Drug Discovery

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Inhibition of Lipid A Stimulated Activation of Human Dendritic Cells and Macrophages by Amino and Hydroxylamino Monosaccharides

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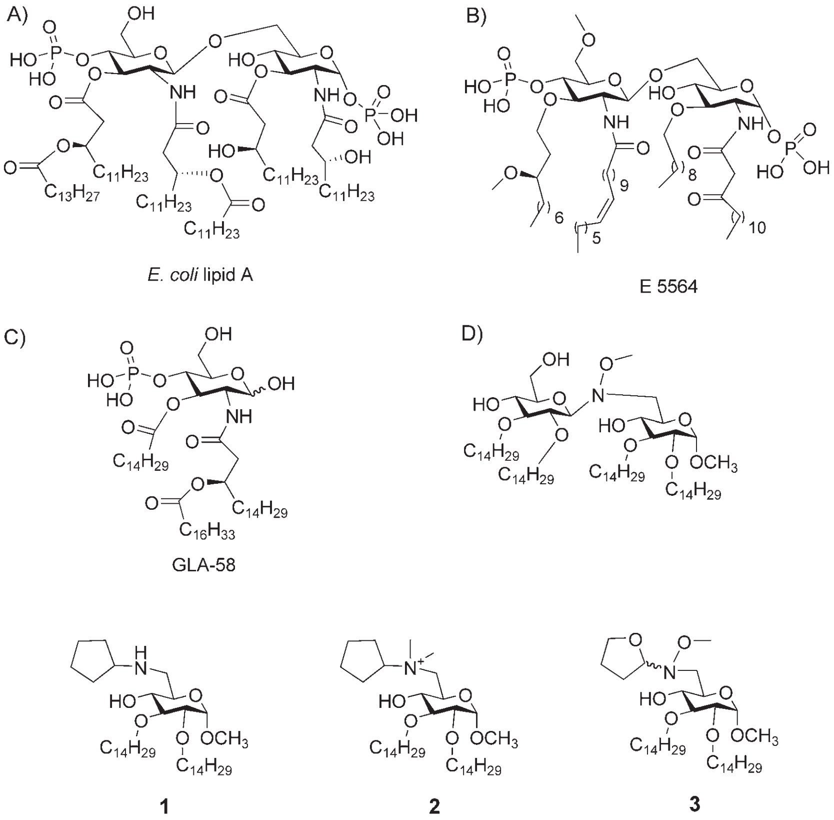
Sepsis and septic shock are serious clinical syndromes linked with high mortality rates[1] and caused by the presence of circulating microbial antigens in the blood of affected patients.[2] The development of septicaemia is often associated with a systemic inflammatory response to bacterial lipopolysaccharides (LPSs). The toxicity of LPSs lies in the membrane-anchoring moiety named lipid A.[3] Lipid A is the lipophilic portion of bacterial LPSs. Its general chemical structure consists of a β-(1→6)-linked disaccharide core with two phosphate esters (at positions C1 and C4′) and an *R*-3-hydroxy fatty acid at ester and amide linkages; some of these fatty acids are acylated at the 3-hydroxy group (Scheme 1 A).

Lipid A units of different bacterial origin differ in their chemical structure (mainly in the ramification of lipophylic chains) and display a variety of biological activities. There are highly toxic forms (for example, hexa-acylated lipid A from *Escherichia coli* or *Salmonella* species) that stimulate massive cytokine production, less toxic forms, and even variants that have antagonistic properties towards cytokine stimulation. Biophysical studies on the three-dimensional conformation adopted by different lipid A structures have revealed that under physiological conditions the most active (toxic) forms have a conical shape, whereas a lack of activity or antagonistic properties are associated with a cylindrical structure.[4] The different 3D structures of lipid A are thought to influence the geometry of the complex with the cellular target, the Toll-like receptor 4 (TLR4), and therefore the intensity and the nature of the biological activity of the LPS.



Scheme 1. Structures of A) *Escherichia coli* lipid A, B) the synthetic antagonist E 5564, C) the monosaccharide mimic GLA-58, and D) a synthetic disaccharide antagonist. Glucose derivatives **1**, **2**, and **3**.

Great effort has been devoted to the synthesis of lipid A mimics, which have shown activity as agonists or antagonists in the TLR4-activation process. The phosphorylated disaccharide core plays a fundamental role in the biological activity; it is therefore present in the majority of lipid A synthetic mimics, as in the case of compound E 5564 (Scheme 1 B). This potent lipid A antagonist developed by the Eisai Group was found to be a good antisepsis drug in phase II clinical trials.[5] Very few compounds are known that lack the disaccharide structure but maintain activity as lipid A agonists or antagonists.[6] Monosaccharide lipid A analogues, including compound GLA-58 (Scheme 1 C), preserved LPS-mimetic activity despite the absence of the second, non-reducing monosaccharide moiety.[7] A series of reducing *N*-acylated glucosylamines with a phosphate group at C4 and linear or branched 3-hydroxytetradecanoic acid ester and amide chains at C2 and C3 were recognized by murine

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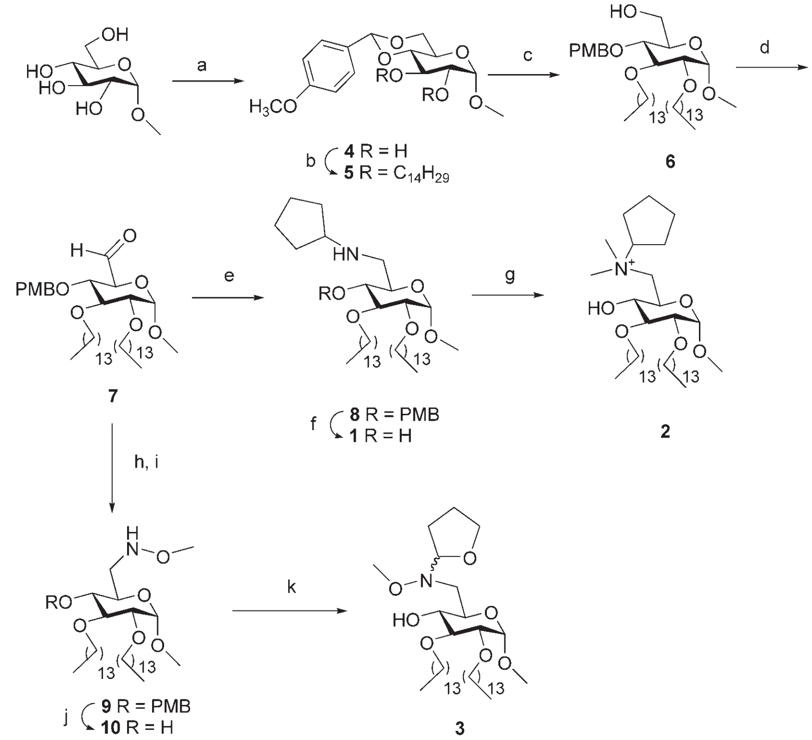
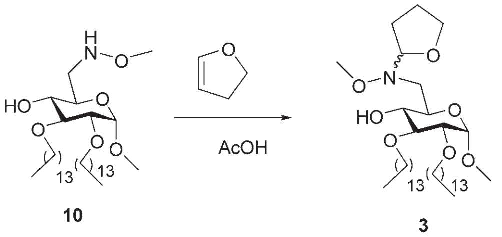
macrophages as both LPS agonists and antagonists. Furthermore, compound GLA-58 was shown to be active as an LPS antagonist in human cells. Interestingly, it was found recently that some substances with chemical structures totally unrelated to that of lipid A, such as taxol, flavolipin,[8] and vitamin D,[9] have LPS-mimetic activity towards TLR4.

Herein we present the synthesis and unexpected biological activity of the glucose-derived amine **1**, ammonium species **2**, and hydroxylamine **3** (Scheme 1). These compounds, unlike other lipid A mimics synthesized so far, have a monosaccharide structure and are devoid of phosphate esters. In a previous report, we showed that a nonphosphorylated N(OMe)-linked disaccharide (Scheme 1 D) inhibits lipid A induced cytokine production in MT2 macrophages in a dose-dependent manner.[10]

Compound **3** was first obtained serendipitously when we attempted the chemoselective synthesis of this disaccharide by the direct condensation of two unprotected monosaccharides. Unexpectedly, the reaction of the methoxyamino monosaccharide **10** (Scheme 2) with traces of dihydrofuran (DHF) present in the solvent (THF) led to the formation of the tetrahydro adduct **3** in very low quantities. The ability of **3** to inhibit lipid A activity was tested on dendritic cells (DCs) and macrophages (MΦs). Modest activity was found, unfortunately accompanied by significant chemical instability, particularly under acidic conditions, owing to the presence of an N,O-acetal group.[11] We then developed a synthetic route for the gram-scale synthesis of compounds **1** and **2**, two chemically stable analogues of **3** (Scheme 3). Compound **1** has a cyclopentylamine group linked to C6, and compound **2** is the corresponding dimethyl ammonium salt, with a permanent positive charge on the nitrogen atom.



Scheme 3. Synthesis of monosaccharides **1**–**3**: a) anisaldehyde dimethylacetal, CSA, DMF, 94%; b) $C_{14}H_{29}Br$, NaH, DMF, 74%; c) $LiAlH_4$, $AlCl_3$, CH_2Cl_2/Et_2O, 86%; d) Dess–Martin periodinane, CH_2Cl_2; e) cyclopentylamine, $NaBH_3CN$, AcOH, CH_2Cl_2, MeOH, 75% (two steps); f) TFA, CH_2Cl_2, 80%; g) CH_3I, Na_2CO_3, DMF, 94%; h) *O*-methylhydroxylamine hydrochloride, pyridine, 65% from **6**; i) $NaBH_3CN$, glacial AcOH, 92%; j) TFA, CH_2Cl_2, 90%; k) DHF, PPTS, CH_2Cl_2, 65%. DMF = *N*,*N*-dimethylformamide, TFA = trifluoroacetic acid.



Scheme 2. Unexpected reaction of the monosaccharide **10** with dihydrofuran impurities present in THF.

The synthesis of all compounds started with the conversion of commercially available methyl α-D-glucopyranoside into the methyl 4,6-*O*-(4-methoxybenzylidene)-α-D-glucopyranoside **4** in 94% yield by treatment with anisaldehyde dimethylacetal and camphorsulfonic acid (CSA). The alkylation of **4** with tetradecyl bromide in the presence of NaH furnished **5** (74% yield), the benzylidene ring in which was opened regioselectively by treatment with $LiAlH_4$ and $AlCl_3$ to give **6** in 86% yield.[10] The oxidation of **6** with Dess–Martin periodinane gave the corresponding aldehyde **7**, which was converted directly into **8** by reductive amination with cyclopentylamine and $NaBH_3CN$ (75% yield over two steps). Acidic cleavage of the *p*-methoxybenzyl (PMB) group at C4 of **8** gave **1** in 80% yield; the secondary cyclopentylamine at C6 was converted into the quaternary ammonium ion by treatment with methyl iodide and sodium carbonate to afford compound **2** in 94% yield. Alternatively, aldehyde **7** was treated with *O*-methylhydroxylamine hydrochloride in pyridine to give the corresponding C6 methyloxime in 65% yield from **6**. The methyloxime was then reduced to the methylhydroxylamine **9** by treatment with sodium cyanoborohydride in glacial acetic acid (92% yield). The PMB ether was cleaved with TFA/CH_2Cl_2 to afford **10** (90% yield), which was finally treated with DHF in the presence of the acid catalyst pyridinium *p*-toluenesulfonate (PPTS) to give the target monosaccharide **3** in 65% yield.

The biological activities of compounds **1**–**3** were investigated by analyzing their ability to interfere with lipid A induced DC and MΦ activation. These two special classes of leukocytes are involved directly in boosting and controlling inflammatory responses and, following encounter with microbial products, such as LPSs, produce large amounts of TNFα,

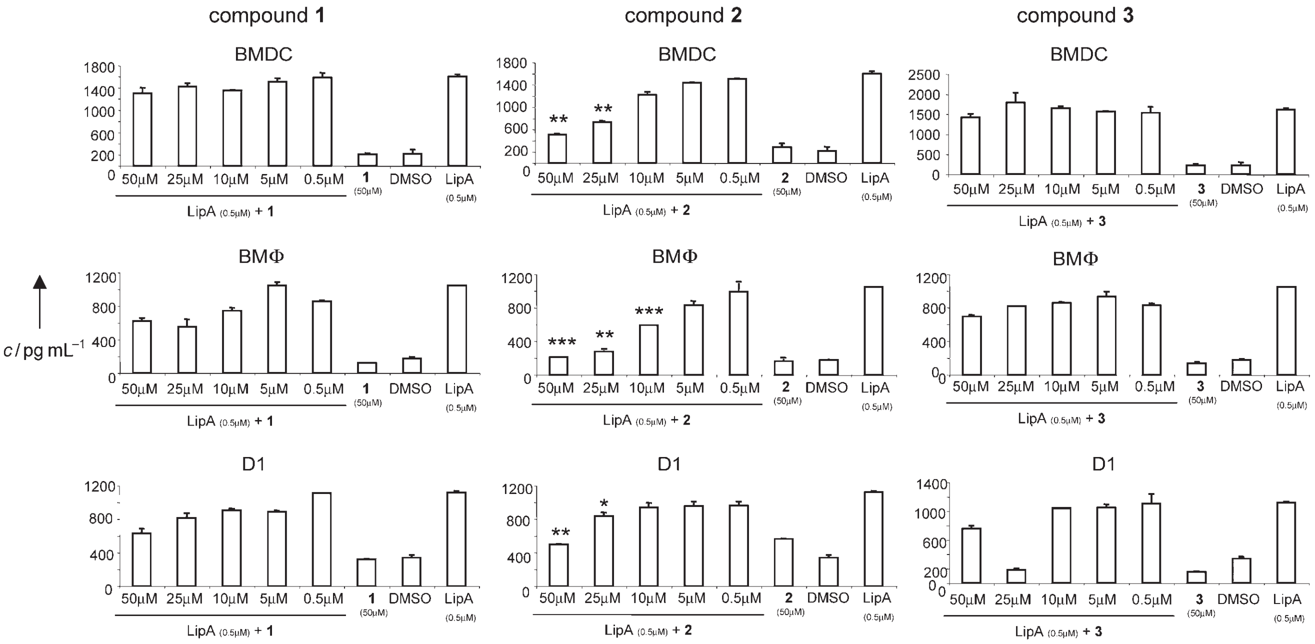


Figure 1. Antagonistic activity of monosaccharides **1**–**3** towards BMDC, BMΦ, and D1 cells. TNFα production was measured in the supernatants after 24 h. From right to left: lipid A (0.5 μM): cells treated with lipid A alone; DMSO: cells incubated in the presence of the complete medium plus DMSO; inhibitor (50 μM): cells treated only with monosaccharides **1**–**3**; other columns: lipid A plus compounds **1**–**3** in increasing doses. The data represent the mean and standard deviation for triplicate wells. The results are representative for three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, where p is the statistical probability value.

one of the principal mediators of inflammation and septic shock. In particular, DCs are distributed in tissues that interface with the external environment, such as the skin, gut, and lungs, where they can perform a sentinel function for incoming pathogens, and have the capacity to recruit and activate cells of the innate immune system.[12] After microbial uptake has occurred, DCs undergo a complex process of maturation with the sequential acquisition of different immune-regulatory functions. Once the maturation process initiated by direct exposure to TLR ligands has been completed, DCs become efficient activators of acquired immune responses. In this context, LPS activation of TLR4 is one of the most potent stimuli for DC priming.[13]

Because of the pivotal role of early activated DCs and MΦs in directing inflammatory reactions, we tested whether the two compounds could antagonize the stimulatory activity of lipid A on bone-marrow-derived macrophages (BMΦs) and DCs (BMDCs) by interfering with their capacity to induce TNFα production. As a source of DCs, we also used D1 cells, a DC line that is dependent on the long-term growth factor GM-CSF (granulocyte macrophage colony-stimulating factor).[14] BMΦ, BMDC, and D1 cells were stimulated with lipid A (0.5 μM) after preexposure to compounds **1**–**3**, and the amounts of TNFα released in the supernatant were measured by ELISA. As shown in Figure 1, monosaccharides **1** and **2** interfered with lipid A activity in a dose-dependent manner, whereas compound **3** showed consistently lower activity. Monosaccharide **10**, which lacks the tetrahydrofuran ring, was totally inactive. In general, monosaccharides **1**–**3** did not show any direct inflammatory function, as they are not capable of stimulating cytokine production. Compound **2** was more potent than **1**, which was in turn a better inhibitor than **3**. Moreover, compound **2** had the highest solubility in the aqueous media used in our tests. For these reasons, amines **1** and **2** seemed the best candidates for drug development, whereas the chemical instability, sparing solubility, and lower potency of hydroxylamine **3** render it a less interesting lead.

The activity of the most promising lead, compound **2**, was then analyzed in more detail by monitoring the production of a second inflammatory cytokine, IL-1β. IL-1β and TNFα synthesis was inhibited in BMΦ and D1 cells, and the inhibitory effect was proportional to the concentration of the inhibitor. To evaluate the selectivity for TLR4, TNFα production was investigated in the presence of compound **2** in response to stimulation by the CpG motif of bacterial DNA, which is recognized by TLR9,[15] and the tripalmitoyl cysteine (Pam$_3$Cys-SK$_4$) moiety, which is specific for TLR2.[16] Cytokine production was not inhibited by **2** in either the TLR9- or the TLR2-mediated inflammatory cascade (Figure 2), thus indicating a relevant level of selectivity.

We then investigated the cytotoxic potential of the monosaccharides. Compounds **1**–**3** are composed of a polar part (the sugar moiety) linked to lipophilic chains, and can therefore act as detergents and kill cells by generating pores in the plasma membranes. The toxicity of compounds **1**–**3** was investigated with the PI test, the apoptotic potential with the annexinV test. Neither for the DCs nor for the MΦs was an appreciable percentage of dead cells found after incubation for 48 h with **2** at concentrations between 10 and 100 μM (Figure 3).

Further direct evidence that the activity of compound **2** is selective for the TLR4 receptor was obtained with experi-

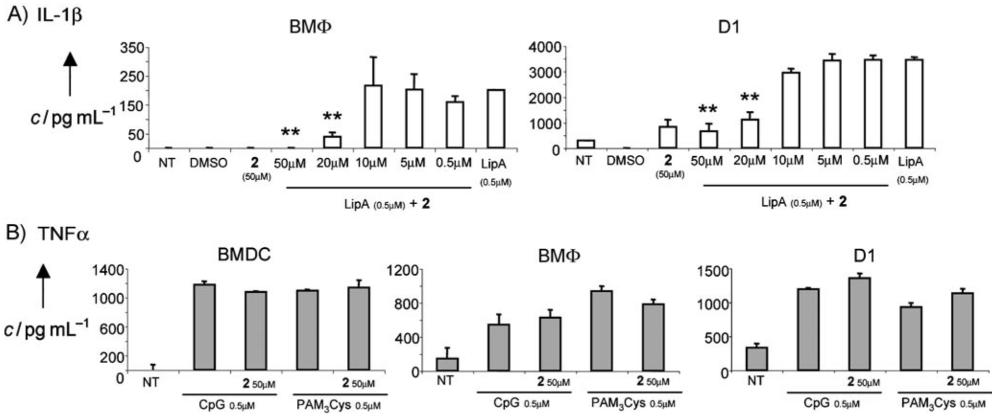


Figure 2. Selectivity of the monosaccharide **2** for TLR4. A) The antagonistic activity of compound **2** was investigated by testing its ability to interfere with IL-1β production 24 h after stimulation with lipid A. The quantity of IL-1β was measured in the supernatants by ELISA. Lipid A (0.5 μM): cells treated with lipid A alone; NT: cells incubated in the presence of the complete medium; DMSO: cells incubated in the presence of the complete medium plus DMSO; inhibitor (50 μM): cells treated only with the monosaccharide **2**; other columns: lipid A plus compound **2** in increasing doses. The data represent the mean and standard deviation for triplicate wells. The results are representative for three independent experiments. B) TNFα production measured by ELISA 24 h after stimulation with CpG or PAM$_3$Cys in the presence of compound **2**. NT: cells incubated in the presence of the complete medium plus DMSO; other columns: cells treated with CpG or Pam$_3$Cys-SK$_4$ in the presence or absence of the inhibitor **2** (50 μM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ments on a TLR4- and TLR9-transfected HEK 293 cell system.[17] In these cells, TLR-dependent activation of NF-kB (nuclear factor κB) can be measured readily after stimulation. The activation of the TLR4 signaling pathway leads to NF-kB nuclear translocation and inflammatory cytokine production.[18] TLR4- and TLR9-transfected HEK 293 cells were incubated with lipid A (0.5 μM) and CpG (0.5 μM), respectively, after preexposure to compound **2**, and the activation of NF-kB was measured 2 h later. Monosaccharide **2** was able to counteract significantly the effect of lipid A in TLR4-transfected cells, whereas it was inactive at the maximal dose of 50 μM in influencing the effect of CpG on TLR9-transfected HEK 293 (Figure 4).

The biological data presented allow some preliminary considerations on the structure–activity relationship of this new class of lipid A antagonists. The presence of a five-membered ring attached to the nitrogen atom at C6 appears to be fundamental to the activity of these compounds; compound **10**, which lacks this ring, is completely inactive. The permanent positive charge on the nitrogen atom of **2** has a positive effect on both activity and solubility.

Monosaccharides **1**–**3** and the other lipid A mimics described to date display remarkable structural diversity. Therefore, the experimental evidence for the interaction of these monosaccharides with TLR4 could open interesting perspectives in both drug discovery and the characterization

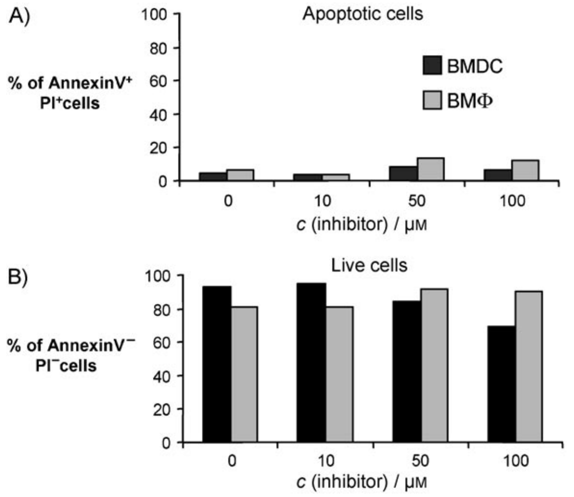


Figure 3. Compound **2** does not induce the death of MΦs or DCs. Mouse DCs and MΦs were either left untreated or treated for 24 h with compound **2** at the indicated concentrations. Cells were then analyzed by FACS for the presence of annexinV and propidium iodide (PI) double-positive cells. A) percentage of annexinV and PI double-positive cells; B) percentage of annexinV and PI double-negative cells.

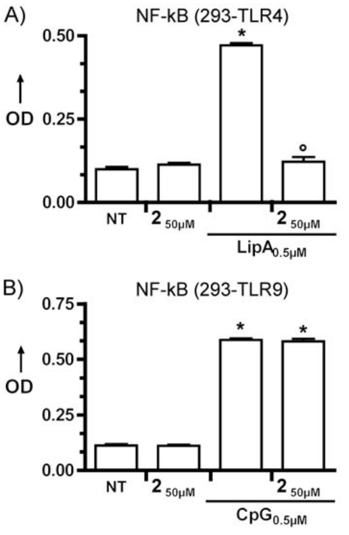


Figure 4. Antagonistic activity of monosaccharide **2**. HEK 293 cells transfected stably with the human TLR4A or TLR9 gene were treated with lipid A alone or lipid A in the presence of monosaccharide **2**, and with CpG alone or CpG in the presence of monosaccharide **2**, respectively. NF-kB activation was evaluated in the nuclear extract 2 h later, and is expressed as optical density (OD) at 450 nm. NT: nontreated cells. The data represent the mean and standard error for duplicate wells. The results are representative for three independent experiments. *, $p < 0.001$ versus NT cells; °, $p < 0.001$ versus lipid A alone (ANOVA (analysis of variance); Tukey test).

of TLR4–ligand molecular recognition and binding. We are currently testing the activity of **2** and of molecules with similar structures in different biological contexts. Recent publications outlined the central role of TLR4 in a number of important pathological events, such as the modulation of a series of immunological and allergic reactions[19] and pain transmission.[20] We are therefore convinced that compound **2** and other molecules capable of binding TLR4 could be used as antisepsis drugs as well as leads for the development of pain-relief and antiallergic drugs with innovative structures.

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