Haverford College

Annual Progress Report: 2010 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

The Haverford College received $30,855 in formula funds for the grant award period January 1, 2011 through December 31, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Studies on the Bacterial RNA Polymerase Inhibitor Ripostatin A: Efforts to Synthesize an Orally Bioactive Antibiotic for Treatment of Tuberculosis - The purpose of the work described in this project is to prepare and to study molecules that could lead to new drug treatments for tuberculosis (TB), particularly in cases of rifamycin-resistant strains. Compounds such as myxopyronin A and ripostatin A have been isolated, and recent research shows that these molecules inhibit bacterial growth through a novel binding action with the bacterial RNA polymerase enzyme. Thus, they serve as excellent lead candidates for new drug discovery in the treatment of TB, and the goal of this work is to synthesize ripostatin A, and its close relative, ripostatin B. Another goal is to prepare synthetic analogs of the ripostatins in order to more fully understand their structure activity relationship with bacterial RNA polymerase, and improve their pharmacokinetic profile, potency and bioavailability.

Anticipated Duration of Project

1/1/2011 - 12/31/2014

Project Overview

The broad research objective of this work is to discover new knowledge that will lead to the development of novel drug therapies to treat tuberculosis (TB), a bacterial infection that affects millions of people each year and kills approximately 2 million people each year. At present there are FDA approved antibiotics available to treat tuberculosis, but health care professionals and the scientific community report that there is an increase in multidrug-resistant (MDR) strains of Mycobacterium tuberculosis, the causative agent of TB, and traditional antibiotic regimens are no longer successful in combating the disease. Consequently, there is concern that a global public health crisis could be imminent.

The antibiotics currently in use, particularly the rifamycin antibiotic and its semi-synthetic analogs, work by binding to the bacterial RNA polymerase (RNAP) near its active site, and when

bound, these drugs prevent transcription of bacterial DNA to RNA. However, recent literature has shown that there are new natural products, in particular ripostatin A, which specifically binds to the Mycobacterium tuberculosis RNAP at a different site than the rifamycin antibiotics, and there does not appear to be cross-resistance between the two classes of compounds. Ripostatin A then has the potential to serve as a new drug or drug lead to treat rifamycin resistant bacteria.

The specific aims of the project are to first synthesize pieces of ripostatin A, and then assemble those pieces to make the actual drug candidate in the lab. A second aim is to work with the individual pieces to introduce modifications in order to improve the pharmacokinetic profile of the molecule, and also to study potency and the structural requirements for binding to the bacterial RNAP. The project will involve synthetic organic reactions and techniques to produce the individual fragments, and to assemble the ripostatin A molecule as a whole. Modifications include introducing polar groups to improve aqueous solubility, studying alkene substitution patterns, and examining the stereochemical requirements of the asymmetric centers. It is envisioned that the results will lead to useful information regarding structural and functional group criteria for the development of drugs employed to combat MDR strains of tuberculosis.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

There are several benefits to this research project in addition to the goal stated previously. The experiments in this research project will be conducted by the principal investigator and by undergraduate students at Haverford College. These investigations will provide an excellent education for the students. From their experiences in the laboratory, they will be trained to think as independent scientists, to work on research problems that contribute new knowledge, and to use their creativity, intellect and skills to solve relevant scientific questions. At least half of the students in the Blase laboratory pursue Ph.D. degrees at large graduate research universities, and these students therefore become the future scientists in both industrial and academic communities. The students also will present their work at department seminars and professional meetings, and the presentations will provide opportunities for students to interact with their peers in the discipline and with experts in the larger scientific community. Another benefit is that this project will allow the principal investigator to establish collaborations with colleague(s) who can test the biological activity of the molecules synthesized, and to conduct studies with the synthetic molecules and their designated biological target. Successful outcomes could lead to future publications and continued collaboration, and also could be the starting point for larger grant

support and funding. Outcomes of the work also will be reported by the principal investigator at professional meetings and invited talks.

Summary of Research Completed

Molecular modeling studies on the switch region of the RNA polymerase enzyme revealed that the natural product, ripostatin A, in Figure 1, binds in an enzyme pocket that actually is a three sub-pocket domain consisting of a left-side hydrophobic subpocket, a cleft-region subpocket in the center and a hydrogen-bonding subpocket on the right side, as shown in Figure 2. Protein ligand binding revealed that key interactions with the amino acids leucine in the hydrophobic pocket, serine and lysine in the cleft-region pocket, and lysine and tryptophan in the hydrogen-bonding pocket were important for binding and activity. However our modeling studies also revealed that the central 14-membered macrolactone did not anchor into the binding pocket tightly, thus productive protein-ligand contact was not maximized. The large central ring was too big and too 'floppy' to reside tightly in the cleft-region subpocket. Also, the two-carbon chain containing the carboxylic acid in ripostatin A did not extend into the hydrogen bonding region. Consequently, work began to synthesize compounds that contained smaller, highly conjugated rings containing a carbonyl moiety along with functionalized side arms capable of hydrophobic and hydrogen bonding interactions.

Synthetic studies preparing analogs of ripostatin A that contained a smaller central ring were attempted. The six-membered pyrone ring was chosen as the central cyclic unit because it is the backbone cyclic system in the myxopyronin and corallopyronin antibiotics, depicted in Figure 1. The first analog, 4, contains the *E*-alkenyl side arm of the ripostatin A molecule, a central smaller, conjugated pyrone ring and a longer side arm carboxylic acid chain, illustrated in Figure 3. Molecular modeling studies revealed that a key interaction in the binding pocket involves a hydrogen bond interaction with both lysine and tryptophan residues, and it was envisioned that the carboxylic acid could provide both hydrogen bond donor and acceptor capabilities to these amino acid residues.

The synthesis of analog 4 began with the commercially available 4-bromopropanol. Protection with *t*-butyldimethylsilyl chloride led to the isolation of the protected alcohol. Finkelstein reaction of (3-bromopropoxy)*tert*-butyldimethylsilyl ether with sodium iodide in dry acetone led to *tert*-butyl(3-iodopropoxy)dimethylsilane, 7, in high yield, shown in Scheme 1. Commercially available dehydroacetic acid was then treated with slightly more than three molar equivalents of lithium di*iso*propylamide (LDA) to yield the trianion, 9, and careful addition of one equivalent of *tert*-butyl(3-iodopropoxy)dimethylsilane, 7, led to the isolation of the mono-alkylated product 10. The structure of the alkylated product was confirmed by ^{1}H and ^{13}C NMR spectroscopy. Deprotonation of pyrone 10, this time with approximately two molar equivalents of lithium di*iso*propylamide (LDA), led to the formation of the dianion, and subsequent alkylation with *(E)*-(4-bromo-3-methylbut-2-en-1-yl)benzene, prepared by a two step reaction sequence, proceeded in good yield to produce the dialkylated pyrone, 13. Deprotection of the silyl protecting group followed by oxidation of the primary alcohol to the carboxylic acid will produce the ripostatin A analog containing the smaller, more rigid central cyclic core and the longer carboxylic acid side arm.

A second analog, 5, as shown in Figure 3, contains a *para*-methoxyphenyl side arm. The *para*-methoxy benzene ring could provide a hydrophobic interaction with the tryptophan residue, while the oxygen atom in the *para*-methoxy substituent could provide a hydrogen bond acceptor. The synthesis of this analog proceeded smoothly in a similar manner as described above. First, the commercially available 3-(4-methoxyphenyl)propan-1-ol was converted to its corresponding bromide by reaction with phosphorous tribromide (PBr_3) in anhydrous diethyl ether. Finkelstein reaction with sodium iodide in acetone led to the isolation of 1-(3-iodopropyl)-4-methoxybenzene, 16, in high yield, depicted in Scheme 2. Commercially available dehydroacetic acid was then treated with slightly more than three molar equivalents of lithium di*iso*propylamide (LDA) to yield the trianion, and careful addition of one equivalent of 1-(3-iodopropyl)-4-methoxybenzene led to the isolation of the mono-alkylated product 18. The structure of the alkylated product again was confirmed by ^{1}H and ^{13}C NMR spectroscopy. Deprotonation of pyrone 18 with two molar equivalents of lithium di*iso*propylamide (LDA) led to the formation of the dianion, 19, and alkylation with the previously prepared *(E)*-(4-bromo-3-methylbut-2-en-1-yl)benzene proceeded to yield the dialkylated pyrone 5. These two analogs could be tested for antibiotic activity against bacterial strains, particularly, multi-drug resistant *mycobacterium* tuberculosis.

Figure 1: RNA Polymerase (RNAP) Inhibitors

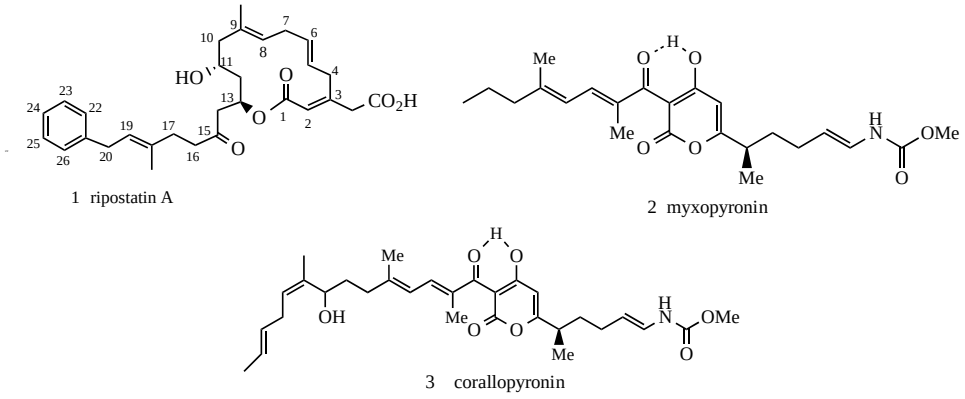


Figure 2: Molecular Modeling Studies of Ligand-Protein Binding in the RNAP Switch Pocket

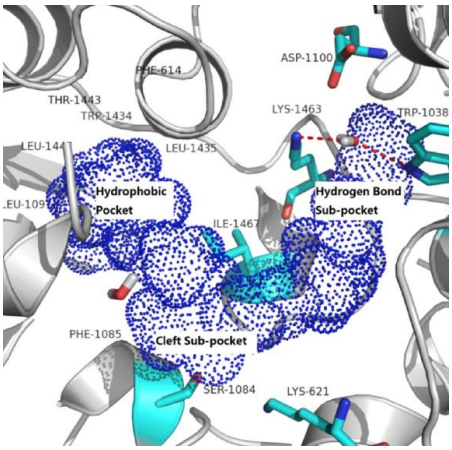
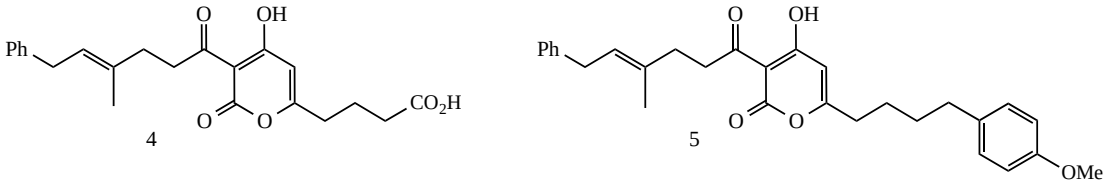
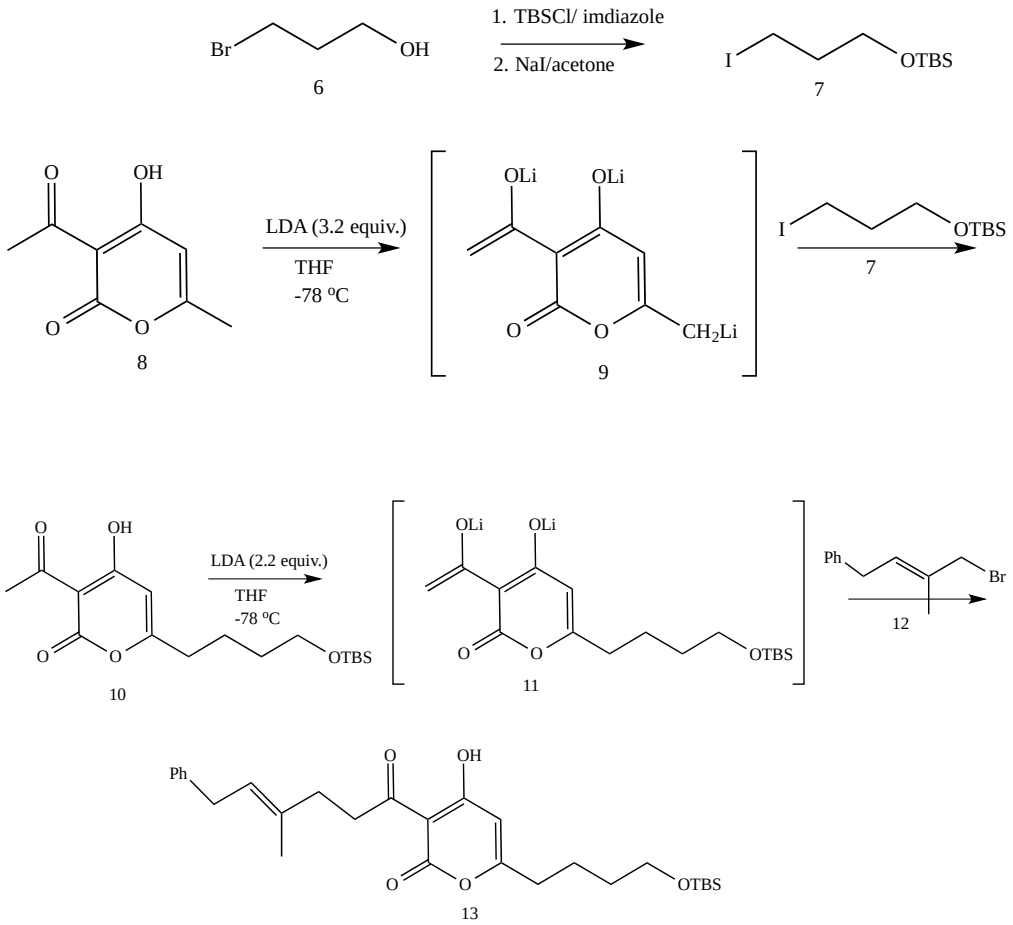


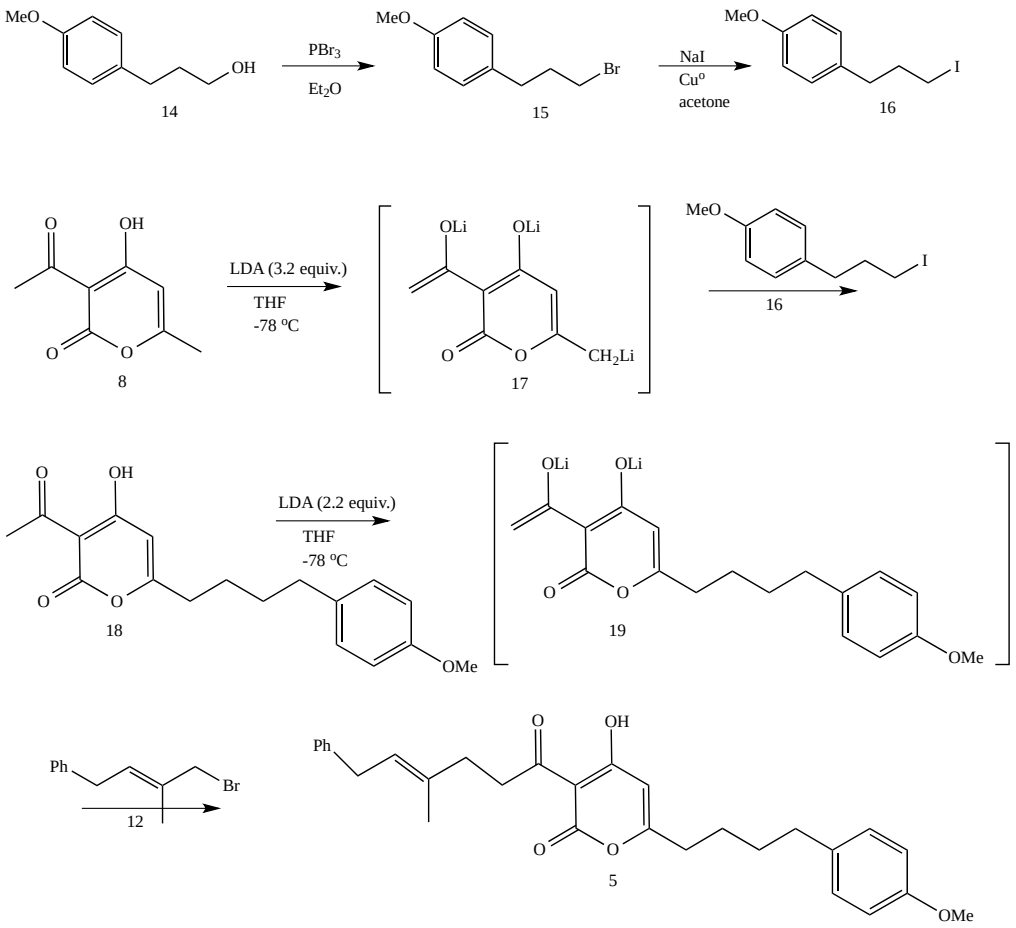
Figure 3: Two Analogs for RNAP Inhibitors



Scheme 1: Synthetic Studies to Prepare RNAP Analog Inhibitor 4



Scheme 2: Synthetic Studies to Prepare RNAP Analog 5



Research Project 2: Project Title and Purpose

A Network of Chromosomal Proteins in the Germline of C. elegans - The purpose of this research is to understand the logic by which genes interact in complicated biological processes. Most of the life-threatening diseases in American adults are biologically complex, with both genetic and environmental risk factors. The interactions of these risk factors that result in disease are hard to predict. From the analysis of the interactions of a relatively small group of genes in *C. elegans*, a well-studied model organism, in a controlled laboratory environment, I will develop predictions about which genes are the most likely to have functional interactions and which are not. These predictions can be readily tested and refined to give us better insights into the logical network by which gene interactions produce complex phenotypes.

Anticipated Duration of Project

1/1/2011 - 12/31/2014

Project Overview

In previous work, my students and I identified and characterized him-5, a key gene needed for normal meiosis in the germline in C. elegans. We also found 12 other genes and their protein products that are capable of physical interactions with the HIM-5 protein, and have tested each protein for its physical interactions with all of the other twelve proteins. Using approaches based on graph theory, my students and I predicted which of these proteins will have functional interactions as well as physical interactions. All possible functional interactions were tested among this network, and our predictions of which genes will interact were largely verified. In the current research, I will expand this interaction network by using another key gene in meiosis as the starting point, the gene xnd-1. XND-1 does not interact directly with HIM-5 but does interact with some of the other proteins in the network. My first specific aim is to use XND-1 as the bait protein in a yeast two-hybrid screen, first against all of the proteins that physically interact with HIM-5 and then against a library that includes most of the genome of C. elegans. The second specific aim will determine all of the physical interactions among this new and larger set of proteins. The third specific aim will predict and test which of the newly identified physical interactions will have genetic interactions with xnd-1 and him-5 using known mutations of these genes as well as RNAi. Two molecular steps of meiosis have recently been shown to be altered in xnd-1 and him-5 mutants; these are the ability of SPO-11 to make the double-stranded DNA breaks that initiate recombination, and a particular post-translational modification of the H2A histone protein. These two molecular processes provide us with additional assays to examine the effects of him-5, xnd-1, and the network of their interactions. Thus, the fourth specific aim will be to examine both of these molecular processes in our interacting genes.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Two outcomes and health benefits could arise from this research project. The first expected outcome and potential health benefit comes from meiosis itself, the biological process that we are studying. Meiosis, the process affected by *him-5* and *xnd-1*, is a defining characteristic of eukaryotic organisms. Meiotic errors are the origin of trisomies and other chromosome non-disjunction events, which are the most common cause of first trimester miscarriages in humans. The most familiar trisomy that survives in humans is Trisomy 21 or Down Syndrome. While neither *him-5* nor *xnd-1* has an exactly homologous gene in humans, the human genome has other genes that play functionally equivalent roles. Thus, this work will provide insights into the

molecular processes by which normal meiosis occurs, which can help to explain how trisomies and other meiotic errors occur.

The second expected outcome and potential health benefit arises from the experimental approach that I am pursing. While much of molecular biology and modern medicine has focused on the actions of individual molecules, all biological molecules function as parts in complicated networks of interactions. The extended network of interactions can potentially be used to predict new approaches to therapies, to anticipate and avoid side effects, and to identify other molecules that are part of the same process. However, our understanding of the logic of these interaction networks is rudimentary at best, so most of these benefits are not yet realized. Thus, by taking a network approach to the action of these genes in a relatively simple model organism, I hope to refine our ability to analyze and understand the logic of biological networks in higher organisms.

Summary of Research Completed

In addition to our previous work with a network of 14 genes that affect the germline in *C. elegans,* we are analyzing another network of approximately twenty genes that affect cuticle formation. Cuticle formation involves proteins with well-studied biochemical properties, such as collagens, which has provided a better background for understanding the interactions in detail; in addition, while germline functions occur more or less continuously, cuticle formation occurs at discrete stages of the life cycle with distinct steps. The core set of six genes (referred to as the bli-1 through bli-6 genes) used to construct the network of interactions result in fluid-filled blisters on the adult cuticle when mutated. Notably, while mutations in any of these genes often result in the formation of blisters on some worms, genetically identical siblings may not have blisters; this phenomenon, known as reduced penetrance, is well-known in genetics but little understood. It is thought to arise, in part, from small fluctuations in gene activity and interactions with other genes within the network. This reduced penetrance is seen even when the activity of a gene is completely knocked out by mutation, so it cannot be readily explained from partial loss of function mutations; it likely arises from interactions with other genes and their products.

Three of these six core genes (bli-1, bli-2, and bli-6) encode collagens, the main structural protein of the cuticle, while the other three genes encode enzymes known or thought to be involved in modifications of collagen proteins. From known and predicted interactions among the genes, three distinct subnetworks of interactions with other collagen proteins and collagen-related proteins have been found. Subnetworks are defined as a set of genes that have many interactions with each other, but few interactions with other genes. (An analogy using social networks is helpful. The subnetwork consisting of our family members are very likely to be "friends" with each other, but unlikely to have many interactions with the subnetwork of our co-workers.) Using a combination of genetic mutations that knock out the function of a gene and RNA interference (RNAi) which reduces the function of a gene, we have been studying the interactions among the genes in these three subnetworks. Our primary experiment is to knock out or knock down two (or more) genes simultaneously and analyze the effect on cuticle formation, both by counting the number of worms with blisters and by observing other phenotypes related to the cuticle. While most of our work has been with interactions that increased the fraction of blisters (known as enhanced penetrance), we also found a broader range of interactions and phenotypes than expected. We have now tested the interactions among dozens of different pairs

of genes, and can draw some tentative conclusions.

In general, genes within the same subnetwork do not enhance one another, whereas genes in different subnetworks are more likely to enhance one another. For example, bli-1 and bli-2 are collagen genes that form part of a subnetwork including several other collagen genes; knocking out two of these genes together results in roughly the same percentage of worms with blisters as knocking out of one of them. I interpret this to mean that once the function of a subnetwork has been compromised, additional knockdowns or knockouts have little effect. Conversely, bli-5 defines a different subnetwork of interactions from either bli-1 or bli-2; knocking out bli-5 and bli-2 together results in a much higher percentage of blisters than either bli-2 or bli-5 alone. I interpret this to mean that the knockouts are affecting related but distinct functions, so that knocking out both functions simultaneously has an increased effect. These effects are seen with genes that encode collagens (including ones that do not affect blister formation) and genes with other functions in the cuticle; the key seems to be the functional subnetwork in which the gene acts. This overall result is consistent with what has been observed in analogous experiments with networks affecting other biological processes in other organisms; enhancement interactions, or increased risk in terms of human diseases, are more commonly observed between genes involved in different functional subnetworks rather than with genes acting within one subnetwork.

However, in the past year we also uncovered exceptions to this general rule, which we are pursuing. For example, some pairs of interactions within a subnetwork greatly reduce the number of worms with blisters (suppression); we find that suppression is characteristic of particular pairs of genes rather than being a general property of the network or the particular mutations we are using, and thus probably reflects some specific interaction between the gene products. These can be interpreted as “anti-risk” factors that reduce the likelihood of developing the mutant phenotype, and are much less common than enhancement interactions. Because the biochemistry of collagen is understood, we have been able to predict, with some accuracy, other genes that might act as suppressors. Other interactions result in completely novel cuticle phenotypes, unlike either of the original genes or any other mutant in the subnetwork; these are quite puzzling. Gene interactions resulting in suppression and novel phenotypes lie outside what network models have been able to predict in any organism. In order to understand all of these interactions in more detail, we are constructing and analyzing reporter gene constructs for several genes; these constructs allow us to see indications of an interaction before the blister phenotype itself occurs or to determine if the gene products are acting in the same tissues at the same time. One of the novel phenotypes, in which the cuticle appears to separate from the body of the worm, seems to arise from genes acting at two different times; other novel phenotypes are under analysis.