Exploration of Toll-like Receptor 7 and 8 Agonists as Potential Vaccine Adjuvants

By

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Submitted to the Graduate Degree program in Medicinal Chemistry and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

Toll-like receptors (TLRs)-7/-8 are among pathogen recognition receptors (PRRs) present in the endosomal compartment that are activated by viral single-stranded RNA (ssRNA) as well as synthetic small molecules. TLR7/8 agonists hold promise as potential vaccine adjuvants, since they directly activate antigen-presenting cells and enhance T helper 1-driven immune responses.

A general introduction to TLRs, with an emphasis on the role of TLR7/8 activation in innate and adaptive immune responses is presented in Chapter 1.

Structure-activity relationship (SAR) studies in small molecule TLR8/7-agonistic ligands showed that thiazolo[4,5-c]quinolines display mixed TLR8/7 agonistic activities with the optimal C2-alkyl chain length being butyl (Chapter 2).

In an ongoing search toward exploring alternative chemotypes, furo[2,3-c]pyridines with pyridoxal as the aldehyde component in a one-pot multicomponent Groebke–Blackburn–Bienaymé reaction were obtained and found to exhibit TLR8-dependent NF-κB activation and strong adjuvanticity without proinflammatory cytokine induction (Chapter 3).

Combinatorial libraries using the Groebke–Blackburn–Bienaymé reaction have also yielded TLR7/8-inactive, but antibacterial imidazo[1,2-a]pyridines (Chapter 4).

Based on the previously reported SARs on imidazoquinolines, the syntheses and biological evaluation of novel imidazo[4,5-c]pyridine analogues were undertaken, with modifications at the N4- and C6 positions, which afforded strong Type I IFN inducers in conjunction with attenuated proinflammatory profiles (Chapter 5).
With the goal of defining structural requisites governing activity and selectivity at TLR7 and/or TLR8, we undertook scaffold-hopping approach, quantum chemical calculations followed by linear discriminant analyses that permitted the classification of inactive, TLR8-active, and TLR7/8 dual-active compounds, confirming the critical role of partial charges in determining biological activity (Chapter 6).

Molecular conjugation of TLR7/8 agonists to hyaluronic acid (HA) was evaluated to enhance selective and targeted delivery of vaccine construct to draining lymph nodes while limiting systemic exposure. The superior adjuvanticity evoking affinity-matured high-avidity immunoglobulins after a single boost was observed with HA conjugate bearing dual TLR7/8 agonist (Chapter 7).

Extensive SAR investigations in several TLR7/8 agonistic scaffolds and exploration as vaccine adjuvant candidates have incrementally improved our understanding of how these molecules activate innate and adaptive immune responses and also catalyzed novel approaches to vaccine design and development.
Acknowledgements

There are a lot of people without whose help this thesis would not have been possible.

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Euna Yoo

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Abbreviations

AIBN  azobisisobutyronitrile
APC   antigen-presenting cell
Be-1  B-effector-1-cells
CCR   chemokine receptor
cDC   conventional dendritic cell
CDMT  2-chloro-4,6-dimethoxy-1,3,5-triazine
CIK   cytokine-induced killer
CLR   C-type lectin receptor
CRP   C-reactive protein
CXCL  chemokine ligand
DC    dendritic cell
DFT   density functional theory
DIPEA diisopropylethylamine
DMF   dimethylformamide
DMSO  dimethylsulfoxide
DS    degree of substitution
dsRNA double stranded RNA
EC₅₀  half maximal effective concentration
ECM   extracellular matrix
EDC   N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide
ELISA enzyme-linked immunosorbent assay
ESI   electrospray ionization
GlcNAc N-acetyl glucosamine
GlcUA glucuronic acid
HA    hyaluronic acid
HAase hyaluronidase
HBTU  N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
HCV   hepatitis C virus
HEK   human embryonic kidney
HIV   human immunodeficiency virus
hPBMC human peripheral blood mononuclear cell
HPV   human papilloma virus
IFN   interferon
Ig    immunoglobulin
IL    interleukin
IP    interferon gamma-induced protein
IRF   IFN regulatory factor
ITAM  immunoreceptor tyrosine-based activation motif
LC    liquid chromatography
LDH   lactate dehydrogenase
LPS   lipopolysaccharides
LRR   leucine-rich repeats
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYVE-1</td>
<td>lymphatic vessel endothelial hyaluronan receptor-1</td>
</tr>
<tr>
<td>MBC</td>
<td>minimum bactericidal concentration</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemoattractant protein</td>
</tr>
<tr>
<td>mCPBA</td>
<td>m-chloroperoxybenzoic acid</td>
</tr>
<tr>
<td>MDA</td>
<td>melanoma differentiation-associated protein</td>
</tr>
<tr>
<td>MES</td>
<td>2-((N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MESP</td>
<td>molecular electrostatic potential</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MPL</td>
<td>monophosphoryl lipid A</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MS</td>
<td>mass spectroscopy</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NALP</td>
<td>NACHT, LRR and PYD domains-containing protein</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NMM</td>
<td>N-methylmorpholine</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methyl-2-pyrrolidone</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide oligomerization domain</td>
</tr>
<tr>
<td>ORTEP</td>
<td>oak ridge thermal ellipsoid plot</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PLVAP</td>
<td>plasmalemma vesicle-associated protein</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>QSAR</td>
<td>quantitative structure-activity relationship</td>
</tr>
<tr>
<td>RIG</td>
<td>retinoic acid inducible gene</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I-like receptor</td>
</tr>
<tr>
<td>sAP</td>
<td>secreted alkaline phosphatase</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
</tr>
<tr>
<td>SEC</td>
<td>size exclusion chromatography</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded RNA</td>
</tr>
<tr>
<td>TB</td>
<td>tuberculosis</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetra-n-butylammonium fluoride</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper 2</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TIR</td>
<td>toll/IL-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
</tbody>
</table>
Chapter 1.

Introduction

TLR-7/8 Agonists
1.1. Vaccine Adjuvants

There can be no greater substantiation of Benjamin Franklin’s adage, ‘An ounce of prevention is worth a pound of cure’ than the resounding impact that vaccines have had in preventing morbidity and mortality due to infectious diseases.\(^1\) Vaccines have resulted in the eradication or dramatic reduction in number of cases such as smallpox, polio, and tetanus. Nevertheless, there is still a pressing need for new vaccines for diseases for which sufficiently effective vaccines do not exist, but also to replace reactogenic vaccines with safer alternatives.\(^2\)

In order to achieve a high level of efficacy and safety, many newer vaccines with more defined composition that is often linked to lower immunogenicity rely on potent immunostimulants (vaccine adjuvants).\(^3\) The Food and Drug Administration (FDA) considers an adjuvant to a substance added to vaccines to enhance the immune response in vaccinated individuals. Adjuvants also serve to reduce the amount of antigen needed for the induction of a robust immune response (‘dose-sparing effect’) or the number of immunizations needed for protective immunity. The ability of adjuvants to broaden antibody responses could be crucial for the success of vaccines against many pathogens that display substantial antigenic drift and/or strain variations including influenza viruses, human immunodeficiency virus (HIV), human papilloma virus (HPV), and the malaria parasite.\(^4\) Adjuvants also help improve the efficacy of vaccines in newborns, the elderly or immunocompromised persons, or can be used as antigen delivery systems for the uptake of antigens.\(^5\)

Several hundred natural and synthetic compounds have been identified to have adjuvantic activity (Table 1).\(^5-6\) Alum, principally aluminum phosphate or hydroxide, was originally identified in the 1920s and has been the most widely used human adjuvants.\(^7\) Studies have shown that alum enhances antigen uptake by dendritic cells (DCs), recruitment of immune-competent cells
to the injection site, and stimulation of immune cells via the inflammasome pathway,⁸ although further details and precise mechanisms need to be elucidated.⁹ A second adjuvant that could be considered a success is the MF59 squalene oil in water emulsion (O/W). MF59 is licensed in most of Europe for use with seasonal flu vaccines in the elderly. MF59 induces the recruitment of neutrophils and monocytes to the site of immunization and potentiates both cellular and humoral immune responses.¹⁰ Virosomes are liposomes that contain fusogenic viral proteins and have been licensed as a component of an influenza vaccine.¹¹ They can be classified more as delivery systems since their main function is to promote more effective delivery of vaccine antigens and immunostimulants. The most advanced of adjuvants, termed the AS series, are generally combinations of alum (or emulsions or liposomes) with immune potentiators.¹² The immune potentiator component(s) is added to increase antibody titers or to induce more potent and focused T cell responses. The more complex formulations, comprising of three or more adjuvant components, are particularly designed to induce more potent T helper 1 (Th1) cellular immune responses.¹³

Despite the impressive success of currently approved adjuvants for generating immunity to viral and bacterial infections, an understanding of the mechanism of action of vaccine adjuvants has remained rudimentary until recently, and there is a compelling need for the rational development of adjuvants that not only provide protective antibody responses, but also generate strong T cell immunity, especially in subunit vaccine constructs incorporating highly purified, recombinantly expressed proteins as immunogens.¹⁴ Adjuvants that stimulate cellular immunity (antigen-specific CD4 and CD8 responses) have been particularly important in protective immunity against intracellular pathogens, and it is worth noting that none of the currently approved adjuvants uniformly and/or sufficiently enhance cell-mediated immune responses. The availability of adjuvants with defined mechanisms of action would not only permit a greater insight into the interface between innate and adaptive immunity, but could perhaps also help
circumvent some of the potential deficiencies associated with current vaccines and catalyze the development of highly efficacious, yet safe vaccines for infectious diseases.

**Table 1. Major adjuvant formulations tested in humans.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Class</th>
<th>Receptors or Pathway</th>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Licensed Adjuvants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alum</td>
<td>Mineral salt</td>
<td>NALP3, Ag delivery</td>
<td>Various</td>
</tr>
<tr>
<td>MF59</td>
<td>Oil/water emulsion</td>
<td>Immune cell recruitment, Ag uptake</td>
<td>Influenza (Fraud), Pandemic flu</td>
</tr>
<tr>
<td>Liposome</td>
<td>Lipid vesicle</td>
<td>Ag delivery</td>
<td>HAV, Flu</td>
</tr>
<tr>
<td>AS03</td>
<td>Oil/water emulsion + α-tocopherol</td>
<td></td>
<td>Pandemic flu (Pandemic)</td>
</tr>
<tr>
<td>AS04</td>
<td>Monophosphoryl Lipid A (MPL) + Alum</td>
<td>TLR4</td>
<td>HBV (Fendrix), HPV (Cevarix)</td>
</tr>
<tr>
<td><strong>Widespread Experimental Use or in Late Stage Clinical Development</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Montanide</td>
<td>Water/oil emulsion</td>
<td></td>
<td>Malaria, cancer</td>
</tr>
<tr>
<td>PLG</td>
<td>Polymeric microparticle</td>
<td></td>
<td>DNA vaccine (HIV)</td>
</tr>
<tr>
<td>Flagellin</td>
<td>Flagellin from S. typhimurium</td>
<td>TLR5</td>
<td>Flu</td>
</tr>
<tr>
<td>QS21</td>
<td>Saponin</td>
<td></td>
<td>Various</td>
</tr>
<tr>
<td>AS01</td>
<td>MPL + liposomes + QS21</td>
<td>TLR4</td>
<td>Malaria, TB</td>
</tr>
<tr>
<td>AS02</td>
<td>MPL + Oil/water emulsion + QS21</td>
<td>TLR4</td>
<td>Malaria</td>
</tr>
<tr>
<td>RC-529</td>
<td>Synthetic MPL + Alum</td>
<td>TLR4</td>
<td>HBV</td>
</tr>
<tr>
<td>ISCOMs</td>
<td>Saponins + cholesterol + phospholipids</td>
<td></td>
<td>Various</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>Synthetic derivative of dsRNA</td>
<td>TLR3, MDA5</td>
<td></td>
</tr>
<tr>
<td>IC31</td>
<td>Peptide + oligonucleotide</td>
<td>TLR9</td>
<td>TB</td>
</tr>
<tr>
<td>CpG 7909</td>
<td>Oligonucleotide + Alum or MF59</td>
<td>TLR9</td>
<td>HBV, malaria, HCV</td>
</tr>
<tr>
<td>ISS</td>
<td>Oligonucleotide Alum</td>
<td>TLR9</td>
<td>HBV</td>
</tr>
<tr>
<td>MF59 + MTP-PE</td>
<td>Lipidated MDP + Oil/water emulsion</td>
<td></td>
<td>HIV, Flu</td>
</tr>
<tr>
<td>Imiquimod</td>
<td>Imidazoquinoline derivatives</td>
<td>TLR7/8</td>
<td></td>
</tr>
<tr>
<td>CAF01</td>
<td>Trehalose dimycolate</td>
<td></td>
<td>Mincl</td>
</tr>
</tbody>
</table>

1.2. Innate Immunity and Toll-like Receptors (TLRs)

The human immune system can be conceived of as comprising of two mutually non-exclusive subsystems: the innate and the adaptive components; these two limbs work cooperatively to afford protection from numerous pathogenic microorganisms and toxins. Until recently, innate immunity was thought of as rather crude and unsophisticated first line of defense, providing non-
specific microbicidal activity and early inflammatory signals and merely allowing downstream adaptive immune responses more time to develop. However, it is now clear that the adaptive immune responses are largely predicated upon the level and specificity of the initial signals perceived by innate immune cells following infection and/or vaccination, determining whether or not specific and sustained response and protection will ensue.  

Vaccine adjuvant research has expanded rapidly in the past decade and has directly benefited from our evolving understanding of immunology, beginning with the recognition of the cellular elements involved in innate immunity, and growing to encompass the elucidation of the mechanisms of recruitment of adaptive immune effector pathways. Knowledge of the molecular mechanism of innate immune activation has also afforded a large number of potential new targets for immune stimulators. Numerous receptors and signaling pathways in the innate immune system have been defined. Unlike adaptive immunity, the initial innate immune responses rely on a limited number of germline-encoded pattern recognition receptors (PRRs), which recognize specific molecular patterns present in molecules that are broadly shared by pathogens but are sufficiently different so as to be distinguishable from host molecules, and are collectively referred to as pathogen-associated molecular patterns (PAMPs). PRRs are classified according to their structural homology: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs).

The TLR family is one of the well studied targets in terms of ligands, downstream signaling pathways, and functional relevance. There are 10 TLRs in the human genome; these are transmembrane proteins with an extracellular domain having leucine-rich repeats (LRR) and a cytosolic domain called the Toll/IL-1 receptor (TIR) domain. The ligands for these receptors are highly conserved microbial molecules such as lipopolysaccharides (LPS) (recognized by TLR4), lipopeptides (TLR2 in combination with TLR1 or TLR6), flagellin (TLR5), single stranded
RNA (TLR7 and TLR8), double stranded RNA (TLR3), CpG motif-containing DNA (recognized by TLR9), and profilin present on uropathogenic bacteria (TLR11). TLR1, -2, -4, -5, and -6 recognize extracellular stimuli, while TLR3, -7, -8 and -9 function within the endolysosomal compartment. The activation of TLRs by their cognate ligands leads to production of inflammatory cytokines, and up-regulation of major histocompatibility complex (MHC) molecules and co-stimulatory signals in antigen-presenting cells as well as activating natural killer (NK) cells (innate immune response), in addition to priming and amplifying T-, and B-cell effector functions (adaptive immune responses). TLR stimuli serve to link innate and adaptive immunity and therefore there is considerable interest in utilizing TLR agonists as vaccine adjuvants.

The discovery of TLRs and identification of natural TLR ligands have intensified the search for synthetic agonists that can target TLRs with greater specificity and selectivity than pathogen-derived ligands. Interestingly, many immunostimulants that had been identified previously were later found to be TLR agonists such as imidazoquinolines for TLR7/8 (vide infra). Indeed, the only TLR agonist approved by the FDA as an adjuvant (3-O-desacyl-4′-monophosphoryl lipid A, derived from hydrolytic treatment of lipopolysaccharide isolated from *Salmonella Minnesota* Re595; MPL) was indentified to be a TLR4 agonist long after its adjuvantic properties had been established.

A detailed understanding of the structural and mechanistic bases of adjuvanticity and toxicity is pivotal in rationally developing novel adjuvants. In an effort to explore and develop strategies for the rapid and simultaneous evaluation of both adjuvant potency and proinflammatory activities of relatively large focused libraries of compounds being generated by our group, we have examined representative members of virtually the entire compendium of known TLR agonists in a series of hierarchical assays. Of all the innate immune stimuli examined, we found that TLR2
(thioacylglycerol lipopeptide chemotype), TLR4 (LPS and MPL), TLR5 (flagellin), and TLR7 (imidazoquinoline chemotype) were immunostimulatory; the imidazoquinoline class of TLR7 agonists was found to be extraordinarily immunostimulatory, stimulating virtually all subsets of lymphocytes, and yet without inducing dominant proinflammatory cytokine responses.\(^{30}\)

1.3. Intracellular TLR Activation – TLR7/8

The prototypical intracellular TLRs (TLR3 and TLR7-9) are generally associated with sensing nucleic acids released within endosomal compartments. Their activation leads to the production of a variety of nuclear factor (NF)-κB-mediated cytokines and Type I interferons (IFNs). TLR7 and TLR8 are phylogenetically and structurally related. TLR7 is expressed in plasmacytoid dendritic cells (pDC) and B cells, whereas TLR8 is mainly expressed in conventional/myeloid dendritic cells (cDCs), monocytes, macrophages, and neutrophils. Single stranded RNA (ssRNA) molecules of viral as well as nonviral origin (poly(dT); double-stranded thymidine homopolymer) induce the production of inflammatory cytokines, mediated by the recognition in the endosomal compartment by TLR7 and TLR8.\(^{31}\) TLR7 preferentially recognizes GU-rich RNA sequences and TLR8 has more affinity for AU-rich sequences.\(^{32}\) The activation by ssRNA induces the recruitment of the adapter protein Myeloid Differentiation primary response gene 88 (MyD88) via its TIR domain. TIR domains initiate the signaling cascade through TIR adapters, leading to downstream responses to specific pathogens. This results in the activation of NF-κB and IFN regulatory factors (IRFs) 3 and 7, which in turn induce the production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 and Type I IFNs, respectively.\(^{33}\) TLR7 and TLR8 can also detect a variety of synthetic chemical agonists. Small molecule, non-polymeric, synthetic agonists include the imidazoquinolines
(imiquimod, resiquimod [R-848], and gardiquimod), as well as guanosine analogues such as loxoribine (Fig. 1).

Long before endosomal TLR7 was discovered to serve as the primary sensor for short, single-stranded, GU-rich RNA sequences (ssRNA), mainly of viral origin, a number of small molecules were synthesized and evaluated in the 1970s and '80s for antiviral activities owing to their pronounced Type I interferon (IFN-α and -β) inducing properties. Members of the 1H-imidazo[4,5-c]quinolines were found to be good Type I IFN inducers in human cell-derived assays, and FDA approval was obtained in 1997 for Imiquimod (commercialized under the brand name Aldara™) for the treatment of basal cell carcinoma and actinic keratosis. The drug was later discovered to be a TLR7 agonist. The induction of IFN-α, IL-6 and TNF-α by imiquimod has been observed in vitro and in both human and animal studies. Imiquimod also activates NK lymphocytes and cells of the monocyte/macrophage lineage, and induces B lymphocytes to proliferate and differentiate. Resiquimod (R-848), another structurally similar compound, stimulates immune responses via TLR7 in mice and via TLR7 and TLR8 in humans. Resiquimod also triggers significant cytokine secretion, macrophage activation, and enhancement of cellular immunity. Resiquimod reached Phase III clinical trials for the topical treatment of genital herpes; however, development was suspended due to lack of adequate efficacy. CL097 and 3M-003 are derivatives of resiquimod that activate both TLR7 and TLR8. 3M-001, selective TLR7 agonist, stimulates plasmacytoid dendritic cells to produce cytokines such as IFN-α and IFN-γ inducible protein (IP)-10. Gardiquimod is another agonist of human and mouse TLR7 and more potent than imiquimod. At higher concentration, it is also known to activate TLR8. CL075 (3M-002) is a thiazoquinoline derivative that triggers TLR8 in human peripheral blood mononuclear cells (PBMCs). Agonistic activity of CL075 at TLR8 is known to activate NF-κB and trigger secretion of TNF-α and IL-12. This compound also
activates TLR7, however, to a lesser extent.\textsuperscript{45} Loxoribine is a guanosine ribonucleoside analogue that activates human and mouse TLR7. This compound enhances anti-tetanus-specific IgG antibody anamnestic responses in a dose-dependent manner in human PBMCs, as well as strongly activating NK cells in IL-12-dependent manner.\textsuperscript{35c} Isatoribine (ANA245) and ANA975 (an oral prodrug of isatoribine) are other examples of guanosine nucleoside analogues. Isatoribine, like imiquimod, was also identified as an immune potentiator before its mechanism of action was elucidated to be through activation of TLR7. These agents were developed for treatment of HCV infection;\textsuperscript{46} however, ANA975 has been discontinued due to unacceptable toxicity.\textsuperscript{47} Bropirimine is an aryl pyrimidinone analogue, an antineoplastic compound that induces IFN-\(\alpha\) and is used for treatment of carcinoma.\textsuperscript{48} The mechanism of action of bropirimine is likely associated with direct anti-tumor effect rather than cytokine mediated anti-tumor activity.\textsuperscript{49}

\textit{Fig. 1. Chemical structures of representative synthetic TLR7/8 agonists.}
Much of what is known about the vaccine adjuvant potential of TLR7 and/or TLR8 agonists has been derived from preclinical studies in animal models using the imidazoquinolines. These compounds, as mentioned earlier, have shown to directly activate antigen-presenting cells resulting in the induction of costimulatory molecules and numerous cytokines that modulate adaptive immunity. In terms of differences between human TLR7 and TLR8, TLR7-selective agonists were known to be more effective at inducing IFN-α and IFN-regulated cytokines than TLR8-selective agonists. On the other hand, TLR8 is functional in monocytes and myeloid DC, and its engagement results in the production of dominant proinflammatory cytokines such as TNF-α, IL-12, and MIP-1α. Together with IL-12, Type I IFNs appear to be required for optimal Th1 and CD8+ T cell responses following the administration of TLR7 and TLR8 agonists. Thus, TLR7 and TLR8 agonists, as well as TLR7/8 dual agonists could hold potential applications as vaccine adjuvants, especially in regard to their propensity for promoting Th1-type immune responses.

Fig. 2. A. Target receptors on APCs for adjuvants and their downstream signaling (adapted from Reed, S. G. et al, Nature medicine 2013, 19 (12), 1597). B. Expression of TLRs in human DCs and their ability to produce cytokines.
Desirous of specifically identifying chemotypes with strong Th1-biased immunostimulatory signatures, we implemented screens examining the induction of Type I and Type II IFNs (IFN-α/β and IFN-γ, respectively), IL-12, and IL-18 using human PBMCs, all of which are strongly associated with dominant Th1 outcomes. These studies enabled us to determine that of all of the diverse chemotypes of our rapidly expanding libraries and identify N1-(4-aminomethyl) benzyl-substituted TLR7/8 dual-agonistic imidazoquinoline (Fig. 3) that displayed a prominent Th1 bias, orders of magnitude higher than that of even lipopolysaccharide. This lead compound has been pivotal in exploring a variety of concepts such as TLR7/8 modulators and antagonists, model self-adjuvanting subunit vaccine constructs, a dendrimeric adjuvant, and cell-permeable, endosome-localizing, fluorescent analogues that retain agonistic activity (Fig. 3). The covalently coupled TLR7/TLR8 dual-agonistic imidazoquinoline to antigens using mild, nondenaturing conditions induced high antibody titers indicative of Th1 immunity. The dendrimeric adjuvant consisting of six linked TLR7/8 agonists (hexamer) lost TLR8 activity but retained the TLR7 agonistic effects, and it was found to be superior to the imidazoquinoline monomer in inducing high titers of high-affinity antibodies to bovine α-lactalbumin in rabbits. Additionally, epitope mapping experiments showed that the dendrimer induced immunoreactivity to more contiguous peptide epitopes along the amino acid sequence of the model antigen.
Fig. 3. Utility and diversification of $N^1$-(4-aminomethyl)benzyl-substituted imidazoquinoline.

Self-adjuvanting Subunit Vaccines

Fluorescent Analogues

TLR7/8 Modulators

Dendrimeric Adjuvants
Excessive or uncontrolled innate immune responses could potentially result in reactogenicity, and even undermine subsequent adaptive immune responses, and it is of particular importance to simultaneously take into consideration aspects of immune potentiation alongside safety issues associated with local or systemic immune activation and inflammation.

The premise of this thesis is that a careful and systematic exploration of chemical space around TLR7/8 chemotypes, as well as the development of standardized panels of bioassays that permit a detailed insight into the immunopharmacology of such molecules would not only allow the determination of the structural correlates governing adjuvant activity (Chapters 2-6), but also allow the rational exploration of novel strategies to dissociate adjuvanticity from reactogenicity (Chapter 7).
Chapter 2.

**TLR8/7-agonistic 2-alkylthiazolo[4,5-c]quinolines**
2.1. Introduction

We decided to extend our investigations toward delineating structure-activity relationships (SAR) in small-molecule agonistic ligands of TLR8 (thiazolo[4,5-c]quinolines) for the following reasons: First, the thiazoloquinolines are of interest because like the imidazoquinolines, these compounds were identified in antiviral assays long before the discovery of the endosomal TLR7 and TLR8 receptors,36, 59 and other than the original landmark studies performed by investigators at 3M Pharmaceuticals,60 SAR of the thiazoloquinoline chemotype remains poorly explored; qualitative assays for TNF-α and IFN-α induction in human blood were performed in these initial studies as surrogate biomarkers of immunostimulation, and no data on TLR-7 and -8 specific agonistic activities existed in the literature. Second, TLR8 agonists, both single-stranded RNA as well as small molecule imidazoquinoline ligands such as R-848 (resiquimod, Chapter 1)61 and thiazoloquinolines such as 3M-002 (CL075, Chapter 1)45, 62 appear uniquely potent in activating costimulatory responses in neonatal antigen-presenting cells (APCs), inducing robust production of the Th1-polarizing cytokines TNF-α and IL-12, which are not observed upon stimulation by TLR-2, -4, or -7 agonists. Such Th1-biasing compounds are of particular interest as candidate vaccine adjuvants in the newborn.63 During the first few weeks of life, newborns rely almost entirely on maternal IgG antibodies acquired by passive transplacental passage,64 and remain susceptible to a wide range of pathogens until early infancy.65 Neonates and infants, in whom vaccines could — and perhaps should — have the greatest impact, do not mount adequate adaptive immune responses, and therefore are most vulnerable; consequently, even the most efficacious vaccines that confer excellent protection in adults may fail to elicit strong immune responses in them.66 There is mounting evidence pointing to significant differences between adult and infant innate immune responses.67 The neonatal immunophenotype is characterized by decreased production of both Type I and Type II IFNs, IL-
12, IL-18, IL-23 and other proinflammatory cytokines such as TNF-α, the preferential induction of memory B lymphocytes rather than immunoglobulin-secreting plasma cells, as well as a pronounced Th2 skewing of T-cell responses.\textsuperscript{65-66, 68} TLR8 agonists induce the production of IL-12, IL-18 and IFN-γ, and may therefore be of value in developing vaccines for the neonate. We therefore sought to explore SAR in the thiazoloquinolines (typified by CL075, 8c in Scheme 1).

Our studies began with examining the optimal alkyl chain length at the C2 position. We found, as expected, that the C2-alkyl thiazoloquinolines exhibit mixed TLR8/7 agonistic activities in primary reporter gene assays with the optimal chain length being butyl. We observed unexpectedly strict length dependence with only the C2-butyl, but none of the other analogues, inducing IFN-α in human PBMCs. Examination of analogues with branched alkyl groups at C2 suggested poor tolerance of terminal steric bulk. We noted, however, that certain C2-branched analogues were substantially more TLR8-selective than their corresponding straight-chain analogues. Virtually all modifications at C8 led to abrogation of agonistic activity. Alkylation on the C4-amine was not tolerated, whereas N-acyl analogues with short acyl groups (other than acetyl) retained activity.

### 2.2. Results and Discussion

In our previous work on the TLR7-active imidazoquinolines, we had observed a distinct relationship between C2-alkyl chain length and TLR7-agonistic potency,\textsuperscript{69} and we therefore thought it logical to begin our SAR studies on the thiazoloquinolines by examining analogues with C2-alkyl groups of varying chain lengths. These analogues (8a-h, Scheme 1) were synthesized in parallel from the 3-aminoquinolin-4-ol precursor 4.\textsuperscript{69}

\[
\begin{align*}
&\text{Scheme 1. Synthesis of C2-alkylthiazoloquinoline analogues.} \\
&\text{Fig. 1. TLR7 and TLR8 agonistic potencies of the C2-alkyl thiazoloquinoline homologues. Data points} \\
&\text{represent means and standard deviations of EC}_{50}\text{ values derived from dose-response profiles and are} \\
&\text{computed on quadruplicates.}
\end{align*}
\]
In primary screens using human TLR7 and TLR8-specific reporter gene assays, these analogues exhibited, as anticipated, mixed TLR8/TLR7 agonism; the EC$_{50}$ values of 8c (CL075) were found to be 1.32 $\mu$M and 5.48 $\mu$M, respectively (Fig. 1, Table 1). As in our earlier SAR studies on the imidazoquinolines, we observed a clear dependence of agonistic potency on the C2-alkyl chain length. The C2-methyl, -ethyl, and -propyl analogues (8a-c) displayed comparable potencies in the hTLR8 and hTLR7 reporter gene assays (Fig. 1). Maximal TLR8-agonistic potency was observed in the butyl analogue (8d). Increasing the chain length to pentyl (8e) led to attenuation of potency, and higher homologues (8f-h) showed abrogation of activity (Fig. 1, Table 1). The C2-butyl analogue 8d appeared to exhibit substantially higher TLR7-agonistic potency than 8c (Figs. 1 and 3), suggesting that subtle variations at C2 could modulate TLR8 versus TLR7 specificity.

We therefore synthesized several additional analogues of both 8c (C2-n-propyl) and 8d (C2-n-butyl) with branched alkyl groups at C2 (8i-o, Scheme 1) and examined their activities (Fig. 2). The introduction of an isopropyl group at C2 (8i) led to an approximately ten-fold reduction in TLR8-agonistic potency (Table 1), while the attenuation of its activity in TLR7-specific reporter gene assays was more modest (about two-fold). Rather dramatic differences were noted between 8j (C2-isobutyl)/8k (C2-neopentyl) and 8l (C2-isopentyl)/8m (C2-neohexyl) congenic pairs. Whereas 8j was as potent as 8c in TLR8 agonism assays, 8k was inactive (Fig. 2), which suggested poor tolerance of terminal steric bulk. However, 8l and 8m were both inactive, pointing to additional length requirements in C2-branched alkyl substituents. These results led us to evaluate analogues 8n (2-methylpropyl substituent at C2) and 8o (2-methylbutyl substituent). Both these compounds retained TLR8 agonistic properties readily comparable to their parent compounds 8c and 8d, respectively, lending support to the premise that steric bulk at the $\omega$-position of the C2-alkyl group was not tolerated. We also evaluated bioisosteric
analouges 8p and 8q (Scheme 1) with terminal trifluoromethyl groups. A decrease in potency at TLR8 was observed in the longer homologue (8q). We noted that, in general, analogues with branched C2-alkyl groups of optimal chain length and bulk (such as 8j, 8n, and 8o) are substantially more TLR8-selective than their corresponding straight-chain analogues (Fig. 2).

Fig. 2. TLR8- and TLR7-specific NF-κB induction profiles of branched-chain and trifluoromethyl analogues of 8c and 8d. Means and SD obtained from quadruplicate samples are shown.
However, in secondary cytokine induction screens using human PBMCs, the branched-chain analogues behave differently in that they were neither as potent as their straight-chain parent compounds (Fig. 3), nor did they show enhanced IL-12, IL-18, and IFN-γ production (data not shown) as would be expected for TLR8-selective compounds. We do not yet understand the basis of attenuated activity of the C2-branched analogues, and we are currently exploring whether differential plasma protein binding behavior of these compounds could be contributory.70

*Fig. 3. EC₅₀ values of proinflammatory cytokine induction in human PBMCs by analogues of 8c and 8d. Representative data from three independent experiments are shown.*
Noting that previous studies in the patent literature\textsuperscript{60, 71} had been performed largely on the C7 position, and C8 and C4 analogues remain unexplored. We therefore first examined substituents on the quinoline at the C8 position of \(8c\) (CL075). Electrophilic substitution on the quinoline ring selectively afforded the 8-nitro analogue \(9\) (Scheme 2), and the 8-bromo analogue \(14\) (Scheme 4), both of which were inactive. The 8-amino (10) and 8-azido (11) analogues were obtained from \(9\) (Scheme 2). Compound 10 showed attenuated activity relative to \(8c\), and 11 was inactive. The triazole derivatives 12a-g were synthesized from 11 (Scheme 2) using conventional copper-catalyzed 'click' chemistry; of these analogues, the triazolo analogue 12e displayed feeble, but selective TLR8 agonism (Table 1). Selective C8 \(N\)-alkylation and \(N\)-acylation of the 8-amino analogue 10 provided analogues 13a and 13b (Scheme 3), both of which were inactive (Table 1).

\textit{Scheme 2.} Modification at the C8 position.

![Diagram](image_url)

\textbf{Reagents:} i. HNO\(_3\), H\(_2\)SO\(_4\); ii. Zn, NH\(_4\)COOH, MeOH; iii. NaNO\(_2\), CH\(_3\)COOH, NaN\(_3\); iv. alkyne, CuSO\(_4\), sodium ascorbate, THF, H\(_2\)O; v. TBAF, THF.
Next, the C4-amine of 8c was alkylated or acylated, yielding analogues 15a-l (Scheme 5). The C4-N-alkylated compounds 15a and 15b were devoid of any agonistic activity, while some, but not all of the C4-N-acylated derivatives with short acyl groups were found to be active. Specifically, the formyl (15d), and butyryl (15f) analogues, but not the acetyl (15e) compound, were active. Cytokine induction profiles in hPBMCs mirrored these findings with a near-complete loss of activity for the acetyl compound 15e (Fig. 5A). Our provisional interpretation that a stringent length requirement exists for the acyl substituents on the C4-amine was borne out in compounds 15j-l (azidoacetamide, azidopropionamide and pentynamide analogues, respectively), which displayed weak, TLR8-selective agonistic activity (Table 1). Aromatic amides at this position appear not to be tolerated, since the benzamide analogue 15i was entirely inactive. A series of carbamates (15m-p) and sulfonamides (15r-v) were also
synthesized (Scheme 5). The carbamate derivatives showed feeble activity with very low area-under-curves in primary TLR8 screens, while the sulfonamide analogues were completely inactive (Table 1). Several other analogs including urea (15q; Scheme 5), phosphoramidate (15w; Scheme 5) and guanidine (17; Scheme 6) functional groups at C4 were also examined, but were found to be inactive.

**Scheme 5.** Synthesis of C4 N-alkyl and N-acyl analogues.

The peculiar SAR, with only the acetamide analogue (15e) showing loss of activity was unexpected and, desiring to confirm this pattern, we also synthesized selected amide analogues of 8d (18a-c, formamide, acetamide, and butyramide derivatives, respectively; Scheme 7). As the 8c derivatives, a virtually identical pattern was observed, with a selective loss of activity for the acetamide analogue 18b (Figs. 4B and 5B, Table 1).
Scheme 7. Synthesis of C4 N-alkyl and N-acyl analogues of 8d.

\[
\begin{align*}
\text{NH}_2 & \quad \text{NHR} \\
\text{8d} & \quad \text{i} \\
\text{18a-c} & \quad R = \\
& \quad 18a: \text{CHO} \\
& \quad 18b: \text{COCH}_3 \\
& \quad 18c: \text{COC}_2\text{H}_7
\end{align*}
\]

Reagents: i. For 18a, 2,2,2-trifluoroethylformate, Et$_3$N; For 18b and 18c, RCOCI, pyridine.

Fig. 4. TLR8 induction by C4-amides of 8c (Panel A) and 8d (Panel B). Data points represent means and standard deviations on quadruplicates.

![Graphs showing TLR8 induction](image)
Fig. 5. Dose-response profiles of proinflammatory cytokine induction in hPBMCs by C4-amides of 8c (Panel A) and 8d (Panel B). Representative data from three independent experiments are presented. Vehicle controls (not shown) elicited undetectable levels of cytokines.

Our studies aimed at understanding structure-activity relationships in the thiazoloquinolines have yielded two compounds of interest: 8c and 8d, possessing differential TLR8/TLR7-agonistic properties. All of the other analogues were either of lower potency, or exhibited very poor aqueous solubility (such as the amide analogues 15d and 18a, for instance). We therefore elected to directly evaluate the adjuvantic properties of 8c and 8d. In light of the fact that murine
TLR8 was thought to be functionally inactive,\textsuperscript{72} we chose to examine the adjuvantic activities of 8c, 8d directly in a rabbit model using bovine $\alpha$-lactalbumin as a model subunit vaccine antigen, which is a small (14 kDa), soluble protein which we have adopted as our test-antigen in ongoing humoral and cellular immune response assays.\textsuperscript{56}

We were gratified to find that both 8c and 8d were highly adjuvantic in evoking high antigen-specific IgG titers (Fig. 6). Importantly, no evidence of local or systemic toxicity was apparent in any of the cohorts. The data suggest that 8d, with its dual TLR7/TLR8-agonistic properties, elicits adjuvanticity with greater consistency and uniformity, as evidenced by narrower confidence intervals of antibody titers (Fig. 6).

\textit{Fig. 6.} Box-plots of anti-bovine $\alpha$-lactalbumin IgG titers in cohorts of three rabbits immunized with $\alpha$-lactalbumin adjuvanted with either 8c or 8d. Means and medians of titers are represented by $\Box$ and $\boldsymbol{-}$ symbols within the box, respectively, and the $\times$ symbols indicate the 1\% and 99\% percentile values.
Table 1. EC₅₀ values of compounds in human TLR7/8-specific reporter gene assay.

<table>
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<th>No.</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Agonistic Activity (µM)</th>
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<th>TLR7</th>
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<td>H</td>
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<td>0.41 0.86</td>
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<td>H</td>
<td>NH₄C₁₁H₁₃</td>
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2.3. Conclusion

TLR8 agonists are thought to be uniquely potent in activating adaptive immune responses by inducing robust production of T helper 1-polarizing cytokines, and may be promising candidate vaccine adjuvants, especially for neonatal vaccines. Thiazoloquinoline analogues with methyl, ethyl, propyl and butyl groups at C2 displayed comparable TLR8-agonistic potencies; however, the C2-butyl compound \( 8d \) was unique in possessing substantial TLR7-agonistic activity. Analogues with branched alkyl groups at C2 displayed poor tolerance of terminal steric bulk. C4-\( N \)-acyl analogues with short acyl groups (other than acetyl) retained TLR8 agonistic activity, but were substantially less water-soluble. Immunization in rabbits with a model subunit antigen adjuvanted with the most potent TLR8 agonist showed dramatic enhancements of antigen-specific antibody titers.

2.4. Experimental

**Chemistry.** All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 °C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf ‘Gold’ high performance silica columns on CombiFlash Rf instrument unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel (200 \( \mu \)m) CCM pre-coated aluminum sheets. Purity for all final compounds was confirmed to be greater than 98% by LC-MS using a Zorbax Eclipse Plus 4.6 mm × 150 mm, 5 \( \mu \)m analytical reverse phase C18 column with \( \text{H}_2\text{O–isopropanol} \) or \( \text{H}_2\text{O–CH}_3\text{CN} \) gradients and either an Agilent ESI-
TOF mass spectrometer (mass accuracy of 20 ppm) or an Agilent 6520 ESI-QTOF mass spectrometer (mass accuracy of <10 ppm) operating in the positive ion (or negative ion, as appropriate) acquisition mode.

**Synthesis of compound 2: 2-(2-Nitroethylideneamino)benzoic acid.** Nitromethane (4.32 mL, 80 mmol) was added dropwise to a solution of NaOH (9.6 g, 250 mmol) in water (10 mL) at 0 ºC. The mixture was then warmed to 40 ºC and nitromethane (4.32 mL, 80 mmol) was again added slowly at 40-45 ºC. The temperature was maintained until a clear solution was obtained. The reaction mixture was then heated to 55 ºC for 2-5 minutes, cooled to 30 ºC, poured onto crushed ice and acidified with conc. HCl (11 mL). The resultant solution of methazoic acid was added immediately to a filtered solution of anthranilic acid \( 1 \) (10 g, 73 mmol) and conc. HCl (3.3 mL) in water (75 mL). The reaction mixture was allowed to stand at room temperature for 12 h. After filtration, the residue obtained was washed with water, and dried to yield compound 2 (12.94 g, 85%). \(^1\)H NMR (400 MHz, MeOD) \( \delta \) 8.14 (d, \( J = 7.7 \) Hz, 1H), 7.83 (d, \( J = 6.1 \) Hz, 1H), 7.68 – 7.59 (m, 2H), 7.22 (t, \( J = 7.4 \) Hz, 1H), 6.73 (d, \( J = 6.3 \) Hz, 1H). \(^{13}\)C NMR (101 MHz, MeOD) \( \delta \) 168.6, 141.2, 136.6, 134.3, 132.0, 123.2, 116.7, 114.5, 100.0. MS (ESI) calculated for \( C_9 H_8 N_2 O_4 \), m/z 208.05, found 209.06 [M+H]⁺.

**Synthesis of compound 3: 3-Nitroquinolin-4-ol.** A solution of compound 2 (12.94 g, 62.2 mmol) in acetic anhydride (50 mL) was placed in a 2-neck flask fitted with a reflux condenser. It was stirred and heated to 105 ºC until a clear solution was obtained. Heating was then discontinued and potassium acetate (6.22 g, 63.5 mmol) was added. The mixture was then refluxed for 15 min with vigorous stirring, until a solid started to precipitate. The reaction mixture was then slowly cooled to room temperature. The residue was filtered, washed with glacial acetic acid until the washings were colorless, then suspended in water, filtered, washed with
water and dried at 110 °C to get 3-nitroquinolin-4-ol 3 (4.68 g, 40%). ^1H NMR (500 MHz, DMSO) δ 13.04 (s, 1H), 9.21 (s, 1H), 8.25 (dd, J = 8.1, 1.1 Hz, 1H), 7.83 – 7.77 (m, 1H), 7.74 – 7.70 (m, 1H), 7.52 (ddd, J = 8.1, 7.1, 1.1 Hz, 1H). ^13C NMR (126 MHz, DMSO) δ 167.6, 142.5, 138.3, 133.2, 130.9, 128.1, 125.9, 125.8, 119.5. MS (ESI) calculated for C_9H_6N_2O_3, m/z 190.04, found 191.05 [M+H]^+.

**Synthesis of compound 4: 3-Aminoquinolin-4-ol.** To a solution of compound 3 (1.89 g, 9.93 mmol) in DMF (25 mL), was added 5% Pt on carbon (20%, 0.38 g). The reaction mixture was allowed to react in a Parr hydrogenation apparatus at 60 psi H_2 pressure for 3.5 h with vigorous shaking. The reaction mixture was filtered through celite with several washes of methanol. The filtrate was concentrated by evaporation to get compound 4 (1.5 g, 94%). ^1H NMR (500 MHz, MeOD) δ 8.90 (d, J = 8.1 Hz, 1H), 8.33 (d, J = 5.8 Hz, 1H), 8.30 – 8.23 (m, 2H), 7.97 (ddd, J = 8.1, 6.0, 1.9 Hz, 1H). ^13C NMR (126 MHz, MeOD) δ 146.9, 140.8, 138.9, 134.2, 131.2, 130.6, 128.7, 127.5. MS (ESI) calculated for C_9H_8N_2O, m/z 160.06, found 161.07 [M+H]^+.

**Synthesis of compound 5a: N-(4-hydroxyquinolin-3-yl)acetamide.** Compound 4 (200 mg, 1.25 mmol) was dissolved in a mixture of CH_2Cl_2 (20 mL) and DMF (2 mL) and stirred at room temperature for 5 min. Acetyl chloride (133 μL, 1.875 mmol) was added to the stirring reaction mixture at 0 °C and the solution was allowed to react for 1.5 h. Solvents were removed and the crude residue was purified using silica gel column chromatography (0-10% MeOH in CH_2Cl_2) to obtain the compound 5a (106 mg, 42%). ^1H NMR (500 MHz, CDCl_3) δ 9.20 (d, J = 5.5 Hz, 1H), 8.86 (s, 1H), 8.46 (s, 1H), 8.41 (dd, J = 8.3, 1.0 Hz, 1H), 7.62 (ddd, J = 8.3, 7.1, 1.3 Hz, 1H), 7.41 – 7.32 (m, 2H), 2.26 (s, 3H). ^13C NMR (126 MHz, CDCl_3) δ 169.1, 137.9, 132.1, 126.6, 126.3, 123.6, 122.9, 117.6, 24.6. MS (ESI) calculated for C_{11}H_{10}N_2O_2, m/z 202.07, found 203.08 [M+H]^+.
Compounds 5b-5q were synthesized similarly as compound 5a.

5b: N-(4-hydroxyquinolin-3-yl)propionamide. 132 mg, 43%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 9.29 (s, 1H), 8.71 (s, 1H), 8.42 (d, \(J = 7.7\) Hz, 1H), 7.64 (t, \(J = 7.4\) Hz, 1H), 7.49 (s, 1H), 7.38 (s, 1H), 2.56 (dd, \(J = 14.2, 7.0\) Hz, 2H), 1.31 (t, \(J = 7.5\) Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 137.9, 132.2, 126.1, 126.1, 124.5, 124.1, 124.0, 123.9, 118.0, 30.6, 9.9. MS (ESI) calculated for C\(_{12}\)H\(_{12}\)N\(_2\)O\(_2\), m/z 216.09, found 217.10 [M+H]+.

5c: N-(4-hydroxyquinolin-3-yl)butyramide. \(^1\)H NMR (500 MHz, MeOD) \(\delta\) 9.02 (s, 1H), 8.28 (dd, \(J = 8.3, 1.3\) Hz, 1H), 7.68 (ddd, \(J = 8.4, 7.0, 1.4\) Hz, 1H), 7.57 (d, \(J = 8.4\) Hz, 1H), 7.42 – 7.35 (m, 1H), 2.46 (t, \(J = 7.5\) Hz, 2H), 1.74 (dd, \(J = 14.9, 7.4\) Hz, 2H), 1.01 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (126 MHz, MeOD) \(\delta\) 174.6, 172.2, 139.7, 133.1, 131.3, 126.2, 124.8, 124.7, 122.8, 119.4, 39.6, 20.3, 14.0. MS (ESI) calculated for C\(_{13}\)H\(_{14}\)N\(_2\)O\(_2\), m/z 230.11, found 231.11 [M+H]+.

5d: N-(4-hydroxyquinolin-3-yl)pentanamide. 155 mg, 52%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 10.61 (s, 1H), 9.18 (s, 1H), 8.98 (s, 1H), 8.19 (d, \(J = 8.0\) Hz, 1H), 7.72 – 7.62 (m, 2H), 7.37 – 7.30 (m, 1H), 2.44 (t, \(J = 7.4\) Hz, 2H), 1.60 – 1.52 (m, 2H), 1.32 (dq, \(J = 14.7, 7.4\) Hz, 2H), 0.89 (dd, \(J = 12.5, 5.1\) Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 171.6, 168.9, 138.0, 131.4, 129.1, 124.0, 123.0, 122.9, 121.4, 118.5, 27.4, 21.9, 13.8. MS (ESI) calculated for C\(_{14}\)H\(_{16}\)N\(_2\)O\(_2\), m/z 244.12, found 245.13 [M+H]+.

5e: N-(4-hydroxyquinolin-3-yl)hexanamide. 141 mg, 44%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 9.65 (s, 1H), 8.44 (d, \(J = 8.2\) Hz, 1H), 8.31 (d, \(J = 7.2\) Hz, 1H), 7.88 (t, \(J = 7.4\) Hz, 1H), 7.68 (t, \(J = 7.3\) Hz, 1H), 2.82 (t, \(J = 6.5\) Hz, 2H), 1.88 – 1.76 (m, 2H), 1.48 – 1.35 (m, 4H), 0.92 (t, \(J = 7.0\) Hz, 3H).
$^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 177.9, 137.1, 133.7, 128.0, 124.8, 122.0, 120.7, 120.1, 36.1, 31.3, 25.6, 22.5, 14.1. MS (ESI) calculated for C$_{15}$H$_{18}$N$_2$O$_2$, m/z 258.14, found 259.15 [M+H]$^+$. 

5f: N-(4-hydroxyquinolin-3-yl)heptanamide. 105 mg, 31%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.24 (d, $J = 6.3$ Hz, 1H), 8.88 (d, $J = 1.6$ Hz, 1H), 8.46 (s, 1H), 8.43 – 8.40 (m, 1H), 7.62 (ddd, $J = 8.4$, 7.1, 1.4 Hz, 1H), 7.40 – 7.33 (m, 2H), 2.49 – 2.44 (m, 2H), 1.80 – 1.72 (m, 2H), 1.44 – 1.36 (m, 2H), 1.32 (td, $J = 7.1$, 3.5 Hz, 4H), 0.89 (dd, $J = 9.7$, 4.3 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.3, 170.7, 138.0, 132.0, 126.5, 126.3, 123.5, 123.3, 122.8, 117.6, 37.7, 31.7, 29.1, 25.8, 22.6, 14.2. MS (ESI) calculated for C$_{16}$H$_{20}$N$_2$O$_2$, m/z 272.15, found 273.16 [M+H]$^+$. 

5g: N-(4-hydroxyquinolin-3-yl)octanamide. 263 mg, 73%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.25 (d, $J = 6.4$ Hz, 1H), 8.99 (s, 1H), 8.46 (s, 1H), 8.43 – 8.40 (m, 1H), 7.62 (ddd, $J = 8.4$, 7.0, 1.4 Hz, 1H), 7.40 – 7.33 (m, 2H), 2.49 – 2.44 (m, 2H), 1.81 – 1.73 (m, 2H), 1.41 – 1.24 (m, 8H), 0.88 (t, $J = 6.9$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.3, 170.8, 138.0, 132.0, 126.6, 126.3, 123.5, 123.3, 122.8, 117.6, 37.7, 31.9, 29.4, 29.2, 25.9, 22.8, 14.2. MS (ESI) calculated for C$_{17}$H$_{22}$N$_2$O$_2$, m/z 286.17, found 287.18 [M+H]$^+$. 

5h: N-(4-hydroxyquinolin-3-yl)decanamide. 231 mg, 59%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.24 (s, 1H), 8.47 (s, 1H), 8.41 (dd, $J = 8.2$, 1.0 Hz, 1H), 7.63 – 7.58 (m, 1H), 7.40 (d, $J = 8.4$ Hz, 1H), 7.37 – 7.32 (m, 1H), 2.50 – 2.43 (m, 2H), 1.80 – 1.72 (m, 2H), 1.43 – 1.20 (m, 12H), 0.87 (t, $J = 7.0$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.4, 170.8, 138.0, 132.0, 126.8, 126.2, 123.5, 123.3, 122.7, 117.7, 37.7, 32.0, 29.6, 29.5, 29.4, 29.4, 25.9, 22.8, 14.3. MS (ESI) calculated for C$_{19}$H$_{36}$N$_2$O$_2$, m/z 314.20, found 315.21 [M+H]$^+$. 

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Synthesis of compound 6a: 2-Methylthiazolo[4,5-c]quinoline. To a solution of compound 5a (100 mg, 0.49 mmol) in pyridine (10 mL), was added phosphorous pentasulfide (109 mg, 0.49 mmol) and reaction mixture was refluxed for 2 h. The resulting solution was cooled to room temperature and pyridine was removed under reduced pressure. The residue was dissolved in water and pH was adjusted to 8 with saturated sodium bicarbonate solution and extracted in ethyl acetate. The organic layer was dried over sodium sulfate and concentrated to afford compound 6a (64 mg, 65%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.42 (s, 1H), 8.23 (d, $J = 8.3$ Hz, 1H), 7.94 (dd, $J = 8.1$, 0.9 Hz, 1H), 7.73 (ddd, $J = 8.4$, 7.0, 1.4 Hz, 1H), 7.62 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 2.94 (s, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 167.5, 148.0, 145.6, 144.3, 141.1, 130.6, 128.8, 127.6, 125.0, 123.5, 20.3. MS (ESI) calculated for C$_{11}$H$_8$N$_2$S, m/z 200.04, found 201.05 [M+H]$^+$.  

Compounds 6b-6q were synthesized similarly as compound 6a.

6b: 2-Ethylthiazolo[4,5-c]quinoline. 90 mg, 70%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.45 (s, 1H), 8.24 (d, $J = 8.3$ Hz, 1H), 7.99 – 7.94 (m, 1H), 7.73 (ddd, $J = 8.4$, 7.0, 1.4 Hz, 1H), 7.64 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 3.26 (q, $J = 7.6$ Hz, 2H), 1.54 (dd, $J = 10.6$, 4.5 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 174.2, 147.9, 145.8, 144.2, 140.6, 130.6, 128.8, 127.6, 125.0, 123.6, 28.0, 14.0. MS (ESI) calculated for C$_{12}$H$_{10}$N$_2$S, m/z 214.06, found 215.08 [M+H]$^+$. 

6c: 2-Propylthiazolo[4,5-c]quinoline. 950 mg, 95%. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.45 (s, 1H), 8.24 (d, $J = 8.3$ Hz, 1H), 7.97 (dd, $J = 8.1$, 0.9 Hz, 1H), 7.74 (ddd, $J = 8.4$, 7.0, 1.4 Hz, 1H), 7.69 – 7.59 (m, 1H), 3.26 – 3.11 (m, 2H), 2.04 – 1.92 (m, 2H), 1.10 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.8, 147.9, 145.8, 144.3, 140.7, 130.6, 128.8, 127.6, 125.0, 123.6, 36.4, 23.3, 13.8. MS (ESI) calculated for C$_{13}$H$_{12}$N$_2$S, m/z 228.07, found 229.08 [M+H]$^+$. 


6d: 2-Butylthiazolo[4,5-c]quinoline. 108 mg, 73%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.44 (s, 1H), 8.24 (d, $J = 8.4$ Hz, 1H), 7.96 (dd, $J = 8.1$, 0.9 Hz, 1H), 7.73 (ddd, $J = 8.4$, 5.4, 1.4 Hz, 1H), 7.63 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 3.25 – 3.19 (m, 2H), 1.97 – 1.89 (m, 2H), 1.55 – 1.46 (m, 2H), 1.00 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 173.0, 147.9, 145.8, 144.2, 140.6, 130.6, 128.8, 128.8, 127.6, 125.0, 123.6, 34.2, 32.0, 22.4, 13.9. MS (ESI) calculated for C$_{14}$H$_{14}$N$_2$S, m/z 242.09, found 243.10 [M+H]$^+$. 

6e: 2-Pentylthiazolo[4,5-c]quinoline. 104 mg, 76%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.45 (s, 1H), 8.24 (d, $J = 8.3$ Hz, 1H), 7.96 (dd, $J = 8.1$, 0.9 Hz, 1H), 7.73 (ddd, $J = 8.4$, 7.0, 1.4 Hz, 1H), 7.63 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 3.24 – 3.17 (m, 2H), 1.95 (dt, $J = 15.3$, 7.7 Hz, 2H), 1.50 – 1.36 (m, 4H), 0.93 (t, $J = 7.2$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 173.1, 147.9, 145.8, 144.2, 140.7, 130.6, 128.8, 127.6, 125.0, 123.6, 34.4, 31.4, 29.6, 22.5, 14.1. MS (ESI) calculated for C$_{15}$H$_{16}$N$_2$S, m/z 256.10, found 257.14 [M+H]$^+$. 

6f: 2-Hexylthiazolo[4,5-c]quinoline. 93 mg, 96%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.45 (s, 1H), 8.24 (d, $J = 8.4$ Hz, 1H), 7.97 (dd, $J = 8.1$, 0.9 Hz, 1H), 7.74 (ddd, $J = 8.4$, 7.0, 1.4 Hz, 1H), 7.66 – 7.62 (m, 1H), 3.25 – 3.19 (m, 2H), 1.98 – 1.90 (m, 2H), 1.48 (dd, $J = 10.5$, 4.5 Hz, 2H), 1.36 (ddd, $J = 16.3$, 7.0, 4.9 Hz, 4H), 0.90 (dd, $J = 9.4$, 4.7 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 173.1, 147.9, 145.8, 144.3, 140.7, 130.6, 128.8, 127.6, 125.0, 123.6, 34.5, 31.6, 29.9, 28.9, 22.6, 14.2. MS (ESI) calculated for C$_{16}$H$_{18}$N$_2$S, m/z 270.12, found 271.14 [M+H]$^+$. 

6g: 2-Heptylthiazolo[4,5-c]quinoline. 108 mg, 42%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.44 (s, 1H), 8.23 (d, $J = 8.4$ Hz, 1H), 7.96 (dd, $J = 8.1$, 0.9 Hz, 1H), 7.73 (ddd, $J = 8.4$, 7.0, 1.4 Hz, 1H), 7.65 – 7.60 (m, 1H), 3.22 – 3.17 (m, 2H), 1.94 (dt, $J = 15.3$, 7.6 Hz, 3H), 1.50 – 1.43 (m, 3H), 1.40 – 1.35 (m, 2H), 1.31 – 1.27 (m, 4H), 0.88 (t, $J = 7.0$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 173.1,
147.9, 145.8, 144.2, 140.6, 130.6, 128.8, 127.6, 125.0, 123.6, 34.5, 31.8, 29.9, 29.2, 29.1, 22.7, 14.2. MS (ESI) calculated for C₁₁H₂₀N₂S, m/z 284.13, found 285.17 [M+H]+.

6h: 2-Nonylthiazolo[4,5-c]quinoline. 157 mg, 52%. ¹H NMR (500 MHz, CDCl₃) δ 9.45 (s, 1H), 8.24 (d, J = 8.4 Hz, 1H), 7.96 (d, J = 8.1 Hz, 1H), 7.73 (dd, J = 8.4, 5.3, 1.4 Hz, 1H), 7.63 (t, J = 7.5 Hz, 1H), 3.23 – 3.18 (m, 2H), 1.94 (dt, J = 15.4, 7.7 Hz, 2H), 1.51 – 1.43 (m, 2H), 1.40 – 1.34 (m, 2H), 1.31 – 1.24 (m, 8H), 0.87 (t, J = 6.9 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 173.1, 147.9, 145.8, 144.2, 130.6, 128.8, 128.7, 127.6, 125.0, 123.6, 120.3, 34.5, 32.0, 29.9, 29.5, 29.4, 29.4, 29.2, 22.8, 14.2. MS (ESI) calculated for C₁₇H₂₄N₂S, m/z 312.17, found 313.19 [M+H]+.

6i: 2-Isopropylthiazolo[4,5-c]quinoline. 164 mg, 83%. ¹H NMR (500 MHz, CDCl₃) δ 9.46 (s, 1H), 8.24 (d, J = 8.2 Hz, 1H), 8.01 – 7.96 (m, 1H), 7.73 (dd, J = 8.4, 7.0, 1.4 Hz, 1H), 7.64 (ddd, J = 8.1, 7.0, 1.2 Hz, 1H), 3.58 – 3.48 (m, 1H), 1.56 (d, J = 6.9 Hz, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 179.3, 147.8, 145.9, 144.2, 140.2, 130.6, 128.8, 127.6, 125.0, 123.7, 34.3, 23.1. MS (ESI) calculated for C₁₃H₁₂N₂S, m/z 228.10, found 229.08 [M+H]+.

6j: 2-Isobutylthiazolo[4,5-c]quinoline. 150 mg, 72%. ¹H NMR (500 MHz, MeOD) δ 9.31 (s, 1H), 8.18 (d, J = 8.3 Hz, 1H), 8.11 (dd, J = 8.1, 0.9 Hz, 1H), 7.81 (ddd, J = 8.4, 7.0, 1.4 Hz, 1H), 7.73 (ddd, J = 8.1, 7.1, 1.2 Hz, 1H), 2.30 (m, J = 13.7, 6.8 Hz, 1H), 1.08 (d, J = 6.6 Hz, 6H). ¹³C NMR (126 MHz, MeOD) δ 174.5, 148.7, 146.0, 144.7, 142.6, 130.5, 130.4, 129.3, 126.2, 124.7, 43.7, 31.1, 22.6. MS (ESI) calculated for C₁₄H₁₄N₂S, m/z 242.09, found 243.09 [M+H]+.

6l: 2-Isopentylthiazolo[4,5-c]quinoline. 165 mg, 75%. ¹H NMR (500 MHz, CDCl₃) δ 9.44 (s, 1H), 8.26 – 8.22 (m, 1H), 7.98 – 7.95 (m, 1H), 7.73 (ddd, J = 8.4, 7.0, 1.4 Hz, 1H), 7.64 (ddd, J =
= 8.1, 7.0, 1.2 Hz, 1H), 3.26 – 3.20 (m, 2H), 1.88 – 1.82 (m, 2H), 1.75 (dd, J = 13.3, 6.7 Hz, 1H), 1.01 (d, J = 6.6 Hz, 6H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 173.2, 147.9, 145.8, 144.3, 140.6, 130.6, 128.8, 127.6, 125.0, 123.6, 38.8, 32.5, 27.9, 22.5. MS (ESI) calculated for C$_{15}$H$_{16}$N$_2$S, m/z 256.10, found 257.10 [M+H]$^+$. 

6o: (S)-2-(2-methylbutyl)thiazolo[4,5-c]quinoline. 170 mg, 77%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.45 (s, 1H), 8.24 (d, J = 8.3 Hz, 1H), 7.96 (dd, J = 8.1, 0.9 Hz, 1H), 7.73 (ddd, J = 8.4, 7.0, 1.4 Hz, 1H), 7.63 (ddd, J = 8.1, 7.1, 1.1 Hz, 1H), 3.21 (dd, J = 14.6, 6.2 Hz, 1H), 3.01 (dd, J = 14.6, 8.2 Hz, 1H), 2.12 – 2.02 (m, 1H), 1.58 – 1.48 (m, 1H), 1.40 – 1.30 (m, 1H), 1.03 (d, J = 6.7 Hz, 3H), 0.98 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.1, 147.9, 145.8, 144.2, 140.8, 130.6, 128.8, 127.6, 125.0, 123.6, 41.4, 36.3, 29.4, 19.2, 11.5. MS (ESI) calculated for C$_{15}$H$_{16}$N$_2$S, m/z 256.10, found 257.10 [M+H]$^+$. 

6p: 2-(3,3,3-Trifluoropropyl)thiazolo[4,5-c]quinoline. 180 mg, 74%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.45 (s, 1H), 8.26 (d, J = 8.4 Hz, 1H), 7.99 – 7.95 (m, 1H), 7.76 (ddd, J = 8.4, 7.0, 1.4 Hz, 1H), 7.66 (ddd, J = 8.1, 7.0, 1.2 Hz, 1H), 3.51 – 3.45 (m, 2H), 2.91 – 2.80 (m, 2H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 168.0, 147.7, 145.8, 144.4, 140.8, 130.7, 129.2, 127.8, 126.4 (q, 1JCF = 276.5 Hz), 125.0, 123.4, 33.0 (q, 2JCF = 29.8 Hz), 27.0 (q, 3JCF = 3.5 Hz). MS (ESI) calculated for C$_{15}$H$_{16}$F$_3$N$_2$S, m/z 282.04, found 283.04 [M+H]$^+$. 

6q: 2-(4,4,4-Trifluorobutyl)thiazolo[4,5-c]quinoline. 190 mg, 75%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.46 (s, 1H), 8.26 (d, J = 8.2 Hz, 1H), 8.00 – 7.95 (m, 1H), 7.76 (ddd, J = 8.4, 7.0, 1.4 Hz, 1H), 7.66 (ddd, J = 8.1, 7.0, 1.2 Hz, 1H), 3.32 (t, J = 7.2 Hz, 2H), 2.37 – 2.22 (m, 4H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 170.4, 147.9, 145.8, 144.4, 140.7, 130.7, 129.1, 127.8, 127.0 (q, 1JCF = 276.4
Hz), 125.0, 123.5, 33.9 (q, 2JCF = 28.9 Hz), 33.0, 21.1 (q, 3JCF = 3.0 Hz). MS (ESI) calculated for C_{14}H_{11}F_{3}N_{2}S, m/z 296.05, found 297.06 [M+H]^+.

**Synthesis of compound 7a: 2-Methylthiazolo[4,5-c]quinoline 5-oxide.** To a solution of compound 6a (57 mg, 0.28 mmol) in CHCl₃ (10 mL), was added m-chloroperoxybenzoic acid (95 mg, 0.43 mmol) and the reaction mixture was stirred at room temperature for 18 h. The solvent was then removed under reduced pressure and the crude residue was purified using silica gel column chromatography (5% MeOH/CH₂Cl₂) to obtain compound 7a (57 mg, 94%). ¹H NMR (500 MHz, CDCl₃) δ 9.13 (s, 1H), 8.89 (d, J = 8.9 Hz, 1H), 7.94 (dd, J = 7.9, 1.1 Hz, 1H), 7.81 – 7.72 (m, 2H), 2.93 (s, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 169.7, 147.2, 139.0, 131.5, 131.1, 129.9, 129.7, 125.6, 123.8, 121.6, 20.4. MS (ESI) calculated for C_{11}H₈N₂OS, m/z 216.04, found 217.05 [M+H]^+.

Compounds 7b-7q were synthesized similarly as compound 7a.

**7b: 2-Ethylthiazolo[4,5-c]quinoline 5-oxide.** 81 mg, 88%. ¹H NMR (500 MHz, CDCl₃) δ 9.15 (s, 1H), 8.90 (dd, J = 8.6, 0.8 Hz, 1H), 7.95 (dd, J = 8.0, 1.0 Hz, 1H), 7.82 – 7.72 (m, 2H), 3.23 (q, J = 7.6 Hz, 2H), 1.53 (d, J = 15.1 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 176.4, 147.1, 139.0, 131.6, 130.7, 129.9, 129.6, 125.6, 123.9, 121.6, 28.0, 13.9. MS (ESI) calculated for C_{12}H_{10}N₂OS, m/z 230.05, found 231.06 [M+H]^+.

**7c: 2-Propylthiazolo[4,5-c]quinoline 5-oxide.** 950 mg, 88%. ¹H NMR (400 MHz, CDCl₃) δ 9.14 (s, 1H), 8.90 (d, J = 8.7 Hz, 1H), 7.95 (dd, J = 7.9, 1.1 Hz, 1H), 7.82 – 7.71 (m, 2H), 3.17 (d, J = 7.6 Hz, 2H), 1.95 (dt, J = 14.9, 7.4 Hz, 2H), 1.09 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 175.0, 147.1, 139.0, 131.6, 130.7, 129.9, 129.6, 123.9, 121.6, 36.4, 23.2, 13.8. MS (ESI) calculated for C_{13}H_{12}N₂OS, m/z 244.07, found 245.07 [M+H]^+. 

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7d: 2-Butylthiazolo[4,5-c]quinoline 5-oxide. 82 mg, 75%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.13 (s, 1H), 8.90 – 8.86 (m, 1H), 7.94 (dd, $J$ = 7.9, 1.1 Hz, 1H), 7.80 – 7.71 (m, 2H), 3.21 – 3.16 (m, 2H), 1.94 – 1.86 (m, 2H), 1.53 – 1.45 (m, 2H), 0.99 (t, $J$ = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 175.2, 147.1, 139.0, 131.6, 130.6, 129.9, 129.6, 125.6, 123.9, 121.6, 34.2, 31.8, 22.4, 13.9. MS (ESI) calculated for C$_{14}$H$_{14}$N$_2$OS, m/z 258.08, found 259.09 [M+H]$^+$. 

7e: 2-Pentylthiazolo[4,5-c]quinoline 5-oxide. 90 mg, 87%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.13 (s, 1H), 8.88 (d, $J$ = 8.1 Hz, 1H), 7.94 (dd, $J$ = 8.0, 1.2 Hz, 1H), 7.81 – 7.70 (m, 2H), 3.22 – 3.13 (m, 2H), 1.92 (dt, $J$ = 15.4, 7.7 Hz, 2H), 1.48 – 1.36 (m, 4H), 0.92 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 175.3, 147.0, 138.9, 131.6, 130.7, 129.9, 129.6, 125.6, 123.9, 121.6, 34.5, 31.3, 29.5, 22.4, 14.0. MS (ESI) calculated for C$_{15}$H$_{16}$N$_2$OS, m/z 272.10, found 273.11 [M+H]$^+$. 

7f: 2-Hexylthiazolo[4,5-c]quinoline 5-oxide. 65 mg, 76%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.15 (s, 1H), 8.90 (d, $J$ = 8.5 Hz, 1H), 7.96 (dd, $J$ = 8.0, 1.0 Hz, 1H), 7.82 – 7.72 (m, 2H), 3.22 – 3.17 (m, 2H), 1.92 (dd, $J$ = 15.3, 7.7 Hz, 2H), 1.51 – 1.44 (m, 2H), 1.40 – 1.30 (m, 4H), 0.90 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 175.3, 147.1, 139.0, 131.7, 130.8, 129.9, 129.6, 125.6, 123.9, 121.6, 34.5, 31.6, 29.8, 28.9, 22.6, 14.2. MS (ESI) calculated for C$_{16}$H$_{18}$N$_2$OS, m/z 286.11, found 287.12 [M+H]$^+$. 

7g: 2-Heptylthiazolo[4,5-c]quinoline 5-oxide. 81 mg, 75%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.13 (s, 1H), 8.88 (dd, $J$ = 8.7, 0.8 Hz, 1H), 7.94 (dd, $J$ = 7.9, 1.1 Hz, 1H), 7.80 – 7.71 (m, 2H), 3.21 – 3.15 (m, 2H), 1.91 (dt, $J$ = 15.3, 7.6 Hz, 2H), 1.49 – 1.42 (m, 2H), 1.41 – 1.35 (m, 2H), 1.32 – 1.26 (m, 4H), 0.88 (t, $J$ = 7.0 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 175.3, 147.0, 138.9, 131.6, 130.7, 129.9, 129.6, 125.6, 123.9, 121.6, 34.5, 31.7, 29.8, 29.2, 29.0, 22.7, 14.2. MS (ESI) calculated for C$_{17}$H$_{20}$N$_2$OS, m/z 300.13, found 301.14 [M+H]$^+$. 

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7h: 2-Nonylthiazolo[4,5-c]quinoline 5-oxide. 105 mg, 66%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.13 (s, 1H), 8.88 (d, $J$ = 8.5 Hz, 1H), 7.93 (dd, $J$ = 7.9, 1.1 Hz, 1H), 7.81 – 7.69 (m, 2H), 3.19 – 3.14 (m, 2H), 1.91 (dt, $J$ = 15.3, 7.6 Hz, 2H), 1.45 (dt, $J$ = 14.9, 7.0 Hz, 2H), 1.35 (dd, $J$ = 14.2, 6.9 Hz, 2H), 1.29 – 1.21 (m, 8H), 0.86 (t, $J$ = 6.9 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 175.3, 147.0, 138.9, 131.6, 130.7, 129.8, 129.6, 125.6, 123.9, 121.5, 34.5, 31.9, 29.8, 29.5, 29.3, 29.2, 22.8, 14.2. MS (ESI) calculated for C$_{19}$H$_{24}$N$_2$OS, m/z 328.16, found 329.17 [M+H]$^+$. 

7i: 2-Isopropylthiazolo[4,5-c]quinoline 5-oxide. 150 mg, 62%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.15 (s, 1H), 8.90 (dd, $J$ = 8.7, 1.0 Hz, 1H), 7.99 – 7.95 (m, 1H), 7.79 (ddd, $J$ = 8.6, 7.0, 1.5 Hz, 1H), 7.77 – 7.72 (m, 1H), 3.54 – 3.45 (m, 1H), 1.54 (d, $J$ = 6.9 Hz, 6H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 181.5, 147.0, 138.9, 131.7, 130.3, 129.9, 129.6, 125.6, 124.0, 121.6, 34.4, 23.0. MS (ESI) calculated for C$_{13}$H$_{12}$N$_2$OS, m/z 244.07, found 245.07 [M+H]$^+$. 

7j: 2-Isobutylthiazolo[4,5-c]quinoline 5-oxide. 80 mg, 75%. $^1$H NMR (500 MHz, MeOD) $\delta$ 9.26 (s, 1H), 8.79 (dd, $J$ = 8.6, 0.9 Hz, 1H), 8.25 – 8.21 (m, 1H), 7.95 (ddd, $J$ = 8.7, 7.1, 1.5 Hz, 1H), 7.90 (ddd, $J$ = 8.2, 7.1, 1.4 Hz, 1H), 3.14 (d, $J$ = 7.2 Hz, 2H), 2.35 – 2.25 (m, 1H), 1.09 (d, $J$ = 6.6 Hz, 6H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 176.8, 147.8, 139.0, 135.3, 133.5, 131.7, 131.6, 127.2, 125.2, 121.5, 43.7, 31.0, 22.6. MS (ESI) calculated for C$_{14}$H$_{14}$N$_2$OS, m/z 258.08, found 259.08 [M+H]$^+$. 

7k: 2-Neopentylthiazolo[4,5-c]quinoline 5-oxide. 80 mg, 75%. $^1$H NMR (500 MHz, MeOD) $\delta$ 9.31 (s, 1H), 8.79 (dd, $J$ = 8.7, 0.9 Hz, 1H), 8.26 – 8.22 (m, 1H), 7.99 – 7.88 (m, 2H), 3.16 (s, 2H), 1.13 (s, 9H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 174.7, 147.8, 138.8, 135.9, 133.8, 131.8, 131.7, 127.3, 125.1, 121.4, 48.3, 33.2, 29.9. MS (ESI) calculated for C$_{14}$H$_{16}$N$_2$OS, m/z 272.10, found 273.10 [M+H]$^+$. 


7l: 2-Isopentylthiazolo[4,5-c]quinoline 5-oxide. 110 mg, 69%. $^1$H NMR (500 MHz, MeOD) $\delta$ 9.23 (s, 1H), 8.77 (dd, $J = 8.6, 0.9$ Hz, 1H), 8.21 – 8.18 (m, 1H), 7.93 (ddd, $J = 8.7, 7.0, 1.5$ Hz, 1H), 7.89 (ddd, $J = 8.2, 7.1, 1.3$ Hz, 1H), 3.27 (dd, $J = 8.4, 7.4$ Hz, 2H), 1.88 – 1.82 (m, 2H), 1.78 – 1.69 (m, 1H), 1.02 (d, $J = 6.6$ Hz, 6H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 178.1, 147.7, 138.9, 135.1, 133.5, 131.6, 131.6, 127.1, 125.1, 121.5, 39.6, 33.1, 28.8, 22.6. MS (ESI) calculated for C$_{15}$H$_{16}$N$_2$OS, m/z 272.10, found 273.10 [M+H]$^+$. 

7m: 2-(3,3-Dimethylbutyl)thiazolo[4,5-c]quinoline 5-oxide. 80 mg, 76%. $^1$H NMR (500 MHz, MeOD) $\delta$ 9.22 (s, 1H), 8.77 (dd, $J = 8.7, 0.9$ Hz, 1H), 8.21 – 8.18 (m, 1H), 7.93 (ddd, $J = 8.7, 7.1, 1.5$ Hz, 1H), 7.89 (ddd, $J = 8.2, 7.1, 1.3$ Hz, 1H), 3.27 – 3.22 (m, 2H), 1.89 – 1.84 (m, 2H), 1.04 (s, 9H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 178.7, 147.7, 138.9, 135.1, 133.4, 131.6, 131.6, 127.1, 125.1, 121.4, 44.7, 31.5, 30.9, 29.5. MS (ESI) calculated for C$_{16}$H$_{18}$N$_2$OS, m/z 286.11, found 287.11 [M+H]$^+$. 

7n: (S)-2-(sec-butyl)thiazolo[4,5-c]quinoline 5-oxide. 117 mg, 78%. $^1$H NMR (500 MHz, MeOD) $\delta$ 9.23 (s, 1H), 8.77 (dd, $J = 8.6, 1.0$ Hz, 1H), 8.22 – 8.18 (m, 1H), 7.93 (ddd, $J = 8.7, 7.1, 1.5$ Hz, 1H), 7.88 (ddd, $J = 8.2, 7.1, 1.4$ Hz, 1H), 3.35 (dd, $J = 13.9, 6.9$ Hz, 1H), 2.04 – 1.94 (m, 1H), 1.92 – 1.82 (m, 1H), 1.52 (d, $J = 6.9$ Hz, 3H), 1.02 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 183.1, 147.7, 138.9, 134.7, 133.5, 131.6, 131.6, 127.1, 125.2, 121.5, 42.3, 31.6, 20.9, 12.0. MS (ESI) calculated for C$_{14}$H$_{14}$N$_2$OS, m/z 258.09, found 259.08 [M+H]$^+$. 

7o: (S)-2-(2-methylbutyl)thiazolo[4,5-c]quinoline 5-oxide. 103 mg, 74%. $^1$H NMR (500 MHz, MeOD) $\delta$ 9.24 (s, 1H), 8.78 (dd, $J = 8.7, 0.8$ Hz, 1H), 8.23 – 8.19 (m, 1H), 7.94 (ddd, $J = 8.7, 7.1, 1.5$ Hz, 1H), 7.89 (dd, 1H), 3.26 (dd, $J = 14.7, 6.2$ Hz, 1H), 3.06 (dd, $J = 14.7, 8.0$ Hz, 1H), 2.14 – 2.03 (m, 1H), 1.60 – 1.50 (m, 1H), 1.44 – 1.30 (m, 1H), 1.04 (d, $J = 6.7$ Hz, 3H), 1.00 (t, $J$
= 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 177.0, 147.8, 139.0, 135.3, 133.5, 131.7, 131.6, 127.1, 125.1, 121.5, 41.8, 37.4, 30.2, 19.4, 11.6. MS (ESI) calculated for C$_{15}$H$_{16}$N$_{2}$OS, m/z 272.10, found 273.10 [M+H]$^+$. 

7p: 2-(3,3,3-Trifluoropropyl)thiazolo[4,5-c]quinoline 5-oxide. 120 mg, 75%. $^1$H NMR (500 MHz, MeOD) $\delta$ 9.24 (s, 1H), 8.76 (dd, $J$ = 8.8, 0.7 Hz, 1H), 8.17 (dd, 1H), 7.93 (ddd, $J$ = 8.6, 7.0, 1.4 Hz, 1H), 7.88 (ddd, $J$ = 8.2, 7.1, 1.3 Hz, 1H), 3.54 (t, 2H), 2.99 – 2.87 (m, 2H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 173.8, 147.6, 139.1, 135.2, 133.6, 131.8, 131.6, 128.1 (q, $J$$_{CF}$ = 275.6 Hz), 127.1, 125.0, 121.5, 33.2 (q, $J$$_{CF}$ = 29.7 Hz), 27.7 (q, $J$$_{CF}$ = 3.5 Hz). MS (ESI) calculated for C$_{13}$H$_{9}$F$_{3}$N$_{2}$OS, m/z 298.04, found 299.04 [M+H]$^+$. 

7q: 2-(4,4,4-Trifluorobutyl)thiazolo[4,5-c]quinoline 5-oxide. 128 mg, 81%. $^1$H NMR (500 MHz, MeOD) $\delta$ 9.26 (s, 1H), 8.80 – 8.74 (m, 1H), 8.22 – 8.18 (m, 1H), 7.94 (ddd, $J$ = 8.6, 7.1, 1.5 Hz, 1H), 7.89 (ddd, $J$ = 8.2, 7.1, 1.3 Hz, 1H), 3.36 (t, $J$ = 7.5 Hz, 2H), 2.48 – 2.34 (m, 2H), 2.27 – 2.17 (m, 2H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 176.0, 147.8, 139.0, 135.2, 133.6, 131.7, 131.6, 128.6 (q, $J$$_{CF}$ = 275.4 Hz), 127.1, 125.1, 121.5, 33.6 (q, $J$$_{CF}$ = 29.0 Hz), 33.5, 22.6 (q, $J$$_{CF}$ = 3.2 Hz). MS (ESI) calculated for C$_{14}$H$_{11}$F$_{3}$N$_{2}$OS, m/z 312.05, found 313.05 [M+H]$^+$. 

Synthesis of compound 8a: 2-Methylthiazolo[4,5-c]quinolin-4-amine. Compound 7a (50 mg, 0.23 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (5 mL). Benzoyl isocyanate (68 mg, 0.46 mmol) was added to the reaction mixture and refluxed for 30 min. The solvent was then removed under vacuum and the residue was dissolved in anhydrous methanol (5 mL). Excess of sodium methoxide was added and the reaction mixture was refluxed for 2 h. After evaporating solvents under reduced pressure, the crude residue was purified using silica gel column chromatography (0-7% MeOH in CH$_2$Cl$_2$) to obtain compound 8a as a white solid (41 mg, 82%).
$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.77 (dd, $J = 8.4, 0.5$ Hz, 1H), 7.72 (dd, $J = 8.0, 1.0$ Hz, 1H), 7.55 (ddd, $J = 8.4, 7.1, 1.5$ Hz, 1H), 7.31 (ddd, $J = 8.1, 7.1, 1.2$ Hz, 1H), 5.61 (s, 2H), 2.89 (s, 3H).

$^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 165.9, 151.5, 144.8, 141.0, 138.1, 129.1, 126.8, 124.8, 123.2, 120.1, 20.1. MS (ESI) calculated for C$_{11}$H$_9$N$_3$S, m/z 215.05, found 216.06 [M+H]$^+$. Compounds 8b-8q were synthesized similarly as compound 8a.

**8b: 2-Ethylthiazolo[4,5-c]quinolin-4-amine.** White solid (36 mg, 47%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.77 (dd, $J = 8.4, 0.5$ Hz, 1H), 7.74 (dd, $J = 8.0, 1.1$ Hz, 1H), 7.56 (ddd, $J = 8.4, 7.1, 1.5$ Hz, 1H), 7.31 (ddd, $J = 8.1, 7.1, 1.1$ Hz, 1H), 5.58 (s, 2H), 3.20 (q, $J = 7.6$ Hz, 2H), 1.51 (t, $J = 7.6$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.6, 151.6, 144.8, 140.4, 138.0, 129.0, 126.8, 124.8, 123.2, 120.2, 27.8, 14.1. MS (ESI) calculated for C$_{12}$H$_{11}$N$_3$S, m/z 229.07, found 230.08 [M+H]$^+$.  

**8c: 2-Propylthiazolo[4,5-c]quinolin-4-amine.** White solid (450 mg, 90%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.77 (d, $J = 8.4$ Hz, 1H), 7.73 (dd, $J = 8.0, 1.3$ Hz, 1H), 7.55 (ddd, $J = 8.4, 7.1, 1.4$ Hz, 1H), 7.34 – 7.27 (m, 1H), 5.67 (s, 2H), 3.13 (t, $J = 7.6$ Hz, 2H), 1.94 (dd, $J = 15.0, 7.4$ Hz, 2H), 1.09 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 171.2, 151.7, 144.7, 140.5, 138.0, 129.0, 126.8, 124.8, 123.1, 120.2, 36.2, 23.4, 13.8. MS (ESI) calculated for C$_{13}$H$_{13}$N$_3$S, m/z 243.08, found 244.09 [M+H]$^+$.  

**8d: 2-Butylthiazolo[4,5-c]quinolin-4-amine.** White solid (25 mg, 32%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.77 (dd, $J = 8.4, 0.6$ Hz, 1H), 7.73 (dd, $J = 8.0, 1.0$ Hz, 1H), 7.56 (ddd, $J = 8.5, 7.1, 1.5$ Hz, 1H), 7.31 (ddd, $J = 8.1, 7.1, 1.2$ Hz, 1H), 5.56 (s, 2H), 3.20 – 3.13 (m, 2H), 1.90 (dt, $J = 15.3, 7.6$ Hz, 2H), 1.50 (dq, $J = 14.7, 7.4$ Hz, 2H), 1.00 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 171.2, 151.7, 144.7, 140.5, 138.0, 129.0, 126.8, 124.8, 123.1, 120.2, 36.2, 23.4, 13.8. MS (ESI) calculated for C$_{14}$H$_{15}$N$_3$S, m/z 257.10, found 258.11 [M+H]$^+$.  

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CDCl₃ δ 171.5, 151.6, 144.7, 140.5, 138.0, 129.0, 126.8, 124.8, 123.2, 120.2, 34.0, 32.0, 22.4, 13.9. MS (ESI) calculated for C₁₄H₁₅N₃S, m/z 257.10, found 258.11 [M+H]⁺.

**8e: 2-Pentylthiazolo[4,5-c]quinolin-4-amine.** White solid (38 mg, 45%). ¹H NMR (400 MHz, CDCl₃) δ 7.77 (d, J = 8.1 Hz, 1H), 7.73 (dd, J = 8.0, 1.1 Hz, 1H), 7.55 (ddd, J = 8.4, 7.1, 1.4 Hz, 1H), 7.34 – 7.29 (m, 1H), 5.59 (s, 2H), 3.18 – 3.12 (m, 2H), 1.96 – 1.86 (m, 2H), 1.50 – 1.35 (m, 4H), 0.94 (t, J = 7.1 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 171.5, 151.6, 144.7, 140.5, 138.0, 129.0, 126.8, 124.8, 123.2, 120.2, 34.3, 31.4, 29.7, 22.5, 14.1 MS (ESI) calculated for C₁₅H₁₇N₃S, m/z 271.11, found 272.12 [M+H]⁺.

**8f: 2-Hexylthiazolo[4,5-c]quinolin-4-amine.** White solid (25 mg, 44%). ¹H NMR (400 MHz, CDCl₃) δ 7.77 (dd, J = 8.4, 0.5 Hz, 1H), 7.73 (dd, J = 8.0, 1.0 Hz, 1H), 7.55 (ddd, J = 8.4, 7.1, 1.5 Hz, 1H), 7.31 (ddd, J = 8.1, 7.1, 1.1 Hz, 1H), 5.56 (s, 2H), 3.21 – 3.11 (m, 2H), 1.91 (dt, J = 15.3, 7.5 Hz, 2H), 1.46 (dt, J = 9.2, 7.0 Hz, 2H), 1.39 – 1.30 (m, 4H), 0.91 (t, J = 7.1 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 171.6, 151.6, 144.7, 140.51, 138.0, 129.1, 126.8, 124.8, 123.2, 120.2, 34.3, 31.6, 30.0, 29.0, 22.6, 14.2. MS (ESI) calculated for C₁₆H₁₉N₃S, m/z 285.13, found 286.14 [M+H]⁺.

**8g: 2-Heptylthiazolo[4,5-c]quinolin-4-amine.** White solid (34 mg, 42%). ¹H NMR (400 MHz, CDCl₃) δ 7.77 (dd, J = 8.4, 0.5 Hz, 1H), 7.73 (dd, J = 8.0, 1.0 Hz, 1H), 7.55 (ddd, J = 8.4, 7.1, 1.5 Hz, 1H), 7.31 (ddd, J = 8.1, 7.1, 1.2 Hz, 1H), 5.56 (s, 2H), 3.20 – 3.08 (m, 2H), 1.91 (dt, J = 15.3, 7.6 Hz, 2H), 1.50 – 1.42 (m, 2H), 1.38 (ddd, J = 14.0, 8.2, 5.2 Hz, 2H), 1.30 (dt, J = 11.5, 3.9 Hz, 4H), 0.89 (t, J = 6.9 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 171.6, 151.6, 144.7, 140.5, 138.0, 129.0, 126.8, 124.8, 123.2, 120.2, 34.3, 31.8, 30.0, 29.2, 29.1, 22.8, 14.2. MS (ESI) calculated for C₁₇H₂₁N₃S, m/z 299.15, found 300.16 [M+H]⁺.
8h: 2-Nonylthiazolo[4,5-c]quinolin-4-amine. White solid (26 mg, 26%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.77 (dd, $J = 8.4$, 0.5 Hz, 1H), 7.73 (dd, $J = 8.0$, 1.0 Hz, 1H), 7.55 (ddd, $J = 8.5$, 7.1, 1.5 Hz, 1H), 7.31 (ddd, $J = 8.1$, 7.1, 1.2 Hz, 1H), 5.57 (s, 2H), 3.21 – 3.09 (m, 2H), 1.91 (dt, $J = 15.3$, 7.6 Hz, 2H), 1.46 (ddd, $J = 14.9$, 8.7, 6.5 Hz, 2H), 1.36 (dd, $J = 13.7$, 6.7 Hz, 2H), 1.28 (s, 8H), 0.88 (t, $J = 6.9$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 171.6, 151.6, 144.7, 140.5, 138.0, 129.0, 126.8, 124.8, 123.2, 120.2, 34.3, 32.0, 30.0, 29.6, 29.4, 29.2, 22.8, 14.3. MS (ESI) calculated for C$_{19}$H$_{25}$N$_3$S, m/z 327.18, found 328.19 [M+H]$^+$.  

8i: 2-Isopropylthiazolo[4,5-c]quinolin-4-amine. White solid (40 mg, 67%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.77 (dd, $J = 8.4$, 0.5 Hz, 1H), 7.75 – 7.71 (m, 1H), 7.55 (ddd, $J = 8.4$, 7.0, 1.5 Hz, 1H), 7.31 (ddd, $J = 8.1$, 7.1, 1.2 Hz, 1H), 5.66 (s, 2H), 3.51 – 3.41 (m, 1H), 1.52 (d, $J = 6.9$ Hz, 6H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 177.7, 151.8, 144.7, 140.0, 137.9, 129.0, 126.8, 124.8, 123.1, 120.3, 34.2, 23.2. MS (ESI) calculated for C$_{13}$H$_{13}$N$_3$S, m/z 243.09.10, found 244.09 [M+H]$^+$.  

8j: 2-Isobutylthiazolo[4,5-c]quinolin-4-amine. White solid (38 mg, 77%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.77 (dd, $J = 8.4$, 0.5 Hz, 1H), 7.73 (dd, $J = 8.0$, 1.0 Hz, 1H), 7.55 (ddd, $J = 8.4$, 7.1, 1.5 Hz, 1H), 7.31 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 5.63 (s, 2H), 3.02 (d, $J = 7.2$ Hz, 2H), 2.30 – 2.20 (m, 1H), 1.06 (d, $J = 6.6$ Hz, 6H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 170.3, 151.7, 144.8, 140.6, 138.1, 129.1, 126.8, 124.9, 123.1, 120.2, 43.1, 30.0, 22.5. MS (ESI) calculated for C$_{14}$H$_{15}$N$_3$S, m/z 257.10, found 258.10 [M+H]$^+$.  

8k: 2-Neopentylthiazolo[4,5-c]quinolin-4-amine. White solid (45 mg, 76%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.77 (dd, $J = 8.4$, 0.5 Hz, 1H), 7.74 (dd, $J = 8.0$, 1.0 Hz, 1H), 7.55 (ddd, $J = 8.4$, 5.5, 1.5 Hz, 1H), 7.31 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 5.60 (s, 2H), 3.04 (s, 2H), 1.10 (s, 9H). $^{13}$C
NMR (126 MHz, CDCl$_3$) $\delta$ 168.1, 151.7, 144.8, 140.7, 138.2, 129.1, 126.8, 124.9, 123.2, 120.1, 47.8, 32.3, 29.7. MS (ESI) calculated for C$_{14}$H$_{17}$N$_3$S, m/z 271.11, found 272.11 [M+H]$^+$. 

**8l: 2-Isopentylthiazolo[4,5-c]quinolin-4-amine.** White solid (40 mg, 68%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.77 (dd, $J = 8.4$, 0.5 Hz, 1H), 7.73 (dd, $J = 8.0$, 1.0 Hz, 1H), 7.55 (ddd, $J = 8.4$, 7.1, 1.5 Hz, 1H), 7.31 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 5.61 (s, 2H), 3.20 – 3.13 (m, 2H), 1.84 – 1.78 (m, 2H), 1.77 – 1.69 (m, 1H), 1.00 (d, $J = 6.5$ Hz, 6H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 171.7, 151.6, 144.7, 140.5, 138.0, 129.0, 126.8, 124.8, 123.2, 120.2, 38.9, 32.3, 27.9, 22.5. MS (ESI) calculated for C$_{15}$H$_{16}$N$_2$OS, m/z 271.11, found 272.11 [M+H]$^+$. 

**8m: 2-(3,3-Dimethylbutyl)thiazolo[4,5-c]quinolin-4-amine.** White solid (45 mg, 65%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.77 (dd, $J = 8.4$, 0.5 Hz, 1H), 7.74 – 7.70 (m, 1H), 7.55 (ddd, $J = 8.4$, 7.0, 1.5 Hz, 1H), 7.31 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 5.64 (s, 2H), 3.17 – 3.09 (m, 2H), 1.86 – 1.79 (m, 2H), 1.02 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.2, 151.6, 144.7, 140.4, 138.0, 129.0, 126.8, 124.8, 123.1, 121.0, 44.1, 30.8, 30.1, 29.3. MS (ESI) calculated for C$_{16}$H$_{19}$N$_3$S, m/z 285.13, found 286.14 [M+H]$^+$. 

**8n: (S)-2-(sec-butyl)thiazolo[4,5-c]quinolin-4-amine.** White solid (45 mg, 76%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.79 – 7.75 (m, 1H), 7.75 – 7.72 (m, 1H), 7.55 (ddd, $J = 8.4$, 7.0, 1.5 Hz, 1H), 7.31 (ddd, $J = 8.1$, 7.1, 1.2 Hz, 1H), 5.63 (s, 2H), 3.30 – 3.21 (m, 1H), 2.00 – 1.90 (m, 1H), 1.88 – 1.76 (m, 1H), 1.49 (d, $J = 6.9$ Hz, 3H), 1.00 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 177.0, 151.7, 144.7, 140.0, 137.9, 129.0, 126.8, 124.8, 123.1, 120.3, 41.0, 30.9, 21.0, 11.9. MS (ESI) calculated for C$_{14}$H$_{15}$N$_3$S, m/z 257.10, found 258.10 [M+H]$^+$. 


8o: (S)-2-(2-methylbutyl)thiazolo[4,5-c]quinolin-4-amine. White solid (46 mg, 77%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.77 (dd, $J = 8.4$, 0.5 Hz, 1H), 7.73 (dd, $J = 8.0$, 1.0 Hz, 1H), 7.55 (ddd, $J = 8.4$, 7.1, 1.5 Hz, 1H), 7.31 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 5.66 (s, 2H), 3.14 (dd, $J = 14.6$, 6.2 Hz, 1H), 2.96 (dd, $J = 14.6$, 8.1 Hz, 1H), 2.08 – 1.97 (m, 1H), 1.57 – 1.47 (m, 1H), 1.38 – 1.28 (m, 1H), 1.02 (d, $J = 6.7$ Hz, 3H), 0.97 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 170.5, 151.7, 144.7, 140.6, 138.1, 129.0, 126.8, 124.8, 123.1, 120.2, 41.2, 36.3, 29.3, 19.2, 11.5. MS (ESI) calculated for C$_{15}$H$_{17}$N$_3$S, m/z 271.11, found 272.11 [M+H]$^+$. 

8p: 2-(3,3,3-Trifluoropropyl)thiazolo[4,5-c]quinolin-4-amine. White solid (70 mg, 78%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.80 – 7.76 (m, 1H), 7.73 (dd, $J = 8.0$, 1.0 Hz, 1H), 7.58 (ddd, $J = 8.4$, 7.1, 1.4 Hz, 1H), 7.35 – 7.30 (m, 1H), 5.63 (s, 2H), 3.45 – 3.39 (m, 2H), 2.87 – 2.76 (m, 2H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 166.5, 151.6, 145.0, 140.8, 137.9, 129.4, 126.9, 126.4 (q, $1$JCF = 276.5 Hz), 124.9, 123.4, 119.9, 33.1 (q, $2$JCF = 29.8 Hz), 26.8 (q, $3$JCF = 3.4 Hz). MS (ESI) calculated for C$_{13}$H$_{10}$F$_3$N$_3$S, m/z 297.05, found 298.05 [M+H]$^+$. 

8q: 2-(4,4,4-Trifluorobutyl)thiazolo[4,5-c]quinolin-4-amine. White solid (50 mg, 72%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.77 (dd, $J = 8.4$, 0.4 Hz, 1H), 7.73 (dd, $J = 8.0$, 1.1 Hz, 1H), 7.57 (ddd, $J = 8.4$, 7.1, 1.4 Hz, 1H), 7.32 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 5.62 (s, 2H), 3.25 (t, $J = 7.2$ Hz, 2H), 2.36 – 2.26 (m, 2H), 2.26 – 2.18 (m, 2H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 168.8, 151.6, 144.9, 140.6, 138.1, 129.3, 127.0 (q, $1$JCF = 276.4 Hz), 126.9, 124.9, 123.3, 120.0, 33.1 (q, $2$JCF = 29.7 Hz), 32.7, 21.9 (q, $3$JCF = 3.0 Hz). MS (ESI) calculated for C$_{14}$H$_{12}$F$_3$N$_3$S, m/z 311.07, found 312.07 [M+H]$^+$. 

**Synthesis of compound 9: 8-Nitro-2-propylthiazolo[4,5-c]quinolin-4-amine.** To a stirred solution of compound 8c (200 mg, 0.82 mmol) in H$_2$SO$_4$ (0.5 mL), was added HNO$_3$ (0.5 mL)
and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was neutralized with 1N NaOH and extracted with EtOAc (3 × 15 mL). The combined organic layer was dried over Na₂SO₄, concentrated under reduced pressure, and the crude residue was purified using silica gel column chromatography (0-5% MeOH in CH₂Cl₂) to obtain compound 9 as a yellow solid (171 mg, 72%). ¹H NMR (400 MHz, CDCl₃) δ 8.68 (d, J = 2.5 Hz, 1H), 8.35 (dd, J = 9.2, 2.6 Hz, 1H), 7.80 (d, J = 9.2 Hz, 1H), 6.09 (s, 2H), 3.17 (dd, J = 8.7, 6.4 Hz, 2H), 1.97 (dd, J = 15.0, 7.5 Hz, 2H), 1.11 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 173.3, 153.6, 148.3, 142.5, 141.2, 138.7, 127.3, 123.2, 121.5, 119.1, 36.2, 23.3, 13.8. MS (ESI) calculated for C₁₃H₁₂N₄O₂S, m/z 288.07, found 289.08 [M+H]+.

**Synthesis of compound 10: 2-Propylthiazolo[4,5-c]quinoline-4,8-diamine.** To a stirred solution of compound 9 (200 mg, 0.69 mmol) in methanol (5 mL), were added zinc dust (453 mg, 6.94 mmol) and ammonium formate (437 mg, 6.94 mmol), and the reaction mixture was stirred at room temperature for 30 min. The reaction mixture was then filtered through celite, the filtrate concentrated under reduced pressure, and purified over silica gel column chromatography (0-10% MeOH in CH₂Cl₂) to afford compound 10 as a yellow solid (185 mg, 88%). ¹H NMR (400 MHz, CDCl₃) δ 8.71 (s, 1H), 8.46 (s, 2H), 7.70 (d, J = 8.9 Hz, 1H), 7.00 (dd, J = 8.9, 2.5 Hz, 1H), 6.87 (d, J = 2.4 Hz, 1H), 3.17 – 3.09 (m, 2H), 1.99 – 1.88 (m, 2H), 1.08 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 173.1, 170.3, 149.2, 143.9, 142.1, 137.1, 129.5, 121.9, 120.7, 118.7, 107.4, 36.1, 23.2, 13.8. MS (ESI) calculated for C₁₃H₁₄N₄S, m/z 258.09, found 259.10 [M+H]+.

**Synthesis of compound 11: 8-Azido-2-propylthiazolo[4,5-c]quinolin-4-amine.** To a stirred solution of compound 10 (25 mg, 0.096 mmol) in a 1:1 mixture of acetic acid and water (2 mL), was added sodium nitrite (20 mg, 0.29 mmol) and the reaction mixture was stirred at room
temperature for 1 h. Sodium azide (18.8 mg, 0.29 mmol) was then added to the reaction mixture and stirred for additional 1 h. The reaction mixture was neutralized with 1N NaOH and extracted with EtOAc (3 × 10 mL). The combined organic layer was dried over Na₂SO₄, concentrated under reduced pressure, and the crude compound was purified on silica gel column chromatography (0-5% MeOH in CH₂Cl₂) to obtain compound 11 as a brown solid (18 mg, 67%). ¹H NMR (400 MHz, CDCl₃) δ 7.76 (d, J = 8.9 Hz, 1H), 7.31 (d, J = 2.5 Hz, 1H), 7.26 – 7.23 (m, 1H), 5.60 (s, 2H), 3.17 – 3.12 (m, 2H), 2.01 – 1.90 (m, 2H), 1.09 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 172.0, 151.3, 139.5, 138.5, 134.8, 128.4, 121.0, 120.8, 113.5, 36.2, 23.4, 13.8. MS (ESI) calculated for C₁₃H₁₂N₆S, m/z 284.08, found 285.09 [M+H]⁺.

Synthesis of compound 12a: 2-(1-(4-Amino-2-propylthiazolo[4,5-c]quinolin-8-yl)-1H-1,2,3-triazol-4-yl)propan-2-ol. To a stirred solution of compound 11 (12 mg, 0.042 mmol) in THF (1 mL), were added CuSO₄.5H₂O (1.3 mg, 0.005 mmol, in 0.5 mL water), sodium ascorbate (2.1 mg, 0.010 mmol, in 0.5 mL water), 2-methylbut-3-yn-2-ol (8.4 μL, 0.084 mmol) and the reaction mixture stirred at room temperature for 1 h. The reaction mixture was diluted with water and extracted with EtOAc (3 × 10 mL). The combined organic layer was dried over Na₂SO₄, concentrated under reduced pressure, and the crude residue was purified using silica gel column chromatography (0-10% MeOH in CH₂Cl₂) to obtain compound 12a as a white solid (14 mg, 92%). ¹H NMR (400 MHz, CDCl₃) δ 8.12 (d, J = 2.0 Hz, 1H), 7.97 (s, 1H), 7.91 – 7.81 (m, 2H), 5.74 (s, 2H), 3.49 (s, 1H), 3.19 – 3.13 (m, 2H), 1.96 (dd, J = 15.0, 7.5 Hz, 2H), 1.74 (s, 6H), 1.10 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 172.5, 156.5, 152.2, 144.5, 140.2, 138.7, 132.0, 128.3, 121.5, 120.3, 117.8, 116.4, 68.9, 36.2, 30.7, 23.4, 13.8. MS (ESI) calculated for C₁₈H₂₀N₆OS, m/z 368.14, found 369.15 [M+H]⁺.

Compounds 12b-12f were synthesized similarly as compound 12a.
12b: (1-(4-Amino-2-propylthiazolo[4,5-c]quinolin-8-yl)-1H-1,2,3-triazol-4-yl)methanol. White solid (15 mg, 83%). \(^1\)H NMR (500 MHz, DMSO) \(\delta\) 8.84 (s, 1H), 8.33 (d, \(J = 2.5\) Hz, 1H), 8.06 (dd, \(J = 9.0, 2.5\) Hz, 1H), 7.76 (d, \(J = 9.0\) Hz, 1H), 7.13 (s, 2H), 5.37 (t, \(J = 5.6\) Hz, 1H), 4.64 (d, \(J = 5.9\) Hz, 2H), 3.19 (t, \(J = 7.5\) Hz, 2H), 1.88 (dt, \(J = 14.8, 7.4\) Hz, 2H), 1.04 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 171.8, 152.7, 149.2, 144.5, 139.1, 138.2, 130.8, 127.3, 121.2, 120.9, 118.9, 115.2, 55.1, 35.2, 22.8, 13.5. MS (ESI) calculated for C\(_{16}\)H\(_{16}\)N\(_6\)OS, m/z 340.11, found 341.11 [M+H]\(^+\).

12c: 8-(4-Phenyl-1H-1,2,3-triazol-1-yl)-2-propylthiazolo[4,5-c]quinolin-4-amine. Solid (19 mg, 93%). \(^1\)H NMR (500 MHz, DMSO) \(\delta\) 9.44 (s, 1H), 8.36 (d, \(J = 2.5\) Hz, 1H), 8.12 (dd, \(J = 9.0, 2.5\) Hz, 1H), 7.97 (dd, \(J = 8.2, 1.1\) Hz, 2H), 7.80 (d, \(J = 9.0\) Hz, 1H), 7.53 (dd, \(J = 10.6, 4.8\) Hz, 2H), 7.44 – 7.37 (m, 1H), 7.17 (s, 2H), 3.21 (t, \(J = 7.5\) Hz, 2H), 1.90 (dd, \(J = 14.9, 7.4\) Hz, 2H), 1.04 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 171.8, 152.8, 147.3, 144.7, 139.0, 138.2, 130.6, 130.4, 129.1, 128.3, 127.4, 125.3, 120.9, 119.8, 118.9, 115.3, 35.2, 22.8, 13.5. MS (ESI) calculated for C\(_{21}\)H\(_{18}\)N\(_6\)S, m/z 386.13, found 387.14 [M+H]\(^+\).

12d: 2-Propyl-8-(4-propyl-1H-1,2,3-triazol-1-yl)thiazolo[4,5-c]quinolin-4-amine. Yellow solid (16 mg, 86%). \(^1\)H NMR (500 MHz, DMSO) \(\delta\) 8.72 (s, 1H), 8.28 (d, \(J = 2.4\) Hz, 1H), 8.04 (dd, \(J = 9.0, 2.5\) Hz, 1H), 7.75 (d, \(J = 9.0\) Hz, 1H), 7.12 (s, 2H), 3.19 (t, \(J = 7.5\) Hz, 2H), 2.71 (t, \(J = 7.5\) Hz, 2H), 1.89 (dd, \(J = 14.9, 7.4\) Hz, 2H), 1.72 (dd, \(J = 14.9, 7.4\) Hz, 2H), 1.04 (t, \(J = 7.4\) Hz, 3H), 0.98 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 171.8, 152.7, 148.0, 144.4, 139.0, 138.2, 130.9, 127.3, 120.8, 120.3, 118.9, 115.0, 35.2, 27.1, 22.8, 22.1, 13.7, 13.5. MS (ESI) calculated for C\(_{18}\)H\(_{20}\)N\(_6\)S, m/z 352.15, found 353.15 [M+H]\(^+\).
12e: 8-(4-Pentyl-1H-1,2,3-triazol-1-yl)-2-propylthiazolo[4,5-c]quinolin-4-amine. Solid (19 mg, 91%). $^1$H NMR (500 MHz, DMSO) δ 8.71 (s, 1H), 8.27 (d, $J = 2.5$ Hz, 1H), 8.04 (dd, $J = 9.0$, 2.5 Hz, 1H), 7.75 (d, $J = 9.0$ Hz, 1H), 7.12 (s, 2H), 3.19 (t, $J = 7.5$ Hz, 2H), 2.72 (t, $J = 7.6$ Hz, 2H), 1.89 (dd, $J = 14.9$, 7.4 Hz, 2H), 1.69 (dt, $J = 15.3$, 7.6 Hz, 2H), 1.42 – 1.26 (m, 6H), 1.04 (t, $J = 7.4$ Hz, 3H), 0.87 (dd, $J = 9.2$, 4.9 Hz, 3H). $^{13}$C NMR (126 MHz, DMSO) δ 171.8, 152.7, 148.2, 144.4, 139.0, 138.2, 130.9, 127.3, 120.8, 120.2, 118.9, 114.9, 35.2, 31.1, 28.8, 28.3, 25.1, 22.8, 22.1, 14.0, 13.5. MS (ESI) calculated for C$_{21}$H$_{26}$N$_6$S, m/z 394.19, found 395.20 [M+H]$^+$.  

12f: 2-Propyl-8-(4-(trimethylsilyl)-1H-1,2,3-triazol-1-yl)thiazolo[4,5-c]quinolin-4-amine. White solid (18 mg, 90%). $^1$H NMR (500 MHz, DMSO) δ 8.98 (s, 1H), 8.31 (d, $J = 2.4$ Hz, 1H), 8.07 (dd, $J = 9.0$, 2.5 Hz, 1H), 7.76 (d, $J = 9.0$ Hz, 1H), 7.13 (s, 2H), 3.19 (t, $J = 7.5$ Hz, 2H), 1.89 (dd, $J = 14.8$, 7.4 Hz, 2H), 1.04 (t, $J = 7.4$ Hz, 3H), 0.36 – 0.33 (m, 9H). $^{13}$C NMR (126 MHz, DMSO) δ 171.8, 152.7, 146.1, 144.4, 139.1, 138.2, 130.7, 128.9, 127.3, 121.3, 118.9, 115.3, 35.2, 22.8, 13.5, -0.9. MS (ESI) calculated for C$_{18}$H$_{22}$N$_6$SSi, m/z 382.14, found 383.14 [M+H]$^+$.  

Synthesis of compound 12g: 2-Propyl-8-(1H-1,2,3-triazol-1-yl)thiazolo[4,5-c]quinolin-4-amine. To a stirred solution of compound 12f (20 mg, 0.052 mmol) in THF (1 mL) was added TBAF (20 mg, 0.078 mmol), and the reaction mixture was stirred at room temperature for 10 h. The reaction mixture was then diluted with water and extracted with EtOAc (3 × 10 mL). The organic layer was dried over Na$_2$SO$_4$, concentrated under reduced pressure, and the crude material purified using silica gel column chromatography (0-5% MeOH in CH$_2$Cl$_2$) to obtain compound 12g as a white solid (14 mg, 87%). $^1$H NMR (500 MHz, DMSO) δ 8.94 (d, $J = 1.1$ Hz, 1H), 8.29 (d, $J = 2.4$ Hz, 1H), 8.04 (dd, $J = 9.0$, 2.5 Hz, 1H), 7.98 (d, $J = 1.1$ Hz, 1H), 7.75 (d, $J =$
9.0 Hz, 1H), 7.12 (s, 2H), 3.17 (t, \( J = 7.5 \) Hz, 2H), 1.91 – 1.83 (m, 2H), 1.02 (t, \( J = 7.4 \) Hz, 3H).

\(^{13}\)C NMR (126 MHz, DMSO) \( \delta \) 171.7, 152.8, 144.6, 139.1, 138.2, 134.4, 130.7, 127.4, 123.4, 121.2, 118.9, 115.5, 35.23, 22.8, 13.5. MS (ESI) calculated for C\(_{15}\)H\(_{14}\)N\(_6\)S, m/z 310.10, found 311.10 [M+H]+.

**Synthesis of compound 13a: \( N^8 \)-hexyl-2-propylthiazolo[4,5-c]quinoline-4,8-diamine.** To a stirred solution of compound 10 (25 mg, 0.096 mmol) in DMF (2 mL) were added 1-iodohexane (20.5 mg, 0.096 mmol) and potassium carbonate (26.7 mg, 0.19 mmol), and the reaction mixture was stirred at room temperature for 4 h. The reaction mixture was diluted with water and extracted with EtOAc (3 × 10 mL). The organic layer was dried over Na\(_2\)SO\(_4\), concentrated under reduced pressure, and the crude compound was purified using silica gel column chromatography (0-5% MeOH in CH\(_2\)Cl\(_2\)) to obtain 13a as a solid (16 mg, 61%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 7.69 (d, \( J = 8.9 \) Hz, 1H), 6.96 (dd, \( J = 8.9, 2.6 \) Hz, 1H), 6.67 (d, \( J = 2.5 \) Hz, 1H), 6.15 (s, 2H), 3.87 (s, 1H), 3.18 (t, \( J = 7.1 \) Hz, 2H), 3.15 – 3.11 (m, 2H), 1.99 – 1.89 (m, 2H), 1.68 (dt, \( J = 14.7, 7.2 \) Hz, 2H), 1.49 – 1.41 (m, 2H), 1.35 (td, \( J = 7.2, 3.7 \) Hz, 4H), 1.09 (t, \( J = 7.4 \) Hz, 3H), 0.92 (t, \( J = 7.1 \) Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 172.1, 148.2, 145.5, 141.2, 137.4, 124.8, 120.3, 119.8, 102.9, 44.3, 36.2, 31.8, 29.4, 27.0, 23.3, 22.8, 14.2, 13.8. MS (ESI) calculated for C\(_{19}\)H\(_{26}\)N\(_4\)S, m/z 342.19, found 343.20 [M+H]+.

**Synthesis of compound 13b: \( N^8 \)-(4-amino-2-propylthiazolo[4,5-c]quinolin-8-yl)butyramide.** To a stirred solution of compound 10 (20 mg, 0.077 mmol) in CH\(_2\)Cl\(_2\) (2 mL) were added triethylamine (30 \( \mu \)L, 0.164 mmol) and butyryl chloride (8.7 \( \mu \)L, 0.082); the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was then diluted with water and extracted with EtOAc (3 × 10 mL). The combined organic layer was dried over Na\(_2\)SO\(_4\), concentrated under reduced pressure, and the crude compound was purified using silica gel.
column chromatography (0-10% MeOH in CH₂Cl₂) to furnish compound 13b as a white solid (22 mg, 87%). ¹H NMR (400 MHz, CDCl₃) δ 8.34 (d, J = 2.2 Hz, 1H), 7.71 (d, J = 8.9 Hz, 1H), 7.37 (dd, J = 8.9, 2.4 Hz, 2H), 5.67 (d, J = 1.2 Hz, 2H), 3.13 (t, J = 7.4 Hz, 2H), 2.41 (t, J = 7.4 Hz, 2H), 1.94 (dd, J = 15.0, 7.5 Hz, 2H), 1.85 – 1.74 (m, 2H), 1.11 – 1.02 (m, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 172.0, 171.5, 150.9, 140.6, 138.2, 133.4, 126.8, 122.1, 120.2, 114.6, 39.8, 36.2, 23.3, 19.2, 13.9, 13.8. MS (ESI) calculated for C₁₇H₂₀N₄OS, m/z 328.14, found 329.15 [M+H]⁺.

**Synthesis of compound 14: 8-Bromo-2-propylthiazolo[4,5-c]quinolin-4-amine.** To a stirred solution of compound 8c (50 mg, 0.205 mmol) in CH₃CN (1 mL) were added N-bromosuccinimide (44 mg, 0.246 mmol), ammonium acetate (1.6 mg, 0.02 mmol) and the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was then diluted with water and extracted with EtOAc (3 × 10 mL). The combined organic layer was dried over Na₂SO₄, concentrated under reduced pressure, and the crude material was purified using silica gel column chromatography (0-5% MeOH in CH₂Cl₂) to obtain compound 14 as a white solid (40 mg, 67%). ¹H NMR (500 MHz, CDCl₃) δ 7.86 (dd, J = 2.0, 0.6 Hz, 1H), 7.61 (dd, J = 3.9, 1.3 Hz, 2H), 5.68 (s, 2H), 3.16 – 3.11 (m, 2H), 1.95 (dt, J = 15.0, 7.4 Hz, 2H), 1.09 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 172.1, 151.8, 143.4, 139.3, 138.4, 132.1, 128.4, 126.9, 121.5, 115.9, 36.2, 23.4, 13.8. MS (ESI) calculated for C₁₃H₁₂BrN₃S, m/z 320.99, found 322.00 [M+H]⁺.

**Synthesis of compound 15a: N-butyl-2-propylthiazolo[4,5-c]quinolin-4-amine.** To a stirred solution of compound 8c (50 mg, 0.205 mmol) in THF (1 mL) were added sodium hydride (10 mg, 0.41 mmol) and 1-iodobutane (35 µL, 0.308 mmol), the resulting mixture was stirred at room temperature for 6 h. The reaction mixture was then diluted with water and extracted with CH₂Cl₂ (3 × 10 mL). The organic layer was dried over Na₂SO₄, concentrated under reduced
pressure, and the crude compound was purified using silica gel column chromatography (0-5% MeOH in CH2Cl2) to obtain compound 15a (45 mg, 70%). 1H NMR (500 MHz, CDCl3) δ 7.81 (dd, J = 8.3, 0.5 Hz, 1H), 7.70 – 7.66 (m, 1H), 7.51 (ddd, J = 8.4, 7.1, 1.5 Hz, 1H), 7.23 (ddd, J = 8.0, 7.1, 1.1 Hz, 1H), 5.96 (t, J = 4.8 Hz, 1H), 3.77 – 3.69 (m, 2H), 3.15 – 3.08 (m, 2H), 1.99 – 1.89 (m, 2H), 1.75 (ddd, J = 14.9, 11.1, 7.5 Hz, 2H), 1.52 (dq, J = 14.7, 7.4 Hz, 2H), 1.08 (t, J = 7.4 Hz, 3H), 1.00 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, CDCl3) δ 170.7, 151.2, 145.3, 138.9, 138.3, 128.6, 126.9, 124.5, 122.1, 119.3, 40.6, 36.0, 31.9, 23.3, 20.3, 14.0, 13.7. MS (ESI) calculated for C17H21N3S, m/z 299.15, found 300.14 [M+H]+.

Compounds 15b and 15c were synthesized similarly as compound 15a.

15b: N-hexyl-2-propylthiazolo[4,5-c]quinolin-4-amine. White solid (9.9 mg, 72%, based on starting material recovery). 1H NMR (400 MHz, CDCl3) δ 7.81 (d, J = 8.3 Hz, 1H), 7.68 (dd, J = 7.9, 1.1 Hz, 1H), 7.51 (ddd, J = 8.5, 7.1, 1.5 Hz, 1H), 7.23 (ddd, J = 8.1, 7.2, 1.1 Hz, 1H), 5.96 (t, J = 4.6 Hz, 1H), 3.71 (dt, J = 7.2, 5.8 Hz, 2H), 3.11 (dd, J = 8.7, 6.5 Hz, 2H), 1.94 (dd, J = 15.0, 7.5 Hz, 2H), 1.76 (t, J = 7.3 Hz, 2H), 1.53 – 1.44 (m, 2H), 1.36 (ddd, J = 6.9, 6.2, 3.3 Hz, 4H), 1.08 (t, J = 7.4 Hz, 3H), 0.91 (dd, J = 9.1, 5.1 Hz, 3H). 13C NMR (101 MHz, CDCl3) δ 170.9, 151.3, 145.5, 139.1, 138.5, 128.8, 127.1, 124.6, 122.3, 119.5, 41.1, 36.2, 31.8, 29.9, 27.1, 23.5, 22.8, 14.2, 13.8. MS (ESI) calculated for C19H25N3S, m/z 327.18, found 328.18 [M+H]+.

15c: N-hexadecyl-2-propylthiazolo[4,5-c]quinolin-4-amine. White solid (26 mg, 68%). 1H NMR (400 MHz, CDCl3) δ 7.81 (d, J = 7.9 Hz, 1H), 7.68 (dd, J = 7.9, 1.1 Hz, 1H), 7.51 (ddd, J = 8.5, 7.1, 1.5 Hz, 1H), 7.23 (td, J = 7.1, 3.6 Hz, 1H), 5.96 (s, 1H), 3.71 (dd, J = 12.9, 7.1 Hz, 2H), 3.11 (dd, J = 8.7, 6.5 Hz, 2H), 1.98 – 1.88 (m, 2H), 1.76 (dt, J = 14.8, 7.4 Hz, 2H), 1.51 – 1.43 (m, 2H), 1.30 – 1.21 (m, 24H), 1.08 (t, J = 7.4 Hz, 3H), 0.88 (t, J = 6.8 Hz, 3H). 13C NMR (126
MS (ESI) calculated for C$_{29}$H$_{45}$N$_3$S, m/z 467.33, found 468.35 [M+H]$^+$.  

**Synthesis of compound 15d:** *N-(2-propylthiazolo[4,5-c]quinolin-4-yl)formamide.* To a stirred solution of compound 8c (50 mg, 0.205 mmol) in 1,4-dioxane (1 mL) were added 2,2,2-trifluoroethylformate (60 µL, 0.616 mmol) and triethylamine (86 µL, 0.616 mmol). The resulting mixture was stirred at 80 °C for 12 h. The reaction mixture was then diluted with water and extracted with CH$_2$Cl$_2$ (3 × 10 mL). The combined organic layer was dried over Na$_2$SO$_4$, concentrated under reduced pressure, and the crude compound was purified using silica gel column chromatography (0-3% MeOH in CH$_2$Cl$_2$) to furnish compound 15d as a white solid (26 mg, 46%). $^1$H NMR (500 MHz, CDCl$_3$) δ 9.84 (d, $J$ = 10.8 Hz, 1H), 8.86 (d, $J$ = 10.3 Hz, 1H), 8.03 – 7.94 (m, 1H), 7.84 (ddd, $J$ = 8.0, 1.4, 0.5 Hz, 1H), 7.67 (ddd, $J$ = 8.5, 7.0, 1.4 Hz, 1H), 7.50 (ddd, $J$ = 8.1, 7.1, 1.2 Hz, 1H), 3.24 – 3.08 (m, 2H), 2.05 – 1.87 (m, 2H), 1.09 (t, $J$ = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 172.7, 162.0, 143.8, 143.2, 141.7, 137.5, 129.5, 128.8, 125.8, 124.8, 121.9, 36.2, 23.1, 13.8. MS (ESI) calculated for C$_{14}$H$_{13}$N$_3$OS, m/z 271.08, found 272.09 [M+H]$^+$.  

**Synthesis of compound 15e:** *N-(2-propylthiazolo[4,5-c]quinolin-4-yl)acetamide.* To a stirred solution of compound 8c (20 mg, 0.082 mmol) in pyridine (1 mL) was added acetyl chloride (5.8 µL, 0.082 mmol), the resulting mixture was stirred at room temperature for 1 h. The solvent was evaporated under reduced pressure and extracted with CH$_2$Cl$_2$ (3 × 10 mL). The combined organic layer was dried over Na$_2$SO$_4$, concentrated under reduced pressure, and the crude compound was purified using silica gel column chromatography (0-5% MeOH in CH$_2$Cl$_2$) to furnish compound 15e as a white solid (18 mg, 78%). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.03 (d, $J$
= 8.3 Hz, 1H), 7.88 (dd, J = 8.1, 0.9 Hz, 1H), 7.72 (ddd, J = 8.4, 7.2, 1.3 Hz, 1H), 7.59 – 7.54 (m, 1H), 3.17 (td, J = 7.6, 2.3 Hz, 2H), 2.77 (s, 3H), 1.96 (dd, J = 15.0, 7.5 Hz, 2H), 1.09 (t, J = 7.3 Hz, 3H). 13C NMR (126 MHz, CDCl3) δ 173.0, 144.7, 138.3, 131.6, 130.0, 127.2, 126.5, 125.9, 125.3, 125.0, 121.2, 36.2, 25.7, 23.2, 13.8. MS (ESI) calculated for C15H15N3OS, m/z 285.09, found 286.11 [M+H]+.

Compounds 15f-15i were synthesized similarly as compound 15e.

15f: N-(2-propylthiazolo[4,5-c]quinolin-4-yl)butyramide. White solid (20 mg, 78%). 1H NMR (500 MHz, CDCl3) δ 8.96 (s, 1H), 8.04 (d, J = 8.3 Hz, 1H), 7.82 (dd, J = 8.0, 1.0 Hz, 1H), 7.65 (ddd, J = 8.4, 7.1, 1.4 Hz, 1H), 7.49 (ddd, J = 8.1, 7.1, 1.1 Hz, 1H), 3.21 – 3.04 (m, 4H), 2.01 – 1.92 (m, 2H), 1.92 – 1.82 (m, 2H), 1.10 (td, J = 7.4, 6.1 Hz, 6H). 13C NMR (126 MHz, CDCl3) δ 172.1, 144.5, 143.5, 140.8, 138.6, 129.2, 129.2, 125.7, 124.6, 121.4, 39.9, 36.2, 23.2, 18.6, 14.1, 13.8. MS (ESI) calculated for C17H19N3OS, m/z 313.12, found 314.13 [M+H]+.

15g: N-(2-propylthiazolo[4,5-c]quinolin-4-yl)octanamide. White solid (25 mg, 83%). 1H NMR (400 MHz, CDCl3) δ 8.95 (s, 1H), 8.04 (d, J = 8.3 Hz, 1H), 7.82 (dd, J = 8.0, 0.9 Hz, 1H), 7.65 (ddd, J = 8.4, 7.1, 1.4 Hz, 1H), 7.52 – 7.44 (m, 1H), 3.15 (t, J = 7.5 Hz, 4H), 1.96 (dd, J = 14.9, 7.4 Hz, 2H), 1.83 (dd, J = 15.2, 7.7 Hz, 2H), 1.54 – 1.45 (m, 2H), 1.39 – 1.23 (m, 6H), 1.09 (t, J = 7.4 Hz, 3H), 0.89 (t, J = 6.9 Hz, 3H). 13C NMR (126 MHz, CDCl3) δ 172.1, 144.5, 143.5, 140.8, 138.6, 129.3, 129.2, 125.7, 124.7, 121.4, 38.1, 36.2, 31.9, 29.8, 29.6, 29.3, 25.2, 23.2, 22.8, 14.2, 13.8. MS (ESI) calculated for C21H27N3OS, m/z 369.19, found 370.22 [M+H]+.

15h: N-(2-propylthiazolo[4,5-c]quinolin-4-yl)palmitamide. Yellow solid (33 mg, 84%). 1H NMR (500 MHz, CDCl3) δ 8.95 (s, 1H), 8.04 (d, J = 8.3 Hz, 1H), 7.83 (dd, J = 8.0, 1.0 Hz, 1H),
7.65 (ddd, \( J = 8.4, 7.1, 1.4 \) Hz, 1H), 7.49 (ddd, \( J = 10.4, 5.8, 2.2 \) Hz, 1H), 3.20 – 3.10 (m, 4H), 2.01 – 1.91 (m, 2H), 1.84 (dt, \( J = 15.2, 7.6 \) Hz, 2H), 1.53 – 1.44 (m, 2H), 1.43 – 1.34 (m, 2H), 1.34 – 1.19 (m, 20H), 1.09 (t, \( J = 7.4 \) Hz, 3H), 0.88 (t, \( J = 7.0 \) Hz, 3H). \(^{13}\text{C} \) NMR (126 MHz, CDCl\(_3\)) \( \delta \) 172.4, 144.7, 143.8, 140.9, 139.3, 135.0, 132.4, 130.1, 129.2, 129.0, 127.7, 126.1, 124.5, 121.8, 36.2, 23.4, 13.9. MS (ESI) calculated for C\(_{20}\)H\(_{17}\)N\(_3\)OS, m/z 347.11, found 348.12 [M+H]\(^{+}\).

15i: \( N\)-(2-propylthiazolo[4,5-c]quinolin-4-yl)benzamide.\) White solid (24 mg, 96%). \(^{1}\text{H} \) NMR (400 MHz, CDCl\(_3\)) \( \delta \) 9.70 (s, 1H), 8.24 (d, \( J = 8.5 \) Hz, 1H), 8.07 (d, \( J = 7.4 \) Hz, 2H), 7.86 (d, \( J = 7.6 \) Hz, 1H), 7.69 (t, \( J = 7.5 \) Hz, 1H), 7.62 – 7.51 (m, 4H), 3.19 (t, \( J = 7.6 \) Hz, 2H), 1.98 (dt, \( J = 14.9, 7.5 \) Hz, 2H), 1.12 (t, \( J = 7.4 \) Hz, 3H). \(^{13}\text{C} \) NMR (126 MHz, CDCl\(_3\)) \( \delta \) 172.4, 164.7, 144.7, 143.8, 140.9, 139.3, 135.0, 132.4, 130.1, 129.2, 129.0, 127.7, 126.1, 124.5, 121.8, 36.2, 23.4, 13.9. MS (ESI) calculated for C\(_{20}\)H\(_{17}\)N\(_3\)OS, m/z 347.11, found 348.12 [M+H]\(^{+}\).

**Synthesis of compound 15j:** 2-Azido-\( N\)-(2-propylthiazolo[4,5-c]quinolin-4-yl)acetamide. To a stirred solution of 2-azidoacetic acid (18.6 mg, 0.123 mmol) in DMF (1 mL) were added triethylamine (23 \( \mu \)L, 0.164 mmol) and HBTU (46.6 mg, 0.123 mmol), and the resulting mixture was stirred at room temperature for 15 min. Compound 8c (20 mg, 0.082 mmol) was then added, and stirring was continued for 4 h. The reaction mixture was diluted with water and extracted with CH\(_2\)Cl\(_2\) (3 \( \times \) 10 mL). The organic layer was dried over Na\(_2\)SO\(_4\), concentrated under reduced pressure, and the crude residue was purified by silica gel column chromatography (0-5% MeOH in CH\(_2\)Cl\(_2\)) to obtain compound 15j as a white solid (50 mg, 96%). \(^{1}\text{H} \) NMR (500 MHz, CDCl\(_3\)) \( \delta \) 9.25 (d, \( J = 0.6 \) Hz, 1H), 8.03 (d, \( J = 8.1 \) Hz, 1H), 7.84 (dd, \( J = 8.0, 1.0 \) Hz, 1H), 7.68 (ddd, \( J = 8.4, 7.1, 1.4 \) Hz, 1H), 7.52 (ddd, \( J = 8.1, 7.2, 1.1 \) Hz, 1H), 4.86 (s, 2H), 3.17 (t, \( J = 7.5 \) Hz, 2H), 2.01 – 1.92 (m, 2H), 1.10 (t, \( J = 7.4 \) Hz, 3H). \(^{13}\text{C} \) NMR (126 MHz,
Compounds 15k-15l were synthesized similarly as compound 15j.

**15k: 3-Azido-N-(2-propylthiazolo[4,5-c]quinolin-4-yl)propanamide.** White solid (59 mg, 85%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 9.07 (s, 1H), 8.03 (d, \(J = 8.3\) Hz, 1H), 7.84 (dd, \(J = 8.0, 0.9\) Hz, 1H), 7.67 (ddd, \(J = 8.4, 7.1, 1.4\) Hz, 1H), 7.53 – 7.48 (m, 1H), 3.82 (t, \(J = 6.6\) Hz, 2H), 3.59 (s, 2H), 3.16 (t, \(J = 7.5\) Hz, 2H), 2.01 – 1.91 (m, 2H), 1.09 (dd, \(J = 9.3, 5.4\) Hz, 3H). \(^13\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 172.4, 144.1, 143.3, 141.1, 138.3, 129.4, 129.1, 125.9, 124.7, 121.5, 46.9, 37.6, 36.2, 23.2, 13.8. MS (ESI) calculated for C\(_{16}\)H\(_{14}\)N\(_6\)OS, m/z 340.11, found 341.12 [M+H]\(^+\).

**15l: N-(2-propylthiazolo[4,5-c]quinolin-4-yl)pent-4-ynamide.** White solid (60 mg, 90%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 9.02 (s, 1H), 8.05 (d, \(J = 8.3\) Hz, 1H), 7.82 (dd, \(J = 8.0, 0.9\) Hz, 1H), 7.66 (ddd, \(J = 8.4, 7.1, 1.4\) Hz, 1H), 7.49 (ddd, \(J = 8.1, 7.1, 1.1\) Hz, 1H), 3.49 (s, 2H), 3.15 (dd, \(J = 8.6, 6.5\) Hz, 2H), 2.75 (ddd, \(J = 9.0, 6.9, 2.7\) Hz, 2H), 2.03 (t, \(J = 2.7\) Hz, 1H), 2.00 – 1.90 (m, 2H), 1.09 (t, \(J = 7.4\) Hz, 3H). \(^13\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 172.2, 144.2, 143.4, 141.0, 138.4, 129.3, 129.2, 125.8, 124.7, 121.5, 83.6, 68.9, 37.2, 36.2, 23.2, 14.3, 13.8. MS (ESI) calculated for C\(_{18}\)H\(_{17}\)N\(_3\)OS, m/z 323.11, found 324.13 [M+H]\(^+\).

**Synthesis of compound 15m: Methyl (2-propylthiazolo[4,5-c]quinolin-4-yl)carbamate.** To a stirred solution of 8c (50 mg, 0.205 mmol) in CH\(_2\)Cl\(_2\) (2 mL) were added triethylamine (57 \(\mu\)L, 0.410 mmol) and methyl chloroformate (32 \(\mu\)L, 0.3075 mmol), and the resulting mixture was stirred at room temperature for 5 h. The reaction mixture was diluted with water and extracted with CH\(_2\)Cl\(_2\) (3 × 10 mL). The organic layer was dried over Na\(_2\)SO\(_4\), concentrated under reduced
pressure, and the crude residue was purified by silica gel column chromatography (0-5% MeOH in CH₂Cl₂) to obtain compound 15m as a white solid (58 mg, 94%). ¹H NMR (500 MHz, CDCl₃) δ 8.51 (s, 1H), 8.16 (d, J = 8.4 Hz, 1H), 7.82 (dd, J = 8.0, 0.9 Hz, 1H), 7.69 – 7.63 (m, 1H), 7.52 – 7.46 (m, 1H), 3.91 (s, 3H), 3.17 – 3.11 (m, 2H), 1.95 (dt, J = 14.8, 7.4 Hz, 2H), 1.09 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 172.2, 152.2, 144.3, 143.8, 140.8, 138.6, 129.7, 129.2, 125.7, 124.5, 121.4, 52.9, 36.2, 23.3, 13.8. MS (ESI) calculated for C₁₅H₁₅N₃O₂S, m/z 301.09, found 302.17 [M+H]⁺.

Compounds 15n-15p were synthesized similarly as compound 15m.

15n: Ethyl (2-propylthiazolo[4,5-c]quinolin-4-yl)carbamate. Yellow solid (50 mg, 77%). ¹H NMR (500 MHz, CDCl₃) δ 8.48 (s, 1H), 8.17 (t, J = 8.3 Hz, 1H), 8.17 (t, J = 8.3 Hz, 1H), 7.81 (dd, J = 8.0, 1.0 Hz, 1H), 7.81 (dd, J = 8.0, 1.0 Hz, 1H), 7.69 – 7.61 (m, 1H), 7.51 – 7.45 (m, 1H), 4.36 (q, J = 7.1 Hz, 2H), 3.18 – 3.12 (m, 2H), 2.01 – 1.90 (m, 2H), 1.39 (t, J = 7.1 Hz, 3H), 1.09 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 172.1, 151.6, 144.5, 143.8, 140.8, 138.6, 129.8, 129.1, 125.7, 124.5, 121.3, 61.8, 36.2, 23.3, 14.7, 13.8. MS (ESI) calculated for C₁₆H₁₇N₃O₂S, m/z 315.10, found 316.19 [M+H]⁺.

15o: Butyl (2-propylthiazolo[4,5-c]quinolin-4-yl)carbamate. White solid (52 mg, 74%). ¹H NMR (500 MHz, CDCl₃) δ 8.47 (s, 1H), 8.18 – 8.13 (m, 1H), 7.81 (dd, J = 8.0, 0.9 Hz, 1H), 7.66 (qd, J = 8.5, 2.6 Hz, 1H), 7.52 – 7.45 (m, 1H), 4.31 (t, J = 6.7 Hz, 2H), 3.19 – 3.11 (m, 2H), 2.02 – 1.91 (m, 2H), 1.80 – 1.70 (m, 2H), 1.54 – 1.44 (m, 2H), 1.13 – 1.06 (m, 3H), 0.98 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 172.1, 151.8, 144.5, 143.7, 140.8, 138.6, 129.8, 129.1, 125.7, 124.5, 121.3, 65.7, 36.2, 31.0, 23.3, 19.2, 13.9, 13.8. MS (ESI) calculated for C₁₈H₂₁N₃O₂S, m/z 343.14, found 344.14 [M+H]⁺.
15p: Octyl (2-propylthiazolo[4,5-c]quinolin-4-yl)carbamate. White solid (60 mg, 73%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.47 (s, 1H), 8.17 – 8.13 (m, 1H), 7.81 (dd, $J$ = 8.0, 1.0 Hz, 1H), 7.68 – 7.61 (m, 1H), 7.47 (ddd, $J$ = 8.1, 7.2, 1.1 Hz, 1H), 4.30 (t, $J$ = 6.7 Hz, 2H), 3.15 (dd, $J$ = 9.7, 5.5 Hz, 2H), 2.02 – 1.90 (m, 3H), 1.80 – 1.71 (m, 2H), 1.51 – 1.39 (m, 3H), 1.38 – 1.22 (m, 10H), 1.09 (dd, $J$ = 9.0, 5.8 Hz, 5H), 0.88 (t, $J$ = 7.0 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.1, 151.8, 144.5, 143.9, 140.8, 138.6, 129.7, 129.1, 125.6, 124.5, 121.3, 66.0, 36.2, 31.9, 29.4, 29.0, 26.0, 23.3, 22.8, 14.2, 13.8. MS (ESI) calculated for C$_{22}$H$_{29}$N$_3$O$_2$S, m/z 399.20, found 400.21 [M+H]$^+$.

Synthesis of compound 15q: 1-(2-Propylthiazolo[4,5-c]quinolin-4-yl)urea. To a stirred solution of 8c (50 mg, 0.205 mmol) in acetonitrile (2 mL) were added NaHCO$_3$ (26 mg, 0.307 mmol) and chlorosulfonyl isocyanate (27 $\mu$L, 0.307 mmol), and the resulting mixture was stirred at room temperature for 1 h. Then, one more equivalent of chlorosulfonyl isocyanate (27 $\mu$L, 0.307 mmol) was added and the reaction mixture was stirred at room temperature for additional 1h. The reaction mixture was diluted with water and extracted with EtOAc (3 × 10 mL). The organic layer was dried over Na$_2$SO$_4$, concentrated under reduced pressure, and the crude residue was purified by silica gel column chromatography (0-5% MeOH in CH$_2$Cl$_2$) to obtain compound 15q as a white solid (26 mg, 45% starting material was recovered). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.84 (d, $J$ = 10.8 Hz, 1H), 8.86 (d, $J$ = 10.3 Hz, 1H), 8.03 – 7.94 (m, 1H), 7.84 (ddd, $J$ = 8.0, 1.4, 0.5 Hz, 1H), 7.67 (ddd, $J$ = 8.5, 7.0, 1.4 Hz, 1H), 7.50 (ddd, $J$ = 8.1, 7.1, 1.2 Hz, 1H), 3.24 – 3.08 (m, 2H), 2.05 – 1.87 (m, 2H), 1.09 (t, $J$ = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.7, 162.0, 143.8, 143.2, 141.7, 137.5, 129.5, 128.8, 125.8, 124.8, 121.9, 36.2, 23.1, 13.8. MS (ESI) calculated for C$_{14}$H$_{14}$N$_4$OS, m/z 286.09, found 287.09 [M+H]$^+$. 
Synthesis of compound 15r: \( N-(2\text{-propylthiazolo[4,5-c]quinolin-4-yl})\text{-methanesulfonamide} \).

To a stirred solution of 8c (40 mg, 0.146 mmol) in dichloromethane (2 mL) was added methyl methanesulfonyl chloride (26 \( \mu \)L, 0.292 mmol), and the resulting mixture was stirred at room temperature for 12 h. The reaction mixture was diluted with water and extracted with CH\(_2\)Cl\(_2\) (3 \( \times \) 10 mL). The organic layer was dried over Na\(_2\)SO\(_4\), concentrated under reduced pressure, and the crude residue was purified by silica gel column chromatography (0-5% MeOH in CH\(_2\)Cl\(_2\)) to obtain compound 15r as a white solid (34 mg, 65%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 11.91 (s, 1H), 7.73 (d, \( J = 7.8 \) Hz, 1H), 7.58 (t, \( J = 7.4 \) Hz, 1H), 7.40 (t, \( J = 7.6 \) Hz, 2H), 3.29 (s, 3H), 3.20 – 3.15 (m, 2H), 1.97 – 1.87 (m, 2H), 1.07 (t, \( J = 7.4 \) Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 173.0, 148.8, 143.4, 141.6, 133.5, 130.6, 125.4, 125.2, 117.5, 116.9, 43.2, 36.3, 23.7, 13.8. MS (ESI) calculated for C\(_{14}\)H\(_{15}\)N\(_3\)O\(_2\)S\(_2\), m/z 321.06, found 322.06 [M+H]\(^+\).

Compounds 15s-15v were synthesized similarly as compound 15r.

**15s: \( N-(2\text{-propylthiazolo[4,5-c]quinolin-4-yl})\text{-ethanesulfonamide} \).** White solid (35 mg, 63%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 12.05 (s, 1H), 7.72 (d, \( J = 6.7 \) Hz, 1H), 7.58 (t, \( J = 7.1 \) Hz, 1H), 7.40 (d, \( J = 7.3 \) Hz, 2H), 3.34 (d, \( J = 5.0 \) Hz, 2H), 3.18 (t, \( J = 7.7 \) Hz, 2H), 1.99 – 1.87 (m, 2H), 1.46 (t, \( J = 7.4 \) Hz, 3H), 1.07 (t, \( J = 7.4 \) Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 172.9, 149.9, 143.3, 141.9, 133.5, 130.7, 125.5, 125.0, 117.7, 116.8, 49.4, 36.3, 23.8, 13.8, 8.3. MS (ESI) calculated for C\(_{15}\)H\(_{17}\)N\(_3\)O\(_2\)S\(_2\), m/z 335.08, found 336.08 [M+H]\(^+\).

**15t: \( N-(2\text{-propylthiazolo[4,5-c]quinolin-4-yl})\text{-propane-1-sulfonamide} \).** White solid (35 mg, 61%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 12.05 (s, 1H), 7.72 (d, \( J = 6.7 \) Hz, 1H), 7.58 (t, \( J = 7.1 \) Hz, 1H), 7.40 (d, \( J = 7.3 \) Hz, 2H), 3.34 (d, \( J = 5.0 \) Hz, 2H), 3.18 (t, \( J = 7.7 \) Hz, 2H), 1.99 – 1.87 (m, 2H), 1.46 (t, \( J = 7.4 \) Hz, 3H), 1.07 (t, \( J = 7.4 \) Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 172.9,
149.9, 143.3, 141.9, 133.5, 130.7, 125.5, 125.0, 117.7, 116.8, 49.4, 36.3, 23.8, 13.8, 8.3. MS (ESI) calculated for C_{16}H_{19}N_{3}O_{2}S_{2}, m/z 349.09, found 350.09 [M+H]^+.

15u: **N-(2-propylthiazolo[4,5-c]quinolin-4-yl)butane-1-sulfonamide.** White solid (37 mg, 62%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 12.04 (s, 1H), 7.71 (d, \(J = 6.7\) Hz, 1H), 7.57 (t, \(J = 7.0\) Hz, 1H), 7.39 (d, \(J = 6.2\) Hz, 2H), 3.32 (s, 2H), 3.18 (t, \(J = 7.7\) Hz, 2H), 2.00 – 1.82 (m, 4H), 1.55 – 1.40 (m, 2H), 1.07 (t, \(J = 7.4\) Hz, 3H), 0.94 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 172.8, 149.7, 143.3, 141.9, 133.5, 130.7, 125.5, 125.0, 117.6, 116.8, 54.8, 36.3, 25.5, 23.8, 21.7, 13.8, 13.8. MS (ESI) calculated for C_{17}H_{21}N_{3}O_{2}S_{2}, m/z 363.11, found 364.11 [M+H]^+.

15v: **4-Methyl-N-(2-propylthiazolo[4,5-c]quinolin-4-yl)benzenesulfonamide.** White solid (40 mg, 61%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 12.04 (s, 1H), 7.71 (d, \(J = 6.7\) Hz, 1H), 7.57 (t, \(J = 7.0\) Hz, 1H), 7.39 (d, \(J = 6.2\) Hz, 2H), 3.32 (s, 2H), 3.18 (t, \(J = 7.7\) Hz, 2H), 2.00 – 1.82 (m, 4H), 1.55 – 1.40 (m, 2H), 1.07 (t, \(J = 7.4\) Hz, 3H), 0.94 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 172.8, 149.0, 143.4, 143.0, 142.1, 139.9, 133.5, 130.7, 129.4, 126.7, 125.5, 125.1, 117.7, 117.0, 36.2, 23.67, 21.7, 13.8. MS (ESI) calculated for C_{20}H_{19}N_{3}O_{2}S_{2}, m/z 397.09, found 398.10 [M+H]^+.

**Synthesis of compound 15w: Diethyl (2-propylthiazolo[4,5-c]quinolin-4-yl) phosphoramidate.** To a stirred solution of 8c (50 mg, 0.205 mmol) in CH\(_2\)Cl\(_2\) (2 mL) was added diethyl chlorophosphate (60 \(\mu\)L, 0.810 mmol), and the resulting mixture was stirred at room temperature for 12 h. The reaction mixture was diluted with water and extracted with CH\(_2\)Cl\(_2\) (3 \(\times\) 10 mL). The organic layer was dried over Na\(_2\)SO\(_4\), concentrated under reduced pressure, and the crude residue was purified by silica gel column chromatography (0-5% MeOH in CH\(_2\)Cl\(_2\)) to obtain compound 15w as a white solid (45 mg, 56%). \(^1\)H NMR (500 MHz, DMSO) \(\delta\) 8.41 (d, \(J = \))
10.1 Hz, 1H), 7.96 (d, J = 7.8 Hz, 1H), 7.83 (d, J = 8.3 Hz, 1H), 7.68 (dd, J = 11.2, 4.0 Hz, 1H), 7.49 (t, J = 7.4 Hz, 1H), 4.24 (p, J = 7.2 Hz, 4H), 3.20 (t, J = 7.5 Hz, 2H), 1.94 – 1.85 (m, 2H), 1.27 (t, J = 7.0 Hz, 6H), 1.02 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, DMSO) δ 171.9, 147.0, 143.0, 140.3, 138.0 (d, J = 10.4 Hz), 131.2, 129.2 (d, J = 207.2 Hz), 127.8 (d, J = 94.8 Hz), 124.9 (d, J = 12.8 Hz), 120.2, 63.1 (d, J = 5.6 Hz), 35.2, 63.1 (d, J = 5.6 Hz), 16.2 (d, J = 6.8 Hz) 13.5. MS (ESI) calculated for C$_{17}$H$_{22}$N$_{3}$O$_{3}$PS, m/z 379.11, found 380.12 [M+H]$^+$. 

Synthesis of compound 17: 1-(2-Propylthiazolo[4,5-c]quinolin-4-yl)guanidine. Compound 7c (100 mg, 0.411 mmol) was dissolved in POCl$_3$ (3 mL) and stirred at 100 °C for 1 h. POCl$_3$ was evaporated under reduced pressure, ice cold water was added and the residue was extracted with CH$_2$Cl$_2$ (3 × 10 mL). The organic layer was dried over Na$_2$SO$_4$, concentrated under reduced pressure to give crude 16 (100 mg, 93%) which was used further without purification. To a stirred solution of guanidine (62 mg, 0.615 mmol) in 1,4-dioxane (2 mL) was added NaH (12 mg, 0.492 mmol), the reaction mixtures was stirred at 60 °C for 30 min. Compound 16 (30 mg, 0.123 mmol) in DMF (2 mL) was added and heating was continued at 90 °C for 12 h. The reaction mixture was diluted with water and extracted with CH$_2$Cl$_2$ (3 × 10 mL). The organic layer was dried over Na$_2$SO$_4$, concentrated under reduced pressure, and the crude residue was purified by silica gel column chromatography (0-10% MeOH in CH$_2$Cl$_2$) to obtain compound 17 as a yellow solid (25 mg, 71%). $^1$H NMR (500 MHz, MeOD) δ 8.05 (dd, J = 8.4, 0.5 Hz, 1H), 8.00 – 7.96 (m, 1H), 7.75 (ddd, J = 8.4, 7.1, 1.4 Hz, 1H), 7.62 (ddd, J = 8.2, 7.1, 1.2 Hz, 1H), 3.25 (dd, J = 8.7, 6.4 Hz, 2H), 2.06 – 1.97 (m, 2H), 1.11 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) δ 175.2, 157.5, 146.0, 143.5, 142.7, 138.9, 131.0, 129.3, 128.0, 125.9, 122.5, 36.9, 24.2, 14.0. MS (ESI) calculated for C$_{14}$H$_{15}$N$_{5}$S, m/z 285.10, found 286.11 [M+H]$^+$. 

Compound 18a was synthesized similarly as compound 15c.
18a: $N$-(2-butylthiazolo[4,5-c]quinolin-4-yl)formamide. White solid (32 mg, 71%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.85 (d, $J = 10.8$ Hz, 1H), 8.86 (d, $J = 10.3$ Hz, 1H), 7.99 (d, $J = 8.4$ Hz, 1H), 7.85 (dd, $J = 8.0$, 0.9 Hz, 1H), 7.67 (ddd, $J = 8.4$, 7.1, 1.4 Hz, 1H), 7.50 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 3.20 – 3.16 (m, 2H), 1.91 (dt, $J = 15.2$, 7.6 Hz, 2H), 1.55 – 1.45 (m, 2H), 1.01 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.8, 161.9, 143.7, 143.2, 141.6, 137.4, 129.4, 128.7, 125.7, 124.7, 121.8, 33.8, 31.6, 22.2, 13.8. MS (ESI) calculated for C$_{15}$H$_{15}$N$_3$OS, m/z 285.09, found 286.10 [M+H]$^+$. Compounds 18b and 18c were synthesized similarly as compound 15d.

18b: $N$-(2-butylthiazolo[4,5-c]quinolin-4-yl)acetamide. White solid (50 mg, 85%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.97 (s, 1H), 8.02 (d, $J = 8.3$ Hz, 1H), 7.82 (ddd, $J = 8.0$, 1.4, 0.5 Hz, 1H), 7.65 (ddd, $J = 8.4$, 7.1, 1.4 Hz, 1H), 7.49 (ddd, $J = 8.1$, 7.1, 1.2 Hz, 1H), 3.21 – 3.13 (m, 2H), 2.82 (s, 3H), 1.96 – 1.85 (m, 2H), 1.55 – 1.44 (m, 2H), 1.04 – 0.96 (m, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.4, 144.4, 143.4, 140.9, 138.5, 129.2, 129.1, 125.7, 124.7, 121.5, 34.0, 31.8, 25.9, 22.3, 13.9. MS (ESI) calculated for C$_{16}$H$_{17}$N$_3$OS, m/z 299.11, found 300.11 [M+H]$^+$. 

18c: $N$-(2-butylthiazolo[4,5-c]quinolin-4-yl)butyramide. White solid (55 mg, 87%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.95 (s, 1H), 8.05 (d, $J = 8.3$ Hz, 1H), 7.82 (ddd, $J = 8.0$, 1.4, 0.4 Hz, 1H), 7.65 (ddd, $J = 8.4$, 7.1, 1.4 Hz, 1H), 7.49 (ddd, $J = 8.1$, 7.1, 1.2 Hz, 1H), 3.22 – 3.06 (m, 4H), 1.95 – 1.83 (m, 4H), 1.54 – 1.45 (m, 2H), 1.10 (t, $J = 7.4$ Hz, 3H), 1.00 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 172.2, 144.3, 143.4, 140.7, 138.4, 129.1, 129.0, 125.6, 124.5, 121.3, 39.8, 33.8, 31.7, 22.2, 18.5, 14.0, 13.8. MS (ESI) calculated for C$_{18}$H$_{21}$N$_3$OS, m/z 327.14, found 328.14 [M+H]$^+$. 

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Human TLR-7/-8 reporter gene assays (NF-κB induction). The induction of NF-κB was quantified using HEK-Blue-7 (hTLR7-specific) and HEK-Blue-8 (hTLR8-specific) cells as previously described by us.30, 55b, 69 HEK293 cells stably co-transfected with human TLR7 or human TLR8 and secreted alkaline phosphatase (sAP), were maintained in HEK-Blue™ Selection medium containing zeocin and normocin. Stable expression of secreted alkaline phosphatase (sAP) under control of NF-κB/AP-1 promoters is inducible by appropriate TLR agonists, and extracellular sAP in the supernatant is proportional to NF-κB induction. HEK-Blue cells were incubated at a density of ~10^5 cells/ml in a volume of 80 μl/well, in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluency was achieved, and subsequently stimulated with graded concentrations of stimuli. sAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen (present in HEK-detection medium as supplied by the vendor) at 620 nm.

Immunoassays for Interferon (IFN)-α, IFN-γ, Interleukin (IL)-12, IL-18, and cytokines. Fresh human peripheral blood mononuclear cells (hPBMCs) were isolated from human blood obtained by venipuncture with informed consent and as per institutional guidelines on Ficoll-Hypaque gradients as described elsewhere.73 Aliquots of PBMCs (10^5 cells in 100 μL/well) were stimulated for 12 h with graded concentrations of test compounds. Supernatants were isolated by centrifugation, and were assayed in triplicates using either high-sensitivity analyte-specific ELISA kits (PBL Interferon Source, Piscataway, NJ and R&D Systems, Inc., Minneapolis, MN), or analyte-specific multiplexed cytokine/chemokine bead array assays as reported by us previously.69 PBMC supernatants were also analyzed for 41 chemokines and cytokines (EGF, Eotaxin, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1ra, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB,
RANTES, TGF-α, TNF-α, TNF-β, VEGF, and sCD40L) using a magnetic bead-based multiplexed assay kit (Milliplex MAP Human Cytokine/Chemokine kit). Data were acquired and processed on a MAGPIX instrument (EMD Millipore, Billerica, MA) with intra-assay coefficients of variation ranging from 4 to 8% for the 41 analytes.

**Rabbit immunization and antigen-specific ELISA.** All experiments were performed at Harlan Laboratories (Indianapolis, IN) in accordance with institutional guidelines (University of Kansas IACUC permit # 119-06). Cohorts of adult female New Zealand White rabbits (n = 4 per cohort) were immunized intramuscularly in the flank region with (a) 100 μg of bovine α-lactalbumin in 0.2 mL saline, or (b) 100 μg of bovine α-lactalbumin plus 100 μg of either 8c or 8d in 0.2 mL saline. Pre-immune test-bleeds were first obtained on Day 1 via venipuncture of the marginal vein of the ear. Animals were immunized on Days 1 and 15. A final test-bleed was performed via the marginal vein of the ear on Day 28. Sera were stored at -80 °C until used. Bovine α-lactalbumin-specific ELISAs were performed in 384-well format using automated liquid handling methods as described by us.74
Chapter 3.

**TLR8-agonistic 2,3-diamino-furo[2,3-c]pyridines**

[Diagram showing the structure and reactions of Imidazo[1,2-a]pyridine, Groebke-Blackburn-Bienaymé multicomponent reaction, and Furo[2,3-c]pyridine.]

- Imidazo[1,2-a]pyridine: No TLR activation
- Groebke-Blackburn-Bienaymé multicomponent reaction
- Furo[2,3-c]pyridine: TLR8-agonist, No proinflammatory cytokine induction, Strong adjuvant activity
3.1. Introduction

As mentioned earlier, small molecule TLR7/8 activators constitute a small set of compounds occupying a very small chemical space. The identification of simpler molecules as TLR7/8 agonists may pave the way for inexpensive vaccine constructs, and we are therefore keenly interested in exploring alternative chemotypes that are synthetically less complex. A detailed structural characterization of the mode of binding of TLR7 ligands is not yet available to guide scaffold-hopping approaches.75 We speculated that 3,8-diamino-imidazo[1,2-a]pyrazines 4 may bear sufficient structural similarities to the known TLR7/8 ligands (Fig. 1). These molecules are, in principle, readily accessible in two steps (one-pot synthetic process) via the Groebke-Blackburn-Bienaymé multicomponent reaction,76 and we envisaged a rapid elaboration and screening of a library of compounds for TLR7/8 agonistic activities.

**Fig. 1.** TLR7/8 agonistic scaffolds. R_1 is typically alkyl or O-alkyl, R_2 is alkyl or benzyl, and R_3 is usually alkyl and OH in oxoadenines.

We began with the syntheses of small test-libraries of 3,8-diamino-imidazo[1,2-a]pyrazines as well as 3-amino-imidazo[1,2-a]pyridine/pyrazines (Schemes 1 and 2). Most of these compounds (5a-d, 6-23) were inactive in NF-κB reporter gene assays specific for human TLR-3, -7, -8, and -9; however compounds 26-29 obtained with pyridoxal as the aldehyde component were found to specifically activate NF-κB signaling in TLR8-transfected HEK293 cells. Detailed spectroscopic
analyses confirmed the formation of a hitherto unknown 2,3-diamino-furo[2,3-c]pyridine skeleton via a non-canonical pathway, which was unambiguously confirmed by single crystal X-ray analysis. The TLR8-specific agonistic properties of this novel and unexpected chemotype warranted a systematic SAR, which is presented herein.

3.2. Results and Discussion

We have previously described extensive SAR on the 1,2-disubstituted-(1H-imidazo[4,5-c]quinoline-4-amines) class of compounds (1a, Fig. 1) as TLR7/8 agonists, and their application in designing self-adjuvanting vaccine constructs. In our ongoing search toward identifying novel and synthetically simpler candidate vaccine adjuvants, we hypothesized that the imidazo[1,2-a]pyrazines would possess sufficient structural similarity with the known small molecule TLR7/8 ligands such as 1-3 (Fig. 1). These molecules are readily accessible in a one-pot, two-step process using the Groebke-Blackburn-Bienaymé multicomponent reaction as a key step. An acid-catalyzed (HCl in dioxane), microwave-mediated (400 W, 110 ºC, 10 min) reaction using 2-amino-3-chloropyrazine (amidine component), isocyanocyclohexane (isonitrile component) and benzaldehyde (aldehyde component) resulted, as expected, in 8-chloro-N-cyclohexyl-2-phenylimidazo[1,2-a]pyrazin-3-amine (Scheme 1). Subsequent microwave-mediated ipso-chloro displacement using ammonium hydroxide was unsuccessful, but conventional heating in a sealed tube (110 ºC, 16 h) furnished the desired N3-cyclohexyl-2-phenylimidazo[1,2-a]pyrazine-3,8-diamine 5a (Scheme 1) in 30% yield over two steps. Using this one-pot process, a small set of 8-amino-imidazo[1,2-a]pyrazines 5b-d was synthesized by varying the aldehyde and isonitrile components (Scheme 1).
Scheme 1. A two step one-pot synthetic process.

\[
\begin{align*}
\text{Cl} + R_1\text{NC} + R_2\text{-CHO} & \xrightarrow{i} \text{H}_2\text{N} \quad 5a: R_1 = \text{cyclohexyl}, R_2 = \text{Ph} \\
& \text{H}_2\text{N} \quad 5b: R_1 = \text{cyclohexyl}, R_2 = \text{biphenyl} \\
& \text{H}_2\text{N} \quad 5c: R_1 = \text{Bn}, R_2 = \text{C}_4\text{H}_9 \\
& \text{H}_2\text{N} \quad 5d: R_1 = \text{PhOMe}, R_2 = \text{C}_4\text{H}_9
\end{align*}
\]

Reagents and conditions: i. (a) HCl in 1,4-dioxane, CH₃CN, MW 400 W, 110 °C, 20 min (b) NH₄OH, 110 °C, 16h.

Scheme 2. Twenty-four membered diverse test-library.

<table>
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<th>CHO</th>
<th>CHO</th>
<th>CHO</th>
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Reagents and conditions: i. HCl in 1,4-dioxane, CH₃CN, MW 400 W, 110 °C, 20 min.
Simultaneously, a diverse test-library comprising of twenty-four compounds was also synthesized using two amidines (2-aminopyridine and 2-aminopyrazine), three isonitriles (2-isocyano-2-methylpropane, isocyanocyclohexane, (isocyanomethyl)benzene), and four aldehydes (benzaldehyde, isonicotinaldehyde, salicylaldehyde, and pyridoxal) (Scheme 2). The syntheses of 6-23 proceeded smoothly. However, the typical Groebke reaction, carried out at 110 ºC for 20 min in CH$_3$CN, was found to be excessively harsh for reactions using pyridoxal (24-29), leading to low yields and charring of reaction mixtures. Reactions for this subset of compounds progressed rapidly in 2 min under microwave conditions at 80 ºC and 600 W power in CH$_3$CN or MeOH. We noticed that only compounds 24-29 (synthesized using pyridoxal) were fluorescent on TLC under long-wave ultraviolet radiation.

The compounds were screened in NF-κB reporter gene assays specific for human TLR-3, -4, -5, -7, -8, and -9. The 3,8-diamino-imidazo[1,2-a]pyrazines 5a-d as well as 3-amino-imidazo[1,2-a]pyridine/pyrazine library members 6-23 did not display any activity in these assays up to concentrations of 250 μM. However, compounds 26-29 obtained with the use of pyridoxal as the aldehyde component, were found to specifically activate NF-κB signaling in human TLR8-transfected HEK293 cells (Table 1, Fig. 2), but not human TLR-3, -4, -5, -7, and -9.

The NMR spectra of compounds 26-29 as well as their fluorescence properties alerted us to the possibility of the formation of a new chemical entity with a heterocyclic system other than the classical imidazo[1,2-a]pyridine/pyrazines during the Groebke-Blackburn-Bienaymé multi-component reaction. In $^1$H NMR spectra, the aliphatic CH of the cyclohexyl group in compounds 8/9, 14/15, and 20/21 (Scheme 2) appeared in the range of 3.0 to 3.1 δ ppm, whereas a pronounced downfield shift of this CH proton (up to 3.85 δ ppm) was observed in compounds 26 and 27. Similar observations were noted in another set in which the aliphatic benzylic CH$_2$ in
compounds 10/11, 16/17, and 22/23 appeared in the range of 4.2 to 4.3 δ ppm, whereas a pronounced downfield shift of this CH₂ (up to 4.76 δ ppm) was observed in compounds 28 and 29. ¹³C NMR spectra of compounds 24-29 showed an unusual upfield shift of one of the aromatic quaternary carbons in the region (90-96 δ ppm) (Fig. 3). These observations suggested a different heterocyclic system in 24-29. Initial efforts to crystallize these molecules were unsuccessful. Pending continuing crystallization efforts, we sought to elucidate the structures of these compounds via alternate routes.

**Fig. 2.** Dose-response profiles of TLR8 agonism by select 2,3-diamino-furo[2,3-c]pyridines. Top: TLR8 agonism by compounds derived from Schemes 2 and 5. Bottom: TLR8 agonism by compounds derived from Schemes 6 and 7.
Three possible cyclization products appeared plausible in this acid-catalyzed multicomponent reaction (Panel A in Scheme 3). As mentioned earlier, NMR spectroscopic observations for compounds 24-29 were not congruent with the 4-(3-(cyclohexylamino)imidazo[1,2-a]pyridin-2-yl)-5-(hydroxymethyl)-2-methylpyridin-3-ol 32, the expected product via the canonical Groebke mechanism (‘Path A’, Panel A in Scheme 3). In order to test whether the phenolic or benzylic hydroxyl groups of pyridoxal may be involved in an alternate pathway of cyclization, we carried out a reaction using a pyridoxal derivative 34 with its phenolic hydroxyl protected with a benzyl group (Scheme 4). This resulted in a product with a spectral signature entirely consistent with
the classical Groebke product (5-(benzyloxy)-4-(3-(cyclohexylamino)imidazo[1,2-a]pyridin-2-yl)-6-methylpyridin-3-yl)methanol 35, indirectly also ruling out the possibility of cyclization involving the benzylic hydroxyl group (‘Path B’, Panel A in Scheme 3). We reasoned that annulation via ‘Path C’ ought to lead to a furo[2,3-c]pyridine skeleton 26, and that if this were indeed the case, this cyclization could proceed even if the amidine were to be replaced with an aniline.

**Scheme 3. Possible cyclization pathways and proposed mechanism.**
Scheme 4. Synthesis of compound 35 using a pyridoxal derivative 34 with its phenolic hydroxyl protected with a benzyl group.

We were gratified that a multicomponent reaction involving aniline, benzyl isonitrile and pyridoxal (Scheme 5) yielded the fluorescent compound 36, which $^1$H and $^{13}$C NMR spectra resembled those of compounds 24-29. Interestingly, 36 was also found to be weakly active (EC$_{50}$ = 1.68 μM) in primary TLR8 screens (Table 1, Fig. 2). Our investigations suggested the formation of a hitherto unknown furo[2,3-c]pyridine structure, exclusively when pyridoxal was used as the aldehyde component in the Groebke-Blackburn-Bienaymé multicomponent reaction.

Scheme 5. Synthesis of compound 36 using aniline.

Although a definitive elucidation of the reaction mechanism leading to the unexpected furo[2,3-c]pyridine was not an immediate goal, understanding plausible mechanisms was of interest, and
was probed in some detail. Formation of the pyrano[3,4-c]pyridine 33 via nucleophilic attack of the benzylic hydroxyl group, and its subsequent rearrangement to the furo[2,3-c]pyridine 26 (Path D, Panel A in Scheme 3) appeared improbable. Salicylaldehyde, which lacks the bulky hydroxymethylene group, yielded the classical imidazo[1,2-a]pyridine 20 (confirmed by single crystal X-ray analysis, see below), rather than the benzofuran derivative B.3 (Panel B in Scheme 3). We reasoned, therefore, that the benzylic hydroxyl in the transition state 31 could assist cyclization via Path C (Panel A in Scheme 3) due to steric reasons, apposing the phenolic hydroxyl with the electrophilic carbon. In addition to the direct formation of the furo[2,3-c]pyridine 26 via path C, a plausible alternate mechanism for this unusual cyclization route, involving the pyridine ring system is proposed in Scheme 3 (Panel C).

After many unsuccessful attempts, a hydrochloride salt of compound 28 was crystallized as multiply-twinned bundles in acetonitrile. A multi-domain specimen of 28 was cut from one bundle which gave a set of diffracted intensities, permitting a crystal structure solution (non-centrosymmetric, triclinic P1-C1 space group with eight crystallographically-independent molecules in the asymmetric unit), but not a satisfactory refinement (Fig. 4). The structure of 28 unambiguously confirmed the furo[2,3-c]pyridine chemotype. The structure of compound 20 (obtained with salicylaldehyde, which also possesses a phenolic OH; Scheme 2) was also elucidated, which established the formation of a classic Groebke product 2-(3-(cyclohexylamino)imidazo[1,2-a]pyridin-2-yl)phenol (Fig. 4). These observations clearly emphasize the significance of the additional substituents of pyridoxal, directing the unique cyclization route leading to the furo[2,3-c]pyridine scaffold.

Thus, our initial attempts toward the synthesis of twenty-four membered imidazo[1,2-a]pyrazine/pyridine test-library unexpectedly resulted in the formation of densely substituted furo[2,3-c]pyridines 24-29. Pyridoxal was found to be an indispensable component for this cyclization
reaction. Four of the six compounds obtained (26-29, Scheme 2) were found to be active in our primary screens using TLR8-transfected HEK293 cells, while compounds 24 and 25, synthesized using 2-isocyno-2-methylpropane as one of the components, were found to be inactive, warranting detailed structure based activity relationship investigations for this new chemotype.

**Fig. 4.** Crystal structures of the salicylaldehyde-derived classic Groebke product (imidazo[1,2-a]pyridine, 20), and a non-Groebke, pyridoxal-derived furo[2,3-c]pyridine, 28.

Among the active compounds 26-29, we observed that compounds 26 and 28 (derived from 2-aminopyridine) were more active than 27 and 29 (from 2-aminopyrazine; Table 1, Fig. 2). We therefore selected 2-aminopyridine and pyridoxal as the invariant components and varied the isonitrile component. We explored thirteen different isonitriles (Scheme 6), including linear aliphatic (as in 37a and 37b), branched aliphatic (37c-e), linear aliphatic with silyl (37f), heteroaromatic ring (37g), ester (37h and 37i), and phosphate ester (37j) termini, as well as aromatic substituents (37k-m). Maximal activity was observed in 37b, with a pentylene substituent on the C2 amine (Scheme 6, Fig. 2). Diminishing the chain length by one methylene unit (37a) decreased activity, and potency was further attenuated in compounds with alpha-
branched substituents (37d). Compound 37n, with a free NH₂ at C2 obtained by N-dealkylation of the tert-octylamine group\(^{78}\) of 37e with trifluoroacetic acid, as well as compounds with aromatic substituents (37k-m) were devoid of TLR8-stimulatory activity (Table 1). Thus, a distinct dependence of the nature of the C2 amino substituent on the activity profiles was observed in the furo[2,3-c]pyridines, with the C2-N-pentyl (37b) and C2-N-(trimethylsilyl)methyl analogues (37f) displaying dominant TLR8 agonism.


**Reagents and conditions:** i. HCl in 1,4-dioxane, CH₃CN, MW 600W, 80 °C, 2 min; ii. 50% TFA/CH₂Cl₂, 25 °C, 6h.
As mentioned earlier, our earlier efforts at unambiguously confirming the structure of 28 did not allow for satisfactory crystal structure refinement because of the intrinsic properties of the crystal space group. Having synthesized thirteen additional furo[2,3-c]pyridines (37a-m) for purposes of delineating SAR, a parallel crystallization of these compounds was attempted using various solvents. Suitable crystals of compound 37e were obtained as pale yellow crystals by slow evaporation of a super-saturated solution of 37e in CH₃CN/CH₃OH mixtures at room temperature. A single-domain specimen was selected and the X-ray diffraction data was collected. The ORTEP diagram of 37e is shown in Fig. 5, confirming the non-Groebke furo[2,3-c]pyridine.

![ORTEP diagram of 37e](image_url)

**Fig. 5.** Crystal structure (ORTEP view) of the non-Groebke furo[2,3-c]pyridine, 37e, obtained with the use of pyridoxal as the aldehyde component.

Previous mention was made that 36 (Scheme 5) was found to be weakly active (EC₅₀ = 1.68 μM, Table 1, Fig. 2) relative to the lead compound 28, suggesting that the 2-aminopyridine core could be substituted by anilines in this multicomponent reaction. Having optimized the C2 group as pentylamine (derived from 1-isocyanopentane, Scheme 6), three more furo[2,3-c]pyridines were synthesized using aniline 38a, 3-fluoroaniline 38b, and 3-nitroaniline 38c in combination with 1-isocyanopentane and pyridoxal (Scheme 7).
The C2 \( N \)-pentyl analogue 38a was found to be more active than the C2 \( N \)-benzyl analogue 36 (Fig. 2, Table 1). The nitro derivative 38c was found to be as potent as the parent compound 38a, whereas substantial gain in TLR8 activity was noticed for the fluoro-substituted compound 38b in TLR8-specific functional assays. However, these compounds exhibited a poorer dose-response profile (lower area-under the-curve, Fig. 2). It is pertinent to note that no stable product could be obtained by the replacement of 2-aminopyridine with aliphatic amines.

Several TLRs are thought to signal via ligand-induced dimerization, as evident in the crystal structures of TLRs. Therefore, the dimeric compound 39 was also synthesized using 1,6-diisocyanohexane (Scheme 8); the activity of this analogue was comparable in its TLR8-agonistic potency to the most active compounds, 28, 37a, 37b and 37f (Table 1).
Table 1. EC_{50} values of compounds in human TLR8-specific reporter gene assay.

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<th>No.</th>
<th>Structure</th>
<th>TLR8 Agonistic Activity (μM)</th>
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We examined the cytokine-inducing properties\textsuperscript{25a, 30} of a subset of compounds that were maximally active (28, 37a, 37b and 37f), all of which showed robust dose-response profiles in primary TLR8-agonistic screens (Fig. 2). We used 2-propylthiazolo[4,5-c]quinolin-4-amine 40 (CL075) as a reference TLR8 agonist,\textsuperscript{45, 62} which exhibited an EC\textsubscript{50} of 1.32 \(\mu\)M (Fig. 2). In human PBMCs, only the reference thiazoloquinoline 40, but none of the furo[2,3-c]pyridines showed any proinflammatory cytokine induction (Fig. 6).

\textbf{Fig. 6.} Dose-response profiles of proinflammatory cytokine induction in hPBMCs by compounds 28, 37a, 37b and 37f. Representative data from three independent experiments are presented.
We do not yet know if the dissociation between TLR8-specific NF-κB induction on the one hand, and lack of cytokine induction on the other is ascribable to a non-myeloid differentiation primary response gene 88 (MyD88)-independent mechanism. However, mindful of recent observations that proinflammatory activity is not an absolute prerequisite for adjuvantic properties, and because we had previously observed potent NF-κB transactivation in a bis-quinoline, 7-chloro-N-(4-(7-chloroquinolin-4-ylamino)butyl)quinolin-4-amine (RE-660) unaccompanied by any proinflammatory cytokine induction, we decided to examine two representative compounds (37b and 37f) in transcriptomal profiling experiments. Consistent with the cytokine assays, there were no transcriptional signatures of inflammation; however, both compounds upregulated several chemokine ligand (both CXCL and CCL) genes. Although entirely bereft of any proinflammatory activity, the bis-quinoline compound 41 was found to be a potent adjuvant which appears to be related to its functional agonism at CCR1. Given some similarities in activity profiles between the furo[2,3-c]pyridines and 41, we decided to evaluate and compare the adjuvant activity of 37b alongside the reference compounds, 40 and 41. Rabbits were immunized using bovine α-lactalbumin as a model subunit antigen.

Anti-α-lactalbumin IgG titers in immune sera clearly showed an adjuvant effect of 37b, with a rise-in-titer values of >1000, comparable to the adjuvant activities of the reference compounds, 40 and 41 (Fig. 7). The complete lack of proinflammatory cytokine induction coupled with strong adjuvant activity of the novel furo[2,3-c]pyridines render this hitherto unknown chemotype an exceedingly attractive class of compounds which are expected to be devoid of local or systemic reactogenicity.
Fig. 7. Anti-bovine \( \alpha \)-lactalbumin-specific IgG titers in rabbits adjuvanted with 37b, 40, and 41 (\( n=4 \), for each cohort). Box-plots of ratios of immune/pre-immune titers yielding absorbance values of 1.0 are shown for the individual samples.

3.3. Conclusion

In our ongoing search toward identifying novel and synthetically simpler candidate vaccine adjuvants, we hypothesized that the imidazo[1,2-\( \alpha \)]pyrazines, readily accessible via the Groebke–Blackburn–Bienaymé multicomponent reaction, would possess sufficient structural similarity with TLR7/8-agonistic imidazoquinolines. With pyridoxal as the aldehyde component, furo[2,3-\( \alpha \)]pyridines, rather than the expected imidazo[1,2-\( \alpha \)]pyridines, were obtained, which were characterized by NMR spectroscopy and crystallography. Several analogues were found to activate TLR8-dependent NF-\( \kappa \)B signaling. In a focused library of furo[2,3-\( \alpha \)]pyridines, a
distinct SAR was observed with varying substituents at C2. In human PBMCs, none of the furo[2,3-c]pyridines showed any proinflammatory cytokine induction but upregulated several chemokine ligand genes. In immunization studies in rabbits, the most active compound showed prominent adjuvantic effects. The complete lack of proinflammatory cytokine induction coupled with strong adjuvantic activity of the novel furo[2,3-c]pyridines render this hitherto unknown chemotype an attractive class of compounds which are expected to be devoid of local or systemic reactogenicity.

3.4. Experimental

Chemistry. All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 °C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf “Gold” high performance silica columns on a CombiFlash Rf instrument unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel (200 \( \mu \)m) CCM precoated aluminum sheets. The purity of all final compounds was confirmed to be greater than 95% by HPLCMS using a Zorbax Eclipse Plus 4.6 mm × 150 mm, 5 \( \mu \)m analytical reverse phase C18 column with either H\(_2\)O–isopropanol or H\(_2\)O–CH\(_3\)CN gradients, a diode-array detector operating in the 190–500 nm range (2 nm bandpass), and an Agilent ESI-TOF mass spectrometer (integration on total ion intensity counts, with a mass accuracy of 10 ppm) operating in the positive ion acquisition mode.
Synthesis of compound 5a: $N^3$-cyclohexyl-2-phenylimidazo[1,2-a]pyrazine-3,8-diamine.

To a solution of 2-amino-3-chloropyrazine (64 mg, 0.50 mmol) in anhydrous acetonitrile (1 mL), were added benzaldehyde (60 µL, 0.60 mmol), 4N HCl/dioxane (10 µL) and cyclohexylisonitrile (74 µL, 0.60 mmol). The reaction mixture was then heated under microwave conditions (400 W, 110 °C) in a sealed vial for 20 min. The reaction mixture was cooled to room temperature; ammonium hydroxide (NH₃ content 28-30%, 0.5 mL) was added and further heated at 110 °C in a sealed vial for overnight. After cooling the reaction mixture to room temperature, the solvents were removed and the residue was purified using column chromatography to obtain compound 5a (47 mg, 30%). $^1$H NMR (500 MHz, MeOD) $\delta$ 8.02 (dd, $J$ = 8.2, 1.3 Hz, 2H), 7.64 (d, $J$ = 4.8 Hz, 1H), 7.46 (dd, $J$ = 10.9, 4.6 Hz, 2H), 7.39 – 7.28 (m, 1H), 7.21 (d, $J$ = 4.8 Hz, 1H), 2.96 – 2.83 (m, 1H), 1.76 (d, $J$ = 11.6 Hz, 2H), 1.71 – 1.62 (m, 2H), 1.57-1.51 (m, 1H), 1.31 – 1.19 (m, 2H), 1.19 – 1.03 (m, 3H).$^{13}$C NMR (126 MHz, MeOD) $\delta$ 151.14, 136.46, 135.17, 129.95, 129.55, 129.44, 128.67, 128.24, 128.11, 109.02, 57.87, 35.05, 26.86, 25.99. MS (ESI) calcd for C₁₈H₂₁N₅, m/z 307.1797, found 308.1923 [M+H]$^+$. 

Compounds 5b-5d were synthesized similarly as compound 5a.

5b: 2-([1,1'-Biphenyl]-4-yl)-$N^3$-cyclohexylimidazo[1,2-a]pyrazine-3,8-diamine. 44 mg, 23%.

$^1$H NMR (500 MHz, MeOD) $\delta$ 8.13 (d, $J$ = 8.2 Hz, 2H), 7.72 (d, $J$ = 8.3 Hz, 2H), 7.71 – 7.67 (m, 2H), 7.64 (d, $J$ = 4.8 Hz, 1H), 7.45 (t, $J$ = 7.7 Hz, 2H), 7.38 – 7.31 (m, 1H), 7.22 (d, $J$ = 4.8 Hz, 1H), 3.02 – 2.84 (m, 1H), 1.80 (d, $J$ = 12.2 Hz, 2H), 1.69 (dd, $J$ = 8.2, 5.3 Hz, 2H), 1.55 (s, 1H), 1.35 – 1.23 (m, 2H), 1.24 – 1.01 (m, 3H).$^{13}$C NMR (126 MHz, MeOD) $\delta$ 151.16, 141.92, 141.53, 136.10, 134.18, 130.07, 129.93, 129.56, 128.57, 128.45, 128.13, 128.02, 127.86, 109.03, 57.99, 35.12, 26.88, 26.03. MS (ESI) calcd for C₂₄H₂₅N₅, m/z 383.2110, found 384.2382 [M+H]$^+$. 
5c: \(N^3\)-benzyl-2-butylimidazo[1,2-a]pyrazine-3,8-diamine. 10 mg, 7%. \(^1\)H NMR (500 MHz, MeOD) \(\delta\) 7.44 (d, \(J = 4.8\) Hz, 1H), 7.29 – 7.17 (m, 5H), 7.12 (d, \(J = 4.8\) Hz, 1H), 4.13 (s, 2H), 2.51 – 2.42 (m, 2H), 1.54 – 1.42 (m, 2H), 1.31 (dq, \(J = 14.8, 7.4\) Hz, 2H), 0.91 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (126 MHz, MeOD) \(\delta\) 150.52, 141.10, 138.98, 130.14, 129.57, 129.49, 128.81, 128.40, 127.74, 108.93, 53.28, 32.76, 27.26, 23.73, 14.26. MS (ESI) calcd for C\(_{17}\)H\(_{21}\)N\(_5\), m/z 295.1797, found 296.1952 [M+H]\(^+\).

5d: 2-Butyl-\(N^3\)-(4-methoxyphenyl)imidazo[1,2-a]pyrazine-3,8-diamine. 36 mg, 23%. \(^1\)H NMR (500 MHz, MeOD) \(\delta\) 7.26 (d, \(J = 4.7\) Hz, 1H), 7.16 (d, \(J = 4.7\) Hz, 1H), 6.80 – 6.69 (m, 2H), 6.49 – 6.38 (m, 2H), 3.70 (s, 3H), 2.67 (t, \(J = 7.6\) Hz, 2H), 1.73 – 1.62 (m, 2H), 1.41 – 1.23 (m, 2H), 0.88 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (126 MHz, MeOD) \(\delta\) 154.65, 150.75, 142.09, 140.98, 129.90, 128.28, 124.98, 115.97, 115.50, 109.10, 56.07, 32.40, 27.41, 23.46, 14.15. MS (ESI) calcd for C\(_{17}\)H\(_{21}\)N\(_5\)O, m/z 311.1746, found 312.1905 [M+H]\(^+\).

Synthesis of compound 6: \(N\)-(tert-butyl)-2-phenylimidazo[1,2-a]pyridin-3-amine. To a solution of 2-aminopyridine (24 mg, 0.25 mmol) in anhydrous acetonitrile, were added benzaldehyde (28 \(\mu\)L, 0.28 mmol), 4N HCl/dioxane (5 \(\mu\)L) and tert-butyl isonitrile (27 \(\mu\)L, 0.24 mmol). The reaction mixture was then heated under microwave conditions (400 W, 110 \(^\circ\)C) in a sealed vial for 20 min. After cooling the reaction mixture to room temperature, the solvents were removed and the residue was purified using column chromatography to obtain compound 6 (44 mg, 70%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.23 (dt, \(J = 6.9, 1.2\) Hz, 1H), 7.90 (dt, \(J = 8.1, 1.6\) Hz, 2H), 7.55 (dt, \(J = 9.0, 1.0\) Hz, 1H), 7.43 (t, \(J = 7.6\) Hz, 2H), 7.31 (t, \(J = 7.4\) Hz, 1H), 7.13 (ddd, \(J = 9.0, 6.6, 1.3\) Hz, 1H), 6.77 (td, \(J = 6.8, 1.1\) Hz, 1H), 3.12 (s, 1H), 1.04 (s, 9H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 142.15, 135.42, 135.40, 128.43, 128.32, 127.52, 124.17, 123.64, 117.48,
Compounds 7-29 were synthesized similarly as compound 6.

7: \textit{N-(tert-butyl)-2-phenylimidazo[1,2-a]pyrazin-3-amine}. 47 mg, 74\%. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.00 (d, $J = 1.4$ Hz, 1H), 8.14 (dd, $J = 4.6$, 1.5 Hz, 1H), 7.91 (dd, $J = 8.3$, 1.3 Hz, 2H), 7.86 (d, $J = 4.6$ Hz, 1H), 7.46 (t, $J = 7.5$ Hz, 2H), 7.37 (t, $J = 7.4$ Hz, 1H), 3.19 (s, 1H), 1.05 (s, 9H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 143.54, 142.40, 137.43, 134.38, 129.04, 128.66, 128.38, 128.33, 125.17, 116.49, 57.11, 30.45. MS (ESI) calcd for C$_{16}$H$_{18}$N$_4$, m/z 266.1531, found 267.1588 [M+H]$^+$.  

8: \textit{N-cyclohexyl-2-phenylimidazo[1,2-a]pyridin-3-amine}. 64 mg, 86\%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.16 (d, $J = 6.8$ Hz, 1H), 8.05 (dd, $J = 8.3$, 1.2 Hz, 2H), 7.62 (d, $J = 9.0$ Hz, 1H), 7.45 (t, $J = 7.8$ Hz, 2H), 7.32 (t, $J = 7.4$ Hz, 1H), 7.17 (ddd, $J = 8.9$, 6.7, 1.2 Hz, 1H), 6.82 (td, $J = 6.8$, 0.9 Hz, 1H), 3.32 (s, 1H), 3.07 – 2.85 (m, 1H), 1.81 (d, $J = 13.1$ Hz, 2H), 1.68 (dd, $J = 9.1$, 3.6 Hz, 2H), 1.60 – 1.54 (m, 1H), 1.28 – 1.12 (m, 5H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 141.02, 135.43, 133.45, 128.74, 127.74, 127.20, 125.17, 125.04, 123.07, 116.94, 112.30, 57.02, 34.27, 25.81, 24.94. MS (ESI) calcd for C$_{19}$H$_{21}$N$_3$, m/z 291.1735, found 292.1832 [M+H]$^+$.  

9: \textit{N-cyclohexyl-2-phenylimidazo[1,2-a]pyrazin-3-amine}. 25 mg, 36\%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.99 (d, $J = 1.4$ Hz, 1H), 8.01 (ddd, $J = 4.6$, 3.2, 1.8 Hz, 3H), 7.85 (d, $J = 4.6$ Hz, 1H), 7.48 (t, $J = 7.7$ Hz, 2H), 7.38 (t, $J = 7.4$ Hz, 1H), 3.26 (s, 1H), 3.00 (m, 1H), 2.22 (s, 1H), 1.82 (dd, $J = 6.6$, 5.4 Hz, 2H), 1.70 (dd, $J = 9.3$, 3.3 Hz, 2H), 1.62 – 1.55 (m, 1H), 1.34 – 1.07 (m, 5H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 143.37, 139.08, 136.82, 133.62, 129.01, 128.90, 128.30,
127.42, 126.70, 115.73, 57.02, 34.41, 25.69, 24.91. MS (ESI) calcd for C₁₈H₂₀N₄, m/z 292.1688, found 293.1775 [M+H]+.

10: N-benzyl-2-phenylimidazo[1,2-a]pyridin-3-amine. 62 mg, 86%. ¹H NMR (500 MHz, CDCl₃) δ 7.98 (ddt, J = 3.7, 3.0, 1.6 Hz, 3H), 7.57 (dt, J = 9.0, 1.0 Hz, 1H), 7.45 (t, J = 7.7 Hz, 2H), 7.39 – 7.26 (m, 6H), 7.13 (ddd, J = 9.0, 6.7, 1.3 Hz, 1H), 6.74 (td, J = 6.8, 1.1 Hz, 1H), 4.20 (d, J = 6.1 Hz, 2H), 3.52 (t, J = 6.0 Hz, 1H), 2.45 (s, 1H). ¹³C NMR (126 MHz, CDCl₃) δ 141.57, 139.06, 136.04, 134.13, 128.84, 128.30, 127.82, 127.65, 127.16, 125.77, 124.32, 122.50, 117.52, 111.94, 52.57. MS (ESI) calcd for C₂₀H₁₇N₃, m/z 299.1422, found 300.1490 [M+H]+.

11: N-benzyl-2-phenylimidazo[1,2-a]pyrazin-3-amine. 45 mg, 62%. ¹H NMR (500 MHz, CDCl₃) δ 8.98 (d, J = 1.3 Hz, 1H), 7.94 (dt, J = 8.1, 1.6 Hz, 2H), 7.82 (dd, J = 4.6, 1.4 Hz, 1H), 7.77 (d, J = 4.6 Hz, 1H), 7.47 (t, J = 7.6 Hz, 2H), 7.39 (t, J = 7.4 Hz, 1H), 7.34 – 7.27 (m, 5H), 4.23 (d, J = 2.4 Hz, 2H), 3.66 (s, 1H), 2.20 (s, 1H). ¹³C NMR (126 MHz, CDCl₃) δ 143.46, 138.76, 138.56, 136.79, 133.34, 129.06, 129.03, 128.99, 128.46, 128.24, 128.08, 127.42, 127.18, 115.38, 52.39. MS (ESI) calcd for C₁₉H₁₆N₄, m/z 300.1375, found 301.1494 [M+H]+.

12: N-(tert-butyl)-2-(pyridin-4-yl)imidazo[1,2-a]pyridin-3-amine. 50 mg, 78%. ¹H NMR (500 MHz, CDCl₃) δ 8.65 (d, J = 5.9 Hz, 2H), 8.20 (dt, J = 6.9, 1.0 Hz, 1H), 7.96 (dd, J = 4.7, 1.4 Hz, 2H), 7.56 (d, J = 9.1 Hz, 1H), 7.18 (ddd, J = 9.0, 6.6, 1.2 Hz, 1H), 6.81 (td, J = 6.8, 1.0 Hz, 1H), 3.07 (s, 1H), 1.10 (s, 9H). ¹³C NMR (126 MHz, CDCl₃) δ 149.69, 142.82, 142.39, 136.46, 124.86, 124.77, 123.38, 122.17, 117.68, 111.82, 56.78, 30.38. MS (ESI) calcd for C₁₆H₁₈N₄, m/z 266.1531, found 267.1747 [M+H]+.
13: *N*-(*tert*-butyl)-2-(pyridin-4-yl)imidazo[1,2-a]pyrazin-3-amine. 49 mg, 76%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.02 (d, $J = 1.4$ Hz, 1H), 8.69 (dd, $J = 4.5$, 1.6 Hz, 2H), 8.11 (dd, $J = 4.7$, 1.5 Hz, 1H), 7.94 (dd, $J = 4.5$, 1.6 Hz, 2H), 7.88 (d, $J = 4.7$ Hz, 1H), 3.12 (s, 1H), 1.10 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 150.22, 144.25, 141.96, 139.25, 137.72, 129.39, 126.24, 122.42, 116.42, 57.46, 30.61. MS (ESI) calcd for C$_{15}$H$_{17}$N$_5$, m/z 267.1484, found 268.1705 [M+H]+.

14: *N*-cyclohexyl-2-(pyridin-4-yl)imidazo[1,2-a]pyridin-3-amine. 69 mg, 98%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.64 (dd, $J = 4.6$, 1.6 Hz, 2H), 8.07 (dt, $J = 6.9$, 1.2 Hz, 1H), 8.00 (dd, $J = 4.6$, 1.6 Hz, 2H), 7.54 (dt, $J = 9.1$, 1.0 Hz, 1H), 7.17 (ddd, $J = 9.1$, 6.6, 1.3 Hz, 1H), 6.81 (td, $J = 6.8$, 1.1 Hz, 1H), 3.11 (d, $J = 3.9$ Hz, 1H), 2.98 (td, $J = 10.3$, 4.1 Hz, 1H), 1.83 (d, $J = 10.8$ Hz, 2H), 1.71 (dd, $J = 9.4$, 3.3 Hz, 2H), 1.59 (dd, $J = 7.3$, 1.4 Hz, 1H), 1.32 – 1.09 (m, 5H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 150.03, 142.26, 142.11, 133.78, 126.92, 124.92, 122.87, 121.21, 117.99, 112.31, 57.26, 34.42, 25.75, 24.97. MS (ESI) calcd for C$_{18}$H$_{20}$N$_4$, m/z 292.1688, found 293.1854 [M+H]+.

15: *N*-cyclohexyl-2-(pyridin-4-yl)imidazo[1,2-a]pyrazin-3-amine. 56 mg, 80%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.01 (d, $J = 1.5$ Hz, 1H), 8.70 (dd, $J = 4.5$, 1.6 Hz, 2H), 8.03 – 7.93 (m, 3H), 7.88 (d, $J = 4.6$ Hz, 1H), 3.21 (d, $J = 5.9$ Hz, 1H), 3.06 – 2.97 (m, 1H), 1.84 (d, $J = 10.5$ Hz, 2H), 1.72 (dd, $J = 9.7$, 2.6 Hz, 2H), 1.63 – 1.58 (m, 1H), 1.31 – 1.14 (m, 5H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 150.42, 144.32, 141.25, 137.17, 135.99, 129.45, 128.03, 121.37, 115.69, 57.35, 34.55, 25.62, 24.93. MS (ESI) calcd for C$_{17}$H$_{19}$N$_5$, m/z 293.1640, found 294.1894 [M+H]+.

16: *N*-benzyl-2-(pyridin-4-yl)imidazo[1,2-a]pyridin-3-amine. 54 mg, 75%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.62 (dd, $J = 4.6$, 1.6 Hz, 2H), 7.94 (dt, $J = 6.9$, 1.1 Hz, 1H), 7.90 (dd, $J = 4.6$, 1.6 Hz, 2H), 7.55 (dt, $J = 9.1$, 1.0 Hz, 1H), 7.34 – 7.27 (m, 5H), 7.17 (ddd, $J = 9.1$, 6.6, 1.3 Hz, 1H), 6.76
(td, \(J = 6.8, 1.1\) Hz, 1H), 4.22 (d, \(J = 6.1\) Hz, 2H), 3.52 (t, \(J = 6.1\) Hz, 1H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 150.12, 142.11, 141.94, 138.64, 133.41, 128.98, 128.31, 128.06, 127.46, 125.05, 122.50, 121.13, 118.06, 112.42, 52.64. MS (ESI) calcd for C\(_{19}\)H\(_{16}\)N\(_4\), \(m/z\) 300.1375, found 301.1599 [M+H]+.

17: \(N\)-benzyl-2-(pyridin-4-yl)imidazo[1,2-a]pyrazin-3-amine. 47 mg, 65%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 9.01 (d, \(J = 1.4\) Hz, 1H), 8.67 (dd, \(J = 4.5, 1.6\) Hz, 2H), 7.87 (dd, \(J = 4.5, 1.6\) Hz, 2H), 7.78 (dt, \(J = 4.6, 3.0\) Hz, 2H), 7.30 (dd, \(J = 5.1, 1.9\) Hz, 3H), 7.24 (dd, \(J = 6.9, 2.6\) Hz, 2H), 4.25 (d, \(J = 6.2\) Hz, 2H), 3.66 (t, \(J = 6.2\) Hz, 1H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 150.46, 144.33, 140.94, 138.23, 137.12, 135.70, 129.45, 129.14, 128.45, 128.32, 128.25, 121.34, 115.35, 52.59. MS (ESI) calcd for C\(_{18}\)H\(_{15}\)N\(_5\), \(m/z\) 301.1327, found 302.1474 [M+H]+.

18: 2-(3-(tert-butylamino)imidazo[1,2-a]pyridin-2-yl)phenol. 20 mg, 30%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 12.47 (bs, 1H), 8.19 (d, \(J = 6.9\) Hz, 1H), 8.12 (dd, \(J = 7.8, 1.6\) Hz, 1H), 7.51 (d, \(J = 9.0\) Hz, 1H), 7.24 – 7.17 (m, 2H), 7.07 – 6.97 (m, 1H), 6.95 – 6.87 (m, 1H), 6.83 (td, \(J = 6.8, 0.9\) Hz, 1H), 3.16 (bs, 1H), 1.16 (s, 9H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 157.07, 148.51, 140.76, 138.42, 134.51, 131.35, 129.43, 128.13, 124.82, 123.01, 118.69, 117.76, 116.96, 112.09, 109.94, 57.22, 30.50. MS (ESI) calcd for C\(_{18}\)H\(_{19}\)N\(_3\)O, \(m/z\) 281.1528, found 281.1528 [M+H]+.

19: 2-(3-(tert-butylamino)imidazo[1,2-a]pyrazin-2-yl)phenol. 40 mg, 59%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 11.87 (s, 1H), 8.98 (d, \(J = 1.4\) Hz, 1H), 8.16 – 8.08 (m, 2H), 7.92 (d, \(J = 4.6\) Hz, 1H), 7.31 – 7.24 (m, 1H), 7.05 (dd, \(J = 8.2, 1.0\) Hz, 1H), 6.93 (td, \(J = 7.9, 1.2\) Hz, 1H), 3.22 (bs, 1H), 1.17 (s, 9H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 157.08, 142.79, 140.85, 135.93, 130.39, 129.72, 128.31, 123.47, 119.03, 118.04, 117.65, 115.85, 57.73, 30.54. MS (ESI) calcd for C\(_{18}\)H\(_{18}\)N\(_4\)O, \(m/z\) 282.1481, found 283.1690 [M+H]+.
20: 2-(3-(Cyclohexylamino)imidazo[1,2-a]pyridin-2-yl)phenol. 30 mg, 41%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 13.16 (s, 1H), 8.17 (d, $J = 6.8$ Hz, 1H), 8.04 (dd, $J = 7.8$, 1.3 Hz, 1H), 7.50 (dt, $J = 9.0$, 0.9 Hz, 1H), 7.25 – 7.17 (m, 2H), 7.04 (dd, $J = 8.2$, 1.1 Hz, 1H), 6.95 – 6.89 (m, 1H), 6.87 (td, $J = 6.8$, 1.0 Hz, 1H), 3.17 – 2.99 (m, 2H), 1.83 (d, $J = 12.5$ Hz, 2H), 1.72 (dd, $J = 9.5$, 3.1 Hz, 2H), 1.64 – 1.57 (m, 1H), 1.32 (dd, $J = 22.7$, 11.7 Hz, 2H), 1.26 – 1.13 (m, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 157.67, 139.51, 136.00, 129.25, 126.41, 124.88, 123.55, 122.62, 118.82, 117.84, 117.40, 116.64, 112.46, 57.08, 34.19, 25.83, 24.96. MS (ESI) calcd for C$_{19}$H$_{21}$N$_3$O, m/z 307.1685, found 308.1995 [M+H]$^+$. 

21: 2-(3-(Cyclohexylamino)imidazo[1,2-a]pyrazin-2-yl)phenol. 54 mg, 73%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 12.52 (bs, 1H), 8.96 (d, $J = 1.4$ Hz, 1H), 8.05 (ddd, $J = 9.5$, 6.2, 1.5 Hz, 2H), 7.94 (d, $J = 4.6$ Hz, 1H), 7.30 – 7.26 (m, 1H), 7.07 (dd, $J = 8.2$, 1.1 Hz, 1H), 6.94 (td, $J = 8.0$, 1.2 Hz, 1H), 3.31 – 2.99 (m, 2H), 1.83 (d, $J = 12.3$ Hz, 2H), 1.77 – 1.70 (m, 2H), 1.62 (dd, $J = 7.5$, 2.3 Hz, 1H), 1.33 (dd, $J = 21.3$, 10.5 Hz, 2H), 1.27 – 1.15 (m, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 157.70, 142.41, 138.53, 134.72, 130.19, 129.97, 126.50, 125.10, 119.13, 118.09, 116.60, 115.35, 57.17, 34.34, 25.69, 24.92. MS (ESI) calcd for C$_{16}$H$_{20}$N$_4$O, m/z 308.1637, found 309.1856 [M+H]$^+$. 

22: 2-(3-(Benzylamino)imidazo[1,2-a]pyridin-2-yl)phenol. 10 mg, 13%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 13.02 (s, 1H), 8.06 – 7.95 (m, 2H), 7.51 (d, $J = 9.0$ Hz, 1H), 7.42 – 7.29 (m, 5H), 7.25 – 7.17 (m, 2H), 7.06 (dd, $J = 8.2$, 1.0 Hz, 1H), 6.95 – 6.89 (m, 1H), 6.80 (td, $J = 6.8$, 0.9 Hz, 1H), 4.24 (bs, 2H), 3.41 (s, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 157.75, 139.49, 138.91, 135.58, 129.38, 128.96, 128.43, 127.97, 125.95, 124.95, 124.41, 122.19, 119.04, 117.88, 117.21, 116.74, 112.51, 52.43. MS (ESI) calcd for C$_{20}$H$_{17}$N$_3$O, m/z 315.1372, found 316.1611 [M+H]$^+$. 


23: 2-(3-(Benzylamino)imidazo[1,2-a]pyrazin-2-yl)phenol. 43 mg, 57%. 
$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 12.40 (bs, 1H), 8.95 (d, $J = 1.4$ Hz, 1H), 8.02 (dd, $J = 7.9$, 1.6 Hz, 1H), 7.82 (d, $J = 4.6$ Hz, 1H), 7.78 (dd, $J = 4.6$, 1.4 Hz, 1H), 7.37 – 7.27 (m, 6H), 7.09 (dd, $J = 8.3$, 1.2 Hz, 1H), 6.94 (ddd, $J = 7.9$, 7.3, 1.2 Hz, 1H), 4.26 (d, $J = 5.1$ Hz, 2H), 3.53 (s, 1H). 
$^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 157.70, 142.41, 138.44, 138.07, 134.64, 130.35, 129.95, 129.12, 128.39, 128.24, 126.21, 125.76, 119.36, 118.13, 116.33, 114.97, 52.35. MS (ESI) calcd for C$_{19}$H$_{16}$N$_4$O, m/z 316.1324, found 317.1545 [M+H]$^+$. 

24: (2-(tert-butylamino)-7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol. 13 mg, 17%. 
$^1$H NMR (400 MHz, MeOD) $\delta$ 7.97 (d, $J = 4.1$ Hz, 1H), 7.87 (s, 1H), 7.59 (ddd, $J = 8.7$, 7.2, 1.8 Hz, 1H), 6.75 (ddd, $J = 7.0$, 5.2, 0.7 Hz, 1H), 6.67 (d, $J = 8.4$ Hz, 1H), 4.61 (s, 2H), 2.72 (s, 3H), 1.53 (s, 9H). 
$^{13}$C NMR (126 MHz, MeOD) $\delta$ 165.65, 159.92, 147.97, 143.43, 140.55, 139.89, 132.15, 131.86, 125.53, 115.57, 110.65, 96.72, 59.04, 55.33, 30.04, 13.08. MS (ESI) calcd for C$_{18}$H$_{22}$N$_4$O$_2$, m/z 326.1743, found 327.1782 [M+H]$^+$. 

25: (2-(tert-butylamino)-7-methyl-3-(pyrazin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol. 14 mg, 18%. 
$^1$H NMR (500 MHz, MeOD) $\delta$ 8.01 (d, $J = 1.1$ Hz, 1H), 7.88 (dd, $J = 2.8$, 1.4 Hz, 1H), 7.80 (s, 1H), 7.77 (d, $J = 2.8$ Hz, 1H), 4.56 (s, 2H), 2.65 (s, 3H), 1.43 (s, 9H). 
$^{13}$C NMR (126 MHz, MeOD) $\delta$ 165.60, 156.50, 143.30, 143.00, 140.79, 134.76, 134.39, 132.05, 131.74, 125.39, 95.43, 58.99, 55.50, 29.98, 12.92. MS (ESI) calcd for C$_{17}$H$_{21}$N$_5$O$_2$, m/z 327.1695, found 328.1836 [M+H]$^+$. 

26: (2-(Cyclohexylamino)-7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol. 19 mg, 22%. 
$^1$H NMR (500 MHz, MeOD) $\delta$ 8.07 (t, $J = 7.4$ Hz, 1H), 7.96 (s, 1H), 7.90 (d, $J = 5.9$ Hz, 1H), 7.29 (d, $J = 8.4$ Hz, 1H), 7.07 (t, $J = 6.7$ Hz, 1H), 4.64 (s, 2H), 3.91 – 3.79 (m, 1H), 2.75
(s, 3H), 2.05 (d, $J = 8.8$ Hz, 2H), 1.87 – 1.77 (m, 2H), 1.68 (d, $J = 13.1$ Hz, 1H), 1.48 – 1.33 (m, 4H), 1.21 (dd, $J = 12.1, 9.2$ Hz, 1H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 164.86, 155.98, 145.65, 143.70, 142.10, 137.34, 133.72, 132.85, 124.81, 115.77, 115.45, 89.79, 59.50, 54.25, 34.33, 26.28, 26.16, 12.97. MS (ESI) calcd for C$_{20}$H$_{24}$N$_{4}$O$_{2}$, $m/z$ 352.1899, found 353.2023 [M+H]$^+$. 

27: (2-(Cyclohexylamino)-7-methyl-3-(pyrazin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol. 24 mg, 28%. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.10 (s, 1H), 7.97 (d, $J = 1.4$ Hz, 1H), 7.86 (s, 2H), 4.64 (s, 2H), 3.86 – 3.78 (m, 1H), 2.71 (s, 3H), 2.03 – 1.96 (m, 2H), 1.80 (dd, $J = 5.3, 3.1$ Hz, 2H), 1.66 (d, $J = 13.0$ Hz, 1H), 1.38 (t, $J = 9.6$ Hz, 4H), 1.23 – 1.15 (m, 1H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 165.01, 156.57, 143.09, 142.98, 141.82, 134.49, 132.10, 131.50, 125.12, 94.07, 58.98, 53.94, 34.42, 26.31, 26.20, 12.80. MS (ESI) calcd for C$_{19}$H$_{23}$N$_{5}$O$_{2}$, $m/z$ 353.1852, found 354.1902 [M+H]$^+$. 

28: (2-(Benzylamino)-7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol. 13 mg, 15%. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.06 – 8.01 (m, 1H), 8.00 (s, 1H), 7.89 (d, $J = 5.9$ Hz, 1H), 7.44 – 7.39 (m, 2H), 7.34 (t, $J = 7.5$ Hz, 2H), 7.28 (t, $J = 7.3$ Hz, 1H), 7.23 (d, $J = 8.6$ Hz, 1H), 7.05 (t, $J = 6.7$ Hz, 1H), 4.76 (s, 2H), 4.65 (s, 2H), 2.77 (s, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 165.45, 156.13, 145.31, 143.75, 142.15, 138.61, 138.18, 133.67, 133.22, 130.23, 130.00, 129.91, 128.97, 128.77, 125.32, 115.82, 115.01, 90.66, 59.42, 47.33, 12.98. MS (ESI) calcd for C$_{21}$H$_{20}$N$_{4}$O$_{2}$, $m/z$ 360.1586, found 361.1830 [M+H]$^+$. 

29: (2-(Benzylamino)-7-methyl-3-(pyrazin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol. 15 mg, 16%. $^1$H NMR (500 MHz, MeOD) $\delta$ 7.99 (s, 1H), 7.94 (dd, $J = 2.8, 1.5$ Hz, 1H), 7.87 (s, 1H), 7.81 (d, $J = 2.8$ Hz, 1H), 7.37 – 7.32 (m, 2H), 7.29 (dd, $J = 10.3, 5.0$ Hz, 2H), 7.21 (t, $J = 7.3$ Hz, 1H), 4.64 (s, 2H), 4.61 (s, 2H), 2.61 (s, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 163.44, 156.59,
Synthesis of compound 34: (2-(Benzylationo)-7-methyl-3-(phenylamino)furo[2,3-c]pyridin-4-yl)methanol. To a solution of aniline (45 µL, 0.5 mmol) in anhydrous methanol, were added pyridoxal hydrochloride (112 mg, 0.55 mmol), 4N HCl/dioxane (10 µL) and benzyl isonitrile (68 µL, 0.55 mmol). The reaction mixture was then heated under microwave conditions (600 W, 80 °C) in a sealed vial for 2 min. After cooling the reaction mixture to room temperature, the solvents were removed and the residue was purified using column chromatography to obtain compound 34 (84 mg, 47%). ¹H NMR (500 MHz, MeOD) δ 7.88 (s, 1H), 7.34 (d, J = 7.8 Hz, 2H), 7.29 (t, J = 7.6 Hz, 2H), 7.21 (t, J = 7.2 Hz, 1H), 7.09 (t, J = 7.9 Hz, 2H), 6.66 (td, J = 7.3, 0.9 Hz, 1H), 6.58 – 6.51 (m, 2H), 4.57 (s, 2H), 4.52 (s, 2H), 2.54 (s, 3H). ¹³C NMR (126 MHz, MeOD) δ 160.73, 150.14, 141.35, 141.10, 138.40, 137.10, 130.29, 129.51, 128.35, 128.23, 119.02, 114.15, 94.89, 60.27, 47.02, 16.66. MS (ESI) calcd for C₂₂H₂₁N₃O₂, m/z 359.1634, found 360.1832 [M+H]⁺.

Synthesis of compound 35: 3-(Benzyloxy)-5-(hydroxymethyl)-2-methylisonicotin aldehyde. To a solution of pyridoxal hydrochloride (500 mg, 2.45 mmol) in anhydrous DMF, were added potassium carbonate (408 mg, 2.95 mmol), and benzyl bromide (0.35 mL, 2.95 mmol). The reaction mixture was stirred at room temperature for overnight. After the completion of reaction, water (20 mL) was added and the crude product obtained was extracted in ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulfate and evaporated. The residue was purified using column chromatography to obtain compound 35 (100 mg, 15%). ¹H NMR (500 MHz, CDCl₃) δ 8.05 (s, 1H), 7.45 – 7.32 (m, 5H), 6.64 (d, J = 1.7
Hz, 1H), 5.34 (d, J = 11.2 Hz, 1H), 5.22 (d, J = 12.7 Hz, 1H), 5.19 (d, J = 11.2 Hz, 1H), 4.99 (d, J = 12.7 Hz, 1H), 2.50 (s, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 150.88, 148.63, 136.84, 136.17, 135.53, 135.42, 128.80, 128.47, 127.74, 100.10, 74.13, 69.95, 19.38. MS (ESI) calcd for C$_{15}$H$_{16}$NO$_3$, m/z 257.1052, found 258.1279 [M+H]$^+$.  

**Synthesis of compound 36: (5-(Benzyloxy)-4-(3-(cyclohexylamino)imidazo[1,2-a]pyridin-2-yl)-6-methylpyridin-3-yl)methanol.** To a solution of 2-aminopyridine (24 mg, 0.25 mmol) in anhydrous acetonitrile, were added 3-(benzyl oxy)-5-(hydroxymethyl)-2-methylisonicotinaldehyde 34 (68 mg, 0.28 mmol), 4N HCl/dioxane (5 μL) and cyclohexylisonitrile (30 μL, 0.24 mmol). The reaction mixture was then heated under microwave conditions (400 W, 110 ºC) in a sealed vial for 20 min. After cooling the reaction mixture to room temperature, the solvents were removed and the residue was purified using column chromatography to obtain compound 36 (19 mg, 17%). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.41 (s, 1H), 8.07 (d, J = 6.9 Hz, 1H), 7.54 (d, J = 9.1 Hz, 1H), 7.26 – 7.15 (m, 4H), 7.02 – 6.97 (m, 2H), 6.90 (td, J = 6.8, 1.0 Hz, 1H), 4.59 (bs, 1H), 4.43 (s, 2H), 4.36 (bs, 1H), 3.88 (d, J = 7.9 Hz, 1H), 2.61 (bs, 1H), 2.58 (s, 3H), 1.85-1.75 (m, 1H), 1.70-1.65 (m, 1H), 1.50-1.40 (m, 2H), 1.37-1.28 (m, 1H), 1.22-1.05(s, 2H), 1.05-0.85 (m, 2H), 0.60 (bs, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 152.95, 150.53, 145.20, 141.29, 135.58, 129.62, 129.25, 128.76, 128.58, 128.57, 128.40, 126.28, 125.05, 123.16, 117.47, 112.53, 76.73, 61.64, 56.36, 34.32, 33.74, 25.60, 25.03, 24.53, 19.58. MS (ESI) calcd for C$_{27}$H$_{30}$N$_4$O$_2$, m/z 442.2369, found 443.2410 [M+H]$^+$.  

**Synthesis of compound 37a: (2-(Butylamino)-7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol.** To a solution of 2-aminopyridine (24 mg, 0.25 mmol) in anhydrous acetonitrile, were added pyridoxal hydrochloride (56 mg, 0.28 mmol), 4N HCl/dioxane (5 μL) and $n$-butyl isonitrile (25 μL, 0.24 mmol). The reaction mixture was then heated under microwave
conditions (600 W, 80 °C) in a sealed vial for 2 min. After cooling the reaction mixture to room temperature, solvents were removed and the residue was purified using column chromatography to obtain compound 37a (32 mg, 41%). $^1$H NMR (500 MHz, MeOD) $\delta$ 8.06 (t, $J = 8.0$ Hz, 1H), 7.96 (s, 1H), 7.90 (d, $J = 5.8$ Hz, 1H), 7.29 (d, $J = 8.5$ Hz, 1H), 7.07 (t, $J = 6.6$ Hz, 1H), 4.65 (s, 2H), 3.56 (t, $J = 7.1$ Hz, 2H), 2.75 (s, 3H), 1.75 – 1.61 (m, 2H), 1.49 – 1.35 (m, 2H), 0.96 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 165.68, 155.98, 145.61, 143.66, 142.08, 137.58, 133.67, 132.87, 124.84, 115.82, 115.31, 90.01, 59.50, 43.64, 32.79, 20.93, 13.99, 12.96. MS (ESI) calcd for C$_{18}$H$_{22}$N$_4$O$_2$, m/z 326.1743, found 327.1925 [M+H]$^+$. Compounds 37b-37m were synthesized similarly as compound 37a.

37b: (7-Methyl-2-(pentylamino)-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol. 27 mg, 33%. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.06 (t, $J = 8.0$ Hz, 1H), 7.96 (s, 1H), 7.91 (d, $J = 5.9$ Hz, 1H), 7.28 (d, $J = 8.5$ Hz, 1H), 7.07 (t, $J = 6.6$ Hz, 1H), 4.65 (s, 2H), 3.56 (t, $J = 7.1$ Hz, 2H), 2.75 (s, 3H), 1.75 – 1.64 (m, 2H), 1.38 (d, $J = 3.6$ Hz, 4H), 0.92 (t, $J = 6.9$ Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 165.68, 156.04, 145.55, 143.66, 142.09, 137.74, 133.66, 132.85, 124.85, 115.82, 115.25, 90.09, 59.49, 43.90, 30.42, 29.98, 23.31, 14.32, 12.95. MS (ESI) calcd for C$_{19}$H$_{24}$N$_4$O$_2$, m/z 340.1899, found 341.2058 [M+H]$^+$.  

37c: (2-(Isopropylamino)-7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol. 20 mg, 27%. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.05 (t, $J = 8.0$ Hz, 1H), 7.95 (s, 1H), 7.90 (d, $J = 5.9$ Hz, 1H), 7.26 (d, $J = 8.6$ Hz, 1H), 7.05 (t, $J = 6.7$ Hz, 1H), 4.63 (s, 2H), 4.25 (dt, $J = 13.0$, 6.5 Hz, 1H), 2.74 (s, 3H), 1.33 (d, $J = 6.5$ Hz, 6H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 164.96, 156.16, 145.41, 143.72, 142.06, 137.79, 133.63, 132.82, 124.86, 115.77, 115.24, 90.04, 59.48, 47.16, 23.12, 12.94. MS (ESI) calcd for C$_{17}$H$_{20}$N$_4$O$_2$, m/z 312.1586, found 313.1740 [M+H]$^+$.  

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37d: (7-Methyl-2-(pentan-2-ylamino)-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol. 34 mg, 42%. $^1$H NMR (500 MHz, MeOD) $\delta$ 7.99 (t, $J = 8.0$ Hz, 1H), 7.88 (s, 1H), 7.83 (d, $J = 5.2$ Hz, 1H), 7.20 (d, $J = 7.3$ Hz, 1H), 7.00 (t, $J = 6.7$ Hz, 1H), 4.56 (s, 2H), 4.03 (dq, $J = 12.9$, 6.5 Hz, 1H), 2.66 (s, 3H), 1.60 – 1.41 (m, 2H), 1.38 – 1.26 (m, 2H), 1.23 (d, $J = 6.6$ Hz, 3H), 0.86 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 165.14, 155.98, 145.72, 143.66, 142.17, 137.38, 133.77, 132.84, 124.79, 115.82, 115.41, 89.73, 59.52, 51.18, 39.97, 21.53, 20.53, 14.15, 12.94. MS (ESI) calcd for C$_{19}$H$_{24}$N$_4$O$_2$, m/z 340.1899, found 341.2077 [M+H]$^+$.  

37e: (7-Methyl-3-(pyridin-2-ylamino)-2-((2,4,4-trimethylpentan-2-yl)amino)furo[2,3-c]pyridin-4-yl)methanol. 33 mg, 36%. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.08 (t, $J = 8.0$ Hz, 1H), 7.99 (s, 1H), 7.90 (s, 1H), 7.27 (s, 1H), 7.08 (dd, $J = 7.0$, 6.4 Hz, 1H), 4.63 (s, 2H), 2.78 (s, 3H), 1.95 (d, $J = 12.9$ Hz, 2H), 1.60 (s, 6H), 1.00 (s, 9H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 165.37, 156.14, 145.96, 141.40, 137.05, 133.87, 132.93, 124.92, 115.77, 115.62, 90.82, 59.88, 59.51, 53.31, 32.62, 31.82, 30.73, 13.06. MS (ESI) calcd for C$_{22}$H$_{30}$N$_4$O$_2$, m/z 382.2369, found 383.2541 [M+H]$^+$.  

37f: (7-Methyl-3-(pyridin-2-ylamino)-2-(((trimethylsilyl)methyl)amino)furo[2,3-c]pyridin-4-yl)methanol. 24 mg, 28%. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.03 (t, $J = 8.0$ Hz, 1H), 7.91-7.90 (m, 2H), 7.24 (d, $J = 8.7$ Hz, 1H), 7.04 (t, $J = 6.6$ Hz, 1H), 4.62 (s, 2H), 3.11 (s, 2H), 2.72 (s, 3H), 0.12 (s, 9H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 165.66, 156.25, 145.27, 143.32, 142.02, 138.19, 133.57, 132.16, 124.28, 115.77, 115.00, 90.25, 59.52, 34.64, 12.94, -2.60. MS (ESI) calcd for C$_{18}$H$_{24}$N$_4$O$_2$Si, m/z 356.1669, found 357.1815 [M+H]$^+$.  

37g: (7-Methyl-2-((2-morpholinoethyl)amino)-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol. 9 mg, 10%. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.03 (s, 2H), 7.93 (d, $J = 5.8$ Hz, 1H), 7.27
(d, J = 7.3 Hz, 1H), 7.05 (t, J = 6.5 Hz, 1H), 4.66 (s, 2H), 4.06-3.95 (m, 6H), 3.54 (bs, 2H), 3.42 (bs, 4H), 2.80 (s, 3H). $^{13}$C NMR (126 MHz, MeOD) δ 165.51, 156.26, 145.10, 143.92, 141.95, 138.27, 134.09, 133.62, 126.01, 115.97, 115.07, 90.35, 64.80, 59.39, 57.37, 53.45, 37.84, 13.21. MS (ESI) calcd for C$_{20}$H$_{25}$N$_5$O$_3$, m/z 383.1957, found 384.2132 [M+H]$^+$. 

37h: Ethyl 2-((4-(hydroxymethyl)-7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-2-yl)amino)acetate. 21 mg, 25%. $^1$H NMR (500 MHz, MeOD) δ 8.07 (t, J = 8.0 Hz, 1H), 8.03 (s, 1H), 7.91 (d, J = 6.1 Hz, 1H), 7.29 (d, J = 8.9 Hz, 1H), 7.08 (t, J = 6.7 Hz, 1H), 4.66 (s, 2H), 4.36 (s, 2H), 4.18 (q, J = 7.1 Hz, 2H), 2.74 (s, 3H), 1.25 (t, J = 7.1 Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) δ 170.68, 165.42, 155.75, 145.81, 143.87, 142.15, 137.71, 133.94, 133.89, 125.93, 115.98, 115.25, 91.02, 62.95, 59.42, 44.55, 14.48, 13.03. MS (ESI) calcd for C$_{18}$H$_{20}$N$_4$O$_4$, m/z 356.1485, found 357.1738 [M+H]$^+$. 

37i: tert-Butyl 3-((4-(hydroxymethyl)-7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-2-yl)amino)propanoate. 20 mg, 21%. $^1$H NMR (500 MHz, MeOD) δ 8.08 (t, J = 8.0 Hz, 1H), 8.00 (s, 1H), 7.91 (d, J = 5.8 Hz, 1H), 7.29 (d, J = 7.8 Hz, 1H), 7.08 (t, J = 6.7 Hz, 1H), 4.66 (s, 2H), 3.80 (t, J = 6.4 Hz, 2H), 2.78 (s, 3H), 2.67 (t, J = 6.7 Hz, 2H), 1.42 (s, 9H). $^{13}$C NMR (126 MHz, MeOD) δ 171.99, 165.49, 155.86, 145.77, 143.76, 142.08, 137.49, 133.79, 133.30, 125.17, 115.86, 115.36, 90.25, 82.28, 59.49, 39.67, 36.03, 28.31, 13.02. MS (ESI) calcd for C$_{21}$H$_{26}$N$_4$O$_4$, m/z 398.1954, found 399.2128 [M+H]$^+$. 

37j: Diethyl (((4-(hydroxymethyl)-7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-2-yl)amino)methyl)phosphonate. 13 mg, 13%. $^1$H NMR (500 MHz, MeOD) δ 8.08 – 8.00 (m, 2H), 7.95 (d, J = 5.7 Hz, 1H), 7.23 (d, J = 4.9 Hz, 1H), 7.06 (t, J = 6.5 Hz, 1H), 4.69 (s, 2H), 4.24 – 4.16 (m, 4H), 4.09 (d, J = 10.6 Hz, 2H), 2.81 (s, 3H), 1.33 (t, J = 7.0 Hz, 6H). $^{13}$C NMR (126
MHz, MeOD) δ 164.98, 156.41, 145.04, 143.68, 142.06, 139.12, 133.83, 133.71, 125.96, 115.97, 114.67, 91.93, 64.56 (d, J = 6.9 Hz), 59.37, 39.02 (d, J = 158.9 Hz), 16.78 (d, J = 5.6 Hz), 13.09. MS (ESI) calcd for C_{19}H_{25}N_{4}O_{5}P, m/z 420.1563, found 421.1672 [M+H]^+.

37k: (2-((4-Methoxyphenyl)amino)-7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl) methanol. 13 mg, 14%. ¹H NMR (500 MHz, MeOD) δ 7.99 – 7.87 (m, 2H), 7.62 (t, J = 7.8 Hz, 1H), 7.33 (d, J = 8.8 Hz, 2H), 6.90 (d, J = 8.8 Hz, 2H), 6.77 (t, J = 6.3 Hz, 2H), 4.66 (s, 2H), 3.77 (s, 3H), 2.71 (s, 3H). ¹³C NMR (126 MHz, MeOD) δ 162.79, 159.21, 158.92, 146.57, 143.34, 141.31, 140.61, 132.84, 132.22, 130.80, 126.51, 124.33, 115.64, 115.43, 111.46, 96.53, 62.30, 58.97, 55.97, 13.06. MS (ESI) calcd for C_{21}H_{20}N_{4}O_{3}, m/z 376.1535, found 377.1694 [M+H]^+.

37l: (2-((2-Chloro-6-methylphenyl)amino)-7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl) methanol. 27 mg, 28%. ¹H NMR (500 MHz, MeOD) δ 8.10 (s, 1H), 7.90 (dd, J = 20.4, 6.6 Hz, 2H), 7.28 – 7.15 (m, 3H), 7.03 (t, J = 6.6 Hz, 1H), 6.95 (s, 1H), 4.70 (s, 2H), 2.75 (s, 3H), 2.32 (s, 3H). ¹³C NMR (126 MHz, MeOD) δ 162.57, 155.17, 145.80, 144.21, 142.68, 139.93, 136.98, 134.45, 133.96, 133.78, 132.14, 130.74, 128.65, 126.80, 115.93, 114.82, 91.45, 59.30, 18.71, 13.11. MS (ESI) calcd for C_{21}H_{19}ClN_{4}O_{2}, m/z 394.1197, found 395.1349 [M+H]^+.

37m: (S)-(7-methyl-2-((1-phenylethyl)amino)-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl) methanol. 28 mg, 31%. ¹H NMR (500 MHz, MeOD) δ 7.92 (s, 1H), 7.89 (t, J = 8.0 Hz, 1H), 7.90 – 7.81 (m, 1H), 7.38 (d, J = 7.6 Hz, 2H), 7.29 (t, J = 7.4 Hz, 2H), 7.21 (t, J = 7.2 Hz, 1H), 7.05 (d, J = 6.1 Hz, 1H), 6.94 (t, J = 6.4 Hz, 1H), 5.20 (q, J = 6.8 Hz, 1H), 4.58 (s, 2H), 2.68 (s, 3H), 1.60 (d, J = 6.9 Hz, 3H). ¹³C NMR (126 MHz, MeOD) δ 164.81, 157.13, 144.72, 143.97, 143.61, 142.11, 140.61, 133.20, 132.80, 129.92, 128.71, 126.98, 125.52, 115.70, 113.93, 91.91, 59.24, 54.49, 23.31, 12.88. MS (ESI) calcd for C_{22}H_{22}N_{4}O_{2}, m/z 374.1743, found 375.1931 [M+H]^+.
Synthesis of compound 37n: ((2-Amino-7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridin-4-yl)methanol. The solution of compound 37e (95 mg, 0.25 mmol) in TFA/CH₂Cl₂ (50%, 4 mL) was stirred at room temperature for 6 h. The solvents were removed and the residue obtained was column purified to afford compound 37n (56 mg, 84%). ¹H NMR (500 MHz, MeOD) δ 7.91 – 7.86 (m, 2H), 7.81 (t, J = 7.9 Hz, 1H), 6.96 (d, J = 8.6 Hz, 1H), 6.87 (t, J = 6.5 Hz, 1H), 4.61 (s, 2H), 2.66 (s, 3H). ¹³C NMR (126 MHz, MeOD) δ 167.39, 157.68, 143.36, 143.14, 142.12, 132.82, 132.44, 125.47, 115.59, 113.10, 92.54, 59.18, 12.80. MS (ESI) calcd for C₁₄H₁₄N₄O₂, m/z 270.1117, found 271.1310 [M+H]+.

Compounds 38a-38c were synthesized similarly as compound 34.

38a: (7-Methyl-2-(pentylamino)-3-(phenylamino)furo[2,3-c]pyridin-4-yl)methanol. 117 mg, 70%. ¹H NMR (500 MHz, MeOD) δ 7.85 (s, 1H), 7.14 (dd, J = 8.6, 7.4 Hz, 2H), 6.71 (tt, J = 7.4, 1.0 Hz, 1H), 6.60 (dd, J = 8.6, 1.0 Hz, 2H), 4.65 – 4.59 (m, 2H), 3.51 (t, J = 7.1 Hz, 2H), 2.68 (s, 3H), 1.72 – 1.57 (m, 2H), 1.41 – 1.28 (m, 4H), 0.95 – 0.84 (m, 3H). ¹³C NMR (126 MHz, MeOD) δ 165.54, 149.56, 143.22, 141.35, 133.46, 132.44, 130.44, 125.33, 119.45, 114.08, 96.74, 59.31, 43.42, 31.00, 29.94, 23.33, 14.34, 13.48. MS (ESI) calcd for C₂₀H₂₅N₃O₂, m/z 339.1947, found 340.2087 [M+H]+.

38b: (3-((3-Fluorophenyl)amino)-7-methyl-2-(pentylamino)furo[2,3-c]pyridin-4-yl)methanol. 70 mg, 82%. ¹H NMR (500 MHz, MeOD) δ 7.81 (s, 1H), 7.06 (td, J = 8.2, 6.7 Hz, 1H), 6.42 – 6.32 (m, 2H), 6.23 (d, J = 11.5 Hz, 1H), 4.60 (s, 2H), 3.46 (t, J = 7.1 Hz, 2H), 2.65 (s, 3H), 1.59 (p, J = 7.1 Hz, 2H), 1.31 – 1.23 (m, 4H), 0.83 (t, J = 7.0 Hz, 3H). ¹³C NMR (126 MHz, MeOD) 166.34, 165.62 (d, J = 242.1 Hz), 151.55 (d, J = 10.4 Hz), 142.79, 142.04, 131.84 (d, J = 10.1 Hz), 131.65, 131.28, 125.42, 110.01, 105.58 (d, J = 21.8 Hz), 100.74 (d, J = 25.8
Hz), 96.47, 58.95, 43.43, 30.83, 29.93, 23.33, 14.35, 12.79. MS (ESI) calcd for C$_{20}$H$_{24}$FN$_3$O$_2$, m/z 357.1853, found 358.2013 [M+H]$^+$.  

38c: (7-Methyl-3-((3-nitrophenyl)amino)-2-(pentylamino)furo[2,3-c]pyridin-4-yl)methanol.  
34 mg, 37%. $^1$H NMR (500 MHz, MeOD) δ 7.90 (s, 1H), 7.49 (dd, J = 8.1, 2.2 Hz, 1H), 7.34 (s, 1H), 7.31 (t, J = 8.1 Hz, 1H), 6.95 (d, J = 8.0 Hz, 1H), 4.54 (s, 2H), 3.41 (t, J = 7.0 Hz, 2H), 2.56 (s, 3H), 1.65 – 1.52 (m, 2H), 1.30 (dd, J = 8.5, 4.8 Hz, 4H), 0.86 (t, J = 6.5 Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) δ 160.86, 151.59, 150.89, 144.93, 141.84, 138.47, 136.71, 131.12, 124.51, 120.06, 113.05, 107.81, 92.23, 60.13, 43.34, 31.50, 30.02, 23.40, 16.73, 14.35. MS (ESI) calcd for C$_{20}$H$_{24}$N$_4$O$_4$, m/z 384.1798, found 385.1950 [M+H]$^+$.  

Synthesis of compound 39: 2,2'-(Hexane-1,6-diylbis(azanediyl))bis(7-methyl-3-(pyridin-2-ylamino)furo[2,3-c]pyridine-4,2-diyl))dimethanol. To a solution of 2-aminopyridine (48 mg, 0.50 mmol) in anhydrous acetonitrile, were added pyridoxal hydrochloride (112 mg, 0.56 mmol), 4N HCl/dioxane (10 μL) and 1,6-diisocyanohexane (36 μL, 0.24 mmol). The reaction mixture was then heated under microwave conditions (600 W, 80 °C) in a sealed vial for 2 min. After cooling the reaction mixture to room temperature, solvent was removed and the residue was purified using column chromatography to obtain compound 39 (21 mg, 7%). $^1$H NMR (500 MHz, MeOD) δ 8.07 (t, J = 8.0 Hz, 2H), 7.97 (s, 2H), 7.90 (d, J = 5.6 Hz, 2H), 7.34 (d, J = 5.6 Hz, 2H), 7.07 (t, J = 6.7 Hz, 2H), 4.64 (s, 4H), 3.57 (t, J = 7.0 Hz, 4H), 2.75 (s, 6H), 1.78 – 1.65 (m, 4H), 1.46 (s, 4H). $^{13}$C NMR (126 MHz, MeOD) δ 165.67, 155.95, 145.66, 143.71, 142.10, 137.48, 133.70, 132.91, 124.87, 115.83, 115.43, 90.05, 59.50, 43.86, 30.65, 27.48, 13.02. MS (ESI) calcd for C$_{34}$H$_{38}$N$_8$O$_4$, m/z 622.3016, found 623.3187 [M+H]$^+$.  

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X-ray crystallographic structural studies of 20, 28, and 37e. Crystals for compound 28 utilize the non-centrosymmetric triclinic space group P1-C1 with eight crystallographically-independent molecules in the asymmetric unit. They also invariably form multiply-twinned bundles. After many unsuccessful attempts, a multi-domain specimen of 28 was cut from one of these bundles that gave a set of diffracted intensities that permitted a crystal structure solution but not a satisfactory refinement. Full sets of unique diffracted intensities were measured for single-domain specimens of compounds 20 and 37e using monochromated CuKα radiation (λ = 1.54178 Å) on a Bruker Proteum Single Crystal Diffraction System equipped with Helios multilayer optics, an APEX II CCD detector and a Bruker MicroStar microfocus rotating anode x-ray source operating at 45kV and 60mA. Diffracted intensities were obtained with the Bruker program SAINT and the structures were solved using “direct methods” techniques incorporated into the Bruker SHELXTL Version 2010.3-0 software package. All stages of weighted full-matrix least-squares refinement were performed using the SHELXTL software with F₂ data. The final structural model for compounds 20 and 37e incorporated anisotropic thermal parameters for all non-hydrogen atoms and isotropic thermal parameters for all hydrogen atoms. The respective asymmetric units consist of four molecules of 20; one molecule of 37e. All hydrogen atoms for 37e and hydrogen atoms of 20 that are bonded to amine nitrogens (one in each molecule) were located in a difference Fourier map and included in the structural model as independent isotropic atoms whose parameters were allowed to vary in least-squares refinement cycles. All hydroxyl hydrogens for 20 were placed at idealized sp³-hybridized positions with an O-H bond length of 0.84 Å; the hydroxyl group was then allowed to rotate about its O-C bond during least squares refinement cycles. The remaining hydrogen atoms for 20 were included in the structural model as idealized atoms (assuming sp²- or sp³-hybridization of the carbon or nitrogen atoms and C-H bond lengths of 0.95 to 1.00 Å or N-H bond lengths of 0.88 Å). The isotropic thermal parameters of all idealized hydrogen atoms for 20 were fixed at values 1.2 (nonmethyl) or 1.5
(methyl) times the equivalent isotropic thermal parameter of the carbon, nitrogen or oxygen atom to which they are covalently bonded.

**Human TLR-3/-4/-5/-7/-8/-9 reporter gene assays (NF-κB induction).** As described in Chapter 2.

**Immunoassays for cytokines.** As described in Chapter 2.

**Rabbit immunization and antigen-specific ELISA.** As described in Chapter 2.

**Transcriptomal profiling in human PBMCs.** Detailed procedures for transcriptomal profiling have been described by us previously.\(^3^0\) Briefly, fresh human PBMC samples were stimulated with 10 \(\mu\)g/mL of 37b and 37f for two hours, and total RNA was extracted from treated and negative control blood samples with QIAamp RNA Blood Mini Kit (Qiagen). Subsequently, 160 ng of each of the RNA samples was used. The Human Genome GeneChip U133 plus 2.0 oligonucleotide array (Affymetrix, Santa Clara, CA) was employed. Established standard protocols at the KU Genomics Facility were performed on cRNA target preparation, array hybridization, washing, staining and image scanning. The microarray data was first subjected to quality assessment using the Affymetrix GeneChip Operating Software (GCOS). QC criteria included low background, low noise, detection of positive controls, and a 5’/3’ ratio of < 3.0. To facilitate direct comparison of gene expression data between different samples, the GeneChip data were first subjected to preprocessing. This step involved scaling (in GCOS) data from all chips to a target intensity value of 500, and further normalizations steps in GeneSpring GX (Agilent Technologies, Santa Clara, CA). Prior to identifying target genes, genes that were detected as non-expressed in all samples, i.e., those with absence calls, were filtered out. To identify genes whose expression was changed by our compounds, a fold change threshold of 2.0 between the compound treatment and the negative control was used.
Chapter 4.

Antibacterial activities of Groebke-Blackburn-Bienaymé-derived imidazo[1,2-\textit{a}]pyridin-3-amines

\[ \text{MIC: 3.91} \, \mu\text{g/mL (MRSA)} \]
4.1. Introduction

The introduction of antibiotics into the therapeutic armamentarium in the early 20th century revolutionized the management of microbial infections. Once considered ‘wonder drugs’, antibiotics have perhaps become victims of their own success, and resistance to these drugs has almost invariably followed on the heels of their widespread use (and misuse). The incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) infections continues to increase alarmingly not only in hospital-associated settings (nosocomial infections), but in recent times, also in community settings in the United States, and throughout the globe. The increase in morbidity and mortality due to *S. aureus* infections is a reflection of increased invasive procedures, indwelling devices, older age, and comorbidities, as well as the acquisition of resistance to commonly-used antimicrobial agents. Of particular concern is the emergence of multidrug-resistant strains of Gram-positive bacteria, with the loss of susceptibility to a wide range of reserve antibiotics such as vancomycin. The need for the development of effective antibiotics is urgent, especially in the face of a diminishing pipeline of drugs for antimicrobial chemotherapy.

As mentioned in Chapter 3, some of the initial compounds that were synthesized using the one-pot multicomponent Groebke-Blackburn-Bienaymé reaction with the aim to explore new TLR7/8-modulatory chemotype were proved to be neither agonistic nor antagonistic at TLR7/8 but found to have antibacterial activity against *S. aureus* with a clear indication of possible SARs. Subsequent focused libraries of compounds were synthesized and these latter compounds, too, were not active in TLR7 and TLR8 screens, but displayed prominent bacteriostatic activity against several Gram-positive bacteria, including MRSA. To investigate the mechanism of antibacterial activity of this new chemotype, a resistant strain of *S. aureus* was generated by serially passaging the organism in escalating doses of the most active analogue. A comparison
of minimum inhibitory concentrations (MICs) of known bacteriostatic agents in wild-type and resistant strains indicates a novel mechanism of action. These findings served as a point of departure for further exploration of SAR and mechanisms of bacteriostatic activity in this chemotype.

4.2. Results and Discussion

Our initial test-library comprising of twenty-four compounds was synthesized (Scheme 1) using two amidines (2-aminopyridine and 2-aminopyrazine), three isonitriles (2-isocyano-2-methylpropane, isocyanocyclohexane, (isocyanomethyl)benzene), and four aldehydes (benzaldehyde, 2-phenylacetaldehyde, 1-naphthaldehyde, anthracene-9-carbaldehyde). The syntheses of 1a-3h (Scheme 1) proceeded smoothly. All compounds were tested in TLR7 and TLR8 agonism and antagonism assays using specific reporter gene-based cellular assays as described earlier.58, 77 To our disappointment, none of the compounds displayed any activity in these assays up to concentrations of 250 μM (data not shown). The assay plates were stored in the autoclave room at room temperature prior to disposal, and, quite by accident, we observed a dose-dependent inhibition of a bacterial contaminant in such plates. We therefore decided to examine the antibacterial activities of these compounds in antibacterial screens (E. coli ATCC 9637 and S. aureus ATCC 13709) routinely employed in our laboratory.84 Four compounds were identified to be inhibitory to S. aureus ATCC 13709 but not E. coli ATCC 9637. Maximal antibacterial activity resided in compounds derived from 2-aminopyridine, bearing either a bulky N-tert-butyl, or cyclohexyl groups at C3, and a large aromatic pendant group (naphthyl or anthracenyl) at C2 (1e, 1g, 2e, 2g). The MICs of both 1g and 2g for S. aureus ATCC 13709 and
MRSA ATCC 33591 were 3.91 \( \mu g/mL \) (Table 1), and 7.81 \( \mu g/mL \) for coagulase-negative \( S. \) epidermidis ATCC 35983 (data not shown).

**Scheme 1.** Synthesis of a library of compounds using Groebke multicomponent reaction.

Reagents and conditions: MW, 400W, 110 °C, 20 min, HCl/dioxane, CH₃CN.
Fig. 1. Comparison of antimicrobial susceptibility of wild-type S. aureus ATCC 13709 (hatched bars) and 5e-resistant organism.
The active compounds listed above were all determined to be bacteriostatic rather than bactericidal by conventional microplate MBC assays.\textsuperscript{85} The outer membrane of Gram-negative bacteria serves as a permeability barrier for hydrophobic antimicrobials,\textsuperscript{86} and we wondered if the lack of activity of these compounds against Gram-negative bacteria could be attributable to its bulky, nonpolar nature. We therefore performed additional assays using polymyxin B nonapeptide,\textsuperscript{87} which is commonly used to permeabilize the outer-membranes of Gram-negative bacteria, rendering the organisms susceptible to otherwise impermeable antimicrobials;\textsuperscript{88} however, the imidazopyridines did not exert any significant antibacterial effect even at high concentrations of polymyxin B nonapeptide (Fig. 1), confirming that the antibacterial spectrum of these compounds is specific to Gram-positive organisms.

Given that $1g$ and $2g$ demonstrated identical potencies, we arbitrarily selected isocyanocyclohexane and anthracene-9-carbaldehyde as the invariant components, and varied the amidine component; we chose 2-aminopyrimidine, 2,3-diaminopyridine, and 2-amino-3-chloropyrazine (Scheme 2). The 2-amino-3-chloropyrazine-derived compound $4c$ was inactive, while $4a$ and $4b$ exhibited lower activities than $1g$ (Table 1).

\textit{Scheme 2.} Groebke reaction using different amidines.

\begin{center}
\begin{tikzpicture}[scale=0.8]
\node at (0,0) {\includegraphics[width=\textwidth]{scheme2.png}};
\end{tikzpicture}
\end{center}

\textbf{Reagents and conditions:} i. MW, 400 W, 110 °C, 30 min, HCl/dioxane, CH$_3$CN.
Next, we varied the isonitrile component. We explored nine different isonitriles (Scheme 3) chosen to include linear aliphatic (as in 5a and 5b), branched aliphatic (5c-e), silyl-containing (5f), adamantyl (5g), and aromatic substituents (5h and 5i). We observed that the \( N-2,4,4 \)-trimethylpentan-2-yl group of 5e, could be dealkylated under strongly acidic conditions as has been reported recently,\(^7\) affording the possibility of introducing alkyl or acyl substituents (represented by 7a-c, Scheme 4) that were not accessible through commercially available isonitriles. Optimal activity profiles appeared to correspond to compounds with branched-chain substituents (5d and 5e), while compounds with aromatic substituents (5h and 5i) were bereft of antibacterial activity (Table 1).

**Scheme 3.** Groebke reaction using various isonitriles.

\[
\begin{align*}
\text{Reagents and conditions: } & \text{i. MW, 400 W, 110 ^\circ C, 20 min, HCl/dioxane, CH}_3\text{CN.}
\end{align*}
\]
Scheme 4. Derivatization of the C3-amine.

\[
\begin{align*}
\text{5e} & \xrightarrow{i} \text{6} \xrightarrow{\text{ii or iii}} \text{7a: } R_1 = \text{C}_9\text{H}_{17}; R_2 = \text{H} \\
\text{7b: } R_1 = R_2 = \text{C}_9\text{H}_{17} \\
\text{7c: } R_1 = \text{COCl}_3; R_2 = \text{H}
\end{align*}
\]

Reagents and conditions: i. HCl/dioxane; ii. For 7a and 7b, C_9H_{15}CHO, CH_3COOH, NaCNBH_3, MeOH; iii. For 7c, (CF_3CO)_2O, CH_2Cl_2.

Noting that bulky, aldehyde-derived aromatic substituents at C2 corresponded to maximal activity (exemplified by the anthracenyl group in 1g, 2g, and 5a-g), it was of interest to explore other similar functional groups. Compounds with biphenyl (8a), phenanthrenyl (8b), and pyrenyl (8c) substituents were therefore synthesized and evaluated (Scheme 5). Compounds 8b and 8c exhibited lower activity than the anthracenyl-bearing compound, while the biphenyl compound 8a was inactive, pointing to the necessity of a large polycyclic, aromatic group at C2.

Scheme 5. Groebke reaction using various aldehydes.

\[
\begin{align*}
\text{8a-8c} & \xrightarrow{i} \text{N}_{\text{H}} \text{R} \\
\text{R} &= \text{8a, 8b, 8c}
\end{align*}
\]

Reagents and conditions: i. MW, 400W, 110 °C, 20 min, HCl/dioxane, CH_3CN.
The compound that showed maximal activity thus far was the very lipophilic 5e, and it was desirable to explore more polar analogues for future evaluation in vivo models. We therefore attempted to introduce \( N,N \)-dimethylaminopropylamino groups on both the amidine- and aldehyde-derived portions of 5e. This was achieved in a straightforward manner using appropriate halo-substituted components, followed by Buchwald-Hartwig coupling as depicted in Scheme 6. The antibacterial activities of 10 and 12 were found to be identical to that of 5e (Table 1).

**Scheme 6. Synthesis of polar derivatives of 5e.**

---

**Reagents and conditions:** i. MW, 400W, 110 °C, 30 min, HCl/dioxane, CH\(_3\)CN; ii. K(OrBu), Pd\(_2\)(dba)\(_3\), DavePhos, NH\(_2\)(CH\(_2\))\(_3\)N(CH\(_3\))\(_2\), dioxane, 80 °C.

Although the lead compounds of this chemotype exhibit narrow-spectrum bacteriostatic activity against Gram-positive organisms, the substantial potency against MRSA warranted an attempt at understanding the mechanism of action. Our preliminary studies have been to examine the
antibiograms of wild-type and 5e-resistant S. aureus organisms, comparing a variety of antimicrobials with known mechanisms of action. 5e-resistant S. aureus organisms were generated by exposing the bacterium to escalating doses of the compound. Within about 10 serial passages, organisms that withstood 5e up to concentrations of 100 µg/mL emerged. The MICs of various classes of bacteriostatic and bactericidal antibiotics were found to be identical within experimental error for both the wild-type and 5e-resistant S. aureus, suggesting that the molecular target of 5e and related compounds were distinct and unique (Fig. 2).

Fig. 2. Comparison of antimicrobial susceptibility of wild-type S. aureus ATCC 13709 (hatched bars) and 5e-resistant organism (solid bars) to a range of bacteriostatic antibiotics with known mechanisms of action, and 5e. Bacteriostatics include trimethoprim and sulfamethoxazole (dihydrofolate reductase pathway), doxycycline and amikacin (30S ribosomal subunit), chloramphenicol (23S ribosomal subunit), erythromycin and tylosin (50S ribosomal subunit), nisin (lipid II), nitrofurantoin (bacterial DNA); also included were the bactericidal controls, ciprofloxacin, amoxicillin, and cefotaxime.
We noticed, however, that upon prolonged storage (~ 2 weeks), aqueous solutions of hydrochloride salts of both 10 and 12 were degrading gradually, giving rise to N-dealkylated products as was discussed earlier. Furthermore, we were desirous of introducing a functional group that would permit facile coupling of probes such as fluorophores or biotin in order to identify the possible molecular target(s) of 5e. We elected to replace the N-2,4,4-trimethyl pentan-2-yl group with the more stable tert-butyl group, and the N,N-dimethylaminopropylamino group with a 1,8-diaminooctane, using the Buchwald-Hartwig coupling strategy employed earlier (Scheme 7). The introduction of the primary amine-bearing 1,8-diaminooctanyl group on either the anthracenyl or pyridinyl portions of the molecule did not result in significant attenuation of antibacterial activity relative to 1g (Table 1), allowing the possibility of exploring the binding partners for these compounds.

**Scheme 7. Synthesis of 1g derivatives bearing terminal primary amines.**

![Scheme 7](image-url)
Table 1. Minimum Inhibitory Concentration values (µg/mL) of compounds.\textsuperscript{a}

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<th>Compound Number</th>
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Table 1. (continued)

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Table 1. (continued)

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*ND denotes no activity detected at 100 μg/mL and NT denotes not tested.
4.3. Conclusions

Our attempts at exploring imidazo[1,2-a]pyridin-3-amines for TLR7 (or 8)-modulatory activities have unexpectedly led to the identification of a novel chemotype with substantial bacteriostatic activity against Gram-positive bacteria, including methicillin-resistant *S. aureus* (MRSA). Our preliminary results suggest that the mechanism of action may be distinct from known bacteriostatics. Structure-activity relationship studies have led to the identification of positions on the scaffold for additional structural modifications that should allow for the introduction of probes designed to examine cognate binding partners and molecular targets, while not significantly compromising antibacterial potency.

4.4. Experimental

**Chemistry.** All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 °C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf ‘Gold’ high performance silica columns on CombiFlash Rf instrument unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel CCM pre-coated aluminum sheets. Microwave reactions were done in Synthos 3000 instrument (Anton Paar). IR spectra were recorded on Shimadzu 8400 series FTIR instrument and values are reported in cm⁻¹. Purity for all final compounds was confirmed to be greater than 95% by LC–MS using a Zorbax Eclipse Plus 4.6 × 150 mm, 5 μm analytical reverse phase C18 column with H₂O–isopropanol or H₂O–CH₃CN gradients and an Agilent ESI-TOF mass spectrometer.
accuracy of 3 ppm) operating in the positive ion acquisition mode. Unless otherwise mentioned, the compounds synthesized were obtained as yellow solids.

**Synthesis of compound 1a: N-(tert-butyl)-2-phenylimidazo[1,2-a]pyridin-3-amine.** To the solution of 2-aminopyridine (24 mg, 0.25 mmol) in anhydrous acetonitrile, were added benzaldehyde (28 µL, 0.28 mmol), a catalytic amount of 4N HCl/dioxane (10 µL) and tert-butyl isonitrile (27 µL, 0.24 mmol). The reaction mixture was then heated under microwave conditions (400 W, 110 °C, 20 min). After the reaction mixture was cooled to room temperature, solvent was removed and the residue was purified using column chromatography to obtain compound 1a (44 mg, 69%). 

1H NMR (400 MHz, CDCl₃) δ 8.23 (dt, J = 6.9, 1.2 Hz, 1H), 7.90 (dt, J = 8.1, 1.6 Hz, 2H), 7.55 (dt, J = 9.0, 1.0 Hz, 1H), 7.43 (t, J = 7.6 Hz, 2H), 7.31 (t, J = 7.4 Hz, 1H), 7.13 (ddd, J = 9.0, 6.6, 1.3 Hz, 1H), 6.77 (td, J = 6.8, 1.1 Hz, 1H), 3.12 (s, 1H), 1.04 (s, 9H). 13C NMR (101 MHz, CDCl₃) δ 135.42, 128.43, 128.32, 127.52, 124.17, 123.64, 117.48, 111.46, 101.91, 56.59, 30.43. MS (ESI) calculated for C₁₇H₁₉N₃, m/z 265.16, found 266.17 [M+H]+.

Compounds 1b-f, 2a-f, and 3a-f were synthesized similarly as compound 1a.

**1b: N-(tert-butyl)-2-phenylimidazo[1,2-a]pyrazin-3-amine.** 45 mg, 62%. 1H NMR (400 MHz, CDCl₃) δ 9.00 (d, J = 1.4 Hz, 1H), 8.14 (dd, J = 4.6, 1.5 Hz, 1H), 7.91 (dd, J = 8.3, 1.3 Hz, 2H), 7.86 (d, J = 4.6 Hz, 1H), 7.46 (t, J = 7.5 Hz, 2H), 7.37 (t, J = 7.4 Hz, 1H), 3.19 (s, 1H), 1.05 (s, 9H). 13C NMR (101 MHz, CDCl₃) δ 143.54, 142.40, 137.43, 134.38, 129.04, 128.66, 128.38, 128.33, 125.17, 116.49, 100.13, 57.11, 30.45. MS (ESI) calculated for C₁₆H₁₈N₄, m/z 266.15, found 267.16 [M+H]+.

**1c: 2-Benzyl-N-(tert-butyl)imidazo[1,2-a]pyridin-3-amine.** 41 mg, 61%. 1H NMR (400 MHz, CDCl₃) δ 8.16 (dt, J = 6.9, 1.1 Hz, 1H), 7.47 (dt, J = 9.0, 1.0 Hz, 1H), 7.28 – 7.23 (m, 4H), 7.21 –
7.14 (m, 1H), 7.08 (ddd, J = 9.0, 6.7, 1.3 Hz, 1H), 6.72 (td, J = 6.8, 1.1 Hz, 1H), 4.20 (s, 2H), 2.57 (s, 1H), 1.18 (s, 9H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 139.83, 139.62, 128.68, 128.48, 126.06, 123.41, 123.35, 117.06, 110.93, 101.78, 98.20, 55.71, 34.36, 30.45. MS (ESI) calculated for C\(_{18}\)H\(_{21}\)N\(_3\), \(m/z\) 279.17, found 280.19 [M+H]\(^+\).

1d: 2-Benzyl-N-(tert-butyl)imidazo[1,2-a]pyrazin-3-amine. 31 mg, 43%. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.92 (d, J = 1.4 Hz, 1H), 8.06 (dd, J = 4.6, 1.5 Hz, 1H), 7.81 (d, J = 4.6 Hz, 1H), 7.32 – 7.27 (m, 2H), 7.25 – 7.18 (m, 3H), 4.24 (s, 2H), 2.62 (s, 1H), 1.20 (s, 9H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 142.95, 142.78, 138.88, 137.32, 128.72, 128.67, 126.45, 116.28, 56.29, 34.39, 30.48. MS (ESI) calculated for C\(_{17}\)H\(_{20}\)N\(_4\), \(m/z\) 280.17, found 281.18 [M+H]\(^+\).

1e: N-(tert-butyl)-2-(naphthalen-1-yl)imidazo[1,2-a]pyridin-3-amine. 56 mg, 74%. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.34 (dt, J = 6.9, 1.1 Hz, 1H), 8.02 – 7.85 (m, 3H), 7.68 – 7.44 (m, 5H), 7.19 (ddd, J = 9.0, 6.7, 1.3 Hz, 1H), 6.83 (td, J = 6.8, 1.1 Hz, 1H), 3.00 (s, 1H), 0.78 (s, 9H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 133.83, 132.94, 131.98, 128.48, 128.25, 128.15, 126.57, 125.73, 125.70, 125.34, 123.97, 123.64, 117.45, 111.37, 101.79, 100.00, 55.95, 29.76. MS (ESI) calculated for C\(_{21}\)H\(_{21}\)N\(_3\), \(m/z\) 315.17, found 316.19 [M+H]\(^+\).

1f: N-(tert-butyl)-2-(naphthalen-1-yl)imidazo[1,2-a]pyrazin-3-amine. 29 mg, 38%. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 9.07 (d, J = 1.4 Hz, 1H), 8.24 (dd, J = 4.6, 1.5 Hz, 1H), 7.97 – 7.87 (m, 4H), 7.67 – 7.42 (m, 4H), 3.08 (s, 1H), 0.80 (s, 9H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 143.57, 141.83, 133.85, 131.76, 131.71, 128.98, 128.95, 128.67, 128.27, 126.94, 126.02, 125.31, 125.19, 116.54, 105.36, 98.21, 56.51, 29.81. MS (ESI) calculated for C\(_{20}\)H\(_{20}\)N\(_4\), \(m/z\) 316.17, found 317.17 [M+H]\(^+\).
1g: 2-(Anthracen-9-yl)-N-(tert-butyl)imidazo[1,2-a]pyridin-3-amine. 55 mg, 63%. IR (CHCl₃) ν max (cm⁻¹): 2970, 1500, 1473, 1342, 1311. ¹H NMR (400 MHz, CDCl₃) δ 8.52 (s, 1H), 8.40 (dt, J = 6.9, 1.2 Hz, 1H), 8.09 – 8.02 (m, 2H), 7.93 – 7.86 (m, 2H), 7.66 (dt, J = 9.0, 1.1 Hz, 1H), 7.51 – 7.38 (m, 4H), 7.29 – 7.20 (m, 1H), 6.89 (td, J = 6.8, 1.1 Hz, 1H), 2.69 (s, 1H), 0.64 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 142.71, 131.45, 130.94, 128.75, 127.60, 126.80, 126.34, 126.19, 125.06, 124.00, 123.62, 117.57, 111.40, 101.78, 97.01, 55.59, 29.72. MS (ESI) calculated for C₂₅H₂₃N₃, m/z 365.19, found 366.20 [M+H]⁺.

1h: 2-(Anthracen-9-yl)-N-(tert-butyl)imidazo[1,2-a]pyrazin-3-amine. 50 mg, 57%. ¹H NMR (400 MHz, CDCl₃) δ 9.14 (d, J = 1.5 Hz, 1H), 8.57 (s, 1H), 8.31 (dd, J = 4.6, 1.5 Hz, 1H), 8.12 – 8.05 (m, 2H), 7.98 (d, J = 4.6 Hz, 1H), 7.83 – 7.75 (m, 2H), 7.53 – 7.42 (m, 4H), 2.75 (s, 1H), 0.65 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 143.65, 139.88, 138.08, 131.37, 130.86, 129.05, 128.96, 128.35, 127.48, 126.81, 125.52, 125.24, 116.55, 101.78, 56.14, 29.77. MS (ESI) calculated for C₂₄H₂₂N₄, m/z 366.18, found 367.19 [M+H]⁺ and 389.18 [M+Na]⁺.

2a: N-cyclohexyl-2-phenylimidazo[1,2-a]pyridin-3-amine. 51 mg, 73%. ¹H NMR (500 MHz, CDCl₃) δ 8.16 (d, J = 6.8 Hz, 1H), 8.05 (dd, J = 8.3, 1.2 Hz, 2H), 7.62 (d, J = 9.0 Hz, 1H), 7.45 (t, J = 7.8 Hz, 2H), 7.32 (t, J = 7.4 Hz, 1H), 7.17 (ddd, J = 8.9, 6.7, 1.2 Hz, 1H), 6.82 (td, J = 6.8, 0.9 Hz, 1H), 3.32 (s, 1H), 3.07 – 2.85 (m, 1H), 1.81 (d, J = 13.1 Hz, 2H), 1.68 (dd, J = 9.1, 3.6 Hz, 2H), 1.60 – 1.54 (m, 1H), 1.28 – 1.12 (m, 5H). ¹³C NMR (126 MHz, CDCl₃) δ 141.02, 135.43, 133.45, 128.74, 127.74, 127.20, 125.17, 125.04, 123.07, 116.94, 112.30, 57.02, 34.27, 25.81, 24.94. MS (ESI) calculated for C₁₉H₂₁N₃, m/z 291.17, found 292.18 [M+H]⁺.

2b: N-cyclohexyl-2-phenylimidazo[1,2-a]pyrazin-3-amine. 25 mg, 36%. ¹H NMR (500 MHz, CDCl₃) δ 8.99 (d, J = 1.4 Hz, 1H), 8.01 (ddd, J = 4.6, 3.2, 1.8 Hz, 3H), 7.85 (d, J = 4.6 Hz, 1H),
7.48 (t, J = 7.7 Hz, 2H), 7.38 (t, J = 7.4 Hz, 1H), 3.26 (s, 1H), 3.00 (s, 1H), 1.82 (dd, J = 6.6, 5.4 Hz, 2H), 1.70 (dd, J = 9.3, 3.3 Hz, 2H), 1.62 – 1.55 (m, 1H), 1.34 – 1.07 (m, 5H). $^{13}$C NMR (126 MHz, CDCl₃) δ 143.37, 139.08, 136.82, 133.62, 129.01, 128.90, 128.30, 127.42, 126.70, 115.73, 57.02, 34.41, 25.69, 24.91. MS (ESI) calculated for C₁₈H₂₀N₄, m/z 292.17, found 293.18 [M+H]⁺.

2c: 2-Benzyl-N-cyclohexylimidazo[1,2-a]pyridin-3-amine. 51 mg, 70%. $^1$H NMR (500 MHz, CDCl₃) δ 8.04 (dt, J = 6.8, 1.1 Hz, 1H), 7.52 – 7.46 (m, 1H), 7.31 – 7.26 (m, 4H), 7.19 (qd, J = 5.3, 2.7 Hz, 1H), 7.10 (ddd, J = 9.0, 6.7, 1.3 Hz, 1H), 6.76 (td, J = 6.8, 1.1 Hz, 1H), 4.16 (s, 2H), 2.77 (s, 1H), 2.60 (s, 1H), 1.79 – 1.67 (m, 4H), 1.59 (s, 1H), 1.32 – 1.04 (m, 5H). $^{13}$C NMR (126 MHz, CDCl₃) δ 141.11, 139.62, 137.49, 128.68, 128.53, 126.20, 125.44, 123.43, 122.59, 116.93, 111.41, 57.32, 34.17, 34.11, 25.74, 24.85. MS (ESI) calculated for C₂₀H₂₃N₃, m/z 305.19, found 306.20 [M+H]⁺.

2d: 2-Benzyl-N-cyclohexylimidazo[1,2-a]pyrazin-3-amine. 37 mg, 50%. $^1$H NMR (500 MHz, CDCl₃) δ 8.92 (d, J = 1.4 Hz, 1H), 7.93 (dd, J = 4.6, 1.5 Hz, 1H), 7.81 (d, J = 4.6 Hz, 1H), 7.33 – 7.25 (m, 4H), 7.22 (ddd, J = 6.2, 3.3, 1.6 Hz, 1H), 4.20 (s, 2H), 2.81 (s, 1H), 2.68 (s, 1H), 1.84 – 1.65 (m, 4H), 1.60 (d, J = 6.1 Hz, 1H), 1.24 – 0.95 (m, 5H). $^{13}$C NMR (126 MHz, CDCl₃) δ 141.19, 139.22, 137.30, 135.01, 127.27, 127.20, 127.17, 125.71, 125.04, 114.00, 55.65, 32.84, 32.76, 24.09, 23.31. MS (ESI) calculated for C₁₉H₂₂N₄, m/z 306.18, found 307.20 [M+H]⁺.

2e: N-cyclohexyl-2-(naphthalen-1-yl)imidazo[1,2-a]pyridin-3-amine. 24 mg, 29%. $^1$H NMR (500 MHz, CDCl₃) δ 8.20 (d, J = 6.8 Hz, 1H), 7.95 – 7.88 (m, 3H), 7.72 – 7.64 (m, 2H), 7.59 – 7.54 (m, 1H), 7.53 – 7.45 (m, 2H), 7.25 – 7.20 (m, 1H), 6.90 (t, J = 6.8 Hz, 1H), 3.15 (s, 1H), 2.63 (t, J = 9.6 Hz, 1H), 1.58 (d, J = 12.6 Hz, 2H), 1.50 – 1.36 (m, 3H), 1.04 – 0.77 (m, 5H). $^{13}$C
NMR (126 MHz, CDCl₃) δ 141.06, 133.76, 131.95, 128.57, 128.49, 128.15, 126.76, 126.65, 125.86, 125.50, 125.36, 124.30, 122.89, 117.21, 112.14, 56.32, 33.70, 25.47, 24.51. MS (ESI) calculated for C₂₃H₂₃N₃, m/z 341.19, found 342.20 [M+H]⁺.

2f: N-cyclohexyl-2-(naphthalen-1-yl)imidazo[1,2-a]pyrazin-3-amine. 50 mg, 61%. ¹H NMR (500 MHz, CDCl₃) δ 9.05 (d, J = 1.5 Hz, 1H), 8.05 (dd, J = 4.6, 1.5 Hz, 1H), 7.96 – 7.93 (m, 2H), 7.92 – 7.89 (m, 2H), 7.61 (ddd, J = 15.2, 7.5, 4.2 Hz, 4H), 7.55 – 7.48 (m, 1H), 3.27 (s, 1H), 2.73 (s, 1H), 1.61 (dd, J = 9.5, 3.2 Hz, 2H), 1.44 (ddd, J = 14.5, 11.6, 4.6 Hz, 3H), 1.07 – 0.82 (m, 5H). ¹³C NMR (126 MHz, CDCl₃) δ 143.40, 138.38, 136.73, 133.80, 131.90, 130.80, 129.03, 128.84, 128.58, 128.53, 128.07, 126.87, 126.08, 125.30, 125.27, 115.67, 56.12, 33.85, 25.35, 24.50. MS (ESI) calculated for C₂₂H₂₂N₄, m/z 342.18, found 343.19 [M+H]⁺ and 365.17 [M+Na]⁺.

2g: 2-(Anthracen-9-yl)-N-cyclohexylimidazo[1,2-a]pyridin-3-amine. 83 mg, 88%. ¹H NMR (500 MHz, CDCl₃) δ 8.54 (s, 1H), 8.23 (dt, J = 6.8, 1.2 Hz, 1H), 8.06 (d, J = 8.4 Hz, 2H), 7.83 (dd, J = 8.7, 0.8 Hz, 2H), 7.66 (dt, J = 9.0, 1.0 Hz, 1H), 7.49 – 7.44 (m, 2H), 7.40 (ddd, J = 8.6, 6.5, 1.3 Hz, 2H), 7.23 (ddd, J = 9.0, 6.7, 1.3 Hz, 1H), 6.90 (td, J = 6.8, 1.1 Hz, 1H), 2.80 (d, J = 7.4 Hz, 1H), 2.62 – 2.49 (m, 1H), 1.47 (d, J = 12.6 Hz, 2H), 1.35 – 1.24 (m, 3H), 0.89 – 0.77 (m, 3H), 0.68 (dt, J = 12.8, 6.6 Hz, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 142.02, 134.01, 131.38, 131.20, 128.59, 128.34, 128.10, 127.64, 126.25, 126.12, 125.10, 123.40, 122.81, 117.72, 111.60, 56.18, 33.62, 25.39, 24.38. MS (ESI) calculated for C₂₇H₂₅N₃, m/z 391.20, found 392.22 [M+H]⁺.

2h: 2-(Anthracen-9-yl)-N-cyclohexylimidazo[1,2-a]pyrazin-3-amine. 17 mg, 18%. ¹H NMR (500 MHz, CDCl₃) δ 9.12 (d, J = 1.4 Hz, 1H), 8.59 (s, 1H), 8.13 – 8.06 (m, 3H), 7.97 (d, J = 4.6 Hz, 1H), 7.72 (dd, J = 8.7, 0.6 Hz, 2H), 7.46 (dddd, J = 10.0, 7.8, 6.5, 1.1 Hz, 4H), 2.91 (d, J =
6.2 Hz, 1H), 2.62 (s, 1H), 1.48 (d, J = 12.6 Hz, 2H), 1.29 (dd, J = 24.7, 8.3 Hz, 3H), 0.91 – 0.76 (m, 3H), 0.76 – 0.63 (m, 2H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 142.63, 136.28, 135.36, 130.29, 130.12, 128.74, 128.01, 127.76, 127.36, 125.75, 125.54, 124.62, 124.25, 114.68, 54.95, 32.71, 24.20, 23.32. MS (ESI) calculated for C$_{26}$H$_{24}$N$_4$, m/z 392.20, found 393.20 [M+H]$^+$. 

3a: N-benzyl-2-phenylimidazo[1,2-a]pyridin-3-amine. 62 mg, 86%. $^1$H NMR (500 MHz, CDCl$_3$) δ 7.98 (ddt, J = 3.7, 3.0, 1.6 Hz, 3H), 7.57 (dt, J = 9.0, 1.0 Hz, 1H), 7.45 (t, J = 7.7 Hz, 2H), 7.39 – 7.26 (m, 6H), 7.13 (ddd, J = 9.0, 6.7, 1.3 Hz, 1H), 6.74 (td, J = 6.8, 1.1 Hz, 1H), 4.20 (d, J = 6.1 Hz, 2H), 3.52 (t, J = 6.0 Hz, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 141.57, 139.06, 136.04, 134.13, 128.84, 128.30, 127.82, 127.65, 127.16, 125.77, 124.32, 122.50, 117.52, 111.94, 52.57. MS (ESI) calculated for C$_{20}$H$_{17}$N$_3$, m/z 299.14, found 300.15 [M+H]$^+$. 

3b: N-benzyl-2-phenylimidazo[1,2-a]pyrazin-3-amine. 45 mg, 62%. $^1$H NMR (500 MHz, CDCl$_3$) δ 8.98 (d, J = 1.3 Hz, 1H), 7.94 (dt, J = 8.1, 1.6 Hz, 2H), 7.82 (dd, J = 4.6, 1.4 Hz, 1H), 7.77 (d, J = 4.6 Hz, 1H), 7.47 (t, J = 7.6 Hz, 2H), 7.39 (t, J = 7.4 Hz, 1H), 7.34 – 7.27 (m, 5H), 4.23 (d, J = 2.4 Hz, 2H), 3.66 (s, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 143.46, 138.76, 138.56, 136.79, 133.34, 129.06, 129.03, 128.99, 128.46, 128.24, 128.08, 127.42, 127.18, 115.38, 52.39. MS (ESI) calculated for C$_{19}$H$_{16}$N$_4$, m/z 300.14, found 301.15 [M+H]$^+$. 

3c: N,2-dibenzylimidazo[1,2-a]pyridin-3-amine. 33 mg, 44%. $^1$H NMR (500 MHz, CDCl$_3$) δ 7.95 (dt, J = 6.8, 1.1 Hz, 1H), 7.49 (dt, J = 9.1, 0.9 Hz, 1H), 7.38 – 7.15 (m, 10H), 7.09 (ddd, J = 9.0, 6.7, 1.3 Hz, 1H), 6.72 (td, J = 6.7, 1.1 Hz, 1H), 4.01 (s, 2H), 3.94 (d, J = 5.5 Hz, 2H), 2.99 (s, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 141.18, 139.68, 139.24, 137.57, 128.69, 128.62, 128.57, 128.28, 127.59, 126.25, 125.93, 123.36, 122.14, 117.15, 111.44, 52.83, 34.13. MS (ESI) calculated for C$_{21}$H$_{19}$N$_3$, m/z 313.16, found 314.18 [M+H]$^+$. 

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3d: **N,2-dibenzylimidazo[1,2-a]pyrazin-3-amine.** 19 mg, 25%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.83 – 7.72 (m, 2H), 7.35 – 7.25 (m, 5H), 7.25 – 7.18 (m, 4H), 7.16 (dd, $J = 7.0$, 2.2 Hz, 2H), 4.03 (s, 2H), 3.96 (d, $J = 3.9$ Hz, 2H), 3.15 – 3.05 (m, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 141.87, 139.52, 137.76, 137.68, 135.44, 128.01, 127.89, 127.75, 127.71, 127.66, 127.63, 127.59, 127.56, 127.26, 127.19, 126.97, 126.84, 126.75, 125.57, 114.09, 51.49, 51.46, 33.19. MS (ESI) calculated for C$_{20}$H$_{18}$N$_4$, m/z 314.15, found 315.17 [M+H]$^+$.

3e: **N-benzyl-2-(naphthalen-1-yl)imidazo[1,2-a]pyridin-3-amine.** 42 mg, 50%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.15 – 8.10 (m, 1H), 8.01 (d, $J = 8.3$ Hz, 1H), 7.94 – 7.86 (m, 2H), 7.61 (d, $J = 9.0$ Hz, 1H), 7.55 – 7.44 (m, 4H), 7.18 (ddd, $J = 9.0$, 6.7, 1.3 Hz, 1H), 7.15 – 7.11 (m, 3H), 7.02 (dd, $J = 6.5$, 3.0 Hz, 2H), 6.83 (td, $J = 6.8$, 1.0 Hz, 1H), 3.92 (d, $J = 4.9$ Hz, 2H), 3.53 (s, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 141.35, 138.75, 135.57, 133.79, 132.14, 131.50, 128.45, 128.37, 128.33, 127.88, 127.34, 127.31, 126.49, 125.88, 125.82, 125.27, 123.69, 122.50, 117.60, 111.80, 52.42. MS (ESI) calculated for C$_{24}$H$_{19}$N$_3$, m/z 349.16, found 350.18 [M+H]$^+$.

3f: **N-benzyl-2-(naphthalen-1-yl)imidazo[1,2-a]pyrazin-3-amine.** 12 mg, 14%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.01 (d, $J = 1.5$ Hz, 1H), 7.97 – 7.89 (m, 4H), 7.83 (d, $J = 4.6$ Hz, 1H), 7.49 (tddd, $J = 10.0$, 6.8, 3.6, 1.4 Hz, 4H), 7.17 – 7.08 (m, 3H), 7.01 – 6.94 (m, 2H), 3.96 (d, $J = 5.8$ Hz, 2H), 3.78 (t, $J = 5.8$ Hz, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 143.50, 138.21, 137.83, 136.53, 133.78, 131.90, 130.44, 129.08, 128.95, 128.89, 128.49, 128.46, 128.02, 127.77, 127.73, 127.58, 126.78, 126.06, 125.45, 125.21, 115.35, 51.83. MS (ESI) calculated for C$_{23}$H$_{18}$N$_4$, m/z 350.15, found 351.18 [M+H]$^+$.

3g: **2-(Anthracen-9-yl)-N-benzylimidazo[1,2-a]pyridin-3-amine.** 47 mg, 49%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.55 (s, 1H), 8.18 (dt, $J = 6.9$, 1.2 Hz, 1H), 8.07 (d, $J = 8.5$ Hz, 2H), 7.82 (dd, $J =
8.8, 0.9 Hz, 2H), 7.66 (dt, J = 9.0, 1.1 Hz, 1H), 7.50 – 7.43 (m, 2H), 7.39 (ddd, J = 8.7, 6.5, 1.3 Hz, 2H), 7.22 (ddd, J = 9.0, 6.7, 1.3 Hz, 1H), 7.05 – 6.95 (m, 3H), 6.90 – 6.82 (m, 3H), 3.74 (d, J = 6.3 Hz, 2H), 3.25 (t, J = 6.3 Hz, 1H). 13C NMR (126 MHz, CDCl3) δ 140.88, 137.71, 132.57, 130.36, 130.23, 127.77, 127.54, 127.15, 127.02, 126.76, 126.63, 126.10, 125.31, 125.10, 124.09, 122.54, 121.46, 116.74, 110.71, 51.19. MS (ESI) calculated for C28H21N3, m/z 399.17, found 400.20 [M+H]+.

3h: 2-(Anthracen-9-yl)-N-benzylimidazo[1,2-a]pyrazin-3-amine. 8 mg, 8%. 1H NMR (500 MHz, CDCl3) δ 9.09 (d, J = 1.4 Hz, 1H), 8.59 (s, 1H), 8.08 (d, J = 8.5 Hz, 2H), 8.01 (dd, J = 4.6, 1.5 Hz, 1H), 7.91 (d, J = 4.6 Hz, 1H), 7.69 (dd, J = 8.8, 0.7 Hz, 2H), 7.52 – 7.45 (m, 2H), 7.41 (ddd, J = 8.6, 6.5, 1.2 Hz, 2H), 7.06 – 6.94 (m, 3H), 6.83 – 6.77 (m, 2H), 3.78 (d, J = 5.3 Hz, 2H), 3.44 (s, 1H). 13C NMR (126 MHz, CDCl3) δ 143.66, 138.12, 137.10, 135.78, 131.32, 131.19, 130.28, 129.06, 128.75, 128.50, 128.33, 127.55, 127.41, 126.56, 126.53, 125.74, 125.28, 115.37, 51.61. MS (ESI) calculated for C27H20N4, m/z 400.17, found 401.19 [M+H]+.

Compounds 4a-4c were synthesized similarly as compound 1a.

4a: 2-(Anthracen-9-yl)-N-cyclohexylimidazo[1,2-a]pyrimidin-3-amine. 40 mg, 43%. 1H NMR (500 MHz, CDCl3) δ 8.61 (dd, J = 4.1, 2.1 Hz, 1H), 8.56 (s, 1H), 8.54 (dd, J = 6.8, 2.1 Hz, 1H), 8.07 (d, J = 8.4 Hz, 2H), 7.83 (dd, J = 8.7, 0.8 Hz, 2H), 7.49 – 7.44 (m, 2H), 7.41 (ddd, J = 8.6, 6.5, 1.3 Hz, 2H), 6.97 (dd, J = 6.8, 4.1 Hz, 1H), 2.83 (s, 1H), 2.47 (s, 1H), 1.43 (d, J = 12.9 Hz, 2H), 1.29 (d, J = 7.4 Hz, 3H), 0.82 (t, J = 6.6 Hz, 3H), 0.75 – 0.62 (m, 2H). 13C NMR (126 MHz, CDCl3) δ 148.91, 144.93, 135.89, 131.34, 131.05, 130.37, 128.66, 128.12, 127.26, 126.67, 126.29, 126.07, 125.18, 108.17, 56.46, 33.55, 25.28, 24.28. MS (ESI) calculated for C26H24N4, m/z 392.20, found 393.22 [M+H]+.
4b: **2-(Anthracen-9-yl)-N\(^3\)-cyclohexylimidazo[1,2-a]pyridine-3,8-diamine.** 15 mg, 15%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.54 (s, 1H), 8.06 (d, \(J = 8.4\) Hz, 2H), 7.86 (dd, \(J = 8.7, 0.7\) Hz, 2H), 7.70 (dd, \(J = 6.7, 0.9\) Hz, 1H), 7.49 – 7.44 (m, 2H), 7.43 – 7.37 (m, 2H), 6.74 (t, \(J = 7.0\) Hz, 1H), 6.40 (dd, \(J = 7.3, 0.9\) Hz, 1H), 4.57 (s, 2H), 2.77 (s, 1H), 2.56 (s, 1H), 1.49 (d, \(J = 12.4\) Hz, 2H), 1.29 (d, \(J = 5.7\) Hz, 3H), 0.94 – 0.75 (m, 3H), 0.75 – 0.61 (m, 2H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 135.97, 135.69, 132.06, 131.45, 131.37, 129.03, 128.57, 128.36, 127.62, 126.37, 126.06, 125.12, 113.22, 112.69, 101.65, 56.09, 33.63, 25.41, 24.41. MS (ESI) calculated for C\(_{27}\)H\(_{26}\)N\(_4\), m/z 406.22, found 407.23 [M+H]\(^+\).

4c: **2-(Anthracen-9-yl)-8-chloro-N-cyclohexylimidazo[1,2-a]pyrazin-3-amine.** 20 mg, 20%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.59 (s, 1H), 8.10 – 8.06 (m, 3H), 7.77 (d, \(J = 4.5\) Hz, 1H), 7.70 (dd, \(J = 8.7, 0.7\) Hz, 2H), 7.52 – 7.47 (m, 2H), 7.46 – 7.41 (m, 2H), 2.93 (d, \(J = 6.8\) Hz, 1H), 2.66 – 2.58 (m, 1H), 1.48 (d, \(J = 12.4\) Hz, 2H), 1.32 (d, \(J = 6.9\) Hz, 3H), 0.92 – 0.79 (m, 3H), 0.69 (dd, \(J = 22.4, 11.8\) Hz, 2H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 143.61, 136.76, 134.02, 131.59, 131.27, 131.26, 128.77, 128.58, 127.43, 126.61, 126.17, 125.63, 125.29, 115.63, 56.13, 33.74, 25.16, 24.33. MS (ESI) calculated for C\(_{26}\)H\(_{23}\)ClN\(_4\), m/z 426.16, found 427.17 [M+H]\(^+\).

Compounds 5a-5i were synthesized similarly as compound 1a.

5a: **2-(Anthracen-9-yl)-N-butylimidazo[1,2-a]pyridin-3-amine.** 22 mg, 25%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.55 (s, 1H), 8.19 (dt, \(J = 6.8, 1.2\) Hz, 1H), 8.06 (d, \(J = 8.4\) Hz, 2H), 7.84 (dd, \(J = 8.7, 0.8\) Hz, 2H), 7.67 (dt, \(J = 9.1, 1.0\) Hz, 1H), 7.48 – 7.44 (m, 2H), 7.40 (ddd, \(J = 8.6, 6.5, 1.3\) Hz, 2H), 7.22 (ddd, \(J = 9.0, 6.7, 1.3\) Hz, 1H), 6.90 (td, \(J = 6.8, 1.1\) Hz, 1H), 2.89 (t, \(J = 6.1\) Hz, 1H), 2.64 (q, \(J = 6.6\) Hz, 2H), 1.09 – 0.97 (m, 2H), 0.86 – 0.72 (m, 2H), 0.43 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 141.97, 133.07, 131.52, 131.47, 129.40, 128.68, 128.39, 127.80,
5b: 2-(Anthracen-9-yl)-N-pentylimidazo[1,2-a]pyridin-3-amine. 35 mg, 38%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.55 (s, 1H), 8.19 (dt, \(J = 6.8, 1.2\) Hz, 1H), 8.06 (d, \(J = 8.4\) Hz, 2H), 7.84 (dd, \(J = 8.7, 0.8\) Hz, 2H), 7.67 (dt, \(J = 9.0, 1.1\) Hz, 1H), 7.49 – 7.44 (m, 2H), 7.40 (ddd, \(J = 8.6, 6.5, 1.3\) Hz, 2H), 7.22 (ddd, \(J = 9.0, 6.7, 1.3\) Hz, 1H), 6.90 (td, \(J = 6.8, 1.1\) Hz, 1H), 2.91 (t, \(J = 6.4\) Hz, 1H), 2.65 (q, \(J = 6.7\) Hz, 2H), 1.02 (dt, \(J = 14.3, 7.0\) Hz, 2H), 0.82 – 0.65 (m, 4H), 0.52 (t, \(J = 7.1\) Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 141.85, 132.95, 131.40, 131.34, 129.24, 128.56, 128.26, 127.67, 126.32, 126.10, 125.13, 123.31, 122.48, 117.81, 111.67, 48.05, 29.68, 28.58, 22.07, 13.70. MS (ESI) calculated for C\(_{26}\)H\(_{25}\)N\(_3\), m/z 379.20, found 380.22 [M+H]\(^+\).

5c: 2-(Anthracen-9-yl)-N-isopropylimidazo[1,2-a]pyridin-3-amine. 15 mg, 18%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.54 (s, 1H), 8.25 (dt, \(J = 6.8, 1.2\) Hz, 1H), 8.08 – 8.04 (m, 2H), 7.84 (dd, \(J = 8.7, 0.9\) Hz, 2H), 7.66 (dt, \(J = 9.0, 1.1\) Hz, 1H), 7.49 – 7.44 (m, 2H), 7.41 (ddd, \(J = 8.6, 6.5, 1.3\) Hz, 2H), 7.23 (ddd, \(J = 9.0, 6.7, 1.3\) Hz, 1H), 6.90 (td, \(J = 6.8, 1.1\) Hz, 1H), 2.86 (dq, \(J = 12.5, 6.3\) Hz, 1H), 2.71 (d, \(J = 6.4\) Hz, 1H), 0.69 (d, \(J = 6.3\) Hz, 6H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 142.10, 134.36, 131.39, 131.13, 128.63, 128.31, 128.29, 127.69, 126.23, 126.17, 125.11, 123.56, 122.78, 117.69, 111.64, 49.20, 23.15. MS (ESI) calculated for C\(_{24}\)H\(_{21}\)N\(_3\), m/z 351.17, found 352.19 [M+H]\(^+\).

5d: 2-(Anthracen-9-yl)-N-isopropylimidazo[1,2-a]pyridin-3-amine. 37 mg, 41%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.54 (s, 1H), 8.23 (dd, \(J = 6.8, 1.0\) Hz, 1H), 8.06 (d, \(J = 8.3\) Hz, 2H), 7.89 – 7.83 (m, 2H), 7.67 (dt, \(J = 9.0, 1.0\) Hz, 1H), 7.50 – 7.37 (m, 4H), 7.23 (ddd, \(J = 9.0, 6.7, 1.3\) Hz, 1H), 6.90 (td, \(J = 6.8, 1.1\) Hz, 1H), 2.79 – 2.61 (m, 2H), 1.12 – 0.95 (m, 2H), 0.92 – 0.82 (m,
2H), 0.79 – 0.67 (m, 1H), 0.63 (d, J = 6.2 Hz, 2H), 0.39 (t, J = 7.1 Hz, 2H), 0.29 (t, J = 7.4 Hz, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 142.03, 134.21, 131.41, 131.39, 131.36, 131.19, 131.13, 128.62, 128.61, 128.59, 128.36, 128.34, 127.64, 127.61, 126.26, 126.15, 126.13, 125.09, 123.45, 123.34, 122.68, 122.60, 117.74, 117.71, 111.62, 59.63, 53.06, 39.14, 26.01, 20.85, 18.53, 13.58, 9.01. MS (ESI) calculated for C$_{26}$H$_{25}$N$_3$, m/z 379.20, found 380.22 [M+H]$^+$.  

5e: 2-(Anthracen-9-yl)-N-(2,4,4-trimethylpentan-2-yl)imidazo[1,2-a]pyridin-3-amine. 51 mg, 50%. IR (CHCl$_3$) $\nu_{\text{max}}$ (cm$^{-1}$): 2956, 1519, 1481, 1365, 1340. $^1$H NMR (500 MHz, CDCl$_3$) δ 8.53 (s, 1H), 8.41 (d, J = 6.9 Hz, 1H), 8.05 (d, J = 7.7 Hz, 2H), 7.88 (d, J = 8.5 Hz, 2H), 7.66 (d, J = 9.0 Hz, 1H), 7.50 – 7.38 (m, 4H), 7.24 (ddd, J = 8.9, 6.7, 1.2 Hz, 1H), 6.88 (td, J = 6.8, 1.0 Hz, 1H), 2.89 (s, 1H), 0.97 (s, 2H), 0.68 (s, 6H), 0.52 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 142.71, 137.08, 131.44, 131.01, 129.27, 128.71, 127.53, 126.78, 126.32, 126.24, 125.05, 123.94, 123.70, 117.51, 111.35, 59.61, 55.82, 31.20, 30.95, 28.61. MS (ESI) calculated for C$_{29}$H$_{31}$N$_3$, m/z 421.25, found 422.27 [M+H]$^+$.  

5f: 2-(Anthracen-9-yl)-N-((trimethylsilyl)methyl)imidazo[1,2-a]pyridin-3-amine. 33 mg, 35%. $^1$H NMR (500 MHz, CDCl$_3$) δ 8.55 (s, 1H), 8.15 (dt, J = 6.8, 1.1 Hz, 1H), 8.06 (d, J = 8.4 Hz, 2H), 7.89 (dd, J = 8.7, 0.7 Hz, 2H), 7.69 – 7.65 (m, 1H), 7.49 – 7.44 (m, 2H), 7.43 – 7.38 (m, 2H), 7.22 (ddd, J = 9.0, 6.7, 1.3 Hz, 1H), 6.91 (td, J = 6.8, 1.0 Hz, 1H), 2.60 (s, 1H), 2.15 (s, 2H), -0.31 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 141.55, 132.06, 131.73, 131.42, 131.23, 128.58, 128.10, 127.65, 126.45, 126.02, 125.11, 123.20, 122.44, 117.79, 111.61, 38.69, -3.37. MS (ESI) calculated for C$_{29}$H$_{31}$N$_3$Si, m/z 395.18, found 396.20 [M+H]$^+$.  

5g: N-(adamantan-1-yl)-2-(anthracen-9-yl)imidazo[1,2-a]pyridin-3-amine. 46 mg, 43%. $^1$H NMR (500 MHz, CDCl$_3$) δ 8.52 (s, 1H), 8.45 (dt, J = 6.9, 1.1 Hz, 1H), 8.08 – 8.03 (m, 2H), 7.87
(d, J = 8.8 Hz, 2H), 7.65 (dt, J = 9.0, 1.0 Hz, 1H), 7.44 (ddddd, J = 10.0, 7.9, 6.5, 1.3 Hz, 4H),
7.23 (ddd, J = 9.0, 6.7, 1.3 Hz, 1H), 6.88 (td, J = 6.8, 1.1 Hz, 1H), 2.66 (s, 1H), 1.65 (s, 3H),
1.34 (d, J = 12.1 Hz, 3H), 1.19 (d, J = 11.4 Hz, 3H), 1.13 (d, J = 2.5 Hz, 6H). $^{13}$C NMR (126
MHz, CDCl$_3$) $\delta$ 142.80, 137.17, 131.51, 131.15, 129.27, 128.82, 127.68, 126.39, 126.35,
125.99, 125.17, 123.99, 123.87, 117.61, 111.43, 55.78, 43.30, 36.03, 29.50. MS (ESI)
calculated for C$_{31}$H$_{29}$N$_3$, m/z 443.24, found 444.26 [M+H]$^+$.  

5h: 2-(Anthracen-9-yl)-N-(4-methoxyphenyl)imidazo[1,2-a]pyridin-3-amine. 36 mg, 36%. $^1$H
NMR (500 MHz, CDCl$_3$) $\delta$ 8.50 (s, 1H), 8.01 (d, J = 8.5 Hz, 2H), 7.90 – 7.83 (m, 3H), 7.74 (d, J =
9.1 Hz, 1H), 7.46 – 7.39 (m, 2H), 7.38 – 7.28 (m, 3H), 6.87 (td, J = 6.8, 1.0 Hz, 1H), 6.61 – 6.56
(m, 2H), 6.37 – 6.29 (m, 2H), 5.29 (s, 1H), 3.63 (s, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 153.58,
142.85, 137.63, 136.25, 131.45, 131.34, 128.69, 128.04, 127.61, 126.28, 126.18, 125.19,
124.47, 123.39, 123.21, 118.11, 115.45, 114.84, 112.22, 55.69. MS (ESI) calculated for
C$_{28}$H$_{21}$N$_3$O, m/z 415.17, found 416.19 [M+H]$^+$.  

5i: 2-(Anthracen-9-yl)-N-(2-chloro-6-methylphenyl)imidazo[1,2-a]pyridin-3-amine. 11 mg,
11%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.38 (s, 1H), 8.21 (dt, J = 6.8, 1.1 Hz, 1H), 7.94 (d, J = 8.4
Hz, 2H), 7.75 (ddd, J = 15.6, 8.3, 4.8 Hz, 3H), 7.41 – 7.35 (m, 2H), 7.34 – 7.28 (m, 3H), 6.97 (td,
J = 6.8, 1.1 Hz, 1H), 6.61 (dd, J = 7.9, 1.0 Hz, 1H), 6.41 – 6.35 (m, 1H), 6.29 (t, J = 7.7 Hz, 1H),
5.43 (s, 1H), 1.48 (s, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 142.20, 139.32, 137.26, 131.22,
129.58, 128.96, 128.51, 127.69, 127.36, 126.96, 126.32, 125.86, 124.98, 124.43, 124.33,
122.77, 121.59, 118.07, 112.53, 18.14. MS (ESI) calculated for C$_{28}$H$_{20}$ClN$_3$, m/z 433.13, found
434.15 [M+H]$^+$.  

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Synthesis of compound 6: 2-(Anthracen-9-yl)imidazo[1,2-a]pyridin-3-amine. Compound 5\textit{e} (50 mg, 0.12 mmol) was stirred in a solution of 4M HCl/dioxane for 3 h, followed by removing the solvent under vacuum. The residue was then purified using column chromatography to obtain compound 6 in quantitative yield. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.55 (s, 1H), 8.12 (d, $J$ = 6.8 Hz, 1H), 8.07 (d, $J$ = 8.4 Hz, 2H), 7.87 (d, $J$ = 8.8 Hz, 2H), 7.66 (d, $J$ = 9.1 Hz, 1H), 7.49 – 7.45 (m, 2H), 7.44 – 7.38 (m, 2H), 7.23 – 7.17 (m, 1H), 6.91 (td, $J$ = 6.8, 0.9 Hz, 1H), 3.07 (s, 2H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 141.49, 131.62, 131.32, 130.44, 128.77, 127.97, 127.79, 126.54, 126.32, 125.88, 125.33, 123.00, 122.10, 117.82, 111.89. MS (ESI) calculated for C$_{21}$H$_{15}$N$_3$, m/z 309.13, found 310.14 [M+H]$^+$. 

Syntheses of compounds 7\textit{a} and 7\textit{b}: To a solution of compound 6 (20 mg, 0.065 mmol) in anhydrous methanol, were added octyl aldehyde (15 $\mu$L, 0.098 mmol), 4 drops of acetic acid and sodium cyanoborohydride (6 mg, 0.098 mmol). The reaction mixture was stirred for 18h and the solvent was then removed under vacuum. The residue was purified using column chromatography to obtain compounds 7\textit{a} (0-3% MeOH in CH$_2$Cl$_2$) and 7\textit{b} (0-2% MeOH in CH$_2$Cl$_2$).

7\textit{a}: 2-(Anthracen-9-yl)-N-octylimidazo[1,2-a]pyridin-3-amine. 10 mg, 36%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.54 (s, 1H), 8.20 (dt, $J$ = 6.8, 1.1 Hz, 1H), 8.06 (d, $J$ = 8.4 Hz, 2H), 7.84 (dd, $J$ = 8.7, 0.7 Hz, 2H), 7.67 (d, $J$ = 9.0 Hz, 1H), 7.50 – 7.43 (m, 2H), 7.42 – 7.37 (m, 2H), 7.23 (ddd, $J$ = 9.0, 6.7, 1.3 Hz, 1H), 6.91 (td, $J$ = 6.8, 1.1 Hz, 1H), 2.92 (s, 1H), 2.70 – 2.61 (m, 2H), 1.21 – 1.11 (m, 2H), 0.99 (ddd, $J$ = 15.6, 8.8, 7.1 Hz, 4H), 0.90 – 0.84 (m, 2H), 0.82 (t, $J$ = 7.3 Hz, 3H), 0.73 – 0.66 (m, 4H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 141.95, 132.96, 131.54, 131.48, 129.37, 128.73, 128.28, 127.86, 126.44, 126.28, 125.28, 123.54, 122.65, 117.92, 111.86, 48.16, 31.79,
30.06, 29.11, 29.10, 26.56, 22.70, 14.22. MS (ESI) calculated for C$_{29}$H$_{31}$N$_3$, m/z 421.25, found 422.27 [M+H]$^+$. 

7b: 2-(Anthracen-9-yl)-N,N-dioctylimidazo[1,2-a]pyridin-3-amine. 7 mg, 20%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.54 (s, 1H), 8.30 (d, $J$ = 6.8 Hz, 1H), 8.04 (d, $J$ = 8.5 Hz, 2H), 7.63 (d, $J$ = 8.7 Hz, 3H), 7.46 – 7.40 (m, 2H), 7.31 (ddd, $J$ = 8.6, 6.5, 1.1 Hz, 2H), 7.26 – 7.21 (m, 1H), 6.90 (td, $J$ = 6.8, 1.0 Hz, 1H), 2.51 – 2.45 (m, 4H), 1.32 – 1.19 (m, 8H), 1.19 – 1.08 (m, 8H), 1.08 – 0.93 (m, 8H), 0.85 (t, $J$ = 7.2 Hz, 6H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 142.02, 135.49, 132.12, 131.84, 131.45, 129.90, 128.41, 127.72, 127.10, 125.49, 125.20, 123.93, 123.19, 117.77, 111.74, 54.71, 31.92, 29.51, 29.38, 29.16, 27.09, 22.75, 14.25. MS (ESI) calculated for C$_{37}$H$_{47}$N$_3$, m/z 533.38, found 534.38 [M+H]$^+$. 

Synthesis of compound 7c: N-(2-(anthracen-9-yl)imidazo[1,2-a]pyridin-3-yl)-2,2,2-trifluoro acetamide. To a solution of compound 6 (10 mg, 0.024 mmol) in anhydrous CH$_2$Cl$_2$, was added trifluoroacetic anhydride (4 µL, 0.03 mmol) and the reaction mixture was stirred for 12 h. The solvent was then removed under vacuum and the residue was purified using column chromatography (0-3% MeOH in CH$_2$Cl$_2$) to obtain compound 7c (10 mg, 73%). IR (CHCl$_3$) $\nu_{max}$ (cm$^{-1}$): 1730, 1494, 1355, 1315, 1240, 1195, 1157. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.89 (s, 1H), 8.76 (d, $J$ = 6.8 Hz, 1H), 8.28 – 8.14 (m, 3H), 8.10 (d, $J$ = 9.0 Hz, 1H), 7.77 (d, $J$ = 8.5 Hz, 2H), 7.72 (dd, $J$ = 10.0, 4.0 Hz, 1H), 7.59 (ddt, $J$ = 9.9, 6.6, 3.9 Hz, 4H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 161.71, 161.41, 161.11, 160.82, 159.55, 159.23, 158.92, 158.61, 140.74, 135.66, 132.62, 132.61, 132.47, 130.22, 129.81, 129.06, 127.34, 127.00, 125.58, 118.91, 118.54, 113.88. MS (ESI) calculated for C$_{23}$H$_{14}$F$_3$N$_3$O, m/z 405.11, found 406.12 [M+H]$^+$. 

Compound 8a-8c were synthesized similarly as compound 1a.
8a: 2-[[1,1'-Biphenyl]-4-yl]-N-(2,4,4-trimethylpentan-2-yl)imidazo[1,2-a]pyridin-3-amine. 60 mg, 63%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.27 (d, $J = 6.9$ Hz, 1H), 7.99 – 7.95 (m, 2H), 7.71 – 7.65 (m, 4H), 7.61 (d, $J = 9.0$ Hz, 1H), 7.46 (dd, $J = 10.5$, 4.8 Hz, 2H), 7.38 – 7.33 (m, 1H), 7.21 – 7.15 (m, 1H), 6.82 (dd, $J = 6.6$, 6.2 Hz, 1H), 3.29 (s, 1H), 1.61 (s, 2H), 1.05 (s, 9H), 0.99 (s, 6H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 140.75, 140.14, 128.92, 128.78, 128.70, 127.31, 127.27, 127.26, 127.00, 126.96, 124.63, 123.66, 123.48, 117.04, 111.69, 60.86, 57.09, 31.86, 31.76, 29.02. MS (ESI) calculated for C$_{27}$H$_{31}$N$_3$, m/z 397.25, found 398.25 [M+H]$^+$.  

8b: 2-(Phenanthren-9-yl)-N-(2,4,4-trimethylpentan-2-yl)imidazo[1,2-a]pyridin-3-amine. 79 mg, 78%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.78 (d, $J = 8.2$ Hz, 1H), 8.74 (d, $J = 8.1$ Hz, 1H), 8.37 (dt, $J = 6.9$, 1.1 Hz, 1H), 8.04 (dd, $J = 8.2$, 1.0 Hz, 1H), 7.92 (dd, $J = 7.9$, 1.2 Hz, 1H), 7.90 (s, 1H), 7.68 (dddd, $J = 8.3$, 6.9, 4.2, 1.4 Hz, 2H), 7.65 – 7.56 (m, 3H), 7.21 (ddd, $J = 9.0$, 6.7, 1.3 Hz, 1H), 6.86 (td, $J = 6.8$, 1.1 Hz, 1H), 3.21 (s, 1H), 1.26 (s, 2H), 0.78 (s, 6H), 0.73 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 142.35, 139.07, 131.88, 131.67, 131.27, 130.82, 130.44, 129.27, 128.91, 127.17, 126.89, 126.83, 126.72, 126.63, 125.55, 124.24, 123.90, 123.18, 122.72, 117.53, 111.59, 60.24, 56.39, 31.63, 31.45, 28.78. MS (ESI) calculated for C$_{29}$H$_{31}$N$_3$, m/z 421.25, found 422.26 [M+H]$^+$ and 444.25 [M+Na]$^+$.  

8c: 2-(Pyren-4-yl)-N-(2,4,4-trimethylpentan-2-yl)imidazo[1,2-a]pyridin-3-amine. 47 mg, 44%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.40 (dt, $J = 6.9$, 1.1 Hz, 1H), 8.28 (d, $J = 7.8$ Hz, 1H), 8.23 – 8.16 (m, 4H), 8.11 (dd, $J = 12.1$, 5.0 Hz, 3H), 8.02 (t, $J = 7.6$ Hz, 1H), 7.67 (dt, $J = 9.0$, 0.9 Hz, 1H), 7.23 (ddd, $J = 9.0$, 6.7, 1.3 Hz, 1H), 6.87 (td, $J = 6.8$, 1.1 Hz, 1H), 3.24 (s, 1H), 1.20 (s, 2H), 0.73 (s, 9H), 0.67 (s, 6H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 142.45, 139.49, 131.39, 131.00, 130.94, 130.62, 129.17, 128.39, 128.17, 127.55, 127.46, 125.92, 125.48, 125.20, 125.05,
Compound 9 was synthesized similarly as compound 1a.

9: 2-(10-Chloroanthracen-9-yl)-N-(2,4,4-trimethylpentan-2-yl)imidazo[1,2-a]pyridin-3-amine. 128 mg, 59%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.60 (d, $J = 8.8$ Hz, 2H), 8.41 (d, $J = 6.9$ Hz, 1H), 7.90 (d, $J = 8.8$ Hz, 2H), 7.67 (d, $J = 9.0$ Hz, 1H), 7.62 – 7.57 (m, 2H), 7.50 – 7.45 (m, 2H), 7.28 – 7.25 (m, 1H), 6.90 (t, $J = 6.8$ Hz, 1H), 2.83 (s, 1H), 0.96 (s, 2H), 0.68 (s, 6H), 0.52 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 142.73, 136.53, 131.45, 129.61, 129.14, 128.64, 127.24, 127.01, 126.69, 126.58, 126.55, 125.21, 124.28, 123.73, 117.53, 111.57, 59.62, 55.90, 31.20, 31.00, 28.67. MS (ESI) calculated for C$_{29}$H$_{30}$ClN$_3$, m/z 455.21, found 456.23 $[M+H]^+$.

Synthesis of compound 10: $N^1,N^1$-dimethyl-$N^3$-(10-(3-((2,4,4-trimethylpentan-2-yl)amino)imidazo[1,2-a]pyridin-2-yl)anthracen-9-yl)propane-1,3-diamine. To a solution of compound 9 (50 mg, 0.11 mmol) in anhydrous dioxane, were added potassium tert-butoxide (38 mg, 0.34 mmol), $N^1,N^1$-dimethylpropane-1,3-diamine (69 $\mu$L, 0.55 mmol) and catalytic amounts of tris(dibenzylideneacetone)dipalladium [Pd$_2$(dba)$_3$] and 2-dicyclohexylphosphino-2'-(N,N-dimethylamino)biphenyl [DavePhos]. The reaction was heated in a sealed tube at 80 °C for 4 h. The solvent was then removed under vacuum and the residue was purified using column chromatography to obtain compound 10 (15 mg, 26%). $^1$H NMR (500 MHz, MeOD) $\delta$ 8.53 (dt, $J = 6.9$, 1.0 Hz, 1H), 8.47 (d, $J = 8.7$ Hz, 2H), 7.74 (d, $J = 8.5$ Hz, 2H), 7.60 – 7.56 (m, 1H), 7.51 – 7.45 (m, 2H), 7.44 – 7.35 (m, 3H), 7.04 (td, $J = 6.8$, 1.1 Hz, 1H), 3.45 (t, $J = 7.1$ Hz, 2H), 2.57 – 2.52 (m, 2H), 2.30 (s, 6H), 2.02 – 1.91 (m, 2H), 0.95 (s, 2H), 0.74 (s, 6H), 0.45 (s, 9H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 144.90, 143.81, 137.83, 133.18, 128.54, 127.84, 127.32, 126.52, 126.46,
125.51, 125.31, 125.08, 124.09, 117.18, 113.25, 60.30, 58.65, 56.77, 51.56, 45.43, 44.86, 32.49, 31.81, 31.76, 29.47, 29.44. MS (ESI) calculated for C_{34}H_{43}N_{5}, m/z 521.35, found 522.37 [M+H]^+.

Compound 11 was synthesized similarly as compound 1a.

11: 2-(Anthracen-9-yl)-6-bromo-N-(2,4,4-trimethylpentan-2-yl)imidazo[1,2-a]pyridin-3-amine. 150 mg, 63%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.54 – 8.51 (m, 2H), 8.08 – 8.04 (m, 2H), 7.82 (dd, $J = 8.6$, 0.9 Hz, 2H), 7.56 (dd, $J = 9.4$, 0.7 Hz, 1H), 7.49 – 7.41 (m, 4H), 7.30 (dd, $J = 9.4$, 1.9 Hz, 1H), 2.91 (s, 1H), 0.96 (s, 2H), 0.68 (s, 6H), 0.50 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 141.22, 138.24, 131.56, 131.08, 128.94, 128.70, 127.97, 127.51, 127.30, 126.66, 126.08, 125.26, 124.04, 118.41, 106.62, 59.88, 55.93, 31.31, 31.07, 28.78. MS (ESI) calculated for C$_{29}$H$_{30}$BrN$_3$, m/z 499.16, found 500.18 [M+H]$^+$. Compound 12 was synthesized similarly as compound 10.

12: 2-(Anthracen-9-yl)-N$_3$-(3-(dimethylamino)propyl)-N$_6$-(2,4,4-trimethylpentan-2-yl)imidazo[1,2-a]pyridine-3,6-diamine. 21 mg, 40%. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.61 (s, 1H), 8.11 (d, $J = 8.2$ Hz, 2H), 7.83 (d, $J = 8.6$ Hz, 2H), 7.65 (d, $J = 1.9$ Hz, 1H), 7.52 – 7.42 (m, 4H), 7.39 (d, $J = 9.5$ Hz, 1H), 7.04 (dd, $J = 9.5$, 2.1 Hz, 1H), 3.18 (t, $J = 6.8$ Hz, 2H), 2.60 – 2.54 (m, 2H), 2.34 (s, 6H), 1.98 – 1.90 (m, 2H), 0.93 (s, 2H), 0.73 (s, 6H), 0.46 (s, 9H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 140.23, 138.50, 136.47, 133.04, 132.49, 130.41, 129.86, 128.71, 128.35, 127.41, 127.33, 126.29, 121.96, 117.07, 103.72, 60.35, 58.54, 56.85, 45.44, 43.56, 31.78, 31.70, 29.50, 27.45. MS (ESI) calculated for C$_{34}$H$_{43}$N$_5$, m/z 521.35, found 522.36 [M+H]$^+$. Compound 13 was synthesized similarly as compound 1a.
13: \textit{N-(tert-butyl)-2-(10-chloroanthracen-9-yl)imidazo[1,2-a]pyridin-3-amine}. 242 mg, 84%.

\textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \(\delta\) 8.60 (d, \(J = 8.8\) Hz, 2H), 8.40 (dt, \(J = 6.9, 1.1\) Hz, 1H), 7.91 (d, \(J = 8.8\) Hz, 2H), 7.66 (d, \(J = 9.0\) Hz, 1H), 7.60 (ddd, \(J = 8.8, 6.5, 1.1\) Hz, 2H), 7.48 (ddd, \(J = 8.7, 6.5, 1.1\) Hz, 2H), 7.28 – 7.23 (m, 1H), 6.90 (td, \(J = 6.8, 1.1\) Hz, 1H), 2.63 (s, 1H), 0.65 (s, 9H). \textsuperscript{13}C NMR (126 MHz, CDCl\textsubscript{3}) \(\delta\) 142.88, 136.60, 131.47, 129.77, 129.12, 128.75, 127.13, 126.76, 126.72, 126.67, 125.36, 124.42, 123.78, 117.69, 111.71, 55.76, 29.90. MS (ESI) calculated for C\textsubscript{25}H\textsubscript{22}ClN\textsubscript{3}, m/z 399.15, found 400.17 [M+H]\textsuperscript{+}.

Compound 14 was synthesized similarly as compound 10.

14: \textit{N\textsuperscript{1}-(10-(3-(tert-butylamino)imidazo[1,2-a]pyridin-2-yl)anthracen-9-yl)octane-1,8-diamine}. 20 mg, 32%. \textsuperscript{1}H NMR (500 MHz, MeOD) \(\delta\) 9.01 (d, \(J = 6.8\) Hz, 1H), 8.55 (d, \(J = 8.8\) Hz, 2H), 8.15 – 8.08 (m, 1H), 7.99 (dd, \(J = 13.0, 4.8\) Hz, 3H), 7.85 (dd, \(J = 8.4, 7.0\) Hz, 2H), 7.80 – 7.71 (m, 2H), 7.67 (dd, \(J = 10.0, 4.0\) Hz, 1H), 3.79 (dd, \(J = 16.7, 8.6\) Hz, 2H), 2.94 (dd, \(J = 14.0, 6.6\) Hz, 2H), 2.00 – 1.91 (m, 2H), 1.72 – 1.63 (m, 2H), 1.46 – 1.38 (m, 6H), 0.81 (s, 9H). \textsuperscript{13}C NMR (126 MHz, MeOD) \(\delta\) 139.74, 135.47, 135.36, 132.83, 130.52, 129.36, 129.01, 128.07, 127.63, 127.26, 127.03, 125.59, 123.03, 118.21, 113.15, 56.34, 54.29, 40.75, 30.28, 30.13, 29.99, 28.55, 27.62, 27.38, 27.35. MS (ESI) calculated for C\textsubscript{33}H\textsubscript{41}N\textsubscript{5}, m/z 507.34, found 508.35 [M+H]\textsuperscript{+}.

Compound 15 was synthesized similarly as compound 1a.

15: \textit{2-(Anthracen-9-yl)-6-bromo-N-(tert-butyl)imidazo[1,2-a]pyridin-3-amine}. 260 mg, 81%.

\textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \(\delta\) 8.54 – 8.52 (m, 2H), 8.08 – 8.04 (m, 2H), 7.83 (d, \(J = 8.6\) Hz, 2H), 7.56 (dd, \(J = 9.4, 0.6\) Hz, 1H), 7.49 – 7.42 (m, 4H), 7.30 (dd, \(J = 9.4, 1.9\) Hz, 1H), 2.68 (s, 1H),
0.64 (s, 9H). $^{13}$C NMR (126 MHz, CDCl₃) $\delta$ 141.20, 138.13, 131.52, 130.96, 128.97, 128.47, 128.04, 127.60, 127.27, 126.67, 125.99, 125.25, 123.94, 118.41, 106.67, 55.85, 29.83. MS (ESI) calculated for C₂₅H₂₂BrN₃, m/z 443.10, found 444.10 [M+H]$^+$. Compound 16 was synthesized similarly as compound 10.

16: $N^6$-(8-aminooctyl)-2-(anthracen-9-yl)-$N^3$-(tert-butyl)imidazo[1,2-a]pyridine-3,6-diamine. 55 mg, 47%. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.81 (s, 1H), 8.20 (dd, $J = 6.5, 2.3$ Hz, 2H), 7.85 – 7.80 (m, 2H), 7.72 – 7.56 (m, 7H), 3.23 (t, $J = 7.0$ Hz, 2H), 2.97 – 2.90 (m, 2H), 1.80 (dt, $J = 14.5, 7.1$ Hz, 2H), 1.72 – 1.64 (m, 2H), 1.59 – 1.51 (m, 2H), 1.45 (s, 6H), 0.78 (s, 9H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 141.50, 141.40, 134.23, 132.75, 132.52, 131.64, 130.29, 128.91, 128.20, 127.68, 126.85, 126.05, 121.64, 112.69, 104.55, 56.28, 45.40, 40.78, 30.46, 30.35, 30.30, 29.46, 28.65, 28.27, 27.52. MS (ESI) calculated for C₃₃H₄₁N₅, m/z 507.34, found 508.35 [M+H]$^+$. Microbiological methods. MICs of the compounds were determined by broth microdilution method per CLSI (formerly NCCLS) guidelines as described earlier. Mid-log phase Mueller-Hinton broth (MHB; noncation supplemented) cultures of organisms (40 μL; optical density at 600 nm adjusted to 0.5 AU, and diluted 10-fold) were added to equal volumes of 2-fold serially diluted compounds in a 384-well microtiter plate with the help of a Biotek Precision 2000 automated microplate pipetting system. The MICs of known antibiotics were included as reference compounds for comparison of activity. The microtiter plates were sealed and incubated overnight at 37°C. The plates were read at an absorbance of 600 nm. The lowest concentration of an agent inhibiting growth of the organisms was recorded as the MIC. For MBC determinations, conventional microdilution techniques were employed wherein 0.5 μL of each of
the 384 wells in the parent MIC plate was diluted into 80 μL of fresh MHB using the Biotek Precision 2000 automated liquid handling device. The microtiter plates were incubated overnight at 37°C. The plates were read at an absorbance of 600 nm. $5e$-resistant $S. \text{aureus}$ organisms were generated by exposing $S. \text{aureus}$ ATCC 13709 to escalating doses of the compound. Within about 10 serial passages, organisms that withstood $5e$ up to concentrations of 100 μg/mL emerged.
Chapter 5.

TLR7-agonistic imidazo[4,5-c]pyridines

Potent TLR7-specific agonist (EC<sub>50</sub> = 0.26 μM)
High IFN-α / Low Proinflammatory Cytokine Induction
5.1. Introduction

Occupancy of TLR7\textsuperscript{31b, 51a, 90} or TLR9\textsuperscript{91} in professional antigen-presenting cells (APCs), particularly plasmacytoid dendritic cells (pDCs), leads to the induction of IFN-\(\alpha/\beta\). Although the Type I IFNs are best known historically for their antiviral activities,\textsuperscript{92} recent studies show that they have many essential functions in the control of adaptive immunity.\textsuperscript{93} First, Type I IFNs promote cross-priming through direct stimulation of DCs, leading to specific CD8\(^+\) lymphocytic responses to soluble antigens.\textsuperscript{94} Second, Type I IFNs potently enhance the primary antibody responses to soluble antigens, inducing sustained and durable humoral responses with appropriate isotype switching, as well as the induction of immunological memory.\textsuperscript{95} B lymphocytes can differentiate into two distinct types of functionally polarized effectors: B-effector-1-cells (Be-1), producing a Th1-like cytokine pattern, or Be-2, characterized by a Th2-like profile.\textsuperscript{96} It is of particular interest that recent reports suggest that IFN-\(\alpha\) may serve as an initial trigger for Be-1-biased differentiation pattern.\textsuperscript{97} Third, Type I IFNs secondarily induce Type II IFN (IFN-\(\gamma\)) secretion, also driving Th1-biased adaptive immune responses.\textsuperscript{98} Type I IFN-inducing TLR ligands may therefore hold promise as vaccine adjuvants.

In an effort to identify optimal immunostimulatory chemotypes, we have screened representative members of virtually the entire compendium of known TLR agonists in a series of hierarchical assays including primary TLR-reporter assays, secondary indices of immune activation such as IFN-\(\alpha/\beta/\gamma\) and cytokine induction, activation of lymphocytic subsets in whole human blood, and tertiary screens characterizing transcriptomal activation patterns.\textsuperscript{30} In these assays, small-molecule agonists of TLR7 were uniquely immunostimulatory; they were potent inducers of Type I IFN and, unlike TLR-4, -5, or -8 agonists,\textsuperscript{30} did not evoke dominant proinflammatory cytokine responses, suggesting that they may be effective, yet safe vaccine adjuvants, a
premise that we have been actively exploring. Small molecule TLR7 agonists are also being investigated as orally bioavailable, endogenous Type I IFN inducers for the management of chronic viral diseases, especially hepatitis C and hepatitis B. Current therapeutic regimens for the therapy of hepatitis C and hepatitis B include parenteral IFN-α. Clinical trials of TLR7 agonists for hematological malignancies are also currently underway.

As mentioned earlier, the currently known small molecule agonists of TLR7 occupy a very small chemical space, and are represented by the 1H-imidazo[4,5-c]quinolines, 8-hydroxy- or 8-oxoadenines, and guanine nucleoside analogues. We had previously reported structure-activity relationships (SAR) in the imidazooquinolines with a focus on substituents at the N1, C2, N3 and N4 positions, and we had observed that relatively minor structural modifications at these positions yielded compounds with widely differing immunomodulatory properties.

It was of interest, therefore, to extend our SAR studies to the quinoline ring system. We asked if a part-structure (imidazopyridine) or a benzologue (benzoimidazoquinoline) would alter the biological properties of the parent imidazoquinoline compound. Examination of the structures of 3M-003 and the 8-hydroxy- and 8-oxoadenines (Fig. 1) suggested that the quinoline system may be dispensable, and activity would be retained in imidazopyridines. Indeed, imidazopyridine derivatives with alkyl groups at C6 and C7 positions, hydroxyalkyl, oxime and hydroxylamine-bearing substituents at C2, and alkylsulfonamide substituents at the N1 position have been reported in the patent literature. Detailed activity profiles of these compounds, however, are not available, perhaps owing to the fact that the investigations of such compounds precede the discovery of the TLRs.

Incorporating substituents that we had previously determined to be optimal in the imidazoquinolines (N1-benzyl and C2-butyl; IMDQ, Fig. 1), we embarked on the syntheses and biological evaluation of novel 1H-imidazo[4,5-c]pyridine analogues with modifications at the N4-
and C6 positions. The parent imidazopyridine compound, 1-benzyl-2-butyl-1H-imidazo[4,5-c]pyridin-4-amine, exhibited moderate TLR7-agonistic activity. N4-acyl or -alkyl substitutions abrogated activity. The majority of C6 derivatives bearing aryl groups were also inactive, but analogues with N6-benzyl substituents gained TLR7-specific activity. Particular N6 substituents were found to augment TLR7-specific agonistic potency without compromising specificity at TLR7; consistent with their pure TLR7 activity (and undetectable TLR8 agonism), these compounds potently induced IFN-α in human peripheral blood mononuclear cells (PBMCs), upregulated CD69 in lymphocytic subsets, and yet showed very weak proinflammatory cytokine-inducing activities. Strong Type I IFN inducers, especially in conjunction with attenuated proinflammatory profiles are expected to be potently adjuvantic without inducing prominent local or systemic inflammation.

*Fig. 1.* Structures of small molecule agonists of TLR7 represented by the 8-oxoadenine (SM360320), 8-hydroxyadenines (CL264), 1H-imidazo[4,5-c]quinolines 3M-003, 1-isobutyl-1H-imidazo[4,5-c]quinolin-4-amine (Imiquimod) and 1-benzyl-2-butyl-1H-imidazo[4,5-c]quinolin-4-amine (IMDQ).
5.2. Results and Discussion

Our interest in exploring TLR7 agonists as vaccine adjuvants has been greatly reinforced by our observations that pure TLR7 agonists, unlike other TLR ligands, are potently immunostimulatory without prominently activating inflammatory programs in human whole blood model systems. As mentioned earlier, the structures of 3M-003 and the 8-hydroxy- and 8-oxoadenines (Fig. 1), as well as patent literature suggested that the quinoline system may be dispensable, and activity would be retained in imidazopyridines. Our previous SAR studies on the imidazoquinolines had established that \( N^1 \)-benzyl and \( C^2 \)-butyl substituents were optimal; our point of departure in examining structure-activity relationships in the imidazopyridines consequently began with the evaluation of 1-benzyl-2-butyl-1H-imidazo[4,5-c]pyridin-4-amine (5), following the synthetic strategy described earlier (Scheme 1). Compound 5, itself a novel and unprecedented structure, was found to possess TLR7-specific agonistic activity (EC\(_{50} \): 1.57 \( \mu \)M, Fig. 2, Table 1), with negligible TLR8 activity. The potency of the lead TLR7-specific imidazoquinoline (1-benzyl-2-butyl-1H-imidazo[4,5-c]quinolin-4-amine, structure in Fig. 1) was 0.06 \( \mu \)M (Fig. 2).

\textbf{Scheme 1.} Synthesis of 1-benzyl-2-butyl-1H-imidazo[4,5-c]pyridin-4-amine 5.

\[
\begin{align*}
\text{Reagents:} & \quad \text{i. BnNH}_2, \text{NET}_3, \text{CH}_2\text{Cl}_2; \quad \text{ii. Zn, HCOONH}_4, \text{MeOH}; \quad \text{iii. (a) C}_4\text{H}_9\text{COCl}, \text{NET}_3, \text{THF} \quad \text{(b) NaOH, EtOH; iv. mCPBA, CHCl}_3; \quad \text{v. (a) Benzoyl isocyanate, CH}_2\text{Cl}_2 \quad \text{(b) NaOMe, MeOH.}
\end{align*}
\]
Acylation (6a and 6b, Scheme 2) of the C4-NH2 resulted in complete abrogation of activity (Table 1).

**Scheme 2. Synthesis of C4-N-acylated analogues.**

![Synthesis of C4-N-acylated analogues](image)

Reagents: i. RCOCI, NEt3, CH2Cl2

C6-modified analogues were synthesized via an alternate route. Nitration of 4-amino-2-chloropyridine resulted, as expected, in a mixture of the 3- and 5-nitro intermediates 7a and 7b, which were taken forward to obtain the 4- and 6-chloroimidazopyridines 10a and 10b (Scheme 3). Excellent chromatographic separation of these advanced intermediates was possible. Pd-catalyzed C-N cross-coupling reactions using n-butylamine and benzylamine from intermediate 10a furnished the C4-N-alkylated analogues 11a and 11b, respectively (Scheme 3). A 4-butoxy analogue 11c was also obtained by ipso-chloro displacement with 1-butanol. Compounds 11a-c were, however, inactive (Table 1). We had envisaged utilizing the 6-chloroimidazopyridine intermediate 10b for synthesizing C6-functionalized analogues. However, this intermediate exhibited unexpectedly low reactivity to displacement with nucleophiles or to Buchwald-Hartwig coupling reactions. As outlined in Scheme 4, we utilized 4-amino-2,6-dichloropyridine as the starting material and obtained 13 as a key intermediate which, upon reaction with tert-octylamine provided exclusively the N2-alkylated intermediate 14. The 6-chloro-N-(2,4,4-trimethylpentan-2-yl)-1H-imidazopyridin-4-amine intermediate 16 was obtained without difficulty,
and we were able to synthesize the $N^6$-substituted analogues 19a-o under conventional Buchwald-Hartwig conditions (Scheme 4).

**Scheme 3.** Synthesis of $C^4$-$N$- and $O$-alkylated analogues.

**Scheme 4.** Synthesis of $N^6$-substituted analogues.

Reagents: i. (a) $H_2SO_4$, HNO$_3$ (b) $H_2SO_4$; ii. Br$_2$, NaH, THF; iii. Zn, HCOONH$_4$, MeOH; iv. (a) C$_4$H$_6$COCl, NEt$_3$, THF; (b) NaOH, EtOH; v. For 11a and 11b, amines (n-BuNH$_2$ and BrNH$_2$, respectively); Pd$_2$(dba)$_3$, DavePhos, KOtBu, dioxane; For 11c, BuOH, NaH, THF.

Reagents: i. (a) $H_2SO_4$, HNO$_3$ (b) $H_2SO_4$; ii. Br$_2$, NaH, THF; iii. t-Odylamine, NEt$_3$, CH$_2$Cl$_2$; iv. Zn, HCOONH$_4$, MeOH; v. (a) $C_4H_6$COCl, NEt$_3$, THF; (b) NaOH, EtOH; vi. RNH$_2$, Pd$_2$(dba)$_3$, DavePhos, KOtBu, dioxane; vii. HCl.
TLRs signal via ligand-induced dimerization, but since that the crystal structure of human TLR7 and of its ligand binding modes are as yet unknown, we utilized intermediate 16 in constructing ‘dimeric’ imidazopyridines (using p- and m-xylylenediamine, Scheme 5) to ascertain if such pre-organized dimeric ligands could yield high-potency agonists.

Scheme 5. Synthesis of dimeric compounds.

![Scheme 5](image)

Reagents: i. Pd2(dba)3, DavePhos, KOrBu, dioxane; ii. HCl.

The 6-chloroimidazopyridine 17 (Scheme 4) was inactive (Table 1). Buchwald-Hartwig-derived N6-substituted analogues 19a-q, however, showed a distinctive SAR. Compound 19a with a free NH2 at C6, obtained by coupling the tert-octylamine and subsequent N-dealkylation with HCl (Scheme 4, Table 1) displayed TLR7-specific agonism with a potency comparable to that of the parent C6-unsubstituted compound 5. Modest gains in potency were obtained in analogues with short aliphatic substituents with N6-butyl (19b) and N6-cyclohexymethyl (19d), but potency diminished in the N6-heptyl analogue (19c). The N6-phenyl-substituted compound 19e was marginally weaker than 5; however, the potency of the N6-benzyl analogue 19f was ~7.6 times that of 5 (Table 1, Fig. 2), triggering a detailed SAR investigation on various aryl substituents at N6. Both steric and electronic effects appear to play a role in governing TLR7-agonistic potency, since the biphenylmethyl-substituted compound 19o was active, whereas the naphthylmethyl analogue 19n was quiescent; to a first approximation, electron-rich N6 substituents appear to be preferred, with the methoxybenzyl derivatives (19g and 19h) and the pyridinylmethyl compounds (19l and 19m) being marginally more active than the trifluoromethyl- (19i) or chloro-
(19j) substituted analogues. Compounds 19p and 19q were also active in primary screens, with EC<sub>50</sub> values of 0.26 and 0.37 µM, respectively (Table 1).

For the C6-substituted compounds 23a-j (Scheme 6), we observed mediocre yields in pilot Suzuki coupling reactions with the advanced intermediate 16. We therefore exploited the electron-withdrawing resonance effect of the 3-nitro group in 14. As expected, the classical Suzuki reaction on intermediate 14 using various aliphatic and aromatic boronic acids/boronic esters resulted in the intermediates 20a-j (Scheme 6), which were further derivatized to obtain the desired C6 alky/aryl substituted imidazopyridines 23a-j.

**Scheme 6. Synthesis of C6-substituted analogues.**

In the C<sup>6</sup>-alkyl or -aryl analogues (Scheme 6), the SAR appeared more stringent. Whereas the C<sup>6</sup>-butyl compound 23a was more active than 5, direct aryl-aryl connections at C6 (23b-f) abrogated activity, but TLR7 agonistic properties were restored in the 6-benzyl (23g) and 6-
phenethyl analogues (23j). Taken together with activity data of compounds of the 19 series, we surmised that rotational constraints about the C6-aryl groups may hinder TLR7 occupancy. Unlike TLR2, TLR3, TLR4,110 and TLR5111 for which crystal structures are available as complexes with their cognate ligands, a detailed structural characterization of the mode of binding of TLR7 ligands is not yet available to guide structure-based design of small molecule agonists of TLR7, necessitating classical SAR approaches to refine successive iterations of ligand design.

**Fig. 2.** TLR7 agonistic activities of imidazopyridine compounds. Data points represent means and standard deviations on quadruplicates.

The benzologue 30 was also synthesized as shown in Scheme 7. It showed substantial improvements in potency over the parent imidazopyridine 5 (Fig. 3, Table 1), but the two most potent compounds in the entire series as adjudged by primary screens were the N6-(4-
methoxybenzyl) and \(N^6\)-(furan-2-ylmethyl) analogues (19g and 19k, respectively), both of which were approximately twenty-fold more potent than 5 (Fig. 2, Table 1).

**Scheme 7. Synthesis of benzologue 30.**

![Chemical synthesis scheme](image)

**Reagents:** i. (a) HCl, HON=CHCH\(_2\)NO\(_2\), H\(_2\)O (b) (CH\(_3\)CO\(_2\))\(_2\)O, CH\(_3\)COOK; ii. POCl\(_3\); iii. BrNH\(_2\), NEt\(_3\), CH\(_3\)Cl; iv. Zn, HCOONH\(_4\), MeOH; v. (a) C\(_6\)H\(_5\)COCl, NEt\(_3\), THF (b) NaOH, EtOH; vi. mCPBA, CH\(_2\)Cl\(_2\), CHCl\(_3\), MeOH; vii. (a) Benzoyl isocyanate, CH\(_2\)Cl\(_2\) (b) NaOMe, MeOH.

**Fig. 3.** Dose-response profiles of TLR7 agonistic activity of compounds 5 and 30. Data points represent means and standard deviations on quadruplicates.
Table 1. EC<sub>50</sub> values of compounds in human TLR7/8-specific reporter gene assay.

![Chemical structure](image)

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<sup>a</sup>Inactive: no activity was detected up to a concentration of 500 μg/mL.
We chose the nine most active compounds (19b, 19d, 19f-g, 19k-m, 19p, and 23a) for evaluation in secondary screens using IFN-α and cytokine release in human PBMCs. We used, as reference compounds, imiquimod, a known TLR7 agonist, as well as CL075, a predominantly TLR8-active agonist with an EC₅₀ of 1.32 µM in hTLR8 assays.

**Fig. 4.** Dose-response profiles of Type I interferon (IFN-α) and proinflammatory cytokine (IL-8, IL-1β, and TNF-α) induction by selected imidazopyridine (and reference) compounds. Representative data from three independent experiments are presented.

Given that the imidazopyridine compounds are pure TLR7 agonists, we expected to find prominent IFN-α induction, and this was indeed the case, with 19p, 19m, and 19k being the most potent (EC₅₀: 0.3 µM, 0.4 µM and 0.7 µM, respectively; Fig. 4). CL075 was among the
least potent in IFN-α induction (EC₅₀: 2.6 μM; Fig. 4), and as expected for a TLR8 agonist, CL075 was dramatically more active in inducing proinflammatory cytokines such as TNF-α, IL-1β, and IL-8 (Fig. 4). We do not yet understand the basis for the slight discrepancy between rank-order potency in primary screens (19k≈19g>19f>19p; Fig. 2, Table 1) vis-à-vis IFN-α-inducing potency in human PBMCs (19p≈19m>19k; Fig. 4), and we surmise that analogues with more basic C6 substituents may allow for higher endolysosomal partitioning. The dose-response profiles show characteristic biphasic responses (dose-dependent activation, followed by apparent suppression) as we had previously observed in several chemotypes. We verified that the apparent suppression was not due to cytotoxicity using LDH release and mitochondrial redox-based assays.

We had previously shown that of all the various classes of innate immune stimuli, TLR7 agonists were extraordinarily immunostimulatory, stimulating virtually all subsets of lymphocytes (assessed by quantifying CD69 expression), and yet without inducing dominant proinflammatory cytokine responses, and we wished to confirm the rank-order potency observed in IFN-α induction assays described above.

We observed considerable dissociation between Type I IFN induction on the one hand (Fig. 4), and CD69 upregulation in lymphocytic subsets on the other (Fig. 5). Whereas the subset of active compounds induced IFN-α with similar potencies (EC₅₀ values between 0.3-2 μM; Fig. 4), pronounced differences were observed in CD69 expression in natural killer, cytokine-induced killer and B lymphocytic subsets with 19p being as active as the reference TLR7 agonist IMDQ, and 19d showing virtually no activity (Fig. 5). Possible mechanisms underlying the differential activity in these two compounds are being investigated.
Fig. 5. CD69 upregulation in human natural killer (NK), cytokine-induced killer (CIK) and nominal B lymphocytes by select imidazopyridine (and reference) compounds.
5.3. Conclusions

These findings raise the possibility of utilizing these compounds in selectively targeting Type I IFN induction versus lymphocytic activation, and are being explored in greater detail. The potential advantages of strong Type I IFN inducers as candidate vaccine adjuvants have been discussed earlier. Such compounds, especially in conjunction with attenuated proinflammatory cytokines, are expected to be potently adjuvantic without inducing prominent local or systemic inflammation. As mentioned earlier, the prominent Type I IFN-inducing abilities of the imidazopyridines may also find utility as an alternative therapeutic strategy to address disease states wherein systemic IFN-α is of proven benefit. A clear delineation of structural features that confer TLR specificity not only charts a rational course for the development of effective, yet safe vaccine adjuvants, but also provides tools to understand innate immune function in greater detail.

5.4. Experimental

Chemistry. All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 °C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf ‘Gold’ high performance silica columns on CombiFlash Rf instrument unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel (200 μm) CCM pre-coated aluminum sheets. Purity for all final compounds was confirmed to be greater than 97% by LC-MS using a Zorbax Eclipse Plus 4.6 mm × 150 mm, 5 μm analytical reverse
phase C18 column with H$_2$O-isopropanol or H$_2$O–CH$_3$CN gradients and an Agilent 6520 ESI-QTOF Accurate Mass spectrometer (mass accuracy of 5 ppm) operating in the positive ion (or negative ion, as appropriate) acquisition mode.

**Synthesis of compound 1: N-Benzyl-3-nitropyridin-4-amine.** To a solution of 4-chloro-3-nitropyridine (1.0 g, 6.31 mmol) in 25 mL of CH$_2$Cl$_2$ were added triethylamine (1.32 mL, 9.47 mmol) and benzyl amine (0.83 mL, 7.57 mmol). The reaction mixture was refluxed for 18 h. The solvent was then evaporated under vacuum and H$_2$O was added to the residue. The solution was extracted with CH$_2$Cl$_2$ (3 × 20 mL), washed with water and dried over sodium sulfate. The solvent was evaporated and the residue was purified using silica gel column chromatography (0-5% MeOH in CH$_2$Cl$_2$) to obtain compound 1 as a yellow solid (1.4 g, 94%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.22 (s, 1H), 8.53 (s, 1H), 8.25 (dd, $J = 6.1$, 0.6 Hz, 1H), 7.41 – 7.35 (m, 2H), 7.32 (dd, $J = 7.2$, 5.3 Hz, 3H), 6.69 (d, $J = 6.2$ Hz, 1H), 4.56 (d, $J = 5.7$ Hz, 2H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 153.38, 149.06, 148.68, 135.96, 130.04, 129.21, 128.22, 127.10, 108.25, 46.85. MS (ESI) calculated for C$_{12}$H$_{12}$N$_3$O$_2$, m/z 230.0924, found 230.0949 [M+H]$^+$.

**Synthesis of compound 2: N$_4$-Benzylpyridine-3,4-diamine.** To a solution of compound 1 (1.0 g, 4.36 mmol) in 40 mL of MeOH were added zinc dust (1.4 g, 21.8 mmol) and ammonium formate (1.4 g, 21.8 mmol). The reaction mixture was stirred at room temperature for 10 min and filtered through celite. Then the solvent was evaporated and the residue was dissolved in water. This was extracted with EtOAc (3 × 20 mL), washed with water and dried over sodium sulfate. The solvent was evaporated under vacuum to obtain the compound 2 (0.8 g, 92%).$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.81 (d, $J = 5.4$ Hz, 1H), 7.77 (s, 1H), 7.29 – 7.19 (m, 5H), 6.36 (d, $J = 5.4$ Hz, 1H), 4.87 (s, 1H), 4.28 (d, $J = 5.4$ Hz, 2H), 3.28 (s, 2H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$
Synthesis of compound 3: 1-Benzyl-2-butyl-1H-imidazo[4,5-c]pyridine. To a solution of compound 2 (400 mg, 2.00 mmol) in 20 mL of anhydrous THF were added triethylamine (0.29 mL, 2.10 mmol) and valeryl chloride (0.27 mL, 2.20 mmol). The reaction mixture was refluxed for 2 h. The solvent was then removed under vacuum, and the residue was dissolved in EtOAc and washed with water. The EtOAc fraction was dried using sodium sulfate and evaporated under vacuum to obtain the intermediate amide compound. This was dissolved in 20 mL of EtOH and NaOH (160 mg, 4.00 mmol) in 2 mL of H2O was added. The reaction mixture was refluxed for 4 h. The solvent was then removed under vacuum, and the residue was dissolved in EtOAc and washed with water. The organic layer was dried using sodium sulfate and evaporated and purified using column chromatography (0-5% MeOH in CH2Cl2) to obtain the compound 3 (210 mg, 40%). 1H NMR (500 MHz, CDCl3) δ 9.04 (d, J = 0.6 Hz, 1H), 8.34 (d, J = 5.6 Hz, 1H), 7.34 – 7.28 (m, 3H), 7.14 (dd, J = 5.6, 1.0 Hz, 1H), 7.01 (dd, J = 7.7, 1.8 Hz, 2H), 5.33 (s, 2H), 2.86 – 2.81 (m, 2H), 1.82 (dt, J = 15.5, 7.7 Hz, 2H), 1.46 – 1.36 (m, 2H), 0.91 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, CDCl3) δ 157.30, 142.14, 142.00, 140.48, 140.01, 135.26, 129.28, 128.37, 126.27, 105.12, 47.25, 29.44, 27.38, 22.61, 13.85. MS (ESI) calculated for C17H20N3, m/z 266.1652, found 266.1715 [M+H]+.

Synthesis of compound 4: 1-Benzyl-2-butyl-1H-imidazo[4,5-c]pyridine 5-oxide. To a solution of compound 3 (210 mg, 0.79 mmol) in 15 mL of was added m-chloroperoxybenzoic acid (443 mg, 1.98 mmol), and the solution was refluxed at 45-50 ºC for 1 h. The solvent was then removed and the residue was purified using column chromatography (0-10% MeOH in CH2Cl2) to obtain the N-oxide derivative (188 mg, 85%). 1H NMR (500 MHz, CDCl3) δ 8.71 (d, J =...
= 1.3 Hz, 1H), 8.05 (dd, J = 7.0, 1.6 Hz, 1H), 7.36 – 7.30 (m, 3H), 7.01 (dd, J = 10.4, 4.3 Hz, 3H), 5.31 (s, 2H), 2.85 – 2.80 (m, 2H), 1.79 (dt, J = 15.4, 7.6 Hz, 2H), 1.44 – 1.34 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 160.72, 140.89, 134.42, 134.32, 133.94, 131.52, 129.48, 128.75, 126.24, 106.63, 47.75, 29.25, 27.49, 22.52, 13.78. MS (ESI) calculated for C$_{17}$H$_{20}$N$_3$O, m/z 282.1601, found 282.1612 [M+H]$^+$. 

**Synthesis of compound 5: 1-Benzyl-2-butyl-1H-imidazo[4,5-c]pyridin-4-amine.** To a solution of compound 4 (188 mg, 0.67 mol) in 15 mL of CH$_2$Cl$_2$ was added benzoyl isocyanate (197 mg, 1.34 mmol) and heated at 45 $^\circ$C for 2 h. The solvent was then removed under vacuum, and the residue was dissolved in 15 mL of anhydrous MeOH, followed by the addition of excess sodium methoxide. The reaction mixture was then heated at 80 $^\circ$C for 1 h. The solvent was removed under vacuum and the residue was purified using column chromatography (0-7% MeOH in CH$_2$Cl$_2$) to obtain the compound 5 (56 mg, 30%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.78 (d, J = 5.8 Hz, 1H), 7.34 – 7.28 (m, 3H), 7.03 (d, J = 6.4 Hz, 2H), 6.59 (d, J = 5.8 Hz, 1H), 5.27 (s, 2H), 5.15 (s, 2H), 2.83 – 2.75 (m, 2H), 1.72 (ddd, J = 13.0, 9.0, 7.7 Hz, 2H), 1.44 – 1.34 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 154.06, 151.00, 140.61, 140.41, 135.76, 129.21, 128.23, 126.33, 97.74, 47.52, 30.10, 27.42, 22.68, 13.89. HRMS (ESI) calculated for C$_{17}$H$_{21}$N$_4$, m/z 281.1761, found 281.1795 [M+H]$^+$. 

**Synthesis of compound 6a: N-(1-Benzyl-2-butyl-1H-imidazo[4,5-c]pyridin-4-yl)acetamide.** To a solution of compound 5 (30 mg, 0.11 mmol) in 2 mL of CH$_2$Cl$_2$ were added triethylamine (17 $\mu$L, 0.12 mmol) and acetyl chloride (8 $\mu$L, 0.11 mmol). The reaction mixture was stirred at room temperature for 3 h and purified using column chromatography (5% MeOH/CH$_2$Cl$_2$) to obtain the compound 6a as white solid (6 mg, 16%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 11.60 (s, 1H), 8.20 (s, 1H), 7.37 (dd, J = 7.7, 5.5 Hz, 3H), 7.20 (d, J = 5.6 Hz, 1H), 7.05 (dd, J = 6.3, 2.5 Hz,
2H), 5.46 (s, 2H), 3.03 – 2.94 (m, 2H), 2.55 (s, 3H), 1.85 (dt, J = 15.0, 7.6 Hz, 2H), 1.43 (dq, J = 14.6, 7.3 Hz, 2H), 0.92 (t, J = 7.3 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 141.48, 129.64, 129.40, 129.17, 126.30, 126.25, 48.47, 29.06, 24.87, 22.44, 13.60. HRMS (ESI) calculated for C$_{19}$H$_{23}$N$_{4}$O, m/z 323.1866, found 323.1911 [M+H]$^+$. Compound 6b was synthesized similarly as compound 6a.

6b: N-(1-Benzyl-2-butyl-1H-imidazo[4,5-c]pyridin-4-yl)butyramide. Butyryl chloride was used as a reagent. 4 mg, 14 %. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 11.31 (s, 1H), 8.30 (d, J = 4.8 Hz, 1H), 7.38 – 7.34 (m, 3H), 7.27 (s, 1H), 7.04 (dd, J = 6.4, 2.6 Hz, 2H), 5.50 (s, 2H), 2.94 (t, J = 7.5 Hz, 2H), 2.78 (t, J = 7.4 Hz, 2H), 1.80 (dp, J = 22.1, 7.5 Hz, 4H), 1.41 (dt, J = 14.7, 7.4 Hz, 2H), 1.01 (t, J = 7.4 Hz, 3H), 0.91 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 174.32, 141.35, 133.50, 129.69, 129.16, 126.46, 103.38, 48.58, 39.27, 29.16, 27.45, 22.55, 18.38, 13.75. HRMS (ESI) calculated for C$_{21}$H$_{27}$N$_{4}$O, m/z 351.2179, found 351.2240 [M+H]$^+$.  

Synthesis of compound 10a: 1-Benzyl-2-butyl-4-chloro-1H-imidazo[4,5-c]pyridine. 4-Amino-2-chloropyridine (2.0 g, 15.6 mmol) was taken in 20 mL of conc. H$_2$SO$_4$ in an ice-bath to which was added 10 mL of conc. HNO$_3$ slowly. The reaction mixture was gradually brought to room temperature and stirred for 1 h. The reaction was quenched by pouring the reaction mixture on ice. Ammonium hydroxide solution was slowly added until a pH of 3 was reached. A white solid was obtained which was filtered, washed with water, and dried. This (N-nitro)aminopyridine intermediate was taken up in 10 mL of conc. H$_2$SO$_4$ and the reaction solution was heated at 90 °C for 30 min. It was cooled to room temperature and poured into ice. It was slowly neutralized with ammonium hydroxide solution until a pH of 7 and the formed yellow solid was filtered, washed with water and dried to obtain compound 7 as a mixture of 2-
chloro-3-nitropyridin-4-amine and 2-chloro-5-nitropyridin-4-amine intermediates. Sodium hydride (275 mg, 6.90 mmol) was carefully added to 20 mL of THF under N\textsubscript{2} and compound 7 (1.0 g, 5.76 mmol) was slowly added to the solution at 0 °C. The reaction mixture was stirred for 1 h, followed by the addition of benzyl bromide (0.75 mL, 6.34 mmol). The reaction mixture was stirred at room temperature for 2 h and poured into ice water. Then it was extracted with EtOAc (3 × 20 mL), washed with water, dried over sodium sulfate. The solvent was removed and the crude residue was purified using column chromatography (0-20% EtOAc in hexane) to obtain the compound 8 as a mixture of regioisomeric N-benzyl-2-chloro-3-nitropyridin-4-amine and N-benzyl-2-chloro-5-nitropyridin-4-amine intermediates. To this regioisomeric mixture (1.0 g, 4.2 mmol) in 20 mL of MeOH were added zinc dust (1.4 g, 21.0 mmol) and ammonium formate (1.4 g, 21.0 mmol). The reaction mixture was stirred at room temperature for 10 min and filtered through celite. Then the solvent was evaporated and the residue was dissolved in water. This was extracted with EtOAc (3 × 20 mL), washed with water and dried over sodium sulfate. The filtrate evaporated under vacuum, and chromatographed (0-20% EtOAc in hexane) to obtain the required N\textsuperscript{4}-benzyl-2-chloropyridine-3,4-diamine compound 9a. Also obtained was N\textsuperscript{4}-benzyl-6-chloropyridine-3,4-diamine as a side-product. To a solution of compound 9a (495 mg, 2.12 mmol) in 20 mL of anhydrous THF were added triethylamine (0.31 mL, 2.23 mmol) and valeryl chloride (0.28 mL, 2.33 mmol). The reaction mixture was refluxed for 1 h. The solvent was then removed under vacuum, and the residue was dissolved in 20 mL of EtOH and NaOH (170 mg, 4.24 mmol) in 2 mL of H\textsubscript{2}O was added. The reaction mixture was refluxed for 2 h. The solvent was then removed under vacuum, and the residue was dissolved in EtOAc and washed with water. The EtOAc fraction was dried using sodium sulfate and evaporated and purified using column chromatography (0-5% MeOH in CH\textsubscript{2}Cl\textsubscript{2}) to obtain the compound 10a (203 mg, 32%).

\[^1\text{H} \text{NMR (500 MHz, CDCl}_3\] δ 8.10 (d, J = 5.6 Hz, 1H), 7.35 – 7.30 (m, 3H), 7.08 (d, J = 5.6 Hz, 1H), 7.01 (dd, J = 7.4, 2.0 Hz, 2H), 5.34 (s, 2H), 2.91 – 2.86 (m, 2H), 1.78 (dt, J = 15.7, 7.7 Hz, 2H), 1.45 – 1.36 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H). \[^{13}\text{C} \text{NMR (126 MHz, CDCl}_3\] δ 158.03, 141.88,
141.75, 141.12, 137.03, 134.84, 129.39, 128.58, 126.25, 105.18, 47.90, 30.04, 27.63, 22.73, 13.82. MS (ESI) calculated for C$_{17}$H$_{19}$ClN$_3$, m/z 300.1262, found 300.1159 [M+H$^+$].

**Synthesis of compound 11a: 1-Benzyl-N,2-dibutyl-1H-imidazo[4,5-c]pyridin-4-amine.** To a solution of compound 10 (50 mg, 0.17 mmol) in 1 mL of dioxane were added potassium tert-butoxide (57 mg, 0.51 mmol), catalytic amount of 2-dicyclohexylphosphino-2'-(N,N-dimethyl amino)biphenyl (DavePhos) and tris(dibenzylideneacetone)dipalladium(0) (Pd$_2$(dba)$_3$) and butyl amine (83 µL, 0.83 mmol). The reaction mixture was then heated under microwave conditions (500 W, 100 ºC) in a sealed vial for 1 h. It was cooled to room temperature and filtered through celite and washed with MeOH. The solvent was removed and the crude residue was purified using column chromatography (0-5% MeOH in CH$_2$Cl$_2$) to obtain the compound 11a (21 mg, 36%). $^1$H NMR (500 MHz, CDCl$_3$) δ 7.84 (d, $J = 5.9$ Hz, 1H), 7.33 – 7.27 (m, 3H), 7.03 (dd, $J = 4.5$, 3.6 Hz, 2H), 6.48 (d, $J = 5.9$ Hz, 1H), 5.41 (s, 1H), 5.25 (s, 2H), 3.60 (dt, $J = 12.9$, 6.5 Hz, 2H), 2.80 – 2.74 (m, 2H), 1.73 – 1.67 (m, 4H), 1.49 (dt, $J = 15.0$, 7.4 Hz, 3H), 1.38 (dd, $J = 15.0$, 7.5 Hz, 2H), 0.96 (t, $J = 7.4$ Hz, 3H), 0.89 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 153.15, 151.39, 135.95, 129.24, 129.15, 128.15, 127.25, 126.34, 126.18, 96.16, 47.43, 41.09, 32.22, 30.27, 27.41, 22.69, 20.47, 14.11, 13.89. HRMS (ESI) calculated for C$_{21}$H$_{29}$N$_4$, m/z 337.2387, found 337.2451 [M+H$^+$].

Compound 11b was synthesized similarly as compound 11a.

**11b: N,1-Dibenzyl-2-butyl-1H-imidazo[4,5-c]pyridin-4-amine.** Benzyl amine was used as a reagent. 33 mg, 52%. $^1$H NMR (500 MHz, CDCl$_3$) δ 7.86 (d, $J = 5.9$ Hz, 1H), 7.45 (dd, $J = 7.9$, 0.9 Hz, 2H), 7.35 – 7.27 (m, 6H), 7.03 (d, $J = 6.4$ Hz, 2H), 6.54 (d, $J = 5.9$ Hz, 1H), 5.76 (s, 1H), 5.26 (s, 2H), 4.83 (d, $J = 5.6$ Hz, 2H), 2.79 – 2.74 (m, 2H), 1.72 – 1.67 (m, 2H), 1.37 (dq, $J =$
14.8, 7.4 Hz, 2H), 0.88 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 153.39, 150.92, 140.51, 139.81, 139.78, 135.88, 129.18, 128.62, 128.23, 128.19, 127.24, 126.36, 126.24, 96.73, 47.46, 45.40, 30.14, 27.38, 22.67, 13.88. HRMS (ESI) calculated for C$_{24}$H$_{27}$N$_4$, m/z 371.2230, found 371.2303 [M+H]$^+$. 

**Synthesis of compound 11c: 1-Benzyl-4-butoxy-2-butyl-1H-imidazo[4,5-c]pyridine.** To a suspension of sodium hydride (48 mg, 2.00 mmol) in 2 mL of anhydrous THF was added 1-butanol (0.18 mL, 2.00 mmol). It was stirred at room temperature for 1 h, followed by the addition of compound 10 (100 mg, 0.33 mmol). The reaction mixture was heated at 60 °C for 18 h and then solvent was evaporated under vacuum. The residue was extracted with EtOAc (3 × 10 mL), washed with water and dried over sodium sulfate. The solvent was removed and the crude residue was purified using column chromatography (0-5% MeOH in CH$_2$Cl$_2$) to obtain the compound 11c (61 mg, 55%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.85 (d, $J$ = 5.8 Hz, 1H), 7.33 – 7.28 (m, 3H), 7.01 (dd, $J$ = 7.7, 1.7 Hz, 2H), 6.77 (d, $J$ = 5.8 Hz, 1H), 5.30 (s, 2H), 4.52 (t, $J$ = 7.0 Hz, 2H), 2.87 – 2.82 (m, 2H), 1.90 (dd, $J$ = 15.0, 7.1 Hz, 2H), 1.75 (dt, $J$ = 15.7, 7.7 Hz, 2H), 1.58 – 1.48 (m, 2H), 1.38 (dq, $J$ = 14.7, 7.4 Hz, 2H), 0.97 (t, $J$ = 7.4 Hz, 3H), 0.88 (t, $J$ = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 156.04, 155.18, 142.02, 138.95, 135.59, 129.21, 128.27, 127.41, 126.27, 100.47, 66.25, 47.61, 31.34, 30.21, 27.44, 22.75, 19.45, 14.09, 13.85. HRMS (ESI) calculated for C$_{21}$H$_{28}$N$_3$O, m/z 338.2227, found 338.2307 [M+H]$^+$. 

**Synthesis of compound 12: 2,6-Dichloro-3-nitropyridin-4-amine.** 4-Amino-2,6-dichloropyridine (2.0 g, 12.27 mmol) was added to 20 mL of conc. H$_2$SO$_4$. The mixture was cooled to 0 °C and 10 mL of conc. HNO$_3$ was dropwise at 0 °C. The reaction mixture was stirred at room temperature for 1 h and then poured into crushed ice. The white solid was filtered, washed with water and dried. This intermediate was dissolved in 10 mL of conc. H$_2$SO$_4$ and the reaction
solution was heated at 90 °C for 30 min. It was cooled to room temperature and poured into ice. It was slowly neutralized with ammonium hydroxide solution until a pH of 9 and the formed yellow solid was filtered, washed with water and dried to obtain compound 12 as light yellow solid. \(^1\text{H} \text{NMR} (500 \text{ MHz}, \text{MeOD}) \delta 6.84 \text{ (s, 1H)}. \:^{13}\text{C} \text{NMR} (126 \text{ MHz}, \text{MeOD}) \delta 152.53, 151.18, 144.57, 111.12.

**Synthesis of compound 13: N-Benzyl-2,6-dichloro-3-nitropyridin-4-amine.** Sodium hydride (138 mg, 5.77 mmol) was carefully suspended in 10 mL of dry THF under \(N_2\). The grey suspension was cooled to 0 °C and compound 12 (1.0 g, 4.81 mmol) was slowly added to the suspension at 0 °C. The reaction mixture was stirred for 1 h, followed by the addition of benzyl bromide (0.5 mL, 5.29 mmol). The reaction mixture was stirred at room temperature for 2 h and poured into ice water. Then it was extracted with EtOAc (3 × 20 mL), washed with water, dried over sodium sulfate. The solvent was removed and the crude residue was purified using column chromatography (0-20% EtOAc in hexane) to obtain the compound 13 as yellow solid. \(^1\text{H} \text{NMR} (500 \text{ MHz}, \text{CDCl}_3) \delta 7.44 – 7.34 \text{ (m, 3H)}, 7.32 – 7.27 \text{ (m, 2H)}, 6.99 \text{ (s, 1H)}, 6.67 \text{ (s, 1H)}, 4.47 \text{ (d, } J = 5.4 \text{ Hz, 2H}). \:^{13}\text{C} \text{NMR} (126 \text{ MHz}, \text{CDCl}_3) \delta 152.07, 149.97, 145.68, 135.01, 129.48, 128.71, 127.32, 106.62, 47.74.

**Synthesis of compound 14: \(N^4\)-Benzyl-6-chloro-3-nitro-\(N^2\)-(2,4,4-trimethylpentan-2-yl)pyridine-2,4-diamine.** To a solution of compound 13 (300 mg, 1.02 mmol) in 20 mL of CH\(_2\)Cl\(_2\) were added triethylamine (0.21 mL, 1.53 mmol) and tert-octylamine (0.5 mL, 3.06 mmol). The reaction mixture was refluxed for 48 h and the solvent was removed under vacuum. The crude residue was purified using column chromatography (0-20% EtOAc in hexane) to obtain the compound 14 as yellow solid (360 mg, 91%). \(^1\text{H} \text{NMR} (500 \text{ MHz}, \text{CDCl}_3) \delta 9.57 \text{ (s, 1H)}, 9.51 \text{ (s, 1H)}, 7.39 \text{ (ddd, } J = 7.5, 4.4, 1.3 \text{ Hz, 2H)}, 7.36 – 7.30 \text{ (m, 3H)}, 5.92 \text{ (s, 1H)}, 4.44 \text{ (d, } J = 5.4 \text{ Hz,
2H), 1.95 (s, 2H), 1.56 (s, 6H), 0.98 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 155.05, 154.25, 153.44, 136.10, 129.24, 128.24, 127.43, 115.80, 94.21, 57.32, 51.54, 47.64, 31.94, 31.66, 29.84. MS (ESI) calculated for C$_{20}$H$_{28}$ClN$_4$O$_2$, m/z 391.1895, found 391.1901 [M+H]$^+$. 

**Synthesis of compound 16:** 1-Benzyl-2-butyl-6-chloro-$N$-(2,4,4-trimethylpentan-2-yl)-1$H$-imidazo[4,5-c]pyridin-4-amine. To a solution of compound 14 (260 mg, 0.66 mmol) in 20 mL of MeOH were added zinc dust (434 mg, 6.60 mmol) and ammonium formate (416 mg, 6.60 mmol). The reaction mixture was stirred at room temperature for 10 min and filtered through celite. Then the solvent was evaporated and the residue was dissolved in water. This was extracted with EtOAc (3 × 20 mL), washed with water and dried over sodium sulfate. The solvent was removed under vacuum to obtain compound 15, brown oil (184 mg, 77%). To a solution of compound 15 (184 mg, 0.50 mmol) in 10 mL of anhydrous THF were added triethylamine (74 µL, 0.52 mmol) and valeryl chloride (62 µL, 0.50 mmol). The reaction mixture was refluxed for 1 h. The solvent was then removed under vacuum, and the residue was dissolved in 10 mL of EtOH and NaOH (40 mg, 1.00 mmol) in 1 mL of H$_2$O was added. The reaction mixture was refluxed for 5 h. The solvent was then removed under vacuum, and the residue was dissolved in EtOAc and washed with water. The EtOAc fraction was dried using sodium sulfate and evaporated and purified using column chromatography (0-20% EtOAc in hexane) to obtain the compound 16 as yellow solid (120 mg, 56%). $^1$H NMR (500 MHz, CDCl$_3$) δ 7.34 – 7.28 (m, 3H), 7.02 (d, $J = 6.5$ Hz, 2H), 6.42 (s, 1H), 5.38 (s, 1H), 5.16 (s, 2H), 2.76 – 2.69 (m, 2H), 2.02 (s, 2H), 1.71 – 1.65 (m, 4H), 1.60 (s, 6H), 1.37 (dd, $J = 15.0$, 7.5 Hz, 2H), 1.01 (s, 9H), 0.89 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 153.37, 149.45, 141.67, 140.77, 135.64, 129.21, 128.22, 126.31, 125.50, 93.95, 55.74, 51.42, 47.42, 31.91, 31.73, 30.12, 29.96, 27.37, 22.66, 13.87. MS (ESI) calculated for C$_{25}$H$_{36}$ClN$_4$, m/z 427.2623, found 427.2635 [M+H]$^+$. 

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Compound 16 (34 mg, 0.082 mmol) was dissolved in 1 mL of HCl (4M in dioxane) and stirred at room temperature for 30 min. Then the solvent was removed under vacuum to obtain compound 17 (11 mg, 42%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.36 – 7.30 (m, 3H), 7.01 (d, $J = 6.4$ Hz, 2H), 6.58 (s, 1H), 5.23 (s, 2H), 5.21 (s, 2H), 2.80 – 2.73 (m, 2H), 1.72 (dt, $J = 15.5$, 7.7 Hz, 2H), 1.39 (dq, $J = 14.8$, 7.4 Hz, 2H), 0.90 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 154.83, 149.94, 142.31, 141.73, 135.26, 129.32, 128.57, 128.41, 126.26, 96.29, 47.59, 29.92, 27.36, 22.63, 13.87. HRMS (ESI) calculated for C$_{17}$H$_{20}$ClN$_4$, m/z 315.1371, found 315.1422 [M+H]$^+$. 


To a solution of compound 16 (70 mg, 0.16 mmol) in 1 mL of dioxane were added potassium tert-butoxide (92 mg, 0.82 mmol), catalytic amount of DavePhos and Pd$_2$(dba)$_3$ and tert-octylamine (83 $\mu$L, 0.83 mmol). The reaction mixture was then heated under microwave conditions (500 W, 100 ºC) in a sealed vial for 1 h. It was cooled to room temperature and filtered through celite and washed with MeOH. The solvent was removed and the crude residue was purified using column chromatography (0-20% EtOAc in hexane) to obtain the compound 18a, brown solid (41 mg, 49%). Compound 18a (33 mg, 0.063 mmol) was dissolved in 1 mL of HCl (4M in dioxane) and stirred at room temperature for 30 min. Then the solvent was removed under vacuum to obtain compound 19a as brown solid (11 mg, 58%). $^1$H NMR (500 MHz, DMSO) $\delta$ 7.37 – 7.31 (m, 2H), 7.30 – 7.25 (m, 1H), 7.09 – 7.04 (m, 2H), 6.41 (s, 2H), 5.65 (s, 1H), 5.24 (s, 2H), 2.73 – 2.67 (m, 2H), 1.59 (dt, $J = 15.3$, 7.5 Hz, 2H), 1.31 (dq, $J = 14.7$, 7.4 Hz, 2H), 0.83 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 151.81, 147.80, 144.08, 136.91, 128.81, 128.77, 127.47, 126.29, 117.96, 76.19, 46.11, 29.27, 26.17, 21.80, 13.68. HRMS (ESI) calculated for C$_{17}$H$_{22}$N$_5$, m/z 296.1870, found 296.1906 [M+H]$^+$. 

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Compounds 19b-19o were synthesized similarly as compound 19a.

19b: 1-Benzyl-\(N^6,2\)-dibutyl-1\(H\)-imidazo[4,5-c]pyridine-4,6-diamine. Butyl amine was used as a reagent. Brown solid. 23 mg, 79%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.31 (ddd, \(J = 8.6, 6.4, 3.4\) Hz, 3H), 7.05 (d, \(J = 6.7\) Hz, 2H), 5.46 (s, 1H), 5.26 (s, 1H), 5.16 (s, 2H), 3.03 (t, \(J = 7.1\) Hz, 2H), 2.72 – 2.66 (m, 2H), 1.68 (dd, \(J = 15.5, 7.6\) Hz, 2H), 1.55 (dd, \(J = 14.8, 7.2\) Hz, 2H), 1.43 – 1.33 (m, 4H), 0.90 (dt, \(J = 16.4, 7.4\) Hz, 6H). \(^1\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 152.42, 148.50, 144.42, 135.99, 129.14, 128.06, 126.33, 74.80, 47.20, 43.09, 31.41, 30.03, 27.37, 22.66, 20.40, 14.02, 13.89. HRMS (ESI) calculated for C\(_{21}\)H\(_{30}\)N\(_5\), m/z 352.2496, found 352.2515 [M+H]^+.

18c: 1-Benzyl-2-butyl-\(N^6\)-heptyl-\(N^4\)-(2,4,4-trimethylpentan-2-yl)-1\(H\)-imidazo[4,5-c]pyridine-4,6-diamine. Heptylamine was used as a reagent. Brown oil. 43 mg, 65%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.32 – 7.26 (m, 3H), 7.05 (d, \(J = 6.7\) Hz, 2H), 5.39 (s, 1H), 5.13 (s, 1H), 5.11 (s, 2H), 3.13 (t, \(J = 7.2\) Hz, 2H), 2.68 – 2.64 (m, 2H), 2.04 (s, 2H), 1.65 – 1.55 (m, 11H), 1.41 – 1.22 (m, 12H), 1.01 (s, 9H), 0.89 – 0.84 (m, 6H). \(^1\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 154.28, 150.38, 149.22, 142.39, 136.63, 128.97, 128.05, 127.76, 126.40, 120.44, 95.52, 73.57, 55.27, 51.72, 47.00, 43.63, 31.97, 31.94, 31.90, 31.79, 30.47, 30.29, 29.97, 29.32, 27.44, 27.39, 22.77, 22.71, 14.25, 13.90. MS (ESI) calculated for C\(_{32}\)H\(_{52}\)N\(_5\), m/z 506.4217, found 506.4209 [M+H]^+.

19c: 1-Benzyl-2-butyl-\(N^6\)-heptyl-1\(H\)-imidazo[4,5-c]pyridine-4,6-diamine. Light brown solid. 24 mg, 80%. \(^1\)H NMR (500 MHz, DMSO) \(\delta\) 8.03 (s, 2H), 7.38 – 7.33 (m, 2H), 7.32 – 7.28 (m, 1H), 7.12 (d, \(J = 7.2\) Hz, 2H), 6.84 (s, 1H), 5.98 (s, 1H), 5.39 (s, 2H), 3.08 (t, \(J = 7.0\) Hz, 2H), 2.73 – 2.68 (m, 2H), 1.59 (dt, \(J = 15.3, 8.2\) Hz, 2H), 1.50 (dd, \(J = 14.3, 7.2\) Hz, 2H), 1.33 – 1.21 (m, 12H), 0.85 (t, \(J = 7.0\) Hz, 3H), 0.81 (t, \(J = 7.4\) Hz, 3H). \(^1\)C NMR (126 MHz, DMSO) \(\delta\) 154.62, 145.66, 145.15, 136.32, 128.96, 128.86, 127.73, 126.64, 126.55, 74.21, 46.34, 42.24,
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18d: 1-Benzyl-2-butyl-N\(^6\)-(cyclohexylmethyl)-N\(^4\)-(2,4,4-trimethylpentan-2-yl)-1H-imidaz[4,5-c]pyridine-4,6-diamine. \(N\)-Cyclohexylmethylamine was used as a reagent. Yellow solid. 50 mg, 62%. \(^1^H\) NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.32 – 7.27 (m, 3H), 7.06 (d, \(J = 6.7\) Hz, 2H), 5.37 (s, 1H), 5.10 (s, 2H), 2.99 (d, \(J = 6.7\) Hz, 2H), 2.69 – 2.64 (m, 2H), 2.04 (s, 2H), 1.79 (d, \(J = 13.2\) Hz, 2H), 1.73 – 1.60 (m, 9H), 1.58 (s, 6H), 1.35 (dd, \(J = 15.0, 7.5\) Hz, 2H), 1.27 – 1.13 (m, 4H), 1.01 (s, 9H), 0.98 – 0.91 (m, 2H), 0.86 (t, \(J = 7.4\) Hz, 3H). \(^{13}C\) NMR (126 MHz, CDCl\(_3\)) \(\delta\) 154.37, 150.36, 149.19, 142.41, 136.65, 128.97, 127.76, 126.44, 120.35, 73.62, 55.26, 51.67, 50.18, 47.02, 37.88, 31.91, 31.80, 31.48, 30.51, 30.31, 27.46, 26.80, 26.17, 22.72, 13.91. MS (ESI) calculated for C\(_{32}\)H\(_{50}\)N\(_5\), m/z 504.4061, found 504.41 \([\text{M+H}]^+\).

19d: 1-Benzyl-2-butyl-N\(^6\)-(cyclohexylmethyl)-1H-imidazo[4,5-c]pyridine-4,6-diamine. Light brown. 28 mg, 80%. \(^1^H\) NMR (500 MHz, DMSO) \(\delta\) 7.35 (t, \(J = 7.3\) Hz, 2H), 7.31 – 7.27 (m, 1H), 7.10 (d, \(J = 7.2\) Hz, 2H), 6.34 (d, \(J = 3.1\) Hz, 1H), 5.85 (s, 1H), 5.33 (s, 2H), 2.92 (t, \(J = 6.1\) Hz, 2H), 2.71 – 2.66 (m, 2H), 1.75 – 1.64 (m, 4H), 1.57 (dt, \(J = 15.3, 7.6\) Hz, 3H), 1.49 (dtd, \(J = 11.2, 7.4, 3.7\) Hz, 1H), 1.30 (dt, \(J = 14.8, 7.4\) Hz, 2H), 1.21 – 1.09 (m, 4H), 0.91 (dd, \(J = 22.3, 10.5\) Hz, 2H), 0.81 (t, \(J = 7.4\) Hz, 3H). \(^{13}C\) NMR (126 MHz, DMSO) \(\delta\) 153.32, 146.28, 145.05, 136.69, 128.79, 127.61, 126.53, 116.94, 74.21, 48.49, 46.22, 36.76, 30.47, 29.02, 26.21, 26.05, 25.43, 21.75, 13.65. HRMS (ESI) calculated for C\(_{24}\)H\(_{36}\)N\(_5\), m/z 394.2965, found 394.3001 \([\text{M+H}]^+\).

18e: 1-Benzyl-2-butyl-N\(^6\)-phenyl-N\(^4\)-(2,4,4-trimethylpentan-2-yl)-1H-imidazo[4,5-c]pyridine-4,6-diamine. Aniline was used as a reagent. Yellow solid. 51 mg, 55%. \(^1^H\) NMR (500 MHz,
CDCl₃ δ 7.31 (dt, J = 16.5, 5.5 Hz, 3H), 7.22 (t, J = 7.8 Hz, 2H), 7.16 (d, J = 7.6 Hz, 2H), 7.05 (d, J = 6.7 Hz, 2H), 6.90 (t, J = 6.9 Hz, 1H), 6.16 (s, 1H), 6.02 (s, 1H), 5.23 (s, 1H), 5.12 (s, 2H), 2.74 – 2.67 (m, 2H), 2.03 (s, 2H), 1.66 (dd, J = 15.8, 8.2 Hz, 4H), 1.60 (s, 6H), 1.38 (dd, J = 15.0, 7.5 Hz, 2H), 1.02 (s, 9H), 0.89 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 151.49, 149.42, 149.11, 142.46, 141.51, 136.32, 129.16, 129.06, 127.93, 126.53, 121.98, 120.91, 118.83, 55.42, 51.80, 47.26, 31.93, 31.80, 30.44, 30.19, 27.46, 22.71, 13.91. MS (ESI) calculated for C₃₁H₄₂N₅, m/z 484.3435, found 484.3459 [M+H]⁺.

19e: 1-Benzyl-2-butyl-N⁶-phenyl-¹H-imidazo[4,5-c]pyridine-4,6-diamine. Light yellow solid. 28 mg, 76%. ¹H NMR (500 MHz, DMSO) δ 9.10 (s, 1H), 8.08 (s, 1H), 7.39 – 7.35 (m, 2H), 7.33 – 7.28 (m, 3H), 7.11 (t, J = 7.3 Hz, 4H), 7.01 (t, J = 7.3 Hz, 1H), 6.41 (s, 1H), 5.44 (s, 2H), 2.82 (t, J = 7.5 Hz, 2H), 1.63 (dt, J = 15.3, 7.6 Hz, 2H), 1.33 (dd, J = 14.9, 7.4 Hz, 2H), 0.84 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, DMSO) δ 146.34, 140.41, 136.06, 129.52, 128.92, 127.84, 126.69, 122.44, 118.87, 46.68, 28.89, 26.15, 21.72, 13.63. HRMS (ESI) calculated for C₂₃H₂₆N₅, m/z 372.2183, found 372.2219 [M+H]⁺.

18f: N⁶,¹-Dibenzyl-2-butyl-N⁴-(2,4,4-trimethylpentan-2-yl)-¹H-imidazo[4,5-c]pyridine-4,6-diamine. Benzyl amine was used as a reagent. Light brown solid. 30 mg, 46%. ¹H NMR (500 MHz, CDCl₃) δ 7.35 (d, J = 7.4 Hz, 2H), 7.26 (dddd, J = 9.4, 7.2, 6.1, 1.6 Hz, 6H), 7.03 (d, J = 6.8 Hz, 2H), 5.44 (s, 1H), 5.17 (s, 1H), 5.07 (s, 2H), 4.42 (s, 2H), 2.70 – 2.64 (m, 2H), 1.67 – 1.60 (m, 2H), 1.55 (s, 6H), 1.35 (dq, J = 14.7, 7.4 Hz, 2H), 1.02 – 0.94 (m, 9H), 0.87 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 153.83, 150.58, 149.20, 142.27, 140.52, 136.49, 128.98, 128.56, 127.80, 127.68, 126.97, 126.46, 126.39, 74.17, 55.29, 51.62, 47.59, 47.07, 31.87, 31.76, 30.45, 30.28, 30.20, 27.42, 22.70, 13.90. MS (ESI) calculated for C₃₂H₄₄N₅, m/z 498.3591, found 498.3599 [M+H]⁺.
19f: $N^6$-Dibenzyl-2-butyl-$1H$-imidazo[4,5-c]pyridine-4,6-diamine. White solid. 22 mg, 85%. $^1$H NMR (500 MHz, DMSO) δ 8.07 (s, 2H), 7.38 – 7.35 (m, 2H), 7.34 – 7.29 (m, 5H), 7.29 – 7.25 (m, 1H), 7.07 (d, $J$ = 6.3 Hz, 2H), 6.06 (s, 1H), 5.33 (s, 2H), 4.38 (s, 2H), 2.75 – 2.69 (m, 2H), 1.58 (dt, $J$ = 15.3, 7.6 Hz, 2H), 1.30 (dt, $J$ = 14.8, 7.4 Hz, 2H), 0.81 (t, $J$ = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, DMSO) δ 154.68, 145.29, 137.99, 136.12, 128.85, 128.45, 127.78, 127.51, 127.23, 126.74, 75.08, 46.43, 45.67, 28.76, 26.16, 21.70, 13.62. HRMS (ESI) calculated for C$_{24}$H$_{28}$N$_5$, m/z 386.2339, found 386.2389 [M+H]+.

19g: 1-Benzyl-2-butyl-$N^6$-(4-methoxybenzyl)-$1H$-imidazo[4,5-c]pyridine-4,6-diamine. Light yellow solid. 22 mg, 69%. $^1$H NMR (500 MHz, DMSO) δ 8.07 (s, 2H), 7.34 – 7.28 (m, 5H), 7.08 (d, $J$ = 6.4 Hz, 2H), 6.87 (d, $J$ = 8.7 Hz, 2H), 6.04 (s, 1H), 5.34 (s, 2H), 4.29 (s, 2H), 3.72 (s, 3H), 2.75 – 2.70 (m, 2H), 1.58 (dt, $J$ = 15.3, 7.6 Hz, 2H), 1.29 (dd, $J$ = 14.9, 7.4 Hz, 2H), 0.81 (t, $J$ = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, DMSO) δ 158.48, 154.67, 145.28, 145.27, 136.13, 129.73, 128.92, 128.84, 127.78, 126.74, 113.82, 75.11, 55.08, 46.43, 45.18, 28.76, 26.16, 21.71, 13.62. HRMS (ESI) calculated for C$_{25}$H$_{30}$N$_5$O, m/z 416.2445, found 416.2490 [M+H]$^+$.

19h: 1-Benzyl-2-butyl-$N^6$-(3-methoxybenzyl)-$1H$-imidazo[4,5-c]pyridine-4,6-diamine. Brown solid. 27 mg, 69%. $^1$H NMR (500 MHz, DMSO) δ 7.32 – 7.27 (m, 3H), 7.23 (t, $J$ = 7.9 Hz, 1H), 7.06 (d, $J$ = 6.1 Hz, 2H), 6.94 (dd, $J$ = 12.3, 4.8 Hz, 2H), 6.83 (dd, $J$ = 8.1, 2.2 Hz, 1H), 6.03 (s, 1H), 5.31 (s, 2H), 4.33 (d, $J$ = 5.8 Hz, 2H), 3.71 (s, 3H), 2.70 (t, $J$ = 7.6 Hz, 2H), 1.57 (dt, $J$ = 15.3, 7.6 Hz, 2H), 1.29 (dd, $J$ = 14.9, 7.4 Hz, 2H), 0.81 (t, $J$ = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, DMSO) δ 159.37, 136.32, 129.52, 128.80, 127.72, 126.70, 119.55, 113.13, 112.50, 75.05, 55.00, 46.33, 45.63, 40.11, 40.02, 39.94, 28.89,
26.21, 21.72, 13.63. HRMS (ESI) calculated for C_{25}H_{30}N_{5}O, m/z 416.2445, found 416.2506 [M+H]⁺.

19i: 1-Benzyl-2-butyl-N⁶-(4-(trifluoromethyl)benzyl)-1H-imidazo[4,5-c]pyridine-4,6-diamine. 4-(Trifluoromethyl)benzylamine was used as a reagent. Brown solid. 17 mg, 77%. ¹H NMR (500 MHz, DMSO) δ 7.60 (d, J = 8.2 Hz, 2H), 7.51 (d, J = 8.1 Hz, 2H), 7.29 – 7.21 (m, 3H), 7.02 (d, J = 6.7 Hz, 2H), 6.23 (s, 1H), 5.70 (s, 2H), 5.57 (s, 1H), 5.15 (s, 2H), 4.41 (d, J = 6.3 Hz, 2H), 2.69 – 2.63 (m, 2H), 1.56 (dt, J = 15.3, 7.5 Hz, 2H), 1.29 (dd, J = 14.9, 7.4 Hz, 2H), 0.82 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, DMSO) δ 153.45, 150.32, 149.41, 146.48, 143.12, 137.24, 128.58, 127.82, 127.31, 126.51, 124.88, 124.85, 118.91, 75.32, 46.04, 44.94, 29.43, 26.19, 21.82, 13.69. HRMS (ESI) calculated for C_{25}H_{27}F_{3}N_{5}, m/z 454.2213, found 454.2284 [M+H]⁺.

19j: 1-Benzyl-2-butyl-N⁶-(4-chlorobenzyl)-1H-imidazo[4,5-c]pyridine-4,6-diamine. 4-Chlorobenzylamine was used as a reagent. Yellow solid. 15 mg, 63%. ¹H NMR (500 MHz, DMSO) δ 7.33 – 7.25 (m, 8H), 7.02 (d, J = 6.6 Hz, 2H), 6.18 (s, 1H), 5.83 (s, 2H), 5.59 (s, 1H), 5.16 (s, 2H), 4.30 (d, J = 6.0 Hz, 2H), 2.68 – 2.64 (m, 2H), 1.56 (dt, J = 15.3, 7.5 Hz, 2H), 1.29 (dd, J = 14.9, 7.4 Hz, 2H), 0.82 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, DMSO) δ 150.57, 149.14, 143.25, 140.18, 137.18, 130.77, 129.08, 128.63, 127.96, 127.37, 126.56, 118.72, 75.34, 46.06, 44.71, 40.11, 40.02, 39.94, 29.40, 26.20, 21.82, 13.69. HRMS (ESI) calculated for C_{24}H_{27}ClN_{5}, m/z 420.1950, found 420.1976 [M+H]⁺.

18k: 1-Benzyl-2-butyl-N⁶-(furan-2-ylmethyl)-N⁴-(2,4,4-trimethylpentan-2-yl)-1H-imidazo[4,5-c]pyridine-4,6-diamine. Furfurylamine was used as a reagent. Yellow oil. 53 mg, 54%. ¹H NMR (500 MHz, CDCl₃) δ 7.30 (ddd, J = 10.9, 4.6, 1.1 Hz, 4H), 7.04 (d, J = 6.6 Hz, 2H), 6.28 (dd, J = 3.2, 1.9 Hz, 1H), 6.15 (dd, J = 3.2, 0.6 Hz, 1H), 5.50 (s, 1H), 5.10 (s, 2H), 4.43 (s, 2H), 2.72 –
2.64 (m, 2H), 2.03 (s, 2H), 1.68 – 1.59 (m, 4H), 1.58 (s, 6H), 1.35 (dt, J = 14.7, 7.4 Hz, 2H), 1.00 (s, 9H), 0.87 (t, J = 7.4 Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 153.92, 153.28, 150.77, 149.15, 142.19, 141.69, 136.47, 129.00, 127.83, 126.42, 110.36, 106.53, 74.85, 55.32, 51.68, 47.09, 40.78, 31.89, 31.77, 30.47, 30.31, 27.43, 22.71, 13.90. MS (ESI) calculated for C\(_{30}\)H\(_{42}\)N\(_5\)O, m/z 488.3384, found 488.3446 [M+H]+.

19k: 1-Benzyl-2-butyl-\(N^6\)-(furan-2-ylmethyl)-1\(H\)-imidazo[4,5-c]pyridine-4,6-diamine. Brown solid. 21 mg, 55%. \(^1\)H NMR (500 MHz, DMSO) \(\delta\) 7.55 (s, 1H), 7.34 (t, J = 7.3 Hz, 2H), 7.31 – 7.27 (m, 1H), 7.10 (d, J = 7.3 Hz, 2H), 6.36 (dd, J = 3.0, 1.9 Hz, 1H), 6.29 (d, J = 2.6 Hz, 1H), 5.99 (s, 1H), 5.31 (s, 2H), 4.35 (d, J = 5.8 Hz, 2H), 2.73 – 2.67 (m, 2H), 1.58 (dt, J = 15.2, 7.6 Hz, 2H), 1.30 (dd, J = 14.9, 7.4 Hz, 2H), 0.82 (t, J = 7.4 Hz, 3H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 142.17, 136.66, 128.79, 127.62, 126.61, 110.39, 107.43, 75.34, 60.19, 46.26, 38.71, 29.07, 26.21, 21.76, 13.65. HRMS (ESI) calculated for C\(_{22}\)H\(_{26}\)N\(_5\)O, m/z 376.2132, found 376.2184 [M+H]+.

18l: 1-Benzyl-2-butyl-\(N^6\)-(pyridin-4-ylmethyl)-\(N^4\)-(2,4,4-trimethylpentan-2-yl)-1\(H\)-imidazo[4,5-c]pyridine-4,6-diamine. 4-Picolylamine was used as a reagent. Yellow solid. 25 mg, 31%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.48 (dd, J = 4.5, 1.6 Hz, 2H), 7.29 – 7.25 (m, 3H), 7.24 (dd, J = 4.5, 1.5 Hz, 2H), 7.01 (d, J = 6.1 Hz, 2H), 5.40 (s, 1H), 5.06 (s, 2H), 4.49 (s, 2H), 2.70 – 2.64 (m, 2H), 1.92 (s, 2H), 1.63 (dt, J = 15.5, 7.7 Hz, 2H), 1.48 (s, 6H), 1.36 (dt, J = 14.9, 7.4 Hz, 2H), 0.95 (s, 9H), 0.87 (t, J = 7.4 Hz, 3H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 149.83, 136.31, 129.01, 127.89, 126.40, 122.20, 74.67, 55.25, 51.56, 47.13, 46.09, 31.81, 31.75, 31.71, 30.44, 30.23, 27.41, 22.69, 13.89. MS (ESI) calculated for C\(_{31}\)H\(_{43}\)N\(_6\), m/z 499.3544, found 499.3475 [M+H]+.
19l: 1-Benzyl-2-butyl-N6-(pyridin-4-ylmethyl)-1H-imidazo[4,5-c]pyridine-4,6-diamine. Brown solid. 7 mg, 47%. 1H NMR (500 MHz, DMSO) \( \delta \) 8.43 (dd, \( J = 4.5, \ 1.5 \) Hz, 2H), 7.27 (ddd, \( J = 9.7, \ 6.8, \ 4.4 \) Hz, 5H), 7.03 (d, \( J = 6.7 \) Hz, 2H), 6.50 – 6.28 (m, 2H), 5.65 (s, 1H), 5.18 (s, 2H), 4.37 (d, \( J = 6.3 \) Hz, 2H), 2.69 – 2.65 (m, 2H), 1.57 (dt, \( J = 15.3, \ 7.5 \) Hz, 2H), 1.30 (dd, \( J = 14.9, \ 7.4 \) Hz, 2H), 0.81 (d, \( J = 7.4 \) Hz, 3H). 13C NMR (126 MHz, DMSO) \( \delta \) 149.28, 137.09, 130.51, 128.65, 127.43, 126.53, 122.35, 113.97, 75.37, 46.09, 44.38, 29.34, 26.19, 21.80, 13.68. HRMS (ESI) calculated for C\(_{23}\)H\(_{27}\)N\(_6\), m/z 387.2292, found 387.2299 [M+H]+.

19m: 1-Benzyl-2-butyl-N6-(pyridin-3-ylmethyl)-1H-imidazo[4,5-c]pyridine-4,6-diamine. 3-Picolylamine was used as a reagent. Light brown solid. 33 mg, 63%. 1H NMR (500 MHz, DMSO) \( \delta \) 8.92 (s, 1H), 8.80 (d, \( J = 5.3 \) Hz, 1H), 8.46 (d, \( J = 8.0 \) Hz, 1H), 8.12 (s, 2H), 7.94 (dd, \( J = 7.9, \ 5.7 \) Hz, 1H), 7.69 (s, 1H), 7.33 – 7.25 (m, 3H), 7.04 (d, \( J = 6.9 \) Hz, 2H), 6.03 (s, 1H), 5.32 (s, 2H), 4.64 (s, 2H), 2.72 (t, \( J = 7.6 \) Hz, 2H), 1.56 (dt, \( J = 15.2, \ 7.6 \) Hz, 2H), 1.28 (dd, \( J = 14.9, \ 7.4 \) Hz, 2H), 0.80 (t, \( J = 7.4 \) Hz, 3H). 13C NMR (126 MHz, DMSO) \( \delta \) 154.65, 145.80, 145.01, 143.55, 141.29, 141.12, 138.17, 136.10, 128.83, 127.76, 126.68, 126.52, 75.49, 46.45, 42.51, 28.77, 26.12, 21.68, 13.60. HRMS (ESI) calculated for C\(_{23}\)H\(_{27}\)N\(_6\), m/z 387.2292, found 387.2296 [M+H]+.

19n: 1-Benzyl-2-butyl-N6-(naphthalen-1-ylmethyl)-1H-imidazo[4,5-c]pyridine-4,6-diamine. 1-Naphthylmethylamine was used as a reagent. Light brown solid. 28 mg, 70%. 1H NMR (500 MHz, DMSO) \( \delta \) 12.12 (s, 1H), 8.10 (d, \( J = 8.0 \) Hz, 1H), 8.04 (s, 2H), 7.99 – 7.96 (m, 1H), 7.88 (d, \( J = 8.2 \) Hz, 1H), 7.61 – 7.53 (m, 3H), 7.45 (dd, \( J = 8.1, \ 7.1 \) Hz, 1H), 7.33 – 7.28 (m, 3H), 7.08 (d, \( J = 6.4 \) Hz, 2H), 6.22 (s, 1H), 5.35 (s, 2H), 4.83 (s, 2H), 2.74 – 2.68 (m, 2H), 1.58 (dt, \( J = 15.3, \ 7.6 \) Hz, 2H), 1.29 (dd, \( J = 14.9, \ 7.4 \) Hz, 2H), 0.81 (t, \( J = 7.4 \) Hz, 3H). 13C NMR (126 MHz, DMSO) \( \delta \) 154.69, 145.51, 145.31, 136.23, 133.44, 132.90, 130.92, 128.83, 128.63, 127.99,
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127.76, 126.67, 126.39, 125.97, 125.49, 125.43, 123.63, 74.91, 46.39, 43.99, 28.80, 26.20, 21.71, 13.62. HRMS (ESI) calculated for C_{28}H_{30}N_{5}, m/z 436.2496, found 436.2549 [M+H]^+.

19o: \(N^6\)-[1,1'-Biphenyl]-4-ylmethyl]-1-benzyl-2-butyl-1\(H\)-imidazo[4,5-c]pyridine-4,6-diamine. [1,1'-biphenyl]-4-yl methane amine was used as a reagent. Light brown solid. 21 mg, 51%. \(^1\)H NMR (500 MHz, DMSO) \(\delta\) 12.36 (s, 1H), 8.04 (s, 2H), 7.63 (dd, \(J = 12.4, 7.8\) Hz, 4H), 7.50 – 7.42 (m, 4H), 7.40 – 7.33 (m, 1H), 7.28 (t, \(J = 7.3\) Hz, 2H), 7.23 (t, \(J = 7.2\) Hz, 1H), 7.07 (d, \(J = 7.3\) Hz, 2H), 6.07 (s, 1H), 5.33 (s, 2H), 4.42 (s, 2H), 2.71 (t, \(J = 7.6\) Hz, 2H), 1.57 (dt, \(J = 15.2, 7.7\) Hz, 2H), 1.29 (dq, \(J = 14.6, 7.3\) Hz, 2H), 0.81 (t, \(J = 7.3\) Hz, 3H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 154.65, 145.40, 145.35, 139.80, 139.07, 137.24, 136.17, 128.95, 128.80, 128.06, 127.67, 127.43, 126.71, 126.69, 126.57, 75.11, 46.38, 45.31, 28.81, 26.18, 21.69, 13.61. HRMS (ESI) calculated for C_{30}H_{32}N_{5}, m/z 462.2652, found 462.2698 [M+H]^+.

Synthesis of compound 19p and 19r: \(N^6\)-(4-(aminomethyl)benzyl]-1-benzyl-2-butyl-1\(H\)-imidazo[4,5-c]pyridine-4,6-diamine. To a solution of compound 16 (70 mg, 0.16 mmol) in 1 mL of dioxane were added potassium tert-butoxide (90 mg, 0.80 mmol), catalytic amount of DavePhos and Pd_{2}(dba)_{3} and p-xyllylenediamine (109 mg, 0.80 mmol). The reaction mixture was then heated under microwave conditions (500 W, 100 °C) in a sealed vial for 1 h. It was cooled to room temperature and filtered through celite and washed with MeOH. The solvent was removed and the crude residue was purified using column chromatography (0-20% EtOAc in hexane) to obtain the compounds 18p and 18r. Compound 18p (33 mg, 0.063 mmol) was dissolved in 1 mL of HCl (4M in dioxane) and stirred at room temperature for 30 min. Then the solvent was removed under vacuum to obtain compound 19p as light yellow solid (5 mg, 63%). \(^1\)H NMR (500 MHz, DMSO) \(\delta\) 8.37 (s, 2H), 8.06 (s, 2H), 7.42 (q, \(J = 8.5\) Hz, 4H), 7.36 – 7.30 (m, 3H), 7.08 (d, \(J = 6.8\) Hz, 2H), 6.08 (s, 1H), 5.34 (s, 2H), 4.40 (s, 2H), 3.98 (q, \(J = 5.8\) Hz, 2H),
2.75 – 2.67 (m, 2H), 1.57 (dt, \( J = 15.3, 7.6 \text{ Hz}, 2H \)), 1.28 (dd, \( J = 14.9, 7.4 \text{ Hz}, 2H \)), 0.80 (t, \( J = 7.4 \text{ Hz}, 3H \)). \(^{13}\text{C NMR (126 MHz, DMSO) }\delta 154.71, 145.41, 145.34, 138.43, 136.19, 132.98, 129.02, 128.89, 127.79, 127.75, 126.65, 46.40, 45.31, 41.89, 28.78, 26.18, 21.70, 13.62. \) HRMS (ESI) calculated for C\(_{25}\)H\(_{31}\)N\(_6\), m/z 415.2605, found 415.2606 [M+H]+.

19r: \( N^6,N^6\text{-}(1,4\text{-phenylenebis(methylene))bis(1-benzyl-2-butyl-1H-imidazo[4,5-c]pyridine-4,6-diamine}). \) Compound 18r (35 mg, 0.038 mmol) was dissolved in 1 mL of HCl (4M in dioxane) and stirred at room temperature for 30 min. Then the solvent was removed under vacuum to obtain compound 19r as light yellow solid (12 mg, 45%). \(^1\text{H NMR (500 MHz, MeOD) }\delta 7.27 \text{ (s, 4H)}, 7.21 – 7.17 \text{ (m, 6H)}, 7.02 – 6.98 \text{ (m, 4H)}, 5.20 \text{ (s, 4H)}, 4.33 \text{ (s, 4H), }2.78 – 2.73 \text{ (m, 4H)}, 1.67 \text{ (dt, } J = 15.3, 7.6 \text{ Hz, 4H)}, 1.37 \text{ (dd, } J = 15.0, 7.5 \text{ Hz, 4H}), 0.89 \text{ (t, } J = 7.4 \text{ Hz, 6H}). \(^{13}\text{C NMR (126 MHz, MeOD) }\delta 155.94, 150.96, 148.16, 146.47, 138.77, 137.12, 130.00, 129.03, 128.70, 127.70, 118.63, 48.01, 47.26, 30.28, 27.76, 23.29, 14.05. \) HRMS (ESI) calculated for C\(_{42}\)H\(_{49}\)N\(_{10}\), m/z 693.4136, found 693.4331 [M+H]+.

Compounds 19q and 19s were synthesized similarly as compounds 19p and 19r.

19q: \( N^6\text{-}(3\text{-aminomethyl)benzyl)-1-benzyl-2-butyl-1H-imidazo[4,5-c]pyridine-4,6-diamine.} \) \( m\)-Xylylenediamine was used as a reagent. Light yellow solid. \(^1\text{H NMR (500 MHz, MeOD) }\delta 7.49 \text{ (s, 1H)}, 7.41 \text{ (dd, } J = 3.9, 1.5 \text{ Hz, 2H)}, 7.39 – 7.35 \text{ (m, 1H)}, 7.32 – 7.28 \text{ (m, 3H)}, 7.05 – 7.02 \text{ (m, 2H)}, 5.31 \text{ (s, 2H)}, 4.44 \text{ (s, 2H)}, 4.07 \text{ (s, 2H)}, 2.79 – 2.75 \text{ (m, 2H)}, 1.70 \text{ (dt, } J = 15.3, 7.6 \text{ Hz, 2H)}, 1.38 \text{ (dq, } J = 14.8, 7.4 \text{ Hz, 2H}), 0.89 \text{ (t, } J = 7.4 \text{ Hz, 3H}). \(^{13}\text{C NMR (126 MHz, MeOD) }\delta 157.36, 147.23, 146.79, 140.00, 136.86, 134.96, 130.63, 130.12, 129.26, 129.19, 129.14, 129.00, 127.62, 48.10, 47.15, 44.18, 30.00, 27.75, 23.24, 14.03. \) HRMS (ESI) calculated for C\(_{25}\)H\(_{31}\)N\(_6\), m/z 415.2605, found 415.2610 [M+H]+.
\[N^6,N^6'-(1,3-\text{phenylenebis(methylene)})\text{bis}(1-\text{benzyl}-2-\text{butyl}-1H-\text{imidazo}[4,5-c]\text{pyridine}-4,6-\text{diamine})\]. Light yellow solid. \(^1\text{H NMR}\ (500 \text{ MHz, DMSO}) \delta 12.38 (s, 2H), 8.04 (s, 4H), 7.38 (s, 1H), 7.30 – 7.22 (m, 9H), 7.04 (d, \(J = 6.8 \text{ Hz, 4H}\)), 6.01 (s, 2H), 5.28 (s, 4H), 4.34 (s, 4H), 2.73 – 2.68 (m, 4H), 1.56 (dd, \(J = 15.3, 7.7 \text{ Hz, 4H}\)), 1.29 (dd, \(J = 14.9, 7.4 \text{ Hz, 4H}\)), 0.81 (t, \(J = 7.4 \text{ Hz, 6H}\)). \(^{13}\text{C NMR}\ (126 \text{ MHz, DMSO}) \delta 154.62, 145.36, 145.31, 138.22, 136.15, 128.80, 128.61, 127.72, 126.66, 126.56, 126.38, 46.36, 45.73, 28.80, 26.19, 21.71, 13.62. \text{HRMS (ESI)} \text{calculated for C}_{42}\text{H}_{49}\text{N}_{10}, m/z \text{ 693.4136, found 693.4131 [M+H]}^+.

**Synthesis of compound 23a:** To a solution of compound 14 (120 mg, 0.31 mmol) in 1 mL of dioxane were added cesium carbonate (303 mg, 0.93 mmol) in \(\text{H}_2\text{O}\) (0.5 mL), \([1,1'-\text{bis(diphenylphosphino)ferrocene}]\text{dichloropalladium(II)}\) (Pd(dppf)Cl\(_2\)) (15 mg, 0.019 mmol) and \(n\)-butylboronic acid (98 \(\mu\text{L}, 0.46 \text{ mmol}) under \(\text{N}_2\). The reaction mixture was then heated at 90 \(^\circ\text{C}\) in a sealed vial for 18 h. It was cooled to room temperature and filtered through celite and washed with MeOH. The solvent was removed and the crude residue was purified using column chromatography (0-15% EtOAc in hexane) to obtain the compound 20a (97mg, 76%). To a solution of compound 20a (94 mg, 0.23 mmol) in 10 mL of MeOH were added zinc dust (149 mg, 2.30 mmol) and ammonium formate (145 mg, 2.30 mmol). The reaction mixture was stirred at room temperature for 10 min and filtered through celite. Then the solvent was evaporated and the residue was dissolved in water. This was extracted with EtOAc (3 \(\times\) 20 mL), washed with water and dried over sodium sulfate. The solvent was removed under vacuum to obtain compound 21a (45 mg, 51%). To a solution of compound 21a (42 mg, 0.11 mmol) in 7 mL of anhydrous THF were added triethylamine (16 \(\mu\text{L}, 0.12 \text{ mmol}) and valeryl chloride (13 \(\mu\text{L}, 0.11 \text{ mmol}). The reaction mixture was refluxed for 1 h. The solvent was then removed under vacuum, and the residue was dissolved in 5 mL of EtOH and NaOH (10 mg, 0.22 mmol) in 1 mL of \(\text{H}_2\text{O}\) was added. The reaction mixture was refluxed for 18 h. The solvent was then removed under
vacuum, and the residue was dissolved in EtOAc and washed with water. The EtOAc fraction was dried using sodium sulfate and evaporated and purified using column chromatography (0-20% EtOAc in hexane) to obtain the compound 22a (25 mg, 51%). Compound 22a (22 mg, 0.049 mmol) was dissolved in 1 mL of HCl (4M in dioxane) and stirred at room temperature for 30 min. Then the solvent was removed under vacuum to obtain compound 23a (11 mg, 69%).

20a: \( N^4\)-Benzyl-6-butyl-3-nitro-\( N^2\)-(2,4,4-trimethylpentan-2-yl)pyridine-2,4-diamine. Yellow oil. 97 mg, 76%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 9.51 (t, \( J = 4.4 \) Hz, 1H), 9.40 (s, 1H), 7.37 (mmm, \( J = 7.1, 4.4, 1.6 \) Hz, 2H), 7.34 – 7.30 (m, 3H), 5.74 (s, 1H), 4.45 (d, \( J = 5.4 \) Hz, 2H), 2.44 (t, \( J = 7.5 \) Hz, 2H), 2.01 (s, 2H), 1.67 – 1.61 (m, 2H), 1.30 (dd, \( J = 15.0, 7.4 \) Hz, 2H), 0.96 (s, 9H), 0.89 (t, \( J = 7.4 \) Hz, 3H). \(^13\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 166.49, 154.69, 152.58, 136.94, 129.07, 127.92, 127.42, 115.70, 94.41, 56.59, 51.17, 47.44, 38.87, 31.95, 31.64, 30.61, 30.19, 22.43, 14.11. MS (ESI) calculated for C\(_{24}\)H\(_{37}\)N\(_4\)O\(_2\), m/z 413.2911, found 413.3267 [M+H]\(^+\).

23a: 1-Benzyl-2,6-dibutyl-1\( H \)-imidazo[4,5-c]pyridin-4-amine. White solid. 11 mg, 69%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 7.37 – 7.31 (m, 3H), 7.10 (d, \( J = 6.9 \) Hz, 2H), 6.81 (s, 1H), 5.47 (s, 2H), 2.85 (t, 2H), 2.72 (t, 2H), 1.74 (dd, \( J = 15.3, 7.6 \) Hz, 2H), 1.67 (ddd, \( J = 15.3, 10.4, 7.0 \) Hz, 2H), 1.38 (ddq, \( J = 14.8, 10.2, 7.4 \) Hz, 4H), 0.94 (t, \( J = 7.4 \) Hz, 3H), 0.90 (t, \( J = 7.4 \) Hz, 3H). \(^13\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 157.75, 150.13, 143.74, 137.10, 130.16, 129.22, 127.59, 124.62, 97.63, 35.61, 32.84, 30.27, 27.85, 23.31, 23.22, 14.12, 14.04. HRMS (ESI) calculated for C\(_{21}\)H\(_{29}\)N\(_4\), m/z 337.2387, found 337.2476 [M+H]\(^+\).

Compounds 23b-23j were synthesized similarly as compound 23a.
20b: $N^4$-Benzyl-3-nitro-6-phenyl-$N^2$-(2,4,4-trimethylpentan-2-yl)pyridine-2,4-diamine.

Phenylboronic acid was used as a reagent. Yellow solid. 28 mg, 57%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$9.68 (s, 1H), 9.54 (s, 1H), 7.92 (ddd, $J = 4.4, 2.5, 1.4$ Hz, 2H), 7.45 – 7.42 (m, 3H), 7.40 – 7.38 (m, 3H), 7.34 – 7.31 (m, 1H), 7.26 (s, 1H), 6.39 (s, 1H), 4.58 (d, $J = 5.4$ Hz, 2H), 2.09 (s, 2H), 1.64 (s, 6H), 0.99 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 159.01, 154.74, 153.13, 138.88, 136.87, 130.20, 129.19, 128.66, 128.06, 127.50, 127.44, 92.16, 56.72, 51.69, 47.62, 32.02, 31.74, 30.15. MS (ESI) calculated for C$_{28}$H$_{33}$N$_4$O$_2$, m/z 433.2598, found 433.2615 [M+H]$^+$. 

22b: 1-Benzyl-2-butyl-6-phenyl-$N$-(2,4,4-trimethylpentan-2-yl)-1$H$-imidazo[4,5-c]pyridin-4-amine. Yellow solid. 31 mg, 47%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.02 (dd, $J = 8.3, 1.2$ Hz, 2H), 7.39 (t, $J = 7.7$ Hz, 2H), 7.34 – 7.27 (m, 4H), 7.07 (d, $J = 6.7$ Hz, 2H), 6.94 (s, 1H), 5.34 (s, 1H), 5.28 (s, 2H), 2.79 – 2.73 (m, 2H), 2.18 (s, 2H), 1.73 – 1.66 (m, 8H), 1.39 (dd, $J = 15.0, 7.5$ Hz, 2H), 1.03 (s, 9H), 0.89 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 153.22, 150.01, 148.43, 141.28, 140.32, 136.16, 129.61, 129.42, 129.15, 128.41, 128.07, 127.67, 127.54, 126.72, 126.36, 126.28, 126.22, 91.77, 55.45, 51.63, 47.28, 31.98, 31.81, 30.33, 30.25, 27.50, 22.72, 13.90. MS (ESI) calculated for C$_{31}$H$_{41}$N$_4$, m/z 469.3326, found 469.3350 [M+H]$^+$. 

23b: 1-Benzyl-2-butyl-6-phenyl-$1H$-imidazo[4,5-c]pyridin-4-amine. White solid. 13 mg, 73%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.91 (d, $J = 7.2$ Hz, 2H), 7.54 – 7.44 (m, 3H), 7.42 – 7.34 (m, 3H), 7.05 (dd, $J = 6.4, 1.1$ Hz, 2H), 6.83 (s, 1H), 5.37 (s, 2H), 2.88 – 2.78 (m, 2H), 1.78 (dd, $J = 15.2, 7.8$ Hz, 2H), 1.42 (dq, $J = 14.7, 7.4$ Hz, 2H), 0.93 (t, $J = 7.3$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 157.54, 149.18, 142.11, 141.93, 134.08, 132.07, 130.82, 129.68, 129.66, 129.01, 127.38, 126.25, 95.25, 48.13, 29.31, 27.40, 22.52, 13.84. HRMS (ESI) calculated for C$_{23}$H$_{25}$N$_4$, m/z 357.2074, found 357.2155 [M+H]$^+$. 

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20c: 4-(4-(Benzylamino)-5-nitro-6-((2,4,4-trimethylpentan-2-yl)amino)pyridin-2-yl)benzonitrile. 4-Cyanophenylboronic acid was used as a reagent. Yellow solid. 30 mg, 55%. 1H NMR (500 MHz, CDCl₃) δ 9.72 (s, 1H), 9.51 (s, 1H), 7.97 (d, J = 8.6 Hz, 2H), 7.72 (d, J = 8.6 Hz, 2H), 7.44 – 7.31 (m, 5H), 6.38 (s, 1H), 4.59 (d, J = 5.4 Hz, 2H), 2.06 (s, 2H), 1.63 (s, 6H), 0.99 (s, 9H). 13C NMR (126 MHz, CDCl₃) δ 156.75, 154.69, 153.26, 143.14, 136.53, 132.50, 129.28, 128.23, 127.93, 127.35, 118.79, 113.38, 93.05, 56.87, 51.72, 47.68, 32.01, 31.72, 30.10. MS (ESI) calculated for C₂₇H₃₂N₅O₂, m/z 458.2551, found 458.2571 [M+H]+.

22c: 4-(1-Benzyl-2-butyl-4-((2,4,4-trimethylpentan-2-yl)amino)-1H-imidazo[4,5-c]pyridin-6-yl)benzamide. Cyanopropylpropionic acid was converted into amide in the basic condition. Yellow solid. 22 mg, 41%. 1H NMR (500 MHz, CDCl₃) δ 8.10 (d, J = 8.5 Hz, 2H), 7.84 (d, J = 8.5 Hz, 2H), 7.34 – 7.28 (m, 3H), 7.08 – 7.04 (m, 2H), 6.99 (s, 1H), 6.14 (s, 1H), 5.79 (s, 1H), 5.41 (s, 1H), 5.29 (s, 2H), 2.81 – 2.74 (m, 2H), 2.17 (s, 2H), 1.72 – 1.66 (m, 8H), 1.43 – 1.33 (m, 2H), 1.03 (s, 9H), 0.89 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, CDCl₃) δ 169.46, 153.74, 150.08, 147.00, 144.87, 140.11, 135.97, 131.83, 129.20, 128.15, 127.62, 126.76, 126.74, 126.31, 92.63, 55.50, 51.55, 47.31, 31.95, 31.79, 30.25, 30.17, 27.24, 22.69, 13.89. MS (ESI) calculated for C₃₂H₄₂N₅O, m/z 512.3384, found 512.3408 [M+H]+.

23c: 4-(4-Amino-1-benzyl-2-butyl-1H-imidazo[4,5-c]pyridin-6-yl)benzamide. White solid. 11 mg, 79%. 1H NMR (500 MHz, MeOD) δ 8.09 – 8.05 (m, 2H), 7.89 – 7.85 (m, 2H), 7.51 (s, 1H), 7.41 – 7.32 (m, 3H), 7.17 (d, J = 7.0 Hz, 2H), 5.62 (s, 2H), 3.76 – 3.72 (m, 1H), 3.68 – 3.64 (m, 1H), 3.58 (dd, J = 7.0, 2.7 Hz, 1H), 2.94 – 2.89 (m, 2H), 1.78 (dt, J = 15.3, 7.6 Hz, 2H), 1.42 (dd, J = 15.0, 7.5 Hz, 2H), 0.92 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, MeOD) δ 171.03, 159.91, 149.79, 143.90, 141.45, 137.23, 136.75, 136.73, 130.29, 129.74, 129.43, 128.43, 127.76,
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125.80, 98.94, 73.57, 72.45, 62.18, 43.73, 30.01, 27.99, 23.28, 14.04. HRMS (ESI) calculated for C_{24}H_{26}N_5O, m/z 400.2132, found 400.2177 [M+H]^+.

20d: \( N^4\)-Benzy1-5-nitro-\( N^6\)-(2,4,4-trimethylpentan-2-yl)-[2,3'-bipyridine]-4,6-diamine. 4-Pyridinylboronic acid was used as a reagent. Orange solid. 135 mg, 82%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 9.74 (t, \( J = 5.1 \) Hz, 1H), 9.54 (s, 1H), 9.10 (dd, \( J = 2.2, 0.6 \) Hz, 1H), 8.65 (dd, \( J = 4.8, 1.6 \) Hz, 1H), 8.21 – 8.16 (m, 1H), 7.42 – 7.36 (m, 5H), 7.35 – 7.31 (m, 1H), 6.38 (s, 1H), 4.59 (d, \( J = 5.5 \) Hz, 2H), 2.07 (s, 2H), 1.63 (s, 6H), 0.99 (s, 9H). \(^1\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 156.52, 154.78, 153.22, 150.86, 148.93, 136.60, 134.75, 134.36, 129.26, 128.17, 127.35, 123.54, 116.37, 92.38, 56.86, 51.65, 47.65, 32.01, 31.72, 30.11. MS (ESI) calculated for C\(_{25}\)H\(_{32}\)N\(_5\)O\(_2\), m/z 434.2551, found 434.2563 [M+H]^+.

23d: 1-Benzyl-2-butyl-6-(pyridin-3-yl)-1\( H\)-imidazo[4,5-c]pyridin-4-amine. Light yellow solid. 10 mg, 72%. \(^1\)H NMR (500 MHz, MeOD) \( \delta \) 9.28 (d, \( J = 1.9 \) Hz, 1H), 8.94 (dd, \( J = 5.5, 1.2 \) Hz, 1H), 8.83 (d, \( J = 8.2 \) Hz, 1H), 8.11 (ddd, \( J = 8.2, 5.6, 0.5 \) Hz, 1H), 7.72 (s, 1H), 7.40 – 7.32 (m, 3H), 7.18 (d, \( J = 6.9 \) Hz, 2H), 5.65 (s, 2H), 2.98 – 2.91 (m, 2H), 1.78 (dd, \( J = 15.3, 7.7 \) Hz, 2H), 1.42 (dd, \( J = 15.0, 7.5 \) Hz, 2H), 0.92 (t, \( J = 7.4 \) Hz, 3H). \(^1\)C NMR (126 MHz, MeOD) \( \delta \) 150.19, 143.97, 143.57, 143.11, 136.48, 130.41, 130.32, 129.52, 128.03, 127.74, 127.73, 100.47, 48.55, 29.93, 27.90, 23.26, 14.02. HRMS (ESI) calculated for C\(_{22}\)H\(_{24}\)N\(_5\), m/z 358.2026, found 358.2067 [M+H]^+.

20e: \( N^4\)-Benzy1-6-(furan-3-yl)-3-nitro-\( N^2\)-(2,4,4-trimethylpentan-2-yl)pyridine-2,4-diamine. 3-Furylboronic acid was used as a reagent. Yellow solid. 95 mg, 70%. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 9.68 (t, \( J = 4.8 \) Hz, 1H), 9.49 (s, 1H), 7.95 (dd, \( J = 1.5, 0.8 \) Hz, 1H), 7.45 (t, \( J = 1.7 \) Hz, 1H), 7.41 – 7.34 (m, 4H), 7.34 – 7.30 (m, 1H), 6.69 (dd, \( J = 1.9, 0.8 \) Hz, 1H), 6.07 (s, 1H), 4.53 (d, \( J = 3.2 \) Hz, 2H), 2.07 (s, 2H), 1.63 (s, 6H), 0.99 (s, 9H). \(^1\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 151.75, 144.73, 144.68, 143.38, 136.47, 136.43, 130.59, 130.45, 129.46, 128.23, 127.90, 127.72, 125.24, 124.08, 123.83, 123.00, 116.37, 92.38, 56.86, 51.65, 47.65, 32.01, 31.72, 30.11. MS (ESI) calculated for C\(_{25}\)H\(_{32}\)N\(_5\)O\(_2\), m/z 434.2551, found 434.2563 [M+H]^+.
= 5.4 Hz, 2H), 2.05 (s, 2H), 1.60 (s, 6H), 0.98 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 154.88, 153.66, 153.09, 143.99, 143.68, 136.85, 129.19, 128.06, 127.39, 127.36, 108.83, 91.66, 56.68, 51.51, 47.61, 31.99, 31.71, 30.13. MS (ESI) calculated for C$_{24}$H$_{31}$N$_4$O$_3$, m/z 423.2391, found 423.2401 [M+H]$^+$. 

22e: 1-Benzyl-2-butyl-6-(furan-3-yl)-N-(2,4,4-trimethylpentan-2-yl)-1H-imidazo[4,5-c]pyridin-4-amine. Brown oil. 30 mg, 41%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.94 (dd, $J = 1.6$, 0.7 Hz, 1H), 7.41 (t, $J = 1.7$ Hz, 1H), 7.34 – 7.27 (m, 3H), 7.05 (d, $J = 6.6$ Hz, 2H), 6.76 (dd, $J = 1.8$, 0.8 Hz, 1H), 6.61 (s, 1H), 5.30 (s, 1H), 5.24 (s, 2H), 2.76 – 2.71 (m, 2H), 2.13 (s, 2H), 1.70 – 1.65 (m, 2H), 1.64 (s, 6H), 1.36 (dt, $J = 14.7$, 7.4 Hz, 2H), 1.02 (s, 9H), 0.88 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 152.80, 150.15, 143.24, 142.70, 142.70, 140.71, 140.01, 136.16, 129.15, 128.83, 128.06, 126.33, 125.88, 108.87, 91.20, 55.44, 51.45, 47.25, 31.93, 31.79, 30.29, 30.16, 27.45, 22.71, 13.90. MS (ESI) calculated for C$_{29}$H$_{39}$N$_4$O, m/z 459.3118, found 459.3168 [M+H]$^+$. 

23e: 1-Benzyl-2-butyl-6-(furan-3-yl)-1H-imidazo[4,5-c]pyridin-4-amine. Light yellow solid. 12 mg, 64%. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.23 – 8.19 (m, 1H), 7.73 – 7.70 (m, 1H), 7.42 (s, 1H), 7.40 – 7.32 (m, 3H), 7.15 (d, $J = 6.9$ Hz, 2H), 6.97 (dd, $J = 2.0$, 0.9 Hz, 1H), 5.58 (s, 2H), 2.90 – 2.85 (m, 2H), 1.75 (dt, $J = 15.3$, 7.6 Hz, 2H), 1.40 (dq, $J = 14.8$, 7.4 Hz, 2H), 0.91 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 159.50, 149.42, 146.46, 144.24, 142.92, 136.72, 135.38, 130.27, 129.41, 127.69, 124.89, 121.45, 109.21, 96.82, 48.27, 29.95, 27.91, 23.26, 14.03. HRMS (ESI) calculated for C$_{21}$H$_{23}$N$_4$O, m/z 347.1866, found 347.1921 [M+H]$^+$. 

20f: $N^6$-Benzyl-3-nitro-6-(thiophen-3-yl)-$N^2$-(2,4,4-trimethylpentan-2-yl)pyridine-2,4-diamine. 3-Thienylboronic acid was used as a reagent. Yellow solid. 107 mg, 76%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.69 (s, 1H), 9.52 (s, 1H), 7.89 (dd, $J = 3.0$, 1.2 Hz, 1H), 7.49 (dd, $J = 5.1$, 1.2 Hz, 1H), 7.35 – 7.27 (m, 3H), 7.15 (d, $J = 6.9$ Hz, 2H), 6.95 (dd, $J = 2.0$, 0.9 Hz, 1H), 5.58 (s, 2H), 2.90 – 2.85 (m, 2H), 1.75 (dt, $J = 15.3$, 7.6 Hz, 2H), 1.40 (dq, $J = 14.8$, 7.4 Hz, 2H), 0.91 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 159.50, 149.42, 146.46, 144.24, 142.92, 136.72, 135.38, 130.27, 129.41, 127.69, 124.89, 121.45, 109.21, 96.82, 48.27, 29.95, 27.91, 23.26, 14.03. HRMS (ESI) calculated for C$_{21}$H$_{23}$N$_4$O, m/z 347.1866, found 347.1921 [M+H]$^+$. 

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1.2 Hz, 1H), 7.42 – 7.30 (m, 6H), 6.26 (s, 1H), 4.56 (d, J = 5.4 Hz, 2H), 2.08 (s, 2H), 1.63 (s, 6H), 0.99 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 154.88, 154.61, 153.25, 142.21, 136.90, 129.19, 128.05, 127.38, 126.59, 126.56, 126.28, 91.99, 56.68, 51.59, 47.61, 32.02, 31.72, 30.16. MS (ESI) calculated for C$_{24}$H$_{31}$N$_4$O$_2$S, m/z 439.2162, found 439.2183 [M+H]+.

22f: 1-Benzyl-2-butyl-6-(thiophen-3-yl)-N-(2,4,4-trimethylpentan-2-yl)-1H-imidazo[4,5-c]pyridin-4-amine. Brown solid. 25 mg, 35%. $^1$H NMR (500 MHz, CDCl$_3$) δ 7.79 (dd, J = 3.1, 1.2 Hz, 1H), 7.58 (dd, J = 5.0, 1.2 Hz, 1H), 7.33 – 7.27 (m, 4H), 7.06 (d, J = 6.6 Hz, 2H), 6.79 (s, 1H), 5.35 (s, 1H), 5.25 (s, 2H), 2.78 – 2.70 (m, 2H), 2.17 (s, 2H), 1.70 (dd, J = 8.7, 7.0 Hz, 3H), 1.67 (s, 6H), 1.38 (dq, J = 14.7, 7.4 Hz, 2H), 1.26 (dd, J = 8.7, 5.6 Hz, 1H), 1.02 (s, 9H), 0.89 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 153.04, 150.02, 144.89, 144.32, 140.11, 136.13, 129.14, 128.07, 126.45, 126.34, 125.94, 125.41, 121.55, 91.54, 55.43, 51.49, 50.96, 47.26, 31.96, 31.80, 30.26, 30.21, 27.46, 22.71, 13.89. MS (ESI) calculated for C$_{29}$H$_{39}$N$_4$S, m/z 475.2890, found 475.2922 [M+H]+.

23f: 1-Benzyl-2-butyl-6-(thiophen-3-yl)-1H-imidazo[4,5-c]pyridin-4-amine. White solid. 9 mg, 53%. $^1$H NMR (500 MHz, MeOD) δ 8.04 (dd, J = 2.9, 1.4 Hz, 1H), 7.66 (dd, J = 5.1, 2.9 Hz, 1H), 7.58 (dd, J = 5.1, 1.4 Hz, 1H), 7.48 (s, 1H), 7.41 – 7.32 (m, 3H), 7.17 (d, J = 6.9 Hz, 2H), 5.59 (s, 2H), 2.94 – 2.86 (m, 2H), 1.76 (dt, J = 15.3, 7.6 Hz, 2H), 1.41 (dq, J = 14.8, 7.4 Hz, 2H), 0.91 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) δ 159.63, 149.38, 144.24, 137.90, 136.73, 135.24, 130.28, 129.42, 129.31, 127.74, 126.73, 126.03, 124.88, 97.21, 49.07, 29.96, 27.94, 23.26, 14.03. HRMS (ESI) calculated for C$_{21}$H$_{23}$N$_4$S, m/z 363.1638, found 363.1686 [M+H]+.

20g: $N^4$-Dibenzyl-3-nitro-$N^2$-(2,4,4-trimethylpentan-2-yl)pyridine-2,4-diamine. Benzyl boronic acid pinacol ester was used as a reagent. Orange oil. 135 mg, 91%. $^1$H NMR (500 MHz,
CDCl₃ δ 9.54 (t, J = 5.0 Hz, 1H), 9.37 (s, 1H), 7.36 (ddd, J = 7.4, 4.5, 1.4 Hz, 2H), 7.31 (dt, J = 9.8, 4.4 Hz, 1H), 7.27 (ddd, J = 6.7, 3.3, 1.4 Hz, 5H), 7.22 – 7.18 (m, 3H), 5.75 (s, 1H), 4.40 (d, J = 5.4 Hz, 2H), 3.75 (s, 2H), 1.89 (s, 2H), 1.49 (s, 6H), 0.90 (s, 9H). ¹³C NMR (126 MHz, CDCl₃) δ 164.70, 154.68, 152.84, 138.77, 136.80, 129.45, 129.07, 128.54, 127.91, 127.46, 126.50, 124.96, 115.72, 94.56, 56.63, 51.05, 47.44, 45.61, 31.87, 31.60, 30.09, 24.86. MS (ESI) calculated for C₂₇H₃₅N₄O₂, m/z 447.2755, found 447.2737 [M+H]⁺.

22g: 1,6-Dibenzyl-2-butyl-N-(2,4,4-trimethylpentan-2-yl)-1H-imidazo[4,5-c]pyridin-4-amine. Light brown solid. 55 mg, 60%. ¹H NMR (500 MHz, CDCl₃) δ 7.33 – 7.27 (m, 5H), 7.24 (t, J = 7.6 Hz, 2H), 7.14 (t, J = 7.3 Hz, 1H), 7.01 (d, J = 6.4 Hz, 2H), 6.31 (s, 1H), 5.22 (s, 1H), 5.16 (s, 2H), 3.96 (s, 2H), 2.74 – 2.67 (m, 2H), 2.02 (s, 2H), 1.66 – 1.61 (m, 2H), 1.56 (s, 6H), 1.35 (dd, J = 15.0, 7.5 Hz, 2H), 0.93 (s, 9H), 0.86 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 152.37, 151.90, 150.06, 141.57, 140.10, 136.28, 129.31, 129.06, 28.89, 26.28, 21.69, 13.61. MS (ESI) calculated for C₃₂H₄₃N₄, m/z 483.3482, found 483.3526 [M+H]⁺.

23g: 1,6-Dibenzyl-2-butyl-1H-imidazo[4,5-c]pyridin-4-amine. White solid. 23 mg, 58%. ¹H NMR (500 MHz, DMSO) δ 13.63 (s, 1H), 8.27 (s, 2H), 7.37 – 7.29 (m, 7H), 7.27 – 7.23 (m, 1H), 7.15 (s, 1H), 7.11 (d, J = 6.9 Hz, 2H), 5.52 (s, 2H), 4.08 (s, 2H), 2.86 – 2.79 (m, 2H), 1.61 (dt, J = 15.3, 7.6 Hz, 2H), 1.31 (dt, J = 14.9, 7.4 Hz, 2H), 0.81 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, DMSO) δ 156.72, 148.03, 142.12, 141.72, 137.39, 135.93, 128.96, 128.72, 128.70, 127.92, 126.98, 126.63, 123.29, 97.60, 46.84, 40.11, 38.20, 28.89, 26.28, 21.69, 13.61. HRMS (ESI) calculated for C₂₉H₂₇N₄, m/z 371.2230, found 371.2285 [M+H]⁺.
20h: 1-Benzyl-6-(4-methylbenzyl)-3-nitro-N²-(2,4,4-trimethylpentan-2-yl)pyridine-2,4-diamine. 4-Methyl benzylboronic acid pinacol ester was used as a reagent. Yellow oil. 130 mg, 91%. 1H NMR (500 MHz, CDCl₃) δ 9.52 (t, J = 5.0 Hz, 1H), 9.37 (s, 1H), 7.37 – 7.30 (m, 3H), 7.28 – 7.24 (m, 3H), 7.10 – 7.03 (m, 7H), 5.74 (s, 1H), 4.39 (d, J = 5.4 Hz, 2H), 3.70 (s, 2H), 2.31 (s, 3H), 1.90 (s, 2H), 1.51 (s, 6H), 0.90 (s, 9H). 13C NMR (126 MHz, CDCl₃) δ 165.00, 154.68, 152.83, 136.82, 135.98, 135.70, 135.50, 134.26, 129.28, 129.23, 129.12, 129.05, 128.98, 127.89, 127.47, 115.70, 94.47, 83.50, 56.63, 51.03, 47.44, 45.22, 31.87, 31.57, 30.12, 24.87, 21.19, 21.11. MS (ESI) calculated for C₂₈H₃₇N₄O₂, m/z 461.2911, found 461.3003 [M+H]⁺.

23h: 1-Benzyl-2-butyl-6-(4-methylbenzyl)-1H-imidazo[4,5-c]pyridin-4-amine. White solid. 19 mg, 66%. 1H NMR (500 MHz, MeOD) δ 7.38 – 7.32 (m, 3H), 7.16 (d, J = 7.9 Hz, 2H), 7.13 – 7.08 (m, 4H), 6.89 (s, 1H), 5.49 (s, 2H), 4.08 (s, 2H), 2.92 – 2.87 (m, 2H), 2.32 (s, 3H), 1.76 (dt, J = 15.3, 7.6 Hz, 2H), 1.40 (dt, J = 14.8, 7.4 Hz, 2H), 0.91 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, MeOD) δ 159.11, 149.35, 144.27, 143.93, 138.37, 136.53, 134.52, 130.68, 130.25, 129.85, 129.42, 127.74, 124.41, 99.41, 39.40, 30.01, 27.84, 23.26, 21.08, 14.03. HRMS (ESI) calculated for C₂₅H₂₉N₄, m/z 385.2387, found 385.2451 [M+H]⁺.

20i: 1-Benzyl-3-nitro-6-(4-(trifluoromethoxy)benzyl)-N²-(2,4,4-trimethylpentan-2-yl)pyridine-2,4-diamine. 4-(Trifluoromethoxy)benzylboronic acid pinacol ester was used as a reagent. Yellow solid. 115 mg, 70%. 1H NMR (500 MHz, CDCl₃) δ 9.57 (t, J = 5.0 Hz, 1H), 9.37 (s, 1H), 7.38 – 7.29 (m, 3H), 7.28 (t, J = 1.8 Hz, 2H), 7.21 – 7.17 (m, 2H), 7.10 (d, J = 7.9 Hz, 2H), 5.74 (s, 1H), 4.43 (d, J = 5.5 Hz, 2H), 1.83 (s, 2H), 1.45 (s, 6H), 0.88 (s, 9H). 13C NMR (126 MHz, CDCl₃) δ 163.87, 154.70, 152.91, 137.54, 136.71, 130.77, 129.10, 127.95, 127.33, 121.09, 115.75, 94.57, 56.63, 50.96, 47.44, 44.69, 31.82, 31.55, 30.02. MS (ESI) calculated for C₂₈H₃₄F₃N₄O₃, m/z 531.2578, found 531.2738 [M+H]⁺.
23i: 1-Benzyl-2-butyl-6-(4-(trifluoromethoxy)benzyl)-1H-imidazo[4,5-c]pyridin-4-amine. White solid. 21 mg, 47%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.37 – 7.29 (m, 5H), 7.21 (d, $J = 8.0$ Hz, 2H), 7.08 (d, $J = 6.5$ Hz, 2H), 6.79 (s, 1H), 5.45 (s, 2H), 4.09 (s, 2H), 2.87 (dd, $J = 9.3$, 6.1 Hz, 2H), 1.74 (dt, $J = 15.3$, 7.6 Hz, 2H), 1.41 (dt, $J = 15.0$, 7.4 Hz, 2H), 0.91 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 157.96, 150.50, 149.43, 149.42, 143.48, 138.54, 136.96, 131.59, 130.16, 129.25, 127.66, 124.97, 122.37, 99.02, 40.76, 30.29, 27.86, 23.31, 14.05. HRMS (ESI) calculated for C$_{25}$H$_{26}$F$_3$N$_4$O, m/z 455.2053, found 455.2131 [M+H]$^+$.

20j: N$^4$-Benzyl-3-nitro-6-phenethyl-N$^2$-(2,4,4-trimethylpentan-2-yl)pyridine-2,4-diamine. 2-Phenylethylboronic acid was used as a reagent. Yellow oil. 85 mg, 59%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.52 (t, $J = 4.9$ Hz, 1H), 9.43 (s, 1H), 7.36 (t, $J = 7.2$ Hz, 2H), 7.33 – 7.28 (m, 4H), 7.27 – 7.25 (m, 3H), 7.19 (d, $J = 7.3$ Hz, 1H), 7.17 (d, $J = 7.0$ Hz, 2H), 5.69 (s, 1H), 4.39 (d, $J = 5.4$ Hz, 2H), 2.99 (dd, $J = 9.1$, 6.8 Hz, 2H), 2.75 (dd, $J = 9.1$, 6.8 Hz, 2H), 2.04 (s, 2H), 1.58 (s, 6H), 0.98 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 165.06, 154.74, 152.65, 141.77, 136.85, 129.09, 128.52, 128.49, 127.95, 127.39, 126.10, 115.78, 94.52, 56.68, 51.35, 47.41, 40.87, 34.61, 31.97, 31.69, 30.20. MS (ESI) calculated for C$_{28}$H$_{37}$N$_4$O$_2$, m/z 461.2911, found 461.3096 [M+H]$^+$.

23j: 1-Benzyl-2-butyl-6-phenethyl-1H-imidazo[4,5-c]pyridin-4-amine. Yellow solid. 11 mg, 42%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.36 – 7.30 (m, 3H), 7.23 – 7.18 (m, 2H), 7.13 (ddd, $J = 5.7$, 3.7, 1.5 Hz, 3H), 7.02 (d, $J = 6.5$ Hz, 2H), 6.68 (s, 1H), 5.38 (s, 2H), 2.99 (s, 4H), 2.85 – 2.79 (m, 2H), 1.69 (ddd, $J= 13.2$, 8.5, 6.7 Hz, 2H), 1.37 (dq, $J = 14.8$, 7.4 Hz, 2H), 0.89 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 157.35, 150.48, 148.64, 143.39, 141.89, 137.14, 130.14, 129.53, 129.43, 129.16, 127.59, 127.22, 124.68, 97.93, 48.17, 38.42, 36.84, 30.28, 27.83, 23.31, 14.03. HRMS (ESI) calculated for C$_{29}$H$_{29}$N$_4$, m/z 385.2387, found 385.2446 [M+H]$^+$.
Synthesis of compound 24: 3-Nitrobenzo[g]quinolin-4-ol. Nitromethane (0.96 mL, 18 mmol) was added dropwise to a solution of NaOH (2.2 g, 54 mmol) in water (5 mL), at 0 °C. The mixture was then warmed to 40 °C and nitromethane (0.96 mL, 18 mmol) was again added slowly at 40-45 °C. The temperature was maintained until a clear solution was obtained. The reaction mixture was then heated to 55 °C for 2-5 minutes, cooled to 30 °C, poured onto crushed ice and acidified with conc. HCl (5 mL). The resultant solution of methazoic acid was added immediately to a filtered solution of 3-amino-2-naphtholic acid (3 g, 16 mmol) and conc. HCl (1 mL) in water (20 mL). The reaction mixture was allowed to stand at room temperature for 12 h. After filtration, the residue obtained was washed with water, and dried (1.1 g, 90%). A solution of intermediate (2 g, 7.75 mmol) in acetic anhydride (10 mL) was placed in a 2-neck flask fitted with a reflux condenser. It was stirred and heated to 105 °C until a clear solution was obtained. Heating was then discontinued and potassium acetate (0.77 g, 7.90 mmol) was added. The mixture was then refluxed for 15 min with vigorous stirring, until a solid started to precipitate. The reaction mixture was then slowly cooled to room temperature. The residue was filtered, washed with glacial acetic acid until the washings were colorless, then suspended in water, filtered, washed with water and dried at 110 °C to get compound 24 (0.93 g, 50%). \(^1\)H NMR (500 MHz, DMSO) δ 13.12 (s, 1H), 9.28 (s, 1H), 8.95 (s, 1H), 8.27 – 8.21 (m, 2H), 8.11 (d, J = 8.4 Hz, 1H), 7.69 (t, J = 7.1 Hz, 1H), 7.61 (t, J = 7.1 Hz, 1H). \(^13\)C NMR (126 MHz, DMSO) δ 168.61, 144.40, 135.05, 134.63, 130.14, 129.54, 128.94, 128.71, 127.46, 127.41, 126.65, 126.48, 116.78. MS (ESI) calculated for C\(_{13}\)H\(_8\)N\(_2\)O\(_3\), m/z 240.05, found 263.04 [M+Na]\(^+\).

Synthesis of compound 25: 4-Chloro-3-nitrobenzo[g]quinoline. A suspension of compound 24 (2.0 g, 8.30 mmol) in phosphorus(V) oxychloride was placed in a pressure vessel and it was heated at 150 °C. After a clear solution was obtained, the reaction mixture was kept at 150 °C for 1 h. Then it was slowly cooled to room temperature and the solvent was evaporated under
vacuum. The residue was poured over crushed ice while stirring and the formed solid was filtered, washed with water and dried to obtain compound 25 (1.95 g, 91%). $^1$H NMR (500 MHz, DMSO) $\delta$ 13.34 (d, J = 6.9 Hz, 1H), 9.26 (d, J = 7.3 Hz, 1H), 8.95 (s, 1H), 8.25 (d, J = 9.4 Hz, 2H), 8.11 (d, J = 8.4 Hz, 1H), 7.72 – 7.67 (m, 1H), 7.61 (ddd, J = 8.1, 6.8, 1.1 Hz, 1H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 168.64, 144.23, 134.96, 134.64, 130.16, 129.56, 128.98, 128.70, 127.48, 127.43, 126.69, 126.48, 116.74.

**Synthesis of compound 26:** *N*-Benzyl-3-nitrobenzo[g]quinolin-4-amine. To a solution of compound 25 (1.0 g, 3.90 mol) in 20 mL of CH$_2$Cl$_2$ was added triethylamine (0.81 mL, 5.80 mmol) and benzylamine (0.50 mL, 4.60 mmol). The reaction mixture was refluxed for 2 h. The solvent was then evaporated under vacuum and H$_2$O was added to the residue. The solution was extracted with CH$_2$Cl$_2$ (3 × 20 mL), washed with water and dried over sodium sulfate. The solvent was evaporated and the residue was purified using silica gel column chromatography (0-5% MeOH in CH$_2$Cl$_2$) to obtain compound 26 as a yellow solid (1.1 g, 88%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 10.57 (s, 1H), 9.41 (s, 1H), 8.87 (s, 1H), 8.50 (s, 1H), 8.02 (d, J = 8.4 Hz, 1H), 7.83 (d, J = 8.4 Hz, 1H), 7.65 – 7.60 (m, 1H), 7.55 – 7.51 (m, 1H), 7.50 – 7.47 (m, 4H), 7.42 (ddd, J = 11.0, 5.4, 3.0 Hz, 1H), 5.28 (d, J = 5.9 Hz, 2H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 152.50, 147.73, 145.88, 136.85, 135.48, 130.53, 129.64, 129.12, 129.03, 128.98, 128.83, 128.11, 128.04, 127.29, 126.86, 124.21, 118.09, 53.19. MS (ESI) calculated for C$_{20}$H$_{16}$N$_3$O$_2$, m/z 330.1237, found 330.1304 [M+H]$^+$.  

**Synthesis of compound 30:** 1-Benzyl-2-butyl-1H-benzo[g]imidazo[4,5-c]quinolin-4-amine. To a solution of compound 26 (300 mg, 0.91 mmol) in 20 mL of MeOH were added zinc dust (594 mg, 9.10 mmol) and ammonium formate (574 mg, 9.10 mmol). The reaction mixture was stirred at room temperature for 30 min and filtered through celite. Then the solvent was
evaporated and the residue was dissolved in water. This was extracted with EtOAc (3 × 20 mL), washed with water and dried over sodium sulfate. The solvent was removed under vacuum to obtain compound 27 (100 mg, 37%). To a solution of compound 27 (98 mg, 0.33 mmol) in 10 mL of anhydrous THF were added triethylamine (48 μL, 0.35 mmol) and valeryl chloride (40 μL, 0.33 mmol). The reaction mixture was refluxed for 2 h. The solvent was then removed under vacuum, and the residue was dissolved in 10 mL of EtOH and NaOH (26 mg, 0.66 mmol) in 1 mL of H₂O was added. The reaction mixture was refluxed for 2 h. The solvent was then removed under vacuum, and the residue was dissolved in EtOAc and washed with water. The EtOAc fraction was dried using sodium sulfate and evaporated and purified using column chromatography (0-10% MeOH in CH₂Cl₂) to obtain the compound 28 (76 mg, 63%). To a solution of compound 28 (76 mg, 0.21 mmol) in a solvent mixture of MeOH:CH₂Cl₂:CHCl₃ (1:10:10) was added 3-chloroperoxy benzoic acid (443 mg, 1.98 mmol), and the solution was refluxed at 45-50 ºC for 1 h. The solvent was then removed and the residue was purified using column chromatography (0-10% MeOH in CH₂Cl₂) to obtain the N-oxide derivative 29 (64 mg, 80%). To a solution of compound 29 (64 mg, 0.17 mol) in 10 mL of CH₂Cl₂ was added benzoyl isocyanate (37 mg, 0.25 mmol) and heated at 45 ºC for 18 h. The solvent was then removed under vacuum, and the residue was dissolved in 15 mL of anhydrous MeOH, followed by the addition of excess sodium methoxide. The reaction mixture was then heated at 80 ºC for 2 h. The solvent was removed under vacuum and the residue was purified using column chromatography (0-10% MeOH in CH₂Cl₂) to obtain the compound 30 (20 mg, 30%). ¹H NMR (500 MHz, DMSO) δ 8.40 (s, 1H), 8.05 (s, 1H), 7.88 (d, J = 8.3 Hz, 1H), 7.77 (d, J = 8.3 Hz, 1H), 7.38 (d, J = 7.2 Hz, 1H), 7.33 (dd, J = 12.9, 5.2 Hz, 3H), 7.24 (d, J = 7.4 Hz, 1H), 7.16 (d, J = 7.4 Hz, 2H), 6.91 (s, 2H), 6.02 (s, 2H), 3.00 – 2.92 (m, 2H), 1.74 (dt, J = 15.4, 7.6 Hz, 2H), 1.40 (dd, J = 14.9, 7.4 Hz, 2H), 0.88 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, DMSO) δ 136.73, 131.80,
HRMS (ESI) calculated for C_{25}H_{25}N_{4}, m/z 381.2074, found 381.2089 [M+H]^+.

**Human TLR-7/-8 reporter gene assays (NF-κB induction).** As described in Chapter 2.

**Immunoassays for Interferon (IFN)-α, and cytokines.** As described in Chapter 2.

**Flow-cytometric immunostimulation experiments.** CD69 upregulation was determined by flow cytometry using protocols published by us previously,\textsuperscript{30} and modified for rapid-throughput. Briefly, heparin-anticoagulated whole blood samples were obtained by venipuncture from healthy human volunteers with informed consent and as per guidelines approved by the University of Kansas Human Subjects Experimentation Committee. Serial dilutions of selected imidazopyridine compounds (and imiquimod, used as a reference compound) were performed using a Bio-Tek Precision 2000 XS liquid handler in sterile 96-well polypropylene plates, to which were added 100 μL aliquots of anticoagulated whole human blood. The plates were incubated at 37°C for 16.5 h. Negative (endotoxin free water) controls were included in each experiment. Following incubation, fluorochrome-conjugated antibodies (CD3-PE, CD56-APC, CD69-PE-Cy7, 10 μL of each antibody, Becton-Dickinson Biosciences, San Jose, CA) were added to each well with a liquid handler, and incubated at 37 °C in the dark for 30 min. Following staining, erythrocytes were lysed and leukocytes fixed by mixing 200 mL of the samples in 2 mL pre-warmed Whole Blood Lyse/Fix Buffer (Becton-Dickinson Biosciences, San Jose, CA) in 96 deep-well plates. After washing the cells twice at 200 g for 8 minutes in saline, the cells were transferred to a 96-well plate. Flow cytometry was performed using a BD FACSArray instrument in the tri-color mode (tri-color flow experiment) and two-color mode (two-
color flow experiment) for acquisition on 100,000 gated events. Compensation for spillover was
computed for each experiment on singly-stained samples. CD69 activation in the major
lymphocytic populations, viz., natural killer lymphocytes (NK cells: CD3−CD56+), cytokine-
induced killer phenotype (CIK cells: CD3−CD56+), B lymphocytes (B cells: CD3−CD19+).
Chapter 6.

Determinants of activity at human TLR7 and 8: QSAR of diverse heterocyclic scaffolds
6.1. Introduction

Extensive SARs on several TLR7/8-agonistic scaffolds such as imidazo[4,5-c]quinolines,\textsuperscript{55b, 69} thiazolo[4,5-c]quinolines (Chapter 2),\textsuperscript{113} furo[2,3-c]pyridines (Chapter 3),\textsuperscript{114} imidazo[4,5-c]pyridines (Chapter 5),\textsuperscript{115} and furo[2,3-c]quinolines\textsuperscript{116} have been reported from our laboratory (Fig. 1). Recently, a C2-butyl furo[2,3-c]quinoline (5) having pure TLR8 agonistic activity was cocrystallized with the human TLR8 ectodomain.\textsuperscript{117} This served as the point of departure toward a focused structure-based ligand design study, leading to the identification of 3-pentylquinoline-2-amine (6, Fig. 1) as a novel, structurally simple, and highly potent human TLR8-specific agonist.\textsuperscript{118}

Fig. 1. Structures of representative TLR7 and TLR8 agonists.
Our SAR investigations in several of these scaffolds, while continuing to incrementally improve our understanding of the structural features required for the TLR7/8 activity, pointed strongly also to the strict dependence of the selectivity for TLR7 vis-à-vis TLR8 on the electronic configurations of the heterocyclic systems, the nuances of which we desired to examine quantitatively with the goal of developing ‘heuristics’ to clearly define structural requisites governing activity at TLR7 and/or TLR8. In order to systematically examine the effect of electronic properties on the activity profiles, we undertook a scaffold hopping approach\textsuperscript{119} which entailed the syntheses and biological evaluations of thirteen different chemotypes including oxazolo[4,5-c]quinoline, thiazolo/oxazolo[4,5-c]quinolin-2-amines, thiazolo/oxazolo[5,4-c]quinolines, imidazo[1,2-c]quinazoline, [1,2,4]triazolo[1,5-c]quinazoline, imidazo[1,2-a]quinoxaline, [1,2,4]triazolo[1,5-a]quinoxaline, [1,2,4]-triazolo[4,3-a]quinoxaline, and pyrazolo[3,4-c]quinoline.

Crystal structures of TLR8 in complex with two most active compounds confirmed important binding interactions playing a key role in ligand occupancy and biological activity. Reasoning that stereo-electronic effects of heterocyclic ring systems could have a profound effect on the biological activity of TLR7/8 modulators, we undertook studies of three-dimensional molecular electrostatic potential (MESP) in an effort to obtain complementary and/or mechanistic information in characterizing active molecules.\textsuperscript{120} Density functional theory (DFT) based quantum chemical calculations and linear discriminant analyses were therefore performed. These studies allowed, for the first time, a clear delineation of inactive, TLR8-active and TLR7/8 dual active compounds, confirming the critical role of partial charges in determining biological activity.
6.2. Results and Discussion

As mentioned earlier, a number of leads including pure TLR7 agonists (1 and 2), dual TLR7/8 agonist (3), and pure TLR8 agonists (4, 5, and 6) are undergoing preclinical evaluation as vaccine adjuvants in our laboratory. Our earlier structure-activity relationship studies on the imidazo[4,5-c]quinolines,\textsuperscript{55b, 69} thiazolo[4,5-c]quinolines,\textsuperscript{113} furo[2,3-c]pyridines,\textsuperscript{114} as well as furo[2,3-c]quinolines\textsuperscript{116} had all converged on the optimal chain length for the C2 alkyl substituent being butyl. Our goal was therefore to examine the electronic effects of heterocyclic modifications, while holding the substituent at the C2 position invariant at 4 atoms.

We envisioned that a reagent-based diversification approach\textsuperscript{121} could allow us to access to several different heterocyclic scaffolds (including the thiazolo[4,5-c]quinolines) with substantial variations in the electronic configurations (Scheme 1). Employing this diversification strategy, the previously described 2-butylthiazolo[4,5-c]quinoline was synthesized from aminoquinolin-4-ol and valeroyl chloride via a one-pot, sequential reaction involving acylation and subsequent microwave-accelerated (120 °C, 600 W) cyclization using P\textsubscript{2}S\textsubscript{5} (7, Scheme 1), while replacement of P\textsubscript{2}S\textsubscript{5} with P\textsubscript{2}O\textsubscript{5} in this reaction resulted in its congener 8 (2-butyloxazolo[4,5-c]quinoline) in moderate yield. Microwave-assisted cyclization also yielded N-propylthiazolo[4,5-c]quinolin-2-amine 10 using propyl isothiocyanate, whereas conventional heating was unsuccessful. The synthesis of N-propyloxazolo[4,5-c]quinolin-2-amine 12 using P\textsubscript{2}O\textsubscript{5} led to the formation of a mixture of compounds with very poor yields; substituting N-(3-dimethylamino propyl)-N’-ethylcarbodiimide (EDC) for P\textsubscript{2}O\textsubscript{5} in this reaction not only worked as a sulfur scavenger, but greatly enhanced yields of the desired oxazolo analog 12 (Scheme 1). The C4 amine functionality was then installed using conventional methods\textsuperscript{69, 113} to furnish the 2-butyloxazolo[4,5-c]quinolin-4-amine 9, the N-propylthiazolo[4,5-c]quinoline-2,4-diamine 11, and the N-propyloxazolo[4,5-c]quinoline-2,4-diamine 13.

The 2-butyloxazolo[4,5-c]quinolin-4-amine 9 displayed more potent dual TLR7/8-agonistic activity compared to the thiazolo[4,5-c]quinolin-4-amine 3, with EC₅₀ values of 0.55 μM and 0.18 μM in TLR7 and TLR8 assays, respectively (Fig. 2). The N-propythiazolo[4,5-c]quinoline-2,4-diamine 11, however, exhibited comparable TLR7-agonistic activity (EC₅₀: 0.73 μM), but a ten-fold reduction in TLR8 potency (EC₅₀: 3.94 μM). Astonishingly, the N-propyloxazolo[4,5-c]quinoline-2,4-diamines 13 was entirely devoid of any detectable TLR7- or TLR8-agonistic activity. The C2 N-acyl derivative, N-(4-aminothiazolo[4,5-c]quinolin-2-yl)propionamide 16 and its des-acyl analog 17 were synthesized from commercially-available thiazolo[4,5-c]quinolin-2-amine (Scheme 2). These compounds were also found to be inactive in cell based assays (Fig. 2).
**Fig. 2.** TLR7- and TLR8-agonistic potencies (EC<sub>50</sub> values) of the compounds determined in TLR-specific reporter gene assays. Means and standard deviations on quadruplicate samples are depicted.

Reagents and conditions: i. C₂H₅COCl, Pyridine, 25 °C, 1 h; ii. mCPBA, CHCl₃, 25 °C, 4 h; iii. (a) Benzoyl isocyanate, CH₂Cl₂, 45 °C, 1.5 h (b) CH₃ONa, MeOH, 70 °C, 30 min.

The dramatic (and rather unexpected) differences in activity profiles of the closely-related congeners warranted a detailed investigation of analogues with variable electronic properties. We first synthesized and evaluated the regioisomeric 2-butylthiazolo[5,4-c]quinolin-4-amine 22a and 2-butyloxazolo[5,4-c]quinolin-4-amine 22b (Scheme 3). The thiazolo[5,4-c]quinoline derivative (22a) was completely inactive in both TLR7 and TLR8 agonism assays and the oxazolo[5,4-c]quinoline derivative (22b) was found to possess negligibly low TLR8-agonistic activity. This result, too, was unexpected, given that we had observed prominent and selective TLR8 agonism in the 2-butylfuro[2,3-c]quinolin-4-amine 5,¹¹⁶ but further strengthened the case for a systematic exploration of the role of electron densities in the heterocyclic core in determining TLR7/8 activity.


Reagents and conditions: i. C₄H₉COCl, Pyridine, 65 °C, 1.5 h; ii. NBS, AIBN, Benzene, 85 °C, 5 h; iii. For a, Lawesson's reagent, Pyridine, MW, 140 °C, 35 min; For b, C₂H₅, K₂CO₃, Pyridine, MW, 140 °C, 35 min; iv. mCPBA, CH₂Cl₂, 25 °C, 4 h; v. (a) Benzoyl isocyanate, CH₂Cl₂, 45 °C, 3 h (b) CH₃ONa, CH₃OH, 70 °C, 8 h.
Further scaffold modifications were therefore carried out based on the pure TLR7-agonistic lead molecule 1-benzyl-2-butyl-1\textit{H}-imidazo[4,5-c]quinolin-4-amine 1 (Fig.1). 'Repositioning' of the nitrogen atoms in the imidazole ring as well as triazole analogues were designed and synthesized (Schemes 4-8).\textsuperscript{122} The novel analogues 2,3-dihydroimidazo[1,2-c]quinazoline 27 and imidazo[1,2-c]quinazoline 29, with an altered imidazole fused ring (Scheme 4) were entirely inactive (Fig. 2). We sought to examine if activity could be restored by incorporating an additional nitrogen atom in ring system, but the triazole analogue 33 (2-butyl-[1,2,4]triazolo[1,5-c]quinazolin-5-amine, Scheme 5) was also inactive. On the other hand, the 1,2-dihydroimidazo[1,2-a]quinoxaline 37 and the imidazo[1,2-a]quinoxaline 39 shown in Scheme 6 were found to be selective TLR8 agonists with EC\textsubscript{50} values of 3.05 \(\mu\text{M}\) and 7.99 \(\mu\text{M}\), respectively (Fig. 2).


![Scheme 4](image)

Reagents: i. Urea; ii. DIPEA, POCl\textsubscript{3}; iii. DL-2-amino-1-hexanol, DMAP, DIPEA, DMF; iv. NEt\textsubscript{3}, CH\textsubscript{3}SO\textsubscript{2}Cl, CH\textsubscript{2}Cl\textsubscript{2}; v. 2M NH\textsubscript{3} in CH\textsubscript{3}OH; vi. MnO\textsubscript{2}, toluene; vii. 2M NH\textsubscript{3} in CH\textsubscript{3}OH.


![Scheme 5](image)

Reagents: i. Methyl chloroformate, Na\textsubscript{2}CO\textsubscript{3}; ii. Valeric acid hydrazide, NMP; iii. DIPEA, POCl\textsubscript{3}; iv. 2M NH\textsubscript{3} in CH\textsubscript{3}OH.

Reagents and conditions: i. POCb, DMF, 100 °C, 1.5 h; ii. DL-2-amino-1-hexanol, EtOH, 100 °C, 18 h; iii. SOCl₂, CHCl₃, 0-65 °C, 2 h; iv. 2M NH₃ in CH₃OH, 90 °C, 20 h; v. MnO₂, toluene, 115 °C, 72 h; vi. 2M NH₃ in CH₃OH, 90 °C, 20 h.

Transitioning from the imidazo[1,2-a]quinoxaline scaffold to two other triazolo analogues (46 in Scheme 7 and 49 in Scheme 8) also resulted in complete loss of activity.

Scheme 7. Synthesis of [1,2,4]triazolo[1,5-a]quinoxaline.

Reagents: i. C₆H₅COCl, NMP, CH₂Cl₂; ii. POC₅; iii. 2M NH₃ in CH₃OH; iv. Ethyl oxalyl chloride, ether, toluene; v. Fe, AcOH; vi. DIPEA, POC₅; vii. 2M NH₃ in CH₃OH.


Reagents: i. Hydrazine hydrate, EtOH; ii. Trimethyl orthoformate; iii. 2M NH₃ in CH₃OH.
Our scaffold-hopping approach also led us to synthesize 2-butyl-2H-pyrazolo[3,4-c]quinolin-4-amine 53 (Scheme 9). Compound 53 was found to be extraordinarily potent as a TLR7 agonist (EC$_{50}$: 0.19 μM), significantly greater than that of the thiazoloquinoline 3 (EC$_{50}$: 0.86 μM), the oxazoloquinoline 9 (EC$_{50}$: 0.55 μM), and the aminothiazoloquinoline 11 (EC$_{50}$: 0.73 μM), and approaching that of our best-in-class, pure TLR7 agonistic imidazoquinoline 1 (EC$_{50}$: 0.059 μM). Furthermore, the pyrazolo[3,4-c]quinoline 53 was also found to be the most potent in TLR8 agonism assays (EC$_{50}$: 0.056 μM) among all TLR8-active compounds that we had hitherto characterized (Fig. 2).

**Scheme 9. Synthesis of pyrazolo[3,4-c]quinoline.**

![Scheme 9](image)

*Reagents:* i. Ethyl chlorooxocacetate, pyridine, Et$_2$O; ii. Butylhydrazine.HCl, EtOH, CH$_3$COOH; iii. PCl$_5$, POCl$_3$; iv. 2M NH$_2$ in CH$_3$OH.

We confirmed TLR7/8 selectivity and potency of the active compounds in secondary screens including cytokine-inducing properties in human PBMCs, as well as cellular activation in ex vivo whole human blood. In IFN-α induction assays, as expected and in accordance with our previous SAR, compounds with TLR7 agonistic activity (9, 11, and 53) showed IFN-α inducing ability and the TLR8 selective compound 39 did not (Fig. 3).
Fig. 3. Dose-response profiles of IFN-α induction by the active compounds in human PBMCs. Means on triplicate samples of a representative experiment are shown.

We also found strong Type II IFN (IFN-γ), cytokine (IL-1α, IL-1β, IL-6, IL-10, TNF-α), and chemokine (IP-10/CXCL-10, MCP-1, MIP-1β) induction by the active compounds consistent with their TLR7/8 selectivity profiles (Fig. 4). The extraordinary potency of 53 was also manifested in CD69 expression in whole blood assays, showing dramatically enhanced expression in cytokine-induced killer-, natural killer-, T-, and B-lymphocytic subsets (Fig. 5).
Fig. 4. Cytokine and chemokine induction profiles in human PBMCs stimulated with 10 μM of select compounds. Means on triplicate samples of a representative experiment are shown.
We were fortunate in also being able to obtain the crystal structures of TLR8 in complex with the two most active compounds: the oxazoloquinoline 9 and the pyrazoloquinoline 53. An examination of TLR8 liganded with 9 and 53 confirmed near-identical binding geometries of the
two compounds (Fig. 6). Major interactions include hydrogen bonding of the amidine group with Asp543 and the N atom of the oxazole/pyrazole ring with Thr574, π-π interactions of the quinoline ring with Phe405, and hydrophobic interactions of the alkyl chain in a pocket formed by Tyr348, Val378, and Phe405.

**Fig. 6.** Crystal structures of human TLR8 ectodomain complexed with compound 9 (Panel A) and compound 53 (Panel B), showing key interactions in the binding pocket. PDB codes for compounds 9 and 53 are, respectively, 4QBZ and 4QC0.
Whereas the steric properties of most of these analogues are very similar, their activity profiles are considerably different and the data, taken together, strongly pointed to electronic densities of ring system(s) being dominant determinants of occupancy and activation of TLR7 and TLR8 by these analogues. We therefore undertook quantum chemical calculations of electron densities and of Mulliken atomic charges with the objective of obtaining insights into the properties of these molecules, which we hoped would lead to quantitative predictors of selectivity and potency at TLR7 and TLR8.

As described earlier, the crystal structures of TLR8 complexed with active analogues showed key H-bonds between the amidine group of the quinoline moiety with the side chain of Asp543, and the N atoms of the oxazole or pyrazole moieties of compounds 9 and 53, respectively, with Thr574, providing major contributions to overall binding interactions (Fig. 6). Consistent with our expectation that the strength and geometry of the H-bonds are modulated by electron densities and Mulliken charges on appropriate heteroatoms, we observed clear differences in atoms known to be involved in H-bonding interactions. The active compounds 3, 9, 11, 39, and 53 (Fig. 7) display pronounced negative charges (-0.24, -0.24, -0.29, -0.27, and -0.23 electron units, respectively) at position M1 of the five-membered ring. Compounds that do not have the electronegative atom at position M1 (22a and 29; +0.15, 0.0) were found to be inactive. We also noticed a higher partial positive charge at the M2 position in the oxazolo[4,5-c]quinoline-2,4-diamine 13 (0.33 electron unit, Fig. 7c) compared to other compounds, attributable to adjacent electronegative heteroatoms (N and O), possibly explaining the lack of activity. The quinazoline analogues 29 and 33 were unique in that the presence of an additional electronegative nitrogen atom at position M5 (-0.25 and -0.22, respectively) resulted in strong positive charge at position M6 (+0.26 and +0.29 electron units, respectively), again correlating with absence of agonistic activities.
Fig. 7. Molecular electrostatic potential surfaces of selected compounds.
Given the importance of hydrogen bonding of the amidine group with Asp543, we examined electrostatic potentials at M7. Active compounds could be differentiated from inactive compounds (Fig. 8A) with three exceptions: compounds 13, 16 and 22a. The absence of TLR7/8-agonistic activity in 16 and 22a could be explained readily; the presence of a polar amide sidechain in 16 is expected to disfavor interactions in the hydrophobic pocket, while the regio-isomeric thiazoloquinoline 22a possesses a bulky sulfur atom at M1. The misclassification of 13 as an active compound may, as mentioned earlier, be related to the higher partial positive charge at the M2 position.

The availability of electron density and charge parameters for all atoms in all of the analogues prompted us to examine if a formal classification of active vis-à-vis inactive compounds could be arrived at, with our goal of being able to utilize such methodology in prospectively designing ‘bespoke’ compounds with predefined selectivity. Stepwise linear discriminant analyses were performed with the 13 compounds shown in Fig. 2 as a training set. A linear combination of the variables corresponding to M1-M6 explained 100% of the variance in two dimensions (Discriminant Functions 1 and 2, see Fig. 8B), allowing a clear-cut classification of inactive (coded ‘0’), TLR8-active (coded ‘1’), and TLR7/8 dual-active (coded ‘2’) compounds (Fig. 8B). The discriminant functions were utilized to examine a test set of compounds which included 5116 (Fig. 1), CL097,43a as well as nine ‘hypothetical’ compounds (Fig. 8B). Compound 5 and CL097 were correctly classified as being TLR8 (Group 1) and TLR7/8 dual-active (Group 2). All of the proposed analogues with the exception of H3 were predicted to be active. It is noteworthy that the isomeric compounds H8 and H9 (2-butyl-cyclopentaquinolin-4-amines) could be considered as conformationally-constrained analogues of 3-alkyl-quinoline-2-amines118 (Fig. 1) which we have recently designed and characterized as pure TLR8 agonists. The thienoquinolines H1 and H2 as well as the pyrroloquinolines H3 and H4 are of particular interest, and we are currently evaluating such analogues.
Fig. 8. A: Classification of inactive and active compounds based on electrostatic potentials at M7. B: Linear discriminant analysis. Demarcation of Group 0 (inactive), Group 1 (TLR8-specific), and Group 2 (TLR7/8-dual active) compounds obtained via linear discriminant analyses of Mulliken charges.
6.3. Conclusion

We undertook a scaffold-hopping approach, which entailed the syntheses and biological evaluations of 13 different chemotypes. Density functional theory based quantum chemical calculations on these compounds followed by linear discriminant analyses permitted the classification of inactive, TLR8-active, and TLR7/8 dual-active compounds, confirming the critical role of partial charges in determining biological activity. The question as to why the activity profiles of the oxazoloquinoline 9 and its 2-amino analogue 13 are completely divergent remains unclear, however, crystallographic observations of the complex of 9 with TLR8 even in conjunction with electronic structure calculations only allow us to speculate at the present time as to the role of the water molecule by virtue of its permanent dipole moment and polarizability on stabilizing (or destabilizing interactions) depending on electron densities around the five-membered ring. We are gratified, nonetheless, that quantum chemical calculations in conjunction with rigorous multivariate analyses may afford an empirical, but accessible means to evaluating analogues de novo.

6.4. Experimental

Chemistry. All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 °C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf “Gold” high performance silica columns on CombiFlash Rf instrument unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel CCM precoated aluminum sheets. Purity for all final compounds was confirmed to be greater than
97% by LC–MS using a Zorbax Eclipse Plus 4.6 mm × 150 mm, 5 μm analytical reverse phase C18 column with H2O–CH3CN gradients and an Agilent 6520 ESIQTOF Accurate Mass spectrometer (mass accuracy of 5 ppm) operating in the positive ion acquisition mode.

**Synthesis of compound 7: 2-Butylthiazolo[4,5-c]quinoline.** To a solution of 3-aminoquinolin-4-ol (12 mg, 0.075 mmol) in pyridine (0.5 mL) was added valeroyl chloride (11 μL, 0.09 mmol) and the resulting mixture was heated in a sealed vial at 50 °C for 1 h. P2S5 (33 mg) was added and the mixture was heated at 120 °C for 1 h under microwave irradiation. The solvents were removed and the crude residue was purified by flash chromatography (SiO2, 0-5% MeOH in CH2Cl2) to give compound 7 (13.4 mg, 79%) as reddish brown solid. 1H NMR (500 MHz, CDCl3) δ 9.44 (s, 1H), 8.24 (d, J = 8.4 Hz, 1H), 7.96 (dd, J = 8.1, 0.9 Hz, 1H), 7.73 (ddd, J = 8.4, 5.4, 1.4 Hz, 1H), 7.63 (ddd, J = 8.1, 7.1, 1.1 Hz, 1H), 3.25–3.19 (m, 2H), 1.97–1.89 (m, 2H), 1.55–1.46 (m, 2H), 1.00 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, CDCl3) δ 173.0, 147.9, 145.8, 144.2, 140.6, 130.6, 128.8, 128.8, 127.6, 125.0, 123.6, 34.2, 32.0, 22.4, 13.9. MS (ESI-TOF, m/z): calculated for C14H14N2S 243.0950; found 243.0976 [M+H]+ (Data from Org. Biomol. Chem. 2013, 11, 1179).

**Synthesis of compound 8: 2-Butyloxazolo[4,5-c]quinolone.** To a solution of 3-aminoquinolin-4-ol (12 mg, 0.075 mmol) in pyridine (0.5 mL) was added valeroyl chloride (11 μL, 0.09 mmol) and the resulting mixture was heated in a sealed vial at 50 °C for 1 h. P2O5 (22 mg) was added and the resulting mixture was heated at 120 °C for 1 h under microwave irradiation. The solvents were removed and the crude residue was purified by flash chromatography (SiO2, 0-5% MeOH in CH2Cl2) to give compound 8 as pale yellow solid (11.5 mg, 63%). 1H NMR (500 MHz, CDCl3) δ 9.27 (s, 1H), 8.25 (d, J = 8.4 Hz, 1H), 8.20 (ddd, J = 8.1, 1.5, 0.6 Hz, 1H), 7.75 (ddd, J = 8.5, 7.0, 1.5 Hz, 1H), 7.68 (ddd, J = 8.1, 7.0, 1.1 Hz, 1H), 3.08 (dd, J = 9.0, 6.3 Hz,
2H), 1.96 (dd, J = 15.2, 7.6, 6.0 Hz, 2H), 1.51 (dt, J = 14.8, 7.4 Hz, 2H), 1.01 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 167.5, 152.1, 145.9, 143.9, 134.9, 130.1, 128.7, 127.3, 120.1, 116.3, 28.9, 28.4, 22.3, 13.7. MS (ESI-TOF, m/z): calculated for C$_{14}$H$_{14}$N$_2$O 227.1179; found 227.1216 [M+H]$^+$. 

**Synthesis of 2-Butyloxazolo[4,5-c]quinoline 5-oxide.** To a solution of compound 8 (68 mg, 0.30 mmol) in CHCl$_3$ (5 mL), was added m-CPBA (â‰¥ 77%, 100 mg, 0.45 mmol) and the reaction mixture was stirred at room temperature for 4 h. The solvent was removed under reduced pressure and the crude residue was purified by flash chromatography (SiO$_2$, 0-5% MeOH in CH$_2$Cl$_2$) to give 2-butyloxazolo[4,5-c]quinoline 5-oxide. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.97 (s, 1H), 8.92 – 8.87 (m, 1H), 8.18 (ddd, J = 6.4, 2.2, 0.6 Hz, 1H), 7.83 – 7.77 (m, 2H), 3.06 (dd, J = 9.0, 6.3 Hz, 2H), 1.97 – 1.90 (m, 2H), 1.55 – 1.47 (m, 2H), 1.01 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 169.5, 144.3, 139.8, 134.6, 130.0, 129.7, 129.6, 121.5, 120.8, 116.6, 28.8, 28.4, 22.3, 13.7. MS (ESI-TOF, m/z): calculated for C$_{14}$H$_{14}$N$_2$O$_2$ 243.1128; found 243.1146 [M+H]$^+$. 

**Synthesis of compound 9: 2-Butyloxazolo[4,5-c]quinolin-4-amine.** 2-Butyloxazolo[4,5-c]quinoline 5-oxide (60 mg, 0.25 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (2 mL). Benzoyl isocyanate (74 mg, 0.50 mmol) was added and the resulting mixture was refluxed for 1.5 h. The solvent was removed and the residue was dissolved in anhydrous methanol (2 mL). Sodium methoxide (27 mg, 0.50 mmol) was added and the resulting mixture was refluxed for 30 min. The solvent was removed under reduced pressure and the crude residue was purified by flash chromatography (SiO$_2$, 0-10% MeOH in CH$_2$Cl$_2$) to give compound 9 as white solid (37 mg, 61%). $^1$H NMR (500 MHz, MeOD) $\delta$ 7.95 (ddd, J = 8.0, 1.5, 0.5 Hz, 1H), 7.70 – 7.65 (m, 1H), 7.56 (ddd, J = 8.5, 7.0, 1.5 Hz, 1H), 7.35 (ddd, J = 8.1, 7.0, 1.1 Hz, 1H), 3.09 – 3.03 (m, 2H),
1.92 (ddd, J = 13.7, 8.2, 6.9 Hz, 2H), 1.55 – 1.46 (m, 2H), 1.02 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, MeOD) δ 168.0, 154.0, 152.9, 146.4, 130.2, 126.3, 125.6, 124.0, 121.1, 114.3, 30.0, 28.9, 23.3, 14.0. MS (ESI-TOF, m/z): calculated for C14H15N3O 242.1288; found 242.1313 [M+H]+.

Synthesis of compound 10: N-Propylthiazolo[4,5-c]quinolin-2-amine. To a solution of 3-aminoquinolin-4-ol (32 mg, 0.20 mmol) in pyridine (1 mL) was added propyl isothiocyanate (31 μL, 0.30 mmol) and the resulting mixture was heated in a sealed vial at 50 °C for 30 min. P2S5 (89 mg) was added and the resulting mixture was heated at 120 °C for 1 h under microwave irradiation. The solvent was removed and the crude residue was purified by flash chromatography (SiO2, 0-5% MeOH in CH2Cl2) to give compound 10 as pale brown solid (34 mg, 70%). 1H NMR (400 MHz, CDCl3) δ 9.12 (s, 1H), 8.15 (dd, J = 8.3, 0.8 Hz, 1H), 7.78 – 7.71 (m, 1H), 7.60 (ddd, J = 8.4, 7.0, 1.7 Hz, 1H), 7.55 (ddd, J = 8.2, 7.0, 1.4 Hz, 1H), 5.85 (s, 1H), 3.47 (dd, J = 11.7, 7.0 Hz, 2H), 1.78 (dd, J = 14.4, 7.3 Hz, 2H), 1.06 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, CDCl3) δ 168.3, 147.0, 143.1, 143.1, 134.0, 130.3, 127.1, 127.0, 123.7, 123.6, 47.8, 22.7, 11.4. MS (ESI-TOF, m/z): calculated for C13H13N3S 244.0903; found 244.0946 [M+H]+.

11: N2-Propylthiazolo[4,5-c]quinoline-2,4-diamine. 1H NMR (500 MHz, MeOD) δ 7.57 (d, J = 8.4 Hz, 1H), 7.50 (d, J = 8.0 Hz, 1H), 7.42 – 7.36 (m, 1H), 7.26 – 7.19 (m, 1H), 3.42 (t, J = 7.0 Hz, 2H), 1.72 (h, J = 7.3 Hz, 2H), 1.02 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, MeOD) δ 169.3, 152.0, 143.2, 137.6, 133.7, 128.3, 125.8, 124.4, 123.9, 121.0, 47.8, 23.5, 11.8. MS (ESI-TOF, m/z): calculated for C13H14N4S 259.1012; found 259.1054 [M+H]+.
Synthesis of compound 12: N-Propyloxazolo[4,5-c]quinolin-2-amine. To a solution of 3-aminoquinolin-4-ol (32 mg, 0.20 mmol) in pyridine (1 mL) was added propyl isothiocyanate (31 μL, 0.30 mmol) and the resulting mixture was heated in a sealed vial at 50 °C for 30 min. EDC (77 mg, 0.4 mmol) was added and the resulting mixture was heated at 120 °C for 30 min under microwave irradiation. The solvent was removed and the crude residue was purified by flash chromatography (SiO₂, 0-5% MeOH in CH₂Cl₂) to give compound 12 as brown solid (25 mg, 59%). ¹H NMR (500 MHz, MeOD) δ 8.83 (s, 1H), 8.06 – 8.02 (m, 1H), 8.01 – 7.97 (m, 1H), 7.67 – 7.58 (m, 2H), 3.42 (t, J = 7.1 Hz, 2H). ¹³C NMR (126 MHz, MeOD) δ 164.7, 150.1, 145.1, 141.2, 137.8, 129.7, 128.7, 128.6, 120.2, 116.7, 45.9, 23.6, 11.6. MS (ESI-TOF, m/z): calculated for C₁₃H₁₃N₃O 228.1131; found 228.1164 [M+H]⁺.

2-(Propylamino)oxazolo[4,5-c]quinoline 5-oxide. ¹H NMR (500 MHz, DMSO-d₆) δ 8.83 (s, 1H), 8.58 (t, J = 5.8 Hz, 1H), 8.56 (d, J = 8.8 Hz, 1H), 7.97 (dd, J = 8.3, 0.6 Hz, 1H), 7.75 (ddd, J = 8.2, 6.9, 1.1 Hz, 1H), 7.69 – 7.64 (m, 1H), 3.36 – 3.30 (m, 10H), 1.69 – 1.59 (m, 2H), 0.94 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 163.8, 139.3, 137.2, 136.8, 129.4, 127.7, 127.3, 120.5, 119.6, 115.3, 44.4, 22.0, 11.3. MS (ESI-TOF, m/z): calculated for C₁₃H₁₃N₃O 244.1081; found 244.0974 [M+H]⁺.

13: N²-Propyloxazolo[4,5-c]quinoline-2,4-diamine. ¹H NMR (500 MHz, DMSO-d₆) δ 11.96 (s, 1H), 8.16 (dd, J = 8.0, 1.2 Hz, 1H), 7.50 – 7.45 (m, 1H), 7.44 (dd, J = 8.2, 1.0 Hz, 1H), 7.16 (ddd, J = 8.1, 6.6, 1.5 Hz, 1H), 7.06 (t, J = 5.9 Hz, 1H), 3.24 (dd, J = 14.1, 6.3 Hz, 2H), 1.63 – 1.48 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 161.7, 155.7, 150.2, 137.4, 129.0, 124.9, 124.0, 120.2, 117.0, 112.8, 43.9, 22.6, 11.3. MS (ESI-TOF, m/z): calculated for C₁₃H₁₄N₄O 243.1240; found 243.1141 [M+H]⁺.
Synthesis of compound 15: 2-Propionamidothiazolo[4,5-c]quinoline 5-oxide. To a solution of thiazolo[4,5-c]quinolin-2-amine (100 mg, 0.497 mmol) in pyridine (2 mL) was added propionyl chloride (54 μL, 0.62 mmol) and the resulting mixture was stirred at room temperature for 1 h. The solvent was removed and the crude residue was purified by flash chromatography (SiO₂, 0-5% MeOH in CH₂Cl₂) to give compound N-(thiazolo[4,5-c]quinolin-2-yl)propionamide 14 as impure solid, which was dissolved in CHCl₃ (4 mL). m-CPBA (≤ 77%, 224 mg, 1.0 mmol) was added and the reaction mixture was stirred at room temperature for 4 h and then concentrated. The crude residue was purified by flash chromatography (SiO₂, 0-5% MeOH in CH₂Cl₂) to give compound 15 as white solid (98 mg, 72%). ¹H NMR (500 MHz, DMSO-d₆) δ 12.75 (s, 1H), 9.11 (s, 1H), 8.68 – 8.62 (m, 1H), 8.24 – 8.17 (m, 1H), 7.86 – 7.76 (m, 2H), 2.56 (q, J = 7.5 Hz, 2H), 1.14 (t, J = 7.5 Hz, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 173.3, 160.0, 142.9, 137.8, 129.8, 129.7, 128.7, 125.1, 124.6, 123.6, 120.3, 28.4, 8.8. MS (ESI-TOF, m/z): calculated for C₁₃H₁₁N₃O₂S 274.0645; found 274.0667 [M+H]+.

Synthesis of compound 16: N-(4-Aminothiazolo[4,5-c]quinolin-2-yl)propionamide. Compound 15 (55 mg, 0.20 mmol) was dissolved in anhydrous CH₂Cl₂ (2 mL). Benzoyl isocyanate (59 mg, 0.40 mmol) was added and the resulting mixture was refluxed for 1.5 h. The solvent was removed under reduced pressure and the residue was dissolved in anhydrous MeOH (2 mL). Sodium methoxide (22 mg, 0.40 mmol) was added and the reaction mixture was refluxed for 30 min. The solvent was removed and the crude residue was purified by flash chromatography (SiO₂, 0-10% MeOH in CH₂Cl₂) to give compound 16 as white solid (41 mg, 79%). ¹H NMR (500 MHz, DMSO-d₆) δ 12.53 (s, 1H), 7.80 (dd, J = 8.0, 1.0 Hz, 1H), 7.61 (dd, J = 8.3, 0.6 Hz, 1H), 7.48 (ddd, J = 8.4, 7.0, 1.4 Hz, 1H), 7.26 (ddd, J = 8.1, 7.0, 1.2 Hz, 1H), 2.55 (q, J = 7.5 Hz, 2H), 1.14 (t, J = 7.5 Hz, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 173.1, 157.0, 151.5, 144.2, 133.8, 133.1, 127.8, 125.9, 123.7, 122.2, 119.1, 28.3, 9.0. MS (ESI-TOF, m/z):
calculated for C_{13}H_{12}N_{4}OS 273.0805; found 273.0832 [M+H]^+. The sodium methoxide solvolysis also resulted in small amount of thiazolo[4,5-c]quinoline-2,4-diamine 17 which was isolated from the crude mixture as white solid (4 mg, 9%). \(^1\)H NMR (500 MHz, MeOD) \(\delta\) 7.58 (ddd, \(J = 8.4, 1.0, 0.5\) Hz, 1H), 7.52 (ddd, \(J = 8.0, 1.4, 0.5\) Hz, 1H), 7.41 (ddd, \(J = 8.4, 7.0, 1.5\) Hz, 1H), 7.24 (ddd, \(J = 8.1, 7.1, 1.1\) Hz, 1H). \(^{13}\)C NMR (126 MHz, MeOD) \(\delta\) 169.6, 152.0, 143.6, 137.3, 134.7, 128.4, 126.0, 124.4, 123.9, 121.1. MS (ESI-TOF, \(m/z\)): calculated for C_{10}H_{8}N_{4}S 217.0542; found 217.0569 [M+H]^+.

**Synthesis of compound 18: \(N\)-(Quinolin-4-yl)valeramide.** To a solution of 4-aminoquinoline (302 mg, 2.1 mmol) in pyridine (6 mL) was added valeroyl chloride (0.28 mL, 2.4 mmol). The mixture was heated at 65 °C for 90 min and then concentrated. The crude residue was purified by flash chromatography (SiO\(_2\), 0-10\% MeOH in CH\(_2\)Cl\(_2\)) to give compound 18 as a dark brown solid (382 mg, 80%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 11.11 (s, 1H), 9.07 (d, \(J = 6.5\) Hz, 1H), 8.95 (dd, \(J = 8.7, 1.1\) Hz, 1H), 8.70 (d, \(J = 6.4\) Hz, 1H), 8.32 (dd, \(J = 8.5, 1.1\) Hz, 1H), 8.12 (ddd, \(J = 8.4, 6.9, 1.1\) Hz, 1H), 7.93 (ddd, \(J = 8.4, 6.9, 1.1\) Hz, 1H), 2.80 (t, \(J = 7.4\) Hz, 2H), 2.00 (tt, \(J = 7.5\) Hz, 2H), 1.39 (tq, \(J = 7.4\) Hz, 2H), 0.94 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (126 MHz, DMSO-\(d_6\)) \(\delta\) 174.2, 149.6, 145.0, 139.0, 133.8, 128.1, 123.8, 121.2, 119.0, 108.9, 36.4, 26.6, 21.6, 13.7. MS (ESI-TOF, \(m/z\)): calculated for C\(_{14}\)H\(_{16}\)N\(_2\)O 229.1341; found 229.1306 [M+H]^+.

**Synthesis of compound 19: \(N\)-(3-Bromoquinolin-4-yl)valeramide.** To a suspension of compound 18 (151 mg, 0.66 mmol) in benzene (anhydrous, 10 mL) was added \(N\)-bromosuccinimide (NBS, 143 mg, 0.81 mmol) and azobisisobutyronitrile (AIBN, 99 mg, 0.60 mmol). The mixture was refluxed for 5 h and then concentrated. The crude residue was purified by flash chromatography (SiO\(_2\), 0-30\% EtOAc in hexane) to give compound 19 as a light brown solid (86 mg, 42%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 10.31 (s, 1H), 9.05 (s, 1H), 8.08 (dd, \(J=...
8.5, 1.2 Hz, 1H), 7.95 (dd, J = 8.4, 1.4 Hz, 1H), 7.84 (ddd, J = 8.4, 6.9, 1.5 Hz, 1H), 7.70 (ddd, J = 8.2, 6.8, 1.3 Hz, 1H), 2.54-2.46 (m, 2H), 1.68 (quin, J = 7.5 Hz, 2H), 1.43 (sex, J = 7.4 Hz, 2H), 0.95 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 171.4, 152.1, 147.1, 141.5, 130.2, 129.1, 127.8, 126.6, 123.8, 116.8, 35.2, 27.3, 21.9, 13.8. MS (ESI-TOF, m/z): calculated for C₁₄H₁₅BrN₂O 307.0446; found 307.0254 [M+H]^⁺.

Synthesis of compound 20a: 2-Butylthiazolo[5,4-c]quinoline. To a solution of compound 19 (60.8 mg, 0.20 mmol) in pyridine (4 mL) was added Lawesson’s reagent (234 mg, 0.58 mmol). The resulting mixture was heated in a sealed vial under microwave irradiation (500 W, 140 °C) for 35 min and then concentrated. The crude residue was purified by flash chromatography (SiO₂, 0-10% EtOAc in hexanes) to give compound 20a as brown oil (15 mg, 31%). ¹H NMR (500 MHz, CDCl₃) δ 9.33 (s, 1H), 8.71 (dd, J = 8.1, 1.5 Hz, 1H), 8.22 (brd, J = 8.3 Hz, 1H), 7.76 (dd, J = 8.3, 7.0, 1.5 Hz, 1H), 7.70 (ddd, J = 8.1, 7.0, 1.2 Hz, 1H), 3.27 (t, J = 7.7 Hz, 2H), 1.95 (tt, J = 7.6, 7.7 Hz, 2H), 1.52 (qt, J = 7.3, 7.4 Hz, 2H), 1.01 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 177.3, 147.8, 145.9, 145.9, 143.8, 129.4, 128.7, 127.8, 127.2, 123.6, 123.4, 34.3, 32.0, 22.3, 13.8. MS (ESI-TOF, m/z): calculated for C₁₄H₁₄N₂S 243.0956; found 243.1019 [M+H]^⁺.

Synthesis of compound 21a: 2-Butylthiazolo[5,4-c]quinoline 5-oxide. To a solution of compound 20a (14.5 mg, 0.060 mmol) in CH₂Cl₂ (1 mL) was added m-CPBA (≤ 77%, 35.2 mg). The reaction mixture was stirred at room temperature for 2.5 h and then concentrated. The crude residue was purified by flash chromatography (SiO₂, MeOH in CH₂Cl₂: 0 to 5%) to give compound 21a (13.5 mg, 87%) as brown oil. ¹H NMR (500 MHz, CDCl₃) δ 9.04 (s, 1H), 8.90-8.76 (m, 1H), 8.78-8.58 (m, 1H), 7.95-7.72 (m, 2H), 3.25 (t, J = 7.7 Hz, 2H), 1.94 (tt, J = 7.6, 7.7 Hz, 2H), 1.63-1.44 (m, 2H), 1.02 (t, 3H, J = 7.3 Hz). ¹³C NMR (126 MHz, CDCl₃) δ 176.4, 147.8,
Synthesis of compound 22a: 2-Butylthiazolo[5,4-c]quinolin-4-amine. To a solution of compound 21a (10.1 mg, 0.039 mmol) in CH₂Cl₂ (0.2 mL) was added benzoyl isocyanate (13.2 mg, 0.090 mmol). The mixture was heated to reflux for 3 h and then concentrated. The crude residue was purified by flash chromatography (SiO₂, 0-15% EtOAc in hexanes) to give intermediate as light brown oil (8.8 mg, 63%). Benzamide (8.8 mg, 0.024 mmol) was dissolved in NaOMe solution (0.5 mL, 0.5 M in MeOH). The mixture was heated to reflux overnight and then concentrated. The crude residue was purified by flash chromatography (SiO₂, 0-5% MeOH in CH₂Cl₂) to give compound 22a as a yellow solid (5.6 mg, 89%). ^1H NMR (500 MHz, CDCl₃) δ 8.50 (dd, J = 8.1, 1.2 Hz, 1H), 7.82 (brd, J = 8.4 Hz, 1H), 7.62 (ddd, J = 8.4, 7.0, 1.5 Hz, 1H), 7.44 (ddd, J = 8.0, 7.0, 1.1 Hz, 1H), 5.15 (brs, 2H), 3.25 (t, J = 7.7 Hz, 2H), 2.00-1.87 (m, 2H), 1.61-1.43 (m, 2H), 1.02 (t, J = 7.4 Hz, 3H). ^13C NMR (126 MHz, CDCl₃) δ 175.7, 157.0, 150.9, 145.8, 129.2, 125.7, 123.6, 123.5, 120.2, 116.7, 34.2, 32.0, 22.3, 13.8. MS (ESI-TOF, m/z): calculated for C₁₄H₁₄N₂OS 259.0827; found 259.0755 [M+H]^+.

Synthesis of compound 20b: 2-Butyloxazolo[5,4-c]quinoline. To a solution of compound 19 (50.4 mg, 0.16 mmol) in pyridine (2 mL) was added CuI (66 mg, 0.35 mmol) and K₂CO₃ (47 mg, 0.34 mmol). The resulting mixture was heated in a sealed vial under microwave irradiation (500 W, 140 °C) for 35 min and then concentrated. The crude residue was purified by flash chromatography (SiO₂, 0-10% EtOAc in hexanes) to give compound 20b as a brown solid (28 mg, 76%). ^1H NMR (500 MHz, CDCl₃) δ 9.20 (bs, 1H), 8.46 (bs, 1H), 8.25 (bs, 1H), 7.76 (bt, J = 6.0 Hz, 1H), 7.72 – 7.64 (m, 1H), 3.09 (t, J = 7.6 Hz, 2H), 2.00 – 1.91 (m, 2H), 1.55 – 1.43 (m, 2H), 1.00 (t, J = 7.4 Hz, 3H). ^13C NMR (126 MHz, CDCl₃) δ 169.8, 145.5, 143.7, 135.1, 129.9,
Synthesis of compound 21b: 2-Butyloxazolo[5,4-c]quinoline 5-oxide. To a solution of compound 20b (28 mg, 0.124 mmol) in CH₂Cl₂ (2 mL) was added m-CPBA (≤ 77%, 83.5 mg). The reaction mixture was stirred at room temperature for 4 h and then concentrated. The crude residue was purified by flash chromatography (SiO₂, 0-5% MeOH in CH₂Cl₂) to give compound 21b as a red solid (22 mg, 73%). ¹H NMR (500 MHz, CDCl₃) δ 8.93 (s, 1H), 8.87-8.79 (m, 1H), 8.46-8.41 (m, 1H), 7.84-7.75 (m, 2H), 3.06 (t, J = 7.6 Hz, 2H), 1.93 (tt, J = 7.7, 7.6 Hz, 2H), 1.50 (qt, J = 7.4, 7.3 Hz, 2H), 1.01 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 169.9, 143.9, 140.1, 135.7, 129.6, 129.3, 123.6, 122.8, 121.8, 120.8, 28.9, 28.5, 22.3, 13.7. MS (ESI-TOF, m/z): calculated for C₁₄H₁₄N₂O₂ 243.1134; found 243.1032 [M+H]+.

Synthesis of compound 22b: 2-Butyloxazolo[5,4-c]quinolin-4-amine. To a solution of compound 21b (17.8 mg, 0.073 mmol) in CH₂Cl₂ (0.5 mL) was added benzoyl isocyanate (38.4 mg, 0.26 mmol). The mixture was heated to reflux for 4 h and then concentrated. The crude residue was purified by flash chromatography (SiO₂, 0-20% EtOAc in hexanes) to give intermediate as colorless oil (8.7 mg, 34%). Benzamide (6 mg, 0.017 mmol) was dissolved in NaOMe solution (0.5 mL, 0.5 M in MeOH). The mixture was heated to reflux for 8 h and then concentrated. The crude residue was purified by flash chromatography (SiO₂, 0-5% MeOH in CH₂Cl₂) to give compound 22b as a yellow solid (3.3 mg, 79%). ¹H NMR (500 MHz, CDCl₃) δ 8.22 (dd, J = 8.2, 1.5 Hz, 1H), 7.80 (dd, J = 8.7, 1.5 Hz, 1H), 7.59 (ddd, J = 8.5, 7.0, 1.6 Hz, 1H), 7.42 (ddd, J = 8.2, 7.0, 1.1 Hz, 1H), 5.15 (bs, 2H), 3.05 (t, J = 7.7 Hz, 2H), 2.01-1.85 (m, 2H), 1.58-1.43 (m, 2H), 1.00 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 168.6, 144.9, 144.0,
135.2, 128.5, 127.3, 126.2, 123.5, 122.0, 119.0, 29.2, 28.6, 22.3, 13.7. MS (ESI-TOF, m/z): calculated for C_{14}H_{15}N_{3}O 242.1293; found 242.1240 [M+H]^+.

**Synthesis of compound 23: Quinazoline-2,4(1H,3H)-dione.** The mixture of anthranilic acid (500 mg, 3.65 mmol) and urea (2.2 g, mol) was heated at 150 °C for 6 h. The reaction mixture was cooled to room temperature and then water (50 mL) was added to quench the reaction. The crude product was obtained by filtration, and then washed with water (20 mL × 3). The residue was dissolved in hot aq. NaOH and cooled to 0 °C and pH was adjusted to 5-6 using dilute HCl and stirred for 30 min. The crude mixture was filtered and washed with water and dried under vacuum to yield compound 23 as white solid (500 mg, 85%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 11.28 (s, 1H), 11.14 (s, 1H), 7.88 (dd, $J$ = 7.8, 1.1 Hz, 1H), 7.65 – 7.60 (m, 1H), 7.17 (ddd, $J$ = 8.2, 6.1, 1.8 Hz, 2H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 162.9, 150.3, 140.9, 135.0, 127.0, 122.4, 115.3, 114.4. MS (ESI-TOF, m/z): calculated for C_{8}H_{6}N_{2}O_{2} 163.0502; found 163.0491 [M+H]^+.

**Synthesis of compound 25: 2-((2-Chloroquinazolin-4-yl)amino)hexan-1-ol.** POCl$_3$ (5 mL) and DIPEA (430 µL, 2.47 mmol) were added to compound 23 (200 mg, 1.23 mmol) and the reaction mixture was heated to reflux for 4 h. The excess POCl$_3$ was removed by evaporation. The residue was dissolved in ice water, and then the suspension was filtered and washed with water to afford compound 24 as white solid (220 mg, 90%). To a solution of compound 24 (200 mg, 1 mmol) in DMF (5 mL), was added DL-2-amino-1-hexanol (194 µL, 1.5 mmol), the resulting mixture was heated at 100 °C for 1 h, and allowed to cool, and concentrated. The residue was purified by flash chromatography (SiO$_2$, 0-50% EtOAc in Hexane) to give compound 25 as a yellow solid (95 mg, 34 %). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.37 (dd, $J$ = 8.4, 0.8 Hz, 1H), 8.24 (d, $J$ = 8.4 Hz, 1H), 7.78 (ddd, $J$ = 8.3, 7.0, 1.3 Hz, 1H), 7.60 (dd, $J$ = 8.3, 0.8 Hz, 1H), 7.52 (ddd, $J$ = 8.2, 7.0, 1.2 Hz, 1H), 4.80 (t, $J$ = 5.7 Hz, 1H), 4.35 (qd, $J$ = 5.7, 10.6 Hz, 1H), 3.56 –
3.46 (m, 2H), 1.74 – 1.65 (m, 1H), 1.62 – 1.52 (m, 1H), 1.29 (dd, d, J = 14.8, 10.3, 8.2, 3.2 Hz, 4H), 0.85 (t, J = 6.7 Hz, 3H). 13C NMR (126 MHz, DMSO-d6) δ 161.4, 157.1, 150.4, 133.6, 126.6, 125.8, 123.5, 113.6, 62.8, 52.9, 30.1, 27.8, 22.1, 14.0. MS (ESI-TOF, m/z): calculated for C14H18ClN3O 280.1211; found 280.1279 [M+H]+.

**Synthesis of compound 27: 2-Butyl-2,3-dihydimidazo[1,2-c]quinazolin-5-amine.** To a solution of compound 25 (50 mg, 0.18 mmol) in CH2Cl2 (2 mL) was added triethylamine (38 μL, 0.27 mmol) and methanesulfonyl chloride (17 μL, 0.22 mmol) and the resulting mixture was stirred at room temperature overnight. CH2Cl2 (20 mL) was added and the organic layer was washed with water (10 mL x 2), dried over anhydrous sodium sulfate and evaporated to furnish the crude residue of compound 26 (45 mg, 96% crude yield). Ammonia in methanol (2M, 1 mL) was added to compound 26 (10 mg, 38 μmol) and the reaction mixture was heated at 80 ºC for 2 h and concentrated. The residue was purified by flash chromatography (SiO2, 0-10% MeOH in CH2Cl2) to give compound 27 as a yellow solid (6 mg, 66%). 1H NMR (500 MHz, MeOD) δ 7.88 (dd, J = 8.0, 1.2 Hz, 1H), 7.53 (ddd, J = 8.5, 7.2, 1.5 Hz, 1H), 7.20 (d, J = 8.0 Hz, 1H), 7.12 (ddd, J = 8.1, 7.2, 1.0 Hz, 1H), 4.42 – 4.35 (m, 1H), 4.21 (t, J = 10.4 Hz, 1H), 3.77 (dd, J = 10.3, 7.6 Hz, 1H), 1.87 – 1.78 (m, 1H), 1.66 (ddd, J = 11.0, 6.6, 3.7 Hz, 1H), 1.50 – 1.37 (m, 4H), 0.97 (t, J = 7.1 Hz, 3H). 13C NMR (126 MHz, MeOD) δ 156.9, 151.8, 150.6, 135.4, 126.5, 124.6, 123.6, 113.2, 64.5, 51.9, 37.3, 28.6, 23.7, 14.4. MS (ESI-TOF, m/z): calculated for C14H18N4 243.1604; found 243.1589 [M+H]+.

**Synthesis of compound 29: 2-Butylimidazo[1,2-c]quinazolin-5-amine.** To a solution of compound 26 (28 mg, 0.11 mmol) in toluene (2 mL) was added MnO2 (47 mg, 0.54 mmol) and heated at reflux for 20 h. Additional MnO2 was added and the reaction mixture was refluxed for another 48 h. The mixture was allowed to cool, filtered and purified by column chromatography.
(SiO₂, 0-50% EtOAc in Hexane) to obtain compound 28 as a pale yellow solid (12 mg, 41%).

Compound 28 (10 mg) in ammonia solution (2M in ammonia, 1 mL) was heated at 80 °C for 2 h, concentrated and purified by column chromatography (SiO₂, 0-10% MeOH in CH₂Cl₂) to obtain compound 29 as a pale yellow solid (4 mg, 36%). ¹H NMR (500 MHz, MeOD) δ 8.28 (dd, J = 8.0, 0.9 Hz, 1H), 7.70 (s, 1H), 7.58 – 7.51 (m, 2H), 7.35 (ddd, J = 8.1, 6.8, 1.5 Hz, 1H), 2.84 – 2.78 (m, 2H), 1.79 (ddd, J = 13.1, 8.5, 6.6 Hz, 2H), 1.48 (dq, J = 14.8, 7.4 Hz, 2H), 1.01 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, MeOD) δ 131.5, 125.5, 124.7, 123.5, 108.3, 32.4, 29.1, 23.5, 14.2. MS (ESI-TOF, m/z): calculated for C₁₄H₁₆N₄ 241.1448; found 241.1466 [M+H]⁺.

Synthesis of compound 30: Methyl (2-cyanophenyl)carbamate. 2-Aminobenzonitrile (200 mg, 1.69 mmol) and sodium carbonate (359 mg, 3.39 mmol) was heated to reflux in methyl chloroformate (7 mL) for 4 h. The reaction mixture was concentrated and purified by column chromatography (SiO₂, 0-20% EtOAc in Hexane) to obtain compound 30 as a white solid (276 mg, 93%). ¹H NMR (500 MHz, DMSO-d₆) δ 9.77 (s, 1H), 7.79 (dd, J = 7.8, 1.3 Hz, 1H), 7.67 (ddd, J = 8.2, 7.6, 1.6 Hz, 1H), 7.52 (d, J = 8.1 Hz, 1H), 7.33 (td, J = 7.6, 1.1 Hz, 1H), 3.69 (s, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 154.5, 140.5, 133.9, 133.3, 125.5, 125.2, 116.8, 107.3, 52.2. MS (ESI-TOF, m/z): calculated for C₉H₈N₂O₂ 177.0659; found 177.0694 [M+H]⁺.

Synthesis of compound 31: 2-Butyl-[1,2,4]triazolo[1,5-c]quinazolin-5(6H)-one. To a solution of compound 30 (200 mg, 1.14 mmol) in N-methyl-2-pyrrolidone (NMP, 5 mL) was added valeric acid hydrazide (158 mg, 1.36 mmol) and the reaction mixture was heated at 180 °C for 3 h. Crushed ice was added to the reaction mixture and the solid obtained was filtered and purified by column chromatography (SiO₂, 0-50% EtOAc in hexanes) to obtain compound 31 as white solid (270 mg, 98%). ¹H NMR (500 MHz, DMSO-d₆) δ 12.21 (s, 1H), 8.11 (dd, J = 7.9, 1.1 Hz, 1H), 7.69 – 7.64 (m, 1H), 7.41 (d, J = 8.2 Hz, 1H), 7.39 – 7.33 (m, 1H), 2.82 (t, J =
7.6 Hz, 2H), 1.79 – 1.72 (m, 2H), 1.43 – 1.33 (m, 2H), 0.92 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 166.5, 152.7, 143.8, 136.9, 132.6, 124.0, 123.5, 116.0, 110.3, 29.6, 27.7, 21.8, 13.7. MS (ESI-TOF, $m/z$): calculated for C$_{13}$H$_{14}$N$_4$O 243.1240; found 243.1350 [M+H]$^+$.  

**Synthesis of compound 32:** 2-Butyl-5-chloro-[1,2,4]triazolo[1,5-c]quinazoline. To a suspension of compound 31 (18 mg, 74 µmol) in POCl$_3$ (1 mL) was added DIPEA (29 µL, 0.15 mmol) and the resulting mixture was heated at 110 ºC for 18 h. The mixture was cooled to room temperature and concentrated. The residue was purified by flash chromatography (SiO$_2$, 0-30% EtOAc in hexane) to give compound 32 as a pale yellow solid (15 mg, 79%). $^1$H NMR (500 MHz, DMSO-$d_6$) δ 8.41 (d, $J = 8.0$ Hz, 1H), 7.99 (d, $J = 8.3$ Hz, 1H), 7.94 (t, $J = 7.7$ Hz, 1H), 7.82 (t, $J = 7.5$ Hz, 1H), 2.93 (t, $J = 7.6$ Hz, 2H), 1.84 – 1.76 (m, 2H), 1.47 – 1.37 (m, 2H), 0.94 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 167.0, 152.0, 142.3, 135.3, 132.7, 129.1, 127.6, 123.6, 116.8, 29.7, 27.8, 21.8, 13.7. MS (ESI-TOF, $m/z$): calculated for C$_{13}$H$_{13}$ClN$_4$ 261.0902; found 261.1021 [M+H]$^+$.  

**Synthesis of compound 33:** 2-Butyl-[1,2,4]triazolo[1,5-c]quinazolin-5-amine. Compound 32 (10 mg, 38 µmol) was treated with 2M ammonia in methanol (1 mL), heated at 80 ºC for 20 h and concentrated. The residue was purified by flash chromatography (SiO$_2$, 0-10% MeOH in CH$_2$Cl$_2$) to give compound 33 as a white solid (5 mg, 55%). $^1$H NMR (500 MHz, MeOD) δ 8.21 (ddd, $J = 8.0$, 1.5, 0.5 Hz, 1H), 7.65 (ddd, $J = 8.5$, 7.1, 1.5 Hz, 1H), 7.57 (dd, $J = 8.4$, 0.5 Hz, 1H), 7.37 (ddd, $J = 8.1$, 7.1, 1.1 Hz, 1H), 2.97 – 2.89 (m, 2H), 1.86 (dt, $J = 15.3$, 7.6 Hz, 2H), 1.50 – 1.38 (m, 2H), 0.98 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) δ 167.9, 153.1, 146.3, 146.1, 133.4, 126.0, 124.8, 124.5, 114.4, 114.4, 31.4, 29.2, 23.4, 14.1. MS (ESI-TOF, $m/z$): calculated for C$_{13}$H$_{15}$N$_5$ [242.1400; found 242.1399 M+H]$^+$. 

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Synthesis of compound 34: 2,3-Dichloroquinoxaline. Quinoxaline-2,3-diol (500 mg, 3.08 mmol) and POCl₃ (5 mL) in DMF (5 mL) were heated at 100 ºC for 1.5 h, allowed to cool, and concentrated. The residue was treated with ice water and filtered to give compound 34 as off white solid (418 mg, 68%). ¹H NMR (500 MHz, DMSO-d₆) δ 8.10 – 8.06 (m, 2H), 7.96 – 7.91 (m, 2H). ¹³C NMR (126 MHz, DMSO-d₆) δ 144.7, 140.1, 131.8, 128.0. MS (ESI-TOF, m/z): calculated for C₈H₄Cl₂N₂ 198.9824; found 198.9853 [M+H]+.

Synthesis of compound 35: 2-((3-Chloroquinoxalin-2-yl)amino)hexan-1-ol. To a solution of compound 34 (200 mg, 1 mmol) in EtOH (5 mL), was added DL-2-amino-1-hexanol (194 µL, 1.5 mmol) in EtOH (3 mL), heated at 90 ºC for 18 h, allowed to cool, and concentrated. The residue was purified by flash chromatography (SiO₂, 0-20% EtOAc in Hexane) to give compound 35 as a yellow solid (160 mg, 57%). ¹H NMR (500 MHz, CDCl₃) δ 7.80 (dd, J = 8.2, 1.0 Hz, 1H), 7.66 (dd, J = 8.3, 0.9 Hz, 1H), 7.58 (ddd, J = 8.4, 7.0, 1.4 Hz, 1H), 7.40 (ddd, J = 8.3, 7.0, 1.4 Hz, 1H), 5.65 (d, J = 6.6 Hz, 1H), 4.30 – 4.22 (m, 1H), 4.18 (m, 1H), 3.90 (dd, J = 11.1, 2.8 Hz, 1H), 3.76 (dd, J = 11.1, 6.3 Hz, 1H), 3.60 (s, 1H), 1.78 – 1.64 (m, 2H), 1.48 – 1.35 (m, 4H), 0.93 (t, J = 7.1 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 148.5, 140.6, 138.1, 136.6, 130.5, 128.1, 125.7, 125.4, 66.8, 54.6, 31.3, 28.5, 22.7, 14.1. MS (ESI-TOF, m/z): calculated for C₁₄H₁₈ClN₃O 280.1211; found 280.1296 [M+H]+.

Synthesis of compound 36: 2-Butyl-4-chloro-1,2-dihydroimidazo[1,2-a]quinoxaline. To a solution of compound 35 (100 mg, 0.36 mmol) in CHCl₃ (1 mL), was added thionyl chloride (1 mL) at 0 ºC, and heated to reflux for 2 h. The mixture was cooled to room temperature and concentrated. The residue was purified by flash chromatography (SiO₂, 0-10% MeOH in CH₂Cl₂) to give compound 36 as a yellow solid (75 mg, 80%). ¹H NMR (500 MHz, DMSO-d₆) δ 8.02 (dd, J = 8.1, 0.7 Hz, 1H), 7.94 – 7.89 (m, 1H), 7.67 (dd, J = 14.6, 7.6 Hz, 2H), 4.90 (m, 1H), 4.62 –
4.51 (m, 2H), 1.90 – 1.82 (m, 1H), 1.78 – 1.70 (m, 1H), 1.47 – 1.33 (m, 4H), 0.92 (t, J = 7.1 Hz, 3H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 147.5, 136.5, 134.3, 133.3, 129.0, 128.8, 126.8, 115.5, 57.2, 53.4, 33.9, 26.3, 21.9, 13.9. MS (ESI-TOF, m/z): calculated for C$_{14}$H$_{16}$ClN$_3$ 262.1106; found 262.1173 [M+H]$^+$. 

**Synthesis of compound 37: 2-Butyl-1,2-dihydroimidazo[1,2-a]quinoxalin-4-amine.** Compound 36 (29 mg, 0.11 mmol) was dissolved in 2 M ammonia in methanol (1 mL) and heated in a sealed vial at 90 ºC for 20 h and concentrated. The residue was purified by flash chromatography (SiO$_2$, 0-10% MeOH in CH$_2$Cl$_2$) to give compound 37 as a yellow solid (18 mg, 67%). $^1$H NMR (500 MHz, MeOD) δ 7.25 (dd, J = 7.9, 1.2 Hz, 1H), 7.13 (td, J = 7.9, 1.4 Hz, 1H), 7.00 (td, J = 7.8, 1.3 Hz, 1H), 6.85 (dd, J = 8.0, 1.2 Hz, 1H), 4.38 – 4.30 (m, 1H), 4.26 – 4.20 (m, 1H), 3.76 (dd, J = 10.3, 8.6 Hz, 1H), 1.79 – 1.71 (m, 1H), 1.68 – 1.57 (m, 1H), 1.53 – 1.37 (m, 4H), 0.95 (t, J = 7.1 Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) δ 149.4, 147.7, 134.7, 130.9, 125.9, 125.2, 123.0, 112.8, 65.8, 52.8, 37.8, 28.9, 23.8, 14.4. MS (ESI-TOF, m/z): calculated for C$_{14}$H$_{18}$N$_4$ 243.1604; found 243.1677 [M+H]$^+$. 

**Synthesis of compound 38: 2-Butyl-4-chloroimidazo[1,2-a]quinoxaline.** To a solution of compound 37 (357 mg, 1.36 mmol) in toluene (15 mL) was added MnO$_2$ (593 mg, 6.82 mmol) and heated to reflux for 24 h. Additional MnO$_2$ (593 mg, 6.82 mmol) was added and after another 48 h, the mixture was allowed to cool and filtered. The solvent was evaporated and the crude product was purified by flash chromatography (SiO$_2$, 0-50% EtOAc in hexane) to give compound 38 as a yellow solid (99 mg, 28%). $^1$H NMR (500 MHz, DMSO-$d_6$) δ 8.73 (s, 1H), 8.33 (dd, J = 8.3, 0.9 Hz, 1H), 7.97 (dd, J = 8.2, 1.2 Hz, 1H), 7.78 – 7.73 (m, 1H), 7.67 – 7.62 (m, 1H), 2.80 (t, J = 7.6 Hz, 2H), 1.72 (dd, J = 15.2, 7.6 Hz, 2H), 1.44 – 1.34 (m, 2H), 0.94 (t, J = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 158.1, 150.8, 144.0, 143.5, 138.8, 138.4, 136.3,
136.3, 125.5, 122.8, 40.3, 37.6, 31.4, 23.3. MS (ESI-TOF, m/z): calculated for C$_{14}$H$_{14}$ClN$_{3}$ 260.0949; found 260.0900 [M+H]$^+$. 

**Synthesis of compound 39: 2-Butylimidazo[1,2-a]quinoxaline-4-amine.** Compound 38 (70 mg, 0.27 mmol) was dissolved in 2M ammonia in methanol (2 mL), heated in a sealed vial at 90 °C for 20 h and concentrated. The residue was purified by flash chromatography (SiO$_2$, 0-100% EtOAc in hexane) to give compound 39 as a pale yellow solid (21 mg, 33%). $^1$H NMR (500 MHz, MeOD) $\delta$ 8.18 (s, 1H), 7.94 (dd, $J$ = 8.1, 1.2 Hz, 1H), 7.60 (dd, $J$ = 8.1, 1.2 Hz, 1H), 7.46 – 7.41 (m, 1H), 7.36 (ddd, $J$ = 8.5, 7.3, 1.4 Hz, 1H), 2.86 – 2.80 (m, 2H), 1.80 (ddd, $J$ = 13.1, 8.4, 6.7 Hz, 2H), 1.47 (dd, $J$ = 15.0, 7.5 Hz, 2H), 1.00 (t, $J$ = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 149.5, 148.1, 137.3, 132.9, 127.7, 126.7, 125.8, 125.0, 116.2, 112.2, 32.8, 29.2, 23.5, 14.2. MS (ESI-TOF, m/z): calculated for C$_{14}$H$_{16}$N$_{4}$ 241.1448; found 241.1462 [M+H]$^+$. 

**Synthesis of compound 40: N’-(2-Nitrophenyl)pentanehydrazide.** To a solution of (2-nitrophenyl)hydrazine (500 mg, 3.26 mmol) in CH$_2$Cl$_2$ (10 mL) was added N-methylmorpholine (298 µL, 4.24 mmol), followed by valeroyl chloride (415 µL, 3.43 mmol). The reaction was stirred at room temperature for 1 h and concentrated. The residue was purified by flash chromatography (SiO$_2$, 0-10% MeOH in CH$_2$Cl$_2$) to give compound 40 as a yellow solid (564 mg, 73%). $^1$H NMR (500 MHz, DMSO-d$_6$) $\delta$ 10.10 (s, 1H), 9.21 (s, 1H), 8.09 (dd, $J$ = 8.5, 1.5 Hz, 1H), 7.61 – 7.56 (m, 1H), 7.05 (dd, $J$ = 8.6, 1.1 Hz, 1H), 6.85 (ddd, $J$ = 8.4, 7.0, 1.3 Hz, 1H), 2.23 (t, $J$ = 7.5 Hz, 2H), 1.56 (dt, $J$ = 15.0, 7.5 Hz, 2H), 1.33 (dq, $J$ = 14.6, 7.4 Hz, 2H), 0.90 (t, $J$ = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, DMSO-d$_6$) $\delta$ 171.9, 145.5, 136.5, 131.6, 125.8, 117.7, 114.6, 33.0, 27.0, 21.8, 13.7. MS (ESI-TOF, m/z): calculated for C$_{11}$H$_{15}$N$_{3}$O$_{3}$ 238.1186; found 238.1189 [M+H]$^+$. 

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Synthesis of compound 41: $N'$-(2-Nitrophenyl)pentanehydrazonoyl chloride. Compound 40 (82 mg, 0.35 mmol) in POCl₃ (3 mL) was heated at 110 °C for 2 h. The mixture was cooled to room temperature and concentrated. The residue was treated with ice water and extracted with CH₂Cl₂. The organic layer was washed with brine, dried over Na₂SO₄, concentrated and the crude product was purified by flash chromatography (SiO₂, 0-10% EtOAc in hexane) to give compound 41 as yellow oil (68 mg, 76%). ¹H NMR (500 MHz, DMSO-d₆) δ 10.82 (s, 1H), 8.15 (dt, $J = 8.6, 0.9$ Hz, 1H), 7.71 (dd, $J = 4.5, 1.0$ Hz, 2H), 7.03 – 6.99 (m, 1H), 2.76 – 2.71 (m, 2H), 1.68 (dt, $J = 20.6, 7.5$ Hz, 2H), 1.42 – 1.32 (m, 2H), 0.92 (t, $J = 7.4$ Hz, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 140.1, 137.0, 134.1, 131.4, 125.9, 119.5, 115.8, 37.9, 28.1, 21.1, 13.6.

Synthesis of compound 42: $N'$-(2-Nitrophenyl)pentanehydrazonamide. Compound 41 (60 mg, 0.23 mmol) was treated with 2 M ammonia in methanol (1 mL) and stirred at room temperature for 20 h. The residue was concentrated to give compound 42 as a red solid (38 mg, 70%). ¹H NMR (500 MHz, DMSO-d₆) δ 9.73 (s, 1H), 8.11 (dd, $J = 8.5, 1.4$ Hz, 1H), 7.61 (ddd, $J = 8.5, 7.2, 1.4$ Hz, 1H), 7.22 (d, $J = 8.5$ Hz, 1H), 6.89 (t, $J = 7.6$ Hz, 1H), 2.44 (t, $J = 7.6$ Hz, 2H), 1.71 – 1.62 (m, 2H), 1.38 (dt, $J = 14.7, 7.4$ Hz, 2H), 0.93 (t, $J = 7.4$ Hz, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 142.5, 136.4, 125.9, 118.1, 114.5, 30.8, 28.6, 21.6, 13.6. MS (ESI-TOF, m/z): calculated for C₁₁H₁₆N₄O₂ 237.1346; found 237.1344 [M+H]⁺.

Synthesis of compound 43: Ethyl 3-butyl-1-(2-nitrophenyl)-1H-1,2,4-triazole-5-carboxylate. To a solution of compound 42 (78 mg, 0.33 mmol) in ether (3 mL) was added dropwise ethyl oxalyl chloride (110 µL, 0.99 mmol) in ether (2 mL). Toluene (5 mL) was added, and the mixture was heated at 110 °C for 3 h, and concentrated. The residue was purified by flash chromatography (SiO₂, 0-50% EtOAc in hexane) to give compound 43 as yellow oil (60 mg, 57%). ¹H NMR (500 MHz, DMSO-d₆) δ 8.26 (dd, $J = 8.5, 1.4$ Hz, 1H), 7.94 (ddd, $J = 8.0,
7.4, 1.5 Hz, 1H), 7.87 – 7.83 (m, 2H), 4.20 (q, \(J = 7.1\) Hz, 2H), 2.73 (t, \(J = 7.4\) Hz, 2H), 1.71 –
1.64 (m, 2H), 1.37 – 1.29 (m, 2H), 1.13 (t, \(J = 7.1\) Hz, 3H), 0.90 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR
(126 MHz, DMSO-\(d_6\)) \(\delta\) 164.6, 161.0, 156.5, 144.2, 134.6, 131.5, 131.2, 130.1, 125.3, 62.1,
29.6, 27.1, 21.5, 13.8, 13.6. MS (ESI-TOF, \(m/z\)): calculated for \(\text{C}_{15}\text{H}_{18}\text{N}_{4}\text{O}_{4}\) 319.1401; found
319.1410 [M+H]\(^+\).

**Synthesis of compound 44: 2-Butyl-[1,2,4]triazolo[1,5-a]quinoxalin-4(5H)-one.** To a
solution of compound 43 (58 mg, 0.18 mmol) in acetic acid (2 mL) was added iron powder (100
mg). The mixture was heated at 90 °C for 1 h, allowed to cool, filtered, and concentrated. The
residue was purified by flash chromatography (SiO\(_2\), 0-10% MeOH in CH\(_2\)Cl\(_2\)) to give compound
44 as a white solid (19 mg, 43%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 12.31 (s, 1H), 8.04 (dd, \(J =
8.2, 1.0\) Hz, 1H), 7.50 – 7.43 (m, 2H), 7.36 (ddd, \(J = 8.5, 7.1, 1.6\) Hz, 1H), 2.90 – 2.83 (m, 2H),
1.76 (ddd, \(J = 13.3, 8.4, 6.7\) Hz, 2H), 1.39 (dq, \(J = 14.7, 7.4\) Hz, 2H), 0.93 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C
NMR (126 MHz, DMSO-\(d_6\)) \(\delta\) 166.0, 152.2, 144.3, 128.6, 127.8, 123.5, 122.4, 116.6, 115.4,
29.9, 27.6, 21.7, 13.7. MS (ESI-TOF, \(m/z\)): calculated for \(\text{C}_{13}\text{H}_{14}\text{N}_{4}\text{O}\) 243.1240; found 243.1227
[M+H]\(^+\).

Syntheses of compounds 45 and 46 were carried out as reported for 32 and 33, respectively.

**45: 2-Butyl-4-chloro-[1,2,4]triazolo[1,5-a]quinoxaline.** \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\)
8.35 (dd, \(J = 8.3, 1.0\) Hz, 1H), 8.13 (dd, \(J = 8.2, 1.0\) Hz, 1H), 7.90 (ddd, \(J = 8.4, 7.3, 1.4\) Hz, 1H), 7.79
(ddd, \(J = 8.5, 7.3, 1.4\) Hz, 1H), 3.00 – 2.93 (m, 2H), 1.81 (ddd, \(J = 13.3, 8.4, 6.7\) Hz, 2H), 1.47 –
1.36 (m, 2H), 0.94 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (126 MHz, DMSO-\(d_6\)) \(\delta\) 167.0, 142.7, 140.3,
134.7, 130.9, 129.1, 128.0, 127.5, 115.3, 29.9, 27.8, 21.8, 13.7. MS (ESI-TOF, \(m/z\)): calculated
for \(\text{C}_{13}\text{H}_{13}\text{ClN}_{4}\) 261.0902; found 261.1890 [M+H]\(^+\).
46: 2-Butyl-[1,2,4]triazolo[1,5-a]quinoxalin-4-amine. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.17 (dd, $J$ = 8.2, 1.0 Hz, 1H), 7.68 (dd, $J$ = 8.2, 1.0 Hz, 1H), 7.54 – 7.50 (m, 1H), 7.44 (ddd, $J$ = 8.5, 7.3, 1.3 Hz, 1H), 3.00 – 2.97 (m, 2H), 1.88 (dt, $J$ = 15.2, 7.6 Hz, 2H), 1.48 (dq, $J$ = 14.8, 7.4 Hz, 2H), 1.00 (t, $J$ = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 167.8, 149.1, 140.2, 137.9, 128.6, 126.8, 126.5, 125.6, 116.0, 31.6, 29.2, 23.4, 14.1. MS (ESI-TOF, $m/z$): calculated for C$_{13}$H$_{15}$N$_5$ 242.1400; found 242.1391 [M+H]$^+$. 

Synthesis of compound 47: 2-Chloro-3-hydrazinylquinoxaline. To a solution of 2,3-dichloroquinoxaline 34 (160 mg, 0.81 mmol) in MeOH (4 mL), hydrazine hydrate (100 $\mu$L, 3.24 mmol) was added and the reaction mixture was stirred at room temperature overnight. The solvent was evaporated to obtain compound 47 as a pale yellow solid (148 mg, 95%). $^1$H NMR (500 MHz, MeOD) $\delta$ 7.78 – 7.71 (m, 2H), 7.61 (ddd, $J$ = 8.4, 7.1, 1.5 Hz, 1H), 7.42 (ddd, $J$ = 8.4, 7.1, 1.3 Hz, 1H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 150.9, 142.2, 138.3, 137.7, 131.4, 128.6, 126.7, 126.3. MS (ESI-TOF, $m/z$): calculated for C$_8$H$_7$ClN$_4$ 195.0432; found 195.0344 [M+H]$^+$. 

Synthesis of compound 48: 1-Butyl-4-chloro-[1,2,4]triazolo[4,3-a]quinoxaline. Trimethyl orthovalerate (0.5 mL) was added to compound 47 (50 mg, 0.257 mmol) and the resulting mixture was heated at 100 °C for 1 h. The solvent was removed and the product obtained was purified by flash chromatography to obtain compound 48 as a white solid (38 mg, 57%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.12 (dd, $J$ = 8.4, 1.1 Hz, 1H), 8.09 (dd, $J$ = 8.0, 1.4 Hz, 1H), 7.74 (ddd, $J$ = 8.4, 7.4, 1.7 Hz, 1H), 7.68 (ddd, $J$ = 7.9, 7.4, 1.3 Hz, 1H), 3.53 – 3.45 (m, 2H), 2.08 – 1.99 (m, 2H), 1.67 – 1.54 (m, 2H), 1.04 (t, $J$ = 7.4 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 152.8, 143.3, 142.9, 135.8, 130.6, 130.0, 128.1, 126.4, 115.6, 28.8, 28.5, 22.6, 13.9. MS (ESI-TOF, $m/z$): calculated for C$_{13}$H$_{13}$ClN$_4$ 261.0902; found 261.1004 [M+H]$^+$. 

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Synthesis of compound 49: 1-Butyl-[1,2,4]triazolo[4,3-a]quinoxalin-4-amine. Compound 48 (25 mg, 0.096 mmol) was dissolved in ammonia solution (2M in methanol, 1 mL) and heated in a sealed vial at 60 °C for 4 h. Concentration and purification by column chromatography (SiO₂, 0-5% MeOH in CH₂Cl₂) to give compound 49 as a white solid (16 mg, 70%). ¹H NMR (500 MHz, CDCl₃) δ 7.96 (dd, J = 8.4, 1.1 Hz, 1H), 7.71 (dd, J = 8.1, 1.4 Hz, 1H), 7.53 – 7.47 (m, 1H), 7.38 (ddd, J = 8.7, 7.3, 1.5 Hz, 1H), 5.87 (bs, 2H), 3.48 – 3.39 (m, 2H), 2.07 – 1.98 (m, 2H), 1.60 (dq, J = 14.8, 7.4 Hz, 2H), 1.04 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 152.3, 147.1, 139.9, 137.6, 127.7, 127.4, 124.6, 124.5, 115.4, 28.8, 28.4, 22.6, 13.9. MS (ESI-TOF, m/z): calculated for C₁₃H₁₅N₅ 242.1400; found 242.1426 [M+H]+.

Synthesis of compound 50: Ethyl 2-(1H-indol-3-yl)-2-oxoacetate. To a solution of indole (1.01 g, 8.66 mmol) in anhydrous Et₂O (17 mL) and pyridine (0.95 mL, 11.7 mmol) was added a solution of ethyl chlorooxoacetate (1.2 mL, 10.7 mmol) in anhydrous Et₂O (3 mL) at 0 °C over a period of 15 min. The reaction mixture was stirred at 0 °C for 2 h and filtered. The resulting solid was then wash with cold Et₂O and water, dried over high vacuum to give compound 50 as a pale yellow powder (1.52 g, 81%). ¹H NMR (500 MHz, DMSO-d₆) δ 12.39 (bs, 1H), 8.42 (d, J = 3.3 Hz, 1H), 8.18-8.13 (m, 1H), 7.57-7.53 (m, 1H), 7.32-7.24 (m, 2H), 4.36 (q, J = 7.1 Hz, 2H), 1.34 (t, J = 7.1 Hz, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 179.1, 163.6, 138.3, 136.7, 125.5, 123.9, 122.9, 121.1, 112.8, 112.4, 61.6, 14.0. MS (ESI-TOF, m/z): calculated for C₁₂H₁₁NO₃ 218.0812; found 218.0796 [M+H]+.

Synthesis of compound 51: 2-Butyl-2H-pyrazolo[3,4-c]quinolin-4(5H)-one. To a solution of butylhydrazine hydrochloride salt (288 mg, 2.31 mmol) in absolute EtOH (20 mL) was added compound 50 (352 mg, 1.62 mmol) and acetic acid (0.4 mL). The reaction mixture was heated to reflux and stirred for 24 h, cooled to room temperature and concentrated. The crude product
was purified by flash chromatography (SiO₂, 0-3% MeOH in CH₂Cl₂) to give compound 51 as a grey powder (243 mg, 62%). ¹H NMR (500 MHz, DMSO-d₆) δ 11.32 (s, 1H), 8.69 (s, 1H), 7.85 (d, J = 7.8 Hz, 1H), 7.36-7.29 (m, 2H), 7.17 (m, 1H), 4.37 (t, J = 7.0 Hz, 2H), 1.94-1.80 (m, 2H), 1.35-1.20 (m, 2H), 0.91 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 157.0, 139.9, 135.9, 127.2, 125.5, 123.5, 122.0, 121.2, 115.9, 115.3, 52.5, 31.8, 19.2, 13.4. MS (ESI-TOF, m/z): calculated for C₁₄H₁₅N₃O 242.1288; found 242.1272 [M+H]+.

**Synthesis of compound 52: 2-Butyl-4-chloro-2H-pyrazolo[3,4-c]quinoline.** To a mixture of compound 51 (163 mg, 0.675 mmol) and PCl₅ (33.3 mg, 0.160 mmol) was added POCl₃ (3 mL). The reaction mixture was heated to reflux, stirred for 2 h and then concentrated. The residue was diluted with CH₂Cl₂ and washed with saturated NaHCO₃, dried over Na₂SO₄, filtered and concentrated. The crude product was purified by flash chromatography (0-20% EtOAc in Hexanes) to give compound 52 as a yellow solid (152 mg, 86%). ¹H NMR (500 MHz, CDCl₃) δ 8.31 (s, 1H), 8.08-8.04 (m, 1H), 8.02-7.98 (m, 1H), 7.63-7.55 (m, 2H), 4.53 (t, J = 7.3 Hz, 2H), 2.12-2.00 (m, 2H), 1.46-1.37 (m, 2H), 0.99 (t, J = 7.4 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 143.4, 142.1, 141.3, 129.4, 127.4, 127.4, 123.5, 123.1, 122.5, 121.9, 54.3, 32.7, 19.9, 13.6. MS (ESI-TOF, m/z): calculated for C₁₄H₁₄ClN₃ 260.0949; found 260.0989 [M+H]+.

**Synthesis of compound 53: 2-Butyl-2H-pyrazolo[3,4-c]quinolin-4-amine.** Ammonia solution (2M in MeOH, 1 mL) was added to compound 52 (60 mg, 0.23 mmol) and the reaction mixture was heated in a sealed vial at 100 °C for overnight and then concentrated. The crude product was purified by flash chromatography (0-10% MeOH in CH₂Cl₂) to give compound 53 as a pale yellow powder (27 mg, 48%). ¹H NMR (500 MHz, DMSO-d₆) δ 8.82 (s, 1H), 7.95 (dd, J = 7.8 Hz, J = 1.5 Hz, 1H), 7.53 (dd, J = 8.2, 1.2 Hz, 1H), 7.39 (ddd, J = 8.3, 7.1, 1.5 Hz, 1H), 7.29-7.22 (m, 1H), 4.46 (t, J = 7.0 Hz, 2H), 3.42 (s, 2H), 2.00-1.84 (m, 2H), 1.37-1.24 (m, 2H), 0.92 (t, J =
7.4 Hz, 3H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 150.1, 140.3, 135.5, 126.9, 125.3, 123.4, 123.3, 122.5, 120.6, 118.1, 52.8, 32.0, 19.2, 13.5. MS (ESI-TOF, $m/z$): calculated for $C_{14}H_{16}N_4$ 241.1448; found 241.1470 [M+H]$^+$. 

Human TLR-7/-8 reporter gene assays (NF-$\kappa$B induction). As described in Chapter 2.

Immunoassays for Interferon (IFN)-$\alpha$ and cytokines. As described in Chapter 2.

Flow-cytometric immunostimulation experiments. As described in Chapter 5.

Protein expression, purification and crystallization. The extracellular domain of human TLR8 (hTLR8, residues27–827) was prepared as described previously,$^{113}$ and was concentrated to 16 mg/mL in 10 mM MES (pH 5.5), 50 mM NaCl. The protein solutions for the crystallization of hTLR8/compound complexes contained hTLR8 (8.5 mg/mL) and compound (protein:compound molar ratio of 1:10) in a crystallization buffer containing 7 mM MES (pH 5.5), 35 mM NaCl. Crystallization experiments were performed with sitting-drop vapor-diffusion methods at 293K. Crystals of hTLR8/compound were obtained with reservoir solutions containing 9-12% (w/v) PEG3350, 0.3 M potassium formate, and 0.1 M sodium citrate (pH 4.8-5.2).

Data collection and structure determination. Diffraction datasets were collected on beamlines PF-AR NE3A (Ibaraki, Japan) and SPring-8 BL41XU under cryogenic conditions at 100 K. Crystals of hTLR8/compound were soaked into a cryoprotectant solution containing 15% (w/v) PEG3350, 0.23 M potassium formate, 75 mM sodium citrate pH 4.8-5.2, 7.5 mM MES pH 5.5, 38 mM NaCl, and 25% glycerol. Datasets were processed using the HKL2000 package.$^{119}$
or imosflm.\textsuperscript{120} HTLR8/compound structures were determined by the molecular replacement method using the Molrep program\textsuperscript{121} with the hTLR8/CL097 structure (PDB ID: 3W3J) as a search model. The model was further refined with stepwise cycles of manual model building using the COOT program\textsuperscript{122} and restrained refinement using REFMAC\textsuperscript{123} until the R factor was converged. Compound molecule, N-glycans, and water molecules were modeled into the electron density maps at the latter cycles of the refinement. The quality of the final structure was evaluated with PROCHECK.\textsuperscript{124} The figures representing structures were prepared with PyMOL.\textsuperscript{125} Coordinates have been deposited in the Protein Data Bank of the Research Collaboratory for Structural Bioinformatics; PDB codes for compounds 9 and 53 are, respectively, 4QBZ and 4QC0.

**Quantum chemical computations and linear discriminant analyses.** Calculations were performed using the NWChem\textsuperscript{126} quantum chemical software for the electronic structure, electrostatic charge and property calculations. All the compounds were fully optimized at the density functional theory (DFT) level of theory using the M06-2X\textsuperscript{127} functional and correlation consistent cc-pVDZ basis set. The optimized structures were verified as minima by calculating the second-order Hessian matrices. The molecular electrostatic potentials were calculated on the DFT-optimized geometries and superimposed onto a constant electron density (0.002 e/Å\textsuperscript{3}) to provide a measure of the electrostatic potential at roughly the van der Waals surface of the molecules using the Gauss View03 software. The color-coded surface provides a location of the positive (blue, positive) and negative (red, negative) electrostatic potentials. The regions of positive charge indicate relative electron deficiency, and regions of negative potential indicate areas of excess negative charge. Fisher’s linear discriminant analyses were performed using SPSS v22 (IBM, Armonk, NY); classification function coefficients.
Chapter 7.

Hyaluronic acid conjugation of TLR7/8 agonists for lymphoid tissue delivery
7.1. Introduction

The lymphatic system provides for unidirectional transport for interstitial fluid and proteins exiting microcirculation due to hydrostatic pressure, and returning then back to blood circulation. Lymphatic fluid drains from the initial lymphatics or lymphatic capillaries first into lymph nodes, which are highly organized secondary lymphoid tissues specialized in immune surveillance of the contents of afferent lymph. Immune cells located in specialized zones within lymph nodes not only respond to antigens arriving from distal sites of infection, but also receive and orchestrate appropriate immune responses to migrating antigen-presenting cells (APCs) bearing antigenic epitopes. This is of particular relevance in immunization, wherein, peripheral APCs, such as dendritic cells (DCs) and macrophages capture antigens from the injection site and then migrate into the lymphoid tissues to trigger downstream T- and B-lymphocyte activation as well as memory cell differentiation. The lymph nodes also contain a large number of resident APCs, which can actively process antigens and serve as major reservoir for long-lived memory B cells and central memory T cells, therefore playing a crucial role in generating long-term immunological memory.

The flow of interstitial fluid also brings fragments of extracellular matrix (ECM) macromolecules into lymph. Important among the constituents of ECM is hyaluronic acid (hyaluronan, HA), a linear glycosaminoglycan polymer with a molecular weight that can reach 10^7 Daltons, and composed of repeating polymeric disaccharides of d-glucuronic acid (GlcUA) and N-acetyl glucosamine (GlcNAc) linked by β-1,4 and β-1,3 glucosidic bonds (Fig. 1). HA serves to maintain a hydrated and stable extracellular space, not only by virtue of its ability to absorb a large amount of water expanding up to 1000 times its solid volume, but also due to its distinct hydrodynamic properties, such as very high viscosity. Depending on the molecular size, HA shows the capacity to interact with many different binding proteins and important group of
receptors. The two most abundant of these receptors are CD44, which is a single chain, transmembrane glycoprotein expressed on leukocytes that traffic through the lymphatics, and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), which is expressed almost exclusively on lymphatic endothelium. CD44–HA interaction is known to be involved in a variety of cellular functions, including cell–cell interactions, receptor-mediated internalization/degradation of HA, and cell migration. LYVE-1 participates in HA-mediated leukocyte trafficking, adhesion, and transmigration.

**Fig. 1. Chemical structure of hyaluronic acid.**

The efficient delivery of antigen/adjuvant has been a major challenge in the development of subunit vaccines, and enhancing vaccine delivery to secondary lymphoid organs might be a promising approach for improving vaccine efficacy. Several studies have addressed enhanced or targeted delivery of antigens to secondary lymphoid tissue, including the use of depot-forming adjuvants, nanoparticulate carriers that are preferentially internalized by APCs, or intralymphatic immunization, but strategies that could use well-defined molecular conjugates would be more attractive. We envisioned that we could take advantage of the characteristics of HA — biodegradability, biocompatibility, high potential loading, and most of all, selective interaction with receptors — for targeted delivery of small molecule Toll-like receptor (TLR) agonists to lymph nodes.

A number of lead adjuvants including the pure TLR7 agonist (a, Chapter 5), the pure TLR8 agonist (b) and the dual TLR7/8 agonists (c, d) are undergoing preclinical evaluation as
vaccine adjuvants in our laboratory (Fig. 2). The approach of targeted delivery of these adjuvants may also serve to minimize systemic exposure of these small molecules with molecular properties that portend a large volume of distribution. For proof-of-concept studies, we selected these four compounds with an amine functional group (Fig. 2) that can be utilized in a straightforward manner for direct coupling with carboxylic acid group on HA. There are numerous methods that have been reported for HA conjugation,\textsuperscript{127} we eschewed methods entailing harsh reaction conditions such as strongly alkaline or acidic pH, prolonged heating, or conditions calling for ultra-sound or microwave irradiation which are all known to potentially induce significant HA degradation, and elected to use the recently-reported triazine-activated amidation strategy, since it allows for a highly controlled substitution under aqueous/mixed-solvent conditions, at room temperature, and at neutral pH.\textsuperscript{135}

*Fig. 2. Chemical structures of TLR7 and TLR8 agonists used for hyaluronic acid conjugation.*

The conjugation reactions proceeded uneventfully, and spectroscopic and analytical characterization of the products suggested efficient hyaluronic acid conjugation with small molecules, as described below.
7.2. Results and Discussion

The reaction of 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) with carboxylic acids of HA and amines of small molecule TLR agonists in the presence of N-methylmorpholine (NMM) proceeded smoothly as reported in the literature\(^{135}\) (Scheme 1).

\textit{Scheme 1.}

All reactants were soluble in a mixture of water and acetonitrile (3:2) and the pH values of the reaction mixtures after addition of all reactants were about 7~8. After overnight stirring at room temperature, the reaction mixtures were treated with Dowex ion exchange resin before dialysis to remove the morpholinium species, as well as unreacted amines which could otherwise act as counter-ions in their protonated form toward non-modified carboxylic acid groups of HA. Dialysis against 0.1 N NaCl solutions also helped eliminate electrostatically bound amines from anionic
polymers. As could be expected, the benzylic amine, and not the amidine amine participates in the formation of the amide bond, since the reaction with analogues that do not have the benzylic amine group did not yield detectable substitution (such as compound 6 in Chapter 6; data not shown). In addition to coupling the individual TLR7-, TLR8-, and TLR7/8-agonistic compounds on HA (HA–a, HA–b, HA–c, respectively), we also carried out the simultaneous coupling of HA with a mixture of three amine compounds — (HA–a+b+c) with the aim being to examine the relative adjuvanticity of a construct carrying multiple TLR agonists (conjugate 5). The product yields were calculated based on the average molecular weight of the disaccharide repeating units considering the degree of substitution and found similar between the products ranging 71–75% (Table 1).

All products were characterized using 1H NMR spectroscopy and the spectra for products 1-4, as well as that of native hyaluronic acid and of the small molecules a-d are presented in Fig. 3. The poorly-resolved broad multiplet between c.a. 3.2 – 4.0 ppm corresponds to the resonances of the protons in the sugar ring of HA; a broad peak at 4.5 ppm could be assigned to the two anomeric protons attached to the carbons adjacent to two oxygen atoms, and the CH3 protons of the N-acetyl group of HA could be readily identified at 1.95 ppm (Fig. 3). New peaks associated with aliphatic (butyl or pentyl chain) protons at 0.8 – 3.0 ppm and aromatic protons at 7.0 – 8.5 ppm, both of which are diagnostic of the conjugated small molecules, were observed. The degrees of substitution (DS) were determined by comparing integrated signals of the ω-CH3 resonance of the butyl/pentyl side chain of the small molecules (triplet, 0.86 ppm, shown as α–δ in Fig. 3); this resonance could be clearly resolved from that of the CH3 signal arising from the N-acetylglucosamine moiety (singlet, 1.95 ppm, shown as ε in Fig. 3) of native HA. As shown in Table 1, the DS ranged from 29 to 39%. The slight difference in the observed DS could be attributable to differential steric accessibility of the amines and/or solubility.
Fig. 3. $^1$H NMR spectra of hyaluronic acid, small molecules a-d, and conjugates 1-4.
While the ratios of peak integrals by $^1$H NMR spectroscopy showed high batch-to-batch repeatability and consistency, we were mindful of the broad signals due to highly viscous sample solutions, as well as sample microheterogeneity, we sought to quantify the ‘loading’ also using spectroscopic measurements at 260 nm. All the TLR7/8 agonistic small molecules used for conjugation exhibit a distinct and intense absorption profile in the UV spectral region, and it was therefore straightforward to measure small molecule loading. The highest loading amount was found in conjugate 3 and the lowest in conjugate 2 (31% and 10%, respectively, Table 1).

**Table 1. Properties of the conjugates.**

<table>
<thead>
<tr>
<th>Conjugate No.</th>
<th>TLR agonist (SM)</th>
<th>Yield (%)</th>
<th>DS (%)</th>
<th>SM Content (w/w %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>a</td>
<td>75</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>b</td>
<td>75</td>
<td>35</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>c</td>
<td>71</td>
<td>39</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>d</td>
<td>74</td>
<td>36</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>a + b + c</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

DS, degree of substitution; SM, small molecule

Taking advantage of the UV absorptivity of compounds, the conjugates were also analyzed by gel electrophoresis. Samples were loaded on agarose gel and visualized by UV illumination. After a few minutes of running, we noticed that free small molecules (a and b) tend to travel toward the cathode due to its positive charges of the protonated amine (Figs. 4A and 4B; Lanes 8 and 9), which was not observed from conjugate samples. The conjugates 1-5, on the other hand, clearly showed UV-absorbing bands (Fig. 4; Lanes 3-7), indicating covalent conjugation of the small molecules with hyaluronic acid, with an apparent molecular weight corresponding to 500 kDa, as adjudged by comparing the electrophoretic mobility of rhodamine-labeled HA (Fig.
4C, Lane 2). This technique was particularly useful in characterizing conjugate 5 (HA–a+b+c; Lane 7 in Fig. 4), which was not amenable to quantification of the loading either by $^1$H NMR, or spectrophotometry owing to degeneracy of signals and spectral overlaps, respectively.

**Fig. 4.** Agarose gel electrophoresis. Lane 1: HA; Lane 2: rhodamine-labeled HA; Lane 3: conjugate 1 (HA–a); Lane 4: conjugate 2 (HA–b); Lane 5: conjugate 3 (HA–c); Lane 6: conjugate 4 (HA–d); Lane 7: conjugate 5 (HA–a+b+c); Lane 8: a; Lane 9: b; Lane 10: c; Lane 11: d.

The conjugates were also confirmed by size exclusion chromatography (SEC) analysis as shown in Fig. 5A. HA has no significant absorption in the UV and the conjugates were clearly observed to elute with a retention time of 50 min.

Reverse-phase HPLC analyses were also carried out to ensure the purity of conjugates. With a polymeric PRP-3 column, conjugate 3, for example, eluted at 32 min, while that of its corresponding small molecule c appeared at a retention time of 14 min. Again, the spectroscopic signatures of the small molecules enabled unambiguous identification of the conjugates and, importantly, verified that no unconjugated small molecules were detected in the conjugate samples (Fig. 5B).
**Fig. 5.** A: Size exclusion chromatography. B: Reverse-phase HPLC with polymeric PRP-3 column. Shown below are detected at 320 nm.
In vitro enzymatic degradation was performed using hyaluronidase (HAase) from bovine testes (400-1000 units/mg), and analyzed by LC-MS. After 45 h incubation of conjugates with hyaluronidase, oligosaccharides of HA covalently adducted with the small molecules were identified. Common major oligosaccharide species throughout the conjugates are tetra- and decasaccharides with one small molecule and dodecasaccharides with two small molecules on them (Table 2). From the HAase treated conjugate 5, these major oligosaccharides with each small molecule as well as with the combination of different small molecules were found.

Table 2. Major oligosaccharide species found by LC-MS analysis after in vitro hyaluronidase enzymatic degradation of hyaluronic acid conjugates.

<table>
<thead>
<tr>
<th>Conjugate No.</th>
<th>RT (min)</th>
<th>Species</th>
<th>Conjugate No.</th>
<th>RT (min)</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugate 1 (HA–a)</td>
<td>7.262</td>
<td>[GlcUA-GlcNAc]$_5$$\rightarrow$-a</td>
<td>Conjugate 3 (HA–c)</td>
<td>6.234</td>
<td>[GlcUA-GlcNAc]$_2$$\rightarrow$-c</td>
</tr>
<tr>
<td></td>
<td>8.885</td>
<td>[GlcUA-GlcNAc]$_6$$\rightarrow$-[a]$_2$</td>
<td></td>
<td>7.549</td>
<td>[GlcUA-GlcNAc]$_5$$\rightarrow$-c</td>
</tr>
<tr>
<td>Conjugate 2 (HA–b)</td>
<td>6.966</td>
<td>[GlcUA-GlcNAc]$_2$$\rightarrow$-b</td>
<td>Conjugate 4 (HA–d)</td>
<td>6.059</td>
<td>[GlcUA-GlcNAc]$_2$$\rightarrow$-d</td>
</tr>
<tr>
<td></td>
<td>8.505</td>
<td>[GlcUA-GlcNAc]$_5$$\rightarrow$-b</td>
<td></td>
<td>7.247</td>
<td>[GlcUA-GlcNAc]$_5$$\rightarrow$-d</td>
</tr>
<tr>
<td>Conjugate 5 (HA–a, b, c)</td>
<td>6.243</td>
<td>[GlcUA-GlcNAc]$_2$$\rightarrow$-c</td>
<td>Conjugate 5 (HA–a, b, c)</td>
<td>7.900</td>
<td>[GlcUA-GlcNAc]$_6$$\rightarrow$-[a][c]</td>
</tr>
<tr>
<td></td>
<td>6.977</td>
<td>[GlcUA-GlcNAc]$_2$$\rightarrow$-b</td>
<td></td>
<td>7.911</td>
<td>[GlcUA-GlcNAc]$_2$$\rightarrow$-a</td>
</tr>
<tr>
<td></td>
<td>7.278</td>
<td>[GlcUA-GlcNAc]$_5$$\rightarrow$-[b][c]</td>
<td></td>
<td>8.161</td>
<td>[GlcUA-GlcNAc]$_6$$\rightarrow$-[a][b][c]</td>
</tr>
<tr>
<td></td>
<td>7.376</td>
<td>[GlcUA-GlcNAc]$_6$$\rightarrow$-[b][c]</td>
<td></td>
<td>8.293</td>
<td>[GlcUA-GlcNAc]$_6$$\rightarrow$-[a][b]</td>
</tr>
<tr>
<td></td>
<td>7.517</td>
<td>[GlcUA-GlcNAc]$_5$$\rightarrow$-c</td>
<td></td>
<td>8.528</td>
<td>[GlcUA-GlcNAc]$_5$$\rightarrow$-b</td>
</tr>
</tbody>
</table>
Fig. 6. LC-MS spectra of hyaluronidase treated conjugate 3.
Next, the conjugates were screened in NF-κB reporter gene assays specific for human TLR7 and TLR8. Whereas all small molecule TLR-7 and/or -8 agonists (a-d) showed, as expected, agonistic activities, the HA conjugates did not display any detectable NF-κB induction up to concentrations of 50 μg/mL in both TLR7 and TLR8 agonism assays. Hyaluronic acid (used as control) was also verified to be quiescent in these assays (Fig. 7).

**Fig. 7. Dose-response profiles of TLR7/8 agonism.**

![Dose-response profiles of TLR7/8 agonism.](image)

We examined the cytokine/chemokine-inducing properties of these conjugates using human PBMCs and they did not show any proinflammatory cytokine induction (represented by IL-1β, IL-6, IL-8, and TNF-α in Fig. 8). It is important to note that the hyaluronic acid conjugates become “silent”, presumably because the polyanionic character of the conjugates precludes significant internalization and access to the endolysosomal compartment. These results, importantly, augur very weak (if any) reactogenicity for the HA conjugates vis-à-vis the unconjugated agonists and, therefore, are predicted to have a very high safety profile in addition to their being highly adjuvantic.
The adjuvantic activities of these conjugates were evaluated in New Zealand White rabbits and compared to a variety of candidate adjuvants under evaluation in our laboratory in a standardized manner. All candidate adjuvants were designed to be entirely water soluble, with a purity of at least 99.5% as judged by LC-MS profiles. The adjuvant dose was held constant at 100 μg/dose. The antigen used was Diphtheria toxoid (CRM197) at 10 μg/dose. The vaccine construct was formulated under excipient-free conditions in sterile, pyrogen-free saline. Rabbits (n = 4 per cohort) were pre-bled on Day 0 for estimation of preimmune titers, and then primed with the first dose of vaccine. Animals were boosted on Days 15 and 28, and bled on Days 25 (for Immune-1 sera) and 38 (for Immune-2 sera), respectively. All ELISA assays were carried
out in a 384-well plate format with automated liquid handling instrumentation as described in the Experimental Section. Reference hyperimmune sera were used in each plate for quality control purposes.

As shown in Fig. 9, conjugates 3 and 4 were found to be highly adjuvant in evoking high antigen-specific IgG titers even after a single boost; titers were higher than that obtained using the corresponding unconjugated small molecule TLR7/8 agonist c (Fig. 9B). Particularly, conjugate 4 was shown to be most adjuvant among all TLR active compounds that we had characterized in our laboratory with a rise-in-titer values of >1000 (Fig. 10, overleaf). Considering very small dose of the TLR agonist in the conjugate, the strong adjuvant activity of the conjugate after a single boost, coupled with the complete lack of proinflammatory cytokine induction in ex vivo models, as mentioned earlier, presages a highly effective and safe vaccine adjuvant.

Fig. 9. Adjuvant activities of HA conjugates after a single boost. Antigen: Diphtheria toxoid (CRM197), 10 μg/dose; Adjuvant: 100 μg/dose in NZ rabbits; Formulation: excipient-free saline. A. Antigen-specific titers in HA conjugate-adjuvanted rabbits. Preimmune values are in open symbols. B. Box-and-whisker plots of ratios of immune/preimmune titers yielding absorbance values of 1.0.
**Fig. 10.** Induction of high anti-CRM197 IgG levels after a single boost (Immune-1 sera) by HA conjugate 4 and Infanrix (left panel), compared to titers after two boosts (Immune-2 sera) for the HA conjugates, as well as a variety of experimental, candidate adjuvants. Also shown in red are titers obtained by immunization with a full human dose of Infanrix and unadjuvanted controls (right panel).

Whilst measurements of serum antibody titers employing an immunoassay such as ELISA serve to quantify antigen-specific antibody concentrations, antibody ‘avidity’ (functional affinity; the sum of affinities of multiple antigen-binding sites in polyclonal antibodies simultaneously interacting with their cognate antigenic epitopes) is an important characteristic of protective
immune responses. In order to also assess the quality of antibody, the strength of the interaction between CRM197 and antigen-specific IgG antibodies obtained 10 days after a single boost (Immune-1) was determined using the chaotropic ELISA. Replicate wells containing antibody bound to antigens were exposed to increasing concentrations of the chaotropic thiocyanate ion that disrupts antibody-antigen complexes and the concentration of sodium thiocyanate (NaSCN) that decreases antibody binding by 50% was used as the measurement of avidity. As shown in Fig. 11B, the highest avidity was found in the conjugate 4-adjuvant sera with 50% attenuation of absorbance elicited at 1.0 M NaSCN. This result unambiguously indicates higher quality IgG elicited by the hyaluronic acid conjugates.

Fig. 11. A. Chaotropic ELISA with increasing concentration of sodium thiocyanate (NaSCN) after a single boost (Immune-1 sera). B. Quantitation of antibody avidity in chaotropic ELISAs with NaSCN concentration eliciting 50% attenuation of absorbance.

The observation of extraordinary adjuvantic potency completely dissociated with biomarkers of inflammation and reactogenicity is unprecedented in the field, and warranted confirmation. In an
independent experiment, cohorts of rabbits (n=4 per group) were immunized with either graded
doses of antigen (human vaccine-grade formalin-inactivated Diphtheria toxoid, Statens Serum
Institute, Denmark) with a fixed dose of conjugate 4 to examine dose-sparing effects, or graded
doses of adjuvant with a fixed dose of Diphtheria toxoid. As shown in Fig. 12A, conjugate 4
elicits very high titers even at an antigen concentration of 1 µg/dose, confirming the excellent
dose-sparing effect of the adjuvant. Although 100 µg/dose of conjugate 4 is optimal, robust
humoral responses are observed even with 10 µg of conjugate 4, as depicted in Fig. 12B.

Fig. 12. Dose-sparing effects of conjugate 4 as adjuvant in rabbits (n=4 per group) immunized with:
Graded doses of formalin-inactivated Diphtheria toxoid with fixed dose (100 µg/dose) of conjugate 4 (A);
graded doses of conjugate 4 with fixed dose (10 µg/dose) of antigen (B); unadjuvanted and compound d-
adjuvanted (100 µg/dose) controls (C).
7.3. Conclusion

A potential drawback in using small molecule TLR agonists as vaccine adjuvants is their propensity to diffuse out of the vaccination site into systemic circulation, thereby not only limiting their adjuvantic properties, but perhaps also enhancing the risk of systemic reactogenicity. The administration of R-848 (Chapter 1) was poorly tolerated in human preclinical trials, with systemic side effects including fever, headache, malaise, and myalgia, likely due to systemic immune activation. Indeed, intravenous injections of large doses of TLR7/8 agonists (described in earlier chapters; listed in Fig. 13) as a bolus in rabbits induce C-reactive protein, an acute-phase bio-marker of systemic inflammation (Fig. 13).

Fig. 13. Induction of C-reactive protein (CRP) in New Zealand White Rabbits (n=5 per cohort) by TLR7/8 agonists. Animals were bled two days before challenge to obtain baseline CRP levels, and were injected with either 1 mg of candidate adjuvant (or 100 μg of LPS, used as control) formulated in saline intravenously as a bolus, into the marginal vein of the ear, on Day 0. Successive bleeds were performed on Day 1, 2, 5 and 9 after injection. Rabbit CRP-specific ELISAs were performed to quantify CRP in the sera.
Limiting systemic exposure has recently been addressed by adsorbing small molecules incorporating phosphonate groups on ‘alum’ [Al(OH)₃]. Whilst alum has been the mainstay of vaccines since the 1920s, aluminum exposure has been implicated in neurotoxicity, especially in the preterm and infant populations and our understanding of innate immune signaling may have reached a threshold as to allow considering rational alternatives.

Targeted delivery of the TLR agonists to secondary lymphoid organs using molecular conjugation to HA was therefore evaluated to simultaneously enhance selective delivery to draining lymph nodes while limiting systemic exposure. As mentioned earlier, both CD44 and LYVE-1 are known to interact with HA. Soluble antigens in the interstitial fluid are carried by bulk lymphatic via afferent lymphatic ducts into the subcapsular sinus of the draining lymph node. Recent studies on the microanatomic architecture of the subcapsular sinus of the lymph node receiving afferent lymphatic fluid show that in the subcapsular sinus, plasmalemma vesicle-associated protein (PLVAP; also known as PV-1, PAL-E or MECA-32 antigen) forms a physical sieve that limits the entry of molecules smaller than 70 kDa from accessing lymph node parenchyma, and confining such large molecules to the sinus (Fig. 14).

*Fig. 14. The subcapsular sinus of the lymph node, lined by lymphatic endothelial cells, is directly connected to the afferent lymphatic vessels. The fibroblastic reticular cells (FRCs) constitute a network interconnecting the subcapsular sinus and vasculature, forming a conduit system for direct egress of fluid and small molecules arriving from the subcapsular sinus to the blood circulation. Molecules of size >70 kDa are retained by a diaphragm made up of PLVAP, which forms a cartwheel structure. Adapted from: The lymph node filter revealed. M. Hons & M. Sixt, Nature Immunology 16, 338–340 (2015).*
Sinus-lining macrophages are known to transfer antigens from the sinus lumen to the B cell follicles. Given that the size of HA that was used in these studies is approximately 500 kDa, we speculate that the retention of the HA conjugates in the subcapsular sinus may facilitate and enhance sampling by the sinus-resident macrophages, augment localized innate immune signaling, and marshal subsequent adaptive immune responses in a spatiotemporally far more efficient manner.

As noted earlier, the hyaluronic acid conjugates entirely lose their TLR-agonistic activities, both in respect to TLR recognition as probed by the reporter gene assays, as well as in their ability to induce cytokines in secondary screens. One of these ‘silent’ conjugates has, however, yielded the best adjuvant, capable of evoking, with a single boost regimen, affinity-matured high-avidity immunoglobulins whose titers rival and surpass all of the best-in-class small molecule adjuvants that have been examined to date. As mentioned earlier, significant hyaluronidase activity is associated with macrophages, possibly rendering the conjugate into small oligosaccharide fragments bearing the TLR agonist(s). The high adjuvanticity observed with several of the conjugates tested perhaps additionally implies that amidases in the subcapsular macrophage may release the free agonist, which may act in the milieu where the antigen is also being sampled. These are questions yet to be formally addressed. Studies, for instance, have already begun on conjugates obtained with carboxymethylcellulose to probe the contribution of the receptor-mediated ‘uptake’ of hyaluronic acid vis-à-vis passive, but contemporaneous delivery of antigen and adjuvant via bulk lymphatic flow. Finally, the superior adjuvanticity observed with conjugate 4 suggests that HA conjugates bearing dual TLR7/8 agonists may be necessary for optimal innate immune signaling and subsequent adaptive immune recruitment, since neither conjugate 1 (bearing a pure TLR7 agonist), nor conjugate 2 (decorated with a pure TLR8 agonist) were found to be as strongly adjuvant as conjugate 4. These results, made possible by extensive SAR on a variety of chemotypes described in the preceding chapters, have laid the
foundation and point the way forward for many other experimental approaches designed to probe the consequences of rational, targeted vaccine delivery that take into consideration not only the medicinal chemistry and immunology of small molecule innate immune stimuli, but also incorporate elements of the microanatomical organization and physiology of the immune system.

7.4. Experimental

Materials
Sodium hyaluronate (HA) from *Streptococcus equi* (bacterial glycosaminoglycan polysaccharide) was purchased from Sigma-Aldrich (USA) and used as received. All other reagents and solvents including 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT, 97%), N-methylmorpholine (NMM, 99%), as well as Dowex®50WX8 ion exchange resin (hydrogen form, 50-100 mesh) were purchased from Sigma-Aldrich. Hyaluronate Rhodamine (500 kDa) was purchased from Creative PEGWorks (USA). Hyaluronidase from bovine testes (55 kDa, 400-1000 units/mg) was purchased from Sigma-Aldrich and used for enzymatic degradation. For dialysis, Slide-A-Lyzer G2 Dialysis Cassettes, 3.5 K MWCO (Thermo Scientific) were used.

Synthesis of hyaluronic acid conjugates

**General procedure for triazine-activated amidation of hyaluronic acid (HA).** Sodium hyaluronate (1.0 eq., 100.0 mg, 0.25 mmol –COO–) was dissolved in 20 mL of water under gentle stirring. The solution was cooled in an ice bath and 14 mL of acetonitrile were added dropwise. 2-Chloro-4,6-dimethoxy-1,3,5-triazine (1.0 eq., 44 mg, 0.25 mmol) and N-methylmorpholine (1.5 eq., 41 μL, 0.38 mmol) were added to the solution and the mixture was subsequently allowed to come to room temperature and was stirred for 1 h. Thereafter the
amine compound (1.0 eq., 0.25 mmol) was added and the mixture was stirred at room temperature overnight. The crude mixture was stirred with Dowex®50WX8 ion exchange resin (1 g) for 1 h, then filtered and dialyzed (3.5 kDa MW cutoff) against aq. NaCl (0.1 N) for 2 days and against dH₂O for 3 days and lyophilized. The solids obtained were thoroughly washed with acetone. The product yields were calculated based on the average molecular weight of the disaccharide repeating units considering the degree of substitution.

For the synthesis of conjugate 5, the mixture of small molecules a, b and c (each 0.4 eq., 0.1 mmol) was used.

Characterization of HA conjugates

Proton nuclear magnetic resonance spectroscopy (¹H NMR). ¹H NMR spectra of the samples (5 mg/mL in D₂O) were recorded on a Bruker Advance 400 MHz spectrometer. The chemical shifts are given in parts per million (ppm) and are calibrated relative to the residual solvent protons (HDO: 4.79 ppm). Degrees of substitution (DS) were calculated from comparing the integrals of the methyl protons (1.95 ppm) of the N-acetylglucosamine moiety of HA and distinctive peaks of the introduced methyl group of small molecules (0.86 ppm) and are given in % per 100 disaccharide units.

Size exclusion chromatography (SEC). A Shimadzu analytical HPLC system was used with HiPrep 16/60 Sephacryl S-200 HR (GE Healthcare, PA, USA) size exclusion chromatography (SEC) column for analytical characterization. Samples of the conjugates (1 mg/mL, 50 μL) were injected using PBS (pH 7.4) buffer containing 0.05% NaN₃ as a mobile phase at a flow rate of 1.5 mL/min.

High performance liquid chromatography (HPLC). A Shimadzu analytical gradient reversed-phase HPLC system was used with PRP-3 (cross-linked polystyrene/poly-divinylbenzene
polymeric stationary phase, Hamilton Laboratory Products, Reno, NV) column for analytical characterization. Gradient elution was carried out at constant flow of 1 mL/min, from 90% A to 50% A (corresponding to 10% B to 50% B) for 50 min and from 50% A to 0% A (corresponding to 50% B to 100% B) for 5 min followed by an isocratic elution at 100% B for 5 min. Mobile phase compositions were (A) water with 0.1% TFA and (B) acetonitrile with 0.1% TFA.

**Quantification of small molecule loading in HA conjugates.** The compound (TLR7/8 agonist; \(a, b, c, d\)) stock solutions at a concentration of 1 mg/mL were used to prepare standard solutions with concentrations of 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 \(\mu\)g/mL, respectively. The conjugate solutions were also prepared by dissolving 100\(\mu\)g of each conjugate sample in 1 mL of water. Percentage of small molecule loading was determined by UV measurement at 260 nm by correlating with the standard curve.

**Gel electrophoresis.** Samples were subjected to electrophoresis using 1.2% Clear E-Gel\textsuperscript{®} agarose gel (Life Technologies). 20 \(\mu\)L of prepared samples (1mg/mL) were loaded into each well and gel was run for 40 min. Bands were visualized in the gel by UV illumination. The size of the oligosaccharides was compared with rhodamine labeled, commercially available HA of defined size (500 kDa).

**In vitro enzymatic degradation.** The reaction solutions were prepared by mixing 200 \(\mu\)L of HA conjugates (1 mg/mL in H\(_2\)O) with 200 \(\mu\)L of hyaluronidase solution (HAase, 400-1000 U/mL in H\(_2\)O). The solutions were incubated at 37 °C for 48 h then analyzed by LC-MS.

**Human TLR-7/-8 reporter gene assays (NF-\(\kappa\)B Induction).** As described in Chapter 2.
Immuoassays for cytokines. As described in Chapter 2.

Rabbit immunization and antigen-specific ELISA. All experiments were performed at Harlan Laboratories (Indianapolis, IN) in accordance with institutional guidelines (University of Kansas IACUC permit # 119-06). Adult New Zealand White rabbits are immunized intramuscularly in the flank region with (a) $10 \mu g$ of antigen (CRM197) in 0.2 mL saline ($n = 4$ for unadjuvanted, antigen+saline control cohort), or (b) $10 \mu g$ of antigen plus $100 \mu g$ of adjuvant in 0.2 mL saline ($n = 4$ for antigen+adjuvant test cohorts). Pre-immune test-bleeds were first obtained via venipuncture of the marginal vein of the ear on Day 1. Animals are immunized on Days 1, 15 and 28. A test-bleed is performed via the marginal vein of the ear on Days 25 and 38. Sera are banked at -80 °C for antigen-specific IgG quantitation by ELISA.

C-reactive protein (CRP) measurements. Cohorts of New Zealand White Rabbits ($n=5$ per cohort) were bled two days before administration of the test compounds to obtain baseline CRP levels. Animals were injected with either $1 mg$ of candidate adjuvant (or $100 \mu g$ of LPS, used as control) formulated in saline intravenously as a bolus, into the marginal vein of the ear, on Day 0. Successive bleeds were performed on Day 1, 2, 5 and 9 after injection. Sera were stored at -80 °C until used and rabbit CRP-specific ELISAs were performed to obtain the concentrations of CRP in the sera samples.
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