Haverford College

Annual Progress Report: 2010 Formula Grant

Reporting Period

January 1, 2010 – June 30, 2011

Formula Grant Overview

The Haverford College received $30,855 in formula funds for the grant award period January 1, 2011 through December 31, 2012. 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 - 12/31/2012

Project Overview

The broad research objective of this work is 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

Working with undergraduate students, my laboratory has made steady progress in the chemical assembly of ripostatins A and B, macrolactones isolated from *Sorangium cellulosum* (Figure 1). Ripostatin A, in particular, has been reported to inhibit bacterial RNA polymerase (RNAP), binding to a site distinctly different from the rifamycin antibiotic, which is currently used as a first line treatment for tuberculosis. Thus ripostatin A, ripostatin B and their synthetic analogs are promising new leads in the development of multi-drug resistant tuberculosis drugs. Scheme 1 shows our retrosynthetic strategy. The initial retrosynthesis (focusing on ripostatin B) dissected the molecule into 3 major fragments: compounds 4, 5, and 6. Further analysis revealed that fragment 4 could be derived in enantiomerically pure form from the ring opening of chiral epoxide 8 with the (*E*)-tri-substituted allylic Grignard reagent 7.

As shown in Scheme 2, it was possible to convert bromide 9 to its Grignard reagent, 7, using Rieke magnesium or even elemental magnesium turnings, but all attempts to open the oxirane in high efficiency failed. Even attempts with (*E*)-4-phenyl-1-bromo-2-butene, 11, a simple alkenyl analog, also shown in Scheme 2, did not produce reasonable yields of the compound 12. Despite many varied reaction conditions, and numerous attempts, the copper-catalyzed Grignard ring opening reaction was not successful in producing acceptable quantities of desired products. However, we were able to carry out these synthetic transformations with the 4-phenylbutyl analog, as illustrated in Scheme 3. Reaction of the Grignard reagent derived from 4-phenyl bromobutane with the enantiomerically pure epoxide 8 in the presence of a catalytic amount of copper (I) iodide, CuI, proceeded in quite good yield. We then were able to protect the newly formed hydroxyl group as its *p*-methoxybenzyl (PMB) ether. Deprotection of the TBS ether under mild acidic conditions, followed by a Dess Martin periodinane oxidation reaction led to the desired methyl ketone 16 in good overall yield. This was a phenyl substituted alkyl chain analog for fragment 4. Even though compound 16 could be successfully synthesized via this route, it was decided to employ an alternate approach because reactions with allylic Grignard reagents simply were not viable, and the latter were the more relevant reagents for the synthesis of the natural products.

After careful analysis of fragment 4, it was determined that a new strategy could employ compound 17 as an attractive target, in Scheme 4. Molecule 17 posesses the necessary 1,3-oxygenated functionality (at carbons 13 and 15) in the form of a β-ketoester, and this 1,3-oxygen functionality is a key feature for the ripostatin fragment 4. Compoud 17, in turn, could be derived from bromide 9 (which our lab has made numerous times and in high yield via a 2 step process) and ethyl acetoacetate, 18. The strikingly attractive feature of this alternate strategy is that it would lead to ripostatin A by selective protection of the keto-carbonyl, and would lead to ripostatin B by selective enantioselective reduction of the keto-carbonyl. It also could provide an easy route to analogs because 9, in theory, could be any alkyl or allylic halide. Thus, this was an efficient, flexible, relatively straightforward approach to our synthetic endeavors.

As described in Scheme 5, treatment of freshly distilled, inexpensive and commercially available ethyl acetoacetate, 18, with 2 equivalents of lithium diisopropylamide (LDA) resulted in the corresponding dianion that could be selectively and cleanly mono-alkylated with bromide 9 in 89% yield. No significant side products were observed. For the ripostatin A synthesis, the ketone carbonyl of the β-ketoester was protected as its ketal, 21, and conversion of the ester carbonyl to the Weinreb amide proceeded efficiently. Finally, treatment of this Weinreb amide with the simple Grignard reagent, methylmagnesium bromide, led to the formation of the C12-C20 fragment of ripostatin A, 22, in 5 steps, but only 4 one-pot reactions.

For the synthesis of ripostatin B, it was important to establish the absolute stereochemistry of the secondary alcohol (at C15) through enantioselective reduction of the ketone carbonyl. After searching the chemical research literature, it was decided to first investigate the enantioselective reduction reaction employing the Corey-Bakshi-Shibata (CBS) catalyst, along with different borane reducing agents as shown in Scheme 6. The methyl-CBS catalyst (Me-CBS) and the butyl-CBS catalyst (Bu-CBS) were prepared according to published procedures. For the actual reducing agent, borane –dimethyl sulfide complex and catecholborane were employed. Table 1 shows the results from some of the attempts at the stereoselective reduction reaction. Despite varying amounts of (*R*)-CBS catalyst, different borane reductants, a range of reaction temperatures, different solvents, and various reaction times, this reaction was not successful. In fact, it gave consistently low yields of the desired chiral alcohol, 23. Enantiomeric excesses (*ee* ‘s) of the isolated alcohol were extremely low (6% *ee* was the best result obtained) as determined by chiral high performance liquid chromatography (chiral HPLC). The reaction was clean in that only two compounds were observed by thin layer chromatography (TLC), the starting material and the alcohol product. However, after careful examination of molecular models, it was concluded that the two groups flanking the keto-carbonyl were not sterically different enough to warrant high facial selectivity with this particular chiral catalyst. Thus, it was decided to investigate other means to produce 23.

One promising area was enzyme mediated resolution of secondary alcohols using lipase enzymes, in particular. Consequently, as depicted in Scheme 7, sodium borohydride ($NaBH_4$) reduction of 17 led to the racemic secondary alcohol, 24, in excellent yield. Resolution of 24 with Lipase PS “Amano” IM (generously supplied free of charge by AMANO ENZYME, INC USA) in anhydrous ether led to the isolation of chiral alcohol 23 and acetate 25 in 45% and 46% yield, respectively. The exact numerial values for the *ee*‘s of alcohol 23 and the acetate 25 have yet to be determined, but chiral HPLC on two trials of this reaction showed a preponderance of one enantiomer for 23. This is an extremely promising result, and the exact *ee* value can be calculated after several more trials in order to obtain a statistically valid number. As shown in Scheme 8, it was possible to convert the achiral secondary alcohol 24 to the desired ripostatin B fragment 4 (with the *t*-butyldimethylsilyl, TBS, protecting group) through reactions already described above. If the racemic alcohol can be converted to compound 28, then it is clear that chiral alcohol 23 should be converted smoothly to the desired fragment 4.

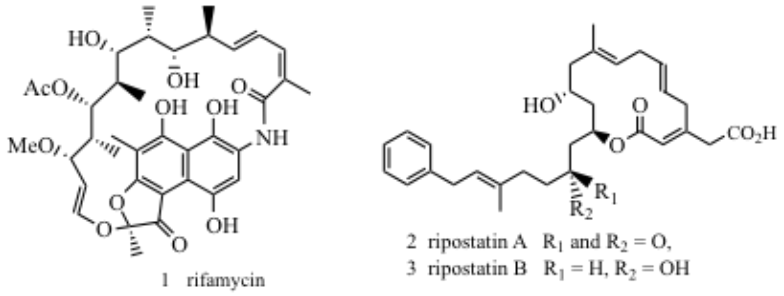
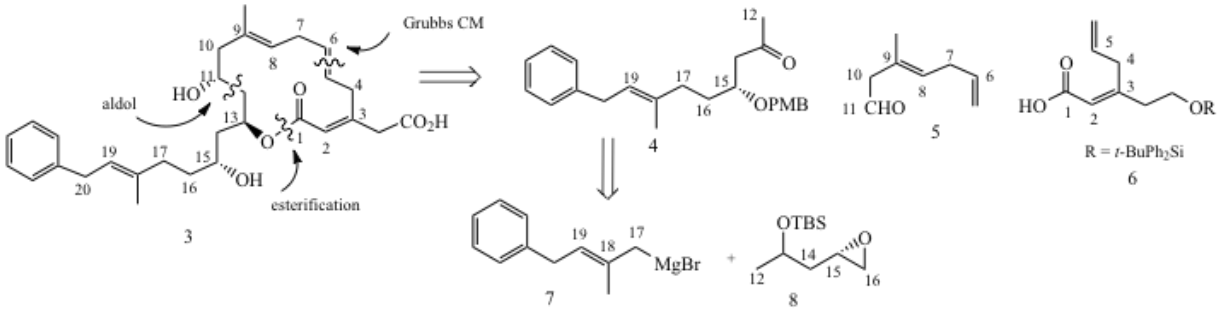
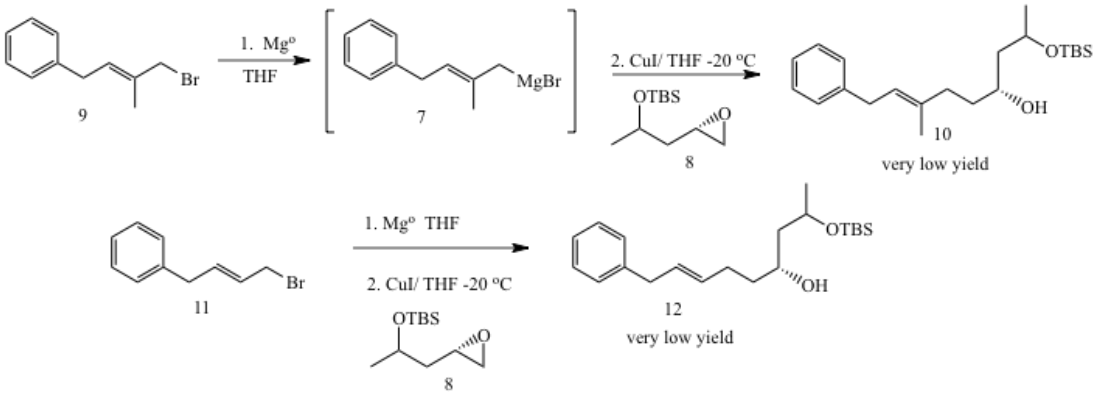


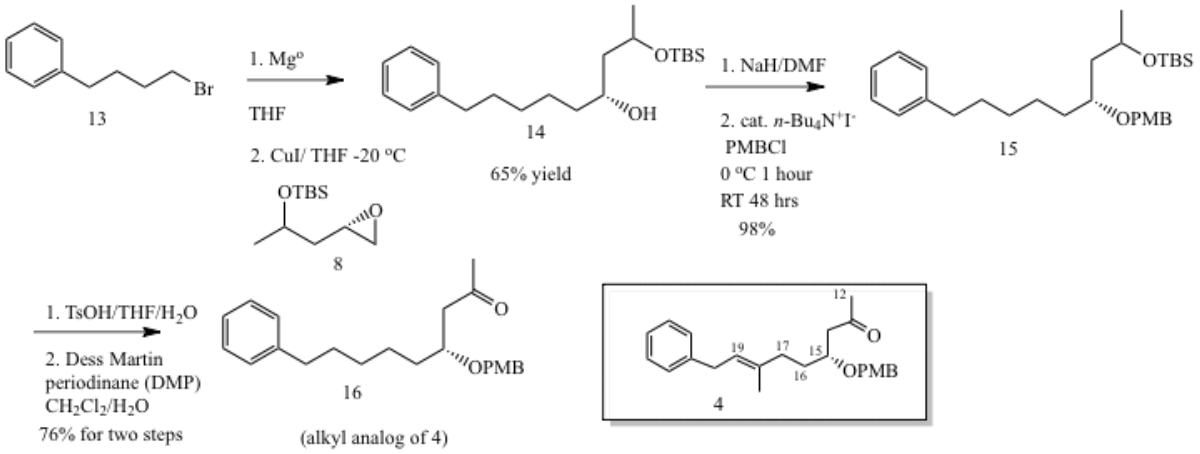
Figure 1: Rifamycin (1), clinically approved to treat tuberculosis and Ripostatins A, (2) and Ripostatin B, (3)



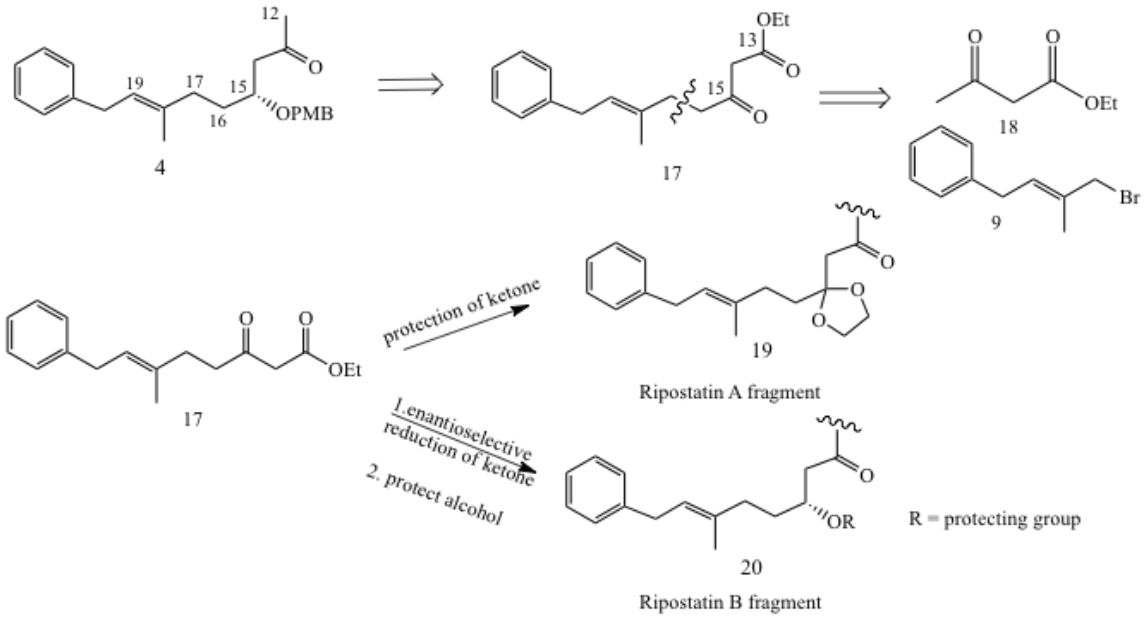
Scheme 1: Retrosynthetic analysis of the ripostatin B antibiotic



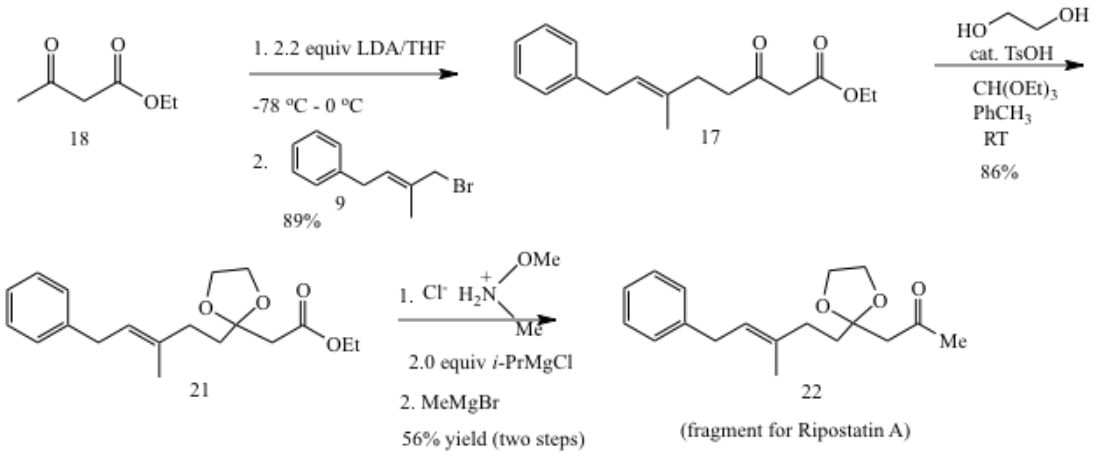
Scheme 2: Attempts at copper-catalyzed ring opening reactions of epoxide 8



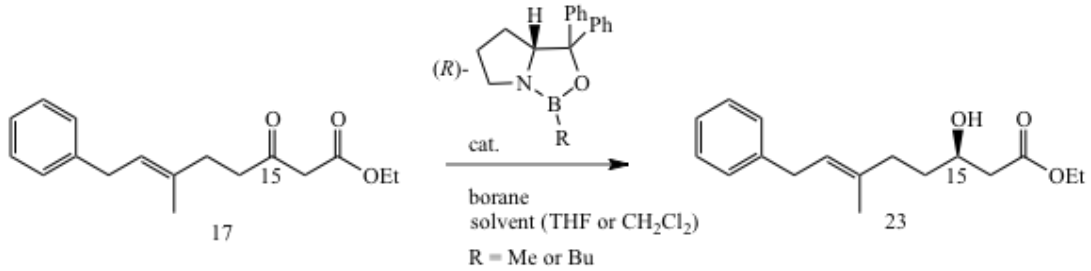
Scheme 3: Successful synthesis of an alkyl analog



Scheme 4: Revised retrosynthetic analysis for fragment 4: pathway to ripostatin A, ripostatin B



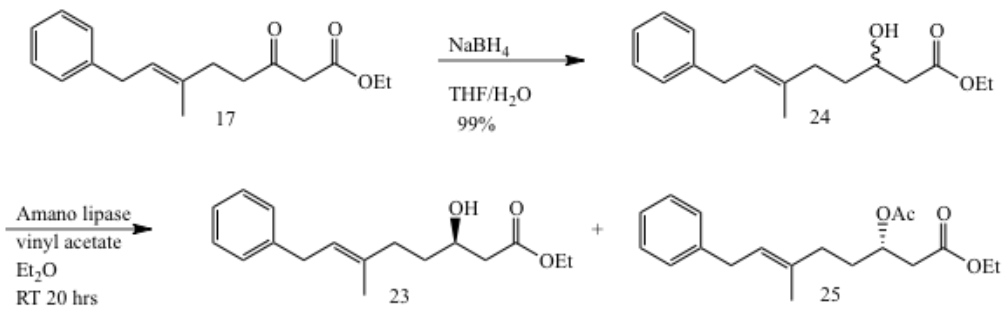
Scheme 5: Successful synthesis of ripostatin A fragment 4



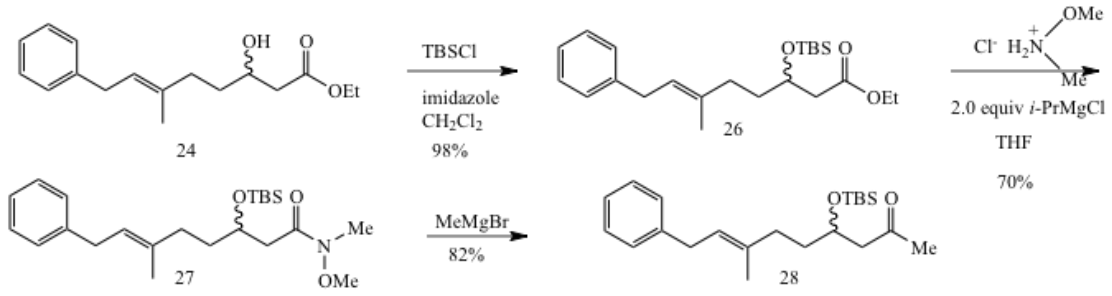
Scheme 6: Attempts at enantioselective ketone reduction with (*R)*-oxazaborolidine catalyst

Catalyst (equiv.)	Reducing Agent (equiv.)	Reaction Temperature	Reaction Time	Yield	*ee*%
Me-CBS (0.22)	$BH_3 SMe_2$ (0.60)	-78 °C - 0 °C to RT	12 hr	53%	0 %
Me-CBS (0.30)	$BH_3 SMe_2$ (1.20)	0 °C to RT	12 hr	25%	0%
Me-CBS (0.50)	$BH_3 SMe_2$ (2.0)	0 °C	8 hr	12%	0%
Me-CBS (0.25)	$BH_3 SMe_2$ (1.0)	-78 °C - 0 °C to RT	24 hr	36%	0%
Bu-CBS (0.10)	Catecholborane (1.0)	-78 °C - -45 °C	24 hr	38%	<10%

Table 1: Results for (*R*)-CBS oxazaborolidine-catalyzed reduction reactions



Scheme 7: Enzyme mediated resolution of racemic secondary alcohol with lipase



Scheme 8: Completion of the synthesis of achiral Fragment 4 for ripostatin B

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 - 12/31/2012

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.

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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

My undergraduate students and I are analyzing the interactions among proteins that govern meiosis and germline functions in the model organism C. elegans. When this project began, we had a network of twelve proteins that physically interact with the product of the him-5 gene; except for him-5, none of the other genes has a mutant phenotype on its own, but several of them show mutant phenotypes and genetic interactions in combination with him-5 mutants or with each other. During January – June, 2011, we expanded this network to test the physical interactions among these proteins and the products of xnd-1, him-17, and spo-11, three other genes that affect meiosis in C. elegans. These four genes, him-5, xnd-1, him-17, and spo-11, encode the core of our meiotic proteins; none of their gene products physically interact with each other.

Our principal method to test physical interactions among these genes is to use the yeast two-hybrid system (Y2H), a well-established and versatile assay that is easy for my students to learn and use. As proposed in my first aim originally, we determined all of the physical interactions among XND-1 and the 12 proteins that interact with HIM-5; three of the proteins from the HIM-5 network also interact with XND-1, forming a physical link between these two genes whose functions are distinct but related. In order to test the interactions of these proteins with HIM-17 and SPO-11, all of the original genes in the HIM-5 network had to be cloned into different vectors. The Y2H method relies on the interactions of the so-called “bait” and “prey” proteins to re-constitute the GAL4 transcription factor in yeast. Thus, one of the controls for Y2H assays is to ensure that the bait protein, in this case, HIM-5, XND-1, HIM-17, or SPO-11, requires an interaction with another protein to activate transcription of the target gene; in the jargon of Y2H, this is testing that the bait protein does not auto-activate. Both HIM-17 and SPO-11 do auto-activate as bait proteins, which meant that we needed to re-clone all of the genes in the HIM-5 network as baits so that they could be tested with HIM-17 and SPO-11 prey. This time-consuming step has now been done, so all of the genes have been cloned as both a bait and a prey for subsequent experiments. We have found that two proteins from the HIM-5 network also have interactions with XND-1, SPO-11, and HIM-17, as well as with each other. These two proteins link the four genes of the meiotic core. Four more proteins in the HIM-5 network have interactions with at least two of the other proteins in the meiotic core, as well as with HIM-5. The interactions with SPO-11 are particularly important since its function in meiosis is well-known; it produces the double-stranded DNA break that initiates recombination. Genetic evidence has indicated that the role of xnd-1, him-5, and him-17 is to recruit SPO-11 to sites on the chromosomes or to allow SPO-11 to make the double-stranded breaks. These gene products do not directly contact SPO-11 themselves, but based on the evidence from Y2H, we suggest that the HIM-5 network includes some proteins that serve as intermediaries between these genes and

SPO-11. In support of this hypothesis, the network of interactions is highly connected, such that a protein that interacts with one of a connected group also interacts with several other members of the group.

None of the genes or the gene products that interact with HIM-5, HIM-17, XND-1, or SPO-11 has been studied in any detail by us or by others, and mutant alleles for these other genes are not yet available. From data compiled as part of the C. elegans genome and modENCODE projects, the two genes that interact with all four of the core meiotic genes are predicted to be other DNA binding proteins (as are HIM-5, HIM-17, and XND-1). Microarray expression analysis done by others indicates that transcription of these genes begins prior to meiosis. Thus, these two genes are candidates for playing a role in the structure of meiotic chromosomes that allows SPO-11 to act. The four genes that encode proteins that interact with SPO-11, HIM-5, and one other member of the core network are more variable in structure and expression. Two of these four have extensive interactions with other proteins among the network and have genetic interactions with him-5 and other genes in the network. The other two have few physical interactions with other proteins, but one of them has exhibited genetic interactions with other genes in the network. The relationship, if any, between physical and genetic interactions in a network is one of the major unresolved questions and a key goal.

We have also used XND-1 as a bait protein to identify additional proteins that might be part of this interaction network. This screen yielded a very large number of candidate genes, which we are currently confirming and characterizing. This is a slow process. Thus, most of my first specific aim has been successfully accomplished, and several promising candidate genes for further investigation have been identified. In addition to my own work on the project, these experiments have been done by four undergraduate students at Haverford College, two of them women and one of those a member of an under-represented group.