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The 'Ethereal' nature of TLR4 agonism and antagonism in the AGP class of lipid A mimetics

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ABSTRACT

To overcome the chemical and metabolic instability of the secondary fatty acyl residues in the AGP class of lipid A mimetics, the secondary ether lipid analogs of the potent TLR4 agonist CRX-527 (**2**) and TLR4 antagonist CRX-526 (**3**) were synthesized and evaluated along with their ester counterparts for agonist/antagonist activity in both in vitro and in vivo models. Like CRX-527, the secondary ether lipid **4** showed potent agonist activity in both murine and human models. Ether lipid **5**, on the other hand, showed potent TLR4 antagonist activity similar to CRX-526 in human cell assays, but did not display any antagonist activity in murine models and, in fact, was weakly agonistic. Glycolipids **2**, **4**, and **5** were synthesized via a new highly convergent method utilizing a common advanced intermediate strategy. A new method for preparing (*R*)-3-alkyloxytetradecanoic acids, a key component of ether lipids **4** and **5**, is also described.

The discovery of toll-like receptors (TLRs) on cells of the immune system together with the identification of natural TLR ligands has led to a great deal of interest in developing synthetic TLR agonists and antagonists to manipulate innate and adaptive immune responses.[1–3] Targeting TLR receptors and cognate intracellular pathways could potentially lead to more effective vaccines and novel therapeutic approaches for the treatment of immune and inflammatory diseases. For example, certain variants of lipopolysaccharide (LPS), the main cell-surface component of Gram-negative bacteria, are potent stimulators of host defense systems via their interaction with TLR4 and accessory molecules such as MD-2, but the pathophysiology of LPS and its active principle, lipid A (**1**, Fig. 1), have precluded their medicinal use.[4] Thus, considerable effort has been directed towards the development of synthetic lipid A mimetics with simplified structures and improved toxicity/activity profiles for use as vaccine adjuvants and stand-alone immunotherapeutics.[5]

In the course of our own structure–activity studies on lipid A,[6,7] we identified a new class of potent monosaccharide immunomodulators known as aminoalkyl glucosaminide 4-phosphates (AGPs), in which the less-conserved reducing sugar unit of lipid A is substituted with a flexible *N*-acyloxyacyl aglycon unit (e.g., **2** and **3**, Fig. 1).[7] The flexible AGP motif likely permits energetically favored close packing[8] of fatty acid moieties, facilitating intercalation of the lipids into the hydrophobic pocket of the TLR4 accessory molecule MD-2. Further, the carboxyl group of seryl-based AGPs serves as a stable bioisostere of the labile anomeric phosphate of lipid A, and—along with the sugar 4-phosphate—presumably binds electrostatically to lysines 128 and 132 along the edge of the hydrophobic pocket of MD-2.[9] Crystal structures of disaccharide lipid A antagonists bound to human MD-2 or the TLR4–MD-2 complex show that these and other positively charged amino acids at the edge of the hydrophobic pocket interact electrostatically with the phosphate groups and that the pocket has evolved to accommodate large and structurally diverse fatty acyl moieties.[10,11] Both the spacial arrangement of the acyl moieties and distance between anionic groups appear to be critical determinants for binding of TLR4 agonists and antagonists to MD-2.[12] Secondary fatty acid chain length in the AGP series, on the other hand, appears to determine the ability of the putative[9] AGP:MD-2 complexes to subsequently activate TLR4 and trigger oligomerization of TLR4 molecules.[12,13] Oligomerization—aggregation of TLR4 molecules into lipid rafts—is thought to be prerequisite to signal transduction and the release of pro-inflammatory cytokines and chemokines associated with the innate immune response.[14]

Among seryl-based AGPs, CRX-527 (**2**) containing 10-carbon secondary acyl residues and CRX-526 (**3**) possessing 6-carbon acyl groups have been shown to exhibit potent TLR4 agonist and antagonist activity, respectively, in both murine and human mod-

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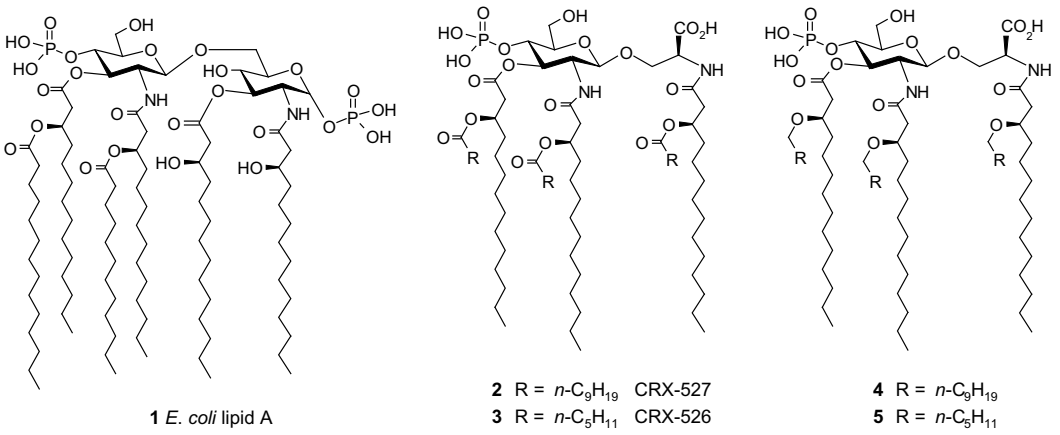


Figure 1. Structures of *Escherichia coli* lipid A (**1**) and AGP lipid A mimetics **2**–**5**.

els.[12,13,15] For example, the anti-infective effects of CRX-527 were demonstrated recently in a preclinical model for respiratory syncytial virus (RSV), a significant pathogen in humans—particularly infants, immunocompromised patients, and the elderly.[16] In contrast, CRX-526 was recently shown to inhibit moderate-to-severe disease in murine models of inflammatory bowel disease (IBD).[15] Increased TLR4 expression on the intestinal epithelium of human IBD patients may play a key role in the etiology of this disease.[17]

In an the effort to overcome the inherent chemical and metabolic instability of the ester-linked secondary fatty acids present in lipid A mimetics[18,19] such as CRX-527 (**2**) and CRX-526 (**3**) as well as to further evaluate structural modifications in the AGP series, the corresponding ether lipid analogs **4** and **5** (Fig. 1) were synthesized and evaluated for agonist/antagonist activity in both in vitro and in vivo models.

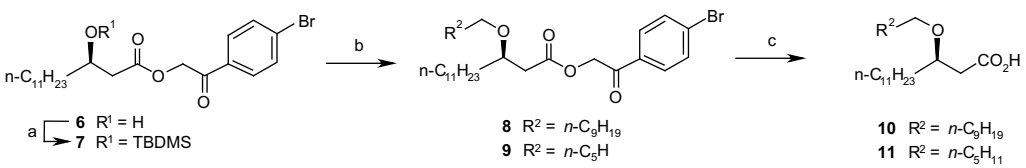
Earlier, we described the synthesis of CRX-526 and CRX-527 in 15 steps from β-D-glucosamine pentaacetate,[7] but the synthesis was low yielding and suffered from a number of drawbacks. Most notably, incorporation of two of the three (*R*)-3-alkanoyloxytetradecanoyl residues onto the AGP scaffold early in the synthesis led to multiple synthetic and chromatographic steps and precluded the use of a common advanced intermediate (CAI) for the synthesis of AGPs comprising the same sugar and aglycon units but different fatty acyl substituents.

Herein, we describe a new, efficient synthesis of CRX-527 (**2**) as well as ether lipid analogs **4** and **5** in 11 linear steps from readily available 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranoside (**12**)[20] and benzyl *N*-(2,2,2-trichloroethoxycarbonyl)-L-serine (**13**).[21] This new synthesis, which is convergent with respect to the AGP backbone and the fatty acid moieties, utilizes a CAI strategy to introduce the fatty acyl groups near the end of the synthesis. As part of the effort to make the synthesis of ether lipids **4** and **5** more amenable to large scale, a new method for preparing the (*R*)-3-alkyloxytetradecanoic acids **10** and **11** was also developed (Scheme 1).

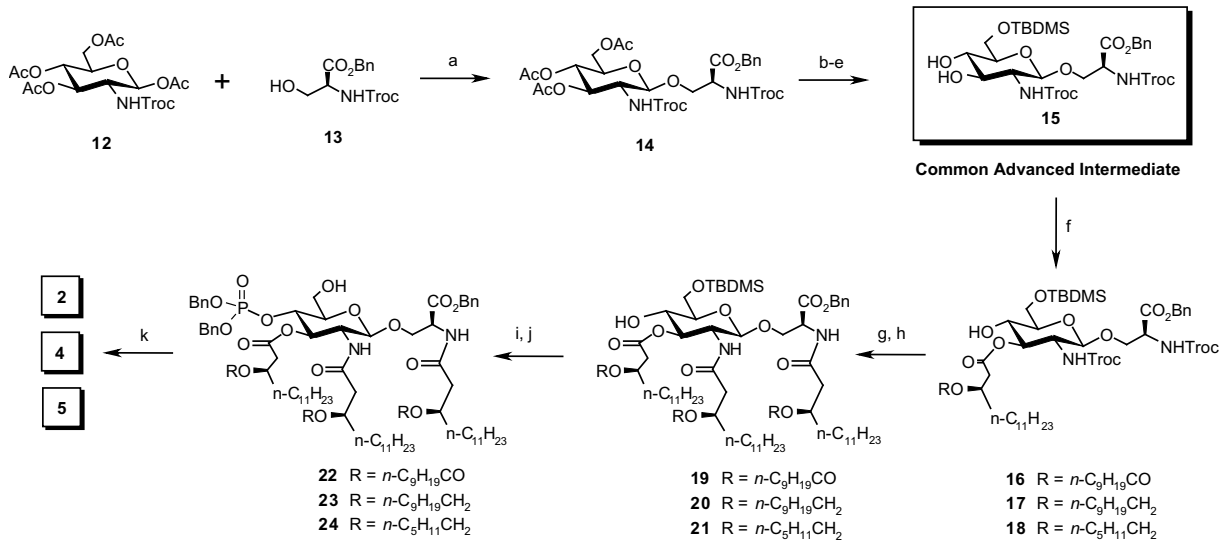
Although several methods have been reported[22–24] for the synthesis of (*R*)-3-alkyloxytetradecanoic acids we found these to be multi-step and/or low yielding. Application of the Bajwa–Komatsu reductive alkylation procedure[25,26] to silyl ester **7** derived from hydroxy ester **6**[27] using decanal or hexanal in the presence of bismuth tribromide and triethylsilane (in situ-formation of bromotriethylsilane)[26] reproducibly gave ether esters **8** and **9** in 70–80% yield on a multi-gram scale along with minor amounts of (separable) hydroxy ester **6**. Base hydrolysis (or reductive deprotection)[27] of the *p*-bromophenacyl esters **8** and **9** then gave the desired acids **10** and **11** in >90% yield.[28]

The target compounds **2**, **4**, and **5** were assembled from the glycosyl donor/acceptor pair **12**/**13** using the *N*-2,2,2-trichloroethoxycarbonyl (Troc) method for stereoselective β-glycosylation in the first step to give β-glycoside **14** in high yield (Scheme 2). To circumvent transesterification during O-deacetylation, the benzyl group of **14** was removed hydrogenolytically prior to base-induced deacetylation and subsequently re-installed under phase-transfer conditions; 6-O-silylation then gave CAI **15** in 59% overall yield from **12**. Selective carbodiimide-mediated 3-O-acylation of **15** with (*R*)-3-decanoyloxytetradecanoic acid,[27] **10** or **11**, followed by reductive removal of the *N*-Troc groups and N,N-diacylation with the appropriate fatty acid, provided triacyl derivatives **19**–**21**. Compounds **19**–**21** could also be prepared directly from **15** via Troc-deprotection and selective[27] N,N,O-triacylation, but Troc removal with Zn/AcOH typically led to emulsions during aqueous workup (required to remove complexed zinc) and low recoveries of the intermediate diamino diol. Phosphorylation of **19**–**21** and silyl deprotection gave final intermediates **22**–**24**, which were debenzylated by catalytic hydrogenation to afford the desired seryl AGPs **2**, **4**, and **5** in >95% purity after silica gel chromatography and lyophilization of the free acids from aqueous *tert*-butanol containing triethylamine.[29]

CRX-527 (**2**) and its ether analog **4** were compared for their ability to induce cytokine production in human primary monocytic cells as well as their ability to enhance non-specific resistance in mice to *Listeria monocytogenes* and influenza infections. The activities of CRX-527 and ether lipid **4** were similar in these models, as exemplified by the induction of TNF-α in human monocytes (Table 1), the reduction of splenic bacteria following Listeria challenge



Scheme 1. Reagents: (a) TBDMS-Cl, imid, DMAP, CH_2Cl_2, 97%; (b) R^2CHO, $BiBr_3$, Et_3SiH, CH_3CN, 70% (**8**), 82% (**9**); (c) aq LiOH, THF, 90% (**10**), 100% (**11**).



Scheme 2. Reagents and conditions: (a) BF_3–OEt_2 (2 equiv), CH_2Cl_2, 87%; (b) 5% Pd/C, H_2, THF; (c) NH_4OH, MeOH, 76% (2 steps); (d) BnBr, *n*-Bu_4NBr, CH_2Cl_2, aq $NaHCO_3$; (e) TBDMS-Cl, pyr, 89% (2 steps); (f) (*R*)-3-decanoyloxytetradecanoic acid, **10**, or **11**, (1 equiv), EDC–MeI (1 equiv), DMAP (0.03 equiv), CH_2Cl_2, 0 °C → rt, 2 h, 85% (**16**), 83% (**17**), 69% (**18**); (g) Zn, AcOH, rt, aq THF; (h) (*R*)-3-decanoyloxytetradecanoic acid, **10**, or **11** (2 equiv), EDC–MeI (2 equiv), DMAP (0.03 equiv), CH_2Cl_2, rt, 66% (**19**), 42% (**20**), 48% (**21**) (2 steps); (i) i—$(BnO)_2PN$*i*-Pr_2 (1.4 equiv), 4,5-dicyanoimidazole (1.4 equiv), CH_2Cl_2, rt; ii—*m*-chloroperbenzoic acid (3 equiv), 0 °C; (j) TFA, CH_2Cl_2, rt, 71% (**23**), 59% (**24**), 85% (**25**) (2 steps); (k) 5% $Pd(OH)_2$/C, H_2, THF, 65% (**2**), 85% (**4**), 80% (**5**).

Table 1
Comparison of the TLR4 agonist activities of CRX-527 (**2**) and ether lipid **4** and the antagonist activities of CRX-526 (**3**) and **5** in primary human monocytes

Compound	TNF-α induction in monocytes[a] ED_{50}, nM (95% CI)	TNF-α inhibition in monocytes[b] IC_{50}, nM (95% CI)
2	0.39 (0.11–0.89)	—
4	0.17 (0.07–0.41)	—
3	—	2.40 (1.34–6.13)
5	—	5.30 (2.87–13.46)

[a] Human PBMCs were isolated from whole blood by ficoll-hypaque (Sigma Histopaque™-1077) density gradient. Monocytes were isolated by 2 h adherence to tissue culture treated plated and stimulated with 0–10,000 nM of compound in 0.2% aq triethanolamine (TEOA) for 18 h. Supernatants were analyzed for TNF-α induction by sandwich ELISA. The results are shown from three replicate samples of one of three donors tested with similar results. ED_{50} values were calculated by fitting data to a four parameter logistic, dose–response model in XL*fit*™ (IBDS).

[b] Monocytes were incubated with compound for 30 min and then stimulated with 100 ng/mL *E. coli* LPS. TNF-α levels were determined in supernatants taken after 18 h by ELISA and IC_{50} values were determined as for ED_{50} values above.

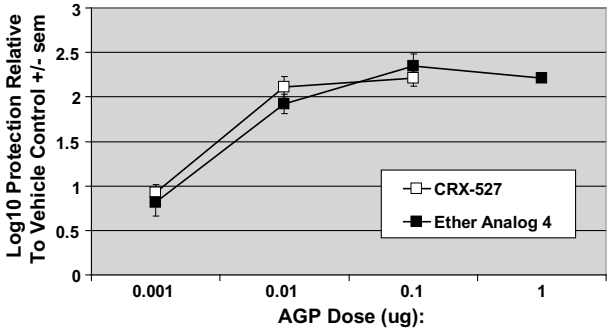


Figure 2. Potency comparison of CRX-527 to ether analog **4** in murine intravenous *L. monocytogenes* protection model. BALB/c mice received AGP in 100 μL of 0.2% aq TEOA by iv administration 2 days prior to iv challenge with 10^5 *L. monocytogenes*; CFUs were determined 2 days later by serial dilutions of splenic homogenates. $\log_{10}$ relative protection was calculated by subtracting CFUs/spleen ($\log_{10}$ value) in test group from CFUs/spleen ($\log_{10}$ value) in control group.

(Fig. 2), and survival to lethal influenza challenge (Table 2). Notably, intranasal administration of 5 μg of CRX-527 or **4** two days prior to lethal influenza challenge provided 100% protection in each case, and significant protection at lower doses.

In contrast to CRX-527 (**2**) and its ether analog **4**, neither CRX-526 (**3**) nor its ether analog **5** induced detectable cytokines in human cell assays (data not shown). However, both of these AGPs equally inhibited the LPS-induced production of TNF-α in human monocytes (Table 1).

TLR4 antagonist CRX-526 (**3**) and its ether lipid analog **5** were also evaluated for their ability to inhibit LPS induction of TNF-α in mice and for their ability to antagonize CRX-527-mediated protection against intravenous *L. monocytogenes* challenge in mice. CRX-526 (**3**) effectively inhibited serum TNF-α production when co-administered intravenously with LPS but **5** did not demonstrate any antagonist activity (Fig. 3).

Similarly, CRX-526, but not its ether analog **5**, antagonized CRX-527-mediated protection against intravenous bacterial challenge in mice in a dose-dependent manner (Fig. 4). A dose–response comparison of ether lipid **5** and CRX-527 in the Listeria protection model showed that **5** was about 100 times less potent (i.e., moderately protective) than CRX-527 in reducing splenic bacteria following Listeria challenge (data not shown). The weak TLR4 agonist activity of ether lipid **5** in mice is further exemplified by its ability

Table 2
Comparison of the protection from influenza challenge by administration of CRX-527 (**2**) or **4**

Compound	Dose[a] (μg)	% Survival[b]
0.2% TEOA	—	0
2	5.0	100
2	0.5	70
2	0.05	70
2	0.005	30
4	5.0	100
4	0.5	80
4	0.05	60
4	0.005	20

[a] BALB/c mice (8/group) received indicated dose of AGP in 20 μL of 0.2% aq TEOA by intranasal (in) administration 2 days prior to in influenza challenge ($2 \times LD_{50}$ infectious influenza A/HK/68).

[b] Survival was recorded for 21 days post-viral challenge.

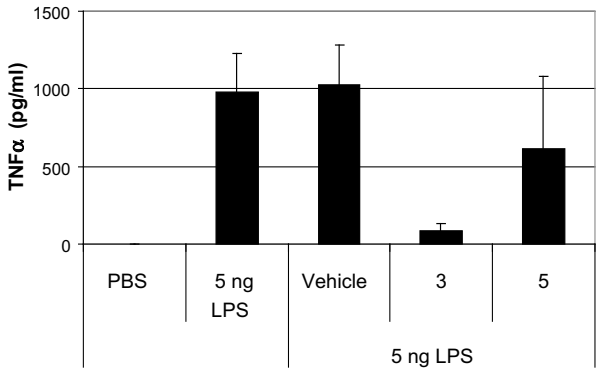


Figure 3. Capacity of CRX-526 (**3**) and ether lipid **5** to inhibit LPS-induced serum TNF-α production in BALB/c mice ($n = 3$) challenged with LPS. BALB/c mice were administered LPS alone or with 0.2% TEOA vehicle, **3** or **5** via iv administration. Serum was collected at 1.5 h post-injection and evaluated for TNF-α by ELISA.

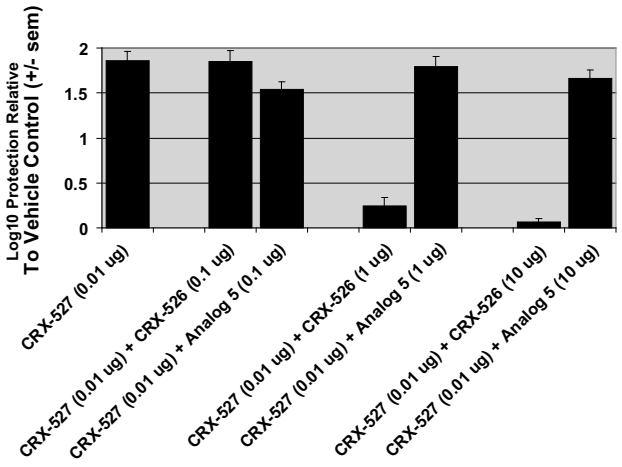


Figure 4. Inhibition of CRX-527-mediated protection against intravenous *L. monocytogenes* challenge in mice by CRX-526 and ether analog **5**. BALB/c mice received AGPs in 100 μL of 0.2% aq TEOA by iv administration 2 days prior to iv challenge with 10^5 *L. monocytogenes*; CFUs were determined 2 days later by serial dilutions of splenic homogenates. $\log_{10}$ relative protection was calculated by subtracting CFUs/spleen ($\log_{10}$ value) in test group from CFUs/spleen ($\log_{10}$ value) in control group.

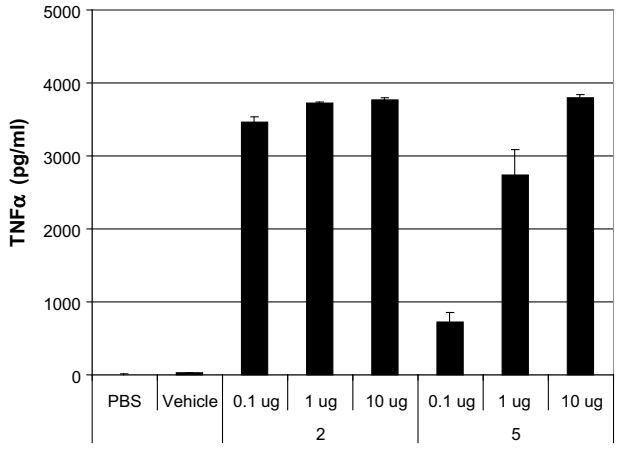


Figure 5. TLR4 agonist activity of CRX-527 (**2**) and **5** in mice. BALB/c mice ($n = 3$) were administered (iv) various amounts of AGP in 0.2% TEOA and serum TNF-α was measured by ELISA 1.5 h post-injection.

to induce TNF-α when administered iv to mice: a dose–response comparison of serum TNF-α levels induced by CRX-527 and ether lipid **5** showed **5** was between 10 and 100 times less active than CRX-527 as a TLR4 agonist in mice (Fig. 5).

The observed species-specific agonist/antagonist activity of ether lipid **5** is reminiscent of what has been reported for lipid IVa, a biosynthetic precursor of lipid A, and is likely attributable to species differences in the structure of MD-2,[30,31] particularly amino acids 57, 61, and 122 located in close proximity to each other along the edge of the hydrophobic pocket.[32] In fact, the co-crystal structure of human MD-2 and lipid IVa shows hydrogen bonding between Lys122 and the 2′-acyl carbonyl oxygen of lipid IVa as well as hydrogen bonds between the nearby Ser120 residue and the 2′-and 1-acyl groups.[10] Thus, it is possible that one or more of the secondary ester carbonyls of CRX-526 (**3**), which are not present in lipid IVa or ether lipid **5**, affect H-bonding of the acyl groups to this domain in murine MD-2 (which possesses Glu122 in place of Lys122 among other substitutions) and prevent conformational changes in MD-2 required for TLR4 activation/oligomerization.

In summary, an improved synthesis of the potent TLR4 agonist CRX-527 (**2**) has been developed in 11 linear steps and 15% overall yield from β-acetate **12** and serine **13**. This synthesis, which employs common advanced intermediate **15**, was also applied to the synthesis of novel ether lipids **4** and **5**. A new method for preparing (*R*)-3-alkyloxytetradecanoic acids, a key component of ether lipids **4** and **5**, was also developed using Bajwa–Komatsu reductive alkylation of silyl ether **7** in the key step.

Ether lipids **4** and **5** exhibited similar TLR4 agonist and antagonist activities, respectively, to their ester analogs **2** and **3** in human cell assays; however, unlike the potent TLR4 antagonist CRX-526, ether lipid **5** was a weak agonist in murine models, suggesting that one or more of the ester carbonyls in CRX-526 plays a pivotal role in binding to murine MD-2 at the entrance to the hydrophobic pocket and preventing TLR4 activation. As with subtle structural changes in MD-2,[11] slight modifications to the TLR4 ligand can also have a significant impact on the activation of the TLR4–MD-2 complex.

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28. *General procedure for the preparation of ether acid **11**:* A solution of **6** (15.0 g) in CH_2Cl_2 (150 mL) was treated with TBDMS-Cl (10.8 g), imidazole (4.86 g), and DMAP (415 mg) and allowed to stir at room temperature for 24 h. Aqueous workup followed by flash chromatography on silica gel afforded 18.6 g (98%) of **7** as an oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.78 (d, J = 8.8 Hz, 2H), 7.63 (d, J = 8.8 Hz, 2H), 5.30 (d, J = 16.4 Hz, 1H), 5.25 (d, J = 16.4 Hz, 1H), 4.18 (m, 1H), 2.63 (d, J = 6.4 Hz), 1.52 (m, 2H), 1.26 (m, 18H), 0.87 (m, 12H), 0.07 (s, 3H), 0.05 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 191.5, 171.4, 133.2, 132.4, 129.5, 129.4, 69.5, 65.9, 42.5, 37.7, 32.2, 29.9, 29.8, 29.6, 26.0, 25.2, 22.9, 18.2, 14.4, −4.3, −4.5. To a solution of **7** (500 mg) in MeCN (1.2 mL) at 0 °C was added Et_3SiH (0.17 mL) followed by $BiBr_3$ (100 mg). The resulting black suspension was treated with hexanal (0.13 mL) and allowed to stir for 10 min. Additional Et_3SiH (0.03 mL), $BiBr_3$ (10 mg) and hexanal (0.03 mL) were then added sequentially and the mixture was stirred for 10 min, filtered, and concentrated. Flash chromatography on silica gel afforded 0.39 gm (82%) of **9** as a colorless oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.78 (d, J = 8.8 Hz, 2H), 7.63 (d, J = 8.8 Hz, 2H), 5.32 (d, J = 16.4 Hz, 1H), 5.25 (d, J = 16.4 Hz, 1H), 3.76 (m, 1H), 3.46 (m, 2H), 2.72 (dd, J = 15.2, 7.2 Hz, 1H), 2.59 (dd, J = 15.2, 5.6 Hz, 1H), 1.50–1.60 (m, 4H), 1.26–1.29 (m, 24H), 0.88 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 191.5, 171.5, 133.2, 132.4, 129.5, 129.3, 76.3, 69.8, 66.0, 39.8, 34.7, 32.2, 31.9, 30.3, 29.9, 29.9, 29.8, 29.6, 26.1, 25.5, 22.9, 22.9, 14.4, 14.3. A solution of **9** (945 mg) in a mixture of THF (45 mL) and 1 M aq LiOH (9 mL) was stirred at room temperature for 1 h. Aqueous workup (EtOAc–10% aq HCl) gave an oily yellow solid which was triturated with hexanes and purified by flash chromatography to provide 590 mg (quant) of **11** as a colorless oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.2 (br, 1H), 3.69 (m, 1H), 3.48 (t, J = 6.4 Hz, 2H), 2.53 (m, 2H), 1.5–1.6 (m, 4H), 1.25–1.29 (m, 24H), 0.88 (t, J = 6.8 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 176.7, 76.3, 69.8, 54.9, 39.6, 34.3, 32.2, 32.1, 31.8, 30.1, 29.9, 29.9, 29.8, 29.6, 29.3, 26.0, 25.4, 22.9, 22.8, 14.4, 14.3; negative ES TOF-MS calcd for $[M-H]^-$ 327.2899, found 327.2892.
29. Spectral data for compounds **4** and **5**. *Compound **4**:* ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.08 (d, J = 6.8 Hz, 1H), 6.80 (br, 1H), 5.25 (t, J = 10.0 Hz, 1H), 4.67 (m, 1H), 4.49 (m, 1H), 4.30 (q, J = 9.6 Hz, 1H), 4.22 (d, J = 10.4 Hz, 1H), 4.00 (m, 1H), 3.85 (m, 1H), 3.71–3.64 (m, 2H), 3.55–3.42 (m, 2H), 3.39–3.29 (m, 2H), 3.06 (q, J = 7.2 Hz, 6H), 2.67 (dd, J = 15.6, 5.6 Hz, 1H), 2.48–2.41 (m, 2H), 2.36 (dd, J = 14.8, 4.8 Hz, 1H), 2.29–2.17 (m, 2H), 1.52–1.42 (m, 12H), 1.37 (t, J = 7.2 Hz, 9H), 1.25–1.20 (m, 96H), 0.84 (t, J = 6.8 Hz, 12H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.9, 171.7, 101.8, 77.5, 76.4, 69.6, 60.6, 53.8, 52.2, 46.7, 46.1, 41.4, 39.2, 34.5, 32.2, 32.2, 30.4, 30.2, 30.2, 30.1, 30.1, 30.0, 30.0, 30.0, 30.0, 29.9, 29.9, 29.9, 29.9, 29.8, 29.8, 29.7, 29.7, 29.7, 29.6, 29.6, 26.5, 26.3, 25.8, 25.7, 25.5, 25.4,14.4, 8.9; negative ES TOF-MS calcd for $[M-H]^-$ 1444.1193, found 1444.1152.*Compound **5**:* ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.95 (d, J = 9.6 Hz, 1H), 6.67 (d, J = 9.6 Hz, 1H), 5.29 (m, 1H), 4.69 (m, 1H), 4.38–4.26 (m, 2H), 4.16 (m, 1H), 3.96 (dd, J = 12.2, 5.2 Hz, 1H), 3.79 (m, 2H), 3.67 (m, 2H), 3.50 (m, 4H), 3.40–3.29 (m, 4H), 3.06 (q, J = 7.2 Hz, 6H), 2.85 (m, 1H), 2.51–2.44 (m, 3H), 2.36 (dd, J = 14.4, 6.0 Hz, 1H), 2.14 (m, 2H), 1.54–1.47 (m, 12H), 1.35–1.19 (m, 81H), 0.89–0.86 (m, 12H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.4, 171.2, 77.5, 76.7, 70.0, 69.7, 60.5, 53.4, 46.2, 43.3, 41.7, 39.2, 35.8, 34.7, 32.2, 32.2, 32.2, 32.0, 32.0, 31.9, 30.5, 30.2, 30.2, 30.1, 30.1, 30.0, 30.0, 30.0, 29.9, 29.9, 29.7, 29.7, 29.6, 26.2, 26.2, 26.0, 25.8, 22.9, 22.9, 14.4, 14.3, 8.9, 0.2. negative ES TOF-MS calcd for $[M-H]^-$ 1275.9315, found 1275.9253.
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