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**THE DISCOVERY OF SMALL-MOLECULE INHIBITORS OF
KEAP1-NRF2 INTERACTION WITH HOMOGENEOUS
FLUORESCENCE-BASED HIGH THROUGHPUT SCREENING
ASSAYS**

By

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ABSTRACT OF THE DISSERTATION

THE DISCOVERY OF SMALL-MOLECULE INHIBITORS OF

KEAP1-NRF2 INTERACTION WITH HOMOGENEOUS FLUORESCENCE-

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Keap1-Nrf2 interaction is a key protein-protein interaction involved in the activation of antioxidant response element which regulates the expression of cytoprotective enzymes in response to oxidative stress conditions. To identify direct inhibitors of Keap1-Nrf2 interaction, a series of fluorescently-labeled Nrf2 peptides were synthesized and evaluated as tracers in the development of a fluorescence polarization (FP) assay. The optimized tracer, FITC-9mer Nrf2 amide, was determined to have the highest binding affinity and dynamic range of all tracers evaluated. The FP assay has considerable tolerance towards DMSO and was capable of distinguishing the inhibitory Nrf2 peptides with a Z' -factor of 0.70 as determined under the FP assay conditions. Taken together, these results demonstrate that the FP assay is suitable for application in high-throughput screening (HTS).

In addition, a series of novel lanthanide chelates were designed, synthesized, and evaluated for the development of a time-resolved fluorescence resonance energy transfer (TR-FRET) assay using an *in situ* labeling approach. The cyclam and EDTA-based tris-NTA were found to have the strongest affinity to the His₆-tag of Keap1 Kelch domain with a K_d of 1.6 nM and 2.2 nM, respectively. Among the tris-NTA conjugates evaluated as donors of TR-FRET, the EDTA-tris-

NTA Acp₆-linker conjugate was found to have the highest TR-FRET signal. The *in situ* labeling technique was also evaluated for Eu(III) chelate and through biotin-streptavidin interaction, increasing the choice of fluorophores and extending the application of the assay to biotinylated biomolecules. Thus, the novel labeling reagent provides an excellent solution for facile development of TR-FRET assays in drug discovery.

The FP assay was adapted for HTS of the NIH MLPCN library. Eight confirmed hits were obtained and one of the promising hits (LH602) was targeted for preliminary structure-activity relationship (SAR) studies. Among the synthesized compounds, 2,4,6-trimethylbenzenesulfonamide substituted analog **81** showed the highest affinity to Keap1 Kelch domain. However, LH602 was found to be unstable due to its naphthoquinone structure with a half-life of 6 hours in the assay buffer. Further SAR studies are needed to improve the binding affinity as well as stability of the compound in our effort to identify potent inhibitors of Keap1-Nrf2 interaction.

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DEDICATION

*To my wife, my son, and my parents,
for their encouragement throughout
my graduate studies*

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ABBREVIATIONS

<i>A</i>	Anisotropy
Ac	Acetyl
Acp	6-Aminocaproic acid
ACN	Acetonitrile
Ala	Alanine (A)
AMQ	7-Amino-4-methyl-quinolin-2-one (Cs124)
Ap ₃	Propargyl amine
ARE	Antioxidant response element
Arg	Arginine (R)
Asp	Asparagine (N)
Bn	Benzyl (Bzl)
Boc	<i>tert</i> -Butoxycarbonyl
Bodipy	Boron-dipyrromethene
tBu	<i>tert</i> -Butyl
Cbz	Benzyloxycarbonyl
Cs124	Carbostyryl-124 (AMQ)
Cy	Cyanine
Cys	Cysteine (C)
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane (Methylene chloride)
DEA	Diethylamine
DIPEA	Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	<i>N, N</i> - Dimethylformamide

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTPA	Diethylenetriamine pentaacetic acid
EDA	Ethylenediamine
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediamine tetraacetic acid
EtOAc	Ethyl acetate
EpRE	Electrophile response element
FITC	Fluorescein-5-isothiocyanate
Fmoc	9-Fluorenylmethoxycarbonyl
FP	Fluorescence polarization
FRET	Fluorescence (Föster) resonance energy transfer
Gln	Glutamine (Q)
Glu	Glutamic acid (E)
Gly	Glycine (G)
GSH	Glutathione
GST	Glutathione S-transferase
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
His	Histidine (H)
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole
HOSu	<i>N</i> -Hydroxysuccinimide
HPLC	High-performance Liquid Chromatography
HTS	High-throughput screening

Ile	Isoleucine (I)
IMAC	Immobilized metal ion affinity column
iPr	Isopropyl
ISC	Intersystem crossing
ITC	Isothermal titration calorimetry
Keap1	Kelch-like ECH-associated protein 1
Leu	Leucine (L)
Lys	Lysine (K)
mCPBA	m-chloroperoxybenzoic acid
Met	Methionine (M)
NBC	<i>N</i> -Boc-cysteine
NMP	<i>N</i> -Methyl-2-pyrrolidone
Nrf2	Nuclear Factor-Erythroid 2 (NF-E2) related factor 2
NTA	Nitrilotriacetic acid
OPA	<i>O</i> -Phthaldialdehyde
<i>P</i>	Polarization
Phe	Phenylalanine (F)
PyAOP	(7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
SA	Streptavidin (SA _v)
SD	Standard deviation
Ser	Serine (S)
SPPS	Solid-phase peptide synthesis
SPR	Surface plasmon resonance
TEA	Triethylamine
TFA	Trifluoroacetic acid

THF	Tetrahydrofuran
Thr	Threonine (T)
TIS	Triisopropylsilane
TMT	Terpyridine -bis(methylenamine)tetraacetic acid
TR-FRET	Time-resolved fluorescence (Föster) resonance energy transfer
TRL	Time-resolved luminescence
Trp	Tryptophan (W)
Trt	Trityl
Tyr	Tyrosine (Y)
Val	Valine (V)
Z	Benzylloxycarbonyl (Cbz)

CHAPTER ONE

INTRODUCTION

I. Chemoprevention as a strategy against cancer

Cancer is a group of various diseases characterized by uncontrolled growth and spread of malicious cells. In cancer, untreated malignant tumors invade and destroy nearby tissues and organs, leading to serious illness and death. It is a growing health problem and one of the leading causes of death in the world.^{1,2} The cancer statistics reported in 2013 by National Cancer Institute (NCI) estimated that approximately 13.7 million Americans live with or have a history of cancer.¹ In 2012, the World Health Organization (WHO) reported about 8.2 million cancer-related deaths worldwide, which accounted for about 13% of all deaths.² In the same report, WHO projected that annual cases of cancer will rise from 14 million to 22 million within the next two decades. According to the report by WHO, at least 30% of all cancer cases can be prevented and prevention is the single most cost effective long-term strategy against cancer. The underlying causes of cancer are complex but the primary preventive strategy against cancer is to avoid exposure to known carcinogens such as tobacco smoke, radiation, and pollutants.^{3,4} It is also reported that unhealthy diet (i.e. obesity) and lack of exercise can increase one's risk for developing cancer. While many of the risk factors can be controlled by having a healthy life style, avoiding exposure to carcinogens is becoming increasingly difficult due to environmental pollution as a result of urbanization.³ For some specific groups of working populations such as industrial workers, high risk of carcinogen exposures may also be from their working environments and are difficult to completely avoid exposures. Therefore, increasing the activity of body's natural defense against carcinogens is attracting more efforts in cancer research as a potential therapeutic tool for preventing and reducing the burden of cancer.

Recent studies demonstrated that DNA and cellular damages induced by oxidation, and

inflammation are closely associated with mutation and carcinogenesis.⁵⁻⁷ Redox reaction is a vital element of many of the natural physiological processes and as a result, human body is constantly exposed to oxidative stress. The oxidative stress in our body can be from both exogenous and endogenous oxidative sources.⁵ Exogenous oxidative sources include carcinogenic chemicals, environmental carcinogens, and radiations. Endogenous oxidative sources include intracellular processes such as signalings, metabolic processes, and inflammations that produce oxidative conditions within our body.^{5,8,9} Examples of endogenous sources of oxidative stress include reactive oxygen species (ROS) (e.g. hydroxyl radical, superoxide, and hydrogen peroxide) and reactive nitrogen species (RNS) (e.g. nitric oxide and peroxynitrite) that are generated as reactive byproducts of oxidative metabolism. Cellular production of ROS and RNS is associated with the regulation of redox homeostasis, while other metabolic byproducts are constantly generated as the result of natural physiological processes such as aerobic respiration in mitochondria and during inflammatory responses that protect our body from foreign pathogens. Since sustained oxidative conditions are damaging to DNA and cellular structures necessary for viability, human body has developed antioxidative and cytoprotective mechanisms against various kinds of oxidative stress.⁹⁻¹³

Over the years, epidemiological studies have shown that consumption of fruits and vegetables that are rich in antioxidants may be associated with reduced risk of developing cancer and number of other diseases.¹⁴⁻¹⁷ Antioxidants are compounds that work to neutralize oxidative stress by either directly participating in biochemical processes to inactivate free radicals (e.g. ROS) or acting indirectly to induce the expression of cytoprotective and antioxidative detoxication enzymes (e.g. glutathione S-transferase).¹⁸ Vitamin C (ascorbic acid), Vitamin E (tocopherols), β -carotene, isothiocyanates (e.g. sulforaphane), Michael-acceptors (e.g. curcumin), polyphenols and flavanoids (e.g. catechins) are some of the antioxidants commonly found in human diet. An antioxidant can be classified as either direct- or indirect-acting antioxidant based on how the

molecule exerts its antioxidative action.¹⁹ Compounds such as ascorbic acid, tocopherols, ubiquinol (CoQ₁₀), and phylloquinone (Vitamin K) participate in redox reactions under physiological conditions and directly scavenge ROS and RNS. These antioxidants are designated as direct-acting compounds and therefore need to be replenished once they are consumed or modified upon exerting their antioxidative action.¹⁹

Studies have shown that variety of compounds act as inhibitors of carcinogenesis and protect the body against various metabolic carcinogens and mutagens.^{20,21} Evidence suggests these inhibitors of carcinogenesis act by the induction of detoxication enzymes, and antioxidants such as sulforaphane and curcumin that do not participate in redox reactions under physiological conditions have been demonstrated to also act through the similar mechanism.^{18,19,22} Such compounds belong to the indirect class of antioxidants and the hallmark of these antioxidants is the ability to induce expression of detoxication enzymes that have relatively long half-lives as compared to direct antioxidants.¹⁹ Detoxication enzymes are catalysts that are not consumed during antioxidative action and involved in a variety of chemical reactions that lead to detoxication of mutagens and carcinogens. Enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (GPx) are classical detoxication enzymes that directly sequester ROS and RNS.²³ Other enzymes with antioxidant activities such as NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione reductase (GSR), heme oxygenase-1 (HO1), and thioredoxin reductase are involved in regeneration of endogenous antioxidants such as glutathione and NAD(P)H.^{11,19,24} Enzymes that catalyze biotransformation reactions of metabolism such as glutathione S-transferase (GST), aldo-keto reductases (AKRs), and uridine diphosphate (UDP)-glucuronyl transferase are also involved in detoxication of various toxins and metabolites.^{19,25} Observations that induction of detoxication enzymes reduces the risk of cancer have laid the foundation in strategy for prevention of cancer in which the elevated expression of cytoprotective genes by chemical agents protects the body of carcinogens and mutagens.

II. Discovery of antioxidant response element

As discussed in the preceding section, a variety of compounds have been found to induce the expression of detoxication enzymes. Elevation in the cellular concentrations of xenobiotic metabolizing enzymes and antioxidative proteins such as GST, NQO1, AKRs, and HMOX1 confers cells the ability to respond to oxidative and cytotoxic conditions by restoring redox balance and increasing the rate of detoxifying reactions. The expression of many of the cytoprotective proteins was found to be regulated at the transcriptional level by a common transcriptional enhancer sequence called Antioxidant Response Element (ARE). The ARE is a *cis*-acting promoter sequence that was first identified by Pickett and co-workers while investigating the gene expression of Ya subunit of rat GST (GST-Ya) by xenobiotics.²⁶⁻²⁹ During their investigation, at least two regulatory regions within 5' flanking region of Ya subunit gene between nucleotides 1651 and 663 have been discovered to be responsible for the induction of GST-Ya expression by xenobiotics.²⁶

Sequential and mutational analysis of the 5' region revealed that the regulatory regions are distinct in their contribution to control the expression of GST-Ya. One of the regulatory regions localized between nucleotides 908 and 899 was found to contain the core Xenobiotic Response Element (XRE) sequence found in 5' flanking region of cytochrome P-450 IA1 (CYP1A1).^{28,29} The second region between nucleotides 867 and 857 was identified as the hepatocyte nuclear factor 1 (HNF1) recognition motif found in several liver-specific genes and demonstrated to contribute to the maximal basal expression of GST-Ya.²⁸ The third region localized between nucleotides 722 and 682 is referred as ARE and found to be responsible for the inducible expression of detoxication enzyme by planar aromatic and phenolic antioxidants such as β -naphthoflavone (β NF) and 3-methyl-cholanthrene.^{28,29} Around the same time, Daniel and co-

workers reported their investigation on the homologous regulatory regions of mouse GST-Ya subunit gene.³⁰ They observed a region that was responsible for the induction of GST-Ya by electrophiles such as *tert*-butylhydroquinone (*t*-BHQ) and tentatively named this region electrophile responsive element (EpRE).³⁰ They later found that two regions, 5'-GTGACATTGC-3' and 5'-GTGACAAAGC-3' separated by 6 base pairs, are required for the induction of GST-Ya and that two sequences shared sequence similarity to the core 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response element (TRE), a recognition site for activator protein-1 (AP-1).³¹ AP-1 is a heterodimer composed of Jun and Fos which acts as a transcription enhancer that upregulates the expression of genes regulated by TRE. Thus, two regions within EpRE were concluded to be imperfect TRE sequences that required both sequences to work synergistically to recruit AP-1 by supporting the induction as one functional TRE sequence (5'-GATGAGTCAGC-3').³¹ However, later research demonstrated that the TRE-like regions reported by Daniel *et al.* is not a classical TRE sequences albeit their resemblance to the AP-1 recognition site.³²⁻³⁴

Mutational and sequential analysis of the region by Pickett and co-workers demonstrated that a change of dinucleotide AA (underlined) to TC in the TRE-like sequences (e.g. 5'-GTGACAAAGC-3') confers the sequence to function as high affinity AP-1 recognition site while retaining inducibility by both xenobiotics and TPA.³⁴ The change of dinucleotide GC (italicized) to TC in the sequence abolishes inducibility by xenobiotics, but retains affinity to AP-1 and response to TPA.³⁴ The GC dinucleotide was found to be essential for the induction of genes by ARE, thus it was concluded that the core nucleotide sequence of ARE is comprised of the sequence 5'-RGTGACNNNGC-3' where R is A or G and N is any base (IUPAC nucleotide abbreviations).^{27,32,34,35} Additionally, the sequence which lacks affinity to Jun/Fos AP-1 complex was speculated to mediate transcriptional activation in response to xenobiotics and TPA phorbol ester through a mechanism that is distinct from the classical TRE that binds AP-1.³³ Itoh and co-workers reported on the similarity between ARE and nuclear factor erythroid 2 (NF-E2) binding

sequence 5'-RTGASTCAGCA-3' where R is A or G, S is G or C, and Y is C or T.³⁵⁻³⁷ Research over the years demonstrated that a member of NF-E2 heterodimerizes with a small Maf protein to bind to ARE and activates transcription of detoxication enzymes, providing insights into the mechanism of ARE gene activation.

III. NF-E2 related factor 2

NF-E2 related factor 2 (Nrf2) is a 66-kDa Cap n' Collar (CNC) protein with a basic leucine zipper (bZIP) DNA binding motif that is characteristic of NF-E2.³⁸ A CNC family of transcription factors also includes proteins such as NF-E2 p45, Nrf1, Nrf3, and BTB (Broad-complex, tramtrack, and brick a brac) and CNC homology (Bach) proteins.³⁹ This family of transcription factors heterodimerize with small Maf protein to bind to Maf recognition element (MARE) also referred to as the NF-E2 binding sequence. It has been found that Nrf1 and Nrf2 are expressed ubiquitously in various organs, while Nrf3 is mainly expressed in human placenta.³⁹ The expression of NF-E2 p45 in human was reported to be high in hematopoietic progenitor, erythroid, megakaryocytic, and mast cells.⁴⁰ Bach1 is expressed ubiquitously, while Bach2 is expressed mainly in monocytes and neuronal cells.⁴¹ Yamamoto and coworkers demonstrated using electrophoretic mobility shift assay (EMSA) that a heterodimer of Nrf2 and small Maf protein interacts with ARE and that this Nrf2/Maf complex forms specifically even under competing conditions. More importantly, Yamamoto and coworkers observed that following treatment of Nrf2-null mutant mice with *t*-butylhydroxy anisole (BHA), inducible expression of four GST subunits (Ya1, Ya3, Yp, and Yb) and NQO1 was diminished due to the absence of Nrf2 activity.³⁷ Furthermore, the role of Nrf2 in up-regulation of ARE genes encoding various detoxication enzymes is reported in many literatures and thus, the disruption of Nrf2 activity impairs the expression of detoxicating and metabolizing enzymes that occurs as a response to various antioxidant signals.⁴²⁻⁵⁰ Among members of the NF-E2 transcription factor family, Nrf2 is accepted to be the central transcription factor in the activation of cytoprotective enzyme

expression through interaction with the ARE and the potential of targeting Nrf2 pathway in cancer chemoprevention has been highlighted in many reviews.⁵¹⁻⁵⁵

Comparison of Nrf2 amino acid sequences across species identified 6 highly conserved regions named Nrf2-ECH homology (Neh) domains and the domain structure of Nrf2 is shown in Figure 1.⁵⁶ The first domain, Neh1, is highly conserved and was found to contain both the CNC and the bZIP domain required for dimerization with Maf and binding to DNA. Additionally, the DNA binding domain within Neh1 (residues 484-516) was reported to contain nuclear localization sequence (NLS, residues 494-511) essential for nuclear transport of Nrf2 upon treatment with inducers of cytoprotective genes such as *t*-BHQ.⁵⁷ Neh2 and Neh3 domain of Nrf2 are also highly conserved domains that are located at the N-terminal and the C-terminal region of the protein, respectively. The N-terminal Neh2 domain can be divided into two regions; the hydrophobic amino end and the hydrophilic carboxyl end of the domain. The hydrophobic region (32 residues) was found to be conserved among the CNC family, while the hydrophilic region (40 residues) of Neh2 is unique to Nrf2 but conserved across species.⁵⁶ It has been found that the function of Neh2 domain is involved in negative regulation of Nrf2. In order to determine the cellular factor that associates with Neh2 domain of Nrf2, a fusion protein of Gal4-Nrf2-Neh2 was used to screen and isolate the negative regulator which had a structural similarity to Kelch, a *Drosophila* cytoskeleton binding protein. Thus, the isolated protein was referred to as Kelch-like ECH-associate protein 1 (Keap1) and the protein was identified to be the cytoplasmic redox sensor protein that interacts with Neh2 domain to repress the activity of Nrf2.⁵⁶ Keap1 protein and its interaction with Nrf2 will be discussed in detail in the succeeding section.

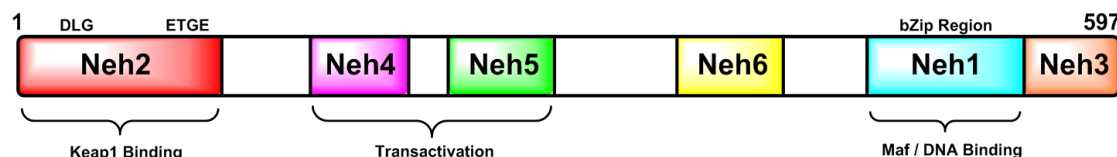


Figure 1. The domain structure of Nrf2 showing the 6 evolutionary conserved domains, Neh1 - Neh6. The Neh2 domain contains the 2 evolutionary conserved motifs necessary for interaction with Keap1 Kelch domain.

Neh3 domain of Nrf2 is another domain of Nrf2 that is highly conserved across species and among members of the CNC bZIP transcription factors. Site-directed mutagenesis revealed a short sequence of amino acids within Neh3 domain that is required for transcriptional activity of Nrf2.⁵⁸ The mutant Nrf2 lacking this amino acid sequence was found to retain the ability to localize to the nucleus and bind to DNA, but lacked transcription activity. Furthermore, this region was found to interact with an important partner protein chromo-ATPase/helicase DNA binding protein (CHD6). Thus, it was concluded that the Neh3 domain is one of the essential domains required for the transactivation of ARE gene by Nrf2. In addition to Neh3 domain, two transcriptional activation domains were identified within Nrf2 that interact with essential partner proteins and are indispensable for the activity of the protein. These domains of Nrf2, namely Neh4 and Neh5 domain, are highly conserved and was found to cooperatively bind to cAMP Response Element Binding (CREB) Protein (CBP) which has been shown to be essential co-activator for many transcription factors. A deletion mutant lacking both Neh4 and Neh5 was found to have diminished transactivation of firefly luciferase (*LUC*) reporter gene.⁵⁹ Thus, these transactivation domains are essential for complexation with protein partners that are necessary for Nrf2 activity and appear to act synergistically to attain maximum level of transcription activation. Lastly, Neh6 homology domain of Nrf2 has been reported to be involved in redox-regulated turnover of Nrf2 and its function more closely related to Neh2 domain.⁶⁰ On the contrary to Neh2 which is associated with redox-sensitive degradation of Nrf2, Neh6 domain has been found to be associated with redox-insensitive degradation of the protein.⁶⁰ Keap1-mediated degradation of

Nrf2 is accepted to be the major regulatory mechanism for Nrf2 activation in cells that are not oxidatively stressed, while the less rapid redox-insensitive degradation of Nrf2 is believed to be the major contributor to Keap1-independent degradation of Nrf2 in oxidatively stressed cells.

IV. Kelch-like ECH-associated protein 1 and its interaction with Nrf2

Yamamoto and his colleagues reported that Neh2 domain deleted mutant of Nrf2 is a potent transactivator of a *LUC* reporter gene in both QT6 quail fibroblasts and HD3 chicken proerythroblasts, while the wild-type Nrf2 was found to be a potent transactivator of the reporter gene in only QT6 cells.⁵⁶ Yamamoto hypothesized that Neh2 domain of Nrf2 interacts with a novel cellular protein partner that is responsible for cell-type specific negative regulation of Nrf2 transactivation activity. Attempts to identify the negative regulator of Nrf2 using the yeast two-hybrid system led to the discovery of a novel protein that shares homology to *Drosophila* Kelch protein involved in organization of ring canal actins.^{56,61-63} Due to its structural similarity to Kelch, Yamamoto named the isolated protein Kelch-like ECH-associated protein 1 (Keap1) and analysis of murine Keap1 sequence revealed approximately 94% sequence identity with a human KIAA0132 gene, the human homolog of Keap1.^{56,64} Keap1 is a 69 kDa BTB-Kelch cytoplasmic protein which possess five domains that are believed to be functionally distinct.⁶³ As shown in Figure 2, these domains of Keap1 are referred to as the N-terminal region (NTR), BTB domain, intervening region (IVR), Kelch repeats, and the C-terminal region (CTR).

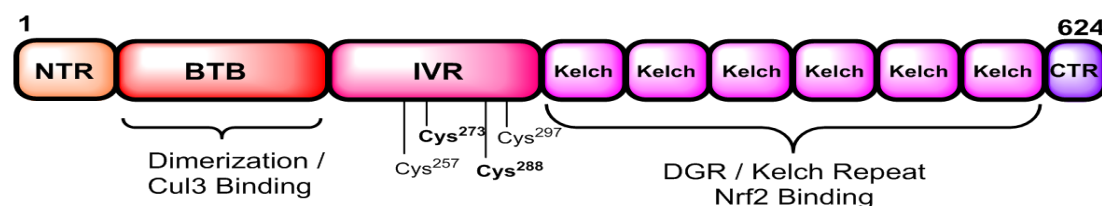


Figure 2. The organization and the domain structure of Keap1. The reactive Cys residues within the IVR region are shown in bold.

The X-ray crystal structure of Keap1 Kelch domain showed that it is a 6-bladed β -propeller structure, where each blade of the propeller is made of 4 β -strands.⁶⁵ Two-hybrid interaction assay demonstrated that the Kelch domain of Keap1 binds a hydrophilic C-terminal region within the Neh2 domain of Nrf2.^{56,66} The Neh2 domain of Nrf2 contains 2 highly conserved regions known as DLG and ETGE motif that are found in amino acids 17 - 32 and 77 - 82 of Neh2 domain, respectively.⁶⁷ While both of these motifs within the Neh2 domain bind to the same site in Kelch domain of Keap1, the affinity of Keap1 for the ETGE motif was reported to be approximately 100-fold higher than the DLG motif.⁶⁸ The X-ray crystallography analysis of the human Keap1 Kelch domain in complex with 16mer Nrf2 peptide (H-⁶⁹AFFAQLQLDEETGEFL⁸⁴-OH) determined that the Nrf2 binding pocket is on the top face of the Kelch domain, which is defined by the longer loops that connect the β -strands of the β -propellers.⁶⁷ All 6 blades of the β -propeller has been demonstrated to contribute to the binding of the Nrf2 peptide where residues Arg415, Arg483, and Ser508 interact with E79 of the peptide and residues Ser363, Arg380, and Asn382 interact with E82 of the peptide.

Yamamoto's group demonstrated that the transactivation activity of wild-type Nrf2 was repressed by Keap1, while Neh2 deletion mutant of Nrf2 was not affected by Keap1. Thus, the interaction between two proteins is believed to be dependent on the presence and integrity of both Neh2 domain of Nrf2 and Kelch domain of Keap1. They also demonstrated that Keap1 functions by

retaining Nrf2 in the cytoplasm under homeostatic conditions and that electrophilic agents release Nrf2 from Keap1, leading to translocation of Nrf2 into the nucleus.⁵⁶ While the model proposed by Yamamoto's group may account for the lower expression of cytoprotective genes that are driven by induction of ARE, it does not account for the accumulation of Nrf2 in response to oxidative stress. Nguyen and coworkers reported that the change in level of Nrf2 in response to electrophiles is mediated by a post-transcriptional mechanism, suggesting that the increased stability of Nrf2 (e.g. longer $t_{1/2}$) results in its increased cellular concentration during oxidative stress.⁶⁹ They reported that the cellular level of Nrf2 is regulated by the ubiquitin-dependent degradation involving 26 S proteasome and by the stabilization of Nrf2 which is believed to be promoted as a result of phosphorylation mediated by the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) pathway. In fact, the role of MAPK pathway in activation of ARE genes has been initially identified by Yu et al, where the expression of extracellular-signal-regulated kinase kinase kinase 1 (MEKK1), transforming growth factor- β -activated kinase (TAK1), and apoptosis signal-regulating kinase (ASK1) mediates ARE gene expression by phosphorylation of Nrf2.⁷⁰ In addition, McMahon and coworkers demonstrated that Nrf2 undergoes ubiquitin-dependent proteasomal degradation under both homeostatic and oxidative stress conditions, however the rate of proteasomal degradation under homeostatic conditions was found to be significantly faster due to direct interaction between Keap1 and Nrf2.⁷¹ For example, the half life of Nrf2 in oxidatively stressed RL34 cell was reported to be ~31 min, while the half life in non-stressed RL34 cell could not be calculated (e.g. Nrf2 not detected).⁷¹ Kobayashi and coworkers identified that the mechanism in which Keap1 contributes to the rapid degradation of Nrf2 involves the Cul3-based ubiquitin ligase (E3) system.⁷² Keap1 was demonstrated to be the stress sensor protein that functions as an adaptor protein that bridges the interaction between Nrf2 and the Cul3-based E3 ligase complex through its N-terminal domains (BTB and IVR) and C-terminal Kelch domain.^{66,72-75}

BTB and IVR domains are believed to be necessary for Keap1-dependent ubiquitination of Nrf2, while only the BTB domain of Keap1 was found to be necessary for dimerization.^{72,74,76} Keap1 has been found to form dimers spontaneously *in vitro* and thus is believed to also exist as dimers under basal conditions *in vivo* in regulation of Nrf2. A mutational analysis revealed that the highly conserved serine residue (Ser¹⁰⁴) in the BTB domain of Keap1 is essential for dimerization of the protein. The wild-type Keap1 sequesters Nrf2 in the cytoplasm, while the S104A mutant Keap1 failed to affect Nrf2 localization.⁷⁶ This suggests that the dimerization of Keap1 may play a critical role for the ability of Keap1 to associate with Nrf2 for sequestration and ubiquitination. In addition, multiple cysteine residues in Keap1 were shown to react with thiol-specific reagents such as isothiocyanates and lead to conformational changes in Keap1. The changes results in disruption of Keap1-Nrf2 interaction and induction of ARE genes *in vitro*. In the IVR domain of Keap1, 4 especially reactive cysteines (Cys²⁵⁷, Cys²⁷³, Cys²⁸⁸, and Cys²⁹⁷) were identified and of these 4 residues, Cys²⁷³ and Cys²⁸⁸ are essential for Keap1-dependent ubiquitination of Nrf2 and Keap1-mediated repression of Nrf2 activity.^{74,77,78} It is believed that modification at these reactive cysteine residues lead to conformational changes that allow Nrf2 to dissociate from Keap1 dimer and translocate to the nucleus. In light of these findings, Keap1 is believed to be the major negative regulator of the expression of cytoprotective genes.

V. Mechanism of Nrf2 regulation

Recent model of Keap1-dependent regulation of Nrf2 involves interaction of Keap1-dimer with a single molecule of Nrf2. Two evolutionary conserved binding motifs known as high-affinity ETGE and low-affinity DLG in the Neh2 domain of Nrf2 collaboratively binds to both members of a Keap1 dimer in a "hinge" and "latch" mechanism.⁷⁹ The Keap1-Nrf2 complex cycles through an "open" and a "closed" conformation, where in the "open" conformation only the ETGE motif of Nrf2 binds to Keap1 (Figure 3). Nrf2 is not targeted for ubiquitin-dependent degradation when

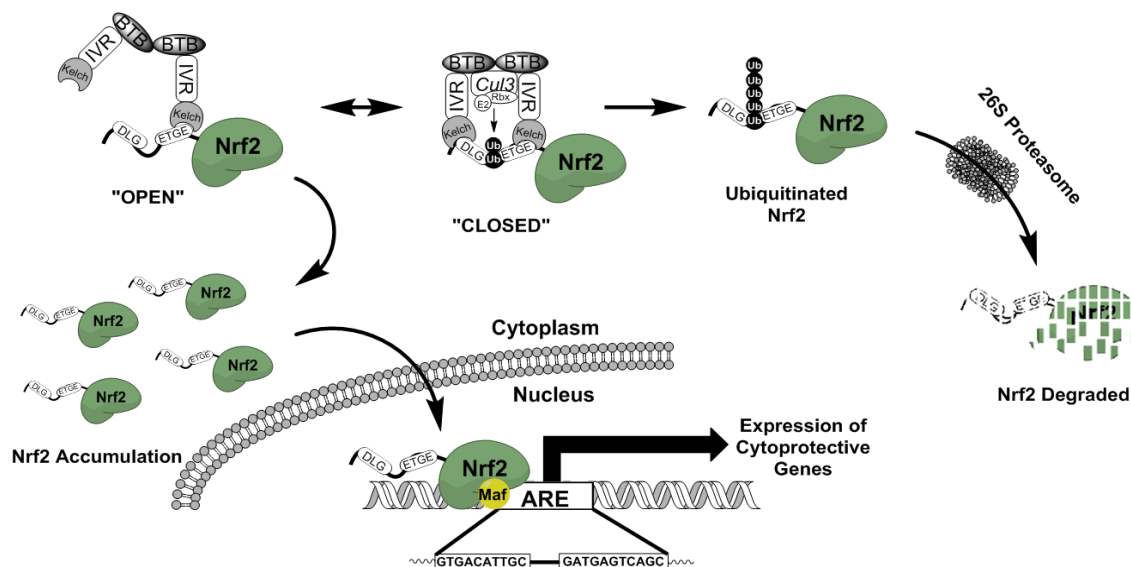


Figure 3. The Keap1-Nrf2 pathway in activation of antioxidant response element. In the "hinge" and "latch" mechanism of Nrf2 regulation, the high affinity ETGE motif of Nrf2 initially binds to Kelch domain of Keap1 and the lower affinity affinity DLG motif binds to the second Keap1 to close the conformation. Nrf2 is polyubiquitinated at its Lys rich (7K) region and targeted for subsequent degradation by the 26 S proteasome.

only the ETGE motif is bound to the Keap1 dimer.⁸⁰ However, once the low-affinity DLG motif binds to the Kelch domain of the other member of the Keap1 dimer to form the "closed" conformation, the Lys residues within the Neh2 domain is correctly positioned and the ubiquitination of Nrf2 is facilitated.⁸⁰ The polyubiquitinated Nrf2 is subsequently released from the dimer for degradation by the 26 S proteasome.⁷⁹

Oxidative stress conditions are believed to cause conformational changes in Keap1 by covalent modifications at the sensitive cysteine residues.⁸¹ These conformational changes disrupt the binding of the low-affinity DLG motif, while the high-affinity ETGE motif remains intact with the Keap1 Kelch domain.⁶⁸ In this state, the Nrf2 is no longer oriented properly for ubiquitination and newly synthesized Nrf2 accumulates in the cell as it escapes the Keap1-mediated proteasomal degradation.^{80,81} These Nrf2 translocate into the nucleus and bind to ARE to induce the expression of cytoprotective genes. In the other model of Keap1-mediated regulation of Nrf2, the

covalent modification of cysteine residues lead to the dissociation of Keap1 and Cul3 interaction.⁸² The disruption of Keap1-Cul3 E3 ligase function results in overall stabilization of Nrf2 and newly synthesized Nrf2 accumulate in the nucleus for ARE activation. Clearly, covalent modifications of sensitive cysteine residues of Keap1 is of relevance in the mechanism of Nrf2 regulation. Regardless of the mechanism, it has been demonstrated through research that Keap1 acts as the key cytosolic redox sensor protein which regulates the activation of ARE by preventing the nuclear accumulation of Nrf2. On the other hand, the evasion of Keap1-mediated ubiquitination by Nrf2 during oxidative conditions leads to accumulation of Nrf2 in the cell and the expression of cytoprotective genes. Therefore, Keap1-Nrf2 protein-protein interaction is regarded as the key interaction in mediating cytoprotective gene expressions in various cells and tissues under oxidative stress.^{37,56,83}

At the time of the present study, almost all known inducers of ARE genes were indirect inhibitors of Keap1-Nrf2 interaction. The indirect inhibitors are believed to exert their effect by forming adducts or modification of the sulfhydryl group of Keap1.⁸⁴ Thus, many of the indirect inhibitors that regulate the ARE induction are electrophiles that are chemically reactive towards nucleophiles such as sulfhydryl group of glutathione (GSH) and exposed cysteine residues of a protein. Inhibitors that are chemically inert and directly interfere with the Keap1-Nrf2 protein-protein interaction to activate ARE should be a safer alternative to the indirect inducers. Direct inhibitors of Keap1-Nrf2 interaction at the time of the present study were peptide inhibitors based on the sequence of the ETGE motif, and no small-molecule direct inhibitors of Keap1-Nrf2 interaction had been reported in literature. Therefore, it is the goal of the present study to discover small-molecule direct inhibitors of Keap1-Nrf2 interaction that can induce the expression of cytoprotective genes by the activation of ARE mediated by the Keap1-Nrf2 pathway.

CHAPTER TWO

THE DEVELOPMENT OF A FLUORESCENCE POLARIZATION ASSAY FOR KEAP1-NRF2 INTERACTION

I. Fundamental Theory of Fluorescence Polarization

Fluorescence polarization (FP) is an intrinsic property of a fluorescent molecule and the FP assay is a powerful technique for studying molecular interactions by measuring the change in the size of the fluorescent molecule, either inherent or labeled. The fluorescent molecule is often referred as the tracer or probe and the FP measurement is taken in real-time to provide nearly instantaneous analysis of bound and free tracer ratio. FP is first described by Perrin in 1926 and is based on the observation that when a fluorescent molecule is excited by a plane-polarized light, its fluorescence emission is polarized as long as the molecule remains stationary during excitation and emission.⁸⁵ However, molecules in solution are never stationary and instead tumble rapidly during its fluorescence lifetime. As a result, the emitted light from the tracer contains both parallel and perpendicular components with respect to the plane of polarized excitation light.⁸⁶ Thus, the degree of polarization (P) and anisotropy (A) of the emitted light can be expressed as the ratio of fluorescence intensity in the parallel and perpendicular plane as shown in the following equations (1) and (2):

$$P = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + F_{\perp}} \quad (1)$$

$$A = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + 2F_{\perp}} \quad (2)$$

Where $F_{\parallel}$ is the parallel component of the emitted light, and $F_{\perp}$ is the perpendicular component of the emitted light.

The polarization (P) and anisotropy (A) are both unitless ratios that are independent of tracer concentration and the intensity of the emitted light. In addition, anisotropy is related to polarization by equation (3):

$$A = \frac{2P}{3-P} \quad (3)$$

Where the values of P and A are both derived from the intensities of the parallel and the perpendicular component of emitted light as described above.

Due to this mathematical relationship, the values can be easily interconverted. Theoretical values of polarization (P) can range from -0.33 to 0.5 and anisotropy (A) values range from -0.25 to 0.4.⁸⁶ Typical values of observed P ranges from 0.01 to 0.3 and therefore, P and A are often reported in mP ($mP = 1000 \times P$) and mA ($mA = 1000 \times A$) to give a range of 10 to 300 mP and 25 to 400 mA . The observed polarization is related to the fluorescence lifetime and the rotational diffusion of a molecule by the Perrin Equation (4):

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(1 + \frac{RT}{\eta V} \tau \right) \quad (4)$$

Where P is the observed polarization, P_0 is the intrinsic polarization, V is the molecular volume, R is the gas constant, T is the temperature, η is the viscosity of the medium, and τ is the fluorescence lifetime.^{85,87}

Fundamentally, the polarization of a molecule is proportional to the Debye rotational relaxation time of the molecule.⁸⁷ Therefore, Perrin equation (4) can be rewritten using equation (5) which describes the Debye rotational relaxation time:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(1 + \frac{3}{\rho} \tau \right) \quad (5)$$

Where ρ is the Debye rotational relaxation time. It is the time the molecule takes to rotate through an angle of $\arccos e^{-1}$, which is approximately 68.42° .^{86,87}

Stokes-Einstein equations (6 and 7) describe the Debye rotational relaxation time (ρ) in which:

$$\rho = \frac{3\eta V}{RT} \quad \text{for a spherical molecule} \quad (6)$$

$$\rho = \frac{3\eta M(v+h)}{RT} \quad \text{for a protein} \quad (7)$$

Where M is the molecular weight, v is the partial specific volume, and h is the degree of hydration which takes into account the shape of a protein.⁸⁷

In practice, the viscosity and the temperature of the assay are held constant, therefore the rotational relaxation time is directly proportional to the molecular volume or size. Since polarization is proportional to the Debye rotational relaxation time, it is also directly proportional to the size of the molecule as shown below.

$$\text{Polarization } (P) \propto \rho = \frac{3\eta V}{RT}$$

This means that the rotational relaxation time of a larger molecule (high V value) such as protein is inherently slower and have higher value of polarization (e.g. more polarized emission) due to slower rate of molecular rotation. Conversely, a small molecule such as tracer will have faster rotational relaxation time and lower value of polarization. When the tracer binds to a protein, the degree of polarization increases as a result of changes in rotational relaxation time and the size of the molecule as a protein complex (Figure 4). Such change in polarization can be used to study the molecular interaction and also develop assays that can determine the extent of binding interaction from the observed polarization values.

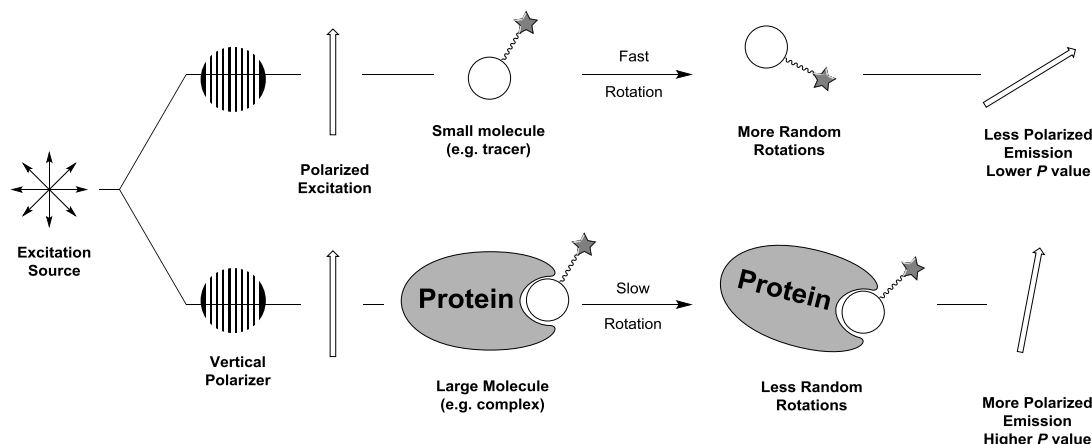


Figure 4. Illustrated example of differences in FP for a small tracer molecule and a larger complex. A larger fluorescent molecule or complex has a slower rotation, which results in higher polarization value of the emitted light when excited by a linear polarized light. The difference in anisotropy between the free tracer and the complex can be used to directly measure the fraction of tracer bound to the target.

While both polarization and anisotropy values represent the weight average of the bound and free tracer in solution as ratio quantities, anisotropy values are often preferred due to the additivity that makes them mathematically simple. The Perrin equation (4) was extended by Gregorio Weber to describe the polarization of a mixture of molecules in which a portion of molecules in the mixture possess a different rotational relaxation time due to change in their size (e.g. tracer bound to a protein).⁸⁸ A group of identical molecules in solution will have same polarization values, however in a mixture containing more than one species of molecule in solution, the observed polarization P is the average of the individual components P_i given by Weber's equation (8):

$$\left(\frac{1}{P} - \frac{1}{3}\right)^{-1} = \sum_{i=1}^n f_i \left(\frac{1}{P_i} - \frac{1}{3}\right)^{-1} \quad (8)$$

Where f_i is the contribution of individual fluorescent species to the total fluorescence intensity of the mixture. Perrin had mentioned the term $\frac{2P}{3-P}$ in his earlier work, but Aleksander Jabłoński later referred to the value as anisotropy and defined its additivity. Although the additivity of anisotropy follows the same law of additivity as the polarization values, anisotropy values are directly additive and the observed anisotropy (A) is given by a simpler equation (9):

$$A = \sum_{i=1}^n f_i A_i \quad (9)$$

Where A_i is the anisotropy of the individual components and f_i is the fractional contribution to the total intensity of the mixture. It is interesting to note that the manipulation of the polarization values by simple additivity only results in less than 1 to 2.5% error in results.⁸⁷ However, because the two values can be easily interconverted, there is no reason not to use anisotropy values for the analysis of fluorescence polarization data. The simple additivity of anisotropy is particularly useful in determining values of molecular species, such as fraction bound of a ligand in deriving its binding affinity. Thus, we will use anisotropy in treatment of the FP assay data to calculate the binding and inhibition constants. In order to facilitate the discovery of small-molecule direct

inhibitors of Keap1-Nrf2 interaction, the FP-based homogeneous competition assay was developed and adapted for applications in high-throughput screening (HTS) of chemical libraries. FP is an intrinsic property of a fluorescent molecule, therefore FP-based assay is more robust and better suited for HTS than other fluorescence-based assays.

II. The Development of Keap1-Nrf2 FP Assay

A. Selection of the FP Assay Conditions

As discussed in the preceding chapter, the DLG and the ETGE motifs within the Neh2 domain are two evolutionary conserved motifs of Nrf2 (Figure 5). In the "hinge" and "latch" mechanism of Keap1-Nrf2 interaction, both motifs bind to the same site in the Kelch domain of Keap1 but the weaker binding DLG motif has been reported to be necessary for ubiquitination of Nrf2.⁸⁰ The high affinity ETGE motif has been reported to be essential for the initial interaction with Keap1 dimer, ultimately leading to the regulation of Nrf2 activity. The ETGE motif was reported to be approximately 100 times stronger in binding affinity to Keap1 as compared to the lower affinity DLG motif.⁶⁸ Thus, a series of ETGE-containing Nrf2 peptides have been reported as inhibitors of Keap1-Nrf2 interaction that are capable of directly displacing Nrf2 from Keap1. The ETGE-containing 16mer Nrf2 peptides with the sequence H-AFFAQLQLDEETGEFL-OH is the longest reported Nrf2 peptide. It effectively displaces the Nrf2 protein from the Keap1-Nrf2 complex with a reported K_d as low as 20 nM determined by the isothermal titration calorimetry (ITC).^{67,68} Although the shorter 10mer Nrf2 peptide (H-LDEETGEFLP-OH) has also been shown to inhibit the Keap1-Nrf2 protein complex, it is reported to be much weaker in affinity as compared to the longer Nrf2 peptides.⁶⁷ At the time of this research, there were no known small molecules that can directly interfere with the protein-protein interaction between Keap1 and Nrf2 with the exception of these Nrf2-derived peptides.

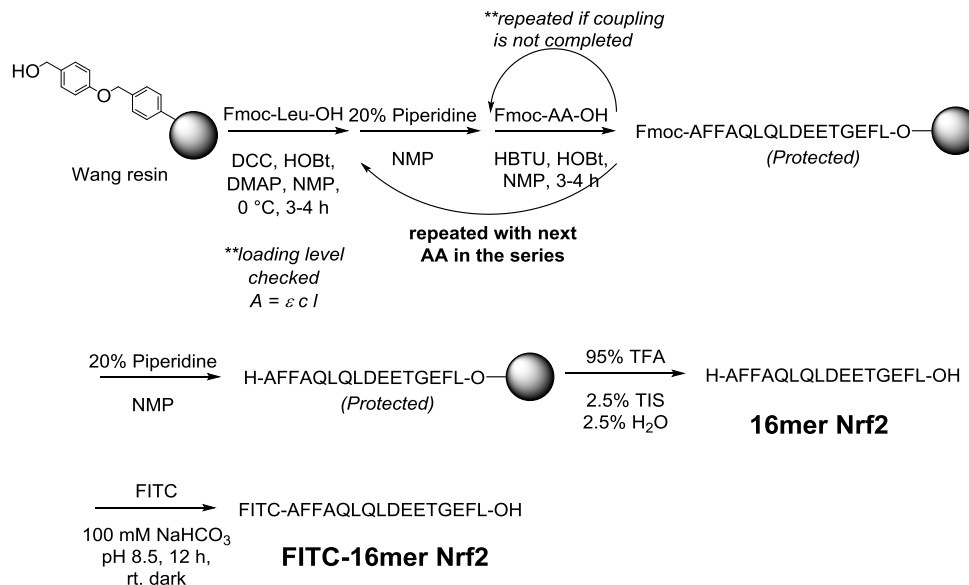
Based on the reported binding affinity, the FITC-labeled 16mer Nrf2 peptide was selected as the

Drosophila	KLKALEELQQDKDKNNENQLEDITNEWNGIPFTID ⁷⁵ NETGEYIRLPLDELLNDVLK
Zebrafish	KEVELRRRREQEEQELQERLQ-EQEKTLAQLQL ⁷⁵ DEETGEFLPRSTPLTHTPEAD
Human Nrf2	KEYELEKQKKLE-KERQEQQLQKEQEKAFFAQLQL ⁷⁵ DEETGEFLP-IQPAQHIQSET
16mer Nrf2	⁶⁹ AFFAQLQL ⁷⁵ DEETGEFL ⁸⁴
10mer Nrf2	⁷⁶ LDEETGEFLP ⁸⁵

Figure 5. The aligned sequences of ETGE motif of Nrf2 Neh2 domain from 3 different species and the Nrf2 peptides derived from human Nrf2 sequence. The Nrf2 peptides derived from the sequence of human Nrf2 ETGE motif is used in the present study for the development of the FP assay.

initial tracer used to explore conditions for the FP assay. The peptide was synthesized using Fmoc chemistry on Wang resin in solid-phase peptide synthesis (SPPS) as shown in Scheme 1. The Nrf2 peptides synthesized were cleaved from the resin prior to FITC labeling in order to avoid the Edman degradation of the FITC-labeled Nrf2 peptides during TFA cleavage of the peptides from the resin.⁸⁹ The resulting FITC-labeled peptide was purified to >95% purity on a reversed-phase high performance liquid chromatography (RP-HPLC) system as described in the experimental section.

The optimal concentration of the probe for use in the FP binding assays is related to the fluorescence intensity of the fluorophore and the sensitivity of the instrument, which can be determined by measuring the polarization value of FITC-16mer Nrf2 peptide as a function of its concentration. As discussed in the previous section, polarization and anisotropy values are proportional to the molecular volume or size and are not affected by the concentration of the tracer. However, when the tracer concentration is too low such that the vertical and the horizontal fluorescence components cannot be accurately detected by the instrument, errors in the measurements become prominent due to the background noise and the linearity of the measurement is lost. The anisotropy measurements of FITC-labeled 16mer-Nrf2 were performed as described in the experimental section. As shown in Figure 6A, approximately 10 nM of the



Scheme 1. The solid-phase peptide synthesis of FITC-labeled 16mer Nrf2 peptide on the Wang resin. Standard Fmoc chemistry is used to sequentially assemble the Nrf2 peptide on the resin.

FITC-16mer Nrf2 peptide is needed to give a reliable anisotropy measurement that is within the fluorescence linear range. The anisotropy signals fluctuate at concentrations lower than 10 nM and the errors are larger as the background noise become prominent in the fluorescence measurements.

In order to determine the equilibration time required before the FP measurement, the change in anisotropy signal was monitored over 3 hour period following the mixture of the tracer and the protein. The assay plate was incubated at rt covered from light on a plate shaker and centrifuged at 370×g for 2 min prior to FP measurement to ensure all the solutions is free of air bubbles. The time it took for the anisotropy signal to plateau was within the first 15 min and was not affected over the 3-h incubation period. For this reason, the incubation time for the assay was set at 30 min to ensure consistent and thorough mixing of the assay mixture. The observed anisotropy of the FITC-labeled 16mer Nrf2 peptide as a function of Keap1 Kelch domain concentration is shown in

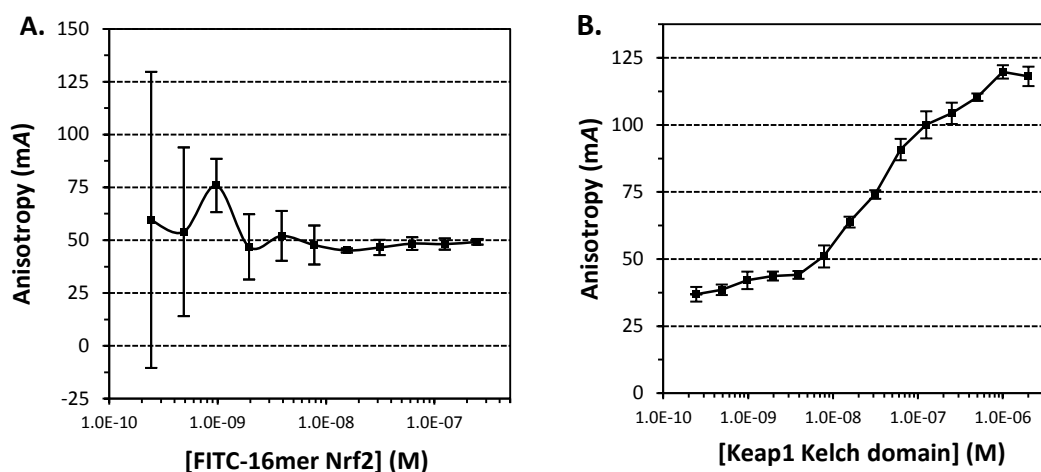
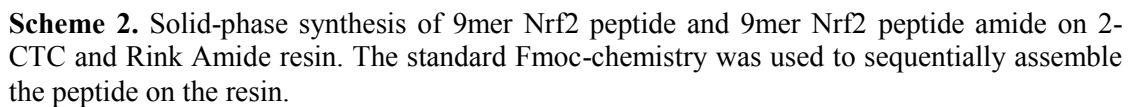


Figure 6. (A) The anisotropy measurements of the FITC-16mer Nrf2 peptide and (B) the binding curve of FITC-16mer Nrf2 peptide obtained by the FP assay.⁹³ For the FP assay shown in (B), 10 nM FITC-16mer Nrf2 peptide was incubated with a range of concentrations of Keap1 Kelch domain. The P values obtained were converted to A values using equation (3). The error bars in Figure 6A shows the standard deviation (SD) of the triplicates at each concentration and is the variability of the measurement around the mean. The A values in (B) were adjusted with the G -value determined from the literature value of free fluorescein.

Figure 6B. The dynamic range (ΔmA) for the assay using the FITC-labeled 16mer Nrf2 peptide was determined by measuring the anisotropy (mA) of the tracer bound (A_{bound}) to the protein and tracer free in solution (A_{free}). This is because based on the additivity of anisotropy values, the dynamic range of the assay is simply the difference between the values of A_{bound} and A_{free} . The A_{bound} of the tracer is the highest anisotropy value at the upper plateau end of the binding curve. The dynamic range of the FP assay using FITC-16mer Nrf2 peptide was determined to be 80.1 mA. The FP binding assay shown in Figure 6B was used to derive the binding affinity (K_d) of FITC-labeled 16mer Nrf2 peptide, which was determined to be 28.7 ± 5.7 nM. The K_d by the FP assay is in good agreement with the value that was obtained using the SPR assay (Table 2, FITC-16mer Nrf2, $K_d = 14.5$ nM), as well as to the value of the unlabeled 16mer Nrf2 peptide ($K_d = 23.9$ nM) that we have previously reported for the analysis of the Keap1-Nrf2 interaction using

While polarization is a property of a fluorophore, the excess amino acids on the tracer can increase flexibility of the fluorescent dye on a tracer. The additional rotational motion arising



Scheme 2. Solid-phase synthesis of 9mer Nrf2 peptide and 9mer Nrf2 peptide amide on 2-CTC and Rink Amide resin. The standard Fmoc-chemistry was used to sequentially assemble the peptide on the resin.

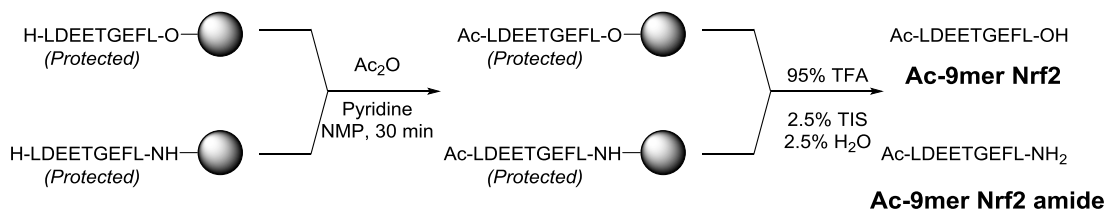
Table 1. The solution dissociation equilibrium constants of Nrf2 peptides determined by the SPR Competition Assay.⁹⁰

	Peptide	K _d (nM)
H-7mer Nrf2	H-EETGEFL-OH	>>1000
H-8mer Nrf2	H-DEETGEFL-OH	>>1000
H-9mer Nrf2	H-LDEETGEFL-OH	352
H-10mer Nrf2	H-QLDEETGEFL-OH	27.3
H-11mer Nrf2	H-LQLDEETGEFL-OH	31.3
H-12mer Nrf2	H-QLQLDEETGEFL-OH	23.8
H-14mer Nrf2	H-FAQLQLDEETGEFL-OH	22.5
H-16mer Nrf2	H-AFFAQLQLDEETGEFL-OH	23.9

The SPR binding assay was performed as described under Experimental section. The K_d were determined by regression analysis using equation (14).

from increased flexibility of the fluorophore is known as the "propeller effect," and it depolarizes (lowers) the FP signal of the bound complex.^{91,92} In principle, the rigidity of the tracer should be increased by attaching the fluorescent dye as close as possible to the peptide binding site without adversely affecting the binding affinity of the tracer. In fact, the X-ray crystal structure of 16mer Nrf2 peptide-Keap1 Kelch domain complex has shown that the 16mer Nrf2 peptide is much longer than the DxETGE motif and several residues were exposed to the solvent as an undefined structure.⁶⁷ Thus, the shorter Nrf2 peptides containing the sequence of the ETGE motif could have improvements over the 16mer Nrf2 peptide probe. In light of this, the optimization of the tracer for the FP assay was performed by successively deleting the N-terminal residues in the 16mer-Nrf2 peptide sequence to design the shortened Nrf2 peptide tracers with potentially higher dynamic range.

A series of shortened Nrf2 peptides were synthesized following the same solid-phase Fmoc chemistry as the 16mer Nrf2 peptide as shown in Scheme 2. The Nrf2 peptides were synthesized on either the Wang or the 2-chlorotrityl chloride (2CTC) resin, while the Rink amide resin was used for the peptide amides. Amino acids were protected at their side chains by Boc or t-butyl protecting group where necessary. As with the 16mer Nrf2 peptide, all of the peptides were



Scheme 3. The N-terminal capping of the 9mer Nrf2 peptides performed on the resin-bound 9-mer Nrf2 peptides using acetic anhydride. The progress of the N-terminal capping was monitored by ninhydrin (Kaiser) test.

cleaved from the resin prior to FITC labeling to circumvent the Edman degradation around the thiourea linkage.

First, the binding affinity of the unlabeled Nrf2 peptides were determined and confirmed by the SPR competition assay as shown in Table 1.⁹⁰ The SPR assay, along with the present study, demonstrated that the 9mer Nrf2 peptide is the minimal sequence required for binding to Keap1 Kelch domain due to the fact that the binding affinities between the Nrf2 8mer and 9mer Nrf2 peptides were significantly different. The large difference in binding affinity between the 9mer Nrf2 (352 nM) and 10mer Nrf2 peptide (27.3 nM) in the SPR assay suggested that the free amino end of the 9mer places its positive charge too close to the Nrf2 binding pocket of the Kelch domain and adversely affected the binding. This is because the Nrf2 binding pocket possesses multiple positively charged residues such as Arg380, Arg415, and Arg483. The capping of the N-terminal end of the 9mer Nrf2 peptide by the Acetyl (Ac) group should eliminate the electric charge that could adversely affect the binding of the Nrf2 peptide. In fact, N- and C-terminal capping are strategies often used in peptide chemistry to generate peptides that better resemble the peptide backbone of a protein by removing the ionizable end groups of the peptide.

A series of 9mer Nrf2 peptides were synthesized starting from a resin bound Nrf2 9mers as shown in Scheme 3. For the N-acetylated peptides, the N-terminal end capping was performed on

Table 2. The Binding affinity of 9mer Nrf2 peptides determined by the SPR competition assay.⁹⁰

Nrf2 Peptides	Peptide Sequence	K _d (nM)
H-9mer-OH	H-LDEETGEFL-OH	352
H-9mer-NH ₂	H-LDEETGEFL-NH ₂	355
Ac-9mer-OH	Ac-LDEETGEFL-OH	23.1
Ac-9mer-NH ₂	Ac-LDEETGEFL-NH ₂	21.4

The SPR binding assay was performed as described under Experimental section. The K_d were determined by regression analysis using equation (14).

a resin-bound peptide using acetic anhydride (5 eq. relative to resin loading) and pyridine (5eq. relative to resin loading) in *N*-methyl-2-pyrrolidinone (NMP) for 30 min. The binding affinity of the 9mer Nrf2 peptide series determined using the SPR competition assay are summarized in Table 2.⁹⁰ The effect of the amidation of the C-terminal end was negligible and the binding affinity of the 9mer Nrf2 (352 nM) and the 9mer Nrf2 amide (355 nM) was similar. On the other hand, the effect of N-terminal end capping by the acetyl group was found to be over 10-fold improvement in the binding affinity, which is similar to the effect of labeling the 9mer Nrf2 peptide with FITC as discussed later. The binding affinity of *N*-acetyl 9mer peptide and peptide amide was determined to be 23.1 nM and 21.4 nM, respectively. Based on the SPR assay results, the *N*-acetyl 9mer peptide amide is the strongest inhibitory peptide derived from the sequence of the ETGE motif and parallels the affinity of much longer 16mer Nrf2 peptide (23.9 nM).

Next, the affinity and the assay dynamic range of each fluorescent labeled peptides (tracers) were determined by analyzing the binding curves of each tracer in the FP assay. The results of the FP assays are summarized in Table 3 and Figure 7.⁹³ Based on the results, the FITC-labeled 9mer Nrf2 peptide is the shortest peptide capable of binding to Keap1 Kelch domain with high affinity, where the K_d was determined to be 65.1 nM. The result of the FP assay was in agreement with the SPR assay on the unlabeled Nrf2 peptides.⁹⁰ The dynamic range of the FITC-labeled 9mer Nrf2 peptide was 97.3 mA, a 21.5% increase from the FITC-labeled 16mer Nrf2 peptide. Shortening

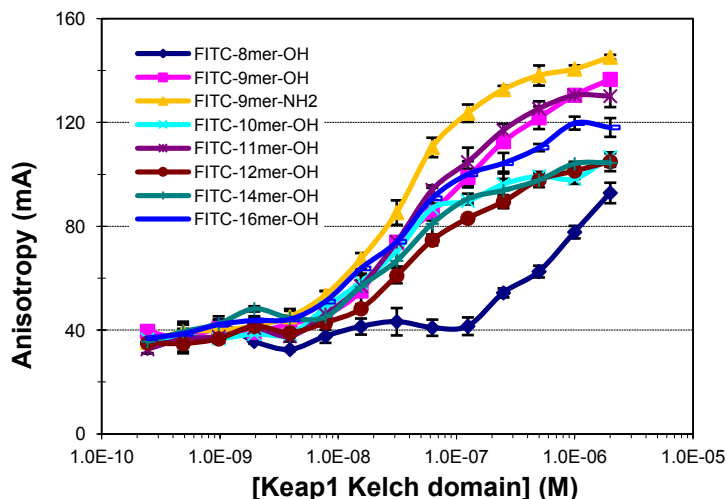


Figure 7. The fraction bound of various FITC-labeled Nrf2 peptides as a function of Keap1 Kelch domain concentrations.⁹³ Each of the fluorescent probes (10 nM) were incubated with 0.2 nM to 2 μ M Keap1 Kelch domain for 30 min at rt before anisotropy measurement. The A values were adjusted with the G-value determined from the literature value of free fluorescein.

the sequence by another amino acid deactivates the peptide probe and the K_d could not be determined by the regression analysis for the FITC-labeled 8mer Nrf2 peptide. This suggests that the fluorescent dye is too close to the ETGE binding site and adversely affected the binding. The binding affinity of the longer 10- to 14mer Nrf2 peptides were similar to that of the FITC-labeled 9mer Nrf2 peptide. This is consistent with the observations that the most of the binding interactions between Keap1 Kelch domain and Nrf2 resides within the DxETGE sequence. Therefore, the binding affinity of the tracer is not significantly affected as long as it contains the 9mer Nrf2 peptide sequence.

Finally, binding affinities of FITC-labeled Nrf2 peptides were confirmed by the SPR binding assay.⁹³ As shown in Table 4, the general trend for the binding affinity of the FITC-Nrf2 peptides was consistent with the results obtained by the FP assay. With the exception of the FITC-labeled 10mer and the 12mer, the binding affinity increases with increasing peptide length. Therefore, the SPR data also supports that most of the bonding interaction between Keap1 Kelch domain resides within the 9mer Nrf2 peptide sequence as the binding affinity of the FITC-labeled 9mer and the

longer Nrf2 peptides were similar. The binding affinity of the FITC-labeled 8mer Nrf2 peptide was significantly weaker than the FITC-9mer Nrf2 peptide as seen in the FP assay, where the deletion of one residue from the 9mer Nrf2 peptide sequence results in more than 20-fold decrease in affinity.

In order to obtain a peptide tracer that improves on FITC-9mer Nrf2 peptide, the effect of C-

Table 3. The binding affinity and dynamic range of FITC-labeled Nrf2 peptides determined by the FP assay.⁹³

	Peptide	K_d (nM)	Dynamic Range (ΔmA)
FITC-8mer Nrf2	FITC-DEETGEFL-OH	~ 750	-
FITC-8mer Nrf2-NH ₂	FITC-DEETGEFL-NH ₂	~ 1000	-
FITC-9mer Nrf2	FITC-LDEETGEFL-OH	65.1 $\pm$ 9.7	97.3
FITC-9mer Nrf2-NH ₂	FITC-LDEETGEFL-NH ₂	25.6 $\pm$ 10.8	109.8
FITC-10mer Nrf2	FITC-QLDEETGEFL-OH	30.1 $\pm$ 6.1	73.5
FITC-11mer Nrf2	FITC-LQLDEETGEFL-OH	47.7 $\pm$ 7.4	96.3
FITC-12mer Nrf2	FITC-QLQLDEETGEFL-OH	44.5 $\pm$ 12.9	70.6
FITC-14mer Nrf2	FITC-FAQLQLDEETGEFL-OH	61.9 $\pm$ 16.5	64.2
FITC-16mer Nrf2	FITC-AFFAQLQLDEETGEFL-OH	28.7 $\pm$ 5.7	80.1

Anisotropy measurements for the FP binding assay were performed as described under methods section. K_d values were determined using equation (14) and the dynamic range was calculated as the difference in the free and the bound anisotropy of the respective ligand.

Table 4. The SPR Competition Assay for the Confirmation of Binding Affinity of the FITC-Nrf2 Peptides.⁹³

	Peptide	K_d (nM)
FITC-8mer Nrf2	FITC-DEETGEFL-OH	~835
FITC-9mer Nrf2	FITC-LDEETGEFL-OH	33.2 $\pm$ 4.4
FITC-9mer Nrf2-NH ₂	FITC-LDEETGEFL-NH ₂	22.3 $\pm$ 4.2
FITC-10mer Nrf2	FITC-QLDEETGEFL-OH	7.6 $\pm$ 2.5
FITC-11mer Nrf2	FITC-LQLDEETGEFL-OH	24.4 $\pm$ 9.8
FITC-12mer Nrf2	FITC-QLQLDEETGEFL-OH	2.8 $\pm$ 1.6
FITC-14mer Nrf2	FITC-FAQLQLDEETGEFL-OH	22.8 $\pm$ 6.0
FITC-16mer Nrf2	FITC-AFFAQLQLDEETGEFL-OH	14.5 $\pm$ 4.4

The SPR binding assay was performed as described under Experimental section. The K_d were determined by regression analysis using equation (14).

terminal end capping of the FITC-labeled 9mer Nrf2 peptide was also investigated.⁹³ The FITC-9mer and -8mer Nrf2 peptide amides were synthesized as shown in Scheme 2 using the Rink Amide resin. Although the effect of C-terminal end capping for unlabeled Nrf2 peptide were negligible, the C-terminal end-capping of FITC-9mer Nrf2 peptide as the amide increased the binding affinity of the probe by 2-fold as compared to the FITC-9mer Nrf2 peptide. The binding affinity of the FITC-labeled 9mer Nrf2 peptide amide was determined to be 25.6 nM (Table 1). Interestingly, the dynamic range of the assay for the amide was also better with approximately 12.8% increase in the assay range at 109.8 *nM*. The increase in the assay dynamic range over the FITC-16mer Nrf2 peptide was 37%. However, the similar increase in binding affinity to Keap1 Kelch domain was not observed between the FITC-8mer Nrf2 peptide and the FITC-8mer Nrf2 amide. By shortening the sequence and capping the C-terminal end of the peptide as the amide, a tracer with high dynamic range and binding affinity to Keap1 Kelch domain was successfully obtained.

C. Comparison of fluorophores for the FP Assay

To determine the fluorophore best suited for the Keap1-Nrf2 FP assay, the 9mer Nrf2 peptide amide was labeled with 3 additional fluorophores; Bodipy-FL, Cy3B, and Cy5. The Bodipy-FL is a popular replacement for fluorescein owing to its higher extinction coefficient ($>80,000 \text{ M}^{-1} \text{ cm}^{-1}$), high quantum yield (1.0 in water), insensitivity to environment such as pH,⁹⁴ and sharp excitation and emission spectra. The cyanine (Cy) dyes, Cy3B and Cy5, are also known for their excellent fluorescence properties and high water solubility, which is advantageous for labeling compound that dissolve poorly in aqueous assay medium.⁹⁵ The Cy3B dye is an analog of Cy3 that is extremely bright and much higher quantum yield than any other Cy dyes. It is also stable and the properties of Cy3B make it well suited for FP application. The ranking of the fluorophores for the FP assay was determined by the analysis of K_d and dynamic range of each probe to Keap1 Kelch

domain. The same titration conditions developed for FITC-labeled Nrf2 peptides was used for the analysis where 10 nM of each probe was incubated with Keap1 Kelch domain at a range of concentrations for 30 min at room temperature.

As shown in Table 5 and Figure 8, Bodipy-FL labeled 9mer Nrf2 peptide amide gave the lowest dynamic range of the four fluorescently-labeled peptides with an assay window of 66.5 mA. The FITC-labeled 9mer Nrf2 amide gave the second lowest assay window with a anisotropy change of 109.8 mA, while the Cy3B and Cy5-labeled peptide gave assay windows of 167.7 and 143.2 mA, respectively. Interestingly, the cyanine labeled peptides had slightly higher binding affinity to Keap1 Kelch domain, while Bodipy-FL-9mer Nrf2 peptide amide showed similar binding affinity to the FITC-labeled peptide. In order to compare the performance of fluorophores for our assay, Z' -factor was calculated for each probe.⁹⁶ The Z' -factor evaluates the performance of assay by incorporating the assay window and the random error present in the FP measurement. Thus, Z' -factor is a statistical parameter that can be used to evaluates the quality of any given assay and can be useful for assay development and optimization.⁹⁶ As shown in Table 5, all of the fluorophores perform well under our assay conditions with the Z' -factors greater than 0.8. Despite

Table 5. The dynamic ranges and affinities of 9mer Nrf2 peptide amides labeled by different fluorophores determined by the FP assay.⁹³

Fluorophore	Filter set	ΔmA	Z' -factor	K_d (nM)
Fluorescein	485/535	109.8	0.915	25.6 ± 10.8
Bodipy-FL	485/535	66.6	0.862	20.0 ± 4.18
Cy3B	530/590	167.7	0.933	6.07 ± 4.4
Cy5	620/680	143.2	0.821	11.4 ± 7.2

Anisotropy measurements for the FP binding assay were performed as described under methods section. K_d values were calculated from anisotropy of ligands using equation (14) and ΔmA values were calculated as the difference in the free and the highest observed anisotropy of the respective ligands. The Z' -factors were calculated as described by Zhang et al using the top and bottom replicates of the dose-response curves.⁹⁶

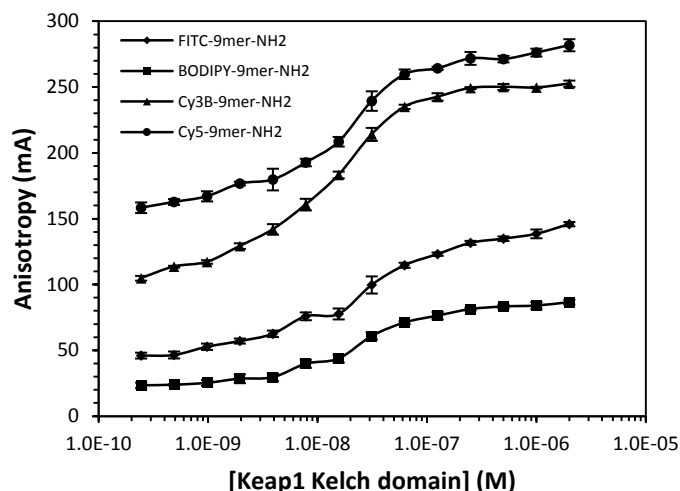


Figure 8. The anisotropy of four 9mer Nrf2 peptide probes plotted as a function of Keap1 Kelch domain concentrations. Each fluorescent probe at a fixed concentration (10 nM) was incubated with varying concentrations of Keap1 Kelch domain (0.24 nM to 2 μ M) for 30 min at rt.

the advantages of using Cy dyes, the cost of the labeling reagents make the FITC-labeled peptide as the viable option for HTS application as evidenced by the relatively high Z' -factor.

III. Competition FP Assay Using the Optimized Tracer

A. Evaluation of Inhibitory Activities of the Nrf2 Peptides

The tracer used for the competition FP assay was obtained by the optimization of the FITC-labeled Nrf2 peptide. Based on the affinity and the dynamic range of the assay, FITC-9mer Nrf2 peptide amide was selected as the tracer for the competition assay. Shown in Figure 9 is the binding curve of FITC-9mer Nrf2 peptide amide, which hereon forth will be referred to as the tracer or the probe. The binding affinity of the tracer has been determined ($K_d = 25.6$ nM) by titrating with Keap1 Kelch domain as shown in Figures 7 and 9. The concentration of the tracer that is appropriate for the assay has been previously determined by the anisotropy measurement of the tracer alone and is fixed at $L_0 = 10$ nM. The selected concentration of the tracer is lower than twice its K_d to ensure that the stoichiometric titration conditions, which decrease the assay

sensitivity, are avoided for the competition FP assay.⁹⁷ In addition, the concentration of Keap1 Kelch domain was selected such that the fraction bound of the tracer (f_b) is between 0.5 and 0.8.⁹⁷ This is because based on the IC_{50} equation (10) for a FP competition assay, f_b close to 1.0 will result in large value of IC_{50} for an inhibitor:

$$IC_{50} = \left(\frac{f_b - K_d}{(1 - f_b)(2 - f_b)} + \frac{f_b \times L_0}{2} \right) \left(\frac{K_i(2 - f_b)}{K_d \times f_b} + 1 \right) \quad (10)$$

Where K_i is the affinity of the inhibitor. The f_b is selected so that the anisotropy measurement fall in the sensitive region of the curve (e.g. steeper slope) and not when the binding plateaus. It is problematic when the IC_{50} is underestimated as many potential inhibitors will be missed when the assay is used to evaluate a compound or screen a library of compounds. For this reason, the concentration of Keap1 Kelch domain was set to 100 nM to achieve sufficient anisotropy level and the f_b of the tracer at the protein concentration is approximately 0.75 as shown in Figure 9.

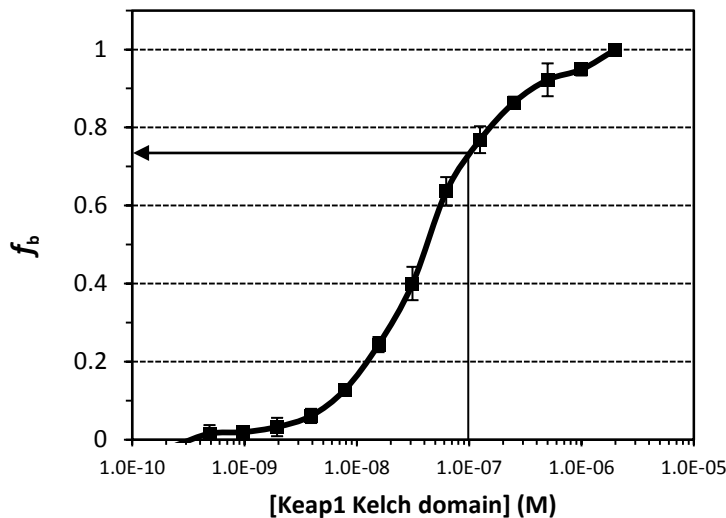


Figure 9. The fraction of FITC-9mer Nrf2 peptide amide bound (f_b) to Keap1 Kelch domain as a function of protein concentrations. The tracer (10 nM) was incubated with varying concentrations of Keap1 Kelch domain for 30 min at room temperature before anisotropy measurement. The fraction bound (f_b) for each point was calculated using equation (13).

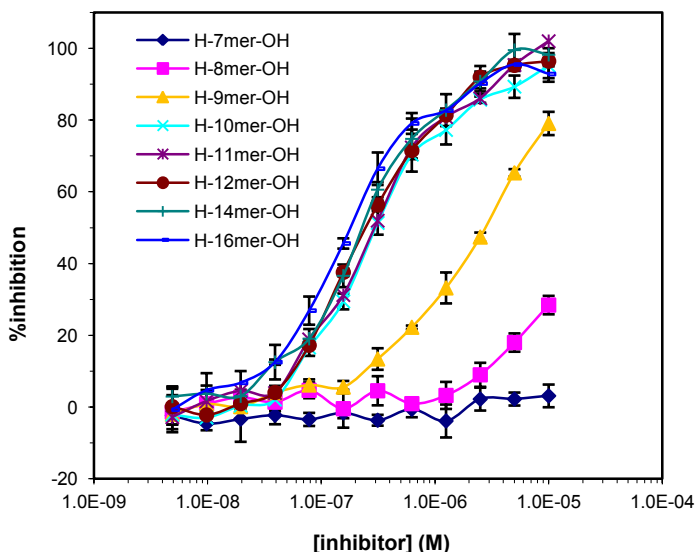


Figure 10. The inhibition of binding of the tracer to Keap1 Kelch domain by a series of Nrf2 peptides determined by the competition FP assay.⁹³ The assay was performed using 10 nM tracer and 100 nM Keap1 Kelch domain in presence of varying concentrations of the Nrf2 peptides.

FP competition assay under the selected conditions was used to differentiate a series of Nrf2 peptide as inhibitors of Keap1-Nrf2 interaction. The results from the competition assay are summarized in Figure 10 and Table 6. The binding affinities of the Nrf2 peptides against Keap1 Kelch domain determined by the FP competition assay were in agreement with the binding affinities for the series of Nrf2 peptides obtained by the SPR method (tables 3 and 4). The general trend in the inhibitory activity of the Nrf2 peptide is that it increases with increasing peptide length as higher IC_{50} values for the shorter 8mer and 9mer Nrf2 peptides ($IC_{50} = 30.5$ and $3.48 \mu M$, respectively) and lower IC_{50} values were observed for the longer 10mer to the 16mer Nrf2 peptides. The inhibitory activity of the 16mer Nrf2 peptide (H-AFFAQLQLDEETGEFL-OH) was determined to be 163 nM and a calculated affinity K_i of 37.4 nM by the FP assay. The FP data for the 16mer Nrf2 peptide was also in good agreement with the reported value obtained by ITC.⁶⁷

It has been reported that for a FP assay under nonstoichiometric conditions, the resolution of inhibitor potencies will be limited by the K_d of the tracer when the inhibitor potency is much stronger relative to the affinity of the tracer ($K_i \lll K_d$).⁹⁷ This means that in such scenario, the IC_{50} of an inhibitor will be directly proportional to the K_d of the tracer used. The binding affinities of Nrf2 peptide inhibitors determined by the FP assay were in good agreement with the results obtained by the SPR assay, which indicates that the resolution of binding affinities between the Nrf2 peptides is not limited by the FP competition assay conditions. In addition, the binding affinity of the 16mer Nrf2 peptide is in good agreement with the reported value. This proves that the assay is able to determine the binding affinity of Nrf2 peptides of varying length. The

Table 6. The inhibition of Keap1 Kelch domain by the Nrf2-peptides determined by the FP assay.⁹³

	Peptide	IC_{50} (μ M)	K_i (nM)
7mer Nrf2	H-EETGEFL-OH	$>>100$	-
8mer Nrf2	H-DEETGEFL-OH	21.7 ± 20.1	7010
8mer Nrf2-NH ₂	H-DEETGEFL-NH ₂	30.5 ± 22.7	9870
9mer Nrf2	H-LDEETGEFL-OH	3.48 ± 0.919	865
9mer Nrf2-NH ₂	H-LDEETGEFL-NH ₂	3.57 ± 2.2	1140
Ac-9mer Nrf2	Ac-LDEETGEFL-OH	0.194 ± 0.049	47.4
Ac-9mer Nrf2-NH ₂	Ac-LDEETGEFL-NH ₂	0.196 ± 0.032	48.1
10mer Nrf2	H-QLDEETGEFL-OH	0.272 ± 0.026	72.7
11mer Nrf2	H-LQLDEETGEFL-OH	0.298 ± 0.033	81.1
12mer Nrf2	H-QLQLDEETGEFL-OH	0.249 ± 0.022	65.2
14mer Nrf2	H-FAQLQLDEETGEFL-OH	0.243 ± 0.020	63.3
16mer Nrf2	H-AFFAQLQLDEETGEFL-OH	0.163 ± 0.011	37.4

The fluorescence polarization measurements for the competition assay were performed as described in the methods section. The reported IC_{50} values were determined by regression analysis using equation (10)

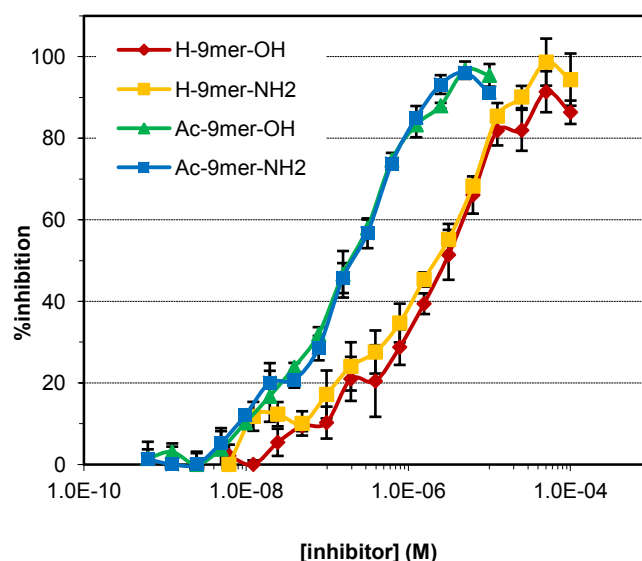
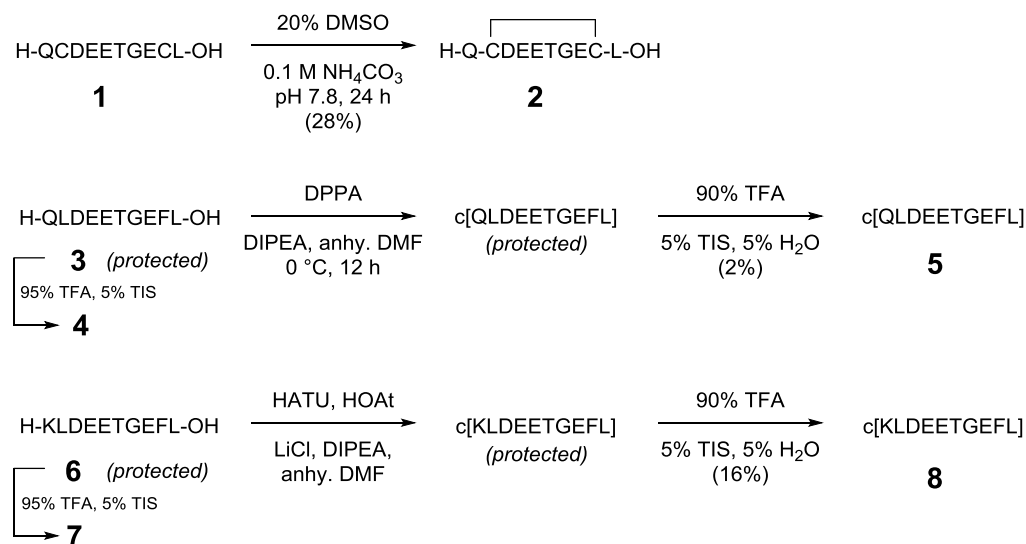


Figure 11. Inhibition of Keap1-Nrf2 interaction by a series of 9mer Nrf2 peptides. The assay was performed at rt using 10 nM FITC-9mer Nrf2 peptide amide as the tracer and 100 nM Keap1 Kelch domain in the presence of varying concentrations of 9mer Nrf2 peptides.

inhibitory activity of the N- and or C-terminal capped 9mer Nrf2 peptides were also determined by the FP competition assay and compared to the results obtained by the SPR assay. As shown in Figure 11, a significant 14× increase was observed as a result of N-terminal acetylation of 9mer Nrf2 peptide ($IC_{50} = 194$ nM). A similar increase of 18× was observed for the N-acetylation of 9mer Nrf2 peptide amide ($IC_{50} = 196$ nM). While the effect of neutralizing the positive charge at the N-terminus of 9mer Nrf2 peptides by acetylation was obvious, the C-terminal capping as an amide had negligible effect on the binding affinity. These results were in agreement with the results obtained by the SPR assay.⁹⁰

B. Synthesis and Evaluation of Cyclic Nrf2 Peptides

The crystal structure of human Keap1 Kelch domain bound to the 16mer Nrf2 peptide determined



Scheme 4. The synthesis route for 3 cyclic Nrf2 peptides

that the peptide binds to the Kelch domain binding pocket as a β -hairpin motif, in which two anti-parallel β -strands are connected by overlapping β -turns.⁶⁷ The β -turn is stabilized by the hydrogen-bonding interaction between the side chains of residues E77 and T80.⁶⁷ Analysis of the crystal structure revealed that the side chains of residues Q75, L76, F83 and L84 have no direct contact with the residues of Keap1 and could be replaced by residues such as cysteine to restrict the peptide into β -turn conformation through cyclization. Conformational restriction of peptides to mimic the secondary structures is a strategy often used in peptide chemistry to improve specificity and binding affinity.⁹⁸ In addition, the cyclic peptides could provide insight into the design of peptidomimetics by identifying pharmacophores necessary for strong affinity to Keap1. In light of this, several cyclic peptides were targeted to evaluate the effect of conformational restriction in hopes to obtain a stronger inhibitory Nrf2 peptide.

As shown in scheme 4, 3 cyclic peptides were synthesized as the inhibitor of Keap1-Nrf2 interaction. The disulfide linked cyclic peptide **2** can be readily synthesized by the DMSO

Table 7. The binding affinity of Nrf2 peptides determined by the AlphaScreen assay

Peptide	Sequence	K _d (nM)
1	H-QCDEETGECL-OH	29.9
2	H-Q-c[CDEETGEC]-L-OH	64.8
4	H-QLDEETGEFL-OH	87.3
5	c[QLDEETGEFL]	301.2
7	H-KLDEETGEFL-OH	98.7
8	c[KLDEETGEFL]	558.8
11mer Nrf2	H-LQLDEETGEFL-OH	69.4
16mer Nrf2	H-AFFAQLQLDEETGEFL-OH	58.6

oxidation of the linear peptide **1** in dilute solution. The concentration of the reaction was kept low to prevent intermolecular disulfide bond formation. The progress of the disulfide formation was monitored using Ellman's test. The head-to-tail cyclizations of the peptides **3** and **6** were also performed in diluted solution and either DPPA or HATU was used as the coupling reagent. For the cyclo-Lys-10mer peptide **8**, it was envisioned that the fluorescent labeling by a reactive dye at the ϵ -amino group could be performed to give the tracer with high affinity with minimal propeller effect.

The evaluation of the cyclic peptides using AlphaScreen was performed by our collaborator, Dr. Xinyi Huang, using the recombinant His₆-tagged Keap1 Kelch domain and the biotin-labeled 16mer Nrf2 peptide. The concept of AlphaScreen assay is discussed in Chapter 4. As shown in Figures 12 and 13, the disulfide-linked peptide showed almost 2-fold less affinity to Keap1 Kelch domain than its linear counterpart where the binding affinity of the linear peptide **1** and disulfide linked peptide **2** was 29.9 nM and 64.8 nM, respectively. Similarly, the other two cyclic peptides have worse binding affinity to Keap1 Kelch domain than its linear analog where the difference in binding affinity between linear and cyclic analog was approximately 4-fold for the 10mer Nrf2 (peptides **4** and **5**) and more than 5-fold for the Lys-containing 10mer Nrf2 peptide (peptides **7** and **8**). This means that the conformational restriction of the peptide puts the peptide in less ideal

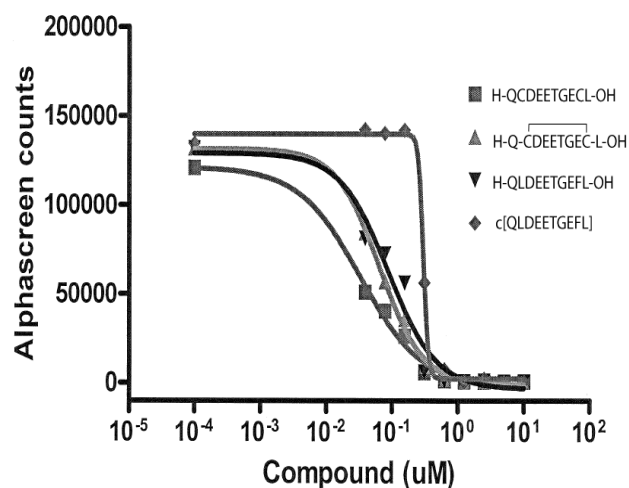


Figure 12. The binding curves of cyclic and linear Nrf2 peptides determined by the AlphaScreen assay

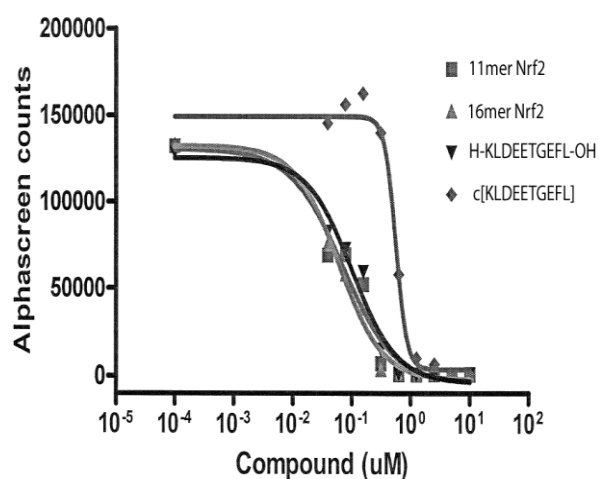


Figure 13. The binding of 11-, 16-mer, and the Lys-containing Nrf2 peptides determined by the AlphaScreen assay

conformation for binding to Keap1 Kelch domain. While the cysteine-containing linear peptide **2** showed strong affinity to Keap1, there is a concern of using this peptide in the development of the FP assay due to the free thiols that could potentially form a disulfide bond with the protein. In light of these data, it was determined that linear peptides based on the sequence of ETGE motif are still the best peptide inhibitors of Keap1-Nrf2 interaction as well as for the fluorescently-

labeled peptides for the FP assay.

IV. High-Throughput Screening of Pilot Libraries

A. The DMSO Tolerance and Performance of the FP Screening Assay

Compounds in chemical libraries are often dissolved in DMSO and the typical DMSO concentration for high-throughput screening (HTS) FP assays range from 0.5 – 5%. The concentration of DMSO in FP-based assays are kept relatively low as not to unnecessarily increase the viscosity (η) of the assay medium. The polarization value of the fluorescent molecules increases due to slower rotational relaxation time in viscous medium and is not desirable for FP measurement. This, again is given by the Stokes-Einstein equation for the Debye rotational relaxation time (ρ):

$$\text{Polarization } (P) \propto \rho = \frac{3\eta V}{RT}$$

Where increase in viscosity (η) increases the rotational relaxation time (ρ), which is directly proportional to the polarization of the molecule. As shown in Figure 14, the bound state of the tracer is less sensitive to the increasing concentration of DMSO and the change in anisotropy value even at 20% DMSO was negligible. On the other hand, the anisotropy of the free tracer increases with increasing concentration of DMSO. Figure 14 also shows that the FP assay tolerates fairly high concentrations of DMSO with less than 7 mA increase in the anisotropy of the free tracer at 10% DMSO concentration. Despite this, the decrease in the assay dynamic range due to unnecessary high concentration of DMSO will negatively impact the Z' -factor of the assay and should still be avoided. The concentration of DMSO in typical HTS assays fall under 5% and that DMSO concentration for the HTS FP assay is acceptable for high throughput applications considering the almost negligible change in the dynamic range of the assay.

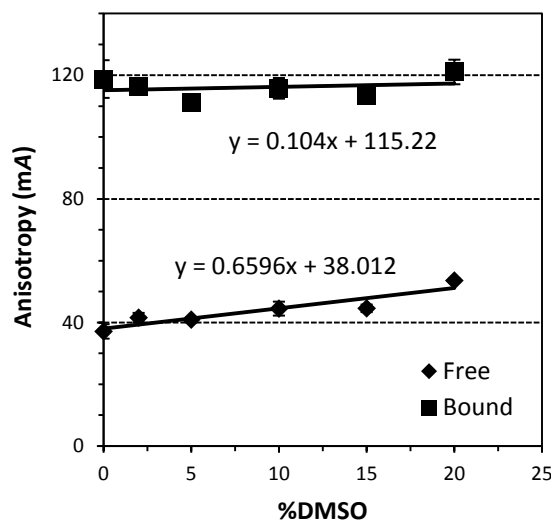


Figure 14. The effect of DMSO concentration on the dynamic range of the FP assay was evaluated by measuring the anisotropy of the free tracer and bound tracer in the assay buffer containing 0 to 20% DMSO.

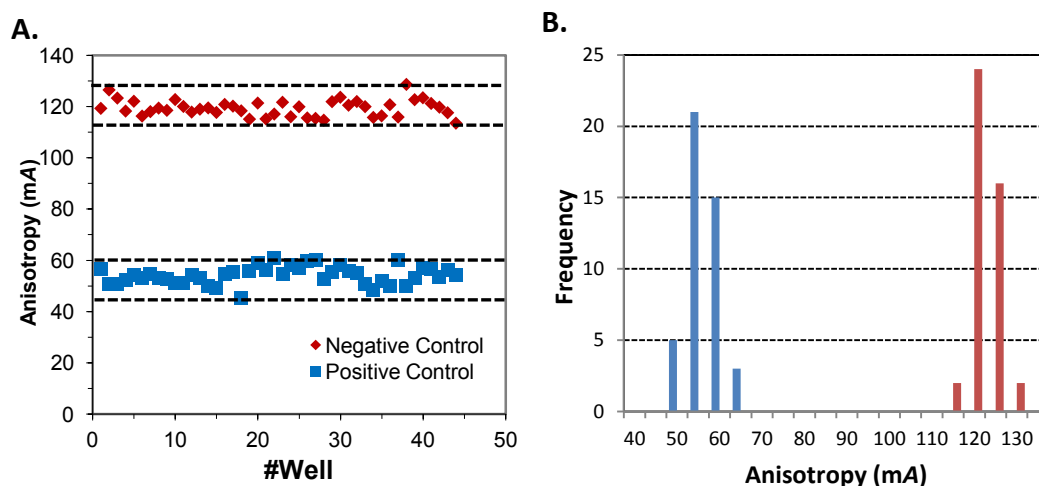


Figure 15. (A) The single FP assay data showing 44 replicates of the positive and negative control wells and (B) the histogram of the assay data showing the separation of the bands. The dotted horizontal lines show the data variability of the controls. The Z' -factor for the assay was calculated as described in the Experimental section using equation (17).⁹⁶

In order to evaluate the performance of the FP assay for HTS, Z' -factor was calculated using equation (17) as described in the Experimental section. According to the Z -factor model, values greater than 0.5 indicate excellent assays which may be readily adapted to high throughput applications.⁹⁶ Using 1 μ M *N*-acetyl 9mer Nrf2 amide as the test compound (positive control,

~80% inhibition) and 0.75 fraction of the tracer bound in the absence of the test compound as the negative control, a Z' -factor of 0.70 was obtained for the assay. As shown in Figure 15, the Z' -factor indicates that the separation between the top and bottom data variability band is large, and FP assay in the 384-well format is an excellent assay suitable for HTS application.

B. HTS FP Assay of NCI Diversity Set II and NIH Clinical Collection

The high-throughput screening assay focused on the discovery of inhibitors of Keap-Nrf2 interaction was performed on the NCI Diversity Set II, which consists of 1364 compounds, and the NIH Clinical Collection (NCC), which consists of 446 compounds with history of use in human clinical trials, for a combined total of 1810 compounds. The NCI Diversity Set II is a set of compounds selected from DTP repository of 140,000 compounds based on criteria such as availability of the compound and their pharmacophores. The compounds in the NCI Diversity Set II are selected by the 3-point pharmacophore method centered around H-bond acceptor/donor, positive charge, aromatic, hydrophobic, and acid/base using Chem-X (Oxford Molecular Group) program to increase the number of unique pharmacophores in the set. Thus, screening of the diversity set may provide some structure-based hypothesis from the hits. The primary screening assay of the pilot libraries was performed in duplicate under the FP competition assay conditions as determined in the present study using 100 nM Keap1 Kelch domain and 10 nM FITC-9mer Nrf2 amide (tracer) in presence of 100 μ M samples with final DMSO concentration of 10% to ensure samples are dissolved in solution. Relatively high concentration for the primary screening was used such that weak inhibitors at this concentration will not be ideal for further medicinal chemistry work. The positive control wells included in each assay plate were octuplicate of 1 μ M *N*-acetyl-9mer Nrf2 peptide amide containing 10% DMSO, which provides approximately 80% inhibition of Keap1 Kelch domain and can be used to calculate the Z' -factor of each assay plate.

For the primary screen at 100 μ M sample concentration, the inhibition cutoff of $\text{mean} \pm 3 \times \text{standard deviations (SD)}$ was applied to determine whether a compound is a hit. A compound that has fluorescence or quenches the fluorescence signal of the tracer causes interference in the polarization measurement. Therefore in order to minimize the inclusion of unreliable data in the analysis, the assay data were filtered using the fluorescence cutoff of tracer $\text{fluorescence} \pm 2\text{SD}$. Based on the fluorescence, 170 compounds were removed due to interference of the fluorescence signal as compared to the control wells. With the above hit calling criteria, a hit frequency of 2.0% (36 / 1810 compounds) was observed for the primary screening assay at 100 μ M (Figure 16A) and the Z'-factor for this pilot screen averaged 0.6 ± 0.05 . The secondary screening assay at 10 μ M sample concentration gave 2 compounds that were hits based on the $\text{mean} \pm 3\text{SD}$ inhibition cutoff as shown in Table 7 and Figure 16B. The confirmatory dose-response analysis of the two compounds using the FP assay showed that only one of the compounds inhibit the Keap1-Nrf2 interaction at IC_{50} lower than 10 μ M (Figure 18A).

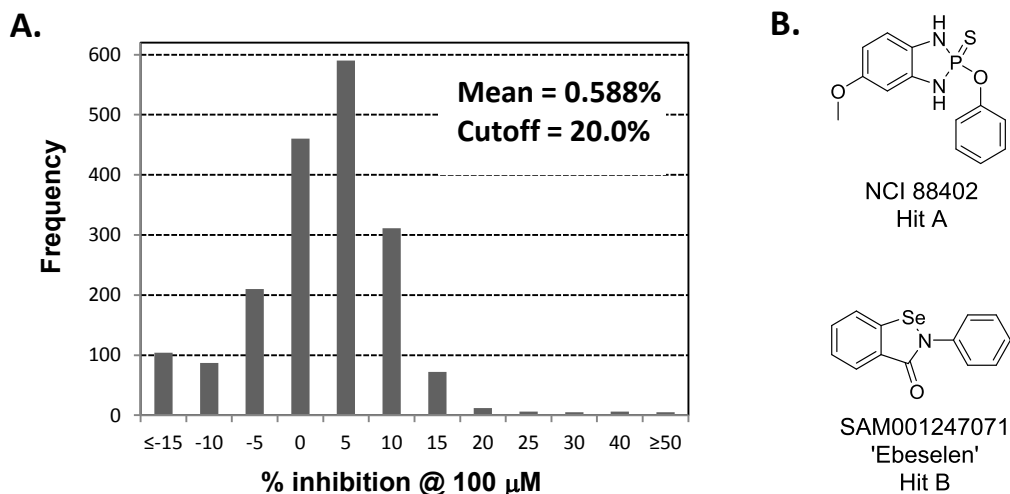
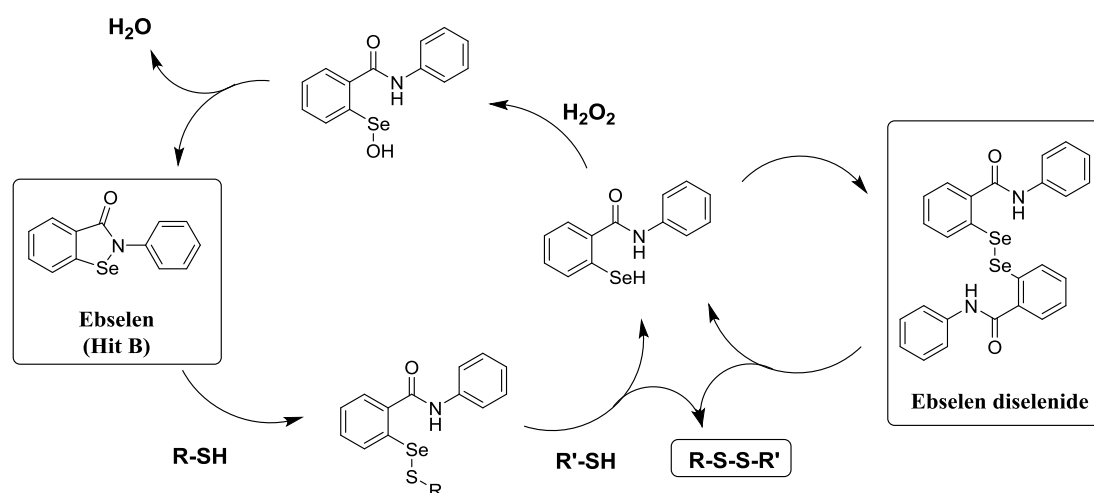


Figure 16. (A) The combined histogram of Keap1-Nrf2 inhibitory activity for NCI Diversity Set II and NIH Clinical Collection and (B) the structures of 2 compounds considered as hits in the HTS assay.

Table 7. Results of the pilot library screening by the FP assay

NCI Diversity Set II and NIH Clinical Collection	1810 compounds
Inhibition cutoff (mean $\pm$ 3SD)	20.0%
Fluorescent samples removed	170
Primary hits (100 μ M)	36 (2.0%)
Secondary hits (10 μ M)	2
Hit compound with IC ₅₀ < 10 μ M	1

As shown in Figure 16B, Hit B is ebselen (2-phenyl-1, 2-benzisoselenazol-3(2H)-one) which is a mimetic of Glutathione peroxidase reported to be a potent scavenger of peroxides.^{99,100} Based on the mechanism of action of ebselen against Glutathione S-transferase (GST), it is believed that cysteine(s) of Keap1 Kelch domain may be covalently modified by ebselen, and thus irreversibly inhibited from binding to the tracer by conformational change or denaturation of the protein (Figure 17).¹⁰⁰⁻¹⁰³ The addition of fresh Keap1 Kelch domain into the assay wells temporary restores the fluorescence polarization signal (i.e. the tracer bound state), however the polarization signal decreases over time to give the similar dose-response curve. This suggests that when additional Keap1 Kelch domain is added to the wells, tracer can bind to the fresh unmodified Keap1 Kelch domain. After extended incubation with ebselen, Keap1 Kelch domain is prevented from binding to the tracer due to irreversible or covalent modification of the protein.

**Figure 17.** The structure of ebselen and its glutathione peroxidase-like catalytic activity.

In light of these results, it was hypothesized that other thiol-reactive electrophiles known for its anti-oxidative effect, such as PEITC, sulforaphane and curcumin, could exhibit similar inhibitory activity against Keap1 Kelch domain in the FP assay. Therefore, these compounds were tested together for their activity against Keap1 by the dose-response assay. The results of the assay is summarized in Figure 18B. After 24 hr incubation, only Ebselen showed inhibition of Keap1-Nrf2 interaction, while sulforaphane and PEITC had no inhibition. Curcumin did not give reliable data as its strong yellow color severely interfered with the fluorescence polarization signal, and therefore, it was excluded from further analysis. While electrophiles such as PEITC, sulforaphane, and curcumin are consumed in the reaction with a nucleophile to form an adduct, organoselenium compounds such as Ebselen has been reported to quickly form diselenides in absence of peroxides, which can continue to react with proteins in a catalytic mechanism (Figure 17).¹⁰⁴ Based on the fact that isothiocyanates (PEITC, sulforaphane) and Michael-acceptor (curcumin) did not show direct inhibition of Keap1- Nrf2 interaction, it is possible that the catalytic mechanism of ebselen is more effective in inhibition of Keap1 Kelch domain under the assay conditions. While the pilot screening may have not discovered a direct inhibitor of Keap1-Nrf2

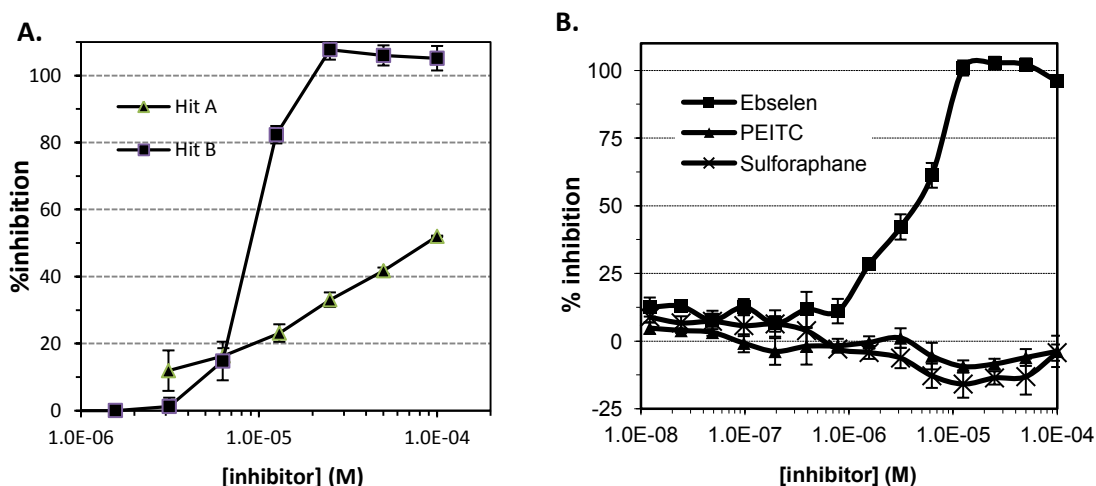


Figure 18. (A) Dose-response curves for the two hit compounds at 10 μ M using the FP assay and (B) the dose-response curves of 2 known inducers of ARE and Ebselen. The competition FP assay was performed using 10 nM FITC-9mer Nrf2 peptide amide as the probe and 100 nM Keap1 Kelch domain in the presence of varying concentrations of inhibitors.

interaction, the FP assay applied to screen library of compounds was successful in identifying a molecule capable of inhibiting Keap1-Nrf2 interaction regardless of the mechanism of inhibition.

V. Summary

In the present study, a method for screening library of compounds was investigated to facilitate the discovery of small molecule inhibitors of Keap1-Nrf2 interaction. The optimization of fluorescent labeled Nrf2 peptides based on the sequence of high affinity ETGE motif of human Nrf2 was successfully performed to obtain the tracer for the development of FP assay. The 16mer Nrf2 peptide was sequentially shortened and found that the shortest FITC-labeled Nrf2 peptide that retains a strong binding affinity to Keap1 Kelch domain was FITC-9mer Nrf2 peptide. Further modification of FITC-9mer Nrf2 peptide lead to the FITC-9mer Nrf2 peptide amide, which was selected as the tracer in developing the fluorescence polarization assay. FITC-9mer Nrf2 peptide amide was shown to have the largest assay window and a binding affinity that is equal to the FITC-16mer Nrf2 peptide. In addition, the 9mer Nrf2 peptide amide was modified with additional fluorophores and found that the assay windows of cyanine-labeled 9mer Nrf2 peptide amides were greater than that of the FITC-9mer Nrf2 amide. These cyanine-labeled tracers could be used as the secondary probe to provide better signal-to-noise ratio and also in confirmatory FP assays to rule out the false positives by evaluating the hits at a much higher wavelength.

The competition FP binding assay using the optimized tracer was performed to evaluate the binding affinity of 7mer to 16mer Nrf2 peptide inhibitors. The binding affinity of the 16mer Nrf2 peptide (H-AFFAQLQLDEETGEFL-OH, $K_i = 37.4$ nM) was consistent with the reported value and was in fact the strongest Nrf2 peptide in terms of affinity to Keap1.⁶⁸ The assay results indicated a overall trend that the binding affinity increases as the peptide length increase. In the efforts to find a potent peptide inhibitor of Keap1-Nrf2 interaction, the 9mer Nrf2 peptides was modified at the N- and C-terminal ends. The results showed that the N-acetylated 9mer peptides

can bind to Keap1 Kelch domain with affinity similar to the 16mer Nrf2 peptide. The cyclic Nrf2 peptides were also investigated, but the conformational restriction of the peptide was found to result in decrease of inhibitory activity. It was concluded that the conformation of cyclic peptides were likely restricted in undesirable conformations for binding to Keap1 Kelch domain. In a separate project, a colleague in the group investigated the penetration of Nrf2 peptide inhibitors into the cell by attaching the Nrf2 peptide to cell-penetrating peptide sequences such as Cys-TAT, as peptide-based inhibitors are known to have limitations on drug-like properties such as cell permeability. Cellular activity of these Nrf2 peptides were not detected and our research was focused on the discover of small-molecule inhibitors of Keap1.

The competition FP assay was extended to screen NCI Diversity Set II and NIH Clinical Collection for a total of 1810 unique compounds. The HTS of the libraries at a sample concentration of 100 μ M identified 36 compounds as initial hits. These hits were screened at a lower 10 μ M concentration to obtain 2 hit compounds with potential activity against Keap1-Nrf2 interaction. The dose-response analysis of the 2 compounds has shown that Hit A was not a confirmed hit. On the other hand, Hit B (Ebselen) showed dose-dependent inhibition of Keap1-Nrf2 interaction at an IC_{50} lower than 10 μ M. However, the inhibitory effect from Hit B (Ebselen) appears to stem from the covalent modification of Keap1 Kelch domain, which prevents the tracer from binding, and therefore it is likely to be not a direct inhibitor of Keap1. Although the NCI Diversity Set II and NIH Clinical Collection did not contain a direct inhibitor of Keap1-Nrf2 interaction, it demonstrated that the FP competition assay using FITC-9mer Nrf2 peptide amide is robust and suitable for high throughput applications for the discovery of small molecules that can disrupt the interaction between Keap1 and Nrf2. Nrf2 is the principle mediator of ARE expression, thus the disruption of Keap1-Nrf2 complex is believed to be an effective strategy in inducing the expression of antioxidant and cytoprotective genes.

CHAPTER THREE

THE SITE SPECIFIC *IN SITU* LABELING OF RECOMBINANT PROTEINS FOR TR-FRET ASSAY

I. Theory of TR-FRET

Fluorescence or Förster resonance energy transfer (FRET) and time-resolved FRET (TR-FRET) share the same fundamental theory. It is a distance-dependent nonradiative transfer of excited state energy from the donor fluorophore to the acceptor fluorophore by a dipole-dipole interaction that occurs when the pair of fluorophores with a spectra overlap is brought within close proximity of one another as shown in Figure 19.^{105,106} This principle theory of FRET was first developed by Theodor Förster in 1946. As shown in Figure 20, the donor fluorophore at singlet ground state (S_0) absorbs the energy from incident light, resulting in the excitation of the donor to a singlet excited state (S_1).¹⁰⁷ Multiple level of excited states (S_n) exists for the donor but the vibrational relaxation to the singlet state S_1 is very rapid and almost all emissions occur from the singlet excited state.¹⁰⁶ The donor in S_1 state can return to the S_0 state spontaneously by either radiative or nonradiative processes. When the acceptor is within the close proximity of the donor, then the fluorescence energy transfer can occur by the resonance of the emission transition dipole of the donor and the absorption transition dipole of the acceptor.

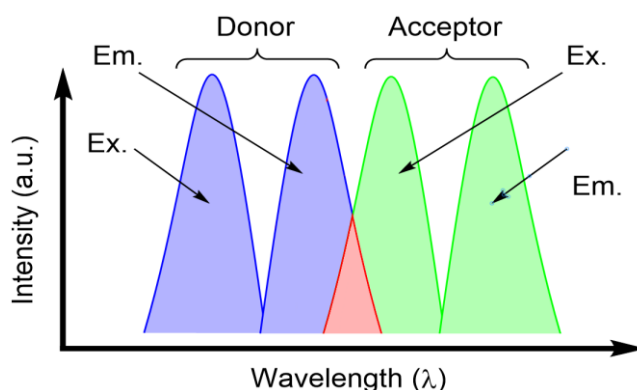


Figure 19. The schematic diagram showing the spectral overlap (red region) of the donor and the acceptor. When there is no spectral overlap between the donor emission and the acceptor excitation, no FRET can occur.

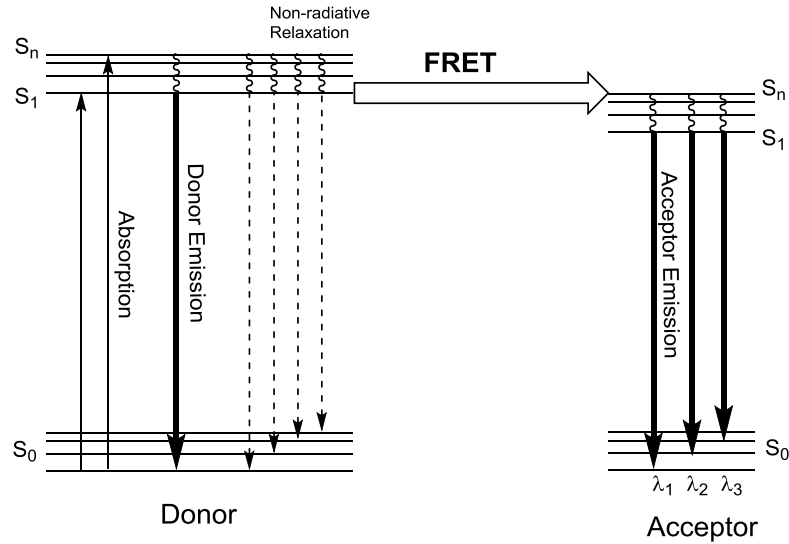


Figure 20. The simplified Jablonski energy diagram of FRET process between a donor-acceptor pair.

The efficiency of energy transfer (Φ_T) between the two transition dipoles are given by the equation:

$$\Phi_T = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} \quad (11)$$

Where R is the separation distance between donor-acceptor pair and R_0 is the Förster radius, the distance at which the rate of energy transfer between donor and acceptor has an efficiency of 50%.^{106,108} The Förster distance is dependent on the spectral properties of the donor and the acceptor, as well as on the relative orientation of the transition dipole moments of the pair.¹⁰⁹ The Förster radius in angstrom is given by the equation:

$$R_0 = 8.79 \times 10^{-5} (\kappa^2 n^{-4} Q_d J)^{1/6} \quad (12)$$

Where Q_d is the quantum yield of the donor, J is the integral of the spectral overlap, n is the refractive index of the medium, and κ is the factor describing the relative orientation of the transition dipole moments of the donor-acceptor pair. Due to the uncertainty in the orientation of transition dipoles and the value of κ , it is often estimated as $\kappa^2 = 2/3$ without significant error in

calculation of R_0 .¹⁰⁵ However, the orientation dependence of FRET efficiency makes the precise determination of the distance difficult, where the error in measured distance can be as high as $\pm 12\%$ in the worst case.^{105,110} In addition, the quantum yield of the donor is affected by the components of a medium (e.g. ions), therefore the efficiency of the energy transfer is not only dependent on the spectral properties of the fluorophore but also on the environment in which the FRET is analyzed.¹¹¹ Also, the donor-acceptor pairs typically have R_0 that are less than ideal for the study of biological molecules.¹¹² While the maximum reported FRET distance is approximately 80 Å, at this range of distance FRET signal is very low due to relatively small R_0 of the organic fluorophores and the fast inverse-sixth power of distance decay ($1/R^6$) in FRET signal.¹¹² Therefore, these limitations make FRET less ideal for accurate measurement of many subcellular membranes and large biological molecules.

Time-resolved FRET (TR-FRET) improves on the traditional FRET (i.e. between two organic fluorescence dye) by using a lanthanide chelate as the donor to overcome drawbacks of FRET.¹¹² It is important to note here that the emission arising from resonance energy transfer involving lanthanide is technically called *luminescence* and not *fluorescence*.^{110,113} A commonly accepted mechanism of FRET involving a lanthanide ion is shown in Figure 21.¹¹³ Fluorescence is an emission from singlet-to-singlet transition. In a singlet state, the electrons are spin-paired and are anti-parallel to each other. But for the lanthanide, a sensitizer molecule is excited to the singlet excited state, then passes to a triplet state by the process known as intersystem crossing (ISC). In the triplet excited state the electrons are parallel to each other. Therefore, spin transition to the triplet state is parity forbidden and results in the slow decay to the ground state upon excitation. When the lanthanide ion is in the proximity of a sensitizer molecule, the parity forbidden transition becomes partly allowed as induced or forced transition.¹¹⁴ Then, the energy of the triplet state is passed from the sensitizer molecule to a lanthanide, which then results in the triplet-to-singlet emission known as luminescence. The similar emission of triplet-to-singlet

emission by an organic molecule is known as *phosphorescence*.^{115,116}

Certain literatures refers to the FRET involving a lanthanide as LRET (luminescence resonance energy transfer), although the LRET and FRET share the same underlying mechanism. For this reason, resonance energy transfer involving lanthanides are still commonly referred to as FRET. Therefore, in this dissertation the term FRET (and TR-FRET) will continue to be used to describe the process, but the emission arising from sensitized lanthanide emission will be referred to as luminescence.

The advantage of TR-FRET over the traditional FRET is that the luminescence lifetime of lanthanide metals used as the donor fluorophore are in milliseconds range, while background fluorescence scattering and fluorescence lifetime of organic dyes are within nanosecond range.¹¹⁰ This magnitudes of difference in fluorescent lifetime allows the signal due to energy transfer to be detected using time-resolved measurement after all interfering signals have decayed as shown in Figure 22.

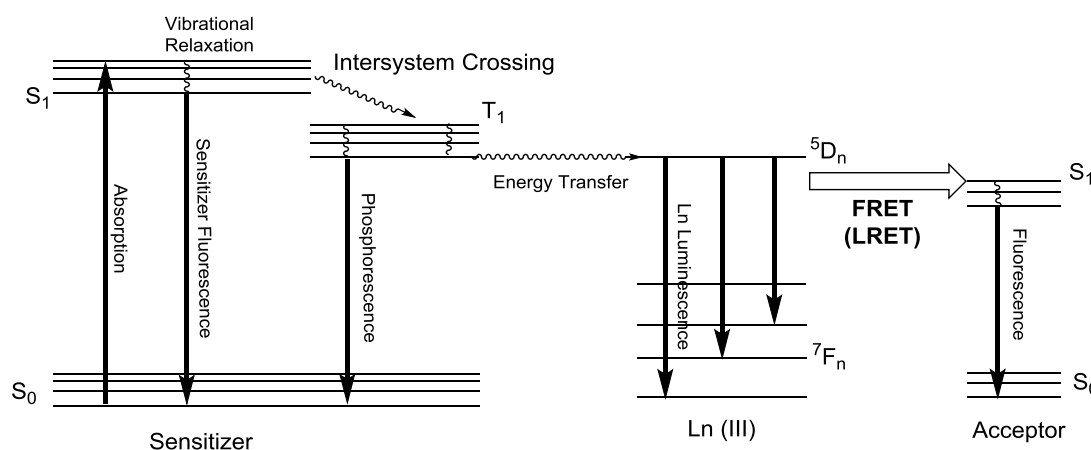


Figure 21. The simplified Jablonski energy diagram of the TR-FRET process involving a lanthanide chelate and the acceptor fluorophore.

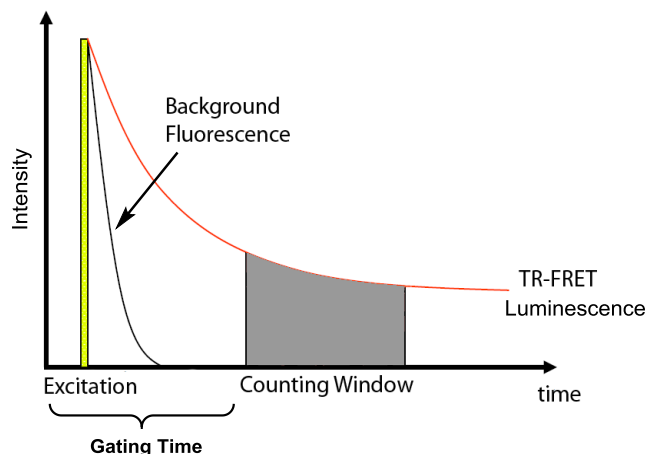


Figure 22. The diagram showing the time-gated measurement of TR-FRET, where the background interferences and emission from organic molecules decay within the gating time.

The distance at which the FRET can be measured using lanthanides is also greatly increased (>100 Å) due to the long luminescence lifetime and the Förster distance of lanthanides that can easily exceed >70 Å.¹¹⁷ Lanthanides also possess desirable luminescence properties such as near unity (~ 1) quantum yield in aqueous media and non-polarized millisecond lifetime emission that makes the error in measurements due to orientation dependence negligible.^{105,110} In traditional FRET, the signal-to-noise (S/N) ratio of the FRET emission is low due to the small Stokes shifts of donor-acceptor pair that cause interfering fluorescence signal from the donor and the emission due to direct excitation of the acceptor. On the other hand, the lanthanides have large Stokes shifts where the excitation and emission wavelengths are separated by hundreds of nanometers. For example, the sensitizer is excited at 340 nm and the main emission of Eu(III) is over 600 nm. This separation increases the signal resolution of TR-FRET as the interference from excitation light and the emission arising from direct excitation of the acceptor are unlikely in the signal, as well as the time-resolution of other interfering signals improves the S/N ratio of the acceptor emission.¹¹⁶ These superior properties of lanthanide provide greater sensitivity as a probe and have attracted interests in a variety of biological and biochemical applications such as in immunoassays, bioimaging, and in detection of protein-ligand interactions including screening

assays.

II. The Application of TR-FRET and a New Approach to Assay Development

The use of lanthanide probes for detection of protein-ligand binding is becoming increasingly popular in drug discovery owing to the properties of lanthanide probes and the development of homogeneous TR-FRET assays that is ideal for high-throughput screening of compound libraries. The major advantage of TR-FRET is that the luminescence lifetime of a lanthanide is in millisecond range, while common organic fluorescent dyes are no longer than few nanoseconds. The enormous difference in the emission lifetime allows the time-resolved detection of acceptor fluorescence arising from TR-FRET, which negates interferences from autofluorescence and background scattered light. In addition, the requirement between the donor-acceptor pair to be in close proximity for the TR-FRET to occur minimizes false positive and false negatives that are commonly encountered in HTS using traditional fluorescence techniques such as FP. In addition, the ratiometric nature of the assay, where the TR-FRET signal is calculated as a ratio of acceptor and donor emissions, minimizes interwell variations as well as the effect from quenching samples.

The application of TR-FRET in the evaluation of protein-ligand interaction requires the target protein to be also labeled by a fluorescent probe in addition to the ligand. For example some commercial TR-FRET assay kits such as LanthaScreen, DELFIA, and Eu(TMT)ITC, are available in which the target protein is labeled with either the Eu(III) or Tb(III) chelate as the donor of TR-FRET. In this method, the lanthanide chelate is linked to the target protein via a specific biomolecular interaction, a primary or secondary antibody, or covalent modification by a reactive labeling reagent. A lanthanide chelate anti-epitope antibodies, such as anti-GST antibodies and anti-His₆-tag antibodies, are examples of primary antibodies that are used for the non-covalent labeling of the protein. These primary antibodies are useful in cases in which the

covalent modification of the target protein affects the activity of the protein or when working with a partially pure protein as these primary antibodies target affinity tags which minimizes cross-labeling of impurities.

Like primary antibodies, secondary antibodies can be used to non-covalently label the target protein with a lanthanide chelate. Commercial secondary antibodies for TR-FRET are reported to bind to their corresponding species IgG with low nM binding affinities and can be used to easily develop TR-FRET assays when their corresponding species IgG is available. Additionally, biomolecules can also be expressed as a fusion with streptavidin, with biotinylation tag, or with a streptavidin binding tag such as Strep-Tag for affinity purification. Biotin-streptavidin interaction is known to be one of the strongest biological interaction known with a binding affinity in the order of 10^{-15} .¹¹⁸ Biomolecules that were designed to take advantage of the exceptionally strong biotin-streptavidin interaction can repurpose these tags to non-covalently attach a lanthanide chelate by the Tb-labeled streptavidin or anti-biotin antibody. While development of TR-FRET assay using these antibody kits may be simple, the use of these TR-FRET antibody kits can be costly for application in high-throughput screening. For example, at the time of this dissertation research 25 μ g (0.17 nmol) of LanthaScreen Tb-anti-His tag antibody will cost about \$1000 and 50 μ g (1.1 nmol) of Tb-streptavidin costs more than \$1200.

Alternatively, covalent modification of the target protein using reactive labeling reagents, such as amine-reactive (ITC and NHS) or thiol-reactive (maleimides) lanthanide chelates are available for modification of free amino group or sulfhydryl group of a cysteine residue in peptides and proteins. Following the covalent modification of proteins by a reactive chelate labeling reagent, the unreacted dye is often necessary to be removed, and therefore the modified protein needs to be purified. The additional labor-intensive protein purification results in loss of precious protein sample. The cost of the antibody reagents and the drawbacks of covalent modification methods has prompted us to investigate a new and convenient approach to TR-FRET. In this dissertation

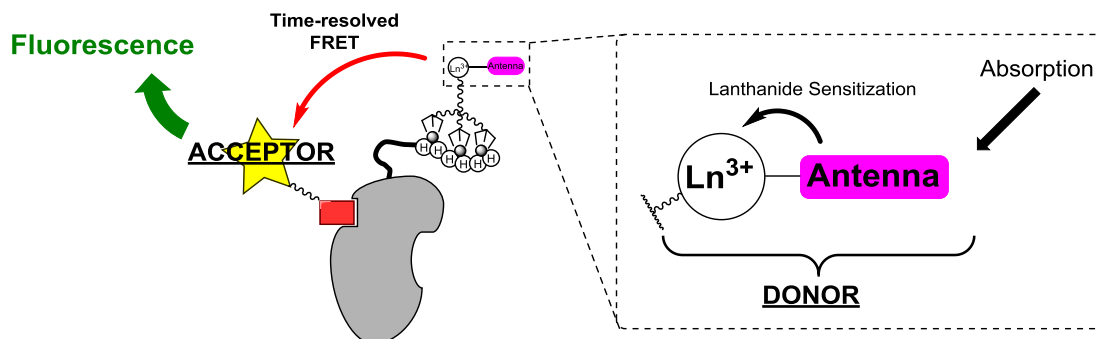


Figure 23. The schematic diagram of site-specific *in situ* labeling strategy in TR-FRET assay development. The His₆-tag of a recombinant protein is utilized in the new approach to label the protein with a lanthanide complex.

research, the His₆-tag commonly present in recombinant proteins as the affinity tag for purification of the protein is utilized in a new approach to attach *in situ* the lanthanide probe like Eu TMT chelate or LanthaScreen Tb chelate (Figure 23). This is the same site the commercial AlphaScreen Ni(II) Chelate Histidine Detection Kit uses to attach to acceptor beads and the LanthaScreen anti-His₆ Tag antibody uses to attach lanthanide probes.

The new approach to attaching a lanthanide probe *in situ* through the His₆ affinity tag will circumvent the purification steps required following the covalent modification of the protein using amine or thiol reactive reagents. This addresses some of the drawbacks in development of traditional TR-FRET and allows for a facile transition of commonly used FP assay into TR-FRET. The site-specific *in situ* labeling approach in development of TR-FRET will facilitate the drug discovery process by providing easier access to the superior assay technology and accelerating the discovery of novel leads as potential preventive and therapeutic agents in many areas of research.

III. Design and Synthesis of *in situ* Lanthanide Labeling Reagent

A. Tris-NTA Ni(II) ligands

Nitrilotriacetic acid (NTA) is a tetradentate ligand which has long been used as a chelating agent of divalent cations such as Ni^{2+} , Zn^{2+} , Co^{2+} , and Cu^{2+} . Its polymer-bound variant has gained popularity in immobilized metal ion affinity column (IMAC) used for isolation of recombinant proteins. Hochuli et al pioneered the most widely used IMAC methodology, the Ni-NTA column. In this IMAC, nickel (II) ions are immobilized by NTAs that are bound to the surface of a polymer resin.¹¹⁹ The protein purification by the Ni-NTA column is based on the high affinity of neighboring 3 Ni-NTAs to cooperatively bind proteins containing a polyhistidine tag consisting of consecutive histidine residues. More recently, Knecht et al reported that the hexahistidine (His_6) tag has the highest binding affinity of all polyhistidine tags to Ni-NTA surface where K_d was determined to be 14 ± 1 nM by a Biacore SPR assay.¹²⁰ The purification using Ni-NTA is highly selective and up to 95% homogeneity in a single step for a target recombinant protein can be achieved.¹²¹ This high selectivity is due to the rarity of exposed and naturally occurring polyhistidine sequence. It is important to note that the His_6 -tag has been reported to be non-immunogenic and rarely affects the structure or the function of the tagged protein.¹²² For this reason, many recombinant proteins are synthesized with N- or C-terminal His_6 -tag for purification as the tag does not need to be removed after purification.

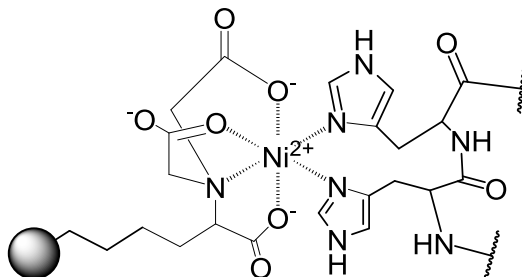


Figure 24. The interaction between Ni-NTA and 2× His at the coordination sites of Ni(II) in the Ni-NTA IMAC

The His₆-tag of recombinant proteins has been previously targeted for non-covalent radiolabeling of proteins for fluorescence assays and in immobilization of proteins for protein-protein interaction study.^{123,124} In the design of novel *in situ* labeling reagent for TR-FRET assay, similar approach on the His₆-tag was taken and utilized as the site of attachment of the lanthanide complex. As reported in several sources, the His₆-tag requires 3× Ni-NTA complex for optimal binding affinity, where the ratio of histidine to Ni-NTA complex is 2:1 as shown in Figure 24. At least two successful tris-NTA structures have been reported in literature for binding to His₆-tag with high affinity; one using a lysine dendrimer approach and the other utilizing cyclam, a tetraaza macrocycle, as its core structure (Figure 25). The lysine-based dendron tris-NTA (trivial name, Lys-tris-Lys-NTA) was reported to have K_d of 3.0 ~ 17.3 nM depending on protein structure.^{123,125-127} On the other hand, the tris-NTA based on tetraaza cyclam (H₄-cyclam) scaffold using glutamic acid for its NTA subunit (trivial name, cyclam-tris-Glu-NTA) was reported to have K_d of 20 nM and 23 nM to the hexahistidine peptide and His₆-tagged maltose binding protein (MBP-H₆), respectively.^{124,128} The effect of the linker length of tris-NTA has been reported by Huang et al using Lys-tris-Lys-NTA where the linker length between individual NTA subunit was varied at the highlighted lysine residue from C2 (diaminobutric acid) to C4 (lysine).¹²⁷ Authors concluded that the shorter C2 linker yields better binding to the hexahistidine peptide, where sub-nanomolar binding affinities were reported for the diaminobutyric acid (Dab)-based dendron tris-NTA (trivial name, Lys-tris-Dab-NTA). Taking these binding data into consideration, these tris-NTAs were selected as targets to test their binding to the His₆-tag of Keap1 Kelch domain (Figure 25).

Based on reported data, the binding affinity of tris-NTA to His₆-tag of any protein is highly dependent on the structure of a protein. Therefore in addition to the 3 tris-NTAs reported in literature, a new tris-NTA using ethylenediamine tetraacetic acid (EDTA) as the scaffold was designed for the present study, which will be referred to by its trivial name EDTA-tris-Lys-NTA

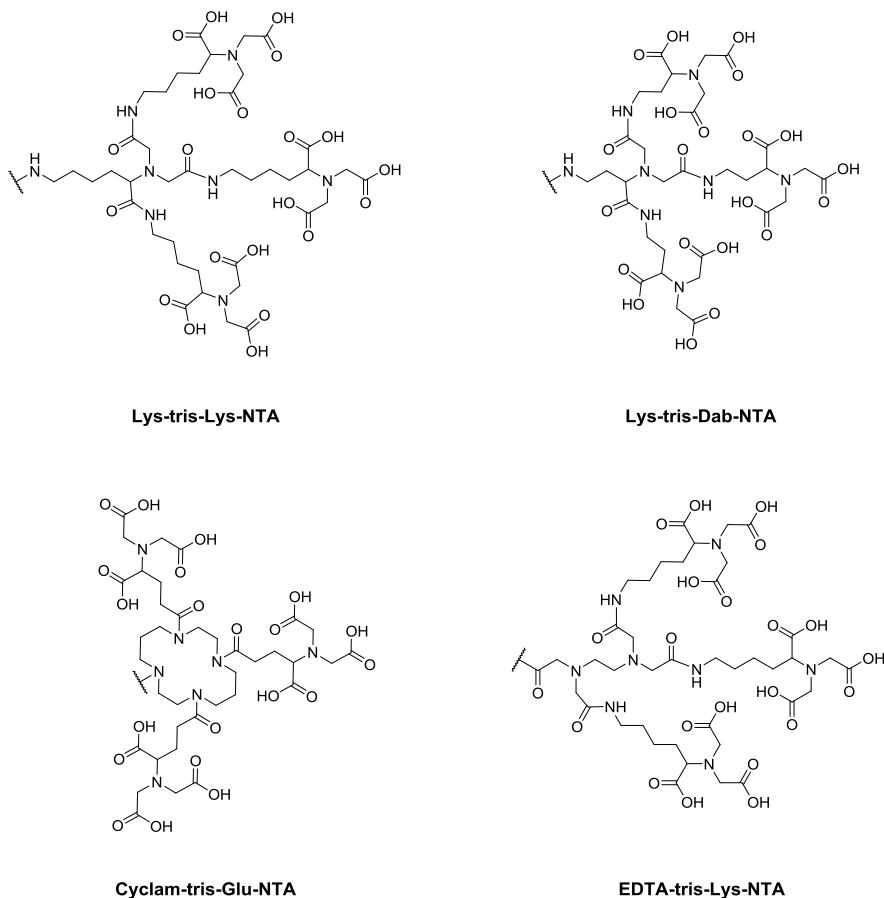
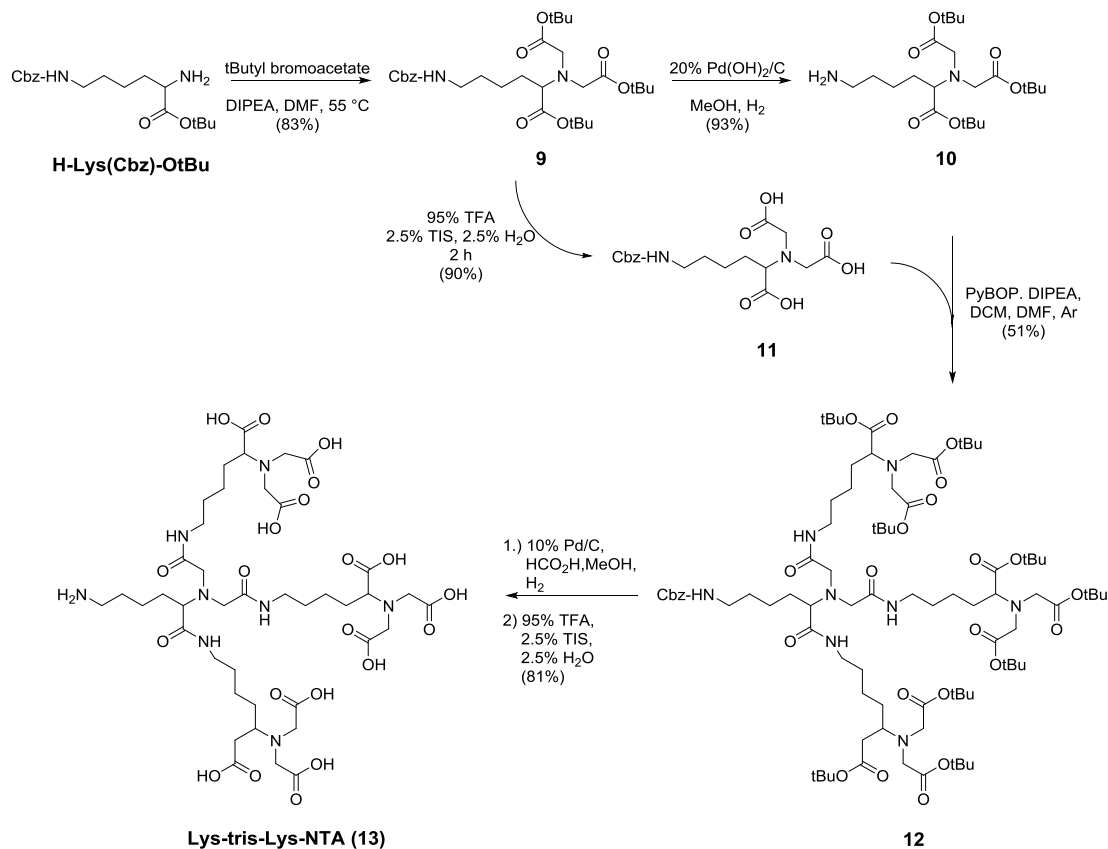


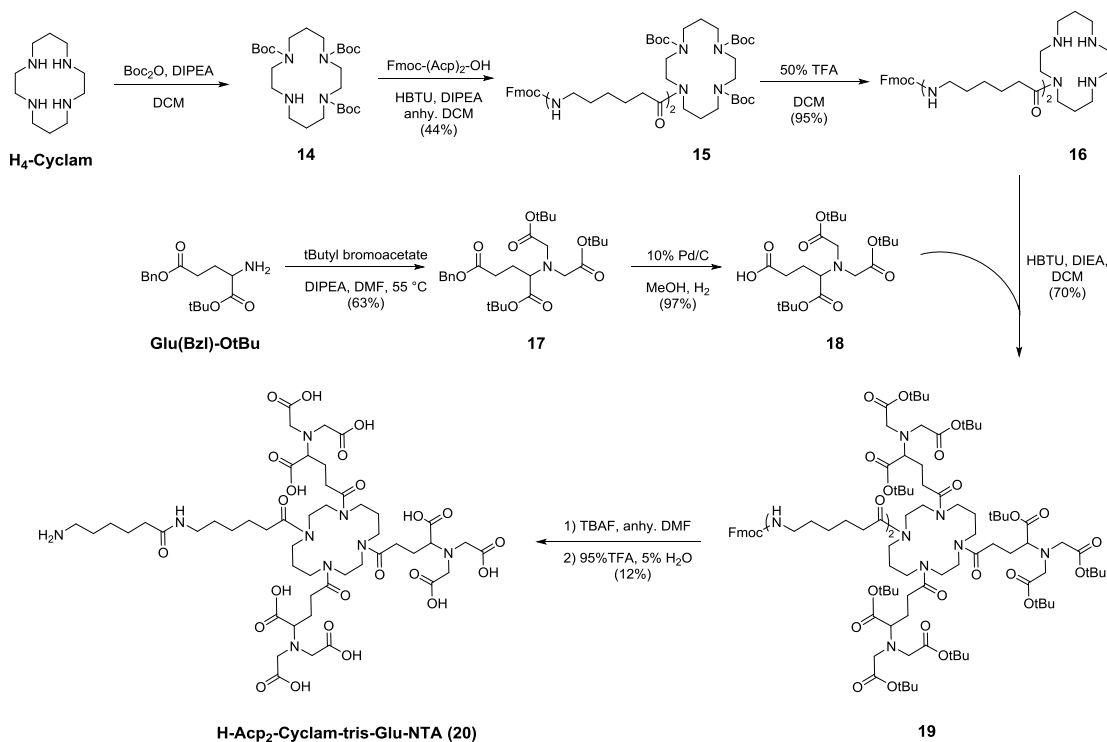
Figure 25. The structure of 4 tris-NTA synthesized and evaluated for His₆-tag affinity.

(Figure 25). As opposed to the dendrimer approach of the Lys-tris-Lys-NTA, EDTA scaffold allows for facile synthesis of the core triacetic acids using readily available material as compared to H-Lys(Z)-OtBu which is used as the starting material for Lys-tris-Lys-NTA. Structurally, EDTA-tris-Lys-NTA resembles Lys-tris-Lys-NTA, however shifting of the third NTA side chain creates additional space between individual NTA subunits. While Huang et al reported the effect of linker length, the spatial arrangement of 3 NTA subunits should theoretically impact the binding affinity of the tris-NTA to the His₆-tag of Keap1 Kelch domain.¹²⁷ In order to determine tris-NTA suitable for the development of new TR-FRET method, the synthesis of 4 tris-NTAs were targeted to test the binding to Keap1 Kelch domain (Figure 25).



Scheme 5. The synthesis route of Lys-tris-Lys-NTA starting from the Z-protected lysine using dendrimer approach.¹²³

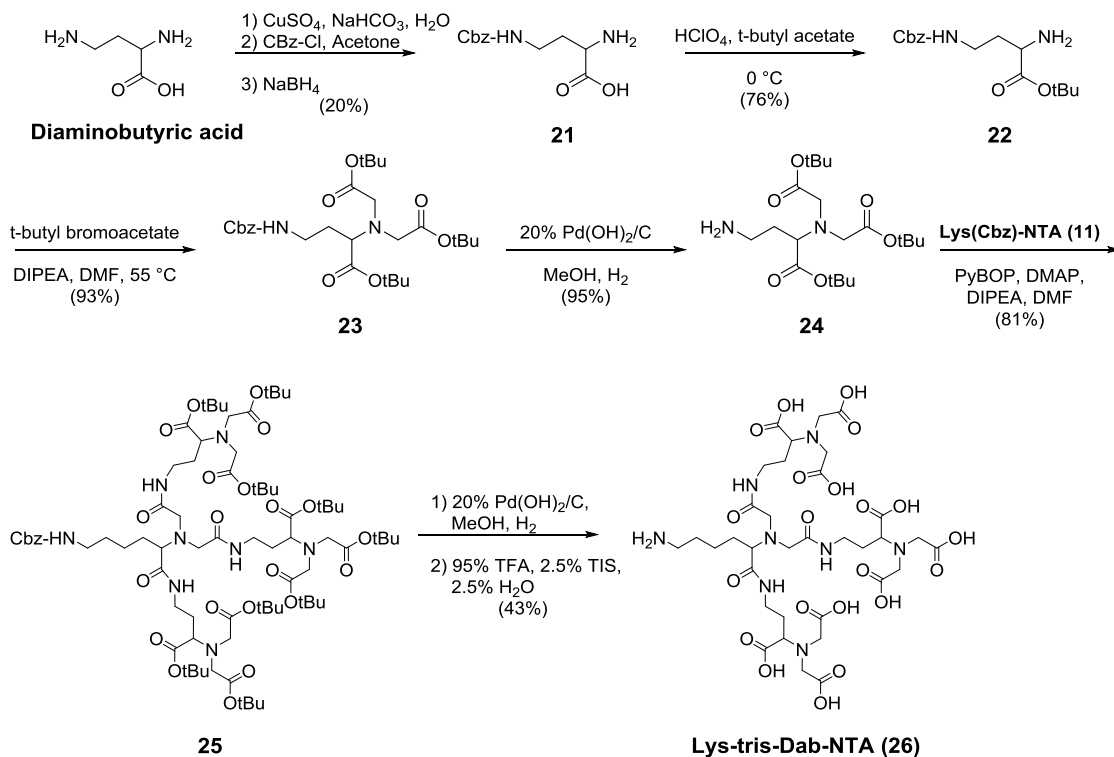
Lys-tris-Lys-NTA **13** was synthesized following the literature outlined in Scheme 5.¹²³ First, H-Lys(Z)-OtBu is reacted with *t*-butyl bromoacetate to make benzyloxycarbonyl (Cbz) protected NTA *t*-butyl ester (**9**). This protected NTA was deprotected selectively at the amine and the carboxylate end to make either the free amine **10** or the free tris-acetates **11** of NTA. The hydrogenolysis of benzylcarbamate of **9** was performed using Pearlman's catalyst (20% Pd(OH)₂/C) in MeOH under H₂ to give the free amine **10**. The *t*-butyl ester cleavage of **9** was performed in 95% TFA using triisopropylsilane (TIS) and water as cation scavengers to give the NTA free acid **11**. The protected tris-NTA **12** was synthesized by the coupling of **10** and **11** upon activation of **11** as OBt esters by PyBOP in the presence of base. The protecting groups were



Scheme 6. The synthesis route of H-Acp₂-cyclam-tris-Glu-NTA starting from H₄-cyclam following the modified literature procedure.¹²⁴ The synthesis of cyclam-tris-Glu-NTA was performed by Dr. Yanhui Yang.

removed in the final steps and Lys-tris-Lys-NTA **13** was obtained.

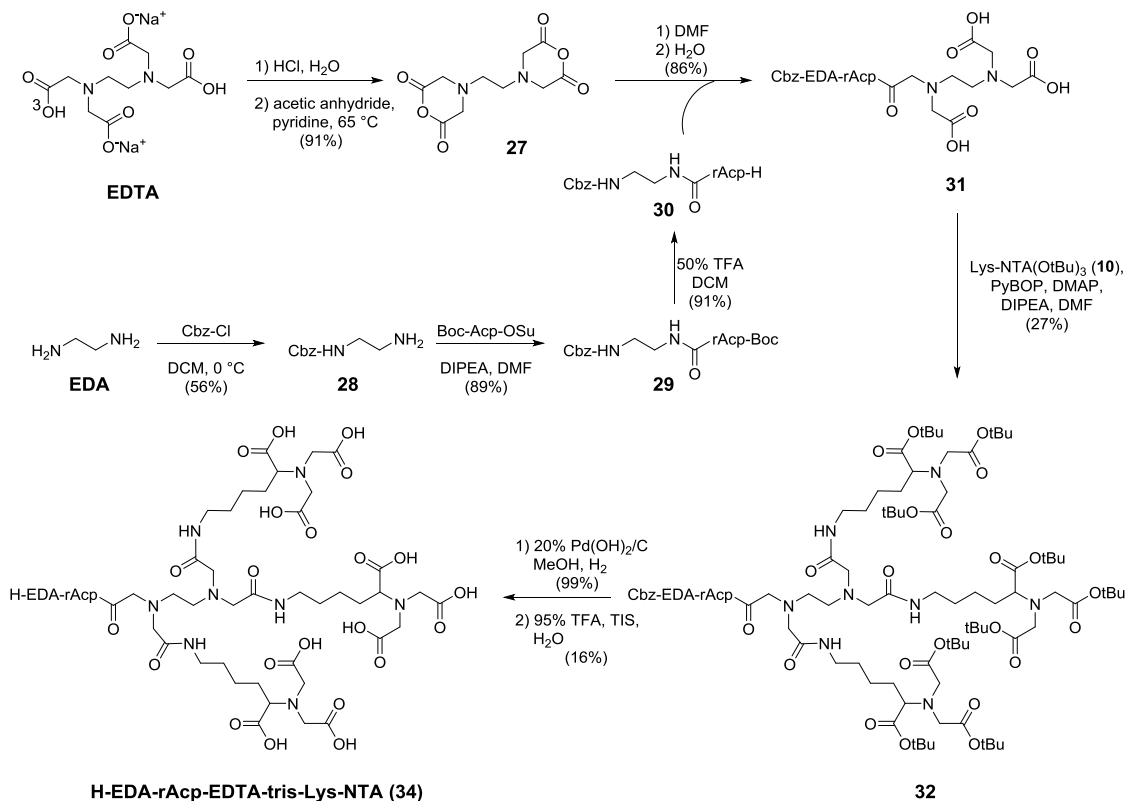
Similarly, cyclam-tris-Glu-NTA was synthesized following the reported procedures as outlined in Scheme 6.¹²⁴ Cyclam-tris-Glu-NTA **20** was synthesized by a colleague, Dr. Yanhui Yang. Tetraaza cyclam (H₄-cyclam) was first protected by Boc (t-butoxy carbonyl) using Boc anhydride (Boc₂O) to make tris-Boc H-cyclam **14**. Then **14** was conjugated to Fmoc-protected aminocaproic (Acp) acid linker (Fmoc-Acp₂-OH) using HBTU as the activating agent under basic condition to give compound **15**. The deprotection of Boc protecting groups by TFA gave H₃-cyclam **16**, which was subsequently coupled with Glu-NTA(OtBu)₃ **18** to give protected cyclam-tris-Glu-NTA **19**. Fmoc was deprotected by tetrabutylammonium fluoride (TBAF), followed by the cleavage of t-butyl ester by TFA to give the target compound H-Acp₂-cyclam-tris-Glu-NTA (**20**). The use of secondary amine for deprotection lead to either diethylamine or piperidine adduct formation in



Scheme 7. The synthesis of Lys-tris-Dab NTA (**26**) performed by following a modified literature procedures.¹²⁷

the later coupling reaction with OSu ester of the DTPA Tb(III) chelate as described later.

The synthesis of Lys-tris-Dab-NTA was performed starting from the commercially available diaminobutyric acid (Dab) as shown in Scheme 7 with modifications to the published preparation method to simplify side chain protection of Dab.¹²⁷ Copper (II) complex formation at the α -amino and carboxylate using CuSO_4 allowed selective protection of diaminobutyric acid at ω -amino group with Cbz-Cl. Upon reduction of the Cu(II)-complex with NaBH_4 , the transesterification catalyzed by HClO_4 in t-butyl acetate converted compound **21** to Dab t-butyl ester **22**.¹²⁹ Subsequently, compound **22** was alkylated with t-butyl bromoacetate to give the protected NTA compound **23**. The fully protected tris-NTA **25** was assembled by the reaction of Lys(Z)-NTA (**11**) and compound **24** with PyBOP. The hydrogenolysis using Pearlman's catalyst, followed by t-butyl ester cleavages in TFA afforded the target compound Lys-tris-Dab-NTA (**26**).



Scheme 8. The synthesis of Cbz-protected linker and EDTA-tris-Lys-NTA (**34**) from EDTA.

The tris-NTA designed with EDTA scaffold was synthesized as shown in Scheme 8 from EDTA bisanhydride **27**, which was formed by the treatment of EDTA in acetic anhydride and pyridine at 65°C . It is important to note that more commonly available EDTA disodium salt failed to directly form the bisanhydride. EDTA disodium needed to be converted to the free acid using concentrated HCl to adjust the pH of EDTA solution below 1 as the lowest pKa of EDTA is reported to be around 2. EDTA has poor solubility in water and precipitates as white solid, which can be dried and used to form the bisanhydride. EDTA bisanhydride **27** was reacted with a Cbz-protected ethylenediamine (EDA) Acp linker **30** and then hydrolyzed by water to give the triacetic acid **31**. The coupling reaction of **31** with Lys-NTA(OTBu)₃ **10** using PyBOP gave the protected tris-NTA **32**. Final deprotections using the previously introduced methods afforded the

target compound EDTA-tris-Lys-NTA (**34**).

In order to determine which tris-NTA structure is optimal for interaction with the His₆-tag of Keap1 Kelch domain, a competition SPR assay using the NTA chip was performed to evaluate the affinity of each tris-NTA to the His₆-tag of Keap1 Kelch domain. The concentration of the tris-NTA samples were determined by the fluorescence intensity of the tris-NTA sample solution upon derivatization with *O*-phthaldialdehyde (OPA) and *N*-Boc cysteine (NBC).¹³⁰ OPA/NBC derivatization reaction forms an isoindole derivative at the free amine of tris-NTA, which the fluorescence can be measured at 460 nm upon excitation at 355 nm. The fluorescence intensity of each sample was used to determined the concentration based on the standard curve generated by the derivatized NTA stock solution (Figure 26 and Table 10).

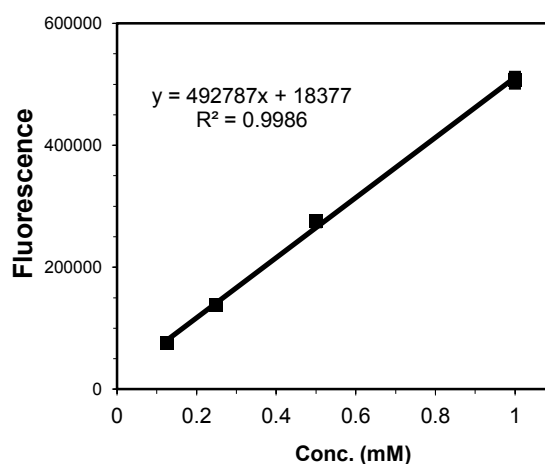


Figure 26. The standard curve generated by the fluorescence analysis of OPA/NBC derivatized NTA. The OPA/NBC derivatization of NTA samples were performed as described in the Experimental section.

Table 9. The concentration of tris-NTA determined by OPA/NBC derivatization

	Conc. (mM) by wt	Calc. Conc. (mM) NTA
Lys-tris-Dab-NTA (26)	1.26	1.127
Lys-tris-Lys-NTA (13)	1.22	0.722
EDA-rAcp-EDTA-tris-Lys-NTA (34)	0.822	0.441
Acp-Acp-cyclam-tris-Glu-NTA (20)	0.887	0.508

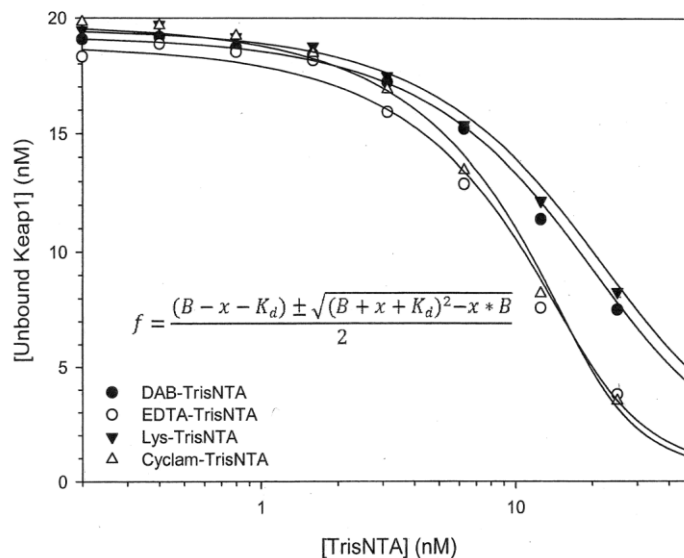


Figure 27. The binding curves of tris-NTA samples to His₆-tag of Keap1 Kelch domain obtained by the SPR assay. The K_d for each tris-NTA was derived by fitting the binding data to the quadratic equation using SigmaPlot.

Table 10. The binding affinity of tris-NTA to His₆-tag of Keap1 Kelch domain determined by the SPR assay

	K_D (nM)
Lys-tris-Dab-NTA (26)	9.3 ± 1.0
Lys-tris-Lys-NTA (13)	11.1 ± 1.3
EDA-rAcp-EDTA-tris-Lys-NTA (34)	2.2 ± 0.7
Acp-Acp-cyclam-tris-Glu-NTA (20)	1.6 ± 0.4

A fixed concentration of Keap1 Kelch domain was allowed to form His₆-Ni-tris-NTA complex with varying concentration of tris-NTA samples for 30 min. The amount of unbound Keap1 Kelch domain remaining in each sample solution was determined on the Ni-loaded NTA chip. The effect of NiCl₂ in solution during complex formation on Keap1 Kelch domain and its binding to Ni-NTA chip was taken into account by adjusting the binding of Keap1 Kelch domain using standard curves generated in presence and in absence of NiCl₂.¹³¹ Obtained SPR data were fitted to a quadratic equation to derive the K_d of each tris-NTA to His₆-tag of Keap1 Kelch domain using SigmaPlot (Figure 27).

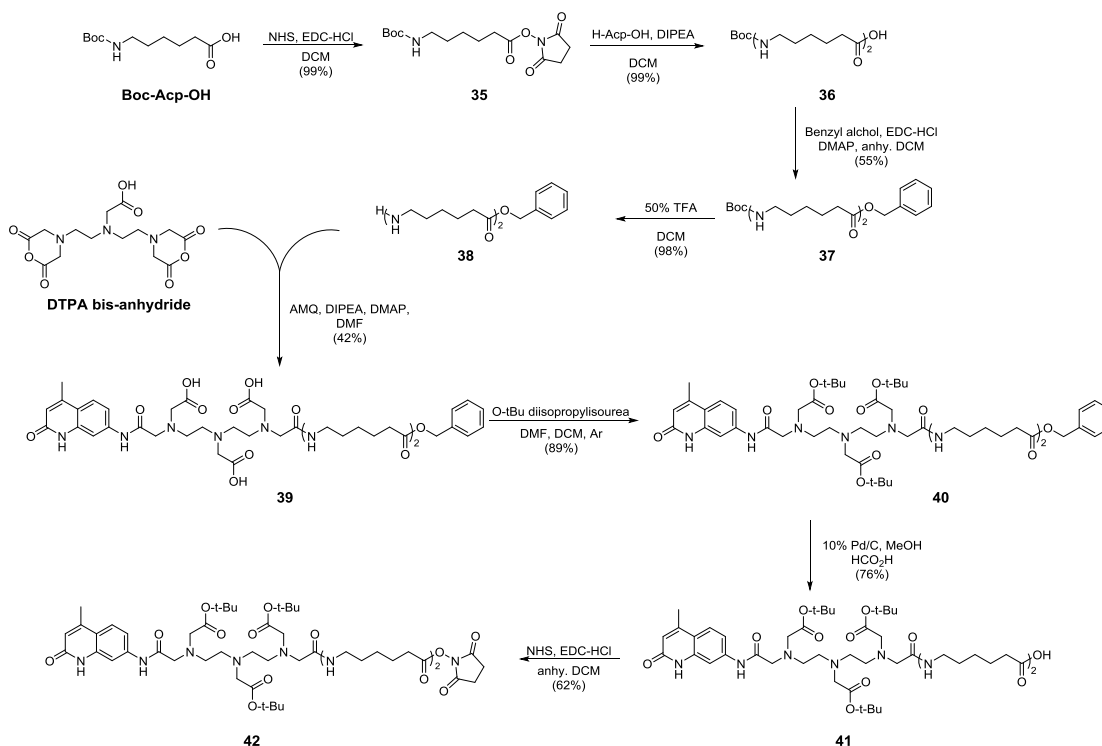
As shown in Figure 27, the binding affinities of cyclam-tris-NTA and EDTA-tris-Lys-NTA were similar and strongest of the four tris-NTAs investigated. The binding affinity of cyclam-tris-Glu-NTA and EDTA-tris-Lys-NTA was determined to be 1.6 ± 0.4 nM and 2.2 ± 0.7 nM, respectively. Surprisingly, two Lysine dendron-based tris-NTA were as much as 5-fold weaker in affinity to Keap1 Kelch domain where the K_d of Lys-tris-Dab-NTA was determined to be 9.3 ± 1.0 nM. While the shorter NTA linker tris-NTA such as Lys-tris-Dab-NTA was reported to be stronger in affinity to His₆-tag, this was not the case for Keap1 Kelch domain.¹²⁷ The longer C4-linker counterpart, Lys-tris-Lys-NTA, had a K_d of 11.1 ± 1.3 , which was similar to the affinity observed for the shorter C2 -linker Lys-Dab-tris-NTA. Huang et al studied the affinity of tris-NTA with a short hexahistidine peptide, which may not be an accurate representation of tris-NTA associating to the His₆-tag of a protein.¹²⁷ Conformation of short peptides are known to be at random in solution.¹³² In such case, it is possible that the protein structures are not considered and the environment surrounding the His₆-tag including steric effects could be ignored. In either case, the binding affinity of the cyclam and EDTA-based tris-NTAs were clearly higher than the two Lysine dendron-based tris-NTAs for Keap1 Kelch domain. Therefore, EDTA-tris-Lys-NTA and cyclam-tris-Glu-NTA were selected as the high affinity His₆-tag complexes for the design of the novel labeling reagents for the development of Keap1-Nrf2 TR-FRET assay.

B. Terbium (III) and Europium (III) Complex

Terbium (Tb), dysprosium (Dy), europium (Eu), samarium (Sm), and gadolinium (Gd) are lanthanides used as donor fluorophore species due to their long emission lifetimes ideal for time-resolved fluorescence measurements. However, unlike the main emission peaks of terbium, dysprosium, europium, and samarium that fall in the visible region, the $\lambda_{\max}$ of gadolinium falls in the UV region (312 nm). For this reason gadolinium has seen less application in TR-FRET but more so in other imaging technologies and research.¹³³ The order of $\lambda_{\max}$ luminescence intensities

of lanthanides used in TR-FRET assay is $\text{Eu} > \text{Tb} > \text{Sm} > \text{Dy}$.¹³⁴ Huhtinen et al investigated the luminescence properties of lanthanide chelates and reported that the relative sensitivity in detection of human prostate-specific antigen (PSA) by lanthanide chelates was 1.0 : 0.67 : 0.16 : 0.01 for Eu, Tb, Sm, and Dy, respectively.¹³⁵ Based on their luminescence intensity, europium chelates produce the highest sensitivity in a TR-FRET assay, followed by terbium chelates. Terbium has multiple emission peaks (489, 545, 582, and 621 nm) with large Stokes shifts, which offers advantage to TR-FRET assay development by allowing flexibility of acceptor fluorophore selection and ease of detection due to lower background signals. On the other hand, the emission of europium at λ_{max} of 612 nm, also with large Stokes shifts, is most intense and longest lifetime fluorescence of all lanthanides, making it the ideal choice for TR-FRET application.¹³⁴ For these reasons, most applications of lanthanides in fluorescence research exploit the luminescence properties of terbium and europium chelates, as are commercially available kits such as LANCE, DELFIA (Perkin Elmer, Waltham, MA), Eu (TMT) ITC (GE Healthcare, Buckinghamshire, UK) and LanthaScreen (Invitrogen, Carlsbad, CA). Likewise, it was determined that the chelates of terbium and europium can be applied to the design of the site-specific protein label for TR-FRET assay.

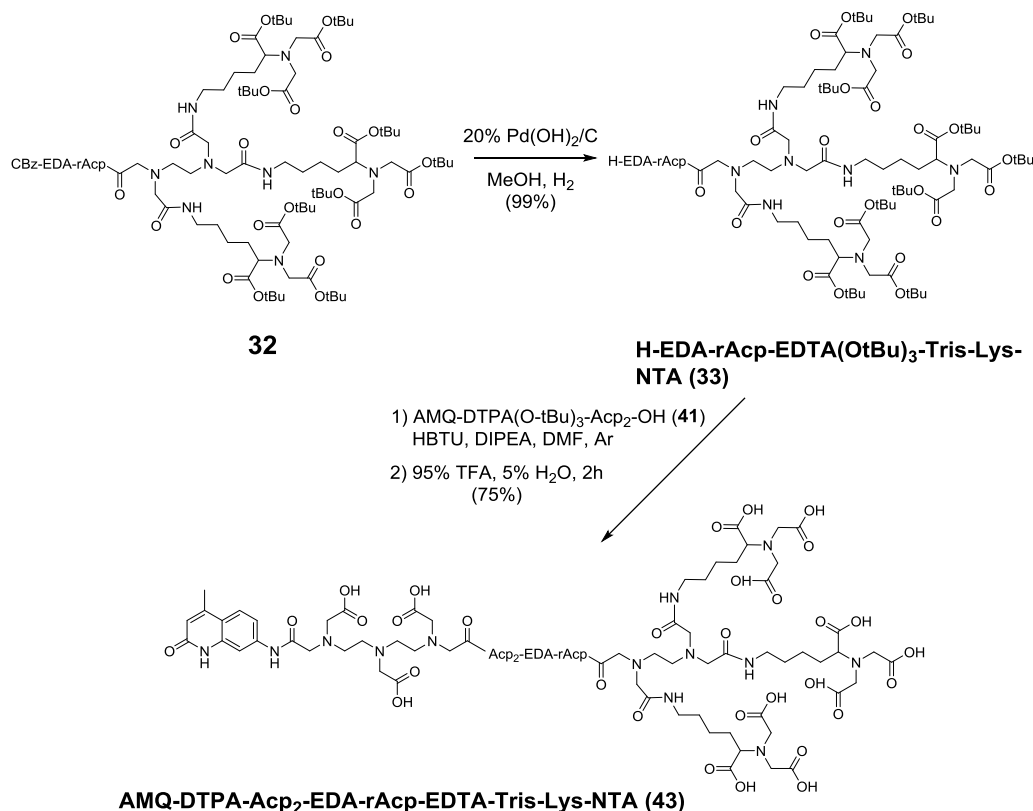
Lanthanide ions absorb light poorly due to forbidden transition of electrons to unfilled 4f electron shells and as a result, direct excitation results in low luminescence.¹³⁶ Therefore, lanthanide ions require a covalently attached chromophore known as "antenna" that can photosensitize the ion.^{134,137} Antenna molecule (donor) absorbs light and transfers its excited energy to the overlapping excitation energy level of a lanthanide ion (acceptor), allowing the forbidden transition of electrons to 4f electron shells to occur (Figure 21).¹³⁶ For this reason, the photosensitization of lanthanide ions or the antenna effect by a chromophore is far more efficient than direct excitation of these ions. Water molecules are known to deactivate lanthanide ions, thus lanthanide chelators must be able to saturate the coordination shell (>6 and usually $8 \sim 9$) of a



Scheme 9. The synthesis of Tb chelate intermediate **41** and its OSu ester **42** based on DTPA.

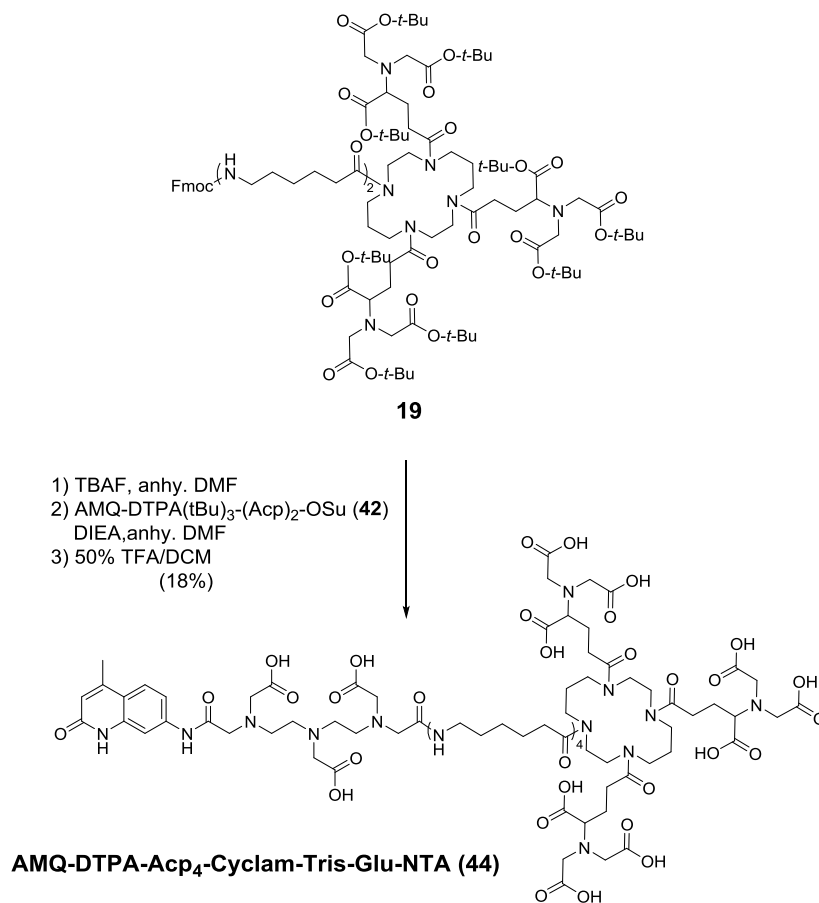
lanthanide to shield it from water.¹¹⁶ Therefore antenna such as carbostyryl-124 (Cs124 or AMQ, 7-amino-4-methyl quinolin-2-one) are often conjugated to a lanthanide chelate of polyaminocarboxylates such as diethylenetriamine pentaacetic acid (DTPA) and triethylenetetramine hexaacetic acid (TTHA).^{105,117,138} Polyaminocarboxylate are some of the common chelators capable of saturating the coordination shell of a lanthanide ion. In the case of DTPA, it can provide an additional carboxylic acid that can be modified without affecting affinity to the lanthanide ion. In addition, polyaminocarboxylates are stable and hydrophilic, which makes them ideal for use in forming lanthanide complex. Thus, the Tb(III) chelate using DTPA and AMQ were designed for their proven application in lanthanide sensitized emission.

The synthesis of the DTPA-based terbium chelate using AMQ as the antenna is outlined in Scheme 9. Syntheses of AMQ and DTPA dianhydride were performed following previously



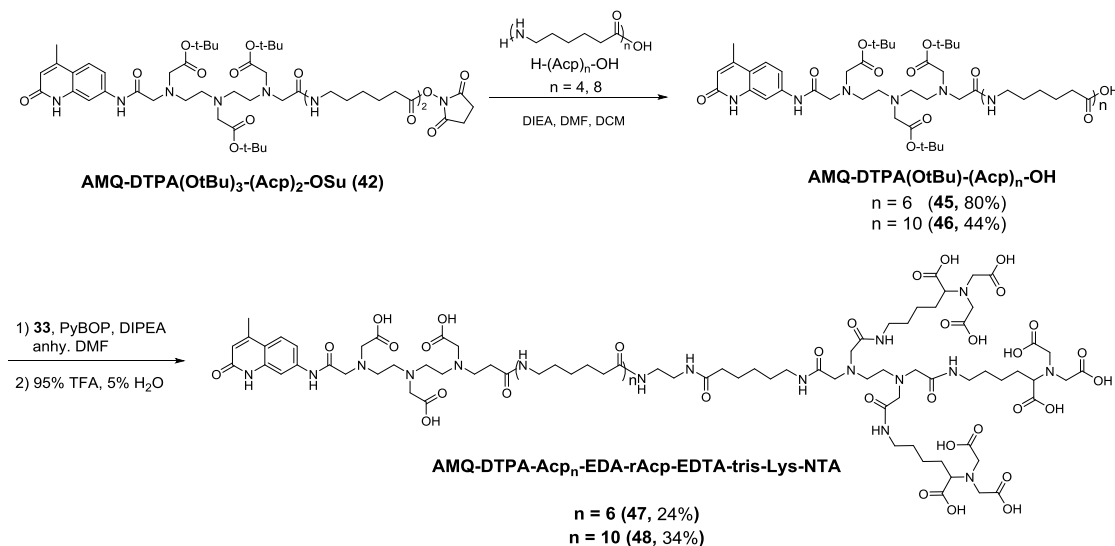
Scheme 10. The preparation of Tb labeling reagent **43** from compound **32** using PyBOP as the coupling agent.

reported conditions.^{139,140} Di-Acp benzyl ester **38** was synthesized starting from Boc-protected Acp by forming the Boc-Acp-OSu ester **35** using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in DCM. The OSu ester **35** is reacted with the second H-Acp-OH, followed by the reaction with benzyl alcohol (BnOH) in the presence of EDC to give the Benzyl protected di-Acp linker **37**. Then, the Boc protecting group is removed by the treatment with a 50% solution of TFA in DCM to give the di-Acp linker **38**. The intermediate compound **39** is synthesized by the reaction of AMQ, DTPA dianhydride, and the benzyl ester of the di-Acp linker **38** under pseudo-high dilution conditions.¹⁴¹ This technique mimics the high dilution conditions without slowing the reaction, where a solution of AMQ is added dropwise into a solution of DTPA dianhydride using a syringe pump. After some time, di-Acp linker **38** is added



Scheme 11. The synthesis of cyclam DTPA conjugate from compound **44** using the OSu ester of DTPA **42**.

using a separate syringe pump to convert the reaction AMQ conjugated intermediate to the product **39**. Using this pseudo high dilution method, the yield of this step can be increased to as high as 48%. Then, the DTPA Tb chelate **39** is converted to the tri-*t*-butyl ester **40** using *O*-*t*-butyl *N,N*-diisopropylisourea that is freshly prepared from the reaction of DIC and *t*-butanol. Other *t*-butylating reagents such as *t*-butyl 2,2,2-trichloroacetimidate failed to convert **39** to **40**. Transfer hydrogenolysis of the benzyl ester from compound **40** afforded compound **41** at quantitative yield. The *t*-butyl protected intermediate **41** was then activated as the OSu ester **42** using EDC for the reaction with the tris-NTA ligand for the synthesis of the AMQ-DTPA-Acp₄-cyclam-tris-Glu-NTA ligand **44** or used directly for the coupling with the tris-NTA ligand using

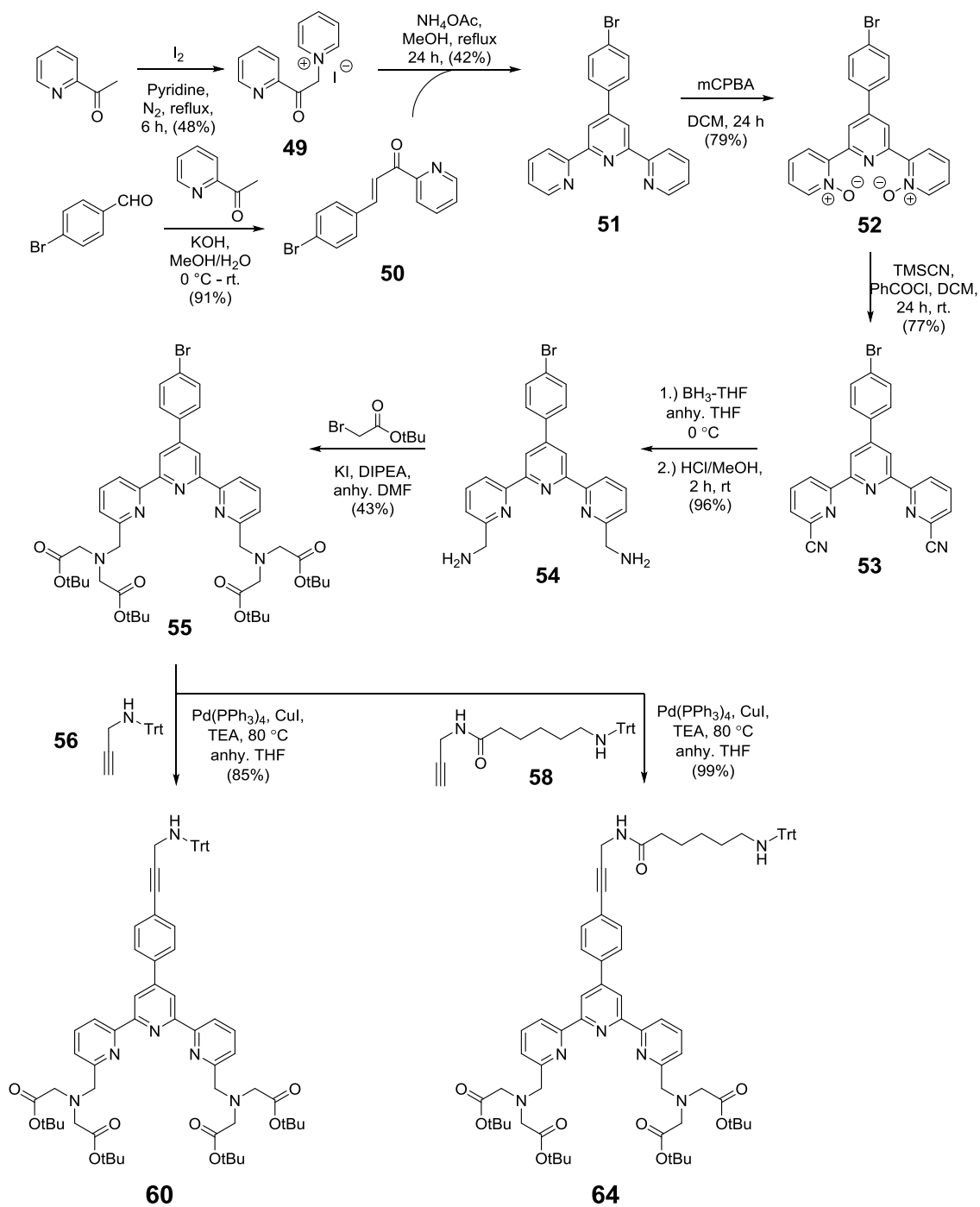


Scheme 12. The syntheses of EDTA-tris-Lys-NTA ligands with modified Acp linkers.

PyBOP as the coupling agent for the synthesis of AMQ-DTPA-Acp₂-EDTA-rAcp-EDTA-tris-Lys-NTA ligand **43** as shown in Schemes 10 and 11.

In order to study the effect of the separation distance between the terbium and the nickel-NTA complex, the length of the Acp linker was extended by 4 or 8 Acp for a total of 6 and 10 Acp linker. As shown in Scheme 12, the OSu ester **42** was reacted with tetra- or octa-Acp linker to give compounds **45** or **46** with either the 6- or the 10-Acp linker, respectively. The tetra- and octa-Acp linkers were prepared by Dr. Yanghui Yang. The compounds were subsequently deprotected in a solution of trifluoroacetic acid containing 5% deionized water to give the desired products **47** and **48**. These final products were purified on a reversed phase HPLC and tested against the di-Acp linker EDTA tris-NTA ligand **43** in TR-FRET efficiency.

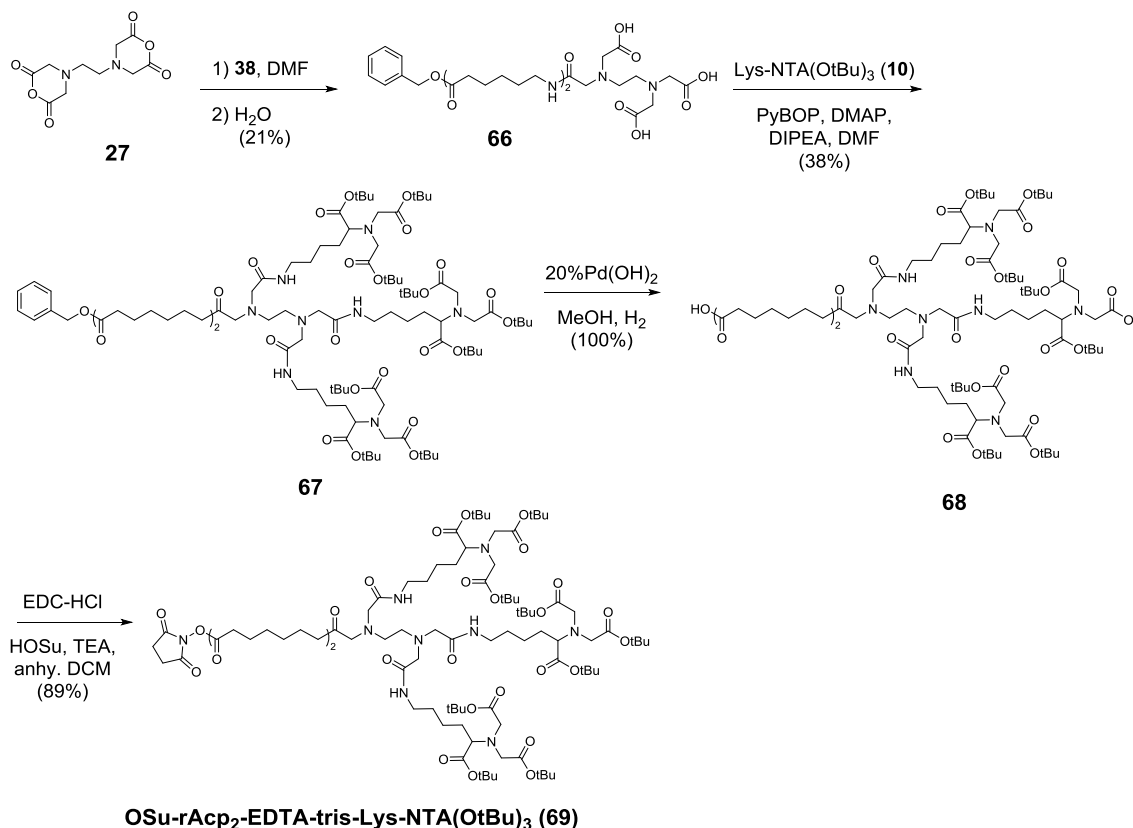
It has been demonstrated that heterocyclic ligands, such as terpyridine, can form highly luminescent complex with lanthanides.^{142,143} In these type of structures, the aromatic rings that



Scheme 13. The synthesis of N-Trt-TMT-Ap3 **60** was performed as previously described in the literature.^{143,144} Compound **64** was synthesized by using the Acp-extended linker **58** in the final Sonogashira coupling reaction.

cage the lanthanide ion sensitizes it and the excitation energy that is absorbed by the aromatic groups is transferred to the lanthanide ion. Recently, terpyridine containing ligands, terpyridine bis(methyl-enamine) tetraacetic acid (TMT), have been demonstrated to be highly luminescent chelates of Eu(III) ion with long luminescence lifetime and high quantum yield.^{142,144,145} The structure is also capable of completely occupying the coordination sites of Eu(III) ion, thus forming a strong complex that prevents contact of other ligands and ions with the lanthanide ion.¹⁴² Considering these properties of this chelate, TMT Eu(III) conjugate was designed and targeted for development of Eu(III) TR-FRET. The synthesis of amino-functionalized TMT for the attachment to biomolecules was previously reported in the literature.^{145,146} It was envisioned that this amine could be used to react with the active ester of the tris-NTA ligand to obtain the desired conjugate. Therefore, *N*-trityl-protected aminopropargyl TMT intermediates **60** and **64** were synthesized in 8-steps as described in literatures using either the Trt-protected propargylamine or Trt-protected Acp extended propargylamine linker as shown in Scheme 13.^{144,145}

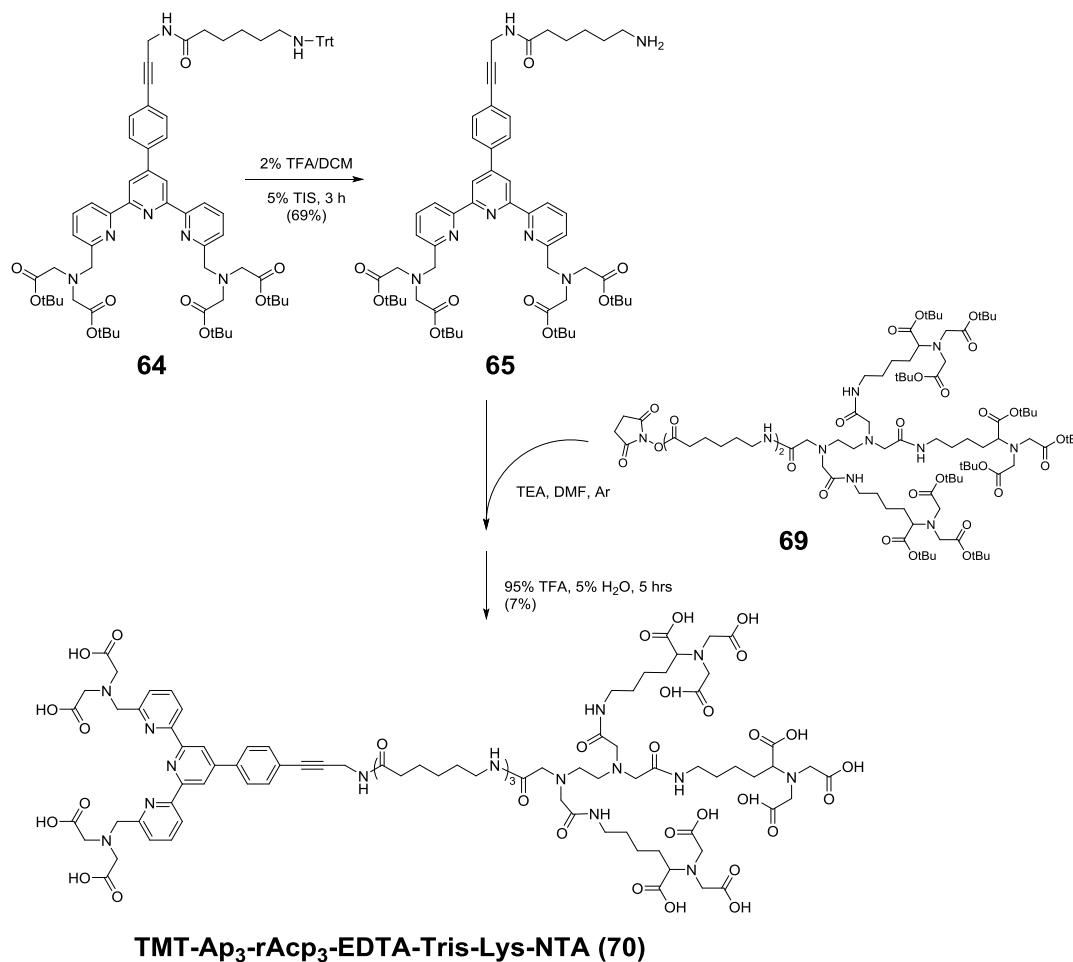
Following the literature procedure, a Claisen-Schmidt condensation of 4-bromobenzaldehyde and 2-acetylpyridine gave compound **50** in 91% yield.¹⁴⁴ It was subsequently reacted under Krohnke synthesis conditions in refluxing methanol containing ammonium acetate (NH₄OAc) with pyridinium compound **49** to give the bromo-terpyridine **51**. Terpyridine **51** was oxidized at the terminal pyridine moieties by *m*-chloroperoxybenzoic acid (mCPBA) to give the *N,N'*-dioxide **52** in 79% yield. The modified Reissert-Henze reaction of **52** gave rise to the dicyanitrile **53**, which was subsequently reduced with borane to give the bis(methyl-enamine) **54**. The compound was alkylated with *t*-butyl bromoacetate to give the tetra-*t*-butyl ester **55**. The reaction of the TMT bromide **55** with an alkyne in presence of Pd(II) catalyst and CuI under Sonogashira conditions gave the Trt-protected intermediates **60** and **64** in high yields.



Scheme 14. The synthesis of EDTA-tris-Lys-NTA OSu ester **69** starting from EDTA bisanhydride.

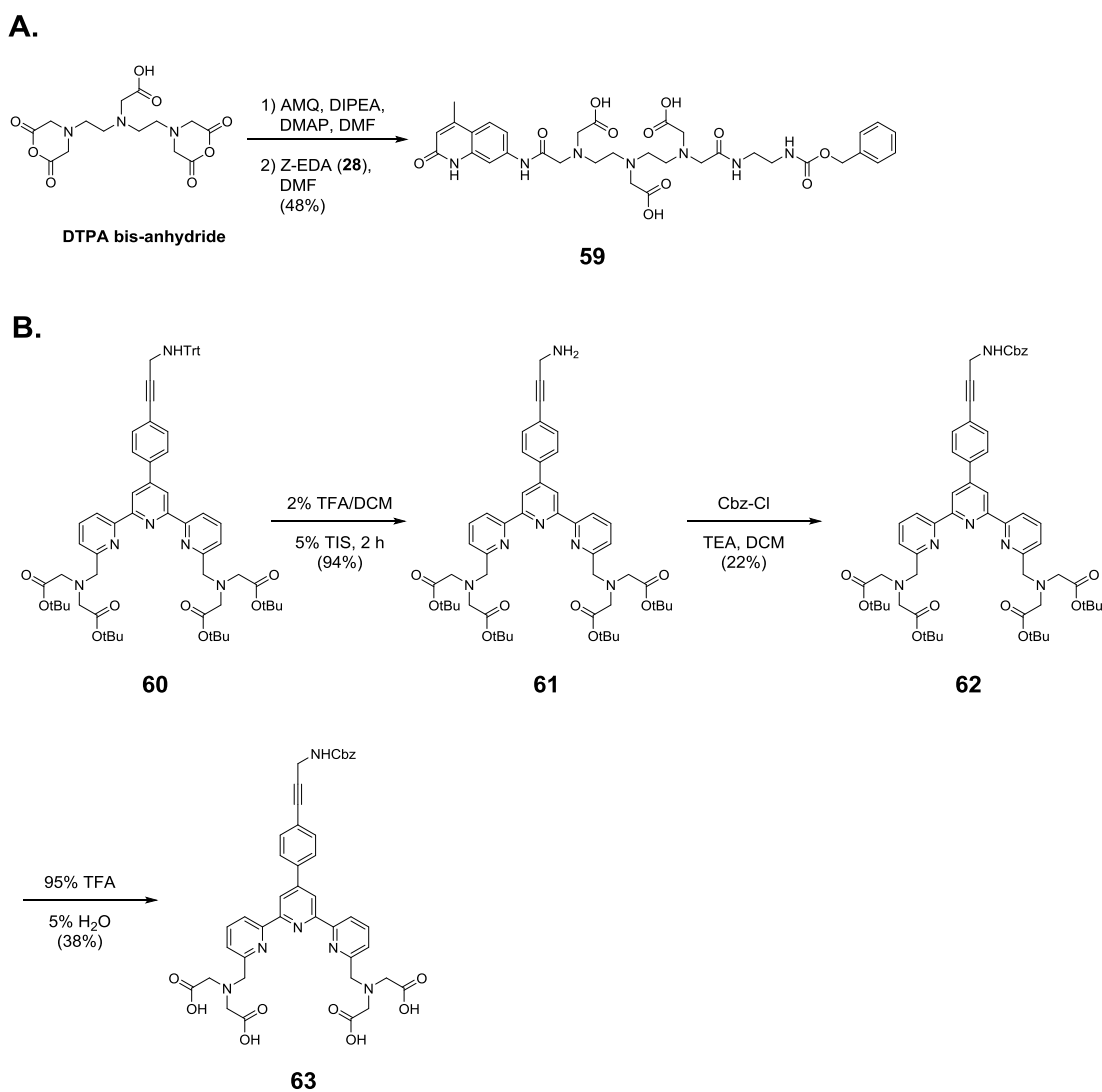
Then, the EDTA tris-NTA with an appropriate linker was synthesized for the conjugation to the TMT-Ap₃ chelate as shown in Scheme 14. First, EDTA bisanhydride **27** was reacted with the benzyl ester of di-Acp linker **38** and then hydrolyzed by water to give the trisacetic acid **66**. The coupling reaction of **66** with Lys-NTA(OTBu)₃ **10** using PyBOP gave the Bzl-protected tris-NTA **67**. Debenzylation of the Acp-linker using Pearlman's catalyst smoothly converted **67** to the free carboxylate of the rAcp₂-EDTA-tris-NTA **68**. Then, compound **68** was activated as the OSu-ester **69** using EDC-HCl and HOSu in DCM and used fresh for the reaction with the TMT(OTBu)₄-Ap₃-rAcp **65**.

As shown in Scheme 15, TMT(OtBu)₄-Ap₃-rAcp **65** was prepared by the selective deprotection of the trityl group from the Trityl-protected TMT-Ap₃-rAcp **64**, which was achieved by the 3-hour treatment of the compound with 2% solution of TFA in DCM containing 5% TIS as the cation scavenger. The amine **65** was then stirred overnight with the OSu-ester **69** in anhydrous DMF with TEA under Ar to obtain the t-butyl protected intermediate, which was subsequently deprotected without purification by the 5-hour treatment with 95% TFA solution containing deionized water. The final product TMT-Ap₃-rAcp₃-EDTA-tris-Lys-NTA **70** was purified on a reversed-phase HPLC using YMC-Pack ODS-AQ column suited for separation of hydrophilic compounds due to low retention time and the broad peak of the product on a regular C₁₈ column.



Scheme 15. The synthesis of the TMT-Ap₃ ligand conjugate **70** starting from the *N*-trityl TMT-Ap₃ intermediate **64**.

The methodology development for performing the TR-FRET assay using the *in situ* labeling technique was initially explored with the novel ligand conjugates synthesized in the present study, EDTA-tris-Lys-NTA and cyclam-tris-Glu-NTA conjugates **43** and **44**. Further optimization of the assay conditions were performed by selecting the best ligand conjugate as described in the following section. In addition to these conjugates, negative control compounds derived from the lanthanide chelates were synthesized as shown in Scheme 16. The control compounds **59** and **63** lack the protein-binding ligand moiety, thus even in presence of the protein and the fluorescently-



Schemes 16. The synthesis of control compounds for (A) Tb(III) TR-FRET and (B) Eu(III) TR-FRET.

labeled tracer the TR-FRET should not occur. Therefore, these compounds could be used to calculate the background luminescence from the sensitized lanthanide emission not involved in the TR-FRET process.

IV. TR-FRET Assay Development Using *in situ* Protein Labeling

In the preceding section, it was determined that the EDTA and the cyclam based ligands had highest binding affinity to the hexahistidine tag of Keap1 Kelch domain. These ligands were selected and conjugated to the appropriate lanthanide chelate for either Tb(III) or Eu(III) TR-FRET assay development. The initial methodology development was performed using the conjugates of Tb(III) DTPA chelate, which was synthesized before the TMT Eu(III) chelate in the present study. It has been shown that polyaminocarboxylates such as EDTA and NTA can also form a complex with terbium, albeit weaker than that of DTPA.^{117,147} Therefore, due to the potential competing complexation of Tb(III) ion between the DTPA and the tris-NTA sites within the conjugate, the amount of Tb(III) required to reach the optimal Tb(III) sensitized luminescence

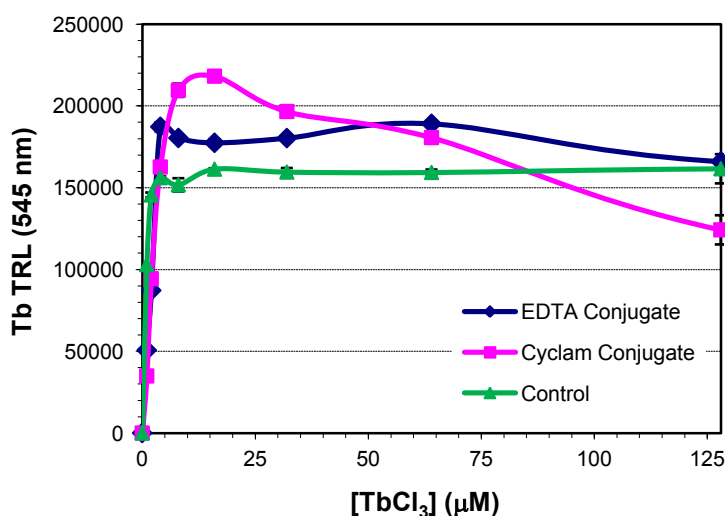


Figure 28. The effect of TbCl₃ concentration on Tb(III) sensitized luminescence. The concentration of the conjugates were fixed at 1 μM during complex formation and diluted 100-fold to 10 nM for Tb TRL measurement at 545 nm.

was investigated. The efficiency of Tb(III) complex formation of the conjugates was first analyzed by measuring the time-resolved luminescence (TRL) of the Tb(III) complex as a function of TbCl₃ concentration. The concentration of conjugates are fixed at 1 μ M during Tb(III) complex formation and the time delay before the counting window was set to 200 μ s for both Tb TRL and TR-FRET unless otherwise specified.

As shown in Figure 28, the amount of Tb(III) ion required to reach the highest TRL signal of sensitized Tb(III) at $\lambda = 545$ nm is approximately 3 - 4 equivalents of Tb(III) ion for the EDTA and cyclam conjugates (**43** and **44**). On the other hand, the control compound **59** reaches its maximum level of TRL at 545 nm with about 2 equivalents of TbCl₃. The additional amount of Tb(III) required to reach the highest TRL for the conjugates could potentially be due to the presence of tris-NTA in the molecule, which is likely to compete for Tb(III) ions with DTPA. Addition of excess Tb(III) ion seems to be detrimental to the TRL signal and is more prominent for cyclam conjugate **44** as the signal decreases rapidly over 16 equivalents of Tb(III). While the mechanism of this fluorescence quenching is currently not known, it is likely due to the additional Tb(III) complex that forms at the tris-NTA end of the molecule. The more prominent effect of excess Tb(III) for cyclam conjugate **44** may be due to its structure where the cyclam-tris-NTA may bind tighter to the Tb(III) at its tris-NTA end than the EDTA-tris-NTA conjugate **43**.¹⁴⁷ Nevertheless, the Tb(III) complex is likely to be predominantly forming at the DTPA moiety at low concentrations of TbCl₃ as DTPA is known to have higher affinity to Tb(III) ion than other polyaminocarboxylates.^{117,147} The concentration of 2 μ M TbCl₃ (2 equivalents Tb(III) ion) was selected in order to avoid the tris-NTA site from complexing with Tb(III) ions since the control compound **59** without the tris-NTA group reaches its maximum level of Tb(III) luminescence with 2 μ M TbCl₃ solution.

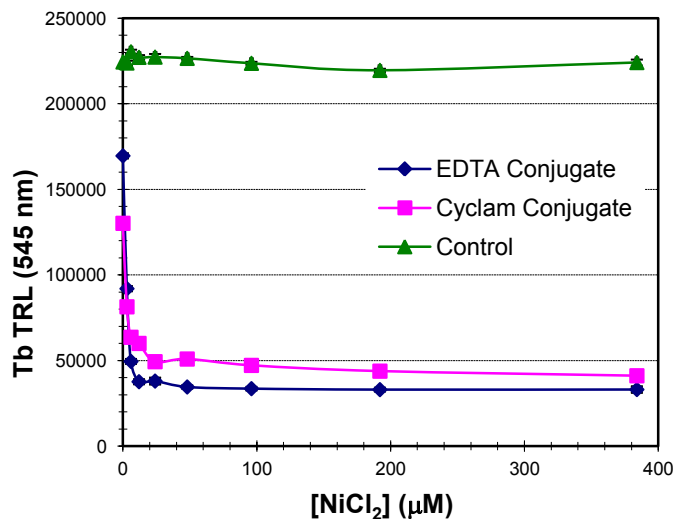


Figure 29. The effect of Ni(II) ion loading on the terbium sensitized luminescence at 545 nm. A fixed concentration of Tb-complex (1 μM) was allowed to form a Ni(II) complex for 60 min. The Ni-complex solution was diluted to 10 nM and Tb TRL measured at 545 nm.

Next, the amount of NiCl₂ that is required for loading of Ni(II) ions onto the tris-NTA was investigated by analyzing the effect of NiCl₂ on the sensitized luminescence of Tb(III). Again, the concentration of the conjugates were fixed at 1 μM during complex formation. As shown in Figure 29, the terbium luminescence of the control compound **59** was not affected by the presence of NiCl₂ even at concentrations as high as 0.384 mM. This indicates that the DTPA-Tb(III) complex is stable upon formation and addition of other ions will not disrupt the complex or affect the luminescence intensity. However, the presence of Ni(II) ions have a profound effect on the terbium TRL of the conjugates **43** and **44**, where the addition of NiCl₂ causes a rapid drop in the luminescence intensity. With the addition of 12 equivalents of NiCl₂, the signal reaches the lower end of the TRL signal where further increase in NiCl₂ does not seem to affect the luminescence significantly. This means that free Ni(II) ions in solution does not affect the terbium luminescence, while Ni(II) ion complexed to an organic molecule act as a quencher (e.g. non-radiative) of triplet-state and energy transfer.¹⁴⁸⁻¹⁵⁰ Although the underlying mechanism of this intramolecular energy quenching process is not quite understood, it appears that the Ni(II) ion in

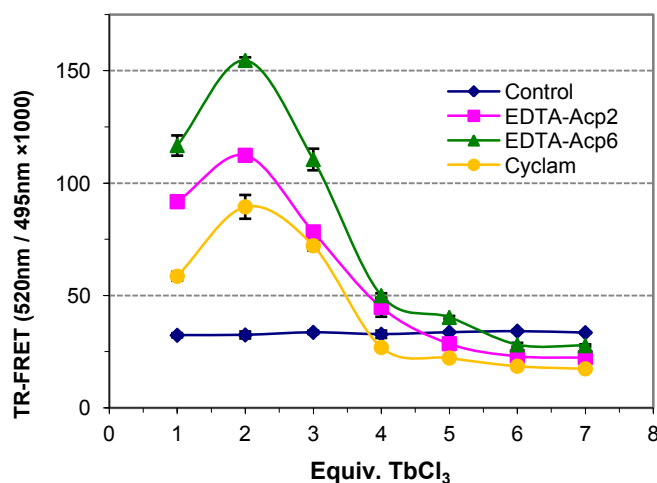
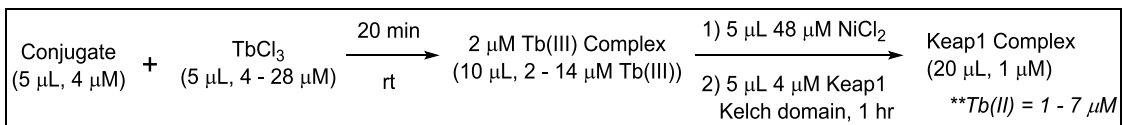


Figure 30. The effect of Tb(III) concentration on the TR-FRET efficiency of the conjugates. The protein complexes were prepared as shown in Scheme 17 and the complex solutions were diluted to 10 nM. The Tb TR-FRET signal was measured in the presence of 20 nM tracer.

close proximity of the Tb(III) complex interferes with the lanthanide sensitization involving the triplet-state and affects the overall TR-FRET signal. Technically, $3 \times$ Ni(II) ions are needed to form a complex with the tris-NTA, therefore the actual equivalents of Ni(II) used is equal to 4 equivalents with respect to the tris-NTA. Since the presence of Ni(II) ions in solution does not affect the Tb(III) complex, 12 equivalents of NiCl₂ (4 equivalents with respect to tris-NTA) was selected to make sure 3 Ni(II) ions and the tris-NTA forms a complex.

For the further development of the TR-FRET assay using the labeling reagents, recombinant Keap1 Kelch domain, which was expressed with the His₆-tag, and the FITC-9mer Nrf2 amide tracer from the Keap1-Nrf2 FP assay was used. The FP assay optimized in the present study provided an excellent platform to explore conditions for the development of TR-FRET assay by *in situ* labeling of the target protein. First, the effect of TbCl₃ concentration on the TR-FRET assay signal was evaluated as the excess TbCl₃ poses concern due to the propensity of the tris-NTA group of the conjugates to complex with Tb(III) ions.



Scheme 17. The Tb(III) and 3 $\times$ Ni(II)-NTA complex formation steps for the preparation of the protein complex in evaluating the effect of TbCl₃ concentration on the assay signal.

As shown in Scheme 17, the concentration of the conjugates was fixed at 4 μ M and the TbCl₃ concentration range between 4 - 28 μ M (1 - 7 eq. TbCl₃) during the Tb(III) complexation step. The Tb(III) complex was allowed to form for 20 min and then added 12 equivalents of NiCl₂ solution, followed by 1 equivalent of Keap1 Kelch domain to form the 3 $\times$ Ni-NTA complex that binds to the His₆-tag of the protein. The protein complex solution was diluted to final concentration of 10 nM containing 20 nM of FITC-9mer Nrf2 amide.

As shown in Figure 30, the amount of Tb(III) required for highest TR-FRET assay signal was determined to be 2 equivalents. This was consistent with the previous results of Tb(III) TRL experiments where 2 equivalents of Tb(III) ion was required to reach highest level of Tb sensitized emission and that Tb(III) complex appear to form predominantly at the DTPA portion of the conjugates. The control compound **59** lacks the tris-NTA moiety, therefore TR-FRET cannot occur as it cannot bind to the His₆-tag of the protein. Due to the sensitivity of the assay signal to the TbCl₃ concentration between 1 - 3 equivalents of Tb(III), the experiment was repeated with closer data points to determine the optimal TbCl₃ concentration. As shown in Figure 31A, the control compound **59** again is not affected by the TbCl₃ concentration as TR-FRET cannot occur for this compound. The EDTA-Acp₂ linker conjugate **43** shows the highest TR-FRET signal at 1.5 equivalent, while the EDTA-Acp₆ linker conjugate **47** and the cyclam conjugate **44** reach the highest signal with 2 equivalents of TbCl₃. The optimal amount of TbCl₃ for EDTA-Acp₂ linker conjugate **43** was confirmed by 3 $\times$ triplicates at 1.5 and 2.0 equivalents of Tb(III) (Figure 31B).

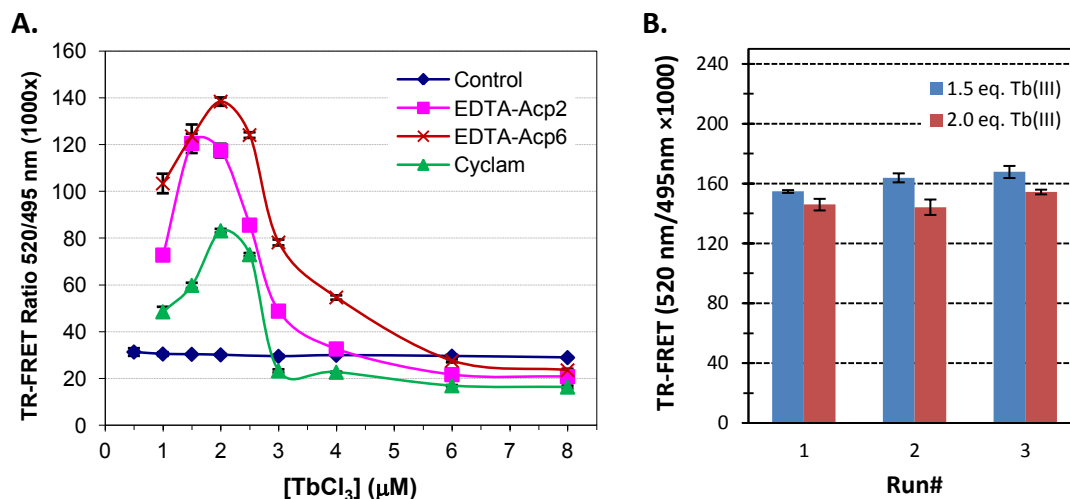
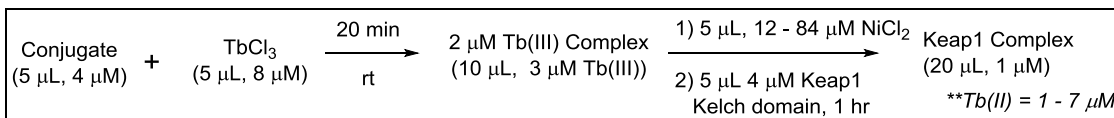


Figure 31. (A) The effect of TbCl₃ on TR-FRET signal repeated with closer data points and (B) testing TR-FRET signal of EDTA-Acp₂ conjugate at 1.5 and 2 equivalents of Tb(III).

Next, the optimal amount of the Ni(II) ion for tris-NTA complex was evaluated by performing the TR-FRET assay with a range of NiCl₂ concentrations. In order for the TR-FRET to occur, the donor chelate needs to be in proximity of the tracer peptide, meaning that the tris-NTA-His₆ interaction needs to form between the conjugate and the target protein. The extent of formation of this complex should be dependent on the amount of Ni(II) successfully loaded onto the tris-NTA group of the molecule.

The complex formation steps are shown in Scheme 18, where the concentration of NiCl₂ during the protein complex formation step was 3 to 21 μM (1 to 7 eq. with respect to tris-NTA). The Figure 32 shows that all conjugates require at least 12 mM NiCl₂ or 4 equivalents of Ni(II) with respect to tris-NTA for the signals to reach the plateau. Any further increase in NiCl₂ does not affect the TR-FRET, therefore 12 μM NiCl₂ was determined to be the optimal amount of Ni(II) for the assay. As expected, the control compound **59** which does not bind to Ni(II) was not affected by the presence of Ni(II) ions in solution.



Scheme 18. The Tb(III) and 3 $\times$ Ni(II)-NTA complex formation steps for the preparation of the protein complex in determining the optimal amount of NiCl₂ for the assay.

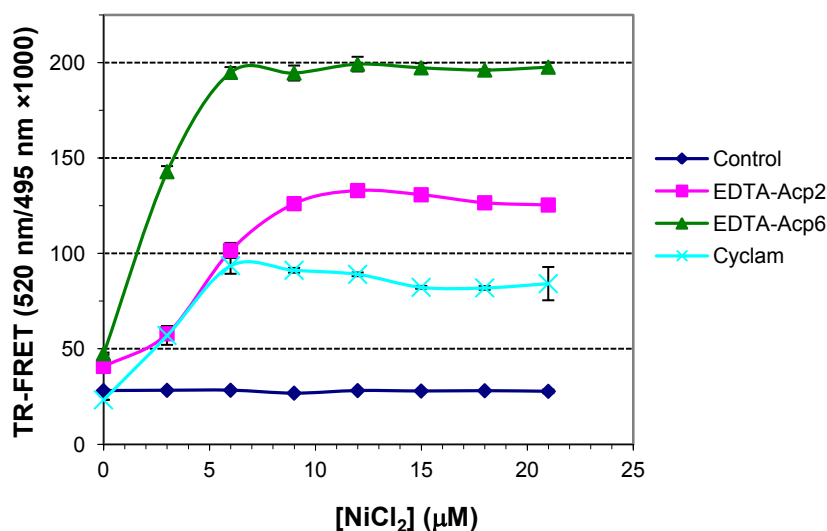
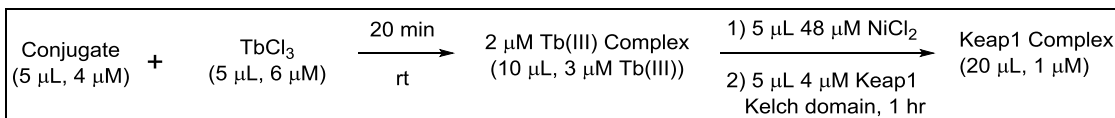


Figure 32. The graph showing the effect of Ni(II) on TR-FRET signal of the conjugates as a function of NiCl₂ concentration. The protein complexes were prepared as shown in Scheme 18 and the complex solutions were diluted to 10 nM. The Tb TR-FRET signal was measured in the presence of 20 nM tracer.

Finally, The effect of tracer concentration on the TR-FRET signal was investigated as the intensity of assay signal depends on the amount of donor-acceptor interactions formed (i.e. the fraction of tracer bound). Earlier, the amount of Tb(III) required for the Tb(III) complex formation was determined to be 1.5 - 2.0 equivalents of TbCl₃, while the amount of Ni(II) required for the conjugates was determined to be 12 equivalent of NiCl₂ during complexation to form the 3 $\times$ Ni-NTA complexes at the tris-NTA end. For this assay, Acp₆ and Acp₁₀ linker conjugates **47** and **48** were also added to evaluate the effect of the linker length on TR-FRET



Scheme 19. The Tb(III) and 3 $\times$ Ni(II)-NTA complex formation for the preparation of the protein complex to determine the effect of tracer concentration

assay signal. These compounds were designed with the hope to increase the distance of separation between the DTPA Tb(III) chelate and the Ni(II)-tris-NTA complex to minimize the effect of quenching. However, this may also increase overall distance from the acceptor. Since the TR-FRET efficiency has the inverse-sixth power dependence on the distance, the optimal distance between the two complexes should allow the balance of quenching by Ni(II) and FRET decay due to distance.

As shown in Scheme 19, the concentration of the conjugates and TbCl₃ during the Tb(III) complexation step was 4 μ M and 6 μ M (1.5 eq. TbCl₃), respectively. The Tb(III) complex was allowed to form for 20 min and then added 12 equivalents of NiCl₂ solution, followed by 1 equivalent of Keap1 Kelch domain to form the 3 $\times$ Ni-NTA complex that binds to the His₆-tag of the protein. The protein complex solution was diluted to final concentration of 10 nM containing 100 nM to 0.05 nM of FITC-9mer Nrf2 amide.

The background of the TR-FRET signal was determined for each sample well by the addition of excess inhibitory peptide (1 μ M Ac-9mer Nrf2 amide) to completely displace the tracer from Keap1 Kelch domain. After subtracting the background signal, the binding curves of the tracer in Figure 33A were obtained. As expected, the binding affinity of the tracer obtained by the TR-FRET assay was very similar (Figure 33B) to each other, where the binding curves overlap when plotted using fraction of tracer bound (f_b) as a function of the tracer concentration. The binding affinity of the tracer was determined to be 2.83 ± 0.53 nM using the EDTA-Acp₂ conjugate **43**.

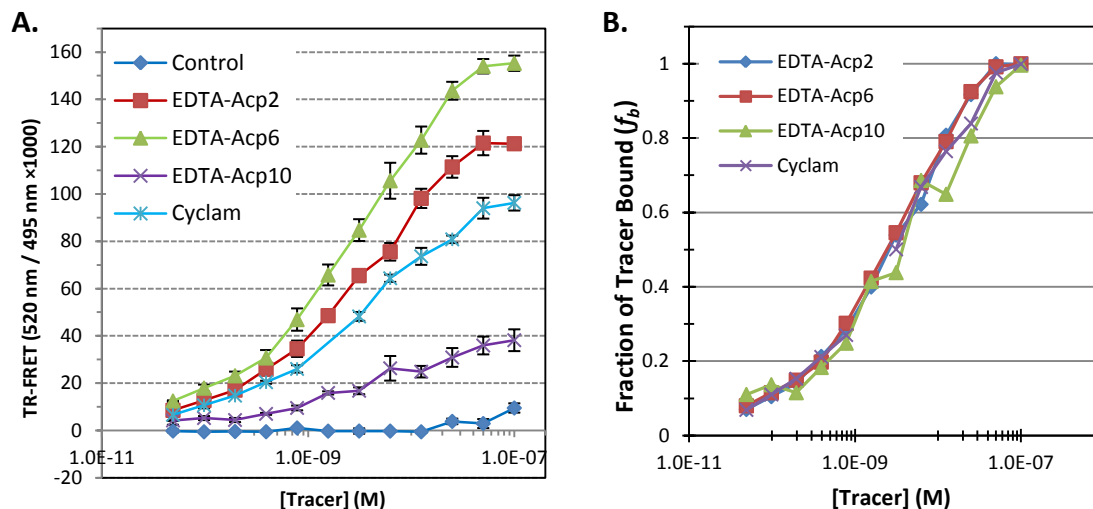


Figure 33. (A.) The TR-FRET binding curves of the tracer to Keap1 Kelch domain using the conjugates and (B.) the fraction of tracer bound to Keap1 Kelch domain. For Figure A, the background signal of TR-FRET was subtracted by determining the TR-FRET signals in presence of the inhibitors peptide (Ac-9mer Nrf2 amide).

The assay range of the EDTA-Acp₆ conjugate **47** was the highest, followed by the EDTA-Acp₂ conjugate **43**. The Acp₁₀-linker conjugate **48** had a significantly lower signal, which implies that the Acp₁₀ linker may be too long for efficient energy transfer due to the separation distance between the donor and the acceptor. This means that EDTA-Acp₆ conjugate **47** achieves the best balance of Ni(II) quenching effect and the signal decay due to separation distance. The tracer concentration of 20 μ M used for exploring the conditions of the assay gives >0.8 fraction of the tracer bound to Keap1 Kelch domain and can be used for performing a competition assay using the TR-FRET assay developed.

The (Bodipy-FL)-9mer Nrf2 amide tracer has been synthesized for the optimization of FP assay and, in the FP assay, Bodipy-FL dye is often used as a substitute for fluorescein due to its superior fluorescence properties. It has higher extinction coefficient and quantum yield approaching unity even in aqueous media. It is insensitive to pH and polarity of the solvent due to lack of ionizable group, but more importantly it has sharper excitation and emission at 503 nm

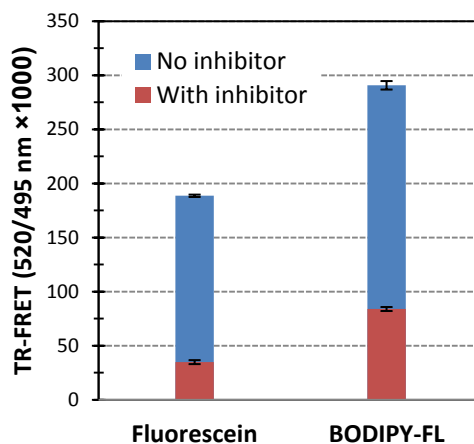


Figure 34. The graph comparing fluorescein and Bodipy-FL as the acceptor of terbium FRET. The protein complexes were prepared as shown in Scheme 18 and the complex solutions were diluted to 10 nM. The Tb TR-FRET signal was measured in the presence of 20 nM of either FITC-9mer Nrf2 amide or (Bodipy-FL)-9mer-Nrf2 amide.

and 512 nm, respectively. For these reasons, it was hypothesized that the Bodipy-FL dye may be the better acceptor of the terbium FRET.

The TR-FRET assay was performed using the selected conditions for TbCl_3 , NiCl_2 , and the tracer concentration.¹⁰² As shown in Figure 34, the signal range using Bodipy-FL was found to be approximately 34.5% greater, where the range for Bodipy-FL and fluorescein was determined to be 206.78 and 153.74, respectively. The background signal measured after the addition of the inhibitor was significantly higher for Bodipy-FL, which is potentially due to its brightness and longer fluorescence lifetime ($>5 \mu\text{s}$) as compared to fluorescein. Nevertheless, the use of Bodipy-FL dye in Tb(III) TR-FRET gives a significant increase in the assay dynamic range due to its superior fluorescence properties.

The assay conditions for Eu(III) TR-FRET assay was also explored using Cy5-labeled 9mer Nrf2 amide as the acceptor, which was also synthesized for the Keap1-Nrf2 FP assay. The Eu(III) loading on the TMT-tris-NTA conjugate **70** was performed by incubating a fixed concentration of

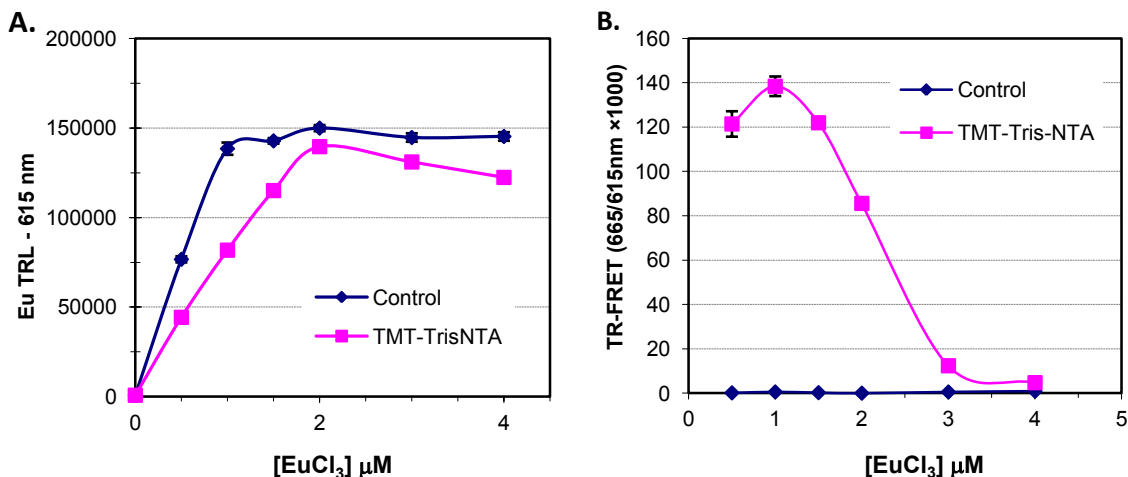


Figure 35. (A) The plot of Eu(III) TRL at 615 nm as a function of EuCl_3 concentration and (B) the effect of EuCl_3 concentration on the Eu TR-FRET signal. The TR-FRET assay shown in (B) was performed similarly to the Tb TR-FRET assay in Scheme 17.

TMT-tris-NTA conjugate with a range of concentrations of EuCl_3 for 20 min at rt. Similar to the results of Tb(III) complex formation, TMT-tris-NTA conjugate **70** required twice as much lanthanide to reach the highest level of Eu(III) TRL as compared to the control TMT compound **63** (Figure 35A). Addition of more than 2 equivalents of Eu(III) ions result in gradual decrease in the Eu TRL signal. The Eu(III) complex formation for the control compound **63** required 1 equivalent of Eu(III) ion to reach the plateau. This may mean that the Eu(III) complex formation by a TMT chelate is more efficient than the formation of Tb(III) complex by a DTPA chelate. The effect of EuCl_3 concentration on the TR-FRET assay signal was also evaluated, where it was found that the TR-FRET assay using Eu(III) also has similar sensitivity to excess Eu(III) ion used during the complex formation (Figure 35B). The assay signal reaches the highest level with 1 equivalent of Eu(III) and decreases as the concentration of Eu(III) ion is increased. This was consistent with the result from Eu(III) loading experiment that 1 equivalent of EuCl_3 is sufficient for Eu(III) complex formation. In light of these results, 1 equivalent of EuCl_3 was selected as the optimal concentration for Eu(III) complex formation.

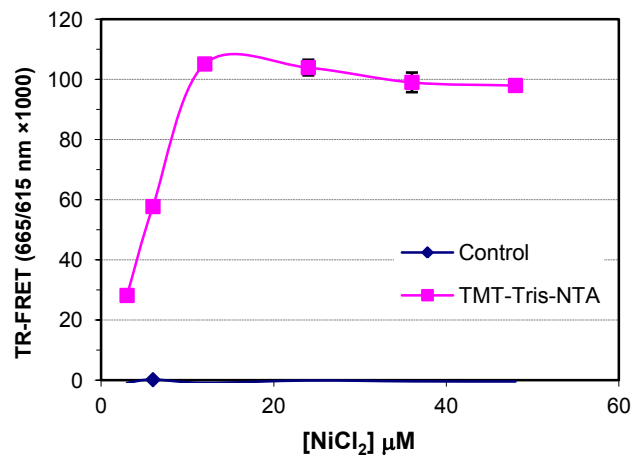


Figure 36. The effect of NiCl_2 on the Eu(III) TR-FRET signal. The background signal after addition of the inhibitory peptide has been subtracted from the TR-FRET signal.

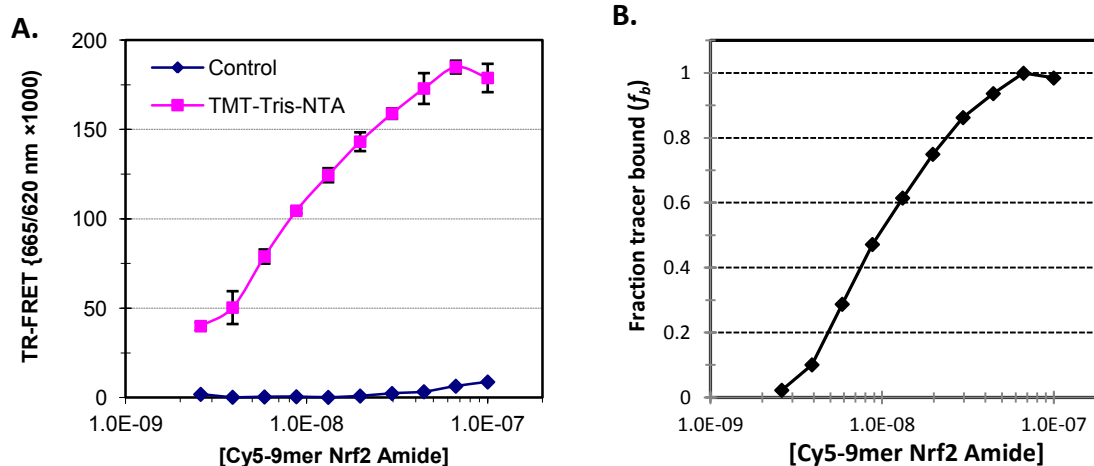


Figure 37. (A.) The TR-FRET binding curves of the tracer to Keap1 Kelch domain using the TMT-tris-NTA conjugate and (B.) the fraction of tracer bound to Keap1 Kelch domain. For Figure A, the background signal of TR-FRET was subtracted by determining the TR-FRET signals in presence of the inhibitor peptide (Ac-9mer Nrf2 amide).

The concentration of NiCl_2 required for the formation of the Ni-tris-NTA complex was also evaluated for the TMT-tris-NTA conjugate **70** (Figure 36). The structure of the tris-NTA of conjugate **70** is identical to the EDTA-Acp_n-tris-NTA conjugates, thus the same concentration of NiCl_2 should be required to reach the highest level of Eu(III) TR-FRET signal. The amount of Ni(II) ion required for Eu TR-FRET was determined to be also 12 equivalents of NiCl_2 and was

consistent with the results from Tb TR-FRET experiments. The effect of concentration of the tracer on TR-FRET assay signal was also evaluated by analyzing the Eu TR-FRET signal for a range of Cy5-labeled tracer concentration. At 20 μM tracer concentration, the fraction of the tracer bound was determined to be approximately 0.75, which was similar to the result obtained in the Tb TR-FRET experiment (Figure 37). Based on the binding curve of the tracer, the concentration of the tracer can probably be increased to 30 μM without a problem to improve the assay dynamic range.

In this section, the conditions for performing TR-FRET assay using the *in situ* labeling approach was explored using the FP assay that was optimized in the present study as the foundation. By using the novel lanthanide chelate conjugates, the facile transition of the Keap1-Nrf2 FP assay into TR-FRET assay was attempted and succeeded by the attachment of the lanthanide complex *in situ*. The present study demonstrated that the use of this novel reagent allows easy entry into a time-resolved assay in which the FRET from the lanthanide can be captured by the tracer developed for the FP assay. Further optimization for improving the assay range and the adaptability of the novel TR-FRET reagent to other protein-ligand interactions will be the future focus of this research.

V. Application of *in situ* Protein Labeling through Biotin-Streptavidin Interaction

In order to determine the applicability of the *in situ* labeling reagent for TR-FRET synthesized in the present study, the biotin-streptavidin (SA or SAV) interaction was targeted as another test platform. Biotin-streptavidin is considered to be the strongest non-covalent interaction known ($K_d \sim 10^{-15}$ M) and the interaction has seen vast applications in many areas of research as a way to attach biomolecules.¹⁵¹ For example, Amplified Luminescent Proximity Homogeneous Assay (ALPHA) or AlphaScreen is a bead-based chemical assay in which a biotinylated biomolecule is

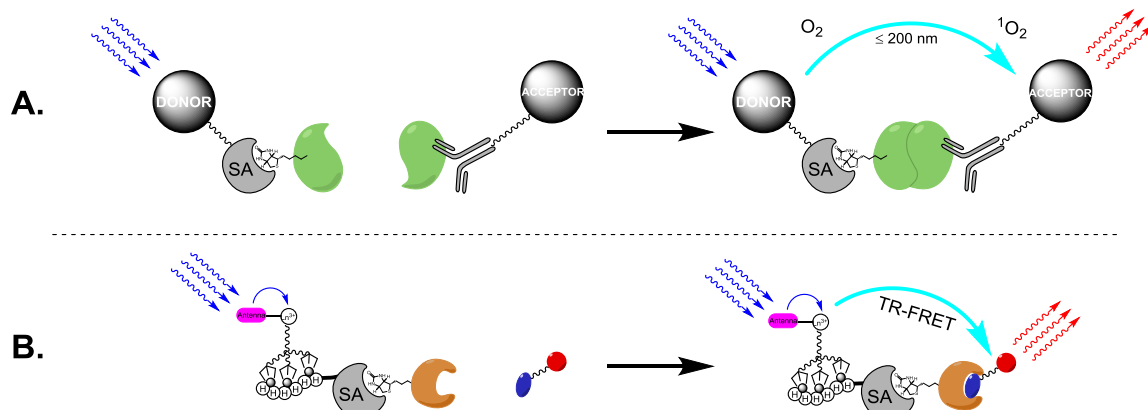
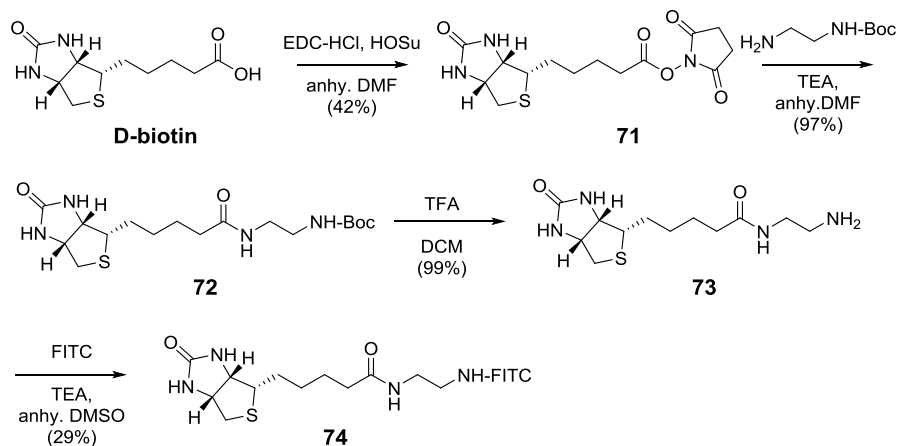


Figure 38. The schematic diagram of (A.) AlphaScreen and (B.) the TR-FRET assay targeting biotinylated biomolecules through SA-mediated interaction. Streptavidin is shown as a monomer.

labeled by a SA-attached donor bead (Figure 38A). The assay can be used to study variety of biomolecular interactions. Similar to FRET in theory, the assay uses the donor that photosensitizes ambient O_2 and converts it into a singlet excited state. The singlet oxygen diffuses and can transfer energy to acceptor beads if the acceptor is in close proximity. The acceptor bead is primarily linked by an antibody that recognizes primary antibodies, chemical labels (e.g. FITC, phosphotyrosine), or epitope tags (e.g. GST, His₆) of a target protein or a ligand as in LanthaScreen. In addition, many biomolecules can be safely biotinylated post-translation as a method for providing a tag for the attachment of the donor and acceptor bead. Therefore, the use of the conjugates for *in situ* labeling of biotinylated biomolecules mediated through the His₆-tagged streptavidin interaction, similar to the AlphaScreen, will expand the applicability of this labeling strategy (Figure 38B).

The test platform was simplified to focus the study on the evaluation of TR-FRET signal mediating through the biotin-SA interaction. D-biotin was directly labeled by the acceptor fluorophore essentially removing the primary protein-ligand interaction. As shown in Scheme 20, FITC-labeled biotin **74** was synthesized by the reaction of D-biotin-OSu ester **71** with Boc-ethylenediamine, followed by deprotection and fluorescent labeling with FITC.



Scheme 20. The synthesis route for FITC-labeled biotin **74** used as the tracer for biotin-SA assays.

The interaction between biotin and streptavidin, once formed, has been reported to be difficult to reverse due to the unusually high binding affinity ($K_d \sim 10^{-15}$ M). In order to determine the amount of FITC-labeled biotin to use for the assay, the FP assay was performed to analyze the binding between the biotin-FITC and streptavidin. Streptavidin is known to exist as a tetramer in solution, and therefore each tetramer is capable of binding 4 biotin.¹¹⁸ The concentration of biotin-EDA-FITC **74** was 4 nM as it was determined to be the lowest concentration of the tracer that is within the linear range of FP with fluorescence high enough for accurate FP measurement. In the absence of competing biotin, streptavidin appears to stoichiometrically titrate biotin-FITC due to its high affinity as 1 nM of streptavidin tetramer gives more than 0.8 fraction of biotin-EDA-FITC tracer bound at a fixed concentration of 4 nM as shown in Figure 39. This was expected as the L_0 used for the FP assay is much larger in magnitude than $2 K_d$ of biotin, and therefore the FP assay is performed under stoichiometric titration condition. In addition, the fluorescence of the sample is quenched when more than one biotin-FITC begins to bind to the streptavidin tetramer. At 1 nM streptavidin tetramer concentration, almost 95% of fluorescence is quenched, which is consistent with stoichiometric titration of biotin by streptavidin tetramer. In

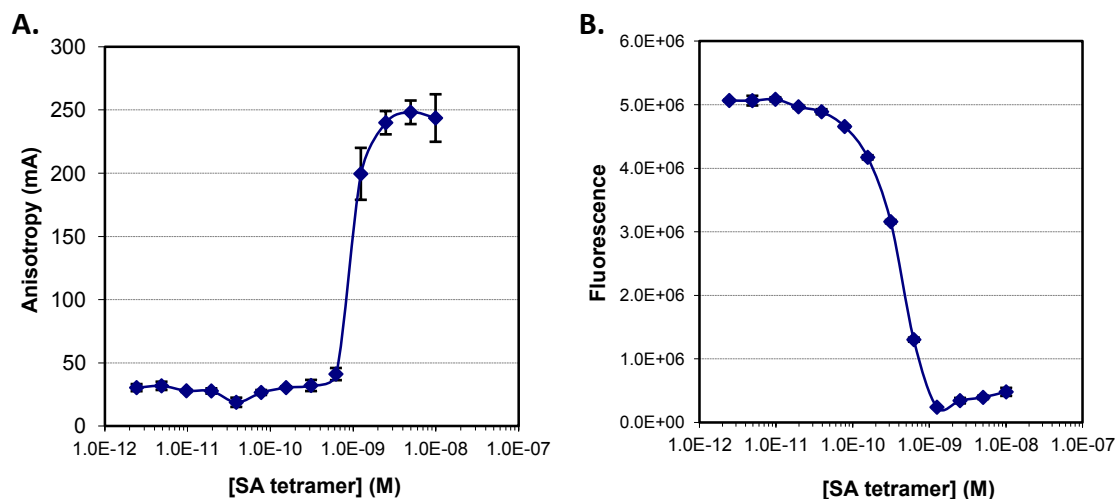
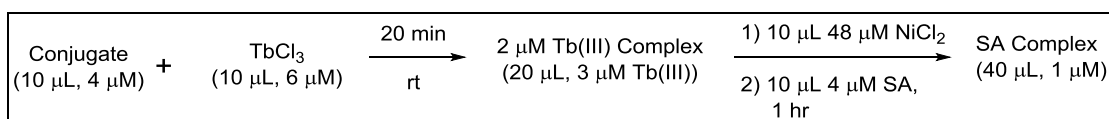


Figure 39. (A) The FP binding curve of biotin-FITC and streptavidin tetramer and (B) the total fluorescence intensity as a function of streptavidin concentration.

addition, this suggests that biotin-streptavidin binds cooperatively where the tetramer forms predominantly than the partially bound tetramer. This is evidenced by the gradual decrease in total fluorescence due to quenching, however no change in FP is detected until almost all of the biotin-FITC is bound due to the lack of contribution to total fluorescence signal by the quenched tetramer. Also the error in the FP measurements becomes prominent at higher concentration of streptavidin due to the decrease in fluorescence intensity from quenching. The TR-FRET assay for biotin-SA was performed using 10 nM streptavidin (2.5 nM tetramer) complex which was formed as shown in Scheme 21.

Since the interaction between biotin-FITC and streptavidin is likely to be irreversible unless the protein is denatured, the excess amount of unlabeled biotin was used to block the biotin binding



Scheme 21. The preparation steps of Tb-SA-complex for the TR-FRET assay.

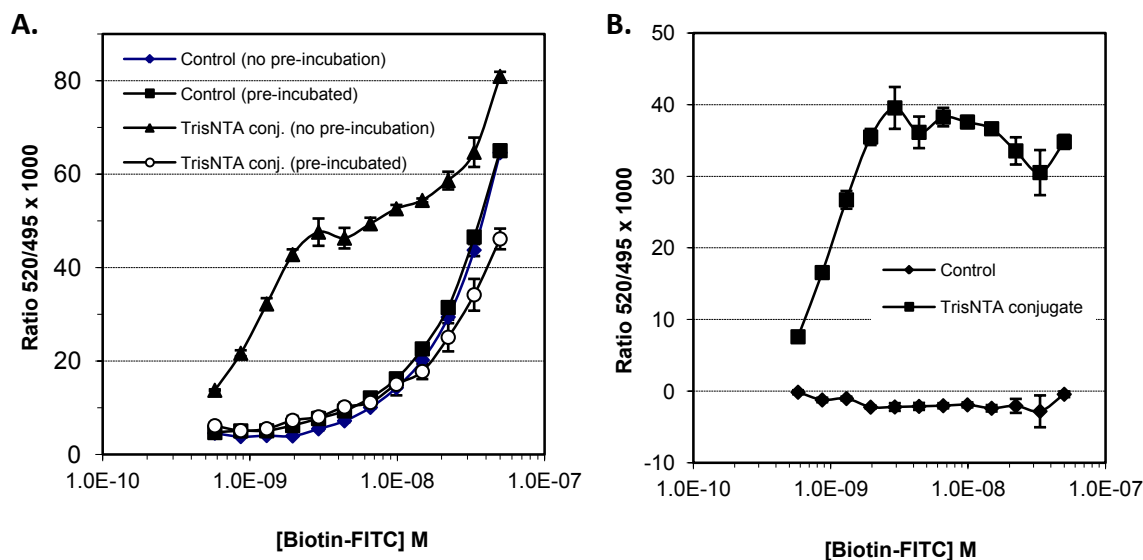


Figure 40. (A.) The Tb TR-FRET curve of biotin-FITC and streptavidin interaction before background subtraction and (B.) the TR-FRET curve after background subtraction. The TR-FRET signal of the wells pre-incubated with biotin was used as the TR-FRET background signals.

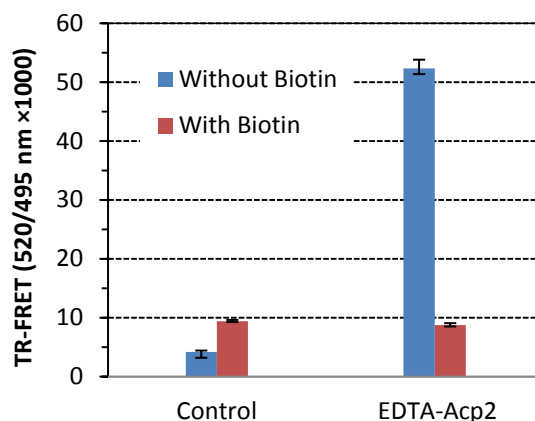


Figure 41. The TR-FRET assay range for biotin-streptavidin interaction using the EDTA-Acp₂ conjugate **43**.

site of streptavidin for the calculation of background TR-FRET signal. Based on the results from the FP assay and the concentration of the streptavidin complex (2.5 nM tetramer) used, approximately 2.5 nM of the biotin-FITC should give the highest TR-FRET signal. As shown in Figure 40, the amount of biotin-FITC required to reach the highest TR-FRET signal was indeed about 2.5 nM, where further increase does not benefit the signal intensity. As shown in Figure 41,

the assay range for the biotin-streptavidin TR-FRET assay was 42 and the relatively low TR-FRET signal as compared to the Keap1-Nrf2 TR-FRET assay is likely due to the quenching effect from the binding of multiple biotin-FITC. In a real TR-FRET assay where biotin-FITC is replaced by a biotinylated protein and acceptor ligand, the separation distance between the acceptor fluorophores are expected to be much greater. Therefore, the quenching effect on the acceptor fluorescence could be minimized and the TR-FRET signal is expected to be much greater. Nevertheless, the TR-FRET assay for biotin-streptavidin interaction has been demonstrated to be working and implies the potential application of this approach in targeting biotinylated biomolecules in addition to the His₆-tagged proteins.

V. Summary

TR-FRET assay is often considered to be superior to other fluorescence assay owing to the time-resolved measurement of the assay signal. The lifetime of luminescence resulting from a lanthanide probe sensitized by the antenna effect is in the millisecond range, while the autofluorescence is in the nanosecond range. This allows a delay in signal measurement, where all interfering and background autofluorescence dissipate during this delay time and the signal can be detected with high signal-to-noise (S/N) ratio. Another useful feature of TR-FRET assay is that the FRET signal is measured as a ratio of the donor and the acceptor emission, which minimizes interwell variations. The ratiometric nature of the assay is also helpful to counteract the interferences from quenching sample. However, the adaptation of TR-FRET has been limited due to high reagent costs and labor-intensive preparations, such as protein modification and purification, involved in the development of assay conditions.

Herein, novel reagents that address these drawbacks of TR-FRET assay were designed and evaluated for their efficiency to perform TR-FRET. The potential for facile development of the

assay was demonstrated by transitioning the existing FP assay platform into TR-FRET assay. FP is commonly utilized in high-throughput screening of chemical libraries because of the robustness and real-time measurement of the polarization signal. However, FP suffers from interference due to autofluorescence, and as a result, has relatively low S/N ratio as well as more susceptible to have false positives and negatives in the result. This limits the hit calling in HTS as compounds that interfere with the FP must be excluded from further analysis even if these samples could be hits. Assays such as SPR that is not affected by the autofluorescence is relatively low-throughput and screening compounds that have been excluded due to fluorescence using such assay will be a labor intensive task. The novel *in situ* labeling reagent presented in this study uses an established FP assay as a platform, which should allow the developed assay to be easily adapted to HTS format. Such novel reagent that allows facile development of HTS TR-FRET assay will be useful in facilitating drug discovery research by allowing access to superior assay technology that improves the sensitivity of screening assay.

A new approach for *in situ* attachment of a lanthanide probe was developed by combining the His₆-tag binding tris-NTA Ni(II) complex with a lanthanide chelate. The *in situ* labeling of the protein at its affinity tag circumvents the labor intensive preparation steps involved in the development of traditional TR-FRET. The His₆-tag is often present in recombinant proteins as a tag for affinity purification following the synthesis and allows a much more convenient approach in labeling of the target protein. It was demonstrated that lanthanide complex and Ni(II) complex form predominantly at their respective coordinating sites. However, the binding of the Ni(II) ions to the tris-NTA of the molecule results in significant quenching of the lanthanide luminescence. In order to counteract the quenching effect, Acp linker between the Tb(III) chelate and tris-NTA moiety was extended from 2 Acp to 6 and 10 Acp linkers. The most efficient donor of TR-FRET was determined to be the Acp₆ linker EDTA tris-NTA conjugate. An Eu(III) chelate based on the TMT was synthesized and similarly tested in a TR-FRET assay. It was found that the *in situ*

labeling approach also works for the Eu(III) TR-FRET and the signal range of the two lanthanides were quite similar. The ability to utilize different lanthanide chelates allows much broader choice of acceptor fluorophores in the development of TR-FRET assay.

In addition, the ability of the assay to utilize the biotin-streptavidin interaction was also investigated. Many biomolecules can be safely and selectively biotinylated post-translation and the ability of the reagent to form a complex with the recombinant streptavidin will expand the use of this reagent beyond the His₆-tagged proteins by providing an alternative method to the costly streptavidin lanthanide complex. Streptavidin exists as a tetramer and the fluorescence signal of the acceptor is significantly quenched due to multiple copies of acceptor labeled biotin binding to streptavidin. It was shown that the TR-FRET signal mediating through the biotin-streptavidin interaction is relatively low. This is likely due to the quenching of the acceptor fluorescence since the TR-FRET signal is a ratiometric value calculated as the quotient of the acceptor and the donor emission. Despite the low signal, it clearly demonstrated that the *in situ* labeling could be applied to biotinylated proteins. The ease of assay development using the novel reagent described in this dissertation was demonstrated and the future research to improve the application of the *in situ* labeling approach to other protein targets should facilitate drug discovery research.

CHAPTER FOUR

THE DISCOVERY OF SMALL-MOLECULE INHIBITORS OF KEAP1-NRF2 INTERACTION FROM THE SCREENING OF MLPCN LIBRARY

I. Hits Identified By the HTS FP Assay of MLPCN Library

High-throughput screening (HTS) of the NIH's Molecular Libraries Probe Production Centers Network (MLPCN) small molecule library of 337,116 compounds at 10 μ M concentration by the FP assay identified 489 initial hits with inhibitory activity higher than 12%, which corresponds to inhibition greater than 3 standard deviations from the means of vehicle treated wells (PubChem Assay ID: 504523). Fluorescent compounds were excluded from the initial hits (29 fluorescent compounds) and hits were cherry-picked for further testing in eight-point dose-response FP assay and in a secondary thermal shift assay. Further evaluations confirmed 8 compounds to have the capacity to inhibit the Keap1-Nrf2 protein-protein interaction (Figure 42). Among the 8 confirmed hits, 2 of the compounds with the highest inhibitory activity were chosen for preliminary structure-activity-relationship (SAR) study.

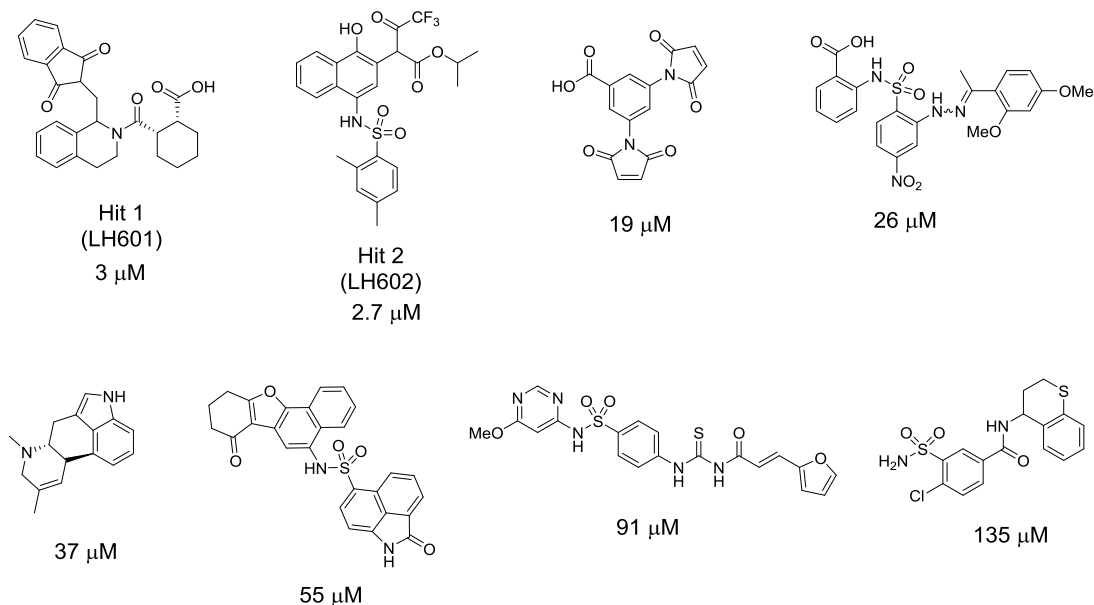


Figure 42. The IC_{50} and the structures of 8 confirmed hits from the HTS FP assay of the MLPCN library at the Broad Institute

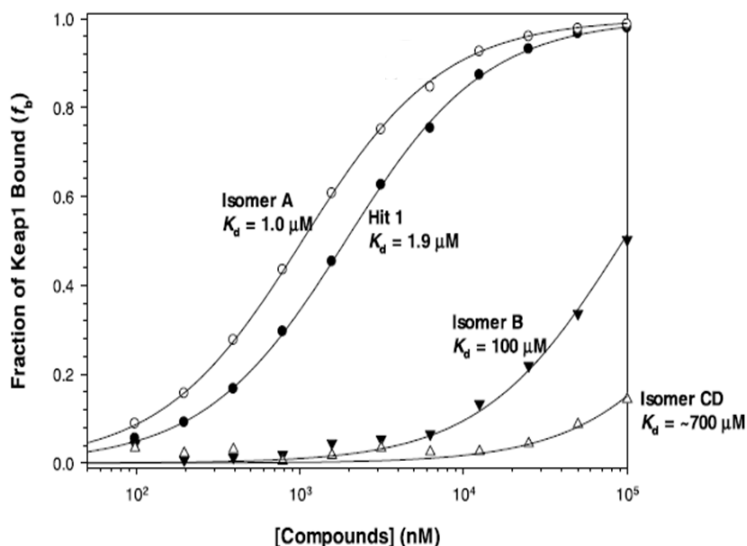


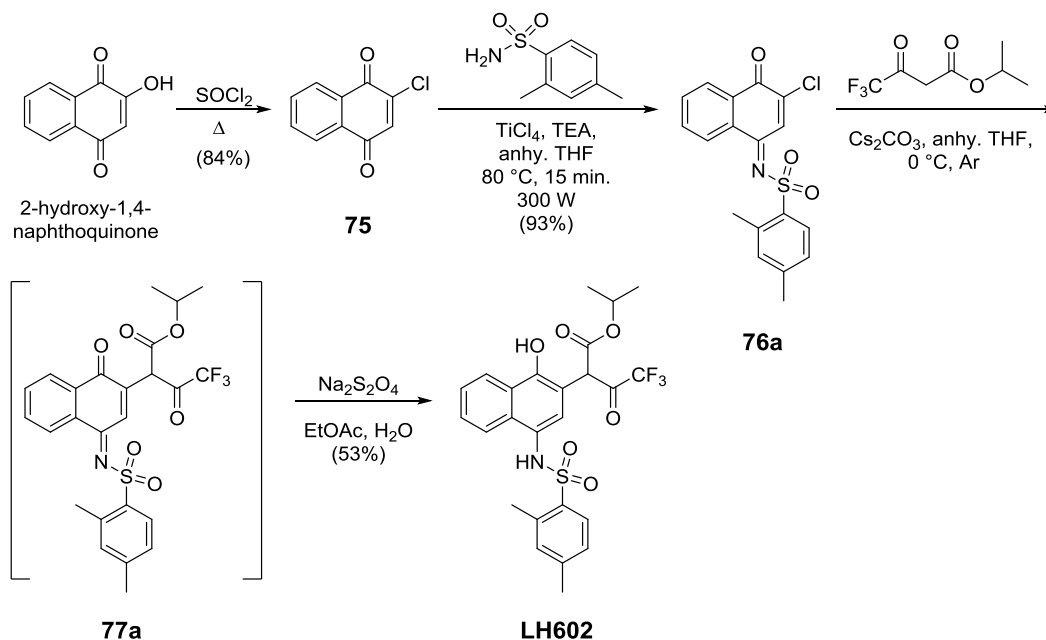
Figure 43. The SPR competition assay on LH601A and its isomers.¹⁴⁹ The isomer A with the absolute stereoconfiguration of *SRS* was determined to be the most active isomer of LH601. The SPR assay was performed in singlicate.

As shown in Figure 42, the phthalimide containing hit compound 1 (**LH601**) possessing three stereogenic centers has an IC_{50} of 3 μM , while hit 2 (**LH602**) with one chiral center has an IC_{50} of 2.7 μM . Activity confirmation of LH601 by the resynthesis of the compound in our group as a mixture of diastereomers showed activity that was over 10-fold less than the MLPCN sample ($IC_{50} > 50 \mu M$). The results of the FP assay led us to believe that a single isomer of LH601 is responsible for most of the activity and that the sample in the MLPCN library may also be a mixture of diastereomers but at a different ratio. The absolute stereoconfigurations of all isomers were assigned by the X-ray crystallography of one enantiomer that was able to crystallize out of solution. The enantiomer with a *SRS* absolute configuration was found to be the most active isomer of LH601 and this isomer is referred to as the isomer A.¹⁵² Pure isomer of LH601A was determined to have a K_d of 1.0 μM by the SPR competition assay (Figure 43). Although SAR analysis of both compounds are ongoing efforts in our group, the synthesis and confirmation of activity of LH602 as well as its preliminary SAR analysis will be the focus of this section.

II. Synthesis and Activity Confirmation of LH602

The compound LH602 is one of the most active inhibitors discovered as a result of screening MLPCN library with the assay sample showing an IC_{50} of 2.7 μ M. In order to validate the activity of LH602 in house, the synthesis of LH602 was carried out as outlined in Scheme 22. A two-step synthesis of a variety of naphthoquinone sulfonimide has been previously reported for the synthesis of proteasome inhibitors starting from 2-chloro-1,4-naphthoquinone **75**.¹⁵³ It was envisioned that LH602 can be prepared similarly by following steps to synthesize the corresponding naphthoquinone sulfonimide intermediate **76a**. The compound **75** was prepared by a chlorination of 2-hydroxy-naphthoquinone in refluxing $SOCl_2$ at 84% yield.¹⁵⁴ A nucleophilic addition of 2,4-dimethylbenzene sulfonamide using $TiCl_4$ under microwave-assisted heating conditions in dry THF gave the 2-chloro-naphthoquinone sulfonimide **76a** in 89% yield. It has been reported that dry THF gives highest yield and is therefore the solvent of choice for this particular step.¹⁵³

Initial attempt to displace the chloride from sulfonimide intermediate **76a** with commercially available isopropyl-4,4,4-trifluoroacetoacetate using NaH or DBU as a base gave complex mixture on LC/MS chromatogram. When K_2CO_3 is used, the reaction did not proceed at reduced temperature (e.g. 0 $^{\circ}C$), however slowly increasing the temperature to room temperature led to a complex mixture similar to the conditions using NaH and DBU. On the other hand, when Cs_2CO_3 was used as the base at reduced temperature, the reaction proceeded cleanly with LC/MS showing the desired product as the major peak and only one side product, which appears to be from hydrolysis. It was speculated that the increased solubility of Cs_2CO_3 allows the reaction to proceed at reduced temperature. The reaction mixture containing the naphthoquinone sulfonimide intermediate **77a** was directly reduced with sodium dithionite ($Na_2S_2O_4$) to the desired product LH602. The blue reaction mixture upon neutralization of the base turned red, where after the



Scheme 22. Synthesis route to HTS hit compound LH602

addition of the reducing agent turned the solution to pale yellow. The solution turning clear with a slight tint of yellow marked the completion of the reduction step. The yield of reactions after 2 steps followed by a reversed-phase HPLC purification on a Gilson HPLC was 41% upon lyophilization of the collected product.

The activity of the LH602 was evaluated by the FP and the SPR competition assays and the results of the SPR assays is shown in Figure 44. The competition FP assay was performed at 5% DMSO concentration with 12 concentrations of the sample in triplicates from 50 μM to 10 nM. The details of competition assay procedures are described in the Experimental section. The FP assay showed that the IC_{50} of the sample is $15.3 \pm 4.2 \mu\text{M}$ and the binding affinity (K_i) calculated from the IC_{50} of LH602 using equation (10) was 2.84 μM . The IC_{50} obtained from the FP assay for LH602 have some discrepancy with the value reported from the screening of the MLPCN library. Nonetheless, results showed that LH602 is indeed a direct inhibitor of Keap1-Nrf2

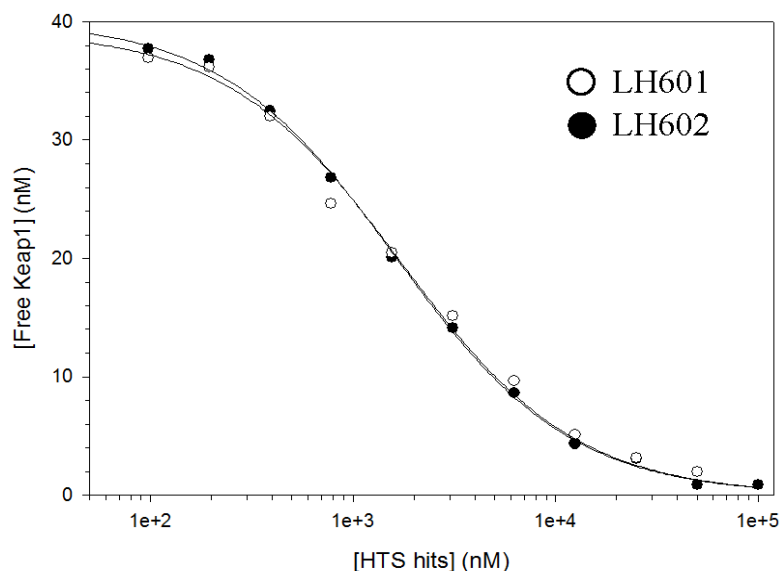
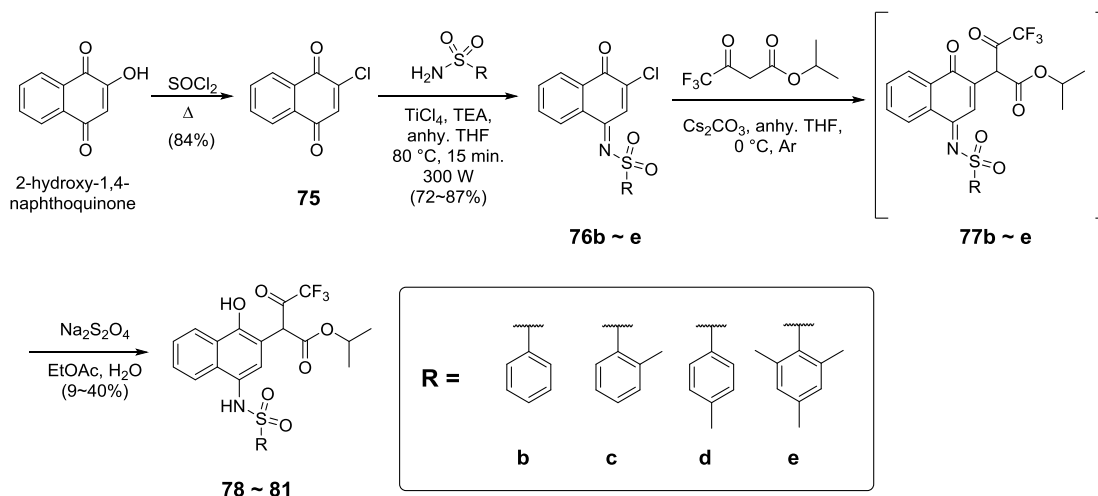


Figure 44. The binding of LH601 and LH602 were determined by the competition SPR assay performed in singlicate as described in the Experimental section.

interaction. Additionally, the binding affinity (K_d) of LH602 obtained by the SPR assay was 1.71 μ M, which is in good agreement with the calculated binding affinity obtained by the FP assay (Figure 44). Interestingly, the inhibitory activity of LH602 determined by the SPR assay shows that it is equal in potency to LH601, which stipulates that the synthesized sample has activity comparable to that of the MLPCN library sample (Figure 44). Therefore, we validated the activity of the library sample by the activity confirmation of the sample synthesized in our group and demonstrated that LH602 is in fact one of the first-in-class inhibitor of Keap1-Nrf2 interaction discovered through HTS. Thus, the analogs of LH602 were targeted in the effort to discover potent inhibitor of Keap1-Nrf2 interaction.

III. The Preliminary SAR for LH602

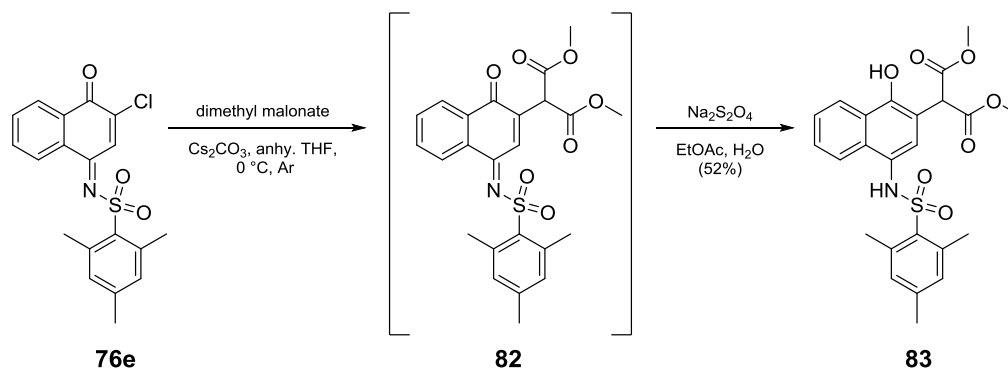
In the preliminary SAR analysis of LH602, the modifications of the benzenesulfonamide moiety was targeted to determine the effect of substituents on binding affinity. By substituting the



Scheme 23. The synthesis of substituted benzenesulfonamide analogs (**78-81**) of LH602

benzenesulfonamide used in the coupling step involving 2-chloronaphthoquinone **75**, other corresponding sulfonamides can be synthesized as shown in Scheme 23. Commercially available sulfonamides were used unless necessary sulfonamides were not readily available. In such case, sulfonamides were simply prepared from their corresponding sulfonylchloride following the literature procedure.¹⁵⁴ The initial targets, as shown in Scheme 23, were designed to evaluate the effect of substitutions on the phenyl ring of the sulfonamide. By varying the methyl substitution on the benzene ring, the effect of the substituents of the ring could be determined. As expected, the microwave-assisted coupling step for different sulfonamides proceeded similarly to LH602 and obtained reaction yields were comparable to that of 2,4-dimethyl-benzenesulfonamide. The trifluoroacetoacetate coupling and the final reduction steps using sodium dithionite were performed without isolation of intermediates **77b-e** as performed for LH602. The purification of final products after reduction was performed on a Gilson reversed-phase HPLC as described in the experimental section.

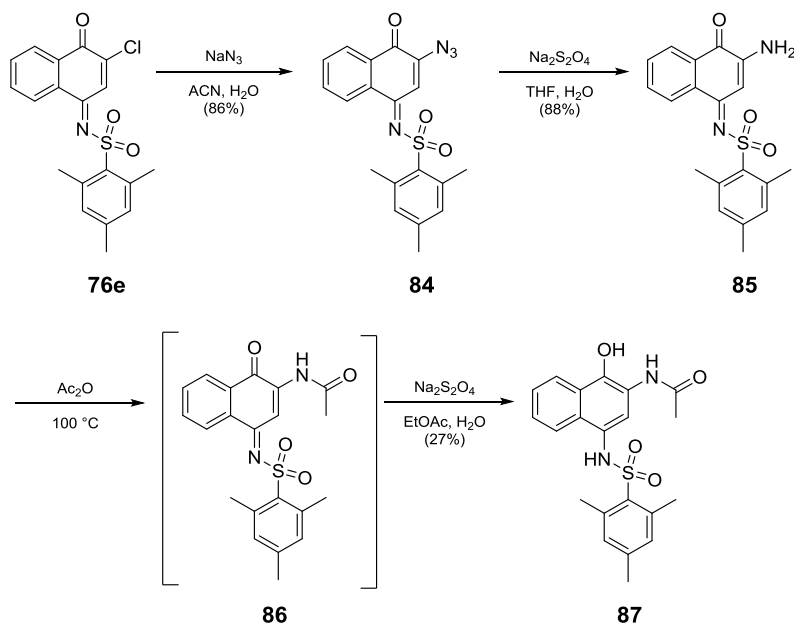
The substitution of isopropyl 4,4,4-trifluoroacetoacetate by other carbonyl groups such as



Scheme 24. The synthesis of dimethyl malonate analog **83** starting from intermediate **76e**

acetamide and dimethyl malonate was targeted to evaluate the requirement of a malonate-like carbonyl moiety for binding to Keap1 Kelch domain. The replacement of the isopropyl trifluoroacetoacetate by symmetric di-carbonyl compounds, such as dimethyl malonate, will also allow simplification of the overall structure by removing the chiral center. In addition, simple malonate such as dimethyl malonate is commercially available from various sources at much high purity ($\geq 98\%$) at significantly reasonable cost. As shown in Scheme 24, dimethyl malonate analog **83** was synthesized starting from sulfonimide **76e** the common intermediate from the preparation of compound **81**. The substitution step using dimethyl malonate did not proceed with 1 equivalent of base and LC/MS showed only the starting material after 30 minutes at 0 °C. The reaction required additional base as compared to isopropyl 4,4,4-trifluoroacetoacetate and completed after 3 additional equivalents of Cs_2CO_3 was added to the mixture. This may be due to the lower acidity of the α -H of dimethyl malonate. The reduction of the naphthoquinone intermediate to the target molecule **83** was performed without isolating intermediate **82** and subjected to final purification by Gilson reversed-phase HPLC. The yield of the substitution-reduction step after HPLC purification was 52% and is comparable to the yields obtained for other analogs of LH602.

In order to further evaluate the effect of modification of isopropyl 4,4,4-trifluoroacetoacetate

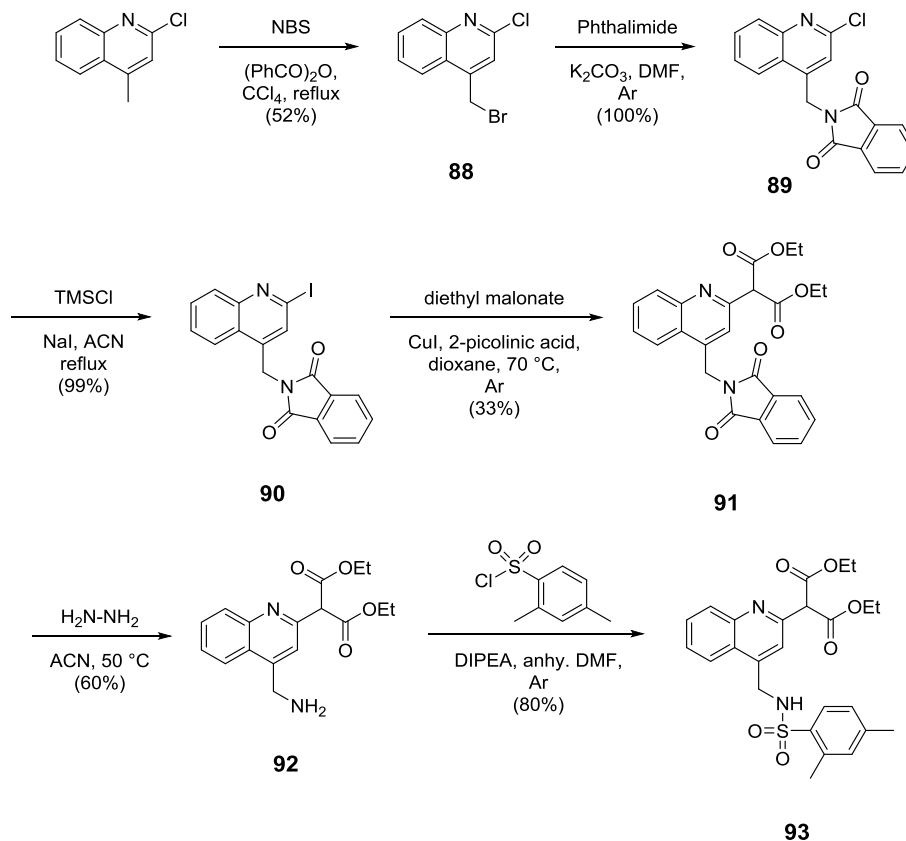


Scheme 25. The synthesis of acetamide analog **87** starting from intermediate **76e**

moiety, the target compound **87** was designed where isopropyl 4,4,4-trifluoroacetate is replaced by the acetamide group. The synthetic route to compound **77** is summarized in Scheme 25. It was postulated that the introduction of an amine group at the 2-position will allow modifications at the amine to rapidly generate analogs if it is tolerated. The common sulfonimide intermediate **76e** is first reacted with sodium azide (NaN_3) to give the azide **84**. It was initially envisioned that Staudinger reaction could be used for coupling the acids to the intermediate. However upon treatment of the azide intermediate **84** with $\text{Na}_2\text{S}_2\text{O}_4$ only the azide was reduced to the amine to give compound **85**. The expected usual reduction of naphthoquinone to the hydroxy naphthalene sulfonamide was not observed. It appears that having the heteroatom at the 2-position of naphthoquinone makes the reduction of such compound difficult. The acetylation of the amine intermediate **85** with acetic anhydride (Ac_2O) at $100\text{ }^\circ\text{C}$ cleanly converted the compound to the acetylated intermediate **86**, which was directly reduced by sodium dithionite without purification to the target compound **87**.

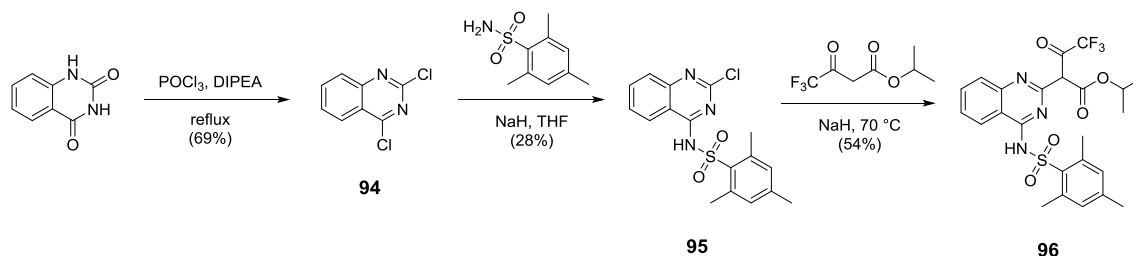
In the effort to discover potent direct inhibitor of Keap1-Nrf2 interaction, it was determined that the hydroxy naphthalene moiety may raise concerns as it could readily oxidized back to the naphthoquinone form. The β -unsaturated carbonyl compounds such as naphthoquinone are electrophiles that undergo 1,4-addition (Michael addition). In order to eliminate such concerns, the isosteric replacements of the hydroxy naphthalene moiety with other non-reactive aromatic heterocycles were targeted. One such target compound has the naphthoquinone moiety replaced by the quinoline. As shown in Scheme 26, the target quinoline compound **93** can be prepared starting from commercially available 2-chlorolepidine. First, 2-chlorolepidine is brominated to 2-chloro-4-(bromomethyl)quinoline **88** following a literature procedure using N-bromo succinimide (NBS) and benzoyl peroxide.^{155,156} Substitution of the bromide with a sulfonamide under basic condition did not proceed as expected and resulted in formation of dimers where 2 molecules of **88** reacted with the sulfonamide. Instead, a phthalimide was introduced at the position to generate the primary amine by Gabriel synthesis.

The phthalimide-protected compound **89** was synthesized by the reaction of **88** with phthalimide under basic condition. Model reaction using 2-chlorolepidine to displace the chloride by a malonate in Ullman-type reaction presented challenge as the halide at the 2-position was far less reactive than it was anticipated as the reaction does not proceed even with CuI at elevated temperature.¹⁵⁷ Elevating the temperature further to perform the reaction resulted in rapid decomposition of the reaction mixture. It was determined that increasing the reactivity at the 2-position should allow the reaction to proceed with milder conditions. Schlosser et al reported the chlorine-iodine halide exchange on heterocycles using iodotrimethylsilane generated *in situ* from trimethylsilylchloride and NaI, which could be applied to compound **89** to perform the halide exchange.¹⁵⁸ Using this method, the 2-chloroquinoline **89** was successfully converted to the 2-iodoquinoline **90** after refluxing overnight in acetonitrile. Despite the 2-iodo substitution,



Scheme 26. The synthesis of compound **93** from 2-chlorolepidine

Ullman-type arylation of malonate by **90** still could not be achieved without decomposition of the mixture. While Ullman method involving an aryl iodide with enolates carried out at mild conditions remains a challenge, improvements in such coupling reaction for aryl halides were accomplished by the use of Cu-chelating ligands such as ethylene glycol, 2-phenylphenol, neocuproine, and L-proline in addition to the catalytic amount of Cu(I) salt.¹⁵⁹⁻¹⁶² The procedure was further improved by Yip et al by the use of 2-picolinic acid as the ligand to achieve room temperature Ullman reactions.¹⁶³ Using this protocol, compound **90** was conjugated with diethyl malonate to give compound **91** at 33% yield. Finally, the phthalimide protecting group was removed by the treatment with hydrazine and the obtained free amine was reacted with 2,4-dimethylbenzenesulfonyl chloride in a Gabriel-type reaction to give the desired quinoline analog **93** in 80% yield.



Scheme 27. Synthesis of compound **96** starting from 2,4(1H,3H)-quinazolinedione

The replacement of the naphthoquinone moiety by the quinazoline ring was also targeted as the analog of LH602. The target quinazoline compound **96** was synthesized from 2,4(1H,3H)-quinazolinedione as show in Scheme 27. The starting quinazolinedione was chlorinated following a literature procedure, in which the treatment of the starting material in refluxing phosphoryl chloride (POCl_3) yielded 2,4-dichloroquinazoline **94**.^{164,165} Compound **94** was subsequently reacted with 2,4-dimethylbenzenesulfonamide under basic condition in THF to give the sulfonamide conjugate **95** in 28% yield. Attempts to conjugate the malonate to compound **95** was not successful when the malonate was used in slight excess. The target compound **96** was prepared by reacting **95** in neat isopropyl 4,4,4-trifluoroacetoacetate at 70 °C. Elevating the temperature above 70 °C resulted in complex reaction mixture with lower product yield. The diethyl malonate analog **97** was synthesized by the same route as **96** except diethyl malonate was used in the final step instead of isopropyl-4,4,4-trifluoroacetoacetate.

IV. Results and Discussions

The comparison of the binding affinity between LH602 and its analogs have been performed using the SPR assay following the method used to determine the binding affinity of LH602. Two concentrations, high (50 μM) and low (5 μM), were selected to analyze their potency as inhibitors

of Keap1-Nrf2 interaction. The inhibitory activity of LH602 and its analogs are summarized on Figure 45 and Table 11. The inhibitory activity of LH602 at 5 μ M and 50 μ M was 57.8% and 97.0%, respectively, and these figures were used as benchmarks to determine whether a modification increases or decreases the binding affinity of the compound. The benzenesulfonamide analog **78** showed inhibitory activity that was approximately 10-fold weaker than LH602 where the inhibitory activity of compound **78** at 5 μ M and 50 μ M was 12.7% and 59.0%, respectively. Thus, the removal of methyl substituents from the benzenesulfonamide moiety was a deactivating modification. Compounds **79** and **80** having the single methyl substituent either at the ortho or para position have activities that is markedly higher than **78**. The inhibitory activity of compound **79** at 5 μ M and 50 μ M was 49.5% and 86.5%, respectively, while compound **80** showed 30.2% and 80.3% inhibition at 5 μ M and 50 μ M, respectively. LH602 which has ortho and para methyl substituents on the benzenesulfonamide moiety has activity higher than compounds **78** ~ **80**, suggesting that multiple methyl substituents on the benzenesulfonamide moiety is preferred for the activity against Keap1-Nrf2 interaction.

Based on the SPR assay results, the ortho substituent is preferred over the para substituent as the inhibitory activity of compound **80** was slightly higher than that of compound **79**. In light of this, it was hypothesized that the activity of LH602 could be improved by the presence of a third methyl substituent at the 6-position (i.e. the 2nd ortho position) of the benzenesulfonamide moiety. The SPR assay results of compound **81** at 5 μ M and 50 μ M showed marked improvement over LH602 where inhibitory activities at these concentrations were determined to be 79.7% and 105.2%, respectively. The result suggests that alkyl substituents at 2-,4-, and 6-position are preferred for increasing the activity of LH602. In addition, trimethyl substituents may suggest that a bulkier alkyl or a electron-donating substituent could also be preferable at these positions.

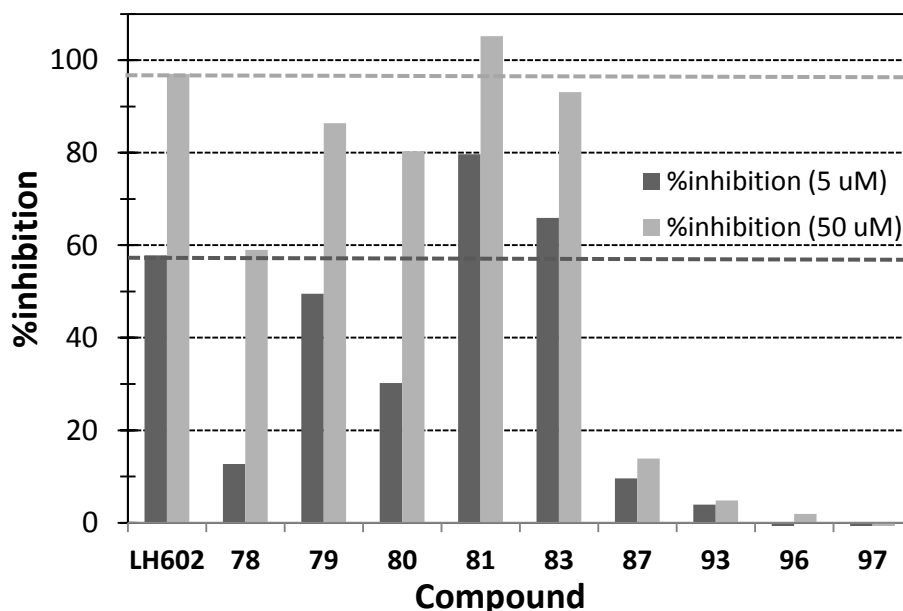


Figure 45. The inhibitory activity of LH602 and its analogs at 5 μ M and 50 μ M concentrations

In the preliminary SAR analysis of LH602, two modifications at the isopropyl 4,4,4-trifluoroacetoacetate moiety have been targeted. The modification at this position was aimed to determine whether an isosteric replacement of trifluoroacetoacetate by other dicarbonyl compounds such as malonates is tolerated. Compound **83** having the replacement of trifluoroacetoacetate moiety by dimethyl malonate was analyzed by the SPR assay. The inhibition of Keap1-Nrf2 interaction at 5 μ M and 50 μ M was determined to be 65.9% and 93.1%, respectively. The inhibitory activity of the dimethyl malonate compound **83** was slightly lower than its parent compound **81** as a result of the modification. It seemed possible that the replacement of trifluoroacetoacetate moiety by a simple carbonyls such as acetamide could also provide compounds that are still active. In order to determine the effect of removing one carbonyl group on the activity of the compound, acetamide compound **87** was designed and targeted for synthesis. The analysis of the compound by the SPR assay showed that the 5 μ M and 50 μ M inhibitory activity was 9.6% and 13.9% respectively. Clearly, the activity of the compound was

lost and the modification has deactivated the compound. This means that a dicarbonyl moiety could be a requirement at this position.

The replacement of the naphthoquinone moiety in LH602 was attempted in order to convert the structure to one that is not reactive. Two analogs of LH602 were targeted in the preliminary SAR analysis where the hydroxynaphthalene moiety was replaced by an heterocycle. The quinoline compound **93** was determined to be inactive as the 5 μ M and the 50 μ M inhibitory activity determined by the SPR assay was 3.9% and 4.8% respectively. Due to a complex reaction mixture and the difficulty in isolating the product when isopropyl 4,4,4-trifluoroacetoacetate was used, compound **93** had to be synthesized using diethyl malonate in addition to the replacement of the core aromatic ring. Based on the SAR analysis of compound **93**, malonate analogs should possess activity similar to that of LH602 containing the trifluoroacetate moiety. Therefore, the compound is not expected to be deactivated due to the use of diethyl malonate at this position.

Similarly, two quinazoline containing analogs **96** and **97** were synthesized where compound **96** has isopropyl 4,4,4-trifluoroacetoacetate like LH602 and compound **97** has diethyl malonate replacement at this position. The SPR assay determined that both quinazoline analogs are inactive and showed no inhibitory activity even at 50 μ M concentration. Based on these results, the replacement of the naphthoquinone ring by a quinoline or quinazoline ring inactivates the compound and other aromatic rings may need to be explored to obtain analogs of LH602 that are less likely to be reactive. It is worthy to mention here that in the effort to improve the affinity of compound **96** in the research group, the compound which has replacement of trimethylbenzenesulfonamide by tosyl-hydrazide was synthesized (not shown). Although this compound has very low activity compared to the parent compound, **LH602**, it showed 28.3%

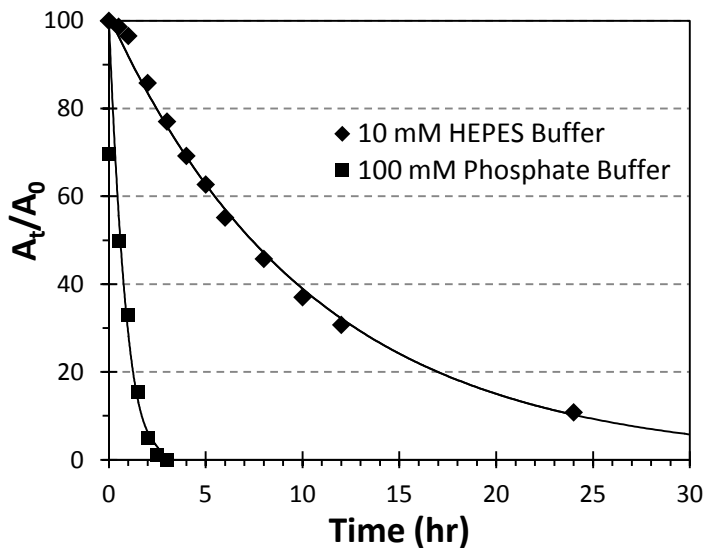


Figure 46. The stability of LH602 determined in 10 mM HEPES and 100 mM phosphate buffer at pH 7.4 containing 5% DMSO at 37 °C

inhibition at 50 μ M concentration against Keap1 Kelch domain. This means that the homologization of the sulfonamide linker to the sulfonyl hydrazide allowed the quinazoline compound **96** to regain some of the activity against Keap1. The potential modification using trimethylbenzenesulfonyl hydrazide may improve the activity further for this series and the future SAR effort for the quinazoline series could be focused in this direction.

The preliminary SAR analysis of LH602 to replace the naphthoquinone core resulted in loss of activity and was not successful. As quinone containing structures are known electrophiles that are likely to be reactive, the stability of LH602 was investigated starting with the assay conditions. The compound dissolved in DMSO was serially diluted to 5% DMSO concentration in 10 mM HEPES or 100 mM phosphate buffer at pH 7.4. The sample solution was incubated at 37 °C and the amount of compound remaining in solution was determined by analyzing the peak area from HPLC chromatogram. As shown in Figure 46, the half-life of LH602 in 10 mM HEPES buffer

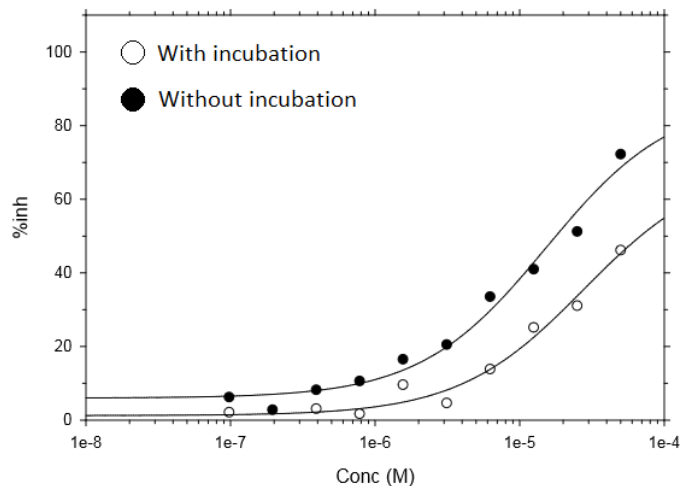


Figure 47. The effect of pre-incubation of LH602 in the assay buffer on its activity as determined by the competition FP assay.

was determined to be 6.08 hr, while a significantly shorter half-life at 42.3 min was observed in 100 mM phosphate. In order to determine the effect of decomposition, LH602 was incubated to its half-life at 37 °C and tested by FP assay to compare the activity to a fresh solution of LH602. The pre-incubated sample of LH602 showed decreased activity where IC_{50} of the sample was determined to be 27.9 μ M, while a fresh sample of LH602 had an IC_{50} of 15.3 μ M (Figure 47).

Also, it is important to note that the half life of the more active 2,4,6-trimethyl benzenesulfonamide analog **81** was merely 19.7 min in 10 mM HEPES buffer at pH 7.4. Taken together, these results suggest that LH602 and its analogs containing the naphthoquinone core are likely to be unstable due to their inherent reactivity. A quinone is a common structural feature often found in compounds that are reactive towards nucleophiles such as sulfhydryl group of cysteine residue and glutathione. The replacement of the trifluoroacetate and the naphthoquinone moiety by non-reactive structures should help improve the stability of the compound. The success of this replacement in the future SAR effort will be important in order to

avoid the reactivity concerns as well as the likeliness to be an indirect inhibitor. The future work in this project will be to discover such analogs with successful replacements of potentially reactive moieties and improve upon its activity.

Table 11. The inhibitory activities of LH602 analogs

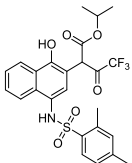
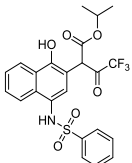
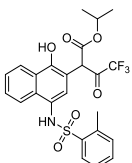
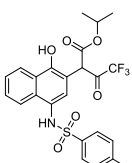
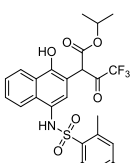
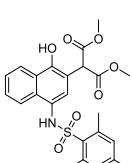
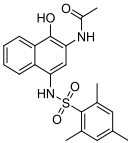
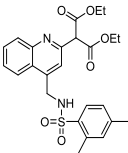
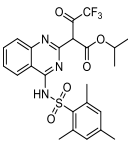
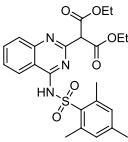
Compound	Structure	%inh (5 μ M)	%inh (50 μ M)
LH602		57.8	97.0
78		12.7	59.0
79		49.5	86.4
80		30.2	80.3
81		79.7	105.2
83		65.9	93.1

Table 11 (cont.). The inhibitory activities of LH602 analogs

Compound	Structure	%inh (5 μ M)	%inh (50 μ M)
87		9.6	13.9
93		3.9	4.8
96		-4.5	1.9
97		-6.0	-2.5

EXPERIMENTAL

General

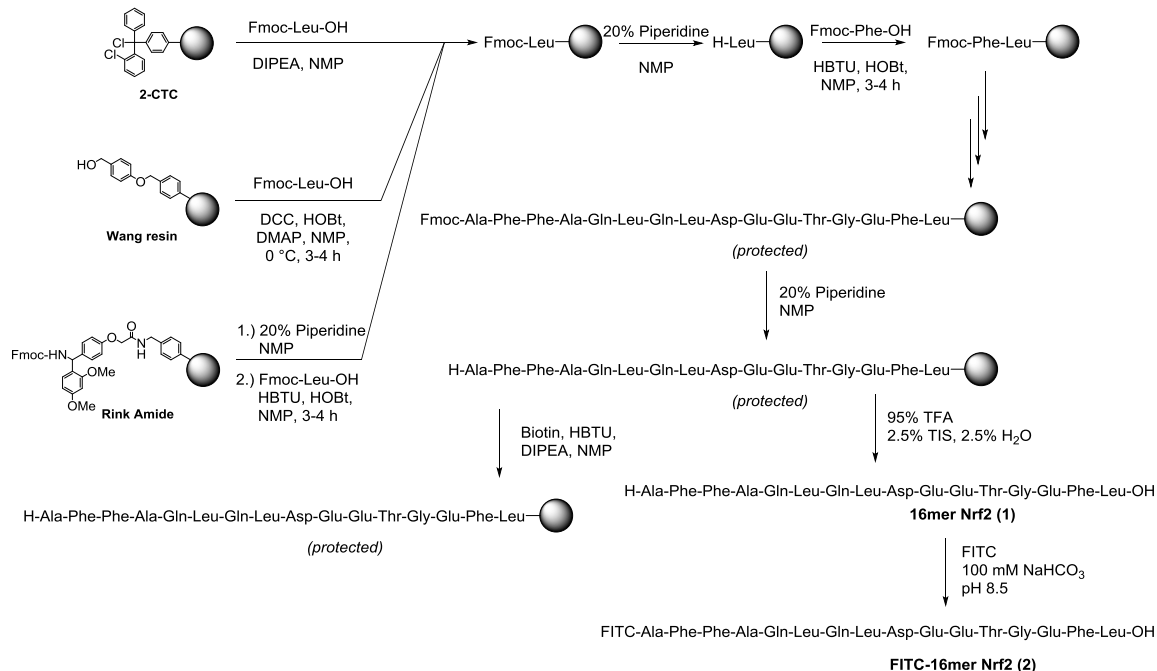
All reactions were performed in oven-dried glassware unless other stated. Syntheses of tris-NTAs and Ln chelates for TR-FRET were conducted using acid-washed glassware unless otherwise noted. Moisture- and air-sensitive reactions were carried out under argon or nitrogen. Dry solvents used for the reactions were obtained from commercial sources unless otherwise stated. Anhydrous acetonitrile was obtained by distillation from calcium hydride. Dichloromethane was obtained by either distillation from calcium hydride or purchased from commercial sources. All solvents used for reactions and purifications were HPLC grade unless otherwise noted. All starting materials and reagents that were commercially available are used directly without purification unless otherwise noted. Normal phase purifications were performed on Teledyne ISCO CombiFlash Companion using prepacked silica gel columns. Reversed-phase purifications were performed on Gilson preparative HPLC system or on Teledyne ISCO CombiFlash Companion using a prepacked C₁₈ column. All reactions were monitored by thin-layer chromatography (TLC) and on LC/MS systems (Shimadzu 2010, Finnigan LCQ Deca XP+, Finnigan LTQ). TLC plates were visualized using either an aqueous KMnO₄ solution, iodine, *p*-anisaldehyde, or sulfuric acid/methanol solution as necessary. LC/MS systems were equipped with a C₁₈ analytical column (50 × 4.6mm) with a flow rate of 1 mL/min using a gradient of acetonitrile in water containing 0.1% TFA as the mobile phase. Proton (¹H) and carbon (¹³C) NMR spectra were recorded on a Varian Gemini 2000 or Bruker Avance III spectrometer.

All aqueous solutions for assays were prepared using deionized water collected from a Millipore water purification system. The buffers were filtered through a 0.45 μm filter membrane upon preparation and degassed for 30 min prior to use. Fluorescein-5-isothiocyanate (FITC) and boron-dipyrromethene (BODIPY) FL C₅-NHS were purchased from Molecular Probes (Carlsbad, CA).

The cyanine dyes such as Cy3B NHS and Cy5 NHS were purchased from GE Healthcare (Piscataway, NJ). TbCl_3 and EuCl_3 were purchased from Sigma Aldrich. All FP, TRL, and TR-FRET assay data were recorded using Perkin Elmer Victor 3V plate reader equipped with filters appropriate for the label detected. Assays were performed using either a non-binding surface (NBS) black bottom polystyrene Corning 3650 96-well plate or Corning 3575 384-well plate unless otherwise noted. HTS FP assay of pilot libraries were performed using Perkin Elmer MultiProbe II HT EXpanded automated liquid handler equipped with low retention liquid sensing tips. All assay plates were centrifuged for 2 min at $370\times g$ prior to recording unless otherwise stated. The FP assay data were analyzed using Accelrys Pipeline Pilot.

I. Peptide Synthesis and Purification

A. Solid-phase peptide synthesis



Scheme 28. The solid-phase peptide synthesis of 16mer Nrf2 peptides showing the first attachment and subsequent conjugations of amino acids on a resin to assemble the target peptides.

All peptides were synthesized using solid-phase techniques on a Model 90 automated peptide synthesizer from Advanced ChemTech (now AAPPTec, Louisville, KY). Standard Fmoc chemistry was utilized for the synthesis of Nrf2 peptides with amino acid side chains protected by either Boc or *tert*-butyl esters where necessary. The series of Nrf2 peptides were synthesized on either Wang or 2-chlorotrityl chloride (2CTC) resin, while the Nrf2 peptide amides were synthesized on Rink Amide resin (Scheme 28). For the attachment of first amino acid to Wang resin, a solution of N^α -Fmoc-amino acid (3 eq. relative to resin substitution), DIC (3 eq.), DMAP (0.1 eq.), and DIPEA (4 eq.) in 10% NMP / DCM was transferred to a reaction vessel containing the pre-swollen resin. The reaction was shaken overnight under N_2 and the unreacted hydroxyl groups on the Wang resin were capped with acetic anhydride (2 eq. relative to resin substitution) in presence of pyridine (2 eq. relative to resin substitution). For the attachment of first amino acid to Rink-amide resin, the resin was first deprotected using 20% piperidine/NMP solution for 30 minutes and Fmoc-amino acid was loaded onto the resin using HBTU/HOBt coupling method. The attachment of the first amino acid was confirmed by negative ninhydrin test. For the attachment of first amino acid to 2CTC resin, a solution of Fmoc-amino acid (1 eq. relative to resin substitution) and DIPEA (1.2 eq. relative to amino acid) in NMP was added to the pre-swollen resin. The reaction was shaken for 3 h at room temperature under N_2 and the residual chloride groups on the 2CTC resin were deactivated by MeOH in the presence of pyridine (1 eq. relative to resin substitution) for 30 minutes.

The Fmoc protecting group was removed with 20% piperidine in NMP for 30 min at room temperature and the subsequent Fmoc-protected amino acids (3 eq. relative to resin loading) were coupled to the deprotected resin through HOBt-activated esters. The reaction was stirred with N_2 bubbling for 3 h at room temperature and the completion of the coupling reactions were confirmed by the ninhydrin test. After successful coupling of all amino acids, the assembled peptides were cleaved from the resin with a trifluoroacetic acid (TFA) solution containing 2.5%

triisopropylsilane (TIS) and 2.5% water. The acid-sensitive side chain protecting groups were removed simultaneously during the cleavage step. The crude peptide was obtained by concentrating the TFA solution with N₂ followed by precipitation with dropwise addition of diethyl ether at 0 °C. The precipitate was collected by centrifugation and washed 3 times with fresh ice-cold diethyl ether.

Peptides assembled on 2CTC resin were cleaved using 1% TFA in DCM when the side chain protecting groups were necessary for cyclization of cleaved peptides. Following the standard peptide synthesis protocol, TFA was neutralized with a solution of 10% pyridine in methanol upon cleavage of the protected peptide and the peptide solution was concentrated to remove volatile organic solvents. Then, crude protected peptide was obtained by triturating with ice cold water and the peptide was dried on lyophilizer to give the protected peptide which was used for the cyclization step without further purification.

B. Reversed-phase HPLC purification

All peptides were purified on a Gilson preparative HPLC system using a reversed-phase BDS-Hypersil C₁₈ column (150 × 20 mm). In general, the crude peptide sample obtained after cleavage from the resin was redissolved in 50% acetonitrile in water for injection into the HPLC system. An acetonitrile gradient containing 0.1% TFA was used to elute the peptides from the column with the exception of 16mer Nrf2 peptide (H-AFFAQLQLDEETGEFL-OH), which lacked sufficient solubility under the acidic conditions. For this peptide, an acetonitrile gradient containing 10 mM ammonium acetate pH 7.6 was used to elute the product from the column. The peptides were obtained at >95% purity and their identities confirmed by LC-MS and high-resolution mass spectrometry.

C. Peptide modification and labeling

Fluorescein-labeled peptides were prepared by modification of purified peptides with FITC according to the manufacturer's standard protocol. Briefly, the peptide (1 mg) was dissolved in 0.5 mL of 100 mM sodium carbonate buffer at pH 8.5 and FITC (2 eq.) was added to the solution. The reaction was stirred at room temperature for 12 h while protected from light. The progress of the labeling reaction was monitored by the disappearance of the starting peptide using LC-MS. A 1 N solution of sodium bisulfate (NaHSO_4) was added to reduce the pH of the reaction to 2 and the precipitated crude modified peptide was collected by centrifugation. The product was purified by reversed-phase HPLC as described above. For labeling of the 9mer Nrf2 peptide amide (H-LDEETGEFL-NH₂) with *N*-hydroxy succinimide (NHS) esters of BODIPY-FL, Cy3B and Cy5, the peptide was first dissolved in anhydrous DMSO containing 5 eq. of TEA, which was then followed by the addition of the NHS ester (1.2 eq.) in one portion. The reaction mixture was stirred for 12 h at room temperature protected from light. The reaction was acidified with formic acid before the mixture was diluted with water for reversed-phase HPLC purification.

The *N*-biotinyl 16mer Nrf2 peptide for the SPR binding assay was prepared on solid phase. Briefly, 2CTC resin-bound 16mer Nrf2 peptide (20.0 mg) was reacted with a 1 mL solution of biotin (12.4 mg, 5 eq.) pre-activated with HBTU (19.3 mg, 5 eq.) in NMP containing DIPEA (10.7 μL , 6 eq.). The biotinylated 16mer Nrf2 peptide was cleaved from the resin by a 3-h treatment of the resin-bound peptide with 95% TFA containing 2.5% TIS and 2.5% H₂O. The crude peptide obtained after ether precipitation was redissolved in 50% acetonitrile in 10 mM NH₄OAc and purified on a reversed-phase HPLC using the same conditions as in the purification of the free 16mer Nrf2 peptide as described earlier.

The *N*-acetylation of the 9mer Nrf2 peptide and 9mer Nrf2 peptide amide was performed on the resin-bound 9mer Nrf2 peptides. Briefly, the resin was pre-swollen in DCM for 30 min and

washed 3 times with NMP. The resin was treated with acetic anhydride (5 eq.) and pyridine (5 eq.) in NMP for 30 min and the capping reaction was monitored by ninhydrin test. The crude peptide was cleaved from the resin with trifluoroacetic acid (TFA) containing 2.5% triisopropylsilane (TIS) and 2.5% water as the scavengers. The peptide was purified on a reversed-phase HPLC using the conditions described earlier.

D. Peptide cyclization

Gln-cyclo[Cys-Asp-Glu-Glu-Thr-Gly-Glu-Cys]-Leu (2)



The linear peptide Gln-Cys-Asp-Glu-Glu-Thr-Gly-Glu-Cys-Leu (**1**) was assembled on Wang resin and cleaved from the resin using the standard TFA-mediated peptide cleavage procedure. A solution of the linear peptide **1** (11.3 mg, 0.010 mmol) was dissolved in 20 mL 0.1 M NaHCO₃ (pH 8.5) and 5 mL DMSO was added to the solution. The pH of the solution was adjusted to 7.5 and the reaction was stirred at rt open to the atmosphere for 1.5 days. The progress of the disulfide formation was monitored using Ellman test. The peptide solution was concentrated by lyophilization and the crude cyclic peptide was purified by RP-HPLC (Phenomenex Jupiter C₁₈ 250×10 mm) using 30-60% methanol in water containing 0.1%TFA as the mobile phase. The peptide solution was lyophilized to give 2.1 mg white powder in 28% yield. LC/MS (ESI-) *m/z*: 1122.4 [M-H]⁻. HRMS (ESI+): *m/z* calc'd for C₄₂H₆₅N₁₁O₂₁S₂: [M+H]⁺=1124.3871, found: 1124.3844.

cyclo[Gln-Leu-Asp-Glu-Glu-Thr-Gly-Glu-Phe-Leu] (5)

The linear protected peptide Gln-Leu-Asp-Glu-Glu-Thr-Gly-Glu-Phe-Leu (**3**) was assembled on 2CTC resin and cleaved from the resin by following standard 2CTC resin cleavage procedure using 1% TFA in DCM. To a solution of peptide **3** (57.6 mg, 0.0394 mmol) in 75 mL anhydrous DMF was added DIPEA (54.9 μ L, 0.315 mmol) and stirred for 5 min. Then, DPPA (35.4 μ L, 0.158 mmol) was added to the solution and the reaction was continued to stir at rt for 36 h. The reaction was quenched with water and concentrated under vacuum. The crude residue was dissolved in 3 mL DMF and triturated with water. The solid was collected by filtration and dried under vacuum to give 53.4 mg of cyclized protected peptide. The side chain protecting groups were deprotected by 4-h treatment of the cyclized peptide in 10 mL TFA solution containing 2.5% water and 2.5% triisopropyl silane (TIS). The TFA solution was concentrated with N₂ and the crude peptide was precipitated by a dropwise addition of cold ether. The crude peptide was dried to give 39.9 mg white solid, which was dissolved in 4 mL 30% ACN/water and subjected to RP-HPLC purification using 50% ACN in water (isocratic elution) as the mobile phase. The fraction containing the desired peptide was lyophilized to give 0.92 mg white powder in 2% yield. LC/MS (ESI-) *m/z*: 1161.4 [M-H]⁻. HRMS (ESI+) *m/z* calc'd for C₅₁H₇₅N₁₁O₂₀: [M+H]⁺=1162.5263, [M+2H]²⁺=581.7668, found: 581.7645.

cyclo[Lys-Leu-Asp-Glu-Glu-Thr-Gly-Glu-Phe-Leu] (8)

The linear protected peptide Lys-Leu-Asp-Glu-Glu-Thr-Gly-Glu-Phe-Leu (**6**) was assembled on 2CTC resin and cleaved from the resin using 1% TFA in DCM. To a solution of HOAt (3.73 mg, 0.0274 mmol) and DIPEA (28.6 mL, 0.164 mmol) in 3 mL anhydrous DMF were added a solution of peptide **6** (40 mg, 0.0274 mmol) and LiCl (23.2 mg, 0.55 mmol) in 9 mL anhydrous

DMF over the period of 24-h using a syringe pump. A solution of HATU in 3 mL anhydrous DMF was also added over the period of 24-h simultaneously using a syringe pump. The solution was concentrated and then the crude peptide precipitated with ice cold water. The peptide was lyophilized to give 10.9 mg white solid. The solid was dissolved in 1 mL TFA solution containing 5% water and 5% TIS and stirred at rt for 3 h. The TFA solution was concentrated with N₂ and the crude peptide precipitated by a dropwise addition of cold diethyl ether. The peptide was collected by centrifugation and purified by RP-HPLC (BDS-Hypersil C₁₈ 150×50mm) using 25% acetonitrile in water containing 0.1%TFA (isocratic elution) as the mobile phase. The product was lyophilized to give 1.36 mg white solid in 16% yield. LC/MS (ESI+) *m/z*: 1162.2 [M+H]⁺. HRMS (ESI+) *m/z* calc'd for C₅₂H₇₉N₁₁O₁₉: [M+H]⁺=1162.5627, [M+2H]²⁺=581.7850, found: 581.7800.

E. Peptide concentration determination

The concentrations of unlabeled Nrf2 peptide solutions were determined by amino acid analysis through acid hydrolysis and *o*-phthaldialdehyde (OPA)/*N*-Boc-L-cysteine (NBC) derivatization as described previously.¹³⁰ Briefly, 10 µL of peptide solutions were dried using a Savant Speedvac for 2 h at 45 °C and hydrolyzed by 6 N constant boiling HCl in a sealed hydrolysis vial under vacuum at 110 °C for 24 h with Cbz-L-phenylalanine as the control and L-tyrosine as the internal standard. After the hydrolysis reaction, the sample tubes were dried for 2 h at 45 °C using a Savant Speedvac prior to derivatization. The OPA/NBC derivatization solutions were prepared fresh by dissolving 11 mg of *o*-phthaldialdehyde (OPA) and 20 mg of *N*-Boc-L-cysteine (NBC) each in 1 mL of methanol. For derivatization, 10 µL of 1.0 mM L-tyrosine, 50 µL of 0.1 M Na₂B₂O₇ (pH 9.6), 50 µL deionized water, 20 µL OPA solution, and 20 µL NBC solution were added to each test tube containing the hydrolyzed peptide sample. The mixture was vortexed for 5 min at room temperature, then immediately analyzed by HPLC on a reversed-phase C₁₈ column

(Waters Symmetry, 150×4.6 mm, 4 μ m) using methanol and 10 mM phosphate buffer, pH 7.0 as the mobile phases at a flow rate of 0.5 mL/min. The elution started with an isocratic step of 30% methanol for 5 min, followed by a gradient from 30 to 60% methanol in 25 min. The concentration of the peptide was determined by calculating the ratio of the peak areas ($\lambda=340$ nm) of the OPA/NBC derivatized phenylalanine and the internal standard, L-tyrosine.

The concentrations of fluorescently labeled Nrf2 peptides were determined based on the absorbance of the fluorophores at their characteristic λ_{max} . Each of the fluorescently labeled Nrf2 peptides was dissolved in 10 mM HEPES buffer to give an estimated concentration of 1.0 mM. For fluorescein-labeled peptides, the UV absorbance of a diluted sample (50 – 100× dilution) was measured in triplicate at $\lambda_{\text{max}}=494$ nm ($\epsilon=68,000$ M⁻¹cm⁻¹). For BODIPY, Cy3B, and Cy5-labeled Nrf2 peptides, the UV absorbances were measured at 504 nm, 563 nm, and 646 nm, respectively; the concentrations were calculated using molar extinction coefficients of 87,000 M⁻¹cm⁻¹, 130,000 M⁻¹cm⁻¹, and 250,000 M⁻¹cm⁻¹, respectively. These diluted samples were used as stock solutions for the fluorescence polarization assays.

II. FP assay procedures

A. Anisotropy measurements

All FP-assays were performed on a Wallac Victor 3V multilabel counter/plate reader (Perkin Elmer, Shelton, CT) using the excitation and emission filters appropriate for each fluorophore used in the binding experiment. The plates used for the FP measurements were the black NBS (nonbinding surface) Corning 3650 96-well or Corning 3575 384-well plates, loaded with 80 or 40 μ L of assay solution per well. For fluorescein and BODIPY, 485 nm excitation and 535 nm emission filters were used. For Cy3B, excitation and emission filters of 560 nm and 650 nm were

used, respectively. For Cy5, 579 nm excitation and 670 nm emission filters were used. The buffer used for the FP assays was 10 mM HEPES pH 7.4 buffer containing 50 mM EDTA, 150 mM NaCl, and 0.005% Tween-20. All aqueous solutions were prepared using deionized water collected from a Millipore water purification system.

Fluorescence polarization was determined by measuring the parallel and perpendicular fluorescence intensity ($F_{\parallel}$ and $F_{\perp}$) with respect to the linearly polarized excitation light. As previously discussed, the fluorescence polarization (P) and the anisotropy (A) values are expressed by equations (1) and (2), respectively, and the anisotropy A is related to the P value by equation (3).

$$P = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + F_{\perp}} \quad (1)$$

$$A = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + 2F_{\perp}} \quad (2)$$

$$A = \frac{2P}{3 - P} \quad (3)$$

In the quantitative analysis of the polarization signal, anisotropy values were used for arithmetical simplicity. In addition, total fluorescence ($F_{\parallel} + 2F_{\perp}$) was calculated for each well to ensure that the fluorescence intensity was consistent with the amount of fluorescent probes used across all assay wells.

B. Measurement of binding of fluorescent peptide probes to Keap1 Kelch domain

The binding of the fluorescently labeled Nrf2 peptides to Keap1 Kelch domain were determined by dose titration of the fluorescent peptide probes with varying concentrations of Keap1 Kelch

domain protein. The maximum anisotropy achieved at a high concentration of Keap1 Kelch domain protein defines the upper end of the dynamic range for the peptide probe and the total fluorescence ($F_{\parallel} + 2F_{\perp}$) at the high concentration of Keap1 Kelch domain protein can be used to calculate the Q-value, the quantum yield ratio of the bound and the free fluorescent peptide probe. Equation (13) is used to calculate f_b , the fraction of the bound probe, at a given concentration of Keap1 Kelch domain protein:

$$f_b = \frac{A_{obs} - A_{free}}{(A_{bound} - A_{obs})Q + (A_{obs} - A_{free})} \quad (13)$$

Where A_{obs} , A_{bound} , A_{free} refer to the observed anisotropy at the given concentration of Keap1 Kelch domain protein, the anisotropy of probe when it is bound to Keap1 Kelch domain protein, and the anisotropy of the free probe, respectively. The f_b data obtained can be fitted to the quadratic equation (14) to derive K_d using SigmaPlot (SPSS, USA):

$$f_b = \frac{K_d + L_t + R_t - \sqrt{(K_d + L_t + R_t)^2 - 4L_tR_t}}{2L_t} \quad (14)$$

Where L_t is the concentration of total peptide probe used, which was fixed at 10 nM, and R_t is the concentration of Keap1 Kelch domain protein, which varied between 0 and 2 μ M.

C. FP competition assays to determine inhibitory potency of Keap1-Nrf2 interaction

A competition assay using the conditions described above was established and miniaturized to 384-well plate format to determine the potency of inhibitors of Keap1-Nrf2 interaction. A Packard Bioscience (now Perkin Elmer) Multiprobe II HT EXpanded liquid handling robot was

used to deliver small volumes of solutions to each of the 384 wells. Each well had a final volume of 40 μL that consisted of 10 μL of 40 nM FITC-9mer Nrf2 peptide amide and 10 μL of 400 nM Keap1 Kelch domain protein, 10 μL of HEPES buffer, and 10 μL of an inhibitor sample of varying concentrations. The binding experiments were performed in triplicates with initial concentration of the inhibitor typically set between 10 μM and 100 μM depending on the inhibitor potency and serially diluted 2-fold to give a concentration range down to 0.5 nM or 5 nM. The plate was centrifuged at 370 $\times g$ for 2 min to get rid of any air bubbles in the assay solution and to ensure thorough mixing. The plate was covered and allowed to equilibrate for 30 min at room temperature. The plate was centrifuged again prior to fluorescence polarization measurements.

The % inhibition of the competitor at each concentration point were determined by using equation (15) and the IC_{50} of an inhibitor was determined from the plot of %inhibition against inhibitor concentration using equation (16).

$$\%inhibition = \frac{A_{top} - A_{obs}}{A_{top} - A_{bottom}} \times 100\% \quad (15)$$

$$A = A_{bottom} + \frac{A_{top} - A_{bottom}}{1 + 10^{n(\log IC_{50} - x)}} \quad (16)$$

IC_{50} is the concentration of an inhibitor needed to inhibit 50% binding of the fluorescently labeled peptide probe, x is the log of the inhibitor concentration, and n is the Hill slope that describes the steepness of the curve. The values of A_{top} , A_{bottom} , and A_{obs} in the equations refer to the anisotropy of the wells containing Keap1 and the probe, the anisotropy of the free probe, and the observed anisotropy for the wells containing the inhibitors at a range of concentrations under the assay conditions. The IC_{50} values determined were used to derive K_i of the inhibitors using equation (10) with f_b set at 0.75.⁹⁷

$$IC_{50} = \left(\frac{f_b - K_d}{(1 - f_b)(2 - f_b)} + \frac{f_b \times L_0}{2} \right) \left(\frac{K_i(2 - f_b)}{K_d \times f_b} + 1 \right) \quad (10)$$

D. The effect of DMSO on anisotropy measurement

The effect of DMSO on our FP assay was determined by measuring the anisotropy change of the Keap1-bound and free fluorescent probe in presence of DMSO in the assay solutions at concentrations up to 20%. Briefly, 20 μ L of the assay buffer containing varying concentrations of DMSO and 10 μ L of 40 nM FITC-9mer Nrf2 peptide amide was added to a Corning 3575 384-well plate. Then, either 10 μ L of 400 nM Keap1 Kelch domain protein or 10 μ L of buffer were added to each well and the plate was centrifuged for 2 min at 370 $\times$ g. The plate was incubated for 30 min in the dark at room temperature prior to the anisotropy measurement.

E. The effect of DMSO on anisotropy measurement

For the measurement of Z' -factor, a 384-well plate was prepared with 10 nM FITC-9mer Nrf2 amide and 100 nM Keap1 Kelch domain in 10 mM HEPES buffer in the presence or absence of 1 μ M of *N*-acetyl 9mer Nrf2 amide (44 replicates each). From the anisotropy of 10 nM FITC-9mer Nrf2 peptide amide in the presence of 100 nM Keap1 Kelch domain (~75% bound, as the negative control) and the anisotropy of 10 nM FITC-9mer Nrf2 peptide amide in the presence of 100 nM Keap1 Kelch domain and 1 μ M *N*-acetyl-9mer-Nrf2 peptide amide (~80% inhibition, as the positive control), the Z' -factor was calculated by the equation:

$$Z' = 1 - \frac{3\sigma_{c1} + 3\sigma_{c2}}{|\mu_{c1} - \mu_{c2}|} \quad (17)$$

where μ_{c1} and μ_{c2} are the mean values of the positive control and negative control wells, respectively, and σ_{c1} and σ_{c2} are the standard deviation of the positive and negative control wells, respectively. When comparing the different fluorescent probes, only the top and bottom replicates were used to derive the Z' -factor in absence of the peptide inhibitor.

F. SPR Competition Binding Assay

The SPR competition binding assay was carried out on a Biacore 3000 biosensor (GE Healthcare, Piscataway, New Jersey) using the immobilized *N*-biotinylated 16mer Nrf2 peptide as the ligand and Keap1 Kelch domain as the analyte.^{90,93} Briefly, the surface of a CM5 chip was first activated using the standard amine coupling method.¹⁶⁶ Equal volumes of 0.1 M NHS and 0.4 M EDC were mixed and injected into flow cell (Fc) 1 and 2 at a flow rate of 10 μ L/min for 7 min, followed by the injection of streptavidin (200 μ g/mL) for 7 min. Finally, ethanolamine (0.1 M) was injected through the flow cell to deactivate the remaining NHS esters on the chip surface. Around 7000 RU of streptavidin was immobilized on each flow cell. A solution of *N*-biotinyl 16mer Nrf2 peptide was diluted to 10 nM using 10 mM HEPES buffer, pH 7.4 containing 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween-20 (HBS running buffer) and injected through Fc2 at a flow rate of 10 μ L/min. The streptavidin surface was slowly saturated by the biotin-labeled Nrf2 peptide and the maximum immobilization level of ~300 RU was finally achieved. Fc1 of the chip without the Nrf2 peptide was used as the blank surface.

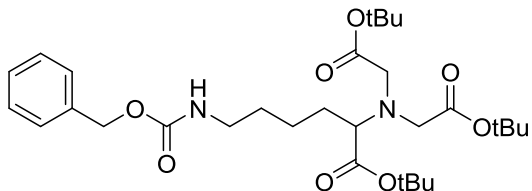
All interactions between Keap1 Kelch domain and the immobilized biotin-16mer Nrf2 peptide were carried out with a 1-min association time and a 3-min dissociation time at a flow rate of 30 μ L/min. The sensor chip surfaces were regenerated with a 0.5-min injection of 1M NaCl at a flow rate of 30 μ L/min. The regeneration step was followed by two buffer washes of IFC and the needle. The data analysis was performed using BIAevaluation software v4.1 by measuring the slope of the initial association phase from the SPR sensograms after double subtraction of

responses from the reference surface and the zero blank in the absence of Keap1 Kelch domain. To calculate the concentrations of free (i.e. unbound) Keap1 Kelch domain, a standard curve was constructed using serially diluted solutions of Keap1 Kelch domain with concentrations covering the observed Keap1 Kelch domain concentrations in the competition assay. For competition binding assay, a solution of Keap1 Kelch domain at a fixed concentration of 20 nM or 40 nM and various concentrations of inhibitory Nrf2 peptides ranging from 0 nM to 1 μ M or LH602 analogs at either 5 μ M or 50 μ M concentration was introduced over each surface (Fc1 and Fc2). The slopes of initial association phase obtained from sensograms were used to calculate the concentrations of free Keap1 Kelch domain and the fraction of Keap1 Kelch domain (f_b) bound to the inhibitory peptides. The f_b data obtained was fitted to the quadratic equation (14) to derive K_d using, where R_t is the concentration of Keap1 kelch domain used, which was fixed at 20 nM or 40 nM, and L_t is the concentration of each inhibitory Nrf2 peptide.

III. Synthesis of Reagents for TR-FRET Assay Development

Di-tert-butyl2,2'-((6-(((benzyloxy)carbonyl)amino)-1-(tert-butoxy)-1-oxohexan-2-yl)azanediyl)diacetate

Lys(Cbz)-NTA(OtBu)₃ (9)

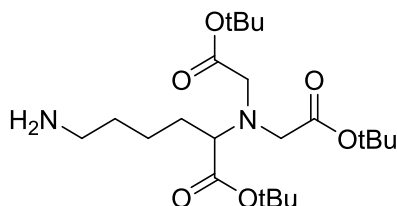


Lys(Cbz)-NTA(OtBu)₃ (**9**) was synthesized as previously described in the literature.¹²³ Briefly, t-butyl bromoacetate (887 μ L, 6 mmol) was added to a solution of H-Lys(Cbz)-OtBu (558 mg, 1.5 mmol) and DIPEA (1.57 mL, 9 mmol) dissolved in 8 mL of anhydrous DMF. The reaction was

heated to 55°C and stirred for 12 h under N₂. The reaction mixture was concentrated under vacuum and the residue was washed 3 times with 15 mL of 30% EtOAc/hexane. The organic fractions were concentrated and purified by silica gel flash column chromatography. The product was eluted with a 0-25% gradient of EtOAc/hexane in 25 min at a flow rate of 35 mL/min. The product was dried under vacuum to give 700 mg as a clear oil (83%). ¹H NMR (CDCl₃, 400 MHz): δ 7.26 (m, 5H), 5.06 (s, 2H), 3.42 (ABq, 4H, Δδ_{AB} = 0.04, J = 17.3), 3.27 (t, 1H, J = 7.4), 3.17 (m, 2H), 1.61 (m, 2H), 1.51 (m, 4H), 1.43 (s, 9H), 1.39 (s, 18H). ¹³C NMR (CDCl₃, 100 MHz): δ 172.37, 170.68, 156.46, 136.82, 128.42, 128.05, 127.92, 81.07, 80.66, 66.43, 65.14, 53.94, 40.83, 30.12, 28.22, 28.10, 23.01. LC/MS (ESI+) *m/z*: 565.6 [M+H]⁺, 587.7 [M+Na]⁺.

Di-tert-butyl 2,2'-((6-amino-1-(tert-butoxy)-1-oxohexan-2-yl)azanediyl)diacetate

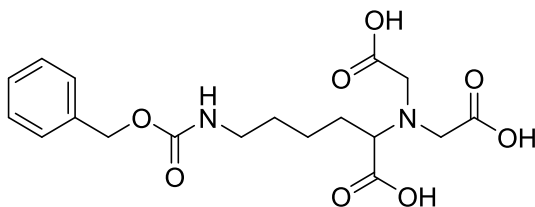
Lys-NTA(OtBu)₃ (10)



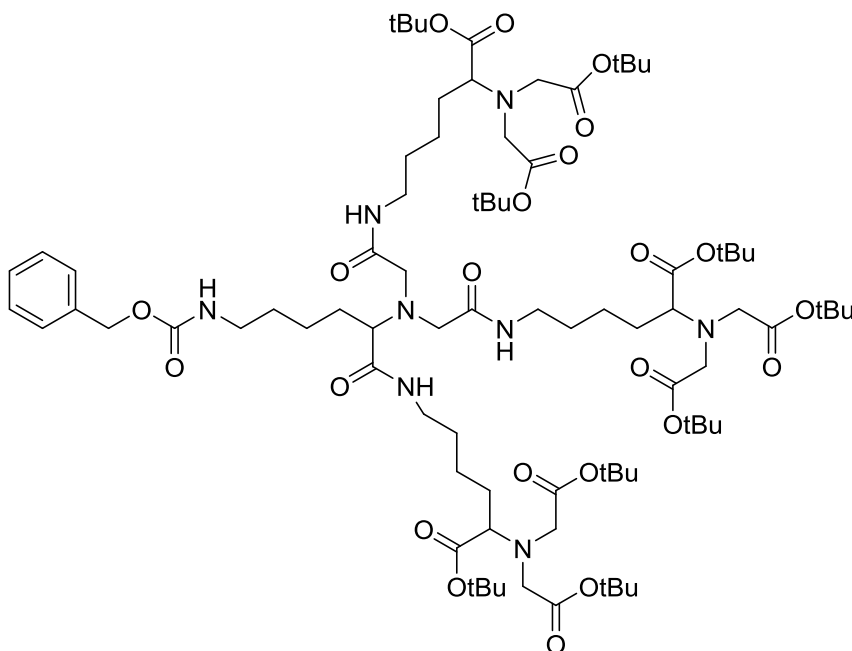
Lys-NTA(OtBu)₃ (**10**) was synthesized as previously described in the literature.¹²³ Lys(Cbz)-NTA(OtBu)₃ (**9**, 450 mg, 0.80 mmol) was dissolved in 10 mL of MeOH and deaerated with N₂. A catalytic amount of 20% Pd(OH)₂/C (45 mg) was added to the solution in one portion and the reaction mixture was purged with H₂. The reaction was stirred for 1 h under H₂ and filtered through a pad of celite upon completion of the reaction. The filtrate was concentrated under vacuum to give 316 mg of the product as a clear oil in 93% yield. ¹H NMR (MeOH-d₄, 400 MHz): δ 3.22 (ABq, 4H, Δδ_{AB} = 0.04, J = 17.4), 3.07 (t, 1H, J = 7.3), 2.44 (t, 2H, J = 6), 1.40 (m, 2H), 1.30 (m, 4H), 1.22 (s, 9H), 1.20 (s, 18H). ¹³C NMR (CDCl₃, 100 MHz): δ 172.45, 170.68, 80.97, 80.58, 65.41, 53.78, 42.07, 38.76, 30.60, 28.14, 23.77, 23.28, 22.97. LC/MS (ESI+) *m/z*: 431.6[M+H]⁺, 453.5[M+Na]⁺, 375.4[(M-tBu)+H]⁺, 319.4[(M-2tBu)+H]⁺, 263.3[(M-3tBu)+H]⁺.

2,2'-((5-(((benzyloxy)carbonyl)amino)-1-carboxypentyl)azanediyl)diacetic acid

Lys(Cbz)-NTA (11)

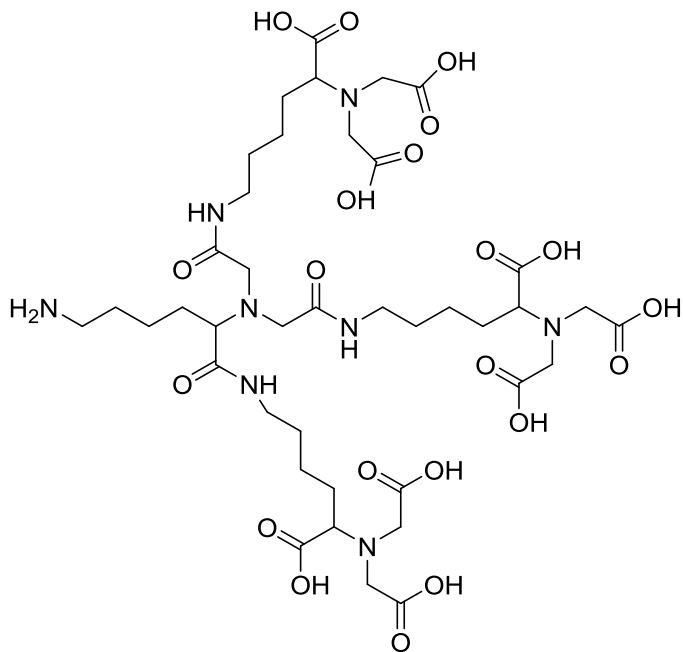


Lys(Cbz)-NTA (**11**) was synthesized as previously described in the literature.¹²³ To a solution of **9** (200 mg, 0.354 mmol) in 2 mL DCM was added 100 μ L triisopropylsilane (TIS) and stirred briefly. Then, 2 mL TFA was slowly added to the solution and stirred for 5 h at rt. The reaction was concentrated under reduced pressure and the product triturated with cold ether. The product was washed with cold ether and dried under vacuum to give 126 mg white solid in 90% yield. ¹H NMR (MeOH-d₄, 400 MHz): δ 7.37 (m, 5H), 5.08 (s, 2H), 3.78 (ABq, 4H, $\Delta\delta_{AB}$ = 0.04, J = 16), 3.62 (t, 1H, J = 7), 3.15 (t, J=7, 2H), 1.84 (m, 1H), 1.73 (m, 1H), 1.48-1.58 (m, 4H). ¹³C NMR (CDCl₃, 100 MHz): δ 175.09, 174.84, 158.99, 138.50, 129.46, 129.06, 128.94, 128.78, 67.37, 66.94, 55.26, 41.41, 30.52, 30.28, 24.61. LC/MS (ESI+) *m/z*: 397.2 [M+H]⁺, 419.2 [M+Na]⁺.

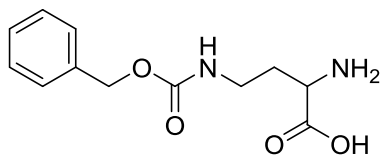
Lys(Cbz)-tris-Lys-NTA(OtBu)₃ (12)

To a solution of **11** (50 mg, 0.126 mmol) in anhydrous DMF under Ar were added DIPEA (300 μ L, 1.72 mmol) and PyBOP (330 mg, 0.634 mmol). Then a solution of **10** in 8 mL anhydrous DCM was added in one portion and the reaction was stirred overnight at rt. The reaction mixture was diluted with 20 mL DCM and washed with water, followed by brine. The organic layer containing the product was dried over Na₂SO₄ and concentrated under reduced pressure. The residue was loaded onto a 24 g silica gel column and purified by flash column chromatography (0 to 10% MeOH in DCM containing 0.1% TEA) to give the product as 106 mg clear oil (51.4%).

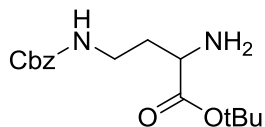
¹H NMR (MeOH-d₄, 400 MHz): δ 7.32 (m, 5H), 5.06 (s, 2H), 3.42 (m, 16H), 3.25 (m, 14H), 1.63 (m, 8H), 1.49 (m, 16H), 1.44 (s, 27H), 1.43 (s, 54H).

Lys-tris-Lys-NTA (13)

Compound **12** (12 mg, 7.34 μmol) was dissolved in 2 mL methanol containing 5 μL formic acid (132 μmol) and catalytic amount of 10% Pd/C was added in one portion. The reaction was stirred for 3 h at rt. Pd/C was removed by filtration through a celite bed and the filtrate was concentrated to oil under vacuum. Then, the oil was treated with 95% TFA containing 2.5% TIS and 2.5% H_2O for 2 h at rt. The volatiles were removed by N_2 and concentrated to approximately 20% volume. The product was triturated with cold ether and the crude product was subjected to purification by RP-HPLC (Phenomenex Jupiter C18, 21 $\times$ 250 mm) with water and acetonitrile containing 0.1% TFA as the mobile phase to give 6 mg (81%) of the desired product **13**. LC/MS (ESI+) m/z : 995.1 $[\text{M}+\text{H}]^+$, 1017.2 $[\text{M}+\text{Na}]^+$.

2-amino-4-(((benzyloxy)carbonyl)amino)butanoic acid**H-Dab(Cbz)-OH (21)**

To a 1.0N NaOH (52.4 mmol) solution containing 4.32 g K₂CO₃ (26.2 mmol) and 3.28 g CuSO₄ (13.1 mmol) was added 2,4-diaminobutyric acid (5.0g, 26.2 mmol). Then the solution was cooled to 0 °C and benzylchloroformate (4.83 mL, 34.1 mmol) in 5 mL anhydrous DCM was added dropwise while stirring vigorously. The reaction was kept at 0 °C for 60 min, then slowly warmed to rt and continued to stir for 2 h. The solid was filtered and washed with 5 mL ice water (×2), cold methanol (×2), and again by ice water. The product was suspended in 50 mL deionized water with 4.87 g di-sodium EDTA (Titriplex, 13.1 mmol) and the mixture was brought to reflux. The solution was allowed to slowly cool to 4 °C and then filtered to collect the solid. The solid was washed with ice cold water (×2) and cold methanol (×2) to give 1.85 g crude solid. The product was recrystallized from H₂O to give 1.28 g white powder (19.5%) ¹H NMR (DMSO-d₆, 400 MHz): δ 7.35 (m, 5H), 5.01(s, 2H), 3.14 (m, 3H), 1.87 (m, 1H), 1.70(m, 1H). ¹³C NMR (DMSO-d₆, 100 MHz) 169.44, 137.23, 128.31, 127.70, 99.45, 65.14, 52.36, 37.94, 32.18. LC/MS (ESI⁺): 253.3 [M+H]⁺.

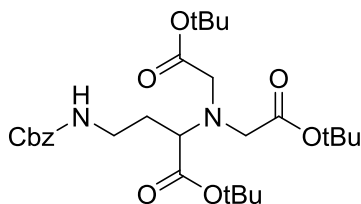
Tert-butyl 2-amino-4-(((benzyloxy)carbonyl)amino)butanoate**H-Dab(Cbz)-OtBu (22)**

A solution of H-Dab(Cbz)-OH (**21**, 600 mg, 2.38 mmol) in 25 mL t-butyl acetate was cooled to 0 °C and 286 μL perchloric acid (4.76 mmol) was added dropwise while stirring vigorously. The

reaction was left on ice for 30 min and then allowed to warm to rt. Reaction was left for overnight and then neutralized with 1N NaOH. The product was extracted three times with 25 mL EtOAc and the organic fractions were pooled and dried over Na₂SO₄. Single product on LC/MS and TLC (R_f = 0.5, 10% MeOH/DCM) and no purification was necessary. The product was dried under vacuum to give 559 mg white solid in 76.3% yield. ¹H NMR (CDCl₃, 400 MHz): δ 7.27 (m, 5H), 5.02 (s, 2H), 3.38 (m, 1H), 3.25 (m, 2H), 1.85 (m, 1H), 1.56(m, 1H). ¹³C NMR (CDCl₃, 100 MHz): 174.85, 156.39, 136.69, 128.49, 128.10, 128.05, 81.39, 65.59, 53.89, 38.83, 34.1 28.02. LC/MS (ESI+): 309.6 [M+H]⁺, 331.6 [M+Na]⁺

Di-tert-butyl 2,2'-((4-(((benzyloxy)carbonyl)amino)-1-(tert-butoxy)-1-oxobutan-2-yl)azanediyl)diacetate

Dab(Cbz)-NTA(OtBu)₃ (23)

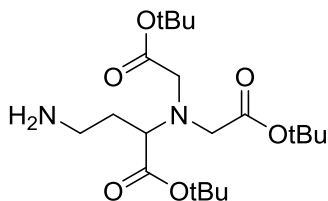


A solution of H-Dab(Cbz)-OtBu (**22**, 1.18 g, 3.84 mmol) in anhydrous DMF under Ar was added DIPEA (3.42 mL, 19.2 mmol) and t-butyl bromoacetate (2.27 mL, 15.4 mmol). The reaction was heated to 55 °C and left to stir overnight. DMF was removed under vacuum and the residue dissolved in 40 mL EtOAc. The organic layer was washed with 15 mL water (×3) and dried over Na₂SO₄. The solution was concentrated under vacuum and purified by a flash column chromatography using 0 - 30% EtOAc in Hexane. The product eluted at 20% EtOAc/Hexane as a broad peak. The fractions were combined and dried under vacuum to give 1.92 g oil in 93.3% yield. ¹H NMR (CDCl₃, 400 MHz): δ 7.23 (m, 5H), 5.01 (s, 2H), 3.39-3.19 (m, 7H), 1.85 (m, 1H), 1.58(m, 1H), 1.34 (s, 9H), 1.33 (s, 18H). ¹³C NMR (CDCl₃, 100 MHz): 171.82, 170.75, 156.90,

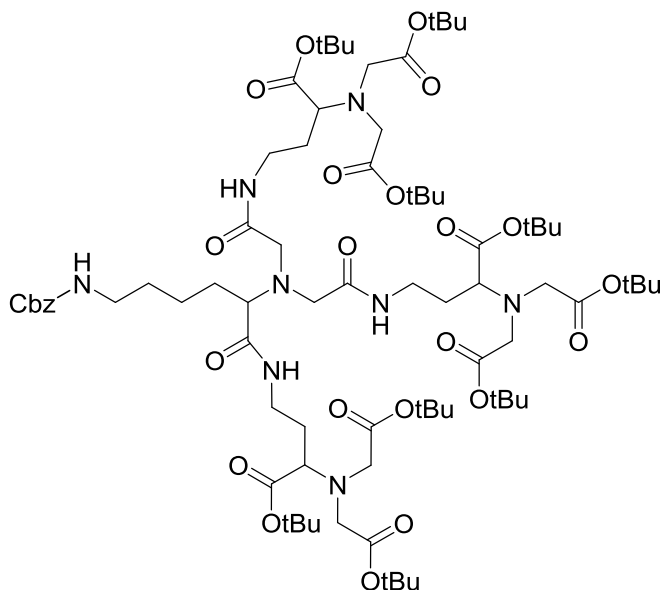
137.26, 128.26, 127.57, 81.50, 81.23, 65.98, 62.40, 53.81, 37.31, 29.30, 28.22, 28.05. LC/MS (ESI+): 537.6 [M+H]⁺, 559.6 [M+Na]⁺.

Di-tert-butyl 2,2'-((4-amino-1-(tert-butoxy)-1-oxobutan-2-yl)azanediyl)diacetate

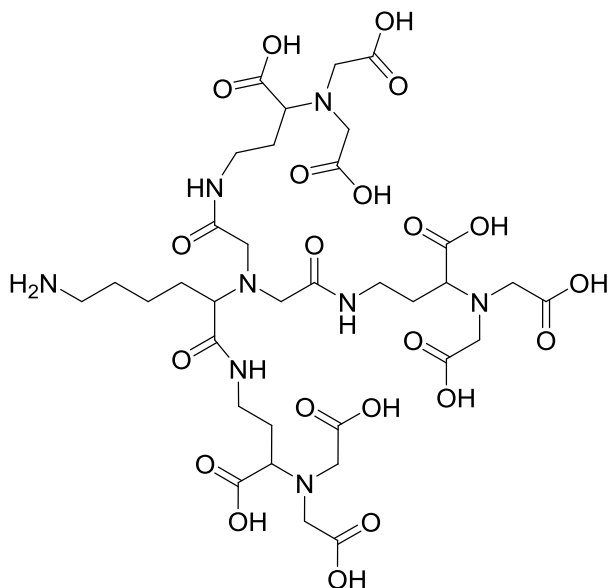
Dab-NTA(OtBu)₃ (58)



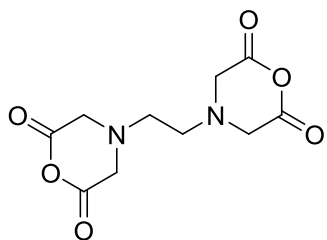
Dab(Cbz)-NTA(OtBu)₃ (**23**, 500 mg, 0.93 mmol) was dissolved in 10 mL of MeOH and deaerated with N₂. A catalytic amount of 20% Pd(OH)₂/C (50 mg) was added to the solution in one portion and the reaction mixture was purged with H₂. The reaction was stirred for 2 h under H₂ and filtered through a celite bed upon completion of the reaction. The filtrate was concentrated under vacuum to give 357 mg of the product as a clear oil in 95% yield. ¹H NMR (CDCl₃, 400 MHz): δ 3.52 (m, 1H), 3.47 (s, 4H), 2.92 (m, 1H), 2.83 (m, 1H), 1.79 (m, 2H), 1.62 (br, 2H), 1.48 (s, 9H), 1.47 (s, 18H). ¹³C NMR (CDCl₃, 100 MHz): δ 172.42, 170.59, 81.14, 80.75, 62.63, 53.89, 38.86, 33.68, 28.23, 28.11. LC/MS (ESI+): 403.7 [M+H]⁺.

Lys(Cbz)-Dab-tris-NTA(OtBu)₃ (25)

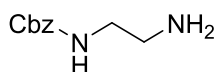
Lys(Cbz)-NTA (**11**, 86.0 mg, 0.217 mmol), Dab-NTA(OtBu)₃ (**24**, 350 mg, 0.868 mmol), PyBOP (452 mg, 0.868 mmol), DMAP (8.0 mg, 0.065 mmol), and DIPEA (0.31 mL, 1.74 mmol) were dissolved in 3 mL anhydrous DMF and the reaction stirred overnight under Ar. Then, solvent was removed under vacuum and the residue resuspended in 40 mL EtOAc. The organic layer was washed with 15 mL saturated NaHCO₃ solution (×3) and concentrated for purification on a silica gel flash column using 0 - 10% MeOH in DCM containing 0.1% TEA as the mobile phase. The fractions containing the product were combined and dried under vacuum to give 276 mg oil (81.4%). *Tert*-butyl esters were deprotected and the m/z of $[M+H]^+ = 1044.9$ corresponding to the free acid form was detected on a LC/MS system. ¹H NMR (CDCl₃, 400 MHz): δ 7.35 (m, 5H), 5.08 (s, 2H), 3.45 (s, 12H), 3.41-3.31 (m, 16H), 1.92 (m, 6H), 1.74 (m, 6H), 1.48-1.41 (m, 81H).

Lys-Dab-tris-NTA (26)

- (1) A solution of Lys(Cbz)-Dab-tris-NTA(OtBu)₃ (**25**, 20.0 mg 0.0128 mmol) in 5 mL methanol was deaerated with N₂. Then, catalytic amount of 20% Pd(OH)₂/C was added in one portion and the reaction vessel purged with H₂. The reaction was stirred vigorously for 2 h and filtered through a bed of celite to remove the catalyst. The filtrate was concentrated under vacuum to give 18.3 mg clear oil in 100% yield. LC/MS (ESI+) calc'd for C₇₀H₁₂₆N₈O₂₁: found 1439.77 [M+Na]⁺
- (2) A solution of product from step (1) was dissolved in 1 mL TFA containing 5% deionized water and vortexed vigorously for 2 h. The reaction was concentrated by N₂ and the product triturated with ice cold ether. The precipitate was washed 2 times with ice cold ether and then dissolved in 30% acetonitrile in deionized water for purification by RP-HPLC using 5-50% acetonitrile in water containing 0.1% TFA as the mobile phase. The peak corresponding to the product was collected and lyophilized to give 5 mg white solid (43%), which immediately absorbed water after removal from vacuum to become sticky solid. LC/MS (ESI+) *m/z*: 911.0 [M+H]⁺. HRMS (ESI+): *m/z* calc'd for C₃₄H₅₄N₈O₂₁: [M+H]⁺=911.3477, [M+Na]⁺=933.3296, [M+2H]²⁺=456.1775, found 911.3503, 933.3325, 456.1784

4,4'-(ethane-1,2-diyl)bis(morpholine-2,6-dione)**EDTA bisanhydride (27)**

EDTA bisanhydride was synthesized as previously described.¹⁶⁷ Briefly, EDTA (0.5 g, 1.7 mmol) was suspended in 2 mL of anhydrous pyridine under Ar. To this suspension was added 2 mL of acetic anhydride (2 mL, 17 mmol). After stirring at 65 °C for 24 hr, the reaction mixture was slowly cooled to rt. The suspension was filtered with N₂ and the crude solid was rinsed 5 times with 5 mL of Ac₂O and 5 times with 5 mL of diethyl ether. The solid was collected and dried under vacuum to give 800 mg of EDTA bisanhydride (**27**) as white powder in 91% yield. ¹H-NMR (DMSO-*d*₆): δ 3.71 (s, 8H), 2.68 (s, 4H). ¹³C-NMR (DMSO-*d*₆): δ 165.7, 52.3, 51.2.

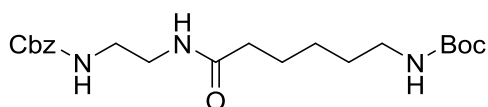
Benzyl (2-aminoethyl)carbamate**Cbz-EDA (28)**

A solution of ethylenediamine (6.67 mL, 100 mmol) in 120 mL of anhydrous DCM was cooled to 0 °C under Ar and a solution of benzylchloroformate (1.4 mL, 10 mmol) in 50 mL of anhydrous DCM was added at a rate of 10 mL/h. The reaction was allowed to slowly warm up to rt and then stirred overnight. The reaction mixture was filtered to remove precipitates and the filtrate concentrated under reduced pressure. The crude product was suspended in 50 mL of 2 N NaOH and extracted 4 times with 25 mL of DCM. The organic fractions were combined and dried over Na₂SO₄ and the volatiles were removed to give a crude oil. The crude oil was subjected to purification on a flash column with 0 to 15% gradient of MeOH/DCM in 16 min at a flow rate of

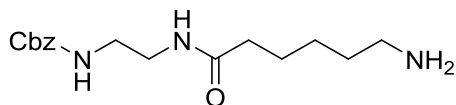
40 mL/min to give the product as 1.08 g of Cbz-EDA (**28**) as a waxy solid in 56% yield. ^1H NMR (DMSO- d_6 , 400 MHz): δ 7.40-7.30 (m, 5H), 7.22 (s, 1H), 5.02 (s, 2H), 3.00 (q, 2H, $J = 8.0$), 2.57 (t, 2H, $J = 8.0$), 1.76 (br, 2H). ^{13}C NMR (DMSO- d_6 , 100 MHz): δ 156.2, 137.3, 128.3, 127.7, 65.1, 44.1, 41.5. LC/MS (ESI+) m/z : 195.2 $[\text{M}+\text{H}]^+$, 217.2 $[\text{M}+\text{Na}]^+$.

Benzyl (2-(6-((tert-butoxycarbonyl)amino)hexanamido)ethyl)carbamate

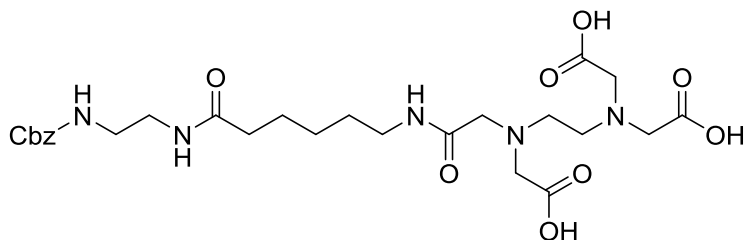
Cbz-EDA-rAcp-Boc (29)



Boc-Acp-OSu (**35**, 987 mg, 3.0 mmol) was added to a solution of Cbz-EDA (**28**, 700 mg, 3.6 mmol) in 10 mL of anhydrous DMF containing 2.1 mL of DIPEA (2.09 mL, 12 mmol). The reaction was stirred overnight under Ar and the reaction mixture was concentrated under vacuum. The crude residue was re-dissolved in 50 mL of DCM and the organic phase was extracted 3×20 mL water, followed by a wash with 10 mL of brine. The organic phase was dried over anhydrous Na_2SO_4 and purified on a flash column using 0-15% gradient of MeOH/DCM in 16 min with a flow rate of 40 mL/min. The product was dried under vacuum to give 1.08 g of Cbz-EDA-rAcp-Boc (**29**) as a clear oil in 89% yield. ^1H NMR (CDCl_3 , 400 MHz): δ 7.26 (br, 5H), 6.18 (s, 1H), 5.37 (s, 1H), 5.02 (s, 2H), 4.54 (s, 1H), 3.26 (m, 4H), 3.01 (m, 2H), 2.06 (t, 2H, $J = 6.7$), 1.53 (m, 2H), 1.36 (s, 9H), 1.24 (m, 4H). ^{13}C NMR (CDCl_3 , 100 MHz): δ 173.73, 157.26, 156.08, 136.43, 128.52, 128.40, 128.16, 128.05, 79.09, 66.81, 41.05, 40.34, 40.16, 36.35, 29.71, 28.42, 26.28, 25.14. LC/MS (ESI+) m/z : 308.4 $[(\text{M}-\text{Boc})+\text{H}]^+$, 408.4 $[\text{M}+\text{H}]^+$, 430.4 $[\text{M}+\text{Na}]^+$.

Benzyl (2-(6-aminohexanamido)ethyl)carbamate**Cbz-EDA-rAcp-H (64)**

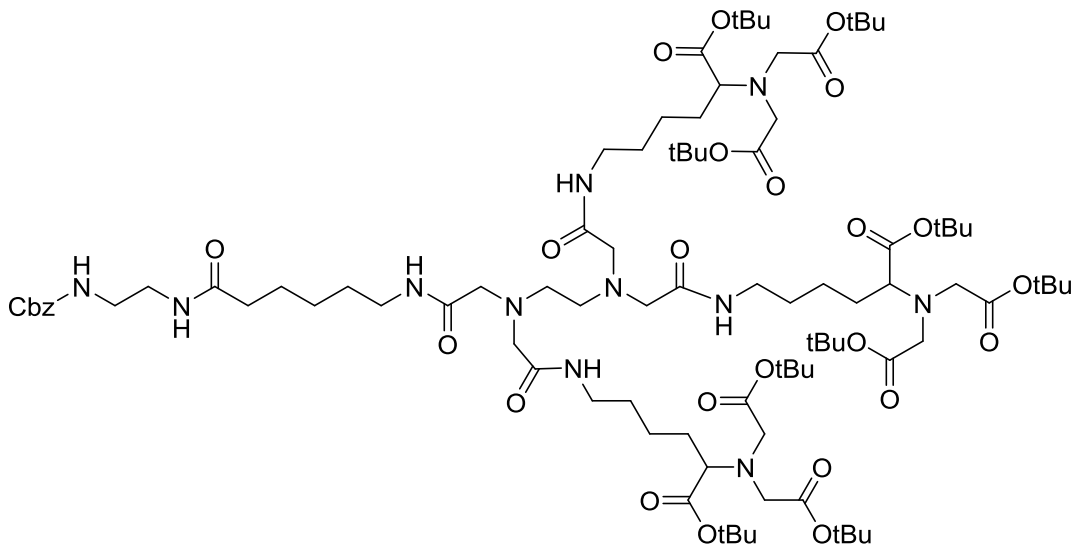
Cbz-EDA-rAcp-Boc (**29**, 500 mg, 1.23 mmol) was treated for 2 h with 20 mL of 50% TFA/DCM solution containing 0.5 mL of water and 0.5 mL of triisopropyl silane. After removal of TFA and DCM with N₂, the product was precipitated with dropwise addition of diethyl ether at 0 °C, washed 3 times with 12 mL of diethyl ether, and dried under vacuum to give 472 mg of Cbz-EDA-rAcp-H (**30**) as a white solid in 91% yield. LC/MS (ESI+) *m/z*: 308.4 [M+H]⁺.

17,20-bis(carboxymethyl)-3,8,15-trioxo-1-phenyl-2-oxa-4,7,14,17,20-pentaazadocosan-22-oic acid**Cbz-EDA-rAcp-EDTA (31)**

To a solution of EDTA bisanhydride (**27**, 1.87 g, 7.31 mmol) in 200 mL of anhydrous DMF under Ar was added DIPEA (1.78 mL, 10.2 mmol). Then, a solution of Cbz-EDA-rAcp-H (**30**, 450 mg, 1.06 mmol, TFA salt) in 50 mL of anhydrous DMF was added at a rate of 3.5 mL/h and the reaction was quenched by the addition of deionized water. The mixture was concentrated to orange residue under vacuum and the residue was dissolved in 20 mL 50% acetonitrile/water and subjected to reversed-phase purification on a C₁₈ column using a 30-90% gradient of acetonitrile containing 0.1% TFA at a flow rate of 35 mL/min to give 538 mg of Cbz-EDA-rAcp-EDTA (**31**) as a white solid after lyophilization in 86% yield. ¹H NMR (MeOH-d₄, 400 MHz): δ 7.35 (m, 5H), 5.08 (s, 2H), 3.97 (s, 2H), 3.95 (s, 4H), 3.89 (s, 2H), 3.30-3.19 (m, 10H), 2.19 (t, 2H, J =

6.9), 1.62 (m, 2H), 1.54 (m, 2H), 1.35 (m, 2H). ^{13}C NMR (MeOH- d_4 , 100 MHz): δ 176.52, 172.21, 171.46, 169.05, 129.50, 129.01, 128.80, 67.49, 55.78, 55.63, 53.19, 52.46, 40.40, 40.29, 36.87, 29.89, 27.41, 26.44. LC/MS (ESI+) m/z : 582.1[M+H] $^+$.

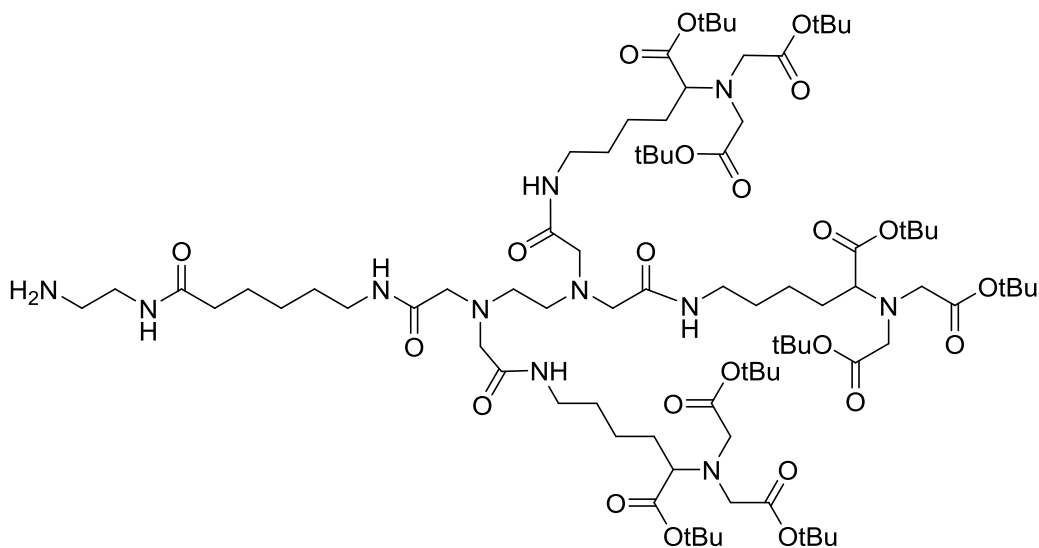
Cbz-EDA-rAcp-tris-NTA(OtBu) $_3$ (32)



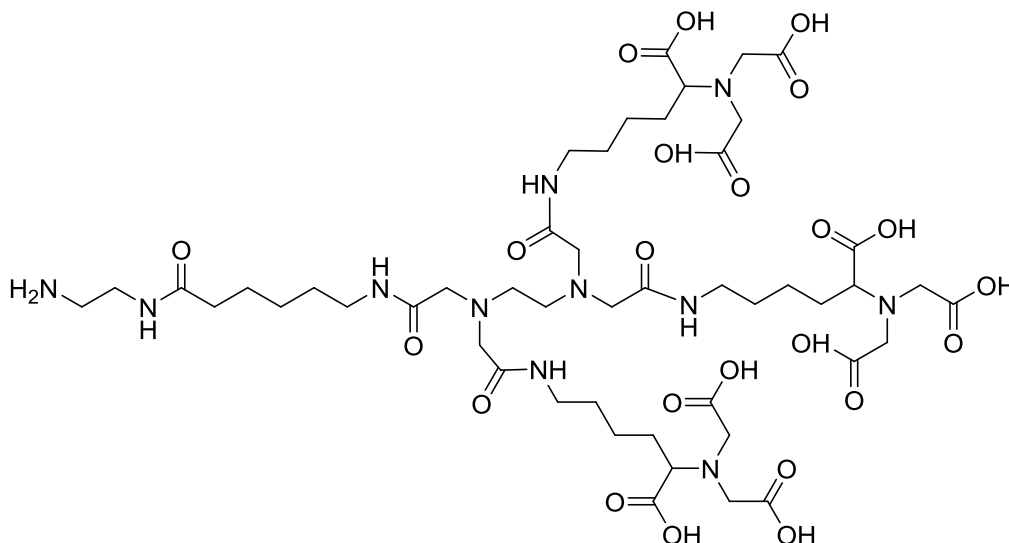
Cbz-EDA-rAcp-EDTA (**31**, 443 mg, 0.68 mmol), Lys-NTA(OtBu) $_3$ (**10**, 1.31 g, 3.05 mmol), PyBOP (1.59g, 3.05 mmol), DMAP (24.4 mg, 0.2 mmol), and DIPEA (1.18 mL, 6.8 mmol) were dissolved in 8 mL of anhydrous DMF and the reaction mixture was stirred under Ar for 24 h. The turbid mixture slowly turned clear as the reaction progressed. The reaction mixture was concentrated under vacuum and the crude residue was redissolved in 100 mL of EtOAc. The organic fraction was washed 4 times with 25 mL of 0.5 N NaOH and then dried over anhydrous Na $_2$ SO $_4$. The product was purified on a flash silica gel column using a 0-10% gradient of MeOH/DCM containing 0.1% TEA in 30 min at a flow rate of 60 mL/min. The product was dried under vacuum to give 338 mg clear oil in 27% yield. ^1H NMR (CDCl $_3$, 400 MHz): δ 7.64 (br, 1H), 7.49 (br, 3H), 7.3 (m, 5H), 6.88 (br, 1H), 6.01 (br, 1H), 5.05 (s, 2H), 3.46-3.35 (m, 16H), 3.27-3.12 (m, 18H), 2.62 (br, 4H), 2.14 (t, 2H, J = 7.0), 1.78 (m, 1H), 1.60 (m, 8H), 1.49 (m, 8H), 1.43 (s, 27H), 1.40 (s, 54H) 1.34-1.20 (m, 8H). ^{13}C NMR (CDCl $_3$, 100 MHz): δ 173.91, 172.30, 172.27,

170.86, 170.84, 170.73, 170.71, 156.99, 136.69, 128.44, 128.07, 128.00, 81.14, 80.76, 80.74, 66.52, 65.06, 59.21, 58.76, 58.68, 53.77, 53.47, 46.29, 46.25, 39.79, 39.20, 39.14, 38.78, 36.13, 30.05, 28.12, 27.91, 26.35, 25.08, 23.19, 23.11. LC/MS (ESI+) m/z : found 1818.3[M+H]⁺.

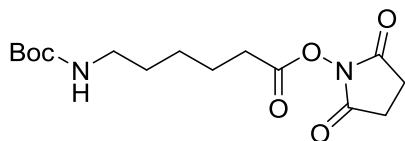
H-EDA-rAcp-tris-NTA(OtBu)₃ (33)



Cbz-EDA-rAcp-tris-NTA(OtBu)₃ (**32**, 105.4 mg, 0.058 mmol) was dissolved in 1 mL of MeOH and the solution was deaerated with N₂. A catalytic amount of 20% Pd(OH)₂/C (10 mg) was added to the solution and the reaction was purged with H₂. The reaction was stirred under H₂ for 3 h and then filtered through a celite bed to remove the catalyst. The filtrate was concentrated under vacuum to give 98.0 mg clear oil in 99% yield. ¹H NMR (MeOH-d₄, 400 MHz): δ 3.59-3.47 (m, 18H), 3.46-3.28 (m, 18H), 3.16 (t, 2H, J = 5.9), 2.34 (t, 2H, J = 7.4), 1.93 (m, 1H), 1.72 (m, 8H), 1.63 (m, 8H), 1.55 (s, 27H), 1.54 (s, 54H), 1.46 (m, 8H). ¹³C NMR (MeOH-d₄, 100 MHz): δ 177.24, 173.75, 172.54, 172.44, 82.51, 82.27, 82.13, 66.76, 55.01, 54.92, 54.19, 54.16, 41.06, 40.30, 40.14, 38.27, 36.80, 31.34, 30.16, 28.54, 28.34, 27.61, 26.27, 24.57, 23.76. LC/MS (ESI+) m/z : 1684.33[M+H]⁺, 842.9 [M+2H]²⁺, 1706.3 [M+Na]⁺.

H-EDA-rAcp-tris-NTA (34)

H-EDA-rAcp-tris-NTA(OtBu)₃ (**33**, 8.9 mg, 0.0468 mmol) was treated 2 h with 95% TFA solution containing TIS and H₂O. The solution was concentrated with N₂ and the product triturated with cold ether. The precipitate washed with cold ether and dried under vacuum. The product was purified on a RP-HPLC column (Phenomenex Jupiter C18, 21×250 mm) with water and acetonitrile containing 0.1% TFA as the mobile phase to give 0.7 mg desired product. LC/MS (ESI+) *m/z*: 1180.27 [M+H]⁺, 591.0 [M+2H]²⁺.

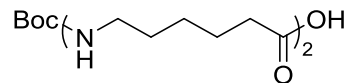
2,5-dioxopyrrolidin-1-yl 6-((tert-butoxycarbonyl)amino)hexanoate**Boc-Acp-OSu (35)**

N-Hydroxysuccinimide (0.985 g, 8.6 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, 2.5 g, 13.0 mmol) were added to a solution of Boc-6-amino-hexanoic acid (2.0 g, 8.6 mmol) in anhydrous DCM (10 mL). The mixture was stirred at rt for 4 h. After dilution with DCM (20 mL), the solution was washed with brine (10 mL×3). The organic layer

was concentrated to give 2.8 g (8.5 mmol) of the product in 98.8% yield. LC-MS (ESI+) m/z : 351.3 $[M+Na]^+$, 229.2 $[M-Boc]^+$.

6-(6-((tert-butoxycarbonyl)amino)hexanamido)hexanoic acid

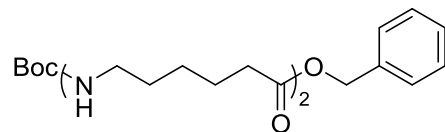
Boc-(Acp)₂-OH (36)



H-Acp-OH (144.0 mg, 1.096 mmol) and DIPEA (382 μ L, 2.193 mmol) were added to a solution of Boc-Acp-OSu (**35**, 240.2 mg, 0.732 mmol) in anhydrous DCM (10 mL). The mixture was stirred at rt for 3 h. After the solvent was removed under vacuum, deionized water (10 mL) was added to the residue and the pH of the solution was adjusted to 4-5 by acetic acid. Then, the solution was extracted with ethyl acetate (5 mL $\times$ 3) and organic layer was washed with brine (5 mL). The organic layer was concentrated to give 250.1 mg (0.726 mmol) of Boc-(Acp)₂-OH (**36**) in 99.2% yield. ¹H NMR (CDCl₃, 400 MHz): δ 9.57(s br, 1H), 6.47(s, 1H), 4.92(s, 1H), 3.16(dd, 2H), 3.01(d, 2H), 2.26(t, 2H), 2.12(t, 2H), 1.56(p, 4H), 1.36-1.48(m, 13H), 1.24-1.31(m, 4H). ¹³C NMR (CDCl₃, 100 MHz): δ 177.3, 175.1, 156.3, 79.1, 40.2, 39.2, 36.3, 33.8, 29.5, 28.9, 28.3, 26.3, 26.2, 25.3, 24.2. LC/MS (ESI+) m/z : 367.3 $[M+Na]^+$, 245.2 $[M-Boc]^+$.

Benzyl 6-(6-((tert-butoxycarbonyl)amino)hexanamido)hexanoate

Boc-(Acp)₂-OBzl (37)

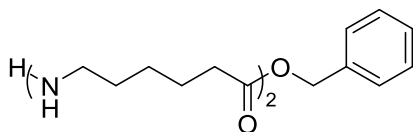


EDC-HCl (561.7 mg, 2.930 mmol) was added to a solution of Boc-(Acp)₂-OH (**36**, 501.4 mg, 1.457 mmol) in anhydrous DCM (5 mL), and the reaction mixture was stirred for 30 min. Then, benzyl alcohol (226.1 μ L, 2.185 mmol) and DMAP (25.0 mg, 0.205 mmol) were added to the

mixture. The mixture was continually stirred at rt overnight. Millipore water (3 mL) was added to the mixture and DCM layer was separated and purified on silica gel flash column chromatography using 0-20% Methanol in DCM as the mobile phase to afford 350.9 mg of the product in 55.4% yield. ^1H NMR (CDCl_3 , 400 MHz): δ 7.28(s, 5H), 5.57(s, 1H), 5.04(s, 2H), 4.59(d, 1H), 3.15(d, 2H), 3.03(s, 2H), 2.29(t, 2H), 2.07(t, 2H), 1.20-1.80(m, 21H). ^{13}C NMR (CDCl_3 , 100 MHz): δ 173.4, 172.8, 156.0, 136.0, 128.2, 127.0, 79.1, 66.2, 40.3, 39.2, 36.6, 34.1, 29.8, 29.3, 28.4, 26.4, 26.3, 25.3, 24.5. LC/MS (ESI+) m/z : 435.5 $[\text{M}+\text{H}]^+$, 457.5 $[\text{M}+\text{Na}]^+$, 335.4 $[\text{M}-\text{Boc}]^+$.

Benzyl 6-(6-aminohexanamido)hexanoate

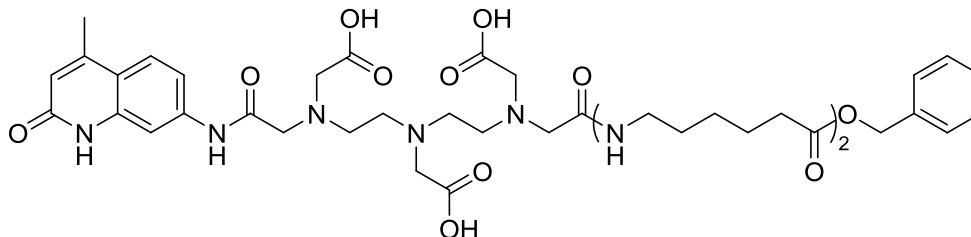
H-(Acp) $_2$ -OBzl (38)



Boc-(Acp) $_2$ -OBzl (**37**, 350.9 mg, 0.807 mmol) was treated with 50% TFA/DCM (6 mL) at rt for 2 h. The volatiles were removed by N_2 and the product was triturated with cold diethyl ether (2 mL $\times$ 3) to give 340.2 mg H-(Acp) $_2$ -OBzl as a TFA salt in 97.6% yield. ^1H NMR (TFA salt, CDCl_3 , 400 MHz): δ 12.2(s, 4H), 7.36(m, 5H), 5.12(s, 2H), 3.26(d, 2H), 3.05(d, 2H), 2.37(p, 4H), 1.31-1.74(m, 12H). ^{13}C NMR (CDCl_3 , 100 MHz): δ 176.4, 175.0, 160.7(TFA), 135.5, 129.3, 128.6, 128.0, 115.3(TFA), 66.7, 40.3, 40.2, 34.8, 34.0, 28.1, 26.4, 25.9, 25.1, 24.8, 24.2. LC/MS (ESI+) m/z : 335.3 $[\text{M}+\text{H}]^+$.

19,22-bis(carboxymethyl)-25-(2-((4-methyl-2-oxo-1,2-dihydroquinolin-7-yl)amino)-2-oxoethyl)-3,10,17-trioxo-1-phenyl-2-oxa-9,16,19,22,25-pentaazaheptacosan-27-oic acid

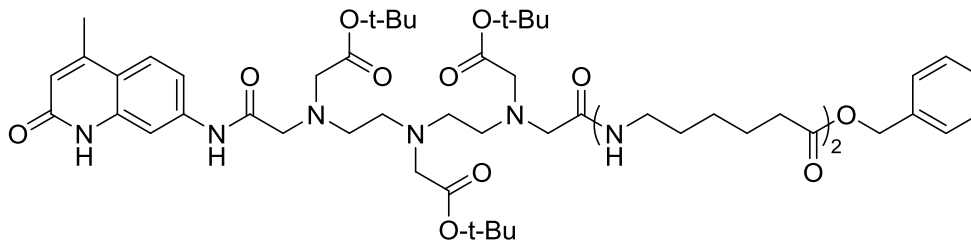
AMQ-DTPA-(Acp)₂-OBzl (39)



DTPA bisanhydride (357 mg, 1.0 mmol) dissolved in 100 mL anhydrous DMF was added DIPEA (695 μ L, 4 mmol) and DMAP (12 mg, 0.1 mmol) in 1 mL anhydrous DMF. Then 7-amino-4-methylquinolin-2-one (AMQ, 87 mg, 0.5 mmol) in 10 mL DMF was added at a rate of 2 mL/hr using a syringe pump. After 3 h of AMQ addition, H-Acp₂-OBzl (**38**, 501 mg, 1.5 mmol) in 10 mL DMF was added at a rate of 2.5 mL/hr using a second syringe pump. The reaction was left to stir overnight. DMF was removed under vacuum and the residue dissolved in 12 mL 50% methanol / water. The insoluble solid was removed by centrifugation at 10,000 rpm and purified on a reversed-phase C₁₈ column using 50 - 90% methanol in water containing 0.1% TFA as the mobile phase. The fractions containing the product was combined and lyophilized to give 183.5 mg (42.4%) white powder. ¹H-NMR (MeOH-d₄, 400 MHz): δ 7.99(s, 1H), 7.71(d, 1H, J=8), 7.34(m, 6H), 6.45(s, 1H), 5.10(s, 2H), 4.27(s, 2H), 3.91(s, 2H), 3.82(s, 2H), 3.74(s, 2H), 3.67(s, 2H), 3.50(s, 4H), 3.37(m, 2H), 3.25(m, 2H), 3.13(m, 4H), 3.00(s, 3H), 2.87(s, 3H), 2.48(s, 3H), 2.37(t, 3H, J=8), 2.19(m, 1H), 2.11(m, 2H), 1.10-1.70(m, 18H). ¹³C-NMR (MeOH-d₄, 100MHz): 176.0, 175.2, 173.7, 173.6, 172.0, 171.1, 170.0, 165.3, 164.9, 151.7, 141.8, 140.1, 137.7, 129.6, 129.2, 126.6, 119.1, 118.7, 118.5, 116.7, 115.8, 107.4, 67.2, 59.0, 58.0, 56.4, 56.2, 54.2, 51.8, 40.3, 40.2, 37.0, 36.9, 35.0, 31.7, 30.0, 29.9, 27.5, 27.4, 26.7, 26.6, 19.1. LC/MS (ESI+) *m/z*: 866.2 [M+H]⁺.

25-benzyl 1-(tert-butyl) 6,9-bis(2-(tert-butoxy)-2-oxoethyl)-3-(2-((4-methyl-2-oxo-1,2-dihydroquinolin-7-yl)amino)-2-oxoethyl)-11,18-dioxo-3,6,9,12,19-pentaazapentacosanedioate

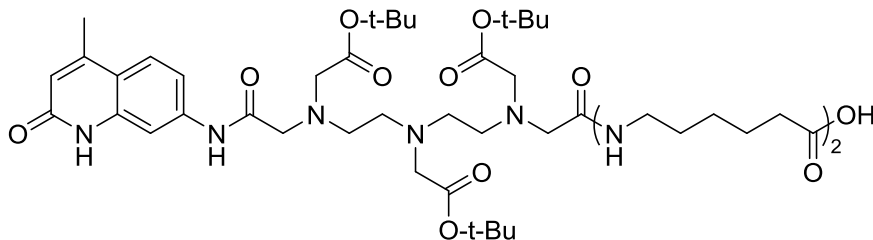
AMQ-DTPA(tBu)₃-(Acp)₂-OBzl (40)



To a solution of AMQ-DTPA-(Acp)₂-OBzl (**39**, 56.2 mg, 0.065 mmol) in 10% anhydrous DMF in DCM (2 mL) was added *O*-tert-butyl *N,N'*-diisopropylisourea (130.0 mg, 0.649 mmol) and the reaction mixture was stirred for 48 h at rt. The reaction was diluted with 3 mL DCM and extracted with 3 mL deionized water (×2) and brine (3 mL). The organic layer was dried over sodium sulfate and concentrated. The crude residue was purified by flash column chromatography using 0-20% methanol in DCM as the mobile phase to give 60.3 mg of AMQ-DTPA(tBu)₃-(Acp)₂-OBzl (**40**) in 89.2% yield. ¹H NMR (CDCl₃, 400 MHz): δ 10.0(s, 1H), 7.99(d, 1H, J=4), 7.70(d, 1H, J=8), 7.66(m, 1H), 7.60(dd, 1H, J=4), 7.27(s, 5H), 6.49(s, 1H), 5.94(s, 1H), 5.03(s, 2H), 4.46(d, J=4, 4H), 3.37(s, 2H), 3.34(s, 2H), 3.19(s, 2H), 3.08-3.16(m, 4H), 2.60-2.80(m, 6H), 2.47(s, 3H), 2.27(t, J=8, 2H), 2.05(m, 2H), 1.50-1.60(m, 2H), 1.41(s, 9H), 1.36(s, 9H), 1.34(s, 9H), 1.18-1.50(m, 12H), 1.00-1.16(m, 1H). ¹³C NMR (CDCl₃, 100 MHz): 173.4, 172.9, 171.1, 170.8, 170.5, 170.3, 169.9, 162.3, 157.2, 147.3, 145.6, 138.5, 136.0, 128.5, 128.2, 124.1, 121.8, 116.7, 116.6, 113.9, 82.0, 81.6, 81.4, 79.8, 66.1, 59.9, 59.1, 57.6, 57.3, 56.0, 53.8, 53.6, 52.7, 41.8, 39.2, 38.8, 36.5, 34.1, 29.3, 28.7, 28.2, 28.1, 26.5, 26.4, 25.3, 24.5, 23.5, 18.3. LC/MS (ESI+) *m/z*: 1034.1 [M+H]⁺.

9,12-bis(2-(tert-butoxy)-2-oxoethyl)-2,2-dimethyl-6-(2-((4-methyl-2-oxo-1,2-dihydroquinolin-7-yl)amino)-2-oxoethyl)-4,14,21-trioxo-3-oxa-6,9,12,15,22-pentaazaooctacosan-28-oic acid

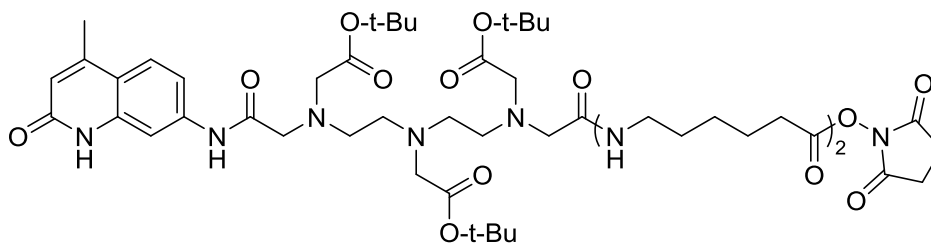
AMQ-DTPA(tBu)₃-(Acp)₂-OH (41)



To a solution of AMQ-DTPA(tBu)₃-(Acp)₂-OBzl (**40**, 57.0 mg, 55.1 μ mol) in methanol (20 mL) containing formic acid (50.7 μ L, 2% in methanol) under N₂ was added 10% Pd/C (10 mg) and the reaction mixture was vigorously stirred for 1.5 h at rt. The Pd/C was removed by filtration over celite bed and the filtrate was concentrated under reduced pressure to give 39.6 mg product as clear oil in 76% yield. LC/MS (ESI+) *m/z*: 944.1 [M+H]⁺.

1-(tert-butyl) 25-(2,5-dioxopyrrolidin-1-yl) 6,9-bis(2-(tert-butoxy)-2-oxoethyl)-3-(2-((4-methyl-2-oxo-1,2-dihydroquinolin-7-yl)amino)-2-oxoethyl)-11,18-dioxo-3,6,9,12,19-pentaazapentacosanedioate

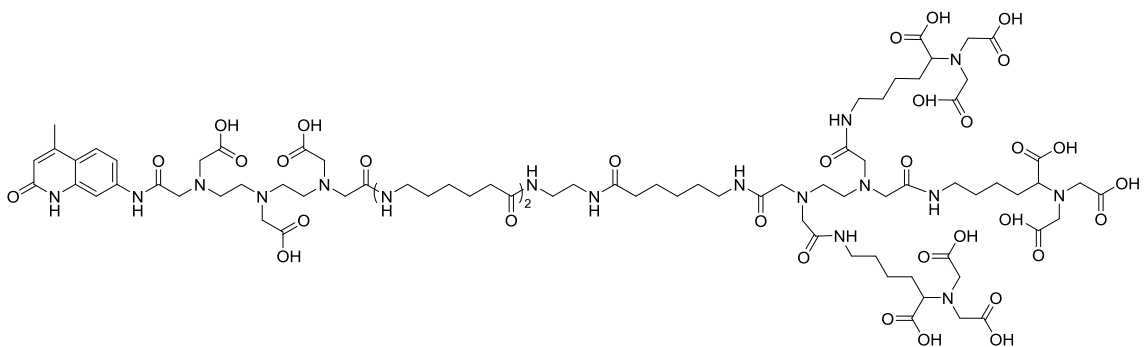
AMQ-DTPA(tBu)₃-(Acp)₂-OSu (42)



To a solution of AMQ-DTPA(tBu)₃-(Acp)₂-OH (**41**, 55.0 mg, 58.3 μ mol) in 2 mL anhydrous DCM were added NHS (22.4 mg, 194.6 μ mol) and EDC·HCl (56.0 mg, 292.1 μ mol). The reaction mixture was stirred at rt for overnight and then deionized water (3 mL) was added to the mixture. The product was extracted with 3 mL DCM ($\times$ 3) and organic fractions were combined and dried over Na₂SO₄. The solution was concentrated and purified by silica gel flash column

chromatography using 0-20% methanol in DCM as the mobile phase to give 37.6 mg of AMQ-DTPA(tBu)₃-(Acp)₂-OSu (**42**) as a clear oil in 61.9% yield. ¹H NMR (CDCl₃, 400 MHz): δ 10.0(s, 1H), 7.99(d, 1H, J=1), 7.74(m, 1H), 7.71(d, 1H, J=6), 7.58(dd, 1H, J₁=12, J₂=4), 6.50(s, 1H), 6.05(m, 1H), 5.94(m, 1H), 3.65(s, 1H), 3.47(q, 2H, J=4), 3.39(s, 2H), 3.36(s, 2H), 3.10-3.25(m, 6H), 2.70-2.85(m, 6H), 2.66(s, 4H), 2.60(s, 4H), 2.54(m, 3H), 2.47(s, 3H), 2.10(m, 2H), 1.10-1.72(m, 12H), 1.41(s, 9H), 1.36(s, 9H), 1.35(s, 9H). LC/MS (ESI+) *m/z*: 1041.0 [M+H]⁺.

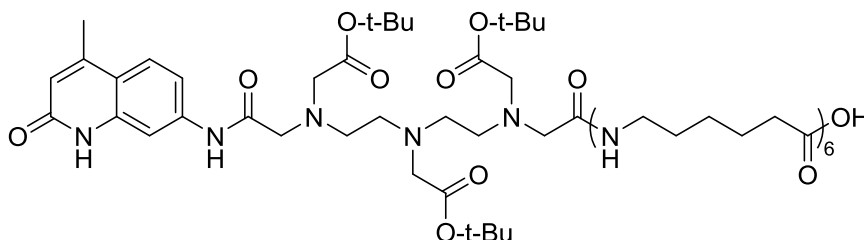
AMQ-DTPA-(Acp)₂-EDA-rAcp-EDTA-tris-Lys-NTA (43**)**



To a solution of AMQ-DTPA(tBu)₃-(Acp)₂-OH (**41**, 34.0 mg, 0.036 mmol) in 1 mL anhydrous DCM under Ar were added HBTU (13.7 mg, 0.036 mmol) and DIPEA (19 μ L, 0.108 mmol). The reaction was stirred for 1 h and H-EDA-rAcp-tris-NTA(OtBu)₃ (**33**, 73.0 mg, 0.043 mmol) in anhydrous DMF (3 mL) was added to the mixture. The reaction was stirred for additional 12 h and then concentrated under vacuum. The crude residue was dissolved in acetonitrile (1 mL) and then triturated by dropwise addition of 0.5 N NaOH (6 mL). The solid was washed with deionized water and treated for 2 h with 3 mL of 95%TFA solution containing 5% deionized H₂O. The solution was concentrated by N₂ and the residue triturated by dropwise addition of diethyl ether at 0 °C. The crude solid was dried under vacuum to give 52.0 mg (74.5%). Then, 8.8 mg of product was purified on a C₁₈ RP-HPLC column (BDS-Hypersil C₁₈ 150×50 mm 5 μ) using 10-50% gradient of acetonitrile containing 0.1% TFA in 16 min at a flow rate of 10 mL/min. The product was obtained as 4.6 mg white powder after lyophilization (purification yield 52.3%). LC/MS

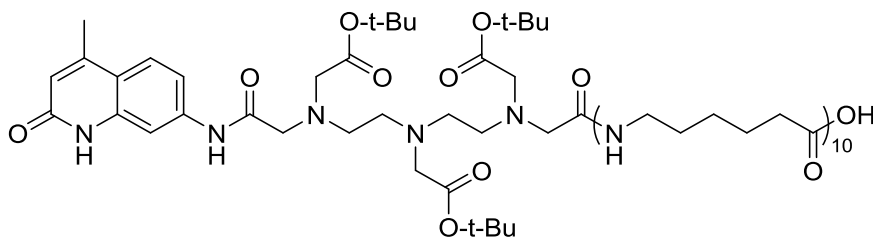
(ESI+) m/z : 1938.4[M+H]⁺, 969.4[M+2H]²⁺. HRMS (ESI+) m/z calc'd for C₈₄H₁₃₂N₁₈O₃₄: [M+H]⁺=1937.9226, [M+2H]²⁺=969.4649, [M+3H]³⁺=646.6457, found 969.4711, 646.6493.

AMQ-DTPA(tBu)₃-(Acp)₆-OH (45)



To a solution of AMQ-DTPA-(Acp)₂-OSu (**42**, 19.0 mg, 18.25 μmol) and DIPEA (16 μL, 91.85 μmol) in anhydrous DCM (1 mL) was added H-(Acp)₄-OH TFA salt (17.0 mg, 29.95 μmol) in anhydrous DMF (1 mL). The reaction was mixed at rt for overnight and then solvent was removed under reduced pressure. The product was triturated by the addition of DCM (5 mL) and 20.4 mg (80%) white solid was obtained after filtration. LC/MS (ESI+) m/z : 1396.4 [M+H]⁺, 698.9 [M+2H]²⁺.

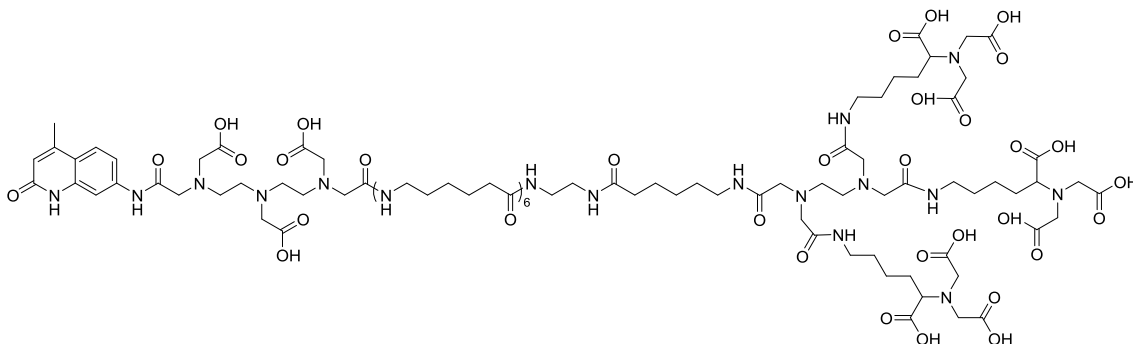
AMQ-DTPA(tBu)₃-(Acp)₁₀-OH (46)



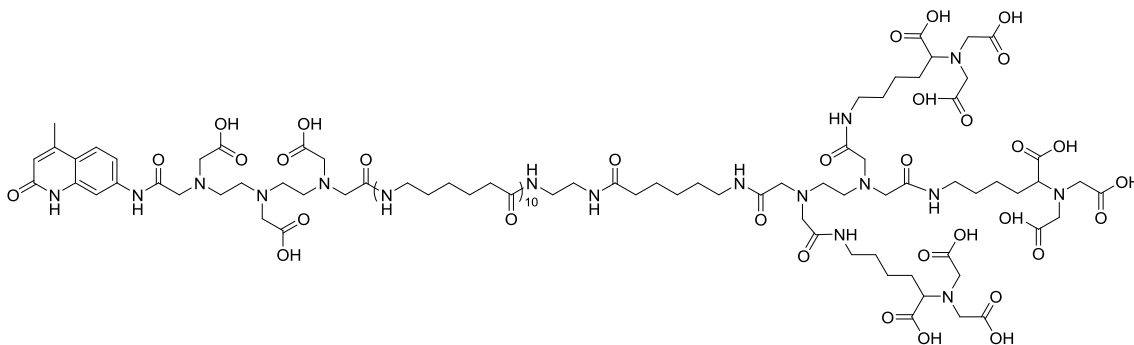
To a solution of AMQ-DTPA-(Acp)₂-OSu (**42**, 39.0 mg, 37.5 μmol) and DIPEA (32.6 μL, 188 μmol) in anhydrous DMSO (3 mL) was added H-(Acp)₈-OH (60.0 mg, 65.0 μmol). The suspension was mixed at rt for 1 day under Ar. After the disappearance of the OSu ester, the product was triturated by the addition of water (10 mL) and the solid washed 2× 5 mL water. The crude product was lyophilized to give 72 mg white solid, which was purified on RP-HPLC (BDS-

Hypersil-C₁₈ 150 x 50 mm, 5 μ) using 35-95% acetonitrile in water containing 0.1% TFA as the mobile phase. The fractions containing the product was lyophilized to give 32.0 mg white powder in 43.8% yield. LC/MS (ESI+) m/z : 1849.3 $[M+H]^+$, 925.0 $[M+2H]^{2+}$, 617.1 $[M+3H]^{3+}$.

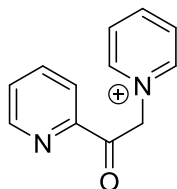
AMQ-DTPA-(Acp)₆-EDA-rAcp-EDTA-tris-NTA (47)



AMQ-DTPA(tBu)₃-(Acp)₆-OH (**45**, 20.5 mg, 14.7 μ mol), H-EDA-rAcp-tris-NTA(OtBu)₃ (**33**, 32.9 mg, 19.5 μ mol), and PyBOP (7.6 mg, 14.7 μ mol) were added to a flask and dissolved in 2 mL of anhydrous DMF containing DIPEA (10.2 μ L, 58.8 μ mol). The reaction was stirred under Ar for 12 h and the clear solution became turbid overnight. The reaction mixture was concentrated under vacuum and the residue was directly treated for 3 h with 0.5 mL of 95% TFA solution containing 5% deionized H₂O. TFA was removed by N₂ and the crude product was triturated with dropwise addition of diethyl ether at 0 °C and then subjected to RP-HPLC purification (Phenomenex Jupiter C₁₈ 200 $\times$ 20 mm 5 μ) using a 10-50% gradient of acetonitrile containing 0.1% TFA in 25 min at a flow rate of 20 mL/min. The product was obtained as a clear oil (8.5 mg, 24% after 2 steps). LC/MS (ESI+) m/z : 1195.6 $[M+2H]^{2+}$, 1196.5 $[M(^{13}\text{C})+2H]^{2+}$, 797.6 $[M+3H]^{3+}$. HRMS (ESI+) m/z calc'd for C₁₀₈H₁₇₆N₂₂O₃₈: $[M(^{13}\text{C})+H]^+=2391.2622$, $[M(^{13}\text{C})+2H]^{2+}=1196.1348$, $[M(^{13}\text{C})+3H]^{3+}=797.7589$, found 1196.1395, 797.7618.

AMQ-DTPA-(Acp)₁₀-EDA-rAcp-EDTA-tris-NTA (48)

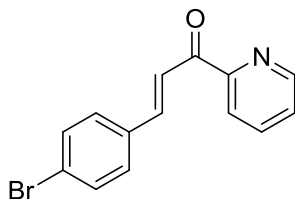
AMQ-DTPA(tBu)₃-(Acp)₁₀-OH (**46**, 16 mg, 8.2 μ mol) and PyBOP (6.4 mg, 12.3 μ mol) were dissolved in 1 mL of anhydrous DMSO containing DIPEA (7.1 μ L, 41.1 μ mol). The reaction was stirred under Ar for 30 min and then added H-EDA-rAcp-tris-NTA(OtBu)₃ (**33**, 20 mg, 11.9 μ mol). The reaction was stirred overnight under Ar, and then the crude product precipitated by deionized water. The precipitate was treated for 3 h with 2.5 mL of 95% TFA solution containing 5% deionized H₂O. TFA was removed by N₂ and the crude product was triturated with dropwise addition of diethyl ether at 0 °C and then subjected to RP-HPLC purification (BDS-Hypersil C₁₈ 150 $\times$ 50 mm 5 μ) using a 20-35% gradient of acetonitrile containing 0.1% TFA in 16 min at a flow rate of 15 mL/min. The product was obtained as 7.9 mg clear oil (34%) after lyophilization. LC/MS (ESI+) *m/z*: 1421.7 [M+2H]²⁺, 948.3 [M+3H]³⁺, 711.6 [M+4H]⁴⁺, 569.5 [M+5H]⁵⁺.

1-(2-oxo-2-(pyridin-2-yl)ethyl)pyridin-1-ium (49)

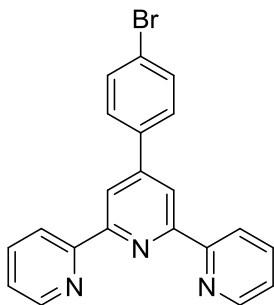
Compound **49** was synthesized following the literature procedure.¹⁴⁴ Iodine (12.6 g, 50.0 mmol) and 2-acetylpyridine (5.6 mL, 50.0 mmol) were mixed in 40 mL anhydrous pyridine and heated to reflux for 6 h. Then, the reaction was allowed to slowly cool to rt and then transferred to the refrigerator to cool to 4 °C to precipitate the product. The solid was collected by filtration and

recrystallized from ethanol (50 mL) to give 7.70 g off-white solid in 47% yield. ^1H NMR (DMSO- d_6 , 400 MHz): δ 9.07 (d, 2H, $J=5.4$), 8.79 (tt, 1H, $J_A=7.9$, $J_B=1.2$), 8.35 (d, 1H, $J=6.7$), 8.33 (d, 1H, $J=6.7$), 8.20 (td, 1H, $J_A=7.9$, $J_B=1.6$), 8.14 (m, 1H), 8.01 (m, 1H), 7.90 (m, 1H), 6.56 (s, 2H). LC/MS (ESI+) m/z : 200.1 $[\text{M}+\text{H}]^+$.

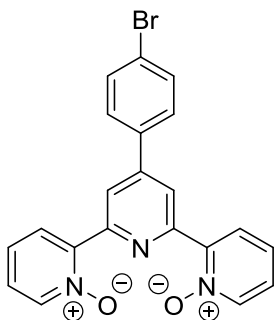
(E)-3-(4-bromophenyl)-1-(pyridin-2-yl)prop-2-en-1-one (50)



Compound **50** was synthesized following the literature procedure.¹⁴⁴ To a solution of 4-bromobenzaldehyde (7.5g, 40.5 mmol) in a mixture of 80 mL MeOH and 17 mL water at 0 °C was added dropwise an aqueous solution of KOH (2.28g, 40.5 mmol). Then, 2-acetylpyridine (4.54 mL, 40.5 mmol) was added dropwise to the mixture and the product precipitated from the mixture as 2-acetylpyridine was added. The reaction was slowly warmed to rt and then left to stir overnight. The precipitates were collected by filtration and the solid washed with ice cold methanol to give 10.6 g in 91% yield. ^1H NMR (CDCl_3 , 400 MHz): δ 8.75 (dm, 1H, $J=4.5$), 8.32 (s, 1H), 8.19 (d, 2H, $J=7.9$), 7.88 (td, 2H, $J_A=7.7$, $J_B=1.7$), 7.86 (d, 2H, $J=16$), 7.50 (m, 2H). LC/MS (ESI+) m/z : 288.0 $[\text{M}(^{79}\text{Br})+\text{H}]^+$, 290.0 $[\text{M}(^{80}\text{Br})+\text{H}]^+$.

4'-(4-bromophenyl)-2,2':6',2''-terpyridine (51)

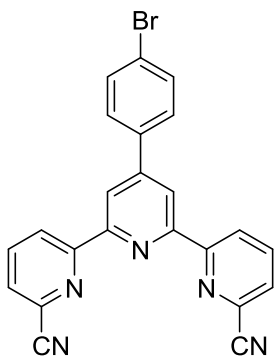
The Krohnke synthesis of compound **51** was performed following the literature procedure.¹⁴⁴ Compound **49** (5.66 g, 17.4 mmol), compound **50** (5.0 g, 17.4 mmol), and ammonium acetate (NH₄OAc, 33.5 g, 435 mmol) were dissolved in 150 mL methanol and refluxed overnight (12 h). The reaction was slowly cooled to rt and then transferred to a refrigerator to cool to 4 °C. The precipitate was collected by filtration and the crude solid washed with ice cold methanol. The resulting yellow solid was dried under vacuum to give 2.85 g of the desired product in 42% yield. ¹H NMR (MeOH-d₄, 400 MHz): δ 8.72 (dm, 2H, J=4.5), 8.69 (d, 2H, J=7.6), 8.68 (s, 2H), 8.04 (td, 2H, J_A=7.7, J_B=1.7), 7.89 (d, 2H, J=8.6), 7.76 (d, 2H, J=8.6), 7.52 (m, 2H). ¹³C NMR (MeOH-d₄, 100 MHz): δ 159.57, 153.14, 152.62, 141.53, 140.94, 136.13, 132.63, 128.19, 127.59, 125.84, 122.59. LC/MS (ESI+) *m/z*: 388.3 [M(⁷⁹Br)+H]⁺, 390.3 [M(⁸¹Br)+H]⁺.

4'-(4-bromophenyl)-[2,2':6',2''-terpyridine] N,N''-dioxide (52)

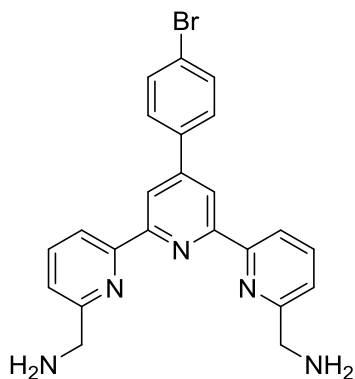
Compound **52** was synthesized following the literature procedure.¹⁴⁴ To a solution of **51** (2.70 g, 6.96 mmol) in 50 mL DCM was added *m*-chloroperoxybenzoic acid (mCPBA, 4.79g, 27.8 mmol).

The reaction was stirred at rt for 1 day and the suspension dissolved completely. The reaction mixture was washed with 25 mL saturated NaHCO_3 ($\times 3$) and brine solution. The organic layer was dried over Na_2SO_4 and then concentrated. The crude product was dissolved in DCM and purified by a silica gel flash column chromatography using 0-15% MeOH in DCM as the mobile phase. The product fractions were combined and concentrated under vacuum to give 2.31 g white solid in 79% yield. LC/MS (ESI+) m/z : 420.1 $[\text{M}(^{79}\text{Br})+\text{H}]^+$, 422.1 $[\text{M}(^{81}\text{Br})+\text{H}]^+$.

4'-(4-bromophenyl)-[2,2':6',2''-terpyridine]-N,N'-dicarbonitrile (53)

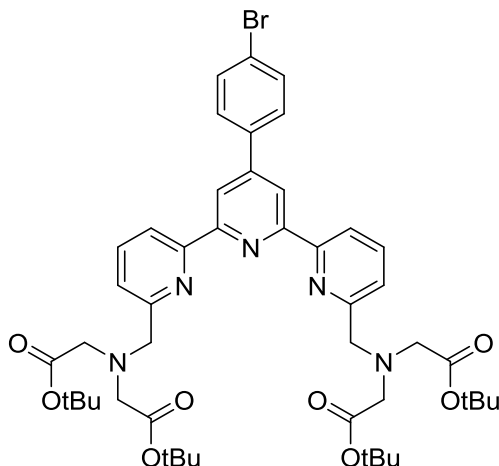


The modified Reissert-Henze reaction of compound **52** was performed following the literature procedure.¹⁴⁴ To a suspension of **52** (1.89 mg, 4.5 mmol) in 75 mL anhydrous DCM under N_2 was added TMS-CN (6.0 mL, 45.0 mmol) and stirred for 5 min at rt. Benzoyl chloride (2.1 mL, 18.0 mmol) was added dropwise to the mixture and the reaction was stirred overnight. Then, 5 mL 10% K_2CO_3 solution was added and stirred for additional 15 min and the precipitate was filtered. The solid was washed with water and ice cold DCM. The solid was dried under vacuum to give 1.51 g white powder in 77% yield. ^1H NMR ($\text{DMSO}-d_6$, 400 MHz): δ 9.03 (d, 2H, $J=8.0$), 8.74 (s, 2H), 8.36 (t, 2H, $J=7.8$), 8.25 (d, 2H, $J=7.8$), 8.02 (d, 2H, $J=7.6$), 7.86 (d, 2H, $J=8.6$). ^{13}C NMR ($\text{DMSO}-d_6$, 100 MHz): δ 156.13, 154.16, 149.12, 139.50, 135.98, 132.39, 132.36, 129.66, 129.29, 125.11, 123.45, 119.08, 117.44. LC/MS (ESI+) m/z : 438.3 $[\text{M}(^{79}\text{Br})+\text{H}]^+$, 440.3 $[\text{M}(^{81}\text{Br})+\text{H}]^+$.

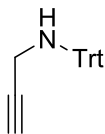
(4'-(4-bromophenyl)-[2,2':6',2''-terpyridine]-6,6''-diyl)dimethanamine (54)

The reduction of **53** using borane was performed as described in the literature.¹⁴⁴ A suspension of dicyanonitrile **53** (1.51 g, 3.46 mmol) in 20 mL anhydrous THF at 0 °C was deaerated with Ar. Then, a 1 M solution of borane-THF complex in THF (1 M BH₃-THF, 69 mL, 69 mmol) was added dropwise to the solution over 20 min. After stirring for additional 2.5 h, the excess borane was quenched by the addition of methanol. The reaction was concentrated and dissolved in 100 mL methanol saturated with HCl and stirred for 2 h at rt. The mixture was concentrated and the HCl salt of the product was suspended in THF. The solid was collected by filtration and washed with THF. The product was dried under vacuum to give 1.71 g white solid in 96% yield. ¹H NMR (MeOH-d₄, 400 MHz): δ 9.06 (s, 2H), 8.76 (d, 2H, J=7.8), 8.15 (t, 2H, J=8.0), 8.07 (d, 2H, J=8.0), 7.82 (d, 2H, J=8.3), 7.64 (d, 2H, J=8.0), 4.49 (s, 4H). ¹³C NMR (MeOH-d₄, 100 MHz): δ 155.07, 153.85, 140.24, 140.21, 134.16, 133.68, 133.63, 130.84, 130.61, 124.81, 122.77, 121.01, 44.11. LC/MS (ESI+) *m/z*: 446.1 [M(⁷⁹Br)+H]⁺, 448.1 [M(⁸¹Br)+H]⁺.

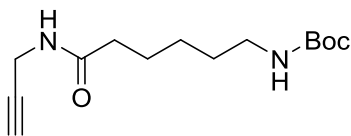
Tetra-tert-butyl 2,2',2'',2'''-(((4'-(4-bromophenyl)-[2,2':6',2''-terpyridine]-6,6''-diyl)bis(methylene))bis(azanetriyl))tetraacetate (55**)**



The tetra-*t*-butyl ester **55** was synthesized following the literature procedure.¹⁴⁴ Compound **54** (1.69 g, 3.26 mmol) in 10 mL anhydrous DMF were added KI (596 mg, 3.59 mmol) and DIPEA (9.08 mL, 52.2 mmol). Then, *t*-butyl bromoacetate (3.86 mL, 26.1 mmol) was added and the reaction was stirred overnight under Ar. The mixture was concentrated and dissolved in DCM. The solution was washed with saturated NaHCO₃ and then dried over Na₂SO₄. The crude product was subjected to purification by a silica gel flash column chromatography using 0 - 50% EtOAc in hexane containing 2% TEA as the mobile phase. The fractions containing the product was concentrated to give 1.26 g yellow sticky solid in 43% yield. ¹H NMR (CDCl₃, 400 MHz): δ 8.69 (s, 2H), 8.52 (d, 2H, *J*=7.6), 7.85 (t, 2H, *J*=7.8), 7.75 (d, 2H, *J*=8.3), 7.69 (d, 2H, *J*=7.6), 7.63 (d, 2H, *J*=8.3), 4.15 (s, 4H), 3.54 (s, 8H), 1.44 (s, 36H). ¹³C NMR (CDCl₃, 100 MHz): δ 170.64, 158.86, 156.25, 155.22, 148.79, 137.88, 137.42, 132.06, 128.92, 123.34, 123.22, 119.62, 118.52, 80.99, 59.94, 55.83, 28.20. LC/MS (ESI+) *m/z*: 901.8 [M(⁷⁹Br)+H]⁺, 903.8 [M(⁸¹Br)+H]⁺.

N-trityl propargylamine (56)**N-Trt-Ap₃**

To a solution of propargylamine (67.2 μ L, 1.05 mmol) and TEA (139 μ L, 1.0 mmol) in anhydrous THF at 0 $^{\circ}$ C was added dropwise a solution of trityl chloride (278 mg, 1.0 mmol) in 1 mL anhydrous THF. After stirring for 1 h at 0 $^{\circ}$ C, the reaction was allowed to slowly warm to rt. The reaction mixture was concentrated and the residue purified by a silica gel flash column chromatography using 0 - 50% DCM in hexane containing 0.1% TEA as the mobile phase. The product was concentrated to give 95.5 mg oil in 31% yield. ^1H NMR (CDCl_3 , 400 MHz): δ 7.41 (m, 6H), 7.21 (m, 6H), 7.13 (m, 3H), 2.87 (d, 2H, $J=2.5$), 2.1 (t, 1H, $J=2.5$), 1.91 (bs, 2H). ^{13}C NMR (CDCl_3 , 100 MHz): δ 145.18, 128.56, 128.00, 126.55, 82.69, 70.97, 70.67, 33.54. LC/MS (ESI+) m/z : 243.7 $[\text{M}(-\text{Trt})+\text{H}]^+$.

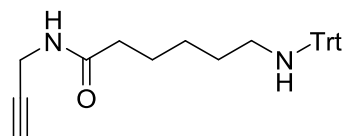
Tert-butyl (6-oxo-6-(prop-2-yn-1-ylamino)hexyl)carbamate (57)**Ap₃-rAcp-Boc**

Boc-Acp-OH (1.16 g, 5 mmol), HBTU (2.08 g, 5.5 mmol), and TEA (1.4 mL, 10 mmol) were dissolved in 5 mL anhydrous DMF and stirred for 1 h under Ar. Then, propargylamine (0.35 mL, 5.5 mmol) was added in one portion and the reaction was stirred for overnight. The mixture was concentrated and dissolved in EtOAc. The solution was washed with 0.5 N NaHSO_4 and dried over Na_2SO_4 . The solvent was removed and the crude product triturated with DCM to give 631 mg white crystal in 47% yield. ^1H NMR (CDCl_3 , 400 MHz): δ 3.95 (d, 2H, $J=2.5$), 3.02 (m, 4H),

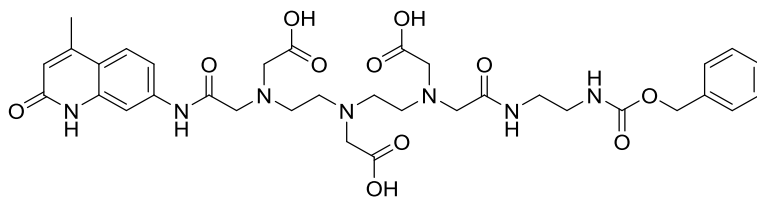
2.20 (t, 1H, $J=2.5$), 2.15 (t, 2H, $J=8.0$), 1.59 (m, 2H), 1.44 (m, 2H), 1.39 (s, 9H), 1.28 (m, 2H). ^{13}C NMR (CDCl_3 , 100 MHz): δ 173.38, 156.46, 79.57, 71.12, 40.14, 35.83, 29.41, 28.79, 28.28, 26.07, 24.98. LC/MS (ESI+) m/z : 269.2 $[\text{M}+\text{H}]^+$, 169.2 $[\text{M}(-\text{Boc})+\text{H}]^+$.

N-(prop-2-yn-1-yl)-6-(tritylamino)hexanamide (58)

Ap₃-rAcp-Trt



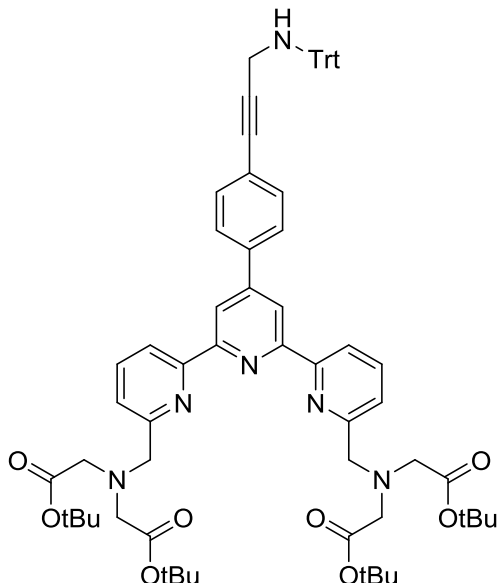
Compound **57** (300 mg, 1.12 mmol) was dissolved in 10 mL 50% TFA in DCM and stirred at rt for 2 h. The reaction was concentrated and the product triturated by a dropwise addition of cold ether. The product was collected by centrifugation and then dried under vacuum to give 296 mg of the deprotected intermediate in 94% yield. Then the white solid was dissolved in 3 mL anhydrous DMF and TEA (775 μL , 5.6 mmol) and trityl chloride (342 mg, 1.23 mmol) were added to the solution. After stirring for overnight at rt, DMF was removed under vacuum and the crude residue was dissolved in EtOAc. The solution was washed with saturated NaHCO_3 and then dried over Na_2SO_4 . The product was purified by a flash column chromatography using 0 - 50% EtOAc in hexane as the mobile phase. The product was concentrated to give 406 mg clear oil in 95% yield (88% yield over 2 steps). ^1H NMR (CDCl_3 , 400 MHz): δ 7.49 (m, 6H), 7.29 (m, 6H), 7.20 (m, 3H), 4.06 (dd, 2H, $J_A=5.0$, $J_B=2.5$), 2.23 (t, 1H, $J=2.5$), 2.17 (m, 4H), 1.62 (m, 2H), 1.52 (m, 2H), 1.37 (m, 2H). ^{13}C NMR (CDCl_3 , 100 MHz): δ 172.50, 146.14, 128.66, 127.76, 126.22, 79.63, 71.55, 71.01, 43.47, 36.32, 30.48, 29.14, 26.94, 25.45. LC/MS (ESI+) m/z : 411.2 $[\text{M}+\text{H}]^+$.

AMQ-DTPA-EDA-Cbz (59)

DTPA bisanhydride (479 mg, 1.34 mmol), DMAP (16.4 mg, 0.13 mmol), and DIPEA (1.4 mL, 8.0 mmol) was dissolved together in 100 mL anhy. DMF and a solution of AMQ (117 mg, 0.67 mmol) in 10 mL anhy. DMF was added to this solution at a rate of 1 mL/hr. After 5 hr of AMQ addition, a solution of Z-EDA **28** (671 mg, 2.0 mmol) in 10 mL anhy. DMF was added to the reaction at a rate of 1 mL/hr. The reaction was left to stir under Ar overnight and then quenched by the addition of excess deionized water. The solution was concentrated under reduced pressure and redissolved in methanol for reversed-phase purification using a 30 to 90% gradient of methanol containing 0.1% TFA in 45 min at a flow rate of 32 mL/min. The product was lyophilized to give 279 mg (48.1%) off-white powder. ^1H NMR (DMSO- d_6 , 400 MHz): δ 11.55 (br, 1H), 10.23 (br, 1H), 8.13 (br 1H), 7.69 (s, 1H), 7.56 (d, 1H, $J=9$), 7.26 (m, 6H), 6.20 (s, 1H), 4.92 (s, 2H), 4.27 (s, 2H), 3.51 (m, 6H), 3.33 (br, 6H), 3.02 (m, 8H), 2.30 (s, 3H). LC/MS (ESI+) m/z : 726.5 $[\text{M}+\text{H}]^+$, 592.5 $[(\text{M}-\text{Cbz})+\text{H}]^+$, 363.9 $[\text{M}+2\text{H}]^{2+}$.

Tetra-tert-butyl 2,2',2'',2'''-(((4'-(4-(3-(tritylamino)prop-1-yn-1-yl)phenyl)-[2,2':6',2''-terpyridine]-6,6''-diyl)bis(methylene))bis(azanetriyl))tetraacetate

TMT(OtBu)₄-Ap₃-Trt (60)

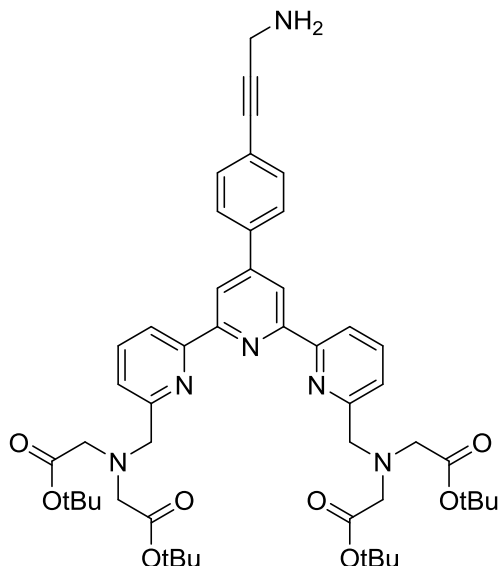


N-Trt-TMT(OtBu)₄-Ap₃-Trt (**60**) was synthesized according to the literature procedure.^{144,145}

Briefly, a solution of TMT(OtBu)₄-bromide (208 mg, 230 μ mol) in 2 mL TEA was deaerated with Ar. Then, tetrakis-triphenylphosphine palladium (Pd(PPh₃)₄, 6.4 mg, 5.54 μ mol) and CuI (1.3 mg, 6.65 μ mol) was added to the solution, followed by the dropwise addition of N-Trt-propargylamine (79 mg, 266 μ mol) in 0.5 mL deaerated TEA. The reaction was heated to 80 °C and stirred overnight and TEA was removed under vacuum. The residue was dissolved in 50 mL EtOAc and the organic layer washed 3 times with 20 mL saturated NaHCO₃, followed by 20 mL brine. The organic fraction was dried over sodium sulfate and purified by flash column chromatography using 0-25% ethyl acetate in hexane containing 5% TEA. The product was concentrated under vacuum to give 171 mg off-white crystalline solid in 85% yield. *Tert*-butyl esters and Trityl-group were deprotected and the m/z of $[M+H]^+$ = 653.0 corresponding to the free carboxylic acid form was detected on a LC/MS system. ¹H NMR (CDCl₃, 400 MHz): δ 8.72 (s, 2H), 8.53 (d, 2H, J=7.6), 7.85 (m, 4H), 7.70 (d, 2H, J=7.6), 7.54 (m, 8H), 7.32 (m, 6H), 7.22 (m, 4H), 4.18 (s, 4H), 3.56 (s, 8H), 3.25 (s, 2H), 1.46 (s, 36H).

Tetra-tert-butyl 2,2',2'',2'''-(((4'-(4-(3-aminoprop-1-yn-1-yl)phenyl)-[2,2':6',2''-terpyridine]-6,6''-diyl)bis(methylene))bis(azanetriyl))tetraacetate

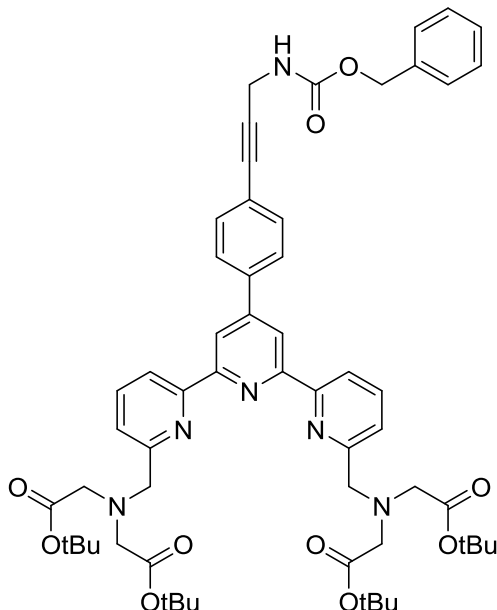
TMT(OtBu)₄-Ap₃ (61)



A solution of compound **60** (50 mg, 0.0447 mmol) in 2.5 mL 2% TFA/DCM solution containing 5% triisopropylsilane was stirred vigorously for 5 h at rt. The reaction mixture was neutralized by the addition of excess TEA (100 μ L) and concentrated to brown oil. The crude product was purified by silica gel flash column chromatography using 0-50% ethyl acetate in hexane containing 2% TEA as the mobile phase. The fraction containing the product was combined and concentrated to give 39.3 mg off-white solid in 94% yield. ¹H NMR (CDCl₃, 400 MHz): δ 8.71 (s, 2H), 8.53 (d, 2H, J=7.6), 7.85 (m, 4H), 7.70 (d, 2H, J=7.6), 7.55 (m, 2H), 4.17 (s, 4H), 3.71 (s, 2H), 3.56 (s, 8H), 1.46 (s, 36H). LC/MS (ESI+) *m/z*: 876.9 [M+H]⁺.

Tetra-tert-butyl 2,2',2'',2'''-(((4'-(4-(3-(((benzyloxy)carbonyl)amino)prop-1-yn-1-yl)phenyl)-[2,2':6',2''-terpyridine]-6,6''-diyl)bis(methylene))bis(azanetriyl))tetraacetate

TMT(OtBu)₄-Ap₃-Cbz (62)

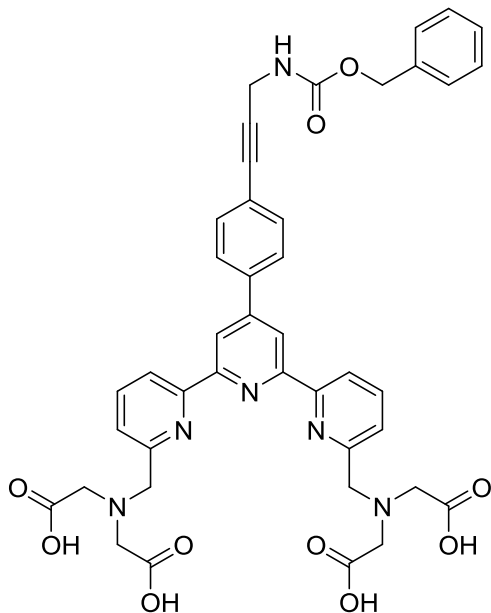


A solution of compound **61** (16.5 mg, 0.0224 mmol) in 2 mL anhydrous DCM added 15.5 μ L TEA (0.112 mmol) and 4.8 μ L benzyl chloroformate (0.269 mmol). The reaction was stirred overnight at rt and then concentrate under reduced pressure. The residue was dissolved in 4 mL ethyl acetate and washed 3 times with 1 mL deionized water, followed by brine wash. The organic layer was dried over sodium sulfate and the product was purified by flash column chromatography using 0-50% ethyl acetate in hexane containing 5% TEA as the mobile phase. The broad peak corresponding to the product was collected and concentrated to give 5 mg clear oil in 22.1% yield. *Tert*-butyl esters were deprotected from a small amount of the sample and the m/z of $[M+H]^+ = 787.6$ corresponding to the free carboxylic acid form was detected on a LC/MS system. ^1H NMR (CDCl_3 , 400 MHz): δ 8.72 (s, 2H), 8.54 (d, 2H, $J=7.6$), 7.86 (m, 4H), 7.71 (d, 2H, $J=7.6$), 7.55 (m, 2H), 7.41-7.31 (m, 5H), 5.17 (s, 2H), 4.28 (br, 2H), 4.19 (s, 4H), 3.57 (s, 8H), 1.46 (s, 36H). ^{13}C NMR (CDCl_3 , 100 MHz): δ 170.50, 156.09, 155.22, 149.05, 138.78, 137.54,

136.30, 132.35, 132.25, 128.56, 128.18, 127.35, 127.22, 123.40, 123.11, 119.72, 118.66, 86.17, 83.27, 81.12, 67.14, 59.86, 55.81, 28.19.

2,2',2'',2'''-(((4'-(4-(3-(((benzyloxy)carbonyl)amino)prop-1-yn-1-yl)phenyl)-[2,2':6',2''-terpyridine]-6,6''-diyl)bis(methylene))bis(azanetriyl))tetraacetic acid

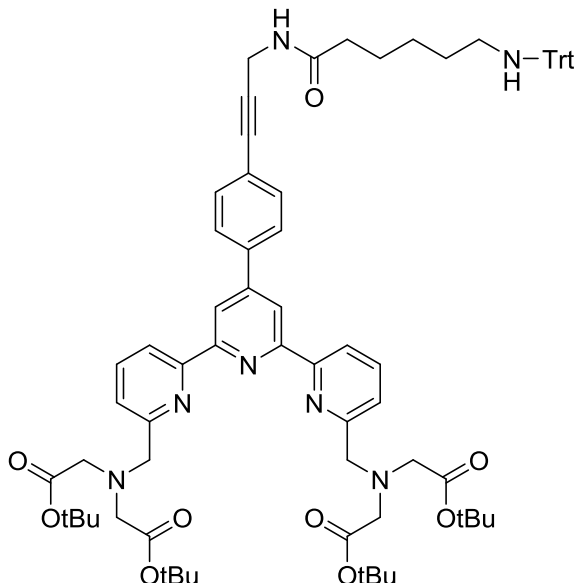
TMT-Ap₃-Cbz (63)



The compound **62** (5 mg, 0.005 mmol) was treated with 1 mL 95% TFA solution containing 5% deionized water for 3 h at rt. After disappearance of the starting material on the LC/MS UV chromatogram, the solution was gently concentrated to 10% volume by N₂. The product was triturated by the dropwise addition of cold ether and the precipitate was collected by centrifugation. The crude product was dissolved in 100 μ L 50% ACN/H₂O and subjected to purification by RP-HPLC system using 10-90% acetonitrile in water containing 0.1%TFA as the mobile phase. The fractions containing the product were combined and lyophilized to give 2.3 mg product as white powder in 59% yield. In addition to the desired product, 1.2 mg (38%) of Cbz-deprotected side product was collected. LC/MS (ESI+) *m/z*: 787.6 [M+H]⁺. HRMS (ESI+) *m/z* calc'd for C₄₂H₃₈N₆O₁₀: [M+H]⁺=787.2722, found 787.2738.

Tetra-tert-butyl 2,2',2'',2'''-(((4'-(4-(3-(6-(tritylamino)hexanamido)prop-1-yn-1-yl)phenyl)-[2,2':6',2''-terpyridine]-6,6''-diyl)bis(methylene))bis(azanetriyl))tetraacetate,

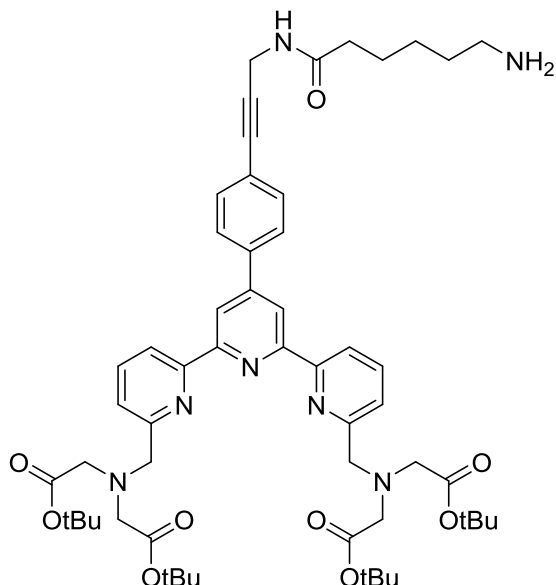
TMT(OtBu)₄-Ap₃-rAcp-Trt (64)



N-Trt-TMT(OtBu)₄-Ap₃-rAcp (**64**) was synthesized according to the literature procedure described for **60** except using the Acp extended propargyl amine linker for the Pd-catalyzed Sonogashira coupling.^{144,145} The product **64** was obtained as 279 mg yellow crystals in 99% yield. ¹H NMR (CDCl₃, 400 MHz): δ 8.71 (s, 2H), 8.54 (d, 2H, $J=7.6$), 7.85 (m, 4H), 7.70 (d, 2H, $J=7.6$), 7.55-7.38 (m, 10H), 7.28-7.14 (m, 6H), 4.17 (s, 4H), 3.55 (s, 8H), 2.23-2.00 (m, 4H), 1.64 (m, 2H), 1.46 (s, 36H), 1.26 (m, 6H), 0.83 (m, 1H). LC/MS (ESI+) m/z : 990.6 [(M-Trt)+H]⁺, 496.3 [(M-Trt)+2H]²⁺.

Tetra-tert-butyl 2,2',2'',2'''-(((4'-(4-(3-aminoprop-1-yn-1-yl)phenyl)-[2,2':6',2''-terpyridine]-6,6''-diyl)bis(methylene))bis(azanetriyl))tetraacetate

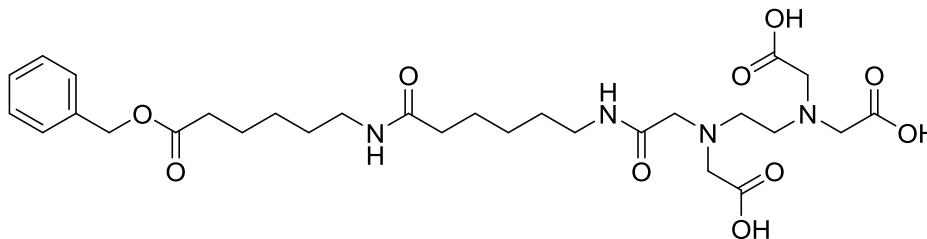
TMT(OtBu)₄-Ap₃-rAcp (65)



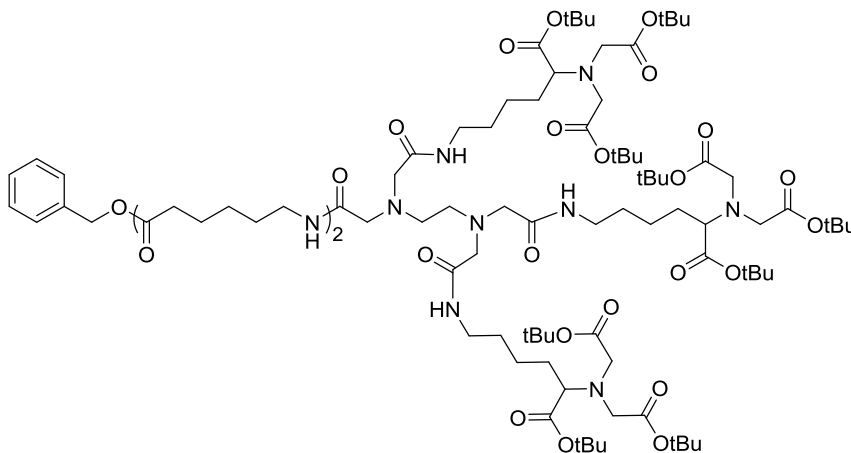
Compound **64** (50 mg, 0.045 mmol) in 1.5 mL 2% TFA/DCM solution containing 5% deionized water was stirred vigorously for 3 h at rt. The reaction mixture was neutralized by the addition of TEA (62 μ L, 0.447 mmol) and concentrated to brown oil. The crude product was purified by silica gel flash column chromatography using 0-50% ethyl acetate in hexane containing 2% TEA as the mobile phase. The fraction containing the product was combined and concentrated to give 27 mg yellow oil in 69% yield. ¹H NMR (CDCl₃, 400 MHz): δ 8.64 (s, 2H), 8.46 (d, 2H, J=7.9), 7.80-7.76 (m, 4H), 7.63 (d, 2H, J=7.9), 7.53-7.47 (m, 2H), 4.10 (s, 4H), 3.64 (s, 2H), 3.48 (s, 8H), 1.39 (s, 36H), 1.33-1.17 (m, 8H), 0.83 (m, 2H). ¹³C NMR (CDCl₃, 100 MHz): δ 170.65, 158.81, 156.17, 155.28, 149.09, 137.42, 132.25, 132.23, 132.08, 127.23, 127.18, 123.32, 119.63, 118.58, 80.96, 59.92, 55.81, 32.24, 29.68, 28.19, 28.02. LC/MS (ESI+) *m/z*: 991.1 [M+H]⁺, 496.2 [M+2H]²⁺.

19,22-bis(carboxymethyl)-3,10,17-trioxo-1-phenyl-2-oxa-9,16,19,22-tetraazatetracosan-24-oic acid

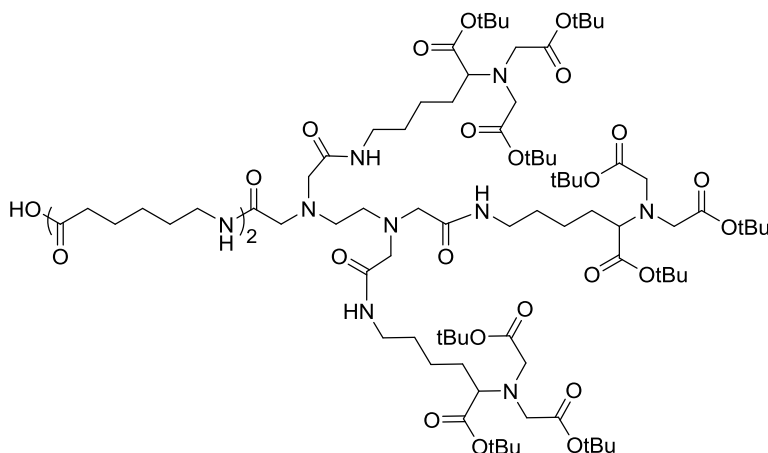
BzlO-rAcp₂-EDTA (66)



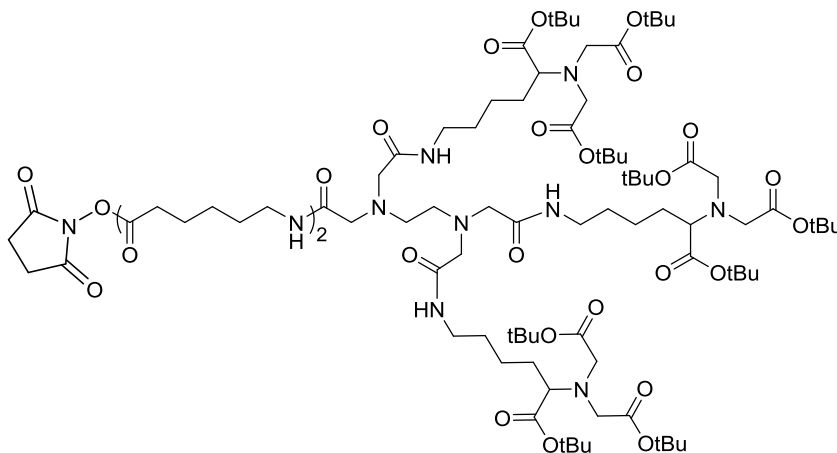
To a solution of EDTA bisanhydride (**27**, 1.40 g, 5.47 mmol) in 30 mL anhydrous DMF under Ar was added 1.23 mL TEA (8.83 mmol). Then, a solution of H-Acp₂-OBzl (**38**, 789 mg, 1.77 mmol) in 30 mL anhydrous DMF added over a period of 6 h using a syringe pump (5 mL/h). After the addition of **72**, the reaction was continued to stir for 2 h and then 20 mL deionized water was added. The reaction was stirred overnight. The solvent was removed under vacuum and the residue was dissolved in 10 mL 20% acetonitrile /water. The precipitate was removed by centrifugation at 10,000 rpm and the supernatant was subjected to purification by the reversed-phase C₁₈ flash column chromatography using 30-70% acetonitrile in water containing 0.1%TFA as the mobile phase. The fractions containing the product were combined and lyophilized to give 216 mg white powder in 21% yield. ¹H NMR (MeOH-d₄, 400 MHz): δ 7.36 (m, 5H), 5.13 (s, 2H), 3.94 (s, 4H), 3.83 (s, 2H), 3.78 (s, 2H), 3.25 (m, 6H), 3.16 (m, 2H), 2.40 (t, 2H, J=7), 2.20 (t, 2H, J=7), 1.69-1.47 (m, 8H), 1.36 (m, 4H). ¹³C NMR (MeOH-d₄, 100 MHz): δ 176.11, 175.12, 172.18, 171.90, 169.86, 137.74, 129.56, 129.19, 67.18, 57.62, 55.85, 52.86, 52.76, 40.25, 40.16, 36.95, 34.94, 30.04, 29.93, 27.49, 27.41, 26.65, 25.68. LC/MS (ESI+) *m/z*: 609.5 [M+H]⁺.

BzlO-rAcp₂-EDTA-tris-Lys-NTA(OtBu)₃ (67**)**

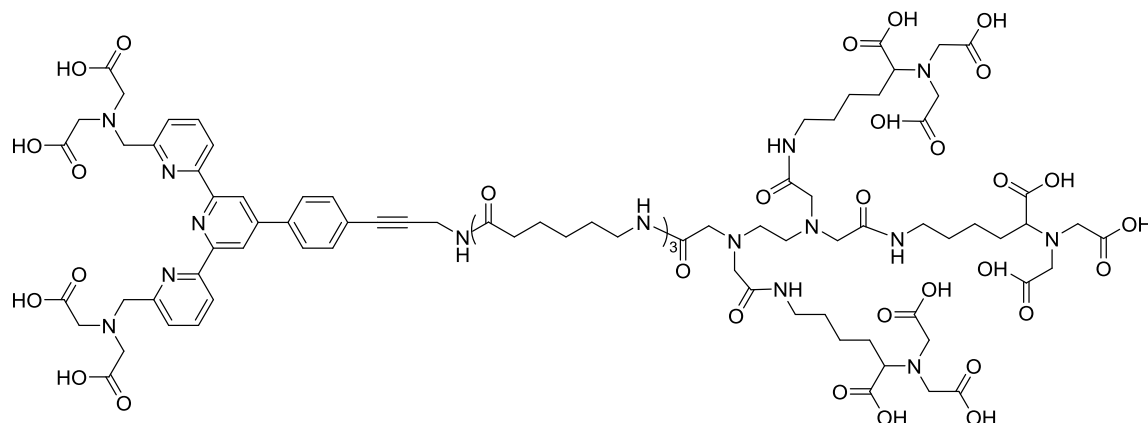
To a solution of BzlO-rAcp₂-EDTA (**66**, 250 mg, 0.41 mmol), Lys-NTA(OtBu)₃ (**10**, 707 mg, 1.64 mmol), PyBOP (855 mg, 1.64 mmol), and DMAP (15 mg, 0.12 mmol) in 10 mL anhydrous DMF was added TEA (0.46 mL, 3.3 mmol) under Ar. The reaction was stirred vigorously for 12 h at rt. The reaction was concentrated under vacuum and the residue dissolved in 25 mL EtOAc. The organic layer washed with 15 mL deionized water (×3) and brine, and the organic layer was dried over sodium sulfate. The solvent was removed under vacuum and the crude residue subjected to silica gel flash column chromatography using 0-20% methanol in DCM. Fractions containing the product were combined and concentrated to give 291 mg yellow oil in 38% yield. Small amount of the product was deprotected using TFA and m/z (1342.1 [M(-9×tBu)+H]⁺, 672.2 [M(-9×tBu)+2H]²⁺) corresponding to the free carboxylic acid of **67** was detected on a LC/MS system. ¹H NMR (CDCl₃, 400 MHz): δ 8.15 (s, 1H), 7.37 (m, 5H), 6.07 (s, 1H), 5.13 (s, 2H), 3.44 (m, 16H), 3.33-3.23 (m, 18H), 2.38 (t, 2H, J=7.5), 2.16 (t, 2H, J=7.5), 1.67 (m, 12H), 1.55 (m, 12H), 1.48 (s, 27H), 1.46 (s, 54H), 1.43-1.32 (m, 9H).

HO-rAcp₂-EDTA-tris-Lys-NTA(OtBu)₃ (68)

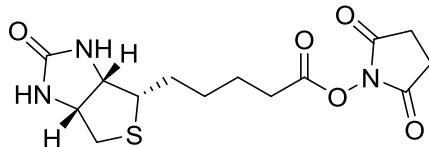
A solution of BzlO-rAcp₂-EDTA-tris-Lys-NTA(OtBu)₃ (**67**, 100 mg, 0.054 mmol) in 5 mL MeOH was deaerated with N₂. Then, catalytic amount of 20% Pd(OH₂)/C was added in one portion and the reaction vessel charged with H₂. The reaction was hydrogenated overnight and the catalyst removed by filtration through a celite bed. The filtrate was concentrated to give 97 mg pure product **51** in 100% yield. ¹H NMR (MeOH-d₄, 400 MHz): δ 3.43-3.03 (m, 32H), 2.44 (m, 4H), 2.10 (m, 2H), 1.72-1.46 (m, 24H), 1.38 (m, 81H) 1.27-1.15 (m, 9H). LC/MS (ESI+) *m/z*: 1755.1 [M+H]⁺.

SuO-rAcp₂-EDTA-tris-Lys-NTA(OtBu)₃ (69)

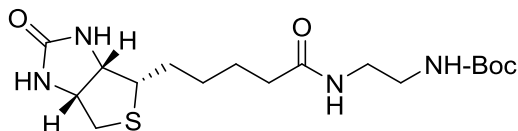
A solution of HO-rAcp₂-EDTA-tris-Lys-NTA(OtBu)₃ (**68**, 97 mg, 0.054 mmol), EDC (16 mg, 0.083 mmol), and HOSu (7.0 mg, 0.061) in 1.5 mL DCM was stirred overnight at rt under Ar. Then, the reaction mixture was washed with water (1 mL ×3), followed by brine. The solution was dried over sodium sulfate and concentrated to give 91 mg clear oil in 89% yield. The product was used directly for the next step. A small amount of the product was derivatized with benzylamine and t-butyl esters were deprotected for analysis on a LC/MS system. The LC/MS detected *m/z* corresponding to the t-butyl deprotected derivatized product 1340.1 [M+H]⁺.

TMT-Ap₃-rAcp₃-EDTA-tris-Lys-NTA (70)

To a solution of TMT(OtBu)₄-Ap₃-rAcp (65, 30 mg, 0.030 mmol) and SuO-rAcp₂-EDTA-tris-Lys-NTA(OtBu)₃ (69, 61 mg, 0.033 mmol) in 0.5 mL anhydrous DMF added 33 μ L TEA (0.24 mmol). The reaction was stirred for 2 days at rt and then concentrated under reduced pressure. The remaining residue was treated with 1.5 mL TFA solution containing 5% water (v/v). The solution was mixed vigorously for 3 h and the volatiles removed by N₂. The crude product triturated by cold diethyl ether and the precipitate collected by centrifugation (10.7 mg crude). The solid was dissolved in 10 mM HEPES buffer and purified on using reversed-phase HPLC (YMC-Pack ODS-AQ 150x4.6 5 μ) using 20-30% acetonitrile in water containing 0.1%TFA as the mobile phase. The fractions containing the product was combined and lyophilized to give 4 mg brown crystalline solid (6.7% yield). LC/MS (ESI+) m/z 1000.5 [M+2H]²⁺, 668.1 [M+3H]³⁺. HRMS (ESI+) m/z calc'd for C₉₂H₁₂₇N₁₇O₃₃: [M+H]⁺=1998.8855, [M+2H]²⁺=999.9464, [M(¹³C)+2H]²⁺=1000.4481, found 999.9510, 1000.4521.

D-biotin-OSu (71)

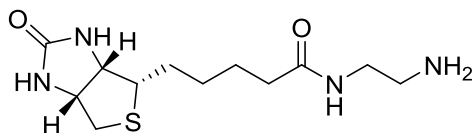
D-biotin (25.3 mg, 0.109 mmol), EDC (25.2 mg, 0.131 mmol), and HOSu (13.9 mg, 0.121 mmol) were dissolved in 1 mL anhydrous DMF and stirred overnight at rt. DMF was removed under reduced pressure and the residue dissolved in 10 mL DCM. The organic layer washed 3 times with 5 mL deionized water, followed by 3 mL brine. The DCM layer was dried over Na₂SO₄ and concentrated to give 13 mg white powder in 42% yield. The OSu ester was used directly for the next step.

Tert-butyl (2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethyl)carbamate**D-biotin-EDA-Boc (72)**

To a solution of D-biotin-OSu ester (**71**, 13 mg, 0.0454 mmol) and Boc-ethylenediamine (8.7 mg, 0.0545 mmol) in 1 mL anhydrous DMF was added 17.7 μ L TEA (0.136 mmol). The reaction was stirred overnight at rt and the solvent removed under reduced pressure. The crude residue was dissolved in 5 mL water and the aqueous phase washed 3 times with DCM. The water was removed by lyophilization and the white powder was obtained. The powder was dissolved in 20 μ L methanol and then triturated with ether. The trituration was repeated 3 times. The white solid was dried under vacuum to give 17 mg product in 97% yield. ¹H NMR (MeOH-d₄, 400 MHz): δ 4.51 (m, 1H), 4.32 (m, 1H), 3.26 (m, 2H), 3.16 (m, 2H), 2.95 (dd, 1H, J_A=12.5, J_B=5), 2.73 (m, 2H), 2.22 (t, 2H, J=8), 1.80-1.57 (m, 4H), 1.46 (m, 12H). ¹³C NMR (MeOH-d₄, 100 MHz): δ

176.38, 166.13, 158.60, 80.16, 63.37, 61.66, 56.94, 41.03, 40.52, 36.82, 29.75, 29.47, 28.77, 28.71, 26.76, 26.19. LC/MS (ESI+) m/z : 387.7.

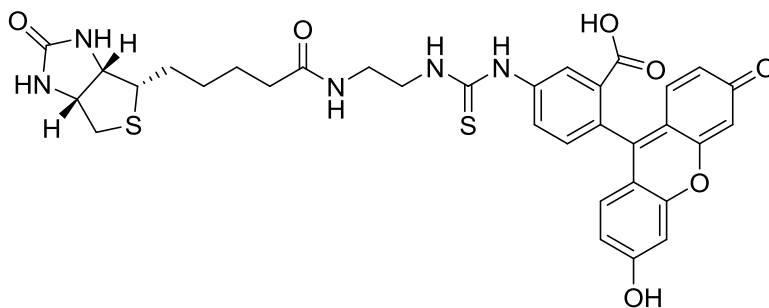
D-biotin-EDA (73)



To a solution of D-biotin-EDA-Boc (**72**, 10 mg, 0.0259 mmol) in 400 μ L DCM was added 400 μ L TFA. The reaction was stirred for 2 h at rt and then concentrated to 30% volume using N_2 . The product was triturated by the dropwise addition of cold diethyl ether and the precipitate was collected by centrifugation at 10,000 rpm to give 7.4 mg solid. The product was used directly for the next step. LC/MS (ESI+) m/z : 287.3 $[M+H]^+$.

2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-(3-(2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethyl)thioureido)benzoic acid

D-biotin-EDA-FITC (74)



To a solution of D-biotin-EDA (**73**, 7.4 mg, 0.025 mmol) in 50 μ L anhydrous DMSO were added FITC (12 mg, 0.031 mmol) in 50 μ L anhydrous DMSO and TEA (7.3 μ L, 0.052 mmol). The reaction was stirred overnight at rt while covered from light. The reaction mixture was diluted 10 $\times$ with 50% methanol in water and subjected to RP-HPLC purification (Phenomenex Jupiter C_{18} 250 $\times$ 10 mm 5 μ) using a gradient of 20-40% acetonitrile in water containing 0.1%TFA. The

product was lyophilized to give 5.1 mg yellow powder in 29% yield. ^1H NMR (MeOH-d_4 , 400 MHz): δ 8.23 (s, 1H), 7.85 (d, 1H, $J=8.4$), 7.28 (d, 1H, $J=8$), 7.00 (m, 2H), 6.90 (s, 2H), 6.76 (d, 1H, $J=8.4$), 4.47 (m, 1H), 4.28 (m, 1H), 3.49 (m, 2H), 3.20 (m, 2H), 2.90 (dd, 1H, $J_A=12.5$, $J_B=5$), 2.73 (m, 2H), 2.25 (t, 2H, $J=8$), 1.77-1.54 (m, 4H) 1.44 (m, 2H), 1.30 (m, 1H). LC/MS (ESI+) m/z : 676.3 $[\text{M}+\text{H}]^+$.

V. TR-FRET Assay Procedures.

A. Derivatization of tris-NTA samples to determine the concentration

A 1 mM solution of tris-NTA samples were prepared in the 10 mM HEPES assay buffer. Each sample were derivatized separately in order to minimize the sample storage time which was found to decrease the accuracy. The OPA/NBC derivatization solutions were prepared fresh by dissolving 11 mg of *o*-phthaldialdehyde (OPA) and 20 mg of *N*-Boc-L-cysteine (NBC) each in 1 mL of methanol. The derivatization was performed in triplicates by adding 50 μL of 0.1 M $\text{Na}_2\text{B}_2\text{O}_7$ (pH 9.6), 50 μL deionized water, 20 μL OPA solution, and 20 μL NBC solution to each tris-NTA sample solution. The solution was mixed vigorously for 5 min, centrifuged, and transferred (80 μL) to a Corning 3650 96-well NBS black plate. The fluorescence of the derivatized adduct was measured using Victor 3V plate reader with excitation and emission wavelengths set at 355 and 460 nm, respectively. The concentration of the tris-NTA sample was calculated based on the standard curve of generated using a known concentration of NTA and Acp.

B. The time-resolved luminescence measurement to determine:

1. The effect of LnCl_3 concentrations on lanthanide complexes formation

The conjugate (5 μ L, 2 μ M) and LnCl_3 (5 μ L, $\text{Ln} = \text{Tb}$ or Eu , concentration depending on the assay range) were mixed and incubated for 20 min at rt on a shaker. Then, the Ln(III) -complex was diluted 100 $\times$ with the HEPES buffer to give 10 nM solution. The diluted solution was seeded to a Corning 3650 black NBS (non-binding surface) 96-well plate and centrifuged at 1500 rpm for 2 min. The plate was read using Victor 3V plate reader set to 340 nm excitation and the emitted photons were counted for 200 μ s at 545 nm for Tb TRL and 615 nm for Eu TRL after 100 μ s gating time.

2. The effect of NiCl_2 concentrations on lanthanide luminescence

The conjugate (25 μ L, 10 μ M), LnCl_3 (25 μ L, $\text{Ln} = \text{Tb}$ or Eu , 20 μ M), and buffer (175 μ L) were mixed and incubated for 20 min at rt on a shaker. The Tb-complex solution was separated into 9 aliquots (18 μ L each), and NiCl_2 solution (2 μ L, 3840 μ M to 30 μ M by 2-fold serial dilutions, and 0 μ M) was added to each aliquot. The solutions were incubated for additional 60 min at rt on the shaker. The resulting Ni(II) complex were diluted 100 $\times$ with buffer and then seeded to Corning 3650 black NBS 96 wells plate and centrifuged at 1500 rpm for 2 min. The plate was read using Victor 3V plate reader set to 340 nm excitation and the emitted photons were counted for 200 μ s at 545 nm for Tb TRL and 615 nm for Eu TRL after 100 μ s gating time.

3. The effect of LnCl_3 concentrations on TR-FRET assay signal

The conjugate (5 μ L, 4 μ M) and LnCl_3 (5 μ L, $\text{Ln} = \text{Tb}$ or Eu , 4 - 28 μ M) were mixed and incubated at rt for 20 mins. Then, 10 μ L of each Ln-complex solution were added Keap 1 (5 μ L, 4 μ M) and NiCl_2 (5 μ L, 48 μ M) and the resulting mixtures were incubated at rt for 60 mins. The Ni(II) -complex solutions (10 μ L) were diluted 75 $\times$ with buffer (740 μ L). Then, 30 μ L of the

diluted solution was seeded to Corning 3575 black NBS 384- wells plate in triplicate, followed by 10 μ L of 80 nM peptide tracer solution to each well. The plate was centrifuged at 1500 rpm for 2 min and read using Victor 3V plate reader set to 340 nm excitation and the emitted photons were counted for 200 μ s at 520 and 545 nm for Tb TR-FRET. For the Eu TR-FRET the counting window was set to 300 μ s and the emitted photons were measured at 615 and 670 nm. The gating times for Tb TR-FRET and the Eu TR-FRET were set to 200 μ s and 50 μ s, respectively.

4. The effect of NiCl₂ concentrations in TR-FRET assay

The conjugate (5 μ L, 4 μ M) and LnCl₃ (5 μ L, Ln = Tb or Eu, 6 μ M for Tb and 4 μ M for Eu) were mixed and incubated at rt for 20 mins. Then, 10 μ L of each Ln-complex solution were added Keap 1 (5 μ L, 4 μ M) and NiCl₂ (5 μ L, 0 - 84 μ M) and the resulting mixtures were incubated at rt for 60 mins. The Ni(II)-complex solutions (10 μ L) were diluted 75 $\times$ with buffer (740 μ L). Then, 30 μ L of the diluted solution was seeded to Corning 3575 black NBS 384- wells plate in triplicate, followed by 10 μ L of 80 nM peptide tracer solution to each well. The plate was centrifuged at 1500 rpm for 2 min and read using Victor 3V plate reader set to 340 nm excitation and the emitted photons were counted for 200 μ s at 520 and 545 nm for Tb TR-FRET. For the Eu TR-FRET the counting window was set to 300 μ s and the emitted photons were measured at 615 nm and 670 nm. The gating times for Tb TR-FRET and the Eu TR-FRET were set to 200 μ s and 50 μ s, respectively.

5. The effect of probe concentrations in TR-FRET assay

The conjugate (5 μ L, 4 μ M) and LnCl_3 (5 μ L, $\text{Ln} = \text{Tb}$ or Eu , 6 μ M for Tb and 4 μ M for Eu) were mixed and incubated at rt for 20 mins. Then, 10 μ L of each Ln-complex solution were added Keap 1 (5 μ L, 4 μ M) and NiCl_2 (5 μ L, 48 μ M) and the resulting mixtures were incubated at rt for 60 mins. The Ni(II) -complex solutions (20 μ L) were diluted 75 $\times$ with buffer (1480 μ L). Then, 30 μ L of the diluted solution was seeded to Corning 3575 black NBS 384- wells plate, followed by 10 μ L (0.2 - 400 nM) of peptide tracer solutions in triplicate. The plate was centrifuged at 1500 rpm for 2 min and read using Victor 3V plate reader set to 340 nm excitation and the emitted photons were counted for 200 μ s at 520 and 545 nm for Tb TR-FRET. For the Eu TR-FRET the counting window was set to 300 μ s and the emitted photons were measured at 615 nm and 670 nm. The gating times for Tb TR-FRET and the Eu TR-FRET were set to 200 μ s and 50 μ s, respectively. Finally, the inhibitor (Ac-9mer Nrf2, 4 μ L, 11 μ M) was added to each well to disrupt the donor-acceptor pair, and the TR-FRET signal was recorded as the background of TR-FRET.

B. Comparison of acceptor fluorophores for Tb TR-FRET

The conjugate (5 μ L, 4 μ M) and TbCl_3 (5 μ L, 6 μ M) were mixed and incubated at rt for 20 mins. Then, 10 μ L of each Ln-complex solution were added Keap 1 (5 μ L, 4 μ M) and NiCl_2 (5 μ L, 48 μ M) and the resulting mixtures were incubated at rt for 60 mins. The Ni(II) -complex solutions (10 μ L) were diluted 75 $\times$ with buffer (740 μ L). Then, 30 μ L of the diluted solution was seeded to Corning 3575 black NBS 384- wells plate in triplicate, followed by 10 μ L of 80 nM peptide tracer (FITC-9mer Nrf2 amide or Bodipy-FL-9mer Nrf2 amide) solution to each well. The plate was centrifuged at 1500 rpm for 2 min and read using Victor 3V plate reader set to 340 nm excitation and the emitted photons were counted for 200 μ s at 520 and 545 nm. Then, the

inhibitor (Ac-9mer Nrf2, 4 μ L, 11 μ M) was added to each well to disrupt the donor-acceptor pair, and the TR-FRET signal was recorded as the background of TR-FRET.

C. Application of TR-FRET to Biotin-SA interaction

1. FP assay to determine the binding between biotin-EDA-FITC and streptavidin

The FP assay was performed as described in the FP assay procedure (Experimental, Section II) and the buffer used for the FP assays was 10 mM HEPES pH 7.4 buffer containing 50 mM EDTA, 150 mM NaCl, and 0.005% Tween-20. The stock solution of recombinant His₆-tag streptavidin (1 mg/mL) was diluted to 80 nM (monomer concentration) and then diluted from 80 nM to 20 pM over 13 concentrations by 2-fold serial dilutions. The tracer (biotin-EDA-FITC) concentration used for the FP assay was 4 nM as it was determined to be the lowest concentration of the tracer that provides FP measurement in the linear range. To the assay plate (Corning 3575) were transferred in triplicates 20 μ L of the SA solutions at various concentrations and 20 μ L of tracer solution. The plate was incubated on the shaker for 30 min at rt and then centrifuged at 1500 rpm for 2 min. The plate was read using Victor 3V plate reader with excitation and emission set for fluorescein at 485 and 535 nm, respectively.

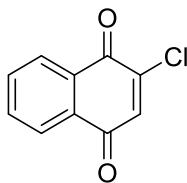
2. TR-FRET assay for biotin-SA interaction

The Tb-complex and the subsequent complexation with the target protein (His₆-SA) was performed as described for Tb TR-FRET (Experimental, Section V) and in Scheme 21. Briefly, 10 μ L of the conjugate (4 μ M) and 10 μ L TbCl₃ (6 μ M) solutions were mixed and incubated on a shaker for 20 min. To the resulting Tb-complex were added 10 μ L of NiCl₂ (48 μ M) and 10 μ L of SA (4 μ M monomer) and the solution was incubated on a shaker for additional 60 min at rt. The

SA-complex obtained were split into 2 aliquots of 20 μL , where one aliquot is diluted 50 $\times$ with the buffer (980 μL) and the other aliquot diluted 50 $\times$ with 200 nM biotin solution (980 μL). The aliquot treated with biotin prior to addition of the tracer is used as the background of TR-FRET signal as the biotin-SA interaction is irreversible under assay conditions. Twenty μL of the diluted SA-complex solution was added to the assay plate wells (Corning 3575), and then 20 μL of the tracer solution at a range of concentrations (50 - 0 nM) was added to the plate in triplicates. The assay plate was briefly mixed on a shaker and then centrifuged at 1500 rpm for 2 min. The Tb TR-FRET signal was read using Victor 3V plate reader set to 340 nm excitation and the emitted photons were counted for 200 μs at 520 and 545 nm. The signal from the pre-incubated solutions were used as the background of the TR-FRET signal and subtracted from the sample wells without biotin.

IV. Synthesis of HTS hit compound and analogs

2-Chloro-1,4-naphthoquinone (75)



The compound was synthesized following the previously reported procedure using 2-hydroxy-1,4-naphthoquinone.¹⁵⁴ Firstly, 2-hydroxy-1,4-naphthoquinone (2.0 g, 1.49 mmol) was suspended in 35 mL SOCl_2 and refluxed overnight. Then, SOCl_2 was removed under reduced pressure and the remaining residue suspended in DCM. The organic layer was first washed 3 times with water, followed by a wash with brine, and finally dried over sodium sulfate. The solution was concentrated and the product recrystallized from ethanol (30 mL) to give 1.84 g gold crystalline

solid in 84% yield. ^1H NMR (CDCl_3 , 400 MHz) δ 8.19 (m, 1H), 8.11 (m, 1H), 7.79 (m, 2H), 7.24 (s, 1H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 182.65, 177.95, 146.37, 135.93, 134.46, 134.10, 131.83, 131.38, 127.51, 126.76.

General procedure (A) for synthesis of compounds 76a ~ e.

The general procedure for the nucleophilic addition of aryl sulfonamides to 2-chloro-1,4-naphthoquinone was performed following the previously reported procedure.¹⁵³ A solution of 2-chloro-1,4-naphthoquinone (**75**, 100 mg, 0.52 mmol, 1 equiv.) and Aryl sulfonamide (0.52 mmol, 1 equiv.) were cooled to 0 °C. Then, TiCl_4 (57.2 μL , 0.52 mmol, 1 equiv.) and TEA (160 μL , 1.15 mmol, 2.2 equiv.) were added to the cooled solution and the reaction color changed to dark green-grey. The mixture was heated in a microwave reactor for 15 min at 60 °C with 300 W power. The reaction mixture was concentrated under reduced pressure and the residue was suspended in 50% EtOAc/Hexane. The precipitate was collected by filtration and the solid dried under vacuum to give the product.

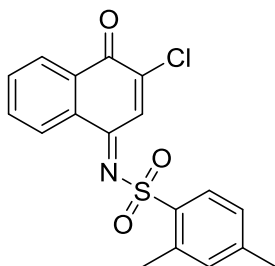
General procedure (B) for synthesis of compounds 77a ~ e

A solution of compound **76a ~ e** (1 equivalent) in anhydrous THF was cooled to 0 °C. Then, CsCO_3 (4 equivalent) was added in one portion, followed by a dropwise addition of isopropyl 4,4,4-trifluoroacetoacetate or a malonate (1 equivalent) into the solution. The mixture was stirred at 0 °C for additional 30 - 60 min. The reaction was allowed to slowly rise to rt and then the solution changed color from brown to blue. The reaction was stirred for additional 0 - 3 h at rt and then neutralized with 0.5 N NaHSO_4 to give a deep red solution. The volatiles were removed under reduced pressure and the remaining solution was added EtOAc. The organic phase was washed with 0.5N NaHSO_4 and then dried over Na_2SO_4 . The product in EtOAc was taken directly to the general procedure (C) for the reduction of the naphthoquinone moiety.

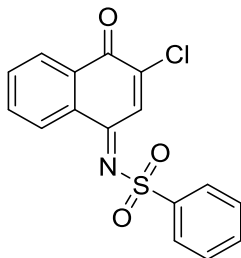
General procedure (C) for reduction of intermediates (77a ~ e) using Na₂S₂O₄.

The general procedure for the reduction of intermediates using sodium dithionite (Na₂S₂O₄) was performed following the previously reported procedure.¹⁵³ An ethyl acetate solution of the naphthoquinone intermediate for the synthesis of compounds **77a ~ e** was added deionized water and Na₂S₂O₄ (1 equivalent) and stirred for 30 min at rt. Additional equivalent of Na₂S₂O₄ was added every 30 min over the period of 2.5-h while monitoring the progress of the reduction using LC/MS. The aqueous layer was discarded and then the EtOAc layer washed 3 times with 0.5 N NaHSO₄, followed by brine. The organic solution was dried over sodium sulfate and concentrated to give the crude product which was either subjected to purification by silica gel flash column chromatography, RP-HPLC purification, or recrystallized from a mixture of DCM and hexane to obtain the pure product.

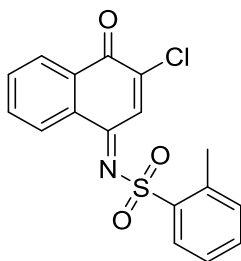
(E)-N-(3-chloro-4-oxonaphthalen-1(4H)-ylidene)-2,4-dimethylbenzenesulfonamide (76a)



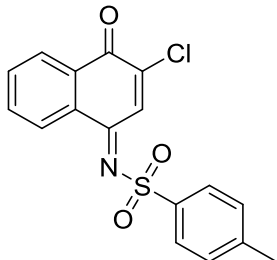
The general procedure (A) was used with 40 mg of 2-chloro-1,4-naphthoquinone (0.208 mmol) and 38.5 mg of 2,4-dimethylbenzenesulfonamide (0.208 mmol). The product was recrystallized from 50% EtOAc/Hex to give 69.4 mg product in 93% yield. ¹H NMR (CDCl₃, 400 MHz) δ 8.71 (s, 1H), 8.25 (dd, 1H, *J*_A=7.8, *J*_B=1.3), 8.18 (dd, 1H, *J*_A=7.8, *J*_B=1.3), 8.05 (d, 1H, *J*=7.8), 7.75 (m, 2H), 7.25-7.24 (s, 2H), 2.74 (s, 3H), 2.47 (s, 3H). LC/MS (ESI+) *m/z*: 359.9 [M+H]⁺.

(E)-N-(3-chloro-4-oxonaphthalen-1(4H)-ylidene)benzenesulfonamide (76b)

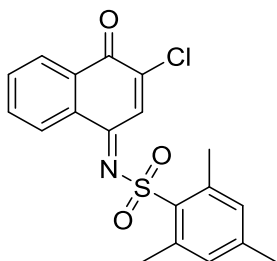
The general procedure (A) was used with 100 mg of 2-chloro-1,4-naphthoquinone (0.52 mmol) and 81.8 mg of benzenesulfonamide (0.52 mmol). The product was recrystallized from 50% EtOAc/Hex to give 122 mg yellow powder in 72% yield. ^1H NMR (CDCl_3 , 400 MHz) δ 8.70 (s, 1H), 8.20 (dd, 2H, $J_A = 8$ Hz, $J_B = 2$ Hz), 8.11 (m, 2H), 7.73 (m, 3H), 7.63 (m, 2H). LC/MS (ESI) m/z : 331.4 $[\text{M}+\text{H}]^+$.

(E)-N-(3-chloro-4-oxonaphthalen-1(4H)-ylidene)-2-methylbenzenesulfonamide (76c)

The general procedure (A) was used with 64 mg of 2-chloro-1,4-naphthoquinone (0.33 mmol) and 57 mg of 2-methylbenzenesulfonamide (0.33 mmol). The product was recrystallized from 30% EtOAc/Hex to give 89 mg yellow powder in 78% yield. ^1H NMR (CDCl_3 , 400 MHz) δ 8.65 (s, 1H), 8.18 (m, 3H), 7.73 (m, 2H), 7.58 (m, 1H), 7.42 (m, 2H), 2.74 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 176.92, 161.35, 144.87, 138.50, 138.19, 134.09, 133.81, 133.01, 131.41, 129.79, 128.50, 127.90, 127.79, 127.52, 126.64, 126.29, 20.62. LC/MS (ESI) m/z : 345.9 $[\text{M}+\text{H}]^+$.

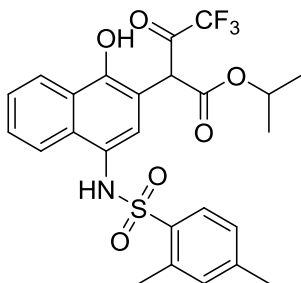
(E)-N-(3-chloro-4-oxonaphthalen-1(4H)-ylidene)-p-toluenesulfonamide (76d)

The general procedure (A) was used with 64 mg of 2-chloro-1,4-naphthoquinone (0.33 mmol) and 57 mg of p-toluenesulfonamide (0.33 mmol). The product was recrystallized from 30% EtOAc/Hex to give 82 mg yellow powder in 73% yield. ^1H NMR (CDCl_3 , 400 MHz) δ 8.66 (s, 1H), 8.15 (m, 2H), 7.93 (d, 2H, $J=8.3$), 7.68 (m, 2H), 7.38 (d, 2H, $J=8.3$), 2.45 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 176.98, 160.61, 144.92, 144.66, 137.37, 134.09, 133.81, 132.96, 131.35, 129.60, 127.79, 127.51, 127.45, 126.84, 21.67. LC/MS (ESI) m/z : found 345.9 $[\text{M}+\text{H}]^+$.

(E)-N-(3-chloro-4-oxonaphthalen-1(4H)-ylidene)-2,4,6-trimethylbenzenesulfonamide (76e)

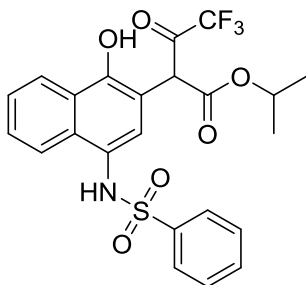
The general procedure (A) was used with 192 mg of 2-chloro-1,4-naphthoquinone (1.0 mmol) and 199 mg of p-toluenesulfonamide (1.0 mmol). The product was recrystallized from 50% EtOAc/Hex to give 325 mg yellow powder in 87% yield. ^1H NMR (CDCl_3 , 400 MHz) δ 8.67 (s, 1H), 8.24 (dd, 1H, $J_A=7.8$, $J_B=1.5$), 8.16 (dd, 1H, $J_A=7.8$, $J_B=1.3$), 7.73 (m, 2H), 7.05 (s, 2H), 2.72 (s, 6H), 2.38 (s, 3H). LC/MS (ESI) m/z : 373.78 $[\text{M}+\text{H}]^+$.

Isopropyl 2-(4-((2,4-dimethylphenyl)sulfonamido)-1-hydroxynaphthalen-2-yl)-4,4,4-trifluoro-3-oxobutanoate (LH602)



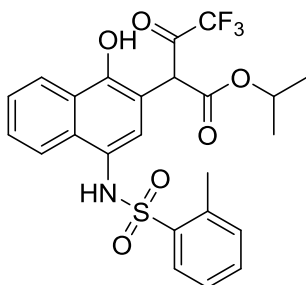
LH602 was prepared by following the general procedure (B) with compound **76a** (500 mg, 1.39 mmol), CsCO₃ (1.81 mg, 5.6 mmol), and isopropyl 4,4,4-trifluoroacetoacetate (229 μ L, 1.39 mmol). The intermediate **77a** was reduced using Na₂S₂O₄ (240 mg, 1.39 mmol) following the general procedure (C) and the reduced product was subjected to purification by flash column chromatography using 0-50% EtOAc in hexane as the mobile phase. The fractions containing the product were combined and concentrated to give 478 mg yellow solid. The solid was recrystallized from a mixture of DCM and hexane to give 386 mg white solid in 53% yield. ¹H NMR (CDCl₃, 400 MHz) δ 8.04 (d, 1H, J=8), 7.81 (br, 1H), 7.75 (d, 1H, J=8), 7.72 (d, 1H, J=8.3), 7.49 (m, 2H), 7.31 (s, 1H), 7.06 (s, 1H), 7.02 (d, 1H, J=8), 6.67 (s, 1H), 5.20 (m, 1H), 2.52 (s, 3H), 2.34 (s, 3H), 1.41 (d, 3H, J=6.3), 1.31 (d, 3H, J=6.3). ¹³C NMR (CDCl₃, 100 MHz) δ 170.39, 152.88, 143.79, 137.11, 133.27, 129.81, 126.98, 126.01, 122.42, 120.42, 120.08, 114.49, 105.72, 105.38, 71.76, 47.99, 21.62, 21.53, 21.24, 20.54. LC/MS (ESI) *m/z*: 523.5 [M+H]⁺.

Isopropyl 4,4,4-trifluoro-2-(1-hydroxy-4-(phenylsulfonamido)naphthalen-2-yl)-3-oxobutanoate (78)



The compound **78** was prepared by following the general procedure (B) with compound **76b** (20 mg, 0.0604 mmol), CsCO₃ (60 mg, 0.185 mmol), and isopropyl 4,4,4-trifluoroacetoacetate (12 μ L, 0.0604 mmol). The naphthoquinone intermediate **77b** was reduced using Na₂S₂O₄ (63 mg total, 0.3624 mmol) following the general procedure (C) and the reduced product was subjected to purification by reversed-phase HPLC (Phenomenex Jupiter 250x20mm 5m) using 50-90% acetonitrile in water containing 0.1% TFA as the mobile phase. The product was lyophilized to give 2.6 mg white powder in 8.7% yield after 2 steps. ¹H NMR (CDCl₃, 400 MHz) δ 8.04 (d, 1H, J=8), 7.82 (br, 1H), 7.65 (m, 2H), 7.49 (m, 4H), 7.34 (m, 4H), 6.57 (s, 1H), 5.21 (m, 1H), 1.42 (d, 3H, J=6.3), 1.37 (d, 3H, J=6.3). LC/MS (ESI) *m/z*: 495.6 [M+H]⁺.

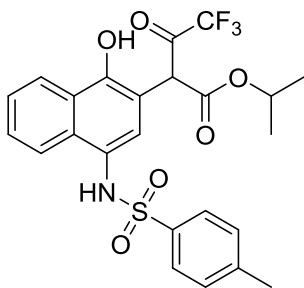
Isopropyl 4,4,4-trifluoro-2-(1-hydroxy-4-((2-methylphenyl)sulfonamido)naphthalen-2-yl)-3-oxobutanoate (79)



The compound **79** was prepared by following the general procedure (B) with compound **76c** (15 mg, 0.0434 mmol), CsCO₃ (56.4 mg, 0.174 mmol), and isopropyl 4,4,4-trifluoroacetoacetate (7.2 μ L, 0.0434 mmol). The intermediate **77c** was reduced using Na₂S₂O₄ (46 mg total, 0.26 mmol)

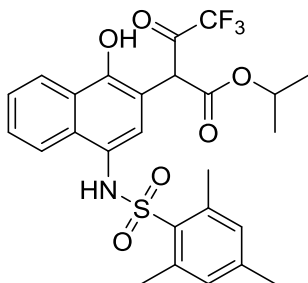
following the general procedure (C) and the reduced product was subjected to purification by reversed-phase C₁₈ flash column chromatography using 50-90% acetonitrile in water containing 0.1% TFA as the mobile phase. The product was lyophilized to give 8.9 mg white powder in 40% yield after 2 steps. ¹H NMR (CDCl₃, 400 MHz) δ 8.04 (d, 1H, J=8.3), 7.84 (d, 1H, J=8.3), 7.80 (br, 1H), 7.70 (d, 1H, J=8.3), 7.57-7.40 (m, 4H), 7.33-7.21 (m, 3H), 6.60 (s, 1H), 5.20 (m, 1H), 2.56 (s, 3H), 1.42 (d, 3H, J=6.3), 1.37 (d, 3H, J=6.3). LC/MS (ESI) *m/z*: 509.6 [M+H]⁺.

Isopropyl 4,4,4-trifluoro-2-(1-hydroxy-4-(p-toluenesulfonamido)naphthalen-2-yl)-3-oxobutanoate (80)



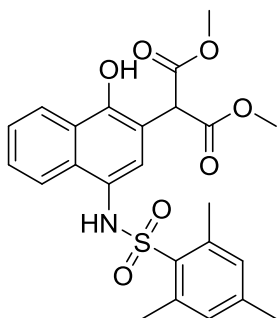
The compound **80** was prepared by following the general procedure (B) with compound **76d** (10 mg, 0.029 mmol), CsCO₃ (37.7 mg, 0.116 mmol), and isopropyl 4,4,4-trifluoroacetoacetate (4.8 μ L, 0.029 mmol). The intermediate **77d** was reduced using Na₂S₂O₄ (30 mg total, 0.174 mmol) following the general procedure (C) and the reduced product was subjected to purification by reversed-phase C₁₈ flash column chromatography using 50-90% acetonitrile in water containing 0.1% TFA as the mobile phase. The product was lyophilized to give 2.1 mg white powder in 14% yield after 2 steps. ¹H NMR (CDCl₃, 400 MHz) δ 8.01 (d, 1H, J=8.2), 7.82 (br, 1H), 7.54 (m, 3H), 7.49-7.44 (m, 2H), 7.39-7.31 (m, 2H), 7.14 (d, 2H, J=8), 6.55 (s, 1H), 5.22 (m, 1H), 2.33 (s, 3H), 1.42 (d, 3H, J=6.3), 1.37 (d, 3H, J=6.3). LC/MS (ESI) *m/z*: 509.7 [M+H]⁺.

Isopropyl 4,4,4-trifluoro-2-(1-hydroxy-4-((2,4,6-trimethylphenyl)sulfonamido)naphthalen-2-yl)-3-oxobutanoate (81)



The compound **81** was prepared by following the general procedure (B) with compound **76e** (15 mg, 0.0402 mmol), CsCO₃ (52.3 mg, 0.161 mmol), and isopropyl 4,4,4-trifluoroacetoacetate (6.6 μL, 0.0402 mmol). The intermediate **77e** was reduced using Na₂S₂O₄ (42 mg total, 0.24 mmol) following the general procedure (C) and the reduced product was subjected to purification by reversed-phase C₁₈ flash column chromatography using 50-90% acetonitrile in water containing 0.1% TFA as the mobile phase. The product was lyophilized to give 6.9 mg white powder in 33% yield after 2 steps. ¹H NMR (CDCl₃, 400 MHz) δ 8.01 (d, 1H, J=8.4), 7.74 (d, 1H, J=8.4), 7.52-7.4 (m, 3H), 7.22 (s, 1H), 6.87 (s, 2H), 6.57 (s, 1H), 6.00 (br, 1H), 5.14 (m, 1H), 2.45 (s, 6H), 2.26 (s, 3H), 1.38 (d, 3H, J=6.3), 1.30 (d, 3H, J=6.3). LC/MS (ESI) *m/z*: 535.8 [M+H]⁺.

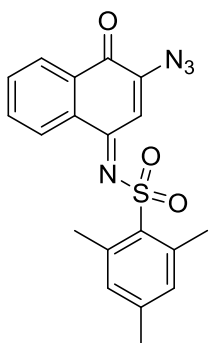
Dimethyl 2-(1-hydroxy-4-((2,4,6-trimethylphenyl)sulfonamido)naphthalen-2-yl)malonate (83)



The compound **83** was prepared by following the general procedure (B) with compound **76e** (2 mg, 5.36 μmol), CsCO₃ (7.0 mg, 21.4 μmol), and dimethyl malonate (0.62 μL, 5.36 μmol). The

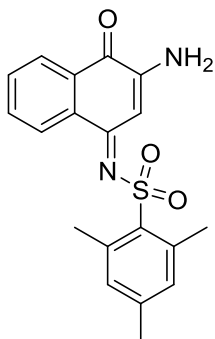
intermediate **82** was directly reduced using $\text{Na}_2\text{S}_2\text{O}_4$ (5.5 mg total, 32 μmol) following the general procedure (C) and the reduced product was subjected to purification by reversed-phase C_{18} flash column chromatography using 50-90% acetonitrile in water containing 0.1% TFA as the mobile phase. The product was lyophilized to give 1.3 mg white powder in 52% yield after 2 steps. ^1H NMR (CDCl_3 , 400 MHz) δ 8.91 (br, 1H), 8.31 (m, 1H), 7.81 (m, 1H), 7.47 (m, 2H), 6.87 (s, 2H), 6.80 (s, 1H), 6.61 (s, 1H), 4.48 (s, 1H), 3.79 (s, 6H), 2.44 (s, 6H), 2.27 (s, 3H). LC/MS (ESI) m/z : 471.6 $[\text{M}+\text{H}]^+$.

N-(3-azido-4-oxonaphthalen-1(4H)-ylidene)-2,4,6-trimethylbenzenesulfonamide (84)



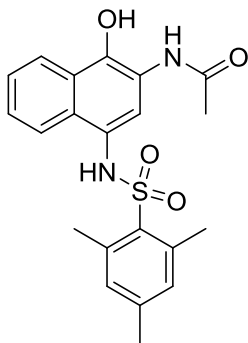
To a solution of compound **76e** (25 mg, 0.067 mmol) in 1 mL ACN was added aqueous solution of 500 μL NaN_3 (5.7 mg, 0.0871 mmol) in one portion.. The reaction was stirred for 10 min and concentrated under reduced pressure. The remaining solution was extracted with ethyl acetate (2 mL $\times$ 3). The organic fractions were combined and dried over sodium sulfate. The solution was concentrated and 22 mg (86%) of pure product was obtained without purification. ^1H NMR (CDCl_3 , 400 MHz) δ 8.15 (m, 2H), 7.70 (s, 1H), 7.69 (m, 2H), 7.01 (s, 2H), 2.68 (s, 6H), 2.34 (s, 3H). LC/MS (ESI) m/z : 380.6 $[\text{M}+\text{H}]^+$.

N-(3-amino-4-oxonaphthalen-1(4H)-ylidene)-2,4,6-trimethylbenzenesulfonamide (85)



To a solution of compound **84** (22mg, 0.0579 mmol) in 4 mL ethyl acetate was added 2 mL aqueous solution of $\text{Na}_2\text{S}_2\text{O}_4$ (20 mg, 0.116 mmol) and stirred for 2 h at rt. Another 20 mg $\text{Na}_2\text{S}_2\text{O}_4$ was added and stirred for additional 1 h at which time the solution was diluted with EtOAc and washed with brine. The organic solution was concentrated and then dissolved in 5 mL 50% acetonitrile in water. After stirring overnight at rt, the solution was lyophilized to give 18 mg red solid in 88% yield. ^1H NMR (CDCl_3 , 400 MHz) δ 8.14-8.04 (m, 2H), 7.59 (m, 2H), 7.20 (s, 1H), 6.98 (s, 2H), 5.60 (br, 2H), 2.69 (s, 6H), 2.33 (s, 3H). LC/MS (ESI) m/z : 355.0 $[\text{M}+\text{H}]^+$.

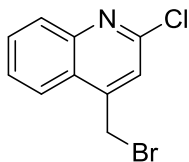
N-(1-hydroxy-4-((2,4,6-trimethylphenyl)sulfonamido)naphthalen-2-yl)acetamide (87)



Compound **85** (2 mg, 5.65 μmol) in 0.5 mL acetic anhydride was stirred overnight at 100 $^\circ\text{C}$. The reaction was concentrated under vacuum and the remaining residue dissolved in 50% acetonitrile in water. The crude solution was subjected to RP-HPLC purification using 50-90% acetonitrile in water containing 0.1% TFA as the mobile phase. The fraction containing intermediate **86** was

lyophilized to give white powder, which was dissolved in 2 mL 50% EtOAc/water. Then, sodium dithionite (6 mg, 0.034 mmol) was added to the suspension and the mixture was stirred vigorously for 2 h. The aqueous phase was discarded and the EtOAc layer washed 3 times with water, followed by brine wash. The crude solution was concentrated and subjected to reversed-phase C₁₈ flash column chromatography using 30-90% acetonitrile in water containing 0.1% TFA as the mobile phase. The fraction containing the product was lyophilized to give 0.6 mg white solid in 27% yield. ¹H NMR (CDCl₃, 400 MHz) δ 8.24 (d, 1H, J=8.5), 7.41 (d, 1H, J=8.5), 7.33 (m, 1H), 7.19 (m, 1H), 7.1 (s, 1H), 6.77 (s, 2H), 2.36(s, 6H), 2.23 (s, 3H), 2.18 (s, 3H). LC/MS (ESI) *m/z*: 398.9 [M+H]⁺.

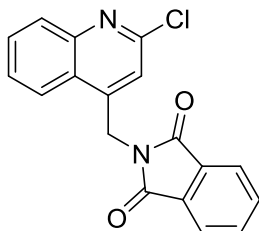
4-(bromomethyl)-2-chloroquinoline (88)



The synthesis of compound **88** was performed as previously reported in literatures.^{155,156} To a solution of 2-chlorolepidine (2.0 g, 11.24 mmol) in 25 mL CCl₄ were added N-bromosuccinimide (NBS, 2.2 g, 12.4 mmol), and benzoyl peroxide ((PhCO)₂O, 272 mg, 1.12 mmol). The reaction was refluxed overnight and then diluted with DCM. The organic solution was washed with saturated NaHCO₃ solution, followed by brine and the organic solution was dried over sodium sulfate. The solution was concentrated and the crude residue was purified by flash column chromatography using 0-25% EtOAc in hexane as the mobile phase. The fraction containing the product was concentrated to give 1.47 g of the desired product in 52% yield. ¹H NMR (CDCl₃, 400 MHz) δ 8.07 (m, 2H), 7.76 (m, 1H), 7.65 (m, 1H), 7.43 (s, 1H), 4.78 (s, 2H).

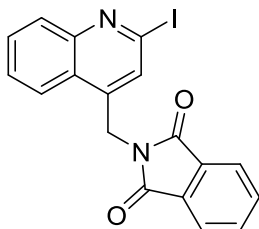
^{13}C NMR (CDCl_3 , 100 MHz) δ 150.34, 148.39, 145.49, 130.85, 129.52, 127.39, 124.73, 123.40, 122.43. LC/MS (ESI) m/z : 256.1 $[\text{M}(^{79}\text{Br})+\text{H}]^+$, 258.1 $[\text{M}(^{81}\text{Br})+\text{H}]^+$.

2-((2-chloroquinolin-4-yl)methyl)isoindoline-1,3-dione (89)



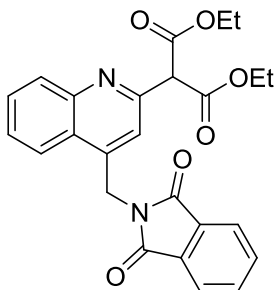
A solution of compound **88** (250 mg, 0.98 mmol), phthalimide (145 mg, 0.98 mmol), and K_2CO_3 (136 mg, 0.98 mmol) in 4 mL anhydrous DMF was stirred at rt overnight under Ar. DMF was removed under vacuum and the residue dissolved in DCM. The organic solution was washed with 1 N HCl, followed by brine. The organic fraction was dried over Na_2SO_4 and the concentrated solution was purified by flash column chromatography using 0-50% EtOAc in hexane as the mobile phase. The product was concentrated under vacuum to give 319 mg tan solid at quantitative yield (100%). ^1H NMR (CDCl_3 , 400 MHz) δ 8.19 (d, 1H, $J=8.3$), 8.01 (d, 1H, $J=8.3$), 7.87-7.79 (m, 2H), 7.73-7.68 (m, 3H), 7.59 (m, 1H), 7.19 (s, 1H), 5.23 (s, 2H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 167.75, 150.52, 147.95, 144.45, 134.48, 134.31, 131.82, 130.73, 129.28, 127.50, 124.98, 123.19, 121.53, 38.02. LC/MS (ESI) m/z : 323.2 $[\text{M}+\text{H}]^+$.

2-((2-iodoquinolin-4-yl)methyl)isoindoline-1,3-dione (90)



To a solution of compound **89** (135 mg, 0.42 mmol) and NaI (378 mg, 2.52 mmol) in 15 mL anhydrous ACN was added TMSCl (108 μ L, .84 mmol) and the reaction was refluxed for 8 h. The mixture was concentrated and the residue suspended in 50 mL EtOAc. The organic phase was washed with deionized water and brine. The organic fraction was dried over Na_2SO_4 and purified by flash column chromatography using 0-100% EtOAc in hexane as the mobile phase. The product was concentrated to give 170 mg white solid in 99% yield. ^1H NMR (CDCl_3 , 400 MHz) δ 8.24 (d, 1H, $J=8.3$), 8.06 (d, 1H, $J=8.3$), 7.90 (m, 2H), 7.77 (m, 2H), 7.73 (m, 1H), 7.67 (s, 1H), 7.64 (m, 1H), 5.23 (s, 2H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 167.76, 149.44, 142.29, 134.46, 134.35, 131.81, 129.43, 127.65, 125.33, 123.77, 123.44, 118.70, 37.58. LC/MS (ESI) m/z : 415.2 $[\text{M}+\text{H}]^+$.

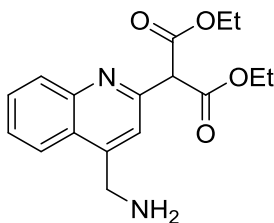
Diethyl 2-(4-((1,3-dioxisoindolin-2-yl)methyl)quinolin-2-yl)malonate (91)



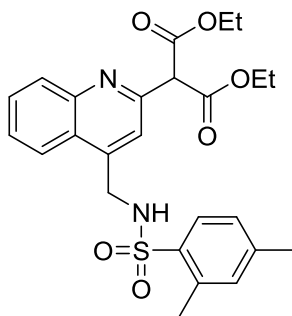
Diethyl malonate (39 μ L, 0.242 mmol), Cs_2CO_3 (157 mg, 0.484 mmol), CuI (9.2 mg, 0.048 mmol), and 2-picolinic acid (11.8 mg, 0.096 mmol) were dissolved in 5 mL dioxane and stirred at rt for 30 min. Then, compound **90** (50 mg, 0.121 mmol) was added, and the mixture was heated to

70 °C and stirred overnight. The reaction mixture was concentrated and dissolved in 5 mL EtOAc. The organic solution was washed with saturated NaHCO₃ solution, followed by 0.5 N NaHSO₄ and brine. Then, the solution was concentrated and purified by flash column chromatography using 0-50% EtOAc in hexane as the mobile phase. The fraction containing the product were combined and dried under vacuum to give 18 mg yellow solid in 33% yield. ¹H NMR (CDCl₃, 400 MHz) δ 8.31 (d, 1H, J=8.3), 8.07 (d, 1H, J=8.3), 7.89 (m, 2H), 7.74 (m, 2H), 7.65 (m, 2H), 7.52 (m, 1H), 5.32 (s, 1H) 5.08 (s, 2H), 4.15 (q, 4H, J=7.0), 1.32 (t, 6H, J=7.0). LC/MS (ESI) *m/z*: 447.0 [M+H]⁺.

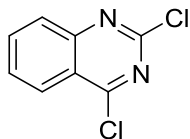
Diethyl 2-(4-(aminomethyl)quinolin-2-yl)malonate (92)



The compound **91** (5 mg, 0.0112 mmol) was dissolved in 0.5 mL ACN and hydrazine (3.6 μ L, 0.112 mmol) was added to the solution. The reaction mixture was heated to 50 °C and stirred for 24 h. The reaction was concentrated and purified by reversed-phase flash column chromatography using 10-90% acetonitrile in water containing 0.1%TFA as the mobile phase. The product was lyophilized to give 2.1 mg yellow powder in 60% yield. ¹H NMR (MeOH-d₄, 400 MHz) δ 7.81 (d, 1H, J=8), 7.75 (s, 1H), 7.67 (m, 1H), 7.52 (d, 1H, J=8), 7.40 (m, 1H), 4.50 (s, 2H), 4.26 (q, 4H, J=7.0), 1.32 (t, 6H, J=7.0). LC/MS (ESI) *m/z*: 317.1 [M+H]⁺.

Diethyl 2-(4-(((2,4-dimethylphenyl)sulfonamido)methyl)quinolin-2-yl)malonate (93)

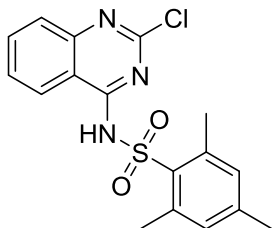
To a solution of compound **92** (2.0 mg, 6.33 μ mol) in 300 μ L DMF were added DIPEA (3.3 μ L, 0.019 mmol) and 2,4-dimethylbenzenesulfonyl chloride (1.5 mg, 7.6 μ mol). The reaction was mixed at rt for 4 h and then diluted 10-fold with EtOAc. The organic solution was washed with saturated NaHCO_3 solution and then dried over Na_2SO_4 . The product was purified by flash column chromatography using 0-50% acetone in hexane as the mobile phase. The product was concentrated under vacuum to give 2.4 mg yellow solid in 80% yield. ^1H NMR (MeOH-d_4 , 400 MHz) δ 7.73 (dd, 1H, $J_{\text{A}}=8.4$, $J_{\text{B}}=1.0$), 7.65 (d, 1H, $J=8.0$), 7.54 (dt, 1H, $J_{\text{A}}=8.4$, $J_{\text{B}}=1.2$), 7.46 (s, 1H), 7.34 (d, 1H, $J=8$), 7.27 (dt, 1H, $J_{\text{A}}=7.6$, $J_{\text{B}}=1.0$), 6.97 (d, 1H, $J=8.4$), 6.90 (s, 1H), 4.36 (s, 2H), 4.27 (q, 4H, $J=7$), 2.37 (s, 3H), 2.27 (s, 3H), 1.35 (t, 6H, $J=7$). LC/MS (ESI) m/z : 485.0 $[\text{M}+\text{H}]^+$.

2,4-dichloroquinazoline (94)

The synthesis of 2,4-dichloroquinazoline **94** was performed following literature procedures.^{164,165} Briefly, to a suspension of 2,4(1H,3H)-quinazolinedione (5.0 g, 30.9 mmol) in 18.3 mL phosphoryl chloride (POCl_3 , 200 mmol) was added DIPEA (5.9 mL, 34.0 mmol) and the mixture was heated to reflux ($\sim 120^\circ\text{C}$) overnight. POCl_3 was removed under vacuum and the residue

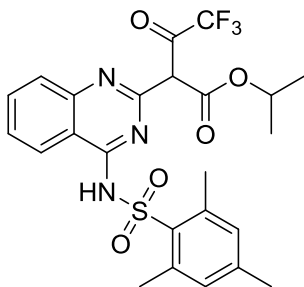
dissolved in 50 mL DCM. The organic layer was washed with deionized water (15 mL $\times$ 3) and brine. The crude solution was dried over Na₂SO₄ and purified by flash column chromatography using 0-50% EtOAc in hexane. The product was concentrated to give 4.20 g white powder in 69% yield. ¹H NMR (CDCl₃, 400 MHz) δ 8.21 (m, 1H), 8.13 (m, 1H), 7.81 (m, 2H), 7.24 (s, 1H). ¹³C NMR (CDCl₃, 100 MHz) δ 182.65, 177.95, 146.37, 135.93, 134.10, 131.83, 127.51, 126.76. LC/MS (ESI) *m/z*: 199.2 [M+H]⁺.

N-(2-chloroquinazolin-4-yl)-2,4,6-trimethylbenzenesulfonamide (95)



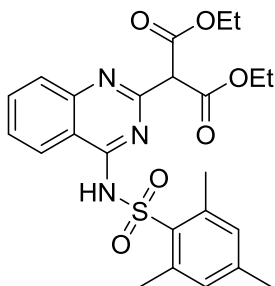
To a solution of compound **94** (200 mg, 1.0 mmol) in 5 mL anhydrous THF was added 2,4,6-trimethylbenzenesulfonamide (220 mg, 1.1 mmol) and NaH (60% dispersion in oil, 44 mg, 1.1 mmol) in 5 mL NMP at 0 °C. The reaction was stirred for additional 2 h at rt and then diluted 10-fold with 0.5 N NaHSO₄. The aqueous solution was extracted 4 times with 10 mL EtOAc. The organic fractions were combined and concentrated to crude oil. The crude oil was dissolved in ACN and triturated by the dropwise addition of H₂O with final ACN concentration at about 30%. The precipitate was collected by centrifugation and the solid washed with deionized water. The white solid was dried under vacuum to give 102 mg of the product in 28% yield. ¹H NMR (CDCl₃, 400 MHz) δ 11.97 (s, 1H), 8.23 (d, 1H, J=8), 7.81 (t, 1H, J=7.5), 7.68 (d, 1H, J=7.5), 7.52 (t, 1H, J=8), 7.00 (s, 2H), 2.78 (s, 6H), 2.33 (s, 3H). ¹³C NMR (CDCl₃, 100 MHz) δ 152.96, 152.69, 147.14, 147.05, 142.66, 135.90, 135.47, 131.85, 128.31, 127.34, 126.88, 28.98, 22.43, 20.99. LC/MS (ESI) *m/z*: 361.9 [M+H]⁺.

Isopropyl 4,4,4-trifluoro-3-oxo-2-(4-((2,4,6-trimethylphenyl)sulfonamido)quinazolin-2-yl)butanoate (96)



To a solution of compound **95** (5 mg, 0.0138 mmol) in 1.5 mL isopropyl 4,4,4-trifluoroacetoacetate was added sodium hydride (60% dispersion in mineral oil, 1.1 mg, 0.0277). The reaction was quickly sealed and heated to 70 °C. The reaction was stirred for overnight, and then isopropyl 4,4,4-trifluoroacetoacetate was removed under reduced pressure. The crude oil dissolved in EtOAc and washed with deionized water, followed by brine. The organic solution was dried over Na₂SO₄ and purified by reversed-phase flash column chromatography using 50-90% acetonitrile in water containing 0.1% TFA as the mobile phase. The product was lyophilized to give 3.9 mg white solid in 28% yield. ¹H NMR (CDCl₃, 400 MHz) δ 11.80 (s, 1H), 8.19 (d, 1H, J=8), 7.70 (t, 1H, J=7.5), 7.46 (d, 1H, J=7.5), 7.37 (t, 1H, J=8), 6.97 (s, 2H), 6.44 (s, 1H), 4.90 (m, 1H), 2.77 (s, 6H), 2.30 (s, 3H), 1.08 (d, 6H, J=5.4). LC/MS (ESI) *m/z*: 523.9 [M+H]⁺.

Diethyl 2-(4-((2,4,6-trimethylphenyl)sulfonamido)quinazolin-2-yl)malonate (97)



To a solution of compound **95** (4 mg, 0.011 mmol) in 0.4 mL diethyl malonate was added NaH (60% dispersion in oil, 1.8 mg, 0.044 mmol) and the reaction heated to 90 °C in a sealed tube.

The reaction was stirred for 2 days at which time it was concentrated under vacuum. The crude residue was purified by flash column chromatography using 0-50% EtOAc in hexane as the mobile phase. The product was concentrated under vacuum to give 2.5 mg white solid in 47% yield. ^1H NMR (CDCl_3 , 400 MHz) δ 12.50 (s, 1H), 8.29 (d, 1H, $J=8.1$), 7.79 (t, 1H, $J=7.6$), 7.67 (d, 1H, $J=8.1$), 7.48 (t, 1H, $J=7.6$), 6.97 (s, 2H), 4.39-4.24 (m, 4H), 3.89 (s, 1H), 2.78 (s, 6H), 2.32 (s, 3H), 1.39-1.28 (m, 6H). LC/MS (ESI) m/z : 485.8 $[\text{M}+\text{H}]^+$.

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