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Design and Development of Stable, Water-soluble, Human Toll-like Receptor 2-Specific, Monoacyl Lipopeptides as Candidate Vaccine Adjuvants

Deepak B. Salunke, **Seth W. Connelly**, **Nikunj M. Shukla**, **Alec R. Hermanson**, **Lauren M. Fox**, and **Sunil A. David***
Department of Medicinal Chemistry, University of Kansas

Abstract

Antigens in modern subunit vaccines are largely soluble and poorly immunogenic proteins inducing relatively short-lived immune responses. Appropriate adjuvants initiate early innate immune responses, amplifying subsequent adaptive immune responses. Agonists of TLR2 are devoid of significant pro-inflammatory activity in *ex vivo* human blood models, and yet potently adjuvantic, suggesting that this chemotype may be a safe and effective adjuvant. Our earlier work on the monoacyl lipopeptide class of TLR2 agonists led to the design of a highly potent lead, but with negligible aqueous solubility, necessitating the reintroduction of aqueous solubility. We explored several strategies of introducing ionizable groups on the lipopeptide, as well as the systematic evaluation of chemically stable bioisosteres of the ester-linked palmitoyl group. These studies have led to a fully optimized, chemically stable, and highly water-soluble, human TLR2-specific agonist, which was found to have an excellent safety profile and displayed prominent adjuvantic activities in rabbit models.

Keywords

TLR2; TLR2 agonists; Vaccine adjuvants; Innate immunity

Introduction

The overall goal of vaccination is the generation of specific, robust, and durable immune responses against the antigen to provide long-term protection against pathogens. Early vaccines which frequently utilized killed whole organisms are reactogenic, and are associated with local and systemic adverse reactions, whole cell pertussis vaccines being an example.[1–4] Modern vaccines such as acellular pertussis vaccines[1] use highly purified antigens. Such ‘subunit vaccines’ have a much more defined composition, facilitating not only the ease of production and quality control, but importantly, are also considerably less reactogenic. However, subunit antigens are largely soluble proteins which are intrinsically poorly immunogenic, and do not induce long-lived immune responses. The recent reemergence of pertussis in the United States[5–7] has served to highlight the rapid waning of protective immunity following vaccination with acellular subunit pertussis vaccines,[8,9] and emphasizes the need for safe and effective adjuvants.

*Corresponding Author Address: Sunil A. David, Department of Medicinal Chemistry, University of Kansas, Multidisciplinary Research Building, Room 320D, 2030 Becker Drive, Lawrence KS 66047. Tel: 785-864-1610; Fax: 785-864-1961, sdavid@ku.edu.

Supporting Information Available: Characterization data (^{1}H, ^{13}C, mass spectra) and LC-MS analyses of final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

Adjuvants initiate early innate immune responses which subsequently lead to the induction of robust and long-lasting adaptive immunity.[10] Aluminum salts (primarily phosphate and hydroxide), discovered by Glenny and coworkers,[11] have been the only adjuvants in clinical use until the recent introduction of 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL).[12] Aluminum salts (used as adjuvants in acellular pertussis vaccines) are weak adjuvants for antibody induction, promoting a T helper 2 (Th2)-skewed, rather than a Th1 response,[13,14] and are virtually ineffective at inducing cytotoxic T lymphocyte or mucosal IgA antibody responses. They also appear to promote the induction of IgE isotype switching which has been associated with allergic reactions in some subjects.[13,14]

Our knowledge of the molecular mechanisms of innate immunity has expanded rapidly since the discovery of Toll-like receptors (TLRs)[15–17] and of their role in induction and amplification of adaptive immune responses.[18,19] Innate immune afferent signals activated by vaccine adjuvants include those originating from Toll-like receptors (TLRs), as well as RIG-I-like receptors[20] and NOD-like receptors (NLRs).[21,22] There are 10 functional TLRs encoded in the human genome, which are trans-membrane proteins with an extracellular domain having leucine-rich repeats (LRR) and a cytosolic domain called the Toll/IL-1 receptor (TIR) domain.[23] The ligands for these receptors are highly conserved molecules such as lipopolysaccharides (LPS) (recognized by TLR4), lipopeptides (TLR2 in combination with TLR1 or TLR6), flagellin (TLR5), single stranded RNA (TLR7 and TLR8), double stranded RNA (TLR3), CpG motif-containing DNA (recognized by TLR9), and profilin present on uropathogenic bacteria (TLR11).[24] TLR1, -2, -4, -5, and -6 recognize extracellular stimuli, while TLR3, -7, -8 and -9 function within the endolysosomal compartment.[23] The engagement of TLRs by their cognate ligands lead to the production of inflammatory cytokines, and up-regulation of major histocompatibility complex (MHC) molecules and co-stimulatory signals in antigen-presenting cells as well as activating natural killer (NK) cells (innate immune response). These responses result in the priming and amplification of T-, and B-cell effector functions (adaptive immune responses).[25–28]

We have been systematically exploring detailed structure-activity relationships (SAR) of several immunostimulatory TLR agonists,[29–37] with a particular focus on TLR2 agonists. Unlike other TLR-active compounds, agonists of TLR2, first identified in a mycoplasmal lipopeptide, S-[2,3-*bis*(palmitoyloxy)-(2*R*)-propyl]-*R*-cysteinyl-GNNDESNISFKEK, termed Macrophage-Activating Lipopeptide-2 **1** (MALP-2)[38–40] (Fig. 1) and exemplified by the S-[2,3-*bis*(palmitoyloxy)-(2*RS*)-propyl]-*R*-cysteinyl-*S*-serine **2** (PAM_2CS) chemotype[30,36] are of particular interest to us, for although the lipopeptide is devoid of any detectable pro-inflammatory activity in *ex vivo* human blood models (as defined by the production of detectable levels of TNF-α, IL-1β, IL-6, or IL-8),[41] or of local reactogenicity and pyrogenicity in rabbit models,[31] it is potently adjuvantic in murine models of immunization,[41] suggesting that this chemotype may be a safe and effective adjuvant.

The exposure of bone marrow-derived dendritic cells of C57/BL6 mice to TLR2-agonistic lipopeptides results in an upregulation of MHC Class II and CD80/CD86 costimulatory molecules, with enhanced expression of CD11b and CD11c, associated with the production of TNF-α and IL-12.[42] These results have been confirmed in BALB/c mice using **1**, and extended to show that the lipopeptide also upregulates immunoproteasome (LMP2, LMP7 and MECL1) activity in a dose-dependent manner, suggesting that TLR2 agonists may indirectly enhance MHC Class I-restricted responses by accelerated antigen processing and peptide presentation.[43] In a recent report comparing the adjuvanticity of several ligands of TLRs in *Chlamydia* major outer membrane protein vaccine constructs, TLR2 agonists were found to be superior in inducing protective responses against a challenge of *Chlamydia trachomatis*.[44]

Importantly, the effects of lipopeptides on APC maturation and antigen presentation have also been demonstrated in human dendritic cells (DCs).[45] Plasmacytoid and myeloid dendritic cells (pDCs and mDCs, respectively) constitute the most potent professional antigen presenting cells, and play a pivotal role in the induction and polarization of antigen-specific immune responses.[46] Human myeloid dendritic cells comprise of two major subsets: $CD1c^+$ mDCs and $CD141^+$ mDCs, both of which express TLR2.[47,48] Furthermore, TLR2 agonists induce isotypic switching and differentiation of naïve human B lymphocytes to IgG-secreting plasma cells,[49] indicating a functional association between BCR stimulation and TLR activation.[49]

It is pertinent to highlight anecdotal human data on TLR2 agonists in the context of tolerability and safety of these compounds. In one study, ten patients with incompletely resectable pancreas carcinomas were injected intra-tumorally during surgery with 20–30 μg of **1** followed by postoperative chemotherapy. As expected, the investigators observed influx of lymphocytes and monocytes in wound secretions, but no systemic side effects were noted. The study concluded that "up to 20 μg of **1** was well tolerated".[50] In another study, 0.125–1.0 μg of **1** was directly applied on punch biopsy lesions of the skin in twelve patients; other than transient and self-limited erythema, no systemic side effects were noted.[51] The lack of proinflammatory activity, excellent safety profile in animal models and human subjects, the expression of the TLR2 in multiple DC subsets, and its strong adjuvantic activity have been compelling reasons for our focus on agonists of TLR2.

Our SAR studies on the **2** series of compounds[36] led to a simplified second-generation monoacyl lipopeptide **3** (Fig. 1), in which the spacing between the ester-linked acyl group and the thioether was found to play a crucial role in determining activity;[30] further SAR studies led to the identification of a cysteine *N*-acetyl analogue **4**, which retained exquisite human TLR2 (hTLR2)-specificity, with a substantial gain in potency, rivaling that of **2**.[52] Although highly potent and hTLR2-specific, the acetylation of the cysteine amine in **4** led to loss of the lone ionizable group and, consequently, to the complete loss of aqueous solubility. An important component of our work on vaccine adjuvant design and development has been to engineer complete water solubility into our candidate adjuvants so as to obviate the need for any excipients, and it therefore became necessary to reintroduce aqueous solubility in **4**. Drawing from our previous SAR studies that the sidechain of the terminal amino acid (Ser) of the dipeptide unit was not a key determinant of TLR2 activity,[30,36,52] an obvious and straightforward approach was to replace it with lysine, the ε-amine of which was anticipated to restore solubility to the lipopeptide. However, we observed hydrolytic lability in the analogue, with consequent deterioration of activity. This observation prompted us to explore various strategies of introducing ionizable groups on the lipopeptide, as well as the systematic evaluation of stable bioisosteres of the ester-linked palmitoyl group. Analogues with the serine hydroxyl functionality of the lipopeptide esterified with L-lysine, succinic acid or nicotinic acid retained hTLR2-specific agonistic activity, but progressively hydrolyzed to **4** upon prolonged storage, indicating that ester groups were contributing to lability. These initial observations prompted us to first explore replacing the ester-linked palmitoyl group with amide, triazole, and carbamate linked long-chain alkyl groups. A carbamate derivative was found to be more potent than the parent compound. Aqueous solubility in this analogue was restored by appending a N^1,N^1-dimethylpropane-1,3-diamine moiety to the carboxyl group of serine via an amide linkage, culminating in a fully optimized, stable, and highly water-soluble, human TLR2-specific agonist with very high potency. The optimized lead compound was found to have an excellent safety profile in rabbit models, and displayed prominent adjuvantic activities.

Results and Discussion

To restore water solubility to **4**, we synthesized an analogue with the terminal serine methyl ester replaced with a lysine methyl ester (Compound **10**, Scheme 1). While the lysine analogue **10** was indeed as active as **4** (EC_{50} = 1.50 nM, Table 1, 10Fig. 2) and highly water soluble, we noticed significant loss of activity of aqueous stocks in less than a week. Mass spectrometry revealed hydrolysis of the methyl ester, presumably via an intramolecular attack of the ε-amine of lysine, which presaged inadequate shelf-life of vaccine constructs incorporating as a candidate adjuvant. We next explored analogues with the serine hydroxyl functionality of **4** esterified with L-lysine (**12**), sulfated (**13**), converted to the hemisuccinate (**14**) or esterified with nicotinic acid (**15**) (Scheme 2). All the compounds retained hTLR2-specific agonistic activity (Table 1, 12Fig. 2), and the lysine conjugate (EC_{50} = 1.50 nM), the sulfate **13** (EC_{50} = 5.84 nM), as well as hemisuccinate **14** (EC_{50} = 0.65 nM) were found to be highly water soluble. As observed for compound **10**, aqueous stocks of compound **12** were also found to be unstable, and a white precipitate of its parent compound **4** was observed (confirmed by TLC and LC-MS) upon prolonged storage, indicating that compound **12** was behaving as a water-soluble, but relatively unstable prodrug of compound **4**.

Given that the mono-acyl lipopeptides are human TLR2-specific, murine models of immunization that we had used previously[34] to benchmark adjuvantic activity were inappropriate, and it was of importance to verify whether the rabbit model[34,53] that we had subsequently adopted as a screen would be suitable to evaluate the adjuvanticity of this chemotype. We elected first to evaluate the adjuvanticity of the highly water-soluble **12** using bovine α-lactalbumin as a model subunit antigen[34,53] and under excipient-free conditions, reasoning that degradation *in vivo* of **12** would yield **4**, which would retain TLR2-agonistic activity. We were gratified to find that the water soluble monoacyl lipopeptide **12** showed excellent induction of anti-bovine α-lactalbumin IgG responses in rabbits using a prime+dual-boost model (Fig. 3), validating the relevance of the animal model, and demonstrating excellent adjuvanticity.

Having observed lability of two ester groups, both of which undermine and compromise the activity and shelf-life, we set out to design out all of the labile groups in our lead compound **4**, beginning with the replacement of the ester-linked palmitoyl group with amide- (compound **22**, Scheme 3), triazole- (compounds **24** and **27**, Scheme 4) and carbamate- (compounds **31** and **34**, Scheme 5) linked long-chain alkyl groups.

Starting from L-cystine, the advanced intermediate **18** was synthesized as reported by us earlier.[30,52] The hydroxyl functionality in **18** was mesylated and displaced by sodium azide to furnish the azido compound **19** (73% over two steps, Scheme 3). A Staudinger azide reduction protocol was used for the synthesis of amino compound **20,** which was *N*-palmitoylated to obtain **21**. Global deprotection and controlled acetylation (yielding predominantly the cystine *N*-acetylated product) furnished the desired amide-linked derivative **22**. The triazole linked derivatives **24** and **27** were designed based on the fact that substituted 1,2,3-triazoles are popular functionalities used in drug discovery for the bioisosteric replacement of key functional groups. They are also known to readily associate with biological targets through hydrogen-bonding and dipole interactions.[54,55] The azido intermediate **19** described above was a convenient synthon for the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction for the synthesis of the 4-alkyl-1*H*-1,2,3-triazol-1-yl compound **24**. Pentadec-1-yne was selected as an alkyne component in this reaction to maintain the overall length of the molecule. The regioisomeric 1-alkyl-1*H*-1,2,3-triazol-4-yl analogue **27** was also synthesized by swapping the alkyne and azide functionalities in the respective synthons (Scheme 4); the alkyne component **25** was

synthesized by alkylation of mercapto-dipeptide **17** with 4-bromobut-1-yne, while 1-azidohexadecane was synthesized from 1-bromohexadecane using sodium azide in DMF. The synthesis of the carbamate-linked analogue **29** was achieved by treatment of the primary alcohol functionality in compound **18** with 1,1′-carbonyldiimidazole (CDI) in dichloromethane (DCM), and reacting *in situ* the imidazolecarboxylate intermediate **28** with hexadecylamine (Scheme 5). The desired derivative **31** was synthesized as reported for the synthesis of compound **22**. The regioisomeric carbamate derivative **34** was synthesized simply by the reaction of **20** with cetylchloroformate and further elaboration as reported for compounds **22** or **31**.

The triazole derivatives **24** (EC_{50} = 0.29 μM, Table 1, 27Fig. 4) and (EC_{50} = 1.02 μM, Table 1, 22Fig. 4) were found to be the least active in TLR2 agonism assay. The loss in activity was also observed in amide derivative (EC_{50} = 12.78 nM, Table 1, 31Fig. 4). We were delighted to observe in our cell-based reporter gene assays that the carbamate derivative was as potent (EC_{50} = 0.32 nM, Table 1, 4Fig. 4) as , and its regioisomer **34** was highly active (**34**: EC_{50} = 65 pM; **4**: EC_{50} = 0.66 nM; Table 1, Fig. 4).

Aqueous solubility in the highly potent and stable carbamate derivative **34** was restored by appending a N^1,N^1-dimethylpropane-1,3-diamine moiety to the carboxyl group of serine via a stable amide linkage (compound **38**, Scheme 6), an approach that is similar to attaching a polar polyethylene glycol unit to the terminal amino acid residue.[56] Our initial attempts at hydrolyzing the methyl ester in compound **32** for coupling the N^1,N^1-dimethylpropane-1,3-diamine using conventional lithium hydroxide protocols resulted in epimerization of the stereocenters, yielding the target compound in its racemic form (EC_{50} = 1.29 nM). We successfully utilized the mild and selective trimethytin hydroxide ester hydrolysis method developed by Nicolaou[57] and obtained compound **35** in enantiopure form. Subsequent amidation of **35** using N^1,N^1-dimethylpropane-1,3-diamine and further elaboration resulted in the highly water soluble (>10 mg/mL) and highly potent (EC_{50} = 0.25 nM, Table 1) enantiopure final compound **38**, which retained excellent chemical stability under accelerated stability testing conditions.

The combination of highly desired attributes of high potency (in primary screens), excellent aqueous solubility, and chemical stability in **38** warranted a careful evaluation of this compound in secondary screens. Consistent with our earlier findings that **2** induced virtually undetectable levels of proinflammatory mediators such as TNF-α, IL-1β and IL-18,[31] we could not detect any significant proinflammatory cytokine signatures in human PBMCs or human whole blood stimulated with **38** using our standard 5-plex cytometric bead assays[35,41] (data not shown). In an effort to understand in greater detail the basis of the potent adjuvanticity of TLR2 agonists that appears to be entirely dissociated from the induction of proinflammatory cytokines, we examined **38** in a 41-plex immunoassay that we have recently implemented. We observed prominent biphasic induction of the chemokines monocyte chemotactic protein-1 (MCP-1), and MCP-3, macrophage-derived chemokine (MDC), as well as interleukin-8 (IL-8, also a chemokine; Fig. 6). The rather unexpected finding of strong macrophage-derived or -targeted chemokine induction prompted us to examine markers of monocytic activation in *ex vivo* flow cytometric assays using whole human blood. As previously reported,[31] TLR2 and TLR4 agonists induce CD11b upregulation in human granulocytes, and we found that **38** indeed upregulated CD11b very potently, relative to **2** (Fig. 6, left panel) in neutrophils; however, we also observed strong monocytic CD11b upregulation (Fig. 6, right panel), which has not been previously reported for agonists of TLR2. Although we cannot as yet establish a causal relationship between monocytic activation and chemokine production on the one hand, and adjuvanticity on the other, these biomarkers will likely prove useful in evaluating several other chemotypes which we are currently examining.

Cautious of the very high potency of **38**, we elected to first evaluate the safety of this lipopeptide in rabbit models. A dose-escalation study in rabbits up to 100 μg/animal (administered intramuscularly, or as an intravenous bolus) did not result in any observable adverse effects. Whereas a dose of 1 μg of LPS evoked prominent leucopenia, lymphocytopenia (Fig. 7a–b) and febrile responses (Fig. 7c) in the animals, 100 μg of **38** did not induce any such effects (Fig. 7).

Encouraged by the excellent safety profile, we proceeded to evaluate the adjuvanticity of **38**. Non-alum-adsorbed, toxoided pertussis antigens are not commercially available, and we therefore chose CRM197, a nontoxic mutant of diphtheria toxin as a test-antigen.[58] The excellent solubility of the lipopeptide allowed excipient-free formulation of the test-antigen and adjuvant in sterile saline. Pre-immune test-bleeds were first obtained via venipuncture of the marginal vein of the ear. Rabbits were then immunized intramuscularly on Days 1, 15, and 28 with antigen-sparing doses (10 μg/dose) of unadjuvanted CRM197 in saline, or adjuvanted with 100 μg/dose of **38** in a total volume of 0.2 mL. A test bleed was performed on Day 25 (ten days after the first boost) and a final bleed was performed via the marginal vein of the ear on Day 38. Sera were stored at −80 °C until used. CRM197-specific IgG titers in sera were quantified by conventional antibody-capture ELISA techniques performed in 384-well format using automated liquid handling methods as described by us.[34,53] We were gratified to find rapid and robust anti-CRM197 IgG titers in animals receiving the adjuvant (Fig. 8), as compared to unadjuvanted controls.

We have been fortunate in being able to successfully apply principles of classical medicinal chemistry and rational drug optimization to an unexpected problem of chemical stability, achieving in the process augmented potency, excellent aqueous solubility and, importantly, preserving safety and efficacy. The identification of key chemokine biomarkers for TLR2 agonists will likely prove useful as we continue to develop this and other chemotypes as candidate vaccine adjuvants.

Experimental Section

Chemistry

All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 °C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf 'Gold' high performance silica columns on CombiFlash Rf instrument unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel CCM pre-coated aluminum sheets. Purity for all final compounds was confirmed to be greater than 97% by LC-MS using a Zorbax Eclipse Plus 4.6 mm × 150 mm, 5 μm analytical reverse phase C_{18} column with H_2Oisopropanol or H_2O-CH_3CN gradients and an Agilent ESI-TOF mass spectrometer (mass accuracy of 3 ppm) operating in the positive ion (or negative ion, as appropriate) acquisition mode.

Synthesis of Compound 6: (2S,2′S)-Dimethyl 2,2′-(((2R,2′R)-3,3′-disulfanediylbis(2-acetamidopropanoyl))bis(azanediyl))bis(6-((((9H-fluoren-9-yl)methoxy)carbonyl)amino) hexanoate)—To a solution of L-cystine (500 mg, 2.08 mmol) in water (10 mL) were added triethylamine (870 μL, 6.24 mmol) and di-*tert*-butyldicarbonate (1.35 g, 6.24 mmol). The reaction mixture was stirred at room temperature for 2 h. After completion of the reaction, the solvent was evaporated under reduced pressure. The residue was dissolved in ethyl acetate and washed with 10% HCl. The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to obtain the crude product which was purified using column chromatography (15% MeOH/

CH_2Cl_2) to obtain compound *Nα,Nα′*-di-Boc-L-cystine (870 mg, 95%). To a solution of *Nα,Nα′*-di-Boc-L-cystine (500 mg, 1.13 mmol) in anhydrous DMF (15 mL) were added H-Lys(Fmoc)-OMe·HCl (1.05 g, 2.50 mmol), HOBt (338 mg, 2.50 mmol), and pyridine (411 μL, 4.50 mmol). The reaction mixture was stirred at 0 °C for 30 min, followed by addition of EDCI (958 mg, 5.00 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 16 h, followed by evaporation of the solvent under reduced pressure. The residue was then dissolved in ethyl acetate and washed with water. The organic solvent was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to obtain the crude product which was purified using column chromatography (50% EtOAc/hexanes) to obtain compound **5** (1.10 g, 83%). MS (ESI-TOF) for $C_{60}H_{76}N_6O_{14}S_2$ $[M+H]^+$ Found 1169.5011, Calculated 1169.4934; $[M+Na]^+$ Found 1191.4834, Calculated 1191.4753. Compound **5** (600 mg, 0.51 mmol) was dissolved in hydrogen chloride solution (10 mL, 4M in dioxane) and the reaction mixture was stirred at room temperature for an hour and the volatilities were removed to afford (2S,2′S)-dimethyl 2,2′-(((2R,2′R)-3,3′-disulfanediylbis(2-aminopropanoyl))bis(azanediyl))bis(6-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)hexanoate) dihydrochloride salt (535 mg, 0.51 mmol). The crude product was dissolved in CH_2Cl_2 (5 mL) and pyridine (5 mL). Acetic anhydride (291 μL, 3.08 mmol) was added and the reaction mixture was stirred at room temperature for 2h, followed by evaporation of the solvent under reduced pressure. The residue was then dissolved in ethyl acetate and washed with water. The organic solvent was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to obtain the crude product which was purified using column chromatography (50% EtOAc/hexanes) to obtain compound **6** (425 mg, 79% over two steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.46 (d, *J* = 7.1 Hz, 2H), 7.74 (d, *J* = 7.5 Hz, 4H), 7.57 (d, *J* = 7.4 Hz, 4H), 7.38 (t, *J* = 7.4 Hz, 4H), 7.29 (t, *J* = 7.5 Hz, 4H), 6.72 (d, *J* = 9.1 Hz, 2H), 5.45 (td, *J* = 9.9, 3.2 Hz, 2H), 4.94 (t, *J* = 5.4 Hz, 2H), 4.48 – 4.32 (m, 6H), 4.18 (t, *J* = 6.8 Hz, 2H), 3.71 (s, 6H), 3.20 – 3.03 (m, 6H), 2.94 – 2.83 (m, 2H), 2.02 (s, 6H), 1.91 – 1.68 (m, 4H), 1.58 – 1.31 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.4, 170.7, 156.5, 144.1, 141.4, 127.8, 127.2, 125.1, 120.1, 66.6, 53.2, 52.7, 52.5, 47.4, 46.2, 40.8, 31.2, 29.5, 23.5, 22.8. MS (ESI-TOF) for $C_{54}H_{64}N_6O_{12}S_2$ $[M+H]^+$ Found 1053.4234, Calculated 1053.4096; $[M+Na]^+$ Found 1075.4048, Calculated 1075.3916.

Synthesis of Compound 9: (9S,12R)-12-Acetamido-1-(9H-fluoren-9-yl)-9-(methoxycarbonyl)-3,11-dioxo-2-oxa-14-thia-4,10-diazahexadecan-16-yl palmitate—To a solution of **6** (375 mg, 0.356 mmol) in CH_2Cl_2 (5 mL) were added water (100 μL) and tributylphosphine (356 μL, 1.43 mmol). The reaction mixture was stirred at room temperature for 2 h. After completion of the reaction, the solvent was removed under reduced pressure to obtain the crude product, which was purified using column chromatography (50% EtOAc/hexanes) to obtain compound **7** (250 mg, 66%). MS (ESI-TOF) for $C_{27}H_{33}N_3O_6S$ $[M+H]^+$ Found 528.2232, Calculated 528.2163; $[M+Na]^+$ Found 550.2061, Calculated 550.1988. To a solution of compound **7** (245 mg, 0.465 mmol) in DMF (5 mL) were added 2-iodoethanol (182 μL, 2.33 mmol) and potassium carbonate (320 mg, 2.33 mmol). The reaction mixture was stirred at room temperature for an hour. After completion of the reaction, the solid potassium carbonate was filtered out and the solvent was removed under reduced pressure to obtain the crude product, which was purified using column chromatography (5% MeOH/CH_2Cl_2) to obtain compound **8** as a white solid (111 mg, 42%). MS (ESI-TOF) for $C_{29}H_{37}N_3O_7S$ $[M+H]^+$ Found 572.2427, Calculated 572.2425; $[M+Na]^+$ Found 594.2255, Calculated 594.2244. Compound **8** (100 mg, 0.175 mmol) was then dissolved in CH_2Cl_2 (1 mL) and pyridine (1 mL). Palmitoyl chloride (80 μL, 0.262 mmol) was added and the reaction mixture was stirred at room temperature for 1 h. After completion of the reaction, the solvents were removed under reduced pressure to obtain the crude product, which was purified using column chromatography (50% EtOAc/

hexanes) to obtain compound **9** (133 mg, 94%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.4 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 7.07 (d, *J* = 7.7 Hz, 1H), 6.54 (d, *J* = 7.2 Hz, 1H), 5.12 (t, *J* = 5.7 Hz, 1H), 4.63 – 4.50 (m, 2H), 4.46 – 4.35 (m, 2H), 4.31 – 4.16 (m, 3H), 3.73 (s, 3H), 3.17 (td, *J* = 13.6, 6.9 Hz, 2H), 2.92 (qd, *J* = 14.0, 6.4 Hz, 2H), 2.82 (td, *J* = 6.6, 1.9 Hz, 2H), 2.30 (t, *J* = 7.6 Hz, 2H), 2.01 (s, 3H), 1.89 (ddd, *J* = 14.9, 10.8, 5.7 Hz, 1H), 1.79 – 1.66 (m, 1H), 1.65 – 1.56 (m, 2H), 1.56 – 1.47 (m, 2H), 1.45 – 1.33 (m, 2H), 1.33 – 1.21 (m, 24H), 0.87 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.05, 172.33, 170.53, 170.38, 156.76, 144.11, 144.08, 141.44, 127.81, 127.17, 125.17, 120.11, 66.67, 62.76, 52.68, 52.64, 52.39, 47.39, 40.53, 34.34, 34.26, 32.06, 31.64, 31.15, 29.83, 29.79, 29.76, 29.62, 29.50, 29.42, 29.36, 29.28, 25.02, 23.20, 22.83, 22.40, 14.27. MS (ESI-TOF) for $C_{45}H_{67}N_3O_8S$ $[M+H]^+$ Found 810.4754, Calculated 810.4722; $[M+Na]^+$ Found 832.4591, Calculated 832.4541.

Synthesis of Compound 10: 2-(((R)-2-Acetamido-3-(((S)-6-amino-1-methoxy-1-oxohexan-2-yl)amino)-3-oxopropyl)thio)ethyl palmitate—To a solution of compound **9** (81 mg, 0.1 mmol) in DMF (1 mL) was added polymer-bound piperazine (1–2 mmol/g loading) (333 mg, ~ 0.5 mmol) and the reaction mixture was stirred at room temperature for 4 h. After completion of the reaction, the resin was filtered out and the solvents were removed under reduced pressure to obtain the crude product, which was purified using column chromatography (20% MeOH/CH_2Cl_2) to obtain compound **10** as a white solid (32 mg, 55%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.03 (d, *J* = 7.9 Hz, 1H), 7.05 (d, *J* = 8.4 Hz, 1H), 4.84 (td, *J* = 8.0, 5.5 Hz, 1H), 4.54 (dd, *J* = 13.8, 7.4 Hz, 1H), 4.31 – 4.15 (m, 2H), 3.73 (s, 3H), 3.06 (ddd, *J* = 19.2, 13.6, 6.2 Hz, 3H), 2.92 – 2.72 (m, 3H), 2.34 – 2.26 (m, 2H), 2.06 (s, 3H), 1.97 – 1.88 (m, 1H), 1.85 (dd, *J* = 15.0, 7.7 Hz, 2H), 1.77 – 1.67 (m, 1H), 1.59 (dd, *J* = 14.3, 7.2 Hz, 4H), 1.40 – 1.17 (m, 24H), 0.87 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.01, 172.17, 171.42, 170.90, 63.08, 52.63, 52.40, 39.05, 34.97, 34.36, 32.07, 31.19, 30.21, 29.85, 29.82, 29.81, 29.79, 29.66, 29.51, 29.46, 29.33, 26.58, 25.06, 23.41, 22.84, 22.07, 14.28. MS (ESI-TOF) for $C_{30}H_{57}N_3O_6S$ $[M+H]^+$ Found 588.4086, Calculated 588.4041; $[M+Na]^+$ Found 610.3914, Calculated 610.3860.

Synthesis of 12: 2-(((R)-2-Acetamido-3-(((S)-3-(((S)-2,6-diaminohexanoyl)oxy)-1-methoxy-1-oxopropan-2-yl)amino)-3-oxopropyl)thio)ethyl palmitate—To a solution of *N*,*N*′-di-boc-L-lysine (63 mg, 0.183 mmol) and compound **4** (50 mg, 0.091 mmol) in anhydrous CH_2Cl_2 (5 mL), were added *N*-methylmorpholine (20 μL, 0.183 mmol) and catalytic amount of DMAP. The reaction mixture was stirred at 0 °C and EDCI (28 mg, 0.183 mmol) was added after 15 min. The reaction mixture was then stirred at room temperature for 4 h. After the completion of reaction, water (10 mL) was added and the product was extracted in CH_2Cl_2. The organic layer was washed with water (10 mL × 2), brine (10 mL) and dried over anhydrous sodium sulfate. The solvent was removed under vacuum and the residue was purified using column chromatography (5 % MeOH/CH_2Cl_2) to obtain the diboc protected intermediate **11** as white solid (40 mg, 50%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35 (d, *J* = 7.6 Hz, 1H), 6.74 (d, *J* = 7.6 Hz, 1H), 5.23 (d, *J* = 6.6 Hz, 1H), 4.83 (dt, *J* = 7.7, 3.9 Hz, 1H), 4.77 (s, 1H), 4.66 (d, *J* = 6.3 Hz, 1H), 4.58 (d, *J* = 10.0 Hz, 1H), 4.45 (dd, *J* = 11.2, 3.9 Hz, 1H), 4.32 – 4.14 (m, 3H), 3.78 (s, 3H), 3.18 – 3.07 (m, 2H), 2.97 (qd, *J* = 13.9, 6.4 Hz, 2H), 2.85 – 2.74 (m, 2H), 2.31 (t, *J* = 7.6 Hz, 2H), 2.07 (s, 3H), 1.86 – 1.55 (m, 6H), 1.55 – 1.41 (m, 18H), 1.41 – 1.20 (m, 24H), 0.87 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.95, 172.59, 170.84, 170.57, 169.19, 156.49, 155.86, 80.25, 79.42, 63.83, 62.93, 53.54, 53.15, 52.66, 52.15, 39.84, 34.35, 33.95, 32.07, 31.67, 31.11, 29.84, 29.80, 29.77, 29.63, 29.51, 29.44, 29.30, 28.59, 28.50, 25.04, 23.25, 22.84, 22.48, 14.28. MS (ESI-TOF) for $C_{43}H_{78}N_4O_{12}S$ $[M+H]^+$ Found 875.5417, Calculated 875.5410; $[M+Na]^+$ Found 897.5232, Calculated 897.5229. Compound **11** was then dissolved in HCl/dioxane (4 M solution, 2 mL) and the reaction

mixture was stirred at room temperature for 15 min, followed by removal of the solvent under vacuum to obtain compound **12** as a white solid in quantitative yield. ^{1}H NMR (500 MHz, DMSO) δ 8.80 (d, *J* = 8.1 Hz, 1H), 8.63 (s, 3H), 8.31 (d, *J* = 8.3 Hz, 1H), 7.95 (s, 3H), 4.72 (ddd, *J* = 7.9, 6.3, 4.9 Hz, 1H), 4.46 (ddd, *J* = 15.7, 10.0, 4.7 Hz, 2H), 4.38 (dd, *J* = 11.2, 6.4 Hz, 1H), 4.18 – 4.11 (m, 2H), 3.97 (bs, 1H), 3.73 – 3.69 (m, 1H), 3.69 – 3.64 (m, 4H), 3.52 – 3.48 (m, 1H), 3.46 (ddd, *J* = 6.1, 3.9, 1.2 Hz, 1H), 2.93 (dd, *J* = 13.7, 4.8 Hz, 1H), 2.83 – 2.72 (m, 4H), 2.67 (dd, *J* = 13.7, 9.4 Hz, 1H), 2.28 (t, *J* = 7.4 Hz, 2H), 1.88 (s, 3H), 1.84 – 1.77 (m, 2H), 1.62 – 1.32 (m, 8H), 1.30 – 1.18 (m, 24H), 0.85 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 172.81, 170.76, 169.68, 169.23, 168.91, 72.17, 70.53, 63.95, 62.88, 60.18, 52.44, 51.67, 50.78, 43.63, 38.23, 33.53, 33.41, 31.31, 30.01, 29.11, 29.06, 29.03, 29.02, 29.00, 28.90, 28.72, 28.46, 26.19, 24.44, 22.55, 22.12, 21.13, 13.99. MS (ESI-TOF) for $C_{33}H_{62}N_4O_8S$ $[M+H]^+$ Found 675.4181, Calculated 675.4361.

Synthesis of 13: 2-(((R)-2-Acetamido-3-(((S)-1-methoxy-1-oxo-3-(sulfooxy)propan-2-yl)amino)-3-oxopropyl)thio)ethyl palmitate—To a solution of compound **4** (60 mg, 0.110 mmol) in anhydrous pyridine was added sulfur trioxide pyridine complex (175 mg, 1.10 mmol). The reaction mixture was heated at 80 °C for 16 h. The solvent was removed under reduced pressure to obtain the residue which was purified using column chromatography (10% MeOH/CH_2Cl_2), to furnish compound **13** as a white solid (23 mg, 33%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.20 (d, *J* = 5.3 Hz, 1H), 7.30 (d, *J* = 1.6 Hz, 1H), 4.92 – 4.79 (m, 2H), 4.32 (dd, *J* = 33.8, 6.7 Hz, 2H), 4.26 – 4.14 (m, 2H), 3.76 (s, 3H), 3.00 (d, *J* = 9.1 Hz, 1H), 2.89 (dd, *J* = 12.8, 6.8 Hz, 1H), 2.78 (t, *J* = 6.4 Hz, 2H), 2.36 – 2.26 (m, 3H), 2.02 (s, 3H), 1.59 (dd, *J* = 14.3, 7.2 Hz, 2H), 1.35 – 1.22 (m, 24H), 0.88 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.00, 172.28, 171.43, 170.29, 67.45, 62.92, 53.15, 52.60, 52.19, 34.36, 32.08, 30.97, 29.87, 29.86, 29.82, 29.71, 29.52, 29.51, 29.38, 25.08, 23.01, 22.85, 14.28. MS (ESI-TOF, Negative Mode) for $C_{27}H_{50}N_2O_{10}S_2$, $[M-H]^-$ Found 625.2633, Calculated 625.2834.

Synthesis of 14: (7S,10R)-10-Acetamido-7-(methoxycarbonyl)-4,9,16-trioxo-5,15-dioxa-12-thia-8-azahentriacontan-1-oic acid—To a solution of compound **4** (50 mg, 0.091 mmol) in anhydrous THF were added triethylamine (25 μL, 0.183 mmol) and succinic anhydride (18 mg, 0.183 mmol). The reaction mixture was stirred at 50 °C for 2 h. The solvent was removed under vacuum to obtain the residue which was purified using column chromatography (10% MeOH/CH_2Cl_2), to furnish compound **14** (37 mg, 62%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25 (d, *J* = 7.8 Hz, 1H), 6.77 (d, *J* = 8.5 Hz, 1H), 4.87 – 4.78 (m, 2H), 4.65 (dd, *J* = 11.4, 3.5 Hz, 1H), 4.39 (dd, *J* = 11.4, 3.4 Hz, 1H), 4.34 – 4.19 (m, 2H), 3.79 (s, 3H), 2.99 (dd, *J* = 14.0, 5.9 Hz, 1H), 2.87 (dd, *J* = 14.0, 7.2 Hz, 1H), 2.81 (td, *J* = 6.8, 1.0 Hz, 2H), 2.75 – 2.53 (m, 4H), 2.37 – 2.29 (m, 2H), 2.08 (s, 3H), 1.66 – 1.56 (m, 2H), 1.35 – 1.21 (m, 24H), 0.87 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.28, 174.54, 171.84, 171.65, 170.25, 169.41, 63.29, 63.04, 53.10, 52.40, 52.03, 34.58, 34.39, 32.06, 30.90, 29.84, 29.80, 29.76, 29.67, 29.61, 29.50, 29.41, 29.39, 29.27, 25.00, 23.36, 22.83, 14.27. MS (ESI-TOF) for $C_{31}H_{54}N_2O_{10}S$ $[M+H]^+$ Found 647.3649, Calculated 647.3572; $[M+Na]^+$ Found 669.3476, Calculated 669.3391.

Synthesis of 15: (S)-2-((R)-2-Acetamido-3-((2-(palmitoyloxy)ethyl)thio)propanamido)-3-methoxy-3-oxopropyl nicotinate—To a solution of compound **4** (50 mg, 0.091 mmol) and nicotinic acid (23 mg, 0.183 mmol) in anhydrous CH_2Cl_2 (5 mL), were added *N*-methylmorpholine (20 μL, 0.183 mmol) and catalytic amount of DMAP. The reaction mixture was stirred at 0 °C and EDCI (28 mg, 0.183 mmol) was added after 15 min. The reaction mixture was then stirred at room temperature overnight. After completion of the reaction, water (10 mL) was added and the product was extracted in CH_2Cl_2. The organic layer was washed with water (10 mL × 2),

brine (10 mL) and dried over anhydrous sodium sulfate. The solvent was removed under vacuum and the residue was purified using column chromatography (5 % MeOH/CH_2Cl_2) to obtain product **15** as a white solid (41 mg, 68%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.19 (dd, *J* = 2.2, 0.8 Hz, 1H), 8.80 (dd, *J* = 4.9, 1.7 Hz, 1H), 8.30 – 8.23 (m, 1H), 7.48 (d, *J* = 7.6 Hz, 1H), 7.41 (ddd, *J* = 8.0, 4.9, 0.8 Hz, 1H), 6.44 (d, *J* = 7.1 Hz, 1H), 4.95 (dt, *J* = 7.7, 3.8 Hz, 1H), 4.77 – 4.65 (m, 2H), 4.61 (td, *J* = 7.2, 5.7 Hz, 1H), 4.32 – 4.18 (m, 2H), 3.82 (s, 3H), 2.99 (dd, *J* = 14.0, 5.6 Hz, 1H), 2.93 – 2.75 (m, 3H), 2.34 – 2.24 (m, 2H), 2.01 (s, 3H), 1.62 (s, 3H), 1.62 – 1.53 (m, 2H), 1.36 – 1.18 (m, 24H), 0.88 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.05, 170.51, 170.44, 169.36, 164.88, 154.04, 151.18, 137.36, 125.39, 123.56, 64.41, 62.61, 53.28, 52.55, 52.23, 34.35, 33.99, 32.07, 31.20, 29.84, 29.80, 29.77, 29.62, 29.51, 29.42, 29.29, 25.02, 23.21, 22.84, 14.28. MS (ESI-TOF) for $C_{33}H_{53}N_3O_8S$ $[M+H]^+$ Found 652.3672, Calculated 652.3626; $[M+Na]^+$ Found 674.3472, Calculated 674.3446.

Synthesis of 19: (S)-Methyl 2-((R)-3-((2-azidoethyl)thio)-2-((*tert*-butoxycarbonyl)amino) propanamido)-3-(*tert*-butoxy)propanoate—To a solution of compound **18** (500 mg, 1.185 mmol) in CH_2Cl_2 (5 mL) were added triethylamine (0.5 mL, 3.56 mmol) and methanesulfonyl chloride (276 μL, 3.55 mmol) and the reaction mixture was stirred at room temperature for 3 h. After the completion of the reaction, water (10 mL) was added and the product was extracted in CH_2Cl_2. The organic layer was washed with water (10 mL × 2) and brine (10 mL), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain the crude mesylate. This crude product was dissolved in DMF (5 mL) and sodium azide (385 mg, 5.925 mmol) was added and the reaction mixture was stirred at 60 C for 4 h. After completion of the reaction, water (20 mL) was added to the reaction and the product obtained was extracted in EtOAc. The organic layer was washed with water (10 mL × 3) and brine (10 mL), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain the crude product which was purified using column chromatography (20% EtOAc/hexanes) to obtain compound **19** (390 mg, 73% over two steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.14 (d, *J* = 7.8 Hz, 1H), 5.49 (s, 1H), 4.66 (dt, *J* = 8.2, 3.0 Hz, 1H), 4.32 (d, *J* = 4.9 Hz, 1H), 3.83 (dd, *J* = 9.1, 2.9 Hz, 1H), 3.74 (s, 3H), 3.57 (dd, *J* = 9.1, 3.2 Hz, 1H), 3.51 (ddd, *J* = 12.5, 9.0, 6.2 Hz, 2H), 3.02 (dd, *J* = 14.0, 5.1 Hz, 1H), 2.90 (dd, *J* = 14.0, 7.0 Hz, 1H), 2.79 (ddd, *J* = 12.1, 9.5, 3.7 Hz, 2H), 1.46 (s, 9H), 1.14 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.59, 170.41, 155.40, 80.47, 73.70, 61.76, 53.93, 53.31, 52.60, 51.14, 34.89, 31.86, 28.43, 27.41. MS (ESI-TOF) for $C_{18}H_{33}N_5O_6S$ $[M+Na]^+$ Found 470.1919, Calculated 470.2044.

Synthesis of 20: (S)-Methyl 2-((R)-3-((2-aminoethyl)thio)-2-((tert-butoxycarbonyl)amino) propanamido)-3-(tert-butoxy)propanoate—To a solution of compound **19** (237 mg, 0.53 mmol) in dry THF (5 mL) was added triphenylphosphine (208 mg, 0.795 mmol) and the reaction mixture was heated to reflux for 3 h. Water (1 mL) was added and the heating was continued for 2 more hours. After the completion of reaction, the solvent was removed under reduced pressure to obtain the crude product which was purified using column chromatography (10% MeOH/CH_2Cl_2) to obtain compound **19** (200 mg, 90%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57 (bs, 1H), 5.73 (d, *J* = 7.5 Hz, 1H), 4.74 – 4.61 (m, 1H), 4.35 (bs, 1H), 3.82 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.73 (s, 3H), 3.57 (dd, *J* = 9.1, 3.3 Hz, 1H), 3.03 – 2.80 (m, 4H), 2.79 – 2.63 (m, 2H), 1.45 (s, 9H), 1.13 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.7, 170.6, 155.5, 80.3, 73.6, 61.9, 54.0, 53.3, 52.5, 41.4, 36.4, 34.9, 28.5 (3C), 27.4 (3C). MS (ESI-TOF) for $C_{18}H_{35}N_3O_6S$ $[M+H]^+$ Found 470.1919, Calculated 470.2044.

Synthesis of 21: (S)-Methyl 3-(*tert*-butoxy)-2-((R)-2-((tert-butoxycarbonyl)amino)-3-((2-palmitamidoethyl)thio)propanamido)propanoate

—To a solution of compound **20** (100 mg, 0.238 mmol) in dry CH_2Cl_2 (2 mL) were added triethylamine (50 mL, 0.356 mmol) and palmitoyl chloride (109 mL, 0.356 mmol) and the reaction mixture was stirred at room temperature for 30 min. After the completion of reaction, water (10 mL) was added to the reaction and the product obtained was extracted in CH_2Cl_2. The organic layer was washed with water (10 mL × 3) and brine (10 mL), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain the crude product which was purified using column chromatography (40% EtOAc/ hexanes) to obtain compound **21** (105 mg, 69%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.14 (d, *J* = 8.1 Hz, 1H), 6.25 (s, 1H), 5.48 (d, *J* = 4.1 Hz, 1H), 4.66 (dt, *J* = 8.2, 3.1 Hz, 1H), 4.32 (d, *J* = 5.6 Hz, 1H), 3.83 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.74 (s, 3H), 3.57 (dd, *J* = 9.1, 3.2 Hz, 1H), 3.48 (td, *J* = 9.3, 6.0 Hz, 2H), 2.97 (dd, *J* = 13.9, 5.5 Hz, 1H), 2.88 (dd, *J* = 13.9, 6.9 Hz, 1H), 2.82 – 2.66 (m, 2H), 2.24 – 2.13 (m, 2H), 1.67 – 1.56 (m, 2H), 1.45 (s, 9H), 1.37 – 1.19 (m, 24H), 1.14 (s, 9H), 0.87 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.53, 170.71, 170.50, 155.50, 80.47, 73.75, 61.76, 53.88, 53.30, 52.62, 38.58, 36.86, 34.55, 32.51, 32.06, 29.84, 29.80, 29.78, 29.71, 29.67, 29.54, 29.50, 28.45, 27.43, 25.88, 22.83, 14.27. MS (ESI-TOF) for $C_{34}H_{65}N_3O_7S$ $[M+H]^+$ Found 660.4560, Calculated 660.4616; $[M+Na]^+$ Found 682.4373, Calculated 682.4435.

Synthesis of 22: (S)-Methyl 2-((R)-2-acetamido-3-((2-palmitamidoethyl)thio)propanamido)-3-hydroxypropanoate—To compound **21** (95 mg, 0.183 mmol) was added TFA (2 mL). The reaction mixture was stirred at room temperature for 30 min and then dried by blowing nitrogen through the solution. The crude product was used directly for the next step. To a solution of the crude intermediate in CH_2Cl_2 (2 mL) was added pyridine (16 μL, 0.2 mmol) and acetic anhydride (19 μL, 0.2 mmol). The reaction mixture was stirred at room temperature for 30 min and then concentrated. The residue was purified by a flash chromatography (5% MeOH/CH_2Cl_2) to give product **22** (70 mg, 84%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.8 Hz, 1H), 6.75 (d, *J* = 7.3 Hz, 1H), 6.07 (s, 1H), 4.71 (d, *J* = 7.1 Hz, 1H), 4.62 (dd, *J* = 7.7, 3.3 Hz, 1H), 4.01 (s, 2H), 3.78 (s, 2H), 3.63 – 3.48 (m, 2H), 2.95 (dd, *J* = 22.3, 6.2 Hz, 2H), 2.85 (dt, *J* = 14.2, 5.7 Hz, 1H), 2.72 (dt, *J* = 14.5, 7.4 Hz, 1H), 2.23 – 2.19 (m, 1H), 2.06 (s, 2H), 1.63 – 1.56 (m, 2H), 1.34 – 1.22 (m, 18H), 0.88 (t, *J* = 6.9 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.10, 170.66, 170.61, 170.55, 62.88, 55.43, 52.78, 52.28, 39.22, 37.01, 35.36, 32.75, 32.07, 29.85, 29.84, 29.81, 29.77, 29.63, 29.51, 29.45, 29.40, 25.80, 23.35, 22.84, 14.28. MS (ESI-TOF) for $C_{27}H_{51}N_3O_6S$ $[M+H]^+$ Found 546.3474, Calculated 546.3571; $[M+Na]^+$ Found 568.3293, Calculated 568.3391.

Synthesis of Compound 23: (S)-methyl 3-(tert-butoxy)-2-((R)-2-((tert-butoxycarbonyl)amino)-3-((2-(4-tridecyl-1H-1,2,3-triazol-1-yl)ethyl)thio)propanamido)propanoate—To a stirred solution of compound **19** (60 mg, 0.134 mmol) and pentadec-1-yne (39 μL, 0.147 mmol) in THF (2 mL), were added $CuSO_4.5H_2O$ (3 mg in 0.25 mL water, 0.013 mmol) and sodium ascorbate (5 mg in 0.25 mL water, 0.003 mmol) and the reaction mixture was stirred at room temperature for overnight. After the completion of reaction, water (10 mL) was added and the product obtained was extracted in EtOAc. The organic layer was washed with water (10 mL × 2) and brine (10 mL), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain the crude product. The residue was further purified by a flash chromatography (5% MeOH/CH_2Cl_2) to give product **23** (48 mg, 55%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43 (s, 1H), 7.12 (d, *J* = 7.6 Hz, 1H), 5.51 (d, *J* = 4.1 Hz, 1H), 4.64 (dt, *J* = 8.2, 3.1 Hz, 1H), 4.54 (t, *J* = 7.0 Hz, 2H), 4.32 (d, *J* = 4.4 Hz, 1H), 3.82 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.73 (s, 3H), 3.56 (dd, *J* = 9.1, 3.2 Hz, 1H), 3.12 (dd, *J* = 13.3, 6.5 Hz, 1H), 3.02 (ddd, *J* = 14.1, 13.1, 5.9 Hz, 2H), 2.84 (dd, *J* = 14.1, 7.2 Hz, 1H), 2.74 – 2.64 (m, 2H), 1.71 – 1.59 (m, 3H), 1.46 (s, 9H), 1.39 – 1.21 (m, 20H), 1.13 (s, 9H), 0.87 (t, *J* = 7.0 Hz,

3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.56, 170.36, 155.36, 148.63, 121.30, 80.54, 73.75, 61.67, 53.85, 53.35, 52.62, 49.64, 34.44, 32.24, 32.07, 29.84, 29.82, 29.80, 29.72, 29.62, 29.55, 29.50, 29.47, 28.44, 27.42, 25.86, 22.84, 14.28. MS (ESI-TOF) for $C_{33}H_{61}N_5O_6S$ $[M+H]^+$ Found 656.4403, Calculated 656.4415; $[M+Na]^+$ Found 678.4223, Calculated 678.4235.

Synthesis of Compound 24: (S)-Methyl 2-((R)-2-acetamido-3-((2-(4-tridecyl-1H-1,2,3-triazol-1-yl)ethyl)thio)propanamido)-3-hydroxypropanoate— The global deprotection of compound **23** using TFA and further *N*-acetylation was carried out similarly as described earlier (synthesis of **22**) to furnish compound **24** as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.86 (d, *J* = 7.8 Hz, 1H), 7.38 (s, 1H), 6.78 (d, *J* = 6.8 Hz, 1H), 5.21 (s, 1H), 4.92 (ddd, *J* = 14.2, 8.9, 4.1 Hz, 1H), 4.63 (ddd, *J* = 7.7, 4.6, 3.0 Hz, 1H), 4.61 – 4.52 (m, 2H), 4.03 (dd, *J* = 12.2, 3.7 Hz, 2H), 3.75 (s, 3H), 3.24 (ddd, *J* = 14.8, 5.9, 4.2 Hz, 1H), 3.07 (dd, *J* = 9.0, 4.7 Hz, 1H), 2.90 (dd, *J* = 14.5, 4.1 Hz, 1H), 2.72 (dd, *J* = 14.5, 9.1 Hz, 1H), 2.67 (dd, *J* = 8.5, 6.8 Hz, 2H), 2.03 (s, 3H), 1.73 – 1.54 (m, 2H), 1.39 – 1.17 (m, 20H), 0.87 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.40, 170.23, 169.96, 149.51, 121.78, 62.52, 55.79, 53.11, 52.67, 50.05, 36.03, 34.69, 32.06, 29.83, 29.81, 29.79, 29.69, 29.50, 29.48, 29.39, 29.37, 25.57, 23.31, 22.84, 14.28. MS (ESI-TOF) for $C_{26}H_{47}N_5O_5S$ $[M+H]^+$ Found 542.3378, Calculated 542.3371; $[M+Na]^+$ Found 564.3201, Calculated 564.3190.

Synthesis of Compound 27: (S)-Methyl 2-((*R*)-2-acetamido-3-((2-(1-hexadecyl-1H-1,2,3-triazol-4-yl)ethyl)thio)propanamido)-3-hydroxypropanoate —To a solution of compound **17** (200 mg, 0.529 mmol) in dry DMF (5 mL) were added 4-bromobut-1-yne (248 μL, 2.65 mmol) and triethylamine (147 μL, 1.06 mmol) and the reaction mixture was heated to 90 °C for 1 h. After the completion of reaction, water (20 mL) was added to the reaction and the product obtained was extracted in EtOAc. The organic layer was washed with water (10 mL × 3) and brine (10 mL), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain the crude product **25** as a white solid. The crude product was further washed with hexanes to remove excess 4-bromobut-1-yne, dried and used as it is for the next step. To a stirred solution of compound **25** (150 mg, 0.35 mmol) and 1-azidohexadecane (139 mg, 0.52 mmol) in THF (3 mL), were added $CuSO_4.5H_2O$ (9 mg in 0.5 mL water, 0.035 mmol) and sodium ascorbate (14 mg in 0.5 mL water, 0.07 mmol) and the reaction mixture was stirred at room temperature overnight. After the completion of reaction, water (20 mL) was added and the product obtained was extracted in EtOAc. The organic layer was washed with water (10 mL × 2) and brine (10 mL), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain the crude product **26**. MS (ESI-TOF) for $C_{36}H_{67}N_5O_6S$ $[M+H]^+$ Found 698.5075, Calculated 698.4885. The product **26** was used as it is for the next step. The global deprotection using TFA and further *N*-acetylation was carried out similarly as described earlier for synthesis of **22** to furnish compound **27** as white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 (d, *J* = 7.5 Hz, 1H), 7.38 (s, 1H), 6.93 (d, *J* = 7.2 Hz, 1H), 4.66 (td, *J* = 7.6, 4.5 Hz, 1H), 4.62 (dt, *J* = 7.3, 3.5 Hz, 1H), 4.29 (t, *J* = 7.3 Hz, 2H), 4.27 (bs, 1H), 4.02 (bs, 2H), 3.77 (s, 3H), 3.16 (ddd, *J* = 13.9, 7.7, 5.4 Hz, 1H), 3.09 (dd, *J* = 14.3, 4.4 Hz, 1H), 3.06 – 2.90 (m, 3H), 2.79 (dd, *J* = 14.3, 7.9 Hz, 1H), 2.06 (s, 3H), 1.92 – 1.82 (m, 2H), 1.30 (bs, 2H), 1.25 (bs, 20H), 0.87 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.61, 170.56, 170.36, 146.09, 121.88, 62.41, 55.56, 52.76, 52.68, 50.67, 35.02, 32.56, 32.07, 30.37, 29.85, 29.83, 29.80, 29.75, 29.68, 29.53, 29.51, 29.14, 26.64, 25.54, 23.37, 22.85, 14.29. MS (ESI-TOF) for $C_{29}H_{53}N_5O_5S$ $[M+H]^+$ Found 584.3889, Calculated 584.3840; $[M+Na]^+$ Found 606.3692, Calculated 606.3660.

Synthesis of Compound 30: (2S,5R)-methyl 5-amino-2-(hydroxymethyl)-4,11-dioxo-10-oxa-7-thia-3,12-diazaoctacosan-1-oate—To a solution of compound **18** (100 mg, 0.237 mmol) in CH_2Cl_2 (1 mL) was added carbonyldiimidazole (58 mg, 0.355 mmol) and the reaction mixture was stirred at room temperature. Hexadecylamine (146 mg, 0.593 mmol) and DMF (1 mL) were added after 4 h and the reaction was kept stirring for 1 h. After the completion of reaction, water (10 mL) was added and the product obtained was extracted in CH_2Cl_2. The organic layer was washed with water (10 mL × 3) and brine (10 mL), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain the crude product. The residue was further purified by a flash chromatography (5% MeOH/CH_2Cl_2 to yield product **29** (110 mg, 67%) as a white solid. MS (ESI-TOF) for $C_{35}H_{67}N_3O_8S$ $[M+H]^+$ Found 690.4659, Calculated 690.4722; $[M+Na]^+$ Found 712.4478, Calculated 712.4541. The global deprotection of compound **29** using TFA resulted in compound **30** as a TFA salt in quantitative yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.53 (d, *J* = 7.6 Hz, 1H), 5.41 (t, *J* = 5.7 Hz, 1H), 4.72 – 4.63 (m, 1H), 4.36 (t, *J* = 6.5 Hz, 1H), 4.22 (ddd, *J* = 23.1, 11.5, 5.7 Hz, 2H), 3.89 (dt, *J* = 11.7, 7.2 Hz, 2H), 3.75 (s, 3H), 3.24 – 2.96 (m, 4H), 2.80 (t, *J* = 5.5 Hz, 2H), 1.46 (m, 2H), 1.25 (s, 26H), 0.88 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.45, 168.42, 156.76, 63.91, 61.89, 55.41, 52.94, 52.89, 41.32, 33.41, 32.08, 31.40, 29.94, 29.87, 29.86, 29.82, 29.77, 29.52, 26.97, 22.84, 14.28. MS (ESITOF) for $C_{26}H_{51}N_3O_6S$ $[M+H]^+$ Found 534.3524, Calculated 534.3571.

Synthesis of Compound 31: (2S,5R)-Methyl 5-acetamido-2-(hydroxymethyl)-4,11-dioxo-10-oxa-7-thia-3,12-diazaoctacosan-1-oate—To a solution of compound **30** (56 mg, 0.087 mmol) in $CH2Cl_2$ (1 mL) were added pyridine (8 μL, 0.1 mmol) and acetic anhydride (9 μL, 0.1 mmol). The reaction mixture was stirred at room temperature for 30 min and then concentrated. The residue was purified by a flash chromatography (5% MeOH/CH_2Cl_2) to yield product **31** (32 mg, 64%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.48 (d, *J* = 7.6 Hz, 1H), 6.66 (d, *J* = 7.1 Hz, 1H), 5.25 (t, *J* = 5.5 Hz, 1H), 4.67 (dd, *J* = 14.1, 7.1 Hz, 1H), 4.63 (dd, *J* = 7.5, 3.6 Hz, 1H), 4.32 (dt, *J* = 12.2, 6.2 Hz, 1H), 4.25 (dt, *J* = 11.7, 6.0 Hz, 1H), 3.97 (q, *J* = 11.4 Hz, 2H), 3.79 (s, 3H), 3.31 (bs, 1H), 3.15 (dd, *J* = 13.4, 6.8 Hz, 2H), 2.96 (qd, *J* = 14.1, 6.6 Hz, 2H), 2.89 – 2.78 (m, 2H), 2.05 (s, 3H), 1.52 – 1.44 (m, 2H), 1.34 – 1.20 (m, 26H), 0.88 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.83, 170.68, 170.63, 156.79, 64.06, 62.80, 55.23, 52.91, 52.78, 41.31, 34.86, 32.07, 31.87, 29.98, 29.85, 29.81, 29.76, 29.72, 29.51, 29.46, 26.94, 23.29, 22.84, 14.28. MS (ESI-TOF) for $C_{28}H_{53}N_3O_7S$ $[M+H]^+$ Found 576.3594, Calculated 576.3677; $[M+Na]^+$ Found 598.3414, Calculated 598.3496.

Synthesis of Compound 32: (2S,5R)-Methyl 5-((*tert*-butoxycarbonyl)amino)-2-(*tert*-butoxymethyl)-4,11-dioxo-12-oxa-7-thia-3,10-diazaoctacosan-1-oate—To a solution of compound **20** (100 mg, 0.238 mmol) in dry CH_2Cl_2 (5 mL) were added triethylamine (50 μL, 0.356 mmol) and cetyl chloroformate (117 μL, 0.356 mmol) and the reaction mixture was stirred at room temperature for 30 min. After the completion of reaction, water (10 mL) was added to the reaction and the product obtained was extracted in CH_2Cl_2. The organic layer was washed with water (10 mL × 2) and brine (10 mL), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain the crude product which was purified using column chromatography (50% EtOAc/hexanes) to obtain compound **32** (120 mg, 74%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.15 (d, *J* = 8.0 Hz, 1H), 5.47 (s, 1H), 5.26 (s, 1H), 4.66 (dt, *J* = 8.2, 3.1 Hz, 1H), 4.32 (s, 1H), 4.03 (t, *J* = 6.6 Hz, 2H), 3.82 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.74 (s, 3H), 3.57 (dd, *J* = 9.1, 3.2 Hz, 1H), 3.39 (d, *J* = 5.4 Hz, 2H), 2.97 (dd, *J* = 13.9, 5.4 Hz, 1H), 2.87 (dd, *J* = 13.8, 6.8 Hz, 1H), 2.80 – 2.65 (m, 2H), 1.63 – 1.54 (m, 2H), 1.45 (s, 9H), 1.33 – 1.23 (m, 26H), 1.14 (s, 9H), 0.87 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.68, 170.52, 156.92, 155.44, 80.44, 73.73, 65.28, 61.78, 53.79, 53.30, 52.60, 40.15, 34.50, 32.73, 32.06, 29.84, 29.82,

29.80, 29.75, 29.71, 29.50, 29.47, 29.16, 28.44, 27.42, 26.01, 22.83, 14.27. MS (ESI-TOF) for $C_{35}H_{67}N_3O_8S$ $[M+Na]^+$ Found 712.4461, Calculated 712.4547.

Synthesis of Compound 33: (2S,5R)-Methyl 5-amino-2-(hydroxymethyl)-4,11-dioxo-12-oxa-7-thia-3,10-diazaoctacosan-1-oate—To compound **32** (110 mg, 0.159 mmol) was added TFA (2 mL). The reaction mixture was stirred at room temperature for 30 min and then dried by blowing nitrogen through the solution. The residue was purified by a flash chromatography (10% MeOH/CH_2Cl_2) to yield product **33** in quantitative yield. ^{1}H NMR (500 MHz, DMSO) δ 8.96 (d, *J* = 7.8 Hz, 1H), 8.09 (s, 2H), 7.98 (t, *J* = 5.7 Hz, 1H), 5.26 (t, *J* = 5.4 Hz, 1H), 4.47 – 4.41 (m, 1H), 4.02 (dd, *J* = 8.9, 4.6 Hz, 1H), 3.79 (dt, *J* = 10.4, 5.1 Hz, 1H), 3.65 (s, 3H), 3.64 – 3.60 (m, 1H), 3.24 (tt, *J* = 13.7, 7.0 Hz, 2H), 3.03 (dd, *J* = 14.4, 4.5 Hz, 1H), 2.74 (dd, *J* = 14.4, 8.9 Hz, 1H), 2.65 (td, *J* = 6.6, 2.6 Hz, 2H), 2.06 (t, *J* = 7.4 Hz, 2H), 1.51 – 1.44 (m, 2H), 1.32 – 1.18 (m, 24H), 0.85 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 172.57, 170.35, 167.92, 61.06, 54.76, 52.13, 51.68, 38.12, 35.41, 32.82, 31.36, 31.32, 29.08, 29.04, 28.98, 28.84, 28.73, 28.70, 25.28, 22.12, 13.99. MS (ESI-TOF) for $C_{26}H_{51}N_3O_6S$ $[M+H]^+$ Found 534.3530, Calculated 534.3571; $[M+Na]^+$ Found 556.3351, Calculated 556.3391.

Synthesis of Compound 34: (2S,5R)-Methyl 5-acetamido-2-(hydroxymethyl)-4,11-dioxo-12-oxa-7-thia-3,10-diazaoctacosan-1-oate—To a solution of compound **33** (60 mg, 0.093 mmol) in dichloromethane (2 mL) was added pyridine (8 μL, 0.102 mmol) and acetic anhydride (10 μL, 0.102 mmol). The reaction mixture was stirred at room temperature for 30 min and then concentrated. The residue was purified by a flash chromatography (5% MeOH/CH_2Cl_2) to yield product **34**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 (d, *J* = 7.4 Hz, 1H), 6.75 (d, *J* = 6.7 Hz, 1H), 5.20 (d, *J* = 5.2 Hz, 1H), 4.74 – 4.58 (m, 2H), 4.10 – 3.89 (m, 4H), 3.79 (s, 3H), 3.42 (dd, *J* = 12.5, 6.2 Hz, 3H), 2.94 (d, *J* = 6.1 Hz, 2H), 2.83 – 2.67 (m, 2H), 2.05 (s, 3H), 1.66 – 1.53 (m, 2H), 1.36 – 1.20 (m, 26H), 0.88 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.87, 170.66, 157.61, 65.69, 62.77, 55.23, 52.90, 52.58, 40.34, 34.48, 32.92, 32.07, 29.85, 29.84, 29.81, 29.75, 29.71, 29.51, 29.45, 29.09, 26.00, 23.27, 22.84, 14.28. MS (ESI-TOF) for $C_{28}H_{53}N_3O_7S$ $[M+H]^+$ Found 576.3602, Calculated 576.3677; $[M+Na]^+$ Found 598.3416, Calculated 598.3496.

Synthesis of Compound 35: (2S,5R)-5-((*tert*-Butoxycarbonyl)amino)-2-(*tert*-butoxymethyl)-4,11-dioxo-12-oxa-7-thia-3,10-diazaoctacosan-1-oic acid—To a solution of compound **32** (100 mg, 0.145 mmol) in dichloroethane (2.5 mL) was added trimethyltin hydroxide (79 mg, 0.435 mmol) and the reaction mixture was heated to reflux for 6 h. After the completion of reaction, the solvent was removed under reduced pressure to obtain the crude product which was purified using column chromatography (50% CH_2Cl_2/EtOAc) to obtain compound **35** (72 mg, 73%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.23 (d, *J* = 6.8 Hz, 1H), 5.55 (d, *J* = 7.3 Hz, 1H), 5.28 (s, 1H), 4.70 – 4.60 (m, 1H), 4.34 (s, 1H), 4.04 (t, *J* = 6.4 Hz, 2H), 3.90 (dd, *J* = 9.0, 3.5 Hz, 1H), 3.57 (dd, *J* = 8.5, 4.9 Hz, 1H), 3.36 (dd, *J* = 16.2, 10.1 Hz, 2H), 2.93 (qd, *J* = 13.9, 6.2 Hz, 2H), 2.78 – 2.61 (m, 2H), 1.64 – 1.54 (m, 2H), 1.45 (s, 9H), 1.35 – 1.22 (m, 26H), 1.19 (s, 3H), 0.88 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.38, 171.01, 157.13, 155.65, 80.69, 74.63, 65.46, 61.26, 53.86, 52.90, 40.17, 34.31, 32.65, 32.07, 29.85, 29.81, 29.76, 29.72, 29.51, 29.48, 29.15, 28.44, 27.45, 26.01, 22.84, 14.28. MS (ESI-TOF) for $C_{34}H_{65}N_3O_8S$ $[M+Na]^+$ Found 698.4272, Calculated 698.4385.

Synthesis of Compound 36: *tert*-Butyl hexadecyl ((8S,11R)-8-(*tert*-butoxymethyl)-2-methyl-7,10-dioxo-13-thia-2,6,9-triazapentadecane-11,15-diyl)dicarbamate—To a solution of acid **35** (563 mg, 0.833 mmol) and N^1,N^1-

dimethylpropane-1,3-diamine (115 μL, 0.916 mmol) in DMF (5 mL) were added triethylamine (232 μL, 1.67 mmol) and *N*-hydroxybenzotriazole (HOBt, 56 mg, 0.417 mmol). The reaction mixture was cooled to 0 °C and EDCI (258 mg, 1.67 mmol) was added after 30 min. The resulting mixture was stirred at room temperature overnight. After the completion of reaction, water (20 mL) was added to the reaction and the product obtained was extracted in EtOAc. The organic layer was washed with water (10 mL × 3) and brine (10 mL), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain the crude product which was purified using column chromatography (10% MeOH/CH_2Cl_2) to obtain compound **36** (530 mg, 84%). MS (ESITOF) for $C_{39}H_{77}N_5O_7S$ $[M+H]^+$ Found 760.5486, Calculated 760.5616.

Synthesis of Compound 38: Hexadecyl ((8S,11R)-11-acetamido-8-(hydroxymethyl)-2-methyl-7,10-dioxo-13-thia-2,6,9-triazapentadecan-15-yl)carbamate—To compound **36** (521 mg, 0.685 mmol) was added TFA (5 mL). The reaction mixture was stirred at room temperature for 30 min and then dried by blowing nitrogen through the solution. The crude product was used directly for the next step. To a solution of the crude intermediate **37** in CH_2Cl_2 (5 mL) were added pyridine (61 μL, 0.754 mmol) and acetic anhydride (71 μL, 0.754 mmol). The reaction mixture was stirred at room temperature for 30 min and then concentrated. The residue was purified by a flash chromatography (20% MeOH/CH_2Cl_2) to give product **38** as a white solid. ^{1}H NMR (500 MHz, MeOD) δ 4.49 (dd, *J* = 8.0, 5.9 Hz, 1H), 4.28 (t, *J* = 5.0 Hz, 1H), 4.02 (t, *J* = 6.6 Hz, 2H), 3.88 (dd, *J* = 11.0, 5.1 Hz, 1H), 3.79 (dd, *J* = 11.0, 5.0 Hz, 1H), 3.40 – 3.31 (m, 4H), 3.17 – 3.10 (m, 2H), 3.01 (dd, *J* = 13.7, 5.7 Hz, 1H), 2.89 (s, 6H), 2.83 (dd, *J* = 13.7, 8.1 Hz, 1H), 2.68 (td, *J* = 13.5, 6.6 Hz, 2H), 2.03 (s, 3H), 1.93 (dt, *J* = 13.2, 6.4 Hz, 2H), 1.68 – 1.55 (m, 2H), 1.42 – 1.25 (m, 26H), 0.90 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, MeOD) δ 173.97, 173.33, 173.25, 159.35, 66.05, 62.44, 57.50, 56.40, 54.92, 43.59, 41.16, 36.65, 33.94, 33.09, 33.02, 30.80, 30.78, 30.72, 30.49, 30.44, 30.23, 27.00, 25.97, 23.75, 22.48, 14.45. MS (ESI-TOF) for $C_{32}H_{63}N_5O_6S$ $[M+H]^+$ Found 646.4426, Calculated 646.4572.

TLR2-specific NF-κB induction

The induction of NF-κB in a human TLR2-specific reporter gene assay was quantified using HEK-Blue™ cells as previously described by us.[30,36,52] Occupancy of TLR2 by cognate ligands leads to nuclear translocation of NF-κB in a MyD88-dependent manner,[59–61] and consequent transactivation of the secreted alkaline phosphatase (seAP) reporter gene. Extracellular seAP in the supernatant is proportional to NF-κB induction, and was quantified spectrophotometrically.

Immunoassays for cytokines and chemokines

Fresh human peripheral blood mononuclear cells (hPBMC) were isolated from human blood obtained by venipuncture with informed consent and as per institutional guidelines on Ficoll-Hypaque gradients as described elsewhere.[62] Aliquots of PBMCs (10^5 cells in 100 μL/well) were stimulated for 12 h with graded concentrations of test compounds. Supernatants were isolated by centrifugation, and were assayed in duplicates for 41 chemokines and cytokines (EGF, Eotaxin, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1ra, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, PDGF-AA, PDGFAB/BB, RANTES, TGFα, TNF-α, TNF-β, VEGF, sCD40L) using a magnetic bead-based multiplexed assay kit (Milliplex MAP Human Cytokine/Chemokine kit). Data were acquired and processed on a MAGPIX instrument (EMD Millipore, Billerica, MA) with an intra-assay coefficients of variation ranging from 4–8% for the 41 analytes.

Flow-cytometric immunostimulation experiments

CD11b upregulation was determined by flow cytometry using protocols published by us previously,[41] modified for rapid-throughput using an automated liquid-handling system. Briefly, heparin-anticoagulated whole blood samples were obtained by venipuncture from healthy human volunteers with informed consent and as per guidelines approved by the University of Kansas Human Subjects Experimentation Committee. Serial dilutions of **38** (as well as **2** and LPS, used as reference compounds) were performed using a Bio-Tek Precision 2000 XS liquid handler in sterile 96-well polypropylene plates, to which were added 100 μL aliquots of anticoagulated whole human blood. Negative (fRPMI) controls were included in each experiment. The plates were incubated at 37 °C for 1 h. Following incubation, 10 μL of each fluorochrome-conjugated antibody (CD11b-PE, CD14-APC, Becton-Dickinson Biosciences, San Jose, CA) were added to each well with a liquid handler, and incubated at 4 °C in the dark for 30 min. Following staining, erythrocytes were lysed and leukocytes fixed by mixing 200 μL of the samples in 2 mL prewarmed Whole Blood Lyse/Fix Buffer (Becton-Dickinson Biosciences, San Jose, CA) in 96 deep-well plates. After washing the cells twice at 200 g for 8 minutes in saline, the cells were transferred to a 96-well plate. Flow cytometry was performed using a BD FACSArray instrument with acquisition on 100,000 gated events. Granulocytes were gated by forward- and side-scatter, while monocytes were gated on the basis of $CD14^{-}$APC staining.

Immunization and safety evaluation in rabbits

All experiments were performed at Harlan Laboratories (Indianapolis, IN) in accordance with institutional guidelines (University of Kansas IACUC permit # 119-06). In experiments designed to evaluate the adjuvanticity of **12**, cohorts of adult female New Zealand White rabbits were immunized intramuscularly in the flank region with either 100 μg of bovine α-lactalbumin in 0.2 mL saline (unadjuvanted control), or 100 μg of bovine α-lactalbumin plus 100 μg of **12** in 0.2 mL saline. The adjuvantic property of **38** (100 μg/dose) was evaluated using CRM197 as antigen (10 μg/dose). Pre-immune test-bleeds were first obtained via venipuncture of the marginal vein of the ear. Animals were immunized on Days 1, 15 and 28. A test bleed was performed on Day 25 and a final test-bleed was performed via the marginal vein of the ear on Day 38. Sera were stored at −80 °C until used. Antigen-specific ELISAs were performed in 384- well format using automated liquid handling methods as described by us.[34,53] Safety evaluation in rabbit models included examination of clinical hematology parameters (total and differential leukocyte counts, enumeration of band-forms (immature neutrophils), hemoglobin, and mean corpuscular hemoglobin), as well as core-temperature monitoring. Cohorts of animals (n=3 per group) received either **38 (**100 μg/dose), LPS (1 μg/dose, positive control) or vehicle (saline) formulated in sterile saline without any excipients in a volume of 0.5 mL, administered as an intravenous bolus in the marginal vein of the ear. Blood was obtained at 0h (pre-challenge), and at 2h and 6h via venipuncture on the contralateral ear. Core temperature was monitored by rectal thermistor probes connected to a temperature logging device as described by us.[31]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

APCs	antigen-presenting cells
CD	cluster of differentiation
DCs	dendritic cells
EC_{50}	Halfmaximal effective concentration for agonism
ESI-TOF	Electrospray ionization-time of flight
HEK	Human embryonic kidney
Ig	Immunoglobulin
IL	Interleukin
MPL	3-*O*-desacyl-4′-monophosphoryl lipid A
MCP	monocyte chemotactic protein
mDC	myeloid dendritic cell
MHC	Major histocompatibility complex
NF-κB	Nuclear factor-κB
pDC	plasmacytoid dendritic cell
SAR	Structure activity relationship
seAP	secreted alkaline phosphatase
Th1	Helper T lymphocyte, type 1
Th2	Helper T lymphocyte, type 2
TLR	Toll like receptor

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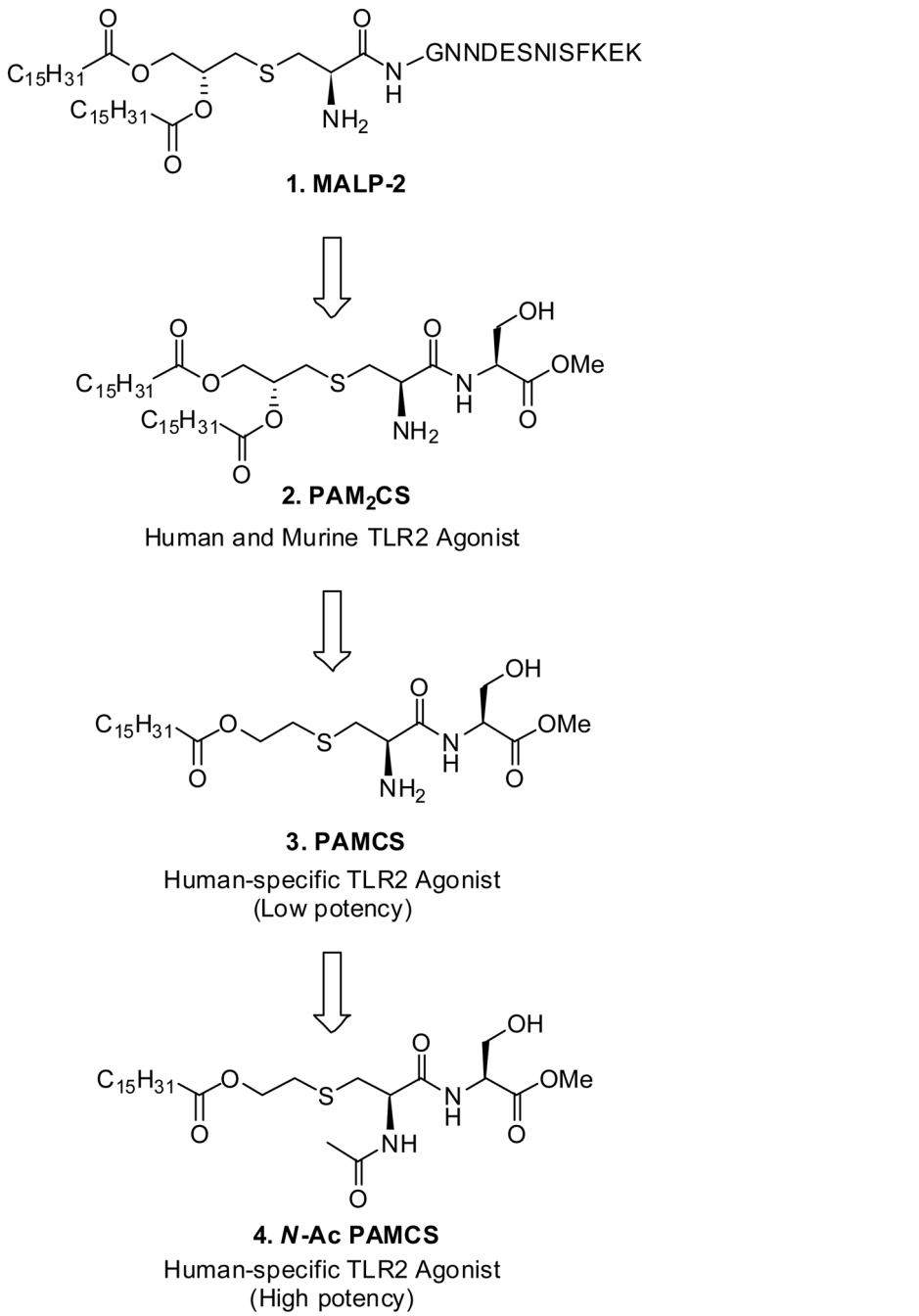


Figure 1.
Structures of the human/murine TLR2 agonists (**1**, **2**) human TLR2-specific low potency (**3**) and high potency (**4**) lipopeptides.

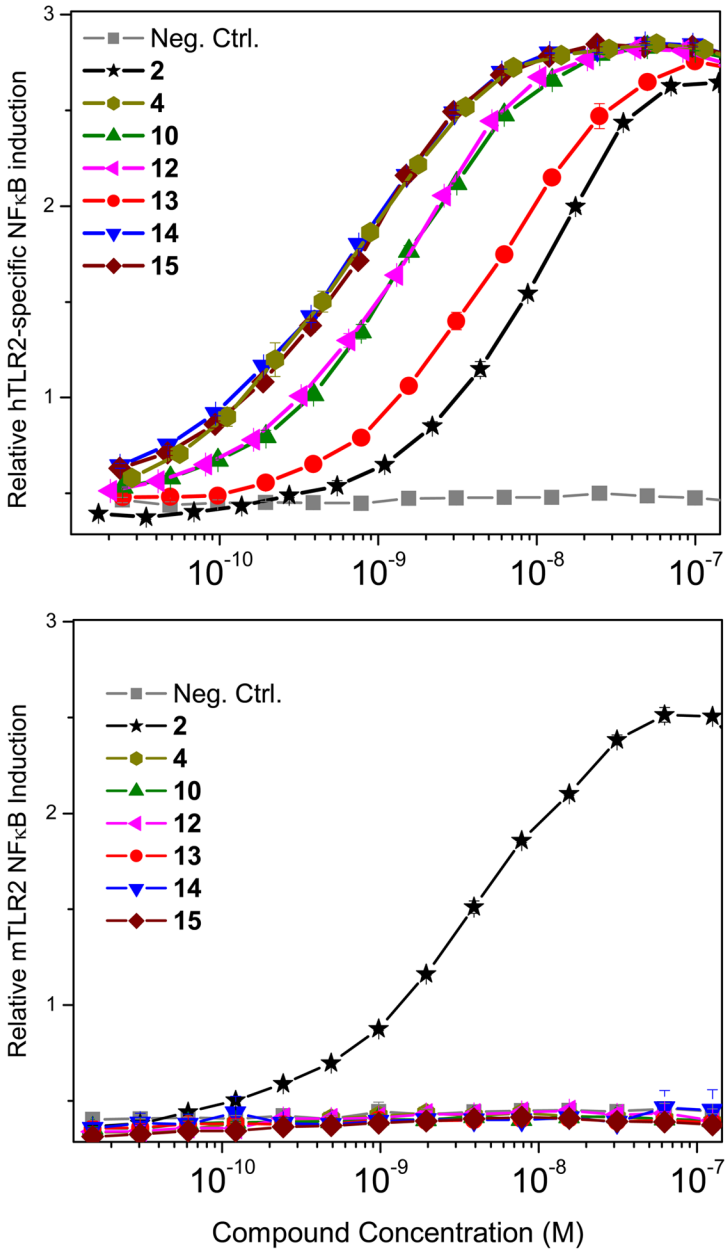


Figure 2.

Dose-response profiles of TLR2-agonistic activities of water soluble derivatives of **4** (Compounds **10**-**15**) in reporter gene assays specific for human TLR2 (top) and murine TLR2 (bottom). Compounds **2** and **4** were used as comparators. Means and s.d. on quadruplicate samples are shown.

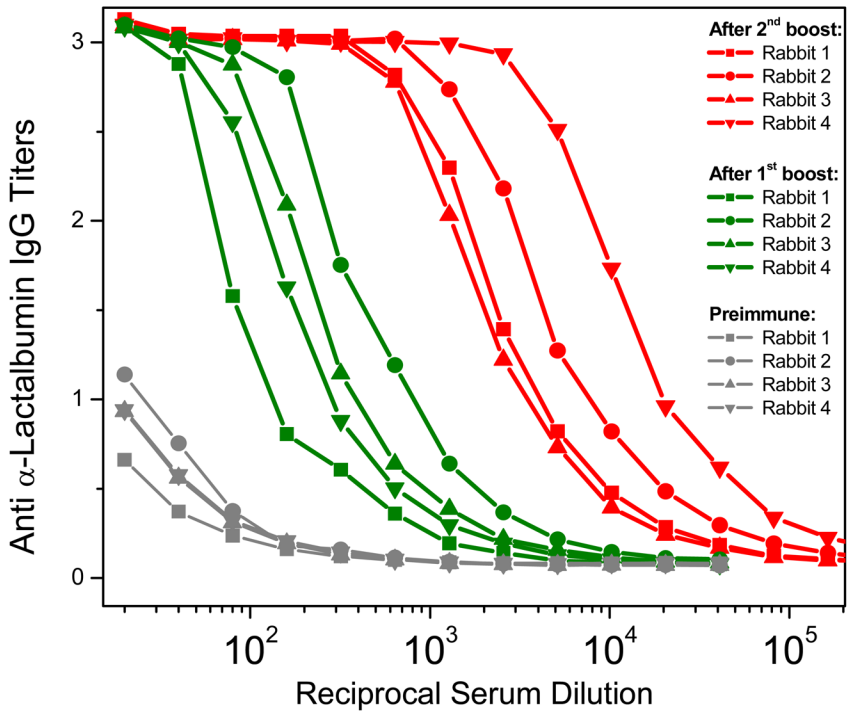


Figure 3.

Rise in antigen-specific IgG levels after primary and secondary immunizations in rabbits (n=4 per cohort) receiving 100 μg/dose of bovine α-lactalbumin, adjuvanted with 100 μg/dose of **12**, formulated in saline, with no excipients.

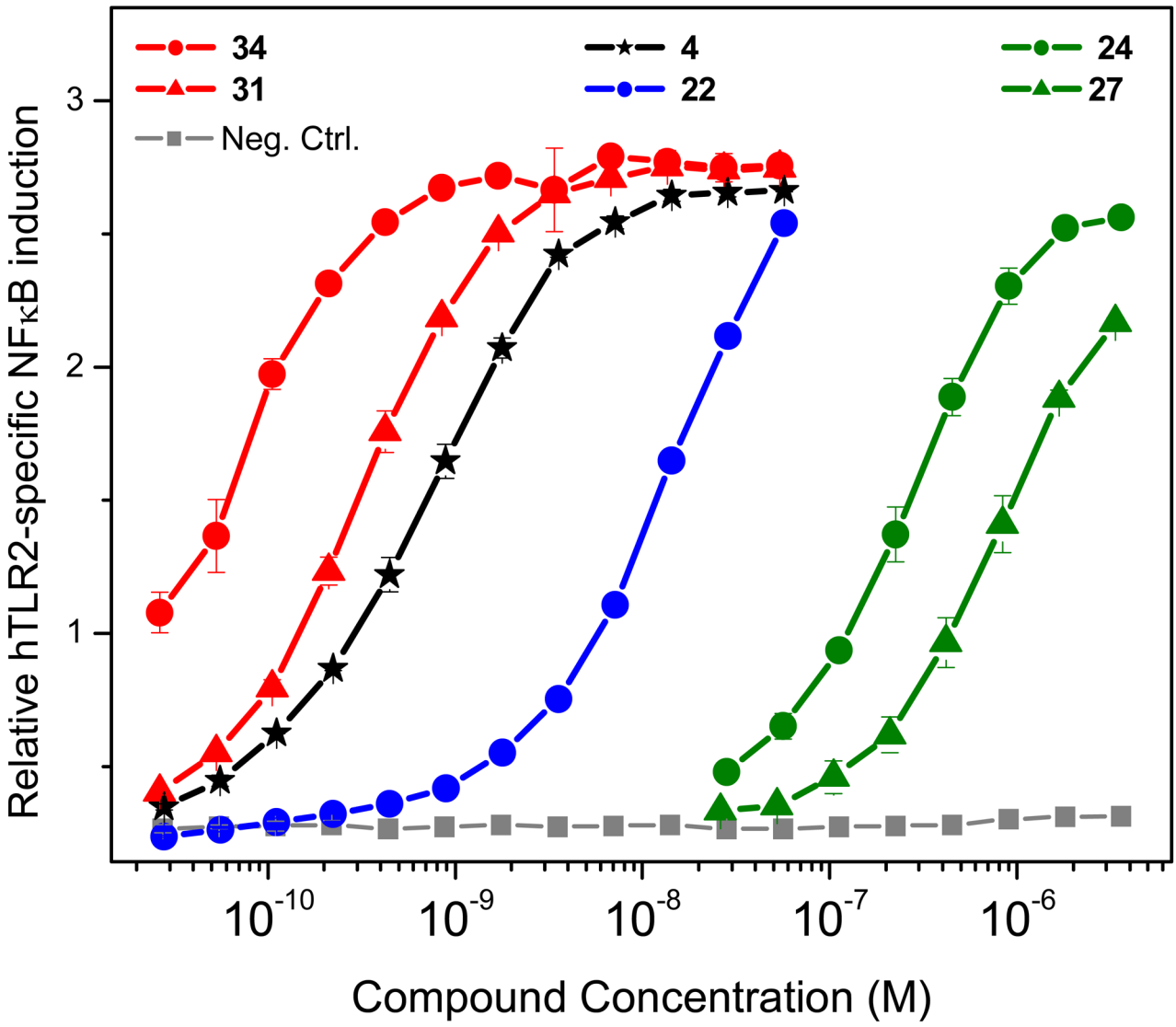


Figure 4.
Dose-response profiles of human TLR2-specific agonistic activities of the triazole, amide and carbamate analogues of **4** in hTLR-specific reporter gene assays. Means and s.d. on quadruplicate samples are shown.

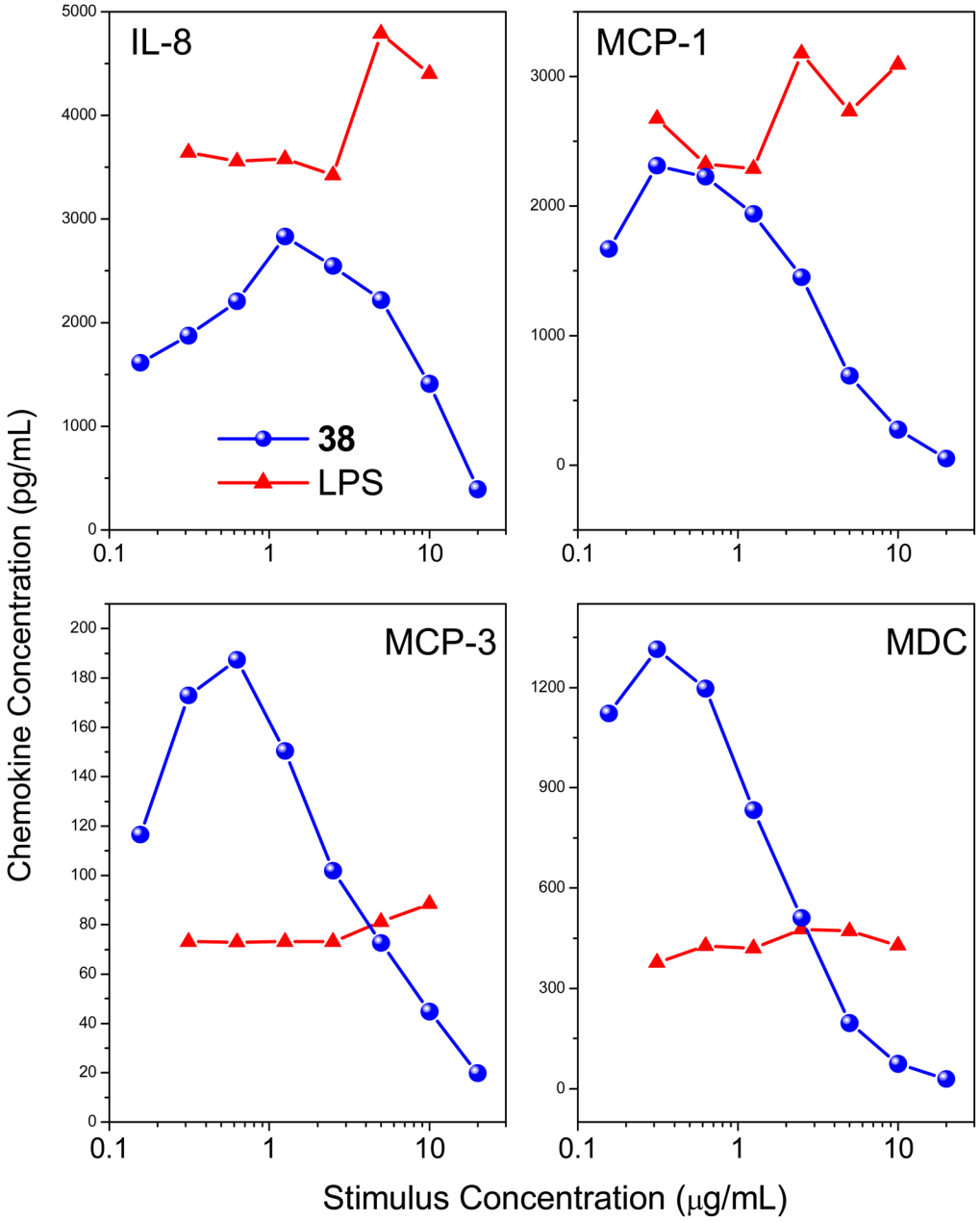


Figure 5.

Induction of select chemokines by **38** in human PBMCs quantified by 41-plex immunoassays. LPS was used as reference. Means of duplicates of a representative experiment are shown. Chemokine concentrations were below detection limits in negative controls, and are not shown.

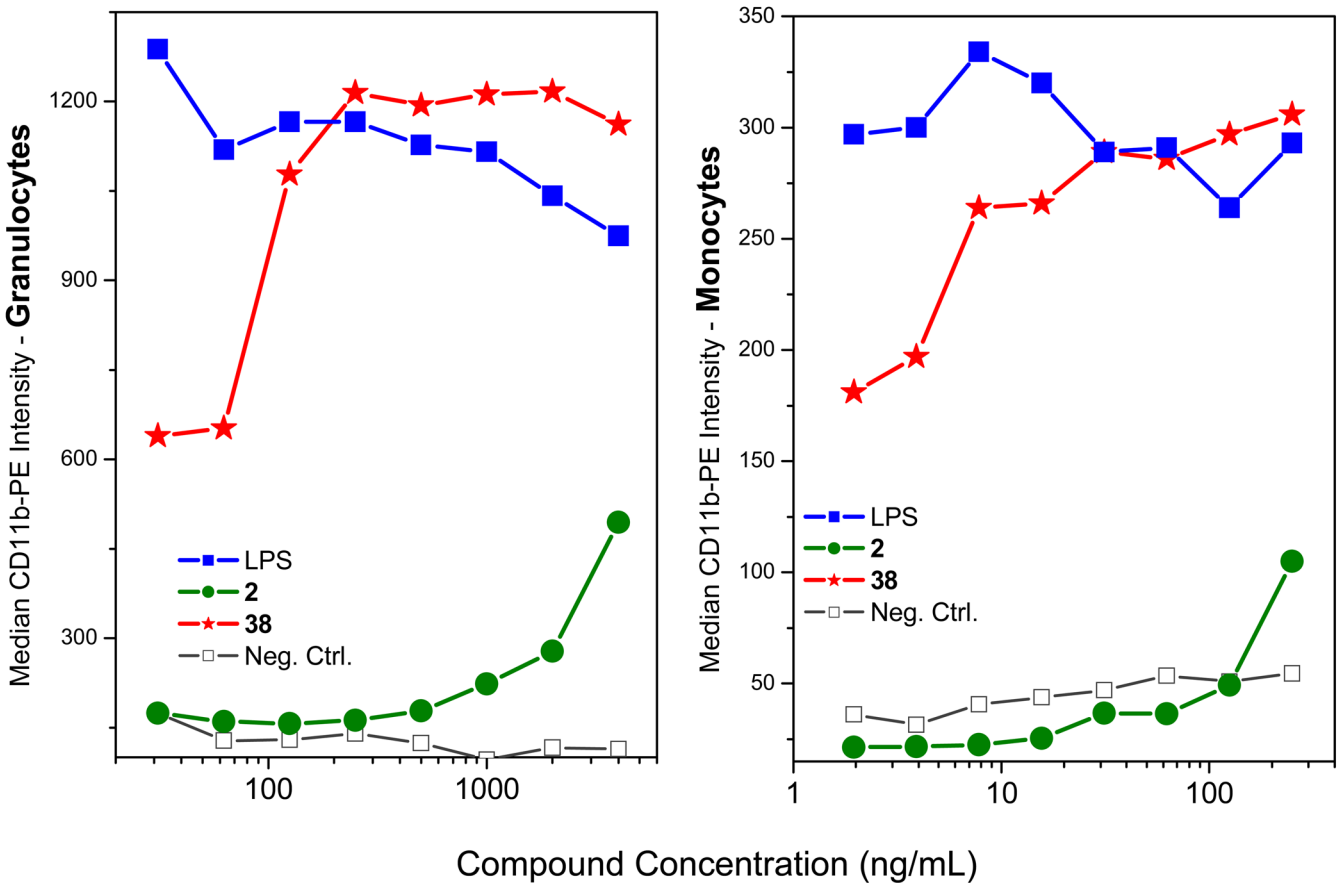


Figure 6.

CD11b upregulation (dose-response profiles) in granulocytes (left) and monocytes (right) induced by **38** in human whole blood. Compound **2** and LPS were used as comparator/positive controls. Insets show flow cytometric gating strategies (forward- and side-scatter for granulocytes, left) and $CD14^+$ cells for monocytes (right).

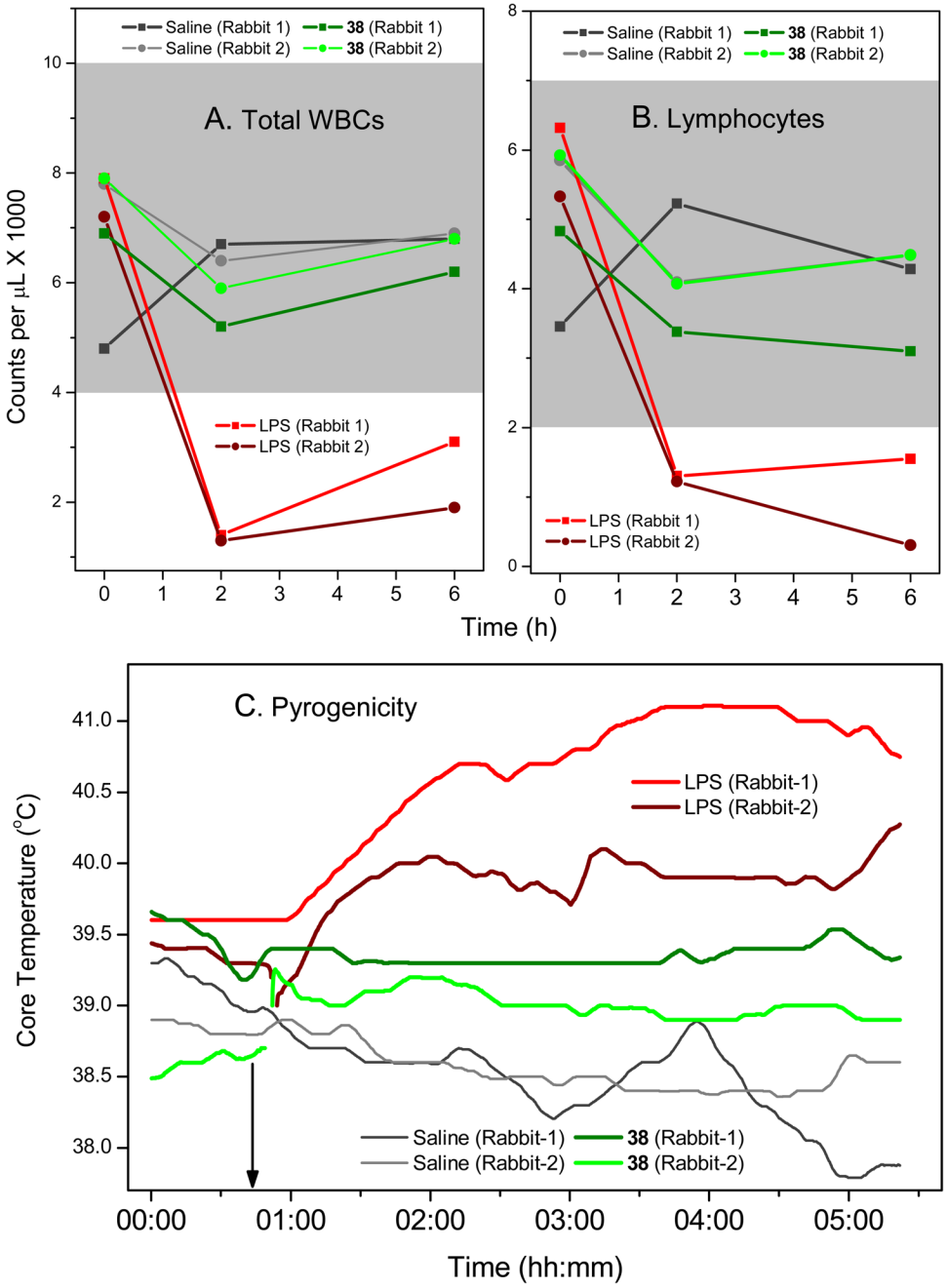


Figure 7.

Panels A and B: Effect of a single intravenous bolus injection of **38** (100 μg/dose) and LPS (1 μg/dose) on total leukocyte and lymphocyte counts in peripheral blood in rabbits (n=3 per cohort). Gray regions indicate normal values. Panel C: Pyrogenicity in rabbits (n=3 per cohort) administered either **38** (100 μg/dose) and LPS (1 μg/dose). Core temperature was monitored using rectal thermistor probes. One animal in each cohort was invalidated due to unforeseen problems: coagulated blood samples (A, B) or expulsion of temperature probes (C).

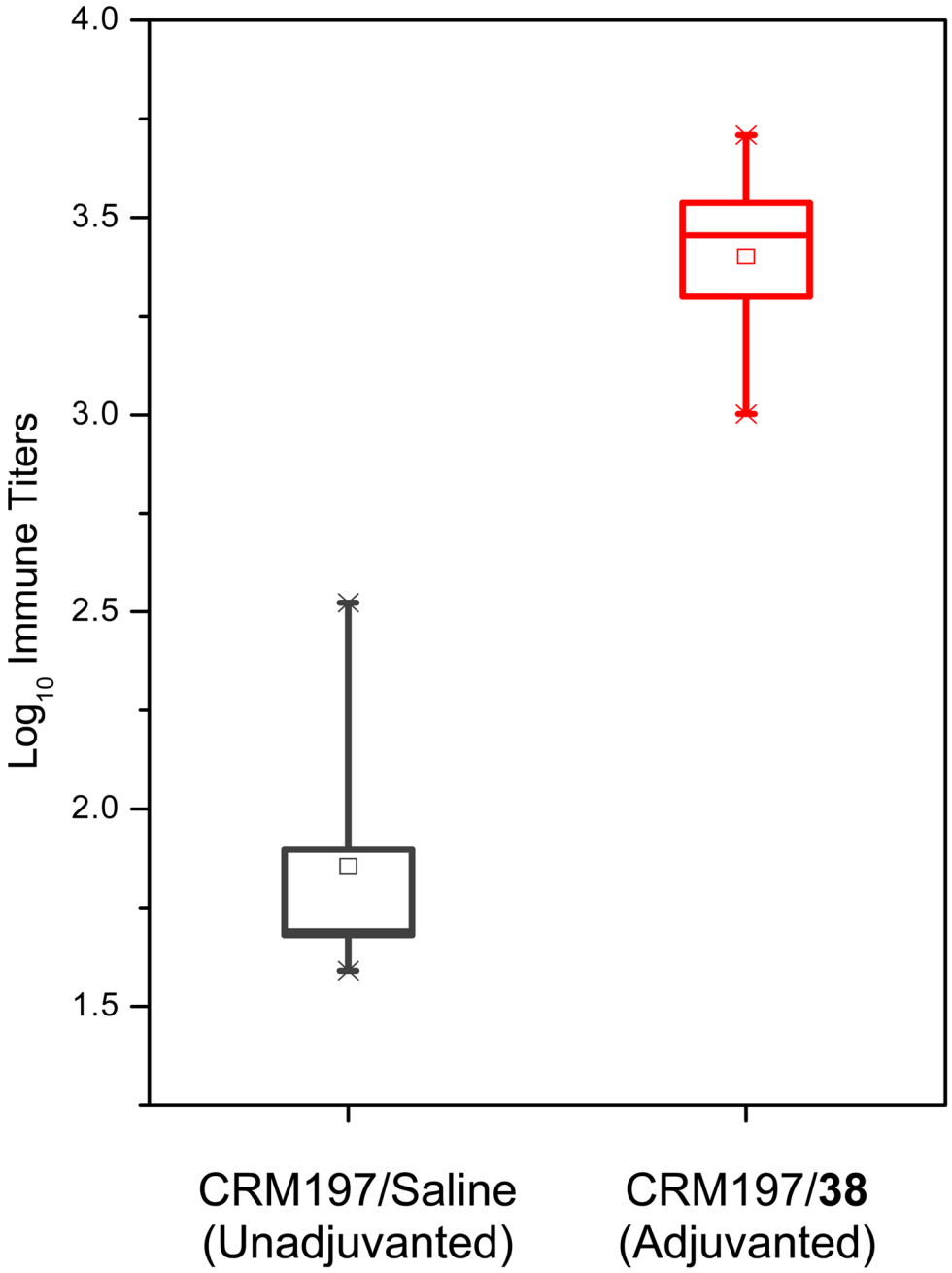
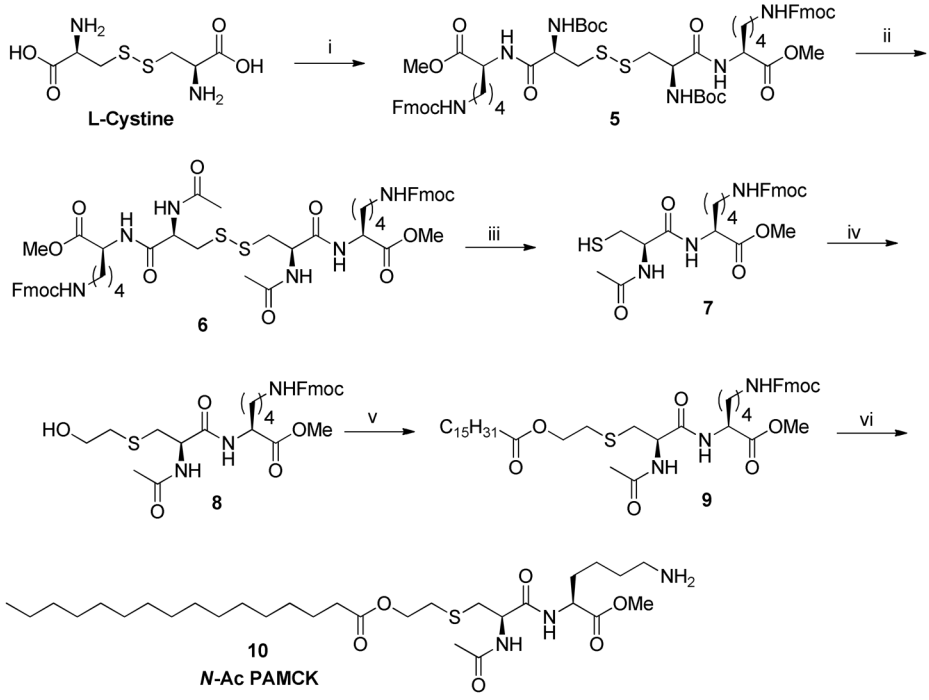


Figure 8.

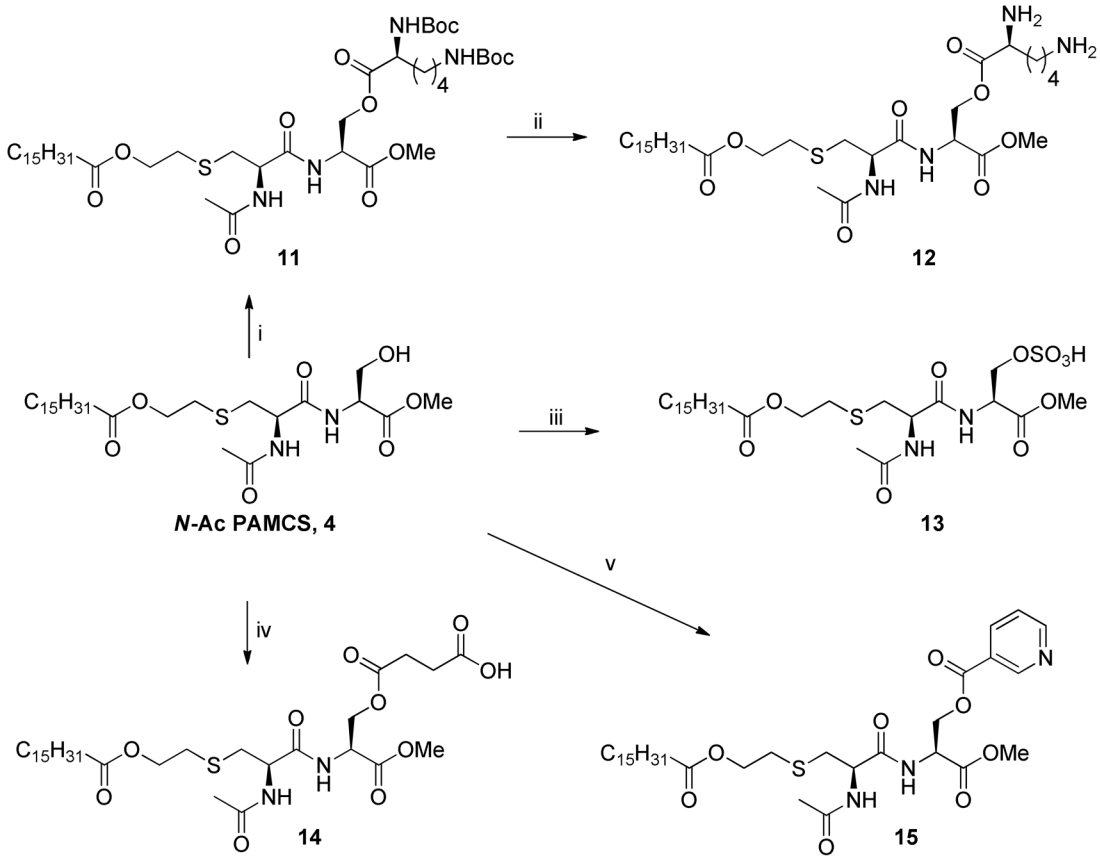
Anti-diphtheria toxoid (CRM197) IgG titers in rabbits (n=5 per cohort) immunized with 10 μg/dose of CRM197 in saline (controls), or CRM197 adjuvanted with 100 μg/dose of **38**. Box-plots of immune titers yielding absorbance values of 1.0 are shown for the individual samples. Means and medians of titers are represented by □ and — symbols within the box, respectively, and the X symbols indicate the 1% and 99% percentile values.



Scheme 1.

Syntheses of the water soluble analogue **10**.

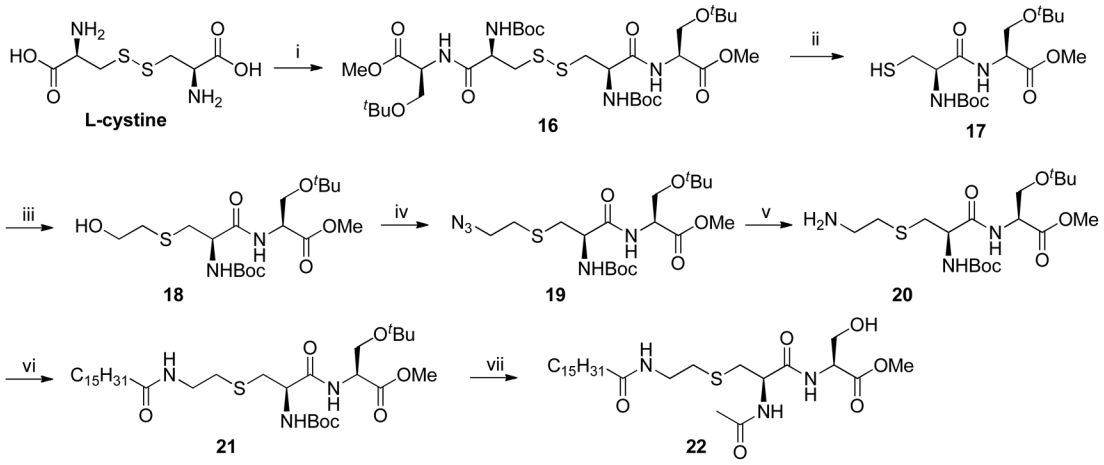
Reagents and Conditions: i. (a) Boc_2O, Et_3N, H_2O (b) H-Lys(Fmoc)-OMe.HCI, EDCI, HOBt, Pyridine, DMF; ii. (a) HCI, dioxane (b) Ac_2O, Pyridine, DCM; iii. Bu_3P, CH_2CI_2, H_2O; iv. 2-lodoethanol, K_2CO_3, DMF; v. $C_{15}H_{31}COCI$, Pyridine, DCM; vi. Piperazine polymer bound, DMF.



Scheme 2.

Attempts towards new water soluble monoacyl analogues.

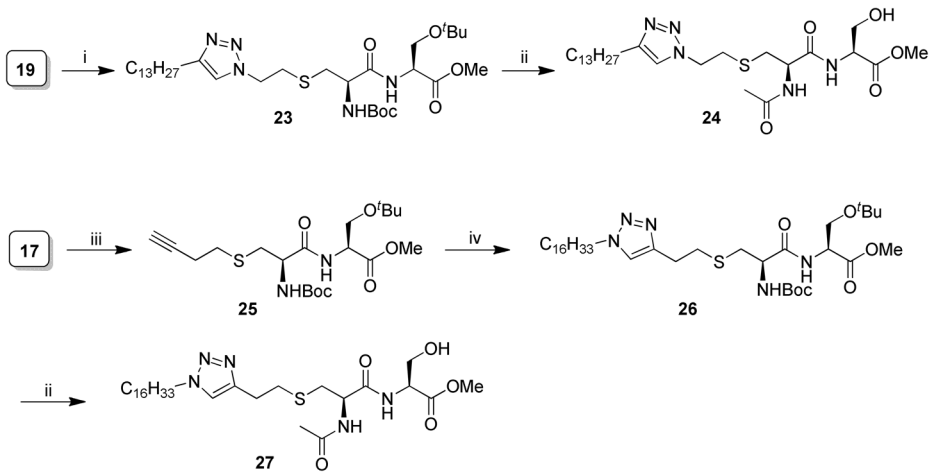
Reagents and Conditions: i. *N*,*N*′-Di-Boc-L-lysine, EDCI, NMM, DMAP, DCM; ii. HCl, dioxane; iii. SO_3-Pyridine, pyridine; iv. Succinic anhydride, Et_3N, THF; v. nicotinic acid, EDCI, DMAP, NMM.



Scheme 3.

Synthesis of amide derivative of **4**.

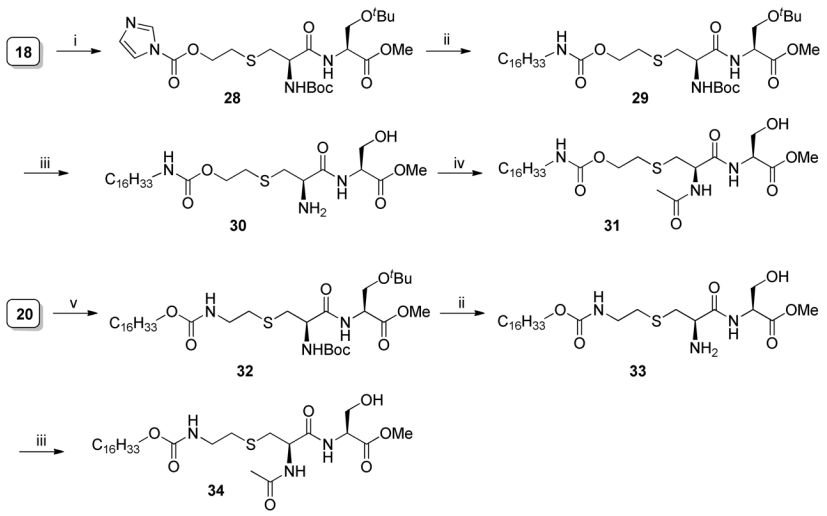
Reagents and Conditions: i. (a) Boc_2O, Et_3N, H_2O (b) *H*-Ser(^{t}Bu)-OMe.HCl, EDCI, HOBt, Et_3N, DMF; ii. Bu_3P, CH_2Cl_2; iii. 2-Iodoethanol, Et_3N, DMF; iv. (a) MsCl, Et_3N, DCM (b) NaN_3, DMF; v. PPh_3, THF, H_2O; vi. $C_{15}H_{31}COCl$, DCM; vii. (a) TFA (b) Ac_2O, Et_3N, DCM.



Scheme 4.

Synthesis of 1,2,3-triazole derivatives of **4**.

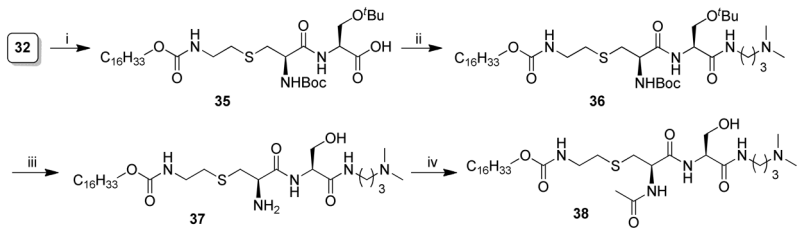
Reagents and Conditions: i. pentadec-1-yne, $CuSO_4$, sodium ascorbate, THF, H_2O. ii. (a) TFA (b) Ac_2O, pyridine, DCM; iii. 4-bromobut-1-yne, Et_3N, DMF; iv. 1-azidohexadecane, $CuSO_4$, sodium ascorbate, THF, H_2O.



Scheme 5.

Synthesis of carbamate derivatives of **4**.

Reagents and Conditions: i. CDI, DCM; ii. $C_{16}H_{33}NH_2$, DCM; iii. TFA; iv. Ac_2O, pyridine, DCM; v. cetyl chloroformate, Et_3N, DCM.



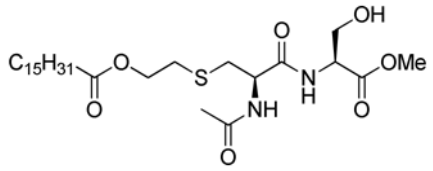
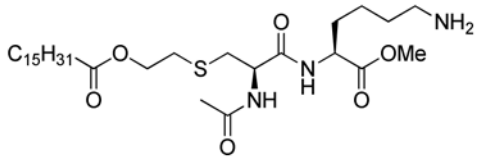
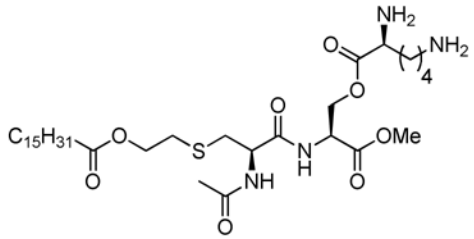
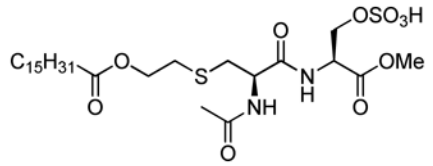
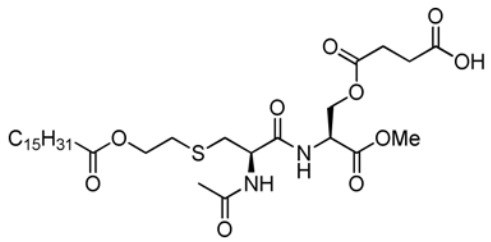
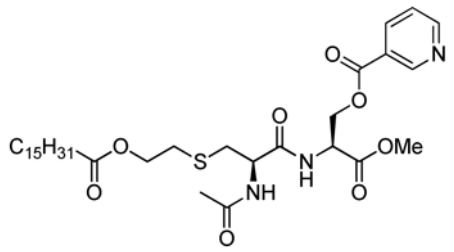
Scheme 6.

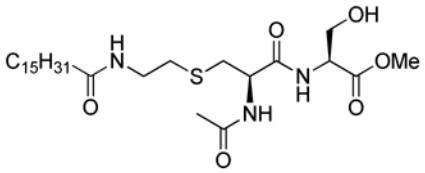
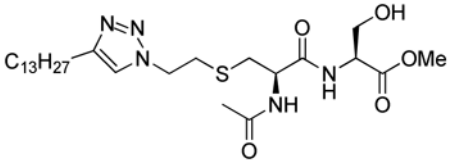
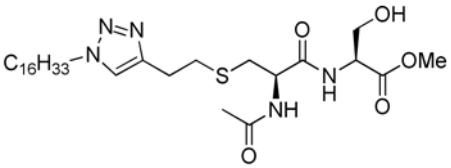
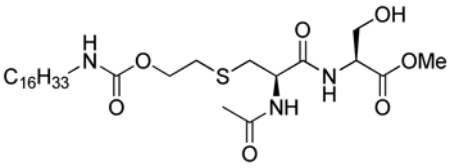
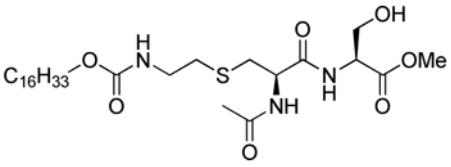
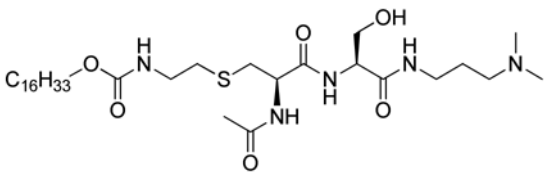
Synthesis of the water soluble carbamate derivative **38**.

Reagents and Conditions: i. $(CH_3)_3SnOH$, DCE; ii. N^1,N^1-dimethylpropane-1,3-diamine, EDCI, HOBT, NMM, DMF; iii. TFA; iv. Ac_2O, Et_3N, DCM.

Table 1

EC50 values of compounds in human TLR2-specific reporter gene assay.

Structure	Compound Number	hTLR2-Agonistic Activity (EC_{50} in nM)
	**4**	0.66
	**10**	1.50
	**12**	1.50
	**13**	5.84
	**14**	0.65
	**15**	0.75

Structure	Compound Number	hTLR2-Agonistic Activity (EC_{50} in nM)
	**22**	12.78
	**24**	290.00
	**27**	1021.00
	**31**	0.32
	**34**	0.065
	**38**	0.25