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Synthetic Ceramide Analogues as Skin Permeation Enhancers: Structure–Activity Relationships

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Abstract—The study presents new information about the structure–activity relationships of the skin permeation enhancers. A series of ceramide analogues including eight different polar head groups and six different chain lengths was synthesised. The compounds were evaluated as permeation enhancers in vitro using porcine skin. The physico-chemical parameters of the tested compounds obtained by computer modelling were used to evaluate, by multiple linear regression, the enhancement ratios (ERs) of the compounds. The regression analysis suggests that the hydrogen bonding ability of the compounds is inversely related to the ER values and that the molecular size and lipophilicity must be well balanced. In the studied enhancers having the same chain length, the enhancement activity is dependent only on their permeability coefficients. This finding confirms the Warner's hypothesis that the polar head of an enhancer is responsible for the permeation and anchoring of the molecule into the stratum corneum lipids and that it does not influence the mechanism of action. For the specific action of enhancers, that is disordering of the intercellular lipid packing, the length of the hydrophobic chain(s) and not the lipophilicity is important. Furthermore, the examination of the FTIR spectra indicated that the most active substances possess the most ordered chains. The described relationships could bring more rational approaches in designing new potent enhancers for transdermal formulations.

Introduction

One of the approaches to facilitation of transdermal drug delivery is the use of chemical permeation enhancers. Pharmaceutically acceptable enhancers decrease the skin barrier properties through various mechanisms;[1] the interaction with the stratum corneum (SC) intercellular lipids seems to be the most effective one.[2] Particularly, ceramides are believed to be the target of their action because they determine the level of skin permeability. Similarly to ceramides, many synthetic enhancers have a polar head and a hydrophobic chain in their molecule, which enables them to insert themselves into the ceramide matrix. At the same time, enhancers must be able to disrupt the ceramide packing, which subsequently leads to easier drug permeation.

The effort to obtain more information about the structure–activity relationships of permeation enhancers could bring more rational approaches in designing new potential enhancers for transdermal formulations. In our previous paper, a series of novel permeation enhancers designed as ceramide analogues with different polar head size and structure was reported.[3,4] The results of this initial study lead to a suggestion that the increased hydrogen bonding ability of the evaluated enhancers decreases their enhancing properties and also that the increased polar head size decreases the enhancement ability. The purpose of the present investigation was to examine these trends further by extending the studies to the barrier interactions of another ceramide analogues to obtain a larger dataset for a QSAR study. We evaluate here the skin enhancement potential of ceramide analogues of the same chain length to test what is the influence of the polar head structure in the enhancement process. The importance of lipophilicity in comparison with the hydrophobic chain length is also estimated, by comparing the analogues with the

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same polar head structure and different chain lengths. The another aim was to examine the FTIR spectra of the ceramide analogues to get information about the chain packing order. The compounds synthesised and tested are listed according to their polar head structure in Table 1.

Results and Discussion

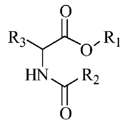
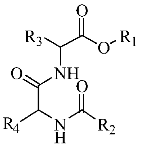
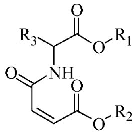
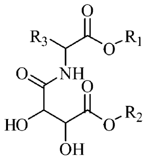
The initial study on ceramide analogues[3] lead to a hypothesis that both the polar head size and the hydrogen bonding ability are negatively related to their enhancement ratios (ERs). However, the number of compounds estimated was small to perform a QSAR study proving this hypothesis. In the present study, a larger series of ceramide analogues was synthesised including eight different polar head groups and six different chain lengths. The enhancement ability of the compounds was evaluated in vitro using full thickness porcine skin and theophylline as the model drug. ER values are listed in Table 2.

When analogues of the same chain length are compared, the best enhancement showed the compounds having glycine and glycine-maleic acid as the polar head. These compounds have the lowest hydrogen bonding ability. However, the polar head of **12GM12** is relatively large, comparable with the dipeptide derivatives, which are inactive. It means that the hydrogen bonding is of greater importance than the polar head size and/or that the *cis*-double bond is of some special importance.

As was previously noted, the optimal chain length of enhancers is about 10–12 carbons.[5] This chain length could be either ideally suited to create disorder between the much longer hydrophobic chains of ceramides or it could only give the molecule an optimal lipophilicity to penetrate into the SC. In the present study, the ERs decrease in the following order: $12 > 10 > 8 > 11 > 9$ carbons in each chain. The lipophilicity of these compounds with two chains is much higher than the lipophilicity of enhancers having only one hydrophobic chain. Therefore, it can be concluded, that the cause of the greatest activity of compounds having 12 carbon chains is the length of the hydrophobic part and not the lipophilicity. It is also remarkable that the permeation enhancing activity tends to be higher in compounds having chains with even carbon number.

In general, the interaction of the permeation enhancers with the intercellular skin lipids can be divided into two stages: the penetration of the enhancer into the SC lipid lamellae and its consecutive specific action within these lipids. Both stages of action may be determined by a combination of steric, hydrophobic and electron parameters of the enhancer. The steric parameters describing the molecular size can be modelled by molecular weight, molecular volume (e.g., van der Waals volume V_W), polarisability (π) and molar refractivity. The

Table 1. The ceramide analogues synthesized and tested as skin permeation enhancers

Polar head	Formula	R_1	R_2	R_3	R_4	Abbrev.
Amino acid		$C_{12}H_{25}$	$C_{11}H_{23}$	H		**12G12**
		$C_{11}H_{23}$	$C_{10}H_{21}$	H		**11G11**
		$C_{10}H_{21}$	C_9H_{19}	H		**10G10**
		C_9H_{19}	C_8H_{17}	H		**9G9**
		C_8H_{17}	C_7H_{15}	H		**8G8**
		$C_{12}H_{25}$	$(CH_2)_7CH{=}CH(CH_2)_7CH_3$	H		**12G18**
		$C_{12}H_{25}$	$C_{11}H_{23}$	CH_2OH		**12S12**
		$C_{12}H_{25}$	$(CH_2)_7CH{=}CH(CH_2)_7CH_3$	CH_2OH		**12S18**
Dipeptide		$C_{12}H_{25}$	$C_{11}H_{23}$	H	H	**12GG12**
		$C_{12}H_{25}$	$C_{11}H_{23}$	H	CH_2OH	**12GS12**
		$C_{12}H_{25}$	$C_{11}H_{23}$	CH_2OH	CH_2OH	**12SS12**
Amino acid + maleic acid		$C_{12}H_{25}$	$C_{12}H_{25}$	H		**12GM12**
Amino acid + tartaric acid		$C_{12}H_{25}$	$C_{12}H_{25}$	H		**12GT12**
		$C_{12}H_{25}$	$C_{12}H_{25}$	CH_2OH		**12ST12**

For the purpose of clarity, abbreviations were used instead of compound numbers (G, glycine; S, serine; M, maleic acid; T, tartaric acid; number, chain length).

Table 2. Enhancement ability (ER and logER values), physico-chemical characteristics used in the multiple regression analysis and the permeability coefficients of the studied ceramide analogues

Compd	ER ± SD	logER	Σα	Σβ	V_W	LogP	π	HE	kp_1	kp_2
12G12	4.53 ± 2.51*	0.66	0.40	1.16	474.0	7.36	50.64	14.97	0.85	10.05
11G11	2.26 ± 0.60*	0.35	0.40	1.16	440.2	6.57	46.97	13.20	0.35	8.61
10G10	3.19 ± 1.12*	0.50	0.40	1.16	406.4	5.78	43.30	10.65	−0.15	7.18
9G9	2.16 ± 0.21*	0.33	0.40	1.16	372.8	4.98	39.63	8.95	−0.64	5.75
8G8	2.35 ± 0.88	0.37	0.40	1.16	338.9	4.19	35.96	6.42	−1.14	4.30
12G18	0.45 ± 0.08	−0.35	0.40	1.23	570.0	9.48	61.46	18.85	1.97	13.85
12S12	1.24 ± 0.31	0.09	0.77	1.64	497.0	7.11	53.12	−8.05	−0.19	8.50
12S18	0.45 ± 0.09	−0.35	0.77	1.71	593.2	9.23	63.93	−4.30	0.95	12.31
12GG12	1.00 ± 0.07	0.00	0.80	1.87	520.1	6.28	55.75	5.02	−0.73	8.53
12GS12	0.94 ± 0.17	−0.03	1.17	2.35	543.8	6.04	58.22	−17.55	−1.46	7.01
12SS12	0.94 ± 0.23	−0.03	1.54	2.83	567.7	5.79	60.70	−40.39	−1.70	5.50
12GM12	6.64 ± 1.85**	0.82	0.40	1.68	544.7	7.79	58.52	6.75	0.20	11.01
12GT12	1.04 ± 0.08	0.02	1.14	2.57	563.1	6.51	59.98	−19.84	−0.66	7.02
12ST12	0.83 ± 0.02	−0.08	1.51	3.05	586.3	6.26	62.45	−40.18	−1.11	5.48

$n = 3$–6; * $p < 0.05$; ** $p < 0.01$.

Σα and Σβ, scaled values of hydrogen bond donor and acceptor potentials; V_W, Van der Waals volume (Å^3); logP, logarithm of the octanol–water partition coefficient; π, polarizability (Å^3); MR, molar refractivity (Å^3); HE, hydration energy (kJ/mol).

kp values were calculated according to Pugh[12] (kp_1) and Potts[9] (kp_2).

hydrophobicity is well modelled by the logarithm of the octanol–water partitioning coefficient (logP), and, the electron parameters include the donor and acceptor hydrogen-bonding ability (Σα and Σβ), hydration energy (HE), HOMO (highest occupied molecular orbital) and LUMO (lowest unocupied molecular orbital) energies.

The physico-chemical parameters of the prepared ceramide analogues obtained by computer modelling (listed in Table 2) were used to evaluate, by multiple linear regression, the ER values obtained by the permeation experiments. Only those terms, which had statistically significant regression coefficients and resulted in the maximal value of *F*-statistics and the maximal regression coefficient, were included in the regression analysis; all other terms were omitted. Finally, we obtained the following equation:

$$\begin{array}{lllll} \mathrm{logER} = & -4.00\Sigma\alpha & -4.42\Sigma\beta & +0.29V_\mathrm{w} & -1.56\mathrm{logP} \\ & (0.61) & (1.12) & (0.06) & (0.32) \\ -2.30\pi & -0.09\mathrm{HE} & -2.29 & & \\ (0.49) & (0.02) & (0.79) & & \end{array} \tag{1}$$

$$n = 14;\ r^2 = 0.92;\ s = 0.13;\ F = 19;\ p < 0.001$$

The figures in parentheses are the standard errors of the regression coefficients, n is the number of compounds, r^2 is the determination coefficient, s is the standard error of estimate F is the significance test (F-test) and p is the probability error.

Eq 1 implies that the enhancement effect of the substances tested is primarily controlled by the hydrogen bonding ability, molecular size, lipophilicity and hydration energy, that is by the physico-chemical parameters that are relevant and interpretable. No relationship was found between the parameters describing the chemical reactivity of the ceramide analogues, that is HOMO and LUMO energies. This finding is consistent with the view of enhancers' action. The first important suggestion is that decreasing both donor and acceptor hydrogen bonding ability of the enhancers could lead to an increase of activity. The regression analysis could serve as a predictive tool under the condition of a larger dataset. We used eq 1, however, as a practical tool of making directions for future syntheses. The logical direction for the next syntheses of the amino acid based enhancers is towards sarcosine and proline derivatives as they have decreased hydrogen bonding. Proline derivatives have already been reported as permeation enhancers, supporting this hypothesis.[6–8]

The influence of the molecular size on the enhancing activity of the tested ceramide analogues is modelled by the van der Waals volume and polarisability. While volume is a positive determinant in eq 1, the polarisability is a negative one. The lipophilicity is also negatively related to the ER value. The possible explanation is that both the size and lipophilicity of an enhancer have an impact on different processes and must be well balanced. In the process of permeation of any compound (not only enhancer) into the SC lipids, the increasing size increases the hydrophobic surface and this will enhance partitioning into and permeability through a lipid membrane. Conversely, larger molecules require more space and diffuse more slowly.[9] Furthermore, the optimal chain length of an enhancer to create disorder in the ceramide lamellae has been found to be about 10–12 carbons.[5] The hydration energy characterising free energy of interaction of the molecule with the surrounding water is also a negative determinant of the enhancing activity of these compounds.

Previously, Warner[10,11] hypothesised that for enhancer with the same chain length, the alkyl group of an enhancer is able to penetrate and to intercalate into the lipid bilayer to the same depth, thus inducing the same extent of regional fluidisation, while the polar head group locates in the polar/semipolar microenvironment.

He also suggested that the polar head group does not contribute to enhancer action in any local/specific manner; it assists in the translocation of the enhancer to the site of action via its free energy of transfer (partitioning) from the bulk aqueous phase to the interfacial polar/semipolar microenvironment. Thus, the enhancement ratios of compounds with the same chain length should be related only to their permeability coefficients into the SC from an aqueous phase (kp). The permeability coefficients were calculated according to Pugh[12] (kp_1) and according to Potts[9] (kp_2); these values are listed in Table 2. These kp values were developed for application to human skin, however, their use in our calculation with ERs obtained using porcine skin is appropriate, because we aimed to find a relationship, not to develop a quantitative predictive model. For the evaluation of the above-mentioned hypothesis, only compounds with the chain length of 12 carbons have been included in this regression analysis. The analysis revealed the following relationships:

$$\log ER(12C) = \underset{(0.11)}{0.12(\log kp_1)^2} \underset{(0.14)}{+0.45 \log kp_1} \underset{(0.11)}{+0.32} \tag{2}$$

$$n = 8;\quad r^2 = 0.74;\quad s = 0.21;\quad F = 17;\quad p < 0.01$$

$$\log ER(12C) = \underset{(0.01)}{0.04(\log kp_2)^2} \underset{(0.17)}{-0.56 \log kp_2} \underset{(0.69)}{+1.71} \tag{3}$$

$$n = 8;\quad r^2 = 0.95;\quad s = 0.10;\quad F = 103;\ p < 0.0001$$

Eqs 2 and 3 show that the differences in the ER values of the tested enhancers with the same chain lengths may be explained simply by the different permeabilities of those enhancers into their site of action. The differences in these two equations could be explained by the different methods of permeability coefficient calculation.

The relationship described by eq 3 is shown graphically in Figure 1. This finding supports the hypothesis of Warner that the polar head does not contribute to the local mechanism of action of enhancers. Although the dataset is small, the value of this support is that it was obtained using different enhancers, different type of permeation experiment and different data analysis. The action of this type of skin permeation enhancers could be hypothesised as described in Figure 2. However, for a relevant proof, more substances should be evaluated.

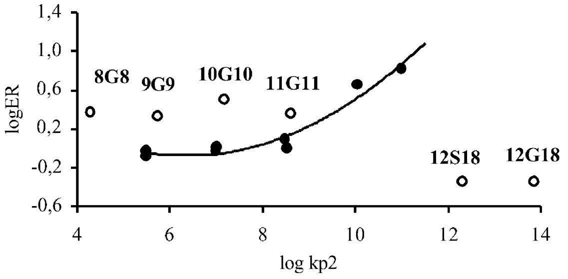


Figure 1. The enhancement ability (logER) of the studied permeation enhancers in relation to their permeability coefficients calculated according to Potts[9] ($\log kp_2$). ● enhancers having the same chain length and different polar head structure, ○ enhancers having different chain length. The figure shows that the differences in the ER values in the ceramide analogues with different polar head structure could be attributed only to their different permeability into the site of their action.

With respect to the FTIR measurements it was found in the spectra of the symmetric glycine-based ceramide analogues, the CH_2 rocking mode is split into two bands at 719 and 730 cm^{-1} in **12G12–9G9** and 720 and 729 cm^{-1} in **8G8**. This indicates orthorhombic chain packing.[13] The CH_2 scissoring mode is also split (1473 and 1463 cm^{-1}) and the symmetric methylene stretching mode is 2849 cm^{-1} in **12G12** and 2850 cm^{-1} in **11G11–8G8**. The maleic acid derivative **12GM12** has the methylene stretching mode at 2850 cm^{-1} and the scissoring mode is split into two bands. No splitting and the symmetric CH_2 stretching higher than 2850 cm^{-1} were found in other ceramide analogues, indication less ordered chain packing. The ceramide analogues with the highest permeation enhancing activity possess the most ordered chains, thus we may speculate that the chain order is important for the action of enhancers. This finding was observed during examination of routine IR spectra and detailed investigation of the thermotropic phase behaviour of these ceramide analogues is necessary.

Experimental

Chemicals and instrumentation

All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany). Silica gel 60 (230–400 mesh) and Lichroprep RP-18 (15–25 μm) for column chromatography, TLC plates (silica gel 60 F_{254}) and HPLC columns (see in HPLC determination section) were obtained from Merck (Darmstadt, Germany).

The structure and purity of the synthesised compounds was confirmed by FTIR (Nicolet Impact 400 spectrophotometer) and ^1H and ^{13}C NMR spectra (Varian Mercury-Vx BB 300 instrument, operating at 300 MHz for ^1H, 75 MHz for ^{13}C). Optical rotations were measured using ADP Bellingham and Stanley Polarimeter; 0.5 dm cell. Melting points were measured using the Kofler apparatus and are uncorrected.

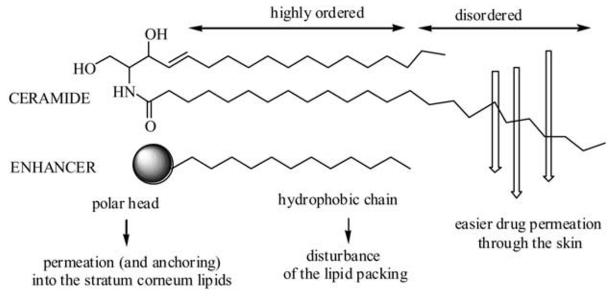


Figure 2. The schematic representation of the hypothesised mechanism of action of the studied permeation enhancers.

Chemistry

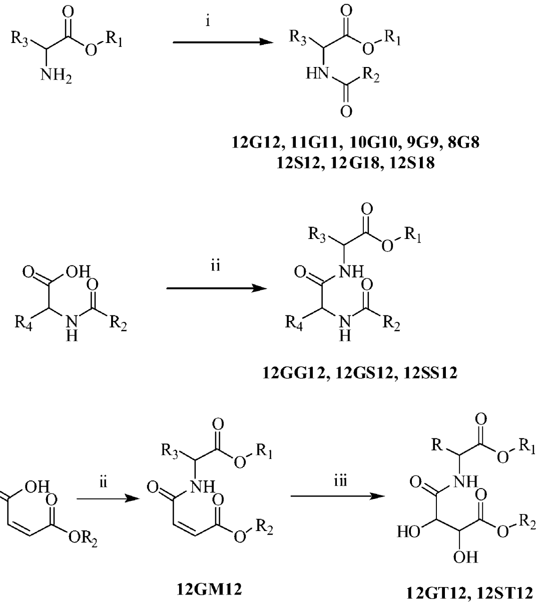
Scheme 1 outlines the synthesis of the ceramide analogues.

General procedure for the preparation of the alkoxycarbonylmethylammonium chlorides (glycine esters hydrochlorides)[14]

50 g of glycine was dissolved in water and 60 mL of concentrated HCl was added. The mixture was stirred for 1 h and then evaporated to dryness. White crystalline glycine hydrochloride was dried in vacuo over KOH (mp = 184–185 °C, the same as reported[14]). 0.1 mol of glycine hydrochloride and 0.3 mol of the pertinent alcohol were stirred at 110–120 °C. After 30 min, dry hydrogen chloride was introduced into the mixture for 15 min and this was repeated every 2 h. The reaction mixture was kept under nitrogen. After 8 h, the mixture was crystallised from hot acetone several times and the resulting white crystals were dried in vacuo over KOH.

Dodecyloxycarbonylmethylammonium chloride. $C_{14}H_{30}NO_2Cl$; 279.85 g mol^{-1}; Yield: 91%; white crystals; mp = 93.5–95 °C (mp 94–95 °C was reported[14]); IR (KBr): ν_{max} 3447, 2919, 2851, 1744, 1467, 1241 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ 8.52 (3H; s; NH_3^+); 4.12 (2H; t; J = 6.6 Hz; OCH_2); 3.75 (2H; s; NCH_2); 1.65–1.50 (2H; m; CH_2); 1.40–1.15 (18H; m; 9 CH_2); 0.84 (3H; t; J = 6.7 Hz; CH_3); ^{13}C NMR (75 MHz, DMSO): δ 167.9; 65.6; 31.5; 29.3; 29.2; 29.2; 28.9; 28.9; 28.2; 25.4; 22.3; 14.2.

Undecyloxycarbonylmethylammonium chloride. $C_{13}H_{28}NO_2Cl$; 265.82 g mol^{-1}; Yield: 86%; white crystals; mp = 84–86 °C; IR (KBr): ν_{max} 3432, 2922, 2853, 1750, 1467, 1248 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ 8.53 (3H; s; NH_3^+); 4.11 (2H; t; J = 6.6 Hz; OCH_2); 3.74 (2H; s; NCH_2); 1.65–1.50 (2H; m; CH_2); 1.40–1.15 (16H; m; 8 CH_2); 0.84 (3H; t; J = 6.7 Hz; CH_3); ^{13}C NMR (75 MHz, DMSO): δ 167.8; 65.5; 31.5; 29.2; 29.2; 28.9; 28.9; 28.2; 25.4; 22.3; 14.2.



Scheme 1. (i) Alkanoyloxysuccinimide, $CHCl_3$; (ii) TEA, THF, ClCOOEt, amino acid ester; (iii) $KMnO_4$, DH-18-C-6, CH_2Cl_2.

Decyloxycarbonylmethylammonium chloride. $C_{12}H_{26}NO_2Cl$; 251.80 g mol^{-1}; Yield: 89%; white crystals; mp = 81–84 °C (mp 87–89 °C was reported[14]); IR (KBr): ν_{max} 3432, 2922, 2853, 1745, 1468, 1245 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ 8.56 (3H; s; NH_3^+); 4.12 (2H; t; J = 6.6 Hz; OCH_2); 3.74 (2H; s; NCH_2); 1.70–1.50 (2H; m; CH_2); 1.40–1.15 (14H; m; 7CH_2); 0.84 (3H; t; J = 6.5 Hz; CH_3); ^{13}C NMR (75 MHz, DMSO): δ 167.9; 65.5; 31.5; 29.2; 28.9; 28.9; 28.2; 25.4; 22.3; 14.2.

Nonyloxycarbonylmethylammonium chloride. $C_{11}H_{24}NO_2Cl$; 237.77 g mol^{-1}; Yield: 85%; white crystals; mp = 74–77 °C; IR (KBr): ν_{max} 3447, 2923, 2854, 1749, 1465, 1254, 1243 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ 8.52 (3H; s; NH_3^+); 4.12 (2H; t; J = 6.6 Hz; OCH_2); 3.74 (2H; s; NCH_2); 1.65–1.50 (2H; m; CH_2); 1.40–1.15 (12H; m; 6CH_2); 0.84 (3H; t; J = 6.7 Hz; CH_3); ^{13}C NMR (75 MHz, DMSO): δ 167.8; 65.6; 31.5; 29.2; 28.9; 28.2; 25.4; 22.3; 14.2.

Octyloxycarbonylmethylammonium chloride. $C_{10}H_{22}NO_2Cl$; 223.74 g mol^{-1}; Yield: 88%; white crystals; mp = 65–68 °C (mp 68–70 °C was reported[14]); IR (KBr): ν_{max} 3432, 2926, 2856, 1752, 1468, 1249 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ 8.52 (3H; s; NH_3^+); 4.12 (2H; t; J = 6.6 Hz; OCH_2); 3.75 (2H; s; NCH_2); 1.65–1.50 (2H; m; CH_2); 1.40–1.15 (10H; m; 5CH_2); 0.84 (3H; t; J = 6.7 Hz; CH_3); ^{13}C NMR (75 MHz, DMSO): δ 167.9; 65.6; 31.4; 28.8; 28.2; 25.4; 22.3; 14.2

(L) - 1 - Dodecyloxycarbonyl - 2 - hydroxyethylammonium chloride. $C_{15}H_{32}NO_3Cl$; 309.87 g mol^{-1}. The procedure was analogous to that for preparation of glycine esters hydrochlorides. L-Serine was used in the reaction (not the hydrochloride) and the temperature was kept at 70 °C. Yield: 85%; white crystals; mp = 92–95 °C; $[\alpha]_D^{25°C} = -7{,}7°$ (1,0; ethanol); IR (KBr): ν_{max} 2923, 2853, 1747, 1468, 1245, 1024 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ 8.55 (3H; s; NH_3^+); 5.62 (1H; s; OH); 4.12 (2H; t; J = 6.5 Hz; $COOCH_2$); 4.05 (1H; t; J = 3.7 Hz; CHN); 3.89 (2H; t; J = 3.7 Hz; CH_2OH); 1.65–1.50 (2H; m; CH_2); 1.40–1.15 (18H; m; 9 CH_2); 0.83 (3H; t; J = 6.7 Hz; CH_3); ^{13}C NMR (75 MHz, DMSO): δ 168.3; 65.7; 59.7; 54.5; 31.5; 29.3; 29.2; 29.2; 29.2; 28.9; 28.9; 28.2; 25.4; 22.3; 14.2

General procedure for the preparation of the *N*-hydroxysuccinimide esters[15]

150 mmol of the carboxylic acid (C8–C12) and 150 mmol of *N*-hydroxysuccinimide were suspended in dry ethyl acetate. A solution of 165 mmol of dicyclohexyl carbodiimide (DCC) and 15 mmol of 4-pyrrolidinopyridine (PP) in dry ethyl acetate was added. The mixture was stirred at room temperature overnight. Then 1.5 mL of glacial acetic acid was added and the mixture was stirred for further 2 h. The resulting dicyclohexylurea was filtered off and washed with ethyl acetate. The filtrate was allowed to stand at 4 °C overnight

and eventual dicyclohexylurea was filtered off. The filtrate was washed with water and brine and then evaporated to dryness. Pure esters were obtained by crystallisation from ethanol.

***N*-Dodecanoyloxysuccinimide.** $C_{16}H_{27}NO_4$; 297.40 g mol^{-1}. Yield: 83%; white crystals; mp = 75.5–77.5 °C (mp 75 °C was reported[15]); IR (KBr): ν_{max} 3436, 2922, 2852, 1827, 1819, 1787, 1742, 1728, 1211, 1071, 867, 722, 654 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.83 (4H; s; $2CH_2CON$); 2.59 (2H; t; *J* = 7.4 Hz; $COCH_2$); 1.80–1.65 (2H; m; CH_2); 1.50–1.15 (16H; m; $8CH_2$); 0.87 (3H; t; *J* = 6.6 Hz; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.2; 168.7; 31.9; 30.9; 29.5; 29.5; 29.3; 29.3; 29.1; 28.8; 25.6; 24.5; 22.6; 14.1.

***N*-Undecanoyloxysuccinimide.** $C_{15}H_{25}NO_4$; 283.37 g mol^{-1}. Yield: 72%; white crystals; mp = 59–61 °C; IR (KBr): ν_{max} 3436, 2923, 2853, 1823, 1787, 1742, 1728, 1210, 1072, 868, 722, 654 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.83 (4H; s; $2CH_2CON$); 2.59 (2H; t; *J* = 7.4 Hz; $COCH_2$); 1.80–1.65 (2H; m; CH_2); 1.50–1.15 (16H; m; $8CH_2$); 0.87 (3H; t; *J* = 6.6 Hz; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.2; 168.7; 31.8; 30.9; 29.5; 29.3; 29.2; 29.1; 28.7; 25.6; 24.5; 22.6; 14.1.

***N*-Decanoyloxysuccinimide.** $C_{14}H_{23}NO_4$; 269.34 g mol^{-1}. Yield: 72%; white crystals; mp = 67–69 °C (mp 62–65 °C was reported[16]); IR (KBr): ν_{max} 3436, 2923, 2853, 1823, 1787, 1742, 1728, 1210, 1072, 868, 722, 654 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.83 (4H; s; $2CH_2CON$); 2.59 (2H; t; *J* = 7.4 Hz; $COCH_2$); 1.80–1.65 (2H; m; CH_2); 1.50–1.15 (16H; m; $8CH_2$); 0.87 (3H; t; *J* = 6.6 Hz; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.2; 168.7; 31.8; 30.9; 29.3; 29.2; 29.1; 28.7; 25.6; 24.5; 22.6; 14.1.

***N*-Nonanoyloxysuccinimide.** $C_{13}H_{21}NO_4$; 255.31 g mol^{-1}. Yield: 74%; white crystals; mp = 52–54 °C; IR (KBr): ν_{max} 3436, 2924, 2854, 1821, 1787, 1743, 1728, 1211, 1072, 867, 722, 654 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.83 (4H; s; $2CH_2CON$); 2.59 (2H; t; *J* = 7.4 Hz; $COCH_2$); 1.80–1.65 (2H; m; CH_2); 1.50–1.15 (16H; m; $8CH_2$); 0.87 (3H; t; *J* = 6.6 Hz; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.2; 168.7; 31.7; 30.9; 29.1; 29.0; 28.7; 25.6; 24.5; 22.6; 14.1.

***N*-Octanoyloxysuccinimide.** $C_{12}H_{19}NO_4$; 241.29 g mol^{-1}. Yield: 76%; white crystals; mp = 56–57 °C (mp 63 °C was reported[15]); IR (KBr): ν_{max} 3436, 2923, 2856, 1819, 1787, 1743, 1728, 1212, 1072, 867, 723, 654 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.83 (4H; s; $2CH_2CON$); 2.59 (2H; t; *J* = 7.4 Hz; $COCH_2$); 1.80–1.65 (2H; m; CH_2); 1.50–1.15 (16H; m; $8CH_2$); 0.87 (3H; t; *J* = 6.6 Hz; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.2; 168.7; 31.5; 30.9; 28.7; 28.7; 25.5; 24.5; 22.5; 14.1.

***N*-Oleoyloxysuccinimide.** $C_{22}H_{37}NO_4$; 379.54 g mol^{-1}. 4.24 g (15 mmol) of oleic acid (99% purity), 3.40 g (16.5 mmol) of DCC and 0.22 g (1.5 mmol) of PP were dissolved in 50 mL of dry tetrahydrofurane (THF) at 0 °C. 3.45 g (30 mmol) of *N*-hydroxysuccinimide in dry THF was added and the mixture was stirred 2 h at 0 °C and then 16 h at room temperature. The reaction was monitored by TLC (chloroform/methanol 9:1). The precipitated dicyclohexylurea was filtered off and washed with ethyl acetate. The filtrate was evaporated, dissolved in ethyl acetate and allowed to stand at 4 °C overnight. Precipitated dicyclohexylurea was filtered off and the filtrate was washed with water and brine. The organic layer was dried with Na_2SO_4 and the solvent was evaporated. The purification was finished after this step in the reference,[17] however, two spots were still present on TLC chromatogram. We added 1.5 mL of triethylamine (TEA) dropwise to the residuum until the spot of oleic acid disappeared. Pure product was obtained by column chromatography using silicagel and diethyl ether as the eluent. Yield: 4.47 g (76%); colourless oil; IR ($CHCl_3$): ν_{max} 3028, 3007, 2927, 2856, 1816, 1787, 1743 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.35–5.25 (2H; m; 2CH=); 2.81 (4H; s; $2CH_2CON$); 2.57 (2H; t; *J* = 7.4 Hz; $COCH_2$); 2.05–1.85 (4H; m; 2C*H*$_2$CH=); 1.80–1.50 (2H; m; CH_2); 1.45–1.15 (20H; m; $10CH_2$); 0.85 (3H; t; *J* = 6.7 Hz; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.2; 168.6; 130.0; 129.7; 31.9; 30.9; 29.7; 29.6; 29.5; 29,3; 29.0; 28.7; 27.2; 27.1; 25.6; 24.5; 22.7; 14.1.

General procedure for the preparation of the *N*-acylamino acids[15]

30 mmol of glycine (or L-serine) was dissolved in 200 mL of solution of 30 mmol of $NaHCO_3$ in water. After 15 min, 30 mmol of *N*-alkanoyloxysuccinimide in 200 mL of THF was added. The mixture was stirred for 16 h at room temperature and then was acidified with 1 M HCl to pH 2. THF was evaporated and another 100 mL of water was added to the mixture. Resulting crystals were filtered off and recrystallised from methanol/water.

***N*-Dodecanoylglycine.** $C_{14}H_{27}NO_3$; 257.37 g mol^{-1}. Yield: 90%; white crystals; mp = 116–118 °C (mp 117 °C was reported[15]); IR (KBr): ν_{max} 3313, 2919, 2850, 1701, 1645, 1560 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ 12.45 (1H; bs; COOH); 8.09 (1H; t; *J* = 5.9 Hz; NH); 3.70 (2H; d; *J* = 5.8 Hz; C*H*$_2$NH); 2.08 (2H; t; *J* = 7.4 Hz; $COCH_2$); 1.55–1.40 (2H; m; CH_2); 1.30–1.15 (16H; m; $8CH_2$); 0.84 (3H; t; *J* = 6.7 Hz; CH_3); ^{13}C NMR (75 MHz, DMSO): δ 172.8; 171.7; 35.3; 31.6; 29.3; 29.3; 29.2; 29.1; 29.0; 25.4; 22.3; 14.2.

***N*-Dodecanoyl-(L)-serine.** $C_{15}H_{29}NO_4$; 287.40 g mol^{-1}. Dimethoxyethane (DME) was used instead of THF. Yield: 93%; white crystals; mp = 85.5–88 °C; $[\alpha]_D^{25°C} = 9.2°$ (0.9; ethanol) (mp 87 °C[15] was reported); IR (KBr): ν_{max} 3339, 2853, 1739, 1614, 1537, 1471, 1212, 1037, 722 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ 7.91 (1H; t; *J* = 8.0 Hz; NH); 4.25 (1H; dt; J_1 = 8.0 Hz; J_2 = 4.8 Hz; CHN); 3.70–3.50 (2H; m; C*H*$_2$OH); 3.35 (1H; bs; OH); 2.12 (2H; t; *J* = 7.4 Hz; $COCH_2$); 1.55–1.35 (2H; m; CH_2); 1.35–1.15 (16H; m; $8CH_2$); 0.84 (3H; t; *J* = 6.6 Hz; CH_3); ^{13}C NMR (75 MHz, DMSO): δ 172.6; 172.4; 61.7; 54.7; 35.2; 31.6; 29.3; 29.3; 29.3; 29.2; 29.1; 29.0; 28.9; 25.5; 22.4; 14.4.

General procedure for the preparation of the *N*-acyl-amino acid esters

3.3 mmol of TEA was added to a suspension of 3 mmol of amino acid ester hydrochloride in ca. 20 mL of chloroform and stirred for 1 h. The mixture was washed several times with brine, the organic layer was dried with Na_2SO_4 and evaporated to dryness. The resulting oil (glycine or L-serine ester) was kept overnight in vacuo over sulphuric acid to remove the residual TEA. The amino acid ester and 3 mmol of *N*-acyloxysuccinimide were dissolved in 25 mL of dry chloroform and stirred at room temperature. The reaction was monitored by TLC (chloroform/methanol 9:1). After 20 h, the reaction mixture was washed with water and 5% HCl. The organic layer was dried by Na_2SO_4 and evaporated. Pure products were crystallised from chloroform: diethyl ether.

***N*-Dodecanoylglycine dodecyl ester (12G12).** The data were reported earlier.[3]

***N*-Undecanoylglycine undecyl ester (11G11).** $C_{24}H_{47}NO_3$; 397.63 g mol^{-1}. Yield: 90%; white crystals; mp = 76.5–77.5 °C; IR (KBr): ν_{max} 3421, 3333, 2919, 2850, 1740, 1642, 1547, 1473, 1463, 1245, 730, 719 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 5.97 (1H; bs; NH); 4.14 (2H; t; *J* = 6.9 Hz; COO–CH_2); 4.03 (2H; d; *J* = 4.6 Hz; CH_2NH); 2.23 (2H; t; *J* = 7.7 Hz; $COCH_2$); 1.75–1.50 (4H; m; 2 CH_2); 1.40–1.15 (30H; m; 15CH_2); 0.87 (6H; t; *J* = 6.6 Hz; 2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.2; 170.2; 65.7; 41.3; 36.4; 31.9; 29.6; 29.5; 29.5; 29.4; 29.3; 29.2; 28.5; 25.8; 25.6; 22.7; 14.1; CHN analysis (% calculated/found): C 72.49/72.16; H 11.91/12.15; N 3.52/3.33

***N*-Decanoylglycine decyl ester (10G10).** $C_{22}H_{43}NO_3$; 369.58 g mol^{-1}. Yield: 76% white crystals; mp = 70.5–72 °C; IR (KBr): ν_{max} 3428, 3332, 2919, 2850, 1740, 1641, 1548, 1473, 1464, 1243, 729, 719 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 5.98 (1H; bs; NH); 4.14 (2H; t; *J* = 6.7 Hz; COO–CH_2); 4.03 (2H; d; *J* = 5.0 Hz; CH_2NH); 2.23 (2H; t; *J* = 7.7 Hz; $COCH_2$); 1.75–1.50 (4H; m; 2CH_2); 1.40–1.10 (26H; m; 13CH_2); 0.95–0.75 (6H; m; 2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.2; 170.2; 65.7; 41.3; 36.4; 31.9; 29.6; 29.5; 29.5; 29.4; 29.3; 29.2; 28.5; 25.8; 25.6; 22.7; 14.1; CHN analysis (% calculated/found): C 71.50/71.40; H 11.73/11.89; N 3.79/3.62

***N*-Nonanoylglycine nonyl ester (9G9).** $C_{20}H_{39}NO_3$; 341.53 g mol^{-1}. Yield: 84%; white crystals; mp = 61.5–63 °C; IR (KBr): ν_{max} 3421, 3333, 2919, 2850, 1740, 1642, 1547, 1473, 1463, 1245, 730, 719 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 5.98 (1H; bs; NH); 4.14 (2H; t; *J* = 6.7 Hz; COO–CH_2); 4.03 (2H; d; *J* = 4.9 Hz; CH_2NH); 2.23 (2H; t; *J* = 7.6 Hz; $COCH_2$); 1.80–1.55 (4H; m; 2CH_2); 1.45–1.15 (22H; m; 11CH_2); 0.95–0.80 (6H; m; 2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.2; 170.2; 65.7; 41.3; 36.4; 31.9; 29.6; 29.5; 29.5; 29.4; 29.3; 29.2; 28.5; 25.8; 25.6; 22.7; 14.1; CHN analysis (% calculated/found): C 70.33/70.09; H 11.51/11.74; N 4.10/3.96

***N*-Octanoylglycine octyl ester (8G8).** $C_{18}H_{35}NO_3$; 313.48 g mol^{-1}. Yield: 70%; white crystals; mp = 55.5–56 °C; IR (KBr): ν_{max} 3428, 3331, 2923, 2851, 1740, 1641, 1548, 1473, 1463, 1243, 729, 720 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 5.97 (1H; bs; NH); 4.14 (2H; t; *J* = 6.7 Hz; COO–CH_2); 4.03 (2H; d; *J* = 5.2 Hz; CH_2NH); 2.23 (2H; t; *J* = 7.7 Hz; $COCH_2$); 1.75–1.55 (4H; m; 2CH_2); 1.40–1.15 (18H; m; 9CH_2); 0.95–0.75 (6H; m; 2 CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.2; 170.2; 65.7; 41.3; 36.4; 31.7; 31.6; 29.2; 29.1; 29.0; 28.5; 25.8; 25.6; 22.6; 22.6; 14.1; CHN analysis (% calculated/found): C 68.97/68.69; H 11.25/11.51; N 4.47/4.42

***N*-Oleoylglycine dodecyl ester (12G18).** $C_{32}H_{61}NO_3$; 507.83 g mol^{-1}. The reaction was carried out in dichloromethane and the product was obtained by column chromatography using silicagel and diethyl ether as the eluent followed by crystallisation from petroleum ether. Yield: 78%; white crystals; mp = 53.5–54.5 °C; IR (KBr): ν_{max} 3428, 3313, 3006, 2918, 2850, 1740, 1646, 1551, 1469, 1214, 719 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 5.95 (1H; bs; NH); 5.40–5.20 (2H; m; CH=CH); 4.12 (2H; t; *J* = 6.7 Hz; COO–CH_2); 4.01 (2H; d; *J* = 4.7 Hz; CH_2NH); 2.21 (2H; t; *J* = 7.6 Hz; $COCH_2$); 2.05–1.85 (4H; m; 2 CH_2CH=); 1.70–1.50 (4H; m; 2CH_2); 1.45–1.10 (38H; m; 19 CH_2); 0.95–0.75 (6H; m; 2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.2; 170.2; 130.0; 129.7; 65.7; 41.3; 36.4; 31.9; 29.7; 29.7; 29.6; 29.6; 29.5; 29.5; 29.5; 29.5; 29.3; 29.2; 29.2; 29.1; 28.5; 27.2; 27.1; 25.8; 25.6; 22.7; 14.1; CHN analysis (% calculated/found): C 75.68/75.16; H 12.11/12.45; N 2.76/2.33.

***N*-Dodecanoyl-L-serine dodecyl ester (12S12).** $C_{27}H_{53}NO_4$; 455.72 g mol^{-1}. Yield: 55%; white crystals; mp = 85–86 °C; $[\alpha]_D^{25\,°C} = 12.5°$ (1.0; chloroform); IR (KBr): ν_{max} 3436, 3315, 2919, 2850, 1734, 1649, 1548, 1467, 1282, 1242, 1082, 721 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 6.51 (1H; d; *J* = 7.1 Hz; NH); 4.70–4.60 (1H; m; CHNH); 4.16 (2H; t; *J* = 6.7 Hz; COO–CH_2); 3.94 (2H; d; *J* = 3.6 Hz; CH_2OH); 2.96 (1H; bs; OH); 2.26 (2H; t; *J* = 7.7 Hz; $COCH_2$); 1.75–1.55 (4H; m; 2 CH_2); 1.40–1.10 (34H; m; 17 CH_2); 0.87 (6H; t; *J* = 6.6 Hz; 2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.9; 170.6; 66.1; 63.8; 54.8; 36.5; 31.9; 29.6; 29.5; 29.5; 29.5; 29.3; 29.2; 29.2; 28.4; 25.8; 25.6; 22.7; 14.1; CHN analysis (% calculated/found): C 75.92/76.16; H 12.55/13.02; N 2.60/2.70.

***N*-Oleoyl-L-serine dodecyl ester (12S18).** $C_{33}H_{63}NO_4$; 537.86 g mol^{-1}. The same procedure as for the preparation of *N*-oleoylglycine dodecyl ester. Yield: 75%; white crystals; mp = 71.5–72 °C; $[\alpha]_D^{25\,°C} = 11.0°$ (1.0; chloroform); IR (KBr): ν_{max} 3478, 3308, 3007, 2921, 2851, 1718, 1651, 1546, 1465, 1281, 1085, 721 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 6.46 (1H; bs NH); 5.40–5.20 (2H; m; CH=CH); 4.70–4.60 (1H; m; CHNH); 4.15 (2H; t; *J* = 6.3 Hz; COO–CH_2); 4.00–3.85 (2H; m; CH_2OH); 2.62 (1H; bs OH); 2.25 (2H; t; *J* = 7.4 Hz; $COCH_2$); 2.10–1.90 (4H; m; 2 CH_2CH=); 1.75–1.50 (4H; m; 2 CH_2); 1.40–1.00 (38H; m; 19 CH_2); 0.95–0.80 (6H; m; 2 CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.8; 170.6; 130.0; 129.7; 66.1; 63.9; 54.9; 36.5; 31.9; 29.7; 29.7; 29.6;

29.5; 29.5; 29.3; 29.2; 29.2; 29.1; 28.5; 27.2; 27.2; 25.8; 25.5; 22.7; 14.1; CHN analysis (% calculated/found): C 73.69/74.17; H 11.81/12.14; N 2.60/2.01.

General procedure for the preparation of the dipeptide derivatives 12GG12, 12SG12 and 12SS12

2 mmol of *N*-dodecanoylamino acid was dissolved in dry DME and 2 mmol of TEA was added dropwise. The flask was closed with a silicone septum and kept 15 min at −20 °C. 2 mmol of ethylchloroformate was added into the mixture through the septum and the mixture was allowed to stand at −20 °C for additional 15 min. 2 mmol of amino acid dodecyl ester (prepared from its hydrochloride, see above) in dry DME was added and after 15 min the mixture was allowed to warm up to room temperature and stirred for 1.5 h. The purification is described in the pertinent compounds.

***N*-Dodecanoylglycylglycine dodecyl ester (12GG12).** $C_{28}H_{54}N_2O_4$; 482.74 g mol^{-1}. The solvent was evaporated, dissolved in chloroform and washed with 10% HCl, 5% $NaHCO_3$ and water. The organic layer was dried with Na_2SO_4 and evaporated. The product was crystallised from glacial acetic acid. Yield: 35%; white crystals; mp = 120.5–121 °C; IR (KBr): ν_{max} 3420, 3308, 3085, 2918, 2850, 1746, 1637, 1559, 1467, 1215, 1037, 721 cm^{-1}; ^{1}H NMR (300 MHz, CF_3COOD): δ 4.55–4.15 (6H; m; $2CH_2N$, CH_2O); 2.65 (2H; t; *J* = 7.1 Hz; $COCH_2$); 1.90–1.65 (4H; m; 2 CH_2); 1.60–1.15 (34H; m; 17 CH_2); 1.00–0.80 (6H; m; 2 CH_3); ^{13}C NMR (75 MHz, CF_3COOD): δ 174.5; 173.3; 70.2; 45.4; 43.6; 36.9; 33.7; 31.4; 31.3; 31.3; 31.2; 31.1; 30.9; 30.7; 29.9; 27.6; 27.3; 24.3; 14.6. In the HMBC spectrum, the signals of CH_2N protons at 4.3 ppm showed strong correlations to the carbonyl group carbons at 174.5 and 173.3 ppm. CHN analysis (% calculated/found): C 69.66/69.32; H 11.27/11.65; N 5.80/5.37.

***N*-Dodecanoyl-L-serylglycine dodecyl ester (12SG12).** $C_{29}H_{56}N_2O_5$; 512.77 g mol^{-1}. The product was purified by column chromatography using silicagel RP-18 and methanol as the mobile phase. The fractions were monitored by TLC (chloroform/methanol 9:1). Yield: 60%; white crystals; mp = 86–87 °C; $[\alpha]_D^{25^\circ C} = -5.9^\circ$ (1.0; chloroform); IR (KBr): ν_{max} 3424, 3307, 2920, 2851, 1734, 1637, 1546, 1467, 1210, 1060, 721 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35 (1H; bs; N*H*CH_2); 6.65 (1H; d; *J* = 6.3 Hz; N*H*CH); 5.98 (1H; bs; OH); 4.54 (1H; bs; CHN); 4.12 (2H; t; *J* = 6.7 Hz; $COOCH_2$); 4.01 (2H; d; *J* = 5.0 Hz; C*H*$_2$OH); 3.62 (2H; s; CH_2N); 2.25 (2H; t; *J* = 7.6 Hz; $COCH_2$); 1.70–1.55 (4H; m; 2 CH_2); 1.40–1.15 (34H; m; 17 CH_2); 0.87 (6H; t; *J* = 6.6 Hz; 2 CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.2; 171.5; 169.7; 65.8; 62.8; 53.7; 41.3; 36.4; 31.9; 29.6; 29.6; 29.5; 29.5; 29.3; 29.2; 29.2; 28.5; 25.8; 25.5; 22.7; 14.1; CHN analysis (% calculated/found): C 67.93/67.59; H 11.01/11.48; N 5.46/5.29.

***N*-Dodecanoyl-L-seryl-L-serine dodecyl ester (12SS12).** $C_{30}H_{58}N_2O_6$; 542.80 g mol^{-1}. Purified in the same manner as 12SG12. Yield: 19%; white crystals; mp = 126–129 °C; $[\alpha]_D^{25^\circ C} = 10.0^\circ$ (0.5; chloroform); IR (KBr): ν_{max} 3417, 3320, 2922, 2852, 1737, 1638, 1625, 1542, 1467, 1211, 1057, 721 cm^{-1}; ^{1}H NMR (300 MHz, pyridine): δ 9.41 (1H; d; *J* = 8.0 Hz; NH); 8.84 (1H; d; *J* = 7.7 Hz; NH); 7.09 (2H; bs; 2 OH); 5.55–5.40 (1H; m; CHN); 5.30–5.15 (1H; m; CHN); 4.55–4.00 (6H; m; $COOCH_2$; 2C*H*$_2$OH); 2.50–2.40 (2H; m; $COCH_2$); 1.90–1.65 (2H; m; CH_2); 1.65–1.45 (2H; m; CH_2); 1.40–1.00 (34H; m; 17CH_2); 0.84 (6H; t; *J* = 6.7 Hz; 2CH_3); ^{13}C NMR (75 MHz, pyridine): δ 174.3; 174.3; 171.2; 66.3; 66.2; 62.8; 55.2; 54.3; 36.5; 31.9; 29.6; 29.6; 29.6; 29.5; 29.5; 29.3; 29.3; 29.2; 29.2; 28.5; 25.8; 25.6; 22.7; 14.1; CHN analysis (% calculated/found): C 65.18/64.65; H 10.57/11.07; N 5.07/4.95.

General procedure for the preparation of the maleic acid derivatives

Maleic acid monododecyl ester was prepared according to Huber.[18] 50 mmol of maleic anhydride and 50 mmol of dodecanol was stirred 10 min at 130–140 °C. Product was obtained by crystallisation from hexane. Yield: 81; white crystals; mp = 55–57 °C. 5 mmol of maleic acid monododecylester was dissolved in dry THF and 5 mmol of TEA was added dropwise. The flask was closed with a silicone septum and kept 15 min at −20 °C. 5 mmol of ethylchloroformate was added into the mixture through the septum and the mixture was allowed to stand at −20 °C for additional 15 min. 5 mmol of amino acid dodecyl ester (prepared from its hydrochloride, see above) in dry THF was added and after 15 min the mixture was allowed to warm up to room temperature and stirred for 1.5 h. The precipitated triethylammonium chloride was filtered off and the filtrate was evaporated to dryness. The residuum was dissolved in chloroform and washed with water. The organic layer was dried with Na_2SO_4, evaporated and dried in vacuo over P_2O_5.

***N*-(Dodecyloxycarbonylmethyl)maleinamic acid dodecyl ester (12GM12).** $C_{30}H_{55}NO_5$; 509.77 g mol^{-1}. Yield: 91%; white crystals; mp = 50–53 °C; IR (KBr): ν_{max} 3433, 3366, 3058, 2919, 2851, 1747, 1730, 1668, 1635, 1538, 1472, 1465, 1244, 1198, 720 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.87 (1H; s; NH); 6.32 (1H; d; *J* = 13.2 Hz; CH=); 6.15 (1H; d; *J* = 13.2 Hz; CH=); 4.20–4.05 (6H; m; 2 $COOCH_2$; CH_2N); 1.70–1.55 (4H; m; 2 CH_2); 1.40–1.15 (36H; m; 18 CH_2); 0.84 (6H; t; *J* = 6.7 Hz; 2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.6; 166.2; 164.0; 137.7; 126.1; 65.9; 65.6; 41.7; 31.9; 29.6; 29.5; 29.5; 29.3; 29.2; 28.4; 28.3; 25.8; 25.8; 22.7; 14.1; CHN analysis (% calculated/found): C 70.68/70.31; H 10.88/11.02; N 2.75/2.49

***N*-(1-Dodecyloxycarbonyl-2-hydroxyethyl)maleinamic acid dodecyl ester (12SM12).** $C_{31}H_{57}NO_6$; 539.79 g mol^{-1}. Pure product was obtained by crystallisation from methanol. Yield: 78%; white crystals; mp = 60–61 °C; $[\alpha]_D^{25^\circ C} = 58.8^\circ$ (1.0; chloroform); IR (KBr): ν_{max} 3458, 2920, 2851, 1739, 1705, 1647, 1535, 1468, 1211, 1071, 721 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.76 (1H; d; *J* = 6.9 Hz; NH); 6.45 (1H; d; *J* = 12.5 Hz; CH=); 6.14 (1H; d; *J* = 12.5 Hz; CH=); 4.75–4.60 (1H; m; CH–N); 4.30–4.05 (6H; m; 2$COOCH_2$); 3.95 (2H; dd;

$J_1 = 11.7$ Hz; $J_2 = 2.6$ Hz; CH_2OH); 3.11 (1H; bs; OH); 1.75–1.55 (4H; m; 2 CH_2); 1.40–1.15 (36H; m; 18 CH_2); 0.87 (6H; t; $J = 6.7$ Hz; 2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.2; 165.8; 164.7; 138.2; 125.2; 66.1; 65.9; 62.7; 55.5; 31.9; 29.6; 29.5; 29.5; 29.3; 29.2; 28.4; 28.3; 25.8; 25.7; 22.7; 14.1; CHN analysis (% calculated/found): C 68.98/69.40; H 10.64/11.01; N 2.59/2.54

General procedure for the preparation of the tartaric acid derivatives (according to Mukaiyama,[19] modified)

2 mmol of **12GM12** or **12SM12** was dissolved in 10 mL of dry dichloromethane and cooled to −50 °C (solid CO_2/ethanol). 2.2 mmol of $KMnO_4$ and 0.4 mmol of crown-ether DH-18-C-6 in dry dichloromethane was added dropwise and the mixture was stirred 3 h at −50 °C. The reaction mixture was allowed to warm up to 0 °C slowly and 10 mL of saturated aqueous Na_2SO_3 was added. The mixture was transferred to a separatory funnel and 5% H_2SO_4 was added in small portion with vigorous shaking until the brown mixture decolourised. The resulting emulsion was extracted with dichloromethane several times, the organic part was evaporated to dryness. Product was obtained by column chromatography using silicagel and chloroform/ethyl acetate 8:2.

***N*-(Dodecyloxycarbonylmethyl)-2,3-dihydroxysuccinamic acid dodecyl ester (12GT12).** $C_{30}H_{57}NO_7$; 543.78 g mol^{-1}. Yield: 26%; white crystals; mp = 67–70 °C; IR (KBr): ν_{max} 3416, 2922, 2852, 1740, 1668, 1540, 1534, 1217, 1110, 721 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.35 (1H; t; $J = 5.5$ Hz; NH); 4.60–4.45 (2H; m; C*H*$_2$NH); 4.19 (2H; t; $J = 6.9$ Hz; COO–CH_2); 4.14 (2H; t; $J = 6.7$ Hz; COO–CH_2); 4.07 (1H; d; $J = 5.5$ Hz; C*H*OH); 4.04 (1H; d; $J = 5.5$ Hz; C*H*OH); 3.92 (1H; bs; OH); 3.77 (1H; bs; OH); 1.75–1.55 (4H; m; 2CH_2); 1.40–1.15 (36H; m; 18 CH_2); 0.87 (6H; t; $J = 6.7$ Hz; 2 CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.6; 170.4; 169.8; 73.6; 72.5; 66.4; 65.9; 40.9; 31.9; 29.6; 29.6; 29.6; 29.5; 29.3; 29.2; 28.5; 28.4; 25.8; 25.7; 22.7; 14.1; CHN analysis (% calculated/found): C 66.26/66.01; H 10.57/10.91; N 2.58/2,47

***N*-(1-Dodecyloxycarbonyl-2-hydroxyethyl)-2,3-dihydroxysuccinamic acid dodecyl ester (12ST12).** $C_{31}H_{59}NO_8$; 573.81 g mol^{-1}. Yield: 22%; white crystals; mp = 93.5–96 °C; IR (KBr): ν_{max} 3412, 2922, 2852, 1736, 1663, 1534, 1467, 1217, 721 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.75 (1H; d; $J = 7.7$ Hz; NH); 4.85–4.65 (1H; m; C*H*NH); 4.65–4.50 (3H; m; 3 OH); 4.50–4.35 (1H; m; C*H*OH); 4.30–4.05 (4H; m; 2 COO–CH_2); 4.05–3.85 (2H; m; C*H*$_2$OH); 3.85–3.65 (1H; m; C*H*OH); 1.75–1.50 (4H; m; 2CH_2); 1.40–1.10 (36H; m; 18CH_2); 0.87 (6H; t; $J = 6.6$ Hz; 2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.5; 170.7; 170.3; 73.8; 72.8; 66.4; 66.2; 62.6; 54.7; 31.9; 29.6; 29.6; 29.6; 29.5; 29.3; 29.2; 28.4; 28.4; 25.8; 22.7; 14.1; CHN analysis (% calculated/found): C 64.89/65.24; H 10.36/10.61; N 2.44/1.96

Permeation experiments

Porcine ears were obtained from the local slaughterhouse. The full-thickness dorsal skin was carefully separated from the underlying tissue by a scalpel and the hairs were clipped off. Then the skin was soaked in 0.005% sodium azide in saline for 5 min for preservation. The skin was vacuum-sealed and stored at −18 °C for maximum 2 months.

The enhancement activities of the compounds were evaluated in vitro using the Franz diffusion cells and theophylline as a model permeant. Donor samples were prepared as 5% aqueous suspensions of theophylline with 1% of the tested enhancer. All donor samples were saturated with the pertinent enhancer at this concentration. The suspensions were stirred for 5 min at 50 °C and then they were left standing at 37 °C for 24 h to equilibrate. Control samples containing 5% of theophylline were prepared likewise. Porcine skin was slowly thawed directly before use and cut into ca. 2 × 2 cm pieces. The skin samples were mounted into the Franz diffusion cells dermal side down to leave an area of 1 cm^2. The acceptor compartment of the cell was filled with phosphate buffer saline pH 7.4 with 0.03% sodium azide as a preservative. The precise volume of the acceptor compartment (16–18 mL) was taken and included into calculation. The Franz cells were put into a 32 °C water bath for 30 min to equilibrate. Then 200 μL of the donor sample was applied and occluded with a cover glass. In each experiment, three controls were run. The acceptor phase was stirred and tempered at 32 °C throughout the experiment. Sink conditions were maintained. Samples of the acceptor phase (0.6 mL) were taken at seven predetermined intervals during 48 h and replaced with fresh acceptor phase.

HPLC determination and data analysis

Theophylline in the acceptor phase samples was determined by HPLC using the LCP high-pressure pump (ECOM, Prague, Czech Republic), autosampler (ECOM, Prague, Czech Republic), LiChroCART 250-4 column (LiChrospher 100, RP 18, 5 μm, Merck, Darmstadt, Germany), an SP 8440 UV detector (Spectra Physics) and CSW 1.7 integrating software. Methanol/0.1 M NaH_2PO_4 6:4 v/v was used as the mobile phase at a flow rate of 1.2 mL/min. The effluent was monitored at 272 nm. The retention time of theophylline was 3.3 ± 0.1 min.

The cumulative amount of theophylline permeated across the skin corrected for acceptor sample replacement was plotted against time. The steady state flux was calculated from the linear region of the plot (Fig. 3). All the tested compounds are characterised by the enhancement ratio (ER, ratio of the means of the pertinent flux values and the control ones). Statistical analysis was performed using Student's *t*-test.

Computer calculations and QSAR study

Quantum-chemical calculations were run on a PC computer using software HyperChem for Windows v. 7.1, Hypercube. The models of compounds were formed on RHF/AM1 level. We calculated conformations of compounds with parallel aliphatic chains. We used the

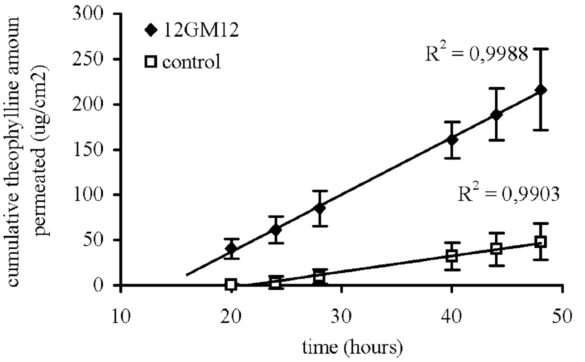


Figure 3. An example of the plot of cumulative amount of theophylline permeated through the skin versus time from a sample containing 1% **12GM12** and the control donor sample (without enhancer).

method of restraint forces for this purpose. For instance for the compound 12G12 we used the restrained distance between the carbon atom C2 of the alcoholic part of the molecule and the carbon atom C4 of the acyl part of the molecule (distance 4 Å, force constant 7) and for the corresponding atoms C8 and C10 we used the same distance and the force constant 0.5 and 20 points on cube side.

Calculations of logP values were carried out using atomic parameters derived by Ghose[20] and later extended by Viswanadhan.[21] Van der Waals volume V_W were calculated using the grid method described by Bodor[22] using the atomic radii of Gavezotti.[23] Hydration energy is calculated by the method published by Scheraga[24] and polarizability is estimated from an additivity scheme presented by Miller.[25]

The ability to form hydrogen bonds was calculated as summed fragmental values[12] of H-bond donor and acceptor properties (primary alcohol: $\alpha = 0.37$, $\beta = 0.48$; secondary alcohol: $\alpha = 0.33$, $\beta = 0.56$; secondary amide $\alpha = 0.40$, $\beta = 0.71$; ester $\alpha = 0$, $\beta = 0.45$; double bond $\alpha = 0$, $\beta = 0.07$).[26]

Correlation and regression analyses of the QSAR study were run on a PC computer using the Microsoft Excel program. Multiple regression analysis which involves finding the best fit of dependent variable (enhancement ratios) to a linear combination of independent variables (physico-chemical descriptors) were used by the least squares method.

Permeability coefficients calculations

The permeability coefficients (kp) of the ceramide analogues were calculated according to Pugh[12] (kp_1) and according to Potts[9] (kp_2) by the following equations.

$$\log kp_1 = -1.324 + 0.59 \log P - 0.761\,\mathrm{RC} - 0.00463 M_r \qquad (4)$$

$$\mathrm{RC} = -0.0678 + 1.17\ \mathrm{RC}^* - 0.0807(\mathrm{RC}^*)^2$$

$$\mathrm{RC}^* = 0.0024 + 1.36(\Sigma\alpha - \Sigma\beta) + 3.18\Sigma\beta$$

$$\log kp_2 = 0.256\,\mathrm{MV} - 1.72\,\Sigma\alpha - 3.93\,\Sigma\beta - 4.85 \qquad (5)$$

In eq 4, M_r is the molecular weight (g mol^{-1})and RC and RC* values are calculated from the hydrogen bonding donor and acceptor ability.[12] In eq 5, the molecular volume (MV) is given in cm^3 mol^{-1}. We used the V_W values for the calculation, where MV cm^3 mol^{-1} = 1.66 Å^3 per 1 molecule.

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