40 ml), MeOH (30 ml) and 10% Pd/C (0.11 g) was stirred under argon for 2 h. The mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by gradient F_c (CH_2Cl_2/MeOH/AcOH 300:5:1). This provided an oily liquid. Yield: 1.0 g (85%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.73 (s, 3H, C*H*$_3$), 0.79 (d, 3H, C*H*$_3$, J = 6.17 Hz), 0.82–0.92 (m, 27H, 9 × C*H*$_3$), 0.95–2.40 (m, 95H), 2.50–2.65 (m, 4H, 2 × C*H*$_2$Ar), 2.72–3.50 (m, 8H, 2 × COC*H*$_2$C*H*$_2$CO), 3.38 (m, 1H, C$_{(3)}$*H*), 4.96 (m, 1H, C$_{(7)}$*H*), 5.15 (m, 1H, C$_{(12)}$*H*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 11.9, 12.0, 12.2, 12.3, 13.3, 17.5, 19.6, 19.7, 19.8, 19.9, 20.0, 20.7, 21.2, 22.6, 22.7, 22.8, 23.0, 24.6, 24.9, 25.6, 27.3, 28.1, 28.8, 28.9, 29.0, 29.5, 29.6, 30.4, 30.5, 30.8, 31.2, 31.5, 32.8, 32.9, 34.4, 34.7, 35.1, 37.4, 37.5, 37.6, 37.7, 38.1, 39.0, 39.5, 41.1, 43.4, 45.3, 47.5, 71.6, 71.6, 75.2, 76.0, 117.5, 117.6, 123.1, 125.1, 125.2, 126.9, 140.6, 140.7, 149.6, 171.0 (*C*OOR), 171.1 (*C*OOR), 171.9 (*C*OOR), 178.8 (*C*OOH). IR (cm^{-1}): ν(CH) 2923, 2865, ν(C=O) 1730, ν(CO) 1140, 1109, 1072. Anal. Calc. for $C_{90}H_{144}O_{13}$ (1434.10): 75.38% C, 10.12% H; found: 74.87% C, 10.59% H. HR-MS: for $C_{90}H_{143}O_{13}$ $[M-H]^-$ calculated: 1432.0529 m/z; found: 1432.0514 m/z.

*4.1.37. Benzyl (+/−)-3α,7α,12α-tris(3-{[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl]oxycarbonyl}propanoyloxy)-5β-cholan-24-oate (**41**)*

The mixture of benzyl cholate (**17**, 1.00 g, 2.01 mmol), BTEAC (0.18 g, 0.79 mmol), CaH_2 (0.51 g, 12.11 mmol) and toluene (20 ml) was mixed with the solution of acyl chloride **16** (6.65 g, 12.11 mmol) in toluene (10 ml) and refluxed under argon for 3.5 h. The mixture was poured into the mixture of aqueous AcOH (3%) and toluene. After shaking the aqueous layer was washed with toluene. The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (toluene/AcOEt 20:1). This provided an oily liquid. Yield: 3.5 g (86%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.68 (s, 3H, C*H*$_3$), 0.76 (d, 3H, C*H*$_3$, J = 5.99 Hz), 0.80–0.93 (m, 39H, 13 × C*H*$_3$), 0.95–2.40 (m, 129H), 2.50–3.05 (m, 18H, 3 × COC*H*$_2$C*H*$_2$CO, 3 × C*H*$_2$Ar), 4.58 (m, 1H, C$_{(3)}$*H*), 4.94 (m, 1H, C$_{(7)}$*H*), 5.07 (2H, PhC*H*$_2$), 5.15 (m, 1H, C$_{(12)}$*H*), 7.29–7.35 (m, 5H, *Ph*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 12.0, 12.3, 12.4, 13.1, 17.5, 19.6, 19.7, 19.8, 19.9, 20.7, 21.2, 22.7, 22.8, 22.9, 24.6, 25.0, 25.9, 27.0, 28.1, 28.7, 29.0, 29.2, 29.5, 30.8, 31.3, 32.8, 32.9, 34.5, 34.7, 34.8, 37.5, 37.6, 37.7, 37.8, 38.0, 39.5, 41.1, 43.6, 45.4, 47.7, 66.2, 71.4, 74.6, 75.2, 75.9, 123.1, 125.0, 125.1, 126.9, 128.3, 128.4, 128.7, 136.3, 140.7, 149.6, 170.9 (*C*OOR), 171.1 (*C*OOR), 171.2 (*C*OOR), 171.8 (*C*OOR), 171.9 (*C*OOR), 173.9 (*C*OOR).

*4.1.38. (+/−)-3α,7α,12α-Tris(3-{[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl]oxycarbonyl}propanoyloxy)-5β-cholan-24-oic acid (**42**)*

The solution of $HCOONH_4$ (0.42 g, 6.66 mmol) in MeOH (30 ml) and 10% Pd/C (0.10 g) were added to the solution of benzyl ester **41** (3.15 g, 1.55 mmol) in AcOEt (50 ml), and the mixture was stirred under argon for 1.5 h. Then the mixture was filtered through celite and evaporated under reduced pressure. The crude product was purified by F_c (hexane/acetone/AcOH 80:10:1). This provided an oily liquid. Yield: 2.6 g (87%). ^{1}H NMR (250 MHz, $CDCl_3$), δ: 0.73 (s, 3H, C*H*$_3$), 0.79 (d, 3H, C*H*$_3$, J = 6.07 Hz), 0.83–0.95 (m, 39H, 13 × C*H*$_3$), 1.00–2.40 (m, 129H), 2.50–3.05 (m, 18H, 3 × COC*H*$_2$C*H*$_2$-CO, 3 × C*H*$_2$Ar), 4.59 (m, 1H, C$_{(3)}$*H*), 4.97 (m, 1H, C$_{(7)}$*H*), 5.18 (m, 1H, C$_{(12)}$*H*). ^{13}C NMR (62.5 MHz, $CDCl_3$), δ: 11.9, 12.2, 12.4, 13.1, 17.5, 19.6, 19.7, 19.8, 19.9, 20.0, 20.7, 21.2, 22.7, 22.8, 22.9, 24.6, 24.9, 25.9, 26.9, 27.3, 28.1, 28.6, 29.0, 29.1, 29.5, 30.5, 30.8, 31.2, 32.8, 32.9, 34.5, 34.7, 34.8, 37.4, 37.5, 37.6, 37.7, 38.0, 39.5, 41.0, 43.5, 45.3, 47.5, 71.3, 74.6, 75.2, 75.8, 117.5, 123.1, 125.0, 125.1, 126.8, 140.6, 149.5, 170.9 (*C*OOR), 171.0 (*C*OOR), 171.1 (*C*OOR), 171.8 (*C*OOR), 171.8 (*C*OOR), 179.4 (*C*OOH). IR (cm^{-1}): ν(CH) 2924, 2866, ν(C=O) 1753, 1731, 1708, ν(CH) 1377, ν(CO) 1137. Anal. Calc. for $C_{123}H_{196}O_{17}$ (1946.86): 75.88% C, 10.15% H; found: 74.83% C, 10.36% H. HR-MS: for $C_{123}H_{195}O_{17}$ $[M-H]^-$ calculated: 1944.4394 m/z; found: 1944.4269 m/z.

4.2. Lipophilicity determination and calculation of molecular descriptors

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 × 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 can be used as the lipophilicity index converted to log P scale [48].

Log P values (*i.e.*, the logarithm of the partition coefficient for octanol/water) were predicted using ACD/LogP 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/LogS DB software (ver. 12.01, Advanced Chemistry Development, Inc., Toronto, ON, Canada, 2010). ACD/Solubility DB (ver. 12.01, Advanced Chemistry Development, Inc., Toronto, ON, Canada, 2010) 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. Molar volume (MV [cm^3]) was calculated by means of ACD/ChemSketch (ACD/Labs, ver. 12.01, Advanced Chemistry Development, Inc., Toronto, ON, Canada, 2010). Polar surface area (PSA [Å^2]) was calculated using CambrigeSoftware ChemBio3D (ver. 12, CambridgeSoft, Cambridge, MA, USA). All the results are shown in Table 1.

4.3. In vitro screening of penetration enhancing effect 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 effect 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 five determinations was performed using skin fragments from a minimum of 2 animals for each compound. The samples were immediately 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 × 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 ($R^2 \geqslant 0.98$), and steady state fluxes (μg/cm^2/h) were calculated using the linear region of the plots. Enhancement ratios (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 effect of newly synthesized final compounds was evaluated *in vitro*, using a vertical PAMPA (Parallel Artificial Membrane Permeability Assay) system (BD GentestTM-Pre-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 ambient 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 × 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. Enhancement ratios (ERs) were calculated as ratios of theophylline flux with and without the enhancer. The results are summarized in Table 2.

4.5. In vitro cytotoxicity/anti-proliferative 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, 10000 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 1.0×10^5 cells/ml (0.5×10^5 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 by dividing the intensity of the fluorescence signals from the exposed wells by the intensity of signals from control wells and multiplying by 100. These ratios were then used to construct dose-response curves from which IC_{50} values were calculated, and the results obtained for the respective compounds are shown in Table 2.

4.6. Statistical analysis

All experiments were carried out fivefold (ERs data for skin), fourfold (PAMPA) or threefold (anti-proliferative activity). Data were expressed as means ± SD. Differences were evaluated by one-way analysis of the variance (ANOVA) test completed by the Bonferroni's multicomparison test (ORIGIN PRO7). The differences were considered significant at $P = 0.05$.

Acknowledgments

This study was supported by the Czech Science Foundation (the Grant Agency of the Czech Republic) in projects no. P304/11/2246, 304/10/1951 and P503/11/0616, by grant no. ED0007/01/01 Centre of the Region Hana for Biotechnological and Agricultural Research, by the Ministry of Education of the Czech Republic MSM6046 137305 and by Sanofi-Aventis Pharma Slovakia.

References

[1] Junghanns JUAH, Muller RH. Nanocrystal technology, drug delivery and clinical applications. Int J Nanomed 2008;3:295.
[2] Payghan SA, Bhat M, Savla A, Toppo E, Purohit S. Solubility of active pharmaceutical ingredients (API) has always been a concern for formulators, since inadequate aqueous solubility may hamper development of parenteral products and limit bioavailability of oral products. <http://www.pharmainfo.net/reviews/potential-solubility-drug-discovery-and-development/>.
[3] Kerns EH, Li D. Drug-like properties: concept, structure design and methods. San Diego: Elsevier; 2008.
[4] Hofmann AF, Mysels KJ. Bile salts as biological surfactants. Colloid Surf B 1988;30:145.

[5] Kim KH, Singh BN. Drug delivery – oral route. In: Swarbrick J, Boylan JC, editors. Encyclopedia of Pharmaceutical Technology. New York: Marcel Dekker; 2002. p. 899–900.
[6] Smith EW, Maibach HI. Percutaneous Penetration Enhancers. 2nd ed. Boca Raton: CRC Press; 2006.
[7] Sharma P, Chawla HPS, Panchagnula R. The role of sorption promoters in increasing the bioavailability of drugs in oral preparations. Drugs Fut 1999;24:1221.
[8] Mrozek L, Dvorakova L, Mandelova Z, Rarova L, Rezacova A, Placek L, Opatrilova R, Dohnal J, Paleta O, Kral V, Drasar P, Jampilek J. Investigation of new acyloxy derivatives of cholic acid and their esters as drug absorption modifiers. Steroids 2011;76:1082.
[9] Reddy SM, Sinha VR, Reddy DS. Novel oral colon-specific drug delivery systems for pharmacotherapy of peptide and nonpeptide drugs. Drugs Today 1999;35:537.
[10] Michael S, Thole M, Dillmann R, Fahr A, Drewe J, Fricker G. Improvement of intestinal peptide absorption by a synthetic bile acid derivative, cholylsarcosine. Eur J Pharm Sci 2000;10:133.
[11] Cernea S, Kidron M, Wohlgelernter J, Modi P, Raz I. Comparison of pharmacokinetic and pharmacodynamic properties of single-dose oral insulin spray and subcutaneous insulin injection in healthy subjects using the euglycemic clamp technique. Clin Ther 2004;26:2084.
[12] Sharma P, Varma MVS, Chawla HPS, Panchagnula R. Relationship between lipophilicity of BCS class III and IV drugs and the functional activity of peroral absorption enhancers. Farmaco 2005;60:870.
[13] Mikov M, Fawcett JP, Kuhajda K, Kevresan S. Pharmacology of bile acids and their derivatives: absorption promoters and therapeutic agents. Eur J Drug Metab Pharmacokinet 2006;31:237.
[14] Kim SK, Lee DY, Lee E, Lee YK, Kim CY, Moon HT, Byun Y. Absorption study of deoxycholic acid-heparin conjugate as a new form of oral anti-coagulant. J Control Release 2007;120:4.
[15] Mikov M, Al-Salami H, Golocorbin-Kon S, Skrbic R, Raskovic A, Fawcett JP. The influence of 3alpha,7alpha-dihydroxy-12-keto-5beta-cholanate on gliclazide pharmacokinetics and glucose levels in a rat model of diabetes. Eur J Drug Metab Pharmacokinet 2008;33:137.
[16] Yang L, Zhang H, Fawcett JP, Mikov M, Tucker IG. Effect of bile salts on the transport of morphine-6-glucuronide in rat brain endothelial cells. J Pharm Sci 2011;100:1516.
[17] Yang L, Fawcett JP, Ostergaard J, Zhang H, Tucker IG. Mechanistic studies of the effect of bile salts on rhodamine 123 uptake into RBE4 cells. Mol Pharm 2012;9:29.
[18] Duchateau GSMJE, Zuidema J, Merkus FWHM. Bile salts and intranasal drug absorption. Int J Pharm 1986;31:196.
[19] Zhou XH, Po ALW. Effects of cholic acid and other enhancers on the bioavailability of insulin from a subcutaneous site. Int J Pharm 1991;69:29.
[20] Barry BW. Modern methods of promoting drug absorption through the skin. Mol Aspects Med 1991;12:195.
[21] Carelli V, Di Colo G, Nannipieri E, Serafini MF. Bile acids as enhancers of steroid penetration through excised hairless mouse skin. Int J Pharm 1993;89:81.
[22] Hadgraft J. Pharmaceutical skin penetration enhancers. New York: Marcel Dekker; 1993.
[23] Lassmann-Vague V, Raccah D. Alternatives routes of insulin delivery. Diabetes Metab 2006;32:513.
[24] Posa M, Kevresan S, Mikov M, Cirin-Novta V, Kuhajda K. Effect of cholic acid and its keto derivatives on the analgesic action of lidocaine and associated biochemical parameters in rats. Eur J Drug Metab Pharmacokinet 2007;32:109.
[25] Hassan N, Ahad A, Ali M, Ali J. Chemical permeation enhancers for transbuccal drug delivery. Exp Opin Drug Deliv 2010;7:97.
[26] Jampilek J, Brychtova K. Azone analogues: classification, design, and transdermal penetration principles. Med Res Rev 2012;32:907.
[27] Hofmann AF, Roda A. Physicochemical properties of bile acids and their relationship to biological properties: an overview of the problem. J Lipid Res 1984;25:1477.
[28] Araki YI, Lee S, Sugihara G, Furuichi M, Yamashita S, Ohseto F. New cationic surfactants derived from bile acids: synthesis and thermodynamic and biophysicochemical properties such as membrane perturbation and protein solubilizing abilities. Colloid Surf B 1996;8:81.
[29] Hishikawa Y, Sada K, Watanbe R, Miyata M, Hanabusa K. A novel class of organogelator based on n-isopropylcholamide and the first observation of fibrous colloidal aggregates. Chem Lett 1998;1998:795.
[30] Valenta C, Nowack E, Bernkop-Schnurch A. Deoxycholate-hydrogels: novel drug carrier systems for topical use. Int J Pharm 1999;185:103.
[31] Willemen HM, Vermonden TA, Marcelis TM, Sudholter EJR. *N*-cholyl amino acid alkyl esters – a novel class of organogelators. Eur J Org Chem 2001;2001:2329.
[32] Willemen HM, Vermonden T, Marcelis ATM, Sudholter EJR. Alkyl derivatives of cholic acid as organogelators: one-component and two-component gel. Langmuir 2002;18:7102.
[33] Dukh M, Saman D, Kroulik J, Cerny I, Pouzar V, Kral V, Drasar P. Metal coordination as a tool for controlling the self-assembling and gelation properties of novel type cholic amide-phenanthroline gelating agent. Tetrahedron 2003;59:4069.
[34] Valkonen A, Lahtinen M, Virtanen E, Kikkonen S, Kolehmainen E. Bile acid amidoalcohols: simple organogelators. Biosens Bioelectron 2004;20:1233.
[35] Sayyed-Ahmad A, Lichtenberger LM, Gorfe AA. Structure and dynamics of cholic acid and dodecylphosphocholine-cholic acid aggregates. Langmuir 2010;26:13407.
[36] Brychtova K, Jampilek J, Opatrilova R, Raich I, Csollei J. Synthesis, physicochemical properties and penetration activity of alkyl-6-(2,5-dioxopyrrolidin-1-yl)-2-(2-oxopyrrolidin-1-yl)hexanoates as potential transdermal penetration enhancers. Bioorg Med Chem 2010;18:73.
[37] Brychtova K, Opatrilova R, Raich I, Kalinowski DS, Dvorakova L, Placek L, Csollei J, Richardson DR, Jampilek J. Investigating the activity of 2-substituted alkyl-6-(2,5-dioxopyrrolidin-1-yl)hexanoates as skin penetration enhancers. Bioorg Med Chem 2010;18:8556.
[38] Brychtova K, Dvorakova L, Opatrilova R, Raich I, Kacerova S, Placek L, Kalinowski DS, Richardson DR, Jampilek J. Investigation of substituted 6-aminohexanoates as skin penetration enhancers. Bioorg Med Chem 2012;20:86.
[39] Franz TJ. Percutaneous absorption on the relevance of in vitro data. J Invest Dermatol 1975;64:190.
[40] Kansy M, Senner F, Gubernator K. Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption processes. J Med Chem 1998;41:1007.
[41] Avdeef A, Tsinman O. PAMPA – a drug absorption in vitro model 13. Chemical selectivity due to membrane hydrogen bonding: in combo comparisons of HDM-, DOPC-, and DS-PAMPA models. Eur J Pharm Sci 2006;28:43.
[42] Tam KY, Velicky M, Dryfe RAW. The importance of and different approaches to permeability determination. In: Mandic Z, editor. Physico-chemical methods in drug discovery and development. Zagreb: IAPC Publishing; 2012. p. 121–64.
[43] Anelli LP, Lattuada L, Uggeri F. One-pot mitsunobu-staudinger preparation of 3-aminocholan-24-oic acid esters from 3-hydroxycholan-24-oic acid esters. Synth Commun 1998;28:109.
[44] Larsson U, Carlson R. Synthesis of amino acids with modified. Principal properties 3: sulfur-containing amino acids. Acta Chem Scand 1994;48:511.
[45] Fourneau EJ. Amino alcohols. Derivatives of glycerol and phenol ethers. Pharm Chim 1910;1:97.
[46] Cunningham C, Wenzel SW, Decker C, Seidling JR. (Kimberly-Clark Worldwide, Inc.). Tain-discharging and removing system. WO 2009133518 A2, 05.11.2009.
[47] Delaire S, Adao A, Desmurs JR, Gelo-Pujic M, Saint-Jalmes L, Kassem T. (Rhodia Chimie & Chanel Parfums Beaute). Polyphenol-based bioprecursor. WO 2006134282 A1, 21.12.2006.
[48] Mannhold R, Dross K, Sonntag C. Estimation of lipophilicity by reversed-phase thin-layer chromatography. In: Pliska V, Testa B, van de Waterbeemd H, editors. Lipophilicity in drug action and toxicology. Weinheim: VCH; 1996. p. 141–56.
[49] Dewitte RS. Understanding polyelectrolytes. Modern Drug Discov 2004;7:41.
[50] Japertas P, Didziapetris R, Petrauskas A. Fragmental methods in the analysis of biological activities of diverse compound sets. Mini-Rev Med Chem 2003;3:797–808.
[51] Ni N, El-Sayed MM, Sanghvi T, Yalkowsky SH. Estimation of the effect of NaCl on the solubility of organic compounds in aqueous solutions. J Pharm Sci 2000;89:1620.
[52] Katz M, Ben-Shlush I, Kolusheva S, Jelinek R. Rapid colorimetric screening of drug interaction and penetration through lipid barriers. Pharm Res 2006;23:580.
[53] Fang JY, Tsai TH, Hung CF, Wong WW. Development and evaluation of the essential oil from Magnolia fargesii for enhancing the transdermal absorption of theophylline and cianidanol. J Pharm Pharmacol 2004;56:1493.
[54] Sloan KB, Beall HD, Taylor HE, Getz JJ, Villaneuva R, Nipper R, Smith K. Transdermal delivery of theophylline from alcohol vehicles. Int J Pharm 1998;171:185.
[55] Evrard D, Touitou E, Kolusheva S, Fishov Y, Jelinek R. A new colorimetric assay for studying and rapid screening of membrane penetration enhancers. Pharm Res 2001;18:943.
[56] Williams AC, Barry BW. Urea analogues in propylene glycol as penetration enhancers in human skin. Int J Pharm 1989;56:43.
[57] Yamane MA, Williams AC, Barry BW. Terpene penetration enhancers in propylene glycol/water co-solvent systems: effectiveness and mechanism of action. J Pharm Pharmacol 1995;47:978.
[58] Jacobi U, Kaiser M, Toll R, Mangelsdorf S, Audring H, Otberg N, Sterry W, Lademann J. Porcine ear skin: an in vitro model for human skin. Skin Res Technol 2007;13:19.
[59] Herkenne C, Naik A, Kalia YN, Hadgraft J, Guy RH. Pig ear skin ex vivo as a model for in vivo dermatopharmacokinetic studies in man. Pharm Res 2006;23:1850.
[60] Meyer W, Schwarz K, Neurand KT. The skin of domestic mammals as a model for the human skin, with special reference to the domestic pig. Curr Probl Dermatol 1978;7:39.
[61] Wilkinson SC, Maas WJM, Nielsen JB, Greaves LC, Sandt JJM, Williams FM. Interactions of skin thickness and physicochemical properties of test compounds in percutaneous penetration studies. Int Arch Occup Environ Health 2006;79:405.
[62] Brain KR, Walters KA. Molecular modeling of skin permeation enhancement by chemical agents. In: Walters KA, Hadgraft J, editors. Pharmaceutical skin penetration enhancement. New York: Marcel Dekker; 1993. p. 389–416.
[63] Suzuki H, Sunada H. Mechanistic studies on hydrotropic solubilization of nifedipine in nicotinamide solution. Chem Pharm Bull 1998;46:125.

[64] Da Silva RC, Spitzer M, Da Silva LHM, Loh W. Investigations on the mechanism of aqueous solubility increase caused by some hydrotropes. Thermochim Acta 1999;328:161.
[65] Devinsky F, Kopecka-Leitmanova A, Sersen F, Balgavy P. Cut-off effect in antimicrobial activity and in membrane perturbation efficiency of the homologous series of *N*,*N*-dimethylalkylamine oxides. J Pharm Pharmacol 1990;42:790.
[66] Balgavy P, Devinsky F. Cut-off effects in biological activities of surfactants. Adv Coll Interface Sci 1996;66:23.
[67] Sarapuk J, Kubica K. Cut-off phenomenon. Cell Mol Biol Lett 1998;3:261.
[68] Kralova K, Sersen F, Devinsky F, Lacko I. Photosynthesis-inhibiting effects of cationic biodegradable gemini surfactants. Tenside Surfactants Deter 2010;47:288.