THE SYNTHESIS OF NOVEL AGENTS TARGETING *SALMONELLA ENTERICA* TYPHIMURIUM

by
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DEDICATION

To my cherished family and friends, your support has been the cornerstone of my journey, providing me with the courage to pursue my dreams and the strength to overcome life's challenges. Your presence in my life is a constant reminder of the power of love and friendship, and I am deeply grateful for the countless ways you've uplifted me.

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ABSTRACT

Foodborne illness resulting from non-typhoidal *Salmonella enterica* is a major contributor to global diarrheal diseases[1], with the potential to induce life-threatening morbidity within afflicted patients. In addition to its widespread impact, *S. enterica* has developed significant antimicrobial resistance, challenging the efficacy of current antibiotics. Through high-throughput screening (HTS), a promising hit compound was identified as an inhibitor of a unique Fructose-Asparagine (F-Asn) metabolic pathway utilized by *S. enterica*. This pathway converts F-Asn into Glucose-6-Phosphate (G6P) through a multi-step pathway involving several enzymes, including FraB[2].

[1] "Salmonella (Non-Typhoidal)." World Health Organization, World Health Organization, www.who.int/news-room/fact-sheets/detail/salmonella-(non-typhoidal). Accessed 26 Sept. 2023.

[2] Sabag-Daigle, A., Blunk, H., Sengupta, A. et al. A metabolic intermediate of the fructose asparagine utilization pathway inhibits growth of a Salmonella fraB mutant. Sci Rep 6, 28117 (2016). https://doi.org/10.1038/srep28117

Inhibiting FraB leads to cell death due to the accumulation of a toxic intermediate, 6-Phosphofructose-Aspartate (6-P-F-Asp). Building upon the original hit scaffold, an extensive exploration of structure-activity relationships (SAR) has been undertaken in an attempt to enhance the potency of the original hit and create a novel narrow-spectrum antibiotic. The original hit offers a multitude of functionality that can be easily modified to explore the SAR, which allowed for the synthesis of 26 unique derivatives. While the SAR panel may not have yielded significantly more potent compounds, ongoing HTS efforts explore alternative chemical cores to effectively inhibit this crucial metabolic pathway.

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LITERATURE REVIEW[3]

The inception of novel antibiotics marked a significant milestone in medical science, fundamentally transforming the landscape of disease therapeutics. Nonetheless, the triumph of these medical breakthroughs was coupled with an intricate predicament: microorganisms, notably bacteria, exhibited a remarkable propensity to undergo adaptive transformations, ultimately resulting in the acquisition of resistance mechanisms that could attenuate the efficacy of antibiotics. This phenomenon of microbial adaptation, underpinned by genetic mutations and horizontal gene transfer, remains a formidable contemporary concern, particularly exacerbated by the prevalent issue of antibiotic overutilization. The injudicious employment of antibiotics imparts selective pressure on microbial populations, expediting the emergence of mutational events that confer resistance advantages. Consequently, the pace of microbial evolution is expedited, leading to a perpetual arms race between therapeutic innovation and microbial resistance.

Many contemporary therapeutic antibiotics belong to the category of broad-spectrum agents. These antibiotics possess the capacity to target both Gram-positive and Gram-negative bacteria that are implicated in various disease processes. Broad-spectrum antibiotics typically exert their effects by specifically interfacing with crucial cellular components, such as the 30s or 50s ribosomal subunits, or by intercalating within deoxyribonucleic acid (DNA). While this approach offers substantial advantages, including the rapid treatment of infections without the need for precise identification of the infectious agent's Gram-staining characteristics, it also harbors a significant drawback—its potential to induce genetic mutations in bacteria. These mutations equip bacteria with the means to evade the effects of small-molecule antibiotics through diverse

[3]Sabag-Daigle, A., Blunk, H., Sengupta, A. et al. A metabolic intermediate of the fructose-asparagine utilization pathway inhibits growth of a Salmonella fraB mutant. Sci Rep 6, 28117 (2016). https://doi.org/10.1038/srep28117

mechanisms. The concern surrounding broad-spectrum antibiotics extends not only to their mutagenic potential but also to the adverse effects they can induce in patients.

Broad-spectrum antibiotics, when appropriately utilized, can be invaluable therapeutic tools. Nonetheless, their usage carries certain side effects that pose substantial risks to patients. One of these risks involves the potential of broad-spectrum antibiotics to disrupt the delicate balance of microbial populations within the patient, leading to a state known as dysbiosis. This disruption can create opportunities for other microorganisms to transition into opportunistic pathogens. In addition to the threat posed by these opportunistic pathogens, dysbiosis can render the patient more susceptible to bacterial shedding, particularly in the context of *Salmonella* transmission. Treating a patient with a broad-spectrum antibiotic is often a therapeutic intervention that extends beyond the specific needs of the infection and can yield more harm than benefit. The risk-to-benefit ratio associated with this treatment approach encompasses numerous potential outcomes that could ultimately result in greater harm than good. This underscores the critical need for the development and utilization of narrow-spectrum antibiotics, which can target specific pathogens with greater precision and minimize the collateral damage caused by broad-spectrum counterparts.

Salmonella enterica Typhimurium is a Gram-negative facultative anaerobic bacterium and one of the three pathogens most commonly responsible for causing foodborne illnesses in humans. This bacterium has evolved a sophisticated defense mechanism in the form of efflux proteins, which function to expel antimicrobial agents from within the bacterial cell. Much like any other living organism, *S. enterica* Typhimurium relies on various metabolic pathways to obtain the necessary energy for its survival. However, this particular species possesses a distinctive capability to harness a unique precursor molecule, fructose-asparagine (F-Asn), to generate glucose-6-

phosphate (G-6-P) for its metabolic processes. This metabolic transformation of F-Asn yields not only G-6-P but also aspartate, both of which serve as biologically inert molecules within the

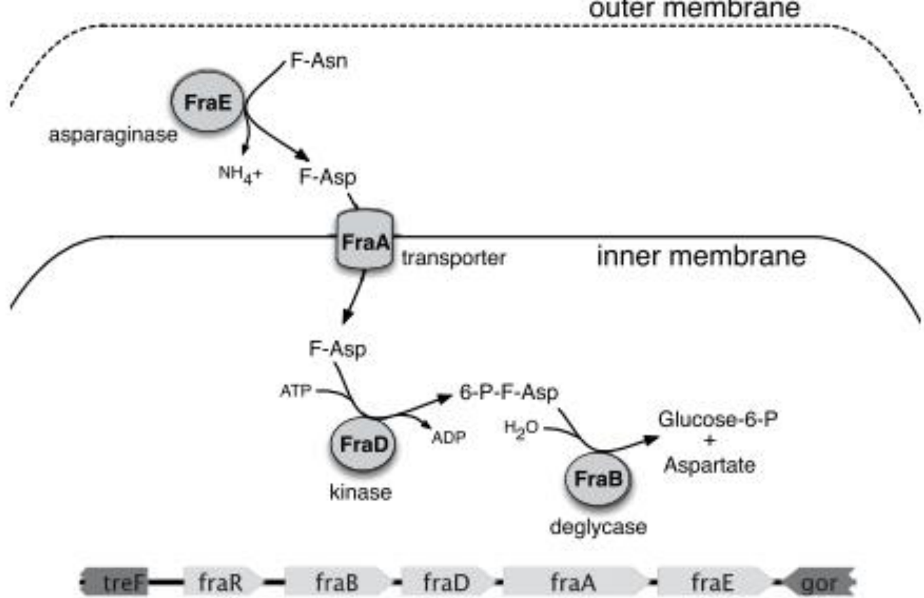


Figure 1. Proposed pathway for F-Asn in *S. enterica*

Sabag-Daigle, A., Blunk, H., Sengupta, A. et al. *A metabolic intermediate of the fructose-asparagine utilization pathway inhibits growth of a* Salmonella fraB *mutant.* Sci Rep ***6***, *28117 (2016). https://doi.org/10.1038/srep28117*

bacterium's metabolic pathways (Sabag-Daigle et al.).

Salmonella enterica harbors a set of five distinct *fra* genes responsible for orchestrating the intricate metabolic conversion of fructose-asparagine (F-Asn). By conducting a comprehensive proteomic investigation of subcellular fractions, it was ascertained that FraE is predominantly situated within the periplasmic compartment, positioned between the outer and inner cellular membranes. In contrast, FraB is primarily localized within the intracellular space. The functional roles of these enzymes have been elucidated through a comparative analysis of their homologs in *Escherichia coli*, specifically the FrlD and FrlB proteins, which participate in the fructose-lysine metabolic pathway (Sabag-Daigle et al.). Notably, it has been observed that *S. enterica* strains

carrying mutations in the *fraB* gene exhibit a significant growth impairment when cultivated in an enriched culture with fructose-asparagine (Sabag-Daigle et al).

Utilizing a combination of enzymatic assays and mass spectrometry analyses, it was unequivocally verified that FraD catalyzes the conversion of fructose-aspartic acid (F-Asp) into the intermediate compound known as 6-phosphofructose-aspartic acid (6-P-F-Asp). Subsequently, this intermediate undergoes further enzymatic processing facilitated by FraB, ultimately resulting in the production of glucose-6-phosphate (G-6-P) and aspartate. Intriguingly, when *Salmonella enterica*, specifically the *fraD* mutant strain, is cultivated in a medium supplemented with fructose-asparagine (F-Asn), a conspicuous absence of 6-P-F-Asp accumulation is observed, concomitant with only minimal cellular growth.

In stark contrast, when *Salmonella enterica*, exemplified by the *fraB* mutant variant, is cultivated in an F-Asn-enriched medium, a notable accumulation of 6-P-F-Asp becomes evident, and this metabolic perturbation precipitates cytotoxic cell death. The toxic nature of this intermediate was rigorously substantiated, as the wild-type *S. enterica* exhibited a complete absence of detectable 6-P-F-Asp, while the *fraB* mutant displayed quantifiable levels of this intermediary (Sabag-Daigle et al). This groundbreaking metabolic insight unveils a potential avenue for exploitation, whereby the distinctive toxicity associated with the accumulation of 6-P-F-Asp can be leveraged as a novel target for the development of narrow-spectrum antibiotics.

CHEMISTRY

The concept of a narrow spectrum is the future of antibiotics because of the rising antimicrobial resistance to current broad-spectrum antibiotics. This section aims to explain the original hit and the chemistry achieved to explore the SAR and the pharmacophore relationships.

HIGH THROUGHPUT SCREENING

The initial High Throughput Screening (HTS) involved the investigation of a comprehensive set of over 200,000 chemical compounds. The primary objective was to examine various structural cores to ascertain the specific chemical moieties required for inhibiting the FraB enzyme. Out of this extensive pool of compounds, a limited subset was carefully selected for subsequent in-house testing. This secondary evaluation aimed to delve deeper into the compounds' potency and their dependency on the Fra mechanism. Among the chosen compounds, only a singular candidate exhibited exceptional promise and was subjected to further scrutiny. This promising compound was designated as "K19" and was characterized by a diphenylamine core, a carboxylic acid, and an aniline moiety. The chemical framework of K19 was particularly intriguing due to its potential for modification to explore the SAR of the compound. The SAR investigation strategy entailed systematic alterations to the chemical structure of K19. Specifically, it involved exploring substituting the carboxylic acid with lipophilic and hydrophilic amide couplings, varying the core terminal ring, and eliminating the aniline component. This systematic approach allowed for a comprehensive assessment of the compound's structure-activity relationship, thereby facilitating the development of more potent derivatives.

AMIDE COUPLINGS

K19	IC_{50}	
	Wild type	109 μM
	Δ fra mutant	252 μM
	Δ tolC mutant	49 μM

OSUAB-0330-01	IC_{50}	
	Wild type	139 μM
	Δ fra mutant	4,800 μM
	Δ tolC mutant	4.7 μM

Figure 2 Primary amide comparison to K19 hit.

The initial pursuit of the SAR was to change the functionality of the carboxylic acid to amides to determine how important that position is for activity. We initially pursued a primary amide before deciding to pursue more complex couplings because of the known importance of hydrogen-bound acceptors (HBA) and hydrogen-bond donors (HBD) in binding activity across all facets of small molecule inhibitors. What was the most exciting about the primary amide analog was not a comparable IC_{50}, but the fact that the primary amide analog showed an over 480-fold increase in FraB dependency, as seen in Figure 2 above. This shows that creating amide analogs can create a small molecule that works mechanistically and not just by toxicity. With this knowledge, we pursued a small catalog of amide couplings that fall into two categories: hydrophobic amide couplings and hydrophilic amide couplings. These categories aimed to determine how changing the HBA and HBD aspects could impact the potency of these compounds.

The generation of novel 3-amino-4-(phenylamino)benzamides relied on amide couplings by activating the carboxylic acid with 1,1'-carbonyldiimidazole (CDI) to generate a highly reactive (3-amino-4-(phenylamino)phenyl)(1H-imidazol-1-yl)methanone intermediate. After the electrophilic center is generated, adding a nucleophilic amine will generate the amide product. The general pathway is highlighted in Scheme 1.1.

Scheme 1.1 Amide coupling synthesis.

The 3-amino-4-(phenylamino)benzamide coupling scheme is shown in Scheme 1.2, which was used to generate an intermediate before the final compound was synthesized. The reaction of **1** with aniline in ethanol yielded **2** via a nucleophilic aromatic substitution (SN_{Ar}) to yield a nitro intermediate. To get to **K19**, the nitro had to be reduced to an amine. While **K19** is commercially available, it was inexpensive to synthesize this intermediate due to how readily available the starting materials were to purchase. The reaction of **K19** with boc anhydride and triethyl amine yielded an important intermediate **3**. This boc protection was a key step utilized to successfully generate an amide coupling. We chose to boc protect the aniline due to issues with generating the (3-amino-4-(phenylamino)phenyl)(1H-imidazol-1-yl)methanone intermediate. We often found that if the aniline was left unprotected, there was a generation of undesired by-products that were very hard to separate. Once there was an abundance of **3**, we were able to generate 10 amides that fell into the hydrophobic or the hydrophilic category to yield **4**. The reaction between **4** and hydrochloric acid yielded **5**. This yielded the final amide product as a salt which helped eliminate

any solubility issues. The purity of these compounds was determined using ultra-high-performance liquid chromatography (UPLC) and ranged between 65-95%.

Scheme 1.2 The synthesis of 3-amino-4-(phenylamino)benzamides for desired amide coupling analogs.

The final analogs were synthesized using acidic conditions. The acidic conditions were utilized based on Boc deprotection conditions to yield a final 3-amino-4-(phenylamino) benzamide as a salt. The purity issues with this series of compounds came from possible degradation of the amide bond due to hydrolysis from thawing and freezing compounds from the freezer. The compounds shown in Figure 3 utilized the synthetic steps shown in Scheme 1.2.

Figure 3 Final Compounds of Amide Coupling Series

Compound	**Wild type IC_{50}**	***fra* mutant IC_{50}**	***tolC* mutant IC_{50}**
350	> 100 µM	> 100 µM	> 100 µM
351	> 100 µM	> 100 µM	> 100 µM
352	> 100 µM	> 100 µM	> 100 µM
353	> 100 µM	> 100 µM	> 100 µM
354	> 100 µM	> 100 µM	> 100 µM
355	> 100 µM	> 100 µM	> 100 µM
356	> 100 µM	> 100 µM	> 100 µM
357	> 100 µM	> 100 µM	> 100 µM

359	> 100 µM	> 100 µM	> 100 µM
360	> 100 µM	> 100 µM	> 100 µM

Table 1 The Half maximal inhibitory concentration (IC_{50}) of amid coupling analogs tested.

The selection of these analogs was methodically undertaken to elucidate Structure-Activity Relationships (SAR) and to empirically illustrate the influence of specific molecular attributes on their efficacy across a spectrum of assays. The strains used in these assays helped establish the activity of the compounds. The first strain was the wild-type strain. This strain is the kind of bacteria that is found in nature and has all the defense mechanisms and the target. The second strain was the *fra* mutant. In this strain, the genes coding for this metabolic pathway were knocked out to help show a *fra* dependency. This needs to be established so that we can support a mechanism of action and not induce toxicity due to the compound. The third and final strain was the *tolC knockout*. This is where the TolC protein is removed from the genome to prevent the efflux of these compounds. Each analog underwent rigorous testing against the three distinct strains of *Salmonella enterica* Typhimurium at varying concentrations, thereby generating dose-response curves. The compound denoted as K19 was employed as the positive control throughout these experimental trials.

Despite the initial enthusiasm surrounding the potential of amide couplings, this series of compounds failed to yield statistically significant results. This outcome suggests that the extension of molecular moieties at this particular position may indicate the presence of a highly constricted binding pocket. Even in the case of compound **357**, where no extension with carbon chains was introduced, no activity was observed, even in the wild-type and tolC mutant strains. This analog further demonstrated the impact of the absence of hydrogen bond donor functionality. As anticipated, this compound exhibited a lack of activity, underscoring the pivotal role of both HBD and HBA characteristics within the conjectured binding pocket.

Furthermore, compounds **359** and **360**, which incorporate primary alcohols and primary amines, possessing HBD and HBA attributes, also failed to elicit any discernible activity. These observations point to a persistent issue related to the constrained nature of the binding pocket, which appears to preclude any molecular elongation at the amide position.

AMIDE COUPLING EXPERIMENTALS

3-nitro-4-(phenylamino)benzoic acid

To a flask charged with **1** and aniline (1.5 equiv.) was suspended in ethanol (0.9 M) and reacted at 78ºC for 4 h. The solution went from yellow to dark orange. The reaction was cooled to 23ºC and poured into a 125-mL Erlenmeyer flask. To the wet ethanol suspension was then added an equal volume amount of aqueous 1M HCl. The suspension was filtered via vacuum filtration, rinsing water (3 x 5 mL), and then was azeotropically dried with toluene to yield **2** (average yield 86%) as an orange solid. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 12.99 (br s, 1H), 9.79 (s, 1H), 8.63 (d, *J* = 2.1 Hz, 1H), 7.93 (dd, *J* = 9.0, 2.0 Hz, 1H), 7.47 (td, *J* = 8.3, 1.9 Hz, 2H), 7.37 (d, *J* = 9.0, 2H), 7.29 (m, 1H), 7.13 (d, *J* = 8.9 Hz, 1H).

3-amino-4-(phenylamino)benzoic acid

To a stirred solution of methyl **2** in methanol (333 mmol/L):water (333 mmol/L), iron powder (7 equiv.), and ammonium chloride (7 equiv.) were combined and reacted at 23ºC for 24 h. The reaction suspension was filtered through a celite bed and washed with EtOAc. The solutions were evaporated under reduced pressure and quenched with sat. $NaHCO_3$, was extracted with EtOAc (3

x 5 mL). The combined organic extracts were washed with brine (1 x 10 mL), dried over sodium sulfate, and filtered through a cotton pug. The reaction solution was concentrated directly with silica gel and purified via Combiflash (12.0 g column), eluting with 0-40% EA:Hexanes. The fractions were evaporated under reduced pressure to yield **K19** (90% yield) as a purple solid. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 12.22 (br s, 1H), 7.35, (s, 1H), 7.33 (d, *J* = 2.0 Hz), 7.23 (td, *J* = 8.9, 2.0 Hz, 2H), 7.15 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.06 (d, *J* = 8.3 Hz, 1H), 6.98 (dd, *J* = 8.5, 1.0 Hz, 2H), 6.83 (tt, *J* = 14.6, 7.3, 1.2 Hz) 4.97 (br s, 2H).

O HO NH$_2$ NH **K19** → O HO NHBoc NH **4**

3-((tert-butoxycarbonyl)amino)-4-(phenylamino)benzoic acid

To a stirred solution of **3** (1 equiv.) in 2:1 dioxane:water (0.8 M : 0.4 M) was added triethylamine (1.5 equiv.) and boc anhydride (1.5 equiv.) at 23°C and stirred for 24 h. Then dioxane was evaporated under reduced pressure, and the resulting aqueous residue was diluted water (9.0 mL) The aqueous residue was adjusted to a pH of 2 with aqueous 1M hydrochloric acid. The thick pink mixture was separated via vacuum filtration, rinsing the flask with acidified water (3 x 1mL), and azeotropically dried with toluene to yield **4** (average yield 75-80%) as a purple solid. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 8.91 (s, 1H), 7.67 (dd, *J* = 8.5, 2.1 Hz, 1H), 7.41 (dt, *J* = 16.0, 8.0, 2.1 Hz, 2H), 7.21 (m, 2H), 7.08 (dd, *J* = 8.6, 1.2 Hz, 2H), 6.77 (d, *J* = 8.6 Hz, 1H), 1.55 (s, 9H).

tert-butyl (5-carbamoyl-2-(phenylamino)phenyl)carbamate

To a flask charged with **4** (50.5 mg, 1 equiv.), CDI (1.5 equiv.), THF (180 mmol/L) under nitrogen pressure was added. After 1 h an amine R_2 (1.5 equiv.) was added and stirred for 1 h at 23ºC. After 30 min TLC was run on reaction solution in 80% EtOAc:HX and saw the separation of intermediate and desired product with an Rf ≈ 0.38-0.45. The reaction mixture was concentrated directly with silica gel and purified via Combiflash (4.0 g column), eluting with 1:1 EtOAc:HX. The product eluted at about 2-3.5 min. The fractions were evaporated under reduced pressure to yield **5** (average yield as 40-85%) as a purple flaky solid.

The following compound analog was prepared using the general procedure listed above to yield **350a** 59% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.49 (s, 1H), 8.41 (d, *J* = 7.8 Hz, 1H), 7.95 (s, 1H), 7.64 (s, 1H), 7.51 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.24 (m, 2H), 7.00 (d, *J* = 7.9 Hz, 2H), 6.88 (t, *J* = 14.9, 7.4 Hz), 5.76 (s, 1H), 3.18 (q, 2H), 1.51 (q, 2H), 1.45 (s, 9H), 0.88 (t, 3H).

The following analog was prepared using the general procedure listed above to yield **351a** in 75% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.49 (s, 1H), 8.41 (d, *J* = 7.8 Hz, 1H), 7.95 (s, 1H), 7.64 (s, 1H), 7.51 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.24 (m, 2H), 7.00 (d, *J* = 7.9 Hz, 2H), 6.88 (t, *J* = 14.9, 7.4 Hz), 3.22 (q, 2H), 1.48 (q, 2H), 1.45 (s, 9H), 1.31 (q, 2H), 0.89 (t, 3H).

The following analog was prepared using the general procedure listed above to yield **352a** in 55% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.49 (s, 1H), 8.41 (d, J = 7.8 Hz, 1H), 7.95 (s, 1H), 7.64 (s, 1H), 7.51 (dd, J = 8.7, 2.2 Hz, 1H), 7.24 (m, 2H), 7.00 (d, J = 7.9 Hz, 2H), 6.88 (t, J = 7.6, 7.4 Hz), 3.05 (t, 2H), 1.83 (m, 1H), 1.45 (s, 9H), 0.87 (d, 6H).

The following analog was prepared using the general procedure listed above to yield **353a** in 90% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.49 (s, 1H), 8.41 (d, J = 7.8 Hz, 1H), 7.95 (s, 1H), 7.64 (s, 1H), 7.51 (dd, J = 8.7, 2.2 Hz, 1H), 7.24 (m, 2H), 7.00 (d, J = 7.9 Hz, 2H), 6.88 (t, J = 7.6, 7.4 Hz), 4.06 (m, 1H), 1.45 (s, 9H), 1.14 (d, 6H).

The following analog was prepared using the general procedure listed above to yield **354a** in 87 % yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.49 (s, 1H), 8.41 (d, J = 7.8 Hz, 1H), 7.95 (s, 1H), 7.64 (s, 1H), 7.51 (dd, J = 8.7, 2.2 Hz, 1H), 7.24 (m, 2H), 7.00 (d, J = 7.9 Hz, 2H), 6.88 (t, J = 7.6, 7.4 Hz), 4.39 (m, 1H), 2.18 (t, 2H), 2.05 (t, 2H), 1.65 (m, 2H), 1.45 (s, 9H).

The following analog was prepared using the general procedure listed above to yield **355a** in 76% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.49 (s, 1H), 8.41 (d, J = 7.8 Hz, 1H), 7.95 (s, 1H), 7.64 (s, 1H), 7.51 (dd, J = 8.7, 2.2 Hz, 1H), 7.24 (m, 2H), 7.00 (d, J = 7.9 Hz, 2H), 6.88 (t, J = 7.6, 7.4 Hz), 3.73 (m, 1H), 1.77 (m, 4H), 1.46 (m, 2H), 1.45 (s, 9H), 1.21 (m, 4H).

The following analog was prepared using the general procedure listed above to yield **356a** in 70% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.49 (s, 1H), 8.41 (d, J = 7.8 Hz, 1H), 7.95 (s, 1H), 7.64

(s, 1H), 7.51 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.24 (m, 2H), 7.00 (d, *J* = 7.9 Hz, 2H), 6.88 (t, *J* = 7.6, 7.4 Hz), 3.25 (q, 2H), 1.45 (s, 9H), 1.09 (t, 3H).

The following analog was prepared using the general procedure listed above to yield **357a** in 48% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.49 (s, 1H), 8.41 (d, *J* = 7.8 Hz, 1H), 7.95 (s, 1H), 7.64 (s, 1H), 7.51 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.24 (m, 2H), 7.00 (d, *J* = 7.9 Hz, 2H), 6.88 (t, *J* = 7.6, 7.4 Hz), 2.96 (s, 6H), 1.45 (s, 9H).

The following analog was prepared using the general procedure listed above to yield **359a** in 47% yield. ^{1}HNMR (300 MHz, (CD_3OD)) δ: 8.49 (s, 1H), 8.41 (d, *J* = 7.8 Hz, 1H), 7.95 (s, 1H), 7.64 (s, 1H), 7.51 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.24 (m, 2H), 7.00 (d, *J* = 7.9 Hz, 2H), 6.88 (t, *J* = 7.6, 7.4 Hz), 3.72 (t, 2H), 3.5 (t, 2H), 1.5 (s, 9H).

The following analog was prepared using the general procedure listed above to yield **360a** in 61% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.49 (s, 1H), 8.41 (d, *J* = 7.8 Hz, 1H), 7.95 (s, 1H), 7.64 (s, 1H), 7.51 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.24 (m, 2H), 7.00 (d, *J* = 7.9 Hz, 2H), 6.88 (t, *J* = 7.6, 7.4 Hz), 3.27 (t, 2H), 3.08 (t, 2H), 1.45 (s, 9H), 1.37 (s, 9H).

3-amino-4-(phenylamino)benzamide hydrogen chloride

To a flask containing **5** (1 equiv.)**,** hydrochloric acid (4 M, 0.5 mL) was added and stirred for 24 h at 23ºC. The reaction suspension was diluted with methanol (2 mL) and was evaporated under reduced pressure to yield **6** (average yield 93-99%) as an off-white flaky solid.

The following final analog was prepared using the general procedure listed above to yield **350** in 52% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.31 (d, *J* = 8.2 Hz, 1H), 7.61 (s, 1H), 7.48 (d, *J* = 7.3 Hz, 1H), 7.25 (m, 3H), 7.01 (d, *J* = 7.6 Hz, 2H), 6.89 (t, *J* = 7.6, 7.1 Hz, 1H), 5.76 (s, 1H), 3.18 (q, 2H), 1.51 (q, 2H), 0.88 (t, 3H).

The following final analog was prepared using the general procedure listed above to yield **351** in 65% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.31 (d, *J* = 8.2 Hz, 1H), 7.61 (s, 1H), 7.48 (d, *J* = 7.3 Hz, 1H), 7.25 (m, 3H), 7.01 (d, *J* = 7.6 Hz, 2H), 6.89 (t, *J* = 7.6, 7.1 Hz, 1H), 3.22 (q, 2H), 1.48 (q, 2H), 1.31 (q, 2H), 0.89 (t, 3H).

The following final analog was prepared using the general procedure listed above to yield **352** in 75% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.31 (d, *J* = 8.2 Hz, 1H), 7.61 (s, 1H), 7.48 (d, *J* = 7.3 Hz, 1H), 7.25 (m, 3H), 7.01 (d, *J* = 7.6 Hz, 2H), 6.89 (t, *J* = 7.4, 7.1 Hz, 1H), 3.05 (t, 2H), 1.83 (m, 1H), 1.45 (s, 9H), 0.87 (d, 6H).

The following final analog was prepared using the general procedure listed above to yield **353** in 90% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.31 (d, *J* = 8.2 Hz, 1H), 7.61 (s, 1H), 7.48 (d, *J* = 7.3 Hz, 1H), 7.25 (m, 3H), 7.01 (d, *J* = 7.6 Hz, 2H), 6.89 (t, *J* = 7.4, 7.1 Hz, 1H), 4.06 (m, 1H), 1.14 (d, 6H).

The following final analog was prepared using the general procedure listed above to yield **354** in 77% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.31 (d, J = 8.2 Hz, 1H), 7.61 (s, 1H), 7.48 (d, J = 7.3 Hz, 1H), 7.25 (m, 3H), 7.01 (d, J = 7.6 Hz, 2H), 6.89 (t, J = 7.4, 7.1 Hz, 1H), 4.39 (m, 1H), 2.18 (t, 2H), 2.05 (t, 2H), 1.65 (m, 2H).

The following final analog was prepared using the general procedure listed above to yield **355** in 70% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: : 8.31 (d, J = 8.2 Hz, 1H), 7.61 (s, 1H), 7.48 (d, J = 7.3 Hz, 1H), 7.25 (m, 3H), 7.01 (d, J = 7.6 Hz, 2H), 6.89 (t, J = 7.4, 7.1 Hz, 1H), 3.73 (m, 1H), 1.77 (m, 4H), 1.46 (m, 2H), 1.21 (m, 4H).

The following final analog was prepared using the general procedure listed above to yield **356** in 88% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.31 (d, J = 8.2 Hz, 1H), 7.61 (s, 1H), 7.48 (d, J = 7.3 Hz, 1H), 7.25 (m, 3H), 7.01 (d, J = 7.6 Hz, 2H), 6.89 (t, J = 7.4, 7.1 Hz, 1H), 3.25 (q, 2H), 1.09 (t, 3H).

The following final analog was prepared using the general procedure listed above to yield **357** in 90% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.31 (d, J = 8.2 Hz, 1H), 7.61 (s, 1H), 7.48 (d, J = 7.3 Hz, 1H), 7.25 (m, 3H), 7.01 (d, J = 7.6 Hz, 2H), 6.89 (t, J = 7.4, 7.1 Hz, 1H), 2.96 (s, 6H).

The following final analog was prepared using the general procedure listed above to yield **359** in 98% yield. ^{1}HNMR (300 MHz, (MeOD)) δ: 8.36 (d, J = 8.2 Hz, 1H), 7.66 (s, 1H), 7.52 (d, J = 7.3

Hz, 1H), 7.25 (m, 3H), 7.06 (d, *J* = 7.6 Hz, 2H), 6.91 (t, *J* = 7.4, 7.1 Hz, 1H), 3.72 (t, 2H), 3.5 (t, 2H).

The following final analog was prepared using the general procedure listed above to yield **360** in 92% yield. ^{1}HNMR (300 MHz, (DMSO-d_6)) δ: 8.31 (d, *J* = 8.2 Hz, 1H), 7.61 (s, 1H), 7.48 (d, *J* = 7.3 Hz, 1H), 7.25 (m, 3H), 7.01 (d, *J* = 7.6 Hz, 2H), 6.89 (t, *J* = 7.4, 7.1 Hz, 1H), 3.27 (t, 2H), 3.08 (t, 2H).

TERMINAL RING ANALOGS

The goal of this chapter is to explain the design and synthesis of terminal ring analogs that were made via nucleophilic aromatic substitution and Suzuki couplings.

NUCLEOPHILIC AROMATIC SUBSTITUTION

Figure 4 General terminal ring analog synthesis

Following the exploration of amide couplings, our pursuit of SAR led us to alter the functionality of the terminal ring, enabling the generation of a wide array of compounds. The creation of novel terminal ring analogs involved the utilization of SNAr, which was applied to generate a critical methyl 3-nitro-4-(linker)benzoate intermediate. This intermediate assumes significant importance, as it represents the initial stage where functionality varies among each terminal ring analog. The rationale for developing SAR for the terminal ring lies in its capacity to accommodate HBD, and HBA functionalities, and its potential for conformational flexibility. The terminal linkers, situated at the 4-R_1 position, exhibit structural diversity by incorporating different elements, such as nitrogen, oxygen, and carbon. Concurrently, the terminal rings vary in terms of the aromatic systems attached to the linker. The primary objective of diversifying the aromatic regions is to investigate the impact of binding pocket interactions on compound activity. The

overarching goal of this section is to highlight the optimization of the synthetic processes used to produce terminal ring analogs and to establish the SAR specific to this segment of the molecule.

Figure 5 General terminal ring analog synthesis with primary amide.

To elucidate the SAR in mind, a series of terminal ring analogs were synthesized, falling into three distinct categories: N-linker, O-linker, and C-linker. Within these categories, matched series were methodically crafted to robustly establish the SAR by systematically alternating between various linkers and aromatic systems. These matched series were further investigated through the synthesis of primary amides of the terminal ring analogs, as illustrated in the general synthesis depicted in Figure 5. The motivation for delving deeper into primary amide couplings stemmed from the intention to substantiate the concept of FraB dependency, which had been observed in the case of compound **330**.

The total synthesis of this extensive collection of compounds necessitated careful consideration and meticulous preparation. The chemical processes involved proved to be particularly challenging, especially as the complexity of the terminal ring increased. Notably, as the aromatic system became more intricate, the nucleophilicity of the linker (in the cases of oxygen or nitrogen) diminished. While employing a sulfur linker might have mitigated this issue, we opted against this approach, as it had the potential to introduce toxicity and was deemed an imprudent pursuit.

This subsequent section of the chapter defines three distinct methodologies employed for the attachment of the terminal ring to the core benzene. The overarching aim of employing these

three methods was to enhance the first step's efficiency in terms of purity, reaction kinetics, and reaction conditions. The rationale behind the development of these three distinct approaches is, in part, attributable to the inherently acidic nature of the carboxylic acid. To improve this issue, methyl esterification of the carboxylic acid was performed, as illustrated in Reaction Scheme 2.1 below.

$NaHCO_3$, ICH_3
DMF
3 h
23°C

Scheme 2.1Reaction Scheme of methyl 4-fluoro-3-nitrobenzoate

In Reaction Scheme 2.1, iodomethane was selected for its well-established efficacy as a potent methylating agent, primarily due to the weak carbon–iodine bond which makes iodine a good leaving group. The deprotonation of the carboxylic acid allowed the carboxylate to serve as a proficient nucleophile, facilitating an S_N2 attack on iodomethane. The choice of an aprotic solvent was deliberate, aimed at avoiding potential side reactions that might occur if the reaction were conducted in a protic solvent like ethanol. Remarkably, this reaction consistently yielded approximately 80% of the desired product at a 27 mmol scale, and it did not require purification for further incorporation into the synthesis of terminal ring analogs. This methyl ester is later saponified to obtain the carboxylic acid as a final product and an intermediate in synthesizing primary amides. The methyl ester played a crucial role as a means of monitoring the completion of the reaction due to its distinctive singlet signal in spectra. This singlet is prominently observed at a chemical shift of 3.98 ppm in MeOD.

Once the synthesis of the methyl 4-fluoro-3-nitrobenzoate was established. I developed three methods of attaching a terminal ring analog via SNAr. In scheme 2.2, there will be method

A, method B, and method C. Each one of these methods utilizes different reagents and conditions to optimize each reaction.

Method A

R, $NaHCO_3$
H_2O
18 h
85ºC

Method B

R, $NaHCO_3$
H_2O
0.33 h
150ºC

Method C

R, DIEP
MNP
120 h
120ºC

Scheme 2.2 Methods of SNAr terminal ring analogs

Method A can be characterized as employing "classic" SNAr conditions. These conditions are considered classic due to the utilization of sodium bicarbonate and water, creating a solution that enables the deprotonation of the nucleophile, thereby enhancing its nucleophilicity. In the context of Method A, R represents a substituent resembling aniline or phenol. The deprotonation step is pivotal in influencing reaction kinetics. Following deprotonation, methyl 4-fluoro-3-nitrobenzoate is introduced to the reaction flask and heated to expedite the reaction rate. A waterless air condenser is employed to prevent the evaporation of the solvent system over time.

Although these conditions demonstrated consistency and reliability, yielding an average of around 80%, they did entail a lengthy and time-consuming work-up process. Moreover, the generation of analogs was slow.

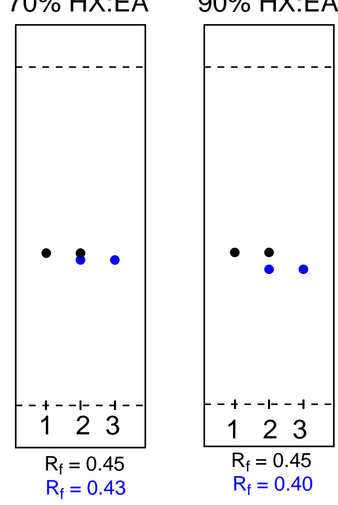
Given the need for efficient and rapid synthesis of numerous analogs, a monowave was introduced. This approach capitalizes on the principle that a monowave can handle intense pressure from heat, resulting in accelerated reaction times. Method B, thus developed, retained the same reagents as Method A. Remarkably, Method B exhibited a remarkable 50-fold increase in reaction speed and an elevated average yield of 90%. This substantial enhancement in reaction speed enabled multiple SNAr reactions to be conducted in a single day. Additionally, the work-up process for Method B proved to be considerably simpler, as it merely necessitated transferring the reaction mixture into 1 M HCl, as opposed to the delicate pH adjustment required for Method A, where the pH needed to be adjusted to 3.

A direct comparison of Method A (1_a) and Method B (I_b) is presented in Table 2.1. It is evident that Method B surpasses Method A in terms of both time and yield, as previously discussed. Furthermore, Method B required only 1.1 equivalents of the R group (phenol-like or aniline-like nucleophiles) to attain completion, likely attributable to the higher concentration of the solvent in which Method B maintained a more concentrated reaction suspension.

Method	Conditions	Time (h)	Temperature (ºC)	Yield (%)
A	4-Fluoro-3-nitrobenzoate(1.0 eq) Sodium carbonate (2.0 eq) R (2.0 eq) Water (0.2 M)	18	85	80-90
B	4-Fluoro-3-nitrobenzoate (1.0 eq) Sodium carbonate (2.1 eq) R (1.1 eq) Water (0.33 M)	0.33	120	88-95

Table 2.1 Comparison of Method A to Method B

The development of method C was needed as the aromatic system became more intricate and the nucleophilicity of the linker (in the cases of oxygen or nitrogen) diminished. We classified this method as “heavy duty” SNAr as the reagents and conditions were more robust and side reactions became more prevalent. The use of N-methyl-2-pyrrolidone (NMP) was picked because of how polar it is and because it is an aprotic solvent which removed the need to worry about the solvent being more nucleophilic than the R group. Using this same logic, Diisopropylethylamine (DIPEA) is a common non-nucleophilic base used in substitution reactions. This method reacted in a sealed pressure tube to ensure that the heat would not cause the pressure to break a standard flask. This method averaged a 30% yield and was very difficult to purify because the starting material and the final product had very similar rf values as seen in Figure 6 below. The TLC plate



1 = Starting material
2 = Reaction after 96 h
3 = Purfied Product

Figure 6 TLC of Method C

on the left in Figure 6 was run in 70% Hexanes: EtOAc and the black spot is the starting material, and the blue spot is the desired product. The retention factors (Rf) of the starting material and the desired product are identical, making purification very difficult. When changing the eluent to 90% HX:EtOAc there is a slight separation that when purified via Combiflash (24 g column) can separate the desired product from the starting material. The best way to observe the conversion of starting material to the desired product for these SNAr reactions is by color. The starting material, methyl 4-fluoro-3-nitrobenzoate, is a bright yellow solid, and the desired product is a bright neon orange. This color change can be monitored over the course of the reaction and can be used as a gauge of how the reaction is going. This observation is seen in all of the SNAr methods (A, B, and C) and

is common with aniline-like nucleophiles. When a phenol-like nucleophile is employed, the desired product is a muted orange color.

Scheme 2.3 Total Synthesis of Amide Couplings

In reaction scheme 2.3 a series of 6 reactions can yield **VII** and **IV** as final compounds that were tested against wild-type *S. enterica* Typhimurium, a *fraB* mutant, and a *tolC* mutant. The rest of scheme 2.3 utilized previously established procedures that were used from the amide coupling scheme 1.2. The final compounds **IV** and **VII** are in a neutral and salt formulation and did not experience any solubility issues. It was noted that as the aromatic rings got greasier and bulkier, there were some solubility issues with the final compound **IV** neutral formulations. The final compounds in Figure 7 and Figure 8 below were synthesized according to Scheme 2.3.

361 362 363 364

383 386

Figure 7 Final carboxylic acid SNAr terminal ring analogs

367 368 369

Figure 8 Final primary amide SNAr terminal ring analogs

Compound	Wild type IC_{50}	*fra* mutant IC_{50}	*tolC* mutant IC_{50}
361	> 100 µM	> 100 µM	100 µM
362	> 100 µM	> 100 µM	30.13 µM
363	> 100 µM	> 100 µM	> 100 µM
364	> 100 µM	> 100 µM	11.64 µM
367	> 100 µM	> 100 µM	> 100 µM
368	> 100 µM	> 100 µM	> 100 µM
369	> 100 µM	> 100 µM	69.26 µM

383	> 100 µM	> 100 µM	52 µM
386	> 100 µM	> 100 µM	> 100 µM

Table 2.2 The Half maximal inhibitory concentration (IC_{50}) of SNAr terminal ring analogs tested.

These SNAr terminal ring analogs with the oxygen and nitrogen linkers were selected to elucidate the SAR and start the generation of matched series. The intention of synthesizing matched series was to be able to comment directly on how changing a singular atom affected the activity of the compounds in assays. Each compound was tested against wild-type *S. enterica* Typhimurium, a *fraB* mutant, and a *tolC* mutant.

Unlike the amide coupling analogs, there was an improvement in activity in the *tolC* mutants compared to **K19**. While this *tolC* mutant activity is promising, there was no wild-type activity. That being said, the *tolC* mutant allowed for a new insight regarding the SAR of **K19**. The most potent *tolC* mutant IC_{50} belonged to compound **364**. This terminal ring analog contained the 1-naphthol aromatic system. This planar system sheds a little light on what kind of binding interactions may be going on at the target. The increase in activity may indicate that the binding pocket contains phenylalanine, tyrosine, or tryptophan amino acids. These amino acids contain aromatic systems and could indicate that pi-pi stacking is a possible key binding interaction.

The second most *tolC* mutant IC_{50} was compound **362**, which contained a *m*-chlorophenol. Halogenated compounds have a history of being antimicrobials and the ability to disinfect surfaces. While it was not as potent as **364**, the activity of **362** can help further confirm the potential of polar amino acids within the binding pocket. Amino acids like aspartic acid, asparagine, or tyrosine have the potential to interact with the chlorine at the meta position on the terminal ring from an inductive affect.

Now looking at **362** and **364** matched series, we see that they outperformed **368** and **369**, which were the primary amide derivatives. This led us to no longer go after the primary amide

couplings because of the lack of wild-type activity and a decrease in the *tolC* mutant IC_{50}. The other matched series compared **362** and **364** to **383** and **386**. This comparison showed that the oxygen linker outperformed the nitrogen linker. Although it is important to know that the oxygen linker performed better than the nitrogen linker, the lack of a crystal structure can prevent a full understanding of what is happening at the linker position within the binding pocket. It is hard to comment due to the fact that both the nitrogen linker and oxygen linker can act as a HBA with amino acids.

SUZUKI COUPLINGS

Figure 9 General Suzuki coupling scheme.

After the synthesis of terminal rings via SNAr that contain a nitrogen or oxygen linker, we decided to pursue the synthesis of terming ring analogs that contained carbon–carbon linker. The reason for this investigation is to determine if HBA is needed and if having a more rigid biphenyl core could improve activity. The pursuit of the terminal ring analogs with a carbon-carbon linker was obtained via Suzuki couplings. The carbon-carbon linker would make the third and final category of terminal ring analogs. Each of the final compounds was designed to complete the matched series between the N-linker, O-linker, and C-linker categories. With the knowledge of a less active primary amide derivative of the terminal ring analog, we decided not to pursue them any further with the carbon-carbon linker analogs.

Scheme 3.1 Synthesis of carbon-carbon linker terminal ring analogs

The following scheme 3.1 was used to synthesize the final carbon linker terminal ring analogs. This scheme utilizes a very similar synthetic pathway that was used to synthesize the nitrogen and oxygen linker final compounds. The final biphenyl core compounds are seen in Figure 11 and were tested against wild-type *S. enterica* Typhimurium, a *fraB* mutant, and *tolC* mutants.

Figure 11 Final Suzuki analogs

Compound	Wild type IC_{50}	*fra* mutant IC_{50}	*tolC* mutant IC_{50}
374	> 100 µM	> 100 µM	> 100 µM
375	> 100 µM	> 100 µM	> 100 µM
376	> 100 µM	> 100 µM	> 100 µM
382	> 100 µM	> 100 µM	> 100 µM

Table 3.1 The Half maximal inhibitory concentration (IC_{50}) of Suzuki terminal ring analogs tested.

Despite the interesting results from the SNAr terminal rings, the C-linker terminal ring analogs proved to not be as effective as the heteroatom linkers that were synthesized via SNAr. While none of the C-linker terminal rings were active, the matched series allowed for more constructive SAR feedback. Compounds **374, 375,** and **382** have a matched pair with a N-linker and an O-linker. These matched pairs allowed us to see that having a heteroatom linker is better than having the C-linker. We believe that this may be due to a high amount of polar amino acids that have a key HBA bond at the terminal linker. With this in mind, no other carbon-carbon terminal ring analogs were synthesized.

TERMINAL RING ANALOG EXPERIMENTALS

Methyl 4-fluoro-3-nitrobenzoate

A flask charged with a solution of 4-fluoro-3-nitrobenzoic acid (5.0 g), and dry DMF (0.864 M) was stirred at 23ºC while NaHCO3 (5.0 equiv.) was added. Then iodomethane (2 equiv.) was added dropwise over 10 min and reacted for 3 h at 23ºC. The reaction suspension was evaporated under reduced pressure and then diluted with water (200 mL) and extracted with EtOAc (3 x 200 mL). The organic layers were combined and washed with brine (2 x 150 mL) and the combined organic layers were dried with sodium sulfate and poured over a cotton plug. The solution was evaporated under reduced pressure to yield **I** (4.0 g, 75% yield) as a yellow solid. ^{1}HNMR (400 MHz, (MeOD)) δ: 8.68 (dd, J = 7.3, 2.3 Hz, 1H), 8.36 (m, 1H), 7.57 (m, 1H), 3.97 (s, 3H).

Methyl 3-nitro-4-(R_1)benzoate (Method A)

To an oven-dried 50-mL two neck RBF charged with **I** (185 mg, 1 mmol) and R (2.0 equiv.) was added with an oven-dried stir bar. Then the mixture was suspended in water (0.2 M) and then sodium carbonate (2.0 equiv.) was added in small portions. The reaction suspension was heated to 85°C for 23 hr. The suspension went from yellow to a dark orange. The solution was then extracted with EtOAc (3 x 5 mL). The aqueous layer was drained off and collected. The solution was then

acidified to a pH of 3 with 1 M HCl, with evident solids crashing out of the solution. The acidified aqueous layer was transferred into the same separatory funnel and was extracted with EtOAc(3 x 5 mL) and washed with Brine (1 x 10 mL) and then the combined organic layers were dried with sodium sulfate. The solution was evaporated under reduced pressure to yield **II** (average yield of 80-90%) as a pale yellow flakey solid.

The following compound analog was prepared using the general procedure listed above to yield **361a** in 95% yield. ^{1}HNMR (400 MHz, (DMSO-d$_6$)) δ: 8.49 (d, *J* = 2.2 Hz, 1H), 8.15 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.50 (m, 2H), 7.31 (m, *J* = 1H), 7.21 (m, 2H), 7.10 (d, *J* = 8.8 Hz. 1H), 3.78 (s, 3H).

O HO NO_2 F **I** → O HO NO_2 R_1 **II**

Methyl 3-nitro-4-(R_1)benzoate (Method B)

To a 5-mL pressure tube, **I** (1 mmol, 185 mg) was added with an oven-dried stirbar. Then water (0.33 M) was added followed by sodium bicarbonate (2.0 equiv.) in small additions. Then R (1.1 equiv.) was added in one slow addition to the solution. Upon addition, the solution turned orange. The pressure tube was placed in the monowave programmed at 150ºC for 20 min. After reaction time a thick orange mixture was present. Then the mixture was poured into 40 mL of 1 M HCl, and the aqueous layer was extracted with EtOAc (3 x 5 mL). The combined organic layers were washed with brine (1 x 10 mL) and then the organic solution was poured over a cotton plug and the solvent was removed under reduced pressure to yield **II** (average yield of 88-95%) as a bright orange fluffy solid.

The following compound analog was prepared using the general procedure listed above to yield **362a** in 85% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 8.69 (d, *J* = 2.0 Hz, 1H), 8.22 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.38 (t, *J* = 8.6. 8.3 Hz, 1H), 7.14 (t, *J* = 4.2, 2.1 Hz, 1H), 7.04 (m, 2H), 4.01 (s, 3H).

The following compound analog was prepared using the general procedure listed above to yield **363a** in 88% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 8.97 (d, *J* = 2.1 Hz, 1H), 8.77 (t, *J* = 6.0, 5.7 Hz, 1H), 8.04 (dd, *J* = 9.0, 2.2 Hz, 1H), 7.36 (m, 5H), 6.88 (d, *J* = 9.2 Hz, 1H), 4.66 (d, 2H), 4.01 (s, 3H).

The following compound analog was prepared using the general procedure listed above to yield **364a** in 82% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 8.72 (d, *J* = 2.2 Hz, 1H), 8.08 (dd, *J* = 8.9, 2.2 Hz, 1H), 7.99 (d, *J* = 8.0 Hz, 1H), 7.93 (d, *J* = 8.2 Hz, 1H), 7.82 (d, *J* = 8.4 Hz, 1H), 7.53 (m, 3H), 7.23 (m, 1H), 6.84 (d, *J* = 8.8 Hz, 1H), 4.01 (s, 3H).

Methyl 3-nitro-4-(R_1)benzoate (Method C)

To a pressure tube, **I**, R (2.5 equiv.) was suspended in NMP (0.63 M). Then DIPEA (2.8 equiv.) was added dropwise at 23ºC. The reaction solution was heated to 120ºC for 96 h. The color changes from yellow to orange to very dark orange. The reaction mixture was diluted with EtOAc (15 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with brine (1 x 15 mL) and dried with sodium sulfate, then poured over a cotton plug. The reaction solution was

concentrated directly with silica gel and purified via Combiflash (24.0 g column), eluting with 0-10% EA:Hexanes. The fractions were evaporated under reduced pressure to yield **II** (20-40% yield) as an orange solid.

The following compound analog was prepared using the general procedure listed above to yield **383a** in 33% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 9.79 (s, 1H), 8.64 (d, *J* = 2.1 Hz, 1H), 7.98 (dd, *J* = 9.0, 2.1 Hz, 1H), 7.46 (m, 2H), 7.34 (m, 2H), 7.2 (d, *J* = 9.0 Hz, 1H), 3.85 (s, 3H).

The following compound analog was prepared using the general procedure listed above to yield **386a** in 21% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 10.15 (br s, 1H), 8.72 (d, *J* = 2.1 Hz, 1H), 8.06 (d, *J* = 8.3 Hz, 1H), 7.89 (d, *J* = 8.3 Hz, 1H), 7.82 (dd, *J* = 9.0 Hz, 2.0 Hz, 1H), 7.59 (m, 4H), 6.55 (d, *J* = 9.1 Hz, 1H), 3.85 (s, 3H).

Methyl 3-nitro-4-(R_1)benzoate (Suzuki)

A flask charged with a mixture of methyl-4-chloro-5-nitrobenzoate, boronic acid (1.1 equiv.), and $Pd(PPh_3)_4$ (0.02 equiv.) in a pressure tube and purge cycle with N_2 was backfilled three times. Then dry Toluene (0.667 M) was added and sparged with N_2 gas for 10 min and sparged 2 M K_2CO_3 (2 equiv.) for 10 min. Then the sparged base was added to the solution drop-wise portions and then heated to 110ºC for 24 h. Then filtered over celite and concentrated to dryness and extracted with EtOAc (3 x 5 mL). Then the combined organic layers were washed with water and brine (3 x 10 mL). The combined organic layers were dried with sodium sulfate and poured over a cotton plug.

The reaction solution was concentrated directly with silica gel and purified via Combiflash (4.0-12.0 g column), eluting with 0-100% EA:HX. The fractions were evaporated under reduced pressure to yield **II** (50-70% yield) as an off-white solid.

The following compound analog was prepared using the general procedure listed above to yield **374a** in 68% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 8.51 (d, *J* = 1.7 Hz, 1H), 8.28 (dd. *J* = 8.1, 1.8 Hz, 1H), 7.57 (d, *J* = 8.1 Hz, 1H), 7.47 (m, 3H), 7.36 (m, 2H), 4.01 (s, 3H).

The following compound analog was prepared using the general procedure listed above to yield **375a** in 50% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 8.51 (d, *J* = 1.7 Hz, 1H), 8.28 (dd. *J* = 8.1, 1.8 Hz, 1H), 7.57 (d, *J* = 8.1 Hz, 1H), 7.42 (m, 1H), 7.37 (t, *J* = 15.4, 7.5 Hz, 1H), 7.34 (t, *J* = 3.6, 2.3 Hz, 1H), 7.19 (dt, *J* = 7.5, 3.3, 1.5 Hz, 1H), 4.01 (s. 3H).

The following compound analog was prepared using the general procedure listed above to yield **376a** in 69% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 8.43 (d, *J* = 1.6 Hz, 1H), 8.23 (dd, *J* = 8.1, 1.8 Hz, 1H), 7.52 (d, *J* = 8.2 Hz, 1H), 7.27 (m, 2H), 6.97 (m, 2H), 4.01 (s, 3H), 3.85 (s, 3H).

The following compound analog was prepared using the general procedure listed above to yield **382a** in 25% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 8.71 (d, *J* = 1.7 Hz, 1H), 8.34 (dd. *J* = 8.1, 1.8 Hz, 1H), 7.92 (dd, *J* = 8.3, 3.6 Hz, 2H), 7.59 (d, *J* = 7.9 Hz, 1H), 7.52 (m, 2H), 7.41 (m, 2H), 7.34 (dd, *J* = 7.1, 1.2 Hz, 1H), 4.01 (s, 3H).

Methyl 3-amino-4-(R_1)benzoic acid

To a stirred solution of **II** in methanol (333 mmol/L):water (333 mmol/L), iron powder (7 equiv.), and ammonium chloride (7 equiv.) were combined and reacted at 23ºC for 24 h. The reaction suspension was filtered through a celite bed and washed with EtOAc. The solutions were evaporated under reduced pressure and quenched with sat. $NaHCO_3$, was extracted with EtOAc (3 x 5 mL). The combined organic extracts were washed with brine (1 x 10 mL), dried over sodium sulfate, and filtered through a cotton pug. The reaction solution was concentrated directly with silica gel and purified via Combiflash (12.0 g column), eluting with 0-40% EA:Hexanes. The fractions were evaporated under reduced pressure to yield **III** (20-90% yield) as a purple solid.

The following final compound analog was prepared using the general procedure listed above to yield **361** in 40% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.58 (d, *J* = 2.2 Hz, 1H), 7.48 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.39 (m, 2H), 7.18 (m, 1H), 7.07 (m, 2H), 6.82 (d, *J* = 8.8 Hz. 1H).

The following final compound analog was prepared using the general procedure listed above to yield **362** in 33% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.57 (d, *J* = 2.0 Hz, 1H), 7.48 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.29 (t, *J* = 8.6. 8.3 Hz, 1H), 7.13 (t, *J* = 4.2, 2.1 Hz, 1H), 7.04 (m, 2H).

The following final compound analog was prepared using the general procedure listed above to yield **363** in 20% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 12.3 (br s, 1H), 7.33 (m, 5H), 7.22

(m, 2H). 7.16 (d, *J* = 2.1 Hz, 1H), 7.08 (dd, *J* = 9.0, 2.2 Hz, 1H), 6.88 (d, *J* = 9.2 Hz, 1H), 5.88 (t, *J* = 6.0, 5.7 Hz, 1H), 4.75 (br s, 2H), 4.66 (d, 2H), 4.01 (s, 3H).

The following final compound analog was prepared using the general procedure listed above to yield **364** in 25% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 12.3 (br s, 1H), 8.04 (m, 2H), 7.73 (d, *J* = 8.1 Hz, 1H), 7.57 (m, 2H), 7.47 (m, 2H), 6.95 (d, *J* = 7.7 Hz, 1H), 6.64 (d, *J* = 8.3 Hz, 1H), 5.34 (br s, 2H).

Methyl 3-amino-4-(R_1)benzoate

To a stirred solution of **II** in methanol (333 mmol/L):water (333 mmol/L), iron powder (7 equiv.), and ammonium chloride (7 equiv.) were combined and reacted at 23°C for 24 h. The reaction suspension was filtered through a celite bed and washed with EtOAc. The solutions were evaporated under reduced pressure and quenched with sat. $NaHCO_3$, was extracted with EtOAc (3 x 5 mL). The combined organic extracts were washed with brine (1 x 10 mL), dried over sodium sulfate, and filtered through a cotton pug. The reaction solution was concentrated directly with silica gel and purified via Combiflash (12.0 g column), eluting with 0-40% EA:Hexanes. The fractions were evaporated under reduced pressure to yield **III** (20-90% yield) as a purple solid.

The following general compound **III** was prepared using the general procedure listed above to yield **374c** in 44%. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.45 (m, 6H), 7.38 (m, 1H), 7.17 (d, *J* = 7.7 Hz, 1H), 3.91 (s, 3H), 3.86 (br s, 2H).

The following compound analog was prepared using the general procedure listed above to yield **375c** in 40% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.64 (m, 5H), 7.37 (m, 1H), 7.16 (m, 1H), 3.91 (s, 3H), 3.86 (br s, 2H).

The following compound analog was prepared using the general procedure listed above to yield **376c** in 20% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.46 (dd, *J* = 7.9, 1.8 Hz, 1H), 7.42 (d, *J* = 1.6 Hz, 1H), 7.38 (m, 1H), 7.15 (d, *J* = 7.6 Hz, 1H), 6.99 (m, 1H), 3.91 (s, 3H), 3.85 (s, 3H).

The following compound analog was prepared using the general procedure listed above to yield **382c** in 54% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.94 (m, 2H), 7.57 (m, 5H), 7.46 (m, 2H), 7.25 (d, *J* = 7.9 Hz, 1H), 3.91 (s, 3H), 3.63 (br s, 2H).

The following compound analog was prepared using the general procedure listed above to yield **383c** in 95% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 7.66 (s, 1H), 7.38 (d, *J* = 2.1 Hz, 1H), 7.19 (m, 2H), 7.46 (m, 2H), 7.10 (d, *J* = 8.1 Hz, 1H), 6.84 (m, 2H), 6.80 (m, 1H), 5.08 (br s, 2H) 3.78 (s, 3H).

The following compound analog was prepared using the general procedure listed above to yield **386c** in 63% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 8.04 (d, *J* = 8.8 Hz, 1H), 7.92 (d, *J* = 8.8 Hz, 1H), 7.50 (m, 6H), 7.10 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.03 (d, *J* = 7.4 Hz, 1H), 6.64 (d, *J* = 8.3 Hz), 5.12 (br s, 2H), 3.78 (s, 3H).

O NH2 O R1 III → HO NH2 R1 IV

3-amino-4-(R_1)benzoic acid

A flask charged with **III** was suspended in a 1:1 H_20 MeOH (0.2 M). Then KOH (2 equiv.) was added to the flask and a water waterless reflux condenser was put on top and reacted at 100ºC. The reaction suspension was monitored via TLC and reacted until the starting material was consumed. The reaction solution was reduced to dryness and acidified by 1 M HCl to a pH of 3. The suspension was extracted with DCM or EtOAc (Depending on solubility) and then the organic layers were combined and dried with sodium sulfate. The organic solution was poured over a cotton plug and evaporated under reduced pressure to yield **IV** (average yield of 80%) as an off-white solid.

The following general compound **IV** was prepared using the general procedure listed above to yield 374 in 82%. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.40 (m, 6H), 7.20 (dd, *J* = 7.9, 1.8 Hz, 1H), 7.07 (d, *J* = 7.7 Hz, 1H), 3.91 (s, 3H), 3.86 (br s, 2H).

The following final compound analog was prepared using the general procedure listed above to yield **375** in 41% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.64 (m, 5H), 7.41 (m, 1H), 7.18 (m, 1H), 3.86 (br s, 2H).

The following final compound analog was prepared using the general procedure listed above to yield **376** in 40% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.53 (dd, *J* = 7.9, 1.8 Hz, 1H), 7.47 (d, *J* = 1.6 Hz, 1H), 7.40 (m, 2H), 7.19 (d, *J* = 7.6 Hz, 1H), 7.00 (m, 1H), 3.85 (s, 3H).

The following final compound analog was prepared using the general procedure listed above to yield **382** in 89% yield. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.94 (m, 2H), 7.57 (m, 5H), 7.46 (m, 2H), 7.25 (d, *J* = 7.9 Hz, 1H), 3.63 (br s, 2H).

The following final compound analog was prepared using the general procedure listed above to yield **383** in 76% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 12.2 (br s, 1H), 7.66 (s, 1H), 7.38 (d, *J* = 2.1 Hz, 1H), 7.19 (m, 2H), 7.46 (m, 2H), 7.10 (d, *J* = 8.1 Hz, 1H), 6.84 (m, 2H), 6.80 (m, 1H), 5.08 (br s, 2H).

The following final compound analog was prepared using the general procedure listed above to yield **386** in 80% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 12.2 (br s, 1H), 8.08 (d, *J* = 8.8 Hz, 1H), 7.92 (d, *J* = 8.8 Hz, 1H), 7.50 (m, 6H), 7.10 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.03 (d, *J* = 7.4 Hz, 1H), 6.64 (d, *J* = 8.3 Hz), 5.12 (br s, 2H).

3-((tert-butoxycarbonyl)amino)-4-(R_1)benzoic acid

To a stirred solution of **IV** (1 equiv.) in 2:1 dioxane:water (0.8 M : 0.4 M) was added triethylamine (1.5 equiv.) and boc anhydride (1.5 equiv.) at 23°C and stirred for 24 h. Then dioxane was evaporated under reduced pressure, and the resulting aqueous residue was diluted water (9.0 mL) The aqueous residue was adjusted to a pH of 2 with aqueous 1M hydrochloric acid. The thick pink

mixture was separated via vacuum filtration, rinsing the flask with acidified water (3 x 1mL), and azeotropically dried with toluene to yield **V (**average yield 75-80%) as a purple solid.

The following compound analog was prepared using the general procedure listed above to yield **367a** in 53% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: : 7.58 (d, *J* = 2.2 Hz, 1H), 7.48 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.39 (m, 2H), 7.18 (m, 1H), 7.07 (m, 2H), 6.82 (d, *J* = 8.8 Hz. 1H), 1.46 (s, 3H).

The following compound analog was prepared using the general procedure listed above to yield **368a** in 74% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 7.57 (d, *J* = 2.0 Hz, 1H), 7.48 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.29 (t, *J* = 8.6, 8.3 Hz, 1H), 7.13 (t, *J* = 4.2, 2.1 Hz, 1H), 7.04 (m, 2H), 1.46 (s, 3H).

The following compound analog was prepared using the general procedure listed above to yield **369a** in 40% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 12.3 (br s, 1H), 8.04 (m, 2H), 7.73 (d, *J* = 8.1 Hz, 1H), 7.57 (m, 2H), 7.47 (m, 2H), 6.95 (d, *J* = 7.7 Hz, 1H), 6.64 (d, *J* = 8.3 Hz, 1H), 1.46 (s, 3H).

tert-butyl (5-carbamoyl-2-(R_1)phenyl)carbamate

To a flask charged with 4 (50.5 mg, 1 equiv.), CDI (1.5 equiv.), THF (180 mmol/L) under nitrogen pressure was added. After 1 h ammonia (1.5 equiv.) was added and stirred for 1 h at 23ºC. After 30 min TLC was run on reaction solution in 80% EA:HX and saw the separation of intermediate

and desired product with an Rf ≈ 0.38-0.45. The reaction mixture was concentrated directly with silica gel and purified via Combiflash (4.0 g column), eluting with 1:1 EA:Hexanes. The product eluted at about 2-3.5 min. The fractions were evaporated under reduced pressure to yield 5 (average yield as 40-85%) as a purple flaky solid.

The following compound analog was prepared using the general procedure listed above to yield **367a** in 94% yield. ^{1}HNMR (400 MHz, (MeOD)) δ: 8.02 (d, *J* = 2.2 Hz, 1H), 7.87 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.54 (m, 2H), 7.33 (m, 1H), 7.21 (m, 2H), 6.91 (d, *J* = 8.8 Hz. 1H). 1.46 (s, 3H).

The following compound analog was prepared using the general procedure listed above to yield **369a** in 55% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 12.3 (br s, 1H), 8.08 (m, 2H), 7.77 (d, *J* = 8.1 Hz, 1H), 7.57 (m, 2H), 7.47 (m, 2H), 6.95 (d, *J* = 7.7 Hz, 1H), 6.64 (d, *J* = 8.3 Hz, 1H), 1.46 (s, 3H).

O
H_2N NHBoc R
VI
→
O HCl
H_2N NH_2 R
VII

3-amino-4-(R_1)benzamide hydrochloride

To a flask containing **VI** (1 equiv.)**,** hydrochloric acid (4 M, 0.5 mL) was added and stirred for 24 h at 23ºC. The reaction suspension was diluted with methanol (2 mL) and was evaporated under reduced pressure to yield **VII** (average yield 93-99%) as an off-white flaky solid.

The following final compound analog was prepared using the general procedure listed above to yield **367** in 94% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 8.02 (d, *J* = 2.2 Hz, 1H), 7.87 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.54 (m, 2H), 7.33 (m, 1H), 7.21 (m, 2H), 6.91 (d, *J* = 8.8 Hz. 1H).

The following final compound analog was prepared using the general procedure listed above to yield **368** in 94% yield. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 7.81 (br s, 1H), 7.44 (d, *J* – 1.6 Hz, 1H), 7.38 (t, *J* = 8.4, 8.1 Hz, 1H), 7.18 (m, 2H), 6.98 (t, *J* = 5.8, 4.2 Hz, 1H), 6.92 (dd, *J* = 8.4, 2.6 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 1H), 4.25 (br s, 2H).

The following final compound analog was prepared using the general procedure listed above to yield **369** in 100%. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 12.3 (br s, 1H), 8.08 (m, 2H), 7.77 (d, *J* = 8.1 Hz, 1H), 7.57 (m, 2H), 7.47 (m, 2H), 6.95 (d, *J* = 7.7 Hz, 1H), 6.64 (d, *J* = 8.3 Hz, 1H), 5.34 (br s, 2H).

EXPLORATION OF THE 3-AMINO GROUP

The goal of this chapter is to explain the design and the synthesis of a series of compounds that explored the results when the 3-amino was removed or altered as seen in Figure 12 below.

Figure 12 SAR exploration of the 3-Amino group

After the exploration of the terminal ring analogs, the only area left of the original **K19** hit to explore was the 3-amino position. We wanted to know if this functionality was needed for the compound to be active because of how aromatics amines, like the 3-amino group, have been known to cause problems. These problems range from being a possible carcinogen to endocrine disruptors that may increase the risk of breast cancer in patients.[4] These possible risks fueled our investigation of the 3-amino group in the original hit.

The methods of altering the 3-amino group by completely removing the group from the molecule and altering its HBD capabilities. To make these compounds, two unique synthetic

[4] Trakoli, Anna. "IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 99: Some Aromatic Amines, Organic Dyes, and Related Exposures. International Agency for Research on Cancer." *Occupational Medicine* 62, 3 (2012): 232.

pathways were employed. The first of these two pathways used Buchwald-Hartwig couplings, and the second pathway generated a triazole as the final product. The use of the Buchwald-Hartwig couplings was intentionally done to make a matched pair to the original **K19** hit so that we could directly comment on the activity with and without the 3-amino group.

Through the synthesis of these compounds, it was found that the saponification step needed to be optimized to better maximize the purity and reaction rate of the final step. This led to the development of two methods of saponification: method D and method E. These methods utilize very similar conditions which can be found in Figure 13 below.

Scheme 4.1 Methods of Saponification

The biggest difference between these two methods is the base used and the temperature at which the reaction was conducted. With the removal of the 3-amino group, the core benzene ring was not as activated, which became a problem when using lithium hydroxide as a base. The issue with this base is that lithium does not dissociate completely due to the presence of some covalent

bond character. Building on this step, there was also no heat used to help the speed of this reaction. This issue was further confirmed by 1HNMR as seen in Figure 14 below.

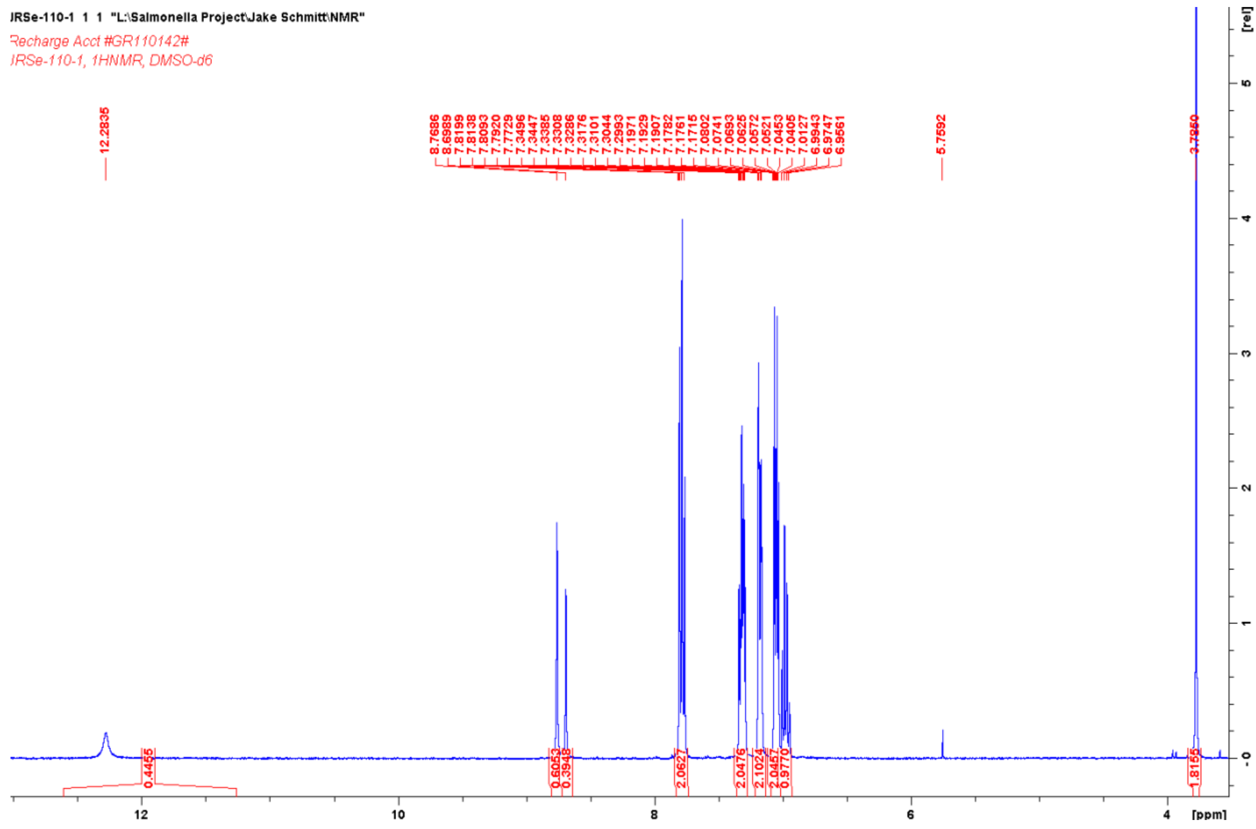


Figure 14 1HNMR of 4-(phenylamino)benzoic acid after 24 h

There are three areas of Figure 14 that hint that there is a mixture of starting material and desired product. The first sign of unreacted starting material is the presence of a methyl ester peak just below 4.0 ppm. The presence of this and a sharp-broad -OH peak above 12.0 ppm can hint at this suspected mixture. But to further support this, there are two NH singlets at 8.75 ppm. The presence of two NH singlets confirms the presence of unreacted starting material and the desired product.

The same saponification seen in Scheme 4.1 was performed using method E. These conditions used a potassium hydroxide, which is completely ionic, methanol, and water as the reagents with heat. The presence of a stronger base and heat allowed for reaction times to decrease

and for the purity of the final compound to improve. The progress of the reaction was monitored by ^{1}HNMR and yielded the spectrum seen below in Figure 15 after 2 hours.

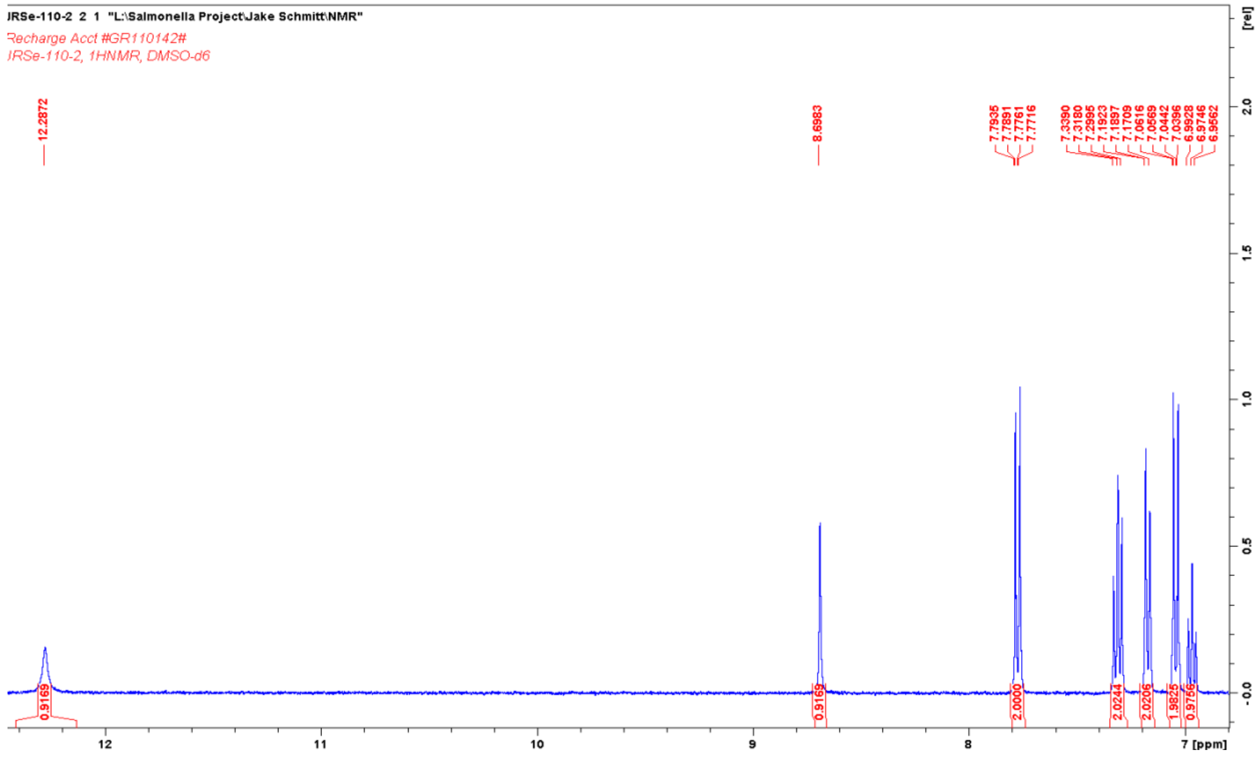


Figure 15 1HNMR of 4-(phenylamino)benzoic acid after 2 h

The disappearance of two NH singlets at 8.75 ppm indicates that there was full conversion of the starting material to the desired product in Figure 15. From these two methods, a lot of insight was gained about the modest transformations of saponification mechanisms.

After pursuing the removal of the 3-amino group, we decided that converting the benzene core of **K19** to a triazole would be a great way to investigate the HBD abilities of the 3-amino group. The introduction of a triazole simply added one step to the synthesis and proved to be rather convenient. The synthesis of **392** used the synthetic pathway laid out in scheme 4.3. The final compounds that explored the 3-amino group were synthesized using scheme 4.2 and scheme 4.3 to yield **378** and **392** as seen in Figure 16. These compounds were then tested against wild-type *S. enterica* Typhimurium, a *fraB* mutant, and *tolC* mutants.

Scheme 4.2 Synthetic route of 4-(phenylamino)benzoic acid

Scheme 4.3 Synthetic route of 1-(3-chlorophenyl)-1H-benzo[d][1,2,3]triazole-5-carboxylic acid

Figure 16 Final 3-amino analogs

Compound	**Wild type IC_{50}**	***fra* mutant IC_{50}**	***tolC* mutant IC_{50}**

378	> 100 µM	> 100 µM	92 µM
392	> 100 µM	> 100 µM	> 100 µM

Table 4.1 The Half maximal inhibitory concentration (IC_{50}) of 3-amino analogs tested.

Even with the activity seen with the original hit and other 1,2,3 triazole hits, there was no improvement in activity. While this may seem disappointing, the data is actually very helpful as it establishes a clear SAR. It can be seen that the 3-amino group is very essential to the activity. Even with a nitrogen at the 3 position, the HBA most likely does not have a strong enough interaction with possible polar amino acids within the binding pocket. This is further supported by the matched series. Compound **378** is paired with **K19,** which differs with the presence or absence of the 3-amino group. This removal shows that HBD or HBA is needed for the activity. This is further narrowed down to a key HBD by the matched series of **392** and **383**. This pair shows a free 3-amino group in **383** to a locked nitrogen in a triazole ring as seen in **392**. The free 3-amino group in **383** can HBD in the binding pocket whereas **392** can only HBA. This hints that the polar amino acids have a negative charge and could be aspartate or glutamate.

3-AMINO GROUP EXPERIMENTALS

Methyl 4-(phenylamino)benzoate

To a 5-mL pressure tube, **I** (1.05 mmol, 226.0 mg) was added with an oven-dried stir bar. Then aniline (1 equiv.), palladium bis(acetate) (0.01 equiv.), Xantphos (0.01 equiv.) and cesium carbonate (1.3 equiv.) were added. Then the purge cycle with N_2 was backfilled three times before dry 1,4 dioxane (0.44 M) was added. The pressure tube was placed in the monowave programmed at 120ºC for 3 h. After reaction time a thick orange mixture was present. Then the mixture was poured into 10 mL of DCM and was evaporated under reduced pressure. The crude solid was suspended in 50 mL of water and then extracted with DCM (3 x 20 mL). The combined organic layers were washed with saturated NaHCO3 (1 x 10 mL) and brine (1 x 10 mL). The reaction solution was concentrated directly with silica gel and purified via Combiflash (4.0 g column), eluting with 0-20% EA:Hexanes. The fractions were evaporated under reduced pressure to yield **II** (142.0 mg, 63% yield) as an orange solid. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.92 (dt, *J* = 8.7, 6.8, 2.0 Hz, 2H) 7.33 (td, *J* = 8.5, 2.1 Hz, 2H) 7.17 (m, 2H), 7.06 (m, 1H), 6.98 (m, 2H), 6.00 (br s, 1H), 3.88 (s, 3H).

4-(phenylamino)benzoic acid

To a flask charged with **II** was suspended in a 1:1 H_20 MeOH (0.2 M). Then KOH (2 equiv.) was added to the flask and a water waterless reflux condenser was put on top and reacted at 100ºC. The reaction suspension was monitored via TLC and reacted until the starting material was consumed. The reaction solution was reduced to dryness and acidified by 1 M HCl to a pH of 3. The suspension was extracted with DCM or EtOAc (Depending on solubility) and then the organic layers were combined and dried with sodium sulfate. The organic solution was poured over a cotton plug and evaporated under reduced pressure to yield **III** (80% yield) as an off-white solid. ^{1}HNMR (400 MHz, ($CDCl_3$)) δ: 7.98 (m, 2H) 7.35 (td, *J* = 8.7, 8.5 Hz, 2H) 7.19 (m, 2H), 7.09 (m, *J* = 1H), 7.00 (dt, *J* = 8.7, 6.8 Hz, 2H), 6.06 (br s, 1H).

NO_2 F I NO_2 NH II Cl

methyl 4-((3-chlorophenyl)amino)-3-nitrobenzoate

To a pressure tube, **I**, *m*-chloroaniline (2.5 equiv.) was suspended in NMP (0.63 M). Then DIPEA (2.8 equiv.) was added dropwise at 23ºC. The reaction solution was heated to 120ºC for 96 h. The color changes from yellow to orange to very dark orange. The reaction mixture was diluted with EtOAc (15 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with brine (1 x 15 mL) and dried with sodium sulfate, then poured over a cotton plug. The reaction solution was concentrated directly with silica gel and purified via Combiflash (24.0 g column), eluting with 0-10% EA:Hexanes. The fractions were evaporated under reduced pressure to yield **II** (30% yield) as an orange solid. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 9.79 (s, 1H), 8.64 (d, *J* =

2.1 Hz, 1H), 7.98 (dd, $J = 9.0$, 2.1 Hz, 1H), 7.46 (m, 2H), 7.34 (m, 2H), 7.2 (d, $J = 9.0$ Hz, 1H), 3.85 (s, 3H).

methyl 3-amino-4-((3-chlorophenyl)amino)benzoate

To a stirred solution of II in methanol (333 mmol/L):water (333 mmol/L), iron powder (7 equiv.), and ammonium chloride (7 equiv.) were combined and reacted at 23℃ for 24 h. The reaction suspension was filtered through a celite bed and washed with EtOAc. The solutions were evaporated under reduced pressure and quenched with sat. $NaHCO_3$, was extracted with EtOAc (3 x 5 mL). The combined organic extracts were washed with brine (1 x 10 mL), dried over sodium sulfate, and filtered through a cotton pug. The reaction solution was concentrated directly with silica gel and purified via Combiflash (12.0 g column), eluting with 0-40% EA:Hexanes. The fractions were evaporated under reduced pressure to yield **III** (90% yield) as a purple solid. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 7.66 (s, 1H), 7.38 (d, $J = 2.1$ Hz, 1H), 7.19 (m, 2H), 7.46 (m, 2H), 7.10 (d, $J = 8.1$ Hz, 1H), 6.84 (m, 2H), 6.80 (m, 1H), 5.08 (br s, 2H) 3.78 (s, 3H).

methyl 1-(3-chlorophenyl)-1H-benzo[d][1,2,3]triazole-5-carboxylate

To a stirred solution of **III** in THF (0.34 M) at 0ºC, a solution of 1:1 sulfuric acid (460 µL) and water (460 µL) was added dropwise. Then a solution of 0.34 M NaNO2 (1.65 equiv.) was added to the reaction suspension. The reaction suspension was brought to 23ºC and stirred for 2 h. The reaction suspension was diluted with EtOAc (10 mL) and extracted with EtOAc (3 x 10 mL). The organic layers were combined and washed with saturated NaHCO3 (1 x 10 mL) and brine (1 x 10 mL). The recombined organic layers were poured through a cotton plug and n was concentrated directly with silica gel and purified via Combiflash (4.0 g column), eluting with 0-40% EA:Hexanes. The fractions were combined and evaporated under reduced pressure to yield **IV** (35.0 mg, 38% yield) as an off-white solid. ^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 8.78 (s, 1H), 8.22 (dd, *J* = 8.8, 1.5 Hz, 1H), 8.08 (d, *J* = 8.8 Hz, 1H), 8.04 (t, *J* = 4.0, 3.7 Hz, 1H), 7.91 (dt, *J* = 7.7, 6.1 Hz, 1H) 7.75 (t, *J* = 8.1, 7.9 Hz, 1H), 7.71 (dt, *J* = 8.3, 6.5 Hz, 1H), 3.95 (s, 3H).

O O N N N IV Cl → HO N N N V Cl

1-(3-chlorophenyl)-1H-benzo[d][1,2,3]triazole-5-carboxylic acid

To a flask charged with **IV** was suspended in a 1:1 H_20 MeOH (0.2 M). Then KOH (2 equiv.) was added to the flask and a water waterless reflux condenser was put on top and reacted at 100ºC. The reaction suspension was monitored via TLC and reacted until the starting material was consumed. The reaction solution was reduced to dryness and acidified by 1 M HCl to a pH of 3. The suspension was extracted with DCM or EtOAc (Depending on solubility) and then the organic layers were combined and dried with sodium sulfate. The organic solution was poured over a cotton plug and evaporated under reduced pressure to yield **V** (30% yield) as an off-white solid.

^{1}HNMR (400 MHz, (DMSO-d_6)) δ: 8.72 (s, 1H), 8.2 (d, *J* = 8.5 Hz, 1H), 8.03 (m, 2H), 7.91 (d, *J* = 7.9 Hz, 1H), 7.73 (m, 2H).

CONCLUSION

The principal challenge encountered in this project pertained to the deficiency in wild-type activity, attributed to the efflux capabilities of *Salmonella enterica* Typhimurium. This phenomenon potentially elucidates the observed inactivity of tested analogs in the assay. The hypothesis gained support from the discerned activity of compounds in *tolC* knockouts. Ongoing HTS is focused on exploring new cores that specifically target the FraB enzyme. Promisingly, these novel hits exhibit enhanced wild-type activity.

Concurrently, the Ahmer lab at The Ohio State University is actively engaged in investigating alternative metabolic pathways. The objective is to identify pathways whose inhibition could induce effects akin to those observed with the inhibition of the FraB enzyme. This multifaceted approach aims to deepen our understanding of the underlying mechanisms and broaden the potential avenues for therapeutic intervention.