Acylated Sulfa Drugs: A Phthesis on the Synthesis of Acylated Sulfa Compounds in Connection with Antitubercular Drug Design

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Acylated Sulfa Drugs
A Phthesis on the Synthesis of Acylated Sulfa Compounds in Connection with Antitubercular Drug Design

Catherine Deitsch Pugh

Submitted in Partial Fulfillment
of the Prerequisite for Honors
in the Chemistry Department
under the advisement of Professor Michael J Hearn

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Abstract

Tuberculosis (TB) is an infectious disease caused by the organism *Mycobacterium tuberculosis* (MTB). Improved sanitation and successful chemotherapy have largely eliminated TB in industrialized countries, but it remains a serious threat to lower socio-economic classes, the immunocompromised population, and developing countries where industrialization is just taking hold. Although two new drugs have surfaced in the last decade, they are largely reserved for compassionate cases because of the severity of their side effects. The first-line TB treatment prescribed by the World Health Organization and the Center for Disease Control and Prevention remains the comparatively small number of drugs synthesized or isolated in the 1950’s and 1960’s. These drugs, while currently effective, can cause adverse side effects such as liver toxicity. Additionally, the emergence of strains of drug-resistant TB threatens to completely undermine existing treatment regimens and enable TB to reach a level of prevalence not experienced for the past century.

In the past decade, studies have suggested intriguing antitubercular possibilities offered by applying modern methods to a very early class of drugs: the sulfa drugs. Sulfa drugs work by mimicking the natural molecule *para*-aminobenzoic acid, inhibiting dihydropteroate synthase (DHPS) and resulting in cell death. Arylamine N-acetyltransferases (NATs) de-activate sulfa drugs inside the body by catalyzing the transfer of an acetyl group onto position N⁴ of the sulfa molecule. NATs also appear in *M. tuberculosis*, where they act as a defense mechanism for MTB against chemotherapy.

My work in the Hearn lab has been guided by recent crystallographic images of the DHPS binding pocket and the deactivating mechanism of NATs, as well as by previous studies in our lab that demonstrated that acylation can improve activity and bioavailability while
reducing toxicity. Our work began with the development of a reliable protocol to prepare the acetylated NAT metabolites. We then explored a number of different procedures for increasing acyl chain length at $N^4$, focusing on derivatives of sulfamethazine; and then continued to selective di-acylation at both $N^4$ and $N^1$, varying both chain length and steric bulk for a number of different sulfa drug scaffolds. Several of our acylated sulfa derivatives possess improved biological and anti-tubercular properties over their parent drugs. Moreover, the compounds serve as probes of the structural factors influencing biological activity such as fit within DHPS, resistance to the de-activating effects of NATs, and permeability.
**Introduction**

**TUBERCULOSIS IN HISTORY: PHTHESIS AND CONSUMPTION**

Tuberculosis is an infectious disease caused by the organism *Mycobacterium tuberculosis*. It usually affects the lungs, but can also spread to other areas of the body such as the skin, bones or kidneys. The characteristic symptoms of pulmonary tuberculosis are chest pain, fatigue, chills/fever, night sweats, and a persistent cough that often includes expectorating blood or sputum.\(^1\) Biological research estimates that the modern strain of *M. tuberculosis* (MTB) surfaced approximately 20,000 years ago. The disease recurs frequently throughout history: archaeologists have found skeletal evidence of tuberculosis in 5,000-year-old Egyptian mummies, and ancient Greek philosophers and doctors wrote at great length of tuberculosis-like symptoms, possible causes, and suggested treatments. In the Middle Ages, the touch of a member of the royal family was thought to cure scrofula, the swollen lymph nodes that were often a sign of a tuberculosis infection.\(^2\) The prevalence of tuberculosis waxed and waned throughout the centuries. Though painful, debilitating, and almost always fatal by the time outward symptoms manifested, tuberculosis did not reach the peak of its reign as a terrifying plague until the nineteenth century, when the rise of industrialization created the perfect conditions for infection to flourish.\(^3\)

The Industrial Revolution, which swept across Europe and the United States at the end of the 18\(^{th}\) century, shifted the population from small farming communities to burgeoning urban environments. In that era, cities grew far too rapidly to maintain any kind of reasonable sanitation, and the idea that disease was contagious was mostly dismissed by medical practitioners of the time. Laborers were crammed together at work and at home in buildings that were poorly ventilated, filthy and overcrowded. With such exposure to the exhalations and bodily fluids of others, contagions of all kinds ran rampant. Today we understand that physical
and psychological stress can increase susceptibility to sickness, and there was a high quotient of misery in the new industrial lifestyle. Dubos writes about the “ubiquity of contagion” in the Industrial Era, adding that the higher social classes were by no means immune: “Physiological misery and crowding permitted the explosive spread of the disease among the labor classes, and from this huge focus the infection spread through society by means of countless unavoidable contacts.”

Terrorized by the omnipresence of the illness and impressed by its lethality, society christened tuberculosis with several euphemisms, including “the Captain of All the Men of Death” and the “White Plague.” While medical science had made great strides since the Renaissance, tuberculosis was still poorly understood. Due to its varying presentations in different areas of the body, tuberculosis was frequently classified as many different diseases. In fact, as physicians grew more adept at distinguishing between different symptoms and classifying them accordingly, the single disease we would now identify as tuberculosis acquired many different diagnostic profiles.

Unlike a straightforward cold or influenza, the contagious nature of tuberculosis was difficult to discern because enormous swaths of the population were likely infected with the latent form of the disease. Dubos asserts “[i]t is certain that during the eighteenth and nineteenth centuries all dwellers in large cities of Europe became infected at an early age and remained in contact with heavily contaminated objects, sputum, food and dust throughout their life.” As a result, it was not a question of whether any given person was infected but who manifested the symptoms of an active infection, and how quickly and severely the disease progressed. Although the idea that tuberculosis was contagious had been proposed by an Italian scientist named Hyeronymus Fracastorius in 1596, and the idea briefly held sway in Southern Europe, it was by
the nineteenth century rejected by most physicians, especially in the northern parts of Europe. Instead, physicians believed that if a patient developed tuberculosis – or pulmonary phthisis, as it was called in its most familiar form – it was due to an inherent, constitutional susceptibility. The putative hereditary weakness frequently presented itself in one’s early twenties, or after some traumatic event or stressor. Apparent evidence for this theory was provided by the many members of family lines who developed tuberculosis at around the same point in their lives, often wiping out entire households. Due to tuberculosis’ long incubation period before becoming active and causing symptoms, the alternate explanation of a single source of infection or indeed an entire house permeated with the bacteria could not be conceptualized for many more years. For example, the tragic demise of the six siblings of the Brontë family and later their father, almost all from clear symptoms of tuberculosis, was simply attributed to their hereditary profile and not the effects of being confined together to a tiny, damp and poorly ventilated house.3,4

While the infectious aspect of tuberculosis was denied, physicians struggled to treat its devastating symptoms. By the time a patient started coughing up blood, often the first recognizable sign of pulmonary infection, tuberculosis had already wreaked its destruction in the body and the patient faced a painful march toward death. With no coherent idea of the progress of tuberculosis inside the body, the prescribed treatment for symptoms varied wildly from physician to physician and from trend to trend. Common features included protection from fresh air by sealing the windows of the sickroom, an aggressive starvation diet to deprive the disease – and, by unfortunate side effect, the patient – of nutrients, and travel to milder climates for the calming and healing effects of balmy weather. Later in the nineteenth century, physicians prescribed horseback riding or other fresh-air exercise, high-fat diets, and total rest as treatment protocols.3 As medical science progressed and the value of rest for damaged lungs became
clearer, the concept was taken to the extreme – beginning in 1882 and continuing for about twenty-five years, the preferred technique for slowing the progress of tuberculosis was inducing pulmonary collapse through crude surgery.\(^5\)

These treatments were nominally informed by advances in medical knowledge regarding the various manifestations of tuberculosis and its pathogenesis. Gaspard Bayle and his student René Laënnec contributed to the existing repository of information about disease by performing hundreds of dissections on corpses with pulmonary phthisis. They meticulously detailed the types and consistencies of tubercles, the spherical nodules that form in the lungs around sites of tuberculosis infection. Before his own early death of tuberculosis, Laënnec proposed his inspired unified theory of tuberculosis.\(^3\) In 1804, decades before it would be accepted by the medical world at large, Laënnec outlined how infiltration, tubercles and open cavity ulcers were consecutive stages of a single illness. With no knowledge of germ theory, he nonetheless suggested that many diseases of the skin, lungs and other body parts which we would today recognize as caused by MTB were in fact multiple forms of one causative agent.\(^3\) In the study of his newly defined disease, Laënnec invented and promoted the use of the stethoscope, allowing a previously unparalleled level of auditory insight into a patient’s body cavities, especially the lungs. He was partially inspired by another advance made at the turn of the nineteenth century: Jean-Nicolas Corvisart’s resurrection of a technique written about by Josef Auenbrugger thirty years earlier, in which the quantity of fluid in a patient’s lungs could be determined by rapping on their sternum.\(^3\) This technique, called percussion, and the use of the stethoscope, called mediate auscultation, became the primary diagnostic tools for a physician to identify tuberculosis before its outward symptoms manifested, and remained crucial to the field for over a century.
Although tuberculosis was pervasive and incurable except by luck and happy accident, it nevertheless came to symbolize a certain brand of sickly beauty that became emblematic of the Romantic Era’s art and literature. Protagonists in the Victorian novel and in the plethora of operas being composed at the time frequently suffered from tuberculosis – notable examples include *La Bohème* and *La Traviata*. Poets idolized the short and painful lives of tuberculosis patients, incorporating the concept into the imagery and ideals expressed in their poetry. The pale and languid consumptive, with a feverish pallor and emaciated limbs, appeared frequently in Romantic paintings as an ideal of feminine beauty.

Lord Byron once joked, “I should like to die of consumption… because the ladies would all say, ‘Look at that poor Byron – how interesting he looks in dying.’” Alexandre Dumas commented that it was “the fashion to suffer from the lungs; everybody was consumptive, poets especially; it was good form to spit blood after any emotion that was at all sensational, and to die before reaching the age of thirty.”

As these assertions suggest, there was also an association between tuberculosis and artistic genius, a view that was compounded by the suffering of an extraordinary number of poets, writers and other luminaries of the time. Since tuberculosis was viewed as a hereditary defect, it followed that it could be especially prevalent in those gifted with extraordinary mental faculties. The early death of John Keats at the age of 26 was heavily romanticized by PB Shelley and his contemporaries; the suffering of the self and of others was synonymous with mental exertion and creation. The Brônte family is the quintessential example, but tuberculosis also afflicted Frédéric Chopin, Henry David Thoreau, Ralph Waldo Emerson, and many others.
THE BIRTH OF BACTERIOLOGY

In the last two decades of the nineteenth century, a number of extraordinary discoveries revolutionized the field of medicine. Primary among them was the bacteriological work performed by Robert Koch, a German scientist. Through the development of new microscopy and inoculation techniques, he was able to isolate the bacteria responsible for a number of major diseases, including tuberculosis.\(^8\) In 1880, Koch published and presented his work demonstrating the existence of \(M.\) \(\text{tuberculosis}\), along with a list of four postulates regarding bacterial science that still underpin the field today.\(^9\) With Koch’s definitive evidence of his discovery, different manifestations of tuberculosis could be traced back to the same bacteria, and germ theory entered the medical lexicon as a substantive concept.

Despite this stunning achievement, for a few decades the discovery had surprisingly little impact on the practical applications of identifying and treating tuberculosis. The omnipresence of the disease presented a great difficulty – although tuberculosis had been proven to be quite infectious, a strategy of isolation and quarantine was functionally useless when most of the population already carried the bacteria in some form.\(^3\) Unfortunately, Koch’s stellar reputation was later tarnished when he announced his development of tuberculin, a non-infectious substance which he had isolated and believed to be an effective vaccine for increasing resistance to the destructive form of the disease. While tuberculin did have an application under a very narrow set of circumstances, in most of the population it caused a severe allergic reaction in the body, and in some cases actually worsened a patient’s tuberculosis.\(^3\) Despite this failure, a modified form of tuberculin is still used today as a simple test for the presence of latent tuberculosis. In a method developed by Clemens von Pirquet in 1907, tuberculin is injected
intravenously under the skin; the area is monitored for redness and swelling, which together
indicate the presence of the tuberculosis bacteria.\textsuperscript{10}

In a curious blend of the previous century’s treatment protocols and a new understanding
of tuberculosis as an infectious disease, a movement swept across America and parts of Europe
in the early 1900’s to open tuberculosis sanatoria. A tuberculosis sanatorium was designed as a
restful haven for patients, simultaneously isolating them from the general population and
allowing them the fresh air and mild climate that many believed were beneficial to the healing
process. They also created an environment where tuberculosis patients had constant access to
knowledgeable and specific medical care. While they did unquestionably ease a patient’s
suffering during the lengthy progression of the disease, they were not a miracle cure and
ultimately did not meaningfully affect the overall fatality rate for tuberculosis.\textsuperscript{3}

As a more precise understanding of tuberculosis filtered into the public consciousness
and the social conscience corrected itself from the industrial age, humanitarians pioneering
improvements in sanitation and working conditions found their goals aligned with those seeking
to understand and prevent tuberculosis epidemics. As a result, private collectives in America
launched one of the first real public health initiatives in history, hoping to prevent the spread of
and increase diagnosis rates for tuberculosis. In 1904, the National Association for the Study and
Prevention of Tuberculosis was founded with a mission statement to “embrace not only the
scientific study of the disease of tuberculosis, but a study of all its relations to man, social and
economic, and all measures for its prevention, eradication and cure.”\textsuperscript{3} This movement was the
first appearance of the idea that the public as a whole had a responsibility to enact conditions that
promoted the safety and well-being of its members, and that a contagious individual had the
moral obligation to seek treatment and attempt to control the spread of their illness.
Though rates of tuberculosis had been in decline over the previous century, they peaked again when the World Wars raised the collective levels of stress and violence. During this time, X-rays became a valuable way to examine body cavities without invasive surgery, allowing identification of tuberculous patients before they started exhibiting symptoms. Combined with auscultation, X-rays became the primary method of diagnosis. In the US in the 1940’s, X-rays were used to examine the lungs of potential soldiers who wished to enlist to fight in World War II, and vans with portable machines were set up on street corners to screen civilians en masse.

PATHOGENESIS

Once the bacteriological origin of tuberculosis had been discovered, rapid progress towards characterizing and treating the disease became possible, uniting scientific disciplines as diverse as biology, chemistry, and the newborn field of bacteriology. Among the many advances in the 1930’s and 1940’s were the discovery of the highly lipoid nature of the cell wall of M. tuberculosis and the first scientifically rigorous attempts at tuberculosis chemotherapy. This progress laid the groundwork for a modern understanding of its pathogenesis and treatment.

Today, we know that tuberculosis infection begins with the introduction of its characteristic bacteria, usually through the inhalation of contaminated sputum. Unlike many bacteria-borne illnesses, however, tuberculosis is often neutralized rapidly by the immune system. Macrophages cordon off pockets of the bacteria, forming thick-walled nodules that prevent the infection from spreading but also keep the tuberculosis bacteria alive within the cellular environment. These nodules range in size from microscopic to visible to the naked eye, and it is the advanced form of these nodules that earlier practitioners of medicine identified as tubercles. A healthy body can frequently exist in this stalemate for years or even decades, until a
trauma or other sudden change in the immune environment precipitates the rupture of these nodules and the release of their contents into the bloodstream.\textsuperscript{13,14} This long period of inactive incubation is one of the reasons tuberculosis has historically been more difficult than other bacterial infections to identify and treat. Once this “sneak attack” floods the body with the necrotic contents of the tubercles, the bacteria have multiplied to a point where containment is impossible.\textsuperscript{14} The patient enters the active stage of tuberculosis and begins manifesting symptoms as the lungs and/or other infected tissues are destroyed. Left untreated, tuberculosis is fatal in 70\% of cases.\textsuperscript{12}

Another notable feature of tuberculosis is the natural defense characteristics its bacteria employ. The \textit{Mycobacterium} genus possesses an exceptionally thick and waxy cell wall, which surrounds the bacterium and protects it from both the body’s immune system and the destructive effects of traditional antibiotics.\textsuperscript{3,12} Its inactive period may also be related to its extremely slow cell growth rate, which is distinctive to MTB.\textsuperscript{15} Its unique structure and infectious process have raised a formidable challenge to scientists who attempt to develop safe and effective drug or vaccine treatments.

**MODERN TREATMENT**

Chemotherapy was precipitated by the discovery of penicillin and the sulfa class of drugs in 1928 and 1932, respectively. Unfortunately, these otherwise versatile drugs were not effective against tuberculosis.\textsuperscript{16} The unique difficulties presented by MTB required several more decades of experimentation before the first effective anti-tubercular drugs were developed. The initial foray into the field yielded thioacetazone, otherwise known as conteben, which was prepared by German scientist Gerhard Domagk in 1945. Around the same time, Jorgen Lehmann first
reported the synthesis of *para*-aminosalicylic acid, or PAS.\textsuperscript{17,18} Though results for the two drugs were initially promising, they eventually proved to be bacteriostatic, preventing the multiplication of MTB without killing it (Fig. 1). In 1944, Allied scientists demonstrated the efficacy of streptomycin against MTB. While the drug caused a few miraculous recoveries, it also proved to have serious side effects. In addition, when it was administered as the only treatment for tuberculosis, bacterial resistance to the drug would quickly develop.\textsuperscript{3}

\begin{figure}
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\includegraphics[width=\textwidth]{chemical_structures.png}
\caption{Chemical structure of thioacetzone and *para*-aminosalycylic acid. Both discovered 1945 and bacteriostatic.}
\end{figure}

The turning point in anti-tubercular drug design occurred in 1952, when multiple laboratories simultaneously synthesized isonicotinylhydrazide, commonly known as isoniazid or INH.\textsuperscript{17} In contrast to previous attempts at chemotherapy, isoniazid was safe and effective, and it became the first reliable cure for tuberculosis. With its widespread use, sanatoria became obsolete and patients were able to return home to receive treatment. Further experimentation demonstrated the optimal treatment of the time was “triple therapy,” a combination of INH, PAS and streptomycin (Fig. 2). This remained the first-line treatment for over a decade.\textsuperscript{17}
In 1957, a laboratory in Italy isolated the rifamycin class of drugs from cultures of *Streptomyces mediterranei*; further optimization of these drugs provided Rifampin. Along with the 1961 discovery of ethambutol, Rifampin became an excellent replacement for PAS, and the new standard became Rifampin, isoniazid and ethambutol (Fig. 2). This combination shortened treatment to six months and also proved to be effective against strains of the bacteria that had become resistant to isoniazid and streptomycin. Pyrazinamide had been explored previously and abandoned due to side effects but at a lower dose was found to be another good option for combination therapy.

With the advent of combination therapy, the incidence of tuberculosis fell rapidly in developed countries throughout the 1960’s, and research into new drugs to treat the disease was deprioritized as other health crises took precedence. However, in the late 1980’s there was an abrupt resurgence of tuberculosis and a new form of the disease emerged: multidrug-resistant
tuberculosis, abbreviated to MDR-TB.\textsuperscript{17,19} While improved sanitation and aggressive chemotherapy treatment have largely eliminated tuberculosis in the middle and upper classes of industrialized countries, it remains a serious threat to lower socio-economic classes and the immunocompromised population.\textsuperscript{20}

With the rise of HIV/AIDS, tuberculosis has resurfaced as the lethal plague that it once was; tuberculosis is the leading cause of death in patients with AIDS.\textsuperscript{17} In addition, the same conditions of poverty and neglect that once characterized the industrializing cities of America and Europe are now prevalent in other countries embracing a modern level of urban development. Tuberculosis has increased proportionally in response in those cities, the same way it once plagued cities like London, Paris and New York.\textsuperscript{20} In its yearly \textit{Global Tuberculosis Report}, the World Health Organization (WHO) shares a few chilling statistics:

In 2017, TB caused an estimated 1.3 million deaths (range, 1.2–1.4 million) among HIV-negative people and there were an additional 300,000 deaths from TB (range, 266,000–335,000) among HIV-positive people…. Globally, the best estimate is that 10.0 million people (range, 9.0–11.1 million) developed TB disease in 2017: 5.8 million men, 3.2 million women and 1.0 million children.\textsuperscript{20}

As the focus of wealthier countries has shifted to other illnesses, the development of anti-tuberculosis drugs has slowed to a trickle. The first-line combination therapy currently prescribed by the WHO and the Center for Disease Control and Prevention (CDC) remains the comparatively small number of drugs synthesized or isolated in the 1950’s and 1960’s.\textsuperscript{20,21} This treatment is not without substantial flaws. Tuberculosis’ ability to persist for long periods of time inside the body requires a correspondingly lengthy treatment regimen; while a typical course of
antibiotics for another bacterial infection ranges typically ranges between ten days and four weeks, the prescribed treatment time for tuberculosis is six months at a minimum. This leads to poor patient compliance in finishing the treatment, especially because bacteria can persist long after the symptoms have subsided.\textsuperscript{22} Additionally, the current drugs prescribed can cause adverse side effects, including liver toxicity.\textsuperscript{23} Lastly and most compellingly, the emergence of multidrug-resistant tuberculosis, extensively drug-resistant (XDR-TB), and total drug-resistant tuberculosis (TDR-TB) threatens to completely undermine current treatment regimens and enable tuberculosis to reach a level of prevalence not experienced for the past century.\textsuperscript{20}
**Background**

It is against this alarming backdrop that the pressure increases to develop new forms of drugs to combat tuberculosis. The advent of computational modeling combined with sensitive X-ray crystallography techniques has allowed the characterization of the processes through which bacteria develop resistance to specific types of anti-tubercular drugs. Guided by this exploration process, we can develop new chemotherapy agents specifically designed to counter those resistance mechanisms, with increased activity against TB, and able to endure the attempts of bacterial evolution to deactivate them. As strains of TB resistant to first-line treatment become more prevalent, a few studies suggest the intriguing possibilities offered by applying modern methods to a very early class of drugs: the sulfa drugs.

**SULFA DRUGS**

Sulfa drugs were some of the very first antibiotic agents ever discovered and remain a cornerstone of medical science. Also called sulfonamides, they are characterized by this eponymous group, which consists of a sulfanyl singly bonded to an amine. The sulfonamide is positioned adjacent to a phenyl ring, as seen in sulfanilamide, one of the simplest sulfa drugs (Fig. 3).

![Figure 3](image-url)  
*Figure 3. Left: general structure of a sulfa drug, with sulfonamide shown in blue. Right: structure of sulfanilamide, first sulfa drug discovered. N¹ & N⁴ labeled.*
Sulfanilamide was the progenitor of the rest of the class, discovered by German scientist Gerhard Domagk in 1932 as part of a foray into the antibacterial properties of chemicals in the dye industry. Domagk won the Nobel Prize in 1939 for this discovery, an achievement which the Nazi Party recognized by arresting and jailing him. The red dye he had singled out was called “Prontosil,” and it was soon marketed by Bayer as the first broadly applicable antibacterial agent.\(^{24}\) Testing afterward in several other labs revealed Prontosil's activity within the body resulted from metabolism into the active component sulfanilamide, a molecule that was inexpensive, easily synthesized, and out of patent.\(^{25}\) A “sulfa craze” swept across the globe, lasting just over a decade, and sulfa derivatives were generated by the hundreds. Between 1935 and 1945, organic chemists synthesized over 5,000 variations on the original sulfa structure, though “only about 20 were shown to have any medical value” (Fig. 4).\(^{26}\) The United States was by no means immune: in 1937 alone, the US exported 350,000 pounds of sulfa drugs!\(^{27}\)

\begin{center}
\begin{tabular}{c}
\includegraphics[width=\textwidth]{figures/figure_4.png}
\end{tabular}
\end{center}

\textit{Figure 4. The chemical structures of a few synthesized sulfa drugs with medicinal value.}\(^{26}\)

The fervor cooled slightly as newer, more powerful antibacterial agents were discovered and popularized, among them the “wonder drug” penicillin. Sulfa drugs possessed a number of unfortunate properties, most notably their tendency to crystallize in the kidneys and urinary tract, causing excruciating pain.\(^{28}\) Moreover, sulfa drugs in monotherapy were not effective against tuberculosis, the scourge of the previous century and still a major health concern. Sulfa drugs eventually came to be classified as bacteriostatic: while they inhibited further growth of harmful
bacteria, they were unable to destroy bacteria extant in the body. Drugs were developed to treat specific illnesses, and a broadly applicable antibacterial agent lost some appeal. Additionally, resistance to the sulfa drugs materialized, making treatment more difficult. The pharmaceutical field moved on, and by the end of the 1950's the drugs that originated modern chemotherapy had been largely supplanted.\(^\text{29}\)

It was not until the twenty-first century that a handful of observations rekindled interest in the sulfa drugs. When combined with trimethoprim, they showed unexpected efficacy against tuberculosis, offering a new avenue to combat strains that had developed resistance to more traditional chemotherapy. A pioneering paper in this area was published in 2009 by Pierre Forgacs and colleagues, in *Antimicrobial Agents and Chemotherapy*. The paper details the case study of an immunocompromised patient admitted to the Lahey Clinic Medical Center, who was initially believed to be infected with nocardiosis but was later diagnosed with tuberculosis. Following procedure when doctors suspected the former infection, he received an intravenous combination of trimethoprim (TMP) and sulfamethoxazole (SMX) for two and a half weeks. In that time, before tuberculosis was detected and the patient was switched to traditional TB chemotherapy, the patient defervesced and showed “soft” symptoms of improvement; the paper outlines how this “was unlikely to be due to factors other than a response of his tuberculosis infection to [TMP-SMX] therapy.”\(^\text{29}\)

This was a very surprising result – since the 1950’s tuberculosis had been thought to be unaffected by TMP-SMX, and indeed by sulfa drugs as a class. Forgacs and colleagues collected the strain of MTB that had been isolated from the patient, as well as 44 other isolates. Using guidelines modified from the Clinical & Laboratory Standards guidelines for “related organisms,” they tested the isolates’ susceptibility to TMP-SMX. Results showed that “[f]or 43
of 44 isolates of *M. tuberculosis*, including 4 of 4 multidrug-resistant (MDR) isolates, there was at least an 80% inhibition of growth at a MIC of $\leq 1/19 \, \text{µg/mL}$ of TMP-SMX… For 36 of 44 isolates (82%), there was at least a 99% inhibition by $\leq 2/38 \, \text{µg/mL}$ of TMP/SMX.”

Since the scientific consensus on TMP-SMX combination therapy seemed to be incorrect, Forgacs and colleagues trawled through past literature to ascertain how the idea had come to be accepted as medical gospel. They found that very early literature on the sulfa drugs demonstrated they were weakly active against tuberculosis, in the sense that they worked to a small degree and no better antibacterial agents had yet been discovered. The authors narrowed down the source of the TMP-SMX confusion to one study on one isolate of MTB, performed by Wellcome Laboratories in 1968. The pivotal aspect is that this 1968 experiment was performed on an egg yolk medium, which today is not recommended for TMP-SMX testing because it may “produce false resistance.” The single test was by no means comprehensive, yet it resulted in the neglect of an entire class of drugs for decades. Forgacs and colleagues close this revolutionary paper by suggesting further research into TMP-SMX as a drug to treat MTB, including strains that have become resistant to first-line tuberculosis treatments.”

In the five years that followed, several other laboratories followed up on this rediscovery. Leslie, Ong, and Sievers wrote eight months later to the same journal that had published Forgacs’ research, confirming the original results and expanding on them with twelve new *in vitro* studies. Leslie and colleagues suspected the sulfa moiety to be the source of the combination therapy’s effect, but Forgacs countered with a 1982 study that demonstrates trimethoprim increases the efficacy of sulfamethoxazole by two to four times in one-third of *M. fortuitum* isolates, a close relative of tuberculosis.”

The text of the 1982 study, however, concludes that *M. fortuitum* is largely resistant to TMP despite being affected by SMX. It also
notes “marked differences in susceptibility among the various species” of the *Mycobacterium* genus. At this point in time, the value of TMP in combination therapy had not been definitively ascertained.

In 2014, Davies Forsman in Sweden continued the research into sulfa combination therapy, leading a team of researchers in a large-scale study of the efficacy of sulfamethoxazole against MDR- and XDR-TB. The new study cited a number of studies done in the intervening years identifying sulfamethoxazole as the active component of TMP-SMX therapy, and reported its efficacy in combination with trimethoprim. This portion of the study found “[n]o significant differences in the MIC distributions between MDR-TB, XDR-TB, or isolates with other resistance patterns…. All isolates had MICs of 38 mg/liter of SMX….” Referring both to their own results and a large number of other studies, Davies Forsman and colleagues conclude that TMP-SMX combination therapy is active against even highly drug-resistant strains of MTB, with the caveat that it is most effective when MTB bacteria are found extracellularly.

In a short correspondence with *Clinical Infectious Diseases* regarding a 2016 review, Didier Raoult concisely summarizes the struggles facing current research into tuberculosis chemotherapy. He mentions several “old” antibiotics, including sulfadiazine, and writes, “In practice, it is necessary to use the patrimony of old antimicrobial compounds, even in fields in which segregation has eventually led to the thinking that only specific drugs could be effective.” His commentary was received with traces of resentment by the original authors of the review, who penned a response arguing that Raoul’s “suggestion that [they] were advocating an abandonment of older antibiotics in order to use newer molecules is not accurate.” They also call for a “comprehensive approach to the development of new regimens,” the value of which
any scientist in or adjacent to the field of medicine can see. In order to make progress against such a dangerous disease, we must make use of the entire array of tools at our disposal.

**DHPS AND FOLATE SYNTHESIS**

A modern usage for drugs from a previous century requires a modern understanding of their mechanism. Sulfa drugs target the process by which bacteria synthesize folate products, which are essential for the “biosynthesis of a diverse range of cellular components.”

**Folate Pathway**

Living cells require folate products in one form or another, and prokaryotic cells are no exception. While mammals obtain folate in their diet and utilize it through an active transport system, plants and microorganisms such as tuberculosis bacteria must synthesize it through their own biochemical pathways. This dichotomy offers an excellent opportunity for targeted drug action, as drugs inhibiting folate synthesis can prevent further bacterial growth without harming the human host.

The step of interest for sulfa drug action is that of para-aminobenzoic acid, or pABA. pABA binds through condensation with 6-hydroxymethyl-7,8-dihydropterinpyrophosphate (DHPP), producing a covalently bonded single molecule of 7,8-dihydropteroate. This reaction occurs when both pABA and DHPP are bound to the catalytic enzyme dihydropteroate synthase, or DHPS. X-ray crystallography provides a closer look into the minutiae of this mechanism.
Crystallographic analysis of the binding pocket of DHPS reveals two “flexible, organized” loops that interact with the key substrates. Three substrates coordinate in this area: DHPP, pABA, and an octahedrally coordinated Mg\(^{2+}\) ion. Loop 1 creates a lid over the active site to form a “restricted entrance” matching pABA’s precise shape, while loop 2 wraps around pABA, stabilizing it in the pocket. The reaction itself follows an S\(_{N}\)1-like mechanism: pyrophosphate is removed from DHPP, a cationic resonance structure forms that is stabilized by the binding pocket as well as delocalization around the pterin ring, and then the amino nitrogen of pABA attacks at the C\(^9\) atom to generate 7,8-dihydropteroate.\(^{36}\)
**Sulfa’s Mimicry**

Since the Woods-Filde Theory of Competitive Inhibition was proposed in 1940, it has been understood that sulfa drugs mimic pABA in the folate pathway. The exact details, however, were not elucidated until much more recently. Using cutting-edge crystallography and computational tools, scientists Stephen White and colleagues have provided detailed pathways and structural illustrations of the individual steps involved in sulfa drugs' mechanism of action.

Sulfa drugs inhibit the folate pathway by binding to dihydropteroate synthase (DHPS) in the place of its natural substrate pABA. The structural explanation for sulfa drugs’ mimicry of pABA in this process is abundantly clear from three-dimensional modeling. The figure below (Fig. 6) uses sulfamethoxazole as an example: the sulfa drug matches the structure of pABA nearly perfectly, with its sulfonamide acting as an isostere to pABA’s carboxylate region and a similar phenyl group interacting with the lipophilic loops nearby.

![Figure 6](image)

**Figure 6.** Left: Crystal structure of sulfamethoxazole bound in DHPS, from Lee et al. 2012. Right: chemical structure of sulfamethoxazole, top, and pABA, bottom.

Unfortunately, resistance to sulfa drugs can develop rapidly, and the flexible nature of pABA binding ensures the endogenous function of the enzyme can still be performed even when sulfa drugs are rejected. Sulfamethoxazole and its sibling sulfa drugs bind in a hinged
conformation, with the methoxazole ring (or other N₁ substituent) sticking out of the pocket at the solvent front (Fig. 6). Mutations in DHPS that result in resistance to sulfa drugs occur near this protrusion, “relegating sulfonamide-based therapies to second- or third-line options.”

Because of this discrepancy between sulfa and pABA structure, any further extensions out of the binding pocket should be avoided.

On the other hand, the DHPP (“pterin”) portion of the binding pocket is deeper in the DHPS protein, and structurally specific such that any mutations would also critically affect the function of the enzyme. These observations point to structural modification on the other side of the sulfa drug molecule, near the arylamine and away from the varying ring structure – a thiazole ring in the case of sulfathiazole, for example. Although the bulk of this crystallographic work was performed using DHPS enzymes from *Bacillus anthracis* and *Yersinia pestis*, it can nonetheless guide hypotheses for structural modifications to fight MTB. Further work in the White lab has already demonstrated this principle with the use of pterin-sulfa conjugates, discussed in more detail in the next section.

*Drug Design*

Several small molecules have been designed to take advantage of the pterin binding site. Another option is “dual-site binders” that interact with both the pterin and pABA binding site while not extending too far out of the binding pocket. The concept of dual-site binders recurs throughout the literature; multiple authors muse on the value of “designing a molecule that is able to bridge between the two substrate sites.” However, much of the dual-site binder work occurred prior to the 2012 paper identifying the S_N₁-like mechanism of DHPS catalysis. As such, the molecular design relied on incorrect assumptions and the drugs were inactive.
Stephen White and colleagues not only elucidated the DHPS catalysis mechanism but were also among the first to synthesize drugs that took advantage of this new information. In 2016, the laboratory published a paper regarding pterin-sulfa conjugates as DHPS inhibitors. Their novel synthetic methods yielded compounds that can engage both sulfa drugs' original binding site as a pABA mimic and the pterin binding site deeper in the cleft of DHPS. Assays demonstrate that these compounds have genuine antibacterial activity, inhibiting DHPS and acting as antagonists to the folate pathway, in addition to their ability to interfere by conjugating with DHPP to form “dead-end” products. While these compounds are around ten times less active than their parent sulfa drugs, they are excellent candidates for lead compounds. Their conjugation of the reactive arylamine may also offer a decrease in sulfonamide anaphylaxis, always a concern in sulfa drug treatment.

N-ACETYLTRANSFERASES

Overview

The primary metabolic pathway through which sulfa drugs are deactivated inside the body involves arylamine N-acetyltransferases, or NATs. NATs are responsible for catalyzing the transfer of an acetyl group from acetyl-Coenzyme A (AcCoA) onto the terminal amine of an arylamine substrate. They are found in many species, including humans and M. tuberculosis. Studies focusing on exclusively on a single species can resolve mysteries regarding the immune system and metabolism. In contrast, examining the polymorphisms – structural variants within different forms of the same enzyme – as they occur across multiple species can provide insight into the structure and function of the NAT enzyme class as a whole.
**Human NATs and TB-NATs**

NATs in MTB participate in the synthesis of the mycobacterial cell wall, which is exceptionally thick and tough and allows MTB to survive attack by the immune system. It may be possible to exploit NAT mutations in MTB that make the cell wall permeable to antibiotics that would usually have no effect. This could provide a potential method to treat drug-resistant tuberculosis in parts of the world where this mutation is found. Unfortunatley, experiments regarding metabolism of drugs by TB-NATs are difficult; TB-NATs are notoriously intractable and unstable, and strict biological controls are required to study live tuberculosis bacteria. Several analogues of MTB are therefore employed, including *M. marinum* and the attenuated form of *M. bovis* known as Bacille Calmette-Guérin (BCG). NAT enzymes in MTB are identical to the enzymes in BCG, indicating efficacy studies against the latter bacteria should apply to MTB, at least as far as metabolism is concerned.

Humans possess two isoenzymes of NATs, designated NAT1 and NAT2. The two human NAT enzymes (H-NATs) are 87% similar but act on different substrates. Human NAT1 has a wide selectivity profile which includes pABA and *para*-aminosalicylic acid; its endogenous purpose seems to be acetylating the folate derivative p-aminobenzoyleglutamate. NAT2, found primarily at “traditional sites of drug metabolism” such as in the liver and gut, has a more selective profile that includes isoniazid and the sulfa drugs.

In drug design, we consider the action of both TB-NATs and H-NATs. Much of this information relies on NAT studies done on a variety of species, including “mice, men and microorganisms.”
Structural Elucidation through Polymorphisms

The mechanism of action of NATs is a central question that must be answered to target these enzymes with small molecules. Crystal structure analysis of NAT enzymes across multiple species suggests action at all three domains of the enzyme, engaging in a “universal reaction mechanism” involving a catalytic triad that does not vary by species: a cysteine residue, a histidine residue, and an aspartic acid residue.\(^{39}\) The C-terminal region is partially responsible for the high specificity of these enzymes, as it is “involved in interactions with substrates in the active site cleft.”\(^{40}\)

Kinetic studies on a variety of both prokaryotic and eukaryotic NATs have demonstrated that substrate specificity differs between species, implying that NATs may have different roles in different organisms.\(^{42}\) Although this diversity is intriguing, all studies to date have shown an identical mechanism for acetyl transfer. Early studies with bird NATs helped pave the way towards a complete mechanism; the work was culminated by Blanchard and colleagues, who “provide[d] a detailed understanding of the kinetic and chemical mechanism” of TB-NAT and illustrated their results with the figure below (Fig. 7).\(^{40,42}\) It is easy to see the catalytic triad interacting with AcCoA, stabilizing the acetyl group before transferring it to the amine of the arylamine substrate.
After years of hypothesizing the differences between Human NAT1 and NAT2 based on less complicated species, a crystal structure was solved for both variants in high resolution, including NAT2 complexed with Coenzyme A. The human models are similar to their prokaryotic analogues in that the protein is divided into the traditional three domains, but include a 17-residue insertion that affects the structure of the C-terminus. As in previous studies, the catalytic triad that drives NAT activity is clearly composed of Cysteine-68, Histidine-107, and Aspartate-122, maintained in an identical position in each crystal structure by “a complex network of non-bonding, polar interactions.”

The endogenous role of Human NAT1 is to metabolize folate derivatives by transferring the acetyl group from AcCoA. However, an examination of the interaction of folate itself with Human NAT1 (and its mouse analogue) reveals an additional folate-dependent mechanism for hydrolysis of the AcCoA molecule without transferring the acetyl to an arylamine substrate. This reaction is proposed to also be endogenous. Structural analysis suggests a mechanism of action where folate binds to the catalytic active site and affects the conformation of the enzyme,
allowing water to enter the normally lipophilic binding pocket. This hydrolysis reaction in the presence of folate was not observed in Human NAT2 (nor its mouse analogue).\textsuperscript{44}

In examining substrate specificity, crystallography indicates that NAT2’s phenylalanine at position 93 creates a hydrophobic “lid” that critically affects its selectivity by altering the “interactions with the pocket surface at this position.” This Phe-93 ensures that sulfa drugs bind almost exclusively to NAT2, by conforming into a hinged structure around the sulfonyl group (Fig. 8). The authors of the paper also identified “key features” that can structurally affect some of the polymorphisms of NAT1 and NAT2 and cause “defective function,” which can lead to differences in speed and efficacy of drug metabolism.\textsuperscript{43}

![Figure 8. Sulfamethazine in NAT2 binding pocket. Shown in stick-and-ball representation (left) and schematic representation (right). From Plotnikov et al. 2007.\textsuperscript{43}]

\textit{Metabolism & Drug Design}

While NAT1 has a clear endogenous purpose related to the folate pathway, NAT2 is thus far only understood as a “drug metabolizing enzyme.” Some of the earliest NAT research demonstrated the existence of two distinct phenotypes of NAT2, a “fast” and a “slow”
acetylation type. The slow acetylation phenotype decreases metabolism of isoniazid and other arylamines, including sulfa drugs. While this increases exposure, it also increases risk of adverse reaction, hepatotoxicity and certain types of cancer. These concerns suggest treatment regimens guided by genetic testing of NAT2 to improve efficacy and control toxicity risk.\textsuperscript{40,45}

Though the existence of this division in the population has been known for decades, modern kinetic analysis was required to shed some light on isoniazid and its interaction with NAT enzymes. For example, it has long been hypothesized that MTB's ability to develop resistance to isoniazid is related to over-expression of NATs. This theory was recently determined to be unlikely; while substituted arylamines in general are excellent substrates for TB-NATs, isoniazid is an “extremely poor” substrate relative to others in the class.\textsuperscript{42}

As research into the metabolism and biological pathways of anti-tuberculosis drugs continues, we can use the last decade's elucidation of NAT polymorphisms to conceive of personalized chemotherapy adapted to suit a person's specific genetic makeup. In a representative study, Azuma and co-authors propose a “pharmacogenetically stratified” approach to tuberculosis treatment to reduce the frequency of INH-induced liver damage. Currently, there is a single recommended dose of isoniazid for all patients, which is “too high for slow acetylators and insufficient for rapid acetylators.”\textsuperscript{23} By genotyping patients before they begin treatment, physicians can determine if they fall into the slow acetylation or rapid acetylation category. In the trial of 172 Japanese patients, some patients were assigned to standard treatment without genotyping, while another group was sorted genetically according to their markers for speed of acetylation. All patients received the internationally recommended treatment for adult pulmonary tuberculosis, aside from the variable dosage of isoniazid: a low dosage for slow acetylators, a standard dosage for the control group and intermediate acetylators, and a high dosage for rapid
acetylators. The genetically stratified group experienced “much lower incidences of unfavorable events” such as liver damage or treatment failure, while maintaining – and in some cases improving – therapeutic efficacy across all groups. The authors posit “personalized medicine” of this kind to be “obviously safer and more efficacious,” and “expect it to become the standard of care.” While there is some delay required in order to genotype patients before beginning treatment, this is compelling evidence that attention should be paid to the role of NAT acetylation when considering drug dosage and metabolism.

The Sim research group, which has conducted much of the pivotal research on NAT enzymes, recently applied these principles in their examination of piperidinol derivatives. This class of compounds has been earmarked for further research due to its potential antimycobacterial properties and inhibition of NATs. Once synthesized or acquired, the derivatives were tested for their antimycobacterial activity against *Mycobacterium marinum*. These compounds inhibit NAT by a “prodrug-like mechanism” where the piperidinol is activated to a phenyl vinyl ketone (PVK); the PVK fragment then forms a covalently-bonded adduct with NAT’s active-site cysteine. Obviously, a complex understanding of NAT mechanism was necessary to explore this class of compounds. It is important to note that potency measures were carried out with MM-NAT, in place of TB-NAT. While results appear “comparable” for most compounds, there is some discrepancy. Crystal structures and computer modelling suggest the piperidinols bond to an area in the binding pocket that is significantly “more charged and polar” in MM-NAT. Lastly, a few more potential NAT inhibitors were suggested through computer modelling, based on the 3D shape of the selected compounds. While operating in a slightly different area of research to the modification of sulfa drugs, this paper nevertheless presents a
cogent approach to modification and testing of derivatives using modern laboratory and computational techniques.

**PREVIOUS WORK IN THIS LAB**

The Hearn lab has been investigating the organic chemistry of antitubercular drug design for over two decades. Our ongoing partnership with Dr. Michael Cynamon began in 2003 with the design, preparation, and biological testing of acylated isoniazid (INH) derivatives. This work contributed to emerging literature on the interaction of NAT with INH by probing with acylation the primary amine (N$^2$) that makes up the hydrazine portion of the molecule. Six different compounds were prepared in our lab, including the acetylated form of INH that is produced after its deactivation by NAT. As expected, the N$^2$-acetyl derivative was significantly less active than its progenitor against tuberculosis, but some of the other acyl derivatives showed a higher activity than expected. Among them was the N$^2$-COCF$_3$ derivative, which was substantially more lipophilic than INH and demonstrated an MIC within an order of magnitude of INH's value.$^{46}$

![Chemical structure](image)

**Figure 9.** N$^2$-acyl derivatives prepared and tested by Hearn & Cynamon, 2003.$^{46}$

Dr. Cynamon also tested the N$^2$-propionyl derivative *in vivo*, comparing it to an identical dose of isoniazid. The N$^2$-propionyl derivative provided “significant levels of protection,” an unexpected response due to both its activity *in vitro* and its similarity to the N$^2$-acetyl metabolite
compound. This result suggests the value in the “study of such close structural congeners of metabolites.”

Our lab has focused on the modification of antitubercular drugs for many years, in an effort to elaborate on their properties and discover potential new antitubercular agents. Our research into the modification of sulfa drugs is the latest piece in the larger puzzle of the fight against tuberculosis, especially strains resistant to traditional chemotherapy.

ROADMAP

We can craft a forward direction for antitubercular drug design that is shaped by this abundance of information about sulfa drugs, their mechanism of action, and their metabolism by enzymes. Any novel drug would ideally accomplish several improvements over the original sulfa compounds: (1) increased efficacy, i.e. activity against MTB; (2) increased bioavailability, including resistance to the de-activating effects of NATs; and (3) reduced frequency and severity of undesirable side effects.

The interaction of hydrophobic loop 2 with pABA and therefore with its sulfa drug mimics implies that increasing lipophilicity would increase the strength of this interaction and perhaps correspondingly boost activity against tuberculosis. Lipophilicity would also improve permeability through cell membranes and overall pharmacokinetic properties, a worthy consideration in drug design. Based on the shape of the binding pocket, modifications should be made at the nitrogen of the arylamine (N4). As White and colleagues propose, modifications that extend into the DHPP pocket could prevent resistance from developing as easily; they note that while the pABA site is tolerant of mutations, the DHPP pocket is “highly conserved and
unlikely to tolerate mutations that might decrease binding affinity.\textsuperscript{38} Such modifications at N\textsuperscript{4} maintain the possibility of decreasing allergic reaction to sulfa drug treatment.

Blocking N\textsuperscript{4} with a substituent such as an acyl group should also prevent de-activation by NATs. We know such compounds are stable because they are similar to the metabolized form of the drug; our lab’s work with INH derivatives suggests we may be able to achieve a surprising level of increased activity. Also note that the hydrazine moiety is implicated in INH’s mechanism of action – it must be removed to form the active structure – which may explain the decrease in activity when it is acylated.\textsuperscript{46} While sulfa drugs’ N\textsuperscript{4} atom is the nexus for their conjugation to DHPP, sulfa drugs are not a pro-drug like INH and therefore may experience a different effect on their activity. When this possibility to avoid NATs is taken together with our knowledge of the mechanism of DHPS, acylation seems like an excellent starting place to probe the reaction of sulfa drugs with electrophiles.
Experimental

MATERIALS AND METHODS

**General methods.** Elemental analyses were carried out by Galbraith Laboratories, Knoxville, Tennessee, or by Robertson Microlit Laboratories, Ledgewood, New Jersey. All new compounds gave satisfactory elemental analyses for carbon, hydrogen and nitrogen. In a number of cases, it was also useful to obtain satisfactory elemental analyses for known compounds. Melting points (mp, °C) were taken in open capillary tubes using a Mel-Temp apparatus (Laboratory Devices, Cambridge, Massachusetts), and are uncorrected. Due to recently implemented institutional safety requirements on the use of mercury, some melting points were taken using alcohol thermometers and thus recorded only as high as 260 °C. From the earliest days of research on their preparation, it has been well-known that sulfa compounds display polymorphisms and often form solvates of crystallization; these phenomena may influence the apparent physical properties of compounds prepared in different laboratories.\(^{48}\) Infrared (IR) spectra were recorded on a Perkin-Elmer Spectrum One Fourier transform spectrophotometer fitted with a universal attenuated total reflectance sampling accessory, reported in wavenumbers (\(\bar{\nu}, \text{cm}^{-1}\)). Nuclear magnetic resonance (NMR) spectra were taken on a Bruker Avance 500 Fourier transform instrument as dilute solutions in dimethyl sulfoxide-\(d_6\) (DMSO-\(d_6\)) or chloroform-\(d\), recorded at 500 megahertz (\(^1\text{H NMR}\)) or 125 megahertz (\(^{13}\text{C NMR}\)) and are reported in parts per million delta (\(\delta\)) downfield from internal tetramethylsilane (TMS) as reference; common vicinal aromatic coupling constants (\(J\)) were in the range of 7-9 Hz; common vicinal aliphatic coupling constants were in the range of 5-7 Hz. In some proton spectra, only signals in the region 0-10 ppm are reported. Appropriate solvent blanks were recorded to account for water and DMSO. Hard copies of original infrared spectra, magnetic resonance spectra and
elemental analysis reports are maintained by the principal investigator and are available upon request.

Reactants, reagents and solvents were obtained from Sigma-Aldrich Chemical Company (Milwaukee, Wisconsin) and Alfa Aesar Incorporated (Windham, New Hampshire) and were used as received. Reactions were conveniently carried out on 10 mmole scale in a 100 mL round bottom flask fitted for reflux with a temperature-controlled heating mantle, magnetic stirrer and reflux condenser, using approximately 20 mL of solvent as specified in the individual procedures. Unless otherwise noted, ethanol refers to absolute ethanol.

Once satisfactory protocols were obtained for a given reaction, no further attempt was made to optimize yields. Calculated values of the logarithms of the partition coefficients (C log P values) were obtained using the QSAR properties function of ChemDraw, following geometry optimization of structures.

Safety Notes: Gloves and eye protection were worn during the chemical syntheses, and the reactions were carried out in the hood. In general, any scale-up of preparations of compounds with relatively high proportions of nitrogen and oxygen was done with due caution. No specific safety problems were encountered with our methods.

**Biological assessments.** The assessments were performed through the collaboration of Dr. Michael H. Cynamon, Chief, Infectious Diseases, Veterans Affairs Medical Center, Syracuse, New York. For initial screening, Kirby-Bauer disk diffusion testing was used. As a representative example, we give here in brief the protocol for screening against *M. bovis BCG Tice*. The test compounds (20 mg) were dissolved in enough DMSO to prepare solutions that had a concentration of 20 mg/mL. The solutions were then applied to 6-mm filter paper disks such
that the total weight of test compound per disk was 200 micrograms. The disks were laid on
7H10 agar plates having a cell density of *M. bovis* BCG *Tice* of three MacFarland units. *M. bovis* 
BCG *Tice* was obtained from the American Type Culture Collection (ATCC, Manassas,
Virginia). The plates were incubated at 37 °C for seven days and then read using transmitted
light. The antimicrobial activity of the compound was measured by the dimensions of the
circular clear zone surrounding the disk in which no growth occurred, while the remainder of the
plate showed a luxuriant bacterial lawn, as did control plates containing no drug.

For the determination of minimum inhibitory concentrations, the experimental protocols
have been fully documented.\(^5^0\) In brief, *M. tuberculosis* ATCC 35801 (strain Erdman) was
obtained from the ATCC. The reference standard isonicotinic acid hydrazide (INH) was
purchased from Sigma Chemical Company (St. Louis, Missouri). For testing, a given compound
was dissolved in dimethyl sulfoxide and subsequently diluted in distilled water. The positive
control INH was dissolved in distilled water. Stock solutions were filter-sterilized by passage
through a 0.22 µm-pore-size membrane filter and stored at -20 °C until use. The drug solutions
were prepared each morning, before experimentation. With respect to testing against the above
isolate, the minimum inhibitory concentrations (MICs) of all antimicrobial agents were
determined in modified 7H10 broth (7H10 agar formulation with agar and malachite green
omitted; pH 6.6) supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase
(OADC) enrichment (Difco Laboratories, Detroit, Michigan) and 0.05% Tween 80.\(^5^1\) The
activities of the antimicrobial agents were determined by a broth dilution method.\(^5^2\) The
organism was grown in the modified 7H10 broth with 10% OADC enrichment and 0.05% Tween
80 on a rotary shaker at 37 °C for 5 days. The culture suspension was diluted in modified 7H10
broth to yield 100 Klett units/mL (Photoelectric Colorimeter, Manostat Corporation, New York,
New York), or approximately $5 \times 10^7$ cfu/mL, where cfu is the abbreviation for colony-forming units. The size of the inoculum was determined by titration and counting from triplicate 7H10 agar plates (BBL Microbiology Systems, Cockeysville, Maryland) supplemented with 10% OADC enrichment. The plates were incubated at 37 °C in ambient air for 4 weeks before counting of the colonies. The use of *M. tuberculosis Erdman* for determinations of MIC values is regarded as a very rigorous challenge of antitubercular behavior.53
SYNTHETIC PROTOCOLS

PREPARATION OF SULFA DRUG METABOLITES

1  N⁴-Acetylsulfabenzamide

In a procedure representative of the preparation of the following sulfa drug metabolites, glacial acetic acid (5 mL) was placed in a 100 mL round bottom flask fitted with a magnetic stirrer and voltage-regulated heating mantle. The liquid was warmed to 85 °C. Sulfabenzamide (1.38 g, 5.00 mmol) was added to the warm acid with rapid stirring to form a clear colorless solution (1.0 M). Slow dropwise addition of acetic anhydride (0.56 g, 5.50 mmol, 1.1 equiv) in acetic acid (5 mL) over 20 minutes was followed by warming and stirring for another hour. Distilled water (40 mL) was added, and the mixture was cooled to room temperature. Filtration and drying of the abundant white crystalline product led to the title compound, the N⁴-acetyl metabolite, 1.52 g (96%). The analytical sample was readily obtained by recrystallization from ethanol, mp 245 °C.

FT-IR: $\nu_{\text{max}}$ 3312, 3276, 3120, 3058, 1696, 1604, 1589, 1265, 1161 cm⁻¹

$^1$H-NMR (DMSO-$d_6$): $\delta$ 12.43 (brs, 1H), 10.40 (s, 1H), 7.95-7.93 (m, 2H), 7.85-7.87 (m 2H), 7.81-7.80 (m, 2H), 7.65-7.60 (m, 1H), 7.50-7.57 (m, 2H), 2.09 (s, 3H)

$^{13}$C-NMR (ppm, DMSO-$d_6$): $\delta$ 168.52, 164.74, 143.27, 132.60, 132.13, 132.05, 128.53, 127.98, 127.76, 117.77, 23.55

*Anal.* Calcd for C$_{15}$H$_{14}$N$_2$O$_4$S: C, 56.59; H, 4.43; N, 8.80.

Found: C, 56.63; H, 4.49; N, 8.67.
II  \( N^4 \)-Acetylsulfacetamide

![Chemical Structure]

Yield: 1.33 g (86%), mp 248 °C, lit mp 249-251 °C^{54}

FT-IR: \( \nu_{\text{max}} \) 3312, 3284, 3060, 2871, 1696, 1604, 1187, 1158 cm\(^{-1}\)

\(^1\)H-NMR (DMSO-\( d_6 \)): \( \delta \) 11.96 (brs, 1H), 10.38 (brs, 1H), 7.84 (d, 2H), 7.77 (d, 2H), 2.09 (s, 3H), 1.91 (s, 3H)

\(^13\)C-NMR (ppm, DMSO-\( d_6 \)): \( \delta \) 168.55, 168.07, 143.17, 132.11, 128.26, 117.79, 23.53, 22.58.

*Anal.* Calcd for C\(_{10}\)H\(_{12}\)N\(_2\)O\(_4\)S: C, 46.87; H, 4.72; N, 10.93.

Found: C, 47.08; H, 4.84; N, 10.78.
III  N\textsuperscript{4}-Acetylsulfamethazine

Yield: 1.04 g (81%), mp 248-250 °C, lit mp 249-251 °C\textsuperscript{55}

FT-IR: $\nu_{\text{max}}$ 3342, 3020, 2727, 1671, 1591, 1528, 1492, 1299, 1154 cm$^{-1}$

$^1$H-NMR (DMSO-$d_6$): $\delta$ 11.58 (brs, 1H), 10.28 (s, 1H), 7.90 (d, 2H), 7.72 (d, 2H), 6.75 (s, 1H), 2.24 (s, 6H), 2.07 (s, 3H)

$^{13}$C-NMR (ppm, DMSO-$d_6$): $\delta$ 168.36, 166.85, 155.65, 142.32, 133.64, 128.69, 117.23, 112.96, 23.53, 22.30.
IV  N^4-Acetylsulfamerazine

\[
\begin{array}{c}
\text{CH}_3 \\
\text{N} \\
\text{SO} \\
\text{NH} \\
\text{C}_6\text{H}_5 \\
\text{O} \\
\text{CH}_3
\end{array}
\]

Yield: 1.43 g (94%), mp 246-247 °C, lit mp 245-246 °C\(^6\)

FT-IR: \( v_{\text{max}} \) 3536, 3087, 3042, 2719, 1661, 1590, 1565, 1540, 1493, 1398, 1373, 1271, 1241, 1187, 1160 cm\(^{-1}\)

\(^1\)H-NMR (DMSO-\(d_6\)): \( \delta \) 11.60 (brs, 1H), 10.30 (s, 1H), 8.30 (d, \( J = 4 \) Hz, 1H), 7.93 (d, 2H), 7.74 (d, 2H), 6.89 (d, \( J = 4 \) Hz, 1H), 2.30 (s, 3H), 2.07 (s, 3H)

\(^1^3\)C-NMR (ppm, DMSO-\(d_6\)): \( \delta \) 169.47, 168.70, 158.00, 157.04, 143.53, 134.43, 129.53, 118.50, 115.30, 24.58, 23.71
V  N₄-Acetylsulfadiazine

![Chemical Structure]

Yield: 1.37 g (94%), mp 253-254 °C (ethanol), lit mp 253-254 °C

FT-IR: $\nu_{\text{max}}$ 3603, 3309, 3036, 2943, 2730, 1674, 1580, 1537, 1486, 1444, 1405, 1373, 1333, 1324, 1272, 1264, 1159, 1091, 1040 cm⁻¹

$¹$H-NMR (DMSO-$d_6$): $\delta$ 11.68 (brs, 1H), 10.31 (s, 1H), 8.49 (d, $J = 4$ Hz, 2H), 7.92 (d, 2H), 7.75 (d, 2H), 7.03 (m, 1H), 2.06 and 2.07 (overlapping singlets, 3H)

$¹³$C-NMR (ppm, DMSO-$d_6$): $\delta$ 169.50, 158.80, 157.40, 143.65, 134.26, 129.33, 118.67, 116.13, 24.58
VI  \(N^4\)-Acetyl sulfathiazole

\[
\begin{array}{c}
\text{N} \quad \text{S} \\
\text{H} \quad \text{O} \\
\text{CH}_3
\end{array}
\]

Yield: 1.35 g (91%), mp 262-263 °C (ethanol), lit mp 250 °C\textsuperscript{58}

FT-IR: \(\nu_{\text{max}}\) 3288, 3246, 3153, 3108, 3064, 2979, 2906, 1670, 1604, 1590, 1518, 1491, 1407, 1396, 1372, 1321, 1300, 1264, 1173, 1144, 1117, 1088 cm\textsuperscript{-1}. This spectrum was a good match with that of an authentic sample, as reported.\textsuperscript{59}

\(^1\)H-NMR (DMSO-\textit{d}_6): \(\delta\) 12.66 (brs, 1H), 10.25 (s, 1H), 7.72 (aromatic pseudo-quartet, two overlapping doublets, 4H), 7.24 (d, 1H), 6.81 (d, 1H), 2.07 (s, 3H)

\(^{13}\)C-NMR (ppm, DMSO-\textit{d}_6): \(\delta\) 169.33, 142.91, 136.59, 127.39, 124.81, 118.88, 108.51, 24.56
VII  \(N^4\)-Acetylsulfanilamide

\[
\begin{array}{c}
\text{H}_2\text{N} \quad \text{S} \\
\text{O} \\
\text{O} \\
\text{N} \\
\text{H} \\
\text{CH}_3
\end{array}
\]

Yield: 0.62 g (59%), mp 214 °C, lit mp 211-212 °C\(^60\), lit mp 215-216 °C\(^61\)

FT-IR: \(\nu_{\text{max}} 3342, 3296, 3248, 1661, 1591, 1520, 1399, 1370, 1289, 1264, 1153, 1096, 1074, 908\ \text{cm}^{-1}\). This spectrum was a good match with that of an authentic sample, as reported.\(^62\)

\(^1\)H-NMR (DMSO-\(d_6\)): \(\delta 10.26\) (s, 1H), 7.76 (aromatic pseudo-quartet, two overlapping doublets, 4H), 7.23 (s, 2H), 2.08 (s, 3H)

\(^13\)C-NMR (ppm, DMSO-\(d_6\)): \(\delta 169.38, 142.66, 138.53, 127.15, 118.91, 24.58\)
PREPARATION OF N⁴-ACYLATED SULFA DRUGS

These materials constitute the family of N⁴-acylated sulfa compounds that are not NAT metabolites. Thus the N⁴-acyl group is other than acetyl.

VIII  N⁴-Valerylsulfamethazine

In a procedure representative of the preparation of the following N⁴-acylated sulfa compounds (VIII-XIV), valeric acid (10 mL) was placed in a 100 mL round bottom flask fitted with a magnetic stirrer and voltage-regulated heating mantle. The liquid was warmed to 80 °C and rapid stirring was begun. To the warm stirred liquid was added sulfamethazine (1.39 g, 5.00 mmol). The sulfamethazine did not all dissolve, but it did form a tractable slurry. Valeric anhydride (1.02 g, 5.50 mmol, 1.1 equiv) was added dropwise at a rapid rate. Within less than 2 minutes after the completion of the addition of the anhydride, all of the white slurry had dissolved, and there was a homogeneous yellow solution. During the addition of the anhydride, the temperature had risen; it was now maintained at 105 °C over the course of an hour. Heating was stopped, and the reaction mixture was cooled to near room temperature using a cool water bath. The mixture was poured onto 50 g of chipped ice in a large beaker and allowed to stand overnight. The resulting white solid was filtered off, and a second crop was obtained from the mother liquor, 1.41 g (78%). The analytical sample was obtained by recrystallization from ethanol, mp 171-172 °C.
FT-IR: $\nu_{\text{max}}$ 3278, 3237, 3178, 3120, 3098, 3066, 2955, 2924, 1658, 1624, 1586, 1540, 1399, 1271, 1141, 1119, 1076 cm$^{-1}$

$^1$H-NMR (DMSO-$d_6$): $\delta$ 11.57 (brs, 1H), 10.22 (s, 1H), 7.94-7.92 (d, 2H), 7.75-7.73 (d, 2H), 6.72 (s, 1H), 2.32 (t, 2H), 2.24 (s, 6H), 1.55 (quint, 2H), 1.30 (sextet, 2H), 0.87 (t, 3H).

$^{13}$C-NMR (ppm, DMSO-$d_6$): $\delta$ 172.42, 156.69, 143.41, 134.61, 129.73, 118.36, 114.02, 36.63, 27.52, 23.33, 22.23, 14.14

Anal. Calcd for C$_{17}$H$_{22}$N$_4$O$_3$S: C, 56.33; H, 6.12; N, 15.46.

Found: C, 56.53; H, 6.26; N, 15.20.
IX \(\text{N}^4\)-Valeroylsulfamerazine

![Chemical structure of \(\text{N}^4\)-Valeroylsulfamerazine]

Yield: 2.99 g (86%, 10.0 mmol scale), mp 191-193°C, lit mp 189-190°C\textsuperscript{63}

FT-IR: \(\nu_{\text{max}}\) 3325, 3200, 3020, 2958, 2949, 2936, 2866, 1672, 1586, 1560, 1531, 1475, 1436, 1397, 1334, 1313, 1247, 1189, 1156, 1092, 1036, 960 cm\textsuperscript{-1}

\(^1\)H-NMR (DMSO-\(d_6\)): \(\delta\) 11.59 (brs, 1H), 10.25 (s, 1H), 8.32 (d, 1H), 7.92 (d, 2H), 7.75 (d, 2H), 6.90 (d, 1H), 2.35-2.31 (m consisting of triplet and singlet, 5H), 1.55 (quintet, 2H), 1.32 (sextet, 2H), 0.88 (t, 3H)

\(^{13}\)C-NMR (ppm, DMSO-\(d_6\)):

\(\delta\) 172.45, 168.69, 158.0, 157.03, 143.56, 134.36, 129.51, 118.55, 115.31, 36.63, 27.52, 23.71, 22.22, 14.15

\textit{Anal.} Calcd for C\textsubscript{16}H\textsubscript{20}N\textsubscript{4}O\textsubscript{3}S: C, 55.16; H, 5.79; N, 16.08.

Found: C, 54.87; H, 5.83; N, 15.90.
N\textsuperscript{4}-Valeroylsulfadiazine

\[
\begin{array}{c}
\text{\includegraphics[width=0.8\textwidth]{structure.png}}
\end{array}
\]

Yield: 3.17 g (95%, 10.0 mmol scale), mp 218-219 °C, lit mp 222-223 °C\textsuperscript{63}

FT-IR: \(v_{\text{max}}\) 3376, 3083, 3031, 2949, 2926, 2867, 2855, 2807, 2731, 1685, 1580, 1484, 1441, 1401, 1333, 1304, 1266, 1251, 1187, 1164, 1092, 937 cm\(^{-1}\)

\(^{1}\)H-NMR (DMSO-\(d_6\)): \(\delta\) 11.67 (brs, 1H), 10.26 (s, 1H), 8.50 (d, 2H), 7.92 (d, 2H), 7.77 (d, 2H), 7.04 (t, 1H), 2.34 (t, 2H), 1.57 (quint, 2H), 1.32 (sextet, 2H), 0.88 (t, 3H).

\(^{13}\)C-NMR (ppm, DMSO-\(d_6\)): \(\delta\) 171.43, 157.75, 156.34, 142.63, 133.14, 128.27, 117.66, 115.19, 35.59, 26.47, 21.16, 13.10

\textit{Anal.} Calcd for C\textsubscript{15}H\textsubscript{18}N\textsubscript{4}O\textsubscript{3}S \cdot 0.25 H\textsubscript{2}O: C, 53.16; H, 5.50; N, 16.53.

Found: C, 53.45; H, 5.48; N, 16.72.
XI  N\textsuperscript{4}-Valeroylsulfathiazole

\[
\text{CH}_2\text{CH}_2\text{CH}_3
\]

Yield: 3.27 g (96%, 10.0 mmol scale), mp 220-221 °C, lit mp 212-213°C\textsuperscript{63}

FT-IR: \(v_{\text{max}}\) 3346, 3312, 3146, 3129, 3109, 3096, 3020, 2969, 2860, 2807, 1667, 1519, 1416, 1397, 1335, 1308, 1281, 1250, 1141, 1115, 1083, 938 cm\(^{-1}\)

\(^1\)H-NMR (DMSO-\(d_6\)): \(\delta\) 12.3 (very brs), 10.21 (s, 1H), 7.75 (apparent s, 4H), 7.25 (d, 2H), 6.82 (d, 2H), 2.35 (t, 2H), 1.57 (quint, 2H), 1.33 (sextet, 2H), 0.89 (t, 3H).

\(^{13}\)C-NMR (ppm, DMSO-\(d_6\)): \(\delta\) 172.34, 169.15, 142.94, 136.50, 127.38, 124.83, 118.95, 108.52, 36.63, 27.55, 22.23, 14.15

\textit{Anal.} Calcd for C\textsubscript{14}H\textsubscript{17}N\textsubscript{3}O\textsubscript{3}S\textsubscript{2}: C, 49.54; H, 5.05; N, 12.38.

Found: C, 49.32; H, 5.04; N, 12.31.
**XII  N⁴-Valeroylsulfacetamide**

![Chemical Structure](image)

**Yield:** 0.78 g (52%), mp 216 °C

**FT-IR:** $\nu_{\text{max}}$ 3310, 3276, 3188, 3083, 2954, 2872, 2807, 1714 (sh), 1676, 1590, 1529, 1467, 1400, 1337, 1310, 1233, 1152, 1092, 996 cm⁻¹

$^1$H-NMR (DMSO-$d_6$): $\delta$ 11.95 (burs, 1H), 10.32 (s, 1H), 7.84 (d, 2H), 7.79 (d, 2H), 2.35 (t, 2H), 1.91 (s, 3H), 1.57 (quint, 2H), 1.35 (sextet, 2H), 0.89 (t, 3H)

$^{13}$C-NMR (ppm, DMSO-$d_6$): $\delta$ 172.55, 169.09, 144.29, 133.12, 129.32, 118.88, 36.66, 27.51, 23.64, 22.22, 14.16

*Anal. Calcd for C_{13}H_{18}N_{2}O_{4}S: C, 52.33; H, 6.08; N, 9.39.*

*Found: C, 51.93; H, 5.98; N, 9.23.*
XIII  N⁴-Valeroylsulfabenzamide

Yield: 1.63 g (91%), mp 219 °C

FT-IR: $\nu_{\text{max}}$ 3344, 3070, 3059, 2957, 2928, 2861, 1669, 1587, 1534, 1468, 1401, 1337, 1250, 1187, 1159, 1093, 965 cm⁻¹

$^1$H-NMR (DMSO-$d_6$): $\delta$ 12.44 (brs, 1H), 10.34 (s, 1H), 7.91 (d, 2H), 7.84-7.46 (m, 7H), 2.35 (t, 2H), 1.57 (quint, 2H), 1.32 (sextet, 2H), 0.87 (t, 3H)

$^{13}$C-NMR (ppm, DMSO-$d_6$): $\delta$ 172.60, 165.82, 144.38, 133.64, 133.15, 132.01, 129.59, 129.03, 128.82, 118.90, 36.68, 27.51, 22.22, 14.14

*Anal.* Calcd for C$_{18}$H$_{20}$N$_2$O$_4$S: C, 59.98; H, 5.59; N, 7.77.

Found: C, 59.66; H, 5.70; N, 7.77.
XIV  N⁴-Valeroylsulfanilamide

\[
\begin{align*}
\text{H} & \quad \text{SO} \quad \text{N} \quad \text{O} \\
\text{N} & \quad \text{CH}_2\text{CH}_2\text{CH}_3
\end{align*}
\]

Yield: 1.55 g (61%, 10.0 mmol scale), mp 208-209 °C

FT-IR: \( \nu_{\text{max}} \) 3364, 3318, 3199, 3128, 2958, 2874, 2859, 1669, 1591, 1527, 1401, 1328, 1151, 1096 cm \(^{-1} \)

\(^1\)H-NMR (DMSO-\(d_6\)): \( \delta \) 10.20 (s, 1H), 7.74 (apparent singlet, 4H), 7.23 (s, 2H), 2.35 (t, 2H), 1.58 (quint, 2H), 1.33 (sextet, 2H), 0.87 (t, 3H)

\(^13\)C-NMR (ppm, DMSO-\(d_6\)): \( \delta \) 172.36, 142.67, 138.49, 127.11, 118.96, 36.62, 27.55, 22.25, 14.18

*Anal.* Calcd for C\(_{11}\)H\(_{16}\)N\(_2\)O\(_3\)S:  C, 51.54; H, 6.29; N, 10.93.

Found:  C, 51.52; H, 6.22; N, 10.87.
Pyridine (10 mL) was placed in a 100 mL round bottom flask fitted with a magnetic stirrer and voltage-regulated heating mantle. The liquid was warmed to 70 °C and rapid stirring was begun. To the warm stirred liquid was added sulfamethazine (1.39 g, 5.00 mmol) in several portions, creating a homogeneous yellow solution. The warm solution was stirred vigorously as benzoyl chloride (0.70 g, 5.00 mmol) was added dropwise in a rapid manner. There was a distinct exotherm. The mixture was stirred for 30 minutes, at the end of which time the temperature was 70 °C. The mixture was cooled to near ambient temperature in a cool water bath, then poured onto 75 g of chipped ice. This produced a white precipitate. The mixture was allowed to stand several hours. The solid was filtered off and allowed to dry for several days. The solid was then recrystallized from ethanol (105 mL), dried on the filter and then dried in vacuo.

Yield: 1.77 g (93%), mp 237 °C, lit mp 233-235 °C The 1H-NMR spectrum was identical to the spectrum obtained online on SciFinder under the entry for the title compound (see below).

FT-IR: ν max 3345, 3067, 2948, 2784, 1661, 1593, 1526, 1400, 1316, 1187, 1156, 1090, 1071 cm⁻¹

1H-NMR (DMSO-d6): δ 11.65 (very brs, 1H), 10.57 (s, 1H), 8.01-7.52 (m, 9H), 6.73 (s, 1H), 2.25 (s, 6H)

13C-NMR (ppm, DMSO-d6): δ 167.84, 166.49, 156.72, 143.32, 135.42, 134.91, 132.39, 129.57, 128.92, 128.25, 119.69, 113.99, 23.34
Sulfamethazine (1.39 g, 5.00 mmol) was weighed into a 50 mL pear-shaped flask fitted with a heating mantle and mixed with pyridine (4 mL). The mixture was brought to 80 °C to produce a clear slightly yellow solution. To this warm mixture was added 4-phenylbenzoyl chloride (1.09 g, 5.00 mmol) in several portions with swirling after each addition. Finally, the last of the acid chloride was washed in with pyridine (2 mL). The mixture was maintained at 80 °C for 45 minutes. Heating was stopped, and the mixture was poured onto 150 mL of chipped ice. The material was allowed to stand overnight to produce a voluminous white crystalline solid. The solid was filtered off and washed with ether (10 mL, then 3 x 5 mL) and allowed to dry to a white free-slowing solid. The analytical sample was easily recrystallized from ethanol (45 mL).

Yield: 2.20 g (96%), mp 242-243 °C

FT-IR: $\nu_{\text{max}}$ 3280, 3031, 1651, 1590, 1522, 1511, 1397, 1381, 1306, 1159, 1089, 1072 cm$^{-1}$

$^1$H-NMR (DMSO-$d_6$): $\delta$ 11.5 (very brs, 1H), 10.6 (s, 1H), 8.08-7.41 (m, 13H), 6.75 (s, 1H), 2.25 (s, 6H)

$^{13}$C-NMR (ppm, DMSO-$d_6$): $\delta$ 164.98, 155.68, 142.85, 139.40, 132.56, 128.50, 128.47, 127.92, 127.60, 126.34, 126.04, 118.61, 22.30. There was considerable fortuitous overlap of peaks in the aromatic region. The number of peaks and relative positions, however, corresponded to the calculated values at the website of cheminfo.org.$^{64}$

*Anal.* Calcd for C$_{25}$H$_{22}$N$_4$O$_3$S: C, 65.48; H, 4.84; N, 12.22.

Found: C, 65.35; H, 4.87; N, 12.19.


**XVII  N<sup>4</sup>-Benzoylsulfathiazole**

![Chemical Structure](image)

Pyridine (20 mL) was placed in a 100 mL round bottom flask fitted with a magnetic stirrer and voltage-regulated heating mantle. The liquid was warmed to 70 °C and rapid stirring was begun. To the warm stirred liquid was added sulfathiazole (2.55 g, 10.0 mmol) in several portions, creating a homogeneous yellow solution. The warm solution was stirred vigorously as benzoyl chloride (1.40 g, 10.0 mmol) was added dropwise in a rapid manner. There was a distinct exotherm. The mixture was stirred and warmed for 30 minutes, at the end of which time the temperature was 70 °C. The mixture was cooled to near ambient temperature in a cool water bath, then poured onto 150 g of chipped ice. This produced a white precipitate. The mixture was allowed to stand overnight. The solid was filtered off and gave 3.72 g of a slightly damp mass. The solid was then washed with 150 mL of boiling ethanol for 10 minutes, then filtered and dried. A small amount of material also crystallized from the ethanol.

Yield: 3.28 g (91%), mp >260 °C

FT-IR: \( \nu_{\text{max}} \) 3392, 3323, 3144, 3116, 3053, 3019, 2962, 2899, 2808, 1674, 1654 (sh), 1590, 1574, 1520 (sh), 1599 1323, 1292, 1256, 1144, 1116, 1086, 935 cm\(^{-1}\)

\(^1\)H-NMR (DMSO-\(d_6\)): \( \delta \) 12.71 (s, 1H), 10.56 (s, 1H), 7.97-7.53 (m, 9H), 7.26 (d, 1H), 6.83 (d, 1H)

\(^13\)C-NMR (ppm, DMSO-\(d_6\)): \( \delta \) 169.22, 166.43, 142.89, 137.20, 134.98, 132.36, 128.92, 128.25, 127.27, 124.89, 120.26, 108.59

**Anal.** Caled for C\(_{16}\)H\(_{13}\)N\(_3\)O\(_3\)S\(_2\): C, 53.47; H, 3.65; N, 11.69.
Found: C, 53.30; H, 3.62; N, 11.49.
XVIII N\textsuperscript{4}-Lauroylsulfathiazole

\begin{center}
\begin{tikzpicture}
\node at (0,0) {\includegraphics[width=0.5\textwidth]{compound.png}};
\end{tikzpicture}
\end{center}

The preparation of this compound was done from the reaction of sulfathiazole with lauroyl chloride and was carried out in a manner similar to that of compound XVII.

Yield: 3.18 g (73\%, 10.0 mmol scale), mp 167-168 °C, lit mp 165-167 °C\textsuperscript{63}, lit mp 166 °C\textsuperscript{65}

FT-IR: \( \nu_{\max} \) 3311, 3145, 3004, 2954, 2915, 2847, 1666, 1596, 1565, 1517, 1398, 1296, 1247, 1176, 1146, 1089, 937 cm\textsuperscript{-1}

\(^1^H\)-NMR (DMSO-\( d_6 \)): \( \delta \) 12.8 (brs, 1H), 10.2 (s, 1H), 7.73 (apparent singlet, 4H), 7.24 (d, 1H), 6.80 (d, 1H), 2.32 (t, 2H), 1.56 (quintet, 2H), 1.24 (m, 16H), 0.83 (t, 3H)

\(^1^3^C\)-NMR (ppm, DMSO-\( d_6 \)): \( \delta \) 172.26, 169.13, 142.96, 136.51, 127.35, 124.82, 118.90, 108.47, 36.92, 31.76, 29.49, 29.46, 29.39, 29.26, 29.18, 29.09, 25.43, 22.56, 14.38
XIX  N^4-3-Chlorobenzoylsulfathiazole

![Chemical structure of N^4-3-Chlorobenzoylsulfathiazole](image_url)

The preparation of this compound was done from the reaction of sulfathiazole with 3-chlorobenzoyl chloride and was carried out in a manner similar to that of compound XVII.

Yield: 3.43 g (87%, 10.0 mmol scale), mp 224-226 °C

FT-IR: $\nu_{\text{max}}$ 3360, 3092, 3058, 3013, 1664, 1588, 1571, 1519, 1314, 1248, 1142, 1077, 938 cm$^{-1}$

$^1$H-NMR (DMSO-$d_6$): $\delta$ 12.71 (brs, 1H), 10.63 (s, 1H), 8.01-7.56 (m, 8H), 7.25 (d, 1H), 6.83 (d, 1H)

$^{13}$C-NMR (ppm, DMSO-$d_6$): $\delta$ 169.25, 164.93, 142.57, 137.48, 136.92, 133.72, 132.17, 130.92, 127.99, 127.29, 127.09, 124.85, 120.37, 108.60

Anal. Calcd for C$_{16}$H$_{12}$ClN$_3$O$_3$S$_2$: C, 48.79; H, 3.07; N, 10.67.

Found: C, 49.10; H, 3.12; N, 10.62.
**XX  N^4-Lauroylsulfacetamide**

![Chemical structure](image)

The preparation of this compound was done from the reaction of sulfacetamide with lauroyl chloride and was carried out in a manner similar to that of compound **XVII**. The material was easily recrystallized from ethanol.

Yield: 3.11 g (78%, 10.0 mmol scale), mp 151-153 °C

FT-IR: $\nu_{\text{max}}$ 3346, 3284, 2952, 2919, 2850, 1723 (sh), 1690, 1590, 1514, 1433, 1400, 1331, 1209, 1154, 1091, 941 cm$^{-1}$

$^1$H-NMR (DMSO-$d_6$): $\delta$ 11.95 (brs, 1H), 10.31 (s, 1H), 7.85 (d, 2H), 7.79 (d, 2H), 2.34 (t, 2H), 1.90 (s, 3H), 1.58 (quintet, 2H), 1.22 (m, 16H), 0.84 (t, 3H)

$^{13}$C-NMR (ppm, DMSO-$d_6$): $\delta$ 172.50, 169.03, 144.31, 133.11, 129.29, 118.83, 36.94, 31.77, 29.49, 29.47, 29.40, 29.26, 29.19, 29.08, 25.39, 23.62, 22.56, 14.36

*Anal.* Calcd for C$_{20}$H$_{32}$N$_2$O$_4$S: C, 60.58; H, 8.13; N, 7.06.

Found: C, 60.84; H, 8.04; N, 6.99.
XXI  \(N^4\)-Cyclobutanecarbonylsulfathiazole

![Chemical structure of XXI](image)

The preparation of this compound was done from the reaction of sulfathiazole with cyclobutanecarbonyl chloride and was carried out in a manner similar to that of compound XVII.

Yield: 2.78 g (82%, 10.0 mmol scale), mp >260 °C

FT-IR: \(v_{\text{max}}\) 3320, 3145, 3117, 2990, 2941, 1672, 1593, 1559, 1528, 1518, 1399, 1293, 1179, 1145, 1085, 935 cm\(^{-1}\)

\(^1\)H-NMR (DMSO-\(d_6\)): \(\delta\) 12.66 (brs, 1H), 10.04 (s, 1H), 7.76-7.71 (aromatic pseudo-quartet, overlapping doublets, 4H), 7.24 (d, 1H), 6.81 (d, 1H), 3.20 (quintet, 1H), 2.25-1.76 (m, 6H)

\(^{13}\)C-NMR (ppm, DMSO-\(d_6\)): \(\delta\) 173.89, 169.12, 143.01, 136.49, 127.36, 124.85, 119.03, 108.51, 25.00, 18.14 (one high field signal is coincident with the solvent peak, calculated value 39.7\(^{64}\))

*Anal.* Calcd for \(C_{14}H_{15}N_3O_3S_2\): C, 49.83; H, 4.48; N, 12.45.

Found: C, 49.71; H, 4.54; N, 12.37.
XXII  N⁴-Cyclohexanecarbonylsulfathiazole

The preparation of this compound was done from the reaction of sulfathiazole with
cyclohexanecarbonyl chloride and was carried out in a manner similar to that of compound
XVII.

Yield: 2.57 g (70%, 10.0 mmol scale), mp 227-228 °C

FT-IR: ν_max 3315, 2021, 1665, 1586, 1571, 1539, 1522, 1398, 1289, 1146, 1120 cm⁻¹

¹H-NMR (DMSO-d₆): δ 12.66 (brs, 1H), 10.13 (s, 1H), 7.75-7.71 (aromatic pseudo-quartet,
overlapping doublets, 4H), 7.24 (d, 1H), 6.81 (d, 1H), 2.33 (m, 1H), 1.80-1.20 (m, 10H)

¹³C-NMR (ppm, DMSO-d₆): δ 175.28, 169.14, 143.13, 136.42, 127.35, 124.86, 118.97, 108.51,
45.34, 29.48, 25.81, 25.62


Found:   C, 52.57; H, 5.13; N, 11.59.
XXIII N\textsuperscript{4}-Lauroylsulfabenzamide

The preparation of this compound was done from the reaction of sulfabenzamide with lauroyl chloride and was carried out in a manner similar to that of compound XVII. The material was easily recrystallized from ethanol.

Yield: 3.60 g (79%, 10.0 mmol scale), mp 208 °C

FT-IR: $\nu_{\text{max}}$ 3327, 3298, 3243, 3114, 3068, 3042, 2953, 2915, 2848, 1708 (sh), 1685, 1590, 1528, 1497, 1457, 1437, 1403, 1350, 1322, 1188, 1163, 1117, 1087, 927 cm$^{-1}$

$^1$H-NMR (DMSO-$d_6$): $\delta$ 12.42 (brs, 1H), 10.32 (s, 1H), 7.94-7.46 (m, 9H), 2.34 (t, 2H), 1.58 (quintet, 2H), 1.22 (m, 16H), 0.83 (t, 3H)

$^{13}$C-NMR (ppm, DMSO-$d_6$): $\delta$ 172.53, 165.79, 144.35, 133.60, 133.19, 132.08, 129.57, 129.00, 128.82, 118.84, 36.95, 31.75, 29.47, 29.44, 29.37, 29.24, 29.17, 29.06, 25.38, 22.55, 14.39

Anal. Calcd for C\textsubscript{25}H\textsubscript{34}N\textsubscript{2}O\textsubscript{4}S: C, 65.47; H, 7.47; N, 6.10.

XXIV  N^4-3-Chlorobenzoysulfabenzamide

The preparation of this compound was done from the reaction of sulfabenzamide with 3-chlorobenzoyl chloride and was carried out in a manner similar to that of compound XVII.

Yield: 3.93 g (95%, 10.0 mmol scale), mp 251 °C

FT-IR: $\nu_{\text{max}}$ 3291, 3062, 1690 (sh), 1656, 1591, 1515, 1496, 1452, 1420, 1398, 1316, 1166, 1056 cm$^{-1}$

$^1$H-NMR (DMSO-$d_6$): $\delta$ 12.50 (brs, 1H), 10.76 (s, 1H), 8.05-7.47 (m, 13H)

$^{13}$C-NMR (ppm, DMSO-$d_6$): $\delta$ 165.87, 165.14, 144.06, 136.81, 134.14, 133.75, 133.70, 132.28, 132.01, 130.93, 129.46, 129.06, 128.87, 128.06, 127.16, 120.30

*Anal.* Calcd for C$_{20}$H$_{15}$ClN$_2$O$_4$S: C, 57.90; H, 3.64; N, 6.75.

Found: C, 58.01; H, 3.85; N, 6.73.
PREPARATION OF N₁,N₄-DIACYLATED SULFA DRUGS

In general, these compounds were prepared by the controlled sequential acylation, in order, of N⁴ and N¹. The N⁴-acyl materials were isolated and fully characterized, then subjected to a second acylation at N¹. This permitted considerable variation of the acyl groups at each position. The exceptions to this were the preparations of XXV and XXVI, in which a large excess of acylating agent was used under vigorous conditions to prepare the diacyl compound (N¹,N⁴ substituents identical) in one pot.

**XXV  N¹,N⁴-Diacetylsulfanilamide**

![Chemical structure of XXV](image)

In a 100 mL round bottom flask fitted for magnetic stirring and reflux, acetic anhydride (20 mL) was mixed with sulfanilamide (1.72 g, 10.0 mmol). Upon stirring and good mixing, the beige chunky sulfanilamide was rapidly replaced with a white microcrystalline solid. The mixture was brought to reflux. After 10 minutes at the boil, the white crystalline solid had dissolved to form a clear homogeneous solution. The mixture was refluxed for 90 minutes, cooled and the excess anhydride evaporated to give a white solid. This was washed with ether (20 mL), then covered with a fresh portion of ether (20 mL) and allowed to stand for 5 hours. The solid was filtered to give the title compound (2.15 g, 84%), identical (FT-IR) to the authentic specimen prepared by the N⁴-acetylation of sulfacetamide (compound II, thus the metabolite of sulfacetamide), as previously described, and to the compound described in SciFinder under CAS Registry Number 5626-90-4. If desired, the compound could be readily recrystallized from ethanol; however, the
purity was not further improved by recrystallization; \textit{viz.}, the ether washes were sufficient to give excellent purity.
XXVI  \(N^1,N^4\)-Diacetylsulfamethazine

\[
\text{[Chemical Structure Image]}
\]

Sulfamethazine (1.00 g, 3.6 mmoles) was weighed into a 50 mL pear-shaped flask fitted with a heating mantle. Pyridine (4 mL) was added and the mixture was warmed until a homogeneous slightly yellow solution was obtained. Acetic anhydride (1.84 g, 18.0 mmol, 5.00 equiv) was added to the warm solution in several portions and washed in with further pyridine (1 mL). The mixture was brought to the boil. Within several minutes, the mixture became milky in appearance and a solid was suspended within the liquid. After 10 minutes, a white solid had deposited. Refluxing was continued for an hour. Heating was stopped and the mixture allowed to cool and stand overnight. The solvent was evaporated. The resulting beige mass was washed with portions of ether (20 mL, then 5 mL) and dried. The analytical sample was obtained by recrystallization from ethanol. The following characterization data made it clear that \(N^1,N^4\)-diacetylation had indeed occurred and that the compound was not merely \(N^4\)-monoacetylated (compare data for compound III).

Yield: 1.08 g (83%), mp 239-240 °C, lit mp 237-238 °C (dimethylacetamide, water)\(^{66}\)

FT-IR: \(\nu_{\text{max}}\) 3320, 3281, 3187, 3118, 1705 (sh), 1688, 1600, 1583, 1527, 1400, 1362, 1315, 1260, 1186, 1164, 1113, 1086, 1023 cm\(^{-1}\)

\(^1\)H-NMR (DMSO-\(d_6\)): \(\delta\) 10.47 (s, 1H), 8.03 (d, 2H), 7.84 (d, 2H), 7.47 (s, 1H), 2.54 (s, 6H), 2.11 (s, 3H), 1.81 (s, 3H)

\(^13\)C-NMR (ppm, DMSO-\(d_6\)): \(\delta\) 170.32, 169.75, 168.90, 156.31, 144.82, 132.46, 130.49, 121.12, 118.77, 24.66, 24.28, 23.72
Compound VIII (N^4-valeroylsulfamethazine 0.58 g, 1.60 mmol) was weighed into a 50 mL pear-shaped flask fitted with a heating mantle. Pyridine (4 mL) was added and the mixture was warmed until a homogeneous slightly yellow solution was obtained. Acetic anhydride (1.84 g, 18.0 mmol, 11.25 equiv) was added to the warm solution in several portions and washed in with further pyridine (1 mL). The mixture was brought to the boil. Refluxing was continued for an hour. Heating was stopped and the mixture allowed to cool and stand overnight. The solvent was evaporated. The resulting beige mass was washed with portions of ether (3 x 10 mL) and dried. Yield: 0.60 g (93%), mp 165 °C

FT-IR: $\nu_{\text{max}}$ 3313, 3279, 3120, 2954, 2935, 2871, 1686, 1594, 1582, 1526, 1364, 1256, 1184, 1163, 1115, 920 cm$^{-1}$

$^1$H-NMR (DMSO-$d_6$): $\delta$ 10.40 (s, 1H), 8.04 (d, 2H), 7.87 (d, 2H), 7.45 (s, 1H), 2.54 (s, 6H), 2.38 (t, 2H), 1.81 (s, 3H), 1.59 (quintet, 2H), 1.33 (sextet, 2H), 0.90 (t, 3H)

$^{13}$C-NMR (ppm, DMSO-$d_6$): $\delta$ 170.9, 168.87, 156.35, 144.88, 132.40, 130.50, 121.08, 118.81, 39.45 (obscured by solvent), 36.71, 27.50, 24.26, 23.70, 22.23, 14.15

Anal. Calcd for C$_{19}$H$_{24}$N$_4$O$_4$S: C, 56.42; H, 5.98; N, 13.85.

Found: C, 56.38; H, 6.17; N, 13.83.
Compound VIII (N⁴-valeroylsulfamethazine 0.58 g, 1.60 mmol) was weighed into a 50 mL pear-shaped flask fitted with a heating mantle. Pyridine (4 mL) was added and the mixture was warmed until a homogeneous slightly yellow solution was obtained. Propionic anhydride (1.04 g, 8.00 mmol, 5.00 equiv) was added to the warm solution in several portions in a rapid dropwise manner and washed in with further pyridine (1 mL). The mixture was brought to the boil. Refluxing was continued for an hour. Heating was stopped and the mixture allowed to cool and stand overnight. The solvent was evaporated. The resulting tan mass was washed with portions of ether (3 x 10 mL) and dried.

Yield: 0.59 g (88%), mp 167-168 °C

FT-IR: \( \nu_{\text{max}} \) 3313, 3279, 3120, 2954, 2935, 2871, 1686, 1594, 1582, 1526, 1364, 1256, 1184, 1163, 1115, 920 cm\(^{-1}\)

\(^1\)H-NMR (DMSO-\(d_6\)): \( \delta \) 10.41 (s, 1H), 8.03 (d, 2H), 7.85 (d, 2H), 7.46 (s, 1H), 2.51 (s, 6H), 2.38 (t, 2H), 2.00 (quartet, 2H), 1.59 (quintet, 2H), 1.33 (sextet, 2H), 0.90 (t, 3H), 0.81 (t, 3H)

\(^13\)C-NMR (ppm, DMSO-\(d_6\)): \( \delta \) 172.71, 172.14, 170.30, 156.04, 144.81, 132.53, 130.50, 121.07, 118.80, 36.70, 29.32, 27.51, 23.72, 22.23, 14.16, 8.35


Found: C, 57.56; H, 6.16; N, 13.41.
XXIX  

N¹-Acetyl-N⁴-benzoysulfamethazine

This compound was prepared in a manner similar in procedure and scale to compound XXVIII, from the reaction of XV with acetic anhydride.

Yield: 0.61 g (94%), mp 220-221 °C

FT-IR: ν max  3343, 3109, 3068, 1678, 1590, 1526, 1363, 1348, 1315, 1257, 1186, 1161, 1090 cm⁻¹

¹H-NMR (DMSO- d₆): δ  10.73 (s, 1H), 8.11-7.55 (m, 9H),  7.48 (s, 1H), 2.56 (s, 6H), 1.83 (s, 3H)

¹³C-NMR (ppm, DMSO- d₆): δ  170.35, 168.94, 166.73, 156.31, 144.86, 134.80, 133.10, 132.58, 130.32, 128.99, 128.35, 121.15, 120.09, 24.30, 23.74


Found:  C, 59.35; H, 4.40; N, 13.10.
XXX \( N^1 \)-Butyryl-\( N^4 \)-valeroylsulfamethazine

This compound was prepared in a similar way and on the same scale as compound XXVIII from the reaction of compound VIII with butyric anhydride.

Yield: 0.67 g (96%), mp 176-177 °C

FT-IR: \( \nu_{\text{max}} \) 3263, 3190, 3115, 2963, 2873, 1709 (sh), 1674, 1589, 1529, 1363, 1161, 1090 cm\(^{-1}\)

\(^1\)H-NMR (DMSO-\( d_6 \)): \( \delta \) 10.39 (s, 1H), 8.03 (d, 2H), 7.85 (d, 2H), 7.46 (s, 1H), 2.54 (s, 6H), 2.38 (t, 2H), 1.96 (t, 2H), 1.59 (quintet, 2H), 1.37-1.31 (overlapping m, 4H), 0.90 (t, 3H), 0.68 (t, 3H)

\(^{13}\)C-NMR (ppm, DMSO-\( d_6 \)): \( \delta \) 172.68, 171.31, 170.27, 156.05, 144.80, 132.59, 130.47, 121.07, 118.76, 37.41, 36.70, 27.50, 23.73, 22.24, 17.41, 14.16, 13.50

*Anal.* Calcd for C\(_{21}\)H\(_{28}\)N\(_4\)O\(_4\)S: C, 58.31; H, 6.52; N, 12.95.

Found: C, 58.20; H, 6.36; N, 12.95.
XXXI  \( N^1\)-\textit{iso}-Butyryl-\textit{N}^4\text{-valeroylsulfamethazine}

An attempt to prepare this compound was made in a similar way and on the same scale as compound XXX from the reaction of compound VIII with \textit{iso}-butyric anhydride. The reaction failed, and the information is included here for the sake of completeness. Following the usual procedure, a mixture was obtained, comprised mostly of recovered VIII. Notably, we observed the \( ^1\text{H}-\text{NMR} \) signal of VIII near \( \delta 11.8 \) as still being present. We speculate that the introduction of additional substitution at the alpha-position of the anhydride disfavors the reaction, even under the fairly vigorous conditions which we used.
XXXII  \(N^{1},N^{4}\)-Divaleroylsulfamethazine

\[
\begin{align*}
\text{N} & \text{C} \text{O} \text{N} \\
\text{H} & \text{H} \text{H} \\
\text{CH}_3 & \text{CH}_3 \\
\end{align*}
\]

This compound was prepared in a similar way and on the same scale as compound XIII from the reaction of compound VIII with valeric anhydride.

Yield: 0.50 g (86%), mp 169-170 °C

FT-IR: \(v_{\text{max}}\) 3262, 3193, 3117, 3058, 2955, 2929, 2871, 1710 (sh), 1674, 1589, 1530, 1364, 1162, 1091 cm\(^{-1}\)

\(^1\)H-NMR (DMSO-\(d_6\)): \(\delta\) 10.39 (s, 1H), 8.03 (d, 2H), 7.86 (d, 2H), 7.46 (s, 1H), 2.54 (s, 6H), 2.38 (t, 2H), 1.97 (t, 2H), 1.59 (quintet, 2H), 1.32 (overlapping m, 4H), 1.06 (sextet, 2H), 0.90 (t, 3H), 0.69 (t, 3H)

\(^{13}\)C-NMR (ppm, DMSO-\(d_6\)): \(\delta\) 172.68, 171.41, 170.25, 156.08, 144.80, 132.56, 130.46, 121.05, 118.76, 36.70, 35.27, 27.50, 26.03, 23.72, 22.23, 21.62, 14.16, 13.90

\text{Anal.}  \text{Calcd for C}_{22}\text{H}_{30}\text{N}_4\text{O}_4\text{S:  C, 59.17; H, 6.77; N, 12.55.}

\text{Found:  C, 59.10; H, 6.54; N, 12.49.}
XXXIII  \(\text{N}^1\)-Propionyl-\(\text{N}^4\)-benzoylsulfamethazine

This compound was prepared in a manner similar in procedure and scale to compound XXVIII, from the reaction of XV with propionic anhydride.

Yield: 0.74 g (97%), \(\text{mp} 204-205 \degree\text{C}\)

FT-IR: \(\nu_{\text{max}} \) 3375, 3110, 3045, 2987, 2038, 2880, 1692 (sh), 1676, 1590, 1522, 1499, 1312, 1163, 1090, 1074 cm\(^{-1}\)

\(^1\)H-NMR (DMSO-\(d_6\)): \(\delta\) 10.75 (s, 1H), 8.12-7.58 (m, 9H), 7.48 (s, 1H), 2.55 (s, 6H), 2.03 (quartet, 2H), 0.83 (s, 3H)

\(^{13}\)C-NMR (ppm, DMSO-\(d_6\)): \(\delta\) 172.18, 170.35, 166.72, 156.03, 144.82, 134.82, 133.21, 132.56, 130.36, 128.98, 128.36, 121.12, 120.07, 29.35, 23.74, 8.37

\textit{Anal.} Calcd for \(\text{C}_{22}\text{H}_{22}\text{N}_4\text{O}_4\text{S}\): \(\text{C}, 60.26; \text{H}, 5.06; \text{N}, 12.78\).

Found: \(\text{C}, 59.97; \text{H}, 4.96; \text{N}, 12.74\).
XXXIV  N\textsuperscript{1}-Butyryl-N\textsuperscript{4}-benzoylsulfamethazine

This compound was prepared in a manner similar in procedure and scale to compound XXVIII, from the reaction of XV with butyric anhydride.

Yield: 0.68 g (87%), mp 204 °C

FT-IR: \( \nu_{\text{max}} \) 3321, 3112, 3064, 2965, 2932, 2873, 1709 (sh), 1656, 1591, 1519, 1164, 1109, 1089, 832 cm\(^{-1}\)

\(^1\)H-NMR (DMSO-\(d_6\)): \( \delta \) 10.73 (s, 1H), 8.12-7.56 (m, 9H), 7.48 (s, 1H), 2.56 (s, 6H), 1.99 (t, 2H), 1.38 (sextet, 2H), 0.70 (t, 3H). Each of the three high-field multiplets appeared to be the very close overlap of two equal-sized signals, possibly indicating hindered rotation.

\(^{13}\)C-NMR (ppm, DMSO-\(d_6\)): \( \delta \) 171.36, 170.31, 166.71, 156.05, 144.80, 134.83, 133.31, 132.55, 130.31, 128.97, 128.35, 121.12, 120.05, 37.43, 23.76, 17.43, 13.53

Anal. Calcd for C\textsubscript{23}H\textsubscript{24}N\textsubscript{4}O\textsubscript{4}S: C, 61.05; H, 5.35; N, 12.38.

Found: C, 61.02; H, 5.40; N, 12.47.
An attempt to prepare this compound was made in a similar way and on the same scale as compound XXVIII from the reaction of compound XV with valeric anhydride. Although some of the characteristics of the compound were suitable for the proposed structure, we believe that the compound was air or moisture sensitive. The data are included here for the sake of completeness. Following the usual procedure, a beige solid was obtained, much in keeping with the expectations of previous examples. The material was easily recrystallized from ethanol, initially yielding a white microcrystalline solid. Upon standing in the recrystallization solvent, however, the white solid changed into larger brown crystals, which were filtered off and dried. Yield: 0.60 g (87%; caveat: based on the assumed structure), mp 182 °C

FT-IR: $\nu_{\text{max}}$ 3322, 3062, 2967, 2929, 2903, 2853, 1710 (sh), 1655, 1591, 1518, 1358, 1316, 1162 cm$^{-1}$

$^1$H-NMR (DMSO-$d_6$): Indicative of a mixture

$^{13}$C-NMR (ppm, DMSO-$d_6$): Indicative of a mixture

Thin-Layer Chromatography (silica gel; ethyl acetate:ether, 1:1): Two spots, $R_f$ 0.55 and $R_f$ 0.70.

*Anal.* Calcd for C$_{24}$H$_{26}$N$_4$O$_4$S · 0.20 H$_2$O: C, 61.31; H, 5.66; N, 11.92.

Found: C, 61.10; H, 5.64; N, 12.22.
Results and Discussion

In their function as xenobiotic-metabolizing enzymes, arylamine \(N\)-acetyltransferases are able to acetylate the terminal amine of all common sulfa drugs, producing a metabolite with a significantly decreased activity against tuberculosis. Any modification of sulfa drugs should first consider strategies to block or avoid this de-activating action by NATs, and will therefore require a uniform, reliable, and reproducible protocol to synthesize the sulfa metabolites on gram-scale. Since no such protocol was found in the literature, our lab began our exploration into the sulfa drugs by testing a number of conditions, arriving at a method with excellent yield and purity.

SULFA METABOLITE PROTOCOL

Our initial protocol to produce the sulfa metabolites attempted the reaction of 10 mmol of our chosen sulfa drug in a large excess of neat acetic anhydride, such that the anhydride acted as both the solvent and the electrophilic reagent. While this reaction was successful in high yield and good purity when performed with sulfacetamide, further reactions with other sulfa drugs under the same conditions yielded impure product. Analysis of the NMR spectra of the impure substances revealed two distinct acetyl signals, suggesting di-acetylation at both the desired arylamine nitrogen (\(N^4\)) and the nitrogen of the sulfonamide (\(N^1\)). The latter was an unexpected reaction, as we assumed \(N^1\) would be significantly less nucleophilic; conjugation draws electron density into the electronegative sulfonyl unit and the carbonyl or heterocycle substituent. The acetylation of sulfadiazine yielded a product with a broad melting point approximately 100 °C below the expected product’s melting point. This could indicate we produced a different product entirely or some combination of products; it is also worth noting that the melting point of crystalline sulfa compounds can be highly variable due to polymorphisms in crystal structure. \(^{48}\)
We returned to sulfamethazine to refine conditions and combat di-acetylation, switching to pyridine as a solvent. This choice was suggested to us by general literature on acylation procedures, as pyridine is well known for its activating effect on anhydrides. The lone pair on pyridine’s basic nitrogen interacts with the carbonyl group of the anhydride in solution, increasing the carbon's electrophilicity. Instead of using excess acetic anhydride, we limited the quantity added such that the molar ratio of sulfa:anhydride was 1:1. While this gave a yield of 96% and NMR spectra which largely suggested a mono-acetylated compound, there were some small peaks in the downfield region of the carbon NMR which could represent impurities, the di-acetylated product or even unreacted starting material. We performed one more near-identical reaction with a sulfa:anhydride ratio of 1:1.1 to ensure all starting material was consumed, but found similar results and spectra. We did not pursue the characterization of the minor impurities.

Returning to the literature for guidance, we switched solvents again and performed a reaction on sulfamethazine on the same scale in glacial acetic acid. Pyridine can produce a number of side products, including acetylated pyridine from its electrophilic aromatic substitution and other unsatisfactory results; additionally, its removal from the reaction requires thoughtful treatment. In contrast, the by-product from acetic anhydride after the acetyl moiety has been transferred is acetic acid, which is easily removed. The switch to acidic conditions also strongly favors mono-acetylation at the more nucleophilic N^4 atom. As suggested by these principles, the reaction with sulfamethazine and acetic anhydride in acetic acid at 85 °C did not need to be recrystallized and yielded clean, definitive spectra that implied a pure mono-acetylated compound with a yield of 83%. The material was fully characterized and the data were entirely consistent with the proposed structure.
Experimentation with choice of solvent and several other factors resulted in a clean and reliable synthetic protocol in good yield for the acetylation of sulfa drugs to produce their corresponding metabolites. Using this established protocol, we prepared a series of seven different acetylated sulfa metabolites (Compounds I-VII). The seven sulfa drugs chosen for metabolite preparation are easily obtained in good purity from commercial sources and are bench-stable, allowing large quantities to be stored for future reaction. They also exhibit the varied N\textsuperscript{1} substituents possible in sulfa drugs. Our selection consisted of (1) sulfanilamide, the original sulfa drug discovered and the simplest in structure; (2) sulfabenzamide and (3) sulfacetamide, which are both N\textsuperscript{1}-acylated; (4) sulfadiazine, (5) sulfamerazine, (6) sulfamethazine, and (7) sulfathiazole, all of which have heterocyclic aromatic rings. The similarity between the heterocycles in sulfadiazine, sulfamerazine and sulfamethazine allows evaluation of the effect of methyl addition onto the ring, which may have implications for the fit of the sulfa drug into the DHPS binding pocket.

A number of refinements were made to the metabolite protocol as it was applied to the rest of the sulfa drugs. We found N\textsuperscript{1},N\textsuperscript{4}-di-acetylation seemed to occur more frequently when the existing N\textsuperscript{1} substituent was an aromatic heterocycle, e.g. sulfadiazine. We hypothesize that electron density is shared more equally across N\textsuperscript{1} and the heterocycle than it is in the N\textsuperscript{1}

**Figure 10.** Representative reaction; the acetylation of sulfathiazole to yield its metabolite, N\textsuperscript{4}-acetylsulfathiazole (Compound VI).
acetamide compounds. The acetamides are less sensitive to di-acetylation because the
electrophilic carbon of the carbonyl draws electron density from N1.

In earlier reactions we added the anhydride portion-wise with a disposable glass pipet. To
address the more sensitive compounds, we set up an addition funnel with the stopcock adjusted
so that a single drop of anhydride was added approximately every five seconds. While the
solvent switch was the major factor in obtaining a clean reaction, implementing a slower addition
of anhydride to the reaction encouraged mono-acetylation by providing a limited amount of
electrophile in solution at any given time.

When clean samples of our compounds had been prepared in reasonable quantity on
gram scale, they were shipped to our physician collaborator, Dr. Michael Cynamon, Chief of
Infectious Diseases at the Syracuse, New York, Veterans Affairs Medical Center. Dr. Cynamon’s
group performed antibacterial testing in whole-cell assays. Since the compounds prepared in this
section are chemically identical to the metabolites produced through NAT deactivation of sulfa
drugs, they are inactive against mycobacteria.

![Figure 11. Structure of metabolites prepared, Compounds I-VII. Added N\textsuperscript{4} acetyl group shown in blue.](image-url)
VALEROYL SERIES AT N⁴
Guided by crystallographic images of the pterin-sulfa hybrid in the active site of DHPS, we modified the metabolite protocol and began the process of active drug design. Our lab's previous acylations of isoniazid suggested that acyl chains can lend antitubercular drugs interesting antimycobacterial properties. Applying the same logic to sulfa drugs, we hypothesize that longer acyl substituents at the N⁴ atom will block de-activation of the drug by NATs. In addition, the lipophilicity may lead to more favorable biological properties.

We made our first foray into novel pharmaceutical space with the synthesis of the corresponding valeroyl derivatives of our seven original sulfa drugs (Compounds VIII-XIV). These syntheses were again convenient and high-yielding, though the visual appearance of the reactions differed slightly from that of the acetylation procedure. Once again, we used a symmetric carboxylic anhydride and the corresponding acid: valeric anhydride was added dropwise to a slurry of sulfa drug in valeric acid at 80 °C. Although these reactants smelled remarkably foul, within two minutes of addition of anhydride the white slurry had cleared to a homogeneous yellow solution. The solution also spontaneously heated upon addition of the anhydride, reaching 105 °C. After several minutes at this higher temperature, the solution became opaque again, eventually returning to a slurry texture. The temperature was maintained for an hour. The homogenization of the solution suggests that although the original sulfa drug may not have perfect solubility in valeric acid at 85 °C, it was rapidly converted to product. If this was indeed the case, solubility was not an issue – the product was obtained as a white solid in excellent yield after cooling to room temperature, quenching over chipped ice, and drying. Spectral analysis indicated the desired compound was formed. The analytical sample was obtained by hot wash recrystallization with ethanol.
**Figure 12.** Representative reaction: reaction of sulfamethazine with valeric anhydride to yield its N⁴-valeroyl derivative (Compound XIII).
*Added N⁴ valeroyl group shown in blue.*

**BULKIER N⁴ ACYLATIONS**
We followed our work with straight-chain acylations by increasing the steric bulk of the N⁴ substituents; these compounds furthered our exploration of the lipophilicity and three-dimensional shape in this area of the DHPS binding pocket. We focused on several promising sulfa drugs as our starting materials, mostly sulfamethazine and sulfathiazole. Acyl groups of several types were used, including benzoyl rings, long straight-chains, and various sizes of saturated carbon rings. A number of compounds were prepared in the pursuit of this objective, summarized in the figure below.
Figure 13. “Bulkier” \( N^4 \)-acyl substituents on sulfathiazole and sulfamethazine scaffolds.

Unlike the valeroyl or acetyl reactions, anhydrides of these acyl substituents would be difficult to obtain or work with. In addition, using the parent carboxylic acid as solvent was generally impractical; for example, the use of benzoic anhydride (a solid) in benzoic acid (a solid at room temperature and at 85 °C) was not suitable. For this reason, we turned to more reactive electrophiles in the use of the acid chlorides, and switched to pyridine as a solvent. Pyridine is hypothesized to form a complex with the electrophilic carbon of the acid chloride’s carbonyl group, stabilizing the carbon atom’s partial positive charge with the pyridine nitrogen's lone pair and increasing overall reactivity of the electrophile.\(^67\)

While the acid chloride/pyridine reactions are not as convenient as the anhydride/acid reactions, they have the benefit of easily dissolving the reactants. Solutions were homogenous even before the addition of the electrophile, at temperatures as low as 70 °C. An exotherm was
still observed upon the addition of the anhydride, and quenching over ice yielded voluminous white precipitate, often immediately. With solubility definitively eliminated as a concern and continuing high yields, we were able to obtain pure samples for biological analysis through a number of purification procedures, used as needed.

Among these techniques was trituration with ether, a process designed to remove impurities from a solid through careful choice of solvents. The solid is ground into a suspension in the solvent; impurities dissolve into the ether, which is then filtered off.\(^{68}\) For some compounds, we washed the material with hot ethanol. Other compounds were purified by the classic technique of recrystallization from ethanol. Occasionally, we dried our analytical samples in a dessicator under house vacuum (350 torr). The Experimental section contains a detailed account of which techniques were applied to each compound.

**SELECTIVE DIACYLATION**

Our laboratory’s progression from metabolite protocol to valeroyl series to bulkier acylations allowed us to make meaningful deductions about the sensitivity of the DHPS binding pocket and its relationship to anti-tubercular activity, as discussed further below. We next addressed a related question that had already surfaced during the preparation of previous compounds: the relative nucleophilicities of the two nitrogen atoms in sulfa drugs' common scaffold. We knew from our initial experiments in metabolite preparation that when an excess of acetic anhydride was present, spectral analysis indicated the presence of the di-acetylated compound as a significant impurity. While acetylation at N\(^4\) is still preferred and occurs first, this result suggests that subsequent acetylation at N\(^1\) is preferred over a second acetylation at N\(^4\). This pattern was observed in several different sulfa drugs. In order to gather more information about the relative reactivity of these amines, we performed a series of experiments to selectively di-acylate
compounds that had already been acylated at N\textsuperscript{4}. The products also provided insight into the role of N1 in anti-tubercular activity.

The reactants for this group of N1 acylations were the characterized and isolated products of previous N\textsuperscript{4} acylation reactions in our laboratory. A representative example is shown below, where N\textsuperscript{4}-valeroysulfamethazine (Compound VIII) is reacted with acetic anhydride in pyridine to produce N\textsuperscript{1}-acetyl-N\textsuperscript{4}-valeroysulfamethazine (Compound XXVII). Nine compounds were prepared in this fashion (Compounds XXVII-XXXV), and two additional compounds were intentionally di-acetylated using the excess anhydride method discovered during metabolite preparation (Compounds XXV and XXVI). The former compound, di-acetylated sulfanilamide (Compound XXV) is chemically identical to the N\textsuperscript{4} metabolite of sulfacetamide (Compound II) and should appear as so in spectral analysis. This was indeed confirmed to be the case.

\begin{center}
\includegraphics[width=0.8\textwidth]{reactionDiagram.png}
\end{center}

**Figure 14.** Representative reaction; N\textsuperscript{1} acetylation of N\textsuperscript{4}-valeroysulfamethazine to produce N\textsuperscript{1}-acetyl-N\textsuperscript{4}-valeroysulfamethazine (Compound XXVII). Added N\textsuperscript{1} acetyl group shown in blue.

To enable direct comparison among biological results, the same sulfa scaffold was used for all nine compounds in this series. With limited biological data available at the time, we
selected sulfamethazine, suggested by a 1947 study of acetylating enzymes by Hans Krebs. Krebs found that sulfamethazine responded differently than other sulfa drugs, hinting that it might produce the most biochemically active compounds. While this did not turn out to be the case, our consistency nevertheless equipped us to comment on the relative merits of different acyl chains.

The distribution of the electron density in the environment surrounding N\textsuperscript{1} decreases its nucleophilicity in comparison to N\textsuperscript{4}. Although N\textsuperscript{4} is an aniline nitrogen with some electron density drawn into the adjacent aromatic ring, N\textsuperscript{1} has two substituents stripping electron density: the electronegative sulfur of the sulfonyl group and the aromatic diazine ring. In other sulfa drugs, this is still often a heterocycle, acyl or other electron-withdrawing substituent. Earlier experiments demonstrated the variable reactivity of N\textsuperscript{1}, such that a shift to an acidic solvent prevented the addition of acyl substituents at N\textsuperscript{1}. With this in mind, we performed our sequential acylation reactions in pyridine, both to actuate the desired N\textsuperscript{1} reaction site and to benefit from pyridine’s activating effect on anhydrides.

As expected, the second acylation was more difficult than the first. More vigorous conditions were required to obtain the product, i.e. the N\textsuperscript{1},N\textsuperscript{4}-di-acylated sulfamethazine. In addition to the use of pyridine for its activating effect, the electrophile was added in at least five-fold molar excess. N\textsuperscript{4}-Valeroylsulfamethazine (Compound VIII) and N\textsuperscript{4}-benzoylsulfamethazine (Compound XV) were selected as starting materials. Both compounds were reacted with acetic through valeric anhydride to add acyl chains from two to five carbon atoms in length. All compounds in both series were successfully prepared, isolated, characterized, and shipped to our physician collaborators for biological testing.
As interesting as the successful preparations are those that were more difficult or even failed entirely. An attempt was made to prepare N\textsuperscript{1}-isobutyryl-N\textsuperscript{4}-valeroylsulfamethazine (Compound XXXI) using isobutyric anhydride and similar conditions to the other di-acylated compounds. Even under these fairly vigorous conditions, a mixture was obtained, comprised mostly of starting material. This acyl group is similar to propionyl and butyryl, both of which were successfully prepared; we hypothesize the steric bulk of its branched chain interfered with its ability interact with N\textsuperscript{1}, which is already sterically hindered from the sulfonyl and methazine groups adjacent to it. The alpha carbon of this acyl electrophile is substituted, i.e. a tertiary carbon, unlike the other electrophiles used.

The N\textsuperscript{1}-acyl-N\textsuperscript{4}-benzoyl series, formed by performing successively longer acylations at N\textsuperscript{1} on Compound XV, produced some unique results as the acyl substituent increased in length. While two-carbon and three-carbon acylation proceeded as expected (Compounds XXIX and XXXIII), N\textsuperscript{1}-butyryl-N\textsuperscript{4}-benzoylsulfamethazine (Compound XXIV) showed unexpected details in its H-NMR spectrum. Each of the three high-field multiplets associated with the aliphatic chain of the butyryl substituent appeared as two nearly identical sets of peaks, slightly offset from one another. The low-field signals and the methyl signal of the methazine were unaffected and appeared as predicted, implying that this abnormality was caused by a segment of an individual compound, not the presence of starting material or other impurity. The splitting pattern suggests restricted rotation of the hydrogens in the N\textsuperscript{1}-acyl chain, which increased our curiosity about the steric environment around N\textsuperscript{1}. 
Intrigued by the difficulties encountered in Compound XXIV’s preparation, we attempted to increase the length of the N\textsuperscript{1} acyl chain. The reaction of Compound XV with valeric anhydride did not proceed to completion; spectral analysis revealed a mix of starting material and valeroylated product. We attempted recrystallization with ethanol, which initially yielded a white solid. However, after standing in ethanol, the white crystals merged into larger beige crystals. This transformation, as well as the difficulty separating the mixture, suggests the compound produced was air- or water-sensitive. Indeed, when the sample was sent for elemental analysis, the data were consistent with a hydrate. When recrystallization and trituration failed to separate the compounds, a variety of thin layer chromatography (TLC) conditions were tested to determine if thick-layer chromatography could be employed. In the end, TLC on silica gel (ethyl acetate:ether, 1:1) produced the best separation. Two spots were observed with R\textsubscript{f} values of 0.55 and 0.70, but these were judged to be too close for a thick-layer plate. In the end, the compound was deemed as intractable, but the experience of this reaction indicated to us that it was quite different from those with shorter N\textsuperscript{1} chain lengths.
The NMR details observed with N\textsuperscript{1}-butyryl-N\textsuperscript{4}-benzoylsulfamethazine and the difficulty of the N\textsuperscript{1}-valeroyl reaction indicate N\textsuperscript{1} acylation becomes less favorable as the length of the attempted N\textsuperscript{1} chain increases. These issues were prominent in the N\textsuperscript{4}-benzoyl series, so we reasonably assume that the N\textsuperscript{4} substituent plays a role in the reactivity of N\textsuperscript{1}. Electron-withdrawing inductive effects seem unlikely – though the benzoyl substituent is aromatic, it is approximately seven atoms away from N\textsuperscript{1}. All reactions in this series were performed in pyridine and so were homogeneous and transparent at reaction temperature. Quenching with water was necessary to obtain solid product precipitate, demonstrating solubility was not an issue, nor did the visual appearance of these two reactions differ significantly from the rest of the series. We return, then, to the crystal structures discussed in the background section of this paper.

Regardless of environment, sulfa drugs are observed to take on the “hinged” conformation described earlier.$^{36,43}$ This sharp bend at the sulfonyl occurs in an otherwise long and relatively planar molecule. Though the benzoyl substituent is also two-dimensional, it nevertheless fills more space sterically than a flexible straight-chain acyl group like valeroyl. We propose the N\textsuperscript{4}-benzoyl substituent contributes to steric hindrance at N\textsuperscript{1}, blocking access to N\textsuperscript{1} by the acyl electrophile.

Taking a first glance at the molecule drawn on paper, the idea may seem counterintuitive. To begin with, most carbon-skeleton structures of the sulfa drugs draw the sulfonyl group as linear, when of course it is acting as the tetrahedral hinge. Most of the rest of the molecule is conjugated, with the lone pairs of both nitrogen atoms participating in the pi delocalization throughout the two aromatic rings and beyond. N\textsuperscript{4}-Benzoyl further contributes to this conjugation, with its sp\textsuperscript{2}-hybridized carbon and phenyl ring. Figure 16 offers an energy-minimized model for the three-dimensional model of the molecule in space. However, note that
during an $N^1$ acylation, the nitrogen's lone pair must twist out of conjugation to be free for bond formation. This hints at the possibility of a higher energy conformation which is the reactive form, where the various chemical moieties may not be as linearly aligned as in the conformation expected to predominate. That is, the stabllest conformation may not be the reactive conformation. We can imagine a situation where the benzoyl group, though many atoms apart from $N^1$, might curl under the main body of the sulfa drug in a lobster-tail-like fashion and block access to $N^1$ (Fig. 16). $N^1$ is already heavily hindered by the methazine ring and the sulfonyl group. This appears to be the most plausible explanation for the phenomenon which was observed empirically and therefore requires justification.

![Figure 16. Left: Space-filling, energy-minimized structure of $N^4$-isobutyryl-$N^4$-benzoylsulfamethazine. Right: image of a lobster with its tail curled under its body.](image)

We further note that similar results were obtained when we attempted to acetylate $N^1$ of $N^4$-4-phenylbenzoylsulfamethazine (Compound XVI). Again, the reactant (Compound XVI) had been fully characterized without complication, but the putative product was intractable. These results seem to align with the above observations, reinforcing the hypothesis that steric bulk at $N^4$ affects ease of acylation at $N^1$. 
BIOLOGICAL ASSAY RESULTS

These experiments are useful from an organic chemistry perspective because they expand our knowledge about the chemical properties of sulfa drugs and synthesis of their derivatives. With these data, our laboratory and others can continue optimization of pharmacokinetic properties, fine-tune the fit into the binding pocket, and increase antibacterial effect against tuberculosis.

Our compounds were tested in whole-cell assays against BCG and the virulent strain *M. tuberculosis* Erdman; the results lay the foundation for more extensive testing, including toxicity studies and assays in animal models. We can draw several reasonable conclusions about the relative merits of our sulfa drug modifications.

The N^4^-acetyl compounds, or sulfa drug metabolites, are inactive against *M. tuberculosis* as expected.

We were heartened to discover, thanks to Dr. Cynamon's efforts in biological testing, that several of our new compounds were significantly active against tuberculosis. The valeroyl derivatives of sulfathiazole (XI) and of sulfacetamide (Compound XII) had MIC values of 16 µg/mL against TB Erdman, and both cleared the plate of bacterial growth in the BCG tests.

These compounds are significantly more active than their parent drugs.

Many of the functionalized sulfa drugs prepared in these experiments were derivatives of sulfamethazine. Given our first intuition about the early information supplied by Krebs, this was a reasonable approach to our work, while we waited for biological data to shape our further efforts. Nonetheless, out of the seven different ring substituents tested, sulfathiazole derivatives are in fact more effective against tuberculosis than the sulfamethazine compounds. For example, N^4^-valeroylsulfamethazine (Compound XIII) has MIC values of 64, 64 µg/mL against MTB; the
corresponding sulfathiazole derivative, $N^4$-valeroylsulfathiazole (Compound XI) has MIC values of 32, 16 $\mu$g/mL against MTB.

The reason for this is not immediately obvious – Lee’s crystallographic image of the DHPS binding pocket places the heterocycle close to the solvent front, extending slightly from the binding pocket and far from the likely locus of chemical activity. This protrusion is due to the minor structural inconsistency between sulfa drugs and the $para$-aminobenzoic acid molecules for which they act as isosteres. It is certainly reasonable, however, to suggest that the unique chemical structure of each ring might affect the positioning of the sulfa drug at this point, and so shift its conformation in the rest of the pocket as a result. Recall that sulfathiazole is characterized by a five-membered heterocycle containing sulfur and nitrogen, while sulfamethazine has a six-membered heterocycle containing only nitrogen, with two bulky methyl substituents extending at positions $meta$ to its connection to the rest of the sulfa drug.

The impact of methyl substituents can be specifically examined by comparing the structure-activity relationship of corresponding sulfamethazine, sulfamerazine and sulfadiazine derivatives. For example, the $N^4$-valeroyl compounds of these three sulfa drugs were tested against MTB and displayed varying activities, shown in the table below. Compound VIII was the most active and Compound X was the least active, demonstrating the importance of the methyl substituents. More detailed SAR would need to performed to determine if this effect is due to fit within the binding pocket, lipophilic interactions, permeability, or another cause.
Perhaps the most striking result from biological testing of these compounds is the increased activity of longer acyl chains at N⁴. Although N⁴ acetylation produces the sulfa metabolites, which are significantly less active than their parent drugs, five of the seven N⁴-valeroyl compounds demonstrated improvement in activity over their parent drugs. This cannot unequivocally confirm a hypothesis, but it certainly aligns with our guiding principles that longer acylations might be (1) improving affinity for the binding pocket and (2) preventing deactivation by NAT enzymes. Another possible, associated benefit is that the increased lipophilicity could improve permeability and thus bioavailability.

Lastly, we investigated acylating both N¹ and N⁴ of sulfa drugs with substituents of different lengths. The di-acylated sulfamethazine series showed decreased biological activity.
when compared to their parent sulfa drugs. Since several of the N\textsuperscript{4}-acylated compounds were more active than their parents, this suggests that N\textsuperscript{1} acylation significantly impairs anti-tubercular action. N\textsuperscript{1} is therefore still important to activity despite not being directly implicated in the sulfa drug/DHPS mechanism of action. This decrease contributes to the evidence that sulfa drugs fit into the DHPS binding pocket very precisely, and even a short carbon chain at N\textsuperscript{1} can sufficiently ruin their three-dimensional shape such that they can no longer mimic pABA. This decrease in activity provides further information about drug-bacillus interaction. Thus our series acted as a chemical probe into the importance of N\textsuperscript{1} in relation to N\textsuperscript{4}, which is valuable information for any attempted modification of sulfa drugs in the future.
Conclusion

Medical science has made enormous strides in the last few centuries. Thanks to the pioneering work of scientists such as Gerhard Domagk and Alexander Fleming, we possess chemical compounds that can vanquish diseases once thought to be death sentences. Many of the plagues of the past have been reduced to footnotes in history: polio, smallpox, and even the Black Plague that obliterated enormous swathes of the population in Europe in the fourteenth century.

Tuberculosis has never been relegated to the background in the same way. It possesses several unique qualities that make it difficult to prevent or treat: among them, its thick and waxy cell wall and its stasis period lurking inside the body, protected from the immune system. With no reliable vaccine and drug resistance rampant in the population, the need for novel antitubercular chemotherapy agents is clear.

Though sulfa drugs have been well-known for over eighty years now, their application to treat tuberculosis is a surprisingly unaddressed area of research. Fortunately, in 2007 a medical anomaly prompted Pierre Forgacs and his research team to scrutinize the long-held assumption that sulfa drugs were not effective against tuberculosis. With a wealth of modern technology at our disposal, the scientific community has been able to elucidate sulfa drugs' mechanism of action, which stems at least in part from its inhibition of dihydropteroate synthase. We also have a deeper understanding of its metabolism by N-acetyltransferases. Previous studies in the Hearn lab have enumerated the merits of acylation of antitubercular drugs to avoid de-activation by NATs and improve biological properties such as permeability. This thesis presents the results of applying that strategy of acylation to a collection of pre-existing sulfa drugs.

We have developed a reliable, high-yield synthetic protocol for a series of acetylated sulfa drugs to enable further study of the NAT metabolites and their properties. One
complication during this process was the tendency for a second acetylation to occur at the N\(^1\) atom, which was addressed with careful control of variables such as rate of addition, temperature and stoichiometry. As expected, the sulfa NAT metabolites were lower in activity than their parent drugs, and we ascertained conditions that provided easy separation and high yield.

With this foundation, we probed the fit of sulfa drugs into the binding pocket of DHPS and NATs by experimenting with increasing acyl chain length at N\(^4\). We explored a number of different procedures for N\(^4\) acylation, focusing on derivatives of sulfamethazine. After we had successfully prepared a rich variety of N\(^4\)-acylated sulfa drugs, we continued to selective di-acylation at both N\(^4\) and N\(^1\), varying both chain length and steric bulk. We were hopeful that di-acyl compounds might fit differently into the active site and show different biological activities. These reactions were easily accomplished with click chemistry, and furnished data with fascinating implications for the relative nucleophilicities of the two amines.

The results of whole-cell assays against BCG, TB Erdman, and others suggest the following themes for our compounds:

1. Sulfathiazole derivatives seem to produce the most biologically active compounds out of the sulfa drugs surveyed.

2. While the N\(^4\)-acetylated derivatives produced by NAT de-activation are inactive against tuberculosis, acylation at N\(^4\) with longer groups such as valeroyl does produce active compounds. This may be due to such compounds' escaping the deactivating action of NAT, to a better fit within the pABA binding pocket of DHPS, to better penetration of the drug due to enhanced lipophilicity, or perhaps all three.

3. Acylation at N\(^1\) leads to lower activity or even to no activity at all.
4. Differences in activity among the sulfamethazine derivatives prepared suggest the factors governing results are in delicate balance.

FUTURE DIRECTIONS
As indicated above, our lab continues to utilize the ongoing biological feedback on our compounds, which includes the preparation of sulfathiazole derivatives. We were able to draw a number of conclusions based on sulfamethazine which hopefully apply broadly enough to guide research with the other sulfa derivatives as well – for example, the deactivation noted after N\(^1\) acylation is dramatic enough to bespeak an effect throughout the entire binding pocket. However, in the pursuit of promising compounds for further development, we will seek to maximize efficacy by selecting the optimal sulfa framework.

This is an exciting area of research limited only by one's imagination and the variety of sulfa drugs and electrophiles available. While this thesis concerned acyl substitutions, it is easy to envision exploring the surrounding chemical space with more diverse electrophiles. Our laboratory has already initiated research into N\(^4\) Schiff bases of sulfa drugs, which involve reaction with an aldehyde and therefore involve very similar chemistry. This is another avenue pursued by our lab previously with other anti-tubercular drugs to some success.\(^{50}\)

We eagerly await the biological results from the remaining compounds that were shipped to our physician collaborators. The current literature is somewhat ambiguous about the role of trimethoprim in augmenting sulfa drugs' activity; evaluating how our compounds act against tuberculosis in the presence of trimethoprim versus on their own would certainly be useful information to bring to the debate. A sample of our compounds have been tested against infectious organisms other than tuberculosis, including \textit{S. aureus}, \textit{E. coli}, \textit{C. albicans} and others.
Any hits could lead to an entirely new avenue of drug discovery. Lastly, promising compounds from our research would benefit from *in vivo* studies in mice.

It would be illuminating to understand the three-dimensional positioning of the reactants in our intriguing N\(^1\) acylation. X-ray crystallography could capture the shape of N\(^4\)-benzoylsulfamethazine and provide insight into the steric factors at play as the electrophile approaches N\(^1\). Another possibility would be computational modeling of the reactive conformation and/or transition state involved in the acylation of N\(^1\). Such a study could illustrate the orientation of the lone pair on N\(^1\), the steric environment around N\(^1\), and the source of the interaction with the N\(^4\) acyl group. We would then be able to confirm or deny our hypothesis, and propose an explanation for a phenomenon which so far has only been directly observed at the macro level.

Lastly, we have justified our observations about the biological activity of our compounds with the best data available to us: the Lee group's crystallographic snapshot of sulfa drugs binding in the DHPS active site. In order to rationalize how the binding pocket is affected by our new sulfa compounds, it would be useful to perform docking studies, and our lab has a future external collaboration in mind for this. This could point to the reason activity is so sensitive to N\(^1\) acylation, and perhaps fill in some of the details about the mechanism of action of our successful compounds. For example, is our molecule still conjugating with DHPP the way sulfamethoxazole is illustrated to in the Lee paper? Or is the lipophilic acyl chain interacting with the DHPS active site in its own unique way? Understanding the mechanism better would guide us in future modifications, allowing us to make reasoned decisions to maximize the beneficial interactions.
The threat drug-resistant tuberculosis poses to the global community is undeniable. Though this frightening disease has been menacing humanity for many centuries, the scientific disciplines have made extraordinary discoveries to combat it in return. This small slice of organic chemistry will hopefully contribute to our accelerating progress towards a world free of tuberculosis.
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(64) Predict 1H NMR spectra


(68) Performing a Trituration
