

**I. Synthesizing and Identifying Small Molecule Probes for Targeting
Transcriptional Co-factors**
**II. Design and Implementation of a Peer-Led Practical Research Ethics Module for
Teaching Graduate Research Ethics**

by

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Table of Contents

Acknowledgements	ii
List of Figures.....	ix
List of Tables.....	xi
List of Boxes.....	xii
List of Abbreviations	xiii

Chapter I

Introduction

A. Project Overview.....	1
B. Introduction to Transcription.....	2
C. Activator Proteins.....	4
C.1. DNA Binding Domains (DBDs).....	6
C.2. Transcriptional Activation Domains (TADs).....	7
C.3. Mis-regulation of Transcription in Diseases.....	9
D. Roles of CBP/p300 in Regulating Transcription	9
D.1. CBP/p300 in Cancer Initiating Cells (CICs).....	12
E. Small Molecules as Tools for Probing Transcription.....	14
E.1. Traditional Challenges in Designing Small Molecules Probes for Activator-Co-activator Interactions.....	14
E.1.A. Novel Methodology for the Synthesis of Allenamides as a Building Block Scaffold.....	16
E.2. The Use of Small Molecules as Probes for Gaining Insight into Transcription.....	17
E.2.A. Small Molecules that Bind CBP/p300.....	18
E.2.B. Small Molecules that Interact with Med23.....	21
E.2.C. Implications of Using Small Molecules as Tools for Probing the Roles of Proteins Involved in Transcription.....	21
F. Thesis Summary	22
G. References.....	25

Chapter II

A Palladium-Catalyzed [3,3]-Rearrangement for the Facile Synthesis of

Allenamides

A. Abstract.....	32
B. Introduction.....	33
B.1. Synthesis of Building Block Molecules that can be used in the Formation of Synthetic Probes of Transcriptional Processes.....	33
B.2. Allenamides as Substrates for the Formation of Cyclic Small Molecules.....	34
B.3. Established Routes for Accessing Allenamides.....	35
B.4. A Novel Methodology for the Formation of Allenamides.....	38
C. Optimization Studies.....	39
C.1. Optimization of the Protecting Group.....	39
C.2. Optimizing the Phosphorous Substituents.....	40
C.3. Optimizing Temperature and Solvent Conditions.....	40
C.4. Optimizing for Steric Considerations.....	41
D. Discovery of a Competing Reaction Pathway.....	42
E. Optimization for Application to Internal Alkynes.....	44
F. Defining the Scope of the Rearrangement.....	45
G. Defining the Stereoselectivity of the Rearrangement.....	48
H. Utilizing Allenamides as Substrates in Further Reactions.....	50
I. Experimental Methods and Data.....	52
J. References.....	65

Chapter III

Utilizing p300/CBP Ligands to Disrupt Transcription of Genes Governing Pluripotency and Self-Renewal

A. Abstract.....	69
B. Introduction.....	70
B.1. Cancer Initiating Cell (CIC) Model.....	70
B.1.A. Origins of the Model.....	70
B.1.B Therapeutic Implications of the CIC Model.....	71
B.2 Transcription in CICs.....	73
B.2.A. The Roles of Nanog, Sox2 and Oct4 in ESCs.....	74
B.3. The Role of CBP/p300 in ESCs.....	75
B.4. Small Molecules that Target CBP/p300.....	77
B.5. Scope of This Study.....	80
C. RNAi Studies of Dependence of CBP and p300 on Nanog-Driven Gene Expression.....	81
D. Effects of Synthetic Ligands of CBP/p300 on Nanog-Driven Transcription.....	85
D.1. Synthesis of Amphipathic Isoxazolidine 444.....	86
D.2. Results of Synthetic Probes on Nanog-Driven Luciferase Expression.....	87
E. Effects of Synthetic Ligands of p300 on Cellular Viability in a CIC Model System.....	89

E.1. Initial Studies of Effectiveness of 444 in Altering Cellular Viability in a CIC Model System.....	89
E.2. Effects of 444 in a Heterogeneous Tumor System.....	93
F. Conclusions and Future Directions	95
G. Experimental.....	100
H. References.....	105

Chapter IV

Design and Implementation of a Peer-Led Module for Teaching Practical Research Ethics to Graduate Students

A. Abstract.....	110
B. Introduction.....	111
B.1. Examples of Scientific Misconduct.....	111
B.2. Defining Scientific Misconduct	112
B.3. Addressing Issues of Scientific Misconduct and Research Ethics.....	113
C. Rationale for Utilizing Peer-Leaders.....	115
D. Module Goals and Design.....	116
E. Module Layout and Content	119
F. Student Discussions of Case Studies.....	122
G. Student Responses to the Data Interpretation Exercise.....	128
H. Using Student Feedback to Improve the Curriculum.....	130
I. Response from the Chemical Community.....	131
J. Conclusions and Future Directions.....	132
K. Course Material.....	135
K.1. Case Studies.....	135
K.2. Data Interpretation Exercise.....	139
L. References.....	141

Chapter V

Conclusions and Future Directions

A. Conclusions	143
A.1. Synthesizing and Identifying Small Molecule Probes for Targeting Transcriptional Co-factors	143
A.2. Design and Implementation of a Peer-Led Practical Research Ethics Module for Teaching Graduate Research Ethics.....	145
B. Future Directions.....	146
B.1. Determining Domains of p300 Responsible for Nanog-Driven Gene Expression	146
B.2. Determining the Effects of Inhibiting p300 Functionality in Maintaining Pluripotency and Self-Renewal.....	147
B.3. Determining the <i>In Vivo</i> Functionality of p300 Ligands.....	148
B.4. Synergistic Approach for Targeting CICs.....	149

B.5. Strategies for Developing Small Molecule Ligands that Target Transcription Co-factors	150
B.5.A Synthesizing Small Molecules from the Allenamide Scaffold	151
B.5.B. High Throughput Screens for Identifying Natural Product Ligands	152
B.6. Improvements to Current Methodologies for Teaching Research Ethics to Graduate Students	153
C. References	154

Appendix

Design and Synthesis of a Focused Library of Spirooxindoles to Modulate the Function of Transcription Co-factors

A. Abstract	156
B. Introduction	157
B.1. The role of CBP/p300 in Activated Transcription	157
B.2. Small Molecule Ligands of the CH1 Domain of p300/CBP	157
B.3. Designing Small Molecule Ligands of the CH1 Domain of p300/CBP	158
C. Synthesis of a Focused Library of Spirooxindoles	159
D. Determining the Ability of Spirooxindoles to Inhibit Activator-Co-activator Interactions	162
E. Conclusions	164
F. Experimental	165
G. References	176

List of Figures

Figure I-1	Schematic of activated transcription.....	3
Figure I-2	DBD·DNA interactions.....	7
Figure I-3	TAD-target interactions.....	8
Figure I-4	Schematic of CBP/p300.....	11
Figure I-5	Schematic of inhibition of a TAD·co-activator interaction	14
Figure I-6	p53 binding with two distinct proteins	15
Figure I-7	Amphipathic heterocycles that can be synthesized from an allenamide precursor.	17
Figure I-8	Small probes for studying transcription.....	31
Figure II-1	Small molecule building blocks for the formation of amphipathic heterocycles.....	34
Figure II-2	Nitrogen substituted allenes	35
Figure II-3	Base-catalyzed isomerization of a propargylic amide to form an allenamide	36
Figure II-4	Example of copper-catalyzed coupling of an allenyl halide with an amide to form an allenamide.. ..	37
Figure II-5	Examples of [2,3]-sigmatropic rearrangements of propargylic sulfides to form allenamides.....	37
Figure II-6	Rearrangement of tricholoacetimidates to form allenamides.....	38
Figure II-7	Novel methodology for the formation of allenamides	39
Figure II-8	Desired versus undesired reaction pathway	43
Figure II-9	Applications of fully-protected allenamides	52
Figure III-1	Cancer initiating cell model	71
Figure III-2	Therapeutic paradigm in light of the CIC theory	73
Figure III-3	Schematic of the role of CBP and/or p300 in maintaining pluripotency and self-renewal.....	77
Figure III-4	Structure of amphipathic isoxazolidine (444).	79
Figure III-5	Structure of a hydrogen bond surrogate (HBS).	79
Figure III-6	Schematic of Nanog.	82
Figure III-7	Nanog CD2-driven gene expression is dependant on p300 but not CBP	83
Figure III-8	Effects on mRNA levels of <i>NANOG</i> and <i>OCT4</i> upon knockdown of p300.	85
Figure III-9	Effect of synthetic ligands of p300/CBP on Nanog-driven gene expression	88
Figure III-10	Effect of 444 on CIC model system.....	90
Figure III-11	Effect of isoxazolidine that does not target CBP on CIC model system	91
Figure III-12	Response of NCCIT cells to treatment with 444 over 72 hours.. ..	92
Figure III-13	ALDH+ CICs from head and neck cancer cell lines are resistant to cis-platinum treatment but not treatment with 444.....	94

(continued)

Figure III-14	Dependence on p300 and CBP for Oct4-mediated transcription.....	99
Figure IV-1	Flow chart detailing how students who are faced with a dilemma can, through their own poor decision making, become set on a path leading them to commit gross misconduct.....	117
Figure V-1	Determining the reliance of Nanog –driven gene expression on individual domains of p300.....	147
Figure V-2	Small molecules that could potentially be synthesized from an electron-neutral allenamide.	152
Figure V-3	Structure of sekikaic acid.	153
Figure A-1	Structure of chetomin.	158
Figure A-2	Spirooxindole scaffold	159
Figure A-3	Synthetic route for the formation of spirooxindole A-1	160
Figure A-4	Spirooxindole library.....	161
Figure A-5	Spirooxindoles that inhibited HIF-1 α -driven transcription.....	163
Figure A-6	Spirooxindoles do not specifically inhibit the function of the CH1 domain of p300/CBP.....	164

List of Tables

Table II-1	Conditions examined in initial optimization studies.....	42
Table II-2	Steric effects of phosphorous substituents.....	45
Table II-3	Scope of the rearrangement.	47
Table II-4	Enantiopurity decreases with time exposed to palladium.....	50

List of Boxes

Box IV-1	A Format for Ethical Decision Making	121
Box IV-2	Example of a case study presented to students	125
Box IV-3	Example of a case study presented to students	126
Box IV-4	Example of a case study presented to students	128
Box IV-5	Example of a situation presented to students as part of the data interpretation exercise	130

List of Abbreviations

A	Alanine
ACS	American Chemical Society
ALDH	Aldehyde dehydrogenase
ALS	Amyotrophic lateral sclerosis
ATCC	American Type Culture Collection
AML	Acute myeloid leukemia
Bn	Benzyl
BF ₃ ·OEt	Boron trifluoride diethyl etherate
^t BuOH	<i>tert</i> -butanol
bZIP	Basic region leucine zipper
cAMP	Cyclic adenosine monophosphate
C	Cysteine
CAN	Cerium ammonium nitrate
CBP	Creb binding protein
Cbz	Carbobenzoyl
CDCl ₃	Chloroform-d
ChIP	Chromatin immunoprecipitation
CIC	Cancer initiating cells
CREB	cAMP response binding protein
CSC	Cancer stem cells
D	Aspartic acid
DBD	DNA binding domain
DCM	Dichloromethane
DMEM	Dubelco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dr	Diastereomeric ratio
DTT	Dithiothreitol
E	Glutamate
ee	Enantiomeric excess
EC	Embryonal carcinoma
ESI	Electrospray injection
Et	Ethyl
Et ₃ N	Triethylamine
Et ₂ O	Diethyl ether
EtOAc	Ethyl acetate
ETP	Epipolythiodiketopiperazine
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum

FP	Fluorescence polarization
GAANN	Graduate Assistance in Areas of National Need
GAPDH	Glyceradleyde-3-phosphatate dehydrogenase
GSI	Graduate Student Instructor
GTF	General transcription factor
H ₂ NOH·HCl	Hydroxylamine hydrochloride
H ₂ O	Water
HAT	Histone acetyltransferase
HBS	Hydrogen bond surrogate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia inducible factor
HMG	High mobility group protein
HNSCC	Head and neck squamous cell carcinoma
HOAc	Acetic acid
HPLC	High pressure liquid chromatography
HRMS	High resolution mass spectrometry
HSC	Hematopoietic stem cell
HSQC	Heteronuclear single quantum coherence
IPA	Isopropanol
IR	Infra-red
kDa	Kilodalton
JACS	Journal of the American Chemical Society
M	Molar
Me	Methyl
MeCN	Acetonitrile
MDM2	Murine double mutant 2
Med	Mediator
MHz	Megahertz
MOPS	3-(N-Morpholino)propanesulfonic acid
Mp	Masking protein
MsCl	Methanesulfonyl chloride
N	Asparagine
Na ₂ CO ₃	Sodium carbonate
NaBH ₄	Sodium borohydride
NaBH(OAc) ₃	Sodium triacetoxyborohydride
NaIO ₄	Sodium periodate
NaN ₃	Sodium azide
NaOCl	Sodium hypochlorite
NIH	National Institutes of Health
NMO	N-methylmorpholine-N-oxide
NMR	Nuclear magnetic resonance
NOD	Non-obese diabetic
NOESY	Nuclear overhauser effect spectroscopy
ORI	Office of Research Integrity
OsO ₄	Osmium tetroxide
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PDB	Protein Data Bank
PG	Protecting group
Ph	Phenyl
PNAS	Proceedings of the National Academy of Sciences
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
R	Arginine
RCR	Responsible Conduct of Research
REU	Research Experience for Undergraduates
RID	Receptor interacting domain
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SCID	Severe combined immunodeficient
SDOM	Standard deviation of the mean
shRNA	Short hairpin RNA
SID	SRC-1 interacting domain
T	Temperature
TAD	Transcriptional activation domain
TAF	TBP associated factors
TBP	TATA binding protein
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Tol	Toluene
Ts	Tosyl
Y	Tyrosine

Chapter I

Introduction

A. Project Overview

Activated transcription is a complex process that is governed by the interactions of several different proteins. One essential protein-protein interaction that governs transcription is that of a DNA bound transcriptional activator with co-activator proteins. Two co-activators, CBP and p300, are highly homologous proteins that play key roles in several cellular processes including cell growth, differentiation, cell-cycle regulation and apoptosis. As these proteins are involved in so many processes, considerable interest has been devoted to identifying small molecules that can modulate the function of these proteins in defined ways. These small molecules can act as probes for dissecting the roles of CBP and p300 in various cellular processes.

My thesis work has focused in part on the design, synthesis and application of small molecules that can be used to modulate the functions of CBP and p300. This work has taken on several forms. First, I will discuss a novel methodology for the synthesis of a class of building block substrates that can be used in the synthesis of a wide variety of highly functionalized heterocycles which can be assayed for their ability to modulate transcription. Second, I will discuss the use of small molecule modulators of transcription in altering the phenotype of a specific set of cancer cells. Finally, I will

discuss the design and synthesis of a focused library of small molecules that were designed to modulate the activity of a specific domain of CBP/p300.

B. Introduction to Transcription

Transcription is the process by which DNA is converted to messenger RNA (mRNA) which leads to the synthesis of proteins that are regulated by a specific gene.¹ In order for activated transcription to proceed, transcription factors must bind to a specific site on DNA. Once these transcription factors are in place, they recruit the proteins necessary to modify chromatin structure and initiate mRNA synthesis.^{1,2} One of the proteins recruited during this event is RNA polymerase II (RNA Pol II), the enzyme directly responsible for the formation of mRNA.²

One type of transcription, activated transcription, relies on a modular protein termed an activator protein that must be present to activate transcription. Activator proteins consist minimally of a transcription activation domain (TAD) which is responsible for binding members of the transcriptional machinery and a DNA binding domain (DBD) which binds to specific sites on DNA. The DBDs bind to sites on the DNA known as enhancers which are located upstream of the promoter region.^{2,3,4} The TADs can then activate transcription by binding with co-factors which aid in the assembly of the pre-initiation complex (PIC) at the promoter of the gene to be transcribed. There are a wide variety of proteins involved in activated mammalian transcription including: bridging co-factors such as the Creb Binding Protein (CBP) and its paralog p300, complexes that modulate histone acetylation such as CBP/p300 (histone

acetyltransferases) and Swi/Snf, SAGA (histone deacetylases) the mediator complex, and members of the general transcription machinery such as general transcription factors (GTFs), the TATA binding protein (TBP), TBP associated factors (TAFs) and RNA Pol II.^{2,4}

Activated transcription begins in response to intra-or extra-cellular stimuli. These stimuli can trigger several changes within the cell that allow transcription to occur. One common initial response to these stimuli involves bringing the activator protein into a state where it can effectively interact with both DNA and its relevant co-factors in the transcriptional machinery (Figure I-1).^{1,2}

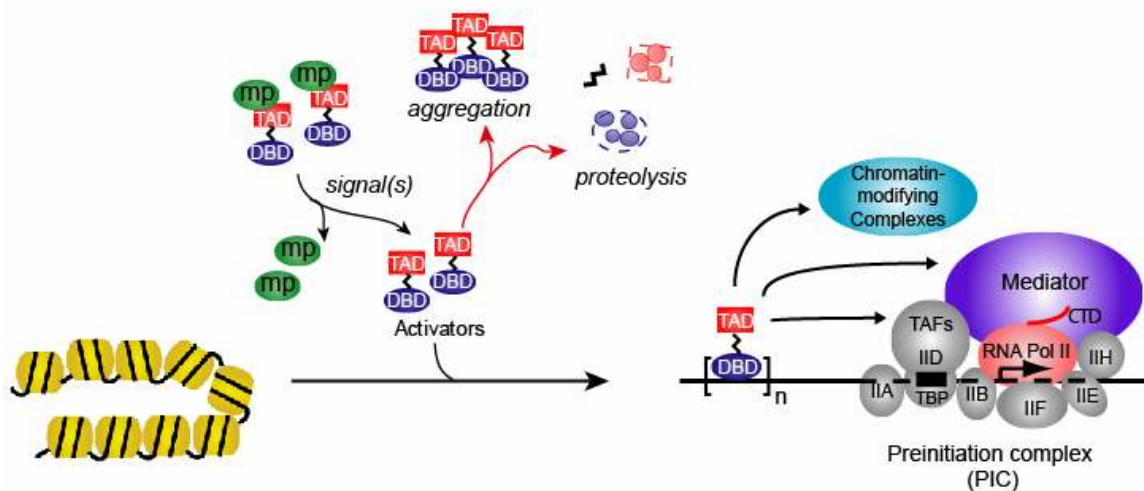


Figure I-1⁵. Schematic of activated transcription. Activator proteins are responsible for initiating activated transcription. The activators consist minimally of two domains. The transcriptional activation domain (TAD) (here a red square) is responsible for contacting members of the transcriptional machinery. The DNA binding domain (DBD) (here a blue oval) is responsible for contacting specific sites on DNA. These domains work together to promote binding of cofactors and other members of the transcriptional machinery to specific sites on DNA. When active transcription is not necessary, activator proteins are rendered inoperable by the cell via a variety of mechanisms including: interactions with masking proteins (mps), aggregation, and proteolytic degradation.

Upon binding to DNA, the transcriptional activator then recruits co-factors via direct binding interactions.^{6,7} Co-factors (or co-activators) are proteins or protein

complexes that do not bind directly to DNA but serve as a bridge between DNA-bound activators and members of the general transcriptional machinery.^{1,2} There are a large number of co-factors which serve diverse functions including histone modification and bridging the transcriptional activator and RNA Pol II holoenzyme.^{2,3} The bridging co-factors serve a key role in transcription as it has been shown in a reconstituted yeast system that when activators are in the presence of RNA Pol II and GTFs but in the absence of other co-factors, transcription does not occur. Upon the introduction of the co-factor Mediator, transcription function is restored.^{1,2,3, 8}

Additional co-factors such as the Swi/Snf complex and the SAGA complex have been shown to modify the nucleosomes in both yeast and mammalian cells.^{1,2,9,10}

Chromatin modification is necessary for transcription as interactions between DNA and the nucleosomes may inhibit transcription by blocking sites on DNA necessary for binding by activators and other members of the transcriptional machinery.^{9,10} Other important co-factors in mammalian cells are the Creb binding protein (CBP) and its paralog p300. These co-factors have no known homologs in yeast, but have been implicated in the transcription of many mammalian genes. They have been shown in mammalian cells to contain acetyl transferase activity and to directly interact with transcriptional activation domains.^{1,10}

While it is generally accepted that recruitment of co-activators via activator proteins is an essential step in activated transcription, the exact timing and nature of these interactions is poorly understood.

C. Activator Proteins

As the name suggests, activated transcription is largely governed by a class of proteins known as transcriptional activators. These proteins are modular in nature and are minimally composed of a transcriptional activation domain (TAD) and a DNA binding domain (DBD). This modularity has interesting implications for activator construction as it has been shown that functional activators can be synthesized by exchanging the DBD of one activator with the TAD of another to form a chimeric molecule.^{1,2}

One piece of evidence for the role of activators in recruiting the transcriptional machinery to DNA comes from activator bypass experiments. In these experiments, portions of the RNA Pol II holoenzyme (the term given to the mega-complex formed between RNA Pol II and the transcription co-factors) are directly fused to a protein capable of binding the DNA without the incorporation of an activating region.^{1,2, 11} It has been shown in several different studies that the level of activation achieved by these fusion proteins equals that achieved by endogenous activators, thus suggesting the role of the transcriptional activator protein as a link between the DNA and the transcriptional machinery.^{2,3,4,5,6,7}

The binding of activators to their co-factors have been linked to the maintenance of disease states. One example is the hypoxia inducible factor-1 α (HIF-1 α). This transcription factor is necessary for the expression of genes involved in maintaining homeostasis under hypoxic conditions, such as those found in solid tumors.¹² The TAD of HIF-1 α has been shown by a variety of methods to interact with the CH1 domain of the co-factor p300.^{13,14} Livingston and co-workers have shown that by disruption of the

interaction of HIF-1 α with p300 is sufficient to cause a decrease in cellular survival under hypoxic conditions.¹³ Additionally, disruption of this interaction suppresses tumor growth in a mouse xenograft model.¹³

C. 1. DNA Binding Domains (DBDs)

The DNA binding domain is responsible for binding to DNA in a gene-specific manner.^{1,2} In general, the DBD acts by interacting with either the major or minor groove of B-form DNA.¹⁵ While there is no exact “code” for DNA recognition, several general features that contribute to binding specificity and stability have been identified. The DBD presents a complementary face to its target DNA strand and imparts specificity through van der Waals forces, water-mediated hydrogen bonds, amino acid·base pair hydrogen bonding and favorable electrostatic interactions. Additional stability is imparted by interactions with the negatively charged phosphate backbone, specifically through salt bridges between positively charged amino-acid side chains and by hydrogen bonding.^{12,16}

Nuclear magnetic resonance (NMR) and x-ray crystallographic studies of DBDs reveal that they are globular proteins that can fold into a wide array of different structural motifs that are responsible for providing the specificity and stability of protein/DNA interactions (see Figure I-2 for examples). The most commonly used secondary structure is the α -helix. This structure is employed in a variety of motifs including the homeodomain motif and the basic region leucine zipper (bZIP).^{2,15} Additionally, several types of DBD motifs (including the zinc-finger motifs and the zinc-cluster motifs)

coordinate to one or more zinc molecules.¹⁵ Because of this wealth of structural information, the formation of artificial DBDs has proven to be a tractable target for exogenous regulation of transcription.

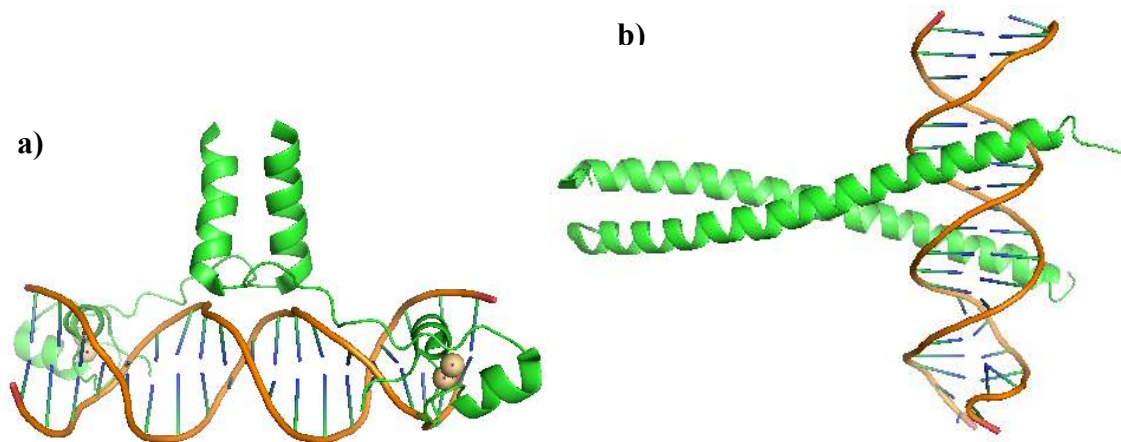


Figure I-2. DBD·DNA interactions. a) Ribbon diagram of the crystal structure of the Gal4-DNA binding domain bound to DNA as a dimer. The Gal4-DNA binding domain utilizes a zinc cluster binding motif (PDB ID: 1D66). b) Ribbon diagram of the crystal structure of GCN4-DBD bound to DNA using a basic leucine zipper (bZIP) motif (PDB ID: 1YSA).

C. 2. Transcriptional Activation Domains (TADs)

In contrast to DBDs, which are characterized by their structural motifs, transcriptional activation domains are characterized by their amino acid sequences.^{1,2} The most abundant class of activators is the amphipathic class (sometimes referred to as the acidic class) which consists of an interspersion of hydrophobic and polar residues; however, glutamine and proline rich activators have also been identified.^{1,2,17}

Amphipathic transcription activators are intrinsically unstructured in solution.^{2,18} NMR structures of several amphipathic activators including ESX, VP16, c-Myb, CREB, MLL, p53, and HIF-1 α , have shown that these activators adopt a helical structure upon binding their endogenous co-factors (Figure I-3).^{2,14,19,20,21} The hydrophobic amino acids

are displayed along one face of the helix. It is this hydrophobic face that is generally responsible for making contacts with co-factors.^{22,23}

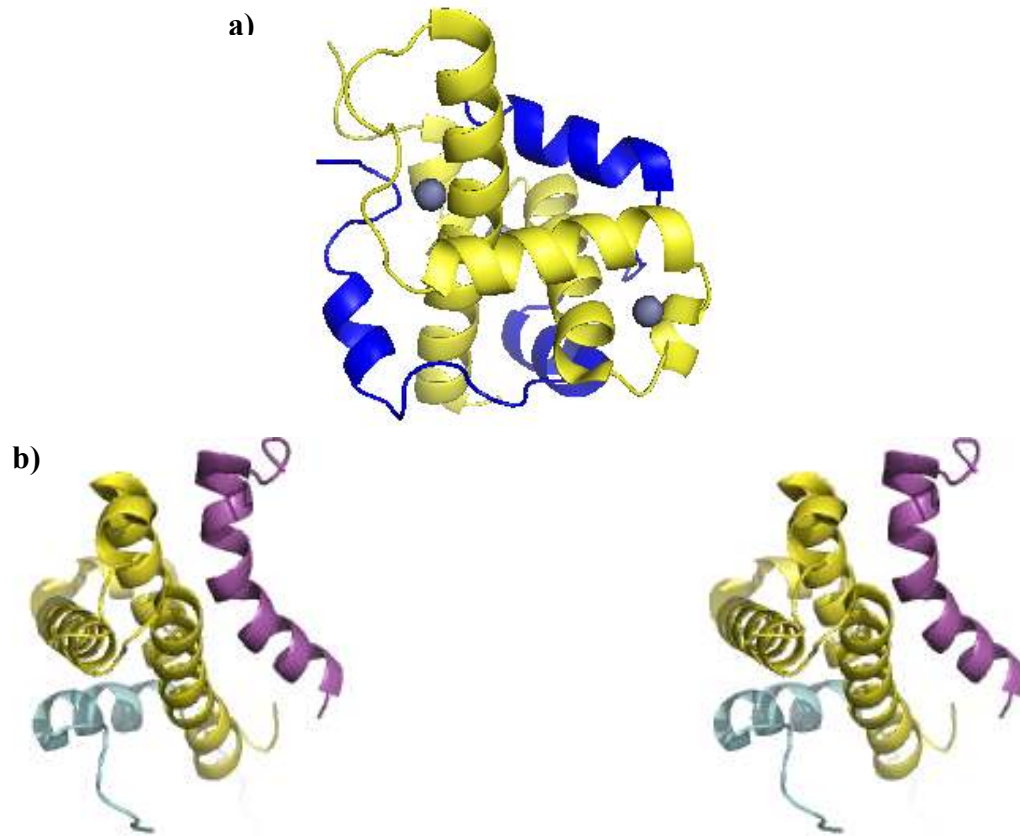


Figure I-3. TAD-target interactions. a) Ribbon diagram of the NMR structure of the minimal activation domain of the HIF-1 α TAD (blue) bound to the CH1 domain of CBP (yellow) (PDB ID: 1L8C). b) Stereoview of the ribbon diagram of the NMR structure of the MLL TAD (cyan) and the Myb TAD (magenta) co-bound to the KIX domain of CBP (yellow) (PDB ID: 2AGH).

The TAD is responsible for contacting members of the transcriptional machinery to localize them to DNA. Using a potent amphipathic activator derived from the herpes simplex virus (VP16) as a model of amphipathic activators, several groups have demonstrated the ability of these activators to bind to the GTF TFIIB and to TBP.^{24,25} It is postulated that the binding of amphipathic activators to TFIIB is critical for the stable formation of the PIC.²⁵ There are also several examples of amphipathic TADs interacting

with other members of the transcriptional machinery including CBP/p300, other GTFs and RNA Pol II.² Indeed, amphipathic transcriptional activators are known to exhibit a multi-partner binding profile with many of these interactions being transient and of medium affinity. This makes it difficult to identify and characterize all of the relevant binding partners of a given activator.^{1,2}

C.3. Mis-regulation of Transcription in Diseases

The mis-regulation of transcription has been implicated in many disease states including several types of cancers, schizophrenia, diabetes and sickle cell anemia.^{26,27,28,29,30} In order for effective therapeutics to be developed, the interactions between transcriptional activators implicated in diseases and the co-factors that bind them must be better understood.²⁶ Two co-factors that have been shown to bind several transcription factors implicated in disease states are the Creb Binding Protein (CBP) and its paralog p300.^{31,32} Studies have shown that disrupting the binding between CBP/p300 and HIF-1 α (a transcription factors involved several types of solid-tumor cancers) has induced a decrease in tumor-size in a mouse model.¹³ The study of the interactions between CBP/p300 and TADs could provide essential information for the formation of effective therapeutics.

D. Roles of CBP/p300 in Regulating Transcription

The mammalian co-activators CBP and p300 are large, multi-domain proteins that play key roles in several cellular processes including cell growth, differentiation, cell-cycle regulation and apoptosis.^{31,32,33,34,35} The importance of CBP is underscored by its role in embryogenesis and certain diseases. It has been found in mice studies that the *CBP*^{-/-}, *p300*^{-/-} and *CBP*^{+/-} and *p300*^{+/-} genotypes impart embryonic lethality.³⁶ Haploinsufficiency for CBP in humans is the cause of Rubinstein-Taybi syndrome (a genetic disorder characterized by mental retardation, skeletal abnormalities and neoplasia) and sequestering of CBP has been linked with poly glutamine disorders such as Huntington's Disease.^{31,37} Additionally, while p300 and CBP are highly homologous proteins (and are often referred to interchangeably) with certain overlapping functions, they also have distinct cellular roles. For example in both embryonal carcinoma (EC) cells and in hematopoietic stem cells (HSCs), CBP has been implicated in self renewal whereas p300 has been shown to play a key role in differentiation.^{33,38}

CBP and p300 share several functional domains that are responsible for interacting with a large number of transcriptional activators. These domains include an N-terminal receptor interacting domain (RID), three cysteine-histidine rich domains (CH1-3), a KIX domain, a bromo-domain (BD), a steroid receptor coactivator-1 interacting domain (SID) and a Glutamine rich domain (QP)^{31,32} (Figure I-4). The region of CBP/p300 encompassing the BD and CH2 domains also acts as a histone acetyltransferase (HAT). These domains have been shown to bind a number of different activator proteins, with some activators such as p53, interacting with several different domains.³²

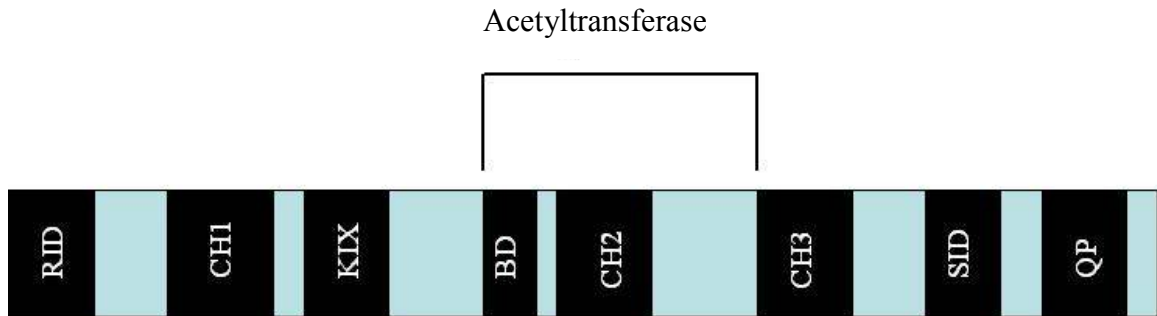


Figure I-4. Schematic of CBP/p300. These proteins each contain a number of highly conserved domains. These domains have been shown to bind to a myriad of different proteins. The region responsible for acetyltransferase activity is indicated.

As CBP and p300 have the ability to bind to many different transcription activators, one of their main roles in regulating transcription is to serve as a bridge between DNA-bound activators and the basal transcriptional machinery.³⁹ Several transcription factors that are linked with disease states such as the hypoxia inducible factor 1 (HIF-1 α), the tumor suppressor protein p53, and the viral proteins Tat, Tax and E1A have been shown to bind to CBP/p300.^{31,32,34,35,39} Additionally, it has been shown that both CBP and p300 interact with TFIIB (a GTF) and TBP, thus providing a link between activators and the transcriptional machinery.⁴⁰

In addition to this bridging functionality, CBP and p300 have acetyl transferase activity.³³ Studies in the Roeder laboratories utilizing chromatin immunoprecipitation (ChIP) have shown that following or concomitant with activator binding, CBP/p300 specifically acetylates histones proximal the promoters at which they are bound.⁴¹ It was also found that this acetylation was necessary for transcription to occur as ablation of HAT activity (either by addition of a small molecule HAT inhibitor or removal of the co-factor acetyl-CoA) resulted in a decrease in levels of activated transcription.²¹

CBP/p300 play another role in regulating transcription via their ability to acetylate other proteins, including certain transcription factors.³² One example of this function is in regulating the expression of the interferon- β (IFN β) gene. This gene is regulated by the assembly of several distinct proteins, including the high mobility group protein (HMG1) which acts as a scaffold for the binding of the other proteins. CBP has been shown to acetylate HMG1 at a key lysine residue, causing HMG1 to de-associate from DNA, resulting in down regulation of the IFN β gene expression.⁴²

Most of what is known about the roles of CBP/p300 comes from studies performed in differentiated adult cells. Evidence is emerging, however, that CBP/p300 may play an important role in regulating the transcription of undifferentiated cells such as embryonic stem cells, hematopoietic stem cells, and cancer imitating cells.

D.1. CBP/p300 in Cancer Initiating Cells (CICs)

Cancer initiating (CI) cells (also referred to as cancer stem cells (CSCs)) are a sub-population of cancer cells that exhibit stem cell-like qualities such as pluripotency and self-renewal and are postulated to be largely responsible for tumor growth.⁴³ These CI cells are biochemically distinct from differentiated adult cells; however, they display a gene expression signature similar to embryonic stem cells (ESCs) including reliance on distinct regulatory networks and transcription factors.^{44,45,46} Cancers that exhibit a greater degree of ESC-like gene set enrichment are generally more poorly differentiated and are associated with a poorer prognosis.⁴⁴

Three key transcription factors, Oct4, Nanog, and Sox2, are central in governing the expression of genes that promote pluripotency and self renewal in ESCs.⁴⁷ These activators also tend to be over-expressed in several types of cancers.⁴⁴ To date, very little is known about the mechanisms by which these three transcription factors regulate pluripotency and self renewal. Emerging evidence suggests, however, that CBP and/or p300 may play a role in regulating these factors. The discussion in Chapter 3 will further address the roles of these co-factors in regulating cancer initiating cells.

Chen and co-workers used chromatin immunoprecipitation coupled with ultra-high-throughput DNA sequencing (ChIP-seq) in ESCs to map the locations of stem cell activators including Oct4, Nanog and Sox2, as well as the location of p300. They found that p300 co-occurred with Oct4-Nanog-Sox2 binding sites, suggesting that one or more of these factors may be capable of recruiting p300. Accordingly, they found that when RNA interference (RNAi) targeted against any of these three factors was introduced to the cell, there was a reduction in p300 binding at these sites.⁴⁸ Additionally, work carried out in mouse ESCs suggests that p300 aids in Nanog-driven gene expression by acetylating a distal regulatory region of the Nanog promoter.⁴⁹

In cellulo studies indicate a role of p300 in Sox2 mediated transcription. Using chimeric fusion proteins comprised of the Gal4 DBD and the Sox2 TAD, Knowlins and co-workers examined the effects of p300 on a Sox2-driven reporter system. They found an increased level of transcription when p300 was introduced into the cell. Conversely, they found that when a protein capable of sequestering endogenous p300 (E1A) was introduced, transcription levels decreased in a dose dependant manner.⁵⁰ Activator bypass experiments performed in a follow up study in which p300 was fused directly to the

Gal4 DBD resulted in increased levels of transcription in a similar reporter system.⁵¹ These experiments indicate that p300 plays a role in Sox2-mediated transcription.

E. Small Molecules as Tools for Probing Transcription

Mis-regulation of transcription has been implicated in several disease states including cancer, diabetes and schizophrenia.^{27,29,30} As such, there has been much effort focused on finding exogenous modulators of transcription.^{52,53,54} In this section, the design of artificial probes for studying TAD-co-activator interactions will be discussed (Figure I-5).

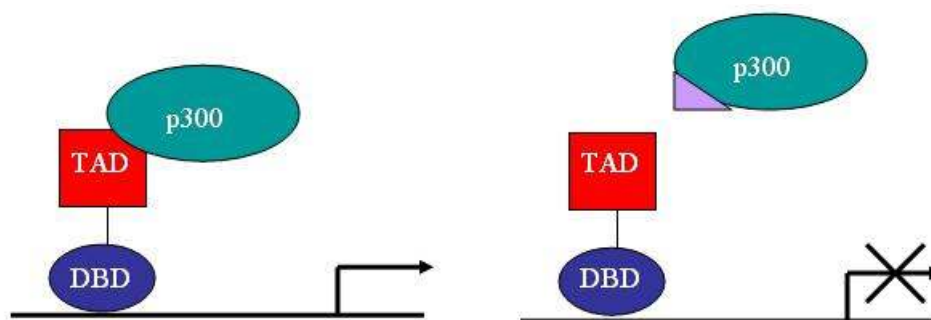


Figure I-5. Schematic of inhibition of a TAD-co-activator interaction. *Left:* Under endogenous conditions, the TAD of the DNA bound activator is able to interact with its relevant co-factor and transcription of the gene proceeds. *Right:* When a small molecule ligand of the co-factor (here a purple wedge) is introduced, it binds to the co-factor thus preventing the co-factor from interacting with the TAD. Small molecules that can perturb the functionality of specific co-factors can be used to study the role(s) of these co-factors in specific transcriptional processes and help to determine the role(s) they play in maintaining certain cellular phenotypes.

E.1. Traditional Challenges in Designing Small Molecules Probes for Activator-Co-activator Interactions

One of the complicating factors in designing small molecules that can perturb activator-co-activator interactions is the ability of endogenous TADs to interact with several different proteins, or several different regions of the same protein.² For example, the TAD of the tumor suppressor protein, p53, has been shown to bind CBP/p300 at four distinct sites (the CH1, CH3, KIX, and SID domains). p53 has also been observed to bind several members of the basal transcriptional machinery as well as its suppressor protein, MDM2 (Figure I-6).⁵⁵ This promiscuity is owed in large part to the intrinsically unstructured TADs' ability to bind to several different targets and may help provide a mechanism for regulating transcription.⁵⁶

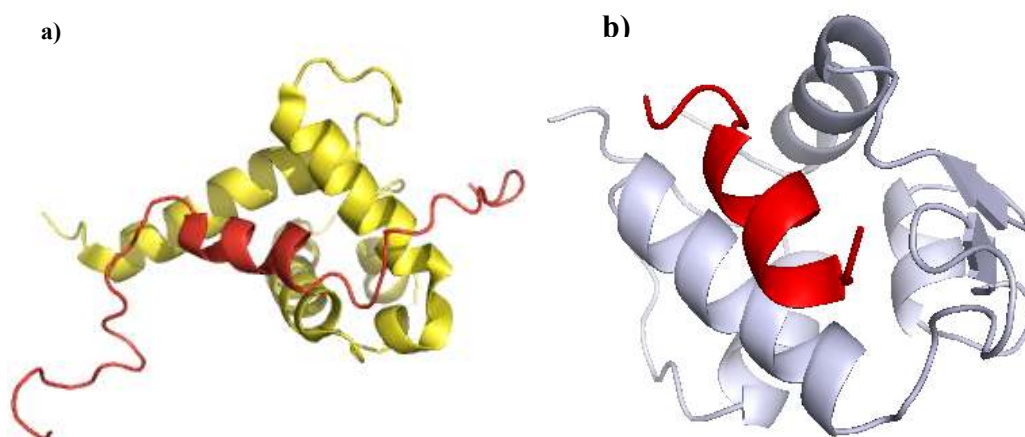


Figure I-6. p53 binding with two distinct proteins. a) Ribbon diagram of the NMR structure of the p53 TAD (red) bound as an α -helix to the CH3 domain of p300 (yellow) (PDB ID: 2K8F). b) Ribbon diagram of the crystal structure of the p53 TAD (red) bound as an α -helix to its suppressor protein MDM2 (silver) (PDB ID: 1YCR).

Another factor that complicates the development of small molecule modulators of transcription is that several co-factors are able to interact with multiple different TADs. The KIX domain of CBP, for example, has been shown to interact with over 12 different amphipathic TADs.⁵² To further complicate matters, it has been shown that many of these TAD-co-factor interactions are of moderate affinity and the structure and specificity

of the interacting proteins are poorly defined.⁵³ These types of transient, low affinity interactions make the design of artificial modulators of transcription exceedingly challenging. In order to meet these this challenge, a broad approach to small molecule design must be taken as it seems unlikely that a single class or type of scaffold will be able to selectively modulate all of these disparate transcription events. One way to efficiently synthesize a diverse library of structures is to utilize a common building block molecule that can be further transformed to a wide variety of structures.

E.1.A. Novel Methodology for the Synthesis of Allenamides as a Building Block Scaffold

Allenamides are an especially promising building block substrate as they have been used as substrates in the synthesis of a variety of highly substituted heterocycles including: dihydrofurans,^{57,58} pyranyl heterocycles,⁵⁹ isoxazolines and bis-isoxazolines,⁶⁰ diazines and imidazolidanones,^{61,62} and cyclopentenones⁶³ (Figure I-7). Thus, significant efforts have been devoted towards general methodologies to access allenamides; however, these methodologies are often lengthy or require complex precursor syntheses.⁶⁴ One intriguing route for the formation of allenamides involves the [3,3]-rearrangement of propargylic trichloroacetimidates.^{65,66} While this reaction proceeded in poor yields (10-20%) it showed that a [3,3]-rearrangement could be used in the formation of allenamides.^{65,66} Previous work carried out in the Mapp laboratories focused on a novel [3,3]-rearrangement of a phosphorimidate to a phosphoramidate for the synthesis of allylic amides.^{67,68} Work discussed in Chapter 2 will demonstrate that

this strategy can be applied to a propargylic system for the formation of highly functionalized allenamides.

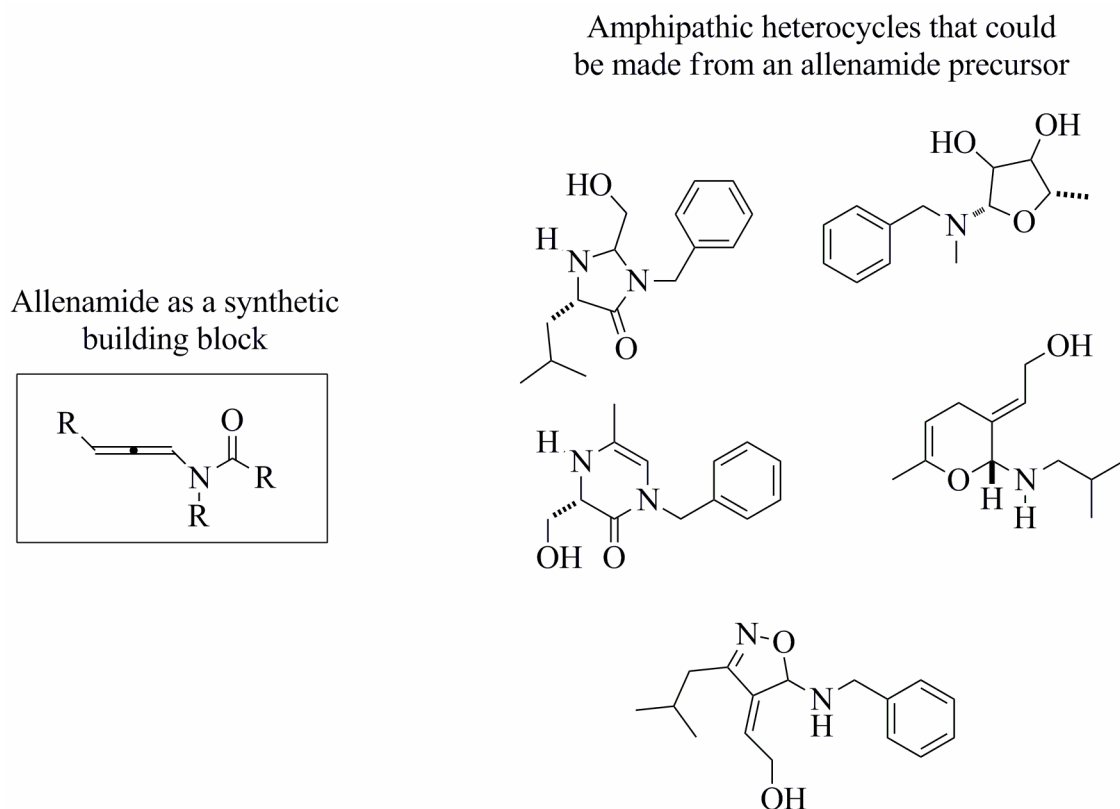


Figure I-7. Amphipathic heterocycles that can be synthesized from an allenamide precursor. By varying the substrates on the allenamide and its reaction partners, a variety of highly functionalized heterocycles can be formed. These molecules could be screened for their ability to modulate transcription.

E.2. The Use of Small Molecules as Probes for Gaining Insight into Transcription

Small molecules such as the types that can be formed from allenamides can be used as probes for perturbing TAD-co-activator interactions. By preventing co-factors from interacting with their endogenous binding partners, small molecules can allow for the dissection of the role(s) that individual proteins play in complex events. Additionally,

small molecule ligands that bind specific co-factors can be useful in determining if these co-factors are associated with specific cellular phenotypes that are linked to disease states. Some small molecule probes that perturb the binding of specific co-factors have already been discovered.

E.2.A. Small Molecules that Bind CBP/p300

One type of small molecule that has been shown to bind CBP is an amphipathic isoxazolidine that was developed by the Mapp laboratories.⁶⁹ This small molecule was first identified as an artificial activator of transcription; however, it was discovered that when not linked to a DNA binding moiety, it can function as a transcriptional inhibitor.^{70,71,72} When not linked to a DNA binding moiety, the amphipathic isoxazolidine has been used to inhibit transcription of two endogenous CBP-KIX ligands (MLL and c-Jun) in a luciferase reporter system.⁷² NMR data showing that the isoxazolidine binds to the same site on the KIX domain as these transcription factors indicates that this small molecule is likely competing with MLL and Jun for binding the KIX domain.⁶⁹

Another small molecule that inhibits the binding to the KIX domain of CBP was discovered by the Montminy laboratories from an NMR-based screen. This screen identified a small molecule (KG-501) that is able to inhibit the interaction of CBP and one of its endogenous ligands (CREB) *in cellulo*.⁷³ A derivative of KG-501, naphthol AS-E, was shown by Li and Xiao to bind to CBP with a significantly lower IC₅₀ than KG-501 (Figure I-8a).⁷⁴

The binding of transcription factors to the CH1 domain of CBP/p300 has also proven to be a target for small molecule inhibition. Kung and co-workers discovered a dimeric epipolythiodiketopiperazine (ETP), chetomin, which is capable binding to the CH1 domain of p300 (Figure I-8b).⁷⁵ More recently, the Olenyuk laboratories have designed a series of ETPs to inhibit the interactions of HIF-1 α and CBP/p300 (Figure I-8b).^{76,77}

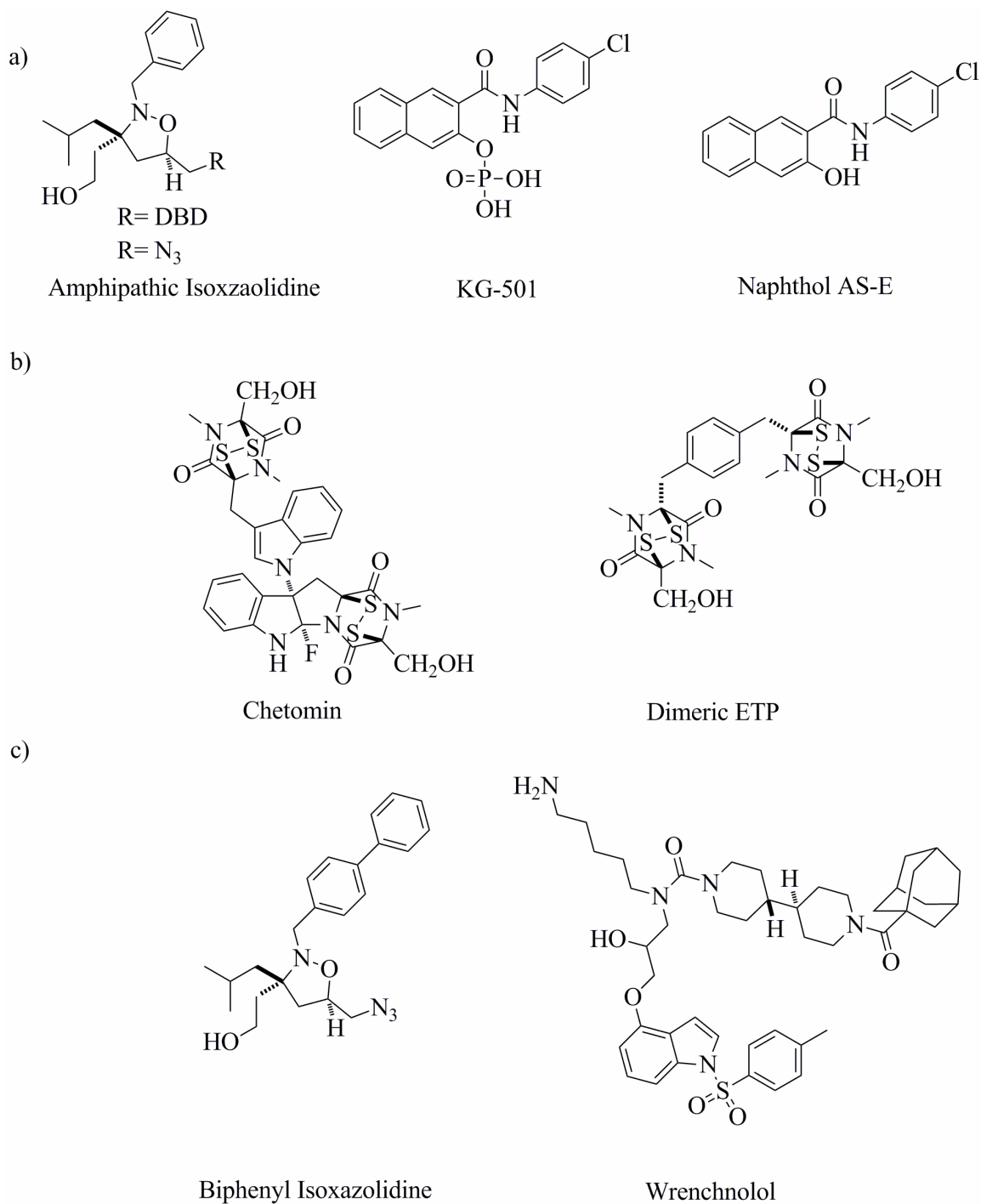


Figure I-8. Small probes for studying transcription. a) Small molecules that bind the CBP-KIX domain. b) Small molecules that bind the CBP-CH1 domain. c) Small molecules that bind Med23.

E.2.B. Small Molecules that Interact with Med23

Med23 (Sur2) is another co-factor found in mammalian cells. There is evidence that the transcriptional activator ESX binds Med23 and in doing so, up-regulates expression of the ErbB2 (Her2) gene. ErbB2 is an important therapeutic target as over-expression of this protein is seen in approximately 20-30% of breast cancers.⁷⁸ To date, there have been few examples of small molecules capable of inhibiting the ESX·Med23 interaction. One example, from the Mapp laboratories, is a modified version of the amphipathic isoxazolidine previously discussed. Cells treated with this isoxazolidine, which contains a biphenyl group attached to the isoxazolidine nitrogen, display a significant decrease in ErbB2 mRNA levels relative to vehicle control (Figure I-8c).⁷⁹ Additionally, wrenchnolol and a related compound, adamanolol have been shown to inhibit the binding of ESX to Med23 (Figure I-8c).^{74,80}

E.2.C. Implications of Using Small Molecules as Tools for Probing the Roles of Proteins Involved in Transcription

These small molecules serve as valuable tools for probing the relation between activators and their target co-activators. Small molecules that bind to specific domains of co-factors such as CBP/p300 are a valuable tool for studying transcription. Small molecule probes such as those described above can effectively inhibit binding of endogenous transcription activators to their co-factors. By employing small molecules such as the ones discussed, the roles of specific domains of specific co-factors can effectively be studied for a variety of transcription events. Work presented in chapter 3 of this thesis will address the use of synthetic probes that bind the KIX and CH1 domains of

p300 in studying the roles of p300 in maintaining pluripotency and self-renewal in cancer initiating cells.

F. Thesis Summary

As seen, there are some known examples of small molecules that alter transcriptional events. While these small molecules have shown promise as probes for understanding transcriptional processes, they only modify a small subset of protein-protein interactions involved in transcription. Because different protein classes require different types of small molecule modulators, it is essential to create diverse small molecule libraries that could be used to modulate a variety of different functions.⁸¹ As the synthesis of large libraries can be expensive and time consuming, employing common substrates in early stages of synthesis that can be diversified at later points decreases the length of syntheses without decreasing the diversity of the final library. Synthetic strategies for forming substrates that can be easily taken on to form a large number of small molecule classes would be highly valuable in this endeavor. As such, Chapter 2 addresses the development of a novel synthetic strategy for forming fully protected allenamides. As allenamides are pre-cursors in the formation of a wide range of small heterocycles, they could serve as a valuable tool in library synthesis. The work accomplished in this study reveals a straight-forward approach to forming highly functionalized allenamides from commonly available starting materials. These allenamides can be used as substrates in further transformations and display a different reactivity pattern than previously identified allenamides. Thus, the fully protected

allenamides described in this chapter could be used in the formation of a wide range of small molecules.

The work presented in Chapter 3 highlights the importance of using small molecules as probes for studying transcriptional processes. This chapter centers on understanding the roles of CBP and p300 in maintaining pluripotency and self-renewal in cancer initiating cells (CICs). RNAi and small molecule studies were carried out to determine if CBP and p300 interacted with key transcription factors that have been implicated in maintaining pluripotency and self-renewal. Preliminary results indicate that small molecules that are known to bind to the KIX and CH1 domains of CBP/p300 can affect the transcriptional output of a Nanog-driven luciferase reporter. Additional studies indicate that Nanog shows at least some dependency on p300, but not CBP, for transcription. Finally, it has been shown that the amphipathic isoxazolidine selectively decreases the population of cancer initiating cells over bulk tumor cells, suggesting that perturbing transcription could prove to be a good strategy in designing therapeutics that target CICs.

The Appendix of this document gives one further example of a small molecule scaffold that can modulate transcription. Presented here is a focused library of small molecules based on the common spirooxindole scaffold. *In cellulo* studies reveal a subset of this library that inhibits the activation of HIF-1 α in a luciferase reporter assay. It was also found that these molecules inhibit the activation of a variety of transcriptional activators that bind to diverse partners in the transcriptional machinery. These small molecules could be used in the future as general inhibitors of CBP/p300 functionality.

Finally, Chapter 4 takes a step back from transcription and addresses a topic that concerns all scientists: research ethics. In this chapter, the development and implementation of a practical research ethics course is discussed. This course derives both its novelty and its utility from the fact that it is designed and taught almost exclusively by senior graduate students and is presented to incoming graduate students at the start of their first year of graduate school. Student evaluations and reactions from the broader chemical community indicate that this course serves as a valuable addition to the way that research ethics is taught to graduate students.

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Chapter II

A Palladium-Catalyzed [3,3]-Rearrangement for the Facile Synthesis of Allenamides^a

A. Abstract

Allenamides are used as substrates in the synthesis of a variety of different compounds, including many highly substituted heterocycles. As part of our ongoing quest to identify small molecule ligands for transcription proteins, we are interested in synthesizing a variety of highly substituted cyclic small molecules. This interest initially led us to examine the feasibility of using allenamides as a common substrate to build a diverse library of small molecules. When examining the known routes to allenamide formation, we found that these routes were often lengthy and synthetically challenging.

It was envisioned that a more effective route toward allenamide synthesis could be developed. Accordingly, a methodology for the straightforward synthesis of highly substituted allenamides was developed. This system relies on a palladium-catalyzed phosphorimidate to phosphoramidate rearrangement. The phosphorimidate can be readily synthesized from a propargylic alcohol, a chlorophosphite and a Cbz-azide. The subsequent rearrangement generates allenamides in good yields. By varying the substitution patterns of the propargylic alcohol, mono-, di- and tri-substituted allenamides

^a Portions of the chapter are reported in Danowitz, A.M.; Taylor, C.E.; Shrikian, T.M.; Mapp, A.K. A Palladium-Catalyzed [3,3]-Rearrangement for the Facile Synthesis of Allenamides . *Org. Lett.*, **2010**, *12*, 2574-2577.

can be formed. Additionally, use of an enantiomerically enriched propargylic alcohol, allows for enantioenriched allenamide formation.

B. Introduction

B.1. Synthesis of Building Block Molecules that can be used in the Formation of Synthetic Probes of Transcriptional Processes

One of the goals of our laboratory is the design and synthesis of novel ligands for transcription co-factors that can act as a tool for studying transcription. As many transcriptional activator proteins exhibit a multi-partner binding profile and bind to their co-factors in the transcriptional machinery with moderate affinity, it is likely that a wide variety of synthetic scaffolds will be needed to modulate individual binding events.^{1,2} Therefore, we have an ongoing interest in developing straightforward strategies for the synthesis of building block molecules that can be further modified to form structures, such as amphipathic heterocycles, which can mimic the functionality of endogenous activators and bind to co-factors within the transcriptional machinery. The allenamide scaffold is ideal for this purpose as it has been used as a substrate in the synthesis of a wide variety of highly substituted amphipathic heterocycles (Figure II-1).

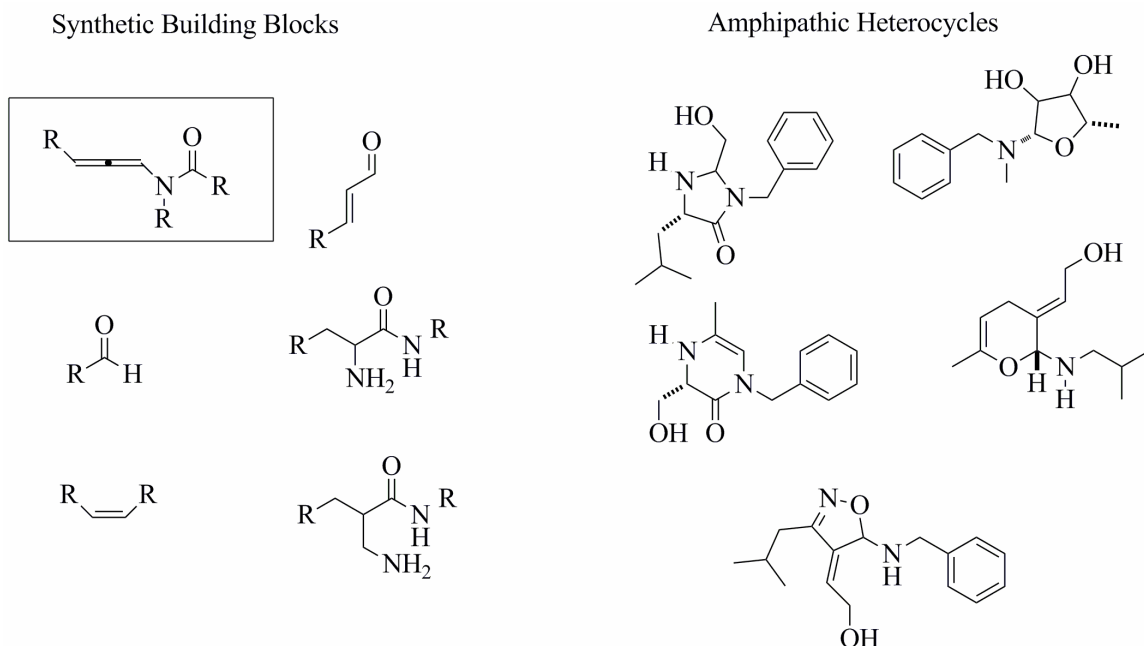


Figure II-1. Small molecule building blocks for the formation of amphipathic heterocycles. The structures shown on the left are building blocks that have been shown to undergo further transformations to form heterocycles such as those shown on the right. By varying the substituents on the building blocks and their reaction partners, a variety of amphipathic heterocycles could be formed. The allenamide (top left in a box) has been shown to form all of the classes of heterocycles shown on the right. It is therefore a promising precursor molecule for the synthesis of libraries of amphipathic heterocyclic compounds.

B.2. Allenamides as Substrates for the Formation of Cyclic Small Molecules

Allenes, or 1,2-dienes, have the ability to act as either nucleophiles or electrophiles, thus allowing for their use in the preparation of a variety of synthetic precursors or end products.^{3,4} The reactivity pattern of the allene is heavily influenced by heteroatoms that are appended to the allenic system. One such example is the allenamine, an allene with a nitrogen atom appended directly to the allenic system. As the lone electron pairs on the nitrogen can be donated into the diene system, allenamines react with electrophiles and nucleophiles in a highly regioselective manner.⁵ This electron rich nature, however, leads to allenamines being generally unstable compounds that are highly

subject to degradation. Allenamides are analogous structures to allenamines with a similar reactivity pattern; however, as the amide moiety has less electron donating capabilities, these compounds tend to have an increased stability relative to their allenamine counterparts (Figure I-2).⁵

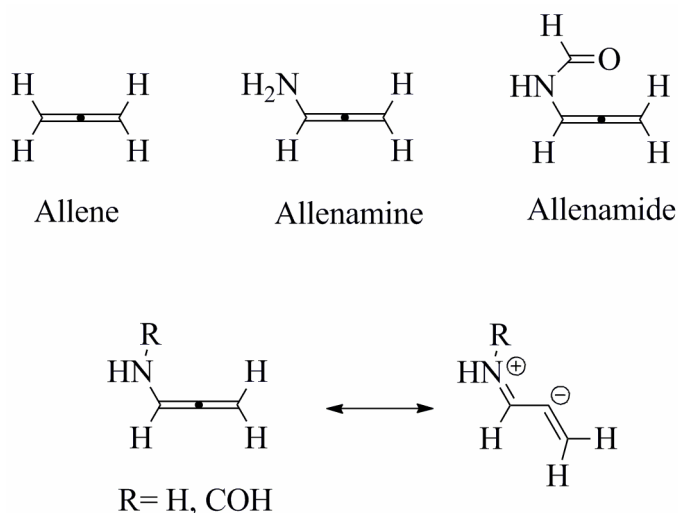


Figure II-2 Nitrogen substituted allenes. *Top*: Structures of an allene, an allenamine and an allenamide are shown. *Bottom*: The electronic distribution in the allenic moiety of a nitrogen substituted allene is shown. The electron pair donated by the nitrogen allows allenamines and allenamides to react with nucleophiles and electrophiles in a highly regioselective manner.

Because of their unique electronic characters, allenamides are used in the synthesis of a variety of interesting cyclic compounds. For example, they have been used in the synthesis of cyclobutanes,⁶ dihydrofurans,^{7,8} pyranyl heterocycles,⁹ isoxazolines and bis-isoxazolines,¹⁰ diazines and imidazolidanones,^{11,12} and cyclopentenones.¹³ It is therefore possible that allenamides could be used as a general precursor in the synthesis of highly diverse small molecule libraries that could be screened for their effectiveness at modulating transcription.

B.3. Established Routes for Accessing Allenamides

There are a variety of known routes for synthesizing allenamides. These routes, however, often require complex and lengthy substrate synthesis, with the formation of enantioenriched allenamides posing a particular challenge.^{5,14-21} One popular route for the formation of allenamides involves the iterative addition of functionality to a mono-substituted allenamide. This allenamide is formed by base-catalyzed isomerization of a propargylic amide to allenamide. Alkyl functionality can then be appended to this mono-substituted allenamide in a stepwise fashion. In this methodology, stereocontrol can only be obtained with a chiral auxiliary⁵ (Figure II-3).

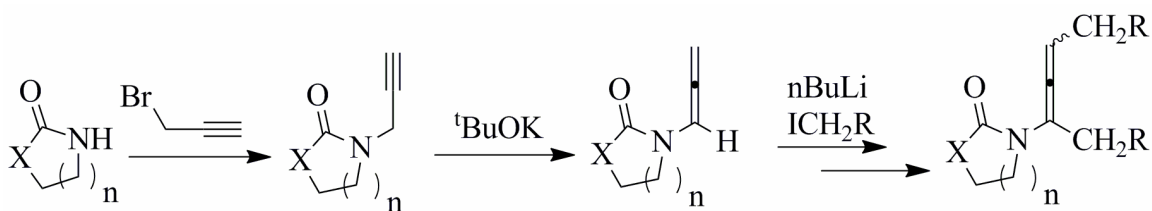


Figure II-3. Base-catalyzed isomerization of a propargylic amide to form an allenamide. While this is the most commonly used method for forming allenamides, it requires a chiral auxiliary to impart stereocontrol and heavily substituted allenamides must be formed by iterative addition of functionality to a mono-substituted allenamide.

In addition to the base-catalyzed isomerization, several metal-catalyzed reactions to form allenamides have been developed. Specifically, the Trost¹⁷ and Hsung¹⁸ laboratories have demonstrated the utility of a copper-catalyzed coupling of allenyl halides to amides in forming allenamides (Figure II-4). The primary drawback to this methodology is the use of allenyl halides which tend to be difficult to synthesize and handle, especially in enantiopure form. As the stereochemistry of the product allenamide is set by that of the allenyl halide, this is a sub-optimal route for forming highly enantiomerically enriched allenamides.

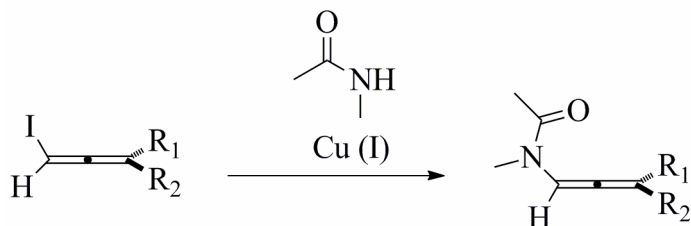


Figure II-4. Example of copper-catalyzed coupling of an allenyl halide with an amide to form an allenamide.

Another method for the formation of allenamides is a [2,3]-rearrangement of propargylic sulfides.^{20,21,22} This method allows for the predictable transfer of stereochemistry to the allenamide. However, the preparation of propargylic sulfides, especially enantioenriched versions, requires several difficult synthetic manipulations (Figure II-5).

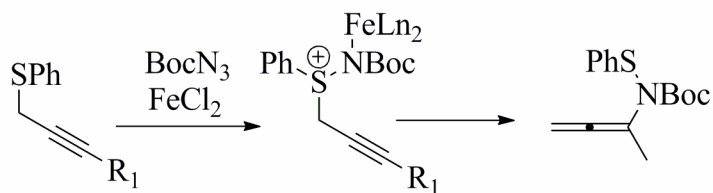
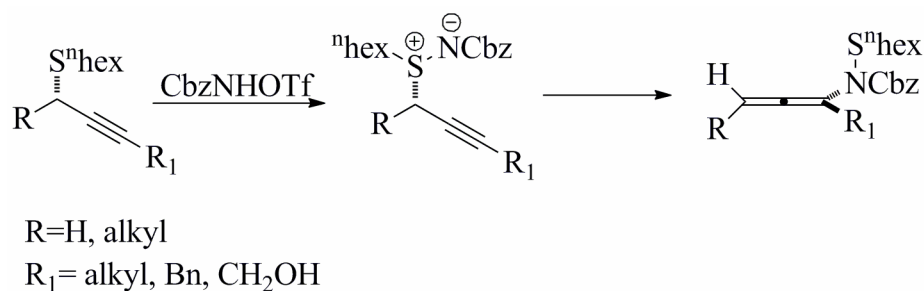


Figure II-5. Examples of [2,3]-sigmatropic rearrangements of propargylic sulfides to form allenamides.

Finally, there have been limited examples of the use of [3,3]-rearrangements to form allenamides.^{23,24,25,26} One especially intriguing route for the formation of allenamides was reported by Overman and co-workers in the 1970s.^{23,24} This route

featured the rearrangement of propargylic trichloroacetimidates to form allenamides. While the use of this [3,3]-rearrangement is intriguing, these reactions proceeded in poor (10-20%) yields (Figure II-6). These compounds were mainly synthesized as intermediates in the formation of 1,3-dienes and the properties and scope of this rearrangement were not fully explored.

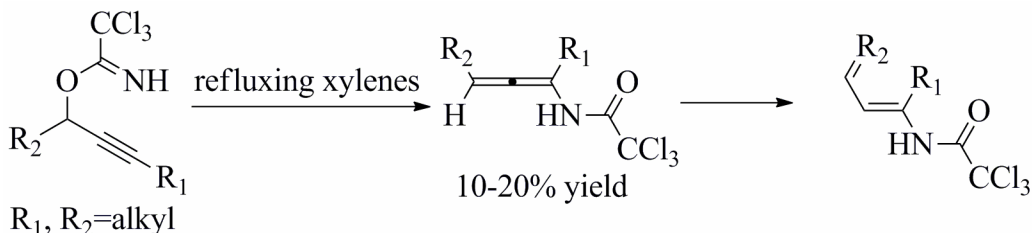


Figure II-6. Rearrangement of trichloroacetimidates to form allenamides. These allenamides readily isomerize to 1,3-dienes.

B.4. A Novel Methodology for the Formation of Allenamides

Upon examining these reactions, it was noted that a [3,3]-rearrangement had the potential to circumvent many of the difficulties associated with the other methods of allenamide formation. We postulated that a known allylic phosphorimidate to phosphoramidate rearrangement could be extended to the formation of allenamides from propargylic alcohols.^{27,28,29} The envisioned reaction pathway would rely on three readily available starting materials: a propargylic alcohol, a protected azide and a chlorophosphite (Figure II-7). In a one-pot procedure, the propargylic alcohol could be reacted with the chlorophosphite to form a trivalent phosphorous species that could then undergo a Staudinger reduction with the azide for form a phosphorimidate. This phosphorimidate could then undergo a thermal or transition-metal catalyzed rearrangement to form the

allenamide product. It is also postulated that if a chiral propargylic alcohol is employed in this reaction, the stereochemical information should carry through to the allenamide. If successful, this methodology would allow for the synthesis of highly enantioenriched allenamides in a small number of straightforward synthetic steps.

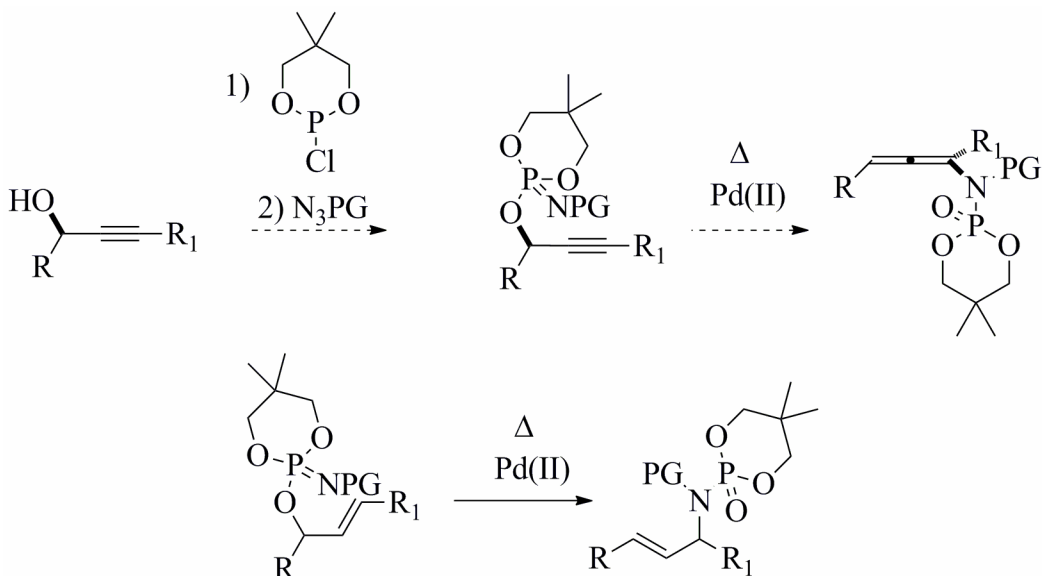


Figure II-7. Novel methodology for the formation of allenamides. *Top:* Proposed reaction pathway for a phosphorimidate to phosphoramidate rearrangement to form allenamides. *Bottom:* Known allylic phosphorimidate to phosphoramidate rearrangement.

C. Optimization Studies

In testing the hypothesis that this rearrangement could be applied to a propargylic system, the protecting group, substituents on the phosphorous, use of thermal versus metal-catalyzed conditions, and the solvent concentration were varied.

C.1. Optimization of the Protecting Group

Using propargyl alcohol as the initial substrate, the protecting group used in the rearrangement was the first variable examined. It was found that when either a Cbz or

tosyl (Ts) protecting group was appended to the azide in combination with a palladium catalyst, we formed the desired allenamide (Table II-1 entries 2-11). It was also found that the Cbz protecting group afforded higher yields over the tosyl group when the reactions were run under the same conditions (Table II-1, entries 3 and 7). Attempts at using a benzyl protecting group resulted in no allenamide formation (Table II-1, entry 1). Upon closer inspection, it was discovered that the phosphorimidate was not forming from the trivalent phosphorous species formed during the first step of this reaction.

C.2. Optimizing the Phosphorous Substituents

As shown in Table II-1, initial attempts were carried out using the cyclic ether substituent on the phosphorous that had been most effective in the allylic rearrangement.^{27,28} It was postulated that an increased yield of allenamide could be achieved by changing to either amine or hydrocarbon substituents. However, when using either diethyl-amino or phenyl groups in this position, no allenamide was formed (Table II-1, Entries 12-13). As was the case when using the benzyl protecting group, the phosphorimidate was not formed.

C.3. Optimizing Temperature and Solvent Conditions

It was initially noted that using dichloromethane (DCM) at room temperature gave the highest yields (Table II-1, entries 7 and 10). Use of toluene in this reaction also worked, albeit with a lower yield (Table II-1, entry 8). Attempts to heat the reaction

caused a decrease in yield (Table II-1, entry 6). Decreasing the concentration of phosphorimidate from 0.1 M to 10 μ M, led to an increase in yield from 49 to 66% (Table II-1, entries 5 and 7).

This finding indicated that the palladium might be causing the degradation or polymerization of the product allenamide. Additionally, if the reaction was allowed to proceed any longer than was necessary to completely deplete the starting material, a decrease in yield was observed.

C.4. Optimizing for Steric Considerations

The final point of examination in the initial optimization studies was the degree of substitution of the alcohol substrate. It was found that when using the optimized conditions (cyclic ether substituents on the phosphorous, Cbz-azide, 10 μ M DCM at room temperature) allenamides were formed in good yields using either a primary or secondary propargylic alcohol (Table II-1, Entries 7 and 10). When tertiary alcohols were used as the substrate no allenamide products were formed. Once again, this was because the phosphorimidate did not form.

Table II-1. Conditions examined in initial optimization studies.

Entry	R1	R2	R3	PG	Catalyst	Solvent	T (°C)	Conc	%yield
1	H	H		Bn	None	Xylenes	120	0.1M	0*
2	H	H	“ “	Ts	None	Xylenes	120	0.1M	0
3	H	H	“ “	Ts	PdCl ₂ (CH ₃ CN) ₂	DCM	RT	0.1M	35
4	H	H	“ “	Ts	PdCl ₂ (CH ₃ CN) ₂	DCM	35	0.1M	35
5	H	H	“ “	Cbz	PdCl ₂ (CH ₃ CN) ₂	DCM	RT	0.1M	49
6	H	H	“ “	Cbz	PdCl ₂ (CH ₃ CN) ₂	Toluene	100	0.1M	16
7	H	H	“ “	Cbz	PdCl ₂ (CH ₃ CN) ₂	DCM	RT	10μM	66
8	H	H	“ “	Cbz	PdCl ₂ (CH ₃ CN) ₂	Toluene	RT	10μM	58
9	H	H	“ “	Cbz	PdCl ₂ (CH ₃ CN) ₂	DCM	RT	5 μM	36
10	H	Me	“ “	Cbz	PdCl ₂ (CH ₃ CN) ₂	DCM	RT	10μM	60
11	Me	Me	“ “	Cbz	PdCl ₂ (CH ₃ CN) ₂	DCM	RT	10μM	0*
12	H	H	(NEt ₂) ₂	Cbz	PdCl ₂ (CH ₃ CN) ₂	DCM	RT	10μM	0*
13	H	H	Ph	Cbz	PdCl ₂ (CH ₃ CN) ₂	DCM	RT	10μM	0*

* Phosphorimidate is not formed

D. Discovery of a Competing Reaction Pathway

The conditions that resulted in a lack of formation of the phosphorimidate were initially intriguing. It was postulated that a [2,3]-rearrangement of the propargylic trivalent phosphorous species was occurring.³⁰ We believed that during the course of the reaction, the trivalent phosphorous species could either undergo the Staudinger reduction or this spontaneous [2,3]-rearrangement depending on which reaction was kinetically favored (Figure II-8). Based on the conditions tried in during the initial optimization, it was discovered that formation of the phosphorimidate was favored under the following conditions: the Staudinger reduction was rapid (as is the case when using an electron withdrawing azide substituent such as Cbz), when an ether was used as the substituent on the phosphorous group, and when a primary or secondary alcohol was used. The formation of the phosphorimidate was disfavored under the following conditions: the Staudinger reduction was slow (as is the case when using benzyl azide), an amine or hydrocarbon substituent was used on the phosphorous, or when a tertiary alcohol was used. This discovery informed the types of substrates that were used in the subsequent optimization and reaction scope studies.

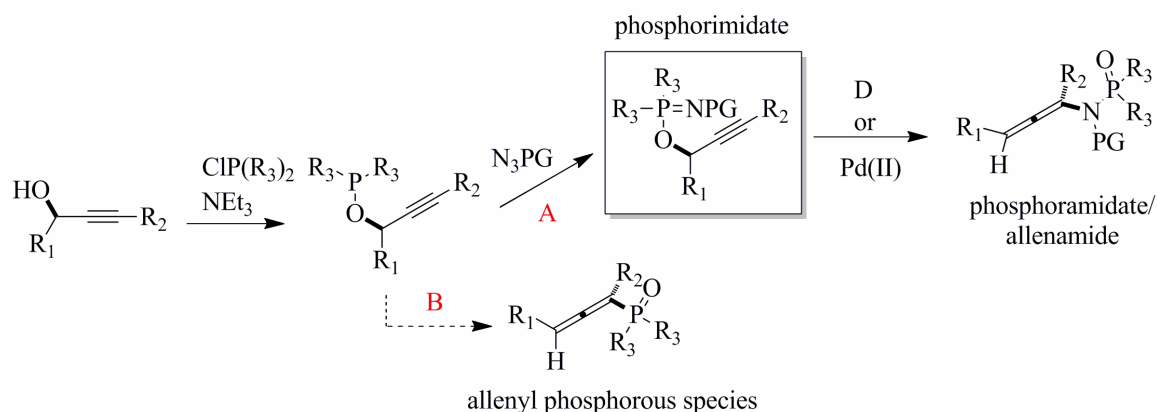
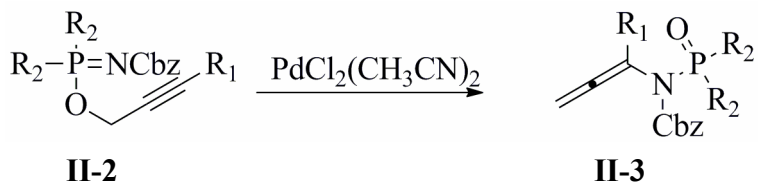


Figure II-8. Desired versus undesired reaction pathway. The trivalent phosphorous species could either undergo a Staudinger reduction and proceed via pathway A to the formation of the phosphorimidate or it could undergo a spontaneous [2,3]-sigmatropic rearrangement to form the allenyl phosphorous species via pathway B.

E. Optimization for Application to Internal Alkynes

The possibility of introducing functionality at the opposite alkyne terminus was examined. Initially, a substrate with a methyl group appended at this position was used. Unfortunately, this reaction resulted in only 16% yield of product allenamide (Table II-2, entry 2). Upon changing the solvent to toluene and heating the reaction to 100 °C, the allenamide was obtained in 13% yield (Table II-2, entry 3). It was postulated that the increased steric bulk caused by the methyl at the 3-position was negatively interacting with the phosphorous substituents during the transition state. To mitigate this effect, the cyclic ether substituent on the phosphorous was replaced with ethyl ether substituents. While the reaction still proceeded in low yields at room temperature (Table II-2, entries 4 and 5), a 60% yield of allenamide was obtained when the reaction was heated to 100 °C (Table II-2, entry 6). Additionally a 10% increase in yield was obtained when using the ethyl ether substituent in combination with propargyl alcohol (Table II-2, entry 7).

Table II-2. Steric effects of phosphorous substituents

Entry ^{a,b}	R ₁	R ₂	Solvent	Temp (°C)	Yield
1	H		DCM	25	66% ^c II-2b
2	Me		DCM	25	16% ^c II-2d
3	Me		toluene	100	13% ^d II-2d
4	Me	OEt	DCM	25	29% ^d II-2e
5	Me	OEt	toluene	25	29% ^d II-2e
6	Me	OEt	toluene	100	60% ^c II-2e
7	H	OEt	DCM	25	76% ^c II-2f

^aConditions: 1) propargylic alcohol (1.6 equiv), 1.3 equiv of R₂PCl and 1.3 equiv of Et₃N in Et₂O, 0 °C, 20 min 2) Cbz azide (1.0 equiv), rt, 2 h. ^bPhosphorimidate (1.0 equiv), PdCl₂(CH₃CN)₂ (3 mol%), solvent to a final concentration of 0.01 M.

^cIsolated yields. ^dNMR yields.

F. Defining the Scope of the Rearrangement

Upon identifying conditions that worked for both internal and terminal alkynes, the method was applied to a broader range of propargylic alcohols to better define the

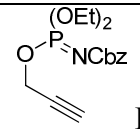
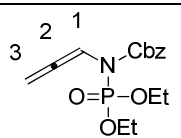
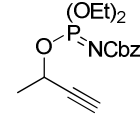
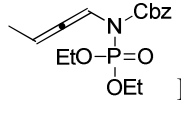
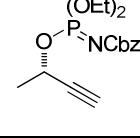
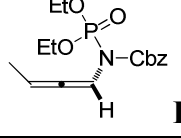
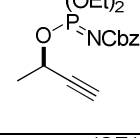
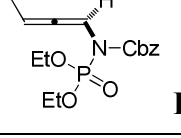
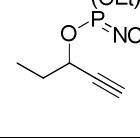
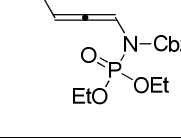
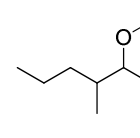
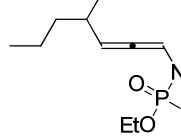
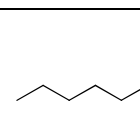
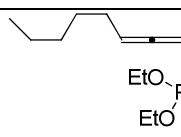
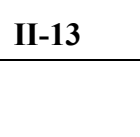
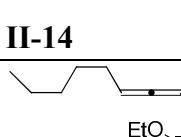
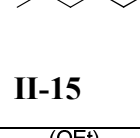
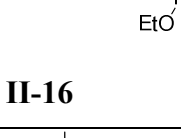
scope of this rearrangement. Of particular utility would be substitution patterns that would allow for the formation of di- and tri-substituted allenamides.

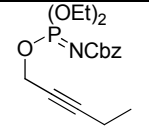
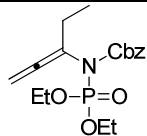
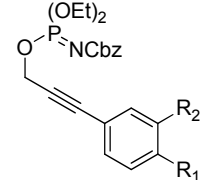
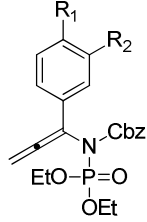
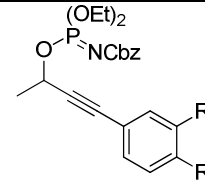
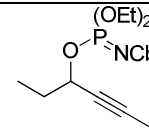
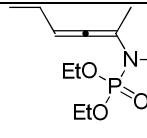
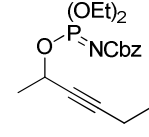
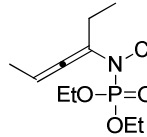
Secondary propargylic alcohols were first examined as they would produce disubstituted allenamides functionalized at the 1 and 3 positions. As shown in Table II-3 (entries 2-8), secondary alcohols with both straight chain and branched alkyl substituents are excellent substrates for the rearrangement. Tertiary propargylic alcohols were not, however, good substrates for the reaction as the intermediate propargylic phosphite underwent rapid conversion to the corresponding allenyl phosphonate.³⁰

1,1-Disubstituted allenamides can also be prepared via rearrangement of internal alkynes. Alkyl groups are well-tolerated at this position (Table II-3, entries 9 and 10). Appending aryl groups at this position, however, proved to be more challenging. Primary alcohols with less electron-rich aryl substituents were able to react under these conditions, albeit with a decrease in yield relative to their alkyl counterparts (Table II-3 entry 11). The phosphorimidates formed from secondary alcohols or alcohols with electron donating aryl substituents decomposed rapidly, making these alcohols unsuitable substrates for this rearrangement (Table II-3 entries 11 and 12).

Tri-substituted allenamides can also be prepared through the reaction of more substituted propargylic alcohols (Table II-3, 13 and 14). Tri-substituted allenamides such as these would be of particular use as substrates in further synthetic transformations, such as the formation of highly substituted heterocycles.⁷⁻¹³

Table II-3. Scope of the rearrangement.

Entry ^a	Phosphorimidate	Allenamide	Yield ^b
1	 II-1f	 II-2f	76%
2	 II-3	 II-4	68%
3 ^c	 II-5	 II-6	67% 92% ee ^d
4	 II-7	 II-8	70% 92% ee
5	 II-9	 II-10	68%
6	 II-11	 II-12	65%
7	 II-13	 II-14	70%
8	 II-15	 II-16	70% 80% ee
9	 II-1e	 II-2e	60%

10	 II-17	 II-18	58%
11	 II-19a-d	 II-20a-d	R ₁ =H, R ₂ =H 45% a R ₁ =Cl, R ₂ =H 27% b R ₁ =H, R ₂ =OMe 35% c R ₁ =OMe, R ₂ =H decomposed phosphorimidate d
12	 II-21a-c	Phosphorimidate decomposed	R ₁ =H, R ₂ =H a R ₁ =OMe, R ₂ =H b R ₁ =Cl, R ₂ =H c
13	 II-22	 II-23	58%
14	 II-24	 II-25	60%

^aConditions as described in Table 1. ^bIsolated yields. ^cee of commercially available starting alcohol found to be <95% in each case. ^dee determined by chiral HPLC.

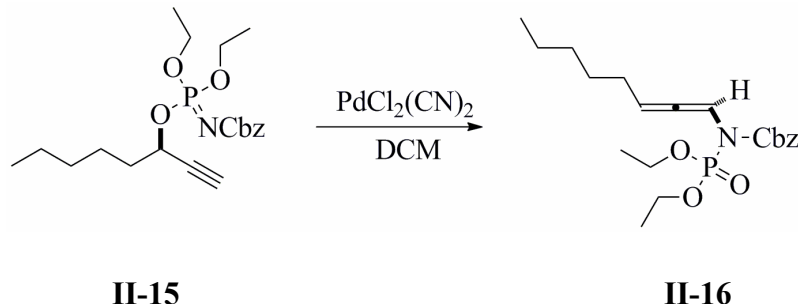
G. Defining the Stereoselectivity of the Rearrangement

As was mentioned earlier, the synthesis of enantiomerically enriched allenamides is particularly challenging.^{5,14-22} This rearrangement was thus examined for its utility in forming enantiomerically enriched allenamides. It was envisioned that stereochemical information could be imparted by using an enantiomerically enriched propargylic alcohol as the initial reaction substrate. Enantiomerically enriched propargylic alcohols are both

commercially available and easily synthesized.³¹⁻³³ For this study, three commercially available propargylic alcohols were employed. Either enantiomer of the allenamide derived from 3-butyne-2-ol can be readily prepared, both in 92% ee (Table II-3, entries 3 and 4). Additionally, an enantiomerically enriched allenamide derived from (R)-(+)-1-octyne-3-ol was also prepared in 80% ee (Table II-3, entry 8). The indicated stereochemistry is predicted based upon the mechanism of closely related allylic rearrangements.²⁷⁻²⁹

Upon further investigation, it was determined that the attenuated enantiopurity is due to a Pd-catalyzed epimerization step that the allenamides undergo. When the allenamide derived from (R)-(+)-1-octyne-3-ol was re-subjected to reaction conditions (toluene, palladium, heating to 100 °C), a sharp decrease in enantiopurity occurred. The enantiopurity decreased from 80% to 51% after re-subjection to reaction conditions for an additional five hours (Table II-4). Allenamides of higher stereochemical purity can be obtained simply by running the reaction for a shorter period of time, albeit in lower yields.

Table II-4. Enantiopurity decreases with time exposed to palladium



Reaction time (hours)	%ee	% yield
5	80	60
7.5	72	70
7.5 + 5 additional (12.5 total)	51	80% recovered

H. Utilizing Allenamides as Substrates in Further Reactions

Despite their utility in a variety of reactions, the lability of nitrogen-substituted allenenes is a recurring concern.²⁻⁵ The fully protected allenamides produced in the phosphorimidate rearrangement exhibit considerable stability, and can be stored neat at 0 °C for over two months with little decomposition. In comparison, other allenamides have been reported to have limited stability when stored neat at -20 °C.¹⁶ It is postulated that the remarkable stability of these allenamides is due to the two electron-withdrawing protecting groups on the nitrogen. By depleting the electron density in the allenic system these groups cause a reduction in the reactivity of the allenenes.

The lack of electron density in the allene system has interesting implications for using these allenamides as substrates in further reactions. With electron rich allenamides,

the lone electron pair on the nitrogen can donate into its adjacent double bond, thus raising the energy of its HOMO and LUMO. This activated double bond is then poised for 1,3-dipolar cycloadditions and inverse-demand Diels Alder reactions as the higher energy HOMO is closer in energy to the LUMO of the binding pair. This allows for a distinct reactivity pattern between the two cumulated double bonds in the allene system. An electron-neutral allenic system, such as a hydrocarbon allene, has a different reactivity pattern. In this case, the HOMOs and the LUMOs of both double bonds are relatively low in energy. Thus, these bonds are both poised to undergo Diels-Alder and other reactions where their LUMO will react with the HOMO of the binding partner. As the two allenic double bonds are electronically identical, steric considerations tend to determine the relative reactivity of the two olefins.^{3,4}

The fully-protected allenamides were unreactive towards a variety of reactions that other, more electron rich allenamides have been shown to engage in. For example, these allenamides failed to undergo a cycloaddition reaction with methyl vinyl ketone to form a pyranyl heterocycle. They also failed to produce an isoxazoline when reacted under the conditions reported by the Zecchi lab on a more electron rich allenamide.¹⁰ Finally, they were unable to undergo an acid-or thermal catalyzed rearrangement to form a 1,3-diene as reported by the Hsung lab for their allenamides.³⁴

These allenamides displayed a reactivity pattern more similar to electron neutral allenes than to other allenamides. For example, allenamide **II-2f** readily undergoes a Diels-Alder reaction to produce enamide **II-26** (Figure II-8). Using conditions similar to those reported for use with allenes,³⁵ allenamide **II-2f** can also be converted to a densely functionalized allyl boronate (**II-27**), which is poised for further transformations.^{36,37} To

our knowledge, this is the first reported example of this reaction using an allenamide (Figure II-9). Both the types and regioselectivity of these reactions indicate that these allenamides have reactivity properties more similar to electron neutral allenes than to electron-rich allenamides.

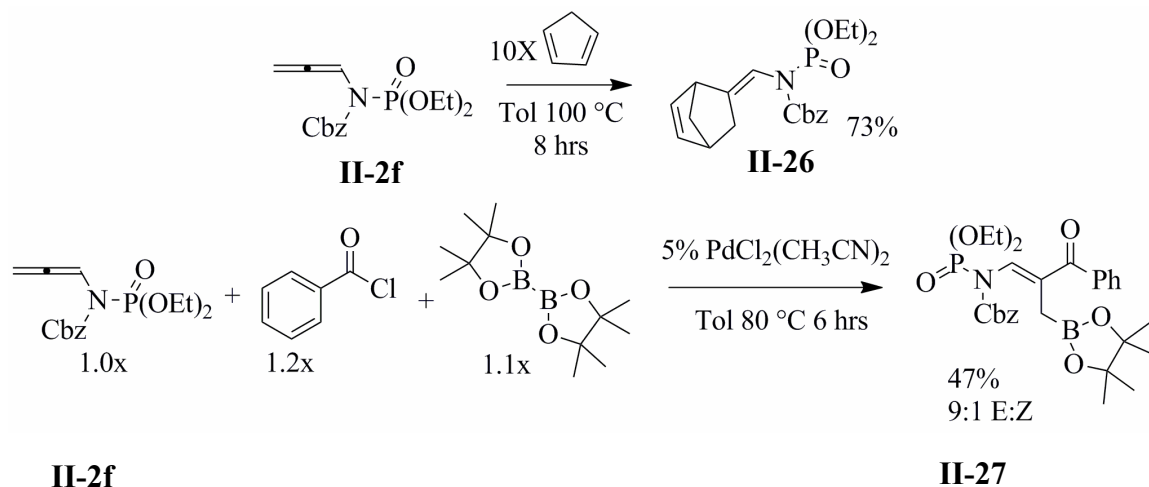


Figure II-9. Applications of fully-protected allenamides. Top: Diels-Alder reaction. Bottom: Formation of boron-substituted enamide. See section I for experimental details.

I. Experimental Methods and Data

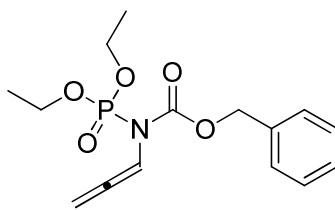
Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. DCM, Et₂O, and toluene were dried by passage through activated alumina columns and degassed by stirring under a dry nitrogen atmosphere. Purification by flash chromatography was carried out with E. Merck Silica Gel 60 (230-400 mesh) according to the procedure of Still, Kahn, and Mitra.³⁸ All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere. Cbz azide,³⁹ 3-Phenylprop-2-yn-1-ol, 3-(4-chlorophenyl)prop-2-yn-1-ol, 3-(3-methoxyphenyl)prop-2-yn-1-ol, and 3-(4-methoxyphenyl)prop-2-yn-1-ol, 3-(4-methoxyphenyl)prop-2-yn-1-ol, 4-(4-methoxyphenyl)but-3-yn-2-ol, 4-(4-

chlorophenyl)but-3-yn-2-ol, and 4-phenylbut-3-yn-2-ol were prepared according to literature procedures.⁴⁰ ¹H and ¹³C NMR spectra were recorded in CDCl₃ at 400 or 500 MHz and 125 MHz. IR spectra were measured as thin films on NaCl plates.

General procedure for allenamide preparation via rearrangement:

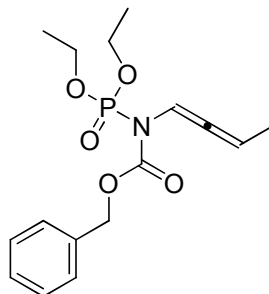
To Et₂O (10 mL) cooled in an ice/H₂O bath was added the propargylic alcohol (1.3 mmol, 1.6 eq) and Et₃N (1.0 mmol, 1.3 eq), followed by dropwise addition of diethyl chlorophosphite (1.0 mmol, 1.3 eq). The solution was allowed to stir for 20 min and the precipitated Et₃N·HCl was removed by filtration. The Et₂O was removed under vacuum and the residue dissolved in 1.7 mL DCM. Cbz azide (0.77 mmol, 1.0 eq) was added dropwise at RT. The reaction was then allowed to stir for 2 h. The DCM was removed under vacuum and the crude mixture was subjected to flash chromatography (gradient 1:5 hexanes/EtOAc to 1:2.5 hexanes/EtOAc). The phosphorimidate decomposed upon standing and was thus used immediately upon isolation.

To a solution of DCM (20 mL) was added the phosphorimidate (0.2 mmol, 1.0 eq) and 3 mol% PdCl₂(CH₃CN)₂. The reaction was stirred at room temperature with monitoring by TLC (1:1 hexanes/EtOAc) for 5-7h, at which time starting material was no longer present. The DCM was then removed under vacuum and the crude mixture was *immediately* subjected to flash chromatography (1:5 hexanes/EtOAc to 1:2.5 hexanes/EtOAc to 1:1 hexanes/EtOAc) to provide the product allenamide. Note that prolonged exposure to palladium salts leads to decomposition and epimerization.



II-2f

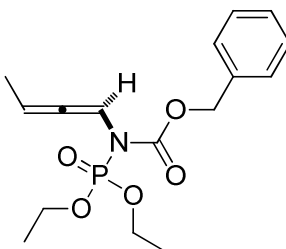
Benzyl diethoxyphosphoryl(propa-1,2-dienyl)carbamate (II-2f): Prepared via the general procedure in 76% yield (57 mg) from the corresponding phosphorimidate (82% yield) as a colorless oil. ^1H NMR: δ 1.27 (t, $J = 6.6$ Hz, 6H), 4.00-4.15 (m, 4H), 5.19 (dd, $J = 3.5, 6.3$ Hz, 2H), 5.22 (s, 2H), 6.33 (dd, $J = 6.4, 13.3$ Hz, 1H), 7.29-7.39 (m, 5H); ^{13}C NMR: δ 15.93 (d, $J = 8.6$ Hz), 64.19 (d, $J = 7.6$ Hz), 68.6, 83.6, 95.6, 135.1, 153.4, (d, $J = 9.5$ Hz), 206.4 (d, $J = 5.8$ Hz); IR (film): 2984, 1731, 1455, 1441, 1383, 1283, 1026 cm^{-1} ; HRMS (ESI) calcd for $[\text{C}_{15}\text{H}_{20}\text{NO}_5\text{P}+\text{Na}]^+$: 348.0977, found: 348.0972.



II-4

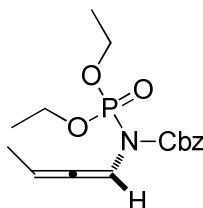
Benzyl buta-1,2-dienyl(diethoxyphosphoryl)carbamate (II-4): Prepared via the general procedure in 68% yield (44 mg) from the corresponding phosphorimidate (71% yield) as a colorless oil. ^1H NMR: δ 1.29 (t, $J = 7.5$ Hz, 6H), 1.72 (dd, $J = 2.5, 7.2$ Hz, 3H), 4.08-4.22 (m, 4H), 5.25 (s, 2H), 5.57-5.60 (m, 1H), 6.22- 6.24 (m, 1H), 7.33-7.42 (m, 5H); ^{13}C NMR δ : 14.3 (d, $J = 2.0$ Hz), 16.0 (d, $J = 3.4$ Hz), 64.1 (d, $J = 3.4$ Hz), 64.1 (d, $J = 3.4$), 68.5, 94.6 (d, $J = 3.4$ Hz), 94.9, 128.2, 128.4, 128.5, 135.3, 153.7 (d, $J = 9.6$

Hz), 202.1 (d, $J = 5.4$ Hz); IR (film): 2984, 1728, 1456, 1381, 1276, 1024 cm^{-1} ; HRMS (ESI) calcd for $[\text{C}_{16}\text{H}_{22}\text{NO}_5\text{P}+\text{Na}]^+$: 362.1133, found: 362.1126.



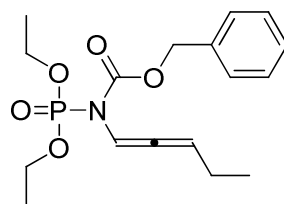
II-8

(S)-Benzyl buta-1,2-dien-1-yl(diethoxyphosphoryl)carbamate (II-8): Prepared via the general procedure in 70% yield from the corresponding phosphorimidate (56% yield) as a colorless oil. ^1H and ^{13}C data identical to that outlined for compound **II-4** [α] $_{\text{D}}^{23} = -53.7$ ($c = 1$, CH_2Cl_2). 92% ee (Chiral HPLC Chiralcel OJ column, flow rate 1 mL/min, 0.4 % hexanes in IPA, detected at 240 nm. $R_t = 16.63$ (maj), 19.58 (min), see supplementary HPLC traces.



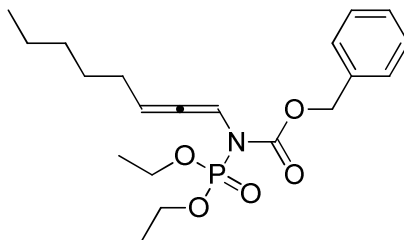
II-6

(R)-Benzyl buta-1,2-dien-1-yl(diethoxyphosphoryl)carbamate (II-6): Prepared via the general procedure in 67% yield from the corresponding phosphorimidate (72% yield) as a colorless oil. ^1H and ^{13}C data identical to that outlined for compound **II-4**. 92% ee (Chiral HPLC Chiralcel OJ column, flow rate 1 mL/min, 0.4 % hexanes in IPA, detected at 240 nm. $R_t = 16.30$ (min), 18.44 (maj), see supplementary HPLC traces.



II-10

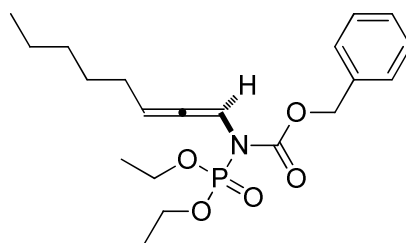
Benzyl diethoxyphosphoryl(penta-1,2-dienyl)carbamate (II-10): Prepared via the general procedure in 68% yield (54 mg) from the corresponding phosphorimidate (25% yield) as a pale yellow oil. ^1H NMR: δ 1.01 (t, $J = 7.0$ Hz, 3H), 1.27 (t, $J = 7.0$ Hz, 6H), 2.03-2.10 (m, 2H), 4.04-4.21 (m, 4H), 5.22 (s, 2H), 5.59-5.65 (m, 1H), 6.22-6.26 (m, 2H), 7.30-7.41 (m, 5H); ^{13}C NMR: δ 12.9, 16.0 (d, $J = 6.9$ Hz), 22.2 (d, $J = 1.5$ Hz), 64.0 (d, $J = 6.1$ Hz), 68.4, 95.5 (d, $J = 2.3$ Hz), 101.6, 128.1, 128.3, 128.4, 135.3, 153.7 (d, $J = 7.7$ Hz), 201.0 (d, $J = 4.6$ Hz); IR (film): 2967, 1728, 1380, 1278 1024 cm^{-1} ; HRMS (ESI) calcd for $[\text{C}_{17}\text{H}_{24}\text{NO}_5\text{P}+\text{Na}]^+$: 376.1290, found: 376.1281.



II-14

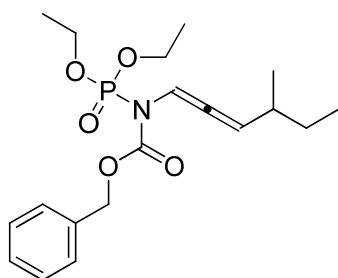
Benzyl diethoxyphosphoryl(octa-1,2-dien-1-yl)carbamate (II-14): Prepared via the general procedure in 70% yield (55 mg) from the corresponding phosphorimidate (67% yield) as a pale yellow oil. ^1H NMR: δ 0.86 (t, $J = 7.2$ Hz, 3H), 1.22-1.30 (m, 10H), 1.40 (m, 2H), 2.04 (m, 2H), 4.02-4.21 (m, 4H), 5.21 (s, 1H), 5.53-5.59 (m, 1H), 6.18-6.22 (m, 1H), 7.30-7.40 (m, 5H); ^{13}C NMR: δ 14.0, 16.0 (d, $J = 8.8$ Hz), 22.4, 28.4, 28.8 (d, $J = 1.3$ Hz), 31.3, 64.1 (d, $J = 7.3$ Hz), 68.4, 95.0 (d, $J = 3.4$ Hz), 100.1, 128.1, 128.3, 128.5, 135.3, 153.7 (d, $J = 10.1$ Hz), 201.3 (d, $J = 5.8$ Hz); IR (film): 2957, 1732, 1456, 1380,

1277, 1026 cm^{-1} ; HRMS (ESI) calcd for $[\text{C}_{20}\text{H}_{24}\text{NO}_5\text{P}+\text{Na}]^+$: 418.1759, found: 418.1755.



II-16

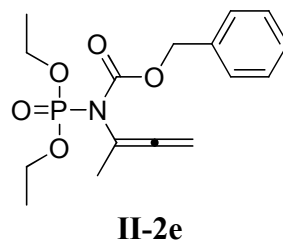
(S)-Benzyl diethoxyphosphoryl(octa-1,2-dien-1-yl)carbamate (II-16): Prepared via the general procedure in 70% yield (44 mg) from the corresponding phosphorimidate (81% yield) as a pale yellow oil. ^1H and ^{13}C data identical to that outlined for compound **II-12**. $[\alpha]_{\text{D}}^{23} = -50.9$ ($c = 1$ CH_2Cl_2). 80% ee (Chiral HPLC Chiralcel OJ column, flow rate 1 mL/min, 0.3% hexanes in IPA, detected at 254 nm. $R_t = 10.97$ (maj), 12.68 (min). The yield increased with extended reaction times but epimerization was observed. See supplementary HPLC traces.



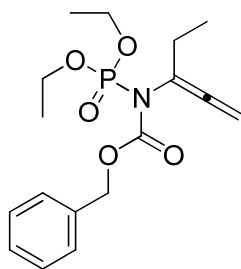
II-12

Benzyl diethoxyphosphoryl(4-methylhepta-1,2-dienyl)carbamate (II-12): Prepared via the general procedure, with the exception that the rearrangement took 12 h for depletion of starting material, in 65% yield (51 mg) from the corresponding phosphorimidate (77% yield) as a pale yellow oil. The reported product is a mixture of 2 diastereomers derived from the 2 diastereomers present as a 1:1 mixture in the starting

alcohol. ^1H NMR: δ 0.86 (t, $J = 6.8$ Hz, 3H), 1.00 (d, $J = 6.8$ Hz, 3H) 1.22-1.36 (m, 8H), 2.19-2.28 (m, 1H), 4.05-4.21 (m, 4H), 5.22 (s, 1H), 5.48-5.55 (m, 1H), 6.22 (dt, $J = 2.6$, 6.2 Hz, 1H), 7.30-7.41 (m, 5H); ^{13}C NMR: δ 14.0, 14.1, 15.9, 16.0, 19.8, 19.9, 20.2, 33.5 (d, $J = 1.4$ Hz), 33.7, 38.3, 38.9, 64.0 (d, $J = 6.3$ Hz), 64.0 (d, $J = 4.8$ Hz), 68.4, 95.5 (d, $J = 3.3$ Hz), 95.6 (d, $J = 3.4$ Hz) 105.7, 105.8, 128.1, 128.2, 128.4, 135.3, 153.7 (d, $J = 10.0$ Hz), 200.4 (d, $J = 4.6$ Hz); IR (film): 2958, 1731, 1456, 1379, 1275, 1026 cm^{-1} ; HRMS (ESI) calcd for $[\text{C}_{20}\text{H}_{24}\text{NO}_5\text{P}+\text{Na}]^+$: 418.1759, found: 418.1749.

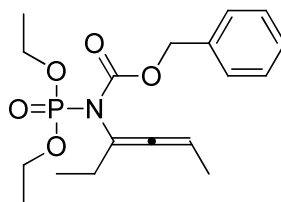


Benzyl buta-2,3-dien-2-yl(diethoxyphosphoryl)carbamate (II-2e): Prepared as stated, with the exception that the rearrangement was carried out in toluene at 100 °C for 12h on a 0.6 mmol scale (0.23 g phosphorimidate), in 58% yield (125 mg) from the corresponding phosphorimidate (75% yield) as a pale yellow oil. ^1H NMR: δ 1.28 (dt, $J = 0.8$, 7.0, 6H), 1.28 (t, $J = 7.0$ 3H), 1.99 (dt, $J = 1.2$, 3.1 Hz 3H), 4.04-4.22 (m, 4H), 5.00-5.04 (m, 2H), 5.22 (s, 2H), 7.32-7.40 (m, 5H); ^{13}C NMR: δ 16.0 (d, $J = 6.9$ Hz), 19.7, 64.0 (d, $J = 5.4$ Hz), 68.2, 80.0 (d, $J = 2.3$ Hz), 105.0, 127.9, 128.2, 128.5, 135.5, 153.7 (d, $J = 9.2$ Hz), 208.4 (d, $J = 4.7$ Hz); IR (film): 2983, 1728, 1497, 1455, 1380, 1271, 1024 cm^{-1} ; HRMS (ESI) calcd for $[\text{C}_{16}\text{H}_{22}\text{NO}_5\text{P}+\text{Na}]^+$: 362.1133, found: 362.1121.



II-18

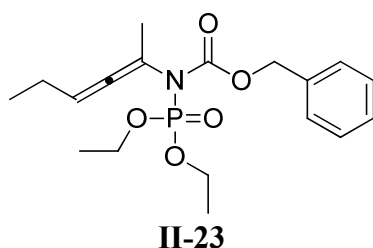
Benzyl diethoxyphosphoryl(penta-1,2-dien-3-yl)carbamate (II-18): Prepared via the general procedure, with the exception that the rearrangement was carried out in toluene at 100°C, in 58% yield (32.5 mg) from the corresponding phosphorimidate (75% yield) as a colorless oil. ^1H NMR: δ 0.99 (t, $J = 7.6$ Hz, 3H), 1.25 (dt, $J = 0.8, 6.8$ Hz, 6H), 2.18-2.28 (m, 2H) 4.00-4.18 (m, 4H), 5.08-5.11 (m, 2H), 5.18 (s, 2H), 7.30-7.40 (m, 5H); ^{13}C NMR: δ 11.0, 16.0 (d, $J = 8.8$ Hz), 25.7, 64.0 (d, $J = 7.3$ Hz), 68.2, 82.1 (d, $J = 2.4$ Hz), 111.0, 127.8, 128.2, 128.4, 135.5, 153.9 (d, $J = 11.6$ Hz), 207.7 (d, $J = 4.9$ Hz); IR (film): 2977, 1727, 1456, 1380, 1278, 1025 cm^{-1} ; HRMS (ESI) calcd for $[\text{C}_{17}\text{H}_{24}\text{NO}_5\text{P}+\text{Na}]^+$: 376.129, found: 376.1277.



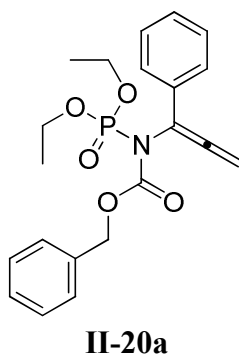
II-25

Benzyl diethoxyphosphoryl(hexa-3,4-dien-3-yl)carbamate (II-25): Prepared via the general procedure, with the exception that the rearrangement was carried out in toluene at 100°C on a 0.1 mmol scale (41 mg phosphorimidate), in 60% yield (25 mg) from the corresponding phosphorimidate (48% yield) as a yellow oil. ^1H NMR: δ 0.98 (t, $J = 7.4$ Hz, 3H), 1.28 (dt, $J = 0.8, 7.2$ Hz, 6H), 1.69 (d, $J = 5.4$ Hz, 3H), 2.21-2.27 (m, 2H), 4.03-4.21 (m, 4H), 5.37 (dd, $J = 12.5, 19.5$ Hz, 2H), 5.46-5.52 (m, 1H), 7.30-7.38 (m, 5H); ^{13}C

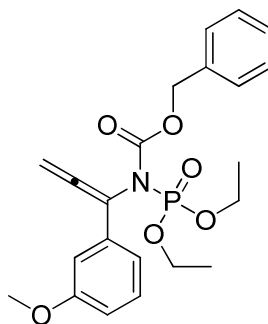
NMR: δ 11.1, 14.1 (d, $J = 2.9$ Hz), 16.0 (d, $J = 7.6$ Hz), 26.2, 63.8 (d, $J = 3.8$ Hz), 63.9 (d, $J = 3.9$ Hz), 68.0, 93.4 (d, $J = 2.9$ Hz), 110.0, 127.8, 128.1, 128.4, 135.6, 154.0, 203.0 (d, $J = 4.9$ Hz); IR (film): 2978, 1726, 1456, 1378, 1272, 1024 cm^{-1} ; HRMS (ESI) calcd for $[\text{C}_{18}\text{H}_{26}\text{NO}_5\text{P}+\text{Na}]^+$: 390.1446, found: 390.1444.



Benzyl diethoxyphosphoryl(hexa-2,3-dien-2-yl)carbamate (II-23): Prepared via the general procedure, with the exception that the rearrangement was carried out in toluene at 100°C, in 58% yield (42.5 mg) from the corresponding phosphorimidate (50% yield) as a yellow oil. ^1H NMR: δ 0.99 (t, $J = 7.6$ Hz, 3H), 1.27 (t, $J = 7.2$ Hz, 6H), 1.97 (dd, $J = 1.2, 2.8$ Hz, 3H), 2.03 (dq, $J = 2.4, 8.6$ Hz, 2H), 4.04-4.19 (m, 4H), 5.20 (s, 2H), 5.40-5.48 (m, 1H), 7.30-7.40 (m, 5H); ^{13}C NMR: δ 13.1, 16.0 (d $J = 9.1$ Hz), 20.3, 22.1 (d, $J = 3.3$ Hz), 63.5 (d, $J = 5.1$ Hz), 68.1, 98.2 (d, $J = 2.4$ Hz), 105.0 (d, $J = 1.0$ Hz), 128.0, 128.2, 128.4, 135.5, 153.9 (d, $J = 11.9$ Hz), 202.7 (d, $J = 5.3$ Hz); IR (film): 2980, 1728, 1456, 1380, 1277, 1026 cm^{-1} ; HRMS (ESI) calcd for $[\text{C}_{18}\text{H}_{26}\text{NO}_5\text{P}+\text{Na}]^+$: 390.1446, found: 390.1445.



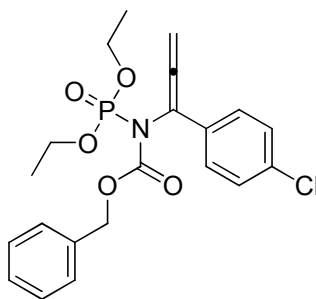
Benzyl diethoxyphosphoryl(1-phenylpropa-1,2-dienyl)carbamate (II-20a): Prepared via the general procedure, with the exception that the rearrangement was carried out in toluene at 100°C on a 0.6 mmol scale, in 41% yield (0.103 g) from the corresponding phosphorimidate (68% yield) as an oil. ¹H NMR: δ 1.25 (t, *J* = 7.0 Hz, 3H), 1.25 (t, *J* = 7.0 Hz, 3H), 4.05-4.13 (m, 2H), 4.15-4.25 (m, 2H), 5.20 (s, 2H), 5.49 (d, *J* = 5.8 Hz, 2H), 7.20-7.25 (m, 3H), 7.27-7.30 (m, 3H), 7.33 (d, *J* = 7.8 Hz, 2H), 7.39 (d, *J* = 7.4 Hz, 2H); ¹³C NMR: δ 16.0 (d, *J* = 6.9 Hz), 64.5 (d, *J* = 6.1 Hz), 68.4, (d, *J* = 1.9 Hz), 83.9 (d, *J* = 2.4 Hz), 110.6, 125.1, 127.5, 128.1, 128.3, 128.5, 133.8 (d, *J* = 1.2 Hz), 135.3, 154.0 (d, *J* = 10.0 Hz), 209.2 (d, *J* = 3.8 Hz); IR (film): 2982, 1731, 1495, 1491, 1380, 1272, 1024 cm⁻¹; HRMS (ESI) calcd for [C₂₁H₂₄NO₅P+Na]⁺: 424.1290, found: 424.1277.



II-20c

Benzyl diethoxyphosphoryl(1-(3-methoxyphenyl)propa-1,2-dienyl)carbamate (II-20c): Prepared via the general procedure, with the exception that the rearrangement was carried out in toluene at 100°C on a 0.6 mmol scale (0.241 g phosphorimidate), in 25% yield (72 mg) from the corresponding phosphorimidate (32% yield) as an oil. ¹H NMR: δ 1.24 (t, *J* = 6.8 Hz, 6H), 3.73 (s, 1H), 4.08-4.13 (m, 2H), 4.13-4.21 (m, 2H), 5.18 (s, 2H), 5.47 (d, *J* = 5.6 Hz 2H), 6.76 (d *J* = 8.4 Hz, 1H), 6.91 (s, 1H), 6.97 (d, *J* = 7.6 Hz 1H), 7.19-7.23 (m, 3H), 7.24-7.28 (m, 2H); ¹³C NMR: δ 16.0 (d, *J* = 8.25 Hz), 55.1, 64.6 (d, *J*

= 6.8 Hz), 68.4, 83.9 (d, $J = 2.9$ Hz), 110.4, 110.5, 115.4, 117.6, 127.8, 128.1, 128.4, 129.5, 135.4, 154.1, 159.8, 209.3 (d, $J = 4.9$ Hz), IR (film): 2980, 1732, 1456, 1379, 1276, 1024 cm^{-1} ; HRMS (ESI) calcd for $[\text{C}_{22}\text{H}_{26}\text{NO}_6\text{P}+\text{Na}]^+$: 454.1395, found: 454.1406.

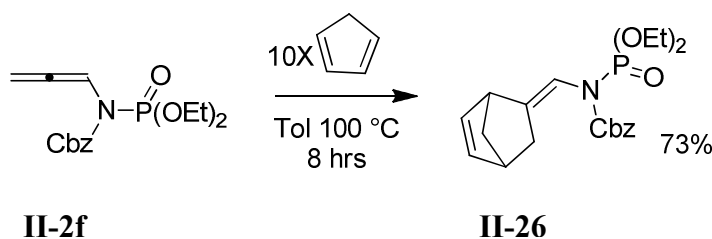


II-20b

Benzyl 1-(4-chlorophenyl)propa-1,2-dienyl(diethoxyphosphoryl)carbamate (II-20b):

Prepared via the general procedure, with the exception that the rearrangement was carried out in toluene at 100°C on a 0.3 mmol scale (0.135 g phosphorimidate), in 27% yield (37 mg) from the corresponding phosphorimidate (77% yield) as an oil. ^1H NMR: δ 1.25 (t, $J = 7.2$ Hz, 6H), 4.01-4.14 (m, 2H), 4.14-4.24 (m, 2H), 5.19 (s, 2H), 5.50 (d, $J = 5.6$ Hz, 2H), 7.21-7.25 (m, 2H), 7.23-7.32 (m, 7H); ^{13}C NMR δ : 16.0 (d, $J = 7.0$ Hz), 64.6 (d, $J = 5.8$ Hz), 68.5, 84.3 (d, $J = 2.3$ Hz), 109.9, 126.3, 127.8, 128.4, 128.7, 132.5, 133.0, 133.3, 135.2, 153.9 (d, $J = 9.7$ Hz), 209.1 (d, $J = 3.9$ Hz); IR (film): 2981, 1732, 1491, 1380, 1277, 1025 cm^{-1} ; HRMS (ESI) calcd for $[\text{C}_{21}\text{H}_{23}\text{ClNO}_5\text{P}+\text{Na}]^+$: 458.0900, found: 458.0886.

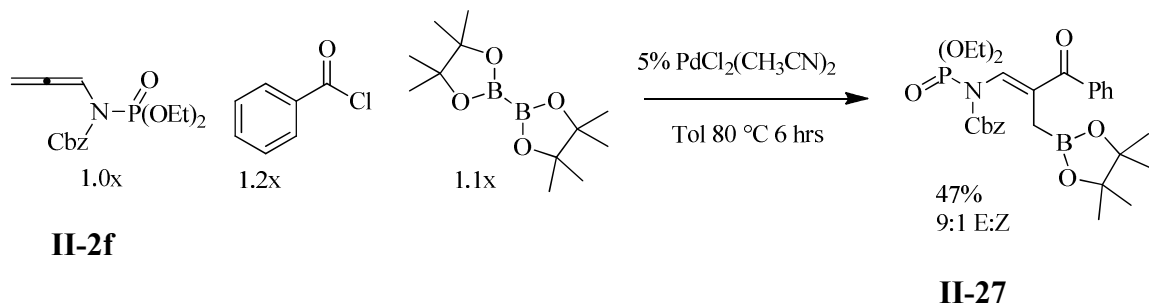
Procedure for Diels-Alder reaction:



(E)-benzyl (bicyclo[2.2.1]hept-5-en-2-ylidenemethyl)(diethoxyphosphoryl)carbamate

(II-26): Allenamide (**II-2f**) (70 mg, 0.22 mmol) was combined with freshly cracked cyclopentadiene (0.18 mL, 2.2 mmol) in toluene (0.4 mL) under N₂. The reaction was sealed and heated to 100° C for 8 hours, after which the toluene was removed under reduced pressure and the crude reaction mixture was purified by flash chromatography (25-50% ethyl acetate in hexanes) to yield **II-26** as a colorless oil (63 mg, 0.16 mmol, 73%). Configuration determined by 1D NOESY. ¹H NMR: δ 1.24 (dt, *J* = 0.4, 6.8 Hz, 3H), 1.25 (dt, *J* = 0.8, 6.8 Hz, 3H), 1.41 (d, *J* = 8.4 Hz, 1H), 1.58 (d, *J* = 10 Hz, 1H), 1.71-1.75 (m, 1H), 2.16-2.20 (m, 1H), 2.92 (s, 1H), 3.27 (s, 1H), 4.00-4.16 (m, 4H), 5.12-5.17 (m, 2H), 5.95 (d, *J* = 2.5 Hz, 1H), 5.99-6.00 (m, 1H), 6.12-6.13 (m, 1H), 7.26-7.32 (m, 5H); ¹³C NMR: δ 16.03 (d, *J* = 2.3 Hz), 16.10 (d, *J* = 2.7), 31.93, 41.42, 48.36, 50.36, 64.02-64.13 (m), 68.08, 114.1, 127.9, 128.0, 128.2, 128.4, 133.3 (d, *J* = 1.9), 135.5, 137.7, 146.8 (d, *J* = 4.67 Hz), 154.1 (d, *J* = 10 Hz), IR (film): 3000, 1731, 1455, 1441, 1383, 1283, 1026 cm⁻¹; HRMS (ESI) calcd for [C₂₀H₂₆NO₅P+Na]⁺: 414.1446, found: 414.1444.

Procedure for formation of boron-substituted enamide:



(E)-benzyl diethoxyphosphoryl(3-oxo-3-phenyl-2-((4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)methyl)prop-1-en-1-yl)carbamate (II-27): To allenamide (**II-2f**) (35 mg, 0.107 mmol) was added bis(pinacolato)diboron (31 mg, 0.12 mmol) and bis(acetonitrile)dichloropalladium(II) (1.4 mg, 0.0055 mmol), and a stir bar. The flask was evacuated and purged with N₂ 3 times, followed by the addition of toluene (0.44 mL) and benzoyl chloride (distilled from calcium chloride, 15 μL, 0.13 mmol). The reaction was heated to 80° C under N₂ for 6 hrs after which the solvent was removed under reduced pressure and the crude reaction was purified by flash chromatography (25-50% ethyl acetate in hexanes) to give **II-27** as a colorless oil (28 mg, 0.050 mmol, 47%) as an inseparable isomeric mixture with (E)-**II-27** as the major diastereomer (dr 9:1 as determined by ¹H NMR integration of the signals at 6.51 and 6.58 ppm). Configuration of major isomer determined by 1D NOESY. ¹H NMR: δ 1.18 (s, 9H), 1.24 (dt, *J* = 1, 6 Hz, 6H), 1.97 (s, 2H), 4.06-4.20 (m, 4H), 5.21 (s, 2H), 6.51-6.52 (m, 1H), 6.58 (m, 1H, *Z* isomer), 7.30-7.39 (m, 7H), 7.44-7.48 (m, 2H), 7.71-7.73 (m, 2H), 8.04-8.06 (m, 2H, *Z* isomer). ¹³C NMR: δ 13.48 (d, *J* = 6.7 Hz), 16.77, 22.21, 62.08 (d, *J* = 6.23 Hz), 66.26, 80.86, 125.5, 125.7, 125.9, 126.0, 126.0, 127.2, 127.6, 129.4, 131.8, 132.5, 135.5, 137.6 (d, *J* = 4.8 Hz), 150.3 (d, *J* = 8.2 Hz), 194.8, IR (film): 2923, 1717, 1685, 1279, 1208, 1142, 1026 cm⁻¹; HRMS (ESI) calcd for [C₂₈H₃₇BNO₈P+Na]⁺: 580.2248, found: 580.2260.

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Chapter III

Utilizing p300/CBP Ligands to Disrupt Transcription of Genes Governing Pluripotency and Self-Renewal

A. Abstract

The cancer initiating cell (CIC) hypothesis is based on the idea that a subpopulation of cancer cells are able to initiate tumorigenesis by undergoing self-renewal and differentiation. CICs have been found in several types of cancers including cancers of the: breast, brain, colon, head and neck, pancreas, skin, liver, lung and ovaries and are often associated with poor patient prognosis. These cells share many biochemical pathways with embryonic stem cells (ESCs) including a reliance on key transcription factors (Nanog, Sox2 and Oct4) that govern pluripotency and self-renewal. Evidence obtained in ESCs indicates that the co-factor p300 plays a role in Nanog driven gene expression; however, no direct evidence has been obtained for this role in CICs. We hypothesize that p300 and/or CBP are required for Nanog-driven gene expression in CICs and that disrupting their function will lead to a decrease the ability of CICs to maintain pluripotency and self renew. Here, the roles of CBP and p300 in Nanog-driven gene expression are examined through a combination of RNAi knockdowns of CBP and p300 and through the use of synthetic ligands that target specific domains of p300. It was found that p300 but not CBP was necessary for Nanog- driven gene expression in a

luciferase reporter context. Additionally, it was found that two synthetic ligands that target the KIX and CH1 domains of p300 (an amphipathic isoxazolidine and a hydrogen bond surrogate respectively) inhibit Nanog-driven gene expression. It was also discovered that the amphipathic isoxazolidine inhibits CIC viability preferentially to bulk tumor cells. These studies are consistent with a model in which p300 is a key protein required for Nanog-driven gene expression and that inhibiting its function may provide insight into how pluripotency and self-renewal are governed in CICs.

B. Introduction

B.1. Cancer Initiating Cell (CIC) Model

B.1.A. Origins of the Model

The cancer initiating cell (CIC) hypothesis (also referred to as the cancer stem cell (CSC) hypothesis) was first proposed in the 1990s by Dick and co-workers. In these studies, it was found that only a small subset of acute myeloid leukemia (AML) cells were able to initiate human AML in non-obese diabetic/severely combined immunodeficient (NOD/SCID) mice. These cells were positive for cell surface markers that are associated with normal stem cells but not with differentiated adult cells ($CD34^+CD38^-$ vs $CD34^+CD38^+$). It was therefore postulated that leukemia-initiating transformation and progression originated from an aberrant undifferentiated cell which then established a hierarchical organization within the cancer whereby only undifferentiated, pluripotent cells had the capacity to extensively proliferate and self renew (Figure III-1).^{1,2} Subsequent to this work, CICs were identified in a variety of

solid tumor cancers including cancers of the: breast, brain, colon, head and neck, pancreas, skin, liver, lung and ovaries.³

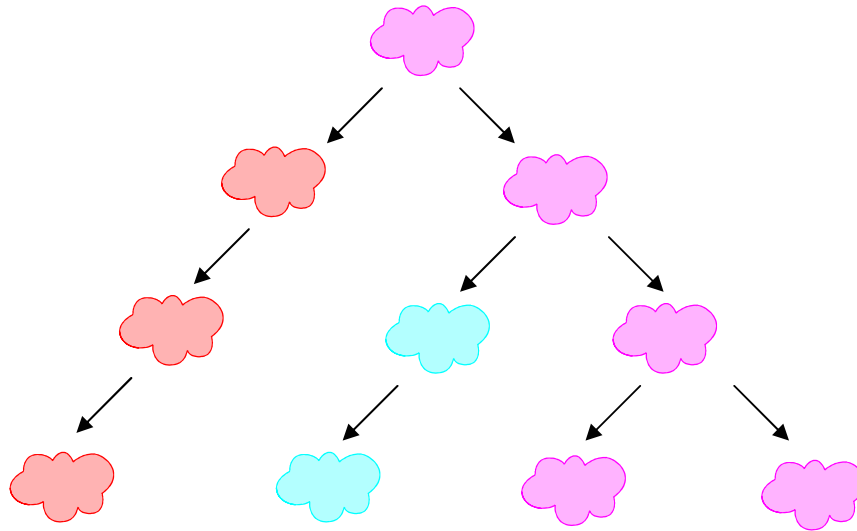


Figure III-1. Cancer initiating cell model. In this model, only cancer initiating cells (purple) are able to proliferate extensively to form new cells. The daughter cells can either become differentiated into bulk tumor cells (blue and red) or be maintained in a pluripotent state as a new generation of cancer initiating cells.

B.1.B Therapeutic Implications of the CIC Model

Despite advances in cancer treatments, the prognoses of many major cancers remain poor. This effect has traditionally been attributed to incomplete elimination of tumor cells, or the re-awakening of “dormant” tumor cells after treatment has ended. However, the CIC hypothesis provides a different theory for how tumors can avoid elimination from traditional chemotherapeutics; namely that by virtue of having distinct cellular processes, CICs are not affected by therapeutics targeted against bulk tumor cells.⁴ As traditional chemotherapeutics are targeted against bulk tumor cells, they may cause an initial decrease in tumor size; however, the unaffected CICs are able to re-grow the original tumor, leading to a re-emergence of the disease (Figure III-2). Understanding

the processes that govern pluripotency and self-renewal in CICs is an essential step in the development of therapeutics designed to target this cell population.

CICs from a variety of tumors have been shown to be immune to traditional chemotherapeutics and ionizing radiation.⁵ In a recent study by Donahoe and co-workers, human ovarian cancers containing a mixed population of bulk tumor and cancer initiating cells were treated with various chemotherapeutic agents.⁶ The relative populations of both types of cells were then analyzed using fluorescence activated cell sorting (FACS). It was shown that doxorubicin, cisplatin and paclitaxel—all traditional chemotherapeutic agents—reduced the relative population of bulk tumor cells while having an insignificant effect on the CIC population.⁶

Additionally, in a study of primary breast cancers, Song and co-workers found that 74% of cells from patients who had undergone chemotherapy displayed stem cell surface markers, whereas only 9% of cells from patients who had not undergone chemotherapy displayed these markers.⁷ Similar findings have been reported in pancreatic cancer cells.⁸ Evidence such as this indicates that CICs are likely to play an important role in cancer initiation and resistance to chemotherapeutics. As CICs rely on a set of transcription factors that govern pluripotency and self-renewal that are largely inactive in differentiated cells, it is likely that by targeting the function of these proteins, therapeutics that specifically target CICs can be developed.⁹

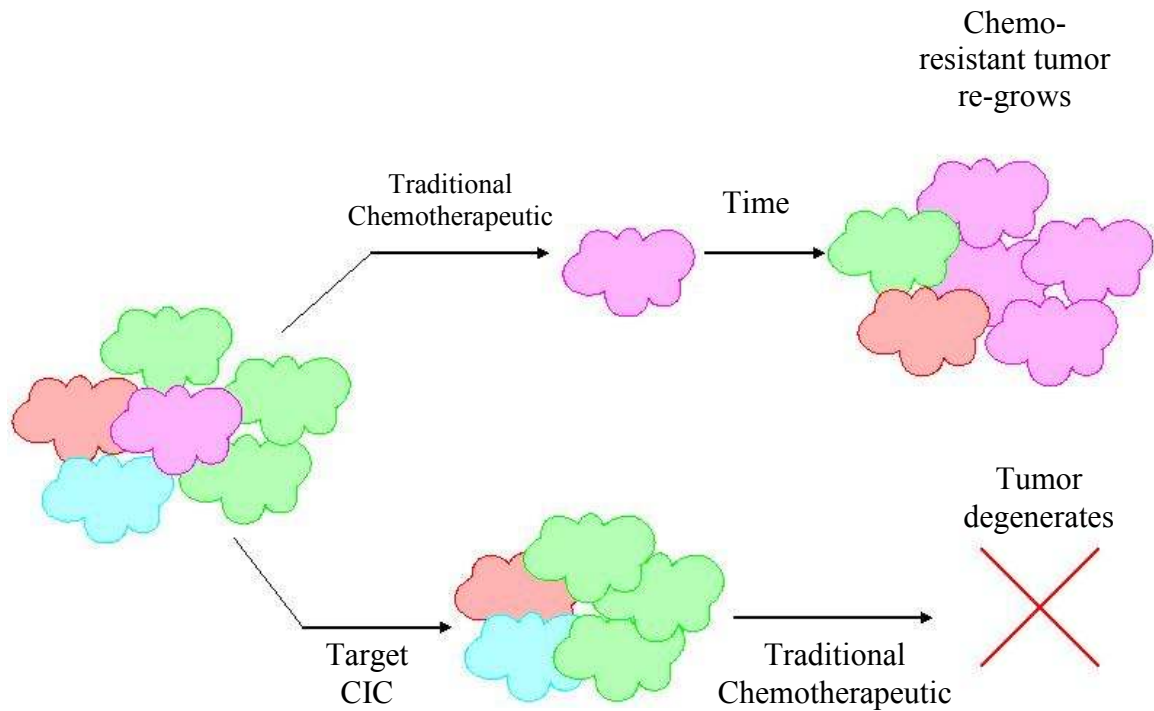


Figure III-2. Therapeutic paradigm in light of the CIC theory. A tumor consisting of CICs (purple) and bulk tumor cells (red, blue, green) is treated by two different approaches. *Top:* Traditional chemotherapeutics are applied to the tumor, resulting in death of the bulk tumor cells and temporary decrease in tumor size; however, the CIC is not affected by treatment. After time, the CIC is able to re-grow the original tumor, resulting in a relatively higher percentage of CIC cells versus bulk tumor cells. *Bottom:* The tumor is treated with an agent capable of eliminating CICs. The remaining bulk tumor cells can then be eradicated with traditional chemotherapeutics resulting in degeneration of the tumor.

B.2 Transcription in CICs

One difference between differentiated and pluripotent cells is the presence of key transcription factors that help maintain pluripotency and self-renewal. It is likely that defining the binding partners within the transcriptional machinery (the proteins

responsible for bridging DNA-bound transcriptional activators with RNA-Polymerase II) for these activators and elucidating the mechanisms by which these partners interact with the transcription factors will give insight into how pluripotency and self-renewal are maintained.

Studies comparing gene expression profiles of embryonic stem cells (ESCs) and CICs reveal that there are many similarities between the two populations including a reliance on a c-Myc-centered regulatory network, and the expression of several transcription factors.^{10,11,12,13} Three of these transcription activators, Oct4, Nanog and Sox2, have been found in ESCs to govern pluripotency and self-renewal and it is postulated that they may fill the same roles in CICs.¹⁰ This finding indicates that studies of transcription factors carried out in ESCs may also hold true in CICs and therefore give insight into mechanisms for disrupting pluripotency and self-renewal in CICs.

B.2.A. The Roles of Nanog, Sox2 and Oct4 in ESCs

In ESCs, it has been found that Oct4, Nanog and Sox2 are key transcription factors involved in the maintenance of pluripotency and self-renewal.¹⁴ A delicate balance of Oct4 levels is necessary for maintenance of pluripotency, with both down-regulation and overexpression of Oct4 in ESCs resulting in differentiation.¹⁵ Similarly, knockdown of Nanog in ESCs causes the cells to lose their ability to self-renew.¹⁶

Mounting evidence suggests that these three factors often work in tandem to synergistically control the expression of genes associated with pluripotency and self-

renewal.¹⁴ A substantial fraction of these genes were found to have binding sites for all three of these transcription factors in close proximity in their promoters; indicating that these factors may work as a complex to recruit additional members of the transcriptional machinery.^{17,18,19} Finally, it has been noted that these factors work together to auto-regulate expression of their own genes, which may help maintain the optimal level of these proteins needed to retain pluripotency.²⁰ As the mechanisms by which these proteins regulate transcription is not fully understood, elucidating the interactions between these proteins and the co-factors they bind will provide a greater understanding of how these factors maintain pluripotency and self-renewal, and may provide insight into how induce differentiation in CICs.¹⁴

B.3. The Role of CBP/p300 in ESCs

The Creb-binding protein (CBP) and its paralog p300 are large, multi-domain proteins that have acetyltransferase activity and can serve as bridges between DNA-bound transcriptional activators and the transcriptional machinery.^{21,22} While CBP and p300 are highly homologous proteins, they have been shown to have distinct roles in several cellular processes. CBP and p300 have been shown to play a role in the transcription of several different genes in differentiated adult cells.^{21,22} Evidence is emerging that these co-factors may also play a critical role in the transcription of genes associated with pluripotency and self-renewal in stem cell models.

A study conducted in embryonal carcinoma cells (a stem cell model system) by Kawasaki and co-workers provided early evidence for distinct roles of CBP and p300 in

maintenance of pluripotency and self-renewal. In this study, hammerhead ribozymes were designed to specifically knock out CBP or p300 mRNA. It was discovered that cells expressing the p300 specific ribozyme became immune to retinoic-acid induced differentiation; however no such effect was observed in the cells expressing CBP specific ribozymes. Additionally, it was found that both CBP and p300 were required for retinoic-acid induced apoptosis.²³ These findings, along with other findings from hematopoietic stem cells, indicate that p300 and CBP play distinct and important roles in maintaining pluripotency and self-renewal in stem cell systems.²⁴

The mechanisms by which p300 and CBP affect differentiation are not well understood. However, there is evidence that these proteins may play a role in Oct4, Nanog and Sox2-mediated transcription (Figure III-3). Chen and co-workers used chromatin immunoprecipitation coupled with ultra-high-throughput DNA sequencing (ChIP-seq) in ESCs to map the locations of Oct4, Nanog and Sox2, and the location of p300. They found that p300 co-occurred with Oct4-Nanog-Sox2 binding clusters, suggesting that one or more of these factors were capable of recruiting p300. Accordingly, they found that when RNA interference (RNAi) targeted against any of these three factors was introduced to the cell, there was a reduction in p300 binding at these sites.²⁵ Additionally, work carried out in mouse ESCs suggests that p300 aids in Nanog-driven gene expression by acetylating a distal regulatory region of the Nanog promoter.²⁶

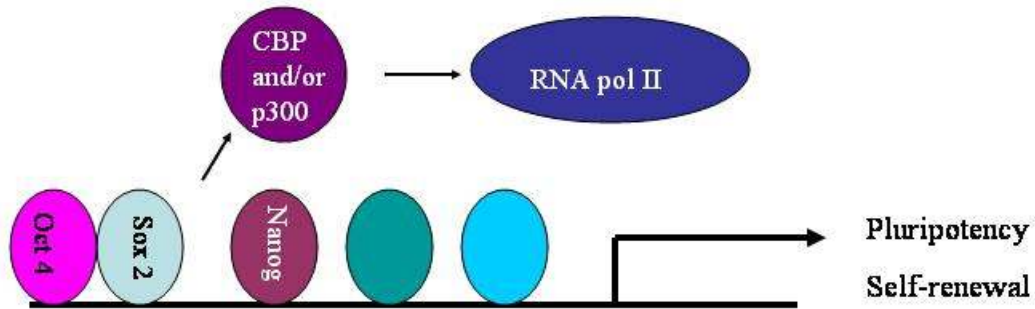


Figure III-3. Schematic of the role of CBP and/or p300 in maintaining pluripotency and self-renewal. Many genes associated with pluripotency and self-renewal have binding sites for Oct4, Sox2 and Nanog upstream of their promoters. Recruitment of CBP and or p300 by these factors is a necessary step for the transcription involved in genes governing pluripotency and self-renewal.

A reliance on p300 for Sox2-mediated transcription has been demonstrated in the Rizzino laboratories. Using chimeric fusion proteins comprised of the Gal4 DBD and the Sox2 TAD, they examined the effects of p300 on a Sox2-driven reporter system. An increased level of transcription was observed when p300 was overexpressed in the cell. Conversely, they found that when a protein capable of sequestering endogenous p300 (E1A) was introduced, transcription levels decreased in a dose dependant manner.²⁷ Activator by-pass experiments performed in a follow up study in which p300 was fused directly to the Gal4 DBD resulted in increased levels of transcription in a similar reporter system.²⁸ Additionally, they found that the activity observed in the activator by-pass experiments could be decreased when the Sox2 TAD was overexpressed in these cells.²⁹ These experiments indicate that p300 serves as a bridging co-activator in Sox2-mediated transcription.

B.4. Small Molecules that Target CBP/p300

As CBP and p300 are important co-factors in regulation transcription of differentiated cells, several small molecule and peptidomimetic scaffolds have been developed that can inhibit the function of specific domains of these molecules. These scaffolds can be used to study the roles of the specific domains of CBP and p300 in a cellular context.^{30,31,32}

Amphipathic isoxazolidines

An amphipathic isoxazolidine (444) with the ability to modulate transcription was discovered by the Mapp laboratories (Figure III-4).³³ NMR, photocrosslinking and *in cellulo* studies indicate that this molecule acts in part by binding to the KIX domain of CBP.^{34,35,36} 444 was originally attached to a DNA-binding domain and used to activate transcription.^{33,34} It was discovered that when not attached to a DNA binding domain, 444 could inhibit transcription.³⁶ Specifically, 444 was shown to elicit a dose-dependent decrease in transcription driven by two natural ligands of the p300/CBP KIX domains, MLL and c-JUN.³⁶ Data obtained by the Mapp laboratories indicates that 444 can elicit biological responses consistent with binding the KIX domain of CBP in a variety of adult human cell lines (HeLa, HEK 293T and MCF7). Additional unpublished data indicates that 444 generally does not affect cellular viability, even at high micro-molar doses. For example, 444 has no effect on cellular viability of MCF7 cells at concentrations as high as 400 μ M. These features make 444 an attractive small molecule probe for studying cellular functions that rely on the KIX domain of CBP.

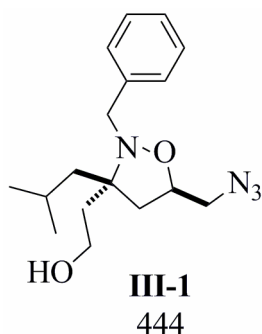


Figure III-4. Structure of amphipathic isoxazolidine (444).

Hydrogen Bond Surrogates (HBS)

A second scaffold that can be used for targeting specific domains of CBP/p300 is the hydrogen bond surrogate (HBS) (Figure III-5). The HBS scaffold overcomes the energetic demands necessary for amino acids to form an α -helix by covalently linking the i and $i + 4^{\text{th}}$ residue of a peptide sequence such that a preorganized α -turn is formed.³⁷

Recently, the Arora laboratories developed an HBS designed to target the CH1 domain of p300. This HBS, which was modeled on the activation domain of an endogenous CH1 ligand (HIF-1 α), has been shown to inhibit the transcription of genes controlled by HIF-1 α *in cellulo*.³⁸

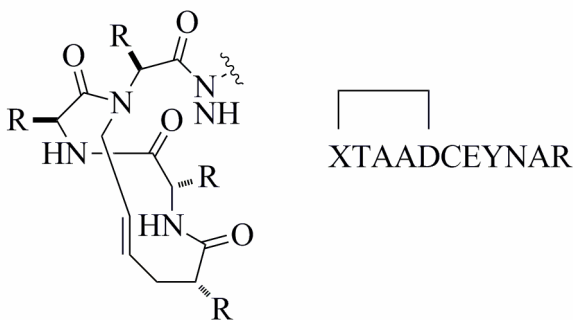


Figure III-5. Structure of a hydrogen bond surrogate (HBS). The sequence of the amino acid residues included in the HBS that binds the CH1 domain of p300/CBP is given to the left. X denotes a pentenoic acid residue.

The HBS is a very versatile scaffold that has also been used to mimic a variety of other alpha helices.^{39,40} The targets of these molecules can be easily tuned by changing the amino acid sequence to reflect that of a native ligand. It is therefore postulated that HBSs can be designed to bind to each domain of p300. These molecules can then be employed to study the effects each of these domains has on the maintenance of pluripotency and self-renewal in CICs.

B.5. Scope of This Study

The CIC model provides an intriguing hypothesis for cancer initiation, metastasis and development of chemotherapeutically resistant cancers. While little is known directly about the biochemical pathways that control pluripotency and self-renewal in CICs, parallels between CICs and ESCs can be drawn. The transcription factors Nanog, Sox2 and Oct4 have been established in ESCs as key proteins responsible for regulating self-renewal and pluripotency. There is emerging evidence suggesting that the co-factors p300 and/or CBP may play a role transcription mediated by these factors. By using exogenous ligands known to disrupt the binding capabilities of specific domains of p300 and/or CBP as well as other biochemical techniques, the functions of these two proteins in CICs can be elucidated.

This study in part aims to determine if CBP and p300 play a role in Nanog-driven gene expression. Additionally, it is aimed at determining if small molecules that modulate CBP/p300 function are able to alter the ability of CICs to self-renew. For initial experiments, we chose to examine the role of CBP and p300 in Nanog-driven

transcription as Nanog is known to be overexpressed in a variety of cancers, and knockdown of Nanog has been shown to cause a loss of self-renewal capabilities in CICs.^{41,42} As no definitive evidence for involvement of CBP or p300 in Nanog-driven gene expression has been obtained, we first wanted to examine if these proteins were indeed involved in this process. To accomplish this, RNAi that is specific for CBP or p300 was used in combination with a dual luciferase reporter assay to determine if either of these proteins has an effect on Nanog-driven gene expression. Additionally, we wanted to determine which domain(s) of CBP and/or p300 were important in this process by using synthetic probes designed to target specific domains of CBP and p300.

Finally, we wanted to determine if interfering with Nanog-driven gene expression would be sufficient to elicit a reduction in self-renewal. As the expression of genes regulating self-renewal in ESCs is tightly controlled with several redundancies in place, it is possible that interfering with Nanog-driven transcription would be insufficient to block CICs' ability to self-renew. As such, the synthetic probes found to interfere with Nanog-driven gene expression were dosed into CICs and the effects on cellular viability were observed as a measure of self-renewal capabilities.

C. RNAi Studies of Dependence of CBP and p300 on Nanog-Driven Gene Expression

Nanog has been shown to contain two transactivation domains, the CD2 and WR domains (Figure III-6).⁴³ In order to better understand the contributions each of these separate domains would have on recruitment of p300 and CBP, plasmids encoding a fusion protein consisting of the DBD of the yeast transcription factor Gal4 and the each

of the Nanog TADs were made by a rotation student in the Mapp laboratories, Victoria Assimon, for use in a luciferase reporter assay. These fusion proteins were then used in combination with short hairpin RNAs (shRNAs) that had been shown to selectively target either CBP or p300 to determine their reliance on CBP and p300 in driving luciferase expression.⁴⁴

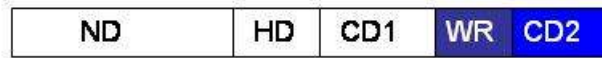


Figure III-6. Schematic of Nanog. The transcriptional activation domains, WR and CD2 are highlighted in blues.

A significant decrease ($p < 0.01$) in Nanog CD2-driven luciferase activity was observed upon treatment with shRNA targeting p300 relative to treatment with a scramble shRNA. However, a decrease in Nanog CD2-driven luciferase activity was not observed upon treatment with shRNA targeting CBP. This indicates that Nanog CD2-driven gene expression is reliant on p300 and not CBP in this system (Figure III-7). The Nanog-WR domain did not activate to sufficient levels in our system to warrant further study (an average of 3-fold activation was observed).

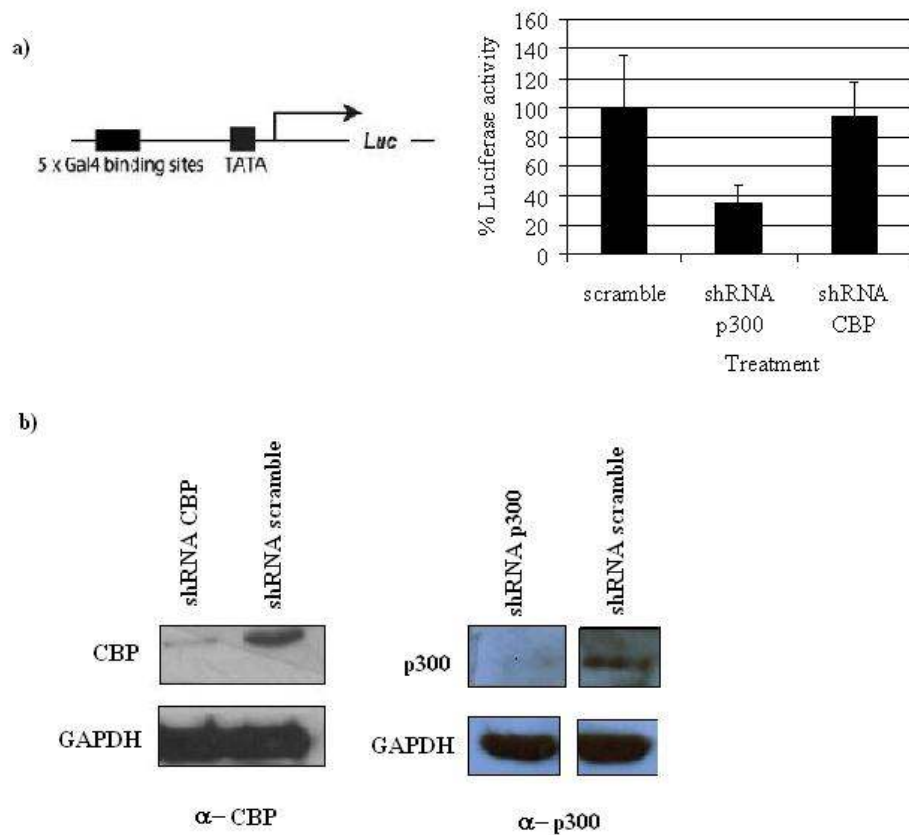


Figure III-7. Nanog CD2-driven gene expression is dependant on p300 but not CBP. a) Effects on Nanog CD2-driven luciferase expression by shRNA targeting p300 and CBP. Briefly, HEK 293T cells were plated in 24 well format (5×10^4 cells/well) and allowed to adhere for 24 hours. Cells were then transfected with a plasmid expressing Gal4 DBD-Nanog CD2, a second plasmid bearing five Gal4-binding sites upstream of a firefly luciferase gene, a third plasmid expressing *Renilla* luciferase as a transfection control and a fourth plasmid expressing an shRNA scramble sequence, or shRNA targeting either CBP or p300 in a solution of Opti-Mem. After 4 hours, the transfection solution was replaced with DMEM supplemented with 10% FBS and allowed to incubate for 48 hours before being read for luciferase activity using the Promega Dual Luciferase Assay according to manufacturer's instructions. Fold activities were then determined by dividing the firefly luciferase activity by that of the *Renilla* luciferase. These values were then normalized so that the wells treated with scramble shRNA represent 100% activity. The values reported are the median of at least 7 trials with the indicated error representing the standard deviation. b) Decrease in amount of CBP and p300 protein upon treatment with shRNA. Treated cells were lysed for 20 minutes at 0 °C with Passive Lysis Buffer (Promega) and a protease inhibitor cocktail (Invitrogen). Lysates were centrifuged for 10 minutes at 14,000 rpm to remove membrane fragments. Supernatant was subjected to PAGE separation, followed by transference to PDVF membrane. The membranes were then subjected to Western-blot analysis using (where appropriate) anti-CBP antibody (1:500), anti-p300 antibody (1:500) or anti-GAPDH antibody (1:1000) and a secondary antibody anti-mouse (1:500), anti-rabbit (1:500) or anti-mouse (1:1000) respectively.

The results obtained from the reporter system provide insight into the reliance of p300 and CBP on transcription mediated by the Nanog CD2 domain. It is possible, however, that p300 and CBP may not be required for Nanog-driven transcription in a native context. It is therefore necessary to determine the effects of perturbing the function of p300 and CBP in a native context. To this end, one of my co-workers in the Mapp laboratories, Conor Doss, transfected embryonal carcinoma cells with shRNA targeting p300 and used qPCR to measure the levels of mRNA of *NANOG* and *OCT4* upon knockdown of p300 (Figure III-8). A significant decrease ($p < 0.05$) in *NANOG* mRNA levels was observed, indicating that p300 may play a role in the transcription of the *NANOG* gene. As Nanog is known to auto-regulate, this may indicate that p300 is required for Nanog-driven gene expression. More experiments are underway to determine how knocking down p300 affects pluripotency and self-renewal in these cells.

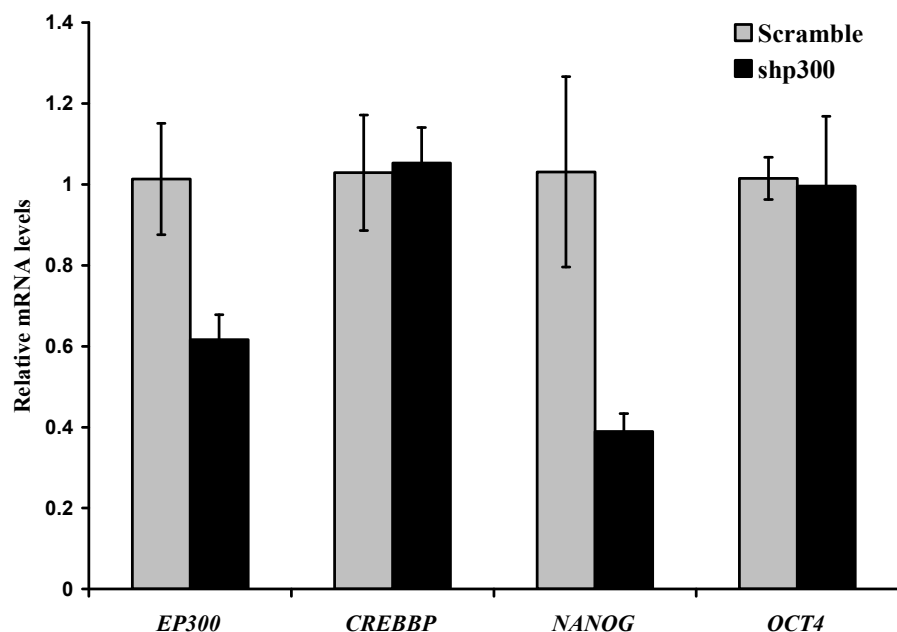


Figure III-8. Effects on mRNA levels of *NANOG* and *OCT4* upon knockdown of p300. Briefly, NCCIT cells (3.5×10^4 cells/well) were plated and allowed to adhere for 12 hours. They were then transfected with pBABE.puro (encoding a puromycin-resistance gene) and either a shRNA plasmid targeting p300 or a scrambled shRNA control. The cells were incubated for 24 hours after which time the transfection solution was removed and fresh RPMI + 10% FBS supplemented with 2.5 $\mu\text{g}/\text{mL}$ puromycin (to select for transfected cells) was added. After an additional 48 hours (72 hours post-transfection), the total RNA was harvested using the RNeasy Kit (Qiagen) and used as a template for the qPCR reactions. The qPCR reactions were prepared following the Path-ID 2X qPCR Master Mix protocol (Applied Biosystems) with the appropriate probe (Applied Biosystems). Each data point represents the mean of three trials with the error representing the standard deviation of the mean. mRNA levels were normalized to the scramble control.

D. Effects of Synthetic Ligands of CBP/p300 on Nanog-Driven Transcription

The discovery that p300 is indeed necessary for Nanog-driven gene expression provided a gratifying initial result. In order to better understand this interaction, however, it was necessary to determine which domain(s) of p300 are responsible for this interaction. As the KIX and CH1 domains of p300 have been shown to be necessary for the maintenance of pluripotency and self-renewal of hematopoietic stem cells (HSCs),

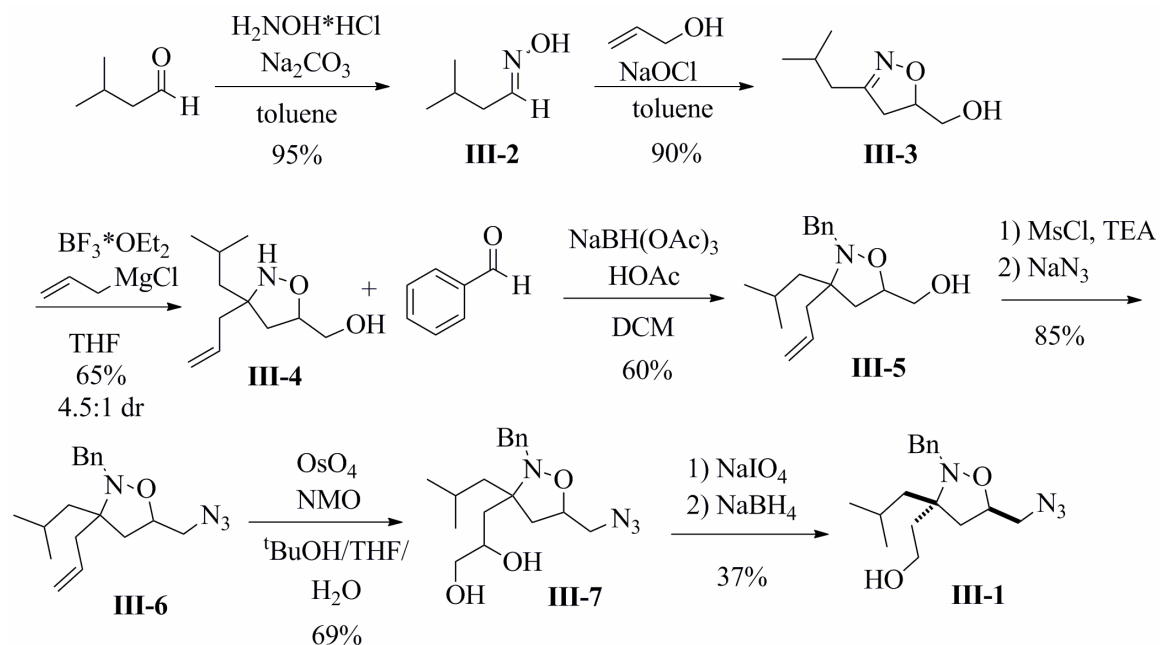
these domains were first examined for their roles in Nanog-driven gene expression.⁴⁵

The amphipathic isoxazolidine that binds the KIX domain of p300/CBP (444) was used as the probe to investigate the role of the KIX domain in Nanog-driven gene expression.

The hydrogen bond surrogate designed to bind the CH1 domain of p300/CBP was used as a probe to investigate the role of the CH1 domain in Nanog-driven gene expression.

D.1. Synthesis of Amphipathic Isoxazolidine 444

Amphipathic isoxazolidine 444 was synthesized by a standard procedure (Scheme III-1). Briefly, nitrile oxide **III-2** undergoes a cycloaddition reaction with allyl alcohol to form isoxazoline **III-3** in 90% yield. This molecule can then undergo a Lewis Acid-catalyzed Grignard addition to form isoxazolidine **III-4** in good yield and moderate diastereoselectivity. This molecule can then undergo a reduction reaction with benzaldehyde in the presence of sodium triacetoxyborohydride to yield **III-5**. The benzylated isoxazolidine can then undergo a mesylation followed by azide displacement to yield the azido compound **III-6** in 85% yield. An oxidation of the olefin by reaction with osmium tetroxide allows for the formation of the dihydroxide **III-7** which can then be cleaved and reduced to the final alcohol **III-1**. The minor diastereomer is removed during the purification following the final reduction, resulting in the isolation of 444.



Scheme III-1. Synthetic route to formation of 444.

D.2. Results of Synthetic Probes on Nanog-Driven Luciferase Expression

These studies were carried out by our collaborators, the Quintin Pan laboratories at Ohio State University, because of their expertise in viral transfections and dealing with head and neck squamous cell carcinomas (HNSCCs) a solid tumor model that contains both bulk tumor and CI cells. For these studies, HNSCCs that were lentivirally transfected with plasmids encoding a Nanog-driven luciferase reporter were treated with 30 μM of 444. A 22% decrease in luciferase activity was observed relative to DMSO control, indicating that Nanog-driven gene expression is effected by 444. The HBS molecule targeting the CH1 domain (provided by the Arora laboratories at New York University) was tested in the same system (Figure III-9). The HBS also elicited a dose-dependent decrease in Nanog-driven luciferase expression

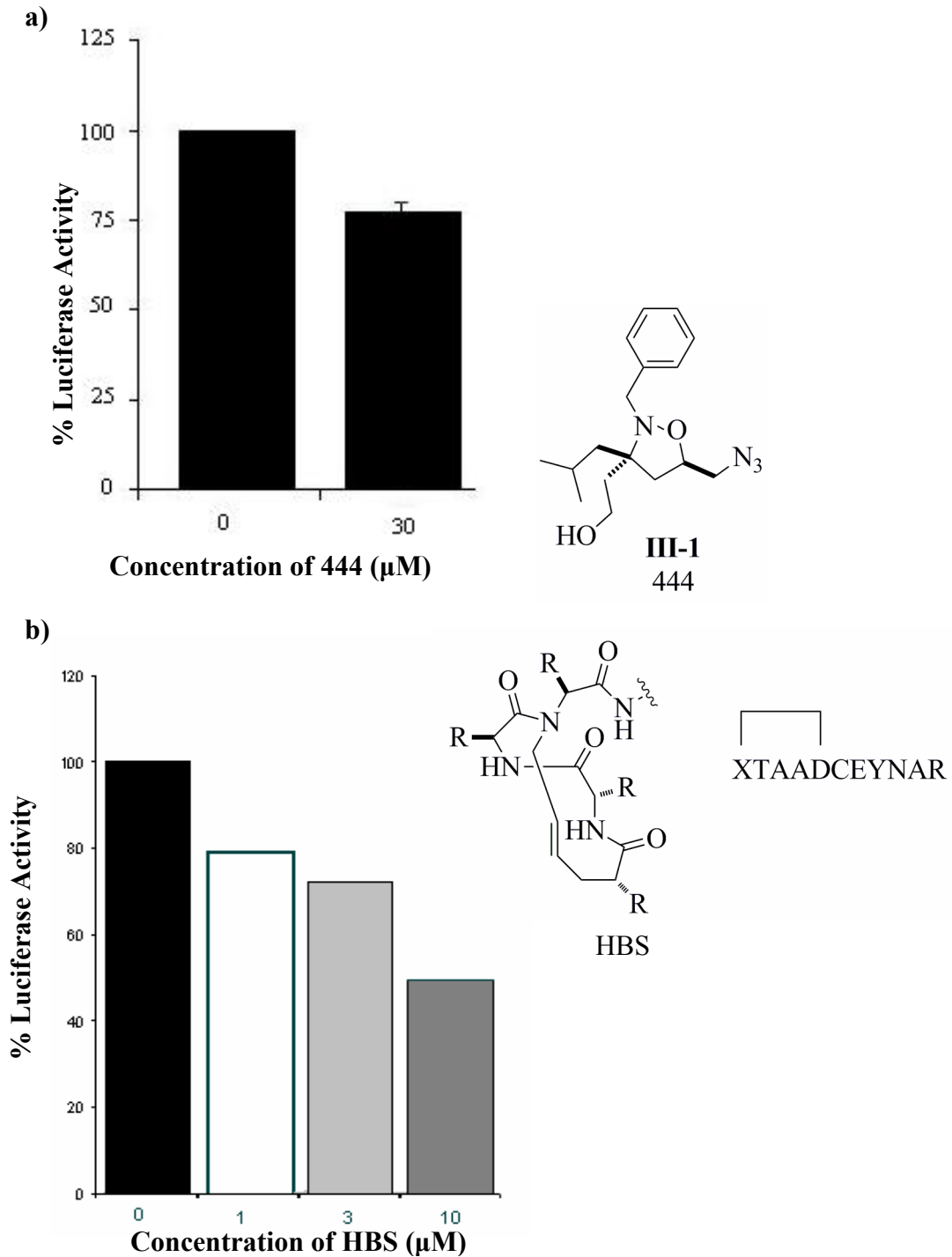


Figure III-9. Effect of synthetic ligands of p300/CBP on Nanog-driven gene expression. a) HNSCC cells treated with 444 exhibit a 22% decrease in luciferase levels relative to vehicle control, indicating a role of the KIX domain of p300 in Nanog-driven gene expression. b) HNSCC cells treated with HBS exhibit a dose-dependent decrease in luciferase levels relative to vehicle control indicating a role of the CH1 domain of p300 in Nanog-driven gene expression. HNSCC cells that express a Nanog-driven luciferase gene were treated with the appropriate compound for 24 hours at which time the luciferase output was read using the Promega luciferase assay kit.

These studies indicate that both the CH1 and KIX domains play a role in Nanog-driven gene expression. As other transcription factors have been shown to bind to multiple domains of p300, it is not surprising that multiple domains would be involved in Nanog-mediated transcription.^{21,22} These initial results were quite exciting as they proved that synthetic ligands of p300 had an effect on Nanog-driven gene expression. In order to determine the effects that these molecules have on self-renewal, whole cell viability studies need to be conducted.

E. Effects of Synthetic Ligands of p300 on Cellular Viability in a CIC Model System

E.1. Initial Studies of Effectiveness of 444 in Altering Cellular Viability in a CIC Model System

Initial studies were carried out in an embryonal carcinoma cell line (NCCIT) which has often been employed as a model stem cell system. NCCIT cells were treated with increasing doses of amphipathic isoxazolidine for 24 hours before the relative viability of the cells were assayed using the WST-1 reagent. It was found that treatment with 444 had a biphasic effect on the viability of the NCCIT cells. No change in viability was observed relative to vehicle control when cells were treated with 0-60 μM of 444. A sharp decrease in the relative viability of the cells was observed between 60-100 μM . Cells treated with 100-140 μM of 444 showed a 50% decrease in viability relative to vehicle control. When both 444 enantiomers were tested individually, similar results were observed (Figure III-10).

a)

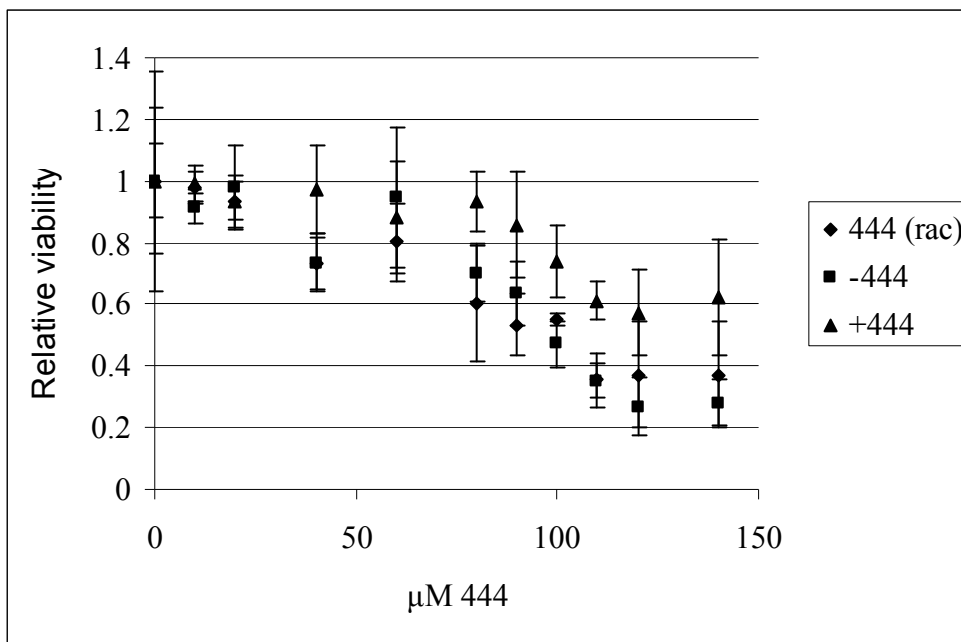
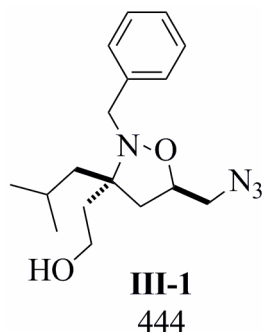
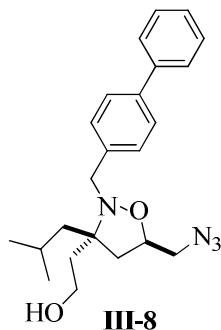


Figure III-10. Effect of 444 on CIC model system. NCCIT cells were plated in 96 well format (2.5×10^3 cells/well) and allowed to adhere for 24 hours, after which time compound was added to the cells such that a final concentration of DMSO in all wells was 1% (v/v). The cells were then incubated for an additional 24 hours at which time WST-1 reagent (Roche) was added to the cells. The cells were incubated for another 4 hours before the assay was read following manufacturer's instructions. The median value of 5 wells is reported for each treatment. NCCIT cells display a biphasic response to treatment with 444 with lower doses of compound (10-60 μM) eliciting no significant difference in viability relative to vehicle control. Doses above 100 μM lead to approximately 50% decrease in cell viability. Each of the two enantiomers were synthesized by a route similar to that of the racemic compound.

NCCIT cells were also treated with a biphenyl-amphipathic isoxazolidine that has previously been shown to bind to Med23 (a component of the human mediator complex)

but not to the KIX domain CBP.⁴⁶ These cells did not exhibit a biphasic response; rather, they exhibited a dose dependant decrease in viability. (Figure III-11).

a)



b)

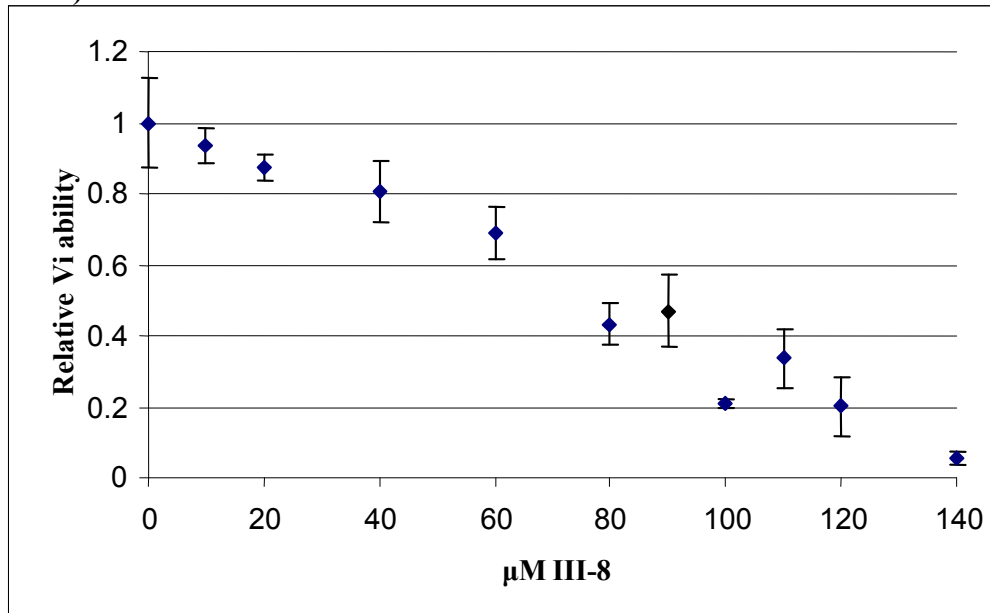


Figure III-11. Effect of isoxazolidine that does not target CBP on CIC model system. The cells were treated as previously described for Figure III-10. Cells exhibit a dose-dependant decrease in viability when treated with **III-8**; a biphenyl-substituted isoxazolidine that has been shown to not bind CBP. **III-8** was synthesized by the same route as was described for **III-7** with the exception that [1,1'-biphenyl]-4-carbaldehyde was used in the place of benzaldehyde during the fourth step of this reaction.

An additional study was carried out to determine the effect of 444 on NCCIT cells over 72 hours. Viability assays performed at 24, 48 and 72 hours post-treatment showed a similar biphasic trend. In this case, cells treated with 10-60 μM 444 showed no growth

inhibition over the course of the study relative to vehicle control. Cells treated with greater than 90 μM 444, however, showed no growth over the course of 72 hours (Figure III-12). These data indicates that 444 may be having a cytostatic effect rather than a cytotoxic effect on the cells. This effect would be consistent with a decrease in the cells' ability to self-renew. As Nanog is known to control self-renewal, it was hypothesized that 444 may disrupt the function of Nanog, possibly by preventing p300 and/or CBP from acting as a co-factor in Nanog-driven transcription. Accordingly, the effects of 444 on Nanog-driven transcription were more directly tested.

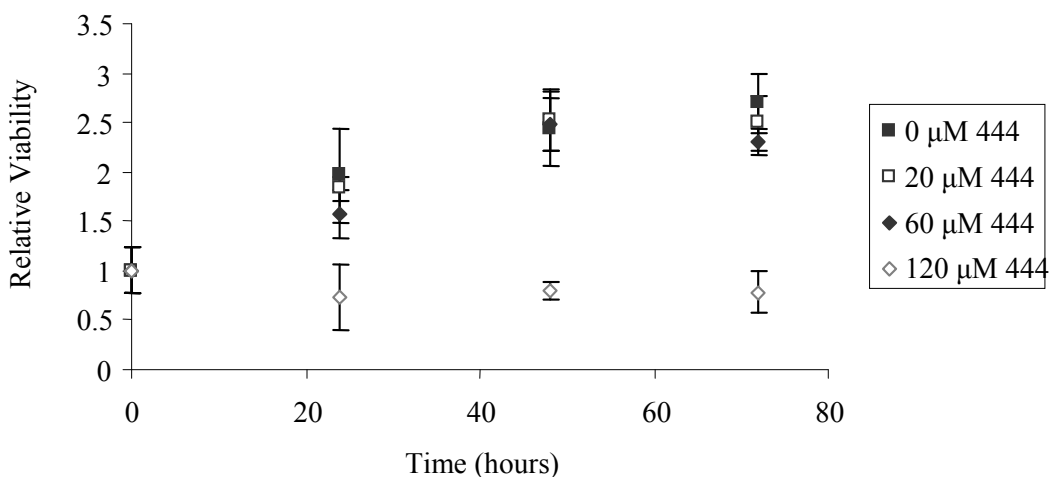


Figure III-12. Response of NCCIT cells to treatment with 444 over 72 hours. Cells treated at concentrations of 444 from 10-60 μM exhibited no change in viability relative to vehicle control over the course of the 72 hour study. Cells treated with 90-120 μM of compound exhibited no change in viability between hour 0 and hour 72. This indicates that 444 may be having a cytostatic rather than cytotoxic effect on these cells. For this study, cells were plated and dosed following the procedure outlined in Figure III-10. The cells were then incubated for either 0, 24, 48 or 72 hours in the presence of 444 before being submitted to the WST-1 assay.

As this sort of a biphasic response to 444 treatment has not been previously observed in other cell lines, these findings suggest that 444 may have a unique effect on

CICs. It was therefore decided that this hypothesis should be tested in a more complicated system.

E.2. Effects of 444 in a Heterogeneous Tumor System

HSNCC cell lines have been shown to contain cells that exhibit surface markers indicative of pluripotency. As such, it was decided that the efficacy of 444 in targeting CICs over bulk tumor cells (cells that do not exhibit surface markers indicative of pluripotency) should be tested in the Pan laboratories to determine how CICs in a more complex, heterogeneous tumor model would respond to treatment with the small molecule.

Head and neck cancer cell lines were treated for 24 hours with 444 (30 and 100 μM) and cis-platinum (10 μM), the drug most commonly prescribed for treating head and neck tumors. In order to determine the effects of treatment on the relative population of CIC versus bulk tumor cells, the relative population of cells expressing a pluripotency marker aldehyde dehydrogenase (ALDH) and the population of cells not expressing this marker were determined by FACS before and after treatment. It was discovered that cis-platinum treatment in two cell lines, SCC15 and SCC25, resulted in a 5 and 3 fold enrichment of CICs, indicating that these cells are resistant to cis-platinum treatment whereas bulk tumor cells are susceptible to this type of treatment (Figure III-13a).

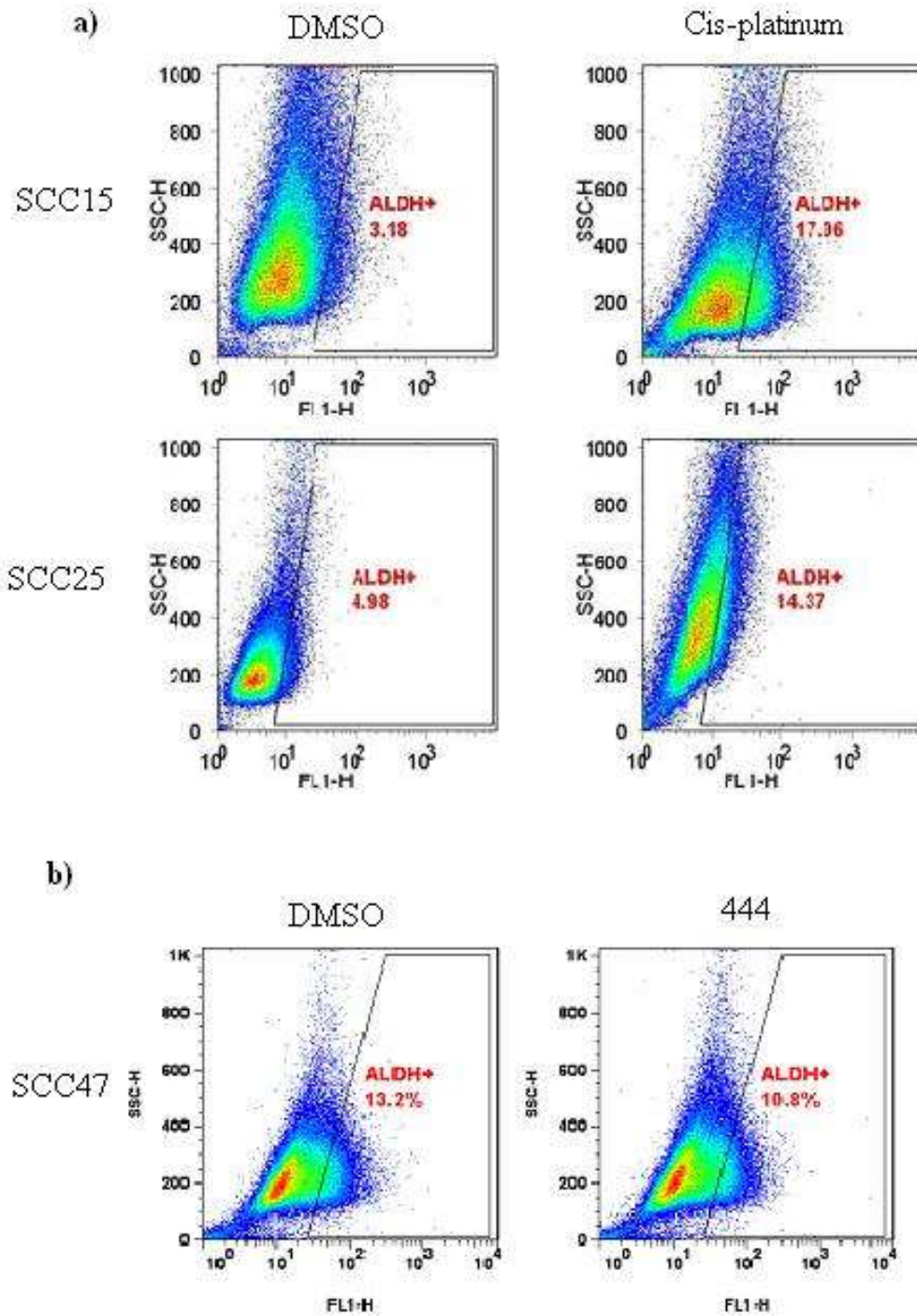


Figure III-13. ALDH+ CICs from head and neck cancer cell lines are resistant to cis-platinum treatment but not treatment with 444. a) Head and neck cancer cell lines SCC15 and SCC25 were treated with DMSO or cis-platin (10 μ M) for 24 hours. Cells were analyzed for ALDH expression by FACS using the ALDEFLUOR assay. A 5 and 3 fold enrichment in CIC populations were observed for the cells treated with cis-platinum in

both cell lines. b) Head and neck cancer cell line SCC47 was treated with DMSO or 444 (30 μ M) for 24 hours. Cells were analyzed for ALDH expression by FACS using the ALDEFLUOR assay. An 18% decrease in the CIC population was observed for cells treated with 444 versus vehicle control.

Conversely, cells treated with 444 exhibited a decrease in ALDH⁺ cells (18% when treated with 30 μ M 444 and 70% when treated with 100 μ M 444) but the population of ALDH⁻ cells remained unchanged (Figure III-13b). This finding confirms that pluripotent cellular viability is more affected than differentiated cellular viability by treatment with 444.

To date, there have been very few examples of small molecules capable of targeting CICs, with no current strategies in place to directly target the functioning of Nanog.^{4,9,47} A small molecule such as 444 that targets CICs preferentially over bulk tumor cells could be of great value in understanding the cellular processes that must be perturbed in order to decrease the population of CICs. If these processes are unique to CICs, it is possible that a therapeutic could be generated that would just target these cells, while leaving differentiated adult cells and normal stem cells in tact. Such an advancement would provide enormously useful in the field of cancer therapeutics.

F. Conclusions and Future Directions

The cancer initiating cell hypothesis provides a novel perspective on the cause and potential treatment for chemotherapeutic-resistant cancers. Several solid tumors have been found to contain cells that exhibit pluripotency markers and overexpress transcription factors and other proteins commonly associated with stem cells. As the

transcription factors Nanog, Oct4 and Sox2 have been found at elevated levels in several types of solid tumors, targeting the functions of these proteins may lead to therapeutic agents capable of inhibiting pluripotency and self-renewal in CICs.

Evidence from embryonic stem cell studies indicate that the co-activator p300 plays a role in transcription mediated by these factors; however no direct evidence of these types of interactions has been obtained in CICs. Work performed in the Mapp laboratories indicates that Nanog-driven gene expression is dependent on p300 but not CBP in a luciferase reporter context. Additionally, knockdown of p300 causes a decrease in levels of *NANOG* mRNA levels. Studies performed with synthetic ligands of p300 indicate that both the KIX and CH1 domains of p300 are involved in Nanog-driven gene expression with a significant ($p < 0.01$) decrease in Nanog-driven luciferase expression upon treatment with 30 μ M 444.

Studies performed in NCCIT cells indicate that 444, a small molecule capable of targeting the KIX domain of CBP causes a decrease in viability at concentrations greater than 60 μ M. A time course study indicates that 444 has a cytostatic effect on NCCIT cells. Follow up studies were performed in a heterogeneous tumor system that consists of both bulk tumor cells and CICs. These studies indicate that treatment with 444 results in an 18% reduction of CICs over the course of 24 hours, with no observed effects on the bulk tumor cell population. Similar studies testing the effects of the HBS on cellular viability are underway.

These findings taken together offer a new insight into the mechanism of Nanog-driven gene expression. This is the first time that a direct correlation between p300 and Nanog-driven gene expression has been established. This is also the first time that a

synthetic molecule has been shown to affect Nanog-driven gene expression. This finding, coupled with the finding that 444 is able to reduce CIC viability in two cellular contexts provides evidence that inhibiting the function of transcription factors such as Nanog may prove to be a valuable strategy for formation of CIC-specific therapeutic agents. These studies, however, have also raised a number of questions about the nature and mechanisms by which p300 is involved in Nanog-driven gene expression and the maintenance of self-renewal and pluripotency.

First, it is unclear if the KIX and CH1 domains of p300 are the only domains responsible for mediating Nanog-driven gene expression. More experiments must be carried out to determine if the remaining domains of p300 are required for Nanog-driven gene expression. To this end the individual domains of p300 are being overexpressed in cells expressing the Nanog-driven luciferase reporter and in NCCIT cells. The critical domains should cause a change in luciferase levels whereas those that are not involved in this process should cause no change.

Additionally, the full effects of 444 and the HBS on self-renewal and pluripotency have yet to be characterized. While the initial studies show a decrease in CIC population, indicative of a loss of self-renewal ability, their effects on pluripotency have yet to be determined. Work in the Mapp and Pan laboratories is currently underway aimed at discovering if these small molecules cause CICs to differentiate. Cells treated with 444 and the HBS are being examined for the expression of key germ-line differentiation markers.

Additionally, it should be determined if 444 has *in vivo* efficacy, and if it affects CICs preferentially to other stem cells in the body such as hematopoietic stem cells. In

order to determine this effect, *in vivo* studies of the effects of 444 on mice are planned. For these studies, HNSCC cells with constitutive luciferase expression will be implanted into NOD/SCID mice. After initial tumors have formed, the mice will be treated intravenously with 444 and the growth of the initial tumor and the extent of lung metastasis will be monitored for 4-6 weeks. At the termination of the experiment, the remaining tumor bulk will be analyzed for its CIC content. Additionally, the effects on normal stem cells will be monitored to determine if 444 preferentially targets CICs. It is likely that some of the probes used *in cellulo* will have to be modified to achieve optimum *in vivo* efficiency.

Also, to expand this study, the dependence on p300 and CBP of Oct4 should be examined. Initial luciferase reporter studies using Gal4-fusion proteins in conjunction with shRNA indicate that neither of the two activation domains of Oct4 are dependant on either p300 or CBP for transcription (Figure III-14). Conversely, it has been previously reported that in similar reporter contexts, Sox2-mediated transcription is dependent on p300. However, it is unknown if either of these activators rely on p300 or CBP in their native promoter contexts in CICs. In order to establish this, experiments similar to those conducted by the Pan laboratories in which the native Nanog promoter was appended upstream of a luciferase gene should be employed for the native Oct4/Sox2 promoter.

a)

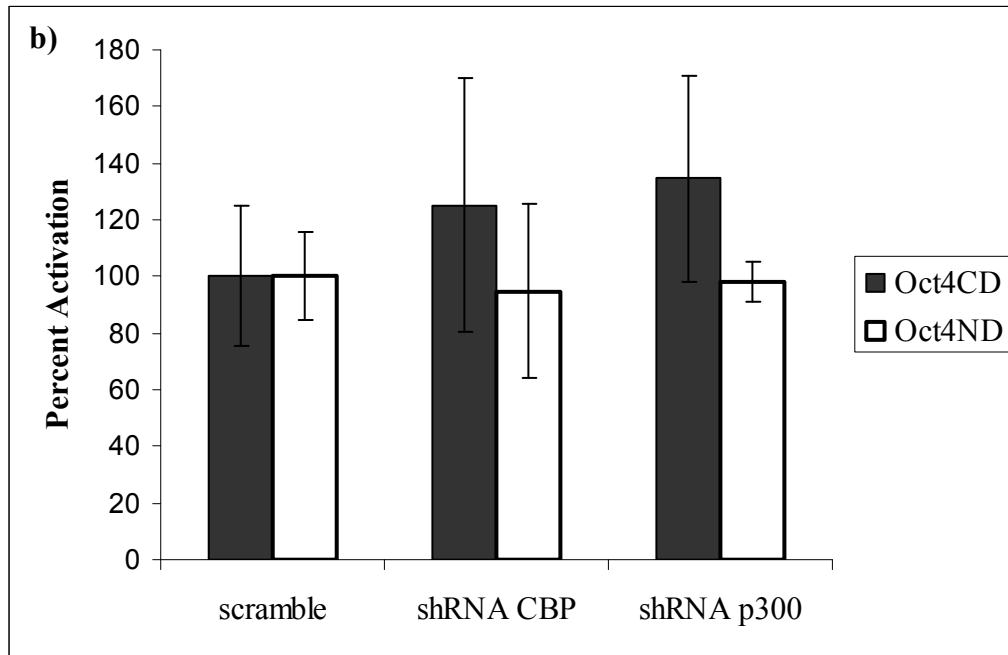


Figure III-14. Dependence on p300 and CBP for Oct4-mediated transcription. a) A scheme of Oct4 is shown with the two activation domains highlighted in blue. b) Plasmids were made encoding fusion proteins of the Gal4 DBD with each of the Oct4 TADs and used in a luciferase reporter assay as previously described. Neither activation domain of Oct4 was dependent on either CBP or p300 for activation.

Finally, the effects of knocking down p300 and CBP on pluripotency and self-renewal in CICs should be monitored. First, these studies will help to further define the disparate roles that p300 and CBP play in differentiation. Secondly, they will provide a measure for how extensively these proteins are involved in the maintenance of pluripotency and self-renewal in a CIC population. CICs have many mechanisms in place for avoiding differentiation. If elimination of p300 and/or CBP is sufficient to induce differentiation, these proteins may prove to have vital therapeutic relevance in the treatment of CICs.

G. Experimental

General.

HBS was provided by the Arora laboratories at New York University. Synthesis of molecule **III-7**, the individual enantiomers of the compound and of molecule **III-8** were performed according to standard procedures.^{33,46,48} These compounds were purified prior to cell studies by passage through a disposable C₈ column in 10% ammonium acetate in methanol after which they were lyophilized until dry. Oct4 and Nanog full length plasmids were purchased from Addgene. CBP and p300 shRNA plasmids were prepared as previously reported.⁴⁴

Assay for Dependence of Nanog-driven Transcription on p300 and CBP.

HEK293T cells were purchased from the American Type Culture Collection (ATCC) and plated onto treated polystyrene Petri dishes (Corning) with 15 mL of DMEM (+ 4.5 g/L d-glucose, + l-glutamine, -sodium pyruvate, + 10% FBS) (Invitrogen). The cells were grown at 37 °C and 5% CO₂ to 80-90% confluence. Upon reaching the desired confluence, the DMEM was removed and 5 mL 0.25% trypsin was added. The cells were incubated with the trypsin solution for 5 minutes at 37 °C and 5% CO₂. Following the incubation, 5 mL of DMEM were added and the resultant solution was gently pipetted several times to ensure removal of all cells from the dish surface. The solution was centrifuged in a 15 mL Falcon tube at 1000 rpm for 4 minutes in a Fisher Centrifuge centrifuge. The supernatant was removed and the cell pellet was resuspended in 10 mL of DMEM. The concentration of cells was calculated using a Hausser Scientific improved Neubauer phase counting chamber hemocytometer. Based on the determined

concentration, the cells were diluted to 5×10^4 cells/mL in DMEM. 1 mL of the cell solution was then added to each well of a Microtest flat-bottom, low-evaporation-lid 24-well plates (Beckton-Dickinson) and the plated cells were incubated for 24 hours at 37 °C and 5% CO₂. The media was removed from the plated cells and they were treated with Opti-Mem (+ HEPES, 2.4 g/L sodium bicarbonate, + l-glutamine) (Invitrogen) and then transfected.

The transfection procedure consisted of first mixing appropriate plasmids and Lipofectamine 2000 together in Opti-MEM media for each well being transfected. After a 20 minute incubation at room temperature 500 µL of the solution was placed in the appropriate wells and the cells were incubated for 4 hours at 37 °C and 5% CO₂. At the end of the 4 hours, the transfection solution was removed and 1 mL of fresh DMEM was added to each well. The cells were then incubated for 48 hours at 37 °C and 5% CO₂.

Following the 48 hour incubation, the media was removed from each well and the cells were washed once with PBS buffer. 100 µL of passive lysis buffer (Promega) was added to each well and the cells were incubated for 20 minutes at room temperature on an orbital shaker. Subsequently, the lysate was mixed by pipetting and 25 µL of lysate was added to a cuvette along with 20 µL Luciferase Assay Reagent II (Promega) and the luminescence was recorded. Then 25 µL Stop & Glo reagent (Promega) was added and the Renilla luminescence was recorded again on a Berthold FB12 single cuvette luminometer.

Western Blot.

HEK293T cells were grown as previously described. For plating, 2×10^5 cells were plated per well in Microtest flat-bottom, low-evaporation-lid 6-well plates (Beckton-Dickinson) and the plated cells were incubated for 24 hours at 37 °C and 5% CO₂. The media was removed from the plated cells and they were transfected with Opti-Mem (+ HEPES, 2.4 g/L sodium bicarbonate, + l-glutamine) (Invitrogen) and then transfected.

The transfection procedure was the same as previously reported with the exception that 2.5 mL of the transfection solution was placed in the appropriate well. At the end of the 4 hours, the transfection solution was removed and 1 mL of fresh DMEM was added to each well. The cells were then incubated for 48 hours at 37 °C and 5% CO₂.

Following the 48 hour incubation, the DMEM was removed. The cells were then incubated with 100 µL 0.25% trypsin for 5 minutes at 37 °C and 5% CO₂. After this time, 500 µL DMEM was added to each well and the resultant solution was gently pipetted several times to ensure removal of all cells from the dish surface. The solution was then transferred to 1.5 mL Eppendorf tubes where the solution was again gently pipetted to ensure homogenization. The concentration of cells harvested from each well was calculated using a Hausser Scientific improved Neubauer phase counting chamber hemocytometer for normalization purposes. The cells were then centrifuged at 14,000 rpm for 1 minute and the supernatant was discarded. The cell pellet was then washed 2x in cold PBS buffer. The cells were then lysed with passive lysis buffer (Promega) with a proEt3Nse inhibitor cocktail (Invitrogen) (volume determined such that there were 2,000 cells/ µL of lysis solution in the final mixture) on ice on an orbital shaker for 20 minutes.

The lysate was cleared by centrifugation at 14,000 rpm for 10 minutes then mixed with loading dye and DTT (final concentration of 1%) and heated at 95 °C for 9 minutes. 20 µL of sample was loaded per well on a 4-12% tris/glycine gel in MOPS buffer (CBP) or a 3-8% tris/acetate gel in tris/acetate buffer (p300) and separated by PAGE for 120 minutes (150V, 200mA). Proteins were transferred to a PVDF membrane for 120 minutes (45V, 200mA).

The membranes were then blocked (10mM PBS, pH 7.4, 1% (v/v) Tween-20, 2% (v/v) non-fat milk) for 1 hour at rt. After 1 hour, the appropriate primary anti-body was added and the membrane was incubated overnight at 4 °C on a shaker. For CBP detection: mouse monoclonal anti-CBP antibody (1:500, Santa Cruz Biotechnology), for p300 detection: rabbit polyclonal anti-p300 antibody (1:500, Santa Cruz Biotechnology), for GAPDH detection: mouse monoclonal anti-GAPDH antibody (1:1,000, Santa Cruz Biotechnology). After incubation with primary antibody, the membrane was washed (3x 10 mins) (10mM PBS, pH 7.4, 1% (v/v) Tween-20) on an orbital shaker at rt. The membranes were then incubated with an appropriate horseradish peroxidase labeled secondary antibody in a buffer (10mM PBS, pH 7.4, 1% (v/v) Tween-20, 2% (v/v) non-fat milk) for 1 hour at rt. (For CBP anti-mouse antibody (1:500, Santa Cruz Biotechnology), for p300 anti-rabbit antibody (1:500, Santa Cruz Biotechnology), for GAPDH anti-mouse antibody (1:1000, Santa Cruz Biotechnology). After 1 hour, the membrane was washed (3x 10 mins) (10mM PBS, pH 7.4, 1% (v/v) Tween-20) on an orbital shaker at rt. Bands were visualized using the ECL plus Western Blot Detection System (GE Healthcare).

Cellular Viability Assays.

NCCIT cells were purchased from the American Type Culture Collection (ATCC) and plated onto treated polystyrene Petri dishes (Corning) with 10 mL of RPMI + 10% FBS (Invitrogen). The cells were grown at 37 °C and 5% CO₂ to 80-90% confluence. Upon reaching the desired confluence, the RPMI was removed and 5 mL 0.25% trypsin was added. The cells were incubated with the trypsin solution for 5 minutes at 37 °C and 5% CO₂. Following the incubation, 5 mL of RPMI were added and the resultant solution was gently pipetted several times to ensure removal of all cells from the dish surface. The solution was centrifuged in a 15 mL Falcon tube at 1000 rpm for 4 minutes in a Fisher Centrifuge centrifuge. The supernatant was removed and the cell pellet was resuspended in 10 mL of RPMI. The concentration of cells was calculated using a Hausser Scientific improved Neubauer phase counting chamber hemocytometer. Based on the determined concentration, the cells were diluted to 2.5×10^4 cells/mL in DMEM. 100 µL of the cell solution was then added to each well of a Microtest flat-bottom, low-evaporation-lid 96-well plates (Beckton-Dickinson) and the plated cells were incubated for 24 hours at 37 °C and 5% CO₂. After 24 hours, compound was added as a solution of DMSO (1% final concentration of DMSO) and incubated for 24-72 hours at 37 °C 5% CO₂. After 24, 48 or 72 hours of incubation, cells were treated with WST-1 (Roche) according to manufacturer's instructions. The plates were read on a Tecan Genios Pro fluorescence, absorbance reader.

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Chapter IV

Design and Implementation of a Peer-Led Module for Teaching Practical Research Ethics to Graduate Students^a

A. Abstract

The need for formal ethics training outside of the student-advisor relationship has been well established. As part of my thesis work, a collaborator (Christopher Taylor) and I have designed and implemented a peer-led module for teaching practical research ethics. We postulated that senior graduate students would be effective as teachers of research ethics as they would bring a unique view of problems facing incoming graduate students. We also hypothesized that incoming graduate students would be more open and honest when discussing ethical issues when they were with a group of peers. In teaching this module, we developed a series of data interpretation exercises and case studies for the students to work through based on dilemmas that we, the peer leaders, or members of our cohorts had faced during our first two years of graduate school. In order to assess the effectiveness of this module, we examined student feedback, responses students gave during large group discussions of case studies, and the reactions of the chemical community as a whole. The students' responses during the large group discussions incorporated many of the ideas that were presented earlier in the module, indicating that

^a Portions of the chapter are reported in Danowitz, A.M. and Taylor, C.E. "Integrating a Peer-Taught Module on Practical Research Ethics into the Graduate Student Orientation Curriculum." *Accepted Manuscript J. Chem. Educ.*

the module was successful as the students were beginning to understand the complexities involved in addressing ethical concerns. Additionally, the positive responses from the student evaluations and the positive feedback from the chemical community indicate that this is a useful module.

B. Introduction

B.1. Examples of Scientific Misconduct

The need for explicit, formal instruction in research ethics and professionalism has been well established.^{1,2} This need has been highlighted in recent years by several major scientific scandals. Cases such as that of Jan Hendrick Schön, the former Bell Laboratories researcher who was found guilty of 16 counts of scientific misconduct in his research relating to nanomaterials, Wu Suk Hwang, the Korean researcher who falsified data regarding the creation of human stem cells, and Marc Hauser, the cognitive scientist who was recently found guilty by Harvard University of engaging in scientific misconduct have garnered international attention.^{3,4,5} While these egregious cases of scientific misconduct are the exceptions rather than the rule, other, less widely publicized forms of misconduct are much more common.

In a study conducted at Arizona State University, 84-91% of undergraduate students in seven biology and zoology courses admitted to “almost always” or “often” manipulating laboratory data to obtain a desired result.⁶ In a study completed in 2005 of 3,247 faculty members and postdocs who received NIH funding (either an R01 grant for faculty or an F32 or T32 grant for postdocs), 33% of respondents admitted to

participating in at least one of ten behaviors that were determined by the designers of the survey to be the most serious questionable research practices. These questionable practices include among others: falsifying or “cooking” research data, overlooking others’ use of flawed data or questionable interpretation of data, and changing the design, methodology or results of a study in response to pressure from a funding source.⁷ A particularly poignant example of scientific misconduct for the University of Michigan is the case of a postdoc, Vipul Bhargava, in the Comprehensive Cancer Center who was arrested for, and pleaded guilty to, charges of malicious destruction of personal property after spending several months sabotaging his colleague’s experiments.⁸ These reports of moral transgressions highlight the prevalence of misconduct in the modern academe, and underline the need for increased awareness of ethical issues and decision making among scientists at all levels.

B.2. Defining Scientific Misconduct

One of the traditional problems facing scientists dealing with ethical dilemmas is that scientific misconduct has been notoriously difficult to define.⁹ In 2004, the Office of Research Integrity (ORI) — a division of the Department of Health and Human Services that oversees and directs Public Health Service research integrity — introduced a series of guidelines for the Responsible Conduct of Research (RCR) in an attempt to define the legal threshold for defining cases of research misconduct. Under this system, research misconduct is defined as:

Fabrication, falsification, or plagiarism in proposing, performing, or reviewing research, or in reporting research results. (a) Fabrication is making up data or results and recording or reporting them. (b) Falsification is manipulating research materials, equipment, or processes, or changing or omitting data or results such that the research is not accurately represented in the research record. (c) Plagiarism is the appropriation of another person's ideas, processes, results, or words without giving appropriate credit. (d) Research misconduct does not include honest error or differences of opinion.¹⁰

While this definition provides a useful standard for gauging research misconduct, several types of questionable research practices fall outside of this definition but still pose a threat to the scientific enterprise.⁷ For example, the sabotage committed by Bhriгу resulted in an estimated loss of \$72,000 in resources and person-hours but does not fall under the RCR definition of misconduct.⁸ Situations like this where ethically dubious acts were clearly committed, highlight the downfall of a narrow definition of scientific misconduct. An effort to incorporate both the RCR guidelines and a broader definition of questionable research practices should be made in designing research ethics courses.

B.3. Addressing Issues of Scientific Misconduct and Research Ethics

Just as scientific misconduct has been traditionally difficult to define, it has been similarly difficult to effectively formally address. Part of this issue stems from identifying the root causes of scientific misconduct. One hypothesis is that scientific misconduct is the action of individual “scoundrels among scientists” who commit ethically dubious acts to further their own goals.¹¹ It would be naïve to assume that any amount of formal ethics training would prevent all individuals who have decided on an ethically dubious course of action from doing so. However, formal ethics training would

provide scientists the tools they need to effectively address problems that arise from their less ethically-minded colleagues before they become large, public scandals.^{11,12} This is highlighted by the case of sabotage that occurred at the University of Michigan last year. While the victim of the sabotage first noted suspicious things occurring with her research in December 2009, a formal investigation into the issue was not launched until six months later, when the victim was first accused of sabotaging her own work.⁸ Had a research environment existed where ethical considerations were prominently discussed, it is possible that the nature of the sabotage could have been uncovered and dealt with much sooner, and with less cost in time and money.

Another school of thought cites the competitive nature of modern science as fostering an environment in which otherwise ethically minded researchers commit ethically dubious acts in response to external pressures.¹¹ While it stands to reason that formal ethics education could help to bring about changes in both the environment in which scientists work and the way that scientists respond to these pressures, there is limited evidence that this is the case.¹¹ It has been noted, however, that formal education programs have the best chance of succeeding if they are “creative,” emphasize practicality in addressing concerns and provide researchers with a support network whom they can contact when issues regarding research ethics arise.¹²

Multiple strategies have been developed for creatively incorporating research ethics into the standard graduate and undergraduate curriculums including: introduction of ethical principles as part of a peer led structured study group,¹³ implementing courses focused specifically on research ethics,^{14,15} integrating ethical discussions into traditional chemistry courses,¹⁶ and the design of a flexible model that can be applied to a variety of

scientists in many different contexts.¹⁷ As researchers who are responsible for generating and publishing original research, and as individuals who are often carrying out research full-time for the first time in their lives, graduate students are a critical audience for these discussions.

After attending the Teaching Research Ethics conference at the Poynter Center at Indiana University,¹⁸ and in consultation with members of the University of Michigan chemistry faculty, we developed a peer-led ethics module to be presented to the entire cohort of incoming chemistry graduate students during graduate orientation. We proposed that a peer-led program would send a positive message about the students' responsibility in taking ownership of their education. Additionally, it would draw the students' attention to these issues in their first few weeks of graduate school. We taught this module for the first time in 2009 and then again in 2010.

C. Rationale for Utilizing Peer-Leaders

The most striking aspect of this approach to teaching research ethics is the use of peer leaders in teaching this module. It was believed that having senior graduate students as instructors would be advantageous for several reasons. First, it was proposed that a peer-led program would send a positive message to the incoming students about the roles that ethics plays in every day research. It was felt that the incoming students would note that ethics is important to their peer group and should therefore, by extension, be important to them. As it has been suggested that a research ethics course is most effective when it influences individuals instead of "merely satisfies an item on a 'check-

off’ list for [an] institution,” the peer-led method should prove to be particularly effective at changing individual view points and behaviors.¹²

Second, we wanted to take advantage of the knowledge that senior graduate students have of the research environment in their own department. Senior students have a wealth of very recent experiences to draw from when discussing ethical issues with younger students. They are well aware of the current difficulties facing students in an ever-changing research environment. This knowledge is especially valuable in writing case studies. We drew on dilemmas faced by ourselves and our peer group when designing the case studies. This ensured that the case studies presented would be relevant to the experiences of first and second year graduate students.

Finally, it was believed that students would be more honest when discussing their views of ethically dubious situations if they were in a group of their peers. There is a real possibility that first year students might censor their responses in order to impress a faculty member.

It must be noted, however, that senior graduate students lack a firsthand faculty perspective on these issues. For this reason, it is extremely useful to consult with faculty members in determining discussion points and potential solutions for each case study. An open and honest dialogue between the peer leaders and members of the faculty is considered to be key in the success of this module.

D. Module Goals and Design

In line with the idea that the drive for personal success and desire to comply with external pressures can outweigh moral concerns, the module was designed around the idea that most students do not start out with the intention of committing morally dubious acts.^{11,13,15} Rather, they might do so in response to the pressures that they face as graduate students (Figure IV-1). This line of reasoning indicates that ethical decision-making and the ability to navigate the conflicts and hazards of graduate school are closely intertwined.¹⁵ The main vehicle employed for facilitating discussions was case studies designed by the peer leaders.

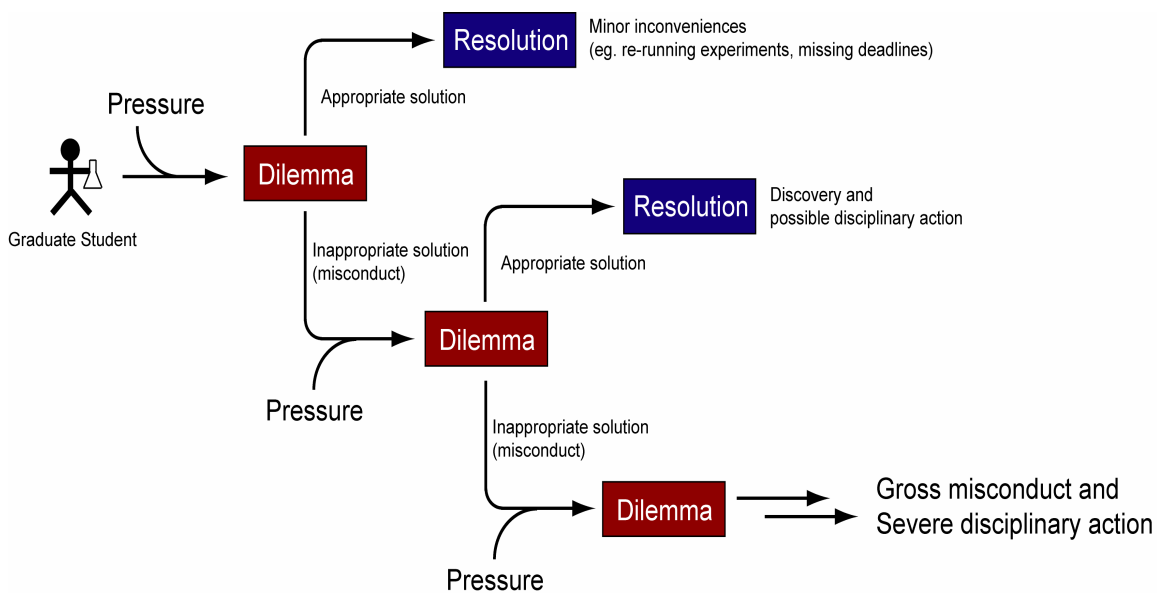


Figure IV-1. Flow chart detailing how students who are faced with a dilemma can, through their own poor decision making, become set on a path leading them to commit gross misconduct.

The first goal in designing this module was to provide students with examples of potential moral dilemmas that they could relate to. As noted by Kovac, "It is essential that students be able to relate to both the context and the characters [of a given situation]. If the people and the scenario are too distant, students will tend to see the situation in

simplistic right-or-wrong terms." ¹⁹ In keeping with this idea, the case studies were based on situations that members of the 2006 and 2007 chemistry cohorts had faced during their first two years of graduate school.

In addition to designing the case studies so that students could relate to them, an emphasis was placed on practical ethical reasoning skills. To begin with, students were encouraged to identify when a situation involved ethical considerations and when it did not. In particular, students were instructed to distinguish between facts and rumor or gossip when examining possible misconduct on the part of a co-worker. Students were also asked to propose several solutions to each of the problems presented. In particular, students were asked to devise solutions that would allow them to succeed in meeting external requirements without committing misconduct.

In order to give students the tools they need for solving moral dilemmas, the final objective was to present students with an over-view of the departmental and campus-wide resources for conflict resolution. As students are often unaware of resources other than their advisors and more experienced lab-mates, their attention was drawn specifically to bodies such as the Graduate Committee and the Ombud's office that can help them with problem solving. Specifically, students were encouraged to begin to differentiate which types of problems would be appropriate to bring to each resource.

In order to meet these goals, we developed six case studies and a data interpretation exercise (see Section K) that were used as vehicles to discuss issues such as authorship, plagiarism, resolution of intra-lab conflict, and data integrity from the perspective of a first or second year graduate student.

E. Module Layout and Content

We implemented this module in two, two-hour blocks over the course of two days. The sequence began with a brief presentation on the resources that are available to graduate students in dealing with conflicts. These discussions were centered on those conflicts that could either lead to misconduct on the students' part or stem from the misconduct of other researchers. Students participated in a brainstorming session that addressed different sorts of conflicts that can arise in graduate school. The peer leaders then guided a large group discussion about the most appropriate venues for resolving these problems. This discussion included potential steps for escalation of unresolved problems, and of alternative courses of action for cases where the usual resource—such as the student's advisor—were not appropriate.

Prior to the discussion of the case studies and data interpretation exercise, the students were given a hand-out titled “A Format for Ethical Decision Making” (Box IV-1). The peer leaders then discussed the issues presented in this framework. Specifically, the students' attention was drawn to the need for postulating several solutions to a given situation. Also, the various ‘tests’ listed in the handout were discussed as a moral barometer for judging these solutions. It was hoped that students would incorporate these ideas when thinking about and discussing moral dilemmas in the future as evidence that they were becoming proficient in identifying and addressing such problems.

Most of the module was based on case studies that focused on dilemmas that students might face as first or second year graduate students. Students discussed cases in small groups (four to six students) with the discussion leaders moving from group to

group in order to facilitate discussion. To ensure that each group contained the expertise necessary to address the data presented in the data interpretation exercise, the students were divided such that each group contained at least one student specializing in each of the different chemistry subdivisions. The entire group then discussed the case, with students volunteering solutions or feedback on other students' solutions. At this stage, the discussion leaders acted as moderators and guided the group towards a general consensus on the best courses of action for each situation. In both the small and large group discussions, students were encouraged to consider the long term consequences of each proposed action and to think about other stakeholders who may be affected by their actions. Peer leaders and students also discussed the differences between professional and unprofessional ways of handling conflicts.

A Format for Ethical Decision Making

Adapted from a handout written by Professor Michael Davis, for the Teaching Research Ethics conference in 2009. Used with permission.

1) Determine the problem.

The initial problem may be an explicitly ethical one (e.g. 'I think this data was fabricated') or it may not be (e.g. 'I am going to miss a research deadline').

2) Determine/check facts.

As much as possible, try to separate objectively verifiable facts from suspicions or rumors. In many cases, the issue may change or disappear upon careful consideration.

3) Consider limitations and objectives.

There will probably be many goals involved in a situation – maintaining personal integrity, advancing your career, making a favorable impression with your advisor or other faculty members, your relationship with your peers, etc.

4) Develop a list of 2-3 options.

Be specific about courses of action – who would you talk to, how would you discuss the issues, etc.

5) Test Options.

The following are good examples of tests that are not necessarily dependent on specific ethical theories.

Harm test – Does this option do less harm than any of the other alternatives?

Publicity test – Would I want my choice of this option known throughout the department?

Defensibility test – Could I defend this choice in front of faculty members or other authorities? If so, what would I say?

Reversibility test – Would I still think this was the best option if I were one of the people adversely affected by it.

6) Choose an option based on steps 1-5.

7) Consider what you can do to make it less likely that this issue will come up again.

Box IV-1. A Format for Ethical Decision Making.

In addition to case studies, we used an exercise and group discussion that dealt with interpreting and presenting ambiguous data to illustrate the ethical dimensions of this process. After a brief presentation on what qualifies as fabrication, falsification and plagiarism, students were presented with examples of data such as synthetic yields obtained when testing the efficacy of two different ligands (see Section K for examples). The students were also given a list of potential conclusions that could be drawn from the data. They then discussed circumstances under which a given conclusion would or would not be valid. In the latter case, students also considered whether inappropriate conclusions represented poor science or misconduct and discussed the factors that determine this distinction. Additionally, students discussed the differences between a reasonable disagreement between scientists and misconduct. The module ended with two 'off the record' question and answer sessions, in which students could ask the discussion leaders questions about graduate school, or broach any unaddressed concerns that they had. In the first session, only the peer leaders were present. For the second session, a faculty member was present so that students could get the faculty's view of any questions they had.

F. Student Discussions of Case Studies

The nature of the responses given by the new graduate students was used as one means to assess the effectiveness of the module. If these discussions were successful, students should assimilate the lessons and identify the larger moral dimensions of the situations presented in the case studies. They should also propose solutions that allow

them to maintain both their ethical and professional integrity, while still meeting the demands of graduate school.

In the case study titled “Key Result” (Box IV-2), students were presented with a situation set during their preparation for candidacy. Many of the student responses referred to ideas from the ‘Format for Ethical Decision Making’ handout that had previously been discussed with them. Several students brought up the potential affects of their actions on their advisor or collaborators who are additional stakeholders in the situation. Other responses included assessment of potential long-term effects—for example, that feigning ignorance of the needed control in situation A could set the student up for further acts of fabrication or falsification if they are faced with the invalidation of their ‘results’ later on.

In addition to recognizing the ethical issues embedded in the case study, students proposed solutions that allowed them to meet the demands of graduate school without committing misconduct or setting themselves up to do so in the future. For example, in situation B, the possibility was raised that students may be able to postpone candidacy until the systematic error has been fixed, rather than present suspect data. For situation A, students discussed ways to present the limitations of the data (i.e. “absent this control we see...”) that might allow them to discuss their data without misrepresenting their results. These responses indicate that students are seeing beyond the simplistic binary dilemma of committing a morally dubious act or failing to meet imposed standards.

For another case study (Box IV-3), students discussed the possibility of misconduct on the part of a coworker. These sorts of problems may arise when multiple scientists contribute to a project. It is therefore important for students to think about how

to determine if misconduct has occurred. Students should also practice professional and courteous ways of broaching these types of concerns with other scientists.

For this exercise, students proposed specific details that would lead them to believe that either (a) misconduct had been committed by the previous student or (b) the previous student had not committed misconduct. This gave students the opportunity to become creatively engaged with the material, and opened up the discussion to a wider variety of circumstances and issues than if they were given a single, completely defined case study. In one instance, a student proposed a situation based directly on his previous research experience. This illustrated both the plausibility of these sorts of situations and the importance of the resulting discussion.

Case Study - “Key Result”

You are a second year student, and you have been working in your thesis lab for several months. You are working on the document that you will submit to your candidacy committee. Your thesis research is quite ambitious and your advisor has warned you that your committee members are likely to be skeptical. Nevertheless, you are confident that it will work and by hard work and luck, have acquired an early result from one of your key experiments that demonstrates proof of concept for your work. While you are writing the paper, a fact comes to light that casts doubt on the veracity of your results (see below). There is not time to repeat the experiment before the deadline for your candidacy document.

In which of these situations (and with what qualifiers) is it permissible to include the questionable experimental results in your document and presentation?

Situation A)

You realize that you did not perform a necessary control to rule out a likely confounding factor in your key experiment.

Situation B)

A serious technical problem is brought to your attention (e.g. the instrument you collected data on was incorrectly calibrated, etc).

Situation C)

You find a paper with an experiment very similar to yours, but which has a significantly different result, and from which the authors draw conclusions which indicate that your idea should not work.

Box IV-2. Example of a case study presented to students. In this situation, students were given an example of a situation where they might be tempted to commit misconduct.

Case Study - “Continuing a Project”

You have inherited a project from a 6th year graduate student who has left the lab. The student's work was published as a communication, and your thesis research will be a series of follow-up studies based on her initial results. Your advisor suggests that you repeat some of her experiments to “test your hands” at the technique she had been using. After running the experiments as written up in the experimental section of her communication for several weeks, you are unable to reproduce the data. You go through her notebooks and contact her via email in order to try and determine what is going wrong with your experiments.

- Propose two situations/results from your actions (emailing and looking through notebooks) which would cause you be concerned about misconduct, and two situations which would not.

(For example, if the student emails back that there is a minor technical issue that she forgot to record, and once you this into account, your experimental results are consistent with those in the paper, misconduct is unlikely).

- For one of the situations in which misconduct may be an issue, propose both a reasonable course of action (who would you talk to, what would you say) and a less reasonable course of action for dealing with the situation.

Box IV-3. Example of a case study presented to students. In this situation, students were given an example of a situation where they suspect misconduct has been committed by a co-worker.

The second portion of this case study centered on appropriate responses to situations where a student believed misconduct may have occurred. Specifically, students proposed professional ways of dealing with this conflict and noted the importance of presenting the facts of the case, rather than their suspicions or opinions about the former student. During these discussions, students again brought up the possible affects on

stakeholders in this situation and suggested courses of action would entail the least harm to those involved. For example, some students initially found it reasonable to first take these concerns to a party outside the lab for a second opinion (in particular, a former advisor or other trusted faculty member). Upon further discussion, they realized that this option has potential consequences for both themselves and their current advisor. They also pointed out that this solution fails the reversibility test, as they would be displeased with the advisor or former student broaching concerns about their work to one of their peers rather than to them.

Finally, case studies that involved conflicts but no overt ethical concerns were also included. In one such case study (Box IV-4), students discussed a potential conflict that they might encounter where no ethical issues were at stake. For this issue, students were quick to realize that even though there were no ethical concerns, they still needed to handle this situation in a professional and respectful manner. Students pointed out that there were certain hierarchies they should follow in getting this problem resolved (ie, first talk to the safety officer, then the advisor, then the departmental head of safety) while still maintaining good relations with their advisor/lab-mates. The students also discussed ways to avoid conflicts with lab-mates, such as asking to be re-located within the lab.

Case study- “Safety Issue”

You are rotating in the lab you wanted to join. Your rotation project is going well, and you get along well with most of your lab-mates. However, the post-doc who works next to you often leaves flasks of the chemicals she is working with uncapped and on the bench. You have politely asked her to deal with them in an appropriate manner, but she has told you not to worry about it – none of them are especially toxic, and besides part of your job as a chemist is putting up with unpleasant smells.

- Propose reasonable and unreasonable courses of action for each of the following conditions:

- 1) The chemical is not particularly toxic (eg. acetone, ethyl acetate).
- 2) The chemical is something potentially toxic or carcinogenic (eg. hexanes, dichloromethane).
- 3) The chemical readily decomposes to hydrogen cyanide (volatile and lethal).
- 4) Changes to any of the above, if the lab's advisor is out of town for an extended period of time.

Box IV-4. Example of a case study presented to students. Students were given an example of a situation where there were no concerns of ethical misconduct; however the situation still needed to be addressed in a courteous and respectful manner.

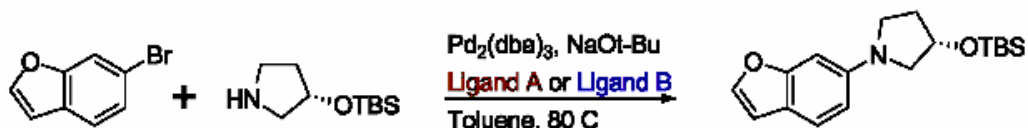
G. Student Responses to the Data Interpretation Exercise

As part of the 2010 module, a new data interpretation exercise was introduced. For this exercise, students were told that during their first rotation, one of their collaborators presented them with a series of data they had collected. The student who had collected this data then proposed a series of conclusions that could be drawn from this data. The students were asked to determine which conclusions they would

accept/reject/amend based on the data presented. They were also asked if any of the conclusions represented ethical concerns (see Section K). When discussing the example shown in Box IV-5, the students had several thoughts on the validity of the conclusions presented. Many students felt that conclusion A posed a problem because it stated that the reaction ran “smoothly” in 95% yield. They believed that without a valid experimental reason as to why the other three trials gave such low yields, the use of the word “smoothly” was misleading. Additionally, students noted that either conclusion C or D (using the mean or the median to represent the average value) would be a valid way to report the data, as long as the person reporting the data included which statistical method was used. One common theme throughout this exercise was the need for transparency in discussing how raw data had been interpreted. These exercises also served as excellent vehicles for discussing the differences between sloppy science, reasonable disagreements among professionals and misconduct.

As was the case with the case studies, we were pleased with the students’ ability to overlay the framework for solving ethical dilemmas onto the data interpretation exercises. The students engaged with the material and applied some of the ideas that had been discussed to these diverse situations. This indicates that the students are beginning to internalize the tools needed to appropriately address complex moral concerns.

The following reaction was run with the reported yields:



Ligand A

Run#	Yield
1	95%
2	26%
3	33%
4	29%

Which of the following conclusions would you accept/amend/reject?

a) Using the most effective ligand (Ligand A) the reaction occurred smoothly in 95% yield.

b) The reaction proceeded in higher yield with Ligand B (48% vs. 33% with Ligand A).

c) Ligand A (mean yield 46%) was more effective at facilitating the reaction than ligand B (mean yield 38%).

d) Ligand B (median yield 48%) was more effective at facilitating the reaction than ligand A (median yield 31%)

Ligand B

Run#	Yield
1	56%
2	48%
3	47%
4	0%

Are there circumstances under which any of these are ethical problems?

Box IV-5. Example of a situation presented to students as part of the data interpretation exercise.

H. Using Student Feedback to Improve the Curriculum

At the end of the module each year, students were given a short questionnaire asking them to identify what they believed to be the most and least useful aspects of the module. The student responses from 2009 indicate that they found the module to generally be valuable and to provide interesting information. In particular, the students appreciated the case studies and the emphasis placed on handling conflicts in a

professional manner. Students also stated that they found the open question and answer sessions to be valuable.

One prevalent suggestion that students gave was to decrease the amount of time allotted for small group discussions. They stated that they found ten minutes to be adequate time for discussion (they had originally been asked to discuss each case for twenty minutes). We agreed with the students comments in this area. In the 2009 session, we had envisioned that we, the peer leaders, would have a greater opportunity to address the small groups. In practice, however, there were too many students (~60) for us to be able to effectively reach all of the groups in a timely manner.

These observations led to changes in the way the case studies were dealt with. First, we re-wrote the cases to give more written details so that the students could have a fuller small group discussion without relying on iterative input from the peer leaders. Second, in decreasing the time spent on the small group discussion, more time was opened up for another activity. In order to fill this gap, the data interpretation exercise was created and incorporated.

The course evaluations from the 2010 indicate that students again found the case studies to be useful. As there were no comments about the time allotted for small group discussions, we concluded that ten minutes of discussion was sufficient. The students also commented that they found the data interpretation exercises to be useful and interesting.

I. Response from the Chemical Community

In evaluating the uniqueness of this module, we wanted to gauge the response by the chemical community. We presented this work at the Spring 2010 National ACS conference where it was met with positive comments from members of the chemical education community.²⁰ This work was also featured as part of the October 2010 ACS graduate student bulletin in an article focused on research ethics in graduate school.²¹ This positive response indicates that a peer-led approach to teaching research ethics is a unique and valuable addition to current methods for accomplishing this task.

J. Conclusions and Future Directions

Recent events have highlighted the need for formal ethics training for all researchers.^{3,4,5} Graduate students are a key audience for this type of training as they are often carrying out full-time research for the first time in their lives. A peer-led approach to teaching research ethics was developed for use as part of the graduate student orientation curriculum. It was postulated that senior graduate students would provide a valuable perspective in teaching research ethics to their junior colleagues as they have unique insight into the issues facing current graduate students. It was also postulated that using senior graduate students as instructors would send the message to the incoming students that research ethics is important to their peer group, and thus should be important to them. This course relied heavily on the use of case-studies and a data interpretation exercise as a means for discussing complex ethical dilemmas. The case studies and data interpretation exercises were designed by the peer-leaders to reflect real-life conundrums that first and second year graduate students were likely to face. The

effectiveness of this module was then gauged by student responses during large group discussion. As the students' responses during the large group discussions incorporated many of the ideas that were presented earlier in the module, it can be concluded that the students were beginning to grasp the complexities inherent in ethical decision making. Additionally, responses from student evaluations and the chemical community at large indicate that the peer-led module is a valuable addition to the curriculum for teaching research ethics.

The future of research ethics training in the Chemistry Department at the University of Michigan will be heavily influenced by the work described here. Starting in the Fall 2011 semester, a 1-credit research ethics course will be taught to chemistry department researchers. This course will be designed and initially co-taught by myself and Dr. Fierke and will likely incorporate many of the positive aspects of the peer-led module including the use of case studies as vehicles for discussion and a focus on immediacy, practicality and identifying resources who can be helpful in advising students facing an ethical dilemma. It is hoped that senior graduate students in the chemistry department will contribute ideas for case studies based on their experiences so that this course will adapt to an ever changing research climate.

Additionally, in the long term, a graduate student instructor (GSI) could be assigned to this course so that senior graduate students would have a direct and continued involvement in the planning and implementation of teaching research ethics. Recitation sections, which are traditionally taught by GSIs, would be an optimal place for senior students to interact with the younger students without a faculty member present. Alternatively, students who have expressed an interest in teaching as a career and are

involved in the GAANN program could work with the faculty member(s) teaching this course to maintain a student presence and to give insight into the every day demands and situations facing current students.

Additionally, it is hoped that ethics training could be more deeply incorporated into the research environment in the chemistry department. While a formal ethics course is certainly valuable, it will not be sufficient to address all student concerns regarding research ethics. This is especially true as the nature of ethical concerns students face tend to change during the course of graduate school. For example, as students become more independent and take on mentorship roles, they are exposed to a variety of concerns such as authorship issues, ethical considerations for engaging in a mentoring relationship, and concerns about forming collaborations that younger students generally will not contend with. Designated senior peer-leaders could act as mediators for formal or informal discussions among other senior graduate students to address ethical issues that arise during the course of research. This would foster an open environment for discussing ethical concerns thus increasing the likelihood that ethical dilemmas will be addressed in a productive manner.

Finally, an effective means for tracking and measuring how effectively students deal with ethical concerns they face outside of the classroom should be established. With the first class of students who were exposed to the peer-led module reaching candidacy in 2011, it would be valuable to chart what effects this module had on student thinking and attitudes as they progressed through their first two years of graduate school. A follow-up survey should be conducted with students in the summer of their second year (soon after most students finish candidacy) to determine which aspects of this course they found

valuable. Specifically, it should be noted how students approached any ethical dilemmas they may have encountered.

As a more long-term way of charting the effects of this course, one could envision discussions with senior faculty members, particularly those who have served or are serving on the Graduate Committee. If formal training in research ethics does elicit a shift in how students approach ethical decisions, faculty members who have mentored several generations of students should note a difference in attitudes of these younger students.

K. Course Material

K.1. Case Studies

Case Study #1: Rotation Project

You are several weeks into a rotation in the research lab that you would like to join. Although the lab's research focus interests you, the senior graduate student you are working with is having you run reactions for his methodology project according to a protocol that he has already developed and using substrates that he has already decided upon.

This lab only will only be accepting a single new student at the end of the year, and has already had three rotators. You do not have much direct interaction with the lab's advisor, and other lab members have told you that whether or not you are selected to join the lab will depend almost entirely on the presentation you give at the end of your rotation. This presentation is given to the entire group, including the advisor, and you will be expected to present and discuss the work you have completed during your rotation. You are concerned that your lack of creative input in your project will negatively affect the quality of your presentation and your chances at being accepted into this lab.

- Are there circumstances under which this would not be a problem (describe them)? How would you go about determining whether or not this is the case?
- Assuming that it turns out to be a problem, propose both a reasonable and unreasonable solution.

Case Study #3: Authorship

As part of your rotation, you run an experiment to identify an unknown protein by MS/MS. You perform the technical aspects of the experiment, under the supervision of your rotation mentor. Your mentor also helps you work up and analyze the data. Based on these results, the protein is tentatively identified. After your rotation ends, you hear that the group is writing up a paper in which the identification of this protein is used as supporting evidence for a proposed regulatory mechanism.

- Under what circumstances is your omission from the authorship list reasonable? Under what circumstances is it a problem? If it is a problem, how could you go about addressing this?
- One of the key questions is how significant of a contribution you made to the experiments resulting in the initial identification of this protein. Propose factors that (1) would clearly indicate that you made a significant contribution to the project, (2) would clearly indicate that you did not make a significant contribution, or (3) fall somewhere in between.

Case Study #6: Inspiration

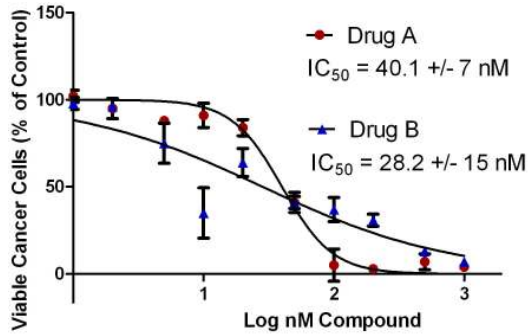
You are finishing up a rotation in the lab you intend to join, and have an upcoming meeting with your future advisor in which the two of you will discuss potential thesis projects. All during your rotation, you had been thinking about a project based on a future direction presented by a graduating PhD student in the lab you worked in as an undergraduate. You think this project fits in nicely with what your grad lab is doing, builds on your current skills and interests, and will be exciting enough to send out as a JACS or perhaps a PNAS paper if it works. You are sure that your new advisor would be as excited about this idea as you are.

- What practical and ethical concerns should you have about proposing this project?
- For the following situations, propose both a reasonable and unreasonable course of action.
 - 1) Your undergraduate advisor is retiring and you know they are no longer carrying out research.
 - 2) You know the project is intended as an REU project for the next summer.
 - 3) The original idea for this project came from either an experimental result of yours (as an undergraduate) or a conversation between you and your undergraduate advisor and/or the graduate student who mentored you.

K.2. Data Interpretation Exercise

The ability of drugs A and B to kill cancer cells was measured

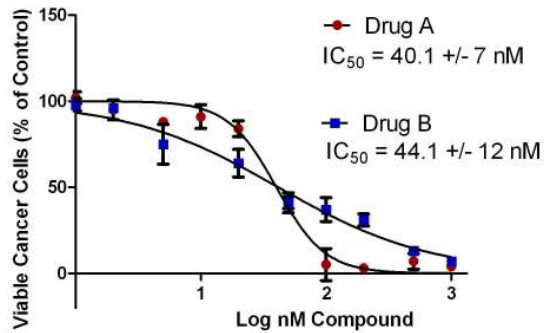
Graph 1



Which of the following conclusions would you accept/amend/reject?

a) Based on graph 1, drug B is more potent than drug A.

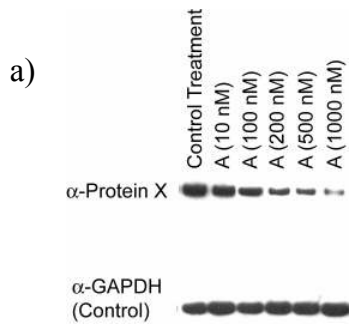
Graph 2 - with offline point removed



b) Removing the off-line data point at X=1 for drug B produces graph 2. Graph 2 is more appropriate for publication.

Are there circumstances under which any of these are ethical problems?

The ability of a 'treatment' (drug, RNAi, etc) to alter expression of protein X was determined by Western Blot

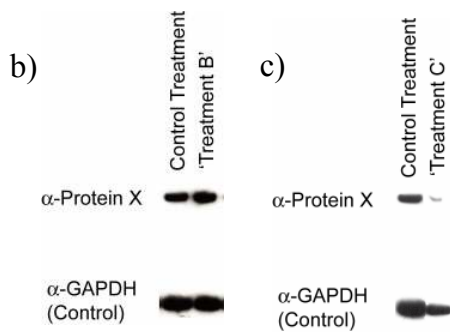


Which of the following conclusions would you accept/amend/reject?

a) Treatment A reduces expression of protein X with an EC_{50} of approximately 100 nM.

b) Treatment B induces a ~20% increase in expression of protein X.

c) Treatment C reduces expression of protein X.



Are there circumstances under which any of these are ethical problems?

This control may be omitted initially for an additional discussion point

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Chapter V

Conclusions and Future Directions

A. Conclusions

A.1. Synthesizing and Identifying Small Molecule Probes for Targeting Transcriptional Co-factors

The first part of my doctoral work focused on synthesizing and identifying small molecule probes for targeting transcriptional co-factors. As endogenous transcriptional activators exhibit a multi-partner binding profile, it is necessary to synthesize a diverse library of small molecules to modulate these interactions.^{1,2,3} In order to swiftly obtain a diverse array of small molecules that can be screened for their ability to modulate transcription, building block molecules that can be further transformed to a variety of more complex structures should be employed.

The allenamide scaffold is one such building block that has been used in the synthesis of a variety of small heterocycles.^{4,5,6,7,8,9} The work presented here includes a novel methodology for the facile synthesis of highly substituted allenamides. This methodology, which can be accomplished in few synthetic steps from readily available starting materials, allows for the formation of mono-, di- and tri-substituted allenamides in good yields. The methodology presented also allows for the formation of enantiomerically enriched allenamides provided that an enantioenriched propargylic alcohol is employed in the first step. It was discovered that due to the unique electronic nature of the allene system, these allenamides display a reactivity pattern more similar to

electron-neutral allenes than to other, more electron-rich allenamides. These allenamides were employed in the synthesis of diverse secondary structures. It is therefore likely that these allenamides could serve as a scaffold for the formation of highly substituted heterocycles that could be screened for their ability to modulate transcription.

My thesis work also focused on using small molecule probes to identify protein co-factors that are essential in maintaining self-renewal in cancer initiating cells (CICs). It was discovered that transcription driven by Nanog (a protein that is over-expressed in CICs and is associated with maintenance of pluripotency and self-renewal) is dependent on the co-factor p300 respectively but not its paralog CBP. In order to elucidate which domain(s) of p300 were essential for Nanog-driven transcription, two synthetic probes (an amphipathic isoxazolidine and a hydrogen bond surrogate) that bind specifically to the KIX and CH1 domains of p300 were tested for their ability to inhibit Nanog-driven gene expression. It was found that both probes caused a reduction in transcription, indicating that both the KIX and CH1 domains of p300 are involved in Nanog-driven gene expression.

In order to examine if disrupting the functionality of these domains was sufficient to elicit a change in the ability of CICs to self-renew, CICs were dosed with amphipathic isoxazolidine and relative viabilities were measured. It was found that at higher doses, the amphipathic isoxazolidine had a cytostatic effect on embryonal carcinoma cells (a CIC model system). Additionally, when the molecule was dosed into a mammalian cell line that contained a mixture of differentiated bulk tumor cells and undifferentiated CICs, a reduction in the CIC population but not the bulk tumor cell population was observed. These findings indicate that the KIX domain of p300 is necessary for Nanog-driven gene

expression and that disruption of KIX function is sufficient to decrease self-renewal in CICs. These findings also indicate that disruption of Nanog functionality could be a novel target for the development of therapeutics targeted against CICs, which as of yet have proven to be largely resistant to traditional chemotherapeutics.

A.2. Design and Implementation of a Peer-Led Practical Research Ethics Module for Teaching Graduate Research Ethics

The need for formal training in research ethics has been well established.^{10,11} It has been noted that formal education in research ethics is most effective when it is taught in a creative manner emphasizing practicality and support networks in addressing ethical concerns.¹² To this end, a portion of my thesis work focused on designing and implementing a peer-led practical research ethics module for teaching graduate research ethics. This module aimed to provide incoming graduate students with the knowledge they would need to address complex ethical concerns they were likely to face in their early graduate careers. In order to facilitate discussions, several case studies were designed by the peer-leaders addressing ethical dilemmas faced by the 2006 and 2007 chemistry department cohorts. The incoming students discussed these case studies in both large and small groups. The student responses given during the large group discussions incorporated many of the topics discussed on effective ways to address ethical concerns. These responses indicate that the module was successful in giving students the tools they need to address complex ethical issues. Responses given in the course evaluation survey indicate that students found the course to be useful. Additionally, the responses from the chemical community indicate that the peer-led

method for teaching research ethics is a valuable addition to the current methods for teaching research ethics to graduate students.

B. Future Directions

B.1. Determining Domains of p300 Responsible for Nanog-Driven Gene Expression

Studies with synthetic ligands of the KIX and CH1 domains of p300 indicate that these domains are necessary for Nanog-driven gene expression. However, it is possible that other p300 domains may play a role in this process. In order to test this, each of the individual domains of p300 will be over-expressed *in cellulo*. These over-expressed domains should effectively compete with p300 for endogenous binding partners, therefore disrupting the ability of p300 to act as a bridge between a DNA-bound activator and the transcriptional machinery (Figure V-1). By mapping out the p300 domains involved in Nanog-driven gene expression, further synthetic probes can then be designed to bind to each of the relevant domains. These probes will be tested for their ability to bind p300. Probes that are found to bind p300 will then be dosed into either a head and neck squamous cell carcinoma (HSNCC) or embryonal carcinoma (EC) cell line. Post-treatment, the levels of messenger RNA (mRNA) levels of Nanog-regulated genes (ex. Nanog, Oct4, Sox2, EBAF1, etc) will be measured by quantitative polymerase chain reaction (qPCR) as a means of determining the *in cellulo* efficacy of these molecules in inhibiting endogenous Nanog-driven gene expression.

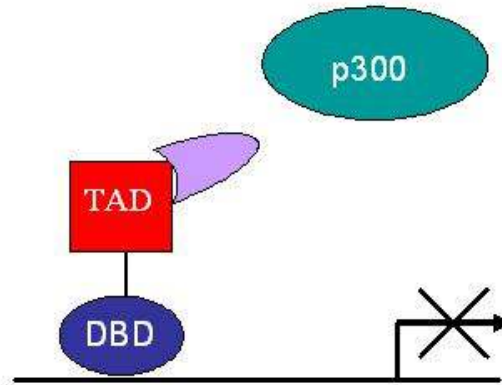


Figure V-1. Determining the reliance of Nanog –driven gene expression on individual domains of p300. Individual domains of p300 (here represented as a purple rounded wedge) will be over-expressed in cells. Domains that bind to the Nanog TAD (here a red square) compete for binding with endogenous p300. This competition causes a decrease in the level of Nanog-driven transcription.

B.2. Determining the Effects of Inhibiting p300 Functionality in Maintaining Pluripotency and Self-Renewal

Results derived from a reporter context will provide valuable insight into the domains of p300 necessary for Nanog-driven gene expression. It will be necessary to determine if perturbing the functionality of p300 will have an effect on the maintenance of self-renewal. To this end, the synthetic molecules will be dosed into HNSCC cells to determine their ability to reduce CIC self-renewal as measured by fluorescence activated cell sorting (FACS) or tumor sphere formation studies. Compounds that are able to selectively inhibit the ability of CICs to self renew will be used in further studies.

Additional studies will be carried out to determine if synthetic ligands of p300 have the ability to disrupt pluripotency in CICs. These studies will be carried out in an embryonal carcinoma cell line, NCCIT, which has been previously used as a model CIC system. NCCIT cells will be dosed with synthetic ligands that bind specific domains of

p300, or short hairpin RNA (shRNA) targeting p300. The messenger RNA (mRNA) levels of genes associated with differentiated (ex. lineage markers such as CDX2, GATA4, BRACHYURY, and MAP2) and undifferentiated (ex. Nanog, Sox2, Oct4, ALDH) cellular phenotypes will be monitored for treated and controlled cells.¹³ Compounds that elicit expression of relatively higher levels of differentiation markers relative to pluripotency markers will be selected for further testing.

In addition to bridging functionality, p300 also has histone acetyltransferase (HAT) activity. Studies carried out by Han and co-workers show that molecules capable of inhibiting histone deacetylation cause a loss of pluripotency and self-renewal in EC cells.¹³ Additionally, it has been shown in embryonic stem cells (ESCs) that p300 plays a role in Nanog-driven gene expression by acetylating a distal regulatory region of the Nanog promoter.¹⁴ It is therefore likely that a highly optimized level of histone acetylation must be achieved for cells to remain in a pluripotent state. It is possible that disrupting the HAT activity of p300 could lead to a decrease in CIC pluripotency and self-renewal. Synthetic molecules that specifically inhibit the HAT activity of p300 and CBP relative to other proteins with HAT capabilities have been discovered in the Cole laboratories.^{15,16} These compounds will be tested by the aforementioned methods to measure their ability to disrupt self-renewal capabilities and pluripotency in CICs, thus determining if p300 HAT activity is necessary for the maintenance of these characteristics in CICs.

B.3. Determining the *In Vivo* Functionality of p300 Ligands

In order to determine the ability of p300 ligands to affectively target CICs *in vivo*, HNSCC cells with constitutive luciferase expression will be implanted into NOD/SCID mice. After initial tumors have formed, the mice will be intravenously dosed with p300 ligands that were effective *in cellulo* at inhibiting pluripotency and self-renewal in CICs. The growth of the initial tumor and the extent of lung metastasis will be monitored for 4-6 weeks. It is likely that some of the probes used in cell culture will have to be modified to maximize their *in vivo* effectiveness.

At the termination of the experiment, the remaining tumor bulk will be analyzed for its CIC content. Additionally, normal stem cells will be examined in mice treated with compounds versus control mice to determine if disrupting p300 functionality will selectively affect CICs rather than normal stem cells. While normal stem cells and CICs share many similar biochemical characteristics, there are limited examples of therapeutics that are able to target CICs without damaging normal stem cells.¹⁷ If these molecules are able to selectively target CICs *in vivo*, it would provide evidence that targeting the functionality of p300 is a tractable means for eliminating this cell population. This would be a large advancement in the field of cancer research as cancer initiating cells have proven to be resistant to traditional chemotherapeutics and ionizing radiation.^{18,19}

B.4. Synergistic Approach for Targeting CICs.

My thesis work has focused on the transcription factors that are responsible for maintaining pluripotency and self-renewal in CICs. Currently, a small number of potential therapeutics have been identified that target pathways and cell surface markers that are present in CICs and not in bulk tumor cells. As some of these therapeutics have

entered phase-III clinical trials, it is likely that at least a subset of them will prove to be effective therapies for treating poorly differentiated cancers in the clinic.¹⁷ It is possible, however, that these therapies in isolation may be insufficient to fully eliminate the CIC population of a given cancer. It is therefore necessary to devise combinatorial approaches to target multiple aspects of the CIC self-renewal and pluripotency pathways.

Current, unpublished work in the Mapp laboratories is focused on using a combinatorial approach to inhibit the growth of erbB2 positive breast cancer cells. In these studies, it was found that combining a small molecule ligand capable of modulating transcription of the erbB2 gene with therapeutics that are currently used in the clinic to target positions in related pathways caused a synergistic decrease in the viability of erbB2 + cancer cells. These results indicate that simultaneously inhibiting transcription of a certain gene and key points in related pathways may prove to be a tractable therapeutic strategy for treating cancers. The synthetic ligands of p300 found elicit a decrease in the ability of CICs to self-renew and maintain pluripotency should therefore be tested in tandem with known inhibitors of CIC-specific pathways for their ability to synergistically affect pluripotency and self-renewal of these cells. Also, combinations of small molecule transcription inhibitors that bind to distinct domains of p300 should be tested to determine if they are able to synergistically inhibit p300 functionality. Similarly, combination strategies involving treatment with active p300 ligands and traditional chemotherapeutics designed to treat bulk tumor cells should be tested for their ability to completely eliminate tumors.

B.5. Strategies for Developing Small Molecule Ligands that Target Transcription Co-factors

B.5.A Synthesizing Small Molecules from the Allenamide Scaffold

Allenamides have proven to be quite versatile as a substrate for the formation of a variety of highly substituted heterocycles. Allenamides are therefore a valuable building block scaffold for the synthesis of a variety of small molecules that could act as ligands for transcription co-factors. The allenamides synthesized in the methodology reported in this thesis differ from previously synthesized allenamides in that the allene moiety is less electron rich. This difference in electronics causes these allenamides to adopt a reactivity pattern analogous to hydrocarbon allenes rather than previously reported allenamides. As such, they could be used as substrates in further reactions that have previously been demonstrated to work with allenes.^{20,21} These reactions could lead to the formation of molecules that could be screened for their ability to bind to transcription co-factors (Figure V-2). Additionally, as these heterocycles are rather small, they could be used included in a fragment library for fragment-based NMR or sulfide-tether screening.

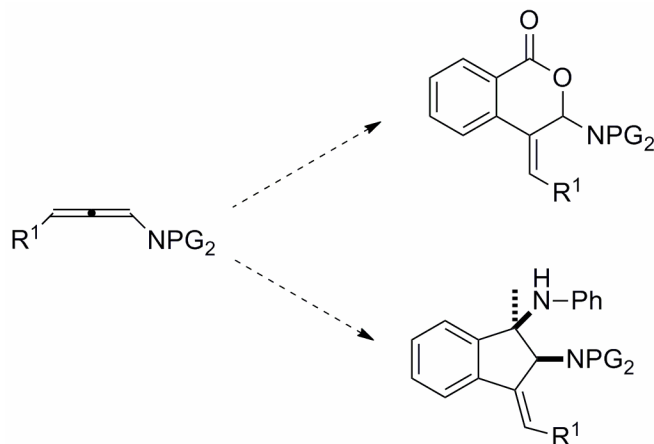


Figure V-2. Small molecules that could potentially be synthesized from an electron-neutral allenamide. Molecules of the nature shown on the left have been previously synthesized from an allene starting material. Due to the unique electronic nature of the allenamides discussed in this thesis, it is likely that they could be used as substrates in these types of reactions to produce a variety of highly substituted small molecules that could be screened for their ability to modulate transcription.

B.5.B. High Throughput Screens for Identifying Natural Product Ligands

One method that is currently being investigated for identifying novel ligands of p300/CBP is the screening of natural product libraries. From an initial screen of 67,000 compounds and natural product extracts, researchers in the Mapp laboratories have identified a class of known natural products, the depsides, which are capable of inhibiting the interaction of the KIX domain of CBP with one of its endogenous ligands (MLL). After initial identification, one molecule, sekikaic acid, was taken on to additional studies. Using fluorescence polarization (FP) and HSQC NMR experiments, it was determined that sekikaic acid is capable of binding in a way that affects both ligand binding domains of KIX (Figure V-3). This initial finding highlights the utility of this approach in identifying potential small molecule ligands of transcription co-factors. As the other compounds that were identified as hits in the original screen were also depsides, non-

natural molecules based on this scaffold could also be synthesized to and tested for their ability to bind to the KIX domain.

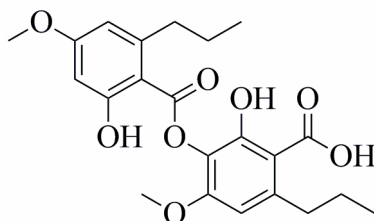


Figure V-3. Structure of sekikaic acid. Sekikaic acid is a natural product that was discovered in the Mapp laboratories to bind to the KIX domain of CBP.

Additional screens on these and other libraries could be carried out using other domains of CBP to identify small molecule ligands capable of binding to them. This will be especially useful for identifying small molecule ligands of CBP domains, such as the CH2 domain, which currently have no known inhibitors.

B.6. Improvements to Current Methodologies for Teaching Research Ethics to Graduate Students

The peer-led method for teaching research ethics has proven to be an effective means for teaching research ethics to first year graduate students in chemistry at the University of Michigan. The use of peer-leaders in teaching research ethics should be expanded to other departments at the University of Michigan and in other chemistry departments across the nation. One way to accomplish a change in the way that ethics is taught in chemistry departments is through partnership with the ACS. By working with the ACS Committee on Ethics, it may be possible to include more graduate students, post-docs and undergraduates directly in developing curriculum for teaching research ethics to their peer groups.

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Appendix

Design and Synthesis of a Focused Library of Spirooxindoles to Modulate the Function of Transcription Co-factors

A. Abstract

Activated transcription is a complicated event that requires the interactions of many different proteins. One type of interaction that is of specific interest to the Mapp laboratories is the interaction of DNA-bound activators with their co-factors in the transcriptional machinery. The co-factors p300 and its paralog the Creb Binding Protein (CBP) have been shown to bind to a number of different transcription factors. In order to better understand the roles of these large, multi-domain proteins, small molecules that bind to specific domains can be employed. To this end, a small focused library of spirooxindoles was synthesized. The spirooxindole scaffold was chosen as spirooxindoles are readily synthesized and highly functionalizable. The library that was synthesized for this work was designed to bind to the CH1 domain of p300/CBP. *In cellulo* studies indicate that four of the spirooxindoles were able to disrupt the functionality of an activator known to bind to the CH1 domain of p300/CBP (HIF-1 α). These compounds also disrupted the functionality of three other amphipathic activators that do not interact with the CH1 domain of p300/CBP. This indicates that these small molecules do not specifically inhibit the function of the CH1 domain; however, they may still be used as probes for studying the general inhibition of amphipathic activators.

B. Introduction

B.1. The role of CBP/p300 in Activated Transcription

Activated transcription is initiated by a class of proteins known as transcriptional activators. These activators are modular in nature consisting of a DNA-binding domain (DBD) and a transcriptional activation domain (TAD). Once the DBD binds to a specific site on DNA, the TAD then makes contacts with other members of the transcriptional machinery that are necessary for the recruitment of RNA Polymerase II (RNA Pol II).¹ The Creb-Binding Protein (CBP) and its paralog p300 have been shown to contact several transcriptional activators that are implicated in disease states.^{2,3} Additionally, there is increasing evidence the p300 may play a role in the maintenance of pluripotency and self-renewal in cancer imitating cells (CICs).^{4,5} In order to better study the roles CBP/p300 play in transcription, small molecule ligands that bind specific domains of these proteins can be employed.

The CH1 domain of CBP/p300 may play an important role in the maintenance of pluripotency and self-renewal in CICs.⁶ Additionally, there is evidence that the binding of the CH1 domain to the hypoxia inducible factor-1 α (HIF-1 α) is necessary for survival of solid tumors and that disruption of this interaction could be sufficient to cause a decrease in the size of such tumors.⁷ As such, synthetic probes that perturb the function of the CH1 domain could provide valuable insight into the underlying cellular mechanisms regulating tumor maintenance and growth.

B.2. Small Molecule Ligands of the CH1 Domain of p300/CBP

In 2006, when the work that will be discussed in this appendix was started, there was only one synthetic molecule that was able to effectively bind the CH1 domain of p300/CBP. This small molecule, the fungal metabolite chetomin, was first found to bind to the CH1 domain by Livingston and co-workers as a part of a high throughput screen of natural products (Figure A-1).⁸ Chetomin proved to bind the p300 CH1 domain with submicromolar affinity. However, there is currently no known synthetic route to chetomin, making it difficult to obtain for further uses as a synthetic probe.

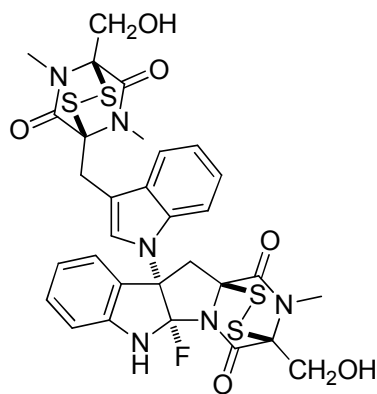


Figure A-1. Structure of chetomin.

B.3. Designing Small Molecule Ligands of the CH1 Domain of p300/CBP

Most of the endogenous TADs that bind the CH1 domain of p300/CBP are amphipathic in nature containing a mix of both polar and hydrophobic residues.¹ Many amphipathic TADs are unstructured in solution but fold into α -helices upon binding to co-activator targets with the hydrophobic residues providing the bulk of the contact with the target protein.⁹ The Mapp laboratories had previously found that amphipathic, conformationally constrained small molecules can effectively bind to members of the

transcriptional machinery.^{10,11,12,13} In order to expand the repertoire of available small molecules that can act as a probes for perturbing specific transcription events, a small focused library based on the spirooxindole scaffold was developed. This scaffold was chosen because spirooxindoles are readily synthesized, highly functionalizable and offer a radial, helix-like display of functionality (Figure A-2).¹⁴ Additionally, spirooxindole derivatives have been shown to effectively inhibit the binding of an amphipathic TAD (p53) to its masking partner MDM2.^{15,16}

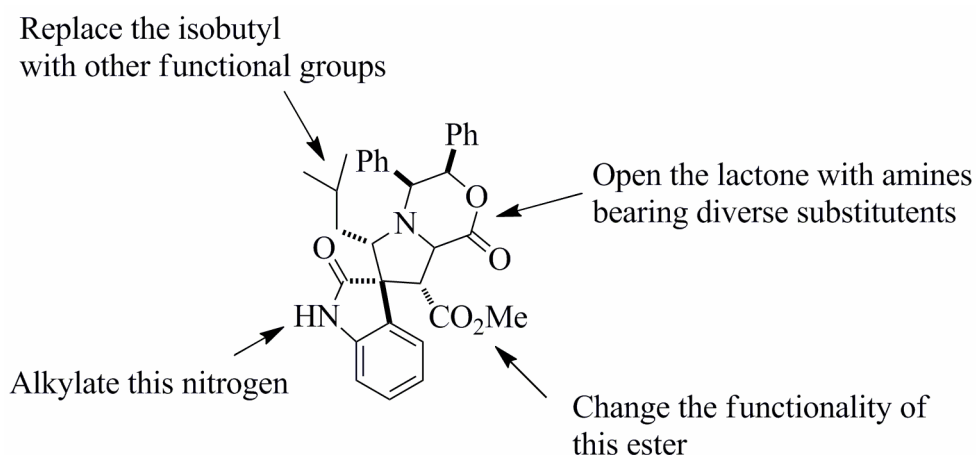


Figure A-2. Spirooxindole scaffold. Sites on the molecule that could be modified to introduce additional functionality are indicated.

C. Synthesis of a Focused Library of Spirooxindoles

The parent spirooxindole for the library was synthesized in good yield (86%) via a route developed by Sebahar and co-workers (Figure A-3).¹⁷ Several sites on the molecule were identified as potential locations for appending additional functionality (Figure A-2). Opening the lactone of the spirooxindole proved to be an accessible way to introduce modifications and the ring-opening reactions proceeded in good to moderate yields (78-53%) (Figure A-4). A number of amines were used to introduce straight-chain and branched hydrophobic functionality at this position. These types of

hydrophobic residues were included as it is known that one of the native CH1 ligands, HIF-1 α , relies on three key leucine residues for binding to the CH1 domain.¹⁸ It was believed that increasing the hydrophobicity of the molecule and including functional groups that would mimic the endogenous leucines would be advantageous.

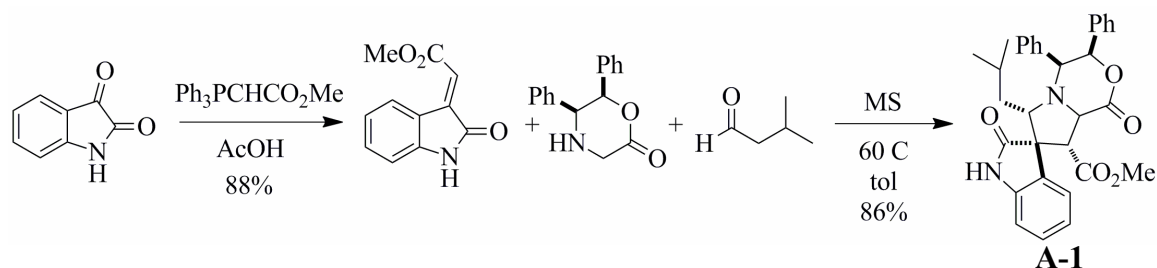


Figure A-3. Synthetic route for the formation of spirooxindole **A-1**. Conditions: 1) isatin (1.0 equiv), methyl (triphenylphosphoranylidene) acetate (1.0 equiv) in acetic acid, 90 °C, 6 hours. 2) Indole (1.5 equiv), morpholine (1.0 equiv), aldehyde (1.2 equiv) molecular sieves, in toluene, 60 °C, 1 hour.

A subset of the molecules that were made for the initial library were further modified by deprotecting the pyrrolidine nitrogen via a radical reaction with cerium ammonium nitrate (CAN) (Figure A-4). This was done to investigate the best balance of hydrophobicity and size for synthesizing effective ligands. The resultant molecules were become quite unstable and they usually decomposed completely on the bench top within 1-2 days of synthesis.

Attempts to modify the parent spirooxindole at other locations were met with limited success. It is possible to introduce phenyl and methoxy-phenyl substituents in place of the isobutyl group simply by using the corresponding aldehyde in the original cycloaddition (Figure A-4). These molecules were then subjected to the same ring-opening conditions as the original parent scaffold. Attempts to alter the ester moiety were unsuccessful, as were attempts to append functionality to nitrogen atom of the indole.

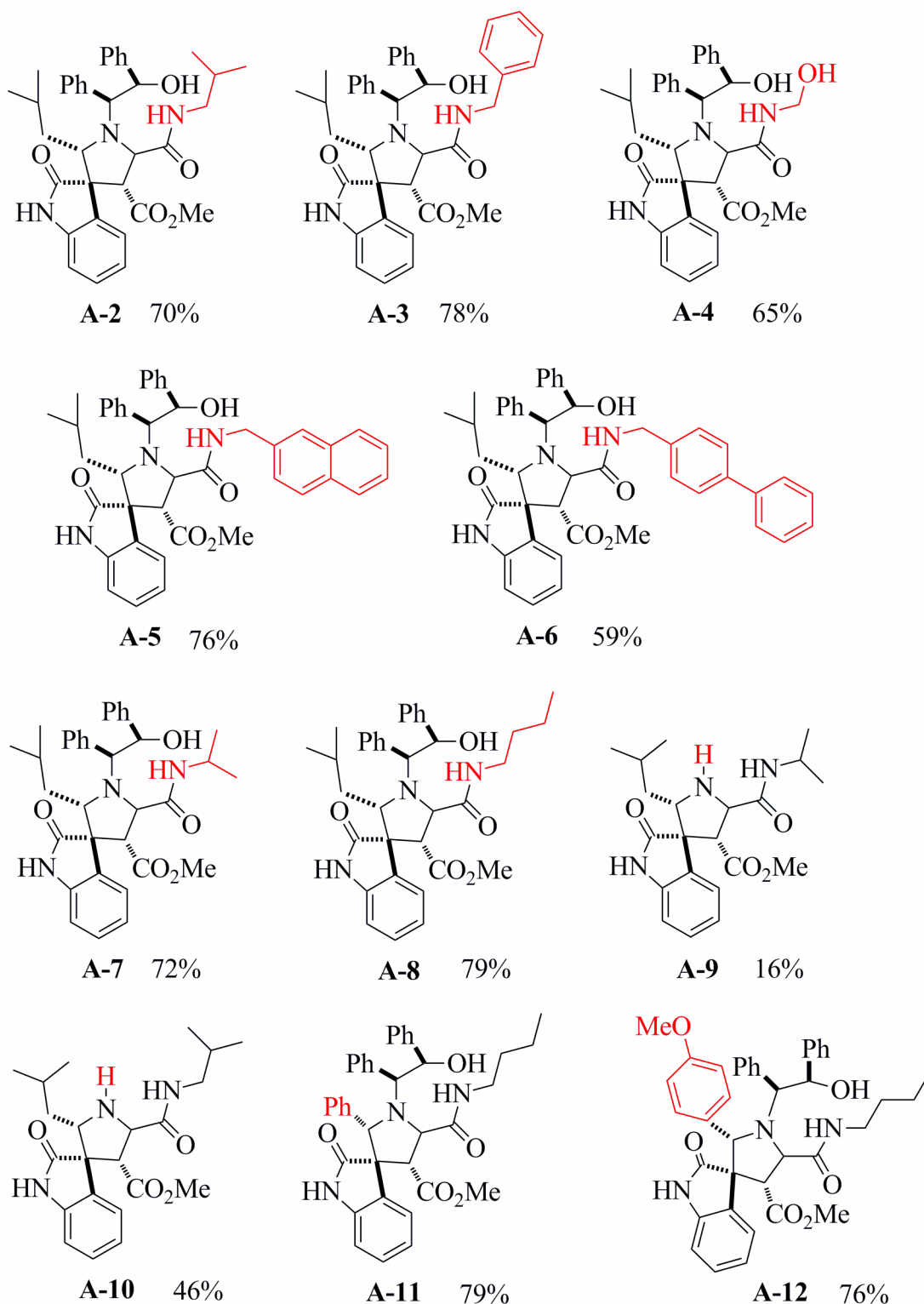


Figure A-4. Spirooxindole library. These spirooxindoles were synthesized to be screened for their ability to inhibit the functionality of the CH1 domain of p300/CBP. Differences in functionality are highlighted in red. The parent spirooxindoles were synthesized as stated in Figure A-2. Conditions for ring opening reaction: spirooxindole (1.0 equiv), primary amine (1.5.0 equiv), Et₃N (1.5.0 equiv) in THF, rt, overnight.

Conditions for the deprotection of the nitrogen: ring opened spirooxindole (1.0 equiv), CAN (2.0 equiv) in MeCN/H₂O, rt, 15 mins. Reported yields are of the final synthetic step required to form the structure.

D. Determining the Ability of Spirooxindoles to Inhibit Activator·Co-activator Interactions

A competitive inhibition experiment was conducted with the minimal activation domain of HIF-1 α (a known endogenous binding partner of the CH1 domain of p300/CBP^{7,16}) and the synthesized spirooxindoles to assess whether these compounds bound overlapping targets. A former post-doc in the Mapp laboratories, Dr. J.P. Desaulniers, synthesized a plasmid that encoded for the Gal4 DBD fused to the minimal HIF-1 α activation domain (amino acids 786-826) to be used in these experiments. For these experiments, Dr. Desaulniers transfected HeLa cells with this plasmid causing the cells to express the fusion protein. Upon binding to the Firefly luciferase reporter plasmid, the HIF-1 α TAD recruited its target proteins (including p300/CBP) and upregulated transcription of the reporter gene. The inhibition of the TAD·co-factor interactions by the small molecules would cause a decrease in reporter gene expression. This is quantified by luminescence of Firefly luciferase, compared to *Renilla* luciferase (used as a transfection control). Four of the spirooxindoles assayed caused a decrease in Firefly luciferase expression (Figure A-5).

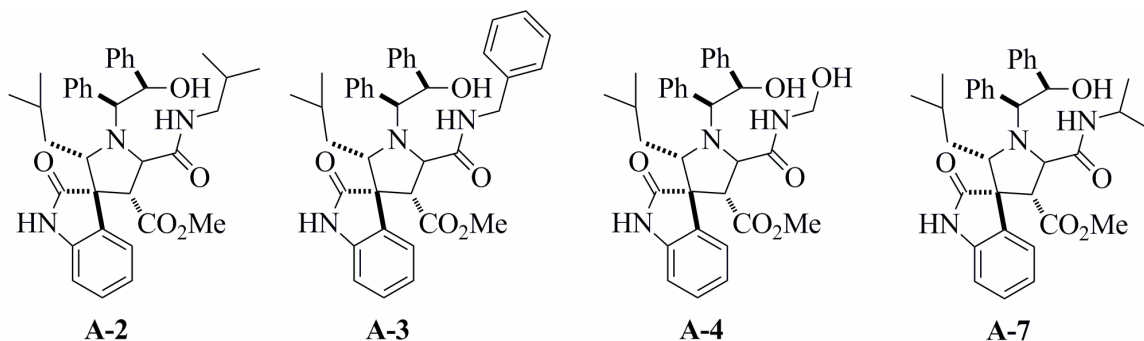
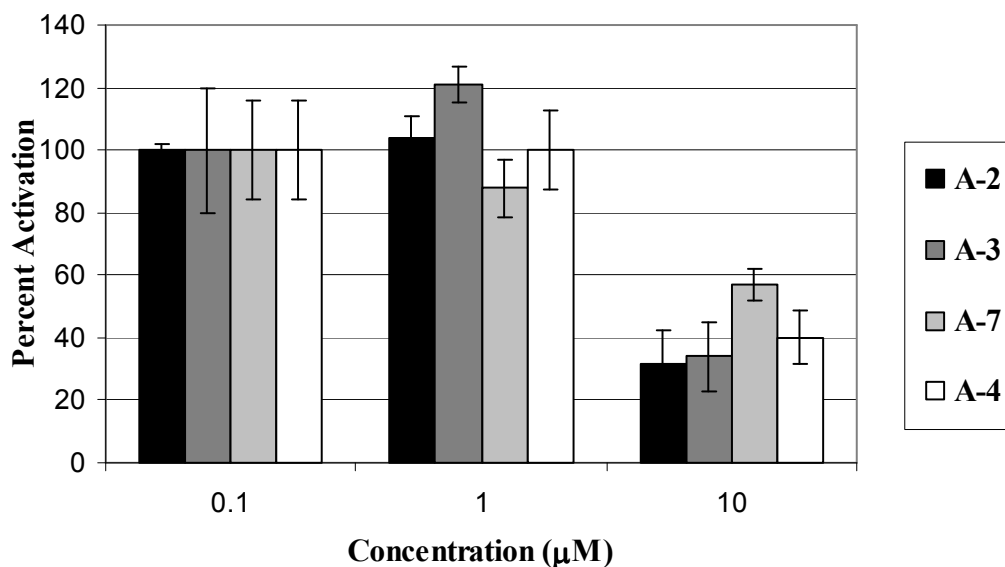
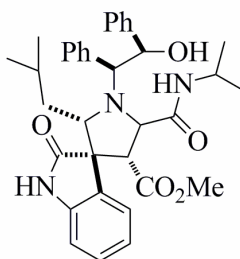
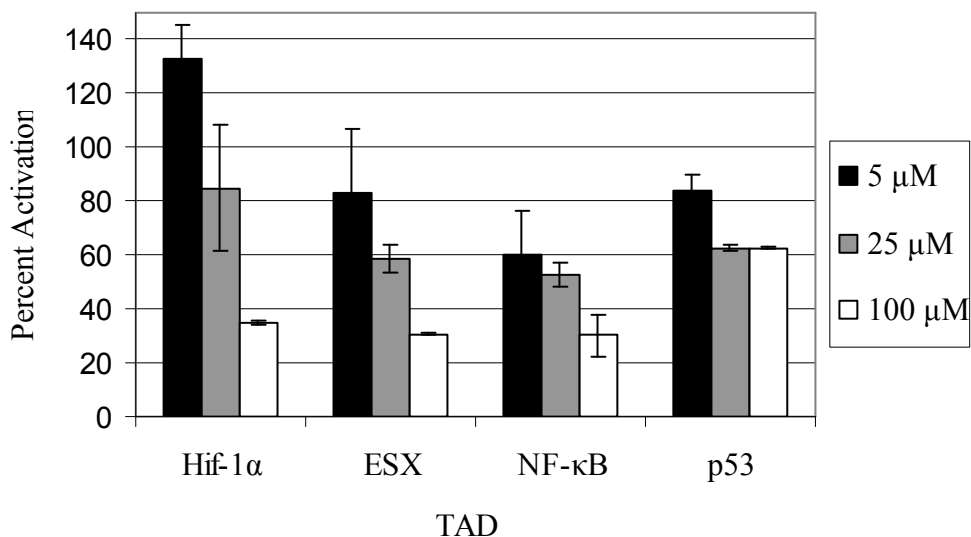


Figure A-5. Spirooxindoles that inhibited HIF-1 α -driven transcription. The four spirooxindoles shown caused a decrease in activation of a HIF-1 α -driven reporter system. Briefly, HeLa cells were transfected with a plasmid expressing the Gal4-HIF-1 α minimal TAD fusion protein, a second plasmid bearing five Gal4-binding sites upstream of a Firefly luciferase reporter gene, and a third plasmid expressing *Renilla* luciferase as a transfection control. Compound was added to the cells as a DMSO solution 3 hours after transfection to a final DMSO concentration of 1% (v/v). The Firefly and *Renilla* luciferase activities were measured 24 hours after compound addition using the Promega Dual Luciferase Kit. Fold activation was determined at each concentration by first dividing the Firefly luciferase by the *Renilla* luciferase. This value was then divided by the activity observed at 0.1 μ M compound. Each value is the mean of three independent experiments with the indicated error (SDOM).

To determine if these compounds bound specifically to the CH1 domain of p300/CBP, competition assays between other TADs known to interact with other proteins besides the CH1 domain were performed by a colleague Jonas Højfeldt. Plasmids

encoding for Gal4-fusions of these activation domains were made and this assay was carried out analogously to HIF-1 α competition assay. It was found that the compounds inhibited the expression of all of the tested activators, indicating that these compounds do not specifically bind to the CH1 domain of p300/CBP (Figure A-6). These compounds could possibly be useful as global inhibitors of co-factors that bind α -helical TADs.



A-7

Figure A-6. Spirooxindoles do not specifically inhibit the function of the CH1 domain of p300/CBP. Spirooxindoles found to inhibit HIF-1 α functionality were assayed for their ability to inhibit the functionality of other amphipathic activators. These compounds inhibited activation driven by these activators to similar levels as they did HIF-1 α indicating they do not specifically affect the function of the CH1 domain of p300/CBP. Experiments were carried out as described in Figure A-4 with the exception that the minimal TADs of ESX, NF- κ B and p53 were used in place of HIF-1 α .

E. Conclusions

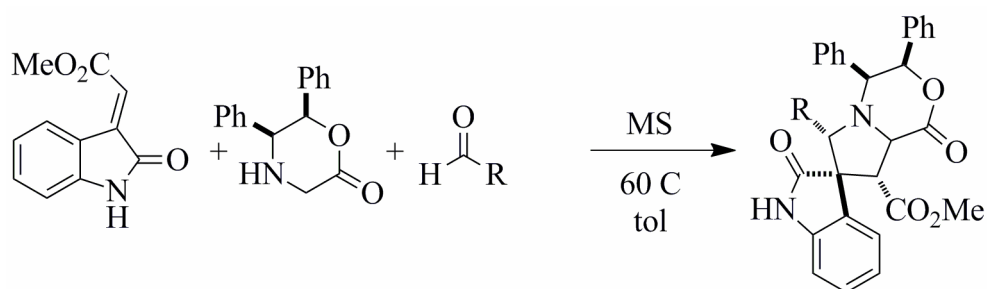
A focused library of spirooxindoles was synthesized and assayed for its ability to inhibit the interactions of HIF-1 α and its endogenous co-factor the CH1 domain of p300/CBP. The spirooxindole scaffold was originally chosen for this work as it is highly functionalizable and allows for a radial display of functionality. Of the 11 spirooxindoles synthesized, four of them were able to down-regulate the expression of a luciferase reporter gene regulated by a fusion protein of the Gal4-DBD with minimal TAD of HIF-1 α . These compounds were also found to inhibit the functionality of three other amphipathic activators that bind to distinct co-factors. This indicates that the spirooxindoles do not specifically inhibit the functionality of the CH1 domain of p300/CBP. These compounds may be used in the future as global inhibitors of the function of amphipathic activators. Additionally, it is possible that these compounds disrupt the interaction of a distinct set of proteins necessary for the transcription of all four of the tested activators and could prove to be useful as probes for understanding the functionality of a different member of the transcriptional machinery.

F. Experimental

Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. THF and toluene were dried by passage through activated alumina columns. Purification by flash chromatography was carried out with E. Merck Silica Gel 60 (230-400 mesh) according to the procedure of Still, Kahn, and Mitra.¹⁹ All reactions involving air- or moisture-sensitive compounds were

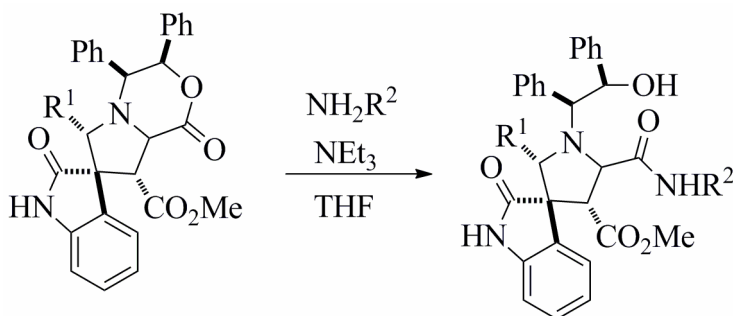
performed under a nitrogen atmosphere. (E)-methyl 2-(2-oxoindolin-3-ylidene)acetate²⁰ and (5R,6S)-5,6-diphenyl-1,3-oxazinan-2-one²¹ were prepared according to literature procedures. ¹H NMR spectra were recorded in CDCl₃ at 400 or 500 MHz.

General procedure for spirooxindole synthesis



To toluene (80 mL) was added (E)-methyl 2-(2-oxoindolin-3-ylidene)acetate (4.8 mmol, 1.5 equiv), (5R,6S)-5,6-diphenyl-1,3-oxazinan-2-one (7.2 mmol, 1.0 equiv), aldehyde (5.8 mmol, 1.2 equiv) and 3 Å activated molecular sieves (0.9g). The solution was heated to 60 °C and stirred for 1 hour with monitoring by TLC (3:1 hexanes/ethyl acetate). The toluene was removed under vacuum and the crude mixture was subjected to flash chromatography (3:1 hexanes/ethyl acetate to 1:1 hexanes/ethyl acetate) to provide the product spirooxindole.

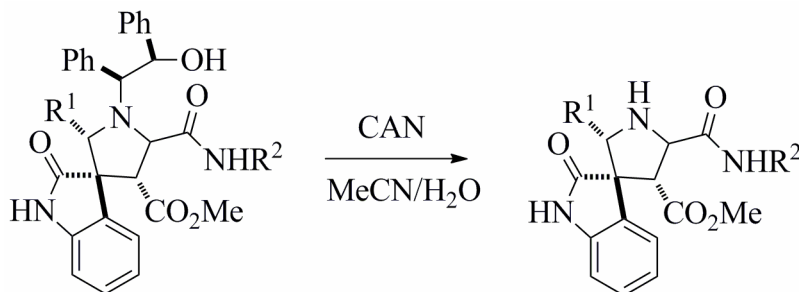
General procedure for opening the spirooxindole lactone



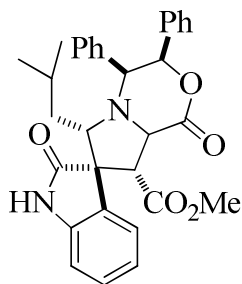
To THF (1.0 mL) spirooxindole (0.1 mmol, 1.0 equiv) primary amine (1.5 mmol, 1.5 equiv) and NEt₃ (1.5 mmol, 1.5 equiv) were added. The reaction was stirred at room

temperature overnight. The THF was removed under vacuum and the crude mixture was subjected to flash chromatography (3:2 hexanes/ethyl acetate).

General procedure for the nitrogen deprotection



To MeCN (0.6 mL) and water (0.3 mL) spirooxindole (0.06 mmol, 1.0 equiv) and CAN (0.1 mmol, 2.0 equiv) were added. The reaction was stirred at room temperature for 15 minutes with monitoring by TLC (3:2 hexanes/ethyl acetate). The reaction was quenched with water, extracted with EtOAc (3x, 1 mL), dried over Na₂SO₄, filtered and concentrated under vacuum. The crude mixture was subjected to flash chromatography (3:2 hexanes/ethyl acetate to 1:1 hexanes/ethyl acetate).

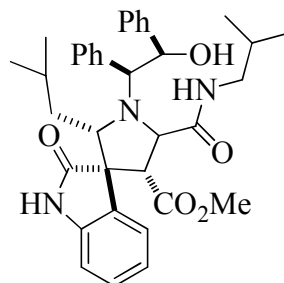


A-1

(3R,3'R,4'S,6'S,8'R)-methyl 6'-isobutyl-1',2-dioxo-3',4'-diphenyl-1',3',4',6',8',8a'-hexahydrospiro[indoline-3,7'-pyrrolo[2,1-c][1,4]oxazine]-8'-carboxylate (A-1):

Prepared via the general procedure in 86% yield as a pale yellow solid. ¹H NMR: δ 0.59

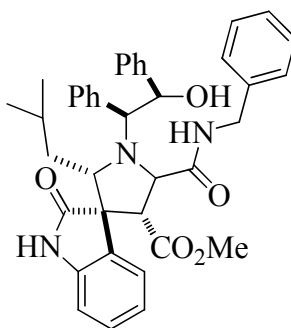
(d, $J = 6.4$ Hz, 3H), 0.73 (d, $J = 6.0$ Hz, 3H), 1.0-1.1 (m, 1H), 1.2-1.3 (m, 2H), 3.2 (s, 3H), 3.8 (dd, $J = 3.2, 7.8$, 1H), 4.0 (d, $J = 8.0$ Hz, 1H), 4.5 (d, $J = 3.2$ Hz, 1H), 4.9 (d, $J = 8.0$ Hz, 1H), 6.1 (d, $J = 3.2$ Hz, 1H), 6.9 (d, $J = 7.6$ Hz, 1H), 7.0 (td, $J = 7.6, 0.8$ Hz, 1H), 7.2-7.3 (m, 14H), 7.8 (s, 1H). HRMS (ESI) calcd for $[C_{32}H_{32}N_2O_5+Na]^+$: 547.2209, found: 547.2222.



A-2

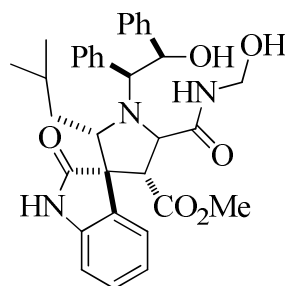
(2'S,3R,4'R)-methyl 1'-((1S,2R)-2-hydroxy-1,2-diphenylethyl)-2'-isobutyl-5'-(isobutylcarbamoyl)-2-oxospiro[indoline-3,3'-pyrrolidine]-4'-carboxylate (A-2):

Prepared via the general procedure in 70% yield as a white solid. 1H NMR: δ 0.56 (d, $J = 7.0$ Hz, 3H), 0.62 (d, $J = 6.5$ Hz, 3H), 0.86 (d, $J = 7.0$ Hz, 6H), 1.1 (t, $J = 7.0$ Hz, 1H), 1.5-1.6 (m, 2H) 1.7 (sept, $J = 7.0$ Hz, 1H), 2.9-3.0 (m, 1H), 3.1-3.2 (m, 1H) 3.2 (s, 3H), 3.7 (d, $J = 9.0$ Hz, 1H), 3.8 (d, $J = 9.0$ Hz, 1H), 4.3 (dd, $J = 3.2, 7.0$ Hz, 1H), 4.6 (d, $J = 5.0$ Hz, 1H), 4.9 (s, 1H), 5.4 (d, $J = 5.0$ Hz, 1H), 5.8 (t, $J = 5.0$ Hz, 1H), 6.9 (t, $J = 7.5$ Hz, 2H), 7.0 (t, $J = 7.5$ Hz, 1H), 7.1 (t, $J = 7.5$ Hz, 1H), 7.2-7.3 (m, 6H), 7.4 (d, $J = 7.0$ Hz, 2H), 7.5 (d, $J = 7.5$ Hz, 2H), 8.0 (s, 1H). HRMS (ESI) calcd for $[C_{36}H_{43}N_3O_5+Na]^+$: 620.3100, found: 620.3107.



A-3

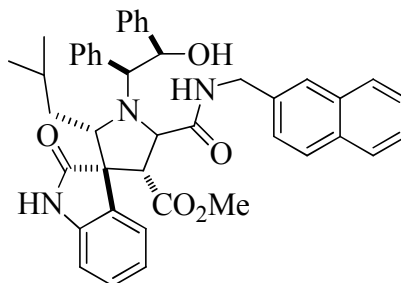
(2'S,3R,4'R)-methyl 5'-(benzylcarbamoyl)-1'-((1S,2R)-2-hydroxy-1,2-diphenylethyl)-2'-isobutyl-2-oxospiro[indoline-3,3'-pyrrolidine]-4'-carboxylate (A-3): Prepared via the general procedure in 78% yield as a white solid. ^1H NMR: δ 0.54 (d, $J = 6.0$ Hz, 3H), 0.57 (d, $J = 6.0$ Hz, 3H), 0.87-0.90 (m, 1H), 1.0-1.1 (m, 2H), 1.4-1.5 (m, 1H), 3.1 (s, 3H), 3.8 (q, $J = 10$ Hz, 2H), 4.3 (d, $J = 11$ Hz, 1H), 4.4 (dd, $J = 6, 15$ Hz, 1H), 4.6 (dd, $J = 6, 15$ Hz, 1H), 4.6 (d, $J = 5.0$ Hz, 1H), 5.4 (d, $J = 5.0$ Hz, 1H), 6.9 (t, $J = 5.0$ Hz, 1H), 6.9 (d, $J = 7.5$ Hz, 2H), 7.1-7.3 (m, 10H), 7.3 (d, $J = 7.5$ Hz, 2H), 7.4 (d, $J = 7.0$ Hz, 2H), 7.5 (s, 2H) 8.3 (s, 1H). HRMS (ESI) calcd for $[\text{C}_{39}\text{H}_{41}\text{N}_3\text{O}_5+\text{Na}]^+$: 654.2944, found: 654.2957.



A-4

(2'S,3R,4'R)-methyl 1'-((1S,2R)-2-hydroxy-1,2-diphenylethyl)-5'-((hydroxymethyl)carbamoyl)-2'-isobutyl-2-oxospiro[indoline-3,3'-pyrrolidine]-4'-carboxylate (A-4): Prepared via the general procedure in 65% yield as a white solid. ^1H NMR: δ 0.25 (d, $J = 6.4$ Hz, 3H), 0.44 (d, $J = 6.4$ Hz, 3H), 0.92 (t, $J = 10.4$ Hz, 2H), 1.2

(t, $J = 14$ Hz, 1H), 3.2 (s, 3H), 3.4 (s, 1H) 3.4-3.5 (m, 2H) 3.6 (m, 1H), 3.7-3.8 (m, 3H), 3.8-3.9 (m, 2H), 4.6 (d, $J = 8.4$ Hz, 1H), 5.4 (d, $J = 8.4$ Hz, 1H), 6.7 (d, $J = 7.2$ Hz, 1H), 6.9 (d, $J = 7.6$ Hz, 1H), 7.0 (t, $J = 6.8$ Hz, 1H), 7.2-7.5 (m, 6H), 7.5 (t, $J = 7.6$ Hz, 2H), 7.7 (d, $J = 6.8$ Hz, 2H), 7.8 (d, $J = 6.8$ Hz, 2H) HRMS (ESI) calcd for $[\text{C}_{33}\text{H}_{37}\text{N}_3\text{O}_6+\text{Na}]^+$: 608.2737, found: 608.2744.

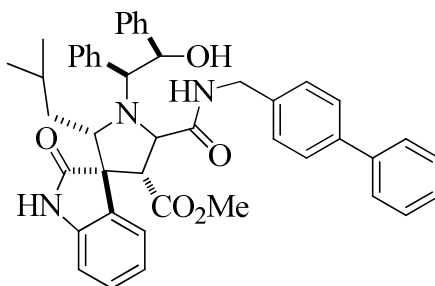


A-5

(2'S,3R,4'R)-methyl 1'-((1S,2R)-2-hydroxy-1,2-diphenylethyl)-2'-isobutyl-5'-

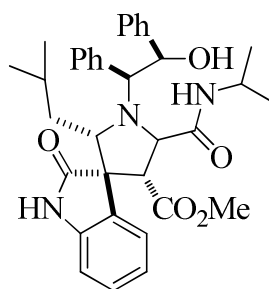
((naphthalen-2-ylmethyl)carbamoyl)-2-oxospiro[indoline-3,3'-pyrrolidine]-4'-

carboxylate (A-5): Prepared via the general procedure in 76% yield as a white solid. ^1H NMR: δ 0.51 (d, $J = 6.5$ Hz, 6H), 0.98-1.0 (m, 2H), 1.4-1.5 (m, 1H), 3.0 (s, 3H), 3.7 (d, $J = 9.5$ Hz, 1H), 3.9 (d, $J = 9.5$ Hz, 1H), 4.2 (dd, $J = 2.5, 10$ Hz, 1H), 4.6 (d, $J = 6.5$ Hz, 1H), 4.8 (dd, $J = 5.0, 15$ Hz, 1H), 5.0 (dd, $J = 6.0, 14$ Hz, 1H), 5.4 (d, $J = 6.0$ Hz, 1H), 5.9 (t, $J = 5.5$ Hz, 1H) 6.8 (d, $J = 7.5$ Hz, 1H), 6.9 (d, $J = 7.5$ Hz, 1H), 6.9 (t, $J = 8.0$ Hz, 1H), 7.1-7.2 (m, 7H), 7.3 (m, 2H), 7.4-7.5 (m, 2H), 7.5 (m, 2H), 7.6 (d, $J = 7.0$ Hz, 2H), 7.8 (dd, $J = 2.5, 6.5$ Hz, 1H), 7.9 (m, 1H), 8.1 (m, 1H), 8.1 (s, 1H). MS (ESI) calcd for $[\text{C}_{43}\text{H}_{43}\text{N}_3\text{O}_5+\text{Na}]^+$: 704.3, found: 704.3.



A-6

(2'S,3R,4'R)-methyl 5'-([1,1'-biphenyl]-4-ylmethyl)carbamoyl-1'-((1S,2R)-2-hydroxy-1,2-diphenylethyl)-2'-isobutyl-2-oxospiro[indoline-3,3'-pyrrolidine]-4'-carboxylate (A-6): Prepared via the general procedure in 59% yield as a white solid. ¹H NMR: δ 0.56 (d, *J* = 6.5 Hz, 3H), 0.59 (d, *J* = 6.5 Hz, 3H), 1.0 (td *J* = 10, 2.5 Hz, 1H), 1.1 (m, 1H), 1.5 (td, *J* = 10, 2.5 Hz, 1H), 3.2 (s, 3H), 3.8 (d, *J* = 9.0 Hz, 1H), 3.8 (d, *J* = 9.0 Hz, 1H), 4.2 (dd, *J* = 3.5, 11 Hz, 1H), 4.3 (dd, *J* = 5.5, 15 Hz, 1H), 4.5 (dd, *J* = 5.5, 15 Hz, 1H), 4.6 (d, *J* = 5.5 Hz, 1H), 5.4 (d, *J* = 5.5 Hz, 1H), 6.1 (t, *J* = 6.0 Hz, 1H), 6.9 (d, *J* = 7.5 Hz, 1H) 6.9 (d, *J* = 7.5 Hz, 1H), 7.0 (t, *J* = 7.5 Hz, 1H), 7.1-7.2 (m, 1H), 7.2-7.3 (m, 6H), 7.3-7.4 (m, 3H), 7.4 (d, *J* = 7.0 Hz, 2H), 7.5 (t, *J* = 7.5 Hz, 2H), 7.6 (dd, *J* = 2.0, 7.5 Hz, 2H), 7.6 (m, 4H), 7.8 (s, 1H). MS (ESI) calcd for [C₄₅H₄₅N₃O₅+Na]⁺: 730.3, found: 730.3.

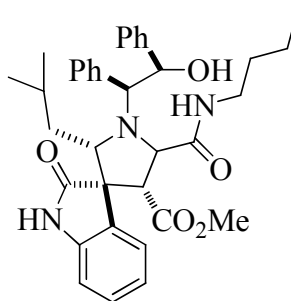


A-7

(2'S,3R,4'R)-methyl 1'-((1S,2R)-2-hydroxy-1,2-diphenylethyl)-2'-isobutyl-5'-(isopropylcarbamoyl)-2-oxospiro[indoline-3,3'-pyrrolidine]-4'-carboxylate (A-7):

Prepared via the general procedure in 72% yield as a white solid. $^1\text{H NMR}$: δ 0.50 (d, $J = 6.4$ Hz, 3H), 0.56 (d, $J = 6.4$ Hz, 3H), 0.98 (d, $J = 6.8$ Hz, 3H), 1.0-1.1 (m, 1H), 1.4 (d, $J = 6.4$ Hz, 3H), 1.2-1.3 (m, 2H), 1.5 (m, 1H), 3.1 (s, 3H), 3.7 (dd, $J = 9.4, 13$ Hz, 2H), 4.0 (m, 1H), 4.2 (dd, $J = 3.2, 10$ Hz, 1H), 4.6 (d, $J = 5.2$ Hz, 1H), 4.7 (s, 1H) (d, $J = 5.2$ Hz, 1H), 5.5 (d, $J = 8.0$ Hz, 1H), 6.9 (d, $J = 7.5$ Hz, 1H) 6.8 (t, $J = 7.2$ Hz, 2H), 6.9 (m, 1H), 7.1-7.2 (m, 7H), 7.3 (d, $J = 7.6$ Hz, 2H), 7.5 (d, $J = 7.6$ Hz, 2H), 8.2 (s, 1H).

HRMS (ESI) calcd for $[\text{C}_{35}\text{H}_{41}\text{N}_3\text{O}_5+\text{Na}]^+$: 606.2944, found: 606.2940.

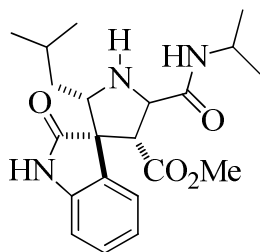


A-8

(2'S,3R,4'R)-methyl 5'-(butylcarbamoyl)-1'-((1S,2R)-2-hydroxy-1,2-diphenylethyl)-

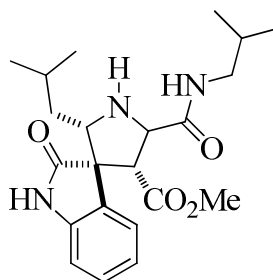
2'-isobutyl-2-oxospiro[indoline-3,3'-pyrrolidine]-4'-carboxylate (A-8): Prepared via

the general procedure in 79% yield as a white solid. $^1\text{H NMR}$: δ 0.55 (d, $J = 6.4$ Hz, 3H), 0.62 (d, $J = 6.0$ Hz, 3H), 0.85-0.93 (m, 4H), 1.0-1.1 (m, 2H), 1.2-1.5 (m, 4H), 3.2 (s, 3H), 3.2 (m, 2H), 3.7 (dd, $J = 9.4, 19$ Hz, 2H), 4.2 (dd, $J = 3.5, 9.4$ Hz, 1H), 4.3 (d, $J = 5.0$ Hz, 1H), 4.8 (s, 1H), 5.4 (d, $J = 5.0$ Hz, 1H), 6.8 (t, $J = 3.5$ Hz, 2H), 6.9 (dd, $J = 7.6, 15$ Hz, 2H), 7.1-7.3 (m, 6H), 7.4 (d, $J = 7.2$ Hz, 2H) 7.5 (d, $J = 7.2$ Hz, 1H) HRMS (ESI) calcd for $[\text{C}_{36}\text{H}_{43}\text{N}_3\text{O}_5+\text{Na}]^+$: 620.3100, found: 620.3111



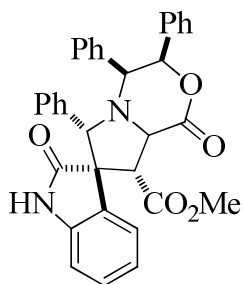
A-9

(2'S,3R,4'R)-methyl 2'-isobutyl-5'-(isopropylcarbamoyl)-2-oxospiro[indoline-3,3'-pyrrolidine]-4'-carboxylate (A-9): Prepared via the general procedure in 16% yield as a pale yellow oil. $^1\text{H NMR}$: δ 0.75 (d, $J = 6.8$ Hz, 6H), 0.83-0.88 (m 1H), 1.1 (d, $J = 6.8$ Hz, 3H), 1.2 (d, $J = 6.4$ Hz, 3H), 1.6 (sept, $J = 6.0$ Hz, 1H) 3.2 (s, 3H), 3.5 (d, $J = 5.6$ Hz, 1H), 3.9 (d, $J = 7.6$ Hz, 1H), 4.0 (m, 1H), 4.5 (d, $J = 7.6$ Hz, 1H), 6.9 (d, $J = 7.6$ Hz, 1H) 7.0 (t, $J = 7.6$ Hz, 1H), 7.1 (s, 1H), 7.1-7.2 (m, 2H), 8.1 (s, 1H). MS (ESI) calcd for $[\text{C}_{21}\text{H}_{29}\text{N}_3\text{O}_4+\text{Na}]^+$: 410.2, found: 410.2.



A-10

(2'S,3R,4'R)-methyl 2'-isobutyl-5'-(isobutylcarbamoyl)-2-oxospiro[indoline-3,3'-pyrrolidine]-4'-carboxylate (A-10): Prepared via the general procedure in 46% yield as a pale yellow oil. $^1\text{H NMR}$: δ 0.79 (d, $J = 3.6$ Hz, 6H), 0.79-0.94 (m, 2H), 0.95 (d, $J = 6.4$ Hz, 6H), 1.5 (sept, $J = 6.8$ Hz, 1H), 1.8 (sept, $J = 6.8$ Hz, 1H) 2.0-3.1 (m, 2H), 3.2 (s, 3H), 3.5 (dd, $J = 4, 9.2$ Hz, 1H), 3.9 (d, $J = 7.6$ Hz, 1H), 4.6 (d, $J = 7.6$ Hz, 1H), 6.9 (d, $J = 7.6$ Hz, 1H) 7.0 (t, $J = 7.6$ Hz, 1H), 7.1 (d, $J = 7.6$ Hz, 1H), 7.2 (d, $J = 7.6$ Hz, 1H), 7.4 (s 1H), 8.3 (s, 1H). MS (ESI) calcd for $[\text{C}_{22}\text{H}_{31}\text{N}_3\text{O}_4+\text{Na}]^+$: 424.2 found: 424.2.

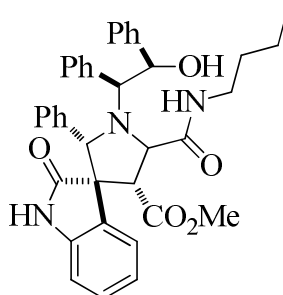


A-13

(3R,3'R,4'S,8'R)-methyl 1',2-dioxo-3',4',6'-triphenyl-1',3',4',6',8',8a'-

hexahydrospiro[indoline-3,7'-pyrrolo[2,1-c][1,4]oxazine]-8'-carboxylate (A-13):

Prepared via the general procedure in 55% yield as a pale yellow solid. $^1\text{H NMR}$: δ 3.3 (s, 3H), 5.1 (s, 1H), 5.2 (d, $J = 10$ Hz, 1H), 6.3 (d, $J = 4.0$ Hz, 1H), 6.6 (d, $J = 10$ Hz, 1H), 6.9 (t, $J = 10$ Hz, 1H), 7.0-7.3 (m, 16H), 7.6 (s, 1H). MS (ESI) calcd for $[\text{C}_{34}\text{H}_{28}\text{N}_2\text{O}_5+\text{Na}]^+$: 567.2 found: 567.2.

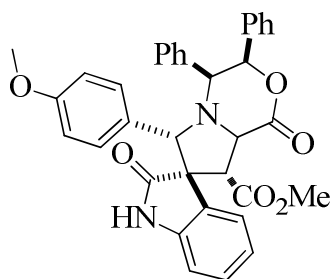


A-11

(2'S,3R,4'R)-methyl 5'-(butylcarbamoyl)-1'-((1S,2R)-2-hydroxy-1,2-diphenylethyl)-

2-oxo-2'-phenylspiro[indoline-3,3'-pyrrolidine]-4'-carboxylate (A-11): Prepared via

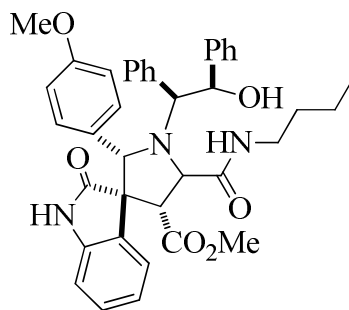
the general procedure in 79% yield as an ivory solid. $^1\text{H NMR}$: δ 0.89 (t, $J = 7.6$ Hz, 3H), 1.2-1.4 (m, 4H), 3.2 (s, 3H), 3.2 (quart, $J = 6.8$ Hz, 2H), 3.9 (d, $J = 8.8$ Hz, 1H), 4.0 (d, $J = 8.4$ Hz, 1H), 4.6 (d, $J = 4.8$ Hz, 1H), 5.1 (d, $J = 4.4$ Hz, 1H), 5.2 (s, 1H), 5.6 (t, $J = 2.5$ Hz, 1H), 6.6 (d, $J = 7.2$ Hz, 1H), 7.0-7.3 (m, 18H), 7.5 (s, 1H), 7.5 (s, 1H). MS (ESI) calcd for $[\text{C}_{38}\text{H}_{39}\text{N}_3\text{O}_5+\text{Na}]^+$: 640.2 found: 640.2.



A-14

(3R,3'R,4'S,6'S,8'R)-methyl 6'-(4-methoxyphenyl)-1',2-dioxo-3',4'-diphenyl-1',3',4',6',8',8a'-hexahydrospiro[indoline-3,7'-pyrrolo[2,1-c][1,4]oxazine]-8'-

carboxylate (A-14): Prepared via the general procedure in 53% yield as a white solid. ^1H NMR: δ 3.2 (s, 3H), 3.7 (s, 3H), 4.1 (d, $J = 8.0$ Hz, 1H), 4.2 (d, $J = 3.2$ Hz, 1H), 4.9 (s, 1H), 5.1 (d, $J = 8.0$ Hz, 1H), 6.2 (d, $J = 3.2$ Hz, 1H), 6.5-6.6 (m, 3H), 7.0-7.3 (m, 15H), 7.6 (s, 1H). MS (ESI) calcd for $[\text{C}_{35}\text{H}_{30}\text{N}_2\text{O}_6+\text{Na}]^+$: 597.2 found: 597.2.



A-12

(2'S,3R,4'R)-methyl 5'-(butylcarbamoyl)-1'-((1S,2R)-2-hydroxy-1,2-diphenylethyl)-2'-(4-methoxyphenyl)-2-oxospiro[indoline-3,3'-pyrrolidine]-4'-carboxylate (A-12):

Prepared via the general procedure in 76% yield as a white solid. ^1H NMR: δ 0.94 (t, $J = 7.5$ Hz, 3H), 1.2-1.5 (m, 4H), 3.2 (s, 3H), 3.2 (dd, $J = 7.0, 13$ Hz, 2H), 3.7 (s, 3H), 3.9 (dd, $J = 8.5, 14$ Hz, 2H), 4.6 (d, $J = 4.5$ Hz, 1H), 5.1 (d, $J = 4.0$ Hz, 1H), 5.2 (s, 1H), 5.6 (t, $J = 6.0$ Hz, 1H), 6.6-6.7 (m, 3H), 6.9-7.0 (m, 4H), 7.0-7.2 (m, 10H), 7.5 (s, 1H), 7.6 (d, $J = 7.0$ Hz, 1H). MS (ESI) calcd for $[\text{C}_{39}\text{H}_{34}\text{N}_3\text{O}_6+\text{Na}]^+$: 670.3 found: 670.3.

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