

DESIGN AND SYNTHESIS OF NON-PEPTIDIC ARTIFICIAL TRANSCRIPTION
FACTORS

by

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To my parents and grandparents

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List of Abbreviations

A	alanine
AEEA	8-amino-3,6,-dioxaoctanoic acid
ATF	artificial transcription factor
BF ₃ •OEt ₂	boron trifluoride diethyl etherate
BnBr	benzylbromide
BnMgCl	benzylmagnesium chloride
Bpa	benzoylphenylalanine
tBuOCl	tert-butyl hypochlorite
tBuOH	tert-butanol
CBP	CREB binding protein
CH ₂ Cl ₂	dichloromethane
CH ₃ CN	acetonitrile
ChIP	chromatin immunoprecipitation
CREB	cAMP response element-binding protein
D	aspartic acid
DBD	DNA binding domain
DHFR	dihydrofolate reductase
DIBAL	diisobutylaluminum hydride
DIEA	diisopropyl ethyl amine
DMF	N, N-dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPPA	diphenylphosphoryl azide
DSG	disuccinimidyl glutarate
E	glutamic acid
EC ₅₀	concentration at half-maximal stimulation
ER	estrogen receptor
EtMgBr	ethyl magnesiumbromide
Et ₂ O	diethyl ether
F	phenylalanine
Fmoc	9-fluorenylmethoxycarbonyl
FP	fluorescence polarization
g	gram
HAT	histone acetyl transferase
HBTU	2-(1H-benzotrizol-1-yl)-1,1,3,3-tetramethyluronium
HeLa	Henrietta Lacks cervical cancer cells hexafluorophosphate
hMDM2	human double-minute 2

HMT	histone methyl transferase
HOBT	N-hydroxybenzotriazole
HPLC	high pressure liquid chromatography
I	isoleucine
IPTG	isopropyl β -D-1-thiogalactopyranoside
Kd	dissociation constant at equilibrium
Kid	kinase inducible domain
L	leucine
M	methionine
Med	Mediator protein
MeOH	methanol
MLL	mixed lineage leukemia
mRNA	messenger ribonucleic acid
MsCl	methane sulfonylchloride
NaH	sodium hydride
NaBH ₄	sodium borohydride
NaIO ₄	sodium periodate
NaN ₃	sodium azide
NMO	N-methylmorpholine-N-oxide
NMR	nuclear magnetic resonance
NSRF	neuron-restrictive silencer factor
OsO ₄	osmium tetroxide
OxDex	oxidized dexamethasone
pKid	phosphorylated kinase inducible domain
p53	protein 53 kilodaltons
PBS	phosphate buffered saline
PEG	polyethylene glycol
PIC	pre-initiation complex
PNA	peptide nucleic acid
PPh ₃	triphenylphosphine
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
REST	response element-1 silencing transcription factor
RNA	ribonucleic acid
RNAPII	RNA polymerase II
SAGA	Spt-Ada-Gcn5-acetyltransferase
S	serine
Srebp	steroid response element binding protein
SWI/SNF	switch/sucrose nonfermentable
T	threonine
TA	transcriptional activator
TAD	transcriptional activation domain
TAF	TBP-associated factor
TBAF	tetrabutylammonium fluoride
TBP	TATA binding protein
TBS	tert-butyl dimethylsilane
TEA	triethyl amine

TFIIB	transcription factor II B
TFIID	transcription factor II D
TFO	triplex forming oligonucleotide
THF	tetrahydrofuran
VP16	viral protein 16
ZF	zinc finger

Abstract

Design and Synthesis of a Non-Peptidic Transcription Factor

By

Ryan J. Casey

Chair: Anna K. Mapp

Transcription is important for the determination of cellular phenotype through the regulation of gene expression and its mis-regulation can lead to abnormal cell function. Transcriptional activators are essential for high fidelity transcription, responsible for seeking out particular genes and up-regulating them to precise levels in a signal-responsive fashion. Molecules that can reconstitute the function of transcriptional activators, artificial transcription activators, are highly desirable commodities as mechanistic tools and transcription-based therapeutics. Transcriptional activators control the specificity and extent of gene upregulation through two domains: the DNA binding domain (DBD) confers specific binding to DNA and the transcriptional activation domain (TAD) dictates the level of gene expression. Many questions surrounding how natural transcriptional activation domains function has hindered the development of TAD replacements despite their likely advantages in terms of stability, delivery, and and/or immunogenic properties.

To address the need for the development and characterization of small molecule

TADs we have employed a combination of organic chemistry and biological evaluations to produce a class of isoxazolidines that functionally mimic natural TADs. We identified the first small molecule, an amphipathic isoxazolidine, that reconstitutes transcription in living cell culture. Additionally, the amphipathic isoxazolidine alone can competitively inhibit the DNA localized-isoxazolidine, indicating that it is the isoxazolidine moiety that makes contacts with the transcriptional machinery that are important for activation. Many different peptide sequences can function as activators and we hypothesized this feature would translate to other suitably functionalized small molecules. Indeed, other isoxazolidine and non-isoxazolidine TADs activated transcription in cell culture. With an array of small molecule TADs, we designed activator artificial transcription factors and tested them for activation in cell culture.

Chapter I

Introduction to Small Molecule Regulation of Transcription

A. Project Focus

Transcription is essential for the determination of cellular phenotype and its mis-regulation can lead to abnormal cell function.¹⁻³ Not surprisingly, malfunctioning transcriptional regulators are correlated with mis-regulated transcription patterns in disease states.¹ Key players in the proper initiation of gene expression are transcriptional activators and thus molecules that mimic the function of these proteins are highly desirable as mechanistic tools and as potential therapeutic agents.² Transcriptional activators are modular proteins and typically carry out their gene-regulating function through two distinct domains: the DNA binding domain (DBD) which recognizes DNA sequence-specificity and the transcriptional activation domain (TAD) that assembles the transcriptional machinery at gene promoters (**Figure I-1**).⁴ Of the two domains, there is far less known about the mechanism or structure of TADs and thus it has been particularly difficult to design small molecule replacements, despite their advantages over peptide-based mimics in terms of stability and delivery.^{2, 5, 6} At the outset of my thesis work there was only one small molecule TAD, identified in our research

group, and only cell-free function had been reported.⁷ Thus the focus of my thesis project was to test if this in vitro activity could translate into cellular activity and to synthesize and evaluate novel analogs for the design of non-peptidic transcription factors.

B. Introduction to Transcription

Transcription is the process by which DNA is transcribed to mRNA and the first step in the production of a protein product from the information encoded in DNA (**Figure I-1**).^{2, 6, 8, 9} In response to extra-cellular or intra-cellular signals, transcriptional activator proteins seek out a specific DNA sequence within the genome and interact with chromatin modifiers and remodelers to alter chromatin structure.⁹⁻¹¹ The transcriptional activator then assembles the RNA polymerase II holoenzyme (RNA pol II and associated transcription factors) through a series of binding interactions with coactivators.^{12, 13} The assembly of the general transcriptional machinery and RNAPII is referred to as the pre-initiation complex or PIC.⁹

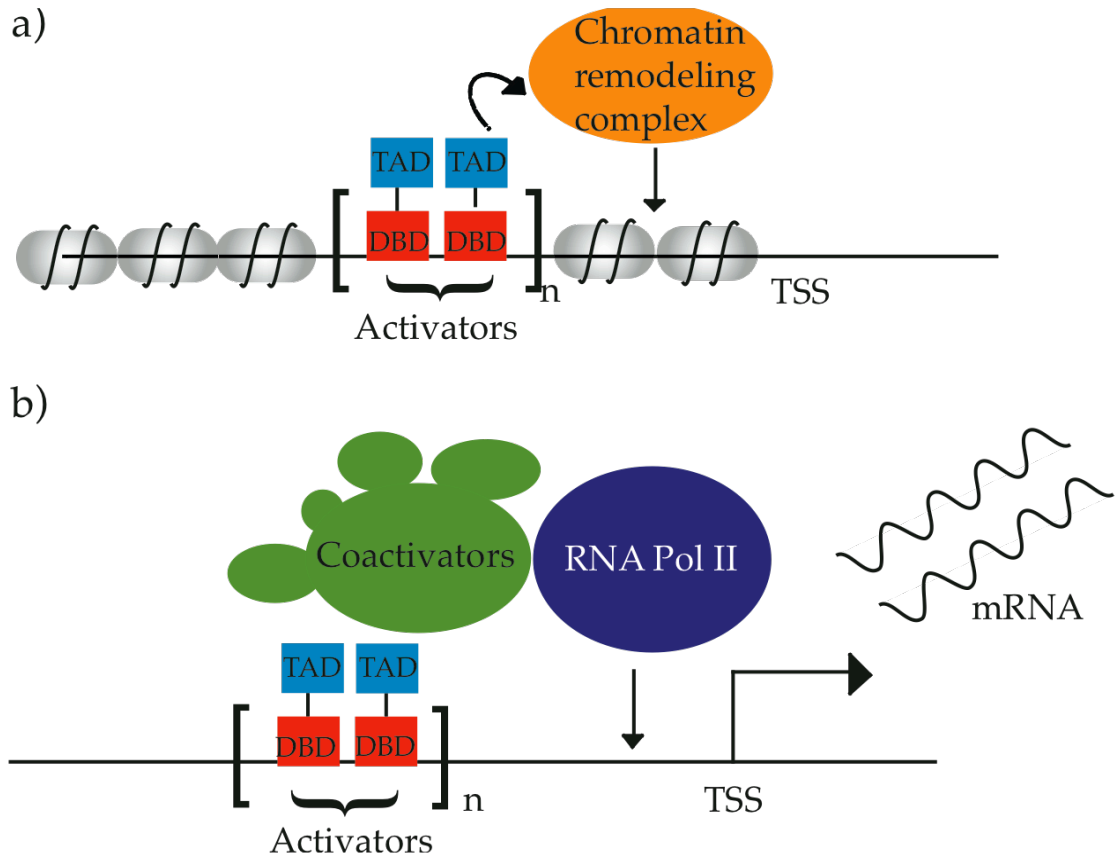


Figure I-1. General scheme of transcriptional activator. a) Chromatin remodeling complexes are either modify histones (silver) so activators can bind or activators bind a region absent from histones and recruit chromatin remodeling complexes. Upon binding, these complexes use ATP to remodel histones by repositioning unwrapping or ejection and expose DNA for further binding of activators or other DNA binding proteins.⁹ b) When bound, activators then bind coactivators to localize RNA Pol II to the correct transcription start site (TSS).

The pre-initiation complex consists of RNAPII and the five general transcription factors TFIIB (Transcription Factor II B), TFIID, TFIIE, TFIIIF, and TFIIH (**Figure II-2**).^{9, 14-18} In vitro, basal transcription can be initiated using only these factors and DNA.¹⁹ TFIIB forms a complex with TFIID and TFIIIF.²⁰ This complex binds to the promoter region of DNA and serves to direct RNAPII to the proper start site.²⁰ TFIIE and TFIIH both function to melt the DNA to create the

transcription bubble and allow RNAPII to transcribe RNA.^{21, 22} TFIIF also has kinase activity and phosphorylates RNAPII to promote transcriptional elongation.²³ While these components can support basal or low levels of transcription, the process of activated transcription, ranging from 10-10000 fold above basal, requires additional components.^{6, 8, 10, 12, 19}

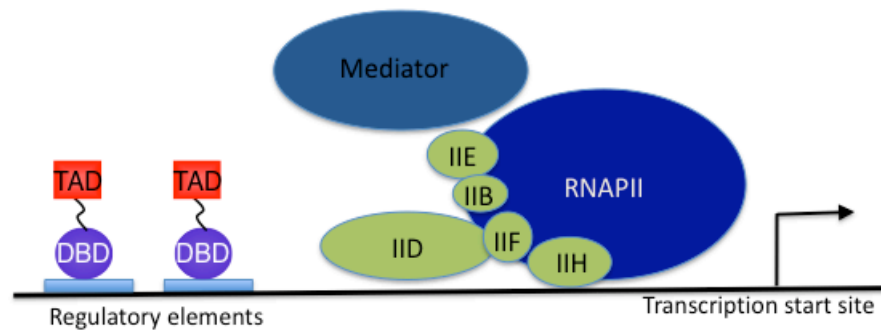


Figure I-2. Transcriptional activation occurs through recruitment of coactivators. Activators are modular proteins composed of a TAD (red box) and DBD (blue circle) and bind to regulatory elements (cyan box) in DNA (black) and recruit coactivators such as mediator (steel). Mediator acts as a bridge between activators and RNA polymerase II. RNA polymerase II (navy) is recruited to transcriptional start sites with the general transcription factors (green) TFIIB, TFIID, TFIIE, TFIIH, TFIIF.²⁴

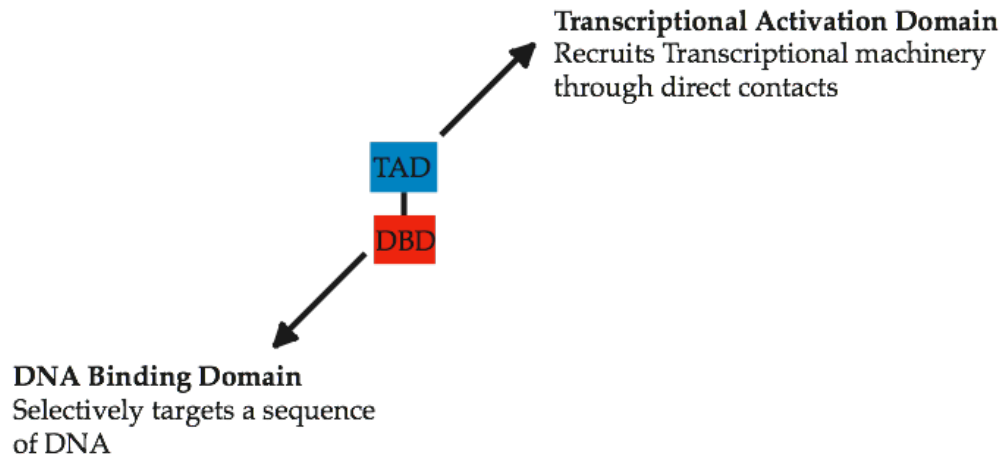
One class of proteins required for activated transcription is that of coactivators, proteins that interact both with transcriptional activators and with other components of the transcriptional machinery.^{10, 12} These proteins lack a DNA binding domain and thus cannot activate transcription in the absence of DNA-bound transcriptional activators. Coactivators perform multiple roles in the transcription process including elongation,²⁵ termination,²⁶ and RNA splicing.²⁷ Mediator is one large coactivator complex that functions as a bridge between DNA-bound activators, RNA polymerase II and other components of the transcriptional machinery (**Figure I-2**).^{12, 24, 28} Additional coactivator complexes are histone acetyl transferase (HAT)

complexes²⁹⁻³³ and other nucleosome remodeling complexes such as COMPASS harbors histone methyltransferase activity.³⁴ Thus, as discussed in more detail later, transcriptional activators have a multi-partner binding profile that represents a considerable challenge to replicate with a small molecule replacement.²

C. Transcriptional Activators

Transcriptional activators contact DNA and the transcriptional machinery and the extent and specificity of these contacts control both the specificity and degree of gene expression.^{2, 13} They accomplish this minimally through use of two domains: a DNA binding domain (DBD), which targets DNA sequence specifically, and a transcriptional activation domain (TAD), responsible for contacts with the transcriptional machinery.^{2, 4, 5, 35} These two domains can exist within a single polypeptide or can associate through non-covalent interactions. A synthetic transcription factor can be constructed using a DBD from one transcriptional activator and attaching it either covalently or non-covalently to a TAD from a different transcriptional activator.^{36, 37} However, for certain activators there can be interactions between TADs and DBDs that can influence activity

a) Anatomy of A Transcription Activator



b) Domain Swapping experiments:

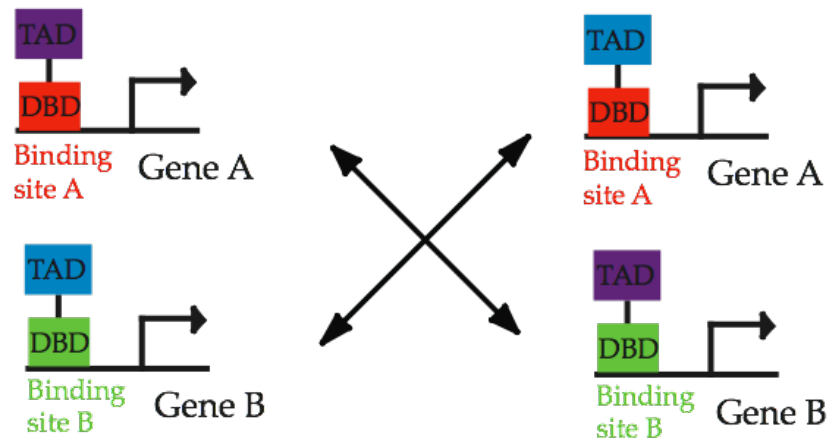


Figure I-3. Transcriptional activators consist of two modular domains. a) The anatomy of a transcriptional activator. b) Transcriptional activators have two modular domains that function with little dependence. The TAD of one transcriptional activator can be fused to a DBD of another transcriptional activator to create a synthetic transcriptional activator.

and are necessary to for function of that specific activator.^{38, 39} In general the modularity of transcriptional activators allows for an independent study of each domain and for developing activator ATFs (**Figure I-3**).⁴

Compared to the transcriptional activation domain, much is known about the DBD in terms of structure and mechanism by which DNA recognition occurs.⁴⁰ The most common folds are zinc fingers,⁴¹ helix-turn-helix,⁴² homeodomains,⁴³ leucine zippers,⁴⁴ and helix-loop-helix.⁴⁵ TADs, however, are described in simpler terms and are categorized as proline-rich,^{46, 47} glutamine-rich,^{48, 49} or amphipathic^{2, 50} based on their amino acid composition. The simplified terms are generally used because of an overall lack of TAD structures. Of the three classes, the amphipathic – or, more historically, the acid-rich - class is the best studied. These transcriptional activators consist of polar residues interspersed with hydrophobic amino acids (**Figure I-4**).²

Insight into the functional groups important for the function of amphipathic TADs has arisen from mutagenesis experiments of VP16, perhaps the most strongly functioning amphipathic activator.^{51, 52} These experiments showed that hydrophobic residues were particularly important for transcriptional activation. A single mutation of phenylalanine 473 to a serine, for example, decreased activation by 80%.⁵² A number of mutants have been tested for binding to TFIID, a target of VP16, and it was found that mutation of a large hydrophobic residue to an alanine or serine severely attenuated binding.⁵³ However, mutations of phenylalanine to leucine only decreased transcriptional activation by 10%.⁵² These observations provided evidence that highly active TADs should consist of large hydrophobic residues and these residues were important for binding. Studies of the orientation of

these hydrophobic and polar residues discovered that this factor was important in some cases but not in others.^{51, 54-57} In one example, both d and l peptides of amphipathic activator ATF29 activated transcription in vitro when localized to DNA.⁵⁵ In another example, an amphipathic helix designed by the Ptashne and coworkers to have an acidic face, a hydrophobic face, and a polar face activated transcription, however, a peptide bearing the same functional groups scrambled was inactive.⁵⁰

VP16

441 AspPheAspLeuAspMetLeuGly 448

p53

9 SerValAspProProLeuSerGlnGluThrPheSerAspLeuTrp 23

CREB

134 TyrArgLysIleLeuAsnAspLeu 145

MLL

2845 LeuProSerAspIleMetProPheValLeuLys 2855

HIV1-Tat

8 LeuGluProTrpLysHisProGlySerGln 17

Figure I-4. Sequences of minimal natural TADs. Polar residues are shown in red and hydrophobic residues in blue. Little sequence homology exists between these activators.

In solution, amphipathic TADs lack a defined structure but adopt a secondary structure upon interacting with binding partners.^{58, 59} However, there has been little progress in obtaining detailed structural information of TAD-coactivator complexes. This is due in part to the modest affinity that TADs have for coactivators as well as

that TADs can often interact with coactivators at more than one site and, evidently, in more than one orientation.⁵⁹⁻⁶⁴ Of the structures of TADs that do exist, majority are of TADs bound to masking proteins, proteins that shield TADs from proteolysis and aggregation.^{2, 39} Interactions between masking proteins and TADs are typically higher affinity and more specific than between TADs and the transcriptional machinery, thus providing more tractable structures. An example of one of these complexes is the interaction between 15 residues of p53 and its natural inhibitor mDM2.⁶⁵ The TAD forms an amphipathic helix, burying hydrophobic residues in the hydrophobic cleft of mDM2.⁶⁵ In fact, the amphipathic helix is the most

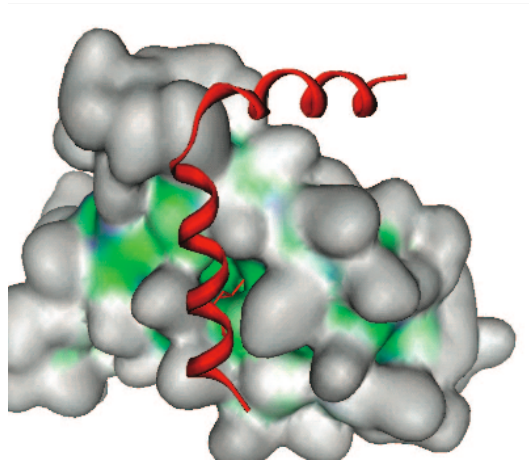


Figure I-5. NMR structure of CREB (red) bound to the KIX domain of CBP (grey). The green patches are hydrophobic surfaces. The hydrophobic residues of CREB face these surfaces and the polar residues are solvent exposed. Adopted from reference 66.

common structure observed for TADs in complex with binding partners.²

Although solid-state characterization of TAD-coactivator complexes has proven challenging, NMR characterization of several complexes has yielded much

insight into how TADs interact with coactivators. One such structure between the activator CREB and the coactivator CBP/p300 demonstrated that the hydrophobic residues of phosphorylated CREB interact with hydrophobic residues present in CBP/p300, consistent with mutagenesis experiments that generally suggest the importance of hydrophobic residues for binding and function (**Figure I-5**).⁶⁶ There is additional evidence that binding site of coactivators are likely flexible. In one example, three peptidic activators (VP2, GCN4, and Gal4) that share little sequence homology bind to the same region of the coactivator Med15.⁶¹

D. Artificial Transcriptional Activators

Artificial transcriptional factors (ATFs) have tremendous utility as therapeutics, for biomanufacturing, and in synthetic biology.² For biomanufacturing applications, ATFs that up-regulate transcription to prescribed levels can improve the amount of protein product or increase the concentrations of biosynthetic enzymes to further boost product yields.^{67, 68} In synthetic biology, transcriptional networks are used to construct networks and cell-based devices. ATFs that function predictably and in an orthogonal fashion relative to natural regulators would be beneficial additions to this field.⁶⁹ This is especially true for the nascent field of eukaryote-based networks, because the complexity of the transcription process increases significantly from prokaryote to eukaryote.⁶⁹ Since the DBD confers selective binding to DNA and thus gene-targeting specificity, significant efforts in the development of ATFs have focused on this module (**Figure I-6**). Of the classes of protein DBDs, the zinc finger proteins, which modularly recognize DNA, have been most extensively studied and have found use in custom transcriptional activators,

even in cell culture.⁴¹ Zinc is chelated by two cysteines and two histidines to create the fold and each finger can recognize three base pairs and target DNA sequence-specifically. Through use of defined pairing rules, custom protein-based transcriptional activators can be designed.⁷⁰ In one example, an artificial zinc finger based transcription activator and repressor have been targeted to the *Oct-4* gene promoter (a major regulator of ES cell pluripotency and self-renewal) in order to regulate its expression. The up and down regulation of the *Oct-4* gene affects the expression of downstream genes dependent on the Oct-4 regulator and thus modulates ES cell differentiation.⁷¹ While mechanistically useful, these protein base activators require gene therapy for delivery and suffer from many of the problems associated with that technology.⁷² Despite these shortcomings, a zinc finger artificial transcriptional activator that up-regulates the VEGF-A gene is in Phase II clinical trials as a treatment for diabetic peripheral sensory motor neuropathy.⁷³

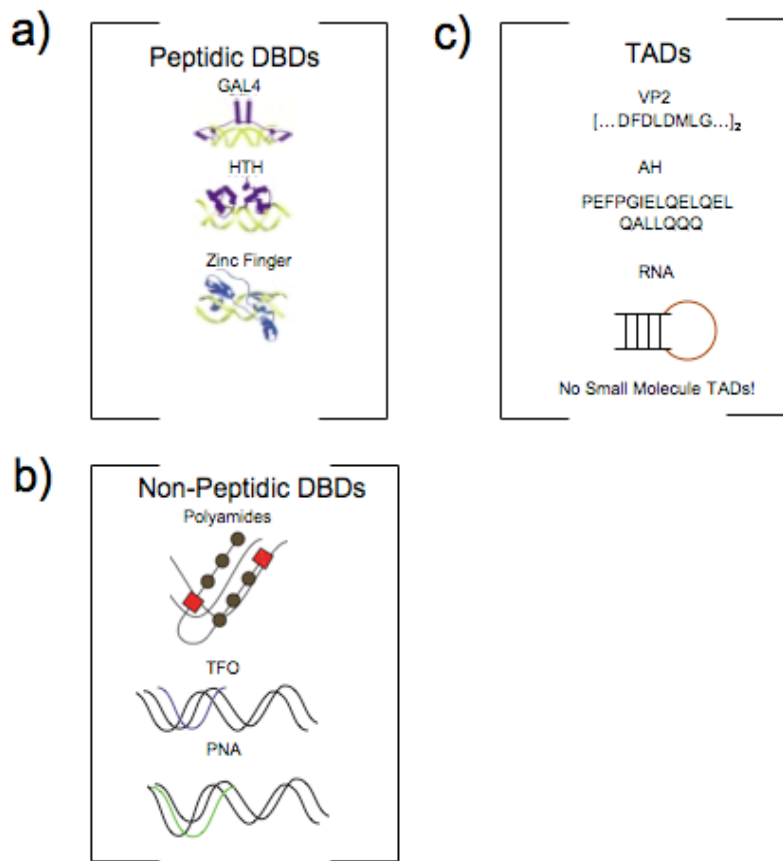


Figure I-6. Domains used in the construction of ATFs.⁴ a) Common peptidic DBDs used in construct artificial ATFs include Gal4, the helix-turn-helix protein, and zinc fingers. a) The most frequently used non-peptidic DBDs are hairpin polyamides, triplex forming oligonucleotides (TFOs), and peptide nucleic acid (PNA). The polyamide binds in the minor groove and TFOs utilize Hoogsteen base pairs. PNA recognizes DNA through strand invasion. c) As of 2002, most TADs had been derived from natural activation domains such as AH and VP2. RNA can also function as a transcriptional activator.

Currently there are three classes of non-peptidic DBDs that have been employed for the construction of activator ATFs: hairpin polyamides,⁷⁴ triplex forming oligonucleotides (TFOs),⁷⁵ and peptide nucleic acids (PNAs)⁷⁶ (**Figure I-6b**). Polyamides are oligomers of pyrrole and imidazole amino acids capable of recognizing the four DNA base pairs and thus recognize specific DNA sequences. Polyamides bind the minor groove to achieve molecular recognition of DNA. TFOs

recognize DNA by the formation of Hoogsteen base pairs and have been utilized to target DNA (**Figure I-7**). Likewise, PNA is similar to single stranded DNA with the exception that the phosphodiester bond has been replaced with an amide bond, targets DNA through strand invasion.

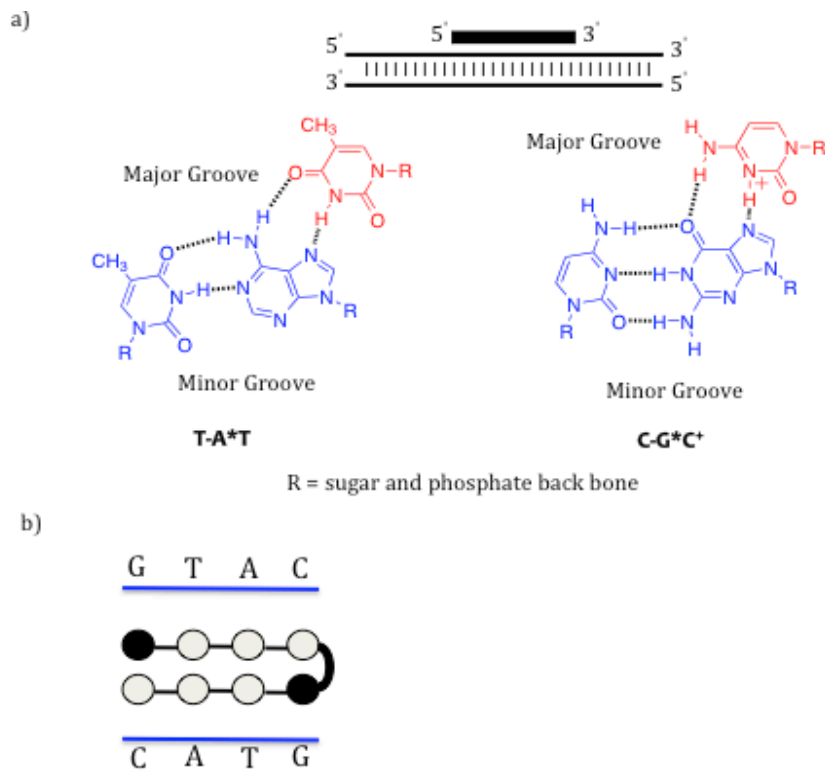


Figure I-7. Synthetic DBDs. a) TFOs target DNA by binding in the major groove of DNA. b) Polyamides bind DNA (blue) by recognition of base pairs through contacts with imidazole (black circles) and pyrrole (white circles) dimmers. Pyrrole/pyrrole dimmers recognize T-A or A-T base pairs and pyrrole/imidazole recognize G-C base pairs. Adopted from ref 75 and 76.

Since the amphipathic class of TADs is well studied, synthetic TADs based on these sequences are most commonly seen in ATFs. The minimal activation domain of the highly active amphipathic activator VP16 has been used in conjunction with many different DBDs, for example.^{77, 78} In one example, VP2 was fused to a TFO to

give rise to a novel ATF capable of activating a predetermined gene in cell culture.⁷⁹ However, when attached to a polyamide, VP2 only functions in cell-free conditions.^{77, 78} While the TFO-VP2 example was active, it required the addition of a transfection agent. This hints at some of the difficulties with the design of ATFs, as generally these molecules are large and have limited bioavailability. Additionally, the propensity of peptidic TADs to proteolytic degradation could diminish the overall activity of the ATF. In deed, both d- and l-peptides of amphipathic activator ATF29 activated transcription in vitro when localized to DNA. However, in cell culture, only the d-peptide activates transcription presumably due to proteolytic degradation of the l-peptide.⁵⁵ Thus a small molecule TAD, which exhibited enhanced stability and bioavailability would be immensely useful.

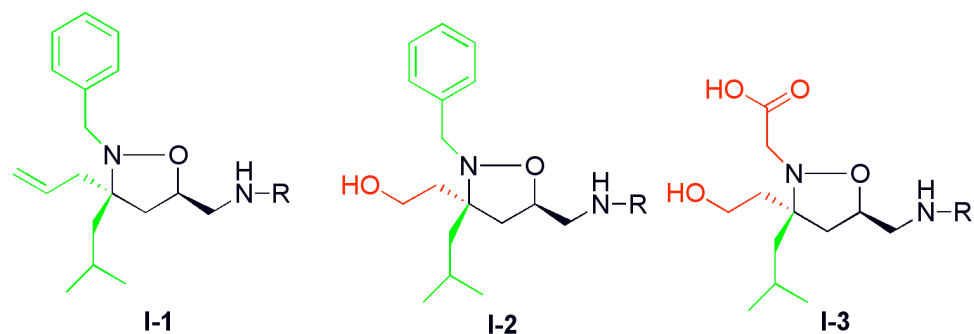
E. Small Molecule TADs

The need for interactions with multiple components of the transcriptional machinery makes traditional methods of small molecule ligand discovery difficult to employ for small molecule TADs. There is considerable evidence that the ability to interact with more than one protein is vital to the function of a transcriptional activation domain.² Indeed, a NMR based screen of the KIX domain of an important coactivator, CBP, yielded a small molecule that can not reconstitute transcriptional activation.⁸⁰ Additionally, because there is only minimal structural data for TADs and TADs in complex with binding partners, there is not enough data for structure-based design. The strategy employed in the Mapp group for small molecule TAD development was rooted in some observations from the most widely studied class of activators, the amphipathic activators.^{7, 56} Firstly, mutagenesis experiments showed

that these activators required hydrophobic residues and an amphipathic balance for high levels of transcriptional activation.^{51, 81, 82} Secondly, activators of the amphipathic class formed a defined secondary structure when bound to targets in the transcriptional machinery.^{58, 59, 83} Thirdly, it is thought that many components of the transcriptional machinery consist of permissive binding sites capable of binding an array of amphipathic polypeptides that lack sequence homology.^{54, 61, 84, 85} Generally these interactions are modest in strength, usually with micromolar affinities.^{63, 86} The hypothesis was that a small molecule that mimics the characteristics of endogenous TADs could interact with a subset of the permissive binding sites for TADs within the transcriptional machinery and by doing so activate transcription. Thus we sought to design a small molecule using these relatively simple design criteria.

F. Isoxazolidine TADs

Former coworkers identified isoxazolidines as a small molecule that fit the above criteria (**Figure I-8**).^{7, 56} The five-membered heterocycle imparts the desired rigidity and the synthetic scheme of this molecule allows for dense and stereodefined functionalization of the isoxazolidine core.^{87, 88} Amphipathic isoxazolidine (**I-2**) was prepared and, when conjugated to a DNA-localizing molecule, activated transcription significantly higher than both the polar (**1-3**) and hydrophobic isoxazolidine (**1-1**) in a cell-free system. This approach proved successful with (**1-2**) as the first small molecule TAD.



R = DNA localizing molecule

Figure I-8. Small molecule isoxazolidines. Isoxazolidine **I-2** activated transcription to comparable levels as a peptidic activator in a cell free assay. Isoxazolidines **I-1** and **I-3** did not.⁷

This was the first example of a small molecule TAD and was an important advance. However, the critical question left unanswered was if this activity could be translated to cellular function. In addition, there was little data regarding the scope of substituents that could be appended to the isoxazolidine core and the impact such substitutions would have on activity. Finally, ideally the small molecule TAD could be appended to a variety of DNA binding modules in order to be able to target endogenous genes. Investigating these points was the thrust of my thesis work.

G. Thesis Outline

To further our understanding of small molecule TADs, we sought to: expand the study of small molecule TADs to a cell-based system; probe the functional group tolerance of the isoxazolidine and the structural importance of the isoxazolidine scaffold; and assemble a non-peptide synthetic activator consisting of a small molecule TAD and a DNA binding domain.

In Chapter II, I will describe the first small molecule that functions similar to natural TADs in cell culture. By amending the original synthetic strategy pioneered by previous group members, we employed a two-hybrid assay to observe a maximal activity of 80-fold over the construct without the small molecule. Similar to the original cell-free study, the hydrophobic isoxazolidine showed activity similar to the construct alone. Additionally, similar to natural TADs, the free small molecule TAD could compete with the small molecule localized to DNA to inhibit transcriptional activation.

In Chapter III examines the functional group amphipathic balance of the isoxazolidine. Though a focused set of isoxazolidines, we derive an optimal logP range of 2.7-3.1 for small molecule TADs. The structural importance of the isoxazolidine ring was further tested and while important, it was not essential to activity. To further test if these observations could translate to design criteria, we examined two natural product scaffolds and observed potent activity.

In Chapter IV, our attempts to design non-peptidic transcriptional activators are described. Employing a simple strategy, I conjugated the small molecule TAD with a polyamide DBD or a TFO to make our non-peptidic transcriptional activator. Neither of these conjugates was able to reconstitute activity in cell culture. We isolated the polyamide DBD to test if it could support activated transcription through a two-hybrid system. Using the constructs in chapter III and chapter II, we targeted endogenous genes and activated transcription nuclear receptors regulated genes.

In sum, evidence suggests that isoxazolidine TADs function similar to natural TADs and display similar limitations upon functional group manipulations. Additional studies by previous group members have demonstrated that isoxazolidine TADs contact CBP by NMR, an important coactivator protein. Coupling NMR studies with our empirically determined design guidelines provides a framework for future development of both inhibitors of transcription and transcriptional activators.

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Chapter II

Small Molecule Isoxazolidines as Transcriptional Activation Domains^a

A. Abstract

Transcription is important for the determination of cellular phenotype through the regulation of gene expression and its mis-regulation can lead to abnormal cell function. Activator artificial transcription factors are synthetic molecules that bind specific sequences in DNA and activate pre-determined genes. They are generally composed of peptidic transcriptional activation domains (TADs) that interact with the transcriptional machinery and a DNA binding domain (DBD) responsible for specific binding to DNA. Small molecule replacements of TADs are desirable tools to parse the mechanism of transcription and have significant potential as therapeutic agents. Towards that end, we examined if molecules that function as TADs in a cell-free assay can similarly function in cell culture. An amphipathic isoxazolidine that displays benzyl, ethanol, and isobutyl functional groups is compared with a hydrophobic isoxazolidine functionalized with three hydrophobic moieties (benzyl, allyl, and isobutyl) using a two-hybrid system. In cell

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culture the amphipathic isoxazolidine activates transcription of a reporter gene 80-fold compared to the DNA binding domain alone or the hydrophobic isoxazolidine, an even more pronounced difference than was observed in cell-free conditions. The amphipathic isoxazolidine alone can competitively inhibit the DNA localized-isoxazolidine, indicating that it is the isoxazolidine moiety that makes contacts with the transcriptional machinery that are important for activation. Since the isoxazolidine interacts with the transcriptional machinery it may also be possible to design inhibitors of transcription based upon this scaffold.

B. Introduction

Transcription is hypothesized to occur through the recruitment of the transcriptional machinery to a transcription start sites by the transcriptional activation domain (TAD) of activators.¹ Generally it is thought that activators recruit chromatin-remodeling complexes, such as SAGA and SWI/SNF that destabilize histone-DNA interactions and allow proteins access to DNA.^{2, 3} Mediator, a large (~25 components) protein complex, is another cofactor recruited and this complex acts as a bridge between the activator and RNA polymerase II (RNAPII) and the general transcription factors.^{2, 4} RNAPII and the general transcription factors form the pre-initiation complex or PIC consisting of RNAPII and the five general transcription factors TFIIB (Transcription Factor II B), TFIID, TFIIE, TFIIF, and TFIIH (**Figure II-1**).^{2, 5-8} TFIID is a large complex consisting of TBP (TATA Binding Protein), TAFs (TBP Associated Factor), and TFIIA.²

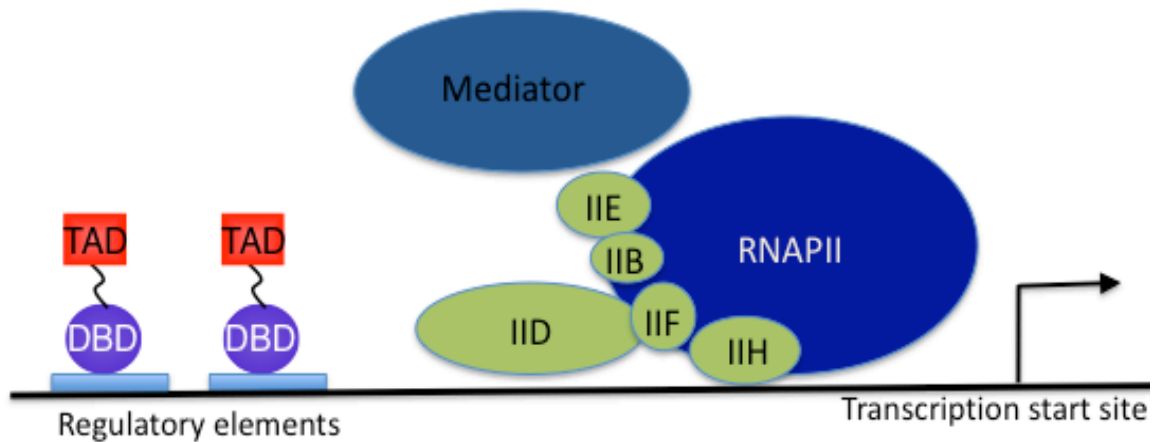


Figure II-1. Transcriptional activation occurs through recruitment of coactivators. Activators are modular proteins composed of a TAD (red box) and DBD (blue circle) and bind to regulatory elements (cyan box) in DNA (black) and recruit coactivators such as mediator (steel). RNA polymerase II (navy) is recruited to transcriptional start sites with the general transcription factors (green) TFIIB, TFIID, TFIIE, TFIIF, TFIIF.

Activators minimally consist of transcriptional activation domains, termed TADs, and DNA binding domains (DBD) (**Figure II-1**).⁹ The DBD confers selective binding to DNA and often acts independently of the TAD.¹ TADs are responsible for the duration of transcription and the quantity of transcript produced.¹ The best-studied class of activators is the acidic or amphipathic class, loosely defined as TADs composed of hydrophobic amino acids interspersed with acidic (Asp, Glu) or polar (Asn, Gln, Ser, Thr, for example) residues.^{10, 11} Early efforts to determine critical targets of amphipathic TADs focused on components of the pre-initiation complex (PIC), composed of RNA polymerase II and associated transcription factors.¹²⁻¹⁵ These studies determined that TBP was a critical target of highly active TADs.^{12, 13} However, later it was demonstrated that addition of TBP and other targets present in the PIC did not result in activated transcription.^{16, 17} Attention shifted to other

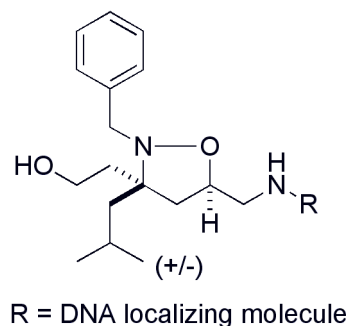
coactivators, as it became apparent that a single interaction was not sufficient for transcriptional activation and instead a multi-partner binding profile was necessary.¹ In yeast, for example, *in vitro* cross-linking has provided evidence that a minimal activation domain of amphipathic activator Gal4 contacts yMED15/Gal11 (a component of the yeast mediator complex), a protein in the SAGA chromatin remodeling complex, Tra1, and Taf12 (a component of TFIID and the SAGA chromatin remodeling complex).^{18, 19} Thus Gal4 contacts at least three different transcriptional complexes, contacts that are important for activated transcription. Interestingly, another amphipathic activator, GCN4, shares little sequence homology yet targets the same set of coactivator targets.^{18, 19} Similar examples exist for metazoan coactivators.^{20, 21} These were puzzling results and questions arose as to whether these two activators shared binding sites or if the two activators target different binding sites within the same protein. A more recent study from our group shows that, in fact, Gal4 and Gcn4 target the identical binding site within yMed15/Gal11.²² Taken together these data suggest proteins in the transcriptional machinery interact with binding sites that are flexible enough to accommodate diverse amphipathic peptide sequences.

As outlined above, amphipathic activators are defined as consisting of hydrophobic and polar functional groups and as a class these activators share little sequence homology. A common early theme in the literature was that large hydrophobic and acidic residues were critical for activation.²³⁻²⁵ For example mutagenesis experiments using the minimal TAD of amphipathic activator p65 revealed that changing a large hydrophobic amino acid (either a phenylalanine,

leucine, or isoleucine) to an alanine abolished activity (**Table II-1**).²³ This study and others defined the importance of these larger hydrophobic residues to activation.²³
²⁵ Based on these examples, we decided that our small molecule TAD should also display a combination of large hydrophobic functional groups and polar groups to activate transcription.

P65	535	-	545											Activity	
535	-	S	I	A	D	M	D	F	S	A	L	L	-	545	100%
		-	-	-	-	-	-	A	-	-	-	-			<1%
		-	-	-	-	-	-	-	-	-	A	-			<1%
		-	-	-	-	-	-	-	D	-	-	-			146%
		D	F	-	-	-	-	-	D	-	-	-			374%
		D	A	-	-	-	-	A	D	-	-	-			<1%

Table II-1. Mutations of the activation domain of p65. Activity normalized to the minimal activation domain of p65 535-545. The triple mutant of two serines to two acids and an isoleucine to a phenylalanine resulted in almost a four fold increase in activity. However, when the large hydrophobic residues are mutated to alanine, resulting peptide is inactive. Adapted from ref 23.



II-1

Figure II-2. Amphipathic isoxazolidine.

The first small molecule TAD, the isoxazolidine TAD (**Figure II-2**) was designed as a generic mimic of natural amphipathic TADs and displays functional groups common to activators, a benzyl, an alcohol, and an isobutyl group.²⁶ The isoxazolidine was chosen for a variety of reasons: firstly, five-membered heterocycles are common structures seen in drugs and peptidomimetics and the ring projects functional groups in a three-dimensional display. Secondly, the nitrogen and oxygen increase water solubility of the small molecule compared to a similarly functionalized cyclopentane ring. Thirdly, the isoxazolidine substituents are easily diversified which allows for the addition of hydrophobic and polar residues characteristic of natural amphipathic TADs. Thus it was hypothesized that a conformationally constrained amphipathic small molecule isoxazolidine would be able to interact with at least a subset of the permissive bindings sites for TADs within the transcriptional machinery and by doing so active transcription. Consistent with this hypothesis, the small molecule isoxazolidine acted as a TAD in nuclear extracts when localized to DNA to comparable levels as a known TAD,

ATF14, and the hydrophobic (**II-2**) and acidic isoxazolidine (**II-5**) did not activate transcription.²⁶ Our goal in the experiments outlined in this chapter was to assess if the function observed in cell-free conditions could be similarly observed in living cell culture to produce the first small molecule TAD(s) with such function.

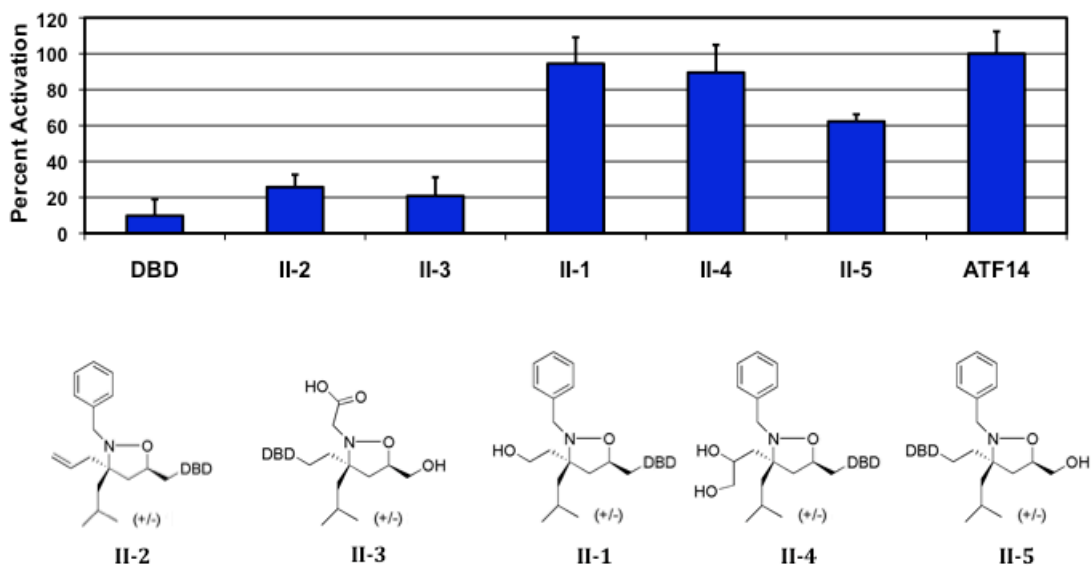


Figure II-3. Cell-free activity of isoxazolidine derivatives. Compounds **II-2** and **II-3** were not active in the cell-free assay. Compounds **II-1** and **II-5** activated to similar levels which demonstrated that the placement of the DBD did not influence activation. The compounds were compared to ATF14 which is an amphipathic activator based on the highly active viral protein VP16.

C. Design and Synthesis of a Small Molecule TAD for Cellular Studies

As outlined above, it had been shown by previous co-workers that isoxazolidines can reconstitute the function of endogenous TADs in a cell-free system; however, in cell-free assays, cellular permeability, nuclear localization and cellular stability are not assessed. An additional complication is that natural and non-natural TADs have been shown to exhibit promiscuous binding, interacting with many different hydrophobic surfaces. In a cell-free assay, there is a more

limited range of potential protein binding partners relative to the cellular environment. Thus it was envisioned that the transition to cellular function could be quite challenging. To test if the small molecules could activate transcription, we evaluated the amphipathic isoxazolidine as well as the hydrophobic isoxazolidine, a molecule which lacked function in the cell free assay, for activity in HeLa cells.²⁷ To localize the small molecule to DNA we took advantage of a 2-hybrid system developed in the Kodadek lab which utilizes oxidized dexamethazone (OxDex), a ligand for the glucocorticoid receptor (**Figure II-4**).²⁸ The glucocorticoid receptor minimal ligand-binding domain was fused to Gal4 DBD that in turn could localize our small molecule to the promoter. There are several advantages with this assay. It is challenging to localize a molecule to a specific sequence of DNA. Since Gal4 is a yeast activator it has no binding sites in the human genome, and it can thus be used to localize proteins to a specific and pre-determined site. One potential problem in many assays is the difference in cell permeability. Since Oxdex is a derivative of a hormone, it has good cell permeability and Oxdex conjugates are more likely to be permeable. In addition, OxDex has a high affinity for GR so the small molecule conjugate is effectively localized to DNA since HeLa cells do not express high levels of GR. The Kodadek group found that attachment of various functional groups to OxDex did not significantly affect binding to GR ligand binding domain.

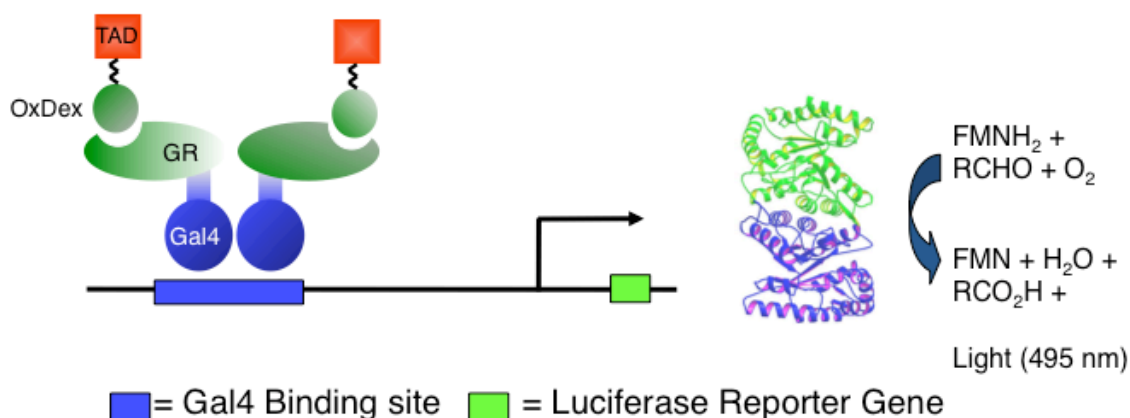


Figure II-4. Two-hybrid system to evaluate our small molecules in HeLa cells. Oxidized dexamethasone (OxDex) localizes our small molecule to the GR ligand binding domain which is fused to the Gal4 DBD. This fusion protein then localizes to DNA by recognition of the Gal4 binding sites.

To synthesize our isoxazolidine analogues, we designed a general synthetic strategy that could be manipulated to accommodate many functional groups; this became particularly important for the studies outlined in Chapter 3.²⁹ It was desirable to introduce functional groups late in the synthesis so that large quantities of a common intermediate could be synthesized and diversified late to save synthetic steps. **Figure II-5** shows our general synthetic strategy and key intermediate. Addition of a nucleophile to an isoxazoline can yield two diastereomers and conditions have been developed by former members of the Mapp lab for selective or non-selective addition of nucleophiles based on solvent and isoxazoline functional groups.^{26, 30-32} After addition, alkylation of the nitrogen of the isoxazolidine ring is possible by addition of an electrophile. These synthetic steps are general and many functional groups have been introduced to yield highly diversified isoxazolidine rings. The common intermediate consists of an isobutyl

group and an alkene. The alkene can be functionalized to alcohols, esters, diols, acids, and amides. Discussion of the synthesis of other analogous will be discussed in depth in Chapter 3. For our initial studies, we synthesized the isoxazolidine bearing an alcohol, isobutyl, and benzyl groups (amphipathic isoxazolidine, **Figure II-5**, bottom left) and an isoxazolidine with an alkene, isobutyl, and benzyl group (hydrophobic isoxazolidine, **Figure II-5** bottom right).

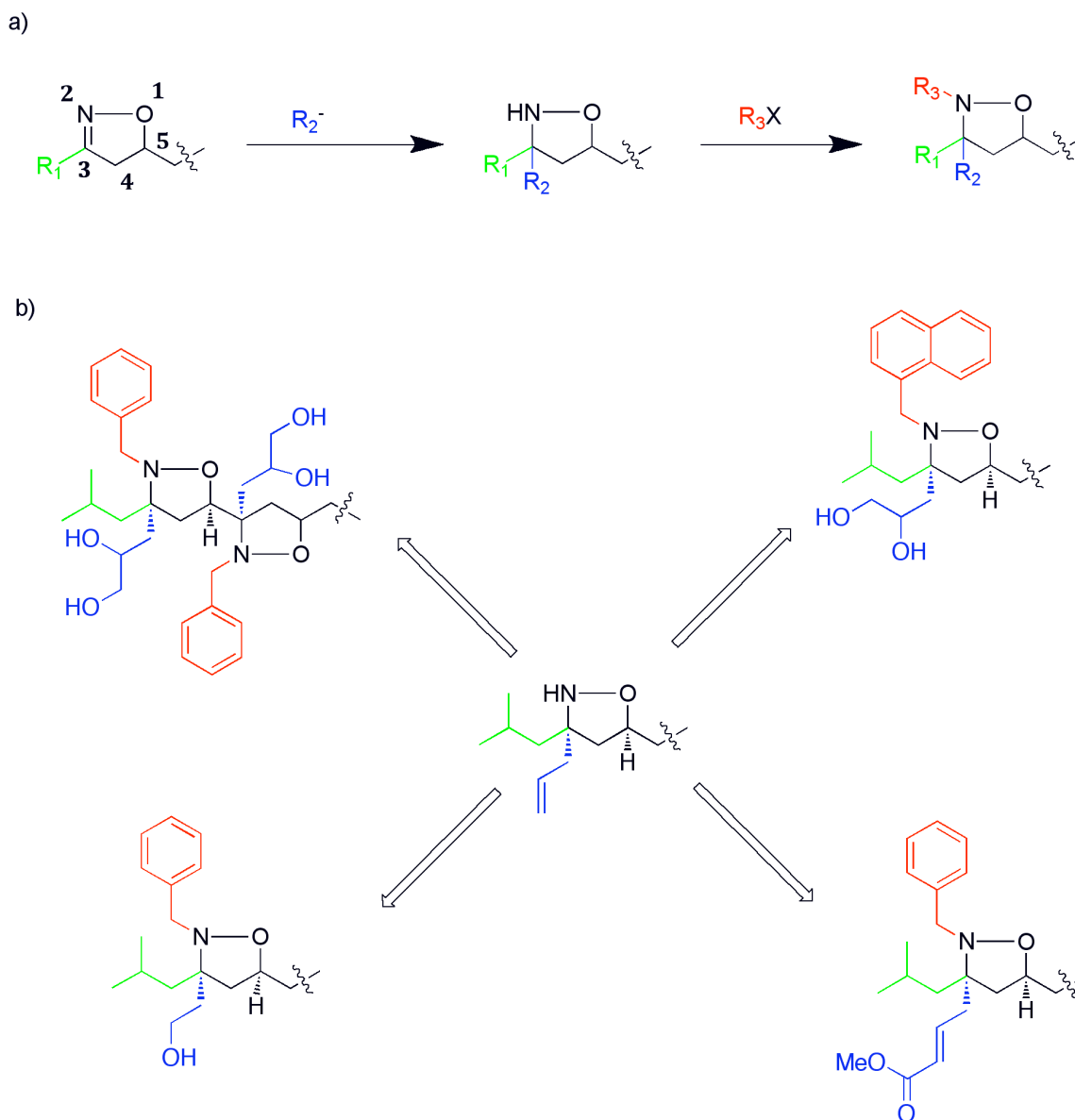


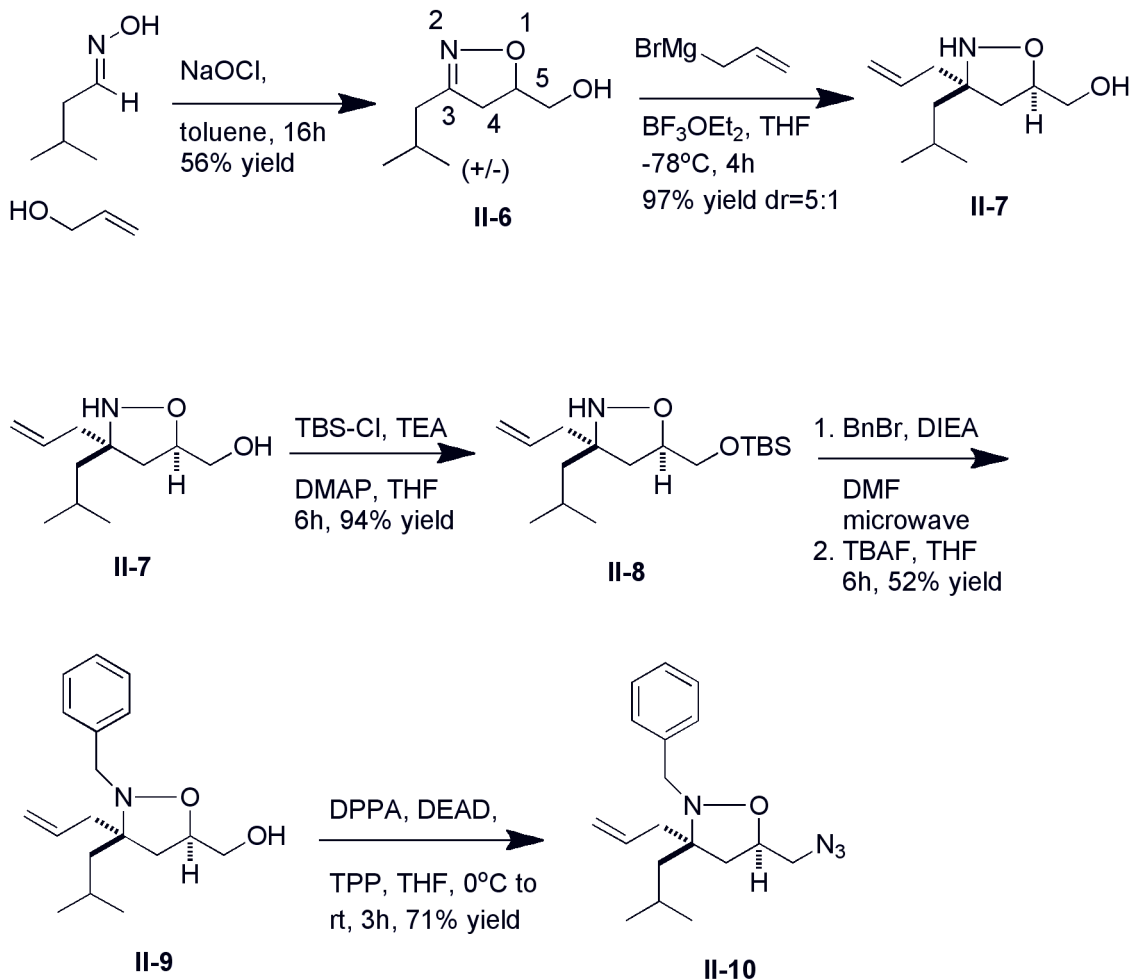
Figure II-5. General strategy for synthesis of isoxazolidines. a) Starting from an isoxazoline a nucleophile is added to generate an isoxazolidine. Addition of an electrophile functionalizes the ring further to incorporate our three functional groups. Ring positions are numbered starting with O1 and ending with C5. b) The common intermediate is shown in the center. The amphipathic isoxazolidine is shown on the bottom left and the hydrophobic isoxazolidine is shown on the bottom right. Functional group manipulations of the alkene can form esters, alcohols, or diols. Dimer isoxazolidines are also possible and will be discussed in Chapter 3.

The synthesis of the hydrophobic isoxazolidine **II-10** is shown below (**Scheme II-1**).

Towards the isoxazoline, the isobutyl oxime was treated with NaOCl to afford the

hydroximinoyl chloride *in situ* that was combined with allyl alcohol to generate isoxazoline **II-6** via a 1,3-dipolar cycloaddition in 57% yield as a racemate.^{refs} Nucleophilic addition to the C=N bond of isoxazoline **II-6** with the Grignard reagent allylmagnesium chloride in the presence of the strong Lewis acid, BF₃•OEt₂ afforded isoxazolidine **II-7**. This reaction yielded a 5:1 mixture of diastereomeric products in a combined 97% yield. The major diastereomer obtained resulted from approach of the nucleophile to the face opposite the bulky C5 sidechain as confirmed by NOE analysis.³⁰

At this point, two of the key functional groups have been introduced onto the ring and the next focus was the N2 substituent. To alkylate the nitrogen of the isoxazolidine ring it was first necessary to protect the free alcohol through a reaction with TBS-Cl, DMAP and TEA to generate the TBS ether **II-8** in 94% yield. The isoxazolidine ring was further functionalized by alkylation with benzyl bromide through microwave-accelerated conditions to afford 53% yield after TBAF deprotection of the crude material. To connect the isoxazolidine to OxDex, an amine handle on the isoxazolidine was needed. To do this, an azide was installed through a Mitsunobu reaction with diphenylphosphoryl azide (DPPA), diethylazodicarboxylate (DEAD), and triphenyl phosphine (TPP) to produce isoxazolidine **II-9** in 71% yield. In more recent syntheses of the isoxazolidine, it is possible to install the azide functionality prior to alkylation though selective mesylation of isoxazolidine **II-7** followed by reaction with sodium azide. Early installation of the azide provides a common intermediate and follows a convergent synthesis for diversification of N2 substituent.

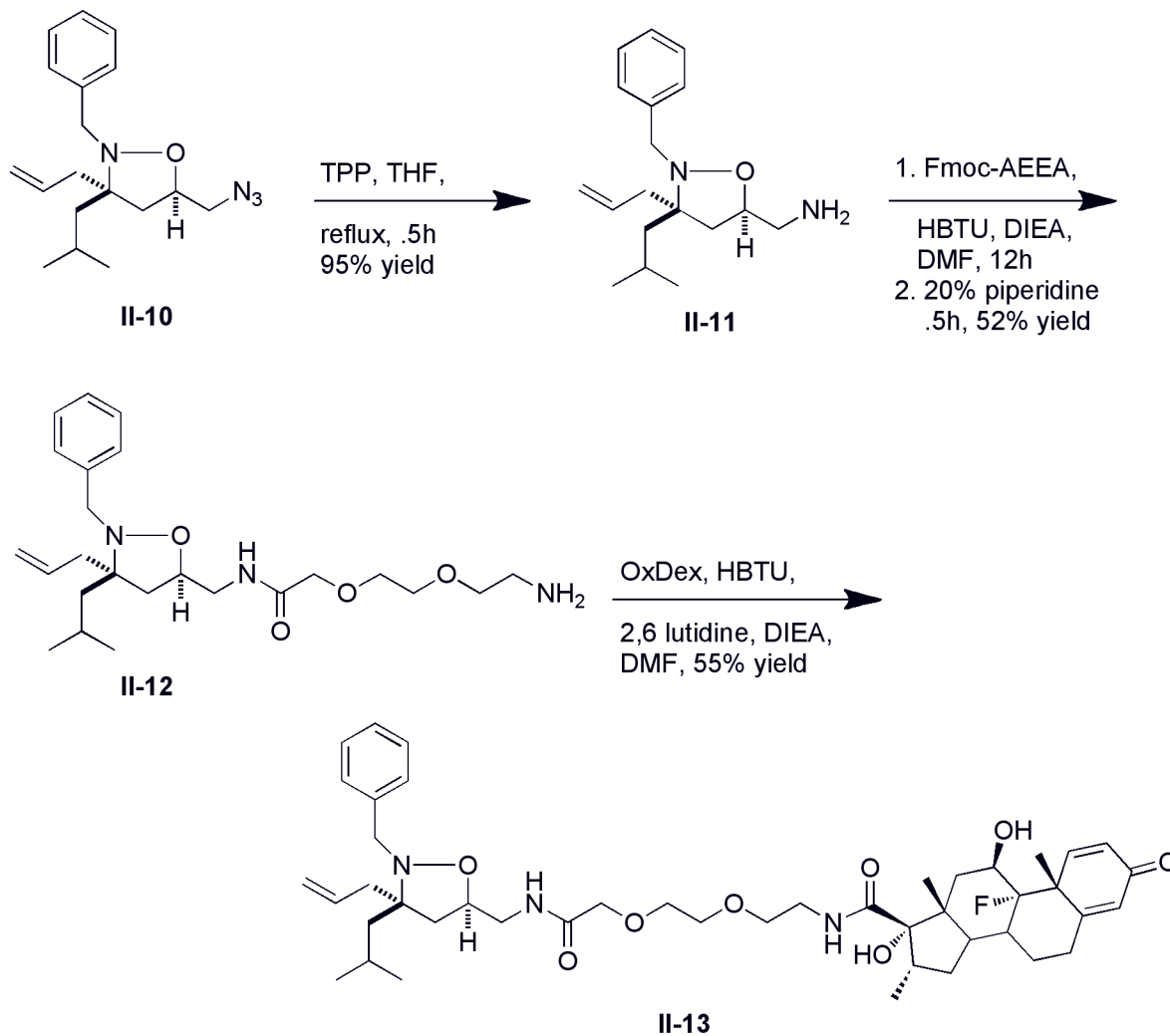


Scheme II-1. Synthesis of isoxazolidine **II-10**. The numbered ring positions of isoxazole **II-2** correspond with the text starting with O1 and ending with C5.

To synthesize the hydrophobic isoxazolidine **II-13**, reduction of the azide **II-10** with triphenylphosphine yielded the amine **II-11** (**Scheme II-2**). Alternatively, the azide can be reduced with tris(2-carboxyethyl)phosphine (TCEP). This amine was reacted through standard amide bond coupling conditions with Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-AEEA), HBTU, and DIEA. The crude material was treated with 20% piperidine in DMF and HPLC purified to afford amine **II-12** in 52% yield. This amine was lyophilized and conjugated to OxDex with HBTU, 2,6-lutidine,

DIEA, and NMP to give the conjugate **II-13** which was HPLC purified in 55% yield.

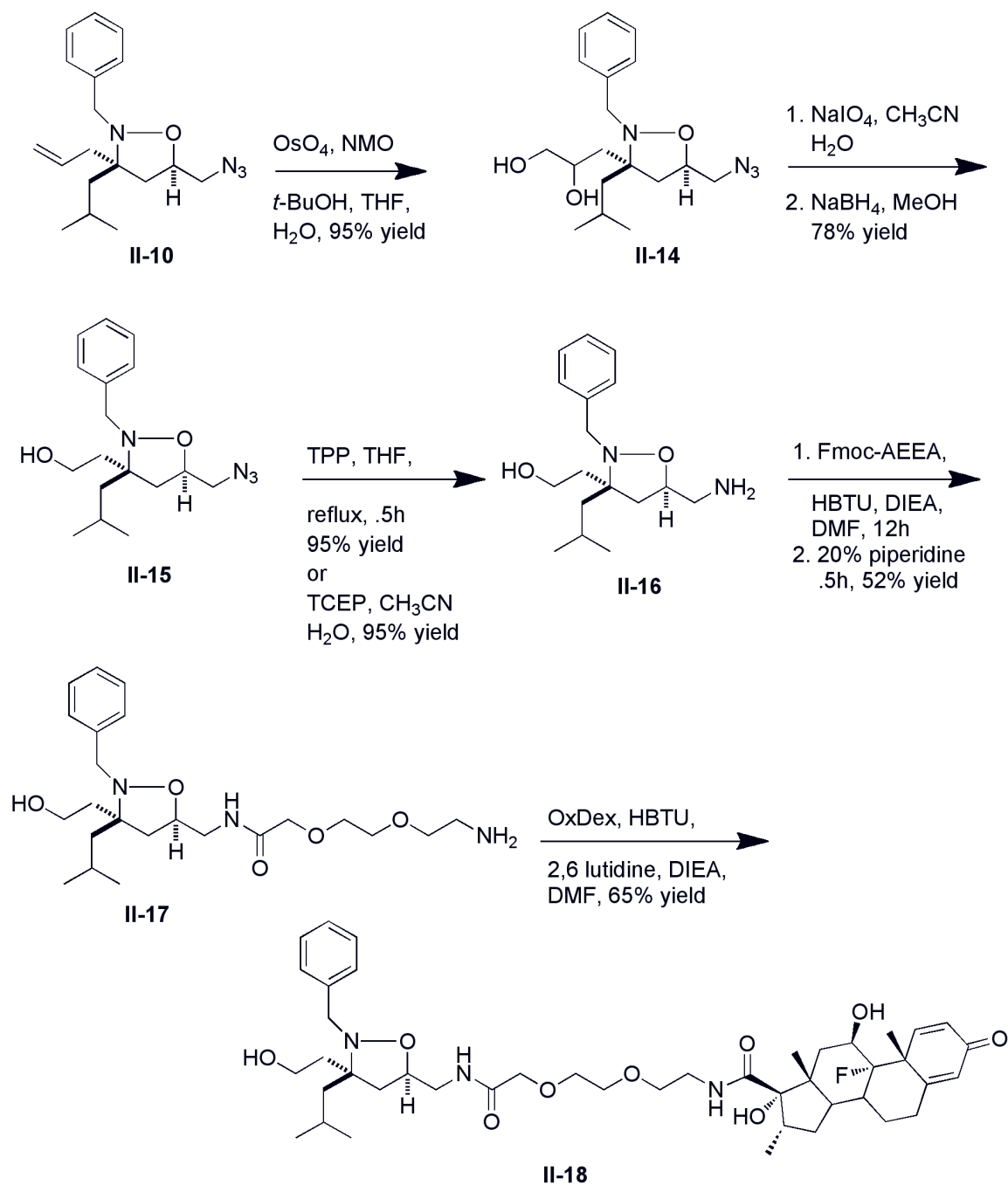
The purity of the compound was confirmed by analytical HPLC.



Scheme II-2. Synthesis of hydrophobic isoxazolidine conjugate.

To prepare the amphipathic isoxazolidine, the double bond was oxidatively cleaved to yield the desired alcohol at that position. This functional group conversion was afforded by an initial dihydroxylation of the double bond followed by treatment

with a catalytic amount of OsO₄ and two equivalents of NMO, affording the diol **II-14** in 95% yield; this was subsequently cleaved to the aldehyde by treatment with NaIO₄. The final alcohol **II-15** was afforded by reduction of the crude aldehyde with NaBH₄ in 78% yield. Similar to the hydrophobic isoxazolidine, azide **II-16** was reduced with triphenylphosphine and the crude material was conjugated to Fmoc-AEEA after extraction with acid, neutralization, and extraction into ethyl acetate. The conjugated was treated with 20% piperidine in DMF and isolated by reverse phase HPLC in 52% yield to provide compound **II-17**. The amine was then linked to OxDex through standard amide bond conditions using 2,6-lutidine, DIEA, HBTU, and NMP and purified by reverse phase HPLC to provide **II-18** in 65% yield. Purity of the molecule was confirmed by analytical HPLC.



Scheme II-3. Synthesis of amphipathic isoxazolidine conjugate **II-18**.

D. Activity of Isoxazolidines in a Cellular System

To test the small molecules in cells, Dr. Steve Rowe and Dr. Brian Brennan utilized a system developed in the Kodadek lab where a fusion protein consisting of Gal4 DBD and the ligand binding domain of the glucocorticoid receptor is transiently transfected on plasmid (**Figure II-4**). In addition, HeLa cells were transfected with a plasmid containing five Gal4 binding sites located upstream of a firefly luciferase reporter gene. As a transfection control and to facilitate assessment of compound toxicity, a third plasmid that expressed *Renilla* luciferase was also included. To calculate fold activation, the firefly luciferase was divided by the *Renilla* luciferase. This number was normalized to the AEEA-OxDex construct at the same concentrations. The isoxazolidines were attached, via an AEEA linker, to OxDex which binds glucocorticoid ligand binding domain, thus localizing the molecule to DNA.

As seen in **Figure II-6**, the amphipathic isoxazolidine exhibited a 80-fold level of activation at 1 μ M and was activate at even low nanomolar concentrations (15-fold at 10nM).²⁷ The calculated EC₅₀ of the isoxazolidine is 33 +/- 6nM. Similar to the cell free results, the hydrophobic isoxazolidine did not activate over the concentration range investigated.

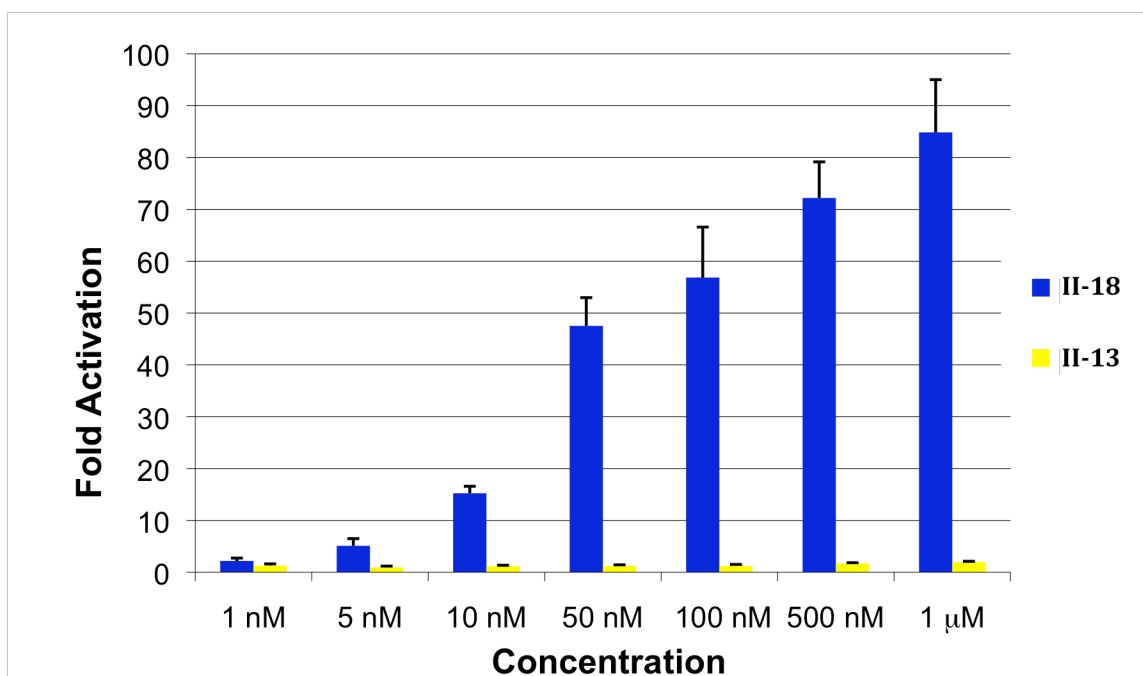


Figure II-6. Transcriptional activation of **II-18** and **II-13**.

This represents the first small molecule TAD reported to function in cells and an important step in the development of a functional tool to study development, differentiation, and determination of cell phenotype. Prior to this example, only one non-peptidic TAD existed: a peptoid developed in the Kodadek lab had been reported to activate transcription in cells; however, it has high molecule weight (greater than 1000 g/mol) and an EC_{50} of 10 μ M (**Figure II-6**). While a big step towards the development of TAD replacement, these limitations curb its use as a biological tool or development as a drug. However, the amphipathic isoxazolidine has a molecular weight of less than 300 g/mol and is a potent activator at low concentrations which make it a more likely drug candidate and potentially more useful to study biological systems.

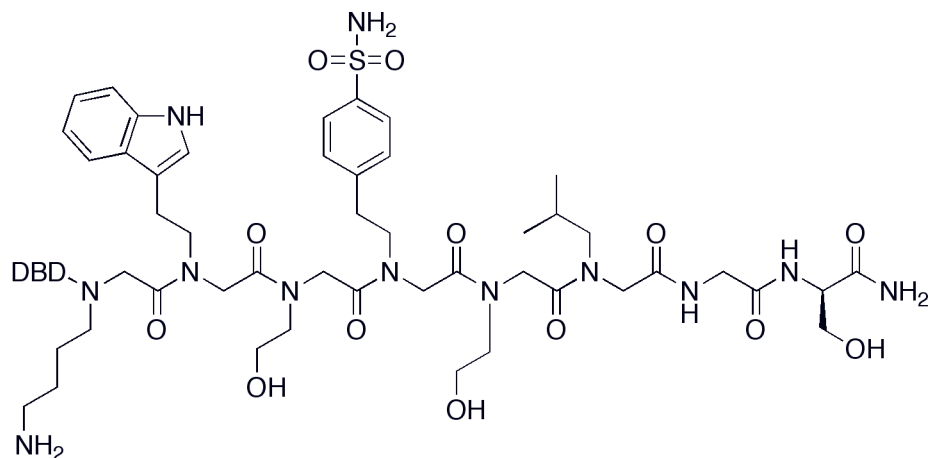


Figure II-7. Peptoid TAD

E. Independent Function of Isoxazolidine TADs

A characteristic of natural transcriptional activators is that the DBD and TAD function independently. However, it is possible that the isoxazolidine could be activating transcription by an alternative mechanism. One possibility is the isoxazolidine causes a conformational change in the GR ligand binding domain and thus exposes a protein surface that interacts favorably with the transcriptional machinery. To this end we conducted a competitive inhibition or ‘squenching’ experiment of the TAD. Increasing concentrations of free TAD **II-16** was added a consistent concentration of construct. As seen in (**Figure II-8**), the construct showed a dose dependent decrease in activation with approximately 70% inhibition when 100 μM of free isoxazolidine was added.²⁷ This is consistent with the DBD being functionally inert.

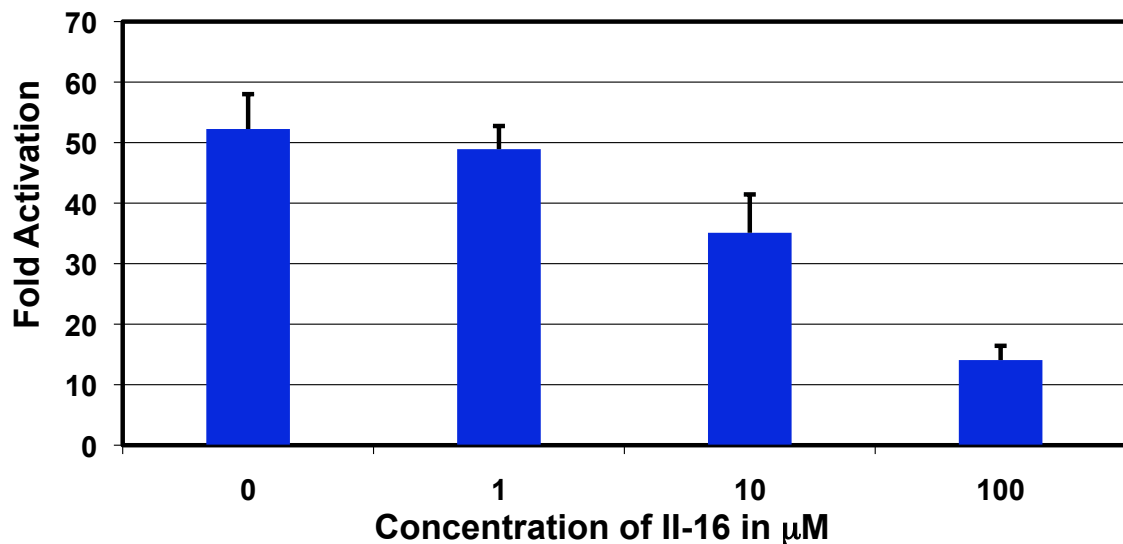


Figure II-8. Inhibition of **II-18**. Increasing concentrations of **II-16** inhibits the transcriptional function of **II-18**.

In a second experiment, we ‘squashed’ DNA localization by increasing concentrations of inactive isoxazolidine in the presence of a constant concentration of isoxazolidine TAD.²⁷ The inactive conjugate occupied a portion of the OxDex binding sites and resulted in a dose dependent reduction in transcription. It is important to note that the hydrophobic isoxazolidine alone does not squelch transcription by the active TAD. Together these experiments suggest that the TAD is an active compound which should be amendable to other DBDs and can act as a non-protein based transcriptional activator. To this end, the Kodadek lab has reported that a peptoid activator modestly upregulates transcription when attached to a hairpin polyamide (4.5 fold at 3 μM).³³

F. Conclusions

A facile synthetic approach for generation of isoxazolidine was utilized to enable the rapid generation of isoxazolidines with the potential to generate diverse isoxazolidines. Using this synthesis, we generated the first small molecule to function as a TAD in cell culture. This molecule elicits a maximal activation of 80-fold and exhibits measurable activity even at low nanomolar concentrations in HeLa cells. This TAD also functions similar to natural TADs as it is portable and has the potential to act as a TAD when attached to a synthetic DBD. The free isoxazolidine can act as an inhibitor of isoxazolidine conjugate, which provides some groundwork for their utility as transcriptional inhibitors. Aberrant transcription is responsible for undesirable cell phenotype and specific regulation of these genes would have enormous utility.

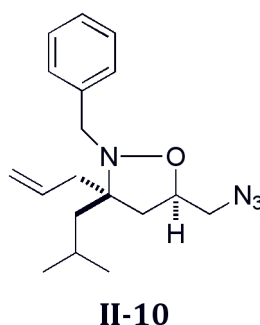
In sum, these experiments demonstrate that artificial transcriptional activators can be generated, using relatively simple criteria that mimic the function and mode of action of endogenous transcriptional activators. Such molecules are important as probes for deciphering mechanistic details of transcription regulatory networks. In Chapter 3, I discuss our approach to expand the scope of activators and if the isoxazolidine is a privileged scaffold or if other scaffolds that meet specified criteria can also function as TADs.

G. Experimental

General synthetic methods

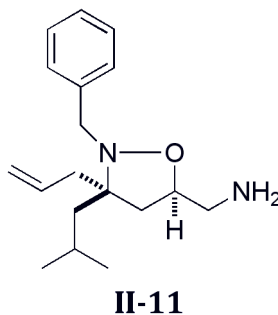
Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. CH₂Cl₂ and THF were dried by passage through activated alumina columns. All reactions were performed under a

dry N₂ atmosphere unless otherwise specified. Et₃N was distilled from CaH₂. Purification by flash chromatography was carried out with E. Merck Silica Gel 60 (230-400 mesh). ¹H and ¹³C NMR spectra were recording in CDCl₃ at 400 MHz and 100 MHz, respectively, unless otherwise specified. IR spectra were measure as thin films on NaCl plates. Reverse-phase HPLC purifications were performed on a Varian ProStar 210 equipped with Rainin Dynamax UV-D II detector ($\lambda = 254$ nm and $\lambda = 214$) using a C18 (8 x 100mm) Radial-PakTM cartridge with a gradient of 0.1% TFA in H₂O and CH₃CN as the mobile phase.



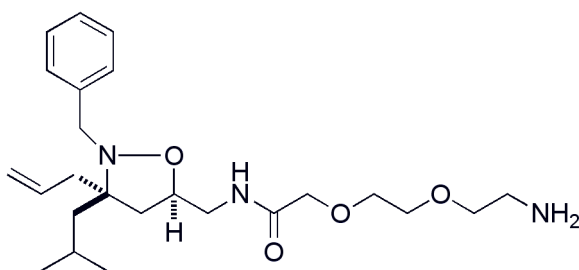
(3-Allyl-2-benzyl-3-isobutyl-isoxazolidine-5-yl)-methylazide (II-10): To a solution of isoxazolidine **II-9** (200 mg, 0.69 mmol, 1.0 eq) in THF (1.4 mL) cooled in an ice-H₂O bath was added a solution of triphenylphosphine (362 mg, 1.38 mmol, 2.0 eq) in THF (1.4 mL). Diphenylphosphoryl azide (0.187 mL, 0.863 mmol, 1.25 eq) was added dropwise and the mixture stirred for 15 min. Diethyl azodicarboxylate (0.15 mL, 0.92 mmol, 1.3 eq) was added dropwise over 5 min. The reaction mixture was stirred for 2 h at which time the reaction was judged complete by ESI-MS analysis. The reaction mixture was then diluted with saturated Na₂CO₃ (2.4 mL) and

stirred for 30 min. The resulting mixture was poured into a biphasic mixture of ether (30 mL) and water (20 mL). The organic layer was separated and the aqueous layer was extracted with ether (2 x 15 mL). The combined organic extracts were washed with water (50 mL), dried over Na₂SO₄, filtered, and concentrated *in vacuo* to a yellow oil. Purification by flash chromatography (97:3 hexanes/EtOAc) provided 154 mg (71% yield) of **II-10** as an oil. IR: 2955, 2869, 2099, 1454, 1278, 916, 732 cm⁻¹; ¹H NMR: δ .96 (d, 3H, *J* = 6.6), .98 (d, 3H, *J* = 6.6), 1.37 (dd, 1H, *J* = 14.7, 6.6), 1.59 (dd, 1H, *J* = 14.5, 5.1), 1.80-1.91 (m, 2H), 2.26 (dd, 1H, *J* = 13.9, 7.4), 2.31 (dd, 1H, *J* = 12.7, 8.6), 2.44 (dd, 1H, *J* = 13.7, 7.0), 3.06 (dd, 1H, *J* = 12.3, 3.3), 3.44 (dd, 1H, *J* = 12.7, 7.3), 3.87 (m, 1H), 3.89 (m, 1H), 4.10-4.15 (m, 1H), 5.06-5.15 (2H, m), 5.85-5.98 (1H, m), 7.19-7.41 (5H, m); ¹³C NMR: δ 24.11, 24.62, 25.22, 38.68, 40.21, 43.56, 53.28, 54.39, 68.29, 74.49, 117.8, 126.7, 128.0, 129.2, 134.9, 138.6; HRMS (ESI) calcd for [C₁₈H₂₆N₄O + H]⁺: 315.2185, found: 315.2171.



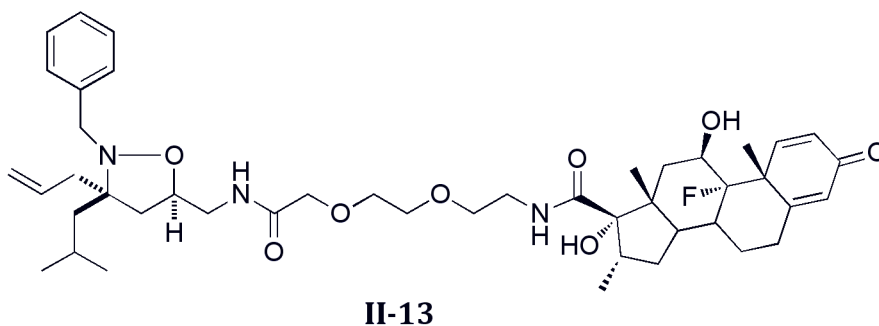
(3-Allyl-2-benzyl-3-isobutyl-isoxazolidine-5-yl)-methylamine (II-11): To a solution of azide **II-10** (15 mg, 0.048 mmol, 1.0 eq) in THF (0.50 mL) was added

triphenylphosphine (25 mg, 0.099 mmol, 2.0 eq) and water (7.7 μ L, 0.480 mmol, 10 eq). The mixture was allowed to stir at reflux for 2 h. The mixture was then poured into a biphasic mixture of 1N HCl (10 mL) and ether (10 mL). The layers were partitioned and the organic layer was extracted with 1N HCl (2 x 10 mL). The combined aqueous layers were basified with 3N NaOH (until pH 10 or greater). The aqueous mixture was then extracted with CH_2Cl_2 (3 x 15 mL) and the combined organics were dried over Na_2SO_4 , filtered, and concentrated *in vacuo*. The crude material was purified by flash chromatography (96:4 CH_2Cl_2 :MeOH) to provide 12.8 mg (92% yield) of **II-11** as an oil. IR: 2955, 2869, 1455, 1278, 916, 732 cm^{-1} ; ^1H NMR: δ .93 (d 3H, J = 6.8), .96 (d 3H, J = 6.6), 1.32 (dd, 1H, J = 14.5, 6.4), 1.38-1.52 (m, 2H), 1.58 (dd, 1H, J = 14.5, 4.9), 1.76-1.89 (m, 2H), 2.21-2.30 (m, 2H), 2.39 (dd, 1H, J = 13.9, 7.4), 2.59-2.69 (m, 1H), 2.75-2.87 (m, 1H), 3.79-3.92 (m, 3H), 5.06-5.15 (m, 2H), 5.85-5.98 (m, 1H), 7.20-7.45 (m, 5H); ^{13}C NMR: δ 24.12, 24.61, 25.20, 38.89, 40.56, 43.72, 46.79, 46.83, 53.48, 68.40, 117.6, 126.7, 127.9, 128.2, 135.2, 139.2; HRMS (ESI) calcd for $[\text{C}_{18}\text{H}_{28}\text{N}_2\text{O} + \text{H}]^+$: 289.2280, found: 289.2271.

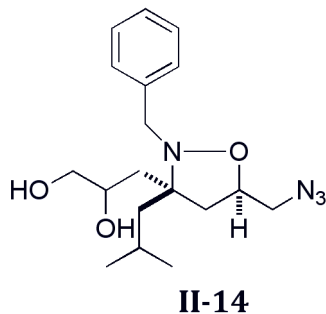


II-12

***N*-(3-Allyl-2-benzyl-3-isobutyl-isoxazolidin-5-ylmethyl)-2-[2-(2-amino-ethoxy)-ethoxy]-acetamide (II-12)**: To a solution of Fmoc-AEEA (52 mg, .14 mmol, 3 eq) dissolved in NMP (.25 mL) were added HOBt (18 mg, .14 mmol, 3 eq) and HBTU (51 mg, .14 mmol, 3 eq). This solution was agitated for 15 min. The solution of activated Fmoc-AEEA was added to amine **II-11** (13 mg, .045 mmol, 1 eq) dissolved in NMP (.25 mL) and the resulting mixture was allowed to stir for 12 h at which time the reaction was complete as judged by ESI-MS analysis. Excess reagents were quenched by addition of 1N HCl (10 mL) and EtOAc (10 mL). The reaction vessel was washed with EtOAc (2 x 2 mL) to remove all residues. The resulting biphasic mixture was separated and the aqueous layer was extracted with EtOAc (3 x 10 mL). The combined organic fractions were dried over Na₂SO₄, filtered, and concentrated *in vacuo*. The resulting oil was dissolved in a solution of 20% piperidine in DMF (.110 mL, .225 mmol piperidine w/v, 5 eq) and was allowed to stir for 30 minutes. The resulting solution was diluted with methanol (10 mL) and extracted with pentane (3 x 30 mL). The methanol layer was concentrated to give crude **II-12**. Reverse-phase HPLC purification provided **II-12** as an oil (52% yield; 6 mg). The identity was verified by mass spectral analysis of the isolated product and NMR analysis. ¹H NMR (CD₃OD): δ .97 (d, 3H, *J* = 6.6), 1.05 (d, 3H, *J* = 6.6), 1.44 (dd, 1H, *J* = 14.3, 6.3), 1.71 (dd, 1H, *J* = 14.5, 5.1), 1.85-2.01 (m, 3H), 2.35-2.55(m, 3H), 3.0-3.08 (m, 2H), 3.35-3.43 (m, 2H) 3.55-3.65 (m, 6H), 3.91-4.10 (m, 4H), 4.15-4.27 (m, 1H), 5.11-5.21 (m, 2H), 5.91-6.02 (m, 1H), 7.20-7.44 (m, 5H); HRMS (ESI) calcd for [C₂₄H₃₉N₃O₄ + H]⁺: 434.3019, found: 434.3015.

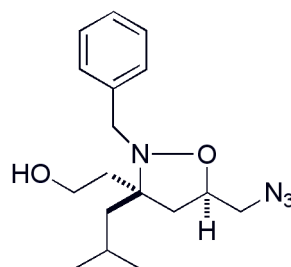


9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,11,12,14,15,16,17-dodecahydro-3H-cyclopenta[*a*]phenanthrene-17-carboxylic acid [2-(2-{{[(3-allyl-2-benzyl-3-isobutyl-isoxazolidin-5-ylmethyl)-carbamoyl]-methoxy}-ethoxy)-ethyl]-amide (II-13): To a solution of OxDex (2.8 mg, 7.3 μ mol, 4.0 eq) dissolved in NMP (50 μ L) was added HOBt (1.1 mg, 7.3 μ mol, 4.0 eq) and HBTU (2.8 mg, 7.3 μ mol, 4.0 eq) and the resulting mixture was agitated for 15 min. To this solution was added a solution of isoxazolidine **II-12** (0.8 mg, 1.8 μ mol, 1.0 eq) dissolved in NMP (50 μ L), 2,6-lutidine (10 μ L, 0.10 mmol, 56 eq), and DIEA (10 μ L, 0.10 mmol, 58 eq). The resulting mixture was stirred for 12 h at rt. The product was isolated by reverse-phase HPLC purification to provide **II-13** as a mixture of diastereomers as a white solid (55% yield; 0.8 mg). The purity of compound **II-13** was confirmed by reverse-phase HPLC analysis. The identity was verified by mass spectral analysis of the isolated compound. HRMS (ESI) calcd for $[C_{45}H_{64}FN_3O_8 + H]^+$: 794.4756, found: 794.4767



3-(2-Benzyl-5-methylazido-3-isobutyl-isoxazolidin-3-yl)propane-1,2-diol (II-14): To a solution of alkene **II-10** (137 mg, .448 mmol, 1.0 eq) dissolved *t*-BuOH (3.1 mL), THF (1.0 mL), H₂O (.25 mL) was added NMO (70 mg, .62 mmol, 1.4 eq). To this solution was added OsO₄ (.44 mL of a 2.5 wt% solution in *t*-BuOH, .044 mmol, .10 eq). The reaction mixture was allowed to stir at ambient temperature until the reaction was judged complete by TLC analysis (typically 5 h). Excess reagents were quenched by addition of Na₂SO₃ (1.0 mL of 10% solution w/v in water) and allowed to stir for 2 h. The mixture was then poured into biphasic mixture H₂O (80 mL) and EtOAc (80 mL). The organic layer was separated and the aqueous layer was extracted EtOAc (3 x 50 mL). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated *in vacuo*. The crude product was subjected to flash chromatography (4:6 hexanes/EtOAc) to provide 156 mg of diol **II-14** (2:1 mixture of diastereomers, indicated as 'maj' and 'min' in the listing of spectral data) in 98% yield as a clear oil. IR: 3374, 2957, 2869, 2100, 1454, 1281, 1069, 918, 735, 697 cm⁻¹; ¹H NMR: δ .89-1.0 (m, 6H, maj, 6H, min), 1.30-1.50 (m, 2H, min, 1H, maj), 1.55-1.76 (m, 3H, maj, 3H, min), 1.85-2.15 (m, 2H, maj, 1H min), 2.26-2.60 (m, 1H, maj, 2H, min), 3.19-3.3 (m, 1H maj), 3.35-3.64 (m, 3H, maj, 4H, min), 3.70-3.91 (m, 4H, maj),

3.96-4.09 (m, 4H, min) 4.10-4.18 (m, 1H, maj) 4.24-4.34 (m, 1H, min), 5.71(br s, 1H, min), 6.30 (br s, 1H, maj), 7.19-7.42 (m, 5H, maj, 5H, min); HRMS (ESI) calcd for $[C_{18}H_{28}N_4O_3 + H]^+$: 349.2240, found: 349.2235.

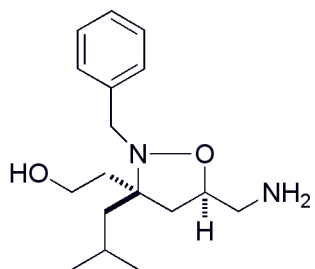


II-15

3-(2-Benzyl-5-methylazido-3-isobutyl-isoxazolidin-3-yl)-ethanol (II-15): **3-(2-Benzyl-5-methylazido-3-isobutyl-isoxazolidin-3-yl)-ethanol (II-15):** To a solution of diol **II-14** (156 mg, .447 mmol, 1.0 eq) in CH_3CN (2.1 mL) and H_2O (2.1 mL) was added $NaIO_4$ (191 mg, .894 mmol, 2 eq). The mixture was allowed to stir at room temperature until the reaction was judged complete by TLC analysis. The reaction mixture was poured into a biphasic solution of H_2O (50 mL) and EtOAc (50 mL). The organic layer was separated and the aqueous layer was extracted with EtOAc (2 x 50 mL). The combined organic layers were dried over Na_2SO_4 , filtered, and concentrated *in vacuo*. The crude material was partially purified on a short silica gel column (9:1 hexanes/EtOAc) and the resulting oil, 121 mg after concentration *in vacuo*, was used immediately in the subsequent step without further purification. IR: 2956, 2869, 2099, 1715, 1455, 1273, 1028, 732 cm^{-1} ; 1H NMR: δ .97 (d, 3H, $J = 6.6$), .96 (d, 3H, $J = 6.6$), 1.4 (dd, 1H, $J = 14.3, 7.1$), 1.64 (dd, 1H,

$J = 14.3, 4.7$), 1.73-1.83 (m, 1H), 2.12 (dd, 1H, $J = 12.8, 5.7$), 2.47 (dd, 1H $J = 13.8, 2.3$), 2.55 (dd, 1H, $J = 12.8, 8.5$), 2.66 (dd, 1H, $J = 13.7, 2.7$), 2.95-3.05 (m, 1H) 3.44 (dd, 1H, $J = 12.7, 7.6$), 3.84 (d, 1H, $J = 14.4$), 3.92 (d, 1H, $J = 14.4$), 3.96-4.05 (m, 1H), 7.17-7.35 (m, 5H), 9.91 (t, 1H, $J = 2.6$); ^{13}C NMR: δ 24.10, 24.66, 25.04, 41.73, 44.80, 48.09, 53.40, 54.13, 69.47, 74.56, 127.0, 128.1, 128.3, 137.6, 200.0; HRMS (ESI) calcd for $[\text{C}_{17}\text{H}_{24}\text{N}_4\text{O}_2 + \text{H}]^+$: 317.1978, found: 317.1966.

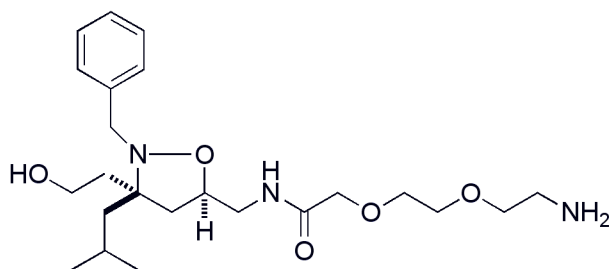
Crude aldehyde (121 mg, .380 mmol, 1 eq) was dissolved in methanol (3.8 mL) and the solution was cooled in an ice- H_2O bath. NaBH_4 (36 mg, .96 mmol, 2.5 eq) was added and the mixture was then allowed to stir until the reaction was judged complete by TLC analysis (typically 30 min). Excess reagents were quenched with H_2O (4 mL) the reaction solution was poured into a biphasic mixture of EtOAc (30 mL) and H_2O (20 mL). The organic layer was separated and the aqueous phase was extracted with EtOAc (2 x 30 mL). The combined organic extracts were dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude material was purified by flash chromatography (1:1 hexanes/EtOAc) to give 106 mg of the title compound **II-15** in 78% yield from diol **II-14** as an oil. IR: 3368, 2955, 2870, 2100, 1455, 1280, 1045, 733, 697 cm^{-1} ; ^1H NMR: δ 1.03 (d, 6H, $J = 6.4$), 1.50-1.60 (m, 1H), 1.65-1.95 (m, 5H), 2.07 (dd, 1H, $J = 12.7, 8.1$), 2.36 (dd, 1H, $J = 12.7, 8.0$), 3.41 (d, 2H, $J = 4.7$), 3.78-3.92 (m, 3H), 3.99 (d, 1H, $J = 13.9$), 4.19-4.27 (m, 1H), 7.22-7.37 (m, 5H); ^{13}C NMR: δ 24.31, 24.89, 25.24, 35.61, 40.82, 43.00, 53.69, 54.49, 59.67, 70.43, 127.2, 128.4, 128.6, 137.6; HRMS (ESI) calcd for $[\text{C}_{17}\text{H}_{26}\text{N}_4\text{O}_2 + \text{H}]^+$: 319.2134, found: 319.2122.



II-16

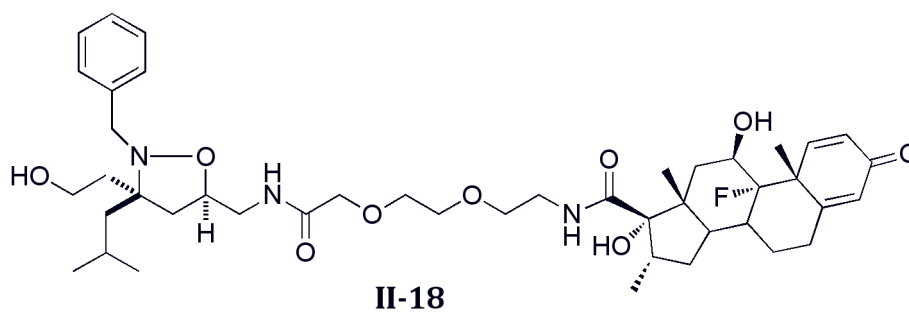
3-(2-Benzyl-5-methylamino-3-isobutyl-isoxazolidin-3-yl)-ethanol (II-16):

Reduction of **II-15** (17 mg) was accomplished under conditions identical to those used for **II-11**. Purification of the crude mixture by flash chromatography (90:10 CH₂Cl₂/MeOH) provided 15 mg of the title compound in 94% yield as clear oil. IR: 3454, 2953, 2867, 1454, 1365, 1045, 733, 697 cm⁻¹; ¹H NMR (CD₃OD): .96, (d, 3H, *J* = 6.6), .99 (d, 3H, *J* = 6.6), 1.35-1.42 (dd, 1H, *J* = 14.1, 6.5), 1.59-1.66 (dd, 1H, *J* = 14.1, 5.1), 1.76-1.94 (m, 5H), 2.39-2.46 (dd, 1H, *J* = 12.7, 8.1), 2.71-2.87 (m, 2H), 3.70-3.79 (m, 2H), 3.86 (s, 2H), 4.02-4.11 (m, 1H), 7.17-7.37 (m, 5H); δ ¹³C NMR: δ 24.85, 25.57, 25.85, 37.58, 42.86, 45.09, 46.70, 55.22, 60.08, 70.45, 127.9, 129.3, 129.5, 140.3; HRMS (ESI) calcd for [C₁₇H₂₈N₂O₂ + H]⁺: 293.2229, found: 293.2218.



II-17

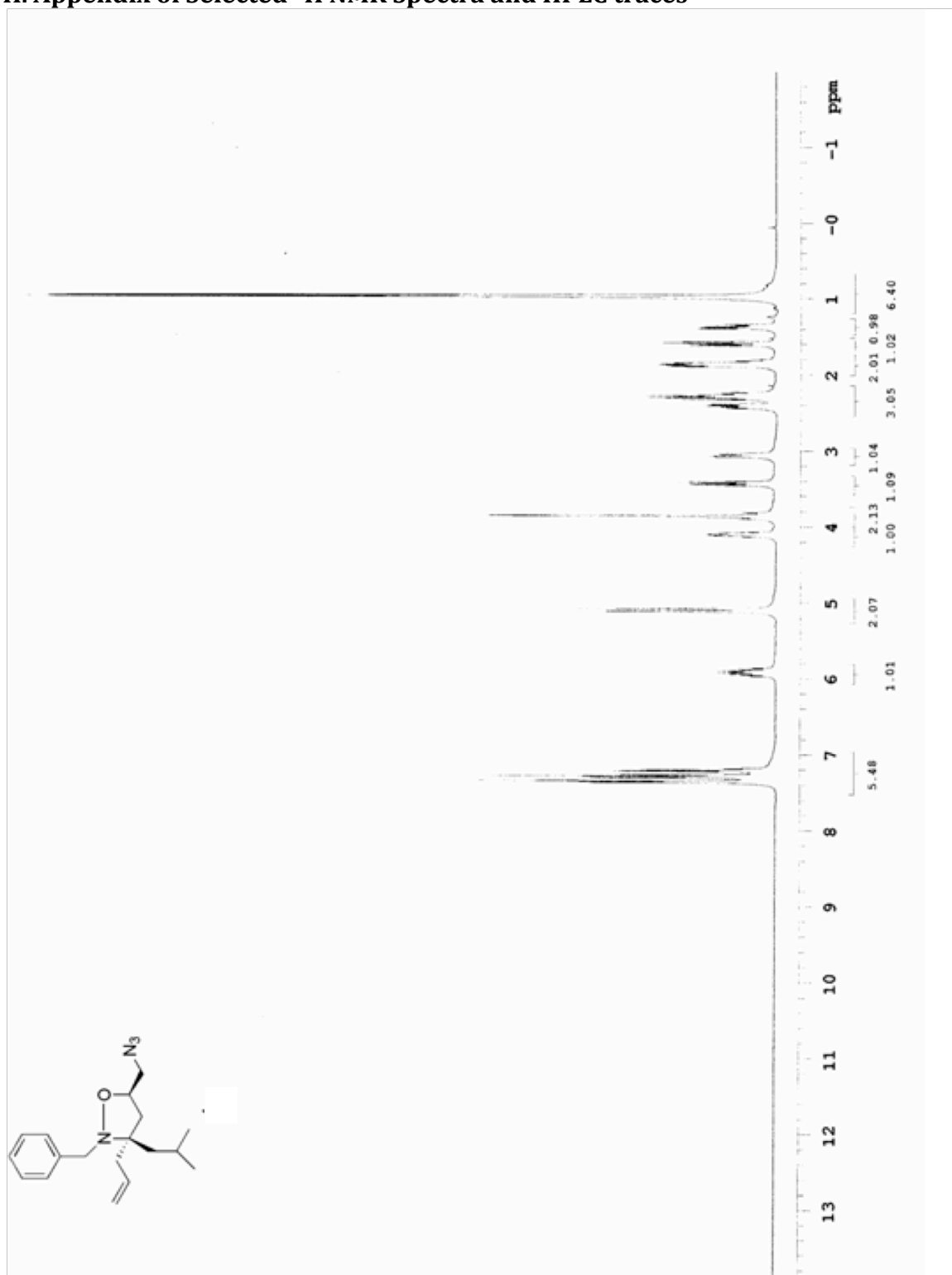
2-[2-(2-Amino-ethoxy)-ethoxy]-N-[2-benzyl-3-(2-hydroxy-ethyl)-3-isobutyl-isoxazolidin-5-ylmethyl]-acetamide (II-17): Preparation of **II-17** was accomplished under conditions identical to those used for **II-12**. Reverse-phase HPLC purification provided 5.0 mg of the title compound in 52% yield as a white solid. ¹H NMR (CD₃OD): .96 (d, 3H, *J* = 6.6), 1.0 (d, 3H, *J* = 6.6), 1.45 (dd, 1H, *J* = 13.7, 6.3), 1.66-1.85 (m, 2H), 1.90 (t, 2H, *J* = 6.8), 1.98-2.07 (m, 1H), 2.45 (dd, 1H, *J* = 12.7, 8.0), 3.00 (t, 2H, *J* = 5.1), 3.37 (dd, 1H, *J* = 13.7, 7.4), 3.43-3.61 (m, 6H), 3.72-3.79 (m, 3H), 3.90-4.00 (m, 4H), 4.17-4.27 (m, 1H), 7.21-7.39 (m, 5H); HRMS (ESI) calcd for [C₂₃H₃₉N₃O₅ + H]⁺: 438.2968, found: 438.2961.

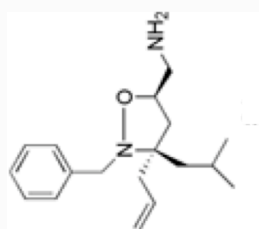


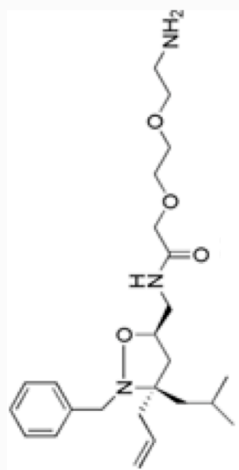
9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,11,12,14,15,16,17-dodecahydro-3H-cyclopenta[*a*]phenanthrene-17-carboxylic acid [2-(2-[[2-(2-benzyl-3-(2-hydroxy-ethyl)-3-isobutyl-isoxazolidin-5-ylmethyl)-carbamoyl]-methoxy]-ethoxy)-ethyl]-amide (II-18): Preparation of **II-18** was accomplished under conditions identical to those used for **II-13**. Reverse-phase HPLC purification provided 4 mg of the title compound in 65% yield as a mixture of diastereomers as a white solid. The purity of compound **II-14** was

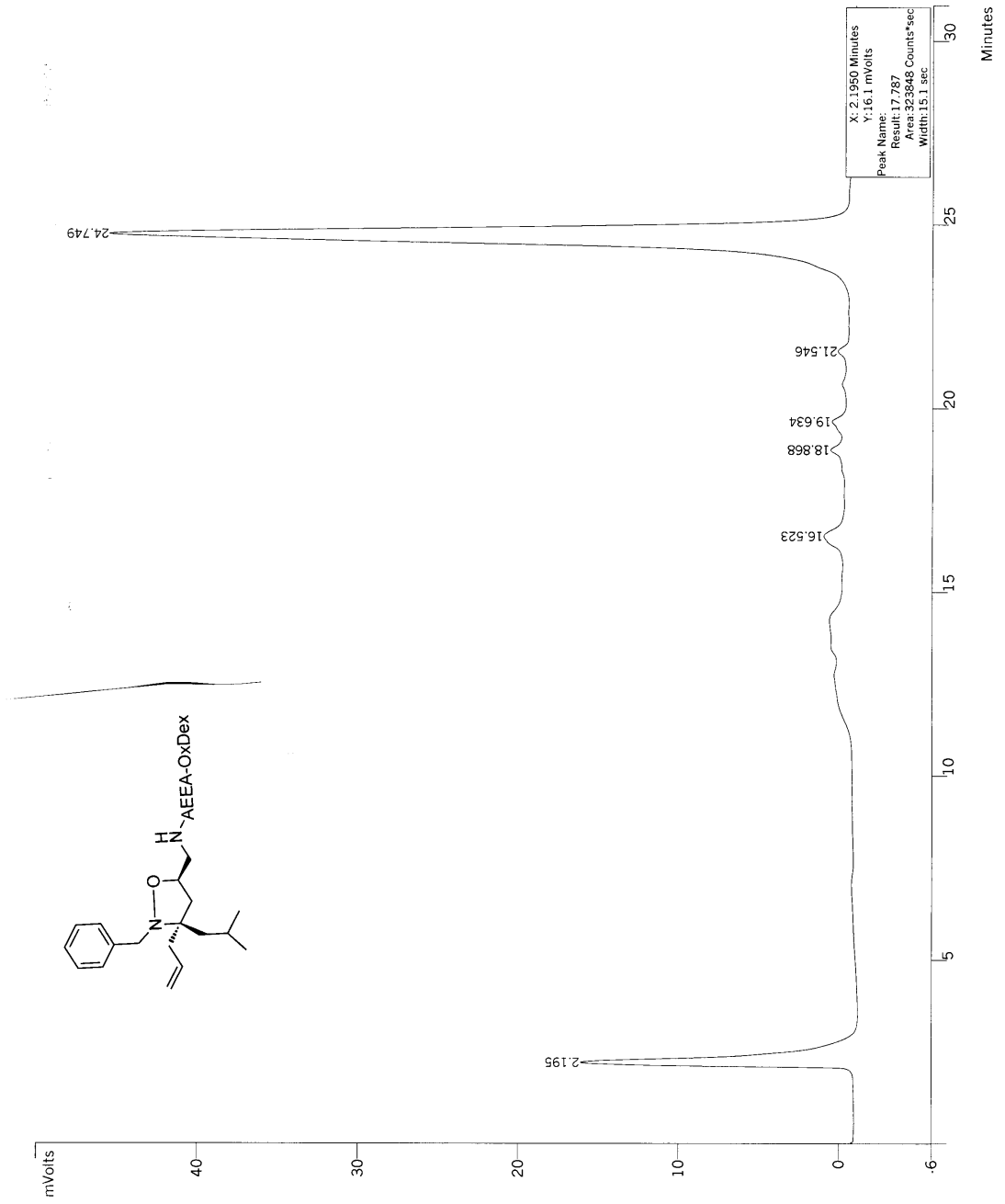
confirmed by reverse-phase HPLC analysis. The identity was verified by mass spectral analysis of the isolated compound. HRMS (ESI) calcd for $[C_{44}H_{64}FN_3O_9 + H]^+$: 798.4705, found: 798.4692.

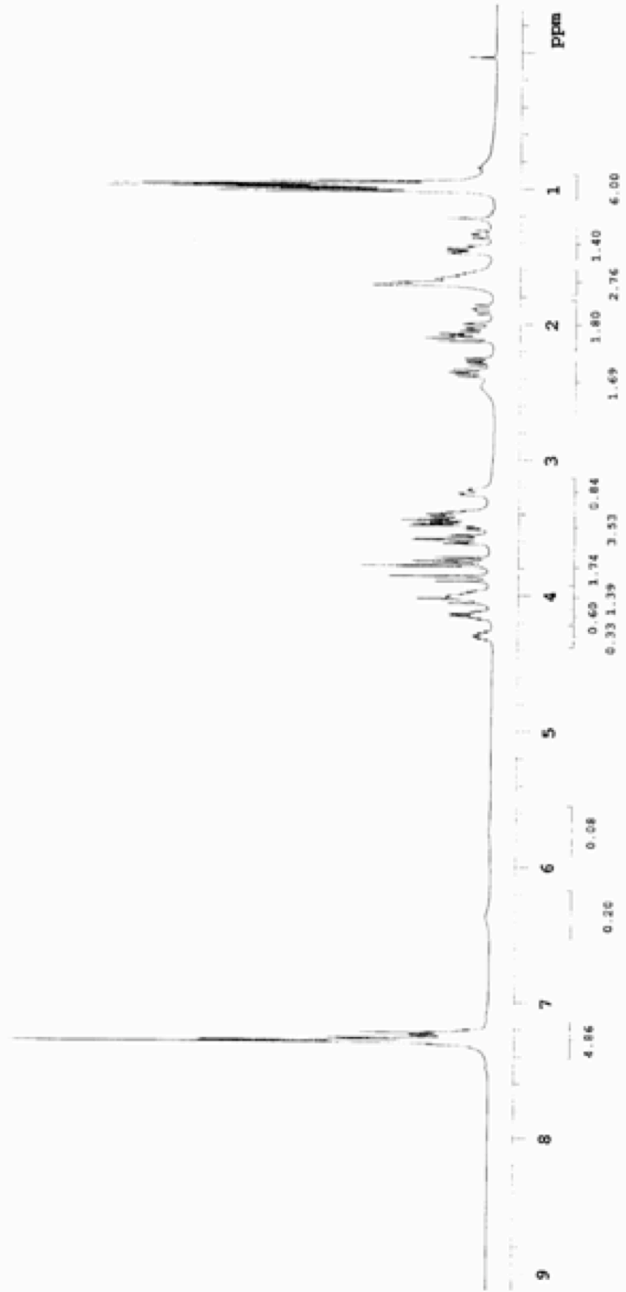
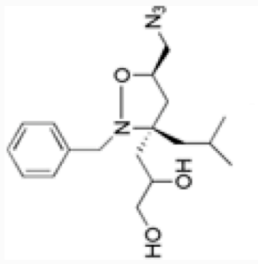
H. Appendix of Selected ^1H NMR Spectra and HPLC traces

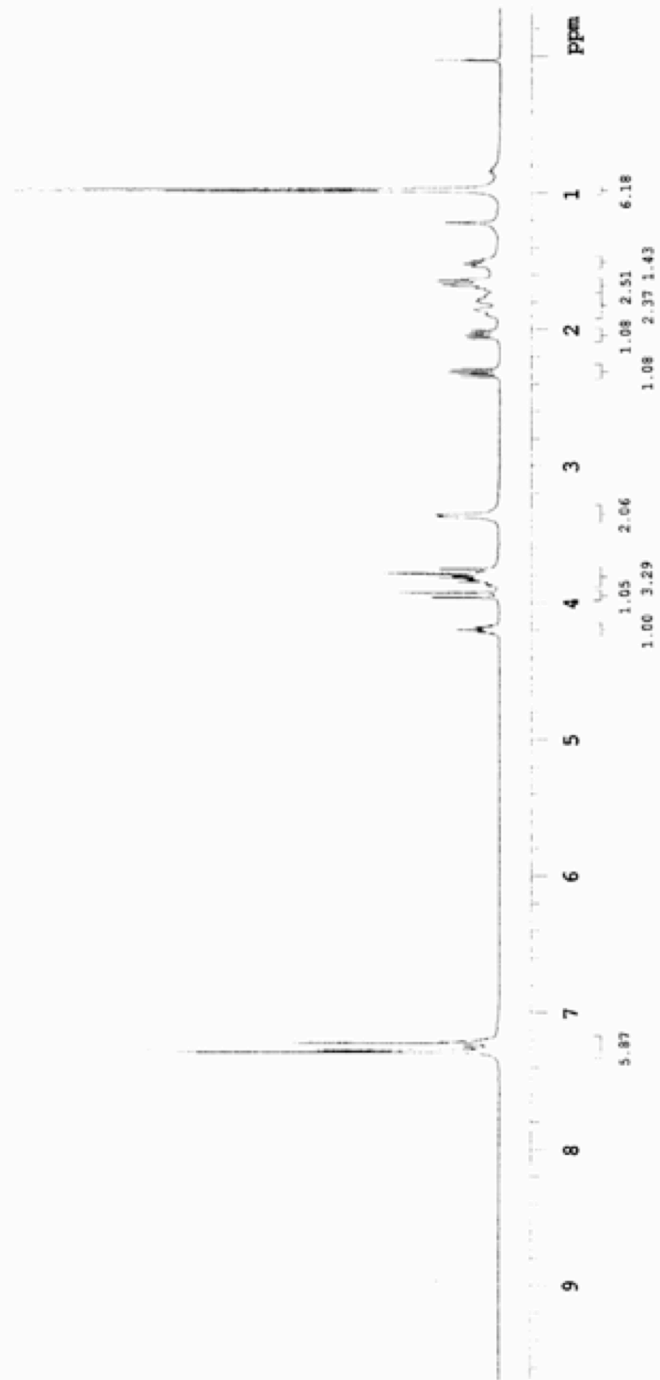
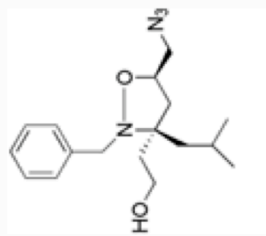


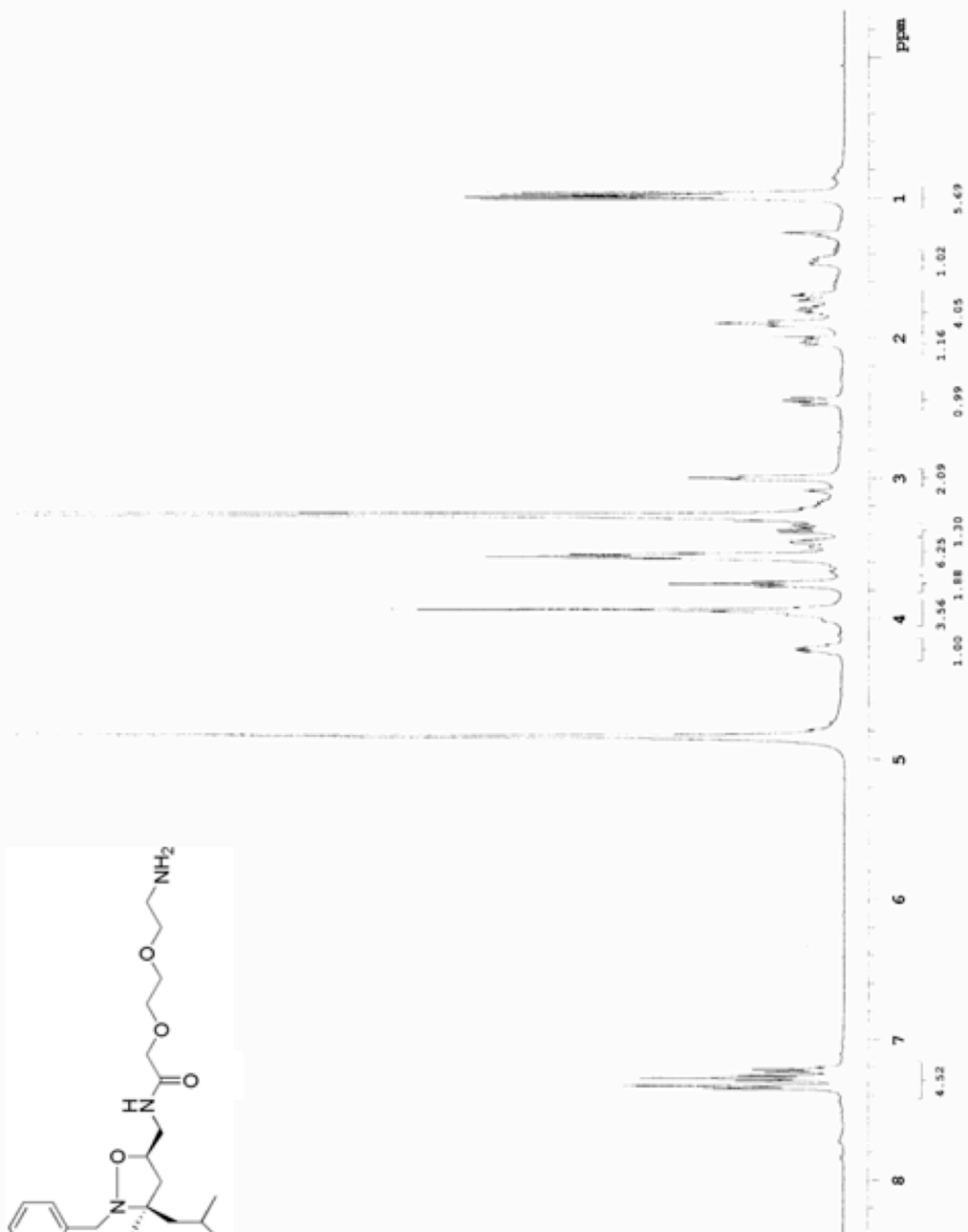
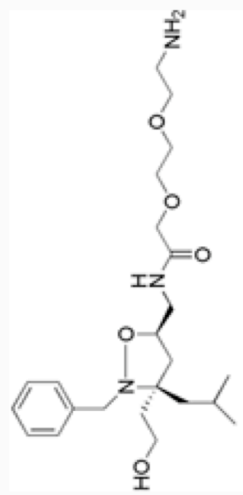


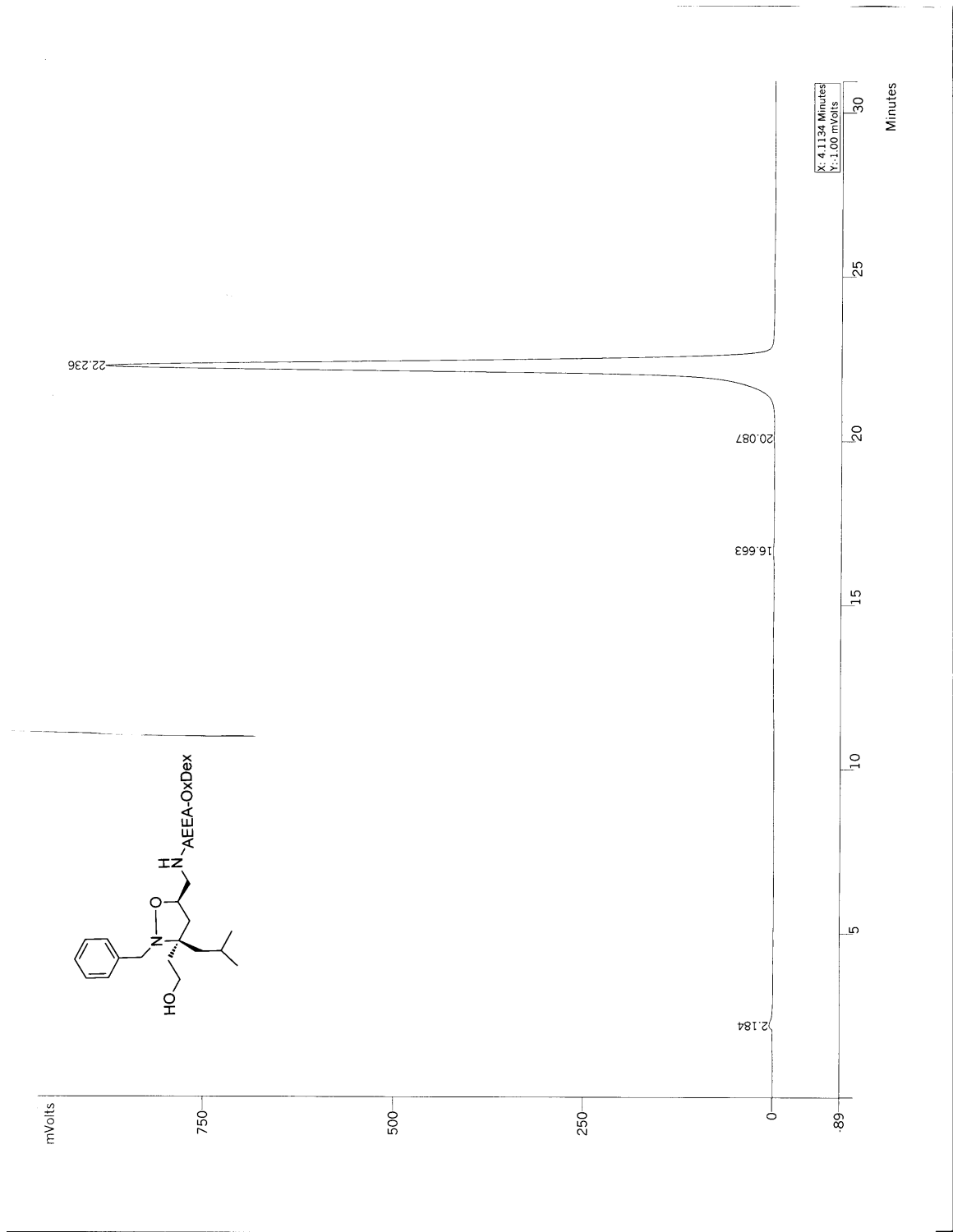












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Chapter III

Expanding the Collection of Small Molecule TADs^a

A. Abstract

Molecules that can reconstitute the function of transcriptional activators hold enormous potential as therapeutic agents and as mechanistic probes. Previously we described an isoxazolidine bearing functional groups similar to natural transcriptional activators that up-regulates transcription up to 80-fold in cell culture. In this Chapter, we analyze analogues of this molecule to define key characteristics of small molecules that function as transcriptional activation domains in cells. Conformational rigidity is an important contributor to function as is an overall amphipathic substitution pattern. Using these criteria, we identified additional molecular scaffolds with excellent activity as transcriptional activation domains. This design strategy will enable the creation of new generations of small molecules with activator function.

B. Introduction

^a Portions of this Chapter are reported in: Casey, R.J.; Desaulniers, J.P.; Hojfeldt, J.W.; Mapp, A.K. *Bioorg. Med. Chem.* **2009**, 17, 1034-1043. Jonas Hojfeldt and JP Desaulniers performed biological evaluation of the compounds. Ryan Casey and JP Desaulniers conducted the chemical synthesis.

We recently reported the first example of a small molecule transcriptional activation domain (TAD).¹ That molecule (**III-1**) was designed to mimic the composition of endogenous TADs with hydrophobic and polar functional groups and display those functional groups in three dimensions.² When localized to the promoter, the TAD activated transcription in cells, with up to 80-fold upregulation observed. Additionally, this molecule was similar to natural TADs as the isoxazolidine alone could compete with the DNA-localized molecule for coactivator targets.¹ The next logical step in this effort was to define the general characteristics of a small molecule TAD and identify a set of guidelines for the future synthesis of small molecule TADs.³

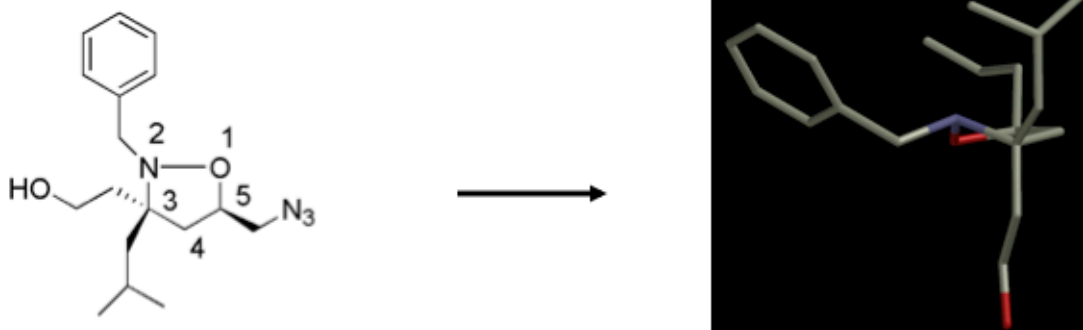


Figure III-1. The isoxazolidine scaffold. This 5-membered heterocycle projects its functional groups in a three dimensional array that allows the isoxazolidine to interact with many partners in the transcriptional machinery, a requirement for TADs.

As discussed in Chapter 2, natural TADs consist of hydrophobic and polar residues.² Shown below are sequences of some minimal natural amphipathic TADs (**Figure III-2**). Despite their diversity these sequences can all act as activators and

upregulate transcription when localized to DNA. Within those sequences, when residues such as alanine are mutated to large hydrophobic residues such as phenylalanine or leucine, a marked increase in activity is observed.⁴⁻⁶ Additionally, in the case of VP16, mutations of large hydrophobic residues to either polar or alanine abrogated binding to TFIID, a target of VP16.⁷ These observations provided evidence that highly active TADs should consist of large hydrophobic residues and these residues were important for binding.⁸⁻¹⁰ Next, researchers studied the location of these hydrophobic and polar residues within the sequence and discovered that residue placement was less important. Based on these observations, Dr. Sara Buhrlage demonstrated that the precise placement of amphipathic functional groups in an isoxazolidine TAD did not influence the ability of the molecule to activate transcription in a cell-free system (**Figure III-3**).¹⁰

VP16

441 AspPheAspLeuAspMetLeuGly 448

p53

9 SerValAspProProLeuSerGlnGluThrPheSerAspLeuTrp 23

CREB

134 TyrArgLysIleLeuAsnAspLeu 145

p65

535 SerIleAlaAspMetAspPheSerAlaLeuLeu 545

Figure III-2. Partial sequences of natural TADs. Polar residues are shown in red and hydrophobic residues are shown in blue.

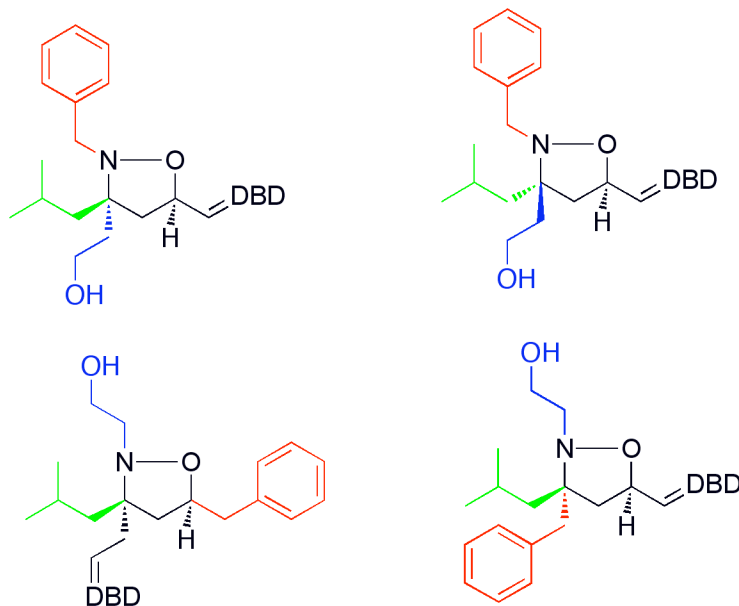


Figure III-3. A sampling of isomeric isoxazolidines designed by Dr. Sara Buhrlage.¹⁰ These isoxazolidines all activated transcription to similar levels in a cell free assay. From these studies it was determined that stereochemistry and the placement of functional groups had little influence in activity. DBD = DNA binding domain.

An idea was gleaned from these studies: the precise residues and placements are not critical determinants of activation but rather it was important to maintain the overall amphipathic composition. Unlike DBDs, residue composition describes classes of natural TADs since no stringent structural motif exists for this class of activators however, there is some shared features.¹¹

In solution, amphipathic TADs lack a defined structure but adopt a secondary structure upon interacting with binding partners and are considered malleable.¹²⁻¹⁴ As mentioned in Chapter 2, the precise binding partners for TADs remain elusive and thus limit the structural characterization of TADs.^{2, 15-17} Additionally, coactivators are often intrinsically disordered and activators can bind to coactivators in more than one binding orientation.¹⁸⁻²⁰ In contrast, the interaction

between transcriptional activation domains and masking proteins, proteins that shield TADs from proteolysis and aggregation^{16, 17}, are typically higher in affinity and specificity and have been more straightforward to characterize.² An example of one of these complexes is the interaction between 15 residues of p53 and its natural inhibitor mDM2. The TAD forms an amphipathic helix, burying hydrophobic residues in the hydrophobic cleft of mDM2.²¹ In fact, the amphipathic helix is the most common structure observed for TADs in complex with coactivators as well.² One such structure between the activator CREB and the coactivator CBP demonstrate that the hydrophobic residues of phosphorylated CREB interact with hydrophobic residues present in CBP (**Figure III-4**).¹³ Additionally, TADs are predicted to be malleable and can bind multiple coactivators. For example amphipathic activator VP16 has been shown to interact with TBP, TFIID, TFIIB, and others.^{12, 22-25} Coactivators can also have flexible binding sites. In one example, the peptidic activator c-Jun and MLL that share little sequence homology bind to the same region of the KIX domain of CBP as amphipathic helices.^{26, 27} This observation not only demonstrates the malleability of activators but also the flexibility of their coactivator targets.

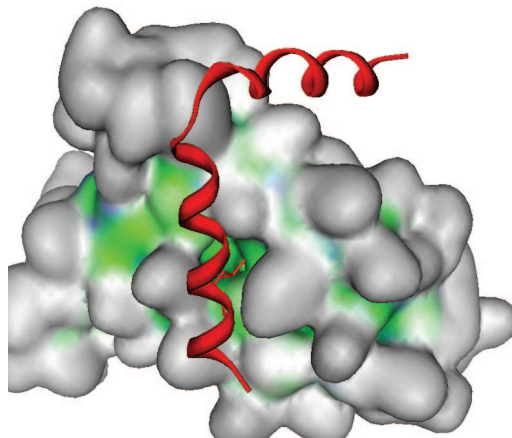


Figure III-4. NMR structure of CREB (red) bound to the KIX domain of CBP (grey). The green patches are hydrophobic surfaces. The hydrophobic residues of CREB face these surfaces whereas the polar residues are solvent exposed. Adopted from reference 13.

The initial small molecule TADs is an isoxazolidine which contains polar and hydrophobic functional groups common to natural activators.¹ The isoxazolidine ring is a five membered heterocycle containing a nitrogen and oxygen which aid in solubility and two stereocenters that project functional groups in a three dimensional display (**Figure III-1**). To evaluate which components of this compound were important for activation, we designed analogues that were more polar and more hydrophobic than our initial isoxazolidine and analogues that were conformationally more flexible yet retained the key functional groups present in the original molecule.³ Since a variety of peptidic TADs can activate transcription, it was unlikely that a single molecule could act as a TAD and we hypothesized that a variety of amphipathic small molecules of different composition should also be able to activate transcription in cell culture. To this end, natural products that meet these specified criteria were tested as TADs.³

C. Design and Synthesis of Small Molecule TADs

To assess whether small molecule TADs mimic the functional profile of natural TADs in the composition of amphipathicity, we designed molecules which contained hydrophobic functional groups of increasing size to test if a more hydrophobic molecule could better activate transcription. Since there are two hydrophobic functional groups present in the molecule, we could increase our hydrophobicity at C3 or N2 (**Figure III-5**). One advantage of introducing functionality at N2 was that previous studies have shown that the functional group at C3 influences the selectivity of the corresponding Grignard addition and thus changing functional groups at this position could complicate synthetic efforts.^{28, 29} Accordingly we choose a late-stage intermediate where functionalization at N2 would be straightforward with the addition of aryl substituted electrophiles. **Figure III-6** shows the diversity of compounds generated using this strategy.

To test the importance of increasing polarity, analogues **III-13-15** were designed. Natural TADs often have acidic residues that are thought to aid in solubility.^{4, 6} We sought to design isoxazolidines bearing amide, and acidic residues as they are common in natural TADs as well as an ester to determine if the hydrogen bond donor was necessary for activation.

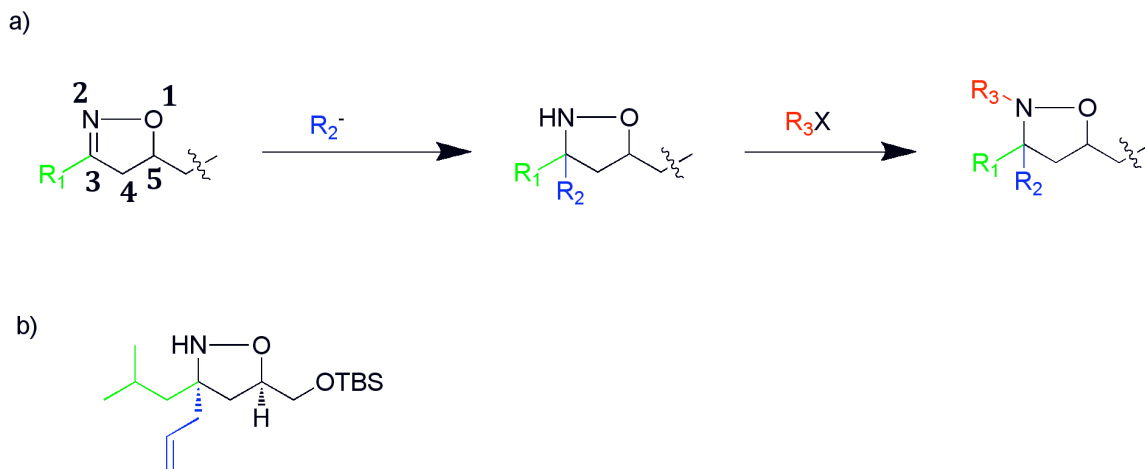


Figure III-5. Schematic of the isoxazolidine synthesis. A) A nucleophile can be added to an isoxazoline at C3 to produce an isoxazolidine. Addition of an electrophile to an isoxazolidine introduces a functional group at N2. b) Electrophiles can be added to the key intermediate shown to introduce functionality at a late stage in the synthesis.

To test the necessity of an organized three dimensional display, the N-O bond of the isoxazolidine ring was cleaved to create an analogue with increased degrees of freedom. To further this study, we made the d and l peptides consisting of serine, phenylalanine, and leucine. As outlined in Chapter II, all molecules were conjugated to oxidized dexamethasone (OxDex) for cell-based assays; OxDex interacts with a protein DNA binding domain (Gal4+GR LBD) that is expressed in our cellular system. (see Chapter 2).

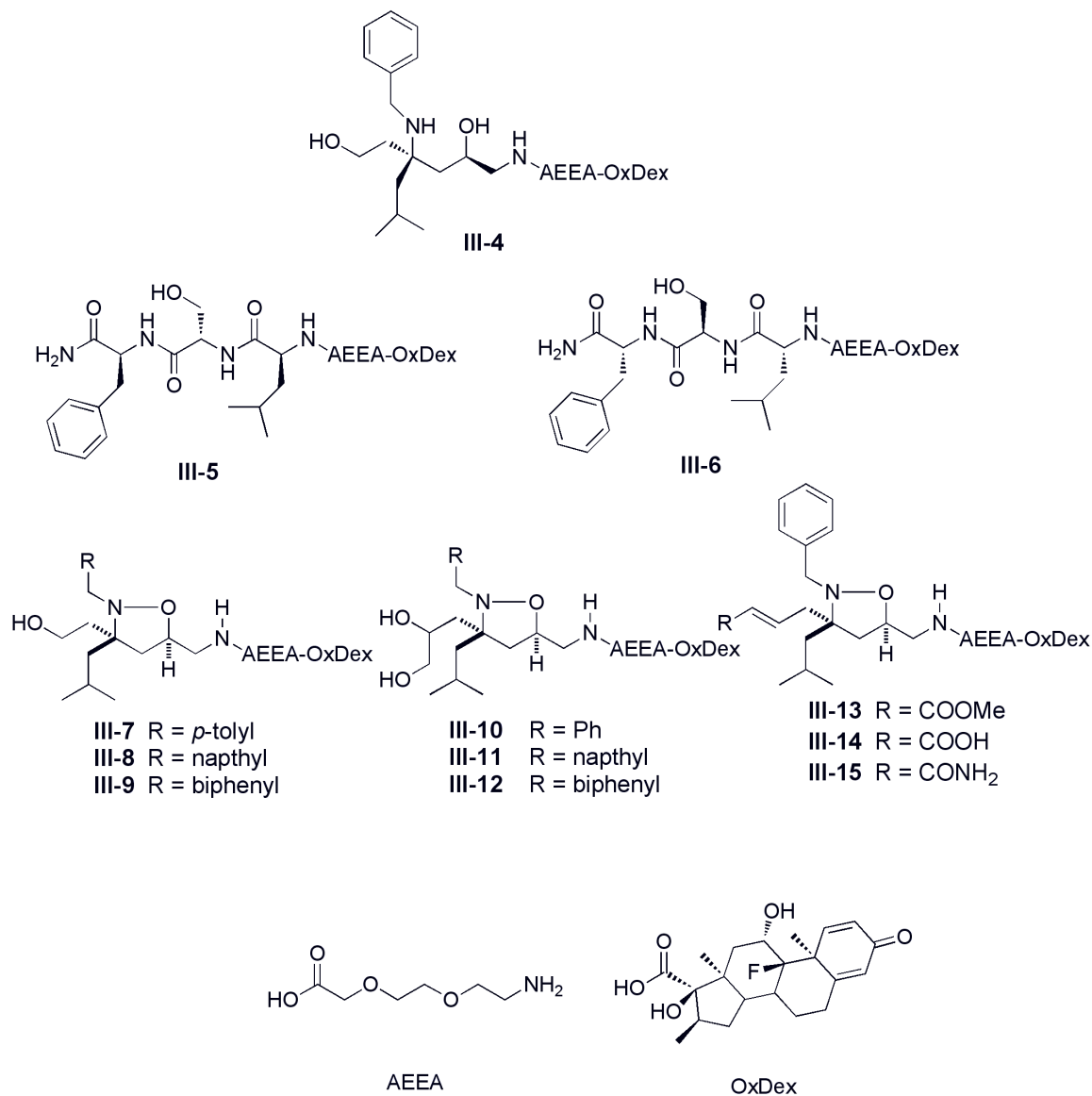
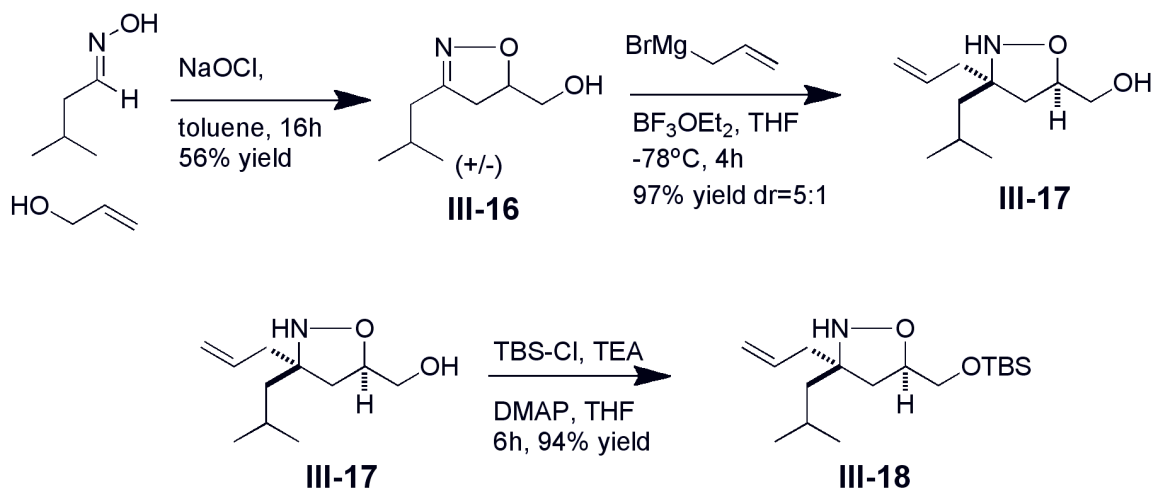


Figure III-6. A focused library to test the requirements of an isoxazolidine TAD.

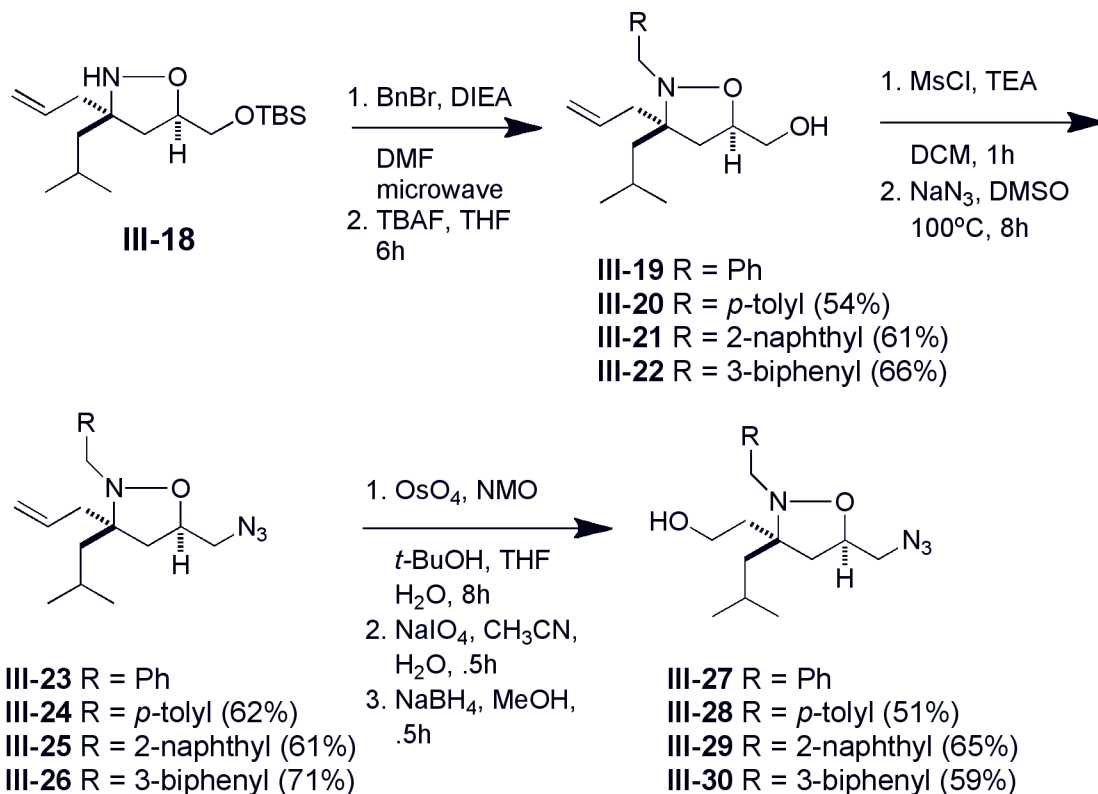
1) Importance of Hydrophobic Balance: Introduction of Hydrophobic Substituents

The first step towards the target molecules was a 1-3 dipolar cycloaddition between a nitrile oxide generated from an oxime and allyl alcohol to produce isoxazoline (III-16) using established protocols (Scheme III-1).^{28,30}



Scheme III-1. Synthesis of key intermediate.

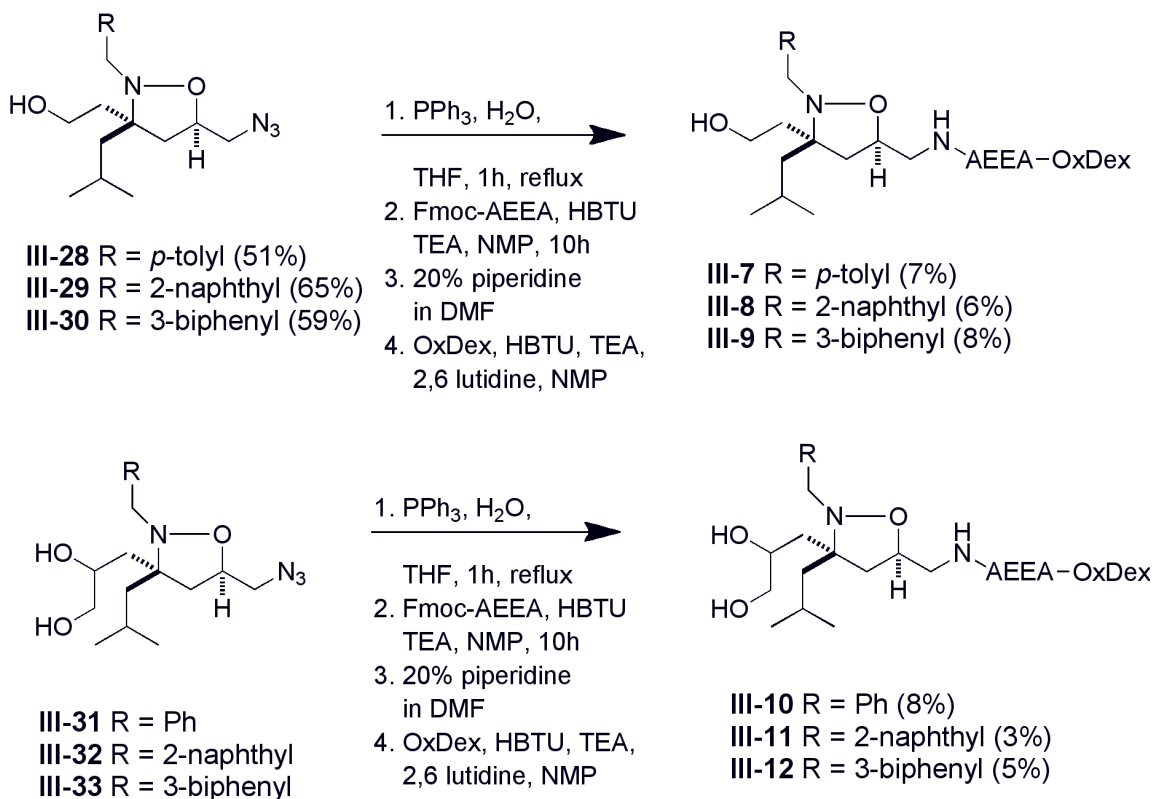
Nucleophilic addition to the C=N bond of the isoxazoline with the Grignard reagent allylmagnesium chloride in the presence of strong Lewis acid ($\text{BF}_3\cdot\text{OEt}_2$) afforded isoxazolidine (**III-17**). This reaction yielded a 5.5:1 mixture of diastereomeric products in a combined yield of 95%. Key isoxazolidine intermediate (**III-18**) was afforded by addition of TBS-Cl in the presence of base. This intermediate was further functionalized by addition of aryl electrophiles using microwave-accelerated conditions. After TBS deprotection with TBAF, the yields of the alkylated products (**II-19-22**) ranged from 54-66% (**Scheme III-2**). Functionalization of the alcohol on C5 to an azide proceeded by mesylation and azide displacement with sodium azide that provided isoxazolidines (**III-23-26**) in good overall yield.



Scheme III-2. Synthesis of isoxazolidines of increased hydrophobicity.

At this point the double bond moiety on C3 of the isoxazolidine was oxidatively cleaved to yield the desired alcohol at that position. The functional group conversion was afforded by an initial dihydroxylation of the olefin by treatment with a catalytic amount of OsO₄ and two equivalents of NMO. The diol was subsequently cleaved to the aldehyde by treatment with NaIO₄ and the final aldehyde was reduced to the primary alcohol by treatment with NaBH₄ to provide isoxazolidines (**III-27-30**). The functionality at C5 served as the point of attachment of the isoxazolidine to OxDex. The azide was reduced by a Staudinger reduction with triphenylphosphine and the free amine was linked to a short PEG linker (Fmoc-AEEA) by standard amide bond coupling conditions (**Scheme III-3**). The crude material was deprotected with 20% piperidine in DMF and purified by reverse

phase HPLC. The OxDex moiety was then coupled to the linker through an amide bond linkage to yield conjugates (**III-7-9**), again using standard amide bond formation conditions. Similarly, a portion of the diol intermediate was subjected to similar conditions to make the diol containing isoxazolidine analogues. All conjugates were purified by reverse phase HPLC prior to evaluation in cells.



Scheme III-3. Synthesis of hydrophobic isoxazolidine conjugates.

2) Introduction of Polar Substituents

A mixture of hydrophobic and acidic residues characterizes natural acidic TADs. Initial *in vitro* results suggested that acidic isoxazolidines could not function as a TAD (**Figure III-7**) however, this had not yet been tested in cells. Functionalization of the isoxazolidine scaffold with acidic residues has been

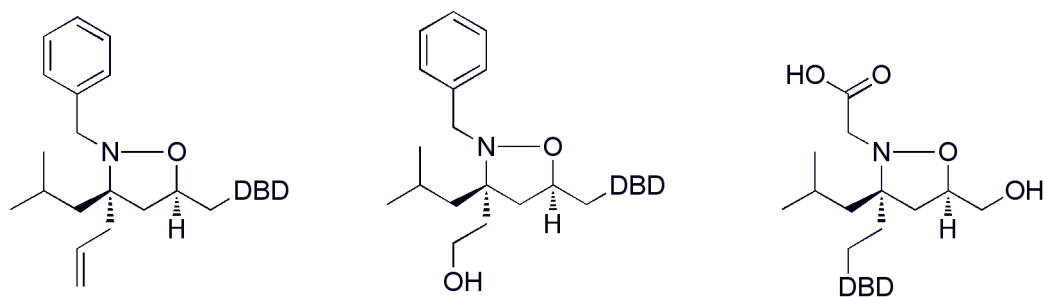
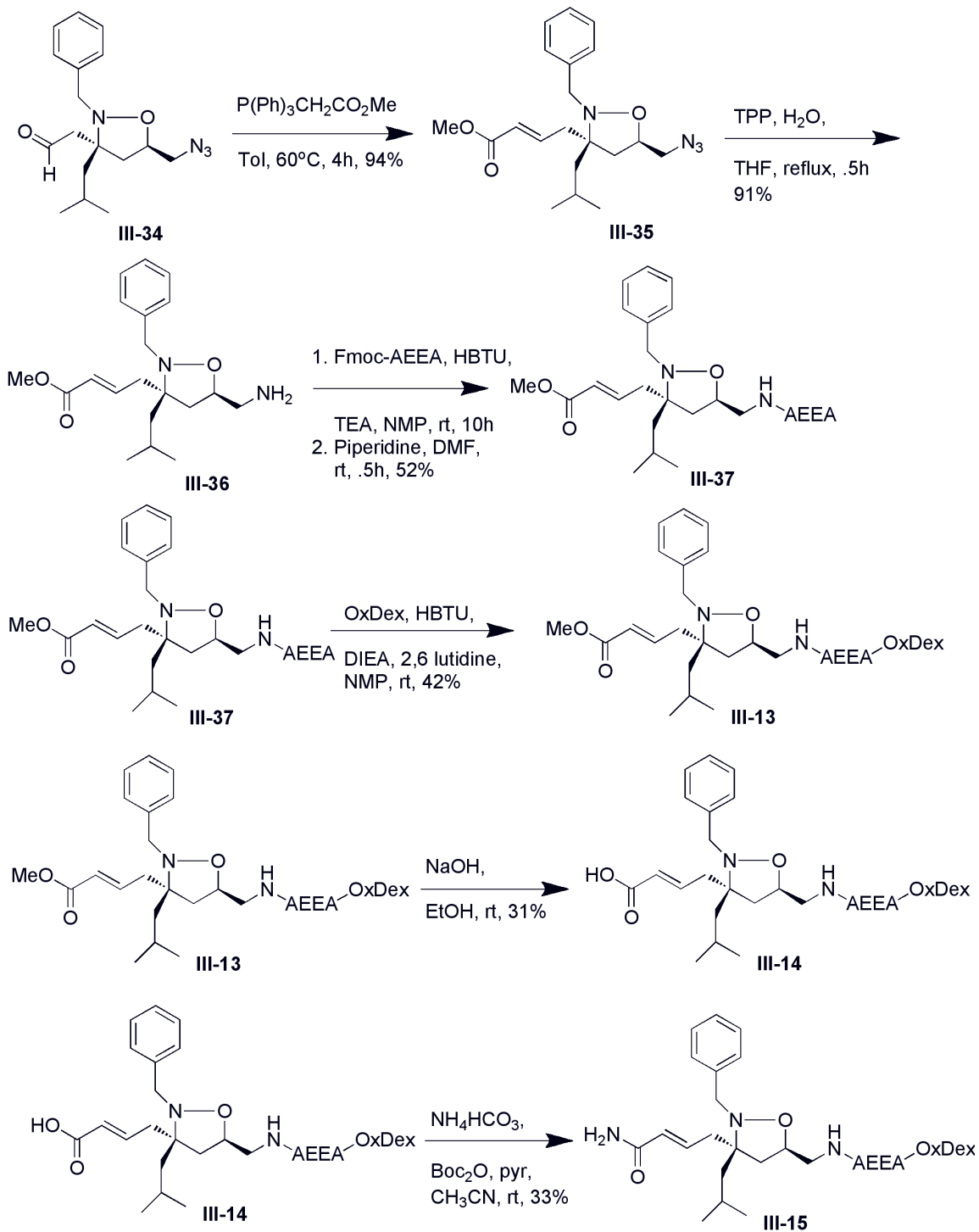


Figure III-7. Compounds tested for in vitro activation. The hydrophobic compound and the acid compound were not active in a in vitro transcription assay. The compound with an alcohol and benzyl functional group was as active as a known amphipathic activator.

challenging until recently (see appendix). To access these acidic isoxazolidines we decided to use a Wittig reaction to give an α,β -unsaturated ester (**Scheme III-4**). To avoid complications with conjugation to the PEG linker, it was decided to leave the functional group as an ester. As shown later, this could later be cleaved to a carboxylic acid.

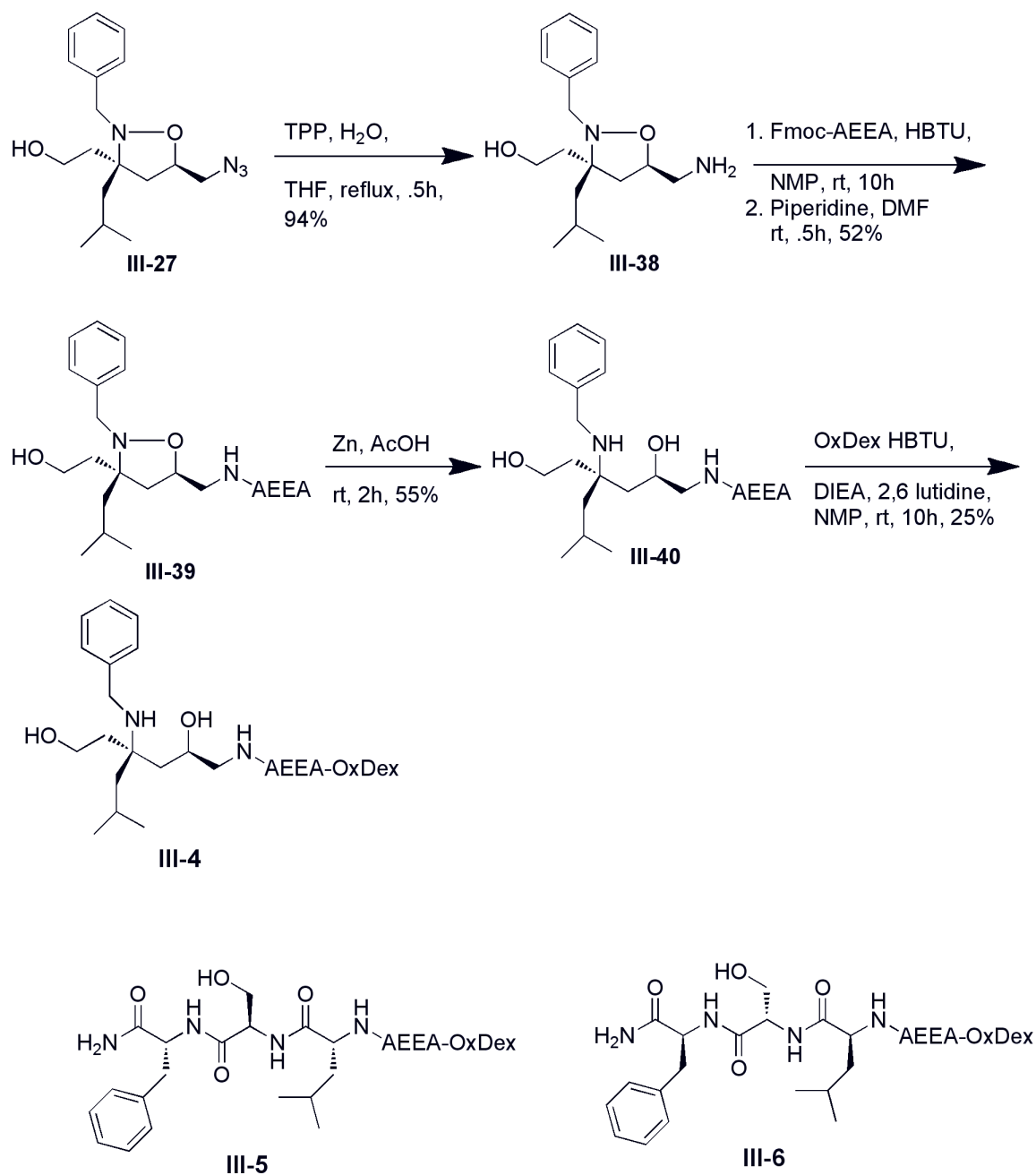


Scheme III-4. Synthesis of amide, acid, and ester analogues.

From compound **(III-34)** a Wittig reaction with methyl triphenylphosphoranylidene acetate gave the desired α,β -unsaturated ester **(III-35)** in good yield and stereoselectivity. Using similar conditions the azide was reduced to give amine **(III-36)** and conjugated to the PEG linker and OxDex and purified by reverse phase HPLC to provide compound **(III-13)**. The ester was saponified to give the acid **(III-14)** and the acid was reacted with acetic acid and ammonium carbonate to provide the amide **(III-15)**. These three compounds were tested in cells for their ability to activate transcription compared to our original isoxazolidine.

3) Analogues to Test the Importance of the Isoxazolidine Scaffold

Starting from intermediate **(III-27)**, the azide was reduced to an amine by a Staudinger reduction to afford the free amine as before (**Scheme III-5**). It was decided to cleave the N-O after coupling to the PEG linker to avoid multiple purifications. The free amine **(III-38)** was coupled to Fmoc-AEEA as described and the Fmoc was removed using 20% piperidine in DMF. The resulting crude material was treated with excess zinc (5.1 eq) in acidic acid to provide the acyclic product **(III-40)**. After filtration through a C8 column to remove excess zinc, the compound was purified by reverse phase HPLC. The amine was then coupled to OxDex as described to afford the N-O cleaved product **(III-4)**. The d and l-peptides were synthesized on solid phase to provide the OxDex conjugates. These three compounds **(III-5** and **III-6)** would serve to test the role of our scaffold and determine if this scaffold was indeed privileged or if our design strategy could be applied generally.



Scheme III-5. Synthesis of compounds to test importance of isoxazolidine scaffold.

C. Cellular Evaluation of Isoxazolidine Analogues

To evaluate the ability of the isoxazolidines to activate transcription we used a system developed in the Kodadek lab in which a fusion protein consisting of a Gal4

DBD and the minimal ligand binding domain of the glucocorticoid receptor is expressed on plasmid (discussed in Chapter 2).³¹ The first series of compounds we evaluated were the compounds of increasing hydrophobicity (**Figure III-7**). The hydrophobic isoxazolidines were tested at 10 μ M compared to amphipathic isoxazolidine (**III-1**) at 1 μ M. Our change in concentration reflected a difference in EC_{50} , with our initial isoxazolidine having a much lower EC_{50} . However, to compare compounds we decided it was best to compare at the concentration at which maximal activity was observed with minimal cytotoxic effects. As hydrophobicity increased we saw a marked decrease in activity. The p-tolyl analogue **III-7** activated about half as much as the original isoxazolidine. Both the naphthyl **III-8** and biphenyl **III-9** isoxazolidines were inactive. From this small subset of compounds it appeared that adding more hydrophobic functional groups inhibited activity of the isoxazolidines. It has been demonstrated that hydrophobic peptides and small molecule function modestly despite exhibiting good affinity for the transcriptional machinery. Some of the loss in activity may be due to non-specific binding events that lead to sequestration.³² To add polarity to these compounds we used the diol intermediate from the naphthyl **III-11** and biphenyl **III-12** series. When we tested the diol compounds we saw a restoration in activity. This provided some evidence that activity was a function of the compounds overall amphipathic characteristic. The diol (**III-10**) was nearly as active as the isoxazolidine **III-1**.

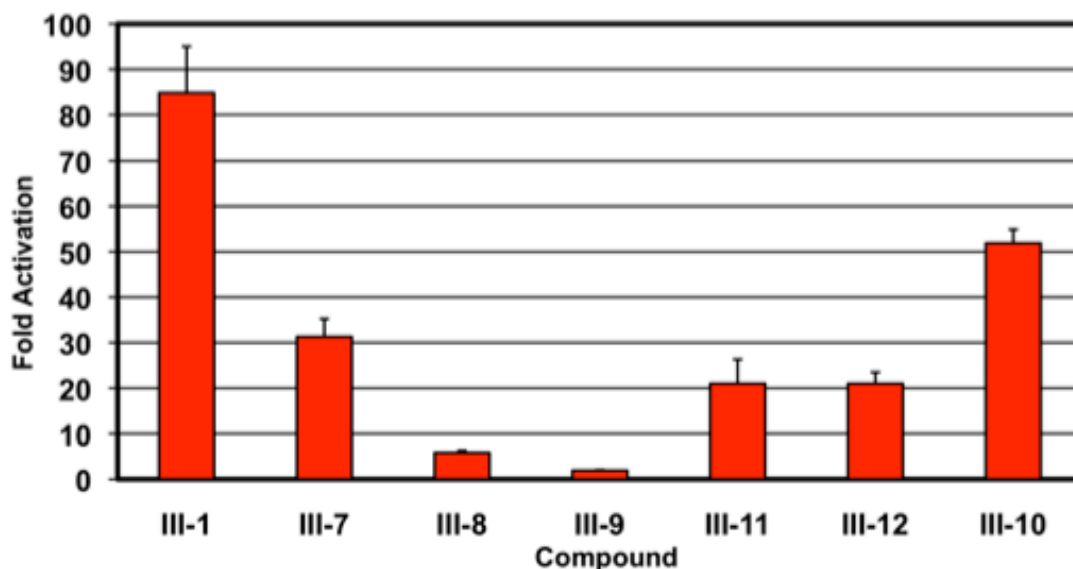


Figure III-7. Cellular activation by small molecule isoxazolidine. A decrease in activity is observed with increasing hydrophobicity. The activity can be rescued upon introduction of polar functionality as shown by **III-11** and **III-12**.

We next tested if more polar substituent would be capable of activating transcription in cell (**Figure III-8**). We compared an ester (**III-13**), acid (**III-14**), and amide (**III-15**) at 10 μ M for maximal activity. The ester activated about half as much as the original isoxazolidine and the acid and amide were inactive. Although acids are commonly found in activators often these residues serve to solubilize the polypeptide rather than for specific interactions with coactivators.⁴ Based on these experiments, we observed that activate molecules fell in a relatively narrow logP range of 2.5-3.1 and molecules outside this range were inactive or activated to low levels. We hypothesized this observation could be extended to other molecules and may be a general design criterion for small molecules activators.

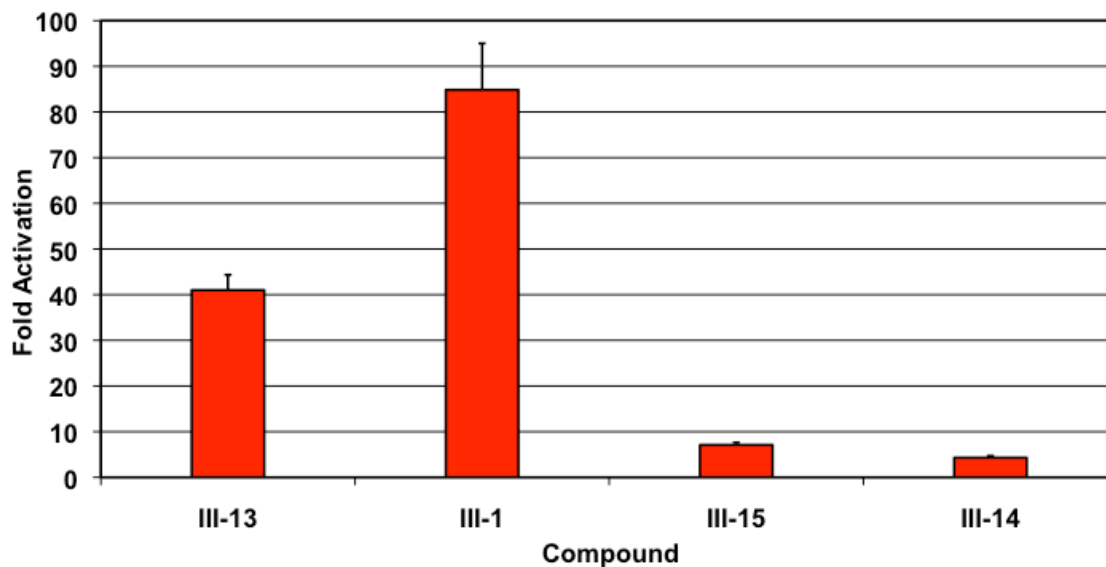


Figure III-8. In cell activity of polar functional groups. As the isoxazolidine analogues become more polar activity decreases.

The last set of analogues tested the importance of the isoxazolidine scaffold (**Figure III-9**). As described, we cleaved the N-O bond in the isoxazolidine ring to provide the 1,3 amino alcohol. This compound (**III-4**) displays the same functionality as the original isoxazolidine but now has increased flexibility. To test compounds of even more flexibility we made the d and l-peptides. The 1,3 amino alcohol (**III-4**) was approximately half as active and the d peptide (**III-5**) was about a third as active. The l peptide (**III-6**) was inactive presumably from degradation. The 1,3 amino alcohol could have an intermolecular hydrogen bond to provide more structure to the molecule and thus restore activity. Nevertheless, this small subset of compounds provides evidence that the isoxazolidine scaffold is important but not required for activity and that activity could be extended to other scaffolds.

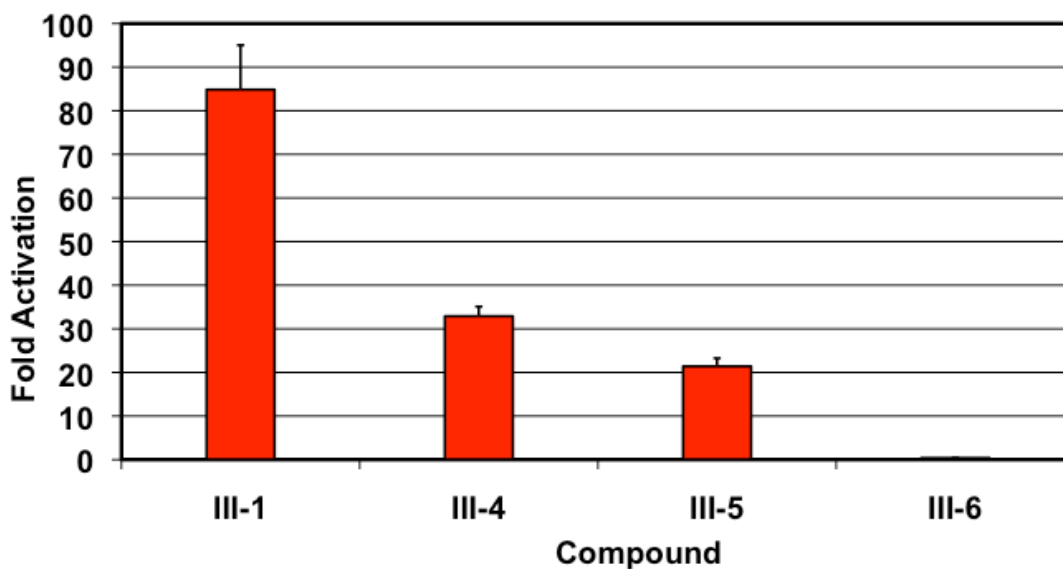


Figure III-9. Compounds to test the importance of the isoxazolidine scaffold. A mark decrease is seen when the N-O bond of the isoxazolidine is cleaved. The d-peptide activates about a fourth as much as the isoxazolidine. The l-peptide is inactive presumably from proteolytic degradation.

E. Additional Small Molecule Activation Domains

Many different amphipathic peptide sequences function as transcriptional activation domains³³ and it seemed likely that more than one amphipathic small molecule scaffold should also function as a transcriptional activation domain. To narrow the search, we sought structures using the design criteria outlined above: a conformationally constrained scaffold and clogP between 2.5-3.1. We looked for compounds that had no known biological function related to transcription and could be easily conjugated to OxDex through a PEG linker (**Scheme III-6**). Compounds that cause DNA damage or localized topoisomerases to DNA may cause a stress response that could impact transcriptional activity by an alternative mechanism. We decided that compounds bearing a five or six member lactone ring or an acid would suffice.

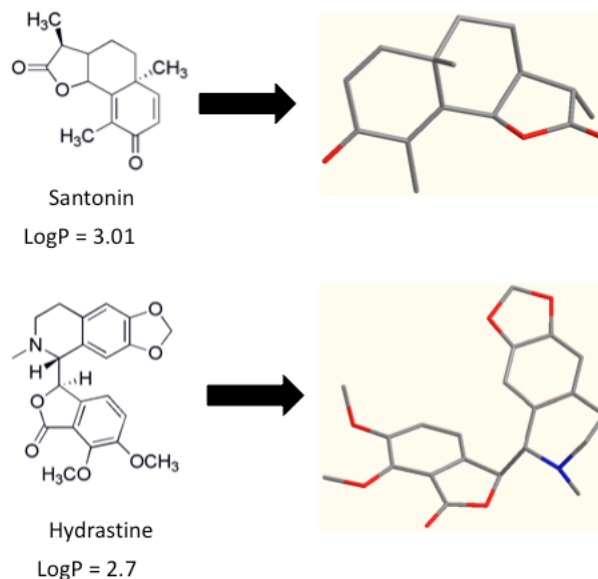
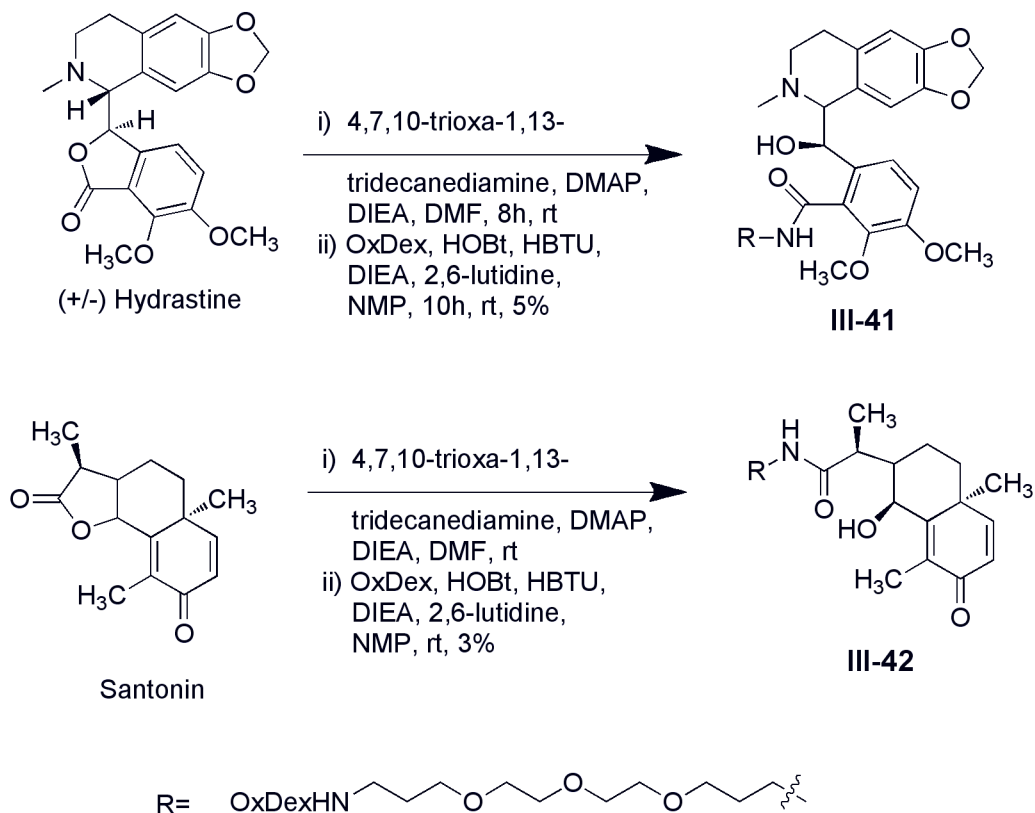


Figure III-10. Hydrastine and santonin. A three-dimensional display of both molecules shows their projection of functional groups and rigid scaffolds. Additionally, both contain a lactone ring that can be functionalized by addition of an amine.

This would allow for conjugation using a diamine linker with one amine opening the lactone and the other amine free to react with OxDex. Two compounds that met those criteria were hydrastine and santonin (**Figure III-10**).^{34, 35} Both compounds contained a lactone ring and when this lactone was opened the resulting compound (**III-41** and **III-42**) had a clogP between 2.5-3.1. Both compounds were constrained in a rigid scaffold and neither had a known biological function related to transcription. We tested additional analogues for activity and while some activated moderately, only santonin and hydrostine activated to levels similar to isoxazolidine (**Figure III-11**). We tested additional compounds which did not meet these criterion that only activated modestly. Most of these compounds fell outside of the defined logP range.



Scheme III-6. Reacting the lactone ring with a diamine functionalized hydrastine and santonin. The resulting free amine was conjugated to OxDex.

Since these compounds were natural products that had biological function, we tested if the free compound could compete for the transcriptional machinery with the DNA bound molecule or “squelch”.³⁶ If these molecules activate transcription by an alternative mechanism, such as acting as an agonist or antagonist for an enzyme involved in transcriptional activation, they would be unable to “squelch”. Figure III-12 depicts a dose dependent decrease in activity upon addition of increasing concentrations of free compound.

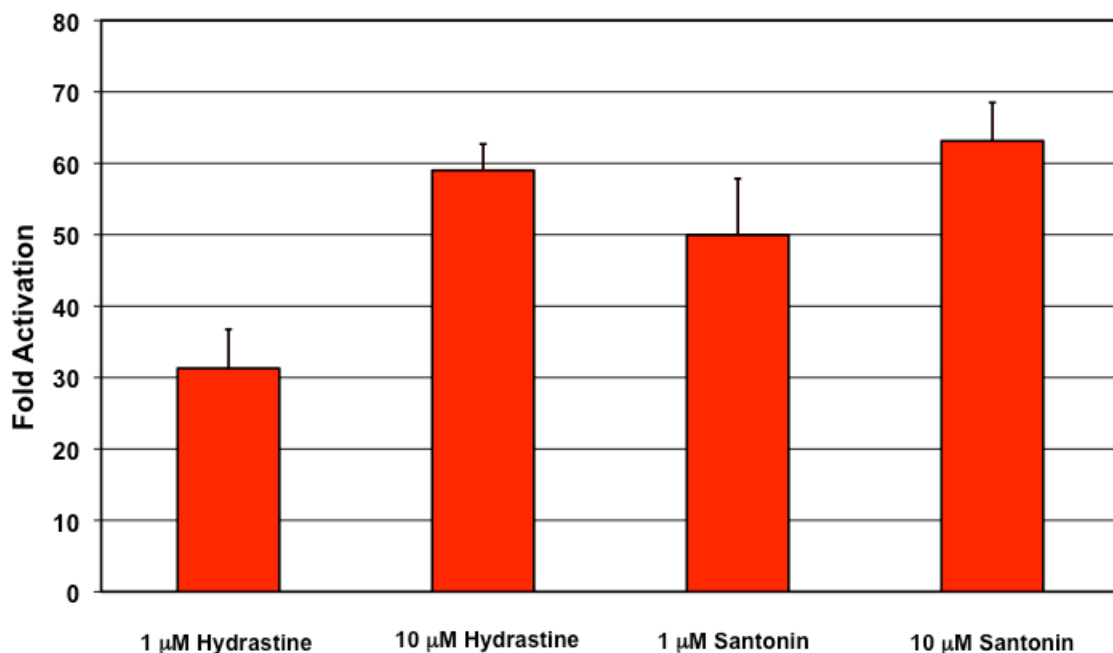


Figure III-11. Hydrastine and santonin activate near 60 fold when localized to DNA. This provides evidence that our guidelines can be extended to other small molecules.

This is consistent with the DBD being a functionality inert scaffold and with the small molecule targeting the transcriptional machinery. Some of the other natural products tested did not “squench” and thus activity could be observed by another mechanism.

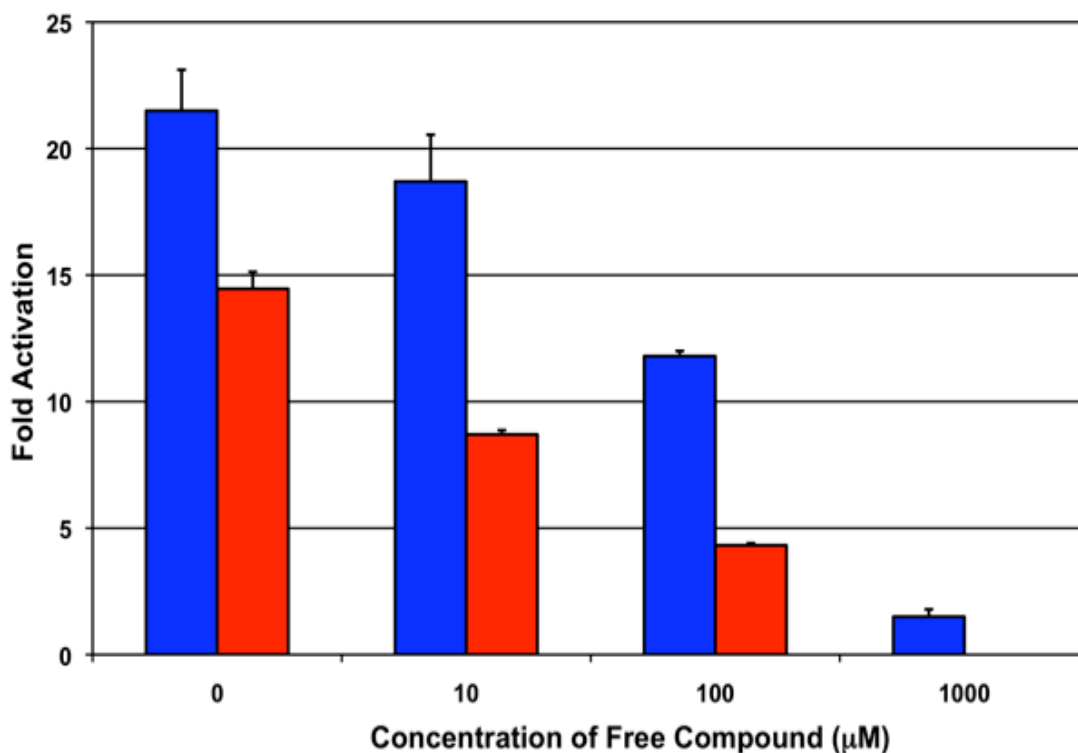
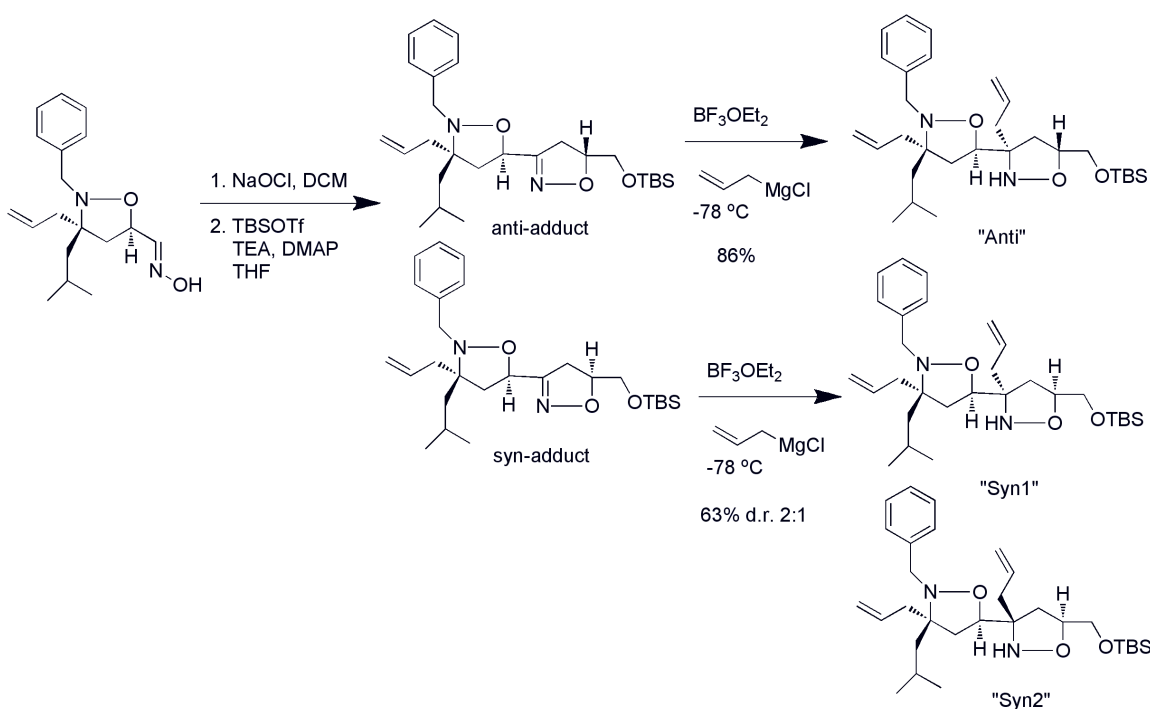


Figure III-12. Free santonin and hydrastine can inhibit santonin (red) and hydrastine (blue) activation. As more santonin and hydrastine are added, a dose dependent decrease in activation is observed. 100nm of both conjugates were added to cells.

Another group of compounds we tested were bis-isoxazolidines. Dr. Sara Buhrlage demonstrated that one of the bis-isoxazolidines could target the KIX domain of CBP and also could activate transcription when localized to DNA.³⁷ With Dr. Ibrahim Bori and Dr. William Pomerantz we synthesized the three dimeric isoxazolidines for biological evaluation by NMR and activation of transcription. We rationalized that while stereochemistry and display of functional groups didn't seem to be a determinate of transcription, it was possible that the spatial relationship between the functional groups might be important as the bis-isoxazolidines are predicted to populate a preferred conformation dictated by the stereochemistry of

the backbone. Thus, I synthesized and tested one of the bis-isoxazolidines termed the syn1 compound (**Scheme III-7**). This bis-isoxazolidine did not activate transcription in cells and did not bind the KIX domain of CBP, however, the anti dimer did bind the KIX domain and activate in cells (W.C.P, J.W.H, R.J.C, I.D.B, A.K.M unpublished results). Thus it is possible that the relationship between the functional groups might influence a molecules ability to activate transcription. The syn2 tetraol will be synthesized and tested for cellular activity and ability to bind the KIX domain of CBP. Further work will involve modeling and docking experiments to rationalize these observations through collaborations with the Brooks and Al-Hashimi groups.



Scheme III-7. Synthesis of “Syn1” tetraol. After cycloaddition, two diastereomers are formed and separated into the syn- and anti-adducts. After Grignard addition to the anti-adduct, one of the diastereomers decomposes. The syn-adduct produces two diastereomers we term Syn1 and Syn2. Syn1 is formed in a 2:1 mixture and thus Syn1 was functionalized as the OxDex conjugated and will be tested in cells.

F. Conclusions

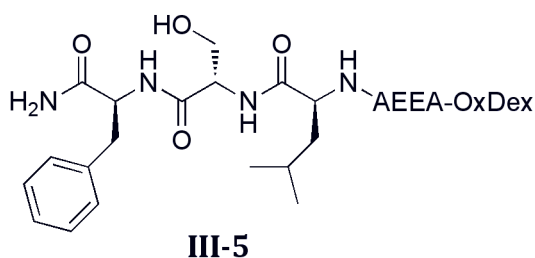
We identified several new isoxazolidine-based transcriptional activation domains as well as two unique TAD scaffolds. The design criteria are simple and should be applicable to the discovery of new TADs. Similar to the natural system it is not possible to predict how active a small molecule TAD will be. This reflects the lack of detailed mechanistic information about how natural and unnatural TADs function in a cellular context. It is not clear which binding partners are critical to activation and this impedes structure-based design and prevents structural characterization of activator complexes. In this regard, small molecules may prove to be valuable tools where cross-linking could elucidate the necessary binding partners for potent activation as well as unproductive binding partners which curb activity.³⁷⁻³⁹ Once partners are identified, a multiplexed screen against a subset of coactivators could yield molecules capable of activating or inhibiting transcription. From the knowledge gained in chapter 2 and chapter 3, chapter 4 describes our efforts to design an activator ATF composed of a small molecule TAD and a non-peptidic DBD.

G. Experimental

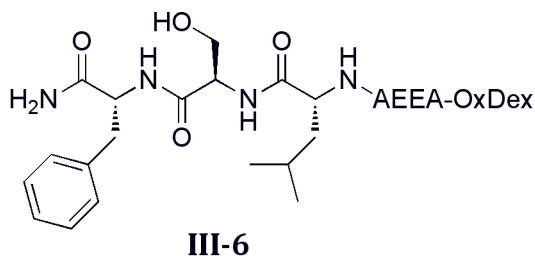
General synthetic methods

Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. CH₂Cl₂ and THF were dried by passage through activated alumina columns. All reactions were performed under a dry N₂ atmosphere unless otherwise specified. Et₃N was distilled from CaH₂. Purification by flash chromatography was carried out with E. Merck Silica Gel 60 (230-400 mesh). ¹H and ¹³C NMR spectra were recording in CDCl₃ at 400 MHz and

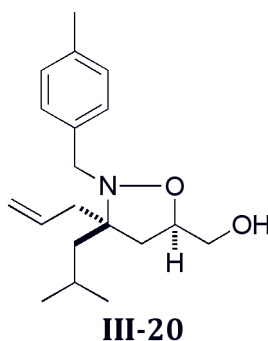
100 MHz, respectively, unless otherwise specified. IR spectra were measured as thin films on NaCl plates. Reverse-phase HPLC purifications were performed on a Varian ProStar 210 equipped with Rainin Dynamax UV-D II detector ($\lambda = 254$ nm and $\lambda = 214$) using a C18 (8 x 100mm) Radial-Pak™ cartridge with a gradient of 0.1% TFA in H₂O and CH₃CN as the mobile phase. All analytical HPLC traces were analyzed at $\lambda = 254$ nm.



OxDex+AEEA+D-LSF (III-5): The tripeptide D-LeuSerPhe was prepared using solid phase peptide synthesis in accordance with standard procedures with the AEEA linker and OxDex added on solid support at the conclusion of the synthesis. The crude product isolated after resin cleavage was purified by reverse phase HPLC to provide 3.5 mg of **III-5** (15% yield) as a white solid. HRMS (ESI) calcd for [C₄₉H₆₈FN₃O₁₀ + Na]⁺: 892.4484, found 892.4493.



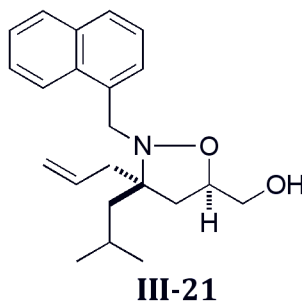
OxDex+AEEA+L-LSF (III-6): Preparation of **III-6** was accomplished under conditions identical to those used for **III-5**. The compound was purified by reverse phase HPLC to provide 2.1 mg of **III-6** (8% yield) as a white solid. HRMS (ESI) calcd for $[C_{49}H_{68}FN_3O_{10} + Na]^+$: 892.4484, found 892.4490.



[3-Allyl-3-isobutyl-2-(4-methyl-benzyl)-isoxazolidin-5-yl]-methanol (III-20):

To a solution of isoxazolidine **III-18** (0.50 g, 1.6 mmol, 1.0 eq; 6:1 mixture of diastereomers) in DMF (7.5 mL) was added α -bromo-p-xylene (1.5 g, 8.0 mmol, 5.0 eq) and *i*-Pr₂NEt (1.4 mL, 8.0 mmol, 5.0 eq). The reaction mixture was irradiated in a 1000 W microwave (6 x 20 s) @ 20% power with mixing between each interval. Once the reaction was judged complete by TLC analysis, the reaction mixture was diluted with water (60 mL) and extracted with EtOAc (3 x 30 mL). The combined organic extracts were washed with water (1 x 30 mL), dried over Na₂SO₄, filtered, and concentrated *in vacuo* to a yellow oil. Following N-alkylation, the crude reaction mixture was dissolved in THF (4.5 mL) and TBAF was added (3.0 mL of a 1M solution in THF, 3.0 mmol, 1.8 eq based on starting material **III-18**). The mixture

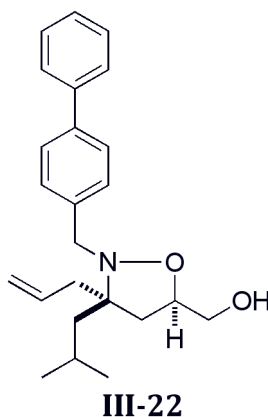
was allowed to stir for 6 h at which time the reaction mixture was diluted with water (60 mL) and extracted with EtOAc (3 x 30 mL). The combined organic extracts were dried over Na₂SO₄, filtered, and concentrated *in vacuo* to a yellow oil. The crude material was purified by flash chromatography (85:15 hexanes/EtOAc) to provide 0.26 g (54% yield) of **III-20**. The major diastereomer was isolated by normal-phase HPLC and used in subsequent steps. ¹H NMR: δ 0.98 (d, 3H, *J* = 3.4), 1.01 (d, 3H, *J* = 3.4), 1.40 (dd, 1H, *J* = 14.7, 7.0), 1.63 (dd, 1H, *J* = 14.4, 4.7), 1.83-1.98 (m, 1H), 2.05 (dd, 1H, *J* = 12.3, 5.9), 2.24-2.51 (m, 7H), 3.51-3.65 (m, 2H), 3.77-3.91 (m, 2H), 4.02-4.12 (m, 1H), 5.08-5.18 (m, 2H), 5.86-6.02 (m, 1H), 7.10-7.16 (m, 2H), 7.22-7.28 (m, 2H); ¹³C NMR: δ 21.09, 24.10, 24.48, 25.29, 38.62, 38.78, 43.92, 53.18, 65.61, 68.55, 75.38, 117.7, 127.8, 129.1, 135.1, 135.7, 136.5; HRMS (ESI) calcd for [C₁₉H₂₉NO₂ + H]⁺: 304.2277, found: 304.2274.



[3-Allyl-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidin-5-yl]methanol (III-21):

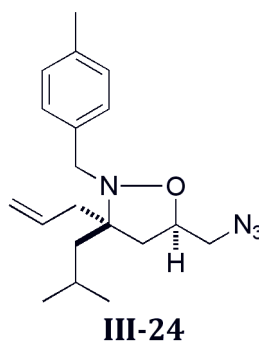
Preparation of **III-21** was accomplished under conditions identical to those used for **III-20** using 2-(bromomethyl)-naphthalene in place of α -bromo-p-xylene.

Purification by flash chromatography (9:1 hexanes/EtOAc) afforded 0.22 g of **III-21** as a colorless oil in 61% yield. $^1\text{H NMR}$: δ 0.98 (d, 3H, $J = 6.8$), 0.99 (d, 3H, $J = 6.8$), 1.41 (dd, 1H, $J = 14.4, 6.8$), 1.64 (dd, 1H, $J = 14.4, 4.8$), 1.88-1.95 (m, 1H), 2.01-2.06 (m, 1H), 2.21 (m, 2H), 2.28 (dd, 1H, $J = 12.5, 8.8$), 2.46 (dd, 1H, $J = 14.0, 7.0$), 3.49-3.56 (m, 2H), 3.95-4.08 (m, 3H), 5.10-5.14 (m, 2H), 5.91-6.01 (m, 1H), 7.40-7.43 (m, 2H), 7.50-7.52 (m, 2H), 7.75-7.79 (m, 3H); $^{13}\text{C NMR}$: δ 24.14, 24.48, 25.27, 38.68, 38.88, 44.00, 53.66, 65.53, 68.54, 75.49, 117.7, 125.5, 125.9, 126.2, 126.5, 127.6, 127.7, 128.0, 132.7, 133.4, 135.1, 136.4; HRMS (ESI) calcd for $[\text{C}_{22}\text{H}_{29}\text{NO}_2 + \text{Na}]^+$: 362.2096, found: 362.2084.



(3-Allyl-2-(biphenyl-4-ylmethyl)-3-isobutylisoxazolidin-5-yl)methanol (III-22): Preparation of **III-22** was accomplished under conditions identical to those used for **III-20** using 4-(bromomethyl)biphenyl in place of α -bromo-p-xylene. Purification by flash chromatography (7:3 hexanes/EtOAc) provided 0.39 g of **III-22** in 66% yield. The major diastereomer was isolated by normal-phase HPLC and used for subsequent steps. $^1\text{H NMR}$ (400 MHz): δ 0.96 (d, 3H, $J = 6.4$), 0.97 (d, 3H, $J = 6.8$),

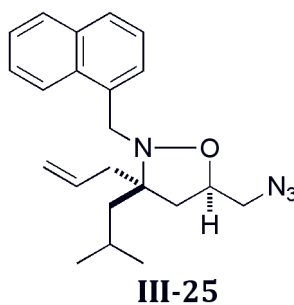
1.38 (dd, 1H, $J = 15.0, 6.8$), 1.61 (dd, 1H, $J = 14.4, 4.8$), 1.86-1.92 (m, 1H), 2.01-2.05 (m, 1H), 2.21 (m, 2H), 2.28 (dd, 1H, $J = 8.2, 4.6$), 2.44 (dd, 1H, $J = 14.0, 7.2$), 3.53-3.63 (m, 2H), 3.85 (d, 1H, $J = 14.4$), 3.92 (d, 1H, $J = 14.4$), 4.04-4.09 (m, 1H), 5.08-5.12 (m, 2H), 5.88-5.98 (m, 1H), 7.28-7.31 (m, 4H), 7.37-7.42 (m, 1H), 7.51-7.56 (m, 4H); ^{13}C NMR δ 24.09, 24.48, 25.26, 38.64, 38.77, 43.29, 53.14, 65.58, 68.51, 117.7, 127.0, 127.2, 128.2, 128.7, 133.9, 135.1, 137.9, 139.8, 141.0; HRMS (ESI) calcd for $[\text{C}_{24}\text{H}_{31}\text{NO}_2 + \text{Na}]^+$: 388.2252, found: 388.2253.



3-Allyl-5-azidomethyl-3-isobutyl-2-(4-methyl-benzyl)-isoxazolidine (III-24):

To a solution of isoxazolidine **III-20** (80 mg, 0.26 mmol, 1.0 eq), in CH_2Cl_2 (2.6 mL) was added Et_3N (70 μL , 0.53 mmol, 2.0 eq). To this solution was added methanesulfonyl chloride (40 μL , 0.53 mmol, 2.0 eq). The solution was stirred at ambient temperature for 4 h at which time the reaction was judged complete by ESI-MS analysis. The reaction mixture was concentrated *in vacuo* and the crude residue was dissolved in EtOAc (20 mL) and water (40 mL). The aqueous and organic layers were separated and the water layer was extracted with EtOAc (3 x 20 mL). The combined organic extracts were washed with water (1 x 30 mL), dried over Na_2SO_4 ,

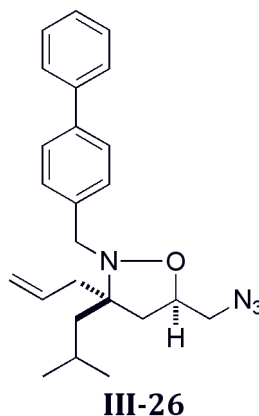
filtered, and concentrated *in vacuo* to a yellow-orange oil. The crude material was dissolved in DMF (2.6 mL) and sodium azide was added (0.17 g, 2.6 mmol, 10 eq based on starting material **III-20**) to the solution. The solution was heated to 85 °C and was allowed to stir for 8 h at which time the reaction was judged complete by TLC analysis. The reaction mixture was diluted with water (15 mL) and the aqueous layer was extracted with EtOAc (3 x 40 mL). The combined organic fractions were washed with water (1 x 30 mL), dried over Na₂SO₄, filtered, and concentrated *in vacuo* to a yellow oil. The crude material was purified by flash chromatography (97:3 hexanes/EtOAc) to provide 53 mg (62% yield) of **III-24** as a colorless oil. ¹H NMR: δ 0.98 (d, 3H, *J* = 2.6), 1.01 (d, 3H, *J* = 2.3), 1.40 (dd, 1H, *J* = 14.7, 7.0), 1.40 (dd, 1H, *J* = 14.7, 6.5), 1.62 (dd, 1H, *J* = 14.7, 5.0), 1.8-1.95 (m, 2H), 2.23-2.50 (m, 7H), 3.11 (dd, 1H, *J* = 12.6, 4.4), 3.45 (dd, 1H, *J* = 12.6, 7.0), 3.76-3.92 (m, 2H), 4.06-4.17 (m, 1H), 5.07-5.19 (m, 2H), 5.86-6.02 (m, 1H), 7.10-7.16 (m, 2H), 7.23-7.30 (m, 2H); ¹³C NMR: δ 21.09, 24.15, 24.67, 25.25, 38.74, 40.31, 45.60, 53.10, 54.45, 68.36, 74.42, 117.8, 128.0, 128.9, 135.0, 135.6, 136.3; HRMS (ESI) calcd for [C₁₉H₂₈N₄O₂ + H]⁺: 329.2341, found: 329.2336.



3-Allyl-5-(azidomethyl)-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidine

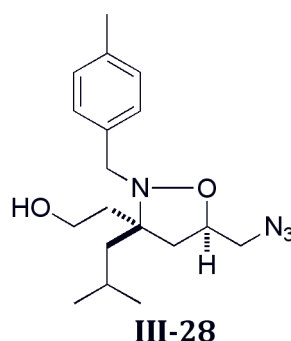
(III-25):

Preparation of **III-25** was accomplished under conditions identical to those used for **III-24** starting with 0.93 mmol of **III-21**. Purification by flash chromatography (9:1 hexanes/EtOAc) afforded 0.22 g of **III-25** as a colorless oil in 61% yield. ^1H NMR: δ 0.98 (d, 3H, $J = 6.8$), 1.0 (d, 3H, $J = 6.8$), 1.41 (dd, 1H, $J = 14.4, 6.4$), 1.63 (dd, 1H, $J = 14.4, 4.8$), 1.84-1.93 (m, 2H), 2.27-2.48 (m, 5H), 3.09 (d, 1H, $J = 8.4$), 3.43 (d, 1H, $J = 12.8, 7.2$), 4.09-4.15 (m, 1H), 5.09-5.12 (m, 2H), 5.91-5.99 (m, 1H), 7.37-7.43 (m, 2H), 7.50-7.52 (m, 2H), 7.75-7.77 (m, 3H); ^{13}C NMR δ 24.18, 24.63, 25.24, 29.67, 38.75, 40.33, 43.66, 53.60, 54.39, 68.47, 74.54, 117.9, 125.3, 125.7, 126.5, 126.6, 127.6, 127.8, 132.7, 133.4, 134.9, 136.2; HRMS (ESI) calcd for $[\text{C}_{22}\text{H}_{28}\text{N}_4\text{O} + \text{Na}]^+$: 387.2008, found 387.2005.



3-Allyl-5-(azidomethyl)-2-(biphenyl-4-ylmethyl)-3-isobutylisoxazolidine (III-26): Preparation of **III-26** was accomplished under conditions identical to those used for **III-24** starting with 0.96 mmol of **III-22**. Purification by flash

chromatography (9:1 hexanes/EtOAc) to afford 0.28 g of **III-26** as a colorless oil in 71% yield. $^1\text{H NMR}$: δ 0.96 (d, 3H, $J = 6.6$), 0.97 (d, 3H, $J = 6.6$), 1.38 (dd, 1H, $J = 14.4$, 6.2), 1.60 (dd, 1H, $J = 14.4$, 4.8), 1.84-1.91 (m, 2H), 2.24-2.33 (m, 2H), 3.10 (dd, 1H, $J = 11.0$, 4.0), 3.44 (dd, 2H, $J = 12.8$, 7.2), 3.89 (s, 2H), 4.08-4.14 (m, 1H), 5.08-5.12 (m, 2H), 5.87-5.97 (m, 1H), 7.22-7.31 (m, 4H), 7.37-7.42 (m, 1H), 7.50-7.56 (m, 4H); $^{13}\text{C NMR}$: δ 24.13, 24.64, 25.22, 38.74, 40.28, 53.04, 54.43, 68.38, 105.0, 117.9, 126.9, 127.0, 127.1, 128.4, 128.6, 134.9, 137.7, 139.7, 141.2; HRMS (ESI) calcd for $[\text{C}_{24}\text{H}_{30}\text{N}_4\text{O} + \text{Na}]^+$: 413.2317, found 413.2312.

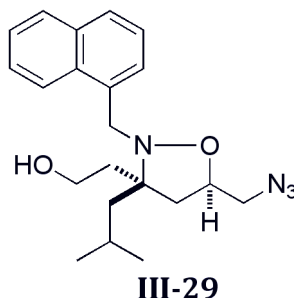


2-(5-Azidomethyl-2-(4-methyl-benzyl)-3-isobutyl-isoxazolidin-3-yl)-ethanol

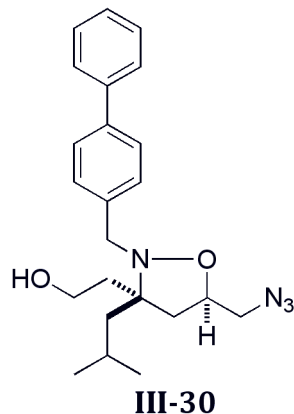
(III-28): To a solution of **III-24** (53 mg, 0.16 mmol, 1.0 eq) dissolved in *t*-BuOH (1.1 mL), THF (0.40 mL), and H₂O (0.080 mL) was added NMO (26 mg, 0.22 mmol, 1.4 eq). To this solution was added OsO₄ (0.16 mL of a 2.5 wt% solution in *t*-BuOH, 0.016 mmol, 0.10 eq). The reaction mixture was allowed to stir at ambient temperature for 5 h at which time the reaction was judged complete by TLC analysis (4:6 hexanes/EtOAc). Excess reagents were quenched by addition of Na₂S₂O₃ (1.0 mL of 10% solution w/v in water) and allowed to stir for 3 h. The mixture was then

poured into a biphasic mixture of H₂O (40 mL) and EtOAc (40 mL). The organic layer was separated and the aqueous layer was extracted with EtOAc (3 x 30 mL). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated *in vacuo* to provide the crude material as a tan oil. The crude product was dissolved in CH₃CN (800 μL) and H₂O (800 μL) and added NaIO₄ (68 mg, 0.32 mmol, 2.0 eq based on starting material **III-24**). The mixture was allowed to stir at room temperature until the reaction was judged complete by TLC analysis (9:1 hexanes/EtOAc). The reaction mixture was poured into a biphasic solution of H₂O (60 mL) and EtOAc (35 mL). Brine (15 mL) was added to separate the emulsion. The organic layer was separated and the aqueous layer was extracted with EtOAc (2 x 35 mL). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated *in vacuo* to provide the crude material as a tan oil. Crude aldehyde was dissolved in methanol (1.6 mL) and the solution was cooled in an ice-H₂O bath. NaBH₄ (14 mg, 0.40 mmol, 2.5 eq based on starting material **III-24**) was added and the mixture was then allowed to stir until the reaction was judged complete by TLC analysis (0.5 h, 1:1 hexanes/EtOAc). Excess reagents were quenched with H₂O (2 mL) and the reaction solution was poured into a biphasic mixture of EtOAc (25 mL) and H₂O (25 mL). The organic layer was separated and the aqueous phase was extracted with EtOAc (2 x 25 mL). The combined organic extracts were dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude material was purified by flash chromatography (1:1 hexanes/EtOAc) to provide 27 mg of the title compound (**III-28**) in 51% yield (from **III-24**) as a colorless oil. ¹H NMR: δ 0.97-1.02 (m, 6H), 1.46-1.57 (m, 1H), 1.59-1.79 (m, 4H), 1.80-1.91 (m, 1H), 1.98-2.07 (m, 1H), 2.23-2.36 (m, 4H), 3.25-3.48 (m,

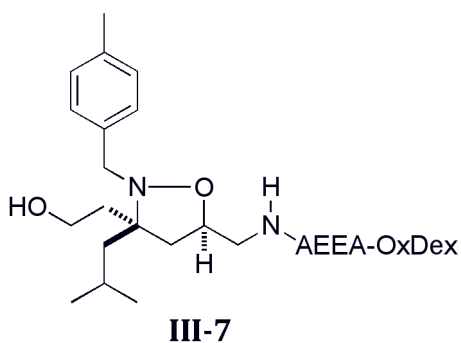
2H), 3.71-3.96 (m, 4H), 4.16-4.27 (m, 1H), 7.06-7.22 (m, 4H); ^{13}C NMR: δ 21.09, 24.29, 24.86, 25.21, 35.49, 39.97, 42.80, 53.59, 54.24, 59.64, 70.65, 73.22, 128.6, 129.0, 134.5, 136.8; HRMS (ESI) calcd for $[\text{C}_{18}\text{H}_{28}\text{N}_4\text{O}_2 + \text{Na}]^+$: 355.2110, found 355.2105.



2-(-5-(Azidomethyl)-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidin-3-yl)ethanol: (III-29): Preparation of **III-29** was accomplished under conditions identical to those used for **III-28** using 0.16 mmol of **III-25**. Purification by flash chromatography (1:1 hexanes/EtOAc) provided 53 mg of **III-29** in 65% yield as a colorless oil. ^1H NMR: δ 0.89-0.90 (m, 6H), 1.41-1.48 (m, 1H), 1.60-1.79 (m, 4H), 1.96-2.00 (m, 2H), 2.21-2.26 (m, 1H), 3.24-3.33 (m, 2H), 3.75-3.85 (m, 4H), 3.98-4.12 (m, 1H), 7.11 (m 2H), 7.29-7.35 (m, 2H), 7.61-7.70 (m, 3H). HRMS (ESI) calcd for $[\text{C}_{21}\text{H}_{28}\text{N}_4\text{O}_2 + \text{Na}]^+$: 391.2110, found 391.2105.



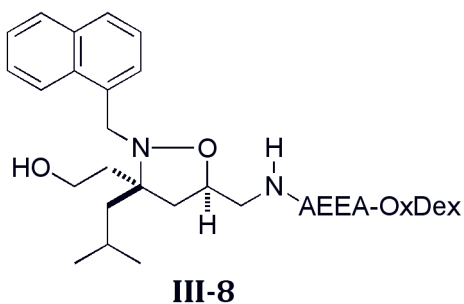
2-(5-(Azidomethyl)-2-(biphenyl-4-ylmethyl)-3-isobutylisoxazolidin-3-yl)ethanol (III-30): Preparation of **III-30** was accomplished under conditions identical to those used for **III-28** using 0.16 mmol of **III-26**. Purification by flash chromatography (1:1 hexanes/EtOAc) provided 50 mg of **III-30** in 59% yield as a colorless oil. ^1H NMR: 0.98-1.0 (m, 6H), 1.52-1.60 (m, 1H), 1.67-1.87 (m, 4H), 2.04-2.09 (m, 2H), 2.31-2.36 (m, 1H), 3.39 (m, 2H), 3.80-4.00 (m, 4H), 4.20-4.24 (m, 1H), 7.27-7.55 (9H); ^{13}C NMR: δ 20.13, 24.28, 24.89, 25.22, 35.54, 40.00, 53.61, 59.67, 124.2, 127.1, 127.2, 128.6, 129.0, 134.6, 136.5, 140.4, 143.2. HRMS (ESI) calcd for $[\text{C}_{23}\text{H}_{30}\text{N}_4\text{O}_2 + \text{Na}]^+$: 417.2266, found 417.2258.



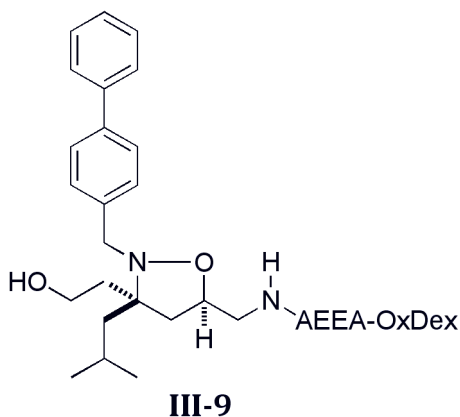
9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,11,12,14,15,16,17-dodecahydro-3H-cyclopenta[*a*]phenanthrene-17-carboxylic acid [2-(2-{{(2-(4-methyl-benzyl-3-(2-hydroxy-ethyl)-3-isobutyl-isoxazolidin-5-ylmethyl)-carbamoyl)-methoxy}-ethoxy)-ethyl]-amide (III-7):

To a solution of azide **III-28** (5.0 mg, 0.015 mmol, 1.0 eq) in THF (0.17 mL) was added triphenylphosphine (8.0 mg, 0.030 mmol, 2.0 eq) and water (2.4 μ L, 0.15 mmol, 10 eq). The mixture was allowed to stir at reflux for 2 h. H₂O (0.17 mL) was added to the reaction and the mixture was allowed to stir at 70 °C for an additional 20 min. The mixture was then poured into a biphasic mixture of 1M HCl (10 mL) and ether (10 mL). The layers were partitioned and the organic layer was extracted with 1M HCl (2 x 10 mL). The combined aqueous layers were basified with 3M NaOH (until pH 10 or greater). The aqueous mixture was then extracted with CH₂Cl₂ (3 x 15 mL) and the combined organics were dried over Na₂SO₄, filtered, and concentrated *in vacuo*. To a solution of 8-(9-fluorenylmethyloxycarbonyl-amino)-3,6-dioxaoctanoic acid (18 mg, 0.045 mmol, 3.0 eq based on starting material **III-28**) dissolved in NMP (0.067 mL) was added HOBt (6.3 mg, 0.045 mmol, 3.0 eq based on starting material **III-28**) and HBTU (17 mg, 0.045 mmol, 3.0 eq based on starting material **III-28**). This solution was agitated for 15 min. The solution of activated ester was added to the crude amine dissolved in NMP (0.067 mL) and the resulting mixture was allowed to stir for 12 h at which time the reaction was complete as judged by ESI-MS analysis. Excess reagents were quenched by addition of 1M HCl (10 mL) and EtOAc (10 mL). The reaction vessel was washed with EtOAc (2 x 2.0 mL) to remove all residues. The resulting biphasic mixture was separated and the

aqueous layer was extracted with EtOAc (3 x 10 mL). The combined organic fractions were dried over Na₂SO₄, filtered, and concentrated *in vacuo*. The resulting oil was dissolved in a solution of 20% piperidine in DMF (0.037 mL, 0.076 mmol piperidine, 5.0 eq based on starting material **III-28**) and was allowed to stir for 30 minutes. The resulting solution was diluted with 0.1% TFA H₂O (0.50 mL) and CH₃CN (0.50 mL) and partially purified by reverse-phase HPLC to remove Fmoc byproducts. The partially purified amine was used immediately in subsequent steps. To a solution of OxDex¹⁴ (17 mg, 0.045 mmol, 3.0 eq) dissolved in NMP (0.15 mL) was added HOBt (6.2 mg, 0.045 mmol, 3.0 eq) and HBTU (17 mg, 0.045 mmol, 3.0 eq) and the resulting mixture was agitated for 15 min. To this solution was added the amine dissolved in NMP (0.15 mL), 2,6-lutidine (35 μL, 0.30 mmol, 6.7 eq), and *i*-Pr₂NEt (49 μL, 0.30 mmol, 6.7 eq). The resulting mixture was stirred for 12 h at ambient temperature. The product was isolated by reverse-phase HPLC purification to provide **III-7** as a white solid (7 % yield from **III-28**; 0.8 mg). The purity of compound **III-7** was confirmed by analytical reverse-phase HPLC analysis. The identity was verified by mass spectral analysis of the isolated compound. HRMS (ESI) calcd for [C₄₅H₆₄FN₃O₈ + H]⁺: 812.4861, found: 812.4885.

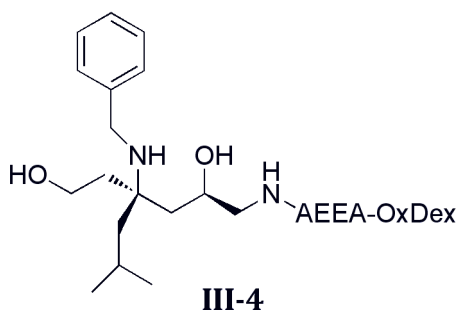


9-Fluoro-11,17-dihydroxy-N-(2-(2-(2-(((5R)-3-(2-hydroxyethyl)-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidin-5-yl)methylamino)-2-oxoethoxy)ethoxy)ethyl)-8,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carboxamide (III-8): Preparation of was accomplished under conditions identical to those used for **III-7**. Purification by reverse phase HPLC providing 0.8 mg of **III-8** (6% yield) as a white solid. HRMS (ESI) calcd for $[C_{48}H_{66}FN_3O_9 + H]^+$: 848.4861, found 848.4865.



N-(2-(2-(2-((2-(biphenyl-4-ylmethyl)-3-(2-hydroxyethyl)-3-isobutylisoxazolidin-5-yl)methylamino)-2-oxoethoxy)ethoxy)ethyl)-9-fluoro-11,17-dihydroxy-8,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carboxamide (III-9): Preparation of was accomplished under conditions identical to those used for **III-7**.

Purification by reverse phase HPLC providing 1.0 mg of **III-9** (8% yield) as a white solid. HRMS (ESI) calcd for $[C_{50}H_{68}FN_3O_9 + H]^+$: 874.5018, found 874.5001.



9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-

6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-

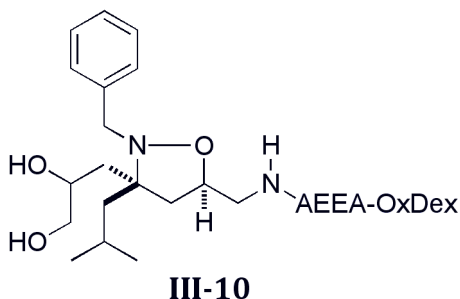
17-carboxylic acid [2-(2-{[4-benzylamino-2-hydroxy-4-(2-hydroxy-ethyl)-6-

methyl-heptylcarbamoyl]-methoxy}-ethoxy)-ethyl]-amide (III-4**):** Compound

III-27 was coupled to Fmoc-AEEA followed by removal of Fmoc by the previously reported procedure. The resulting amine (2.0 mg, 4.6 μ mol, 1.0 eq) was dissolved in AcOH (.10 mL) and to the solution was added zinc (1.5 mg, 23 μ mol, 5.1 eq) to effect reduction of the isoxazolidine N-O bond. The reaction mixture was allowed to stir for 4 h until the reaction was judged complete by ESI mass spectrometry. The reaction mixture was then diluted with 0.1% TFA H₂O (.50 mL) and CH₃CN (.50 mL). The resulting mixture was filtered through a syringe filter and purified by reverse phase HPLC to provide 1.1 mg (54% yield) of the amine as a colorless oil. HRMS (ESI) calcd for $[C_{23}H_{41}N_3O_6 + Na]^+$: 462.2944, found 462.2945. Conjugation of this intermediate to AEEA and OxDex under the conditions described for **III-7** yielded

0.70 mg of **III-4** (23% yield) as a white solid after reverse phase HPLC purification.

HRMS (ESI) calcd for $[C_{44}H_{66}FN_3O_9 + H]^+$: 800.4861, found 800.4887.

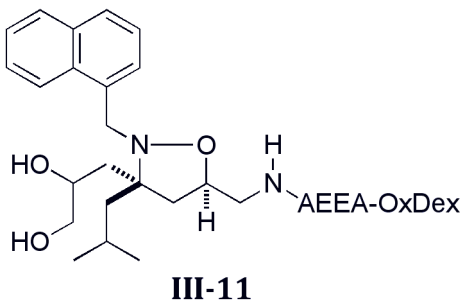


9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-

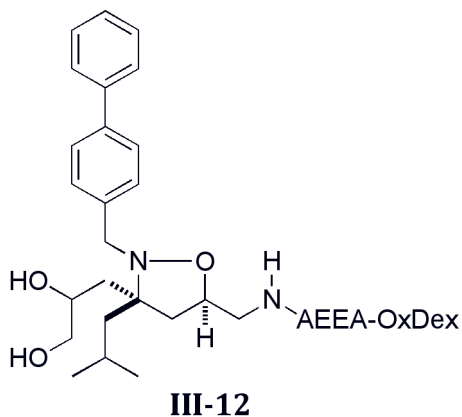
6,7,8,9,11,12,14,15,16,17-dodecahydro-3H-cyclopenta[*a*]phenanthrene-17-

carboxylic acid [2-(2-{{(2-benzyl-3-(2,3-dihydroxy-propyl)-3-isobutyl-isoxazolidin-5-ylmethyl)-carbamoyl}-methoxy}-ethoxy)-ethyl]-amide (III-10**):**

Dihydroxylation of alkene **III-23** has been previously described¹² and the resulting diol (0.062 mmol) was conjugated to AEEA and OxDex using the conditions described for **III-7**. Purification of the crude product thus obtained by reverse phase HPLC providing 4.3 mg of **III-10** (8.0% yield) as a white solid. HRMS (ESI) calcd for $[C_{45}H_{66}FN_3O_{10} + Na]^+$: 850.4830, found 850.4810.

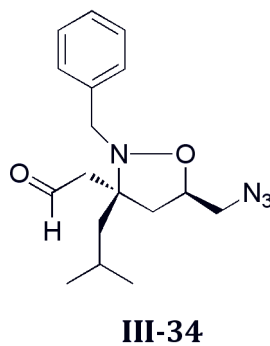


N-(2-(2-(2-((3-(2,3-dihydroxypropyl)-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidin-5-yl)methylamino)-2-oxoethoxy)ethoxy)ethyl)-9-fluoro-11,17-dihydroxy-8,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carboxamide (III-11): To a solution of **III-25** (62 mg, 0.17 mmol, 1.0 eq) in *t*-BuOH (1.2 mL) and THF (0.32 mL) was added NMO (32 mg, 0.26 mmol, 1.5 eq) and H₂O (0.13 mL). To this solution was added OsO₄ (0.17 mL of a 2.5 wt% solution in *t*-BuOH, 0.02 mmol, 0.10 eq). The resulting mixture was stirred for 8 h at which time TLC analysis indicated consumption of **III-25**. Excess reagents were quenched by the addition of Na₂S₂O₃ (50 mg) followed by stirring at ambient temperature for 1 h. To this mixture was added 20 mL of H₂O and the resulting mixture was partitioned with EtOAc (20 mL). The aqueous layer was further extracted with EtOAc (1 x 20 mL). The combined organic extracts were dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product thus obtained was purified by flash chromatography (1:1 hexanes/EtOAc) to yield 57 mg of a diol intermediate as a 1:1 mixture of diastereomers at the newly formed 2° OH in 84% yield. ¹H NMR δ 0.98-1.04 (6H), 1.34-1.50 (2H), 1.58-1.75 (3H), 1.90-2.41 (4H), 3.43-3.64 (3H), 3.78-4.3 (5H), 7.27-7.55 (7H). HRMS (ESI) calcd for [C₂₂H₃₀N₄O₃ + H]⁺: 399.2396, found 399.2392. This material was then conjugated to AEEA and OxDex using the method described for **III-7**. Purification by reverse phase HPLC providing 0.4 mg of **III-11** (3% yield) as a white solid. HRMS (ESI) calcd for [C₄₉H₆₈FN₃O₁₀ + H]: 878.4967, found 878.4972.

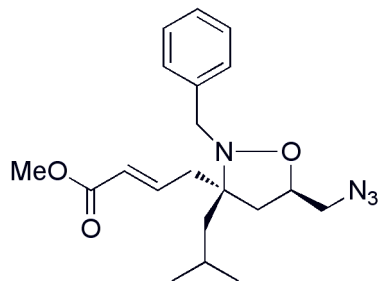


N-(2-(2-(2-((2-(biphenyl-4-ylmethyl)-3-(2,3-dihydroxypropyl)-3-isobutylisoxazolidin-5-yl)methylamino)-2-oxoethoxy)ethoxy)ethyl)-9-fluoro-11,17-dihydroxy-8,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carboxamide (III-12):

Preparation of **III-12** was accomplished under conditions identical to those used for **III-11** starting with 0.17 mmoles of **III-26**. Purification by reverse phase HPLC providing 0.7 mg of **III-12** (5% yield) as a white solid. HRMS (ESI) calcd for $[C_{51}H_{70}FN_3O_{10} + H]^+$: 904.5135, found 904.5123.

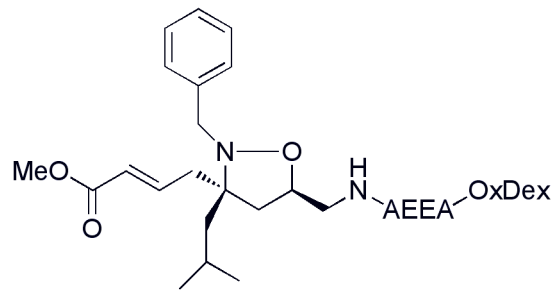


3-(2-Benzyl-5-methylazido-3-isobutyl-isoxazolidin-3-yl)-ethanal (III-34): To a solution of diol **II-23** (156 mg, .447 mmol, 1.0 eq) in CH₃CN (2.1 mL) and H₂O (2.1 mL) was added NaIO₄ (191 mg, .894 mmol, 2 eq). The mixture was allowed to stir at room temperature until the reaction was judged complete by TLC analysis. The reaction mixture was poured into a biphasic solution of H₂O (50 mL) and EtOAc (50 mL). The organic layer was separated and the aqueous layer was extracted with EtOAc (2 x 50 mL). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated *in vacuo*. The crude material was partially purified on a short silica gel column (9:1 hexanes/EtOAc) and the resulting oil, 121 mg after concentration *in vacuo*, was used immediately in the subsequent step in 86% approximate yield. IR: 2956, 2869, 2099, 1715, 1455, 1273, 1028, 732 cm⁻¹; ¹H NMR: δ .97 (d, 3H, *J* = 6.6), .96 (d, 3H, *J* = 6.6), 1.4 (dd, 1H, *J* = 14.3, 7.1), 1.64 (dd, 1H, *J* = 14.3, 4.7), 1.73-1.83 (m, 1H), 2.12 (dd, 1H, *J* = 12.8, 5.7), 2.47 (dd, 1H *J* = 13.8, 2.3), 2.55 (dd, 1H, *J* = 12.8, 8.5), 2.66 (dd, 1H, *J* = 13.7, 2.7), 2.95-3.05 (m, 1H) 3.44 (dd, 1H, *J* = 12.7 7.6), 3.84 (d, 1H, *J* = 14.4), 3.92 (d, 1H, *J* = 14.4), 3.96-4.05 (m, 1H), 7.17-7.35 (m, 5H), 9.91 (t, 1H, *J* = 2.6); ¹³C NMR: δ 24.10, 24.66, 25.04, 41.73, 44.80, 48.09, 53.40, 54.13, 69.47, 74.56, 127.0, 128.1, 128.3, 137.6, 200.0; HRMS (ESI) calcd for [C₁₇H₂₄N₄O₂ + H]⁺: 317.1978, found: 317.1966.



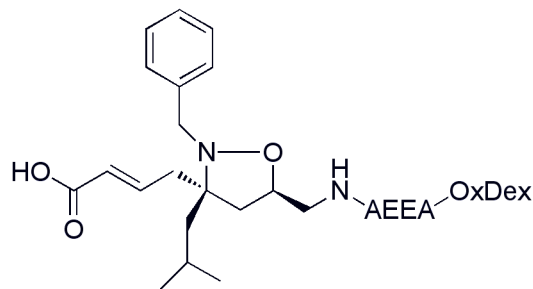
III-35

4-(5-Azidomethyl-2-benzyl-3-isobutyl-isoxazolidin-3-yl)-but-2-enoic acid methyl ester (III-35): Aldehyde **III-34** (21 mg, 0.066 mmol, 1.0 eq) dissolved in toluene (0.66 mL) was added methyl (triphenylphosphoranylidene) acetate (90 mg, 0.26 mmol, 4.0 eq). The mixture was allowed to stir at 50 °C for 6 h at which time the reaction was judged complete by TLC analysis (8:2 hexanes/EtOAc). The solvent was removed *in vacuo* and the crude material was purified by flash chromatography (85:15 hexanes/EtOAc) to provide 24 mg of the title compound **III-35** (97% yield) as a colorless oil (10:1 E/Z mixture). Spectral data for E isomer: $^1\text{H NMR}$: δ 0.92-1.01 (m, 6H), 1.36-1.42 (m, 1H), 1.52-1.63 (m, 2H), 1.74-1.87 (m, 1H), 1.91-1.98 (m, 1H), 2.19-2.29 (m, 1H), 2.30-2.40 (m, 1H), 2.48-2.58 (m, 1H), 2.98-3.09 (m, 1H), 3.36-3.45 (m, 1H), 3.66-3.89 (m, 5H), 4.02-4.11 (m, 1H), 5.85 (d, $J = 14.3$), 7.03 (quintet of doublets, $J = 8.0, 2.7$), 7.14-7.38 (m, 5H); $^{13}\text{C NMR}$: δ 24.22, 24.56, 25.23, 37.21, 40.45, 42.56, 51.56, 53.34, 54.36, 68.53, 74.49, 119.5, 126.9, 128.0, 128.3, 138.1, 145.6, 166.6; HRMS (ESI) calcd for $[\text{C}_{20}\text{H}_{28}\text{N}_4\text{O}_3 + \text{H}]^+$: 373.2240, found 373.2236.



III-13

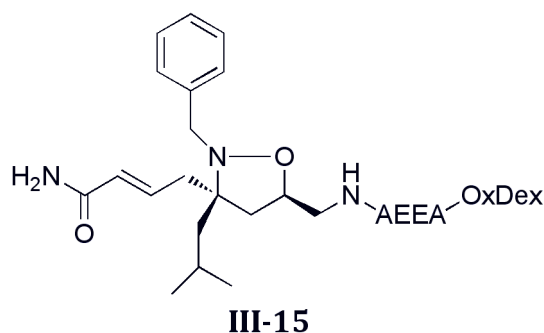
4-(2-Benzyl-5-{{2-(2-{{2-{{(9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,11,12,14,15,16,17-dodecahydro-3*H*-cyclopenta[*a*]phenanthrene-17-carbonyl)-amino]-ethoxy)-ethoxy)-acetylamino]-methyl}-3-isobutylisoxazolidin-3-yl)-but-2-enoic acid methyl ester (III-13): Preparation of **III-13** was accomplished under conditions identical to those used for **5** starting with 0.038 mmol of **III-35**. Purification by reverse phase HPLC providing 6.8 mg of **III-13** (21% yield) as a white solid. HRMS (ESI) calcd for $[C_{47}H_{66}FN_3O_{10} + H]^+$: 852.4810, found 852.4810.



III-14

4-(2-Benzyl-5-{{2-(2-{{2-{{(9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,11,12,14,15,16,17-dodecahydro-3*H*-cyclopenta[*a*]phenanthrene-

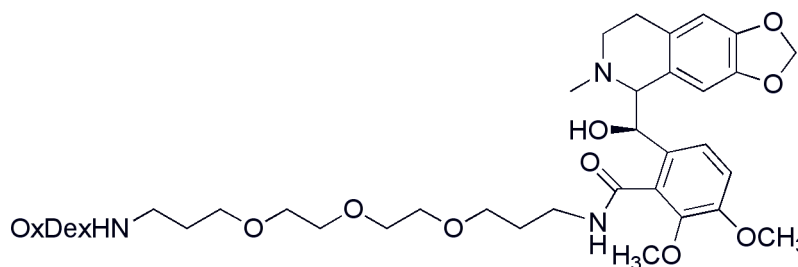
17- carbonyl)-amino]-ethoxy}-ethoxy)-acetylamino]-methyl}-3-isobutyl-isoxazolidin-3-yl)-but-2-enoic acid (III-14): To compound **III-13** (6.0 mg, 12 μ mol, 1.0 eq) was added ethanol (0.13 mL) then sodium hydroxide (1.3 mg, 30 μ mol, 2.5 eq). The mixture was allowed to stir at for 8 h until the reaction was judged complete by ESI mass spectrometry. 0.1% TFA (1.5 mL) and CH₃CN (1.5 mL) were added to the reaction. The resulting mixture was filtered through a syringe filter and purified by reverse phase HPLC providing 3.1 mg of **III-14** (31% yield) as a white solid. HRMS (ESI) calcd for [C₄₆H₆₄FN₃O₁₀ +H]⁺: 838.4654, found 838.4675.



4-(2-Benzyl-5-{[2-(2-{2-[(9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,11,12,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-

17- carbonyl)-amino]-ethoxy}-ethoxy)-acetylamino]-methyl}-3-isobutyl-isoxazolidin-3-yl)-but-2-enoic acid amide (III-15): To a solution of compound **III-14** (2.1mg, 2.5 μ mol, 1.0 eq) in CH₃CN (.030 mL) and pyridine (1.0 μ L, 13 μ mol, 5.0 eq) was added Boc₂O (1.0 mg, 4.6 μ mol, 1.8 eq) and NH₄HCO₃ (1.0 mg, 13 μ mol, 5.0 eq). The mixture was allowed to stir at for 24 h until the reaction was judged complete by ESI mass spectrometry. 0.1% TFA H₂O (0.80 mL) and CH₃CN (0.70 mL)

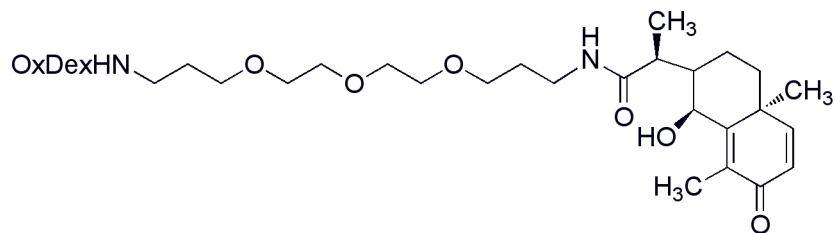
were added to the reaction. The resulting mixture was filtered through a syringe filter and purified by reverse phase HPLC providing 0.70 mg of **III-15** (33% yield) as a white solid. ESI-MS calcd for $[C_{46}H_{65}FN_4O_9 + H]^+$: 837.5, found 837.5.



III-41

9-Fluoro-11,17-dihydroxy-N-(1-(6-(hydroxy(6-methyl-5,6,7,8-tetrahydro-[1,3]dioxolo[4,5-g]isoquinolin-5-yl)methyl)-2,3-dimethoxyphenyl)-1-oxo-6,9,12-trioxa-2-azapentadecan-15-yl)-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carboxamide (III-41): Beta-(+/-)-hydrastine hydrochloride (10 mg, 0.024 mmol, 1.0 eq.) was dissolved in 5.0 mL of DMF, followed by addition of *i*-Pr₂NEt (0.034 mL, 0.24 mmol, 10 eq) and DMAP (3.0 mg, 0.024 mmol, 1.0 eq.). To this solution was added 4,7,10-trioxa-1,13-tridecanediamine (0.11 g, 0.48 mmol, 20 eq.) and the reaction mixture was stirred for 12 h. The reaction was diluted with water (10 mL) and extracted with EtOAc (3 x 10 mL). The organic layer was washed with water (2 x 10 mL), dried over Na₂SO₄, and concentrated under reduced pressure. To this crude mixture was added HOBt (8.0 mg, 0.060 mmol, 2.5 eq.), HBTU (23 mg, 0.060 mmol, 2.5 eq.), *i*-Pr₂Net (8.0 μL, 0.060 mmol, 2.5 eq.) and OxDex (23 mg, 0.060

mmol, 2.5 eq.) and the reaction mixture was stirred overnight. The reaction progress was monitored by ESI-MS. The purification was accomplished by reverse-phase HPLC to yield 1.1 mg of **III-41** in 4.6% yield as a white solid. HRMS (ESI) calcd for $[C_{52}H_{70}FN_3O_{13} + Na]^+$: 986.4790, found: 986.4823.

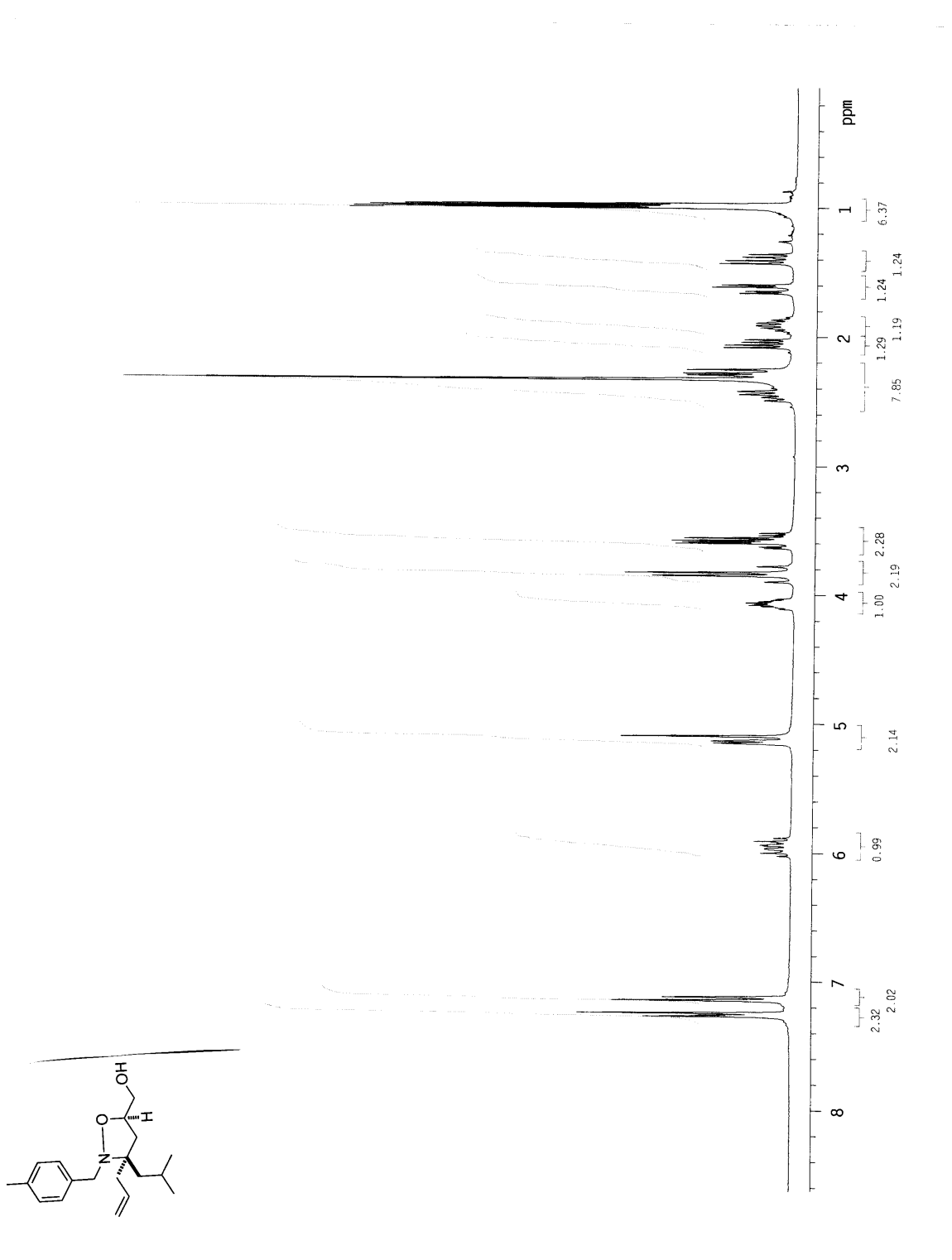


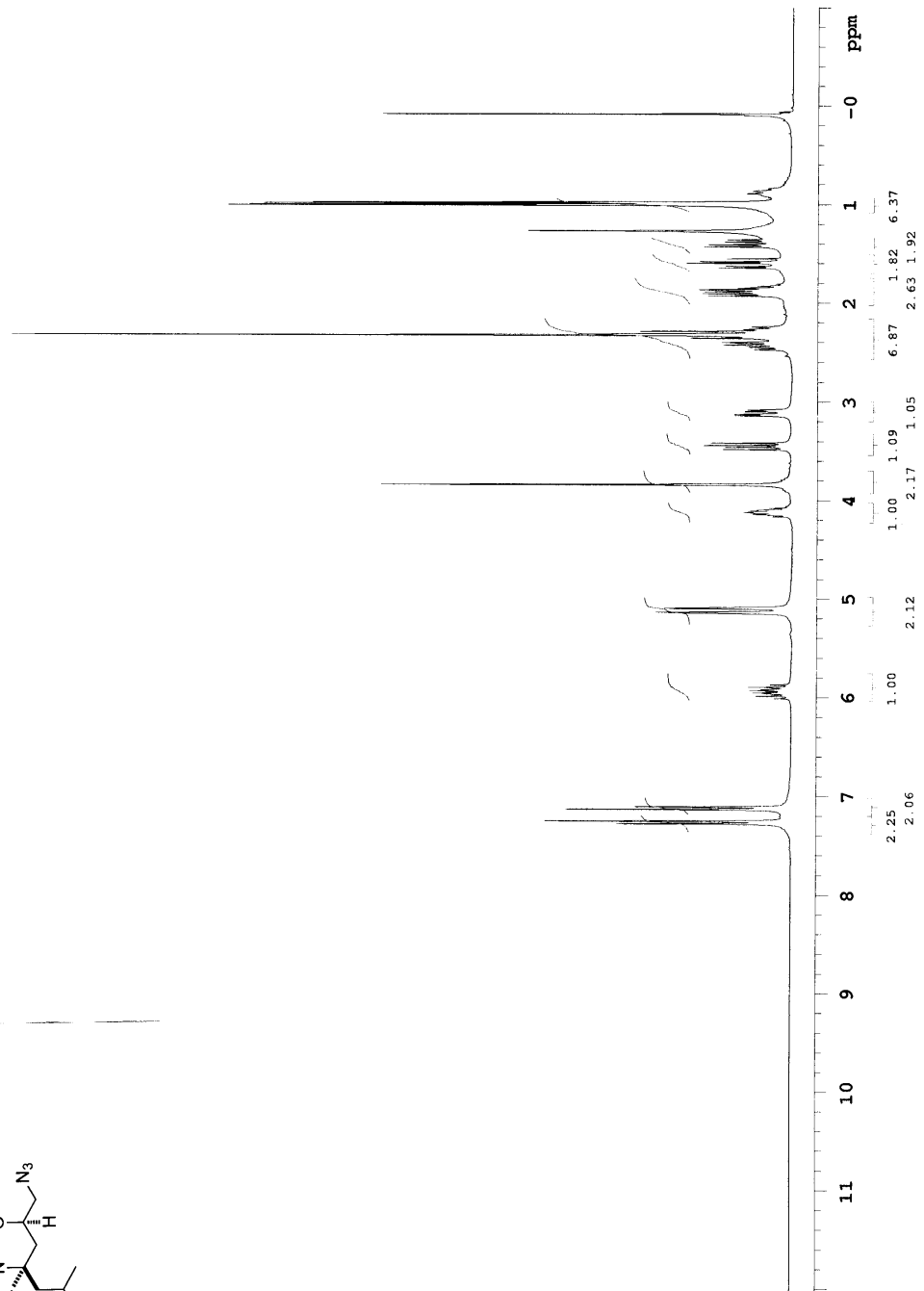
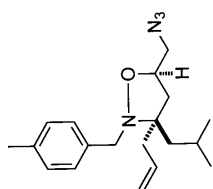
III-42

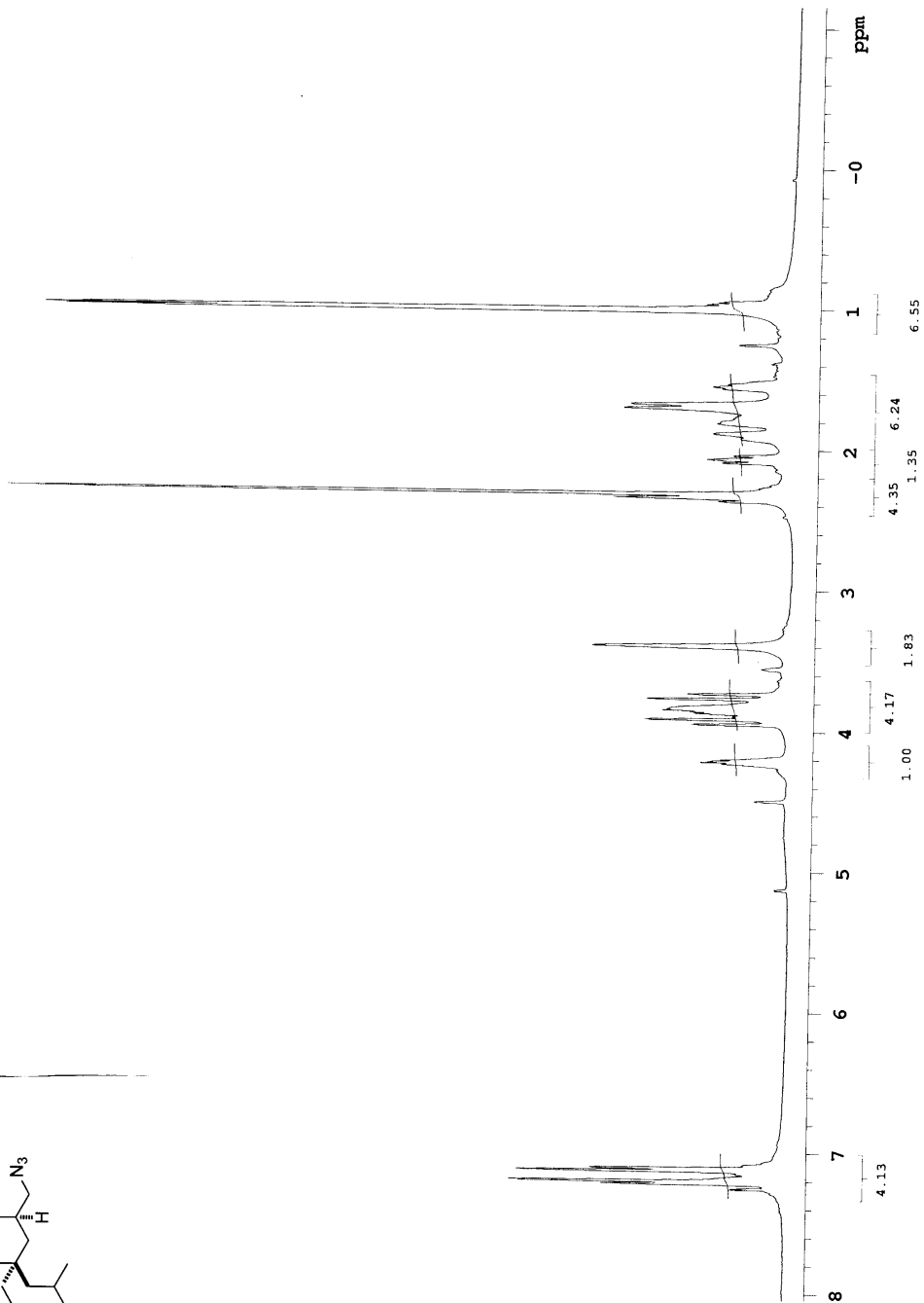
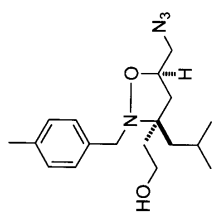
9-Fluoro-11,17-dihydroxy-N-(16-(1-hydroxy-4a,8-dimethyl-7-oxo-1,2,3,4,4a,7-hexahydronaphthalen-2-yl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carboxamide (III-42):

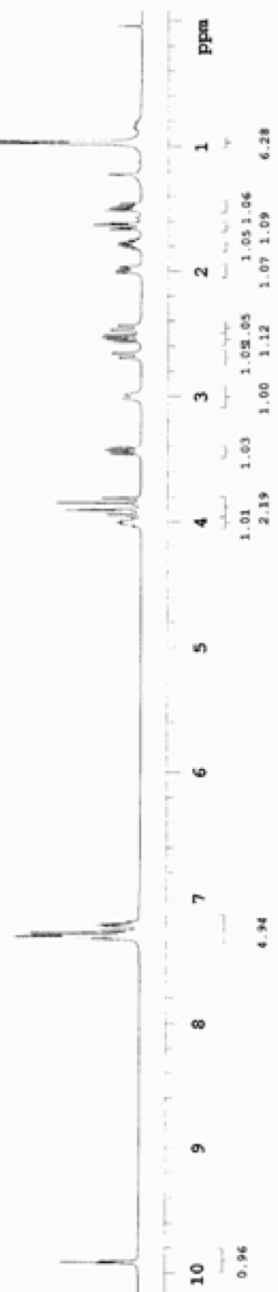
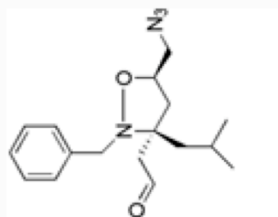
Preparation of was accomplished under conditions identical to those used for **III-41**, except that (-)- α -Santonin was used as the starting material. Purification by reverse phase HPLC provided 0.7 mg (3% yield) of **III-42** as a white solid. HRMS (ESI) calcd for $[C_{46}H_{67}FN_2O_{10} + Na]^+$: 849.4677, found: 849.4660.

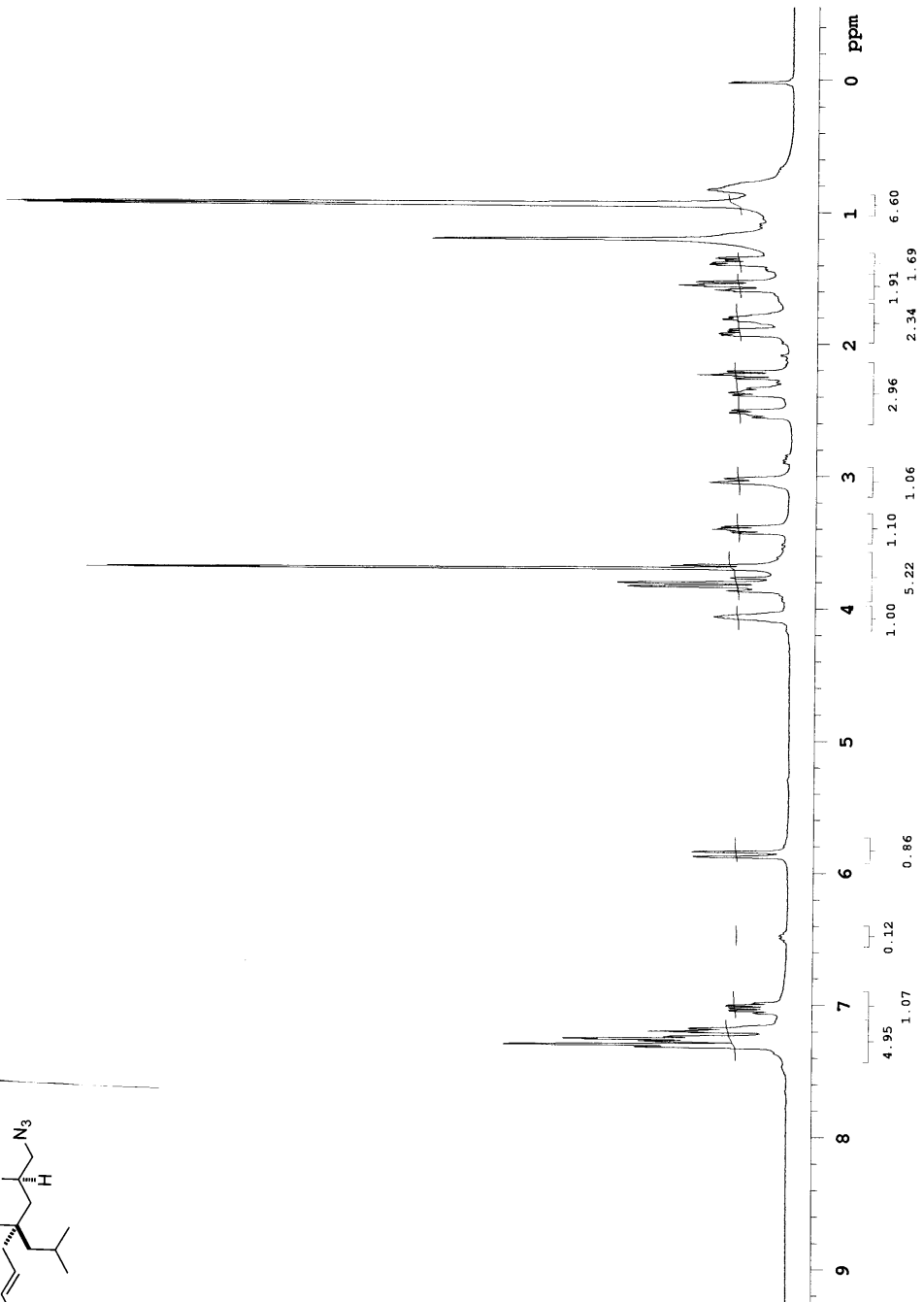
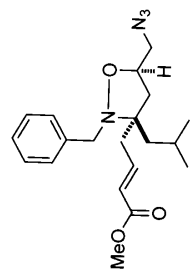
H. Appendix of Selected ^1H NMR Spectra and HPLC traces

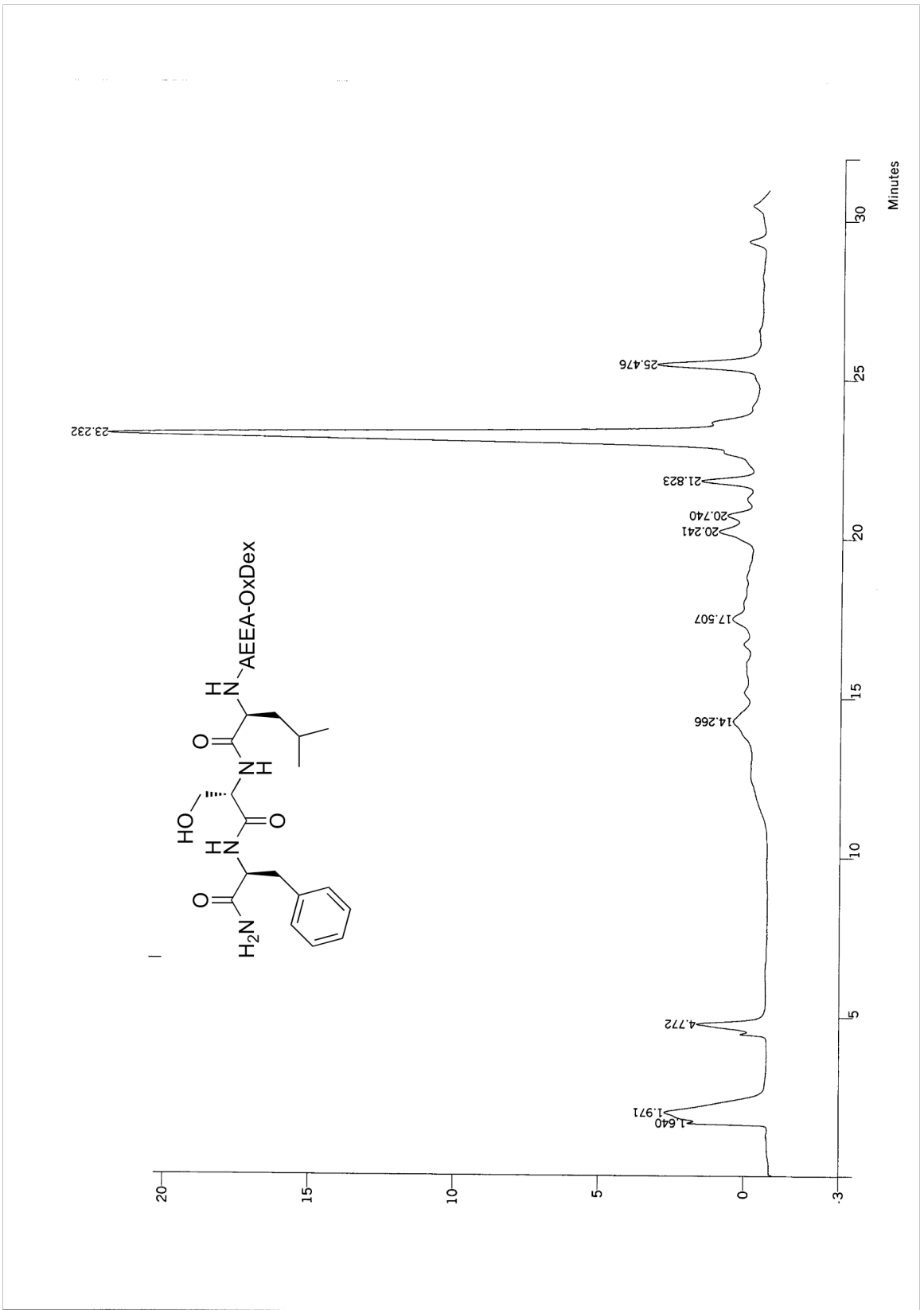


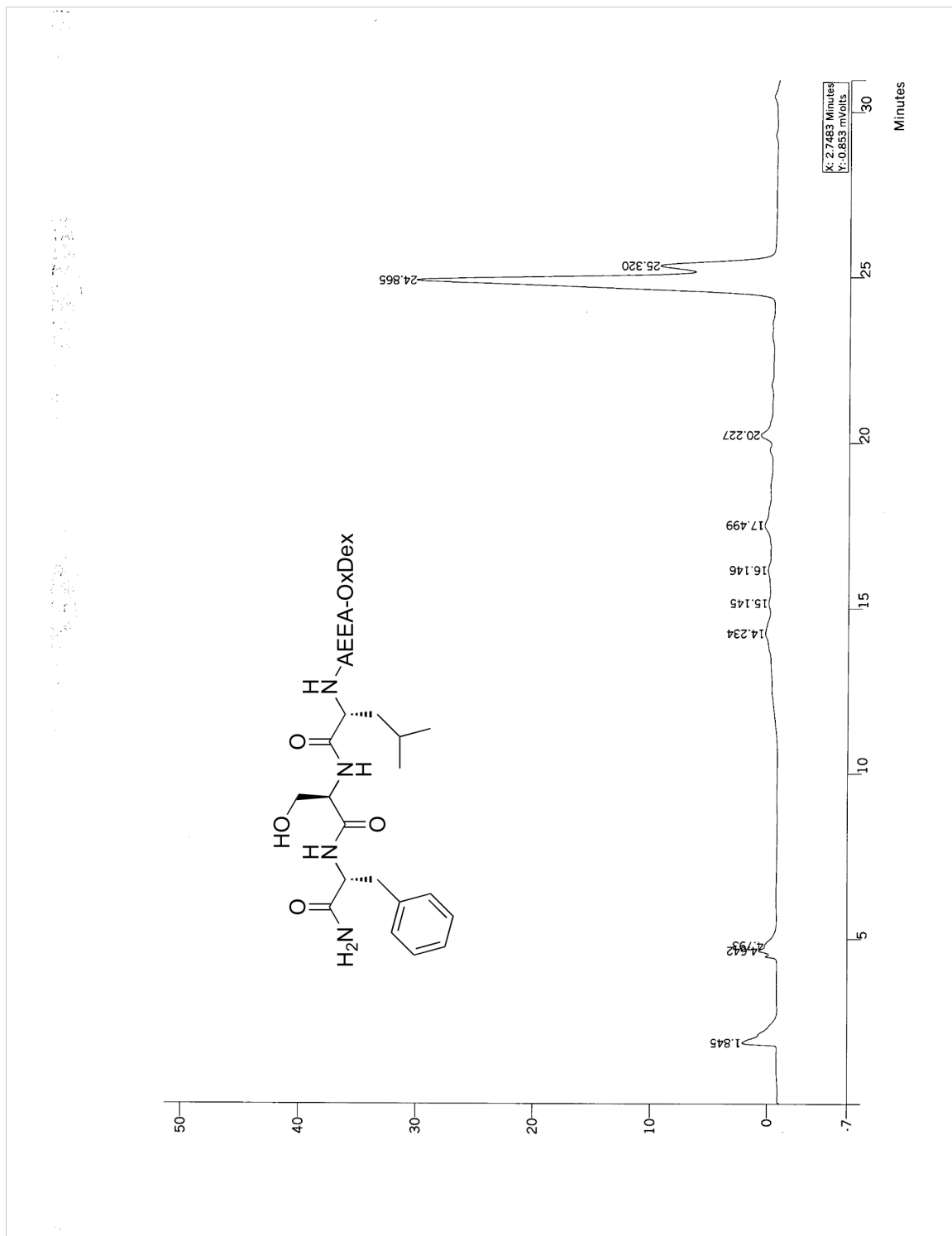


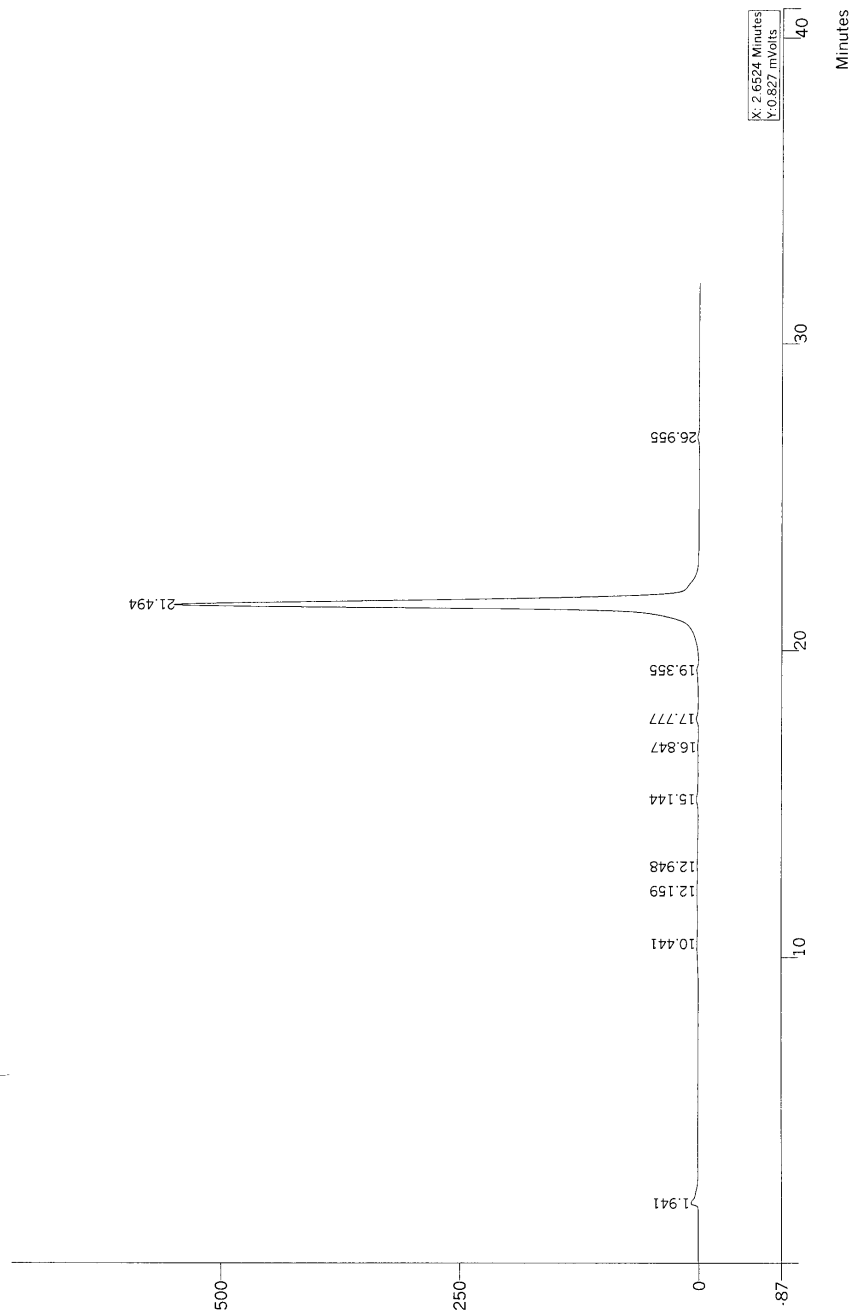
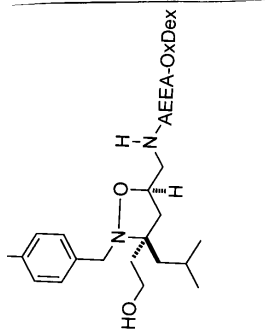


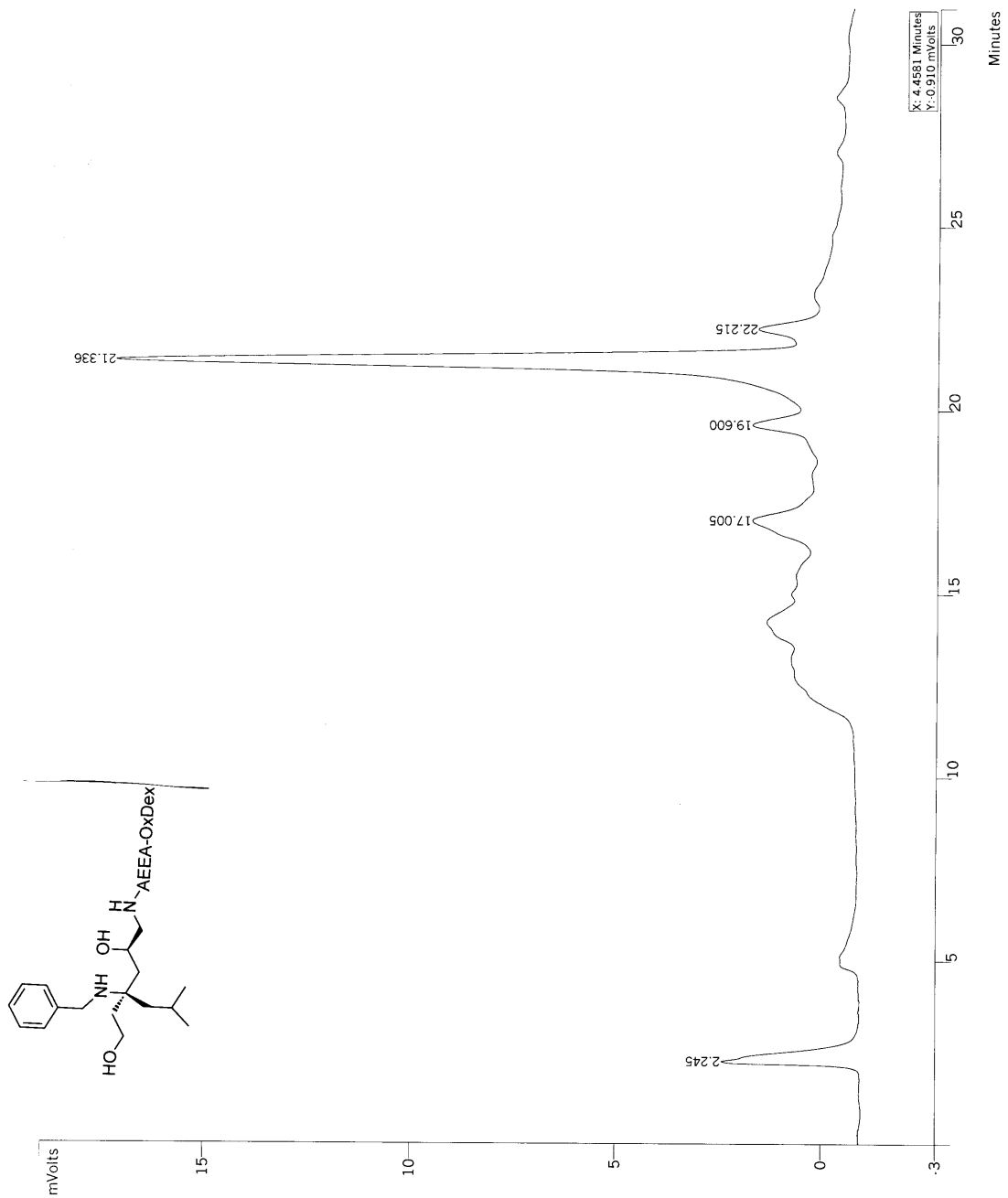


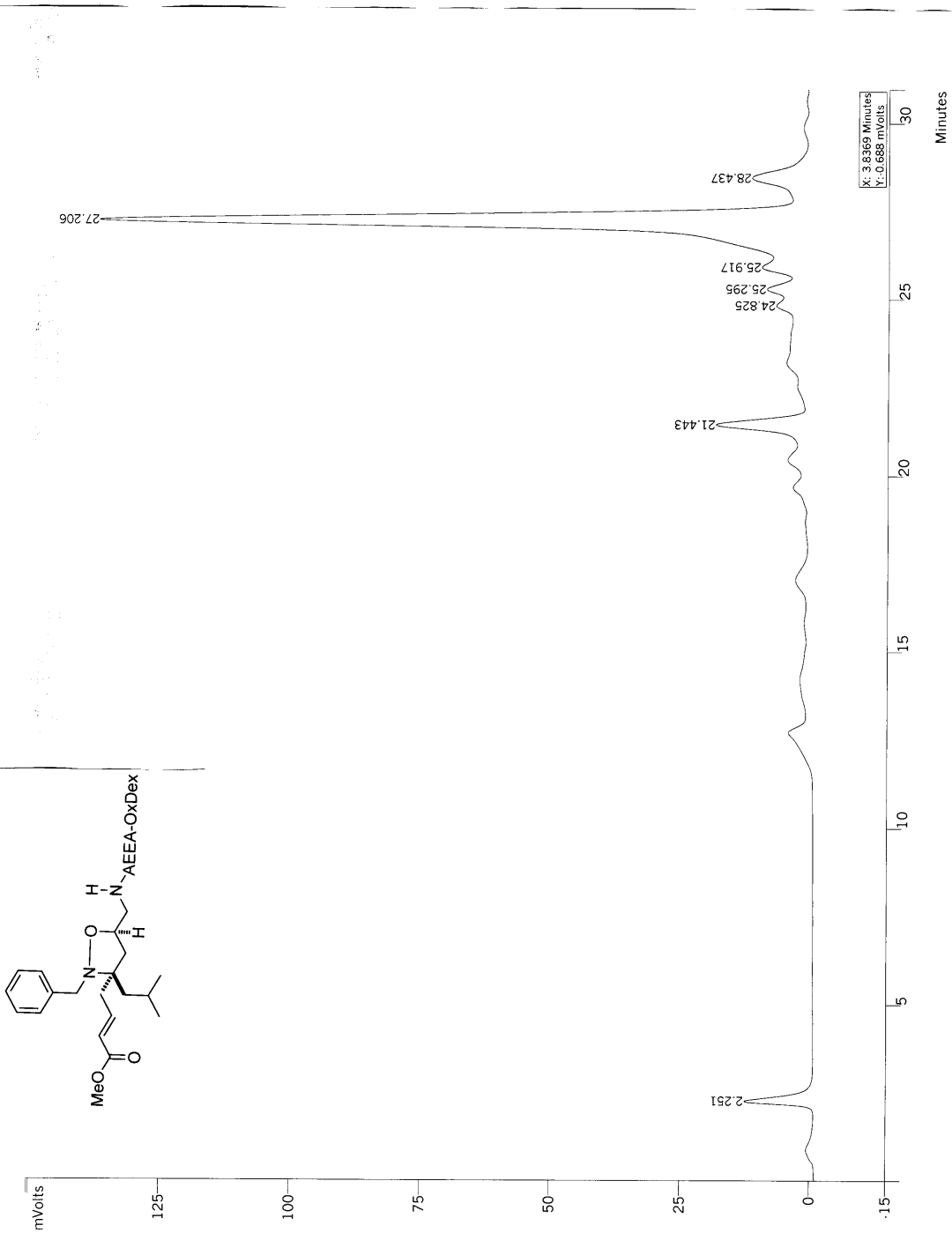


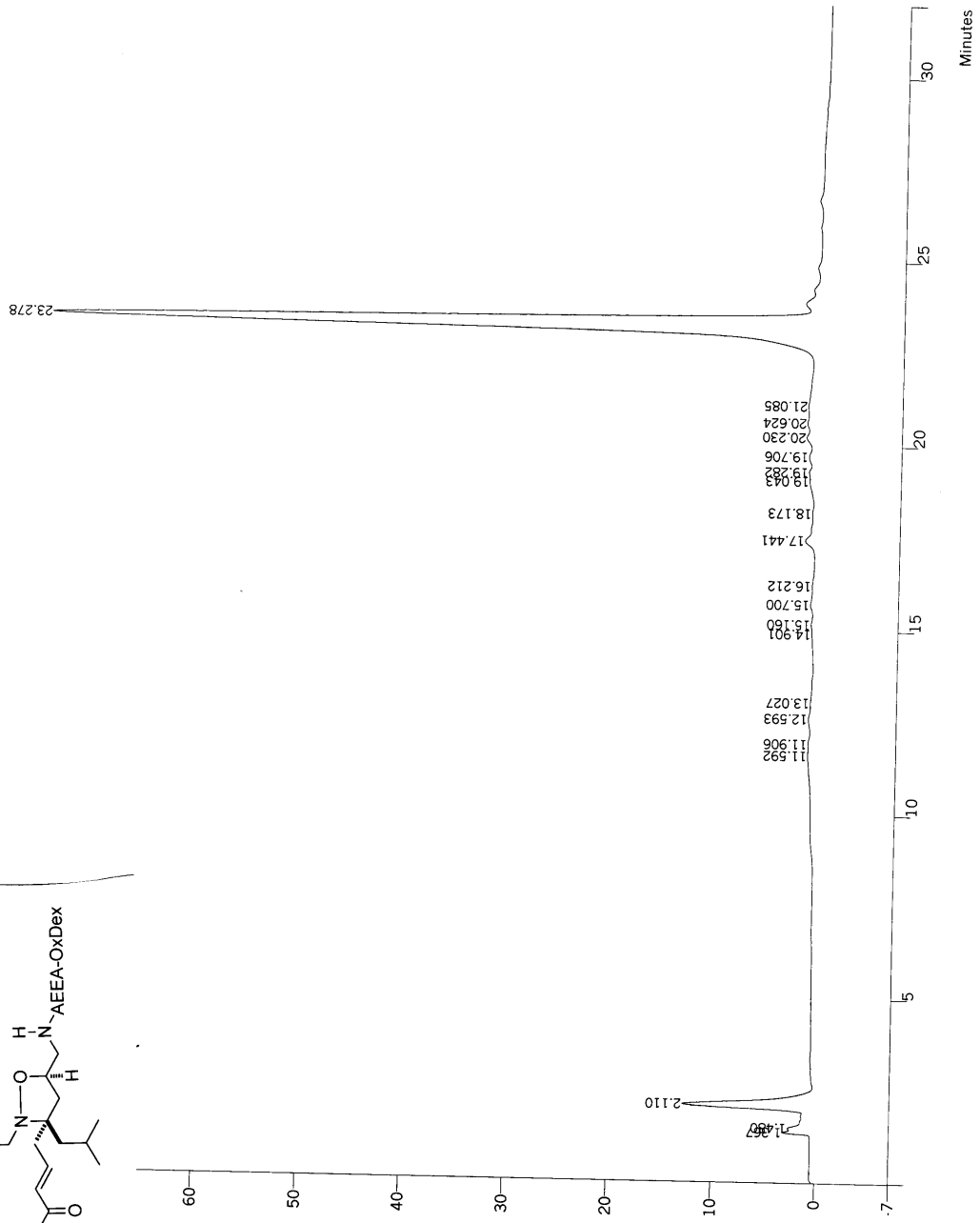
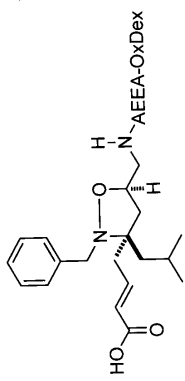












I. References:

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Chapter IV

Design of Activator Artificial Transcription Factors ^a

A. Abstract

Transcriptional activators consist minimally of a DNA binding domain (DBD) and a transcriptional activation domain (TAD). Previously we described an isoxazolidine TAD bearing functional groups similar to natural transcriptional activators that up-regulates transcription 80-fold at 1 μ M in cell culture. Additionally the free activation domain could “squench” or competitively inhibit the DNA-localized TAD, indicating that the functional groups on the isoxazolidine were largely responsible for the observed transcriptional activity. These studies used protein DNA binding domains that ultimately limit the genes that can be regulated by the small molecule. Two non-peptidic DBDs, triplex forming oligonucleotides (TFOs) and polyamides, have been used to design artificial transcription factors (ATFs) capable of activating transcription in vitro and in cells. These molecules can target DNA sequence specifically, which enables the targeting of specific genes. Using polyamides and TFOs we constructed artificial transcription factors with our

^a Ryan Casey conducted chemical synthesis. Jonas Højfeldt performed biological testing. The second generation polyamides were synthesized by David Montgomery at Caltech.

small molecule TAD and tested them for in cell activation. Current work focuses on the study of these in conjunction with the Dervan Lab at Caltech.

B. Introduction

Transcriptional activators are essential molecules in the control of gene expression that function by localizing to specific promoter regions and recruiting the transcriptional machinery to the transcriptional start site.¹ These proteins minimally consist of a transcriptional activation domain (TAD) and DNA binding domain (DBD).² The two domains of an activator are considered to function independently where one domain can be swapped with corresponding domains to create artificial transcription factors.³

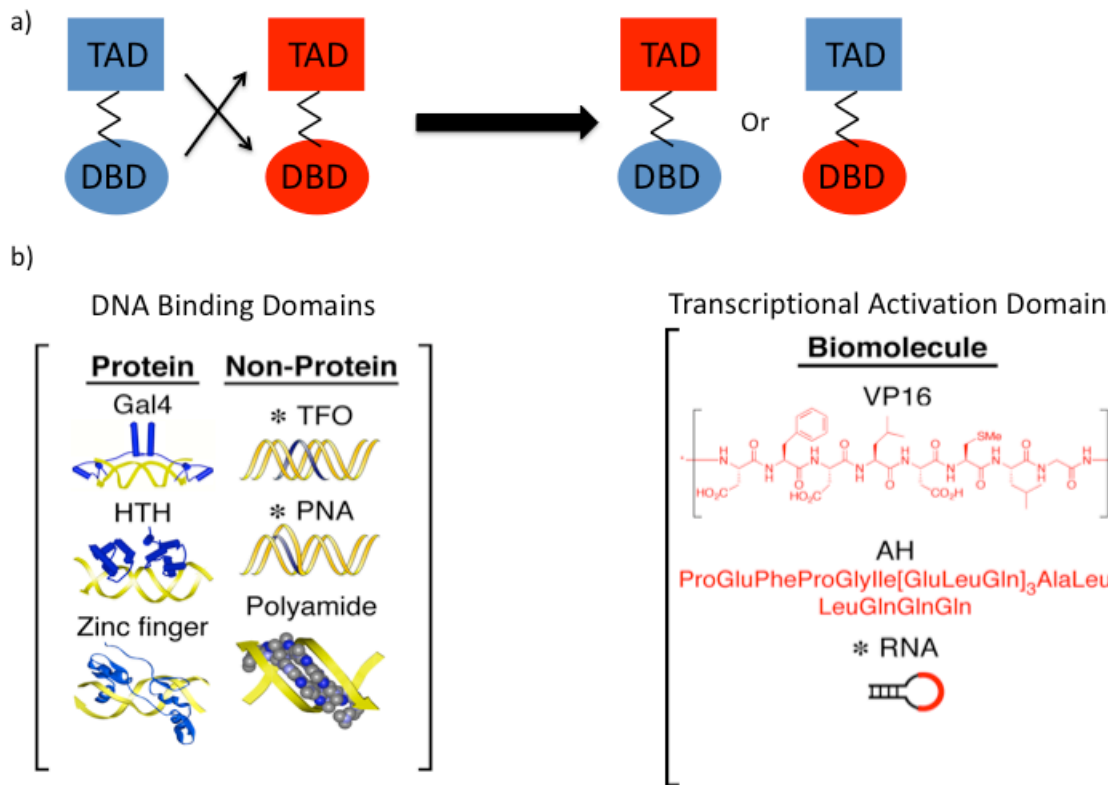


Figure IV-1. Construction of an activator artificial transcription factor (ATF). a) Domain swapping experiments. Having the arrows touch the modules makes it very difficult to understand. A transcriptional activation domain (TAD) from one activator (blue) can be attached to a DNA binding domain (DBD) of a second activator (red) to construct a synthetic activator ATF. This method can localize a potent TAD to a promoter sequence to study the affects of up regulation of a specific subset of genes. b) As of 2003 there were many DBDs to select from but no small molecule TADs and only TADs based on biomolecules. Polyamides bind in the minor groove of DNA where TFOs bind in the major groove of DNA.

(ATFs) (**Figure IV-1**). Of the two domains, far more is known about the DBD than the TAD. Artificial DNA binding proteins, such as synthetic zinc fingers, can target DNA sequence specifically and have been used to make custom activator.⁴ These activator ATFs have tremendous potential for deciphering the details of disease and

other non-biomedical applications (**Table IV-1**).⁵ One example of successful use of a transcriptional therapeutic is the case of medulloblastoma where overexpression of the transcriptional repressor REST/NRSF leads to the suppression of critical genes for proper neuronal cell differentiation. A protein-based activator ATF consisting of a potent TAD and a DBD which recognizes REST/NRSF binding sites can induce tumor cell apoptosis and reverse this phenotype.^{6, 7} However, one drawback of protein-based activator ATFs is they suffer from issues such as immunogenicity and limited bioavailability.⁸ In contrast, non-peptidic activator ATFs could address these concerns since synthetic molecules are less immunogenic than peptides and are more bioavailable than peptides.² Thus molecules that modulate gene expression to prescribed levels would be valuable as potential therapeutics and other applications. Outlined in the next section are the non-peptidic DBDs that have been used to construct activator ATFs and that are the focus of our studies.

Application	Example
Manufacturing	An activator ATF could increase the yield of a desired protein or raise the effective concentration of a necessary enzyme
Synthetic Biology	Activators that are orthogonal to natural activators and regulate genes to prescribed levels could construct synthetic transcription levels
Cancer	In the case of medulloblastoma, over- expression of a repressive protein REST causes decreased expression of genes necessary for proper neural differentiation
Diabetes	An activator ATF could restore insulin homeostasis through activation of either glucose transporters or insulin

Table IV-1. Applications of activator ATFs. Adopted from Ref. 2

1. Non-Peptidic DNA Binding Domains

Polyamides: Polyamides are N-methyl pyrrole (Py) and N-methyl imidazole (Im) polymers joined by amide bonds that recognize DNA sequence selectively by binding the minor groove of DNA.⁹ A Py/Py pair can recognize either an A-T or T-A base pair (W), whereas a Im/Py pair recognizes a G-C base pair (**Figure IV-2**).⁹ By structural studies it was determined that amide of a Im/Py base pair recognizes guanine through specific interactions between the Im and guanine through a hydrogen bond.^{10, 11} Polyamides have found many applications in the inhibition and activation of gene expression and can bind DNA as tightly as some natural proteins. Recent applications include the inhibition of glucocorticoid receptor,¹² HIF 1a,¹³ and androgen receptor.¹⁴ An application towards gene expression was demonstrated by attachment of the peptidic transcriptional activation domain VP2 to a polyamide.

While this molecule activated transcription in vitro, it could not activated transcription in cell culture.^{15, 16} One obvious drawback to polyamides is their specificity to DNA, as they can only target 4-6 base pairs.⁹ This might be one explanation for the loss of activity in cell culture.

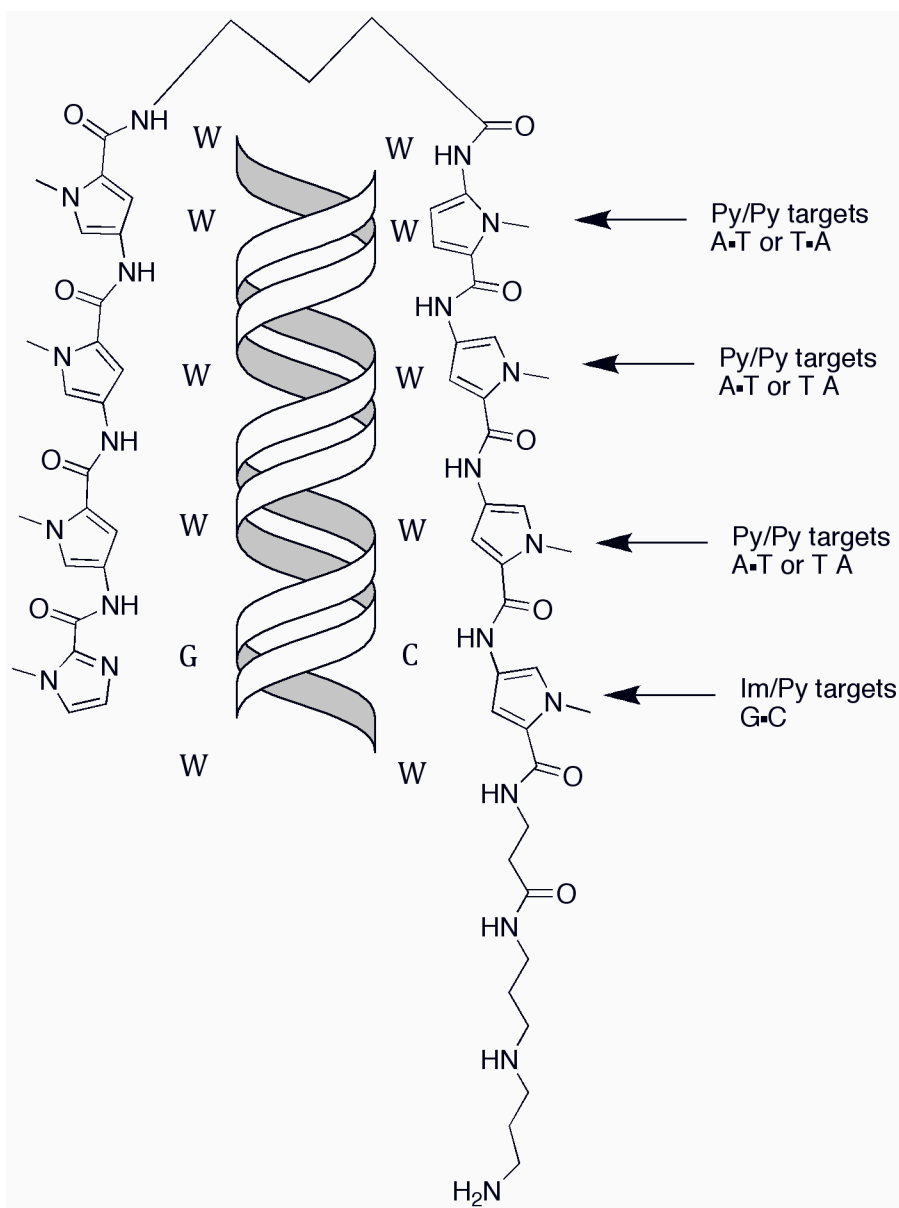


Figure IV-2. Polyamide that targets WGWWWW. Polyamides can target G-C base pairs through Im/Py pairs where as Py/Py can target an A-T or T-A base pair (W). The turn and tail are selective for W over G-C base pairs. Adopted from Dervan 2003.

TFO: Triplex forming oligonucleotides are oligos that target the major groove of DNA through Hoogsteen base pair interactions.¹⁷ They typically target purine or pyrimidine tracts in DNA but recently have been expanded to target almost any sequence in DNA through the incorporation of synthetic nucleosides.¹⁷ Interestingly, there is an over-represented population of purine and pyrimidine tracts in the promoter regions of genes, making TFOs an attractive DBD for ATFs.¹⁸ There are two main triplex motifs that form, GT triplexes and GA triplets.¹⁹ In the GT triplex (not shown), the third strand is either anti-parallel (3' to 5') to the duplex and forms reverse Hoogsteen C-G*G and T-A*T triplets or parallel (5' to 3') and forms Hoogsteen base pairs C-G*G and T-A*T triplets.¹⁹ In the case of a GA triplet, the third strand is oriented anti-parallel to the purine strand of the duplex and forms reverse Hoogsteen base pairs C-G*G and T-A*A triplets (**Figure IV-3**).¹⁹ The CT triplex can also form but requires the protonation of cytosine and thus requires a non-physiological pH.¹⁹ TFOs have tremendous use in molecular biology and biochemistry.²⁰⁻²² They have been used to site-specifically deliver DNA damaging molecules such as psoralen and topoisomerase activators such as camptothecin and etoposide.²³⁻²⁵ By microinjecting TFOs into mouse cell nuclei, scientists were able to introduce genes into a specific chromosomal location through recombination.²¹ However, TFOs require either microinjection or cationic liposomes for delivery which limit their utility.¹⁹ Nevertheless, TFOs have been used in vivo to induce sequence specific mutations in a mouse model. In an example particularly relevant to our studies, an activator ATF was synthesized through attachment of a TFO to a

peptidic TAD derived from the natural activator VP16.²⁶ This conjugate activated transcription both in vitro and in cells, although it required cationic liposomes for delivery.²⁶ Despite limitations in delivery, TFOs have gained widespread use as a biochemical tool.

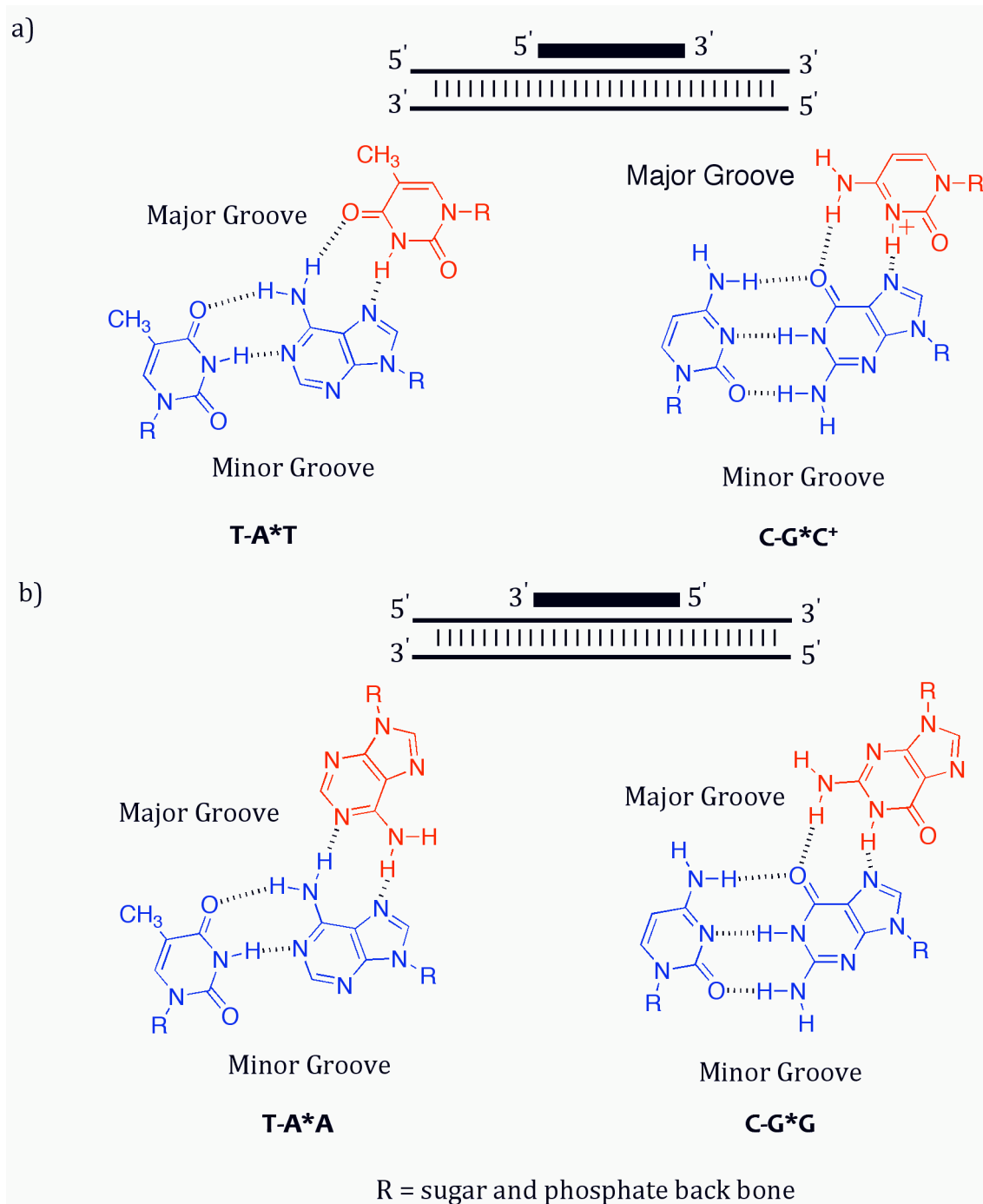


Figure IV-3. Triplex forming oligonucleotides (TFOs) recognize DNA through Hoogsteen base pair in the major groove. a) A CT triplex can form in a parallel orientation (5' to 3') through Hoogsteen base pairs T-A*T and C-G*C⁺ triplets. This requires a high pH to protonate cytosine. b) For a GA triplet, the third strand is anti-parallel to the purine strand (3' to 5') of the duplex and forms reverse Hoogsteen base pairs C-G*G and T-A*A triplets. Adopted from Ref. 17.

2. Non-Peptidic Transcriptional Activation Domains

Activator ATFs are almost solely composed of peptidic TADs, as the DNA binding domain (DBD) has been the more tractable of the two domains to replace with non-natural molecules.^{2, 27} One of the first activator ATFs was designed using a d and l-peptide derivative of the amphipathic activator VP16.²⁸ Both the d- and l-peptide containing ATFs activated transcription similarly *in vitro*; however, only the conjugate with the d-peptide TAD activated in cell culture.²⁸ Presumably this result arises from the enhanced stability of d-peptides to proteolysis. In addition to proteolytic instability, peptidic TADs have other limitations such as low bioavailability and the potential to be immunogenic.² Building on this theme, a screen of a peptoid library against the KIX domain of CBP/p300 yielded a TAD that activated potently in cell culture when localized to DNA by a two-hybrid system.²⁹ When attached to a DNA-binding hairpin polyamide the conjugate only weakly activates transcription, likely due to its large size that limits its bioavailability and cell trafficking.^{30, 31} However, there are still few examples of small molecule TADs and considering the limitations associated with peptidic TADs, there is great interest in the development of small molecule TADs. Some small molecules capable of transcriptional activation are the isoxazolidine (**IV-1**), hydrastine (**IV-2**), santonin (**IV-3**), and wrencholol (**IV-4**) (**Figure IV-4**).³²⁻³⁵ Guidelines to develop such molecules were described in Chapter 3 and the development of the first small molecule TAD was outlined in Chapter 2.^{32, 33}

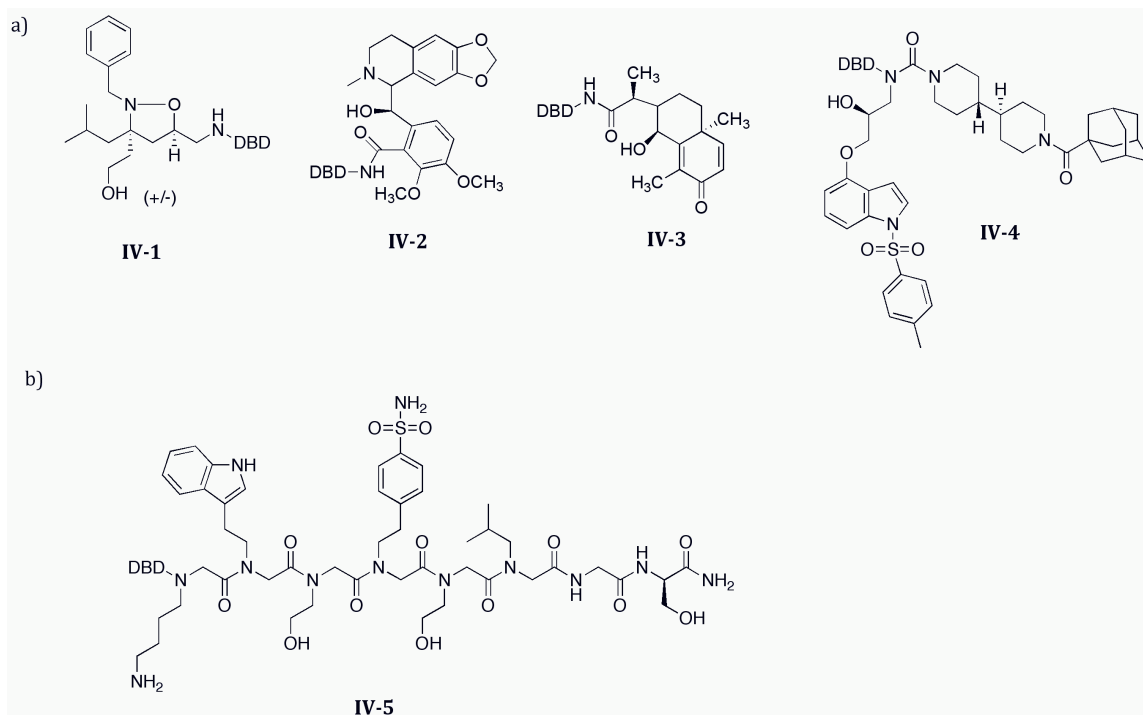


Figure IV-4. Synthetic TADs. a) the scope of small molecule TADs. The amphipathic isoxazolidine (**IV-1**) activates transcription 80-fold in HeLa cells. Two natural products, hydrastine (**IV-2**) and santonin, (**IV-3**) described in the previous chapter are also capable of activating transcription when localized to DNA. Wrenchnolol (**IV-4**) activates transcription when localized to DNA and can function as a TAD for an activator ATF in vitro. b) A peptoid (**IV-5**) described by the Kodadek lab can activate transcription in cells both when localized to DNA in a two hybrid system and as an activator ATF.

3. Design of Non-Peptidic Activator ATFs

On the surface, design of an activator ATF is relatively straightforward: replacement of a DBD and TAD with their synthetic counterparts.^{27, 36, 37} However, translating transcriptional activity from a synthetic system, such as a two-hybrid, to a non-peptidic activator ATF has proven difficult. Studies by the Kodadek lab show that a peptoid (**IV-5**) isolated in a screen against the KIX domain can activate transcription in cells up to 900 fold using a two-hybrid system with an EC_{50} above 10 μ M but only 5 fold at 3 μ M when attached to a hairpin polyamide.^{29, 31} This

reduction in activity could be from a variety of factors such as bioavailability and limited projection of the TAD out of the minor groove of DNA. In the two-hybrid system, the TAD is well projected from DNA by the fusion protein that localizes the TAD to DNA. The protein DBD fusion may also act as a platform and help stabilize complexes that bind to a small molecule. In another example, the Uesugi group employed a structure-based design approach to produce wrencholol, a 10 μ M inhibitor of the MED23-ESX interaction.^{35, 38} When localized to DNA, this molecule activates transcription by a two-hybrid approach in cell culture.³⁹ However, when wrencholol was connected to a polyamide DBD, the conjugate only activated transcription weakly in a cell free system and did not activate in cell culture.³⁴

The Young lab reported an activator ATF composed of a potent TAD termed ATF14 and its dimer ATF29, based on the VP16, conjugated to a TFO.²⁶ This molecule could activate transcription in vitro and in cells at concentrations of 10 and 50 nM. In vitro, both ATF29 and ATF14 activated to similar levels. Interestingly the dimeric ATF29 activated transcription 8 fold while ATF14 activated 30 fold in cells. While cationic liposomes were used in all experiments, it could be that the longer peptide did not cross the cell membrane as effectively as the shorter activation domain. By using a small molecule TAD, the requirements for bioavailability could be overcome and a more potent activator ATF can be developed.² We hypothesized that a small molecule TAD conjugated either to a hairpin polyamide or a TFO should function as an activator ATF. Some of the problems with activator ATFs are low specificity of the DNA binding domain and poor bioavailability of the overall conjugate.^{31, 40} We envision that small molecules

TADs will make the conjugate more bioavailable than the much larger peptoids or peptides.

C. Construction of Activator ATFs

We designed four initial conjugates to test for activity in cells. The DNA binding domains were selected based on success as past activator ATFs and the TADs were the most potent small molecule TADs that we had identified in our earlier studies outlined in Chapter 2 and 3 (**Figure IV-5**).^{15, 16, 26, 32-34} The polyamide selected exhibits good nuclear and cell permeability.⁴⁰ TFOs composed of DNA have uniform bioavailability and require cationic liposomes. The TFO selected is the same TFO used in a previous study to activate transcription.²⁶ To synthesize this conjugate we synthesized the isoxazolidine TAD following procedures previously described in Chapter 2 and Chapter 3. Conjugate **IV-7** bearing an isoxazolidine that was inactive in our previous studies was compared to conjugate **IV-6**.

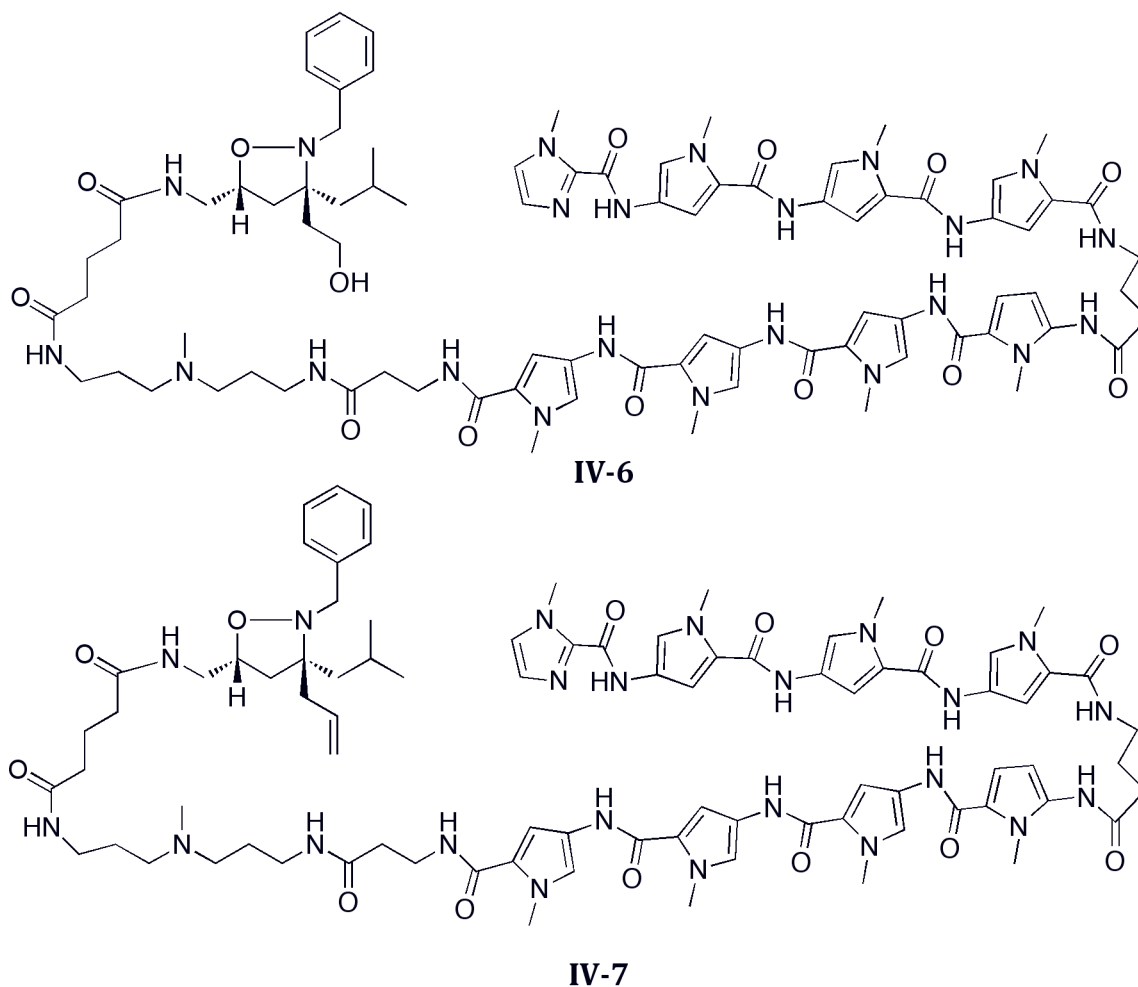
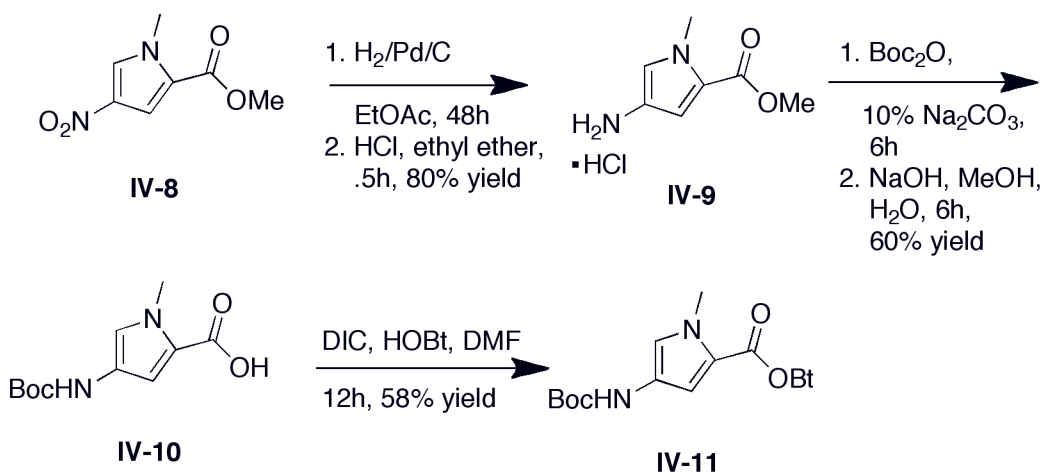


Figure IV-5. Isoxazolidine -olyamide conjugates. The polyamides were selected based on their success as DBDs for construction of ATFs. The TFO (not shown) were identical to those used by Young except with isoxazolidines as TADs instead of peptides. The amphipathic TAD (above) was tested for activity in cells and the hydrophobic isoxazolidine (below) was designed as a negative control.

1. First Generation Polyamide-Small Molecule Conjugate

We chose the best-studied polyamide for our DBD. The polyamide which targets WGWWW, where W = A or T, was used in vitro and in cell culture to support transcriptional activation. This was critical as bioavailability and trafficking of polyamides can depend on the sequence. Nuclear localization studies indicate that some polyamides cannot localize to the nucleus.^{40, 41}

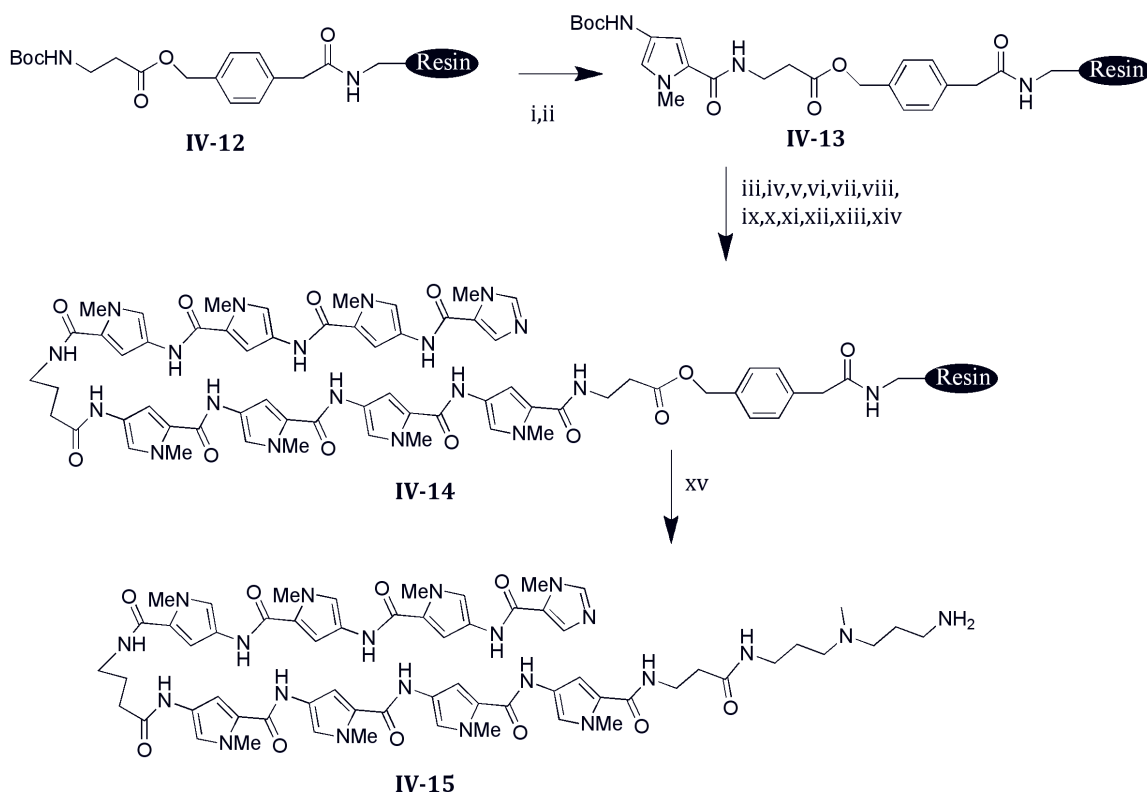
To synthesize this polyamide we started by making 50 grams of the N-methyl pyrrole monomer **IV-11** using established protocols (**Scheme IV-1**).⁴² The synthesis began with methyl 1-methyl-4-nitro-1*H*-pyrrole-2-carboxylate **IV-8** and followed by reduction of the nitro group with palladium on carbon under hydrogen atmosphere (80% yield). The amine **IV-9** was then protected with Boc anhydride to provide carbamate **IV-10** that was reacted immediately combined with NaOH to provide the free acid in 60% yield after crystallization. The white powder was combined with HOBt and DIC to provide the hydroxybenzotriazole ester **IV-11** in 58% yield.



Scheme IV-1. Synthesis of pyrrole monomer for use in polyamide synthesis.

This ester **IV-11** was the monomer used for Boc solid phase synthesis of the WGWWWW polyamide (**Scheme IV-2**). Starting with Boc- β -alanine-Pam-resin, each monomer was sequentially coupled and deprotected. After each step on the solid phase, a small sample of the resin was treated with (dimethylamino)propylamine to liberate polyamide and the resulting solution was monitored by HPLC to assess reaction progress. A shift of 1-2 minutes was observed for each coupling. The final

compound was cleaved to provide the final polyamide in 42% yield after reverse phase HPLC purification. An analytical HPLC verified the purity of the polyamide.

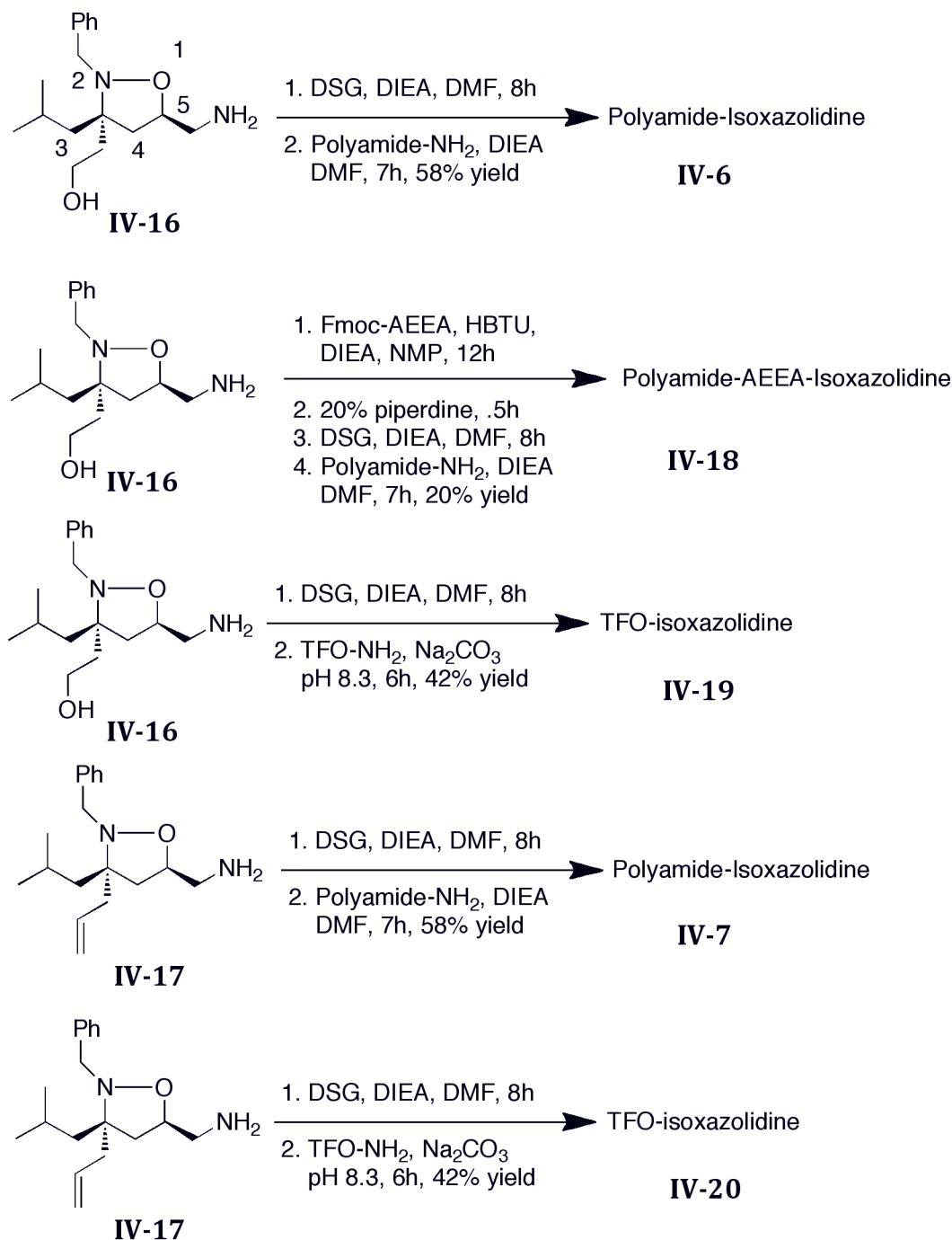


42% yield of final polyamide

Scheme IV-2. Synthesis of polyamide to target WGWWWW. (i) 8:1:1 TFA/DCM/TIPS; (ii) Boc-Py-OBt, DIEA, DMF; (iii) 8:1:1 TFA/DCM/TIPS; (iv) Boc-Py-OBt, DIEA, DMF; (v) 8:1:1 TFA/DCM/TIPS; (vi) Boc-Py-OBt, DIEA, DMF; (vii) 8:1:1 TFA/DCM/TIPS; (viii) Boc- γ -aminobutyric acid, HBTU, (ix) 8:1:1 TFA/DCM/TIPS; (x) Boc-Py-OBt, DIEA, DMF(xi) 8:1:1 TFA/DCM/TIPS; (xii) Boc-Py-OBt, DIEA, DMF; (xiii) 8:1:1 TFA/DCM/TIPS; (xiv) imidazole-2-carboxylic acid, HBTU, DIEA; (xv) (N,N-dimethylamino)propylamine, 55°C. Adopted from Ref. 42.

As can be seen in Scheme IV-2, the polyamide **IV-15** contains a free amine that can be used for attachment of the isoxazolidine. However, the isoxazolidine first needed to be modified to contain a carboxylic acid at the C5 position. This was accomplished through the use of a homo-bifunctional linker ,disuccinimidyl glutarate (DSG). Isoxazolidine **IV-16** or **IV-17** was reacted with DSG and purified by

reverse phase HPLC. The polyamide was then conjugated to the succinimide through the use of base. It was found that the pre-activated ester was easier to handle and reacted to completion with the amine of the polyamide (**Scheme IV-3**).



Scheme IV-3. Synthesis of polyamide and TFO isoxazolidine conjugates. The ring positions are labeled on the first isoxazolidine ring.

2. Second Generation Polyamide-Small Molecule Conjugate

In collaboration with Peter Dervan's lab at California Institute of Technology, we designed polyamides that could target Gal4 binding sites (**Figure IV-6**) We utilized a recently designed polyamide with enhanced binding to DNA and selective targeting to the Gal4 binding site, WGWCA.⁴³ The Dervan lab assembled the polyamide while we assembled the TAD as described for the first generation polyamide. The site of linkage was the free amine of the turn unit. Santonin, hydrastine, and the isoxazolidine TAD with two AEEA linkers were sent to the Dervan lab for attachment at the turn unit.³³

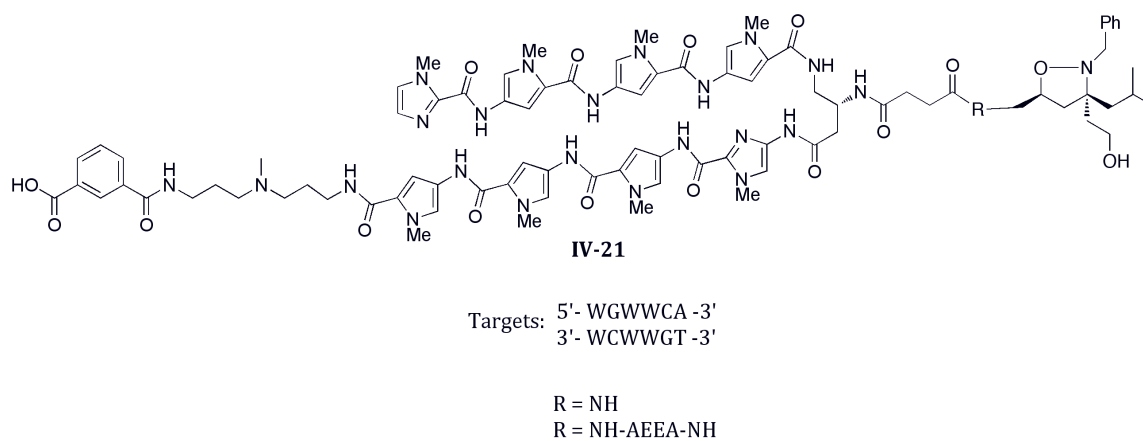


Figure IV-6. Polyamide **IV-21** constructed by the Dervan lab. The polyamide targets WGWCA and has two different linkers: an amide bond and an AEEA linker used in chapter two and three.

3. TFO-Isoxazolidine Conjugate

To synthesize the TFO conjugates we again used the disuccinimidyl glutarate linker to connect our compound to the TFO, which was commercially available

(**Scheme IV-5**). The compounds were isolated by reverse phase HPLC and quantified by UV-vis spectroscopy. These compounds were additionally tested for activity in cells.

D. Activity of Activator ATFs

To test the compounds for activation, we constructed plasmids that bear the DNA sequences necessary for recognition by the polyamide. These sequences were upstream of a TATA box and a luciferase reporter gene. There were five binding sites for the polyamide. The activity was measured in HeLa cells and normalized to the polyamide alone. As a transfection control, a second plasmid that expressed *Renilla* luciferase was additionally transfected. The assays to evaluate the conjugates activity in cells were evaluated by Jonas Højfeldt .

The first generation polyamide conjugate **IV-6** and **IV-7** were tested at 100nM, 1 μ M, 10 μ M, and 20 μ M. Preliminary data suggests that none of the conjugates showed activity in cells and toxicity was seen at 20 μ M. To test if the polyamide could bind DNA and support high levels of activated transcription, we made conjugates of the polyamide attached to oxidized dexamethasone (OxDEX) and an isocyanate derivative of dexamethasone, termed SDex,⁴⁴ which binds to the glucocorticoid ligand binding domain tighter than OxDEX. By transfecting with a plasmid that expresses GR-VP16 conjugate we should be able to localize that fusion protein to DNA by a three-hybrid system and thus activate transcription (**Figure IV-7**). Unfortunately these conjugates also failed to activate transcription. This polyamide can only target four base pairs and achieving sequence specific binding

remains a challenge. Evidence from these studies support that these conjugates are not binding the promoter effectively.

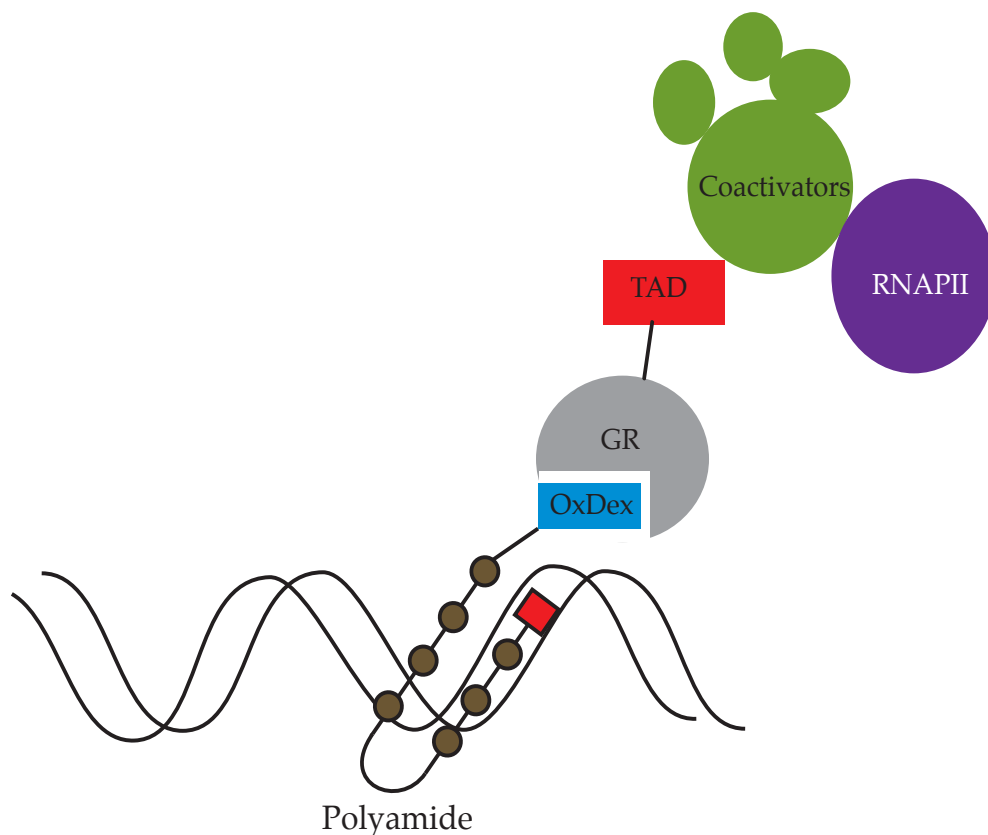


Figure IV-7. Polyamide-OxDex conjugate can localize the transcriptional machinery to DNA through a three hybrid system. The brown circles represent pyrrole monomers (Py) and the red squares represent imidazole monomers (Im).

To test the second-generation polyamide **IV-21** we used the commercially available pG5Luc plasmid which contained five Gal4 binding sites upstream of a TATA box and luciferase reporter gene. Jonas Højfeldt has incorporated the plasmid into a HeLa cell line and can thus we can test for activity in a native chromatin environment. Unfortunately the second-generation free polyamide was cytotoxic at

5 μM and thus limited testing at higher concentrations. While this polyamide is more specific than polyamide **IV-6**, it might lack the specificity to support high levels of transcriptional activation. Jonas Højfeldt tested this idea and attempted to repress Gal4-VP16 activation with this polyamide. Unfortunately, no attenuation in activity was observed in the concentration range tested; however, ten Gal4-VP16 fusion proteins are present at the promoter and they bind tightly to this recognition sequence. A recent report indicates that 5 μM or higher of the polyamide must be used to see transcriptional repression at the GR regulated genes. Thus it seems likely that this construct may not be suitable for transcriptional activation because the polyamide is cytotoxic at the concentrations necessary for activity. Our future studies will aim at other attachment sites on the polyamide. Previous studies in vitro show that attachment at the sixth monomer (**Figure IV-8**) provides optimal activity.^{16, 34} This would be an ideal site for attachment for our small molecule TADs. Through the testing of different attachment sites, longer linkers, and different small molecule TADs, an activator ATF can be synthesized which will activate transcription in cells. Through the testing of different attachment sites, longer linkers, and different small molecule TADs, an activator ATF based on this, or a closely related polyamide, can be synthesized which will activate transcription in cell culture.

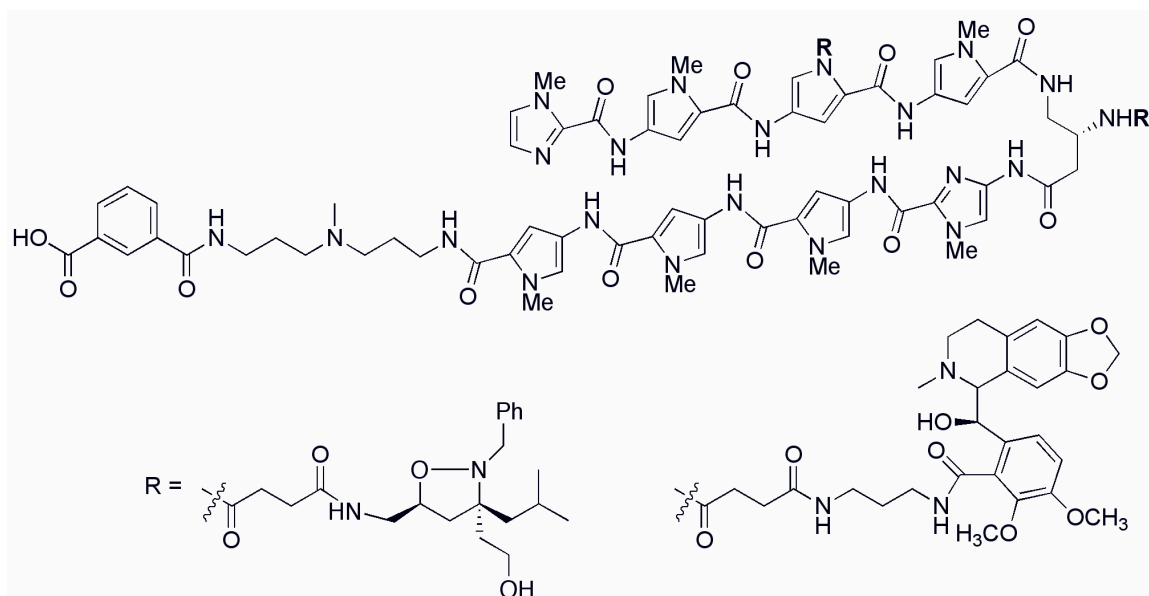


Figure IV-8. Gal4 polyamide with different attachment sites. We propose that attaching a small molecule TAD at the 6th pyrrole ring would be optimal for activity. Additionally, linkers of different lengths may enhance polyamide activation by projecting the TAD further from DNA and thus able to contact the transcriptional machinery.

To test TFO conjugates **IV-19** and **IV-20** for activation, we constructed plasmids that bear the DNA sequences necessary for recognition by the TFO. These sequences were upstream of a TATA box and a luciferase reporter gene. There were five binding sites for the TFO. The activity was measured in HeLa cells and normalized to the TFO alone. As a transfection control, a second plasmid that expressed *Renilla* luciferase was additionally transfected. The assays to evaluate the conjugates activity in cells were evaluated by Jonas Højfeldt .

The TFO conjugate **IV-19** and **IV-20** appeared to repress transcription, as the activity was lower for the conjugate than for the TFO alone. These results were inconclusive and as such the TFO conjugates need to be reconstructed and further analytical methods need to confirm purity and that the TFO correctly binds to the plasmid. Additionally, localizing an activation domain to the TFO through a two-

hybrid system would support that the TFO can support activated transcription. A gel shift to ensure correct triplex formation would validate the TFO correctly binds to the plasmid DNA (**Figure-IV-9**). Additionally, in vitro activity should be tested for the conjugates to limit some of the possible variables associated with in cell assays such as additional genomic binding sites and bioavailability.

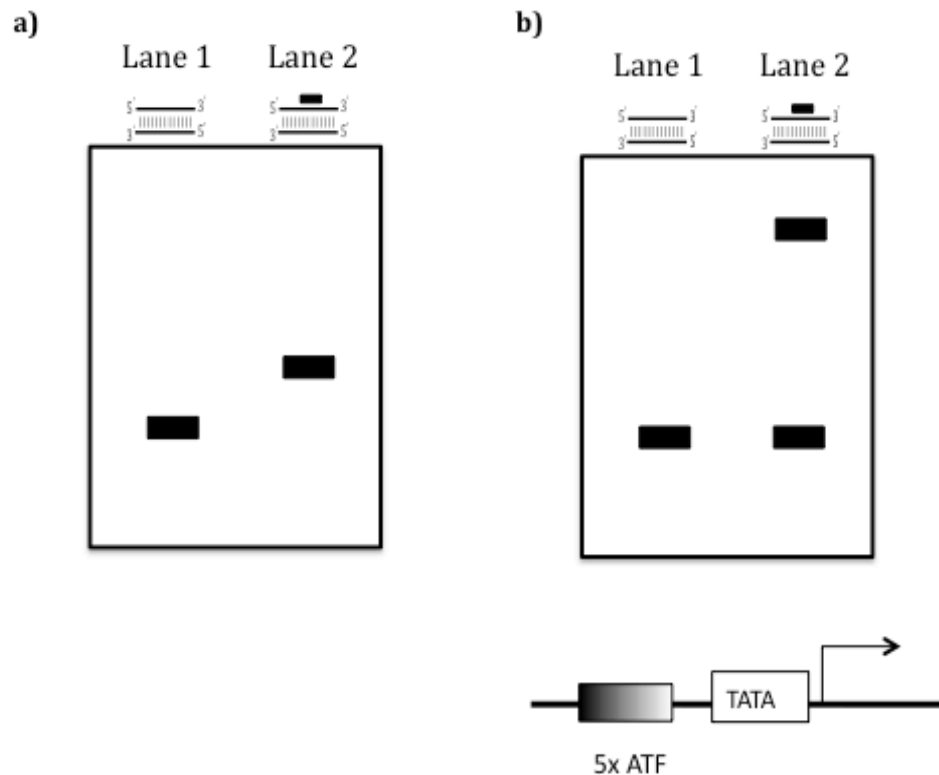


Figure IV-9. Evaluation of TFO based ATF in vitro. a) An electromobility shift assay (EMSA) is used to determine if the TFO binds the correct sequence. DNA with 5 binding sites for the ATF and flanking regions is synthesized with ^{32}P . Lane 1 is this sequence alone (black band). Upon introduction of the TFO (lane 2), the DNA is shifted up the gel. b) An in vitro transcriptional activity assay. DNA with 5 binding sites for the ATF upstream of a TATA box and a sequence of DNA is synthesized with ^{32}P . Addition of the ATF, purified transcriptional machinery, and ^{32}P nucleotides produces transcript (top band) which can be measured by a phosphorimager.

While it was disappointing that none of the conjugates supported activated transcription, valuable lessons were learned. A pre-screening of DBDs through a two hybrid system is a must to ensure proper DNA binding and support of activated transcription. Even if a DBD can repress transcription by blocking a protein-DNA interaction, it could do so by changing conformation of DNA which prevents the binding of necessary transcription factors. Crystallographic evidence suggests that DNA is oriented in a specific conformation during binding of the general transcription factors. To circumvent some of these problems, we decided to directly target nuclear receptors which can activate transcription through ligand-gated activation domains.

E. Activation of Endogenous GR Genes

Glucocorticoid receptor has utility in biology as a model system for nuclear receptors and GR is one of the most common therapeutic targets.^{45, 46} Nuclear receptors such consists of a ligand gated TAD (AF-2) and a non-ligand gated TAD (AF-1) (**Figure IV-10**).⁴⁶ These features allow GR to carry out complex function in cells. The development of molecules that help elucidate the mechanism of and be successful agonists or antagonists for the GR would be tremendously useful. GR can act as a repressor or activator of transcription depending surrounding regulatory elements.⁴⁵ For example, glucocorticoids are used as an immunosuppressant since it can inhibit the expression of pro-inflammatory genes such as IL-6 and other interleukins.^{47, 48} However, some genes such as glucose-6-phosphate are activated in the presence of these ligands and increase gluconeogenesis leading to hyperglycemia.^{49, 50} This prevents diabetics from receiving GR based therapeutics. A

small molecule TAD conjugated to a ligand for GR may be able to influence the activation of these genes differently than a synthetic GR ligand. Mechanistically, GR therapeutic that activation or repression transcription does not do so uniformly across all GR regulated genes.⁵¹ A small molecule can be appended with other functional groups to elucidate the direct targets of GR and may lead to a better understanding of what controls cofactors are involved in repression and activation.

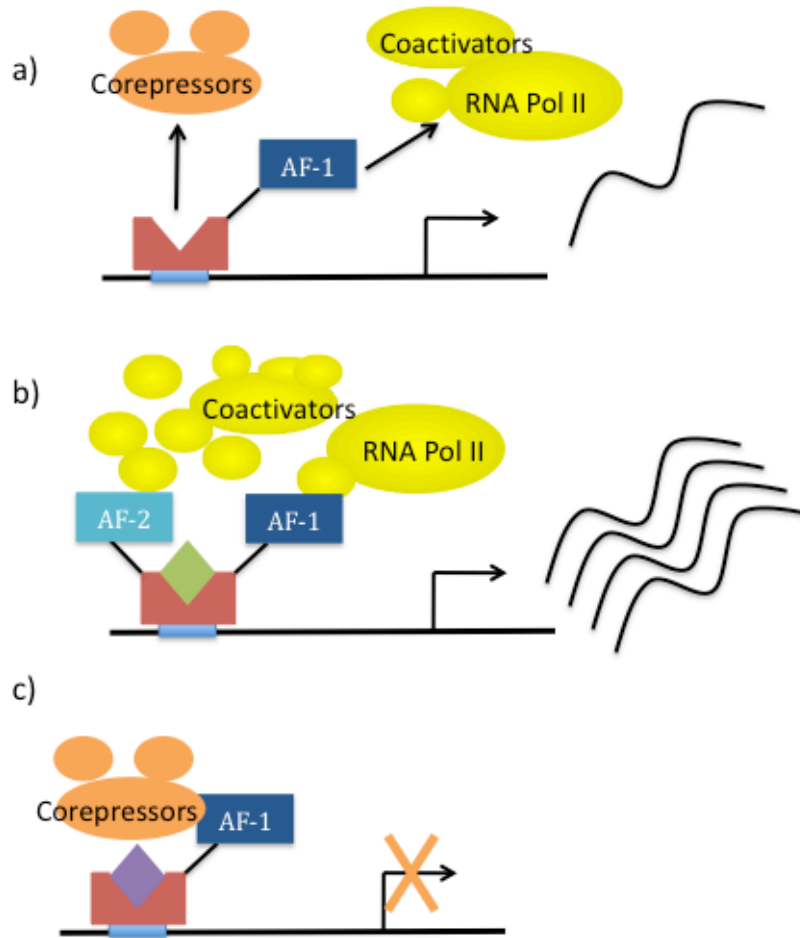
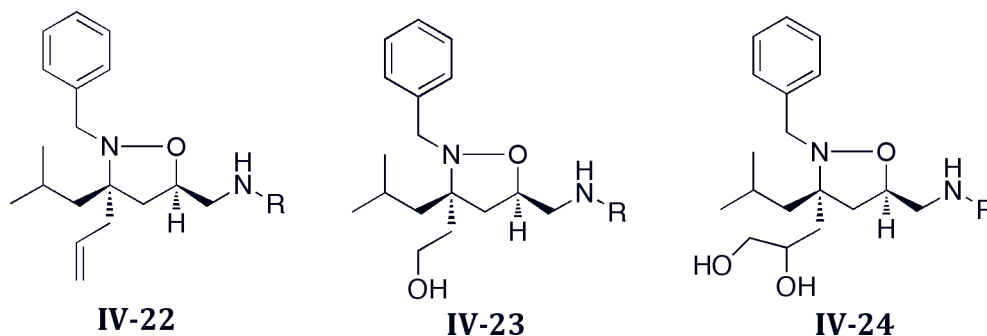
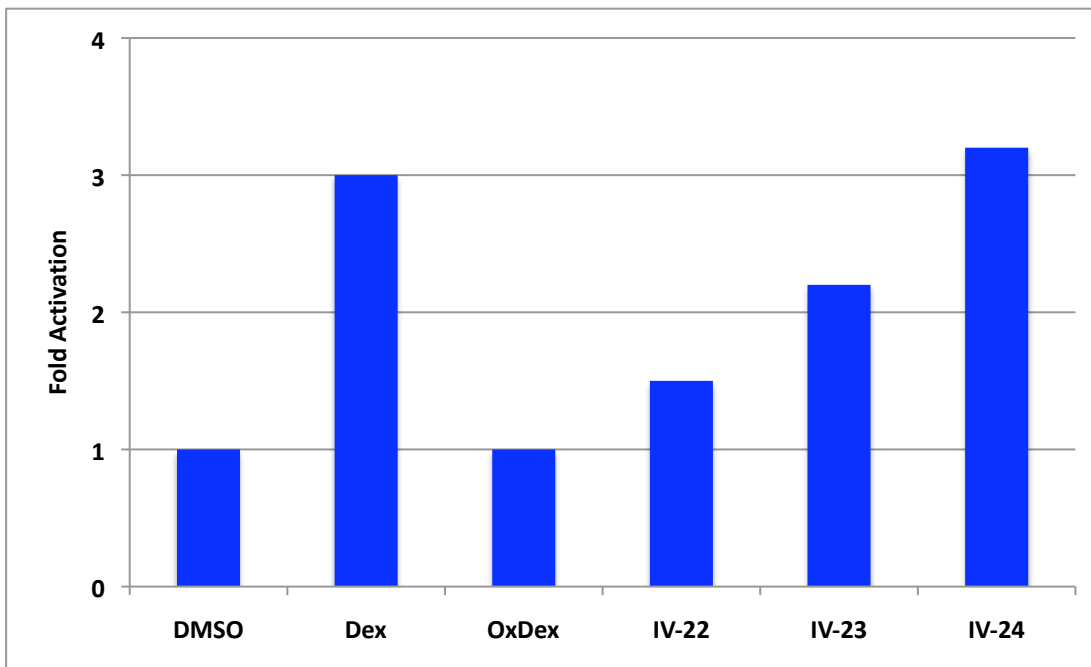


Figure IV-10. Nuclear receptors respond to ligands differently. a) In the absence of a ligand, the nuclear receptor (red box) binds DNA (black line) at regulator elements (blue square) and can either activate transcription weakly through contacts between its ligand independent transcription activation domain AF-1 (navy) and coactivator proteins (yellow) or repress transcription by contact with corepressors. b) In response to an agonist (green diamond), the ligand-gated AF-2 (cyan) interacts with coactivator proteins and synergizes to activate transcription. c) In the case of an antagonist (purple diamond), corepressors localize to the nuclear receptor and repress transcripton. Additionally, the antagonist prevents agonist binding.

Since the small molecule isoxazolidines used GR ligand binding domain to localize to DNA, we collaborated with Prof. Jiandie Lin to test their ability to activate transcription at a GR regulated gene (**Figure IV-11**). As mentioned in the before,

this is a surmountable challenge that needs the localization of many different complexes likely including the SWI-SNF chromatin remodeling complex, mediator, and the elongator complexes as well as individual enzymes including histone methyl transferases (HMTs), histone acetyl transferases (HATs).^{52, 53} Additionally, these complexes must be temporally recruited.⁵⁴ By qPCR, Dr. Lin was able to see activated transcription by our amphipathic isoxazolidine (using a concentration of 1 μ m) to comparable levels a potent therapeutic ligand dexamethasone.



R = AEEA-OxDex

Figure IV-11. Activation of transcription of a GR regulated gene. Dex and OxDex are abbreviations for dexamethasone and oxidized dexamethasone. The amphipathic isoxazolidine (**IV-23**) and diol (**IV-24**) activates nearly as well as a synthetic ligand for GR Dex but significantly better than OxDex. The hydrophobic isoxazolidine (**IV-22**) only weakly activated transcription.

Similar to our earlier results, the hydrophobic isoxazolidine did not activate transcription. Further investigation into this system needs to be conducted to define the isoxazolidines mechanism of action at GR regulated genes. One possibility is the conjugate is acting independently from ligand gated activation domain of GR and

regulating transcription through direct contacts with the transcriptional machinery. Another possibility is that the conjugate is regulating transcription similar to dexamethasone by binding GR and regulating the ligand gated TAD of GR which in turn interacts with the transcriptional machinery. One necessary experiment would be to mutate the ligand gated TAD such that dexamethasone can no longer activate transcription. If the conjugate still activates transcription then it is unlikely the two molecules are regulating transcription by a similar mechanism.

This illustrates the isoxazolidines utility in a biological system as a potential tool to study activated transcription. Current lab member Caleb Bates is now collaborating with Prof. Jorge Iniguez to determine the affects of the isoxazolidines and other small molecules at native GR regulated genes as well as a tool to decipher what coactivators directly bind to GR.

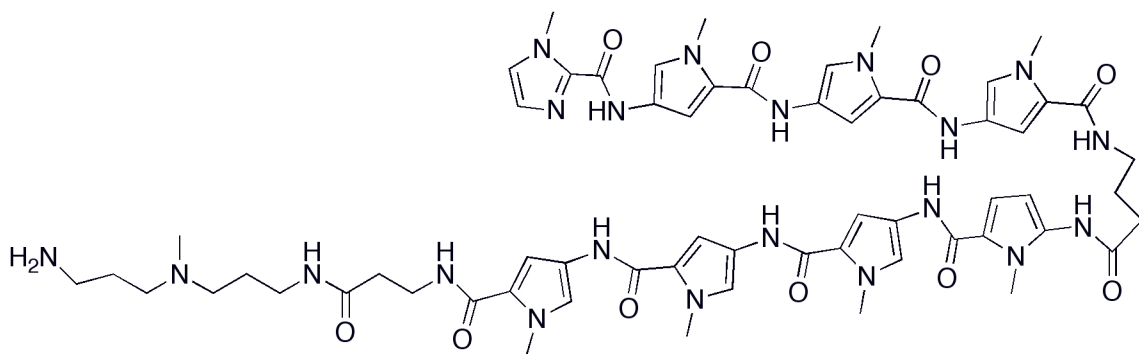
F. Conclusions

A facile synthetic approach was used to generate small molecule TADs and non-peptidic DBDs. By linking the two domains together, we generated activator ATFs and tested them for activity in HeLa cells. We did not observe activation with multiple conjugates. It is expected to see a decrease in activity when assembling an activator ATF. The Kodadek lab's peptoid saw a decrease of almost 300 fold when switching from a two-hybrid system to an activator ATF. It was believed that this decrease in activity may reflect the high molecular weight of the peptoid. If our isoxazolidine TAD experienced a similar loss in activity it would not be measurable by the assay employed. Nevertheless, we predicted that our molecular weight might compensate for some of the problems associated with the first generation

polyamide. However, we did not observe detectable activity in cell culture. We are confident that our experiments with the Dervan lab will provide an activate molecule and will be an enormous step forward in the development of biological tools and potential therapeutics.

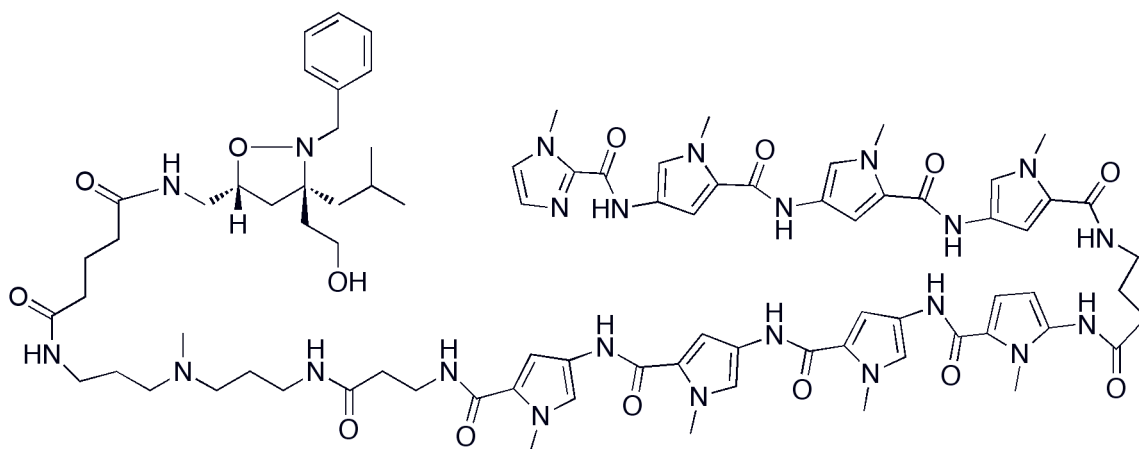
G. Experimental

Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. CH₂Cl₂ and THF were dried by passage through activated alumina columns. All reactions were performed under a dry N₂ atmosphere unless otherwise specified. Et₃N was distilled from CaH₂. The TFOs were purchased from IDT (CYXCTTGTGGTGGGTGGGGTGTGGGT, where X represents the Spacer Phosphoramidite 18 and Y represents the modified T residue bearing the primary amine group on a short tether (Amino-Modifier C2-dT)). Purification by flash chromatography was carried out with E. Merck Silica Gel 60 (230-400 mesh). ¹H and ¹³C NMR spectra were recording in CDCl₃ at 400 MHz and 100 MHz, respectively, unless otherwise specified. IR spectra were measure as thin films on NaCl plates. Reverse-phase HPLC purifications were performed on a Varian ProStar 210 equipped with Rainin Dynamax UV-D II detector ($\lambda = 254$ nm and $\lambda = 304$ for the polyamides and $\lambda = 260$ nm and $\lambda = 214$ for the TFO) using a C18 (8 x 100mm) Radial-Pak™ cartridge with a gradient of 0.1% TFA in H₂O and CH₃CN as the mobile phase.



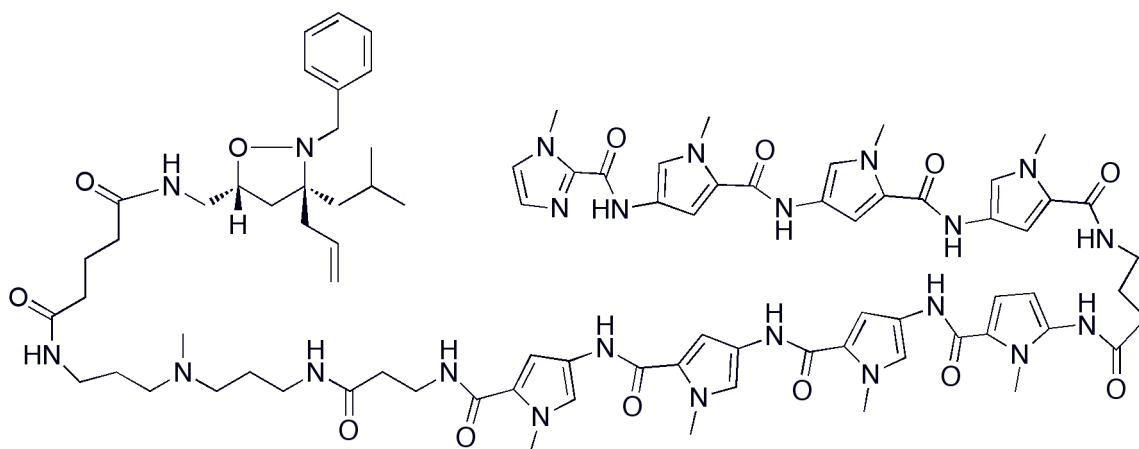
IV-15

Polyamide IV-15: The polyamide monomers were synthesized according to Ref. . The polyamide was synthesized according to Ref. . To monitor each coupling, 5 mg of resin was removed and cleaved accord to Ref. . The product was dissolved 0.5 mL of .1% TFA and .5 mL CH₃CN. The resulting solution was filtered through a .2 μm syringe filter and analyzed by HPLC using a 0-70% CH₃CN gradient over 20 minutes. After each coupling or deprotection a significant shift of at least 1 minute was observed. The final product was seperated into 200 nmol aliquots and stored at -80°C.



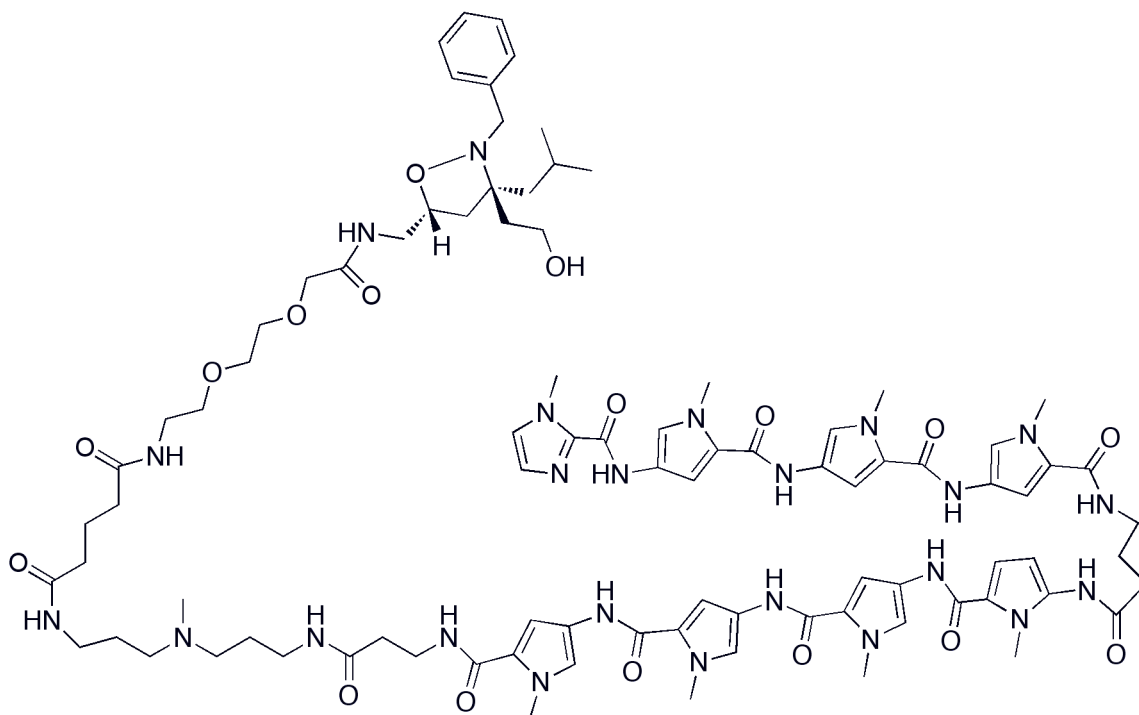
IV-6

Polyamide IV-6 To a solution of isoxazolidine **IV-16** (5.0 mg, 0.017 mmol, 1.0 eq) in DMF (170 μ L) was added disuccinimidyl glutarate (DSG) (14 mg, 0.043 mmol, 2.5 eq) and N,N-diisopropylethylamine (6.0 μ L, 0.034 mmol, 2.0 eq) was added and the mixture stirred for 8 h. The reaction mixture was then diluted with water (5 mL) and poured into a biphasic mixture of EtOAc (15 mL) and water (15 mL). The organic layer was separated and the aqueous layer was extracted with EtOAc (2 x 15 mL). The combined organic extracts dried over Na_2SO_4 , filtered, and concentrated *in vacuo* to a yellow oil. Purification by reverse phase HPLC provided 7.5 mg (88% yield) as a viscous oil after lyophilization. To a portion of the oil (1.0 mg, 2.0 μ mol, 10 eq) in DMF (10 μ L) was added polyamide **IV-15** (.25 mg, .20 μ mol, 1.0 eq) dissolved in DMF (10 μ L) and N,N-diisopropylethylamine (1.0 μ L, 5.7 μ mol, 28 eq). The reaction mixture was stirred for 7 h at which time the reaction was judged complete by ESI-MS analysis. The reaction mixture was diluted with .5 mL .1% TFA and .5mL CH_3CN and purified by reverse phase HPLC to yield 127 nm (64% yield) of product **IV-6**, calculated by UV-vis (UV $\lambda_{\text{MAX}}(\text{H}_2\text{O})$ (ϵ) , 234 (39 300), 304 nm (52 000)), as a yellow powder. ESI-MS calcd for $[\text{C}_{83}\text{H}_{109}\text{N}_{23}\text{O}_1 + \text{H}]^+$: 1652.9, found: 1652.5.



IV-7

Polyamide IV-7: Preparation of **IV-7** was accomplished under conditions identical to those used for **IV-6** starting with 0.016 mmol of **IV-17**. Purification by reverse phase HPLC to yield 124 nm (62% yield) of product **IV-7**, calculated by UV-vis (UV $\lambda_{\text{MAX}}(\text{H}_2\text{O})$ (ϵ), 234 (39 300), 304 nm (52 000)), as a yellow powder. ESI-MS calcd for $[\text{C}_{83}\text{H}_{109}\text{N}_{23}\text{O}_1 + \text{H}]^+$: 1648.8, found: 1649.1.



IV-18

Polyamide IV-18: To a solution of Fmoc-AEEA (52 mg, .14 mmol, 3 eq) dissolved in NMP (.25 mL) were added HOBT (18 mg, .14 mmol, 3 eq) and HBTU (51 mg, .14 mmol, 3 eq). This solution was agitated for 15 min. The solution of activated Fmoc-AEEA was added to amine **IV-16** (13 mg, .045 mmol, 1 eq) dissolved in NMP (.25 mL) and the resulting mixture was allowed to stir for 12 h at which time the reaction was complete as judged by ESI-MS analysis. Excess reagents were quenched by addition of 1N HCl (10 mL) and EtOAc (10 mL). The reaction vessel was washed with EtOAc (2 x 2 mL) to remove all residues. The resulting biphasic mixture was separated and the aqueous layer was extracted with EtOAc (3 x 10 mL). The combined organic fractions were dried over Na₂SO₄, filtered, and concentrated *in vacuo*. The resulting oil was dissolved in a solution of 20% piperidine in DMF

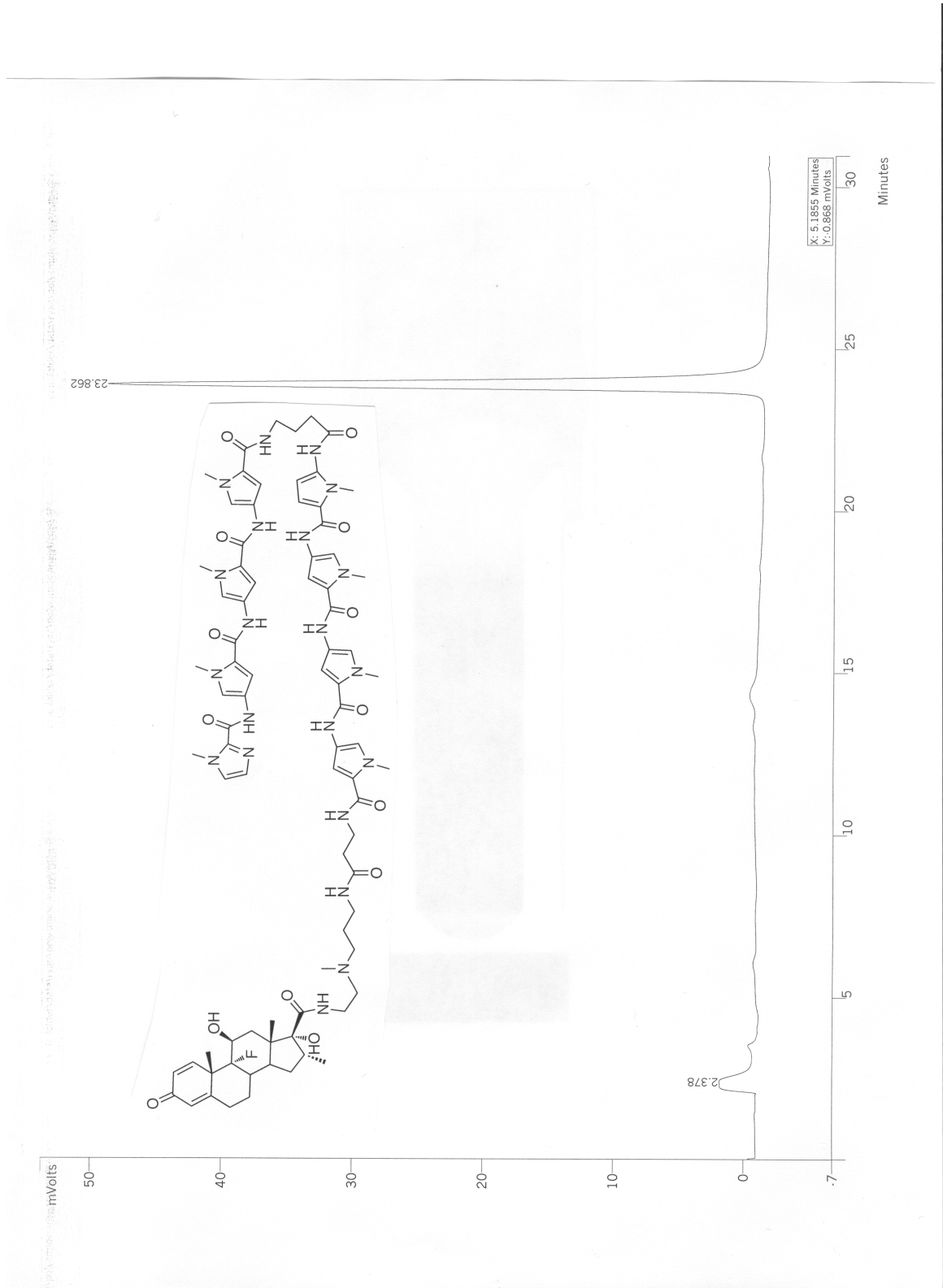
(.110 mL, .225 mmol piperidine w/v, 5 eq) and was allowed to stir for 30 minutes. The resulting solution was diluted with 1.5 mL .1% TFA and 1.5 mL CH₃CN. Reverse-phase HPLC purification provided the product as an oil (52% yield; 6.0 mg). To a solution of the amine (6.0 mg, 0.014 mmol, 1.0 eq) in DMF (140 μL) was added disuccinimidyl glutarate (DSG) (11 mg, 0.043 mmol, 2.5 eq) and N,N-diisopropylethylamine (5.0 μL, 0.027 mmol, 2.0 eq) was added and the mixture stirred for 8 h. The reaction mixture was then diluted with water (3 mL) and poured into a biphasic mixture of ethyl acetate (15 mL) and water (15 mL). The organic layer was separated and the aqueous layer was extracted with ethyl acetate (2 x 15 mL). The combined organic extracts dried over Na₂SO₄, filtered, and concentrated *in vacuo* to a yellow oil. Purification by reverse phase HPLC provided 6.0 mg (85% yield) as a viscous oil after lyophilization. To a portion of the oil (1.0 mg, 2.0 μmol, 10 eq) in DMF (10 μL) was added polyamide **IV-15** (.25 mg, .20 μmol, 1.0 eq) dissolved in DMF (10 μL) and N,N-diisopropylethylamine (1.0 μL, 5.7 μmol, 28 eq). The reaction mixture was stirred for 7 h at which time the reaction was judged complete by ESI-MS analysis. The reaction mixture was diluted with .5 mL .1% TFA and .5mL CH₃CN and purified by reverse phase HPLC to yield 127 nm (45% yield) of product **IV-18**, calculated by UV-vis (UV λ_{MAX}(H₂O) (ε) , 234 (39 300), 304 nm (52 000)), as a yellow powder. ESI-MS calc for [C₈₉H₁₂₀N₂₄O₁₇ +H]⁺ = 1797.9, found = 1797.7.

Synthesis of TFOs IV-19 and IV-20: Preparation of the conjugates was accomplished by following invitrogen's guide for amine reactive probes sections 3.1-3.5. Briefly, dissolve 0.1 mg of oligonucleotide in 100 μL dH_2O and extract three times with an equal volume of chloroform. Precipitate the oligonucleotide by adding one-tenth volume (10 μL) of 3 M NaCl and two and a half volumes 250 μL of cold absolute ethanol. Mix and place at -20°C for 30 minutes. Centrifuge the solution in a microcentrifuge at 14,000 g for 30 minutes. Carefully remove the supernatant, rinse the pellet twice with cold 70% ethanol and dry under vacuum. Dissolve the dry pellet in dH_2O to achieve a final concentration of 25 $\mu\text{g}/\mu\text{L}$. This amine modified oligonucleotide stock solution may be stored frozen at -20°C .

Make a 0.1 M sodium tetraborate buffer by dissolving 0.038 g of sodium tetraborate decahydrate in 1 mL of water. Adjust pH with HCl to 8.5. this labeling buffer should be made as close as possible to the time of labeling. Alternatively, it may be divided into small aliquots and frozen immediately for long-term storage.

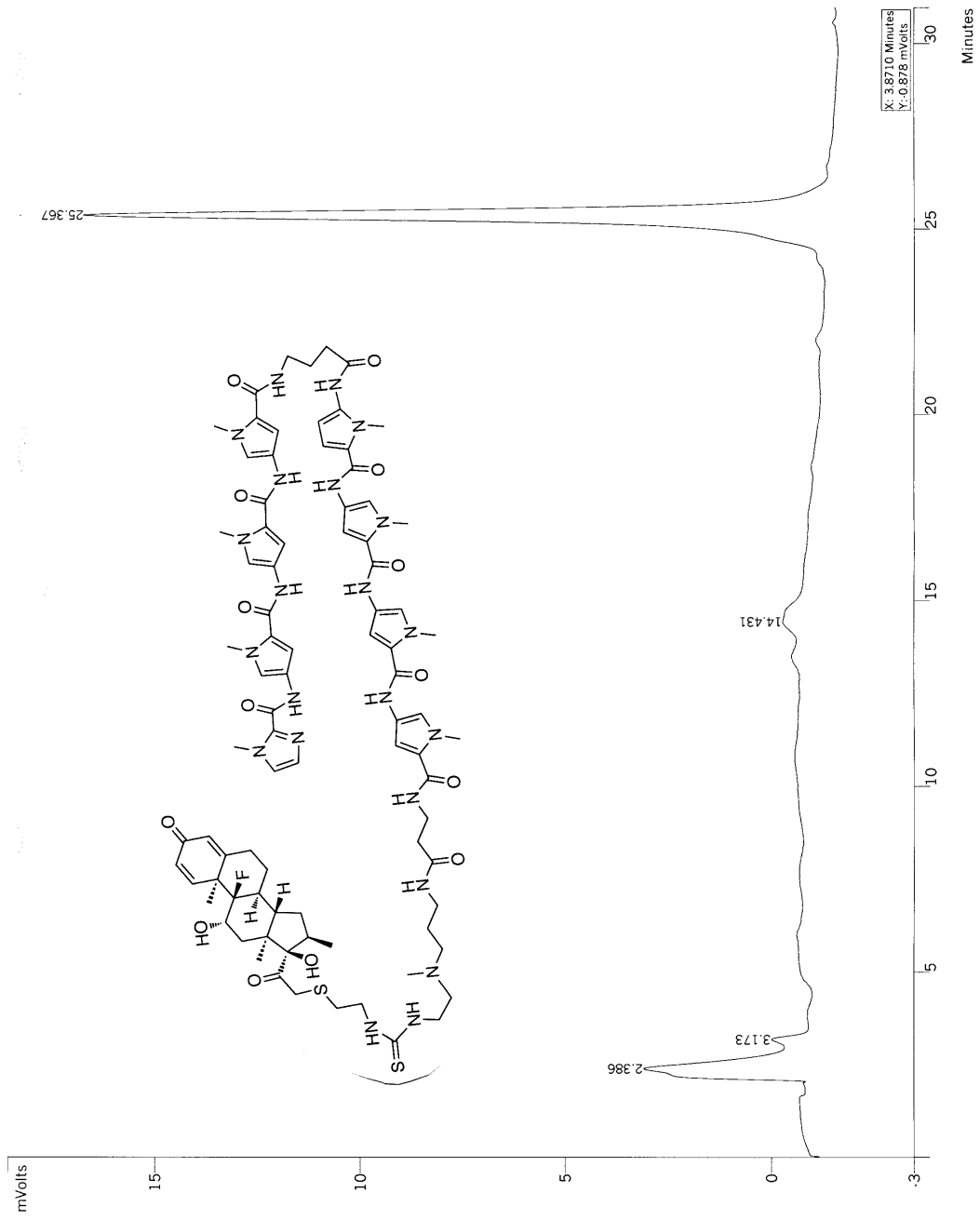
Prepare the succinimidyl ester as described **IV-6** and **IV-7**. Dissolve the 250 mg of the either succinimide in 14 μL of DMSO in a microcentrifuge tube. To this tube add 7 μL of dH_2O , 75 μL of 0.1 M sodium tetraborate buffer, and 4 μL of a 25 $\mu\text{g}/\mu\text{L}$ oligonucleotide stock solution. The reaction mixture will have a grainy appearance, but this will not adversely affect the conjugation. Do not attempt to improve the solubility of the label, because modifying the composition of the mixture can drastically reduce the labeling efficiency. Incubate the reaction for 6 hours to overnight. Agitate the vial every 30 minutes for the first 2 hours.

To purify the conjugate, ethanol precipitate and wash as described above. Dissolve the pellet in 0.1 M TEAA (triethylammonium acetate) made fresh or purchased (0.1 M TEAA buffer made in the lab does not store). Load the dissolved pellet onto the column in 0.1 M TEAA and run a linear 5-65% CH₃CN gradient over 30 minutes. The oligonucleotide will migrate fastest, followed by the conjugate, and finally the succinimide. The oligonucleotide and conjugate will have strong absorption at $\lambda = 260$ nm.



X: 5.1855 Minutes
Y: 0.868 mVolts

Minutes



X: 3.6710 Minutes
Y: -0.878 mVolts

Minutes

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Chapter V

Conclusions and Future Directions

A. Conclusions

My thesis work focused on the successful design, synthesis, and evaluation of small molecule transcriptional activation domains.^{1,2} At the beginning of my PhD work there was a single example of a small molecule transcriptional activation domain (TAD) shown to activate transcription in a cell-free assay.³ In collaboration with several colleagues we showed that small molecule isoxazolidine, **TAD V-1**, also up-regulates transcription in cells.² Further work elucidated some guidelines to design future small molecule TADs. Additionally, using these design criteria, two natural products were selected and could be localized to DNA to activate transcription.¹ Lastly, the isoxazolidine was attached to a synthetic DNA binding domain (DBD) to create a non-peptidic activator artificial transcription factor (ATF). **Figure V-1** lists a sampling of these molecules.

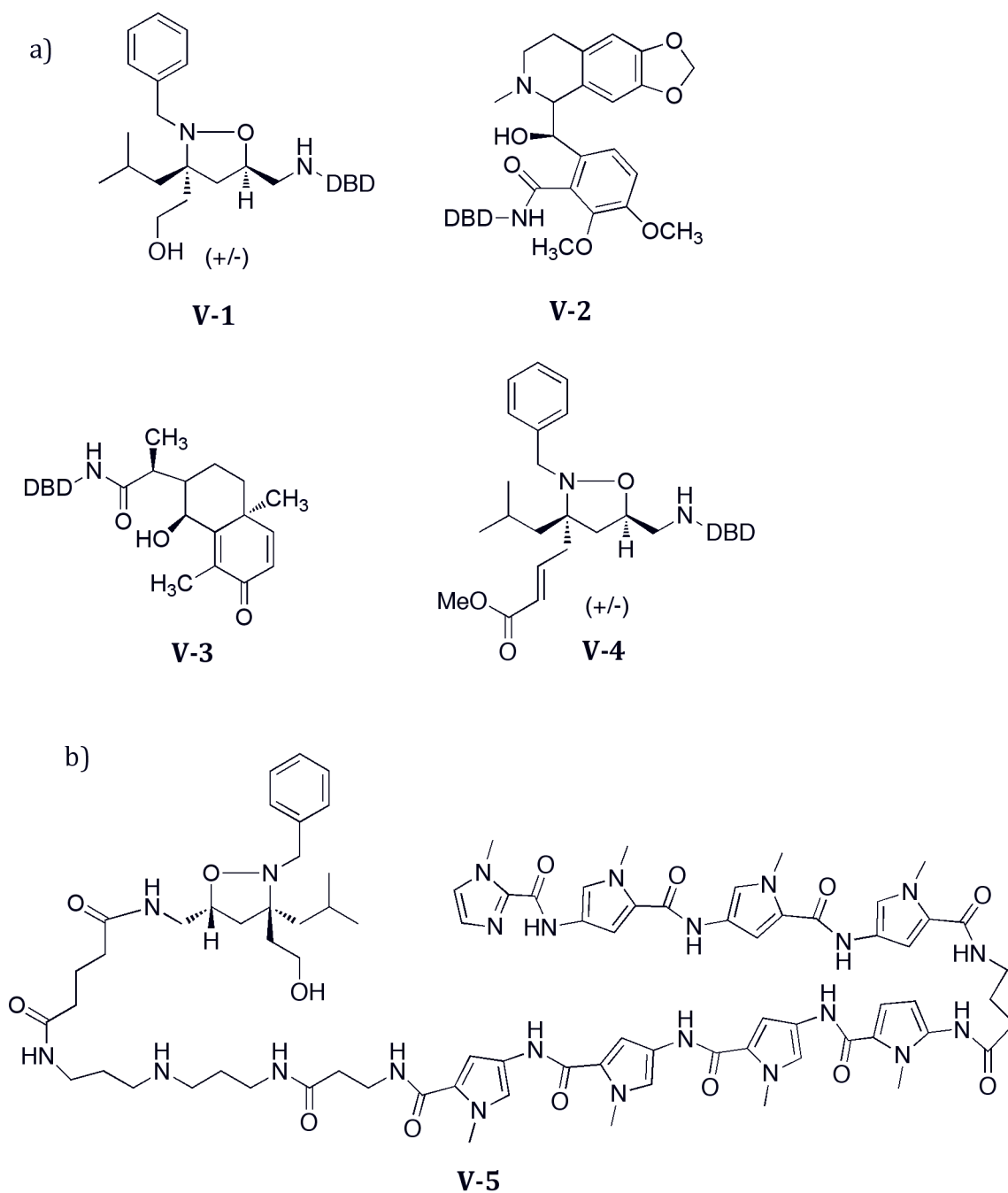


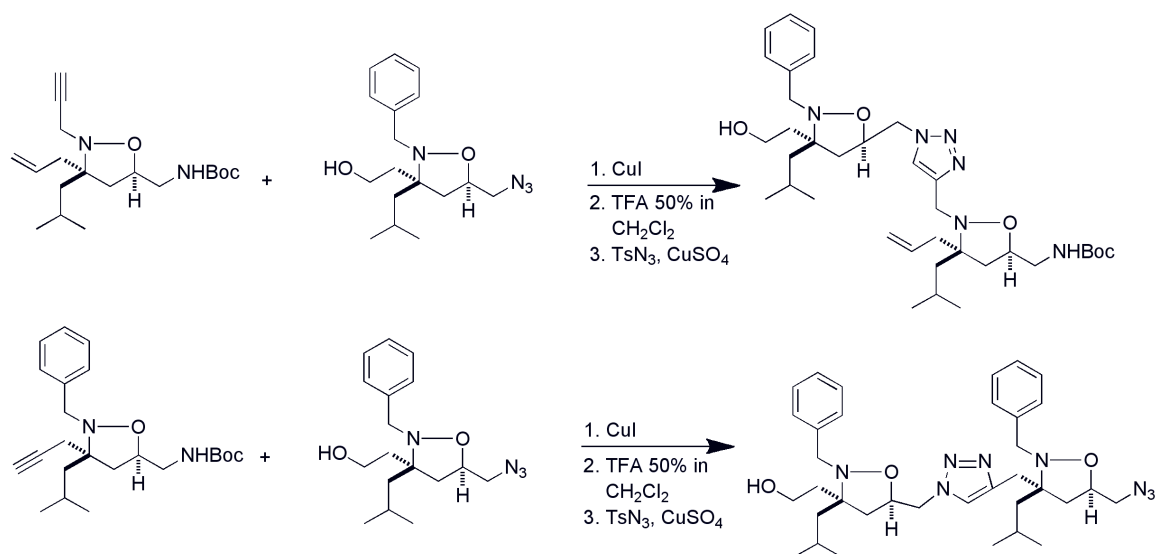
Figure V-1. Many compounds can function as TADs. a) A sampling of small molecule TADs. b) The construction of an activator ATF.

B. Future Directions:

1) Oligomers of Small Molecule TADs

TAD binding sites within coactivators appear to be somewhat permissive, not

requiring a single orientation of particular functional groups on the TAD.⁴⁻⁸ In Chapter 3 it was shown that analogous of **V-1** activated transcription and based on a focused library, some guidelines were designed such as a logP between 2.5-3.1 and a rigid scaffold which projected functional groups in three dimensions.¹ An important implication of these experiments is that isoxazolidines are unlikely to be the only suitable scaffold for the construction of small molecule TADs, but rather a variety of conformationally constrained scaffolds should function as TADs. To test these guidelines we synthesized TADs based on natural products santonin and hydrastine. Additionally, bis-isoxazolidines that met these criteria activated transcription, similar to natural activators where multimers of TADs are typically potent activators of transcription.⁴ One advantage bis-isoxazolidines have over isoxazolidines is an ability to target a large surface area and accordingly make more critical contacts with the transcriptional machinery. While the bis-isoxazolidines represent an interesting class of compounds, their synthesis is linear which results in a long synthesis with a low overall yield. To generate isoxazolidine multimers capable of targeting a large protein surface, triazoles isoxazolidines were synthesized and tested for activity. Triazoles were chosen to link isoxazolidines for



Scheme V-I. Isoxazolidines can be oligomerized by an iterative process using of a triazole linkage.

a number of reasons.⁹ First, the reaction to form a triazole is chemoselective and tolerant of many functional groups. Secondly, it has been suggested the triazoles are isosteres of amide bonds and have a similar dipole moment and water solubility.⁹ **Scheme V-1** shows the general scheme to synthesize oligomers. While initial compounds did not activate transcription, the logP of these conjugates falls outside of the logP range of our active analogues and as such more polar functional groups should be incorporated into the isoxazolidine. In one example, researchers “bundle” TADs to a single DBD and by doing so activate transcription more potently than when the TADs are connected linearly (**Figure V-I**).¹⁰ Using triazoles, one could also generate a “bundling” of small molecule TADs, similar to a dendrimer. Figure V-II illustrates this strategy and an example. These strategies could lead to more potent transcriptional activators.

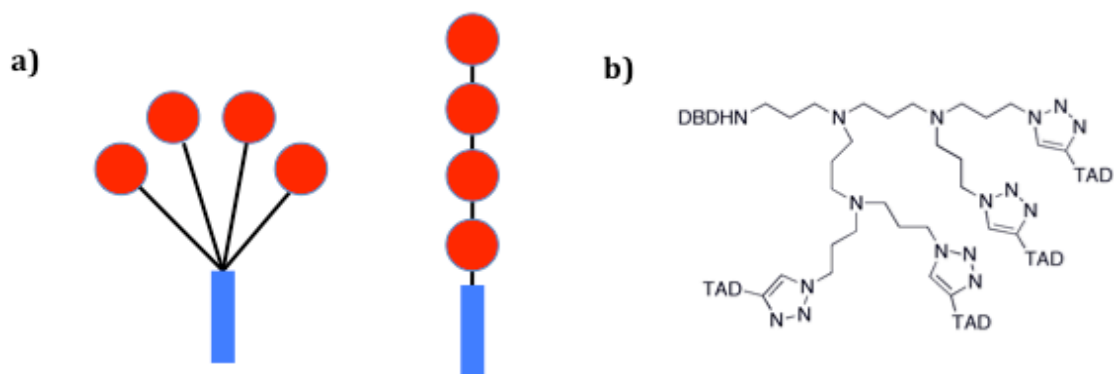


Figure V-II. Strategies to multimerize TADs. a) TADs (red) are either directly linked separated by a short linker (right) and connected to the DBD (blue) or “bundled” through connection to a DBD by individual linkers (left).¹⁰ b) Small molecule TADs bearing an alkyne can be linked to an azide containing scaffold.

2) Quantitation of Cell Permeability

In chapter II, the hydrophobic isoxazolidine was not active in cell culture and the amphipathic activator was highly potent.² To support this observation, the hydrophobic isoxazolidine does not bind the KIX domain of CBP by NMR or FP.⁴ While the KIX domain of CBP is an important target for activators, it is unlikely the sole target of the isoxazolidine as evidence by *in vitro* cross-linking (CAB, RJC, AKM unpublished results). In fact, western blot supports that hydrophobic isoxazolidine and amphipathic isoxazolidines likely share targets. It is then possible that some of the difference in activity may be from a difference in cell permeability or solubility.¹¹

There are many ways to measure cell permeability such as HPLC assays, fluorescence, and radioactivity.¹²⁻¹⁵ In HPLC assays, a compound is incubated in cell culture followed by multiple washings.¹² The cells are extracted with methanol and the extract is run on an HPLC to determine the quantity of compound recovered (**Figure V-**

II). This quantity can be compared to a known “highly permeable” compound.¹⁵ Cell permeability can be looked at by fluorescence by appending a fluorophore to the molecule; however, fluorophores are often larger and may change its bioavailability.¹⁶ One solution to this problem is to use a “pro-fluorophore”.^{14, 17} In this case, a chemical reaction between one of the functional groups of the small molecule and a pro-fluorophore yields a fluorescent molecule. This method is highly powerful as fluorescence microscopy can reveal the intercellular location of a molecule. In collaboration with Will Pomerantz, we made some initial attempts at both of these methods with the isoxazolidines and have not been able to observe permeability of the amphipathic isoxazolidine by either method. Future experiments should focus on improving the solubility of the tetrazole as we could not reproduce previous reports.¹⁸ Another method is radioactivity where a radiolabel must be incorporated into the isoxazolidine. Similar to the HPLC assay, the molecule is incubated with cells and washed. The cells are spun down and the washes are compared to the cell pellet. Radioactivity is highly sensitive meaning this may be the best method to detect small changes in permeability. More straightforward methods include PAMPA (Parallel Artificial Membrane Permeability Assay) and variations where a lipid filled membrane simulates the lipid bilayer of a cell.¹⁹⁻²² These assays can be easily quantified by UV/Vis measurements yet this method of quantification also limits its utility to compounds with extinction coefficients.

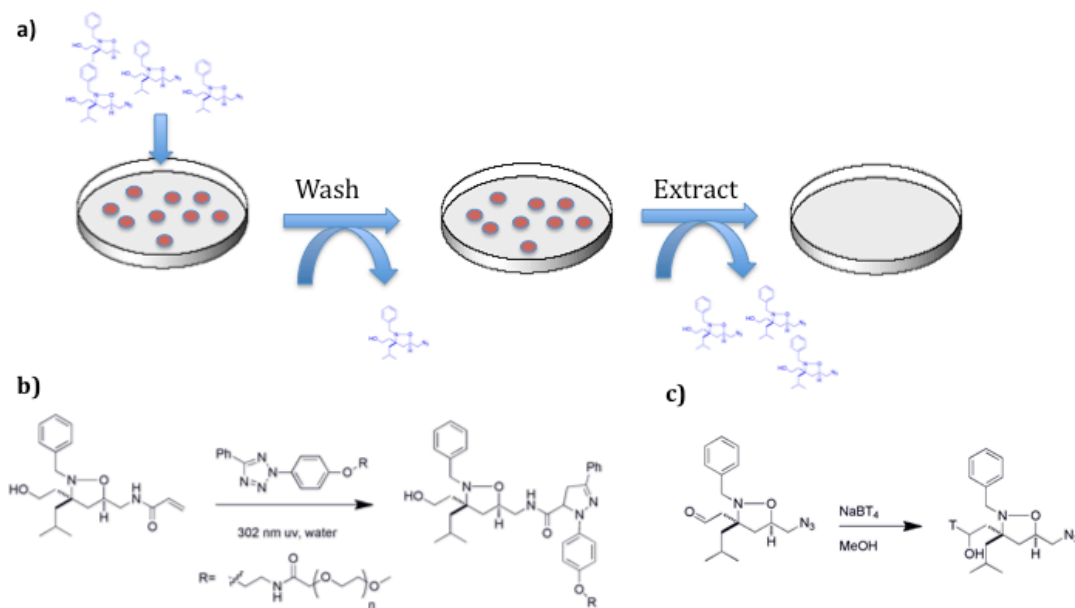


Figure V-III. Methods to detect cell permeability. a) HPLC assay to detect permeability. Molecules are introduced to cells followed by a washing step. Any molecule that doesn't permeate the cells will be washed away. The cells are then extracted and the extracts are quantified by HPLC. b) A method developed by the Lin lab can detect in cell fluorescence. A molecule bearing an alkene is introduced into cells followed by a “pro-fluorophore”. After irradiation at 302nm light, the tetrazole reacts with the alkene to generate a fluorescent molecule. c) Reaction of an aldehyde with NaBT₄ can introduce a tritium into the isoxazolidine making it radioactive.

3) Recruitment of Enzymes to DNA

As discussed in Chapter 4, polyamides bind DNA sequence selectively and as such can localize a small molecule to DNA.^{23, 24} Similar to this idea, researchers have used custom zinc finger proteins to localize specific enzymatic activity to DNA.²⁵ Zinc fingers must be introduced by gene therapy methods. However, an inhibitor of a coactivator, such as the histone acetyl transferase (HAT) CBP, connected to a polyamide could localize CBP to DNA thus activating transcription. The role of the HAT domain of CBP in transcriptional activation is not fully understood but some studies suggest that mutation of this HAT domain does not affect transcription.²⁶ This is similar to an

experiment called an activator bypass in which a coactivator is fused to a DBD. When the coactivator is localized to DNA it activates transcription in the absence of a transcriptional activator.²⁷ One such inhibitor is the lysine-CoA conjugate developed by the Cole lab specifically for CBP.²⁸ It is 1000 times more selective for CBP compared to another HAT PCAF (20 nM for CBP and 20 μ M for PCAF).²⁹ The rationale for this selectivity is not entirely clear as CoA is a cofactor present in HATs. In collaboration with Jonas Hojfeldt, we designed a molecule capable of targeting DNA and potentially activating transcription (**Figure V-III**).

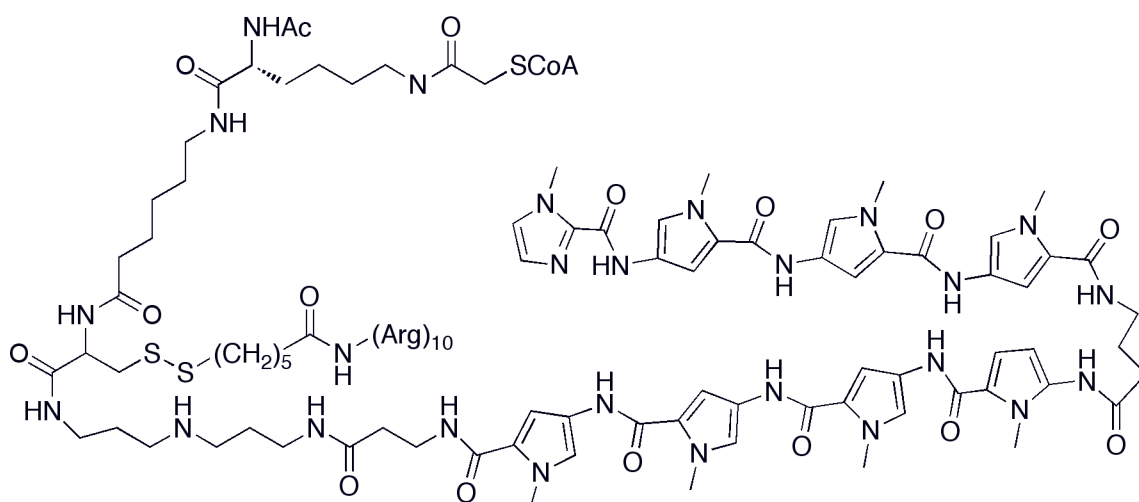


Figure V-IV. Polyamide-Lys-CoA conjugate. A deca-arginine sequence can be added to improve permeability. The Lys-CoA was shown to be selective for the coactivator CBP.

Since the molecule is large, a deca-arginine peptide could be appended through a disulfide bond to aid in cell permeability. The disulfide is cleaved upon entry into the cell. This strategy should be amendable to localize any enzyme to DNA of interest assuming that enzyme has a potent inhibitor.

4) Small Molecule Transcriptional Activators as Tools For Biochemistry

Advances in the field of small molecule transcriptional activators bring us

closer to the construction of a non-peptidic transcriptional activator orthogonal to natural activators.¹¹ An ideal artificial transcriptional activator was defined in Chapter 1 as a molecule that can localize to the nucleus, and upregulate transcription of a specific gene to a prescribed level.

Such tools are highly sought after for the construction of synthetic transcriptional networks and to study the correlation between misregulated transcription and disease.¹¹ The work here demonstrated that small molecules can upregulate transcription in cells by a mode of action that mimics their endogenous counterparts. Synthetic transcriptional activators can now be constructed and pave the way to addressing issues such as delivery and signal responsiveness.

It is important to stress that our guidelines to design highly active small molecule TADs are not translational to peptidic transcriptional activators. Based on our findings that diverse small molecules can act as TADs, it might portray transcription as a loosely regulated network where TADs interact with many proteins at low affinities to activate transcription. Yet this system is tightly regulated and these proteins achieve their specificity in part by masking interactions, intracellular localization, proteolytic degradation, and DNA binding specificity. To further understand this biological process, researchers must elucidate the required binding partners of TADs in cells and understand the function of the coactivators required for transcription.

C. References:

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