Tutor/s

Dr. Fèlix Urpí Tubella
Departament de Química Inorgànica i Orgànica

Dr. José Luis Abad Saiz
CSIC-IQAC

Grau de Química

Treball Final de Grau

Towards the synthesis of (2*S*,3*S*,*E*)-ceramide-1,3-phosphodiester; Phosphosphingolipid analogs as potential modulators of the enzymatic activity.

Cap a la síntesi de la (2*S*,3*S*,*E*)-ceramida-1,3-fosfodièster; Anàlegs de fosfoesfingolípids com a potencials moduladors de l'activitat enzimàtica.

Ariadna Muñoz Nebot

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A mamá,

Agrair l'enorme dedicació, seguiment, esforç i interès a en Fèlix Urpí i en José Luís Abad, l'ajut de l'Eduard Izquierdo i l'Antonio Delgado, i l'incessant suport del meu germà Héctor, els meus avis Emilia i Victorino i el meu pare Javier. Moltíssimes gràcies.

REPORT

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1. SUMMARY

The study of new biologically active molecules for the understanding of the cellular functioning is essential for the advancement of modern medicine. This project is focused on the synthesis of the (2*S*,3*S*,*E*)-ceramide-1,3-phosphodiester, a synthetic molecule inspired by three molecules with a determinant role in the life cycle of animal cells: ceramide, ceramide-1-phosphate and the corresponding cyclic phosphate. Slight changes into the structures of the mentioned molecules may change, and even invert their cellular role. Therefore, and considering that the biological function is very dependent on the structural changes, it has been decided to carry out the insertion of a cyclic phosphodiester into the ceramide backbone, as well as the change to a non-natural (2*S*,3*S*) configuration instead of the natural (2*S*,3*R*), while maintaining the *E* configuration of the double bond.

Keywords: (2*S*,3*S*,*E*)-ceramide-1,3-phosphodiester, stereoselective synthesis, cellular role.

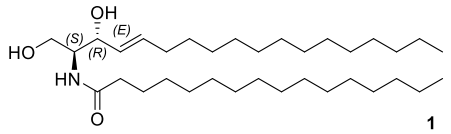
2. RESUM

L'estudi de noves molècules biològicament actives per a la comprensió del funcionament cel·lular és imprescindible per a l'avenç de la medicina moderna. Aquest projecte està enfocat a la síntesi de la (2*S*,3*S*,*E*)-ceramida-1,3-fosfodiester, una molècula sintètica inspirada en tres molècules amb un rol determinant al cicle de vida cel·lular animal: la ceramida, la ceramida-1-fosfat i el corresponent fosfat cíclic. Petits canvis a les estructures de les molècules esmentades poden canviar, i inclús invertir el rol cel·lular d'aquestes. Per això, i atès que la funció biològica és molt depenent dels canvis estructurals, s'ha decidit dur a terme la inserció d'un diéster fosfòric cíclic a la ceramida, així com el canvi a la configuració no natural (2*S*,3*S*) enlloc de la natural (2*S*,3*R*), tot mantenint la configuració *E* del doble enllaç.

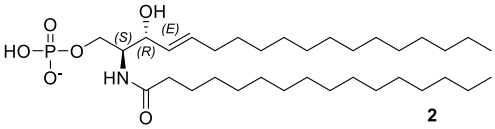
Paraules clau: (2*S*,3*S*,*E*)-ceramida-1,3-fosfodiester, síntesi estereoselectiva, rol cel·lular.

3. INTRODUCTION

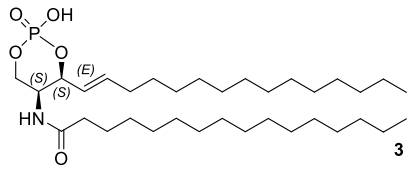
Naturally, our cells control their population by apoptosis, also called programmed cell death, which is a procedure that intervenes in the tissue homeostasis through which the cells inactivate and degrade their own structure and components until death.[1] The integrity of plasma membrane is preserved until the end of the process, allowing the organelles packaging. These will be disintegrated into vesicles attached to the membrane, avoiding the release of toxic intracellular components to the outside. The cell fragments will be phagocytosed by macrophages or neighboring cells, thus the tissue damage will be avoided. Consequently, normal development remains under control and the elimination of unnecessary cells is carried out. Cellular apoptosis is strongly linked to diseases such as cancer, inasmuch as the malignant cells evade apoptotic mechanisms, thus causing an indiscriminate proliferation of the cells with the consequent formation of the tumor.[1] For this reason, the study and use as biomarkers of both the molecules involved in the apoptosis and cell proliferation processes are of vital importance to advance in research and improvement of new therapies and prevention. Among these biomolecules it is included ceramides, which have an apoptotic role.[2] The most common



Natural (2*S*,3*R*) ceramide
Programmed cell death role.



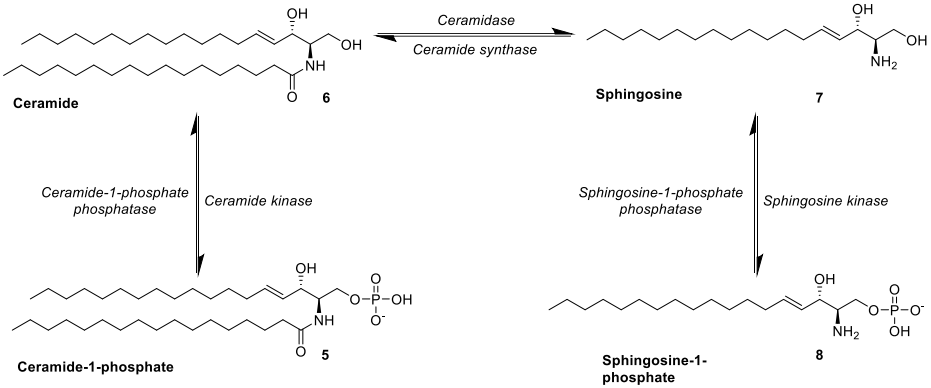
Natural (2*S*,3*R*) ceramide-1-phosphate
Cellular survival role



Synthetic (2*S*,3*S,E*)-ceramide-1,3-phosphodiester
Synthetic target

Figure 1: Natural and synthetic ceramides

ceramides are those that are formed by sphingosine amide-bonding to a fatty acid with usually 16-24 carbons.[1] These long fatty acids make ceramide have a hydrophobic character, since the hydrophilic region of the molecule (only formed by two hydroxyl groups) is minimal. However, it can change with the phosphate insertion in the molecule, making (2*S*,3*S*,*E*)-ceramide-1,3-phosphodiester less polar than the natural phosphate. In addition to a structural function in the cell lipid bilayer, ceramides also play the role of second messengers in cellular division acting as a differentiation messenger, in permeation of membranes, senescence, cell signaling, and as insulin suppressant.[3] But it is worth noting that ceramides have an extremely key role in apoptotic pathways: A very sensitive balance has been established between ceramide-1-phosphate, ceramide, sphingosine, and sphingosine-1-phosphate within the cell, which are easily interconverted.[1,2]

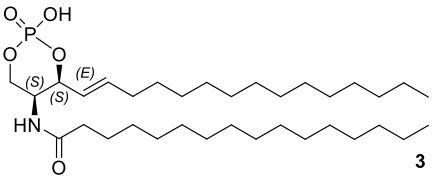


Scheme 1: Sphingolipid rheostat

The intracellular balance of these four sphingolipids, usually referred as “sphingolipid rheostat”, will determine the survival of the cell, where ceramide and sphingosine increase cell apoptosis, and sphingosine-1-phosphate and ceramide-1-phosphate are involved in cell proliferation and survival.[3] This balance occurs naturally in all mammal cells, but can be affected by ionizing radiation, ultraviolet C radiation, stress and others, causing the unbalance of the sphingolipid rheostat.[1,2] Minor changes in the structure of these molecules, as for example the addition of a cyclic phosphate in its primary hydroxyl group, or changing the natural configuration (2*S*,3*R*) to the non-natural (2*S*,3*S*), can cause the molecule carry out a completely different role within the

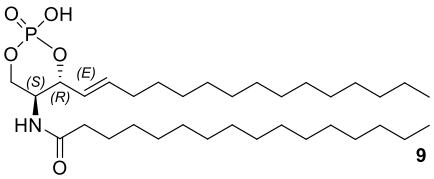
cell. Therefore, although the ceramides have a major apoptotic function, the role of (2*S*,3*S*,*E*)-ceramide-1,3-phosphodiester within the cell (the synthetic target of this project) is difficult to predict. Even so, it would be interesting to know the biological behavior of this molecule, due to its structural similarity to molecules such as natural ceramides like (2*S*,3*R*)-ceramide-1-phosphate and cyclic phosphodiester,[4] both with a biologically active role.[1,3] So, the new molecule could have an interesting therapeutic application; currently, ceramides are already used as inhibitors of cell proliferation in cancer therapies, for example, treatments as used as chemotherapy or radiotherapy increases cell's ceramide concentration.

The fact of finding the cyclic phosphates of ceramide[5] in nature opens a way to study their biological activity, both natural and synthetic ceramides. (*2S,3R,E*)-Ceramide-1,3-phosphodiester (**9**) was previously prepared by the research group. In this project a new synthetic approach will be designed to obtain the (*2S,3S,E*)-ceramide-1,3-phosphodiester (**3**). Finally, the resulting (2*S*,3S,*E*) ceramide phosphodiester will be tested in cell models and compared with the previously obtained (2*S*,3*R*,*E*) isomer.



3

(2*S*,3*S,E*)-ceramide-1,3-phosphodiester
Synthetic target



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(2*S*,3*R,E*)-ceramide-1,3-phosphodiester
previously synthetized in the research group

Figure 2: (2*S*,3*S*,*E*) and (2*S*,3*R*,*E*) ceramides-1,3-phosphodiester

4. OBJECTIVES

The main objective of this project is to carry out the synthesis of the (2*S*,3*S*,*E*)-ceramide-1,3-phosphodiester (**3**), starting from Garner's aldehyde[6] (**10**) taking care of the (2*S*,3*S*) and *E* configurations of the different intermediates. These will be characterized by means of different spectroscopic techniques, like mono and bidimensional ^{1}H NMR (COSY and HSQC), ^{13}C NMR and ^{31}P NMR. The synthetic approach can be divided into minor objectives, as follows:

• The stereoselective C-C bond formation to create the alcohol with *S* configuration with an organometallic reagent.

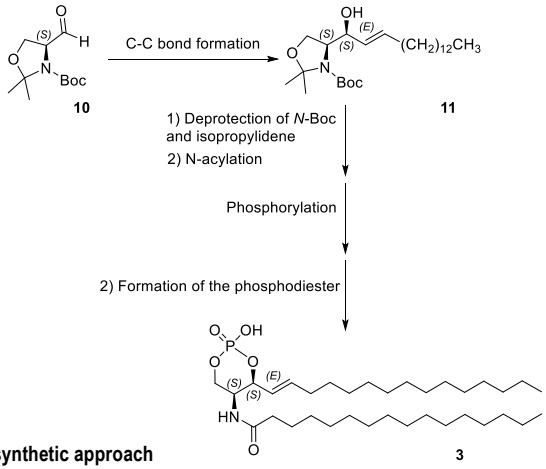
• *N*-Boc and *N*,*O*-isopropylidene deprotection.

• *N*-acylation with palmitic acid.

• Phosphorylation of the C1 (or C3) hydroxyl group.

• (In the phosphorus(III) synthetic route, oxidization from hydrogenphosphonate to phosphonate).

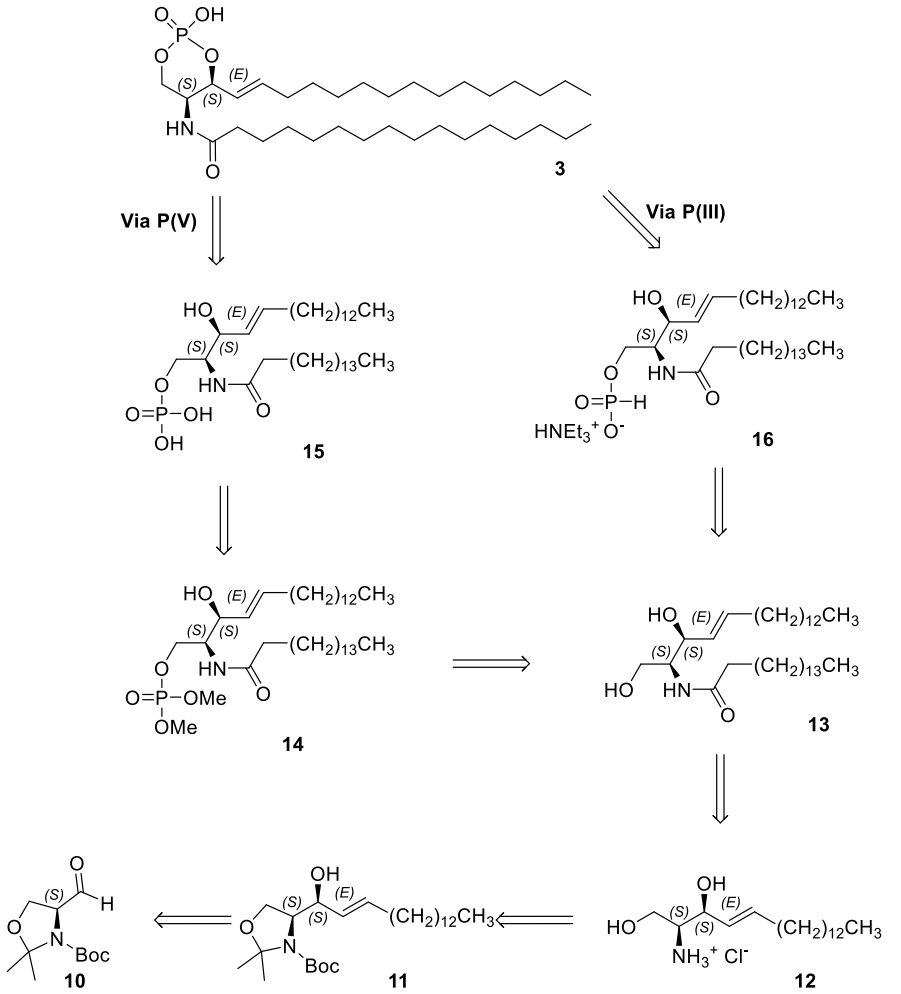
• Cyclization of the phosphonate to obtain the phosphodiester adduct.



Scheme 2: Schematic synthetic approach

4.1. RETROSYNTHETIC ANALYSIS

The proposed retrosynthetic analysis in order to obtain the (2*S*,3*S*,*E*)-ceramide-1,3-phosphodiester (**3**) is represented in the Scheme 3. It was planned to use an organometallic reagent to carry out the addition of the carbon chain through the *syn*-selective addition to the aldehyde, obtaining the desired allylic alcohol with the *S* configuration. Once formed, it was supposed to do the simultaneous deprotection of the Boc and the *N,O*-isopropylidene acetal by an acidic medium, followed by the *N*-acylation with palmitic acid using HOBt and EDC, leading to the formation of (2*S*,3*S*,*E*)-ceramide (**13**). At this point, in order to reach the synthetic target **3**, it has been decided to test two possible synthetic routes. In the first approach, it has been decided to add an hydrogenphosphonate group to the C1 (or C3) alcohol of the ceramide **13** affording the compound **16**, finishing the synthetic route with the oxidation from hydrogenphosphonate to phosphonate and its subsequent cyclization with *tert*-butyl hydroperoxide. On the other hand, through the phosphorus(V) pathway, it has been decided to do the formation of a dimethyl phosphate on the C1 (or C3) hydroxyl group by the addition of dimethyl chlorophosphate, followed by its deprotection using TMSI. Finally, the cyclization of **15** with *N,N*-dicyclohexylcarbodiimide (DCC) would afford the desired compound.

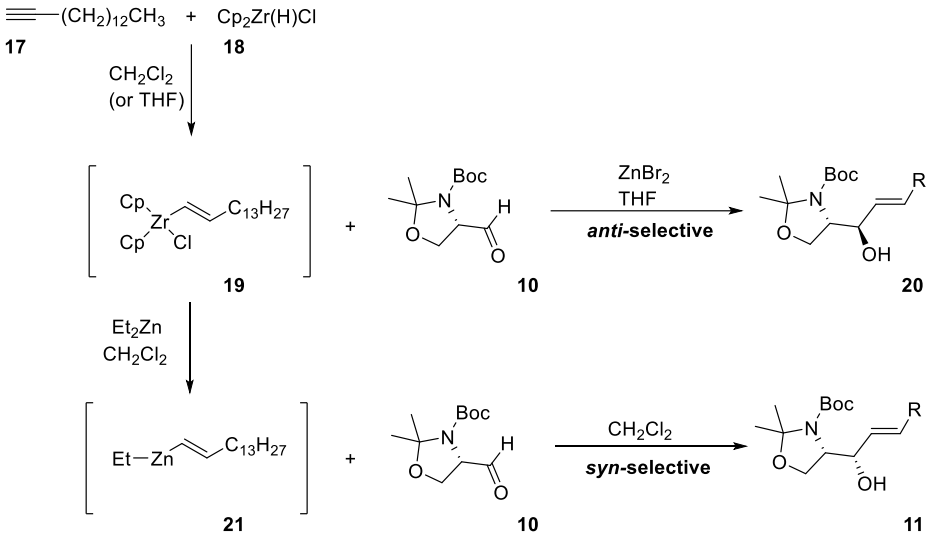


Scheme 3: Retrosynthetic analysis

5. RESULTS AND DISCUSSION

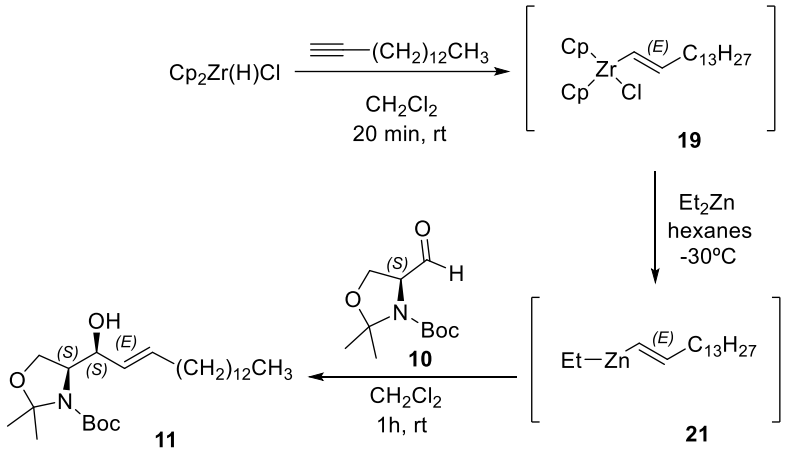
5.1. STEREOSELECTIVE ADDITION OF THE ORGANOMETALLIC REAGENT TO THE GARNER'S ALDEHYDE: SYNTHESIS OF TERT-BUTYL (S)-4-[(*S*,*E*)-1-HYDROXYHEXADEC-2-EN-1-YL]-2,2-DIMETHYLOXAZOLIDINE-3-CARBOXYLATE (11)

According with T. Murakami and K. Furusawa,[8] the new stereocenter configuration can be decided according to scheme 4. The stereoisomer *anti*-(2*S*,3*R*) can be obtained by the reaction of the complexation adduct **19** with Garner's aldehyde (**10**) through zinc bromide in THF, while the *syn*-(2*S*,3*S*) adduct **11** can be obtained by the reaction of Garner's aldehyde with the transmetallation adduct **21**, the product of the reaction by diethyl zinc with the complexation adduct **19**. This complexation adduct is the result of the reaction of the Schwartz reagent[7] (**18**) with 1-pentadecyne (**17**). In general, the hydrozirconation with the Schwartz reagent[7] proceeds rapidly, and more importantly, the former reaction tolerates the presence of certain functional groups, such as alkyl ethers, silyl ethers, and *t*-butyl esters. Although addition of alkenylzirconocenes to aldehydes is sluggish, the reaction is accelerated either by adding a catalyst or by transmetallation with dialkylzinc to afford (*E*)-allylic alcohols in acceptable yields.[8] The reaction mechanism of the zinc bromide catalyzed addition of alkenylzirconocenes remains unclear, and the *syn*-selective addition of pentadecyne-Zn in CH_2Cl_2 can be explained by a chelated transition model of Garner's aldehyde (**10**) with zinc or by a coordinated delivery model.[8]



Scheme 4: Diastereodivergent synthesis of *N*-Boc sphingosine derivatives from 10 and alkenyl-zirconocenes [8]

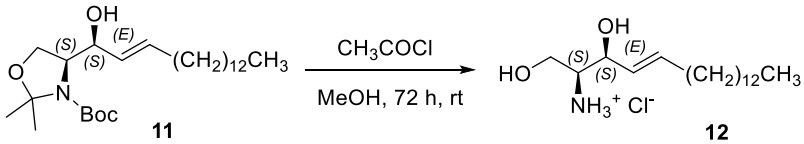
The addition of 1-pentadecyne to Schwartz reagent was run at rt. After 20 min, a change in the coloration of the transparent solution from colorless to yellowish and the TLC control were evidenced the formation of the alkenylzirconium reagent **19**. Then, diethylzinc was added at -30ºC in order to do the transmetallated adduct. Finally, Garner's aldehyde was added to the reaction mixture and the reaction stirred for 1 h at rt. After an aqueous work-up, a white precipitate of zirconium salts was observed. Once filtered with celite, a yellow organic solution corresponding to the addition compound **11** was observed. After a chromatographic purification of the crude reaction mixture two diastereoisomers of **11** could be isolated in 78% yield in a 94:6 ratio by NMR, in agreement with related literature precedents[8].



Scheme 5: Synthesis of 11

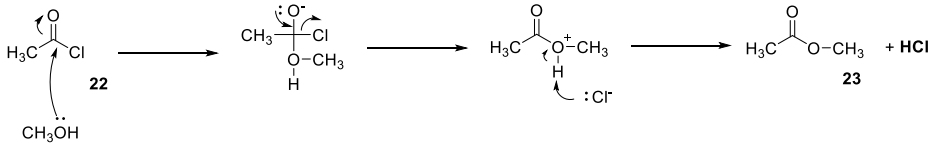
5.2. SYNTHESIS OF (2*S*,3*S*,*E*)-1,3-DIHYDROXYOCTADEC-4-EN-2-AMMONIUM CHLORIDE (12)

The deprotection reaction was run by an acidic medium (HCl) for 48 h at room temperature. After a chromatographic purification, the deprotected adduct **12** was obtained in 72% yield.



Scheme 6: Deprotection of 11

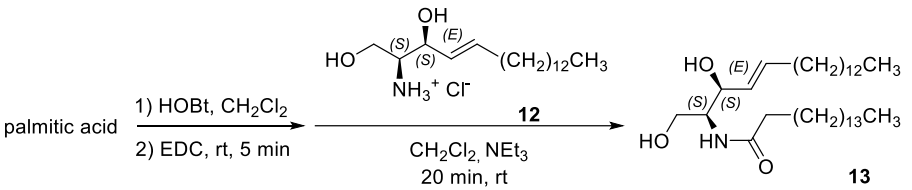
The *N*-Boc and *N,O*-isopropylidene deprotection of **12** was achieved by the generation of hydrochloric acid by the reaction of methanol with acetyl chloride, as indicated in the scheme 7.



Scheme 7: Generation of HCl from MeOH and CH_3COCl

5.3. SYNTHESIS OF (2*S*,3*S*)-CERAMIDE (13)

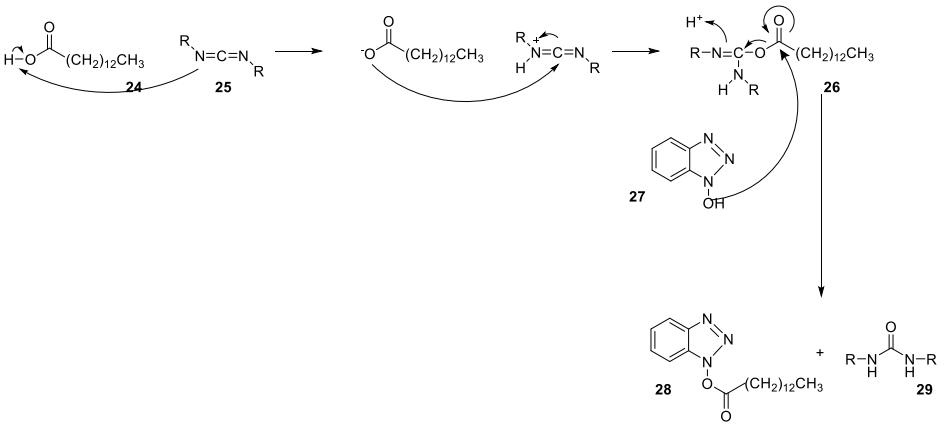
The synthesis of ceramide **13** was carried out by *N*-acylation of **12** with palmitic acid in CH_2Cl_2 in presence of EDC (**25**) and HOBt (**27**) at room temperature, based on precedents of the RUBAM research group. This reaction may be conditioned by the high reactivity of the primary and secondary hydroxyl groups of the starting sphingosine or the resulting ceramide, which would complicate the reaction outcome. Both the primary and the secondary hydroxyl groups are not exempt from reacting, being able to give the formation of the corresponding palmitates. These esters could be saponified in basic medium, but this would suppose an additional reaction step and the probable loss in overall yield. In contrast, ceramide ester formation in the presence of EDC/HOBt has shown to be marginal (see the mechanism in Scheme 9).



Scheme 8: *N*-Acylation of 12

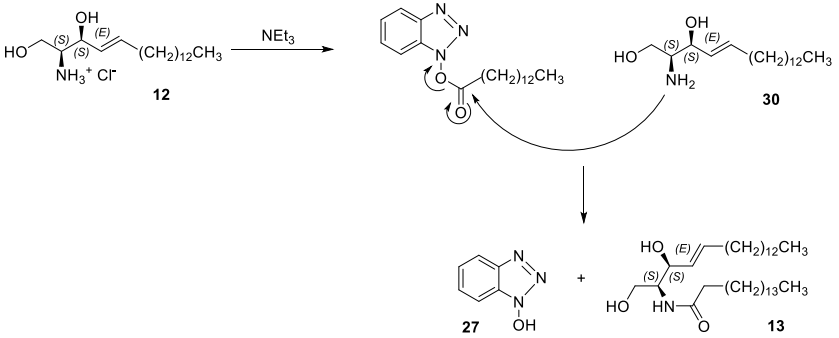
On the work up, the organic phase was washed three times with water. These water washings are required to remove the HOBt (**27**), the *N*-dimethylaminopropil *N*-ethyl urea (derived from EDC) (**29**) and other possible water-soluble impurities. It is advisable to not adding an excess of palmitic acid, due to the objective is to bonding it with the amine to forming amide, but an excess can make bond palmitic acid to the hydroxyl groups, forming an (COCOR) groups. Part of ceramide **13** eluted the column together with different esters of **13**. Those esters were saponified with K_2CO_3 in MeOH at rt for 3 h. Next, the methanol was removed in vacuo, and the amide **13** resulting from the saponification reaction was purified. After purification, the ceramide **13** was obtained in 45% yield.

On the scheme 9, it can be observed the reaction mechanism of the EDC and the HOBt for the formation of the HOBt-palmitic acid intermediate adduct.



Scheme 9: Mechanism of the formation of the intermediate HOBt-palmitic acid

On the scheme 10, it is described the mechanism by which the palmitic acid adds to the sphingosine to form the ceramide, as well as the initial HOBt recovery.



Scheme 10: Formation of (2*S*,3*S*,*E*)-ceramide (13)

5.4. SYNTHESIS OF THE PHOSPHORYLATED CERAMIDES[9]

Three kinds of phosphorus reagents are the most frequently used in the synthesis of phosphate esters: tetracoordinated phosphates with an oxidation state +5, tricoordinated phosphites (or phosphoramidites) with oxidation state +3, and tetracoordinated hydrogenphosphonates with an oxidation state +3. Phosphates have tetrahedral geometry and their chemistry is dominated by the presence of a very stable phosphoryl group (P=O). The phosphorus atom in tetracoordinated phosphates with an oxidation state +5, it is an electrophilic centre and it is subject to reactions with hard nucleophiles. On the contrary, phosphites and phosphoramidites have a trigonal pyramidal geometry with a lone electron pair located on the phosphorus atom. In general, phosphorus(III) derivatives can be easily converted into phosphorus(V) derivatives using different oxidizing reagents (e.g. iodine/water, elemental sulphur, elemental selenium, etc).[9]

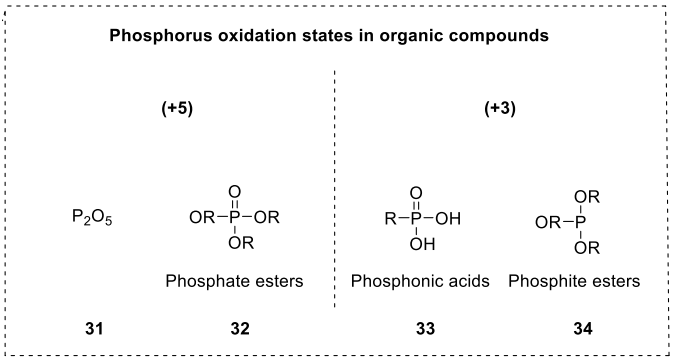
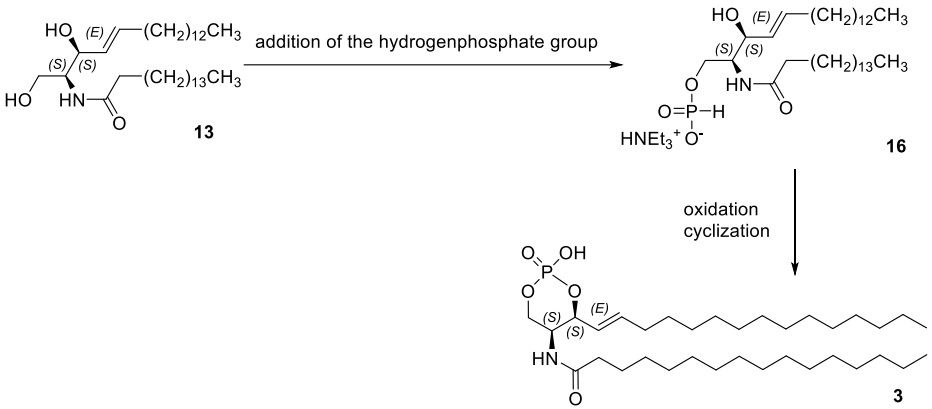


Figure 3: Phosphorus(III) and phosphorus(V) oxidation states

5.4.1. Synthesis of 3 via phosphorus(III)

In order to reach the synthetic target **3** by the phosphorus(III) synthetic pathway it has been decided to add an hydrogenphosphonate group to the C1 alcohol of the ceramide **13** affording to the ammonium phosphonate **16**, finishing the synthetic route with the oxidation with *tert*-butyl hydroperoxide from hydrogenphosphonate to phosphonate and its subsequent cyclization.

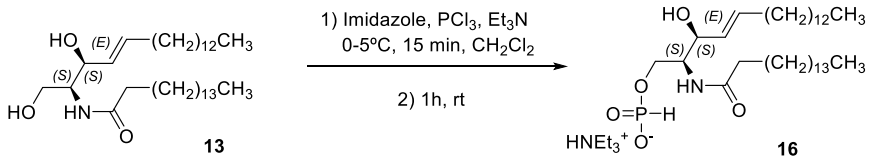


Scheme 11: Phosphorus(III) synthetic pathway

*5.4.1.1. Hydrogenphosphonate addition to the hydroxyl group: Synthesis of (2S,3S,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl phosphonate (**16**)*

For the addition of the hydrogen phosphonate, it is necessary to add a large excess of phosphorus trichloride to ensure its addition into the molecule. The *tris*(imidazol-1-yl)phosphine formed by the reaction of PCl_3 with imidazole can be bonded to both primary or secondary hydroxyl groups, always being bonded only to one of them. Whether it is in the primary hydroxyl or in the secondary hydroxyl group is irrelevant, given the nature of the next reaction step: the cyclization. The method used to prevent the phosphorus bonding in both hydroxyl groups has been the addition of imidazole, which replaced the three chloride bonds by three imidazole bonds, causing steric effect for the unreacted hydroxyl group. When water was added at the end of the reaction, the imidazole groups were substituted forming the hydrogenphosphonate. The

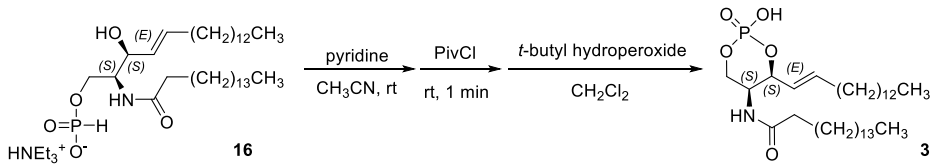
reaction was run at 0 ºC due to the truculence and the high reactivity of the phosphorus trichloride and left to react during 1.25 h warming gradually until room temperature. After purification, the phosphonate **16** was isolated in 80% yield. The ^{1}H NMR and the ^{31}P NMR showed that the hydrogenphosphonate was only added in the C1 carbon.



Scheme 12: Synthesis of phosphonate 16

*5.4.1.2. Cyclization with pivaloyl chloride: Synthesis of N-[(4S,5S)-2-hydroxy-2-oxido-4-((E)-pentadec-1-en-1-yl)-1,3,2-dioxaphosphinan-5-yl]palmitamide (**3**)*

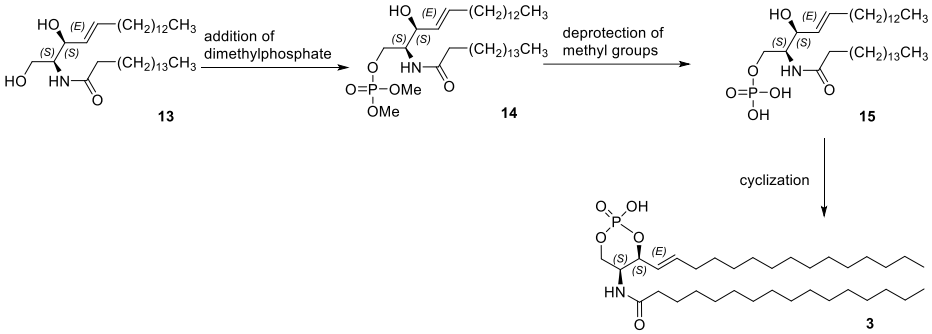
The reaction of **16** with pivaloyl chloride and pyridine formed a phosphorous carboxylic anhydride, which could receive the attack of the alcohol to form the hydrogen phosphonate. In this way, the *tert*-butyl hydroperoxide could cyclize the phosphonate forming the diester bond, thus affording the synthetic target **3**. After the addition of *tert*-butyl hydroperoxide, the reaction mixture was dried with $MgSO_4$ and washed 3 times with a triethylamine:water solution to extract the pyridinium chloride salts. The reaction adduct was situated in the organic phase, instead of the aqueous. The synthesis procedure did not afford the desired compound **3**. Traces of an unknown compound were isolated. This compound presented two peaks in ^{31}P NMR at 4.86 and 3.43 ppm, instead of one peak around zero that we expected.



Scheme 13: First assessment of the cyclization reaction of 16

5.4.2. Synthesis of 3 via phosphorus(V)

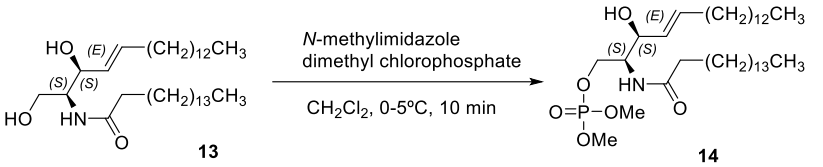
In order to reach the synthetic target **3** by the phosphorus(V) synthetic pathway it has been decided to do the formation of a dimethyl phosphate on the C1 hydroxyl group by the addition of dimethyl chlorophosphate, followed by its deprotection using TMSI. Finally, the cyclization of **15** with *N*,*N*-dicyclohexylcarbodiimide (DCC) would afford the desired compound **3**.



Scheme 14: Phosphorus(V) synthetic pathway

*5.4.2.1. Addition of dimethyl phosphate to the primary hydroxyl group of the ceramide: Synthesis of (2S,3S,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl dimethyl phosphate (**14**)*

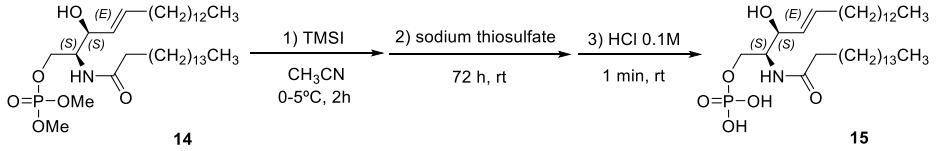
The reaction of diol **13** with dimethyl chlorophosphate in the presence of *N*-methylimidazole was run in CH_2Cl_2 at 0 ºC. After the reaction mixture was stirred for 10 min, it was observed a change of the yellowish reaction mixture from opaque to transparent. The TLC control did not show the presence of starting material **13**. It was necessary to quenching the reaction the sooner the better, due to it was observed that impurities were formed over time. After the solution was quenched by water, the solution took whitish coloring. After purification the phosphate **14** was isolated in 61% yield. The ^{1}H NMR showed that the dimethylphosphate was only added in the C1 carbon.



Scheme 15: Formation of the dimethyl phosphate on the C1 hydroxyl group of 13

*5.4.2.2. Deprotection of phosphate: Synthesis of (2S,3S,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl dihydrogen phosphate (**15**)*

The phosphate **14** was treated with TMSI in acetonitrile at 0°C. As the reaction progressed, it was observed a change in the coloration from yellow to orange, due to the presence of iodine in the reaction mixture. The iodine could produce the loss of the *E*-configuration of the double bond. To solve it, it was decided to add sodium thiosulfate to reduce the iodine to iodide. This reduction made the solution whitish, evidencing the complete elimination of iodine in the solution. Finally, it was added HCl 0.1 M to remove the $SiMe_3$ group of the phosphonate changing it by a hydrogen group, forming $SiMe_3Cl$ and the phosphonate group. Unfortunately, the desired phosphate **15** was only isolated with a very low yield (10%).



Scheme 16: Deprotection of the dimethylphosphate 14

6. EXPERIMENTAL SECTION

6.1. MATERIALS AND METHODS

6.1.1. Reagents and solvents:

Unless otherwise specified, reactions were conducted in oven-dried glassware under inert atmosphere of argon with anhydrous solvents, in a round bottomed flask. All the solvents were anhydride and used without purification. All commercial reagents were obtained from registered suppliers and used without further purification.

6.1.2: Methods and instrumentation:

Analytical thin-layer chromatography (TLC): This method was used to control the reactions and to verify their progress. Thin layers were carried out on DC-Kieselgel ALUGRAM SIL G/UV254 brand. A solution of phosphomolybdic acid hydrate with ethanol was used as stained. The eluent used is specified in each case.

Column Chromatography: This method was used to purify the different substances obtained in each reaction. The stationary phase used was silica gel compacted with hexane. The mobile phase is specified in each case, a mixture of a polar and non-polar (or less polar) solvent. The column were carried out under low pressure (flash conditions) and performed on 230-400 mesh Sigma Aldrich silica gel.

Nuclear magnetic resonance: ^{1}H NMR, ^{13}C NMR, COSY and HSQC spectra data were collected with a Varian 400 spectrometer for the identification of each purified compound. The reference compound is specified in each case (ppm). The coupling constant (*J*) is always in Hz. The chemical shifts (δ) are reported in ppm and referenced to internal TMS (δ 0.00 ppm for ^{1}H NMR) or $CDCl_3$ (δ 77.00 for ^{13}C NMR). Data are reported as follows: s = singlet, d = doublet, t = triplet, q= quartet, m = multiplet, br = broad (and their corresponding combinations).

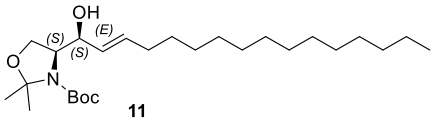
Infrared spectroscopy (IR): This method was used for the characterization of some compounds formed during the synthesis. The instrument used is a FTIR Avatar 360 spectrophotometer. Only the most relevant peaks (cm^{-1}) are described in the experimental section.

Specific optical rotation: This method was used for the characterization of some of the pure compounds that were not an enantiomer mixture and presented optical rotation. The specific rotation ($[\alpha]_D$) were determined at 25 °C on PERKIN ELMER polarimeter 341 polarimeter. They are measured on $CHCl_3$ or a 50:50 $CHCl_3$:MeOH solution, and the wavelength used was the D line of sodium (589 nm).

6.2. SYNTHESIS OF (2*S*,3*S*,*E*)-CERAMIDE -1,3-PHOSPHODIESTER

6.2.1. Synthesis of *tert*-butyl (*S*)-4-[(*S*,*E*)-1-hydroxyhexadec-2-en-1-yl]-2,2-dimethyloxazolidine-3-carboxylate (11)

To an ice-cooled stirred suspension of $Cp_2Zr(H)Cl$ (1.08 g, 3.75 mmol) in CH_2Cl_2 (8 mL) under argon was added neat 1-pentadecyne (1.02 mL, 3.77 mmol). After stirring at room temperature for 20 min, the reaction mixture was cooled to -30ºC and next treated with a 1.0 M solution of Et_2Zn in hexanes (4.40 mL, 4.40 mmol), followed by aldehyde **10** (873 mg, 3.81 mmol) in anhydrous CH_2Cl_2 (2 mL).[6,11] The resulting mixture was allowed to warm gradually and stirred at rt for 1h. Then, the zirconium salts were precipitated with the addition of 5 mL of deionized water and removed by washing with CH_2Cl_2 in the aqueous phase, filtering with a Celite®.[8,12] The solution was dried with $MgSO_4$ and filtered. The solvent was removed in vacuo and the crude oil was analyzed by ^1H NMR to obtain a 15:1 ratio of both two diastereomers of **11**. It was purified by flash column chromatography on silica gel (hexanes/ethyl acetate from 95:5 to 80:20) to afford 1.39 g (78% yield) of the *tert*-butyl (*S*)-4-[(*S*,*E*)-1-hydroxyhexadec-2-en-1-yl]-2,2-dimethyloxazolidine-3-carboxylate (**11**).

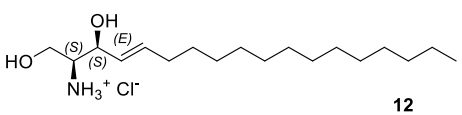


11

White solid; **R_f:** 0.40 (Hexanes:Ethyl acetate 8:2); **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.74 (dt, HOCHCH=C<u>H</u>, *J* = 14.2, 6.6 Hz, 1H), 5.41 (m, HOCHC<u>H</u>=CH, 1H), 4.14 (m, HOC<u>H</u>CH, 1H), 3.98-3.80 (m, $CH_3COC\underline{H_2}$, 2H), 2.04 (q, CH=CHC<u>H_2</u>, *J* = 6.6 Hz, 2H), 1.58 (s, C<u>H_3</u>C(CH_3)N, 3H), 1.50 (s, ($C\underline{H_3}$)$_3$COC(O), 9H), 1.49 (s, $CH_3C(C\underline{H_3})N$, 3H), 1.25 (brs, $CH_2C\underline{H_2}CH_2$, 22H), 0.88 (t, $CH_2C\underline{H_3}$, *J* = 6.6 Hz, 3H).

6.2.2. Synthesis of (2*S*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-ammonium chloride (12)

To a 881 mg (2.07 mmol) of pure **11** in 100 mL of MeOH was added neat acetyl chloride (971 µL, 10 mmol) at room temperature. The reaction mixture was stirred for 72 hours. The solution was dried with $MgSO_4$ and filtered. The solvent was removed in vacuo and the solid was purified by flash column chromatography on silica gel (dichloromethane/methanol from 99:1 to 88:12) to afford 498 mg (72% yield) of (2*S*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-amonium chloride (**12**).



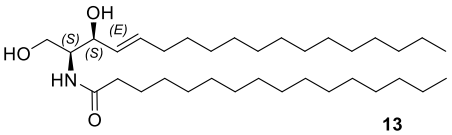
12

White solid; **R_f:** 0.1 (CH_2Cl_2:MeOH, 9:1); **mp:** 68-70 ºC; **[α]$_D$:** -10.76 (*c* 0.97, MeOH); **IR:** 3295, 2954, 2917, 2847, 1461, 1461, 970, 724 cm^{-1}. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.83 (dt, HOCHCH=C<u>H</u>, *J* = 15.7, 7.0 Hz, 1H), 5.44 (dd, HOCHC<u>H</u>=CH, *J* = 15.7, 7.0 Hz, 1H), 4.30 (t, HOC<u>H</u>, *J* = 7.0 Hz, 1H), 3.87 (d, HOC<u>H_2</u>, *J* = 11.4 Hz, 2H), 3.45 (m, $H_3NC\underline{H}$, 1H), 2.02 (q, CHC<u>H_2</u>CH_2, *J* = 7.0 Hz, 2H), 1.26 (brs, $CH_2C\underline{H_2}CH_2$, 22H), 0.88 (t, $CH_2C\underline{H_3}$, *J* = 7.0 Hz, 3H). **^{13}C NMR** (100.6 MHz, $CDCl_3$) δ 137.38, 127.19, 70.52, 59.34, 58.31, 32.64, 32.10, 29.94, 29.87, 29.77, 29.70, 29.56, 29.26, 22.86, 14.27.

6.2.3. Synthesis of *N*-[(2*S*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl]palmitamide (13)

EDC (0.39 mL, 2.19 mmol) was added portion wise to a mixture of 405 mg of palmitic acid (1.58 mmol) and HOBt (295 mg, 2.19 mmol) in 32 mL of CH_2Cl_2 under argon atmosphere. After stirring for 5 min at room temperature, this solution was added dropwise over a CH_2Cl_2 solution (32 mL) of the sphingoid **12** (1.50 mmol) containing NEt_3 (0.45 mL, 3.23 mmol) under argon atmosphere. TLC control showed the evolution of the reaction and, after 20 minutes, the

solution was washed with 3x10 mL of water, dried with $MgSO_4$ and filtered.[13] The solvent was removed in vacuo and the solid was purified by flash column chromatography on silica gel (dichloromethane/methanol from 99:1 to 97:3). The esters formed during the reaction were saponified with a solution of K_2CO_3 (200 mg) in MeOH (40 mL). After 3 hours, the solvent was removed in vacuo and the solid was purified by flash column chromatography on silica gel (dichloromethane/methanol from 99:1 to 97:3), to afford 351 mg (45% yield) of the corresponding amide **13**.



13

Pink solid; **R_f:** 0.5 (CH_2Cl_2:MeOH, 95:5); **mp:** 97-100ºC; **$[\alpha]_D$:** -14.8 (*c* 0.3, 50:50 MeOH:$CHCl_3$); **IR:** 3272, 2955, 2915, 2849, 1645, 1469, 1086, 1049, 964, 719 cm^{-1}; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.11 (d, HNCO, *J* = 7.0 Hz, 1H), 5.75 (dt, HOCHCH=CH, *J* = 14.9, 7.0 Hz, 1H), 5.47 (dd, HOCHCH=CH *J* = 14.9, 7.0 Hz, 1H), 4.39 (dd, HOCH, *J* = 3.5, 7.0 Hz, 1H), 3.92 (m, H_3NCH, 1H), 3.82 (m, $HOCH_2$, 2H), 2.22 (t, $HNCOCH_2$, *J* = 7.0 Hz, 2H), 2.03 (q, $CH=CHCH_2$, *J* = 7.0 Hz, 2H), 1.63 (q, $HNC(O)CH_2CH_2$, *J* = 7.0 Hz, 2H), 1.25 (m, $CH_2CH_2CH_2$, 46H), 0.88 (t, CH_2CH_3, *J* = 7.0 Hz, 6H).

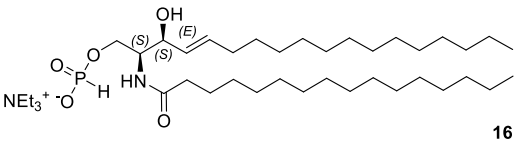
6.2.4. Towards the synthesis of *N*-[(4*S*,5*S*)-2-hydroxy-2-oxido-4-((*E*)-pentadec-1-en-1-yl)-1,3,2-dioxaphosphinan-5-yl]palmitamide (3)

6.2.4.1. Synthesis via phosphorus (III)

6.2.4.1.1. Hydrogenphosphonate addition to the hydroxyl group: synthesis of (2S,3S,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl phosphonate [14,15] (**16**)

Imidazole (0.067 g, 0.98 mmol) was dissolved in 4 mL of CH_2Cl_2 and the solution was kept on an ice-water bath (0-5ºC). Phosphorus trichloride (28 µL, 0.32 mmol) was added under vigorous stirring, followed by 156 µL (1.12 mmol) of triethylamine. The mixture was stirred for 15 min and then a solution of ceramide **13** (50 mg, 0.093 mmol) in 4.5 mL of CH_2Cl_2 was added. After the addition was completed, the cooling bath was removed, and the mixture was kept at room temperature with continuous stirring for 1h. The reaction mixture was quenched by 2 mL of a 0.4 M solution of triethylamine in water at pH 7.5 (using a hydrogencarbonate buffer), forming the triethylammonium hydrogenphosphate salt **16**. The aqueous salt was washed with 3

x 20 mL of CH_2Cl_2.[14] The solvent was removed in vacuo and the solid was purified by flash column chromatography on silica gel (dichloromethane/methanol from 95:5 to 85:15) to afford 73 mg (80**%** yield) of the phosphonate **16**.



16

White solid. **R_f:** 0.1 (CH_2Cl_2:MeOH, 85:15); **^{1}H NMR:** (400 MHz, CD_3OH) δ 6.77 (d, HP(O)ORO$^-$, *J* = 633.0 Hz, 1H), 5.78 (m, HN, 1H), 5.72 (m, HOCHCH=CH, 1H), 5.47 (m, HOCHCH=CH, 1H), 4.33 (m, HOCH, 1H), 4.07 (m, CH(O)NHCH, 1H), 3.97 (m, OPOCH_2, 2H), 3.53 (m, OPOCH_2, 2H), 3.22 (q, NCH_2CH$_3$ (NEt_3), *J* = 7.3 Hz, 6H), 2.23 (m, HNC(O)CH_2, 2H), 2.05 (m, CH=CHCH_2, 2H), 1.63 (m, HNC(O)CH_2CH_2, 2H), 1.35-1.32 (t, NCH_2CH_3 (NEt_3), 9H), 1.30 (brs, CH_2CH_2CH_2, 46H), 0.91 (t, CH_2CH_3, *J* = 7.0 Hz, 6H); **^{31}P RMN:** (122 MHz, CD_3OH) δ 2.20 ppm.

*6.2.4.1.2. Cyclization of **16** with pivaloyl chloride: Towards the synthesis of N-[(4S,5S)-2-hydroxy-2-oxido-4-((E)-pentadec-1-en-1-yl)-1,3,2-dioxaphosphinan-5-yl]palmitamide* (**3**)[14,15]

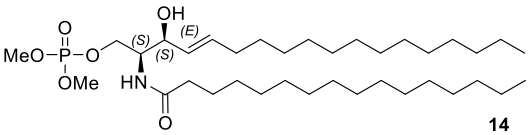
To 50 mg of **16** in 3 mL of dry acetonitrile was added at rt 1 mL (12.4 mmol) of dry, freshly distilled pyridine under argon atmosphere. Next, 0.39 µL (0.32 mmol) of pivaloyl chloride were added dropwise. The solution was stirred by 1 min and 3 mL of dry acetonitrile were added with a 5.2 M solution of *tert*-butyl hydroperoxide in CH_2Cl_2 to cycle the compound. The reaction mixture was dried with $MgSO_4$ and washed 3 times with a triethylamine:water solution 0.7 M. The solvent was removed in vacuo and the organic phase was purified by flash column chromatography on silica gel (dichloromethane/methanol from 95:5 to 87:13). Traces (8 mg) of an unknown compound were isolated

R_f: 0.1 (85:15 CH_2Cl_2:MeOH); **^{31}P RMN:** (122 MHz, CD_3OH) δ 4.86, 3.43 ppm.

6.2.4.2. Synthesis via phosphorus(V)

6.2.4.2.1: Synthesis of (2S,3S,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl dimethyl phosphate (**14**)

Amide **13** (143 mg, 0.27 mmol) in CH_2Cl_2 (6 mL) was cooled to 0-5ºC and *N*-methylimidazole (180 µL, 2.25 mmol), and then dimethyl chlorophosphate (115 µL, 1.07 mmol) were added at same temperature. After the reaction mixture was stirred at rt for 10 min, the reaction mixture was quenched by 10 mL of water.[15,16] The organic layer was removed in vacuo and the solid was purified by flash column chromatography on silica gel (dichloromethane/methanol from 95:5 to 20:70) to afford 83 mg (61% yield) of the phosphate **14**.

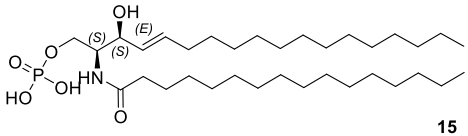


14

White wax. **Rf: 0.50** (CH_2Cl_2:MeOH, 95:5); **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.12 (d, CONH, *J* = 7.0 Hz, 1H), 5.75 (dt, HOCHCH=CH, *J* = 15.0, 7.0 Hz, 1H), 5.42 (dd, HOCHCH=CH, *J* = 15.0, 7.0 Hz, 1H), 4.37 (m, HOCHCH, 1H), 4.12 – 4.07 (m, OP(O)OCH$_2$, OP(O)OCH$_2$CH, 3H), 3.78 (d, CH$_3$OP(CH$_3$), *J* = 7.7 Hz, 3H), 3.76 (d, CH$_3$OP(CH$_3$), *J* = 7.7 Hz, 3H), 2.17 (t, HNC(O)CH$_2$, *J* = 7.0 Hz, 2H), 2.01 (q, CH=CHCH$_2$, *J* = 7.0 Hz, 2H), 1.59 (m, HNCOCH$_2$CH$_2$, 2H), 1.25 (brs, CH$_2$CH$_2$CH$_2$, 46H), 0.87 (t, CH$_2$CH$_3$, *J* = 7.0 Hz, 6H); **^{13}C NMR** (100.6 MHz, $CDCl_3$) δ 133.93, 128.52, 77.48, 76.84, 70.00, 65.98, 54.76, 36.86, 32.47, 32.06, 29.84, 29.30, 25.90, 14.25.

6.2.4.2.2. Synthesis of (2S,3S,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl dihydrogen phosphate[15,16] (**15**)

A solution of phosphate **14** (80 mg, 0.125 mmol) in acetonitrile (4.3 mL) was cooled to 0-5ºC. Iodotrimethylsilane (170 µL, 1.20 mmol) was added to the reaction mixture and stirred at room temperature for 2 h.[16] Next, 1 mL of saturated sodium thiosulfate solution was added. The reaction mixture was stirred for 72 h and treated with 0.1 M aqueous HCl solution until neutralization (pH paper). The solvent was removed in vacuo and phosphate **15** was purified by flash column chromatography on silica gel (dichloromethane/methanol from 95:5 to 80:15). Only traces of compound **15** (8 mg, 10% yield) were isolated.



15

White solid. **R_f:** 0.1 (CH_2Cl_2:MeOH, 85:15); **^{1}H NMR:** (400 MHz, CD_3Cl) δ 6.03 – 5.89 (m, CONH, 1H), 5.89 – 5.69 (m, HOCHCH=CH, 1H), 5.52 – 5.38 (m, HOCHCH=CH, 1H), 4.32 – 4.25 (m, HOCHCH=CH, 1H), 4.19 – 4.02 (m, HOP(O)OCH_2, 2H), 3.98 – 3.89 (m, HOP(O)OCH_2CH, 1H), 3.70 (d, HOP(O)(OH)OCH_2, *J* = 11.0 Hz, 1H), 3.61 (d, HOP(O)(OH)OCH_2, *J* =11.0 Hz, 1H), 2.40 (td, NHC(O)CH_2, *J* = 7.0, 2.6 Hz, 2H), 2.23 (m, NHC(O)CH_2, 2H), 2.11 (q, CH=CHCH_2, *J* = 7.0 Hz, 2H), 2.04 (q, CH=CHCH_2, *J* = 7.0 Hz, 2H), 1.61 (m, NHC(O)CH_2CH_2, 2H), 1.29 (brs, CH_2C$H_2$$CH_2$, 46H), 0.90 (t, CH_2CH_3, *J* = 7.0 Hz, 6H).

7. CONCLUSIONS

A synthetic approach to the (2*S*,3*S*,*E*)-ceramide-1,3-phosphodiester (**3**) have been developed. However, the yields obtained for the synthetic diastereomer **3** of the natural (*2S,3R*,*E*)-ceramide-1,3-phosphodiester (**9**) did not meet the foreseen expectations. Therefore, further studies regarding the cyclization reaction to a cyclic phosphate must be reviewed.

• The *S* configuration of the alcohol and the *E* configuration of the double bond on Garner's aldehyde (**10**) was achieved by a *syn*-selective addition of 1-pentadecyne by a described method, allowing a quantitative yield (78%) of *tert*-butyl (*S*)-4-[(*S*,*E*)-1-hydroxyhexadec-2-en-1-yl]-2,2-dimethyloxazolidine-3-carboxylate (**11**).

• The *N*-Boc and the *N*,*O*-isopropylidene deprotection of the adduct **11** was obtained in 72% yield, allowing the subsequent *N*-acylation of the sphingosine **12** with palmitic acid. The (2*S*,3*S*,*E*)-ceramide (**13**) was obtained in 45% yield.

• Ceramide **13** was the starting point to test both two phosphorus(III) and phosphorus(V) phosphorylation pathways.

• On the first step of phosphorus(III) synthetic pathway it was obtained the hydrogenphosphonate **16** in 80% yield. Unfortunately, the next synthetic step (which would allow the desired compound **3**) was affected by the oxidation and cyclization of the molecule, not providing the desired outcome.

• The first step of phosphorus(V) synthetic pathway obtained the C1 dimethylphosphate adduct **14**, in 61% yield. Unfortunately, the deprotection of the phosphonate methyl groups regarded a very low yield (10%), thus becoming a non-productive reaction step and preventing the continuity on this route.

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9. ACRONYMS

B

Boc: *tert*-butyloxycarbonyl

br: Broad

C

^{13}**C** NMR: carbon nuclear magnetic resonance

COSY: ^{1}H Correlated spectroscopy

D

d: doublet

dt: double triplet

E

EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

H

^{1}**H** NMR: proton nuclear magnetic resonance

HOBt: 1-hydroxybenzotriazole

HSQC: Heteronuclear single quantum coherence spectrometry

M

m: multiplet

M: molar

Mp: melting point

MeOH: methanol

Me: methyl

N

NEt_3: triethylamine

P

^{31}**P** NMR: phosphor nuclear magnetic resonance

P(III): Phosphorus(III)

P(V): Phosphorus(V)

R

rt: room temperature

Rf: Retardation factor

S

s: singlet

T

t: triplet

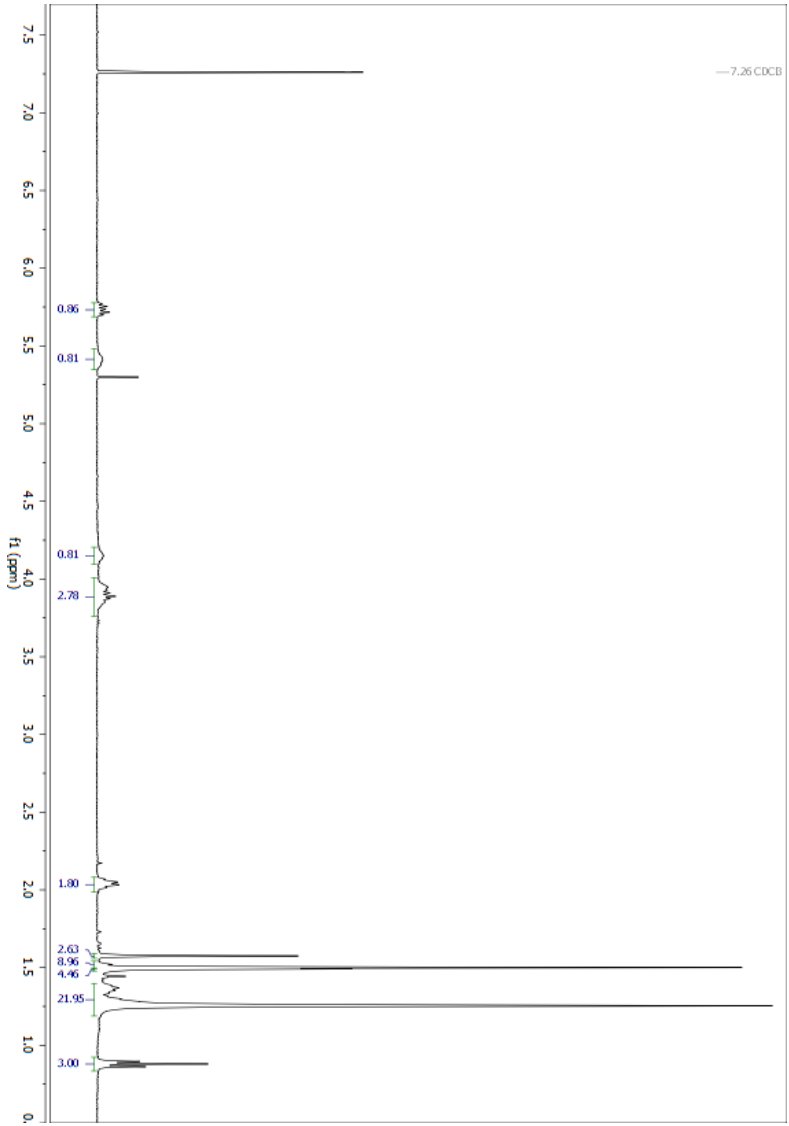
THF: Tetrahydrofuran

TMSI: trimethylsilyliodide

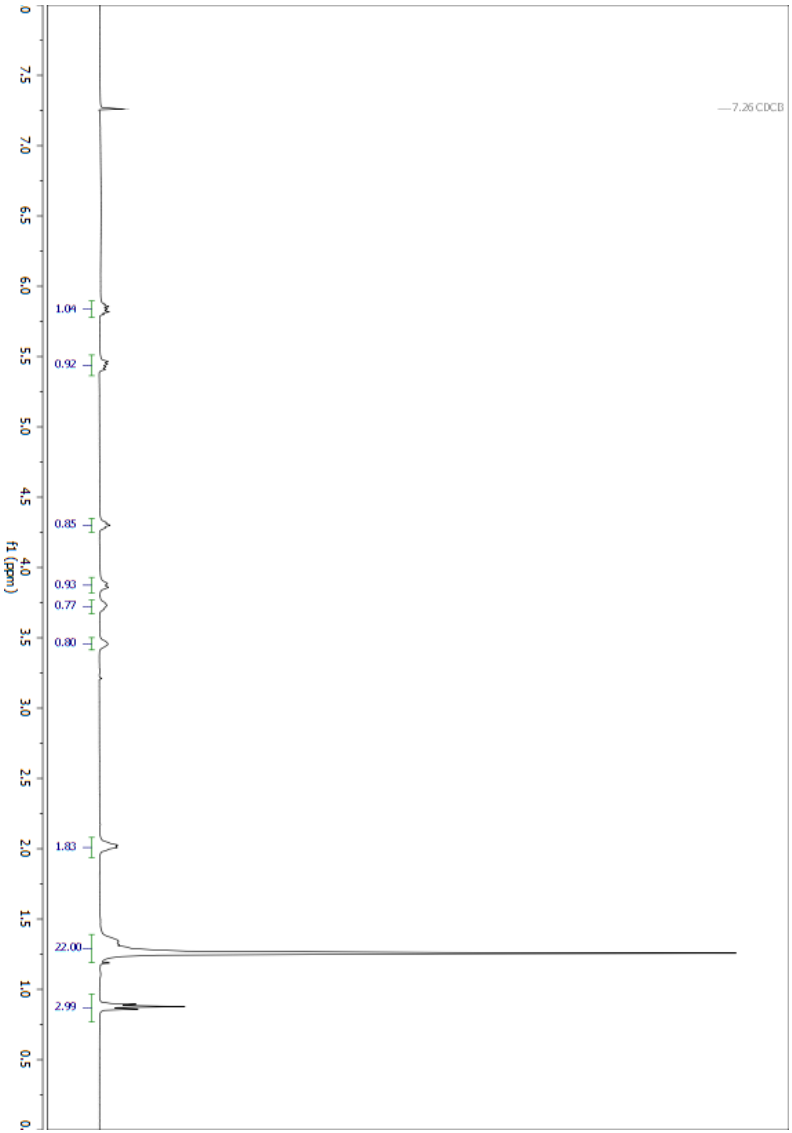
APPENDICES

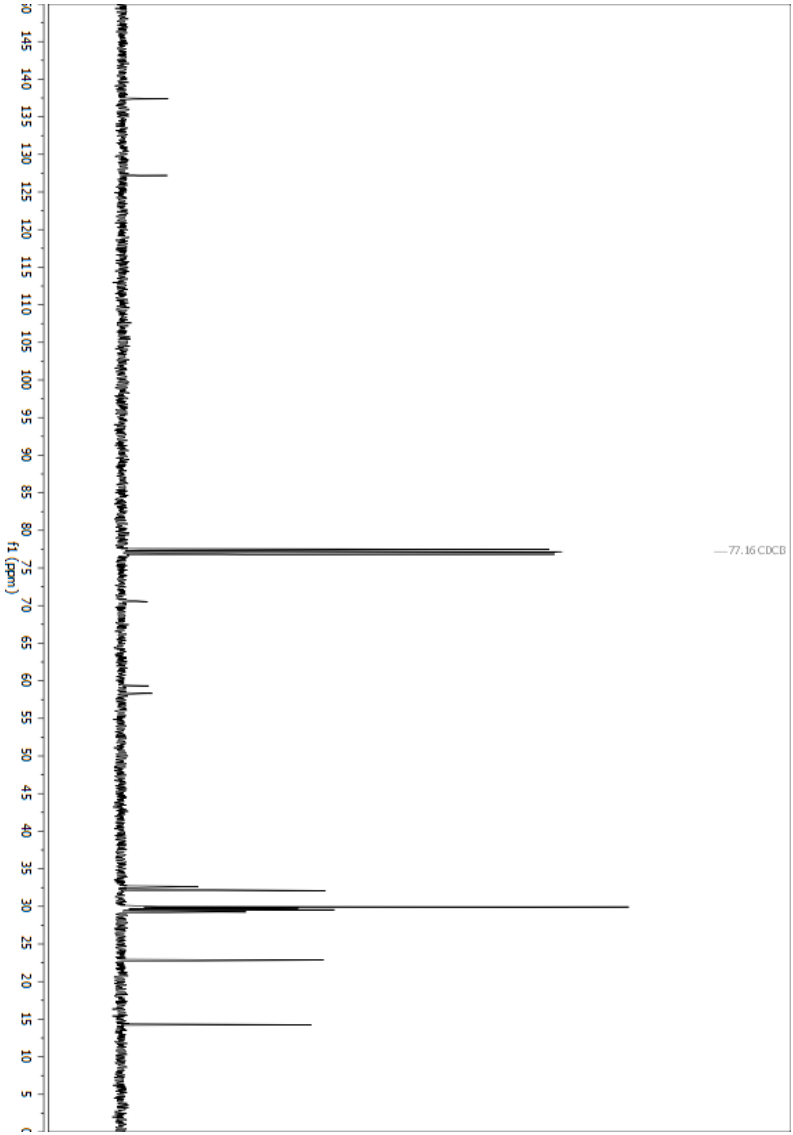
CHARACTERIZATION OF MOLECULES

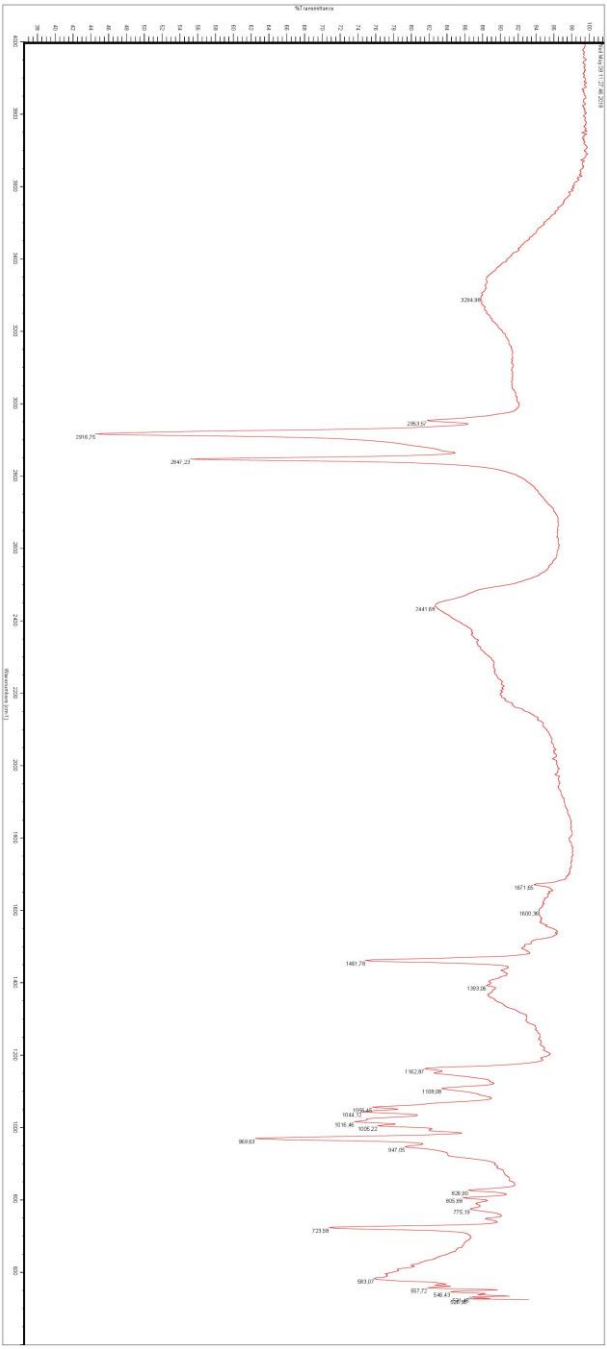
a) *tert-butyl (S)-4-((S,E)-1-hydroxyhexadec-2-en-1-yl)-2,2-dimethyloxazolidine-3-carboxylate (**11**)*



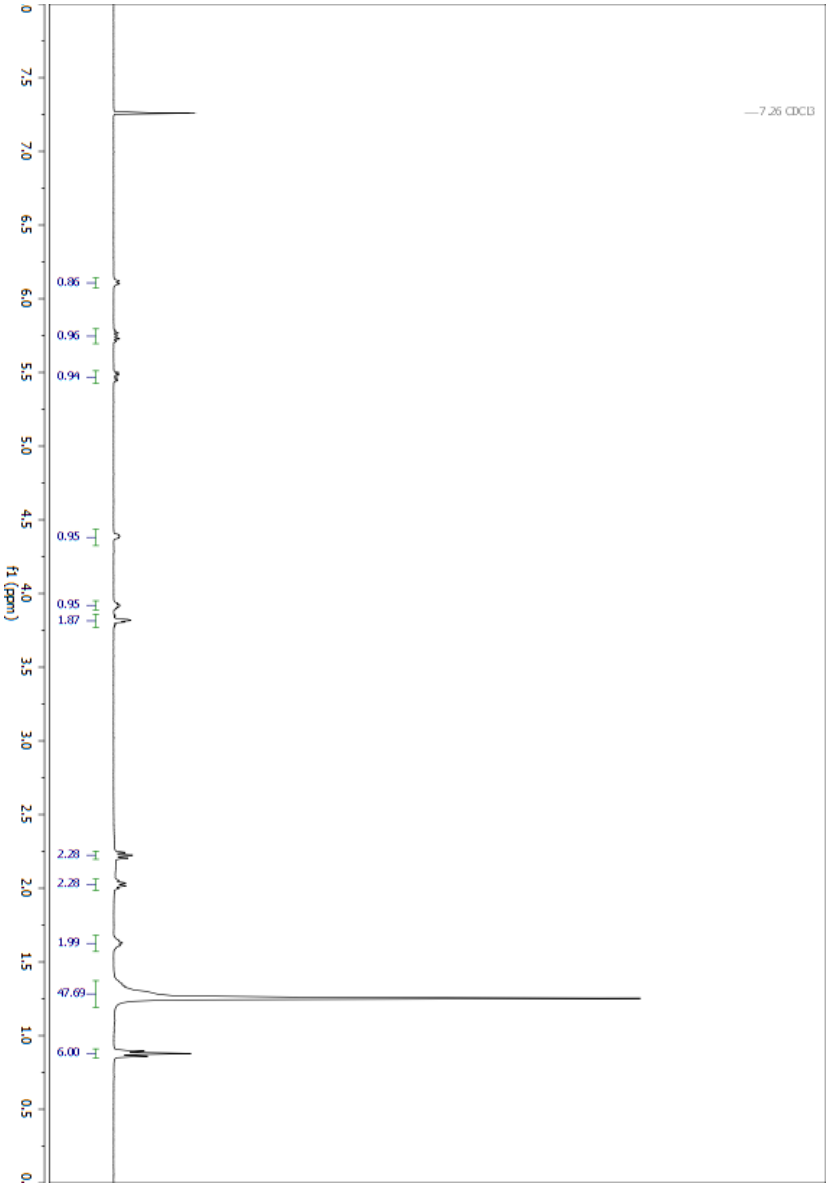
*b) (2S,3S,E)-1,3-dihydroxyoctadec-4-en-2-aminium chloride (**12**)*

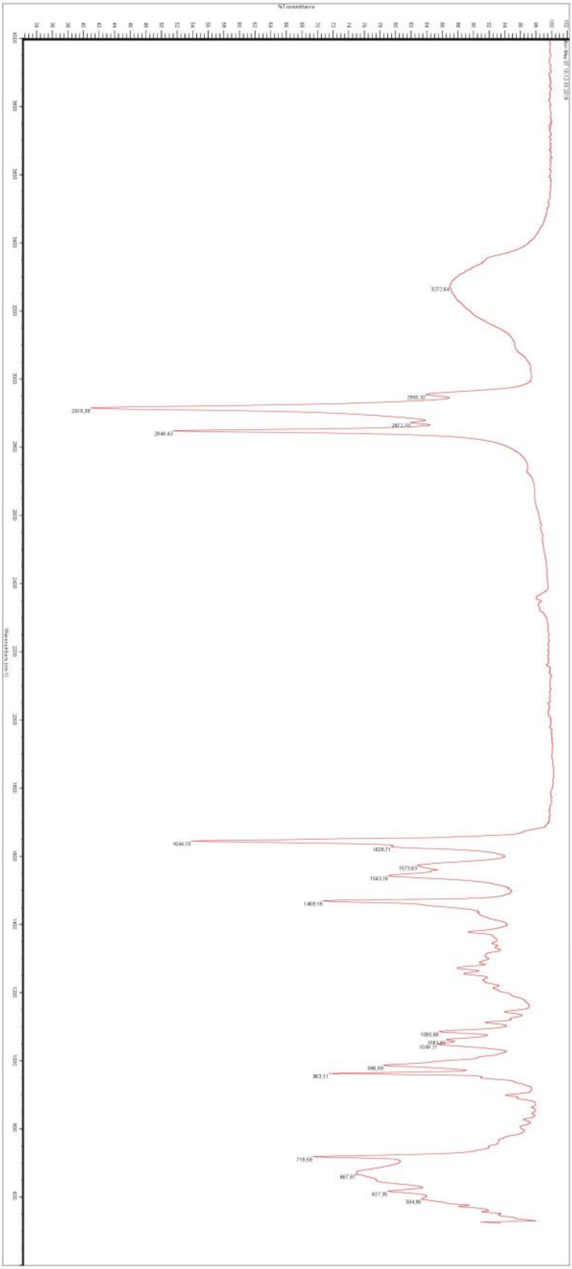



77.16 CDCl3
f1 (ppm)

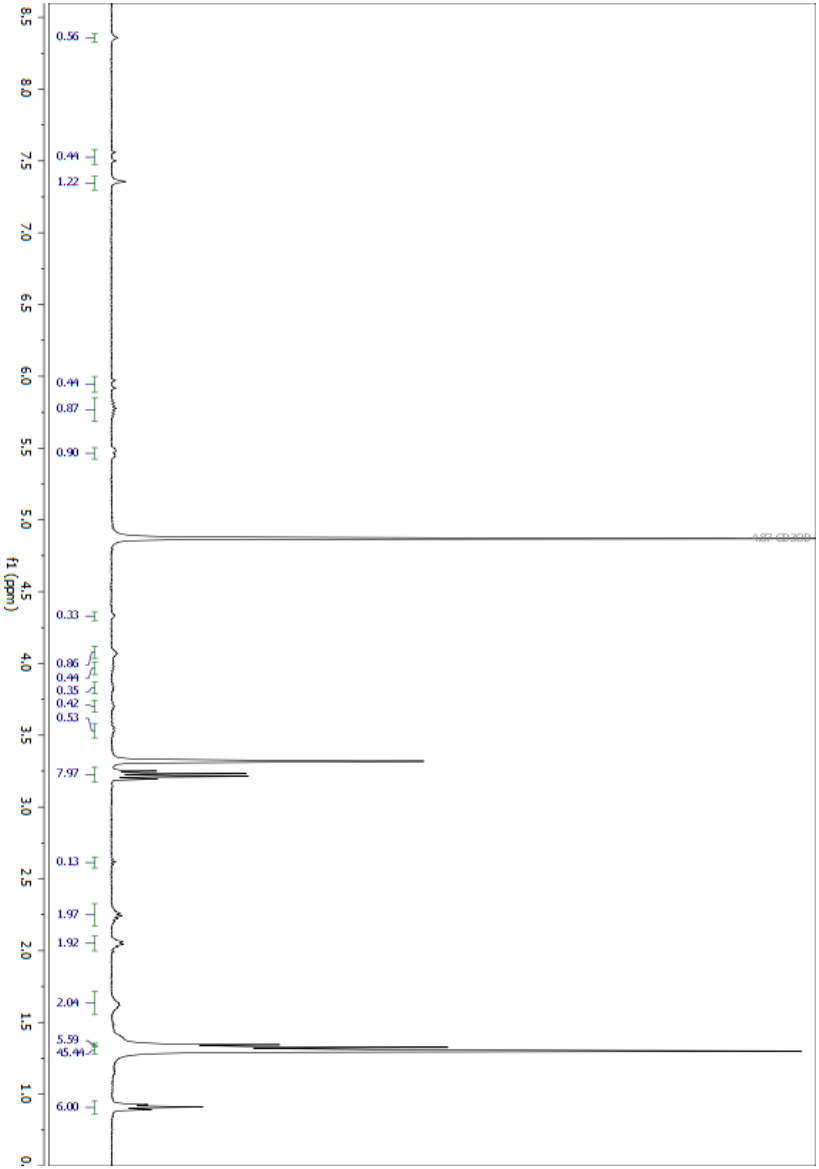

%Transmittance
3294.94
2916.75
2853.32
2847.23
2441.65
1671.65
1600.36
1481.78
1393.26
1162.87
1108.08
1044.12
1016.46
1005.22
969.63
947.05
836.69
805.89
775.19
723.58
583.07
557.72
540.43

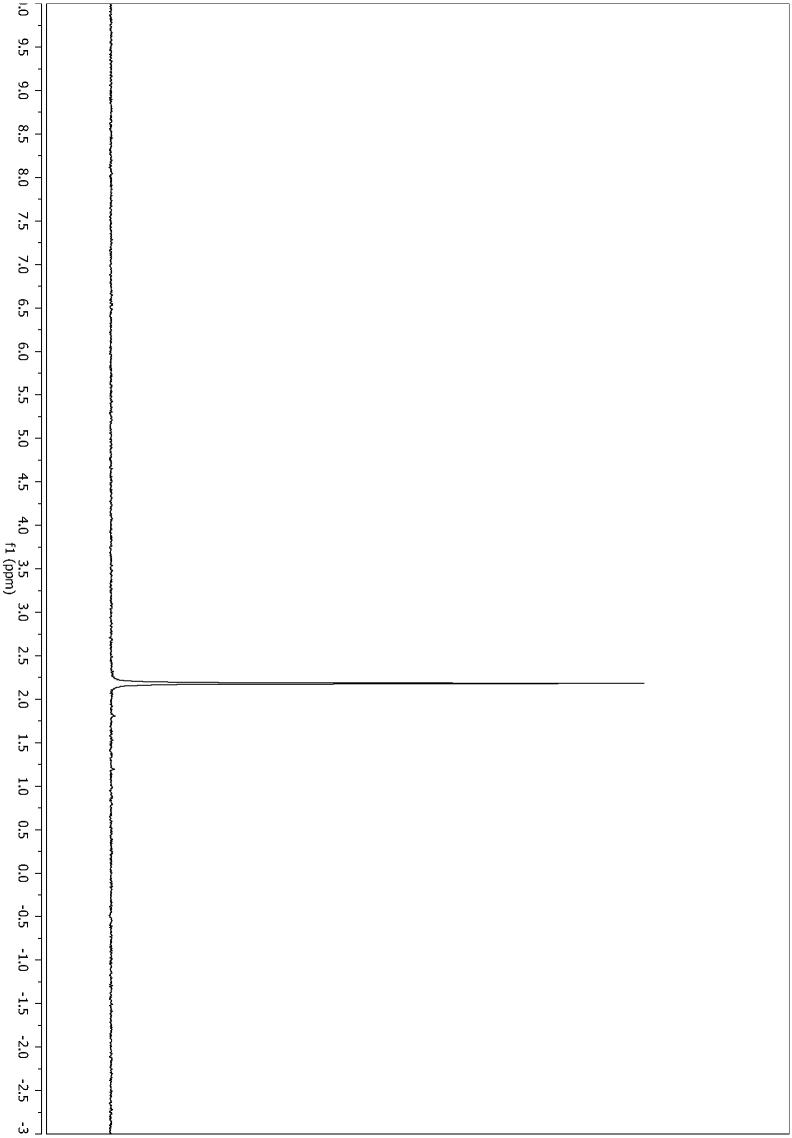
*c) N-((2S,3S,E)-1,3-dihydroxyoctadec-4-en-2-yl)palmitamide (**13**)*



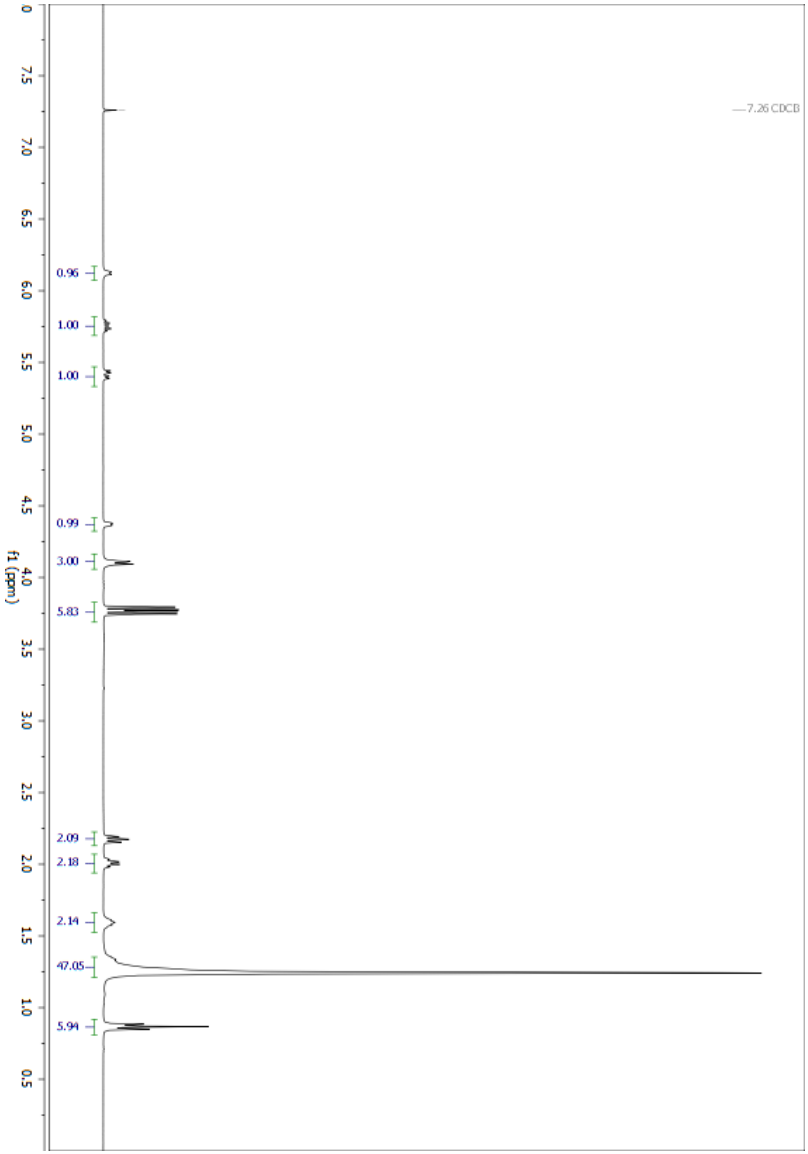

%Transmittance
Wavenumbers (cm-1)
3272.64
2955.32
2915.38
2872.70
2848.43
1644.70
1628.71
1573.63
1543.16
1469.19
1095.68
1049.11
986.99
963.11
718.58
667.97
617.35
594.80

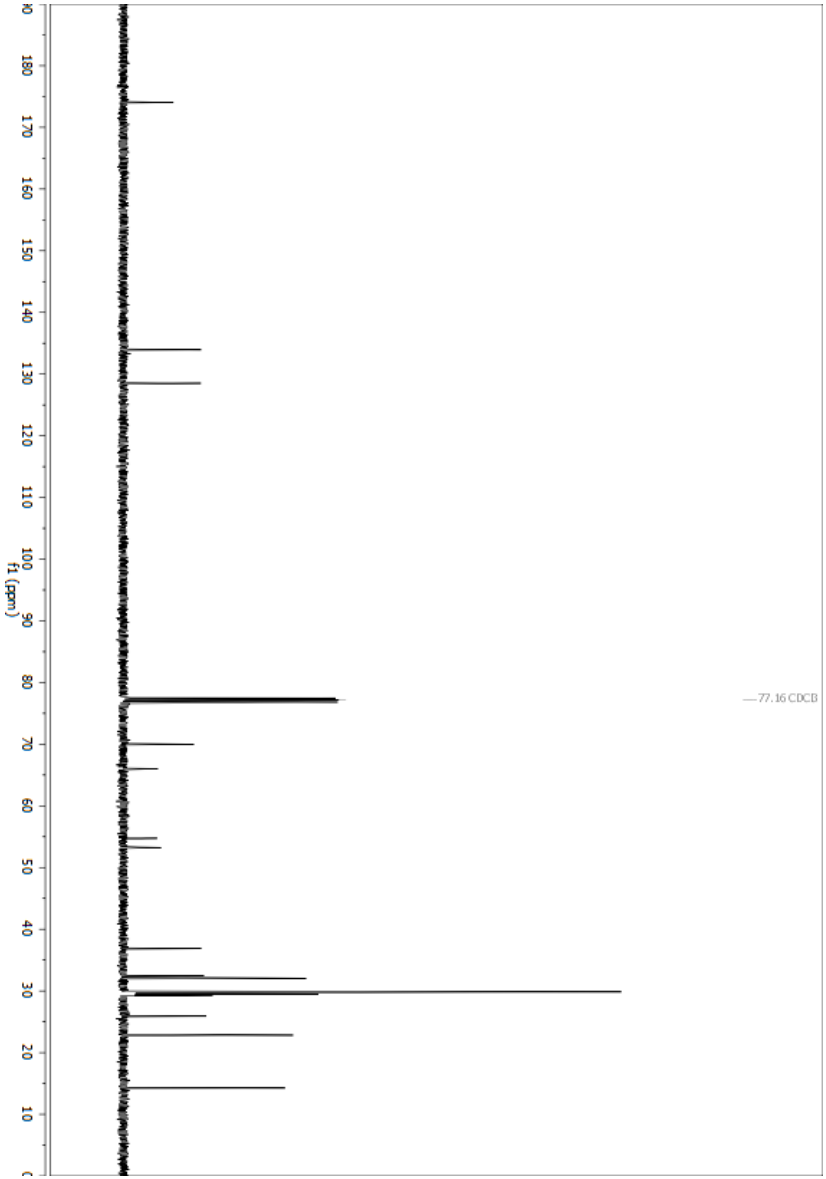
d) *(2S,3S,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl phosphonate* **(16)***:*




10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3
f1 (ppm)

*e) (2S,3S,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl dimethyl phosphate (**14**)*




f1 (ppm)
77.16 CDCl3

*f) (2S,3S,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl dihydrogen phosphate (**15**):*