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Synthesis of Novel 1,2,3-Triazolyl L-Serinol Palmitoyl Muramyl Dipeptide Derivatives

RAMESH ALAGODLA[1,2,#], PATURU RAMA SUBBA REDDY[1,2,#], SRAVANTHI VEMIREDDY[1], DEVDUTT CHATURVEDI[3] and HALMUTHUR M. SAMPATH KUMAR[1,2,*]

[1]Vaccine Immunology Laboratory, OSPC Division, CSIR-Indian Institute of Chemical Technology, Hyderabad-500007, India
[2]Academy of Scientific and Innovative Research (AcSIR), Ghaziabad-201002, India
[3]Department of Chemistry, School of Physical Sciences, Mahatma Gandhi Central University, Motihari (East Champaran)-845401, India
[#]Equal contribution

*Corresponding author: Fax: +91 40 27160387; Tel: +91 40 27191824; E-mail: sampath.iict@gov.in

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In this research, a structural variant of muramyl dipeptide (MDP) is designed wherein, the entire *N*-acetyl group was replaced by a bio-isosteric 1,2,3-triazole moiety with serinol lipid substitution at 4th position. The protecting groups such as benzyl and benzylidine were removed sequentially to evolve three novel derivatives with increasing polarity as seen in the MDP-lipid 21-23 derivatives. A synthesis strategy involving triazolyl click chemistry to combine MDP scaffold to the serinol lipid head group is developed. The new derivatives were characterized using NMR, ESI-MS and MALDI. Preliminary data from *in vitro* screening of the compounds inferred the immuno-potentiating properties.

Keywords: Muramyl dipeptide, 1,2,3-Triazole, Palmitoyl L-Serinol, Triazolyl click chemistry, Amphipathic ligand.

INTRODUCTION

The synthetic immunoreactive peptide known as muramyl dipeptide (MDP) is made up of N-acetyl muramic acid linked to a dipeptide chain of L-Ala-D-isoGln. An active component of Freund's complete adjuvant, it was recently identified in peptidoglycan from bacterium cell walls. Structure and activity relationship (SAR) of this glycopeptide adjuvant has been analyzed *via* numerous structural variations and a few potential MDP derivatives, including murabutide, GMDP, have emerged as powerful vaccine adjuvants [1,2]. The chemical modifications of muramyl peptides offer better ligands with improved pharmacological attributes to evolve new efficacious adjuvants suitable for use with various antigens to develop potential vaccines. Even though the SAR studies reveal dipeptide as the perfect sub-structural part of the MDP to carry out the structural changes, modification at the *N*-acetyl group of muramic acid scaffold is an equally attractive option for deriving better ligands with improved NOD2 binding attributes along with improved pharmacokinetic properties [3]. Again, even though the established SAR of MDP stressed the need for retaining *N*-acetyl moiety on the scaffold to retain the activity. Subsequent reports reveal efforts to change *N*-acetyl moiety with various *N*-acetyl substituent's. In this context, a structural variant of MDP wherein, the entire *N*-acetyl group is replaced by a bio-isosteric 1,2,3-triazole moiety with suitable serinol derived lipid substitution at the 4th position of triazole ring was designed. Thus, replacement with *N*-acetyl part with palmitoyl L-serinol as a hydrophobic entity should present an amphipathic ligand [4].

Based on the above consideration, a novel triazole L-serinol palmitoyl muramyl dipeptide is synthesized by replacing *N*-acetyl group with palmitoyl L-serinol group and the protecting groups such as benzyl and benzylidene were sequentially removed to evolve three novel triazolyl MDP derivatives with increasing polarity as observed in the MDP-lipid 21-23 derivatives. We hypothesize that such derivatives with tuned hydrophobic-lipophilic balance might result in new adjuvants with reduced pyrogenicity and improved efficacy [5,6]. A novel synthesis strategy is devised involving triazolyl click chemistry to combine MDP scaffold to the palmitoyl serinol lipid head group. To achieve this, azido MDP and acetylinic serinol lipid is chosen as partners for click chemistry. The new derivatives

thus synthesized in good yield, were analyzed by NMR, ESI-MS, HRMS and MALDI. *In vitro* evaluation of the novel derivatives in RAW macrophages showed an immuno stimulating activity.

EXPERIMENTAL

All the initial raw materials and reagents were purchased from Spectrochem and Sigma-Aldrich and used as acquired without any further purification. All organic solvents used were of commercial grades and the dry solvents required for reactions were freshly prepared by following standard methods. The solvent tetrahydrofuran (THF) was stirred with CaH_2 at room temperature overnight under argon and distilled at 60 ºC followed by re-distillation over Na-metal and benzophenone. All the moisture sensitive reactions were carried out in oven dried glasswares under argon atmosphere. The progress of chemical reactions were monitored by using TLC, silica coated on glass and aluminium plates which were visualized by charring with suitable charring solutions like PMA, anisaldehyde, ninhydrin and iodine, which were freshly prepared. All compounds were concentrated under reduced pressure by removing excess solvent using a rotary evaporator at 35-48 ºC. Both analytical grade chloroform and methanol were used as eluents in silica gel 60-120 and 100-200 mesh column chromatography. The NMR spectra were obtained in the indicated solvent as solution and at the field strength mentioned. For ESI-MS, *m/z* values were reported in atomic mass units included with Na^+ salt or H^+ and recorded in the Shimadzu instrument. Compound **5** was synthesized over four steps with L-serine following a standard procedure [1,2]. Compounds **13** and **20** were synthesized by the reported methods [3].

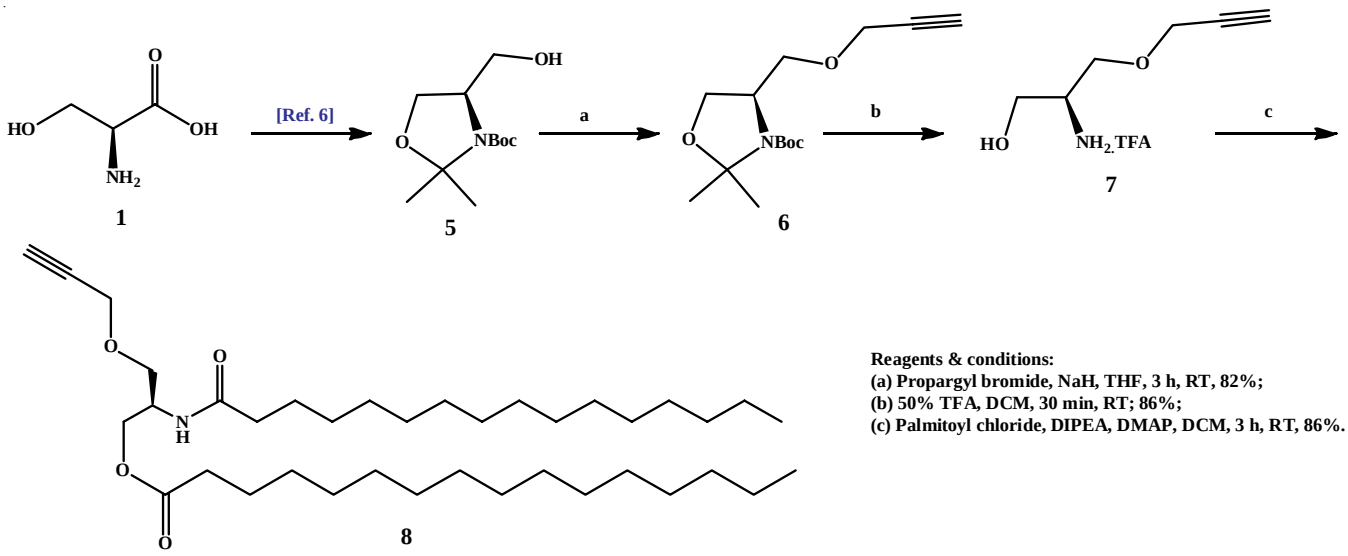
General procedures of synthesized compounds

***tert*-Butyl (*R*)-4-(hydroxymethyl)-2,2-dimethyloxazolidine-3-carboxylate (5):** Compound **5** was synthesized in over four steps with L-serine by using standard procedure [1]. Initially, Boc-L-Ser-OMe (1.6 g, 6.1 mmol) was dissolved in dry THF (20 mL) then lithium aluminium hydride (LAH, 369 mg, 9.26 mmol) in THF was added dropwise over 10 min at 0 ºC, then stirred at room temperature for 30 min. After completion of reaction as monitored by TLC, added 10% KOH solution dropwise at 0 ºC and stirred for 2 h, then the obtained white precipitate was filtered on celite pad. The filtrate was extracted with EtOAc (2 × 50 mL) then, organic layer was washed with brine solution and dried over anhydrous Na_2SO_4. The collected organic fractions were concentrated under reduced pressure to get crude product, which was purified by column chromatography to isolate the compound **5** (1.1 g, 81%) as pale-yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.09 (broad, 1H), 4.05-3.96 (m, 1H), 3.85-3.70 (m, 2H), 3.62 (d, *J* = 7.3 Hz, 1H), 1.54 (s, *J* = 11.5 Hz, 3H), 1.49 (s, 9H), 1.45 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 154.15, 94.13, 81.20, 65.3, 63.48, 59.51, 28.41, 27.17, 24.60; ESI-Mass *m/z* calcd. for $C_{11}H_{21}NO_4$; 231.15; Found $[M + Na]^+$; 254.00.

Synthesis of *tert*-butyl (*R*)-2,2-dimethyl-4-((prop-2-yn-1-yloxy)methyl)oxazolidine-3-carboxylate (6): Suspension of 60% sodium hydride (2 g, 0.085 mol) was taken in a round bottom flask in an argon atmosphere at room temperature. Then, dry THF (10 mL) was added to NaH containing 50 mL round bottom flask at 0 ºC and the suspension was cooled to 0 ºC. Compound **5** (5 g, 0.021 mol) in THF was added dropwise to the stirring suspension, then propargyl bromide (5.7 g, 0.064 mol) was added dropwise to the suspension at 0 ºC. The reaction mixture was allowed to warm to room temperature for 3 h. After completion of all starting material as indicated by thin layer chromatography, the reaction mixture was quenched with ice cooled aq. NH_4Cl solution. The organic layer was extracted with EtOAc (3 × 50 mL) and then combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and then concentrated under reduced pressure. The residue was purified by column chromatography on silica gel to give compound **6** (4.8 g, 82%) yield as colourless oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.16 (d, *J* = 9.9, 5.0 Hz, 2H), 3.95 (m, *J* = 12.2, 9.0, 6.4 Hz, 3H), 3.68 (d, *J* = 5.3 Hz, 1H), 3.43 (dt, *J* = 28.8, 8.4 Hz, 1H), 2.47 (s, 1H), 1.60-1.51 (d, 6H), 1.48 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 151.72, 94.24, 79.98, 75.05, 74.61, 65.48, 63.70 (s), 63.66, 58.53, 28.39, 26.77, 24.37, 23.09; ESI-MASS *m/z* calcd. for $C_{14}H_{23}NO_4$: 269.20 Found $[M + Na]^+$; 292.15.

Synthesis of (*S*)-2-amino-3-(prop-2-yn-1-yloxy)propan-1-ol (7): Compound **6** (3.5 g, 13.01 mmol) was taken in 50 mL round bottom flask and dissolved in acetonitrile (10 mL) and was added with 20% trifluoroacetic acid (TFA) in water (10 mL), then stirred at room temperature for 4 h. After completion of starting material as monitored by thin-layer chromatography acetonitrile was removed under reduced pressure. The reaction mixture was basified with 10% NaOH solution and the resulting reaction mixture was extracted in EtOAc (2 × 50 mL) and washed with water then dried over anhydrous Na_2SO_4, then all organic fraction was concentrated under reduced pressure to give compound **7** as quantitative yield, which was utilized for next reaction without further purification.

Synthesis of (*R*)-2-palmitamido-3-(prop-2-yn-1-yloxy)-propyl palmitate (8): To the stirring solution of compound **7** (1.22 g, 9.4 mmol) in dry DCM (50 mL), DIPEA (8.1 mL, 4.72 mmol), DMAP as catalyst were added to the solution and stirred for 30 min, then palmitic acid (8.54 mL, 28.37 mmol) was added dropwise. The reaction mixture was allowed to stir for 3 h at room temperature. After completion of all staring material as monitored by thin-layer chromatography, the reaction mixture was dissolved and extracted in DCM (2 × 50 mL), washed with $NaHCO_3$ and brine solution. Collected organic fractions dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give crude compound **8**, which was then purified by column chromatography on silica gel (100-200 mesh) to give corresponding compound **8** as white powder in yield (4.9 g, 86%) (**Scheme-I**); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.85 (d, *J* = 8.5 Hz, 1H), 4.45-4.31 (m, 1H), 4.24 (dd, *J* = 11.1, 6.5 Hz, 1H), 4.15 (t, *J* = 2.2, 1.8 Hz, 2H), 4.14-4.06 (dd, 1H), 3.65 (dd, *J* = 9.5, 3.4 Hz, 1H), 3.57 (dd, *J* = 9.5, 4.7 Hz, 1H), 2.44 (t, *J* = 2.4 Hz, 1H), 2.32 (t, *J* = 12.6, 7.6 Hz, 2H), 2.22-2.05 (t, 2H), 1.63 (m, *J* = 14.7, 7.5 Hz, 4H), 1.38-1.16 (m, 48H), 0.88 (t, *J* = 6.9 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$ + CD_3OD): δ 173.82, 173.10, 79.15, 74.93, 68.49, 62.96, 58.56, 47.80, 36.84, 34.07, 31.95, 31.29, 25.73, 24.86, 22.72, 14.14; ESI-

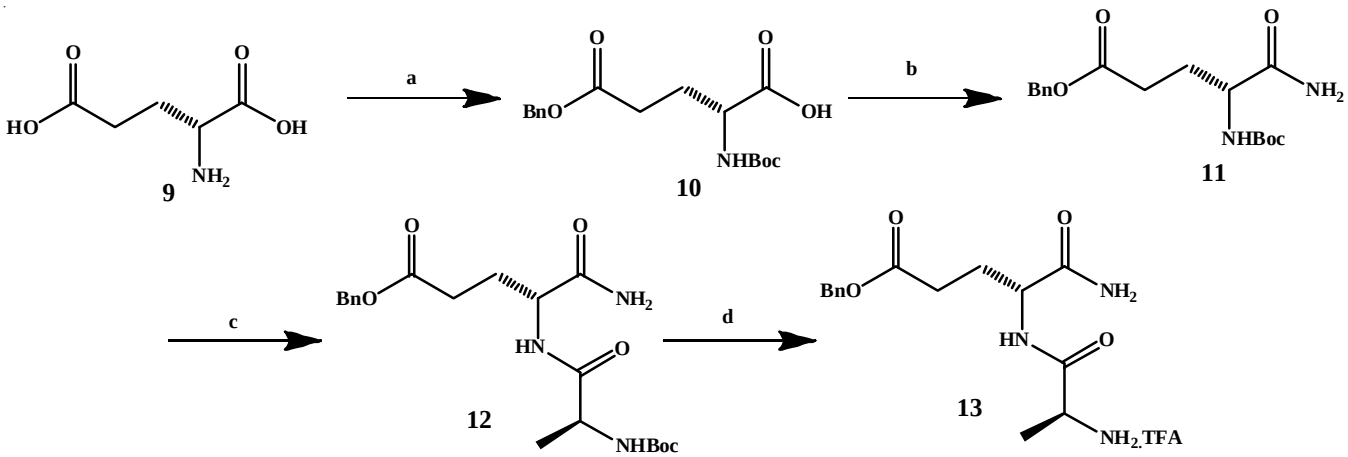


Scheme-I: Synthesis of propargyl L-serinol palmitate (**8**)

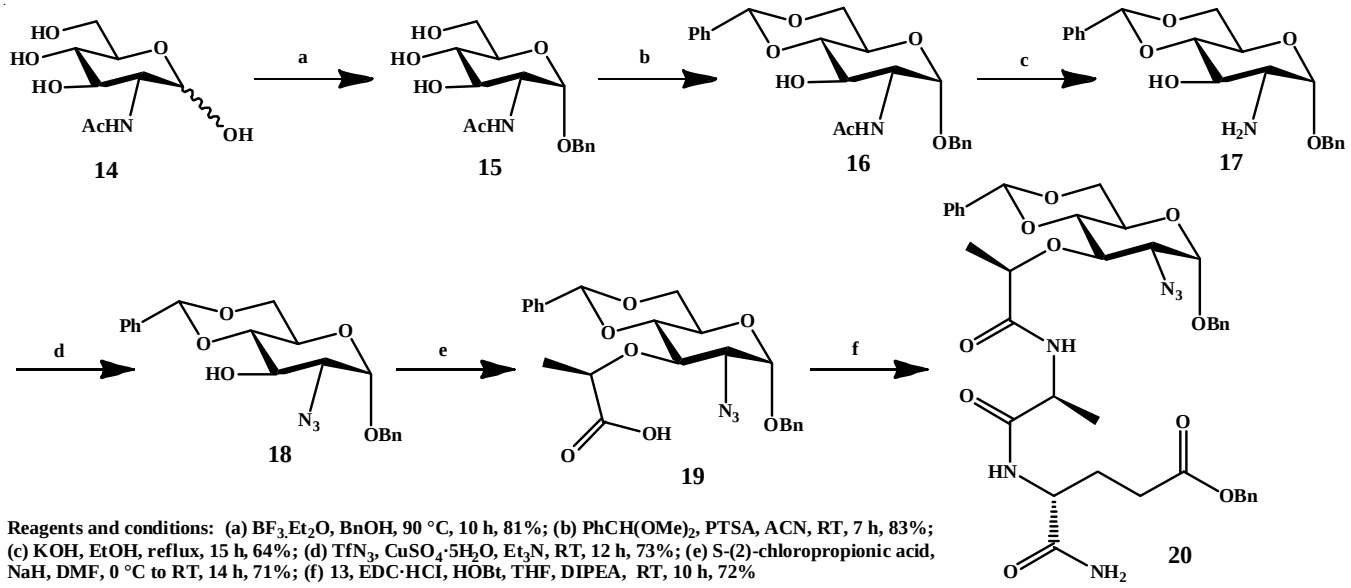
MASS *m/z* calcd. for $C_{38}H_{71}NO_4$: 605.50; Found $[M + Na]^+$; 628.50.

Benzyl(*R*)-5-amino-4-((S)-2-((*tert*-butoxycarbonyl)-amino)propanamido)-5-oxopentanoate (12): The peptide was synthesised by the conventional method of combining benzyl ester of isoglutamine with Boc-L-alanine in trifluoroacetic acid. At each stage of the binding process, we used DIPEA and EDCHCl/HOBt conditions [3]. With a five-step sequence, we were able to acquire fluffy white solid compound **12** in high yields (**Scheme-II**). The spectral and physical properties of this product exactly matched with reported data in the literature. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39-7.30 (m, 5H), 6.81 (br, 1H), 5.78 (br, 1H), 5.20 (d, *J* = 3.2Hz, 1H), 5.12 (q, *J* = 12.3Hz, 2H), 4.49 (dd, *J* = 10.1, 7.6Hz, 1H), 4.07 (dq, *J* = 7.2, 3.2 Hz, 1H), 2.56 (dt, *J* = 16.9, 7.2 Hz, 1H), 2.22 (m, 1H), 2.48 (dt, *J* = 17.0, 6.7 Hz, 1H), 2.26-2.19, (m, 1H), 1.42 (s, 9H), 1.32 (d, *J* = 7.1 Hz, 3H), 2.05-1.98 (m, 1H), 1.41 (s, 9H), 1.32 (d, *J* = 7.1 Hz, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.81, 173.61, 173.28, 155.90, 135.70, 128.59, 128.31, 128.26, 80.26, 66.61, 52.27, 50.58, 30.56, 28.32, 26.91, 18.01; ESI-MASS *m/z* calcd. for $C_{20}H_{29}N_3O_6$; 407.25, Found; $[M + Na]^+$ 430.19.

Synthesis of (*R*)-2-(((2R,4aR,6S,7R,8R,8aS)-7-azido-6-(benzyloxy)-2-phenylhexahydropyrano[3,2-*d*][1,3]dioxin-8-yl)oxy)propanoic acid (19): Compound **19** was synthesized using *N*-acetyl-D-glucosamine as starting material over five steps using standard procedure [3]. After purification through column chromatography in (4% MeOH/DCM) used as eluent to afford compound **19** as white solid (7.6 g, 80%) (**Scheme-III**); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.50-7.41 (m, 2H), 7.41-7.31 (m, 8H), 5.56 (s, 1H), 5.06 (d, *J* = 3.7 Hz, 1H), 4.75 (d, *J* = 11.9 Hz, 1H), 4.62 (d, *J* = 11.9 Hz, 1H), 4.50 (dd, *J* = 13.9, 6.9 Hz, 1H), 4.23 (dd, *J* = 10.3, 4.8 Hz, 1H), 4.05 (t, *J* = 9.5 Hz, 1H), 3.90 (m, 1H), 3.74 (t, *J* = 10.3 Hz, 1H), 3.64 (t, *J* = 9.3 Hz, 1H), 3.42-3.38 (m, 1H), 1.47 (d, *J* = 6.9 Hz, 3H); ^{13}C



Scheme-II: Synthesis of L-alanine-γ-benzyl ester D-glutamine (**13**)



Reagents and conditions: (a) $BF_3.Et_2O$, BnOH, 90 °C, 10 h, 81%; (b) $PhCH(OMe)_2$, PTSA, ACN, RT, 7 h, 83%; (c) KOH, EtOH, reflux, 15 h, 64%; (d) TfN_3, $CuSO_4 \cdot 5H_2O$, Et_3N, RT, 12 h, 73%; (e) S-(2)-chloropropionic acid, NaH, DMF, 0 °C to RT, 14 h, 71%; (f) 13, EDC·HCl, HOBt, THF, DIPEA, RT, 10 h, 72%

Scheme-III: Synthesis of protected 2-azido muramyl dipeptide (**20**)

NMR (75 MHz, $CDCl_3$): δ 175.96, 136.84, 136.16, 129.24, 128.65, 128.42, 128.37, 128.33, 125.90, 101.62, 97.08, 82.46, 76.42, 70.08, 68.79, 62.69, 62.35, 18.98; ESI-MASS: *m/z* calcd. for $C_{23}H_{25}N_3O_7$: 455.43, Found $[M + H]^+$; 456.50, $[M + NH_4]^+$: 473.60.

Benzyl(*R*)-5-amino-4-((*S*)-2-((*R*)-2-(((2R,4aR,6S, 7R,8R,8aS)-7-azido-6-(benzyloxy)-2-phenylhexahydropyrano[3,2-*d*][1,3]dioxin-8-yl)oxy)propanamido)propanamido)-5-oxopentanoate (20): To a solution of compound **19** (6.0 g, 13.19 mmol) in anhydrous THF (80 mL), EDC·HCl (5.0 g, 26.38 mmol), HOBt (3.6 g, 26.38 mmol) and compound **13** (4.8 g, 15.82 mmol) in DIPEA (7.1 mL, 39.4.38 mmol) were added while the mixture was stirred for 10 h at room temperature with the reaction mixture. Once the complete consumption of the starting material was done, the solvent was evaporated under pressure and the remaining residue was extracted with CH_2Cl_2 (100 × 3 mL) and brine solution (50 mL × 2) (**Scheme-III**). In order to obtain a white solid compound **20**, the extract was concentrated after being passed over anhydrous $MgSO_4$ and the crude product was purified *via* column chromatography (5% MeOH/CH_2Cl_2, silica gel). Yield: 7.2 g (75% purity). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.65 (t, *J* = 8.8 Hz, 1H), 7.46-7.40 (m, 2H), 7.40-7.30 (m, 12H), 7.15 (d, *J* = 8.0 Hz, 1H), 6.79 (s, 1H), 5.55 (s, 1H), 5.45 (br, 1H), 5.15-5.05 (m, 3H, α 1-H), 4.76 (d, *J* = 10.0 Hz, 1H), 4.62 (d, *J* = 11.7 Hz, 1H), 4.46 (td, *J* = 8.3, 4.6 Hz, 1H), 4.31-4.19 (m, 3H), 3.96-3.85 (m, 2H), 3.75 (t, *J* = 10.3 Hz, 1H), 3.61 (t, *J* = 9.3 Hz, 1H), 3.41 (dd, *J* = 10.1, 3.7 Hz, 1H), 2.58 (m, 1H), 2.45 (m, 1H), 2.27-2.16 (m, 1H), 2.02 (m, 1H), 1.39 (d, *J* =7.0 Hz, 3H), 1.35 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 174.21, 173.58, 173.36, 172.37, 136.75, 136.05, 135.67, 129.79, 129.26, 129.04, 128.68, 128.63, 128.42, 128.38, 128.31, 125.88, 101.67, 96.86, 82.54, 78.12, 76.37, 70.09, 68.74, 66.73, 62.75, 62.43, 52.45, 49.52, 30.66, 26.68, 19.62, 16.92; ESI-Mass *m/z* Calcd. for $C_{38}H_{44}N_6O_{10}$: 744.24; Found $[M + H]^+$: 745.27, $[M + Na]^+$: 767.25.

(2*R*)-3-((1-((4aR,6S,7R,8R,8aS)-8-(((*R*)-1-(((S)-1-(((*R*)-1-amino-5-(benzyloxy)-1,5-dioxopentan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)oxy)-6-(benzyloxy)-2-phenylhexahydropyrano[3,2-*d*][1,3]dioxin-7-yl)-1*H*-1,2,3-triazol-4-yl)methoxy)-2-palmitamidopropyl palmitate (21): Propargyl L-serinol palmitate (**8**) (81 mg, 0.133 mmol) was dissolved in THF/H_2O (10 mL) and $CuSO_4 \cdot 5H_2O$ (100 mg, 0.402 mmol) along with sodium ascorbate (76 mg, 0.402 mmol) were added then after 10 min, 2-azido muramyl dipeptide (**20**) (100 mg, 0.133 mmol) were added into the stirring solution at room temperature, then stirred the whole mixture for 3 h. After completion of reaction as monitored by TLC, the THF was removed under reduced pressure, the crude residue was then diluted with 10% MeOH/$CHCl_3$ and water, then extracted with 10% MeOH/$CHCl_3$ (2 × 50 mL). The organic layers were combined and dried over Na_2SO_4 and evaporated under reduced pressure to afford the crude product, which was purified by column chromatography (10% MeOH/$CHCl_3$) to afford pure compound **21** as white solid (150 mg, 83%) (**Scheme-IV**); $[\alpha]_D^{20}$: + 8.40 (*c* 0.5, $CHCl_3$, t; 20 ºC; D = 589 nm); m.p.: 190-194 ºC; FT-IR ($CHCl_3$, cm^{-1}): 3305-3199 (NH_2), 2919-2854 (CH-aromatic), 1733-1646 (C=O, amides), 1536-1457 (N-H bending), 1373-1258 (C-N amides), 1162 (ester), 1095-1027 (ether); ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD): δ 7.93 (s, 1H), 7.51-7.43 (m, 2H), 7.41-7.33 (m, 3H), 7.32-7.24 (m, 6H), 7.12 (dd, *J* = 6.4, 3.0 Hz, 2H), 5.64 (s, 1H), 5.12 (dd, *J* = 13.2, 2.7 Hz, 1H), 5.09 (d, *J* = 5.5 Hz, 2H), 5.02 (dd, *J* = 10.5, 3.7 Hz, 1H), 4.72 (d, *J* = 11.8 Hz, 1H), 4.54 (s, 2H), 4.31 (dd, *J* = 14.5, 9.7, 4.7 Hz, 3H), 4.16 (dd, *J* = 11.1, 5.3 Hz, 1H), 4.12-4.01 (m, 2H), 3.98-3.79 (m, 4H), 3.58 (dd, *J* = 9.6, 4.5 Hz, 1H), 3.49 (dd, *J* = 9.5, 5.1 Hz, 1H), 2.51-2.38 (m, 2H), 2.36-2.25 (m, 2H), 2.17 (t, *J* = 7.5 Hz, 2H), 1.98-1.87 (m, 1H), 1.57 (s, 4H), 1.33-1.21 (m, 51H), 0.87 (t, *J* = 6.8 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$ + CD_3OD): δ 178.63, 178.29, 177.65, 148.57, 140.71, 139.82, 139.55, 133, 132.80, 129.86, 127.27, 105.67, 100.80, 85.42, 82.38, 74.23, 73.29, 72.47, 70.46,

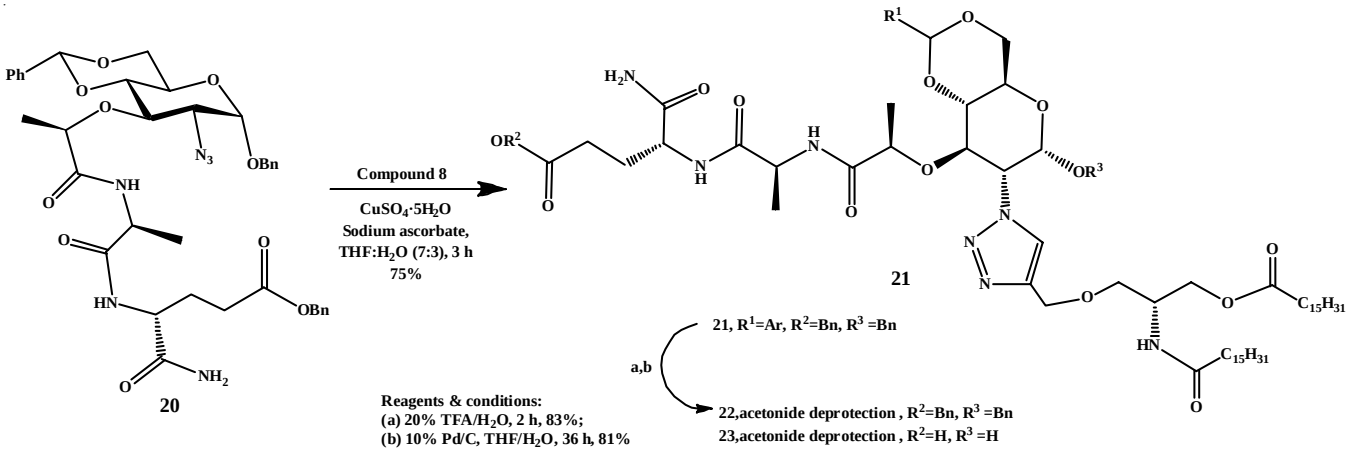
68.04, 67.05, 56.09, 40.19, 38.01, 35.80, 34.38, 33.81, 30.46, 29.79, 28.77, 26.53, 23.13, 20.81, 17.78; MALDI calculated for $C_{76}H_{115}N_7O_{14}$:1349; Found $[M + Na]^+$: 1372. HRMS: calcd. for $C_{76}H_{116}N_7O_{14}$, *m/z* 1349.8235, found; 1350.8530 $[M+H]^+$.

(*R*)-3-((1-((2S,3R,4R,5S,6R)-4-(((*R*)-1-(((*S*)-1-(((*R*)-1-amino-5-(benzyloxy)-1,5-dioxopentan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)oxy)-2-(benzyloxy)-5-hydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-3-yl)-1*H*-1,2,3-triazol-4-yl)methoxy)-2-palmitamidopropyl palmitate (22): Compound **21** (100 mg, 0.074 mmol) was dissolved in TFA in water (8/2 mL) stirred at room temperature for 30 min. After completion of reaction as monitored by TLC, the trifluoro acetic acid and water were evaporated under reduced pressure, co-evaporated with toluene. The residue was washed by *n*-pentane to give compound **22** in 83% yield of crude product (**Scheme-IV**), which was purified by column chromatography using (10% MeOH/$CHCl_3$) as eluent to afford pure compound as white solid (78 mg, 83%); $[\alpha]_D^{20}$: -10.00 (*c* 0.5, $CHCl_3$, t; 20 ºC; D = 589 nm); m.p.: 141-145 ºC; FT-IR ($CHCl_3$, cm^{-1}); 3340 (-OH), 2921-2855 (CH-aromatic), 1729-1662 (C=O, amides), 1537-1457 (N-H bending), 1375-1256 (C-N amides), 1123 (ester), 1040 (C-O vibration of 1°-OH); ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD): δ 7.98 (s, 1H), 7.48 (d, *J* = 1.7 Hz, 1H), 7.32 (d, *J* = 20.6 Hz, 7H), 7.16 (s, 2H), 5.64 (s, 1H), 5.12 (dd, *J* = 13.2, 2.7 Hz, 1H), 5.09 (d, *J* = 5.5 Hz, 2H), 5.02 (dd, *J* = 10.5, 3.7 Hz, 1H), 4.72 (d, *J* = 11.8 Hz, 1H), 4.54 (s, 2H), 4.31 (dd, *J* = 14.5, 9.7, 4.7 Hz, 3H), 4.16 (dd, *J* = 11.1, 5.3 Hz, 1H), 4.12-4.01 (m, 2H), 3.98-3.79 (m, 4H), 3.58 (dd, *J* = 9.6, 4.5 Hz, 1H), 3.49 (dd, *J* = 9.5, 5.1 Hz, 1H), 2.51-2.38 (m, 2H), 2.36-2.25 (m, 2H), 2.17 (t, *J* = 7.5 Hz, 2H), 1.98-1.87 (m, 1H), 1.57 (s, 4H), 1.33-1.21 (m, 51H), 0.87 (t, *J* = 6.8 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$ + CD_3OD): δ 178.72, 177.60, 176.95, 148.41, 147.34, 145.68, 139.86, 132.45, 132.45, 113.79, 100.20, 83.47, 76.60, 73.56, 73.17, 70.47, 67.98, 67.08, 56.16, 40.21, 38.03, 35.78, 34.38, 33.38, 33.05, 30.50, 29.78, 28.76, 26.52, 22.76, 20.66, 17.76; MALDI calculated for $C_{69}H_{111}N_7O_{114}$: 1261; Found $[M+ Na]^+$; 1284. HRMS: calcd. for $C_{69}H_{112}N_7O_{14}$; *m/z* 1261.8252, found: 1262.8262 $[M+H]^+$.

(*R*)-5-Amino-4-((*S*)-2-((*R*)-2-(((2S,3R,4R,5S,6R)-2,5-dihydroxy-6-(hydroxymethyl)-3-(4-(((*R*)-2-palmitamido-3-(palmitoyloxy)propoxy)methyl)-1*H*-1,2,3-triazol-1-yl)tetrahydro-2*H*-pyran-4-yl)oxy)propanamido)propanamido)-5-oxopentanoic acid (23): Compound **22 (**70 mg, 0.055 mmol) was dissolved in THF/H_2O (10 mL) and then 10% Pd/c was added in catalytic amount, then stirred at room temperature for 36 h. After consumption of starting material as monitored by thin-layer chromatography, the reaction mixture was passed through celite pad under continuous vacuum to afford crude product, the filtrate was concentrated under reduced pressure, co-evaporated with toluene (**Scheme-IV**), which was purified by column chromatography using (15% MeOH/$CHCl_3$) as eluents to afford pure compound **23** as white solid (48 mg, 81%). $[\alpha]_D^{20}$; -13.80 (*c* 0.5, $CHCl_3$, t; 20 ºC; D = 589 nm); m.p.: 171-174 ºC; FT-IR ($CHCl_3$, cm^{-1}); 3325 (-OH), 2919-2854 (CH-aromatic), 1660 (C=O, amides), 1542-1456 (N-H bending), 1377-1252 (C-N amides), 1187 (ester), 1093 (ether); ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD): δ 8.25-7.87 (α and β, 1H), 5.22 (α and β,1H),5.09 (d, *J* = 5.5 Hz, 2H), 5.02 (dd, *J* = 10.5, 3.7 Hz, 1H), 4.72 (d, *J* = 11.8 Hz, 1H), 4.54 (s, 2H), 4.31 (dd, *J* = 14.5, 9.7, 4.7 Hz, 3H), 4.16 (dd, *J* = 11.1, 5.3 Hz, 1H), 4.12-4.01 (m, 2H), 3.98-3.79 (m, 4H), 3.58 (dd, *J* = 9.6, 4.5 Hz, 1H), 3.49 (dd, *J* = 9.5, 5.1 Hz, 1H), 2.51-2.38 (m, 2H), 2.36-2.25 (m, 2H), 2.17 (t, *J* = 7.5 Hz, 2H), 1.98-1.87 (m, 1H), 1.57 (s, 4H), 1.33-1.21 (m, 51H), 0.87 (t, *J* = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 178.02, 177.80, 177.73, 171.26, 168.83, 166.49, 129.11, 119.05, 98.72, 95.14, 73.04, 67.95, 67.21, 40.07, 37.94, 35.75, 33.26, 29.80, 28.72, 26.46, 17.57. MALDI calculated for $C_{55}H_{99}N_7O_{14}$: *m/z* 1081.72; Found $[M + Na]^+$; 1104. HRMS: calcd. for $C_{55}H_{100}N_7O_{14}$, *m/z* 1081.8527, found: 1082.7323 $[M+H]^+$.

Cytotoxicity assay: RAW macrophages were seeded at a density of 2×10^4 cells/well and incubated with 1000 µg/mL of test compounds **21**, **22** and **23** for 48 h at 37 ºC with 5% CO_2. After incubation 20 µL MTT reagent was added to check the viability of cells. The addition of MTT reagent was followed by 2 h incubation at 37 ºC with 5% CO_2. Untransformed MTT was replaced by DMSO and absorbance was read at 570 nm [7].

Pro-inflammatory cytokines and NO assay: RAW macrophages 2×10^4 cells/well were seeded and incubated with 20 µg of test compounds **21**, **22** and **23** for 48 h at 37 ºC with 5% CO_2. After incubation, supernatant of the culture was isolated and



Scheme-IV: Synthesis of triazolyl L-serinol palmitoyl muramyl dipeptide analogues (**21-23**)

used for quantification of pro inflammatory cytokines like IL-6 and YNF-α using sandwich ELISA and the rest supernatant was used to quantify NO produced using modified Griess reagent according to the manufactures protocol (Sigma) [7].

RESULTS AND DISCUSSION

Novel triazolyl L-serinol palmitates of muramyl dipeptide derivatives having varying hydrophilic-lipophilic balance have been designed by introducing with 1*H*-1,2,3-triazolyl through copper catalyzed azide-alkyne click reaction between alkynyl L-serinol palmitate with azido muramyl dipeptide in presence of $CuSO_4$ and sodium ascorbate to afford novel triazolyl L-serinol palmitate muramyl dipeptide derivatives (Fig. 1).

The synthesis of 1,2,3-triazolyl L-serinol palmitoyl muramyl dipeptide involves two key intermediates *viz*. propargyl L-serinol palmitate (**8**) and 2-azido muramyl dipeptide (**20**), which was prepared by coupling dipeptide fragment **13** and 2-azido muramic acid (**19**) in EDC·HCl and HOBt in basic condition as followed by known procedure [6]. Compound **13** was synthesized by coupling trifluoroacetic acid salt of isoglutamine benzyl ester and Boc-L-alanine as reported in literature. Initially, D-glutamic acid was orthogonally protected at the carboxyl group end using benzyl alcohol as solvent in presence of Lewis acid $BF_3{\cdot}Et_2O$ to give D-glutaminyl-benzyl ester. Then after, Boc-D-glutaminyl benzyl ester was produced by treating free α-amino group of benzyl ester with $(Boc)_2O$ in presence of $NaHCO_3$. Further acid group was activated by forming mixed anhydride with ethyl chloroformate in triethylamine followed by addition of methanolic NH_4OH solution (4 M) at -15 ºC to form a amidated compound **11**. Boc deprotection was carried in 20% TFA/DCM and coupled with Boc-L-alanine-OH to afford compound **12**. Further Boc deprotection was carried out to afford compound **13**, which was then used for next step without further purification (**Scheme-II**). Compound **20** was carried out by introducing of benzyl ether at C1-anomeric carbon of *N*-acetyl-D-glucosamine. A three-step process is We followed to synthesize compound **17**, which was described in previous reported literature [5]. The synthesis of carbohydrate fragment could be achieved using intermediate **17** through diazo transfer at C2-amine by treating it with TfN_3 in TEA and $CuSO_4{\cdot}5H_2O$ under mild reaction conditions to give 2-azido compound **18**. It was O-alkylated in dry DMF, NaH by treating with (*S*)-2-choloropropionic acid at room temperature to afford lactic acid coupled 2-azido sugar fragment **19**. This was coupled with dipeptide fragment **13** in EDC·HCl/HOBt and DIPEA media to produce compound **20** with 72% yield (**Scheme-III**).

On the other hand, corresponding propargyl L-serinol palmitate **8** was synthesized by using garners aldehyde route here, a known procedure was followed to synthesize compound **5** over four steps [6,8,9]. Propargylation at primary alcohol was carried out by using propargyl bromide [10]. Deprotection of acetonide as well as BOC on adjacent groups such as -OH and $-NH_2$ was carried with 20% TFA in dichloromethane [11]. Palmitoylation of compound **7** with palmitoyl chloride was done in basic condition in the presence of DMAP as catalyst in DCM **(Scheme-I)** [12]. The final compound was synthesized by using Cu(I) catalyzed 1,3-cycloaddition reaction of propargyl L-serinol palmitate and azido muramyl dipeptide (2-azido MDP) to produce covalently linked molecule *via* 1,2,3-triazole-known as "click chemistry" **(Scheme-IV)** [13-16]. All the compounds were synthesized in good yields, which were isolated in high purity using column chromatography. The compounds were characterized by ^1H, ^{13}C NMR, ESI-MS and MALDI-TOF which confirmed the structure.

Biological activity: Novel triazolyl L-serinol palmitate derivatives synthesized were preliminarily evaluated for their cytotoxicity using RAW macrophages wherein the compounds were incubated at 1000 μg/mL concentration and the % viability

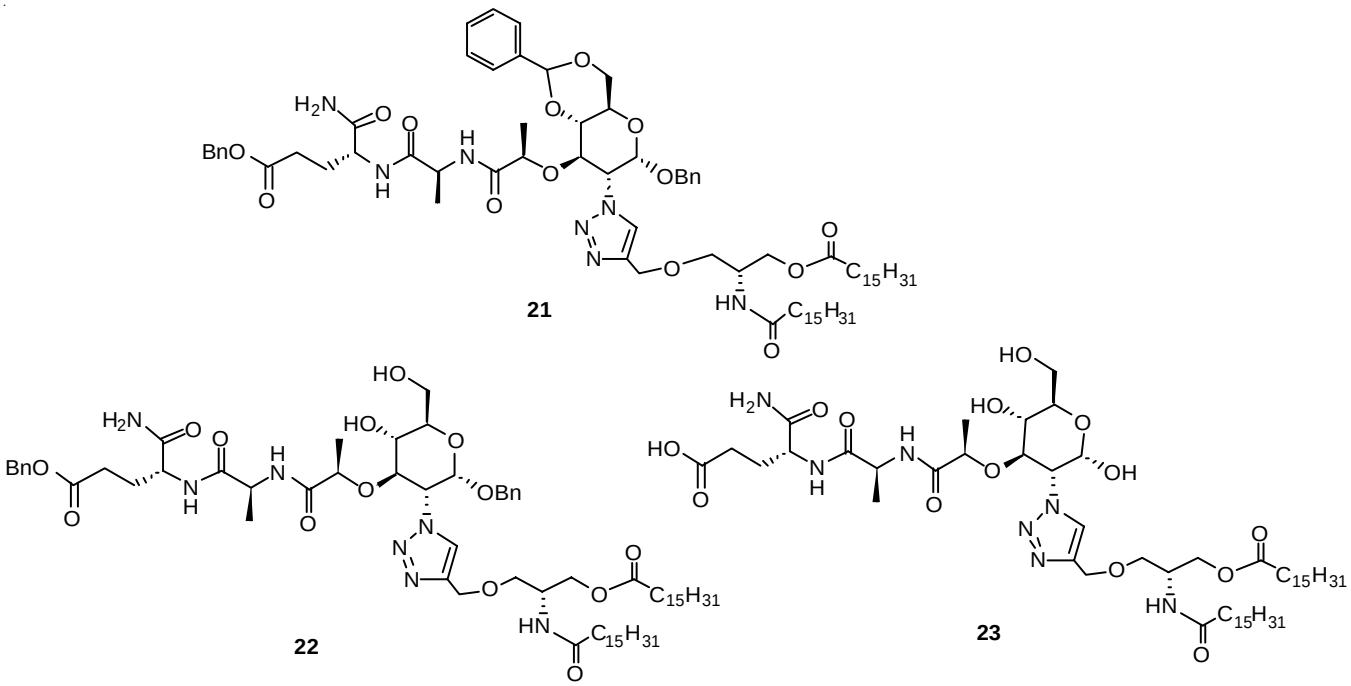


Fig. 1. Novel triazolyl L-serinol palmitate muramyl dipeptide analogues

was determined using MTT assay. From Fig. 2, it is evident that all the three analogues were non-cytotoxic in nature. Further the immunomodulating activity of the analogues was determined by quantifying the NO and IL-6, TNF-α cytokine production upon incubation with RAW macrophages. All the three analogues have elicited a dose dependent production of NO and pro-inflammatory cytokine IL-6, TNF-α (Figs. 3 and 4).

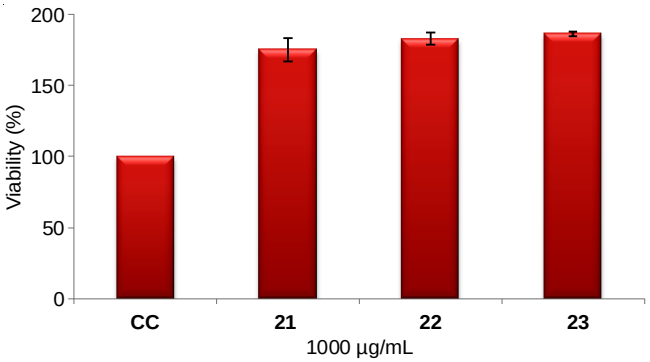


Fig. 2. Cytotoxicity-RAW macrophages were incubated with compounds and viability was assessed using MTT assay. All the experiments were performed in triplicates and bars represent mean ± SD

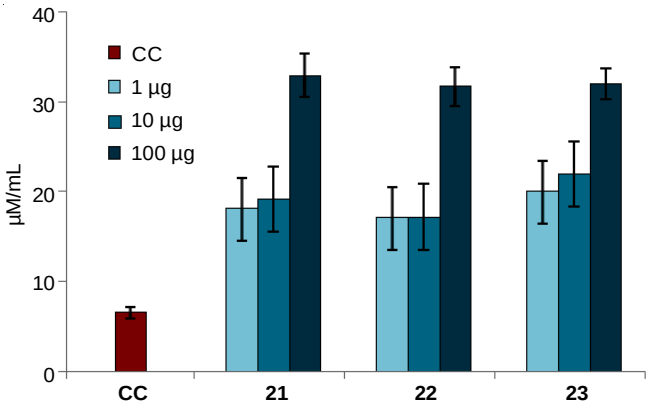


Fig. 3. NO-NO production from RAW macrophages was quantified using Griees reagent, after treating with the compounds at three different concentrations. All the experiments were performed in triplicates and bars represent mean ± SD

The novel analogues were non-toxic in nature and were able to stimulate the innate immune system by augmenting the NO production, which is very much required to tackle the primary infections caused by any bacteria or virus. The pro-inflammatory cytokines produced from stimulated macrophages indicate the immuno potentiating effect of the novel analogues in a dose dependent manner. These pro-inflammatory cytokines act by active recruitment of immune cells to the site of infection/injury. From the preliminary biological data, it is inferred that analogues **21**, **22** and **23** have an immuno stimulating activity and their potential as vaccine adjuvants has to be evaluated against various antigens.

Conclusion

Synthesis of novel triazole tethered serinol derived dipalmitoylated muramyl dipeptide (MDP) derivatives was accomplished by employing click chemistry. All the synthesized compounds were characterized by ^{1}H, ^{13}C NMR, mass spectroscopy. The sequential deprotection of protecting groups yielded novel molecules with various degree of hydrophilic-lipophilic balance (HLB), which may have a potential impact on their adjuvanticity. The novel molecules thus synthesized were also evaluated for their toxicity and immune potentiation ability on RAW macrophages and found to be activated the immune system. The molecules should be further evaluated for their *in vivo* immunogenicity to determine their true potential and their suitability as vaccine adjuvants against various bacterial and viral pathogens.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

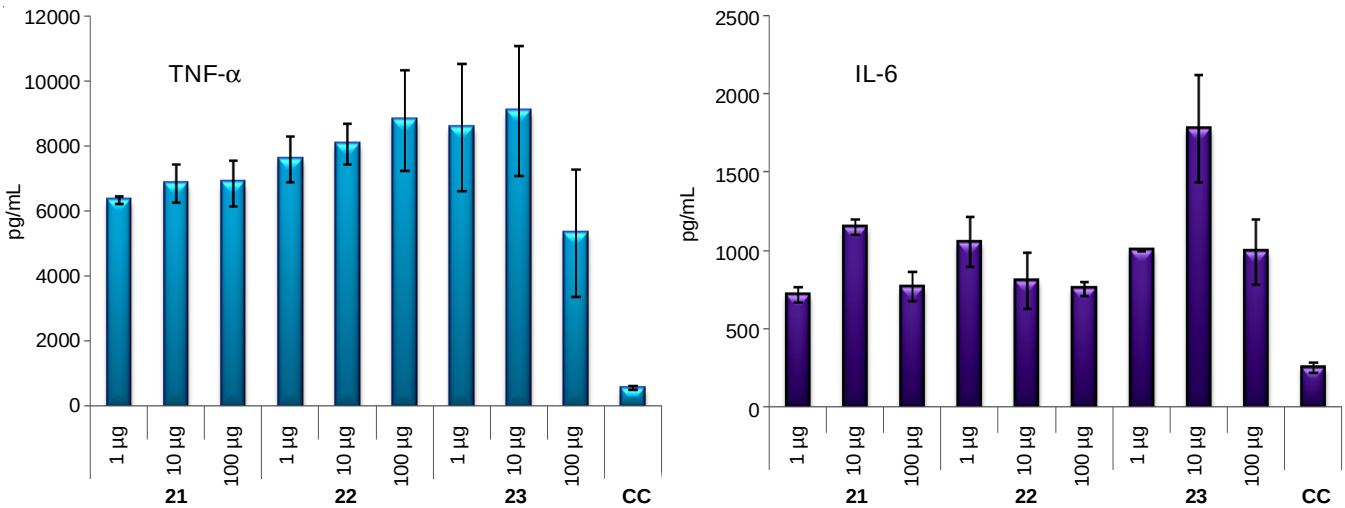

Fig. 4. Pro-inflammatory cytokines TNF-α and IL-6 quantification from stimulated RAW macrophages using sandwich ELISA. All the experiments were performed in triplicates and bars represent mean ± SD

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