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Toll-like receptor 2 antagonists. Part 1: Preliminary SAR investigation of novel synthetic phospholipids

Mark R. Spyvee,[a,*] Huiming Zhang,[a] Lynn D. Hawkins[a] and Jesse C. Chow[b]

[a]*Lead Optimization Department, Eisai Research Institute, 100 Research Drive, Wilmington, MA 01887, USA*
[b]*Target Identification and Assay Development Department, Eisai Research Institute, 4 Corporate Drive, Andover, MA 01810, USA*

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Abstract—Novel synthetic phospholipid compound **1** was discovered to be an antagonist of human toll-like receptor 2 (TLR2) signaling. In a preliminary SAR campaign we synthesized several analogues of **1** and found that considerable structural changes could be made without loss of TLR2 antagonistic activity.

The innate immune system is the first line of defense in humans and protects the body against foreign microorganisms, such as bacteria, fungi, viruses, mycoplasma, and protozoae, resulting in the generation of a multitude of inflammatory responses in the host. A family of innate immune proteins named the toll-like receptors (TLRs) can detect cellular components of invading pathogens and elicit cellular signaling events that lead to the elevation of proinflammatory mediators, including IL-1, IL-6, IL-8, and TNFα.[1] For example, TLR2 recognizes lipoproteins, peptidoglycan, lipoteichoic acid, and zymosan, as well as lipopolysaccharide (LPS) of Gram-positive bacteria; TLR3 recognizes dsRNA of viruses; TLR4 recognizes LPS of Gram-negative bacteria; TLR5 recognizes flagellin of motile bacteria; TLR7 and TLR8 recognize ssRNA; and TLR9 recognizes DNA rich in CpG motifs.[1,2] Certain TLRs can also heterodimerize with one another to provide additional selectively for ligand recognition; for example, TLR2 cooperates with TLR1 to recognize Pam_3CysSK_4 (**2**), or with TLR6 to recognize lipopeptide (*R*)-MALP-2.[1] Hyperstimulation of TLR2-expressing immune cells by microbial products can contribute to pathogen-induced chronic inflammatory joint disease, Gram-positive sepsis, and other inflammatory disorders.[3,4]

In light of the connection between TLR2 and a wide range of inflammatory disorders, we decided to embark upon a high throughput screening (HTS) campaign to identify inhibitors of TLR2 signaling. At the onset of our investigations there were, to our knowledge, no reported small molecule TLR2 antagonists. Our HTS assay system involved measurement of the inhibition of stimulation of TLR2-transfected Hek293 cells, carrying an NF-kB reporter gene, with synthetic Pam_3CysSK_4 (**2**) (Fig. 1).[5] Our HTS effort culminated in the discovery of phospholipid compound **1** (Fig. 2).[6] Lead compound **1** had previously been synthesized on an earlier in-house program investigating TLR4 activity. It interested us that our newly discovered TLR2 antagonist bore structural similarity to the TLR2 agonist ligand **2** (i.e., a lipophilic component, comprising three hydrocarbon chains, attached through a linker to a lipophobic component). Owing to the lability of the malonate stereocenter, **1** was prepared and tested as a ca. 1:1 mixture of diastereomers. In our early investigations, we were eager to test the stereochemical significance of the two non-epimerizable asymmetric centers of **1**. Consequently, we synthesized

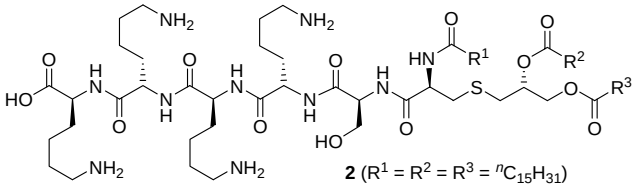

Figure 1. Pam_3CysSK_4, **2**, a highly potent TLR2 agonist.

Keywords: Toll-like receptor; TLR2; TLR2 antagonist; Synthetic phospholipid.
* Corresponding author. Tel.: +1 978 988 7088; fax: +1 978 794 1117; e-mail: mark_spyvee@eisai.com

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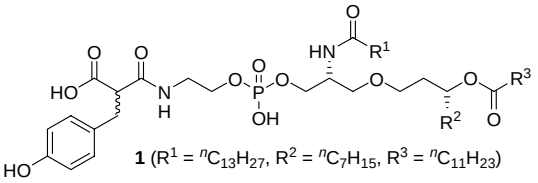
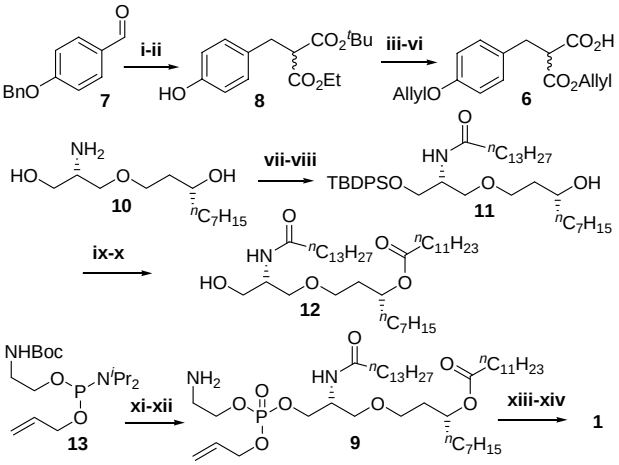


Figure 2. Compound **1**, a novel TLR2 antagonist.

diastereomers **3**–**5** (Table 1). We found that the TLR2 antagonistic potency did not vary greatly between diastereomers, the most potent being **1**, with centers **b** and **c** both having the (*R*)-configuration. The potential stereochemical consequences to TLR4 activity were also of interest to us so the diastereomers were tested in an appropriate assay.[7] In this case, we observed a modest stereochemical significance with respect to TLR4 antagonistic potency, with the (*S*,*S*)-diastereomer being the most potent. It was also noted that without exception these compounds exhibited greater selectivity for TLR4 over TLR2. In addition to demonstrating antagonism in Hek293 cells (Table 1), compound **1** also showed antagonistic activity in human peripheral blood mononuclear cells (PBMCs).[8,9]

For the synthesis of **1**, the requisite left-hand side fragment **6** was prepared via a six-step sequence from commerically available 4-benzyloxybenzaldehyde **7** (Scheme 1). This involved a Knoevenagel condensation[10] of **7** with ethyl *tert*-butyl malonate followed by catalytic hydrogenation to give phenol intermediate **8**. Etherification of **8** with allyl bromide followed by saponification of the ethyl ester, subsequent re-esterification with allyl alcohol, and acid-promoted hydrolysis of the *tert*-butyl ester gave intermediate **6**. The right-hand fragment **9** was derived from amino diol **10**[11] over a separate six-step sequence. This involved myristoylation of **10** with subsequent selective mono-silyl protection of the primary hydroxyl to give **11**. The remaining secondary hydroxyl was then lauryolated and the silyl-protecting group removed to give intermediate **12**. Reaction of **12** with phosphorylating reagent **13**,[11] followed by oxidative workup and subsequent Boc removal, afforded



Scheme 1. Synthesis of the lead TLR2 antagonist **1**. Reagents and conditions: (i) $CH_2(CO_2Et)CO_2{}^tBu$, piperidine, AcOH, PhH, reflux/Dean and Stark; (ii) H_2, Pd/C, MeOH (83% over two steps); (iii) $CH_2{=}CHCH_2Br$, K_2CO_3, AcMe (77%); (iv) LiOH, THF, H_2O (65%); (v) $CH_2{=}CHCH_2Br$, K_2CO_3, $^nBu_4NI$, DMF (99%); (vi) TFA, Et_3SiH, DCM (96%); (vii) $^nC_{13}H_{27}COCl$, $NaHCO_3$, THF (78%); (viii) TBDPSCl, DMAP, NEt_3, DCM (67%); (ix) $^nC_{11}H_{23}CO_2H$, EDCI, DMAP, DCM (80%); (x) TBAF, AcOH, THF (77%); (xi) **12**, pyridinium trifluoroacetate, DCM, −10 °C then 30% H_2O_2 (64%); (xii) TFA, Et_3SiH, DCM then $NaHCO_3$; (xiii) **6**, EDCI, NEt_3, DCM (41% over two steps); (xiv) $Pd(PPh_3)_4$, Ph_3SiH, PPh_3, THF (69%).

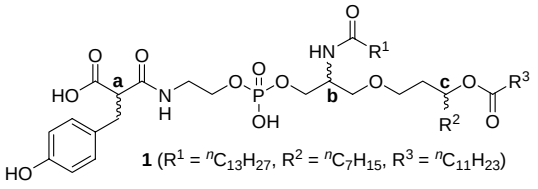
advanced intermediate **9**. Carbodiimide-activated coupling of the left fragment **6** with the right fragment **9**, followed by deallylation, gave final product **1**.

For the synthesis of diastereomer **3**, the starting alcohol **11** was first subjected to a Mitsunobu[12] procedure (Scheme 2). The resultant inverted alcohol intermediate was lauroylated and subsequently desilylated to give alcohol **14**. An identical sequence of steps to that used for **1** was then employed to complete the synthesis of **3** via intermediate **15**.

Diastereomers **4** and **5** were synthesized in analogous fashion to **3** starting from intermediates **16**[11] and **17**,[11] respectively (Schemes 3 and 4).

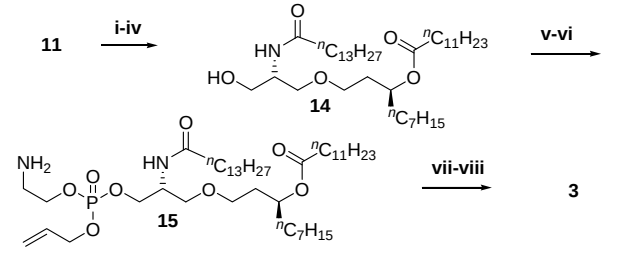
The next phase of our SAR investigations involved examination of the effects of replacing various linkage

Table 1. Diastereomers of **1**

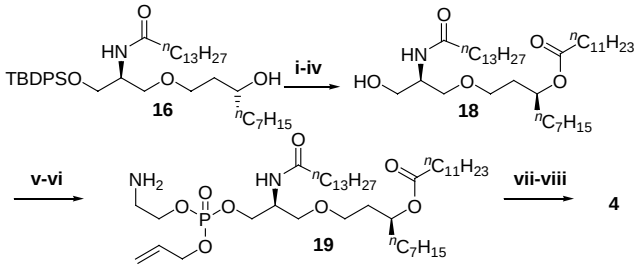


Compound	a	b	c	TLR2 inhibition IC_{50}, μM[a]	TLR4 inhibition IC_{50}, μM[a]
1	*R*/*S*	*R*	*R*	3.07	0.35
3	*R*/*S*	*R*	*S*	6.46	0.16
4	*R*/*S*	*S*	*S*	6.90	0.08
5	*R*/*S*	*S*	*R*	22.00[b]	0.25[b]

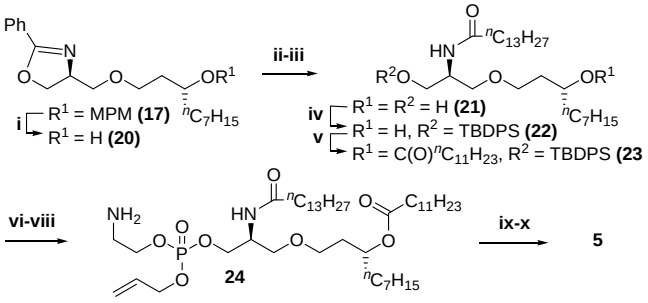
[a] Values are means of at least two experiments.
[b] Value not reliable owing to partial insolubility under assay conditions.



Scheme 2. Synthesis of the (*R*/*S*,*R*,*S*)-diastereomer **3**. Reagents and conditions: (i) DEAD, PPh_3, 4-$NO_2(C_6H_4)CO_2H$, THF (91%); (ii) NaOMe, MeOH, THF (98%); (iii) $^nC_{11}H_{23}CO_2H$, EDCI, DMAP, DCM (96%); (iv) TBAF, AcOH, THF (98%); (v) **13**, pyridinium trifluoroacetate, DCM, MeCN, −10 °C then 30% H_2O_2; (vi) TFA, Et_3SiH, DCM then $NaHCO_3$; (vii) **6**, EDCI, NEt_3, DCM (20% over three steps); (viii) $Pd(PPh_3)4$, Ph_3SiH, PPh_3, THF (40%).



Scheme 3. Synthesis of the (*R*/*S*,*S*,*S*)-diastereomer **4**. Reagents and conditions: (i) DEAD, PPh_3, 4-NO_2(C6H4)CO_2H, THF (77%); (ii) K_2CO_3, MeOH, THF (98%); (iii) $^nC_{11}H_{23}CO_2H$, EDCI, DMAP, DCM (98%); (iv) TBAF, AcOH, THF (94%); (v) **13**, HOAc, MeCN, DCM, H_2O_2; (vi) TFA, Et_3SiH, DCM then $NaHCO_3$; (vii) **6**, EDC, DCM (15% over three steps); (viii) Pd(PPh_3)4, Ph_3SiH, PPh_3, THF (39%).



Scheme 4. Synthesis of the (*R*/*S*,*S*,*R*)-diastereomer **5**. Reagents and conditions: (i) CAN, MeCN, H_2O (83%); (ii) 4 M HCl, MeOH, 65 °C then 4 M NaOH, MeOH, 85 °C (98% over two steps); (iii) $^nC_{13}H_{27}COCl$, $NaHCO_3$, THF; (iv) TBDPSCl, Et_3N, DMAP, DCM (87% over two steps); (v) $^nC_{11}H_{23}CO_2H$, EDCI, DMAP, DCM (75%); (vi) TBAF, AcOH, THF (98%); (vii) **13**, HOAc, MeCN, DCM then H_2O_2; (viii) TFA, Et_3SiH, DCM then $NaHCO_3$; (ix) **6**, EDC, DCM (23% over three steps); (x) Pd(PPh_3)$_4$, Ph_3SiH, PPh_3, THF (40%).

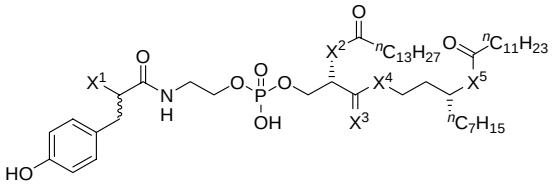
functionalities. Five simple analogues were synthesized (Table 2). It was found that removal of the X^1 carboxyl group was not detrimental to TLR2 antagonistic potency, neither was exchanging the X^2 NH for O (compounds **25** and **26**). Exchanging the X^5 O for NH did appear to reduce potency, though only slightly (compound **27**). More interestingly, analogues **28** and **29** showed that when the X^3/X^4 functionality was changed from an ether to either an amide or an ester this gave moderate improvements in potency. The synthetic routes to the aforementioned linkage analogues are as described below.

Synthesis of des-carboxy analogue **25** was carried out according to the route outlined in Scheme 5. Phenol **30** was subjected to silylation, carbonyl reduction, and subsequent bromination. The intermediate bromide was then displaced with sodio diethylmalonate, desilylated with TBAF, etherified under Mitsunobu conditions,[12] and then saponified to give malonic acid derivative **31**. Copper-catalyzed decarboxylation of **31** gave propionic acid derivative **32**, which was taken through a sequence similar to that described earlier to give **25**.

Glycerol analogue **26** was synthesized according to the route outlined in Scheme 6. Diol **33**[11] was first tosylated to give **34**. The sodium salt of commercially available (*S*)-2,2-dimethyl-1,3-dioxolane-4-methanol was then used to displace the tosyl group of **34**. Lauroylation followed by acidic hydrolysis of the dioxolane gave **35**. Selective mono-silylation of **35** yielded alcohol intermediate **36**. Conversion of **36** into the glycol analogue **26** was achieved using an identical sequence of steps to that described earlier.

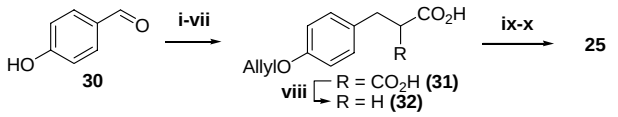
The synthesis of **27** started with a six-step conversion of diol **33**[11] into azido alcohol **38** (Scheme 7). This sequence involved selective mono-silylation, Mitsunobu inversion,[12] mesylation, followed by azide displacement. The hydroxyl of **38** was then mesylated and displaced with (*R*)-serinol benzimide acetal[13] under basic conditions to give intermediate **39**. Hydrolysis of the benzimidine of **39**, myristoylation of the resultant free amino group, and subsequent oxidative phosphorylation with **13** gave **40**. Staudinger reduction[14] of **40** followed by lauroylation, Boc removal, coupling with **6**, and subsequent treatment with palladium(0) gave **27**.

Table 2. Biological data for linkage analogues

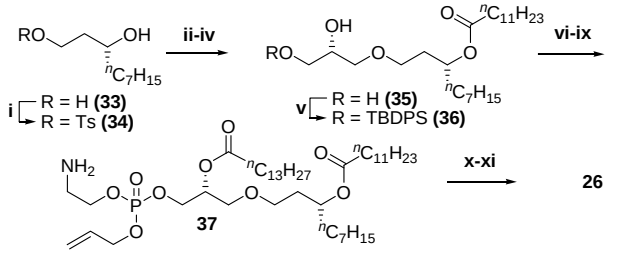


Compound	X^1	X^2	X^3	X^4	X^5	TLR2 inhibition IC_{50}, μM[a]
1	CO_2H	NH	H_2	O	O	3.07
25	H	NH	H_2	O	O	2.15
26	CO_2H	O	H_2	O	O	1.25
27	CO_2H	NH	H_2	O	NH	5.20
28	CO_2H	NH	O	O	O	0.95
29	CO_2H	NH	O	NH	O	0.90

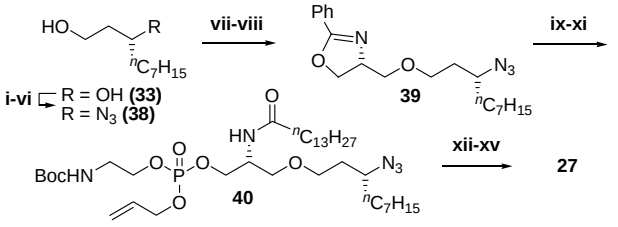
[a] Values are means of at least two experiments.



Scheme 5. Synthesis of the des-carboxy analogue **25**. (i) TBDPSCl, imidazole, DMF (78%); (ii) $NaBH_4$, AcOH, EtOH (83%); (iii) NBS, PPh_3, DCM, 0 °C (58%); (iv) $NaCH(CO_2Et)_2$, DMF, THF; (v) TBAF, THF (93%); (vi) $CH_2{=}CHCH_2OH$, DEAD, PPh_3, THF, 0 °C (84%); (vii) LiOH, H_2O, THF, 70 °C, 2 h (90%); (viii) Cu_2O, MeCN, 105 °C (94%); (ix) **9**, EDCI, DMF, NEt_3; (x) $Pd(PPh_3)_4$, Ph_3SiH, PPh_3, THF (55% over two steps).

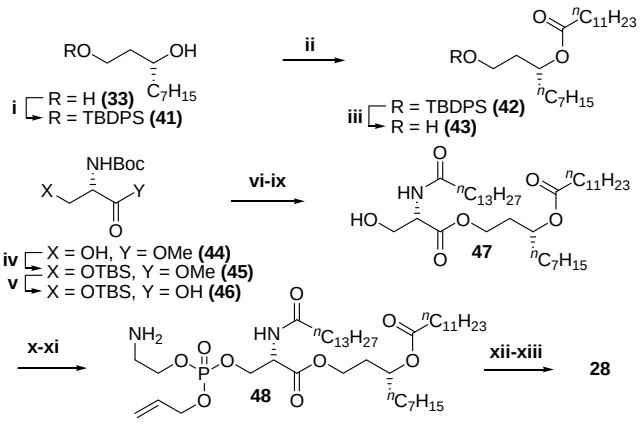


Scheme 6. Synthesis of the glycol analogue **26**. Reagents and conditions: (i) TsCl, pyridine, DCM; (ii) (*S*)-2,2-dimethyl-1,3-dioxolane-4-methanol, NaH, DMF, THF (60% over two steps); (iii) $^nC_{11}H_{23}CO_2H$, EDCI, DMAP, DCM; (iv) AcOH, H_2O (74% over two steps); (v) TBDPSCl, NEt_3, DMAP, DCM (87%); (vi) $^nC13H_{27}CO_2H$, EDCI, DMAP, DCM (38%); (vii) HF, MeCN (70%); (viii) **13**, 1H-tetrazole, DCM, 0 °C then oxone, H_2O (88%); (ix) TFA, Et_3SiH, DCM then $NaHCO_3$; (x) **6**, EDCI, DMF, NEt_3 (79% over two steps); (xi) $Pd(PPh_3)_4$, Ph_3SiH, PPh_3, THF (83%).



Scheme 7. Synthesis of the tail amide analogue **27**. Reagents and conditions: (i) TBDPSCl, Et_3N, DMAP, DCM; (ii) DEAD, PPh_3, 4-$NO_2(C_6H_4)CO_2H$, PPh_3, THF, 0 °C (79%); (iii) K_2CO_3, MeOH, THF (77%); (iv) MsCl, Et_3N, DMAP, DCM, 0 °C; (v) NaN_3, DMF, 140 °C (70%); (vi) TBAF, THF (99%); (vii) MsCl, Et_3N, DMAP, DCM, 0 °C (90%); (viii) (*R*)-serinol benzimidine acetal, KO^{t}Bu, THF, 0 °C (46%); (ix) HCl, H_2O, MeOH, 90 °C; (x) $^nC_{13}H_{27}COCl$, $NaHCO_3$, THF, 0 °C (76%); (xi) **13**, pyridinium trifluoroacetate, DCM, −10 °C then 30% H_2O_2 (46%); (xii) PPh_3, H_2O, THF; (xiii) $^nC_{11}H_{23}CO_2H$, EDCI, HOBt, DCM (39%); (xiv) TFA, Et_3SiH, DCM then $NaHCO_3$; (xv) **6**, EDCI, NEt_3, DMF (69% over two steps); (xvi) $Pd(PPh_3)_4$, Ph_3SiH, PPh_3, THF (71%).

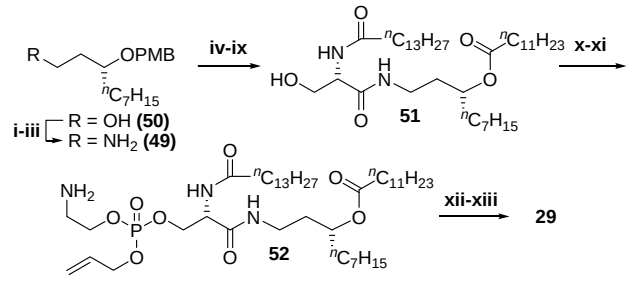
Ester analogue **28** was synthesized according to the route outlined in Scheme 8. The first step was a selective silylation of the primary hydroxyl of diol **33**[11] to give **41**. Lauroylation of the secondary hydroxyl of **41** yielded **42**, which was subsequently desilylated to give alcohol **43**. Silylation of *N*-Boc (*S*)-serine methyl ester **44** gave **45**, which was saponified to give acid intermediate **46**. Esterification of the acid fragment **46** with alcohol **43**, followed by Boc removal, and subsequent



Scheme 8. Synthesis of the ester analogue **28**. Reagents and conditions: (i) TBDPSCl, $^iPr_2NEt$, DMAP, DCM (90%); (ii) $^nC_{11}H_{23}CO_2H$, EDCI, DMAP, DCM (95%); (iii) TBAF, AcOH, THF (82%); (iv) TBSCl, imidazole, DMF (69%); (v) LiOH, H_2O, THF (83%); (vi) **43**, EDCI, DMAP, DCM (58%); (vii) TFA, Et_3SiH, DCM then $NaHCO_3$; (viii) $^nC_{13}H_{27}COCl$, $NaHCO_3$, THF, 0 °C (73% over two steps); (ix) TBAF, AcOH, THF (43%); (x) **13**, 1H-tetrazole, DCM, 0 °C then oxone, H_2O (40%); (xi) TFA, Et_3SiH, DCM then $NaHCO_3$; (xii) **6**, HBTU, $^iPr_2NEt$, NMP (31% over two steps); (xiii) $Pd(PPh_3)_4$, Ph_3SiH, PPh_3, THF (60%).

myristoylation and desilylation, gave intermediate **47**. Oxidative phosphorylation of **47** with **13**, followed by Boc removal, gave advanced intermediate **48**. Amine **48** was then converted into ester analogue **28** via the usual procedure described earlier.

Amide analogue **29** was synthesized according to the route outlined in Scheme 9. Requisite amine **49** was constructed from mono-protected diol **50** via mesylation, azidation, and subsequent Staudinger reduction.[14] Coupling of amine **49** with acid **46**, followed by oxidative removal of the PMB protecting group, lauroylation, Boc removal, myristoylation, and desilylation, gave **51**. Phosphorylation of **51** with **13** with oxidative workup, followed by treatment with TFA, gave advanced amine intermediate **52**. Coupling of



Scheme 9. Synthesis of core amide analogue **29**. Reagents and conditions: (i) MsCl, Et_3N, DMAP, DCM, 0 °C (74%); (ii) NaN_3, DMF, 140 °C (95%); (iii) PPh_3, H_2O, THF; (iv) **46**, EDCI, HOBt, DCM (75% over two steps); (v) DDQ, DCM, H_2O; (vi) $^nC_{11}H_{23}CO_2H$, EDCI, DMAP, DCM; (vii) TFA, Et_3SiH, DCM then $NaHCO_3$; (viii) $^nC_{13}H_{27}COCl$, $NaHCO_3$, H_2O, DCM, 0 °C (69% over four steps); (ix) TBAF, THF; (x) **13**, 1H-tetrazole, DCM, 0 °C then oxone, H_2O (60% over two steps); (xi) TFA, Et_3SiH, DCM then $NaHCO_3$ (98%); (xii) **6**, EDCI, HOBt, $^iPr_2NEt$, DCM (50%); (xiii) $Pd(PPh_3)_4$, Ph_3SiH, PPh_3, THF (50%).

52 with **6**, followed by palladium-catalyzed deallylation, afforded **29**.

In conclusion, from an HTS campaign of ca. one hundred thousand compounds we discovered a novel phospholipid compound **1** as an antagonist of TLR2 signaling. We engaged in preliminary SAR investigations demonstrating that significant structural changes could be made without loss of activity. The finding that amide analogue **29** possessed optimal potency was particularly beneficial because this would pave the way for subsequent analogues that would be more readily accessible. Continuation of our SAR investigations will be reported in due course.

Acknowledgments

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5. Hek293 cells, stably transfected with human TLR2 and an NF-kB reporter gene, were incubated in 10% fetal bovine serum with test compounds for 30 min prior to stimulation with Pam_3CysSK_4 (0.2 ng/ml) for 18 h. Prior to assaying compounds for inhibitory activity, agonism dose–response curves had been generated to establish the EC_{50} of 0.2 ng/ml for Pam_3CysSK_4.
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7. Hek293 cells, stably transfected with human TLR4, MD-2, and an NF-kB reporter gene, were incubated in 10% fetal bovine serum with test compounds for 30 min prior to stimulation with LPS (10 ng/ml) plus soluble CD14 (10 nM) for 18 h. Prior to assaying compounds for inhibitory activity, agonism dose–response curves had been generated to establish the EC_{50} of 10 ng/ml for LPS.
8. PBMCs were purified from heparinized blood of healthy human donors as previously described.[9] Cells were seeded in 96-well plates at a density of 50,000 cells per well. Pam_3CysSK_4 (at its EC_{50} of 50 ng/ml) and compound **1** (at 30, 10, 3, 1, 0.3, 0.1, and 0.03 μM) were added and incubated for 6 h at 37 °C. Culture supernatants were obtained by centrifuging for 5 min at 12,000 rpm and were stored at −20 °C until assayed for TNFα by ELISA. Compound **1** demonstrated an IC_{50} of 1.6 μM (mean value from three donors).
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