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Antiproliferative 3-deoxysphingomyelin analogs: Design, synthesis, biological evaluation and molecular docking of pyrrolidine-based 3-deoxysphingomyelin analogs as anticancer agents

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ABSTRACT

Sphingomyelins and glycerophospholipids are structurally related phospholipids. Nevertheless, glycerophospholipids analogs are known as antitumor agents while sphingomyelin analogs were reported as cytoprotective agents. Herein, we have addressed the development of 3-deoxysphingomyelin analogs as cytotoxic agents possessing modified sphingobases. Thus, pyrrolidine-based 3-deoxysphingomyelin analogs were synthesized and evaluated against a panel of cell lines representing four major types of cancers. Compounds **3d**, **4d** and **6d** elicited better GI_{50} values than the FDA approved drug miltefosine. Investigation of their impact on Akt phosphorylation as a possible mechanism for the antiproliferative activity of this class of compounds revealed that these compounds might elicit a concentration-dependent mechanism via inhibition of Akt phosphorylation at the lower concentration. Molecular docking predicted their binding modes to Akt to involve polar head binding to the Pleckstrin homology domain and hydrophobic tail extension into a hydrophobic pocket connecting the Pleckstrin homology domain and the kinase domain. As a whole, the described work suggests compounds **3d**, **4d** and **6d** as promising pyrrolidine-based 3-deoxysphingomyelin analogs for development of novel cancer therapies.

1. Introduction

Despite the achieved progress in understanding cancer biology, cancer still remains a difficult disease to treat and one of the leading causes of death all over the world [1,2]. According to the statistical analysis of the global burden of disease (GBD), cancers is the second among the major cause of death [3]. Alarmingly, the lifetime risk of cancer has approached up to 53.5% for males and 47.5% for females in some societies [4]. Parenthetically, around half of the population of these societies might develop cancer during their lifetime. The destructive consequences of cancer go beyond the patients' health to the socio-economical scope influencing patients' families, community and economy. There is an indeed need to develop new therapeutic tools and strategies in the fight against cancer.

Glycerophospholipids are a class of lipids that possess a glycerol backbone to which fatty acids and a polar phosphocholine head are attached. In comparison, sphingomyelins (**1**, Fig. 1) possess nitrogenous sphingobases (long chain bases; LCBs) instead of the glycerol backbone of glycerophospholipids [5]. Several synthetic analogs of glycerophospholipids retaining the glycerol backbone such as edelfosine or lacking it such as miltefosine are known as antitumor compounds [6]. In contrast, several sphingomyelin analogs were found to elicit cytoprotective activities via inhibition of sphingomyelinase [7,8]. Interestingly, the literature reports 3-*O*-methylated-sphingomyelin analogs as sphingomyelinase inhibitors, but 3-deoxysphingomyelin analogs in which a hydrogen atom replaces the hydroxyl group at 3-position of the sphingobase were found to be not liable for hydrolytic cleavage by sphingomyelinase nor possessing inhibitory activity against it [9]. This indicates that the oxygen atom at 3-position of the sphingobase is important for sphingomyelinase substrates or competitive inhibitors. This

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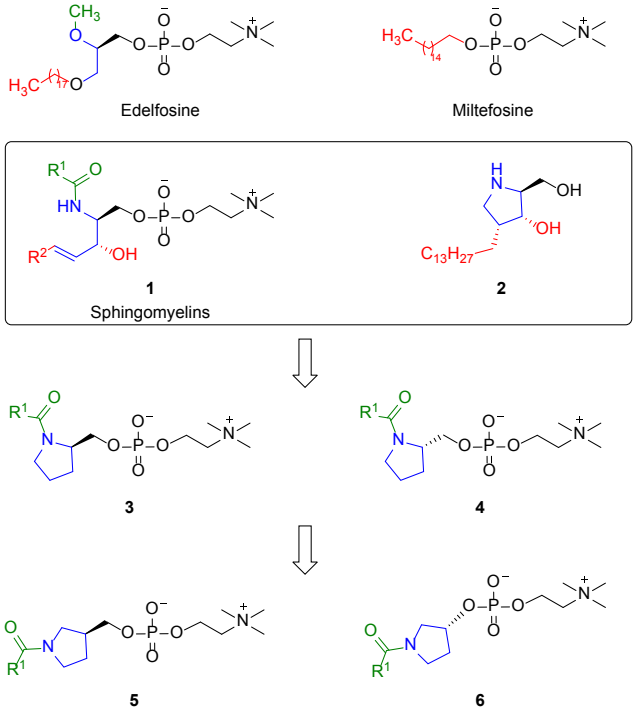


Fig. 1. Reported antitumor alkylphospholipids and the design of pyrrolidine-based 3-deoxysphingomyelin analogs 3, 4, 5 and 6.

suggests that 3-deoxysphingomyelins might be devoid from the cytoprotective activity arising from the reported sphingomyelinase inhibitory activity of sphingomyelin analogs. Furthermore, their structural similarity with glycerophospholipids might suggest 3-deoxysphingomyelins analogs as potential candidates for development of cytotoxic agents. To the best of our knowledge, the literature reports no effort to develop antitumor 3-deoxysphingomyelins analogs despite this apparent structural similarity.

Recently, a novel cytotoxic pyrrolidine-derived sphingomimetic base (**2**) was designed *via* conformational restriction [10]. Considering the pro-apoptotic activity of this pyrrolidine-based derivative as well as the literature reports indicating that 3-deoxysphingomyelins are devoid from the sphingomyelinase inhibitory activity responsible for the cytoprotective activity, in addition to the structural similarity with antitumor alkylphospholipids analogs, we have anticipated that pyrrolidine-based 3-deoxysphingomyelin analogs might elicit a promising antiproliferative activity against cancer cells. Accordingly, novel pyrrolidine-based 3-deoxysphingomyelin analogs belonging to four series were designed, synthesized, biologically evaluated and mechanistically studied. As shown in Fig. 1, the design involved conformational restriction between the 2-amino group and the C-5 carbon atom of the LCB moiety, excision of the alkyl chain of the LCB moiety beyond the introduced pyrrolidine ring, as well as, the hydroxyl group at C-3 carbon atom (highlighted in red color) while retaining the long chain FAs (highlighted in green color) to afford the novel series **3** of pyrrolidine-based 3-deoxysphingomyelin analogs. Series **3** was designed to retain a chiral configuration similar to that of natural sphingomyelins. However, the influence of stereochemistry inversion of the pyrrolidine ring on the biological activity was planned to be assessed after synthesis of series **4**, which represent the opposite enantiomeric form of series **3**. Displacement of the *N*-acylamide group of series **3** by one carbon atom would produce the novel series **5** while displacement of the *N*-acylamide group of series **4** by one carbon atom and direct attachment of the phosphocholine to 2-position of the pyrrolidine ring would provide series **6**. According to the proposed design, the impact of incorporation of various saturated and unsaturated fatty acid moieties on the biological activity would be explored. Herein, we would like to report our results.

2. Results and discussion

2.1. Chemistry

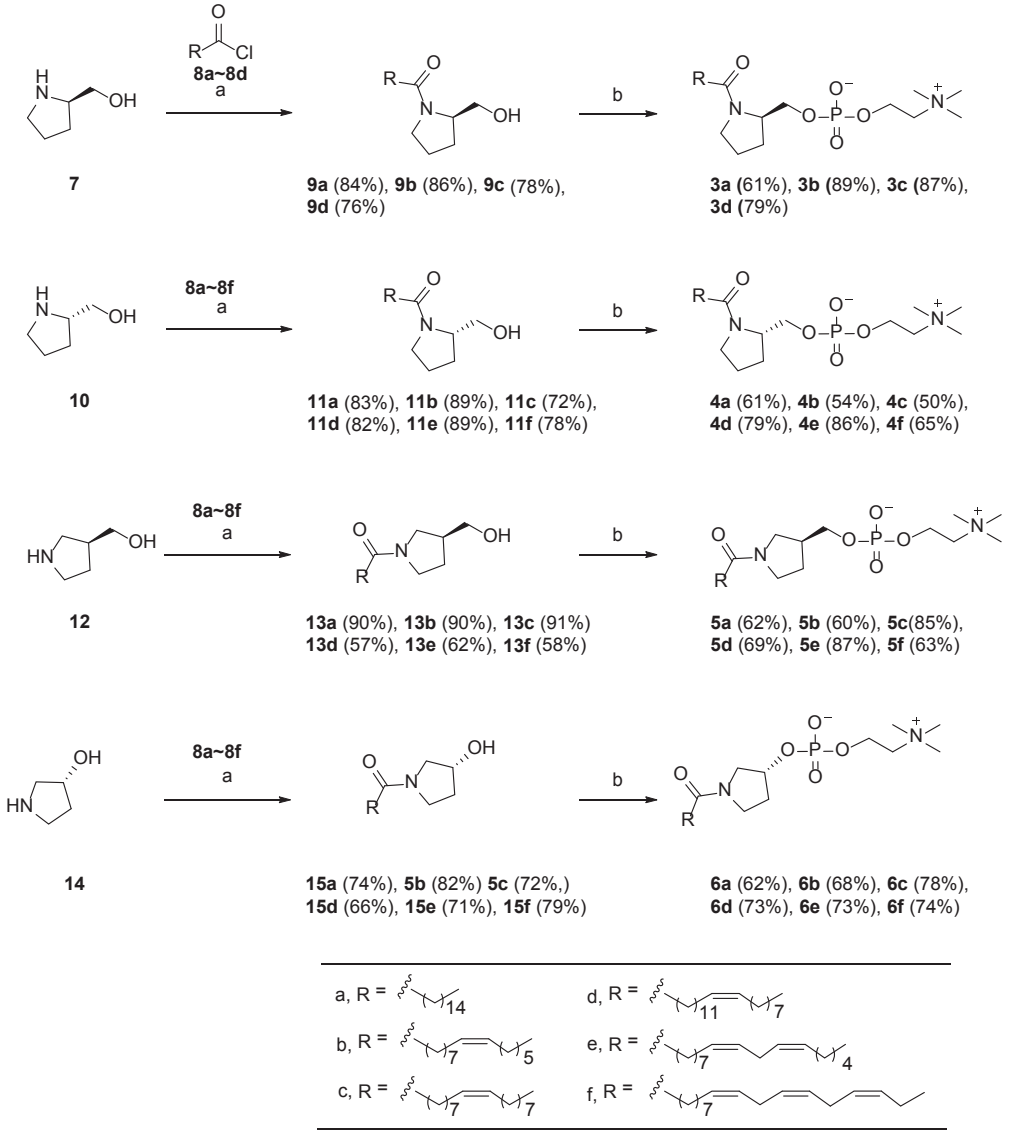
Accessing the desired molecules in few synthetic steps is one of the aims of a good synthetic plan. As concise synthesis would help to approach this ideal goal through step economy, the targeted compounds, as shown in Scheme 1, were synthesized in two pots over three synthetic steps starting from commercially available chiral pool of the starting materials D-prolinol (**7**), L-prolinol (**10**), (*S*)-3-pyrrolidinemethanol (**12**) and (*R*)-3-pyrrolidinol (**14**). The required acid chlorides (**8a–f**) were obtained from palimitic acid, palmitoleic acid, oleic acid, erucic acid, linoleic acid, and α-linolenic acid respectively following a procedure similar to the reported literature method [11]. In the first pot, the starting pyrrolidine derivatives **7**, **10**, **12** and **14** were reacted with acid chlorides **8a–f** in the presence of triethylamine as a base and dimethylformamide as a solvent to afford the corresponding amides **9a–d**, **11a–f**, **13a–f** and **15a–f** respectively. In the second pot, the intermediate amide derivative **9a–d**, **11a–f**, **13a–f** and **15a–f** were first reacted with 2-choloro-1,3,2-dioxaphospholane-2-oxide at 0 °C to afford the corresponding cyclic phospholane esters followed by opening of the ring *via* heating under pressure with trimethylamine to yield the targeted designed compounds.

2.2. Biological evaluation

2.2.1. Inhibition of cell line proliferation:

The antiproliferative activity of the synthesized novel 3-deoxysphingomyelin analogs was evaluated using MTT assay against a panel of four human cancer cells representing breast cancer, non-small-cell lung cancer, liver cancer, and skin cancer. Based on structural similarity of the tested compounds with alkylphospholipids, Akt phosphorylation inhibition was anticipated to be the mechanism of action of these compounds. Therefore, miltefosine (a member of alkylphospholipids that has been approved by FDA for treatment of cutaneous metastatic breast cancer) was used as a reference standard for activity comparisons as it was reported to elicit cytotoxic activity through inhibition of Akt phosphorylation as well as other mechanisms [12]. Except for MCF-7 cell line (breast cancer), miltefosine was found to elicit a limited inhibitory activity on the growth of other used cell lines showing a growth inhibition not exceeding 36.6% at 100 μM concentrations.

Breast cancer is the most common cancer among females and it is ranked the second in regard to the costs associated with premature-mortality [13–15]. In addition, miltefosine; the reference drug used in the conducted biological assay is a FDA approved drug for metastatic breast cancer [12]. Therefore, the impact of the designed 3-deoxysphingomyelin analogs on the proliferation of MCF-7 cell line as a representative for breast cancers was investigated. As displayed in Table 1 and illustrated in Fig. 2, miltefosine elicited an average antiproliferative activity against the employed MCF-7 cell line (69.44% inhibition of growth at 100 μM concentration and GI_{50} value equals to 28.4 μM). Among all evaluated compounds, the saturated palimitic acid and the monounsaturated erucic acid derivatives were the most active compounds whereas the polyunsaturated linoleic and α-linolenic acids derivatives were the least active. The activities were lower for derivatives of oleic acid; a monounsaturated fatty acids shorter than erucic acid, and even more decreased for the more shorter monounsaturated FA; palmitoleic acid. 3-Deoxysphingomyelin analogs possessing pyrrolidine core of type **3** and its enantiomeric antipode **4** were more potent than the corresponding 3-deoxysphingomyelin analogs belonging to types **5** and **6**. This might indicate more activity of 3-deoxysphingomyelin analogs possessing 2-substitutedpyrrolidinyl core than those possessing 3-substitutedpyrrolidinyl core aginst MCF-7 cells. It was noted that



Scheme 1. Reagents and conditions: (a) NEt_3, DMF, rt; (b) (i) 2-chloro-2-oxo-1,3,2-dioxaphospholane, NEt_3, benzene, 0 °C to rt, (ii) NMe_3, CH_3CN, 60 °C.

compounds of core type **3** and the corresponding enantiomeric antipode compounds **4** showed almost identical activity. This might indicate that stereochemistry of the pyrrolidine ring has no significant impact on the elicited biological activity. The most potent 3-deoxysphingomyelin analog was erucic acid derivative **3d** exhibiting GI_{50} value equals to 15.7 μM. Relative to miltefosine whose GI_{50} values was 28.4 μM, this represent 1.8 folds increase in activity.

Out of all cancers, lung cancer causes the highest mortality [3,13–15]. Among lung cancer cases, 85% are non-small lung cancers. Consequently, we investigated the efficacy of the prepared 3-deoxysphingomyelin analogs against A-549 cell line, a non-small-cell lung adenocarcinoma. The reference standard miltefosine elicited a limited inhibition of the growth of the employed A-549 cell line by 36.6% at 100 μM dose. As indicated in Table 1 and outlined in Fig. 3, only derivatives of palmitic and the monounsaturated erucic acids elicited antiproliferative activity comparable to or higher than the reference miltefosine whereas the shorter monounsaturated acids; palmitoleic acid and oleoic acid, and the polyunsaturated linoleic and α-linolenic acids derivatives were of low activity. In addition, compounds possessing type **3** pyrrolidine core and the corresponding enantiomeric antipode compounds having type **4** pyrrolidine core showed almost similar potency confirming that stereochemistry of the pyrrolidine ring has no significant impact on the elicited biological activity. The most active compound (**5a**); a palmitic derivative of type **5** (*S*)-3-pyrrolidinemethanol core elicited inhibition of the growth of A-549 cell line by 71.6%. Whereas, erucic acid derivatives of (*R*)-3-pyrrolidinol (**6d**) and D-prolinol (**3d**) came in the second rank of activity by elicited percent of inhibition of growth by 58.7 and 56.9% respectively. This indicates that the optimum activity against A-549 was related to the FA type more than the type of the pyrrolidine ring.

Among primary liver cancers in adult patients, hepatocellular carcinoma comes to the fore. In addition, it is the third leading cause for mortality among cancer deaths [16]. Surgically unmanageable hepatocellular carcinoma patients show 6–20 months average survival time and 5-years survival less than 5%. Accordingly, we investigated the antiproliferative activity of the designed 3-deoxysphingomyelin analogs against HepG2 cell line as a model for hepatic carcinoma. The employed HepG2 cell line, miltefosine elicited a low efficacy at 100 μM concentration measured as 31.3% inhibition of growth (Fig. 4). Among evaluated 3-deoxysphingomyelin analogs, the most actives were erucic acid derivatives of (*R*)-3-pyrrolidinol (**6d**) and D-prolinol (**3d**) which elicited growth inhibition percentages of 72.8% and 56.9% respectively. The only palmitic acid derivative that elicited more than 50% growth inhibition was **5a**, which was a derivative of (*S*)-3-

Table 1
In vitro cytotoxicity of the synthesized 3-deoxysphingomyelin analogs against selected human cancer cells.

Comp	MCF-7[a]		A-549[b]	HepG2[c]	A-431[d]
	% Inhibition[e]	GI_{50}[f]	% Inhibition[e]	% Inhibition[e]	% Inhibition[e]
3a	86.9	29.6	44.6	22.3	55.6
3b	62.2	ND	4.7	13.3	19.6
3c	76.6	ND	10.1	10.1	27.3
3d	93.0	15.7	56.9	56.9	62.6
4a	87.5	29.6	36.0	30.9	41.5
4b	63.1	ND	8.3	12.6	17.3
4c	72.9	ND	12.9	11.1	24.5
4d	91.0	24.8	49.0	38.5	42.7
4e	38.6	ND	15.7	17.1	7.3
4f	30.1	ND	12.9	17.5	2.4
5a	74.6	42.2	71.6	51.3	74.8
5b	67.3	ND	16.8	26.0	32.6
5c	71.5	51.3	25.3	39.6	55.1
5d	80.5	36.2	49.9	46.6	54.3
5e	10.8	ND	21.6	24.3	NT
5f	25.4	ND	18.7	24.6	3.6
6a	80.9	51.1	42.8	11.1	82.5
6b	63.5	ND	21.6	27.8	20.9
6c	69.4	ND	11.9	10.9	33.8
6d	92.9	23.5	58.7	72.8	67.3
6e	24.4	ND	11.5	21.5	0.3
6f	22.6	ND	11.5	21.2	6.1
Miltefosine	69.4	28.4	36.6	31.3	35.4

[a] Human breast carcinoma cells.
[b] Human lung cancer cells.
[c] Human hepatocellular carcinoma cells.
[d] Human Epidermoid carcinoma cells.
[e] % Inhibition at 100 μM.
[f] GI_{50} was defined as the concentration resulting in 50% inhibition of cell proliferation.

pyrrolidinemethanol. All derivatives of other FAs were less active than the reference miltefosine.

During the last decades, skin cancer mortality rates did not improve despite the progress in cancer's biology research and the development of novel antitumor agents [17]. In A-431 cell line (a cell line model of epidermal squamous cells carcinoma), it was found that evolvement of constitutively active Akt results in acquired resistance to the EGFR inhibitor erlotininb [18]. As the prepared 3-deoxysphingomyelin analogs possess structural similarity with alkylphospholipids, which are known to influence Akt phosphorylation, we investigated the antiproliferative activity of the prepared 3-deoxysphingomyelin analogs against A-431 cell line. The elicited pattern of activity by the prepared 3-deoxysphingomyelin analogs against A-431 cell line was similar to the pattern of activity elicited against other evaluated cell lines. Thus, derivatives of palmitic acid and erucic acid elicited higher activity than the corresponding derivatives of other FAs. Meanwhile, compounds possessing type **3** pyrrolidine core and the corresponding enantiomeric antipode compounds having type **4** pyrrolidine core showed almost similar potency. This confirms that the stereochemistry inversion of the pyrrolidine ring has no significant impact on the elicited biological activity. While miltefosine elicited only 35.4% growth inhibition the most active compounds which were palmitic acid derivatives **6a** and **5a** produced

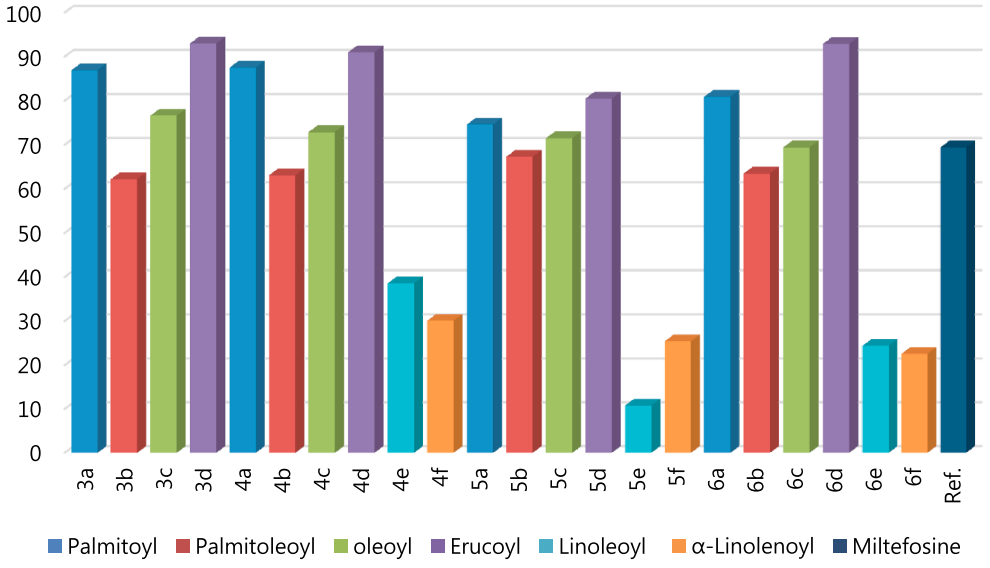


Fig. 2. Percentages inhibition of MCF-7 cell line proliferation after treatment with synthesized 3-deoxysphingomyelin analogs and miltefosine at 100 μM.

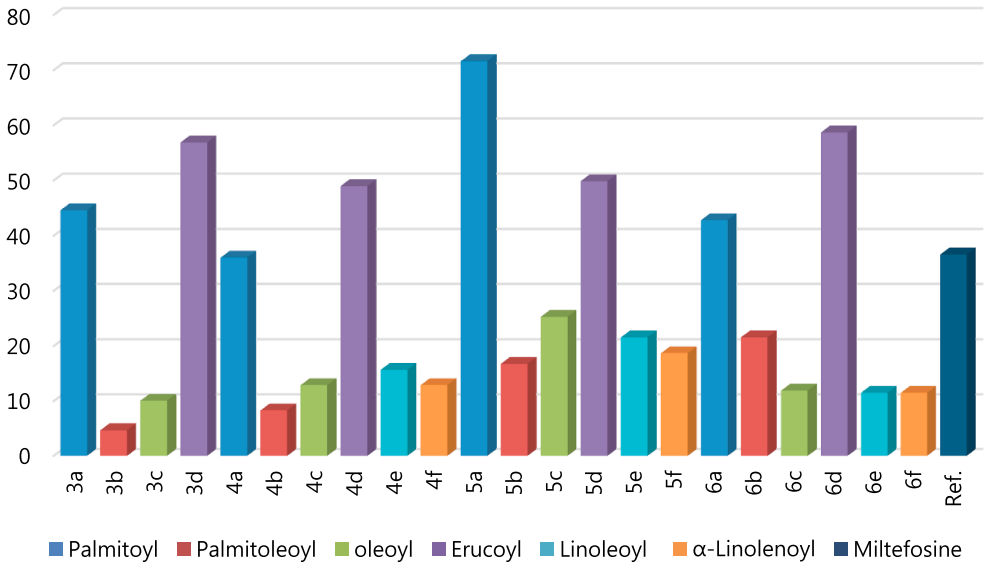


Fig. 3. Percentages inhibition of A-549 cell line proliferation after treatment with synthesized 3-deoxysphingomyelin analogs and miltefosine at 100 μM.

82.5% and 74.8% growth inhibition respectively (Fig. 5). The highest activity of palmitic acid and erucic acid derivatives were for those derivatives with (*R*)-3-pyrrolidinol core rather than other pyrrolidine cores. With exception of oleic acid derivative **5c**, all FAs derivatives other than palmitic acid and erucic acid showed growth inhibition percent lower than 50%. Collectively, the data of the activity of the synthesized 3-deoxysphingomyelin analogs against the employed cell lines indicate that all of the designed pyrrolidine cores can provide antiproliferative 3-deoxysphingomyelin analogs with significant activity when it is substituted the appropriate fatty acid. In addition, the stereochemistry configuration of the pyrrolidine ring has minimal impact on the elicited biological activity whereas the antiproliferative activity is highly impacted by the type of FA. It was found that palmitic and erucic acids derivative yield the most effective antiproliferative 3-deoxysphingomyelin analogs. Similar to miltefosine, the activity of most of the synthesized 3-deoxysphingomyelin analogs were more pronounced against MCF-7 cells than other cell lines.

2.2.2. *Inhibition of Akt phosphorylation assay*

As mentioned in the introduction, antitumor alkylphospholipids, which are structurally similar to the prepared 3-deoxysphingomyelin analogs, inhibit Akt phosphorylation, which can induce cell death. Accordingly, we anticipated that inhibition of Akt phosphorylation might be the mechanism of action of the designed antiproliferative 3-deoxysphingomyelin analogs, at least in part. Therefore, cellular assay of the Akt phosphorylation inhibitory activity of the prepared 3-deoxysphingomyelin analogs, as well as, miltefosine as a reference compound was performed using MCF-7 cell line to check for the validity of the assumption that Akt phosphorylation inhibition is the mechanism of action of the designed antiproliferative 3-deoxysphingomyelin analogs. Among the synthesized 3-deoxysphingomyelin analogs, three compounds; **4a**, **5a** and **6a** were selected. These selected compounds have variable pyrrolidine rings, but with identical alkyl chains. The GI_{50} value of compound **4a** was almost similar to the reference standard miltefosine, while those for **5a** and **6a** were higher. However, at a high dose of 100 μM, all of the three compounds elicited higher antiprolifertive activity against MCF-7 cell line than that of the standard miltefosine. In the course of the experiment, MCF-7 cells pretreated with compounds to be evaluated were stimulated with insulin to induce Akt phosphorylation followed by cell lysis and determination of the

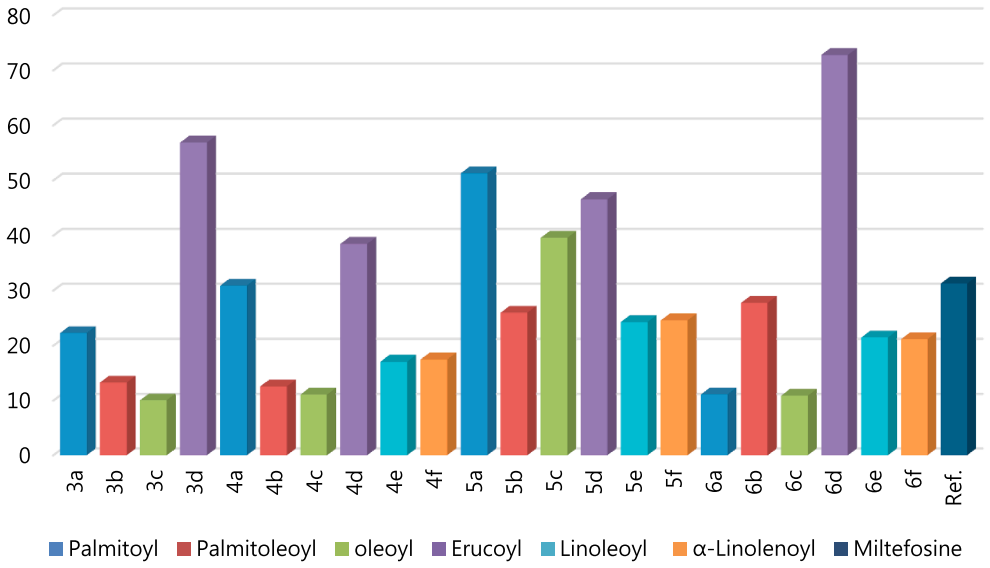


Fig. 4. Percentages inhibition of HepG2 cell line proliferation after treatment with synthesized 3-deoxysphingomyelin analogs and miltefosine at 100 μM.

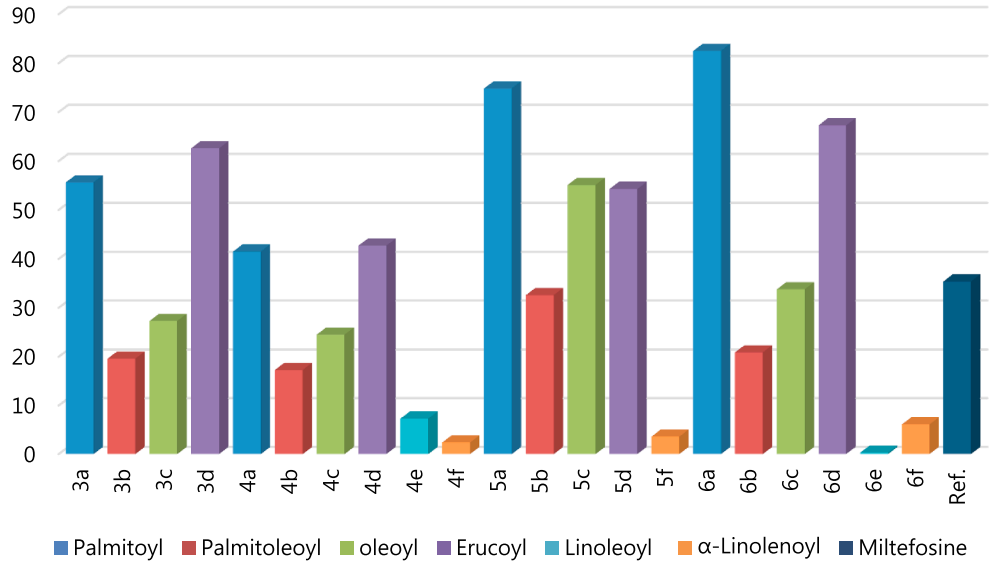


Fig. 5. Percentages inhibition of A-431 cell line proliferation after treatment with synthesized 3-deoxysphingomyelin analogs and miltefosine at 100 μM.

Table 2
Akt phosphorylation inhibitory activities of selected 3-deoxysphingomyelin analogs and their *in silico* estimated energetic terms for the best Akt-docked pose.

Comp	MCF-7 Growth		Akt Phosphorylation		Interaction Energy (kcal/mol)	Full Energy (kcal/mol)	Estimated ΔG (kcal/mol)
	% Inhibition[a]	GI_{50}[b]	% Inhibition[c]	IC_{50}[d]			
4a	87.5	29.6	94.2	21.1	−64.27	−2479.07	−9.43
5a	74.6	42.2	93.6	26.4	−58.34	−2490.84	−9.02
6a	80.9	51.1	73.3	32.5	−58.72	−2476.56	−8.07
Miltefosine	69.4	28.4	100	20.0	−69.27	−2480.24	−8.43
Correl. with Akt Phosphophorylation[e]	0.1704	0.9936	−0.9298	1.0000	0.8419	NC[f]	NC[f]

[a] % Inhibition at 100 μM.
[b] GI_{50} was defined as the concentration resulting in 50% inhibition of cell proliferation.
[c] % Inhibition of AKT phosphorylation at 50 μM in MCF-7.
[d] IC_{50} was defined as the concentration resulting in 50% inhibition of AKT phosphorylation in MCF-7.
[e] The calculated correlation constant with IC_{50} values of Akt phophorylation; [f] Value was not calculated.

amount of phosphorylated Akt. As Table 2 shows, the standard reference miltefosine elicited 100% inhibition of Akt phosphorylation at 50 μM concentration with IC_{50} value of 20.0 μM. On the other hand, compound **4a** elicited incomplete inhibition of Akt phosphorylation measured at 94.2% inhibition of at the same 50 μM concentration. However, compound **4a** exhibited IC_{50} value for inhibition of Akt phosphorylation close to that of the reference miltefosine. In fact, both of compound **4a** and miltefosine also possessed close GI_{50} values for inhibition of growth of MCF-7 cell line despite the higher percent inhibition of growth elicited by compound **4a** at the higher concentrations. This might indicate that the evaluated 3-deoxysphingomyelin analogs elicits a concentration dependent mechanism of action in which the effective mechanism of action inducing the cell cytotoxicity might differ depending on the concentration. It seems that these compounds at the high concentrations might be unselective membrane-disrupting detergents. This conclusion is supported by the assay results of compounds **5a** and **6a** as the results show that these compounds are less efficient inhibitors of Akt phosphorylation (Table 2). Yet, these compounds produces higher percent for inhibition of MCF-7 cells' growth than miltefosine at the higher concentrations despite their higher GI_{50} values relative to the reference miltefosine. To confirm the relation between the mechanism of action and the concentration, the correlation constants were calculated. As shown in Table 2, the statistical analysis of the correlation of the GI_{50} of compounds **4a**, **5a**, **6a** and miltefosine; to the IC_{50} value of Akt phosphorylation inhibition elicited by these compounds showed high correlation value. However, the correlation between the percent inhibitions of growth elicited by the same set of compounds at the higher 100 μM concentration and the IC_{50} value of Akt phosphorylation was very low. This might confirm that the mechanisms contributing to the elicited cytotoxicity at this high concentration might arise from unselective membrane-disruption by these compounds. In summary, these class of compounds might possess concentration-dependent mechanism for their antiproliferative activity where the inhibition of Akt phosphorylation might the mechanism of action for these compounds at the lower concentrations, while other mechanism (possibly membrane-disruption) might possibly contribute to the elicited cytotoxicity at higher concentration.

2.2.3. *In silico simulation study*

Akt is a protein kinase that incorporates a Pleckstrin homology domain (PH domain) which is responsible for binding lipids. The structure of pH domain consists of two antiparallel β-sheets and a terminal α-helix. Despite the fact that the PH domain exists across several kinases, the variation of loops' length and sequence would accounts for the selectivity for binding different lipids. Besides, a single tryptophan in the α-helix is the only conserved amino acid among the whole sequence of the PH domain.

To understand how the tested 3-deoxysphingomyelin analogs might interacts with Akt, a molecular modeling study was conducted using the reported crystal structure of Akt. As mentioned in the introduction, there is a structural similarity between the tested 3-deoxysphingomyelin analogs and alkylphospholipids. In addition, miltefosine

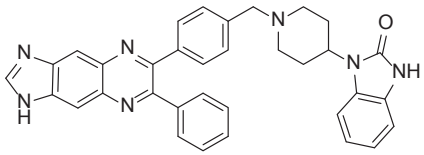


Fig. 6. Structure of the reported cocrystallized allosteric small molecules inhibitors (PDB code: 3O96).

which is a synthetic member of alkylphospholipids was used as a reference standard in the conducted biological evaluations. Therefore, the 3-deoxysphingomyelin analogs **4a**, **5a**, **6a**, as well as, miltefosine were docked blindly to the PH domain of the crystal structure of Akt (PDB code: 3O96) after appropriate preparation employing the SwissDock algorithm that implements CHARMM force field [19,20]. A previous report utilized high resolution field-cycling ^{31}P NMR along with docking study showed that miltefosine binds the PH domain in a site other than the site that binds phosphoinositides, but located in the vicinity of it [21]. In addition, it showed that the positively charged head is directed towards Tyr18 and Glu17 and interacting with Glu17. Considering the whole structure of Akt, this site is adjacent to a hydrophobic site, which is the binding site of some allosteric inhibitors such as shown in Fig. 6. This hydrophobic site connects the PH domain with the kinase domain and, thus, maintains Akt in a closed configuration [22]. This might offer a possibility for the hydrophobic tails of phospholipidic inhibitors of Akt to extend into this site after the ionic head interacts with the crucial Glu17 [21]. In lieu of the above-mentioned reports, analysis was performed for the clustered poses obtained from the *in silico* simulation considering the binding sites, interactions and their calculated energy terms. In the course of the analysis, only clusters showing interaction with Glu17 were considered as this interaction is crucial for binding of phosphoinositides and also for triggering inhibition of Akt by miltefosine and other alkylphospholipids [21]. As shown in Table 2, the calculated interaction energies for the best-selected poses of compounds **4a**, **5a**, **6a**, and miltefosine showed excellent correlation with the experimentally determined IC_{50} values for inhibition of Akt phosphorylation (calculated correlation value equals to 0.8419).

As illustrated in Fig. 7, the positively charged trimethylammonium moiety of miltefosine's head fits in a pocket within the PH domain eliciting polar electrostatic and hydrogen bonding interactions with Glu17 as previously reported. Also, the negatively charged phosphate moiety of the head of miltefosine interacts via polar electrostatic and hydrogen bonding interactions with Arg273 as well as hydrogen bonding interaction with Asn54. Meanwhile, the hydrophobic alkyl chain, as anticipated, extends into the hydrophobic pocket occupied by the hydrophobic small molecule inhibitor. The alkyl chain partially overlaps with the imidazoquinoxaline moiety of the co-crystallized small molecule inhibitor and elicits hydrophobic interaction with Trp80. As shown in Table 2, the *in silico* calculation estimated the interaction between miltefosine and Akt to be stabilized by −69.27 kcal/mol and the ligand-enzyme complex to be stabilized by −2480.24 kcal/mol while the estimated free energy to be −8.43 kcal/mol.

As shown in Fig. 7, the predicted binding modes for compounds **4a** and **5a** were comparable to that of miltefosine, however, with different energetic terms. Thus, both of the polar heads of compounds **4a** and **5a** elicited polar electrostatic and hydrogen bonding interactions via the trimethylammonium moiety with Glu17, polar electrostatic and hydrogen bonding interactions between the phosphate moiety with Arg273, as well as, hydrogen bonding interaction with Asn54. In addition, Tyr326 is involved in two hydrogen bonding interactions with both of the positively charged and the negatively charged moieties of the polar heads of compounds **4a** and **5a**. Both of the alkyl chains of compounds **4a** and **5a** overlap with the imidazoquinoxaline moiety of the co-crystallized small molecule inhibitor and elicit hydrophobic interaction with Trp80. While the substituents' position and the stereochemistry are different between compounds **4a** and **5a**, this did not influence the binding of the polar head nor the orientation of the hydrophobic tail. This might explains why the elicited biological activity is more influenced by the alkyl chain type than by the pyrrolidine ring. Despite binding modes similarity, the calculated energetic terms were different between compounds **4a** and **5a**. For compound **4a**, the interaction energy was significantly higher than compounds **5a**, yet, lower than that for miltefosine. However, the estimated free energy was higher than miltefosine and the ligand-enzyme complex energy was very close to that of miltefosine (Table 2). This might explain the close IC_{50} values for inhibition of Akt phosphorylation of compound **4a** and miltefosine. However in case of compound **5a**, the estimated interaction energy and the estimated free energy were further decreased than that for **4a**. This might explain the lowered potency of compound **5a** relative to that of compound **4a** and miltefosine with regard to inhibition of Akt phosphorylation. Although the polar head of docked compound **6a** elicits similar interactions to compounds **4a**, **5a** and miltefosine, the alkyl chain tail is out of the plane of those of compounds **4a**, **5a** and miltefosine and, accordingly, not overlapping with imidazoquinoxaline moiety of the co-crystallized small molecule inhibitor. Consequently, the *in silico* calculations predicted that compound **6a** possesses lower interaction energy, less ligand-enzyme complex energy and free energy than those for compounds **4a**, **5a** and miltefosine (Table 2). This would explain the lower IC_{50} value for inhibition of Akt phosphorylation of compound **6a**.

3. Conclusion

In this study, twenty two novel pyrrolidine-based 3-deoxysphingomyelin analogs belonging to four series were designed,

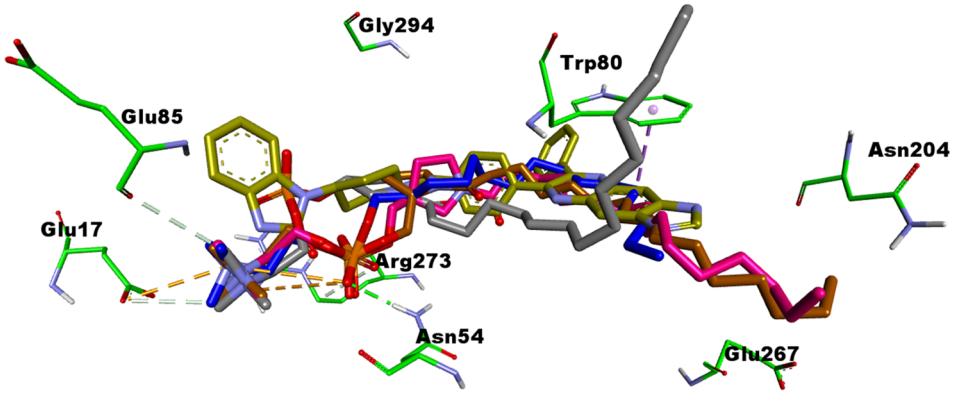


Fig. 7. Overlapping Akt-Docked poses of compounds 4a, 5a 6a and Miltefosine with the co-crystallized small molecule inhibitor (PDB code: 3O96). Blue color is Miltefosine, orange color is 4a, pink color is 5a, gray color is 6a while yellow color for the co-crystallized small molecule inhibitor.

synthesized, evaluated for their antiproliferative activity, and were mechanistically studied employing three highly active compounds out of the screened set of compounds. The mechanistic study was done using cellular assay of inhibition of Akt phosphorylation, in addition to molecular docking study to get insights into the factors that underlie the measured Akt phosphorylation inhibition. The designed 3-deoxysphingomyelin analogs elicit antiproliferative activity in analogy to the structurally similar alkylphospholipids but in contrast to the reported cytoprotective activity of previously developed sphingomyelin analogs. The results showed that the type of the fatty acid moiety in the structure of these compounds has more influence on the biological activity than the variation of the pyrrolidine core. Among evaluated fatty acid, pyrrolidine-based 3-deoxysphingomyelin possessing palmitic or erucic acid were the most active. Similar to the FDA approved reference drug miltefosine, the prepared 3-deoxysphingomyelin analogs were more active against breast cancer cell line than other cell line. Some of palmitic or erucic acid derivatives were more active than miltefosine against MCF-7 cell line, as well as, other cell lines. Particularly, 3-deoxysphingomyelin analogs **3d**, **4d** and **6d** were the most potent with cellular GI_{50} ranges of 15.7–24.8 μM against breast cancer which is lower than that of miltefosine which was 28.4 μM. Cellular assay of Akt-phosphorylation inhibition for the studied compounds revealed that these class of compounds elicits a concentration dependent mechanism of action where Akt phosphorylation might be the prevalent mechanism of action, whereas, at high concentrations other mechanisms of action (possibly membrane-disruption because of a detergent-like property for these compounds) might contribute to the elicited cytotoxicity.

4. Experimental

4.1. Chemistry

Unless otherwise specified, all reactions and manipulations were performed under nitrogen atmosphere using standard Schlenk techniques. Solvent were purified and dried prior to use. For TLC, precoated silica gel (E. Merck Kiesegel 60F254, layer thickness 0.25 mm) was used. For column chromatography Merck Kiesegel 60 Art 9385 (230–400 mesh) was used. NMR spectra were recorded on Bruker AC 400 spectrometer or Agilent 500 spectrometer operating at 400 MHz/100 MHz or 500 MHz/125 MHz for ^{1}H NMR and ^{13}C NMR respectively. Chemical shifts (δ) are reported in ppm, downfield from internal TMS standard. For HRMS data, Jeol accuTOF (JMS-T100TD) with DART ion source (Ionsense, Tokyo, Japan) was employed.

4.1.1. *General procedure for the preparation of acid chlorides 8a–f* [11]

To a stirred solution of the fatty acid (1 eq., 0.78 mmol) and a drop of DMF in dry dichloromethane (5 ml) was added dropwise oxalyl chloride (3 eq., 297 mg, 2.34 mmol) at 0 °C. The reaction mixture was warmed slowly up to rt and stirred for further 3 h. Solvent and excess oxalyl chloride were removed *in vacuo* and the resulting acid chloride was used next step without purification.

4.1.2. *General procedure for the preparation of intermediate compounds 9a–d, 11a–f, 13a–f and 15a–f*

To a solution of the appropriate pyrrolidine derivative (1.17 mmol) and triethylamine (237 mg, 2.34 mmol) in dry DMF (4 ml) was added slowly a solution of the appropriate acid chloride (0.78 mmol) in dry DMF (1 ml) at rt. After stirring for 5 h, the reaction mixture was diluted with EtOAc (100 ml) and washed by water (100 ml × 3). The combined water layer was washed again with EtOAc. The combined organic layer was washed by brine, dried over anhydrous $MgSO_4$ and concentrated. The residue was purified by flash column chromatography (EtOAc/*n*-hexane = 1:5)

4.1.2.1. (R)-1-(2-(Hydroxymethyl)pyrrolidin-1-yl)hexadecan-1-one (9a). Compound **9a** was obtained from **7** and palmitoyl chloride (**8a**) according to the general procedure. Yield 84%. ^{1}H NMR ($CDCl_3$) δ 4.24–4.19 (m, 1H), 3.65 (dd, 1H, J_1 = 11.2 Hz, J_2 = 2.5 Hz), 3.59–3.43 (m, 3H), 2.30 (t, 2H, J = 7.8 Hz), 2.08–1.99 (m, 1H), 1.97–1.91 (m, 1H), 1.89–1.81 (m, 1H), 1.68–1.54 (m, 3H), 1.37–1.20 (m, 24H), 0.88 (t, 3H, J = 7.0 Hz).

4.1.2.2. (R,Z)-1-(2-(Hydroxymethyl)pyrrolidin-1-yl)hexadecan-9-en-1-one (9b). Compound **9b** was obtained from **7** and palmitoleoyl chloride (**8b**) according to general procedure. Yield 86%. ^{1}H NMR ($CDCl_3$) δ 5.38–5.30 (m, 2H), 5.18 (brs, 1H), 4.25–4.19 (m, 1H), 3.66 (d, 1H, J = 11.5 Hz), 3.58–3.42 (m, 3H), 2.30 (t, 2H, J = 7.8 Hz), 2.09–1.97 (m, 5H), 1.96–1.80 (m, 2H), 1.66–1.53 (m, 3H), 1.38–1.22 (m, 16H), 0.88 (t, 3H, J = 7.1 Hz).

4.1.2.3. (R,Z)-1-(2-(Hydroxymethyl)pyrrolidin-1-yl)octadecan-9-en-1-one (9c). Compound **9c** was obtained from **7** and oleoyl chloride (**8c**) according to general procedure. Yield 78%. ^{1}H NMR ($CDCl_3$) δ 5.39–5.30 (m, 2H), 5.22 (dd, 1H, J_1 = 7.6 Hz, J_2 = 1.8 Hz), 4.26–4.19 (m, 1H), 3.69–3.64 (m, 1H), 3.59–3.42 (m, 3H), 2.30 (t, 2H, J = 7.4 Hz), 2.09–1.97 (m, 5H), 1.96–1.83 (m, 2H), 1.65 (t, 2H, J = 6.7 Hz), 1.62–1.53 (m, 1H), 1.30 (d, 20H, J = 20.4 Hz), 0.88 (t, 3H, J = 6.6 Hz).

4.1.2.4. (R,Z)-1-(2-(Hydroxymethyl)pyrrolidin-1-yl)docos-13-en-1-one (9d). Compound **9d** was obtained from **7** and erucoyl chloride (**8d**) according to general procedure. Yield 76%. ^{1}H NMR ($CDCl_3$) δ 5.39–5.31 (m, 2H), 5.23 (d, 1H, J = 6.7 Hz), 4.25–4.19 (m, 1H), 3.69–3.64 (m, 1H), 3.59–3.43 (m, 3H), 2.30 (t, 2H, J = 7.5 Hz), 2.08–1.97 (m, 5H), 1.96–1.83 (m, 2H), 1.65 (t, 2H, J = 7.3 Hz), 1.62–1.53 (m, 1H), 1.37–1.23 (m, 28H), 0.88 (t, 3H, J = 6.5 Hz).

4.1.2.5. (S)-1-(2-(Hydroxymethyl)pyrrolidin-1-yl)hexadecan-1-one (11a). Compound **11a** was obtained from **10** and palmitoyl chloride (**8a**) according to the general procedure. Yield 83%. ^{1}H NMR ($CDCl_3$) δ 5.21 (brs, 1H), 4.24–4.18(m, 1H), 3.65 (d, 1H, J = 11.1 Hz), 3.59–3.43 (m, 3H), 2.30 (t, 2H, J = 7.5 Hz), 2.08–1.99 (m, 1H), 1.98–1.91 (m, 1H), 1.89–1.81 (m, 1H), 1.68–1.55 (m, 3H), 1.35–1.20 (m, 24H), 0.88 (t, 3H, J = 7.0 Hz).

4.1.2.6. (S,Z)-1-(2-(Hydroxymethyl)pyrrolidin-1-yl)hexadecan-9-en-1-one (11b). Compound **11b** was obtained from **10** and palmitoleoyl chloride (**8b**) according to general procedure. Yield 89%. ^{1}H NMR ($CDCl_3$) δ 5.38–5.30 (m, 2H), 5.18 (dd, 1H, J_1 = 7.8 Hz, J_2 = 2.1 Hz), 4.26–4.19 (m, 1H), 3.66 (ddd, 1H, J_1 = 11.04 Hz, J_2 = 7.7 Hz, J_3 = 2.5 Hz), 3.59–3.42 (m, 3H), 2.30 (t, 2H, J = 7.4 Hz), 2.09–1.97 (m, 5H), 1.96–1.80 (m, 2H), 1.67–1.53 (m, 3H), 1.38–1.25 (m, 16H), 0.88 (t, 3H, J = 7.1 Hz).

4.1.2.7. (S,Z)-1-(2-(Hydroxymethyl)pyrrolidin-1-yl)octadecan-9-en-1-one (11c). Compound **11c** was obtained from **10** and oleoyl chloride (**8c**) according to general procedure. Yield 72%. ^{1}H NMR ($CDCl_3$) δ 5.39–5.30 (m, 2H), 5.17 (dd, 1H, J_1 = 7.6 Hz, J_2 = 2.0 Hz), 4.26–4.19 (m, 1H), 3.69–3.64 (m, 1H), 3.59–3.43 (m, 3H), 2.30 (t, 2H, J = 7.4 Hz), 2.09–1.97 (m, 5H), 1.96–1.83 (m, 2H), 1.65 (t, 2H, J = 6.9 Hz), 1.61–1.53 (m, 1H), 1.29 (d, 20H, J = 20.5 Hz), 0.88 (t, 3H, J = 6.6 Hz).

4.1.2.8. (S,Z)-1-(2-(Hydroxymethyl)pyrrolidin-1-yl)docos-13-en-1-one (11d). Compound **11d** was obtained from **10** and erucoyl chloride (**8d**) according to general procedure. Yield 82%. ^{1}H NMR ($CDCl_3$) δ 5.39–5.31 (m, 2H), 5.18 (dd, 1H, J_1 = 7.8 Hz, J_2 = 2.2 Hz), 4.26–4.19 (m, 1H), 3.69–3.64 (m, 1H), 3.59–3.43 (m, 3H), 2.30 (t, 2H, J = 7.4 Hz), 2.08–1.98 (m, 5H), 1.97–1.80 (m, 2H), 1.68–1.53 (m, 3H), 1.35–1.22 (m, 28H), 0.88 (t, 3H, J = 6.3 Hz).

*4.1.2.9. (9Z,12Z)-1-(S)-2-(Hydroxymethyl)pyrrolidin-1-yl)octadeca-9,12-dien-1-one (**11e**)*. Compound **11e** was obtained from **10** and linoleoyl chloride (**8e**) according to general procedure. Yield 89%. ^{1}H NMR ($CDCl_3$) δ 5.37–5.31 (m, 4H), 5.28 (dd, 1H, J_1 = 7.7 Hz, J_2 = 2.2 Hz), 4.25–4.19 (m, 1H), 3.69–3.64 (m, 1H), 3.60–3.44 (m, 3H), 2.77 (t, 2H, J = 6.4 Hz), 2.31 (t, 2H, J = 7.8 Hz), 2.07–2.00 (m, 5H), 1.98–1.83 (m, 2H), 1.68–1.54 (m, 3H), 1.32–1.22 (m, 14H), 0.89 (t, 3H, J = 7.0 Hz).

*4.1.2.10. (9Z,12Z,15Z)-1-((R)-2-(Hydroxymethyl)pyrrolidin-1-yl) octadeca-9,12,15-trien-1-one (**11f**)*. Compound **11f** was obtained from **10** and α-linolenoyl chloride (**8f**) according to general procedure. Yield 78%. ^{1}H NMR ($CDCl_3$) δ 5.41–5.33 (m, 6H), 5.32–5.27 (m, 1H), 4.25–4.19 (m, 1H), 3.69–3.64 (m, 1H), 3.60–3.43 (m, 3H), 2.84–2.78 (m, 4H), 2.30 (t, 2H, J = 7.8 Hz), 2.07–2.00 (m, 5H), 1.98–1.82 (m, 2H), 1.68–1.54 (m, 3H), 1.38–1.23 (m, 8H), 0.89 (t, 3H, J = 6.7 Hz).

*4.1.2.11. (S)-1-(3-(Hydroxymethyl)pyrrolidin-1-yl)hexadecan-1-one (**13a**)*. Compound **13a** was obtained from **12** and palmitoyl chloride (**8a**) according to general procedure. Yield 90%. ^{1}H NMR ($CDCl_3$) δ 3.69–3.50 (m, 4H), 3.46–3.36 (m, 1H), 3.30–3.20 (m, 1H), 2.45 (doublet of septets, 1H, J_1 = 41.7 Hz, J_2 = 7.0 Hz), 2.24 (quartet, 2H, J = 8.4 Hz), 2.11–1.92 (m, 1H), 1.86–1.57 (m, 3H), 1.40–1.09 (m, 24H), 0.88 (t, 3H, J = 7.0 Hz).

*4.1.2.12. (S,Z)-1-(3-(Hydroxymethyl)pyrrolidin-1-yl)hexadec-9-en-1-one (**13b**)*. Compound **13b** was obtained from **12** and palmitoleoyl chloride (**8b**) according to general procedure. Yield 90%. ^{1}H NMR ($CDCl_3$) δ 5.38–5.30 (m, 2H), 3.69–3.50 (m, 4H), 3.46–3.37 (m, 1H), 3.30–3.20 (m, 1H), 2.45 (doublet of septets, 1H, J_1 = 41.4 Hz, J_2 = 7.0 Hz), 2.25 (quartet, 2H, J = 8.3 Hz), 2.11–1.93 (m, 5H), 1.85–1.58 (m, 3H), 1.37–1.24 (m, 16H), 0.88 (t, 3H, J = 7.0 Hz).

*4.1.2.13. (S,Z)-1-(3-(Hydroxymethyl)pyrrolidin-1-yl)octadec-9-en-1-one (**13c**)*. Compound **13c** was obtained from **12** and oleoyl chloride (**8c**) according to general procedure. Yield 91%. ^{1}H NMR ($CDCl_3$) δ5.38–5.30 (m, 2H), 3.76 (brs, 1H), 3.69–3.50 (m, 4H), 3.46–3.37 (m, 1H), 3.30–3.19 (m, 1H), 2.45 (doublet of septets, 1H, J_1 = 41.5 Hz, J_2 = 7.0 Hz), 2.24 (quartet, 2H, J = 8.2 Hz), 2.11–1.92 (m, 5H), 1.85–1.57 (m, 3H), 1.29 (d, 20H, J = 14.9 Hz), 0.88 (t, 3H, J = 7.0 Hz).

4.1.2.14. (S,Z)-1-(3-(Hydroxymethyl)pyrrolidin-1-yl)docos-13-en-1-one (13d). Compound **13d** was obtained from **12** and erucoyl chloride (**8d**) according to general procedure. Yield 57%. ^{1}H NMR ($CDCl_3$) δ 5.38–5.31 (m, 2H), 3.71–3.50 (m, 4H), 3.46–3.38 (m, 1H), 3.30–3.21 (m, 1H), 2.55–2.36 (m, 2H), 2.24 (quartet, 2H, J = 8.0 Hz), 2.11–1.93 (m, 5H), 1.86–1.59 (m, 3H), 1.38–1.22 (m, 28H), 0.88 (t, 3H, J = 7.1 Hz).

*4.1.2.15. (9Z,12Z)-1-((S)-3-(Hydroxymethyl)pyrrolidin-1-yl)octadeca-9,12-dien-1-one (**13e**)*. Compound **13e** was obtained from **12** and linoleoyl chloride (**8e**) according to general procedure. Yield 62%. ^{1}H NMR ($CDCl_3$) δ 5.42–5.28 (m, 4H), 3.71–3.50 (m, 4H), 3.47–3.36 (m, 1H), 3.30–3.21 (m, 1H), 2.77 (t, 2H, J = 6.4 Hz), 2.61 (brs, 1H), 2.50–2.37 (m, 1H), 2.25 (quartet, 2H, J = 6.5 Hz), 2.12–1.93 (m, 5H), 1.85–1.58 (m, 3H), 1.39–1.24 (m, 14H), 0.89 (t, 3H, J = 6.9 Hz).

*4.1.2.16. (9Z,12Z,15Z)-1-((S)-3-(Hydroxymethyl)pyrrolidin-1-yl) octadeca-9,12,15-trien-1-one (**13f**)*. Compound **13f** was obtained from **12** and α-linolenoyl chloride (**8f**) according to general procedure. Yield 58%. ^{1}H NMR ($CDCl_3$) δ 5.43–5.28 (m, 6H), 3.69–3.50 (m, 4H), 3.46–3.37 (m, 1H), 3.30–3.20 (m, 1H), 2.80 (t, 4H, J = 5.6 Hz), 2.45 (doublet of septets, 1H, J_1 = 41.4 Hz, J_2 = 7.0 Hz), 2.25 (quartet, 2H, J = 8.3 Hz), 2.11–1.92 (m, 5H), 1.85–1.58 (m, 3H), 1.38–1.25 (m, 8H), 0.98 (t, 3H, J = 7.6 Hz).

*4.1.2.17. (R)-1-(3-Hydroxypyrrolidin-1-yl)hexadecan-1-one (**15a**)*. Compound **15a** was obtained from **14** and palmitoyl chloride (**8a**) according to general procedure. Yield 74%. ^{1}H NMR ($CDCl_3$) δ 4.50 (d, 1H, J = 18.9 Hz), 3.67–3.41 (m, 4H), 2.87 (brs, 1H), 2.28–2.20 (m, 2H), 2.06–1.90 (m, 2H), 1.63 (quintet, 2H, J = 7.3 Hz), 1.36–1.20 (m, 24H), 0.88 (t, 3H, J = 7.2 Hz).

*4.1.2.18. (R,Z)-1-(3-Hydroxypyrrolidin-1-yl)hexadec-9-en-1-one (**15b**)*. Compound **15b** was obtained from **14** and palmitoleoyl chloride (**8b**) according to general procedure. Yield 82%. ^{1}H NMR ($CDCl_3$) δ 5.38–5.30 (m, 2H), 4.49 (d, 1H, J = 21.3 Hz), 3.67–3.42 (m, 4H), 3.26 (brs, 1H), 2.28–2.21 (m, 2H), 2.06–1.93 (m, 6H), 1.63 (t, 2H, J = 7.0 Hz), 1.38–1.23 (m, 16H), 0.88 (t, 3H, J = 6.8 Hz).

*4.1.2.19. (R,Z)-1-(3-Hydroxypyrrolidin-1-yl)octadec-9-en-1-one (**15c**)*. Compound **15c** was obtained from **14** and oleoyl chloride (**8c**) according to general procedure. Yield 72%. ^{1}H NMR ($CDCl_3$) δ 5.39–5.29 (m, 2H), 4.88 (brs, 1H), 4.52 (m, 1H), 3.69–3.39 (m, 4H), 2.30–2.17 (m, 2H), 2.08–1.88 (m, 6H), 1.72–1.59 (m, 2H), 1.37–1.22 (m, 20H), 0.88 (t, 3H, J = 7.1 Hz).

*4.1.2.20. (R,Z)-1-(3-Hydroxypyrrolidin-1-yl)docos-13-en-1-one (**15d**)*. Compound **15d** was obtained from **14** and erucoyl chloride (**8d**) according to general procedure. Yield 66%. ^{1}H NMR ($CDCl_3$) δ 5.38–5.31 (m, 2H), 4.48 (d, 1H, J = 19.4 Hz), 3.67–3.41 (m, 5H), 2.28–2.20 (m, 2H), 2.05–1.92 (m, 6H), 1.62 (t, 2H, J = 7.2 Hz), 1.37–1.22 (m, 28H), 0.88 (t, 3H, J = 7.0 Hz).

*4.1.2.21. (9Z,12Z)-1-((R)-3-Hydroxypyrrolidin-1-yl)octadeca-9,12-dien-1-one (**15e**)*. Compound **15e** was obtained from **14** and linoleoyl acid chloride (**8e**) according to general procedure. Yield 71%. ^{1}H NMR ($CDCl_3$) δ 5.42–5.30 (m, 4H), 5.26 (brs, 1H), 4.26–4.19 (m, 1H), 3.66 (d, 1H, J = 11.1 Hz), 3.59–3.43 (m, 3H), 2.77 (t, 2H, J = 6.3 Hz), 2.30 (t, 2H, J = 7.8 Hz), 2.09–2.01 (m, 5H), 1.97–1.81 (m, 2H), 1.65 (t, 2H, J = 6.8 Hz), 1.39–1.24 (m, 14H), 0.89 (t, 3H, J = 7.1 Hz).

*4.1.2.22. (9Z,12Z,15Z)-1-((R)-3-Hydroxypyrrolidin-1-yl)octadeca-9,12,15-trien-1-one (**15f**)*. Compound **15f** was obtained from **14** and α-linolenoyl chloride (**8f**) according to general procedure. Yield 79%. ^{1}H NMR ($CDCl_3$) δ 5.42–5.27 (m, 6H), 4.47 (d, 1H, J = 21.5 Hz), 4.11 (brs, 1H), 3.67–3.42 (m, 4H), 2.80 (t, 4H, J = 5.6 Hz), 2.28–2.21 (m, 2H), 2.11–1.99 (m, 5H), 1.97–1.92 (m, 1H), 1.62 (t, 2H, J = 7.1 Hz), 1.32 (brs, 8H), 0.97 (t, 3H, J = 7.5 Hz).

*4.1.3. General procedure for the preparation of compounds **3a–d, 4a–f, 5a–f,** and **6a–f***

To a stirred solution of the appropriate pyrrolidine-amide intermediate (**9a–d**, **11a–f**, **13a–f**, or **15a–f**, 0.35 mmol) and triethylamine (147 mg, 1.05 mmol) in benzene (10 ml) was added 2-chloro-2-oxo-1,3,2-dioxaphospholane (156 mg, 0.35 mmol) at 0 °C and warmed up to rt. After stirring for 5 h, the precipitated triethylamine hydrochloride was filtered off and washed with benzene. The combined filtrate was concentrated, diluted with CH_3CN (10 ml) and transferred into pressure bottle. The mixture was cooled to -78 °C and treated with trimethylamine (2 ml) at the same temperature under nitrogen. The reactor was closed and heated at 65 °C for 15 h. After cooling to rt, the reactor was opened. The solvent and excess TMA were evaporated and the residue was purified by silica gel column chromatography ($CHCl_3$/MeOH/H_2O = 6:25:4).

*4.1.3.1. (R)-(1-Palmitoylpyrrolidin-2-yl)methyl 2-(trimethylammonio) ethyl phosphate (**3a**)*. Compound **3a** was obtained from **9a** according to general procedure. Yield 61%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.38 (brs, 2H), 4.28 (brs, 2H), 4.17–4.10 (m, 1H), 4.00–3.93 (m, 1H), 3.84–3.73 (m, 2H), 3.58–3.49 (m, 1H), 3.47–3.43 (m, 1H), 3.36 (s, 9H), 2.19 (t, 2H, J = 7.6 Hz), 2.08–1.98 (m, 2H), 1.93–1.82 (m, 2H),

1.62–1.50 (m, 2H), 1.25 (brs, 24H), 0.88 (t, 3H, J = 6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.2, 66.2, 64.1, 59.2, 57.0, 54.2 (3C), 47.2, 34.9, 31.9, 29.7 (5C), 29.6, 29.6, 29.5, 29.3, 27.2, 25.6, 24.8, 23.7, 22.7, 14.1; HRMS calcd for $C_{26}H_{54}N_2O_5P$ $[M+H]^+$ m/z 505.3765, found m/z 505.3815.

*4.1.3.2. (R,Z)-(1-Hexadec-9-enoylpyrrolidin-2-yl)methyl 2-(trimethylammonio) ethyl phosphate (**3b**)*. Compound **3b** was obtained from **9b** according to general procedure. Yield 89%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.32 (m, 2H), 4.38–4.25 (m, 2H), 4.18–4.11 (m, 1H), 4.00–3.94 (m, 1H), 3.92–3.75 (m, 2H), 3.58–3.52 (m, 1H), 3.48–3.42 (m, 1H), 3.40 (s, 9H), 2.20 (t, 2H, J = 7.6 Hz), 2.07–1.98 (m, 6H), 1.93–1.83 (m, 2H), 1.59–1.52 (m, 2H), 1.29 (brs, 18H), 0.88 (t, 3H, J = 6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.2, 129.9, 129.7, 66.2, 64.1, 59.2, 57.1, 54.3 (3C), 47.2, 34.9, 31.8, 29.8 (2C), 29.7 (2C), 29.5, 29.4, 29.2, 28.9, 27.2, 24.8, 23.7, 22.6, 14.1; HRMS calcd for $C_{26}H_{52}N_2O_5P$ $[M+H]^+$ m/z 503.3608, found m/z 503.3649.

*4.1.3.3. (R,Z)-(1-Oleoylpyrrolidin-2-yl)methyl 2-(trimethylammonio)ethyl phosphate (**3c**)*. Compound **3c** was obtained from **9c** according to general procedure. Yield 87%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.33 (m, 2H), 4.35–4.20 (m, 2H), 4.18–4.10 (m, 1H), 4.01–3.95 (m, 1H), 3.90–3.76 (m, 2H), 3.59–3.49 (m, 1H), 3.40 (s, 9H), 3.38–3.30 (m, 1H), 2.20 (t, 2H, J = 7.6 Hz), 2.05–1.98 (m, 6H), 1.93–1.81 (m, 2H), 1.62–1.50 (m, 2H), 1.28 (d, 20H, J = 11.0 Hz), 0.88 (t, 3H, J = 7.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.2, 129.9, 129.7, 66.2, 64.1, 59.2, 57.1, 54.2 (3C), 47.2, 34.9, 31.8, 29.7 (2C), 29.6, 29.5, 29.4, 29.4, 29.3 (2C), 29.2, 27.3, 27.2, 24.8, 23.7, 22.6, 14.1; HRMS calcd for $C_{28}H_{56}N_2O_5P$ $[M+H]^+$ m/z 531.3921, found m/z 531.3983.

*4.1.3.4. (R,Z)-(1-Docos-13-enoylpyrrolidin-2-yl)methyl 2-(trimethylammonio) ethyl phosphate (**3d**)*. Compound **3d** was obtained from **9d** according to general procedure. Yield 79%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.31 (m, 2H), 4.40–4.23 (m, 2H), 4.17–4.11 (m, 1H), 4.01–3.94 (m, 1H), 3.90–3.76 (m, 2H), 3.57–3.49 (m, 1H), 3.47–3.42 (m, 1H), 3.40 (s, 9H), 3.37–3.32 (m, 1H), 2.19 (t, 2H, J = 7.5 Hz), 2.07–1.98 (m, 6H), 1.93–1.81 (m, 2H), 1.62–1.50 (m, 2H), 1.27 (s, 28H), 0.88 (t, 3H, J = 6.6 Hz). ^{13}C NMR^{13}C NMR (100 MHz, $CDCl_3$) δ 172.2, 129.9, 129.8, 66.2, 64.2, 59.3, 57.1, 54.3 (3C), 47.2, 34.9, 31.8, 29.8, 29.7, 29.7 (2C), 29.6, 29.6, 29.5 (4C), 29.3, 29.3 (2C), 27.3, 27.2, 24.9, 23.7, 22.6, 14.1; HRMS calcd for $C_{32}H_{64}N_2O_5P$ $[M+H]^+$ m/z 587.4547, found m/z 587.4644.

*4.1.3.5. (S)-(1-Palmitoylpyrrolidin-2-yl)methyl 2-(trimethylammonio) ethyl phosphate (**4a**)*. Compound **4a** was obtained from **11a** according to general procedure. Yield 61%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.29 (brs, 2H), 4.17–4.10 (m, 1H), 4.07 (brs, 2H), 4.01–3.93 (m, 1H), 3.85–3.72 (m, 2H), 3.61–3.51 (m, 1H), 3.48–3.41 (m, 1H), 3.36 (s, 9H), 2.19 (t, 2H, J = 7.6 Hz), 2.10–1.97 (m, 2H), 1.93–1.80 (m, 2H), 1.62–1.49 (m, 2H), 1.25 (brs, 24H), 0.88 (t, 3H, J = 7.2 Hz); HRMS calcd for $C_{26}H_{54}N_2O_5P$ $[M+H]^+$ m/z 505.3765, found m/z 505.3741.

*4.1.3.6. (S,Z)-(1-Hexadec-9-enoylpyrrolidin-2-yl)methyl 2-(trimethylammonio) ethyl phosphate (**4b**)*. Compound **4b** was obtained from **11b** according to general procedure. Yield 54%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.30 (m, 2H), 4.38–4.25 (m, 2H), 4.20–4.11 (m, 1H), 4.02–3.93 (m, 1H), 3.85–3.74 (m, 2H), 3.59–3.50 (m, 1H), 3.48–3.42 (m, 1H), 3.36 (s, 9H), 2.20 (t, 2H, J = 7.6 Hz), 2.07–1.97 (m, 6H), 1.90–1.80 (m, 2H), 1.61–1.50 (m, 2H), 1.29 (brs, 18H), 0.88 (t, 3H, J = 7.0 Hz).

*4.1.3.7. (S,Z)-(1-Oleoylpyrrolidin-2-yl)methyl 2-(trimethylammonio)ethyl phosphate (**4c**)*. Compound **4c** was obtained from **11c** according to general procedure. Yield 50%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.33 (m, 2H), 4.38–4.27 (m, 2H), 4.19–4.12 (m, 1H), 4.01–3.95 (m, 1H), 3.93–3.76 (m, 2H), 3.60–3.50 (m, 1H), 3.42 (s, 9H), 3.38–3.30 (m, 1H), 2.20 (t, 2H, J = 7.6 Hz), 2.06–1.97 (m, 6H), 1.93–1.83 (m, 2H), 1.60–1.49 (m, 2H), 1.28 (d, 20H, J = 10.4 Hz), 0.88 (t, 3H, J = 7.0 Hz); HRMS calcd for $C_{28}H_{56}N_2O_5P$ $[M+H]^+$ m/z 531.3921, found m/z 531.3852.

*4.1.3.8. (S,Z)-(1-Docos-13-enoylpyrrolidin-2-yl)methyl 2-(trimethylammonio) ethyl phosphate (**4d**)*. Compound **4d** was obtained from **11d** according to general procedure. Yield 79%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.30 (m, 2H), 4.40–4.23 (m, 2H), 4.17–4.11 (m, 1H), 4.02–3.94 (m, 1H), 3.89–3.76 (m, 2H), 3.59–3.51 (m, 1H), 3.48–3.41 (m, 1H), 3.38 (s, 9H), 3.35–3.30 (m, 1H), 2.19 (t, 2H, J = 7.6 Hz), 2.07–1.97 (m, 6H), 1.90–1.81 (m, 2H), 1.61–1.50 (m, 2H), 1.26 (s, 28H), 0.88 (t, 3H, J = 7.0 Hz); HRMS calcd for $C_{32}H_{64}N_2O_5P$ $[M+H]^+$ m/z 587.4547, found m/z 587.4674.

*4.1.3.9. ((S)-1-((9Z,12Z)-octadeca-9,12-dienoyl)pyrrolidin-2-yl)methyl (2-(trimethylammonio)ethyl) phosphate (**4e**)*. Compound **4e** was obtained from **11e** according to general procedure. Yield 86%. ^{1}H NMR (400 MHz, CD_3OD) δ 5.30–5.18 (m, 4H), 4.19–4.06 (m, 4H), 3.89–3.85 (m, 1H), 3.74–3.68 (m, 1H), 3.57–3.52 (m, 2H), 3.45–3.36 (m, 1H), 3.13 (s, 9H), 2.68 (t, 2H, J = 6.3 Hz), 2.22 (t, 2H, J = 7.5 Hz), 2.02–1.92 (m, 6H), 1.87–1.80 (m, 2H), 1.54–1.46 (m, 2H), 1.30–1.18 (m, 14H), 0.81 (t, J = 6.5 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 173.4, 129.7, 127.9, 66.2, 64.5, 59.2, 57.3, 53.5 (3C), 45.8, 34.6, 31.8, 29.6, 29.5, 29.4, 29.3 (3C), 29.2, 29.1, 27.1, 27.0, 25.4, 24.9, 23.6, 22.4, 13.3; HRMS calcd for $C_{28}H_{54}N_2O_5P$ $[M+H]^+$ m/z 529.3765, found m/z 529.3826.

*4.1.3.10. ((S)-1-((9Z,12Z,15Z)-octadeca-9,12,15-trienoyl)pyrrolidin-2-yl)methyl (2-(trimethylammonio) ethyl) phosphate (**4f**)*. Compound **4f** was obtained from **11f** according to general procedure. Yield 65%. ^{1}H NMR (400 MHz, CD_3OD) δ 5.31–5.18 (m, 6H), 4.19–4.07 (m, 4H), 3.90–3.85 (m, 1H), 3.75–3.67 (m, 1H), 3.58–3.52 (m, 2H), 3.45–3.36 (m, 1H), 3.13 (s, 9H), 2.71 (t, 4H, J = 5.8 Hz), 2.23 (t, 2H, J = 8.1 Hz), 2.02–1.93 (m, 6H), 1.89–1.80 (m, 2H), 1.57–1.46 (m, 2H), 1.25 (brs, 8H), 0.81 (t, J = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 173.4, 131.5, 129.9, 128.0, 127.7 (2C), 127.6, 127.0, 66.2, 64.5, 59.2, 57.4, 53.5 (3C), 45.8, 34.6, 29.6, 29.3 (2C), 29.1, 27.1, 27.0, 25.4, 24.8, 23.6, 20.3, 13.3; HRMS calcd for $C_{28}H_{52}N_2O_5P$ $[M+H]^+$ m/z 527.3608, found m/z 527.3601.

*4.1.3.11. (S)-(1-Palmitoylpyrrolidin-3-yl)methyl 2-(trimethylammonio) ethyl phosphate (**5a**)*. Compound **5a** was obtained from **13a** according to general procedure. Yield 62%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.40–4.18 (m, 4H), 3.88–3.76 (m, 3H), 3.59–3.50 (m, 2H), 3.32 (s, 9H), 3.25–3.19 (m, 1H), 2.62–2.43 (m, 1H), 2.21 (t, 2H, J = 8.2 Hz), 2.07–1.94 (m, 1H), 1.79–1.65 (m, 1H), 1.63–1.53 (m, 2H), 1.04–1.12 (m, 24H), 0.88 (t, 3H, J = 7.0 Hz). ^{13}C NMR (125 MHz, CD_3OD) δ 172.1, 66.7, 66.1, 64.6, 59.3, 54.2 (3C), 50.1, 48.7, 46.2, 44.9, 34.8, 34.6 31.9, 29.7 (3C), 29.6, 29.5, 29.4, 28.2, 25.0, 24.9, 22.1, 14.1; HRMS calcd for $C_{26}H_{54}N_2O_5P$ $[M+H]^+$ m/z 505.3765, found m/z 505.3792.

*4.1.3.12. (S,Z)-(1-Hexadec-9-enoylpyrrolidin-3-yl)methyl 2-(trimethylammonio)ethyl phosphate (**5b**)*. Compound **5b** was obtained from **13b** according to general procedure. Yield 60%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.30 (m, 2H), 4.43–4.21 (m, 4H), 3.90–3.68 (m, 3H), 3.60–3.49 (m, 2H), 3.32 (s, 9H), 3.22–3.15 (m, 1H), 2.61–2.43 (m, 1H), 2.21 (t, 2H, J = 7.8 Hz), 2.07–1.94 (m, 5H), 1.78–1.66 (m, 1H), 1.63–1.52 (m, 2H), 1.30 (brs, 16H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.0, 129.9, 129.7, 66.7, 66.1, 64.6, 59.3, 54.2 (3C), 48.7, 46.1, 34.7, 34.6, 31.7, 29.8, 29.7, 29.5, 29.4, 29.2, 28.9, 27.2, 25.0, 24.9, 22.1, 14.1; HRMS calcd for $C_{26}H_{52}N_2O_5P$ $[M+H]^+$ m/z 503.3608, found m/z 503.3448.

*4.1.3.13. (S,Z)-(1-Oleoylpyrrolidin-3-yl)methyl 2-(trimethylammonio) ethyl phosphate (**5c**)*. Compound **5c** was obtained from **13c** according to general procedure. Yield 85%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.30 (m, 2H), 4.50–4.21 (m, 4H), 3.90–3.69 (m, 3H), 3.60–3.49

(m, 2H), 3.31 (s, 9H), 3.22–3.16 (m, 1H), 2.59–2.43 (m, 1H), 2.20 (t, 2H, J = 7.7 Hz), 2.06–1.96 (m, 5H), 1.78–1.68 (m, 1H), 1.61–1.53 (m, 2H), 1.28 (d, 20H, J = 13.7 Hz), 0.88 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.0, 129.9, 129.7, 66.7, 66.1, 64.6, 59.2, 54.2 (3C), 48.7, 46.2, 34.7, 34.6, 31.7, 29.8 (4C), 29.5 (4C), 29.3 (5C), 28.9, 27.2, 25.0, 24.9, 22.1, 14.1.

*4.1.3.14. (S,Z)-(1-Docos-13-enoylpyrrolidin-3-yl)methyl 2-(trimethylammonio) ethyl phosphate (**5d**).* Compound **5d** was obtained from **13d** according to general procedure. Yield 69%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.31 (m, 2H), 4.33–4.28 (m, 4H), 3.89–3.72 (m, 3H), 3.60–3.50 (m, 2H), 3.35 (s, 9H), 3.25–3.16 (m, 1H), 2.59–2.46 (m, 1H), 2.20 (t, 2H, J = 7.8 Hz), 2.05–1.96 (m, 5H), 1.78–1.66 (m, 1H), 1.62–1.52 (m, 2H), 1.26 (brs, 28H), 0.88 (t, 3H, J = 6.6 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.0, 129.9, 129.7, 66.7, 66.1, 64.6, 59.3, 54.2 (3C), 48.7, 46.2, 34.7, 34.6, 31.7, 29.8 (2C), 29.5 (2C), 29.3 (3C), 28.9, 27.2, 25.0, 24.9, 22.1, 14.1; HRMS calcd for $C_{32}H_{64}N_2O_5P$ $[M+H]^+$ m/z 587.4547, found m/z 587.4405.

*4.1.3.15. ((S)-1-((9Z,12Z)-Octadeca-9,12-dienoyl)pyrrolidin-3-yl)methyl 2-(trimethylammonio)ethyl phosphate (**5e**).* Compound **5e** was obtained from **13e** according to general procedure. Yield 87%. ^{1}H NMR (400 MHz, CD_3OD) δ 5.29–5.18 (m, 4H), 4.16 (brs, 2H), 3.85–3.70 (m, 2H), 3.61–3.37 (m, 4H), 3.31–3.14 (m, 2H), 3.12 (s, 9H), 2.67 (t, 2H, J = 6.0 Hz), 2.57–2.41 (m, 1H), 2.24–2.19 (m, 2H), 2.03–1.89 (m, 5H), 1.81–1.60 (m, 1H), 1.55–1.45 (m, 2H), 1.32–1.16 (m, 14H), 0.81 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 173.2, 129.7, 127.9, 66.6, 66.4, 66.2, 59.2, 53.5 (3C), 49.3, 46.2, 45.1, 40.1, 38.3, 34.2, 34.0, 29.5, 29.3 (2C), 28.9, 28.0, 27.0, 26.4, 25.4, 24.9, 22.4, 13.2; HRMS calcd for $C_{28}H_{54}N_2O_5P$ $[M+H]^+$ m/z 529.3765, found m/z 529.3634.

*4.1.3.16. ((S)-1-((9Z,12Z,15Z)-Octadeca-9,12,15-trienoyl)pyrrolidin-3-yl)methyl 2-(trimethylammonio)ethyl phosphate (**5f**).* Compound **5f** was obtained from **13f** according to general procedure. Yield 63%. ^{1}H NMR (400 MHz, CD_3OD) δ 5.31–5.16 (m, 6H), 4.16 (brs, 4H), 3.84–3.70 (m, 2H), 3.61–3.38 (m, 3H), 3.31–3.19 (m, 1H), 3.12 (s, 9H), 2.71 (t, J = 5.7 Hz, 2H), 2.57–2.39 (m, 1H), 2.25–2.19 (m, 2H), 2.05–1.89 (m, 5H), 1.81–1.60 (m, 1H), 1.55–1.46 (m, 2H), 1.25 (brs, 8H), 0.88 (t, 3H, J = 7.5 Hz). ^{13}C NMR (125 MHz, CD_3OD) δ 173.2, 131.5, 129.9, 128.0 (2C), 127.7, 127.0, 66.7, 66.4, 66.3, 59.2, 53.5 (3C), 49.3, 46.2, 45.1, 40.1, 38.3, 34.2, 29.5, 29.3, 29.1, 28.0, 27.0, 26.4, 24.8, 20.3, 13.5; HRMS calcd for $C_{28}H_{52}N_2O_5P$ $[M+H]^+$ m/z 527.3608, found m/z 527.3623.

*4.1.3.17. (R)-1-Palmitoylpyrrolidin-3-yl 2-(trimethylammonio) ethyl phosphate (**6a**).* Compound **6a** was obtained from **15a** according to general procedure. Yield 62%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.89–4.80 (m, 1H), 4.36–4.14 (m, 2H), 3.38–3.69 (m, 3H), 3.62–3.53 (m, 2H), 3.51–3.43 (m, 1H), 3.34 (s, 9H), 2.23 (t, 2H, J = 7.0 Hz), 2.22–1.97 (m, 2H), 1.63–1.52 (m, 2H), 1.34–1.20 (m, 24H), 0.88 (t, 3H, J = 7.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.3, 74.7, 73.2, 66.2, 59.2, 54.3 (3C), 52.7, 50.4, 44.6, 43.7, 34.8, 34.6, 33.3, 31.9, 29.8 (2C), 29.7, 29.6, 29.4, 25.0, 24.9, 22.7, 14.1; HRMS calcd for $C_{25}H_{52}N_2O_5P$ $[M+H]^+$ m/z 491.3608, found m/z 491.3638.

*4.1.3.18. (R,Z)-1-Hexadec-9-enoylpyrrolidin-3-yl 2-(trimethylammonio) ethyl phosphate (**6b**).* Compound **6b** was obtained from **15b** according to general procedure. Yield 68%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.29 (m, 2H), 4.89–4.78 (m, 1H), 4.28 (brs, 2H), 3.86–3.72 (m, 2H), 3.65–3.54 (m, 2H), 3.52–3.45 (m, 1H), 3.38 (s, 9H), 2.25–2.16 (m, 2H), 2.08–1.95 (m, 6H), 1.63–1.54 (m, 2H), 1.30 (brs, 16H), 0.88 (t, 3H, J = 6.9 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.0, 129.9, 129.7, 74.7, 73.2, 66.1, 59.3, 54.3 (3C), 52.6, 44.6, 43.7, 34.1, 31.9, 29.7, 29.4, 29.3 (2C), 29.2, 29.1, 26.9, 24.8, 22.6, 14.1; HRMS calcd for $C_{25}H_{50}N_2O_5P$ $[M+H]^+$ m/z 489.3452, found m/z 489.3476.

*4.1.3.19. (R,Z)-1-Oleoylpyrrolidin-3-yl 2-(trimethylammonio) ethyl phosphate (**6c**).* Compound **6c** was obtained from **15c** according to general procedure. Yield 78%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.29 (m, 2H), 4.92–4.79 (m, 1H), 4.29 (brs, 2H), 3.83–3.70 (m, 2H), 3.67–3.54 (m, 2H), 3.51–3.43 (m, 1H), 3.34 (s, 9H), 2.22 (t, 2H, J = 7.0 Hz), 2.06–1.96 (m, 6H), 1.65–1.54 (m, 2H), 1.28 (d, 20H, J = 14.0 Hz), 0.88 (t, 3H, J = 7.0 Hz). ^{13}C NMR (125 MHz, CD_3OD) δ 173.4, 129.9, 129.7, 75.1, 74.0, 66.2, 59.2, 53.5 (3C), 52.7, 44.7, 43.7, 43.6 34.4, 34.1, 31.9, 29.7, 29.4, 29.3 (2C), 29.2, 29.1, 26.9, 24.8, 22.6, 13.3; HRMS calcd for $C_{27}H_{54}N_2O_5P$ $[M+H]^+$ m/z 517.3765, found m/z 517.3726.

*4.1.3.20. (R,Z)-1-Docos-13-enoylpyrrolidin-3-yl 2-(trimethylammonio) ethyl phosphate (**6d**).* Compound **6d** was obtained from **15d** according to general procedure. Yield 73%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38–5.30 (m, 2H), 4.89–4.79 (m, 1H), 4.28 (brs, 2H), 3.79 (brs, 2H), 3.72–3.40 (m, 4H), 3.35 (s, 9H), 2.22 (t, 2H, J = 7.8 Hz), 2.20–2.09 (m, 2H), 2.03–1.98 (m, 6H), 1.63–1.52 (m, 2H), 1.26 (brs, 28H), 0.88 (t, 3H, J = 6.9 Hz). ^{13}C NMR (125 MHz, CD_3OD) δ 173.4, 129.6 (2C), 75.2, 74.0, 66.2, 59.2, 53.5 (3C), 52.7, 44.7, 43.7, 43.7, 34.4, 34.1, 31.9, 29.7 (2C), 29.6 (2C), 29.5, 29.4, 29.3, 29.2, 29.1 (2C), 26.9, 24.8, 22.6, 13.3; HRMS calcd for $C_{31}H_{62}N_2O_5P$ $[M+H]^+$ m/z 573.4391, found m/z 573.4412.

*4.1.3.21. (R)-1-((9Z,12Z)-Octadeca-9,12-dienoyl)pyrrolidin-3-yl 2-(trimethylammonio)ethyl phosphate (**6e**).* Compound **6e** was obtained from **15e** according to general procedure. Yield 73%. ^{1}H NMR (400 MHz, CD_3OD) δ 5.29–5.18 (m, 4H), 4.75–4.71 (m, 1H), 4.15 (brs, 2H), 3.64–3.58 (m, 2H), 3.59–3.47 (m, 3H), 3.41–3.33 (m, 1H), 3.12 (s, 9H), 2.67 (t, 2H, J = 6.0 Hz), 2.28–2.18 (m, 2H), 2.17–2.02 (m, 2H), 2.00–1.91 (m, 4H), 1.54–1.46 (m, 2H), 1.29–1.17 (m, 14H), 0.81 (t, 3H, J = 7.0 Hz). ^{13}C NMR (125 MHz, CD_3OD) δ 173.4, 129.9, 127.9, 75.3, 74.0, 66.2, 59.3, 53.5 (3C), 52.6, 44.7, 43.7, 34.3, 34.0, 33.8, 33.0, 29.6, 29.2 (2C), 29.0, 28.9, 24.8, 22.4, 13.2; HRMS calcd for $C_{27}H_{52}N_2O_5P$ $[M+H]^+$ m/z 515.3608, found m/z 515.3643.

*4.1.3.22. (R)-1-((9Z,12Z,15Z)-Octadeca-9,12,15-trienoyl)pyrrolidin-3-yl 2-(trimethylammonio)ethyl phosphate (**6f**).* Compound **6f** was obtained from **15f** according to general procedure. Yield 74%. ^{1}H NMR (400 MHz, CD_3OD) δ 5.31–5.17 (m, 6H), 4.75–4.71 (m, 1H), 4.16 (brs, 2H), 3.65–3.59 (m, 2H), 3.58–3.48 (m, 3H), 3.41–3.33 (m, 1H), 3.13 (s, 9H), 2.71 (t, 2H, J = 6.0 Hz), 2.27–1.89 (m, 8H), 1.56–46 (m, 2H), 1.25 (br s, 8H), 0.87 (t, 3H, J = 7.5 Hz). ^{13}C NMR (125 MHz, CD_3OD) δ 173.2, 131.4, 130.0, 128.1 (2C), 127.6, 127.0, 75.3, 74.0, 66.2, 59.3, 53.6 (3C), 52.6, 44.8, 43.7, 34.4, 34.0, 33.0, 31.4, 29.6, 29.3 (2C), 29.1, 28.9, 24.8, 22.4, 13.2; HRMS calcd for $C_{27}H_{50}N_2O_5P$ $[M+H]^+$ m/z 513.3452, found m/z 513.3544.

4.2. Biological evaluation

Biological evaluations were conducted according to known literature-reported protocols [23,24] as detailed in the supporting information.

4.3. Molecular docking

In silico calculations were conducted according to known literature-reported protocols [19,20,25] as detailed in the supporting information.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.11.040.

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