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Doctoral Dissertation

**Design and evaluation of novel biomimetic molecules with potential
pharmaceutical applications**
(医薬品応用を指向した新規バイオミメティック分子の設計とその
評価)

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ABSTRACT

Abstract

Stereochemistry dramatically impacts the biological activity of various drugs. In the present report, we investigated the role of stereochemistry of ceramide and its analogues in inducing the release of exosomes, a type of extracellular vesicles, from neuronal cells, with a potential benefit in improving the clearance of Amyloid- β ($A\beta$), an agent of Alzheimer's disease. A library of diverse ceramide analogues was synthesized with the purpose of varying stereochemistry (DE, LT, LE, DT) and hydrophobic tail length (6, 16, 18, 24). The exosome levels were quantified using TIM4-based exosome ELISA after the concentration of the conditioned medium by centrifugal filter devices. The results revealed a pivotal role of stereochemistry in determining the biological activity of ceramide and its analogs, with the superiority of those based on DE and DT

stereochemistry with C16 and C18 tails, which demonstrated a significantly higher exosome release, without a significant change in the particle size of the released exosomes. In transwell experiments with A β -expressed neuronal cells and microglial cells, DE- and DT-ceramides with C16 and C18 tails significantly decreased extracellular A β levels. The results reported here are promising in the design of non-classic therapies for the treatment of Alzheimer's disease and other neurodegenerative disorders.

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1. INTRODUCTION

1. Introduction

1.1) Alzheimer's Disease

Alzheimer's Disease (AD), the most common form of dementia, is clinically characterized by progressive impairment of cognition and memory loss, affecting mainly older people. About 50 million people suffer from AD worldwide, and the number is expected to triple by 2050 if no preventive measures are taken.¹ Pathologically, AD is characterized by the accumulation of Amyloid-beta ($A\beta$) in brains. $A\beta$ accumulation is caused by impaired clearance of $A\beta$ in the sporadic form of AD and from increased production due to genetic mutations of amyloid precursor protein (APP) or $A\beta$ processing enzymes in the less-common familial form of AD.² Hence, decreasing $A\beta$ formation or inducing their clearance can be considered one of the main AD treatment strategies.^{3,4} The FDA's accelerated approval of the anti- $A\beta$ antibody aducanumab and lecanemab for early AD⁵ has swung the additional expectation toward the development of novel drugs and functional food materials against $A\beta$ pathology.⁵

1.2) Exosomes

Exosomes are nano-scaled extracellular vesicles that carry a variety of cargos including proteins, nucleic acids, and bioactive lipids, and are known for their important role in cell-to-cell communication.⁶ A β is one of the cargos carried by exosomes derived from neuronal cells of AD patients.^{7,8} Several reports have linked the increase of exosome release and the decrease of A β accumulation in neurons.^{9–12} Neuronal exosomes were also reported to transport A β to microglia and facilitate its degradation.¹⁰

1.3) Ceramide

Ceramides (Cer), a group of bioactive sphingolipids, have a crucial role in exosome release.¹³ Oral administration of plant-type Cer, mainly composed of sphingadienine, promote neuronal exosome release and alleviate A β accumulation in a human amyloid precursor protein (APP) transgenic mouse.¹⁴ Cer induce the exosome release in a way that is independent of endosomal sorting complex (ESCRT) machinery.¹³ Binding of animal-type and plant-type Cer to lysosome-associated protein transmembrane 4B (LAPTM4B) through its sphingolipid-binding motif was reported to be one of the mechanisms for Cer-induced exosome release.^{14–16} As the mechanism of Cer-induced exosome

release is still not completely understood, it is necessary to study the relation between Cer structure and exosome release activity.

1.4) Stereochemistry

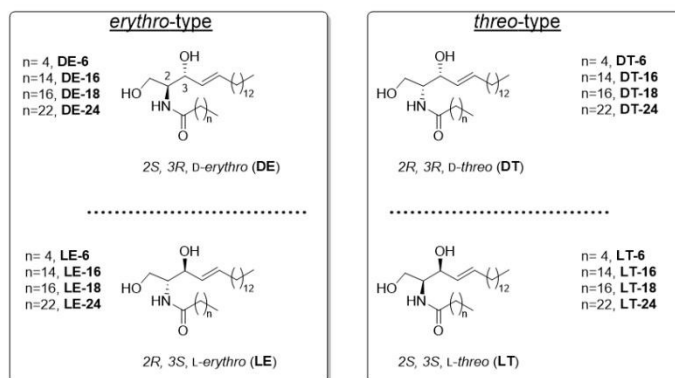
Stereochemistry has been involved in the process of drug design and development as a major detrimental factor for drug pharmacodynamic and pharmacokinetic behavior.¹⁷ Studies comparing the drug action of the different stereoisomers have been well-established for many drugs. Ephedrine versus pseudoephedrine, *R*-ibuprofen versus *S*-ibuprofen, and *S*-warfarin versus *S*-warfarin are famous examples.^{18–20} In harmony with these studies, Cer stereoisomers have been reported to show variable responses with biological targets, like protein phosphatase²¹ and sphingomyelin synthase.²² Hence, in this study, the first report on the effect of the stereochemistry of Cer on exosome release from neuronal cells is clarified.

2. SCOPE OF WORK

2. Scope of work

Sphingosine has two stereocenters on carbons C2 and C3. So, there are an *erythro* enantiomeric pair (D-*erythro* sphingosine, DE and L-*erythro* sphingosine LE) and a *threo* enantiomeric pair (D-*threo* sphingosine, DT and L-*threo* sphingosine LT).²³ So, this study aims at synthesis of the four stereoisomers of ceramide with four acid tails with different length (6, 16, 18, 24), **Figure 1**. The synthesized compounds would be characterized by ¹H-NMR and mass spectroscopy. Measurement of % exosome release would be carried out to study the effect of stereochemistry of ceramide on exosome release. Then, some ceramide derivatives with tail modifications will be synthesized and evaluated for exosome release.

Stereochemistry



Tail modifications

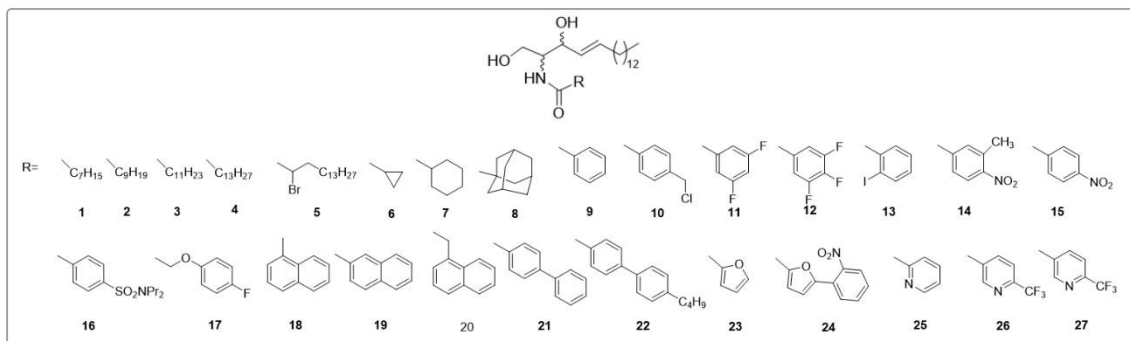


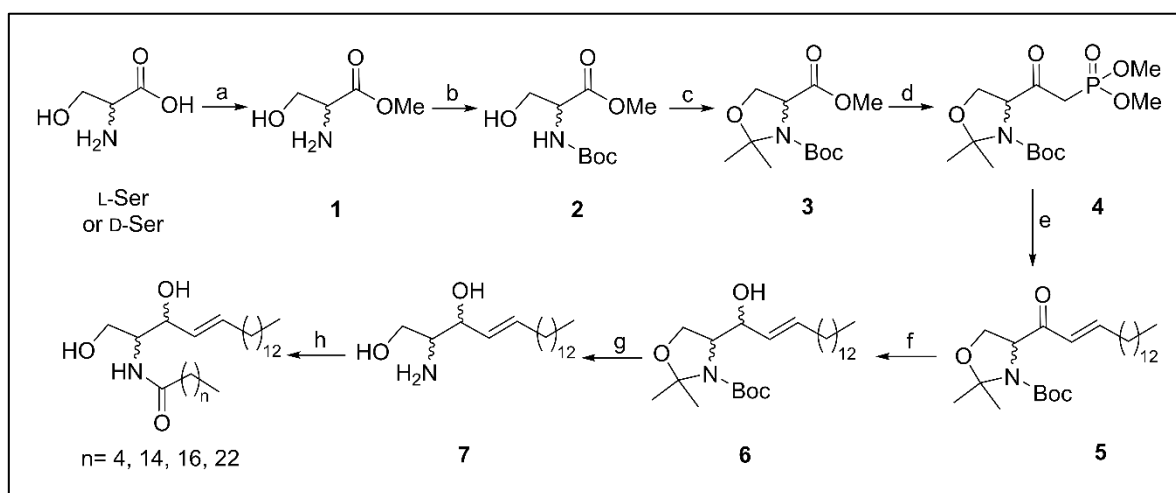
FIGURE 1 Ceramide and its analogues used for this study.

3. RESULTS AND DISCUSSION

3. Results and Discussion

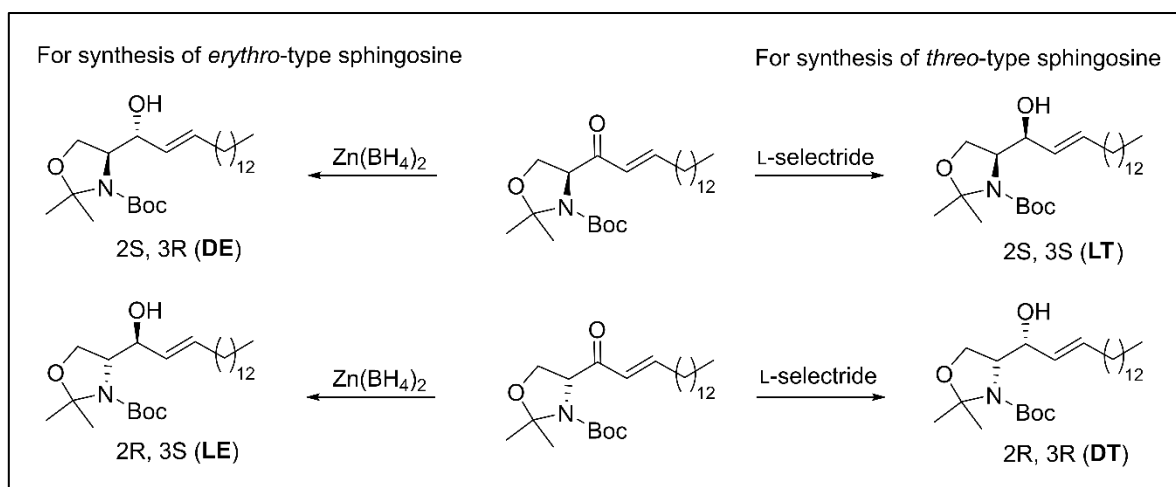
3.1) Stereoselective synthesis of ceramide and its analogues

The four diastereoisomers of sphingosine were synthesized according to the well-established protocol^{22,24–26} with a few variations, **Scheme 1**. The procedure starts with methyl esterification of D- and L-serine, followed by Boc protection of the amino group and acetal protection of the primary hydroxyl and secondary amino group to give a fully protected D- and L-serine **3**. Conversion of the methyl ester group to phosphonate **4** was required for the formation of the trans double bond on C4 through the Horner-Wadsworth-Emmons (HWE) reaction to give a pair of enantiomeric protected β -ketosphingosines **5**.



SCHEME 1 Stereoselective synthesis of ceramide and its analogs; a) $\text{SOCl}_2/\text{MeOH}$, 0°C then rt, 6h; b) Boc_2O , TEA, CH_2Cl_2 , 0°C then rt, overnight (L- 84%, D- 96%); c) $\text{BF}_3\cdot\text{OEt}_2$, DMP, acetone, 0°C then rt, (L- 79%, D- 81%); d) $n\text{-BuLi}$, DMMP, THF, -78°C , 6h, (L- 78%, D- 69%); e) K_2CO_3 , $\text{C}_{13}\text{H}_{27}\text{CHO}$, THF/ H_2O , rt, overnight, (L- 71%, D- 46%); f) $\text{Zn}(\text{BH}_4)_2$, THF, -78°C then 0°C , 3h (L- 91%, D- 90%) or L-Selectride, THF, -78°C then 0°C , 4h, (L- quantitative, D- quantitative); g) 1M HCl , dioxane, 50°C , 3h; g) $\text{CH}_3(\text{CH}_2)_n\text{COOH}$, HBTU, DIPEA, DMF, rt, overnight, 60-80%.

Erythro-type sphingosines were obtained by the stereoselective reduction of the corresponding β -ketosphingosine with zinc borohydride followed by acid hydrolysis. For the synthesis of the *threo*-type sphingosines, L-selectride was used instead of the zinc borohydride, **Scheme 2**. Finally, the desired ceramides were obtained by acylation of the amino group of sphingosines **7** with the appropriate acid. The synthesized compounds were characterized by mass spectroscopy and ^1H -NMR, **Figure 3-18**.



SCHEME 2 Stereoselective reduction of β -ketosphingosines

3.2) Effect of the stereochemistry of ceramide and its analogs on exosome release

SH-SY5Y cells were treated with each Cer species of 10 μ M concentration for 24 h, and exosome levels were measured in the conditioned medium. First, to survey the Cer effects expeditiously, the conditioned medium was concentrated using centrifugal filtration devices. And then, the exosomes in the concentrated medium were measured by the exosome sandwich enzyme-linked immunosorbent assay (ELISA) system with T-cell immunoglobulin and mucin domain-containing protein 4 (TIM4), phosphatidylserine (PS)-binding protein, and antibody against CD63, an exosome marker protein.²⁷ While DE Cer and DT Cer with C16 and C18 tails significantly increased exosome release, all LE Cer and LT Cer did not show any effect, **Figure 2A**. This indicates that exosome release is dependent on the *R* configuration of C3, and to a much lesser extent on the *S* configuration of C2. Furthermore, DE Cer and DT Cer with shorter C6 tails or longer C24 tails did not increase exosome release. Thus, C16 and C18 fatty acid tails are optimal for activity, which comes in agreement with our previous report.¹⁵ The 24h-treatment with ceramide species tested in this study did not show cell toxicity, **Figure 2B**. The measurements of the exosomes

collected by the ultracentrifugation method also showed the effects of DE and DT Cer to induce exosome release, **Figure 2C**. Additionally, consistent with the ELISA results, an analysis with a nanoparticle analyzer also revealed that DE Cer and DT Cer with C16 and C18 tails increased exosome particles, **Figure 2D**. Cer treatment did not alter the size of exosome particles; the diameter remained at 40–160 nm, with a peak at ~100 nm. It has been reported that Cer is endocytosed into cells and interacted with LAPTM4B within lysosomes, leading to exosome production.^{15,16} Future studies are needed to analyze the efficiencies of the Cer species for internalization into the cells or the binding activities on LAPTM4.

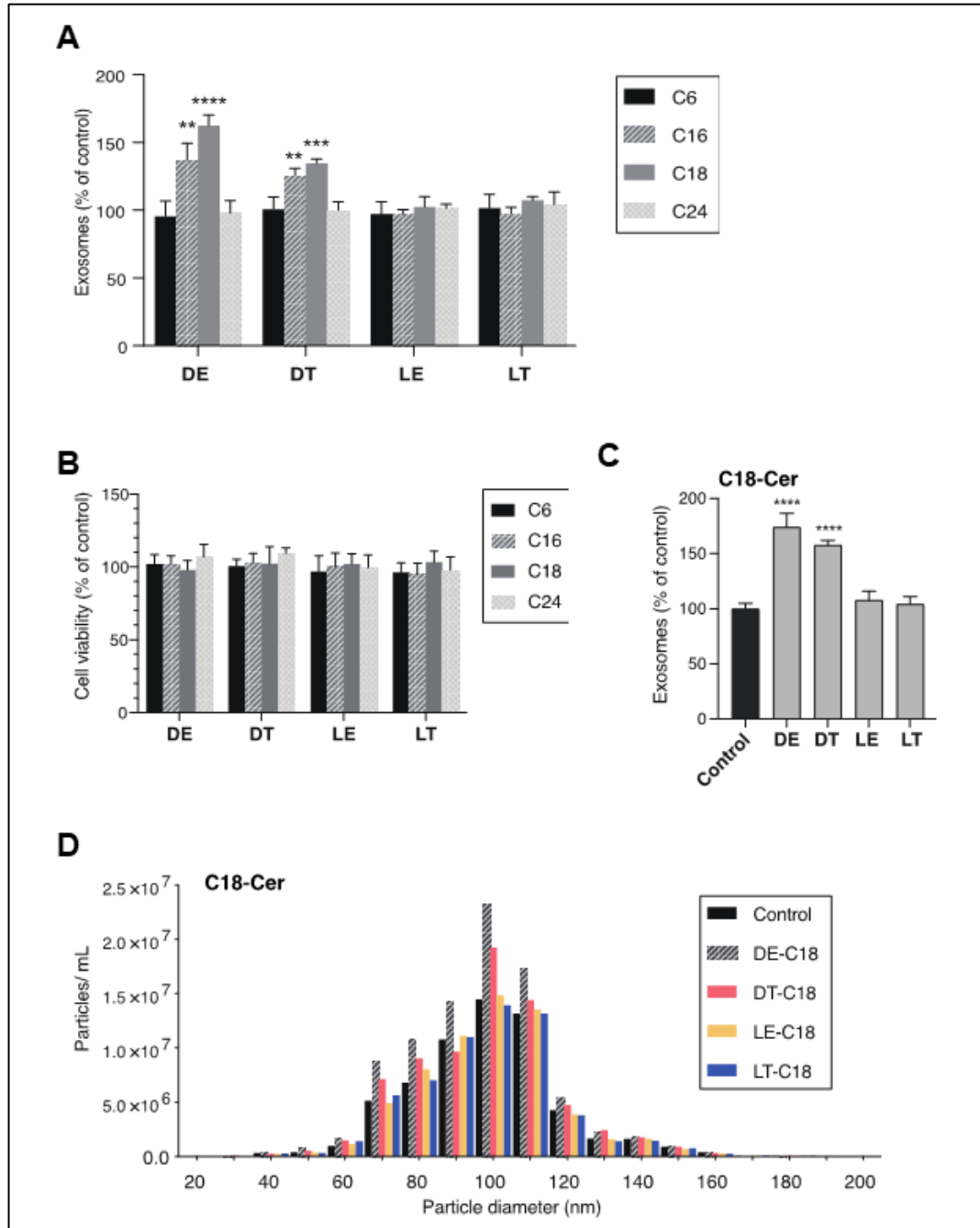


FIGURE 1 (A) Quantification of exosomes released from SH-SY5Y cells treated with ceramide and its analogs for 24 h. After the treatment, the conditioned media were condensed by ultrafiltration, and the levels of exosomes in the concentrated medium were measured using TIM4-Capture Exosome ELISA. Results are normalized against controls and represented as the mean $\pm$ SD ($n=3$; ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$, vs. control by Tukey's multiple comparison test). (B) Cell viabilities of SH-SY5Y cells were measured 24h after the treatment with 10 μ M ceramides. Results were normalized against controls and were represented as the mean $\pm$ SD ($n=4$). (C) The exosomes collected by the ultracentrifugation method were quantified. The cells were treated with the indicated C18 Cer (Cer, 10 μ M) for 24h, the exosomes in the medium were collected and measured by TIM4-Capture Exosome ELISA. ($n=3$; **** $p < 0.0001$ vs. control by one-way ANOVA). (D) Particle size distribution of the exosomes derived from control and C18 Cer treated cell cultures.

3.3) A β clearance reliance on the stereochemistry of ceramide

As previously demonstrated, neuronal cell-derived exosomes could support extracellular A β clearance.¹⁰ A β are associated with the exosomes and transported into microglial cells to be degraded. Next, it was determined whether the increase in exosomes, induced by the treatment with the four C18 Cer, promotes A β clearance using a transwell culture system, **Figure 3A**. The exosomes and A β are secreted from A β -overexpressing SH-SY5Y cells placed on upper inserts that can flow through the membrane into bottom wells with microglial BV-2 cells placed. Under this experimental setting, the ceramide species were added to the cultures at 10 μ M, and after 24 h of co-incubation, the levels of A β in the medium were determined by A β ELISA. Both A β 40 and A β 42 in the culture media decreased following incubation with DE-18 and DT-18, but not LE-18 or LT-18, **Figure 3B**. In addition, the concentrations of A β 40 were significantly lower after treating LE-18 than LT-18, showing that LE-18 has a higher potency of exosome-dependent clearance of extracellular A β .

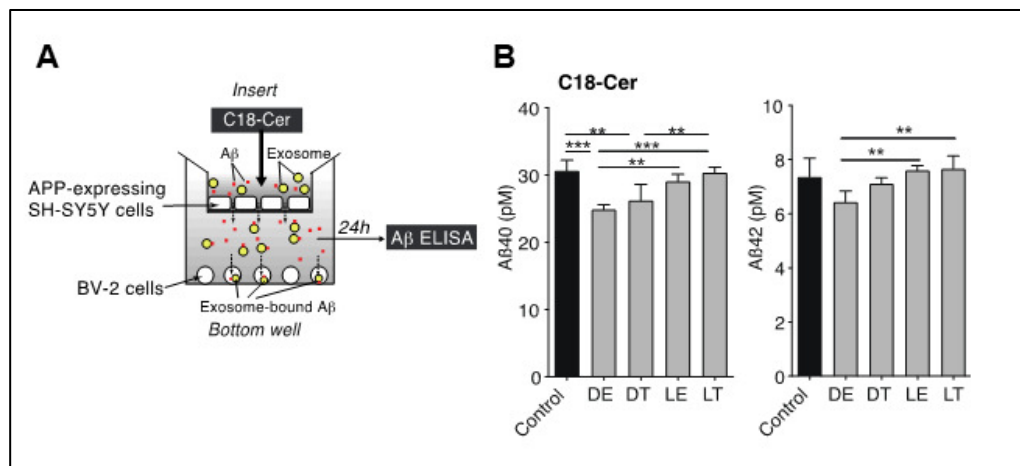


FIGURE 2 Schematic representation of a transwell culture model constructed from human APP-expressed SH-SY5Y and BV-2 cells. Cer (10 μ M) were added to the cultures. After 24 h, the conditioned medium was collected and analyzed by A β ELISA. (B) The levels of A β 40 and A β 42 after the treatment with the indicated Cer. Values are the means $\pm$ SD. (n = 3, * p < 0.05; *** p < 0.001 by Tukey's multiple comparison test).

3.4) Effect of the tail modifications on exosome release

Results of exosome release ELISA have shown that DE promoted exosome release much higher than the other ceramide stereoisomers. Hence, some tail modifications of DE isomer were carried out to study their effect on exosome release, **Figure 1**. These modifications include aliphatic, alicyclic, aromatic, and heterocyclic moieties. The synthesized DE derivatives were evaluated for exosome release using the exosome ELISA kit and the results are shown in **Figure 4**. Comparing the aliphatic moieties of different tail lengths, C18 length showed higher activity than shorter tail lengths. From the alicyclic moieties, DE with cyclopropyl tail showed a significant increase in activity more than DE-18. DE with adamantyl tail showed a slight increase in activity in comparison with DE-18. Alternatively, none of the aromatic moieties tested showed an improvement in activity when compared with DE-18. Different from other heterocyclic tails tested, DE with furanyl tail increased exosome release much more than DE-18.

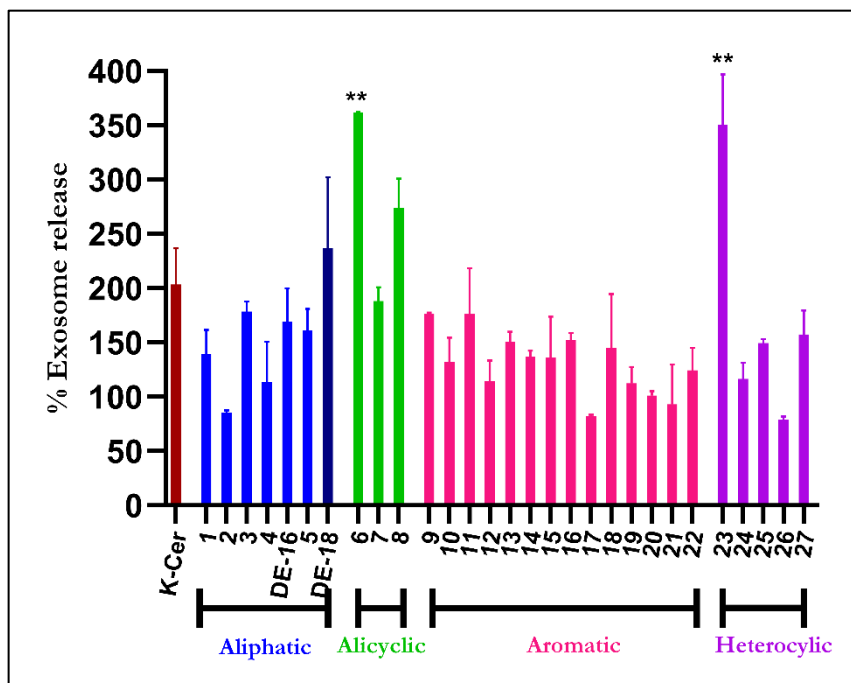


FIGURE 3 Quantification of exosomes released from SH-SY5Y cells treated with ceramide 1-27. After the treatment, the conditioned media were condensed by ultrafiltration, and the levels of exosomes in the concentrated medium were measured using TIM4-Capture Exosome ELISA. Results are normalized against controls and represented as the mean $\pm$ SD (n=2; ** p < 0.0001 by Bonferroni's multiple comparison test).

4. MATERIALS AND METHODS

4. Materials and Methods

4.1) General method for synthesis of the stereoisomers of ceramide from the corresponding sphingosines

HBTU (12 mg, 0.032 mmol) was added to a solution of the fatty acid (0.032 mmol) in DMF (300 μ L). Then, DIPEA (5 mg, 0.032 mmol) was added, and the solution was stirred for 5 minutes at room temperature. As a solution in DMF (300 μ L), the corresponding sphingosine (10 mg, 0.032 mmol) was added to the mixture and allowed to stir overnight at room temperature. The mixture was extracted with EtOAc, washed with brine, and dried over MgSO_4 . The solvent was evaporated, and the crude product was purified by column chromatography (n-hexane: EtOAc, 1:1) to afford the desired products as white or yellowish-white solids (60-80 %).

4.1.1) *N-((2S,3R,E)-1,3-dihydroxyoctadec-4-en-2-yl)hexanamide, (DE-6)*

^1H NMR (500 MHz, CDCl_3 , δ): 6.29 (d, $J=7.0$ Hz, 1H), 5.78 (dt, $J=14.0$, 6.8 Hz, 1H), 5.52 (dd, $J=15.4$, 6.4 Hz, 1H), 4.30 - 4.33 (m, 1H), 3.96 (dd, $J=11.4$, 3.6 Hz, 1H), 3.89 - 3.91 (m, 1H), 3.71 (dd, $J=11.2$, 3.2 Hz, 1H), 2.60 (broad s), 2.23 (t, $J=7.6$ Hz, 2H), 2.03 - 2.06 (m, 2H), 1.63 - 1.67 (m, 2H), 1.27 - 1.38 (m, 26H), 0.89 (t, $J=6.7$ Hz, 6H). (ESI, m/z): $[M + \text{Na}]^+$ calcd for $\text{C}_{24}\text{H}_{47}\text{NO}_3\text{Na}$, 420.3 ; found, 420.3.

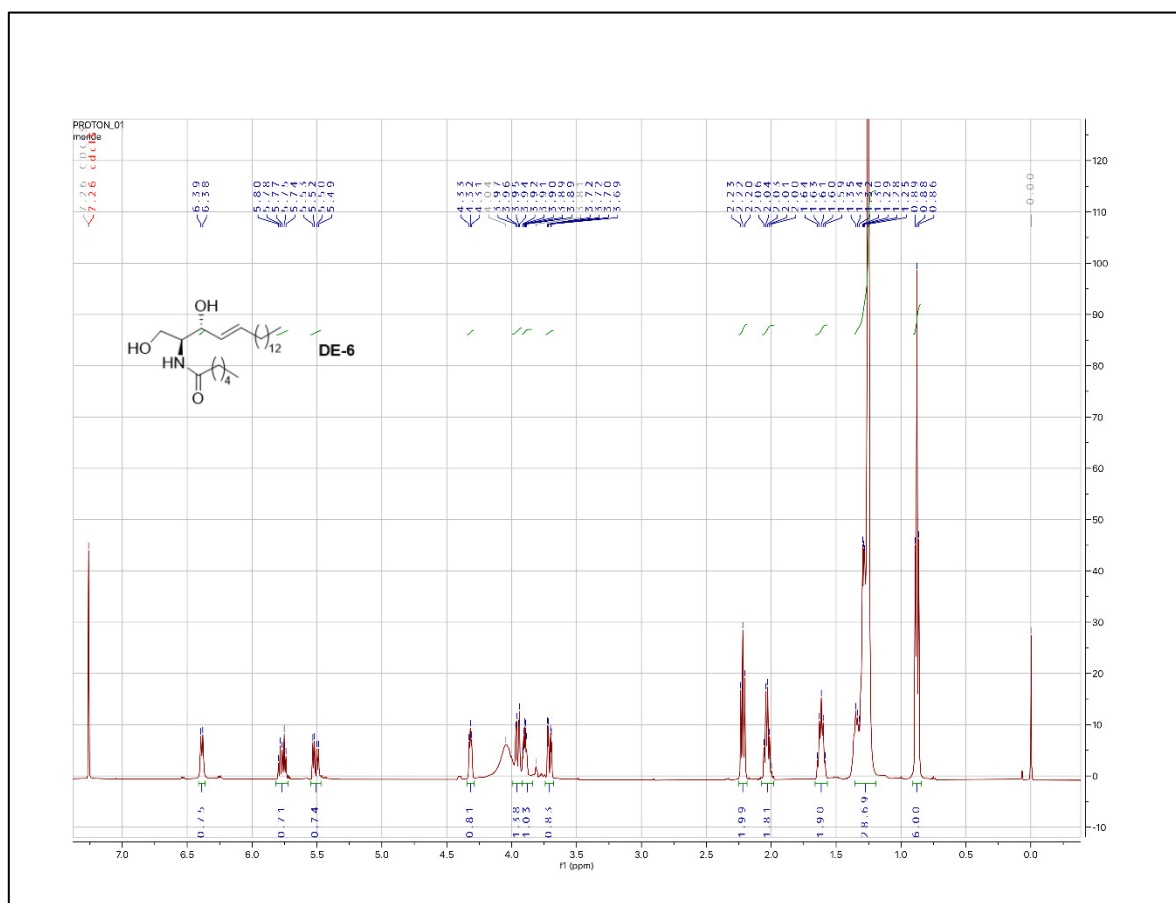


Figure 1 ^1H -NMR spectrum of DE-6

4.1.2 *N-((2S,3S,E)-1,3-dihydroxyoctadec-4-en-2-yl)hexanamide, (LT-6)*

^1H NMR (500 MHz, CDCl_3 , δ): 6.27 (d, $J=7.7$ Hz, 1H), 5.73 (dt, $J=14.2$, 6.8 Hz, 1H), 5.45 (dd, $J=15.4$, 6.4 Hz, 1H), 4.40 (dd, $J=5.9$, 3.4 Hz, 1H), 3.91 (dd, $J=7.9$, 3.4 Hz, 1H), 3.78 – 3.83 (m, 2H), 2.21 (t, $J=7.6$ Hz, 2H), 2.01 - 2.06 (m, 2H), 1.59 - 1.62 (m, 2H), 1.27 - 1.37 (m, 26H), 0.88 (t, $J=6.9$ Hz, 6H). (ESI, m/z): $[M + \text{Na}]^+$ calcd for $\text{C}_{24}\text{H}_{47}\text{NO}_3\text{Na}$, 420.3; found, 420.3.

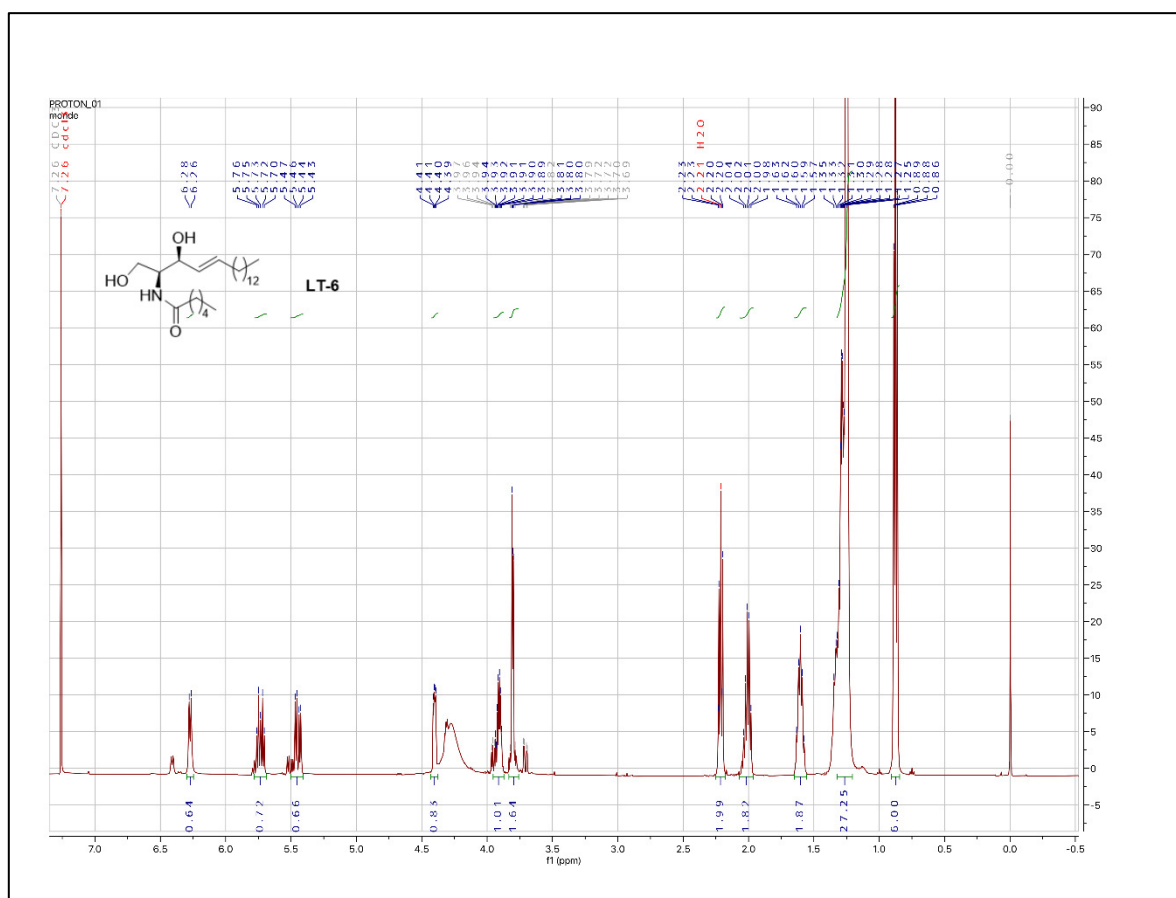


Figure 2 ^1H -NMR spectrum of LT-6

4.1.3 *N-((2R,3S,E)-1,3-dihydroxyoctadec-4-en-2-yl)hexanamide, (LE-6)*

^1H NMR (500 MHz, CDCl_3 , δ): 6.39 (d, $J=7.4$ Hz, 1H), 5.77 - 5.82 (m, 1H), 5.51 (dd, $J=15.5$, 6.4 Hz, 1H), 4.29 - 4.35 (m, 1H), 4.07 (broad s), 3.96 (dd, $J=11.2$, 3.3 Hz, 1H), 3.87 - 3.92 (m, 1H), 3.71 (dd, $J=11.3$, 3.3 Hz, 1H), 2.22 (t, $J=7.6$ Hz, 2H), 2.02 - 2.05 (m, 2H), 1.62 - 1.63 (m, 2H), 1.27 - 1.37 (m, 26H), 0.88 (t, $J=6.8$ Hz, 6H). (ESI, m/z): $[M + \text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{48}\text{NO}_3$, 398.4; found, 398.4.

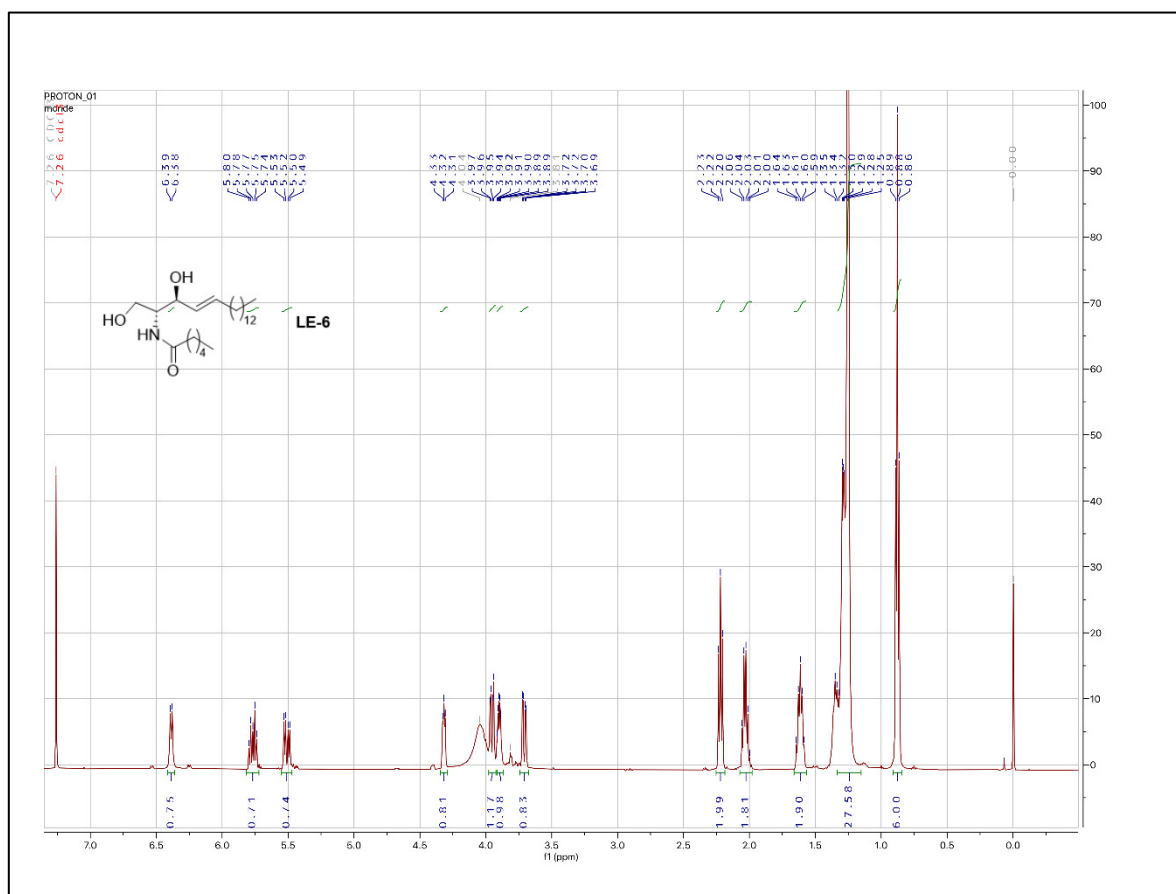


Figure 3 ^1H -NMR spectrum of LE-6

4.1.5 *N-((2S,3R,E)-1,3-dihydroxyoctadec-4-en-2-yl)palmitamide, (DE-16)*

^1H NMR (500 MHz, CDCl_3 , δ): 6.23 (d, $J=7.4$ Hz, 1H), 5.75 - 5.81 (m, 1H), 5.53 (dd, $J=15.5$, 6.4 Hz, 1H), 4.30 - 4.33 (m, 1H), 3.87- 3.97 (m, 2H), 3.71- 3.74 (m, 1H), 2.80 (broad s), 2.23 (t, $J=7.6$ Hz, 2H), 2.03 - 2.06 (m, 2H), 1.60 - 1.67 (m, 2H), 1.22 - 1.40 (m, 46H), 0.88 (t, $J=6.9$ Hz, 6H). (ESI, m/z): $[M + \text{H}]^+$ calcd for $\text{C}_{34}\text{H}_{68}\text{NO}_3$, 538.5; found, 538.1.

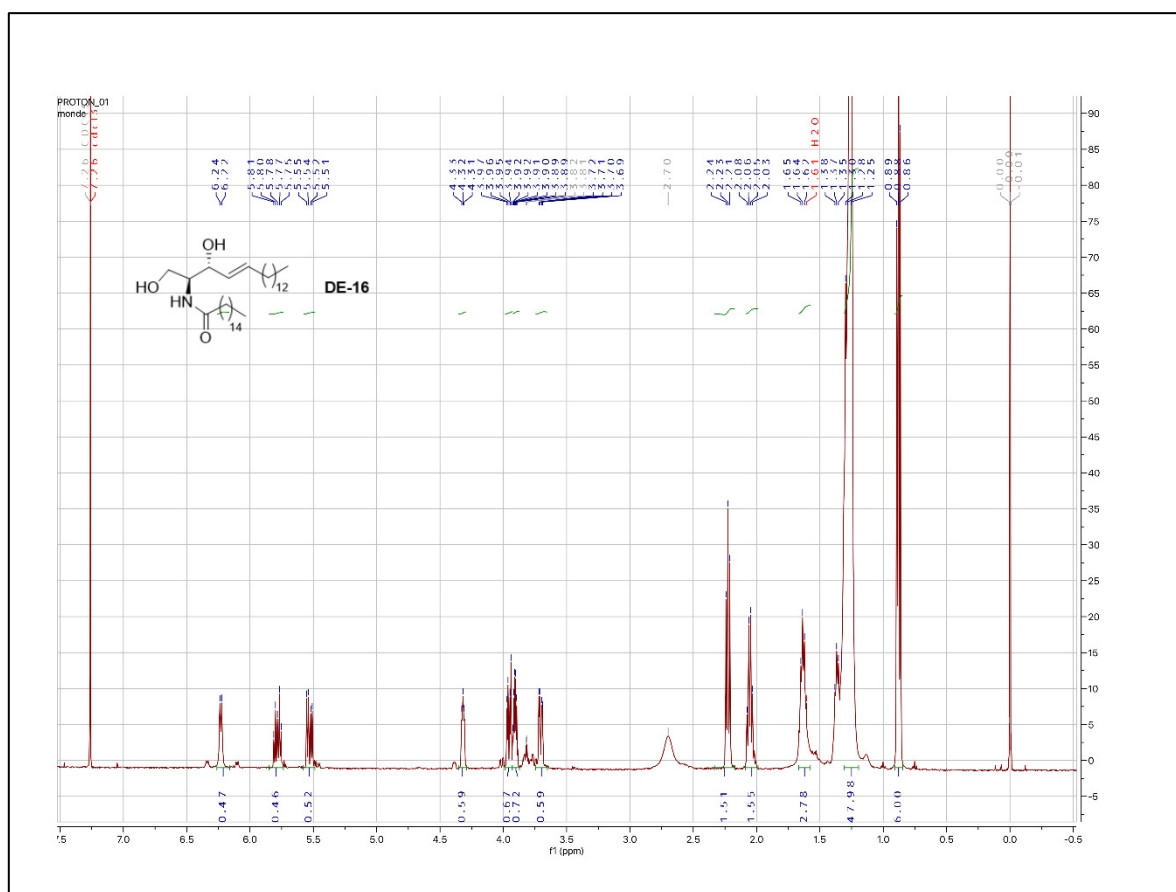


Figure 5 ^1H -NMR spectrum of DE-16

4.1.6 *N-((2S,3S,E)-1,3-dihydroxyoctadec-4-en-2-yl)palmitamide, (LT-16)*

^1H NMR (500 MHz, CDCl_3 , δ): 6.13 (d, $J=7.4$ Hz, 1H), 5.72 - 5.77 (m, 1H), 5.47 (dd, $J=15.4, 6.5$ Hz, 1H), 4.37 - 4.40 (m, 1H), 3.89 - 3.94 (m, 1H), 3.81 - 3.84 (m, 2H), 2.24 (t, $J=7.5$ Hz, 2H), 2.00 - 2.07 (m, 2H), 1.61 - 1.65 (m, 2H), 1.20 - 1.38 (m, 46H), 0.88 (t, $J=7.0$ Hz, 6H). (ESI, m/z): $[M + \text{Na}]^+$ calcd for $\text{C}_{34}\text{H}_{67}\text{NO}_3\text{Na}$, 560.5; found, 560.9.

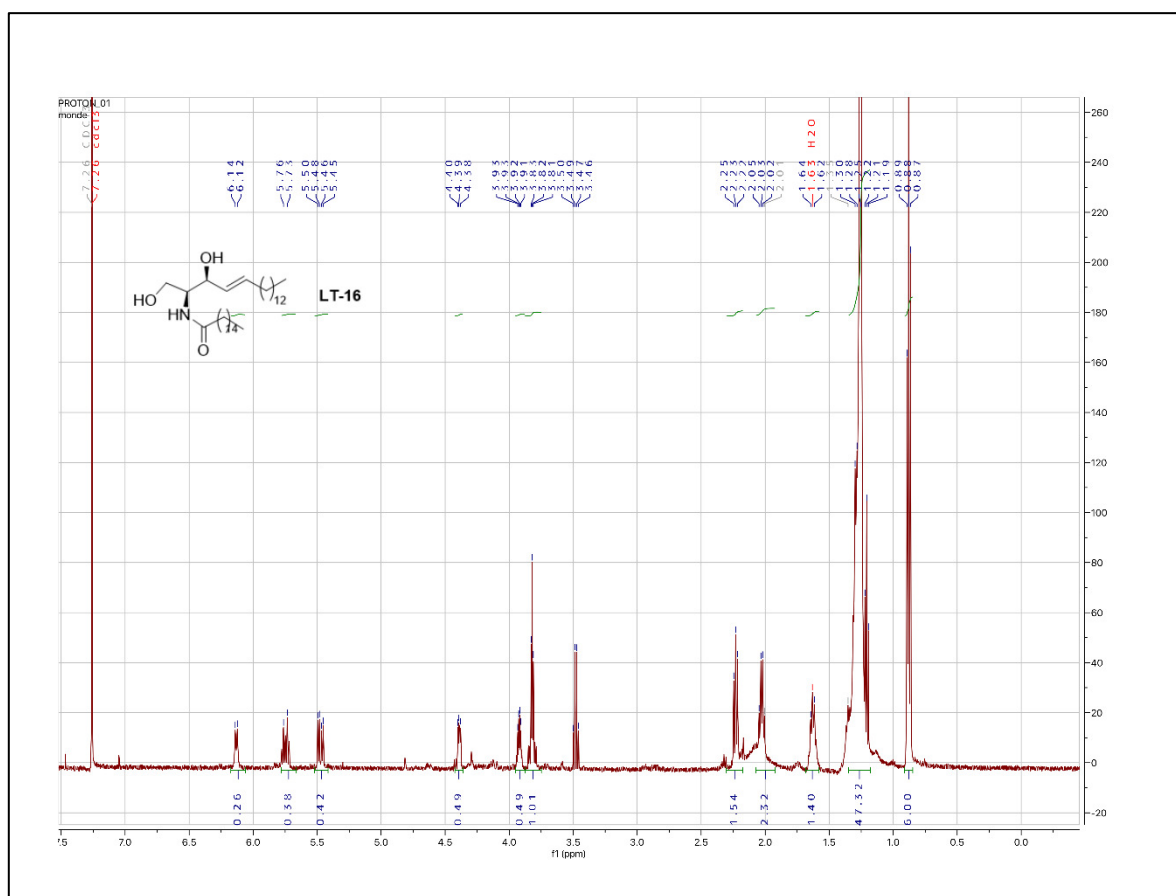


Figure 6 ^1H -NMR spectrum of LT-16

4.1.7 *N-((2R,3S,E)-1,3-dihydroxyoctadec-4-en-2-yl)palmitamide, (LE-16)*

^1H NMR (500 MHz, CDCl_3 , δ): 6.25 (d, $J=7.4$ Hz, 1H), 5.77 - 5.81 (m, 1H), 5.53 (dd, $J=15.4$, 6.2 Hz, 1H), 4.33 - 4.34 (m, 1H), 3.96 (dd, $J=11.3$, 3.8 Hz, 1H), 3.89 - 3.93 (m, 1H), 3.71 (dd, $J=11.2$, 3.3 Hz, 1H), 2.25 (t, $J=7.5$ Hz, 2H), 2.03 - 2.08 (m, 2H), 1.63 - 1.67 (m, 2H), 1.19 - 1.37 (m, 46H), 0.89 (t, $J=7.0$ Hz, 6H). (ESI, m/z): $[M + K]^+$ calcd for $\text{C}_{34}\text{H}_{67}\text{NO}_3\text{K}$, 576.5; found, 575.5.

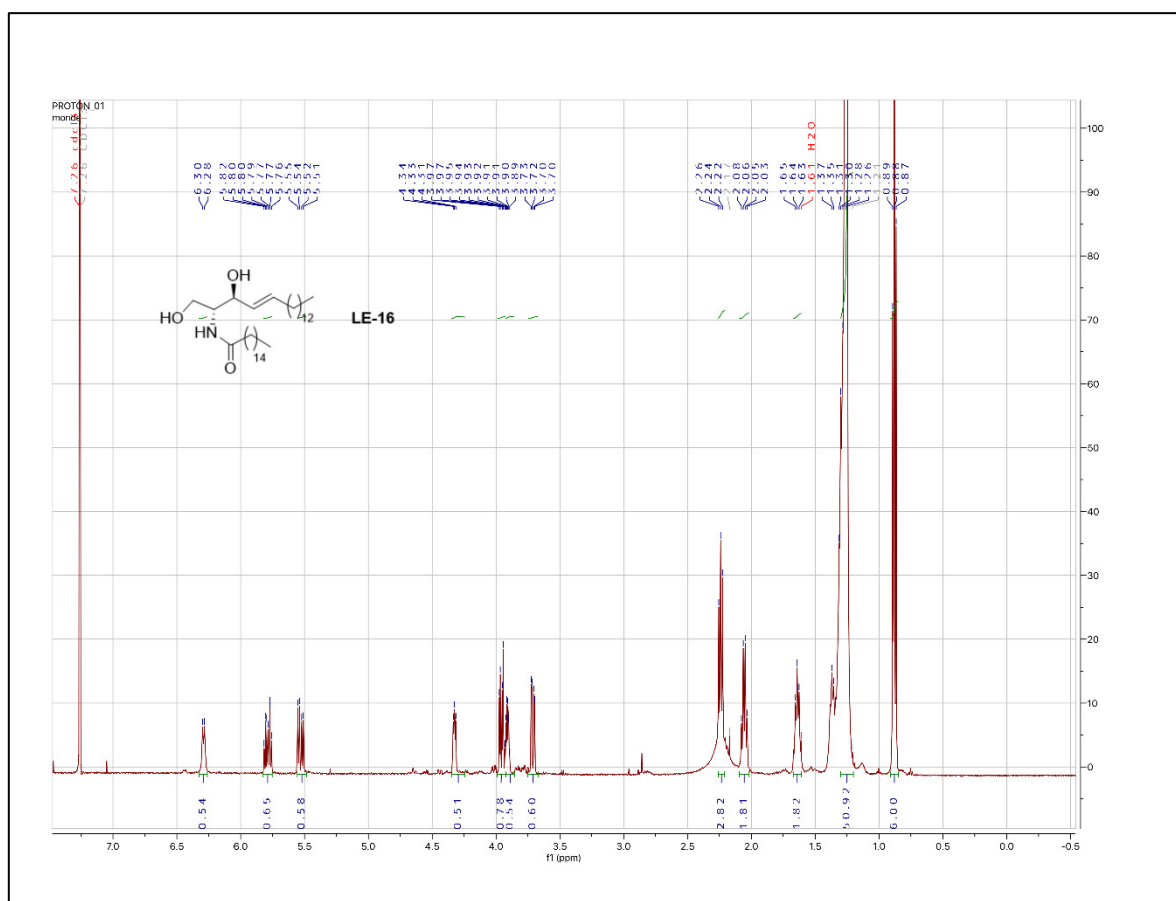


Figure 7 ^1H -NMR spectrum of LE-16

4.1.8 *N-((2R,3R,E)-1,3-dihydroxyoctadec-4-en-2-yl)palmitamide, (DT-16)*

^1H NMR (500 MHz, CDCl_3 , δ): 6.06 (d, $J=7.3$ Hz, 1H), 5.71 - 5.79 (m, 1H), 5.48 (dd, $J=15.5$, 6.4 Hz, 1H), 4.35 - 4.42 (m, 1H), 3.92 (dd, $J=8.0$, 3.9 Hz), 3.76 - 3.85 (m, 2H), 2.22 (t, $J=7.8$ Hz, 2H), 2.00 - 2.07 (m, 2H), 1.59 - 1.68 (m, 2H), 1.16 - 1.42 (m, 46H), 0.89 (t, $J=6.9$ Hz, 6H).). (ESI, m/z): $[M + \text{H}]^+$ calcd for $\text{C}_{34}\text{H}_{68}\text{NO}_3$, 538.5; found, 538.9.

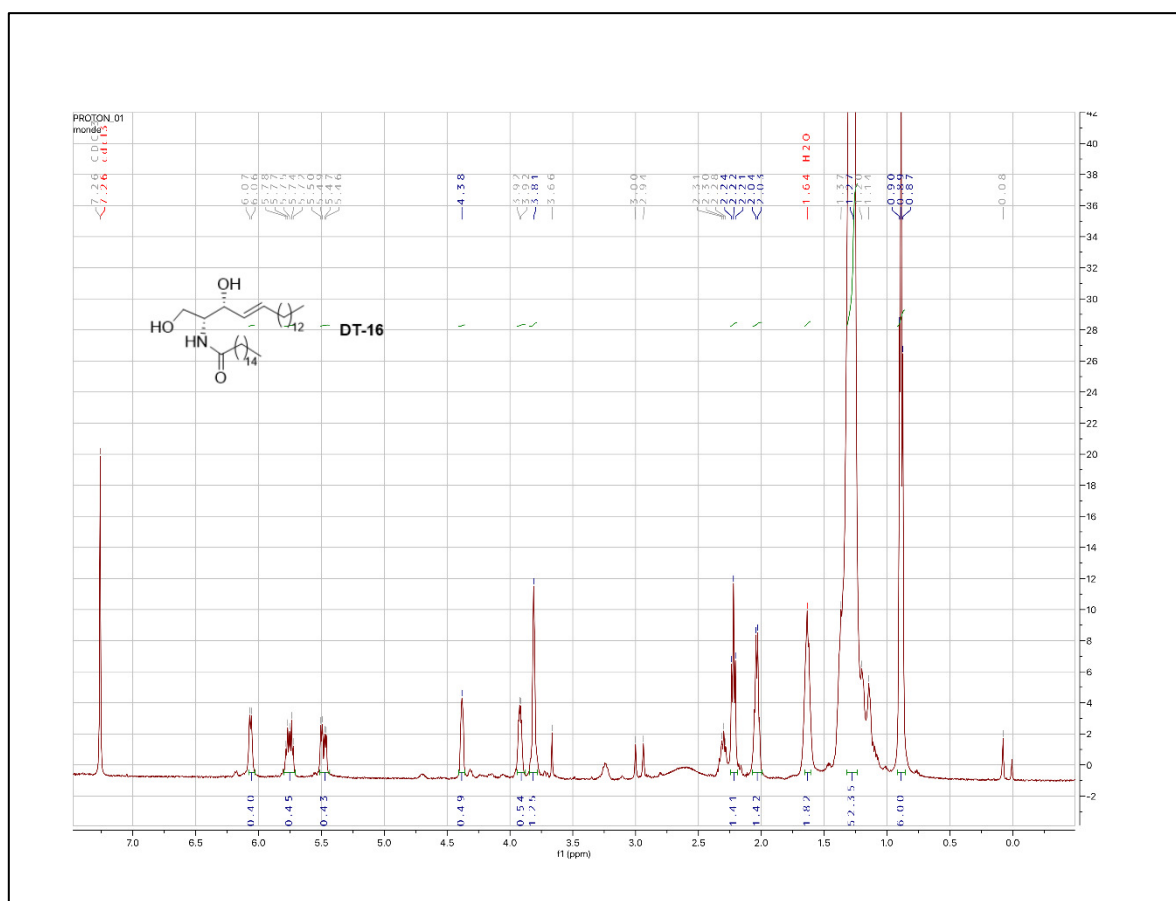


Figure 8 ^1H -NMR spectrum of DT-16

4.1.10) *N*-((2*S*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)stearamide, (LT-18)

^1H NMR (500 MHz, CDCl_3 , δ): 6.14 (d, $J=7.5$ Hz, 1H), 5.72 - 5.77 (m, 1H), 5.47 (dd, $J=15.4$, 6.5 Hz, 1H), 4.38 - 4.40 (m, 1H), 3.90 - 3.94 (m, 1H), 3.79 - 3.84 (m, 2H), 2.23 (t, $J=6.9$ Hz, 2H), 2.01 - 2.06 (m, 2H), 1.60 - 1.64 (m, 2H), 1.23 - 1.40 (m, 50H), 0.88 (t, $J=7.0$ Hz, 6H). (ESI, m/z): $[M + \text{H}]^+$ calcd for $\text{C}_{36}\text{H}_{72}\text{NO}_3$, 566.5; found, 566.6.

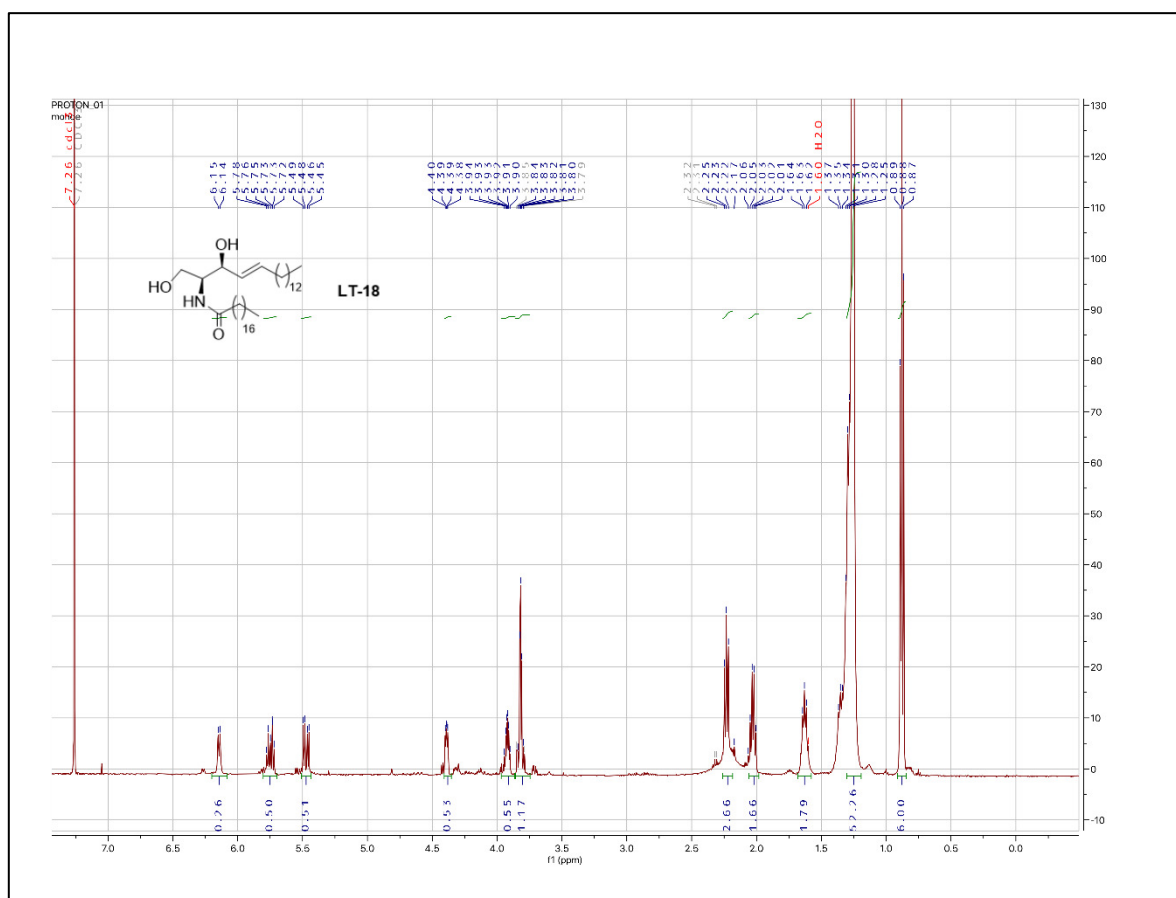


Figure 10 ^1H -NMR spectrum of LT-18

**4.1.13) *N*-((2*S*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)tetracosanamide,
(DE-24)**

^1H NMR (500 MHz, CDCl_3 , δ): 6.18 (d, $J=6.2$ Hz, 1H), 5.75 - 5.83 (m, 1H), 5.53 (dd, $J=15.6, 6.1$ Hz, 1H), 4.30 - 4.33 (m, 1H), 3.88- 3.97 (m, 2H), 3.73 (dd, $J=11.2, 3.3$ Hz, 1H), 2.22 (t, $J=7.6$ Hz, 2H), 2.00 - 2.07 (m, 2H), 1.60 -1.68 (m, 2H), 1.12 - 1.44 (m, 62H), 0.87 (t, $J=6.8$ Hz, 6H). (ESI, m/z): $[M + \text{Na}]^+$ calcd for $\text{C}_{42}\text{H}_{83}\text{NO}_3\text{Na}$, 672.6; found, 672.8.

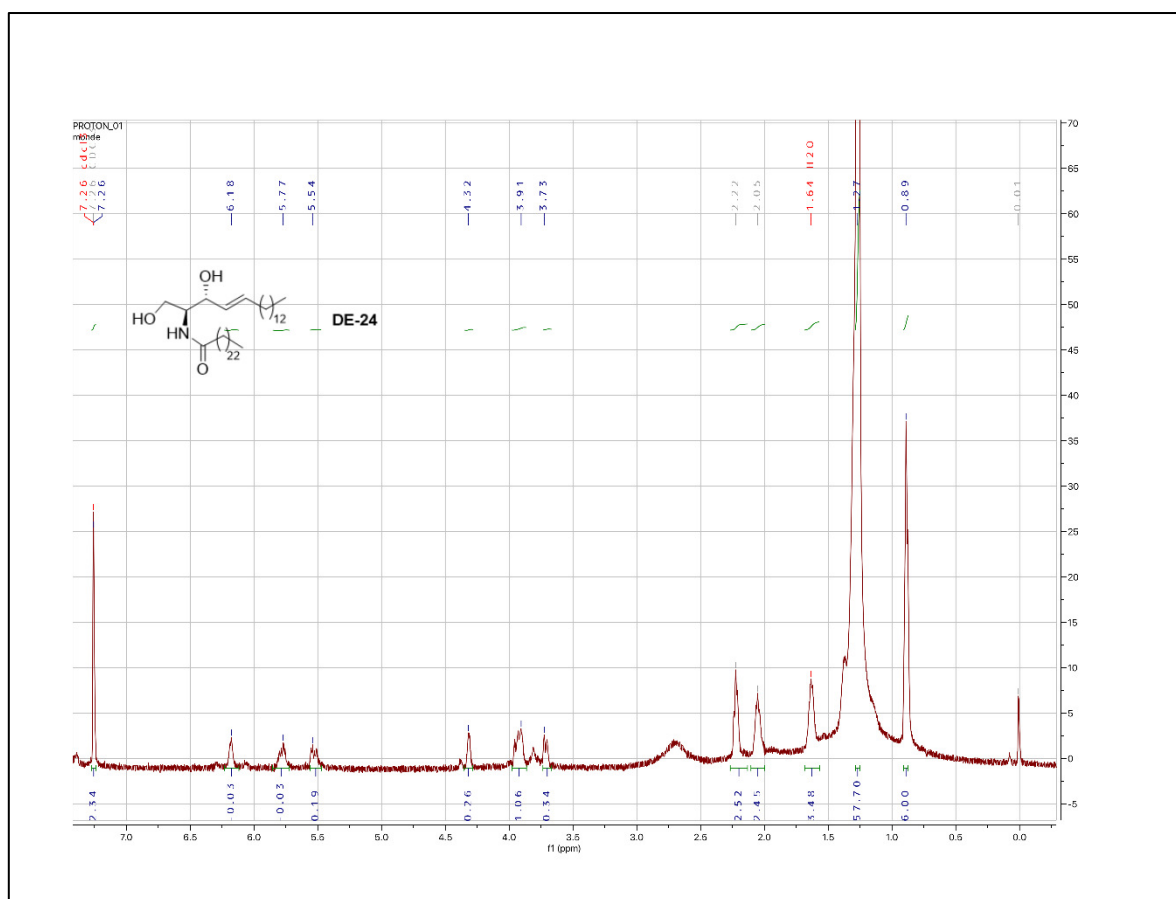


Figure 13 ^1H -NMR spectrum of DE-24

**4.1.14) *N*-((2*S*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)tetracosanamide,
(LT-24)**

^1H NMR (500 MHz, CDCl_3 , δ): 6.23 (d, $J=7.1$ Hz, 1H), 5.73 - 5.80 (m, 1H), 5.53 (dd, $J=15.8$, 6.0 Hz, 1H), 4.37 - 4.41 (m, 1H), 3.93 - 3.97 (m, 1H), 3.81 - 3.84 (m, 2H), 2.23 (t, $J=6.5$ Hz, 2H), 2.01 - 2.07 (dt, $J=14.6$, 7.2 Hz, 2H), 1.60 - 1.69 (m, 2H), 1.22 - 1.41 (m, 62H), 0.89 (t, $J=6.5$ Hz, 6H). (ESI, m/z): $[M + \text{K}]^+$ calcd for $\text{C}_{42}\text{H}_{83}\text{NO}_3\text{K}$, 688.6; found, 688.7.

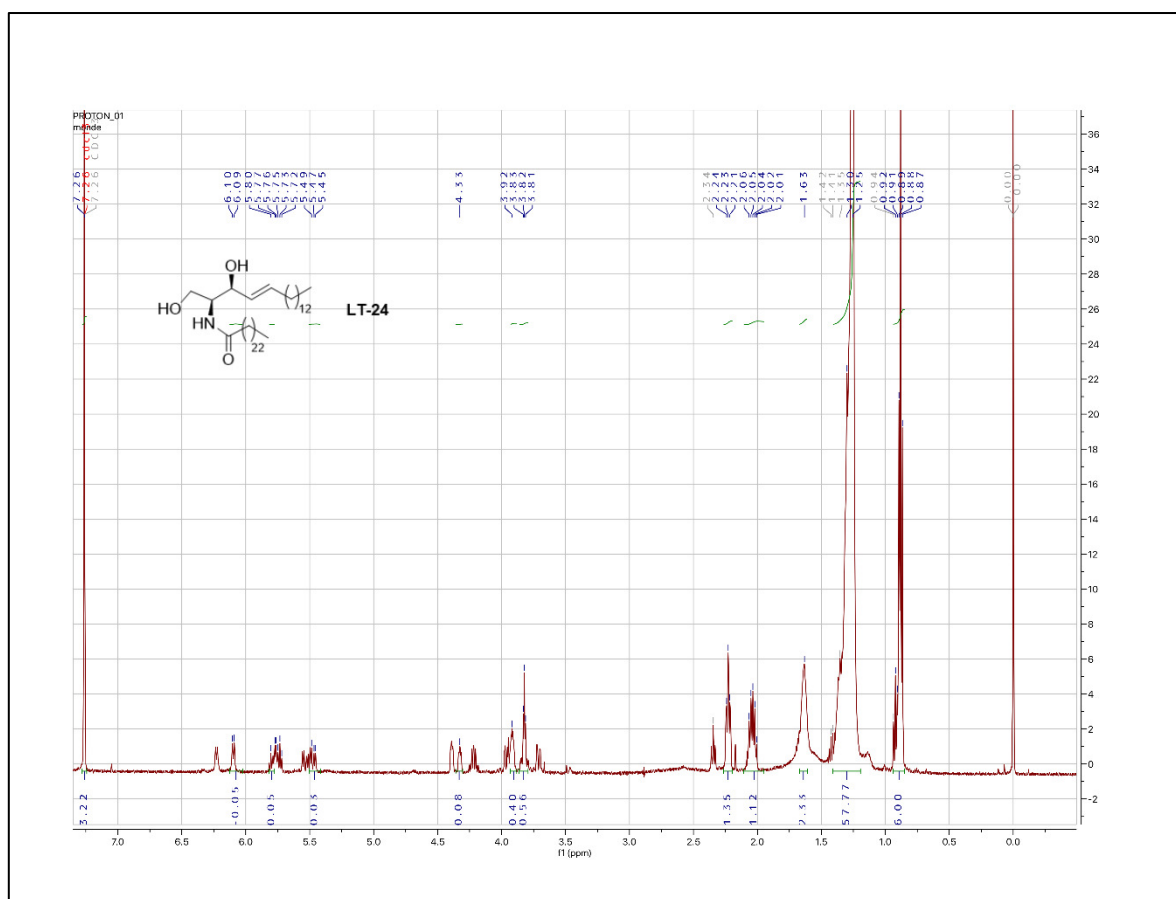


Figure 14 ^1H -NMR spectrum of LT-24

4.1.15) *N*-((2*R*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)tetracosanamide, (LE-24)

^1H NMR (500 MHz, CDCl_3 , δ): 6.17 (d, $J=7.3$ Hz, 1H), 5.74 - 5.82 (m, 1H), 5.57 (dd, $J=15.4$, 6.4 Hz, 1H), 4.30 - 4.34 (m, 1H), 3.95 (dd, $J=11.1$, 4.0 Hz, 1H), 3.88 - 3.93 (m, 1H), 3.72 (dd, $J=11.1$, 3.3 Hz, 1H), 2.23 (t, $J=7.8$ Hz, 2H), 2.02 - 2.08 (m, 2H), 1.60 - 1.67 (m, 2H), 1.22 - 1.41 (m, 62H), 0.89 (t, $J=6.9$ Hz, 6H). (ESI, m/z): $[M + \text{H}]^+$ calcd for $\text{C}_{42}\text{H}_{84}\text{NO}_3$, 650.6; found, 650.5.

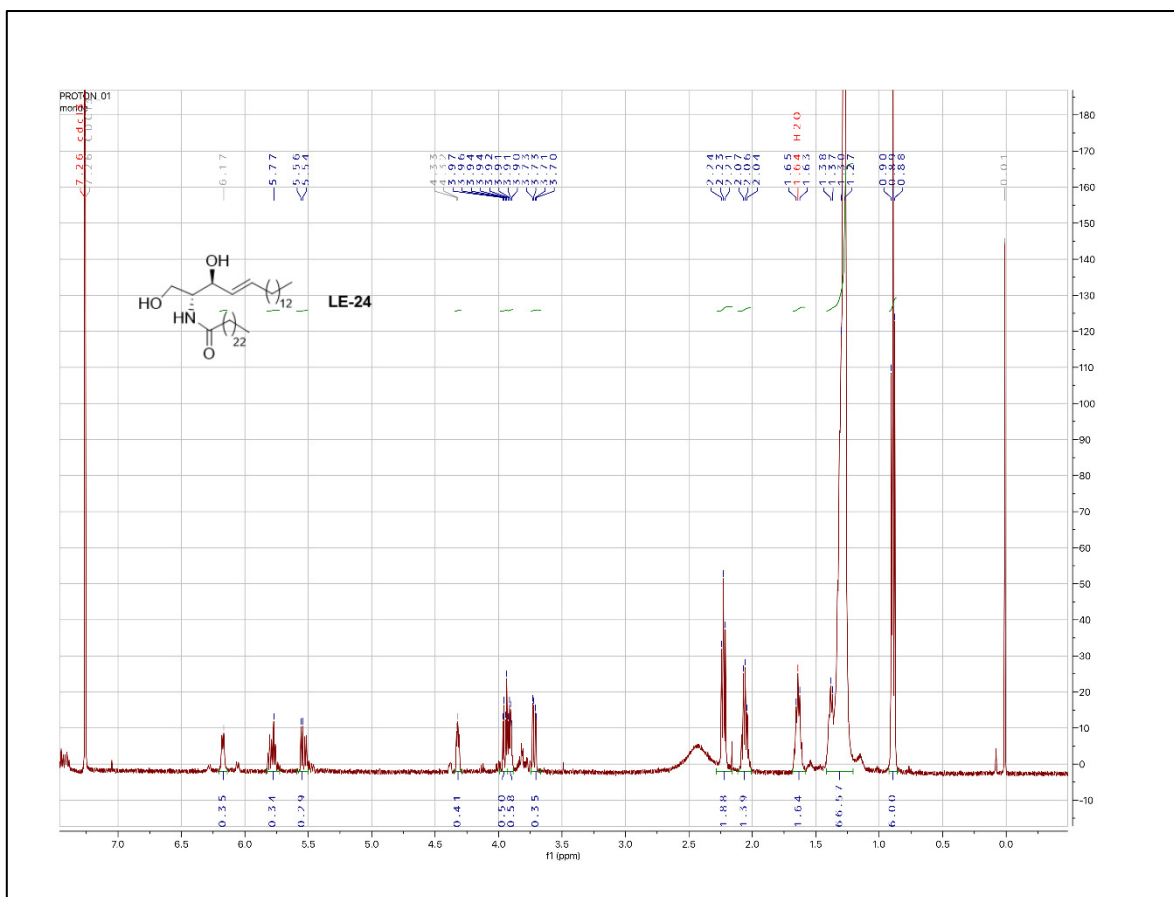


Figure 15 ^1H -NMR spectrum of LE-24

**4.1.16) *N*-((2*R*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)tetracosanamide,
(DT-24)**

^1H NMR (500 MHz, CDCl_3 , δ): 6.10 (d, $J=7.4$ Hz, 1H), 5.71 - 5.78 (m, 1H), 5.47 (dd, $J=15.7$, 6.0 Hz, 1H), 4.35 - 4.40 (m, 1H), 3.89 - 3.94 (m, 1H), 3.77 - 3.83 (m, 2H), 2.22 (t, $J=7.7$ Hz, 2H), 2.00 - 2.06 (m, 2H), 1.59 - 1.67 (m, 2H), 1.20 - 1.42 (m, 62H), 0.89 (t, $J=6.8$ Hz, 6H). (ESI, m/z): $[M + \text{H}]^+$ calcd for $\text{C}_{42}\text{H}_{84}\text{NO}_3$, 650.6; found, 650.8.

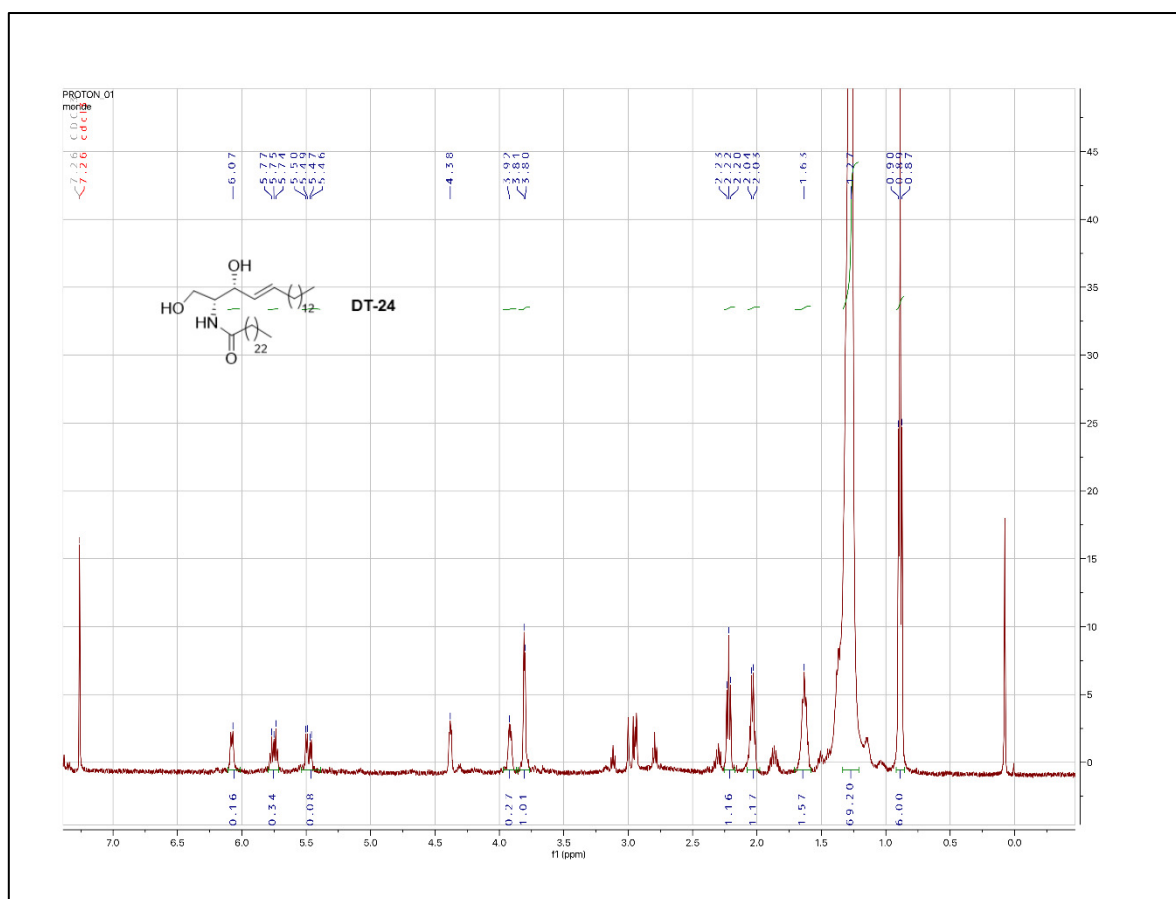


Figure 16 ^1H -NMR spectrum of DT-24

4.2) Synthesis of the library of tail-modified DE Ceramides

Tail-modified DE ceramides were synthesized using the active resin method. The active resins were prepared from carboxylic acids or acid chlorides and nitrophenol on polystyrene as reported.²² Then, the activated resins were coupled with sphingosine to yield the tail-modified library.

4.3) Cell cultures and treatment

4.3.1) Cell cultures

SH-SY5Y human neuroblastoma cells were maintained at 37°C with 5% CO₂ in Eagle's minimum essential medium (E-MEM)/Ham's F-12 medium (ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS). The murine microglial cell line BV-2 was purchased from National Cancer Institute (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy) and cultured in RPMI 1640 medium (ThermoFisher Scientific, USA) supplemented with 10% FBS and L-glutamine.

4.3.2) *Ceramide treatments*

SH-SY5Y Cells ($1 \times 10^5/\text{cm}^2$) cultured on a 12-well plate were treated for 24h with 10 μM ceramide species or 0.1% dimethylsulfoxide (DMSO; control).

Cell viability was assessed using a Cell-counting Kit-8 (Dojindo, Osaka, Japan).

4.3.3) *Transwell study*

SH-SY5Y cells with stable transfection of human amyloid precursor protein APP770 were maintained in E-MEM/Ham's F-12/10% FBS. For Transwell cultures, APP770 expressing SH-SY5Y cells ($5 \times 10^5/\text{cm}^2$) cultured on 24-well plate inserts (0.5 μm pore; Corning, NY) and BV-2 cells (1×10^6) placed on bottom wells were treated for 24 h with 10 μM ceramides in E-MEM/Ham's F-12. A β levels in the transwell-conditioned medium were measured by an A β ELISA (Wako, Osaka, Japan).

4.4) Exosome isolation and quantification

4.4.1) *Concentration of the conditioned medium and exosome quantification*

After the ceramide treatments, the conditioned media were centrifuged at 2,000xg for 10 min, and then the supernatants (0.5 ml) were concentrated using centrifugal filter devices (Amicon Ultra 10 K, Merck) to 50 μ l. Exosomes in the concentrated medium were measured by TIM4-Capture Exosome ELISA (Fujifilm Wako, Osaka, Japan) according to the manufacturer's instructions.

4.4.2) *Exosome collection*

Exosomes in the culture supernatant were collected by the ultracentrifugation method.²⁸ The conditioned culture media were centrifuged at 2,000xg for 10 min, and then centrifuged at 10,000 g for 30 min at 4°C to remove cells and debris. The supernatant was then centrifuged at 100,000 g for 1 h at 4°C to obtain exosomes as pellets. Exosomes were measured as above by Exosome ELISA. The size and number of exosomes were measured using a qNano nanoparticle analyzer (Izon Science, Cambridge, MA). CPC100 served as a calibration sample.

5. CONCLUSION

5. Conclusion

A library of ceramides was synthesized with varying stereochemistry and hydrophobic tail length. Stereochemistry was found to play a crucial role in ceramide-induced exosome production from neurons. DE and DT isomers with C16 and C18 tails showed the best activity without a significant change in the particle size of the released exosomes, implying the essential R configuration of C3. In transwell experiments with A β -expressed neuronal cells and microglial cells, DE- and DT-ceramides with C16 and C18 tails significantly decreased extracellular A β levels. Realizing that DE is the natural diastereoisomer of ceramide, it might be important to develop the natural product-derived Cer as potential functional food materials and natural medicine for the exosome-related AD prevention.

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DEDICATION

Dedication

'All perfect and true praise belongs to Allah who guided us to attain this. We could never have been led right to this if Allah had not guided us.'

[The Holy Quran- 7:43]

To my supporting husband,

who never let me down throughout this tough journey.

To my kids, Halima and Sulaiman, Allah's blessings

To the memory of my mother, my guardian angel,

whom I lost during my Ph.D. journey.

To the memory of my grandmother, my aunts, my uncles

To my father, my siblings, my family, and my friends,

who supported me during my cancer-fighting and Ph.D. time.