Chapter 1
Targeting the Wnt Signaling Pathway to Treat Colon Cancer

Approximately 150,000 people are diagnosed with colorectal cancer every year in the United States, and 1/3 of these die from the disease. Treatment options currently include surgical resection if the tumor has not progressed to an advanced stage and traditional cytotoxic chemotherapy regimens, which have a host of side-effects and often simply prolong the survival period without eliminating the cancer. Fortunately, however, the somatic genetic defects that drive colorectal cancer are among the best characterized of any human tumor, which holds great potential for the development of novel therapeutics that specifically target colorectal cancer cells. The specific pathway that is dysregulated in the majority of colon cancers and many other forms of cancer is the Wnt signaling pathway.

Figure 1-1: Overview of Wnt signaling pathway

The Wnt/β-catenin signaling pathway plays a central role in both normal embryogenesis and development and the initiation and progression of cancer. The name
Wnt derives from two homologous families of genes-Wg (wingless) and Int – and membership in the family derives from amino acid sequence, not biological function.\(^2\) The wingless gene had initially been identified as a segment polarity gene in *Drosophila melanogaster* that functions during embryogenesis and also during adult limb formation during metamorphosis.\(^3\) The Int genes, on the other hand, were originally identified as vertebrate genes near several integration sites of mouse mammary tumor virus (MMTV). As can be inferred by these two examples, the Wnt genes have a diverse role of functions, but they all utilize a signaling pathway with \(\beta\)-catenin as the central effector, as shown in Figure 1-1.

While the list is still growing, there are approximately 20 known *Wnt* genes expressed in humans to date. Highly conserved across species from invertebrates to humans, they encode secreted glycoproteins, which regulate cell growth, motility, and differentiation during embryonic development in the canonical Wnt-pathway.\(^4\) Wnt factors activate diverse signaling cascades by binding to seven transmembrane receptors, called frizzled, which, themselves, must also interact with low density lipoprotein receptor-related protein (LRP) and other co-receptors for full activation.\(^4\) Within the cell, the signal from a Wnt factor is transduced via the protein disheveled, which allows for the accumulation of \(\beta\)-catenin in the cytoplasm and subsequent translocation to the nucleus.

\(\beta\)-catenin, for its part, is a multifunctional protein involved in the binding and activation of the T-cell factor (Tcf)/lymphoid enhancement factor (LEF) family of transcription factors.\(^5\) In the absence of a Wnt signal, most of the \(\beta\)-catenin in the cell is attached to the plasma membrane, in association with E-cadherin in adherence junctions.\(^4\) Meanwhile, the remaining cytosolic \(\beta\)-catenin is trapped in a multiprotein complex called the \(\beta\)-catenin destruction complex, which phosphorylates \(\beta\)-catenin and targets it for subsequent ubiquitination and degradation by the proteasome. Other members of this destruction complex include adenomatous polyposis coli (APC) protein, axin/conductin, casein kinase 1\(\alpha\), and glycogen synthase kinase-3\(\beta\) (GSK-3\(\beta\)).

In the presence of a Wnt signal, however, the destruction complex is inhibited, and the level of \(\beta\)-catenin rises, allowing it to translocate to the nucleus and interact with the Tcf/LEF family of transcription factors. Further co-activators are then recruited for
transcriptional activation of the $\beta$-catenin/Tcf complex. Tcf4 is the factor most commonly expressed in colorectal cancer, and Tcf4/$\beta$-catenin complexes determine cellular responses through the expression of $c$-$\text{Myc}$ and $p21^{\text{CIP1/WAF1}}$. Among the other genes that are turned on by this complex are $c$-$\text{Myc}$, metalloproteinases, and $\text{VEGF}$. This increased level of $\beta$-catenin is invariably present in colorectal tumors, as well as many other cancers, and it often results from loss-of-function mutations in the APC gene.

With the awareness of the prominent role that the Wnt signaling pathway plays in colorectal and many other cancers, it has become a target for many investigations into novel chemotherapeutic drugs in recent years. As there are many proteins involved in the Wnt signaling pathway, a number of approaches have been explored. Nonetheless, since $\beta$-catenin is the central player in this pathway which activates many of the genes that are detrimental in colon cancer when it translocates to the nucleus and interacts with the Tcf/LEF family of cofactors, most approaches aim in either a direct or indirect way to decrease signaling by $\beta$-catenin. Moreover, these ongoing investigations include both biological approaches, such as genomic knockout, RNA interference, and oncolytic viruses, and exploration of small molecules as inhibitors. To date, searches for small molecule inhibitors that inhibit the pathway have utilized the following mechanisms: 1) repression of $\beta$-catenin expression and target genes, 2) induction of $\beta$-catenin degradation by the proteasome, phosphorylation of $\beta$-catenin and subsequent degradation, or stabilization of serine/threonine-phosphorylated, inactive $\beta$-catenin, 3) reduction of nuclear $\beta$-catenin via active export, 4) relocalization of $\beta$-catenin to the plasma membrane, 5) disruption of the interaction between $\beta$-catenin and co-transcription factors, and 6) disruption of the $\beta$-catenin/Tcf interaction. As will be seen, some of these approaches involve further exploration of established drugs used to treat other diseases, while others involve the search for new chemical entities.

In fact, the first of these approaches- repression of $\beta$-catenin expression and target genes- is believed to be one mechanism of action for the well-known class of drugs known as non-steroidal anti-inflammatory drugs (NSAIDs). Specifically, indomethacin (Figure 1-2) has been shown to downregulate aberrant Wnt/\beta-catenin signaling activity.
via repression of β-catenin transcription and induction. This has been demonstrated by the disappearance of nuclear β-catenin\(^6\) and a reduction in β-catenin mRNA levels,\(^7\) both of which preceded indomethacin-induced apoptosis in human colorectal cancer cells. Inhibition of target genes of Wnt/β-catenin signaling was then confirmed by transcriptional repression of the β-catenin/Tcf target gene cyclin D1.\(^8\) Still, this is believed to be only one mechanism of action for indomethacin on Wnt signaling, as it was also shown to induce β-catenin degradation, independent of the β-catenin destruction complex and proteasome machinery.

![Small molecule inhibitors of Wnt signaling pathway](image)

**Figure 1-2: Small molecule inhibitors of Wnt signaling pathway**

Indomethacin is only one of a number of established drugs that have been shown to induce β-catenin degradation, either via the proteasome or apart from it. Others that inhibit the Wnt signaling pathway by increasing the rate of β-catenin degradation include sulindac and sulindac metabolites (\textit{i.e}, sulindac sulfone and sulindac sulfide), cyclooxygenase-2 (COX-2) inhibitors (\textit{i.e}, rofecoxib, celecoxib), aspirin, hexachlorophene, ritonavir, all shown in Figure 1-2, and IWP-1 to -4 as well as IWR-1 to -5, which are all shown in Figure 1-3. Sulindac is an NSAID like indomethacin, but it is actually a prodrug that is converted to the active metabolites of sulindac sulfide and sulindac sulfone \textit{in vivo}.\(^4\) Interesting, both of these metabolites have growth inhibitory
effects on colorectal cancer cells, but only sulindac sulfide inhibits cyclooxygenase (COX) enzyme activities.\textsuperscript{4} When investigating the mechanism for this growth inhibition, it was found that sulindac treatment resulted in decreased levels of nuclear β-catenin, abrogation of β-catenin/Tcf-mediated transcription, and decreased levels of nonphosphorylated β-catenin.\textsuperscript{9} The explanation for the decreased levels of nonphosphorylated β-catenin is not unanimously agreed upon, and several studies suggest a combination of mechanisms, including induction of caspase- and proteasome-dependent degradation\textsuperscript{10} and inhibition of cGMP phosphodiesterases (PDE) which leads to increased cGMP levels, consequent activation of protein kinase G (PKG), and eventual phosphorylation of β-catenin, which signals it for degradation.\textsuperscript{11} In the canonical Wnt signaling pathway, β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK-3β), but its ability to also be phosphorylated by PKG suggests an alternate mechanism for inducing proteasome-mediated degradation in cells where the APC/GSK-3β-mediated phosphorylation is disrupted.

Also increasing the rate of β-catenin phosphorylation and subsequent proteasome degradation, albeit via a different mechanism, are the COX-2 inhibitors, such as rofecoxib and celecoxib. In the case of the COX-2 inhibitors, one target has been shown to be c-Met, which is a receptor tyrosine kinase (RTK) for hepatocyte growth factor and which is often associated with enhanced tumor growth and a poorer prognosis.\textsuperscript{12} It was found that COX-2 inhibitors impair both the autophosphorylation of c-Met and the phosphorylation of substrates by c-Met, resulting in an increase in GSK-3β activity and subsequent increase in β-catenin phosphorylation.\textsuperscript{13} The increase in phosphorylated β-catenin then results in more ubiquitination and degradation and less active β-catenin translocating into the nucleus and activating transcription.

Aspirin, too, has been shown to act by decreasing the amount of active β-catenin for signaling and was, in fact, the first NSAID shown to have cancer preventive properties. Similar to both indomethacin and sulindac, aspirin affects the growth rate of cancer cells via both COX-dependent and COX-independent mechanisms.\textsuperscript{4} Its ability to downregulate β-catenin/Tcf signaling and transcription of target genes is detectable in both cells with APC mutations and with mutationally-activated β-catenin.\textsuperscript{8} Unlike the
other NSAIDS, however, aspirin does not have an effect on β-catenin levels, nuclear localization, or turnover. Rather, aspirin stabilizes β-catenin in a transcriptionally inactive, partially serine/threonine-phosphorylated form, which is not further phosphorylated by GSK-3β or tyrosine kinases to target it for proteasome-mediated degradation but which is also unable to function as a co-transcription factor.14 Hence, the level of active β-catenin falls, diminishing β-catenin/Tcf signaling. The mechanism by which this stabilization of the partially-phosphorylated, inactive state occurs, however, still needs to be fully elucidated.

Hexachlorophene, for its part, is believed to lead to increased β-catenin degradation through a Siah-1-dependent pathway. Siah-1 is a member of the seven in absentia homolog (SIAH) family and a p53-inducible mediator of cell cycle arrest, tumor suppression, and apoptosis.15 It is an E3 ligase that is involved in ubiquitination and proteasome-mediated degradation of β-catenin and other proteins through a unique mechanism by which it interacts with the carboxyl terminus of APC, recruits the ubiquitination complex, and promotes degradation through a pathway independent of both GSK-3β and β-transducin repeat-containing protein (β-TrCP, the F-box protein in the E3 ubiquitin ligase complex).16 Evidence of this mechanism was shown in several ways.16 First, in the presence of LiCl, which is an inhibitor of GSK-3β, hexachlorophene still reduced the level of β-catenin. Secondly, overexpression of β-TrCP failed to block the hexachlorophene induced degradation. Third, hexachlorophene reduced the level of β-catenin in cell lines which express wild-type APC (HCT116 and LS174T) but not in ones with truncated APC (SW480 and DLD-1). Fourth, expression of Siah-1 was induced by hexachlorophene in both HCT116 and HEK293 cell lines, accompanied by decreased β-catenin levels. Finally, overexpression of Siah-1 in the presence of hexachlorophene abrogated the degradation of β-catenin. While Siah-1 is normally induced by p53, however, it should be noted that the results described were found in the absence of p53-dependent transcription and stabilization of the p53 protein, indicating an alternate path to Siah-1 induction which has yet to be elucidated.16

Another established drug currently prescribed for other indications, ritonavir, has not yet been explored for other potential therapeutic uses. Still, it, too, was recently
found to exert an effect on the level of active β-catenin by increasing the amount that is ubiquitinated and degraded. Due to the decreased bone mineral density that afflicts many human immunodeficiency virus (HIV) patients who have been treated with protease inhibitors, investigators studied the mechanism by which ritonavir and other HIV protease inhibitors might be involved in osteoclast differentiation, which has been also shown to involve the Wnt signaling pathway. What they found was that of the protease inhibitors investigated, only ritonavir upregulated the production of transcripts for osteoclast growth factors and suppressed genes involved in canonical Wnt signaling. Accompanying the suppression of Wnt signaling genes was a decrease in nuclear translocation of β-catenin. Moreover, the abrogation of nuclear translocation was found to correlate with ritonavir’s ability to increase the rate of ubiquitination of β-catenin and its subsequent degradation.

Finally, among inhibitors that act via decreasing the amount of active β-catenin, novel compounds IWP-1 to -4 were recently described as inhibitors of Wnt production, while compounds IWR-1 to -5 were reported as inhibitors of Wnt response. The two groups were differentiated by treatment with exogenously supplied Wnt protein, where the inhibitors of Wnt production failed to block the Wnt/β-catenin response. The inhibitors of Wnt production were able to block all Wnt-dependent biochemical changes that were assayed upstream of nuclear translocation of β-catenin, which included phosphorylation of the Lrp6 receptor, phosphorylation of Dvl2, and accumulation of β-catenin. This response was attributed to inhibition of the activity of Porcupine, which is a membrane-bound acyltransferase that adds a palmitoyl group to Wnt proteins, which is necessary for both their signaling ability and secretion. Meanwhile, the inhibitors of Wnt response only affected the levels of β-catenin. For the inhibitors of Wnt response, the mechanism of action was found to be inhibition of axin protein destruction. Axin is another member of the β-catenin destruction complex, and, as such, serves as a suppressor of Wnt pathway signaling. Treatment of the DLD-1 colorectal cancer cell line with compounds IWR-1 to -5 resulted in increased levels of axin, along with elevated levels of β-catenin phosphorylation. Moreover, the compounds did not induce de novo synthesis of Axin2, which is another transcriptional target of the Wnt/β-catenin pathway,
and the proteasome was not inhibited. By inhibiting the destruction of axin protein, compounds IWR-1 to -5 appear to exert their effect by increasing the amount of β-catenin that gets degraded by the β-catenin destruction complex.

**Figure 1-3: Novel small molecule inhibitors of Wnt signaling**
Figure 1-4: Small molecule inhibitors of Wnt signaling via β-catenin sequestration

Also affecting the level of β-catenin in the nucleus but via a different mechanism are curcumin and a closely-related analogue, CHC007, which are shown in Figure 1-4 and which were reported in 2005 as inhibitors of β-catenin/Tcf signaling.20 Upon treatment with curcumin or CHC007, the level of nuclear β-catenin in HCT116 cells (human colorectal cancer cell line) was found to be reduced. Moreover, curcumin’s ability to downregulate β-catenin/Tcf signaling in HEK293 (human embryonic kidney) cells that had been transiently transfected with S33Y mutant β-catenin, which cannot be degraded by the APC/GSK-3β complex, indicated that the mechanism of inhibition was either related to β-catenin itself or downstream of it.20 Further analysis showed that the amount of neither cytosolic nor membranous β-catenin was affected but that the levels of nuclear β-catenin and Tcf4 were markedly reduced.20 While the decreased nuclear levels could be explained either by increased nuclear degradation or increased export from the nucleus, no evidence of nuclear degradation was found. Hence, the decreased levels are most likely the result of increased export, as there are prior reports of β-catenin being exported from the nucleus by both APC-dependent and APC-independent pathways.21,22 The specifics of the nuclear-exporting mechanism remain to be detailed.

The fourth mechanism of inhibiting the Wnt signaling pathway with small molecule inhibitors- increased relocalization to the plasma membrane- has been reported both by an established drug already used to treat chronic myeloid leukemia and a plant flavonoid. These are imatinib (Gleevec) and apigenin, respectively, each of which is
shown in Figure 1-4, and each of which appears to operate by inhibiting a tyrosine kinase. Since imatinib is a known tyrosine kinase inhibitor that acts on the bcr-abl fusion protein, it was investigated in other cancer cell lines and against other panels of tyrosine kinases to see if its role as a cancer treatment could be expanded. Indeed, it was found to inhibit the constitutive activity of β-catenin signaling in human colon cancer cells as well as the Wnt1-induced activation of β-catenin signaling in HOS (human osteosarcoma), HTB-94 (human chondrosarcoma), and HEK 293 cells. The inhibition of β-catenin signaling was then linked to a decreased amount of tyrosine phosphorylation. Tyrosine phosphorylation of β-catenin, for its part, is currently believed to trigger dissociation of β-catenin from E-cadherin at the plasma membrane, allowing active β-catenin to move into the nucleus and activate the target genes. Hence, the inhibition of tyrosine phosphorylation results in increased association of β-catenin with E-cadherin at the plasma membrane and inhibition of the Wnt signaling pathway.

Apigenin, likewise, was shown to decrease tumor volumes and increase the amount of β-catenin associated with the plasma membrane in both transgenic adenocarcinoma of the mouse prostate (TRAMP) and in human prostate cancer cells. Moreover, apigenin had previously been shown to be an inhibitor of several protein tyrosine kinases, including epidermal growth factor and src tyrosine kinase, and the increased association of β-catenin with the plasma membrane was accompanied by a rise in the protein levels of E-cadherin by 24-75%, depending on the dose of apigenin. Also observed were decreased nuclear translocation of β-catenin, less cytosolic β-catenin, and reduced amounts of c-Myc and cyclin D1, two targets of Wnt/β-catenin signaling. All of this data is consistent with apigenin inhibiting Wnt signaling via increased relocalization of β-catenin to the plasma membrane.
The final two categories of small molecule inhibitors of Wnt signaling include more novel chemical entities, which are shown in Figure 1-5, and which are all implicated as inhibitors of the binding between β-catenin and one of its transcription factors. Of those shown, ICG-001 is unique in that it targets the interaction between β-catenin and cyclic AMP response element-binding protein (CBP), which is a co-transcription factor along with Tcf4. Interestingly, ICG-001 inhibits the binding of CBP to β-catenin but not p300, which is highly homologous to CBP and also serves as a co-transcription factor with Tcf4. Moreover, while both CBP and p300 interact with β-catenin via amino acids 1-111, ICG-001 was found to selectively interact with amino acids 1-111 of CPB only. This selective inhibition has important implications for potential clinical uses because the CBP-β-catenin-Tcf complex appears to activate genes that lead to proliferation without differentiation in stem cells and cancer while the p300-β-catenin-Tcf complex appears to activate genes that lead to differentiation with limited proliferative capacity. Hence, inhibiting CBP binding to β-catenin but not p300 binding to β-catenin allows for selective inhibition of the Wnt signaling cascade that is detrimental in cancer. While not currently in use as a drug, ICG-001 was the first
selective small-molecule chemogenomic tool to modulate Wnt/β-catenin signaling and is currently used as a powerful tool for dissecting Wnt signaling.

FH535 also appears to target the interaction between β-catenin and a co-transcription activator, exerting effects on both the Wnt signaling pathway and peroxisome proliferator-activated receptor (PPAR) signaling. While the details are not fully known yet, FH535 appears to inhibit the complex containing PPARγ, β-catenin, and Tcf4 and has been shown to attenuate the expression of genes expressed by the Wnt signaling pathway, including Tcf4 itself. Two possibilities for the mechanism of this inhibition, which are not mutually exclusive, have been proposed and have yet to be deciphered are the following: 1) FH535 inhibits the recruitment of GRIP1 to the PPARγ/β-catenin/Tcf4 complex; 2) FH535 suppresses the transcription of selected PPARγ and PPARδ-targeted genes necessary for Wnt/β-catenin pathway activation.

The effect of FH535 depends on the production and cellular concentration of PPAR agonists that antagonize its activity, and as such, it is emerging as a tool for studying the cross-interaction between the Wnt/β-catenin and the PPAR signaling pathways. In fact, as indomethacin is also a PPARγ-antagonist, this mechanism of action might also have a role there, too.

The other inhibitors shown in Figure 1-5 all target the interaction between β-catenin and Tcf. Since colorectal tumors seem to rely on this dysregulation of the Tcf4/β-catenin complex, disrupting this complex is likely to yield therapeutic benefit. Nevertheless, the number of reports targeting the interaction between β-catenin and Tcf with small molecules is still small, partly because of the belief by some of the difficulty of this approach due to the large surface area (4800 Å²) over which the interaction between the two proteins extends. Regardless, there are at least three recent papers detailing inhibitors that appear to act via this mechanism. The first of these is a study on nitric oxide-donating aspirin (NO-ASA), which had been found to exert a chemopreventive effect in those being treated by it as a safer alternative to traditional NSAIDs. In this paper, NO-ASA was found to inhibit both cell-growth and Wnt/β-catenin signaling in human SW480 (colon cancer) cells, which was linked to decreased β-catenin/Tcf4 interaction. The effect was much more pronounced for both the ortho and
para isomers relative to the meta isomer, and disruption of the β-catenin/Tcf4 complex was not demonstrated either by aspirin itself or by a structural analogue lacking the –NO2 moiety.32

Another report on inhibitors of the β-catenin/Tcf interaction combined virtual and biophysical screens to identify three drug-like, low molecular weight Tcf4-competitive compounds, with the tightest binder having a $K_D$ of 450 nM.33 In this report, six hot spots were first identified using the crystal structures of both the β-catenin/Tcf3 complex and the β-catenin/Tcf4 complex with the PASS34 program. These hot spots are shown on the crystal structure of the β-catenin/Tcf3 complex in Figure 1-6, and the second of these hot spots was chosen for docking based on a more detailed analysis of the six cavities that was carried out. In this more detailed analysis, this second hot spot was found to be sufficiently wide, less solvent-exposed, and containing several small clefts.
that could potentially anchor a small molecular weight inhibitor. After filtering 90,000 compounds from a Pharmacia corporate collection, 17,700 compounds were docked to this hot spot, with the best 22 being put into two biophysical screens that utilized nuclear magnetic resonance spectroscopy (NMR) and isothermal calorimetry (ITC) to determine binding constants. Of the 22, seven compounds were found to bind to β-catenin by NMR, and of these seven, three were found by ITC to inhibit Tcf4 binding to β-catenin by at least 10-fold. The structure and corresponding $K_D$ of 450 nM of only one of these three was reported in the paper and is that of PNU74654, which is shown in Figure 1-5. This compound shows poor physicochemical properties and is difficult to test in vitro because of its very low solubility in water and other common solvents used for assays.

![Figure 1-7: PKF 118-310](image)

Finally, the third paper to describe inhibition of the interaction between β-catenin and Tcf4 with small molecules is the launching point for this project. This report describes the results of a successful high-throughput screen (HTS) in identifying several small-molecule antagonists of the oncogenic Tcf4/β-catenin protein complex. In the assay used for the HTS, β-catenin was coated on microtiter plates and then incubated with GST-Tcf4, goat anti-GST antibody, and alkaline phosphatase conjugated to anti-goat antibody. Compounds that disrupted the Tcf4/β-catenin interaction, therefore, gave reduced alkaline phosphatase signals. Further tests were then carried out to verify that the identified antagonists were specifically inhibiting the Tcf4/β-catenin complex. Several antagonists of this interaction were identified in a screen of over 7000 purified compounds from proprietary and public collections, two of which are PKF115-584 and CGP049090 in Figure 1-5. Neither of these are particularly promising as regards drug development because of their larger size, but there have been follow-up studies on both documenting their ability to induce apoptosis in acute myeloid leukemia cells.
of the screening hits, however, was PKF118-310 or 1,6-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione, which proffers more potential as a drug target and is shown in Figure 1-7. This screening hit was the starting point for the work to be herein described, where the goal was to design an improved analogue with better potency (nM inhibition, if possible) and pharmacokinetic properties amenable to drug development, such as high bioavailability (sub-μM concentration \textit{in vivo}), minimal degradation via metabolism, and reasonable half-life of at least 5 hours. Moreover, as PKF118-310 is also very toxic, another primary goal was to separate activity for the target from general toxicity, hopefully by at least an order of magnitude in the concentration of compound required to effect each.

Towards that end, a number of heterocyclic cores were considered, which are shown in Figure 1-8. First, expanding upon the parent heterocycle of the screen hit, both 1,3,6-substituted and 3,6,8-substituted pyrimidotriazinediones were envisioned in order to

**Figure 1-8: Heterocyclic cores synthesized as potential inhibitors of β-catenin/Tcf**
fully explore all possible structure-activity relationships with this central core and to
determine if a change in the electronics of the central core had any effect. Next,
pyrimidopyridazinediones and pyrimidopyrimidinediones were targets in order to
determine if all of the nitrogens of the pyrimidotriazinedione core were necessary for
activity. Finally, pyrazolopyrimidinediones and pyrrolopyrimidinediones were
considered due to their inability to undergo flavin-like redox chemistry, which is a
potential deleterious physicochemical property of both the pyrimidotriazinediones and
the pyrimidopyridazinediones. If activity could be maintained with fewer nitrogen
atoms in the core and with the lack of redox ability, the hope was that an improved
ADMET (absorption, distribution, metabolism, excretion, toxicity) profile would be
revealed.

While the choice of analogues synthesized was loosely guided by cellular data
that was supplied by Dr. Guido Bommer in Professor Eric Fearon’s laboratory
(University of Michigan Medical School), there were a number of problems and
inconsistencies with the data supplied. Moreover, some key findings in the last months
of work on this project highlighted even greater concerns with the cellular data.
Therefore, the format of this thesis will be to discuss all synthetic work first (Chapters 2-
6), which includes both novel methodology and the use of published procedures to obtain
novel analogues, however rightly or wrongly these were guided by the cellular data. The
final chapters (Chapters 7-9) will then detail all of the biological work that was done to
explore the activity of all compounds synthesized and to formulate what conclusions can
be made about the biological potential of the body of synthetic work overall.
Chapter 2

Synthesis of Pyrimidotriazinedione Analogues

With the screen hit, PKF118-310, embedded with a pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione core, a major focus was on the pyrimidotriazinedione heterocyclic class. Over 50 pyrimidotriazinedione analogues were synthesized using previously described general methods, with the overall strategy shown in Scheme 2-1. Of the analogues synthesized, 8 are previously reported compounds, and another 6 are virtual compounds whose structures are shown in Scifinder but for which there are no literature or patent references in any database. Using 6-chloro-3-methyluracil as a common starting material, this strategy allowed for the synthesis of two separate subgroups of pyrimidotriazinediones (5/8 and 7) where variation at R1, R3, and R8 could all be accomplished. Moreover, this strategy allowed for crossover from the N1-substituted (5,7(1H,6H)-dione) subseries (5 and 8) to the N8-substituted (5,7(6H,8H)-dione) subseries (7) via a demethylation step to N1-H pyrimidotriazinedione 6, capable of isomerization. Table 2-1 summarizes all of the N1-substituted pyrimidotriazinediones synthesized via this route, while Table 2-2 summarizes all of the N8 substituted pyrimidotriazinediones. The synthesis commenced with attack by the more nucleophilic secondary nitrogen of either methylhydrazine or 2-hydroxyethylhydrazine onto 6-chloro-3-methyluracil. While methylhydrazine has been routinely used in this manner in the literature, unique to this work is the use of 2-(hydroxyethyl)hydrazine in this initial step. The next two steps involved formation of the hydrazone (3) between the newly formed hydrazine (2) and the desired aryl or alkyl aldehyde, followed by nitrosation and reduction of the N-oxide (4). In all cases but one, the nitrosation of the hydrazone with NaNO2 in acetic acid produced a mixture of the desired pyrimidotriazinedione and the N-oxide, but the mixture was readily reduced solely to the desired pyrimidotriazinedione. Reduction of the N-oxide with dithiothreitol provided N1-methyl- (5) or N1-(2-hydroxyethyl)- (8) pyrimidotriazinediones for testing. Alternately,
reaction of the N1-methylpyrimidotriazinediones in DMF at 90°C afforded N1-demethylated pyrimidotriazinediones (6), which could then be alkylated with alkyl and benzyl halides in acetone in the presence of cesium carbonate to yield 3,6,8-substituted pyrimidotriazinediones (7).

The one case in which the N-oxide was not isolated in the nitrosation reaction was that where R3 = 2-pyridyl. As the N-oxide is the undesired product, this finding prompted some investigation of the nitrosation procedure to avoid N-oxide formation. This included meticulous exclusion of oxygen, addition of ascorbic acid to provide a reducing environment, addition of pyridine, and addition of HCl for more strongly-dehydrating conditions. Unfortunately, none of these was successful at excluding N-oxide formation, and the only rationale for why the 2-pyridyl substituent avoids N-oxide formation is that there is an electronic effect that affects the reaction mechanism, which is not clearly delineated.
Scheme 2-1: Synthetic strategy for pyrimidotriazinediones

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<td>virtual compound</td>
<td></td>
</tr>
<tr>
<td>5l</td>
<td><img src="structure5l.png" alt="Structure" /></td>
<td>270°C (dec)</td>
<td>Yes\textsuperscript{45}</td>
<td></td>
</tr>
<tr>
<td>5m</td>
<td><img src="structure5m.png" alt="Structure" /></td>
<td>220-223°C</td>
<td>virtual compound</td>
<td></td>
</tr>
<tr>
<td>5n</td>
<td><img src="structure5n.png" alt="Structure" /></td>
<td>223-224°C</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>5o</td>
<td><img src="structure5o.png" alt="Structure" /></td>
<td>298°C (dec)</td>
<td>Yes\textsuperscript{39,40}</td>
<td></td>
</tr>
<tr>
<td>8a</td>
<td><img src="structure8a.png" alt="Structure" /></td>
<td>214-215°C (dec)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>8b</td>
<td><img src="structure8b.png" alt="Structure" /></td>
<td>201-203°C</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>8c</td>
<td><img src="structure8c.png" alt="Structure" /></td>
<td>200-201°C</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>8d</td>
<td><img src="structure8d.png" alt="Structure" /></td>
<td>201-205°C (dec)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>8e</td>
<td><img src="structure8e.png" alt="Structure" /></td>
<td>208-211°C (dec)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>8f</td>
<td><img src="structure8f.png" alt="Structure" /></td>
<td>215-218°C (dec)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>8g</td>
<td><img src="structure8g.png" alt="Structure" /></td>
<td>182-188°C (dec)</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>
Table 2-1: N$_1$-Substituted pyrimidotriazinediones synthesized via strategy in Scheme 1

<table>
<thead>
<tr>
<th>R$_8$</th>
<th>R$_3$</th>
<th>Entry</th>
<th>mp</th>
<th>Known?</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_3$</td>
<td>H$_3$CO-</td>
<td><strong>7a</strong> (AJT-II-76-1)</td>
<td>259-265°C (dec)</td>
<td>Yes$^{41,45,46,47}$</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>N-</td>
<td><strong>7b</strong> (AJT-II-137-1)</td>
<td>195°C (dec)</td>
<td>No</td>
</tr>
<tr>
<td>CH$_2$CH$_2$CH$_3$</td>
<td>Cl-</td>
<td><strong>7c</strong> (AG-154-1)</td>
<td>202-205°C</td>
<td>Yes$^{48}$</td>
</tr>
<tr>
<td>(CH$_2$)$_7$CH$_3$</td>
<td>F-</td>
<td><strong>7d</strong> (AG-156-2)</td>
<td>123-125°C</td>
<td>No</td>
</tr>
<tr>
<td>CH$_2$CH$_2$NEt$_2$</td>
<td>H$_3$CO-</td>
<td><strong>7e</strong> (AF-129-3)</td>
<td>132-134°C</td>
<td>No</td>
</tr>
<tr>
<td>CH$_2$CH$_2$OH</td>
<td>H$_3$CO-</td>
<td><strong>7f</strong> (AJT-IV-71-1)</td>
<td>235-240°C (dec)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>7g</strong> (AG-85-1)</td>
<td>179-180°C</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>7h</strong> (AG-169-1)</td>
<td>221°C (dec)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>7i</strong> (AG-199-1)</td>
<td>161°C (dec)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>7j</strong> (AG-139-1)</td>
<td>dec &gt;240°C</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>7k</strong> (AG-115-1)</td>
<td>212-213°C</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>7l</strong> (AG-164-2)</td>
<td>276-280°C (dec)</td>
<td>No</td>
</tr>
</tbody>
</table>
In an attempt to incorporate better drug-like properties into the pyrimidotriazinediones synthesized, the synthesis depicted in Scheme 2-1 could be extended to incorporate an aqueous solubilizing functionality as shown in Scheme 2-2. By using meta- or para-anisaldehyde as the condensing aryl aldehyde in the second step of the synthesis in Scheme 2-1, the methoxy substituent of the R3 group could be deprotected by BBr3 in CH2Cl2 after alkylation of N8 of the pyrimidotriazinedione. The resulting phenol in 9 could then be alkylated by 2-(diethylamino)ethyl chloride hydrochloride with cesium carbonate in acetone to yield pyrimidotriazinediones 10. Alternately, a fluorobenzaldehyde could be used in the second step of the synthesis of Scheme 2-1, and a methoxy-substituted benzyl bromide could be used in the alkylation step. Then, the same reaction sequence in Scheme 2-2 could be executed, yielding a complementary series of analogues where the position of the fluoro- substituent and the

<p>| | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>F3C</td>
<td></td>
<td></td>
<td></td>
<td>7m (AG-160-1)</td>
<td>225-227°C (dec)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td>7n (AG-161-1)</td>
<td>253-254°C</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>H3CO</td>
<td></td>
<td>7o (AG-200-1)</td>
<td>230-234°C (dec)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td>7p (AG-168-1)</td>
<td>229-232°C</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>F3C</td>
<td></td>
<td>7q (AG-189-3)</td>
<td>156-159°C</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>H3CO</td>
<td></td>
<td>7r (AG-239-1)</td>
<td>240-241°C (dec)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>Cl</td>
<td></td>
<td>7s (AG-146-2)</td>
<td>251-254°C</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td>7t (AG-112-1)</td>
<td>226°C (dec)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>H3CO</td>
<td></td>
<td>7u (AG-187-1)</td>
<td>190°C (dec)</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-2: N8-Substituted pyrimidotriazinediones synthesized via strategy in Scheme 1
water-solubilizing functionality are effectively reversed (11a-11f). Table 2-3 shows all of the N8-substituted pyrimidotriazinediones synthesized via this extended route, along with the yields for the R3 deprotection and alkylation steps. All of these compounds are novel.

Scheme 2-2: Synthesis of 3(8)-(2-(diethylamino)ethoxy)phenyl-8(3)-fluorobenzyl-6-methylpyrimidotriazinediones
Table 2-3: 3(8)-(2-(Diethylamino)ethoxy)phenyl-8(3)-fluorobenzyl-6-methylpyrimidotriazinediones synthesized

<table>
<thead>
<tr>
<th>Structure</th>
<th>Formula</th>
<th>R6</th>
<th>mp (°C)</th>
<th>Decmp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Structure 1" /></td>
<td><img src="image2" alt="Formula 1" /></td>
<td><img src="image3" alt="R6-1" /></td>
<td>41</td>
<td>45</td>
</tr>
<tr>
<td><img src="image4" alt="Structure 2" /></td>
<td><img src="image5" alt="Formula 2" /></td>
<td><img src="image6" alt="R6-2" /></td>
<td>39</td>
<td>47</td>
</tr>
<tr>
<td><img src="image7" alt="Structure 3" /></td>
<td><img src="image8" alt="Formula 3" /></td>
<td><img src="image9" alt="R6-3" /></td>
<td>47</td>
<td>80</td>
</tr>
<tr>
<td><img src="image10" alt="Structure 4" /></td>
<td><img src="image11" alt="Formula 4" /></td>
<td><img src="image12" alt="R6-4" /></td>
<td>50</td>
<td>59</td>
</tr>
<tr>
<td><img src="image13" alt="Structure 5" /></td>
<td><img src="image14" alt="Formula 5" /></td>
<td><img src="image15" alt="R6-5" /></td>
<td>40</td>
<td>48</td>
</tr>
<tr>
<td><img src="image16" alt="Structure 6" /></td>
<td><img src="image17" alt="Formula 6" /></td>
<td><img src="image18" alt="R6-6" /></td>
<td>52</td>
<td>34</td>
</tr>
<tr>
<td><img src="image19" alt="Structure 7" /></td>
<td><img src="image20" alt="Formula 7" /></td>
<td><img src="image21" alt="R6-7" /></td>
<td>65</td>
<td>86</td>
</tr>
<tr>
<td><img src="image22" alt="Structure 8" /></td>
<td><img src="image23" alt="Formula 8" /></td>
<td><img src="image24" alt="R6-8" /></td>
<td>51</td>
<td>45</td>
</tr>
<tr>
<td><img src="image25" alt="Structure 9" /></td>
<td><img src="image26" alt="Formula 9" /></td>
<td><img src="image27" alt="R6-9" /></td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td><img src="image28" alt="Structure 10" /></td>
<td><img src="image29" alt="Formula 10" /></td>
<td><img src="image30" alt="R6-10" /></td>
<td>41</td>
<td>35</td>
</tr>
<tr>
<td><img src="image31" alt="Structure 11" /></td>
<td><img src="image32" alt="Formula 11" /></td>
<td><img src="image33" alt="R6-11" /></td>
<td>48</td>
<td>25</td>
</tr>
<tr>
<td><img src="image34" alt="Structure 12" /></td>
<td><img src="image35" alt="Formula 12" /></td>
<td><img src="image36" alt="R6-12" /></td>
<td>40</td>
<td>37</td>
</tr>
<tr>
<td><img src="image37" alt="Structure 13" /></td>
<td><img src="image38" alt="Formula 13" /></td>
<td><img src="image39" alt="R6-13" /></td>
<td>59</td>
<td>57</td>
</tr>
</tbody>
</table>

Introduction of different substituents at \( R_6 \) was accomplished via the route shown in Scheme 2-3, using barbituric acid (11) as a starting material. Here, barbituric acid was
treated with phosphorus oxychloride to yield 2,4,6-trichloropyrimidine (12), which was then converted to 6-chlorouracil (13) via treatment with sodium hydroxide. The N₁ of 6-chlorouracil could then be selectively protected with a benzyloxymethyl group, followed by alkylation at N₄ to yield chlorouracils with varying substituents at N₄ (N₆ in the final pyrimidotriazinediones). Treatment of these chlorouracils with methylhydrazine yielded N₁-BOM-protected 6-(1-methylhydrazinyl)uracils (16), which were used to form hydrazones as in Scheme 2-1, followed by cyclization with sodium nitrite. Fortunately, the BOM group was removed in situ during the cyclization, obviating the need for a distinct deprotection step. Finally, the N-oxides (18) were reduced with dithiothreitol, as in Scheme 2-1. This last reduction step worked cleanly when the pyrimidotriazinediones remained suspended in solution, but problems were encountered when more soluble analogues were synthesized, as the methyl group at N₁ of the pyrimidotriazinedione was easily removed. Table 2-4 shows all of the pyrimidotriazinediones that were synthesized via this route, none of which has been previously reported in the literature.

Scheme 2-3: Synthesis of pyrimidotriazinediones with variation at R₆
Table 2-4: Pyrimidotriazinediones synthesized with non-methyl $R_6$ substituents

<table>
<thead>
<tr>
<th>$R_3$</th>
<th>$R_6$</th>
<th>Entry (Notebook ID)</th>
<th>mp</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_3$CO-</td>
<td>CH$_2$CH$_2$OH</td>
<td>19a (AJT-18-1)</td>
<td>185°C (dec)</td>
</tr>
<tr>
<td>H$_3$CO-</td>
<td>F-</td>
<td>19b (AJ-291-2)</td>
<td>200-205°C (dec)</td>
</tr>
<tr>
<td>F-</td>
<td>F-</td>
<td>19c (AJ-304-1)</td>
<td>200-202°C (dec)</td>
</tr>
<tr>
<td>F-</td>
<td>H</td>
<td>19d (AJ-274-2)</td>
<td>240-247°C (dec)</td>
</tr>
<tr>
<td>Cl-</td>
<td>H</td>
<td>19e (AJ-290-1)</td>
<td>204-209°C (dec)</td>
</tr>
<tr>
<td>H$_3$CO-</td>
<td>H</td>
<td>19f (AJ-187-1)</td>
<td>267-275°C (dec)</td>
</tr>
</tbody>
</table>

A limiting factor in these synthetic routes outlined above is the inability to incorporate aryl groups at $N_1$, $N_6$, and $N_8$. In the literature, there are examples of phenyl groups incorporated at each of $N_6$ and $N_8$,$^{51}$ and the strategy to do so uses phenylurea as a starting material, as shown in Scheme 2-4. Based on this, our efforts focused next on obtaining $N_8$ phenylpyrimidotriazinediones. Hence, phenylurea was treated with diethylmalonate in the presence of sodium methoxide to form phenyl-substituted barbituric acid (22), which was then treated with phosphorus oxychloride to form a mixture of regioisomers that were easily separated by rinsing with aqueous saturated sodium bicarbonate. Isomer 24 was then used in the synthesis of $N_8$-phenylpyrimidotriazinediones. 6-Chloro-1-phenyluracil (24) was treated with methylhydrazine to form the $\alpha$-methylhydrazinyluracil (25), followed by formation of the hydrazone (26), which was alkylated with dimethylsulfate to intermediate 27. Cyclization with sodium nitrite in glacial acetic acid yielded only the demethylated pyrimidotriazinedione (28), albeit in low yield, without any detectable N-oxide. For $R_3=p$-CH$_3$OC$_6$H$_4$, analogue 28 is a novel compound, and the intention was to incorporate a
water-solubilizing functionality as had been accomplished in Scheme 2-2 for the related analogues. Hence, efforts were made to deprotect the methoxy group with boron tribromide and subsequently alkylate the phenol product. Unfortunately, the methoxy group of analogue 28 resisted deprotection under the same conditions as has been used for the previously-synthesized analogues and also under harsher deprotection conditions, such as higher reaction temperatures and longer reaction times. In each case, starting material was reisolated with the methoxy group still intact. A successful route to pyrimidotriazinedione 30 was eventually accomplished by methylating the N3 position of the uracil prior to hydrazone formation with the aryl aldehyde and by replacement of p-anisaldehyde with p-hydroxybenzaldehyde in the hydrazone formation, which obviated the need for a distinct deprotection step. This successful synthetic route is shown in Scheme 2-5. Here, intermediate 29 was directly obtained from the nitrosation reaction, and the phenolic substituent at R3 was readily alkylated to the desired pyrimidotriazinedione 30.
Scheme 2-4: Initial proposed route to N₈-phenylpyrimidotriazinedione with aqueous solubilizing functionality on R₃
Scheme 2-5: Successful route to N8-phenylpyrimidotriazinedione with aqueous solubilizing functionality on R3

As no N1-phenylpyrimidotriazinediones are known in the literature, new methodology had to be developed to access these analogues. The strategy in Scheme 2-1 is not effective because reaction of 6-chloro-3-methyluracil with phenylhydrazine under the same reaction conditions used with methylhydrazine results in nucleophilic attack via the primary nitrogen instead of the secondary. Alternate conditions were explored to see if there was a way to favor nucleophilic attack by the secondary nitrogen of a mono-aryl hydrazine, but none were found. Table 2-5 summarizes the conditions that were tried to
isolate the 6-(α-phenylhydrazinyl)uracil (35), but in no case was the desired product isolated.

<table>
<thead>
<tr>
<th>Run</th>
<th>R</th>
<th>Hydrazine</th>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td><img src="image1" alt="Hydrazine" /></td>
<td>Hunig’s base, pyridine, Δ</td>
<td>undesired regioisomer</td>
</tr>
<tr>
<td>2</td>
<td>H</td>
<td><img src="image2" alt="Hydrazine" /></td>
<td>pyridine, Δ</td>
<td>undesired regioisomer</td>
</tr>
<tr>
<td>3</td>
<td>H</td>
<td><img src="image3" alt="Hydrazine" /></td>
<td>ethanol, Δ</td>
<td>no starting uracil, no product recovered</td>
</tr>
<tr>
<td>4</td>
<td>H</td>
<td><img src="image4" alt="Hydrazine" /></td>
<td>AlCl₃, THF, Δ</td>
<td>no change by TLC</td>
</tr>
<tr>
<td>5</td>
<td>H</td>
<td><img src="image5" alt="Hydrazine" /></td>
<td>KF, DMF, Δ</td>
<td>no change by TLC</td>
</tr>
<tr>
<td>6</td>
<td>H</td>
<td><img src="image6" alt="Hydrazine" /></td>
<td>Pd(OAc)₂, BINAP, KOrBu, THF, Δ</td>
<td>no change by TLC</td>
</tr>
<tr>
<td>7</td>
<td>BOM</td>
<td><img src="image7" alt="Hydrazine" /></td>
<td>n-butyllithium (2 eq), THF, -78°C to RT</td>
<td>no change by TLC</td>
</tr>
<tr>
<td>8</td>
<td>BOM</td>
<td><img src="image8" alt="Hydrazine" /></td>
<td>n-butyllithium, THF, -78°C to RT</td>
<td>no change by TLC</td>
</tr>
<tr>
<td>9</td>
<td>BOM</td>
<td><img src="image9" alt="Hydrazine" /></td>
<td>NaOMe, methanol, Δ</td>
<td>no starting uracil, no product recovered</td>
</tr>
<tr>
<td>10</td>
<td>H</td>
<td><img src="image10" alt="Hydrazine" /></td>
<td>1,2-dichloroethane, Δ</td>
<td>dimer formation + mix of regioisomers</td>
</tr>
<tr>
<td>11</td>
<td>H</td>
<td><img src="image11" alt="Hydrazine" /></td>
<td>AlCl₃, 1,2-dichloroethane, Δ</td>
<td>recovered starting uracil</td>
</tr>
<tr>
<td>Reaction Conditions</td>
<td>Product</td>
<td>Notes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>---------</td>
<td>-------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 H</td>
<td><img src="#" alt="N1-H pyrimidotriazinedione" /></td>
<td>AlCl₃, THF, Δ</td>
<td>no change by TLC</td>
<td></td>
</tr>
<tr>
<td>13 H</td>
<td><img src="#" alt="N1-H pyrimidotriazinedione" /></td>
<td>BF₃·2Et₂O, 1-2-dichloroethane, Δ</td>
<td>recovered starting uracil</td>
<td></td>
</tr>
<tr>
<td>14 H</td>
<td><img src="#" alt="N1-H pyrimidotriazinedione" /></td>
<td>BF₃·2Et₂O, methanol, Δ</td>
<td>recovered starting uracil</td>
<td></td>
</tr>
<tr>
<td>15 H</td>
<td><img src="#" alt="N1-H pyrimidotriazinedione" /></td>
<td>Pd(PPh₃)₄, Cs₂CO₃, THF, Δ</td>
<td>no change by TLC</td>
<td></td>
</tr>
<tr>
<td>16 H</td>
<td><img src="#" alt="N1-H pyrimidotriazinedione" /></td>
<td>Pd(OAc)₂, NaOtBu, toluene, Δ</td>
<td>no change by TLC</td>
<td></td>
</tr>
<tr>
<td>17 H</td>
<td><img src="#" alt="N1-H pyrimidotriazinedione" /></td>
<td>Pd(OAc)₂, BINAP, KOtBu, THF, Δ</td>
<td>no change by TLC</td>
<td></td>
</tr>
<tr>
<td>18 H</td>
<td><img src="#" alt="N1-H pyrimidotriazinedione" /></td>
<td>Pd(OAc)₂, dppf, KOtBu, toluene, Δ</td>
<td>no change by TLC</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2-5: Reaction conditions attempted to obtain 3-methyl-6-(α-phenylhydrazinyl)uracil**

With the failure to isolate the desired regioisomer in the reaction between phenylhydrazine and a 6-chlorouracil, an investigation of some metal-mediated couplings using a pyrimidotriazinedione substrate was initiated. First, Pd-catalyzed coupling chemistry between an N₁-H pyrimidotriazinedione and an aryl bromide, as shown in Scheme 2-6, was attempted. Unfortunately, Pd-catalyzed couplings of heterocycles are not prevalent in the literature, and not surprisingly, no successful results were obtained. The protocols attempted were based on literature precedent for Buchwald-Hartwig aminations, using the following combinations of catalysts/ligands in the presence of potassium t-butoxide: 1) Pd(OAc)₂, dba; 2) Pd(dba)₂; 3) Pd(dba)₂, dppf. In all of these cases, 3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (31) was consumed, but coupling between this substrate and 1-bromo-4-fluorobenzene (32) was never observed. In addition to these attempts to use Pd-catalyzed coupling chemistry, a coupling protocol using CuCl/Cu powder/Cs₂CO₃ was also tried, but the result was no different. Two other metal-mediated coupling methods that were explored are shown in Scheme 2-7, one with FeCl₃ and another using bismuth chemistry. Unfortunately, both of these also failed.
Scheme 2-6: Attempted Pd-catalyzed coupling of a pyrimidotriazinedione and 1-bromo-4-fluorobenzene

Scheme 2-7: Metal-mediated coupling attempts with an N\textsubscript{1}-H pyrimidotriazinedione

Another approach towards N\textsubscript{1}-phenylpyrimidotriazinediones that was explored is shown in Scheme 2-8. Here, the key transformation that needed to be accomplished was amination of the phenylaminouracil (43). While these aminations are known for the
endocyclic nitrogens of a variety of heterocycles,\textsuperscript{55} there are no reports of amination of exocyclic nitrogens by O-(2,4-dinitrophenyl)hydroxylamine. Unfortunately, after synthesizing O-(2,4-dinitrophenyl)hydroxylamine,\textsuperscript{56} application of it toward the amination of uracil \textsuperscript{43} could not be accomplished. A variety of bases were tried, including NaH, LiH, \textit{n}-butyllithium, and sodium hexamethyldisilazide, but amination of \textsuperscript{43} could never be detected. The amination was also attempted with 6-amino-1,3-dimethyluracil as a model substrate, but here, again, no aminated product was isolated.

![Scheme 2-8: Proposed route to N\textsubscript{1}-phenylpyrimidotriazinediones via amination of phenylaminouracil](image)

One other unsuccessful route towards N\textsubscript{1}-phenylpyrimidotriazinediones that was attempted is shown in Scheme 2-9. Even if successful, this route would be limited to a small subset of substituted-phenyl substituents at N\textsubscript{1}, but the question being asked was whether reaction at N\textsubscript{1}-H or N\textsubscript{8}-H in pyrimidotriazinedione \textsuperscript{36} could be achieved in a nucleophilic aromatic substitution reaction. Moreover, if reactive, would arylation occur at the N\textsubscript{1} or N\textsubscript{8} position? While this route was not extensively explored, a brief investigation of different solvents (DMF, THF, acetonitrile, and acetone) and bases
(Et₃N, Cs₂CO₃) found no reaction to occur between the pyrimidotriazinedione 36 and 1-chloro-2,4-dinitrobenzene (48) to occur.

Scheme 2-9: Attempted nucleophilic aromatic substitution by pyrimidotriazinediones

Despite all of these failed attempts, a successful synthesis of N₁-phenylpyrimidotriazinediones was finally achieved for the first time. Moreover, the methodology that has been developed permits access to these new analogues in an expedient fashion, which is illustrated in Scheme 2-10. First, phenylhydrazine (52), either as a free base or hydrochloride salt, was condensed under reflux with p-anisaldehyde (51) in ethanol or THF to form hydrazone 53, which precipitated out of solution upon cooling. The resulting hydrazone (53) was then treated with AlCl₃ and 6-chloro-3-methyl-5-nitouracil (50), which had been formed by nitration of commercially-available 6-chloro-3-methyluracil according to the published method. The resultant 6-(2-arylidene)-1-phenyl)hydrazinyl-3-methyl-5-nitouracil (54) was then cyclized to 55 upon treatment with zinc dust (4 eq) and ammonium chloride (2 eq) while exposed to the air and with vigorous stirring in refluxing 50% aq EtOH. The N₁-substituted pyrimidotriazinedione was then isolated cleanly, usually in >60% yield, by washing with 1N aq HCl. Other solvents and reducing agents were tried for the zinc-mediated cyclization, but the yields were considerably diminished. Moreover, additional standard methods were evaluated to reduce the nitro group and promote cyclization. These included standard hydrogenation over Pd/C, transfer hydrogenation (Pd/C, ammonium
formate), Sn in EtOAc, SnCl₂·2H₂O in EtOH, Fe in glacial acetic acid, and Zn/HCl in EtOH, but none provided clean product.

Scheme 2-10: Synthesis of N₁-phenylpyrimidotriazinediones

In optimizing the reaction sequence, it was found that hydrazone 53 did not need to be isolated in a separate step and that yields of 54 were often improved by forming the hydrazone in situ. Hence, this became the preferred method, and was particularly beneficial when the formed hydrazone did not precipitate out of ethanol. In this improved protocol, 6-chloro-3-methyl-5-nitouracil (50) and AlCl₃ were added after 1-2 hours of reaction time between phenyl hydrazine 52 and p-anisaldehyde 51. The AlCl₃-catalyzed reaction between hydrazone 53 and uracil 50 was first observed to occur in acetonitrile, but a subsequent investigation of different solvents showed that best yields were attained with THF. Solvents in which the reaction was unsuccessful included 1,2-dichloroethane and methylene chloride.

The method was amenable to a variety of substituents on the phenyl ring for either the hydrazine or the aldehyde. Table 2-6 summarizes the substrates successfully utilized in the reaction and the resultant yields for both steps. Both electron-donating and electron-withdrawing substituents were tolerated on either component, and there was
some variation in overall yield depending on the combination of moieties examined. Also, the 2-pyridyl group was found to be incompatible with the cyclization, whether it was at the R₃ or R₁ position. In the condensation between 6-chloro-3-methyl-5-nitouracil and hydrazone, bromo- and chloro- substituents produced lower yields, perhaps due to interfering complexation of the halogen with AlCl₃.

![Chemical Reaction Diagram]

<table>
<thead>
<tr>
<th>Entry</th>
<th>R₃</th>
<th>R₁</th>
<th>Addition to uracil</th>
<th>Ring closure</th>
<th>Overall yield</th>
</tr>
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<tr>
<td>a</td>
<td>H₃CO-</td>
<td></td>
<td>65% a, b</td>
<td>78%</td>
<td>51%</td>
</tr>
<tr>
<td>b</td>
<td>H₂O-</td>
<td></td>
<td>30% a, 72% b</td>
<td>58%</td>
<td>17%, 41%</td>
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<tr>
<td>c</td>
<td>Cl-</td>
<td></td>
<td>53% a</td>
<td>72%</td>
<td>38%</td>
</tr>
<tr>
<td>d</td>
<td>H₃CO-</td>
<td>F</td>
<td>71% b</td>
<td>62%</td>
<td>44%</td>
</tr>
<tr>
<td>e</td>
<td>H₂O-</td>
<td>F</td>
<td>82% b</td>
<td>82%</td>
<td>67%</td>
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<tr>
<td>f</td>
<td>O</td>
<td>F</td>
<td>38% b</td>
<td>61%</td>
<td>23%</td>
</tr>
<tr>
<td>g</td>
<td>H₃C-</td>
<td>F</td>
<td>73% b</td>
<td>81%</td>
<td>59%</td>
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<td>h</td>
<td>H₃CO-</td>
<td>Br</td>
<td>31% b</td>
<td>76%</td>
<td>24%</td>
</tr>
<tr>
<td>i</td>
<td>F</td>
<td>H₃CO-</td>
<td>73% b</td>
<td>35%</td>
<td>26%</td>
</tr>
</tbody>
</table>

a = hydrazone isolated in separate step;  
b = hydrazone formation in situ and subsequent reaction with uracil and AlCl₃

Table 2-6: Substrates and yields for N₁-aryl substituted pyrimidotriazinediones synthesized via new methodology
In addition to phenylhydrazines, the methodology was investigated for a variety of alkyl hydrazines, and here the hydrazones (53) formed in situ were found to be sufficiently reactive with chlorouracil 50 such that activation with AlCl₃ was unnecessary. Nonetheless, in no case would the hydrazone react with the chlorouracil, with or without AlCl₃, before it had been nitrated. Table 2-7 summarizes reactions for successful alkyl hydrazines. In addition to those shown, the hydrazones of methylhydrazine also reacted quickly and efficiently with 50; however, in the subsequent cyclization with zinc and ammonium chloride, demethylation at N₁ also occurred to a great extent. Varying the reaction time and temperature could minimize this, but it was never completely suppressed. Hence, when R₁ = methyl, the preferred method of synthesis would be one of the standard methods commonly used to make N₁-methylpyrimidotriazinediones,₃₈,₃₉,₄₅ but for most other R₁ substituents, this new methodology is advantageous with regard to both synthesis time and efficiency. Moreover, for these alkyl R₁ substituents where AlCl₃ is not required, the two-step sequence of condensation between the hydrazone and chloronitrouracil and subsequent cyclization can be carried out in one pot with yields comparable to or better than the two-step sequence.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R₃</th>
<th>R₁</th>
<th>Addition to uracil</th>
<th>Ring closure</th>
<th>Overall yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>j</td>
<td>H₃CO-</td>
<td>CH₂CH₂OH</td>
<td>67%</td>
<td>51%</td>
<td>34%</td>
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<td>CH₂CH₂OH</td>
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<td>62%</td>
<td>42%</td>
</tr>
<tr>
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<td>Cl-</td>
<td>CH₂CH₂OH</td>
<td>60%</td>
<td>67%</td>
<td>40%</td>
</tr>
<tr>
<td>m</td>
<td>H₂C-</td>
<td>CH₂CH₂OH</td>
<td>56%</td>
<td>70%</td>
<td>39%</td>
</tr>
<tr>
<td>n*</td>
<td>[\text{H}_3\text{CO} \quad \text{H}_3\text{CO} \quad \text{CH}_2\text{CH}_2\text{OH} ]</td>
<td>[\text{CH}_2\text{CH}_2\text{OH} ]</td>
<td>--</td>
<td>--</td>
<td>39%</td>
</tr>
<tr>
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<td>---</td>
<td>---</td>
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<td>---</td>
<td></td>
</tr>
<tr>
<td>o</td>
<td>[\text{H}_3\text{CO} \quad \text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)_2 ]</td>
<td>[\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)_2 ]</td>
<td>52%</td>
<td>62%</td>
<td>32%</td>
</tr>
<tr>
<td>p</td>
<td>[\text{H}_3\text{CO} \quad \text{CH}(\text{CH}_3)_2 ]</td>
<td>[\text{CH}(\text{CH}_3)_2 ]</td>
<td>80%</td>
<td>51%</td>
<td>41%</td>
</tr>
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</table>

Table 2-7: Substrates and yields for N1-alkyl substituted pyrimidotriazinediones synthesized via new methodology

* synthesized in “one-pot” sequence by adding Zn and NH4Cl to reaction after formation of 58 without isolation of 58

There are some shortcomings to this methodology, however. The reaction between the hydrazones and 6-chloro-3-methyl-5-nitouracil was the more general of the two steps. Examples where the desired Michael-type addition did not occur included hydrazones of t-butylhydrazine (no reaction) and NH2NHCH2CH2NEt2, where adduct 58 appeared to fragment by loss of the CH2CH2NEt2 moiety. For the cyclization, the reaction conditions appeared robust for phenyl, substituted phenyl, and some alkyl substituents at the 6α-position of the uracil. Nevertheless, problems were encountered for both benzyl substituents and methyl substituents (as described above) at this same position. While N1-methylpyrimidotriazinediones could be isolated to some extent despite the demethylation, no desired N1-benzylpyrimidotriazinediones were formed, as debenzylation appeared to compete with the cyclization.

The demethylation that occurred when methylhydrazine was used as a reactant could be used to advantage, however, when the goal was an N8-H pyrimidotriazinedione. While hydrazine itself could be used in the initial condensation of a hydrazone with 6-chloro-3-methyl-5-nitouracil, N8-H pyrimidotriazinediones were never cleanly isolated from the attempted cyclizations with zinc and ammonium chloride. Hence, methylhydrazine could be used as a surrogate to attain the same objective. Table 2-8 lists the substrates that were successfully used to obtain pyrimidotriazinediones without substituents at N1 or N8. Here, slightly lower yields were obtained in the ring closure/demethylation when there was an electron-donating substituent at the para-position of the R3 subsubstituent. Moreover, the highest yields were obtained with a strongly-electron-withdrawing fluoro substituent at the para-position of R3. Both of
these observations are consistent with the ease with which demethylation of N1-methylpyrimidotriazinediones was observed to occur in attempts to prevent it and with the most likely mechanism for demethylation, whereby nucleophilic solvent molecules attack the methyl group and the core pyrimidotriazinedione heterocycle serves as a leaving group.

The demethylated pyrimidotriazinediones could then be alkylated at N8 as shown in Scheme 2-1 to produce a variety of novel pyrimidotriazinedione analogues such as those in Table 2-2. Hence, the new methodology serves as an alternate entry point to the N8-substituted pyrimidotriazinedione series also.
Overall, using both published methods and novel methodology developed in pursuit of N1-phenylpyrimidotriazinediones, more than 80 pyrimidotriazinediones were synthesized. Only 10 of these are previously characterized compounds. Since antibacterial and anti-tumor activity have been shown for this class, the hope is that many of the new compounds described herein may possess useful biological activity, particularly in targeting the interaction between β-catenin and Tcf4 in the Wnt signaling pathway.
Chapter 3
Synthesis of Pyrimidopyridazinedione Analogues

With the screen hit, PKF118-310, embedded with a pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione core, one objective of this work was to determine if all of the nitrogen atoms of the pyrimidotriazinedione core were necessary to maintain activity against the Wnt signaling pathway. If all of the nitrogen atoms of the pyrimidotriazinedione core were not necessary for inhibition of the βcat/Tcf4 complex, this would expand the number of possibilities for improving activity and developing a lead structure. In exploring the possibilities that could exist with this class, pyrimidopyridazinediones both with and without an aryl substituent at either the 3- or 4-position were considered as potential targets. Nonetheless, as the screen hit, PKF118-310, lacked any substituent at the 3- or 4-position, the first pyrimidopyridazinediones synthesized were those without a substituent at either the 3- or the 4-position.

The first pyrimidopyridazinedione synthesized was 1,6-dimethylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (63), as this pyrimidopyridazinedione is the direct analogue of PKF118-310 with methyl groups at both the 1- and 6-positions. This compound has been reported previously and was synthesized according to the literature precedent, which is depicted in Scheme 3-1a. An additional 3,4-unsubstituted analogue was also synthesized with a benzyl group at the 1-position, using similar methodology, as shown in Scheme 3-1b. In both cases, 6-chloro-3-methyluracil (1) was again used as the starting material and treated with either methylhydrazine or benzylhydrazine hydrochloride to give either 2a or 2c, respectively. Each was then cyclized with glyoxal to the desired pyrimidotriazinedione (63 and 64). It should be noted that the conditions for reaction of 6-chloro-3-methyluracil with benzylhydrazine hydrochloride had to be altered from those used for reaction with methylhydrazine because similar conditions (EtOH with either NaOH, Et3N, or Hunig’s base) produced an adduct with the opposite regiochemistry from what was desired. The physical
characterization data for each of these pyrimidopyridazinediones matches that previously reported.

\[
\begin{align*}
\text{Scheme 3-1: Synthesis of 3,4-unsubstituted pyrimidopyridazinediones} \\
\text{Two novel 3,4-unsubstituted pyrimidopyridazinediones towards which synthetic efforts were made are shown in Figure 3-1. The attempted synthetic route to 1-(2-hydroxyethyl)-6-methylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (65) is shown in Scheme 3-2 and involved protection and deprotection of the alcohol since direct cyclization in the presence of the free hydroxyl group yielded a bright orange solid that could not be identified but was not the desired product. This revised synthesis was completed with the exception of the final deprotection step, which was only tried once with TBAF in THF. Unfortunately, these deprotection conditions proved too harsh, resulting in a significant amount of baseline decomposition as observed by TLC and no pure product. If this series was to be pursued further, future synthetic attempts would have examined milder deprotection conditions, but unpromising results in the assay work to be described caused attention to be focused elsewhere.}
\end{align*}
\]

Figure 3-1: Novel 3,4-unsubstituted pyrimidopyridazinediones towards which synthetic efforts were made
Scheme 3-2: Synthesis of 1-(2-hydroxyethyl)-6-methylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione

Much less progress was made in attempted syntheses of pyrimidopyridazinedione 66, however, for which the intention was to use a route similar to that for the other 3,4-unsubstituted pyrimidopyridazinediones. The difficulty here appeared to be due to a presumptive reaction of the desired intermediate 69, as shown in Scheme 3-3. In attempting the synthesis of 66, 2-(diethylamino)ethyl-hydrazine was first prepared from hydrazine and 2-(diethylamino)ethyl chloride hydrochloride.59 This was then treated with 6-chloro-3-methyluracil, but the precipitate collected was not the desired product and was found to be identical to that from the reaction between 6-chloro-3-methyluracil and hydrazine. A mechanism rationalizing formation of this undesired product (70) is shown in Scheme 3-3, and a variety of conditions were attempted to avert this. Conditions tried included changing the solvent to DMF, acetonitrile, pyridine, or 2,2,2-trifluoroethanol, as well as the addition of Lewis acids such as Ti(Oi-Pr)₄ and ZnCl₂. The Bronstead acid, p-toluenesulfonic acid, was also added at both room temperature and elevated temperature. Finally, the reaction was tried neat, at different temperatures, and with the addition of malononitrile, which has been reported to catalyze couplings with conjugated vinyl chlorides.60 Unfortunately, none of these methods was successful in isolating the desired product (69), as each invariably recovered only unreacted 6-chloro-3-methyl uracil or the undesired 6-hydrazino-3-methyl uracil (70).
Scheme 3-3: Undesired reaction pathway in reaction of 2-­
(diethylamino)ethylhydrazine with 6-chloro-3-methyluracil

In addition to the 3,4-unsubstituted pyrimidopyridazinediones, pyrimidopyridazinediones with a substituent at either the 3-position, the 4- position, or both were also synthesized. Two pyrimidopyridazinediones with symmetrical substitution at both the 3- and 4- positions were synthesized as previously reported, one with methyl substituents (72) and one with phenyl substituents (74). The syntheses of these are shown in Schemes 3-4a and 3-4b, and each was carried out in a manner analogous to the 3,4-unsubstituted pyrimidopyridazinediones. After obtaining 6α-­methylhydrazinyluracil (2a) from treatment of 6-chloro-3-methyluracil with methylhydrazine, it was then cyclized either with 2,3-butanedione or benzil to obtain 72 or 74, respectively.

Scheme 3-4: Synthesis of 3,4-disubstituted pyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione

Finally, efforts were directed towards the synthesis of pyrimidopyridazinediones with a single aryl substituent at either the 3- or the 4- position. While there are two
literature reports of 3-substituted pyrimidopyridazinediones, the 4-substituted pyrimidopyridazinediones remain unreported. Since the 3-substituted pyrimidopyridazinediones are most analogous to the series of pyrimidotriazinediones that was synthesized, the first synthetic efforts towards mono-aryl substituted pyrimidotriazinediones focused on this subclass, with the route pursued being modeled on one of the literature reports. The route pursued is shown in Scheme 3-5, and while the literature report involved treatment of 6α-methylhydrazinyluracil with bromoacetophenones to form the desired pyrimidopyridazinediones, the chloroacetophenone 75a was used here due to its availability. Since the initial cyclized adduct between 2a and 75a is the dihydro-congener of the desired pyrimidopyridazinedione, DIAD treatment was added to effect complete oxidation to the desired pyrimidopyridazinedione. The regiochemistry of the pyrimidopyridazinedione product, initially assigned as 76, was presumed to be the same as that reported in the literature from the reaction of 2a with bromoacetophenones, but further studies had to be done in order to substantiate this assignment. In fact, as will be shown, these subsequent studies now provide greater evidence for the actual pyrimidopyridazinedione product in Scheme 3-5 being that of the 4-(4-fluorophenyl)-substituted regioisomer, 77.

Scheme 3-5: Synthesis of pyrimido[4,5-c]pyridazine-5,7(1H,6H)-diones via treatment of hydrazinyluracil (2a) with 2-chloro-4'-fluoroacetophenone (75)

The subsequent studies that were done to verify the regioisomer formed in the cyclization of 2a with 75a can be divided into three broad categories: 1) attempted
isolation of an uncyclized intermediate, 2) treatment of \(2a\) with other chloro- and bromoacetophenones to compare product composition, and 3) synthesis via alternate routes where the regiochemistry could be assigned via comparison of NMR spectra from these vs. earlier routes. Since it would provide the most direct method of comparison, the first attempts at verifying regiochemistry focused on isolating an intermediate adduct where \(2a\) had reacted with \(75a\) but not yet cyclized to the pyrimidopyridazinedione. Unfortunately, all of these proved unsuccessful, as lowering the temperature to RT resulted in isolation of the two starting materials. Moreover, any temperature between RT and reflux that was tried yielded either starting materials or cyclized product, never the desired intermediate.

Another possibility for verifying the regiochemistry of \(76\) or \(77\) came from the same paper\textsuperscript{63} that published the method of Scheme 3-5, where pyrimidopyridazinediones had also been synthesized via an alternate route, which is shown in Scheme 3-6. Here, the regiochemistry of the pyrimidopyridazinedione formed is unambiguous; therefore, this was the method by which the authors had verified the regiochemistry of their products with the bromoacetophenones through comparison of the two reaction products. Unfortunately, in this work the reaction of Scheme 3-6 failed to produce clean products for unambiguous verification of the regiochemistry of \(76\) or \(77\). In each attempt, starting material was either isolated, or a multitude of spots appeared via TLC. Moreover, in all attempts to isolate and purify the numerous products formed, the desired product was never cleanly separated from other contaminants, such that the NMR spectrum could not provide unambiguous verification. The reaction was tried neat and under a variety of solvent conditions, including N,N-dimethylacetamide, ethanol, and N,N-dimethylformamide, and with 1-10 equivalents of triethyl orthoformate. The reaction was also attempted under nitrogen, open to air, and in a sealed tube, as well as varying the reaction time from 2 h to 5 d. It was attempted at a variety of temperatures, and with a variety of substrates, including ones reported in the paper and others that were not, but in no case did clean product crystallize out of ethanol as reported.
Scheme 3-6: Synthesis of pyrimidopyridazinediones via condensation of hydrazone intermediates with triethyl orthoformate

Next, attention turned towards the reaction between 2a and other chloro- and bromo- acetophenones. Assuming they formed pyrimidopyridazinediones as above with 2-chloro-4'-fluoroacetophenone (75a), the products from these reactions were desired in order to increase the number of analogues available for exploring structure-activity relationships against the Wnt signaling pathway, regardless of whether they were able to provide any corroborating evidence for which regioisomer was forming. Nonetheless, the rate of reaction would be expected to vary with the nature of the halogenated acetophenone substrate, making isolation of an intermediate more or less plausible, and making this another viable alternative for determining regiochemistry in the initial reaction of Scheme 3-5. The isolation of an uncyclized intermediate proved fruitless in these endeavors, but elimination of the DIAD oxidation step allowed for isolation of a mixture of both the oxidized (76 or 77) and the dihydro- pyrimidopyridazinedione products (79 or 80) or pure dihydro- pyrimidopyridazinedione product. Table 3-1 summarizes those reactions attempted between 2a and a variety of chloro- and bromo-acetophenones and the product distributions in the absence of DIAD oxidation. Where the chloro- or bromo- acetophenone was not commercially available or prohibitively expensive, the bromoacetophenone was synthesized from the precursor acetophenone by treatment with N-bromosuccinimide and p-toluenesulfonic acid.64 (It should be noted that attempted syntheses of chloroacetophenones by reaction with N-chlorosuccinimide instead of N-bromosuccinimide resulted in mixtures of both mono- and dichloro-acetophenones.) The structure of the cyclized, dihydro congener was characterized by $^1$H NMR (d-DMSO and d-TFA), $^{13}$C NMR, $^{13}$C DEPT NMR, and mass spectrometry for the reaction of 2a with 75e, but these were unable to conclusively prove whether the isolated product was 79 or 80. The most distinguishing feature between 79 and 80 is most likely...
the NH peak, which was found in the $^1$H NMR spectrum in $d$-DMSO to have $\delta = 5.1$ ppm. This is more suggestive of $80e$ than $79e$, as the $\text{N}_1$-H peak in other uracil intermediates and $\text{N}_8$-H analogues in the pyrimidotriazinedione series had $\delta = 10$-12 ppm. Nonetheless, this was not conclusive, necessitating further investigation. It should be noted, however, that in the published work, the spectral analysis included only $^1$H NMR ($d$-TFA only), mass spectrometry, and combustion analysis, none of which would be capable of distinguishing $79$ from $80$.

Scheme 3-7: Formation of pyrimidopyridazinedione (76) or (77) and dihydropyrimidopyridazinedione (79) or (80) via treatment of hydrazinyluracil (2a) with bromo- and chloro-acetophenones in absence of DIAD

<table>
<thead>
<tr>
<th>Entry</th>
<th>Acetophenone</th>
<th>Reaction Yield</th>
<th>Ratio of 76/77 to 79/80</th>
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<td><img src="image1" alt="Acetophenone" /></td>
<td>37%</td>
<td>2:1</td>
</tr>
<tr>
<td>c</td>
<td><img src="image2" alt="Acetophenone" /></td>
<td>41%</td>
<td>1:5</td>
</tr>
<tr>
<td>d</td>
<td><img src="image3" alt="Acetophenone" /></td>
<td>47%</td>
<td>only 79 or 80 isolated</td>
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</tbody>
</table>
Evidence for the regiochemistry of 76 or 77, therefore, came from the exploration of a novel route to the pyrimidopyridazinediones that was done in parallel with the work described above. As glyoxal had been used to cleanly cyclize to the 3,4-unsubstituted pyrimidopyridazinediones, the question arose as to what type of products would be formed from the treatment of hydrazinyluracil 2a with unsymmetrical phenylglyoxals. Hence, hydrazinyluracil 2a was treated with commercially available phenylglyoxal monohydrate as shown in Scheme 3-8. In the initial attempt, the cyclized pyrimidopyridazinedione was not isolated, but rather an uncyclized intermediate (82), whose structure is supported by proton NMR spectra, mass spectrometry analysis, and a negative Tollens’ test for aldehydes. Resubjecting 82 to the reaction conditions and refluxing for an extended period of time (48-72 h) eventually yielded a small amount of the desired pyrimidopyridazinedione, as shown in Scheme 3-9, which could be fractionally recrystallized in ethanol from remaining 82. Because of the isolation of intermediate 82, the regiochemistry of the pyrimidopyridazinedione (83) formed was unambiguous, and δ(H3) = 8.73 ppm in d-DMSO.

<table>
<thead>
<tr>
<th></th>
<th>![Image](213x644 to 227x682)</th>
<th>45%</th>
<th>only 79 or 80 isolated</th>
</tr>
</thead>
</table>

Table 3-1: Reactions between hydrazinyluracil (2a) and chloro- or bromoacetophenones (75b-e)
*synthesized via bromination of acetophenone precursor

Scheme 3-8: Formation of uncyclized adduct between hydrazinyluracil (2a) and phenylglyoxal monohydrate
Scheme 3-9: Formation of 1,6-dimethyl-4-phenylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (83) from unicycled hydrazone intermediate (82)

Upon isolation of 83, a variety of cyclization conditions were tested in order to find conditions that would drive the reaction to completion and be amenable to isolation of pure pyrimidopyridazinedione product without the need for fractional recrystallization. Table 3-2 summarizes the reaction conditions that were investigated and the results that were found. As can be readily seen, there were a number of reaction conditions (entries d, e, f) that yielded solely the 4-phenylpyrimidopyridazinedione (83). Interestingly, however, both 83 and 84 could be formed when NaOAc was added (entry b), and under these conditions, the cyclization occurred most rapidly. While there were no reaction conditions explored that yielded solely 84, the presence of both regioisomers as products when NaOAc was present allowed for determination of $\delta(H_4)$ in the 3-phenylpyrimidopyridazinedione, for which $\delta(H_4) = 8.35$ ppm in $d$-DMSO. Hence, $\delta(H_3)$ is greater (8.73 ppm) in 83 compared to 84, as would be expected based on proximity to the nitrogen atom at the 2-position. (For reference, in 82, $\delta(H_a) = 7.82$ ppm.) Thus, the ability to access an unicycled intermediate and both regioisomers of the pyrimidopyridazinedione via reaction of phenylglyoxals with hydrazinyluracil 2a provided a means for determining the regiochemistry of the product in Scheme 3-5 unambiguously through substitution of 4-fluorophenylglyoxal monohydrate for phenylglyoxal monohydrate.
In order to pursue this course of action, 4-fluorophenylglyoxal monohydrate had to be synthesized. This was readily accomplished using a standard procedure for accessing phenylglyoxals from acetophenones, as shown in Scheme 3-10. As with the commercially available phenylglyoxal monohydrate, the product was isolated almost entirely in the monohydrate form. Hydrazinyluracil 2a was then treated with 4-fluorophenylglyoxal monohydrate (86) under the following two sets of reaction conditions: 1) 1,2-dichloroethane, Δ, 24h and 2) H₂O, NaOAc (2 eq), Δ, 1h. As with phenylglyoxal monohydrate, the former reaction conditions yielded the 4-arylpyrimidopyridazinedione regioisomer, while the latter gave both regioisomers. For, the 4-(4-fluorophenyl)pyrimidopyridazinedione, δ(H₃) = 8.74 ppm, while for the 3-(4-fluorophenyl)pyrimidopyridazinedione δ(H₄) = 8.36 ppm, both in d-DMSO. For the product of the reaction in Scheme 16 between hydrazinyluracil 2a and 2-chloro-4′-fluoroacetophenone, δ(H₃) = 8.75 ppm in d-DMSO, confirming that the product formed was the 4-substituted regioisomer 77.
Scheme 3-10: Synthesis of 4-fluorophenylglyoxal monohydrate

<table>
<thead>
<tr>
<th>Entry</th>
<th>Acetophenone</th>
<th>Phenylglyoxal Monohydrate</th>
<th>Reaction Yield</th>
<th>Purification Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O(\text{CH}_3)F</td>
<td>O(\text{CH}_3)F</td>
<td>50%</td>
<td>Chromatography (hexanes/EtOAc)</td>
</tr>
<tr>
<td>2</td>
<td>O(\text{Cl})</td>
<td>O(\text{Cl})</td>
<td>not determined</td>
<td>Did not purify; used crude product in cyclization</td>
</tr>
<tr>
<td>3</td>
<td>O(\text{CF}_3)CH(_3)</td>
<td>O(\text{CF}_3)OH</td>
<td>73%</td>
<td>Chromatography (hexanes/EtOAc)</td>
</tr>
<tr>
<td>4</td>
<td>O(\text{H}_2\text{O})</td>
<td>O(\text{H}_2\text{O})</td>
<td>39%</td>
<td>Recrystallization (H(_2)O)</td>
</tr>
<tr>
<td>5</td>
<td>O(\text{O}_2\text{N})Cl</td>
<td>O(\text{O}_2\text{N})OH</td>
<td>59%</td>
<td>Chromatography (hexanes/EtOAc)</td>
</tr>
<tr>
<td>6</td>
<td>O(\text{F})CH(_3)</td>
<td>O(\text{F})OH</td>
<td>52%</td>
<td>Chromatography (hexanes/EtOAc)</td>
</tr>
</tbody>
</table>

Table 3-3: Phenylglyoxal monohydrates synthesized via oxidation with SeO\(_2\)

Having established the regiochemistry of 77 and with an efficient route to 4-substituted pyrimidopyridazinediones, additional phenylglyoxal monohydrates were synthesized to further explore the new methodology. Table 3-3 lists all of the phenylglyoxal monohydrates that were synthesized, along with their reaction yields.
Each of these was then treated with hydrazinyluracil 2a under the two same reaction conditions used for 4-fluorophenyl glyoxal monohydrate. Table 3-4 summarizes all of the cyclization reactions that were carried out. In each case, the product(s) were collected by filtration after precipitation from the reaction mixture, and the yield is based on the collected precipitate. In every example shown, conducting the reaction in 1,2-dichloroethane yielded solely the 4-substituted pyrimidopyridazinedione, while conducting the reaction in water with NaOAc (2 eq) yielded a mixture of both regioisomers. In many cases where both regioisomers formed, they were isolated in approximately equal amounts (entries 4b, 5b, 6b), but interestingly, if one regioisomer was favored in the reaction in water with NaOAc, it was always the 3-substituted regioisomer (89) that formed in excess (entries 1b, 2b, 3b, 7b), in contrast to the favored regioisomer with 1,2-dichloroethane as solvent.

The 3-substituted regioisomer would be formed if one of two possibilities occurred in the presence of NaOAc: 1) the hydrazine reacted first with the ketone instead of the aldehyde of the phenylglyoxal, or 2) the hydrazinyluracil 2a reacted with the aldehyde of the phenylglyoxal via nucleophilic attack by C5 instead of the 6β-nitrogen. Due to the much more rapid cyclization in water with 2 equiv of NaOAc, an intermediate, uncyclized product could not be isolated under these conditions to prove which of these possibilities is correct. Nonetheless, since the presence of NaOAc would be more likely to affect the hydrazinyluracil 2a than the phenylglyoxal via its ability to enhance the nucleophilicity of 2a, the latter is the more likely explanation, particularly since 6-hydrazinyluracils like 2a are known to demonstrate nucleophilicity at C5 also.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>Conditions</th>
<th>Reaction Yield (Product Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td><img src="image1.png" alt="Image" /></td>
<td>1,2-dichloroethane, Δ, 24h</td>
<td>56% (88 only)</td>
</tr>
<tr>
<td></td>
<td>Structure</td>
<td>Conditions</td>
<td>Yield (%)</td>
</tr>
<tr>
<td>---</td>
<td>-----------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>1b</td>
<td><img src="image" alt="Structure" /></td>
<td>H₂O, NaOAc (2 eq), Δ, 1h</td>
<td>55%</td>
</tr>
<tr>
<td>2a</td>
<td><img src="image" alt="Structure" /></td>
<td>1,2-dichloroethane, Δ, 24h</td>
<td>*22% (88 only)</td>
</tr>
<tr>
<td>2b</td>
<td><img src="image" alt="Structure" /></td>
<td>H₂O, NaOAc (2 eq), Δ, 1h</td>
<td>62%</td>
</tr>
<tr>
<td>3a</td>
<td><img src="image" alt="Structure" /></td>
<td>1,2-dichloroethane, Δ, 24h</td>
<td>85%</td>
</tr>
<tr>
<td>3b</td>
<td><img src="image" alt="Structure" /></td>
<td>H₂O, NaOAc (2 eq), Δ, 1h</td>
<td>43%</td>
</tr>
<tr>
<td>4a</td>
<td><img src="image" alt="Structure" /></td>
<td>1,2-dichloroethane, Δ, 24h</td>
<td>75%</td>
</tr>
<tr>
<td>4b</td>
<td><img src="image" alt="Structure" /></td>
<td>H₂O, NaOAc (2 eq), Δ, 1h</td>
<td>54%</td>
</tr>
<tr>
<td>5a</td>
<td><img src="image" alt="Structure" /></td>
<td>1,2-dichloroethane, Δ, 24h</td>
<td>42%</td>
</tr>
<tr>
<td>5b</td>
<td><img src="image" alt="Structure" /></td>
<td>H₂O, NaOAc (2 eq), Δ, 1h</td>
<td>51%</td>
</tr>
<tr>
<td>6a</td>
<td><img src="image" alt="Structure" /></td>
<td>1,2-dichloroethane, Δ, 24h</td>
<td>49%</td>
</tr>
<tr>
<td>6b</td>
<td><img src="image" alt="Structure" /></td>
<td>H₂O, NaOAc (2 eq), Δ, 1h</td>
<td>65%</td>
</tr>
<tr>
<td>7a</td>
<td><img src="image" alt="Structure" /></td>
<td>1,2-dichloroethane, Δ, 24h</td>
<td>82%</td>
</tr>
<tr>
<td>7b</td>
<td><img src="image" alt="Structure" /></td>
<td>H₂O, NaOAc (2 eq), Δ, 1h</td>
<td>62%</td>
</tr>
</tbody>
</table>

**Table 3-4: Phenylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-diones synthesized via condensation of hydrazinyluracil (2a) with phenylglyoxal monohydrates**

*crude phenylglyoxal monohydrate used in cyclization reaction*

While more work would need to be done to fully explain the difference in regioselectivity under the two sets of reaction conditions, one hypothesis as to why the uncyclized intermediate fails to cyclize efficiently under some reaction conditions can be postulated from the current data. To do so requires consideration of both the *cis* and
trans forms of the uncyclized hydrazone intermediate, which are depicted in Figure 3-2. While both isomers could be expected to form with the same ease, the energy-minimized three-dimensional renderings shown below each structure in Figure 3-2 demonstrate that the two isomers would be expected to cyclize at different rates. Based on these models, the cis-isomer would be expected to cyclize more efficiently due to the proximity of the ketone and C5 of the uracil, while the trans-isomer would likely be resistant to cyclization in its more extended geometry. Therefore, those reaction conditions that either favor the formation of the cis form over the trans form or that provide a means for the trans form to be converted to the cis form are likely to produce the desired pyrimidopyridazinedione product. Meanwhile, those where the trans form predominates would lead to isolation of the noncyclized hydrazone.

Examination of Table 3-4 reveals that this hypothesis is consistent with the results contained therewithin. For instance, while refluxing in water for 24 h (entry a) yielded only uncyclized hydrazone, addition of p-toluenesulfonic acid (entry f), which would provide a means for isomerizing the C=N bond, yielded cyclized pyrimidopyridazinedione. Moreover, conditions that could favor the cis form either by chelation of the ketone and C4 carbonyl of the uracil with a Lewis acid (entries b and e) or the absence of hydrogen bonding (entries d and e) also lead to the pyrimidopyridazinedione. (Hydrogen-bonding capability might bias the trans form over the cis form due to its inability to form any intramolecular hydrogen bonds and its extended conformation being more accessible for hydrogen-bonding with the solvent. The cis form, in contrast, could form an intramolecular hydrogen-bond between the ketone and the N1-H where there is no opportunity for hydrogen-bonding with the solvent.
In summary, investigation of the pyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione class yielded both expected and unexpected results. For 3,4-unsubstituted pyrimidopyridazinediones, four previously-reported compounds were synthesized according to the published procedures. Inconsistencies with a published procedure for 3-substituted pyrimidopyridazinediones encouraged exploration of new methodology for constructing the pyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione core. In this novel methodology, 6-hydrazinyluracils were treated with phenylglyoxal monohydrates to form 4-phenylpyrimidopyridazinediones, a class of compounds previously not reported in the literature. Seven novel 4-phenylpyrimidopyridazinediones were synthesized in demonstrating the new method.
Chapter 4
Synthesis of Pyrazolopyrimidinedione Analogues

Since both the pyrimidotriazinedione and the pyrimidopyridazinedione analogues are susceptible to flavin-like redox chemistry and since this is often an undesirable characteristic in a potential drug candidate, a series of pyrazolopyrimidinediones was synthesized. In this 5,6-fused ring class of heterocycles, the ability to undergo redox chemistry has been eliminated, and all but one of the nitrogens in the screen hit are still present. There are a number of published methods for the synthesis of pyrazolopyrimidinediones, either cyclization from an initial pyrazole core or from an initial pyrimidine core. As both 6-chloro-3-methyluracil (1) and 3-methyl-6-(1-methylhydrazinyl)pyrimidine-2,4(1H,3H)-dione (2a) were readily available from synthesis within the pyrimidotriazinedione series, the synthetic routes considered for the pyrazolopyrimidinedione analogues were those starting with a pyrimidine core intermediate. The three routes explored are shown in Schemes 4-1, 4-2, and 4-3. The first is simply a thermolysis reaction of the hydrazone intermediates (3) that were utilized in the synthesis of many of the pyrimidotriazinediones. Due to the extreme temperature conditions and the reported lower yields, this route was only attempted for one analogue (R3= p-ClC6H4), and only starting material was isolated after the reported reaction time of 5 minutes. Since the alternate strategies shown in Schemes 4-2 and 4-3 each had much higher reported yields and much milder conditions, these routes were pursued more thoroughly.

Scheme 4-1: Synthesis of pyrazolopyrimidinediones via thermolysis of hydrazones
Scheme 4-2: Synthesis of pyrazolopyrimidinediones via reaction of 6-hydrazinyluracils with dicyanoolefins

Scheme 4-3: Synthesis of pyrazolopyrimidinediones via reaction of 6-hydrazinyluracils with aryl nitrones

In order to utilize the strategy shown in Scheme 4-2, an efficient route to dicyanoolefins (92) had to be established. While initial attempts utilized conventional methods for Knoevenagel condensations of treating aryl aldehydes with malononitrile in refluxing dioxane or DMF, reaction times were very long, and yields were often low. A significantly more expedient route was soon found in the literature, however, which is depicted in Scheme 4-4. Here, an aryl aldehyde and malononitrile, together with benzyltriethylammonium chloride (TEBA), were ground together with a mortar and pestle for 5-10 minutes. The desired dicyanoolefins were then separated cleanly and in
high yields by precipitation from water. Table 4-1 summarizes the dicyanoolefins that were synthesized with this methodology, along with the comparison yields from the literature precedent.

![Scheme 4-4: Synthesis of dicyanoolefins](image)

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>Yield</th>
<th>Reported Yield from Ref.³⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>103a</td>
<td>H</td>
<td>70%</td>
<td>90%</td>
</tr>
<tr>
<td>103b</td>
<td>Cl</td>
<td>99%</td>
<td>97%</td>
</tr>
<tr>
<td>103c</td>
<td>CH₃</td>
<td>90%</td>
<td>95%</td>
</tr>
<tr>
<td>103d</td>
<td>OCH₃</td>
<td>75%</td>
<td>not synthesized</td>
</tr>
</tbody>
</table>

Table 4-1: Reaction yields for synthesis of dicyanoolefins via mortar and pestle Knoevenagel condensations

Two of these dicyanoolefins (R₃= p-ClC₆H₄ and R₃= C₆H₅) were then treated with 3-methyl-6-(1-methylhydrazinyl)pyrimidine-2,4(1H,3H)-dione (2a), as shown in Scheme 4-5. Unfortunately, the desired pyrazolopyrimidine(dione (106) was not isolated in either case, with the product, instead, being the hydrazone (105), which was confirmed in each case by comparison to the product from treatment of 3-methyl-6-(1-methylhydrazinyl)pyrimidine-2,4(1H,3H)-dione (2a) with either benzaldehyde or p-chlorobenzaldehyde. The difference between these attempts and that of the literature precedent is the presence of the methyl substituent at the 6α-position of the uracil.

Closer examination of the mechanism proposed by the authors in Scheme 4-2 suggests that the result with the 6α-methylhydrazinyluracil is not surprising, as the hydrogen at the 6α-position appears to be necessary for proton transfer. Lack of a hydrogen at the 6α-position makes it more favorable for elimination of malononitrile via displacement from the electrons on the 6β-position instead, forming the uncyclized hydrazone (105) and abrogating the pathway to the desired cyclized product.
Scheme 4-5: Hydrazone formation from treatment of 3-methyl-6-(α-methylhydrazinyl)uracil (2a) with dicyanoolefins (103)

With the failure of this methodology to achieve the desired pyrazolopyrimidinediones, efforts then focused on the route shown in Scheme 4-3. This route also utilizes a 6-hydrazinyluracil intermediate. Here, again, the literature precedent does not have a substituent at the 6α-position of the uracil and the mechanism proposed by the authors,70 which is shown in Scheme 4-6, invokes use of the 6α hydrogen. Nonetheless, since there is also a hydrogen at N1 available for proton transfer, the hypothesis was that the uracil could still react via the proposed pathway. If the initial nucleophilic attack could take place at the 5-position, it seemed much more likely to close to the five-membered ring upon nucleophilic attack by the nitrogen at the 6β-position. The reaction was conducted according to the published procedure and is shown in Scheme 4-7.70 In this method, nitrobenzene was first reduced to N-phenylhydroxylamine, followed by condensation with an aryl aldehyde to form the imine oxide (113). Without isolation, the imine oxide was then treated with the 6-hydrazinyluracil (2a or 2b), which cyclized to the reduced form of the desired pyrazolopyrimidinedione with formal loss of N-phenylhydroxylamine (which was not confirmed). Cyclization to a 6,5-membered ring system occurred for both R1 = methyl and R1 = 2-hydroxyethyl; nonetheless, the product isolated was not in the desired oxidation state and in the dihydropyrazolopyrimidinedione form instead (114). Table 4-2 shows all of the dihydropyrazolopyrimidinediones that were synthesized. Surprisingly,
neither these or any analogous dihydropyrazolopyrimidinediones have been reported in the literature.

Scheme 4-6: Proposed mechanism for reaction of 6-hydrazinyluracils with nitrones

Scheme 4-7: Synthesis of pyrazolopyrimidinediones

<table>
<thead>
<tr>
<th>Entry</th>
<th>$R_1$</th>
<th>$R_3$</th>
<th>Yield in Cyclization</th>
<th>mp</th>
</tr>
</thead>
<tbody>
<tr>
<td>114a</td>
<td>CH$_3$</td>
<td></td>
<td>79%</td>
<td>250-254°C</td>
</tr>
<tr>
<td>114b</td>
<td>CH$_3$</td>
<td></td>
<td>85%</td>
<td>227-230°C</td>
</tr>
</tbody>
</table>
Table 4-2: 2,3-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(5H,7H)-diones synthesized

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>114c</td>
<td>CH₃</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>79%</td>
<td>228-230°C</td>
</tr>
<tr>
<td>114d</td>
<td>CH₃</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>75%</td>
<td>220-223°C</td>
</tr>
<tr>
<td>114e</td>
<td>CH₃</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>81%</td>
<td>263-266°C</td>
</tr>
<tr>
<td>114f</td>
<td>CH₃</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>67%</td>
<td>288-290°C</td>
</tr>
<tr>
<td>114g</td>
<td>CH₃</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>86%</td>
<td>288-290°C</td>
</tr>
<tr>
<td>114h</td>
<td>CH₃</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>68%</td>
<td>213-215°C</td>
</tr>
<tr>
<td>114i</td>
<td>CH₂CH₂OH</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>76%</td>
<td>258-261°C</td>
</tr>
<tr>
<td>114j</td>
<td>CH₂CH₂OH</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>82%</td>
<td>224-226°C</td>
</tr>
</tbody>
</table>

A mechanism that hypothesizes why the dihydropyrazolopyrimidinediones were isolated instead of the pyrazolopyrimidinediones (96 in Scheme 4-6) is given in Scheme 4-8, whereby phenylhydroxylamine is released before dehydration to the imine intermediate (109) of Scheme 4-6 occurs. In this proposed mechanism, the absence of a proton at the 6α position would require participation by the N₁-proton of the uracil in the initial nucleophilic attack of the uracil on the nitrone. The resultant intermediate (117) could be less susceptible to the proton shift that is proposed in Scheme 4-6. In Scheme 4-6, intermediate 108 would be thermodynamically favored over intermediate 107 by the conjugation of the double bond that results from the proton shift. Moreover, dehydration of intermediate 108 would be driven by the thermodynamic stability of intermediate 109.
that results, where the imine formed is conjugated with the α,β-unsaturated system of the uracil. In contrast, for proposed intermediate 117 in Scheme 4-8, when R₁ = alkyl instead of proton, there could be less of a driving force for a proton shift from the 5-position of the uracil to the N₁-position as compared to the proton shift in Scheme 4-6 from the 5-position to the 6α-position because the C=N₁ bond is conjugated in intermediate 117. Moreover, without the proton shift, intermediate 117 could be driven to eliminate phenylhydroxylamine instead of water because of the conjugation that results in intermediate 118. Cyclization of intermediate 118 would then result from Michael addition of the 6β-nitrogen. Evidence that the isomer of the dihydropyrazolopyrimidinedione formed is that shown in 114 (with the C=C bond between carbons 3a and 7a), and not one where the C=C bond is between the 3 and 3a positions include the following: 1) the proposed mechanism, 2) a positive NOE signal that is detected in the aryl protons of the R₃ substituent upon irradiation of the Hₐ' peak, and 3) the chemical shift of the N₇-H proton, which is at 10-12 ppm and consistent with the chemical shift of the N₇-H proton in known pyrazolopyrimidinediones, as well as the N₁-H of known uracils.

Scheme 4-8: Proposed mechanism for formation of dihydropyrazolopyrimidinediones (114)

With the isolation of dihydropyrazolopyrimidinediones, an additional step was required to produce the desired oxidation state for both N₁-methyl- and N₁-(2-hydroxyethyl)- analogues. In investigating several oxidants, it was found that pyrazolopyrimidinediones (115 or 116) could be produced by oxidation with N-bromosuccinimide. Oxidation reagents that were tried unsuccessfully included potassium t-butoxide/air, MnO₂, CAN, K₃Fe(CN)₆, NaNO₂, Ca(OCl)₂, and DDQ. While the
oxidation with N-bromosuccinimide appears to be general, it proceeds most rapidly with electron-donating substituents on the R_3 substituent. Moreover, an interesting N_1 substituent effect on the form of the oxidized product was noted. For N_1= CH_3, pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-diones (115) were formed initially, while for the one example where N_1= CH_2CH_2OH, the pyrazolo[3,4-d]pyrimidine-4,6(5H,7H)-dione (116) was formed initially. Determination of the isomer synthesized was made by NMR, as the proton at N_7 of the dihydropyrazolopyrimidinediones has a chemical shift of ~10-12 ppm, while that of the N_2 proton has a chemical shift of ~5-6 ppm. (These assignments were made based on comparison with the uracil intermediates used here and in the synthesis of the pyrimidotriazinediones, where the proton on N_1 of the uracil, which has a shift of 10-12 ppm is analogous to the proton on N_7 of both the dihydro- and pyrazolopyrimidinediones.) It should also be noted that there is a dramatic difference in melting points between the two isomers, as shown in Table 4-3 which summarizes the pyrazolopyrimidinediones that were synthesized via oxidation with N-bromosuccinimide.

All of the pyrazolopyrimidinediones shown are previously unreported in the literature, as there are no published examples of pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-diones, and the pyrazolo[3,4-d]pyrimidine-4,6(5H,7H)-diones that are known have either a methyl substituent or no substituent at the N_1 position.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R_1</th>
<th>R_3</th>
<th>Yield in Oxidation</th>
<th>mp</th>
</tr>
</thead>
<tbody>
<tr>
<td>115a</td>
<td>CH_3</td>
<td><img src="image1.png" alt="Diagram" /></td>
<td>50%</td>
<td>215-217°C</td>
</tr>
<tr>
<td>115b</td>
<td>CH_3</td>
<td><img src="image2.png" alt="Diagram" /></td>
<td>42%</td>
<td>272-274°C (dec)</td>
</tr>
<tr>
<td>115c</td>
<td>CH_3</td>
<td><img src="image3.png" alt="Diagram" /></td>
<td>33%</td>
<td>234-237°C</td>
</tr>
<tr>
<td>115d</td>
<td>CH_3</td>
<td><img src="image4.png" alt="Diagram" /></td>
<td>51%</td>
<td>243-246°C</td>
</tr>
<tr>
<td>116a</td>
<td>CH_3</td>
<td><img src="image5.png" alt="Diagram" /></td>
<td>22%</td>
<td>&gt; 300°C</td>
</tr>
</tbody>
</table>
One hypothesis for the two substituents at N₁ (CH₃ vs. CH₂CH₂OH) forming different isomers can be understood by consideration of the reaction mechanism in Scheme 4-9. Presumably, the mechanism for the oxidation involves nucleophilic attack by the dihydropyrazolopyrimidinedione on the bromine of N-bromosuccinimide, followed by elimination of HBr to the oxidized product. In the case of R₁= CH₃, the isomer isolated is the one that forms most directly from this reaction mechanism. In the case of R₁= CH₂CH₂OH, however, there is hydrogen bonding capability not present with R₁= CH₃, and this provides a mechanism for driving the product to an alternate isomeric form. A possible mechanism for the formation of the pyrazolo[3,4-d]pyrimidine-4,6(5H,7H)-dione isomer from the pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione is shown in Scheme 4-10. A driving force for this isomerization to occur is the resultant hydrogen-bonding between the nitrogen at the 2-position and the proton of the hydroxyethyl group that stabilizes the 4,6(5H,7H)-dione isomer. While the same hydrogen-bonding capacity is not available for the 1-methyl-substituted congeners, these, too, can be driven to isomerize if the reaction is allowed to proceed for an extended period of time. This was demonstrated for the 1-methyl-substituted pyrazolopyrimidinedione where R₃= p-CH₃O-C₆H₄, where both isomeric forms were isolated by varying the reaction time (24 h vs. 4-5 h) to isolate each individually.

<table>
<thead>
<tr>
<th>116b</th>
<th>CH₂CH₂OH</th>
<th>20% a</th>
<th>&gt; 300°C</th>
</tr>
</thead>
</table>

*Solvent used was 2-propanol instead of ethanol. Ethanol was later shown to produce improved yields.

Extended reaction time of >24 h.

**Table 4-3:** 1H-Pyrazolo[3,4-d]pyrimidine-4,6(5H,7H)-diones and 1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-diones synthesized
Scheme 4-9: Proposed mechanism for oxidation by N-bromosuccinimide

Scheme 4-10: Proposed mechanism for isomerization of pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione to pyrazolo[3,4-d]pyrimidine-4,6(5H,7H)-dione

In summary, ten novel 2,3-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(5H,7H)-diones were synthesized utilizing a previously published procedure. However, when employing substrates with substituents present at the 6α-position of the hydrazinyluracil moiety, a different reaction pathway ensued to give unexpected products. The resultant dihydropyrazolopyrimidinediones were then successfully oxidized with N-bromosuccinimide to the corresponding 1H-pyrazolo[3,4-d]pyrimidine-4,6(5H,7H)-diones or 1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-diones in respectable yields. The advantage of obtaining dihydropyrazolopyrimidinediones as stable, isolable intermediates is that it doubles the number of potential analogues available for testing. While neither the pyrazolopyrimidinediones nor their dihydro precursors demonstrated inhibition of the Wnt signaling pathway in the assays to be described for this work, this class is known to have other biological activities, particularly as antitumor agents, and provides an interesting avenue for future investigations to see if the analogous dihydropyrazolopyrimidinediones display the same activities.
Chapter 5
Synthesis of Pyrrolopyrimidinedione Analogues

In addition to the pyrazolopyrimidinediones, another 6,5-heterocyclic class lacking susceptibility to redox chemistry is that of the pyrrolopyrimidinediones. Hence, the synthesis of some pyrrolopyrimidinedione analogues was pursued, with the intention of synthesizing analogues both with and without an aryl substituent at the 5-position (which would be analogous to the 3-position in both the pyrazolopyrimidinediones and the pyrimidotriazinediones). Initially, the plan was to synthesize the 5-aryl substituted pyrrolopyrimidinediones via the unsubstituted intermediates. While pyrrolopyrimidinediones without a substituent at the 5-position are known, those with an aryl substituent are not. Hence, the synthesis of analogues with a substituent at the 5-position would require exploration of new methodology, but synthesis of 5-unsubstituted analogues could be based on published procedures. Investigation of the pyrrolopyrimidinediones, therefore, began with the synthesis of some 5-unsubstituted analogues.

![Scheme 5-1: Synthesis of 5-unsubstituted pyrrolopyrimidinediones](image)

To access the 5-unsubstituted pyrrolopyrimidinediones, 6-chloro-3-methyluracil was once again chosen as a starting material, and the strategy used is shown in Scheme 5-1. Here, 6-chloro-3-methyluracil (1) was first treated with an amine, followed by cyclization with chloroacetaldehyde (124). Using this procedure, pyrrolopyrimidinediones were synthesized where \( R_7 \) = methyl, benzyl, and furan-2-
ylmethyl, all of which are previously-unreported compounds. Table 5-1 summarizes the reaction yields for this small series of pyrrolopyrimidinediones.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R₇</th>
<th>Yield in Amine Addition</th>
<th>Cyclization Yield</th>
<th>mp</th>
</tr>
</thead>
<tbody>
<tr>
<td>124a</td>
<td>CH₃</td>
<td>95%</td>
<td>35%</td>
<td>285°C (dec)</td>
</tr>
<tr>
<td>124b</td>
<td></td>
<td>74%</td>
<td>77%</td>
<td>235-240°C (dec)</td>
</tr>
<tr>
<td>124c</td>
<td></td>
<td>39%</td>
<td>48%</td>
<td>228-230°C (dec)</td>
</tr>
</tbody>
</table>

Table 5-1: 5-Unsubstituted pyrrolopyrimidinediones synthesized

With the 5-unsubstituted pyrrolopyrimidinediones in hand, the intention was to synthesize the 5-aryl-substituted pyrrolopyrimidinediones from these unsubstituted pyrrolopyrimidinediones via halogenation of 124 and subsequent palladium-catalyzed coupling as shown in Scheme 5-2. Unfortunately, all attempts to halogenate selectively at the 5-position proved fruitless. As there is no precedence for halogenation of this ring system at this position, the same methodology⁷⁷ used to iodinate similar ring systems was tried first. Nonetheless, neither the direct treatment of 124 (R₇ = CH₃) with N-iodosuccinimide nor in situ silylation with N,O-bis(trimethylsilyl)acetamide followed by treatment with N-iodosuccinimide yielded an isolable monohalogenated product.

Scheme 5-2: Unsuccessful synthesis of 5-arylpyrrolopyrimidinediones via halogenation and subsequent Pd-catalyzed coupling

The treatment of 124 (R₇ = CH₃) with N-bromosuccinimide after silylation with N,O-bis(trimethylsilyl)acetamide was also attempted, and a monobrominated product was isolated cleanly as shown in Scheme 5-3. Unfortunately, as confirmed by NOE analysis, the 6-bromopyrrolopyrimidinedione (128) was formed instead of the desired 5-bromopyrrolopyrimidinedione (127). The vinyl-type NMR peak is at 6.49 ppm, which is
between the chemical shift of the two pyrrole protons in 124a (6.28 (H5) and 6.67 (H6) ppm). The previous literature\textsuperscript{77} on the iodination of 4(3\textit{H})-oxo-7\textit{H}-pyrrolo[2,3-\textit{d}]pyrimidines (129) showed that monohalogenation causes a downfield shift of \~0.2 ppm of the surviving pyrrole proton, which would be consistent with the product of the bromination here being the 6-bromopyrrolopyrimidinedione. The bromination was also attempted directly with N-bromosuccinimide without prior silylation, but only the dibromopyrrolopyrimidinedione was isolated when using either DMF or glacial acetic acid as solvent. Finally, the bromination was attempted with silylation and in the presence of azoisobutyronitrile, but again, the undesired 6-bromopyrrolopyrimidinedione was isolated.

\textbf{Scheme 5-3: Bromination of 3,7-dimethyl-1\textit{H}-pyrrolo[2,3-\textit{d}]pyrimidine-2,4(3\textit{H},7\textit{H})-dione (124a)}

The next strategy attempted, therefore, is shown in Scheme 5-4. In this synthetic strategy, the goal was to incorporate the desired bromo- substituent in the cyclization reaction of the pyrimidinedione to the pyrrolopyrimidinedione. As such, the desired reacting partner for the pyrimidinedione was dibromoacetaldehyde. While dibromoacetaldehyde is not commercially available, there is a synthesis reported in older literature using direct bromination of acetaldehyde.\textsuperscript{78} Unfortunately,
Dibromoacetaldehyde was not able to be obtained cleanly by this method. In contrast, dibromoacetaldehyde diethyl acetal was isolated cleanly through bromination of bromoacetaldehyde diethyl acetal and subsequent distillation. Therefore, attempts were made to convert the acetal of dibromoacetaldehyde diethyl acetal to the aldehyde, with Table 5-2 listing all of the conditions that were tried. None of these was successful.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% H₂SO₄, CH₂Cl₂</td>
<td>decomposition</td>
</tr>
<tr>
<td>silica gel (300 wt.%), 10% oxalic acid in H₂O, CH₂Cl₂</td>
<td>isolated starting material</td>
</tr>
<tr>
<td>silica gel (300 wt.%), 10% sulfuric acid in H₂O, CH₂Cl₂</td>
<td>isolated starting material</td>
</tr>
<tr>
<td>silica gel (300 wt.%), conc. H₂SO₄, CH₂Cl₂</td>
<td>isolated starting material</td>
</tr>
<tr>
<td>trifluoroacetic acid/H₂O/CHCl₃ (1:1:4)</td>
<td>decomposition</td>
</tr>
<tr>
<td>CHCl₃/1 N HCl (1:1)</td>
<td>isolated starting material</td>
</tr>
<tr>
<td>formic acid</td>
<td>decomposition</td>
</tr>
<tr>
<td>p-toluenesulfonic acid, acetone</td>
<td>isolated starting material</td>
</tr>
<tr>
<td>CHCl₃/conc. HCl (1:1)</td>
<td>decomposition</td>
</tr>
<tr>
<td>Amberlite-120 strongly acidic resin</td>
<td>isolated starting material</td>
</tr>
</tbody>
</table>

Table 5-2: Attempts to convert dibromoacetaldehyde diethyl acetal to aldehyde

Alternately, attempts were made to use the acetal of dibromoacetaldehyde directly in the cyclization reaction with *in situ* deprotection, which was successfully
demonstrated for the condensation between bromoacetaldehyde diethyl acetal and 3-methyl-6-(methylamino)uracil. Table 5-3 lists all of the conditions that were attempted in the direct cyclization of the dibromoacetal with the uracil. Unfortunately, none of the cyclizations proceeded to the desired product, and, in most cases, the only material isolated was the starting uracil.

Another approach to dibromoacetaldehyde that was briefly examined was formylation of dibromomethane. This approach is illustrated in Scheme 5-5. Bases tried included sodium methoxide, sodium hydride, \( n \)-butyllithium, and sodium hexamethyldisilazide. In each case, deprotonation was attempted at -78°C in THF since the sodium salt of the dibromomethane anion has been reported to decompose at -30°C.\(^{79} \) Color changes suggestive of anion formation were only seen with \( n \)-butyllithium and sodium hexamethyldisilazide, but in neither case was there any evidence for formylation of the anion occurring. With these unpromising results and no precedent for such a reaction, this approach was not pursued any further.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOAc, H(_2)O, Δ</td>
<td>isolated starting uracil</td>
</tr>
<tr>
<td>H(_2)O, Δ</td>
<td>isolated starting uracil</td>
</tr>
<tr>
<td>TEBA, grinding</td>
<td>isolated starting uracil</td>
</tr>
<tr>
<td>NaOAc, DMF, Δ</td>
<td>no identifiable products</td>
</tr>
<tr>
<td>DMF, Δ</td>
<td>isolated starting uracil</td>
</tr>
<tr>
<td>trifluoroethanol, Δ</td>
<td>isolated starting uracil</td>
</tr>
<tr>
<td>cat. HCl, trifluoroethanol, Δ</td>
<td>isolated starting uracil</td>
</tr>
<tr>
<td>1) acetal, cat. HCl, H(_2)O, Δ 2) uracil, NaOAc</td>
<td>isolated starting uracil + unidentified product</td>
</tr>
<tr>
<td>Amberlite-120 acidic resin, NaOAc, H(_2)O, Δ</td>
<td>isolated starting uracil</td>
</tr>
<tr>
<td>1) acetal, cat. HClO(_4), H(_2)O, Δ 2) uracil, NaOAc</td>
<td>isolated starting uracil</td>
</tr>
<tr>
<td>1) acetal, ( p )-TsOH, H(_2)O, Δ 2) uracil, NaOAc</td>
<td>isolated starting uracil</td>
</tr>
<tr>
<td>1) BF(_3).(OEt)(_3), NaI, ACN, Δ 2) uracil, NaOAc</td>
<td>isolated starting uracil</td>
</tr>
</tbody>
</table>

**Table 5-3: Attempts to cyclize diethyl acetal with 3-methyl-6-(methylamino)uracil**
A final unsuccessful approach towards 5-arylpyrrolopyrimidinediones that was attempted is shown in Scheme 5-6. In this approach, 3-methyl-6-(methylamino)uracil \((122a)\) was treated with tribromoacetaldehyde, with the hope that intermediate \(137\) would form. Addition of zinc to the reaction mixture should then have allowed for reductive elimination to form the desired pyrrolopyrimidine core. Unfortunately, the desired cyclization and elimination could never be effected, and no identifiable intermediates could be isolated, even after attempts with a variety of solvents and reducing agents.

Nonetheless, aryl substitution at the 5-position was eventually achieved as shown in Scheme 5-7 by treatment of 6-chloro-3-methyluracil \((1)\) with a primary amine, followed by condensation of the resulting adduct with 2-bromo-2-phenylacetaldehyde \((139)\). 2-Bromo-2-phenylacetaldehyde \((139)\) was itself obtained from bromination of phenylacetaldehyde according to a published procedure.\(^{80}\) The regiochemistry of the
cyclization was verified by comparison of the proton peaks for H5 and H6 in analogue 124a with the remaining proton in analogue 140. Synthetic efforts toward more 5-arylpyrrolopyrimidinediones were halted, however, due to negative test results.

![Scheme 5-7: Synthesis of 5-phenylpyrrolopyrimidinediones](image)

In summary, four novel pyrrolopyrimidinediones were synthesized using variations on known methodologies. Three of these lacked a substituent at the 5-position, while one incorporated a phenyl substituent at the 5-position, for which there is no precedent in the literature. While none showed inhibition of the βcat/Tcf4 complex, they also demonstrated no measurable toxicity, making them promising scaffolds for future high-throughput screening campaigns.
Chapter 6

Synthesis of Pyrimidopyrimidinedione Analogues

A final class of heterocycles towards which synthetic efforts were directed was a subclass of the pyrimidopyrimidinediones. Like the pyrimidopyridazinediones, the desired pyrimidopyrimidinediones replace one of the nitrogen atoms of the screen hit, in this case N₁, so they allow for further definition of which nitrogen atoms of the core are necessary for activity. Moreover, they should not be susceptible to the same flavin-like redox chemistry as the screen hit and the pyrimidopyridazinediones, so they could avert potential toxicity issues and be a more promising class to pursue, if active against the target. Specifically, the subclass of pyrimidopyrimidinediones sought was that of the unsymmetrical pyrimido[5,4-d]pyrimidine-2,4(1H,3H)-diones, in contrast to the symmetrical pyrimido[4,5-d]pyrimidine-2,4(1H,3H)-diones, both of which are shown in Figure 6-1. While there are a plethora of syntheses and analogues of the symmetrical pyrimido[4,5-d]pyrimidine-2,4(1H,3H)-diones in the literature (more than 6,000 hits on Scifinder® Scholar), there is a dearth of synthetic routes to the unsymmetrical pyrimido[5,4-d]pyrimidine-2,4(1H,3H)-diones (less than 10 hits. Not surprisingly, therefore, there are far fewer pyrimidopyrimidinediones of the desired subclass in the literature than any of the four previous heterocyclic classes discussed. Moreover, the published routes may not be amenable to alternate substitution patterns, just as the reaction pathway to published routes of the pyrazolopyrimidinediones demonstrated differences with the addition of the methyl group at the 6α-position of the uracil that was to become the eventual N₁ position of the pyrazolopyrimidinedione. Hence, the first synthesized analogue of the pyrimidopyrimidinedione class was one for which there was the most literature precedent. The strategy was to then synthesize analogues more closely resembling the screen hit, PKF118-310, and explore what substituent changes could be tolerated.
The synthesis that was carried out is shown in Scheme 6-1, and while the yields are less than ideal, the route shown yielded the intended pyrimidopyrimidinedione. First, 6-methyluracil (141) was treated with dimethylsulfate and cesium carbonate to form 1,3,6-trimethyluracil (142), which was then nitrated with fuming nitric acid in sulfuric acid, followed by bromination of the 6-methyl group in glacial acetic acid. Nitouracil 144 was then cyclized through nucleophilic displacement of the bromide by benzylamine and subsequent attack on the nitro group to close the ring. The final step required base treatment to induce a rearrangement to the desired pyrimidopyrimidinedione. While the literature reported the use of sodium ethoxide in this last rearrangement step with respectable yields (50-70%), here the best yield ever attained was less than 15%. Hence, sodium hexamethyldisilazide was substituted for sodium ethoxide, with the hypothesis that a stronger base would help drive the reaction in the intended direction. Indeed, the yield was significantly improved with the use of sodium hexamethyldisilazide as base, producing the desired pyrimido[5,4-d]pyrimidinedione with a yield comparable to that of the literature. While pyrimidopyrimidinedione 146 is not a novel compound, few analogues are known, with the only ones reported being the p-methoxyphenyl and 2-furyl derivatives of 146 and the 6-phenyl analogue without methyl substituents at N1 and N3.
Scheme 6-1: Synthesis of pyrimidopyrimidinediones

Having verified the feasibility of this route to pyrimidopyrimidinediones, the intention was to synthesize an analogue or series of analogues where there was a methyl substituent at C₈. As can be seen in Figure 6-2, these analogues (147) would be more closely related to the screen hit, PKF118-310. Moreover, they would be directly analogous to the initial series of pyrimidotriazinediones synthesized, where R₁= CH₃, with the replacement of the nitrogen atom at the 1-position of the pyrimidotriazinediones by the carbon at the 8-position of the pyrimidopyrimidinediones. While the series most analogous to the screen hit would have R₆= R₁ = H, some of the testing data to be described was already suggestive of aryl groups being preferable at the R₆ position, so the intention was to maintain the aryl group that is present in analogue 146 in this new series. Moreover, the methyl group at N₁ of 146 was also maintained in the synthetic efforts towards a novel series of pyrimidopyrimidinediones along with a methyl substituent at C₈ for two reasons: 1) pyrimidotriazinediones do not have the capacity for a substituent at both N₁ and N₈ simultaneously, so assuming some pyrimidopyrimidinediones would exhibit activity, there was no way to predict a priori whether a substituent at both C₈ and N₁ might be preferable or detrimental in this new series; 2) the synthetic route of Scheme 6-1 was more likely to be directly applicable to a new series with a methyl substituent at C₈ by maintaining the methyl group at N₁ also.
The synthetic route investigated toward an 8-methylpyrimidopyrimidinedione is shown in Scheme 6-2. The route is virtually identical to that of Scheme 6-1, with the exception of two additional steps for incorporating the additional methyl substituent. In order to incorporate a methyl substituent at the 8-position (and possibly other substituents in the future), nitouracil 143 was treated with sodium ethoxide to form the sodium salt nitronate 148, which could be isolated according to the literature. Methylation was then accomplished with methyl iodide in DMF, again according to literature precedent. The methylation was also attempted in one step with a variety of solvents and bases, but it never yielded product as cleanly and efficiently as the two-step process depicted in Scheme 6-2.

With 6-ethyl-1,3-dimethyl-5-nitropyrimidine-2,4(1H,3H)-dione (149) synthesized as planned, difficulties were encountered in the proposed synthesis when attempting the bromination of 149. The first attempts were based on a direct standard bromination, as had been accomplished on 143 above. Therefore, 6-ethyluracil 149 was treated with 4 eq of Br₂ in glacial acetic acid and allowed to reflux for 4-5 hours to 2-3 days. Unfortunately, the reaction failed to go to completion regardless of reaction time, and even after attempts at purification by silica gel flash chromatography and recrystallization, the desired product (150) was almost always contaminated with at least 10-20% starting material. Table 6-1 summarizes all of the conditions that were tried for brominating 6-ethyluracil 149. As can be seen, only one set of conditions (entry b) was able to yield pure product, but the yield was low and not consistently reproducible. Hence, a more efficient bromination was still desired.
Scheme 6-2: Proposed synthetic route to 8-methylpyrimidopyrimidinediones

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Br₂ (4.5 eq), AcOH, Δ</td>
<td>mix of starting material and product</td>
</tr>
<tr>
<td>b</td>
<td>Br₂ (8 eq), AcOH, Δ</td>
<td>clean product obtained in 23% yield</td>
</tr>
<tr>
<td>c</td>
<td>AIBN, NBS, CHCl₃, Δ</td>
<td>no reaction</td>
</tr>
</tbody>
</table>
| d     | 1) NaH, THF, 0°C to Δ  
2) Br₂, 0°C to Δ, Δ for 3h | starting material isolated |
| e     | Br₂, AlCl₃, THF, Δ | starting material isolated |
| f     | Br₂, AgO₂CCF₃, AcOH, RT, dark | minimal product formed, decomposition |
| g     | Br₂, AgO₂CCF₃, H₂SO₄, RT, dark | decomposition |
| h     | Br₂, Ag₂CO₃, H₂SO₄, RT, dark | decomposition |
| i     | starting material isolated |

Table 6-1: Bromination attempts of 6-ethyl-1,3-dimethyl-5-nitropyrimidine-2,4(1H,3H)-dione (149)
Another option considered, therefore, was bromination of a more reactive starting material, analogous to sodium salt 148. Formation of the sodium salt of the anion of the 6-ethyluracil 149 was attempted, therefore, as shown in Scheme 6-3. While the yield was not as high as it was for 148, it was still respectable and presented a viable option if the bromination could be carried out more efficiently and cleanly. Next, the bromination was tried. Since bromination of the sodium salt 148 had not been reported, this was attempted first to explore the feasibility of this approach. Table 6-2 summarizes the bromination attempts that were made using the anion of 6-methyl-1,3-dimethyl-5-nitropyrimidine-2,4(1H,3H)-dione (148), and Table 6-3, the bromination attempts that were made with the anion of 6-ethyl-1,3-dimethyl-5-nitropyrimidine-2,4(1H,3H)-dione (153). As with their protonated counterparts, a set of bromination conditions that worked for 148 (Table 6-2, entry c) yielded a mixture of both the desired product and the protonated starting material for 153 (Table 6-3, entry a). Fortunately, however, and somewhat surprisingly, bromination of 153 with pyridinium hydrobromide perbromide (Table 6-3, entry c) yielded clean product in a reasonable yield.

![Scheme 6-3: Formation of isolable salt anion of 6-ethyl-1,3-dimethyl-5-nitropyrimidine-2,4(1H,3H)-dione (149)](image)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>NIS, DMF, Δ</td>
<td>no identifiable product isolated</td>
</tr>
<tr>
<td>b</td>
<td>NIS, THF, Δ</td>
<td>protonated starting material isolated</td>
</tr>
</tbody>
</table>
\[ \text{Br}_2, \text{THF}, \Delta \quad \text{clean product isolated in 58\% yield} \]

\[ \text{I}_2, \text{acetone, } \Delta \quad \text{protonated starting material isolated} \]

Table 6-2: Bromination attempts of sodium salt (148)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Br(_2), THF, (\Delta)</td>
<td>mix of protonated starting material and product</td>
</tr>
<tr>
<td>b</td>
<td>Br(_2), DMF, (\Delta)</td>
<td>protonated starting material isolated</td>
</tr>
<tr>
<td>c</td>
<td>Br(_2), THF, (\Delta)</td>
<td>clean product isolated in 71% yield</td>
</tr>
</tbody>
</table>

Table 6-3: Bromination attempts of sodium salt (153)

With successful bromination conditions determined, two steps remained to the target pyrimidopyrimidinedione. Here, again, there were questions to be answered with respect to how the reaction pathway might be affected by the presence of the methyl group at the 6\(\alpha\)-position of the uracil. Unfortunately, this did seem to exert a profound effect. In no attempt to convert 150 to 151 was the desired pyrazolo[4,3-\(d\)]pyrimidine N-oxide isolated. Table 6-4 summarizes the reaction conditions that were tried with benzylamine in this conversion. What can be gleaned from these results is that the nucleophilic displacement of the bromide group is feasible, but that the desired subsequent intramolecular cyclization into the nitro group fails to occur. In one instance, the only product identified was that occurring from nucleophilic attack by one molecule of benzylamine on two molecules of the 6\(\alpha\)-bromouracil (entry a), but attempts to overcome that by an increased number of equivalents of benzylamine were not successful at isolating the desired product.
Table 6-4: Attempted reactions between 6α-bromouracil (150) and benzylamines

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>H</td>
<td>2 eq benzylamine, Δ, 2h, EtOH</td>
<td>(identified in one of 2 trials) + other unidentified products</td>
</tr>
<tr>
<td>b</td>
<td>H</td>
<td>3 eq benzylamine, Δ, 4h, EtOH</td>
<td>no identifiable product</td>
</tr>
<tr>
<td>c</td>
<td>H</td>
<td>3 eq benzylamine, AlCl3, Δ, 24h, THF</td>
<td>no change by TLC</td>
</tr>
<tr>
<td>d</td>
<td>OCH3</td>
<td>2 eq amine, AlCl3, Δ, 24h, THF</td>
<td>no change by TLC</td>
</tr>
<tr>
<td>e</td>
<td>OCH3</td>
<td>2 eq benzylamine, Δ, 2h, EtOH</td>
<td>no identifiable product</td>
</tr>
</tbody>
</table>

The desired conversion to the pyrazolo[4,3-d]pyrimidine N-oxide (151) was also attempted by combining the amine treatment with the previous bromination step. Evidence of this being a potential alternative had been demonstrated for the non-6α-methyl-substituted congener, as shown in Scheme 6-4. Nonetheless, this, too, failed to produce the desired pyrazolo[4,3-d]pyrimidine N-oxide (151), with the only products isolated being the starting material and the brominated species.
A final alternative that was investigated in order to isolate the desired pyrazolo[4,3-\(d\)]pyrimidine N-oxide (151) involved partitioning the nucleophilic substitution reaction and intramolecular cyclization into two distinct steps. In the literature report of this method,\(^8\) there were examples where the intramolecular cyclization failed to occur in the same step with the nucleophilic displacement, whereby subsequent treatment with triethylamine in methanol allowed for ring closure. Hence, the sequence in Scheme 6-5 was tried with the 6\(\alpha\)-methyl-substituted uracil 150.

Unfortunately, while this approach allowed for isolation of the 6\(\alpha\)-amino-uracil 154, it did not yield the pyrazolo[4,3-\(d\)]pyrimidine N-oxide 151.

**Scheme 6-5: Attempted two-step nucleophilic substitution and cyclization**
With the failure of the two-step nucleophilic substitution and intramolecular cyclization sequence to yield the desired pyrazolo[4,3-d]pyrimidine N-oxide, this route was abandoned. Instead, some preliminary studies were done on an alternative, more novel route, which is shown in Scheme 6-6. Here, the first step proceeded smoothly, with nitroethane being deprotonated by sodium ethoxide generated \textit{in situ}, and subsequently adding via Michael addition into 156. There was evidence by NMR, however, of the resulting dinitrouracil being present in more than one isomeric form (157 and 158), as is also shown in Scheme 6-6. The subsequent reduction, however, is where problems were encountered with this route. While the reduction was not explored exhaustively, Table 6-5 summarizes the conditions that were tried. With both Fe and Zn as reducing agents, decomposition occurred as evidenced by baseline material on TLC, and no identifiable product was isolated. Transfer hydrogenation conditions (NH$_4$HCO$_2$, Pd, EtOH), on the other hand, resulted in no change by TLC. The failure to isolate 159 concluded all efforts to synthesize the desired 8-methylpyrimidopyrimidinedione 161, for by this point, testing results in the various assays to be described were not suggestive of this class of heterocycles holding promise for the intended target.

![Scheme 6-6: Alternate synthetic route to 8-methylpyrimidopyrimidinediones](image)
In conclusion, efforts to synthesize a novel 8-methylpyrimidopyrimidinedione proved unfruitful. Nonetheless, a unique, effective way of brominating 6-ethyl-1,3-dimethyl-5-nitropyrimidine-2,4(1H,3H)-dione at the 6α-position via anion formation and subsequent reaction with pyridinium hydrobromide perbromide was discovered. The resulting α-bromo-α-methyluracil (150) has not been reported in the literature and could be a useful intermediate for other heterocyclic syntheses.

Table 6-5: Attempted reductions of dinitouracil (157)
Chapter 7  
*in vitro* Binding Assay Implementation and Outcome

As the genesis for this work was the report\(^5\) of PKF118-310 as a potent inhibitor of the β-catenin/Tcf4 interaction in the Wnt signaling pathway, and as it had been initially identified in a high throughput binding screen, one goal was to implement this binding assay as a way to identify active heterocyclic classes among those synthesized and to examine structure-activity relationships among those active classes. As described, the assay required plating the armadillo repeat of the β-catenin region (amino acids 134-668, known region of Tcf binding) on microtiter plates and then treating with Tcf4 tagged with glutathione S-transferase (GST) and test compound. After incubation with GST-Tcf4 and compound, the plates were incubated sequentially with goat anti-GST antibody followed by anti-goat alkaline phosphatase (AP)-conjugated secondary antibody. Finally, the plates were treated with a fluorescent substrate for alkaline phosphatase, such that a reduced fluorescence signal would be detected wherever there was inhibition of the interaction between β-catenin and Tcf4. A diagram of the binding assay as described is shown in Figure 7-1.

In order to carry out the assay, both β-catenin and Tcf4 needed to be cloned, expressed, and purified. As in the original paper, Tcf4 was fused to GST, and for ease of purification, β-catenin was expressed with a polyhistidine tag. While a polyhistidine tag had not been used in the work reporting on PKF118-310, there was another report\(^{86}\) of a binding assay to examine the interaction between Tcf4 and β-catenin to determine the minimal length of Tcf4 required for binding, and here a polyhistidine tag had been fused to the armadillo repeat region of β-catenin without affecting its ability to bind Tcf4. Both the plasmid (pCDNA3 S33Y) containing the CTNNB1 gene for β-catenin and the plasmid (pCDNA3 TCF4) containing the TCF7L2 gene for Tcf4 were obtained from Prof. Eric Fearon (University of Michigan Medical School). Each was amplified using the polymerase chain reaction (PCR). The primers used for each are shown in Table 7-1,
and the protocol used is included in the experimental chapter. Plasmids for expression were then prepared using standard protocols, and both β-catenin and Tcf4 were expressed and purified as described in the experimental chapter.

Figure 7-1: Binding assay to assess activity of analogues against βcat/Tcf4

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCF7L2</td>
<td>sense: ATACATGGATCCATGCCGCAGCTGAACGGC</td>
</tr>
<tr>
<td></td>
<td>reverse: ATACATAGAATTCCCGGGATTTGTCTCGGAAACT</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>sense: CGACAGCTAGCGTTGTAAACTTG</td>
</tr>
<tr>
<td></td>
<td>reverse: TTACAGGATCCGCCGTTTCTTTGG</td>
</tr>
</tbody>
</table>

Table 7-1: Primers used to amplify Tcf4 and β-catenin genes
(Key: NheI site; BamHI site; EcoRI site)

Once the proteins were purified, a series of control experiments were carried out to establish a functioning assay. Since the screen hit had been identified with a high-throughput version of this assay, the initial control experiments used DMSO treatment as a positive control to verify interaction between β-catenin and Tcf4 and PKF118-310 as a negative control to verify inhibition of that interaction. The protocol for the assay that was initially used is described in the experimental chapter.
Unfortunately, while obtaining a strong fluorescence signal for interaction between the two proteins with only DMSO present, no inhibition was observed upon treatment with PKF118-310, as shown in Figure 7-2. In fact, if anything, there was a slightly greater fluorescence signal for treatment with PKF118-310 relative to the DMSO control. A number of hypotheses were considered with this disconcerting result, including the following:

1. Binding between β-catenin and Tcf4 occurs extremely rapidly in the absence of inhibitor, so a preincubation period with the compound and either β-catenin or Tcf4 before exposing the two proteins to each other is necessary to observe inhibition.

2. The β-catenin protein is not sufficiently pure, and PKF118-310 is binding to one of the impurities instead of β-catenin, or Tcf4 is binding to both β-catenin and other impurities.

3. The β-catenin protein is aggregating such that the effects observed are the result of nonspecific binding of Tcf4 to β-catenin aggregates, and/or PKF118-310 cannot bind to β-catenin due to its binding site being buried in the aggregate.

4. The concentration of either Tcf4 or β-catenin being used is too high, and PKF118-310 cannot effectively compete.

5. PKF118-310 is binding more strongly to the bovine serum albumin (BSA) that is present in the assay buffer than to β-catenin.

6. PKF118-310 is not a true inhibitor of the β-catenin/Tcf4 interaction.
In order to rule out the first hypothesis— that binding between Tcf4 and β-catenin occurred too rapidly in the absence of inhibitor— the assay was tried with two other variations. As the assay was carried out in Figure 7-2, PKF118-310 was added to the equilibration mixture after GST-Tcf4. In the next two trials, the plates with β-catenin adsorbed were treated in one of the following two ways: 1) addition of PKF118-310 and equilibration with β-catenin at RT for 20-30 minutes, followed by addition of GST-Tcf4, or 2) equilibration of PKF118-310 and Tcf4 for 20-30 minutes at 0°C, followed by addition of the equilibrated mixture to the β-catenin-coated plates. Neither of these variations in equilibration order changed the results, and for all future trials, the assay was conducted according to the second variant, whereby the compounds were equilibrated with Tcf4 before addition of the mixture to the β-catenin-coated plates.

With regards to β-catenin purity, several preparations of β-catenin from unique sources were used in the assay. These multiple preparations included preparations done
by different researchers (Paul Keller/Professor Ron Woodard’s lab and myself/Professor Garry Dotson’s lab) from the plasmid previously described and expression and purification in a different lab (Shaomeng Wang’s lab, courtesy of Steve Kawamoto) from a different plasmid. The β-catenin that was used for the initial experiments had been purified solely with a Ni-NTA column, but subsequent preparations added an additional purification step via anion exchange column. Multiple storage conditions for the β-catenin were also explored, as β-catenin was observed to precipitate easily and is prone to aggregation. Storage conditions that were tried included increasing concentrations of glycerol (10-50%), addition of 1 mM DTT, and addition of 50 mM Arg and Gln to the storage buffer. Among these storage conditions, both 25% glycerol and 50 mM Arg and Gln were able to alleviate precipitation concerns, but the results in the assay were no different with respect to the relative signal between treatment with DMSO and 100 μM PKF118-310. The only change observed with different preparations was a variation in the magnitude of the fluorescence signal, which could vary by 2-5 fold from one preparation to another. While this variation most likely reflects the degree of purity and/or percentage of properly-folded protein, the identical results with DMSO and 100 μM PKF118-310 relative to each other with each different preparation suggested that this was not the primary reason for failing to observe inhibition by PKF118-310.

In order to address potential problems in plating and aggregation that could result, a range of plating conditions were explored. The concentration of β-catenin was varied from 1.25 to 10 μg/mL, and the volume per well during plating was also varied from 50 to 200 μL/well. The salt concentration was adjusted, and multiple buffers were explored, including Tris, HEPES, and phosphate buffers. With none of these modifications, however, could any statistically-significant change in the relative signal from DMSO treatment versus treatment with 100 μM PKF118-310 be observed. The ratios of the fluorescence signals from treatment with 100 μM PKF118-310 to DMSO for different plating concentrations of β-catenin are shown in Table 7-2.

In addition to exploring a range of β-catenin concentrations for plating, the concentration of GST-Tcf4 was also considered. This was important to examine since too high of a concentration would impede the ability of PKF118-310 to effectively
compete. Hence, the concentration of GST-Tcf4 was diluted both 2- and 4-fold from that used in the initial trial. As shown in Table 7-2, however, this change also did not significantly alter the ratio of the fluorescence signals from treatment with 100 μM PKF118-310 to DMSO. Instead, the only difference observed was a decreased signal for both at each lower concentration, suggesting that saturation of the β-catenin present was not occurring below 1 μg/mL GST-Tcf4.

<table>
<thead>
<tr>
<th>β-catenin plating concentration</th>
<th>GST-Tcf4 concentration</th>
<th>1 μg/mL</th>
<th>0.5 μg/mL</th>
<th>0.25 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μg/mL</td>
<td>1.01 ± 0.41</td>
<td>0.80 ± 0.11</td>
<td>1.02 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>5  μg/mL</td>
<td>0.71 ± 0.29</td>
<td>0.96 ± 0.28</td>
<td>1.03 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>2.5 μg/mL</td>
<td>1.07 ± 0.31</td>
<td>0.63 ± 0.17</td>
<td>0.85 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>1.25 μg/mL</td>
<td>1.22 ± 0.35</td>
<td>1.00 ± 0.04</td>
<td>0.85 ± 0.27</td>
<td></td>
</tr>
</tbody>
</table>

Table 7-2: Comparison of treatment with PKF118-310 vs. DMSO at varying concentrations of GST-Tcf4 and plated β-catenin. The assay was performed as described in the experimental chapter. β-catenin was bound to microtiter plate wells. Varying amounts of GST-Tcf4 that had been pre-equilibrated with compound PKF118-310 in DMSO (final concentration = 100 μM) or DMSO was added to each well, followed by successive treatment with goat anti-GST antibody, alkaline phosphatase (AP)-conjugated anti-goat IgG, and Attophos™ fluorescent AP substrate. Fluorescence was measured by excitation at 440 nM and emission at 550 nm. All values were determined in triplicate with the mean values and standard deviations indicated above.

Since varying the concentration of either β-catenin or GST-Tcf4 did not affect the ability of PKF118-310 to inhibit the interaction between these two proteins, the concentration of the presumed “bystander” protein, bovine serum albumin (BSA), was also varied. Normally, as in previous reports, BSA is included to eliminate variation that might occur from non-specific binding and to minimize aggregation/unfolding. However, it seemed possible that PKF118-310 could have a high affinity for BSA. If this affinity were sufficiently high, there was the possibility that PKF118-310’s ability to bind to either β-catenin or Tcf4, and hence inhibit interaction, might be obscured. Figure 7-3 shows the results for DMSO versus 100 μM PKF118-310 with 0, 0.02, 0.2, and 2% BSA. As can be seen, no concentration of BSA tested revealed PKF118-310 as an inhibitor of β-catenin/Tcf4 interaction. In fact, the only difference observed was that the lowest
concentrations of BSA (0% and 0.02%) actually made PKF118-310 appear to be an activator!

![Figure 7-3: Binding data for DMSO control and 100 μM PKF118-310 with varying BSA concentration.](image)

**Figure 7-3: Binding data for DMSO control and 100 μM PKF118-310 with varying BSA concentration.** The assay was performed as described in the experimental chapter. β-catenin was bound to microtiter plate wells. 1 μg/mL GST-Tcf4 that had been pre-equilibrated with compound PKF118-310 in DMSO (final concentration = 100 μM) or DMSO and varying amounts of BSA was added to each well, followed by successive treatment with goat anti-GST antibody, alkaline phosphatase (AP)-conjugated anti-goat IgG, and Attophos™ fluorescent AP substrate. Fluorescence was measured by excitation at 440 nM and emission at 550 nm. All points were determined in triplicate with the mean values and error bars (standard deviation) shown for each point.

With the failure to observe inhibition of β-catenin/Tcf4 interaction by PKF118-310 under any of the conditions attempted, the last hypothesis had to be given consideration, namely, that PKF118-310 was not a true inhibitor of β-catenin/Tcf4 interaction. While this hypothesis seemed highly unlikely given that PKF118-310 was identified via a high-throughput version of this assay, the possibility that the sample used in the original paper contained some type of contaminant to produce the observed effect could not be ruled out. Moreover, later data gathered on this compound that will be discussed in the next chapter suggests that the luciferase reporter assay used in this paper to substantiate PKF118-310’s inhibition of this particular target is actually revealing an off-target effect. Hence, while unlikely, it was not impossible. Nonetheless, as inhibition
by PKF118-310 could not be demonstrated, another method had to be used to show that the assay was capable of detecting inhibition of the β-catenin/Tcf4 complex.

The ability of the assay to detect inhibition of β-catenin/Tcf4 interaction was eventually demonstrated by two complementary methods. The first of these utilized solubilized β-catenin when treating the plates with GST-Tcf4. As shown in Figure 7-4, soluble β-catenin, purified via two different preparation methods in different labs (Prep A and Prep B), was able to compete for Tcf4 binding to the plate-immobilized β-catenin, yielding reasonable binding curves in each case. While the amplitude of the fluorescence varied by ~2-3 fold between the two preparations, each yielded comparable IC₅₀ values for β-catenin under the assay conditions, where the [GST-Tcf4] was 39 nM. For Prep A, the IC₅₀ for competing β-catenin was 75.5 ± 17.3 nM, and for Prep B, the IC₅₀ for competing β-catenin was 59.1 ± 4.2 nM. (IC₅₀ values were determined using sigmoidal curve fits with Microcal Origin 8.0.) The correspondence of the IC₅₀ values for the two different preparations and their being within a 2-fold difference of the [GST-Tcf4] suggested that the assay was functioning properly and that inhibition was detectable, even when there were differences in the purity of β-catenin.
Figure 7-4: Binding assay curves for inhibition of β-catenin/Tcf4 interaction in the presence of soluble β-catenin. The assay was performed as described in the experimental chapter. β-catenin was bound to microtiter plate wells. 1 μg/mL GST-Tcf4 that had been pre-equilibrated with varying amounts of β-catenin was added to each well, followed by successive treatment with goat anti-GST antibody, alkaline phosphatase (AP)-conjugated anti-goat IgG, and Attophos™ fluorescent AP substrate. Fluorescence was measured by excitation at 440 nM and emission at 550 nm. All points were determined in triplicate with the mean values and error bars (standard deviation) shown for each point.

The next data to confirm the ability of the assay to detect inhibition of β-catenin/Tcf4 interaction was garnered from another screen hit reported in the same paper as PKF118-310. Compound PKF115-584, which is shown in Figure 7-5 and known as calphostin C, was reported to have an IC₅₀ of 3.2 μM for inhibiting β-catenin/Tcf4 interaction. Since this compound was commercially available from Sigma and since it was one of two screen hits for which all the subsequent assays verified inhibition of β-catenin/Tcf4 interaction, it was purchased and tested in the binding assay. (Note: For PKF118-310, not all of the subsequent assays in the paper substantiated the β-catenin/Tcf4 interaction as the target.) As can be seen in Figure 7-6, PKF115-584 was an inhibitor of β-catenin/Tcf4 interaction in this work also, with an IC₅₀ = 3.0 ± 0.1 μM, nearly identical to that reported. Hence, this result provided a second piece of solid evidence that the assay was functioning properly.
Figure 7-5: PKF115-584, another reported inhibitor of β-catenin/Tcf4 interaction

Figure 7-6: Binding assay curve for PKF115-584. The assay was performed as described in the experimental chapter. β-catenin was bound to microtiter plate wells. 1 μg/mL GST-Tcf4 that had been pre-equilibrated with varying amounts of PKF115-584 in DMSO or DMSO was added to each well, followed by successive treatment with goat anti-GST antibody, alkaline phosphatase (AP)-conjugated anti-goat IgG, and Attophos™ fluorescent AP substrate. Fluorescence was measured by excitation at 440 nM and emission at 550 nm. Maximum fluorescence with DMSO control was set to a relative value of 1. All points were determined in triplicate with the mean values and error bars (standard deviation) shown for each point.

With these two pieces of evidence to verify the assay’s ability to detect inhibition of β-catenin/Tcf4 interaction, another set of control experiments was performed to confirm that the reported interaction was that between β-catenin and Tcf4 and not a spurious artifact whereby GST was interacting instead. Hence, the β-catenin-coated plates were treated with either GST-Tcf4 or GST. Additionally, some wells were left uncoated with β-catenin, and still other wells that were plated with β-catenin received neither GST-Tcf4 nor GST treatment. These results are shown in Figure 7-7 and are
consistent within expectations. The binding detected with GST alone is less than 10% of that detected with GST-Tcf4 and is only minimally increased above the background fluorescence when either \(\beta\)-catenin or GST-Tcf4 is absent. The conclusion, therefore, was that binding by GST was not a complicating factor. It should be noted that two different concentrations of PKF118-310 were also tested in these controls, and again, no inhibition of \(\beta\)-catenin/Tcf4 interaction by PKF118-310 was detected, since treatment with either concentration of PKF118-310 behaved identically to that of the DMSO control.

**Figure 7-7: Binding data comparison for Tcf4 versus GST only binding.** The assay was performed as described in the experimental chapter. \(\beta\)-catenin was bound to microtiter plate wells, except for the data points for no \(\beta\)-catenin. 1 \(\mu\)g/mL GST-Tcf4 or 1 \(\mu\)g/mL GST or buffer only that had been pre-equilibrated with 100 \(\mu\)M (final conc.) PKF118-310 in DMSO, 0.4 \(\mu\)M (final conc.) PKF118-310 in DMSO, or DMSO was added to each well, followed by successive treatment with goat anti-GST antibody, alkaline phosphatase (AP)-conjugated anti-goat IgG, and Attophos™ fluorescent AP substrate. Fluorescence was measured by excitation at 440 nM and emission at 550 nm. All points were determined in triplicate with the mean values and error bars (standard deviation) shown for each point.

Unable to reproduce the literature findings with PKF118-310 but having supported the functionality of the binding assay through the other means described, testing of all of the synthesized analogues commenced. Rather than produce a full binding curve for every compound, the compounds were initially screened at 100 \(\mu\)M and
300 μM concentrations. Those for which there was a decrease in fluorescence of greater than 60% relative to DMSO at 100 μM were then further investigated, with full binding curves produced and IC₅₀’s determined. Each compound was screened in triplicate, and the average fluorescence relative to DMSO control on each plate was recorded. The negative control on each plate was treatment with 158 nM soluble β-catenin, as that concentration had completely suppressed Tcf4 binding in the control experiment with soluble competing β-catenin. The protocol used for the screen and for all subsequent binding curves shown is detailed in the experimental chapter.
Key: (black= controls)
Figure 7-8: Binding assay data for all compounds screened at 100 μM. The assay was performed as described in the experimental chapter. β-catenin was bound to microtiter plate wells. 1 μg/mL GST-Tcf4 that had been pre-equilibrated with 100 μM (final concentration) compound in DMSO or DMSO was added to each well, followed by successive treatment with goat anti-GST antibody, alkaline phosphatase (AP)-conjugated anti-goat IgG, and Attophos™ fluorescent AP substrate. Fluorescence was measured by excitation at 440 nM and emission at 550 nm. Maximum fluorescence with DMSO control was set to a relative value of 1. All points were determined in triplicate with the mean values and error bars (standard deviation) shown for each point.

The data for all compounds at 100 μM concentration are shown in Figure 7-8. As can be seen, very few potential inhibitors of β-catenin/Tcf4 were detected, and those that appear to inhibit to an appreciable degree (> 60%) are not clustered in any one subclass of all the analogues synthesized. Moreover, for those that show greater than 60% inhibition at 100 μM, only one synthesized compound, AJT-I-229, showed complete elimination (reduction of fluorescence to the same level as that of 158 nM β-catenin) of binding at 300 μM and produced a binding curve representative of true inhibition. The binding curve for AJT-I-229 is shown in Figure 7-9, alongside that of PKF115-584 and two other compounds that had shown promise in other assay work performed in a collaborator’s lab but which demonstrated no significant inhibition in this binding assay. The data shown are based on triplicate measurements for each concentration. From this binding data, the IC₅₀ for AJT-I-229 was determined to be 19.1 ± 3.9 μM- modest inhibition at best.
Figure 7-9: Binding assay curves for AJT-I-229, PKF115-584, AF121, AJT-I-137-1. The assay was performed as described in the experimental chapter. β-catenin was bound to microtiter plate wells. 1 µg/mL GST-Tcf4 that had been pre-equilibrated with varying concentrations of compound in DMSO or DMSO was added to each well, followed by successive treatment with goat anti-GST antibody, alkaline phosphatase (AP)-conjugated anti-goat IgG, and Attophos™ fluorescent AP substrate. Fluorescence was measured by excitation at 440 nM and emission at 550 nm. Maximum fluorescence with DMSO control was set to a relative value of 1. All points were determined in triplicate with the mean values and error bars (standard deviation) shown for each point.

While far from a potent inhibitor and weaker than those from the original report on which this work was based, AJT-I-229 was the best inhibitor of all compounds synthesized. To further validate the assay results, it was important to show that AJT-I-229 was inhibiting Tcf4 at a saturating concentration for the amount of β-catenin plated. Hence, increasing concentrations of GST-Tcf4 were used in the assay, in the presence and absence of 50 µM AJT-I-229 or 158 nM soluble β-catenin. As shown in Figure 7-10, the amount of β-catenin plated saturated at ~1 µg/mL of GST-Tcf4, which is the concentration that was used for all the results described. Both 158 nM soluble β-catenin and 50 µM AJT-I-229 virtually eliminated all binding up to this concentration of GST-Tcf4. Only at 2 µg/mL GST-Tcf4 did an increased amount of binding occur in the presence of 50 µM AJT-I-229. Moreover, the presence of DMSO had negligible effects on either the positive or negative controls, apart from a small artifact that was introduced.
at the lowest concentration of GST-Tcf4 tested in both the presence and absence of soluble β-catenin.

![binding assay curves](image)

**Figure 7-10: Binding assay curves for verifying GST-Tcf4 saturation.** The assay was performed as described in the experimental chapter. β-catenin was bound to microtiter plate wells. GST-Tcf4 at varying concentrations that had been pre-equilibrated in buffer only or with either DMSO, 158 nM β-catenin, 158 nM β-catenin and DMSO, or 50 μM (final conc.) AJT-I-229 in DMSO was added to each well, followed by successive treatment with goat anti-GST antibody, alkaline phosphatase (AP)-conjugated anti-goat IgG, and Attophos™ fluorescent AP substrate. Fluorescence was measured by excitation at 440 nM and emission at 550 nm. Fluorescence with 0.25 μg/mL GST-Tcf4, in the absence of DMSO, was set to a relative value of 1. All points were determined in triplicate with the mean values and error bars (standard deviation) shown for each point.

While seeming to exert little effect on the GST-Tcf4 saturation binding curves, the potential impact of DMSO on the reported results was further investigated by examining its effect on the IC₅₀ curve for AJT-I-229. The DMSO concentration was varied from 1% to 3% because it had been present at a 1% concentration when all of the compounds were tested at a 100 μM concentration and at a 2% concentration when producing the binding curves shown in Figure 7-9. The IC₅₀ values for AJT-I-229 at 1%, 2%, and 3% DMSO concentrations are shown in Table 7-3. While not identical, they are similar, with those at 1% and 2% being statistically the same. Hence, concentrations of DMSO 2% or less have a minimal effect on the assay, but when the DMSO concentration rises to 3%, it may start to abrogate inhibition. It should be noted that the ability of
soluble β-catenin to compete for Tcf4 binding was also tested in the absence and presence of 1%, 2%, and 3% DMSO, and here again, some divergence only started to occur at 3% DMSO.

<table>
<thead>
<tr>
<th>DMSO concentration</th>
<th>IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>11.3 ± 2.6</td>
</tr>
<tr>
<td>2%</td>
<td>14.0 ± 3.6</td>
</tr>
<tr>
<td>3%</td>
<td>22.9 ± 2.2</td>
</tr>
</tbody>
</table>

**Table 7-3: IC_{50} of AJT-I-229 at varying concentrations of DMSO**

Next, the binding curve for AJT-I-229 was measured at increasing concentrations of GST-Tcf4. Here, the anticipated result was that the binding curve would shift to the right at increasing concentrations of GST-Tcf4, as higher concentrations of GST-Tcf4 required higher amounts of AJT-I-229 for inhibition. Indeed, as shown in Figure 7-11, the expected result was confirmed, consistent with competitive inhibition of AJT-I-229 for the β-catenin/Tcf4 binding site.
Figure 7-11: Binding assay curves for AJT-I-229 at increasing GST-Tcf4 concentrations. The assay was performed as described in the experimental chapter. β-catenin was bound to microtiter plate wells. One of four concentrations of GST-Tcf4 that had been pre-equilibrated with varying concentrations of compound AJT-I-229 in DMSO or DMSO was added to each well, followed by successive treatment with goat anti-GST antibody, alkaline phosphatase (AP)-conjugated anti-goat IgG, and Attophos™ fluorescent AP substrate. Fluorescence was measured by excitation at 440 nM and emission at 550 nm. Maximum fluorescence with 1 μg/mL GST-Tcf4 for DMSO control was set to a relative value of 1. All points were determined in triplicate with the mean values and error bars (standard deviation) shown for each point.

Finally, the binding curve for AJT-I-229 was measured at a range of increasing β-catenin plating concentrations. Here, the objective was to observe whether or not there were polydirectional multivalent binding effects as the density of plated β-catenin increased. If so, the binding curve for AJT-I-229 would be expected to shift to the left (producing lower apparent IC₅₀ values) at higher β-catenin concentrations. The range of β-catenin plating concentrations tested varied from 0.5 to 32 μg/mL, and within this range, no variation in the apparent IC₅₀ value was observed, as shown by the binding curves in Figure 7-12. The only difference between the curves was the magnitude of the fluorescence signal, which increased in a linear manner with increased amounts of β-catenin on the plate available for binding, as would be expected.
Figure 7-12: Binding assay curves for AJT-I-229 at increasing β-catenin plating concentrations. The assay was performed as described in the experimental chapter. β-catenin was bound to microtiter plate wells at varying concentrations. 1 μg/mL GST-Tcf4 that had been pre-equilibrated with varying concentrations of compound AJT-I-229 in DMSO or DMSO was added to each well, followed by successive treatment with goat anti-GST antibody, alkaline phosphatase (AP)-conjugated anti-goat IgG, and Attophos™ fluorescent AP substrate. Fluorescence was measured by excitation at 440 nM and emission at 550 nm. Maximum fluorescence with DMSO control for 8 μg/mL β-catenin plated was set to a relative value of 1. All points were determined in triplicate with the mean values and error bars (standard deviation) shown for each point.

All of these results were consistent with AJT-I-229 being an inhibitor of the β-catenin/Tcf4 interaction, albeit a modest one. As AJT-I-229 is a unique pyrimidopyridazinedione in the series of compounds tested, with no closely related analogues, additional pyrimidopyridazinediones were synthesized as described in Chapter 3 and tested, but none of these showed any activity. Concurrently, a re-examination of the spectral data for AJT-I-229 revealed an impurity present from its synthesis, where diisopropyl azodicarboxylate (DIAD) had been used to effect the final oxidation. DIAD, in fact, was still present at a significant molar concentration (~50%) in the sample of AJT-I-229 that had been tested. Hence, AJT-I-229 was recrystallized from ethanol and retested, and unfortunately, all of the activity disappeared. To further confirm that the actual inhibitor was DIAD, binding curves were measured for the following: DIAD, diethyl azodicarboxylate (DEAD), and diisopropyl hydrazine-1,2-dicarboxylate (reduced

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form of DIAD). The results are shown in Figure 7-13 and clearly show that the actual inhibitor of β-catenin/Tcf4 interaction for the data described for AJT-I-229 is DIAD. This is further supported by the observation that DEAD, a close analogue, is nearly as strong of an inhibitor as DIAD. Although full binding curves were not measured for both DIAD and DEAD due to their unexpected potency, the data shown suggests that DIAD inhibits at submicromolar concentrations. Moreover, diisopropyl hydrazine-1,2-dicarboxylate- the reduced form of DIAD and product of the oxidation to AJT-I-229- shows no inhibition. Whether DIAD inhibits the interaction between the two proteins by noncovalent binding at the active site or via reaction with a residue of either Tcf4 or β-catenin cannot be determined from this data. Nonetheless, since DIAD is likely to be quickly reduced in the cellular environment and AJT-I-229 contaminated with DIAD showed no activity in the cellular data to be described, it does not represent a promising lead for further study.

Figure 7-13: Binding assay curves for DIAD, DEAD, and diisopropyl hydrazine-1,2-dicarboxylate. The assay was performed as described in the experimental chapter. β-catenin was bound to microtiter plate wells at varying concentrations. 1 μg/mL GST-Tcf4 that had been pre-equilibrated with varying concentrations of recrystallized AJT-I-229, recrystallized AJT-I-229 and DIAD, DEAD, DIAD, or diisopropyl hydrazine-1,2-dicarboxylate in DMSO or DMSO was added to each well, followed by successive treatment with goat anti-GST antibody, alkaline phosphatase (AP)-conjugated anti-goat IgG, and Attophos™ fluorescent AP substrate. Fluorescence was measured by excitation at 440 nM and emission at 550 nm. Maximum fluorescence for DMSO control was set to a relative value of 1. All points were determined in triplicate with the mean values and error bars (standard deviation) shown for each point.
In conclusion, after cloning, expression, and purification of both β-catenin and GST-Tcf4, an *in vitro* binding assay for measuring inhibition of the interaction between β-catenin and Tcf4 was implemented, based on the previous report of a high-throughput screen. Unfortunately, after screening all synthesized compounds at 100 μM concentrations, few inhibitors were found, and the screen hit upon which this work was based (PKF118-310) also failed to inhibit the interaction. Nonetheless, the reported assay was validated by the following experiments: 1) successful competition of soluble β-catenin for Tcf4, 2) reproduction of the IC$_{50}$ value for another inhibitor (PKF115-584) from the same report, and 3) the finding of another small-molecule inhibitor of the interaction (DIAD), albeit not one of the targets synthesized and not one with promise for future development.
Concurrent with the efforts to implement the *in vitro* binding assay for testing inhibition of the β-catenin/Tcf4 interaction by all of the synthesized analogues was work carried out by Dr. Guido Bommer in Professor Eric Fearon’s lab (University of Michigan Medical School) to test the compounds in a cellular reporter assay. Here, the goal was to measure both the ability of the compounds to inactivate transcription by the β-catenin/Tcf4 complex and the nonspecific toxicity they demonstrated. Figure 8-1 shows a schematic of the overall plan for this functional *in vitro* assay. Initially, two different cell lines were investigated: SW480 human colon adenocarcinoma cells and IEC18 rat epithelial cells. The SW480 cell line was ideal in that it represented the actual target for the compounds synthesized; however, the caveat was that since its survival was dependent on upregulation of β-catenin, compounds that inhibited β-catenin’s action could also cause cell death, as was the intention in investigating these compounds as a potential new treatment for colon cancer. Cell death caused via inhibition of β-catenin/Tcf4 interaction versus nonspecific cell death, however, would not differentiated.

![Figure 8-1: Cellular reporter assay to measure toxicity and to assess ability of compounds to inhibit interaction of β-catenin/Tcf4](image)

**Chapter 8**

**Testing of Synthesized Analogues in Cellular Reporter Assay and Versus Luciferase**
Since differentiating nonspecific cell death from that caused by the intended mechanism of action was vital in this initial screen of all compounds synthesized, efforts soon focused on the IEC18 cell line, which was then used for all of the cellular assay work to be described. The non-transformed rat ileal epithelial cell line IEC18 (Cat. No. CRL-1589, available from American Type Tissue Cultures, Inc.) was used because it possesses several characteristics of normal intestinal epithelial cells and shows virtually no baseline transcriptional activation of β-catenin target genes. The description of the cellular assay used has been provided by Dr. Guido Bommer and is included in the experimental chapter.

![Figure 8-2: Cellular assay results for PKF118-310.](image)

The assay was performed as described in the experimental chapter. Transfected IEC18 cells were incubated with varying concentrations of PKF118-310 for 17-20 h. For toxicity measurements, WST-1 reagent (available from Roche) was added to the cells and the cells were incubated for 1 h at 37 °C, after which time absorption at 550 nm was measured. Following the absorption measurements, the tissue culture medium was aspirated and the cells were lysed. Renilla and firefly luciferase activity were then measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.

For reference, the assay results for the screen hit, PKF118-310, are shown in Figure 8-2. As can be seen, PKF118-310 is reasonably potent (TOPFLASH inhibition), toxic (WST-1 inhibition), and nonspecific, as there is no differentiation between the
TOPFLASH firefly luciferase reporter construct for β-catenin/Tcf4 activation and *Renilla* luciferase activity from CMV-promotion. There were two goals in synthesizing and testing analogues, therefore. The first was to maximize inhibition of the β-catenin/Tcf4 complex via determination of structure-activity relationships for inhibition of the TOPFLASH luciferase reporter. The second was to find analogues that could maintain inhibition of the TOPFLASH reporter construct, signifying inhibition of β-catenin/Tcf4 activation, while decreasing inhibition of CMV-promoted *Renilla* luciferase and generalized toxicity.

The first series of analogues tested was that of the parent compound, PKF118-310, namely, the N1-substituted pyrimidotriazinediones. Unfortunately, as with PKF118-310, the inhibition of CMV-promoted *Renilla* luciferase did not vary significantly from inhibition of the TOPFLASH reporter construct (data not shown), and there was little separation between decreased WST-1 activity and inhibition of either luciferase. The data for inhibition of the TOPFLASH luciferase reporter for the 1-methyl-pyrimidotriazinediones and 1-(2-hydroxyethyl)-pyrimidotriazinediones series are shown in Figures 8-3 and 8-4, respectively. Unfortunately, most of the analogues synthesized were less potent than the screen hit (PKF118-310 = black line on each graph, for reference). Moreover, these data curves do not demonstrate any clear structure-activity relationships with respect to the R3 substituents, as there is no correlation with either steric or electronics, and the relative order of activities of analogues for different R3 substituents varies from one series to the other.

Table 8-1 lists the LD50 and IC50 values calculated for all of the analogues tested in these two series. The values were calculated using MicroCal Origin 8.0 software by fitting sigmoidal curves to the average data for a minimum of two replicates. Comparison of the dimethyl-substituted series (entries 5a-5o) with the 1-(2-hydroxyethyl)-6-methyl-substituted series (entries 8a-8j) reveals that for most R3 substituents, both the LD50 and IC50 values were higher with the 2-hydroxyethyl group at N1 than with a methyl substituent. With both being elevated in the 2-hydroxyethyl series, however, the resulting LD50/IC50 ratios were approximately the same between each series. The trend of higher LD50 and IC50 values in the 2-hydroxyethyl series was not universal, however, as both the 4-dimethylaminophenyl (5l and 8i) and 2-pyridyl (5o and 8j) substituents...
showed the opposite trend between series. Nonetheless, it should be noted that the analogues with these two R₃ substituents were two of the weakest inhibitors in both series. One of the strongest inhibitors of the TOPFLASH luciferase reporter from these two series was 5j (3-(4-(2-(diethylamino)ethoxy)phenyl)-1,6-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione), with an IC₅₀ of 0.136 ± 1 μM. This IC₅₀ is comparable to that of the original screen hit, PKF118-310, but the LD₅₀/IC₅₀ ratio is significantly improved, increasing from 1.81 to 8.32. This promising result stimulated further exploration of the 4-(2-(diethylamino)ethoxy)phenyl substituent at R₃ in some of the other series of pyrimidotriazinediones that were synthesized as described and tested.

Figure 8-3: TOPFLASH luciferase reporter data for 1-methylpyrimidotriazinediones. The assay was performed as described in the experimental chapter. Transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.
Figure 8-4: TOPFLASH luciferase reporter data for 1-(2-hydroxyethyl)pyrimidotriazinediones. The assay was performed as described in the experimental chapter. Transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.

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<th>R₁</th>
<th>R₃</th>
<th>Entry</th>
<th>LD₅₀ (μM)</th>
<th>IC₅₀ (μM)</th>
<th>LD₅₀/IC₅₀</th>
</tr>
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<td>0.19 ± 0.01</td>
<td>0.11 ± 0.21</td>
<td>1.81</td>
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<td>Structure</td>
<td>Formula</td>
<td>Value</td>
<td>Error</td>
<td>Unit</td>
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<td>-----------</td>
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<td>0.136 ± 0.01</td>
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<td>8.32</td>
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<td>1.56 ± 28000</td>
<td>1.51 ± 18000</td>
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<td>CH₂CH₂OH</td>
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<td>8h</td>
<td>2.60 ± 100</td>
<td>1.50 ± 100</td>
<td>1.73</td>
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Within the subclass of 1,3,6-substituted pyrimidotriazinediones, there were a couple of variants from the two series (1,6-dimethyl- and 1-(2-hydroxyethyl)-6-methyl-) described above that had been synthesized and tested. In some of these, there was a non-methyl substituent at N₆, and in others there was either no substituent at N₁ or a substituent other than methyl or 2-hydroxyethyl. Table 8-2 shows the LD₅₀ and IC₅₀ values for a representative set of analogues from these unique 1,3,6-substituted pyrimidotriazinediones. As can be seen, the lack of a substituent at N₁ was clearly detrimental to activity, as only one (6a) of the two N₁-H analogues (6a and 6b) demonstrated any activity, with that activity being only modest (21 μM). Nonetheless, there was also no measurable toxicity below 50 μM with these two analogues. In contrast, for the N₆-H analogues (19d and 19e), both activity and toxicity were comparable to the N₆-methyl analogues. When varying the N₆- substituent from methyl, the results were mixed. When the 6- substituent was 2-hydroxyethyl (19a) or 4-fluorobenzyl (19b), there was no measurable inhibition of luciferase activity or toxicity, but when it was 3,4-difluorobenzyl (19c), it was both potent and toxic. This last analogue (19c), however, also had a different R₃ substituent, so the effects observed are most likely a combined effect of both substituents. Nonetheless, additional analogues were not pursued in this subclass of 1,3,6-substituted pyrimidotriazinediones because there was no example where there was a significant increase in inhibitory activity from the 1,6-dimethyl series or a notable gain in the window between the concentration that showed activity (IC₅₀) and that demonstrating toxicity (LD₅₀).
<table>
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<th>R₁</th>
<th>R₃</th>
<th>R₆</th>
<th>Entry (Notebook ID)</th>
<th>LD₅₀ (μM)</th>
<th>IC₅₀ (μM)</th>
<th>LD₅₀/IC₅₀</th>
</tr>
</thead>
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<td>H</td>
<td>H₆C-</td>
<td>CH₃</td>
<td>6a (AF-127-1)</td>
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<td>21.08 ± 14.14</td>
<td>&gt;2.37</td>
</tr>
<tr>
<td>H</td>
<td>F-</td>
<td>CH₃</td>
<td>6b (AF-97-2)</td>
<td>&gt;50</td>
<td>&gt;50</td>
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<tr>
<td>CH₃</td>
<td>H</td>
<td>CH₃</td>
<td>PKF118-310</td>
<td>0.19 ± 0.01</td>
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<td>4.19 ± 5.98</td>
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<td>CH₂CH₂OH</td>
<td>19a (AJT-18-1)</td>
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<td>&gt;50</td>
<td>NA</td>
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<tr>
<td>CH₃</td>
<td>H₂C-H-</td>
<td>CH₂CH₂OH</td>
<td>19b (AJT-291-2)</td>
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<tr>
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<td>H</td>
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<tr>
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<td>1.09 ± 258</td>
<td>0.11 ± 0.05</td>
<td>9.91</td>
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Table 8-2: IC₅₀ and LD₅₀ values for N₁-H-pyrimidotriazinediones and pyrimidotrazinediones with nonmethyl substituents at N₁ or N₆
Figure 8-5: TOPFLASH luciferase reporter data for some 8-substituted pyrimidotriazinediones. The assay was performed as described in the experimental chapter. Transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.

3,6,8-substituted pyrimidotriazinediones were subsequently tested, and as the data for the TOPFLASH luciferase construct for a small subset shows in Figure 8-5, here the results were significantly different from those for the 1,3,6-substituted analogues, in that activity was significantly reduced, being practically negligible in most cases. While not shown, Renilla luciferase was also not inhibited by any of the analogues from this series. Moreover, there was little to no toxicity for any of these analogues in the WST-1 assay. While the loss of TOPFLASH luciferase inhibition was disappointing, the concurrent lack of toxicity suggested that moving from the 1,3,6-substituted series to the 3,6,8-substituted series might be a way to differentiate inhibition from cell toxicity. Table 8-3 is a compilation of the LD50 and IC50 values that were determined for all 3,6,8-substituted analogues that were tested. It should be noted that analogues 10a-10h were synthesized
with the specific intention of trying to preserve the activity of the 1,3,6-substituted series and still avoid the toxicity of the 3,6,8-substituted series by incorporating one of the most potent R₃ groups of the 1,3,6-substituted series. Unfortunately, this strategy was only marginally successful, for these compounds are the only ones to demonstrate measurable toxicity in this series and their inhibitory activity is not as potent as that of many in the 1,3,6-substituted series.

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<th>Entry (Notebook ID)</th>
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<th>IC₅₀ (μM)</th>
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<td></td>
<td></td>
<td>7h (AG-169-1)</td>
<td>&gt;50</td>
<td>&gt;50</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>7i (AG-199-1)</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>NA</td>
</tr>
<tr>
<td>H₃C</td>
<td>F-</td>
<td>7j (AG-139-1)</td>
<td>&gt;300</td>
<td>0.31 ± 0.01</td>
<td>&gt; 968</td>
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<tr>
<td></td>
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<td>7k (AG-115-1)</td>
<td>&gt;300</td>
<td>0.33 ± 0.01</td>
<td>&gt; 909</td>
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<tr>
<td>O₂N</td>
<td></td>
<td>7l (AG-164-2)</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>NA</td>
</tr>
<tr>
<td>F₃C</td>
<td></td>
<td>7m (AG-160-1)</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>NA</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>7n (AG-161-1)</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>NA</td>
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<td>Compound</td>
<td>Half-life</td>
<td>IC50</td>
<td>Remarks</td>
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<tr>
<td>7o</td>
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<td>&gt;50</td>
<td>undet.</td>
<td>NA</td>
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<tr>
<td>7p</td>
<td>(AG-168-1)</td>
<td>&gt;300</td>
<td>&gt;300</td>
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<td>7r</td>
<td>(AG-239-1)</td>
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<td>&gt;300</td>
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<td>7s</td>
<td>(AG-146-2)</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>7t</td>
<td>(AG-112-1)</td>
<td>&gt;50</td>
<td>1.68 ± 0.91</td>
<td>&gt;29.8</td>
<td></td>
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<tr>
<td>7u</td>
<td>(AG-187-1)</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>10a</td>
<td>(AG-214-2)</td>
<td>12.57 ± 10.99</td>
<td>5.72 ± 4.11</td>
<td>2.20</td>
<td></td>
</tr>
<tr>
<td>10b</td>
<td>(AG-244-2)</td>
<td>21.49 ± 10.42</td>
<td>23.41 ± 2.83</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>10c</td>
<td>(AG-263-2)</td>
<td>9.32 ± 1380</td>
<td>6.11 ± 0.72</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td>10d</td>
<td>(AG-262-2)</td>
<td>36.31 ± 4400</td>
<td>29.77 ± 3.89</td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>10e</td>
<td>(AG-247-1)</td>
<td>46.07 ± 1280</td>
<td>27.38 ± 8.92</td>
<td>1.68</td>
<td></td>
</tr>
<tr>
<td>10f</td>
<td>(AG-291-1)</td>
<td>81.08 ± 14.73</td>
<td>undet.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10g</td>
<td>(AG-258-2)</td>
<td>1.20 ± 1392</td>
<td>92.80 ± 18.02</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>10h</td>
<td>(AJT-213-1)</td>
<td>2.04 ± 0.76</td>
<td>1.10 ± 0.11</td>
<td>1.85</td>
<td></td>
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</tbody>
</table>
When testing the pyrimidotriazinediones, some of the synthetic intermediates were also tested, and these are shown in Figure 8-6, along with the testing results. As can be seen, the hydrazone intermediate showed no activity, whereas the N-oxides were comparable to the analogous reduced ring systems. As the N-oxide was not a significant improvement and N-oxides are generally not favorable for drug development, no additional N-oxides were tested.
After testing the pyrimidotriazinediones against the TOPFLASH reporter construct, representative members of each of the other heterocyclic classes synthesized were also tested. Unfortunately, none of the pyrimidopyridazinediones, pyrazolopyrimidinediones, pyrrolopyrimidinediones, or pyrimidopyrimidinediones that were tested demonstrated any measurable inhibition of TOPFLASH luciferase activity, suggesting that each of the nitrogen atoms of the core structure of the screen hit were vital for its activity and that no alterations could be made. At the same time, however, none of these alternate heterocycles showed any measurable toxicity within the range of concentrations tested. This result is promising should any of these pyrimidopyridazinediones, pyrazolopyrimidinediones, pyrrolopyrimidinediones, or pyrimidopyrimidinediones demonstrate activity versus another target in a future screening campaign, but for this work, these results leave little reason to continue exploring any alternate heterocyclic systems.

Given the discrepancy between the reporter assay results and the in vitro binding assay, there was no evidence that the analogues synthesized were inhibiting the intended target: the interaction between β-catenin and Tcf4. Moreover, since the results from the cellular reporter assay were not consistently reproducible and since whenever there was activity against the TOPFLASH luciferase construct there was also inhibition of CMV-driven Renilla luciferase either at the same concentration or at a slightly higher concentration (usually no larger than 2-3 fold), solid evidence that the Wnt signaling pathway was being selectively inhibited was also lacking. To date, the only supporting data provided by Dr. Fearon’s lab suggestive of the Wnt signaling pathway being targeted are some survival curves produced from the treatment of various cancer cell lines with compound 10a for 48h. This data is shown in Figure 8-7, and important to note is the Wnt signaling pathway dependence of each cell line tested. BxPC3 and MOH1 are both pancreatic cancer cell lines not known to be dependent on β-catenin upregulation, and NCI-H-1299 is a lung cancer cell line, also not dependent on β-catenin upregulation. Meanwhile, HT29, SW480, and DLD1 are all colon cancer cell lines, each of which is dependent on β-catenin upregulation. Finally, IEC18 is the rat intestinal epithelial line used for the reporter assay which should not be dependent on Wnt signaling. If the Wnt signaling pathway is the target of compound 10a, one would expect those cell lines
dependent on β-catenin upregulation \( i.e., \) HT29, SW480, and DLD1) to be more susceptible to treatment by compound 10a. Observation of the data in Figure 8-7 shows that the HT29, SW480, and DLD1 cell lines were three of the five cell lines most susceptible to treatment with 10a and that the BxPC3 and NCI-H-1299 cell lines were the two least susceptible, consistent with the expectation for targeting of the Wnt signaling pathway. Nonetheless, both the IEC18 and MOH1 cell lines were as susceptible as the HT29, SW480, and DLD1 cell lines, which would not be consistent with Wnt pathway targeting. Moreover, the overall difference in compound concentration effecting 50% cell death between the most susceptible cell lines and the least susceptible cell lines is not very large, being less than one log factor. Hence, while this data was presented as corroborating evidence for targeting of the Wnt signaling pathway, it is far from conclusive and raises as many questions as it answers.

![Figure 8-7: Survival curves for various cell lines treated with compound 10a for 48h](image)

Still lacking evidence for the target of the pyrimidotriazinedione analogues that demonstrated activity in the TOPFLASH reporter assay, additional experiments needed to be done. First, the potential of the analogues synthesized to inhibit firefly luciferase itself needed to be evaluated, for our collaborators had never done this experiment. As such, firefly luciferase was obtained from Professor Jason Gestwicki (University of
Michigan, LSI), as well as all necessary buffers and reagents for testing the ability of compounds to inhibit the activity of firefly luciferase. Initially, a small subset of synthesized analogues was tested, including some that had shown activity against the TOPFLASH reporter construct and some that had not, as well as the screen hit, PKF118-310. The test was performed using the protocol detailed in the experimental chapter.

The results from this initial testing set are shown in Figure 8-8a. Of this subset of compounds, AF121 and AC128 are 1,6-dimethylpyrimidotriazinediones that inhibited the TOPFLASH luciferase reporter construct, both with IC$_{50}$ values less than 2 $\mu$M. AG214 is a 3,6,8-substituted pyrimidotriazinedione, also with an IC$_{50}$ value less than 2 $\mu$M in the initial cellular reporter assay data. (It should be noted that this result was not consistently reproducible and was found to vary up to 10-20 $\mu$M.) Finally, AC7 is one of the pyrimidopyridazinediones, which showed no activity in the cellular reporter assay. As can be clearly seen, three of the compounds from this set (AF121, AC128, and PKF118-310) inhibit firefly luciferase very strongly. Moreover, there is a high correlation between the data of this in vitro test against firefly luciferase and the data of the cellular luciferase reporter assay, which is shown for the same compounds in Figure 8-8b. In fact, the only compound for which there is a large discrepancy between the two assays is AG214. These results raise serious questions about the interpretation and utility of the data reported by Dr. Fearon’s lab for the cellular luciferase reporter assay and results in the literature for PKF118-310 based on luciferase reporter data. Due to these alarming results, the decision was made to test all of the compounds that had been tested in the cellular reporter assay against firefly luciferase in vitro.
(a) The assay was performed as described in the experimental chapter. Compounds at varying concentrations in DMSO or DMSO were spotted into each well. Steady-Glo® luciferase substrate was added, followed by firefly luciferase enzyme. Luminescence was recorded after 5-minute incubation period at RT (integration time = 500 ms). Maximum luminescence with DMSO control was set to a relative value of 1. All points were determined in triplicate with the mean values and error bars (standard deviation) shown for each point.

(b) The assay was performed as described in the experimental chapter. Transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point. Figure 8-8: Testing of subset of synthesized analogues: (a) versus firefly luciferase in vitro and (b) in cellular luciferase reporter assay (TOPFLASH construct).

To minimize the cost of reagents that were being generously donated by Professor Gestwicki for this in vitro luciferase work, testing of the complete collection of synthesized analogues was done in 384-well plate format. In doing so, Lyra Chang (graduate student in Professor Gestwicki’s lab) was consulted for scale-up, as she had
previously optimized the assay for high-throughput screening. Additionally, Tom McQuade in the Center for Chemical Genomics (CCG) screening laboratory at the University of Michigan helped automate the compound dilutions. As such, Tom prepared plates with eight two-fold dilutions of each test compound such that the final testing concentrations of the compounds ranged were 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 μM. The eight dilutions for each compound were repeated in quadruplicate, and the volume of the test compound solution in each well was 5 μL. Additionally, since a number of compounds inhibited firefly luciferase at submicromolar concentrations, a second set of dilutions was done subsequently from 3 μM down to 23 nM, again with the volume of the compound solution in each well being 5 μL. The protocol for testing all of the compounds in the 384-well format is included in the experimental chapter.

The results from this *in vitro* testing against firefly luciferase are shown in Figures 8-9 through 8-17. For comparison, alongside each set of *in vitro* testing data are the results from the cellular luciferase reporter assay for the corresponding compounds. As can be seen and will be described, a significant number of analogues were potent inhibitors of firefly luciferase, and in many cases, there is a substantial degree of correspondence between the two sets of data (*in vitro* and cellular reporter data). Taken together, these results are highly indicative of luciferase inhibition playing a major role in the data reported by Dr. Fearon’s lab and raise questions about how much information can be gleaned with respect to the desired target of either the β-catenin/Tcf4 interaction or the Wnt signaling pathway more generally.
(a) The assay was performed as described in the experimental chapter. Compounds at varying concentrations in DMSO or DMSO were spotted into each well. Firefly luciferase was added, followed by Steady-Glo® luciferase substrate. Luminescence was recorded after 10-30 minute incubation period at RT (integration time = 500 ms). Maximum luminescence with DMSO control was set to a relative value of 1. All points were determined in quadruplicate with the mean values and error bars (standard deviation) shown for each point.

(b) The assay was performed as described in the experimental chapter. Transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.

Figure 8-9: Testing of 1,6-dimethylpyrimidotriazinediones:
(a) versus firefly luciferase in vitro
(dashed lines = higher concentration data, distinct data set)
(b) in cellular luciferase reporter assay (data from Fearon lab)
(Note: Legend indicates R3 groups.)
Shown in Figure 8-9 are the data from the two assays for the 1,6-dimethylpyrimidotriazinediones. This is the series of pyrimidotriazinediones which had been the most potent, overall, at inhibiting the TOPFLASH luciferase reporter construct, and which had also demonstrated the greatest degree of toxicity. As can be seen, while there are not direct parallels between the *in vitro* and the cellular data for rank ordering all of the R3 substitutents within the series, there are significant similarities in the two data sets. Notably, most analogues tested were appreciable inhibitors in both assays, having IC$_{50}$ values less than 2 μM, and in both data sets there are some potent inhibitors with submicromolar IC$_{50}$ values. Moreover, the one analogue in this series with virtually no activity in the cellular assay (where R3= 2-pyridyl) also is the only one with no appreciable activity in the *in vitro* assay. IC$_{50}$ values for the *in vitro* luciferase testing were calculated using MicroCal Origin 8.0 software by fitting sigmoidal curves to the average data for four replicates and are shown for this series in Table 8-4.

<table>
<thead>
<tr>
<th>R$_3$</th>
<th>IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2$N phenyl</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>Cl phenyl</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>O$_2$N phenyl</td>
<td>0.26 ± 0.09</td>
</tr>
<tr>
<td>F phenyl</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td>Cl phenyl</td>
<td>0.70 ± 0.02</td>
</tr>
<tr>
<td>H$_2$C phenyl</td>
<td>0.78 ± 0.02</td>
</tr>
<tr>
<td>N[O]O phenyl</td>
<td>0.83 ± 0.05</td>
</tr>
<tr>
<td>phenyl</td>
<td>0.91 ± 0.10</td>
</tr>
</tbody>
</table>
Table 8-4: IC_{50} values for inhibition of firefly luciferase in vitro by 1,6-dimethylypyrimidotriazinediones

<table>
<thead>
<tr>
<th>Substituent</th>
<th>IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-OH</td>
<td>0.96 ± 0.07</td>
</tr>
<tr>
<td>H-O-O</td>
<td>0.97 ± 0.36</td>
</tr>
<tr>
<td>(N_{4}-oxide)</td>
<td>1.26 ± 0.03</td>
</tr>
<tr>
<td>CH_{3}CH_{2}-</td>
<td>1.29 ± 0.27</td>
</tr>
<tr>
<td>C</td>
<td>1.50 ± 0.05</td>
</tr>
</tbody>
</table>

Unlike the cellular reporter assay, however, for which there were no discernible structure activity relationships within the series, a clear trend is observed for the in vitro assay. For this series, all of the phenyl substituents (with the possible exception of phenyl itself) were more potent than the ethyl substituent, and the relative order of inhibition of firefly luciferase was as follows for substituents on phenyl R_{3} (most potent inhibitor to least potent): m-NO_{2} > m-Cl > p-NO_{2} > p-F > p-Cl > p-CH_{3} > p-Et_{2}NCH_{2}CH_{2}O > o-F > p-CH_{3}O > H. This trend clearly shows that analogues with electron-withdrawing substituents on R_{3} are better inhibitors of firefly luciferase, as the most strongly electron-withdrawing substituent, NO_{2}, was the most potent, with the halogenated analogues only slightly less potent and those with electron-donating substituents (p-Et_{2}NCH_{2}CH_{2}O and p-CH_{3}O) being among the weakest. Moreover, there was a clear preference for the meta substitution of the electron-withdrawing substituent over the para and ortho, for each of m-NO_{2} and m-Cl were more potent than their respective para-substituted congeners, and p-F was more potent than o-F substitution.

These data clearly suggest that inhibition of the luciferase reporter itself in the cellular reporter assay is occurring, but the differences within the series between the two data sets could be explained either by the compounds also exerting an effect on the Wnt signaling pathway or differences in compound solubility, cell permeability, or other factors related to the effective concentration achieved inside the cell for each compound. These latter considerations cannot be ruled out based on this data alone, particularly since the most potent inhibitor within this series in the cellular assay was the analogue with the highest water solubility (R_{3}= p-Et_{2}NCH_{2}CH_{2}O-C_{6}H_{4}).
The assay was performed as described in the experimental chapter. Compounds at varying concentrations in DMSO or DMSO were spotted into each well. Firefly luciferase was added, followed by Steady-Glo® luciferase substrate. Luminescence was recorded after 10-30 minute incubation period at RT (integration time = 500 ms). Maximum luminescence with DMSO control was set to a relative value of 1. All points were determined in quadruplicate with the mean values and error bars (standard deviation) shown for each point.

The assay was performed as described in the experimental chapter. Transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.

**Figure 8-10: Testing of 1-(2-hydroxyethyl)-6-methylpyrimidotriazinediones:**

(a) versus firefly luciferase *in vitro*  
(dashed lines = higher concentration data, distinct data set)  
(b) in cellular luciferase reporter assay (data from Fearon lab)  
(Note: Legend indicates R₃ groups.)
The data for the 1-(2-hydroxyethyl)-6-methylpyrimidotriazinediones that were tested in both assays are shown in Figure 8-10. IC\textsubscript{50} values for the \textit{in vitro} assay were calculated the same as for the 1,6-dimethyl series and are shown in Table 8-5. It should be noted that Table 8-5 is expanded relative to Figure 8-10 to include analogues tested \textit{in vitro} which were not tested in the cellular assay or for which the cellular data was questionable. In this series, as with the 1,6-dimethyl series, there is significant inhibition observed in both assays, indicating that inhibition of the luciferase reporter in the cellular assay is an issue. Overall, this series shows slightly less potency in both assays than the 1,6-dimethyl series, and the 2-pyridyl substituent is, again, the weakest inhibitor in both data sets. Moreover, while not as clear, there is some evidence of the same structure activity relationship observed for the 1,6-dimethyl series in the \textit{in vitro} data. For the \textit{in vitro} data, the relative order of inhibition of firefly luciferase was as follows for substituents on phenyl R\textsubscript{3} (most potent inhibitor to least potent): 3,4-di(CH\textsubscript{3}O) > m-Cl > 3,4-diCl > p-CH\textsubscript{3}O > p-CH\textsubscript{3} > p-Cl > Ph > p-(CH\textsubscript{3})\textsubscript{2}N > p-R\textsubscript{2}NCH\textsubscript{2}CH\textsubscript{2}O. Hence, like the 1,6-dimethyl series, those analogues with a substituent at the meta position (3,4-di(CH\textsubscript{3}O), m-Cl, 3,4-diCl) are the most potent inhibitors of firefly luciferase. Unlike the 1,6-dimethyl series, however, there is not the clear preference for electron-withdrawing substituents, as the dimethoxy analogue was more active than the dichloro analogue and the p-CH\textsubscript{3}O- and p-CH\textsubscript{3}– substituted analogues were more active than the p-Cl. Nonetheless, since analogues with the strongly-withdrawing nitro groups were not synthesized for this series, there is not sufficient data to generalize the data trends observed between this subclass of 1,3,6-trisubstituted inhibitors and that above. In considering the juxtaposed data from the two assays in this subseries, there is greater correspondence in the rank ordering of different R\textsubscript{3} substituents, which might be explained by the greater water solubility and likely higher intracellular concentrations achieved by this set over the 1,6-dimethyl series. The relative order of inhibition for the cellular reporter assay was approximately as follows for phenyl-substituted R\textsubscript{3} (most potent inhibitor to least potent): 3,4-di(CH\textsubscript{3}O) > p-Cl > Ph > 3,4-diCl > p-CH\textsubscript{3}O ≈ p-CH\textsubscript{3} > p-(CH\textsubscript{3})\textsubscript{2}N. Here, as with the \textit{in vitro} data, the 3,4-di(CH\textsubscript{3}O)- substituted analogue was the most potent, and the p-(CH\textsubscript{3})\textsubscript{2}N was one of the weakest, with much of the same
clustering in the middle for the \( p-\text{Cl} \), \( p-\text{CH}_3 \), and \( p-\text{CH}_3\text{O} \)-substituted analogues. When viewed together, therefore, the data for both assays within the 1-(2-hydroxyethyl)-6-methyl subseries continue to corroborate the hypothesis that while the cellular reporter assay is reporting out the results for which it was engineered, the data interpretation does not take into account confounding factors due to luciferase inhibition by this series of inhibitors. Thus, an alternate assay using a different reporter construct will eventually be required to supply unequivocal proof that the synthesized compounds bind to the desired target.

<table>
<thead>
<tr>
<th>( R_3 )</th>
<th>( \text{IC}_{50} ) (( \mu \text{M} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>[ \text{H}_3\text{C} ]</td>
<td>0.47 ± 0.04</td>
</tr>
<tr>
<td>[ \text{H}_3\text{CO} ]</td>
<td>0.55 ± 0.12</td>
</tr>
<tr>
<td>[ \text{Cl} ]</td>
<td>0.68 ± 0.21</td>
</tr>
<tr>
<td>[ \text{Cl} ]</td>
<td>0.76 ± 0.09</td>
</tr>
<tr>
<td>[ \text{H}_3\text{C} ]</td>
<td>0.92 ± 0.13</td>
</tr>
<tr>
<td>[ \text{Cl} ]</td>
<td>0.97 ± 0.17</td>
</tr>
<tr>
<td>[ \text{N} ]</td>
<td>1.13 ± 0.32</td>
</tr>
<tr>
<td>[ \text{N} ]</td>
<td>1.32 ± 0.11</td>
</tr>
<tr>
<td>[ \text{N} ]</td>
<td>1.42 ± 0.11</td>
</tr>
<tr>
<td>[ \text{N} ]</td>
<td>3.29 ± 0.59</td>
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</tbody>
</table>

Table 8-5: \( \text{IC}_{50} \) values for inhibition of firefly luciferase \textit{in vitro} by 1-(2-hydroxyethyl)-6-methyrimidotriazinediones
A final set of 1,3,6- substituted pyrimidotriazinediones that were tested against firefly luciferase \textit{in vitro} that had also been tested in the cellular reporter assay were those where the 6-methyl substituent was varied. Figure 8-11 shows the data for both assays for two analogues where the $R_3$ substituent was $o$-fluorophenyl, and Figure 8-12 shows the data for both assays where the $R_3$ substituent was $p$-methoxyphenyl. While there is not complete overlap between the two sets of data, the same general trends are observed, suggestive, again, of luciferase inhibition being a complicating factor in the cellular reporter assay. For $R_3 = o$-fluorophenyl, the 6-unsubstituted analogue was fairly potent in both assays, while the 3,4-difluorobenzyl showed virtually no activity in either. Meanwhile for $R_3 = p$-methoxyphenyl, the 2-hydroxyethyl analogue demonstrated reasonable potency both \textit{in vitro} and in the cellular reporter assay, while the 4-fluorobenzyl analogue was much weaker. Still, it is interesting to note that for all four examples, there was increased potency in the cellular assay relative to the \textit{in vitro} test, which is a trend opposite to that anticipated if luciferase inhibition alone was responsible for the effects seen in the cellular reporter assay. Hence, while demonstrating the likelihood of luciferase inhibition being a confounding factor in the data reported from the cellular assay, these data would also support target modulation within the Wnt signaling pathway.
Figure 8-11: Testing of 3-(2-fluorophenyl)-pyrimidotriazinediones versus firefly luciferase in vitro and in cellular luciferase reporter assay. Both assays were performed as described in the experimental chapter. For in vitro data, compounds at varying concentrations in DMSO or DMSO were spotted into each well. Firefly luciferase was added, followed by Steady-Glo® luciferase substrate. Luminescence was recorded after 10-30 minute incubation period at RT (integration time = 500 ms). Maximum luminescence with DMSO control was set to a relative value of 1. All points were determined in quadruplicate with the mean values and error bars (standard deviation) shown for each point. For cellular assay, transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.
Moving to the 3,6,8- substituted pyrimidotriazinediones, the data for a representative subset from this class in each assay is shown in Figure 8-13. Overall, this series shows much less activity in both assays compared to the 1,3,6- substituted pyrimidotriazinediones. In each case, there are only two analogues with IC\textsubscript{50} values less than 10 μM, and most of the analogues have IC\textsubscript{50} values closer to 100 μM if they inhibit to any measurable degree. Nonetheless, the two most potent compounds in each assay differ from each other, as AF129 (IC\textsubscript{50}= 2.46 ± 0.80 μM) and AG168 (IC\textsubscript{50}= 3.38 ± 0.66 μM) were the most potent of this subset \textit{in vitro} and AG112 (IC\textsubscript{50}= 1.68 ± 0.91 μM) and AG85 (IC\textsubscript{50}= 3.60 ± 2.25 μM) were the most potent in the cellular assay. Since none of
these four analogues share either R₃ or R₈ substituents, there are no clear trends with respect to either subsituent within either set of data. Hence, few conclusions can be drawn from this data, apart from the broad generalization that this subclass of compounds is less active in both assays. The fact that there is a significant decrease in activity in both assays, however, continues to raise questions about whether the cellular data actually reveals anything about modulating the Wnt signaling pathway.

(a) The assay was performed as described in the experimental chapter. Compounds at varying concentrations in DMSO or DMSO were spotted into each well. Firefly luciferase was added, followed by Steady-Glo® luciferase substrate. Luminescence was recorded after 10-30 minute incubation period at RT (integration time = 500 ms). Maximum luminescence with DMSO control was set to a relative value of 1. All points were determined in quadruplicate with the mean values and error bars (standard deviation) shown for each point.
(b) Transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.

<table>
<thead>
<tr>
<th>R₈</th>
<th>R₃</th>
<th>ID</th>
<th>ID</th>
<th>R₈</th>
<th>R₃</th>
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</thead>
<tbody>
<tr>
<td>CH₂CH₂NEt₂</td>
<td>H₃CO—[苯]—</td>
<td>AF129</td>
<td>AG85</td>
<td>CH₂CH₂CH₃</td>
<td>Cl—[苯]—</td>
</tr>
<tr>
<td>F—[苯]</td>
<td>F—[苯]</td>
<td>AG112</td>
<td>AG115</td>
<td>(CH₂)₇CH₃</td>
<td>F—[苯]</td>
</tr>
<tr>
<td>H₃C—[苯]</td>
<td>F—[苯]</td>
<td>AG139</td>
<td>AG146</td>
<td></td>
<td>Cl—[苯]</td>
</tr>
<tr>
<td>F₃C—[苯]</td>
<td>[苯]—</td>
<td>AG169</td>
<td>AG189</td>
<td></td>
<td>F—[苯]</td>
</tr>
</tbody>
</table>

Figure 8-13: Testing of 3,6,8-substituted pyrimidotriazinediones:
(a) versus firefly luciferase in vitro
(b) in cellular luciferase reporter assay (data from Fearon lab)
Within the 3,6,8-substituted pyrimidotriazinediones subclass, several closely-related analogues with a water-solubilizing functionality on the R₃ substituent had been synthesized in an attempt to exploit the potent inhibition that the 1-methyl substituted pyrimidotriazinedione with this R₃ substituent had demonstrated in the cellular reporter assay. While the cellular data results, which are shown in Figure 8-14b, were disappointing in that these analogues, like the rest of the 3,6,8-substituted pyrimidotriazinediones, were far less potent than the 1,6-dimethylpyrimidotriazinediones, the encouraging aspect was that it was the first series of compounds tested in the cellular reporter assay that showed a clear structure activity relationship. First, para substitution of the 2-(diethylamino)ethoxyphenyl substituent at R₃ was clearly more active than meta substitution, as evidenced by AG-214 (10a), AG-263 (10c), AG-244 (10b), and AG-262 (10d) all showing better inhibition than AG-258 (10g) and AG-247 (10e). Meanwhile, regarding the benzyl substituent at R₈, the 4-fluoro substituent seemed to have the best effect on activity. Within both series (where R₃= either p-(2-(diethylamino)ethoxy)phenyl or m-(2-(diethylamino)ethoxy)phenyl), the same trend was observed, with those analogues having a 4-fluoro-substituent on the benzyl ring being more active than those with either a 2-fluoro- or 3-fluoro-substituent.

While these structure activity relationships were initially attributed to binding of the pyrimidotriazinediones to either β-catenin or Tcf4, the in vitro results from testing the other pyrimidotriazinediones against firefly luciferase raised questions about whether this structure activity relationship might simply reflect inhibition of firefly luciferase instead. Therefore, the results from in vitro testing of this series of pyrimidotriazinediones against firefly luciferase were compared to the cellular assay results and is shown in Figure 8-14a. As can be seen, a similar trend is observed in this data as was observed in the reporter assay, and comparison of the two sets of data suggests that the structure activity relationship observed in the reporter assay is largely explained by luciferase inhibition. There is one notable exception, however- AG-214 (10a)- which is nearly ten-fold less potent in the in vitro assay than the reporter assay and suggests that within this series, it is the one most likely to be exerting some effect on the Wnt signaling pathway.
(a) The assay was performed as described in the experimental chapter. Compounds at varying concentrations in DMSO or DMSO were spotted into each well. Firefly luciferase was added, followed by Steady-Glo® luciferase substrate. Luminescence was recorded after 10-30 minute incubation period at RT (integration time = 500 ms). Maximum luminescence with DMSO control was set to a relative value of 1. All points were determined in quadruplicate with the mean values and error bars (standard deviation) shown for each point.

(b) Transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.
Finally, the last compounds that were tested for potential firefly luciferase inhibition as part of the investigation of the pyrimidotriazinedione series were some of the hydrazone intermediates. Here, no inhibition was observed below the highest concentration tested: 100 μM. This, again, is consistent with the cellular reporter assay, as the hydrazone that was tested in the reporter assay also demonstrated no inhibition.

While none of the other heterocyclic cores that were investigated showed any activity in the cellular reporter assay, they were also tested versus firefly luciferase in vitro to further validate the hypothesis that much of the effect being seen in the cellular luciferase assay was attributable to inhibition of the luciferase reporter. The results for the pyrimidopyridazinediones, dihydropyrazolopyrimidinediones, and pyrrolopyrimidinediones/pyrimidopyrimidinedione are shown in Figures 8-15, 8-16, and 8-17, respectively. Overall, minimal inhibition was measured for any of these classes, consistent with the cellular reporter assay. For the pyrimidopyridazinediones, only AC33 showed some activity at 100 μM, which might not have been detected in the cellular assay due to solubility/permeability issues or simply because it is too weak of an inhibitor. For the pyrrolopyrimidinediones and the pyrimidopyrimidinedione that were tested in both assays, no activity was detected for any analogues. For the dihydropyrazolopyrimidinediones, however, some activity was detected at the higher concentrations for one of the three compounds tested, AF111, and it might even be labeled a weak inhibitor with an IC₅₀ of 40.36 ± 4.33 μM. While no inhibition was detected for any of these dihydropyrazolopyrimidinediones in the reporter assay, they actually appeared to increase activity, particularly in the case of AF111. If this apparent
activation is real, it would seem that the data from the two different assays contradict each other in this one case. (It should be noted that there were some irregularities with the cellular assay when this data was obtained, suggesting the increase might be an artifact.) Nonetheless, even this apparent contradiction is reconcilable based on a study in 2008 that showed how luciferase inhibitors could actually appear as activators in reporter assays when the luciferase-inhibitor complex is stablized against degradation relative to luciferase alone, permitting an accumulation of luciferase. Hence, with only one possible exception, these results further demonstrate the parallels between the two assays and support the hypothesis that much of the activity detected in the cellular reporter assay is due to the effect of the synthesized analogues on the reporter itself, rather than the intended pathway.
The assay was performed as described in the experimental chapter. Compounds at varying concentrations in DMSO or DMSO were spotted into each well. Firefly luciferase was added, followed by Steady-Glo® luciferase substrate. Luminescence was recorded after 10-30 minute incubation period at RT (integration time = 500 ms). Maximum luminescence with DMSO control was set to a relative value of 1. All points were determined in quadruplicate with the mean values and error bars (standard deviation) shown for each point.

Transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.

Figure 8-15: Testing of pyrimidopyridazinediones:
(a) versus firefly luciferase in vitro
(b) in cellular luciferase reporter assay (data from Fearon lab)
(a) The assay was performed as described in the experimental chapter. Compounds at varying concentrations in DMSO were spotted into each well. Luciferase was added, followed by Steady-Glo® luciferase substrate. Luminescence was recorded after 10-30 minute at RT (integration time = 500 ms). Maximum luminescence with DMSO control was set to a relative value of 1. All points were determined in quadruplicate with the mean values and error bars (standard deviation) shown for each point.

(b) Transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.

Figure 8-16: Testing of racemic pyrazolopyrimidinediones:
(a) versus firefly luciferase in vitro
(b) in cellular luciferase reporter assay (data from Fearon lab)
(a) The assay was performed as described in the experimental chapter. Compounds at varying concentrations in DMSO or DMSO were spotted into each well. Firefly luciferase was added, followed by Steady-Glo® luciferase substrate. Luminescence was recorded after 10-30 minute incubation period at RT (integration time = 500 ms). Maximum luminescence with DMSO control was set to a relative value of 1. All points were determined in quadruplicate with the mean values and error bars (standard deviation) shown for each point.

(b) Transfected IEC18 cells were incubated with varying concentrations of compound for 17-20 h. After cell lysis, firefly luciferase activity was measured using the Promega Dual Luciferase™ kit. All points were determined in duplicate with the mean values shown for each point.

Figure 8-17: Testing of pyrrolopyrimidinediones and pyrimidopyrimidinedione: (a) versus firefly luciferase in vitro  
(b) in cellular luciferase reporter assay (data from Fearon lab)
In conclusion, after the data from the *in vitro* ELISA binding assay described in the previous chapter failed to correlate with the cellular reporter assay carried out by Dr. Guido Bommer in Professor Fearon’s lab, additional investigations were carried out to better understand this discrepancy. Testing of the compounds synthesized versus firefly luciferase *in vitro* revealed a number of potent inhibitors of luciferase itself, and many correlations were found between the data from the cellular reporter assay and the *in vitro* testing versus luciferase. While not all of the cellular reporter assay data is completely rationalized by the *in vitro* data, the potency and trends observed are highly suggestive of luciferase inhibition being a significant component in the cellular reporter assay. Hence, while there is circumstantial evidence that some of the synthesized inhibitors modulate the Wnt signaling pathway, none points directly to disruption of the β-catenin/Tcf complex. Future studies utilizing cellular assays with non-luciferase reporters or other approaches, such as pull-down experiments, will be necessary to more fully address this question.
Chapter 9
Conclusions and Future Directions

Over 150 pyrimidotriazinedione, pyrimidopyridazinedione, pyrazolopyrimidinedione, pyrrolopyrimidinedione, and pyrimidopyrimidinedione analogues were synthesized in the search for inhibitors of the β-catenin/Tcf interaction as potential agents for the treatment of colon cancer. Published procedures were used to synthesize many novel pyrimidotriazinediones, but new methodology was developed to access 1-phenyl-substituted pyrimidotriazinediones. This new methodology was applicable to a variety of aryl and alkyl substituents, and a variation of it allowed for access to the 3,6,8-substituted pyrimidotriazinediones also.

Pyrimidopyridazinedione analogues, too, were synthesized via a combination of published procedures and development of new methodology. Both 3,4-unsubstituted and 3,4-disubstituted pyrimidopyridazinediones were synthesized according to literature precedent, but 3-unsubstituted-4-substituted pyrimidopyridazinediones were unknown in the literature before this work. The methodology presented allows for clean access to this previously unreported subclass in respectable yields and with a range of aryl substituents at the 4-position.

For the pyrazolopyrimidinedione analogues, the intention was to use a published route, albeit with a different starting material that had a methyl substituent at the 6α-position of the uracil. Unexpectedly, the literature precedent yielded a different result with this change of starting material, producing racemic dihydropyrazolopyrimidinediones in a lower oxidation state than the desired products. No dihydropyrazolopyrimidinediones of this subclass have been reported in the literature, and future work could explore the possibility of making this reaction enantioselective. The dihydropyrazolopyrimidinediones proved difficult to oxidize to the desired pyrazolopyrimidinediones, but a few were successfully oxidized with N-bromosuccinimide. Interestingly, however, two isomeric pyrazolopyrimidinediones were
isolated with slight variations in the oxidation conditions for those dihydropyrazolopyrimidinediones that would oxidize.

Finally, for both the pyrrolopyrimidinediones and pyrimidopyrimidinediones, most of the work was based on literature precedent. For the pyrrolopyrimidinediones, published procedures were used to synthesize three novel 5,6-unsubstituted pyrrolopyrimidinediones and variations on literature precedent were used to make one novel 5-phenyl-pyrrolopyrimidinedione. The pyrimidopyrimidinedione that was successfully synthesized was not novel, but investigation of the same route used to produce it for an analogous 8-methyl-substituted congener revealed significant differences in reactivity with the presence of the additional methyl substituent and forced re-evaluation of much of the proposed route. While this 8-methyl-substituted congener was never successfully attained, significant strides were made towards it, and future attempts could quickly prove fruitful based on preliminary results established here.

While the biological target for all analogues synthesized was that of the β-catenin/Tcf interaction, implementation of an ELISA binding assay with purified β-catenin and Tcf4 revealed no potent inhibitors of this interaction among the compounds synthesized. Nevertheless, both DIAD and DEAD were found serendipitously to be fairly potent inhibitors of the interaction, demonstrating the capacity for the assay to serve as a future tool in finding compounds that inhibit the β-catenin/Tcf interaction.

Finally, while most of the synthetic directions were guided by data obtained from a cellular luciferase reporter assay, recent testing of all of the synthesized analogues against purified luciferase in vitro revealed an alarming degree of luciferase inhibition, particularly with the 1,3,6-substituted pyrimidotriazinediones. This raises significant questions about how best to interpret the data from the cellular reporter assay and does not allow for any conclusive structure-activity-relationships with respect to the desired signaling pathway.

Despite the potent luciferase inhibition measured for many of the 1,3,6-substituted pyrimidotriazinediones that were synthesized, there was significantly less inhibition with the 3,6,8-substituted subseries. Moreover, some of the analogues in this subclass demonstrated reasonable inhibition in the cellular reporter assay (IC$_{50}$’s ~1-10 μM). In this respect, compound AG214 was particularly attractive because it has IC$_{50} = 5.72$ ±
4.11 μM in the cellular assay and IC$_{50} > 50$ μM in the in vitro test against purified luciferase. In addition, of the related compounds in this subseries, it was the only one for which the data from the in vitro testing did not parallel the data from the cellular reporter assay. Moreover, our collaborators were encouraged by the differential response to this compound in the survival tests done on various cancer cell lines.

With this data in hand, a decision was made to pursue further studies toward ascertaining whether AG214 possesses drug-like properties. First, it was subjected to a standard suite of computational calculations to determine how it compares to known drugs. The data are shown in Table 9-1.

<table>
<thead>
<tr>
<th>Computed Property</th>
<th>Non-CNS Drugs$^b$</th>
<th>CNS-Drugs$^b$</th>
<th>AG214</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td>383 ± 210</td>
<td>288 ± 88</td>
<td>496.52</td>
</tr>
<tr>
<td>SLogP</td>
<td>2.1 ± 1.2</td>
<td>2.8 ± 1.4</td>
<td>4.02</td>
</tr>
<tr>
<td>TPSA (Å$^2$) (total polar surface area)</td>
<td>98 ± 84</td>
<td>46 ± 26</td>
<td>91.76</td>
</tr>
<tr>
<td>HBA (hydrogen bond acceptors)</td>
<td>4.8 ± 4.1</td>
<td>2.6 ± 1.3</td>
<td>7</td>
</tr>
<tr>
<td>HBD (hydrogen bond donors)</td>
<td>2.8 ± 3.3</td>
<td>1.0 ± 0.9</td>
<td>0</td>
</tr>
<tr>
<td>RotB (rotational bonds)</td>
<td>6.2 ± 5.5</td>
<td>4.0 ± 2.6</td>
<td>9</td>
</tr>
<tr>
<td>Vdw_polar_SA (Å$^2$)</td>
<td>61 ± 59</td>
<td>25 ± 17</td>
<td>54.16</td>
</tr>
</tbody>
</table>

Table 9-1: Calculated properties$^a$ for CNS vs. non-CNS drugs and AG214
(Areas highlighted in orange indicate properties for AG214 that are at least one standard deviation outside the mean for non-CNS drugs.)

$^a$Values calculated using The Molecular Operating Environment (MOE), version 2008.10, Chemical Computing Group Inc, Montreal, Quebec, Canada. Unpublished data from Dr. Paul Kirchhoff, Department of Medicinal Chemistry, University of Michigan, Ann Arbor.

$^b$Mean values and standard deviations derived from 198 approved CNS drugs and 1015 approved non-CNS drugs.$^{89,90}$

Apart from SLogP, which is an approximation of logP, AG214 falls well within computed properties for marketed non-CNS drugs. AG214 also is compliant with Lipinski’s “Rule of Five”,$^{91}$ which values tend to correlate with drugs possessing good oral absorption. Finally, the HCl salt of AG214 possesses excellent aqueous solubility with a value of 1.2 mg/mL in distilled water.
Hence, a decision was made to proceed to *in vivo* studies with the HCl salt of compound AG214 (shown in Figure 9-1). First, pharmacokinetic studies were carried out to determine if it exhibited drug-like properties with respect to absorption, distribution, metabolism, and excretion to warrant further investigation as a lead compound for future drug development.

![Chemical structure of AG214](image)

**Figure 9-1: Pyrimidotriazine analogue AG214 submitted for *in vivo* studies**

The pharmacokinetic studies were carried out by Charles River Laboratories, Ann Arbor, MI (formerly MIR Discovery and Imaging Services). These initial studies were done on female athymic mice that were 8-9 weeks old and weighed $\geq 19.8$ g on Day 1 of the experiment. They were housed in static cages with Bed-O’Cobbs™ bedding inside Biobubble™ Clean Rooms and were fed LabDiet™ and water *ad libitum*. All treatments and body weight determinations were carried out in the bubble environment, which was controlled to a temperature range of $70^\circ \pm 2^\circ$ F and a humidity range of 30-70%. The mice were acclimated to the environment for 5 days prior to dosing and fasted for 12 h prior to dosing. They were observed for clinical signs at least once daily. The testing was carried out as follows:

1. There were four groups of three mice each. Group 1 served as the untreated control. Mice in Groups 2-4 were treated on Day 1 with a single 10 mg/kg dose of AG214 (lot: AJT-IV-124-1) administered intraperitoneally (IP).
2. At 1 minute post dose, the mice in Group 4 were anesthetized by inhalation of 1.5% isoflurane in air, and a 100 $\mu$L blood sample was collected via retro-orbital bleed.
3. At 15 minutes post dose, the mice in Group 2 were anesthetized by inhalation of 1.5% isoflurane in air, and a 100 $\mu$L blood sample was collected via retro-orbital bleed.
4. At 30 minutes post dose, the mice in Group 3 were anesthetized by inhalation of 1.5% isoflurane in air, and a 100 μL blood sample was collected via retro-orbital bleed.

5. At 3 h post dose, the mice in Group 2 were euthanized by overexposure to carbon dioxide, and a 100 μL blood sample was collected via terminal cardiac puncture.

6. At 6 h post dose, the mice in Group 3 were euthanized by overexposure to carbon dioxide, and a 100 μL blood sample was collected via terminal cardiac puncture.

7. At 12 h post dose, the mice in Group 4 were euthanized by overexposure to carbon dioxide, and a 100 μL blood sample was collected via terminal cardiac puncture.

Blood samples were also obtained from the mice in Group 1, the untreated group.

Analytical data and pharmacokinetic parameters were obtained by Dr. Allen Buhl at PharmOptima, Portage, MI. The results can be summarized as follows:

1. No mice died before being sacrificed and there were no significant findings on the gross necropsies performed. There were also no changes in body mass in any of the mice tested. All of these findings suggest no acute toxicity issues with AG214 at 10 mg/kg single dose.

2. The pharmacokinetic parameters that were determined for compound AG214 are as follows:

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>Measured Value (for 10 mg/kg IP dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum observed concentration (C&lt;sub&gt;max&lt;/sub&gt;)</td>
<td>335 ng/mL</td>
</tr>
<tr>
<td>Time point at C&lt;sub&gt;max&lt;/sub&gt; (T&lt;sub&gt;max&lt;/sub&gt;)</td>
<td>0.25 hr</td>
</tr>
<tr>
<td>Area under the curve (AUC)</td>
<td>3850 ng·hr/mL</td>
</tr>
<tr>
<td>Elimination rate constant (λ)</td>
<td>0.070727 hr&lt;sup&gt;−1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Half-life (t&lt;sub&gt;1/2&lt;/sub&gt;)</td>
<td>9.78 h</td>
</tr>
</tbody>
</table>

Table 9-2: Pharmacokinetic parameters for mice treated with IP dose of compound AG214

Overall, these pharmacokinetic parameters are sufficiently encouraging to permit further exploration of compound AG214. First, compound AG214 appears to achieve a reasonable concentration in the blood. Moreover, it has a half-life well within the range
of many established drugs, suggestive that it is not rapidly cleared by various possible mechanisms including rapid metabolism. As a result of these studies, current plans include testing of compound AG214 in \textit{in vivo} efficacy studies against colon cancer xenograft models (SW480 and then HT-29) in mice. If AG214 does demonstrate efficacy against one or more of these models, elucidation of its target(s) remains a critical priority for any potential development of this compound into a clinical candidate.
Chapter 10
Experimental

All starting materials were obtained from commercial suppliers and were used without further purification. THF was distilled prior to use over Na/benzophenone. Reactions were run under a blanket of N\textsubscript{2} unless specified otherwise. Glassware was oven-dried before use for reactions run under anhydrous conditions. Melting points were determined in open capillary tubes on a Laboratory Devices Mel-Temp apparatus and are uncorrected. The NMR spectra were recorded on a Bruker instrument at 500 MHz for \textsuperscript{1}H, 125 MHz for \textsuperscript{13}C, and 470 MHz for \textsuperscript{19}F spectra. Chemical shift values are recorded in \( \delta \) units (ppm). Mass spectra were recorded on a Micromass TofSpec-2E Matrix-Assisted, Laser-Desorption, Time-of-Flight Mass Spectrometer.

Chapter 2

3-Methyl-6-(1-methylhydrazinyl)pyrimidine-2,4(1\textsubscript{H},3\textsubscript{H})-dione (2a).\textsuperscript{36} 6-Chloro-3-methyluracil (5.0 g, 0.031 mol) was suspended in 45 mL of absolute ethanol. Methylhydrazine (8.3 mL, 0.16 mol, 5 eq) was then added, and the mixture was heated at reflux for 1 h when TLC (20% MeOH/CH\textsubscript{2}Cl\textsubscript{2}) indicated consumption of the starting uracil. The reaction was cooled to RT and the precipitate collected by filtration to yield 2 (3.25 g, 61%): mp 212-215°C (lit\textsuperscript{36} mp 207-209°C), \textsuperscript{1}H NMR (\textit{d}-DMSO) 2.97 (s, 3H), 3.02 (s, 3H), 4.66 (s, 1H), \textsuperscript{13}C NMR (\textit{d}-DMSO) 26.48, 72.74, 150.85, 154.30, 163.38.

6-(1-(2-Hydroxyethyl)hydrazinyl)-3-methylpyrimidine-2,4(1\textsubscript{H},3\textsubscript{H})-dione (2b).
6-Chloro-3-methyluracil (2.0 g, 12.4 mmol) was suspended in 15 mL of absolute ethanol. 2-Hydroxyethylhydrazine (3.37 mL, 49.8 mmol, 4 eq) was added, and the mixture was heated at reflux for 1.5 h wherein TLC (20% MeOH/CH\textsubscript{2}Cl\textsubscript{2}) indicated consumption of the starting uracil. The reaction was cooled to RT, and 10 mL of cold diethyl ether was added to form a cloudy solution. The mixture was concentrated \textit{in vacuo} to yield a
yellow solid residue. This residue was recrystallized from hot ethanol to yield a white precipitate, which was collected by filtration as 9 (1.24 g, 50%): mp 168-170°C, $^1$H NMR ($d$-DMSO) 3.04 (s, 3H), 3.43 (t, $J$= 5.6 Hz, 2H), 3.63 (t, $J$= 5.5 Hz, 2H), 4.74 (s, 1H).

**General Procedure for Synthesis of Hydrazones (3) from**

**Hydrazinylpyrimidines.** (Scheme 2-1) Hydrazinylpyrimidinedione 2 (0.5 mmol) was suspended in absolute ethanol (2 mL) in an oven-dried flask under nitrogen. The substituted aryl aldehyde (0.55 mmol, 1.1 eq) was added, and the mixture was heated at reflux until TLC (5% MeOH/CH$_2$Cl$_2$) indicated complete consumption of starting material. The reaction mixture was cooled to RT, and the precipitate was collected by filtration, rinsed with ethanol, and dried.

6-(2-Ethylidene-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1$H,3H$)-dione ($R_1$= CH$_3$, $R_3$= CH$_3$). 120 mg (mix of cis/trans isomers, 57% yield), mp 201-203°C, $^1$H NMR (CDCl$_3$) 1.17 (t, $J$= 7.5 Hz, 3H), 2.43 (m, 2H), 3.16 (s, 3H), 3.32 (s, 3H), 5.01 (s, 1H), 7.07 (s, 1H), 9.17 (s, 1H), $^{13}$C NMR ($d$-DMSO) 17.68, 18.73, 26.64, 26.89, 31.35, 35.12, 69.81, 71.45, 75.76, 140.73, 148.73, 150.92, 154.03, 163.67.

6-(2-Benzylidene-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1$H,3H$)-dione ($R_1$= CH$_3$, $R_3$= Ph). 126 mg (84% yield), mp 246-251°C, $^1$H NMR ($d$-DMSO) 3.12 (s, 3H), 3.35 (s, 3H), 5.23 (s, 1H), 7.42 (m, 3H), 7.98 (m, 3H), 10.68 (s, 1H), $^{13}$C NMR ($d$-DMSO) 26.75, 32.28, 77.50, 128.20, 128.94, 129.98, 134.86, 140.89, 151.00, 151.08, 163.61.

3-Methyl-6-(1-methyl-2-(4-methylbenzylidene)hydrazinyl)pyrimidine-2,4(1$H,3H$)-dione ($R_1$= CH$_3$, $R_3$= p-$CH_3$-C$_6$H$_4$). 113 mg (70% yield), mp 222-225°C, $^1$H NMR (CDCl$_3$) 2.42 (s, 3H), 3.33 (s, 6H), 5.13 (s, 1H), 7.26 (d, $J$= 7.98 Hz, 2H), 7.58 (d, $J$= 7.23 Hz, 2H), 7.69 (s, 1H), 9.22 (s, 1H), $^{13}$C NMR ($d$-DMSO) 21.51, 26.73, 32.21, 77.27, 128.19, 129.57, 132.17, 139.71, 141.00, 150.99.
6-(2-(4-Fluorobenzylidene)-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₃, R₃= p-F-C₆H₄). 478 mg (92% yield), mp 269-272°C, ¹H NMR (CDCl₃) 3.34 (s, 6H), 5.15 (s, 1H), 7.15 (d, J= 8.6 Hz, 2H), 7.69 (d, J= 8.6 Hz, 2H), 7.70 (s, 1H), 9.16 (s, 1H), ¹³C NMR (d-DMSO) 26.72, 32.28, 77.44, 115.75, 130.37, 130.49, 131.48, 131.52, 139.82, 150.97, 163.59.

6-(2-(2-Fluorobenzylidene)-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₃, R₃= o-F-C₆H₄). 143 mg (88% yield), mp 263-264°C, ¹H NMR (CDCl₃) 3.28 (s, 3H), 3.37 (s, 3H), 5.17 (s, 1H), 7.15 (m, 2H), 7.41 (m, 1H), 7.71 (m, 1H), 7.94 (s, 1H), 9.13 (s, 1H), ¹³C NMR (d-DMSO) 26.78, 32.31, 78.08, 116.03, 116.19, 122.30, 125.03, 128.50, 131.84, 132.90, 150.95, 151.08, 163.62.

6-(2-(4-Chlorobenzylidene)-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₃, R₃= p-Cl-C₆H₄). 119 mg (69% yield), mp 233-235°C, ¹H NMR (CDCl₃) 3.33 (s, 6H), 5.16 (s, 1H), 7.42 (d, J= 6.1 Hz, 2H), 7.62 (d, J= 6.7 Hz, 2H), 7.66 (s, 1H), 9.15 (s, 1H).

6-(2-(3-Chlorobenzylidene)-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₃, R₃= m-Cl-C₆H₄). 288 mg (84% yield), mp 240-242°C, ¹H NMR (CDCl₃) 3.33 (s, 6H), 3.41 (s, 3H), 5.17 (s, 1H), 7.39 (d, J= 6.2 Hz, 2H), 7.56 (d, J= 6.35 Hz, 1H), 7.65 (s, 1H), 7.68 (s, 1H), 9.20 (s, 1H), ¹³C NMR (d-DMSO) 26.70, 32.46, 77.94, 127.11, 127.43, 130.94, 134.02, 137.11, 139.39, 149.83, 151.21, 163.60.

6-(2-(4-Methoxybenzylidene)-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₃, R₃= p-CH₃O-C₆H₄). 57 mg (66% yield), mp 231-233°C, ¹H NMR (CDCl₃) 3.34 (s, 6H), 3.89 (s, 3H), 5.12 (s, 1H), 6.98 (d, J= 8.65 Hz, 2H), 7.63 (d, J= 6.2 Hz, 2H), 7.68 (s, 1H), 9.20 (s, 1H), ¹³C NMR (d-DMSO) 26.71, 32.14, 55.71, 76.93, 114.42, 127.52. 129.84, 140.88, 150.96, 151.08, 160.93, 163.59.

6-(2-(4-(2-(Diethylamino)ethoxy)benzylidene)-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₃, R₃= p-(CH₃CH₂)₂NCH₂CH₂O-
C₆H₄). 134 mg (31% yield), mp 175-176°C, ¹H NMR (d-DMSO) 0.97 (t, J= 7.0, 6H), 2.56 (q, J= 7.1 Hz, 4H), 2.79 (t, J= 7.0 Hz, 2H), 3.11 (s, 3H), 3.32 (s, 3H), 4.06 (t, J= 7.0 Hz, 2H), 5.20 (s, 1H), 6.98 (d, J= 8.4 Hz, 2H), 7.90 (d, J= 8.2 Hz, 2H), ¹³C NMR (d-DMSO) 12.27, 26.71, 32.06, 47.44, 51.74, 66.84, 76.88, 114.92, 127.48, 129.80, 140.73, 151.17, 160.19, 163.64.

6-(2-(4-(Dimethylamino)benzylidene)-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₃, R₃= p-N(CH₃)₂-C₆H₄). 383 mg (90% yield), mp 240-242°C, ¹H NMR (d-DMSO) 2.97 (s, 6H), 3.10 (s, 3H), 3.30 (s, 3H), 5.15 (s, 1H), 6.72 (d, J= 8.7 Hz, 2H), 7.77 (d, J= 8.4 Hz, 2H), 7.86 (s, 1H), 10.44 (s, 1H), ¹³C NMR (d-DMSO) 26.70, 31.98, 76.28, 112.01, 122.31, 129.52, 141.84, 150.81, 151.03, 151.68, 163.58.

3-Methyl-6-(1-methyl-2-(4-nitrobenzylidene)hydrazinyl)pyrimidine-2,4(1H,3H)-dione (R₁= CH₃, R₃= p-NO₂-C₆H₄). 156 mg (84% yield), mp 296-299°C, ¹H NMR (d-DMSO) 3.05 (s, 3H), 3.42 (s, 3H), 5.33 (s, 1H), 8.07 (s, 1H), 8.26 (s, 4H), 10.99 (s, 1H), ¹³C NMR (d-DMSO) 26.79, 32.77, 78.74, 124.14, 129.04, 138.44, 141.31, 147.78, 150.88, 151.17, 163.59.

3-Methyl-6-(1-methyl-2-(3-nitrobenzylidene)hydrazinyl)pyrimidine-2,4(1H,3H)-dione (R₁= CH₃, R₃= m-NO₂-C₆H₄). 161 mg (87% yield), mp 305-308°C, ¹H NMR (d-DMSO) 3.13 (s, 3H), 5.30 (s, 1H), 7.71 (m, 1H), 8.12 (s, 1H), 8.20 (t, J= 6.0 Hz, 1H), 8.47 (t, J= 6.7 Hz, 1H), 8.82 (s, 1H), 11.08 (s, 1H), ¹³C NMR (d-DMSO) 26.77, 32.63, 78.31, 122.56, 124.09, 130.40, 134.24, 136.83, 138.71, 148.70, 151.04, 151.25, 163.61.

3-Methyl-6-(1-methyl-2-(pyridin-2-ylmethylene)hydrazinyl)pyrimidine-2,4(1H,3H)-dione (R₁= CH₃, R₃= o-C₅H₄N). 120 mg (86% yield), mp 261-265°C, ¹H NMR (d-DMSO) 3.13 (s, 3H), 3.40 (s, 3H), 5.34 (s, 1H), 7.38 (m, 1H), 7.86 (m, 2H), 8.52 (d, J= 8.0 Hz, 1H), 8.60 (d, J= 4.25 Hz, 1H), 10.84 (s, 1H), ¹³C NMR (d-DMSO) 26.78, 32.49, 78.46, 121.66, 124.37, 137.06, 140.54, 149.51, 150.89, 151.12, 153.66, 163.63.
6-(2-Benzylidene-1-(2-hydroxyethyl)hydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₂CH₂OH, R₃= Ph). 75 mg (65% yield), mp 238-240°C, $^1$H NMR (d-DMSO) 3.12 (s, 3H), 3.63 (t, J= 5.3 Hz, 2H), 4.03 (t, J= 5.3 Hz, 2H), 5.27 (s, 1H), 7.41 (m, 3H), 7.96 (d, J= 8.1 Hz, 2H), 8.12 (s, 1H), $^{13}$C NMR (d-DMSO) 26.76, 46.63, 57.14, 77.47, 128.23, 128.94, 130.04, 134.81, 140.83, 150.96, 151.02, 163.66.

6-(1-(2-Hydroxyethyl)-2-(4-methylbenzylidene)hydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₂CH₂OH, R₃= p-CH₃-C₆H₄). 140 mg (93% yield), mp 255-257°C, $^1$H NMR (d-DMSO) 2.35 (s, 3H), 3.13 (s, 3H), 3.63 (t, J= 5.5 Hz, 2H), 4.01 (t, J= 5.5 Hz, 2H), 5.04 (br, 1H), 5.25 (s, 1H), 7.25 (d, J= 7.6 Hz, 2H), 7.85 (d, J= 7.6 Hz, 2H), 8.09 (s, 1H), 10.46 (br, 1H), $^{13}$C NMR (d-DMSO) 21.52, 26.73, 46.60, 57.17, 77.23, 128.21, 129.57, 132.12, 139.78, 140.96, 150.95, 151.00, 163.64.

6-(2-(4-Chlorobenzylidene)-1-(2-hydroxyethyl)hydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₂CH₂OH, R₃= p-Cl-C₆H₄). 150 mg (93% yield), mp 258-260°C, $^1$H NMR (d-DMSO) 3.09 (s, 3H), 3.62 (t, J= 5.3 Hz, 2H), 4.02 (t, J= 5.3 Hz, 2H), 5.03 (br, 1H), 5.25 (s, 1H), 7.49 (d, J= 8.4 Hz, 2H), 8.03 (d, J= 8.4 Hz, 2H), 8.12 (s, 1H), 10.65 (br, 1H), $^{13}$C NMR (d-DMSO) 26.74, 46.72, 57.07, 77.69, 128.96, 129.95, 133.80, 134.43, 139.55, 150.93, 151.07, 163.64.

6-(2-(3,4-Dichlorobenzylidene)-1-(2-hydroxyethyl)hydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₂CH₂OH, R₃= 3,4-diCl-C₆H₃). 178 mg (100% yield), mp 252-255°C, $^1$H NMR (d-DMSO) 3.16 (s, 3H), 3.66 (s, 2H), 3.92 (s, 2H), 4.92 (br s, 1H), 5.22 (s, 1H), 7.44 (d, J= 8.85 Hz, 1H), 7.65 (d, J= 7.35, 1H), 7.96 (s, 1H), 8.12 (s, 1H), 10.29 (br s, 1H).

6-(1-(2-Hydroxyethyl)-2-(4-methoxybenzylidene)hydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (R₁= CH₂CH₂OH, R₃= p-CH₃O-C₆H₄). 145 mg (91% yield), mp 234-235°C, $^1$H NMR (d-DMSO) 3.12 (s, 3H), 3.63 (t, 2H), 3.80 (s, 3H), 4.00 (t, 2H), 5.08 (br, 1H), 5.21 (s, 1H), 6.99 (d, J= 8.3 Hz, 2H), 7.93 (d, J= 8.2 Hz, 2H), 8.08 (s, 1H),
6-(2-(3,4-Dimethoxybenzylidene)-1-(2-hydroxyethyl)hydrazinyl)-3-methylpyrimidine-2,4(1\text{H},3\text{H})-dione (R\text{1}= \text{CH}_2\text{CH}_2\text{OH}, R\text{3}= 3,4-di(\text{CH}_3\text{O})-\text{C}_6\text{H}_3). 
301 mg (86% yield), mp 226-228 °C, $^1$H NMR (d-DMSO) 3.12 (s, 3H), 3.63 (s, 2H), 3.81 (s, 3H), 3.85 (s, 3H), 3.99 (s, 2H), 5.23 (s, 1H), 7.01 (d, J= 8.35 Hz, 1H), 7.43 (d, J= 7.9 Hz, 1H), 7.57 (s, 1H), 8.07 (s, 1H), 10.40 (br s, 1H), $^{13}$C NMR (d-DMSO) 26.72, 46.71, 55.98, 56.22, 57.13, 76.99, 110.76, 111.82, 122.39, 127.56, 141.28, 149.30, 150.86, 150.94, 151.01, 163.63.

6-(2-(4-(Dimethylamino)benzylidene)-1-(2-hydroxyethyl)hydrazinyl)-3-methylpyrimidine-2,4(1\text{H},3\text{H})-dione (R\text{1}= \text{CH}_2\text{CH}_2\text{OH}, R\text{3}= p-\text{N(CH}_3)_2-\text{C}_6\text{H}_4). 156 mg (94% yield), mp 244-245 °C, $^1$H NMR (d-DMSO) 2.98 (s, 6H), 3.12 (s, 3H), 3.61 (t, 2H), 3.98 (t, 2H), 5.20 (s, 1H), 7.75 (m, 5H), 8.01 (s, 1H).

6-(1-(2-Hydroxyethyl)-2-(3-nitrobenzylidene)hydrazinyl)-3-methylpyrimidine-2,4(1\text{H},3\text{H})-dione (R\text{1}= \text{CH}_3, R\text{3}= m-\text{NO}_2-\text{C}_6\text{H}_4). 208 mg (79% yield), $^{13}$C NMR (d-DMSO) 26.75, 46.93, 56.96, 78.27, 122.78, 124.10, 130.32, 134.13, 136.73, 138.59, 148.59, 150.95, 151.16, 163.64.

6-(1-(2-hydroxyethyl)-2-(pyridin-2-ylmethylene)hydrazinyl)-3-methylpyrimidine-2,4(1\text{H},3\text{H})-dione (R\text{1}= \text{CH}_2\text{CH}_2\text{OH}, R\text{3}= o-\text{C}_5\text{H}_4\text{N}). 131 mg, mp 256-257 °C, $^1$H NMR (d-DMSO) 3.10 (s, 3H), 3.65 (s, 2H), 4.08 (s, 2H), 5.08 (br s, 1H), 5.31 (s, 1H), 7.37 (t, J= 5.6 Hz, 1H), 7.86 (t, J= 7.8 Hz, 1H), 8.05 (s, 1H), 8.51 (d, J= 7.5 Hz, 1H), 8.59 (s, 1H), 10.70 (br s, 1H), $^{13}$C NMR (d-DMSO) 26.77, 46.96, 57.15, 78.35, 121.70, 124.40, 136.94, 140.83, 149.60, 150.84, 151.08, 153.73, 163.66.

General Procedure for Synthesis of Pyrimidotriazinediones (5) from Hydrazones (3). (Scheme 2-1) A mixture of hydrazone 3 (0.16 mmol) in 1 mL glacial acetic acid and 60 uL H$_2$O was stirred under nitrogen and cooled to 0°C. Sodium nitrite (0.25 mmol, 1.5
eq) was added, and the reaction mixture was allowed to warm to RT while stirring until the reaction was complete, forming a mixture of both the pyrimidotriazinedione and the corresponding N-oxide. Diethyl ether was added to facilitate further precipitation, and the precipitate was collected by filtration. The precipitate was then suspended in 2 mL absolute ethanol under nitrogen, and dithiothreitol (0.5 mmol, 3.1 eq) was added. The mixture was stirred from 24-72 h until mass spectrometry analysis and/or TLC indicated complete conversion of the N-oxide to the reduced pyrimidotriazinedione. The precipitate was collected by filtration and recrystallized from ethanol if necessary.

1,3,6-Trimethylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5a). 30 mg (37% yield), mp 160-170°C (dec), ¹H NMR (CDCl₃/TFA) 2.96 (s, 3H), 3.63 (s, 3H), 4.49 (s, 3H).

3-Ethyl-1,6-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5b). 29 mg (62% yield), mp 174-175°C (dec), ¹H NMR (CDCl₃/TFA) 1.45 (t, J= 7.5 Hz, 3H), 3.22 (q, J= 7.5 Hz, 2H), 3.58 (s, 3H), 4.46 (s, 3H).

1,6-Dimethyl-3-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5c). 20 mg (52% yield), mp 216-218°C (dec), ¹H NMR (d-DMSO) 3.30 (s, 3H), 4.07 (s, 3H), 7.60 (m, 3H), 8.21 (m, 3H), ¹³C NMR (d-DMSO) 28.76, 43.28, 127.03, 129.73, 131.76, 133.41, 150.00, 151.62, 154.84, MS m/z 270.1 (M+H).

1,6-Dimethyl-3-(4-methylphenyl)pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5d). 20 mg (39% yield), mp 223-225°C, ¹H NMR (d-TFA) 2.42 (s, 3H), 3.35 (s, 3H), 3.41 (s, 3H), 7.41 (d, J= 8.9 Hz, 2H), 7.59 (d, J= 8.9 Hz, 2H), ¹³C NMR (d-DMSO) 21.49, 28.75, 43.25, 127.01, 130.33, 141.79, 149.97, 151.74, 154.57, 159.55, MS m/z 284.1 (M+H).

3-(4-Fluorophenyl)-1,6-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5e). 35 mg (66% yield), mp 213-216°C, ¹H NMR (d-DMSO) 3.29 (s, 3H), 4.05 (s, 3H), 7.44 (d, J= 8.6 Hz, 2H), 8.24 (d, J= 8.6 Hz, 2H).
3-(2-Fluorophenyl)-1,6-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5f). 15 mg (28% yield), mp 191-194°C, $^1$H NMR (d-DMSO) 3.28 (s, 3H), 4.04 (s, 3H), 7.44 (m, 2H), 7.65 (m, 1H), 7.98 (d, J= 7.0 Hz, 1H), MS m/z 288.4 (M+H).

3-(4-Chlorophenyl)-1,6-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5g). 12 mg (47% yield), mp 218-223 °C (dec), $^1$H NMR (d-DMSO) 3.33 (s, 3H), 3.92 (s, 3H), 7.52 (d, J= 7.2 Hz, 2H), 7.75 (d, J=7.2 Hz, 2H), MS m/z 304.0, 306.0 (M+H).

3-(3-Chlorophenyl)-1,6-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5h). 41 mg (80% yield), mp 214-217°C, $^1$H NMR (CDCl$_3$/TFA) 3.61 (s, 3H), 4.43 (s, 3H), 7.53 (t, J= 7.9 Hz, 1H), 7.64 (d, J= 7.55 Hz, 1H), 8.25 (d, J= 7.6 Hz, 1H), 8.24 (d, J= 7.6 Hz, 1H), 8.31 (s, 1H).

3-(4-Methoxyphenyl)-1,6-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5i). 30 mg (58% yield), mp 245-250°C (dec), $^1$H NMR (d-TFA) 3.60 (s, 3H), 3.94 (s, 3H), 4.60 (s, 3H), 7.10 (d, J= 9.05 Hz, 2H), 8.34 (d, J= 9.05 Hz, 2H), MS m/z 300.1 (M+H).

3-(4-(2-(Diethylamino)ethoxy)phenyl)-1,6-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5j). 15 mg (87% yield), mp 255-260°C (dec), $^1$H NMR (d-DMSO) 1.25 (t, J= 7.0 Hz, 6H), 3.25 (q, J= 7.0 Hz, 4H), 3.29 (s, 3H), 3.56 (t, J= 7.1 Hz, 2H), 4.05 (s, 3H), 4.41 (t, J= 7.1 Hz, 2H), 7.21 (d, J= 8.3 Hz, 2H), 8.18 (d, J= 7.9 Hz, 2H).

1,6-Dimethyl-3-(5-methylfuran-2-yl)pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5k). 241 mg (73% yield), mp 238°C (dec), $^1$H NMR (CDCl$_3$) 2.40 (s, 3H), 3.52 (s, 3H), 4.20 (s, 3H), 6.25 (s, 1H), 7.37 (s, 1H).

3-(4-(Dimethylamino)phenyl)-1,6-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5l). 25 mg (73% yield), mp 270°C (dec), $^1$H NMR (d-TFA) 3.37 (s, 6H), 3.57 (s, 3H), 4.59 (s, 3H), 7.78 (d, J= 8.1 Hz, 2H), 8.59 (d, J= 8.1 Hz, 2H).
1,6-Dimethyl-3-(4-nitrophenyl)pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5m).
20 mg (39% yield), mp 220-223°C, ¹H NMR (d-TFA) 3.62 (s, 3H). 4.63 (s, 3H), 8.43 (d, J= 7.7 Hz, 2H), 8.61 (d, J= 7.7 Hz, 2H), ¹³C NMR (d-DMSO) 28.82, 43.42, 125.02, 128.21, 138.89, 147.01, 149.36, 150.32, 154.58, 159.55, MS m/z 315.1 (M+H).

1,6-Dimethyl-3-(3-nitrophenyl)pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5n).
21 mg (40% yield), mp 223-224°C, ¹H NMR (d-TFA) 3.63 (s, 3H), 4.66 (s, 3H), 7.84 (m, 1H), 8.54 (d, J= 8.0 Hz, 1H), 8.82 (d, J= 8.0 Hz, 1H), 9.25 (s, 1H), ¹³C NMR (d-DMSO) 28.82, 43.34, 121.31, 126.18, 131.66, 132.94, 134.75, 149.00, 154.56, MS m/z 315.1 (M+H).

1,6-Dimethyl-3-(pyridin-2-yl)pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (5o).
24 mg (24% yield), mp 298°C (dec), ¹H NMR (d-DMSO) 2.51 (s, 3H), 7.51 (d, J= 4.85 Hz, 1H), 7.99 (t, J= 7.20 Hz, 1H), 8.38 (d, J= 7.85 Hz, 1H), 8.75 (s, 1H).

1-(2-Hydroxyethyl)-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (8a).
26 mg (43% yield), mp 214-215°C (dec), ¹H NMR (d-DMSO) 3.29 (s, 3H), 3.86 (s, 3H), 3.93 (t, J= 5.1 Hz, 2H), 4.53 (t, J= 5.2 Hz, 2H), 4.98 (t, 1H), 7.16 (d, J= 8.5 Hz, 2H), 8.15 (d, J= 8.4 Hz, 2H), MS m/z 330.1 (M+H).

1-(2-Hydroxyethyl)-6-methyl-3-(4-methylphenyl)pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (8b).
21 mg (41% yield), mp 201-203°C, ¹H NMR (d-TFA) 2.41 (s, 3H), 3.61 (s, 3H), 4.53 (t, J= 5.2 Hz, 2H), 5.10 (t, J= 5.3 Hz, 2H), 7.36 (d, J= 8.0 Hz, 2H), 8.21 (d, J= 8.1 Hz, 2H), ¹³C NMR (d-DMSO) 21.51, 28.75, 57.13, 58.09, 127.10, 130.31, 130.53, 141.77, 147.01, 150.01, 151.76, 154.61, 159.54, MS m/z 314.1 (M+H).

1-(2-Hydroxyethyl)-6-methyl-3-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (8c).
22 mg (27% yield), mp 200-201°C, ¹H NMR (d-DMSO) 3.22 (s, 3H), 3.95 (t, J= 5.7 Hz, 2H), 7.61 (m, 3H), 8.21 (m, 2H), ¹³C NMR (d-DMSO)
28.78, 57.18, 58.07, 127.12, 128.73, 129.74, 130.29, 131.78, 133.22, 147.09, 150.12, 151.59, 154.65, 159.55, MS m/z 300.1 (M+H).

3-(4-Chlorophenyl)-1-(2-hydroxyethyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (8d). 11 mg (30% yield), mp 201-205°C (dec), \( ^1 \)H NMR (d-DMSO) 3.30 (s, 3H), 3.95 (t, J= 5.5 Hz, 2H), 4.55 (t, J= 5.5 Hz, 2H), 4.99 (t, 1H), 7.69 (d, J= 7.6 Hz, 2H), 8.21 (d, J= 7.3 Hz), \( ^{13} \)C NMR (d-DMSO) 28.77, 57.19, 58.03, 128.85, 129.89, 132.13, 136.56, 147.21, 150.11, 150.88, 154.62, 159.60, MS m/z 334 (M+H).

3-(3,4-Dichlorophenyl)-1-(2-hydroxyethyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (8e). 32 mg (58% yield), mp 208-211°C (dec), \( ^1 \)H NMR (d-TFA) 3.57 (s, 3H), 5.03 (s, 2H), 5.14 (s, 2H), 7.57 (d, J= 9.05 Hz, 1H), 8.13 (s, 1H), 8.36 (s, 1H).

3-(3,4-Dimethoxyphenyl)-1-(2-hydroxyethyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (8f). 26 mg (70% yield), mp 215-218°C (dec), \( ^1 \)H NMR (d-DMSO) 3.29 (s, 3H), 3.87 (s, 6H), 3.94 (s, 2H), 4.54 (s, 2H), 5.00 (t, J= 6.15, 1H), 7.18 (d, J= 8.35 Hz, 1H), 7.69 (s, 1H), 7.84 (d, J= 8.15 Hz, 1H).

1-(2-Hydroxyethyl)-6-methyl-3-(pyridin-2-yl)pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (8g). 21 mg (41% yield), mp 182-188°C (dec), \( ^1 \)H NMR (d-DMSO) 3.30 (s, 3H), 3.94 (t, J= 4.7 Hz, 2H), 4.57 (t, J= 5.0 Hz, 2H), 4.99 (t, J= 6.27, 1H), 7.61 (t, J= 5.5 Hz, 1H), 8.07 (t, J= 7.85 Hz, 1H), 8.25 (d, J= 8.05 Hz, 1H), 8.80 (s, 1H).

3-(4-(Dimethylamino)phenyl)-1-(2-hydroxyethyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (8h). 28 mg (71% yield), mp 170-180°C (dec), \( ^1 \)H NMR (d-TFA) 3.42 (s, 9H), 3.53 (s, 1H), 4.03 (s, 2H), 4.91 (s, 2H), 7.86 (d, J= 5.6 Hz, 2H), 8.05 (d, J= 5.8 Hz, 2H).

1-(2-Hydroxyethyl)-6-methyl-3-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (8i). 54 mg (28% yield), mp 171-179°C (dec), \( ^1 \)H
NMR (d-DMSO) 2.05 (br s, 8H), 3.28 (br s, 5H), 3.68 (br s, 4H), 4.38 (br s, 3H), 7.20 (d, J= 5.5 Hz, 2H), 8.36 (d, J= 5.5 Hz, 2H), MS m/z 413.2 (M+H).

1-(2-Hydroxyethyl)-6-methyl-3-(4-(2-(4-methylpiperazin-1-yl)ethoxy)phenyl)pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (8j). 55 mg (85% yield), mp 180-185°C (dec), 1H NMR (d-DMSO) 2.55 (br s, 8 H), 2.90 (s, 3H), 3.29 (s, 3H), 3.95 (t, J= 5.45 Hz, 2H), 4.54 (t, J= 5.45 Hz, 2H), 7.21 (d, J= 8.35 Hz, 2H), 8.18 (d, J= 8.4 Hz, 2H), MS m/z 442.1 (M+H).

3-(4-Methoxyphenyl)-6-methyl-1-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (8k). 84 mg (78% yield), mp 315-317°C (dec), 1H NMR (d-DMSO) 3.34 (s, 3H), 3.88 (s, 3H), 7.14 (d, J= 8.4 Hz, 2H), 7.67 (m, 3H), 7.76 (m, 2H), 8.15 (d, J= 8.3 Hz, 2H), MS m/z 362.2 (M+H).

General Procedure for Demethylation of Pyrimidotriazinediones N-Oxides (4) to N1-H Pyrimidotriazinediones (6). (Scheme 2-1) The pyrimidotriazinedione N-oxide 4 (1.5 mmol) was suspended in 5 mL dry DMF in an oven-dried flask under nitrogen. The mixture was heated to 90°C for 3-4 hours, during which time it darkened and eventually became homogeneous. It was then cooled to RT, and 6 mL of cold H2O was added, causing the solution to become cloudy. The mixture was refrigerated overnight, and then the formed precipitate was collected by filtration and dried.

6-Methyl-3-p-tolylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (6a). 59 mg (46% yield), 1H NMR (d-DMSO) 2.42 (s, 3H), 3.30 (s, 3H), 7.43 (d, J= 7.65 Hz, 2H), 7.43 (d, J= 7.85 Hz, 2H).

3-(2-Fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (6b). 198 mg (71% yield), mp 248-250°C, 1H NMR (d-DMSO) 3.33 (s, 3H), 7.15 (m, 2H), 7.41 (m, 1H), 7.71 (m, 1H), 12.87 (s, 1H).
6-Methyl-3-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (6c). 647 mg (84% yield), mp > 300°C (dec), $^1$H NMR (d-DMSO) 3.39 (s, 3H), 7.62 (m, 3H), 8.41 (d, J= 4.0 Hz, 2H), 12.86 (s, 1H), $^{13}$C NMR (d-DMSO) 28.34, 127.54, 129.67, 131.70, 132.80, 134.79, 149.98, 150.51, 160.21, 161.15.

3-(4-Chlorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (6d). 53 mg (64% yield), mp 302-304°C, $^1$H NMR (d-DMSO) 3.30 (s, 3H), 7.69 (d, J= 8.3 Hz, 2H), 8.41 (d, J= 8.3 Hz, 2H), 12.91 (s, 1H), $^{13}$C NMR (d-DMSO) 28.36, 129.25, 129.82, 133.72, 136.55, 148.54, 150.51, 161.29.

3-(4-Methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (6e). 278 mg (85% yield), mp 336-340°C (dec), $^1$H NMR (d-DMSO) 3.13 (s, 3H), 3.87 (s, 3H), 7.07 (d, J= 7.5 Hz, 2H), 8.34 (d, J= 7.2 Hz, 2H), 12.77 (s, 1H), $^{13}$C NMR (d-DMSO) 28.32, 55.89, 115.09, 127.22, 129.26, 150.31, 162.28, MS m/z 286.1 (M+H).

**General Procedure for Alkylation of N1-H Pyrimidotriazinediones (6) to 8-Alkylpyrimidotriazinediones (7).** (Scheme 2-1) Pyrimidotriazinedione 6 (0.2 mmol) was dissolved in 2 mL dry acetone in an oven-dried flask under nitrogen. Cesium carbonate (0.3 mmol, 1.5 eq) was then added, followed by the alkyl halide (0.22 mmol, 1.2 eq). The reaction was stirred at RT overnight (benzyl bromides) or heated to 50°C in a sealed vessel overnight (alkyl and benzyl chlorides). H$_2$O was then added to the reaction to precipitate the product. The mixture was cooled in the refrigerator for 3-4 h, and the precipitate was collected and dried at 65°C/20 mm Hg.

3-(4-Methoxyphenyl)-6,8-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7a). 58 mg (43% yield), mp 259-265°C (dec), $^1$H NMR (d-DMSO) 3.35 (s, 3H), 3.70 (s, 3H), 3.88 (s, 3H), 7.18 (d, J= 8.0 Hz, 2H), 8.36 (d, J= 8.0 Hz, 2H), MS m/z 300.1 (M+H).

3-(4-(2-(Diethylamino)ethoxy)phenyl)-6,8-dimethylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7b). 30 mg (63% yield), mp 195°C (dec), $^1$H NMR (d-DMSO) 1.00 (t, J= 6.9 Hz, 6H), 2.55 (s, 3H), 2.59 (q, J= 6.9 Hz, 4H), 2.82 (t, J= 5.65 Hz, 2H), 3.71 (s,
3-(4-Chlorophenyl)-6-methyl-8-propylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7c). 29 mg (47% yield), mp 202-205°C, 1H NMR (d-DMSO) 0.98 (t, J= 7.4 Hz, 3H), 1.76 (m, 2H), 3.37 (s, 3H), 4.32 (t, J= 7.2 Hz, 2H), 7.71 (d, J= 8.05 Hz, 2H), 8.44 (d, J= 7.9 Hz, 2H), 13C NMR (d-DMSO) 11.52, 20.80, 29.20, 44.28, 129.34, 129.91, 133.41, 133.87, 149.91, 150.11, 158.93, 160.50.

3-(4-Fluorophenyl)-6-methyl-8-octylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7d). 8 mg (16% yield), mp 123-125°C, 1H NMR (d-DMSO) 0.86 (t, J= 6.3 Hz, 3H), 1.33 (m, 8H), 1.39 (m, 2H), 1.73 (t, J= 7.3 Hz, 2H), 3.37 (s, 3H), 4.34 (t, J= 7.4 Hz, 2H), 7.48 (d, J= 8.0 Hz, 2H), 8.49 (d, J= 7.6 Hz, 2H).

8-(2-(Diethylamino)ethyl)-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7e). 18 mg (22% yield), mp 132-134°C, 1H NMR (d-DMSO) 0.98 (t, J= 7.0 Hz, 6H), 2.58 (q, J= 7.1 Hz, 4H), 2.73 (t, J= 7.0 Hz, 2H), 3.36 (s, 3H), 3.88 (s, 3H), 4.40 (t, J= 6.8 Hz, 2H), 7.18 (d, J= 8.7 Hz, 2H), 8.38 (d, J= 8.5 Hz, 2H), MS m/z 385.2 (M+H).

8-(2-Hydroxyethyl)-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7f). 32 mg (56% yield), mp 235-240°C (dec), 1H NMR (d-DMSO) 3.33 (s, 3H), 3.70 (d, J= 5.4 Hz, 2H), 3.87 (s, 3H), 4.45 (d, J= 2.9 Hz, 2H), 4.84 (br s, 1H), 7.17 (d, J= 8.6 Hz, 2H), 8.36 (d, J= 8.4 Hz, 2H).

8-Benzyl-3-(2-fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7g). 19 mg (35% yield), 1H NMR (d-DMSO) 3.38 (s, 3H), 5.57 (s, 2H), 7.30 (m, 3H), 7.46 (m, 4H), 7.66 (s, 1H), 8.04 (d, J= 5.85 Hz, 1H), 13C NMR (d-DMSO) 29.32, 45.86, 117.36, 117.64, 125.46, 127.72, 127.80, 128.78, 131.85, 133.62, 134.15, 136.48, 149.67, 150.26, 160.28, MS m/z 364.2 (M+H), 386.1 (M+Na).
6-Methyl-8-phenethyl-3-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7h). 28 mg (39% yield), mp 221°C (dec), $^1$H NMR (d-DMSO) 3.25 (s, 2H), 3.45 (s, 3H), 4.57 (s, 2H), 7.26 (s, 1H), 7.36 (s, 4H), 7.65 (s, 3H), 8.46 (s, 2H), $^{13}$C NMR (d-DMSO) 29.21, 33.39, 43.89, 126.60, 127.04, 127.67, 129.08, 129.20, 129.78, 131.95, 134.53, 138.64, 149.76, 159.99, 160.35, MS m/z 360.1 (M+H).

6-Methyl-3-phenyl-8-(3-phenylpropyl)pyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7i). 16 mg (22% yield), $^1$H NMR (d-DMSO) 2.06 (s, 2H), 2.73 (s, 2H), 3.34 (s, 3H), 4.41 (s, 2H), 7.23 (m, 5H), 7.62 (m, 3H).

3-(2-Fluorophenyl)-6-methyl-8-(4-methylbenzyl)pyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7j). 24 mg (58% yield), $^1$H NMR (d-DMSO) 2.27 (s, 3H), 3.39 (s, 3H), 5.53 (s, 1H), 7.14 (d, J= 7.65 Hz, 2H), 7.36 (d, J= 7.9 Hz, 2H), 7.46 (m, 2H), 7.67 (m, 1H), 8.05 (t, J= 8.2 Hz, 1H), $^{13}$C NMR (d-DMSO) 21.13, 29.31, 45.64, 117.35, 117.64, 125.51, 127.90, 129.31, 131.84, 133.44, 134.06, 136.89, 149.63, 150.22, 160.27.

8-(4-tert-Butylbenzyl)-3-(4-fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7k). 54 mg (71% yield), mp 213-216°C (dec), $^1$H NMR (d-DMSO) 1.25 (s, 9H), 3.39 (s, 3H), 5.53 (s, 2H), 7.34 (d, J= 7.65 Hz, 2H), 7.40 (d, J= 7.8 Hz, 2H), 7.47 (t, J= 8.5 Hz, 2H), 8.49 (d, J= 6.6 Hz, 2H), $^{13}$C NMR (d-DMSO) 29.30, 31.56, 34.65, 40.81, 116.69, 125.53, 127.88, 130.10, 130.22, 133.49, 150.16.

6-Methyl-8-(4-nitrobenzyl)-3-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7l). 32 mg (42% yield), mp 274-280°C (dec), $^1$H NMR (d-DMSO) 3.41 (s, 3H), 5.70 (s, 2H), 7.64 (s, 3H), 7.75 (d, J= 8.5 Hz, 2H), 8.20 (d, J= 8.6 Hz, 2H), 8.43 (s, 2H).

6-Methyl-3-phenyl-8-(4-(trifluoromethyl)benzyl)pyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7m). 32 mg (39% yield), mp 225-227°C (dec), $^1$H NMR (d-DMSO) 3.47 (s, 3H), 5.66 (s, 2H), 7.16 (t, J= 8.7 Hz, 2H), 7.52 (t, J= 8.4 Hz, 2H), 7.63 (m, 3H), 8.43 (d, J= 4.2 Hz, 2H).
8-(4-Fluorobenzyl)-6-methyl-3-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7n).  12 mg (16% yield), mp 253-254°C, $^1$H NMR (d-DMSO) 3.39 (s, 3H), 5.56 (s, 2H), 7.17 (t, J= 8.7 Hz, 2H), 7.52 (d, J= 8.4 Hz, 2H), 7.63 (m, 3H), 8.42 (d, J= 8.0 Hz).

8-(4-Fluorobenzyl)-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7o).  56 mg (81% yield), mp 230-234°C (dec), $^1$H NMR (d-DMSO) 3.37 (s, 3H), 3.87 (s, 3H), 5.53 (s, 3H), 7.16 (m, 4H), 7.51 (m, 2H), 8.37 (d, J= 8.85 Hz, 2H), $^{13}$C NMR (d-DMSO) 29.26, 45.10, 55.90, 115.17, 115.65, 126.78, 129.41, 130.05, 132.76, 134.08, 149.44, 150.15, 160.19, 160.31, 162.48, 163.53, $^{19}$F NMR (d-DMSO) -115.41, MS m/z 394.1 (M+H), 416.1 (M+Na).

8-(3-Fluorobenzyl)-6-methyl-3-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7p).  47 mg (66% yield), mp 229-232°C, $^1$H NMR (d-DMSO) 3.40 (s, 3H), 5.60 (s, 2H), 7.11 (t, J= 7.2 Hz, 1H), 7.31 (m, 2H), 7.39 (m, 1H), 7.63 (d, J= 2.6 Hz, 3H), 8.43 (m, 2H), $^{13}$C NMR (d-DMSO) 29.31, 45.42, 114.42, 123.70, 127.67, 129.77, 130.80, 131.99, 134.42, 139.46, 149.93, 150.19, 159.99, 160.20, 160.51, MS m/z 364.1 (M+H).

8-(3-Fluorobenzyl)-6-methyl-3-(3-(trifluoromethyl)phenyl)pyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7q).  24 mg (33% yield), mp 156-159°C, $^1$H NMR (d-DMSO) 3.39 (s, 3H), 5.59 (s, 2H), 7.10 (m, 1H), 7.32 (m, 2H), 7.39 (m, 1H), 7.90 (m, 1H), 8.01 (d, J= 5.9 Hz, 1H), 8.66 (s, 1H), 8.73 (d, J= 6.0 Hz, 1H).

8-(2-Fluorobenzyl)-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7r).  146 mg (75% yield), mp 240-241°C (dec), $^1$H NMR (d-DMSO) 3.35 (s, 3H), 3.87 (s, 3H), 5.58 (s, 2H), 7.08 (m, 1H), 7.17 (d, J= 8.6 Hz, 2H), 7.25 (m, 1H), 7.32 (m, 1H), 7.38 (m, 1H), 8.37 (d, J= 7.5 Hz, 2H), $^{13}$C NMR (d-DMSO) 29.31, 39.14, 55.91, 115.19, 115.46, 115.74, 123.35, 123.53, 124.77, 126.74, 128.92, 129.44, 129.59, 129.69, 134.18, 149.37, 150.09, 160.31, 160.51, 162.52.
3-(4-Chlorophenyl)-8-(2-fluorobenzyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7s). 39 mg (57% yield), mp 251-254°C, $^1$H NMR (d-DMSO) 3.40 (s, 3H), 5.61 (s, 2H), 7.09 (t, 1H), 7.27 (t, 1H), 7.35 (m, 2H), 7.71 (d, J= 8.1 Hz, 2H), 8.44 (d, J= 7.9 Hz, 2H), $^{13}$C NMR (d-DMSO) 29.35, 115.76, 123.22, 124.78, 128.96, 129.42, 129.65, 129.75, 129.93, 133.25, 134.36, 136.96, 149.94, 150.08, 159.44, 160.31.

8-(3,4-Difluorobenzyl)-3-(4-fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7t). 73 mg (91% yield), mp 226°C (dec), $^1$H NMR (d-DMSO) 3.38 (s, 3H), 5.55 (s, 2H), 7.34 (s, 1H), 7.42 (m, 4H), 8.47 (d, J= 5.6 Hz, 2H), $^{13}$C NMR (d-DMSO) 29.31, 44.91, 116.54, 116.73, 117.02, 117.73, 117.96, 124.61, 130.13, 130.25, 130.97, 134.38, 134.45, 150.16, 159.48, 160.39, MS m/z 400.1 (M+H).

8-(3,4-Difluorobenzyl)-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (7u). 168 mg (65% yield), mp 190°C (dec), $^1$H NMR (d-DMSO) 3.38 (s, 3H), 3.83 (s, 3H), 5.52 (s, 2H), 7.16 (d, J= 8.15 Hz, 2H), 7.31 (s, 1H), 7.41 (d, J= 7.95 Hz, 1H), 7.73 (s, 1H), 8.36 (d, J= 8.55 Hz, 2H), $^{13}$C NMR (d-DMSO) 29.28, 44.83, 55.91, 115.19, 116.54, 116.78, 117.70, 117.93, 124.55, 126.76, 129.43, 134.30, 149.40, 150.15, 160.28, 160.54, 162.52.

**General Procedure for Deprotection of Methoxy Substituents on 3-Aryl Pyrimidotriazinediones (7 to 9). (Scheme 2-2)**

0.6 mL (3 eq) of a 1.0M solution of BBr$_3$ in CH$_2$Cl$_2$ was added dropwise to pyrimidotriazinedione 7 (0.2 mmol) in 2 mL CH$_2$Cl$_2$ at RT under nitrogen. The reaction was stirred at RT until TLC (5% MeOH/CH$_2$Cl$_2$) showed consumption of starting material (2-48 h). Methanol (3-4 mL) was added to the reaction, and it was stirred for an additional 30 minutes. The mixture was concentrated and triturated in methanol to leave a bright yellow precipitate, which was collected, washed with methanol, and dried.

8-(3,4-Difluorobenzyl)-3-(4-hydroxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (9a). 285 mg (73% yield), mp >300°C, $^1$H NMR (d-DMSO) 3.38 (s, 3H), 5.53 (s, 2H), 6.97 (d, J= 6.4 Hz, 2H), 7.31 (s, 1H), 7.35 (m, 1H), 7.48 (m, 1H), 8.27
(d, J= 6.4 Hz, 2H), 10.19 (s, 1H), $^{13}$C NMR (d-DMSO) 29.27, 44.80, 116.55, 116.78, 117.70, 117.93, 124.55, 125.19, 129.59, 134.19, 134.45, 149.19, 150.14, 160.58, 161.20.

3-(4-Fluorophenyl)-8-(3-hydroxybenzyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (9b). 76 mg (45% yield), $^1$H NMR (d-DMSO) 3.39 (s, 3H), 5.47 (s, 2H), 6.65 (d, J= 7.7 Hz, 1H), 6.87 (s, 2H), 7.10 (t, J= 7.7 Hz, 1H), 7.47 (t, J= 7.6 Hz, 2H), 8.47 (m, 2H), 9.38 (s, 1H).

3-(3-Fluorophenyl)-8-(3-hydroxybenzyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (9c). 156 mg (54% yield), $^1$H NMR (d-DMSO) 3.39 (s, 3H), 5.46 (s, 2H), 6.70 (d, J= 8.6 Hz, 2H), 7.30 (d, J= 8.6 Hz, 2H), 7.49 (m, 1H), 7.70 (m, 1H), 8.15 (m, 1H), 8.28 (d, J= 8.0 Hz, 1H), 9.37 (s, 1H).

General Procedure for Alkylation of Phenol (9) with 2-(Diethylamino)ethyl Chloride. (Scheme 2-2) Pyrimidotriazine 9 (0.1 mmol) was dissolved in 1 mL dry acetone in an oven-dried 2-dram vial under nitrogen. Cesium carbonate (0.22 mmol, 2.2 eq) was added, followed by 2-(diethylamino)ethyl chloride hydrochloride (0.11 mmol, 1.1 eq). The reaction vial was sealed under nitrogen and heated at 50°C overnight. The reaction was cooled to RT and filtered. The filtrate was concentrated and triturated in ethanol. The precipitate was collected by filtration and dried.

3-(4-(2-(Diethylamino)ethoxy)phenyl)-8-(3,4-difluorobenzyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (10a). 25 mg (36% yield), mp 145-148°C, $^1$H NMR (d-DMSO) 0.98 (t, J= 5.7 Hz, 3H), 2.57 (m, 4H), 2.82 (t, J= 4.6 Hz, 2H), 3.38 (s, 3H), 4.11 (d, J= 4.6 Hz, 2H), 5.54 (s, 2H), 7.16 (d, J= 6.7 Hz, 2H), 7.33 (s, 1H), 7.40 (d, J= 8.1 Hz, 1H), 7.51 (d, J= 8.1 Hz, 1H), 8.36 (d, J= 6.9 Hz, 2H), MS m/z 497.1 (M+H).

3-(4-(2-(Diethylamino)ethoxy)phenyl)-8-(3-fluorobenzyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (10b). 20 mg (45% yield), mp 115-118°C (dec), $^1$H NMR (d-DMSO) 0.99 (t, J= 6.9 Hz, 6H), 2.57 (t, J= 6.9 Hz, 4H), 2.81 (t, J= 5.5 Hz, 2H), 3.37 (s, 3H), 4.13 (d, J= 5.8 Hz, 2H), 5.57 (s, 2H), 7.08 (t, J= 7.10 Hz, 1H), 7.16 (d, J=
8.8 Hz, 2H), 7.29 (m, 2H), 7.37 (m, 1H), 8.35 (d, J= 6.65 Hz, 2H), $^{13}$C NMR ($d$-DMSO) 12.33, 29.28, 39.14, 45.30, 47.45, 67.11, 114.11, 114.41, 115.69, 116.60, 123.68, 129.44, 130.68, 130.78, 139.63, 150.17, 160.55.

3-(4-(2-(Diethylamino)ethoxy)phenyl)-8-(4-fluorobenzyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (10c). 25 mg (47% yield), mp 170-171°C, $^1$H NMR ($d$-DMSO) 0.99 (t, J= 6.9 Hz, 6H), 2.56 (t, J= 6.9 Hz, 4H), 2.81 (t, J= 5.8 Hz, 2H), 3.33 (s, 3H), 4.12 (d, J= 6.0 Hz, 2H), 5.53 (s, 2H), 7.16 (d, J= 8.5 Hz, 4H), 7.51 (d, J= 8.0 Hz, 2H), 8.35 (d, J= 8.65 Hz, 2H), $^{13}$C NMR ($d$-DMSO) 12.37, 29.26, 47.45, 51.71, 67.10, 115.37, 115.66, 126.72, 129.41, 130.02, 130.13, 132.83, 134.19, 149.41, 150.17, 160.20, 160.50, 161.81.

3-(4-(2-(Diethylamino)ethoxy)phenyl)-8-(2-fluorobenzyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (10d). 48 mg (80% yield), mp 161-163°C, $^1$H NMR (d6-acetone) 1.06 (t, J= 5.3 Hz, 6H), 2.67 (m, 4H), 2.94 (s, 2H), 3.47 (s, 3H), 4.22 (s, 2H), 5.76 (s, 2H), 7.06 (m, 1H), 7.14 (m, 3H), 7.32 (m, 1H), 7.41 (m, 1H), 8.45 (d, J= 6.1 Hz, 2H), $^{13}$C NMR ($d$-DMSO) 12.36, 29.31, 47.45, 51.71, 67.12, 115.46, 115.67, 123.36, 124.77, 126.63, 128.91, 129.44, 129.59, 129.70, 134.18, 149.36, 150.09, 160.32, 160.52, 161.85, MS m/z 479.1 (M+H).

3-(3-(2-(Diethylamino)ethoxy)phenyl)-8-(3,4-difluorobenzyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (10e). 22 mg (59% yield), mp 127-130°C, $^1$H NMR (d6-DMSO) 0.99 (t, J= 5.5 Hz, 6H), 2.56 (m, 4H), 2.81 (t, J= 5.5 Hz, 2H), 3.41 (s, 3H), 4.11 (t, J= 5.5 Hz, 2H), 5.55 (s, 2H), 7.19 (m, 1H), 7.34 (s, 1H), 7.40 (m, 1H), 7.52 (m, 2H), 7.93 (s, 1H), 8.00 (d, J= 6.8 Hz, 1H), $^{13}$C NMR ($d$-DMSO) 9.06, 29.32, 44.95, 47.54, 50.38, 62.97, 113.58, 116.59, 116.83, 117.74, 117.97, 118.17, 120.77, 124.63, 131.16, 134.39, 134.45, 135.87, 149.99, 150.17, 158.73, 159.86, 160.41, $^{19}$F NMR ($d$-DMSO) -136, -134.

3-(3-(2-(Diethylamino)ethoxy)phenyl)-8-(3-fluorobenzyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (10f). 28 mg (48% yield), mp 98-100°C, $^1$H NMR
(d-DMSO) 0.99 (t, J= 6.9 Hz, 6H), 2.57 (m, 4H), 2.82 (s, 2H), 3.47 (s, 3H), 4.13 (s, 2H),
5.58 (s, 2H), 7.09 (m, 1H), 7.20 (s, 1H), 7.31 (m, 2H), 7.38 (d, J= 6.95 Hz, 1H), 7.53 (d, J= 7.0 Hz, 1H), 7.93 (s, 1H), 7.99 (d, J= 6.8 Hz, 1H).

3-(3-(2-(Diethylamino)ethoxy)phenyl)-8-(2-fluorobenzyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (10g). 15 mg (34% yield), mp 143-145 °C, ¹H NMR (d-DMSO) 0.99 (t, J= 6.9 Hz, 6H), 2.57 (q, J= 6.8 Hz, 4H), 2.80 (t, J= 6.35 Hz, 2H), 3.38 (s, 3H), 4.10 (t, J= 5.5 Hz, 2H), 5.58 (s, 2H), 7.07 (t, J= 7.1 Hz, 1H), 7.17 (d, J= 8.2 Hz, 1H), 7.24 (t, J= 8.5 Hz, 1H), 7.32 (m, 1H), 7.36 (m, 1H), 7.51 (t, J= 8.2 Hz, 1H), 7.91 (s, 1H), 7.98 (d, J= 7.8 Hz, 1H), ¹³C NMR (d-DMSO) 12.41, 29.31, 40.81, 47.51, 113.19, 115.67, 124.81, 128.98, 129.71, 130.98, 132.37, 134.19, 135.64, 150.17.

2-(4-(1-(3,4-Difluorobenzyl)-6-methyl-5,7-dioxo-1,5,6,7-tetrahydropyrimido[5,4-e][1,2,4]triazen-3-yl)phenoxy)-N,N-diethylethanaminium chloride (10h). ¹H NMR (CD3OD) 1.42 (t, J= 7.3 Hz, 6H), 3.39 (q, J= 7.3 Hz, 4H), 3.50 (s, 3H), 3.68 (t, J= 4.7 Hz, 2H), 4.50 (t, J= 4.8 Hz, 2H), 5.65 (s, 2H), 7.24 (m, 3H), 7.39 (s, 1H), 7.49 (t, J= 9.2 Hz, 1H), 8.57 (d, J= 8.9 Hz, 2H), ¹³C NMR (d-DMSO) 9.43, 29.29, 44.85, 47.51, 50.40, 67.10, 115.82, 116.55, 116.78, 117.72, 117.94, 124.56, 127.38, 129.47, 134.33, 149.48, 150.15, 160.14, 160.52, 160.90.

8-(4-(2-(Diethylamino)ethoxy)benzyl)-3-(4-fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (11a). 8 mg (45% yield), mp 138-142 °C (dec), ¹H NMR (d-DMSO) 0.98 (m, 6H), 2.58 (m, 4H), 2.77 (d, J= 3.6 Hz, 2H), 3.35 (s, 3H), 4.01 (d, J= 3.6 Hz, 2H), 5.49 (s, 2H), 6.95 (d, J= 8.7 Hz, 2H), 7.39 (d, J= 8.5 Hz, 2H), 7.47 (t, J= 8.8 Hz, 2H), 8.48 (m, 2H).

8-(3-(2-(Diethylamino)ethoxy)benzyl)-3-(4-fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (11b). 29 mg (30% yield), mp 107-112 °C (dec), ¹H NMR (d-DMSO) 1.03 (t, J= 7.0 Hz, 6H), 2.58 (m, 4H), 2.71 (t, J= 6.1 Hz, 2H), 3.39 (s, 3H), 3.96 (t, J= 6.1 Hz, 2H), 5.50 (s, 2H), 6.83 (d, J= 8.1 Hz, 1H), 6.99 (s, 2H), 7.21 (t, J= 8.0 Hz, 1H), 7.46 (t, J= 8.7 Hz, 2H), 8.47 (m, 2H), ¹³C NMR (d-DMSO) 12.26, 29.31,
8-(4-(2-(Diethylamino)ethoxy)benzyl)-3-(3-fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (11c). 10 mg (35% yield), mp 115-118°C (dec), $^1$H NMR (d-DMSO) 1.03 (t, J= 7.0 Hz, 6H), 2.58 (q, J= 7.0 Hz, 4H), 2.71 (t, J= 6.1 Hz, 2H), 3.39 (s, 3H), 3.96 (t, J= 6.1 Hz, 2H), 5.54 (s, 2H), 6.90 (m, 2H), 7.40 (d, J= 8.2 Hz, 2H), 7.70 (t, J= 7.5 Hz, 2H), 8.27 (m, 2H).

8-(3-(2-(Diethylamino)ethoxy)benzyl)-3-(3-fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (11d). 23 mg (35% yield), mp 110-115°C (dec), $^1$H NMR (d-DMSO) 0.96 (t, J= 7.1 Hz, 6H), 2.54 (m, 4H), 2.73 (t, J= 6.3 Hz, 2H), 3.43 (s, 3H), 3.98 (t, J= 6.3 Hz, 2H), 5.54 (s, 2H), 6.85 (d, J= 8.4 Hz, 1H), 7.01 (m, 2H), 7.23 (t, J= 8.1 Hz, 1H), 7.50 (dt, J= 8.5 Hz, J’= 2.4 Hz, 1H), 7.70 (dd, J= 8.0 Hz, J’= 6.0 Hz, 1H), 8.14 (d, J= 9.6 Hz, 1H), 8.28 (d, J= 7.8 Hz), $^{13}$C NMR (d-DMSO) 12.26, 29.32, 45.88, 47.37, 51.86, 66.65, 113.37, 113.87, 114.17, 119.00, 119.86, 123.81, 129.87, 132.09, 134.31, 138.00, 150.19, 159.09, 160.31.

8-Benzyl-3-(4-(2-(diethylamino)ethoxy)-3-fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (11e). 30 mg (37% yield), mp 106-110°C (dec), $^1$H NMR (d-DMSO) 0.99 (t, J= 7.1 Hz, 6H), 2.55 (q, J= 7.1 Hz, 4H), 2.85 (t, J= 5.9 Hz, 2H), 3.39 (s, 3H), 4.21 (t, J= 5.9 Hz, 2H), 5.56 (s, 2H), 7.27 (d, J= 7.2 Hz, 1H), 7.33 (m, 2H), 7.45 (m, 3H), 8.15 (d, J= 12.3 Hz, 1H), 8.21 (d, J= 8.4 Hz, 1H).

8-Benzyl-3-(3-(2-(diethylamino)ethoxy)-4-fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (11f). 56 mg (57% yield), mp 115-120°C (dec), $^1$H NMR (d-DMSO) 0.98 (t, J= 5.2 Hz, 6H), 2.57 (q, J= 5.2 Hz, 4H), 2.85 (t, J= 5.1 Hz, 2H), 3.39 (s, 3H), 4.22 (t, J= 5.1 Hz, 2H), 7.27 (d, J= 6.5 Hz, 1H), 7.33 (t, J= 7.1 Hz, 2H), 7.47 (m, 3H), 8.00 (m, 1H), 8.16 (d, J= 8.3 Hz, 1H), $^{13}$C NMR (d-DMSO) 12.42, 29.31, 45.87, 47.56, 51.57, 68.24, 113.56, 117.22, 117.47, 120.98, 127.72, 127.85, 128.77, 131.22, 134.15, 136.51, 147.48, 147.62, 149.89, 150.19, 159.30, 160.32, MS m/z 479.1 (M+H).
2,4,6-Trichloropyrimidine (12). Barbituric acid (11) (6.0 g, 46.8 mmol) was suspended in phosphorus oxychloride (22.3 mL, 239 mmol, 5.1 eq) in an oven-dried Schlenk tube and then sealed under nitrogen. The mixture was heated at 120°C for 72 h, during which time it became a homogeneous golden-brown solution. The solution was cooled to RT and slowly quenched on ice water. After most fizzing was complete, CH₂Cl₂ was added to partition the product. The organic layer was separated, dried over Na₂SO₄, filtered, and concentrated to a pale yellow oil (12). 8.59 g (76% yield), ¹H NMR (d-DMSO) 8.18 (s, 1H).

6-Chloropyrimidine-2,4(1H,3H)-dione (13). Sodium hydroxide (2.50 g, 61.5 mmol, 4 eq) was dissolved in 30 mL deionized H₂O. 2,4,6-Trichloropyrimidine (2.82 g, 15.4 mmol) was added with vigorous stirring. The suspension was heated at reflux for 1.5 h, during which time it became homogeneous. The mixture was cooled to RT, and 4 mL of concentrated HCl was added to adjust the pH to 2-3. The mixture was refrigerated overnight as precipitate formed. The white precipitate (13) was collected by filtration, rinsing with H₂O. The solid was dried at 70°C/20 mm Hg. 2.26 g (90% yield): mp = 285-290°C (dec); ¹H NMR (d-DMSO) 5.75 (s, 1H), 11.31 (s, D₂O exchangeable, 1H), 12.06 (s, D₂O exchangeable, 1H).

1-(Benzyloxymethyl)-6-chloropyrimidine-2,4(1H,3H)-dione (14). 13 (0.363 g, 2.48 mmol) was suspended in 10 mL dry THF in an oven-dried flask under nitrogen and cooled to 0°C. LiH (30 mg, 3.72 mmol, 1.5 eq) was added, and the reaction was stirred for 40 minutes. Benzyl chloromethyl ether (60%, 690 uL, 2.98 mmol, 1.2 eq) was added dropwise very slowly. The reaction was allowed to gradually warm to RT and then stirred overnight at RT. 7 mL of H₂O was added, and the precipitate was collected by filtration. The white solid was recrystallized from ethanol to yield 14 (341 mg, 52% yield): mp = 180-181°C; ¹H NMR (d-DMSO) 4.61 (s, 2H), 5.41 (s,2H), 5.98 (s, 1H), 7.30 (m, 5H), 11.66 (br s, 1H).
General Procedure for Synthesis of N3-Substituted-1-(Benzyloxymethyl)-6-chloropyrimidinediones (15). (Scheme 2-3) Compound 14 (0.627 g, 2.35 mmol) was suspended in acetone in an oven-dried flask equipped with a drying tube. The benzyl or alkyl bromide (2.59 mmol, 1.1 eq) was added, followed by cesium carbonate (1.148 g, 3.53 mmol, 1.5 eq). The reaction was stirred at RT overnight. The reaction was filtered, and the filtrate was concentrated to an oil. Purification was accomplished by silica gel flash chromatography, eluting with 25-75% EtOAc/hexanes.

1-(Benzyloxymethyl)-6-chloro-3-(2-hydroxyethyl)pyrimidine-2,4(1H,3H)-dione (15a). 365 mg (92% yield), $^1$H NMR (CDCl$_3$) 3.84 (t, J= 5.0 Hz, 2H), 4.13 (t, J= 5.2 Hz, 2H), 4.70 (s, 2H), 5.60 (s, 2H), 5.95 (s, 1H), 7.35 (s, 5H), $^{13}$C NMR (d-DMSO) 43.43, 57.68, 71.33, 75.56, 102.59, 127.90, 128.20, 128.73, 137.83, 145.62, 151.32, 160.95, MS m/z 333.0 (M+Na).

1-(Benzyloxymethyl)-6-chloro-3-(4-fluorobenzyl)pyrimidine-2,4(1H,3H)-dione (15b). 453 g (90% yield), $^1$H NMR (d-DMSO) 4.68 (s, 2H), 5.01 (s, 2H), 5.57 (s, 2H), 5.93 (s, 1H), 7.02 (d, J= 8.6 Hz, 2H), 7.31 (m, 5H), 7.47 (d, J= 8.6 Hz, 2H), $^{13}$C NMR (d-DMSO) 43.89, 71.30, 75.85, 102.67, 115.41, 115.69, 127.91, 128.12, 128.69, 130.35, 130.46, 133.15, 137.97, 145.87, 151.32, 160.48.

1-(Benzyloxymethyl)-6-chloro-3-(3,4-difluorobenzyl)pyrimidine-2,4(1H,3H)-dione (15c). 431 mg (98% yield), $^1$H NMR (CDCl$_3$) 4.69 (s, 2H), 4.97 (s, 2H), 5.57 (s, 2H), 5.96 (s, 1H), 7.28 (m, 8H), $^{13}$C NMR (d-DMSO) 43.74, 62.21, 71.62, 102.44, 115.61, 115.84, 117.17, 117.87, 127.06, 127.43, 127.68, 128.21, 128.67, 137.63, 146.41, 151.16, 161.00, MS m/z 415.0 (M+Na).

General Procedure for Conversion of 6-Chlorouracils (15) to 6α-Methylhydrazinyluracils (16). (Scheme 2-3) Compound 15 (0.95 mmol) was suspended in 2 mL of absolute ethanol. Methylhydrazine (0.35 mL, 6.7 mmol, 7 eq) was then added, and the mixture was heated at reflux for 3 h when TLC (20% MeOH/CH$_2$Cl$_2$) indicated consumption of the starting uracil. The reaction was cooled to RT and
concentrated. The residual oil was dissolved in CH$_2$Cl$_2$ and purified by silica gel flash chromatography (0-2% MeOH/CH$_2$Cl$_2$).

1-(Benzyloxymethyl)-3-(2-hydroxyethyl)-6-(1-methylhydrazinyl)pyrimidine-2,4(1H,3H)-dione (16a). 441 mg (44% yield), $^1$H NMR (d-DMSO) 2.89 (s, 3H), 3.46 (t, J= 6.1 Hz, 2H), 3.83 (t, J= 6.1 Hz, 2H), 4.58 (s, 2H), 5.13 (s, 1H), 5.56 (s, 2H), 7.26 (m, 2H), 7.32 (m, 3H), $^{13}$C NMR (d-DMSO) 42.52, 44.87, 58.07, 71.66, 75.30, 85.52, 127.74, 128.02, 128.70, 159.67, MS m/z 343.1 (M+Na).

1-(Benzyloxymethyl)-3-(4-fluorobenzyl)-6-(1-methylhydrazinyl)pyrimidine-2,4(1H,3H)-dione (16b). 188 mg (51% yield), $^1$H NMR (d-DMSO) 2.86 (s, 3H), 4.74 (s, 2H), 4.94 (s, 2H), 5.17 (s, 1H), 5.58 (s, 2H), 7.15 (d, J= 8.8 Hz), 7.27 (m, 7H), $^{13}$C NMR (d-DMSO) 44.86, 55.29, 71.63, 75.52, 85.21, 115.31, 115.59, 127.74, 127.87, 128.65, 130.32, 130.38, 138.33, 152.69, 159.77, 160.09, 162.12, MS m/z 407.1 (M+Na).

General Procedure for Synthesis of Hydrazones (17) from Hydrazinylpyrimidinediones (16). (Scheme 2-3) Hydrazinylpyrimidinedione 16 (0.5 mmol) was suspended in absolute ethanol (2 mL) in an oven-dried flask under nitrogen. The substituted aryl aldehyde (0.55 mmol, 1.1 eq) was added, and the mixture was heated at reflux until TLC (5% MeOH/CH$_2$Cl$_2$) indicated complete consumption of starting material. The reaction mixture was cooled to RT and concentrated. The residual oil was purified by silica gel flash chromatography (0-50% EtOAc/hexanes).

1-(Benzyloxymethyl)-3-(2-hydroxyethyl)-6-(2-(4-methoxybenzylidene)-1-methylhydrazinyl)pyrimidine-2,4(1H,3H)-dione (17a). 150 mg (50% yield), $^1$H NMR (d-DMSO) 3.33 (s, 3H), 3.45 (t, J= 6.1 Hz, 2H), 3.51 (t, J= 6.2 Hz, 2H), 3.78 (s, 3H), 3.88 (s, 2H), 4.60 (s, 2H), 5.49 (s, 1H), 6.93 (d, J= 7.7 Hz, 2H), 7.28 (m, 5H), 7.61 (d, J= 7.7 Hz, 2H), 7.85 (s, 1H), $^{13}$C NMR (d-DMSO) 36.80, 43.30, 55.67, 58.07, 71.59, 75.85, 89.28, 114.63, 115.37, 115.65, 127.80, 127.95, 128.07, 128.67, 128.75, 130.34, 130.45, 133.90, 133.94, 138.10, 140.51, 152.69, 156.34, 160.21, 160.66, 162.07, 163.42, MS m/z 439.1 (M+H), 461.1 (M+Na).
1-(Benzyloxymethyl)-3-(4-fluorobenzyl)-6-(2-(4-methoxybenzylidene)-1-methylhydrazinyl)pyrimidine-2,4(1H,3H)-dione (17b). 92 mg (25% yield), mp 170-173°C, \(^1\)H NMR (\(d\)-DMSO) 3.21 (s, 3H), 3.77 (s, 3H), 4.56 (s, 2H), 4.96 (s, 2H), 5.43 (s, 1H), 5.50 (s, 2H), 6.92 (d, J= 8.7 Hz, 2H), 7.16 (t, J= 8.8 Hz, 2H), 7.22 (m, 5H), 7.29 (t, J= 8.4 Hz, 2H), 7.60 (d, J= 8.6 Hz, 2H), 7.85 (s, 1H), \(^{13}\)C NMR (\(d\)-DMSO) 37.02, 43.22, 71.63, 75.82, 90.43, 117.72, 117.95, 122.69, 125.26, 127.13, 127.98, 128.10, 128.66, 132.60, 138.07, 152.62, 156.10, MS \(m/z\) 525.1 (M+Na).

1-(Benzyloxymethyl)-3-(3,4-difluorobenzyl)-6-(2-(2-fluorobenzylidene)-1-methylhydrazinyl)pyrimidine-2,4(1H,3H)-dione (17c). 413 mg (47% yield), \(^1\)H NMR (\(d\)-DMSO) 3.28 (s, 3H), 4.68 (s, 2H), 5.06 (s, 2H), 5.45 (s, 1H), 5.60 (s, 2H), 7.10 (d, J= 8.7 Hz, 2H), 7.29 (m, 8H), 7.85 (d, J= 8.8 Hz, 2H), 8.04 (s, 1H), MS \(m/z\) 531.1 (M+Na).

General Procedure for Synthesis of Pyrimidotriazinediones (19) from Hydrazones (17). (Scheme 2-3) A mixture of hydrazone 17 (0.16 mmol) in 1 mL glacial acetic acid and 60 uL H\(_2\)O was stirred under nitrogen and cooled to 0°C. Sodium nitrite (0.25 mmol, 1.5 eq) was added, and the reaction mixture was allowed to warm to RT while stirring until the reaction was complete, forming a mixture of both the pyrimidotriazinedione and the corresponding N-oxide. The precipitate was collected by filtration. The precipitate was then suspended in 2 mL absolute ethanol under nitrogen, and dithiothreitol (0.5 mmol, 3.1 eq) was added. The mixture was stirred from 24-72 h until mass spectrometry analysis and/or TLC indicated complete conversion of the N-oxide to the reduced pyrimidotriazinedione. The precipitate was collected by filtration and recrystallized from ethanol if necessary.

6-(2-Hydroxyethyl)-3-(4-methoxyphenyl)-1-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (19a). 30 mg (10% yield), \(^1\)H NMR (\(d\)-DMSO) 3.56 (t, J= 6.3 Hz, 2H), 3.83 (s, 3H), 3.87 (t, J= 6.3 Hz, 2H), 4.04 (s, 3H), 4.79 (br s, 1H), 7.15 (d, J= 8.6 Hz, 2H), 8.15 (d, J= 8.1 Hz, 2H), \(^{13}\)C NMR (\(d\)-DMSO) 38.08, 43.53, 55.80, 57.90, 114.99, 115.10, 125.47, 129.51, 149.70, 155.01, 159.75, 162.34, MS \(m/z\) 352.1 (M+Na).

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6-(4-Fluorobenzyl)-3-(4-methoxyphenyl)-1-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (19b).  11 mg (36% yield), $^1$H NMR (CDCl$_3$) 3.85 (s, 3H), 4.37 (s, 3H), 5.40 (s, 2H), 7.17 (d, J= 8.7 Hz, 4H), 7.43 (d, J= 5.85 Hz, 2H), 8.15 (d, J= 12.3 Hz, 2H), $^{13}$C NMR ($d$-DMSO) 18.73, 55.84, 56.60, 115.05, 115.30, 115.58, 122.00, 129.31, 130.13, 130.24, 132.69, 133.04, 150.15, 150.53, MS m/z 394.1 (M+H).

6-(3,4-Difluorobenzyl)-3-(2-fluorophenyl)-1-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (19c).  64 mg (66% yield), mp 200-202°C (dec), $^1$H NMR ($d$-DMSO) 4.06 (s, 3H), 5.08 (s, 2H), 7.38 (s, 1H), 7.45 (m, 4H), 7.65 (br s, 1H), 8.00 (s, 1H), $^{13}$C NMR ($d$-DMSO) 43.36, 43.90, 116.79, 117.02, 117.42, 117.61, 117.71, 117.84, 121.60, 121.73, 124.87, 125.49, 125.54, 131.26, 133.62, 133.74, 135.14, 135.19, 147.43, 150.18, 154.17, 158.61, 159.43, 161.99, MS m/z 400.1 (M+H).

3-(2-Fluorophenyl)-1-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (19d).  184 mg (68% yield), mp 240-247°C (dec), $^1$H NMR ($d$-DMSO) 4.33 (s, 3H), 7.43 (s, 2H), 7.79 (s, 1H), 8.16 (s, 1H), 11.61 (br s, 1H).

3-(2,4-Dichlorophenyl)-1-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (19e).  1.0 g (72% yield), $^1$H NMR ($d$-TFA) 4.43 (s, 3H), 7.40 (s, 1H), 7.56 (s, 1H), 7.79 (s, 1H), $^{13}$C NMR ($d$-DMSO) 43.32, 128.43, 130.71, 130.77, 131.72, 133.37, 136.25, 147.94, 150.42, 151.25, 155.09, 159.52, MS m/z 324.0, 326.0 (M+H).

3-(4-Methoxyphenyl)-1-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (19f).  505 mg (84% yield), mp 267-275°C (dec), $^1$H NMR ($d$-TFA) 3.93 (s, 3H), 4.57 (s, 3H), 7.09 (d, J= 8.7 Hz, 2H), 8.32 (d, J= 8.6 Hz), $^{13}$C NMR ($d$-DMSO) 43.32, 55.91, 114.11, 115.09, 125.47, 128.77, 148.06, 151.30, 155.01, 159.75, 162.19, MS m/z 286.1 (M+H).

1-Phenylpyrimidine-2,4,6(1H,3H,5H)-trione (22).  (Scheme 2-4) Sodium metal (380 mg, 16.52 mmol) was finely cut and added to 5 mL anhydrous methanol in an oven-dried flask under nitrogen. After complete dissolution, phenylurea (1.0 g, 7.34 mmol) and
diethylmalonate (1.33 mL, 8.81 mmol) were added. The mixture was heated at reflux for 3 h. It was then cooled to RT, concentrated, and resuspended in 10 mL 3N HCl. After stirring for 15 minutes, the suspension was filtered to collect a white precipitate (22), which was rinsed with water and ethanol. The solid was dried at 70°C/20 mm Hg. 1.41 g (94% yield), $^1$H NMR ($d$-DMSO) 3.74 (s, 2H), 7.24 (d, J= 7.05 Hz, 2H), 7.46 (m, 3H), 11.51 (s, D$_2$O exchangeable, 1H).

6-Chloro-3-phenylpyrimidine-2,4(1H,3H)-dione (23) and 6-Chloro-1-phenylpyrimidine-2,4(1H,3H)-dione (24). Phosphoryl chloride (8.44 mL, 90.6 mmol) was added dropwise to a suspension of 22 in 400 μL water. The mixture was heated at reflux for 3 h, during which time the mixture became homogeneous. It was cooled to RT and concentrated. Ice was added to the residue with rapid stirring, and a precipitate formed. Precipitate was collected by filtration and then resuspended in a saturated solution of sodium bicarbonate. After stirring for 15 minutes, the precipitate was collected by filtration and recrystallized from ethanol to yield 24 (800 mg, 48%): mp 257-260°C (lit $^51$ mp 260-262°C), $^1$H NMR ($d$-DMSO) 6.09 (s, 1H), 7.45 (m, 2H), 7.50 (m, 3H), 11.72 (br s, 1H). The filtrate was acidified with conc. HCl to pH~2, and a second precipitate formed, which was collected by filtration to yield 23 (444 mg, 26%): mp 264-268°C (lit $^51$ mp 268-270°C), $^1$H NMR ($d$-DMSO) 6.09 (s, 1H), 7.45 (m, 2H), 7.50 (m, 3H), 12.50 (br s, 1H).

6-(1-Methylhydrazinyl)-1-phenylpyrimidine-2,4(1H,3H)-dione (25). 6-Chloro-1-phenyluracil (24) (0.50 g, 2.24 mmol) was suspended in 3 mL absolute ethanol. Methylhydrazine (1.1 mL, 20.2 mmol, 9 eq) was then added, and the mixture was heated at reflux for 2 h under nitrogen when TLC (20% MeOH/CH$_2$Cl$_2$) indicated consumption of the starting uracil. The reaction was cooled to RT, concentrated, and triturated with ethanol to yield a white solid (25) (265 mg, 38%): mp 202-205°C, $^1$H NMR ($d$-DMSO) 2.59 (s, 3H), 4.22 (s, D$_2$O exchangeable, 2H), 5.22 (s, 1H), 7.27 (m, 2H), 7.39 (m, 3H), 10.83 (br s, D$_2$O exchangeable, 1H).
6-(2-(4-Methoxybenzylidene)-1-methylhydrazinyl)-1-phenylpyrimidine-2,4(1H,3H)-dione (26). Hydrazinylpyrimidinedione 25 (256 mg, 1.10 mmol) was suspended in absolute ethanol (4 mL) in an oven-dried flask under nitrogen. p-Anisaldehyde (148 mL, 1.21 μmol, 1.1 eq) was added, and the mixture was heated at reflux until TLC (5% MeOH/CH₂Cl₂) indicated complete consumption of starting material. The reaction mixture was cooled to RT, and the precipitate was collected by filtration, rinsed with ethanol, and dried to yield 26 (385 mg, 58%): mp 230-232°C, ¹H NMR (d-DMSO) 3.03 (s, 3H), 3.80 (s, 3H), 5.43 (s, 1H), 6.89 (d, J= 8.7 Hz, 2H), 7.25 (m, 7H), 7.50 (s, 1H), 11.24 (s, 1H).

6-(2-(4-Methoxybenzylidene)-1-methylhydrazinyl)-3-methyl-1-phenylpyrimidine-2,4(1H,3H)-dione (27). Cesium carbonate (279 mg, 0.86 mmol, 1.5 eq) and dimethyl sulfate (65 μL, 0.68 mmol, 1.2 eq) were added to a suspension of 26 (200 mg, 0.57 mmol) in 3 mL dry acetone. The mixture was stirred at RT for 48 h and then filtered to isolate precipitate. The precipitate was resuspended in water, and the remaining white solid was collected to yield 27 (91 mg, 44%). Filtrate from the first filtration was concentrated and then triturated in ethanol to yield a second crop of equally pure 27 (64 mg, 31%): mp 207-209°C, ¹H NMR (d-DMSO) 2.99 (s, 3H), 3.19 (s, 3H), 3.82 (s, 3H), 5.59 (s, 1H), 6.89 (d, J= 8.3 Hz, 2H), 7.28 (m, 7H), 7.51 (s, 1H).

3-(4-Methoxyphenyl)-6-methyl-8-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (28). 27 (125 mg, 0.34 mmol) was suspended in 1 mL glacial acetic acid and 200 μL water and cooled to 0°C. Sodium nitrite (36 mg, 0.51 mmol, 1.5 eq) was added, and the reaction was allowed to gradually warm to RT as it stirred overnight. Bright yellow precipitate was collected by filtration, rinsing with ethanol to yield 27 (25 mg, 18% yield): ¹H NMR (d-DMSO) 3.38 (s, 3H), 3.86 (s, 3H), 7.17 (d, J= 7.1 Hz, 2H), 7.43 (d, J= 5.35 Hz, 2H), 7.55 (t, J= 6.1 Hz, 1H), 7.60 (d, J= 5.9 Hz, 2H), 8.34 (d, J= 7.1 Hz, 2H), MS m/z 384.1 (M+Na).

6-Chloro-3-methyl-1-phenylpyrimidine-2,4(1H,3H)-dione (31). (Scheme 2-5)
6-Chloro-1-phenylpyrimidine-2,4(1H,3H)-dione (24) (1.0 g, 4.49 mmol) was suspended in 15 mL acetone in an oven-dried flask equipped with a drying tube. Dimethyl sulfate (0.51 mL, 5.39 mmol, 1.2 eq) and cesium carbonate (2.19 g, 6.74 mmol, 1.5 eq) were added. The reaction was stirred at RT for 16 h. The mixture was filtered, and the precipitate was washed excessively with water to remove cesium carbonate. The solid was dried at 70°C/20 mm Hg. 890 mg (84% yield), mp 270-275°C (dec), ¹H NMR (d-DMSO) 3.18 (s, 3H), 6.22 (s, 1H), 7.42 (m, 2H), 7.52 (m, 3H), ¹³C NMR (d-DMSO) 28.26, 101.52, 129.60, 129.73, 129.87, 137.48, 145.47, 151.09, 161.26.

3-Methyl-6-(1-methylhydrazinyl)-1-phenylpyrimidine-2,4(1H,3H)-dione (32).
Chlorouracil 31 (0.89 g, 3.76 mmol) was suspended in 5 mL absolute ethanol in an oven-dried flask under nitrogen. Methylhydrazine (1.80 mL, 33.8 mmol, 9 eq) was added, and the reaction was heated at reflux for 2 h. The reaction was cooled to RT and concentrated. The remainder was triturated with isopropanol to yield a white solid. The precipitate was collected by filtration to yield 32 (815 mg, 88% yield): mp 141-142°C, ¹H NMR (d-DMSO) 2.59 (s, 3H), 3.13 (s, 3H), 4.19 (br s, 2H), 5.38 (s, 1H), 7.29 (d, J= 7.2 Hz, 2H), 7.37 (d, J= 7.2 Hz, 1H), 7.44 (m, 2H).

6-(2-(4-Hydroxybenzylidene)-1-methylhydrazinyl)-3-methyl-1-phenylpyrimidine-2,4(1H,3H)-dione (33). Compound 32 (0.77 g, 3.11 mmol) was suspended in 10 mL absolute ethanol in an oven-dried flask under nitrogen. p-Hydroxybenzaldehyde (0.418 g, 3.42 mmol, 1.1 eq) was added, and the reaction was heated at reflux for 1 h. The reaction mixture was cooled to RT, and the white precipitate was collected by filtration to yield 33 (693 mg, 64% yield): ¹H NMR (d-DMSO) 2.97 (s, 3H), 3.20 (s, 3H), 5.59 (s, 1H), 6.73 (d, J= 8.5 Hz, 2H), 7.28 (d, J= 8.4 Hz, 3H), 7.38 (m, 4H), 7.48 (s, 1H), 9.74 (s, 1H), ¹³C NMR (d-DMSO) 28.04, 36.32, 88.49, 115.81, 126.19, 128.13, 128.81, 129.34, 138.51, 139.84, 152.16, 156.34, 158.75, 163.02, MS m/z 351.1 (M+H), 373.1 (M+Na).

3-(4-Hydroxyphenyl)-6-methyl-8-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (29). A mixture of hydrazone 33 (0.693 g, 1.98 mmol) in 8 mL glacial acetic acid and 500 uL H₂O was cooled to 0°C. Sodium nitrite (0.205 g, 2.97 mmol, 1.5 eq) was
added, and the reaction mixture was gradually warmed to RT and stirred overnight. The mixture was returned to 0°C, and the bright yellow precipitate was collected by filtration to yield 29 (300 mg, 44% yield): ¹H NMR (d-DMSO) 3.40 (s, 3H), 6.97 (d, J = 8.7 Hz, 2H), 7.44 (d, J = 7.3 Hz, 2H), 7.55 (t, J = 7.3 Hz, 1H), 7.62 (t, J = 7.3 Hz, 2H), 8.25 (d, J = 8.7 Hz, 2H), 10.19 (s, 1H), MS m/z 348.1 (M+H).

3-(4-(2-(Diethylamino)ethoxy)phenyl)-6-methyl-8-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (30). Pyrimidotriazine 29 (0.070 g, 0.202 mmol) was suspended in 3 mL dry acetone in an oven-dried flask equipped with a drying tube. Cesium carbonate (0.165 g, 0.505 mmol, 2.5 eq) was added, followed by 2-(diethylamino)ethyl chloride hydrochloride (0.042 g, 0.242 mmol, 1.2 eq). The reaction mixture was stirred at RT for 72 h. The mixture was filtered, and the filtrate was concentrated. The precipitate was collected by filtration to yield 30 (40 mg, 44% yield): mp 185-195°C (dec), ¹H NMR (d-DMSO) 0.98 (t, J = 7.1 Hz, 6H), 2.56 (q, J = 7.1 Hz, 4H), 2.81 (t, J = 6.1 Hz, 2H), 3.40 (s, 3H), 4.12 (t, J = 6.1 Hz, 2H), 7.15 (d, J = 8.8 Hz, 2H), 7.43 (d, J = 6.9 Hz, 2H), 7.59 (m, 3H), 8.32 (d, J = 8.8 Hz, 2H), ¹³C NMR (d-DMSO) 12.36, 29.23, 47.44, 51.72, 67.13, 115.67, 126.65, 129.39, 129.44, 129.51, 129.96, 133.28, 135.74, 149.91, 151.09, 160.18, 160.74, 161.83.

6-Chloro-3-methyl-4-nitropyrimidine-2,4(1H,3H)-dione (50).⁵⁷ (Scheme 2-10) Concentrated sulfuric acid (30 mL) was cooled to 0°C. 6-Chloro-3-methyluracil (1) (5.0 g, 31.1 mmol) was added incrementally. After the uracil had completely solubilized, fuming nitric acid (5.0 mL, 119 mmol, 3.8 eq) was added dropwise, and when addition was complete, the mixture was stirred for 10 minutes at 0°C. The mixture was warmed to RT, stirred for an additional 30 minutes, and poured onto ice. The precipitate that formed was collected by filtration after the ice had melted to yield a pale yellow powder as 50 (3.74 g, 59%): ¹H NMR (d-DMSO) 2.99 (s, 3H), 10.11 (br s, 1H), MS 204.3 (M⁺).

1-(4-Methoxybenzylidene)-2-phenylhydrazine (53). A mixture of phenylhydrazine (2.0 mL, 20.3 mmol) and p-anisaldehyde (2.7 mL, 22.3 mmol) was dissolved in 30 mL
absolute ethanol. The solution was heated at reflux for 90 min and then cooled to 25 °C. The precipitated solid was collected by filtration and rinsed thoroughly with ethanol to afford a pale peach product as 53 (3.91 g, 85% yield): mp 226-228 °C, \(^1\)H NMR (\(d\)-DMSO) 3.78 (s, 3H), 6.72 (t, \(J= 7.2\) Hz, 1H), 6.96 (d, \(J= 8.7\) Hz, 2H), 7.06 (d, \(J= 7.75\) Hz, 2H), 7.21 (t, \(J= 7.8\) Hz, 2H), 7.59 (d, \(J= 8.7\) Hz, 2H), 7.84 (s, 1H).

6-(2-(4-Methoxybenzylidene)-1-phenylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1\(H,3H\))-dione (54). Hydrazone 53 (200 mg, 0.88 mmol) was suspended in anhydrous THF under nitrogen. AlCl₃ (118 mg, 0.88 mmol) and 6-chloro-3-methyl-5-nitouracil (5) (164 mg, 0.80 mmol) were added, and the mixture was heated at reflux for 4h, during which time it became homogeneous and dark green. The mixture was cooled to 25 °C and then on ice, and the precipitated solid was collected by filtration and washed with THF and EtOH to leave a pale lime green powder as 54 (490 mg, 65% yield): mp 225-229 °C, \(^1\)H NMR (\(d\)-DMSO) 3.16 (s, 3H), 3.80 (s, 3H), 6.97 (d, \(J= 8.7\) Hz, 2H), 7.39 (d, \(J= 6.05\) Hz, 2H), 7.43 (s, 1H), 7.60 (m, 5H), 11.50 (br s, 1H), \(^13\)C NMR (\(d\)-DMSO) 27.69, 55.80, 114.65, 122.21, 125.98, 128.78, 129.37, 130.57, 130.84, 134.62, 145.07, 147.27, 149.19, 157.44, 161.74.

3-(4-Methoxyphenyl)-6-methyl-1-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(1\(H,6H\))-dione (55). To a suspension of 54 (494 mg, 1.25 mmol) in 6 mL of 50% aq EtOH was added zinc dust (327 mg, 5.0 mmol) and ammonium chloride (134 mg, 2.5 mmol). The suspension was stirred vigorously and heated at reflux overnight with exposure to the air. The mixture was cooled to 25 °C, diluted with 5 mL of 1N aq HCl, and stirred further for 1 h at 25 °C. The precipitated solid was collected by filtration, washed with 1N aq HCl and then EtOH, and dried to leave a red powder as 55 (352 mg, 78% yield): mp 315-317 °C (dec), \(^1\)H NMR (\(d\)-DMSO) 3.34 (s, 3H), 3.88 (s, 3H), 7.14 (d, \(J= 8.4\) Hz, 2H), 7.67 (m, 3H), 7.76 (m, 2H), 8.15 (d, \(J= 8.3\) Hz, 2H); \(^13\)C NMR (\(d\)-TFA) 24.68, 50.69, 117.96, 119.92, 126.77, 127.18, 130.03, 132.30, 137.09, 139.86, 143.29, 153.81, 160.79, MS m/z 362.2 (M+H). 

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General Procedure for \textit{in situ} Generation of Hydrazone and Reaction with 50. (Table 2-6) The aldehyde (1.19 mmol, 1.1 eq) and phenylhydrazine (107 $\mu$L, 1.08 mmol, 1.0 eq) were dissolved in anhydrous THF in an oven-dried flask under nitrogen. The solution was heated at reflux for 1.5 h. The solution was cooled to RT, and 50 (200 mg, 0.97 mmol, 0.9 eq) was added, followed by aluminum trichloride (144 mg, 1.08 mmol, 1.0 eq). The reaction mixture was returned to reflux for 5 h. It was cooled to RT and on ice, and precipitate was collected by filtration, rinsing with THF and ethanol.

6-(2-(4-Methoxybenzylidene)-1-phenylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (58a). Pale lime green powder, 490 mg (65% yield), mp 225-229°C, $^1$H NMR ($d$-DMSO) 3.16 (s, 3H), 3.80 (s, 3H), 6.97 (d, J= 8.7 Hz, 2H), 7.39 (d, J= 6.05 Hz, 2H), 7.43 (s, 1H), 7.60 (m, 5H), 11.50 (br s, 1H), $^{13}$C NMR ($d$-DMSO) 27.69, 55.80, 114.65, 122.21, 125.98, 128.78, 129.37, 130.57, 130.84, 134.62, 145.07, 147.27, 149.19, 157.44, 161.74.

6-(2-(4-Hydroxybenzylidene)-1-phenylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (58b). Pale yellowish-green powder, 805 mg (72% yield), mp 235-240°C (dec), $^1$H NMR ($d$-DMSO) 3.15 (s, 3H), 6.77 (d, J= 8.6 Hz, 2H), 7.39 (m, 3H), 7.48 (d, J= 8.6 Hz, 2H), 7.60 (m, 3H), 10.03 (s, 1H), 11.50 (br s, 1H), $^{13}$C NMR ($d$-DMSO) 27.67, 115.75, 122.25, 124.38, 129.68, 130.43, 130.80, 134.72, 145.57, 147.29, 149.21, 157.46, 160.43.

6-(2-(4-Chlorobenzylidene)-1-phenylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (58c). Yellow powder, 95 mg (53% yield), mp 210-212°C (dec), $^1$H NMR ($d$-DMSO) 3.14 (s, 3H), 7.42 (d, J= 7.7 Hz, 2H), 7.50 (m, 3H), 7.58 (m, 3H), 7.70 (d, J= 7.7 Hz), $^{13}$C NMR ($d$-DMSO) 27.72, 112.52, 122.27, 127.83, 128.78, 129.11, 130.22, 131.17, 132.73, 134.97, 135.29, 142.61, 145.53, 157.75, MS m/z 422.1 (M+Na).

6-(1-(4-Fluorophenyl)-2-(4-methoxybenzylidene)hydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (58d). Yellow powder, 284 mg (71% yield), mp 254-255°C, $^1$H NMR ($d$-DMSO) 3.14 (s, 3H), 3.80 (s, 3H), 6.97 (d, J= 8.9 Hz, 2H), 7.42
(s, 1H), 7.47 (m, 4H), 7.62 (d, J= 8.9 Hz, 2H), $^{13}$C NMR (d-DMSO) 27.65, 55.80, 114.64, 115.80, 117.85, 125.99, 128.87, 130.28, 132.55, 145.26, 147.08, 149.17, 157.48, 161.74, 164.77, $^{19}$F NMR (d-DMSO) -102.22.

6-(1-(4-Fluorophenyl)-2-(4-hydroxybenzylidene)hydrazinyl)-3-methyl-5-nitropyrimidine-$2,4(1H,3H)$-dione (58e). Pale lime green/yellow powder, 316 mg (82% yield), mp > 255°C (dec), $^1$H NMR (d-DMSO) 3.14 (s, 3H), 6.76 (d, J= 8.5 Hz, 2H), 7.35 (s, 1H), 7.45 (m, 6H), 10.04 (br s, 1H), $^{13}$C NMR (d-DMSO) 27.64, 115.77, 117.81, 118.11, 124.38, 130.48, 132.53, 145.78, 147.07, 149.17, 157.48, 160.47, 161.43, 164.71, $^{19}$F NMR (d-DMSO) -102.30.

6-(1-(4-Fluorophenyl)-2-((5-methylfuran-2-yl)methylene)hydrazinyl)-3-methyl-5-nitropyrimidine-$2,4(1H,3H)$-dione (58f). Pale mustard yellow powder, 142 mg (38% yield), mp > 250°C (dec), $^1$H NMR (d-DMSO) 2.33 (s, 3H), 3.16 (s, 3H), 6.27 (d, J= 3.0 Hz, 1H), 6.86 (d, J= 3.0 Hz, 1H), 7.32 (s, 1H), 7.45 (m, 4H), 11.50 (br s, 1H).

6-(1-(4-Fluorophenyl)-2-(4-methylbenzylidene)hydrazinyl)-3-methyl-5-nitropyrimidine-$2,4(1H,3H)$-dione (58g). Bright pastel yellow powder, 280 mg (73% yield), mp 245-248°C, $^1$H NMR (d-DMSO) 2.30 (s, 3H), 3.14 (s, 3H), 7.21 (d, J= 8.1 Hz, 2H), 7.37 (s, 1H), 7.45 (m, 6H), $^{13}$C NMR (d-DMSO) 21.20, 27.68, 116.48, 124.78, 128.47, 129.67, 130.47, 132.51, 141.09, 145.27, 147.16, 149.18, 157.47, 161.50, 164.78, $^{19}$F NMR (d-DMSO) -108.1.

6-(1-(4-Bromophenyl)-2-(4-methoxybenzylidene)hydrazinyl)-3-methyl-5-nitropyrimidine-$2,4(1H,3H)$-dione (58h). Pale orange powder, 96 mg (31% yield), mp 236-238°C (dec), $^1$H NMR (d-DMSO), 3.12 (s, 3H), 3.77 (s, 3H), 6.96 (d, J= 8.8 Hz, 2H), 7.37 (d, J= 8.7 Hz, 2H), 7.51 (s, 1H), 7.60 (d, J= 8.8 Hz, 2H), 7.87 (d, J= 8.7 Hz, 2H), $^{13}$C NMR (d-DMSO) 25.95, 55.66, 114.18, 114.64, 114.98, 127.67, 129.98, 131.27, 132.12, 133.81, 138.02, 145.70, 159.91, 161.82.
6-(2-(4-Fluorobenzylidene)-1-(4-methoxyphenyl)hydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (58i). Bright fluorescent yellow powder, 556 mg (73% yield), mp 228-229°C, $^1$H NMR (d-DMSO), 3.15 (s, 3H), 3.86 (s, 3H), 7.14 (d, J= 8.85 Hz, 2H), 7.23 (dd, J= 8.85 Hz, J’= 8.85 Hz, 2H), 7.33 (d, J= 8.8 Hz, 2H), 7.46 (s, 1H), 7.75 (dd, J= 8.3 Hz, J’= 5.7 Hz, 2H), $^{13}$C NMR (d-DMSO) 27.66, 55.99, 115.83, 124.39, 126.33, 129.68, 130.12, 131.64, 143.71, 147.29, 149.11, 158.83, 160.83, 162.18, 165.48, $^{19}$F NMR (d-DMSO) -107.2.

General Procedure for Cyclization of 58 to Pyrimidotriazinediones (59). (Table 2-6)
To a suspension of 58 (1.25 mmol) in 6 mL of 50/50 ethanol/water was added zinc dust (327 mg, 5.0 mmol, 4.0 eq) and ammonium chloride (134 mg, 2.5 mmol, 2.0 eq). The suspension was heated at reflux overnight. The mixture was cooled to RT, and 5 mL of 1N HCl was added. After stirring for 1 h at RT, precipitate was collected by filtration. Precipitate was washed with 1N HCl and ethanol and dried to yield 59.

3-(4-Methoxyphenyl)-6-methyl-1-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59a). Red powder, 84 mg (78% yield), mp 315-317°C (dec), $^1$H NMR (d-DMSO) 3.34 (s, 3H), 3.88 (s, 3H), 7.14 (d, J= 8.4 Hz, 2H), 7.67 (m, 3H), 7.75 (m, 2H), 8.15 (d, J= 8.3 Hz, 2H), $^{13}$C NMR (d-TFA) 24.68, 50.69, 117.96, 119.92, 126.77, 127.18, 130.03, 132.30, 137.09, 139.86, 143.29, 153.81, 160.79, MS m/z 362.2 (M+H).

3-(4-Hydroxyphenyl)-6-methyl-1-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59b). Deep red powder, 151 mg (72% yield), mp >325°C, $^1$H NMR (d-DMSO) 3.28 (s, 3H), 6.95 (d, J= 7.0 Hz, 2H), 7.60 (d, J= 7.0 Hz, 1H), 7.66 (m, 2H), 7.75 (d, J= 6.2 Hz, 2H), 8.05 (d, J= 7.0 Hz, 2H), $^{13}$C NMR (d-TFA) 31.08, 118.90, 124.79, 126.28, 133.40, 133.58, 136.44, 138.64, 143.56, 146.28, 149.69, 160.09.

3-(4-Chlorophenyl)-6-methyl-1-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59c). Reddish-orange powder, 16 mg (72% yield), mp >305°C, $^1$H NMR (d-DMSO) 3.28 (s, 3H), 6.95 (d, J= 7.0 Hz, 2H), 7.60 (d, J= 7.0 Hz, 1H), 7.66 (m, 2H), 7.87 (d, J= 6.2 Hz, 2H), 8.05 (d, J= 7.0 Hz, 2H), $^{13}$C NMR (d-TFA) 31.31, 126.57, 129.86,
1-(4-Fluorophenyl)-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59d). Bright red/orange solid, 188 mg (72% yield), mp 310-315°C (dec), $^1$H NMR ($d$-DMSO) 3.29 (s, 3H), 3.86 (s, 3H), 7.14 (d, J= 8.8 Hz, 2H), 7.51 (t, J= 8.7 Hz, 2H), 7.82 (dd, J$_1$= 8.8 Hz, J$_2$= 4.9 Hz), 8.14 (d, J= 8.8 Hz, 2H), $^{13}$C NMR (d-DMSO) 26.03, 52.04, 112.45, 115.67, 117.07, 119.19, 124.06, 124.19, 128.12, 129.45, 144.84, 154.96, 162.24, $^{19}$F NMR ($d$-DMSO) -105.6, MS $m/z$ 380.1 (M+H), 402.1 (M+Na).

1-(4-Fluorophenyl)-3-(4-hydroxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59e). Maroon solid, 108 mg (82% yield), mp > 325°C, $^1$H NMR ($d$-TFA) 3.60 (s, 3H), 7.06 (d, J= 8.4 Hz, 2H), 7.40 (d, J= 7.1 Hz, 2H), 7.70 (d, J= 8.3 Hz, 2H), 8.29 (d, J= 8.2 Hz, 2H), $^{13}$C NMR ($d$-TFA) 31.42, 119.25, 121.11, 121.30, 125.05, 129.48, 133.74, 144.21, 144.71, 150.23, 160.37, $^{19}$F NMR ($d$-TFA) -106.3.

1-(4-Fluorophenyl)-6-methyl-3-(5-methylfuran-2-yl)pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59f). Burgundy solid, 80 mg (61% yield), mp >305°C, $^1$H NMR ($d$-TFA) 2.36 (s, 3H), 3.56 (s, 3H), 6.33 (s, 1H), 7.38 (d, J= 5.4 Hz, 2H), 7.63 (s, 1H), 7.67 (t, J= 4.4 Hz, 2H).

1-(4-Fluorophenyl)-3-(4-methylphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59g). Orange solid, 108 mg (82% yield), mp 322-325°C (dec), $^1$H NMR ($d$-TFA) 2.39 (s, 3H), 3.60 (s, 3H), 7.34 (d, J= 8.2 Hz, 2H), 7.41 (dd, J= 8.9 Hz, J’= 7.6 Hz, 2H), 7.71 (dd, J= 8.8 Hz, J’= 3.7 Hz, 2H), 8.20 (d, J= 8.2 Hz, 2H), $^{13}$C NMR ($d$-TFA) 22.41, 31.40, 121.07, 121.26, 128.67, 129.49, 129.57, 131.04, 132.89, 144.40, 146.68, 150.14, 161.44, $^{19}$F NMR ($d$-TFA) -103.8.

1-(4-Bromophenyl)-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59h). Cherry red powder, 89 mg (76% yield), mp 265-268°C (dec),
$^{1}$H NMR (d-DMSO) 3.36 (s, 3H), 3.86 (s, 3H), 7.15 (d, J = 8.5 Hz, 2H), 7.75 (d, J = 8.3 Hz, 2H), 7.88 (d, J = 8.3 Hz, 2H), 8.15 (d, J = 8.6 Hz, 2H), $^{13}$C NMR (d-DMSO) 28.78, 55.96, 115.20, 123.58, 125.24, 128.44, 129.10, 132.67, 140.00, 149.01, 150.09, 154.51, 162.42.

3-(4-Fluorophenyl)-1-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59i). Purplish maroon solid, 65 mg (35% yield), mp 258°C (dec), $^{1}$H NMR (d-DMSO) 3.33 (s, 3H), 3.87 (s, 3H), 7.18 (d, J = 8.1 Hz, 2H), 7.43 (dd, J = 8.8 Hz, J’ = 8.8 Hz, 2H), 7.73 (d, J = 8.1 Hz, 2H), 8.25 (d, J = 8.8 Hz, 2H), $^{13}$C NMR (d-DMSO) 28.79, 56.14, 114.69, 115.58, 116.01, 116.68, 116.96, 127.73, 129.71, 130.06, 130.73, 130.85, 149.97, 151.16, 160.61, $^{19}$F NMR (d-DMSO) -106.8.

General Procedure for in situ Generation of Hydrazone and Reaction with 50. (Table 2-7) The aldehyde (1.19 mmol, 1.1 eq) and hydrazine (1.08 mmol, 1.0 eq) were dissolved in anhydrous THF in an oven-dried flask under nitrogen. The solution was heated at reflux for 1.5 h, and then 50 (200 mg, 0.97 mmol, 0.9 eq) was added. A precipitate began to form within 5-10 minutes after addition of 50. The mixture was heated for an additional 1 h. It was then cooled to RT and on ice, and precipitate was collected by filtration, rinsing with THF and ethanol.

6-(1-(2-Hydroxyethyl)-2-(4-methoxybenzylidene)hydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (58j). Bright pastel yellow powder, 278 mg (67% yield), mp 165-168°C (dec), $^{1}$H NMR (d-DMSO) 3.25 (s, 3H), 3.67 (t, J = 4.55 Hz, 2H), 3.82 (s, 3H), 4.20 (t, J = 4.55 Hz, 2H), 6.91 (d, J = 8.8 Hz, 2H), 7.50 (d, J = 8.8 Hz, 2H), 7.83 (s, 1H), 7.88 (s, 1H), $^{13}$C NMR (d-DMSO) 27.58, 48.35, 55.76, 57.69, 114.76, 115.43, 127.09, 129.45, 143.01, 147.82, 149.07, 157.47, 161.33, MS m/z 362.52 (M+H).

6-(2-(3-Chlorobenzylidene)-1-(2-hydroxyethyl)hydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (58k). Bright pastel yellow powder, 241 mg (68% yield), mp 166-169°C (dec), $^{1}$H NMR (d-DMSO) 3.16 (s, 3H), 3.71 (t, J = 4.35 Hz, 2H), 4.26 (t, J = 4.35 Hz, 2H), 7.50 (m, 3H), 7.61 (s, 1H), 8.15 (s, 1H), $^{13}$C NMR (d-DMSO)
6-(2-(4-Chlorobenzylidene)-1-(2-hydroxyethyl)hydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (58l). Bright pastel yellow powder, 215 mg (60 % yield), mp 202-204°C, $^1$H NMR (d-DMSO) 3.16 (s, 3H), 3.71 (t, J= 4.7 Hz, 2H), 4.26 (t, J= 4.7 Hz, 2H), 7.53 (m, 4H), 8.15 (s, 1H), $^{13}$C NMR (d-DMSO) 27.64, 48.58, 57.50, 115.83, 129.08, 129.42, 133.45, 135.09, 141.77, 148.10, 149.07, 157.41.

6-(1-(2-Hydroxyethyl)-2-(4-methylbenzylidene)hydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (58m). Bright pastel yellow powder, 224 mg (56% yield), mp 175-178°C (dec), $^1$H NMR (d-DMSO) 2.32 (s, 3H), 3.16 (s, 3H), 3.70 (t, J= 4.4 Hz, 2H), 4.26 (t, J= 4.4 Hz, 2H), 7.25 (d, J= 7.9 Hz, 2H), 7.46 (d, J= 7.9 Hz, 2H), 8.10 (s, 1H), $^{13}$C NMR (d-DMSO) 21.48, 27.50, 48.29, 57.51, 115.62, 127.68, 129.75, 131.72, 140.37, 142.98, 147.87, 149.07, 157.46.

6-(1-Isopentyl-2-(4-methoxybenzylidene)hydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (58o). Pale/dull yellow solid, 595 mg (52% yield), mp 264-267°C (dec), $^1$H NMR (d-DMSO) 0.92 (d, J= 6.6 Hz, 6H), 1.46 (q, J= 6.7 Hz, 2H), 1.67 (septet, J= 6.7 Hz, 1H), 3.16 (s, 3H), 3.79 (s, 3H), 4.12 (t, J= 7.55 Hz, 2H), 7.01 (d, J= 8.8 Hz, 2H), 7.53 (d, J= 8.8 Hz, 2H), 7.98 (s, 1H), $^{13}$C NMR (d-DMSO) 25.78, 27.61, 33.89, 37.75, 55.79, 114.75, 127.06, 128.46, 129.52, 143.10, 147.92, 149.66, 157.44, 161.36. MS m/z 388.7 (M+).

6-(1-Isopropyl-2-(4-methoxybenzylidene)hydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (58p). Bright pastel yellow powder, 74 mg (80% yield), mp 280-285°C (dec), $^1$H NMR (d-DMSO) 1.40 (d, J= 6.1 Hz, 6H), 3.20 (s, 3H), 3.79 (s, 3H), 4.76 (m, 1H), 6.97 (d, J= 8.3 Hz, 2H), 8.15 (d, J= 8.2 Hz, 2H), 12.25 (s, 1H), $^{13}$C NMR (d-DMSO) 22.04, 27.57, 49.37, 55.59, 95.54, 113.88, 124.76, 129.86, 143.21, 147.78, 151.18, 158.64, 160.04.
General Procedure for Cyclization of 58 to Pyrimidotriazinediones (59). (Table 2-7)

To a suspension of 58 (1.25 mmol) in 6 mL of 50/50 ethanol/water was added zinc dust (327 mg, 5.0 mmol, 4.0 eq) and ammonium chloride (134 mg, 2.5 mmol, 2.0 eq). The suspension was heated at reflux overnight. The mixture was cooled to RT, and 5 mL of 1N HCl was added. After stirring for 1 h at RT, precipitate was collected by filtration. Precipitate was washed with 1N HCl and ethanol and dried to yield 59.

1-(2-Hydroxyethyl)-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59j). Fluorescent orange solid, 95 mg (51% yield), 215-217°C (dec), \(^1\)H NMR (\(\text{d-DMSO}\)) 3.28 (s, 3H), 3.86 (s, 3H), 3.93 (q, J = 5.8 Hz, 2H), 4.53 (t, J = 5.7 Hz, 2H), 4.98 (t, J = 6.1 Hz, 1H), 7.14 (d, J = 8.9 Hz, 2H), 8.13 (d, J = 8.9 Hz, 2H), \(^1^3\)C NMR (\(\text{d-DMSO}\)) 28.75, 55.93, 57.10, 58.11, 115.12, 125.54, 128.89, 146.89, 149.76, 151.76, 154.58, 159.54, 162.25.

3-(3-Chlorophenyl)-1-(2-hydroxyethyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59k). Bright yellow powder, 56 mg (62% yield), mp 201-204°C (dec), \(^1\)H NMR (\(\text{d-DMSO}\)) 3.28 (s, 3H), 3.94 (t, J = 5.5 Hz, 2H), 4.56 (t, J = 5.5 Hz, 2H), 5.00 (t, J = 6.1 Hz, 1H), 7.66 (m, 2H), 8.16 (s, 1H), 8.34 (d, J = 7.4 Hz, 1H), \(^1^3\)C NMR (\(\text{d-DMSO}\)) 28.77, 57.24, 58.02, 125.71, 126.44, 126.90, 131.50, 132.84, 135.28, 147.09, 150.09, 154.60, 158.81, 161.00.

3-(4-Chlorophenyl)-1-(2-hydroxyethyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59l). Bright orange/yellow powder, 40 mg (67% yield), mp 210-212°C (dec), \(^1\)H NMR (\(\text{d-DMSO}\)) 3.33 (s, 3H), 3.95 (t, J = 5.2 Hz, 2H), 4.55 (t, J = 5.3 Hz, 2H), 4.98 (br s, 1H), 7.68 (d, J = 8.5 Hz, 2H), 8.21 (d, J = 8.5 Hz, 2H), \(^1^3\)C NMR (\(\text{d-DMSO}\)) 28.77, 57.20, 58.04, 128.85, 132.11, 136.57, 147.09, 150.11, 150.63, 154.61, 159.43.

1-(2-Hydroxyethyl)-3-(4-methylphenyl)-6-methyl-pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59m). Bright reddish-orange powder, 57 mg (70% yield), mp 199-201°C (dec), \(^1\)H NMR (\(\text{d-DMSO}\)) 2.41 (s, 3H), 3.34 (s, 3H), 3.93 (t, J = 5.2 Hz, 2H), 4.53
(t, J= 5.3 Hz, 2H), 4.98 (br s, 1H), 7.39 (d, J= 7.3 Hz, 2H), 8.21 (d, J= 7.3 Hz, 2H), $^{13}$C NMR (d-DMSO) 21.50, 28.75, 57.15, 58.09, 127.07, 130.28, 132.64, 141.67, 146.92, 149.95, 151.75, 154.59, 159.49, MS m/z 314.1 (M+H).

3-(3,4-Dimethoxyphenyl)-1-(2-hydroxyethyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59n). Deep maroon solid, 135 mg (39% yield), mp 215-218˚C (dec), $^1$H NMR (d-DMSO) 3.59 (s, 3H), 3.96 (s, 6H), 4.51 (t, J= 5.3 Hz, 2H), 5.08 (t, J= 5.3 Hz, 2H), 5.12 (br s, 1H), 7.08 (d, J= 8.7 Hz, 1H), 7.89 (s, 1H), 8.12 (d, J= 8.7 Hz, 1H), $^{13}$C NMR (d-DMSO) 28.73, 55.86, 57.03, 58.07, 109.57, 111.92, 120.93, 125.56, 146.81, 149.58, 151.69, 152.09, 154.56, 159.51, 161.22.

1-Isopentyl-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59o). Bright orange, fluffy solid, 58 mg (62% yield), mp > 185˚C (dec), $^1$H NMR (d-DMSO) 0.96 (d, J= 6.4 Hz, 6H), 1.68 (m, 1H), 1.76 (m, 2H), 3.21 (s, 3H), 3.85 (s, 3H), 4.44 (t, J= 6.8 Hz, 2H), 7.13 (d, J= 8.9 Hz, 2H), 8.12 (d, J= 8.9 Hz, 2H), $^{13}$C NMR (d-DMSO) 22.67, 25.45, 28.71, 39.16, 52.79, 55.92, 113.91, 115.15, 125.57, 128.79, 147.13, 149.37, 151.74, 154.64, 159.60, 162.24.

1-Isopropyl-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione (59p). Bright orange solid, 45 mg (51% yield), mp 267-270˚C, $^1$H NMR (d-DMSO) 1.48 (d, J= 5.4 Hz, 6H), 3.29 (s, 3H), 3.86 (s, 3H), 5.54 (m, 1H), 7.16 (d, J= 7.5 Hz, 2H), 8.18 (d, J= 7.5 Hz, 2H), $^{13}$C NMR (d-DMSO) 20.76, 22.07, 28.68, 53.38, 55.92, 113.91, 115.14, 125.89, 128.81, 129.86, 149.03, 154.75, 159.70, 162.21.

**General Procedure for in situ Generation of Methylhydrazone and Reaction with 50. (Table 2-8)** The aldehyde (1.19 mmol, 1.1 eq) and methylhydrazine (1.08 mmol, 1.0 eq) were dissolved in anhydrous THF in an oven-dried flask under nitrogen. The solution was heated at reflux for 1.5 h, and then 50 (200 mg, 0.97 mmol, 0.9 eq) was added. A precipitate began to form within 5-10 minutes after addition of 50. The mixture was heated for an additional 1 h. It was then cooled to RT and on ice, and precipitate was collected by filtration, rinsing with THF and ethanol.
6-(2-Benzylidene-1-methylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (62q). 220 mg (75% yield), \(^1\)H NMR (\(d\)-DMSO) 3.15 (s, 3H), 3.44 (s, 3H), 7.42 (m, 3H), 7.63 (d, \(J= 7.2\) Hz, 2H), 8.02 (s, 1H), 12.0 (br s), \(^{13}\)C NMR (\(d\)-DMSO) 27.63, 34.76, 115.40, 127.83, 129.21, 130.42, 134.64, 142.90, 149.27, 150.10, 157.57.

6-(2-(4-Fluoro-3-methoxybenzylidene)-1-methylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (62r). Bright pastel yellow powder, 295 mg (84% yield), mp 218-222°C, \(^1\)H NMR (\(d\)-DMSO) 3.15 (s, 3H), 3.95 (s, 3H), 7.35 (m, 1H), 7.55 (m, 1H), 7.90 (d, \(J= 8.5\) Hz, 1H), 8.71 (s, 1H), 11.30 (br s, 1H), 12.43 (br s, 1H), \(^{13}\)C NMR (\(d\)-DMSO) 27.63, 34.98, 56.34, 111.01, 116.49, 122.42, 131.20, 131.65, 142.36, 149.47, 157.62, \(^{19}\)F NMR (\(d\)-DMSO) -129.7, MS \(m/z\) 336.6 (M\(^+\)).

6-(2-(4-Fluorobenzylidene)-1-methylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (62s). Bright yellow powder, 1.761 g (87% yield), mp 205-206°C, \(^1\)H NMR (\(d\)-DMSO) 3.15 (s, 3H), 3.41 (s, 3H), 7.30 (dd, \(J= 8.8\) Hz, \(J' = 8.8\) Hz, 2H), 7.69 (dd, \(J= 8.0\) Hz, \(J' = 5.9\) Hz, 2H), 8.06 (s, 1H). \(^{13}\)C NMR (\(d\)-DMSO) 27.62, 35.31, 115.34, 116.19, 116.49, 130.05, 130.17, 131.06, 131.10, 142.63, 148.56, 149.44, 157.29, 161.94, 165.23, \(^{19}\)F NMR (\(d\)-DMSO) -107.89.

6-(2-(3-Fluorobenzylidene)-1-methylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (62t). Bright yellow powder, 770 mg (82% yield), mp 215-216°C, \(^1\)H NMR (\(d\)-DMSO) 3.16 (s, 3H), 3.40 (s, 3H), 7.27 (t, \(J= 8.0\) Hz, 1H), 7.48 (m, 3H), 8.07 (s, 1H), \(^{13}\)C NMR (\(d\)-DMSO) 27.65, 35.45, 113.28, 117.49, 124.68, 131.26, 136.88, 142.32, 148.58, 149.41, 157.27, 161.20, 164.43, \(^{19}\)F NMR (\(d\)-DMSO) -110.33.

6-(2-(4-Chlorobenzylidene)-1-methylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (62u). 264 mg (78% yield), \(^1\)H NMR (\(d\)-DMSO) 3.26 (s, 3H), 3.45 (s, 3H), 7.39 (d, \(J= 8.45\) Hz, 2H), 7.65 (d, \(J= 8.45\) Hz, 2H), 7.92 (s, 1H), 12.0 (br s, 1H), \(^{13}\)C NMR (\(d\)-DMSO) 27.65, 35.38, 115.48, 129.37, 129.49, 133.40, 135.11, 142.41, 148.64, 149.45, 157.28.
6-(2-(3-Methoxybenzylidene)-1-methylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (62v). Bright pastel yellow powder, 3.48 g (72% yield), mp 195-196°C, \(^1\)H NMR (\(\text{d-DMSO}\)) 3.15 (s, 3H), 3.43 (s, 3H), 3.79 (s, 3H), 6.97 (d, J= 7.4 Hz, 1H), 7.18 (d, J= 7.8 Hz, 1H), 7.20 (s, 1H), 7.34 (t, J= 7.8 Hz, 1H), 8.00 (s, 1H), \(^{13}\)C NMR (\(\text{d-DMSO}\)) 27.62, 35.07, 55.57, 111.17, 117.12, 121.30, 130.28, 135.89, 143.22, 148.32, 149.48, 157.36, 159.96.

6-(2-(4-Methoxybenzylidene)-1-methylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (62w). Bright yellow powder, 292 mg (90% yield), mp 203-204°C (dec), \(^1\)H NMR (\(\text{d-DMSO}\)) 3.14 (s, 3H), 3.40 (s, 3H), 3.80 (s, 3H), 7.00 (d, J= 6.8 Hz, 2H), 7.59 (d, J= 6.8 Hz, 2H), 7.99 (s, 1H), 12.3 (br s, 1H), \(^{13}\)C NMR (\(\text{d-DMSO}\)) 27.60, 31.15, 55.77, 113.78, 113.96, 114.73, 129.59, 129.77, 132.38, 161.32.

General Procedure for Cyclization and Demethylation to \(N_8\)-H Pyrimidotriazinediones (6). (Table 2-8) To a suspension of 6-(2-benzylidene-1-methylhydrazinyl)-3-methyl-5-nitropyrimidine-2,4(1H,3H)-dione (62) (1.25 mmol) in 15 mL of 50/50 DMF/water was added zinc dust (327 mg, 5.0 mmol, 4.0 eq) and ammonium chloride (134 mg, 2.5 mmol, 2.0 eq). The suspension was stirred vigorously and heated at reflux overnight. The mixture was cooled to RT, and 15 mL of 1N HCl was added. After stirring for 1 h at RT, precipitate was collected by filtration. Precipitate was washed with 1N HCl and ethanol and dried.

6-Methyl-3-phenylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (6q). Olive green/mustard yellow solid, 78 mg (75% yield), mp >300°C, \(^1\)H NMR (\(\text{d-DMSO}\)) 3.38 (s, 3H), 7.46 (m, 3H), 8.41 (d, J= 7.6 Hz, 2H), 12.78 (s, 1H).

3-(4-Fluoro-3-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (6r). Dull yellow powder, 68 mg (86% yield), mp >325°C, \(^1\)H NMR (\(\text{d-DMSO}\)) 3.25 (s, 3H), 3.98 (s, 3H), 7.45 (t, J= 9.2 Hz, 1H), 7.99 (m, 1H), 8.10 (d, J= 8.2 Hz, 1H),
12.78 (br s, 1H), $^{13}$C NMR (d-DMSO) 30.82, 58.66, 115.20, 116.34, 117.71, 122.17, 125.89, 129.61, 136.63, 149.62, 152.29, 163.30, 170.22.

3-(4-Fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (6s).
Mustard yellow solid, 420 mg (87% yield), mp >300°C, $^1$H NMR (d-DMSO) 3.26 (s, 3H), 7.46 (t, J = 8.8 Hz, 2H), 8.45 (dd, J = 8.7 Hz, J’ = 5.6 Hz, 2H), 13.13 (s, 1H), MS m/z 272.4 (M-H).

3-(3-Fluorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (6t).
Mustard yellow powder, 1.350 g (61% yield), mp >325°C, $^1$H NMR (d-TFA) 3.78 (s, 3H), 7.44 (s, 1H), 7.67 (m, 1H), 8.21 (m, 1H), 8.32 (m, 1H), $^{13}$C NMR (d-TFA) 25.94, 112.09, 112.43, 117.81, 121.35, 128.42, 130.55, 131.01, 145.02, 147.56, 162.66, $^{19}$F NMR (d-TFA) -113.8, MS m/z 272.5 (M-H).

3-(4-Chlorophenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (6u).
Mustard yellow powder, 54 mg (78% yield), mp >300°C, $^1$H NMR (d-DMSO) 3.29 (s, 3H), 7.64 (d, J = 8.6 Hz, 2H), 8.36 (d, J = 8.6 Hz, 2H), 12.92 (s, 1H).

3-(4-Methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (6v).
Mustard yellow powder, 1.94 g (72% yield), mp >300°C, $^1$H NMR (d-DMSO) 3.22 (s, 3H), 3.81 (s, 3H), 7.17 (dd, J = 8.1 Hz, J’ = 2.1 Hz, 1H), 7.53 (t, J = 8.0 Hz, 1H), 7.91 (s, 1H), 7.98 (t, J = 8.0 Hz, 1H), 12.75 (s, 1H).

3-(4-Methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7(6H,8H)-dione (6w).
Mustard yellow powder, 897 mg (43% yield), mp >325°C, $^1$H NMR (d-TFA) 3.55 (s, 3H), 3.90 (s, 3H), 7.09 (d, J = 7.2 Hz, 2H), 8.31 (d, J = 7.2 Hz, 2H), $^{13}$C NMR (d-TFA) 19.49, 45.95, 116.54, 121.91, 132.06, 138.08, 147.51, 150.95, 161.72, 166.36.

Chapter 3
1,6-Dimethylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (63).$^{58}$ (Scheme 3-1)
Compound 2a (100 mg, 0.59 mmol) was dissolved in 7 mL water and heated to boiling.
Aqueous glyoxal (40%, 75 uL, 0.65 mmol, 1.1 eq) was added, and the solution was heated at reflux for another 20 minutes. The mixture was cooled to RT and then on ice for 3 h. An orange-brown precipitate was removed by filtration. The mother liquor was extracted with chloroform six times, and the combined organic extracts were dried (Na$_2$SO$_4$) and concentrated in vacuo to yield a yellow solid (110 mg). This solid was purified by recrystallization from ethanol to yield 63 (45 mg, 41%): mp 237-240°C (dec) (lit$^{58}$ mp 239-241°C), $^1$H NMR (CDCl$_3$) 3.44 (s, 3H), 4.22 (s, 3H), 8.21 (d, J= 4.3 Hz, 1H), 8.29 (d, J= 4.3 Hz, 1H), $^{13}$C NMR (d-DMSO) 28.16, 43.58, 122.96, 131.11, 140.73, 155.56, 156.00, 160.84.

1-Benzyl-6-methylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (64). $^{58}$ (Scheme 3-1)
A mixture of 6-chloro-3-methyluracil (200 mg, 1.25 mmol), benzylhydrazine dihydrochloride (488 mg, 2.5 mmol, 2 eq), and diisopropylethylamine (910 mL, 5.5 mmol, 4.4 eq) in 2 mL of pyridine was heated to 100°C for 1.25 h. It was cooled to RT and concentrated in vacuo to yield a yellow oil, which was purified by flash silica gel chromatography with 5% MeOH/CH$_2$Cl$_2$ as eluent to yield 114 mg of 3-methyl-6-(1-benzylhydrazinyl)pyrimidine-2,4(1H,3H)-dione. This compound was dissolved in 4.5 mL of warm absolute ethanol. Aqueous glyoxal (40%, 56 uL, 0.5 mmol, 1.1 eq) was added, and the solution was heated to 70°C for 1 h, during which time a precipitate began to form. The mixture was cooled to RT and then on ice. The precipitate collected was 64 (91 mg, 75%): mp 301-304°C (lit$^{58}$ mp 302-304°C), $^1$H NMR (d-DMSO) 3.08 (s, 3H), 5.17 (s, 2H), 5.20 (s, 1H), 7.14 (d, J= 7.25 Hz, 2H), 7.30 (m, 1H), 7.39 (m, 2H) 8.21 (s, 1H), 10.72 (s, 1H), $^{13}$C NMR (d-DMSO) 26.77, 47.27, 78.11, 126.57, 127.96, 129.37, 134.25, 137.95, 151.00, 163.62.

6-(1-(2-(tert-Butyldimethylsilyloxy)ethyl)hydrazinyl)-3-methylpyrimidine-2,4(1H,3H)-dione (67). (Scheme 3-2) Compound 2b (79 mg, 0.4 mmol) was suspended in 1.6 mL of anhydrous CH$_2$Cl$_2$ in an oven-dried flask under nitrogen and cooled to 0°C. tert-Butyldimethylsilylchloride (181 mg, 1.2 mmol, 3 eq) was added, followed by imidazole (81 mg, 1.2 mmol, 3 eq) 10 minutes later. After 2h, the reaction was diluted with 3 mL of CH$_2$Cl$_2$. The mixture was washed with saturated aqueous NaHCO$_3$, dried over
MgSO$_4$, and concentrated in vacuo. The crude material was purified by silica gel flash chromatography with 1% MeOH/CH$_2$Cl$_2$ as eluent to provide 67 (112 mg, 91%) as a white, crystalline solid: mp 129-131°C, $^1$H NMR (CDCl$_3$) 0.04 (s, 6H), 0.91 (s, 9H), 3.29 (s, 3H), 3.57 (t, J= 5.5 Hz, 2H), 3.91 (t, J= 5.5 Hz, 2H), 4.71 (s, 1H).

1-(2-(tert-Butyldimethylsilyloxy)ethyl)-6-methylpyrimido[4,5-c]pyrazine-5,7(1H,6H)-dione (68). (Scheme 3-2) Compound 67 (112 mg, 0.36 mmol) was suspended in 4 mL H$_2$O, and the mixture was heated to 50°C, at which time aqueous glyoxal (40%, 45 μL, 0.39 mmol, 1.1 eq) was added. The mixture was heated for 3 h wherein TLC (5% MeOH/CH$_2$Cl$_2$) indicated consumption of starting material. The mixture was cooled to RT and the precipitate collected to yield 68 (58 mg, 48%): mp 230°C (dec), $^1$H NMR (CDCl$_3$) 0.05 (s, 6H), 0.92 (s, 9H), 3.36 (s, 3H), 3.92 (t, J= 4.3 Hz, 2H), 3.99 (t, J= 4.3 Hz, 2H), 7.55 (m, 1H), 7.73 (m, 1H).

1,3,4,6-Tetramethylpyrimido[4,5-c]pyrazine-5,7(1H,6H)-dione (72). (Scheme 3-4a) Compound 2a (200 mg, 1.18 mmol) was dissolved in 8 mL of H$_2$O and heated to reflux, open to air. Once reflux temperature was achieved, 2,3-butanedione (113 μL, 1.29 mmol, 1.1 eq) was added, and the reaction was heated at reflux for an additional 25 minutes. The mixture was then cooled to RT, and a pale yellow precipitate began to form. The mixture was further cooled on ice, and the precipitate was collected by filtration, rinsing with H$_2$O, to yield 72 (106 mg, 41% yield): mp 239-241°C (dec) (lit$^5$ m/z 221.1 (M+H), 243.1 (M+Na).

1,6-Dimethyl-3,4-diphenylpyrimido[4,5-c]pyrazine-5,7(1H,6H)-dione (74). (Scheme 3-4b) Compound 2a (100 mg, 0.58 mmol) was dissolved in 3 mL of 2-methoxyethanol, open to air. Benzil (124 mg, 0.58 mmol) was added, and the reaction was heated at reflux for 48 h. The mixture was cooled to RT, and a precipitate formed. The precipitate was collected by filtration, rinsing with 2-methoxyethanol. The collected precipitate was then recrystallized from ethanol to yield 74 (40 mg, 20% yield): mp
342°C (dec) (lit58 mp 308-310°C), 1H NMR (d-DMSO) 3.11 (s, 3H), 4.14 (s, 3H), 7.09 (m, 5H), 7.24 (m, 5H), MS m/z 345.1 (M+H).

2-Bromo-4’-methoxyacetophenone (75c). (Table 3-1) 4'-Methoxyacetophenone (400 mg, 2.66 mmol) was dissolved in 100 mL of acetonitrile under nitrogen. p-Toluenesulfonic acid monohydrate (759 mg, 3.99 mmol, 1.5 eq) was added, followed by N-bromosuccinimide (497 mg, 2.79 mmol, 1.05 eq). The reaction mixture was heated at reflux for 2h and then concentrated in vacuo. The residual oil was dissolved in 25 mL of CH2Cl2 and washed with two X 25 mL of H2O. The organic layer was dried over MgSO4, filtered, and concentrated to a pale, pearly-gray solid (75c, 570 mg, 93% yield): 1H NMR (d-DMSO) 3.91 (s, 3H), 4.43 (s, 2H), 7.00 (d, J= 8.7 Hz, 2H), 7.99 (d, J= 8.7 Hz, 2H).

2-Bromo-4’-(trifluoromethyl)acetophenone (75e). (Table 3-1) 4’-
(Trifluoromethyl)acetophenone (200 mg, 1.06 mmol) was dissolved in 50 mL of acetonitrile under nitrogen. p-Toluenesulfonic acid monohydrate (302 mg, 1.59 mmol, 1.5 eq) was added, followed by N-bromosuccinimide (199 mg, 1.12 mmol, 1.05 eq). The reaction mixture was heated at reflux for 2h and then concentrated in vacuo. The residual oil was dissolved in 25 mL of CH2Cl2 and washed with two X 25 mL of H2O. The organic layer was dried over MgSO4, filtered, and concentrated to a white solid (75e, 233 mg, 82% yield): 1H NMR (d-DMSO) 4.48 (s, 2H), 7.79 (d, J= 8.2 Hz, 2H), 8.13 (d, J= 8. Hz, 2H).

1,6-Dimethyl-4-p-tolyl-2,3-dihydropyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (80d). (Table 3-1) Compound 2a (200 mg, 1.18 mmol) was suspended in 4 mL of absolute ethanol, and 2-bromo-4’-(trifluoromethyl)acetophenone (75d) (275 mg, 1.29 mmol, 1.1 eq) was added. The reaction mixture was stirred at RT for 4 h and then heated at reflux for 10 h, open to air. The reaction mixture was cooled to RT, and the precipitate was collected by filtration, rinsing with ethanol. The precipitate is a fluffy, pastel yellow powder (80d, 156 mg, 47% yield): mp 231-232°C, 1H NMR (d-DMSO) 2.36 (s, 3H), 3.15 (s, 3H), 3.41 (s, 3H), 4.67 (s, 2H), 5.10 (s, 1H), 7.29 (d, J= 7.7 Hz, 2H), 7.66 (d, J= 7.7
Hz, 2H), $^1$H NMR ($d$-TFA) 2.30 (s, 3H), 3.49 (s, 3H), 3.57 (s, 3H), 4.86 (s, 2H), 7.20 (d, $J$= 7.7 Hz, 2H), 7.59 (d, $J$= 7.7 Hz, 2H), $^{13}$C NMR ($d$-DMSO) 21.39, 27.61, 38.04, 76.20, 125.50, 129.83, 130.83, 140.50, 144.25, 145.41, 150.59, 162.03, MS $m/z$ 285.1 (M+H), 307.1 (M+Na).

1,6-Dimethyl-4-(4-(trifluoromethyl)phenyl)-2,3-dihydropyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (80e). (Table 3-1) Compound 2a (135 mg, 0.79 mmol) was suspended in 4 mL of absolute ethanol, and 2-bromo-4’-(trifluoromethyl)acetophenone (75e) (233 mg, 0.87 mmol, 1.1 eq) was added. The reaction mixture was stirred at RT for 4 h and then heated at reflux for 10 h, open to air. A precipitate persisted throughout the duration of the reaction. The precipitate was filtered while the mixture was still warm to collect the precipitate, which was rinsed with ethanol. The precipitate is a bright, pastel yellow powder (80e, 121 mg, 45% yield): mp 276-279°C, $^1$H NMR ($d$-DMSO) 3.15 (s, 3H), 3.45 (s, 3H), 4.71 (s, 2H), 5.15 (s, 1H), 7.68 (d, $J$= 7.2 Hz, 2H), 7.86 (d, $J$= 7.2 Hz, 2H), $^1$H NMR ($d$-TFA) 3.50 (s, 3H), 3.58 (s, 3H), 4.89 (s, 2H), 7.64 (d, $J$= 7.8 Hz, 2H), 7.85 (d, $J$= 7.8 Hz, 2H), $^{13}$C NMR ($d$-TFA) 25.03 (CH3), 34.36 (CH2), 36.10 (CH3), 117.63, 122.52, 123.44 (CH), 129.98, 142.15 (CH), 143.75, 145.25, 160.91, MS $m/z$ 339.9 (M+H).

3-Methyl-6-(1-methyl-2-(2-oxo-2-phenylethylidene)hydrazinyl)pyrimidine-2,4(1H,3H)-dione (82). (Scheme 3-8) Compound 2a (69 mg, 0.41 mmol) was suspended in 2 mL of absolute ethanol, open to air. Phenylglyoxal monohydrate (68 mg, 0.45 mmol, 1.1 eq) was added, and the reaction mixture was heated at reflux for 2 h. The mixture was then cooled to RT, and the precipitate was collected by filtration. The precipitate is a bold yellow powder (82, 76 mg, 65% yield): $^1$H NMR ($d$-DMSO) 3.16 (s, 3H), 3.47 (s, 3H), 5.50 (s, 1H), 7.55 (t, $J$= 7.6 Hz, 2H), 7.66 (d, $J$= 7.5 Hz, 1H), 7.82 (s, 1H), 8.0 (d, $J$= 7.7 Hz, 2H), 10.14 (br s, 1H), MS $m/z$ 287.1 (M+H).

General Procedure for Synthesis of Phenylglyoxal Monohydrates (86) from Acetophenones (85). (Scheme 3-10) The phenylacetophenone (1.5 mmol) was dissolved in 3 mL dioxane and 120 μL of H2O. Selenium dioxide (3.0 mmol, 2 eq) was
added, and the mixture was heated at reflux for 24-72 h. The mixture was cooled to RT and filtered through Celite, rinsing with ethyl acetate. The filtrate was concentrated, dissolved in CH$_2$Cl$_2$ and purified by silica gel flash chromatography (0-20% EtOAc/hexanes).

**4-Fluorophenylglyoxal monohydrate (Table 3-3, Entry 1).** 117 mg (50% yield), $^1$H NMR (d-DMSO) 5.97 (d, J= 9.1 Hz, 1H), 7.33 (m, 2H), 7.53 (d, J= 8.7 Hz, 2H), 8.13 (dd, J= 5.6 Hz, J’= 3.3 Hz, 2H).

**4-(Trifluoromethyl)phenylglyoxal monohydrate (Table 3-3, Entry 3).** 229 mg (73% yield), $^1$H NMR (d-DMSO) 5.68 (s, 1H), 6.98 (d, J= 7.2 Hz, 2H), 7.87 (d, J= 6.9 Hz, 2H), 8.22 (d, J= 6.9 Hz, 2H).

**4-Methoxyphenylglyoxal monohydrate (Table 3-3, Entry 4).** 525 mg (39% yield, recrystallized from H$_2$O), $^1$H NMR (d-DMSO) 3.84 (s, 3H), 5.64 (s, 1H), 6.64 (br s, 2H), 7.04 (d, J= 8.9 Hz, 2H), 8.04 (d, J= 8.9 Hz, 2H).

**4-Chloro-3-nitrophenylglyoxal monohydrate (Table 3-3, Entry 5).** 211 mg (59% yield), 5.76 (s, 1H), 7.95 (br s, 2H), 8.00 (s, 1H), 8.25 (dd, J= 8.4 Hz, J’= 2.0 Hz, 1H), 8.61 (d, J= 2.0 Hz, 1H).

**3-Fluorophenylglyoxal monohydrate (Table 3-3, Entry 6).** 135 mg (52% yield), $^1$H NMR (d-DMSO) 6.38 (s, 2H), 6.96 (s, 1H), 7.55 (m, 4H).

**General Procedure for Synthesis of Pyrimido[4,5-c]pyridazine-5,7(1H,6H)-diones (88) from Hydrazinyluracil (2a) and Phenylglyoxal Monohydrates. (Table 3-4)**

Compound 2a (0.3 mmol) was suspended in 2 mL of 1,2-dichloroethane in a flask equipped with a condenser and open to air. The phenylglyoxal monohydrate (0.3 mmol, 1 eq) was added, and mixture was heated at reflux for 20-24 h. The mixture was cooled to RT, and a bright yellow precipitate was collected by filtration for a first crop of product. The filtrate was concentrated, and the residual was triturated with water to yield
a second crop of bright yellow crystals, which were identical by NMR comparison to the first crop.

4-(4-Fluorophenyl)-1,6-dimethylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (88-1a). 29 mg (56% yield), mp 239-240°C, $^1$H NMR (d-DMSO) 3.26 (s, 3H), 4.13 (s, 3H), 7.39 (t, J = 8.9 Hz, 2H), 8.16 (dd, J = 8.8 Hz, J' = 5.3 Hz, 2H), 8.75 (s, 1H), $^{13}$C NMR (d-DMSO) 28.26, 43.92, 116.49, 116.78, 123.66, 128.85, 129.27, 129.39, 130.22, 146.84, 154.96, 155.47, 160.80, 165.56, MS m/z 287.2 (M+H), 309.1 (M+Na).

4-(4-Chlorophenyl)-1,6-dimethylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (88-2a). 10 mg (22% yield), mp 228-231°C, $^1$H NMR (d-DMSO) 3.12 (s, 3H), 4.12 (s, 3H), 7.61 (d, J = 8.1 Hz, 2H), 8.11 (d, J = 8.1 Hz, 2H), 8.69 (s, 1H), $^{13}$C NMR (d-DMSO) 28.26, 43.95, 123.64, 128.63, 128.78, 129.66, 132.47, 135.78, 146.56, 155.16, 160.80, MS m/z 303.1 (M+H), 325.0 (M+Na).

1,6-Dimethyl-4-(4-(trifluoromethyl)phenyl)pyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (88-3a). 106 mg (85% yield), mp 244-247°C, $^1$H NMR (d-DMSO) 3.28 (s, 3H), 4.16 (s, 3H), 7.92 (d, J = 8.4 Hz, 2H), 8.31 (d, J = 8.3 Hz, 2H), 8.84 (s, 1H), $^{13}$C NMR (d-DMSO) 28.28, 44.06, 123.70, 126.46, 127.67, 129.05, 137.47, 146.16, 155.34, 155.53, 160.80, MS m/z 337.0 (M+H), 359.0 (M+Na).

4-(4-Methoxyphenyl)-1,6-dimethylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (88-4a). 65 mg (75% yield), mp 223-225°C (dec), $^1$H NMR (d-DMSO) 3.26 (s, 3H), 3.84 (s, 3H), 4.11 (s, 3H), 7.10 (d, J = 8.8 Hz, 2H), 8.04 (d, J = 8.7 Hz, 2H), 8.68 (s, 1H), MS m/z 299.1 (M+H), 321.1 (M+Na).

4-(4-Chloro-3-nitrophenyl)-1,6-dimethylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (88-5a). 20 mg (42% yield), mp 285-287°C (dec), $^1$H NMR (d-DMSO) 3.19 (s, 3H), 4.14 (s, 3H), 7.94 (d, J = 8.6 Hz, 1H), 8.41 (dd, J = 8.5 Hz, J' = 2.2 Hz, 1H), 8.80 (d, J = 2.1 Hz, 1H), 8.90 (s, 1H), $^{13}$C NMR (d-DMSO) 28.30, 44.06, 123.62, 123.68, 126.81,
129.06, 131.50, 132.78, 134.02, 144.70, 148.76, 155.33, 155.51, 160.72, MS m/z 348.0 (M+H), 370.0 (M+Na).

4-(3-Fluorophenyl)-1,6-dimethylpyrimido[4,5-c]pyridazine-5,7(1H,6H)-dione (88-6a). 28 mg (49% yield), mp 258-261°C, 1H NMR (d-DMSO) 3.26 (s, 3H), 4.13 (s, 3H), 7.39 (t, J= 5.3 Hz, 1H), 7.59 (dd, J= 7.5 Hz, J’= 5.6 Hz, 1H), 7.96 (d, J= 7.8 Hz, 2H), 8.79 (s, 1H), 13C NMR (d-DMSO) 28.26, 44.00, 113.44, 113.76, 117.50, 117.78, 122.95, 123.67, 128.95, 131.70, 131.81, 136.02, 146.35, 155.23, 155.52, 160.79, MS m/z 348.0 (M+H), 370.0 (M+Na).

Chapter 4
General Procedure for Synthesis of Dicyanoolefins from Knoevenagel Condensations of Aryl Aldehydes with Malononitrile (103). (Scheme 4-4) Aryl aldehyde (3 mmol) and malononitrile (3 mmol, 1 eq) were mixed with benzyltriethylammonium chloride (6 mmol, 2 eq) in a mortar and ground with a pestle for 5-10 minutes. After TLC showed consumption of starting aldehyde, 3-5 mL of H2O was added to precipitate the product. The product was collected by filtration, rinsed with water, and dried.

2-(Benzylidene)malononitrile (103a). 323 mg (70% yield), mp 76-78°C (lit97 mp 81-83°C), 1H NMR (d-DMSO) 7.56 (t, J= 7.7 Hz, 2H ), 7.67 (t, J= 11.7 Hz, 1H), 7.81 (s, 1H), 7.97 (d, J= 7.5 Hz, 2H).

2-(4-Chlorobenzylidene)malononitrile (103b). 564 mg (99% yield), mp 158-162°C (lit97 mp 160-162°C), 1H NMR (d-DMSO) 7.70 (d, J= 8.0 Hz, 2H), 7.94 (d, J= 8.0 Hz,
2H), 8.53 (s, 1H), $^{13}$C NMR ($d$-DMSO) 82.73, 113.51, 114.55, 130.22, 130.57, 132.63, 139.50, 160.60.

**2-(4-Methylbenzylidene)malononitrile (103c).** 456 mg (90% yield), mp 131-133°C (lit$^9$7 mp 132-134°C), $^1$H NMR ($d$-DMSO) 2.40 (s, 3H), 7.42 (d, J= 6.9 Hz, 2H), 7.85 (d, J= 7.2 Hz, 2H), 8.46 (s, 1H), $^{13}$C NMR ($d$-DMSO) 21.93, 80.34, 113.89, 114.86, 129.21, 130.61, 131.15, 146.14, 161.72.

**2-(4-Methoxybenzylidene)malononitrile (103d).** 415 mg (75% yield), mp 114-116°C (lit$^9$8 mp 114-116°C), $^1$H NMR ($d$-DMSO) 3.88 (s, 3H), 7.18 (d, J= 7.6 Hz, 2H), 7.97 (d, J= 7.6 Hz, 2H), 8.39 (s, 1H).

**General Procedure for Synthesis of Dihydropyrazolopyrimidinediones (114).**
(Scheme 4-7) Nitrobenzene (0.10 mL, 1.0 mmol) was suspended in 2 mL H$_2$O with NH$_4$Cl (0.105 g, 2.0 mmol, 2 eq), and the mixture was vigorously stirred. Zinc powder (0.27 g, 4.0 mmol, 2 eq) was added incrementally. After addition was complete, the reaction was stirred for one hour or until TLC (25% EtOAc/hexanes) showed complete consumption of nitrobenzene. The reaction was filtered, rinsing thoroughly with 3-4 mL ethanol. To the filtrate was added the aryl aldehyde (0.95 mmol, 0.95 eq), and the reaction was stirred overnight in the dark. The 6-hydrazinyl-3-methyluracil (2a or 2b) was then added to the mixture, which was stirred for another 10-15 h as precipitate formed. The precipitate was collected by filtration, washed thoroughly with ethanol, and dried.

**1,5-Dimethyl-3-phenyl-7,7a-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (114a).** 178 mg (79% yield), mp 250-254°C, $^1$H NMR ($d$-DMSO) 3.12 (s, 3H), 3.35 (s, 3H), 5.25 (s, 1H), 7.42 (m, 3H), 7.98 (m, 3H), 10.66 (s, 1H), $^{13}$C NMR ($d$-DMSO) 26.75, 32.29, 77.50, 128.19, 128.95, 129.97, 134.87, 140.88, 151.06, 151.11, 163.62, MS m/z 259.1 (M+H), 281.1 (M+Na).
3-(4-Chlorophenyl)-1,5-dimethyl-7,7a-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (114b). 218 mg (85% yield), mp 227-230°C, $^1$H NMR (CDCl$_3$/TFA) 3.85 (s, 6H), 7.84 (d, J= 8.3 Hz, 2H), 8.25 (d, J= 8.6 Hz, 2H), 8.34 (s, 1H), $^{13}$C NMR (d-DMSO) 26.75, 32.36, 77.71, 128.98, 129.89, 134.37, 139.58, 151.19, 163.62, MS m/z 293.1 (M+H)/295.1 (M+H) (3:1 Cl isotope pattern).

3-(4-Methoxyphenyl)-1,5-dimethyl-7,7a-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (114c). 198 mg (79% yield), mp 228-230°C, $^1$H NMR (CDCl$_3$/TFA) 4.95 (s, 3H), 5.02 (s, 3H), 5.43 (s, 3H), 8.53 (d, J= 8.5 Hz, 2H), 9.40 (d, J= 8.6 Hz, 2H), 9.47 (s, 1H), $^{13}$C NMR (d-DMSO) 26.71, 32.11, 55.70, 76.91, 114.40, 127.51, 129.83, 140.85, 150.94, 151.08, 160.93, 163.59, MS m/z 289.1 (M+H), 311.1 (M+Na).

1,5-Dimethyl-3-p-tolyl-7,7a-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (114d). 180 mg (75% yield), mp 220-223°C, $^1$H NMR (d-DMSO) 3.05 (s, 3H), 3.27 (s, 3H), 3.22 (s, 1H), 7.22 (d, J= 13.2 Hz, 2H), 7.90 (d, J= 13.2 Hz, 2H), 10.63 (s, 1H), $^{13}$C NMR (d-DMSO) 26.73, 32.20, 77.26, 128.19, 129.57, 132.18, 139.71, 140.97, 151.10, 163.62, MS m/z 272.3 (M+H).

3-(2-Fluorophenyl)-1,5-dimethyl-7,7a-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (114e). 197 mg (81% yield) mp 263-266°C, $^1$H NMR (d-DMSO) 3.11 (s, 3H), 3.36 (s, 3H), 5.29 (s, 1H), 7.26 (m, 3H), 7.96 (m, 1H), 8.42 (m, 1H), 10.70 (s, 1H), $^{13}$C NMR (d-DMSO) 26.77, 32.30, 78.07, 115.97, 116.24, 122.27, 124.99, 128.48, 131.80, 131.91, 132.85, 151.01, 163.63, MS m/z 277.1 (M+H).

1,5-Dimethyl-3-(4-nitrophenyl)-7,7a-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (114f). 178 mg (67% yield), mp 288-290°C, $^1$H NMR (d-DMSO) 3.13 (s, 3H), 3.40 (s, 3H), 5.34 (s, 1H), 8.08 (s, 1H), 8.21 (s, 4H), 11.00 (s, 1H), $^{13}$C NMR (d-DMSO) 26.79, 32.72, 78.72, 124.14, 129.00, 138.33, 141.34, 147.77, 151.06, 151.28, 163.63.
1,5-Dimethyl-3-(3-nitrophenyl)-7,7a-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (114g). 230 mg (86% yield), mp 288-290°C, \(^1\)H NMR (\(d\)-DMSO) 3.13 (s, 3H), 3.37 (s, 3H), 5.28 (s, 1H), 7.71 (t, J= 7.8 Hz, 1H), 8.11 (s, 1H), 8.19 (d, J= 7.9 Hz, 1H), 8.45 (d, J= 7.4 Hz, 1H), 8.82 (s, 1H), 10.99 (s, 1H), \(^{13}\)C NMR (\(d\)-DMSO) 26.80, 32.59, 78.30, 121.70, 122.55, 124.08, 130.42, 134.24, 149.00, 148.59, 164.03, MS \(m/z\) 304.1 (M+H), 326.0 (M+Na).

3-(3,4-Dimethoxyphenyl)-1,5-dimethyl-7,7a-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (114h). 95 mg (68% yield), mp 213-215°C, \(^1\)H NMR (\(d\)-DMSO) 3.14 (s, 3H), 3.42 (s, 3H). 3.80 (s, 3H), 3.84 (s, 3H), 5.20 (s, 1H), 7.00 (d, J= 6.3 Hz, 1H), 7.40 (d, J= 6.3 Hz, 1H), 7.59 (s, 1H), 7.92 (s, 1H), 10.58 (br s, 1H), \(^{13}\)C NMR (\(d\)-DMSO) 26.73, 32.28, 55.99, 56.29, 77.07, 110.64, 111.81, 122.49, 127.64, 141.31, 149.34, 151.00, 163.59, MS \(m/z\) 319.1 (M+H).

3-(4-Chlorophenyl)-1-(2-hydroxyethyl)-5-methyl-7,7a-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (114i). 73 mg (76% yield), mp 258-261°C, \(^1\)H NMR (\(d\)-DMSO) 3.11 (s, 3H), 3.63 (s, 2H), 4.01 (s, 2H), 5.03 (br s, 1H), 5.27 (s, 1H), 7.49 (d, J= 8.4 Hz, 2H), 8.01 (d, J= 8.2 Hz, 2H), 8.11 (s, 1H), 10.64 (br s, 1H), \(^{13}\)C NMR (\(d\)-DMSO) 26.89, 46.71, 57.07, 78.01, 128.96, 129.96, 133.84, 134.73, 139.57, 151.10, 163.77.

3-(3,4-Dimethoxyphenyl)-1-(2-hydroxyethyl)-5-methyl-7,7a-dihydro-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (114j). 125 mg (82% yield), mp 224-226°C, \(^1\)H NMR (\(d\)-DMSO) 3.11 (s, 3H), 3.66 (s, 2H), 3.99 (s, 3H), 4.02 (s, 3H), 5.04 (br s, 1H), 5.22 (s, 1H), 6.97 (d, J= 8.1 Hz, 1H), 7.43 (d, J= 8.4 Hz, 1H), 7.57 (s, 1H), 8.10 (s, 1H), 10.44 (s, 1H), \(^{13}\)C NMR (\(d\)-DMSO) 26.72, 46.72, 56.00, 56.25, 57.13, 77.01, 110.80, 111.85, 122.40, 127.58, 141.31, 149.31, 150.88, 150.97, 151.02, 163.63, MS \(m/z\) 349.1 (M+H), 371.1 (M+Na).

**General Procedure for Oxidation of Dihydropyrazolopyrimidinedione (114) to Pyrazolopyrimidinedione (115 or 116).** (Scheme 4-7)
Dihydropyrazolopyrimidinedione 114 (0.18 mmol) was mixed with N-bromosuccinimide (0.24 mmol, 1.1 eq) in 2 mL absolute ethanol in an oven-dried flask under nitrogen. The mixture was heated at reflux for 4-5 h, during which time it became a deep purple color. The mixture was cooled to RT. The white precipitate that formed was collected by filtration, rinsing with ethanol.

1,5-Dimethyl-3-phenyl-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (115a). 8 mg (50% yield), mp 215-217°C, $^1$H NMR (d-DMSO) 3.13 (s, 3H), 3.64 (s, 3H), 5.29 (s, 1H), 7.47 (m, 3H), 7.67 (d, J= 6.8 Hz, 2H), $^{13}$C NMR (d-DMSO) 27.73, 34.87, 67.34, 126.24, 128.01, 130.57, 130.78, 149.15, 161.38, MS $m/z$ 257.1 (M+H).

3-(4-Chlorophenyl)-1,5-dimethyl-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (115b). 20 mg (40% yield), mp 249-250°C, $^1$H NMR (d-DMSO) 3.15 (s, 3H), 3.64 (s, 3H), 5.30 (s, D$_2$O exchangeable, 1H), 7.56 (d, J= 8.4 Hz, 2H), 7.70 (d, J= 8.6 Hz, 2H), $^{13}$C NMR (d-DMSO) 27.72, 34.91, 67.44, 125.11, 128.17, 132.43, 135.75, 143.89, 145.53, 149.16, 161.36, MS $m/z$ 291.0 (M+H)/293.1 (M+H) (3:1 Cl isotope pattern).

3-(4-Methoxyphenyl)-1,5-dimethyl-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (115c). 16 mg (33% yield), mp 234-237°C, $^1$H NMR (d-DMSO) 3.14 (s, 3H), 3.61 (s, 3H), 3.83 (s, 3H), 5.26 (s, 1H), 7.01 (d, J= 7.9 Hz, 2H), 7.61 (d, J= 8.4 Hz, 2H), $^{13}$C NMR (d-DMSO) 27.72, 34.80, 55.79, 67.22, 113.49, 118.23, 132.20, 144.81, 149.12, 161.27, 161.34, MS $m/z$ 287.1 (M+H), 309.1 (M+Na).

1,5-Dimethyl-3-p-tolyl-1H-pyrazolo[3,4-d]pyrimidine-4,6(2H,5H)-dione (115d). 25 mg (51% yield), mp 243-246°C, $^1$H NMR (d-DMSO) 2.38 (s, 3H), 3.14 (s, 3H), 3.62 (s, 3H), 5.27 (s, 1H), 7.28 (d, J= 7.7 Hz, 2H), 7.56 (d, J= 8.2 Hz, 2H), $^{13}$C NMR (d-DMSO) 21.49, 27.72, 34.84, 67.29, 123.32, 128.59, 130.47, 140.58, 144.94, 149.19, 161.35, MS $m/z$ 271.1 (M+H), 293.1 (M+Na).

3-(4-Methoxyphenyl)-1,5-dimethyl-1H-pyrazolo[3,4-d]pyrimidine-4,6(5H,7H)-dione (116a). 10 mg (22% yield), mp > 300°C, $^1$H NMR (d-DMSO) 3.20 (s, 3H), 3.34 (s, 3H),
3.80 (s, 3H), 6.97 (d, J= 7.2 Hz, 2H), 8.15 (d, J= 8.5 Hz, 2H), 12.32 (s, 1H). 13C NMR (d-
DMSO) 27.62, 35.61, 55.60, 113.96, 124.46, 129.76, 147.83, 151.21, 158.59, 160.08, MS 
m/z 287.1 (M+H).

3-(3,4-Dimethoxyphenyl)-1-(2-hydroxyethyl)-5-methyl-1H-pyrazolo[3,4-
d]pyrimidine-4,6(5H,7H)-dione (116b). 11 mg (22% yield), mp > 300°C, 1H NMR (d-
DMSO) 3.22 (s, 3H), 3.79 (t, J= 3.7 Hz, 2H), 3.82 (s, 6H), 4.25 (t, J= 3.6 Hz, 2H), 4.92 
br s, 1H), 7.01 (d, J= 8.7 Hz, 1H), 7.83 (d, J= 8.7 Hz, 1H), 7.96 (s, 1H), 12.21 (s, 1H), 
13C NMR (d-DMSO) 27.69, 50.66, 55.89, 55.92, 59.76, 95.68, 111.73, 112.13, 121.10, 
124.67, 145.15, 148.15, 148.61, 149.71, 151.03, 158.71, MS m/z 347.1 (M+H).

Chapter 5

3-Methyl-6-(methylamino)pyrimidine-2,4(1H,3H)-dione (122a).58 (Scheme 5-1) 6-
Chloro-3-methyluracil (500 mg, 3.11 mmol) was suspended in 3.5 mL of a 33% w/w methylamine 
solution in ethanol in a sealed reaction tube. The mixture was heated at reflux for 7 d. The mixture 
was cooled to RT, and the precipitate was removed by filtration. The filtrate was concentrated to 
yield a pale yellow powder (80a): 458 mg (95% yield), mp 225-230°C (lit58 mp 290-295°C), 1H NMR 
(d-DMSO) 2.65 (s, 3H), 3.03 (s, 3H), 4.50 (s, 1H), 6.48 (br s, 1H), 8.56 (br s, 1H), MS m/z 156.0.

General Procedure for Synthesis of 6-Aminouracils (122b/c). (Scheme 5-1) 6-
Chloro-3-methyluracil (0.20 g, 1.25 mmol) was suspended in 5 mL absolute ethanol. The amine 
(8.75 mmol, 7 eq) was added to the mixture, and the reaction vessel was sealed. The reaction was 
heated at 80°C for 36-96 h. When TLC (5% MeOH/CH2Cl2) showed consumption of starting uracil, 
the reaction was cooled to RT, and the precipitate was collected by filtration.

6-(Benzylamino)-3-methylpyrimidine-2,4(1H,3H)-dione (122b).58 White powder, 214 mg 
(74% yield), mp 286-289°C (lit58 mp 302-304°C), 1H NMR (d-DMSO) 3.02 (s, 3H), 4.27 (d, J= 5.65 Hz, 2H), 4.53 (s, 1H), 6.94 (br s, 1H), 7.35 (m, 6H), 13C NMR (d-
DMSO) 26.38, 45.27, 73.84, 127.56, 127.65, 128.97, 134.45, 151.41, 152.85, 163.54, MS 
m/z 232.1.
6-(Furan-2-ylmethylamino)-3-methylpyrimidine-2,4(1\text{H},3\text{H})-dione (122c). 107 mg (39% yield), mp 259-260°C (dec), \textsuperscript{1}H NMR (d-DMSO) 3.03 (s, 3H), 4.29 (s, 2H), 4.72 (s, 1H), 6.31 (s, 1H), 6.41 (s, 1H), 6.49 (s, 1H), 7.63 (s, 1H), 10.28 (s, 1H), \textsuperscript{13}C NMR (d-DMSO) 26.40, 38.66, 73.83, 108.26, 110.96, 143.09, 151.36, 152.64, 163.56, MS m/z 222.1.

**General Procedure for Synthesis of Pyrrolopyrimidinediones (124) from 6-Aminouracils (122).** (Scheme 5-1) The 6-aminouracil (122) (0.65 mmol) was suspended in 2 mL H\textsubscript{2}O with NaOAc (0.105 g, 1.3 mmol, 2 eq). Chloroacetaldehyde (45 wt% H\textsubscript{2}O, 100 uL, 0.7 mmol, 1.1 eq) was added, and the reaction was heated at 80°C for 2 h. The reaction was cooled to RT, and precipitate was collected by filtration, rinsing with H\textsubscript{2}O.

3,7-Dimethyl-1\text{H}-pyrrolo[2,3-d]pyrimidine-2,4(3\text{H},7\text{H})-dione (124a). 144 mg (32% yield), mp 285°C (dec), \textsuperscript{1}H NMR (d-DMSO) 3.18 (s, 3H), 3.58 (s, 3H), 6.28 (d, J= 3.2 Hz, 1H), 6.66 (d, J= 3.2 Hz, 1H), 11.91 (s, 1H), \textsuperscript{13}C NMR (d-DMSO) 27.18, 32.87, 98.55, 103.08, 121.53, 137.93, 151.56, 159.41, MS m/z 180.1 (M+H).

7-Benzyl-3-methyl-1\text{H}-pyrrolo[2,3-d]pyrimidine-2,4(3\text{H},7\text{H})-dione (124b). 49 mg (77% yield), mp 235-240°C (dec), \textsuperscript{1}H NMR (d-DMSO) 3.18 (s, 3H), 5.25 (s, 2H), 6.37 (s, 1H), 6.77 (s, 1H), 7.16 (d, J= 7.25 Hz, 2H), 7.29 (d, J= 7.05 Hz, 1H), 7.35 (t, J= 7.15 Hz, 2H), \textsuperscript{13}C NMR (d-DMSO) 27.37, 48.66, 98.76, 103.86, 121.25, 127.27, 128.27, 129.21, 137.13, 137.61, 151.63, 160.18, MS m/z 256.1.

7-(Furan-2-ylmethyl)-3-methyl-1\text{H}-pyrrolo[2,3-d]pyrimidine-2,4(3\text{H},7\text{H})-dione (124c). 16 mg (48% yield), mp 228-230°C (dec), \textsuperscript{1}H NMR (d-DMSO) 3.18 (s, 3H), 5.25 (s, 2H), 6.32 (d, J= 3.05 Hz, 1H), 6.43 (s, 2H), 6.70 (d, J= 3.2 Hz, 1H), 7.63 (s, 1H), 12.06 (s, 1H), \textsuperscript{13}C NMR (d-DMSO) 27.21, 41.89, 98.64, 103.80, 109.16, 111.08, 120.47, 137.57, 143.87, 150.11, 151.61, 159.39, MS m/z 246.1 (M+H).
3,7-Dimethyl-5-phenyl-1H-pyrrolo[2,3-d]pyrimidine-2,4(3H,7H)-dione (140).
(Scheme 5-7). The 6-aminomethyl-3-methyluracil (122a) (200 mg, 1.29 mmol) was suspended in 5 mL of a 50/50 mixture of acetonitrile/water. Sodium acetate (211 mg, 2.58 mmol, 2 eq) was added, followed by α-bromophenylacetaldehyde (282 mg, 1.42 mmol, 1.1 eq). The mixture was heated at 60°C under nitrogen for 1 h, during which time it turned homogeneous. The reaction was cooled to RT, and precipitate collected by filtration to yield 140 (57 mg, 17%): mp 290-300°C (dec), ¹H NMR (d-DMSO) 3.20 (s, 3H), 3.61 (s, 3H), 7.01 (s, 1H), 7.21 (t, J= 7.2 Hz, 1H), 7.42 (m, 2H), 7.65 (d, J= 7.2 Hz, 2H), 11.7 (br s, 1H), ¹³C NMR (d-DMSO) 27.36, 33.01, 95.66, 119.92, 120.76, 126.48, 128.30, 128.61, 133.97, 139.32, 151.27, 159.42, MS 256.1 (M+H), 278.1 (M+Na).

Chapter 6

1,3,6-Trimethylpyrimidine-2,4(1H, 3H)-dione (142). (Scheme 6-1) 6-Methyluracil (3.0 g, 23.7 mmol) was suspended in 50 mL dry acetone in an oven-dried flask equipped with a drying tube. Cesium carbonate (23.3 g, 71.3 mmol, 3 eq) and dimethyl sulfate (6.76 mL, 71.3 mmol, 3 eq) were added. The mixture was stirred at RT overnight and then filtered. Filtrate concentrated and triturated with ethanol to yield a white, crystalline precipitate (142) (2.719 g, 82%): mp 111-114°C (lit mp 114-115°C), ¹H NMR (d-DMSO) 2.23 (s, 3H), 3.14 (s, 3H), 3.30 (s, 3H).

1,3,6-Trimethyl-5-nitropyrimidine-2,4(1H, 3H)-dione (143). (Scheme 6-1)
Concentrated sulfuric acid (4 mL) was cooled to 0°C, and 142 (500 mg, 3.24 mmol) was added incrementally. After complete dissolution of 142, fuming nitric acid (0.50 mL) was added dropwise. The mixture was stirred for 5 minutes at 0°C and warmed to RT. It was stirred at RT for 40 minutes. Mixture was then poured onto ice, and a precipitate formed. Precipitate collected by filtration, rinsed with water, and dried to yield 143 (451 mg, 70%): mp 149-150°C (lit mp 155°C), ¹H NMR (d-DMSO) 2.38 (s, 3H), 3.14 (s, 3H), 3.30 (s, 3H), 5.63 (s, 1H).

6-(Bromomethyl)-1,3-dimethyl-5-nitropyrimidine-2,4(1H,3H)-dione (144). (Scheme 6-1) Compound 143 (398 mg, 2.00 mmol) was suspended in 5 mL glacial acetic
acid. Bromine (462 μL, 9.00 mmol, 4.5 eq) was added dropwise, and the mixture was heated at 95°C under nitrogen for 3h. Reaction mixture was then cooled to RT, concentrated, and triturated with a 1:2 mixture of ethyl acetate/ether. After standing overnight, yellow precipitate (144) was collected by filtration. 409 mg (74% yield): mp 131-135°C (lit82 mp 143-144°C), 1H NMR (d-DMSO) 3.48 (s, 3H), 3.66 (s, 3H), 4.33 (s, 2H).

2-Benzyl-4,6-dimethyl-5,7-dioxo-4,5,6,7-tetrahydro-2H-pyrazolo[4,3-d]pyrimidine 1-oxide (145). 81 (Scheme 6-1) Compound 144 (80 mg, 0.29 mmol) was suspended in 2.5 mL absolute ethanol under nitrogen. Benzylamine (63 μL, 0.58 mmol, 2 eq) was added, and the mixture was heated at reflux for 3h. It was cooled to RT and then on ice, and precipitate was collected by filtration to yield 145 (28 mg, 34% yield): mp 239-240°C (lit81 mp 239-240°C), 1H NMR (d-DMSO) 3.18 (s, 3H), 3.34 (s, 3H), 5.42 (s, 2H), 7.35 (m, 3H), 7.39 (m, 2H), 7.57 (s, 1H), MS 287.1 (M+H), 309.1 (M+Na).

1,3-Dimethyl-6-phenylpyrimido[5,4-d]pyrimidine-2,4(1H,3H)-dione (146). 81 (Scheme 6-1) Compound 145 (100 mg, 0.349 mmol) was suspended in 1 mL of anhydrous THF in an oven-dried flask under nitrogen. Sodium hexamethyldisilazide (1.0 M, 384 μL, 0.384 mmol, 1.1 eq) was added. The reaction turned a deep purple color upon addition of the sodium hexamethyldisilazide solution. The reaction was heated at reflux for 2 h and then cooled to RT. Ethanol (1 mL) was added to the mixture, and it was further cooled on ice. The precipitate that formed was collected by filtration. The filtrate was concentrated and then triturated with ethanol on ice to recover a second crop of crystals. Both precipitates are pale yellow/peach powders (146): 49 mg (53% yield), mp 271°C (dec) (lit81 mp 263-264°C), 1H NMR (d-DMSO) 2.87 (s, 3H), 3.40 (s, 3H), 7.34 (t, J= 6.8 Hz, 1H), 7.42 (t, J= 7.5 Hz, 2H), 8.20 (s, 1H), 8.25 (d, J= 7.5 Hz, 1H), 8.40 (d, J= 4.8 Hz, 1H), 13C NMR (d-DMSO) 29.22, 38.82, 126.75, 128.59, 128.71, 138.98, 139.47, 141.60, 145.22, 150.58, 169.22.

1,3,6-Trimethyl-5-nitropyrimidine-2,4(1H, 3H)-dione, sodium salt (148). 84 (Scheme 6-2) Sodium metal (481 mg) was dissolved in 30 mL of absolute ethanol in an oven-
dried flask equipped with a reflux condenser and drying tube. After the sodium had completely dissolved, 143 (3.47 g, 17.4 mmol) was added, and the reaction was heated at reflux for 1 h. The mixture was cooled to RT, and the bright orange precipitate was collected by filtration, yielding 148: 3.74 g (98% yield).

6-Ethyl-1,3-dimethyl-5-nitropyrimidine-2,4(1H, 3H)-dione (149).\(^8^5\) (Scheme 6-2)
Compound 148 (500 mg, 2.26 mmol) was suspended in 6 mL of anhydrous DMF in an oven-dried flask under nitrogen. Methyl iodide (169 \(\mu\)L, 2.71 mmol, 1.2 eq) was added, and the mixture was heated at 80\(^\circ\)C for 2 h, during which time the mixture became homogeneous and orange in color. The reaction was cooled to RT and concentrated. The residual oil was triturated with water to yield a yellow solid (149): 276 mg (54% yield), mp 114-117\(^\circ\)C (lit\(^8^5\) mp 119-120\(^\circ\)C), \(^1\)H NMR (\(d\)-DMSO) 1.23 (t, \(J= 7.5\) Hz, 3H), 2.66 (q, \(J= 7.5\) Hz, 2H), 3.22 (s, 3H), 3.43 (s, 3H), \(^13\)C NMR (\(d\)-DMSO) 12.12, 22.91, 28.86, 32.55, 129.08, 150.70, 154.84, 155.61.

6-Ethyl-1,3-dimethyl-5-nitropyrimidine-2,4(1H, 3H)-dione, sodium salt (153).
(Scheme 6-3) Sodium metal (26 mg) was dissolved in 3 mL of absolute ethanol in an oven-dried flask equipped with a reflux condenser and drying tube. After the sodium had completely dissolved, 149 (200 mg, 0.94 mmol) was added, and the reaction was heated at reflux for 2 h. The mixture was cooled to RT and then on ice, and the bright yellow-orange precipitate was collected by filtration, yielding 153: 187 mg (85% yield), \(^1\)H NMR (\(d\)-DMSO) 1.40 (d, \(J= 6.8\) Hz, 3H), 2.95 (s, 3H), 3.04 (s, 3H), 4.95 (q, \(J= 6.8\) Hz, 1H).

6-(1-Bromoethyl)-1,3-dimethyl-5-nitropyrimidine-2,4(1H,3H)-dione (150). (Table 6-3) Pyridinium hydrobromide perbromide (2.17 g, 6.78 mmol, 2 eq) and 153 (797 mg, 3.39 mmol, 1 eq) were suspended in 12 mL of 1,2-dichloroethane in an oven-dried flask under nitrogen. The mixture was heated at reflux for 4 h and then cooled to RT. The reaction was filtered, rinsing with 1,2-dichloroethane. The filtrate was concentrated to an orange residue, which was triturated with ethanol to yield a pale yellow, fine powder (150): 701 mg (71% yield), mp 134-135\(^\circ\)C, \(^1\)H NMR (\(d\)-DMSO) 1.88 (d, \(J= 6.6\) Hz, 3H),
3.20 (s, 3H), 3.52 (s, 3H), 5.44 (q, J= 6.4 Hz, 1H), $^{13}$C NMR ($d$-DMSO) 21.91, 29.19, 37.37, 49.89, 128.70, 148.56, 150.53, 155.87, MS m/z 292.0, 294.0 (M+H, Br isotope), 313.9, 315.9 (M+Na, Br isotope).

6-(1-(Benzylamino)ethyl)-1,3-dimethyl-5-nitropyrimidine-2,4(1H,3H)-dione (154). (Scheme 6-5) Compound 150 (100 mg, 0.342 mmol) was dissolved in 2 mL of ethyl acetate in an oven-dried flask under nitrogen. Benzylamine (75 $\mu$L, 0.68 mmol, 2 eq) was added, and the reaction was stirred at RT for 24-48 h. A precipitate began to form within the first 20 minutes. The precipitate was removed by filtration and verified as the hydrobromide salt of benzylamine. The filtrate was concentrated, dissolved in CH$_2$Cl$_2$, and purified by silica gel flash chromatography (0 to 25% EtOAc/hexanes) to yield 154 (65 mg, 60% yield). Analytically pure 154 was obtained by trituration in ethanol: white powder, $^1$H NMR ($d$-DMSO) 1.81 (d, J= 6.9 Hz, 3H), 3.21 (s, 3H), 3.46 (s, 3H), 5.18 (s, 2H), 5.58 (q, J= 6.8 Hz, 1H), 7.25 (m, 6H).

1,3-Dimethyl-5-nitro-6-(1-nitroethyl)pyrimidine-2,4(1H,3H)-dione (157). (Scheme 6-6) Sodium metal (16 mg, 0.68 mmol, 1.2 eq) was dissolved in 2 mL of absolute ethanol in an oven-dried flask under nitrogen with mild heating (40-50°C). After complete dissolution, nitroethane (41 $\mu$L, 0.57 mmol, 1 eq) was added, and a white precipitate formed almost instantaneously. Compound 156 (100 mg, 0.45 mmol, 0.8 eq) was then added, and the mixture was heated at reflux for 30 minutes. During this time, the mixture turned bright yellow in color, and a bright yellow precipitate formed. The mixture was cooled to RT, and the yellow precipitate was collected by filtration to yield 157 (30 mg, 25% yield): mp 235°C (dec), $^1$H NMR ($d$-DMSO) 1.62 (d, J= 5.6 Hz, 3H), 3.08 (s, 3H), 3.36 (s, 3H), 5.54 (q, J= 5.6 Hz, 1H).

Chapter 7
Polymerase-chain reaction (PCR) conditions for amplification of CTNNB1 and TCF7L2 genes. The following conditions were used: primers (25 pmol each), plasmid (20 ng), dNTPs (200 $\mu$M), Pfu Turbo Hotstart DNA Polymerase (2.5 U), DMSO (3%).
Mg$^{2+}$ (2 mM), brought to a final volume of 50 μL with deionized water. The samples were treated with the following sequence:

1) Denaturation at 95°C for 5 min
2) 10 PCR cycles of: a) 95°C (1 min), b) touchdown from 68°C to 58°C (1 min), c) 72°C (2 min)
3) 30 PCR cycles of: a) 95°C (1 min), b) 58°C (1 min), c) 72°C (2 min)
4) Final extension at 72°C for 10 min

The PCR products were gel-purified (1.5% agarose), visualized by ethidium bromide with ultraviolet light, excised, and extracted using QIAprep gel extraction spin columns (Qiagen) according to the vendor protocol.

**Digestion, purification, and ligation of PCR products.** The purified PCR products for both the Tcf4 gene (TCF7L2) and β-catenin gene (CTNNB1) and pET-23a and pGEX-2T vectors were subjected to double restriction enzyme digests. All digests were done for 1 h at 37°C in 20 μL reaction volumes, with 10 U of each restriction enzyme and ~1 μg of DNA. Table 10-1 lists which restriction enzymes were used for each digestion reaction. Following digestion, purification was accomplished with QIAprep gel extraction spin columns (Qiagen) according to the vendor protocol. The digested TCF7L2 and CTNNB1 genes were then ligated into the digested pGEX-2T (1:16 ratio, 20 μL reaction) and pET-23-a (15:2 ratio, 20 μL reaction), respectively following overnight incubation with T4 DNA ligase (2U) at 4°C.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Restriction Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCF7L2</td>
<td>BamHI, EcoRI</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>BamHI, NheI</td>
</tr>
<tr>
<td>pGEX-2T vector</td>
<td>BamHI, EcoRI</td>
</tr>
<tr>
<td>pET-23-a vector</td>
<td>BamHI, NheI</td>
</tr>
</tbody>
</table>

**Table 10-1: Double restriction enzyme digestion reactions**

**Transformation and harvest of ligated plasmids.** The ligated samples were transformed into *E.coli* XL1-Blue competent cells. Towards Tcf4, 2.5 μL of the plasmid containing TCF7L2 was transformed into 25 μL of XL1-Blue cells, and towards β-
catenin, 0.5 μL of the plasmid containing CTNNB1 was transformed into 20 μL of XL1-Blue cells. In each case, the following protocol was used:

1) Gently mix cells with DNA and let stand on ice for 5 min.
2) Heat-shock at 42°C for 30 sec.
3) Chill on ice for 2 min.
4) Add 80 μL of SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose).
5) Incubate at 37°C in shaker for 45 min.
6) Dilute 10 μL of culture with SOC medium to a final volume of 100 μL.
7) Spread on LB-Agar plates containing 50 μg/mL ampicillin.
8) Grow cells overnight at 37°C.

Individual colonies were isolated, and 3 mL liquid cultures in LB broth (0.5% yeast extract, 1% tryptone, 150 mM NaCl) containing 50 μg/mL ampicillin were inoculated at 37°C overnight with shaking. Plasmids were isolated via QIAprep Spin Miniprep columns (Qiagen) according to vendor protocol. Harvested plasmids were screened by restriction enzyme digestion reactions with BamHI and either EcoRI (TCF7L2) or NheI (CTNNB1), and gene sequences were confirmed with DNA sequencing (University of Michigan DNA Sequencing Core Facilities).

**Expression of β-catenin and GST-Tcf4.** For protein expression, 3 mL cultures in LB broth containing 50 μg/mL ampicillin were inoculated at 37°C overnight with shaking. Overnight cultures (500 μL each) were then used to inoculate 500 mL of fresh LB broth containing 50 μg/mL ampicillin. The cultures were incubated at 37°C with vigorous shaking until the OD₆₀₀ reached ~0.50. Protein expression was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to each culture to a final concentration of 1 mM. The cultures were allowed to incubate at 37°C with vigorous shaking an additional 4 h post-induction, after which time the cells were harvested by centrifugation (5,000 x g, 10 min, 4°C).
Purification of β-catenin. All purification steps were performed at 4°C. The pelleted cells from the 500 mL culture for β-catenin were resuspended in 15 mL lysis buffer (50 mM Na₂HPO₄, 300 mM NaCl, pH 8.0). The cells were lysed with a French press, and the cellular debris was pelleted by centrifugation (20,000 rpm, 30 min, 4°C). Imidazole was added to the supernatant to a final concentration of 10 mM, along with Ni-NTA HisBind® resin (~0.5 mL) that had been equilibrated with lysis buffer. The supernatant was incubated with the resin for 30 min at 4°C while rotating. The mixture was transferred to a column, and the supernatant was removed by gravity filtration. The column was washed with 6 mL of equilibration buffer (50 mM Na₂HPO₄, 300 mM NaCl, 10 mM imidazole), 6 mL of wash buffer (50 mM Na₂HPO₄, 300 mM NaCl, 20 mM imidazole), and 6 mL of elution buffer (50 mM Na₂HPO₄, 300 mM NaCl, 200 mM imidazole). β-catenin partially eluted from the column with the wash buffer and was fully eluted with the first 3 mL of the elution buffer. It was collected in 1 mL fractions. Fractions containing β-catenin were identified by the presence of a 63-kDa band on SDS-PAGE and then diafiltered with Amicon™ Ultra Centrifugal Filter Devices (Millipore™) to remove imidazole. The final concentration of β-catenin was determined via the Bradford method using the Bio-Rad Protein Assay Kit and bovine serum albumin (BSA) as standards. Aliquots (15-30 μL) were stored at -78°C until needed.

Purification of GST-Tcf4. All purification steps were performed at 4°C. The pelleted cells from the 500 mL culture for GST-Tcf4 were resuspended in 15 mL PBS lysis buffer (with added proteinase inhibitor). The cellular suspension was subjected to sonication (3 x 30 s pulses, 1 min. between each), followed by the addition of 25% Triton-X-100 (to a final concentration of 1%). The suspension was incubated at 4°C for 30 min while rotating, and then the cellular debris was pelleted by centrifugation (20,000 rpm, 30 min, 4°C). Glutathione Sepharose beads (0.5 mL sedimented volume) that had been equilibrated with PBS buffer were added to the supernatant, which was incubated with the beads for 30 min at 4°C while rotating. The mixture was transferred to a column, and the supernatant was removed by gravity filtration. The column was washed with 15 mL of PBS buffer, followed by 15 mL of elution buffer (50 mM TrisHCl, 50 mM glutathione (reduced), pH 8.0). GST-Tcf4 was collected in 1 mL fractions. Fractions containing
GST-Tcf4 were identified by the presence of a 35-kDa band on SDS-PAGE. The final concentration of GST-Tcf4 was determined via the Bradford method\textsuperscript{100} using the Bio-Rad Protein Assay Kit and bovine serum albumin (BSA) as standards. Pooled fractions were diluted by a factor of 100 in 50 mM Tris HCl buffer and stored as 100-200 μL aliquots at -78°C until needed.

**Initial protocol for ELISA β-catenin/Tcf4 binding assay.**

1) Microtiter plates (96 well) were plated with 10 μg/mL β-catenin in PBS buffer (100 μL/well) overnight at 4°C.

2) Plates were washed with 8 X 200 μL/well of TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 8.0).

3) To each well was added 200 μL of blocking buffer (Pierce, PBS SuperBlock™).

4) Plates were incubated at 4°C for 2 h.

5) Plates were washed with 8 X 200 μL/well of TBST buffer.

6) To each well was added 98 μL of GST-Tcf4 (1 μg/mL) in assay buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, 2% BSA, pH 8.0), followed by 2 μL of either PKF118-310 in DMSO (concentration range= 10 mM to 313 μM) or 1 μL of DMSO.

7) Plates were incubated at 4°C for 2 h.

8) Plates were washed with 8 X 200 μL/well of TBST buffer.

9) To each well was added 100 μL of goat anti-GST antibody (1 μg/mL) in TBST buffer.

10) Plates were incubated at 4°C for 2 h.

11) Plates were washed with 8 X 200 μL/well of TBST buffer.

12) To each well was added 100 μL of alkaline phosphatase (AP)-conjugated anti-goat IgG (Bio-Rad, 1000 fold dilution) in TBST buffer.

13) Plates were incubated at 4°C overnight.

14) Plates were washed with 8 X 200 μL/well of TBST buffer.

15) To each well was added 100 μL of fluorescent AP substrate Attophos (Roche BioSciences, prepared as directed).

16) Plates were incubated at RT for 5 minutes in the dark.

17) Fluorescence was measured (440 nm excitation, 550 nm emission).
Revised protocol for ELISA β-catenin/Tcf4 binding assay used for testing all synthesized compounds.

(modifications from previous protocol are shown in bold)

1. Microtiter plates (96 well) were plated with 10 μg/mL β-catenin in PBS buffer (50 μL/well) overnight at 4°C.
2. Plates were washed with 6 X 200 μL/well of TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 8.0).
3. To each well was added 200 μL of blocking buffer (Pierce, PBS SuperBlock™).
4. Plates were incubated at 4°C for 2 h.
5. Approximately 1 h after the start of this incubation period, a working solution of GST-Tcf4 in TBST buffer + 2% BSA was prepared on ice. The “recipe” used for a quantity of this working solution sufficient for one 96-well plate was the following:
   - 5928 μL TBST buffer
   - 60 μL GST-Tcf4 stock (~ 0.1 mg/mL)
   - 12 μL BSA stock (10 mg/mL)
6. Solutions of test compound (or control) in this working solution (total volume for each compound = 175 μL for three wells) were then prepared as follows:
   - 2% DMSO and test compound
     - 171.5 μL working solution
     - 3.5 μL test compound diluted in DMSO (DMSO concentration is 50X that being tested, i.e- 10 mM for 200 μM final concentration)
   - 1% DMSO and test compound
     - 173.25 μL working solution
     - 1.75 μL test compound diluted in DMSO (DMSO concentration is 100X that being tested, i.e- 10 mM for 100 μM final concentration)

Control solutions were prepared as follows:

- DMSO control (+)
  - 171.5 μL working solution
  - 3.5 μL DMSO
- Soluble β-catenin control (-)
173.7 μL working solution
1.23 μL β-catenin stock (1.4 mg/mL)

Each of these individual solutions was kept on ice until the 2h incubation period was over. The preparation of the test compound solutions and control solutions with BSA and GST-Tcf4 was completed a minimum of 15 minutes before the solutions were added to the β-catenin coated plate.

7. Plates were washed with 6 X 200 μL/well of TBST buffer.
8. To each well was added 50 μL of the prepared test compound or control solutions with BSA and GST-Tcf4.
9. Plates were incubated at 4°C for 2 h.
10. Plates were washed with 6 X 200 μL/well of TBST buffer.
11. To each well was added 50 μL of goat anti-GST antibody (1 μg/mL) in TBST buffer.
12. Plates were incubated at 4°C for 2 h.
13. Plates were washed with 6 X 200 μL/well of TBST buffer.
14. To each well was added 50 μL of alkaline phosphatase (AP)-conjugated anti-goat IgG (Bio-Rad, 1000 fold dilution) in TBST buffer.
15. Plates were incubated at 4°C overnight.
16. Plates were washed with 6 X 200 μL/well of TBST buffer.
17. To each well was added 50 μL of fluorescent AP substrate Attophos (Roche BioSciences, prepared as directed).
18. Plates were incubated at RT for 5 minutes in the dark.
19. Fluorescence was measured (440 nm excitation, 550 nm emission).

Chapter 8
Cellular assay for testing activity and toxicity of synthesized compounds. (courtesy of Dr. Guido Bommer) IEC18 cells were transduced with a retrovirus driving expression of an activated form of β-catenin (the S33Y mutant) and a stable polyclonal cell line was selected using the antibiotic G418 according to the procedure of F.T. Kolligs et al,\textsuperscript{101} generating the IEC18-S33Y cell line. The S33Y mutant of β-catenin was used because it has an extended in vivo half-life and is capable of translocating to the nucleus
to bind and activate transcription factors of the TCF/LEF family in the absence of Wnt signaling.

As a negative control, IEC18 cells were transduced with the empty retroviral backbone lacking the β-catenin expression cassette and a stable polyclonal cell line was selected, generating the IEC18-NEO cell line. As a control for the general transcriptional status of the cell lines, a lentiviral construct was generated which drives constitutive expression of the Renilla luciferase gene under the control of the CMV promoter. The construct was produced by subcloning the open reading frame of the Renilla luciferase gene (obtained from the pGL4.73 vector, available from Promega, Madison, WI) behind the CMV promoter of a modified version of the LL3.7 lentiviral vector. To measure β-catenin-mediated transcriptional activity, the β-catenin-dependent firefly luciferase expression cassette was subcloned from the TOPFLASH vector (available from Millipore, Billerica, MA) into a self-inactivating lentiviral vector based on the LL3.7 lentiviral vector.

To validate the assay system, IEC18-S33Y and IEC18-NEO cells were infected in parallel with the lentiviral CMV-Renilla construct and the lentiviral β-catenin-dependent firefly luciferase construct. The infections were performed in the presence of 4 μg/mL Polybrene (available from Sigma-Aldrich). The lentivirus used for the infections was produced separately for each construct by transient cotransfection of 293T cells with the lentiviral vector and the packaging plasmids pMDLg/pRRE (containing the viral gag/pol elements), pRSV-REV (driving expression of rev), and pMD.G (driving expression of the VSVG envelope protein). The infected cells were plated in 96-well plates at several cell concentrations varying from 3125 cells/well to 100,000 cells/well. Transcriptional activity of the firefly and Renilla luciferase genes was determined 48 h after plating by measuring luminescence using the Promega Dual Luciferase kit (available from Promega, Madison, WI). The β-catenin-dependent transcriptional activity in the IEC18-S33Y cell line was found to be more than 14-fold increased, as compared to the IEC18-NEO cell line, when cells were plated at a concentration of at least 25,000 cells/well).

IEC18-S33Y cells carrying a β-catenin firefly luciferase cassette and a constitutively active renilla luciferase cassette were seeded at 40,000 cells/well in 96-well
tissue culture plates (BD Biosciences) in a total volume of 80µL of tissue culture medium (Dulbecco’s modified Eagle medium without Phenol-Red (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Hyclone)) and incubated for 24 h. Stock solutions of the compounds were prepared by dissolving the compounds in DMSO at concentrations ranging from 50 mM to 5 mM, depending on individual solubility. Working solutions of the compounds were obtained by further diluting the stock solutions in tissue culture medium by at least 50-fold. Serial twofold dilutions of the working solutions of the compounds were added to the cells in a volume of 40 µL and the cells were incubated in the presence of the compounds for 17-20 h.

Using the above assay system, IC50 and LD50 values were determined for the test inhibitors. LD50 values of the test inhibitors were determined using a WST-1 colorimetric cell proliferation assay. A mixture containing 20 µL of medium and 10 µL of WST-1 reagent (available from Roche) was added to the cells and the cells were incubated for 1 h at 37 °C, after which time absorption at 550 nm was measured using a microplate reader. To determine IC50 values of the test inhibitors, expression of Renilla and firefly luciferase was measured. Following the absorptions measurements, the tissue culture medium was aspirated and the cells were lysed using 100 µL of passive lysis buffer (available from Promega, Madison, WI). Renilla and firefly luciferase activity then was measured using the Promega Dual Luciferase kit.

Protocol for testing of compounds versus luciferase (96-well format).
1) A 5 mL solution of luciferase was prepared according to the following recipe and kept on ice:
   - 500 µL 10X luciferase buffer
     (280 mM HEPES-KOH, 1.2 M KOAc, 12 mM MgOAc, pH= 7.6)
   - 4497.5 µL dd H2O
   - 2.5 µL luciferase (14.9 mg/mL)
2) A 5 mL solution of Steady-Glo® luciferase substrate was prepared according to the following recipe and kept on ice:
   - 500 µL 10X luciferase buffer
     (280 mM HEPES-KOH, 1.2 M KOAc, 12 mM MgOAc, pH= 7.6)
- 25 μL Steady-Glo® reagent
- 200 μL ATP (250 mM)
- 20 μL dithiothreitol (DTT) (1 M)
- 4255 μL dd H2O

3) To each well of a 96-well microtiter plate was added 1 μL of a solution of test compound in DMSO (concentration range = 10 mM to 39 μM).

4) 50 μL of the solution of Steady-Glo® luciferase substrate was added to each well.

5) 50 μL of the solution of luciferase was added to each well.

6) Plates were incubated at RT for 5 minutes, shielded from light.

7) Luminescence was measured (integration time = 500 ms).

**Protocol for testing of compounds versus luciferase (384-well format).**

1) A 35 mL solution of luciferase was prepared according to the following recipe and kept on ice:
   - 3.5 mL 10X luciferase buffer
     (280 mM HEPES-KOH, 1.2 M KOAc, 12 mM MgOAc, pH= 7.6)
   - 31.5 mL dd H2O
   - 3.5 μL luciferase (14.9 mg/mL)

2) A 70 mL solution of Steady-Glo® luciferase substrate was prepared according to the following recipe and kept on ice:
   - 7 mL 10X luciferase buffer
     (280 mM HEPES-KOH, 1.2 M KOAc, 12 mM MgOAc, pH= 7.6)
   - 350 μL Steady-Glo® reagent
   - 2.8 mL ATP (250 mM)
   - 280 μL dithiothreitol (DTT) (1 M)
   - 59.57 mL dd H2O

3) To each well of a 384-well microtiter plate containing 5 μL of a solution of test compound in DMSO was added 5 μL of the solution of luciferase.

4) 10 μL of the solution of Steady-Glo® luciferase substrate was added to each well.

6) Plates were incubated at RT for 10-40 minutes*, shielded from light.
7) Luminescence was measured (integration time = 500 ms).
*It should be noted that the time to read each plate was ~5 minutes, and as a result, the plates had to be tested in two batches for firefly luciferase was found to lose activity when at RT for too long of a period (> 40 minutes).
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