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Structure-Activity Relationships in Toll-like Receptor 2-Agonists Leading to Simplified Monoacyl Lipopeptides

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Abstract

Toll-like receptor 2-agonistic lipopeptides typified by *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*R*-cysteinyl-*S*-serine (PAM_2CS) compounds are potential vaccine adjuvants. In continuation of previously reported structure-activity relationships on this chemotype, we have determined that at least one acyl group of optimal length (C_{16}) and an appropriately orientated ester carbonyl group is essential for TLR2-agonistic activity. The spacing between one of the palmitoyl ester carbonyl and the thioether is crucial to allow for an important H-bond, which observed in the crystal structure of the lipopeptide:TLR2 complex; consequently, activity is lost in homologated compounds. Penicillamine-derived analogues are also inactive, likely due to unfavorable steric interactions with the carbonyl of Ser 12 in TLR2. The thioether in this chemotype can be replaced with a selenoether. Importantly, the thioglycerol motif can be dispensed with altogether, and can be replaced with a thioethanol bridge. These results have led to a structurally simpler, synthetically more accessible, and water-soluble analogue possessing strong TLR2-agonistic activities in human blood.

Keywords

TLR2; TLR2 agonists; Vaccine adjuvants; Innate immunity; Lipopeptides

Introduction

The phenotypic and functional characterization of T lymphocytic responses as Th1 and Th2 are based on their profile of cytokine secretion in both $CD4^+$ T helper (Th) and $CD8^+$ T cytotoxic (Tc) cell subsets. Human Th1 and Th2 lymphocytes not only elaborate distinct sets of cytokines, but also exhibit distinct functional outcomes.[1] Th1 immunity is currently thought to play a central role in the adaptive immune response against intracellular pathogens, while Th2 responses are important in containing parasitic infections, especially intestinal nematodes.[2;3] Several factors determine Th polarization and effector functions.[4–7]

Of particular interest to us is the relationship between the engagement of specific innate immune receptors in the antigen-presenting cell, notably Toll-like receptors (TLRs), and the initiation and development of Th1- or Th2-directed immune responses.[7–11] We are interested equally in TLR-agonistic chemotypes that evoke dominant Th1,[12;13] Th2,[14] or balanced Th1/Th2[15;16] immune responses for purposes of evaluating them as vaccine

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Supporting Information: Experimental methods for all compounds in Schemes 1–4, and 6–7, as well as characterization of intermediates and final compounds (^1H, ^{13}C, mass spectra). This material is available free of charge via the Internet at http://pubs.acs.org.

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adjuvants. We had previously reported a detailed structure-activity relationship on TLR2-agonistic lipopeptides, typified by *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*R*-cysteinyl-*S*-serine (PAM_2CS) analogues,[14] which focused on examining the role of the structurally indispensable Cys residue, its linkage to the diacylthioglycerol backbone, as well as the geometry and stereochemistry of the Cys-Ser dipeptide unit. PAM_2CS distinguishes itself from virtually all other TLR agonists in that although the lipopeptide is devoid of any detectable pro-inflammatory activity in *ex vivo* human blood models,[15] or of local reactogenicity and pyrogenicity in rabbit models,[17] it is potently adjuvantic in murine models of immunization,[15] suggesting that this chemotype may be a safe and effective adjuvant. Although conflicting reports exist as to its Th1- or Th2-polarizing propensities,[18–21] we have observed a distinct Th2 bias (IgG1 > IgG2a)[22;23] relative to glucopyranosyl lipid A (a more potent TLR4-agonistic analogue[24;25] of monophosphoryl lipid A[26;27]), and an TLR7-agonistic imidazoquinoline that we had described earlier,[12;28] in our preliminary murine immunization screens with bovine α-lactalbumin as a model subunit antigen (Fig. 1).

The results obtained illustrate the importance of at least one acyl group of optimal length (C_{16}) and an appropriate orientation of the ester carbonyl group. The spacing between one of the palmitoyl ester carbonyl and the thioether is crucial to allow for an important H-bond, which is observed in the crystal structure of the lipopeptide:TLR2 complex; consequently, activity is lost in homologated compounds. Penicillamine-derived analogues are also inactive, likely due to unfavorable steric interactions with the carbonyl of Ser 12 in TLR2. The thioether in this chemotype can be replaced with a selenoether. The thioglycerol motif can be dispensed with altogether, and can be replaced with a thioethanol bridge. These SAR studies have led to the identification of a structurally simpler, synthetically more accessible, and water-soluble analogue possessing strong TLR2-agonistic activities in human blood.

Results and Discussion

We are specifically interested in examining the adjuvanticity of TLR2-agonistic compounds using formulations that do not require oil-in-water emulsions such as MF59.[29;30] The overarching goal of the structure-activity relationship studies presented herein was therefore to derive entirely water-soluble analogues and part-structures of PAM_2CS, with a focus on the fatty-acyl substituent on the diacylthioglycerol backbone. We began with attempts to examine the consequences of replacing palmitoyl groups with unsaturated fatty acids, hypothesizing that the phase transition temperatures of such analogues would be considerably lower, thereby enhancing aqueous solubility. The synthetic strategy that we had used previously utilized global deprotection of acid-labile protecting groups,[14] which was found to degrade the unsaturated fatty acids, resulting in unacceptably low yields. We therefore elected to replace the terminal serine with glycine ester, eliminating the necessity for an additional protecting group, noting that this substitution is tolerated without any significant loss of activity.[31;32] The syntheses of linoleic- [**8**, *cis*, *cis*-9,12-octadecadienoic acid] and α-linolenic acid (**9**, all-*cis*-9,12,15-octadecatrienoic acid) bearing analogues were carried out (Scheme 1), with (*R*)-2-(*tert*-butoxycarbonyl)-3-(((*R*)-2,2-dimethyl-1,3-dioxolan-4-yl)methylthio)propanoic acid, **1**, as the synthon.[14] Methyl or ethyl esters of the terminal glycine in these analogues resulted in undesired diketopiperazine side products, a problem that was obviated by using the isopropyl ester. Compounds **8** and **9**, as well as the di-palmitoyl analogue with the isopropyl glycine ester (**7**), were all found to be highly active as TLR2 agonists, with mid-picomolar to low nanomolar EC_{50} values (Table 1) in TLR2-specific reporter gene assays, which are comparable to that of PAM_2CS-OMe, the reference compound. These analogues were not water-soluble, however.

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Analogues with the α-amino group of cysteine acylated with long-chain fatty acids (such as PAM_3CSK_4[33]) are also highly potent, but engage TLR1/2 heterodimers rather than TLR2/6 dimerization induced by the diacyl PAM_2CS chemotype.[34;35] We asked if substituting the *N*-acyl moiety with polar or zwitterionic functional groups could result in modulation of TLR2 activity. Our attempts at direct *S*-alklyation of the highly polar glutathione with 4-(iodomethyl)-2,2-dimethyl-1,3-dioxolane did not proceed smoothly, and we found it simpler instead to couple the γ-carboxylic acid of L-glutamic acid directly to the free amine of cysteine (Scheme 1); compound **11**, however, was found to be virtually inactive, indicating that polar appendages at this position are not be tolerated.

We had previously not examined the role of the acyl chain lengths on TLR2-agonistic activity, and limited SAR studies are available only for the triacyl species.[36] Analogues bearing lauroyl (**17**, C_{12}), myristoyl (**18**, C_{14}), and stearoyl (**19**, C_{18}) were synthesized as outlined in Scheme 2, but were found to be lower in activity than the parent PAM_2CS compound (Fig. 2, Table 1), indicating that the C_{16} chain length is optimal. Next, we sought to explore if replacement of one or both of the acyl chains with polyether or polyamine substituents will impart water solubility without significantly affecting activity. A terminal carboxylic acid functional group was installed on commercially-available 3-(2-(2-ethoxyethoxy)ethoxy) propan-1-amine, 3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propan-1-amine (mono-Boc protected) or N^1, N^2, N^3-tri-Boc-spermine[37;38] by sequential reaction with methyl 2-bromoacetate, Boc-protection of the resultant secondary amines, and hydrolysis of the methyl ester (Scheme 3a). The intermediary Boc-protection of the otherwise zwitterionic compounds facilitated extraction and isolation of the carboxylic acids (**22a–c**) into organic layers. Esterification of the **13** proceeded uneventfully only with **22a**, affording the diacylated species **24** (Scheme 3b). Compound **24** was found to be inactive (Table 1). The other two carboxylates (**22b**, **22c**) yielded mixtures of mono- and di-acylated products, even with large excesses of reagents and under long reaction times, which proved intractably difficult to isolate to homogeneity. We therefore attempted palmitoylation of the monoacyl intermediates and obtained **31–33** in which we presume that the first acylation occurred predominantly, if not exclusively on the primary hydroxyl group of the glycerol unit. Compounds **31–33**, all of which were highly water-soluble, were found to be surprisingly active (Fig. 2), which appeared to suggest that a single acyl chain may be sufficient for TLR2 occupancy and signaling.

We therefore systematically evaluated analogues monoacylated on the primary (**35**) and secondary alcohols (**38**) of the glycerol unit (Scheme 4). Compound **38** with the palmitoyl group on the secondary alcohol was synthesized by first protecting the primary hydroxyl group (Scheme 4). Both monoacyl compounds were unexpectedly active in TLR2-specific reporter gene assays, with EC_{50} values comparable to that of PAM_2CS-OMe (Fig. 2). These findings raised the possibility that the glycerol derived backbone could perhaps be dispensed with altogether, and analogues with simpler scaffolds would still retain activity. We therefore directly *S*-alkylated the appropriately-protected Cys-Ser synthon **41** with 2- or 3-iodoalkanol. Acylation of the resulting terminal primary alcohol with palmitoyl chloride, and global deprotection yielded the desired compounds **44** and **47** with di-, and tri-methylene spacers between the ester and the thio-ether functionalities, respectively (Scheme 5). Compound **44** was found to be quite active (EC_{50}: 2.75 ng/mL; 4.4 nM) in primary screens, whilst **47** was completely inactive (Fig. 2). These results strongly indicated that the intervening segment between the long-chain ester and the thioether was a crucial determinant of TLR2 activity. In order to test whether the orientation of the carbonyl group on the palmitoyl ester also played a role, we synthesized **50** and **53** via *S*-alkylation of the protected Cys-Ser synthon with 2- or 3-iodoalkanoic acid, esterification with 1-hexadecanol, and deprotection (Scheme 5). Both compounds were virtually bereft of activity (Fig. 2, Table 1), verifying that the position and orientation of the ester carbonyl is also critical.

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Analogues with L-cysteine replaced with D- or L-penicillamine (**59** and **64**, respectively), or L-homocysteine (**69**) were all inactive, as was the *des*-amino analogue **74** (Table 1).

Taken together, these results illustrate clearly the importance of at least one acyl group of optimal length (C_{16}) and an appropriate orientation of the ester carbonyl group. A two methylene spacer between the palmitoyl ester and the thioether is essential as is evident from the activity profiles of **38** and **44**, since activity is lost in the homologated analogue (**47**). The only apparent exception to this rule would be the observation that **35** with a three-carbon spacing is also active.

All of these observations, including the above-mentioned exception, as well as the loss of activity in the homocysteine (**69**) and penicillamine (**59**, **64**) analogs can be rationalized on the basis of the crystal structure of PAM_2CSK_4 bound to the TLR2/TLR6 heterodimer.[39;40] The overall binding energetics of PAM_2CSK_4 appears to be driven by dominant hydrophobic interactions of the two acyl chains of PAM_2CSK_4 with nonpolar side-chains of residues lining a hydrophobic tunnel, with a relative paucity of multiple H-bonds; the dimensions of this tunnel can easily accommodate a single acyl chain, such as present in compound **44** (Fig. 4). However, a H-bond between the amide NH of Phe 349 of TLR2 and the carbonyl oxygen atom of the palmitoyl ester at the secondary hydroxyl of the thioglycerol backbone appears to be pivotal in orienting the lipopeptide in its binding pocket (Fig. 4). This interaction would be disfavored either if the spacing between the thioether and the carbonyl is lengthened (as would be the case in compound **47**), or if the orientation of the carbonyl group of the ester were to be inverted as in compounds **50** and **53**. There are two additional H-bonds stabilizing the complex, both of which involve the cysteine-derived part of the lipopeptide: The NH_2 on the α-carbon of cysteine is H-bonded with the carbonyl of Phe 317 (not possible in the *des*-amino analog, **74**), and the carbonyl of cysteine is H-bonded with the NH of Phe 319 (disrupted in the homocysteine analog, **69**). It may also be noted that the dimethyl groups on the β-carbon of the cysteine in the penicillamine derivatives (**59**, **64**) would be expected to impose unfavorable steric interactions (Fig. 4).

We had earlier demonstrated the importance of the thioether bridge in determining TLR2 activity since oxoether analogues were found to be inactive.[14] It was therefore of interest to examine if selenocysteine could substitute for cysteine. Compound **79** was synthesized by direct, in situ selenoalkylation (Scheme 7), and was found to be as active as PAM_2CS (Table 1).

We elected to examine the activities of **44** (active in primary screens) and the selenocysteine-derived compound **79** in secondary screens using *ex vivo* whole human blood models. We selected appropriate experimental models based on our earlier characterization of the activity profile of this chemotype, which includes strong and rapid CD11b upregulation and p38 mitogen-activated kinase (p38MAPK) induction in human neutrophils, but not the induction of any significant levels of proinflammatory cytokines in human blood or isolated peripheral blood mononuclear cells.[17]

We observed prominent CD11b upregulation and p38MAPK phosphorylation for **44**, the magnitude of which is greater than that of PAM_2CS (Fig. 3); the apparent higher potency in whole human blood may be a consequence of differential plasma protein binding, and the consequent effects of free (unbound) agonist.[17;41] Our continued SAR studies on the TLR2-agonistic lipopeptide chemotype have led to the identification of a structurally simpler, synthetically more accessible, and water-soluble analogue possessing strong TLR2-agonistic activities in human blood. This compound is now being evaluated in animal models of immunization for potential adjuvantic activity.

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Experimental Section

Chemistry

Experimental methods for all compounds in Schemes 1–4, and 6–7 are described in Supporting Information. 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. 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 200 (Teledyne-Isco, Lincoln, NE) instruments unless otherwise mentioned. Thin-layer chromatography was carried out on silica gel CCM precoated aluminum sheets. Purity for all final compounds was confirmed to be at least 97% by LC-MS using using two systems: (a) a 5 μm Zorbax Eclipse Plus 4.6 mm × 150 mm analytical reverse phase C_{18} column with H_2O-isopropanol (with 0.1% CF_3COOH in both mobile phases), and (b) a 4.6 × 150 mm Hamilton PRP-1 (100 Å pore size) column with H_2O-CH_3CN gradients (with 0.1% CF_3COOH in both mobile phases). An Agilent ESI-TOF Accurate Mass spectrometer (mass accuracy of 5 ppm) operating in the positive ion acquisition mode was used. Total ion current from 150 to 3500 Da was measured.

Synthesis of Compound 40: (2*S*,2'*S*)-dimethyl 2,2'-(((2*R*,2'*R*)-3,3'-disulfanediylbis(2-((*tert*-butoxycarbonyl)amino)propanoyl))bis(azanediyl))bis(3-(*tert*-butoxy)propanoate)

To a solution of L-cystine (500 mg, 2.08 mmol) in water were added triethylamine (870 μL, 6.24 mmol) and Di-*tert*-butyl-dicarbonate (1.35 g, 6.24 mmol). The reaction mixture was stirred at room temperature for 2h. 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 N_α,N_α′-di-Boc-L-cystine (870 mg, 95%). To a solution of N_α,N_α′-di-Boc-L-cystine (500 mg, 1.13 mmol) in anhydrous DMF were added H-Ser(*t*Bu)-OMe.HCl (576 mg, 2.72 mmol), HOBt (460 mg, 3.4 mmol), and triethylamine (475 μL, 3.4 mmol). The reaction mixture was stirred at 0 °C for 30 min, followed by addition of EDCI (652 mg, 3.4 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 **40** (645 mg, 75%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43 (s, 2H), 5.64 (d, *J* = 6.52 Hz, 2H), 4.67 (dd, *J* = 3.80, 7.92 Hz, 4H), 3.76 (dd, *J* = 3.58, 9.12 Hz, 2H), 3.69 (s, 6H), 3.55 (dd, *J* = 3.72, 9.16 Hz, 2H), 3.07 (d, *J* = 4.12 Hz, 4H), 1.42 (s, 18H), 1.09 (s, 18H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.61, 170.34, 155.51, 80.27, 73.60, 61.83, 53.86, 53.21, 52.41, 43.56, 28.45, 27.37. MS (ESI) calculated for $C_{32}H_{58}N_4O_{12}S_2$, m/z 754.34, found 777.36 $(M + Na)^+$.

Synthesis of Compound 41: (*S*)-methyl 3-(*tert*-butoxy)-2-((*R*)-2-((*tert*-butoxycarbonyl)amino)-3-mercaptopropanamido)propanoate

To a solution of **40** (600 mg, 0.79 mmol) in wet dichloromethane was added tributylphosphine (198 μL, 0.79 mmol). The reaction mixture was stirred at room temperature for 30 min. 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 **41** (565 mg, 94%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.93 (d, *J* = 8.03 Hz, 1H), 5.50 (d, *J* = 5.76 Hz, 1H), 4.66 (dt, *J* = 2.59, 5.73 Hz, 1H), 4.38 (s, 1H), 3.82 (dd, *J* = 2.95, 9.11 Hz, 1H), 3.74 (s, 3H), 3.58 (dd, *J* = 3.20,

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9.10 Hz, 1H), 3.07 (ddd, *J* = 4.23, 8.05, 13.78 Hz, 1H), 2.75 (ddd, *J* = 6.46, 10.12, 13.91 Hz, 1H), 1.78 (t, *J* = 8.52 Hz, 1H), 1.45 (s, 9H), 1.13 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.64, 169.96, 155.32, 80.50, 73.73, 61.64, 55.70, 53.27, 52.62, 28.42, 27.44, 27.42. MS (ESI) calculated for $C_{16}H_{30}N_2O_6S$, m/z 378.18, found 401.18 $(M + Na)^+$.

General Procedure for *S*-alkylation (Synthesis of Compound 42): (*S*)-methyl 3-(*tert*-butoxy)-2-((*R*)-2-((*tert*-butoxycarbonyl)amino)-3-((2-hydroxyethyl)thio)propanamido)propanoate

To a solution of compound **41** (100 mg, 0.26 mmol) in DMF were added triethylamine (73 μL, 0.52 mmol) and 2-iodoethanol (24 μL, 0.31 mmol). The reaction mixture was stirred at 90 °C for 2h. After completion of the reaction, the solvent was removed under reduced pressure to obtain the crude product which was purified using column chromatography (70% EtOAc/hexanes) to obtain compound **42** (84 mg, 77%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31–7.23 (m, 1H), 5.59 (d, *J* = 6.79 Hz, 1H), 4.68 (dt, *J* = 3.04, 8.15 Hz, 1H), 4.39 (d, *J* = 4.71 Hz, 1H), 3.83 (dd, *J* = 3.00, 9.17 Hz, 1H), 3.80 (t, *J* = 5.67 Hz, 2H), 3.75 (s, 3H), 3.58 (dd, *J* = 3.21, 9.17 Hz, 1H), 3.02 (dd, *J* = 5.47, 13.97 Hz, 1H), 2.89 (dd, *J* = 7.05, 13.97 Hz, 1H), 2.86–2.74 (m, 3H), 1.46 (s, 9H), 1.15 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.72, 170.60, 155.55, 80.40, 73.74, 61.79, 61.42, 53.95, 53.25, 52.58, 35.76, 35.06, 28.40, 27.37. MS (ESI) calculated for $C_{18}H_{34}N_2O_7S$, m/z 422.20, found 445.21 $(M + Na)^+$.

Synthesis of Compound 43: 2-(((*R*)-3-(((*S*)-3-(*tert*-butoxy)-1-methoxy-1-oxopropan-2-yl)amino)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)thio)ethyl palmitate

Compound **42** (50 mg, 0.11 mmol) was palmitoylated using the general procedure for palmitoylation (see synthesis of compound **28**) to obtain compound **43** (70 mg, 90%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.13 (d, *J* = 7.85 Hz, 1H), 5.44 (s, 1H), 4.64 (dt, *J* = 3.01, 8.12 Hz, 1H), 4.32 (d, *J* = 4.42 Hz, 1H), 4.22 (t, *J* = 6.60 Hz, 2H), 3.81 (dd, *J* = 2.94, 9.11 Hz, 1H), 3.72 (s, 3H), 3.56 (dd, *J* = 3.20, 9.12 Hz, 1H), 2.99 (dd, *J* = 5.57, 13.93 Hz, 1H), 2.90 (dd, *J* = 6.73, 13.93 Hz, 1H), 2.87–2.76 (m, 2H), 2.30 (t, *J* = 7.59 Hz, 2H), 1.64–1.56 (m, 2H), 1.44 (s, 9H), 1.32–1.21 (m, 24H), 1.12 (s, 9H), 0.86 (t, *J* = 6.95 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.77, 170.57, 170.49, 155.35, 80.36, 73.63, 63.11, 61.76, 53.81, 53.30, 52.54, 34.94, 34.30, 32.04, 31.13, 29.81, 29.80, 29.77, 29.74, 29.59, 29.48, 29.40, 29.27, 28.41, 27.41, 25.00, 22.81, 14.25. MS (ESI) calculated for $C_{34}H_{64}N_2O_8S$, m/z 660.43, found 683.44 $(M + Na)^+$.

Synthesis of Compound 44: 2-(((*R*)-2-amino-3-(((*S*)-3-hydroxy-1-methoxy-1-oxopropan-2-yl)amino)-3-oxopropyl)thio)ethyl palmitate, trifluoroacetate

Compound **43** (50 mg, 0.07 mmol) was deprotected with trifluoroacetic acid as described earlier in the general procedure for one step deprotection of *N*-Boc and *O*-*tert*-Butyl (see synthesis of compound **11**) to obtain compound **44** as trifluoroacetate salt (46 mg, quantitative yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.48 (d, *J* = 7.69 Hz, 1H), 4.71–4.64 (m, 1H), 4.34 (t, *J* = 6.43 Hz, 1H), 4.26–4.18 (m, 2H), 3.96–3.90 (m, 1H), 3.86 (dd, *J* = 5.34, 11.71 Hz, 1H), 3.76 (s, 3H), 3.16 (dd, *J* = 5.59, 14.52 Hz, 1H), 3.01 (dd, *J* = 7.16, 14.54 Hz, 1H), 2.81 (t, *J* = 6.49 Hz, 2H), 2.31 (t, *J* = 7.66 Hz, 2H), 1.59 (p, *J* = 7.43 Hz, 2H), 1.37–1.12 (m, 26H), 0.88 (t, *J* = 6.96 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.58, 170.29, 168.27, 62.63, 61.83, 55.40, 52.98, 52.83, 34.32, 33.06, 32.08, 30.90, 29.86, 29.84, 29.82, 29.67, 29.52, 29.45, 29.31, 24.99, 22.84, 14.27. MS (ESI) calculated for $C_{25}H_{48}N_2O_6S$, m/z 504.32, found 505.34 $(M + H)^+$.

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Synthesis of Compound 45: (*S*)-methyl 3-(*tert*-butoxy)-2-((*R*)-2-((*tert*-butoxycarbonyl)amino)-3-((3-hydroxypropyl)thio)propanamido)propanoate

Compound **41** (60 mg, 0.16 mmol) was *S*-alkylated with 3-iodo-1-propanol (18 μL, 0.19 mmol) as described in the general procedure for *S*-alkylation (see synthesis of compound **42**) to obtain compound **45** (50 mg, 72%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.23 (d, *J* = 7.64 Hz, 1H), 5.53 (s, 1H), 4.67 (dt, *J* = 3.01, 8.20 Hz, 1H), 4.34 (d, *J* = 4.72 Hz, 1H), 3.83 (dd, *J* = 2.96, 9.14 Hz, 1H), 3.80–3.70 (m, 5H), 3.58 (dd, *J* = 3.18, 9.14 Hz, 1H), 2.96 (dd, *J* = 5.42, 14.04 Hz, 1H), 2.87 (dd, *J* = 7.03, 14.04 Hz, 1H), 2.80 (d, *J* = 5.68 Hz, 1H), 2.75–2.69 (m, 1H), 2.24 (s, 1H), 1.91–1.82 (m, 2H), 1.46 (s, 9H), 1.15 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.69, 155.50, 80.42, 73.73, 61.82, 60.92, 53.49, 53.25, 52.58, 34.86, 32.04, 28.49, 28.42, 27.40. MS (ESI) calculated for $C_{19}H_{36}N_2O_7S$, m/z 436.22, found 459.23 $(M + Na)^+$.

Synthesis of Compound 46: 3-(((*R*)-3-(((*S*)-3-(*tert*-butoxy)-1-methoxy-1-oxopropan-2-yl)amino)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)thio)propyl palmitate

Compound **45** (40 mg, 0.09 mmol) was palmitoylated using the general procedure for palmitoylation (see synthesis of compound **28**) to obtain compound **46** (50 mg, 81%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.16 (d, *J* = 7.75 Hz, 1H), 5.44 (s, 1H), 4.65 (dt, *J* = 3.02, 8.14 Hz, 1H), 4.29 (d, *J* = 4.64 Hz, 1H), 4.18–4.09 (m, 2H), 3.81 (dd, *J* = 2.94, 9.10 Hz, 1H), 3.73 (s, 3H), 3.56 (dd, *J* = 3.20, 9.10 Hz, 1H), 2.95 (dd, *J* = 5.46, 13.87 Hz, 1H), 2.85 (dd, *J* = 6.94, 13.88 Hz, 1H), 2.64 (td, *J* = 6.72, 13.14 Hz, 2H), 2.28 (t, *J* = 7.61 Hz, 2H), 1.98–1.85 (m, 2H), 1.63–1.54 (m, 2H), 1.44 (s, 9H), 1.30–1.21 (m, 24H), 1.13 (s, 9H), 0.86 (t, *J* = 6.96 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.95, 170.59, 155.32, 80.33, 73.62, 62.70, 61.78, 53.79, 53.27, 52.53, 34.70, 34.39, 32.04, 29.80, 29.77, 29.73, 29.59, 29.48, 29.40, 29.29, 29.01, 28.73, 28.41, 27.40, 25.06, 22.81, 14.25. MS (ESI) calculated for $C_{35}H_{66}N_2O_8S$, m/z 674.45, found 697.45 $(M + Na)^+$.

Synthesis of Compound 47: 3-(((*R*)-2-amino-3-(((*S*)-3-hydroxy-1-methoxy-1-oxopropan-2-yl)amino)-3-oxopropyl)thio)propyl palmitate, trifluoroacetate

Compound **46** (50 mg, 0.07 mmol) was deprotected with trifluoroacetic acid as described earlier in the general procedure for one step deprotection of *N*-Boc and *O*-*tert*-Butyl (see synthesis of compound **11**) to obtain compound **47** as trifluoroacetate salt (47 mg, quantitative yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.35 (d, *J* = 6.83 Hz, 1H), 4.66 (s, 1H), 4.15 (t, *J* = 6.35 Hz, 2H), 4.01–3.87 (m, 3H), 3.79 (s, 3H), 3.05 (dd, *J* = 3.85, 13.59 Hz, 1H), 2.93–2.85 (m, 1H), 2.62 (t, *J* = 7.07 Hz, 2H), 2.30 (dd, *J* = 6.35, 13.88 Hz, 2H), 1.91 (p, *J* = 6.69 Hz, 2H), 1.59 (dd, *J* = 7.17, 14.37 Hz, 2H), 1.45–1.09 (m, 26H), 0.88 (t, *J* = 6.96 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.33, 170.57, 62.72, 62.67, 55.17, 53.43, 52.97, 35.43, 34.42, 32.07, 29.85, 29.81, 29.81, 29.78, 29.65, 29.51, 29.44, 29.32, 28.75, 28.57, 25.08, 22.84, 14.28. MS (ESI) calculated for $C_{26}H_{50}N_2O_6S$, m/z 518.33, found 541.33 $(M + Na)^+$.

Synthesis of Compound 48: 2-(((*R*)-3-(((*S*)-3-(*tert*-butoxy)-1-methoxy-1-oxopropan-2-yl)amino)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)thio)acetic acid

To a solution of compound **41** (100 mg, 0.26 mmol) in DMF were added triethylamine (73 μL, 0.52 mmol) and iodoacetic acid (60 mg, 0.31 mmol). The reaction mixture was stirred at 90 °C for 2h. After completion of the reaction, the solvent was removed under reduced pressure to obtain the residue. The residue was dissolved in ethyl acetate, followed by washing 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 (10% MeOH/CH_2Cl_2) to obtain compound **48** (80 mg, 71%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 (d, *J* = 4.79 Hz, 1H), 5.69 (d, *J* = 5.36 Hz, 1H),

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4.66 (dt, *J* = 3.11, 7.67 Hz, 1H), 4.43 (d, *J* = 4.09 Hz, 1H), 3.80 (dd, *J* = 3.19, 9.23 Hz, 1H), 3.73 (s, 3H), 3.59 (dd, *J* = 3.20, 9.19 Hz, 1H), 3.44–3.26 (m, 2H), 3.03 (d, *J* = 4.67 Hz, 2H), 1.44 (s, 9H), 1.13 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.53, 170.96, 170.63, 155.70, 80.65, 73.91, 61.73, 53.59, 53.39, 52.63, 35.21, 34.08, 28.39, 27.39, 27.35. MS (ESI) calculated for $C_{18}H_{32}N_2O_8S$, m/z 436.18, found 459.18 $(M + Na)^+$.

Synthesis of Compound 49: (*S*)-methyl 3-(*tert*-butoxy)-2-((*R*)-2-((*tert*-butoxycarbonyl)amino)-3-((2-(hexadecyloxy)-2-oxoethyl)thio)propanamido)propanoate

To a solution of **48** (60 mg, 0.13 mmol) in anhydrous DMF were added 1-hexadecanol (50 mg, 0.20 mmol), HOBt (35 mg, 0.26 mmol), and triethylamine (36 μL, 0.26 mmol). The reaction mixture was stirred at 0 °C for 30 min, followed by addition of EDCI (50 mg, 0.26 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 (25% EtOAc/hexanes) to obtain compound **49** (77 mg, 85%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.30 (d, *J* = 7.84 Hz, 1H), 5.55 (d, *J* = 7.44 Hz, 1H), 4.65 (dt, *J* = 2.98, 7.95 Hz, 1H), 4.41 (d, *J* = 6.59 Hz, 1H), 4.12 (t, *J* = 6.86 Hz, 2H), 3.82 (dd, *J* = 3.03, 9.05 Hz, 1H), 3.73 (d, *J* = 3.35 Hz, 3H), 3.57 (dd, *J* = 3.27, 9.06 Hz, 1H), 3.37 (q, *J* = 15.34 Hz, 2H), 2.99 (ddd, *J* = 6.30, 14.18, 33.64 Hz, 2H), 1.67–1.59 (m, 2H), 1.43 (s, 9H), 1.38–1.22 (m, 26H), 1.13 (d, *J* = 4.63 Hz, 9H), 0.87 (t, *J* = 6.95 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.97, 170.63, 170.46, 155.41, 80.23, 73.61, 66.00, 61.73, 53.39, 53.25, 52.53, 35.86, 34.34, 32.05, 29.82, 29.81, 29.79, 29.78, 29.72, 29.65, 29.50, 29.37, 28.61, 28.41, 27.38, 25.94, 22.83, 14.28. MS (ESI) calculated for $C_{34}H_{64}N_2O_8S$, m/z 660.43, found 683.44 $(M + Na)^+$.

Synthesis of Compound 50: (*S*)-methyl 2-((*R*)-2-amino-3-((2-(hexadecyloxy)-2-oxoethyl)thio)propanamido)-3-hydroxypropanoate, trifluoroacetate

Compound **49** (50 mg, 0.07 mmol) was deprotected with trifluoroacetic acid as described earlier in the general procedure for one step deprotection of *N*-Boc and *O*-*tert*-Butyl (see synthesis of compound **11**) to obtain compound **50** as trifluoroacetate salt (46 mg, quantitative yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.74 (d, *J* = 7.57 Hz, 1H), 4.69–4.63 (m, 1H), 4.38 (t, *J* = 6.68 Hz, 1H), 4.16–4.05 (m, 2H), 3.88 (dt, *J* = 7.17, 11.77 Hz, 2H), 3.74 (s, 3H), 3.49–3.39 (m, 3H), 3.24 (dd, *J* = 4.86, 14.44 Hz, 1H), 3.05 (dd, *J* = 8.09, 14.60 Hz, 1H), 1.66–1.58 (m, 2H), 1.36–1.18 (m, 27H), 0.87 (t, *J* = 6.96 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.58, 170.30, 168.27, 66.86, 61.72, 55.38, 52.90, 52.86, 50.84, 34.52, 34.34, 32.06, 29.86, 29.84, 29.81, 29.78, 29.69, 29.52, 29.44, 28.48, 25.94, 22.84, 14.28. MS (ESI) calculated for $C_{25}H_{48}N_2O_6S$, m/z 504.32, found 505.34 $(M + H)^+$.

Synthesis of Compound 51: 3-(((*R*)-3-(((*S*)-3-(*tert*-butoxy)-1-methoxy-1-oxopropan-2-yl)amino)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)thio)propanoic acid

Compound **41** (60 mg, 0.16 mmol) was *S*-alkylated with 3-iodopropionic acid (38 mg, 0.19 mmol) as described for the synthesis of compound **48** to afford compound **51** (55 mg, 78%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (d, *J* = 7.24 Hz, 1H), 5.54 (d, *J* = 6.99 Hz, 1H), 4.69 (dt, *J* = 3.01, 8.19 Hz, 1H), 4.42 (d, *J* = 6.21 Hz, 1H), 3.83 (dd, *J* = 2.99, 9.20 Hz, 1H), 3.75 (s, 3H), 3.58 (dd, *J* = 3.12, 9.19 Hz, 1H), 2.97–2.82 (m, 4H), 2.73–2.61 (m, 2H), 1.46 (s, 9H), 1.15 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.80, 170.92, 170.85, 155.56, 80.49, 73.89, 61.87, 53.50, 53.22, 52.68, 34.65, 34.59, 28.40, 27.37, 26.84. MS (ESI) calculated for $C_{19}H_{34}N_2O_8S$, m/z 450.20, found 473.20 $(M + Na)^+$.

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Synthesis of Compound 52: (*S*)-methyl 3-(*tert*-butoxy)-2-((*R*)-2-((*tert*-butoxycarbonyl)amino)-3-((3-(hexadecyloxy)-3-oxopropyl)thio)propanamido)propanoate

Compound **51** (45 mg, 0.1 mmol) was esterified with 1-hexadecanol using the procedure as described for the synthesis of compound **49** to afford compound **52** (51 mg, 76%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.18 (d, *J* = 7.80 Hz, 1H), 5.48 (d, *J* = 6.07 Hz, 1H), 4.67 (dt, *J* = 2.98, 8.15 Hz, 1H), 4.37 (d, *J* = 5.20 Hz, 1H), 4.08 (t, *J* = 6.81 Hz, 2H), 3.83 (dd, *J* = 2.90, 9.10 Hz, 1H), 3.74 (s, 3H), 3.57 (dd, *J* = 3.19, 9.11 Hz, 1H), 2.98 (dd, *J* = 5.56, 14.00 Hz, 1H), 2.87 (dt, *J* = 6.74, 13.37 Hz, 3H), 2.64 (ddd, *J* = 2.77, 6.13, 10.71 Hz, 2H), 1.69 (s, 1H), 1.65–1.58 (m, 2H), 1.45 (d, *J* = 12.81 Hz, 9H), 1.35–1.22 (m, 25H), 1.14 (s, 9H), 0.88 (t, *J* = 6.93 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.15, 170.62, 170.58, 155.37, 80.32, 75.57, 73.64, 68.95, 65.14, 61.81, 53.64, 53.24, 52.57, 52.02, 35.38, 34.81, 34.72, 32.06, 29.84, 29.82, 29.80, 29.74, 29.67, 29.51, 29.41, 28.68, 28.42, 27.41, 27.29, 26.03, 22.84, 14.29. MS (ESI) calculated for $C_{35}H_{66}N_2O_8S$, m/z 674.45, found 697.45 $(M + Na)^+$.

Synthesis of Compound 53: (*S*)-methyl 2-((*R*)-2-amino-3-((3-(hexadecyloxy)-3-oxopropyl)thio)propanamido)-3-hydroxypropanoate, trifluoroacetate

Compound **52** (40 mg, 0.06 mmol) was deprotected with trifluoroacetic acid as described earlier in the general procedure for one step deprotection of *N*-Boc and *O*-*tert*-Butyl (see synthesis of compound **11**) to obtain compound **53** as trifluoroacetate salt (37 mg, quantitative yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.53 (d, *J* = 7.24 Hz, 1H), 4.68 (s, 1H), 4.38 (s, 1H), 4.08 (t, *J* = 6.77 Hz, 2H), 3.99–3.92 (m, 1H), 3.87 (dd, *J* = 4.49, 11.04 Hz, 1H), 3.75 (s, 3H), 3.49 (s, 1H), 3.13 (dd, *J* = 4.87, 14.36 Hz, 1H), 2.97 (dd, *J* = 7.38, 14.37 Hz, 1H), 2.83 (tq, *J* = 6.79, 13.35 Hz, 2H), 2.63 (t, *J* = 6.84 Hz, 2H), 1.60 (dd, *J* = 6.88, 13.92 Hz, 2H), 1.35–1.20 (m, 27H), 0.88 (t, *J* = 6.95 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.99, 170.32, 65.60, 55.35, 52.92, 52.65, 34.25, 32.07, 29.86, 29.84, 29.82, 29.78, 29.71, 29.52, 29.46, 28.61, 26.50, 26.03, 22.85, 14.29. MS (ESI) calculated for $C_{26}H_{50}N_2O_6S$, m/z 518.33, found 519.35 $(M + H)^+$.

TLR2-specific NF-κB induction

The induction of NF-κB in a TLR2-specific reporter gene assay was quantified using HEK-Blue™ cells as previously described by us.[14] HEK293 cells stably transfected with human TLR2 and alkaline phosphatase (sAP) were obtained from InvivoGen (San Diego, CA), and were maintained in HEK-Blue™ Selection medium containing zeocin and normocin. Stable expression of secreted alkaline phosphatase (sAP) under control of NF-κB promoters is inducible by TLR2 agonists, and extracellular sAP in the supernatant is proportional to NF-κB induction. HEK-Blue cells were incubated at a density of ~10^5 cells/mL in a volume of 80 μL/well, in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluency was achieved, and then stimulated with serially-diluted aliquots of compounds for 12 h. sAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen (present in the HEK-detection medium as supplied by the vendor) at 620 nm.

Experiments involving human blood

Human blood was obtained from healthy adults by antecubital venipuncture in accordance with University of Kansas Human Subjects Experimentation protocols (Protocol # HSCL 12397).

Phosflow™ flow cytometric assay for p38MAPK

Assays were performed as described by us previously.[15;17;37] Briefly, 1 mL aliquots of fresh whole blood, anticoagulated with heparin were incubated with 25 μL an equal volume of graded concentrations of compounds diluted in saline for 15 min at 37°C. Erythrocytes were lysed and leukocytes were fixed in one step by mixing 200 μL of the samples in 4 mL pre-

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warmed Whole Blood Lyse/Fix Buffer (Becton-Dickinson Biosciences, San Jose, CA). After washing the cells at 500 g for 8 min in buffer, the cells were permeabilized in ice-cold methanol for 30 min, washed twice in phosphate-buffered saline and transferred to a Millipore MultiScreen BV 1.2μ filter plate and stained with phycoerythrin (PE)-conjugated mouse anti-p38MAPK (Becton-Dickinson Biosciences; mAb recognizes the conserved dual-phosphorylated pT180/pY182 site of p38α, β, γ, and δ isoforms of p38MAPK) The cells were washed twice in the plate by aspiration as per protocols supplied by the vendor. Cytometry was performed using a BD FACSArray instrument in the single-color mode for PE acquisition on 20,000 gated events. Post-acquisition analyses were performed using FlowJo v 7.0 software (Treestar, Ashland, OR).

CD11b flow cytometric assay

Assays were performed as described by us previously.[15;17;37] Briefly, 1 mL aliquots of fresh anticoagulated whole blood were incubated with 25 μL of graded dilutions of the compounds for 1 hour at 37° C. Negative (saline) controls were included in each experiment. Samples were placed on ice for 15 min before 20 μL of anti-CD11b/Mac-1 antibody (Becton-Dickinson) were added to each sample tube and allowed to incubate on ice for 30 min. This 0° C incubation step prevented internalization of antibody, and ensured staining of only extracellularly expressed CD11b. Erythrocytes were lysed and leukocytes were fixed in one step by mixing 200 μL of the samples in 4 mL pre-warmed Whole Blood Lyse/Fix Buffer (Becton-Dickinson Biosciences, San Jose, CA). After washing the cells twice at 200 g for 5 min in CBA buffer, the cells were transferred to a 96-well plate. Flow cytometry was performed using a BD FACSArray instrument in the single-color mode for PE acquisition on 20,000 gated events. Post-acquisition analyses were performed using FlowJo v 7.0 software.

Animal experiments

All experiments were performed in accordance with animal care protocols approved by the University of Kansas IACUC Committee. Cohorts of 5 outbred CF-1 mice per group were immunized on Day 0 with vehicle (control 1), 10 μg/animal of bovine α-lactalbumin alone (control 2), or 10 μg/animal α-lactalbumin mixed with 50 μg/animal of either GLA[24;25] (TLR4 agonist), PAM_2CS[14] (TLR2 agonist), or imidazoquinoline (compound **1** in Ref. 12; TLR7/8 agonist). All antigen/adjuvant preparations were in sterile, physiological saline (vehicle). A volume of 0.2 mL was injected intramuscularly into the flank region. Animals were boosted once on Day 14, and were bled by terminal cardiac puncture (under isoflurane anesthesia) on Day 21. Sera were stored at −80° C until assayed.

Enzyme-linked immunosorbent assays (ELISA)

A description of the semi-automated 384-well ELISA procedures has been published previously.[12] A Precision 2000 liquid handler (Bio-Tek, Winooski, VT) was used for all serial dilution and reagent addition steps, and a Bio-Tek ELx405 384-well plate washer was employed for plate washes; 100 mM phosphate-buffered saline (PBS) pH 7.4, containing 0.1% Tween-20 was used as wash buffer. Nunc-Immuno MaxiSorp (384-well) plates were coated with α-lactalbumin (10 μg/mL, in a volume of 80 μL/well) in 100 mM carbonate buffer, pH 9.0 overnight at 4° C. After 3 washes, the plates were blocked with 3% bovine serum albumin (in PBS, pH 7.4) for 1 h at room temperature. Serum samples (in quadruplicate) were serially diluted in a separate 384-well plate using the liquid handler. After three additional washes of the assay plate, 30 μL of the serum dilutions were transferred from the dilution plate using the liquid handler, and the assay plate incubated at 37°C for 2 h. The assay plate was washed three times, and 30 μl of 1:10,000 diluted appropriate anti-mouse immunoglobulin isotypes (IgG1, IgG2a) conjugated with horseradish peroxidase was added to all wells. Following an incubation step at 37°C for 1 h, and three

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washes, tetramethylbenzidine substrate was added at concentrations recommended by vendor (Sigma, St. Louis, MO). The chromogenic reaction was terminated at 30 min by the addition of 2M H_2SO_4. Plates were then read at 450 nm using a SpectraMax M4 device (Molecular Devices, Sunnyvale, CA). Data visualization and statistics (Student's T test for significance) were performed using Origin 7.0 (Northampton, MA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

CD	Cluster of differentiation
Cys	Cysteine
DMF	Dimethylformamide
DMAP	4-Dimethylaminopyridine
EC_{50}	Half-maximal effective concentration
EDCI	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ESI-TOF	Electrospray ionization-time of flight
Fmoc-Osu	9-Fluorenylmethyl *N*-succinimidyl carbonate
HEK	Human embryonic kidney
HOBt	1-Hydroxybenzotriazole
IMDQ	Imidazoquinoline
IgG1	Immunoglobulin G subclass 1
IgG2a	Immunoglobulin G subclass 2a
NF-κB	Nuclear factor-κB
p38MAPK	p38 Mitogen activated protein kinase
PBMCs	Peripheral blood mononuclear cells
PE	Phycoerythrin
PAM	Palmitoyl
sAP	Secreted alkaline phosphatase
SAR	Structure activity relationship
Ser	Serine
Th1	Helper T lymphocyte, type 1
Th2	Helper T lymphocyte, type 2
TLR	Toll like receptor

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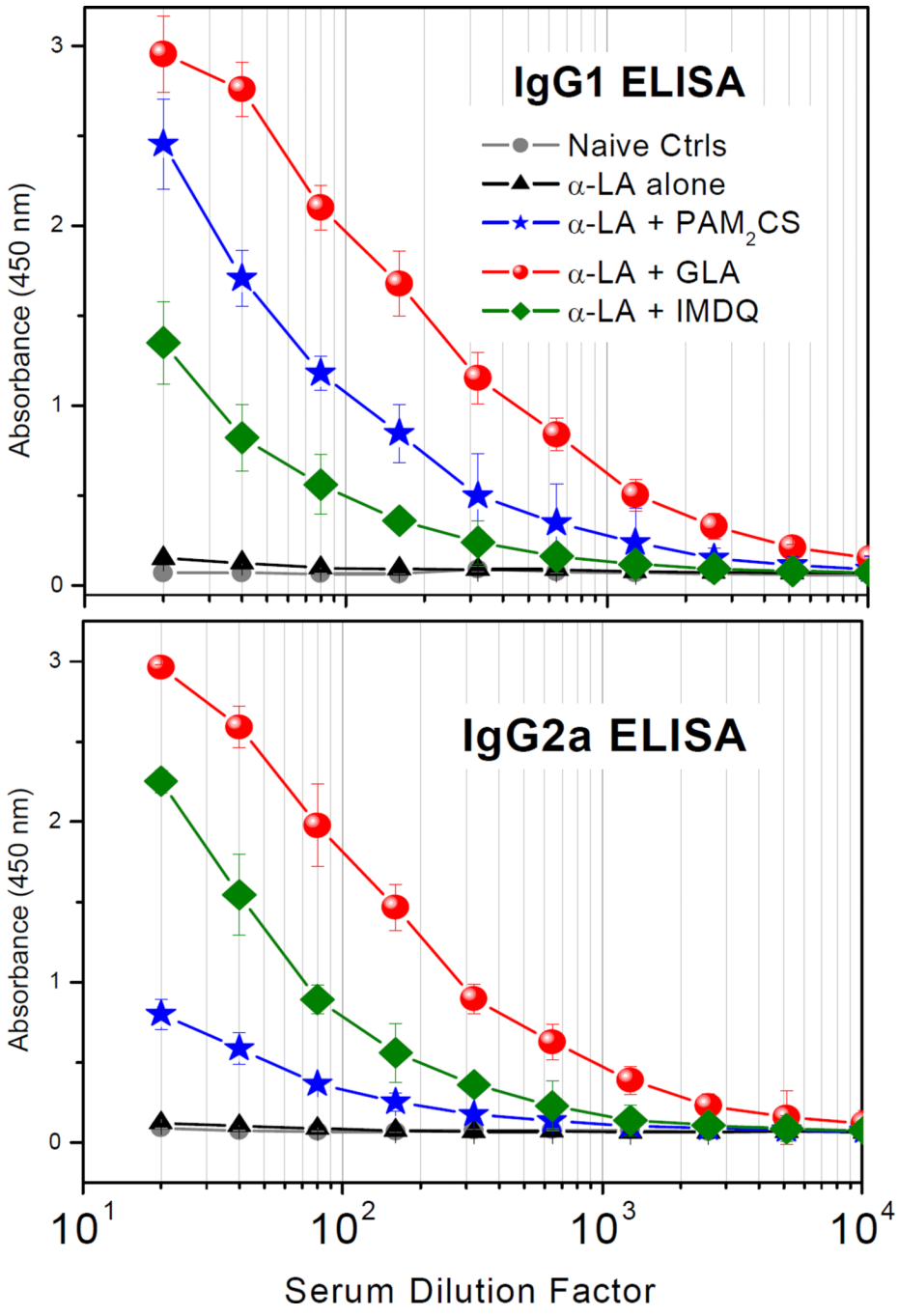

Fig.1.

IgG1 (Th2) and IgG2a(Th1) immunoglobulin isotype titers in outbred CF-1 mice immunized with unadjuvanted α-lactalbumin, or α-lactalbumin adjuvanted with GLA, PAM2CS, or imidazoquinoline. α-lactalbumin-specific immunoglobulin levels were quantified by standard antibody-capture ELISA, performed in liquid handler-assisted 384-well format.

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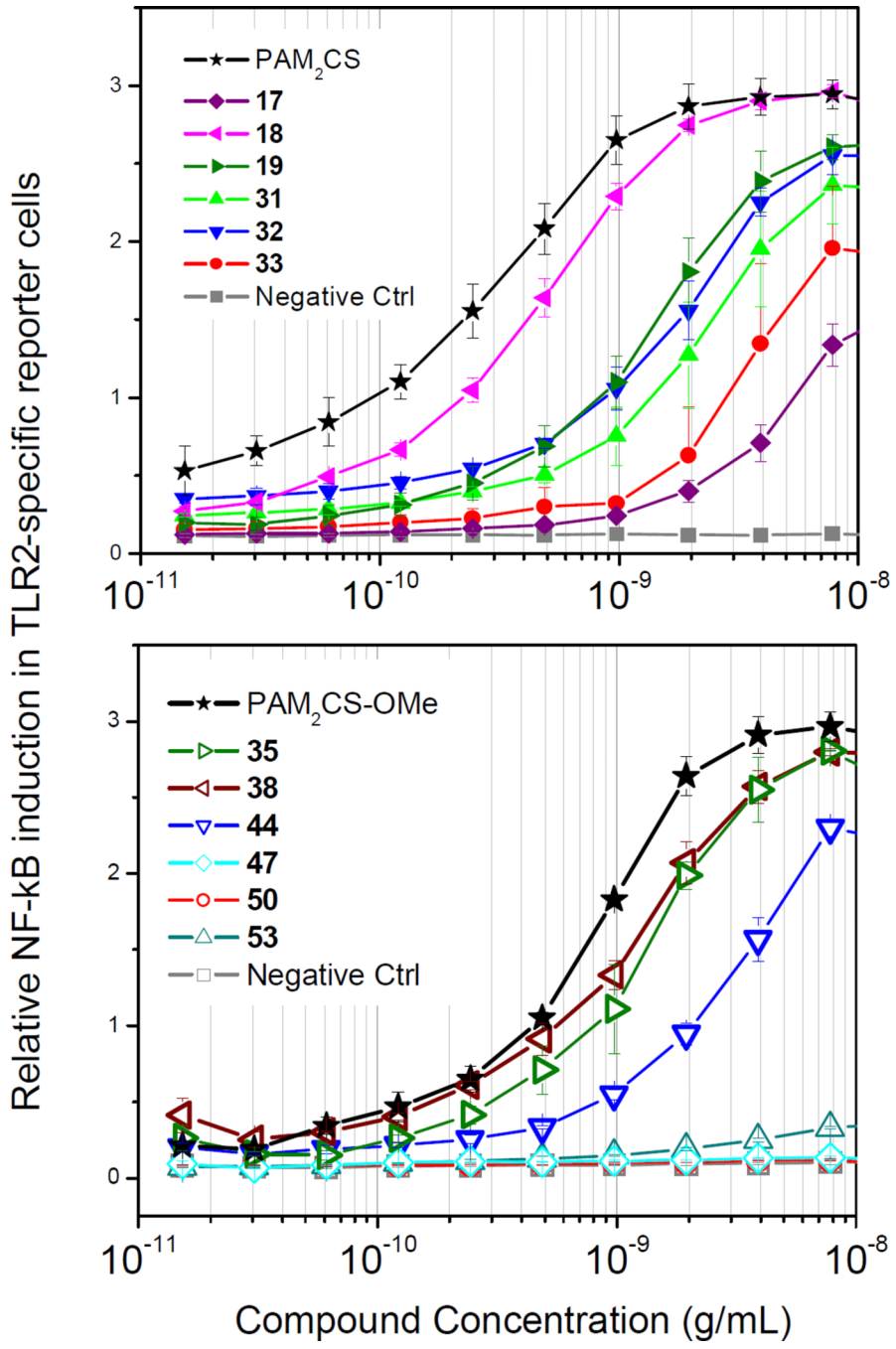

Fig.2.

TLR2-specific NF-κB induction by selected analogues. Means and standard deviations of quadruplicate samples are shown.

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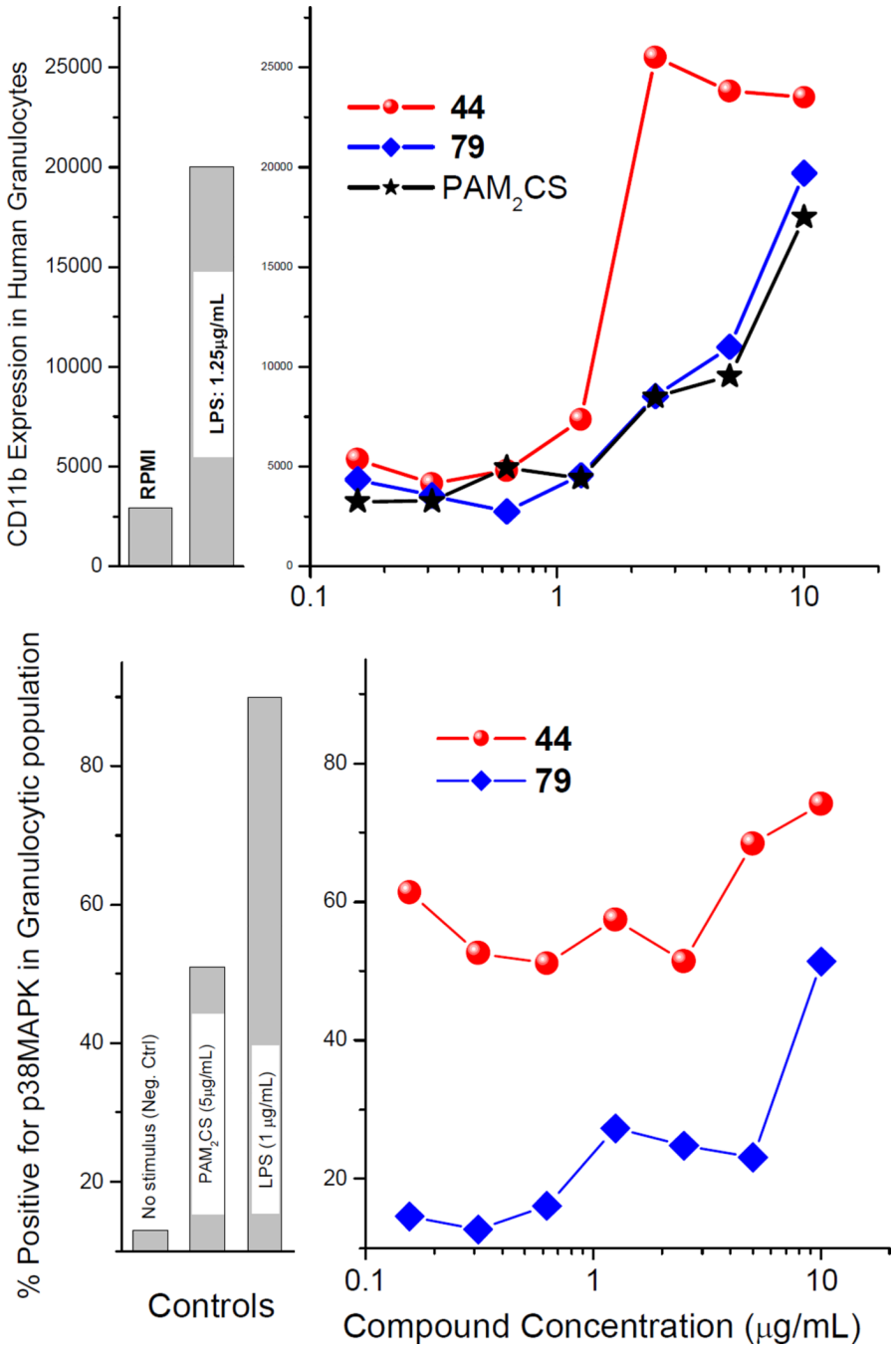

Fig.3.

CD11b (Top) and p38MAPK upregulation in human granulocytes. Whole human blood was stimulated *ex vivo* with lipopeptides, and assayed by flow cytometry. Representative experiment of three independent experiments are shown.

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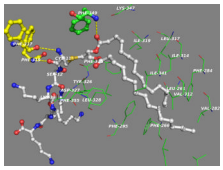

Fig.4.

The crystal structure of PAM2CSK4 bound to TLR2/TLR6 heterodimer, showing crucial H-bond interactions of the lipopeptide with the receptor. The lipopeptide is depticted in ball-and-stick model. Adapted from Ref. 39; PDB code: 3A79.

Scheme -1.

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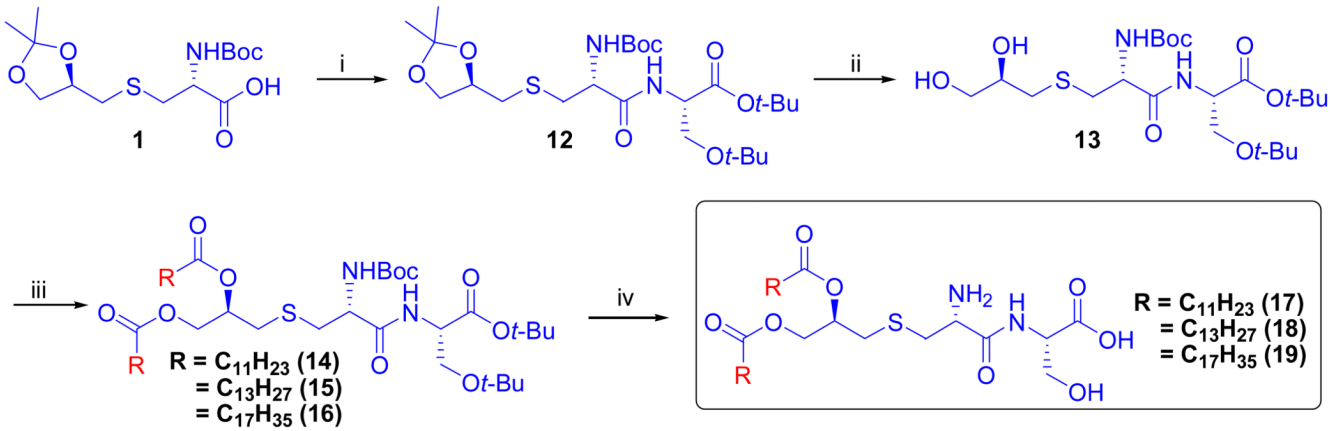

Reagents: i. H-Ser(*t*Bu)-O*t*-Bu.HCl, EDCI, HOBt, TEA, DMF; ii. 70% CH_3COOH; iii. RCOCl, TEA, DMAP, CH_2Cl_2; iv. CF_3COOH.

Scheme -2.

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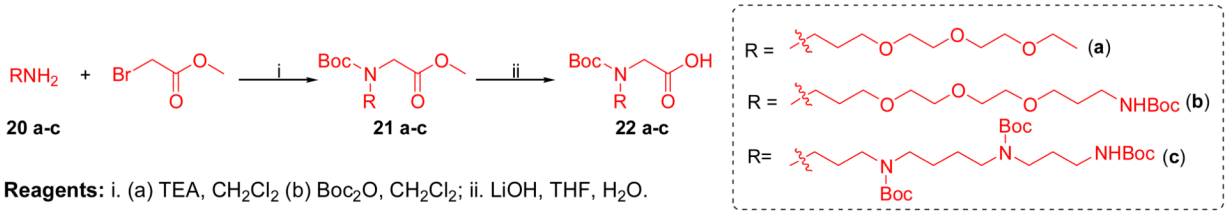
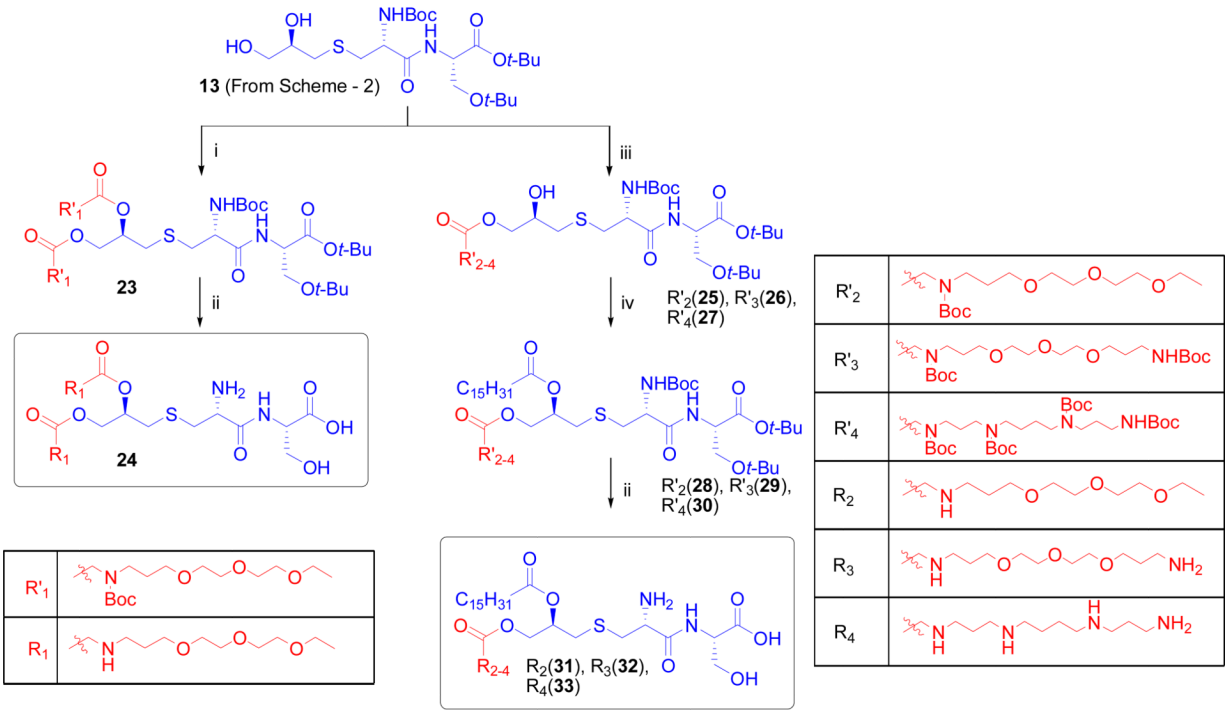
3a



Reagents: i. (a) TEA, CH_2Cl_2 (b) Boc_2O, CH_2Cl_2; ii. LiOH, THF, H_2O.

3b



Reagents: i. R'_1COOH (**22a**, 2 eq.), EDCI, HOBt, TEA, DMF; ii. CF_3COOH; iii. $R'_{2\text{-}4}COOH$ (**22a-c**, 1 eq.), EDCI, HOBt, TEA, DMF; iv. $C_{15}H_{31}COCl$, TEA, DMAP, CH_2Cl_2.

Scheme -3.

Scheme -4.

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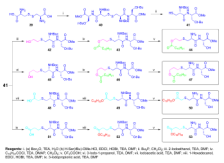

Scheme-5.

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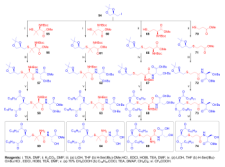

Scheme- 6.

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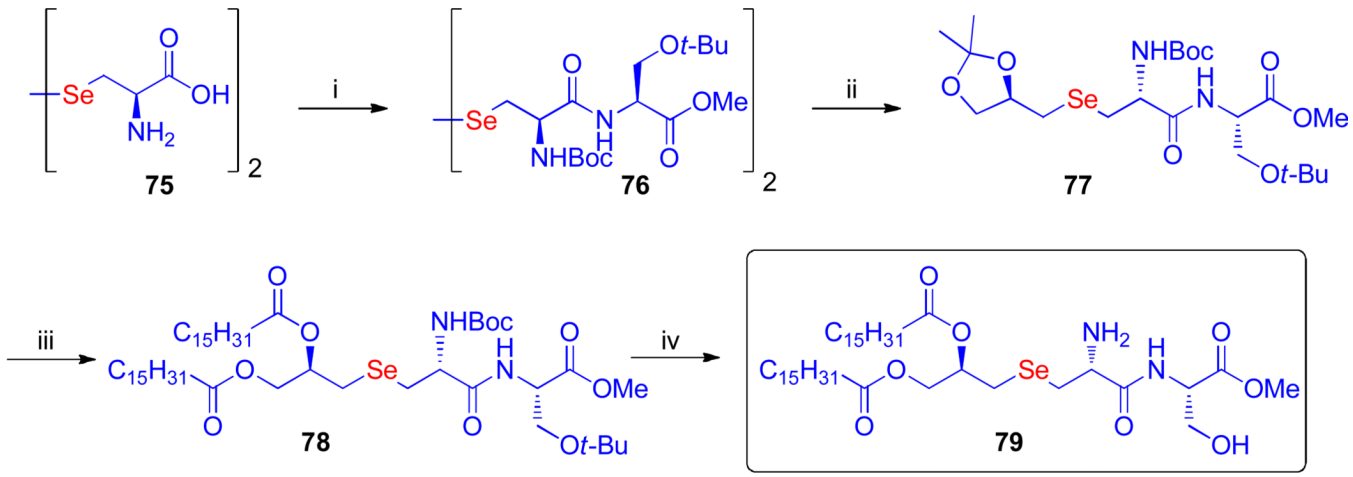

Reagents: i. (a) Boc_2O, TEA, H_2O (b) H-Ser(*t*Bu)-OMe · HCl , EDCI, HOBt, TEA, DMF; ii. (**54**), $NaBH_4$, EtOH; iii. (a) 70% CH_3COOH (b) $C_{15}H_{31}COCl$, TEA, DMAP, CH_2Cl_2; iv. CF_3COOH.

Scheme- 7.

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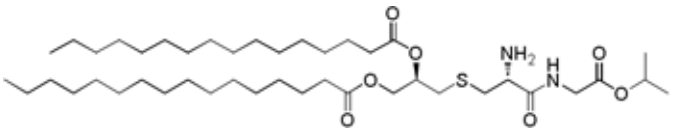
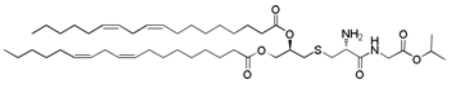
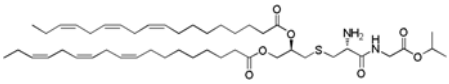
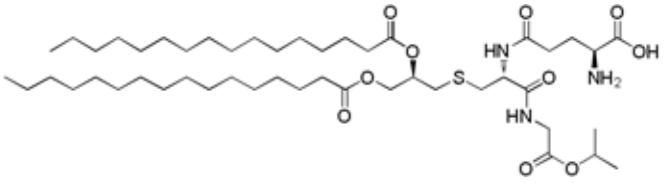
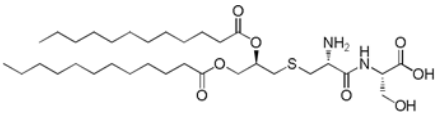
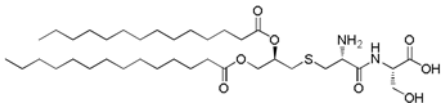
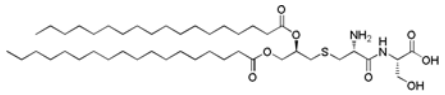
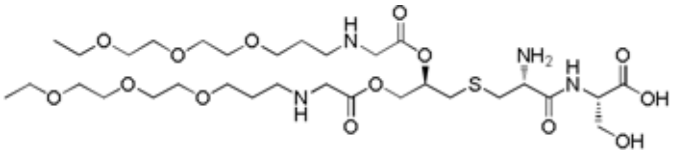
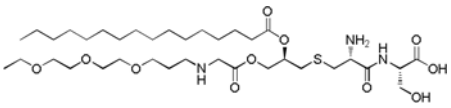
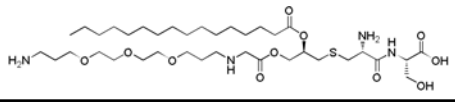
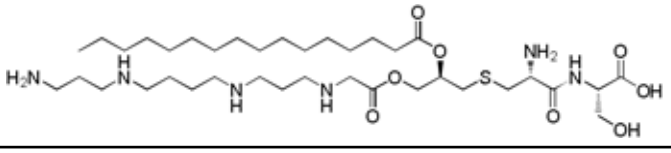
Table 1

Cmpd	Structure	EC_{50} (nM)
7		0.45
8		1.0
9		0.87
11		>1000
17		6.48
18		0.56
19		1.47
24		>1000
31		2.11
32		1.55
33		3.23

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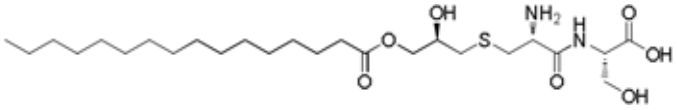
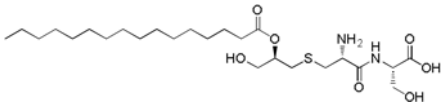
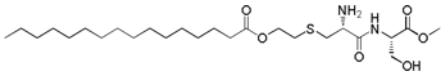
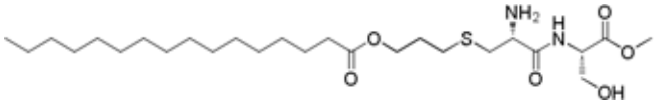
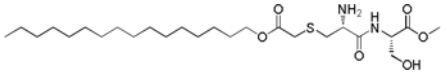
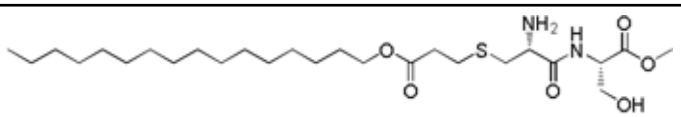
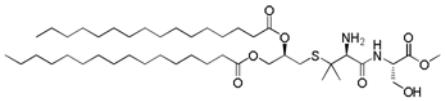
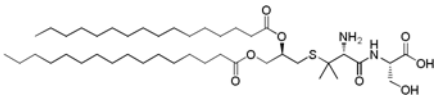
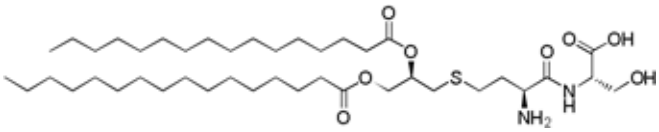
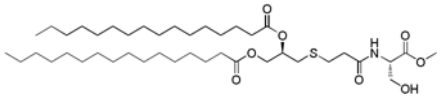
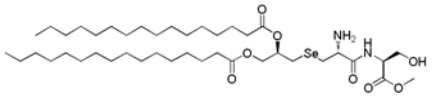
Cmpd	Structure	EC_{50} (nM)
35		1.95
38		1.86
44		4.44
47		>1000
50		>1000
53		>1000
59		>1000
64		>1000
69		>1000
74		>1000
79		0.26