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Structure–Activity Relationship Studies of Pyrimido[5,4-*b*]indoles as Selective Toll-Like Receptor 4 Ligands

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Abstract

Previous high throughput screening studies led to the discovery of two novel, nonlipid-like chemotypes as Toll-like receptor 4 (TLR4) agonists. One of these chemotypes, the pyrimido[5,4-*b*]indoles, was explored for structure–activity relationship trends relative to production of TLR4 dependent cytokines/chemokines, resulting in a semioptimized lead (compound 1) that provided a starting point for further optimization studies. In this report, compounds belonging to three areas of structural modification were evaluated for biological activity using murine and human TLR4 reporter cells, primary murine bone marrow derived dendritic cells, and human peripheral blood mononuclear cells. The compounds bearing certain aryl groups at the C8 position, such as phenyl (36) and β -naphthyl (39), had potencies significantly greater than compound 1. Compound 36 displayed human TLR4 agonist activity at submicromolar concentrations. The computational analysis suggests that the improved potency of these C8-aryl derivatives may be the result of additional binding interactions at the interface of the TLR4/myeloid differentiation protein-2 (MD-2) complex.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b00797.

¹H NMR, ¹³C NMR, and HRMS and LC/MS spectra of all tested compounds **2–41**; ¹H NMR spectra of compounds **23a** and **23c** (expanded aromatic region) (PDF)

Molecular formula strings for all tested compounds **2–41** (CSV)

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Notes

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INTRODUCTION

Receptors on cells of the innate immune system recognize a wide variety of microbial pathogens, such as viruses, bacteria, fungi, and parasites, through engagement with pathogen-associated molecular patterns (PAMPs) present on those pathogens. These receptors are known as pattern-recognition receptors (PRRs) and comprise a great variety of receptor types that may be cytosolic or membrane associated with respect to cellular location and function. One such family of receptors, the Toll-like receptors (TLRs), is the most well understood and well studied family of innate immune receptors,¹ and is known to promote a rapid response to perceived threats as well as to help direct the subsequent adaptive immune response that is most appropriate for the specific pathogenic threat. The innate response to a pathogen can be decisive in determining both the nature and magnitude of the adaptive response.²⁻⁴ Consequently, TLR agonists are being developed for the treatment of cancer, allergies, and infectious diseases, including as adjuvants in prophylactic and therapeutic vaccines.⁵

Most of the microbial ligands for the TLRs are macromolecules such as lipopolysaccharide (LPS), double-stranded RNA, and hypomethylated CpG-containing DNA.⁶ However, several synthetic small molecules have also been found to bind to and activate certain TLRs. Examples include the TLR7/8 agonistic imidazoquinolines,^{7,8} such as imiquimod⁹ and resiquimod,¹⁰ isotorabine,¹¹ and the 9-substituted 8-oxoadenines,¹²⁻¹⁴ as well as the Pam₂CS-type TLR2 agonists.¹⁵ More recently, new classes of non-LPS-like small molecules, the neoseptins,^{16,17} and euodenine A and analogues,¹⁸ have been reported to be myeloid differentiation protein-2 (MD-2) dependent TLR4 agonists, reinforcing the view that strong TLR4/MD-2 agonists need not necessarily mimic LPS. In addition, high throughput screening (HTS) studies from our laboratory using THP-1 NF- κ B CellSensor cells and Förster resonance energy transfer (FRET) assay to identify small molecule activators of innate immune receptors have recently led to the discovery of two new chemotypes that bind and activate TLR4, and these studies were facilitated by the development of novel cluster enrichment analysis methods.¹⁹ Thus, certain pyrimido[5,4-*b*]indoles^{20,21} and 4-substituted aminoquinazolines²² were shown to specifically activate TLR4 in a manner that was MD-2 dependent and CD14 independent in both mouse and human cells. TLR4 agonists are of particular interest because there is extensive human vaccine data suggesting that TLR4 based ligands, such as monophosphoryl lipid A in the licensed vaccine Cervarix, are safe and effective vaccine adjuvants.²³ Moreover, synthetic

small molecules as TLR4 agonists may have distinct advantages over the larger, lipid-like molecules, in that they could more easily be structurally optimized, scaled up, and may have greater stability as a drug product. A disadvantage of synthetic small molecules may be the greater potential for off-target effects.

In our previous structure–activity (SAR) study,²⁰ synthetic modifications of the pyrimido[5,4-*b*]indole scaffold at the carboxamide, N3, and N5 positions revealed differential effects on TLR4 dependent production of NF- κ B and type I interferon associated cytokines, interleukin-6 (IL-6), and interferon gamma-induced protein 10 (IP-10) in primary murine bone marrow-derived dendritic cells (mBMDC), respectively. A subset of the compounds bearing phenyl and substituted phenyl carboxamides induced lower IL-6 release while maintaining IP-10 production at a relatively higher level, suggesting preferential stimulation of the type I interferon pathway. Substitution at N5 with short alkyl substituents reduced the cytotoxicity of the leading hit compound while maintaining TLR4 agonist activity. Thus, the lead compound identified in the preliminary SAR study was the N5 methyl derivative, compound **1**²⁰ (Figure 1).

In the current study, we report the results of further SAR efforts in the pyrimido[5,4-*b*]indole scaffold with the goal of improving the potency of compound **1**. Three categories of modification of this ring system were explored, while independently keeping all other structural features of the lead compound constant as depicted in Figure 1: (1) modifications of the 2-thioacetamide moiety to include replacement of the sulfur by oxygen and nitrogen, lengthening the chain by adding methylene groups between the sulfur and carbonyl functions, and replacing the carboxamide function with methylene groups, (2) truncated derivatives in which the benzo ring of the indole portion was either removed or replaced by methyl groups or cycloalkyl (nonaromatic) groups, and (3) derivatives substituted at the C7 or C8 position of the full ring system.

RESULTS AND DISCUSSION

2-Thioacetamide Analogues and Derivatives

To determine whether the sulfur atom at the C2 position of compound **1** is required for TLR4 activity, we prepared derivatives wherein the sulfur was replaced by oxygen (compound **9**) or nitrogen (compound **6a**) while maintaining all other structural features consistent with compound **1**. Thus, treatment of compound **1** with *m*-chloroperoxybenzoic acid provided the corresponding sulfone (**2**) along with a minor amount of the sulfoxide (**3**). The sulfone was reacted with hydrogen bromide to yield the 2-bromo derivative, compound **4**, which served as a versatile intermediate to provide both the oxygen and nitrogen analogues. Condensation of **4** with preformed side chain substituents *N*-cyclohexyl-2-aminoacetamide (**5a**, prepared from *N*-Boc-glycine) or *N*-cyclohexyl-3-aminopropanamide (**5b**, prepared from *N*-Boc- β -alanine) provided the nitrogen analogue **6a** and its chain extended analogue **6b**, respectively (Scheme 1a). The chain extended analogue **6b** was prepared because a sulfur atom, found in the original hit compound **1**, occupies more than the space of one nitrogen atom, and we desired to cover the equivalent space by adding one more methylene group to the chain, thereby resulting in compound **6b**. For the oxygen

analogue of **1**, compound **4** was reacted with aqueous HCl to yield the C2-hydroxy derivative **7**, which was then reacted with *tert*-butyl bromoacetate to provide the intermediate ester **8**. Deprotection of the ester with TFA followed by 1-[Bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) promoted condensation with cyclohexylamine provided the oxygen analogue **9**.

Concurrently, it was of interest to explore the SAR effects of (1) lengthening the thioacetamide chain of compound **1** and (2) replacing the carboxamide moiety with methylene groups while keeping all other structural features constant (Scheme 1b). Thus, reaction of the 2-thioxo intermediate **10** (reported previously²⁰) with *tert*-butyl 3-bromopropionate or *tert*-butyl 4-bromobutyrate provided the esters **11a** and **11b**, respectively. Deprotection of the esters with trifluoroacetic acid (TFA) followed by HATU promoted condensation with cyclohexylamine yielded the chain lengthened derivatives **12a** and **12b**. Alkylation of intermediate **10** with (3-bromopropyl)-cyclohexane gave the analogue (**13**) containing no carboxamide function but having the equivalent number of atoms in the chain from the sulfur atom to the cyclohexyl group.

We used three cell types to evaluate the potency of the compounds in this series; murine (m) and human (h) TLR4 reporter cells and primary mBMDC (Table 1). In these assays, the cells were incubated with graded doses of compounds and activation of NF- κ B via TLR4 was measured by secreted alkaline phosphatase (SEAP) in the culture supernatants. In TLR4 reporter cell assays, two parameters, area under the curve (AUC) compared to compound **1** (%AUC) and EC₅₀ (concentration of compound at which 50% of maximum activation is achieved), were used to compare the potency of SAR derivatives. Although the EC₅₀ is widely used for this purpose, the %AUC could account for the potency of compounds that did not yield a dose response curve suitable for EC₅₀ calculations due to the shape of the curve and also to take into consideration the relative maximum response of each compound. Compounds were also tested in primary mBMDCs for their ability to induce IL-6 secretion. mBMDCs were incubated with each compound at 5 μ M, and levels of cytokine (IL-6) release in the culture medium were compared to the level of IL-6 release by compound **1** (%IL-6). Results using these parameters showed that all modifications made to the 2-thioacetamide moiety led to essentially total loss of biological activity, suggesting the importance of (1) the presence of both the sulfur atom (in the nonoxidized form) and the carboxamide function and (2) the strict spatial distance between them for activity. These trends are consistent with the observed levels of cytokine (IL-6) release (Table 1). Toxicity was assayed using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based method. Briefly, mBMDC treated with SAR compounds overnight were incubated with an MTT solution for several hours and the reductive state of MTT in the cells was measured by OD_{570–650} (optical density at 570–650 nm). Curiously, all analogues and derivatives in this series were found to be somewhat toxic, with the exception of the nitrogen analogues **6a** and **6b** (Table 1).

Truncated Scaffold Derivatives

One method that can help to determine the pharmacophore, or the minimum structural features required for the desired biological activity, is to systematically remove or truncate

portions of the lead compound while keeping all other structural features constant and observe the effects on biological activity. Indeed, biological activity may even be enhanced in cases where better “fit” or binding to the target occurs as a result of such modifications. In our previous study, we observed that compounds in the original HTS library that were truncated at the benzo ring portion of the pyrimidoindole scaffold were inactive in the THP-1 NF- κ B FRET assay. However, these truncated library compounds did not have the same moieties at the other positions that were later found to be absolutely essential for agonistic activity at the TLR4 receptor. Therefore, definite conclusions could not be drawn relative to the contribution of the benzo ring to the overall activity because the other groups, such as the substituted 2-thioacetamide function and the N3-phenyl group, were missing in the truncated compounds originally tested in the HTS library. With this in mind, we prepared derivatives of compound **1** wherein the benzo ring was removed (Scheme 2) to result in the pyrrolo[3,2-*d*]pyrimidine ring system while the rest of the scaffold was held constant.

For the unsubstituted truncated derivatives, ethyl 3-amino-1*H*-pyrrole-2-carboxylate (**16a**) was used as the starting material and was reacted with phenyl isothiocyanate to form the thiourea intermediate (**17a**), followed by acid-catalyzed ring closure to form the 2-thioxo-pyrrolo[3,2-*d*]pyrimidine (**18a**). Alkylation of the thioxo function with chloroacetic acid provided the 2-mercaptoacetic acid derivative (**19a**). Condensation of the acetic acid intermediate with cyclohexylamine catalyzed by HATU yielded the *N*-cyclohexyl acetamide derivative (**20a**). Finally, the N5-methyl derivative (**21a**) was prepared by reaction of **20a** with sodium hydride followed by methyl iodide. The N5-methyl derivative was prepared so as to allow for determination of the effects of alkylation on the toxicity and activity of the truncated derivative as a direct comparison to the nontruncated lead, compound **1**.

The synthesis of substituted truncated derivatives required the preparation of precursors leading to the appropriate substituted pyrroles because these were not readily available commercially. Thus, the corresponding dimethylpyrrole (**16b**) was prepared in two steps from 3-cyano-2-butanone (**14b**) by reaction with glycine ethyl ester followed by base-catalyzed ring closure of the resulting enamine (**15b**) using sodium ethoxide in ethanol. Once the pyrrole intermediate was formed (**16b**), the same general five-step procedure was followed to prepare the corresponding pyrrolo[3,2-*d*]pyrimidine derivatives **20b** and **21b**. Preliminary TLR4 screening data for these compounds showed no activity for compound **21a** and weak activity for **21b**, suggesting that hydrophobic groups may be preferred at the site of truncation. With this in mind, we extended the synthetic scheme to include derivatives that contain a fused cycloalkyl moiety at the truncation site, namely the cyclohexyl and cycloheptyl derivatives, **20c** and **21c** and **20d** and **21d**, respectively, synthesized as mentioned earlier. The starting material for the cyclohexyl series was 2-oxocyclohexanecarbonitrile (**14c**) and 2-oxocycloheptanecarbonitrile (**14d**) for the cycloheptyl series. The potency of compounds in this series was compared in the same manner we employed in Table 1 (Table 2). In murine and human TLR4 reporter cells, the potency increased (higher %AUC and lower EC₅₀ values) as the hydrophobic groups increased in size at the site of truncation (Table 2). Thus, while the unsubstituted derivative **21a** was inactive, the dimethyl derivative **21b** was weakly active and the cycloalkyl derivatives were yet more active in this series, with the cycloheptyl derivative **20d** being the

most active. Indeed, the cycloheptyl derivative **20d** had about the same activity as the original lead, compound **1**. In connection with this trend was the observation, particularly in the cycloalkyl series, that N5 substitution of hydrogen by methyl (R^3 group) lowers the potency of TLR4 activation significantly with little effect on toxicity, although most derivatives in this truncated series are relatively nontoxic, as indicated by the MTT assay results (Table 2).

This observation contrasts with earlier SAR findings for the nontruncated derivatives in which methylation at N5 did indeed reduce toxicity and had little effect on TLR4 activity relative to the nonmethylated derivatives.²⁰ Interestingly, SAR trends in this series were very similar in both the mouse and human TLR4 reporter cells, although all derivatives including the comparator compound **1** were less potent in human reporter cells relative to mouse reporter cells.

Compounds were also tested in primary mBMDC for their ability to induce IL-6 secretion (Table 2). With the exception of the nonsubstituted derivatives **20a** and **21a**, all derivatives in this series were capable of inducing IL-6 secretion similar to that of compound **1**, with compound **20d** identified as the most active inducer. In general, the levels of IL-6 activity for the derivatives correlated well with their respective levels in the mTLR4 reporter cells. Toxicity results showed all compounds in the truncated series were nontoxic, with the exception of slight toxicity for **20b**.

C7- and C8-Substituted Pyrimido[5,4-*b*]indoles

Our next SAR efforts focused on substitution on the benzo ring of the pyrimido[5,4-*b*]indole scaffold as opposed to truncating this ring. The goal of this effort was to explore the effects on TLR4 activity of substituting a variety of hydrophobic and hydrogen bonding groups at the C7 or C8 position of compound **1** in order to potentially enhance TLR4 interactions. The C7 and C8 positions were chosen based primarily on availability of the commercial starting materials. The first groups considered for substitution were the halogens, not only of interest for probing that site with large, nonhydrogen bonding groups, but also as intermediates for further substitutions at those sites. It was thought that perhaps the easiest method to introduce a bromine or iodine atom would be through electrophilic aromatic substitution directly on compound **1**, as we expected that the site for such substitution would likely be the C8 position. However, treatment of compound **1** with electrophilic bromination reagents, such as *N*-bromosuccinimide in *N,N*-dimethylformamide (DMF) or bromine in water, under a variety of conditions, resulted in complex mixtures of many products with very little of the desired product detected by LC-MS methods. Thus, an alternative route for introduction of a halogen into the C8 position was pursued in which the halogen was already present in the starting material, 5-bromo-2-aminobenzonitrile (**22a**), and carried throughout the entire seven-step synthesis in the same manner that was used to prepare compound **1** originally (Scheme 3). By this method, the C8-bromo (**30a**) and C8-iodo (**30b**) derivatives of compound **1** were prepared. In addition, the C7-bromo (**30c**) derivative was prepared by the same general method but starting instead with 4-bromo-2-aminobenzonitrile (**22c**). To verify the correct assignments of the regioisomers of the C7 and C8 series, proton NMR spectra of the products of the first step (**23a** and **23c**) in the synthetic scheme were compared and

confirmed. Indeed, the spectrum of **23a** was identical to that reported recently for the same structure prepared by a different route.²⁴

The bromo derivatives **30a** and **30c** served as versatile intermediates for further substitutions to introduce a variety of other groups including cyano, azido, amino, acylamino, and aryl. Thus, the C8-cyano (**31a**), C7-cyano (**31c**), and C8-amino (**32**) derivatives were prepared by copper(I) catalyzed nucleophilic aromatic substitution as depicted in Scheme 3.

The C8-amino derivative (**32**) was used as a handle for further modifications at the C8 position, including acylations, to form the benzamido (**33**), octanamido (**34**), and succinamido (**35**) derivatives (Scheme 4). The carboxamides (**33–35**) were prepared to help determine whether acylation of the primary amine of **32** (later found inactive in TLR4 agonism assay) could recover biological activity and also to provide a chemical handle for subsequent conjugations. Examples of three types of carboxamides were prepared: an aromatic (**33**), an aliphatic (**34**), and a dicarboxylic acid amide (**35**).

The C8-aryl derivatives were prepared from **30a** by a Suzuki reaction using the appropriate aryl boronic acid to provide the C8-phenyl (**36**), the C8-biphenyl (**37**), and the C8-naphthyl (**38** and **39**) derivatives (Scheme 5). In addition, the C8-ethynyl (**41**) derivative, for later use in “Click” chemistry applications, was prepared by a Sonogashira procedure through the TMS intermediate (**40**).

An inspection of the %AUC and EC₅₀ values using murine and human TLR4 reporter cells and %IL-6 release by primary mBMDC (Table 3) for this series of compounds revealed a few SAR trends. First, substitution at the C8 position by large, nonhydrogen bonding groups, such as bromo (**30a**) or iodo (**30b**), resulted in enhancement of activity while hydrogen bonding groups at that position, such as amino (**32**), cyano (**31a**, **31c**), or carboxamido (**33–35**), resulted in a significant loss of activity. The potency of the C8-ethynyl derivative (**41**) was similar to that of compound **1**.

Second, substitutions at the C7 position were not as favorable for activity compared to their C8-substituted counterparts (**30a** versus **30c**). Finally, compounds bearing aryl groups at the C8 position, particularly the C8-phenyl (**36**) and C8- β -naphthyl (**39**) derivatives were the most active of all derivatives so far tested in hTLR4 reporter cells. Interestingly, the C8- α -naphthyl derivative (**38**) was less active while the C8-biphenyl derivative (**37**) was much less active and somewhat toxic compared to compound **1**.

An interesting observation was noted relative to species differences in activity profile for the derivatives in this series, particularly the C8-aryl derivatives. The in vitro TLR4 reporter cell data for the mouse versus human receptors clearly shows that the C8-phenyl derivative (**36**) is the most potent derivative in both mouse and human TLR reporter cells with activity at nanomolar concentrations, while the C8- β -naphthyl derivative (**39**) is potent in human reporter cells more so than in mouse reporter cells (Table 3). The other derivatives in the series were only very weakly active in human reporter cells by comparison. In general, immunostimulatory activities in mBMDC showed similar trends with the potencies determined by mTLR4 reporter cells (Table 3).

In vitro toxicity by MTT assay showed most compounds to be nontoxic at 5 μ M, except for the C8-carboxamido (**33–35**) compounds, which were somewhat toxic, and the C8-biphenyl compound (**37**), which was surprisingly toxic as mentioned earlier.

Evaluation of Selected Compounds in Mouse and Human Primary Cells

To follow up on the potency observed for several of the derivatives in mTLR4 and hTLR4 reporter cells, we selected compounds of interest from our SAR studies and evaluated them in primary human cells compared to mouse cells (Figures 2 and 3) for induction of cytokine release. Thus, mTLR4 reporter cells or mBMDC were treated with graded doses of compounds **1**, **21d**, **36**, and **39**, and NF- κ B activation, cytokine (IL-6), and chemokine (CCL5) were assessed by enzyme-linked immunosorbent assay (ELISA) (Figure 2). In three measurements, compound **36** showed the superior potency to **1**, **21d**, or **39**. Next, hTLR reporter cells and human peripheral blood mononuclear cells (hPBMC) were treated with compounds **1**, **21d**, **36**, and **39**, and compared to **1Y136**, a 4-aminoquinazoline derivative was used as a positive control (Figure 3).²² Previous studies from our laboratory²² established **1Y136** as a TLR4 agonist with significant selectivity toward human versus mouse TLR4. After treatment, cell supernatants were assayed for IL-8 and IL-6 by ELISA, and results are displayed in Figure 3. Compounds **36** and **39** that showed enhanced potencies in hTLR4 reporter cells induced significantly higher IL-8 and IL-6 from hPBMCs in comparison to compound **1** (Figure 3A–C). Toxicity measurement by MTT assay in human hepatocellular carcinoma cell line, HepG2 cells, showed there was no significant difference among these compounds (Figure 3D).

Evaluation of Immunostimulatory Potency in Vivo

The observed in vitro activity of the more potent compounds was confirmed in vivo by intravenous (iv) administration of compounds **1**, **21d**, and **36** to mice followed by determination of levels of secreted cytokines/chemokines in the sera. Thus, measured levels of IL-6, KC (keratinocyte chemoattractant; CXCL1), or IP-10 at 3 h post injection indicate that compound **21d** induced about the same level of each relative to compound **1**, whereas **36** induced significantly higher levels of the chemokines KC and IP-10 and showed a trend toward higher IL-6 levels compared to **1** (Figure 4). Thus, addition of the phenyl group at C8 position significantly enhanced the production of cytokines and chemokines relative to compound **1** in both in vitro and in vivo assays.

Computational Studies

Binding of TLR4 agonists such as LPS to the TLR4/MD-2 complex causes dimerization of the extracellular domains to form a TLR4/MD-2/LPS macromolecular complex, a crystal structure of which has been solved at 3.1 Å resolution for the human complex.²⁵ The LPS in the complex binds to a large hydrophobic pocket in the MD-2 protein that is formed by two antiparallel β -sheets. Previous computational studies from our laboratory²⁰ predicted that active small molecules in the pyrimido[5,4-*b*]indole scaffold bind primarily in the LPS pocket of MD-2 in the TLR4/MD-2 complex in such a way as to facilitate oligomerization leading to TLR4 signaling. To examine possible explanations for the significantly improved potency in human cells of the C8-phenyl (**36**) and C8- β -naphthyl (**39**) derivatives relative to

the original lead compound **1** (Table 3), we explored the predicted binding mode(s) of compound **36** with the TLR4/MD-2 complex by conducting molecular docking experiments using the crystal structure of the human complex (PDB 3FXI). We selected the most energy favorable configuration of **36** (shown in magenta) bound to this complex and compared the binding to that of compound **1** (shown in orange) and to LPS (shown in gray) bound to the complex (Figure 5A) using the server based SwissDock software (<http://www.swissdock.ch/docking>),^{26,27} a service provided by the Swiss Institute of Bioinformatics.

Of particular interest is the presence of a subpocket between MD-2 (shown as solid surface in cyan) and part of TLR4 (shown as mesh surface in green) in the LPS binding pocket. Computational docking studies showed that this critical site between MD-2 and TLR4 is occupied by the C8-phenyl substituent (Figure 5B). This is also the same subpocket that is occupied by one of the long-chain acyl groups of the lipid A portion of LPS, shown superimposed with compound **36** in the complex (shown in gray, Figure 5A). Occupation of the subpocket, which is in close proximity to the TLR4 protein, should provide additional binding interaction with TLR4 compared to the reference compound **1**, the original lead. The overlay of binding geometry between compound **1** and **36** shows nearly exact superimposition for the pyrimidoindole ring system (Figure 5C). The extended C8-phenyl substituent of compound **36** is involved in additional interactions with hydrophobic residues Val82, Leu87, Ile124, Phe126, Ser127, and Tyr131 of MD-2 (cyan) and residues of the TLR4 (green) including Asn417, Phe440, Ser441, Leu444, and Phe463. Interestingly, several of these same hydrophobic residues of MD-2 were found to interact with the 4-aminoquinazolines²² (such as 1Y136) in the human TLR4/MD-2 complex, suggesting that both chemotypes bind in the same approximate area of the complex. These interactions could explain the greater potency of **36** for TLR4 signaling compared to that of **1**. In connection with this observation, the C8- β -naphthyl derivative (**39**) seems to be more potent than the reference compound **1** mainly in human cells, although there is a statistically significant increase in the IL-6 release induced by **39** in primary mouse cells. Furthermore, the C8- α -naphthyl derivative (**38**) is significantly less active than its β -isomer in both human and mouse cells (Table 3), probably because the subpocket cannot accommodate the shape of the α -isomer as easily as that of the β -isomer. Indeed, the C8-biphenyl derivative (**37**) is essentially inactive, presumably because the C8-biphenyl group is so large that the compound is excluded from this binding mode altogether and adopts a completely different binding configuration in the complex, one that apparently does not promote dimerization and signaling. Preliminary docking data with compound **37** appears to confirm this suggestion.

CONCLUSIONS

Continuing SAR efforts in the pyrimido[5,4-*b*]indole scaffold aimed to improve the TLR4 agonist activity of compound **1**. Three categories of modifications were assessed: (1) modifications of the 2-thioacetamide moiety to include replacement of the sulfur by oxygen and nitrogen as well as lengthening the chain by adding methylene groups between the sulfur and carbonyl functions, (2) truncated derivatives in which the benzo ring of the indole moiety was either removed or replaced by methyl groups or cycloalkyl (nonaromatic)

groups, and (3) derivatives substituted at the C7 or C8 position of the full ring system. Of these three categories of modifications, compounds bearing certain non-hydrogen bonding groups at the C8 position, such as bromo (**30a**), iodo (**30b**), phenyl (**36**), and β -naphthyl (**39**), had potencies significantly greater than compound **1**, particularly for activating human TLR4 receptor. Compound **36** was a potent inducer of TLR4 dependent cytokines in vitro and in vivo. These observations will form the basis for further optimization efforts in this nonlipid-like chemotype to improve not only the potency for TLR4 activation but also to facilitate formulation strategies that will be important for efficient preclinical and clinical evaluation. Thus, optimized lead compounds in the pyrimido[5,4-*b*]indole class that stimulate innate immune cells with minimal toxicity may be useful as vaccine adjuvants or immunotherapeutic agents.

EXPERIMENTAL SECTION

Chemistry

Materials—Reagents were purchased as at least reagent grade from Sigma-Aldrich (St. Louis, MO) unless otherwise specified and used without further purification. Solvents were purchased from Fischer Scientific (Pittsburgh, PA) and were either used as purchased or redistilled with an appropriate drying agent. Endotoxin levels of active compounds were measured with Endosafe PTS (Charles River, Wilmington, MA) and found to have less than 2 EU/ μ mol. Compounds used for structure–activity studies were synthesized according to methods described below.

Instrumentation—Analytical TLC was performed using precoated TLC silica gel 60 F₂₅₄ aluminum sheets purchased from EMD (Gibbstown, NJ) and visualized using UV light. Flash chromatography was carried out using EMD silica gel 60 (40–63 μ m) or with a Biotage Isolera One (Charlotte, NC) system using the specified solvent. Reaction monitoring and compound purity analysis were done using an Agilent 1260 LC/6420 Triple Quad mass spectrometer (Santa Clara, CA) with either a Supelco Discovery HS C18 (Sigma-Aldrich) or an Onyx Monolithic C18 (Phenomenex, Torrance, CA) column. Purity of all final compounds was above 95% (also see LC-MS spectra in Supporting Information for each compound). All final compounds were analyzed by high resolution MS (HRMS) using an Agilent 6230 ESI-TOFMS (Santa Clara, CA). ¹H NMR spectra were obtained on a Varian Mercury 400 (Varian, Inc., Palo Alto, CA). ¹³C NMR spectra were obtained on a Varian 500 with XSENS probe. The chemical shifts are expressed in parts per million (ppm) using suitable deuterated NMR solvents in reference to tetramethyl silane (TMS) at 0 ppm. NMR spectra and HRMS are included in the Supporting Information.

N-Cyclohexyl-2-((5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)sulfonyl)acetamide (2**) and N-Cyclohexyl-2-((5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)sulfinyl)acetamide (**3**)**—To a solution of compound **1** (500 mg, 1.12 mmol) in a solvent mixture of methanol:chloroform 1:1 was added 3-chloroperoxybenzoic acid (1.4 mg, 8.05 mmol) and heated at 45°C for 1 h. Monitoring the reaction showed formation of predominantly compound **2** while a small amount of compound **3** was also formed due to incomplete oxidation. The solvent was then

removed, and the residue was purified by silica column chromatography (hexanes:EtOAc = 50:50) to obtain 475 mg of compound **2** in 89% yield and 5 mg of compound **3** in 1% yield. ^1H NMR for compound **2** (400 MHz, DMSO- d_6) δ 8.21 (d, J = 7.80 Hz, 1H), 8.17 (d, J = 7.80 Hz, 1H), 7.81 (d, J = 8.78 Hz, 1H), 7.66 (ddd, J = 1.46, 7.19, 8.41 Hz, 1H), 7.50–7.55 (m, 3H), 7.39–7.45 (m, 3H), 4.60 (s, 2H), 4.20 (s, 3H), 3.39–3.51 (m, 1H), 1.54–1.71 (m, 4H), 1.48 (d, J = 11.70 Hz, 1H), 1.02–1.27 (m, 5H). ^{13}C NMR for compound **2** (126 MHz, DMSO- d_6) δ 159.9, 154.7, 147.6, 140.6, 134.0, 133.5, 130.0, 129.8, 128.8, 128.4, 121.8, 121.6, 120.8, 120.3, 111.7, 58.6, 48.1, 32.2, 31.6, 25.3, 24.4. HRMS for compound **2** calcd for $\text{C}_{25}\text{H}_{26}\text{N}_4\text{O}_4\text{S}$ $[\text{M} + \text{Na}]^+$ 501.1577, found 501.1572.

2-Bromo-5-methyl-3-phenyl-3,5-dihydro-4H-pyrimido[5,4-b]indol-4-one (4)—

Compound **2** (475 mg) was dissolved in 5 mL of HBr in acetic acid. The reaction mixture was stirred for 5 min, followed by removal of the solvent to obtain compound **4** in quantitative yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.06 (d, J = 8.29 Hz, 1H), 7.74 (d, J = 8.29 Hz, 1H), 7.51–7.63 (m, 4H), 7.45–7.50 (m, 2H), 7.34 (t, J = 7.40 Hz, 1H), 4.13 (s, 3H). HRMS calcd for $\text{C}_{17}\text{H}_{13}\text{BrN}_3\text{O}$ $[\text{M} + \text{H}]^+$ 354.0237, found 354.0235.

2-Amino-N-cyclohexylacetamide (5a)—To a solution of *N*-Boc glycine (200 mg, 1.14 mmol) in anhydrous DMF were added HATU (477 mg, 1.25 mmol), triethylamine (173 mg, 1.71 mmol), and cyclohexylamine (119 mg, 1.2 mmol). The reaction mixture was stirred for 1 h, followed by addition of water to obtain precipitate. The precipitate was filtered, and the crude residue was purified using column chromatography to yield the intermediate *tert*-butyl (2-(cyclohexylamino)-2-oxoethyl) carbamate (262 mg, 64%). This intermediate compound (120 mg) was then stirred in 3 mL of 4 N HCl/dioxane for 4 h, followed by removal of the solvent to obtain the hydrochloride salt of compound **5a** in quantitative yield. MS calcd for $\text{C}_8\text{H}_{17}\text{N}_2\text{O}$ $[\text{M} + \text{H}]^+$ 157.1, found 157.0.

3-Amino-N-cyclohexylpropanamide (5b)—*N*-Boc- β -alanine (200 mg, 1.06 mmol), cyclohexylamine (127 mg, 1.28 mmol), HATU (1.16 mmol), triethylamine (221 μL , 1.59 mmol), and DMF (5 mL) were reacted similarly to **5a** to give compound **5b** 158.4 mg in 88% yield. MS calcd for $\text{C}_9\text{H}_{19}\text{N}_2\text{O}$ $[\text{M} + \text{H}]^+$ 171.1, found 171.1.

N-Cyclohexyl-2-((5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)amino)acetamide (6a)—To a solution of compound **4** (20 mg, 0.056 mmol) in EtOH was added **5a** (33 mg, 0.169 mmol) and heated at 120°C for 48 h. The solvent was then removed under vacuum, and the residue was purified by flash chromatography on silica (hexanes:EtOAc = 40:60) to obtain 13 mg of compound **6a** in 50% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 7.90 (d, J = 7.80 Hz, 1H), 7.87 (d, J = 7.80 Hz, 1H), 7.53–7.68 (m, 4H), 7.47 (t, J = 7.81 Hz, 1H), 7.35–7.44 (m, 2H), 7.17 (t, J = 7.56 Hz, 1H), 5.38 (t, J = 4.90 Hz, 1H), 4.02 (s, 3H), 3.88 (d, J = 4.88 Hz, 2H), 3.44–3.55 (m, J = 2.00 Hz, 1H), 1.58–1.78 (m, J = 14.40, 14.40 Hz, 4H), 1.46–1.57 (m, J = 10.20 Hz, 1H), 1.00–1.34 (m, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 167.6, 155.5, 149.3, 140.4, 138.7, 135.1, 130.2, 129.4, 129.3, 127.2, 120.4, 120.0, 119.3, 115.9, 110.5, 47.6, 44.7, 32.4, 30.8, 25.2, 24.5. HRMS calcd for $\text{C}_{25}\text{H}_{28}\text{N}_5\text{O}_2$ $[\text{M} + \text{H}]^+$ 430.2238, found 430.2242.

N-Cyclohexyl-3-((5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)amino)propanamide (6b)—Compound **4** (10 mg, 0.028 mmol), compound **5b** (18 mg, 0.106 mmol), triethylamine (24 μ L, 0.168 mmol), and EtOH were reacted similarly to compound **6a** to give 8.1 mg of compound **6b** in 65% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 7.93 (d, J = 7.81 Hz, 1H), 7.70 (d, J = 7.81 Hz, 1H), 7.52–7.65 (m, 4H), 7.47 (t, J = 7.80 Hz, 1H), 7.32 (d, J = 6.83 Hz, 2H), 7.16 (t, J = 7.32 Hz, 1H), 5.33 (t, J = 5.90 Hz, 1H), 4.01 (s, 3H), 3.52 (q, J = 5.90 Hz, 2H), 2.33 (t, J = 6.34 Hz, 2H), 1.59–1.77 (m, J = 10.20 Hz, 4H), 1.48–1.57 (m, J = 12.20 Hz, 1H), 1.00–1.30 (m, 6H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 170.0, 155.7, 149.6, 144.6, 140.5, 139.0, 135.1, 130.2, 129.4, 127.9, 120.8, 119.3, 115.8, 110.5, 47.4, 38.3, 34.7, 32.5, 30.9, 25.3, 24.7, 24.7. HRMS calcd for $\text{C}_{26}\text{H}_{30}\text{N}_5\text{O}_2$ [$\text{M} + \text{H}$] $^+$ 444.2394, found 444.2396.

2-Hydroxy-5-methyl-3-phenyl-3,5-dihydro-4H-pyrimido[5,4-b]-indol-4-one (7)—Compound **4** (65 mg, 0.18 mmol) was dissolved in 18% aqueous HCl solution (5.8M) and heated at 120°C for 10 h. The solvent was then removed under vacuum to obtain compound **7** in quantitative yield. ^1H NMR (400 MHz, DMSO- d_6) δ 12.14 (s, 1H), 8.02 (d, J = 8.30 Hz, 1H), 7.61 (d, J = 8.78 Hz, 1H), 7.39–7.53 (m, 4H), 7.29–7.35 (m, 2H), 7.19 (t, J = 7.32 Hz, 1H), 4.01 (s, 3H). MS calcd for $\text{C}_{17}\text{H}_{14}\text{N}_3\text{O}_2$ [$\text{M} + \text{H}$] $^+$ 292.1, found 291.9.

tert-Butyl 2-((5-Methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)oxy)acetate (8)—To a solution of compound **7** (15 mg, 0.051 mmol) in anhydrous DMF were added *N,N*-diisopropylethylamine (20 mg, 0.155 mmol) and *tert*-butyl bromoacetate (50 mg, 0.255 mmol). The reaction mixture was heated at 60°C for 2 h, followed by removal of the solvent under vacuum to obtain the residue which was purified using silica column chromatography (hexanes:EtOAc = 55:45) to yield 13 mg of compound **8** in 63% yield. MS calcd for $\text{C}_{23}\text{H}_{24}\text{N}_3\text{O}_4$ [$\text{M} + \text{H}$] $^+$ 406.2, found 406.0.

N-Cyclohexyl-2-((5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)oxy)acetamide (9)—Compound **8** was dissolved in 50:50 trifluoroacetic acid:acetonitrile solution and stirred for 4 h. The solvent was then removed and dried under vacuum to obtain the intermediate carboxylic acid derivative. The resulting residue was then dissolved in anhydrous DMF, and to this solution were added sequentially, cyclohexylamine (4.5 mg, 0.046 mmol), triethylamine (7 mg, 0.069 mmol), and HATU (19 mg, 0.051 mmol). The reaction mixture was stirred for 1 h, followed by removal of the solvent under vacuum. The residue was then suspended in methanol with sonication. The precipitate was filtered and washed with excess methanol to give 12 mg of compound **9** in 86.9% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.24 (d, J = 7.81 Hz, 1H), 7.79 (d, J = 8.29 Hz, 1H), 7.67 (d, J = 8.29 Hz, 1H), 7.39–7.58 (m, 4H), 7.30 (d, J = 7.32 Hz, 2H), 7.18 (t, J = 7.56 Hz, 1H), 4.93 (s, 2H), 4.07 (s, 3H), 3.49–3.66 (m, J = 15.10 Hz, 1H), 1.66 (br s, 4H), 1.53 (d, J = 11.22 Hz, 1H), 1.03–1.31 (m, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 165.7, 156.6, 151.2, 139.4, 136.3, 129.2, 129.1, 128.3, 127.8, 127.3, 121.3, 120.3, 114.3, 113.9, 111.3, 47.9, 47.4, 38.4, 32.4, 31.0, 25.2, 24.6. HRMS calcd for $\text{C}_{25}\text{H}_{26}\text{N}_4\text{O}_3$ [$\text{M} + \text{Na}$] $^+$ 453.1897, found 453.1896.

3-((4-Oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)-thio)propanoic Acid (11a)—3-Bromopropanoic acid (28.6 mg, 0.186 mmol) was added to a solution of

compound **10** (25 mg, 0.085 mmol), triethylamine (47.5 μ L, 0.341 mmol), and DMF (3 mL) and heated at 60°C with stirring for 4 h. The solution was concentrated in vacuo and used for the preparation of compound **11a** without further purification. MS calcd for C₁₉H₁₆N₃O₃S [M + H]⁺ 366.1, found 366.0.

4-((4-Oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)-thio)butanoic Acid (11b)—*tert*-Butyl 4-bromobutanoate (28.6 mg, 0.128 mmol), compound **10** (25 mg, 0.085 mmol), and triethylamine (23.8 μ L, 0.170 mmol) were dissolved in DMF (3 mL) and heated to 80°C until the reaction was completed. Organic solvent was removed by rotary evaporator, and the resultant solid was suspended in methanol (MeOH) and filtered. The collected solid was then dissolved in a solution of TFA (2 mL) and ACN (2 mL) and stirred at room temperature overnight. The solution was concentrated in vacuo, and the resulting solid was washed with acetonitrile (ACN) twice to give a white solid. Compound **11b** was used without further purification in the synthesis of compound **12b**. MS calcd for C₂₀H₁₈N₃O₃S [M + H]⁺ 380.1, found 379.9.

N-Cyclohexyl-3-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)propanamide (12a)—Compound **11a** (25 mg, 0.068 mmol), cyclohexylamine (7.9 μ L, 0.068 mmol), and triethylamine (9.53 μ L, 0.137 mmol) were dissolved in DMF (2 mL). HATU (26 mg, 0.068 mmol) was added to the solution and stirred at room temperature for 1 h. The solution was concentrated in vacuo to dryness, and MeOH (3 mL) was added and warmed gently. The suspension was filtered to give 19.5 mg of compound **12a** in 63.8% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.09 (s, 1H), 8.01 (d, *J* = 7.81 Hz, 1H), 7.64–7.79 (m, 2H), 7.35–7.62 (m, 6H), 7.18–7.31 (m, 1H), 3.46–3.55 (m, 1H), 3.34–3.39 (m, 2H), 2.52 (t, *J* = 6.80 Hz, 2H), 1.66 (br s, 4H), 1.53 (d, *J* = 12.20 Hz, 1H), 1.00–1.30 (m, 5H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 169.2, 155.2, 152.7, 139.0, 137.4, 136.3, 129.8, 129.6, 129.5, 127.4, 120.4, 120.3, 120.2, 119.3, 112.9, 47.5, 34.4, 32.5, 28.5, 25.3, 24.6. HRMS calcd for C₂₅H₂₆N₄O₂SNa [M + Na]⁺ 469.1669, found 469.1670.

N-Cyclohexyl-4-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)butanamide (12b)—Compound **11b** (21.8 mg, 0.057 mmol), cyclohexylamine (7.24 μ L, 0.063 mmol), HATU (24.03 mg, 0.063 mmol), triethylamine (8.8 μ L, 0.115 mmol), and DMF (2 mL) were reacted similarly to compound **12a** to give 25.2 mg of compound **12b** in 95.0% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.07 (s, 1H), 8.04 (d, *J* = 7.81 Hz, 1H), 7.71 (d, *J* = 7.81 Hz, 1H), 7.37–7.64 (m, 7H), 7.24 (t, *J* = 7.32 Hz, 1H), 3.45–3.55 (m, *J* = 2.90, 7.30 Hz, 1H), 3.18 (t, *J* = 7.32 Hz, 2H), 2.16 (t, *J* = 7.32 Hz, 2H), 1.89 (quin, *J* = 7.30 Hz, 2H), 1.58–1.75 (m, 4H), 1.53 (d, *J* = 12.20 Hz, 1H), 0.98–1.32 (m, 5H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 170.3, 155.2, 152.8, 139.0, 137.4, 136.3, 129.8, 129.6, 129.6, 127.4, 120.5, 120.3, 120.2, 119.3, 112.9, 47.4, 34.6, 32.6, 31.8, 25.3, 24.8, 24.7. HRMS calcd for C₂₆H₂₉N₄O₂S [M + H]⁺, 461.2006, found 461.2011.

2-((3-Cyclohexylpropyl)thio)-3-phenyl-3H-pyrimido[5,4-b]indol-4(5H)-one (13)—Compound **10** (20 mg, 0.068 mmol) and triethylamine (18.98 μ L, 0.136 mmol) were dissolved in DMF (2 mL) with heat. 3-Cyclohexylpropyl bromide (13.04 μ L, 0.75 mmol) was added to the reaction mixture and stirred at reflux until complete. The solution was

concentrated in vacuo, washed with MeOH, and recrystallized in ethanol to give 31 mg of compound **13** in 72.6%. ^1H NMR (400 MHz, DMSO- d_6) δ 12.07 (s, 1H), 7.99 (d, J = 7.81 Hz, 1H), 7.38–7.63 (m, 7H), 7.24 (t, J = 7.56 Hz, 1H), 3.15 (t, J = 7.32 Hz, 2H), 1.56–1.74 (m, 7H), 1.05–1.32 (m, 6H), 0.85 (q, J = 11.22 Hz, 2H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 155.0, 152.8, 138.8, 137.2, 136.2, 129.6, 129.4, 129.4, 127.2, 120.3, 120.1, 119.9, 119.1, 112.7, 36.4, 36.1, 32.6, 32.1, 26.0, 25.7. HRMS calcd for $\text{C}_{25}\text{H}_{27}\text{N}_3\text{OSNa}$ [$\text{M} + \text{Na}$] $^+$ 440.1767, found 440.1769.

Ethyl (Z)-(3-Cyanobut-2-en-2-yl)glycinate (15b)—To a solution of 3-cyano-2-butanone (**14b**, 1.00 g, 10.3 mmol) in EtOH (20 mL) were added glycine ethyl ester hydrochloride (1.44 g, 10.3 mmol) and Et_3N (1.44 mL, 10.3 mmol). The mixture was stirred at room temperature for 20 h. The solvent was evaporated in vacuo. The residue was treated with Et_2O and water. The water phase was extracted with Et_2O (2 $\times$). The organic phase was dried over Na_2SO_4 and evaporated in vacuo. The residue was crystallized from Et_2O to give compound **15b** (1.05 g, 56%). ^1H NMR (400 MHz, DMSO- d_6) δ ppm 6.33 (t, J = 6.4 Hz, 1H), 4.11 (q, J = 7.0 Hz, 2H), 3.92 (d, J = 6.6 Hz, 2H), 1.60 (s, 3H), 1.93 (s, 3H), 1.19 (t, J = 7.0 Hz, 3H).

Ethyl (2-Cyanocyclohex-1-en-1-yl)glycinate (15c)—To a solution of 2-oxocyclohexanecarbonitrile (**14c**, 1.00 g, 8.55 mmol) in EtOH (16 mL) were added glycine ethyl ester hydrochloride (1.19 g, 8.55 mmol) and Et_3N (1.19 mL, 8.55 mmol). The mixture was stirred at room temperature for 20 h. The solvent was evaporated in vacuo. The residue was treated with Et_2O and water. The water phase was extracted with Et_2O (2 $\times$). The organic phase was dried over Na_2SO_4 and evaporated in vacuo. The residue was purified by crystallization from Et_2O to give compound **15c** (1.52 g, 85%). ^1H NMR (400 MHz, DMSO- d_6) δ ppm 6.14 (t, J = 6.4 Hz, 1H), 4.10 (q, J = 7.3 Hz, 2H), 3.95 (d, J = 6.6 Hz, 2H), 2.09 (br s, 4H), 1.44–1.57 (m, 4H), 1.18 (t, J = 7.2 Hz, 4H).

Ethyl (2-Cyanocyclohept-1-en-1-yl)glycinate (15d)—To a solution of 2-oxocycloheptanecarbonitrile (**14d**, 150 mg, 1.09 mmol) in EtOH (2 mL) were added glycine ethyl ester hydrochloride (152 mg, 1.09 mmol) and Et_3N (150 μL , 1.09 mmol). After stirring for 20 h, the solvent was evaporated in vacuo. The residue was extracted with EtOAc. The mixture was washed with water and brine. The organic layer was dried over Na_2SO_4 , filtered, and evaporated in vacuo. The residue was filtered via silica gel to give compound **15d**. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 8.25 (t, J = 7.3 Hz, 1H), 4.18 (q, J = 7.0 Hz, 2H), 2.17 (t, J = 7.3 Hz, 4H), 1.41–1.53 (m, 7H), 1.18–1.30 (m, 9H). MS calcd for $\text{C}_{12}\text{H}_{19}\text{N}_2\text{O}_2$ [$\text{M} + \text{H}$] $^+$ 223.1, found 223.0.

Ethyl 3-Amino-4,5-dimethyl-1H-pyrrole-2-carboxylate (16b)—To a solution of compound **15b** (946 mg, 5.19 mmol) in EtOH (20 mL) was added a solution of NaOEt in EtOH (3.6 mL, 40 mg/mL, 6.22 mmol). The mixture was stirred at 70°C for 2.5 h and cooled to room temperature. The solvent was concentrated in vacuo. The residue was treated with EtOAc and water. The water phase was extracted with EtOAc. The combined organic phase was dried over Na_2SO_4 and concentrated in vacuo. The residue was roughly purified by filtration on silica gel (hexanes: EtOAc = 1:1) to give **16b**. ^1H NMR (400 MHz, DMSO-

d_6) δ ppm 10.09 (br s, 1H), 4.76 (br s, 2H), 3.99–4.25 (m, 2H), 2.01 (s, 3H), 1.73 (s, 3H), 1.23 (t, $J = 7.0$ Hz, 3H). MS calcd for $C_9H_{15}N_2O_2$ $[M + H]^+$ 183.1, found 183.4.

Ethyl 3-Amino-4,5,6,7-tetrahydro-1H-indole-2-carboxylate (16c)—To a solution of compound **15c** (3.19 mg, 15.3 mmol) in EtOH (51 mL) was added a solution of NaOEt in EtOH (21 mL, 40 mg/mL, 18.7 mmol). The mixture was stirred at 70°C for 40 min and cooled to room temperature. The solvent was concentrated in vacuo. The residue was treated with EtOAc and water. The water phase was extracted with EtOAc. The combined organic phase was dried over Na_2SO_4 and concentrated in vacuo. The residue was roughly purified by filtration on silica gel (hexanes:EtOAc = 1:1) to give **16c** (2.52 g, 79%). 1H NMR (400 MHz, DMSO- d_6) δ ppm 10.00 (br s, 1H), 4.13 (m, $J = 7.10, 7.10, 7.10$ Hz, 2H), 2.33–2.45 (m, 2H), 2.20 (t, $J = 5.1$ Hz, 2H), 1.62 (d, $J = 4.8$ Hz, 4H), 1.22 (t, $J = 7.2$ Hz, 3H). MS calcd for $C_{11}H_{17}N_2O_2$ $[M + H]^+$ 209.1, found 209.4.

Ethyl 3-Amino-1,4,5,6,7,8-hexahydrocyclohepta[b]pyrrole-2-carboxylate (16d)—To a solution of compound **15d** (1.40 g, 6.30 mmol) in EtOH (21 mL) was added a solution of NaOEt in EtOH (21 mL, 40 mg/mL, 7.6 mmol). The mixture was stirred at 70°C for 25 h and cooled to room temperature. The solvent was concentrated in vacuo. The residue was treated with EtOAc and water. The water phase was extracted with EtOAc. The combined organic phase was dried over Na_2SO_4 and concentrated in vacuo. The residue was roughly purified by filtration through silica gel pad (hexanes:EtOAc = 1:1) to give **16d** (748 g, 53%). 1H NMR (400 MHz, DMSO- d_6) δ ppm 10.12 (br s, 1H), 4.13 (q, $J = 7.2$ Hz, 2H), 2.50–2.62 (m, 2H), 2.25–2.36 (m, 2H), 1.71 (br s, 3H), 1.41–1.57 (m, 5 H), 1.11–1.29 (m, 9H). MS calcd for $C_{12}H_{19}N_2O_2$ $[M + H]^+$ 223.1, found 223.0.

Ethyl 3-(3-Phenylthioureido)-1H-pyrrole-2-carboxylate (17a)—To a solution of ethyl 3-amino-1H-pyrrole-2-carboxylate (**16a**, 593 mg, 3.85 mmol) in DMF (5 mL) was added PhNCS (690 μ L, 5.77 mmol) at room temp. The mixture was stirred at 60°C for 24 h and cooled to room temp. The mixture was treated with water and EtOAc. The water phase was extracted with EtOAc (3 $\times$). The combined organic layer was dried over Na_2SO_4 and concentrated in vacuo. The product was partially crystallized and collected. The filtrate was concentrated in vacuo and recrystallized from EtOAc to give compound **17a** as a white solid (593 mg, 53%). 1H NMR (400 MHz, DMSO- d_6) δ ppm 11.60 (br s, 1H), 10.29 (s, 1H), 9.64 (s, 1H), 7.40–7.46 (m, 2H), 7.34–7.40 (m, 2H), 7.27 (t, $J = 2.2$ Hz, 1H), 7.18 (t, $J = 7.3$ Hz, 1H), 6.90 (t, $J = 3.1$ Hz, 1H), 4.16 (q, $J = 7.2$ Hz, 2 H), 2.46–2.51 (m, 1H), 4.03 (br s, 1H), 2.49 (dt, $J = 3.6, 1.7$ Hz, 1H), 1.18 (t, $J = 7.3$ Hz, 3 H). MS calcd for $C_{14}H_{16}N_3O_2S$ $[M + H]^+$ 290.1, found 290.3.

Ethyl 4,5-Dimethyl-3-(3-phenylthioureido)-1H-pyrrole-2-carboxylate (17b)—To a solution of compound **16b** (795 mg, 4.36 mmol) in EtOH was added PhNCS (287 μ L, 4.80 mmol) at room temperature. The mixture was stirred at 70°C for 6 h and cooled to room temperature. The mixture was treated with water and EtOAc. The water phase was extracted with EtOAc (3 $\times$). The combined organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by crystallization from Et₂O to give compound **17b** (1.23 g, 89%). 1H NMR (400 MHz, DMSO- d_6) δ ppm 11.34 (br s, 1H), 9.38 (br s, 1H), 8.91 (s, 1H),

4.15 (q, $J = 7.2$ Hz, 2H), 2.12 (s, 3H), 1.81 (s, 3H), 1.22 (t, $J = 7.2$ Hz, 3H). MS calcd for $C_{16}H_{20}N_3O_2S$ $[M + H]^+$ 318.1, found 318.2.

Ethyl 3-(3-Phenylthioureido)-4,5,6,7-tetrahydro-1H-indole-2-carboxylate (17c)

—To a solution of compound **16c** (854 mg, 4.10 mmol) in EtOH (20 mL) was added PhNCS (737 μ L, 6.15 mmol) at room temperature. The mixture was stirred at 70°C for 15 h and cooled to room temperature. The mixture was treated with water and EtOAc. The water phase was extracted with EtOAc (3 $\times$). The combined organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by crystallization from Et₂O to give compound **17c** (1.12 g, 85%). ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 11.28 (s, 1H), 9.53 (br s, 1H), 8.91 (s, 1H), 7.49 (d, $J = 7.8$ Hz, 2H), 7.29 (t, $J = 8.1$ Hz, 2H), 7.09 (t, $J = 7.3$ Hz, 1H), 4.16 (q, $J = 7.1$ Hz, 2H), 2.34 (t, $J = 5.9$ Hz, 2H), 1.65 (dd, $J = 27.32, 4.39$ Hz, 4H), 1.22 (t, $J = 7.1$ Hz, 3H). MS calcd for $C_{18}H_{22}N_3O_2S$ $[M + H]^+$ 344.1, found 344.1.

Ethyl 3-(3-Phenylthioureido)-1,4,5,6,7,8-hexahydrocyclohepta-[b]pyrrole-2-carboxylate (17d)

—To a solution of compound **16d** (700 mg, 3.15 mmol) in EtOH (15 mL) was added PhNCS (570 μ L, 4.74 mmol) at room temperature. The mixture was stirred at 70°C for 3.5 h and cooled to room temperature. The mixture was treated with water and EtOAc. The water phase was extracted with EtOAc (3 $\times$). The combined organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was coevaporated with toluene to give compound **17d** (641 mg, 57.0%). ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 8.91 (br s, 1H), 7.44–7.49 (m, 3H), 7.28 (t, $J = 7.9$ Hz, 3H), 7.08 (t, $J = 8.1$ Hz, 1H), 4.16 (q, $J = 5.1$ Hz, 2H), 2.66 (m, 2H), 2.25–2.37 (m, 2H), 1.42–1.60 (m, 4H), 1.73 (br s, 4H), 1.22 (t, $J = 7.2$ Hz, 3H). MS calcd for $C_{19}H_{24}N_3O_2S$ $[M + H]^+$ 358.2, found 358.1.

3-Phenyl-2-thioxo-1,2,3,5-tetrahydro-4H-pyrrolo[3,2-d]pyrimidin-4-one (18a)

—A mixture of compound **17a** (300 mg, 1.04 mmol) and PPA (8 g) was stirred at 110°C for 8 h. The mixture was poured into water. The precipitate that formed was collected by filtration and dried in vacuo to give compound **18a** (314 mg, quantitative). ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 12.12 (br s, 1H), 9.87 (br s, 1H), 7.74 (d, $J = 8.4$ Hz, 2H), 7.37–7.45 (m, 1H), 7.31 (t, $J = 7.5$ Hz, 7H), 7.01 (t, $J = 7.3$ Hz, 3H), 6.31 (br s, 3H). MS calcd for $C_{12}H_{10}N_3OS$ $[M + H]^+$ 244.1, found 243.9.

6,7-Dimethyl-3-phenyl-2-thioxo-1,2,3,5-tetrahydro-4H-pyrrolo-[3,2-d]pyrimidin-4-one (18b)

—A mixture of compound **17b** (805 mg, 2.52 mmol) and PPA (40 g) was stirred at 110°C for 8 h. The mixture was poured into ice–water. The mixture was treated with EtOAc and the water phase was extracted with EtOAc (3 $\times$). The residue was concentrated in vacuo. The residue was used for the next reaction without further purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 11.74 (s, 1H), 9.81 (br s, 1H), 7.82 (d, $J = 1.0$ Hz, 2H), 7.30 (t, $J = 1.0$ Hz, 2H), 6.90–7.05 (m, 1H), 2.22 (s, 3H), 2.04 (s, 3H). MS calcd for $C_{14}H_{14}N_3OS$ $[M + H]^+$ 272.1, found 271.9.

3-Phenyl-2-thioxo-1,2,3,5,6,7,8,9-octahydro-4H-pyrimido[5,4-b]indol-4-one (18c)

—A mixture of compound **17c** (600 mg, 1.75 mmol) and PPA (7.0 g) was stirred at 110°C for 24 h. The mixture was poured into ice–water and insoluble materials were collected by filtration. The filtrate was extracted with EtOAc (3 $\times$). The combined organic

layer was washed with satd NaCl aq, dried over MgSO₄, and concentrated in vacuo. The residue was used for the next reaction without further purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 11.66 (br s, 1H), 9.82 (br s, 1H), 7.79 (d, *J* = 8.1 Hz, 2H), 7.29 (d, *J* = 14.3 Hz, 2H), 6.98 (t, *J* = 7.5 Hz, 1H), 2.56 (d, *J* = 19.43 Hz, 4H), 1.60–1.83 (m, 4H). MS calcd for C₁₆H₁₆N₃OS [M + H]⁺ 298.1, found 298.0.

3-Phenyl-2-thioxo-2,3,5,6,7,8,9,10-octahydrocyclohepta[4,5]-pyrrolo[3,2-d]pyrimidin-4(1H)-one (18d)—A mixture of compound **17d** (540 mg, 1.51 mmol) and PPA (27 g) was stirred at 110°C for 4 h. The mixture was poured into ice–water, and insoluble materials were collected by filtration. The filtrate was extracted with EtOAc (3×). The combined organic layer was washed with satd NaCl aq, dried over MgSO₄, and concentrated in vacuo and purified by flash column chromatography to yield 241 mg (51%). ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 11.79 (s, 1H), 9.80 (s, 1H), 7.80 (d, *J* = 8.07 Hz, 2H), 7.32 (d, *J* = 7.33 Hz, 2H), 6.99 (d, *J* = 14.66 Hz, 1H), 2.62–2.79 (m, 4H), 1.61 (m, 4H), 1.80 (br s, 2H). MS calcd for C₁₇H₁₈N₃OS [M + H]⁺ 312.1, found 312.1.

2-((4-Oxo-3-phenyl-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-2-yl)-thio)acetic Acid (19a)—A mixture of compound **18a** (50.0 mg, 0.209 mmol) and KOH (23.1 mg, 0.418 mmol) was dissolved in EtOH (15 mL) at 78°C. After 20 min, a solution of CH₂ClCO₂H (27.1 mg/mL, 717 μL) in EtOH was added to the mixture. The resulting mixture was stirred at 78°C for 12 h. After cooling to room temperature, silica gel (2 g) was added (for silica gel purification). The solvent was evaporated in vacuo. The residue was purified by flash column chromatography (CH₂Cl₂:MeOH = 30:1 → 3:1) to give compound **19a** (41.4 mg, 66%). ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 12.05 (br s, 1H), 7.46–7.64 (m, 3H), 7.19–7.42 (m, 2H), 6.29 (t, *J* = 2.2 Hz, 1H), 3.61 (s, 2H). MS calcd for C₁₄H₁₀N₃O₃S [M – H][–] 300.0, found 299.8.

2-((6,7-Dimethyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrrolo[3,2-d]-pyrimidin-2-yl)thio)acetic Acid (19b)—To a solution of compound **18b** (50.0 mg, 0.184 mmol) in EtOH (3.5 mL) was added a solution of KOH in EtOH (1.0 mL, 20.6 mg/mL, 0.368 mmol) at 78°C. After 15 min, CH₂ClCO₂H (17.43 mg, 0.184 mmol) was added to the reaction mixture at 78°C. The resulting mixture was stirred at 78°C for 24 h. After cooling to room temperature, the reaction mixture was added 1 M HCl aq and volatiles were evaporated in vacuo. The residue was purified by flash column chromatography (CH₂Cl₂:MeOH = 19:1 → 0:1) to give compound **19b** (13.9 mg, 23%). ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 11.72 (br s, 1H), 7.50–7.66 (m, 3H), 7.26–7.41 (m, 2H), 3.83 (s, 2H), 2.26 (s, 3H), 2.06 (s, 3H). MS calcd for C₁₆H₁₆N₃O₃S [M + H]⁺ 330.1, found 330.0.

2-((4-Oxo-3-phenyl-4,5,6,7,8,9-hexahydro-3H-pyrimido[5,4-b]-indol-2-yl)thio)acetic Acid (19c)—To a solution of compound **18c** (143 mg, 0.481 mmol) in EtOH (14 mL) was added a solution of KOH in EtOH (2.4 mL, 20.6 mg/mL, 0.962 mmol) at 78°C. After 15 min, CH₂ClCO₂H (45.4 mg, 0.481 mmol) was added to the reaction mixture at 78°C. The resulting mixture was stirred at 78°C for 24 h. After cooling to room temperature, 1 M HCl aq was added to the reaction mixture and the volatiles were evaporated in vacuo. The residue was purified by flash column chromatography

(CH₂Cl₂:MeOH = 19:1 → 0:1) to give compound **19c** (114 mg, 67%). ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 11.57 (br s, 1H), 7.53 (m, *J* = 4.40 Hz, 3H), 7.28 (dd, *J* = 7.7, 1.8 Hz, 2H), 3.72 (s, 2H), 2.61 (t, *J* = 5.5 Hz, 2H), 2.54 (t, *J* = 5.5 Hz, 2H), 1.75 (dd, *J* = 14.11, 6.78 Hz, 4H). MS calcd for C₁₈H₁₈N₃O₃S [M + H]⁺ 356.1, found 356.0.

2-((4-Oxo-3-phenyl-3,4,5,6,7,8,9,10-octahydrocyclohepta[4,5]-pyrrolo[3,2-d]pyrimidin-2-yl)thio)acetic Acid (19d)—To a solution of compound **18d** (120 mg, 0.385 mmol) in EtOH (13 mL) was added a solution of KOH in EtOH (2.4 mL, 20.6 mg/mL, 0.771 mmol) at 78°C. After 15 min, CH₂ClCO₂H (36.5 mg, 0.385 mmol) was added to the reaction mixture at 78°C. The reaction mixture was stirred at 78°C for 24 h. After cooling to room temperature, 1 M HCl aq was added to the reaction mixture and the volatiles were evaporated in vacuo. The residue was purified by flash column chromatography (CH₂Cl₂:MeOH = 19:1 → 0:1) to give compound **19d** (116 mg, 82%). ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 11.73 (br s, 1H), 7.53–7.61 (m, 3H), 7.33 (d, *J* = 7.3 Hz, 2H), 3.82 (s, 2H), 2.65–2.83 (m, 4H), 1.83 (br s, 2H), 1.62 (br s, 4H). MS calcd for C₁₉H₂₀N₃O₃S [M + H]⁺ 370.1, found 370.0.

N-Cyclohexyl-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-2-yl)thio)acetamide (20a)—To a solution of compound **19a** (190 mg, 0.631 mmol) in DMF (30 mL) were added cyclohexylamine (79 μL, 0.694 mmol), triethylamine (TEA, 241 μL, 1.62 mmol), and HATU (263 mg, 0.694 mmol). The mixture was stirred at room temperature for 30 min and concentrated in vacuo. The residue was purified by flash column chromatography (hexanes:EtOAc = 3:1 → 0:1) to give compound **20a** (200 mg, 83%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.15 (br s, 1H), 8.04 (d, *J* = 7.70 Hz, 1H), 7.50–7.63 (m, 3H), 7.26–7.46 (m, 3H), 6.28 (s, 1H), 3.76 (s, 2H), 3.40–3.52 (m, *J* = 8.10 Hz, 1H), 1.55–1.75 (m, *J* = 4.80, 14.30, 14.30 Hz, 4H), 1.50 (d, *J* = 9.53 Hz, 1H), 1.05–1.24 (m, 5H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.7, 163.8, 153.9, 152.5, 151.9, 143.7, 143.6, 136.2, 129.8, 129.5, 121.4, 114.8, 102.2, 47.8, 36.9, 32.3, 32.1, 25.2, 24.4. HRMS calcd for C₂₀H₂₂N₄O₂ S Na [M + Na]⁺, 405.1356, found 405.1355.

N-Cyclohexyl-2-((6,7-dimethyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-2-yl)thio)acetamide (20b)—To a solution of compound **19b** (49.3 mg, 0.150 mmol) in DMF (2 mL) were added cyclohexylamine (19 μL, 0.165 mmol), TEA (42 μL, 0.300 mmol), and HATU (63 mg, 0.165 mmol). The mixture was stirred for 24 h and treated with H₂O and EtOAc. The water phase was extracted with EtOAc (3×). The combined organic layer was dried over MgSO₄ and concentrated in vacuo. The residue was purified by flash column chromatography (hexanes: EtOAc = 2:1 → 1:2) to give compound **20b** (30.4 mg, 60%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.72 (s, 1H), 8.02 (d, *J* = 7.70 Hz, 1H), 7.51–7.58 (m, 3H), 7.33 (d, *J* = 1.00 Hz, 2H), 3.76 (s, 2H), 3.43–3.54 (m, 1H), 2.26 (s, 3H), 2.10 (s, 3H), 1.59–1.76 (m, 4H), 1.47–1.57 (m, 1H), 1.01–1.31 (m, 5H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.8, 153.3, 151.5, 143.2, 136.4, 136.0, 129.8, 129.6, 129.4, 112.6, 107.4, 48.0, 36.6, 32.4, 25.2, 24.5, 11.5, 7.3. HRMS calcd for C₂₂H₂₇N₄O₂S [M + H]⁺, 411.1849, found 411.1845.

N-Cyclohexyl-2-((4-oxo-3-phenyl-4,5,6,7,8,9-hexahydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (20c)—To a solution of compound **19c** (19 mg, 53 μ mol) in DMF (2.6 mL) were added cyclohexylamine (7.0 μ L, 58 μ mol), TEA (15 μ L, 0.108 mmol), and HATU (20.5 mg, 58.0 μ mol). After stirring for 1 h, the reaction mixture was concentrated in vacuo. The residue was purified by flash column chromatography (hexane:EtOAc = 2:1 $\rightarrow$ 0:1) to give compound **20c** (20.9 mg, 91%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.66 (s, 1H), 8.01 (d, J = 7.70 Hz, 1H), 7.49–7.61 (m, 3H), 7.28–7.37 (m, 2H), 3.75 (s, 2H), 3.42–3.54 (m, 1H), 2.64 (t, J = 5.68 Hz, 2H), 2.59 (t, J = 5.50 Hz, 2H), 1.59–1.87 (m, 8H), 1.52 (d, J = 12.46 Hz, 1H), 1.05–1.30 (m, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 166.3, 153.9, 152.0, 142.2, 138.9, 136.8, 130.2, 130.0, 129.8, 113.6, 110.4, 48.3, 37.0, 32.7, 25.6, 24.9, 23.3, 23.1, 22.9, 20.4. HRMS calcd for $\text{C}_{24}\text{H}_{28}\text{N}_4\text{O}_2\text{SNa}$ [$\text{M} + \text{Na}$] $^+$ 459.1825, found 459.1827.

N-Cyclohexyl-2-((4-oxo-3-phenyl-3,4,5,6,7,8, 9,10-octahydrocyclohepta[4,5]pyrrolo[3,2-d]pyrimidin-2-yl)thio)-acetamide (20d)—To a solution of compound **19d** (50 mg, 0.135 mmol) in DMF (3 mL) were added cyclohexylamine (17 μ L, 0.149 mmol), TEA (37 μ L, 0.271 mmol), and HATU (56.6 mg, 0.149 mmol). The mixture was stirred at room temperature for 30 min, and the reaction mixture was then concentrated in vacuo. The residue was purified by flash column chromatography (hexane:EtOAc = 2:1 $\rightarrow$ 0:1) to give compound **20d** (38.4 mg, 48%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.72 (br s, 1H), 8.00 (d, J = 7.33 Hz, 1H), 7.48–7.59 (m, 3H), 7.31 (d, J = 7.33 Hz, 2H), 3.72 (s, 2H), 3.40–3.53 (m, J = 7.00 Hz, 1H), 2.66–2.82 (m, 4H), 1.45–1.89 (m, 11H), 1.01–1.32 (m, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 165.9, 153.5, 151.7, 142.7, 142.5, 136.4, 129.9, 129.5, 129.4, 114.3, 111.4, 47.9, 36.5, 32.4, 31.9, 28.7, 28.7, 27.2, 25.2, 24.5, 23.2. HRMS calcd for $\text{C}_{25}\text{H}_{30}\text{N}_4\text{O}_2\text{SNa}$ [$\text{M} + \text{Na}$] $^+$ 473.1982, found 473.1984.

N-Cyclohexyl-2-((5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-2-yl)thio)acetamide (21a)—To a solution of compound **20a** (100 mg, 0.261 mmol) in DMF (13 mL) was added NaH (10.4 mg, 0.261 mmol). After stirring for 30 min, methyl iodide (16 μ L, 0.261 mmol) was added to the reaction mixture. After 24 h, the reaction mixture was concentrated in vacuo. The residue was purified by flash column chromatography (hexane:EtOAc = 4:1 $\rightarrow$ 0:1) to give compound **21a** (29.3 mg, 29%). ^1H NMR (400 MHz, DMSO- d_6) δ 8.05 (d, J = 7.70 Hz, 1H), 7.49–7.59 (m, 3H), 7.24–7.36 (m, 3H), 6.23 (d, J = 2.57 Hz, 1H), 3.88 (s, 3H), 3.67 (s, 2H), 3.40–3.47 (m, 1H), 1.53–1.72 (m, 4H), 1.44–1.51 (m, J = 9.20 Hz, 1H), 1.01–1.25 (m, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 165.6, 154.2, 152.8, 143.8, 136.0, 132.8, 129.8, 129.7, 129.4, 114.4, 101.1, 47.8, 36.8, 35.5, 32.3, 25.2, 24.4. HRMS calcd for $\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_2\text{SNa}$ [$\text{M} + \text{Na}$] $^+$ 419.1512, found 419.1512.

N-Cyclohexyl-2-((5,6,7-trimethyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-2-yl)thio)acetamide (21b)—To a solution of compound **20b** (15.0 mg, 36.5 μ mol) in DMF (1.8 mL) was added NaH (2.96 mg, 73.1 μ mol). After stirring for 15 min, a solution of methyl iodide in DMF (10 μ L, 226 μ L/mL) was added to the solution. After stirring for 3 h, the reaction mixture was concentrated in vacuo and purified by PTLTLC (hexanes:EtOAc = 1:1) to give compound **21b** (5.8 mg, 37%). ^1H NMR (400 MHz, DMSO-

d_6) δ 7.92 (d, J = 7.70 Hz, 1H), 7.47–7.54 (m, 2H), 7.36–7.46 (m, 3H), 4.91 (s, 2H), 3.47 (s, 3H), 3.43–3.55 (m, 1H), 2.11–2.18 (m, 3H), 2.07 (s, 3H), 1.62–1.74 (m, 4H), 1.53 (d, J = 12.10 Hz, 1H), 1.08–1.29 (m, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 167.5, 166.0, 158.2, 147.2, 143.2, 140.8, 130.0, 128.2, 128.1, 111.1, 110.6, 47.8, 47.7, 39.6, 32.4, 25.2, 24.5, 9.8, 8.0. HRMS calcd for $\text{C}_{23}\text{H}_{28}\text{N}_4\text{O}_2\text{SNa}$ [$\text{M} + \text{Na}$] $^+$ 447.1825, found 447.1825.

N-Cyclohexyl-2-((5-methyl-4-oxo-3-phenyl-4,5,6,7,8,9-hexahydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (21c)—To a solution of compound **20c** (6.7 mg, 15 μmol) in DMF (1 mL) was added NaH (0.69 mg, 15 μmol). After stirring for 30 min, a solution of methyl iodide in DMF (95 μL , 100 $\mu\text{g/mL}$, 153 μmol) was added to the reaction solution. After stirring for 3 h, reaction mixture was concentrated in vacuo. The residue was purified by flash column chromatography (hexanes:EtOAc = 3:1 $\rightarrow$ 0:1) to give compound **21c** (2.9 mg, 43%). ^1H NMR (400 MHz, DMSO- d_6) δ 7.97 (d, J = 7.70 Hz, 1H), 7.50–7.56 (m, 3H), 7.30 (d, J = 7.70 Hz, 2H), 3.79 (s, 3H), 3.72 (s, 2H), 3.47–3.52 (m, 1H), 2.64 (t, J = 5.90 Hz, 2H), 2.57 (t, J = 5.90 Hz, 2H), 1.58–1.87 (m, 8H), 1.47–1.53 (m, 1H), 1.05–1.21 (m, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 165.7, 153.9, 151.7, 141.1, 140.1, 136.2, 129.8, 129.6, 129.4, 113.0, 109.8, 47.9, 36.6, 32.3, 31.1, 25.2, 24.5, 22.5, 22.4, 21.2, 20.0. HRMS calcd for $\text{C}_{25}\text{H}_{30}\text{N}_4\text{O}_2\text{SNa}$ [$\text{M} + \text{Na}$] $^+$ 473.1982, found 473.1990.

N-Cyclohexyl-2-((5-methyl-4-oxo-3-phenyl-3,4,5,6,7,8,9,10-octahydrocyclohepta[4,5]pyrrolo[3,2-d]pyrimidin-2-yl)thio)-acetamide (21d)—To a solution of compound **20d** (12.1 mg, 26.6 μmol) in DMF (1.5 mL) was added NaH (2.3 mg, 26.6 μmol). After stirring for 30 min, a solution of methyl iodide in DMF (160 μL , 100 $\mu\text{g/mL}$, 0.266 mmol) was added to the reaction solution. After stirring for 20 h, reaction mixture was concentrated in vacuo, and the residue was purified by flash column chromatography (hexanes:EtOAc = 3:1 $\rightarrow$ 0:1) to give compound **21d** (3.8 mg, 35%). ^1H NMR (400 MHz, DMSO- d_6) δ 7.99 (d, J = 7.81 Hz, 1H), 7.44–7.60 (m, 3H), 7.29 (dd, J = 1.95, 7.32 Hz, 2H), 3.83–3.90 (m, 3H), 3.70 (s, 2H), 2.70–2.81 (m, J = 5.40, 10.70, 10.70 Hz, 4H), 1.45–1.88 (m, 11H), 1.04–1.27 (m, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ ppm 165.8, 153.9, 151.7, 143.9, 141.3, 136.2, 129.8, 129.5, 129.3, 114.5, 111.7, 47.9, 36.4, 32.3, 31.3, 31.2, 28.1, 26.4, 25.5, 25.2, 24.4, 22.6. HRMS calcd for $\text{C}_{26}\text{H}_{33}\text{N}_4\text{O}_2\text{S}$ [$\text{M} + \text{H}$] $^+$ 465.2319, found 465.2316.

General Procedure A for Synthesis of Compound 23a–c—Compound **22** (1 equiv), ethyl bromoacetate (1.1 equiv), and sodium bicarbonate (1.2 equiv) were combined in anhydrous EtOH and refluxed for 42 h. After cooling slightly, solution was filtered and concentrated in vacuo and the residue was purified by silica gel column chromatography (hexanes:EtOAc = 88:12) to give compound **23**.

General Procedure B for Synthesis of Compound 24a–c—In a flame-dried flask, a suspension of potassium *tert*-butoxide (1 equiv) in anhydrous tetrahydrofuran (THF) was stirred and maintained below 30°C under argon. To this solution was added a solution of compound **23** (1 equiv) in anhydrous THF over 45 min and stirred for an additional 2 h. The reaction was then poured into ice–water, extracted with EtOAc, and dried over MgSO_4 . The

solvent was concentrated in vacuo, and the resultant residue was purified by silica gel column chromatography (hexanes:EtOAc = 80:20) to give compound **24**.

General Procedure C for Synthesis of Compound 25a–c—To a solution of compound **24** (1 equiv) in warm EtOH was added the appropriate isothiocyanate (1.1 equiv) dropwise with stirring. The reaction was refluxed for 6 h and cooled overnight. Solids were filtered, washed with EtOH, and dried overnight in vacuo to give compound **25**.

General Procedure D for the Synthesis of Compound 26a–c—To a sealed flame-dried flask charged with argon gas and anhydrous EtOH was added acetyl chloride (80 equiv) with stirring. Separately, compound **25** was dissolved in anhydrous EtOH and added to the reaction mixture and stirred at reflux for 12 h. Once complete, the reaction mixture was cooled at 4°C and solids were filtered and washed with cold EtOH. Solids were recrystallized in EtOH to give compound **26**.

General Procedure E for the Synthesis of Compound 27a–c—Compound **26** (1 equiv) and KOH (2 equiv) were suspended in 2 mL of dimethylacetamide (DMA) with stirring. H₂O was added dropwise until KOH was completely dissolved. *tert*-Butyl chloroacetate (1.1 equiv) was added to the reaction mixture and stirred at room temperature, monitoring with thin-layer chromatography (MeOH:dichloromethane (DCM) = 1:99). Upon completion, reaction mixture was extracted with EtOAc and water, dried over MgSO₄, and concentrated in vacuo. EtOH was added to the resulting viscous liquid and pure product was filtered to give compound **27**.

General Procedure F for the Synthesis of Compound 28a–c—NaH 60% dispersion (1 equiv) was added to a solution of compound **27** (1 equiv) in DMF. The reaction mixture was stirred at room temperature for 5 min, and then methyl iodide (1 equiv) was added and stirred until completion. The crude residue was extracted with EtOAc and dried over MgSO₄. Crude material was further purified by silica gel column chromatography (hexanes:EtOAc = 70:30) to give compound **28**.

General Procedure G for the Synthesis of Compound 29a–c—Compound **28** was dissolved in 1:1 ACN)/TFA and stirred at room temperature overnight. Crystals were filtered and collected as pure product, and the filtrate was concentrated in vacuo. The resultant residue was recrystallized with ACN and combined with the previously collected crystals to give compound **29**.

General Procedure H for the Synthesis of Compound 30a–c—Compound **29** (1 equiv), TEA (2 equiv), and cyclohexylamine (1.1 equiv) were dissolved in anhydrous DMF. To this solution, HATU (1.1 equiv) dissolved in DMF was added and stirred at room temperature until complete and concentrated in vacuo. The crude material was then recrystallized in MeOH to give compound **30**.

General Procedure I for the Synthesis of Compound 36–39—Compound **30a** (1 equiv), tetrakis(triphenylphosphine)palladium(0) (0.4 equiv), and an appropriate boronic acid (1.2 equiv) were placed in a thick-walled microwave vessel with a stir bar. The vial

sealed with a septum top and evacuated under vacuum and charged with argon several times. DMF was then added to the vial via syringe. Separately, sodium carbonate (3 equiv) was dissolved in water (1/4 the volume of DMF) and added to the reaction mixture. The reaction mixture was irradiated in the microwave cavity at 110°C for 15 min. After cooling, the crude material was extracted with EtOAc and purified by silica gel column chromatography (DCM:MeOH = 98:2) and then further purified by reverse phase column chromatography under acidic conditions (0.1% TFA in H₂O:0.1% TFA in MeOH = 50:50 to 20:80 gradient) to give compounds **36–39**.

Ethyl 2-((4-Bromo-2-cyanophenyl)amino)acetate (23a)—2-Amino-5-bromobenzonitrile (5.0 g, 25.4 mmol), ethyl bromoacetate (2.9 mL, 26.1 mmol), sodium bicarbonate (2.54 g, 30.23 mmol), and anhydrous EtOH (9 mL) were reacted according to general procedure A to give 2.786 g of compound **23a** in 38.7% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 7.73 (d, *J* = 2.44 Hz, 1H), 7.54 (dd, *J* = 2.20, 9.03 Hz, 1H), 6.63 (d, *J* = 9.27 Hz, 1H), 6.58 (t, *J* = 6.10 Hz, 1H), 4.12 (q, *J* = 7.32 Hz, 2H), 4.05 (d, *J* = 6.34 Hz, 2H), 1.19 (t, *J* = 7.07 Hz, 3H). MS calcd for C₁₁H₁₂BrN₂O₂ [M + H]⁺ 283.0, found 282.9.

Ethyl 2-((2-Cyano-4-iodophenyl)amino)acetate (23b)—2-Amino-5-iodobenzonitrile (5.0 g, 20.5 mmol), ethyl bromoacetate (2.9 mL, 30.8 mmol), sodium bicarbonate (3.44 g, 40.9 mmol), and anhydrous EtOH (20 mL) were reacted according to general procedure A to give 1.51 g of compound **23b** in 22.3% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 7.73 (d, *J* = 2.44 Hz, 1H), 7.54 (dd, *J* = 2.20, 9.03 Hz, 1H), 6.63 (d, *J* = 9.27 Hz, 1H), 6.58 (t, *J* = 6.10 Hz, 1H), 4.12 (q, *J* = 7.32 Hz, 2H), 4.05 (d, *J* = 6.34 Hz, 2H), 1.19 (t, *J* = 7.07 Hz, 3H). MS calcd for C₁₁H₁₂IN₂O₂ [M + H]⁺ 331.0, found 330.8.

Ethyl 2-((5-Bromo-2-cyanophenyl)amino)acetate (23c)—2-Amino-4-bromobenzonitrile (10.0 g, 50.8 mmol), ethyl bromoacetate (5.8 mL, 52.6 mmol), sodium bicarbonate (5.08 g, 60.5 mmol), and anhydrous EtOH (18 mL) were reacted according to general procedure A to give 4.96 g of compound **23c** in 34.5% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 7.45 (d, *J* = 8.29 Hz, 1H), 6.91 (s, 1H), 6.86 (dd, *J* = 1.46, 8.29 Hz, 1H), 6.63 (t, *J* = 6.10 Hz, 1H), 4.08 (d, *J* = 6.34 Hz, 2H), 4.14 (q, *J* = 7.20 Hz, 2H), 1.20 (t, *J* = 7.07 Hz, 3H). MS calcd for C₁₁H₁₂BrN₂O₂ [M + H]⁺ 283.1, found 282.9.

Ethyl 3-Amino-5-bromo-1H-indole-2-carboxylate (24a)—Compound **23a** (3.0 g, 10.6 mmol), potassium *tert*-butoxide (1.2 g, 10.7 mmol), and anhydrous THF (8 mL) were reacted according to general procedure B to give 1.3 g of compound **24a** in 43.3% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 10.60 (s, 1H), 8.02 (s, 1H), 7.30 (dd, *J* = 1.95, 8.78 Hz, 1H), 7.18 (d, *J* = 8.78 Hz, 1H), 5.71 (s, 2H), 4.29 (q, *J* = 6.83 Hz, 2H), 1.33 (t, *J* = 7.07 Hz, 3H). MS calcd for C₁₁H₁₂BrN₂O₂ [M + H]⁺ 283.1, found 282.9.

Ethyl 3-Amino-5-iodo-1H-indole-2-carboxylate (24b)—Compound **23b** (1.0 g, 3.0 mmol), potassium *tert*-butoxide (1.2 g, 10.7 mmol), and anhydrous THF (8 mL) were reacted according to general procedure B to give 1.3 g of compound **24b** in 43.3% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 10.58 (s, 1H), 8.18 (s, 1H), 7.42 (dd, *J* = 1.46, 8.78 Hz,

1H), 7.07 (d, J = 8.78 Hz, 1H), 5.71 (s, 2H), 4.29 (q, J = 7.16 Hz, 2H), 1.32 (t, J = 7.07 Hz, 3H). MS calcd for $C_{11}H_{12}IN_2O_2$ $[M + H]^+$ 331.0, found 330.9.

Ethyl 3-Amino-6-bromo-1H-indole-2-carboxylate (24c)—Compound **23c** (1.0 g, 3.5 mmol), potassium *tert*-butoxide (0.4 g, 3.6 mmol), and anhydrous THF (2.6 mL) were reacted according to general procedure B to give 0.3 g of compound **24c** in 30.2% yield. 1H NMR (400 MHz, DMSO- d_6) δ 10.53 (s, 1H), 7.72 (d, J = 8.78 Hz, 1H), 7.37 (s, 1H), 7.02 (d, J = 8.29 Hz, 1H), 5.77 (s, 2H), 4.29 (q, J = 7.32 Hz, 2H), 1.33 (t, J = 7.07 Hz, 3H). MS calcd for $C_{11}H_{12}BrN_2O_2$ $[M + H]^+$ 283.1, found 282.9.

Ethyl 5-Bromo-3-(3-phenylthioureido)-1H-indole-2-carboxylate (25a)—

Compound **24a** (3.7 g, 13.2 mmol) was reacted with phenyl isothiocyanate (1.7 mL, 14.5 mmol) in ethanol (10 mL) according to general procedure C to give 4.8 g of compound **25a** in 87.8% yield. 1H NMR (400 MHz, DMSO- d_6) δ ppm 12.00 (s, 1H), 9.81 (br s, 1H), 9.36 (s, 1H), 7.69 (s, 1H), 7.48 (d, J = 7.81 Hz, 2H), 7.32–7.43 (m, 2H), 7.31 (t, J = 7.81 Hz, 2H), 7.11 (t, J = 7.30 Hz, 1H), 4.30 (q, J = 6.80 Hz, 2H), 1.30 (t, J = 7.07 Hz, 3H). MS calcd for $C_{18}H_{17}BrN_3O_2S$ $[M + H]^+$ 418.0, found 417.9.

Ethyl 5-Iodo-3-(3-phenylthioureido)-1H-indole-2-carboxylate (25b)—Compound **24b** (530.7 mg, 1.6 mmol) was reacted with phenyl isothiocyanate (221.2 μ L, 1.8 mmol) in ethanol (3 mL) according to general procedure C to give 335.5 mg of compound **25b** in 44.9% yield. 1H NMR (400 MHz, DMSO- d_6) δ ppm 11.99 (s, 1H), 9.80 (br s, 1H), 9.38 (s, 1H), 7.90 (s, 1H), 7.50 (s, 3H), 7.28 (d, J = 8.78 Hz, 1H), 7.33 (t, J = 7.81 Hz, 2H), 7.13 (t, J = 7.30 Hz, 1H), 4.32 (q, J = 7.16 Hz, 2H), 1.32 (t, J = 7.20 Hz, 3H). MS calcd for $C_{18}H_{17}IN_3O_2S$ $[M + H]^+$ 466.0, found 465.9.

Ethyl 6-Bromo-3-(3-phenylthioureido)-1H-indole-2-carboxylate (25c)—

Compound **24c** (700.0 mg, 2.5 mmol) was reacted with phenyl isothiocyanate (324.8 μ L, 2.7 mmol) in ethanol (5 mL) according to general procedure C to give 752.8 mg of compound **25c** in 72.8% yield. 1H NMR (400 MHz, DMSO- d_6) δ ppm 11.93 (s, 1H), 9.78 (br s, 1H), 9.42 (s, 1H), 7.59 (d, J = 1.95 Hz, 1H), 7.53 (d, J = 8.78 Hz, 1H), 7.50 (d, J = 7.40 Hz, 2H), 7.32 (t, J = 7.81 Hz, 2H), 7.22 (dd, J = 1.95, 8.78 Hz, 1H), 7.12 (t, J = 7.30 Hz, 1H), 4.33 (q, J = 7.32 Hz, 2H), 1.32 (t, J = 7.07 Hz, 3H). MS calcd for $C_{18}H_{17}BrN_3O_2S$ $[M + H]^+$ 418.0, found 417.9.

8-Bromo-3-phenyl-2-thioxo-2,3-dihydro-1H-pyrimido[5,4-b]indol-4(5H)-one (26a)—

Compound **25a** (1.2 g, 2.9 mmol) was added to a mixture of acetyl chloride (8.7 mL, 122.3 mmol) and ethanol (50 mL) according to general procedure D to give 839 mg of compound **26a** in 77.1% yield. 1H NMR (400 MHz, DMSO- d_6) δ ppm 13.70 (s, 1H), 12.44 (s, 1H), 8.45 (s, 1H), 7.57 (d, J = 9.27 Hz, 1H), 7.38–7.53 (m, 4H), 7.28 (d, J = 7.81 Hz, 2H). MS calcd for $C_{16}H_{11}BrN_3OS$ $[M + H]^+$ 372.0, found 371.8.

8-Iodo-3-phenyl-2-thioxo-2,3-dihydro-1H-pyrimido[5,4-b]indol-4(5H)-one (26b)—

Compound **25b** (33.6 mg, 0.7 mmol) was added to a mixture of acetyl chloride (4 mL, 56 mmol) and ethanol (15 mL) according to general procedure D to give 177.3 mg of compound **26b** in 59.4% yield. 1H NMR (400 MHz, DMSO- d_6) δ ppm 13.67 (s, 1H), 12.40

(s, 1H), 8.65 (s, 1H), 7.70 (d, $J = 8.80$ Hz, 1H), 7.49 (s, 2H), 7.42 (t, $J = 7.30$ Hz, 1H), 7.33 (d, $J = 8.78$ Hz, 1H), 7.28 (d, $J = 7.81$ Hz, 2H). MS calcd for $C_{16}H_{11}IN_3OS$ $[M + H]^+$ 420.0, found 419.8.

7-Bromo-3-phenyl-2-thioxo-2,3-dihydro-1H-pyrimido[5,4-b]indol-4(5H)-one

(26c)—Compound **25c** (700 g, 1.7 mmol) was added to a mixture of acetyl chloride (5 mL, 70.3 mmol) and ethanol (30 mL) according to general procedure D to give 428.6 mg of compound **26c** in 68.8% yield. 1H NMR (400 MHz, DMSO- d_6) δ ppm 13.77 (s, 1H), 12.38 (s, 1H), 8.17 (d, $J = 8.78$ Hz, 1H), 7.64 (s, 1H), 7.49 (t, $J = 7.80$ Hz, 2H), 7.33–7.45 (m, 2H), 7.28 (d, $J = 7.81$ Hz, 2H). MS calcd for $C_{16}H_{11}BrN_3OS$ $[M + H]^+$ 372.0 found 371.9.

tert-Butyl 2-((8-Bromo-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)thio)acetate (27a)

—*tert*-Butyl chloroacetate (873.3 μ L, 6.1 mmol) was added to a mixture of compound **26a** (2.1 g, 5.6 mmol), KOH (623 mg, 11.2 mmol), DMA (20 mL), and H₂O (2.5 mL) according to general procedure E to give 1.94 g of compound **27a** in 71.9% yield. 1H NMR (400 MHz, DMSO- d_6) δ ppm 12.36 (br s, 1H), 8.05 (s, 1H), 7.54–7.68 (m, 4H), 7.47 (t, $J = 8.05$ Hz, 3H), 3.88 (s, 2H), 1.40 (s, 9H). MS calcd for $C_{22}H_{21}BrN_3O_3S$ $[M + H]^+$ 486.0, found 485.8.

tert-Butyl 2-((8-Iodo-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)thio)acetate (27b)

—*tert*-Butyl chloroacetate (56.3 μ L, 0.39 mmol) was added to a mixture of compound **26b** (150 mg, 0.36 mmol), TEA (100 μ L, 0.72 mmol, in place of KOH), and DMF (1 mL, in place of DMA and H₂O) according to general procedure E to give 160.4 mg of compound **27b** in 84.0% yield. 1H NMR (400 MHz, DMSO- d_6) δ ppm 12.33 (s, 1H), 8.29 (s, 1H), 7.71 (d, $J = 8.78$ Hz, 1H), 7.54–7.66 (m, 3H), 7.43–7.52 (m, 2H), 7.38 (d, $J = 8.78$ Hz, 1H), 3.89 (s, 2H), 1.44 (s, 9H). MS calcd for $C_{22}H_{21}IN_3O_3S$ $[M + H]^+$ 534.0, found 533.9.

tert-Butyl 2-((7-Bromo-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)thio)acetate (27c)

—*tert*-Butyl chloroacetate (84 μ L, 0.59 mmol) was added to a mixture of compound **26c** (200 mg, 0.54 mmol), KOH (60 mg, 1.06 mmol), DMA (mL), and H₂O (250 μ L) according to general procedure E to give 177.7 mg of compound **27c** in 71.9% yield. 1H NMR (400 MHz, DMSO- d_6) δ ppm 12.32 (br s, 1H), 7.88 (d, $J = 7.32$ Hz, 1H), 7.54–7.77 (m, 4H), 7.27–7.53 (m, 3H), 3.91 (br s, 2H), 1.20–1.65 (m, 9H). MS calcd for $C_{22}H_{21}BrN_3O_3S$ $[M + H]^+$ 486.0, found 485.9.

tert-Butyl 2-((8-Bromo-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetate (28a)

—NaH 60% dispersion (176 mg, 4.4 mmol) was added to a solution of compound **27a** (1.941 g, 4.0 mmol) in DMF (5 mL) followed by an addition of methyl iodide (249 μ L, 4.0 mmol) according to general procedure F to give 1.36 g of compound **28a** in 68.0% yield. 1H NMR (400 MHz, DMSO- d_6) δ ppm 8.09 (s, 1H), 7.70 (s, 2H), 7.55–7.65 (m, 3H), 7.45 (d, $J = 7.60$ Hz, 1H), 7.46 (d, $J = 7.60$ Hz, 1H), 4.10 (s, 3H), 3.90 (s, 2H), 1.42 (s, 9H). MS calcd for $C_{23}H_{23}BrN_3O_3S$ $[M + H]^+$ 500.1, found 499.9.

tert-Butyl 2-((8-Iodo-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetate (28b)

—NaH 60% dispersion (13.2 mg, 0.33 mmol) was added

to a solution of compound **27b** (160.4 mg, 0.3 mmol) in DMF (1 mL), followed by an addition of methyl iodide (18.8 μ L, 0.3 mmol) according to general procedure F. Compound **28b** was used without purification for the next step. MS calcd for C₂₃H₂₃BrN₃O₃S [M + H]⁺ 548.0, found 547.9.

tert-Butyl 2-((7-Bromo-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetate (28c)—NaH 60% dispersion (8.1 mg, 0.21 mmol) was added to a solution of compound **27c** (150.0 g, 0.21 mmol) in DMF (1 mL), followed by an addition of methyl iodide (13.1 μ L, 0.21 mmol) according to general procedure F to give 71.42 g of compound **28c** in 46.3.0% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 8.01 (s, 1H), 7.89 (d, *J* = 8.78 Hz, 1H), 7.53–7.67 (m, 3H), 7.45 (d, *J* = 7.81 Hz, 3H), 4.09 (s, 3H), 3.90 (s, 2H), 1.38 (s, 9H). MS calcd for C₂₃H₂₃BrN₃O₃S [M + H]⁺, 500.1; found 500.0.

2-((8-Bromo-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)thio)acetic Acid (29a)—Compound **28a** (50 mg, 0.1 mmol) was dissolved in ACN (1 mL) and TFA (1 mL) according to general procedure G to give compound **29a** in quantitative yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 12.81 (br s, 1H), 8.09 (d, *J* = 1.46 Hz, 1H), 7.65–7.74 (m, 2H), 7.55–7.65 (m, 3H), 7.46 (s, 2H), 4.10 (s, 3H), 3.88–3.98 (m, 2H). MS calcd for C₁₉H₁₅BrN₃O₃S [M + H]⁺ 444.0, found 443.9.

2-((8-Iodo-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)thio)acetic Acid (29b)—Compound **28b** (110 mg, 0.2 mmol) was dissolved in ACN (15 mL) and TFA (3 mL) according to general procedure G. Further purification by preparative TLC was necessary to give 12 mg of compound **29b** in 12.2% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 8.29 (s, 1H), 7.76 (d, *J* = 8.78 Hz, 1H), 7.46–7.64 (m, 4H), 7.37 (d, *J* = 7.81 Hz, 2H), 4.05 (s, 3H), 3.67 (s, 2H). MS calcd for C₁₉H₁₅IN₃O₃S [M + H]⁺ 492.0, found 491.8.

2-((7-Bromo-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)thio)acetic Acid (29c)—Compound **28c** (50 mg, 0.1 mmol) was dissolved in ACN (1 mL) and TFA (1 mL) according to general procedure G to give compound **29c** in quantitative yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 12.82 (br s, 1H), 8.01 (s, 1H), 7.90 (d, *J* = 8.29 Hz, 1H), 7.54–7.68 (m, 3H), 7.40–7.51 (m, 3H), 4.09 (s, 3H), 3.95 (s, 2H). MS calcd for C₁₉H₁₅BrN₃O₃S [M + H]⁺ 444.0, found 443.9.

2-((8-Bromo-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)thio)-N-cyclohexylacetamide (30a)—Compound **29a** (20 mg, 0.045 mmol), HATU (19 mg, 0.05 mmol), triethylamine (13 μ L, 0.09 mmol), cyclohexylamine (6 μ L, 0.052 mmol), and DMF (1 mL) were reacted according to general procedure H to give 17 mg of compound **30a** in 71.9% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 8.32 (s, 1H), 8.23 (d, *J* = 7.80 Hz, 1H), 7.64–7.72 (m, 2H), 7.55–7.64 (m, 3H), 7.39–7.49 (m, 2H), 4.10 (s, 3H), 3.84 (s, 2H), 3.52 (br s, 1H), 1.60–1.89 (m, 4H), 1.46–1.59 (m, 1H), 1.02–1.34 (m, 5H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.8, 155.2, 153.6, 138.5, 136.0, 135.8, 130.0, 129.9, 129.9, 129.9, 129.6, 129.5, 129.4, 122.9, 122.8, 121.2, 119.5, 113.2, 112.6, 47.9, 36.7, 32.5, 31.2, 25.2, 24.5. HRMS calcd for C₂₅H₂₅BrN₄O₂SNa [M + Na]⁺ 547.0774, found 547.0774.

N-Cyclohexyl-2-((8-iodo-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (30b)—Compound **29b** (8.73 mg, 0.018 mmol), HATU (7.42 mg, 0.02 mmol), triethylamine (5.7 μ L, 0.041 mmol), cyclohexylamine (2.3 μ L, 0.02 mmol), and DMF (1 mL) were reacted according to general procedure H to give 7.4 mg of compound **30b** in 72.6% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 8.51 (s, 1H), 8.24 (d, J = 7.81 Hz, 1H), 7.79 (dd, J = 1.46, 8.78 Hz, 1H), 7.52–7.68 (m, 4H), 7.37–7.49 (m, 2H), 4.08 (s, 3H), 3.81 (s, 2H), 3.47–3.61 (m, 1H), 1.60–1.88 (m, 4H), 1.48–1.60 (m, J = 10.70 Hz, 0H), 1.02–1.36 (m, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 166.0, 155.3, 153.7, 139.0, 135.9, 135.9, 135.3, 130.1, 129.7, 129.6, 129.2, 122.1, 119.1, 113.5, 83.9, 48.2, 36.7, 32.7, 31.3, 30.5, 25.4, 24.7. HRMS calcd for $\text{C}_{25}\text{H}_{25}\text{IN}_4\text{O}_2\text{SNa}$ [$\text{M} + \text{Na}$] $^+$ 595.0635, found 595.0634.

2-((7-Bromo-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)thio)-N-cyclohexylacetamide (30c)—Compound **29c** (576.5 mg, 1.3 mmol), HATU (542.7 mg, 1.43 mmol), triethylamine (361.4 μ L, 2.6 mmol), cyclohexylamine (163.7 μ L, 1.43 mmol), and DMF (3 mL) were reacted according to general procedure H to give 600.1 mg of compound **30c** in 88.0% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 8.16 (d, J = 7.80 Hz, 1H), 7.97–8.08 (m, 2H), 7.53–7.73 (m, 4H), 7.37–7.52 (m, 3H), 4.09 (s, 3H), 3.87 (s, 2H), 3.44–3.56 (m, 1H), 1.58–1.78 (m, 3H), 1.52 (d, J = 11.70 Hz, 1H), 1.06–1.30 (m, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 165.6, 155.1, 153.6, 140.4, 136.7, 135.7, 129.9, 129.5, 129.4, 123.3, 122.1, 120.7, 119.1, 118.6, 113.8, 47.9, 36.6, 35.7, 32.3, 31.1, 24.7. HRMS calcd for $\text{C}_{25}\text{H}_{27}\text{BrN}_4\text{O}_2\text{S}$ [$\text{M} + \text{H}$] $^+$ 525.0954, found 525.0952.

2-((8-Cyano-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)thio)-N-cyclohexylacetamide (31a)—Compound **30a** (50 mg, 0.095 mmol), copper(I) cyanide (52 mg, 0.58 mmol), and 1-methyl-2-pyrrolidone (NMP) (1.5 mL) were sealed in a microwave vial and irradiated at 220°C for 20 min. The suspension was cooled to room temperature and extracted with EtOAc and H₂O. The organic layer was dried over MgSO₄ and concentrated in vacuo. The residue was further purified by flash chromatography on silica (hexanes:EtOAc = 70:30 to 65:35 gradient) to give 11 mg of compound **31a** in 24.5% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 8.64 (s, 1H), 8.23 (d, J = 7.81 Hz, 1H), 7.90 (s, 2H), 7.53–7.72 (m, 3H), 7.36–7.52 (m, J = 7.30 Hz, 2H), 4.14 (s, 3H), 3.86 (s, 2H), 3.45–3.60 (m, 1H), 1.59–1.80 (m, 4H), 1.47–1.58 (m, J = 11.20 Hz, 1H), 0.98–1.33 (m, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 165.9, 155.2, 155.0, 140.9, 137.1, 135.7, 130.2, 129.7, 129.5, 129.5, 126.7, 120.4, 119.8, 119.7, 112.7, 102.3, 55.4, 48.1, 36.8, 32.6, 31.6, 25.3, 24.5. HRMS calcd for $\text{C}_{26}\text{H}_{27}\text{N}_5\text{O}_2\text{S}$ [$\text{M} + \text{H}$] $^+$ 472.1802, found 472.1805.

2-((7-Cyano-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)thio)-N-cyclohexylacetamide (31c)—Compound **30c** (50 mg, 0.095 mmol), copper(I) cyanide (52 mg, 0.58 mmol), and 1-methyl-2-pyrrolidone (NMP) (1.5 mL) were combined and conditions similar to **31c** were utilized to give 9.5 mg of compound **31c** in 21.2% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 8.38 (s, 1H), 8.23 (d, J = 8.29 Hz, 1H), 8.10–8.19 (m, J = 7.30 Hz, 1H), 7.52–7.69 (m, 4H), 7.38–7.49 (m, J = 2.20, 7.10 Hz, 2H), 4.14 (s, 3H), 3.86 (s, 2H), 3.43–3.52 (m, 1H), 1.54–1.76 (m, 4H), 1.38–1.54 (m, 1H), 1.03–1.27 (m, J = 10.50, 19.30 Hz, 5H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 165.7, 155.4, 154.3,

138.5, 136.3, 135.7, 130.2, 129.7, 129.5, 122.5, 122.4, 121.8, 121.5, 119.7, 116.9, 108.9, 48.1, 36.8, 32.5, 31.6, 25.3, 24.6. HRMS calcd for C₂₆H₂₇N₅O₂S [M + H]⁺ 472.1802, found 472.1806.

2-((8-Amino-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido-[5,4-b]indol-2-yl)thio)-N-cyclohexylacetamide (32)—Compound **30a** (100 mg, 0.19 mmol), sodium azide (120.4 mg, 1.85 mmol), copper(I) iodide (180.1 mg, 0.94 mmol), and sodium ascorbate (95 mg, 0.48 mmol) were combined in a microwave vial with a stir bar, sealed, and evacuated under vacuum. A mixture of DMSO:H₂O (5:1, 3 mL) was degassed with argon and added to the reaction vial, followed by the addition of *N,N*-dimethylethylenediamine (100.24 μ L, 0.94 mmol). The reaction vial was evacuated under vacuum again, flushed with argon gas, and irradiated at 100°C for 3 h. When the reaction returned to room temperature, the suspension was extracted with EtOAc and brine. The organic layer was dried over MgSO₄ and concentrated in vacuo. The crude residue was purified by flash chromatography on silica (DCM:MeOH = 98:2 to 90:10 gradient) to give 58.4 mg of compound **32** in 66.5% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 8.11 (d, *J* = 7.81 Hz, 1H), 7.53–7.66 (m, *J* = 5.90 Hz, 3H), 7.34–7.47 (m, 3H), 7.14 (s, 1H), 6.97 (d, *J* = 8.80 Hz, 1H), 4.97 (br s, 2H), 4.01 (s, 3H), 3.87 (s, 2H), 3.43–3.56 (m, *J* = 3.40 Hz, 1H), 1.57–1.83 (m, 4H), 1.45–1.57 (m, *J* = 12.70 Hz, 1H), 1.01–1.31 (m, 5H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.7, 155.4, 151.0, 134.3, 129.9, 129.7, 129.6, 129.6, 120.6, 118.9, 118.6, 111.2, 48.0, 36.8, 32.3, 30.9, 25.2, 24.5. HRMS calcd for C₂₅H₂₇N₅O₂SNa [M + Na]⁺ 484.1778, found 484.1774.

N-(2-((2-(Cyclohexylamino)-2-oxoethyl)thio)-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-8-yl)benzamide (33)—Compound **32** (10 mg, 0.022 mmol), benzoic anhydride (9.8 mg, 0.044 mmol), and DMF (500 μ L) were combined and stirred at rt overnight. The solution was concentrated in vacuo, reconstituted with 500 μ L of hot MeOH, and solids were filtered and collected to give 7 mg of compound **33** in 57.1% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 10.37 (s, 1H), 8.68 (s, 1H), 8.20 (d, *J* = 7.32 Hz, 1H), 8.03 (d, *J* = 7.81 Hz, 2H), 7.73–7.82 (m, 1H), 7.66–7.72 (m, 1H), 7.52–7.66 (m, 6H), 7.46 (d, *J* = 7.81 Hz, 2H), 4.12 (s, 3H), 3.93 (s, 2H), 3.42–3.55 (m, 1H), 1.55–1.81 (m, 4H), 1.48 (d, *J* = 11.71 Hz, 1H), 1.02–1.30 (m, 5H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.7, 165.5, 155.6, 152.9, 137.3, 137.2, 136.2, 135.2, 132.6, 131.8, 130.2, 129.8, 129.5, 128.8, 128.7, 127.8, 122.8, 119.7, 119.5, 112.1, 111.1, 48.3, 37.1, 32.5, 31.4, 25.3, 24.7. HRMS calcd for C₃₂H₃₁N₅O₃SNa [M + Na]⁺ 588.2040, found 588.2036.

N-(2-((2-(Cyclohexylamino)-2-oxoethyl)thio)-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-8-yl)octanamide (34)—Compound **32** (10 mg, 0.022 mmol), octanoic acid (3.44 mg, 0.024 mmol), HATU (9.06 mg, 0.024 mmol), TEA (6.03 μ L, 0.044 mmol), and 500 μ L of DMF were reacted similarly to general procedure H (octanoic acid in place of compound **17** and compound **32** in place of cyclohexylamine) to give 4.4 mg of compound **34** in 34% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 9.92 (s, 1H), 8.55 (s, 1H), 8.17 (d, *J* = 7.32 Hz, 1H), 7.52–7.63 (m, 4H), 7.38–7.51 (m, 3H), 4.06 (s, 3H), 3.88 (s, 2H), 3.42–3.52 (m, 1H), 2.32 (t, *J* = 7.32 Hz, 2H), 1.54–1.79 (m, 6H), 1.43–1.53 (m, *J* = 11.70 Hz, 1H), 0.98–1.40 (m, 13H), 0.80–0.89 (m, 3H). ¹³C NMR (126 MHz,

DMSO-*d*₆) δ 171.4, 165.9, 155.7, 152.9, 137.3, 137.1, 136.4, 133.1, 130.0, 130.0, 121.7, 119.9, 119.6, 111.4, 110.6, 48.5, 37.3, 36.7, 32.7, 31.7, 31.5, 29.2, 29.0, 25.7, 25.6, 25.0, 22.6, 14.4. HRMS calcd for C₃₃H₄₁N₅O₃SNa [M + Na]⁺ 610.2822, found 610.2823.

4-((2-((2-(Cyclohexylamino)-2-oxoethyl)thio)-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-8-yl)amino)-4-oxobutanoic Acid (35)—

Compound **32** (20 mg, 0.044 mmol), succinic anhydride (4.34 mg, 0.044 mmol), and DMF (500 μ L) were reacted similarly to compound **33** to give 14 mg of compound **35** in 57.5% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 10.69 (br s, 1H), 8.52 (s, 1H), 8.25 (d, *J* = 7.32 Hz, 1H), 7.36–7.74 (m, 6H), 4.08 (s, 3H), 3.92 (s, 2H), 2.39–2.69 (m, 4H), 1.58–1.83 (m, 4H), 1.43–1.56 (m, *J* = 10.70 Hz, 1H), 0.98–1.37 (m, 5H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.6, 155.5, 152.6, 137.1, 136.8, 136.2, 133.1, 130.1, 129.8, 129.8, 121.3, 119.7, 119.4, 111.2, 110.0, 48.2, 37.3, 33.1, 32.4, 31.3, 25.4, 24.7. HRMS calcd for C₂₉H₃₁N₅O₅SNa [M + Na]⁺ 584.1938, found 584.1941.

N-Cyclohexyl-2-((5-methyl-4-oxo-3,8-diphenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (36)—

Compound **30a** (50 mg, 0.01 mmol), tetrakis(triphenylphosphine)-palladium(0) (44 mg, 0.04 mmol), phenylboronic acid (13.9 mg, 0.012 mmol), sodium carbonate (30 mg, 0.03 mmol), DMF (1.2 mL), and H₂O were reacted according to general procedure I to give 16 mg of compound **36** in 32.2% yield. ¹H NMR (500 MHz, DMSO-*d*₆) δ ppm 8.39 (s, 1H), 8.25 (d, *J* = 7.40 Hz, 1H), 7.89 (d, *J* = 7.40 Hz, 1H), 7.72–7.82 (m, 3H), 7.59 (s, 3H), 7.42–7.54 (m, 4H), 7.38 (t, *J* = 7.40 Hz, 1H), 4.13 (s, 3H), 3.85 (s, 2H), 3.42–3.57 (m, 1H), 1.67–1.79 (m, 2H), 1.52–1.62 (m, 2H), 1.42–1.52 (m, 1H), 1.07–1.29 (m, *J* = 9.20 Hz, 4H), 0.91–1.06 (m, 1H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 166.8, 155.8, 153.5, 140.8, 140.0, 137.9, 136.2, 133.2, 130.5, 130.2, 130.0, 130.0, 129.7, 129.5, 129.3, 127.4, 127.2, 127.1, 120.6, 119.7, 118.9, 118.8, 111.8, 48.7, 36.9, 32.9, 31.7, 29.3, 24.9. HRMS calcd for C₃₁H₃₁N₄O₂S [M + H]⁺ 523.2162, found 523.2160.

2-((8-([1,1'-Biphenyl]-4-yl)-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)-N-cyclohexylacetamide (37)—

Compound **30a** (50 mg, 0.01 mmol), tetrakis(triphenylphosphine)-palladium(0) (44 mg, 0.04 mmol), [1,1'-biphenyl]-4-ylboronic acid (22.5 mg, 0.012 mmol), sodium carbonate (30 mg, 0.03 mmol), and DMF (1.2 mL) and H₂O were reacted according to general procedure I to give 10.3 mg of compound **37** in 18% yield. ¹H NMR (500 MHz, DMSO-*d*₆) δ ppm 8.46 (s, 1H), 8.27 (d, *J* = 8.02 Hz, 1H), 7.96 (d, *J* = 8.60 Hz, 1H), 7.90 (d, *J* = 8.02 Hz, 2H), 7.78–7.84 (m, 3H), 7.75 (d, *J* = 8.02 Hz, 2H), 7.58–7.65 (m, 3H), 7.51 (t, *J* = 7.73 Hz, 2H), 7.46 (d, *J* = 7.40 Hz, 2H), 7.40 (t, *J* = 7.40 Hz, 1H), 4.15 (s, 3H), 3.88 (s, 2H), 3.45–3.55 (m, 1H), 1.68–1.81 (m, *J* = 10.30 Hz, 2H), 1.52–1.62 (m, *J* = 12.60 Hz, 2H), 1.41–1.51 (m, *J* = 12.00 Hz, 1H), 1.12–1.28 (m, 4H), 0.93–1.07 (m, *J* = 11.50 Hz, 1H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 166.0, 155.4, 153.3, 139.7, 139.6, 139.5, 138.7, 137.5, 136.0, 132.1, 130.0, 129.6, 129.6, 129.1, 127.6, 127.3, 127.2, 126.7, 126.6, 120.4, 119.4, 118.4, 111.6, 48.1, 36.8, 32.6, 31.3, 25.2, 24.5. HRMS calcd for C₃₇H₃₅N₄O₂S [M + H]⁺ 599.2475, found 599.2478.

N-Cyclohexyl-2-((5-methyl-8-(naphthalen-1-yl)-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (38)—

Compound **30a** (50 mg, 0.01 mmol),

tetrakis(triphenylphosphine)-palladium(0) (44 mg, 0.04 mmol), naphthalen-1-ylboronic acid (20 mg, 0.012 mmol), sodium carbonate (30 mg, 0.03 mmol) DMF (1.2 mL), and H₂O were reacted according to general procedure I to give 19.1 mg of compound **38** in 35% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 8.17 (s, 1H), 7.94–8.09 (m, 3H), 7.82 (d, *J* = 8.29 Hz, 1H), 7.76 (d, *J* = 8.29 Hz, 1H), 7.52–7.68 (m, 6H), 7.47 (s, 4H), 4.18 (s, 3H), 3.77 (s, 2H), 1.58 (d, *J* = 11.22 Hz, 2H), 1.41 (t, *J* = 13.20 Hz, 3H), 0.90–1.14 (m, 4H), 0.83 (s, 1H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 166.3, 155.7, 153.4, 140.1, 139.6, 137.5, 136.1, 133.7, 132.7, 131.7, 130.3, 129.9, 129.8, 128.6, 127.8, 127.4, 126.7, 126.3, 125.7, 125.7, 121.8, 120.1, 119.5, 111.1, 48.3, 36.8, 32.7, 31.5, 25.3, 24.6. HRMS calcd for C₃₅H₃₃N₄O₂S [M + H]⁺ 573.2319, found 573.2317.

N-Cyclohexyl-2-((5-methyl-8-(naphthalen-2-yl)-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (39)—Compound **30a** (50 mg, 0.01 mmol), tetrakis(triphenylphosphine)-palladium(0) (44 mg, 0.04 mmol), naphthalen-2-ylboronic acid (20 mg, 0.012 mmol), sodium carbonate (30 mg, 0.03 mmol), DMF (1.2 mL), and H₂O were reacted according to general procedure I to give 17.4 mg of compound **39** in 32% yield. ¹H NMR (500 MHz, DMSO-*d*₆) δ ppm 8.58 (s, 1H), 8.35 (br s, 1H), 8.30 (d, *J* = 8.02 Hz, 1H), 8.07 (t, *J* = 8.60 Hz, 2H), 7.95–8.04 (m, 3H), 7.83 (d, *J* = 9.16 Hz, 1H), 7.50–7.65 (m, 5H), 7.44–7.49 (m, 2H), 4.16 (s, 3H), 3.88 (s, 2H), 3.44–3.56 (m, 1H), 1.68–1.82 (m, *J* = 12.60 Hz, 2H), 1.47–1.57 (m, *J* = 3.40, 12.60 Hz, 2H), 1.34–1.45 (m, *J* = 12.60 Hz, 1H), 1.20–1.31 (m, 2H), 1.07–1.19 (m, 2H), 0.87–0.99 (m, 1H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 166.3, 155.5, 153.4, 139.8, 137.8, 137.7, 136.0, 133.6, 132.5, 132.2, 130.1, 129.8, 129.7, 128.6, 128.1, 127.8, 127.2, 126.7, 126.2, 125.5, 125.0, 120.5, 119.5, 118.9, 111.7, 48.4, 36.8, 32.6, 31.4, 25.2, 24.6. HRMS calcd for C₃₅H₃₃N₄O₂S [M + H]⁺ 573.2319, found 573.2318.

N-Cyclohexyl-2-((5-methyl-4-oxo-3-phenyl-8-((trimethylsilyl)-ethynyl)-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (40)—Compound **30a** (30.1 mg, 57.3 μ mol), Pd₂(dba)₃ (5.8 mg, 5.7 μ mol), CuI (1.1 mg, 5.7 μ mol), tri-*tert*-butylphosphonium tetrafluoroborate (6.66 mg, 22.9 μ mol), and cesium carbonate (91.7 mg, 281 μ mol) were added in a Schlenk tube under argon atmosphere. Then, DMF (3 mL) and TMS-acetylene (32.0 μ L, 228 μ mol) were added, and the mixture was allowed to react at 50°C for 1 h. Then, toluene (6 mL) was added to the mixture and filtered through silica gel pad. The filtrate was poured into H₂O, extracted with EtOAc, dried over MgSO₄, and concentrated in vacuo. The residue from the organic layer was purified by silica gel column chromatography (toluene:acetone = 9:1) and preparative HPLC (reversed phase, DAISOPAK SP-120-5-ODS-BP, 250 mm $\times$ 20 mm, MeOH:H₂O = 17:3) to give a white solid (27.9 mg, 90%). ¹H NMR (594 MHz, DMSO-*d*₆) δ 8.25–8.31 (m, 2H), 7.68 (d, *J* = 8.84 Hz, 1H), 7.55–7.63 (m, 4H), 7.42–7.47 (m, *J* = 1.70, 7.80 Hz, 2H), 4.10 (s, 3H), 3.81 (s, 2H), 3.49–3.57 (m, 1H), 1.62–1.79 (m, 4H), 1.50–1.58 (m, *J* = 12.20 Hz, 1H), 1.10–1.31 (m, 6H), 0.22–0.29 (m, 9H). ¹³C NMR (149 MHz, DMSO-*d*₆) δ 165.9, 155.2, 154.0, 139.3, 136.9, 135.8, 130.2, 130.0, 129.6, 129.5, 124.9, 119.6, 119.6, 113.8, 111.5, 105.9, 92.3, 47.9, 36.7, 32.5, 31.3, 25.3, 24.5, 0.1. HRMS calcd for C₃₀H₃₅N₄O₂SSi [M + H]⁺ 543.2245, found 543.2245.

N-Cyclohexyl-2-((8-ethynyl-5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (41)—Compound **40** (14.4 mg, 26.5 μ mol) was placed in a two-neck flask under argon atmosphere. Then, THF (7 mL) and TBAF (29.0 μ L, 29.2 mmol, 1 M THF solution) were added and the mixture was reacted for 2 h. The reaction mixture was poured into H₂O and extracted with EtOAc and dried over MgSO₄. The residue from the organic layer was purified by silica gel column chromatography (toluene:acetone = 10:1) to give a white solid (12.2 mg, 98%). ¹H NMR (594 MHz, DMSO-*d*₆) δ 8.28 (s, 1H), 8.22 (d, *J* = 7.48 Hz, 1H), 7.70 (d, *J* = 8.84 Hz, 1H), 7.56–7.63 (m, 4H), 7.42–7.47 (m, 2H), 4.15 (s, 1H), 4.10 (s, 3H), 3.85 (s, 2H), 3.47–3.55 (m, 1H), 1.60–1.78 (m, 4H), 1.49–1.56 (m, *J* = 11.60 Hz, 1H), 1.06–1.27 (m, 6H). ¹³C NMR (149 MHz, DMSO-*d*₆) δ 206.6, 165.8, 155.2, 153.8, 139.4, 136.8, 135.8, 130.5, 130.0, 129.6, 129.5, 124.7, 119.6, 119.6, 113.4, 111.5, 84.0, 48.0, 36.7, 32.5, 31.3, 30.7, 25.3, 24.5. HRMS calcd for C₂₇H₂₆N₄O₂SNa [M + Na]⁺ 493.1669, found 493.1672.

Biological Studies

In Vitro Assays Using TLR4 Reporter Cell Lines. Murine or human TLR4 reporter cells (TLR4 HEK Blue cells, Invivogen, 2.5 $\times$ 10⁴ cells per well of a 96 well plate) were incubated with graded doses of the test compounds in DMEM with 10% FBS, 1 mM sodium pyruvate, 4 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. Tested compounds were dissolved in dimethyl sulfoxide (DMSO) at 20 mM as a stock solution that were further serially diluted with DMSO and then added to the well (final DMSO concentration at 0.05%). The culture supernatants were harvested after a 20–24 h incubation period. SEAP activity in the culture supernatants was determined by a colorimetric assay, using QuantiBlue (Invivogen), with absorbance read at 630 nm. LPS (100 ng/mL, L4524, Sigma-Aldrich) was used as a positive control.

In Vitro Immune Potency Studies Using Murine and Human Primary Cells— Murine BMDC were prepared from C57BL/6 mice as described.²⁸ Cells (1 $\times$ 10⁵ cells per well) were plated in 96-well plates in 150 μ L of RPMI 1640 (plus 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin). The cells were incubated with a graded dosage of the test compounds for 18 h at 37°C, 5% CO₂. After 18 h incubation, the cell culture supernatants were collected. Ultrapure LPS-EB (10 ng/mL, Invivogen) was used as a positive control. The levels of cytokines IL-6 or CCL5 in the culture supernatants were determined by ELISA (BD Biosciences, La Jolla, CA).²⁹ Minimum detection levels of these cytokines were 15 pg/mL. For the experiments using human primary cells, PBMC were isolated from buffy coats obtained from the San Diego Blood Bank (San Diego, CA) as described previously.²⁹ Human PBMC (1 $\times$ 10⁶/mL) were incubated with various compounds for 18 h at 37°C, 5% CO₂, and culture supernatants were collected. The levels of IL-6 and IL-8 in the supernatants were determined by ELISA.

Cell Viability Assay— Murine BMDC or human liver cells (HepG2 cell line) were plated in 96-well plates (1 $\times$ 10⁴ cells per well) and incubated overnight with 5 μ M concentration of each compound overnight. After 18 h of drug treatment, MTT-based solution (0.5 mg/mL) was added to each well. Six or more hours later, the cells were lysed and absorbance values at 570 and 650 nm were measured.

In Vivo Assays of Immune Potency—Seven-to nine-week-old C57BL/6 (wild-type, WT) mice were purchased from the Jackson Laboratories (Bar Harbor, MA). The mice ($n = 6$) were iv injected with indicated compounds (20 nmol/animal) in 0.1% DMSO, and sera were collected at 3 h post injection. IL-6, IP-10, and KC in sera were measured by Luminex assay (EMD Millipore, Temecula, CA). The procedures used in in vivo studies were in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of University of California, San Diego. Mice were sacrificed by CO₂ inhalation followed by cervical dislocation. All efforts were made to minimize animal suffering.

Statistical Analysis—Data for in vitro and in vivo studies are represented as mean $\pm$ standard error of the mean (SEM). Prism 6 (GraphPad Software, San Diego, CA) statistical software was used to obtain AUC, EC₅₀, and p -values for comparison between groups. Two-tailed Student's t -test was used to compare activities between two compounds. Two-way ANOVA followed by Dunnett's post hoc comparison test were used to compare data for compounds at different concentrations to that of compound **1**. Kruskal–Wallis test with Dunnett's post hoc testing was used to compare multiple groups in in vivo study. $p < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

HTS	high throughput screen
TLR	Toll-like receptor
SAR	structure–activity relationship
IL	interleukin
LPS	lipopoly-saccharide
MD-2	myeloid differentiation protein-2
PBMC	peripheral blood mononuclear cells
ACN	acetonitrile
EtOH	ethanol

EtOAc	ethyl acetate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
DMF	<i>N,N</i> -dimethylformamide
HATU	1-[Bis(dimethylamino)methylene]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i>]-pyridinium 3-oxide hexafluorophosphate
DCM	dichloro-methane
MeOH	methanol
TMS	trimethylsilyl
TEA	triethylamine
mBMDC	mouse bone marrow derived dendritic cells
NF κB	nuclear factor kappa B
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
IFN	interferon
FRET	Förster resonance energy transfer
SEAP	secreted embryonic alkaline phosphatase
ELISA	enzyme-linked immunosorbent assay
KC	keratinocyte chemoattractant
IP-10	interferon gamma-induced protein 10
PAMP	pathogen-associated molecular patterns
PRR	pattern-recognition receptors
DMSO	dimethyl sulfoxide

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Figure 2.

Potencies of synthetic TLR4 ligand SAR derivatives in primary murine BMDC. The potencies of **1**, **21d**, **36**, and **39** were evaluated by mTLR4 reporter cells and mBMDC. (A) Murine TLR4 reporter cells (2.5×10^4 /well) were plated in 96-well plates and were incubated with graded doses of **1**, **21d**, **36**, and **39** overnight. The potency of TLR4 activation was assessed by SEAP assay (OD₆₃₀). (B,C) Murine BMDC (10^5 /well in 96-well plates) were incubated with graded doses of compounds overnight. Activation of mBMDC was evaluated by IL-6 (B) and CCL5 (C) release in the culture supernatants determined by ELISA. *, $p < 0.05$ by two-way ANOVA with Dunnett's post hoc test compared to compound **1**.

Figure 3.

Potencies of synthetic TLR4 ligand SAR derivatives in human cells. The potencies of **1**, **21d**, **36**, and **39** in human cells were measured by hTLR4 reporter cells (A) and primary hPBMC (B,C). Toxicity was evaluated using HepG2 cells (D). (A) Human TLR4 reporter cells (2.5×10^4 /well) were plated in 96-well plates and were incubated with graded doses of **1**, **21d**, **36**, and **39** overnight. The potency was measured by SEAP assay (OD₆₃₀). (B,C) Primary human PBMC obtained from San Diego Blood Bank were plated on 96-well plates (2×10^5) and stimulated with graded doses of the compounds. IL-8 (B) and IL-6 (C) in the culture supernatants were measured by ELISA. (D) HepG2 cells (1×10^4) were incubated with 5 μM compounds overnight, and viabilities of cells were measured by MTT assay (OD₆₃₀). Data shown are mean $\pm$ SEM of representative data of three independent experiments showing similar trends. *, $p < 0.05$ by two way ANOVA with Dunnett's post hoc test compared to compound **1**.

Figure 4.

Immunostimulatory activities of active compounds in vivo. Compounds **1**, **21d**, and **36** (20 nmol in 0.1% DMSO in saline) were administered iv to C57BL/6 mice, and sera were collected 3 h post injection. The levels of IL-6, KC, and IP-10 were measured by Luminex beads assay. The levels of these chemokines and cytokine following injection with vehicle alone were below the detection levels (<2.56 pg/mL). *, $p < 0.05$ by Kruskal–Wallis test with Dunn’s post hoc test compared to compound **1**.

Figure 5.

Computational docking studies of compounds **1** and **36** to TLR4/MD-2 complex. (A) Molecular docking of compound **1** (orange) and **36** (magenta) in the LPS (LPS shown in gray) binding pocket of human TLR4/MD-2 complex (PDB 3FXI). The van der Waals surface representation of MD-2 is shown in cyan, while that of TLR4 interacting with MD-2 is shown in green mesh. (B) Magnified view of subpocket area highlighted in A (yellow square area) showing C8-phenyl substituent of compound **36** (shown without LPS for clarity) in subpocket. (C) Overlay of binding geometry between compound **1** (orange) and **36** (magenta) and additional interactions of the C8-phenyl substituent of compound **36** with the residues (MD-2 residues in cyan and TLR4 residues in green) in the TLR4/MD-2 complex.

Scheme 1. (a) 2-Thioacetamide Analogues and Derivatives; (b) 2-Thioacetamide Derivatives^a

^a(panel a) Reagents and conditions: (a) 3-chloroperoxybenzoic acid, MeOH/CHCl₃, 45°C; (b) HBr/AcOH; (c) (i) HATU, Et₃N, cyclohexylamine, DMF, (ii) 4 N HCl/dioxane; (d) Et₃N, EtOH, 120°C; (e) 5.8 M aqueous HCl, 120°C; (f) *tert*-butyl bromoacetate, *N,N*-diisopropylethylamine, DMF, 60°C; (g) (i) 50% CF₃COOH in CH₃CN, (ii) HATU, Et₃N, cyclohexylamine, DMF. (panel b) (Reagents and conditions: (a) (i) *tert*-butyl 3 bromopropionate for **11a** or *tert*-butyl 4 bromobutyrate for **11b**, Et₃N, DMF, 80°C; (b) (i) 50% CF₃COOH in CH₃CN, (ii) HATU, Et₃N, cyclohexylamine, DMF; (c) 3-cyclohexyl propylbromide, Et₃N, DMF, 80°C.

Scheme 2. Truncated Derivatives^a

^aReagents and conditions: (a) $\text{NH}_2\text{CH}_2\text{COOEt}$, EtOH, NEt_3 ; (b) NaOEt /EtOH reflux; (c) phenyl-NCS, EtOH, reflux; (d) PPA, 110°C ; (e) ClCH_2COOH ; (f) cyclohexyl- NH_2 , HATU, DMF, roomtemp; (g) NaH , DMF, then CH_3I .

Scheme 3. 7- and 8-Substituted Derivatives^a

^aReagents and conditions: (a) BrCH₂COOEt, EtOH, reflux; (b) *tert*-BuOK, THF, <30°C; (c) phenyl-NCS, EtOH, reflux; (d) acetyl chloride, EtOH; (e) BrCH₂COO *t*-Bu; (f) NaH, DMF, then CH₃I; (g) TFA, DCM or CH₃CN; (h) cyclohexyl-NH₂, HATU, DMF, room temp; (i) CuCN, NMP MW 220°C; (j) NaN₃, CuI, sodium ascorbate DMSO/H₂O MW 100°C.

Scheme 4. Substituted 8-Amino Derivatives^a

^aReagents and conditions: (a) benzoic anhydride/DMF, 16 h, room temp; (b) octanoic acid, HATU, NEt₃, DMF, room temp; (c) succinic anhydride/DMF, 16 h, room temp.

Scheme 5. 8-Aryl and Alkynyl Derivatives^a

^aReagents and conditions: (a) phenylboronic acid, Pd(PPh₃)₄, DMF/H₂O, Na₂CO₃, MW110°C, 15 min; (b) biphenylboronic acid, Pd(PPh₃)₄, DMF/H₂O, Na₂CO₃, MW110°C, 15 min; (c) 1- or 2-naphthylboronic acid, Pd(PPh₃)₄, DMF/H₂O, Na₂CO₃, MW 110°C, 15 min; (d) Pd₂(dba)₃, CuI, tri-*tert*-butylphosphoniumBF₄, Cs₂CO₃, TMS-acetylene; (e) TBAF, THF, room temp.

Table 1

2-Thioacetamide Derivatives and Analogues

compd	X	R	mTLR4 ^a		hTLR4 ^b		mBMDC	
			%AUC ^b	EC ₅₀ ^c	%AUC ^b	EC ₅₀ ^c	%IL-6 ^d	MTT ^e
1	-SCH ₂ CONH-	CH ₃	100	3.17 ± 0.65	100	8.26 ± 1.76	100	97
2	-SO ₂ CH ₂ CONH-	CH ₃	2* ^f	>10	16*	>10	0*	50*
6a	-NH(CH ₂)CONH-	CH ₃	2*	>10	10*	>10	1*	113
6b	-NH(CH ₂) ₂ CONH-	CH ₃	2*	>10	8*	>10	0*	92
9	-O(CH ₂)CONH-	CH ₃	1*	>10	9*	>10	0*	58*
12a	-S(CH ₂) ₂ CONH-	H	3*	>10	7*	>10	0*	58*
12b	-S(CH ₂) ₃ CONH-	H	2*	>10	8*	>10	0*	70*
13	-S(CH ₂) ₃ -	H	1*	>10	9*	>10	0*	57*

^aTLR4 reporter cells (2.5×10^4 /well) were plated in 96 well plates and were incubated with graded doses of each compound. The potency of TLR4 activation was assessed by SEAP assay (OD₆₃₀).

^bAUC values in hTLR4 and mTLR4 reporter cells were calculated and normalized to compound **1**. AUC of compound **1** for mTLR4 and hTLR4 reporter cells were 8.11 ± 0.75 units and 3.23 ± 0.89 units, respectively.

^cConcentration in μ M of compound at which 50% of maximum activation is achieved.

^dIL-6 (ng/mL) release by mBMDC following treatment with compounds at 5 μ M and normalized to **1** which gave 6.4 ± 1.0 ng/mL (mean $\pm$ SEM).

^eCell viability of mBMDC as measured by MTT assay following treatment with compounds at 5 μ M. Data was normalized to vehicle control (vehicle OD₅₇₀₋₆₅₀ = 0.8 ± 0.1).

^f*: Statistically significant ($p < 0.05$) compared to **1**.

Table 2

Truncated Derivatives

compd	R ¹	R ²	R ³	mTLR4 ^a			hTLR4 ^b			mBMDC	
				%AUC ^b	EC ₅₀ ^c	EC ₅₀ ^c	%AUC ^b	EC ₅₀ ^c	EC ₅₀ ^c	%IL-6 ^d	MTT ^e
1	-(CH ₂) ₄ -	Me		100	3.17 ± 0.65	100	8.26 ± 1.76	100	106	100	106
20d	-(CH ₂) ₅ -	H		82	2.83 ± 0.65	137* ^f	7.42 ± 0.92	115	84	115	84
21d	-(CH ₂) ₅ -	Me		44*	8.80 ± 3.69	46*	>10	93	95	93	95
20c	-(CH ₂) ₄ -	H		36*	7.12 ± 1.26	27*	>10	80	91	80	91
21c	-(CH ₂) ₄ -	Me		13*	>10	8*	>10	65	106	65	106
20b	Me	Me	H	28*	>10	18*	>10	78*	70	78*	70
21b	Me	Me	Me	22*	>10	12*	>10	81	100	81	100
20a	H	H	H	4*	>10	7*	>10	0*	99	0*	99
21a	H	H	Me	5*	>10	17*	>10	1*	117	1*	117

^aTLR4 reporter cells (2.5 × 10⁴/well) were plated in 96-well plates and were incubated with graded doses of each compound. The potency of TLR4 activation was assessed by SEAP assay (OD₆₃₀).

^bAUC values in hTLR4 and mTLR4 reporter cells were calculated and normalized to compound **1**. AUC of compound **1** for mTLR4 and hTLR4 reporter cells were 8.11 ± 0.75 units and 3.23 ± 0.89 units, respectively.

^cConcentration in μM of compound at which 50% of maximum activation is achieved.

^dIL-6 (ng/mL) release by mBMDC following treatment with compounds at 5 μM and normalized to **1** which gave 6.4 ± 1.0 ng/mL (mean ± SEM).

^eCell viability of mBMDC as measured by MTT assay following treatment with compounds at 5 μM. Data was normalized to vehicle control (vehicle OD₅₇₀₋₆₅₀ = 0.8 ± 0.1).

^f*, Statistically significant (*p* < 0.05) compared to **1**.

Table 3

C7- and C8-Substituted Derivatives

compd	X	mTLR4 ^a			hTLR4 ^a			mBMDc ^a		
		%AUC ^b	EC ₅₀ ^c		%AUC ^b	EC ₅₀ ^c		%IL-6 ^d	MTT ^e	
1	H	100	3.17 ± 0.65		100	8.26 ± 1.76		100	106	
30a	8-Br	103	2.45 ± 0.99		159* ^f	3.58 ± 0.92		120	63	
30b	8-I	104	2.51 ± 0.22		204*	3.75 ± 0.75		74	75	
30c	7-Br	34*	4.89 ± 0.97		40*	>10		73	93	
31a	8-CN	14*	>10		12*	>10		45*	107	
31c	7-CN	6*	>10		20*	>10		3*	65	
32	8-NH ₂	3*	>10		17*	>10		0*	102	
33	8-Bz-NH	2*	>10		26*	>10		7*	43	
34	8-octanoyl-NH	1*	>10		15*	>10		0*	35	
35	8-succinoyl-NH	3*	>10		28*	>10		0*	75	
36	8-Ph	223*	0.82 ± 0.03		1560*	0.43 ± 0.08		123*	93	
37	8-Bi-Ph	1*	>10		31*	>10		0*	23	
38	8-α-naphthyl	38*	2.58 ± 0.07		72*	2.12 ± 0.59		80	102	
39	8-β-naphthyl	78*, ** ^g	2.01 ± 0.25		1300*, ** ^g	0.92 ± 0.20		137*	84	
40	8-C≡CTMS	85*	3.30 ± 1.25		87*	>10		78	73	
41	8-C≡CH	92	2.74 ± 0.32		107	3.27 ± 2.41		92	81	

^aTLR4 reporter cells (2.5×10^4 /well) were plated in 96-well plates and were incubated with graded doses of each compound. The potency of TLR4 activation was assessed by SEAP assay (OD₆₃₀).

^bAUC values in hTLR4 and mTLR4 reporter cells were calculated and normalized to compound **1**. AUC of compound **1** for mTLR4 and hTLR4 reporter cells were 8.11 ± 0.75 units and 3.23 ± 0.89 units, respectively.

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^cConcentration in μM of compound at which 50% of maximum activation is achieved.

^dIL-6 (ng/mL) release by mBMDC following treatment with compounds at 5 μM and normalized to **1** which gave 6.4 ± 1.0 ng/mL (mean $\pm$ SEM).

^eCell viability of mBMDC as measured by MTT assay following treatment with compounds at 5 μM . Data was normalized to vehicle control (vehicle OD_{570–650} = 0.8 ± 0.1).

^f*: Statistically significant ($p < 0.05$) compared to **1**.

^g***: $p < 0.05$ compared to **38**.