



Published in final edited form as:

Med Chem Res. 2021 July ; 30(7): 1377–1385. doi:10.1007/s00044-021-02736-3.

Synthesis, structure-activity relationship studies and evaluation of a TLR 3/8/9 agonist and its analogues

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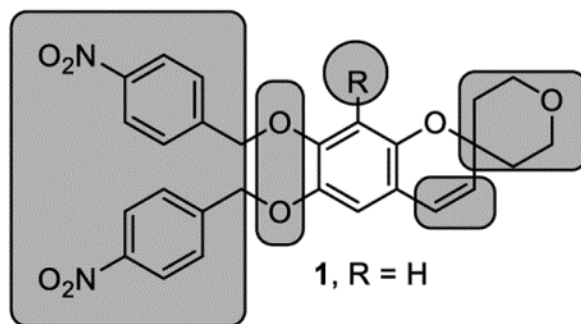
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Abstract

A comprehensive SAR study of a putative TLR 3/8/9 agonist was conducted. Despite the excitement surrounding the potential of the first small molecule TLR3 agonist with a compound that additionally displayed agonist activity for TLR8 and TLR9, compound **1** displayed disappointing activity in our hands, failing to match the potency (EC_{50}) reported and displaying only a low efficacy for the extent of stimulated NF- κ B activation and release. The evaluation of >75 analogs of **1**, many of which constitute minor modifications in the structure, failed to identify any that displayed significant activity and none that exceeded the modest activity found for **1**.

Graphical abstract



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Dedication: This article is dedicated to Professor Gary Grunewald, a treasured longtime friend and early colleague alongside which I had the pleasure of working for many years.

Conflict of Interest: The authors declare that they have no conflict of interest beyond the disclosed interest of Bristol Myers Squibb (coauthors) in supporting and collaborating in the work.

Keywords

Toll-like receptor (TLR); TLR agonists; TLR3 agonist; SAR study

Introduction

Toll-like receptors (TLRs) play a critical role in the mammalian defense against microbial infections.[1] The TLRs are germline-encoded receptors that recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), highly conserved molecular components present in microorganisms or abnormal cells, respectively. [2] Ligand induced TLR dimerization and activation marshals both the initial innate and slower adaptive immune response to protect the host from infectious pathogens[3,4,5] or remove abnormal cells. TLR activation for the treatment of infectious diseases or cancer have been widely explored where TLR agonists serve as attractive and efficacious vaccine adjuvants.[6–10] Despite their potential, there is not yet a wide range of small molecule TLR agonists available. Most bear poor structural, physical, or stability properties found in the identified naturally-occurring agonists that make them unattractive starting points for direct use or even further optimization.[11–17]

Humans possess several TLRs that are expressed heterogeneously in macrophages, dendritic cells, and other compartments. TLRs 1, 2, 4, 5 and 6 are located on the cell membrane and bind bacterial lipids, peptidoglycans, lipoproteins and proteins, whereas TLRs 3, 7, 8 and 9 are situated in the endosome membrane within the cell and are activated by viral or bacterial nucleic acids. Complementing the selective targeting of a single TLR, multi-TLR-based therapies are an emerging area of research in oncology, as the simultaneous activation of multiple TLRs has potential merits. It can boost the immune response by triggering synergy between different TLR pathways.[18] TLRs are distributed heterogeneously and the effectiveness of a single TLR activation may be diminished if the target cells do not express a particular TLR. In contrast, if multiple TLRs are activated, they can complement each other for presentation and cross-presentation of antigens to T cells to enhance immunity. Pertinent to the work detailed herein, maturation of macrophage dendritic cells is promoted by TLR3 agonists in the tumor microenvironment, thus stimulating presentation/cross-presentation to T cells, resulting in expansion of tumor-specific cytotoxic T lymphocytes. On the other hand, TLR9 agonists target only plasmacytoid dendritic cells. As noted below, the first small molecule TLR3 agonist was recently disclosed that also acts on TLR9 and TLR8.[19]

In the recent years, a still limited number of attractive small molecule TLR agonists have been described.[17] We reported the discovery of neoseptins,[20, 21] the first structurally characterized class of murine TLR4 agonists that bear no structural similarity to bacterial lipopolysaccharide (LPS) or its active core Lipid A (LPA). More recently, we disclosed the discovery of the diprovocims,[22,23] a class of exceptionally potent, synthetic small molecule TLR2/TLR1 agonists that show efficacious adjuvant activity in an anticancer intramuscular vaccination against murine melanoma and protect mice from tumor rechallenge in an antigen specific manner.[24] In our continuing efforts, we were

intrigued with the disclosure of compound **1**, derived from a HTS screening lead by Yin.[19] It represents the first and only small molecule TLR3 agonist for a receptor that ordinarily senses viral double-stranded RNA.[25] It also acts as a multi-TLR agonist, activating TLRs 3/8/9.[19] The compound showed strong NF- κ B activation through hTLR 3, 8 and 9 (EC₅₀ = 4.8 μ M, 13.4 μ M and 5.7 μ M, respectively) and negligible or no NF- κ B activation via hTLR 2, 4, 5 and 7, enlisting a SEAP reporter assay and HEK293 cells overexpressing individually each of the hTLRs. It also exhibited significant cytotoxic activity, inhibiting the growth of cervical HeLa cancer cells (IC₅₀ = 2.7 μ M) by inducing apoptosis.

Based on the structure-activity relationship (SAR) studies disclosed by the Yin group,[19] both benzyl ether groups were required for the activity. An electron-withdrawing 4-nitro group was required, as its removal or even its relocation to the *ortho* or *meta* positions resulted in loss of activity. The chromene precursors, a chromanone or chromanol, did not display activity, underscoring the importance of the olefin. The benzyl ether linkage was also found to be important, as a decreased activity was observed when it was replaced with ester. Substitution, steric bulk, and optimally a spirocyclic ring, incorporating a heteroatom (the tetrahydropyran group) on the right side of the molecule was also essential to the activity. By building on the limited SAR reported by the Yin group, we hoped to discover compounds with improved potency and properties (solubility, selectivity, metabolic stability, toxicity, pharmacokinetics, and *in vivo* efficacy). Our initial objective was to confirm the activity and the mechanism of action of **1**, followed by optimization of the compound potency.

Results and Discussion

Chemistry and SAR Study.

To establish insights into the binding of **1** to hTLR8, its structure was compared with resiquimod (**2**, R848), a known TLR8 agonist used as a control in TLR8 and TLR7 assays (Figure 2a), assuming it binds directly to TLR8 at the canonical small molecule agonist site. Resiquimod forms a salt bridge with Asp543 present in the agonist binding site (Supporting Information Figure 1). Docking studies and a simple overlay of **1** with R848 suggested that (1) the oxygen in the tetrahydropyran ring in **1** H-bonds to T574 (TLR8)/G565 (TLR9) backbone NHs, and (2) the two nitro groups are involved in polar interactions with multiple arginines and lysines (Supporting Information Figure 2a and b). Similar conclusions were drawn from a comparison of the putative binding of **1** to hTLR9 with that of 5'-xCx DNA, known TLR9 agonists (Supporting Information Figures 3 and 4),[26] where the cytosine group forms a salt bridge with Asp534 in the binding site (Figure 2d). We also inferred that the nitrophenyl groups in **1** are likely involved in pi-pi stacking interactions with the active site residues in TLR8. No small molecule bound structure is known for TLR3, making it difficult to assign putative binding site(s).[27]

Upon digesting the results of the Yin SAR studies, we elected to independently conduct (1) modifications of the benzyl groups with introduction of a variety of substituents that include nitro group bioisosteres and heterocyclic replacements of the phenyl ring to manipulate its electronic and lipophilic character, diminish its symmetrical nature, and introduce more sp³ character to improve solubility while enhancing activity. Independently we pursued (2) modification of the tetrahydropyran (THP) ring, and (3) examination of alternative

substituted chromene scaffolds with potential introduction of more basic counterparts to enhance receptor binding and endosomal localization.

The synthesis of compounds **9–67** largely followed that detailed by Yin and is outlined in Scheme 1. Friedel-Crafts acylation of 3,4-dimethoxyphenol with acetic anhydride in presence of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (60 °C, 3 h, 90%) yielded the acetophenone **3**, which was condensed with tetrahydro-4*H*-pyran-4-one (methanol, 23 °C, 12 h, 83%) to obtain chromanone **4**. Demethylation of **4** with boron tribromide in anhydrous CH_2Cl_2 (0 °C to 23 °C, 12 h, 85%) furnished catechol **5**, and subsequent reduction with lithium borohydride (LiBH_4 , 2 M in THF, 23 °C, 12 h, 78%) afforded chromanol **6**. The latter reduction with LiBH_4 proved cleaner, easier to implement, and provided **6** in higher yield than that conducted with LiAlH_4 as reported. Dehydration of **6** with 4 N HCl (ethyl acetate, 23 °C, 12 h, 74%) yielded chromene **7** and was much cleaner than the reported dehydration conducted in acetone. Compound **7** was alkylated with the corresponding benzylic bromides (K_2CO_3 , acetone, reflux, 12 h) to furnish compounds **1** and **9–67**.

We replaced the 4-nitro group with bioisosteres, including a nitrile and oxadiazole (compounds **12** and **13**, respectively). In addition, a series of heterocyclic, halogenated and other substituted derivatives (compounds **14–22**, **31–34**, **39–48**) were prepared in efforts to replace the 4-nitro group on the benzyl substituent (Figure 3). Several disubstituted benzyl derivatives containing the 4-nitro group were also synthesized (Figure 4, **23–25**), and the *p*-nitrophenyl group was replaced with a variety of additional bioisosteres (compounds **26–28**). In order to promote possible pi-pi stacking, other electron-deficient aryls and indoles were also examined (compounds **9–11**, **17**, **18**, **38**, **39**, **54**).^[28] In addition, the 4-nitrobenzyl ether was also replaced with a 4-nitrobenzoyl group (**32**). However, all compounds described above displayed little or no activation of TLR 8 and 9 compared to lead compound **1**. As expected, substituents at the *meta*-position did not result in an improvement in activity (e.g., compound **29**). Introduction of alkyl substituents (**49–52**) also did not enhance activation.

5-Nitrofuranyl (**55**, cLogP = 4.0) and 5-nitrothienyl (**56**, cLogP = 5.2) replacements of the 4-nitrophenyl ring with cLogP values [29] more favorable or close to that of the lead molecule (**1**, clogP = 5.5) were synthesized (Figure 5). Additionally, a series of sulfonyl derivatives **59–64** (cLogP ca. 3) were prepared to address the high lipophilicity of the lead molecule (cLogP of compound **1** is 5.5) while simultaneously maintaining potential binding interactions of the nitro group. In order to introduce sp^3 character, and potentially improve solubility, derivatives were prepared that contain each enantiomer of the pyrrolidin-2-one substituent (**57** and **58**). Unfortunately, none of these compounds resulted in activation of TLR 8 and 9.

The necessity of each individual 4-nitro substituent on the two benzyl groups was also defined. Based on docking studies and overlay models of **1** with resiquimod, it is possible only the one nitro group of the 4-nitrophenyl group that potentially interacts with a tyrosine residue (Y348) in the resiquimod binding site is needed (Supplementary Figure 2c). Therefore, analogs of **1** that possess a single 4-nitro group on each of the benzyl substituents were prepared and examined (Figure 6, compounds **65** and **66**). The synthesis of these unsymmetrical derivatives is shown in Figure 6. In addition, we replaced the 4-nitro group

of one of the benzyl groups with a fused oxadiazole as a potential bioisostere for the nitro group while maintaining the one putative key 4-nitrophenyl group (compound **67**). However, compounds **65–67** were inactive, indicating that each 4-nitro group plays an important role in lead compound **1**.

A final set of modifications of the lead candidate **1** targeted the spirocyclic tetrahydropyran (THP) ring. In a subset of these studies, the tetrahydropyran was replaced with two isomeric piperidine rings (Scheme 2 and Figure 7, **68–71**) with the goal to (1) improve solubility, (2) potentially provide endosomal delivery, localization, or trap, (3) improve receptor interactions directly or indirectly through attached side chains, and (4) provide a site for conjugation to neo-antigens and proteins or antibodies for targeted delivery. The introduction of a complementary basic atom could also form a salt bridge with D543 in TLR8 and D534 in TLR9. A 2-hydroxyethyl substituent was appended to the nitrogen to attenuate the amine basicity and potentially mimic the five-atom chain in R848, binding in a narrow pocket in TLR8 (compounds **72** and **73**).

The six-membered 4-THP spirocyclic ring was replaced with spirocyclic rings of altered sizes and modified heteroatoms (Figure 7), including the 4-thiopyran dioxide (**74**), 4-thiopyran (**75**) and its methyl sulfonium salt (**76**), quaternary dimethyl substitution (**77**, original screening lead [19]), regioisomers of 4-THP (**78**), ring contracted versions of the 4-THP (**79** and **80**, tetrahydrofurans **81** and **82**). However, none of the modifications improved TLR activation. Compounds **74–82** or their precursors were prepared by the route outlined in Scheme 1, introducing the modifications in the condensation of **3** with the corresponding ketone.

With the aim of restricting the spatial orientation of the nitrophenyl groups and in efforts to explore a site for additional receptor interactions of the chromene, compound **88** was prepared and contains a C8-methyl substituent attached to the core benzene ring. The known compound **84** was converted to chromanone **86**, which was reduced to chromanol **87**, followed by dehydration to afford chromene **88** (Scheme 3). None of the three compounds (**86**, **87** and **88**), including **88** possessing a single added methyl group relative to **1**, exhibited any TLR activation.

Biological Evaluation.

The Yin group reported that compound **1** showed strong NF- κ B activation in a reporter assay through activation of hTLR 3, 8, and 9 (EC_{50} = 4.8 μ M, 13.4 μ M and 5.7 μ M, respectively).[19] In contrast, compound **1** prepared herein at two different sites and by two different procedures and assessed multiple times only showed weak activity for NF κ B activation in our hands, with EC_{50} s of 22, 27 and 58 μ M for TLRs 3, 8 and 9, respectively. Because of expectations of improvements in the modest activity reported for **1**, the analogues were examined at concentrations up to 5 μ M. However, none of the analogs of **1** prepared showed an improvement in activity, with essentially all compounds being inactive and with all EC_{50} values being greater than 5 μ M (Supporting Information Tables 1 and 2). Selected members of the full set (**13**, **18**, **24**, **55**, **56** and **69**) were rescreened at concentrations up to 100 μ M and, with the exception of **69**, were inactive (EC_{50} >100

μM) and displayed no NF κ B activation. Compound **69** (with a piperidine ring replacing the tetrahydropyran ring) exhibited a weak EC₅₀ of 111 μM in TLR9, but no activity (>125 μM) in TLR8. Consistent with reported results with **1**, [19] neither compound **1** nor any of its analogs showed any activity with TLR7.

We also measured the stimulated release of the cytokine tumor necrosis factor α (TNF- α) by the action of **1** in both mouse macrophages and in partially differentiated human THP-1 cells as described in our prior studies. [20–24] It showed no activity in mouse macrophages and very weak activity in human THP-1 cells, where the weak stimulated TNF- α release continues to rise slowly up to 100 μM , the highest concentration examined (EC₅₀ >100 μM). The extent of this stimulated release was not at a level that we would consider active in this sensitive assay using native cells. This contrasts the reported strong production of TNF- α in THP-1 cells by the Yin group (EC₅₀ 5–10 μM). [19] Thus, although the differences in the potency of **1** in the reporter assays for hTLR 3, 8, and 9 (2- to 10-fold) might be attributable to subtle differences in the assay implementation, those observed with human THP-1 cells cannot.

Conclusions

A comprehensive SAR study of a putative TLR 3/8/9 agonist was conducted. Despite our excitement with the potential of the first small molecule TLR3 agonist with a compound that additionally displayed agonist activity at TLR8 and TLR9, compound **1** displayed disappointing activity in our hands, failing to match the potency (EC₅₀) reported and displaying only a low efficacy for the extent of stimulated NF- κ B release. The evaluation of >75 analogs of **1**, many of which constitute minor modifications in its structure, failed to identify any that displayed significant activity and none that exceeded the modest activity we found for **1** or that was initially reported. Although the authors of the original work [19] have not responded to our requests for guidance on their report of more promising observations with **1**, it is possible that it could be related to the low solubility of the lead candidate compound and potential that compound aggregates may have been assessed in the original work.

Methods and Materials

Experimental.

All reagents and solvents were used as supplied without further purification unless otherwise noted. All reactions were performed in anhydrous solvents unless otherwise noted. CHCl₃ was pre-treated with alumina for at least 24 h prior to use. Preparative TLC (PTLC) and column chromatography were conducted using Millipore SiO₂ 60 F₂₅₄ PTLC (0.5 mm) and Zeochem ZEOprep 60 ECO SiO₂ (40–63 μm), respectively. Analytical TLC was conducted using Millipore SiO₂ 60 F₂₅₄ TLC (0.250 mm) plates. ¹H and ¹³C NMR spectra were obtained using a Bruker Avance III HD 600 MHz spectrometer equipped with either a 5 mm QCI or 5 mm CPDCH probe or a Bruker Avance III 500 MHz spectrometer equipped with a 5 mm BBFO probe. IR spectra were obtained using a Thermo Nicolet 380 FT-IR with a SmartOrbit Diamond ATR accessory. Mass spectrometry analysis was performed by direct sample injection on an Agilent G1969A ESI-TOF mass spectrometer.

Candidate agonist synthesis.

1-(2-Hydroxy-4,5-dimethoxyphenyl)ethan-1-one (3).—A solution of 3,4-dimethoxyphenol (2 g, 12.97 mmol) in acetic anhydride (5 mL) under N₂ was treated with BF₃·Et₂O (1.3 mL mg, 1.3 mmol) at 0 °C for 10 min, and the reaction mixture was stirred at 80 °C for 3 h. After completion of the reaction, the reaction mixture was cooled to room temperature and neutralized with the addition of saturated aqueous sodium bicarbonate solution, followed by extraction with ethyl acetate. The crude product was purified by flash column chromatography (SiO₂, EtOAc/hexane 2:3) to give **3** (2.3 g, 90%) as a white solid: mp 103–104 °C; ¹H NMR (500 MHz, CDCl₃) δ 12.66 (s, 1H), 7.06 (s, 1H), 6.46 (s, 1H), 3.92 (s, 3H), 3.88 (s, 3H), 2.57 (s, 3H). ¹³C NMR (151 MHz, CDCl₃) δ 202.1, 160.1, 156.8, 111.7, 111.6, 100.5, 56.7, 6.2, 26.4. MS (ESI) calcd for C₁₅H₁₉O₅ [M+H]⁺, 197.1; found, 197.1.

6,7-Dimethoxy-2',3',5',6'-tetrahydrospiro[chromane-2,4'-pyran]-4-one (4).—A solution of **3** (2.0 g, 10.2 mmol) in methanol was treated sequentially with pyrrolidine (0.84 mL, 10.2 mmol) and tetrahydro-4*H*-pyran-4-one (3.06 g, 30.6 mmol), and the reaction mixture was warmed at reflux for 12 h. Purification by flash column chromatography (SiO₂, EtOAc/hexane 1:4) gave **4** (2.35 g, 83%) as a yellow solid: mp 124–125 °C: ¹H NMR (500 MHz, CDCl₃) δ 7.29 (s, 1H), 6.49 (s, 1H), 3.96 (s, 3H), 3.89 (s, 3H), 3.89 – 3.77 (m, 4H), 2.71 (s, 2H), 2.05 – 1.99 (m, 2H), 1.79 (ddd, *J* = 14.2, 11.0, 5.0 Hz, 2H). MS (ESI) calcd for C₁₅H₁₉O₅ [M+H]⁺, 279.1; found, 279.1.

6,7-Dihydroxy-2',3',5',6'-tetrahydrospiro[chromane-2,4'-pyran]-4-one (5).—A solution of **4** (0.5 g, 1.8 mmol) in CH₂Cl₂ (5 mL) was slowly treated with boron tribromide (1 M in CH₂Cl₂, 5.4 mL, 5.4 mmol) at 0 °C under nitrogen, and the reaction mixture was stirred at 23 °C for 12 h. The reaction mixture was poured into ice water and extracted with EtOAc, (3x). The combined organic layers were dried with Na₂SO₄, filtered, concentrated, and the residue was purified by flash column chromatography (SiO₂, EtOAc/hexane 4:1) to give **5** (383 mg, 85%) as an off-white solid: ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.22 (s, 1H), 9.05 (s, 1H), 7.03 (s, 1H), 6.36 (s, 1H), 3.68 – 3.61 (m, 4H), 2.65 (s, 2H), 1.81 (dt, *J* = 13.8, 3.5 Hz, 2H), 1.68 (ddd, *J* = 14.4, 9.3, 6.1 Hz, 2H). MS (ESI) calcd for C₁₃H₁₅O₅ [M+H]⁺, 251.1; found, 251.1.

2',3',5',6'-Tetrahydrospiro[chromane-2,4'-pyran]-4,6,7-triol (6).—A mixture of **5** (500 mg, 2 mmol) and 2 M LiBH₄ in THF (2 mL, 4 mmol) in THF (10 mL) was stirred at 23 °C for 6 h. The solution was quenched with the addition of methanol, and the reaction mixture was adjusted to pH 1–2 with the addition of aqueous 2 N HCl prior to removal of solvents under reduced pressure. The residue was extracted with EtOAc (10 mL) and washed sequentially with water and saturated aqueous NaCl. The organic phase was dried with Na₂SO₄, filtered, concentrated, and the residue was purified by flash column chromatography (SiO₂, EtOAc) to afford **6** (393 mg, 78%) as a yellow solid: ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.12 (s, 1H), 9.05 (s, 1H), 7.03 (s, 1H), 6.46 (br s, 1H), 6.36 (s, 1H), 4.78 – 4.75 (m, 1H), 3.68 – 3.61 (m, 4H), 2.65 (s, 2H), 1.81 (dt, *J* = 13.8, 3.5 Hz, 2H), 1.68 (ddd, *J* = 14.4, 9.3, 6.1 Hz, 2H). MS (ESI) calcd for C₁₃H₁₇O₅ [M+H]⁺, 253.1; found, 253.1.

2',3',5',6'-Tetrahydrospiro[chromene-2,4'-pyran]-6,7-diol (7).—Compound **6** (200 mg, 0.79 mmol) was dissolved in ethyl acetate (1 mL), and aqueous 4 N HCl (0.4 mL, 1.58 mmol) was added. The reaction mixture was stirred at 23 °C for 12 h, and then basified with the addition of saturated aqueous NaHCO₃ solution (3 mL) followed by extraction with EtOAc (10 mL). The combined organic layers were dried with Na₂SO₄, filtered, concentrated, and purified by flash column chromatography (SiO₂, EtOAc/hexane 1:3) to afford **7** (137 mg, 74%) as an off white solid: ¹H NMR (400 MHz, CDCl₃) δ 10.15 (s, 1H), 9.15 (s, 1H), 6.56 (s, 1H), 6.48 (s, 1H), 6.29 (d, *J* = 9.7 Hz, 1H), 5.51 (d, *J* = 9.7 Hz, 1H), 3.92 (td, *J* = 11.2, 2.6 Hz, 2H), 3.77 (dt, *J* = 11.5, 4.0 Hz, 2H), 1.96 (d, *J* = 14.0 Hz, 2H), 1.77 (td, *J* = 10.2, 5.4 Hz, 2H). MS (ESI) calcd for C₁₃H₁₅O₄ [M+H]⁺, 235.1; found, 235.1.

Synthesis of compounds 9–64, General procedure.

A mixture of **7** (10 mg, 0.043 mmol) and K₂CO₃ (35.4 mg, 0.26 mmol) in acetone (1 mL) was treated with the aryl bromide (0.093 mmol) and stirred at 60 °C under nitrogen for 12 h. The reaction mixture was filtered, concentrated, and purified by flash column chromatography (SiO₂, EtOAc/hexane) to afford compounds **1** and **9 – 64**.

The characterization for **1, 9–64** is provided in the Supporting Information section. Experimental procedures for adaptations of the general procedure and characterization data for the synthesis **65–67** (Figure 6), **68–73** (Scheme 2), **74–82** (Figure 7), and **86–88** (Scheme 3) are also provided in Supporting Information.

In vitro human TLR cell reporter assays.

Human HEK-blue reporter cells (Invivogen, San Diego, CA) containing a TLR transgene are plated on day 0 at an optimal cell concentration per well of a 384-well plate (20 uL cells/well) and incubated for 16 h at 37 °C, 5% CO₂. Compounds are added (100 nL/well) to each well using an ATS liquid handling instrument (Wagner Life Science, Middleton, MA) and the mixtures are incubated for 24 h at 37 °C, 5% CO₂. Freshly prepared Q-Blue reagent (Invivogen, San Diego, CA; suspended in water) is added to each well (10 uL/well) and the mixtures are incubated at 37 °C, 5% CO₂ for 30 min before measuring the absorbance (620 nm) using an Envision plate reader (PerkinElmer, Hopkinton, MA). Assay baselines are set using relative light unit (RLU) values from DMSO-treated control cells and 100% is set using reference agonist RLU values from cells treated at the highest compound concentration [reference agonists: poly I:C HMW (TLR3), resiquimod (TLR7, TLR8) and ODN-2006 (TLR9)]. Effective concentration values (EC₅₀; compound concentration yielding half-maximal response) are calculated using BMS proprietary data analyses software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements.

We gratefully acknowledge the financial support of NIH (CA042056, D.L.B.) and Bristol Myers Squibb.

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29. cLogP values were calculated with the module within ChemDraw Professional 16.0.

Both benzyl groups required
with nitro group at *p*-position

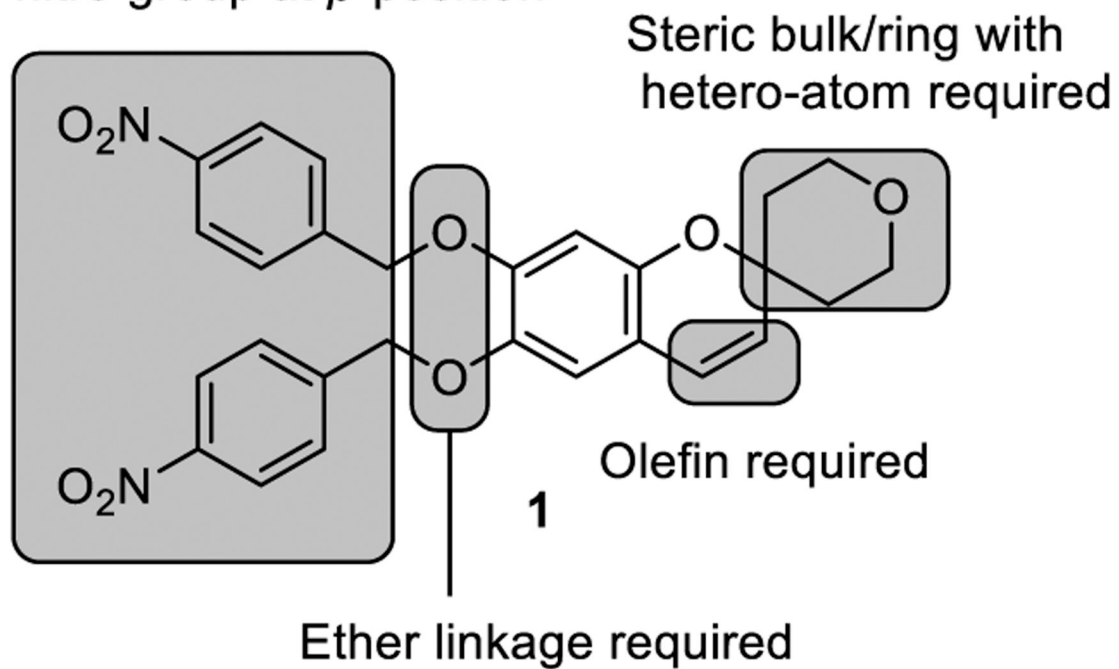


Figure 1.

Structure of reported TLR 3/8/9 agonist and summary of prior SAR (ref 19).

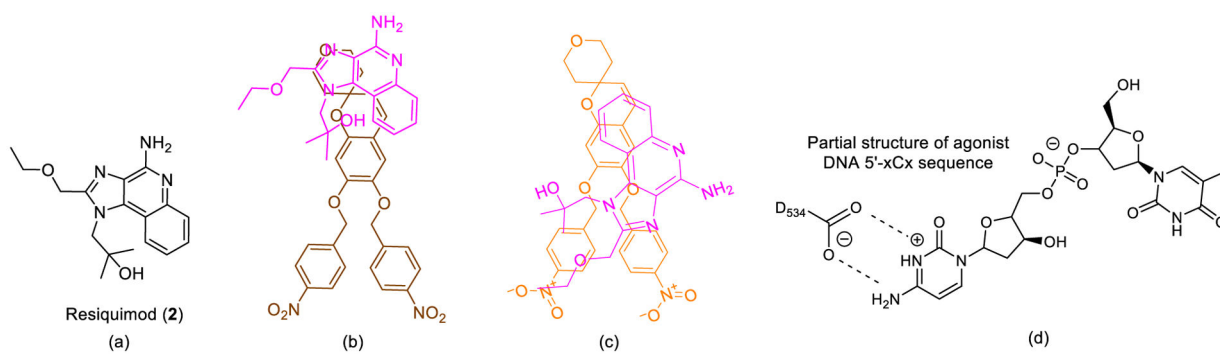


Figure 2.

(a) Structure of resiquimod (**2**, R848). (b) 2D Binding overlay of **1** and resiquimod (pose 1). (c) 2D Binding overlay of **1** and resiquimod (pose 2). (d) Partial structure of 5'-xCx DNA, H-bonding to D₅₃₄ in TLR9 agonist binding site.

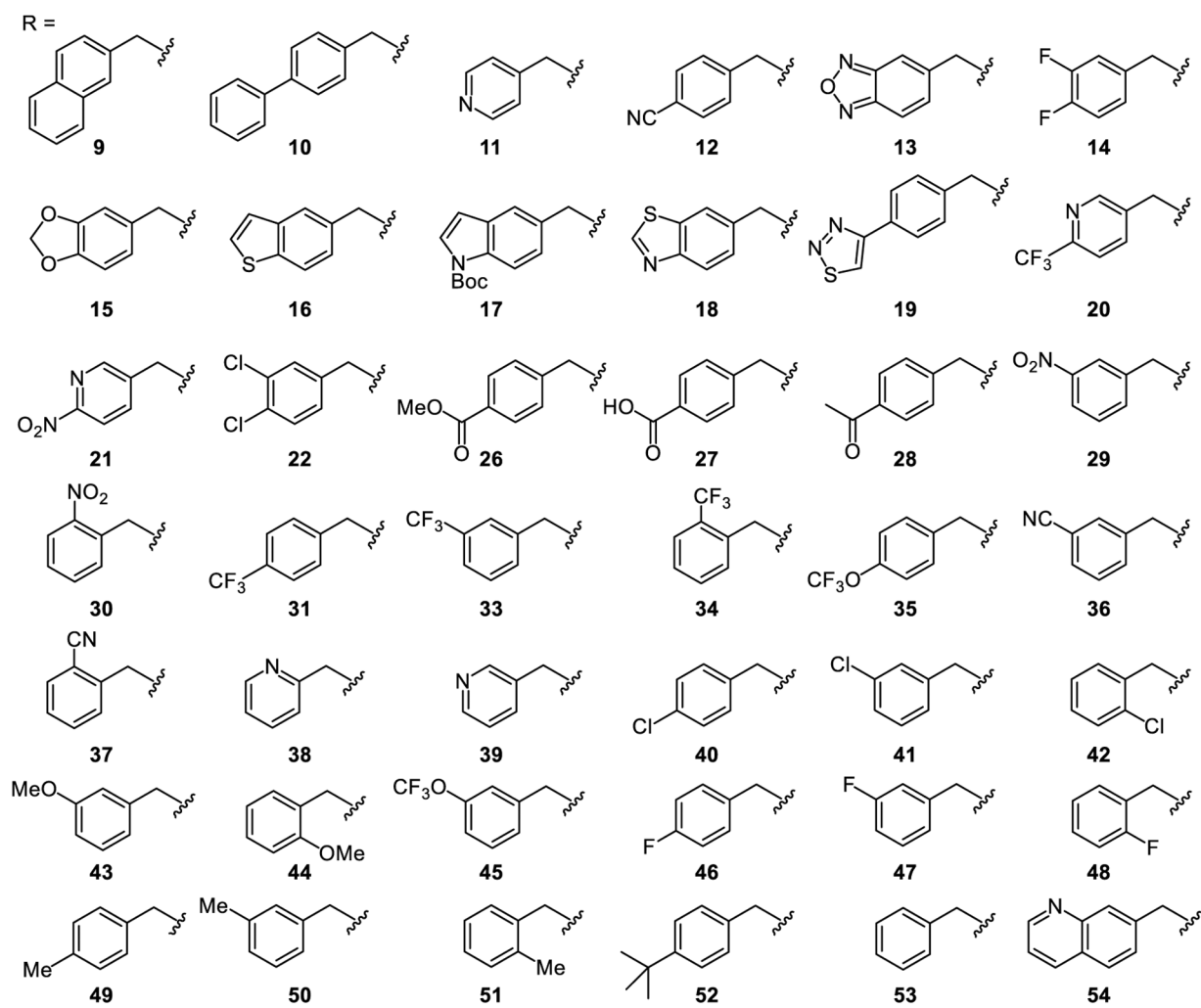


Figure 3.
Structures of compounds 9–22, 26–54.

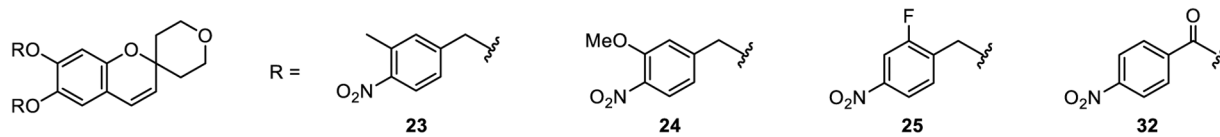


Figure 4. Structures of **23–25**, disubstituted benzyl derivatives containing a 4-nitro group, and 4-nitrobenzoyl derivative **32**.

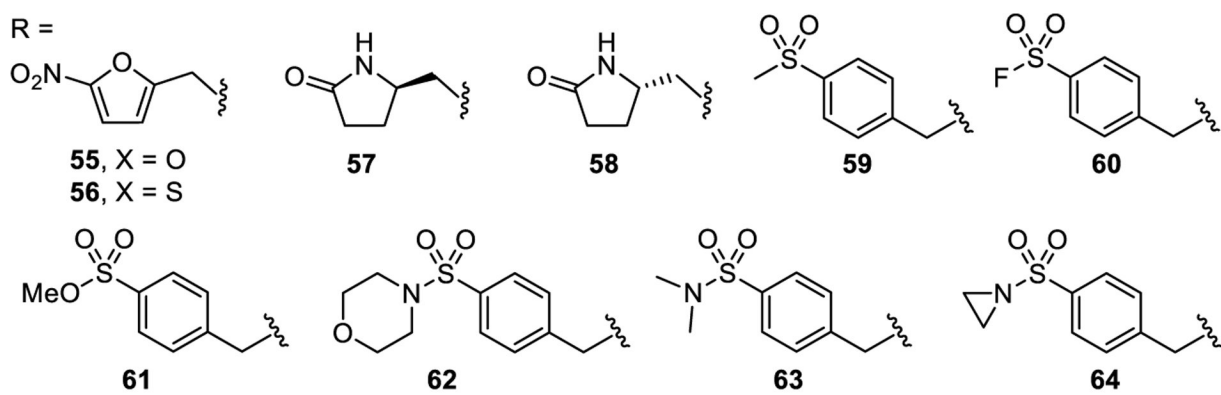


Figure 5.
Structures of compounds **55–64**.

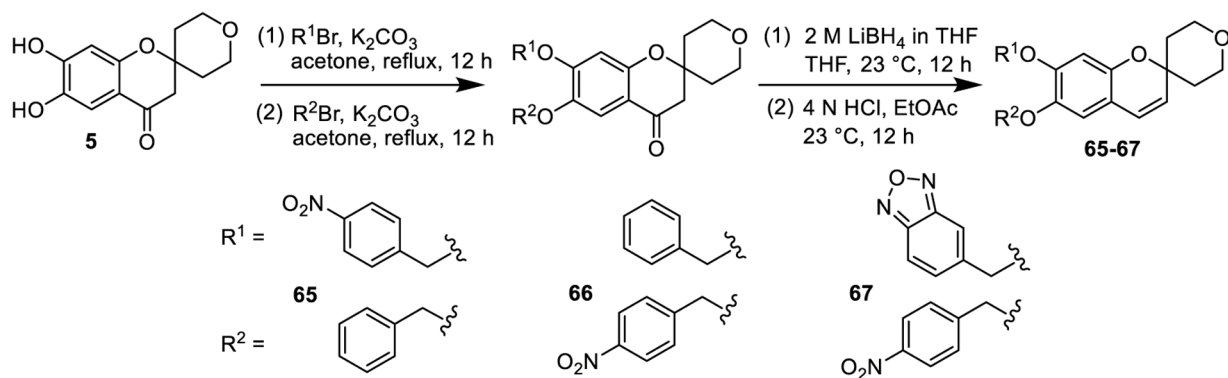
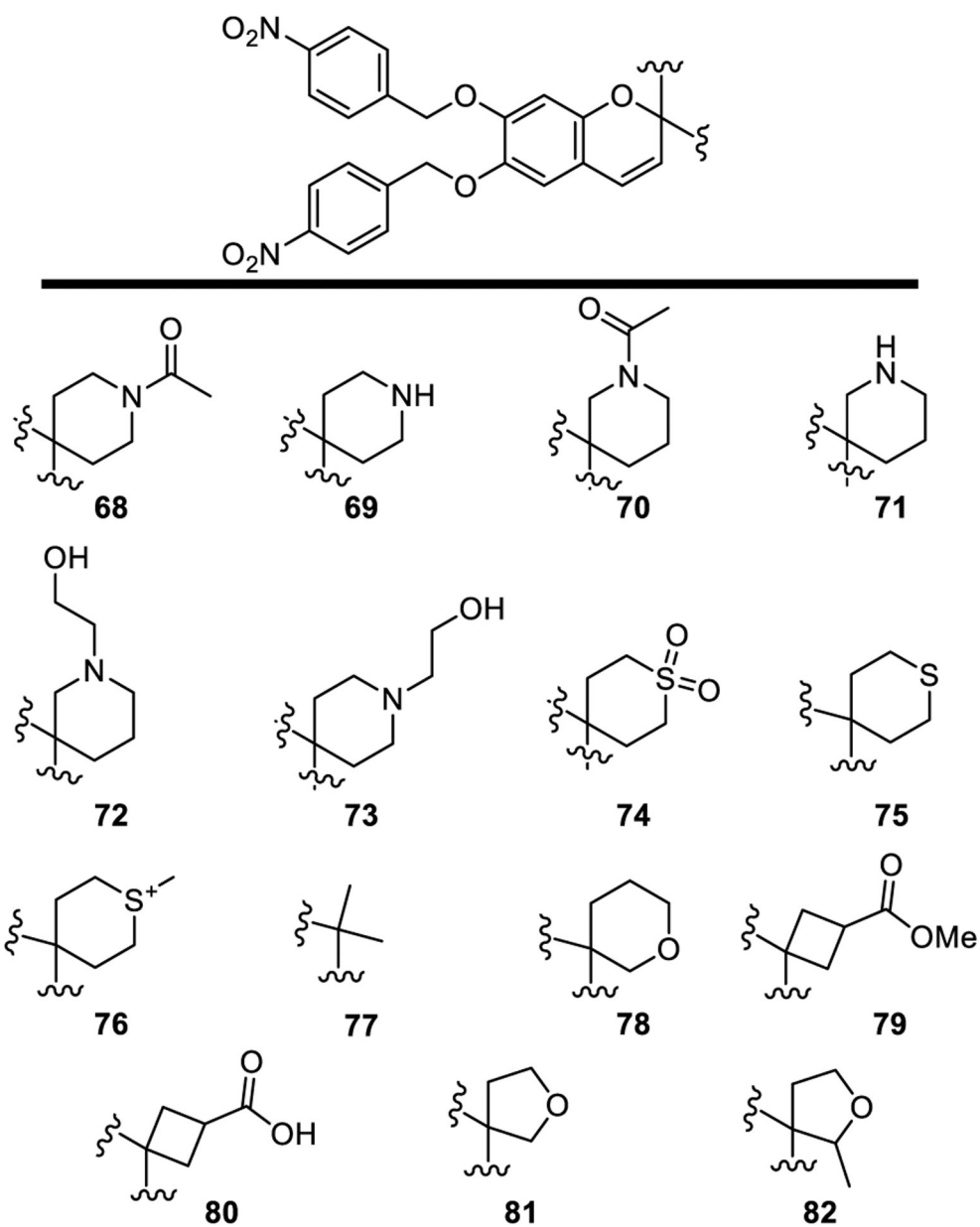
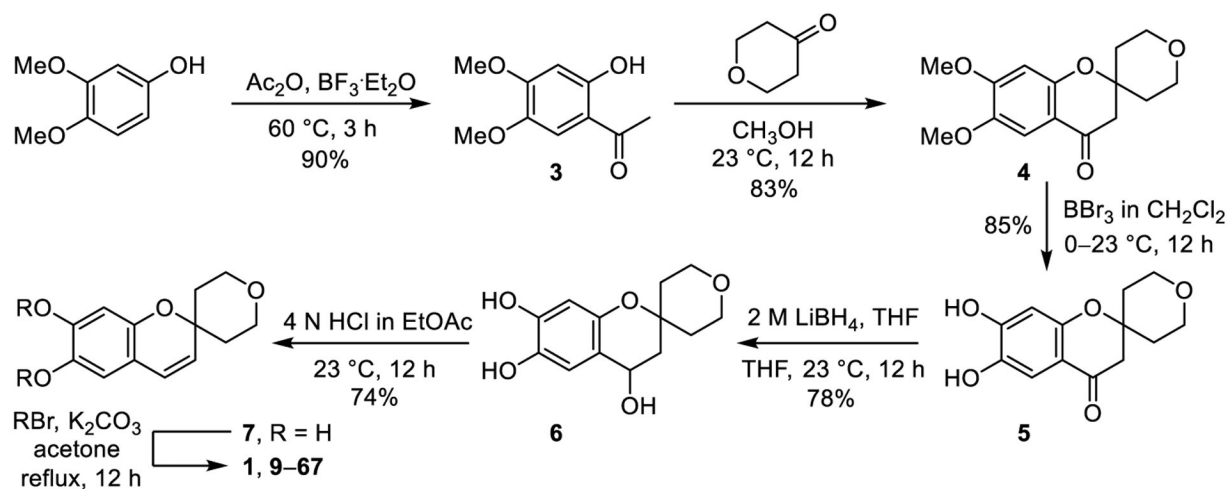
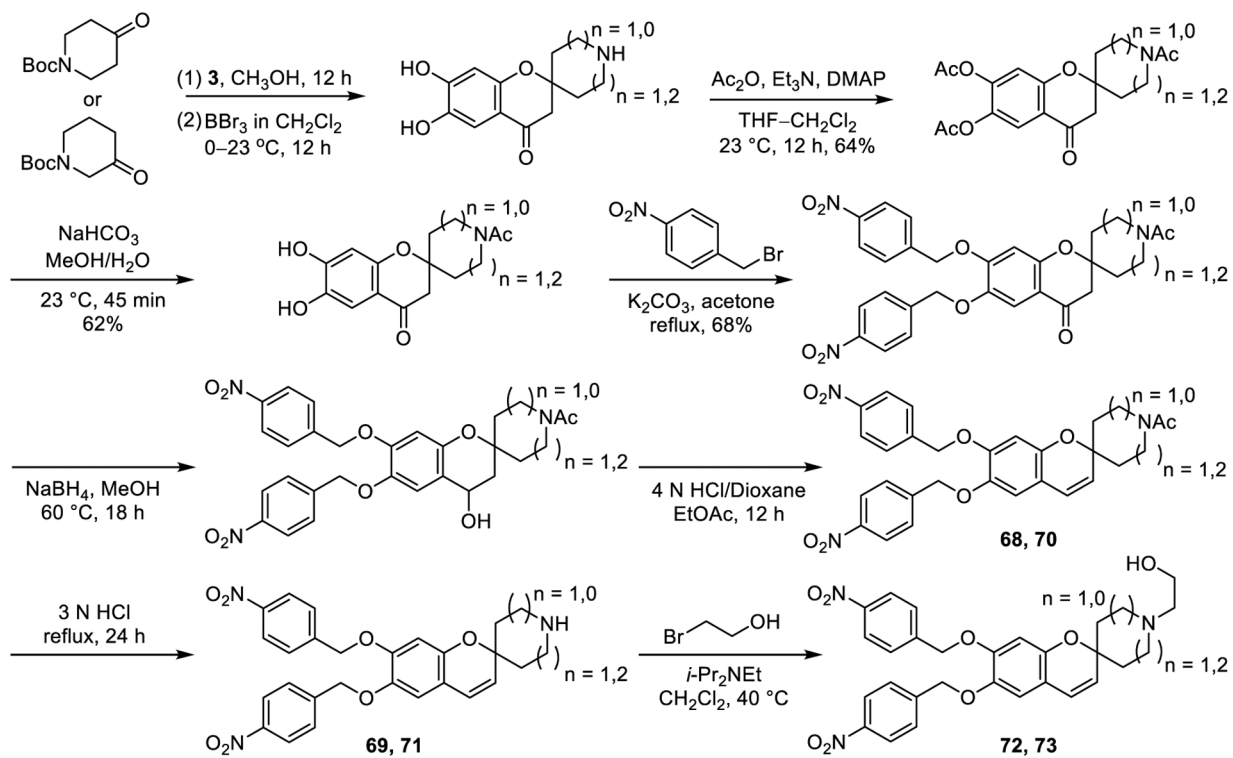


Figure 6.
Preparation and structure of unsymmetrical derivatives.

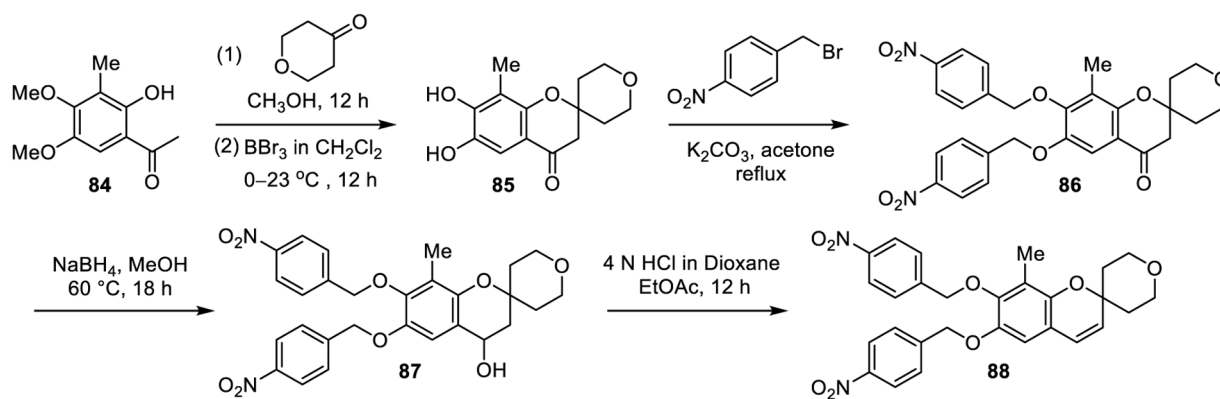




Scheme 1.
Synthesis of compounds **1** and **9-67**



Scheme 2.
Preparation of candidate agonists with a piperidine subunit



Scheme 3.
Synthesis of compound **88**