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SANTA CRUZ

INVESTIGATION OF NATURAL PRODUCTS AS NOVEL ANTIMICROBIAL AGENTS AGAINST THE *PSEUDOMONAS AERUGINOSA* TYPE III SECRETION SYSTEM

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Annalyse Lalljie

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The Thesis of Annalyse Lalljie is approved:

_____
Professor Victoria Auerbuch Stone

_____
Professor Fitnat Yildiz

_____
Professor Roger Linington

_____
Peter Biehl
Vice Provost and Dean of Graduate Studies

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Investigation of Natural Products as Novel Antimicrobial Agents Against the *Pseudomonas aeruginosa* Type III Secretion System

Annalyse Lalljie

ABSTRACT

As antibiotic resistance among bacterial pathogens continues to escalate, posing an immense threat to global health, the discovery and development of alternative antimicrobial therapies has become critical. The type III secretion system (T3SS) is a virulence factor used by dozens of Gram-negative bacterial pathogens to cause disease. The T3SS acts as a needle-like apparatus to modulate host defenses through the injection of effector proteins into target host cells. This extracellular appendage is an attractive target for new antimicrobial therapies due to its high conservation among pathogenic bacteria such as *Salmonella, Chlamydia,* and *Pseudomonas,* its widespread absence in commensal flora, and its critical role in causing disease. In a campaign to discover molecules that inhibit the T3SS, we have screened four unique and diverse natural product-derived libraries for their inhibitory bioactivity against a strain of *P. aeruginosa* harboring a T3SS promoter-controlled luciferase. One marine bacterial extract was shown to inhibit T3SS-driven bioluminescence and, importantly, did not act as a general luciferase inhibitor, affect bacterial viability, or perturb mammalian cell viability. The purified bioactive compound, identified as sebastenoic acid, inhibited T3SS effector protein secretion in a dose-dependent manner. Interestingly, sebastenoic acid was previously found to

affect motility and biofilm formation in *Vibrio cholera*, both of which are linked to cyclic-di-GMP. In *Pseudomonas*, synthesis of the T3SS global regulator, ExsA, is inhibited by cyclic di-GMP through cAMP-Vfr signaling, suggesting that sebastenoic may play a role in modulating cyclic-di-GMP levels. In addition to sebastenoic acid, we also identified marine fungal-derived penicillic acid as a T3SS inhibitor. Penicillic acid inhibited T3SS effector secretion in a dose dependent manner without affecting bacterial viability, however, its known toxicity to mammalian cells complicates its further development as a potential therapeutic. Notably, penicillic acid was previously shown to be bioactive against both biofilm alginate biosynthesis and quorum sensing, two processes which are typically anti-regulated with the T3SS. Lastly, we identified an additional six bioactive extracts from *Burkholderia*-derived natural products and five bioactive extracts from marine-derived natural products for future small molecule inhibitor discovery.

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Combating Multidrug Resistant *Pseudomonas aeruginosa*: Naturally-Derived Inhibitors of the Bacterial Type III Secretion System

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative environmental bacterium and an opportunistic pathogen. As a pathogen, it is a leading cause of hospital-borne acute and chronic infections[1]. Additionally, in 2017 the World Health Organization categorized *P. aeruginosa* as a "priority 1" pathogen for research and development of new antimicrobials because of the increasing abundance of multidrug-resistant *P. aeruginosa*[2]. Multidrug-resistant bacteria are among the top ten threats to global health[3]. In an effort to combat multidrug resistance, one strategy is to develop virulence blockers that target mechanisms crucial for pathogenesis in addition to broad spectrum antibiotics that kill a wide variety of pathogenic and non-pathogenic bacteria. One such mechanism important for pathogenesis utilized by *P. aeruginosa* during infection is the type III secretion system (T3SS).

The T3SS is highly conserved and important for virulence in a variety of Gram-negative pathogens but has little to no role in commensal microbes. The T3SS acts as a syringe to inject effector proteins into eukaryotic host cells[4]. Once translocated, these effector proteins can manipulate host cell defenses or modulate host cell death[5,6]. Because of its major role in virulence and its large absence in commensal flora, the T3SS is an attractive target for alternative antimicrobial therapies[7].

Several novel inhibitors of the T3SS have been discovered through high throughput screening of natural product libraries[8–12], suggesting that T3SS inhibitors may have evolved in nature. High-throughput screening of these chemical libraries contributes to a rapid and optimized pipeline in drug discovery while the vast diversity of natural products continues to serve as a rich source for bioactive small molecule discovery[13–15]. This review aims to highlight the potential for further discovery of natural product-derived inhibitors of the *P. aeruginosa* T3SS as part of an ongoing effort to combat antibiotic resistance.

1.1 *Pseudomonas aeruginosa*

P. aeruginosa is a versatile organism that is able to survive and proliferate in a range of environments from soil and freshwater to saltwater and eukaryotic hosts[16–18]. This amazing adaptability has largely contributed to *P. aeruginosa*'s success as a widespread multidrug-resistant pathogen. Multidrug-resistant pathogens are challenging to treat, leading to severe infections. While *P. aeruginosa* can cause infection in healthy individuals, it is more commonly a causative agent of hospital acquired infections in immunocompromised individuals. Patients with cancer, cardiovascular diseases, cystic fibrosis, and diabetes as well as those on respirators are among the most susceptible to infection[19]. Each year multidrug-resistant *P. aeruginosa* causes an estimated 32,000 hospital acquired infections with 2,7000 resulting deaths[20].

1.1.1 Antibiotic Resistance. Antibiotics are used not only to treat infections but also to prevent infections in chemotherapy, surgery, and chronically ill patients. Treatment of antibiotic resistant infections often requires toxic alternatives and prolonged medical assistance. Antibiotic resistant infections account for 2.8 million infections and over 35,000 deaths every year in the United States alone[20]. *P. aeruginosa* is part of a group of bacteria known as ESKAPE pathogens that are characterized by their large percentage of antibiotic resistant isolates. Together these pathogens are the leading cause of hospital acquired infections worldwide[1]. Not only does *P. aeruginosa* have diverse range of intrinsic antibiotic resistance mechanisms, but its versatility leads to frequent exposure to toxins and the rapid accumulation of acquired resistance mechanisms[21].

Intrinsic resistance in *P. aeruginosa* is conferred by low outer membrane permeability, efflux systems, and antibiotic inactivating enzymes[22]. The outer membrane permeability of *P. aeruginosa* is 12- to 100- fold lower than *E.coli* due to its porin composition[23]. The predominant porin in the *P. aeruginosa* outer membrane is OprF. OprF is involved in non-specific uptake of ions and saccharides. However, lipid bilayer[24] and liposome swelling[25] model membrane studies[23] have shown that OprF has low efficacy for antibiotic permeation. Beyond OprF, *P. aeruginosa* harbors the carbohydrate-specific porin OprB, the phosphate-specific porin OprP, the pyrophosphate-specific porins OprO, and the amino acid-specific porin OprD [26]. OprD is also involved in carbapenem antibiotic uptake, however, it is hypermutable. The carbapenem binding site of OprD is formed by loops 2 and 3 of the protein.

Mutations affecting the conformation of either of these loops can result in carbapenem resistance[27]. Additional antibiotic resistance to polymyxin B and gentamicin is conferred by the overexpression of the smallest *P. aeruginosa* porin, OprH, during nutrient starvation[28]. If an antibiotic is able to permeate the outer membrane, efflux pumps can still expel the molecule from the bacteria before it reaches its target. Efflux pumps function to expel toxins out of the bacterial cytoplasm and are classified into families based on structure and cargo The resistance-nodulation-division (RND) family pumps play a fundamental role in *P. aeruginosa* antibiotic resistance[29]. Four of the twelve RND family efflux pumps expressed by *P. aeruginosa* heavily contribute to intrinsic resistance by exuding drugs, including β-lactams, quinolones, and aminoglycosides, out of the cell[30]. Beyond efflux pumps, antibiotics inside the cytoplasm can be intercepted by antibiotic-inactivating enzymes. *P. aeruginosa* possesses several antibiotic-inactivating enzymes including an inducible β-lactamase and three types of aminoglycoside modifying enzymes[22].

In addition to intrinsic resistance, the nature of *P. aeruginosa* allows the bacteria to easily and rapidly acquire resistance. *P. aeruginosa* has limited nutrient requirements allowing for survival and inhabitation of everything from soil to the ocean, fresh fruits to cosmetics, soaps to toilets, and the lungs to the perineum[17,18]. Furthermore, *P. aeruginosa* has a comparative large genome in which only 1500 of its 5567 genes are required for growth and replication, while the rest are presumed to be responsible for its high adaptability to various environments[21]. Due to the vast

prevalence of antibiotics expelled or distributed into these various environments, *P. aeruginosa* is frequently exposed to toxic compounds. The selective pressure caused by frequent exposure to antibiotics can lead to accumulation of resistance[31].

Acquired resistance has been mapped to several mutations in specific chromosomal genes but can also arise due to acquisition of new genes. Among the most common mutated chromosomal genes are *gyrA* and *gyrB*, which encode DNA gyrase, the target site of quinolone antibiotics[32]. Isogenic mutations in *P. aeruginosa gyrA* and *gyrB* that mimic target alterations observed in resistant clinical isolates can induce strong fluroquinolone resistance[33]. Acquisition of resistance can also occur through procurement of new genes by means of horizontal gene transfer. Horizontal gene transfer involves transmission of plasmids between bacteria. Many of these plasmids encode antibiotic inactivating enzymes and confer resistance to β-lactams and aminoglycosides with varying substrate specificity[22].

1.1.2 T3SS-Mediated Virulence. *P. aeruginosa* has several different virulence factors which aid in colonization and pathogenicity of the bacteria. These virulence factors can be split into two functional groups: those that aid in attachment to host cells and host colonization and those that aid in invasion of tissue and immune response inhibition[34]. The T3SS falls under the latter group.

The T3SS contributes to *P. aeruginosa* virulence in a range of hosts. Unlike some other T3SS-expressing bacteria, such as Yersinia spp., *P. aeruginosa* can express its T3SS under a variety of environmental conditions including diverse

temperatures. *P. aeruginosa* utilizes its T3SS to infect amoebae and insects in addition to mammals[35–38]. In humans, *P. aeruginosa* utilizes the T3SS to cause burn wound, pneumonic, neutropenic, and corneal infections[18,39]. T3SS effector proteins allow *P. aeruginosa* to manipulate host cell defenses to prevent phagocytosis, mediate cytotoxicity, and promote systemic dissemination[6].

1.1.3 Structure and Assembly of the T3SS. The *P. aeruginosa* T3SS is a ~25 protein molecular syringe spanning the cell envelope that is responsible for injection of effector proteins into host-cell cytosol. The T3SS is composed of several substructures including a cytosolic ATPase, a cytoplasmic ring (C-ring), an inner membrane export apparatus, the membrane-spanning basal body, the needle apparatus, and the translocation apparatus (Fig. 1). Assembly of these components is tightly regulated and type III secretion dependent[5].

The cytoplasmic component of the T3SS, important for substrate recruitment and secretion, is composed of the ATPase complex (PscK, PscL, PscN, PscO) and the C-ring (PscQ). Attached above the C-ring is the export apparatus (PscR, PscS, PscT, PscU, PscV) which sits at the base of the basal body. The basal body is composed of several ring structures imbedded in the inner (PscJ and PscD) and outer (PscC) membranes. The basal body encircles an inner rod protein (PscI) and the base of the needle (PscF). The protruding needle is capped by the PcrV tip complex which facilitates the assembly of pores in the host cell membranes upon contact. These pores are formed by translocators (PopD and PopB) secreted from the T3SS itself[5,6,40]. In *Pseudomonas* spp., assembly of these proteins is done in an outside-in manner (**Fig.**

1) in which the outer membrane rings form first without the inner membrane rings. Once the cytoplasmic domain and the basal body have been established, the needle proteins are assembled through helical polymerization as they are secreted out of the forming T3SS. Similarly, the tip complex and translocators are secreted through the T3SS during assembly[5].

Upon host cell contact and the formation of translocation pores in the host cell membrane, direct delivery of T3SS effector proteins to the host cell cytosol begins[5,40]. *P. aeruginosa* T3SS effectors can be split into two group: classical effectors and

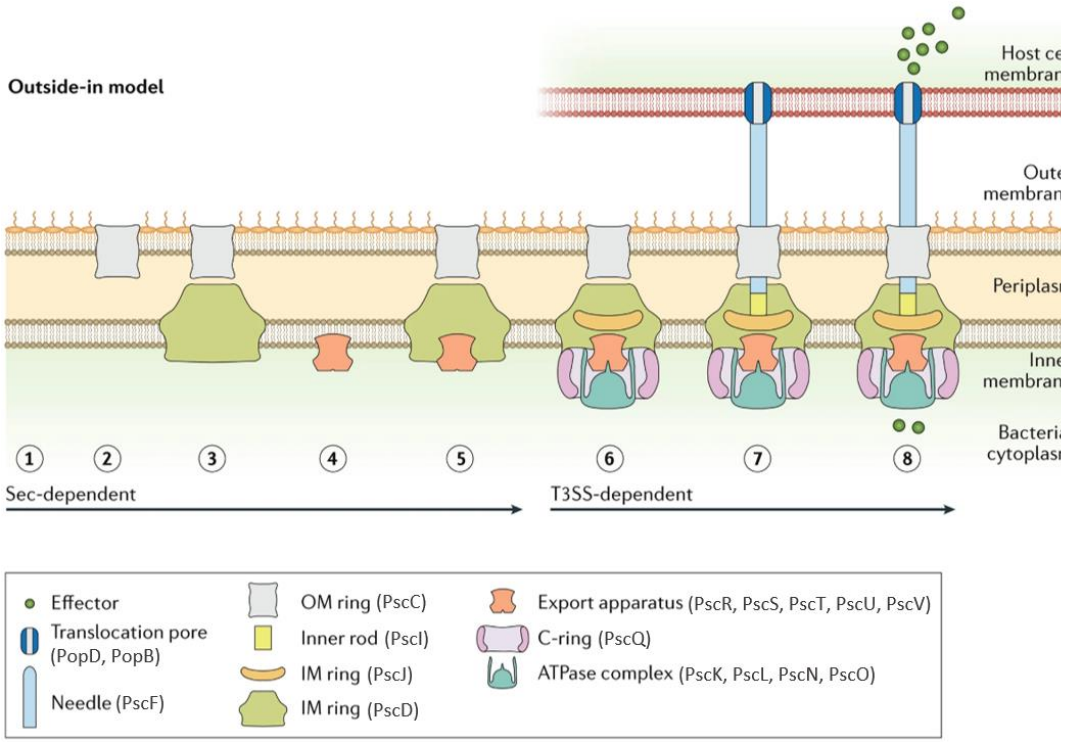


Figure 1. **Structure and assembly of the type III secretion system.** The *P. aeruginosa* T3SS assembles outside-in, with the outer membrane ring forming prior to the inner membrane ring. Following assembly of the cytoplasmic domain and the basal body, the needle filament polymerizes and is capped by the tip complex. (Modified from Deng et al., 2017).

additional effectors. The classical effectors include ExoU, ExoS, ExoT, and ExoY. The additional effectors include FliC (the flagellar filament protein), nuclear diphosphate kinase, and PemA/PemB[41]. Among the classical effectors, ExoU, a potent cytotoxin, is most commonly associated with rapid cell death. In contrast, ExoS causes delayed apoptotic cell death by stimulating production of tumor necrosis factor alpha. ExoU and ExoS heavily contribute to T3SS–mediated pathogenesis in *P. aeruginosa*. In addition, the ExoT GTPase activating enzyme hijacks the host cell actin cytoskeleton while ExoY, an adenylate cyclase, assists with actin cytoskeleton manipulation[6].

1.1.4 Regulation of the T3SS. Expression of *Pseudomonas aeruginosa* T3SS genes is under the control of a negative feedback loop involving a partner switching mechanism and the master regulator/ transcriptional activator ExsA[40–42](**Fig. 2**). The determining component of this feedback loop is the T3SS secretory protein ExsE. Upon host cell contact or in low calcium environments (T3SS inducing conditions), ExsE is secreted out of the cell, via the T3SS, allowing for the release of its intracellular binding partner ExsC. ExsC then becomes free to bind another protein known as ExsD. Under non-secreting conditions, ExsD is bound to ExsA. Therefore, once ExsC binds ExsD, ExsA is freed to bind target promoters to induce transcription[40]. All known *P. aeruginosa* T3SS genes are expressed under one of ten ExsA-dependent promoters[41].

Transcription of ExsA itself is regulated under two promoters: P_{exsC}, for *exsCDABE*, and P_{exsA}, which only regulates *exsA*. P_{exsC} is 400 times more active than

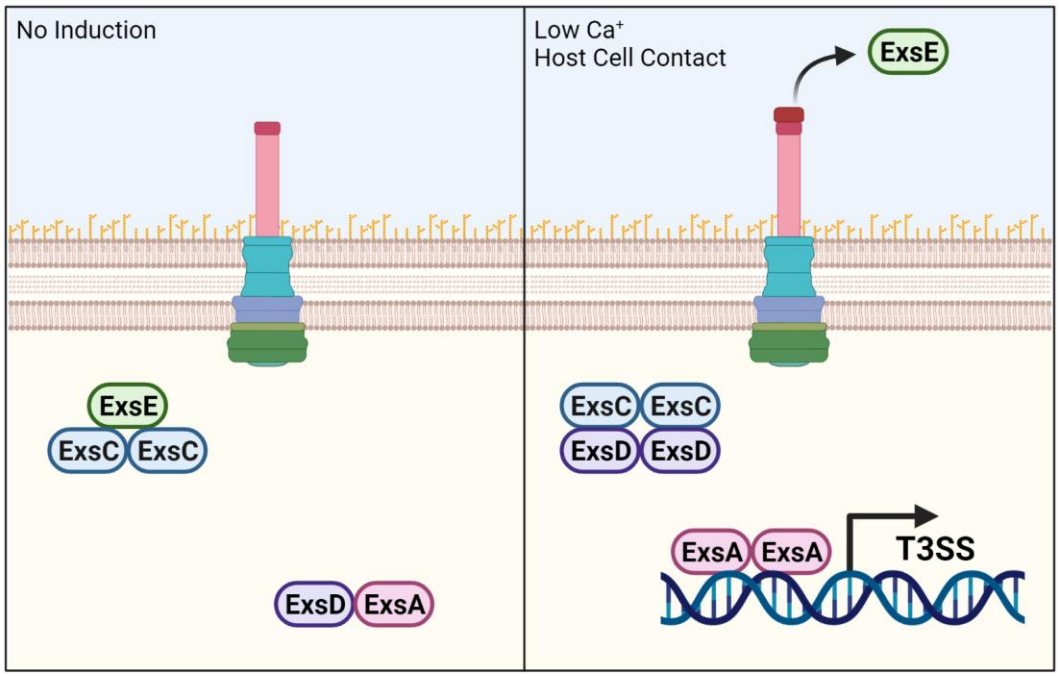


Figure 2. Regulation of the *P. aeruginosa* T3SS by the master regulator ExsA. Under non-T3SS inducing conditions, the negative regulator ExsE sequesters dimerized ExsC and ExsD binds the T3SS master regulator ExsA. Under inducing conditions, ExsE is secreted out of the cell, releasing ExsC to binds ExsD. ExsA is then freed to promote transcription of T3SS genes. (Created with BioRender.com).

P_{exsA} and is activated by ExsA itself. Transcription of *exsA* at both promoters has been found to be regulated by several other proteins including the two component Vrf-cAMP system[41].

1.2 Natural Products

Natural products have high chemical diversity and biochemical specificity which make them a promising source for drug leads[43]. Nearly 68% of the new chemical entities introduced between 1981 and 2019 were natural products, semi-

synthetic natural products analogues, or synthetic compounds based on natural product pharmacophores[44]. In the following years, studies shifted towards defined synthetic compounds because of the reduced time and approaches required. However, in recent years, new research and development strategies have been made to streamline the identification and purification of bioactive natural products[13].

1.2.1 Natural Products as Antimicrobials. In 1929, Alexander Fleming discovered antimicrobial activity from a penicillium fungus[45]. Penicillin is effective against almost all Gram-positive bacteria as well as many Gram-negative bacteria and is still used in clinical practice[46]. Fleming's discovery led to a surge of investigation into natural products in an effort to identify other antimicrobial compounds. Dozens of classes of antibiotics were identified, and many remain in commercial use today[13].

Approximately 58% of antibacterial drugs discovered in the past four decades were natural products, semi-synthetic natural products analogues, or synthetic compounds based on natural product pharmacophores[44]. Antimicrobial natural products have been sourced from plants, animals, bacteria, and fungi[43]. Antimicrobial activity in plant-derived compounds is largely due to secondary metabolites, including phenolics, phenolic acids, quinones, saponins, flavonoids, tannins, coumarins, terpenoids, and alkaloids[47,48]. In animals, a range of compounds with antimicrobial activity have been derived from milk, eggs, exoskeletons, and the mucosal tissue of both vertebrates and invertebrates[43]. Additionally, marine sponges, which display complex chemical scaffolds with potent bioactivity, have shown widespread antimicrobial activity[9,49–51]. A particularly abundant source of

antimicrobial compounds is bacteria-derived natural products. In fact, a significant number of natural product drugs and drug leads in general are derived from microbes or microbial interactions with a host[44]. Antibiotics have been discovered from both soil[52,53] and marine[54] bacteria. Along with fungi, it is thought that bacteria have evolutionarily developed the capacity to produce antimicrobial agents due to natural competition. Even so, in the early 2000s there was a dramatic shift toward synthetic compounds for drug discovery and interest in natural products did not pick back up again until recent years[13].

1.2.2 Natural versus Synthetic. Many pharmaceutical companies reduced their research investments in natural products starting in the late 1990's[13]. Instead, drug discovery from synthetically made molecules became the hot new approach. Compared to synthetic molecules, discovery and development of natural products was considered slow and tedious. In addition, it began to seem like many of the "easy" natural product discoveries had already been made. Once a natural product derivative was discovered with favorable bioactive properties, synthesis and resupply of the desired compound could often present a challenge[13]. However, new discoveries and technology have reduced many of these issues and restored interest in natural products.

Targeted screening and more rapid compound elucidation methods have reopened the door to natural products in drug discovery. Furthermore, screening of combinatorial libraries from parallel synthesis techniques have not been as greatly successful as they were expected to be[13]. One attractive characteristic of natural

products is that the Lipinski Rule of Five[55] does not apply to them. The Lipinski Rule of Five is a set of parameters which predict poor solubility or permeability and was developed as a means for synthetic chemists to create more biophysically optimal compounds for drug candidates. In addition, the continued discovery of novel molecular structures and functionalities alongside well documented interaction with mammalian target receptors serve as a testament for the efficacy of natural products as sources for drug discovery[13].

1.3 T3SS Inhibitors

1.3.1 Inhibitor Discovery. Discovery of T3SS inhibitors follows a common pipeline for both synthetic and natural product-based drug discovery[56]. A wide variety of synthetic and natural product libraries are now readily available for high throughput screening (HTS). HTS-based bioassays have become key for rapid drug discovery[7,13]. Primary screening of libraries for T3SS inhibitors often involves T3SS activity linked to some sort of readout. Luminescence, GFP, or even antibiotic resistance can be linked to T3SS effector secretion or gene transcription. For example, when discovering the MBX 1641 phenoxyacetamide T3SS inhibitor (**Table 1**), a luciferase gene from the luminescing marine bacterium *Photorhabdus luminescens* was placed under the *P. aeruginosa* T3SS *exoT* effector protein promoter. Subsequently, luminescence served as a readout for transcription of T3SS genes[57].

After primary screening for general T3SS activity, inhibitory molecules are tested for mammalian cell cytotoxicity, antibiotic activity, and effects on flagellar motility[7,56]. Compounds must be tested for cytotoxicity to ensure their feasibility as drugs. When looking for specific inhibitors of the T3SS, molecules with general activity against bacterial growth or viability are undesirable. Additionally, motility is often tested because of the evolutionary relationship and structural conservation between the injectisome T3SS and the flagellar apparatus, which consists of a flagellar T3SS basal body and a rotating extracellular flagellar filament[4]. Some identified T3SS inhibitors have been found to also affect motility whereas others are specific to the injectisome T3SS[40,58].

In the case of natural products, extracts must go through cycles of liquid chromatography fractionation and repeated primary screening in order to identify bioactive compounds. The process of elucidating pure bioactive compounds from natural product extracts is a major reason for industry shifts to synthetic libraries. However, methods have been developed to streamline the fractionation, purification, and scale up processes, making natural products realistic options for drug sourcing[13].

Lastly, identified pure bioactive compounds are tested for their breadth and mechanism of activity. Dozens of Gram-negative bacteria, including several human and plant pathogens, harbor a T3SS[4]. While there are high levels of structural and functional conservation among these species, there are also differences[5]. Understanding what species are affected by these compounds is the first step in understanding their potential molecular targets. Some T3SS inhibitors identified only

inhibit T3SS activity in one species whereas others have been shown to have a broader activity[8,58].

1.3.2 Known Inhibitors . Over the past 20 years, a spectrum of T3SS inhibitors have been discovered. This collection includes both synthetically and naturally derived small molecules that have bioactivity against a range of T3SS organisms. Unique molecular targets have been discovered for many of these inhibitors, serving as a testament to the diversity of the T3SS as an antimicrobial target. While several synthetic compounds have been discovered as inhibitors of the *P. aeruginosa* T3SS, there are much fewer compounds derived from natural extracts (**Table 1**) as these are a highly underutilized resource. In addition to small molecule inhibitors, researchers have also developed a vaccine against the *P. aeruginosa* T3SS containing anti-PcrV and anti-Psl exopolysaccharide antibodies[59]. PcrV assists in pore assembly upon contact with host cell membranes and is therefore crucial for effector protein translocation[5]. Importantly, this antibody is in phase 2 clinical trials for prevention of nosocomial pneumonia[60], attesting to the potential of T3SS inhibitors as clinical therapies.

P. aeruginosa synthetic T3SS inhibitors includes salicylidene acylhydrazides, hydroxyquinolines, N-Hydroxybenzimidazoles, phenoxyacetamides, and the epiphepropeptin D (EpD)-derived cyclic peptomers. Salicylidene acylhydrazides were among the first T3SS inhibitors to be discovered[61]. Since the discovery of the first salicylidene acylhydrazide as an inhibitor of the *Y. pseudotuberculosis* T3SS, several other salicylidene acylhydrazides have been identified and characterized for their

activity against the T3SS of numerous bacterial species including *S. enterica*, *C. trachomatis*, and *P. aeruginosa*[62]. Salicylidene acylhydrazides are thought to indirectly interfere with gene transcription by altering cellular metabolism[12,62]. Another major class of synthetic T3SS inhibitors is hydroxyquinolines. Similar to salicylidene acylhydrazides, hydroxyquinolines have bioactivity against several Gram-negative bacterial species including *Pseudomonas*, *Chlamydia*, *Shigella*, and *Salmonella*. Hydroxyquinolines are thought to target the ATPase complex present in the basal body of the injectosome T3SS as well as the flagellar T3SS[62]. N-Hydroxybenzimidazoles were first discovered as T3SS inhibitors in *Y. pseudotuberculosis*. These compounds were initially identified in molecular docking experiments as inhibitors of MAR proteins in *E. coli*. The DNA binding domain of these MAR proteins have high homology to that of LcrF, the T3SS master regulator in *Yersinia* spp., leading researchers to pursue these compounds as potential T3SS inhibitors. Not only were N-hydroxybenzimidazoles found to inhibit LcrF in *Yersinia*, but they also inhibit ExsA as it is also a MAR transcription factor with homology to LcrF[63]. Phenoxyacetamides were discovered in a screen of the Chembridge and Timtec synthetic compound libraries using a luminescent transcriptional reporter in *P. aeruginosa*. Subsequent screening for resistant mutants then showed that these molecules most likely work by targeting the PscF needle protein[64,65].

Few naturally derived inhibitors of the *P. aeruginosa* T3SS have been identified to date. The two well documented inhibitors are the plant-derived TS027 and (-)-hopeaphenol. TS027 was discovered using a fluorescence-based screen that

Compound	Source	Molecular target	IC_{50}	Species
Caminoside A	Marine sponge	--	20	*E. coli*
TS027	Plant	Rsm-ExsA pathway	20	*P. aeruginosa*
(-)-Hopeaphenol	Plant	--	6.6	*P. aeruginosa* *Y. pstb* *Chlamydia*
Piericidin A1	Marine bacteria	Blocks needle assembly	36	*Y. pstb*
Enisorine C	Marine sponge	--	30	*Y. pstb*
INP0341 Salicylidene aclyhydrazide	Synthetic	--		*P. aeruginosa* *Y. pstb* *Chlamydia*
INP1750 hydroxyquinoline	Synthetic	ATPase Complex		*P. aeruginosa* *Y. pstb* *Chlamydia*
Hydroxybenzimidazoles	Synthetic	Transcriptional activator (LcrF)	3.5	*P. aeruginosa* *Y. pstb*
EpD1,2N	Synthetic	--	8.2	*P. aeruginosa* *Y. pstb*
MBX1641 Phenoxyacetamide	Synthetic	Needle protein (PscF)	10	*P. aeruginosa* *Y. pstb* *Chlamydia*
MBX2359 Phenoxyacetamide	Synthetic	Needle protein (PscF)	3	*P. aeruginosa* *Y. pstb*
Anti-PcrV/ LcrV	Antibody	Needle tip cap protein (PcrV)		*P. aeruginosa* *Y. pestis*

Table 1. Type III secretion system inhibitors.

monitored *exoS* promoter activity. As the *exoS* promoter is activated by ExsA, the regulatory pathways that control *exsA* expression were examined through transcriptomics. In the presence of TS027, transcript levels of *exsA* in addition to *rsmY* and *rsmZ* were reduced indicating that this inhibitor targeted the Rsm-ExsA pathway[11]. (-)-Hopeaphenol was discovered from a screen of Papua New Guinean rainforest leaf extracts in *Y. pseudotuberculosis*. A YopE luminescent reporter screen was used to identify (-)-hopeaphenol as a potential T3SS inhibitor. YopE is an effector of the *Y. pseudotuberculosis* T3SS and shares high similarity with ExoS of the *P. aeruginosa* T3SS. Similar to YopE, (-)-hopeaphenol is able to inhibit ExoS expression. Furthermore, (-)-hopeaphenol was able to prevent intracellular growth of *C. trachomatis*[12]. While not many natural product-derived inhibitors of the *P. aeruginosa* T3SS have been identified, several have been discovered for other T3SS+ bacteria such as *Yersinia* species. Using a NF-kB-based screen in *Y. pseudotuberculosis*, two T3SS inhibitors were discovered: piericidins[8] and enisorines[9]. While the inhibitory mechanism of enisorines has yet to be characterized, fluorescent microscopy and biochemical approaches were used to suggest that piericidins inhibit T3SS needle assembly[66].

CONCLUSION

Multi-drug resistant bacteria present a tremendous threat to public health worldwide. Pseudomonas aeruginosa is one of six ESKAPE pathogens which top the

charts for multidrug resistance and nosocomial infections. The urgent need for new antimicrobial therapies against *P. aeruginosa* has led to investigations for T3SS inhibitors. Disarming this important virulence factor can reduce mortality rates, lower healthcare costs, and improve patient immune responses. Currently a vaccine targeting the *P. aeruginosa* T3SS is in clinical trials for prevention of nosocomial pneumonia, serving as a proof of concept for T3SS inhibitors. Natural products have served as a rich source in the discovery of new drug entities over the past four decades, with only a handful of T3SS inhibitors being identified thus far. Despite their vast chemical diversity and biochemical specificity, natural products remain largely unexplored for T3SS inhibitors. Further investigation natural products for inhibitors of the *P. aeruginosa* T3SS is needed and has the potential to yield compounds for clinical application.

REFERENCES

1. Santajit S, Indrawattana N. Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *Biomed Res Int*. 2016;2016. doi:10.1155/2016/2475067

2. WHO publishes list of bacteria for which new antibiotics are urgently needed. Accessed May 27, 2020. https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

3. Ten health issues WHO will tackle this year. Accessed June 13, 2020. https://www.who.int/news-room/feature-stories/ten-threats-to-global-health-in-2019

4. Troisfontaines P, Cornelis GR. Type III Secretion: More Systems Than You Think. *Physiology*. 2005;20(5):326-339. doi:10.1152/physiol.00011.2005

5. Deng W, Marshall NC, Rowland JL, et al. Assembly, structure, function and regulation of type III secretion systems. *Nat Rev Microbiol*. 2017;15(6):323-337. doi:10.1038/nrmicro.2017.20

6. Galle M, Carpentier I, Beyaert R. Structure and function of the Type III secretion system of Pseudomonas aeruginosa. *Curr Protein Pept Sci*. 2012;13(8):831-842. doi:10.2174/138920312804871210

7. Beckham KSH, Roe AJ. From screen to target: insights and approaches for the development of anti-virulence compounds. *Front Cell Infect Microbiol*. 2014;4. doi:10.3389/fcimb.2014.00139

8. Duncan MC, Wong WR, Dupzyk AJ, Bray WM, Linington RG, Auerbuch V. An NF-κB-Based High-Throughput Screen Identifies Piericidins as Inhibitors of the Yersinia pseudotuberculosis Type III Secretion System. *Antimicrob Agents Chemother*. 2014;58(2):1118-1126. doi:10.1128/AAC.02025-13

9. McCauley EP, Lam H, Lorig-Roach N, et al. Investigation of the Physical and Bioactive Properties of Bromo- and Iodo-Containing Sponge-Derived Compounds Possessing an Oxyphenylethanamine Core. *J Nat Prod*. 2017;80(12):3255-3266. doi:10.1021/acs.jnatprod.7b00694

10. Pendergrass HA, May AE. Natural Product Type III Secretion System Inhibitors. *Antibiotics (Basel)*. 2019;8(4). doi:10.3390/antibiotics8040162

11. Yamazaki A, Li J, Zeng Q, et al. Derivatives of Plant Phenolic Compound Affect the Type III Secretion System of Pseudomonas aeruginosa via a GacS-GacA Two-Component Signal Transduction System. *Antimicrob Agents Chemother*. 2012;56(1):36-43. doi:10.1128/AAC.00732-11

12. Zetterström CE, Hasselgren J, Salin O, et al. The Resveratrol Tetramer (-)-Hopeaphenol Inhibits Type III Secretion in the Gram-Negative Pathogens Yersinia pseudotuberculosis and Pseudomonas aeruginosa. *PLoS One*. 2013;8(12). doi:10.1371/journal.pone.0081969

13. Beutler JA. Natural Products as a Foundation for Drug Discovery. *Curr Protoc Pharmacol*. 2009;46:9.11.1-9.11.21. doi:10.1002/0471141755.ph0911s46

14. Koehn FE, Carter GT. The evolving role of natural products in drug discovery. *Nature Reviews Drug Discovery*. 2005;4(3):206-220. doi:10.1038/nrd1657

15. Newman DJ, Cragg GM. Natural Products as Sources of New Drugs from 1981 to 2014. *J Nat Prod*. 2016;79(3):629-661. doi:10.1021/acs.jnatprod.5b01055

16. Barbier M, Damron FH, Bielecki P, et al. From the Environment to the Host: Re-Wiring of the Transcriptome of Pseudomonas aeruginosa from 22°C to 37°C. *PLOS ONE*. 2014;9(2):e89941. doi:10.1371/journal.pone.0089941

17. Kimata N, Nishino T, Suzuki S, Kogure K. Pseudomonas aeruginosa Isolated from Marine Environments in Tokyo Bay. *Microb Ecol*. 2004;47(1):41-47. doi:10.1007/s00248-003-1032-9

18. Moore NM, Flaws ML. Epidemiology and Pathogenesis of *Pseudomonas aeruginosa* Infections. *Clin Lab Sci*. 2011;24(1):43-46. doi:10.29074/ascls.24.1.43

19. Moore NM, Flaws ML. Introduction: Pseudomonas aeruginosa. *American Society for Clinical Laboratory Science*. 2011;24(1):41-42. doi:10.29074/ascls.24.1.41

20. Centers for Disease Control and Prevention (U.S.). *Antibiotic Resistance Threats in the United States, 2019*. Centers for Disease Control and Prevention (U.S.); 2019. doi:10.15620/cdc:82532

21. Lambert PA. Mechanisms of antibiotic resistance in Pseudomonas aeruginosa. *J R Soc Med*. 2002;95(Suppl 41):22-26.

22. Pang Z, Raudonis R, Glick BR, Lin T-J, Cheng Z. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. *Biotechnology Advances*. 2019;37(1):177-192. doi:10.1016/j.biotechadv.2018.11.013

23. Bellido F, Martin NL, Siehnel RJ, Hancock RE. Reevaluation, using intact cells, of the exclusion limit and role of porin OprF in Pseudomonas aeruginosa outer

membrane permeability. *Journal of Bacteriology*. 1992;174(16):5196-5203. doi:10.1128/JB.174.16.5196-5203.1992

24. Benz R, Hancock RE. Properties of the large ion-permeable pores formed from protein F of Pseudomonas aeruginosa in lipid bilayer membranes. *Biochim Biophys Acta*. 1981;646(2):298-308. doi:10.1016/0005-2736(81)90336-9

25. Nikaido H, Nikaido K, Harayama S. Identification and characterization of porins in Pseudomonas aeruginosa. *J Biol Chem*. 1991;266(2):770-779.

26. Hancock REW, Brinkman FSL. Function of pseudomonas porins in uptake and efflux. *Annu Rev Microbiol*. 2002;56:17-38. doi:10.1146/annurev.micro.56.012302.160310

27. Li H, Luo Y-F, Williams BJ, Blackwell TS, Xie C-M. Structure and function of OprD protein in Pseudomonas aeruginosa: from antibiotic resistance to novel therapies. *Int J Med Microbiol*. 2012;302(2):63-68. doi:10.1016/j.ijmm.2011.10.001

28. Bell A, Bains M, Hancock RE. Pseudomonas aeruginosa outer membrane protein OprH: expression from the cloned gene and function in EDTA and gentamicin resistance. *Journal of Bacteriology*. 1991;173(21):6657-6664. doi:10.1128/jb.173.21.6657-6664.1991

29. Li X-Z, Nikaido H. Efflux-Mediated Drug Resistance in Bacteria: an Update. *Drugs*. 2009;69(12):1555-1623. doi:10.2165/11317030-000000000-00000

30. Dreier J, Ruggerone P. Interaction of antibacterial compounds with RND efflux pumps in Pseudomonas aeruginosa. *Front Microbiol*. 2015;6. doi:10.3389/fmicb.2015.00660

31. Cycoń M, Mrozik A, Piotrowska-Seget Z. Antibiotics in the Soil Environment—Degradation and Their Impact on Microbial Activity and Diversity. *Front Microbiol*. 2019;10. doi:10.3389/fmicb.2019.00338

32. Aldred KJ, Kerns RJ, Osheroff N. Mechanism of quinolone action and resistance. *Biochemistry*. 2014;53(10):1565-1574. doi:10.1021/bi5000564

33. Bruchmann S, Dötsch A, Nouri B, Chaberny IF, Häussler S. Quantitative Contributions of Target Alteration and Decreased Drug Accumulation to Pseudomonas aeruginosa Fluoroquinolone Resistance. *Antimicrobial Agents and Chemotherapy*. 2013;57(3):1361-1368. doi:10.1128/AAC.01581-12

34. Moore NM, Flaws ML. Antimicrobial Resistance Mechanisms in Pseudomonas aeruginosa. *American Society for Clinical Laboratory Science*. 2011;24(1):47-51. doi:10.29074/ascls.24.1.47

35. Brannon MK, Davis JM, Mathias JR, et al. Pseudomonas aeruginosa Type III secretion system interacts with phagocytes to modulate systemic infection of zebrafish embryos. *Cell Microbiol*. 2009;11(5):755-768. doi:10.1111/j.1462-5822.2009.01288.x

36. Matz C, Moreno AM, Alhede M, et al. Pseudomonas aeruginosa uses type III secretion system to kill biofilm-associated amoebae. *The ISME Journal*. 2008;2(8):843-852. doi:10.1038/ismej.2008.47

37. Miyata S, Casey M, Frank DW, Ausubel FM, Drenkard E. Use of the Galleria mellonella caterpillar as a model host to study the role of the type III secretion system in Pseudomonas aeruginosa pathogenesis. *Infect Immun*. 2003;71(5):2404-2413. doi:10.1128/iai.71.5.2404-2413.2003

38. Pukatzki S, Kessin RH, Mekalanos JJ. The human pathogen Pseudomonas aeruginosa utilizes conserved virulence pathways to infect the social amoeba Dictyostelium discoideum. *Proc Natl Acad Sci USA*. 2002;99(5):3159-3164. doi:10.1073/pnas.052704399

39. Bodey GP, Bolivar R, Fainstein V, Jadeja L. Infections caused by Pseudomonas aeruginosa. *Rev Infect Dis*. 1983;5(2):279-313. doi:10.1093/clinids/5.2.279

40. Anantharajah A, Mingeot-Leclercq M-P, Bambeke FV. Targeting the Type Three Secretion System in Pseudomonas aeruginosa. *Trends in Pharmacological Sciences*. 2016;37(9):734-749. doi:https://doi.org/10.1016/j.tips.2016.05.011

41. McMackin EAW, Djapgne L, Corley JM, Yahr TL. Fitting Pieces into the Puzzle of Pseudomonas aeruginosa Type III Secretion System Gene Expression. *Journal of Bacteriology*. 2019;201(13). doi:10.1128/JB.00209-19

42. Diaz MR, King JM, Yahr TL. Intrinsic and extrinsic regulation of type III secretion gene expression in Pseudomonas aeruginosa. *Front Microbiol*. 2011;2. doi:10.3389/fmicb.2011.00089

43. Gyawali R, Ibrahim SA. Natural products as antimicrobial agents. *Food Control*. 2014;46:412-429. doi:10.1016/j.foodcont.2014.05.047

44. Newman DJ, Cragg GM. Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *J Nat Prod*. 2020;83(3):770-803. doi:10.1021/acs.jnatprod.9b01285

45. Fleming A. On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ. *Br J Exp Pathol*. 1929;10(3):226-236.

46. James S. Lewis, Bush K. Antibacterial Agents*. *Manual of Clinical Microbiology, Eleventh Edition*. Published online June 1, 2015:1171-1211. doi:10.1128/9781555817381.ch68

47. Ciocan D, Ioan B. PLANT PRODUCTS AS ANTIMICROBIAL AGENTS. In: ; 2007.

48. Nascimento GGF, Locatelli J, Freitas PC, Silva GL. Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Brazilian Journal of Microbiology*. 2000;31(4):247-256. doi:10.1590/S1517-83822000000400003

49. Becerro MA, Lopez NI, Turon X, Uriz MJ. Antimicrobial activity and surface bacterial film in marine sponges. *Journal of Experimental Marine Biology and Ecology*. 1994;179(2):195-205. doi:10.1016/0022-0981(94)90114-7

50. McCaffrey EJ, Endean R. Antimicrobial activity of tropical and subtropical sponges. *Mar Biol*. 1985;89(1):1-8. doi:10.1007/BF00392871

51. Newbold RW, Jensen PR, Fenical W, Pawlik JR. Antimicrobial activity of Caribbean sponge extracts. *Aquatic Microbial Ecology*. 1999;19(3):279-284. doi:10.3354/ame019279

52. Gislin D, Sudarsanam D, Antony Raj G, Baskar K. Antibacterial activity of soil bacteria isolated from Kochi, India and their molecular identification. *J Genet Eng Biotechnol*. 2018;16(2):287-294. doi:10.1016/j.jgeb.2018.05.010

53. Hover BM, Kim S-H, Katz M, et al. Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens. *Nature Microbiology*. 2018;3(4):415-422. doi:10.1038/s41564-018-0110-1

54. Tortorella E, Tedesco P, Palma Esposito F, et al. Antibiotics from Deep-Sea Microorganisms: Current Discoveries and Perspectives. *Mar Drugs*. 2018;16(10). doi:10.3390/md16100355

55. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings1PII of original article: S0169-409X(96)00423-1. The article was originally published in Advanced Drug

Delivery Reviews 23 (1997) 3–25.1. *Advanced Drug Delivery Reviews*. 2001;46(1):3-26. doi:10.1016/S0169-409X(00)00129-0

56. Morgan JM, Lam HN, Delgado J, et al. An Experimental Pipeline for Initial Characterization of Bacterial Type III Secretion System Inhibitor Mode of Action Using Enteropathogenic Yersinia. *Front Cell Infect Microbiol*. 2018;8. doi:10.3389/fcimb.2018.00404

57. Aiello D, Williams JD, Majgier-Baranowska H, et al. Discovery and Characterization of Inhibitors of Pseudomonas aeruginosa Type III Secretion. *Antimicrob Agents Chemother*. 2010;54(5):1988-1999. doi:10.1128/AAC.01598-09

58. Lam HN, Lau T, Lentz A, et al. Developing Cyclic Peptomers as Broad-Spectrum Gram negative Bacterial Type III Secretion System Inhibitors. *bioRxiv*. Published online August 4, 2020:2020.08.03.235622. doi:10.1101/2020.08.03.235622

59. Sawa T, Ito E, Nguyen VH, Haight M. Anti-PcrV antibody strategies against virulent Pseudomonas aeruginosa. *Hum Vaccin Immunother*. 2014;10(10):2843-2852. doi:10.4161/21645515.2014.971641

60. Tabor DE, Oganesyan V, Keller AE, et al. Pseudomonas aeruginosa PcrV and Psl, the Molecular Targets of Bispecific Antibody MEDI3902, Are Conserved Among Diverse Global Clinical Isolates. *The Journal of Infectious Diseases*. 2018;218(12):1983-1994. doi:10.1093/infdis/jiy438

61. Kauppi AM, Nordfelth R, Uvell H, Wolf-Watz H, Elofsson M. Targeting Bacterial Virulence: Inhibitors of Type III Secretion in Yersinia. *Chemistry & Biology*. 2003;10(3):241-249. doi:10.1016/S1074-5521(03)00046-2

62. Anantharajah A, Buyck JM, Sundin C, Tulkens PM, Mingeot-Leclercq M-P, Van Bambeke F. Salicylidene Acylhydrazides and Hydroxyquinolines Act as Inhibitors of Type Three Secretion Systems in Pseudomonas aeruginosa by Distinct Mechanisms. *Antimicrob Agents Chemother*. 2017;61(6). doi:10.1128/AAC.02566-16

63. Kim OK, Garrity-Ryan LK, Bartlett VJ, et al. N-hydroxybenzimidazole Inhibitors of the Transcription Factor LcrF in Yersinia: Novel Anti-Virulence Agents. *J Med Chem*. 2009;52(18):5626-5634. doi:10.1021/jm9006577

64. Bowlin NO, Williams JD, Knoten CA, et al. Mutations in the Pseudomonas aeruginosa Needle Protein Gene pscF Confer Resistance to Phenoxyacetamide Inhibitors of the Type III Secretion System. *Antimicrob Agents Chemother*. 2014;58(4):2211-2220. doi:10.1128/AAC.02795-13

65. Moir DT, Bowlin NO, Berube BJ, et al. A Structure-Function-Inhibition Analysis of the P. aeruginosa Type III Secretion Needle Protein PscF. *J Bacteriol*. Published online June 29, 2020. doi:10.1128/JB.00055-20

66. Morgan JM, Duncan MC, Johnson KS, et al. Piericidin A1 Blocks Yersinia Ysc Type III Secretion System Needle Assembly. *mSphere*. 2017;2(1). doi:10.1128/mSphere.00030-17

67. Lam H, Schwochert J, Lao Y, et al. Synthetic Cyclic Peptomers as Type III Secretion System Inhibitors. *Antimicrob Agents Chemother*. 2017;61(9):e00060-17. doi:10.1128/AAC.00060-17

68. Wong WR, Oliver AG, Linington RG. Development of BioMAP Screening for the Classification and Discovery of Natural Product Antibiotics. *Chem Biol*. 2012;19(11):1483-1495. doi:10.1016/j.chembiol.2012.09.014

69. Ochoa JL, Bray WM, Lokey RS, Linington RG. Phenotype-Guided Natural Products Discovery Using Cytological Profiling. *J Nat Prod*. 2015;78(9):2242-2248. doi:10.1021/acs.jnatprod.5b00455

70. Westerhausen S, Nowak M, Torres-Vargas CE, et al. A NanoLuc luciferase-based assay enabling the real-time analysis of protein secretion and injection by bacterial type III secretion systems. *Molecular Microbiology*. 2020;113(6):1240-1254. doi:https://doi.org/10.1111/mmi.14490

71. Warner CJA, Salinas M, Zamorano-Sánchez D, et al. The Bioactive Lipid (S)-Sebastenoic Acid Impacts Motility and Dispersion in Vibrio cholerae. *Can J Chem*. 2018;96(2):196-203. doi:10.1139/cjc-2017-0334

72. Sanchez LM, Wong WR, Riener RM, Schulze CJ, Linington RG. Examining the Fish Microbiome: Vertebrate-Derived Bacteria as an Environmental Niche for the Discovery of Unique Marine Natural Products. *PLoS One*. 2012;7(5):e35398. doi:http://dx.doi.org/10.1371/journal.pone.0035398

73. Haeckl FPJ, Baldim JL, Iskakova D, Kurita KL, Soares MG, Linington RG. A selective genome-guided method for environmental Burkholderia isolation. *Journal of Industrial Microbiology and Biotechnology*. 2019;46(3-4):345-362. doi:10.1007/s10295-018-02121-x

74. Rasmussen TB, Skindersoe ME, Bjarnsholt T, et al. Identity and effects of quorum-sensing inhibitors produced by Penicillium species. *Microbiology (Reading)*. 2005;151(Pt 5):1325-1340. doi:10.1099/mic.0.27715-0

Investigation of microbial natural products offers promising leads for small molecule inhibitors of the *P. aeruginosa* T3SS

INTRODUCTION

Antibiotic resistance is a rapidly growing global health threat, causing once easily-treatable infections to become deadly. The extensive use of antibiotics in disease control has fostered mobilization of resistance elements throughout bacterial populations[1]. One group of bacteria, known as the ESKAPE pathogens, play a leading role in nosocomial infections worldwide and have become increasingly resistant to current antibiotic treatments[2,3]. Among these ESKAPE pathogens is the Gram-negative opportunistic pathogen *Pseudomonas aeruginosa*.

As an opportunistic pathogen, *P. aeruginosa* causes a handful of hospital-acquired infections including urinary tract infections, bacteremia, pneumonia, meningitis, and burn wound infections[4]. In addition, *P. aeruginosa* has become notorious as a multidrug-resistant pathogen due to its range of intrinsic resistance mechanisms and its ability to easily acquire or develop resistance[5–7]. Multidrug-resistant *P. aeruginosa* is thought to account for 32,000 nosocomial infections and 2,700 deaths in the United States annually[8]. In an effort to combat resistance and create targeted therapies, researchers have begun to explore virulence blockers as alternatives to antibiotics. In *P. aeruginosa*, as well as several other Gram-negative pathogens including *Salmonella*, *Chlamydia*, and *Yersinia*, the type III secretion system (T3SS) is an attractive target for such virulence blockers.

The T3SS is an externally accessible multiprotein needle-like apparatus that mediates virulence through injection of effector proteins into target host cells, modulating host defenses[9,10]. The T3SS is largely conserved among species and has many possible drug targets from transcriptional regulators to structural proteins. *P. aeruginosa* utilizes the T3SS to prevent phagocytosis, mediate cytotoxicity, and promote systemic dissemination[10]. Furthermore, murine and clinical studies revealed significantly higher morbidity and mortality rates in lower respiratory and systemic infections involving *P. aeruginosa* with a functional T3SS[11].

Over the past two decades, a variety of small molecule T3SS inhibitors have been discovered through high-throughput screening of both synthetic and natural product chemical libraries [12–19]. Several synthetic small molecules have been identified as inhibitors of the *P. aeruginosa* T3SS[12–15], however, natural products have remained a largely untapped resource for potential *Pseudomonas* T3SS inhibitors. In addition, while many of these small molecule T3SS inhibitors have shown efficacy *in vitro* and in animal models, none have entered clinical trials. Thus far the only T3SS-target therapy to enter clinical trials is a bispecific antibody targeting both the T3SS needle tip protein, PcrV, and a biofilm exopolysaccharide, Psl, and must be administered intravenously[20,21]. Therefore, the need for small molecule chemical inhibitors of the *P. aeruginosa* T3SS is critical.

In this study, we utilize a high-throughput bioluminescent reporter screen to assay two unique libraries of natural product-derived extracts for inhibitors of the *P. aeruginosa* T3SS. Using this screen, a group of synthetic *P. aeruginosa* T3SS

inhibitors, known as phenoxyacetamides, was previously identified[16]. Further studies suggested that the putative target for phenoxyacetamides is the T3SS needle protein, PscF[22]. During our investigation, we identified an Actinomycete-derived bioactive lipid, sebastenoic acid, along with six lead *Burkholderia*-derived extracts, as inhibitors of the *P. aeruginosa* T3SS.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in **Table 2**. *P. aeruginosa* overnight cultures were grown at 37°C, shaking, in Luria-Bertani (LB) medium, supplemented with antibiotics where noted. All shaking incubations were at 250 rpm. Low calcium LB (LB with 20mM $MgCl_2$ and 5mM EGTA) was used to induce the *P. aeruginosa* T3SS.

Natural product libraries and bioassay guided fractionation. A library of Actinomycete-derived extracts was created from marine sediment samples as described previously[27–29] with the strain producing sebastenoic acid originally isolated on NTF media (1 L Milli-Q water, 20 g agar, 20 g starch, 0.5 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4, 1 g KNO_3, 50 mg nalidixic acid, and 50 mg cycloheximide). A separate library of *Burkholderia*-derived extracts was created from shallow plant root samples collected in British Columbia as detailed by Haeckl et al.[30].

Strain	Description	Reference
P. aeruginosa strains		
PAO1	Wild type PAO1	23
PAO1 *exoT-lux*	PAO1::pGSV3-exoT-luxCDABE	16
PAO1 ΔpscC *exoT-lux*	ΔpscC PAO1-LAC::pGSV3-exoT-luxCDABE	16
PAO1 Xen 41	PAO1 with a stable copy of the *Photorhabdus luminescens* lux operon in the chromosome	PerkinElmer 119229
PA103	Wild type PA103	24,25
PA103 ΔpscJ	PA103 harboring Tn5 Gent insertion in the pscJ gene, defective in T3SS	26
PA103 ExoU-HiBiT	PA103 with the pME6032 exoU D344A Rep plasmid	This study
PA103 ΔpscJ ExoU-HiBiT	PA103 ΔpscJ with the pME6032 exoU D344A Rep plasmid	This study

Table 2. Bacterial strains used in this study

Chemical extracts for both libraries were generated via CombiFlash (Teledyne Isco) automated flash chromatography. A step-wise gradient of methanol/water with ethyl acetate wash was used to create 7 prefractions (water fraction, A-F), at 10% methanol, 20% methanol, 40% methanol, 60% methanol, 80% methanol, 100% methanol, and 100% ethyl acetate. The water fraction is then discarded. Prefractions selected for follow up were then separated via high-performance liquid chromatography (Agilent 1100 series HPLC). Separations were collected into 20-dram scintillation vials and dried under nitrogen. IDRuns determined appropriate gradient conditions for sufficient peak shape. Ten three-minute time chunks were collected via HPLC (Synergi C 4µm analytical column, Phenomenex) with overnight sequences starting at 2 minutes and ending at 32 minutes, including 5-minute post-time. Linear gradient elutions of methanol (Thermo Fisher Scientific, HPLC grade)

with flow rate of 1 ml min^{-1} and 50-75 µl injections were used. Methanol gradient varied for each extract. Samples were dried and redissolved in DMSO.

For the seven hit prefractions from the Actinomycete library, liquid chromatography mass spectrometry (LCMS) was performed (Agilent 1200 series LC, Agilent 6130 mass spectrometer) to elucidate the complexity of the initial extract and determine its viability for purification.

To purify the compound of interest from 1860E, the 1860 extract was grown up and extracted using standard library pipeline mentioned above. After extracts were dried via SpeedVac, the 1860E fraction was obtained and dissolved in 100% methanol. It was purified by HPLC (Phenomenex Kinetix XB-C18 250 mm x 4.6 mm, 5 µm) using an isocratic separation (75% MeOH + 0.02% formic acid, 25% H_2O + 0.02% formic acid, flow rate 1.25 ml min^{-1}) over 48 minutes, before 100% methanol wash. 50 µL injections used. Collections occurred into round bottom flasks. For structure elucidation, a full dataset was obtained for 1860E extracts on two occasions. The first occasion was a full, pure dataset. The second occasion, with one impurity, was used to resolve missing components.

Luciferases transcriptional reporter screen. Overnight cultures of WT *P. aeruginosa exoT-lux* (PAO1 *exoT-lux*) and T3SS mutant *P. aeruginosa exoT-lux* (PAO1 ΔpscC *exoT-lux*) were back diluted 1:40 in LB^{gent10} (10 µg/mL gentamicin) and incubated for 1.5 hours at 37°C. White 384-well plates (Greiner Bio-One 781207) were then prepared with one-half final volume of 2X low calcium LB (2X

$MgCl_2$ and EGTA) supplemented with 10 µg/mL gentamicin and extracts or pure compounds. Extracts/ pure compounds were added using a Janus MDT robot (PerkinElmer). After incubation, the cultures were centrifuged for 5 min at 14,800 rpm. Supernatant was removed and the cell pellets were resuspended in LB^{gent10}. Cultures were then normalized to an OD_{600} of 0.1 and added to the prepared 384-well plates. For the primary screen of both libraries, plates were placed in the plate reader stacker at room temperature and Luminescence was measured every hour for 7 hours (PerkinElmer Envision 2105). An analysis of the z'-factor averages at each time point showed 4 or 5 hours to be most reliable. Data from the primary screen was analyzed at 5 hours. For subsequent screenings, plates were sealed with Breathe-Easy® gas permeable membranes (Sigma-Aldrich) and incubated at ambient room temperature for 4 hours. Luminescence was then measured using the plate reader.

Luciferase inhibitor counterscreen. Following overnight growth, cultures of PAO1 Xen41 were centrifuged for 5 minutes at 14,800 rpm. Supernatant was removed and the cell pellets were resuspended in LB. The cultures were then normalized to an OD_{600} of 0.2. Normalized cultures were added to white with clear bottom 384-well plates (Corning 3763). Extracts or pure compounds were then added by hand or with a Janus MDT robot (PerkinElmer) and plates were placed in a static incubator at 37°C for 3 hours. Luminescence and absorbance at 600nm for each plate were then measured (PerkinElmer Envision 2105).

Bacterial viability counterscreen. WT *P. aeruginosa* (PAO1) was grown overnight and then back diluted 1:40 in LB and grown for 1.5 hours at 37°C. Black untreated

384-well plates (Corning 3573) were then prepared with one-half final volume of LB, extracts, and 10% v/v resazurin-based alamarBlue high-sensitivity (HS) Cell Viability Reagent (catalog number A50101; Invitrogen). Extracts were either added by hand or using Janus MDT robot (PerkinElmer). After incubation, the cultures were centrifuged for 5 min at 14,800 rpm. Supernatant was removed and the cell pellets were resuspended in LB. Cultures were then diluted to an OD_{600} of 0.0005 and added to the prepared 384-well plates. For time course measurements, the plate was incubated in a plate reader (PerkinElmer Envision 2105) at 37°C and fluorescence was measured every hour for 12 hours. For single time point measurements, plates were sealed with Breathe-Easy® gas permeable membranes (Sigma-Aldrich) and placed in a static incubator at 37°C. Fluorescence was then measured on the plate reader at 8 hours post-treatment.

Luciferase secretion reporter assay. A plasmid carrying tetracycline resistance and an inactive ExoU-HiBiT reporter under control of the *exoU* promoter (pME6032 exoU D344A Rep) was provided by Erwin Bohn at the Tübingen University Hospital in Germany. The plasmid was cloned using *E. coli* DH5α competent cells. Isolated plasmid was then electroporated into WT *P. aeruginosa* PA103 and T3SS mutant PA103 ΔpscJ to create the ExoU-HiBiT secretion reporter strains.

Both reporter strains were grown overnight in LB with 50 µg/mL tetracycline. Overnight cultures were centrifuged at 14,800rpm before removing supernatant and resuspending the cell pellet in low calcium LB supplemented with 50µg/mL tetracycline. Cultures were then diluted to an OD_{600} of 0.2 and plated in black

untreated 384-well plates (Corning 3573). 1860E metabolites were then pinned into the cultures using a Janus MDT robot (PerkinElmer). Plates were sealed with Breathe-Easy® gas permeable membranes (Sigma-Aldrich) and incubated at room temperature for 4 hours. A 1:4 dilution of NanoGlo® HiBiT Extracellular Detection System reagent (catalog number N2421; Promega) in 1XPBS was then added to plates at 50% v/v. Plates were then resealed and shook at 26°C for 5 minutes. Luminescence was then measured (PerkinElmer Envision 2105).

Statistical analysis. Prism 9 (GraphPad Software, La Jolla, CA, USA) was used to calculate the mean and standard error of the mean shown.

RESULTS

2.1 The bioactive lipid Sebastenoic Acid inhibits the *P. aeruginosa* T3SS

2.1.1 High-throughput screen of Actinobacteria-derived extracts to identify T3SS inhibitors.

In an effort to identify potential inhibitors of the *P. aeruginosa* T3SS, we adapted a luciferase transcriptional reporter screen originally developed by Aiello et al.[16]. *P. aeruginosa* PAO1 containing a transcriptional fusion of the *Photorhabdus luminescens lux* operon (*luxCDABE*) to the upstream regulatory region of the T3SS effector gene *exoT* (PAO1 *exoT-lux*) allowed for the use of bioluminescence as a readout for transcription of T3SS genes. Because the T3SS

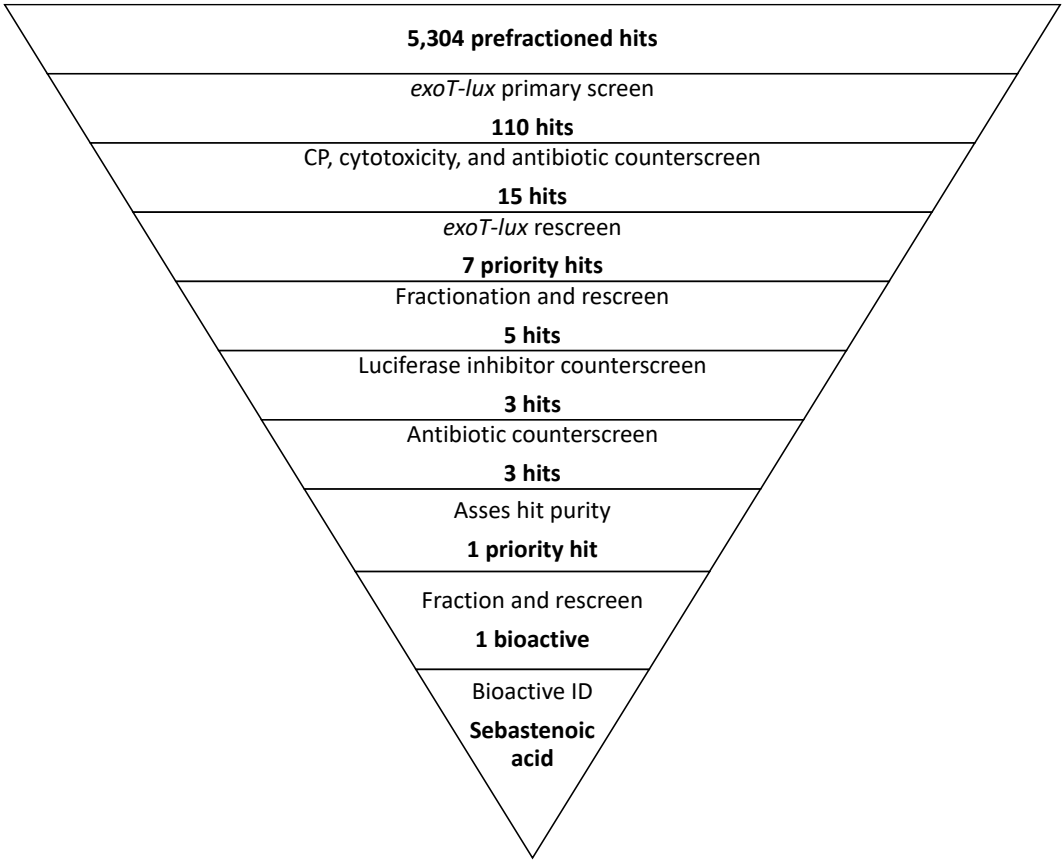


Figure 3. Actinomycete extract library workflow.

secretory protein ExsE serves as a negative regulator of the *P. aeruginosa* T3SS master activator ExsA, decrease in T3SS activity will reduce transcription of all T3SS genes.

Using this PAO1 *exoT-lux* reporter strain, we screened a library of 5,304 marine-derived Actinomycete prefractioned extracts[27,28] in triplicate. 110 prefractions reduced the luminescence by greater than 3 standard deviations and 1.5-fold below the mean of the DMSO-treated control in at least two of the three biological replicates. Data from previous screens for T3SS inhibitors[14,18] suggested that some of

these hit prefractions may contain both T3SS inhibitory compounds and general antibiotics. In addition, it is common for bioactive molecules to have adverse effects on mammalian cells. As part of our campaign to find T3SS inhibitors, we are interested in compounds that may be used for future T3SS research and therapeutics. Therefore, it is important that these compounds have minimal impact on mammalian cells. In studies by Wong et al. and Ochoa et al., this library was screened for antibiotic mode of action profiling in several bacterial strains[31] and cytological profiling in HeLa cells[28]. This library has also been screened for cytotoxic bioactivity in HeLa cells (W. Bray unpublished) and HEK293 cells using an NF-κB-GFP based screen previously describe in Lam et al.[14] (H. Lam unpublished). 95 extracts with antibiotic or cytotoxic bioactivity were eliminated using this data (**Table 3**), leaving 15 prefraction hits for follow up (**Fig. 4a**).

Fresh aliquots of the 15 hit prefractions were rescreened in triplicate using PAO1 *exoT-lux* in order to confirm their activity. All 15 hit prefractions reduced the luminescent signal by 3 standard deviations below the DMSO-treated control in all three biological replicates. Six of the 15 hits had significantly stronger activity and were prioritized for further fractionation. Three of the 15 hit prefractions were from the same parent extract, so the most active prefraction from this extract was also prioritized for further fractionation (**Fig. 4b**).

2.1.2 Identifying sebastenoic acid as an inhibitor of the *P. aeruginosa* T3SS. The seven priority prefractions were separated into more discrete fractions via high performance liquid chromatography. 10 fractions per prefraction were collected in

Prefraction	*exoT-lux* R1	*exoT-lux* R2	*exoT-lux* R3	CP Score	HeLa cell Cytotoxicity	BioMap	HEK239 cell Cytotoxicity	
RLUS-1451B	1.44337	1.20798	1.60350	4.39575	-0.06578		0.96396	
RLUS-1451C	2.36885	1.85465	2.55228	4.46364	0.08151		0.93698	
RLUS-1451D	2.25452	1.89712	2.32295	4.51144	0.14508	BS:1;	0.72042	
RLUS-1462E	1.77029	1.44424	1.26894	7.92933	-0.03012	EF:1;LI:1;	1.24787	
RLUS-1715E	1.52717	1.65770	1.79549	3.26939	0.17922	BS:1;EF:1;LI:1;SE:1;	1.24278	
RLUS-1860E	1.89138	1.71131	1.74214	4.68664	0.24664	BS:1;EF:1;LI:1;SE:1;SA:1;MRSA:1	1.10572	1.28899
RLUS-1881B	1.56850	1.31902	1.38010	5.09884	0.24996		1.80193	0.98943
RLUS-1901E	1.15244	1.61923	1.60820	4.32777	-0.04101	BS:1;EF:1	1.37041	1.00153
RLUS-1912E	1.67710	1.77389	1.11724	4.83048	0.15270		1.02049	1.29230
RLUS-1957A	1.18134	1.58647	1.10114	2.24618	0.09358		1.26606	0.96773
RLUS-1957B	1.23036	1.68499	1.17473	2.22170	0.10425		1.30179	0.97292
RLUS-1959B	1.18121	1.58141	1.13992	3.85743	0.26298		1.06285	1.68548
RLUS-1979D	1.18991	1.89830	1.46918	7.70593	-0.10291	PA:0.5	1.15263	1.03861
RLUS-1985D	1.49093	1.62695	1.43548	3.26962	0.05982		1.04079	1.66296
RLUS-1994D	1.31583	1.60566	1.29450	1.85188	0.04074	BS:1;SA:1;MRSA:1	1.53791	0.99090

Table 3. Counterscreen data of Actinomycete prefraction hits for follow up. 15 prefraction PAO1 *exoT-lux* hits were chosen for follow up based on their low antibacterial and cytotoxic bioactivity. A CP score of less than 10 indicated minimal adverse effects on HeLa cells. A HeLa cytotoxicity score of greater than -0.5 was considered non-cytotoxic. BioMap values range from 0-1 with 0 indicating resistance to the extract and 1 indicating high sensitivity. Extracts with little to no effect on gram-negative bacteria were chosen. BF: *Bacillus subtilis*, EF: *Enterococcus faecium*, LI: *Listeria ivanovii*, SE: *Staphylococcus* epidermis, SA: *Staphylococcus aureus*, MRSA: Methicillin-resistant SA, PA: *Providencia alcalifaciens*. A HEK239-GFP fold change less than or equal to 1.5 was considered non-cytotoxic.

three-minute fragments starting at 2 minutes and ending at 32 minutes. The resulting 70 fractions were then screened against PAO1 *exoT-lux* in triplicate. Six of the 70 fractions reduced the luminescence 3 standard deviations below the mean of the DMSO-treated control in all three biological replicates (**Fig. 5a**).

After using a luciferase-based screen as our primary method for inhibitor discovery, we reasoned that some of our hits may be luciferase inhibitors. In order to screen for luciferase inhibitors, we established a microtiter assay using a strain of *P. aeruginosa* PAO1 that constitutively expresses the *Photorhabdus luminescens lux* operon (PAO1 Xen 41), independent of the T3SS. The six hit fractions were screened against PAO1 Xen 41 in triplicate. Luminesce produced by PAO1 *exoT-lux* and PAO1 Xen 41 after treatment with each hit fraction was normalized as a percentage of the DMSO-treated control. Unpaired t-tests were then used to analyze the normalized values and determine if the signal reduction in the reporter screen was due to luciferase inhibition rather than T3SS inhibition. Only three of the six hit fractions (RLUS-1860E-3, RLUS-1901E-6, and RLUS 1901E-7) had a p-value of less than 0.05, which indicates that the decrease in *exoT* expression is greater than luciferase inhibition (**Fig 5b**). In addition, in order to confirm that these hits did not impact *P. aeruginosa* viability, we carried out a bacterial cell viability and found that none of the three remaining hit fractions affected cell viability (**Fig. 5c**). Notably, two of the three remaining fractions of interest, RLUS-1901E-6 and RLUS 1901E-7, were sequential fractions from the same parent prefraction. It is likely that these fractions

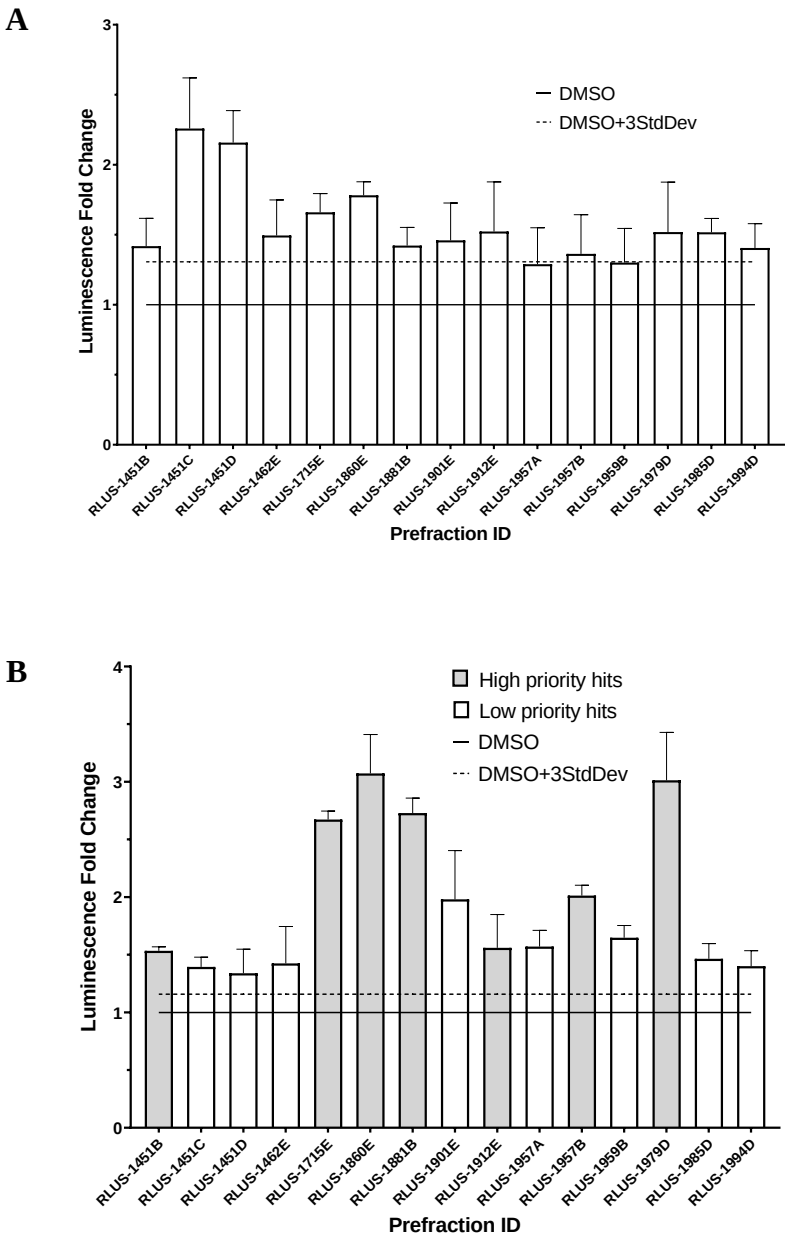


Figure 4. Identification of Actinobacteria-derived prefractions that inhibit T3SS-driven bioluminescence. (A and B) PAO1 *exoT-luxCDABE* was treated with prefractioned extracts and T3SS gene transcription was measured through bioluminescence. Fold change represents the ratio by which a prefraction reduced the luminescent signal in comparison to the DMSO-treated control. Results are a mean of 3 biological replicates. **(A)** Bioactivity of 15 hit prefractions in the primary screen of the Actinomycete library. **(B)** Fresh aliquots of the 15 primary screen hits were rescreened against PAO1 *exoT-lux* to confirm their bioactivity. 7 hits were prioritized for follow up based on their bioactivity.

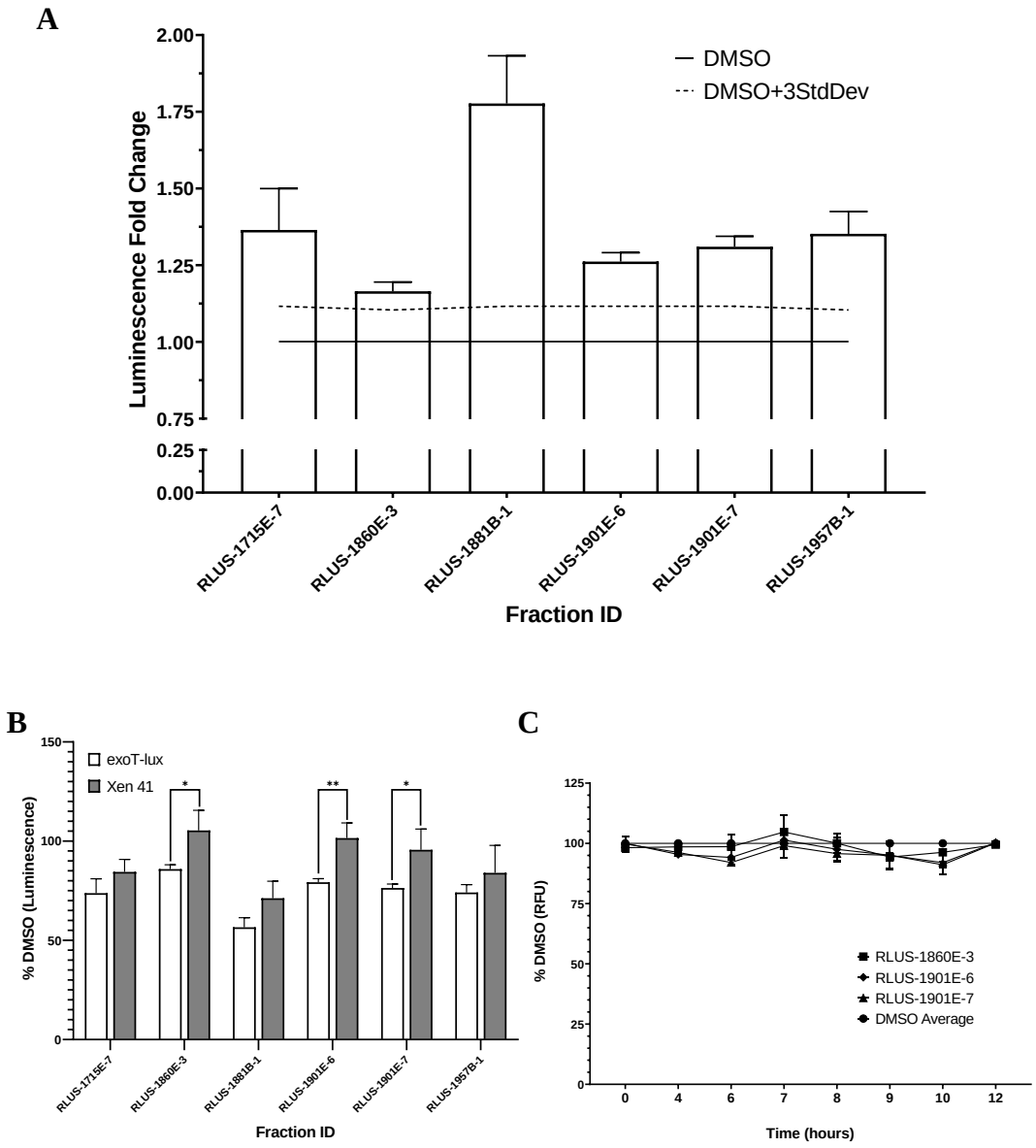


Figure 5. Identification of fractions that inhibit T3SS-driven bioluminescence. **(A)** PAO1 *exoT-luxCDABE* was treated with extract fractions and T3SS gene transcription was measured through bioluminescence. Fold change represents the ratio by which a fraction reduced the luminescent signal in comparison to the DMSO-treated control. **(B)** Hit fractions were counterscreened for general luciferase activity. PAO1 Xen41 was treated with extract fractions and bioluminescence was measured. Values were normalized as a percentage of the DMSO control and compared to normalized PAO1 *exoT-lux* values. Extracts with a p-value > 0.05 were considered luciferase inhibitors. **(C)** PAO1 WT was treated with extract fractions and effects on bacterial viability were measured for 12 hours. Values were normalized as a percentage of the DMSO control. All results are a mean of 3 biological replicates.

contain the same bioactive molecule, with the more active fraction, RLUS-1901E-7, containing a higher concentration of that molecule.

HPLC revealed that the RLUS-1860E-3 fraction contained only two peaks corresponding to potential bioactive molecules (**Fig. 6a**). Compared to the RLUS-1901E fractions, RLUS-1860E-3 was much more pure (**Fig. 6**). Therefore, we chose to focus on RLUS-1860E-3 for further follow up. RLUS-1860E-3 was then reisolated after regrowing the parent Actinobacterium in order to gather more material for structure elucidation. HPLC on the regrown fraction revealed four metabolite peaks (**Fig. 7a**), with a 32-minute peak corresponding to the 13-minute peak observed in the original fraction. All four metabolites were isolated and screened for bioactivity against PAO1 *exoT-lux* in triplicate(**Fig. 7b**). Out of the four metabolites, only the metabolite from the 32-minute peak reduced the luminescent signal 3 standard deviations below the DMSO-treated control. These results suggested that this 32-minute peak indeed contained the molecule responsible for the bioactivity observed in the parent fraction and prefraction.

In order to confirm the 32-minute peak metabolite contained T3SS-inhibitory bioactivity, we took advantage of a reporter plasmid developed by Wagner et al.[28] (generously provided by Erwin Bohn at the Tübingen University Hospital in Germany). This plasmid contains a catalytically inactive effector protein, ExoU, fused to the small fragment of a split luciferase (HiBiT) under the native *exoU* promoter (unpublished). We transformed this plasmid into *P. aeruginosa* PA103. Under T3SS inducing conditions, this ExoU-HiBiT fusion is secreted out of the cell

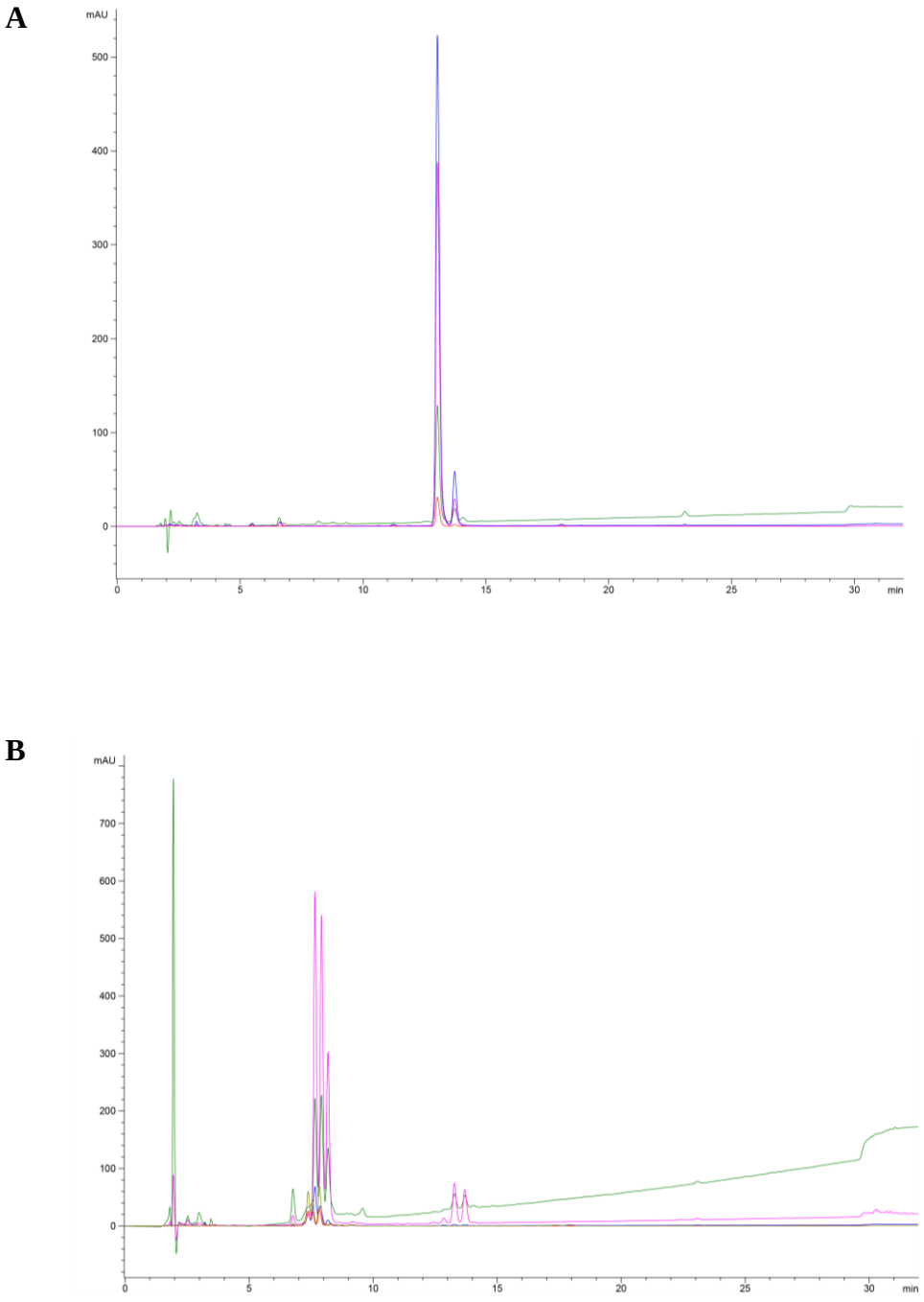


Figure 6. HPLC reveals that the RLUS-1860E-3 fraction contains only 2 potential bioactive molecules. HPLC traces of **(A)** RLUS-1860E-3 and **(B)** RLUS-1901E-7. Based on the HPLC trace, only a simple isolation on RLUS-1860E-3 would be needed for pure compound. One of the two peaks corresponds to the molecule responsible for the bioactivity observed from RLUS-1860E-3

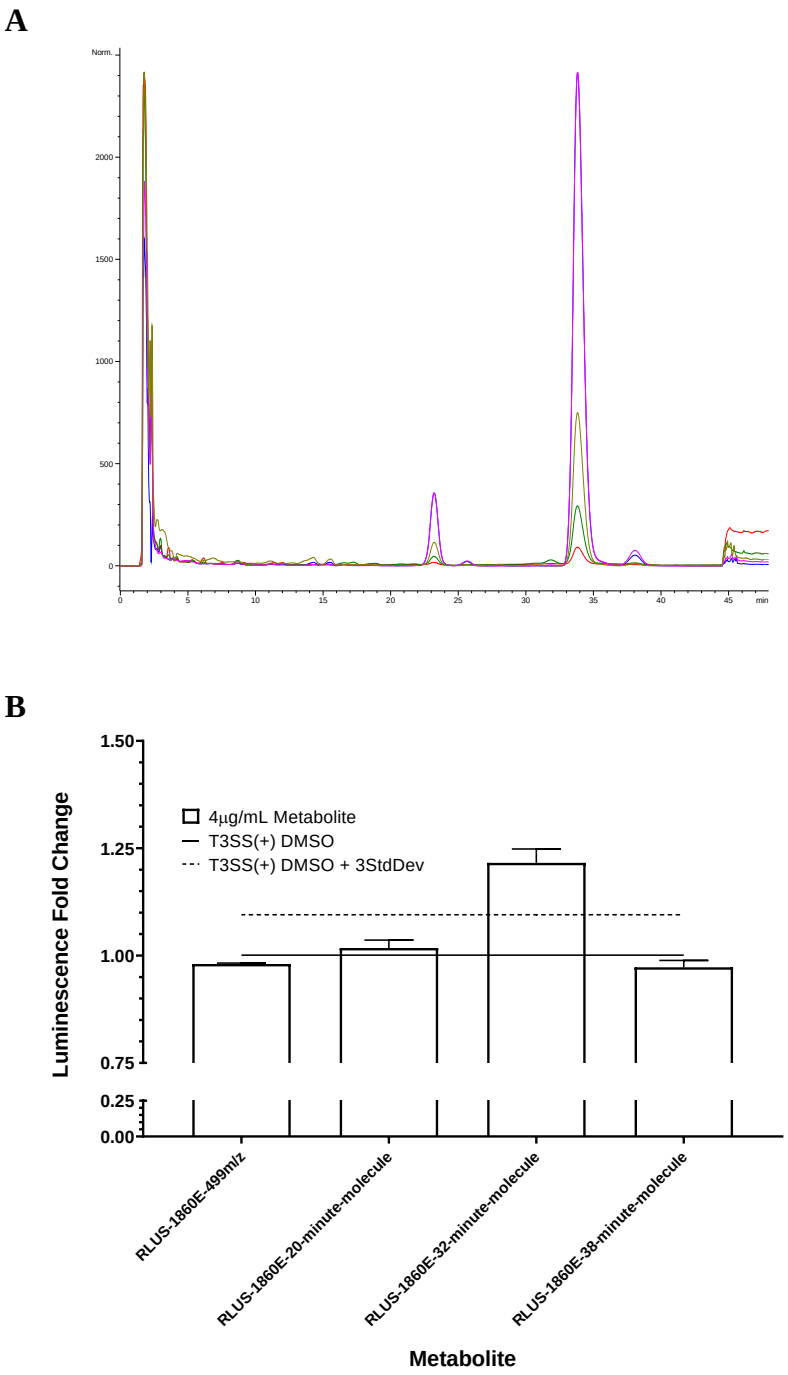


Figure 7. Identification of the bioactive metabolite in the 1860E regrown fraction. **(A)** HPLC data shows 3 major metabolites collected from the regrow of 1860E. An additional molecule with no UV trace, "499m/*z*", was collected. **(B)** PAO1 *exoT-luxCDABE* was treated with 1860E metabolites and T3SS gene transcription was measured through bioluminescence. Fold change represents the ratio by which a metabolite reduced the luminescent signal in comparison to the DMSO-treated control.

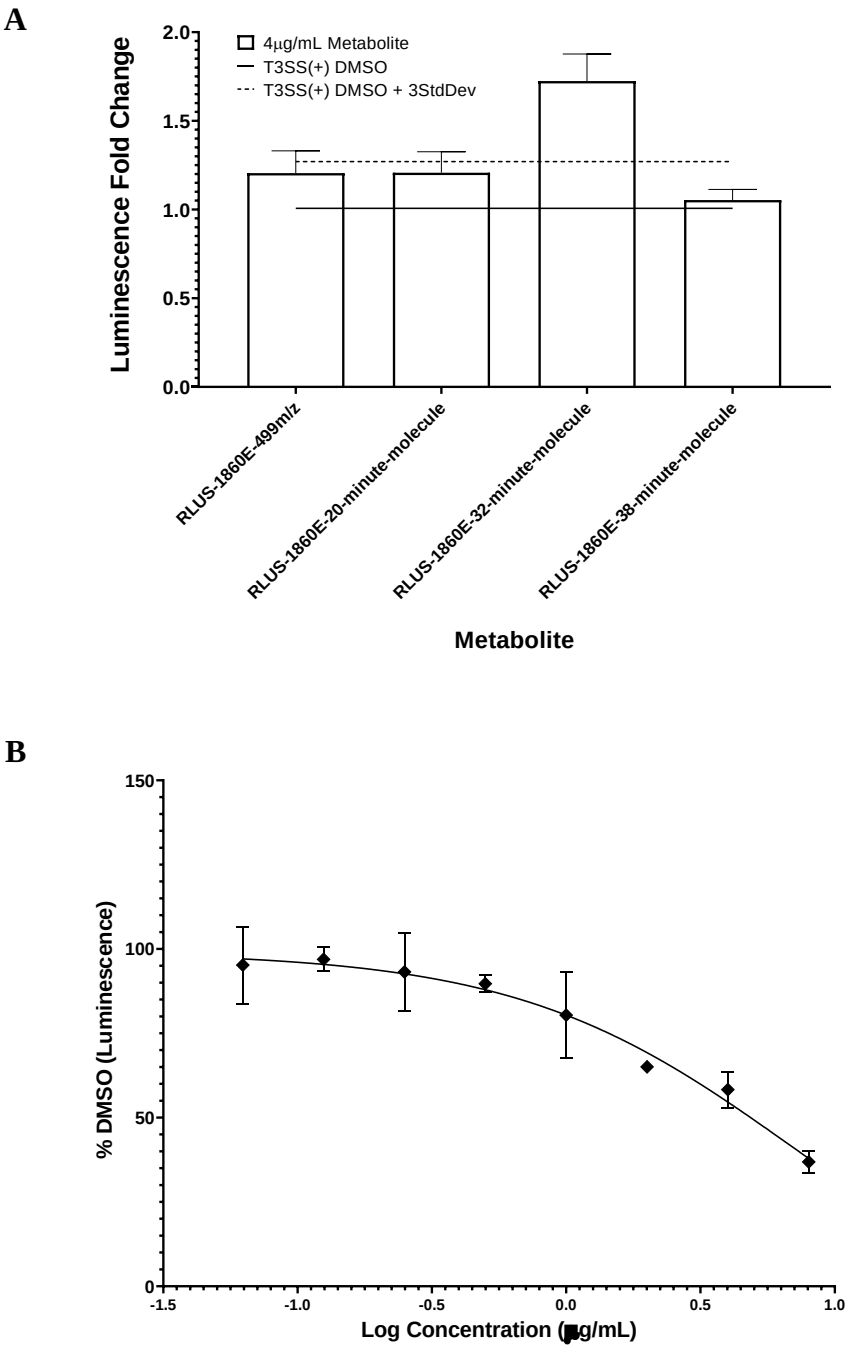


Figure 8. Confirming the T3SS inhibitory bioactivity of the 32-minute molecule using a luciferase secretion reporter assay. (A and B) PAO1 ExoU-HiBiT was treated with 1860E metabolites and T3SS secretion was monitored through bioluminescence. **(A)** Fold change represents the ratio by which a compound reduced the luminescent signal in comparison to the DMSO-treated control. **(B)** The 32-minute molecule inhibits ExoU-HiBiT secretion in a dose-dependent manner. Values were normalized as a percentage of the DMSO-treated control. All results are a mean of 3 biological replicates.

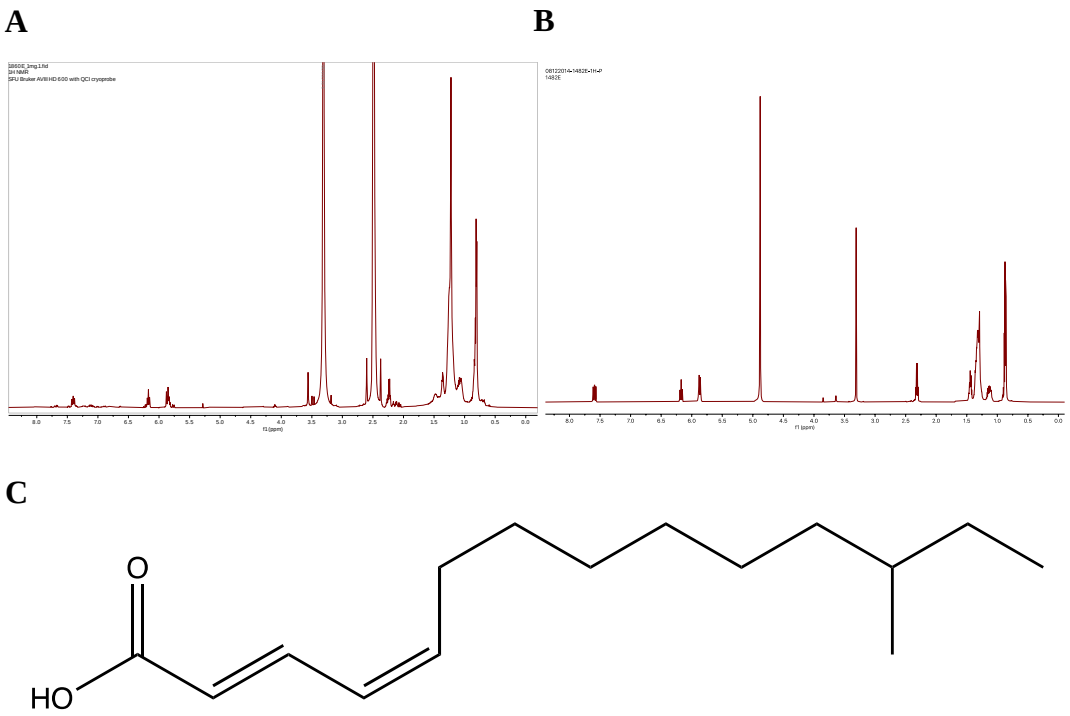


Figure 9. The 32-minute molecule is identified as sebastenoic acid. (A and B) Proton NMR in d4-methonol of **(A)** the 32-minute molecule was matched to **(B)** the NMR of sebastenoic acid published by Warner et al.[29]. Peaks in both traces occur at the same proton chemical shifts. **(C)** Structure of sebastenoic acid.

and can generate bioluminescence if the large fragment (LgBit) of the split luciferase is provided. Using this method as an orthogonal assay, we confirmed the T3SS inhibitory activity of the RLUS-1860E-3 32-minute molecule (**Fig. 8**).

A combination of NMR, LCMS, and HSQC was used to elucidate the structure of the 32-minute molecule. After the initial datasets, half of the structure remained uncertain. However, a carbon NMR provided insight that implied there were carbon atoms at positions that were previously thought to be nitrogen atoms. Provided with this insight, a proton NMR of this 32-minute molecule in d4-methanol was matched to the proton NMR of sebastenoic acid[25], a bioactive lipid (**Fig. 9**).

Interestingly, this novel compound has previously characterized to possess antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecium*, and *Vibrio mimicus*[29] as well as motility and biofilm-related bioactivity in *Vibrio cholerae*[25].

2.2 A screen of *Burkholderia*-derived natural product extracts yields six lead prefractions with T3SS inhibitory bioactivty

2.2.1 High-throughput screen of *Burkholderia*-derived extracts to identify T3SS inhibitors.

A library of approximately 960 prefractioned *Burkholderia* extracts was previously created from plant root isolates collected in British Columbia[30]. To examine if any of these unique extracts possessed bioactivity against the *P. aeruginosa* T3SS, we screened these prefractions using PAO1 *exoT-lux*. 235 prefractions reduced bioluminescence by greater than 3 standard deviations and 1.5-fold below the mean of the DMSO-treated control in at least two of the three biological replicates. Counterscreening these hits for luciferase inhibitors using PAO1 Xen 41 reduced the total hits to 110 prefractions. *P. aeruginosa* viability in the presence of these prefractions was then examined and only six of the 110 prefractions did not reduce the fluorescent signal below 85% of the DMSO-treated control (**Fig. 10c**). As a part of an ongoing investigation, the bioactivity of these hit prefractions will next be confirmed using an orthogonal secretion-based assay prior to fractionation and rescreening (**Fig. 11**).

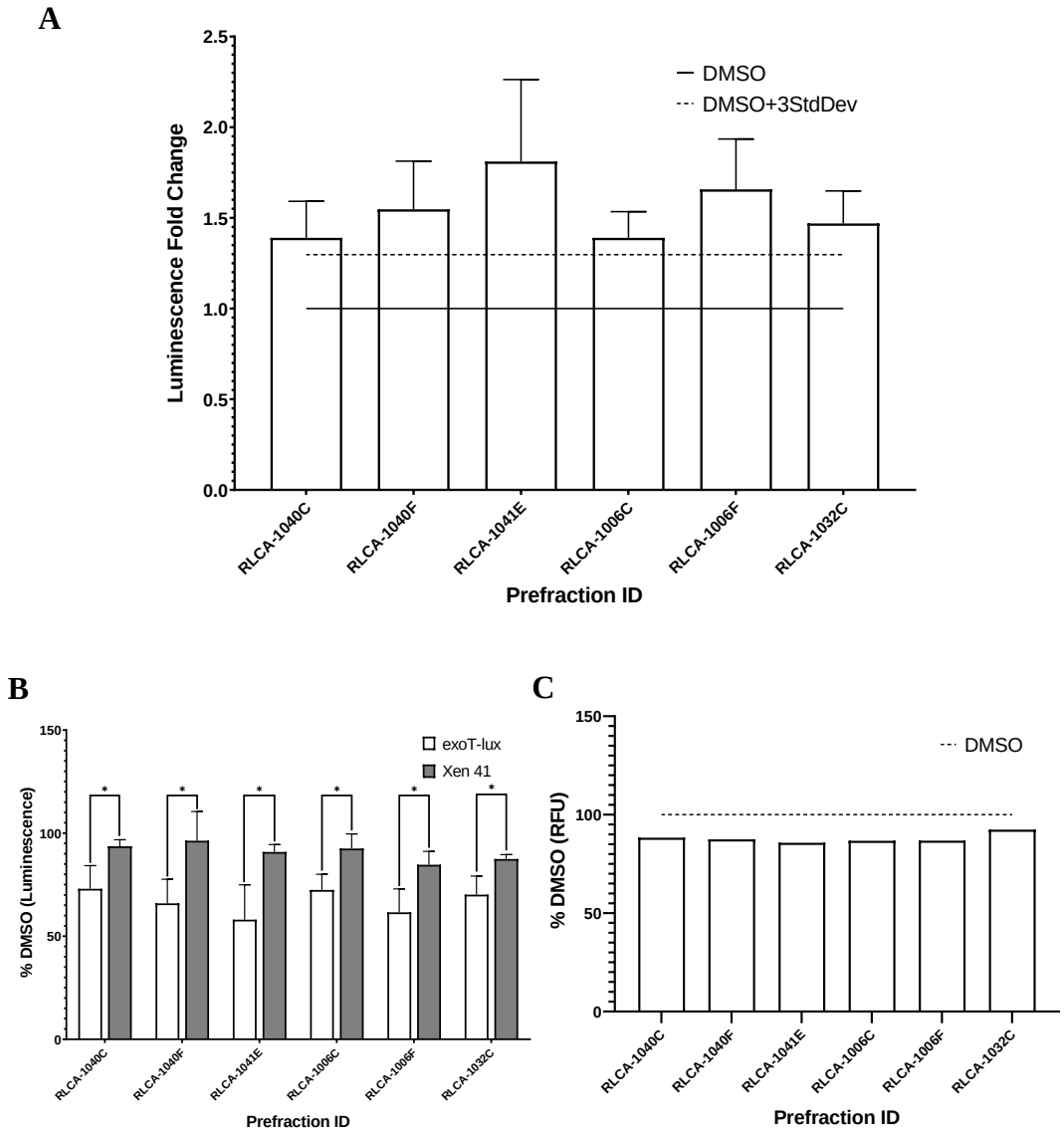


Figure 10. Identification of *Burkholderia*-derived prefractions that inhibit T3SS-driven bioluminescence. (A) PAO1 *exoT-luxCDABE* was treated with prefractioned extracts and T3SS gene transcription was measured through bioluminescence. Fold change represents the ratio by which a prefraction reduced the luminescent signal in comparison to the DMSO-treated control. Results are a mean of 3 biological replicates. **(B)** Hit prefractions were counterscreened for general luciferase activity. PAO1 Xen41 was treated with prefractioned extracts and bioluminescence was measured. Values were normalized as a percentage of the DMSO control and compared to normalized PAO1 *exoT-lux* values. Prefractions with a p-value > 0.05 were considered luciferase inhibitors. Results are a mean of 3 biological replicates. **(C)** PAO1 WT was treated with prefractioned extracts and effects on bacterial viability were measured after 8 hours. Values were normalized as a percentage of the DMSO control. Prefractions which reduced fluorescence below 85% were considered to have general antibiotic activity.

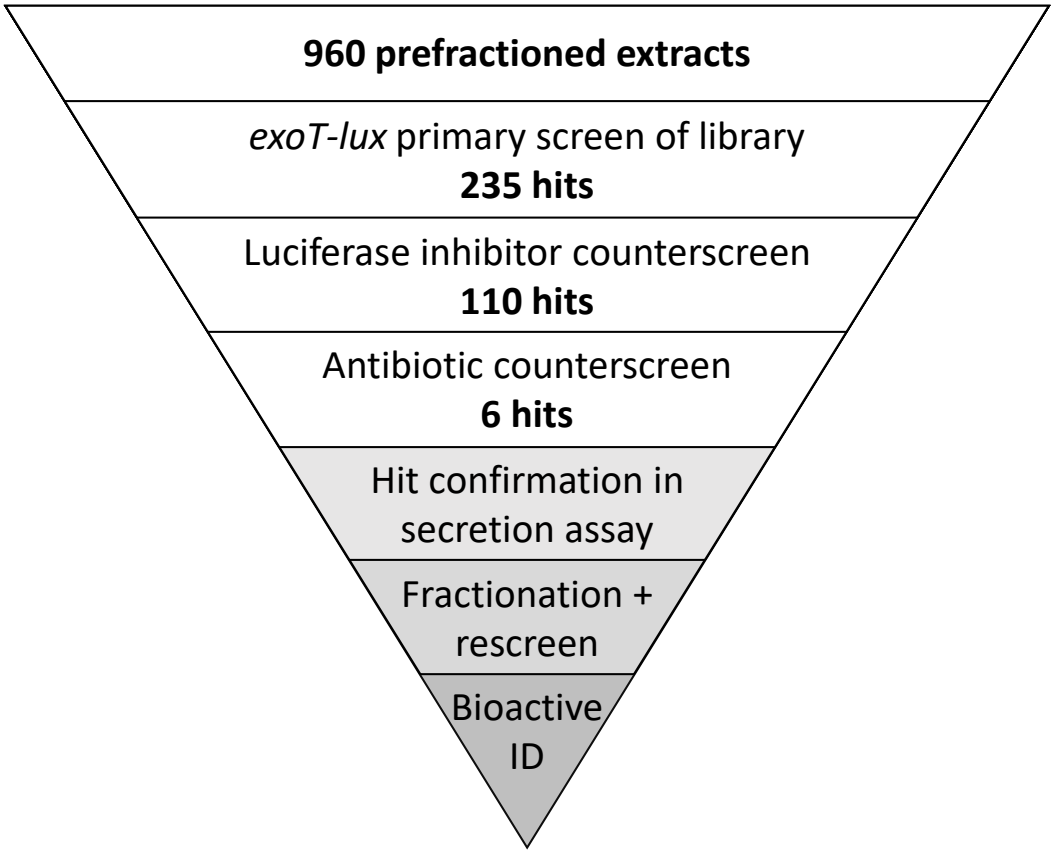


Figure 11. *Burkholderia* extract library workflow

DISCUSSION

In this study, we screened two unique natural product libraries containing a total of 6,264 microbially-derived prefractioned extracts and identified six lead extracts along with one purified small molecule lad inhibitor of the *P. aeruginosa* T3SS, sebastenoic acid. Fractions containing sebastenoic acid and these six lead extracts specifically inhibited T3SS promoter activity without impacting bacterial viability. In addition, the sebastenoic acid parent prefraction did not have a significant impact on

mammalian cells. Furthermore, purified sebastenoic acid showed dose-dependent inhibition of both T3SS gene transcription and effector protein secretion.

Sebastenoic acid. Sebastenoic acid was previously identified as a modulator of motility and biofilm formation in *Vibrio cholerae*[29]. Interestingly, this bioactive lipid promoted motility repressed by cyclic-di-GMP while also enhancing biofilm-associated gene (*vpsL*) levels and biofilm formation. The bacterial secondary messenger, cyclic-di-GMP, has been well characterized as a key signaling molecule in the transition from motile to sessile state in several bacteria, including *Vibrio*. High levels of cyclic-di-GMP are associated with biofilm formation and chronic infections while low levels are associated with motility and virulent acute infections[34]. Thus, the researchers speculated that sebastenoic acid may increase the cyclic-di-GMP pool and may indirectly affect motility.

In addition to promoting biofilm formation[35,36], cyclic-di-GMP inhibits T3SS gene expression in *P. aeruginosa*[37]. High levels of cyclic-di-GMP result in low levels of another secondary messenger, cyclic AMP (cAMP)[34]. cAMP is allosteric activator of the virulence factor regulator Vfr[37,38]. This cAMP-Vfr system acts as a global regulator of virulence gene expression, including the T3SS. As a regulator of the T3SS, Vfr directly activates transcription of the T3SS master regulator ExsA[39]. Thus, inhibition of the T3SS by cyclic-di-GMP is due to repression of cAMP-Vfr-dependent promoter activity.

Based on its bioactivity in *Vibrio* and *Pseudomonas*, it is likely that sebastenoic acid affects T3SS gene expression and secretion indirectly through positive modulation of cyclic-di-GMP levels. Furthermore, it is expected that sebastenoic will increase biofilm formation in *P. aeruginosa* as it does in *V. cholerae*. Excitingly, these findings may allow future use of sebastenoic acid as a molecular probe for better understanding cyclic-di-GMP regulation. For example, in *Yersinia* spp., cyclic-di-GMP has not yet been linked to T3SS gene expression. However, it has been shown that the RNA chaperone Hfq directly represses cyclic-di-GMP levels in *Y. pestis*[40] while also acting as a positive posttranscriptional regulator of the T3SS in both *Y. pseudotuberculosis*[41] and *Y. pestis*[42]. By measuring *Yersinia* T3SS-dependent secretion in the presence of sebastenoic acid, we may be able to test the effect of cyclic-di-GMP in *Yersinia* T3SS gene regulation.

Lead *Burkholderia*-derived Extracts. Six lead extracts for further investigation were identified after screening a library of *Bukholderia*-derived extracts (**Table 4**). *Burkholderia* bacteria have recently garnered attention as sources for natural products due to their considerably large genomes which contribute to broad phenotypic/genotypic diversity and subsequently a vast array of unique natural products[43]. Furthermore, a handful of unusual and novel bioactive structures have been isolated from *Burkholderia* strains[30,43,44]. Lead extracts which repeat their T3SS-inhibitory activity in a secretion-based orthogonal assay will be deconvoluted in order to identify the bioactive molecule. As a relatively new source of interest for natural

products, the *Burkholderia* genus present an opportunity for the discovery of novel structures and novel bioactive molecules.

Hit	16S ribosomal RNA Match
RLCA-1040C	*Paraburkholderia phytofirmans*
RLCA-1040F	*Paraburkholderia phytofirmans*
RLCA-1041E	*Paraburkholderia bryophila*
RLCA-1006C	*Paraburkholderia megapolitana*
RLCA-1006F	*Paraburkholderia megapolitana*
RLCA-1032C	*Paraburkholderia caffeinilytica*

Table 4. *Burkholderia* parent strains of hit prefractions for follow up.

REFERENCES

1. Brown ED, Wright GD. Antibacterial drug discovery in the resistance era. *Nature*. 2016;529(7586):336-343. doi:10.1038/nature17042

2. Navidinia M. The clinical importance of emerging ESKAPE pathogens in nosocomial infections. *1*. 2016;7(3):43-57. doi:10.22037/jps.v7i3.12584

3. Santajit S, Indrawattana N. Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *Biomed Res Int*. 2016;2016. doi:10.1155/2016/2475067

4. Moore NM, Flaws ML. Epidemiology and Pathogenesis of *Pseudomonas aeruginosa* Infections. *Clin Lab Sci*. 2011;24(1):43-46. doi:10.29074/ascls.24.1.43

5. Horcajada JP, Montero M, Oliver A, et al. Epidemiology and Treatment of Multidrug-Resistant and Extensively Drug-Resistant Pseudomonas aeruginosa Infections. *Clinical Microbiology Reviews*. 2019;32(4). doi:10.1128/CMR.00031-19

6. Aloush V, Navon-Venezia S, Seigman-Igra Y, Cabili S, Carmeli Y. Multidrug-Resistant Pseudomonas aeruginosa: Risk Factors and Clinical Impact. *Antimicrobial Agents and Chemotherapy*. 2006;50(1):43-48. doi:10.1128/AAC.50.1.43-48.2006

7. Breidenstein EBM, de la Fuente-Núñez C, Hancock REW. Pseudomonas aeruginosa: all roads lead to resistance. *Trends in Microbiology*. 2011;19(8):419-426. doi:10.1016/j.tim.2011.04.005

8. Centers for Disease Control and Prevention (U.S.). *Antibiotic Resistance Threats in the United States, 2019*. Centers for Disease Control and Prevention (U.S.); 2019. doi:10.15620/cdc:82532

9. Deng W, Marshall NC, Rowland JL, et al. Assembly, structure, function and regulation of type III secretion systems. *Nat Rev Microbiol*. 2017;15(6):323-337. doi:10.1038/nrmicro.2017.20

10. Galle M, Carpentier I, Beyaert R. Structure and function of the Type III secretion system of Pseudomonas aeruginosa. *Curr Protein Pept Sci*. 2012;13(8):831-842. doi:10.2174/138920312804871210

11. Roy-Burman A, Savel RH, Racine S, et al. Type III Protein Secretion Is Associated with Death in Lower Respiratory and Systemic Pseudomonas aeruginosa Infections. *The Journal of Infectious Diseases*. 2001;183(12):1767-1774. doi:10.1086/320737

12. Kauppi AM, Nordfelth R, Uvell H, Wolf-Watz H, Elofsson M. Targeting Bacterial Virulence: Inhibitors of Type III Secretion in Yersinia. *Chemistry & Biology*. 2003;10(3):241-249. doi:10.1016/S1074-5521(03)00046-2

13. Bowser TE, Bartlett VJ, Grier MC, et al. Novel anti-infection agents: Small-molecule inhibitors of bacterial transcription factors. *Bioorganic & Medicinal Chemistry Letters*. 2007;17(20):5652-5655. doi:10.1016/j.bmcl.2007.07.072

14. Lam H, Schwochert J, Lao Y, et al. Synthetic Cyclic Peptomers as Type III Secretion System Inhibitors. *Antimicrob Agents Chemother*. 2017;61(9):e00060-17. doi:10.1128/AAC.00060-17

15. Anantharajah A, Buyck JM, Sundin C, Tulkens PM, Mingeot-Leclercq M-P, Van Bambeke F. Salicylidene Acylhydrazides and Hydroxyquinolines Act as Inhibitors of Type Three Secretion Systems in Pseudomonas aeruginosa by Distinct Mechanisms. *Antimicrob Agents Chemother*. 2017;61(6). doi:10.1128/AAC.02566-16

16. Aiello D, Williams JD, Majgier-Baranowska H, et al. Discovery and Characterization of Inhibitors of Pseudomonas aeruginosa Type III Secretion. *Antimicrob Agents Chemother*. 2010;54(5):1988-1999. doi:10.1128/AAC.01598-09

17. Yamazaki A, Li J, Zeng Q, et al. Derivatives of Plant Phenolic Compound Affect the Type III Secretion System of Pseudomonas aeruginosa via a GacS-GacA Two-Component Signal Transduction System. *Antimicrob Agents Chemother*. 2012;56(1):36-43. doi:10.1128/AAC.00732-11

18. Duncan MC, Wong WR, Dupzyk AJ, Bray WM, Linington RG, Auerbuch V. An NF-κB-Based High-Throughput Screen Identifies Piericidins as Inhibitors of the Yersinia pseudotuberculosis Type III Secretion System. *Antimicrob Agents Chemother*. 2014;58(2):1118-1126. doi:10.1128/AAC.02025-13

19. Pendergrass HA, May AE. Natural Product Type III Secretion System Inhibitors. *Antibiotics (Basel)*. 2019;8(4). doi:10.3390/antibiotics8040162

20. Tabor DE, Oganesyan V, Keller AE, et al. Pseudomonas aeruginosa PcrV and Psl, the Molecular Targets of Bispecific Antibody MEDI3902, Are Conserved Among Diverse Global Clinical Isolates. *The Journal of Infectious Diseases*. 2018;218(12):1983-1994. doi:10.1093/infdis/jiy438

21. Ali SO, Yu XQ, Robbie GJ, et al. Phase 1 study of MEDI3902, an investigational anti–Pseudomonas aeruginosa PcrV and Psl bispecific human monoclonal antibody, in healthy adults. *Clinical Microbiology and Infection*. 2019;25(5):629.e1-629.e6. doi:10.1016/j.cmi.2018.08.004

22. Bowlin NO, Williams JD, Knoten CA, et al. Mutations in the Pseudomonas aeruginosa Needle Protein Gene pscF Confer Resistance to Phenoxyacetamide Inhibitors of the Type III Secretion System. *Antimicrob Agents Chemother*. 2014;58(4):2211-2220. doi:10.1128/AAC.02795-13

23. Stover CK, Pham XQ, Erwin AL, et al. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature*. 2000;406(6799):959-964. doi:10.1038/35023079

24. Kang PJ, Hauser AR, Apodaca G, et al. Identification of Pseudomonas aeruginosa genes required for epithelial cell injury. *Molecular Microbiology*. 1997;24(6):1249-1262. doi:10.1046/j.1365-2958.1997.4311793.x

25. Liu PV. The Roles of Various Fractions of "Pseudomonas aeruginosa" in Its Pathogenesis: III. Identity of the Lethal Toxins Produced in Vitro and in Vivo [with Discussion]. *The Journal of Infectious Diseases*. 1966;116(4):481-489.

26. Shafikhani SH, Morales C, Engel J. The Pseudomonas aeruginosa type III secreted toxin ExoT is necessary and sufficient to induce apoptosis in epithelial cells. *Cellular Microbiology*. 2008;10(4):994-1007. doi:10.1111/j.1462-5822.2007.01102.x

27. Schulze CJ, Donia MS, Siqueira-Neto JL, et al. Genome-directed lead discovery: biosynthesis, structure elucidation, and biological evaluatWion of two families of polyene macrolactams against Trypanosoma brucei. *ACS Chem Biol*. 2015;10(10):2373-2381. doi:10.1021/acschembio.5b00308

28. Ochoa JL, Bray WM, Lokey RS, Linington RG. Phenotype-Guided Natural Products Discovery Using Cytological Profiling. *J Nat Prod*. 2015;78(9):2242-2248. doi:10.1021/acs.jnatprod.5b00455

29. Warner CJA, Salinas M, Zamorano-Sánchez D, et al. The Bioactive Lipid (S)-Sebastenoic Acid Impacts Motility and Dispersion in Vibrio cholerae. *Can J Chem*. 2018;96(2):196-203. doi:10.1139/cjc-2017-0334

30. Haeckl FPJ, Baldim JL, Iskakova D, Kurita KL, Soares MG, Linington RG. A selective genome-guided method for environmental Burkholderia isolation. *Journal of Industrial Microbiology and Biotechnology*. 2019;46(3-4):345-362. doi:10.1007/s10295-018-02121-x

31. Wong WR, Oliver AG, Linington RG. Development of BioMAP Screening for the Classification and Discovery of Natural Product Antibiotics. *Chem Biol*. 2012;19(11):1483-1495. doi:10.1016/j.chembiol.2012.09.014

32. Westerhausen S, Nowak M, Torres-Vargas CE, et al. A NanoLuc luciferase-based assay enabling the real-time analysis of protein secretion and injection by bacterial type III secretion systems. *Molecular Microbiology*. 2020;113(6):1240-1254. doi:https://doi.org/10.1111/mmi.14490

33. Sanchez LM, Wong WR, Riener RM, Schulze CJ, Linington RG. Examining the Fish Microbiome: Vertebrate-Derived Bacteria as an Environmental Niche for the Discovery of Unique Marine Natural Products. *PLoS One*. 2012;7(5):e35398. doi:http://dx.doi.org/10.1371/journal.pone.0035398

34. Römling U, Galperin MY, Gomelsky M. Cyclic di-GMP: the First 25 Years of a Universal Bacterial Second Messenger. *Microbiology and Molecular Biology Reviews*. 2013;77(1):1-52. doi:10.1128/MMBR.00043-12

35. Hickman JW, Tifrea DF, Harwood CS. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *PNAS*. 2005;102(40):14422-14427. doi:10.1073/pnas.0507170102

36. Borlee BR, Goldman AD, Murakami K, Samudrala R, Wozniak DJ, Parsek MR. Pseudomonas aeruginosa uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. *Molecular Microbiology*. 2010;75(4):827-842. doi:10.1111/j.1365-2958.2009.06991.x

37. McMackin EAW, Djapgne L, Corley JM, Yahr TL. Fitting Pieces into the Puzzle of Pseudomonas aeruginosa Type III Secretion System Gene Expression. *Journal of Bacteriology*. 2019;201(13). doi:10.1128/JB.00209-19

38. Almblad H, Harrison JJ, Rybtke M, et al. The Cyclic AMP-Vfr Signaling Pathway in Pseudomonas aeruginosa Is Inhibited by Cyclic Di-GMP. *Journal of Bacteriology*. 2015;197(13):2190-2200. doi:10.1128/JB.00193-15

39. Marsden AE, Intile PJ, Schulmeyer KH, et al. Vfr Directly Activates exsA Transcription To Regulate Expression of the Pseudomonas aeruginosa Type III Secretion System. *Journal of Bacteriology*. 198(9):1442-1450. doi:10.1128/JB.00049-16

40. Bellows LE, Koestler BJ, Karaba SM, Waters CM, Lathem WW. Hfq-dependent, co-ordinate control of cyclic diguanylate synthesis and catabolism in the plague pathogen Yersinia pestis. *Molecular Microbiology*. 2012;86(3):661-674. doi:10.1111/mmi.12011

41. Schiano CA, Bellows LE, Lathem WW. The Small RNA Chaperone Hfq Is Required for the Virulence of Yersinia pseudotuberculosis. *Infection and Immunity*. 2010;78(5):2034-2044. doi:10.1128/IAI.01046-09

42. Schiano CA, Koo JT, Schipma MJ, Caulfield AJ, Jafari N, Lathem WW. Genome-Wide Analysis of Small RNAs Expressed by Yersinia pestis Identifies a Regulator of the Yop-Ysc Type III Secretion System. *J Bacteriol*. 2014;196(9):1659-1670. doi:10.1128/JB.01456-13

43. Kunakom S, Eustáquio AS. Burkholderia as a Source of Natural Products. *J Nat Prod*. 2019;82(7):2018-2037. doi:10.1021/acs.jnatprod.8b01068

44. Liu X, Cheng Y-Q. Genome-guided discovery of diverse natural products from Burkholderia sp. *Journal of Industrial Microbiology and Biotechnology*. 2014;41(2):275-284. doi:10.1007/s10295-013-1376-1

Probing marine natural products for inhibitors of the *P. aeruginosa* T3SS

INTRODUCTION

Antibiotic resistance has become an increasingly rampant threat to global public health. Infections by antibiotic resistant pathogens have increased morbidity and mortality rates and are a significant economic burden on the healthcare system. In the U.S. alone, ~2.8 million people are affected by antibiotic-resistant pathogens annually resulting in >35,000 deaths[1]. Effective antimicrobials are critical in both the treatment and prevention of infections. However, an increasing number of healthcare-associated pathogens are developing resistance to classic antibiotics.

Nosocomial infections in both the developed and developing world are largely caused by so-called ESKAPE pathogens[2]. These ESKAPE pathogens display an increasingly high frequency of multidrug resistance[2–4] and the development of novel therapeutics for associated infections is desperately needed. One of these pathogens is the Gram-negative opportunistic pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* is a common environmental bacterium that takes advantage of hosts with weakened immune systems. An estimated 32,600 hospital patients are infected by multidrug-resistant *P. aeruginosa* annually in the U.S. , resulting in 2,700 deaths and generating $767 million in healthcare costs[1].

P. aeruginosa expresses a number of virulence factors that aid the bacteria in pathogenicity and colonization of the host. Inhibition of virulence factors can disable an invading pathogen, thereby facilitating clearance of the bacteria and recovery of

the host. In addition, targeting virulence factors spares our microbiome and mitigates the selective pressure that often results in resistant mutants. In *P. aeruginosa*, one virulence factor of interest is the type III secretion system (T3SS). The T3SS acts as a molecular syringe, forming a conduit between the bacterial and target host cell cytosol in order to translocate virulence-associated effector proteins into the host cell[5]. This multiprotein syringe-like apparatus is high conserved among dozens of Gram-negative pathogens including *Salmonella*, *Chlamydia*, and *Yersinia* but remains largely absent among commensal flora[6]. In *P. aeruginosa*, the T3SS aids in the prevention of phagocytosis, mediation of cytotoxicity, and promotion of systemic dissemination[7]. In clinical studies, *P. aeruginosa* isolates harboring a functional T3SS are associated with significantly higher morbidity and mortality rates[8].

While a small handful of synthetic-derived inhibitor of the *P. aeruginosa* T3SS have been identified[9–13], natural products have remained a largely untapped resource. Natural products exhibit high levels of chemical diversity and biochemical specificity, making them promising sources for drug leads[14]. In this study, we work to address the need for novel therapeutics against *P. aeruginosa* by assessing two unique libraries of marine natural product derivatives for their bioactivity against the T3SS. Using a bioluminescent-based T3SS reporter assay, we identified five lead extracts in addition to a known mycotoxin, penicillic acid, as inhibitors of the *P. aeruginosa* T3SS.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in **Table 5**. *P. aeruginosa* overnight cultures were grown at 37°C, shaking, in Luria-Bertani (LB) medium, supplemented with antibiotics where noted. Overnight cultures of *Y. pseudotuberculosis* were grown at 26°C, shaking, in 2× yeast extract and tryptone (2×YT) medium. All shaking incubations were at 250 rpm. Low calcium LB (LB with 20mM $MgCl_2$ and 5mM EGTA) was used to induce the *P. aeruginosa* T3SS.

Natural product libraries. An in-house library of natural product-derived pure compounds was created by the Crews research group over the past several decades from marine fungi, sponges, and bacteria. A separate library of natural product extracts was created from sponges and marine bacteria as described previously[19].

Briefly, each sponge was exhaustively extracted with methanol (coded TPE), after which the dried extract was subjected to liquid−liquid extraction between CH_2Cl_2 (coded F) and H_2O (coded W) to remove the majority of the salts. Afterwich, 200 mg of the F fractions was separated into six fractions (coded F1−F6) using preparative HPLC on a Phenomenex Gemini-NX C18 (50 x 21.2 mm, 5 µm) column. Each fraction was then loaded into a 96-well screening plate at 5 mg/ml in DSMO.

Bacterial strains were cultured in a total of 5L of liquid media (5 x 1L flasks) for 21 days, at 250 rpm and room temperature. Culture broths were extracted using EtOAc and dried down under vacuum (coded F fraction). A total of 200 mg of extract

Strain	Description	Reference
P. aeruginosa strains		
PAO1	Wild type PAO1	15
PAO1 *exoT-lux*	PAO1::pGSV3-exoT-luxCDABE	12
PAO1 ΔpscC *exoT-lux*	ΔpscC PAO1-LAC::pGSV3-exoT-luxCDABE	12
PAO1 Xen 41	PAO1 with a stable copy of the *Photorhabdus luminescens* lux operon in the chromosome	PerkinElmer 119229
PA103	Wild type PA103	16,17
Y. pseudotuberculosis strains		
YPIII Xen4 n=1	YPIII with luciferase reporter inserted into the pYV plamid; plasmid copy number = 1	18

Table 5. Bacterial strains used in this study

was resuspended in MeOH and separated into six fractions (coded F1-F6) using preparative HPLC on a Phenomenex Gemini-NX C18 (50 x 21.2 mm, 5 µm) column. Each fraction was then loaded into a 96-well screening plate at 5 mg/ml in DSMO.

384-well plates were prepared for screening from the 96-well bacteria or sponge plates. Active prefractions were subdivided into further fractions using flash chromatography on a Buchi Pure C-815 Flash system equipped with a FP ECOFLEX C18 Column.

Luciferase transcriptional reporter screen. Overnight cultures of PAO1 *exoT-lux* and PAO1 ΔpscC *exoT-lux* were back diluted 1:40 in LB^{gent10} (10 µg/mL gentamicin) and incubated for 1.5 hours at 37°C. White 384-well plates (Greiner Bio-One 781207) were then prepared with one-half final volume of 2× low calcium LB (2× $MgCl_2$ and EGTA) supplemented with 10 µg/mL gentamicin and extracts or pure compounds. Extracts/ pure compounds were added using a Janus MDT robot

(PerkinElmer). After incubation, the cultures were centrifuged for 5 min at 14,800 rpm. Supernatant was removed and the cell pellets were resuspended in LB^{gent10}. Cultures were then normalized to an OD_{600} of 0.1 and added to the prepared 384-well plates. Plates were sealed with Breathe-Easy® gas permeable membranes (Sigma-Aldrich) and incubated at ambient room temperature for 4 hours. Luminescence was then measured using a plate reader (PerkinElmer Envision 2105).

Yersinia luciferase inhibitor counterscreen. Overnight cultures of *Y. pseudotuberculosis* YPIII Xen4 n=1 were normalized to an OD_{600} of 0.2 in high calcium 2×YT (23mM $CaCl_2$) supplemented with 50 µg/mL kanamycin. Normalized culture was then incubated at 26°C, shaking, for 1.5 hours. Culture was then transfer to 96-well white with clear bottom plates (Corning 3610) and pure compounds were added by hand. Plates were then incubated at 37°C, shaking, for 2 hours. Luminescence and absorbance at 600nm for each plate were then measured (PerkinElmer Envision 2105).

Pseudomonas luciferase inhibitor counterscreen. Following overnight growth, cultures of PAO1 Xen41 were centrifuged for 5 minutes at 14,800 rpm. Supernatant was removed and the cell pellets were resuspended in LB. The cultures were then normalized to an OD_{600} of 0.2. Normalized cultures were added to white with clear bottom 384-well plates (Corning 3763). Extracts were then added with a Janus MDT robot (PerkinElmer) and plates were placed in a static incubator at 37°C for 3 hours. Luminescence and absorbance at 600nm for each plate were then measured (PerkinElmer Envision 2105).

Bacterial viability counterscreen. *P. aeruginosa* (PAO1) was grown overnight and then back diluted 1:40 in LB and grown for 1.5 hours at 37°C. Black untreated 384-well plates (Corning 3573) were then prepared with one-half final volume of LB, extracts or pure compounds, and 10% v/v resazurin-based alamarBlue high-sensitivity (HS) Cell Viability Reagent (catalog number A50101; Invitrogen). Extracts/ pure compounds were either added by hand or using a Janus MDT robot (PerkinElmer). After incubation, the cultures were centrifuged for 5 min at 14,800 rpm. Supernatant was removed and the cell pellets were resuspended in LB. Cultures were then diluted to an OD_{600} of 0.0005 and added to the prepared 384-well plates. For time course measurements, the plate was incubated in a plate reader (PerkinElmer Envision 2105) at 37°C and fluorescence was measured every hour for 12 hours. For single time point measurements, plates were sealed with Breathe-Easy® gas permeable membranes (Sigma-Aldrich) and placed in a static incubator at 37°C. Fluorescence was then measured on the plate reader at 8 hours post-treatment.

TCA precipitation of secreted proteins. Visualization and quantification of T3SS secreted protein was performed as described previously[13,20]. Briefly, overnight cultures of WT *P. aeruginosa* PA103 were normalized to an OD_{600} of 0.2 in low calcium LB and grown at 26°C, shaking, for 1.5 hours before adding Crews or commercial penicillic acid and transferring to 37°C, shaking. Cultures were then normalized prior to 15-minute centrifugation at 14,800 rpm. Bovine serum albumin (BSA) was added to samples as a control. Supernatants containing secreted proteins were then transferred to new tubes and mixed with trichloroacetic acid (TCA) at 10%

v/v. Samples were then incubated on ice for one hour before being centrifuged for 15 minutes at 13,200 rpm and 4°C. Supernatant was removed and the pelleted proteins were twice washed with acetone and centrifuged for 15 minutes at 13,200 rpm and 4°C. Dried protein pellets were then resuspended in final sample buffer (FSB) and 20% dithiothreitol (DTT).

Resuspended protein samples were boiled for 15 minutes and then separated via SDS-PAGE. Samples were run for 100 minutes at 110 volts on 7.5% polyacrylamide gels. Gels were stained with Coomasie Blue and imaged using a GelDoc with Image Lab software (Bio-Rad). Image Lab software was used to quantify the relative levels of BSA and T3SS effector protein, ExoU, for each sample relative to the DMSO-treated control.

Statistical analysis. Prism 9 (GraphPad Software, La Jolla, CA, USA) was used to calculate the mean, standard error of the mean, and one-way analysis of variance (ANOVA) values shown.

RESULTS

3.1 The *P. aeruginosa* quorum sensing inhibitor penicillic acid also inhibits the T3SS

3.1.1 High-throughput screen of natural product-derive pure compounds for T3SS inhibitors. As part of our continued search for natural product-derived inhibitors of the T3SS, we took advantage of a small library of ~400 pure compounds derived from marine fungi, sponges, and bacteria over the past several decades. Using our PAO1 *exoT-lux* screen described in chapter 2, we identified five hit compounds which reduced luminescence >3 standard deviations and 1.2-fold below the mean of

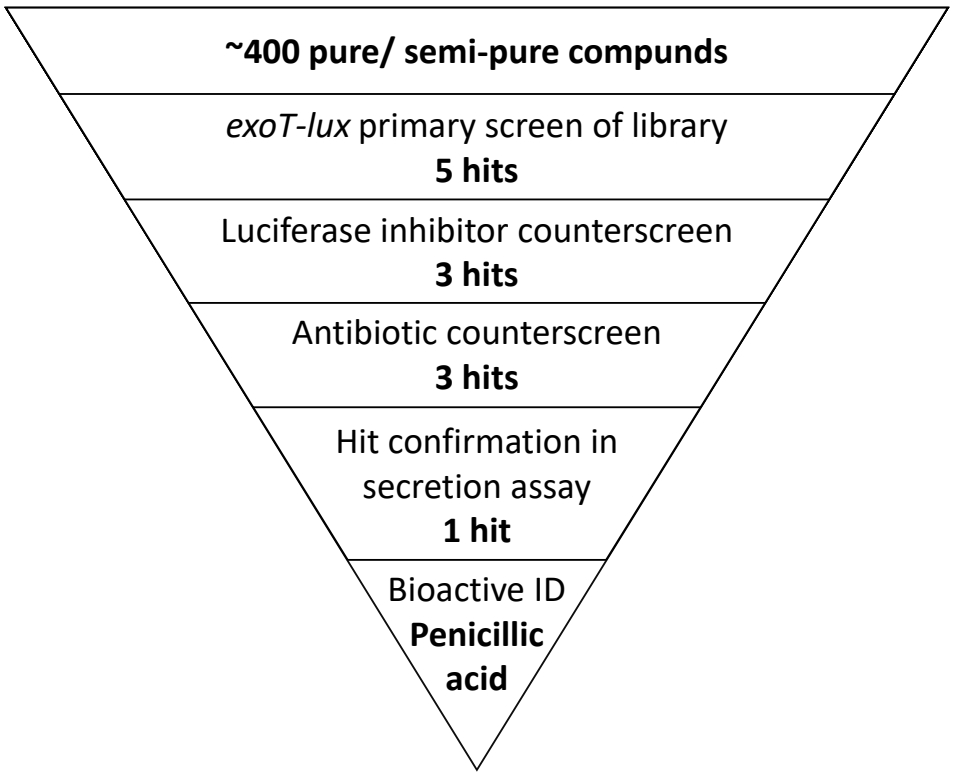


Figure 12. Marine natural product-derived pure compound library workflow.

the DMSO-treated control in all three biological replicates (**Fig. 13a**). These five hit compounds were next screened for luciferase inhibiting bioactivity against a strain of *Yersinia pseudotuberculosis* constitutively expressing luciferase. Compound-treated

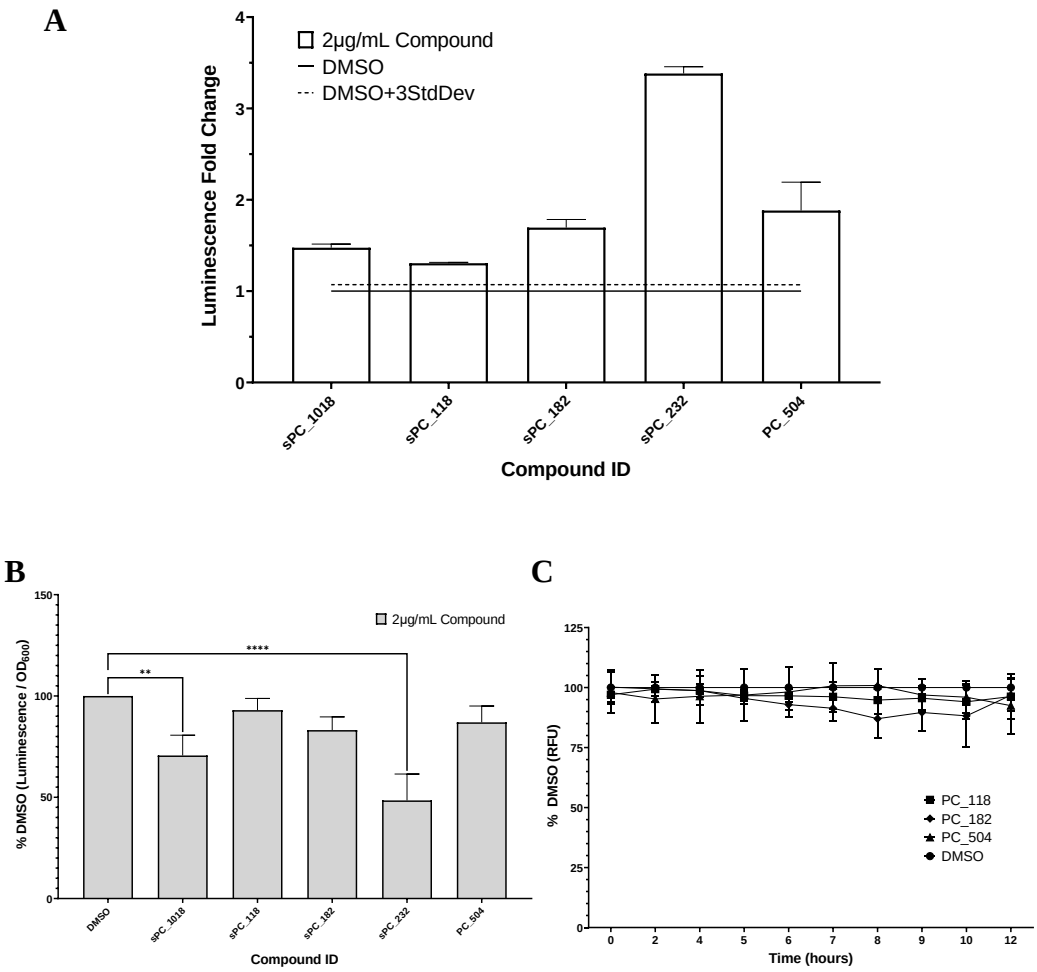


Figure 13. Identification of marine-derived pure compounds that inhibit T3SS-driven bioluminescence. (A) PAO1 *exoT-luxCDABE* was treated with pure compounds and T3SS gene transcription was measured through bioluminescence. Fold change represents the ratio by which a compound reduced the luminescent signal in comparison to the DMSO-treated control. **(B)** Hit compounds were counterscreened for general luciferase activity. PAO1 Xen41 was treated with pure compounds and bioluminescence was measured. Values were normalized as a percentage of the DMSO control and compared to normalized PAO1 *exoT-lux* values. Compounds with a p-value > 0.05 were considered luciferase inhibitors. **(C)** PAO1 WT was treated with pure compounds and effects on bacterial viability were measured for 12 hours. Values were normalized as a percentage of the DMSO control. All results are a mean of 3 biological replicates.

cultures were compared to the DMSO-treated control using a one-way ANOVA analysis. Two of the five hits reduced the constitutive luminescent signal significantly, indicating luciferase inhibitory bioactivity (**Fig. 13b**). The three remaining hits were then screened for antibiotic activity and none of the compounds affected *P. aeruginosa* viability (**Fig 13c**).

3.1.2 Identifying penicillic acid as an inhibitor of the *P. aeruginosa* T3SS. Fresh aliquots of the three hit compounds were rescreened against PAO1 *exoT-lux* in order to confirm their bioactivity. Two of the three fresh aliquots did not maintain the bioactivity observed from their original counterparts but the third sample, PC_504, significantly inhibited bioluminescence (**Fig. 14a**). In order to confirm the T3SS inhibitory bioactivity of PC_504, we evaluated its ability to reduce effector secretion into culture media. *P. aeruginosa* PA103 treated with PC_504 or DMSO was grown in low calcium media at 37ºC in order to induce type III secretion. Secreted protein was then precipitated from the supernatant and assessed using SDS-PAGE. PC_504 inhibited secretion of the effector ExoU in a dose-dependent manner (**Fig. 14b**).

PC_504 was cataloged in the library as the mycotoxin, penicillic acid (**Fig. 15b**). In order to confirm the bioactivity of pencillic acid against the *P. aeruginosa* T3SS, we purchased commercially available penicillic acid. The commercial penicillic acid was then evaluated for its ability to reduce effector secretion into culture media. ExoU secretion was inhibited in a dose-dependent manner with an

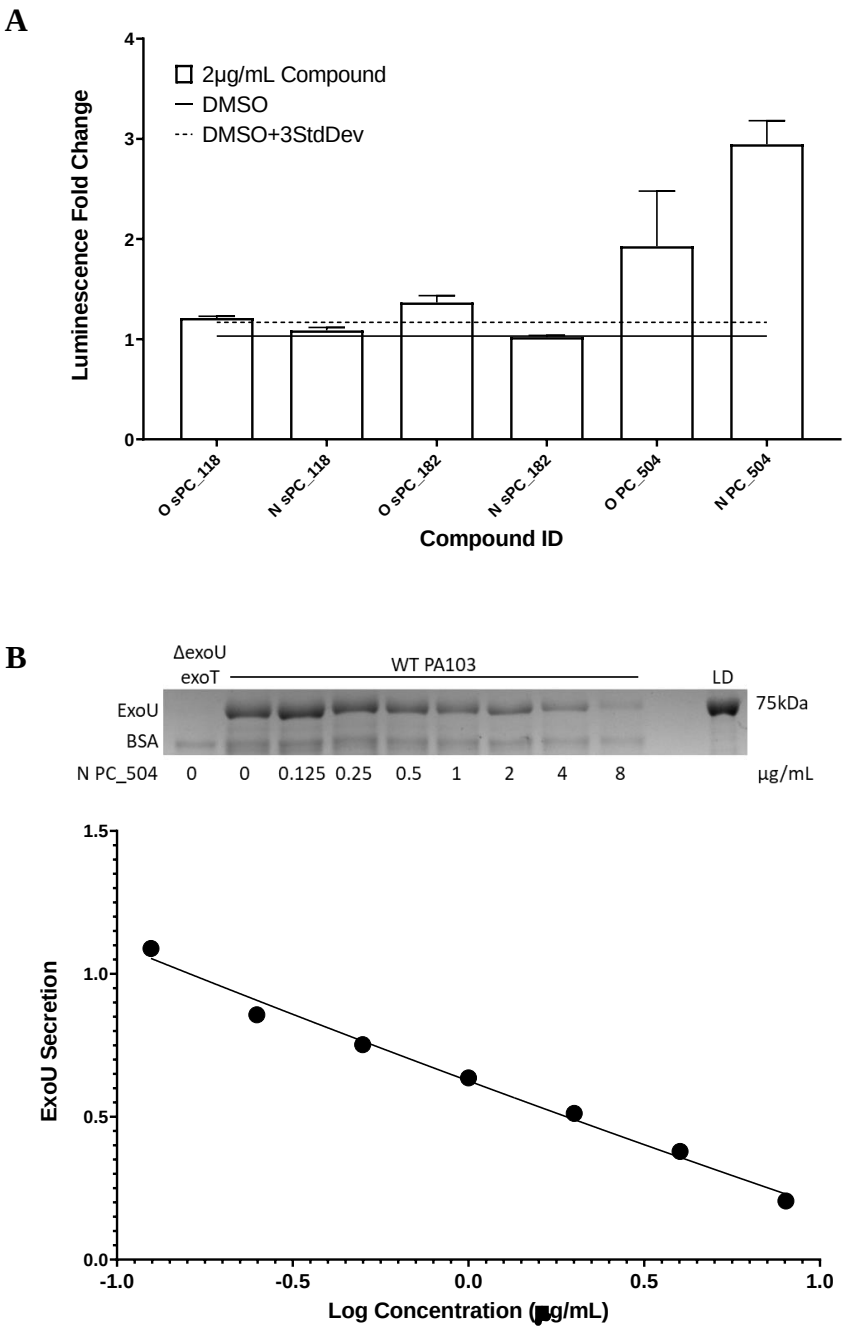
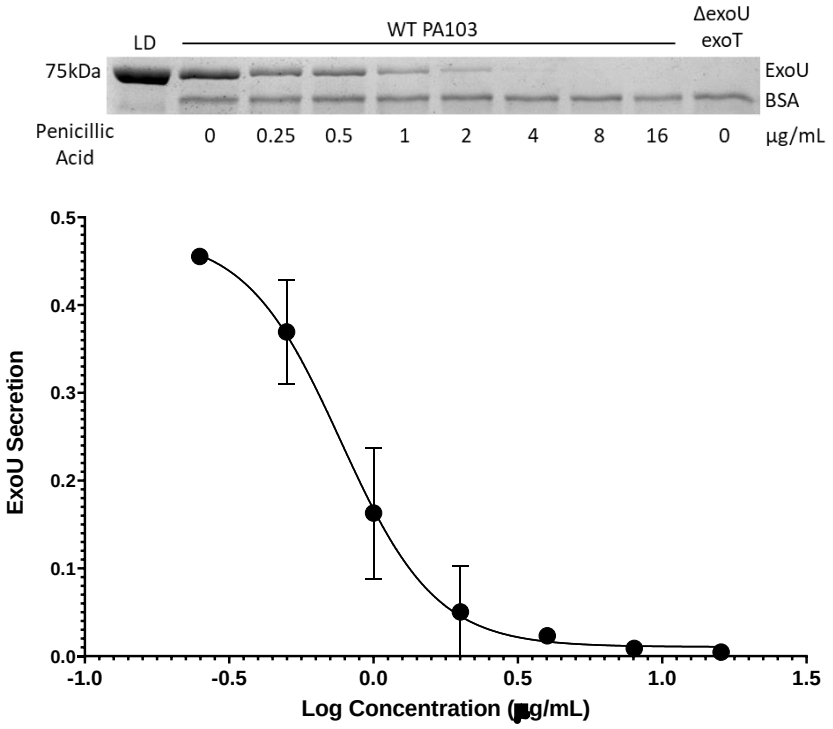
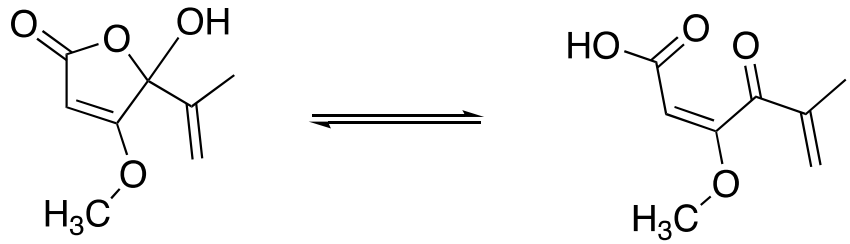


Figure 14. Confirming the T3SS inhibitory bioactivity of PC_504. (A) PAO1 *exoT-lux* screen of new (N) versus original (O) aliquots of primary screen hits to confirm bioactivity. Fold change represents the ratio by which a compound reduced the luminescent signal in comparison to the DMSO-treated control. Results a mean of 3 biological replicates. **(B)** Secretion of the *P. aeruginosa* PA103 T3SS effector ExoU was monitored in the presence of PC_504. BSA was added to supernatant prior to precipitation to act as a control.

A



B



Chemical Formula: $C_8H_{10}O_4$
Exact Mass: 170.0579

Chemical Formula: $C_8H_{10}O_4$
Exact Mass: 170.0579

Figure 15. Penicillic acid inhibits ExoU secretion in a dose-dependent manner. **(A)** Secretion of the *P. aeruginosa* PA103 T3SS effector ExoU was monitored in the presence of Penicillic acid. BSA was added to supernatant prior to precipitation to act as a control. Results are a mean of two biological replicates. **(B)** Penicillic acid structure.

IC50 of 0.77 µg/mL (**Fig 15a**). Intriguingly, penicillic acid has previously been characterized as a quorum sensing inhibitor in *P. aeruginosa*[21]. Due to its cytotoxicity, penicillic acid is not attractive as a pretherapeutic. However, it may help us better understand regulation between quorum sensing, the T3SS, and biofilm formation in *P. aeruginosa*.

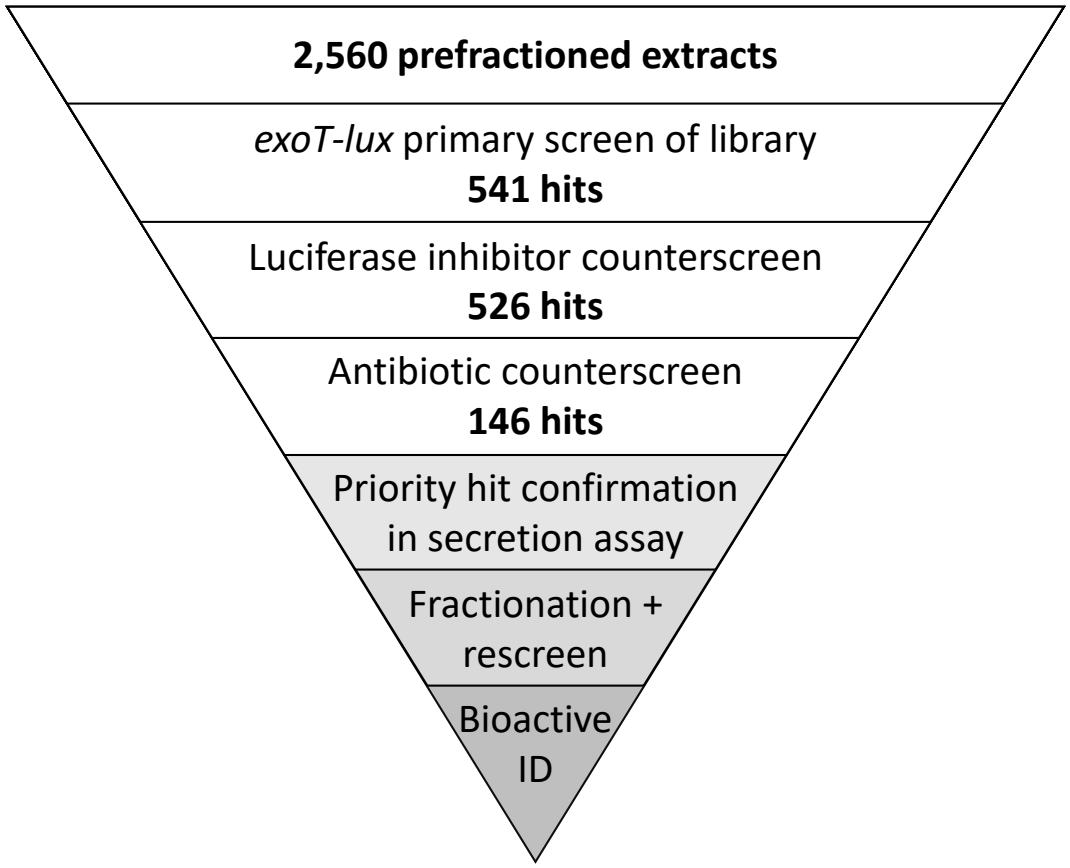


Figure 16. Marine sponge and bacteria extract library workflow.

3.2 A screen of marine natural product extracts for *P. aeruginosa* T3SS inhibitors yields five lead bioactive prefractions

3.2.1 High-throughput screen of sponge and bacterial-derived extracts as T3SS inhibitors. To continue our investigation of marine natural products as a source for T3SS inhibitors, we screened a library of marine sponge and bacterial-derived prefractioned extracts. Bacterial and sponge prefractions were prepared through ethyl acetate or liquid-liquid extraction followed by preparative HPLC fractionation. These prefractions were then tested for bioactivity against PAO1 *exoT-lux*. A total of 541 of the 2,560 prefractions reduced the luminescent signal >3 standard deviations and 1.2-fold below the DMSO-treated control. A counterscreen for luciferase inhibition in PAO1 Xen41 reduced the number of hit prefractions to 526. This number was further reduced to 146 after counterscreening for antibiotics. Hit prefractions were then prioritized by level of complexity, available amount of prefraction, and cytotoxicity. Based on these criteria, six prefractions were chosen for follow up (**Fig. 17**). Based on the ELSD traces, two of the six priority prefractions, 95561-F5 and 96535-F4, appeared to be pure compounds. Proton NMR and HSQC data was collected on these two prefractions in order to confirm their purity (**Fig. 18**). 96535-F4 had high similarity to plasticizers found in other screens and was therefore discarded as a hit. The remaining four prefractions of interest were run on a flash chromatograph. Some fractions were produced from 95103-F5 and 96509-F1. However, 94625-F5 and 96529-F4 had little to no UV trace and will be further fractioned using ELSD. To

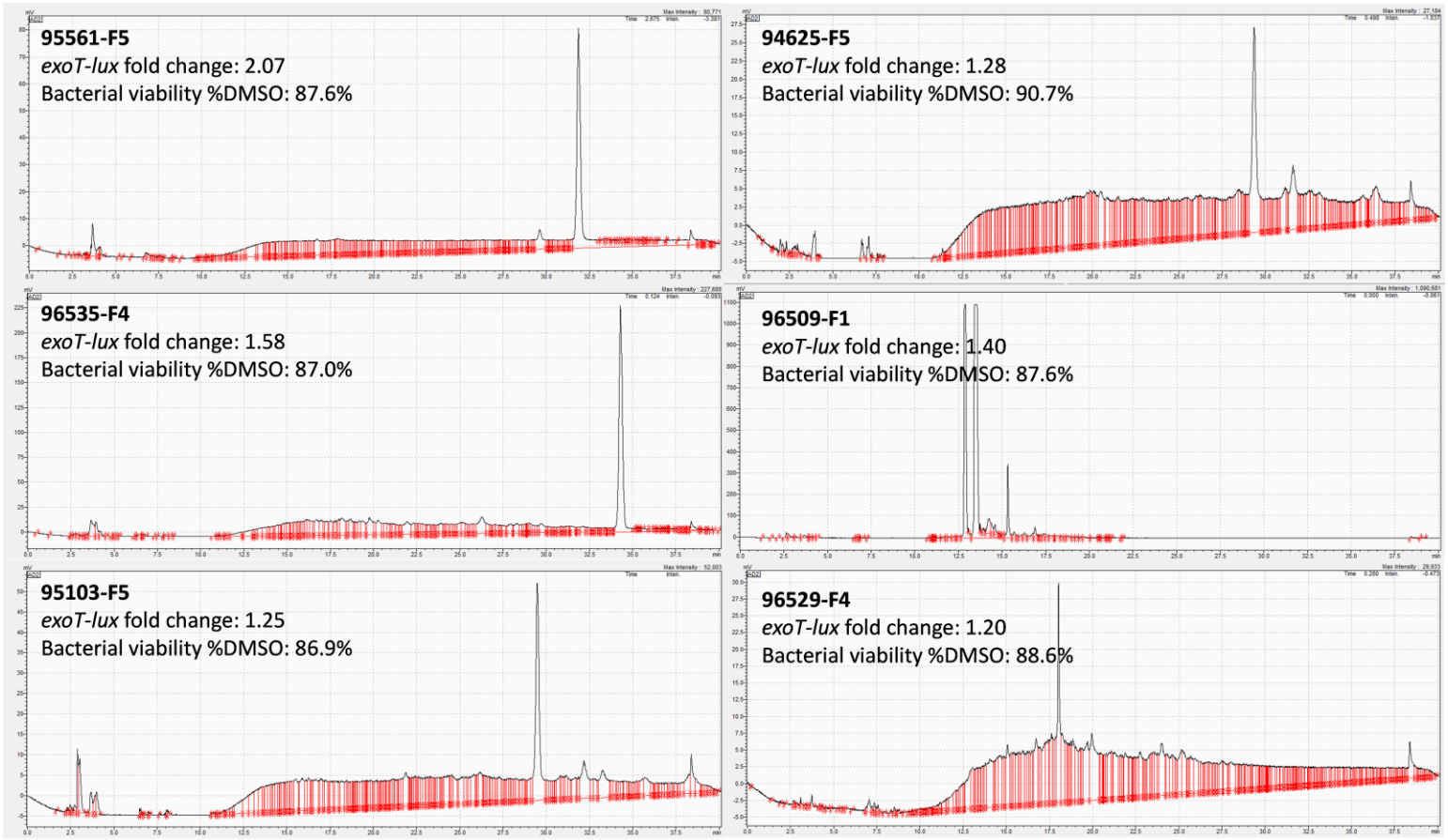


Figure 17. ELSD traces of six priority hit marine-derived prefractions. Six priority hits were chosen based on complexity, available amount, and cytotoxicity. Two of the six priority hits, 95561-F5 and 96535-F4, appeared to be pure compound based on the ELSD chromatogram trace. PAO1 *exoT-lux* fold change represents the ratio by which a compound reduced the T3SS-dependent bioluminescent signal in comparison to the DMSO-treated control. Bacterial viability was measured 8 hours post-treatment and values were normalized as a percentage of the DMSO control.

continue this ongoing investigation, these fractions, alongside the 95561-F5 pure compound, will be probed for their ability to reduce effector secretion into culture media. Samples which reduce ExoU secretion will be subjected to further fractionation followed by structure elucidation of the bioactive molecule (**Fig. 16**).

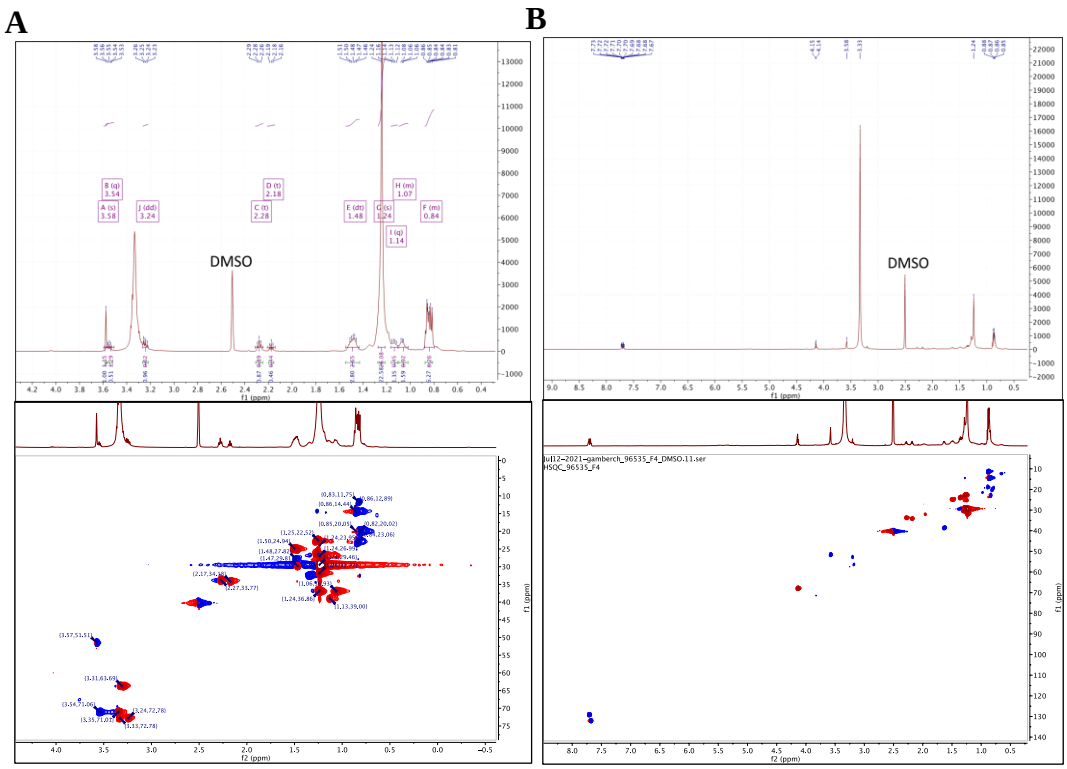


Figure 18. Proton NMR and HSQC of 96535-F4 shows high structural similarity to plasticizer. Proton NMR (top) and HSQC (bottom) data was collected on two priority hit prefractions to confirm their purity. **(A)** 95561-F5 was confirmed to be a pure compound. **(B)** 96535-F4 showed similarity to plasticizers identified in previous screens and was therefore discarded as a hit.

DISCUSSION

In this study, we used a bioluminescent reporter screen to identify five lead extracts and one pure compound inhibitor of the *P. aeruginosa* T3SS, penicillic acid, from two diverse libraries of marine derived natural product pure compounds and prefractioned extracts. Penicillic acid and the five lead extracts inhibited T3SS-dependent bioluminescence without impacting general luciferase activity or bacterial metabolism. In addition, penicillic acid inhibited *P. aeruginosa* T3SS effector secretion into culture supernatant in a dose-dependent manner.

Penicillic acid. The first reported bioactivty of penicillic acid in *P. aeruginosa* was as an inhibitor of alginate biosynthesis[22,23]. Alginate is one of three exopolysaccharides found in the *P. aeruginosa* biofilm matrix[24,25] and is most commonly associated with mucoid isolates from chronically infected cystic fibrosis patients[25]. During the multi-step biosynthesis of alginate, the committed step is catalyzed by an enzyme known as GDP-mannose dehydrogenase. It was later discovered that penicillic acid inhibits alginate biosynthesis through the irreversible inactivation of GDP-mannose dehydrogenase via alkylation of a critical cysteine residue[26]. Transcription of the gene encoding GDP-mannose dehydrogenase, *algD*, is activated by the sigma factor AlgU and the response regulator AlgR. Together AlgU and AlgR were found to reduce T3SS gene expression and secretion by inhibiting expression of *vfr*[27], a transcriptional activator of the T3SS master regulator ExsA[28]. However, deletion of *algD* did not affect T3SS activity[27]. Therefore, the T3SS inhibitory bioactivity we have observed is not due to the inactivation of GDP-mannose dehydrogenase by penicillic acid.

In addition to inhibition of alginate biosynthesis, penicillic acid was also identified as an inhibitor of the *las* and *rhl* quorum sensing (QS) systems in *P. aeruginosa*[21]. Quorum sensing is often used by bacteria to regulate virulence-related gene expression in response to environmental factors[29]. The *rhl* QS system, but not the *las* QS system, downregulates T3SS gene expression and protein secretion under T3SS-inducing conditions[30]. Under non-inducing conditions, *rhl* QS does not impact the level of T3SS gene expression, suggesting that the *rhl* QS system indirectly regulates the T3SS[30]. Inhibition of the *rhl* QS system by penicillic acid should result in upregulation of T3SS gene expression and protein secretion upon induction, however, penicillic acid also inhibits the T3SS. Therefore, the mechanism by which penicillic acid inhibits the T3SS remains unclear.

Interestingly, a DNA microarray revealed that penicillic acid affected expression of ~300 genes in *P. aeruginosa* (3-5% of the genome) but only 34% of the targeted genes were controlled by quorum sensing[21]. It is possible that penicillic acid has multiple molecular targets and therefore is not a good candidate for a virulence blocker in *P. aeruginosa*. However, it may become a useful molecular tool for the elucidation of signaling pathways controlling the T3SS, alginate biosynthesis, and quorum sensing.

Lead marine sponge and bacteria-derived extracts. Five lead extracts for further investigation were identified after screening a library of marine sponge and bacterially-derived extracts. In 2018, 222 sponge-derived and 240 marine bacteria-derived new marine natural products were reported[31]. Marine sponges have long

served as a source for diverse bioactive molecules[19] with a cumulative total of 9231 compounds reporter as of 2020[31]. Additionally, in the past three years the number of marine bacteria-derived compounds reported has increased 22%.

Several T3SS organisms, including *P. aeruginosa*[32], *Vibrio* spp., and *Aeromonas* spp., have been known to occupy marine environments[6]. It is likely that competition among species in this environment has led to the evolutionary biosynthesis of T3SS inhibitors. In fact, two T3SS inhibitors have previously been discovered from marine sponge extracts. Isolated from the *Caminus sphaeroconia* marine sponge, caminoside A was among the first T3SS inhibitors to be identified, inhibiting T3SS effector secretion in enteropathogenic *E. coli*[33]. However, full synthesis of caminosides has proven to be tedious, limiting the amount of follow up studies on this compound[34]. More recently, a class of inhibitors of the *Y. pseudotuberculosis* T3SS, enisorines, was identified from an *Iotrochota* cf. *iota* sponge[19]. While these compounds showed robust activity against the *Yersinia* T3SS, they were inactive against the *Pseudomonas* T3SS. Therefore, investigation of new marine sponge and marine bacterially-derived extracts for inhibitors of the *P. aeruginosa* T3SS is necessary. Following this study, we will continue to deconvolute the bioactive lead extracts in order to identify marine sponge and marine bacterially-derived *P. aeruginosa* T3SS inhibitors.

REFERENCES

1. Centers for Disease Control and Prevention (U.S.). *Antibiotic Resistance Threats in the United States, 2019*. Centers for Disease Control and Prevention (U.S.); 2019. doi:10.15620/cdc:82532

2. Rice LB. Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE. *J Infect Dis*. 2008;197(8):1079-1081. doi:10.1086/533452

3. Mulani MS, Kamble EE, Kumkar SN, Tawre MS, Pardesi KR. Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review. *Front Microbiol*. 2019;10. doi:10.3389/fmicb.2019.00539

4. Navidinia M. The clinical importance of emerging ESKAPE pathogens in nosocomial infections. *1*. 2016;7(3):43-57. doi:10.22037/jps.v7i3.12584

5. Deng W, Marshall NC, Rowland JL, et al. Assembly, structure, function and regulation of type III secretion systems. *Nature Reviews Microbiology*. 2017;15(6):323-337. doi:10.1038/nrmicro.2017.20

6. Troisfontaines P, Cornelis GR. Type III Secretion: More Systems Than You Think. *Physiology*. 2005;20(5):326-339. doi:10.1152/physiol.00011.2005

7. Galle M, Carpentier I, Beyaert R. Structure and function of the Type III secretion system of Pseudomonas aeruginosa. *Curr Protein Pept Sci*. 2012;13(8):831-842. doi:10.2174/138920312804871210

8. Roy-Burman A, Savel RH, Racine S, et al. Type III Protein Secretion Is Associated with Death in Lower Respiratory and Systemic Pseudomonas aeruginosa Infections. *The Journal of Infectious Diseases*. 2001;183(12):1767-1774. doi:10.1086/320737

9. Kauppi AM, Nordfelth R, Uvell H, Wolf-Watz H, Elofsson M. Targeting Bacterial Virulence: Inhibitors of Type III Secretion in Yersinia. *Chemistry & Biology*. 2003;10(3):241-249. doi:10.1016/S1074-5521(03)00046-2

10. Anantharajah A, Buyck JM, Sundin C, Tulkens PM, Mingeot-Leclercq M-P, Van Bambeke F. Salicylidene Acylhydrazides and Hydroxyquinolines Act as Inhibitors of Type Three Secretion Systems in Pseudomonas aeruginosa by Distinct Mechanisms. *Antimicrob Agents Chemother*. 2017;61(6). doi:10.1128/AAC.02566-16

11. Kim OK, Garrity-Ryan LK, Bartlett VJ, et al. N-hydroxybenzimidazole Inhibitors of the Transcription Factor LcrF in Yersinia: Novel Anti-Virulence Agents. *J Med Chem*. 2009;52(18):5626-5634. doi:10.1021/jm9006577

12. Aiello D, Williams JD, Majgier-Baranowska H, et al. Discovery and Characterization of Inhibitors of Pseudomonas aeruginosa Type III Secretion. *Antimicrob Agents Chemother*. 2010;54(5):1988-1999. doi:10.1128/AAC.01598-09

13. Lam H, Schwochert J, Lao Y, et al. Synthetic Cyclic Peptomers as Type III Secretion System Inhibitors. *Antimicrob Agents Chemother*. 2017;61(9):e00060-17. doi:10.1128/AAC.00060-17

14. Beutler JA. Natural Products as a Foundation for Drug Discovery. *Curr Protoc Pharmacol*. 2009;46:9.11.1-9.11.21. doi:10.1002/0471141755.ph0911s46

15. Stover CK, Pham XQ, Erwin AL, et al. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature*. 2000;406(6799):959-964. doi:10.1038/35023079

16. Kang PJ, Hauser AR, Apodaca G, et al. Identification of Pseudomonas aeruginosa genes required for epithelial cell injury. *Molecular Microbiology*. 1997;24(6):1249-1262. doi:10.1046/j.1365-2958.1997.4311793.x

17. Liu PV. The Roles of Various Fractions of "Pseudomonas aeruginosa" in Its Pathogenesis: III. Identity of the Lethal Toxins Produced in Vitro and in Vivo [with Discussion]. *The Journal of Infectious Diseases*. 1966;116(4):481-489.

18. Wang H, Avican K, Fahlgren A, et al. Increased plasmid copy number is essential for Yersinia T3SS function and virulence. *Science*. 2016;353(6298):492-495. doi:10.1126/science.aaf7501

19. McCauley EP, Lam H, Lorig-Roach N, et al. Investigation of the Physical and Bioactive Properties of Bromo- and Iodo-Containing Sponge-Derived Compounds Possessing an Oxyphenylethanamine Core. *J Nat Prod*. 2017;80(12):3255-3266. doi:10.1021/acs.jnatprod.7b00694

20. Auerbuch V, Golenbock DT, Isberg RR. Innate Immune Recognition of Yersinia pseudotuberculosis Type III Secretion. *PLoS Pathog*. 2009;5(12):e1000686. doi:10.1371/journal.ppat.1000686

21. Rasmussen TB, Skindersoe ME, Bjarnsholt T, et al. Identity and effects of quorum-sensing inhibitors produced by Penicillium species. *Microbiology (Reading)*. 2005;151(Pt 5):1325-1340. doi:10.1099/mic.0.27715-0

22. Nakagawa A, Hosoyama T, Chubachi K, Takahashi S, Ohkubo T, Iyobe S. A search for Pseudomonas alginate biosynthesis inhibitors from microbial metabolites. *J Antibiot (Tokyo)*. 1997;50(3):286-288.

23. Hosoyama T, Yuge T, Takahashi S, et al. Inhibitory effect of antimicrobial agents on alginate production in Pseudomonas aeruginosa. *J Antibiot (Tokyo)*. 1999;52(1):65-67. doi:10.7164/antibiotics.52.65

24. Yoon KL and SS. Pseudomonas aeruginosa Biofilm, a Programmed Bacterial Life for Fitness. 2017;27(6):1053-1064. doi:10.4014/jmb.1611.11056

25. Thi MTT, Wibowo D, Rehm BHA. Pseudomonas aeruginosa Biofilms. *International Journal of Molecular Sciences*. 2020;21(22):8671. doi:10.3390/ijms21228671

26. Kimmel JL, Tipton PA. Inactivation of GDP-mannose dehydrogenase from Pseudomonas aeruginosa by penicillic acid identifies a critical active site loop. *Archives of Biochemistry and Biophysics*. 2005;441(2):132-140. doi:10.1016/j.abb.2005.06.028

27. Jones AK, Fulcher NB, Balzer GJ, et al. Activation of the Pseudomonas aeruginosa AlgU Regulon through mucA Mutation Inhibits Cyclic AMP/Vfr Signaling. *Journal of Bacteriology*. 2010;192(21):5709-5717. doi:10.1128/JB.00526-10

28. Marsden AE, Intile PJ, Schulmeyer KH, et al. Vfr Directly Activates exsA Transcription To Regulate Expression of the Pseudomonas aeruginosa Type III Secretion System. *Journal of Bacteriology*. 198(9):1442-1450. doi:10.1128/JB.00049-16

29. Smith RS, Iglewski BH. P. aeruginosa quorum-sensing systems and virulence. *Current Opinion in Microbiology*. 2003;6(1):56-60. doi:10.1016/S1369-5274(03)00008-0

30. Bleves S, Soscia C, Nogueira-Orlandi P, Lazdunski A, Filloux A. Quorum Sensing Negatively Controls Type III Secretion Regulon Expression in Pseudomonas aeruginosa PAO1. *Journal of Bacteriology*. 2005;187(11):3898-3902. doi:10.1128/JB.187.11.3898-3902.2005

31. Carroll AR, Copp BR, Davis RA, Keyzers RA, Prinsep MR. Marine natural products. *Nat Prod Rep*. 2020;37(2):175-223. doi:10.1039/C9NP00069K

32. Kimata N, Nishino T, Suzuki S, Kogure K. Pseudomonas aeruginosa Isolated from Marine Environments in Tokyo Bay. *Microb Ecol*. 2004;47(1):41-47. doi:10.1007/s00248-003-1032-9

33. Linington RG, Robertson M, Gauthier A, Finlay BB, van Soest R, Andersen RJ. Caminoside A, an antimicrobial glycolipid isolated from the marine sponge Caminus sphaeroconia. *Org Lett*. 2002;4(23):4089-4092. doi:10.1021/ol0268337

34. Pendergrass HA, May AE. Natural Product Type III Secretion System Inhibitors. *Antibiotics (Basel)*. 2019;8(4). doi:10.3390/antibiotics8040162