Chapter 5

Synthesis and Evaluation of Polyamide-Isoxazolidine Conjugates as Artificial Transcription Factors

Abstract

Pyrrole-Imidazole hairpin polyamides are a cell-permeable class of DNA-binding small molecules that are able to access chromatin and disrupt the transcription factor-DNA binding interface, thereby downregulating endogenous gene expression in human carcinoma cell lines. Conversely, several polyamide-peptide conjugates have been shown to upregulate transcription *in vitro*, with the polyamide mimicking the DNA-binding domain of a transcription factor and the peptide functioning as the transcriptional activation domain (TAD). Recently, isoxazolidine structures have been used as synthetic TADS, and an isoxazolidine-transcription factor conjugate has yielded an 80-fold induction of gene transcription in HeLa cells. Using a small molecule approach, hairpin polyamides may be utilized to replace the transcription factor-DNA binding domain, yielding polyamide-isoxazolidine conjugates for gene activation studies. Solution-phase polyamide synthesis methods have been developed to generate gram-scale quantities of polyamides, enabling the synthesis of several polyamide-isoxazolidine conjugates. These compounds have been studied using various cell-culture assays for their ability to selectively activate transcription. In all of the studies herein, no significant levels of upregulation were observed.

Introduction

Transcriptional regulation is an important strategy for the modulation of cellular signaling pathways, and molecules that can activate or repress transcription are of therapeutic value.[1-2] Pyrrole-imidazole (Py-Im) hairpin polyamides are a class of sequence-specific DNA-binding small molecules with affinities comparable to transcription factors.[3] Previous studies have shown that polyamides readily enter living cells, traffic to the nucleus, and downregulate endogenous gene expression through disruption of the DNA/transcription factor interface.[4-11] Likewise, polyamide-based upregulation of gene expression is also possible, although the process is somewhat more complicated.

Natural transcription factors can bind to either the wider major groove or narrower minor groove of DNA. These binding events can involve hydrogen bonding, electrostatic interactions, or van der Waals interactions with the base pairs or phosphate backbone of DNA. Proteins that bind to DNA, such as leucine zippers and zinc fingers, can bind as monomers, dimers, or in a combinatorial fashion where multiple DNA-binding proteins are required to regulate a certain gene. In addition to the DNA-binding domain (DBD), natural transcription factors generally contain other structural elements for purposes such as ligand-binding and transcriptional activation. In order to create an artificial transcription factor (ATF) to upregulate transcription, it will be essential to include structural motifs that mimic these domains, particularly the DBD and TAD.

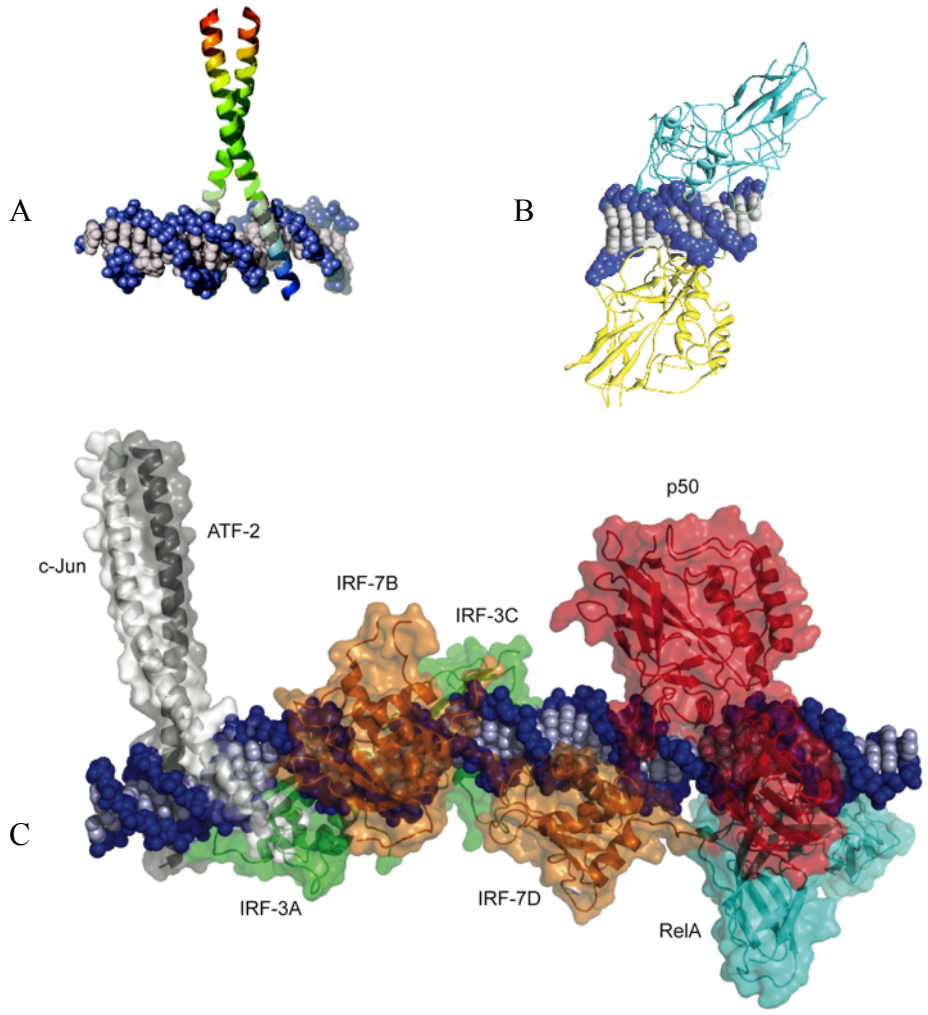

Figure 5.1: Structures of DNA-binding proteins; A) GCN4, a homodimeric leucine zipper; B) NF-κB, a heterodimer of p50/p65; C) Interferon-ß enhanceosome, an example of combinatorial binding.[12]

TADs are structural motifs capable of recruiting and positioning RNA Polymerase II for gene transcription, when localized to specific DNA binding sites. By attaching a TAD to a Py-Im polyamide, it may be possible to selectively activate transcription. In this scenario, the polyamide serves to localize the TAD to a specific sequence in the promoter region of a target gene, thereby directing the assembly of the transcriptional machinery and enabling transcription. In order to allow the activation domain enough conformational flexibility for optimal recruitment of the transcriptional machinery, a stable and flexible linker region is necessary to connect the polyamide to the activation domain.

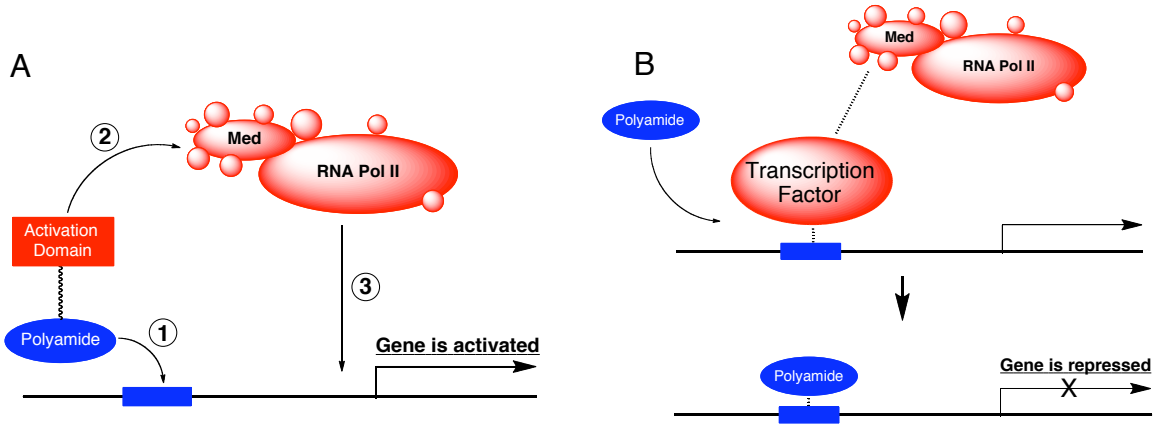

Figure 5.2: Modes of polyamide-based gene regulation. A) Activation: a polyamide attached to a TAD binds to the DNA ①, the TAD recruits the transcriptional machinery ②, which allows assembly of the RNA polymerase II holoenzyme ③, leading to transcription. B) Polyamide can displace natural transcription factors, leading to a downregulation in transcription.

Several polyamide-peptide conjugates have been reported that are capable of upregulating transcription *in vitro*, in which the polyamide mimics the DBD of a transcription factor, and is linked to a peptide known to function as a TAD.[13-15] A polyamide conjugated to a synthetic activation domain, wrenchnolol, was also demonstrated to induce transcription *in vitro*.[16] A lack of efficient cellular uptake, likely due to the large molecular weight of these compounds—which often exceeded 4 kDa—prevented *in vivo* applications.[5,17,18] More recently, a polyamide conjugated to a lower molecular weight synthetic peptoid activation domain showed 5-fold induction of a luciferase reporter in HeLa cells, the first reported ATF that is cell permeable.[19]

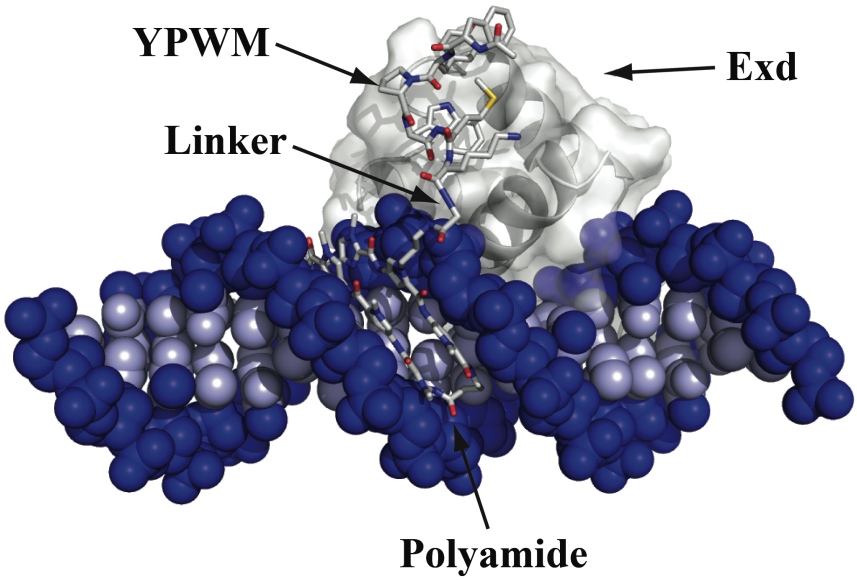

Figure 5.3: An example of a polyamide attached to a peptide activation domain (YPWM) that recruits Exd (a member of the HOX family of transcription factors).

A recent example of a low molecular weight, synthetic TAD is the isoxazolidine structure developed by Mapp and coworkers (Figure 5.4A).[20] The transcriptionally active isoxazolidine derivative contains moieties that mimic amino acids (specifically phenylalanine, leucine, and serine) found in natural transcription factors, and is amphipathic, which has been reported as an important feature in TADs.[1,21]

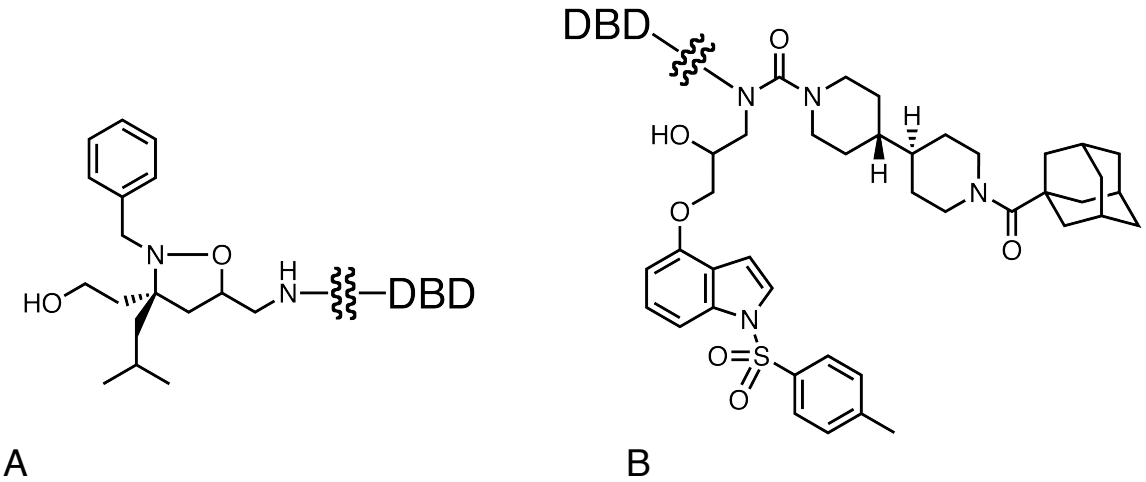

Figure 5.4: Synthetic activation domains. A) Isoxazolidine; B) Wrenchnolol.

Rowe et al. conjugated an isoxazolidine compound to an OxDex steroid—which binds to the glucocorticoid receptor (GR). Gal4, a natural transcription factor, was fused to the functional portion of the GR, which served as the DBD. Transfection of a plasmid containing five Gal4 binding sites upstream of a firefly luciferase reporter gene allowed for the monitoring of transcription *in vivo*.

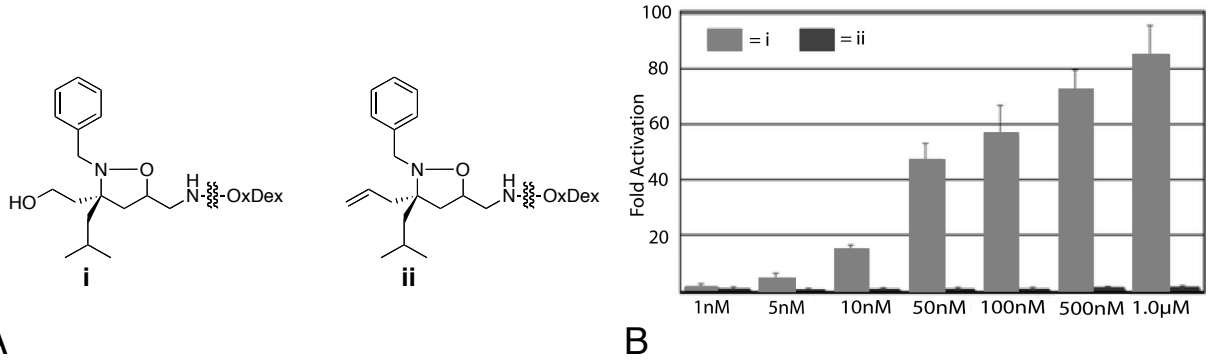

Figure 5.5: a) Structures of active (i) and inactive (ii) isoxazolidine TADs. b) *In vivo* luciferase assay; 80-fold activation at 1.0 μM. Adapted from Rowe et al.

As seen in Figure 5.5, amphipathic **i** containing an alcohol group was found to function as a TAD, whereas hydrophobic **ii** containing an olefin does not. This system utilizing isoxazolidine construct **i** showed an 80-fold induction in HeLa cells over the control construct **ii**.[22] The transcriptional activity of **i** was shown to be comparable to natural peptides used by cells for activation of transcription, making it one of the most active TADs to date. This, combined with its low molecular weight and synthetic accessibility, indicate the great potential for polyamide-isoxazolidine conjugates to function as ATFs.

By directly linking a polyamide to the isoxazolidine TAD, this complicated DBD system can be avoided. The use of a polyamide may be advantageous over DBDs similar to the Gal4/GR/OxDex construct, in that polyamides bind DNA with comparable affinity, and can be programmed to bind many sequences. Therefore, the range of possible targets for

gene activation using polyamides as DBDs is greatly expanded. Py/Im polyamides have also been shown to readily traffic to the nucleus of living cells and modulate endogenous gene expression without the need for lipophilic transfection agents required with other methods.

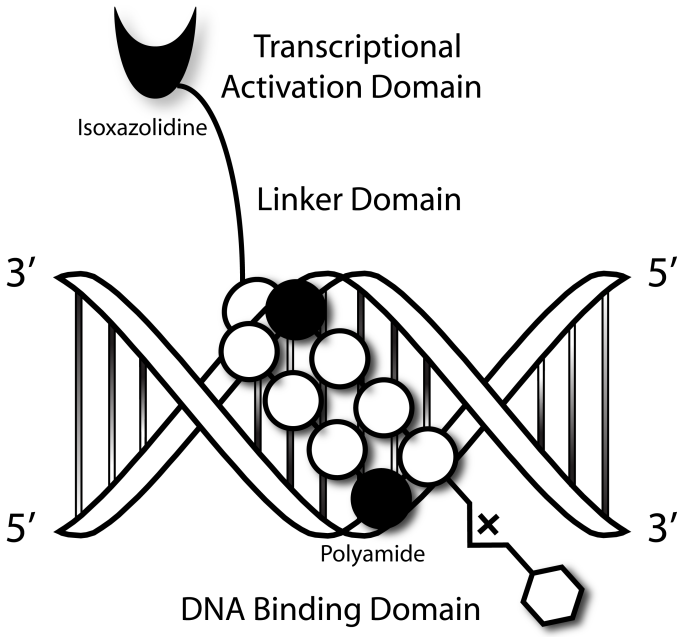

Figure 5.6: General structure of a polyamide-isoxazolidine conjugate, indicating the DBD, linker domain, and TAD.

The polyamide-mediated upregulation of genes has met with limited success in the past, partly due to the small number of molecules that function as general activation domains.[1] In addition, the complication of adding molecular weight to an already large molecule can lead to a decrease in cellular uptake, thereby obstructing *in vivo* applications. Isoxazolidines appear to function as highly active general activation domains, and are relatively low in molecular weight and synthetically accessible. With recent strategies developed in the Dervan lab[17,23] that have been demonstrated to increase cellular uptake, polyamide-isoxazolidine conjugates would seem to have a great potential for transcriptional activation.

In order to compare results for polyamide-isoxazolidine conjugates to previous isoxazolidine studies, the promoter region containing Gal4 binding sites upstream of the firefly luciferase gene in the plasmid (pG5luc) was targeted. There are five Gal4 binding sites in the promoter, each containing a 5'-WGWWCW-3' sequence, a convenient target site for the polyamides shown in Figure 5.7. The geometry of the designed polyamides, in which the isoxazolidine group is conjugated to an (R)-β-amino-γ-turn, was chosen based on recent work in the Dervan laboratory indicating that substituents at this turn are better tolerated and have a relatively small effect on the binding affinity of the polyamide.[23] The length of the linker will be varied to determine whether this has any significant effect on gene activation.

pG5luc Promoter Region:

```
  1 GGTACCGAGT TTCTAGACGG AGTACTGTCC TCCGAGCGGA GTACTGTCCT
 51 CCGACTCGAG CGGAGTACTG TCCTCCGATC GGAGTACTGT CCTCCGCGAA
101 TTCCGGAGTA CTGTCCTCCG AAGACGCTAG CGGGGGGCTA TAAAAGGGGG
151 TGGGGGCGTT CGTCCTCACT CT
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General Structure:

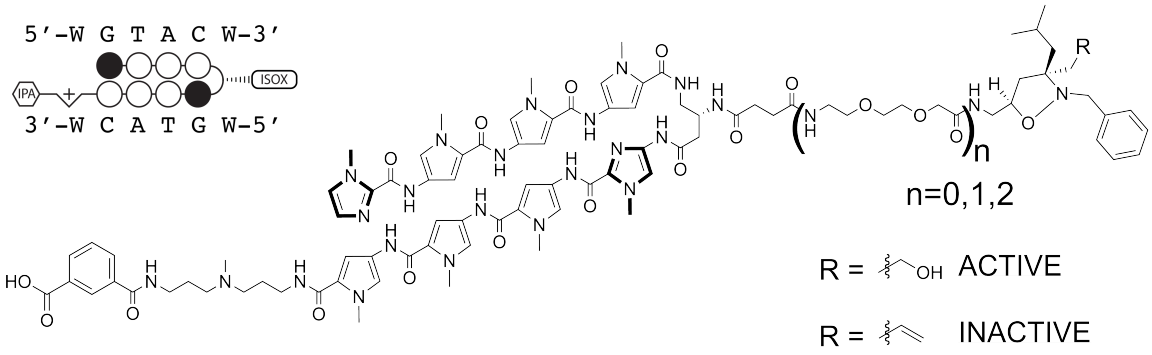


Figure 5.7: Targeting the pG5luc promoter region. There are five binding sites in this promoter, and each contains a 5'-WGWWCW-3' (W=A/T) region (top). The proposed general structure of the polyamide-isoxazolidine conjugates is shown (bottom).

In collaboration with Ryan Casey from Professsor Anna Mapp's laboratory (University of Michigan), a library of six polyamide-isoxazolidine derivatives was chosen.

Initially, Mr. Casey began work synthesizing the compounds listed below, which he would later send to Caltech.

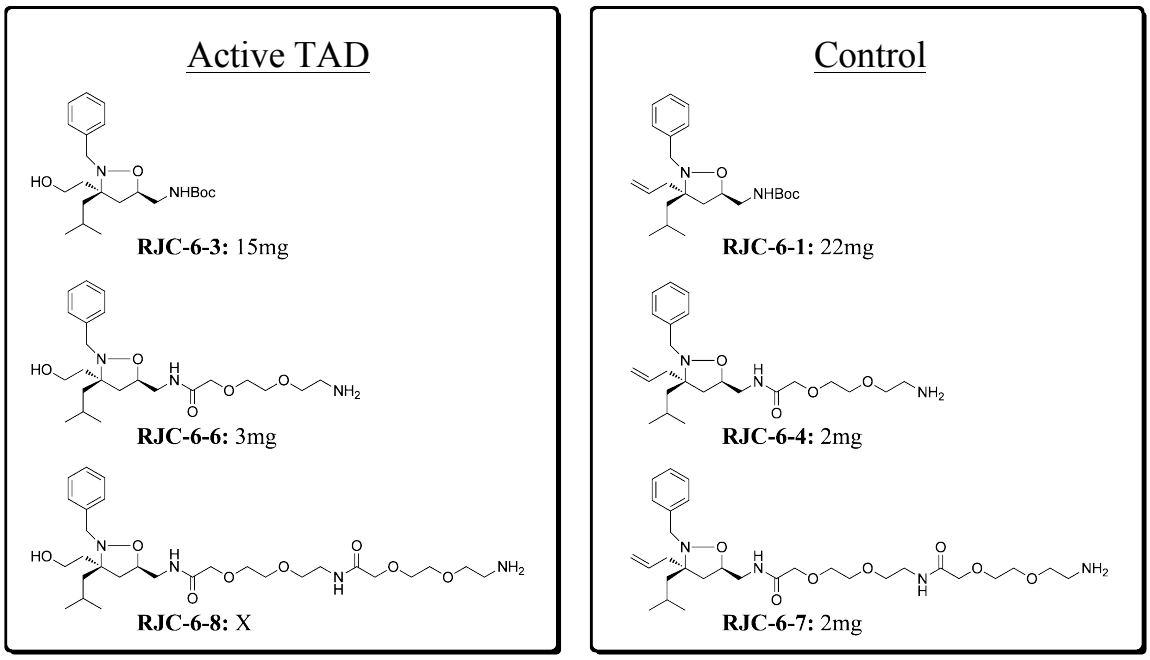


Figure 5.8: Isoxazolidine compounds sent from the Mapp laboratory. Three of these compounds are active (left), and three control compounds are inactive (right).

As seen in Figure 5.8, milligram-scale samples of these compounds were sent to Caltech, including three active TADs, with alcohol moieties, and three inactive control compounds, of varying linker lengths. These compounds can be directly coupled (after t-Boc deprotection for RJC-6-1 and RJC-6-3) to a polyamide bearing a carboxylic acid as discussed below. Unfortunately, it was determined that the compound labeled **RJC-6-8** was not the correct compound. The proposed library of polyamide-isoxazolidine conjugates is shown in Figure 5.9.

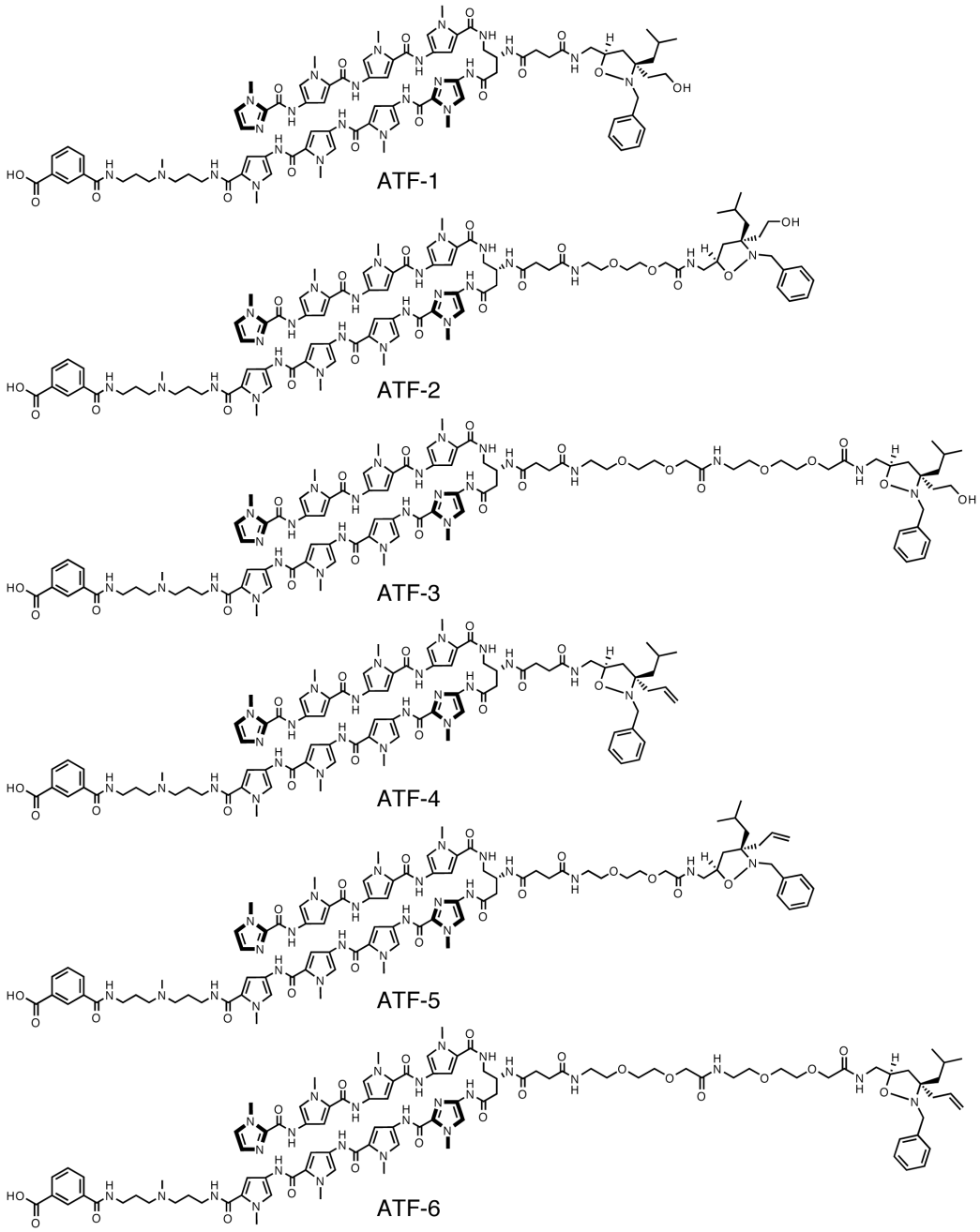


Figure 5.9: Proposed library of polyamide-isoxazolidine conjugates.

Results and Discussion

Solid-phase Polyamide Synthesis

Prior to receiving the isoxazolidine compounds, solid-phase synthesis (SPS)[24,25] methods were employed to generate sufficient quantities of the unsubstituted polyamide, such that the necessary steps to prepare this polyamide for conjugation with isoxazolidines could be worked out.

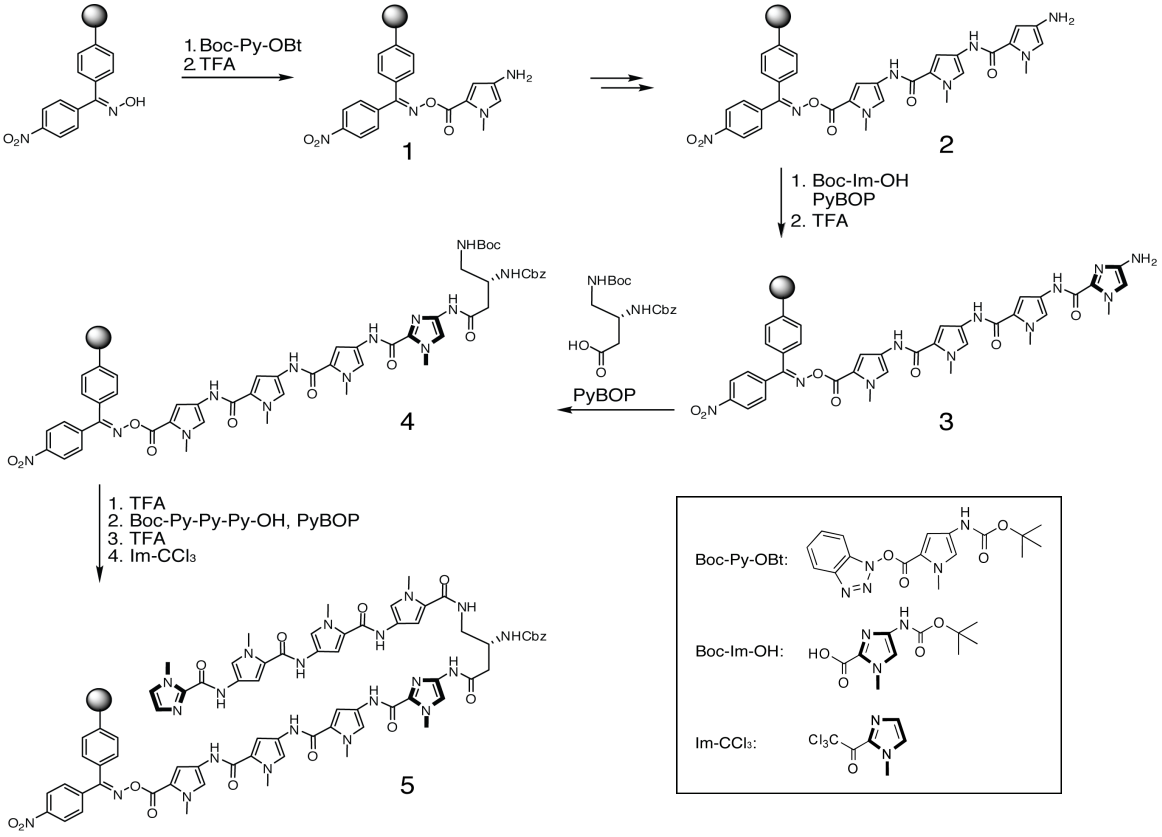


Figure 5.10: *t*-Boc-solid phase synthesis of an eight-ring hairpin polyamide attached to oxime resin, **5**.

This synthesis employed *t*-Boc-SPS with Kaiser Oxime resin, and involved successive PyBOP couplings (or the use of preactivated heterocycles) and TFA deprotections. Each reaction was monitored by the cleavage and analytical HPLC of a

small sample of resin to verify reaction completion. Next, the resin was cleaved with 3,3'-diamino-N-methyl-dipropylamine, as seen in Figure 5.11.

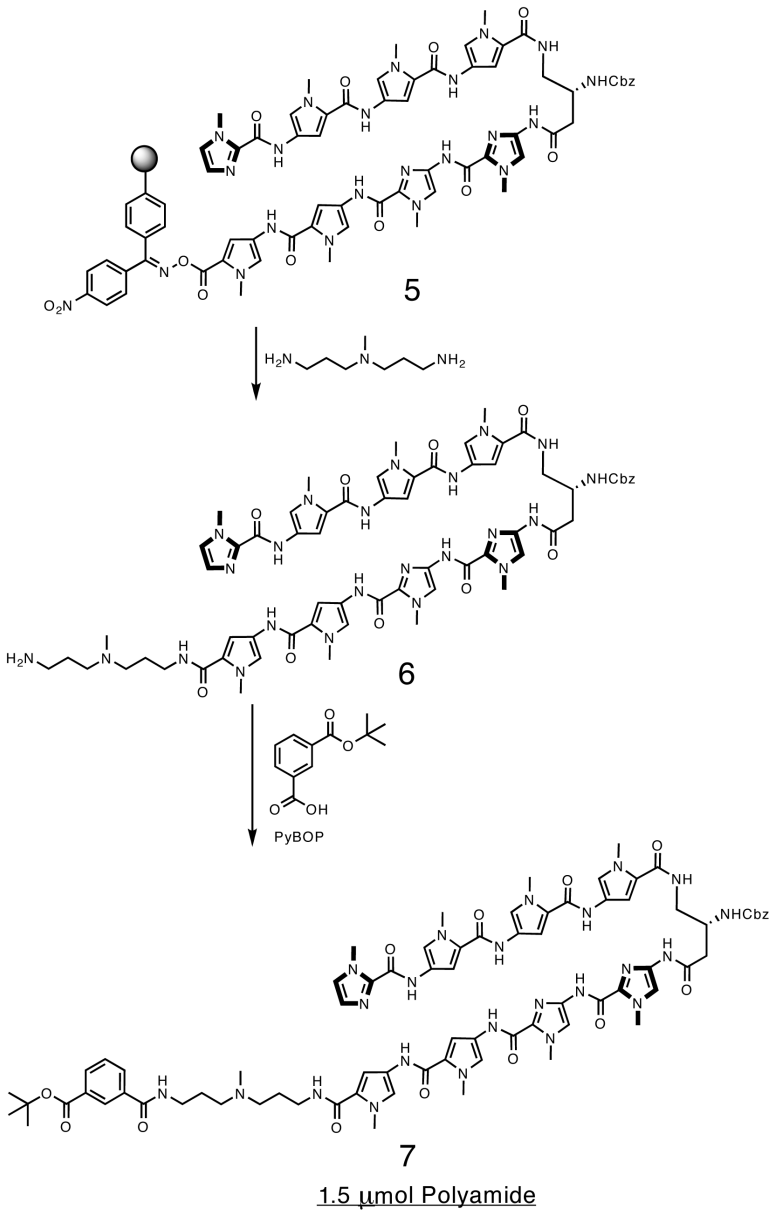


Figure 5.11: Cleavage of polyamide from oxime resin; coupling of mono-*tert*-butyl protected isophthalic acid.

After preparatory HPLC purification to yield polyamide **6**, PyBOP coupling of mono-*tert*-butyl isophthalic acid afforded 1.5μmol of polyamide **7**. This *tert*-butyl ester protecting group was used to later allow regioselective amide coupling of isoxazolidines to

a carboxylic acid-functionalized amino turn. Next, palladium-catalyzed transfer hydrogenation afforded free amine **8**, in low yield. Succinylation of the amine then afforded the corresponding carboxylic acid **9** in moderate yield, which could be coupled directly to the amino-substituted isoxazolidine compounds.

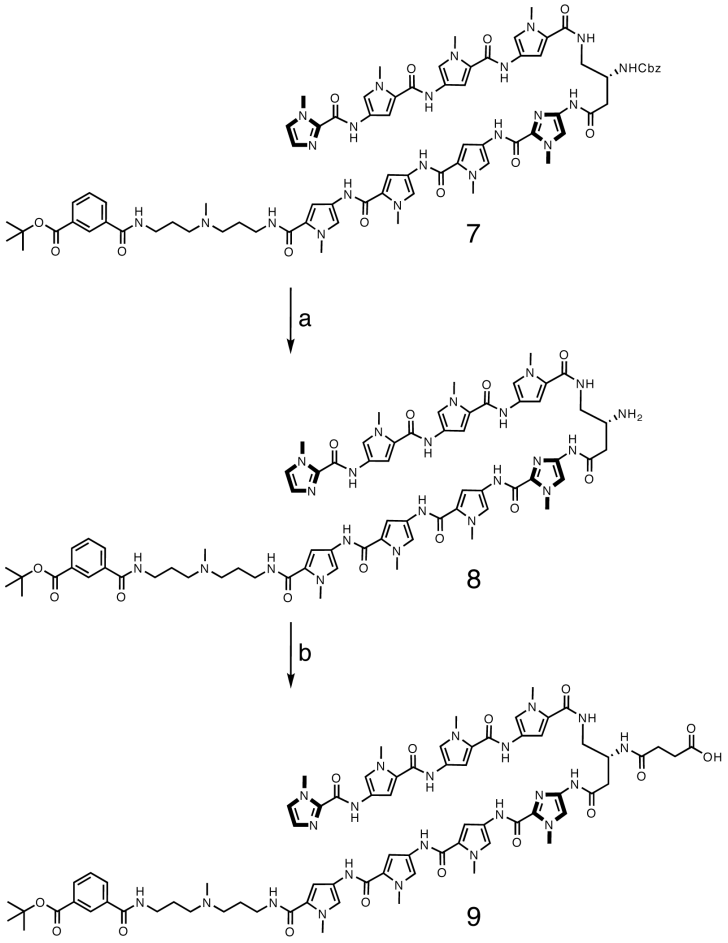


Figure 5.12: Synthesis of carboxylic-acid derivatized polyamide. Conditions: a) $Pd(OAc)_2$, H_4NHCO_2, DMF/H_2O, r.t., 16h, 27%; b) Succinic Anhydride, DIEA, DMF, r.t., 1 hr, 77%.

However, the yield of these reactions (21% over two steps) was very low, despite attempts at optimization. After the isoxazolidines were received, a nanomole-scale (20nmol) isoxazolidine coupling reaction was attempted, but the miniscule amount of remaining material after work-up and purification made analytical characterization

difficult. These results indicated that further optimization would be necessary in order to preserve valuable material, especially considering two additional steps—an isoxazolidine coupling and subsequent *tert*-butyl ester deprotection—would be required to afford the desired structure. This indicated that a large quantity of polyamide **7** would be necessary to synthesize reasonable quantities (~500 nmol) of the six polyamide-isoxazolidine conjugates.

Solution-phase Polyamide Scale-up

As a way to generate a significant quantity of polyamide for isoxazolidine coupling reactions, a solution-phase route was considered.

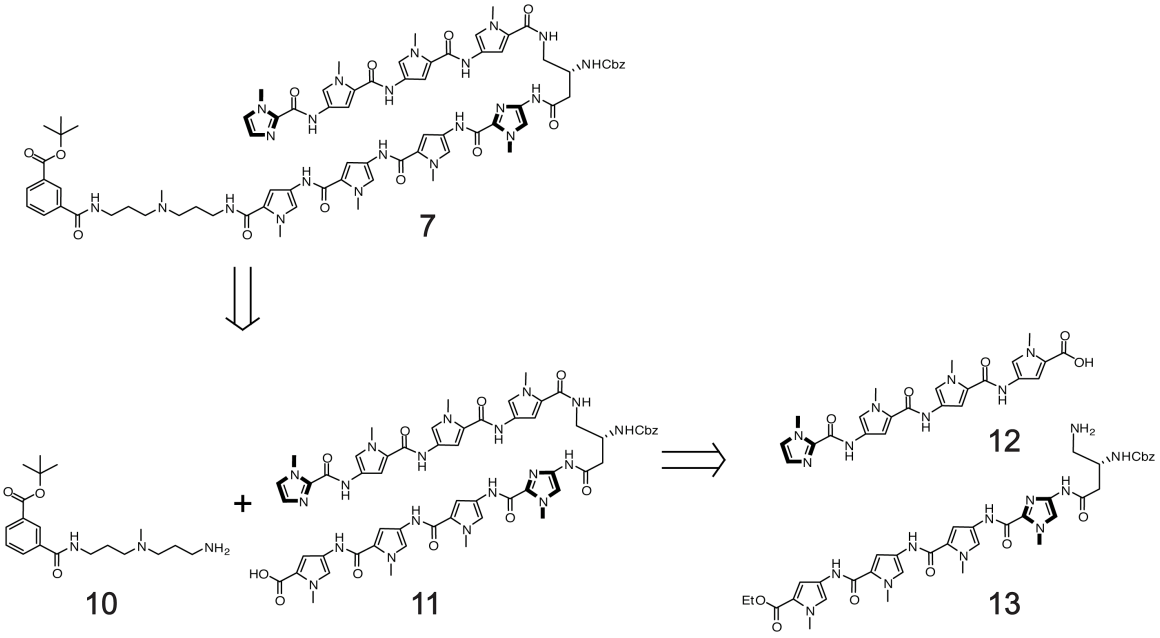


Figure 5.13: Retrosynthesis of polyamide **7**. The tail piece **10** can be coupled to polyamide core **11**, which is generated from tetramer **12**, and pentamer **13** (which contains a hairpin turn unit).

Retrosynthetically, two key amide bond disconnections were envisioned, as seen in Figure 5.13. Polyamide **7** was first disconnected from its tail (**10)** and polyamide core (**11)**

pieces. The polyamide core was further disconnected from its precursors **12** and **13**. Therefore, compounds **10, 12,** and **13** were the initial synthetic targets.

In order to synthesize the tail region **10**, the amide coupling of commercially available triamine **16** with mono-*tert*-butyl isophthalic acid **15** was proposed. While **15** is commercially available, it is relatively expensive. Other methods to synthesize **15** were attempted, involving the conversion of commercially available isophthalic acid to its mono-*tert*-butyl protected form **15**, without much success. However, a literature search also revealed the synthesis of **15** from commercially available isophthaloyl dichloride **14**.[26] This reaction was incorporated to yield **15** in moderate yield, but with extremely inexpensive starting materials. This route allowed the synthesis of large quantities (16 grams) of **15**. Next, DCC-mediated coupling of triamine **16** to carboxylic acid **15** afforded building block **10** in moderate yield.

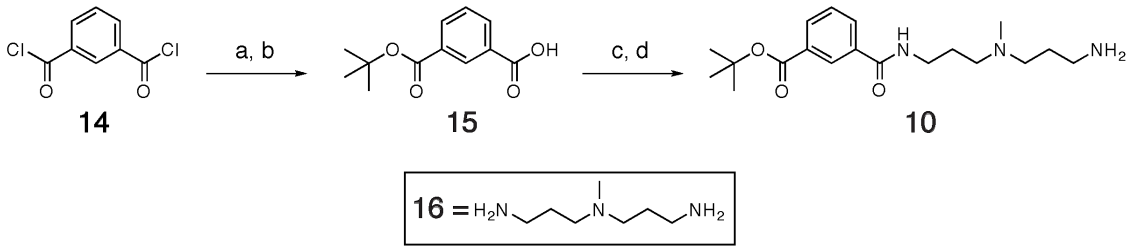


Figure 5.14: Synthesis of **10**. Conditions: a) *tert*-butanol, pyridine, THF, 81°C, 6 hr; b) H_2O, pyridine, THF, 81°C, 6 hr, 52% over two steps; c) DCC, HOBt, DCM, r.t., 1 hr; d) **16**, DCM, r.t., 4 hr, 47% over two steps.

Beginning with trimer **20**, which can be readily synthesized in two steps from Dervan Laboratory stock compounds **17** and **18**, the synthesis of tetramer **12** was completed in two steps by coupling of an activated imidazole unit to **20**, followed by saponification. Tetramer **23** was also synthesized from trimer **20** in two steps through an activated ester coupling of a bifunctional imidazole derivative, followed by acidic

deprotection of the N-*tert*-butoxycarbonyl (Boc) protecting group. PyBOP coupling of an (R)-β-amino-γ-turn to **23** followed by acidic Boc-deprotection yielded pentamer **13**.

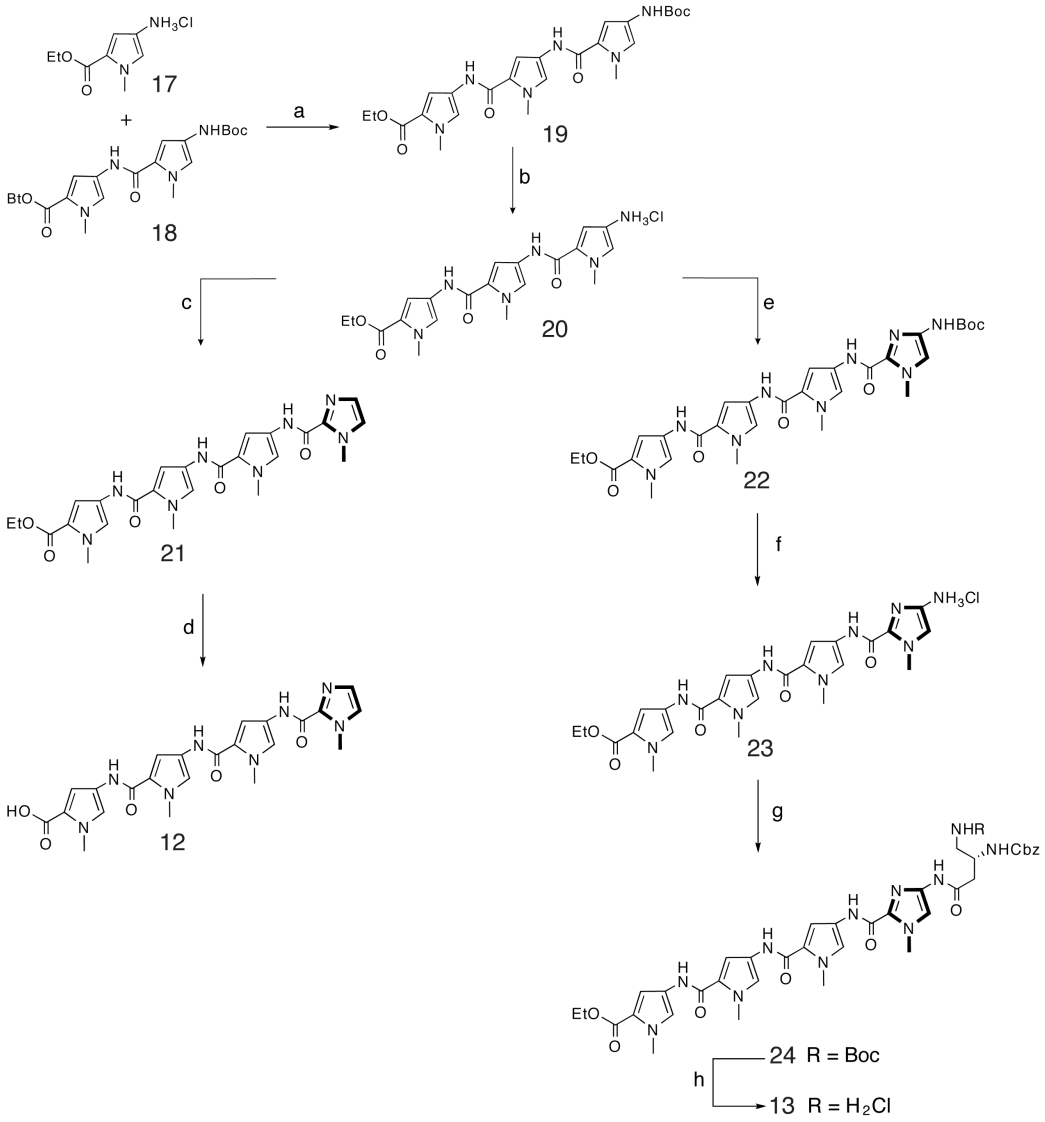


Figure 5.15: Synthesis of **12** and **13** from trimer **20**. Conditions: a) DIEA, DMF, r.t, 16h, 91%; b) 4.0M HCl in 1,4-Dioxane, r.t., 18h, 97%; c) Im-CCl_3, DIEA, DMF, r.t., 16h, 87%; d) 1N NaOH, H_2O, 1,4-Dioxane, 50°, 6h, 98%; e) Boc-Im-COOH, HBTU, DIEA, DMF, r.t., 16h, 81%; f) 2.0M HCl in Et_2O, 1,4-Dioxane, r.t., 3h, 97%; g) Z-ß-Dab(Boc)-OH, PyBOP, DIEA, DMF, r.t., 16h, 78%; h) 2.0M HCl in Et_2O, 1,4-Dioxane, r.t., 3h, 99%.

With polyamide precursors **10**, **12**, and **13** synthesized in gram-scale quantities (0.5-1.0 grams of each compound), polyamide **7** was synthesized in 3 steps: PyBOP coupling of **12** and **13** to yield the polyamide core ethyl ester **25**, saponification to afford

carboxylic acid **11**, and PyBOP coupling of **11** with tail unit **10**, as illustrated in Figure 5.16.

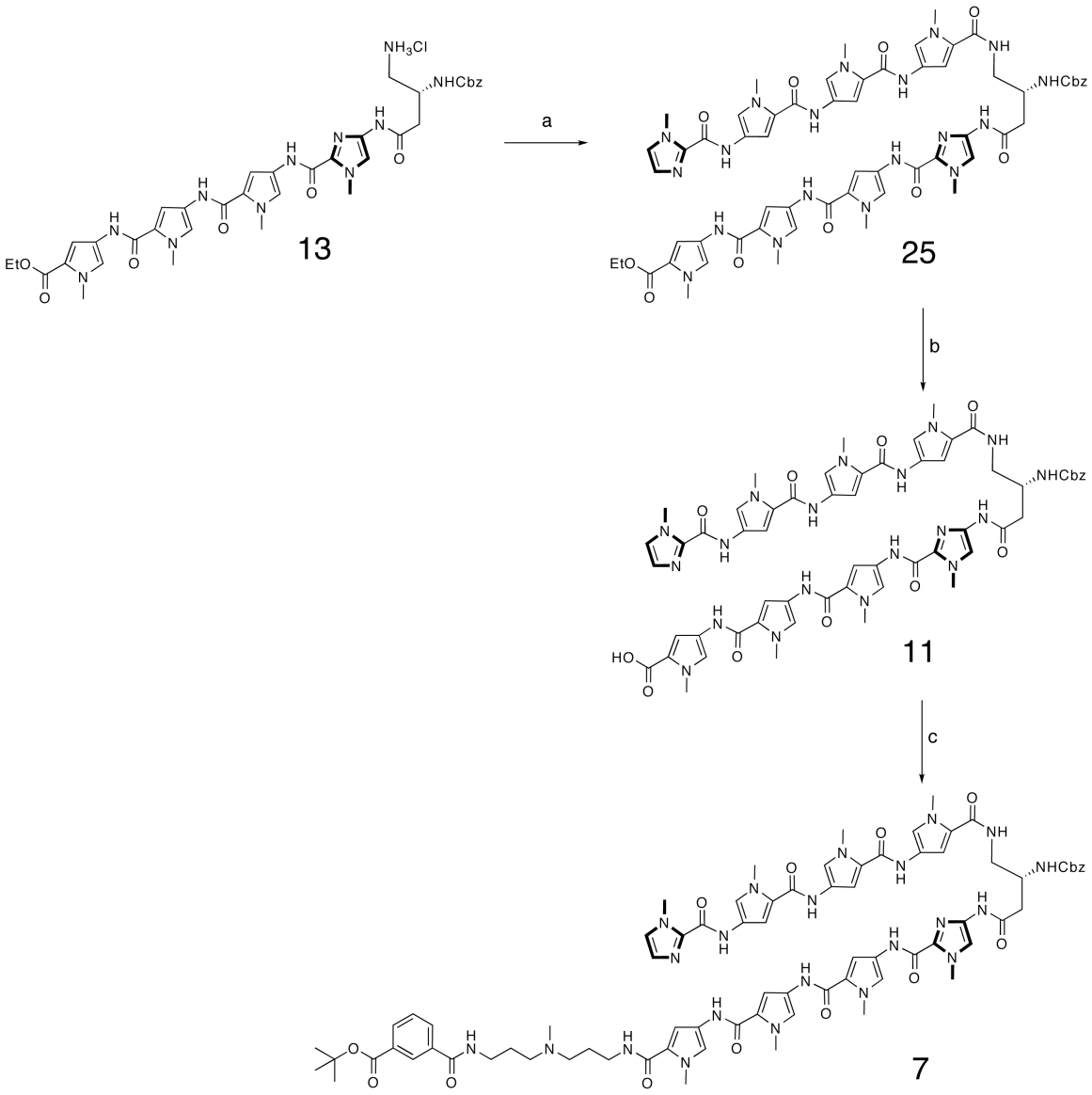


Figure 5.16: Synthesis of Polyamide **7**. Conditions: a) **12**, PyBOP, DIEA, DMF, r.t., 16h, 76%; b) 1N NaOH, H_2O, 1,4-Dioxane, r.t., 15h, 86%; c) **10**, PyBOP, DIEA, DMF, r.t., 2h, 94%.

Using this solution-phase synthesis route, it was possible to synthesize over 350mg (over 200 μmol) of **7**, with more synthetic precursors available if necessary. While this route necessitated chromatography steps and required some optimization, it is anticipated that future syntheses of this nature would be more straightforward and less laborious,

allowing synthesis of large quantities of polyamide where previous solid-phase methods would be inadequate. Indeed, Chenoweth et al. from the Dervan laboratory have since developed a high-yielding chromatography-free route for gram-scale solution phase synthesis of a polyamide.[27]

Synthesis of Polyamide-Isoxazolidine Conjugates

With a large quantity of polyamide **7** available, the next step was derivatization of the polyamide for coupling with the isoxazolidine compounds. Initially, this required deprotection of the Cbz-protected amino turn. Due to the *tert*-butyl protecting group on the polyamide tail, catalytic transfer hydrogenation was chosen as the optimal chemoselective transformation.[28-30] At first, the use of palladium(II) acetate as a catalyst along with ammonium formate (as a source of hydrogen) was attempted, as mentioned above. However, among the various conditions tested, heating was necessary for full conversion and decomposition was always a major drawback, leading to poor yields. Palladium on carbon (Pd/C) was also attempted as a hydrogenation catalyst, and this reaction afforded amino compound **8** with less decomposition. Despite indications that this was a clean reaction, yields were consistently low after filtration and purification. It was found that with too much Pd/C catalyst, yields were lower. It is speculated that some polyamide becomes bound—perhaps irreversibly—to the catalyst, leading to a loss of product. Additionally, it was found that the standard method of filtering Pd/C, using celite, was also a major reason for lost material. Optimization of reaction conditions and purification methods proved crucial, and eventually a high yielding procedure was developed.

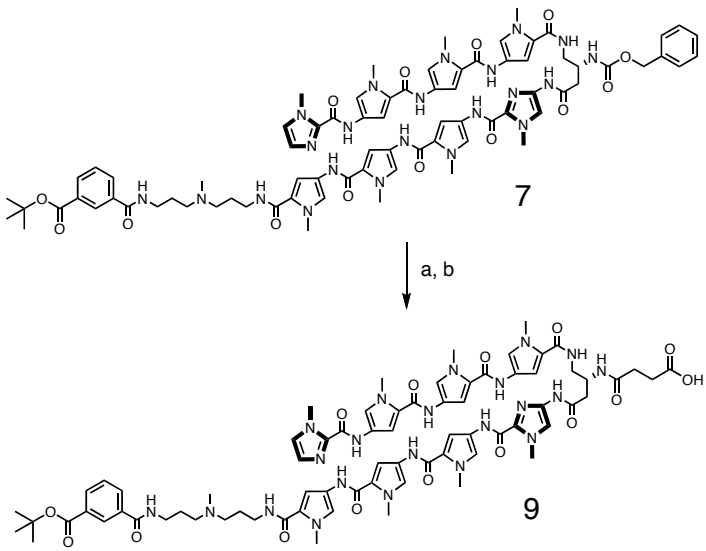


Figure 5.17: Synthesis of acid-functionalized polyamide **9**. Conditions: a) Pd/C, H_4NHCO_2, H_2O, 1,4-Dioxane, 60°, 12 hours; b) Succinic Anhydride, DMF, DIEA, r.t., 30min, 87% over two steps.

As seen in Figure 5.17, the subsequent addition of succinic anhydride gave polyamide **9** in 87% yield over two steps. The final two transformations were the isoxazolidine-polyamide coupling and the subsequent acid deprotection of the *tert*-butyl group. The coupling step was somewhat problematic, as it is difficult to precisely measure stoichiometric quantities of each compound on such a small scale, and this reaction generally involved the formation of minor side products. A general procedure was developed to maximize product formation, but side-products have not been completely eliminated in this procedure. Finally, the *tert*-butyl group on the tail must be deprotected, and this step was also initially challenging. While different acid-catalyzed conditions (TFA/DCM, HCl/Ether, etc.) were attempted, decomposition of these polyamide-isoxazolidine compounds was pervasive among all methods, and yields were extremely low. In order to limit the loss of invaluable isoxazolidine compounds, this reaction was performed on a very small scale, making it difficult to monitor. Compounding this issue was the need to purify the final conjugates via preparatory HPLC, often leading to an

additional significant reduction in yield. However, a milder deprotection procedure proved to be more successful, employing $CeCl_3$ and NaI in refluxing acetonitrile. In general, this reaction minimized decomposition and significantly increased the overall yield.

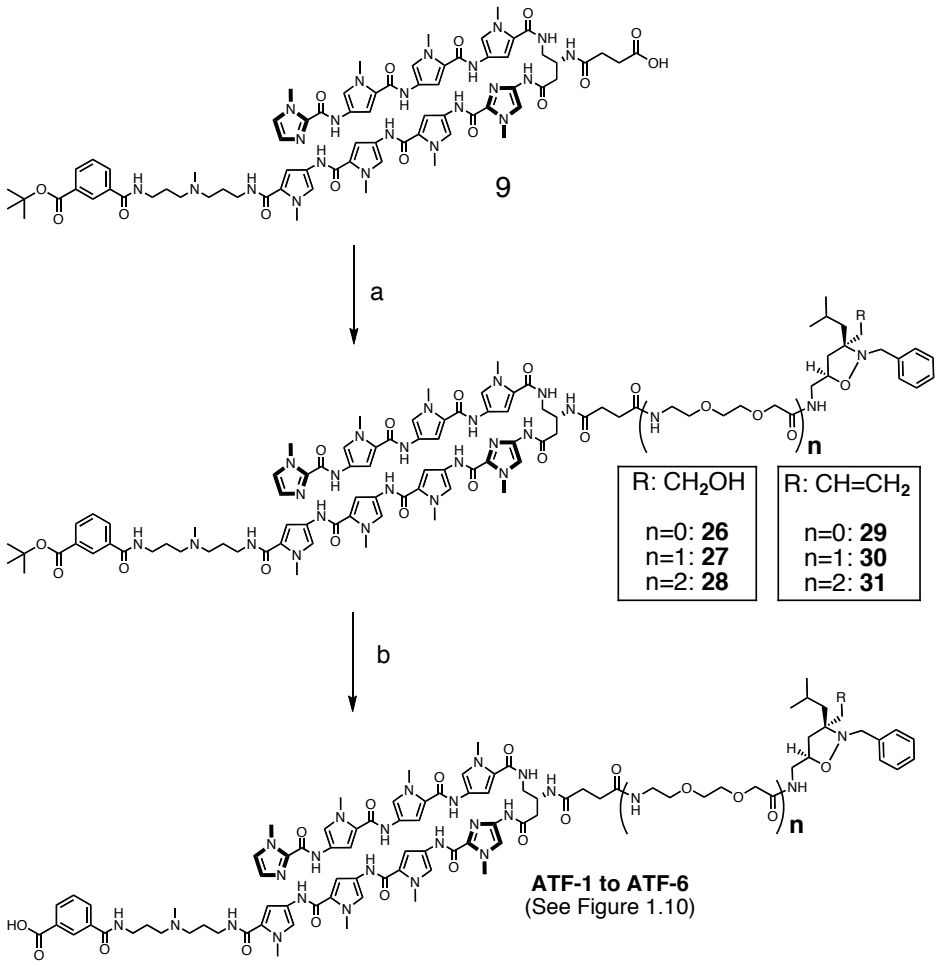


Figure 5.18: Polyamide-isoxazolidine coupling and final *tert*-butyl deprotection. Conditions: a) PyBOP, DIEA, DMF, RJC-6-X (see Figure 5.8), 60°, 3 hours; b) $CeCl_3 \bullet H_2O$, NaI, MeCN, 82°C, 1 hour, 21% yield over two steps.

Using this route, **ATF-1**, **ATF-2**, and **ATF-4** were synthesized. Moderate quantities of **ATF-1** and **ATF-4** were synthesized, while a small amount of **ATF-2** was also synthesized (Figure 5.19). With the excess of isoxazolidine compounds necessary for these coupling reactions, combined with the small amount of available isoxazolidine material, it was possible only to make usable quantities of **ATF-1** and **ATF-4**. However,

these initial stocks allowed preliminary biological investigations to explore the gene activation potential of these conjugates (discussed below).

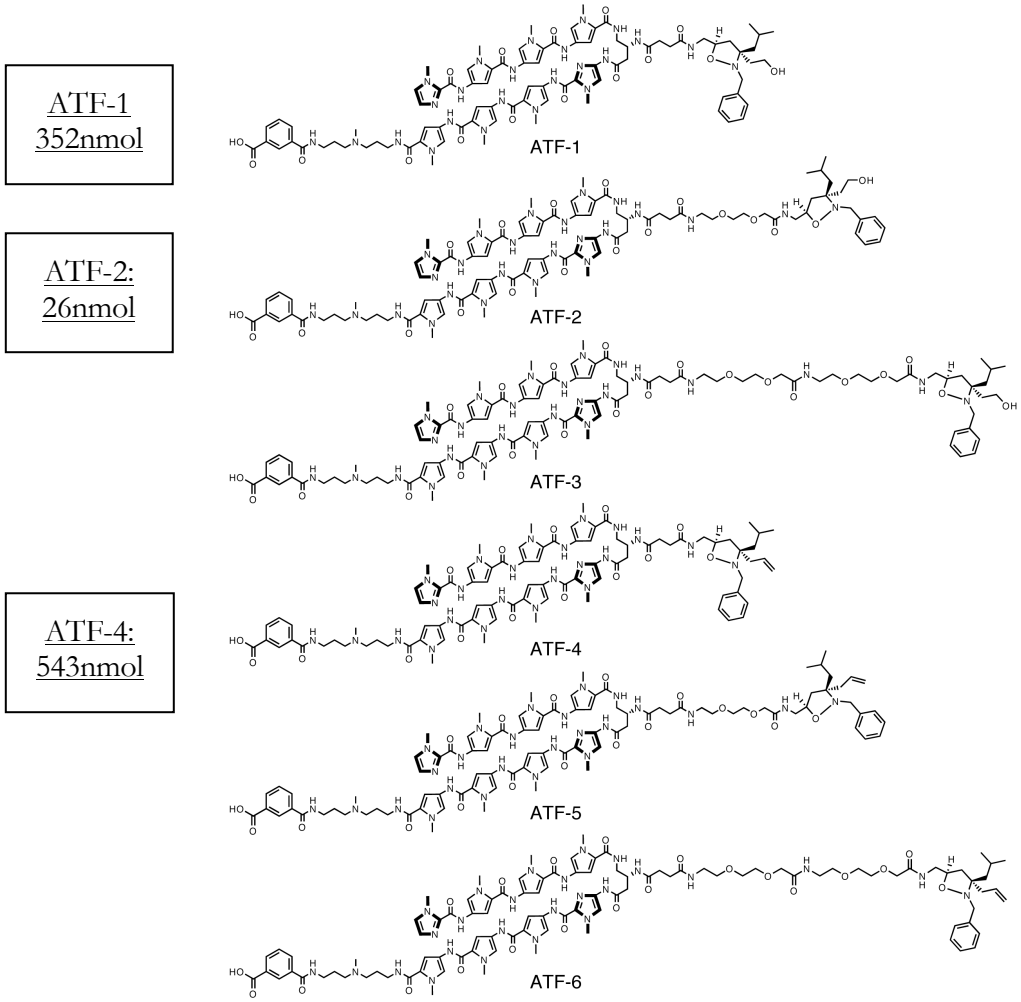


Figure 5.19: Initial synthesized stock of proposed polyamide-isoxazolidine conjugate library.

Linker Synthesis

After a second shipment of isoxazolidine compounds arrived from Mr. Casey in the Mapp laboratory, in somewhat larger quantities than before (Figure 5.20), optimization of the linker couplings was attempted at Caltech, due to low reported yields in previous procedures, which limited the amount of isoxazolidine that was sent.

RJC-6-1dep
24mg

RJC-6-3dep
70mg

Figure 5.20: Second batch of isoxazolidines received from Mapp laboratory.

In considering the optimal route for attachment of a longer length linker, it was deemed that previous methods involving sequential attachment of two short PEG linkers were less than desirable, due to compounding low yield steps. In addition, the previous Fmoc-protected linkers yielded deprotection byproducts, which necessitated preparatory HPLC purification, further decreasing the yield.

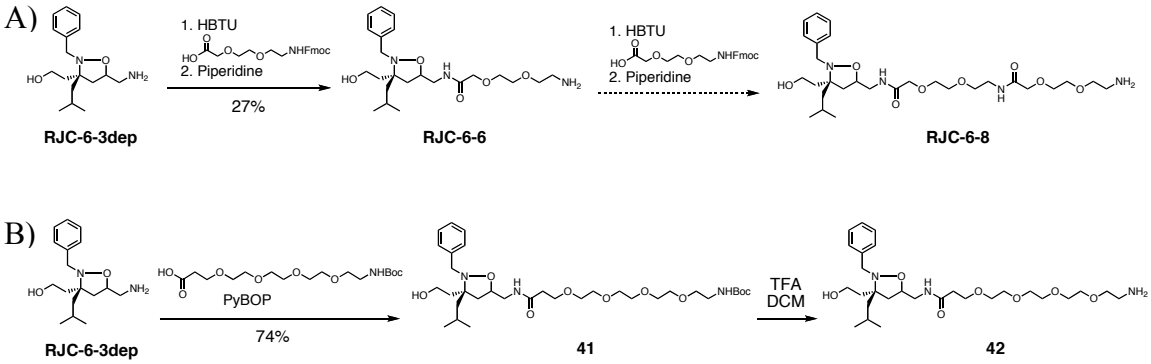


Figure 5.21: Routes for linker attachment (long linker variant). A) Previous strategy, involving sequential attachment of two short PEG linkers. B) Strategy utilizing a single longer linker, with a Boc protecting group.

As seen in Figure 5.21, another envisioned route was to use a single longer linker with a Boc protecting group. This procedure involved a single coupling, providing a relatively non-polar product, which can be purified by preparatory TLC. The linker-isoxazolidine coupling reaction proceeded with relatively high yield. After Boc deprotection, this compound can be directly coupled to a polyamide (Figure 5.22). It was found that the previous $CeCl_3$/NaI t-butyl deprotection procedure caused significant

decomposition in this route, so an improved 95% TFA/5% H_2O protocol was developed allowing significantly improved yields.

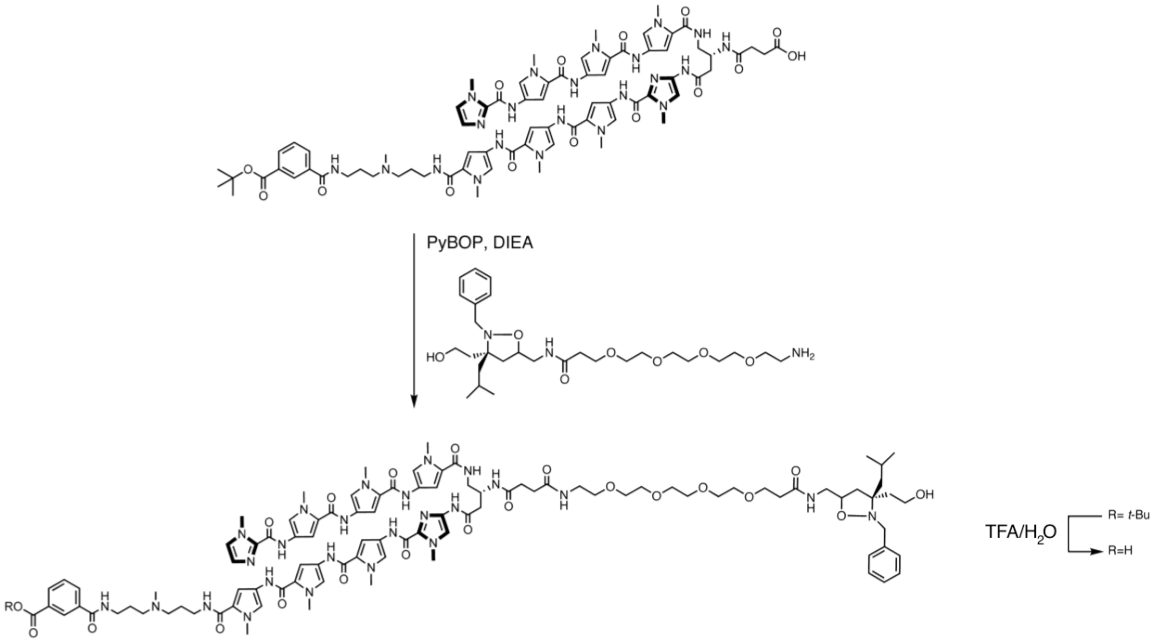


Figure 5.22: Synthesis of polyamide-isoxazolidine with longer linker.

In Vivo Activation Studies

Concurrent with the synthesis of a polyamide-isoxazolidine with a longer linker, the initial completed compounds underwent pilot studies to assay for upregulation of transcription. Samples of **ATF-1, ATF-2,** and **ATF-4** were sent to Michigan, along with parent polyamide **43** (an additional control) to be added to HeLa cells transfected with the pG5luc plasmid, the same system previously used in the Mapp laboratory.

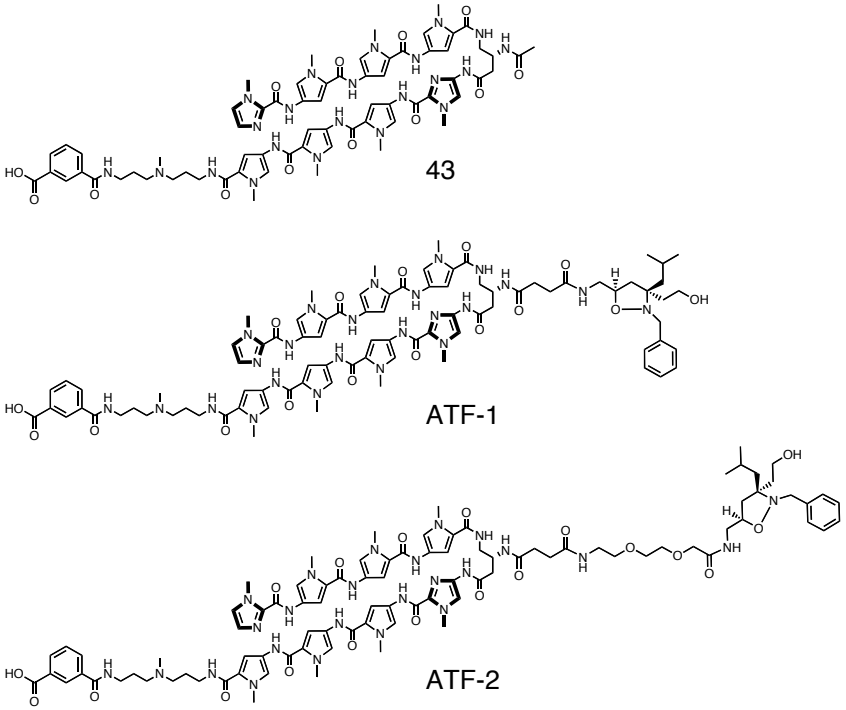


Figure 5.23: Compounds used in initial luciferase assay by Mapp laboratory.

Additionally, a second plasmid containing a Renilla luciferase construct was used, thereby allowing a dual luciferase assay to be performed. Cells were plated, incubated f24 hours, and dosed with polyamide-isoxazolidine conjugates for a 40 hour period.

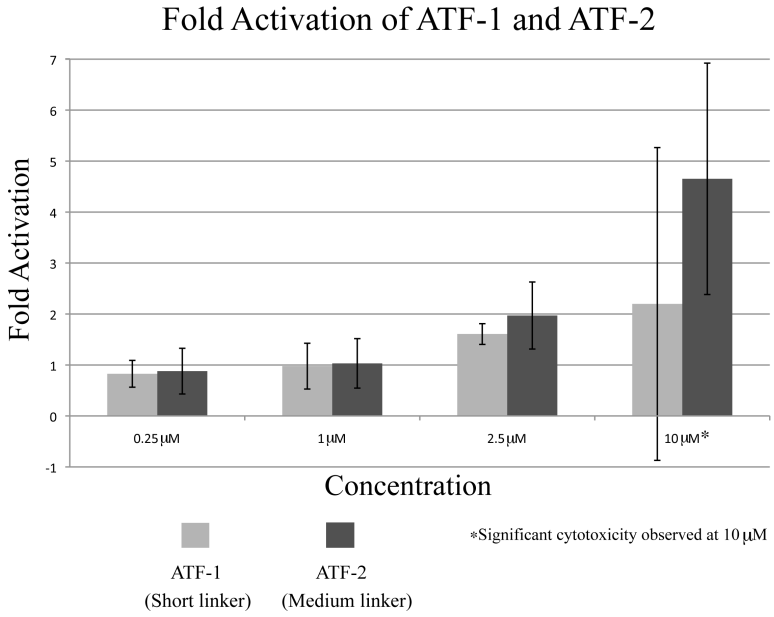


Figure 5.24: Initial results from Mapp laboratory: dual luciferase reporter assay in HeLa cells.

In addition to the Renilla luciferase normalization, the fold activation is also normalized for wells containing no synthetic conjugates, as well as for luciferase signal elicited by the parent polyamide, **43**. At 10μM, significant cytotoxicity was observed, contributing to the large error. These initial results showed some promise, as there was a modest (~2-fold) increase in luciferase signal for cells treated with ATF-1 and ATF-2 at 2.5μM. However, previous studies in the Mapp laboratory exhibited an 80-fold activation with a Gal4-based DBD.

In a later experiment, polyamide **44**, containing a longer linker, was assayed by the Mapp laboratory in a similar dual luciferase reporter assay.

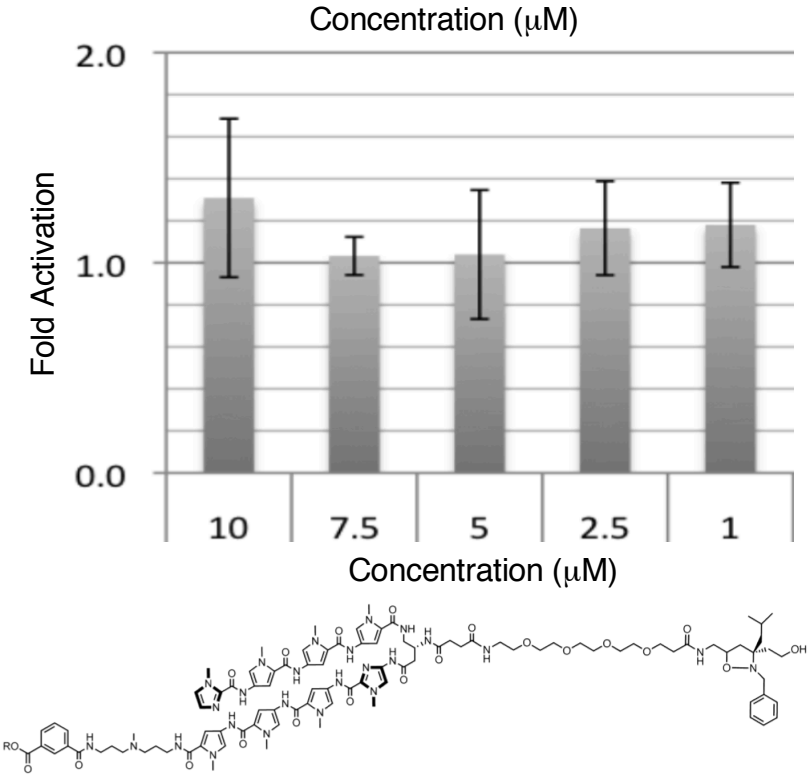


Figure 5.25: Dual luciferase reporter assay for compound **44** in HeLa cells.

As for the results of ATF-1 and ATF-2, no significant activation of luciferase activity was observed in this assay.

In addition to the experiments in HeLa cells run by the Mapp laboratory, experiments were performed at Caltech using the LNAR+ cell line. This cell line was developed by the Charles Sawyer laboratory at Sloan-Kettering. It consists of transformed LNCaP cells that have been stably transfected with a firefly luciferase reporter gene controlled by an Androgen Response Element (ARE) in the promoter region (which likely contains a GWWC sequence). Unfortunately, the sequence of this construct isn't available. However, it was envisioned that this experiment would still be informative as to whether or not these polyamide-isoxazolidine conjugates are activating transcription.

In this experiment, polyamides at varying concentrations were added to cells in quadruplet, on a 96-well plate. Cells were plated at 50,000 cells/mL, incubated for 24 hours, and treated with both active and inactive polyamide-isoxazolidine conjugates, as well as parent polyamide compounds as further controls. Positive and negative controls were set up by the presence and absence of induction by dihydrotestosterone (DHT), respectively. DHT is a potent agonist for androgen receptor activation, so cells induced by DHT should exhibit an increased luciferase signal. In addition to the luminescence assay that indicates firefly luciferase activity, a WST-1 cell proliferation assay was also performed to determine any cytotoxicity caused by the polyamides along the range of concentrations studied. This data can also be used to normalize the luciferase data, in that WST-1 assays provide an estimate of cell viability by measuring mitochondrial activity.

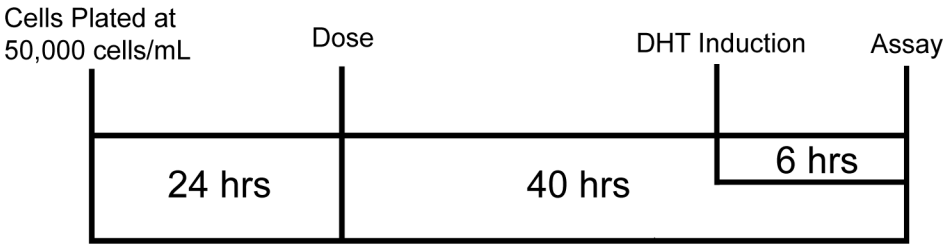


Figure 5.26: Experimental timeline. Cells are plated, incubated 24 hours, dosed with compounds, and incubated for 40 hours. A six-hour DHT induction was also performed in wells with no other added compounds.

After initial pilot studies were performed to optimize the assay (e.g. optimization of incubation time for the WST-1 assay, examination of a wide range of polyamide concentrations, etc.), an experiment consisting of four compounds, and ranging from 3nM to 10μM in concentration was conducted.

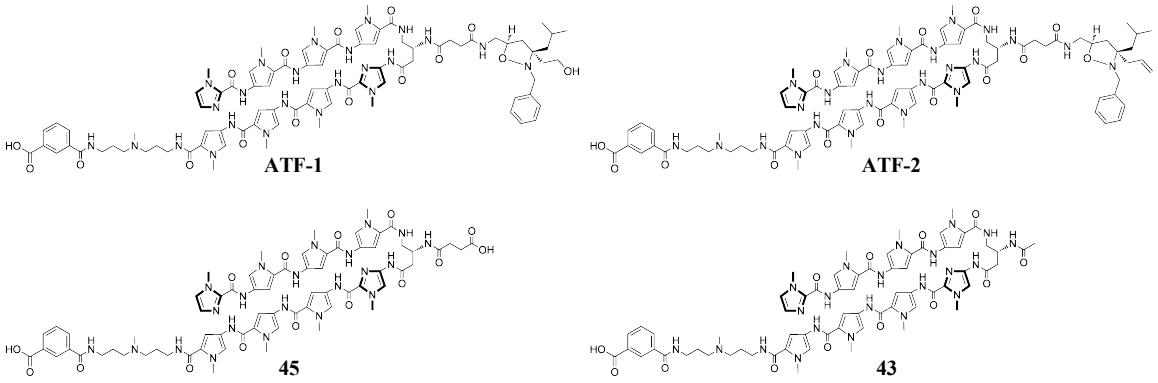


Figure 5.27: Compounds used in LNAR+ assay.

Compounds **43** and **45** can be easily synthesized from intermediates in the solution-phase route detailed above. After the luciferase assay was performed, the data was collected and normalized by subtracting out the background values of wells without any cells. As mentioned above, it was also normalized for WST-1 values, and the final values reported in Relative Luminescence Units (RLU).

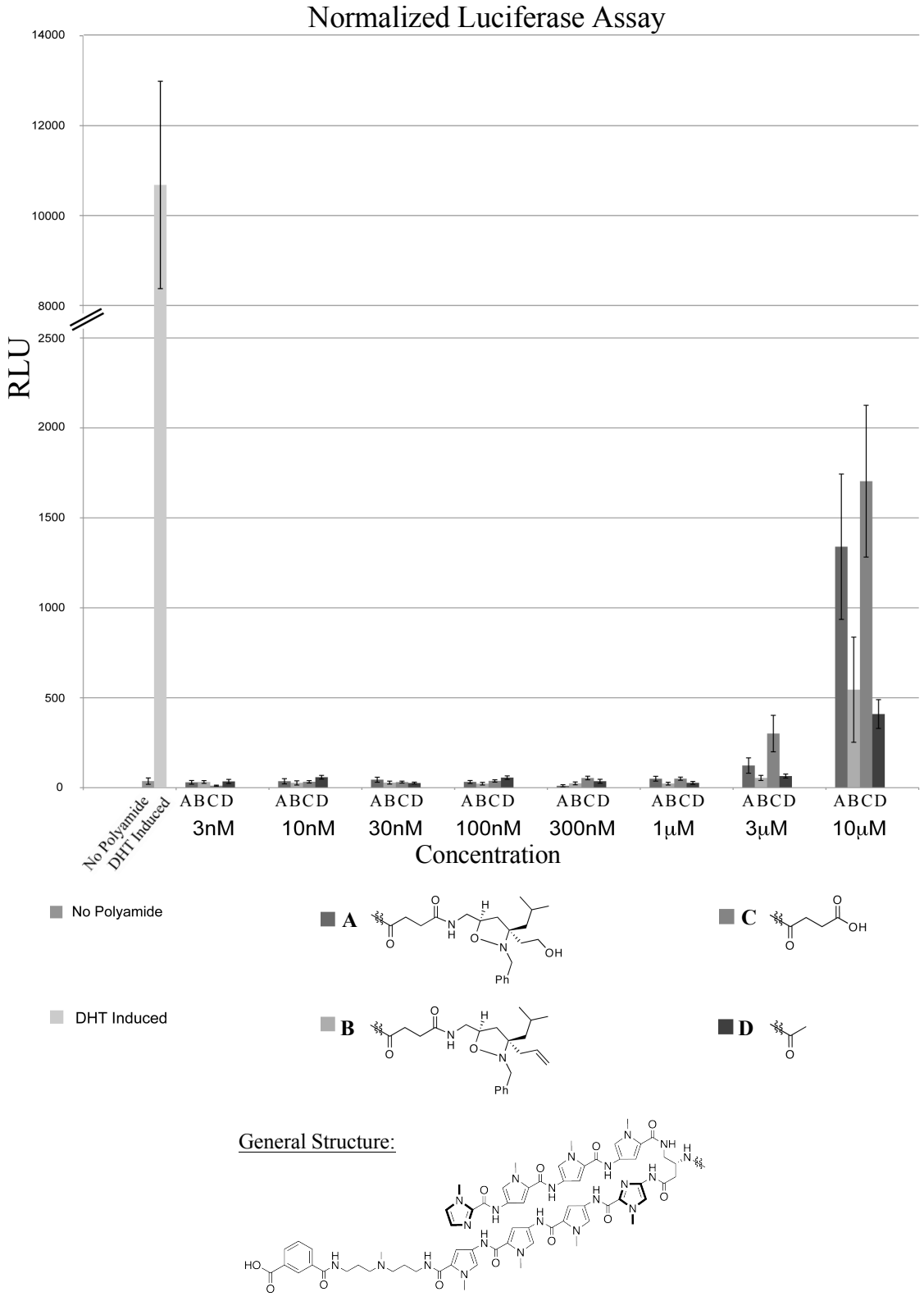


Figure 5.28: LNAR+ assay. The structures of each of the polyamides is indicated below. Concentration is varied from 3nM to 10μM. Note: the y-axis is split.

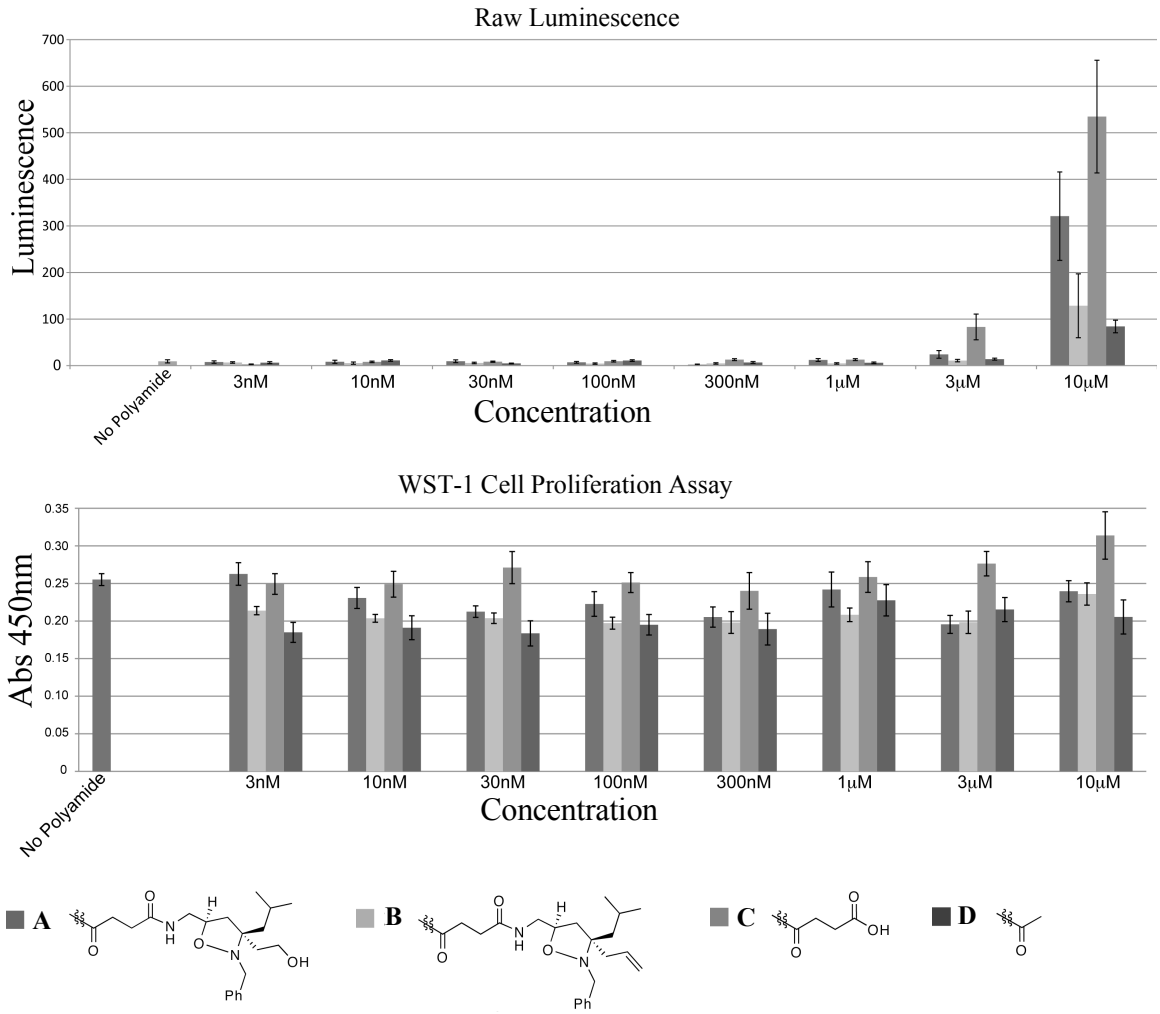


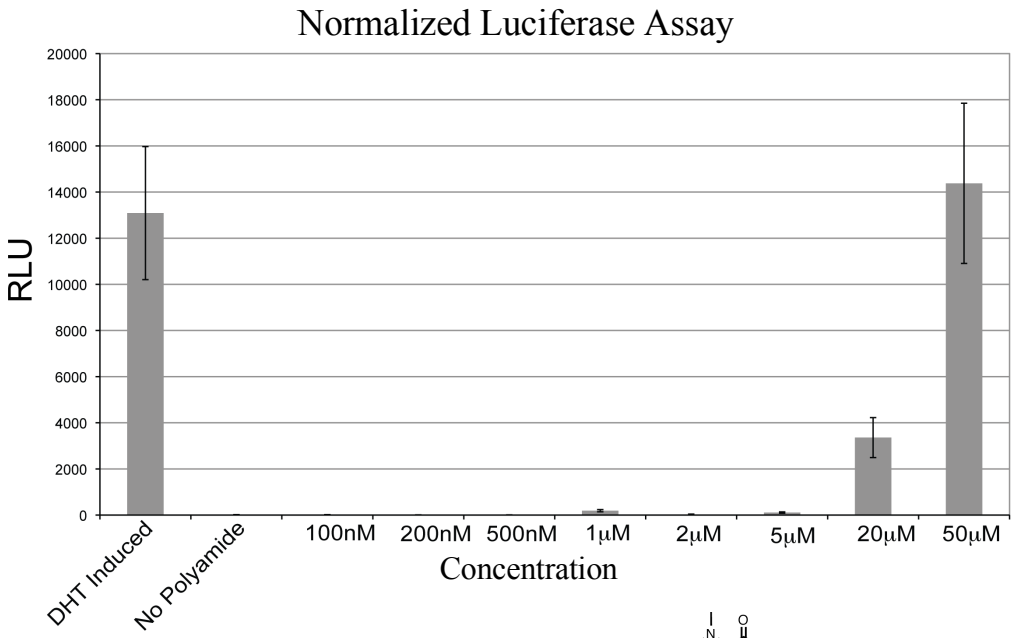
Figure 5.29: LNAR+ assay raw data. It is notable that there is no significant decrease in WST-1 signal for any of the compounds.

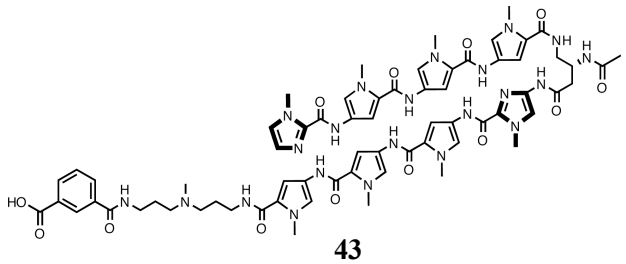
The data seem to indicate that all four compounds cause an increase in luciferase signal to various degrees, which certainly was not anticipated. From this data, the succinylated polyamide **44** (listed as compound C in this study) actually seems to have the most drastic effect, followed by the active polyamide isoxazolidine, **ATF-1** (compound A). At 10μM, however, all four compounds appear to elicit a significant increase in luciferase signal.

To investigate this system further, the concentration could be increased, to determine if the luciferase signal continues to increase, especially with no observable

toxicity via the WST-1 assay. However, an increase in concentration would require significantly more compound, necessitating the use of a sizeable portion of the relatively precious polyamide-isoxazolidine compounds. However, polyamide **43** (compound D) was available in large quantities, making it an ideal candidate to probe these results.

In the next experiment, the concentration of polyamide was varied from 100nM to 50μM, and the procedure was repeated in the same manner as above.





43

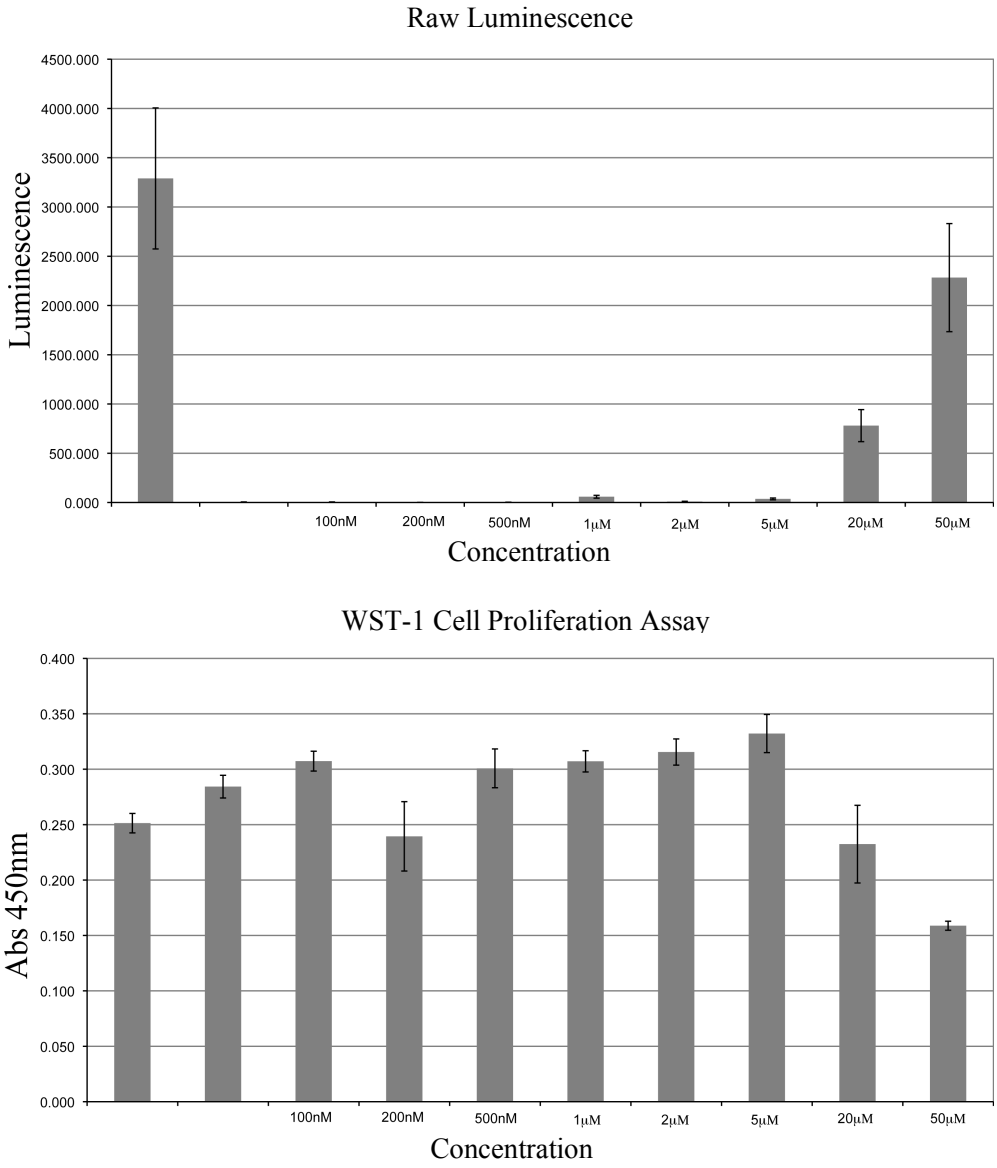


Figure 5.30: LNAR+ assay results for **43**.

In this experiment, **43** elicited an extremely high luciferase signal, comparable to that of DHT at 50μM. In addition, there was observable cytotoxicity at this concentration. However, this polyamide has been previously shown to downregulate AR transcription,[11] so it seems unlikely that it would be directly activating transcription of the luciferase gene at the ARE. It is speculated that perhaps at these high concentrations there is some other global effect, causing downstream events that lead to activation of transcription at the ARE. This is speculation however, as there is no known process by which this occurs. Regardless

of the reason for this effect, it was determined that it was likely not possible to use this system to determine whether or not a polyamide-isoxazolidine conjugate is directly upregulating transcription, if a luciferase signal is elicited for all polyamide compounds tested.

In addition to the luciferase experiments outlined above, an alternative method to assay for transcriptional activation in an endogenous system was envisioned. In previous work by Muzikar et al.,[31] Py-Im polyamides targeted to the glucocorticoid response element (GRE) have shown the ability to disrupt the binding of glucocorticoid receptor (GR) and inhibit expression of GR target genes such as glucocorticoid-induced leucine zipper (GILZ).

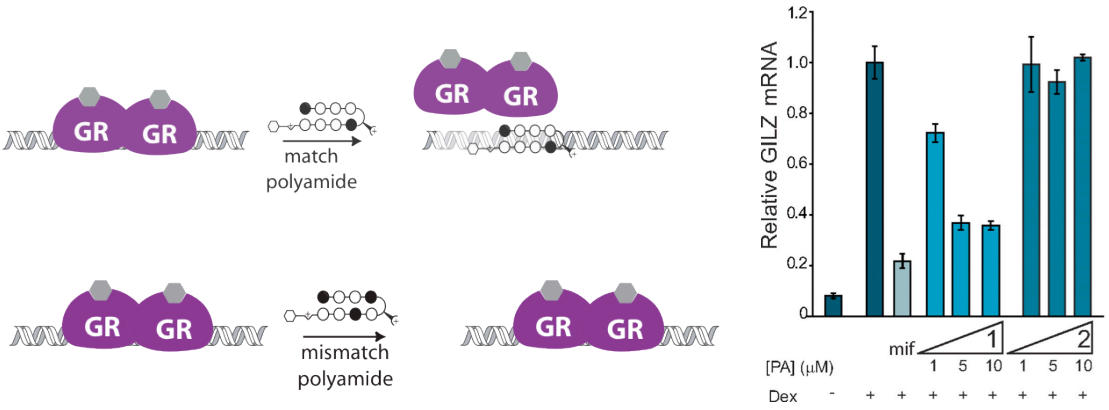


Figure 5.31: Polyamide disruption of glucocorticoid receptor binding, and downregulation of dexamethasone-induced GILZ transcription in A549 lung carcinoma cells.

Conversely, by using a polyamide-isoxazolidine conjugate targeting the same sequence of the GRE, it was envisioned that it might be possible to upregulate the transcription of GILZ. This experiment was performed under similar conditions to those used previously to assess inhibition of GR-mediated expression of GILZ, with 48 hour incubation of A549 cells with polyamide-isoxazolidine conjugates. The levels of GILZ mRNA for both dexamethasone induced and non-induced cells were determined using

quantitative real-time reverse transcriptase PCR relative to β-glucuronidase, an endogenous control, performed in collaboration with Katy Muzikar.

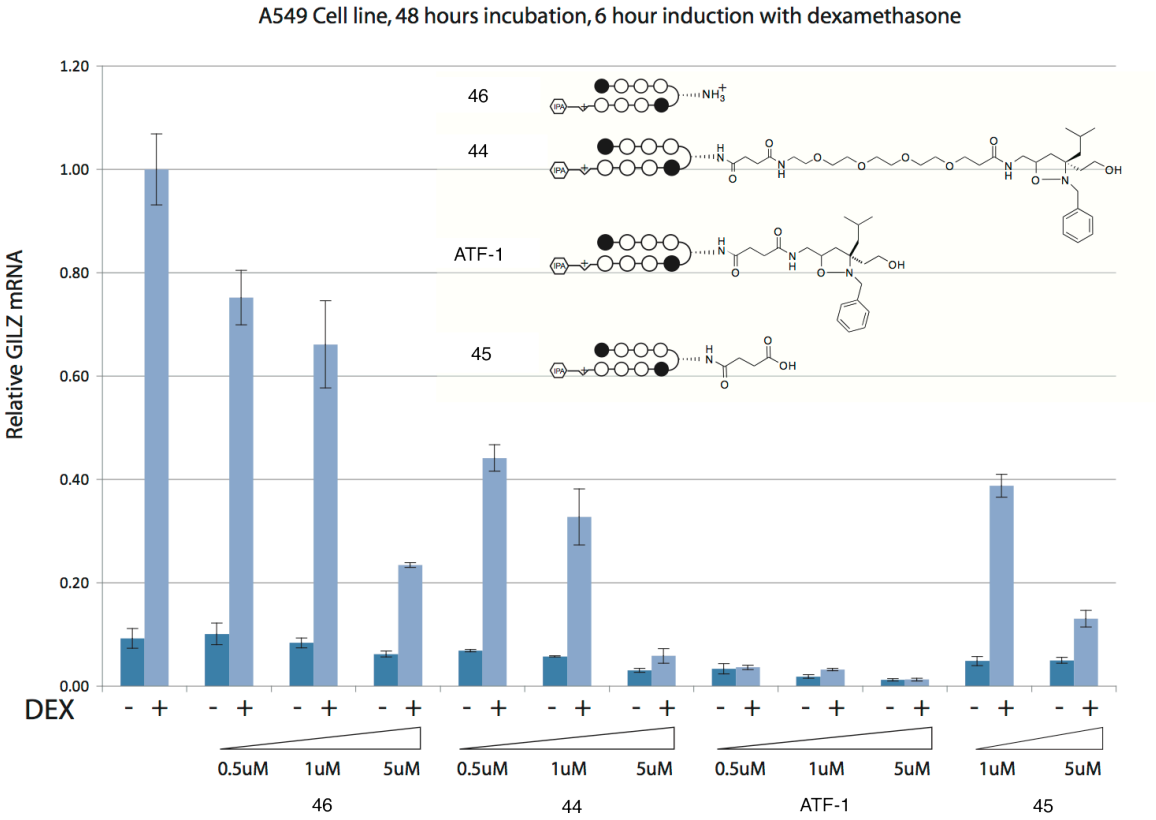


Figure 5.32: Quantitative real-time reverse transcriptase PCR of A549 cells dosed with polyamide-isoxazolidine compounds.

Based on these data, it appears that there is no significant increase in levels of GILZ mRNA in any of the compounds studied, including the polyamide-isoxazolidine conjugates. For the non-induced systems, low levels of GILZ mRNA were seen across the panel of compounds, and for the dexamethasone-induced cells, GILZ mRNA levels were either low or decreased with increasing concentration of polyamide, consistent with polyamide-based disruption of the GR-DNA interface and concentration dependent repression of GILZ expression.

Finally, a cell-free assay was envisioned as another route to determine if polyamide-isoxazolidine conjugates were able to activate transcription *in vitro*. Based on previous studies,[13-15] the plasmid pMLΔ53, containing a g-less cassette reporter gene upstream of a TATA box and known ligation sites, was chosen and obtained via a generous gift of the laboratory of Cheng-Ming Chiang at the University of Texas Southwestern Medical Center. Following previous protocols, construction of a plasmid for gene activation experiments was attempted via insertion of a synthetic oligonucleotide containing the desired WGWWCW binding sites into a Bgl2 restriction site. After initial cloning attempts failed, subsequent sequencing results indicated there was no Bgl2 restriction site in this plasmid, despite it bearing the same name as a previous plasmid with a known Bgl2 site. Another strategy was attempted via insertion of a synthetic oligonucleotide between EcoRI and XmaI restriction sites, which were identified via sequencing, and were upstream of the TATA box and g-less cassette. However, in all further attempts to assay *in vitro* transcription, even basal levels of transcription of the g-less cassette were not observable as expected based on previous studies with the original pMLΔ53 plasmid. Further attempts to obtain the original plasmid with a Bgl2 restriction site were unsuccessful.

Conclusions

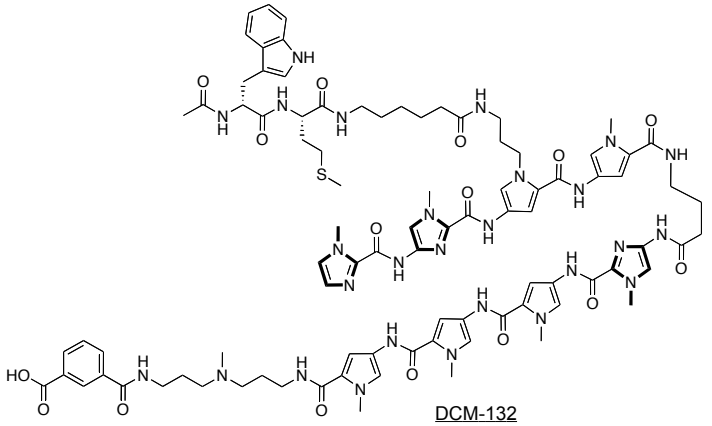
A general method for the synthesis of large quantities of polyamides functionalized for coupling to isoxazolidines has been accomplished. The entire solution-phase synthetic route is mapped out, and is likely applicable to other synthetic modulations for future studies (e.g. different polyamide core, alternative TADs, etc.). Conditions for attachment of various linkers and conjugation of several isoxazolidines moieties to polyamides have been optimized. However, based on multiple cell-culture assays, no significant levels of transcriptional upregulation have been observed to date.

While it is possible that the chosen geometry of the polyamide-isoxazolidine compounds is ineffective at activating transcription, a series of tail-conjugated polyamide-isoxazolidine compounds have been synthesized and studied by Dr. Daniel Gubler. In similar cell culture studies, these compounds also failed to show any levels of transcriptional upregulation. It is currently not fully understood exactly why polyamide-isoxazolidine conjugates were unsuccessful as artificial transcription factors, though it is speculated that the large size of these conjugates may be a negative determinant for cellular uptake, and this could mean that the levels of intracellular concentration are never high enough for significant effects to be observed. Other effects such as geometric constraints or protein binding may also be inhibiting the functionality of these compounds, and further studies will be necessary to deconvolute these various factors.

Section 5B: Synthesis of WM Conjugates

Background

In addition to studying polyamide-isoxazolidine conjugates, another project involving transcriptional activation was also undertaken: a collaboration with Professor Aseem Ansari's laboratory (University of Wisconsin). This involved the synthesis of a "WM" compound (so called because it contains tryptophan and methionine moieties) conjugated to a polyamide. These conjugates can facilitate Exd-DNA dimerization, analogous to the compound shown in Figure 5.3.



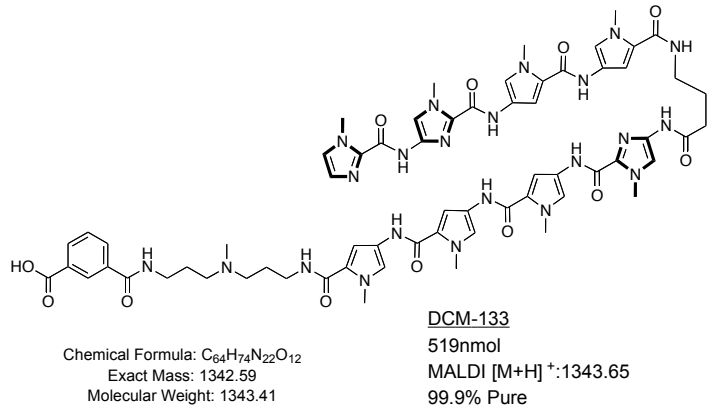


Figure 5.33: The WM compound **DCM-132** and control **DCM-133** that were synthesized. **DCM-132** contains a natural methionine and a tryptophan with D stereochemistry.

These compounds were previously synthesized by Dr. Ryan Stafford of the Dervan group, and were shipped to the Ansari laboratory. Using **RLSiv-279A** (Dr. Stafford's version of **DCM-132**) in Jurkat cells, a reporter luciferase assay was used to assay for gene activation. This method indicated a 2-fold activation of transcription at 1μM. To determine whether this effect was concentration dependent, more material was necessary to allow higher concentrations to be assayed. However, the previously shipped material had nearly all been used in these initial experiments. Leslie Donato of Professor Ansari's group asked if the Dervan laboratory could synthesize more of this compound, to be shipped to the Ansari laboratory.

Following Dr. Stafford's protocol, these compounds were synthesized using Boc-solid-phase polyamide synthesis, as mentioned previously. The synthesis of control compound **DCM-133** was relatively straightforward: after cleavage from resin and preparatory HPLC purification, PyBOP coupling of isophthalic acid yielded the final product, which was again purified by preparatory HPLC. In the case of **DCM-132**, a phthalimide-protected amino group linked to a pyrrole was used as one of the monomers during the synthesis, to provide the handle for peptide conjugation as seen in Figure 5.34. In addition, after cleavage from resin, and preparatory HPLC purification, three more synthetic steps were required: coupling of the peptide fragment, deprotection of the Boc group on the tail, and isophthalic acid coupling. The final products were then purified via preparatory HPLC.

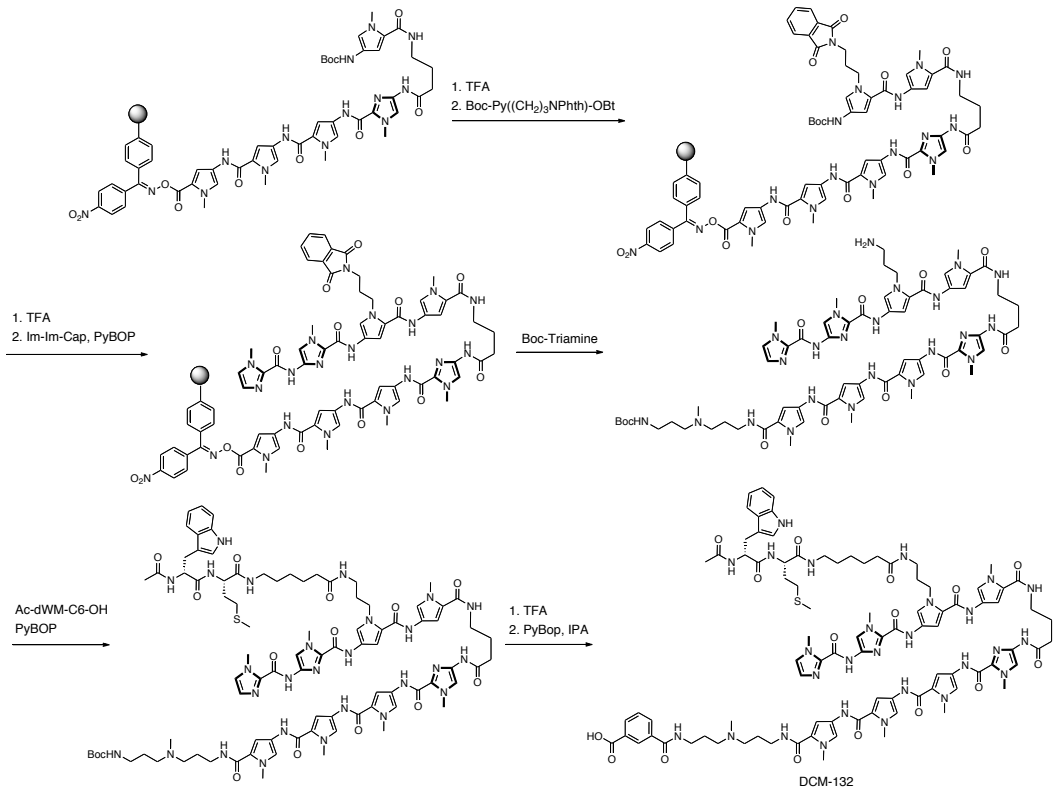


Figure 5.34: Synthesis of WM conjugate.

These compounds were synthesized in reasonable quantities (377nmol and 519nmol for **DCM-132** and **DCM-133** respectively), with comparable yields to those previously reported. After their synthesis, these compounds were sent to the University of Wisconsin. Unfortunately, further luciferase assays employing increased concentrations did not increase the luciferase signal substantially.

Materials and Methods

Polyamides were synthesized using Kaiser oxime resin (0.56 mmol/g) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) from Novabiochem. Z-ß-Dab(Boc)-OH was purchased from Peptides International. Monomers and dimers for solid phase synthesis were synthesized using published protocols.[24,32] *N,N*-dimethylformamide (DMF), Dichloromethane (DCM), methanol (MeOH), trifluoroacetic acid (TFA), acetic anhydride, *N,N*-diisopropylethylamine (DIEA), 2.0M HCl in Ether, succinic anhydride, 1,4-dioxane, and diethyl ether were purchased from Aldrich. 1M aqueous NaOH, 1M aqueous HCl, solid sodium sulfate (Na_2SO_4), and solid sodium bicarbonate ($NaHCO_3$) were purchased from EMD Chemicals.

All NMR spectroscopy data was obtained on a 500MHz Varian instrument. Analytical high-pressure liquid chromatography (HPLC) was performed using a Beckman Gold system with a Phenomenex Gemini C18 column, using 0.1% TFA/water (A) and acetonitrile (B) solvent system. Preparatory HPLC purification was performed using the same solvents on an Agilent Technologies 1200 Series system using a Phenomenex Gemini C18 column. Matrix-assisted laser desportion/ionization time of flight mass spectrometry (MALDI-TOF) was performed on an Applied Biosystems Voyager DE Pro spectrometer. UV-vis were recorded in water and a minimum of acetonitrile using an Agilent 8453 spectrophotometer.

Polyamide synthesis was performed as previously reported.[24,25] Briefly, oxime resin was swelled by flushing with DMF in a peptide synthesis vessel containing a cap, stopcock, and frit. Initial pyrrole loading of the resin occurred overnight (16 hrs) at 37° C. In general, couplings lasted 2 hours. Boc-protecting groups were removed with 20% TFA

in DCM for 25 min at r.t. Carboxylic acids (3 eq) were preactivated with PyBOP (3.5 eq) and DIEA (5 eq) for 30 min in DMF. Cleavage of the polyamide from resin was performed using 1 mL of triamine for 200 mg of resin, allowing the reaction to proceed at 55°C for 12 hours. Preparatory HPLC was used to purify polyamides after cleavage. Mono-*tert*-butyl isophthalic acid or isophthalic acid (10eq) were coupled to polyamide using PyBOP (3eq) and DIEA (5eq).

Solution Phase Polyamide Synthesis

(R)-tert-butyl 3-(3-((3-(4-(4-(4-(4-(3-(benzyloxycarbonylamino)-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)butanamido)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)propyl)-(methyl)amino)propylcarbamoyl)benzoate **(7).** To carboxylic acid **11** (310 mg, 0.255mmol) dissolved in 1mL of DMF was added DIEA (178 µL, 1.02mmol) and PyBOP (265mg, 0.510mmol) and allowed to stir at room temperature for 30 minutes. To this mixture was next transferred a solution of **10** (178mg, 0.510mmol) in 200µL DMF and DIEA (8µL, 2eq) and allowed to stir for 2 hours. Reaction was monitored by HPLC. Upon completion, the reaction mixture was poured into 20mL of H_2O, transferred to Eppendorf tubes, and centrifuged at 13,000rpm for 10 minutes. The tan solid was then washed twice more and centrifuged, providing 368mg of polyamide **7** (94% yield), which was 91% pure by HPLC; MALDI-TOF: Calc. Mass: 1546.70 $[M+H]^+$:1547.71, $[M+Na]^+$:1570.53

(R)-4-(4-(2-(5-(5-(5-(3-((3-(3-(tert-butoxycarbonyl)benzamido)propyl)(methyl)amino)-propylcarbamoyl)-1-methyl-1H-pyrrol-3-ylcarbamoyl)-1-methyl-1H-pyrrol-3-ylcarbamoyl)-1-methyl-1H-pyrrol-3-ylcarbamoyl)-1-methyl-1H-imidazol-4-ylamino)-1-(1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-4-oxobutan-2-ylamino)-4-oxobutanoic acid **(9).** To 4.9mg of Pd/C (1eq) in a glass vial fitted with a teflon septum was added Cbz-protected **7** (5 µmol, 1 eq) dissolved in 850µL of 1,4-Dioxane and 800µL of water.

To this mixture was added ammonium formate (31.5mg, 200eq), and the solution was stirred overnight at 60°. Reaction was monitored by analytical HPLC, and transferred to Eppendorf tubes. After centrifugation at 13,000rpm, supernatant was collected. Pellet was washed twice with 4:1 1,4-dioxane:water, these samples were centrifuged and supernatants combined. After a final centrifuge spin, supernatant was poured into a scintillation vial, and excess solvent was removed via rotary evaporation, then placed on the high vacuum line for 4 hours. Next, 500µL of DMF were added along with DIEA (43.5µL, 50eq). To this mixture was added succinic anhydride (2.5mg, 5eq), and stirred at room temperature for 1 hour. Reaction was monitored via HPLC. Preparatory HPLC purification yielded 3.0 mg of **9** (87%), which was 98% pure by HPLC; MALDI-TOF: Calc. Mass:1512.68 $[M+H]^+$:1513.69.

Tert-butyl 3-(3-((3-aminopropyl)(methyl)amino)propylcarbamoyl)benzoate **(10).** To a solution of **15** in 15mL DCM was added HOBt (334mg, 1.1eq), then DCC (510mg, 1.1eq), and this solution was stirred for 1 hr. Solution filtered to remove urea byproduct, then diluted by the addition of 250mL DCM, and added to an addition funnel. This was dripped into a solution of triamine **16** (7.25mL, 20eq) and 5mL DCM over 3.5 hours. Rotary evaporation, and extraction to remove excess **16,** yielded 362mg of **10**. ^{1}H NMR [499.8 MHz, DMSO-d6]: δ 8.68 (t, 1H), 8.31 (s, 1H), 8.01 (m, 2H), 7.58 (dd, 1H), 3.28 (m, 2H), 2.32 (m, 4H), 2.05 (s, 3H), 1.67 (m, 2H), 1.52 (s, 9H), 1.50 (m, 2H), 1.46 (m, 2H). ESI-MS: Calc. Mass: 349.2 $[M+H]^+$:350.3.

(R)-4-(4-(4-(4-(3-(benzyloxycarbonylamino)-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)butanamido)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxylic acid **(11).** To 50mg of **25** in a scintillation vial was added 1mL 1,4-dioxane, then 1mL 1N aqueous NaOH. Solution was stirred at r.t. for 15 hours, then poured into Falcon tube. 1N aqueous HCl was added until pH<4, and solid **11** precipitates to form a tan brown gel. Centrifugation was performed, the supernatant poured off, and the solid was washed twice with cold water, each time centrifuging and removing supernatant. Product was next lyophilized to yield 42 mg of **11** (86%). 91% pure by HPLC. MALDI-TOF: Calculated Mass: 1215.47 $[M+H]^+$:1216.65 $[M+Na]^+$:1238.63 $[M+K]^+$:1254.59.

1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxylic acid **(12).** See procedure for compound **11**. 98%

yield. ^{1}H NMR [499.8 MHz, DMSO-d6]: δ 10.47 (s, 1H), 9.97 (s, 1H), 9.92 (s, 1H), 7.45 (s, 1H), 7.40 (s, 1H), 7.27 (s, 1H), 7.22 (s, 1H), 7.18 (s, 1H), 7.06 (s, 1H), 7.04 (s, 1H), 6.91 (s, 1H), 3.99 (s, 3H), 3.81 (s, 3H), 3.79 (s, 3H). ESI-MS: Calc. Mass: 492.2 $[M+H]^+$:493.2.

((R)-ethyl 4-(4-(4-(4-(3-(benzyloxycarbonylamino)-4-(tert-butoxycarbonylamino)butanamido)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxylate **(24).** To a solution of Z-ß-Dab(Boc)-OH (350mg, 1.33eq) in 252µL DIEA (2eq) and 10mL DMF was added PyBOP (517mg, 1.33eq), and this solution was stirred for 30 minutes. A separate solution of **23** mixed with 252µL DIEA (2eq) and 5mL DMF was then added to the first solution. Mixture was stirred at room temperature for 16 hours. Rotary evaporation removed as much DMF as possible, and this viscous oil was purified using silica gel chromatography, eluted with 9:1 EtOAc:Hexanes, yielding 506mg of **24** (78%). ^{1}H NMR [499.8 MHz, DMSO-d6]: δ 10.19 (s, 1H), 10.02 (s, 1H), 9.97 (s, 1H), 9.92 (s, 1H), 7.45 (s, 1H), 7.43 (s, 1H), 7.35-7.23 (m, 6H), 7.13 (s, 1H), 7.09-7.02 (m, 2H), 6.90 (s, 1H), 4.98 (s, 2H), 4.20 (q, 2H), 3.96 (s, 3H), 3.89-3.80 (m, 9H), 3.05 (m, 2H), 2.47 (m, 2H), 1.37 (s, 9H), 1.26 (t, 3H). ESI-MS: Calc. Mass: 869.4 $[M+H]^+$:870.3.

(R)-ethyl 4-(4-(4-(4-(4-amino-3-(benzyloxycarbonylamino)butanamido)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxylate **(13).** To a stirred solution of **24** (274mg, 1eq) in DCM (5mL) was added 2M HCl in Et_2O (5mL). The resulting suspension was stirred for 7 hours at room temperature. Reaction mixture was concentrated via rotary evaporation, affording 251mg of **13** (99% yield). ^{1}H NMR [499.8 MHz, DMSO-d6]: δ 10.47 (s, 1H), 9.97 (s, 1H), 9.92 (s, 1H), 7.45 (s, 1H), 7.40 (s, 1H), 7.27 (s, 1H), 7.22 (s, 1H), 7.18 (s, 1H), 7.06 (s, 1H), 7.04 (s, 1H), 6.91 (s, 1H), 3.99 (s, 3H), 3.81 (s, 3H), 3.79 (s, 3H). ESI-MS: Calc. Mass: 769.3 $[M+H]^+$:770.1.

3-(tert-butoxycarbonyl)benzoic acid **(15)** Compound was synthesized as reported in the literature.[26] Briefly, to a solution of isophthaloylchloride (30g, 0.15mol) in 250mL of THF was added *tert*-butanol (13.8mL, 0.15mol) and pyridine (26.3mL, 0.33mol), and this mixture was refluxed for 6 hours. Next, D.I. H_2O was added, and the mixture was refluxed again for 6 hours. After quenching, extraction, and concentration, recrystallization from toluene yielded 16.6g of large needle like crystals. (52% yield). ^{1}H NMR [499.8 MHz,

DMSO-d6]: δ 8.39 (s, 1H), 8.12 (m, 2H), 7.62 (dd, 1H), 1.54 (s, 9H). ESI-MS: Calc. Mass: 222.1 $[M+H]^+$:223.1.

Ethyl 1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxylate **(21).** To trimer **20** (400mg, 1eq) in 5mL DMF and 304μL of DIEA (2eq) was added Im-CCl_3 (228mg, 0.95eq) dissolved in 5mL DMF and 304μL of DIEA (2eq). This solution was stirred 16 hours at room temperature. Reaction mixture was added to 3.6mL 1M aqueous HCl (4eq) in 200mL of water, creating a dark brown precipitate. Solid was filtered and washed with copious amounts of water. Solid was transferred to a separatory funnel along with 80mL of 1%MeOH in EtOAc. Organic layer was washed with 20mL brine, dried over sodium sulfate, filtered, and the solvent removed via rotary evaporation. ^{1}H NMR [499.8 MHz, DMSO-d6]: δ 10.47 (s, 1H), 9.97 (s, 1H), 9.92 (s, 1H), 7.45 (s, 1H), 7.40 (s, 1H), 7.27 (s, 1H), 7.22 (s, 1H), 7.18 (s, 1H), 7.06 (s, 1H), 7.04 (s, 1H), 6.91 (s, 1H), 4.39 (q, 2H), 3.99 (s, 3H), 3.81 (s, 3H), 3.79 (s, 3H), 1.27 (t, 3H). ESI-MS: Calc. Mass: 520.2 $[M+H]^+$:521.4.

Ethyl 4-(4-(4-(4-(tert-butoxycarbonylamino)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxylate **(22).** To a solution of Boc-Im-COOH (102.7mg, 0.95eq) dissolved in 4.5mL DMF and 152μL of DIEA (2eq) was added HBTU (162mg, 0.95eq). Solution stirred at room temperature for 20 minutes. This solution was added to trimer **20** (200mg, 1eq) in 3mL DMF and 152μL of DIEA (2eq). This mixture was stirred 16 hours at room temperature. Reaction mixture was added to 1.8mL 1M aqueous HCl (4eq) in 93mL of water, creating a dark brown precipitate. Solid was filtered and washed with copious amounts of water. Purified via silica gel chromatography, to yield 220mg of **22** (81%). ^{1}H NMR [499.8 MHz, DMSO-d6]: δ 9.95 (s, 3H), 9.92 (s, 1H), 7.43 (s, 1H), 7.27 (s, 1H), 7.24 (m, 2H), 7.14 (s, 1H), 7.08 (s, 1H), 6.94 (s, 1H), 4.20 (q, 2H), 3.93 (s, 1H), 3.82 (m, 3H), 1.46 (s, 9H), 1.27 (t, 3H). ESI-MS: Calc. Mass: 635.3 $[M+H]^+$:636.3.

Ethyl 4-(4-(4-(4-amino-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxylate **(20).** To Boc-protected compound **22** (431mg, 0.678mmol) was added 2.5mL of 1,4-Dioxane. This mixture was added to 40mL of 2.0M HCl/Et_2O, forming a suspension, which was stirred for 3 hours. Solid was collected via vacuum filtration and left under high vacuum to yield 376mg of **23** (97%). ^{1}H NMR [499.8 MHz, DMSO-d6]: δ 9.95 (s, 3H), 7.43

(s, 1H), 7.27 (s, 1H), 7.24 (m, 2H), 7.14 (s, 1H), 7.08 (s, 1H), 6.94 (s, 1H), 4.20 (q, 2H), 3.93 (s, 1H), 3.82 (m, 3H), 1.27 (t, 3H). ESI-MS: Calc. Mass: 448.2 $[M+H]^+$:449.1.

(R)-ethyl 4-(4-(4-(4-(3-(benzyloxycarbonylamino)-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)butanamido)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxylate **(25).** To carboxylic acid **12** (35mg, 1eq) was added 500μL DMF, 25μL DIEA (4eq), and PyBOP (37mg, 1eq), and the mixture was stirred for 20 minutes. To this a solution of **13** (58mg, 1eq) along with 500μL DMF and 25μL DIEA were added. Stirred at room temperature for 16 hours. Reaction mixture poured into a solution of 0.58mL 1M HCl (8eq) in 20mL water. A suspension formed, and the solid was centrifuged and lyophilized to yield 68mg of **25** (76% yield). Compound was 85% pure by HPLC. MALDI-TOF: Calculated Mass: 1243.51, $[M+H]^+$:1244.51, $[M+Na]^+$:1266.49, $[M+K]^+$:1282.46.

Isoxazolidine Coupling Reactions

Tert-butyl 3-(3-((3-(4-(4-(4-(4-((R)-3-(4-(((3S,5R)-3-allyl-2-benzyl-3-isobutylisoxazolidin-5-yl)methylamino)-4-oxobutanamido)-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)butanamido)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)propyl)(methyl)amino)propylcarbamoyl)benzoate **(29).** To isoxazolidine **RJC 6-1** (5.6mg, 5eq) was added 100μL of DCM. 100μL of TFA was next added dropwise, and the resulting mixture was stirred for 5 minutes. After concentration via rotary evaporation, 100μL of DMF was added, followed by DIEA (2.25μL, 5eq). In a separate vial, 2.59μmol of polyamide **9** was dissolved in 200μL DMF, and DIEA (0.9μL, 2 eq) followed by PyBOP (2.7mg, 2eq) were added, and the mixture was stirred for 2 minutes. To this solution was added the solution of deprotected isoxazolidine, and the resulting mixture was stirred for 16 hours at 23°C. After determination of completion by analytical HPLC, the solution was poured into 5mL H_2O. This solution was poured into a Waters C18 Sep-Pak, and flushed with 10% acetonitrile in H_2O with 0.1% TFA to remove any DMF and other highly polar contaminants. A 1:1 acetonitrile: H_2O w/ 0.1% TFA wash

was then used to elute the desired product. The resulting solution was next lyophilized to dryness directly into a ½ dram glass vial, and the crude material was used directly in the following step. Crude **29** was 64% pure by HPLC, and was directly used in the following step.

Tert-butyl 3-(3-((3-(4-(4-(4-(4-(4-((R)-3-(4-(((3S,5R)-2-benzyl-3-(2-hydroxyethyl)-3-isobutylisoxazolidin-5-yl)methylamino)-4-oxobutanamido)-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)butanamido)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)propyl)(methyl)amino)propylcarbamoyl)benzoate **(26).** See procedure for compound **29**. 2.1μmol of **9** used, with 2.9 mg (3.5eq) of **RJC 6-3** used. Crude material carried forward. 55% pure by HPLC.

3-(3-((3-(4-(4-(4-(4-(4-((R)-3-(4-(((3S,5R)-3-allyl-2-benzyl-3-isobutylisoxazolidin-5-yl)methylamino)-4-oxobutanamido)-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)butanamido)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)propyl)(methyl)amino)propylcarbamoyl)benzoic acid **(ATF-4).** To crude **29** lyophilized in a glass vial was added acetonitrile (500μL) and the resulting suspension was stirred. To this suspension was added NaI (5.75mg, 15eq), followed by $CeCl_3 \bullet 7H_2O$ (29mg, 30eq). This solution was refluxed for 1 hour, at which point it was poured into 3mL H_2O, and partially purified via Waters C18 Sep-Pak as in previous step. The resulting solution was concentrated to remove most of the acetonitrile, and taken up in 1:4 acetonitrile:H_2O with 0.1% TFA, and purified via preparatory HPLC, yielding 543nmol (21% yield from **9**) of ATF-4. MALDI-TOF: Calc. Mass: 1726.83, $[M+H]^+$:1727.47. 99% pure by HPLC.

3-(3-((3-(4-(4-(4-(4-(4-((R)-3-(4-(((3S,5R)-2-benzyl-3-(2-hydroxyethyl)-3-isobutylisoxazolidin-5-yl)methylamino)-4-oxobutanamido)-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)butanamido)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)propyl)(methyl)amino)propylcarbamoyl)benzoic acid **(ATF-1).** See procedure for synthesis of

ATF-4. Isolated 352nmol (17% yield from **9**) MALDI-TOF: Calc. Mass: 1730.82, $[M+H]^+$:1731.65. 99% pure by HPLC

Tert-butyl 3-(3-((3-(4-(4-(4-(4-((R)-1-((3S,5R)-2-benzyl-3-(2-hydroxyethyl)-3-isobutylisoxazolidin-5-yl)-17-((1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)methyl)-3,12,15-trioxo-5,8-dioxa-2,11,16-triazanonadecanamido)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)propyl)(methyl)amino)propylcarbamoyl)benzoate **(27)**. See procedure for synthesis of **29**. Used 482nmol of **9**, and 0.32mg isoxazolidine (2eq). Crude material carried forward. 37% pure by HPLC.

3-(3-((3-(4-(4-(4-(4-((R)-1-((3S,5R)-2-benzyl-3-(2-hydroxyethyl)-3-isobutylisoxazolidin-5-yl)-17-((1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-1H-imidazole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)-1H-pyrrole-2-carboxamido)methyl)-3,12,15-trioxo-5,8-dioxa-2,11,16-triazanonadecanamido)-1-methyl-1H-imidazole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)-1-methyl-1H-pyrrole-2-carboxamido)propyl)(methyl)amino) propylcarbamoyl)benzoic acid **(ATF-2).** To lyophilized **27** was added 100μL of 1:1 DCM:TFA. This solution was agitated for 10 minutes, then concentrated via rotary evaporation. To the resulting residue was added 1:4 acetonitrile:H_2O with 0.1% TFA, and preparatory HPLC chromatography afforded 26nmol of **ATF-2** (6% yield from **9**). MALDI-TOF: Calc. Mass:1875.90, $[M+H]^+$:1877.17. 97% pure by HPLC.

Isoxazolidine Scale-up

tert-butyl hypochlorite **(32).** A solution of commercial household bleach (500mL) was stirred at 0°C in a large Erlenmeyer flask. The flask was covered with aluminum foil and lights in the vicinity were turned off, while a solution of *tert*-butanol (37mL, .39mol) and glacial acetic acid (24.5mL, .43mol) was added in a single portion. This solution was stirred for 3 minutes, then washed with 10% $NaHCO_3$ followed by water, and the organic layer was collected and dried over $CaCl_3$, and used directly in subsequent steps without further purification, affording 30g of *tert*-butyl hypochlorite (71% yield). Prepared according to the method of Mintz and Walling.[33]

(E)-3-methylbutanal oxime **(34).** Followed procedure published by Kubanov et al.[34] Briefly, aldehyde **33** (20g, 0.23mol) was refluxed in 200mL Benzene, with hydroxylamine hydrochloride (16.9g, 0.244mol) and Na_2CO_3 (25.819g, 0.244mol) for 5 hours. After extraction, the residue was purified via fractional distillation to yield 11.2g of oxime **34** (48% yield). ^{1}H NMR [499.8 MHz, $CDCl_3$]: δ 7.45, 6.76 (t, 1H), 2.10 (dd, 2H), 1.92-1.81 (m, 1H), 0.96 (m, 6H).

(R)-(3-isobutyl-4,5-dihydroisoxazol-5-yl)methanol **(35).** To a stirring solution of oxime **34** (3g, 29.6 mmol) in 60mL of toluene at -78°C was added **32** (3.35mL, 29.6mmol) drop wise and this mixture was stirred for 2 hours. In a separate flask, allyl alcohol (2.62mL, 38.5mmol) was stirred at 0°C in 120mL of toluene. To this mixture was added *tert*-BuOH (9.27 mL, 97.7 mmol) followed by drop wise addition of a 3.0M solution of EtMgBr in Et_2O (29.6 mL, 88.8mmol), and this mixture was stirred for 1 h. The solution of hydroximinoyl chloride was then transferred via cannula to the allylic alcohol solution and the mixture allowed to warm to room temperature and stirred for 15 h. After quenching with NH_4Cl and H_2O, The organic layer was washed with DCM (3X) and the combined organic extracts were combined, dried, concentrated, and purified via silica gel chromatography. This provided 3.58g of isoxazoline **35** (77% yield). ^{1}H NMR [499.8 MHz, $CDCl_3$]: δ 4.62-4.68 (m, 1H), 3.76 (dd, 1H), 3.54 (dd, 1H), 2.93 (dd, 1H), 2.80 (dd, 1H), 2.21-2.19 (m, 2H), 1.92-1.86 (m, 2H), 0.95 (d, 3H), 0.93 (d, 3H).

((3S,5R)-3-allyl-3-isobutylisoxazolidin-5-yl)methanol **(36).** To a solution of isoxazoline **35** (200mg, 1.27mmol) in 10mL of toluene cooled to -78°C was added BF_3•OEt_2 (484μL, 3.82mmol) drop wise, and the solution was stirred for 30 minutes. A 2.0M solution of allylmagnesium chloride (4.45mL, 8.91mmol) was next added drop wise. After stirring for 4 hours, saturated $NaHCO_3$ was added, and the solution was extracted with EtOAc. The organic extracts were combined, washed with H_2O and brine, dried over Na_2SO_4, concentrated, and purified via flash column chromatography, yielding 230mg of isoxazolidine **36** (91% yield). ^{1}H NMR [499.8 MHz, $CDCl_3$]: δ 5.87-5.80 (m, 1H), 5.12-5.08 (m, 2H), 4.21-4.17 (m, 1H), 3.69 (dd, 1H), 3.53 (dd, 1H), 2.36 (dd, 1H), 2.24-2.19 (m, 2H), 1.84-1.77 (m, 1H), 1.62 (dd, 1H), 1.44 (dd, 1H), 1.38 (dd, 1H), 0.94 (d, 3H), 0.92 (d, 3H). ESI-MS: Calc. Mass: 199.2 $[M+Na]^+$:222.2.

(***3S,5R)-3-allyl-5-((tert-butyldimethylsilyloxy)methyl)-3-isobutylisoxazolidine*** **(37).** To a solution of isoxazolidine **36** (170mg, 0.85mmol) in 50mL of THF at 0°C were added DMAP (5.3mg, 0.0427mmol) and Et_3N (166 μL, 1.19mmol), followed by drop wise addition of TBSOTf (274μL, 1.19mmol). The mixture was

next warmed to room temperature, and stirred for 2 hours. It was next cooled to 0°C and quenched with saturated NH_4Cl, and extracted with Et_2O. The combined organic extracts were next washed with brine, dried, and concentrated via rotary evaporation. Silica gel chromatography yielded 251mg of **37** (94% yield). ^1H NMR [499.8 MHz, $CDCl_3$]: δ 4.63–4.59 (m, 1H), 3.69–3.62 (m, 2H), 3.56 (dd, 1H), 2.88 (d, 2H), 2.37–2.25 (m, 2H), 1.14 (t, 3H), 0.88 (s, 9H), 0.85 (s, 9H), 0.07 (s, 3H), 0.06 (s, 3H), 0.04 (s, 3H), 0.04 (s, 3H). ESI-MS: Calc. Mass: 313.2 $[M+H]^+$:314.2.

((3S,5R)-3-allyl-2-benzyl-3-isobutylisoxazolidin-5-yl)methanol **(38)**. To a solution of **37** (220mg, 0.702mmol) in 2 mL of DMF was added DIEA (367μL, 2.10mmol) followed by BnBr (417μL, 3.508mmol). The solution was next irradiated in a 1000 W microwave (5 x 15 s) @ 30% power with mixing after each cycle. The reaction was then diluted with H_2O, and extracted with Et_2O (3X). The organic extracts were combined and washed with H_2O and brine, dried over Na_2SO_4, filtered, and concentrated via rotary evaporation. The crude residue was next dissolved and stirred in 1mL THF, and a 1.0M solution of TBAF (1.05mL, 1.05mmol) was added drop wise. This solution was stirred for 4 hours, at which point H_2O was added, and it was extracted with EtOAc (3X). The combined organic extracts were washed with brine, dried, and concentrated. Silica gel chromatography provided pure 166mg of pure **38** (82% yield over two steps). ^1H NMR [499.8 MHz, $CDCl_3$]: δ 7.40-7.20 (m, 5H), 5.98-5.88 (m, 1H), 5.09-5.13 (m, 2H), 4.02-4.10 (m, 1H), 3.90 (d, 1H), 3.83 (d, 1H), 3.61-3.54 (m, 2H), 2.45 (dd, 1H), 2.28 (dd, 1H), 2.18-2.24 (br s, 1H), 2.01-2.08 (m, 1H), 1.87-1.93 (m, 1H), 1.62 (dd, 1H), 1.39 (dd, 1H), 0.98 (d, 3H), 0.97 (d, 3H). ESI-MS: Calc. Mass: 289.2 $[M+H]^+$:290.1.

Linker Synthesis

2-(2-(2-aminoethoxy)ethoxy)-N-(((3S)-2-benzyl-3-(2-hydroxyethyl)-3-isobutylisoxazolidin-5-yl)methyl)acetamide **(RJC-6-6).** To a solution of Fmoc-AEEA-COOH (2.6mg, 6.7μmol) in 100μL DMF was added DIEA (3.4μL, 20μmol), followed by PyBOP (3.56mg, 6.8μmol), and this mixture was stirred for 30 minutes at room temperature. Next, a solution of RJC-6-3dep (1mg, 3.4μmol) with DIEA (3.4μL, 20μmol) was added, and this mixture allowed to stir for 16 hours. This mixture was extracted with ethyl acetate, and organic fractions were concentrated. To this crude residue was added 300μL of 20% piperidine in DMF, and

the resulting solution stirred for 30 minutes. Next, the mixture was diluted with methanol, extracted with pentane, and concentrated, followed by preparatory HPLC purification, providing 0.4mg of **RJC-6-6** (27% yield). ^{1}H NMR [499.8 MHz, CD_3OD]: 7.38-7.21 (m, 5H), 4.27-4.17 (m, 1H), 4.00-4.90 (m, 4H), 3.79-3.72 (m, 3H), 3.61-3.43 (m, 6H), 3.37 (dd, 1H), 3.00 (t, 2H), 2.45 (dd, 1H), 2.07-1.98 (m, 1H), 1.90 (t, 2H), 1.85-1.66 (m, 2H), 1.45 (dd, 1H), 1.0 (d, 3H), 0.96 (d, 3H). ESI-MS: Calc. Mass: 437.3 $[M+H]^+$:438.3.

tert-butyl 1-((3S)-2-benzyl-3-(2-hydroxyethyl)-3-isobutylisoxazolidin-5-yl)-3-oxo-6,9,12,15-tetraoxa-2-azaheptadecan-17-ylcarbamate **(41).** To a solution of 2,2-dimethyl-4-oxo-3,8,11,14,17-pentaoxa-5-azaicosan-20-oic acid (12.5mg, 34.3μmol) in 300μL DMF was added DIEA (11.91μL, 68.4μmol) and PyBOP (18.2mg, 35.0μmol). This solution was stirred at 45°C for 30 minutes. Subsequently, a solution of RJC-6-3dep (5mg, 17μmol) with DIEA (5.96μL, 34.2μmol) was added to the activated PEG linker solution. This mixture was allowed to stir overnight. Water and brine were added, and the mixture was extracted with EtOAc (3X), dried, and concentrated. Purification via preparatory TLC afforded 8.1mg of the desired compound **41** (74% yield). ^{1}H NMR [499.8 MHz, $CDCl_3$]: 7.38-7.24 (m, 5H), 4.12-4.05 (s, 1H), 3.94-3.81 (m, 2H), 3.72-3.44 (m, 8H), 3.41-3.30 (m, 2H), 3.28-3.20 (m, 1H), 2.46-2.19 (m, 4H), 1.93-1.80 (m, 2H), 1.68-1.59 (m, 1H), 1.44 (s, 9H), 1.41-1.32 (m, 1H), 1.24 (s, 2H), 0.98 (t, 6H). ESI-MS: Calc. Mass: 639.4 $[M+H]^+$:640.3

Cell Culture

All cell lines were purchased from ATCC (Manassas, VA) and maintained in the following media: A549 cells (F-12K); LNAR+ (RPMI 1640); HCT-116 (McCoy's 5a Medium Modified); MCF-7 (Eagle's Minimum Essential Medium). All media were supplemented with 10% FBS and cultured at 37°C under 5% CO_2.

LNAR+ Luciferase Experiments

LNAR+ cells were plated in 96-well plates at a density of 50,000 cells per mL (5,000 cells/well) in RPMI 1640 medium (ATCC) supplemented with 10% FBS (Irvine Scientific, Santa Ana, CA) and 4 mM penicillin/streptomycin. After 24 h, the medium

was replaced with RPMI 1640 medium containing 10% charcoal-stripped FBS, 4 mM penicillin/streptomycin, and polyamides at varying concentrations in quadruplet. Cells were grown for an additional 34 h before treatment of induced wells with 1nM dihydrotestosterone (DHT) for 6 h. After 40 hours total polyamide treatment, 10μL WST-1 reagent was added, and cells were incubated at 37°C with gentle shaking for 30 min, before measuring the absorbance at 450 nm. Next, cells were lysed by addition of 100μL of Glo Lysis Buffer (Promega), incubated 5 minutes, then 100μL of each well was added to a separate opaque-walled 96-well plate containing 100μL Bright-Glo™ reagent, mixed briefly and gently, and immediately imaged for luminescence.

Measurement of dexamethasone-induced mRNA.

RNA isolation: A549 cells (ATCC) were plated in 24-well plates at a density of 20-25 x 103 cells per well (40-50 x103 cells per mL) in F12-K medium (ATCC) supplemented with 10% FBS (Irvine Scientific, Santa Ana, CA) and 4 mM penicillin/streptomycin. After 24 h, the medium was replaced with F12-K containing 10% charcoal-stripped FBS, 4 mM penicillin/streptomycin, and polyamides or mifepristone at the designated concentrations. Cells were grown for an additional 48 h and then treated with 100 nM dexamethasone for 6 hours. The medium was removed, cells were washed with ice-cold PBS and immediately lysed with RLT buffer from an RNeasy kit (Qiagen). Further RNA isolation was carried out with the RNeasy kit as described in the manufacturer's manual. The isolated total RNA was quantified. The yields were 12-15 μg per well.

Reverse transcription: A 2.5 μg sample of total RNA was used to reverse transcribe cDNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Random hexamers were used as primers. The total volume for each RT reaction was 20 μL.

Real-time quantitative RT-PCR: Analysis was performed using the GILZ gene primers described below, purchased from Integrated DNA Technologies Quantitative real-time RT-PCR was performed with SYBR 66 Green PCR Master Mix (Applied Biosystems, Foster City, CA) following the manufacturer's suggested protocol. Temperature cycling and detection of the SYBR Green emission were performed with an ABI 7300 real-time instrument using Applied Biosystems Sequence Detection System version 1.2. Statistical analysis was performed on three independent experiments. mRNA of the genes of interest were measured relative to β-glucuronidase as an endogenous control. Primer sequences were designed using Primer3.

To amplify the 97-bp fragment from the 3'-translated region of GILZ:

Forward primer: 5'- CTCCCCGTTTGTTTTTCTCA -3'

Reverse primer: 5'- TGCTCCTTCAGGATCTCCAC -3'

To amplify the β-glucuronidase gene as an endogenous control:

Forward primer: 5'- CTCATT TGGAATTTTGCCGATT -3'

Reverse primer: 5'- CCGAGTGAAGATCCCCTTTTTA -3'

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