Application of plant extracts as antimicrobials against *Streptomyces scabies* and characterization of the DNA-binding motif in Scabin toxin

by

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ABSTRACT

Application of plant extracts as antimicrobials against *Streptomyces scabies* and characterization of the DNA-binding motif in Scabin toxin.

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The common scab disease, caused by *Streptomyces* species, is characterized by lesions on tuber crops which causes multi-million dollar annual losses in the potato industry alone. A total of 54 plant tinctures were tested for antimicrobial activity against *S. scabies*. The best plant tinctures for growth inhibition, myrrh and garlic, showed complete cessation of growth in culture for all four *Streptomyces* strains (*S. scabies*, *S. turgidiscabies*, *S. acidiscabies*, and *S. europaeiscabiei*).

A mono-ADP-ribosyltransferase (mART) toxin from *S. scabies*, is termed Scabin. mART toxins are released by pathogenic bacteria as virulence factors that transfer the ADP-ribose group of NAD\(^+\) to a target macromolecule. Characterization of the role of several DNA-binding residues in Scabin was completed to understand the catalytic signature of the enzyme. The corresponding variants were analyzed for enzymatic activity as well as their binding affinity for a dsDNA substrate. It was shown that Tyr129 is an important residue within the Scabin DNA-binding motif that controls substrate binding.
DECLARATION OF WORK PERFORMED

In some cases, experiments performed by other researchers has been included to complete the thesis. The work included in this thesis is my own, with the following exceptions: Genevieve Ramirez developed the *Streptomyces scabies* growth protocol. Nick Tiessen performed all cloning and mutagenesis for the Scabin K130A, L108G, V109G, K181A, Y129E, and Y129H variant. Julia Steckner characterized all the kinetic assays (glycohydrolase, transferase and DNA binding) for Scabin Y129K and Y129F variant, as well as performing the DNA binding and transferase assay for the Scabin Y129H variant.
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LIST OF ABBREVIATIONS

ADP – Adenosine diphosphate
ARTT – ADP-ribosyl-turn-turn
cAMP – Cyclic adenosine monophosphate
CD – Circular dichroism
CFU – Colony forming units
CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CT – Cholera toxin
dG – Deoxyguanosine
DNA – Deoxyribonucleic acid
DT – Diphtheria toxin
ε-AMP – Etheno adenosine monophosphate
ε-NAD – Etheno nicotinamide adenine dinucleotide
EDTA – Ethylenediaminetetraacetic acid
eEF-2 – Eukaryotic elongation factor two
ExoA – Exotoxin A
FPLC – Fast protein liquid chromatography
GH – Glycohydrolase
IMAC – Immobilized metal affinity chromatography

IPTG – Isopropyl β-D-1 thiogalactopyranoside

$K_{0.5}$ – substrate concentration where half maximal velocity or half-saturation is attained

$K_{\text{cat}}$ – turnover rate

$K_M$ – Michaelis-Menten constant

mART – mono adenosine diphosphate ribosyltransferase

NAD – Nicotinamide adenine dinucleotide

P6-E – 4-[8-fluoro-6-oxo 1H,2H,3H,4H,5H,6H-benzo[c]1,6-naphthyridin-2-yl]butanoic acid

PARP – Poly-ADP-ribosylpolymerase

PJ34 – N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino)acetamide hydrochloride

PN – Phosphate-nicotinamide

Rho – Ras homolog gene family

RNA – Ribonucleic acid

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sec – Secretory

$S_N1$ – Unimolecular nucleophilic substitution

Tat – Twin arginine transport
Chapter 1: Introduction
1.1: Antibiotic Resistance

In the early 20th century, the development of antibiotics was thought to be a remarkable success. Shortly thereafter, antibiotic resistance emerged\(^1\). Antibiotics kill or inhibit the growth of an organism, resulting in a non-competitive environment in which resistant strains can thrive\(^2\). Resistance to antibiotics is evolving at a rapid rate resulting in reduced incentives for antibiotic development\(^2\). Therefore, alternative strategies such as natural products and an anti-virulence approach need to be considered.

1.2: Natural Products

Specialized secondary metabolites are abundant in plants\(^3\). It is estimated that at least 200,000 bioactive compounds stem from plants; however, that only accounts for a portion of the compounds they produce\(^4\). One major group of chemical compounds are phytochemicals\(^5\). They have evolved over thousands of years to protect against the effects of viruses, fungi, bacteria, and free radicals\(^5\). They are found in vegetables, legumes, fruits, nuts, whole grains, herbs and spices, fungi and even beverages such as tea and wine\(^5\). One class of phytochemicals are polyphenols, with over 8000 different compounds, and they are used in medicine, pharmacology, and agriculture\(^6,7\). The compounds are part of the plant’s defense mechanisms and are found in the fruits, roots, seeds, wood, leaves and bark\(^6,8\). Within the polyphenol class, two main subclasses include flavonoids and isoprenoids\(^8,9\).

Flavonoids, formerly bioflavonoids, contain phenolic structures that have many functions in the plant. These include acting as messengers between plants and the environment, pigments for leaves, as well as roles in plant reproduction, growth, and predator and pathogen resistance\(^3,10\). The general flavonoid structure consists of a 15-
carbon compound comprising two phenyl rings joined by a heterocyclic ring\(^3\). The position of the substitution (generally present on the C-ring (Figure 1.1)) separates the class into isoflavonoids and flavonoids, which can be further divided into several subclasses such as flavonols, flavones, anthocyanidins, and flavones\(^7\). Flavonoids exhibit anti-inflammatory, hepatoprotective, antioxidative, anticarcinogenic and antiviral effects\(^10\). The strength of the activity depends on the positions and number of -OH groups as well as other modifications include glycosylation and acylation\(^6,11\). The antibacterial activity of flavonoids is due to their ability to form complexes with bacterial membranes, and both soluble and extracellular proteins\(^3\).

Terpenes consist of five-carbon isoprene units that can occur with additional elements, such as oxygen\(^3,5\). The terpenes are classified based on their number of carbons, such as hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), and diterpenoids (C20), to name a few\(^3,12\). Terpenes comprise over 30,000 members\(^12\). They provide protection against pathogens as well as roles in development, reproduction and growth of the plant\(^12,13\).

Other subclasses of the polyphenols are quinones, tannins, saponins and coumarins\(^8,9\). Saponins are derived from triterpenoids or steroids, and have reported antifungal and antibacterial activity\(^14\). By virtue of their ketone-substituted aromatic ring, quinones can react with nucleophilic amino acids, consequently disrupting protein function\(^3\). Tannins are polyphenolic compounds with antimicrobial activity due to such mechanisms as inactivation of enzymes and bacterial adhesions\(^3,5\).
Figure 1.1: The basic structural organization of a flavonoid molecule.\textsuperscript{15}
1.3: Anti-virulence

The anti-virulence strategy focuses on converting a pathogenic population into a harmless, or less virulent state, by targeting virulence factors such as secretion systems, toxins, adhesins and transcription factors\textsuperscript{1,2}. The host immune system can then eliminate the bacteria, reducing the selective pressure that leads to resistance\textsuperscript{1}.

1.4: mART Toxins

Pathogenic bacteria release many virulence factors that can be targets for anti-virulence compounds; one such factor is the mono-ADP-ribosyltransferase (mART) toxin\textsuperscript{16}. This can affect humans (e.g. \textit{Vibrio cholera}), insects (e.g. \textit{Pierisin-1}), and plants (e.g. \textit{Pseudomonas syringae})\textsuperscript{17–19}. mART toxins transfer the ADP-ribose group of NAD\textsuperscript{+} to a target macromolecule, modifying essential proteins such as actin, eukaryotic elongation factor 2 (eEF-2), and Rho (Ras homolog gene family)\textsuperscript{18,20,21}. The modification alters the function of the proteins resulting in an activated, repressed, or inactivated molecule\textsuperscript{20,22}. Many of these modifications inhibit normal eukaryotic protein function and thus result in cell death\textsuperscript{20,22}. Well-studied mART toxins include cholera toxin (\textit{V. cholerae}), diphtheria toxin (\textit{Corynebacterium diphtheriae}), and pertussis toxin (\textit{Bordetella pertussis})\textsuperscript{18,23,24}.

1.4.1: Classification

mART toxins are divided into two groups based on conserved active-site motifs and domain organization: the diphtheria toxin (DT) group and the cholera toxin (CT) group\textsuperscript{16}. The CT group is separated into CT-like, C2-like, and C3-like\textsuperscript{16}.
The DT group consists of single-chain AB toxins with a catalytic A-domain and a binding B-domain\textsuperscript{16} (Figure 1.2A). The B-domain is involved in receptor binding and translocation of the A-domain into the cell\textsuperscript{16}. This group targets the histidine derivative diphthamide on eukaryotic elongation factor 2 (eEF-2) which becomes inactivated by the addition of the ADP-ribosyl moiety\textsuperscript{25}. The inactivation of eEF-2 prevents translocation events on ribosomes, and ultimately peptide chain elongation\textsuperscript{26}. The active site of DT-like toxins includes a His residue, two Tyr residues (in a Tyr-X\textsubscript{10}-Tyr motif) and a Glu residue, which together form an HYE motif\textsuperscript{16} (Figure 1.2B).

The CT-like group contains AB\textsubscript{5} toxins, with the catalytic A-domain surrounded by a pore made by the 5 non-covalently associated B-subunits\textsuperscript{16} (Figure 1.2C). This group ADP-ribosylates the GTP-binding protein, G\textsubscript{as}, leading to high levels of cyclic adenosine monophosphate (cAMP) and fluid secretion\textsuperscript{18}. The C2-like toxins are binary toxins with a catalytic A-domain and receptor binding B-domain\textsuperscript{16}. This group targets actin and once it is ADP-ribosylated; actin polymerization is inhibited which causes the actin cytoskeleton to be compromised\textsuperscript{16}. The C3-like toxins are single-chain proteins with only a catalytic A-domain\textsuperscript{16}. They target Rho-family GTPases which affect the regulation of actin leading to cytoskeletal collapse\textsuperscript{21}. 
Figure 1.2: Oligomeric state of Diphtheria toxin (ISGK) and Cholera toxin (IXTC). (A) Diphtheria toxin crystal structure at 2.3Å resolution is shown as a cartoon highlighting the catalytic domain (green), translocation domain (orange), and receptor-binding domain (cyan). (B) A closer image of the catalytic residue Glu148 as well as other important residues in the reaction mechanism, Tyr54, Tyr65 and His21 are shown as sticks coloured carbon blue, hydrogen grey, nitrogen dark blue, oxygen red and sulfur orange. (C) Cholera toxin crystal structure is shown at 2.4Å resolution as a cartoon with the catalytic domain in red, the translocation domain in blue, and the receptor-binding domain in green. (D) The catalytic residues (Arg7, Ser61-Thr62-Ser63, Glu110 and Glu112) are shown as sticks with the same colouring scheme as image 2B. Images prepared in Pymol version 2.0.
CT group active sites consist of a RSE motif which includes an Arg residue, a Ser-Thr-Ser motif and a Gln(or Glu)-X-Glu motif\(^1^6\) (Figure 1.2D). The active-site cleft is formed by three short regions in both the DT and CT groups\(^2^7\). Region 1 has a β-sheet with the conserved Arg (or His in DT group) that aids in the binding of NAD\(^+\) as well as active-site integrity\(^2^8\). Region 2, which varies in length depending on the toxin, has a β-strand defining the lower face of the cavity and an α-helix as the upper face\(^2^7\). This region forms the binding pocket for the nicotinamide ring throughout the reaction\(^2^7\). For the CT group, the region consists of the Ser-Thr-Ser motif and for the DT group it is the Tyr-X\(_{10}\)-Tyr motif instead that forms the scaffold\(^2^7\). Region 3 has an ADP-ribosylating turn-turn (ARTT) motif with the conserved Glu positioned across from the Arg/His residue in region 1\(^2^7\).

Regarding the CT group in position -2 of the conserved Glu, there is a Glu/Gln residue\(^2^7\).

1.4.2: Mechanism

With the use of the conserved Arg (or His) and Ser-Thr-Ser (or Tyr-X\(_{10}\)-Tyr) motif, the NAD\(^+\) binds to the active site of a mART toxin\(^1^6\). Upon binding, the NAD\(^+\) is in a strained conformation with the glycosidic bond in an unfavourable torsion angle\(^2^2\). Consequently, there is a scission of the C-N bond that relieves this strain, releasing nicotinamide and forming an oxacarbenium intermediate\(^2^9\). The intermediate is stabilized by a hydrogen bond between the 2’OH of N-ribose and the conserved Glu present in both the CT and DT groups\(^2^0\). The second Glu/Gln residue activates a nucleophilic attack by the ADP-ribose acceptor at the C1 of N-ribose\(^2^2\) (Figure 1.3). This produces an ADP-riboseylated molecule, which can be a protein or DNA molecule\(^2^2,2^9\). In the absence of a target molecule, the same reaction occurs, however a water molecule becomes the acceptor; this is termed glycohydrolase\(^2^2,2^9\).
Figure 1.3: Representation of the ADP-ribosylation mechanism. The reaction proceeds with a scission of the glycosidic linkage of NAD$^+$ and then an attack by the nucleophilic transferase substrate leading to the formation of the ADP-ribose adduct (modified from Jorgensen et al. 2005).
1.4.3: Secretion

mART toxins are secreted into either the extracellular medium or straight into the host cell\(^{30}\). This translocation across the membranes is divided into five pathways\(^{31,32}\). In pathways I, III, and IV, the substrate is transported straight through the two membranes\(^{31,32}\). Pathways II and V occur in steps, where the movement into the extracellular medium arises by either the secretory (Sec) pathway or the twin-arginine translocation (Tat) pathway\(^{31,32}\). In the Sec pathway, the proteins are unfolded as they traverse the membrane, whereas proteins transported by the Tat pathway are in their folded state\(^{33}\).

The Tat pathway contains three integral membrane proteins TatA, TatB, and TatC\(^{33}\) (Figure 1.4). Proteins are directed to this pathway by a N-terminal signal peptide with a S/T-R-R-x-F-L-K consensus motif\(^{30}\). TatC comprises six transmembrane helices that recognize the consensus motif resulting in the protein binding to the TatBC complex\(^{33}\). This binding induces the recruitment of TatA forming a translocation site, allowing the toxins to be transported into the extracellular medium\(^{33}\). In order to enter the host cell, the toxin interacts with specific surface receptors such as the low-density lipoprotein receptor-related protein (ExoA toxin from *Pseudomonas aeruginosa*)\(^{16}\).

C2 and C3-like mART toxins such as HopU1 from *Pseudomonas syringae* and SpvB from *Salmonella* spp, are both secreted by the type III secretion system\(^{34,35}\). Type III pathway has the secretion signal in the N-terminus or, as shown by recent research, the signal can also be located in the mRNA\(^{31}\). In comparison to the Tat and Sec pathways, where only a few molecules are transported into the extracellular medium, the type III secretion allows thousands of toxin molecules to be
Figure 1.4: A diagram of the TatABC complex at the translocation site\textsuperscript{33}.
transferred directly into the host cell by means of a pore resembling an injection needle\textsuperscript{31,32}

1.5: DNA and RNA Binding Motifs

DNA-binding proteins include structural motifs that bind to the grooves or the phosphate backbone of DNA by means of β-strands or α-helices\textsuperscript{36}. Some common motifs are the helix-hairpin-helix, helix-turn-helix, helix-loop-helix, leucine zipper, and zinc finger\textsuperscript{36,37}.

Proteins with a helix-loop-helix motif are usually transcriptional regulatory proteins that bind DNA with their N-terminus, while the loop region aids in the protein dimerization\textsuperscript{37}. The helix-hairpin-helix motif is non-sequence-specific and results in hydrogen bonds between the phosphate groups of DNA and the protein backbone\textsuperscript{37}. The motif consists of antiparallel α-helices linked with a hairpin-like loop\textsuperscript{37}. The loop interacts with single-stranded or double-stranded DNA by a consensus GXG sequence, with X being a hydrophobic residue\textsuperscript{37}. The helix-turn-helix recognition of a specific DNA sequence is achieved by hydrogen bonds, van der Waals contacts and water molecules\textsuperscript{38}.

There are some DNA-binding proteins that become ordered once they are bound to DNA, such as GCN4, a yeast transcription factor with a leucine zipper domain\textsuperscript{37}. The C-terminus of the leucine zipper is a parallel α-helical coiled-coil\textsuperscript{39}, whereas the N-terminus is composed of symmetrically placed α-helices that are termed the basic region\textsuperscript{39}.

The DNA-binding protein, poly(ADP-ribose)polymerase 1 (PARP1) is an example of a protein that contains zinc fingers which recognize single- and/or double-stranded
DNA breaks\textsuperscript{40}. The zinc fingers bind the sugar-phosphate backbone and the edges of base pairs either in the minor or major groove, depending on the zinc finger\textsuperscript{40}.

RNA-binding proteins have various functions and therefore a large group of structurally diverse substrates; however, the proteins can be categorized into several groups with domains such as RNA-recognition motif, K-homology (KH) domain, and zinc fingers\textsuperscript{41}.

RNA-recognition motif is the most common domain and includes three conserved residues, Arg or Lys and two aromatic residues\textsuperscript{41}. The Arg or Lys form a salt bridge with the backbone of DNA, while the aromatic residues form stacking interactions with the bases\textsuperscript{41}. The KH domain can bind both single-stranded DNA and RNA using hydrogen bonds and electrostatic interactions\textsuperscript{41}. Some proteins are able to use zinc fingers to not only bind DNA but also RNA\textsuperscript{41}. An example of this is with transcription factor TFIIA which uses certain zinc fingers to form hydrogen bonds with DNA bases in the major groove while the others are used for RNA binding\textsuperscript{41}.

1.6: \textit{Streptomyces scabies}

\textit{Streptomyces} is a filamentous, Gram-positive genus that produces pharmaceutically significant secondary metabolites\textsuperscript{42}. They are soil-dwelling saprophytes, meaning they thrive in aquatic and soil environments where they degrade recalcitrant biological polymers, such as lignin and cellulose\textsuperscript{43,44}. Following spore germination, \textit{Streptomyces} grows into a substrate mycelium by the elongation and branching of hyphae\textsuperscript{42}. Upon nutrient deficiency, a white layer forms by the aerial hyphae on the surface of the colony\textsuperscript{42}. After this, septation of the aerial hyphae results in uni-
genomic compartments, which can later develop into spores for survival and spreading in unfavourable conditions\textsuperscript{42}.

Even though the genus is more notable for pharmaceutical metabolites, there are a few species that cause plant pathogenesis; the most prevalent and economically important one being the common scab disease in tuber crops\textsuperscript{45}. \textit{S. scabies} is the first species described as a cause for the common scab disease, and is the most contributing agent in North America, while other species include \textit{S. turgidiscabies}, \textit{S. acidiscabies}, and \textit{S. europaeiscabiei}\textsuperscript{46–48}. The common scab arises up to 6 weeks after tuber initiation and occurs when the bacteria infects the laterally expanding underground parts of a plant\textsuperscript{46}. This produces lesions that are rounded, pitted, or raised, with a corky texture\textsuperscript{43,46} (Figure 1.5). \textit{Streptomyces} species are neither host- or tissue-specific and therefore can infect a variety of potato and taproot crops such as carrots, turnips, radishes and beets\textsuperscript{42,46}. The common scab disease is one of the top five diseases of potatoes in USA as it greatly affects the quality and visual appeal of the potato. In Canada, the disease resulted in losses up to $16 million dollars for 2002\textsuperscript{46,49}. The severity of the symptoms depends on the susceptibility of the cultivar and other factors, including environmental conditions, pathogen inoculum, and pathogen virulence\textsuperscript{46}.

As the plant is expanding its cell walls, cellobiose and suberin are released stimulating \textit{S. scabies} (as well as \textit{S. acidiscabies} and \textit{S. turgidiscabies}) to produce phytotoxins one of which, thaxtomin A, is the causative agent of the common scab disease\textsuperscript{50–52}. Cellobiose stimulates toxin release by binding to CebE (a lipoprotein) to then cross the bacterial cell membrane using two transmembrane proteins, CebF and CebG\textsuperscript{53}. Cellobiose is then able to bind and
Figure 1.5: Corky lesions observed on a potato tuber infected with the common scab disease\textsuperscript{54}. 
inhibit the cellulose-utilization repressor (CebR)\textsuperscript{53}. When the repressor is inhibited, the downstream thaxtomin biosynthetic gene cluster, \textit{txtA}, \textit{txtB}, \textit{txtC}, \textit{txtD}, \textit{txtE} and \textit{txtR}, can be transcribed\textsuperscript{46,50,53} (Figure 1.6A). Thaxtomin biosynthesis begins with \textit{TxtD} (nitric oxide synthase) catalyzing the production of a nitric oxide from arginine\textsuperscript{44}. \textit{TxtE} (P450 monooxygenase) uses the nitric oxide for nitration of L-tryptophan\textsuperscript{44}. \textit{TxtB} (non-ribosomal peptide synthetase) and \textit{TxTA} (non-ribosomal peptide synthetase) then uses L-4-nitrotryptophan and L-phenylalanine as respective substrates to produce a cyclo-L-4-nitrotryptophyl-L-phenylalanyl intermediate (thaxtomin D)\textsuperscript{44}. The intermediate is N-methylated on both the phenylalanyl and nitrotryptophyl moieties by the S-adenosylmethionine-dependent N-methyltransferase domain on \textit{TxtA} and \textit{TxtB}\textsuperscript{44}. Lastly, \textit{TxtC} (P450 monooxygenase) attaches the additional hydroxyl groups to the phenylalanyl moiety of thaxtomin D forming thaxtomin A\textsuperscript{44} (Figure 1.6B).

1.7: Scabin

The \textit{Streptomyces scabies} pathogenic strain 87.22 contains the Scabin gene, \textit{scab\textunderscore 27771}, which encodes a 22-kDa single-domain enzyme with 200-residues and an N-terminal secretion signal peptide\textsuperscript{30,55}. Joshi \textit{et al.} (2010) proposed that the product of the \textit{scab\textunderscore 27771} gene has a secretion signal peptide that utilizes the Tat secretion pathway as it contains a twin Arg motif\textsuperscript{30}. Lyons \textit{et al.} (2016) generated a multiple-sequence alignment (Figure 1.7) that demonstrates common shared features of Scabin with other mART toxins. These features include the Arg that is critical for NAD\textsuperscript{+} active-site binding, the Ser-Thr-Thr motif that comprises the scaffold of the NAD\textsuperscript{+} binding pocket and the catalytic Gln-X-Glu motif, which is critical for the reaction mechanism\textsuperscript{55}. When the catalytic Gln-X-Glu motif was replaced with Ala (Q128A-X-E160A), a 300-fold decrease
Figure 1.6: Thaxtomin A synthesis. (A) A representation of the *Streptomyces scabies* induction of thaxtomin production by cellobiose\(^5\). (B) The synthesis pathway of thaxtomin A\(^5\). Steps that are not yet understood are labeled with question marks.
Figure 1.7: A multiple sequence alignment comparing Scabin with 14 orthologs demonstrating the conserved Arg, STT, and the QXE motifs.
in turnover number ($k_{cat}$) was observed compared to wild-type$^{55}$. This confirms the importance of both residues in the activity of Scabin. Additionally, the alignment revealed a 40% sequence identity between Scabin and the Pierisin-1 mART toxin$^{55}$. Pierisin-1 is unique as it modify guanine bases in DNA with the ADP-ribose$^{56}$.

Lyons et al. (2016) tested the activity of Scabin with guanine-containing substrates, such as GDP, cGMP, a single-stranded oligonucleotide with 5 dG bases, and a single guanine base in a double-stranded oligonucleotide$^{55}$. Scabin transferase activity was measured using a fluorescence assay as described in Lyons et al. (2016). Transferase activity was observed with all the above substrates, except when the DNA contained no guanine bases$^{55}$. Confirmation was done using mass spectrometry where the added ADP-ribose mass on each substrate was detected$^{55}$. Since Pierisin-1 has been shown to modify the guanine base at the exocyclic N2 substituent$^{57}$, this modification was also tested for Scabin. The absence of activity with a deoxyguanine base lacking an exocyclic NH$_2$ substituent, confirmed that Scabin labels the same site as Pierisin-1$^{55}$. The preferred dsDNA conformation for Scabin was tested by Lyons et al. (2018), using a synthetic cyanine-3 tagged dsDNA$^{58}$. In contrast with blunt-ended dsDNA, Scabin showed a higher affinity for a single-base overhang$^{58}$. The location of the overhang or a second-base overhang did not influence activity$^{58}$.

The crystal structure of Scabin in its apo state (PDB: 5DAZ) and with three competitive inhibitors, PJ34 (PDB: 5EWK), P6-E (PDB: 5EWY), and NADH (PDB: 5TLB) confirm Scabin is a single-domain mART toxin (Figure 1.8A, B, C, and D)$^{55}$. Scabin possesses a central β-sheet core comprised of a four-stranded βI-sheet perpendicular to a three-stranded βII-sheet$^{58}$ (Figure 1.8E). Within the βI-sheet, β4 and β5 are connected via the
Figure 1.8: Surface images of Scabin (5DAZ) highlighting the interaction with (B) P6-E (5EWY), (C) PJ34 (5EWK), and (D) NADH (5TLB). (A) Scabin-apo crystal structure is shown in light blue as a cartoon diagram with a transparent surface. (B) Scabin-P6-E complex structure is shown in beige as a cartoon diagram with a transparent surface and P6-E (black) represented as sticks. (C) Scabin-PJ34 complex structure is shown in pale green as a cartoon diagram with a transparent surface and P6-E (black) represented as sticks. (D) Scabin-NADH complex structure is shown in teal as a cartoon diagram with a transparent surface and NADH (black) represented as sticks. (E) Scabin (5DAZ) shown as ribbons with α-helices and β-strand labeled.\(^{58}\)
ARTT-loop$^{58}$. Regarding the βII-sheet, there is a β6/7-turn that connects β6 with β7-8$^{58}$. The two β-sheets are linked together by the PN-loop and the α1-α2 motif$^{58}$. In Figure 1.8, Scabin can be seen to contain large loop regions. This tertiary structure adds more flexibility to the catalytic core of Scabin facilitating the binding of the DNA substrate.

To determine the possible interaction between Scabin and DNA, Lugo et al. (2018) used experimental (hydrogen deuterium exchange), theoretical (Gaussian network modeling), and simulated (molecular dynamics) approaches. According to the in situ model, the Scabin-DNA interface involves 13 bases on both DNA strands (DNAI and DNAII) and 18 residues on Scabin$^{58}$ (Figure 1.9A,B). The contact area can be divided into three sections: a lower-area, central-area and upper-area (Figure 1.10A, D). The lower-area contains electrostatic interactions between the DNA backbone and the positively charged residues$^{58}$. Salt-bridges to the DNAII strand occur with the side-chains of Lys130 (PN-loop) and the cluster 180-KKxR$^{58}$ (Figure 1.10B). Regarding the middle-area, there are four residues, Asn110, Trp128, Trp155, and Gln158 that interact with the NAD$^+$ molecule as well as both DNA strands$^{58}$. Asn110, Trp128, and Gln158 use hydrogen-bonds and van der Waals forces to interact with the DNAI backbone$^{58}$ (Figure 1.10D). Mutagenesis of Asn110 to an Ala showed impairment to Scabin's ability to bind to dsDNA as the difference in the dissociation constant between N110A, $K_D = 82 \pm 5$ µM, and wild-type, $K_D = 51 \pm 4$ µM, was statistically significant$^{58}$. The Trp128 (PN-loop) variant (W128Y) showed comparable transferase and glycohydrolase activity to wild-type however the affinity for dsDNA is impaired$^{59}$. Trp155 (ARTT-loop) has van der Waals interactions with the 9th base of DNAI, which in the DNA sequence was a guanine base$^{58}$. Trp155 variant (W155A) showed no visible transferase activity and it impaired binding to dsDNA$^{59}$. This
confirms the interaction of Trp155 with both DNA and NAD$^+$ as predicted in the model. Two more residues in this area also contact DNA. Thr127 interacts with the DNAI backbone, but it is the Tyr129 (PN-loop) that plays the most important role\textsuperscript{58}. Tyr129 is located in a minor groove and associates with 4 DNA bases and with the 7\textsuperscript{th} base of the DNAII strand\textsuperscript{58} (Figure 1.10E). Therefore, this residue interacts with both DNA strands. The contacts in the upper-area include a salt-bridge between Arg152 (ARTT-loop) and the DNAII strand, and a hydrogen-bond with Ser105\textsuperscript{58}. The dominate interaction, however, is between the $\alpha$2 residues (Leu108 and Val109) and 5 DNA bases from both strands in a major groove\textsuperscript{58} (Figure 1.10C).

1.8: Research Rationale and Objectives

Scabin is a recently characterized and unique mART toxin that modifies DNA and it is an excellent candidate for inhibitor development. Natural product libraries provide a large and diverse source of potential inhibitor compounds that have the capability to provide treatment for the common scab disease. A natural-based product would offer benefits for treating this disease as potatoes are a food product. The goals of this research were (i) to characterize the DNA-binding motif within the Scabin toxin and (ii) to identify natural product inhibitors against the growth of \textit{Streptomyces scabies}.

First, a methylene blue assay was developed to analyze the inhibition of \textit{S. scabies}'s growth by several plant tinctures. The same plant tinctures were then tested for their ability to reduce the growth of three other \textit{Streptomyces} strains, \textit{S. turgidiscabies}, \textit{S. acidiscabies}, and \textit{S. europaeiscabiei}. Lastly, a kinetic analysis of residues involved in the Scabin DNA-binding motif was performed.
Figure 1.9: Scabin-NAD+-DNA complex model. (A) Scabin-NAD⁺-DNA model is shown as a molecular surface with Scabin in gray ribbons and the contact interface colored according to electrostatic potential. Black sticks represent the NAD⁺ and the dsDNA molecule is shown as green ribbons with the molecular surface colored by electrostatic potential. (B) Scabin-NAD⁺-DNA interactions. A schematic representation of the interactions between 13 bases from both DNA strands and 18 Scabin residues (light blue). The interactions between 4 residues (red) and NAD⁺ are shown.⁵⁸
Figure 1.10: Scabin-NAD+-DNA interaction model. (A) The upper and lower Scabin areas that contact the dsDNA. Scabin is shown as a transparent molecular surface coloured dark gray. The contact points on Scabin with the dsDNA are colored according to the electrostatic potential. The DNAII strand is shown as a green ribbon with the red colour indicating bases that contact Scabin. A closer look at the lower-area (B) and upper-area (C) on Scabin are shown with the same colouring as mentioned above. (D) The middle-area of Scabin that interactions with the DNAI strand and NAD+ molecule. The NAD+ molecule is shown as a cyan stick with a transparent surface coloured according to the electrostatic potential; all other colouring is the same as above. (E) Y129A as a yellow molecular surface and its interactions with the minor groove of DNA. Van der Waals interactions are highlighted as purple shells56.
Chapter 2: Materials and Methods
2.1: Materials

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2: Expression and purification

Scabin was cloned into a pET-TEV vector which encodes a N-terminal His$_6$ tag and a tobacco etch virus (TEV) protease cut site. The Quikchange® mutagenesis kit was used to produce Scabin site-directed mutants. Each plasmid was used to transform chemically competent *Escherichia coli* BL21 λDE3 cells which were grown at 37°C overnight on LB medium containing 30 µg/mL kanamycin. Colonies were then scraped into 50 mL LB supplemented with 30 µg/mL kanamycin and grown at 37°C with shaking at 200 rpm. Once an OD$_{600}$ of 0.6 was achieved, 25 mL of broth was inoculated into 800 mL 2xYT (1.6 w/v (%) tryptone, 1 w/v (%) yeast extract, 0.5 w/v (%) NaCl) medium containing 30 µg/mL kanamycin. The culture was grown to an OD$_{600}$ of 1.2 and then induced with 1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG) for 3 hours. The cells were pelleted at 2000 x g and resuspended in lysis buffer (25 mM Tris-HCl, pH 8.2, and 200 mM NaCl) with the addition of 1 mM ethylenediaminetetraacetic acid (EDTA), 40 µg/mL 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 100 µg/mL DNase and 120 µM phenylmethanesulfonyl fluoride (PMSF). An Emulsifex-C3 high-pressure homogenizer (Avestin Inc., ON, Canada) was used to lyse the cells, followed by centrifugation at 23,700 x g for 50 min at 4°C.

The supernatant was purified by immobilized-metal-affinity chromatography (IMAC) using a HiTrap IMAC HP 5 mL column (GE Healthcare). The column was equilibrated with 5 mM imidazole in binding buffer (50 mM TAPS, pH 8.5, 500 mM NaCl)
and washed with 25 mM imidazole in the binding buffer. The protein was eluted with a gradient of 25 to 250 mM imidazole. To confirm the presence of the protein of interest, fractions were confirmed on a SDS-PAGE gel, then pooled and dialyzed into dialysis buffer 1 (25 mM Tris-HCl (Fisher Scientific (Waltham, MA)), pH 8.2, and 50 mM NaCl).

Anion-exchange chromatography was used to further purify the protein using a HiTrap Q-Sepharose HP column (GE Healthcare) equilibrated with dialysis buffer 1 (with the exception that Tris buffer (Sigma-Aldrich (St. Louis, MO)) was used). A linear gradient of 10 to 500 mM NaCl in 25 mM Tris-HCl, pH 8.2, was used to elute the protein which was then confirmed by SDS-PAGE. The fractions were pooled and dialyzed into buffer 2 (50mM Tris-HCl (Sigma-Aldrich (St. Louis, MO)), pH 8.2, and 100mM NaCl). The protein was then concentrated initially on a bed of poly(ethylene glycol) (PEG) (molecular weight 20,000) at 4°C. When the protein reached a volume of 5 mL, it was further concentrated using a Millipore 0.5-mL 10-kDa spin column at 1,696 x g for 20 min intervals until a concentration of 1.5 mg/mL was achieved as measured with a Nanodrop 2000 spectrophotometer (Thermo Scientific) at a wavelength of 280nm.

2.3: Circular dichroism spectra

The folding integrity of each Scabin variant was confirmed by circular dichroism (CD). This was achieved by using a J-815 CD spectropolarimeter (JASCO, MD, USA) set to scan from 250 – 190 nm and 9 spectra were averaged. The protein (0.2 mg/mL) was in 20 mM Tris-HCl, pH 8.2, and 50 mM NaF and in a 1-mm path length quartz cuvette.
2.4: Glycohydrolase reaction

Glycohydrolase (GH) activity was monitored using a Cary Eclipse fluorescence spectrophotometer (Varian, CA, USA) with an excitation wavelength of 305 nm, emission wavelength of 405 nm, and a band pass of 5 nm. Scabin (final concentration of 50 nM) was added to reaction buffer (20 mM Tris-HCl, pH 7.9, and 50 mM NaCl) and various ε-NAD concentrations (0-450 µM). Triplicate reactions are monitored for 10 min and the initial slope was recorded. A Michaelis-Menten curve was derived in OriginPro version 8 software using a hyperbolic model and an ε-AMP standard curve was used to convert fluorescent units/min to [ε-ADP ribose] formed/min.

2.5: ADP-ribosyltransferase reaction

To determine the transferase activity for the Scabin variants, a few modifications to the “Glycohydrolase reaction” assay described above were performed. Scabin (10 nM) was mixed in reaction buffer containing 2% dimethyl sulfoxide, ε-NAD (200 µM) and various concentrations (0 – 1250 µM) of deoxyguanosine (dG). The data was fit to a sigmoidal dose response model in OriginPro version 8 software.

2.6: DNA binding of Scabin with cyanine-3 tagged oligomer

Two synthetic DNA oligomers were purchased from Sigma-Aldrich. Oligomer 1 (5’-GGAAGAGAGAGAAAGAGAGAG-3’) with a 5’ cyanine-3 tag and oligomer 2 (5’-CTCTCTTTTCTCTCTCTTTCC-3’) were combined in equal molar amounts and annealed together using a Techne TC-512 PCR instrument (Burlington, NJ). The oligomers were heated to 95°C and then cooled to 25°C at a rate of 1.2°C/min. The Cy3-dsDNA (5 µM) were titrated with Scabin in an ultra-micro quartz cuvette (3 mm x 3 mm) and 50 mM Tris-HCl, pH 8.2, 100 mM NaCl.
Data collection was performed as previously described by Lyons et al. (2018). In brief, data was collected using a PTI-Alphascan-spectrofluorometer (Photon Technologies Inc., South Brunswick, NJ) in a 'T-format' configuration. With band-passes set to 4 nm, the anisotropy was measured for 20 s using an excitation wavelength of 550 nm and emission wavelength of 570 nm.

2.7: Culturing Streptomyces strains

The four Streptomyces strains tested were: S. scabies 87.22, S. turgidiscabies MB7, S. acidiscabies ATCC 49003, S. europaeiscabiei CFBP4501.

All strains were cultivated on nutrient agar (0.5 w/v (%) tryptone, 0.3 w/v (%) yeast extract, 0.5 w/v (%) NaCl, and 1.5 w/v (%) agar) at 37°C. Mycelium stocks of each strain were prepared by inoculating a colony into nutrient broth (0.5 w/v (%) tryptone, 0.3 w/v (%) yeast extract, 0.5 w/v (%) NaCl) and incubated for 3 days at 30°C and shaking at 250 rpm in an Erlenmeyer flask with a steel spring (used to shear the filaments that are produced). The culture was then mixed in a 1:1 ratio with 40% glycerol and frozen at -80°C for future use. The colony forming units of each stock was determined by serial diluting the stock on nutrient agar and counting the colonies after incubation for 3 days at 30°C.

Unless otherwise stated growth of all four Streptomyces strains was performed at 30°C and 250 rpm in an Erlenmeyer flask with a steel spring.

2.8: Methylene blue assay

The methylene blue assay reported by Fischer and Sawers (2013), was optimized. Aliquots (1 mL) were taken from cultures at various time points and spun
down for 7 min at 21,230 x g. The supernatant was removed and 1 mL of 1.5 mM filtered methylene blue (Fischer Scientific, MA, USA) was added. The tube was placed in a heat block set at 80°C for 10 min with shaking at 850 rpm. The tube was then chilled on ice for 3 min before another spin at 21,230 x g for 3.5 min. The supernatant was plated in a Corning 96-well, clear, flat-bottom, polystyrene, sterile microplate (Corning Inc., New York City, NY) at a 1/100 dilution. The absorbance at 660 nm was read using a FLUOstar Omega plate reader (BMG LABTECH, Cary, NC) and normalized against the absorbance of pure dye.

2.9: *Escherichia coli* growth curve using the methylene blue assay

*Escherichia coli* was grown in 5 mL LB media overnight at 30°C and 200 rpm. Afterwards, 0.4 mL was inoculated into 50 mL fresh LB and grown again. Every hour, 1 mL of culture was collected and processed first by OD$_{600}$ and then by Abs$_{660}$ (using the methylene blue assay) until a total of 6 hours was achieved.

2.10: Growth curve

The mycelium stocks (1.32 x $10^6$ CFU) for each strain were added to nutrient broth and grown to an Abs$_{660}$ (using the methylene blue assay) of 0.18 was achieved. From these cultures, 0.7 mL was inoculated into 40 mL of fresh nutrient broth and the growth was tracked (using the methylene blue assay) every 2 hours for 28 – 30 hours, depending on the strain.

2.11: Ethanol tolerance

The mycelium stocks (1.32 x $10^6$ CFU) for each strain were grown in nutrient broth until an Abs$_{660}$ of 0.18 was achieved. From this, 0.7 mL was inoculated into 40 mL of fresh
nutrient broth with the addition of either 0%, 1%, 1.5%, 2%, or 2.5% ethanol. The flasks were grown for 16 hours and then analyzed using the methylene blue assay.

2.12: Plant tincture inhibition testing

A total of 56 plant tinctures (Perfect Herbs (Toronto, ON), Mond Trading Co. (Toronto, ON), and Secrets of the Tribe (Nevada Pharm LLC., Las Vegas, NV)) in ethanol (Table 1) were tested against *S. scabies*. Each plant tincture was filtered with a sterile 0.2 um nylon filter (Fischer Scientific, MA, USA) to remove any contamination that may be present. To start the testing, *S. scabies* was grown from mycelium stock until an \( \text{Abs}_{660} \) of 0.18 was achieved. Once complete, 0.7 mL of culture is added to 40 mL nutrient broth with the addition of either plant tincture or 2% ethanol. The flasks are grown for 16 hours, where they are then processed using the methylene blue assay.

2.13: Agar diffusion

Mycelium stocks \((1.32 \times 10^6 \text{ CFU/mL})\) were spread plated on sporulating agar \((0.2 \text{ w/v} \%) \text{ tryptone, 0.1 w/v} \% \text{ yeast extract, 0.0012 w/v} \% \text{ FeSO}_4, 1 \text{ w/v} \% \text{ glucose, and 1.5 w/v} \% \text{ agar})\). Once dried, 20 µL of either 100% ethanol or plant tincture \((100\%, 1/5 \text{ dilution}, 1/10 \text{ dilution}, 1/100 \text{ dilution})\) was added to a disc. All four discs were added to one sporulating plate and incubated at 30°C for 5 days. The zone of inhibition around each disc was measured.
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1 60% ethanol  
2 40% ethanol  
3 50% ethanol  
4 25% ethanol  
5 20% ethanol  
6 100% ethanol  
7 58% ethanol  
8 30% ethanol  
9 45% ethanol  
A Secrets of the Tribe  
B Perfect Herbs  
C Mond Trading Co
Chapter 3: Investigating the susceptibility of four *Streptomyces* strains to plant tinctures.
3.1: Comparing the methylene blue assay to optical density readings

*Streptomyces* strains are difficult to handle because of their filamentous nature. This means their growth can not be tracked with conventional methods such as optical density. Dry cell weight can be used; however, this method requires sufficient mass to be weighed accurately and a drying phase that can last up to 24 hours. The experiments herein were designed to track the extent of growth inhibition caused by a plant tincture which might result in a small cell mass that may be difficult to weigh accurately, therefore another approach was needed to accurately assess *Streptomyces* growth.

Fischer and Sawers (2013) demonstrated the utility of methylene blue dye to track the growth of filamentous bacteria. As *S. coelicolor* M145 strain was used by Fischer and Sawers (2013), adaptations to the method were performed to track the growth of four pathogenic *Streptomyces* strains. As a control, the growth of *Escherichia coli* BL21 cells was studied to compare the methylene blue assay to optical density measurements. The modifications to the assay included the addition of a sonication and vortex step as well as varying incubation times on ice. Briefly, *E. coli* BL21 cells were grown in LB at 37°C and 200 rpm for a total of 5.5 hours with both OD and methylene blue measurements taken every 30 minutes. The addition of sonication and vortex mixing in the methylene blue assay showed measurements that did not align with the OD values for *E. coli* when plotted on the same graph (data not shown). The cooling of the sample for 3 minutes showed the best results and therefore was used in the final version of the protocol. As seen in Figure 3.1, the optical density and methylene blue absorbances showed identical lag phase and similar exponential and stationary phases. This provided the impetus to
Figure 3.1: Comparison of two growth curves of *Escherichia coli* BL21. Absorbances values were taken in triplicates every 30 minutes using both optical density (white circles – $\text{Abs}_{600}$) and the methylene blue assay (black squares – $\text{Abs}_{660}$). *E. coli* BL21 was grown in LB for a total of 5.5 hours at 37°C and 200 rpm.
use the methylene blue assay to measure the growth of the four pathogenic *Streptomyces* strains.

**3.2: Growth curves of *Streptomyces* strains**

The filamentous nature of *Streptomyces scabies* means the culture aggregates when grown in liquid medium (Figure 3.2A). The use of a steel spring added to each flask sheered the filaments resulting in a more even distribution of cells and growth (Figure 3.2B). Once a uniform suspension was achieved, a growth curve of each strain was performed. This was done to ensure that future testing was completed during the exponential growth-phase for each strain. This growth-phase for *Streptomyces* species can vary as seen in a laboratory maintenance article by Shepherd *et al.* (2010) which stated that the growth will double every 4 to 6 hours.62

The use of mycelium from an exponentially growing culture as inoculum allowed *S. scabies* to have a shorter lag phase of 4 hours. This was followed by an exponential growth phase (duration 20 hours; doubling time 6.9 hours) and then after 24 hours, the culture reached stationary phase (Figure 3.3A). The growth of *S. turgidiscabies* shows a lag phase (duration 8 hours), an exponential phase (duration 16 hours; doubling time 6.6 hours), and a stationary phase after 24 hours of growth (Figure 3.3B). The growth of *S. europaeiscabiei* was illustrated by a lag phase (duration 8 hours), a short exponential growth phase (duration 8 hours; doubling time 2.6 hours), and a stationary phase that was reached after 22 hours (Figure 3.3C). Between the 16-hour and 22-hour time period, there was slight growth of the bacteria; however, it proceeded at a slower rate. The growth of *S. acidiscabies* demonstrated a longer lag phase (duration 8 hours) and a similar exponential phase (duration 20 hours; doubling time 5.7 hours) when compared to *S.
Figure 3.2: Culturing of *Streptomycyes scabies*. (A) *S. scabies* filamentous nature results in the culture clumping as observed in 5 mL nutrient broth. The culture was grown at 30°C and 250 rpm. (B) An example flask used to culture the four *Streptomycyes* strains. A steel spring sheered the bacterial filaments to produce a more homogeneous culture.
Figure 3.3: Growth curves of four *Streptomyces* strains. The methylene blue assay was used to measure the absorbance at 660 nm every 2 hours for *S. scabies* (A), *S. turgidiscabies* (B), *S. europaeiscabiei* (C), and *S. acidiscabies* (D). The strains were grown in 40 mL nutrient broth with a steel spring at 30°C and 250 rpm.
scabies and S. turgidiscabies. At 28 hours after inoculation, a death phase commenced (Figure 3.3D).

The variation seen between these four Streptomyces strains is not uncommon. In the literature, Streptomyces strains can vary in their doubling time such as 4 hours for S. griseus and 2 hours for S. viridifaciens. Growth depends on the nutrients available in the medium; therefore, it is not unusual to have one strain with multiple doubling times. Seipke and Loria (2008) worked with S. scabies 87-22 and obtained a doubling time of roughly 2 hours in tryptic soy broth.

3.3: Ethanol tolerance of Streptomyces strains

The commercial plant tinctures were prepared in an ethanol/water solution and, therefore, a tolerance test of the four Streptomyces strains to ethanol was first conducted. This provided a dose range of tincture concentrations that could be added to the growth cultures. As seen in Figure 3.4A, at a dose of 2.5% ethanol, the S. scabies growth was unaffected when compared to the control (0% ethanol). S. turgidiscabies was the most sensitive to exogenous ethanol in the culture with 1.5% ethanol being the most inhibitory; surprisingly, the higher ethanol doses were not as cytotoxic (Figure 3.4B). S. europaeiscabiei was also very sensitive to ethanol in the culture medium. When 2% ethanol was added, the growth decreased by two thirds and almost no growth was observed with 2.5% ethanol (Figure 3.4C). S. acidiscabies showed an increased growth when 1% ethanol was supplied to the culture. The higher doses of ethanol resulted in similar growth levels for this Streptomyces strain (Figure 3.4D).
Figure 3.4: Ethanol tolerance of the *Streptomyces* strains. *S. scabies* (A), *S. turgidiscabies* (B), *S. europaeiscabiei* (C), and *S. acidiscabies* (D) were grown to an Abs$_{660}$ of 0.18 and were then used to inoculate (0.7 mL) 40 mL of nutrient broth. These cultures were then dosed with ethanol at various percentages. The flasks were grown at 30°C and 250 rpm for 16 hours, followed by growth determination with the methylene blue assay.
With these results, it was decided that the maximum dose of ethanol tolerated by *S. scabies*, *S. turgidiscabies* and *S. acidiscabies* was 2% (v/v). For *S. europaeiscabiei*, a maximum of 1.5% ethanol was the limit. These amounts were chosen to ensure that growth inhibition by ethanol was not a factor while determining the tincture dose for curtailing *Streptomyces* growth.

3.4: Plant tinctures growth inhibition of *Streptomyces* strains

From each growth curve, a time point was chosen (*S. scabies* – 16 hour; *S. turgidiscabies* and *S. acidiscabies* – 18 hour; and *S. europaeiscabiei* – 15 hour) to analyze the growth of the culture during the plant tincture test.

A total of 54 plant tinctures were tested against *S. scabies* and there was a range of antimicrobial effects. The 7 best plant tinctures that were the best inhibitors of *S. scabies* growth after 16 hours were myrrh, garlic, cayenne, barberry, frankincense, wild indigo root, and lavender (Figure 3.5). The amount of *S. scabies* in the flask after 16 hours of incubation with the myrrh tincture (absorbance value of 0.0045 ± 0.0038) and the garlic tincture (absorbance value of 0.0085 ± 0.00024) are comparable to the amount of *S. scabies* at the 0-hour (absorbance value of 0.0037 ± 0.0013) and 2-hour (absorbance of 0.0082 ± 0.0078) time point on the growth curve. This means that these tinctures were able to completely inhibit *S. scabies* growth. The best plant tinctures, myrrh and garlic, were also tested on the three other pathogenic *Streptomyces* strains and growth inhibition was observed in all three strains (data not shown).
Figure 3.5: A selection of plant tinctures that exhibited growth inhibition of *Streptomyces scabies*. *S. scabies* was grown to an $\text{Abs}_{660}$ of 0.18 where it was then inoculated (0.7 mL) into 40 mL of nutrient broth with the addition of a plant tincture (2% of the final volume). After 16 hours of growth at 30°C and 250 rpm, the amount of *S. scabies* in each flask was analyzed using the methylene blue assay.
In the literature, sesquiterpenes compound isolated from myrrh resin have been shown to exhibit antimicrobial activity towards several microorganisms such as *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*. Garlic extract exhibits antimicrobial activity towards *E. coli*, *S. aureus*, *Bacillus cereus*, and *Salmonella typhi*. An active component in garlic, allicin, has been shown to inhibit both DNA, RNA and protein synthesis. Therefore, it is evident that these two plant tinctures have a wide range of antimicrobial activity and that identifying the active components may reveal the mode of action of the plant tinctures.

Since the plant tinctures showed a significant effect on *S. scabies*, they were retested against a 10-fold higher *S. scabies* concentration. There was still a wide range of results with some tinctures inhibiting while others promoted growth (Figure 3.6A); however, the best 7 tinctures remained the same (Figure 3.6B).

The inhibitory effect of plant tinctures was also tested against *S. scabies* using an agar diffusion method. *S. scabies* was plated on nutrient agar where four disks were placed on the surface with equal spacing between the disks. Each disc contained either 100% ethanol (as a control) or various dilutions of a plant tincture. The plant tinctures that demonstrated the largest zones of clearing were myrrh and garlic (Figure 3.7). The zones of clearing produced by these two plant tinctures are small when considering that in the broth culture they were able to fully inhibit the growth of the bacteria. This could be due to differences in the amount of *S. scabies* in both experiments. For the agar diffusion experiment, 1 mL of *S. scabies* stock was used as it produced an even lawn of spore growth. Whereas, in the broth culture, *S. scabies* stock was diluted into 40 mL of nutrient broth and then 7 mL of that culture was diluted again in 40 mL of broth. In terms of colony
Figure 3.6: (A) The effect of 47 plant tinctures and (B) the 7 most inhibitory plant tinctures on Streptomyces scabies growth. S. scabies was grown to an Abs$_{660}$ of 0.18 and was then inoculated (7 mL) into 40 mL nutrient broth with the addition of a plant tincture (2% of the final volume). After 16 hours of growth at 30°C and 250 rpm, the amount of S. scabies in each flask was analyzed using the methylene blue assay. The red line indicates the growth of the control.
Figure 3.7: Agar diffusion of myrrh and garlic tinctures against *Streptomyces scabies*. *S. scabies* was spread plated on sporulating agar (see Materials and Methods) with the addition of 4 discs containing 100% ethanol, 100% plant tincture, a 1/10 dilution, and a 1/100 dilution of the plant tincture. The plate was incubated at 30°C for 5 days.
forming units, the agar diffusion had $1.3 \times 10^6$ CFU whereas the broth culture only had $1.9 \times 10^4$ CFU; therefore, the plant tincture on the disc had 100 times more S. scabies to act on compared with the broth culture. This is a significant difference in the experimental setup and the observation of an effect with garlic and myrrh in the agar diffusion experiment means these two tinctures can inhibit a relatively high concentration of S. scabies.

3.5: Conclusions

Currently, the methods for treating S. scabies in a field are reducing soil pH, irrigation management and crop rotation; however, they all have inconsistent results\textsuperscript{47}. The most effective treatment to date is soil fumigation. This is a very expensive process and has a negative impact on the environment; therefore, more environmentally friendly and effective alternatives are needed\textsuperscript{47}.

As seen in both the broth and agar diffusion experiments, both myrrh and garlic show the greatest inhibitory effect on S. scabies. In the broth culture, they can also curtail growth for the other three pathogenic Streptomyces strains. These results provide promise that plant tinctures may be a viable alternative to the treatment of Streptomyces-based scab disease in tuberous crops. The 7 best tinctures, including myrrh and garlic, should be tested in field studies in the quest for the development of more cost-effective and environmentally friendly agents to treat crops diseases.
Chapter 4: Characterizing the DNA-binding signature of Scabin toxin
4.1: Scabin-DNA model

According to the *in situ* model of Scabin in complex with dsDNA, the Scabin-DNA interface involves 13 bases on both DNA strands and 18 residues on Scabin. The lower-area contains salt-bridges to the DNAII strand with the side-chains of Lys130 (PN-loop) and the cluster 180-KKxR. Regarding the middle-area, there are four residues, Asn110, Trp128, Trp155, and Gln158 that interact with the NAD$^+$ molecule as well as both DNA strands. It is the Tyr129 (PN-loop) that plays the most important role. Tyr129 is in a minor groove and associates with 4 DNA bases and with the 7th base of the DNAII strand. The dominate interaction in the upper-area is between the residues Leu108 and Val109 and 5 DNA bases from both strands in a major groove.

4.2: Folded integrity of Scabin variants

The Scabin-DNA model revealed Val109, Lys130, Leu108, and Lys181 as possible residues that interact with bases in the DNA substrate. To determine the role of each active-site residue in the binding of Scabin to DNA, they were replaced with either an Ala or Gly by site-directed mutagenesis. To assess the impact of each replacement on the folded integrity of Scabin, circular dichroism (CD) spectroscopy was conducted. When comparing the CD spectra of the Scabin variants to wild-type, no large differences were observed in the secondary structure (Figure 4.1). This implies that the folded integrity of Scabin did not change significantly upon residue replacement and that any variation in kinetic data for the variants was likely not due to protein conformational changes.
**Figure 4.1:** Circular dichroism spectra of wild-type Scabin and site-directed variants. Wild-type Scabin (black), K130A (orange), K181A (purple), V109G (green), and L108G (maroon). The concentration of each protein was 0.2 mg/mL in 20 mM Tris, 50 mM NaF (pH 8.2) buffer. Each spectrum represents the average of nine independent spectra.
4.3: GH activity of Scabin variants

As reported previously by Lyons et al. (2016), wild-type Scabin shows kinetic activity towards its NAD$^+$ substrate with a $K_M$ of $68 \pm 3 \, \mu$M, a $k_{cat}$ of $94 \pm 2 \, \text{min}^{-1}$, and a catalytic efficiency of $1.38 \times 10^6 \, \text{M}^{-1} \text{ min}^{-1}$. The kinetic data for the four Scabin active-site variants with a fluorescent analog of NAD$^+$, ε-NAD$^+$, are presented in Table 4.1. K130A showed weaker interaction with the substrate compared to wild-type ($K_M = 160 \pm 19 \, \mu$M); however, there was an increase in the $k_{cat}$ value ($141 \pm 5 \, \text{min}^{-1}$). L108G and K181A are overall poorer enzymes for turnover of the ε-NAD$^+$ substrate, although there was a reduction in their $K_M$ values ($49 \pm 8 \, \mu$M and $33 \pm 3 \, \mu$M, respectively), despite the significant reduction in turn-over number as seen for their $k_{cat}$ values ($11 \pm 0.7 \, \text{min}^{-1}$ and $32 \pm 3 \, \text{min}^{-1}$, respectively). V109G showed an increase in $K_M$ ($111 \pm 23 \, \mu$M) and a decrease in the $k_{cat}$ value ($55 \pm 64 \, \text{min}^{-1}$) compared to wild-type Scabin. Overall, the changes in GH activity for these Scabin active-site variants indicated that their residues do not significantly interact with either NAD$^+$, as seen in the DNA-Scabin model, or the NADH inhibitor, as seen in the Scabin-NADH crystal structure.

4.4: Transferase activity of Scabin variants towards a deoxyguanosine substrate

A deoxyguanosine substrate (dG) was used as a model nucleoside to analyze the transferase ability of wild-type Scabin. Lyons et al. (2018) showed the wild-type enzyme in the presence of this nucleoside substrate produced a sigmoidal kinetic curve with a $K_{0.5}$ of $302 \pm 12 \, \mu$M, a $k_{cat}$ of $83 \pm 5 \, \text{min}^{-1}$ and a catalytic efficiency of $2.75 \times 10^5 \, \text{M}^{-1} \text{ min}^{-1}$. This same assay was used to determine the kinetic parameters of the Scabin active-site variants (Table 4.2). The K130A ($K_{0.5} = 58 \pm 3 \, \mu$M; $k_{cat} = 131 \pm 12 \, \text{min}^{-1}$) and K181A
Table 4.1: The kinetic parameters for the glycohydrolase activity of Scabin wild-type and variants.

<table>
<thead>
<tr>
<th>Construct</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>68 ± 3</td>
<td>94 ± 2</td>
<td>1.38 x 10$^6$</td>
</tr>
<tr>
<td>L108G</td>
<td>49 ± 8</td>
<td>11 ± 0.7</td>
<td>2.24 x 10$^5$</td>
</tr>
<tr>
<td>V109G</td>
<td>111 ± 23</td>
<td>55 ± 64</td>
<td>4.95 x 10$^5$</td>
</tr>
<tr>
<td>K130A</td>
<td>160 ± 19</td>
<td>141 ± 5</td>
<td>8.81 x 10$^5$</td>
</tr>
<tr>
<td>K181A</td>
<td>33 ± 3</td>
<td>32 ± 3</td>
<td>0.97 x 10$^6$</td>
</tr>
</tbody>
</table>
Table 4.2: Scabin wild-type and variants transferase activity with a deoxyguanosine substrate.

<table>
<thead>
<tr>
<th>Construct</th>
<th>$K_{0.5}$ (µM)</th>
<th>$k_{cat}$ (min^{-1})</th>
<th>$k_{cat}/K_{0.5}$ (M^{-1} min^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>302 ± 12</td>
<td>83 ± 5</td>
<td>2.75 x 10^5</td>
</tr>
<tr>
<td>L108G</td>
<td>574 ± 56</td>
<td>162 ± 58</td>
<td>2.82 x 10^5</td>
</tr>
<tr>
<td>V109G</td>
<td>281 ± 26</td>
<td>7 ± 1</td>
<td>2.49 x 10^4</td>
</tr>
<tr>
<td>K130A</td>
<td>58 ± 3</td>
<td>131 ± 12</td>
<td>2.26 x 10^6</td>
</tr>
<tr>
<td>K181A</td>
<td>154 ± 16</td>
<td>212 ± 53</td>
<td>1.37 x 10^6</td>
</tr>
</tbody>
</table>


\( (K_{0.5} = 154 \pm 16 \mu M; k_{cat} = 212 \pm 53 \text{ min}^{-1}) \) variants are overall better enzymes than wild-type in terms of dG transferase activity. The L108G variant showed a weaker interaction \( (K_{0.5} = 574 \pm 56 \mu M) \) with the substrate; surprisingly, it gave a higher \( k_{cat} \) \( (162 \pm 58 \text{ min}^{-1}) \). V109G gave an identical \( K_{0.5} \) as the wild-type \( (K_{0.5} = 281 \pm 26 \mu M) \); however, its \( k_{cat} \) \( (7 \pm 1 \text{ min}^{-1}) \) was significantly reduced.

4.5: Scabin variants affect DNA binding

Lyons et al. (2018) showed the ability of wild-type Scabin to bind to a synthetic cyanine-3 tagged double-stranded DNA \( (K_D = 51 \pm 4 \mu M) \) by means of a fluorescence anisotropy-based binding assay\(^{59}\). This same assay was conducted to determine the affinity of K130A, L108G, V109G, and K181A variants for DNA (Table 4.3). The Scabin variants K130A \( (K_D = 75 \pm 4 \mu M) \), L108G \( (K_D = 60 \pm 1 \mu M) \), and K181A \( (K_D = 31 \pm 7 \mu M) \) showed similar or slightly better results for their ability to bind the DNA substrate when compared to the wild-type enzyme. In the case of V109G, the \( K_D \) \( (12 \pm 0.7 \mu M) \) was significantly lower than wild-type Scabin indicating a higher affinity for this substrate.

The K130A and K181A variants, as seen in the Scabin-DNA binding model, are predicted to interact with a limited number of base pairs in the DNA substrate. This information along with the kinetic data indicate that they are not key residues in Scabin transferase activity or DNA substrate binding affinity. A likely explanation is that upon substitution of these two residues, one or more of the 18 residues proposed to interact with the DNA substrate binding can maintain the interaction between Scabin and its DNA substrate.
Table 4.3: Scabin variants binding constant to a cyanine-tagged double-stranded DNA.

<table>
<thead>
<tr>
<th>Construct</th>
<th>$K_D$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>L108G</td>
<td>60 ± 1</td>
</tr>
<tr>
<td>V109G</td>
<td>12 ± 0.7</td>
</tr>
<tr>
<td>K130A</td>
<td>75 ± 4</td>
</tr>
<tr>
<td>K181A</td>
<td>31 ± 7</td>
</tr>
</tbody>
</table>
Glycine is a residue that adds flexibility to a protein’s secondary structure and is also known as a “helix breaker”. In terms of L108G and V109G, both residues are located in helix 3 in Scabin; expectedly, upon replacement with Gly, the corresponding variants showed a different kinetic fingerprint. L108G is located one residue closer to the middle of helix 3; the kinetic data showed that L108G is a comparable enzyme to wild-type Scabin in terms of its transferase activity and in DNA substrate binding number. The GH activity of L108G showed a significant reduction in turn-over ability. Based on these data, helix 3 may not play an important part in DNA substrate binding or transferase activity; however, helix 3 may play a role in NAD\(^+\) hydrolysis activity.

In terms of the V109G variant, the thermodynamic data showed a higher affinity for the dsDNA substrate than the wild-type enzyme; however, its GH and transferase activity were lower. This residue is located at the end of helix 3 and adjacent to a loop region containing Asn110 and Gln111. The Gln111 residue is proposed in the Scabin-DNA model to have van der Waals interactions in the major groove of the dsDNA substrate. The Asn110 residue was previously characterized and it was demonstrated that an N110A variant showed impaired DNA binding\(^{59}\). In the Scabin-DNA model, the Asn110 residue interacts with both the dsDNA and the NAD\(^+\) substrates. As seen with the L108G variant, helix 3 does not participate in transferase activity or DNA substrate binding ability. The change from a Val to a Gly in helix 3 adds flexibility at the residue 109 position and may cause Asn110 and Gln111 to shift in their disposition in binding the DNA substrate. In the crystal structure of Scabin, Asn110 faces towards the NAD\(^+\) binding pocket. Based on the kinetic results of the V109G variant, the replacement of Val109 with Gly may induce Asn110 to shift away from the NAD\(^+\) binding pocket (limiting binding to
NAD\(^+\)) and more towards the DNA substrate. A crystal structure of the V109G variant would help determine if this proposed shift occurs upon residue substitution at the Val109 site within the Scabin active-site.

4.6: Exploring the Tyr129 residue interaction with DNA

Another residue in the active site of Scabin proposed to be involved in DNA-binding and transferase activity is Tyr129. In the Scabin-DNA model, Tyr129 contacts both DNA strands by intercalating into a minor groove in dsDNA\(^{58}\). To elucidate the role of Tyr129 in the kinetic behaviour of Scabin, Lyons et al. (2018) substituted the residue with an Ala\(^{59}\). The Y129A variant showed similar GH activity (\(K_M = 86 \pm 11 \, \mu\text{M}\); \(k_{\text{cat}} = 60 \pm 3 \, \text{min}^{-1}\))\(^{59}\) as the wild-type enzyme and had a similar \(K_{0.5} = 365 \pm 28 \, \mu\text{M}\) as the wild-type enzyme with the dG substrate; however, it gave a higher \(k_{\text{cat}} = 254 \pm 14 \, \text{min}^{-1}\) for the deoxyguanosine substrate\(^{59}\). Intriguingly, the Y129A variant showed the greatest impact on DNA substrate binding\(^{59}\). Some weaker binding to DNA occurred when compared with the wild-type enzyme; however, the data could not be fit to a single-site-binding model as the saturation point was not accurately predicted.

To further investigate this interaction, the Tyr129 residue was replaced with a His, Lys, Phe, Glu, and a Thr. The His substitution was designed to introduce a positive charge while maintaining the ring structure/nonpolar character. Lys also added a positive charge while keeping the hydrogen bonding ability. Phe was introduced at residue 129 to maintain the nonpolar aromatic interaction with DNA bases, but to eliminate the hydrogen bonding ability. Glu was introduced to keep hydrogen bonding intact but remove the nonpolar interactions. Lastly, Thr replaced Tyr since the former residue (as seen in the multiple sequence alignment) is located at position 129 in the Pierisin-1 group members.
in the mART toxin family. Since Scabin shares a relatively high sequence identity with Pierisin-1 (40%), this residue may perform the same function as Tyr in Scabin.

The CD spectrum of the Y129T variant showed disruption in global secondary structure compared to the wild-type enzyme (Figure 4.2A). The DNA-binding assay was performed and no binding of this variant to DNA was observed (Figure 4.3A). Therefore, it cannot confidently be stated that this is due to the residue substitution or disruption in the folded integrity of the protein.

Regarding the other Scabin variants, the CD spectra displayed no major differences in their secondary structure when compared to wild-type protein (Figure 4.2B). Each variant was tested for its GH and transferase activity. Collectively, the variants appear to interact more favourably with both the NAD$^+$ and DNA substrates and showed as good or higher overall activity compared to the wild-type enzyme (Table 4.4). In terms of the DNA-binding ability, none of the variants bound the DNA substrate as well as the wild-type enzyme (Figure 4.3B). The Y129H and Y129F variants did show some binding ability; however, their binding to the DNA substrate was clearly compromised. The Y129K variant showed weak binding to dsDNA, whereas Y129E exhibited no detectable binding.

The similarity between the Y129H and Y129F variants lies in their nonpolar interactions whereas Y129K and Y129E variants both maintain hydrogen bonding. Since the Y129H and Y129F variants showed the strongest DNA binding, nonpolar interactions may be more critical for Tyr129 association with dsDNA than hydrogen bonding. Lugo et al. (2018) proposed that Tyr129 makes important contacts with both DNA strands by interacting with 4 DNA bases and with the side-chain of the 7th base of the DNAII strand.
Figure 4.2: Circular dichroism spectra of wild-type Scabin and site-directed Tyr129 variants. (A) wild-type (black) and Y129T (blue); (B) wild-type (black), Y129K (orange), Y129H (purple), Y129E (green), and Y129F (maroon). Proteins were in 20 mM Tris, 50 mM NaF (pH 8.2) at a concentration of 0.2 mg/mL. Each spectrum is the average of nine independent spectra.
Figure 4.3: DNA-binding curves for Scabin wild-type and variants. (A) wild-type (white circles) and partial-binding curve for Y129T (black squares); (B) wild-type (white circles), Y129H (black squares), Y129F (white squares) Y129E (black triangles), and Y129K (black circles). The proteins were titrated into a cuvette containing 5 µM cyanine-3 tagged blunt-ended dsDNA. The change in anisotropy (Δr) was collected for 20 second intervals with the band passes set to 4 nm and the excitation and emission wavelengths set to 550 and 570 nm, respectively.
Table 4.4: The kinetic parameters for the glycohydrolase and transferase activity of Scabin wild-type and variants.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Glycohydrolase</th>
<th>Transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (µM)</td>
<td>$k_{cat}$ (min$^{-1}$)</td>
</tr>
<tr>
<td>WT</td>
<td>68 ± 3</td>
<td>94 ± 2</td>
</tr>
<tr>
<td>Y129A</td>
<td>86 ± 11</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>Y129E</td>
<td>51 ± 9</td>
<td>117 ± 4</td>
</tr>
<tr>
<td>Y129F</td>
<td>66 ± 9</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>Y129H</td>
<td>25 ± 5</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>Y129K</td>
<td>67 ± 6</td>
<td>61 ± 2</td>
</tr>
</tbody>
</table>
Perhaps the Y129H variant was able to interact with the 4 DNA bases; however, there may be a weaker interaction with the 7\textsuperscript{th} base of DNA\textsubscript{II} strand. Lugo \textit{et al.} (2018) suggested that the interaction between Tyr129 and the 7\textsuperscript{th} base may allow a stable complex to form between Scabin and the DNA substrate.

The kinetic data for the Tyr129 variants demonstrates a flaw in the current transferase assay. If the Tyr129 variants have limited binding to the DNA substrate then why do they show higher transferase activity with the dG substrate than the wild-type enzyme? An enzyme assay with dsDNA as the ADP-ribose acceptor substrate is needed for Scabin. The current method for mART enzymes involves using ε-NAD\textsuperscript{+} as the fluorescent substrate; however, DNA interferes with the fluorescence signal (etheno fluorescence in the εNAD\textsuperscript{+} substrate) in this assay by absorptive screening\textsuperscript{67}. An attempted alternative was a high-performance liquid chromatography (HPLC) assay to measure the transfer of ADP-ribose from the NAD\textsuperscript{+} substrate to dsDNA. The rate of nicotinamide production was measured for the determination of the kinetic constants. However, the nicotinamide product peak is produced by both GH and transferase activities of the Scabin enzyme and separating the two reactions has proved to be difficult (data not shown). Further work to optimize the HPLC assay is required where it may be possible to measure the DNA-ADP-ribose product formation during the transferase reaction.

4.7: Conclusions

Site-directed mutagenesis in combination with kinetic analyses provided new insights into the interaction of Scabin with its DNA substrate. Helix 3, as shown with L108G, is not involved in transferase activity or binding to the DNA substrate. Val109 may
participate as a secondary residue in positioning Asn110 and Gln111 in the DNA binding motif of Scabin. Asn110 was previously implicated for its role in interacting with both the NAD$^+$ and dsDNA substrates. Asn110 uses hydrogen-bonds and van der Waals forces to interact with the DNA backbone and mutagenesis to an Ala showed impairment to Scabin’s ability to bind to the dsDNA substrate. Residue replacements of Tyr129 suggest that non-polar interactions may be the most important contributor at this site for binding the DNA substrate. All in all, this information provides greater insight into the understanding of the DNA binding motif within Scabin toxin and the mechanism of ADP-ribose transfer to the DNA substrate.
Chapter 5: Conclusion and Future Directions
5.1: Conclusions

The common scab disease affects a variety of taproot crops resulting in millions of dollars in lost revenue for Canadian farmers. The lesions are produced 6 weeks after tuber initiation. A principal microbe responsible in North America for this disease is *Streptomyces scabies*. Natural products are being used as an alternative strategy in the food industry as they should generally be less toxic and easier to metabolize in humans upon consumption. An alternative approach is targeting the virulence factors of the pathogenic bacteria. Anti-virulence compounds allow the host immune system time to eliminate the offending bacterium leading to less selective pressure and, in turn, less resistance. One such virulence factor is Scabin, a mART toxin that has been shown to bind and label DNA with an ADP-ribose transferred from the NAD\(^+\) substrate.

In this thesis work, growth curves for *S. scabies*, *S. acidiscabies*, *S. turgidiscabies*, and *S. europaeiscabiei* were acquired. Since *Streptomyces* is a filamentous bacterium, a methylene blue assay was adapted to correlate dye uptake by the cells to bacterial growth. The doubling time for each strain was determined to be 6.9 hours (*S. scabies*), 6.6 hours (*S. turgidiscabies*), 5.7 hours (*S. acidiscabies*), and 2.6 hours (*S. europaeiscabiei*). Next, 54 plant tinctures were tested for antimicrobial activity against *S. scabies* with some tinctures inhibiting the growth, some having no effect and some stimulating growth. The best two plant tinctures for growth inhibition, myrrh and garlic, showed complete cessation of growth of *S. scabies* as well as *S. acidiscabies*, *S. turgidiscabies*, and *S. europaeiscabiei*.

The characterization of Scabin residues involved in DNA binding was performed. There were 4 residues (Val109, Lys130, Lys181, and Leu108) that were analyzed for their
glycohydrolase and transferase activity, and DNA binding ability. The K130A, K181A, and L108G variants showed slight but no significant differences in the GH and transferase activity compared to wild-type. V109G showed the largest impairment in its $k_{\text{cat}}$ value for the deoxyguanosine substrate and this variant also demonstrated the highest affinity for the dsDNA substrate.

One of the most important residues in the binding of Scabin to DNA is Tyr129.$^{58}$ This residue was replaced with Glu, Phe, Lys, and His to discern the important chemical features at the residue 129 site pertaining to binding the DNA substrate. No residue substitution for Tyr129 was able to fully maintain DNA-binding activity as seen for the wild-type enzyme. The two variants that demonstrated the highest binding affinity, Y129H and Y129F, revealed that nonpolar interactions might be necessary for DNA substrate association.

5.2: Future Directions

5.2.1: Characterizing myrrh and garlic growth inhibition

To further characterize the plant tinctures, myrrh and garlic, minimum inhibitory concentrations (MICs) and minimum bacteriostatic concentrations (MBCs) should be determined. In short, MIC is the lowest concentration where there is no visible growth of a bacterium. MBC is the lowest concentration where, following sub culturing, no growth is observed on an antibiotic-free medium.

With the determination of the minimal amount of tincture needed to inhibit and/or kill the bacteria, the question remains, what mode of action do the tinctures have? A method to characterize this is microscopy. The use of both scanning electron microscopy
(SEM) and transmission electron microscopy (TEM) would provide insights into the components of the *Streptomyces* cells that are being affected by each tincture. SEM reveals the surface structure of the filamentous organism whereas TEM shows the inside of the cells. In the literature, garlic extract has been shown by TEM to degrade cell wall and cytoplasm components of *Trichophyton rubrum*. With information gathered from these methods, a better understanding on how these tinctures work may be forthcoming.

5.2.2: Identifying the active compounds of the plant tinctures

With the identification of two plant tinctures that inhibit the growth of *S. scabies*, the active component(s) need to be identified. One method to achieve this is combining solid-phase extraction (SPE) methods with gas chromatography-mass spectrometry (GC-MS) or HPLC (High-performance liquid chromatography).

SPE is used for extraction, clean-up, fractionation, and pre-concentration of samples from biological, clinical, food, environmental, and beverage industries. The procedure involves binding target analytes from a solution on a solid phase, generally a sorbent cartridge. Once unwanted components are removed, the preferred analytes are eluted using organic solvents.

The target analytes can then be processed by GC-MS. The analytes are volatized and then separated by a stationary phase. Once eluted the analytes are ionized and further separated and detected by their mass-to-charge ratios. The identified compounds can then be tested against *S. scabies*, to see if they are antimicrobials. Some compounds may work in synergy and therefore combinations of compounds may need to be tested.
5.2.3: Assay to characterize the transferase activity of Scabin using dsDNA

Currently, the method to analyze Scabin transferase activity employs deoxyguanosine as a model substrate in a fluorescence-based assay. An alternative assay using a high-performance liquid chromatography (HPLC) was attempted; however, separating the nicotinamide product of GH activity from transferase activity proved to be difficult. Other methods that can be used are mass spectrometry and a gel-shift assay. Regarding mass spectrometry, the amount of ADP-ribosylated dsDNA can be directly quantified and related to the reaction rate.

In a gel-shift assay, or electrophoretic mobility shift assay (EMSA), the ability of a protein to bind to DNA is observed when the DNA band on an agarose gel is shifted upwards. This shift correlates to the protein-DNA complex migrating slower than free linear DNA fragments. The interaction between the DNA substrate and protein can then be correlated to binding affinity.
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