CHAPTER-I, SECTION-2

Design and synthesis of novel vaccine adjuvants
1.2.1. Introduction

Finding novel adjuvants with potential to enhance immune responses against target immunogens has been a major issue for the vaccine design. Several potential Th1 modulators have been identified and few of them are approved as adjuvants for human vaccines. However, toxicity and reactogenicity are the major issues associated with such adjuvants and there have been worldwide efforts to evolve safe and efficacious vaccine adjuvants. With the improved knowledge on dendritic cell immunology and the role of dendritic cell surface receptors, several mimics of pathogen associated molecular patterns (PAMPs) have been evolved as possible adjuvants for human use. However, owing to their inherent toxicity, several analogs of muramyl dipeptides (MDP) and some lipopeptides have been developed as non-toxic alternatives. Role of toll like receptors (TLRs) in tightly regulating the host immune response led immunologists to develop powerful TLR ligands and several lipidated analogs of peptides have been studied in this regard. Some potential advantages of lipopeptides as novel alternatives to conventional adjuvant are; (i) their ability to improve efficiency of vaccines, (ii) lack of side effects, (iii) positive synergism in their mixture with antigens, (iv) non-toxic and non-pyrogenic nature and above all their tissue friendly features. In light of these advantages, several novel lipopeptides which proved to be clinically safe and effective adjuvants have been synthesized. Recent reports reveal that the peptide amphiphiles having Nε-lipidated lysine moiety increase uptake and maturation of dendritic cells through a TLR-2 pathway, trigger a Th1-dependent protective immunity and also increase adjuvant activity of minimal CD8 T cell peptides incorporated into lipid-core-peptides. In the lipopeptide, Pam3CSK4 derived from a bacterial lipoprotein, polylysine sequence has shown great potential for its adjuvanticity. Lipidated lysine covalently conjugated with peptide antigens has been used in many self-adjuvanted vaccines and it was found that lipid moiety besides protecting the peptide antigens from enzymatic degradation, also helps peptide antigens in internalization process. Even though palmitoylated cysteine based adjuvants are found to be the effective adjuvants with high TLR specificity, they are mostly used in conjugated form with immunogenic peptide epitopes and are seldom used with exogenous antigens in vaccine formulations.
1.2.2. Present work

In view of the importance of peptides encompassing lysine residues as adjuvants, we explored the possibility of developing small lysine based lipidated molecules which might reveal the pattern of adjuvant activity with regard to the site and extent of lipidation. As an output of these studies, herein we present the synthesis and immunogenicity of a focused library of lipidated lysine peptides tested against a weak antigen ovalbumin (OVA). The effect of lipidation on the adjuvanticity of lysine moiety by varying the site, degree and nature of lipid chain on both lysine and di lysine peptides are also presented. To our knowledge, the present work is first of its kind where suitably substituted lipidated lysine analogs were found to activate immune system to evoke Th1 and Th2 immune response.

A focused library of lipidated lysine, lysine-lysine and lysine-glycine dipeptides was synthesized (Figures 1, 2a, 2b) following HOBT activation procedure for Fmoc chemistry. The synthesized lipidated moieties were purified and characterized by NMR and mass spectrometry and then screened for their possible adjuvant activity. In view of the reported potential advantages of octanoic acid, we synthesized a series of octanoylated and dioctanoylated lysine and lysine-lysine moieties and investigated it for its immunogenicity potential. Myristoylated lysine-lysine dipeptide was also synthesized and investigated for its adjuvanticity. The results from immunogenicity studies for this lipidated dipeptide were compared with those recorded for corresponding octanoylated dipeptide to unravel the impact of acyl group chain length on the immunogenicity potential in these lipidated derivatives. To explore the impact of amino acid moieties in this class of adjuvants we also synthesized octanoylated lysine-glycine dipeptide and screened it for its adjuvanticity.

1.2.3. Experimental Section

1.2.3.1. Materials and methods

Fmoc-Lys (Boc), Fmoc-Gly and 2-chlorotritylchloride resin were purchased from Novabiochem, Switzerland. Peptide synthesis grade solvents viz., dimethylformamide (DMF), piperidine, trifluoroacetic acid (TFA), trifluoroethanol (TFE), N,N-diisopropylethylamine (DIPEA) and dichloromethane (DCM), were purchased from Fluka. Octanoic acid, myristic acid and coupling reagents were purchased from Sigma, USA. Silica gel (230–400 mesh) was supplied by Loba Chemie.
Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes, USA and was used in accordance with the manufacturer's instructions. Muramyl dipeptide (MDP) and ovalbumin (OVA; Grade V, 98% purity) were purchased from Sigma, USA. 96 Wells V shaped microtitration plates, microtissue culture plates (96 U wells) and plastic wares were purchased from Nunc, USA. RPMI-1640 medium (Himedia, India) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin 100 IU/mL, streptomycin 100 IU/mL, 1mM sodium pyruvate and 2-mercaptoethanol (Sigma) was used for cell culture. [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), bovine serum albumin, streptavidin horseradish peroxidase, rabbit anti-mouse IgG peroxidase conjugate, HRP-conjugated swine anti-rabbit immunoglobulin, biotinylated goat anti-mouse IgG1 and IgG2a conjugate, were purchased from Sigma Chemicals, USA. Fluorescein isothiocyanate (FITC)-labeled CD4, CD80 and phycoerytherin (PE)-labeled CD8, CD86, IFN-gamma and IL-4 antimouse monoclonal antibody were purchased from BD Pharmingen.

Solid phase synthesis of peptides was done in Merrifield reactor on solid phase using 2-chlorotrityl chloride resin and Fmoc-protected amino acids. Loading of amino acid at each stage was determined by Perkin-Elmer UV-Spectro-photometer. All solvents were dried and freshly distilled prior to use. IR spectra were recorded on a Bruker Vector 22 instrument using KBr pellets and in CHCl3. ¹H NMR was recorded on a Bruker DPX 200 instrument in CDCl3 using TMS as internal standard for protons. Mass spectra were recorded on ESI-esquire 3000 Bruker Daltonics instrument and MALDI TOF-TOF. Elemental analysis was carried out using Elemental Vario EL III elemental analyser. Chemical shift values are mentioned in δ (ppm) and coupling constants (J) are given in Hz. Mass-spectrometric (MS) data is reported in m/z. Elemental analysis data is reported in % standard. The progress of all reactions was monitored by TLC on 2 cm × 5 cm pre-coated silica gel 60 F254 plates of thickness 0.25 mm (Merck). The chromatograms were visualized under UV 254-366 nm and iodine.
1.2.3.2. Synthesis

A series of orthogonally lipidated lysine and lysine-lysine and lysine-glycine dipeptides (Figures 1, 2a & 2b) were synthesized. A brief discussion about the synthesis of these derivatives is given as under:

Figure 1: Focused library of lipidated (unbranched) lysine & lysine derived peptides.
Figure 2a: Focused library of lipidated (branched) lysine & lysine derived peptides.
A. Synthesis of monoacylated derivatives of lysine and lysine derived peptides

Synthesis of compounds 1-3

Simple solution phase chemistry was used for the synthesis of octanoylated lysine derivatives (1-3). A brief discussion of the steps involved (Scheme 1) is given as under:
Scheme 1: a) C₆H₅COCH₂Br, KF, DMF; b) TFA: DCM (1:9); c) C₇H₁₆COOH, DCC: DMAP, DCM; d) Zn(activated): AcOH; e) Piperidine: DCM (1:9).
2-Amino-6-octanamidohexanoic acid (1):
To a mixture of KF (0.123 g, 2.124 mmol) and phenacyl bromide (0.254 g, 1.28 mmol), was added 10 mL of DMF and the reaction mixture allowed to stir for 15 min. A solution of Fmoclys(Boc)-OH 1a (0.515 g, 1.1 mmol) in DMF (5 mL) was added to the above reaction mixture. The reaction mixture was allowed to stir for 3 h at ambient temperature. After completion of the reaction (monitored by TLC), excess of water was added and the reaction mixture filtered. The precipitate was purified by column chromatography (hexane: EtOAc; 7:3) to afford 1b as a white powder (96%).
A solution of 10% TFA in DCM (3 mL) was added to 1b (0.644 g, 1.1 mmol). After stirring for 1 h, the solvent was removed and the residue completely dried under vacuum to yield 1c as a gummy liquid (90%).

1c (0.534 g, 1.1 mmol) was dissolved in DCM (20 mL) along with octanoic acid (0.158 g, 1.1 mmol) and DMAP (catalytic amount). Next DCC (0.226 g, 1.1 mmol) was added. The solution was stirred for 18 h at ambient temperature. The reaction mixture was then filtered to remove the insoluble urea (DCU) followed by column chromatography (hexane: EtOAc; 7:3) to give 1d as white powder (78%). To a solution of 1d (0.673 g, 1.1 mmol) in AcOH (10 mL), was added activated zinc (0.112 g, 2.2 mmol). The reaction mixture was stirred for 4 h at ambient temperature. It was then filtered and the filtrate completely evaporated and dried under vacuum followed by column chromatography (hexane: EtOAc; 6:4) to give 1e as white powder (87%). A solution of 10% piperidine in DCM (3 mL) was added to 1e (0.543 g, 1.1 mmol). After stirring for 1 h, the solvent was removed and the residue completely dried to vacuum to yield 1 as a gummy liquid (90%).

6-tert-Butoxycarbonylamino-2-octanamidohexanoic acid (2):
To a solution of 10% piperidine in DCM (3 mL), was added 1b (0.543 g, 1.1 mmol). After stirring for 1 h, the solvent was removed and the residue completely dried to vacuum to yield 2a as a gummy liquid (90%). 2a (0.4 g, 1.1 mmol) was dissolved in DCM (20 mL) along with octanoic acid (0.158 g, 1.1 mmol) and DMAP (catalytic amount). Next DCC (0.226 g, 1.1 mmol) was added. The solution was stirred for 18 h at ambient temperature. The reaction mixture was then filtered to remove the insoluble urea (DCU) followed by column chromatography (hexane: EtOAc; 7:3) to give 2b as white powder (78%). To a solution of 2b (0.539 g, 1.1 mmol) in AcOH (10
mL), was added activated zinc (0.112 g, 2.2 mmol). The reaction mixture was stirred for 4 h at ambient temperature. It was then filtered and the filtrate completely evaporated and dried under vacuum followed by column chromatography (hexane: EtOAc; 6:4) to give the product 2 as white powder (87%).

2,6-bis(Octanamido)hexanoic acid (3):
A solution of 10% TFA in DCM (3 mL) was added to 2 (0.409 g, 1.1 mmol). After stirring for 1 h, the solvent was removed and the residue completely dried under vacuum to yield 3a as a gummy liquid (90%). 3a (0.299 g, 1.1 mmol) was dissolved in DCM (20 mL) along with octanoic acid (0.158 g, 1.1 mmol) and DMAP (catalytic amount). Next DCC (0.226 g, 1.1 mmol) was added. The solution was stirred for 18 h at ambient temperature. The reaction mixture was then filtered to remove the insoluble urea (DCU) followed by column chromatography (hexane: EtOAc; 6:4) to give 3 as white powder (87%).

Synthesis of compounds 4-6
Solid phase synthesis was used for the synthesis of lipidated lysine derivatives (4-6). A brief discussion of the steps involved (Scheme 2) is given as under:
Scheme 2: f) DIPEA, DCM; g) Piperidine: DCM (2:8); h) FmocLys(Boc)OH, DIC: HOBT, DCM; i) C_{19}H_{32}COOH, DIC: HOBT, DCM; j) TFE: AcOH: DCM (2:1:7); k) C_{7}H_{16}COOH, DIC: HOBT, DCM; l) TFA: DCM (1:9).
6-<i>tert</i>-Butoxycarbonylamino-2-(6-<i>tert</i>-butoxycarbonylamino-2-tetradecnoyl-
amino-hexanoylamino)-hexanoic acid (4):

I. Loading of Fmoclysine(Boc)-OH to 2-Chlorotrityl chloride resin: Synthesis of 4a

0.5 g of 2-Chlorotrityl chloride resin was charged in the Merrifield reactor. 1a (0.117 g, 0.5 mmol) was dissolved in 5 mL of DCM (and small amount of peptide grade DMF to ensure a clear solution) and introduced to the resin. Further DIPEA (4 eq relative to Fmoc-amino acid) was added and the resin shaken for 3 h under nitrogen atmosphere. The resin was then washed with DCM/MeOH/DIPEA (17:2:1) × 3; DCM/MeOH × 5 (alternatively); DMF, DCM/MeOH × 2; finally with dry Et₂O and dried in vacuum to give 4a. The resin was end capped with 5 mL of AcOH: Pyr (2:3) solvent system for 2 h. Again the wash cycle was repeated and finally dried in vacuum.

2 mg/3 mL of the resin was taken in 20% piperidine solution in DCM, shaken for 10 min and the loading of Fmoc-lysine on resin was determined by its UV analysis using the following conversion factor:

\[
\text{Loading} = \frac{0.181818 \times \text{absorbance} \ (290 \text{ nm}) \times 10}{\text{Wt of resin (mg)} \times \text{volume of solution (mL)}} = \frac{0.181818 \times 1.660 \times 10}{2 \times 3}
\]

\[
= 0.503 \text{ mmol/g of resin.}
\]

II. Fmoc cleavage: Synthesis of 4b

Fmoc cleavage of the resin-bound amino acid was carried out by shaking it in 20% piperidine: DCM solution (10 mL) for half an hour to give 4b. The resin was washed thoroughly with DCM/MeOH × 5; DMF × 1; DCM × 2; finally with dry Et₂O and dried in vacuum for 1 h.

III. Coupling of Fmoclys(Boc)-OH to the N-terminal of resin bound lysine: Synthesis of 4c

0.351 g of Fmoclys(Boc)-OH (3 eq to the loading of previous amino acid) and HOBT (0.013 g, 1 mmol) were dissolved in dry DCM (8 mL). Few drops of DMF were also added in order to make a clear solution. 157 μL (1 mmol) of DIC was added to this solution. The solution was immediately introduced to the resin and shaken for 24 h. After 24 h, the resin was washed with DCM/MeOH × 5; DMF × 1; DCM × 2; finally
with dry Et$_2$O $\times$ 1, and dried in vacuum for half an hour to give 4c. 2 mg/3 mL of the resin was dissolved in 20% piperidine: DCM solution and shaken for 10 min. Loading of Fmoc-lysine was checked by measuring the absorbance at 290 nm which was found to be 1.627. Therefore the loading of Fmoc-lysine was calculated to be 0.49 mmol/g of resin. The resin was end capped with 5 mL of Ac$_2$O: Pyr (2:3) solution for 1 h and the wash cycle repeated as above. Loading was found to be 0.48 mmol/g of resin.

**IV. Fmoc cleavage: Synthesis of 4d**

Fmoc cleavage of the second amino acid was carried out by shaking it in 10 mL of 20% piperidine: DCM solution for half an hour to give 4d. The resin was washed thoroughly with DCM/MeOH $\times$ 5; DMF $\times$ 1; DCM $\times$ 2; finally with dry Et$_2$O $\times$ 1, and dried in vacuum for 1 h.

**V. Myristoylation of Resin-KK-NH$_2$: Synthesis of 4e**

0.3 g (1.31 mmol) of myristic acid (3 eq to the loading of previous amino acid) and HOBT (13.5 mg, 0.1 mmol) were dissolved in 8 mL of dry DCM. 157 μL (1 mmol) of DIC was added to this solution. The solution was immediately introduced to the resin and shaken for 24 h. After 24 h, the resin was washed with DCM/MeOH $\times$ 5; DMF $\times$ 1; DCM $\times$ 2; finally with dry Et$_2$O $\times$ 1, and dried in vacuum for half an hour to give 4e.

**VI. Cleavage of peptide from 2-Chlorotrityl chloride resin: Synthesis of 4**

10 mL solution of AcOH (1 mL), TFE (2 mL) and DCM (7 mL) was prepared. This solution was added to the resin bound peptide which was then allowed to stir for 1 h. The solution was filtered and washed with TFE: DCM (1:9, $\times$ 2) to ensure that the entire product was removed. Solvent was evaporated completely and the product precipitated in HPLC grade MeOH to give the dipeptide 4 (78%), bearing the orthogonal protection.

6-tert-Butyloxycarbonylamino-2-(6-tert-butryloxycarbonylamino-2-octanoylamino-hexanoylamino)-hexanoic acid (5):

**I. Octanoylation of Resin-KK-NH$_2$: Synthesis of 5a**

0.188 g (1.31 mmol) of octanoic acid (3 eq to the loading of previous amino acid) and HOBT (0.013 g, 0.1 mmol) were dissolved in dry DCM (8 mL). 157 μL (1 mmol) of
DIC was added to this solution. The solution was immediately introduced to the resin bound compound 4d, and shaken for 24 h. After 24 h, the resin was washed with DCM/MeOH × 5; DMF × 1; DCM × 2; finally with dry Et₂O × 1, and dried in vacuum for half an hour to give 5a.

II. Cleavage of peptide from 2-chlorotrityl chloride resin: Synthesis of 5
Same procedure as described for compound 4 was used. Yield of the compound was found to be 76%.

6-Amino-2-(6-amino-2-octanoylamino-hexanoylamino)-hexanoic acid (6):
5 mL solution of TFA (0.5 mL) and DCM (4.5 mL) was prepared. This solution was added to the resin bound peptide 5a and stirred for 1 h. The solution was filtered, washed with DCM and the solvent evaporated completely. The product was precipitated in HPLC grade MeOH to give the dipeptide 6 (62%), bearing the orthogonal protection.

Synthesis of compounds 7-9
Solid phase synthesis was used for the synthesis of lipidated amino acid derivatives (7-9). A brief discussion of the steps involved (Scheme 3) is given as under:
Scheme 3: m) DIPEA, DCM; n) Piperidine: DCM (2:8); o) C_{20}H_{38}N_{2}O_{5} (1e), DIC: HOBT, DCM; p) C_{7}H_{16}COOH, DIC: HOBT, DCM; q) TFE: AcOH: DCM (2:1:7).
2-(2,6-Dioctanamidohexanamido)-6-octanamidohexanoic acid (7):
Procedure similar to the one as described for compound 4 was followed. Here N-
octanoylated lysine instead of Boc protected lysine and octanoic acid instead of
myristic acid was used for lipidation. Yield of the compound was found to be 66%.

2-(6-Amino-2-octanamidohexanamido)-6-octanamidohexanoic acid (8):
Procedure similar to the one as described for compound 4 was used. Here instead of
two Boc protected lysine moieties, a combination of one Boc protected and one
N-
octanoylated lysine moieties and octanoic acid instead of myristic acid was used for
lipidation. Yield of the compound was found to be 61%.

6-Octanoylamino-2-(2-octanoylamino-acetylamino)-hexanoic acid (9):
Procedure similar to the one as described for compound 4 was used. Here instead of
two Boc protected lysine moieties, a combination of Fmoc glycine and N-
octanoylated lysine moieties and octanoic acid instead of myristic acid was used for
lipidation. Yield of the compound was found to be 63%.

B. Synthesis of diacylated derivates of lysine and lysine derived peptides
Diacylated derivatives of lysine and its dipeptide were generated from two pathways
using two different reactants namely glyceric acid and glycerol.

B.1. Synthesis of dioctanoylated ester derivatives using glyceric acid
Glyceric acid was synthesized from D-mannitol. Series of reactions that was used for
the synthesis of dioctanolyted ester of glyceric acid is depicted in scheme 4:
Scheme 4: i) DMP, PTSA, DMSO; ii) Na₂CO₃, NaIO₄; iii) MeOH, Br₂, NaHCO₃; iv) KOH, EtOH; v) TFA; vi) C₇H₁₆COCl, TFA.

**(1S,2S)-1,2-bis{(R)-2,2-Dimethyl-1,3-dioxolan-4-yl}ethane-1,2-diol (10a):**

To a mixture of D-mannitol **10** (10 g, 54.94 mmol) and PTSA (catalytic amount, 5 mg) in dry DMSO (20 mL), was added 2,2-dimethoxypropane (14.2 mL) at 0 °C under nitrogen atmosphere. The resulting reaction mixture was gradually warmed to ambient temperature and stirred for 8 h. The complete conversion of the starting material was confirmed by TLC. The reaction mixture was then partitioned between H₂O and EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure to obtain the crude product which was purified by column chromatography (hexane: EtOAc; 8:2) to give the pure product (8.0 g, 55%) as a white solid (**10a**).

**¹H NMR** (200 MHz, CDCl₃): δ 1.34 (s, 6H, (CH₃)₂), 1.40 (s, 6H, (CH₃)₂), 3.68 (t, 2H, CH₂, J = 4.8 Hz), 3.94 (q, 2H), 4.10 (m, 4H).

**IR** (KBr, cm⁻¹): 1068, 1214, 1372, 2935, 2987, 3330.
Mass (ESI-MS): 263 (M⁺ + H).

C, H analysis for C₁₂H₂₂O₆:
Calculated C, 54.95; H, 8.45. Found C, 54.99; H, 8.44.

(R)-2,2-Dimethyl-1,3-dioxolane-4-carbaldehyde (10b):
To a stirred solution of D-mannitol diacetonide (10a) (7.0 g, 0.026 mol) in DCM (50 mL), was added 3 mL of saturated Na₂CO₃ solution at 0 °C and then NaIO₄ (11.7 g, 54.6 mmol) portion wise over 15 min. The reaction mixture was stirred for 8 h at ambient temperature. After completion of reaction, the reaction mixture was filtered and concentrated under reduced pressure to afford the crude product (6.8 g, 98.5%) as colorless oil (10b).

(R)-Methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate (10c):
To the colorless oil (10b) (6.8 g, 52.3 mmol), a solution of MeOH/H₂O (9:1, 100 mL), NaHCO₃ (16.32 g, 194 mmol) and bromine (5.2 mL, 104 mmol) were added slowly. After stirring overnight at ambient temperature, excess of bromine was neutralized by Na₂S₂O₃ and the mixture extracted with DCM (2 × 150 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and evaporated to give ester as a colorless liquid (10c) (6.27 g, 75%).

¹HNMR (CDCl₃, 200 MHz): δ 1.40 (s, 3H, CH₃), 1.49 (s, 3H, CH₃), 3.78 (s, 3H, CH₃), 4.07-4.14 (m, 1H, CH₂°), 4.20-4.28 (m, 1H, CH₂°), 4.85 (m, 1H, CH).

IR (CHCl₃, cm⁻¹): 1056, 1210, 1378, 1741, 2931, 2988.

Mass (ESI-MS): 183 (M⁺ + Na).

C, H analysis for C₇H₁₂O₄:
Calculated C, 52.49; H, 7.55. Found C, 52.41; H, 7.50.

Potassium (R)-2,2-dimethyl-1,3-dioxolane-4-carboxylate (10d):
KOH (3 g, 75 mmol) was dissolved in water (3 mL) in a 50 mL round bottomed flask. Rectified spirit (10 mL) was added to produce a homogeneous solution. 10c (5 g, 31.2 mmol) was added slowly and with shaking. The reaction mixture was allowed to reflux for 3 h. After completion of the reaction, ethanol was evaporated from the reaction mixture. 50 mL of H₂O were further added to the reaction mixture and
washed with EtOAc (2 × 50 mL). Water layer was completely evaporated under reduced pressure to obtain the potassium salt of {2(R), 3-O-isopropylidene} glyceric acid (10d) in quantitative yields. It was used without purification in the next step.

\[ \text{\(^1\)HNMR (CDCl}_3, 200 \text{ MHz):}\]
\[ \delta 1.17 (s, 3\text{H}, \text{CH}_3), 1.22 (s, 3\text{H}, \text{CH}_3), 3.69 (t, 1\text{H}, \text{CH}_2^\prime, J_1 = 8.11 \text{ Hz}, J_2 = 6.94 \text{ Hz}), 4.06 (t, 1\text{H}, \text{CH}_2^\prime, J = 7.87 \text{ Hz}), 4.29 (m, 1\text{H}, \text{CH}). \]

IR (KBr, cm\(^{-1}\)):
1056, 1210, 1378, 1741, 2931, 2988.

(R)-2,3-Dihydroxypropanoic acid (10e):

10d (5 g, 27.17 mmol) was dissolved in TFA (10 mL) and stirred overnight. After completion of the reaction, the reaction mixture was completely evaporated to obtain the crude product 10e (2.88 g), which was directly used in the next step without any purification.

\[ \text{\(^1\)HNMR (CDCl}_3, 200 \text{ MHz):}\]
\[ \delta 3.69 (t, 1\text{H}, \text{CH}_2^\prime, J_1 = 8.11 \text{ Hz}, J_2 = 6.94 \text{ Hz}), 4.06 (t, 1\text{H}, \text{CH}_2^\prime, J = 7.87 \text{ Hz}), 4.29 (t, 1\text{H}, \text{CH}, J = 7.29 \text{ Hz}). \]

IR (KBr, cm\(^{-1}\)):
1056, 1210, 1378, 1741, 2931, 2988.

Mass (ESI-MS): 106 (M\(^{+}\)).

C, H analysis for C\(_3\)H\(_6\)O\(_4\):
Calculated C, 33.97; H, 5.70. Found C, 33.94; H, 5.78.

(R)-2,3-bis(Octanoyloxy)propanoic acid (11):

10e (2.88 g, 27.1 mmol) was dissolved in TFA. Octanoyl chloride (10.59 g, 65.2 mmol) was added slowly to the above solution under nitrogen atmosphere. Reaction mixture was stirred vigorously for 30 min. After completion of the reaction (monitored by TLC), the solvent was completely evaporated under reduced pressure to obtain the crude compound. It was purified on flash chromatography to give the pure compound 11 (8.75 g, 90%).

Specific rotation \([\alpha]_D^{28}\) +6.2 (c 0.021, CH\(_3\)OH).

\[ \text{\(^1\)H NMR (200 MHz, CDCl}_3):}\]
\[ \delta 0.86 (t, 6\text{H}, \text{CH}_3^\prime, \text{CH}_3^\prime\prime, J_1 = 6.67 \text{ Hz}, J_2 = 5.95 \text{ Hz}), 1.21-1.27 (m, 16\text{H}, (\text{CH}_2)_8), 1.57-1.64 (m, 4\text{H}, (\text{CH}_2)_2), 2.29-2.44 (m, 4\text{H}, (\text{CH}_2)_2), 4.37-4.58 (m, 2\text{H}, \text{CH}_2), 5.29-5.37 (m, 1\text{H}, \text{CH}). \]
$^{13}$C NMR (500MHz, CDCl$_3$): δ 14.00, 22.58, 24.72, 24.81, 28.88, 28.96, 29.00, 31.64, 33.82, 33.99, 62.40, 69.91, 171.59, 173.06, 173.53.

Mass (ESI-MS): 381 (M$^+$ + Na).

C, H analysis for 
C$_{19}$H$_{34}$O$_6$:
Calculated C, 63.66; H, 9.56. Found C, 63.64; H, 9.51.

**Synthesis of compounds 12-13**

Solid phase synthesis was used for the synthesis of lipidated lysine derivatives (12-13). A brief discussion of the steps involved (Scheme 5) is given as under:

(13R)-2,2-Dimethyl-13-(octanoyloxy)-4,12,16-trioxo-3,15-dioxa-5,11-diazatricosane-10-carboxylic acid (12):

Procedure similar to the one as described for compound 4 was used. Here Boc protected lysine was lipidated with dioctanoylated derivative of glyceric acid. Yield of the compound was found to be 90%.

6-Amino-2-{(R)-2,3-bis(octanoyloxy)propanamido}hexanoic acid (13):

11a was dissolved in a mixture of TFA: DCM (1:9). After stirring for 2 h, the solvent was removed and the residue washed with Et₂O several times and dried under vacuum to afford 13 with a yield of 71%.

Synthesis of compounds 14-15

Solid phase synthesis was used for the synthesis of lipidated lysine-lysine dipeptide derivatives (14-15). A brief discussion of the steps involved (Scheme 6) is given as under:
Scheme 6: x) DIC: HOBT, DCM; xi) TFE: AcOH: DCM (2:1:7); xii) TFA: DCM (1:9).

(16S)-13-{4-(tert-Butoxycarbonylamino)butyl}-2,2-dimethyl-16-(octanoyloxy)-4,12,15,19-tetraoxo-3,18-dioxa-5,11,14-triazahexacosane-10-carboxylic acid (14): Same procedure as that described for the synthesis of compound 4 was used. Here instead of myristic acid, dioctanoylated derivative of glyceric acid was used for lipidation. Yield of the compound was found to be 76%.

6-Amino-2-{6-amino-2-{((S)-2,3-bis(octanoyloxy)propanamido)hexanamido}hexanoic acid (15):

14a was dissolved in a mixture of TFA: DCM (1:9). After stirring for 2 h, the solvent was removed and the residue washed with Et2O several times and dried under vacuum to afford 15 with a yield of 56%.
Synthesis of compounds 16-18

Steps involved in the synthesis of compounds 16-18 are depicted in scheme 7.
2-Amino-6-\{(S)-2,3-bis(octanoyloxy)propanamido\}hexanoic acid (16):

\(1\epsilon\) (0.5 g, 1.028 mmol) was dissolved in DCM (20 mL) along with \(11\) (0.441 g, 1.233 mmol) and DMAP (catalytic amount). Next DCC (0.232 g, 1.126 mmol) was added. The solution was stirred for 18 h at ambient temperature. The reaction mixture was then filtered to remove the insoluble urea (DCU) followed by column chromatography (hexane: EtOAc; 7:3) to give \(16a\) as a gummy liquid. Activated zinc (0.021 g, 0.326 mmol) was added to a solution of \(16a\) (0.1 g, 0.163 mmol) in AcOH (5 mL). The solution was stirred for 4 h at ambient temperature. The reaction mixture was then filtered and the filtrate completely evaporated and dried under vacuum followed by column chromatography (hexane: EtOAc; 6:4) to give \(16b\) (78%). \(16b\) was dissolved in a solution of 10% piperidine in DMF (3 mL). After stirring for 1 h, the solvent was removed and the residue completely dried under vacuum to yield the product \(16\) (65%).

(20S)-10-Amino-2,2-dimethyl-20-(octanoyloxy)-4,11,19,23-tetraoxo-3,22-dioxa-5,12,18-triazatriacontane-13-carboxylic acid (17):

Same procedure as that described for the synthesis of compound 4 was followed. Here resin bound lysine moiety lipidated with dioctanoylated derivative of glyceric acid at \(\varepsilon\)-N-terminal was coupled with Boc protected lysine. Yield of the compound was found to be 69%.

(20S)-13-Amino-2,2-dimethyl-20-(octanoyloxy)-4,12,19,23-tetraoxo-3,22-dioxa-5,11,18-triazatriacontane-10-carboxylic acid (18):

Procedure similar to the one described for the synthesis of 4 was followed. Here resin bound Boc protected lysine was coupled with lysine moiety lipidated with dioctanoylated derivative of glyceric acid at \(\varepsilon\)-N-terminal. Yield of the compound was found to be 68%.

B.2. Synthesis of dioctanoylated ester derivatives using glycerol

A three step procedure wherein octanoylation of glycerol for the synthesis of dioctanoylated derivative of glycerol which was later used for C-terminal lipidation of lysine and lysine-lysine dipeptide, was followed. Steps involved are depicted in Scheme 8.
Scheme 8: xix) Imidazole (C₃H₄N₂), DMF; xx) C₇H₁₆COOH, DCC: DMAP, DCM; xxi) TBAF, THF.

3-tert-Butyldiphenyl silyl-sn-glycerol (20b):
Glycerol (19) (0.5 g, 5.43 mmol), tert-butyldiphenyl silane chloride (20a) (1.49 g, 5.43 mmol) and imidazole (0.36 g, 5.43 mmol) were dissolved in DMF. The reaction mixture was allowed to stir for 2 days. The solution was filtered and the solvent removed under reduced pressure. The crude material was purified by column chromatography (hexane: EtOAc; 8:2) to afford 20b as a white powder (92%).

¹H NMR (200 MHz, CDCl₃):  δ 1.07 (s, 9H, (CH₃)₃), 3.65-3.74 (m, 5H, (CH₂)₂, CH), 7.39-7.40 (m, 6H, (CH)₆), 7.66 (d, 4H, (CH)₄, J = 7.24 Hz).

Mass (ESI-MS): 353 (M⁺ + Na).

C, H analysis for C₁₉H₂₆O₃Si: Calculated C, 69.05; H, 7.93. Found C, 69.08; H, 7.94.
3-(tert-Butyldiphenylsilyloxy)propane-1,2-diyl dioctanoate (20c):

DCC (0.685 g, 3.3 mmol) was added to a solution of octanoic acid (0.47 g, 3.32 mmol), 20b (0.5 g, 1.51 mmol) and DMAP (catalytic amount) in DCM (20 mL). The solution was stirred for 18 h and then filtered to remove insoluble DCU precipitate. Concentration of the filtrate followed by column chromatography (hexane: EtOAc; 9:1) afforded 20c as a white powder (98%).

\[
\begin{align*}
\text{H NMR (500 MHz, CDCl}_3\text{):} & \delta 0.86 \text{ (t, 6H, (CH}_3)_2\text{, } J = 6.06 \text{ Hz), 1.04 \text{ (s, 9H, (CH}_3)_3\text{)}, 1.26 \text{ (broad m, 16H, (CH}_2)_8\text{)}, 1.59 \text{ (broad m, 4H, (CH}_2)_4\text{)}, 2.23\text{-}2.39 \text{ (m, 4H, (CH}_2)_2\text{)}, 3.67 \text{ (broad m, 1H, CH}_2\text{'\text{),} 3.76 \text{ (d, 1H, CH}_2\text{'\text{, } J = 5.12 \text{ Hz})}, 4.24 \text{ (d, 1H, CH}_2\text{'\text{, } J = 6.19 \text{ Hz})}, 4.37 \text{ (d, 1H, CH}_2\text{'\text{, } J = 3.90 \text{ Hz})}, 5.17 \text{ (m, 1H, CH), 7.38\text{-}7.44 \text{ (m, 6H, (CH}_6\text{)}, 7.65 \text{ (d, 4H, (CH}_4\text{), } J = 5.99 \text{ Hz).}
\end{align*}
\]

Mass (ESI-MS): 367 (M⁺ + Na).

C, H analysis for C₃₅H₅₄O₅Si:
Calculated C, 72.12; H, 9.34. Found C, 72.11; H, 9.36.

3-Hydroxypropane-1,2-diyl dioctanoate (20):

20c (0.5 g, 0.85 mmol) was dissolved in 40 mL of THF. Next tetrabutylammonium fluoride trihydrate (TBAF) (0.89 g, 3.4 mmol) was added and the reaction mixture allowed to stir for 1 h. After 1 h, the reaction was complete as indicated by TLC. The solution was diluted with 10 mL of H₂O and acidified with 1 N HCl to a pH of 3. The product was extracted by DCM, dried over Na₂SO₄ and rotoevaporated to dryness. The residue was purified by column chromatography (hexane: EtOAc; 8:2) to afford 20 as colorless oil (82%).

\[
\begin{align*}
\text{H NMR (200 MHz, CDCl}_3\text{):} & \delta 0.86 \text{ (t, 6H, (CH}_3)_2\text{, } J = 6.62 \text{ Hz), 1.28 \text{ (broad m, 16H, (CH}_2)_8\text{)}, 1.55 \text{ (m, 4H, (CH}_2)_4\text{)}, 2.29\text{-}2.38 \text{ (m, 4H, (CH}_2)_2\text{)}, 3.73 \text{ (d, 1H, CH}_2\text{'\text{, } J = 4.88 \text{ Hz})}, 3.89 \text{ (d, 1H, CH}_2\text{'\text{, } J = 5.01 \text{ Hz})}, 4.14\text{-}4.31 \text{ (m, 2H, CH}_2\text{)}, 5.09 \text{ (m, 1H, CH).}
\end{align*}
\]

Mass (ESI-MS): 605 (M⁺ + Na).

C, H analysis for C₁₉H₃₆O₅:
Calculated C, 66.25; H, 10.53. Found C, 66.23; H, 10.56.
Synthesis of compounds 21-24

C-terminal lipidation of lysine and lysine-lysine dipeptide using dioctanoylated derivative of glycerol, was carried out for synthesis of compounds 21-24 (Scheme 9).

Scheme 9: xxii) DCC: HOBT, DCM; xxiii) Piperidine: DMF (1:9); xxiv) TFA: DCM (1:9); xxv) FmocLys(Boc)-OH, DCC: HOBT, DCM.

(1a) + (20) → (21a)

(21a) → (21) → (22)

(21) → (23a) → (23) → (24)
3-{2-Amino-6-(tert-butoxycarbonylamino)hexanoyloxy}propane-1,2-diyl dioctanoate (21):
DCC (1.1 mmol) was added to a solution containing Fmoclys(Boc)-OH (1 mmol), 20c (1 mmol) and HOBT (catalytic amount) in DCM (20 mL). The solution was stirred for 18 h. The reaction mixture was then filtered to remove the insoluble urea (DCU) followed by column chromatography (hexane: EtOAc; 8:2) to give the product 21a as white powder (78%). 21a was dissolved in a solution of 10% piperidine in DMF (3 mL). After 2 h of stirring, the solvent was removed and the residue washed with Et2O several times and dried under vacuum to afford product 21 (72%).

3-(2,6-Diaminohexanoyloxy)propane-1,2-diyl dioctanoate (22):
21 was dissolved in a mixture of TFA: DCM (1:9). After 2 h of stirring, the solvent was removed and the residue washed with Et2O several times and dried under vacuum to afford product 22 (72%).

10-Amino-13-{4-(tert-butoxycarbonylamino)butyl}-2,2-dimethyl-4,11,14-trioxo-3,15-dioxa-5,12-diazaoctadecane-17,18-diyl dioctanoate (23):
DCC (0.043 g, 0.208 mmol) was added to a solution containing 21 (0.1 g, 0.174 mmol), 1a (0.097 g, 0.208 mmol) and HOBT (catalytic amount) in DCM (10 mL). The solution was stirred for 18 h at ambient temperature. The reaction mixture was then filtered to remove the insoluble urea (DCU) followed by column chromatography (hexane: EtOAc; 7:3) to give the product 23a (60%). 23a was dissolved in a solution of 10% piperidine in DMF (3 mL). After 2 h of stirring, the solvent was removed and the residue washed with Et2O several times and dried under vacuum to afford product 23 (72%).

3-{6-Amino-2-(2,6-diaminohexamido)hexanoyloxy}propane-1,2-diyl dioctanoate (24):
23 was dissolved in a mixture of TFA: DCM (1:9). After 2 h of stirring, the solvent was removed and the residue washed with Et2O several times and dried under vacuum to afford product 24 (72%).
1.2.3.3. Spectral data

2-Amino-6-octanamidohexanoic acid (1):

\[
\text{H}_2\text{N} \quad \text{HN} \quad \text{O} \quad \text{OH}
\]

Specific rotation \([\alpha]_{D}^{28}\) -2.307 (c 0.0052, CH\(_2\)Cl\(_2\)).

\(\lambda_{\text{max}}\) (cm): 231.29.

\(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta\) 0.85 (t, 3H, CH\(_3\), \(J = 6.10\) Hz), 1.26 (m, 10H, (CH\(_2\))\(_5\)), 1.67 (m, 6H, (CH\(_2\))\(_3\)), 2.31 (t, 2H, CH\(_2\), \(J = 7.97\) Hz), 3.15 (broad m, 2H, CH\(_2\)), 3.49 (m, 1H, CH).

IR (CHCl\(_3\), cm\(^{-1}\)): 800, 840, 1022, 1134, 1201, 1316, 1408, 1513, 1619, 1678, 2337, 2361, 2854, 2917, 2924, 3350.

Mass (ESI-MS): 273 (M\(^+\) + H).

C, H, N analysis for C\(_{14}\)H\(_{28}\)N\(_2\)O\(_3\):

Calculated C, 61.73; H, 10.36; N, 10.28. Found C, 61.64; H, 10.41; N, 10.33.

6-\(\text{tert}\)-Butoxycarbonylamino-2-octanamidohexanoic acid (2):

\[
\text{HN} \quad \text{N}
\]

Specific rotation \([\alpha]_{D}^{28}\) -0.740 (c 0.0243, CH\(_2\)Cl\(_2\)).

\(\lambda_{\text{max}}\) (cm): 281.46.

\(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta\) 0.87 (t, 3H, CH\(_3\), \(J = 6.63\) Hz), 1.25 (broad m, 10H, (CH\(_2\))\(_3\)), 1.42 (broad m, 11H, (CH\(_3\))\(_3\), CH\(_2\)), 1.58 (m, 2H, CH\(_2\)), 2.07 (m, 2H, CH\(_2\)), 2.22 (d, 2H, CH\(_2\), \(J = 7.18\) Hz), 2.96 (m, 2H, CH\(_2\)), 4.45 (m, 1H, CH).
IR (KBr, cm\(^{-1}\)): 799, 1072, 1127, 1167, 1381, 1462, 1534, 1559, 1598, 1618, 1699, 1726, 2872, 2929, 2961, 3339.

Mass (ESI-MS): 401 (M\(^{+}\) + K).

C, H, N analysis for C\(_{19}\)H\(_{36}\)N\(_2\)O\(_5\):

Calculated C, 61.26; H, 9.74; N, 7.52. Found C, 61.22; H, 9.79; N, 7.54.

2,6-bis(Octanamido)hexanoic acid (3):

\[
\text{HN} \quad \text{OO}
\]

Specific rotation \([\alpha]_D^{28}\) +4.166 (c 0.0072, CH\(_2\)Cl\(_2\)).

\(\lambda_{\text{max}}\) (cm): 254.01.

\(^1\)H NMR (200 MHz, CDCl\(_3\)):

\(\delta\) 0.88 (t, 6H, (CH\(_3\))^2, \(J = 6.54\) Hz), 1.19-1.45 (m, 18H, (CH\(_2\))^9), 1.65-2.29 (m, 8H, (CH\(_2\))^4), 2.29-2.41 (m, 4H, (CH\(_2\))^2), 3.37 (t, 2H, CH\(_2\), \(J = 6.91\) Hz), 4.51-4.53 (m, 1H, CH).

IR (KBr, cm\(^{-1}\)):

800, 839, 1020, 1135, 1183, 1203, 1423, 1457, 1678, 2850, 2917, 2955, 3420.

Mass (ESI-MS): 437 (M\(^{+}\) + K).

C, H, N analysis for C\(_{22}\)H\(_{42}\)N\(_2\)O\(_4\):

Calculated C, 66.29; H, 10.62; N, 7.03. Found C, 66.22; H, 10.64; N, 7.09.
6-tert-Butoxycarbonylamino-2-(6-tert-butoxycarbonylamino-2-tetradecnoylamino-hexanoylamo)-hexanoic acid (4):

Specific rotation $\left[\alpha\right]_{D}^{28}$: -19.178 (c 0.0073, CH$_2$Cl$_2$).

$\lambda$ max (cm): 229.331.

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 0.89 (t, 3H, CH$_3$, $J$ = 5.86 Hz), 1.14-1.85 (m, 52H, (CH$_3$)$_6$, (CH$_2$)$_{17}$), 2.23 (t, 2H, CH$_2$, $J$ = 7.26 Hz), 3.08 (broad m, 4H, (CH$_2$)$_2$), 4.56 (broad m, 1H, CH), 4.98 (broad m, 1H, CH).

$^{13}$C NMR (500 MHz, CDCl$_3$): $\delta$ 13.99, 19.56, 22.16, 22.64, 25.06, 28.60, 28.96, 29.20, 29.78, 29.89, 31.75, 32.00, 34.38, 42.34, 42.39, 55.36, 79.87, 157.19, 168.09.

IR (KBr, cm$^{-1}$): 801, 1020, 1095, 1172, 1259, 1366, 1392, 1458, 1534, 1654, 1689, 2854, 2925, 3313.

Mass (ESI-MS): 683.6 (M$^+$ - H).

C, H, N analysis for C$_{36}$H$_{68}$N$_4$O$_8$: Calculated C, 63.13; H, 10.01; N, 8.18. Found C, 63.06; H, 10.06; N, 8.26.
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6-tert-Butoxycarbonylamino-2-(6-tert-butoxycarbonylamino-2-octanoylaminohe-xanoylamino)-hexanoic acid (5):

![Chemical structure of compound 5]

Specific rotation $[\alpha]_D^{28}$: -8.0 (c 0.005, CH$_2$Cl$_2$).

λ$_{\text{max}}$ (cm): 242.07.

$^1$H NMR (200 MHz, CDCl$_3$): δ 0.85 (t, 3H, CH$_3$, $J = 6.56$ Hz), 1.28-1.86 (m, 40H, (CH$_3$)$_6$, (CH$_2$)$_{11}$), 2.23 (t, 2H, CH$_2$, $J = 7.08$ Hz), 3.09 (broad m, 4H, (CH$_2$)$_2$), 4.89 (broad m, 1H, CH), 4.90 (broad m, 1H, CH).

$^{13}$C NMR (500 MHz, CDCl$_3$): δ 13.57, 22.11, 22.50, 23.24, 27.95, 28.43, 29.85, 31.21, 38.22, 52.28, 76.72, 151.95, 167.30.

IR (KBr, cm$^{-1}$): 743, 1020, 1073, 1124, 1173, 1262, 1392, 1458, 1526, 1690, 1725, 2859, 2927, 2959, 3314.

Mass (ESI-MS): 623 (M$^+$ + Na).

C, H, N analysis for C$_{30}$H$_{56}$N$_4$O$_8$:


6-Amino-2-(6-amino-2-octanoylamino-hexanoylamino)-hexanoic acid (6):

![Chemical structure of compound 6]

Specific rotation $[\alpha]_D^{28}$: +50.52 (c 0.0019, CH$_2$Cl$_2$).

λ$_{\text{max}}$ (cm): 242.03.
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1H NMR (200 MHz, CDCl3): \( \delta 0.83 \) (t, 3H, \( \text{CH}_3 \), \( J = 6.19 \) Hz), 1.11-1.55 (m, 12H, (CH\(_2\))\(_6\)), 1.97-2.30 (m, 12H, (CH\(_2\))\(_6\)), 3.44 (m, 2H, CH\(_2\)), 3.61 (m, 2H, CH\(_2\)), 5.25 (m, 2H, (CH\(_2\))\(_2\)).

IR (CHCl\(_3\), cm\(^{-1}\)): 799, 1019, 1092, 1169, 1260, 1384, 1458, 1574, 1616, 1684, 2854, 2925, 2963, 3338.

Mass (ESI-MS): 401.3 (M\(^+\) + H).


2-(2,6-Dioctanamidohexanamido)-6-octanamidohexanoic acid (7):

Specific rotation \([\alpha]_D^{28}\) -21.25 (c 0.004, CH\(_2\)Cl\(_2\)).

\( \lambda_{\text{max}} \) (cm): 256.65.

1H NMR (200 MHz, CDCl\(_3\)): \( \delta 0.85 \) (broad t, 9H, (CH\(_3\))\(_3\), \( J = 6.56 \) Hz), 1.11-1.24 (m, 28H, (CH\(_2\))\(_{14}\)), 1.59-1.84 (broad m, 10H, (CH\(_2\))\(_3\)), 2.01 (m, 4H, (CH\(_2\))\(_2\)), 2.16-2.38 (broad m, 6H, (CH\(_2\))\(_3\)), 2.83 (m, 4H, (CH\(_2\))\(_2\)), 4.89 (broad m, 1H, CH), 4.90 (broad m, 1H, CH), 7.37 (broad m, 4H, 4 \( \times \) NH).

IR (KBr, cm\(^{-1}\)): 799, 1020, 1094, 1202, 1260, 1384, 1409, 1455, 1578, 1675, 1695, 2854, 2925, 2962, 3337.

Mass (ESI-MS): 653 (M\(^+\) + H).

C, H, N analysis for C\(_{36}\)H\(_{68}\)N\(_4\)O\(_6\): Calculated C, 66.22; H, 10.50; N, 8.58. Found C, 66.29; H, 10.44; N, 8.53.
2-(6-Amino-2-octanamidohexanamido)-6-octanamidohexanoic acid (8):

Specific rotation $[\alpha]_D^{28}$ -11.616 (c 0.0031, CH$_2$Cl$_2$).

$\lambda$ max (cm): 249.00.

$^1$H NMR (200 MHz, CDCl$_3$): 
$\delta$ 0.90 (t, 6H, (CH$_3$)$_2$, $J = 6.56$ Hz), 1.20-1.27 (m, 20H, (CH$_2$)$_{10}$), 1.40 (s, 9H, (CH$_3$)$_3$), 1.55-1.69 (broad m, 12H, (CH$_2$)$_6$), 2.03-2.10 (broad m, 4H, (CH$_2$)$_2$), 2.84 (m, 2H, CH$_2$), 3.40 (m, 2H, CH$_2$), 4.86 (broad m, 1H, CH), 4.91 (broad m, 1H, CH), 7.37 (broad m, 4H, 4 $\times$ NH).

IR (KBr, cm$^{-1}$): 745, 1040, 1073, 1124, 1274, 1381, 1463, 1580, 1600, 1728, 2858, 2926, 2959, 3407.

Mass (ESI-MS): 627 (M$^+$ + H).

C, H, N analysis for C$_{33}$H$_{62}$N$_4$O$_7$: Calculated C, 63.23; H, 9.97; N, 8.94. Found C, 63.28; H, 9.93; N, 8.96.

6-Octanoylamino-2-(2-octanoylamino-acetylamino)-hexanoic acid (9):

Specific rotation $[\alpha]_D^{28}$ -5 (c 0.0008, CH$_2$Cl$_2$).

$\lambda$ max (cm): 228.50.

$^1$H NMR (200 MHz, CDCl$_3$): 
$\delta$ 0.87 (t, 6H, (CH$_3$)$_3$, $J = 6.11$ Hz), 1.25-1.41 (m, 18H, (CH$_2$)$_9$), 1.62 (broad m, 6H, (CH$_2$)$_3$), 1.84 (broad m, 2H, CH$_2$), 2.42 (m, 4H, (CH$_2$)$_2$), 3.18
(broad m, 2H, CH₂), 4.10 (m, 2H, CH₂), 4.56 (broad m, 1H, CH).

IR (KBr, cm⁻¹): 722, 800, 1020, 1094, 1158, 1186, 1260, 1384, 1409, 1559, 1672, 1696, 2854, 2924, 2957, 3345.

Mass (ESI-MS): 455 (M⁺ + H).


(13R)-2,2-Dimethyl-13-(octanoyloxy)-4,12,16-trioxo-3,15-dioxa-5,11-diazatricosane-10-carboxylic acid (12):

Specific rotation [α]D²⁸  +67.16 (c 0.0006, CH₂Cl₂).

λmax (cm): 260.96.

¹H NMR (200 MHz, CDCl₃): δ 0.87 (t, 6H, (CH₃)₂, J = 8.02 Hz), 1.23 (m, 2H, CH₂), 1.26-1.43 (broad m, 27H, (CH₃)₃, (CH₂)₉), 1.59 (m, 4H, (CH₂)₂), 1.74 (m, 2H, CH₂), 2.04-2.21 (m, 4H, (CH₂)₂), 3.07 (broad m, 2H, CH₂), 4.43 (broad m, 2H, CH₂⁻, CH), 4.96 (broad m, 1H, CH₂⁻), 5.37 (broad m, 1H, CH), 7.00 (broad m, 2H, 2 × NH).

IR (KBr, cm⁻¹): 799, 1019, 1095, 1172, 1260, 1403, 1458, 1534, 1654, 1690, 2856, 2926, 2958, 3315.

Mass (ESI-MS): 625 (M⁺ + K).

6-Amino-2-{(R)-2,3-bis(octanoyloxy)propanamido}hexanoic acid (13):

Specific rotation $[\alpha]_{D}^{28}$: -27.3 (c 0.001, CH$_2$Cl$_2$).
$\lambda_{\text{max}}$ (cm): 252.16.

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 0.88 (t, 6H, (CH$_3)_2$, $J = 8.02$ Hz), 1.23 (m, 2H, CH$_2$), 1.27-1.41 (broad m, 18H, (CH$_2$)$_9$), 1.55 (m, 4H, (CH$_2$)$_2$), 1.74 (m, 2H, CH$_2$), 2.04-2.21 (m, 4H, (CH$_2$)$_2$), 2.65 (broad m, 2H, CH$_2$), 4.43 (broad m, 2H, CH$_2$), 4.56 (t, 1H, CH, $J = 7.1$ Hz), 5.50 (broad m, 1H, CH).

IR (CHCl$_3$, cm$^{-1}$): 800, 1021, 1083, 1260, 1402, 1458, 1743, 2853, 2924, 2957, 3125.

Mass (ESI-MS): 486 (M$^+$ + H).

C, H, N analysis for C$_{25}$H$_{46}$N$_2$O$_7$:
Calculated C, 61.70; H, 9.53; N, 5.76. Found C, 61.76; H, 9.59; N, 5.78.

(16S)-13-{4-(tert-Butoxycarbonylamino)butyl}-2,2-dimethyl-16-(octanoyloxy)-4, 12,15,19-tetraoxo-3,18-dioxa-5,11,14-triazahexacosane-10-carboxylic acid (14):

Specific rotation $[\alpha]_{D}^{28}$: -28.36 (c 0.0011, CH$_2$Cl$_2$).
$\lambda_{\text{max}}$ (cm): 244.86.

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 0.83 (t, 6H, (CH$_3)_2$, $J = 7.81$ Hz), 1.26 (m, 4H, (CH$_2$)$_2$), 1.27-1.41 (broad m, 38H, (CH$_3$)$_6$, (CH$_2$)$_{10}$),
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\[ \text{1.59 (m, 2H, CH}_2\text{), 1.74 (m, 4H, (CH}_2\text{)_2), 2.01-2.04 (m, 4H, (CH}_2\text{)_2), 2.21 (broad m, 4H, (CH}_2\text{)_2), 3.07 (m, 2H, CH}_2\text{), 4.43 (m, 4H, CH}_2\text{´, CH}_2\text{˝, CH, CH), 4.96 (broad m, 1H, CH).} \]

\[ \text{^13C NMR (500 MHz, CDCl}_3\text{: } \delta\text{ 13.03, 21.58, 23.77, 24.69, 27.43, 27.89, 27.91, 27.97, 28.00, 28.05, 28.22, 30.63, 30.65, 30.69, 40.12, 52.60, 62.87, 71.68, 71.55, 152.76, 171.68, 177.27.} \]

\[ \text{IR (KBr, cm}^{-1}\text{: 757, 1091, 1171, 1251, 1366, 1393, 1456, 1522, 1683, 2284, 2857, 2928, 3336.} \]

\[ \text{Mass (ESI-MS): 814.1 (M}^+\text{).} \]

\[ \text{C, H, N analysis for } \text{C}_4\text{H}_7\text{aN}_4\text{O}_{12}\text{: Calculated C, 60.42; H, 9.15; N, 6.87. Found C, 60.41; H, 9.12; N, 6.89.} \]

\[ \text{6-Amino-2-\{6-amino-2-((S)-2,3-bis(octanoyloxy)propanamido)hexanamido\}hexanoic acid (15):} \]

\[ \text{Specific rotation } [\alpha]_D^{28} \text{ -44.57 (c 0.0007, CH}_2\text{Cl}_2\text{).} \]

\[ \text{\lambda_{max} (cm): 245.86.} \]

\[ \text{^1H NMR (200 MHz, CDCl}_3\text{: } \delta\text{ 0.88 (t, 6H, (CH}_3\text{)_2), 1.22 (m, 4H, (CH}_2\text{)_2), 1.25-1.52 (broad m, 20H, (CH}_2\text{)_10), 1.63 (m, 4H, (CH}_2\text{)_2), 1.74-1.79 (m, 4H, (CH}_2\text{)_2), 2.04-2.21 (m, 4H, (CH}_2\text{)_2), 2.61 (broad m, 4H, (CH}_2\text{)_2), 4.42-4.58 (broad m, 4H, CH}_2\text{´, CH}_2\text{˝, CH, CH), 5.47 (t, 1H, CH, } J = 7.0 \text{ Hz).} \]

\[ \text{IR (CHCl}_3\text{, cm}^{-1}\text{: 801, 1021, 1403, 1675, 2361, 2853, 2924, 2961, 3131.} \]

\[ \text{Mass (ESI-MS): 615 (M}^+\text{ + H).} \]

76
C, H, N analysis for
C\textsubscript{31}H\textsubscript{58}N\textsubscript{4}O\textsubscript{8}: Calculated C, 60.56; H, 9.51; N, 9.11. Found C, 60.51; H, 9.55; N, 9.14.

2-Amino-6-{(S)-2,3-bis(octanoyloxy)propanamido}hexanoic acid (16):

![Chemical structure of the compound](image)

Specific rotation $\left[\alpha\right]_D^{28} \quad -26.00 (c \ 0.0008, \text{CH}_2\text{Cl}_2)$.

$\lambda_{\text{max}}$ (cm): 237.38.

$^1\text{H NMR}$ (200 MHz, CDCl$\textsubscript{3}$):
$\delta$ 0.88 (t, 6H, (CH$_3)_2$, $J = 8.5$ Hz), 1.19-1.27 (m, 14H, (CH$_2$)$_7$), 1.32 (m, 4H, (CH$_2$)$_2$), 1.55 (m, 2H, CH$_2$), 1.67 (m, 4H, (CH$_2$)$_2$), 1.77 (m, 2H, CH$_2$), 2.05 (t, 2H, CH$_2$, $J = 7.56$ Hz), 2.28 (t, 2H, CH$_2$, $J = 7.1$ Hz), 3.38 (m, 2H, CH$_2$), 3.73 (broad m, 1H, CH), 4.32 (broad m, 1H, CH$_2$), 4.72 (broad m, 1H, CH$_2$), 5.55 (t, 1H, CH, $J = 7.0$ Hz).

IR (CHCl$_3$, cm$^{-1}$): 758, 1027, 1079, 1161, 1433, 1455, 1585, 2140, 2413, 2512, 2738, 2807, 2855, 2926, 2949, 3446.

Mass (ESI-MS): 487 (M$^+$ + H).

C, H, N analysis for
C\textsubscript{25}H\textsubscript{46}N\textsubscript{2}O\textsubscript{7}: Calculated C, 61.70; H, 9.53; N, 5.76. Found C, 61.72; H, 9.54; N, 5.78.
(20S)-10-Amino-2,2-dimethyl-20-(octanoyloxy)-4,11,19,23-tetraoxo-3,22-dioxa-5, 12,18-triazatriacontane-13-carboxylic acid (17):

Specific rotation \([\alpha]_D^{28}\) +36.83 (c 0.0006, CH$_2$Cl$_2$).

\(\lambda_{\text{max}}\) (cm): 236.42.

$^1$H NMR (200 MHz, CDCl$_3$):

\(\delta\) 0.87 (t, 6H, (CH$_3$)$_2$, \(J = 8.01\) Hz), 1.25 (broad m, 20H, (CH$_2$)$_{10}$), 1.43 (broad m, 13H, (CH$_3$)$_3$, (CH$_2$)$_2$), 1.63 (broad m, 4H, (CH$_2$)$_2$), 1.77 (broad m, 4H, (CH$_2$)$_2$), 2.17 (t, 2H, CH$_2$, \(J = 7.10\) Hz), 2.32 (t, 2H, CH$_2$, \(J = 6.70\) Hz), 3.18-3.35 (broad m, 5H, (CH$_2$)$_2$, CH), 4.41 (m, 3H, CH$_2$, CH), 5.50 (t, 1H, CH, \(J = 7.0\) Hz).

IR (CHCl$_3$, cm$^{-1}$): 799, 1019, 1084, 1260, 1403, 1458, 1636, 2359, 2853, 2924, 2959, 3166.

Mass (ESI-MS): 753 (M$^+$ + K).

C, H, N analysis for C$_{36}$H$_{66}$N$_4$O$_{10}$: Calculated C, 60.48; H, 9.31; N, 7.84. Found C, 6.44; H, 9.33; N, 7.86.
(20S)-13-Amino-2,2-dimethyl-20-(octanoyloxy)-4,12,19,23-tetraoxo-3,22-dioxa-5,11,18-triazatriacontane-10-carboxylic acid (18):

Specific rotation $[\alpha]_{D}^{28}$ +27.85 (c 0.0007, CH$_2$Cl$_2$).

$\lambda_{\text{max}}$ (cm):
233.67.

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 0.87 (t, 6H, (CH$_3$)$_2$, $J$ = 7.98 Hz), 1.25 (broad m, 20H, (CH$_2$)$_{10}$), 1.42 (broad m, 13H, (CH$_3$)$_3$, (CH$_2$)$_2$), 1.58 (broad m, 4H, (CH$_2$)$_2$), 1.77 (broad m, 4H, (CH$_2$)$_2$), 2.07-2.27 (m, 4H, (CH$_2$)$_2$), 2.97 (m, 2H, CH$_2$), 3.22 (m, 2H, CH$_2$), 3.31 (m, 1H, CH), 4.46-4.62 (m, 3H, CH$_2$, CH), 5.32 (t, 1H, CH, $J$ = 7.12 Hz).

IR (CHCl$_3$, cm$^{-1}$): 668, 759, 1102, 1166, 1255, 1397, 1457, 1648, 1704, 2303, 2855, 2926, 3399.

Mass (ESI-MS): 753 (M$^+$ + K).

C, H, N analysis for C$_{36}$H$_{66}$N$_4$O$_{10}$: Calculated C, 60.48; H, 9.31; N, 7.84. Found C, 60.46; H, 9.30; N, 7.87.

3-{2-Amino-6-((tert-butoxycarbonylamino)hexanoyloxy)propane-1,2-diyldioctanoate (21):

Specific rotation $[\alpha]_{D}^{28}$ +15.36 (c 0.0011, CH$_2$Cl$_2$).
\[ \delta 0.86 \text{ (t, 6H, (CH}_3)_2, J = 6.35 \text{ Hz), 1.27 (broad m, 18H, (CH}_2)_9, 1.43 (broad s, 9H, (CH}_3)_3, 1.56-1.63 (m, 6H, (CH}_2)_3, 1.92 (m, 2H, CH}_2), 2.27-2.38 (m, 4H, (CH}_2)_2, 2.58 (t, 2H, CH}_2, J = 7.9 \text{ Hz), 4.05-4.36 (m, 5H, (CH}_2)_2, CH), 5.27 (m, 1H, CH).} \]

IR (CHCl\textsubscript{3}, cm\textsuperscript{-1}): 733, 803, 1020, 1098, 1164, 1260, 1365, 1449, 1511, 1624, 1741, 2852, 2927, 3133, 3323.

Mass (ESI-MS): 595 (\text{M}^+ + \text{Na}).

C, H, N analysis for \( \text{C}_{30}\text{H}_{56}\text{N}_2\text{O}_8 \):

Calculated C, 62.91; H, 9.85; N, 4.89. Found C, 62.94; H, 9.86; N, 4.84.

3-(2,6-Diaminohexanoyloxy)propane-1,2-diyl dioctanoate (22):

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{H}_2\text{N} & \quad \text{O} \\
\text{O} & \quad \text{O}
\end{align*}
\]

Specific rotation [\( \alpha \)]\textsubscript{D}28 52.00 (c 0.0004, CH\textsubscript{2}Cl\textsubscript{2}).

\[ \delta 0.86 \text{ (t, 6H, (CH}_3)_2, J = 6.32 \text{ Hz), 1.24-1.27 (broad m, 18H, (CH}_2)_9, 1.53-1.61 (m, 6H, (CH}_2)_3, 1.90 (m, 2H, CH}_2), 2.20-2.61 (m, 6H, (CH}_2)_3, 3.45 (broad m, 1H, CH), 4.22-4.47 (m, 4H, (CH}_2)_2, 5.16 (broad m, 1H, CH).} \]

IR (CHCl\textsubscript{3}, cm\textsuperscript{-1}): 723, 758, 800, 1136, 1202, 1455, 1676, 2360, 2856, 2927, 3395.

Mass (ESI-MS): 472 (\text{M}^+).

C, H, N analysis for \( \text{C}_{25}\text{H}_{48}\text{N}_2\text{O}_6 \):

Calculated C, 63.53; H, 10.24; N, 5.93. Found C, 63.57; H, 10.25; N, 5.98.
10-Amino-13-{4-(tert-butoxycarbonylamino)butyl}-2,2-dimethyl-4,11,14-trioxo-3,15-dioxa-5,12-diazaoctadecane-17,18-diyldioctanoate (23):

Specific rotation $[\alpha]_D^{28} +13$ (c 0.001, CH$_2$Cl$_2$).

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 0.86 (t, 6H, (CH$_3)_2$, $J = 6.73$ Hz), 1.23-1.29 (broad m, 20H, (CH$_2$)$_{10}$), 1.37 (s, 18H, (CH$_3$)$_6$), 1.54-1.86 (m, 12H, (CH$_2$)$_6$), 2.31 (m, 4H, (CH$_2$)$_2$), 3.12-3.34 (broad m, 5H, (CH$_2$)$_2$, CH), 4.08-4.42 (broad m, 5H, (CH$_2$)$_2$, CH), 5.26 (broad m, 1H, CH).

IR (CHCl$_3$, cm$^{-1}$): 637, 865, 1129, 1169, 1248, 1462, 1522, 1572, 1619, 1682, 2283, 2928, 2966, 3341.

Mass (ESI-MS): 801 (M$^+$ + H).

C, H, N analysis for C$_{41}$H$_{76}$N$_4$O$_{11}$: Calculated C, 61.47; H, 9.56; N, 6.99. Found C, 61.44; H, 9.56; N, 6.96.

3-{6-Amino-2-(2,6-diaminohexanamido)hexanoyloxy}propane-1,2-diyldioctanoate (24):

Specific rotation $[\alpha]_D^{28} -20.22$ (c 0.0009, CH$_2$Cl$_2$).

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 0.87 (t, 6H, (CH$_3)_2$, $J = 7.02$ Hz), 1.24-1.31 (broad m, 20H, (CH$_2$)$_{10}$), 1.52 (m, 4H, (CH$_2$)$_2$), 1.61-1.86 (m, 8H, (CH$_2$)$_4$), 2.33-2.59 (broad m, 8H,
(CH₂)₄, 3.36 (broad m, 1H, CH), 4.14-4.47 (broad m, 5H, (CH₂)$_₂$, CH), 5.14 (broad m, 1H, CH).

IR (CHCl₃, cm⁻¹):
635, 755, 1168, 1202, 1458, 1572, 1614, 1672, 2856, 2927, 2964, 3338.

Mass (ESI-MS):
623 (M$^+$ + Na).

C, H, N analysis for C$_{31}$H$_{60}$N$_4$O$_7$:
Calculated C, 61.97; H, 10.07; N, 9.32. Found C, 61.95; H, 10.04; N, 9.36.

1.2.3.4. Biological Experiments
Initially all the synthesized lipidated amino acids and dipeptides were screened for adjuvant activity (effect on splenocyte proliferation) against muramyl dipeptide and control at the doses of 0.01, 0.1, 1 and 10 μg under in vitro conditions. Among all these compounds, the three most active compounds (4, 5, & 14) were further studied against MDP and control to check their effect on OVA-specific IgG, IgG1 and IgG2a antibodies, splenocyte proliferation (ex vivo), release of Th1 and Th2 cytokines and effect on T-cell sub population. Finally these three selected compounds were subjected to safety studies.

Animals
The study was conducted on male BALB/c mice (18–22 g). The ethical committee of the Indian Institute of Integrative Medicine (IIIM, CSIR) instituted for animal handling approved all the protocols. The animals were housed under standard laboratory conditions (temperature 25 ± 2 °C and 12 h dark/light cycles) and fed with commercial standard pellet diet and tap water ad libitum.

Immunizations and treatment
Animals were immunized subcutaneously with OVA (100 μg) alone dissolved in saline on days 1 and 15. MDP (20 μg) along with OVA (100 μg) was used as positive control throughout the study. In case of ex vivo studies to identify the potential immunoadjuvant, splenocytes from immunized mice were isolated and treated with various concentrations (0.01, 0.1, 1 and 10 μg) of lipidated lysine derivatives along with ovalbumin. For in vivo studies of the active derivatives i.e., compounds 4, 5 and 14, mice were treated with various concentrations (1, 10 and 100 μg) of these compounds along with OVA on the days of immunization. Saline-treated animals
were included as untreated group. A challenging injection was given 2 weeks later. To estimate the expression of cytokine and immunoglobulins, serum samples were taken at various time periods. Effect on Th1/Th2 cytokine expression, T-cell proliferation (CD4⁺, CD8⁺) and co-stimulatory molecules (CD80/CD86) was observed on day 28 after first immunization while immunoglobulin (IgG observed on days 15, 28 and 60) and its isotypes (IgG1 and IgG2a estimated on days 15 and 28) were used to observe initial as well as memory recall response. All experiments were repeated twice.

**Measurement of OVA-specific antibody titre**

OVA-specific IgG, IgG1 and IgG2a antibodies in serum were detected by an indirect ELISA. In brief, microtiter plate wells were coated with 100 μL OVA solution (50, 25 and 50 μg/mL for IgG, IgG1 and IgG2a antibodies, respectively, in 50 mM carbonate–bicarbonate buffer, pH 9.6) for 24 h at 4 °C. The wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS/Tween 20) and then blocked with 5% FCS/PBS at 37 °C for 1 h. After three washings, 100 μL of diluted serum sample (IgG, 1:400; IgG1, 1:100; IgG2b, 1:200) or 0.5% FCS/PBS as control was added to triplicate wells. The plates were then incubated for 1 h at 37 °C, followed by three washings. Aliquots of 100 μL of rabbit anti-mouse IgG horseradish peroxidase conjugate diluted 1:50,000, or goat anti-mouse IgG1 peroxidase conjugate 1:16,000 or IgG2a peroxidase conjugate 1:8000 with 0.5% FCS/PBS were added to each plate. The plates were further incubated for 1 h at 37 °C. After washing, the peroxidase activity was assayed as following: 100 μL of substrate solution (10 mg of o-phenylenediamine and 37.5 μL of 30% H₂O₂ in 25 mL of 0.1 M citrate–phosphate buffer, pH 5.0) was added to each well. The plate was incubated for 10 min at 37 °C and enzyme reaction was terminated by adding 50 μL/well of 2 N H₂SO₄. The optical density (OD) was measured in an ELISA reader at 450 nm.

**Splenocyte isolation and proliferation assay**

Spleen was collected from the immunized BALB/c mice under aseptic conditions and suspended in complete medium RPMI containing 10% FCS for further use. Splenocytes were seeded into 4–5 wells of a 96 well flat-bottom microtiter plate at 5×10⁶ cells/mL in 100 μL complete medium, thereafter OVA (final concentration 10 μg/well), or RPMI-1640 medium was added giving a final volume of 200 μL. The plates were incubated at 37 °C in a humid atmosphere with 5% CO₂. After 72 h, 20
μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h. The plates were centrifuged (1400 × g, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well, 200 μL of a DMSO working solution (180 μL DMSO with 20 μL 1 N HCl) was added and the absorbance evaluated in an ELISA reader at 570 nm after 15 min.

Flow cytometric analysis

I) Lymphocyte immunophenotyping in spleen
The spleen (one-third of the organ) was placed in PBS buffer (without Mg²⁺ and Ca²⁺) and stored on ice prior to preparation of single cell suspensions. Splenic erythrocytes were lysed with red blood cell lysing buffer (BD Pharmingen). Cell suspensions were refrigerated at 4 °C and stained with antibodies. For each sample, 2 × 10⁶ cells were stained with conjugated anti-CD4 FITC and anti-CD8a PE antibodies. After staining with antibodies, cells were washed and resuspended in PBS for flow cytometric analysis, which was performed on a FACS Calibur flow cytometer equipped with Cell Quest software (Becton Dickinson).

II) Estimation of intracellular Th1/Th2 cytokines
Spleen cells (2 × 10⁶ cells/mL) were collected in tubes and Golgi plug (1 μL/mL) was added. 100 μL of the spleen cells were taken in the different falcon tubes. 500 μL of permeabilizing solution (BD Biosciences) was added to each tube. FITC labeled antimouse CD4⁺ T-cell marker and phycoerythrin (PE) labeled IFN-γ and IL-4 monoclonal antibodies were used in two different sets. The tubes were incubated in dark for 30 min at room temperature. 2 mL of 1X FACS lysing solution was then added with gentle mixing followed by incubation for 10 min at room temperature. The samples were centrifuged (300–400 g) for 10 min. The supernatant was aspirated and the sample was resuspended in 500 μL of PBS. Acquisition and the analysis were carried out on flow cytometer using Cell Quest Pro software (BD Biosciences).

III) Flow cytometric analysis of co-stimulatory molecules
For the estimation of co-stimulatory molecules, the single cell suspension of splenocytes was washed twice in PBS. Cells were suspended in RPMI-1640 medium after removing the red blood cell by RBC lysis buffer. 100 μL of splenocytes at 2.0 × 10⁶ cells/mL were stained with FITC labeled anti-CD80 (B7-1) and phycoerythrin (PE) labeled anti-CD86 (B7-2) monoclonal antibodies for antigen presenting cells.
Thereafter the cells were kept in dark for 30 min at 4 °C. After staining, the cells were washed twice with PBS and volume raised up to 500 μL for FACs analysis. Acquisition and the analysis were carried out on flow cytometer using Cell Quest Pro software (BD Biosciences).

**Safety studies**

Safety of the proposed adjuvants *i.e.*, compounds 4, 5 and 14 with weak antigen OVA was proven by their subcutaneous injection to mice. BALB/c mice were divided into groups of 10 animals each and were inoculated with variable doses of compounds 4, 5 and 14 which had been re-suspended in PBS. Control mice, received PBS. After injection, mice were observed daily for a period of 4 weeks. Deviations from their normal behaviour or reactions at the injection site were recorded. The weight of the mice was taken on days 0, 7, 14 and 28 post injection. The weight of the mice as recorded on days 0, 7, 14 and 28 is shown in table 1.

**1.2.4. Results**

**Effect of compounds on splenocyte proliferation (*in vitro*)**

Among all the compounds screened, compounds 4, 5, and 14 significantly enhanced the splenocyte proliferation in comparison to MDP (*Figure 3*). Rest of the compounds did not show any significant enhancement.
Figure 3: Effect of test compounds on mitogen and OVA-stimulated splenocyte proliferation in vitro. Splenocytes were prepared and cultured with Con A (0.25 µg/well), LPS (0.25 µg/well) and OVA (10 µg/well) for 72 h. Splenocyte proliferation was measured by the MTT method. The optical density (OD) at 570 nm was measured and the values are presented as mean ± S.E. (n = 5). Significant differences between the control and treated groups were determined by Bonferroni multiple comparison test and designated as *P < 0.05, **P < 0.01 and *** P< 0.001.

Effect of compounds 4, 5 and 14 on OVA-specific IgG, IgG1 and IgG2a antibody titer

OVA-specific IgG, IgG1 and IgG2a antibody titers in the serum were measured by indirect ELISA as shown in figures 4 and 5. The serum IgG titer in OVA-immunized mice was markedly enhanced by compounds 4, 5 and 14 at a dose of 100 µg, 10 µg and 1 µg as compared to control. Compound 14 was found to be more effective as compared to compounds 4 and 5 at lower dose of 1 µg. Significant enhancements in OVA-specific serum IgG1 titers were observed in compounds 4, 5 and 14 immunized mice compared with control group. Here again compound 14 at a dose of 1 µg was found to be comparatively more active than compounds 4 and 5. Furthermore, compound 14 at a dose of 1 µg significantly enhanced the OVA specific serum IgG2a
titers as compared to compounds 4 and 5 which enhanced the IgG2a titer at a dose of 100 \( \mu g \) and 10 \( \mu g \) respectively.

Figure 4: Effect of compounds 4, 5 and 14 on OVA-specific IgG antibody titre. Groups of five male BALB/c mice were immunized s.c with OVA 100 \( \mu g \) dissolved in saline containing MDP (20 \( \mu g \)) or compounds 4, 5 and 14 (1, 10 and 100 \( \mu g \)) on days 1 and 15. Sera were collected two weeks after the last immunization. OVA-specific IgG antibodies in the sera were measured by an indirect ELISA method. The optical density (OD) at 450 nm was measured and the values are presented as mean ± S.E. (n = 5). The difference between the control and treated groups is determined by Bonferroni multiple comparison test and were designated as *\( P < 0.05 \), **\( P < 0.01 \) and ***\( P < 0.001 \).
Figure 5: Immunoglobulin isotype viz., IgG1 and IgG2a analysis in sera. Groups of five male BALB/c mice were immunized s.c. with OVA 100 μg dissolved in saline containing MDP (20 μg) or compounds 4, 5 and 14 (1, 10 and 100 μg) on Days 1 and 15. Sera were collected two weeks after the last immunization. OVA-specific IgG1 and IgG2a antibodies in the sera were measured by an indirect ELISA method. The optical density (OD) at 450 nm was measured and the values are presented as mean ± S.E. (n = 5). The difference between the control and treated groups was determined by Bonferroni multiple comparison test and were designated as *P < 0.05, **P < 0.01 and *** P< 0.001.

Moreover, IgG2a antibody titre in the mice immunized with compounds 4, 5 and 14 was higher than that in the MDP treated mice. There were, however, no significant differences in the serum IgG1 levels between mice groups immunized with MDP and OVA alone but a significant increase in IgG and IgG2a antibody titre was observed. The results were more promising in case of compound 14 at lower doses. These findings indicate that compound 14 significantly enhances serum OVA-specific antibody production in mice immunized with OVA.
Effect of compounds 4, 5 and 14 on OVA-stimulated splenocyte proliferation ex vivo.

The effect of compounds 4, 5 and 14 on OVA stimulated splenocyte proliferation in mice immunized with OVA is shown in figure 6. Splenocytes isolated from mice immunized with OVA/compounds 4 (100 µg), 5 (10 µg) and 14 (1 µg), stimulated by OVA, showed a greater proliferative response than that observed for the mice immunized with OVA control group. MDP (20 µg), the standard adjuvant used in this test model, enhanced proliferative response to OVA when compared to the control animals. The results indicate that compounds 4, 5 and 14 significantly enhance the splenocyte proliferation assay in comparison to MDP.

Figure 6: Effect of test compounds 4, 5 and 14 on OVA-stimulated splenocyte proliferation ex vivo. Splenocytes were prepared and cultured with OVA (100 µg/mL) for 72 h. Splenocyte proliferation was measured by the MTT method. The optical density (OD) at 570 nm was measured and the values are presented as mean ± S.E. (n = 5). Significant differences between the control and treated groups were determined by Bonferroni multiple comparison test and were designated as *P < 0.05, **P < 0.01 and ***P < 0.001.

Immunophenotyping by flowcytometry (T-cell sub population)

The overall percentage of T cell subsets (CD4 and CD8) was estimated in the splenic lymphocyte population as shown in figure 7. Significant changes were observed in the percentage of T (CD4 and CD8) cell subsets by compounds 5 and 14 at almost all doses in comparison to standard MDP and control. Compounds 4 (100 µg), 5 (10 µg) and 14 (1 µg), stimulated by OVA showed a significant increase in CD4/CD8 population than that observed for the mice immunized with OVA control group. MDP
(20 μg), the standard adjuvant used in this test model, enhanced CD4/CD8 population when compared to the control animals.

Figure 7: Effect of compounds 4, 5 and 14 containing OVA on T cell sub population CD4 and CD8 in the spleen cells. Staining of spleen cells with T cell surface marker CD4 (FITC conjugated monoclonal antibody) and CD8 (PE-conjugated monoclonal antibody).

**Figure 7:** Effect of compounds 4, 5 and 14 containing OVA on T cell sub population CD4 and CD8 in the spleen cells. Staining of spleen cells with T cell surface marker CD4 (FITC conjugated monoclonal antibody) and CD8 (PE-conjugated monoclonal antibody).

**Th1 (IFN-γ) and Th2 (IL-4) cytokine determination in spleen cells by flowcytometry**

The effect of lipidated lysine analogs was tested on the production of Th1 (IFN-γ) and Th2 (IL-4) cytokines in spleen cells as shown in **figure 8**. Results indicate that compounds 4, 5 and 14 significantly stimulated Th1 (IFN-γ) and Th2 (IL-4) cytokine release in comparison to control. Compounds 4 and 5 enhanced the Th1 (IFN-γ) and Th2 (IL-4) cytokines at a dose of 100 μg and 10 μg whereas compound 14 enhanced the same response at a dose of 1 μg. Dose selection was based on the *in vitro* proliferation assay experiments wherein the optimum effects were observed within the dosage range 1-100 μg.
Figure 8: Effect of compounds 4, 5 and 14 containing OVA on Th1 (IFN gamma) and Th2 (IL-4) cytokine production in cells. Staining of spleen cells with IFN gamma and IL-4 (PE-conjugated monoclonal antibody) were used in two different sets. Acquisition and analysis were carried out on flowcytometer using Cell Quest Pro software (BD Biosciences).

Effect of compound 4, 5 and 14 on the expression of CD80 and CD86

Compounds 4, 5 and 14 significantly upregulated the expression of both CD80 and CD86 on splenic macrophages in a dose-dependent manner. Compounds 4, 5 and 14 at the dose of 100 μg, 10 μg and 1 μg (optimum dose range) induced optimum
enhancement of 2-fold in the number of cells expressing CD80/CD86 over the control animals (Figure 9).

Figure 9: Effect of compounds 4, 5 and 14 on co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2). Flow cytometric analysis of the expression of co-stimulatory signal molecules in spleen-derived macrophages. Data represented by percent CD80/CD86 positive cell populations are mean ± SE (n = 5); *P < 0.05, **P < 0.01, ***P < 0.001 (control vs. compounds 4, 5 and 14-treated groups; one-way ANOVA followed by Bonferroni multiple comparison test).
Safety studies

No weight loss was observed for mice when weighed on days 0, 14 and 28 post-injection. Mice were kept under observation for 28 days post-injection for any toxic manifestation and no visible symptoms such as inflammatory, allergic response or any other undesirable effects like edema, induration, granuloma, etc. were observed after treatment with compounds 4, 5 and 14. None of the lipidated lysine/peptides studied here exhibited pyrogenic properties, unlike MDP (a pyrogenic adjuvant) as observed by the intermittent body temperature measurement. In our studies, 8 animals were taken up for safety studies. These animals were kept in an isolator at a temperature of 25 °C and the rectal temperature was measured twice daily. Animal body weights were recorded on 0, 7th, 14th and 28th days. Even though the fluctuation in body weights has been observed (Table 1), no significant fluctuation in body temperature were observed.

<table>
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<th>Groups</th>
<th>Adjuvant dose (μg)</th>
<th>Animal Weight in Grams</th>
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<td></td>
<td>100</td>
<td>21.12 ± 1.60</td>
</tr>
</tbody>
</table>

Table 1: Effect of variable doses of compounds 4, 5 and 14 on mice body weight after different time periods. The results show the groups weight (mean ± S.E.) value in grams.
1.2.5. Discussion

Recently, there has been an increasing emphasis on the development of novel immune adjuvant/immunopotentiator to improve efficacy of vaccine formulations. Although a variety of adjuvants have been used in experimental vaccines, most of these materials only elicit an antibody response and/or have undesirable side effects that limit their potential application in vaccines. Therefore, the development of some novel lipopeptide based immune adjuvants is one of the attractive approaches in this direction. Immunogenicity of lipopeptides though well documented in literature, the relationship between nature, structure and site of lipidation and the immune response derived from such entities are yet to be fully understood.\(^{13,14}\) Recently, Liu et al.,\(^{15}\) reported that the presence of long lipophilic chain is critical for the adjuvant activity. A survey of the published reports in this regard shows that octanyl group has many advantages as lipid moiety of bioactive molecules.\(^{16-19}\) Inspired by these reports; some octanoyl lipidated lysine and its dipeptides were synthesized and investigated as model systems to probe the SAR of lipidated peptides with regard to their adjuvanticity. The adjuvant capacity of acylated analogs of lysine moieties was evaluated along with OVA as compared with MDP, in BALB/c mice. In the screening experiments, lipidation of lysine by octanoic acid was found to be more effective than lipidation with higher fatty acids for desired adjuvant effect. This is well attested from a correlation of the activities of compounds 4 and 5 to their analogs where these compounds are lipidated by myristic acid and octanoic acid respectively. Expecting that enzyme labile carbamate protecting groups may help in improved stability and sustained release/depot formation as encountered in carbamate pro-drug designs,\(^{20}\) we tested some intermediate tert-butyl carbamates synthesized prior to final deprotection for their adjuvanticity. Surprisingly, our preliminary screening results revealed that tert-butyl carbamate compound precursors exhibit greater adjuvant activity in comparison to their free amino counterparts obtainable after final deprotection (Figure 3).

To unravel the SAR vis a vis the cell proliferation potential of these analogs, we tested the lipidated (L)-lysine octanoylated at the $\alpha$-amino group (3a), side chain $\varepsilon$-amino group (1) and also at both these sites (3) for the said property. We also explored lipidated amino acids with the intermediate $\varepsilon$-amino carbamate (2) for said
potential to have a more clear understanding. The pattern of activity emerged was in the order $2 > 3a > 3 > 1$, thereby establishing that lipidation of lysine at the $\alpha$-position gives optimal adjuvanticity whereas the corresponding $\varepsilon$-tert-butyl carbamate intermediate possesses even better cell proliferation potential. However, lipidation on both $\alpha$ and $\varepsilon$-amino groups resulted in slightly lower activity than the corresponding $\alpha$-amino mono-octanoylated lysine whereas octanoylation of $\varepsilon$-amino group resulted in diminished activity. Overall, all the lipidated lysine derivatives showed inferior B/T cell proliferation potential in comparison to MDP. This simple analysis gave interesting insights into the effect of lipidation on the pattern of adjuvanticity of these molecules. When we extended this study to lipidated lys-lys dipeptides, it was observed that dipeptide lipidated at $\alpha$-N-terminus of peptide with side chain protected as tert-butyl carbamates showed highest cell proliferation capacity in comparison to MDP, while least activity was observed with oct-lys-lys peptide deprotected at $\varepsilon$-N-terminals. We also observed that octanoylated peptide exhibited higher magnitude of proliferation in comparison to myristoylated peptide. However, in each case dipeptides show marked proliferation potential often superior to MDP. The study was further extended to dioctanoylated glyceric acid conjugates of lys-lys dipeptides with an aim to examine the effect of branched lipids on the adjuvanticity as expressed by the cell proliferation. The proliferation results were in conformity with the unbranched lipidated dipeptides, where dipeptide bearing branched lipid at the $\alpha$-N-terminus with both the $\varepsilon$-amino groups protected as tert-butyl carbamates exhibited highest activity as compared to free acylated peptide. Lipidation at the side chain keeping $\alpha$-amino terminus deprotected resulted in slightly less active compounds as compared to MDP. These studies seem very useful for generation of SAR model about activity and proliferation. Compounds displaying highest activity in primary screening (Figure 3) were taken up for detailed in vivo studies to estimate the IgG titer and its isotypes viz., IgG1 and IgG2a, Th1 (IFN gamma)/Th2 (IL-4) cytokines expressions and costimulatory molecules (CD80/CD86) to evaluate their adjuvanticity. To have a clear understanding a detailed investigation of immune adjuvant activity of compounds 4, 5 and 14 was carried out with weak antigen (OVA) and the results are summarized above. The type of immune response with these
modified derivatives demonstrates that the variation in chain length and site of fatty acid leads to differences in immune adjuvant activity.

OVA is commonly used as a model for immunogen specific T and B cell mediated immune function. The OVA-specific serum IgG, IgG1 and IgG2a antibody titers in the OVA-immunized mice are shown in figures 4 & 5. Compounds 4 (100 μg), 5 (10 μg), and 14 (1 μg) significantly enhanced OVA specific IgG and its isotypes IgG1 and IgG2a titers were observed in immunized mice compared with OVA control and standard MDP group.

Compounds 5 and 14 gave most striking results which suggest that octanoyl or dioctanoyl group at α-NH₂ group of lysine and tert-butylxycarbonyl groups at ε-NH₂ of both lysine moieties of the dipeptide is optimum for immune adjuvant activity. When splenocytes separated from mice immunized with OVA were exposed to above synthesized peptide amphiphiles, it might be possible that lymphocytes recognize and receive second signals and test candidate viz., compounds 4, 5 and 14 which could facilitate OVA specific lymphocytes to proliferate and differentiate into effector and memory cells.

We evaluated the effect of compounds 4, 5 and 14 on the population of cell surface marker like CD4/CD8. As shown in figure 7, these compounds significantly enhanced the population of CD4 as compared to CD8 but here again compounds 5 and 14 were found to be more active as compared to compound 4. Compound 5 was also found to possess ability for enhancement in immune response but at higher doses. Furthermore, we also determined the possible effect of compounds 4, 5 and 14 on Th1 and Th2 immune response balance. As shown in figure 8, all the three compounds significantly increased the production of Th2 cytokines (IL-4) and Th1 cytokines (IFN-γ) in the OVA immunized mice. Even at lower doses, compounds 5 and 14 were observed to significantly enhance both Th1 and Th2 response.

Furthermore, co-stimulatory signals are the most important secondary signals playing important role in the cell–cell cross-talk for the delivery of the required immune response. CD80 has been found to play a key role in maintaining the state of immune response, whereas CD86 plays a role in maintaining immune memory.21 The expressions of CD80 and CD86 on splenic macrophages of compounds 4, 5 and 14-treated mice were found significantly enhanced, supporting its potential of activating
the antigen presenting cells through co-stimulatory signals that eventually help in the
generation of effective immune response by secreting various signal molecules like
cytokines and chemokines. These results seem quite encouraging in view of the fact
that adjuvants currently available for application in human vaccines, like alum,
predominantly induce type 2 responses and do not induce CTL.\textsuperscript{22} Apart from cellular
responses, we observed that strong protein-specific humoral responses are also
induced by compounds 5 and 14 implying their potency as adjuvants in vaccines
directed against extra-cellular pathogens.

From the above mentioned data, it can be inferred that lipodipeptides possess more
immune adjuvanticity as compared to lipidated amino acids or lipodipeptides. It is
also seen that the presence of a tert-butyloxycarbonyl group on the N\textsuperscript{ε}-lysine moiety
is more effective as compared to that of long acyl chains as can be seen from the
activity of compounds 2, 4, 5, 12, 14, 17 and 18. This in turn depicts that the small
branched acyl group at N\textsuperscript{ε}-lysine moiety is more effective for the HLB as compared to
linear long chain. Similarly compounds 8, 17 and 18 were found to be more active as
compared to compounds 3 and 7 perhaps because of the higher lipophilicity in the
structures of later compounds. Adjuvant study on compound 9 (lipidated dipeptide of
lysine and glycine) reveals that it is less active which in turn indicates that lysine
molecule in itself has a HLB due to polar amino groups and non-polar methylene
groups. So two lysine molecules prove to be better adjuvants than the lysine-glycine
combination. Lipidated lysine is less active than two lysine molecules in combination.
Peptides with free ε-NH\textsubscript{2} group (compounds 6, 13, 15, 22 & 24) are least active.
Peptides with free α-NH\textsubscript{2} (compounds 1, 16, 21 & 23) were also found to show little
enhancement in activity but compounds 17 and 18 showed adjuvanticity almost equal
to that for MDP. From these results we infer that the presence of lipophilic group is
critical for immune adjuvant activity; the minimum structural requirement for activity
is the presence of acyl group at ε-NH\textsubscript{2} group and at α-NH\textsubscript{2} position in the dipeptide
lysine moiety.

Among the two sets of compounds, compounds 5 and 14 seem to be potentially useful
candidates on account of their adjuvanticity. At lower doses compound 14 exhibited
better adjuvant activities compared to compound 5. Since both these compounds have
the same basic dipeptide structure, the differences can be ascribed to the different
structure of their lipid moiety. Hence it can be inferred that branching of lipid structure results in improved activity at lower doses. Use of carbamates as prodrug entities is well established method for improving the stability and bioavailability and achieves controlled release due to enzymatic cleavage of labile carbamate linkage in \textit{vivo}. Superior adjuvanticity of carbamate esters as compared to the corresponding free amino peptides may be attributed to the aforesaid merits of carbamate moiety on adjuvants which might improve depot formation/sustained release of antigen and enhanced antigen delivery into APC. \textit{tert}-Butyl carbamate eventually seems to get cleaved by the cellular carboxy esterase to release the free amino peptide which might be interacting with the complimentary receptors in dendritic cell to evoke observed immune response. The consistency in activity pattern between carbamate and free amino peptides in the whole library studied here clearly shows that the presence of carbamate groups on amino functions improves the adjuvanticity.

\textbf{1.2.6. Conclusion}

In conclusion, our findings on the improved adjuvanticity of carbamate analogs of lysine lipopeptides throw light on the better antigen–adjuvant synergy derived from carbamate functionality that influences the multiple roles played by any vaccine adjuvant. These lipidated peptides are potent inducers of both cellular and humoral immunity, making them suitable for a wide spectrum of prophylactic and therapeutic vaccines. These compounds are well tolerated in mice and exhibit no toxicological consequences and may find useful applications clinically. The need for exploring alternative formulation for each antigen may arise the next step in this evaluation to test these lipopeptides as adjuvant with other protein antigens. Our findings we presage will open up new perspectives in the construction of novel vaccine adjuvants that will facilitate the development of vaccines capable of eliciting potentiating immune response in humans.
1.2.7. References


$^1$H NMR spectrum of compound 5
ESI-MS spectrum of compound 5

$[\text{M}^+ + \text{Na}]$
$^{13}$C NMR spectrum of compound 5
DEPT NMR spectrum of compound 5
IR spectrum of compound 5
ESI-MS spectrum of compound 11
$^{13}$C NMR spectrum of compound 11
DEPT NMR spectrum of compound 11
IR spectrum of compound 11
\(^1\)H NMR spectrum of compound 14
ESI-MS spectrum of compound 14
$^{13}\text{C}$ NMR spectrum of compound 14
DEPT NMR spectrum of compound 14
IR spectrum of compound 14