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DESIGN, SYNTHESIS, AND EVALUATION OF FLUORESCENT SMALL MOLECULE PROBES OF BIOLOGICAL SYSTEMS

A Thesis in
Chemistry
by
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ABSTRACT

Fluorescent derivatives of biologically active molecules are essential tools for studying molecular interactions at the cellular level. Fluorescent tags and/or labels have been used to study protein-protein interactions, intracellular localization of biomolecules and organelles, and small molecule-protein interactions. We report here studies of fluorescent ligands of Peroxisome Proliferator Activated Receptors (PPARs), protein targets under intense investigation for the treatment of human metabolic disorders. Such fluorescent ligands have the potential to enable faster identification of novel therapeutics. To pursue this goal, we synthesized a variety of fluorescent small molecule PPAR ligands for high-throughput examination of potential endogenous and exogenous ligands that bind these proteins. We found that our fluorescein labeled PPAR ligands bound PPAR\(_\gamma\) and PPAR\(_\alpha\) with high selectivity and were good candidates for fluorescence polarization assays. In addition, we also utilized Pennsylvania Green as a more hydrophobic analogue of fluorescein to generate cell-permeable versions of these PPAR probes to develop a novel method for studying PPAR ligand binding in living cells, which can be further extended to other related members of the nuclear hormone receptor family.

To extend previously reported studies on 3\(\beta\)-cholesterylamine derivatives that function as non-natural cell surface receptors, we synthesized a series of Oregon Green labeled symmetrical dimers of 3\(\beta\)-cholesterylamine as potential precursors to membrane spanning artificial cell surface receptors. We examined
these compounds using confocal microscopy, flow cytometry, and spectroscopy to identify the optimal structural requirements for insertion into membranes of living mammalian cells. Modeled after our best compound, we synthesized an asymmetrical 3β-cholesterylamine dimer bearing Oregon Green as an extracellular fluorescent label and biotin as an intracellular ligand of streptavidin expressed in the cytosol. Examination of this dimer using confocal microscopy showed low cellular association, which could be due to the non-protonatable amide bearing biotin. Further studies to generate synthetic analogues bearing protonatable groups on the intracellular motif could project biotin into the cytosol and recruit streptavidin to the inner-leaflet of the membrane. Through intracellular control of protein localization it is postulated that asymmetrical cholesterylamines of this type could be used to influence internal cell signaling and cellular uptake pathways. Artificial cell surface receptors that span cellular membranes could provide a new strategy for controlling therapeutically relevant intracellular pathways.
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Chapter 1
Peroxisome Proliferator-Activated Receptors

1.1 Introduction to Peroxisome Proliferator-Activated Receptors (PPARs)

A sedentary lifestyle and increased consumption of dietary saturated fats, typical of Western diets, has lead to an increased in cardiovascular disease, obesity, and diabetes.\(^1\), \(^2\) Increased mortality and morbidity caused by dietary fat support the proposal that caloric intake plays an important role in the regulation of lipid metabolism, insulin sensitivity, glucose homeostasis, and atherosclerosis.\(^3\) Given the large-scale effect of these diseases, researchers are working to elucidate the mechanisms by which our bodies process fatty acids. From this research, peroxisome proliferator-activated receptors (PPARs) were discovered. PPARs were initially cloned as orphan members of the nuclear receptor gene family.\(^4\) However, reverse endocrinology identified hormones that upregulate these receptors, and PPARs were consequently classified as a part of the superfamily of nuclear receptors that bind steroid, retinoid, and thyroid hormones, among other ligands.\(^5\), \(^6\) This research has led to a greater understanding of fatty acids as hormones and has established the PPARs as molecular targets for the development of drugs to treat human metabolic diseases.
1.2 Structures and Functions of PPAR Domains

There are three PPAR subtypes designated PPAR\(\alpha\), PPAR\(\gamma\), and PPAR\(\beta\), which are found in human, mouse, rat and various other animal species.\(^7\) The PPARs possess a domain structure that is common to other members of the nuclear receptor gene family. Sequence comparison of their DNA binding domains (DBD) and ligand binding domains (LBD) shows that the DBD is relatively conserved, while the LBD have a lower level of conservation across the subtypes (Figure 1.1). Consequently, there is significant sequence variation in the residues that line the ligand binding pockets, which is reflected in the fact that each receptor subtype is pharmacologically distinct.\(^8\)-\(^11\) In addition, the three PPAR subtypes are expressed in specific tissues. PPAR\(\alpha\) is found in brown adipocytes, intestinal mucosa, liver, kidney, skeletal muscle, and retina. PPAR\(\gamma\) originates in brown and white adipose tissue, while PPAR\(\beta\) is ubiquitous. In common with other members of the nuclear receptor gene family, PPARs are

![Figure 1.1](image-url)  
Figure 1.1. Sequence homology between the PPAR subtypes.
ligand-activated transcription factors. PPARs form heterodimers with another nuclear receptor, the Retinod-x-receptor (RXR) and subsequently binds to peroxisome-proliferator response elements (PPREs) on DNA\textsuperscript{12} such as the direct repeat 1 response element (DR-1). In the absence of ligand, PPAR-RXR heterodimers recruit corepressors and associated histone deacetylases and chromatin-modifying enzymes, silencing transcription by so called active repression.\textsuperscript{13, 14} Upon ligand binding, a conformation change is induced in the PPAR-RXR complex, which releases repressors in exchange for coactivators. The newly formed ligand-activated complex then recruits transcriptional machinery to drive enhanced gene expression. Regulation of transcription occurs from this interaction through recruitment of the coactivator steroid receptor coactivator 1 (SRC-1).\textsuperscript{15-17} Based on their expression patterns and the identification of activating natural and synthetic ligands, PPARs have been implicated in the storage and metabolism of fatty acids.

1.3 Mechanisms of PPARs and Disease

PPARs are involved with a variety diseases such as obesity, atherosclerosis, diabetes, cancer, hypertension, and inflammation (Table 1.1). However, these diseases rarely occur alone and are typically part of more complex metabolic syndromes. On the cellular level, PPAR\textsubscript{α}, PPAR\textsubscript{γ}, and PPAR\textsubscript{β} regulate pathways of lipid transport and metabolism. PPAR\textsubscript{α} participates in fatty acid catabolism and increases high-density-lipoprotein (HDL) through
transcription of genes encoding apolipoprotein A-I and A-II.\textsuperscript{18-20} This is of particular importance since atherosclerosis is associated with elevated levels of low-density-lipoprotein (LDL) and low levels of HDL. In addition, PPAR\(\alpha\), and PPAR\(\beta\) reduce inflammation involved with atherosclerosis by negatively regulating cyclooxygenase-2 (COX-2).\textsuperscript{18} However, the insulin sensitizing effects of PPAR\(\gamma\) are of much greater due to increased expression of c-Cbl associated protein, a potential signaling protein in insulin action.\textsuperscript{21} The therapeutic relevance and mechanism of PPAR\(\beta\) is still under investigation. However, there are reports that PPAR\(\beta\) is a key regulator with the potential to be involved in multiple aspects of metabolic syndrome.\textsuperscript{22}

Table 1.1. Summary of PPAR properties.

<table>
<thead>
<tr>
<th>PPAR Subtype</th>
<th>Tissue Distribution</th>
<th>Biological Functions</th>
<th>Therapeutic Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR(\alpha)</td>
<td>liver, kidney, heart</td>
<td>lipid metabolism</td>
<td>obesity, diabetes, atherosclerosis</td>
</tr>
<tr>
<td>PPAR(\gamma)</td>
<td>adipose tissue, immune cells</td>
<td>glucose and lipid homeostasis</td>
<td>diabetes, inflammation, hypertension, cancer</td>
</tr>
<tr>
<td>PPAR(\beta)</td>
<td>ubiquitous</td>
<td>currently under investigation</td>
<td>metabolic syndrome, obesity, diabetes</td>
</tr>
</tbody>
</table>
1.4 Activation of PPARs by Small Molecules

The natural ligands for PPARα include unsaturated, saturated, and essential fatty acids and also eicosanoid derivatives. In addition Pan-agonists, ligands that activate all three subtypes, include linoleic acid (1), linolenic acid (2), palmitic acid (3), and eicosapentaenoic acid (4) (Figure 1.2). It is known that a wide range of fatty acids bind to PPARs with affinities in the micromolar range. However, it is unclear whether the free concentrations of fatty acids in cells are high enough to activate these receptors. Therefore, the relevance of such ligands in vivo has been a topic of debate and researches have sought to generate high-affinity synthetic ligands.

Figure 1.2. Structures of natural fatty acids.
1.4.1 Synthetic Ligands of PPARα

An important class of synthetic compounds that activate PPARα are the fibrate drugs. Compounds such as Wy-14643 (5), benzafibrate (6), clofibrate, and fenofibrate were developed as hypolipidemic agents through optimization of their lipid-lowering activity in rodents (Figure 1.3). Clofibrate and fenofibrate are dual activators of both PPARα and PPARγ with approximately 10-fold selectivity for PPARα. However, dual activation may combine the benefits of insulin sensitization and lipid lowering, and consequently may be effective in diabetic patients that also have high serum triglycerides. Wy-14643 is a micromolar activator of murine PPARα with low activity towards PPARγ, while benzafibrate acts as a pan-agonist. All of these compounds require high micromolar concentrations to maximally activate human PPARα, and this translates into high

![Figure 1.3. Structures of selected PPARα agonists.](image-url)
doses for clinical applications. Recently, tyrosine based agonists such as GW409544 (7), BMS298585 (muraglitazar)\(^\text{33}\) and the chemically different AZ-242 (Tesaglitazar) (8)\(^\text{34, 35}\) have been identified as high-affinity therapeutic candidates for the treatment of dyslipidemia and atherosclerosis.

### 1.4.2 Synthetic Ligands of PPAR\(\gamma\)

PPAR\(\gamma\) is the most extensively studied PPAR subtype and not surprisingly, a vast array of synthetic compounds have been developed to target this receptor. Arguably, the most promising agonists are a class of antidiabetic agents known as thiazolidinediones (TZDs)\(^\text{36}\). TZD based agonists such as troglitazone, pioglitazone, and the more noted rosiglitazone (Avandia) (9) are high-affinity subtype-selective PPAR\(\gamma\) agonists, which are currently used for the treatment of Type II diabetes (Figure 1.4). However, TZD based agonists contain a stereogenic center at C-5 of the heterocyclic headgroup that can undergo racemization under physiological conditions\(^\text{37}\). It has been shown that only the (S)-enantiomer binds to the receptor with high affinity, suggesting that 50% of the drug substance is inactive. To overcome this problem, a series of tyrosine-based PPAR\(\gamma\) agonists, such as GW7845, GW1929, and Gl262570 (farglitazar) (10) were identified as activating at human PPAR\(\gamma\) at subnanomolar concentrations\(^\text{30, 38}\). Currently, farglitazar is in Phase III clinical trials as a potential candidate for the treatment of Type II diabetes.
With the exception of GW501516 \cite{11} there are no known drugs that have been identified as working specifically through PPAR\(\beta\) (Figure 1.4). Thus, part of the challenge in determining the therapeutic relevance of PPAR\(\beta\) has been the identification of potent and selective ligands for use as chemical tools.\cite{4} There have been other ligands published, such as L-631033 and L-783483, but these ligands were only weak activators of PPAR\(\beta\).\cite{41} Nonetheless, there have been documents of PPAR\(\beta\) and its relationship towards fertility, dyslipidemia, and cancer.\cite{42-44}

**Figure 1.4.** Structures of synthetic PPAR\(\gamma\) and PPAR\(\beta\) ligands.

### 1.4.3 Synthetic Ligands of PPAR\(\beta\)

With the exception of GW501516\cite{39,40} (11) there are no known drugs that have been identified as working specifically through PPAR\(\beta\) (Figure 1.4). Thus, part of the challenge in determining the therapeutic relevance of PPAR\(\beta\) has been the identification of potent and selective ligands for use as chemical tools.\cite{4} There have been other ligands published, such as L-631033 and L-783483, but these ligands were only weak activators of PPAR\(\beta\).\cite{41} Nonetheless, there have been documents of PPAR\(\beta\) and its relationship towards fertility, dyslipidemia, and cancer.\cite{42-44}
1.5 Structural Determinants of PPAR Ligand Specificity

Research on PPARs has largely been focused on understanding ligand specificity and binding interactions. In general, each PPAR subtype preferentially binds certain types of synthetic ligands or fatty acid metabolites. X-ray structures of the LBD of human PPARα, PPARγ, and PPARβ bound to various ligands have been solved. These structures provide crystallographic evidence for understanding the differences in the binding pockets of the PPAR subtypes. Two independent studies compared the LBD structures of PPARα and PPARγ complexed to AZ242 (8) and GW409544 (7). The LBD of PPARα is surrounded by helices 3, 5, 7, 11, and 12 and the central cavity spans the area

![Figure 1.5](image_url)
between AF-2 and the antiparallel β-sheet. The entrance to the ligand binding pocket, between helix 3 and the antiparallel β-sheet, is partially covered by a flexible loop that is restricted by a hydrogen bond between Tyr334 in PPARα (Glu341 in PPARγ) and Glu282. This suggests that the LBDs of both PPARγ and PPARα must be flexible in order to accommodate larger ligands. However, GW409544 is a dual agonist of both PPARα and PPARγ whereas GI262570 shows a 1000-fold selectivity for PPARγ. In PPARα, the Tyr314 is bulkier than the equivalent His322 in PPARγ, which allows GW409544 to occupy a deeper position in the PPARα ligand binding pocket than GI262570 (Figure 1.5). In addition, point mutations of Tyr314 to His in PPARα and His232 to Tyr in PPARγ shifted the activity of both agonists, which suggests that these two residues play a crucial role in controlling ligand specificity for these two subtypes. The PPARβ LBD possesses a smaller pocket next to AF-2, which cannot accommodate large ligands and consequently this receptor is harder to target specifically.

1.6 Conclusions

The discovery of PPARs as therapeutic targets has had a widespread impact on the treatment of human metabolic diseases. All three PPAR subtypes function as hormone receptors that affect the storage and catabolism of dietary fats. However, a full appreciation of their therapeutic potential required the identification of synthetic ligands that activate PPARs with high-affinity. Towards this end, pharmaceutical companies have spent the past 16 years optimizing
potential drug candidates from various screening programs. Even though PPARs are now some of the most intensely studied members of the nuclear receptor hormone family, there remains a need for the identification of new potent agonists. Moreover, new technologies that enable faster identification of such ligands would undoubtedly increase the knowledge of the mode of action of PPARs and allow the development of novel therapeutics. On the basis of such need we have endeavored to construct fluorescent PPAR ligands as tools for the discovery and analysis of compounds that bind PPARs.

1.7 References


Chapter 2

Synthesis of a High-Affinity Fluorescent PPARγ Ligand for High-Throughput Fluorescence Polarization Assays

2.1 Introduction

The PPARγ subtype is involved in glucose and lipid homeostasis, adipocyte differentiation, and insulin sensitivity. The binding of endogenous ligand(s) such as Prostaglandin J2 to PPARγ results in the formation of heterodimers with RXR proteins, interaction with co-activators, and regulation of the expression of target genes. Several antidiabetic drugs including the thiazolidinediones and the more recently described GI262570 (10, Figure 2.1) exert their therapeutic effects by targeting the PPARγ isoform. Given the importance of PPARγ in human disease, significant effort is currently focused on

![Figure 2.1. Structures of PPARγ ligands. The novel fluorescent probe 12 was designed from the X-ray crystal structure of PPARγ-bound 10.](image-url)
the discovery of improved therapeutics that modulate activities of this receptor and the identification of natural endogenous ligands. To facilitate these studies, we report here the design, synthesis, binding properties, and utility of the derivative of GI262570 (12) as a fluorescent PPARγ ligand suitable for high-throughput fluorescence polarization (FP) assays directed at the discovery of novel endogenous and exogenous PPARγ ligands.21

2.2 Ligand Design

We employed the X-ray crystal structure of the heterodimeric ligand binding domain (LBD) co-complex of RXRα / 9-cis-retinoic acid, PPARγ / GI262570 and an SRC-1 peptide,22-24 to design the fluorescent ligand 12 as a PPARγ probe. The GI262570 agonist was chosen for modification because of its high affinity (Ki = 1.2 nM)18 for PPARγ and the availability of high-resolution structural

![Image](image_url)

**Figure 2.2.** X-ray crystal structure of the PPARγ GI262570 complex. The solvent-exposed carbon atom meta to the oxazole of bound GI262570 is shown in the ribbon model (A) and electrostatic surface model (B).
information regarding the protein-bound complex (Figure 2.2). Examination of the PPAR\(\gamma\)-GI262570 subunit revealed that the protein-bound drug is nearly completely encapsulated by protein except on one face of the phenyl oxazole moiety. A phenyl carbon atom meta to the oxazole moiety is exposed to the aqueous environment, and this carbon lies at the bottom of a \(~11\) Å deep cavity with minimal dimensions of \(~4\) Å by \(~9\) Å (Figure 2.2). This structural information indicated that a linear functional group might be installed at this meta carbon to project a fluorophore into the adjacent empty protein cavity to minimize unfavorable interactions between the fluorophore-terminated side chain and the protein. Moreover, modeling indicated that the relatively flat fluorophore derived from fluorescein isothiocyanate might be rotationally restricted upon protein binding, potentially providing a strong effect on the fluorescence polarization properties of the probe.

2.3 Synthesis of the Fluorescent PPAR\(\gamma\) Probe

Preparation of 12 commenced with optimization of the synthesis of 3-iodobenzamide \(16\)\(^{25,26}\) as outlined in Figure 2.3. This product was cyclized with bromoketoester 14 prepared from 13 as previously reported\(^{27}\) to afford the meta-iodophenylloxazole 17. Reduction of 17 with lithium borohydride provided the primary alcohol 18, which was subjected to a Mitsunobu reaction\(^{28-30}\) with phenol 19, prepared as previously reported.\(^{18}\) The linked methyl ester product 20 was hydrolyzed to acid 21. This acid (21) was synthesized as an analogue of
GI262570 (10) to provide a high-affinity iodo-modified ligand of PPARγ for subsequent competition binding experiments. Ester 20 was also modified by Sonagashira coupling\textsuperscript{31, 32} with Boc-protected propargylamine (22)\textsuperscript{33} to provide the side-chain coupled product 23. The methyl ester group of 23 was hydrolyzed under basic conditions, the Boc-carbamate removed under acidic conditions, and
the resulting amine acylated with fluorescein isothiocyanate (FITC) to afford the fluorescein-modified GI262570 derivative 12 (Figure 2.3) in 7.8% yield over 10 steps. This compound was isolated as the weakly fluorescent free acid form shown in Figure 2.3. Dissolution of 12 in buffer at pH 7.4 generates the highly fluorescent anionic tautomer shown in Figure 2.1.

2.4 Fluorescence Polarization Assay Development

To examine the potential effectiveness of compound 12 as a fluorescent probe, the FP properties of this compound were initially evaluated as a function of glycerol concentration. This highly viscous solvent slows the molecular tumbling rate, and these solutions provided a method to examine the dynamic range of FP values.34 As shown in Figure 2.4, the FP of 12 was highly sensitive to glycerol, indicating that a reduction in tumbling rate resulting from protein

Figure 2.4. Fluorescence polarization calibration curve of 12 (100 nM) in glycerol / phosphate-buffered saline (pH 7.4). The high viscosity of glycerol decreases the tumbling rate of the fluorescent small molecule to enhance the measured FP value.
binding might be readily detected by FP measurements. This suggested that 12 would comprise a good candidate for FP studies in biochemical systems.

To optimize the conditions required for use of 12 as a fluorescent PPARγ probe, fluorescence intensity and fluorescence polarization (mP) values were quantified as a function of the concentration of 12. As shown in Figure 2.5 (Panel A), fluorescence intensity increased linearly with respect to compound concentration whereas FP values were insensitive to concentrations of 12 at or below 400 nM. These results indicated that the concentration of 400 nM is below the critical aggregation concentration of 12. Furthermore, 100 nM appeared to be the optimal probe concentration for further experiments. To examine binding of PPARγ to this probe, the murine PPARγ LBD was added to buffer containing 12 at 100 nM. The affinity of 12 for the PPARγ LBD was determined to be 71±15 nM by non-linear least squares curve fitting of the data shown in Figure 2.5 (Panel A).

**Figure 2.5.** Optimization of conditions for fluorescence polarization assays. Panel A: Fluorescence intensity and polarization values as a function of the concentration of 12 in PBS (pH 7.4). Panel B: Fluorescence polarization upon addition of mPPARγ LBD to 12 (100 nM) in PBS. In competition experiments, the non-fluorescent compound 21 was added at a final concentration of 50 μM. Data courtesy of Jerry Thompson.
B). Competition with 50 µM of the non-fluorescent iodo-substituted precursor (21) substantially reduced FP values, confirming the specificity of the small molecule-protein interaction. Based on these results, the optimal PPARγ concentration for subsequent competition experiments was determined to be ~400 nM (~15 ng / µl).
2.4.1 High-Throughput Fluorescence Polarization Assays

Figure 2.6. High-throughput competition binding experiments employing fluorescent probe 12 (100 nM). Panel A: Determination of $K_i$ for compound 21. Panel B: Competition assays with known PPARγ ligands. The CLA mixture includes 9Z11E-CLA (35%), 10E12Z-CLA (35%), and 9Z11Z-CLA + 9E11E-CLA (5%). Data courtesy of Jerry Thompson.

Figure 2.7. Structures of conjugated linoleic acid (26-32) ligands of PPARγ.
To validate the high-affinity probe 12 in a competition assay format, the $K_i$ of the iodo precursor 21 for the PPAR$\gamma$ LBD was measured. Displacement of the bound probe 12 by specific ligands such as 21 should diminish observed mP values. As shown in Figure 2.6 (Panel A), titration of 21 provided $K_i = 4.7\pm1.2 \mu M$, which confirmed that specific PPAR$\gamma$ ligands can be identified by competition with 12. Competition studies with a series of known PPAR$\gamma$ ligands such as conjugated linoleic acids (CLA) analyzed in high-throughput format on a Packard Fusion microtiter plate reader provided the displacement curves shown in Figure 2.6 (Panel B). The structures of these ligands are shown in Figure 2.7.

**Table 2.1.** Comparison of fluorescence polarization (FP) and previously reported scintillation proximity (SP) assays with PPAR$\gamma$ in a competitive binding format.

<table>
<thead>
<tr>
<th>PPAR$\gamma$ ligand</th>
<th>FP assay $K_i$ (µM)</th>
<th>SP assay $K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLA mixture</td>
<td>41.03</td>
<td>N/A</td>
</tr>
<tr>
<td>9Z11E-CLA</td>
<td>40.68</td>
<td>6.45</td>
</tr>
<tr>
<td>9Z11Z-CLA</td>
<td>16.15</td>
<td>N/A</td>
</tr>
<tr>
<td>9E11Z-CLA</td>
<td>32.53</td>
<td>4.70</td>
</tr>
<tr>
<td>10E12Z-CLA</td>
<td>20.20</td>
<td>7.10</td>
</tr>
<tr>
<td>11Z13E-CLA</td>
<td>31.27</td>
<td>N/A</td>
</tr>
<tr>
<td>Furan-CLA</td>
<td>65.00</td>
<td>5.50</td>
</tr>
<tr>
<td>CEA</td>
<td>276.62</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The $K_i$ values determined from competition binding experiments were compared with previously reported scintillation proximity (SP) competition assays. As shown in Table 2.1, some similarities were generally observed between the $K_i$ values determined by FP and analogous SP measurements. However, analysis of these compounds by FP assay was found to better predict...
the biological effects of compounds under investigation. For example, the 9Z11Z-CLA isomer is known to exhibit significant activity in a PPARγ-mediated model of adipogenesis.\textsuperscript{36} In contrast, the 9E11E-CLA isomer is known to exhibit much lower activity in this model. Although the SP assay does not indicate significant differences between these structurally similar isomers, the FP assay with \textbf{12} indicated a 200-fold difference in $K_i$ (Table 2.1). These results demonstrate the potential advantages of \textbf{12} as a probe for high-throughput screening by FP, and illustrate the improved sensitivity of this probe to subtle differences in molecular interactions in solution that allow detailed analysis of equilibrium binding events.

\section*{2.5 Conclusion}

We report the synthesis of a fluorescent PPARγ ligand (\textbf{12}) suitable for high-throughput fluorescence polarization (FP) assays. This assay format was investigated because FP has recently emerged as one of the best methods for quantifying molecular interactions in solution.\textsuperscript{34} The FP method enables rapid, non-radioactive determination of the affinities of small molecules for proteins in homogeneous solution-phase assays using either direct or competitive binding experiments. This technique exploits the observation that fluorescent molecules excited with plane-polarized light emit photons in the same plane provided that the molecule remains stationary throughout the excited state (~4 nanoseconds for the fluorescein fluorophore).\textsuperscript{37} Small fluorescent molecules tumble rapidly in solution, which causes photons to be emitted in a different plane from that of the
initial excitation. In contrast, large molecules such as proteins move little during the exited state interval, and emitted photons remain highly polarized with respect to the excitation plane. Hence, small fluorescent molecules are depolarized when free in solution due to rapid tumbling but become highly polarized upon binding to a slowly tumbling protein receptor. This change in FP allows highly accurate determination of the affinities for protein receptors of both fluorescent small molecules and non-fluorescent small molecules that compete for binding with a fluorescent probe to a macromolecular target. The homogeneous format of FP assays facilitates high-throughput screening of small molecule ligands against cognate protein receptors.

Compound 12 was designed based on the X-ray crystal structure of the PPARγ ligand binding domain bound to the small molecule agonist GI262570. A solvent exposed atom of this bound compound at the bottom of an open and narrow protein channel was modified with a linear alkyne linker that was coupled to the fluorescein fluorophore. This modification was designed to minimize loss of affinity upon binding of the fluorescent probe to the protein. Comparison of the $K_i$ value of the related iodo-substituted precursor 21 (4.7 ± 1.3 µM) with the $K_d$ of fluorescent probe 12 (71 ± 15 nM) revealed that modification of this compound with the linear side chain and appended fluorophore resulted in a moderate increase in affinity for the receptor. Because an iodo group is a large as a t-butyl group, the bulky iodo substituent presumably reduces the affinity of the compound compared with the acetylene-derived linker. The $K_i$ values of known PPARγ ligands were measured in high-throughput competition FP assays and
compared with previously reported scintillation proximity (SP) assays. This comparison revealed that $K_i$ values measured by FP better reflected the biological activity of PPAR$_\gamma$ ligands in whole cell assays. The application of fluorescent probe 12 in high-throughput assay formats may significantly facilitate the discovery of endogenous and exogenous PPAR$_\gamma$ ligands. Moreover, the rational ligand design approach described herein holds significant potential for the generation of numerous other fluorescent probes of nuclear hormone receptors.

### 2.6 Experimental Section

#### 2.6.1 General

All reactions, except those in aqueous media, were performed under an argon atmosphere using standard techniques for the exclusion of moisture. All commercial grade reagents for synthesis were purchased from Aldrich or Acros and used without further purification. The protein ligands CLA mixture (Pharmanutrients), and conjugated linoleic acids (Sigma) were obtained from commercial sources. Tetrahydrofuran and diethyl ether were distilled from sodium benzophenone ketyl under nitrogen. Dichloromethane, triethylamine, and hexanes were distilled from calcium hydride under nitrogen. Thin layer chromatography (TLC) was performed on silica gel 60 F$^{254}$ plates (EM Science,
0.25 mm). ICN SiliTech Silica Gel (32—33 µm) was employed for column chromatography. All \(^1\)H and \(^{13}\)C NMR Spectra were recorded using Bruker DPX-300, AMX-360, DRX-400, and AMX-2-500 MHz spectrometers at ambient temperature (22 °C). NMR signals were referenced to internal CHCl\(_3\) (\(\delta_H 7.27\)) and CDCl\(_3\) (\(\delta_C 77.23\)) or d\(_6\)-DMSO (\(\delta_H 2.50\)) and d\(_6\)-DMSO (\(\delta_C 39.5\)) peaks in parts per million (ppm). Mass spectral data was obtained from either The University of Texas at Austin Mass Spectrometry Facility (FAB) or The Pennsylvania State University Mass Spectrometry Facility (ESI and CI). Infrared spectra were obtained with a Perkin Elmer 1600 Series FTIR. Elemental analyses were performed by Midwest Microlab, LLC (Indianapolis, IN). Melting points are uncorrected.

### 2.6.2 Synthetic Procedures and Compound Characterization

![Chemical Structure](image)

**3-iodobenzamide (16)**

3-Iodobenzoic acid (20 g, 80.6 mmol) was refluxed in excess SOCl\(_2\) (150 mL) for 3 h. The reaction was cooled to room temperature and the remaining SOCl\(_2\) was evaporated in vacuo to afford the acid chloride as a yellow liquid that was used immediately without further purification. This acid chloride (21.5 g, 80.6
mmol) was dissolved in THF (300 mL) and cannulated over 45 min into freshly distilled, liquefied ammonia (500 mL) at –78 °C. This mixture was allowed to stir for 45 min and then warmed to –40 °C for 15 h. Excess ammonia was removed by evaporation at 22 °C to yield an off-white solid that was suspended in dH₂O (300 mL), filtered, and washed with dH₂O (4 x 50 mL). Evaporation in vacuo gave 16 as a white solid (19.2 g 97%). mp 185-186 °C; ¹H NMR (DMSO-d₆, 400.13 MHz) δ 8.22 (s, 1H), 8.06 (s, 1H), 7.87 (d, J = 5.8, 2H), 7.47 (s, 1H), 7.26 (t, J = 5.9, 1H); ¹³C NMR (DMSO-d₆, 75.57) δ 166.4, 139.7, 136.3, 135.9, 130.4, 126.8, 94.6; IR (KBr): v max 3337.6, 3158.0, 1665.6, 1561.1, 658.2 cm⁻¹; MS (ESI⁺) calcd. for C₇H₇INO (MH⁺) 248.0, found 248.0 (MH⁺); Anal. Calcd for C₇H₇INO: C, 34.03; H, 2.45; N, 5.67. Found: C, 34.13; H, 2.42; N, 5.55.

**ethyl[2-(3-iodophenyl)-5-methyl-1,3-oxazol-4-yl]acetate (17)**

A solution of Br₂ (5.82 g, 36.4 mmol) in CHCl₃ (10 mL) was added dropwise over 2 h to a stirring solution of ethyl propionylacetate (3) (5.25 g, 36.4 mmol) in CHCl₃ (30 mL) at 0 °C. The reaction mixture was allowed to stir for an additional 30 min and warmed to 22 °C for 17 h. The reaction vessel was exposed to the atmosphere, and air was bubbled in for 1 h. The remaining liquid was dried (Na₂SO₄) and concentrated in vacuo to afford 14 as a crude yellow liquid, which was used immediately without further purification. Compound 16
(5.0 g, 20.2 mmol) was combined with 14 (8.13 g, 36.4 mmol) and the slurry was heated to 125 °C. After 4 h, the resulting dark-red solution was allowed to cool to 22 °C and immediately purified by column chromatography eluting with Et₂O / hexanes (1:9) followed by Et₂O / hexanes (3:10) to afford 17 as a tannish-white solid (4.2 g, 56%). mp 68-69 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 8.34 (t, J = 1.5 Hz, 1H), 7.92 (d, J = 6.7 Hz, 1H), 7.71 (d, J = 6.8 Hz, 1H), 7.14 (t, J = 7.9 Hz, 1H), 4.18 (q, J = 7.2 Hz, 2H), 3.55 (s, 2H), 2.36 (s, 3H), 1.27 (t, J = 7.1 Hz, 3H); ¹³C NMR (CDCl₃, 100.61 MHz) δ 170.4, 157.0, 146.4, 138.8, 134.9, 130.4, 130.0, 129.5, 125.2, 94.4, 61.3, 32.3, 14.4, 10.5; IR (KBr): νₘₐₓ 2983.3, 1730.0, 1648.1, 1543.3, 1413.0, 1372.2, 797.7, 722.9 cm⁻¹; MS (ESI⁺) calcd. for C₁₄H₁₅INO₃ (MH⁺) 372.0, found 372.0; Anal. Calcd for C₁₄H₁₄INO₃: C, 45.30; H, 3.80; N, 3.77. Found: C, 45.24; H, 3.79; N, 3.65.

![Image](image_url)

**2-[2-(3-iodophenyl)-5-methyl-1,3-oxazol-4-yl]ethanol (18)**

A solution of LiBH₄ in THF (2 M, 6.7 mL, 12.0 mmol) was slowly added via a syringe to a stirring solution of 17 (2.21 g, 6.0 mmol) in THF (20 mL) at 0 °C. The reaction mixture was allowed to warm to 22 °C and then heated to 50 °C for 1.5 h. The solution was cooled, poured into ice-water (100 mL), acidified to pH ~ 2 with aqueous HCl (1 M), and extracted with EtOAc (3 x 25 mL). The organic extract was dried (Na₂SO₄) and concentrated to give a yellow oil that was purified
by column chromatography with EtOAc / hexanes (7:3) to afford 18 (1.72 g, 87%) as a white solid. mp 102-103 °C; \(^1\)H NMR (CDCl\(_3\), 400.13 MHz) \(\delta\) 8.31 (t, \(J = 1.6\) Hz, 1H), 7.92 (d, \(J = 6.4\) Hz, 1H), 7.72 (d, \(J = 6.8\) Hz, 1H), 7.15 (t, \(J = 7.9\) Hz, 1H), 3.92 (t, \(J = 5.8\) Hz, 1H), 3.17 (bs, 1H), 2.72 (t, \(J = 5.73\) Hz, 2H), 2.32 (s, 3H); \(^1\)^1C NMR (CDCl\(_3\), 100.61 MHz) \(\delta\) 157.0, 144.0, 138.9, 134.8, 134.4, 130.5, 129.4, 125.2, 94.5, 61.9, 28.4, 10.3; IR (KBr): \(v_{\text{max}}\) 3329.9, 1961.7, 1641.9, 1543.5, 1467.1, 1130.5, 1056.0, 886.1, 732.4 cm\(^{-1}\); MS (ESI\(^+\)) calcd. for C\(_{12}\)H\(_{13}\)INO\(_2\) (MH\(^+\)) 330.0, found 330.0; Anal. Calcd for C\(_{12}\)H\(_{12}\)INO\(_2\): C, 43.79; H, 3.67; N, 4.26. Found: C, 43.91; H, 3.57; N 4.15.

methyl(2S)-2-[(2-benzoylphenyl)amino]-3-(4-{2-[2-(3-iodophenyl-5-methyl-1,3-oxazol-4-yl)ethoxy}phenyl) propanoate (20)

A solution of diethylazodicarboxylate (1.2 g, 7.0 mmol) in THF (70 mL) was added over 0.5 h to a solution of 19 (1.74 g, 4.6 mmol), 18 (1.68 g, 5.1 mmol), and PPh\(_3\) (1.8 g, 7.0 mmol) in THF (170 mL) at 22 °C. The resulting yellow solution was stirred for 24 h and concentrated in vacuo to give a viscous yellow oil that was purified by column chromatography eluting with EtOAc / hexanes (3:17) to afford 20 as a yellowish foam (1.91 g, 61%). mp 104-105 °C; \(^1\)H NMR (CDCl\(_3\), 400.13 MHz) \(\delta\) 8.90 (d, \(J = 8.1\) Hz, 1H), 8.34 (t, \(J = 1.6\) Hz, 1H),
7.94 (d, \( J = 8.7 \) Hz, 1H), 7.73 (d, \( J = 8.8 \) Hz, 1H), 7.62-7.59 (m, 2H), 7.54-7.43 (m, 4H), 7.36-7.32 (m, 1H), 7.19-7.14 (m, 3H), 6.83 (d, \( J = 9.5 \) Hz, 2H), 6.64 (d, \( J = 9.3 \) Hz, 1H), 6.58 (t, \( J = 8.3 \) Hz, 1H), 4.09 (q, \( J = 7.9 \) Hz, 1H), 4.20 (t, \( J = 7.3 \) Hz, 2H), 3.70 (s, 3H), 3.24-3.09 (m, 2H), 2.95 (t, \( J = 7.3 \) Hz, 2H), 2.36 (s, 3H);

\(^{13}\text{C}\) NMR (CDCl\(_3\), 100.61 MHz) \( \delta \) 199.4, 173.2, 157.0, 157.9, 150.4, 145.9, 140.5, 138.8, 135.7, 135.1, 134.8, 133.3, 131.1, 130.5, 130.4, 129.8, 129.3, 128.6, 128.2, 125.2, 118.4, 115.1, 114.8, 111.9, 94.5, 66.7, 58.2, 52.4, 38.3, 26.5, 10.5;

IR (KBr): \( \nu_{\text{max}} \) 3289.7, 2951.2, 2872.1, 1737.8, 1627.7, 1569.8, 936.8, 750.4 cm\(^{-1}\);

MS (APCI\(^{+}\)) calcd. for C\(_{35}\)H\(_{32}\)IN\(_2\)O\(_5\) (MH\(^{+}\)) 687.1, found 687.1; Anal. Calcd for C\(_{35}\)H\(_{31}\)IN\(_2\)O\(_5\): C, 61.23; H, 4.55; N, 4.08. Found: C, 61.11; H, 4.59; N, 4.00.

\[(2S)-2-[(2-benzoylphenyl)amino]-3-(4-{2-[2-(3iodophenyl)-5-methyl-1,3-oxazol-4-yl]ethoxy}phenyl) propanoic acid (21)\]

Aqueous LiOH (2 M, 0.33 mL) was added to a solution of 20 (150 mg, 0.22 mmol) in THF / MeOH (3:1, 8 mL) and the reaction stirred at 22 °C for 3 h. This reaction mixture was diluted with dH\(_2\)O (25 mL), acidified to pH ~ 2 with aqueous HCl (1 M), and extracted with EtOAc (3 x 15 mL). The organic extracts were dried (Na\(_2\)SO\(_4\)) and concentrated to give a yellow oil that was purified by column chromatography eluting with MeOH / CH\(_2\)Cl\(_2\) (1:19) to afford 21 as a
crude yellow oil. Trituration of this with Et₂O / hexanes (1:1, 4 x 25 mL) yielded a yellowish-orange foam that was washed with Et₂O / hexanes (1:1, 3 x 15 mL) to afford 21 as a bright yellow solid (123 mg, 83%). mp 144-145 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 8.86 (bs, 1H), 8.32 (s, 1H), 7.91 (d, J = 7.9 Hz, 1H), 7.72 (d, J = 7.9 Hz, 1H) 7.59 (d, J = 7.1 Hz, 2H), 7.53-7.42 (m, 4H), 7.35 (t, J = 7.7 Hz, 1H), 7.21 (d, J = 8.5 Hz, 2H), 7.13 (t, J = 7.9 Hz, 1H), 6.80 (d, J = 8.4 Hz, 2H), 6.68 (d, J = 8.5 Hz, 1H), 6.61 (t, J = 7.5 Hz, 1H), 4.38 (bs, 1H), 4.14 (t, J = 6.4 Hz, 2H), 3.29-3.13 (m, 2H), 2.93 (t, J = 6.4 Hz, 2H), 2.33 (s, 3H); ¹³C NMR (CDCl₃, 100.61 MHz) δ 199.5, 176.3, 158.03, 158.01, 150.2, 146.0, 140.3, 138.9, 135.7, 135.2, 134.9, 133.2, 131.2, 130.6, 130.5, 129.5, 129.4, 128.4, 128.3, 125.3, 118.7, 115.6, 114.9, 112.3, 94.5, 66.7, 58.1, 37.0, 26.3, 10.5; IR (KBr): νmax 3323.0, 2935.3, 1733.9, 1625.0, 1572.8, 1251.7, 1179.1, 701.0 cm⁻¹; MS (APCI⁻) calcd. for C₃₄H₂₈INO₅ (MH⁻) 671.1, found 671.0; Anal. Calcd for C₃₄H₂₉INO₅: C, 60.72; H, 4.35; N, 4.17. Found: C, 60.73; H, 4.42; N, 4.09.

![Diagram of compound 23](image)

methyl(2S)-2-[(2-benzoylphenyl)amino]-3-(4-{2-[2-(3-{3-[(tert-butoxy carbonyl)amino]-1-propynyl}phenyl)-5-methyl-1,3-oxazol-4-yl]ethoxy} phenyl)propanoate (23)
Compounds 20 (405 mg, 0.59 mmol) and 22 (114 mg, 0.74 mmol) were added to Pd(PPh$_3$)$_2$Cl$_2$ (20.7 mg, 5 mol%) in triethylamine (30 mL) and this solution was extensively degassed under Ar. Cul (11.2 mg, 10 mol%) was added with degassing under Ar, and this mixture was heated to 60 °C for 0.5 h. The reaction was cooled to 22 °C, diluted with dH$_2$O and extracted with EtOAc (3 x 20 mL). This organic extract was dried (Na$_2$SO$_4$) and concentrated in vacuo to afford a faint red oil that was purified by column chromatography eluting with EtOAc / hexanes (3:7) to afford the product as a yellow oil (332 mg, 79%). $^1$H NMR (DMSO-$d_6$, 400.13 MHz) δ 8.65 (d, $J$ = 7.9 Hz, 1H), 7.87 (d, $J$ = 7.9 Hz, 2H), 7.59-7.34 (m, 10H), 7.09 (d, $J$ = 8.4 Hz, 2H), 6.81 (d, $J$ = 8.4 Hz, 3H), 6.62 (t, $J$ = 7.4 Hz, 1H), 4.64 (q, $J$ = 6.4 Hz, 1H), 4.14 (t, $J$ = 6.4 Hz, 2H), 4.01 (d, $J$ = 5.1 Hz, 2H), 3.64 (s, 3H), 3.15-3.03 (m, 2H), 2.88 (t, $J$ = 6.3 Hz, 2H), 2.31 (s, 3H), 1.40 (s, 9H); $^{13}$C NMR (DMSO-$d_6$, 100.61 MHz) δ 198.3, 172.3, 157.4, 157.2, 155.3, 149.5, 145.5, 140.0, 135.1, 134.7, 132.9, 132.4, 131.1, 130.3, 129.6, 128.7, 128.2, 128.1, 128.0, 127.5, 125.2, 123.2, 117.4, 114.9, 114.3, 112.4, 88.6, 80.6, 78.3, 66.0, 56.3, 52.0, 36.7, 30.1, 28.2, 25.5, 9.8; IR (film): $\nu_{\text{max}}$ 3307.1, 2975.9, 1710.9, 1624.8, 1249.3, 735.0, 702.4 cm$^{-1}$; HRMS (APCI$^+$) m/z 714.3152 (MH$^+$, C$_{43}$H$_{44}$N$_3$O$_7$ requires 714.3179).
(2S)-2-[(2-benzoylphenyl)amino]-3-(4-{2-[2-(3-{3-[tert-butoxycarbonyl] amino}-1-propynyl]phenyl}-5-methyl-1,3-oxazol-4-yl]ethoxy}phenyl) propanoic acid (24)

Aqueous LiOH (2 M, 0.55 mL) was added to a solution of 23 (262 mg, 0.37 mmol) in THF / MeOH (3:1, 16 mL) and the reaction stirred at 25 °C for 3 h. This mixture was diluted with dH2O (20 mL), quickly acidified with ice cold aqueous HCl (1 M), and immediately extracted with EtOAc (3 x 15 mL). This organic extract was dried (Na2SO4), the solvent removed in vacuo, and the residue purified by column chromatography eluting with MeOH / hexanes (1:49) to give 24 as a crude yellow oil, which was triturated with a mixture of Et2O / hexanes (1:1, 4 x 15 mL) to afford a yellowish-white solid. This solid was collected and washed with Et2O / hexanes (1:1, 3 x 10 mL) to provide 24 as a bright yellow solid (205 mg, 79%). 1H NMR (DMSO-d6, 400.13 MHz) δ 8.68 (d, J = 7.6 Hz, 1H), 7.87-7.85 (m, 2H), 7.58-7.47 (m, 7H), 7.40 (t, J = 7.3 Hz, 2H), 7.34 (d, J = 7.6 Hz, 1H), 7.11 (d, J = 8.4 Hz, 2H), 6.84-6.79 (m, 3H), 6.58 (t, J = 7.5 Hz, 1H), 4.52 (q, J = 6.3 Hz, 1H), 4.12 (t, J = 6.6 Hz, 2H), 4.02 (d, J = 5.4 Hz, 2H), 3.18-3.01 (m,
2H), 2.87 (t, J = 6.4 Hz, 2H), 2.30 (s, 3H), 1.40 (s, 9H); $^{13}$C NMR (DMSO-$d_6$, 100.61 MHz) δ 198.2, 173.3, 157.4, 157.1, 155.3, 149.7, 145.5, 139.8, 135.0, 134.8, 132.9, 132.4, 131.0, 130.4, 129.6, 128.7, 128.6, 128.2, 128.1, 127.5, 125.2, 123.1, 117.3, 114.5, 114.2, 112.4, 88.6, 80.7, 78.3, 65.0, 56.3, 36.5, 30.2, 28.2, 25.5, 9.8; IR (film): $\nu_{\text{max}}$ 3415.4, 2257.0, 1653.6, 1248.5, 1166.4, 1025.6, 827.1, 765.3 cm$^{-1}$; HRMS (APCI$^+$) m/z 700.3000 (MH$^+$, C$_{42}$H$_{42}$N$_3$O$_7$ requires 700.3023).


Compound 24 (47 mg, 0.07 mmol) was treated with trifluoroacetic acid / “wet” CH$_2$Cl$_2$ (3:47, 10 mL) at 25 °C for 1 h. The solution was diluted with NaOH / dH$_2$O (1:9, 10 mL) and extracted with EtOAc (3 x 10 mL). The organic extract was dried (Na$_2$SO$_4$) and concentrated in vacuo to give the free amine as a crude
oil. This amine (40.3 mg, 0.07 mmol) was added to 5-fluorescein isothiocyanate, isomer I (26 mg, 0.07 mmol) in EtOH / THF (3:2, 5 mL), cooled to 0 °C, and triethylamine (10 µl, 0.07 mmol) was added. The resulting orange reaction mixture was warmed to 22 °C and stirred in the dark for 4 h. The reaction was concentrated in vacuo in the dark to give an orange solid that was purified by column chromatography eluting with AcOH / MeOH / CH₂Cl₂ (2:3:95) to provide 12 as a yellowish-orange solid (37 mg, 55%). 

¹H NMR (DMSO-d₆, 500.13 MHz) δ 8.65 (d, J = 7.0 Hz, 1H), 8.47 (s, 1H), 7.90 (s, 1H), 7.76 (t, J = 8.3 Hz, 2H), 7.56-7.45 (m, 6H), 7.41-7.36 (m, 2H), 7.32-7.31 (m, 1H), 7.16 (d, J = 8.4 Hz, 1H), 7.10 (d, J = 8.3 Hz, 2H), 6.83-6.80 (m, 4H), 6.67-6.66 (m, 2H), 6.60-6.53 (m, 5H), 5.17 (s, 2H), 4.47 (d, J = 5.7 Hz, 1H), 4.13 (t, J = 6.6 Hz, 2H), 3.34 (bs, 4H), 3.16-2.99 (m, 2H), 2.89 (t, J = 6.4 Hz, 2H), 2.33 (s, 3H); 

¹³C NMR (DMSO-d₆, 125.76 MHz) δ 198.1, 173.3, 172.0, 168.8, 159.5, 158.2, 157.1, 152.5, 151.9, 149.8, 145.5, 145.3, 142.6, 139.8, 139.7, 137.1, 135.0, 134.7, 132.8, 131.0, 130.4, 129.5, 129.2, 129.1, 128.8, 128.6, 128.2, 127.6, 127.2, 125.3, 124.4, 123.6, 123.4, 117.2, 116.8, 114.4, 114.2, 112.6, 112.4, 112.0, 109.8, 102.2, 83.1, 69.7, 66.1, 56.4, 36.5, 25.6, 21.1, 9.9; IR (KBr): ᴙ_max 2923.2, 1611.3, 1452.8, 1249.5, 1179.3, 832.0, 752.8 cm⁻¹; HRMS (FAB⁺) m/z 989.2856 (MH⁺, C₅₈H₄₅N₄O₁₀S requires 989.2856).
2.6.3 Production of Bacterially Expressed PPARγ

The DNA encoding the murine PPARγ ligand binding domain (regions D-F, amino acids 203-505, NP_035276) was cloned into the NdeI and BamHI restriction sites downstream of the hexahistidine tag of plasmid pET-15b (Novagen). This construct was confirmed by automated dideoxynucleotide sequencing and expressed in E. coli strain BL21(DE3). Cultures were grown at 37 °C to an OD_{600} = 0.4-0.6. Expression was induced by the addition of 0.4 mM IPTG, and the culture was subsequently grown for 16 h at 23 °C to minimize the formation of inclusion bodies. Bacterial cells from a 1 L culture were harvested by centrifugation and resuspended in 10 mL of loading buffer (20 mM HEPES, pH = 7.9) containing NaCl (250 mM), and imidazole (10 mM). Lysozyme (400 µg/mL) was added and the cell slurry was incubated on ice for 30 min. The slurry was then subjected to 3 cycles of sonication at 4 °C (30 sec, micro-tip installed, duty cycle = 50%, output = 5) with a 30 second rest period between cycles. This extract was frozen in liquid N2 for 2 min followed by warming in a 37 °C water bath until thawed. This lysate was applied to a column of Ni-agarose (1 mL bed volume per liter of bacterial culture) that had been pre-equilibrated with HEPES loading buffer until the OD_{260} was reduced to 0.05. The loaded column was washed with buffer (20 mM HEPES, pH = 7.9) containing imidazole (25 mM) and glycerol (10%) until the OD_{260} was reduced to 0.01. The PPARγ-LBD was eluted in buffer (20 mM HEPES, pH = 7.9) containing imidazole (200 mM) and glycerol (10%). The protein concentration was determined by BCA assay (bovine serum albumin) method.
albumin standard) and the purity was verified by Coomassie staining of SDS-PAGE gels (purity > 90%).

### 2.6.4 Fluorescence Polarization Assays

Assays employed compound 12 (held constant at 100 nM for $K_i$ and $K_d$ measurements) in binding buffer (10 mM HEPES, pH = 7.9, 159 mM NaCl, 2 mM MgCl$_2$, 5 mM DTT). The data shown in Figure 2.5 and the competition data with 21 in Figure 2.6 (Panel A) was obtained with a Panvera Beacon 2000 instrument. The competition data with CLA compounds (26-32) and the CLA mixture (Figure 2.6, Panel B) was obtained on 96-well plates with a Packard Fusion Universal Microplate Analyzer. Values of $K_d$ and $K_i$ were calculated by nonlinear least squares curve fitting using binding models from GraphPad Prism 3.0 (San Diego, CA).

### 2.7 References


Chapter 3
Synthesis and Evaluation of Fluorescent Probes for PPARα/γ and PPARβ

3.1 Introduction

Due to the involvement of PPARα and PPARγ in glucose and lipid homeostasis, adipocyte differentiation, and insulin sensitivity, considerable attention has been focused on the generation of dual PPARα/γ agonists for the treatment of type 2 diabetes. The binding of endogenous and exogenous ligand(s) such as fatty acids to PPARγ and PPARα result in the formation of heterodimers with RXR proteins, interaction with co-activators, and regulation of the expression of target genes. Several antidiabetic drugs and non-thiazolidinedione based agonists such as AZ-242/Tesaglitazar (8), GW409544 (7), and BMS-298585/Muraglitazar (33) (Figure 3.1), are dual PPARα/γ agonists in clinical development, whereas GI262570/farglitazar (10) is selective for PPARγ. Given the importance of PPARα/γ in human disease and the absence of potent drugs that target PPARβ, significant effort is currently focused on the discovery of improved therapeutics that modulate activities of these receptors and the identification of natural endogenous ligands. Based upon our earlier studies, we report the design, synthesis, binding properties, and utility of a new generation of fluorescent ligands targeted toward both
PPARα and PPARγ, which are suitable for fluorescence polarization (FP) assays. In addition, we report the synthesis of a cell-permeable Pennsylvania Green\textsuperscript{30, 31} version of this dual probe and a selective PPARβ probe, linked to Pennsylvania Green for studies of these receptors in living cells.

![Chemical structures](image)

**Figure 3.1.** Structures of dual PPARα/γ ligands. The novel fluorescent probes 34 and 35 were designed from the X-ray crystal structures of PPARα bound to AZ242 and GW409544 respectively.

### 3.2 Ligand Design

We employed X-ray crystal structures of the hPPARα LBD bound to AZ-242\textsuperscript{33} and hPPARα bound to GW409544\textsuperscript{25} to design fluorescent ligands 34 and
as dual PPARα/γ probes. Both AZ-242 and BMS-298585 were chosen for modification because of their high affinities for both PPARγ and PPARα and the availability of high-resolution structural information regarding the protein-bound complexes. As shown in Figure 3.2, examination of the protein-small molecule complexes revealed that the ortho position of phenyl mesylate in AZ-242 is exposed to the aqueous environment, suggesting that modification of the ortho position with a fluorescent appendage should not affect binding of the fluorescent probe to PPARα. Due to structural similarities between BMS-298585 and GW409544, we also postulated that an analogue bearing a fluorophore at the meta position of BMS-298585 should produce similar results and allow maximal binding and strong effects on the FP properties of the probe.

**Figure 3.2.** X-ray crystal structures of PPARα complexed to AZ242 (A) and GW409544 (B). Solvent-exposed carbon atoms of the drugs are visible in these electrostatic surface models.
Due to its smaller binding pocket, PPAR\(\alpha\) requires smaller agonists than PPAR\(\gamma\). However, the more flexible and larger LBD of PPAR\(\gamma\) should also accommodate fluorescent probes that bind PPAR\(\alpha\).\textsuperscript{25, 33-36} In both cases we chose a linear acetylene derived side chain coupled to fluorescein to subdue any “propeller effects” and provide an acceptable distance that should avoid steric clashes with the protein. If the fluorophore was projected far away from the ligand-binding pocket and into the aqueous environment, it could rotate independently with respect to the ligand-protein complex and cause inconsistent FP analyses (propeller effect). Our linear side chain was designed to reduce this effect by retaining the fluorophore fairly close to the protein.

### 3.3 Synthesis of Fluorescent Probes that Bind PPAR\(\alpha\)/\(\gamma\)

#### 3.3.1 Synthesis of a Fluorescein-Labeled AZ242 Analogue

To synthesize a fluorescent derivative of AZ242, we initially alkylated enantiopure 36\textsuperscript{37} by deprotonation with sodium hydride followed by subsequent treatment with ethyl iodide to yield the ethyl ester and ether derivative 37 (Figure 3.3). Standard hydrogenation conditions cleanly removed the benzyl ether of 37 to give 38. Subsequent selective iodination\textsuperscript{38} of 39 using a mixture of sodium hypochlorite and sodium hydroxide produced the ortho iodinated product 40 exclusively, followed by selective protection of the phenol as the corresponding methoxymethyl ether 41. A Mitsunobu reaction\textsuperscript{39-41} between phenol 38 and
primary alcohol 41 yielded the coupled product 42. Acidic removal of the methoxymethyl ether gave phenol 43, which was converted to the methanesulfonate 44 via treatment with methanesulfonyl chloride. Ester 44 was hydrolyzed under basic conditions to give acid 45. Phenol 43 was also modified
by Sonagashira coupling\textsuperscript{42, 43} with Boc-protected propargylamine (22) to afford the side chain coupled product 46. Phenol 46 was then converted to the sulfonate 47, the ester of 47 was hydrolyzed, the Boc-carbamate removed by treatment with acid, and the resulting free amine acylated with fluorescein isothiocyanate (FITC) to afford the fluorescein modified AZ-242 derivative 34 (Figure 3.3).

3.3.2 Synthesis of a Fluorescein-Labeled BMS-298585 Analogue

To prepare a fluorescent derivative of BMS-298585, 4-hydroxybenzaldehyde was alkylated with the phenyl-oxazole mesylate (18) synthesized previously (Figure 3.4).\textsuperscript{32} Standard reductive amination\textsuperscript{44} conditions employing glycine methyl ester hydrochloride with aldehyde 48 afforded the secondary amine 49, which was subsequently acylated with 4-methoxyphenyl chloroformate to yield ester 50. This ester was hydrolyzed to the free acid 51 to provide a high-affinity iodo-modified ligand of PPAR\textalpha/\gamma for subsequent competition binding experiments. Ester 50 was modified further by Sonagashira coupling with 22 to afford the aryl acetylene 52. The ester was hydrolyzed to give acid 53, the Boc-carbamate was deprotected and the free amine was acylated with FITC to furnish 35.
Figure 3.4. Synthesis of the fluorescent BMS-298585 analogue.
3.4 Fluorescence Polarization Assay Development

To examine protein-ligand specificity, competition experiments with the non-fluorescent iodo-substituted precursor (51) were conducted. All three probes were found to bind PPARγ specifically as evidenced by substantial reductions in FP values (Figure 3.5, Panel A). However, competition experiments with 51 revealed that only 35 bound with a high level of specificity to hPPARα (Roche

Figure 3.5. Protein binding and competition experiments. Panel A: Displacement of fluorescent probes (50 nM) from mPPARγ by competition with non-fluorescent 51. Panel B: Displacement of fluorescent probes (50 nM) from hPPARα by competition with non-fluorescent 51. Panel C: Changes in fluorescence polarization values of 35 upon addition of the mPPARγ LBD (100 nM) in PBS. Panel D: Changes in fluorescence polarization values of 35 from addition of hPPARα LBD (100 nM) in PBS. n = 3, Error bars reflect the standard error.
Pharmaceuticals). In contrast, compounds 12 and 34 bound predominantly non-specifically to this receptor (Figure 3.5, Panel B). These results confirm that 12 is a selective PPARγ probe, consistent with the specificity of the unmodified drug GI262570,27-29 and show that 34 is not a useful fluorescent probe of PPARα.

To examine the binding affinity of PPARγ and PPARα for compound 35, the murine PPARγ LBD was added to buffer containing 35 at 50 nM. The affinity of 35 for PPARγ LBD and PPARα LBD was determined to be 91 nM and 191 nM respectively by non-linear least squares curve fitting of data (Figure 3.5, Panels C and D). These values are proportional to the activity of BMS-298585 for PPARγ (IC<sub>50</sub> = 0.19 µM) and PPARα (IC<sub>50</sub> = 0.25 µM).26 Based on these results, we chose to synthesize cell-permeable Pennsylvania Green analogues of 35 and a PPARβ probe derived from GW501516 (11) to study binding to these receptor subtypes in living cells.

3.5 Design of a Pennsylvania Green PPARα/γ Dual Probe and PPARβ Probe

Recently, our lab reported the synthesis and application of a novel cell-permeable fluorophore termed Pennsylvania Green.55 This fluorophore has certain advantages over fluorescein56 and other fluorophores such as Oregon Green57 due to its increased hydrophobicity, photostability and pH-insensitivity. Attachment of this fluorophore to organic molecules can facilitate the detection of binding partners in living cells by confocal microscopy. Because fluorescent small molecules have not been extensively explored for studies of nuclear hormone
Receptors (NHRs) in cell based assays, we sought to synthesize Pennsylvania Green versions of our PPARα/γ dual probe and a related PPARβ probe to further investigate the utility of fluorescent PPAR ligands.

3.6 Synthesis of the Pennsylvania Green PPARα/γ Dual Probe

To prepare the Pennsylvania Green derivative 54 intermediate 53 was treated with acid to remove the Boc-carbamate and liberate the free amine. This amine was acylated with Pennsylvania Green N-hydroxysuccinimidyl ester (PG-OSu)\textsuperscript{55} to give 54 in moderate yield (Figure 3.6).

**Figure 3.6.** Synthesis of a Pennsylvania Green PPARα/γ probe.
3.7 Synthesis of the Pennsylvania Green PPARβ Probe

Figure 3.7. Synthesis of a Pennsylvania Green PPARβ probe.
The synthesis of a PA Green PPARβ probe (71) is illustrated in Figure 3.8. Esterification of commercially available 3-nitro-4-(trifluoromethyl) benzoic acid (55) with thionyl chloride in MeOH gave the desired ester 56, which was hydrogenated to aniline derivative 57. Subsequent diazotization-iodination of the aromatic amine via a Sandmeyer-type reaction58 afforded the aromatic iodide 58 in 83% yield. The ester (58) was saponified with aqueous LiOH to give acid 59, which was converted to benzamide 60 upon treatment with thionyl chloride followed by liquefied ammonia. Treatment of the benzamide (60) with Lawesson’s Reagent59 provided thioamide 61 in 81% yield. This thioamide was condensed with the commercially available ethyl α-chloro-β-keto ester (62) to form the substituted thiazole 63. Reduction of 63 with DIBAL-H afforded alcohol 64, which was subjected to Sonagashira coupling42, 43 with Boc-protected propargylamine (22)60 to provide the side-chain coupled thiazole 65. Treatment with thionyl chloride gave chloromethyl thiazole 66 and displacement with 6761 using Cs₂CO₃ in acetonitrile afforded 68 in 85% yield. In similar fashion methyl bromoacetate (69) was combined with 68 and Cs₂CO₃ in acetonitrile to produce 70 as a slightly impure oil. The methyl ester group of 70 was hydrolyzed under basic conditions, the Boc-carbamate removed under acidic conditions, and the resulting amine acylated with PA Green OSu55 to afford the Pennsylvania Green-modified PPARβ probe 71 (Figure 3.7) in 46% yield over 3 steps.
As shown in Figure 3.8, the non-fluorescent PPARβ derivative 73 was prepared by chlorination of 64 with thionyl chloride to give thiazole 72. Subsequent alkylation with 67 and 69 followed by immediate hydrolysis afforded the PPARβ competitor 73.

![Figure 3.8](image)

**Figure 3.8.** Synthesis of an iodo-PPARβ derivative for competition experiments.

### 3.8 Imaging of PPAR Proteins in Living Cells

To examine the utility of our Pennsylvania Green PPAR probes in cells, we transfected mammalian Chinese hamster ovary (CHO-K1) cells with vectors encoding hPPARγ and hPPARα followed by treatment with the dual PPARα/γ probe 54. As shown in Figure 3.9, compound 54 was highly cell permeable and localized primary in the nucleus of both PPARγ and PPARα transfected cells (Panel A). Competition experiments employing the non-fluorescent derivative 51 and Rosaglitazone (9) show that this compound binds specifically to both PPAR subtypes, further supporting our earlier FP studies (Panels B and C). We also noticed that in the case of PPARα Rosiglitazone does not entirely compete away 54, which is consistent with the known selectivity of this antidiabetic drug for
PPARγ. We also tested the specificity of the Pennsylvania Green PPARβ probe 71 towards PPARγ and PPARα. As shown in Panel D, treatment with 71 shows

**Figure 3.9.** Differential interference contrast (DIC) and confocal laser scanning micrographs of CHO-K1 cells transiently expressing PPARγ and PPARα. Panel A: Treatment with the Pennsylvania Green dual probe 54 (20 μM). Panel B: Treatment with the Pennsylvania Green dual probe 54 (20 μM) and competitor 51 (50 μM). Panel C: Treatment with the Pennsylvania Green dual probe 54 (20 μM) and the antidiabetic drug Rosiglitazone (9) (50 μM). Panel D: Treatment with the Pennsylvania Green PPARβ probe 71 (20 μM). Images contributed by Ewa Maddox.
no nuclear localization indicating that this fluorescent probe does not bind the PPARγ or PPARα subtypes.

(A) Treatment with Pennsylvania Green PPARβ Probe 71

Transfection with PPARβ

(B) Treatment with Pennsylvania Green PPARβ Probe 71 and 73

Transfection with PPARβ

(C) Treatment with Pennsylvania Green PPARβ Probe 71 and 9

Transfection with PPARβ

(D) Treatment with Pennsylvania Green PPARα/γ Probe 54

Transfection with PPARβ

Fluorescence  Overlay  DIC

Figure 3.10. Differential interference contrast (DIC) and confocal laser scanning micrographs of CHO-K1 cells expressing PPARβ. Panel A: Treatment with Pennsylvania Green probe 71 (20 µM). Panel B: Treatment with Pennsylvania Green 71 (20 µM) plus the non-labeled competitor 73 (50 µM). Panel C: Treatment with the Pennsylvania Green probe 71 (20 µM) plus the known antidiabetic drug Rosaglitazone (9) (50 µM). Panel D: Treatment with the Pennsylvania Green PPARα/γ dual probe (20 µM). Images contributed by Ewa Maddox.
We transfected mammalian Chinese hamster ovary (CHO-K1) cells with vectors encoding PPARβ and treated these cells with the synthetic PPARβ probe 71. As shown in Figure 3.10, compound 71 appeared to be more cell permeable than probe 51 and may reveal receptor trafficking from the cytoplasm into the nucleus, providing more widespread cellular fluorescence (Panel A). Competition studies provided good results, and the non-labeled iodo derivative 73 competes away 71 almost completely, whereas the PPARγ drug Rosiglitazone (9) showed no competition (Panels B and C). Similarly, treatment of CHO-K1 cells expressing PPARβ with our earlier PPARα/γ probe 54 showed no nuclear fluorescence, indicating that 54 does not bind PPARβ. Control experiments using non-transfected cells showed no fluorescence.

3.9 Conclusions

To facilitate studies of cell-permeable probes of PPARs, we synthesized fluorescein labeled dual PPARα/γ probes 34 and 35 based on X-ray structural information on the protein-bound complexes. Because only 35 bound PPARα and PPARγ specifically in an FP based assay, we utilized Pennsylvania Green as a more hydrophobic analogue of fluorescein to generate the cell-permeable Pennsylvania Green probe 54. Investigation of this probe in living cells showed nuclear localization and specificity towards both PPARα and PPARγ. In addition, treatment of CHO-K1 cells expressing PPARβ with 54 showed that this probe does not bind PPARβ and only targets the α/γ subtypes. To extend this approach
to the PPARβ subtype, we synthesized the cell-permeable Pennsylvania Green PPARβ probe 71. Cellular experiments employing CHO-K1 cells treated with 71 showed good cellular fluorescence and exclusive specificity for PPARβ. Moreover, this probe also showed enhanced fluorescence within the cytoplasm, which may related to shuttling of PPARβ between the nucleus and cytosol. This new method allows researchers to study the effects of PPAR ligands in living cells and provides yet another method for the identification of potential therapeutic candidates. This latter application could be facilitated by expression of red fluorescent PPAR fusion proteins in cells to control for variations in transfection efficiency. Alternatively, stably transfected cell lines could be used for drug screening with cell permeable fluorescent probes of PPARs.

3.10 Experimental Section

3.10.1 General

All reactions, except those in aqueous media, were performed under a nitrogen atmosphere using standard techniques for the exclusion of moisture. All commercial grade reagents for synthesis were purchased from Aldrich, Acros, TCI, or Alfa Aesar and used without further purification. The protein ligands Prostaglandin J₂ (BioMol), CLA mixture (Pharmanutrients), and conjugated linoleic acids (Sigma) were obtained from commercial sources. Anhydrous solvents were obtained after passage through a drying column of a solvent
puriﬁcation system from GlassContour (Laguna Beach, CA). Thin layer chromatography (TLC) was performed on silica gel 60 F 254 plates (EM Science, 0.25 mm). ICN SiliTech Silica Gel (40—63 µm) was employed for column chromatography. Purification by preparative reverse phase HPLC employed an Agilent 1100 preparative pump / gradient extension instrument equipped with a Hamilton PRP-1 (polystyrene-divinylbenzene) reverse phase column (7 mm particle size, 21.5 mm x 25 cm). The HPLC ﬂow rate was increased from 10 mL/min (t = 0 min) to 20 mL/min (t = 1 min) and maintained at 20 mL/min for the remainder of the run. All 1H and 13C NMR Spectra were recorded using Bruker DPX-300, AMX-360, DRX-400, and AMX-2-500 MHz spectrometers at ambient temperature (22 °C). NMR signals were referenced to internal CDCl3 (dH 7.27) and CDCl3 (dC 77.23) or d6-DMSO (dH 2.50) and d6-DMSO (dC 39.5) peaks in parts per million (ppm). Mass spectral data was obtained from either The University of Texas at Austin Mass Spectrometry Facility or The Pennsylvania State University Mass Spectrometry Facility (ESI and CI). Infrared spectra were obtained with a Perkin Elmer 1600 Series FTIR. Elemental analyses were performed by Midwest Microlab, LLC (Indianapolis, IN). Melting points are uncorrected.
4-(2-hydroxyethyl)-2-iodophenol (40)

To a solution of 4-hydroxyphenethyl alcohol (5.0 g, 36 mmol) and sodium iodide (5.4 g, 36 mmol) in MeOH (100 mL) was added sodium hydroxide (1.5 g, 36 mmol) at 22 °C. Upon dissolution, the reaction mixture was cooled to 0 °C and aqueous sodium hypochlorite (60 mL, 4.0% NaOCl) was added dropwise over a period of 1 h and the mixture was stirred at 0 °C for 0.5 h. Aqueous 10% Na₂S₂O₃ (100 mL) was added and the pH was adjusted to 7 using aqueous 2M HCl. The aqueous layers were then extracted with EtOAc (3 x 40 mL) and the combined organics were dried over Na₂SO₄. Concentration gave an oil that was purified by column chromatography eluting with Et₂O/hexanes (3:2) to afford 40 as a white solid (6.7 g, 70%). Mp 123-124 °C; ¹H NMR (DMSO-d₆, 299.87 MHz) δ 9.2 (bs, 1H), 7.51 (d, J = 2.1 Hz, 1H), 7.03 (dd, J = 2.12 Hz, J = 8.2 Hz, 1H), 6.79 (d, J = 6.6 Hz, 1H), 4.51-4.48 (m, 1H), 3.54 (q, J = 6.7 Hz, 2H), 2.59 (t, J = 6.8 Hz, 2H); ¹³C NMR (DMSO-d₆, 75.40 MHz) δ 154.60, 138.65, 132.22, 129.81, 114.64, 84.09, 62.07, 37.34; IR (film): ν max 3268.1, 2944.1, 1502.0, 1285.9 cm⁻¹; HRMS (Cl⁺) m/z 264.9726 (MH⁺, C₈H₁₀O₂ requires 264.9726).
2-[3-iodo-4-(methoxymethoxy)phenyl]ethanol (41)

A stirring mixture of 40 (4.3 g, 16.3 mmol) and K$_2$CO$_3$ (3.4 g, 24.4 mmol) in CH$_3$CN (100 mL) was treated with chloromethyl methyl ether (1.4 mL, 17.9 mmol). The reaction mixture was stirred at 22 °C for 5 h, poured into aqueous 1M NaOH (30 mL), and extracted with CH$_2$Cl$_2$ (3 x 30 mL). The combined organics were dried over Na$_2$SO$_4$ and concentrated to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:4) followed by EtOAc/hexanes (1:1) to afford 41 as a clear oil (3.2 g, 64%). $^1$H NMR (CDCl$_3$, 300.13 MHz) δ 7.68 (s, 1H), 7.13 (d, $J$ = 7.2 Hz, 1H), 7.00 (d, $J$ = 8.3 Hz, 1H), 5.19 (s, 2H), 3.73 (t, $J$ = 6.5 Hz, 2H), 3.50 (s, 3H), 3.42 (s, 1H), 2.73 (t, $J$ = 6.3 Hz, 2H); $^{13}$C NMR (CDCl$_3$, 75.47 MHz) δ 153.91, 139.14, 133.95, 129.60, 114.41, 94.46, 86.94, 62.66, 55.92, 37.19; IR (film): $\nu_{\text{max}}$ 3310.5, 2938.2, 1498.0, 1034.9 cm$^{-1}$; HRMS (Cl$^+$) m/z 307.9909 (M$^+$, C$_{10}$H$_{13}$IO$_3$ requires 307.9909).

ethyl (2S)-3-[4-(benzyloxy)phenyl]-2-ethoxypropanoate (37)

Sodium hydride (60% dispersion in mineral oil, 504 mg, 21 mmol) was added over two portions to a stirring solution of 36 (2.6 g, 9.5 mmol) at 0 °C.
Iodoethane (7.6 ml, 96 mmol) was subsequently syringed in over a period of 0.5 h and the resulting yellow mixture was allowed to stand at 0 °C for 1.5 h. The reaction mixture was then warmed to 22 °C, allowed to stand for 3 h, and poured into H₂O (50 mL). The aqueous layers were then extracted with CH₂Cl₂ (3 x 30 mL) and the combined organics were washed with aqueous 1M H₃PO₄ (50 mL), saturated aqueous NaCl (50 mL) and dried over Na₂SO₄. Concentration gave an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:9) followed by EtOAc/hexanes (1:4) to afford 37 as a clear oil (2.5 g, 80%). ¹H NMR (CDCl₃, 400.13 MHz) δ 7.47-7.45 (m, 2H), 7.42-7.38 (m, 2H), 7.34-7.32 (m, 1H), 7.23-7.21 (m, 2H), 6.97-6.94 (m, 2H), 5.21 (s, 2H), 4.22-4.17 (m, 2H), 4.06 (t, J = 7.0 Hz, 1H), 3.71-3.64 (m, 1H), 3.44-3.37 (m 1H), 3.01 (d, J = 7.1 Hz, 2H), 1.24 (q, J = 7.1 Hz, 6H); ¹³C NMR (CDCl₃, 75.47 MHz) δ 171.83, 157.16, 136.73, 129.95, 129.01, 128.01, 127.12, 126.93, 114.14, 79.86, 69.35, 65.55, 60.13, 53.10, 30.02, 14.65, 13.72; IR (film): ν max 1746.1, 1511.8, 1242.2, 735.6 cm⁻¹; HRMS (Cl⁺) m/z 328.1675 (M⁺, C₂₀H₂₄O₄ requires 328.1675).

**ethyl (2S)-2-ethoxy-3-(4-hydroxyphenyl)propanoate (38)**

To a flame-dried and degassed schlenk flask was charged 37 (2.2 g, 6.7 mmol) in MeOH (50 mL). 10% palladium on activated carbon was directly added and the reaction mixture was degassed under hydrogen and allowed to stir under
~ 1 atmosphere of hydrogen at 22 °C for 3 h. The reaction mixture was filtered through a pad of Celite and concentrated to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (3:17) to afford 38 as a clear oil (1.5 g, 96%). 

$^1$H NMR (CDCl$_3$, 299.87 MHz) $\delta$ 7.34 (bs, 1H), 7.05-7.02 (m, 2H), 6.78-6.75 (m, 2H), 4.10 (m, 2H), 4.00 (t, $J$ = 9.0 Hz, 1H), 3.65-3.55 (m, 1H), 3.38-3.33 (m, 1H), 2.93 (d, $J$ = 8.9 Hz, 2H), 1.23-1.10 (m, 6H); $^{13}$C NMR (CDCl$_3$, 75.40 MHz) $\delta$ 172.90, 154.68, 130.00, 127.71, 114.95, 80.06, 65.87, 60.76, 37.00, 14.52, 13.66; IR (film): $\nu_{\text{max}}$ 3404.0, 1727.9, 1516.5, 1221.2 cm$^{-1}$; HRMS (Cl$^+$) $m/z$ 239.1283 (MH$^+$, C$_{13}$H$_{19}$O$_2$ requires 239.1283).

Diethylazodicarboxylate (40% in toluene, 4.6 mL, 10.0 mmol) was added dropwise over 10 min to a stirring solution of triphenylphosphine (2.6 g, 10.0 mmol) in THF (60 mL) at 0 °C. This solution was then cannulated over 20 min into a stirring solution of 38 (1.6 g, 6.7 mmol) and 41 (3.0 g, 9.7 mmol) in THF (60 mL) at 0 °C. The reaction mixture was to warmed to 22 °C, stirred for 12 h and concentrated to give an orange oil that was purified by column chromatography eluting with EtOAc/hexanes (3:17) to afford 42 as a pale yellow oil (1.7 g, 49%).
$^1$H NMR (CDCl$_3$, 299.87 MHz) $\delta$ 7.72-7.71 (m, 1H), 7.22-7.12 (m, 3H), 7.02-6.98 (m, 1H), 6.83-6.80 (m, 2H), 5.21 (s, 2H), 4.18 (q, $J$ = 6.6 Hz, 2H), 4.09 (t, $J$ = 6.8 Hz, 2H), 4.00 (t, $J$ = 7.0 Hz, 1H), 3.72-3.57 (m, 1H), 3.50 (s, 3H), 3.31-3.41 (m, 1H), 2.98 (t, $J$ = 6.9 Hz, 4H), 1.24 (t, $J$ = 7.1 Hz, 3H), 1.18 (t, $J$ = 7.0 Hz, 3H); $^{13}$C NMR (CDCl$_3$, 75.40 MHz) $\delta$ 172.15, 157.19, 154.44, 139.48, 133.65, 130.13, 129.78, 129.10, 114.57, 114.05, 94.70, 86.95, 80.09, 68.13, 65.86, 60.45, 56.08, 38.20, 34.14, 14.86, 14.00; IR (film): $\nu_{\text{max}}$ 1744.8, 1511.8, 1241.1, 1034.7 cm$^{-1}$; HRMS (Cl$^+$) m/z 528.1000 (M$^+$, C$_{23}$H$_{29}$I$_6$O$_6$ requires 528.1009).

![Image of compound 43](image)

**ethyl(2S)-2-ethoxy-3-{4-[2-(4-hydroxy-3-iodophenyl)ethoxy]phenyl} propanoate (43)**

To a stirring solution of 42 (1.1 g, 2.1 mmol) in THF (25 mL) was added aqueous 6M HCl (20 mL) at 22 °C. The reaction mixture was stirred for 7 h and slowly quenched with saturated aqueous NaHCO$_3$ (40 mL). The aqueous layers were extracted with CH$_2$Cl$_2$ (3 x 40 mL) and dried over Na$_2$SO$_4$. Concentration gave an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:4) to afford 43 as a clear oil (387 mg, 38%). $^1$H NMR (CDCl$_3$, 400.13 MHz) $\delta$ 7.57-7.56 (m, 2H), 7.13-7.07 (m, 3H), 6.86-6.84 (m, 1H), 6.79-6.77 (m, 2H), 4.15 (q, $J$ = 7.1 Hz, 2H) 4.05 (t, $J$ = 6.9 Hz, 2H), 3.97 (d, $J$ = 6.0 Hz,
1H), 3.60-3.56 (m, 1H), 3.36-3.32 (m, 1H), 2.94-2.91 (m, 4H), 1.20 (t, J = 7.1 Hz, 3H), 1.14 (t, J = 7.0 Hz, 3H); $^{13}$C NMR (CDCl$_3$, 100.61 MHz) δ 172.64, 157.29, 153.67, 138.53, 132.06, 130.39, 130.23, 129.06, 114.84, 114.21, 84.90, 80.18, 68.38, 66.06, 60.79, 38.24, 34.16, 14.89, 14.04; IR (film): $\nu_{\text{max}}$ 3384.0, 1727.2, 1511.3, 1243.4 cm$^{-1}$; HRMS (Cl$^+$) m/z 484.0745 (M$^+$, C$_{21}$H$_{25}$IO$_5$ requires 484.0707).

![Structure of compound 46](image)

**ethyl (2S)-3-{4-[2-(3-{3-[(tert-butoxycarbonyl)amino]prop-1-yn-1-yl}-4-hydroxyphenyl)ethoxy]phenyl}-2-ethoxypyranoate (46)**

To a flame-dried and degassed schlenk flask was charged 43 (387 mg, 0.80 mmol), 22 (161 mg, 1.0 mmol), and Pd(PPh$_3$)$_2$Cl$_2$ (28 mg, 5 mol%) in TEA (20 mL). The mixture was then degassed under argon and Cul (15 mg, 10 mol%) was added. The reaction mixture was degassed once more and stirred vigorously for 3 h at 60 °C. The mixture was then poured into H$_2$O (50 mL) and extracted with EtOAc (3 x 25 mL). The combined organics were dried over Na$_2$SO$_4$ and concentrated to give a faint orange oil that was purified by column chromatography eluting with EtOAc/hexanes (1:4) followed by EtOAc/hexanes (3:7) to afford 46 as a pale yellow oil (208 mg, 51%). $^1$H NMR (CDCl$_3$, 400.13
78 MHz) δ 7.15-7.14 (m, 1H), 7.09-7.05 (m, 3H), 6.84-6.82 (m, 1H), 6.77-6.73 (m, 2H), 6.65 (bs, 1H), 5.23 (bs, 1H), 4.14-4.06 (m, 4H), 4.03 (t, J = 7.0 Hz, 2H), 3.92 (t, J = 6.0 Hz, 1H), 3.57-3.51 (m, 1H), 3.32-3.28 (m, 1H), 2.91-2.88 (m, 4H), 1.42 (s, 9H), 1.22-1.15 (m, 3H), 1.11 (t, J = 7.0 Hz, 3H); 13C NMR (CDCl₃, 100.61 MHz) δ 172.49, 157.38, 156.13, 155.67, 131.68, 130.87, 130.23, 129.58, 129.13, 114.91, 114.18, 108.96, 96.14, 92.03, 80.23, 77.12, 68.47, 68.03, 60.69, 38.31, 34.54, 31.32, 28.23, 14.91, 14.05; IR (film): νmax 3355.1, 2977.3, 1711.0, 1243.3 cm⁻¹; HRMS (Cl⁺) m/z 512.2635 (MH⁺, C₂₉H₃₈NO₇ requires 512.2648).


To a solution of 46 (208 mg, 0.41 mmol) in CH₂Cl₂ (20 mL) at 0 °C was added methanesulfonyl chloride (38 ml, 0.49 mmol) followed by triethylamine (86 ml, 0.61 mmol). The reaction was stirred at 0 °C for 2 h and washed with saturated aqueous NaHCO₃ (10 mL), saturated aqueous NaCl (10 mL), and dried over Na₂SO₄. Concentration gave an oil that was purified by column chromatography eluting with EtOAc/hexanes (3:7) provided 47 as a pale yellow oil (162 mg, 67%). 1H NMR (CDCl₃, 400.13 MHz) δ 7.34 (s, 1H), 7.23-7.21 (m, 2H), 7.08 (d, J = 8.3 Hz, 2H), 6.74 (d, J = 8.3 Hz, 2H), 5.10 (bs, 1H), 4.13-4.00
(m, 6H), 3.91 (t, J = 6.3 Hz, 1H), 3.56-3.52 (m, 1H), 3.31-3.27 (m, 1H), 3.13 (s, 3H), 2.97 (t, J = 6.3 Hz, 2H), 2.88 (d, J = 6.7 Hz, 2H), 1.98 (s, 1H), 1.40 (s, 9H), 1.08-1.21 (m, 6H); $^{13}$C NMR (CDCl$_3$, 100.61 MHz) δ 172.32, 157.16, 155.31, 148.17, 137.71, 133.86, 130.43, 130.31, 129.37, 123.20, 116.64, 114.13, 96.16, 91.43, 80.14, 77.32, 67.70, 65.98, 60.20, 38.33, 38.25, 34.71, 28.18, 14.90, 14.04; IR (film): $\nu_{\text{max}}$ 2977.6, 1712.3, 1512.2, 1173.7 cm$^{-1}$; HRMS (Cl$^+$) m/z 588.2271 (MH$^+$, C$_{30}$H$_{38}$INO$_9$S requires 588.2267).

(2S)-3-[4-(2-{3-[3-[[3',6'-dihydroxy-3-oxo-3H-spiro[2-benzofuran-1,9'-

xanthen]-5-yl]amino]carbonothioyl]amino)prop-1-yn-1-yl]-4-[(methyl
 sulfonyl)oxy]phenyl]ethoxy)phenyl]-2-ethoxypropanoic acid (34)

A stirring solution of 47 (138 mg, 0.23 mmol) in THF: MeOH (3:1, 12 mL) was treated with aqueous 1M LiOH (0.38 mL) and stirred for 1 h 22 °C. The reaction mixture was diluted with H$_2$O (7 mL) and acidified to pH = 2 with aqueous 1M HCl. The aqueous layers were extracted with CHCl$_3$, dried over Na$_2$SO$_4$, and concentrated in vacuo to give the crude free acid as a pale yellow oil. The above free acid (100 mg, 0.18 mmol) was treated with trifluoroacetic
acid/"wet" CH₂Cl₂ (3:47, 20 mL) at 22 °C for 1 h. Removal of solvent in vacuo gave the free amine as a crude brown solid that was taken on immediately without further purification. This amine (83 mg, 0.18 mmol) was combined with triethylamine (0.38 mL, 0.26 mmol) in MeOH/THF (2:3, 20 mL). 5-fluorescein isothiocyanate, isomer I (69 mg, 0.18 mmol) was added and the solution was allowed to stir in the dark at 22 °C for 15 h. The reaction mixture was concentrated and the residue was purified via preparative reverse-phase HPLC (gradient: 20% CH₃CN, 80% H₂O, and 0.1% TFA to 99% CH₃CN, 1% H₂O, and 0.1% TFA over 40 min; retention time = 18.5 min) afforded 34 as an orange solid. (48 mg, 32%). Mp 130-132 °C; ¹H NMR (DMSO-d₆, 400.13 MHz) δ 8.40 (s, 1H), 7.77 (dd, J = 2.0 Hz, J = 8.4 Hz, 1H), 7.48-7.47 (m 1H), 7.38-7.33 (m 2H), 7.21 (d, J = 8.4 Hz, 1H), 7.15-7.12 (m, 2H), 6.90 (t, J = 2.3 Hz, 1H), 6.87 (d, J = 8.6 Hz, 2H), 6.63-6.54 (m, 5H), 5.18 (s, 2H), 4.20 (t, J = 6.6 Hz, 2H), 3.95-3.92 (m, 1H), 3.54-3.48 (m, 1H), 3.45 (s, 3H), 3.33-3.25 (m, 1H), 3.10 (t, J = 6.5 Hz, 2H), 2.90-2.77 (m, 2H), 1.03 (t, J = 7.0 Hz, 3H); ¹³C NMR (DMSO-d₆, 100.62 MHz) δ 173.26, 168.64, 159.58, 156.91, 152.00, 151.93, 144.56, 137.97, 130.31, 129.89, 129.59, 129.13, 128.86, 128.00, 127.35, 126.08, 124.65, 122.89, 114.13, 113.19, 112.63, 111.38, 109.82, 109.53, 102.24, 79.24, 67.88, 64.86, 38.09, 37.50, 34.66, 15.04; IR (film): νmax 3068.1, 1609.6, 1510.2, 1172.1 cm⁻¹; HRMS (Cl⁺) m/z 851.1929 (MH⁺, C₄₄H₃₉N₂O₁₂S₂ requires 851.1944).
ethyl (2S)-2-ethoxy-3-[4-(2-{3-iodo-4-[(methylsulfonyl)oxy]phenyl}ethoxy)phenyl]propanoate (44)

To a solution of 43 (298 mg, 0.62 mmol) in CH₂Cl₂ (20 mL) at 0 °C was added methanesulfonyl chloride (57 ml, 0.74 mmol) followed by triethylamine (0.13 ml, 0.92 mmol). The reaction was stirred at 0 °C for 1 h and washed with saturated aqueous NaHCO₃ (10 mL), saturated aqueous NaCl (10 mL), and dried over Na₂SO₄. Concentration gave an oil that was purified by column chromatography eluting with MeOH/CH₂Cl₂ (1:49) provided 44 as a clear oil (324 mg, 93%).

1H NMR (CDCl₃, 400.13 MHz) δ 7.78 (s, 1H), 7.37-7.28 (m, 2H), 7.14 (d, J = 8.5 Hz, 2H), 6.80 (d, J = 8.6 Hz, 2H), 4.18-4.10 (m, 4H), 3.97 (t, J = 6.3 Hz, 1H), 3.62-3.58 (m, 1H), 3.37-3.33 (m, 1H), 3.25 (s, 3H), 3.02 (t, J = 6.5 Hz, 2H), 2.96-2.94 (m, 2H), 1.22 (t, J = 7.1 Hz, 3H), 1.16 (t, J = 7.0 Hz, 3H); 13C NMR (CDCl₃, 100.61 MHz) δ 172.17, 157.04, 147.84, 140.18, 139.48, 130.31, 130.20, 129.33, 122.56, 114.07, 89.32, 80.03, 67.56, 65.88, 60.51, 38.98, 38.18, 34.32, 14.87, 14.01; IR (film): νmax 2933.5, 1741.7, 1371.6, 1170.3 cm⁻¹; HRMS (Cl⁻) m/z 562.0517 (MH⁻, C₂₂H₂₇I₇O₇S requires 562.0522).
(2S)-2-ethoxy-3-[4-(2-{3-iodo-4-[(methylsulfonyl)oxy]phenyl}ethoxy) phenyl] propanoic acid (45)

A stirring solution of 44 (268 mg, 0.47 mmol) in THF: MeOH (3:1, 24 mL) was treated with aqueous 1M LiOH (0.71 mL) and stirred for 2 h 22 °C. The reaction mixture was diluted with H₂O (7 mL) and acidified to pH = 2 with aqueous 1M HCl. The aqueous layers were extracted with CHCl₃, dried over Na₂SO₄, and the residue was purified via preparative reverse-phase HPLC (gradient: 30% CH₃CN, 60% H₂O, and 0.1% TFA to 99% CH₃CN, 1% H₂O, and 0.1% TFA over 20 min; retention time = 19.5 min) afforded 45 as a white. (210 mg, 84%). Mp 92-93 °C; ¹H NMR (CDCl₃, 299.87 MHz) δ 10.14 (bs, 1H), 7.79-7.78 (m, 1H), 7.38-7.27 (m, 2H), 7.17 (d, J = 8.5 Hz, 2H), 6.81 (d, J = 8.6 Hz, 2H), 4.13 (t, J = 6.5 Hz, 2H), 4.04-4.01 (m, 1H), 3.65-3.60 (m, 1H), 3.44-3.34 (m, 1H), 3.28 (s, 3H), 3.08-2.90 (m, 4H), 1.16 (t, J = 6.9 Hz, 3H); ¹³C NMR (CDCl₃, 100.61 MHz) δ 157.26, 147.97, 140.34, 139.58, 130.44, 129.24, 122.74, 114.27, 114.27, 89.41, 67.72, 66.50, 39.16, 37.86, 34.50, 14.95; IR (film): νmax 2932.8, 1722.5, 1511.9, 1242.0 cm⁻¹; HRMS (Cl⁺) m/z 534.0199 (M⁺, C₂₀H₂₃O₇S requires 563.0209).
4-[2-[2-(3-iodophenyl)-5-methyl-1,3-oxazol-4-yl]ethoxy]benzaldehyde (48)

Methanesulfonyl chloride (0.37 ml, 4.8 mmol) was added dropwise over 0.5 h at 22 °C to a stirring solution of 18 (1.4 g, 4.3 mmol) and triethylamine (0.86 ml, 6.1 mmol) in CH₂Cl₂ (15 mL). The resulting pale yellow solution was stirred for 1 h and diluted with CH₂Cl₂ (50 mL). The organics were then washed with aqueous 1M HCl (2 x 20 mL), H₂O (50 mL), saturated aqueous NaCl (50 mL) and dried over Na₂SO₄. Concentration in vacuo gave the mesylate as a pale brown solid, which was used immediately without further purification. A mixture of the above mesylate x (1.8 g, 4.3 mmol), 4-hydroxybenzaldehyde (641 mg, 5.3 mmol), and K₂CO₃ in CH₃CN (40 mL) were heated to reflux for 15 h and cooled to 22 °C. The reaction was filtered and the filtrate was concentrated in vacuo to give a brown solid that was purified by column chromatography eluting with EtOAc/hexanes (3:17) followed by EtOAc/hexanes (3:7) to afford 48 as a white solid (1.3 g, 70%). Mp 106-107 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 9.84 (s, 1H), 8.31-8.30 (m, 1H), 7.91-7.88 (m, 1H), 7.79-7.77 (m, 2H), 7.70-7.68 (m, 1H), 7.12 (t, J = 7.9 Hz, 1H), 6.97-6.95 (m, 2H), 4.30 (t, J = 6.6 Hz, 2H), 2.97 (t, J = 6.6 Hz, 2H), 2.35 (s, 3H); ¹³C NMR (CDCl₃, 75.41 MHz) δ 190.74, 163.69, 157.83, 145.64, 138.61, 134.57, 132.54, 131.92, 130.30, 129.87, 129.38, 124.89, 114.71, 94.27, 66.85, 26.08, 10.21; IR (film): νmax 1692.1, 1599.2, 1255.4, 1158.8 cm⁻¹;
HRMS (Cl⁺) m/z 434.0264 (MH⁺, C₁₉H₁₇INO₃ requires 343.0253). Anal. calcd. for C₁₉H₁₆INO₃: C, 52.67; H, 3.72; N, 3.23. Found: C, 52.63; H, 3.71; N, 3.20.

methylN-(4-{2-[2-(3-iodophenyl)-5-methyl-1,3-oxazol-4-yl]ethoxy}benzyl) glycinate (49)

A suspended mixture of 48 (1.24 g, 2.9 mmol), glycine methyl ester hydrochloride (478 mg, 3.8 mmol), triethylamine (0.6 mL, 4.0 mmol), 4Å molecular sieves (0.4 g) and anhydrous MgSO₄ (1 g) was stirred at 22 °C for 12 h. The reaction mixture was then cooled to 0 °C and sodium borohydride (141 mg, 3.8 mmol) was added over two portions. The mixture was stirred at 0 °C for 0.5 h and then at 22 °C for 2 h. The reaction mixture was quenched with aqueous 10% NaHCO₃ (10 mL) and the solvent was removed in vacuo. The residue was diluted with H₂O (25 mL) and extracted with EtOAc (3 x 25 mL). The organics were then washed with saturated aqueous NaCl (50 ml), dried over Na₂SO₄ and concentrated to give a pale brown oil that was purified by column chromatography eluting with EtOAc/hexanes (3:7) followed by EtOAc/hexanes (1:1) to afford 49 as a tan solid (865 mg, 59%). Mp 66-67 °C; ¹H NMR (CDCl₃, 299.87 MHz) δ 8.29-8.28 (m, 1H), 7.90-7.86 (m, 1H), 7.68-7.65 (m, 1H), 7.24-7.17 (m, 2H), 7.10 (t, J = 7.9 Hz, 1H), 6.84-6.80 (m, 2H), 4.18 (t, J = 6.7 Hz, 2H),
3.68 (s, 5H), 3.36 (s, 2H), 2.92 (t, J = 6.6 Hz, 2H), 2.33 (s, 3H); $^{13}$C NMR (CDCl$_3$, 75.41 MHz) δ 172.76, 157.77, 157.57, 145.49, 138.41, 134.45, 132.95, 131.48, 130.19, 129.42, 129.35, 124.80, 114.32, 94.19, 66.44, 52.52, 51.64, 49.61, 26.22, 10.17; IR (film): ν$_{max}$ 1739.8, 1511.2, 1243.8, 1174.9 cm$^{-1}$; HRMS (Cl$^+$) m/z 506.0698 (M$^+$, C$_{22}$H$_{23}$IN$_2$O$_7$ requires 506.0703). Anal. calcd. for C$_{22}$H$_{23}$IN$_2$O$_4$: C, 52.19; H, 4.58; N, 5.53. Found: C, 52.52; H, 4.72; N, 5.46.

methyl N-(4-{2-[2-(3-iodophenyl)-5-methyl-1,3-oxazol-4-yl]ethoxy}benzyl)-N-[(4-methoxyphenoxy)carbonyl]glycinate (50)

To a stirring solution of 49 (837 mg, 1.7 mmol) and triethylamine (0.35 ml, 2.5 mmol) in CH$_2$Cl$_2$ (15 mL) at 0 °C was added 4-methoxyphenyl chloroformate (0.3 ml, 2.0 mmol) over 15 min. The reaction was allowed to warm to 22 °C and stirred for 1 h. The solvent was removed in vacuo and the residue was purified by column chromatography eluting with eluting with EtOAc/hexanes (1:4) followed by EtOAc/hexanes (1:1) to afford 50 as a tan oil (860 mg, 77%). $^1$H NMR (CDCl$_3$, 400.13 MHz) δ 8.31-8.30 (m, 1H), 7.90-7.88 (m, 1H), 7.68-7.65 (m, 1H), 7.20 (d, J = 8.2 Hz, 2H), 7.10 (t, J = 7.9 Hz, 1H), 7.05-7.00 (m, 2H), 6.89-6.83 (m, 4H), 4.64 (s, 1H), 4.54 (s, 1H), 4.21 (q, J = 6.5 Hz, 2H), 3.98 (d, J = 3.5 Hz, 2H), 3.74 (s, 3H), 3.69 (s, 3H), 2.96-2.92 (m, 2H), 2.34 (s, 3H); $^{13}$C NMR (CDCl$_3$, 75.48
N-(4-{2-[2-(3-iodophenyl)-5-methyl-1,3-oxazol-4-yl]ethoxy}benzyl)-N-[(4-methoxyphenoxy)carbonyl]glycine (51)

To a stirring solution of 50 (170 mg, 0.26 mmol) in THF (15 mL) was added a solution of LiOH (33 mg, 0.78 mmol) in H₂O (15 mL). The reaction mixture was stirred for 1 h at 22 °C. The mixture was then acidified to pH ~ 2 with aqueous 1M HCl and the THF was removed in vacuo. The remaining aqueous layer was diluted with EtOAc (75 mL) and the mixture was stirred for 0.5 h. The organics were separated and dried over Na₂SO₄ to give an off white solid that was purified by column chromatography eluting with CH₂Cl₂ followed by MeOH/CH₂Cl₂ (1:19) to afford the free acid as a slightly impure white solid. Further purification via preparative reverse-phase HPLC (gradient: 40% CH₃CN, 60% H₂O, and 0.1% TFA to 99% CH₃CN, 1% H₂O, and 0.1% TFA over 30 min;
retention time = 25.8 min) afforded 51 as white foam. (150 mg, 90%). Mp 92-93°C; 1H NMR (CDCl₃, 300.13 MHz) rotomers δ 11.01 (bs, 1H), 8.30 (s, 1H), 7.91 (d, J = 7.8 Hz, 1H), 7.70 (d, J = 7.9 Hz, 1H), 7.24-7.18 (m, 2H), 7.12 (t, , J = 7.9 Hz, 1H), 7.06-6.98 (m, 2H), 6.88-6.80 (m, 4H), 4.64 (s, 1H), 4.54 (s, 1H), 4.19 (q, J = 6.0 Hz, 2H), 4.01 (s, 2H), 3.75/3.74 (s, 3H), 2.97-2.98 (m, 2H), 2.37/2.36 (s, 3H); 13C NMR (CDCl₃, 75.48 MHz) rotomers δ 173.37, 158.34, 157.90, 156.98, 155.41/155.50, 145.88, 144.66, 138.82, 134.70, 132.69, 130.36, 129.83, 129.15, 128.96, 128.34, 125.05, 122.40/122.46, 114.64/114.73, 114.25, 94.26, 66.47, 55.52, 51.12, 25.94, 10.21; IR (film): νmax 1720.9, 1509.2, 1197.7, 1033.4 cm⁻¹; HRMS (Cl⁺) m/z 643.0930 (MH⁺, C₂₉H₂₈N₂O₇ requires 643.0941). Anal. calcd. for C₂₉H₂₇N₂O₇ • H₂O: C, 52.74; H, 4.43; N, 4.24. Found: C, 52.35; H, 4.16; N, 3.84.

methyl N-(4-{2-[2-(3-[( tert-butoxycarbonyl)amino]prop-1-yn-1-yl]phenyl}-5-methyl-1,3- oxazol-4-yl)ethoxy]benzyl)-N-[(4-methoxyphenoxy)carbonyl] glycinate (52)

To a flame-dried and degassed schlenk flask was charged 50 (637 mg, 0.97 mmol), 22 (196 mg, 1.3 mmol), and Pd(PPh₃)₂Cl₂ (34 mg, 5 mol%) in TEA:THF (1:5, 30 mL). The mixture was then degassed under argon and Cul (18
mg, 10 mol%) was added. The reaction mixture was degassed once more and stirred vigorously for 45 min at 22 °C. The mixture was then poured into H₂O (50 mL) and extracted with EtOAc (3 x 25 mL). The combined organics were dried over Na₂SO₄ and concentrated to give a red oil that was purified by column chromatography eluting with EtOAc/hexanes (3:7) followed by EtOAc/hexanes (1:1) to afford X as a tan oil, which upon trituration with Et₂O afforded X as a tan solid (636 mg, 96%). Mp 72-73 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 8.01 (s, 1H), 7.89-7.87 (m, 1H), 7.39-7.37 (m, 1H), 7.31 (t, J = 7.7 Hz, 1H), 7.19 (d, J = 8.0 Hz, 2H), 7.04-6.98 (m, 2H), 6.88-6.82 (m, 4H) 4.98 (bs, 1H), 4.63 (s, 1H), 4.52 (s, 1H), 4.21 (q, J = 6.5 Hz, 2H), 4.11 (d, J = 4.6 Hz, 2H), 3.97 (d, J = 4.4 Hz, 2H), 3.74 (s, 3H), 3.68 (s, 3H), 2.99-2.93 (m, 2H), 2.33 (s, 3H), 1.44 (s, 9H); ¹³C NMR (CDCl₃, 100.63 MHz) rotomers δ 169.69/169.74, 158.48, 158.36, 156.93, 155.23, 145.21, 144.64, 132.72, 132.56, 129.73, 128.99/129.04, 128.58, 128.31, 127.77, 125.46, 123.25, 122.31/122.38, 114.57/114.66, 114.19, 86.16, 82.18, 66.55, 55.44, 52.04, 50.90/51.11, 47.34/47.49, 28.24, 26.19, 10.10; IR (film): νmax 1713.6, 1509.9, 1197.2, 1031.6 cm⁻¹; HRMS (Cl⁺) m/z 684.2915 (MH⁺, C₃₉H₄₁N₃O₉ requires 684.2921)
N-(4-{2-[2-(3-{3-[(tert-butoxycarbonyl)amino]prop-1-yn-1-yl}phenyl)-5-methyl-1,3-oxazol-4-yl]ethoxy}benzyl)-N-[(4-methoxyphenoxy)carbonyl]glycine (53)

To a stirring solution of 52 (614 mg, 0.90 mmol) in THF (20 mL) was added a solution of LiOH (113 mg, 2.7 mmol) in H₂O (20 mL). The reaction mixture was stirred for 1 h at 22 °C. The mixture was then acidified to pH ~ 5 with aqueous 1M HCl and the THF was removed in vacuo. The remaining aqueous layer was diluted with EtOAc (75 mL) and the mixture was stirred for 0.5 h. The organics were separated and dried over Na₂SO₄ to give a tannish oil that was purified by column chromatography eluting with MeOH/CH₂Cl₂ (1:19) to afford 53 as a tan solid. (571 mg, 95%). Mp 82-83 °C; ¹H NMR (CDCl₃, 300.13 MHz) rotomers δ 11.41 (bs, 1H), 8.00 (s, 1H), 7.88 (d, J = 7.7 Hz, 1H), 7.40 (d, J = 7.5 Hz, 1H), 7.31 (t, J = 7.7 Hz, 1H), 7.20-7.17 (m, 2H), 7.04-6.99 (m, 2H), 6.86-6.77 (m, 4H), 5.20 (bs, 1H), 4.63 (s, 1H), 4.55 (s, 1H), 4.21-3.99 (m, 6H), 3.72/3.70 (s, 3H), 2.97-2.94 (m, 2H), 2.34/2.32 (s, 3H), 1.43 (s, 9H); ¹³C NMR (CDCl₃, 75.48 MHz) rotomers δ 172.12, 158.58, 158.18, 156.80, 155.40, 145.49, 144.60, 132.82, 132.43, 129.67, 128.99, 128.64, 128.34, 127.20, 125.54, 123.30, 122.29/122.37, 114.47/114.56, 114.06/114.10, 86.23, 81.96, 66.35, 65.64, 64.24, 55.32/55.34, 51.01, 30.11, 28.16, 25.79, 10.00; IR (film): ν max 2932.9, 1715.1,
HRMS (Cl⁺) m/z 670.2765 (M⁺, C₃₇H₄₀N₅O₉ requires 670.2765). Anal. calcd. for C₃₇H₄₀N₅O₉: C, 66.36; H, 5.87; N, 6.27. Found: C, 66.42; H, 5.96; N, 5.95.

N-[4-[2-(2-{-3-[3-{-[[3',6'-dihydroxy-3-oxo-3H-spiro[2-benzofuran-1,9'-xanthen]-5-yl]amino]carbonothioyl}amino]prop-1-yn-1-yl}phenyl]-5-methyl-1,3-oxazol-4-yl)ethoxy]benzyl}-N-[(4-methoxyphenoxy)carbonyl]glycine (35)

Compound 53 (315 mg, 0.47 mmol) was treated with trifluoroacetic acid/“wet” CH₂Cl₂ (3:47, 25 mL) at 22 °C for 1 h. Removal of solvent in vacuo gave the free amine as a crude brown solid that was taken on immediately without further purification. This amine (315 mg, 0.55 mmol) was combined with triethylamine (0.12 mL, 0.83 mmol) in MeOH/THF (1:2, 30 mL). 5-fluorescein isothiocyanate, isomer I (215 mg, 0.55 mmol) was added and the solution was allowed to stir in the dark at 22 °C for 15 h. The reaction mixture was concentrated and the residue was purified via preparative reverse-phase HPLC (gradient: 30% CH₃CN, 60% H₂O, and 0.1% TFA to 99% CH₃CN, 1% H₂O, and
0.1% TFA over 40 min; retention time = 19.8 min) to afford 35 as an orange solid.

(371 mg, 70%). Mp 148-149 °C; \(^1\)H NMR (DMSO-\(d_6\), 400.13 MHz) rotomers δ 8.39 (s, 1H), 7.90 (s, 1H), 7.80 (d, \(J = 7.7 \text{ Hz}\), 2H), 7.52 (t, \(J = 7.4 \text{ Hz}\), 1H), 7.37 (d, \(J = 7.9 \text{ Hz}\), 1H), 7.30-7.25 (m, 4H), 7.02-6.95 (m, 2H), 6.92-6.87 (m, 6H), 6.76-6.75 (m, 2H), 6.69-6.59 (m, 5H), 5.12 (s, 2H), 4.58 (s, 1H), 4.46 (s, 1H), 4.21 (s, 2H), 4.01 (s, 1H), 3.93 (s, 1H), 3.70/3.69 (s, 3H), 2.94 (bs, 2H), 2.26 (s, 3H); \(^{13}\)C NMR (DMSO-\(d_6\), 100.62 MHz) δ 170.95, 170.66, 168.59, 160.16, 159.34, 158.96, 158.58, 158.31, 158.20, 157.99, 157.94, 156.77, 155.08, 154.75, 152.42, 147.19, 145.56, 144.79, 144.71, 141.01, 136.71, 133.05, 129.62, 129.53, 129.33, 129.23, 127.86, 127.58, 125.21, 124.22, 123.91, 122.75, 122.68, 118.89, 116.97, 115.52, 114.68, 114.62, 114.35, 113.02, 110.04, 102.55, 66.39, 55.44, 48.62, 48.43, 25.86, 10.01; IR (film): \(\nu_{\text{max}}\) 3069.8, 1629.6, 1517.4, 1280.3 cm\(^{-1}\); HRMS (FAB\(^+\)) \(m/z\) 959.2598 (MH\(^+\), \(C_{53}H_{43}N_4O_{12}S\) requires 959.2594).
N-[4-(2-{2-[3-(3-[[4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-
 methylbenzoyl]amino}prop-1-yn-1-yl]phenyl]-5-methyl-1,3-oxazol-4-
 yl)ethoxy]benzyl]-N-[(4-methoxyphenoxy)carbonyl]glycine (54)

Trifluoroacetic acid (2 mL) was added to a stirring solution of Compound
53 (20 mg, 0.03 mmol) in “wet” CH$_2$Cl$_2$ (20 mL) and the reaction was stirred at 22
°C for 1 h. The reaction was concentrated and the crude amine was combined
with PA Green OSu$^{55}$ (14 mg, 0.03 mmol) in DMF (1.5 mL). TEA (10 uL, 0.07
mmol) was added and the reaction was stirred in the dark at 22 °C for 12 h. The
solvent was removed in vacuo and the residue was purified via preparative
reverse-phase HPLC (gradient: 20% CH$_3$CN, 80% H$_2$O, and 0.1% TFA to 99%
CH$_3$CN, 1% H$_2$O, and 0.1% TFA over 25 min; retention time = 20.1 min) afforded
54 as an orange solid (13 mg, 46%). Mp 160-161 °C; $^1$H NMR (DMSO-$d_6$, 400.13
MHz) rotomers δ 8.13 (s, 1H), 7.87 (d, $J = 8.0$ Hz, 1H), 7.80 (d, $J = 7.7$ Hz, 2H),
7.57-7.52 (m, 1H), 7.36 (d, $J = 7.7$ Hz, 1H), 7.31-7.26 (m, 4H), 7.02-6.95 (m, 2H),
6.92-6.87 (m, 6H), 6.69-6.59 (m, 5H), 5.12 (s, 2H), 4.58 (s, 1H), 4.45 (s, 1H),
4.00 (s, 1H), 3.96-3.92 (m, 3H), 3.72/3.70 (s, 3H), 2.94 (bs, 2H), 2.27/2.25 (s,
3H), 2.02 (s, 3H); $^{13}$C NMR (DMSO-$d_6$, 100.62 MHz) δ 170.66, 168.59, 168.24,
methyl 3-nitro-4-(trifluoromethyl)benzoate (56)

To a stirring solution of 3-Nitro-4-(trifluoromethyl)benzoic acid (25.0 g, 106.3 mmol) in MeOH (300 mL) at 0 °C was slowly added thionyl chloride (15.5 mL, 212.7 mmol). The reaction was stirred at 0 °C for 10 min and then at 22 °C for 12 h. The reaction mixture was diluted with EtOAc, washed with H₂O (100 mL), saturated aqueous NaHCO₃ (100 mL) and dried over Na₂SO₄. Concentration gave an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:4) to give 56 as a pale yellow solid (24.3 g, 88%). mp 42-43 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 8.31 (s, 1H), 8.23 (d, J = 8.3 Hz, 1H), 7.82 (d, J = 8.3 Hz, 1H), 3.89 (s, 3H); ¹³C NMR (CDCl₃, 100.62 MHz) δ 163.40, 147.88, 135.00, 133.02, 128.33, 128.27, 128.22, 128.17, 126.90, 126.56, 126.22,
methyl 3-amino-4-(trifluoromethyl)benzoate (57)

To a flame-dried and degassed schlenk flask was charged 56 (21.0 g, 84.3 mmol) in MeOH (100 mL). 10% palladium on activated carbon was directly added and the reaction mixture was degassed under hydrogen and allowed to stir under ~ 1 atmosphere of hydrogen at 22 °C for 3 h. The reaction mixture was filtered through a pad of Celite and concentrated to afford 57 as a white solid (16.4 g, 93%). mp 98-99 °C; ¹H NMR (DMSO-­d₆, 299.87 MHz) δ 7.51 (s, 1H), 7.40 (d, J = 8.3 Hz, 1H), 7.12 (d, J = 8.2 Hz, 1H), 3.83 (s, 3H); ¹³C NMR (DMSO-­d₆, 75.41 MHz) δ 156.80, 146.39, 133.80, 130.11, 126.63, 126.56, 126.49, 126.42, 122.88, 119.27, 117.63, 117.58, 117.54, 115.18, 114.55, 113.37, 113.36, 52.18; IR (film): ν_max 3391.8, 1718.8, 1578.4, 1450.4 cm⁻¹; MS (ESI⁺) calcd. for C₉H₇F₃NO₄ (MH⁺) 250.04, found 250.01. Anal. calcd. for C₉H₆F₃NO₄: C, 43.39; H, 2.43; N, 5.62. Found: C, 43.52; H, 2.60; N, 5.61.
3-iodo-4-(trifluoromethyl)benzoic acid (58)

Compound 57 (16.4 g, 74.8 mmol) was suspended in H₂O (120 mL) and concentrated HCl (105 mL) was carefully added. The mixture was cooled to 0 °C and a solution of sodium nitrite (6.2 g, 89.9 mmol) in H₂O (20 mL) was added over 5 min. The reaction was stirred at 0 °C for 15 min and a solution of potassium iodide (14.9 g, 89.9 mmol) was added and the reaction was heated to 60 °C for 12 h. The reaction was allowed to cool to room temperature and extracted with CH₂Cl₂ (2 x 50 mL). The organics were washed with saturated aqueous NaHCO₃ (100 mL), 10% aqueous Na₂S₂O₃ (100 mL) and dried over Na₂SO₄. Concentration gave an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:9) to give 58 as a tan solid (19.2 g, 83%). mp 40-41 °C; ¹H NMR (CDCl₃, 299.87 MHz) δ 8.55 (s, 1H), 8.00-7.97 (m, 1H), 7.62 (d, J = 8.2 Hz, 1H), 3.89 (s, 3H); ¹³C NMR (CDCl₃, 75.41 MHz) δ 164.16, 142.64, 137.58, 137.17, 136.76, 136.34, 133.95, 128.81, 127.69, 127.47, 127.40, 127.33, 127.25, 124.05, 120.41, 116.77, 90.52, 52.63; IR (film): νₘₐₓ 1735.7, 1562.5, 1437.6, 1310.4 cm⁻¹; MS (ESI⁺) calcd. for C₉H₆F₃IO₂ (M + 2H⁺) 331.95, found 331.16. Anal. calcd. for C₉H₆F₃IO₂: C, 32.75; H, 1.83. Found: C, 32.93; H, 1.94.
3-iodo-4-(trifluoromethyl)benzoic acid (59)

A stirring solution of 58 (15.9 g, 48 mmol) in THF:MeOH (3:1, 150 mL) was treated with aqueous 1M LiOH (100 mL) and stirred for 3 h at 22 °C. The reaction mixture was diluted with H₂O (50 mL) and acidified to pH = 2 with aqueous 2M HCl. The aqueous layers were extracted with EtOAc (3 x 50 mL), dried over Na₂SO₄, and concentrated in vacuo to give the free acid 59 as a pale yellow solid (14.3 g, 91%). mp 179-180 °C; ¹H NMR (DMSO-d₆, 400.13 MHz) δ 13.67 (bs, 1H), 8.52 (s, 1H), 8.05 (d, J = 8.0 Hz, 1H), 7.83 (d, J = 7.4 Hz, 1H); ¹³C NMR (DMSO-d₆, 100.62 MHz) δ 165.09, 142.10, 136.10, 135.17, 134.87, 129.11, 127.98, 127.93, 127.87, 124.10, 121.38, 118.65, 90.44; IR (film): νmax 3076.5, 1697.6, 1307.8, 1178.5 cm⁻¹; MS (ESI⁺) calcd. for C₈H₄F₃IO₂ (MH⁺) 314.91, found 314.95. Anal. calcd. for C₈H₄F₃IO₂: C, 30.41; H, 1.28. Found: C, 30.49; H, 1.40.
3-iodo-4-(trifluoromethyl)benzamide (60)

Compound 59 (14.3 g, 45.2 mmol) was refluxed in excess SOCl$_2$ (100 mL) for 1 h. The reaction was cooled to room temperature and the remaining SOCl$_2$ was evaporated in vacuo to afford the acid chloride as a yellow liquid that was used immediately without further purification. This acid chloride (21.5 g, 80.6 mmol) was dissolved in THF (150 mL) and cannulated over 45 min into freshly distilled, liquefied ammonia (200 mL) at –78 °C. This mixture was allowed to stir for 30 min and then warmed to –40 °C for 3 h. Excess ammonia was removed by evaporation at 22 °C to yield an off-white solid that was suspended in H$_2$O (300 mL), filtered, and washed with H$_2$O (4 x 50 mL). Evaporation in vacuo gave 60 as a slight tan solid (14.1 g 95%). mp 178-179 °C; $^1$H NMR (DMSO-$d_6$, 400.13 MHz) δ 8.53 (s, 1H), 8.25 (s, 1H), 8.01 (d, $J = 8.2$ Hz, 1H), 7.82 (d, $J = 8.2$ Hz, 1H), 7.70 (s, 1H); $^{13}$C NMR (DMSO-$d_6$, 100.62 MHz) δ 165.28, 140.52, 138.77, 134.09, 133.79, 127.75, 127.69, 127.64, 127.49, 124.18, 121.45, 92.38; IR (film): $\nu_{\text{max}}$ 3415.0, 1650.0, 1557.7, 1310.3 cm$^{-1}$; MS (ESI$^+$) calcd. for C$_8$H$_5$F$_3$INO (MH$^+$) 315.94, found 315.96. Anal. calcd. for C$_8$H$_5$F$_3$INO: C, 30.50; H, 1.60; N, 4.45. Found: C, 30.60; H, 1.52; N, 4.49.
3-iodo-4-(trifluoromethyl)benzenecarbothioamide (61)

Compound 60 (12.5 g, 39.7 mmol) was combined with Lawesson’s reagent\textsuperscript{62} (35.3 g, 87.3 mmol) in benzene (150 mL) and the suspension was refluxed for 16 h. The reaction was filtered and the filtrate was concentrated to give an oil that was purified by column chromatography eluting with CH₂Cl₂ to give the product as a yellow solid (10.5 g, 81%). mp 118-119 °C; \textsuperscript{1}H NMR (DMSO-\textit{d}_6, 299.87 MHz) δ 10.17 (s, 1H), 9.76 (s, 1H), 8.47 (s, 1H), 7.97 (d, \(J = 8.7\) Hz, 1H), 7.77 (d, \(J = 8.2\) Hz, 1H); \textsuperscript{13}C NMR (DMSO-\textit{d}_6, 75.41 MHz) δ 197.10, 143.87, 143.86, 143.84, 139.90, 134.00, 133.60, 133.20, 132.79, 128.23, 127.39, 127.32, 127.25, 127.17, 127.02, 124.60, 120.97, 117.34, 91.93; IR (film): \(\nu_{\text{max}}\) 3271.9, 1625.5, 1422.5, 1127.9 cm\textsuperscript{-1}; MS (ESI\textsuperscript{+}) calcd. for C\textsubscript{8}H\textsubscript{6}F\textsubscript{3}INS (MH\textsuperscript{+}) 331.91, found 331.94. Anal. calcd. for C\textsubscript{8}H\textsubscript{5}F\textsubscript{3}INS: C, 29.02; H, 1.52; N, 4.23. Found: C, 29.22; H, 1.43; N, 4.25.
ethyl{2-[3-iodo-4-(trifluoromethyl)phenyl]-4-methyl-1,3-thiazol-5-yl}acetate (63)

Compound 61 (6.0 g, 18.1 mmol) was combined with ethyl 2-chloroacetate (62) (3.0 mL, 21.2 mmol) in absolute EtOH (50 mL) and the reaction was refluxed for 36 h. The solvent was evaporated in vacuo and the residue was purified by column chromatography eluting with Et$_2$O/hexanes (1:7) to afford the product as a slight pink solid (6.2 g, 78%). mp 129-130 °C; $^1$H NMR (CDCl$_3$, 400.13 MHz) δ 8.50 (s, 1H), 7.85 (d, $J$ = 9.2 Hz, 1H), 7.59 (d, $J$ = 8.4 Hz, 1H), 4.30 (q, $J$ = 7.1 Hz, 2H), 2.71 (s, 3H) 1.34 (t, 3H, $J$ = 7.1 Hz); $^{13}$C NMR (CDCl$_3$, 100.62 MHz) δ 165.34, 161.59, 161.18, 139.39, 136.63, 135.10, 134.76, 127.86, 127.80, 127.74, 126.48, 125.73, 123.75, 123.38, 121.03, 118.31, 91.47, 61.45, 17.36, 14.19; IR (film): $\nu_{max}$ 1705.8, 1384.0, 1312.6, 1120.1 cm$^{-1}$; MS (ESI$^+$) calcd. for (MH$^+$) $C_{14}H_{11}F_3INO_2S$ 441.96, found 441.96. Anal. calcd. for $C_{14}H_{11}F_3INO_2S$: C, 38.11; H, 2.51; N, 3.17. Found: C, 38.27; H, 2.55; N, 3.08.
{2-[3-iodo-4-(trifluoromethyl)phenyl]-4-methyl-1,3-thiazol-5-yl}methanol (64)

Diisobutylaluminium hydride (13.5 mL, 1M in THF) was slowly added to a stirring solution of compound 63 (3.0 g, 6.8 mmol) in CH$_2$Cl$_2$ (100 mL) at 0 °C. The reaction was stirred at 0 °C for 1 h and aqueous 1M sodium potassium tartrate (50 mL) was added. The mixture was stirred for 45 min and the aqueous layers were extracted with CH$_2$Cl$_2$ (2 x 50 mL). The combined organics were dried over Na$_2$SO$_4$ and concentrated to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:1) to give the title compound as a white solid (2.6 g, 97%). mp 164-165 °C; $^1$H NMR (DMSO-$d_6$, 400.13 MHz) δ 8.50 (s, 1H), 7.96 (d, $J$ = 8.0 Hz, 1H), 7.76 (d, $J$ = 8.0 Hz, 1H), 5.68-5.65 (m, 1H), 4.65-4.64 (m, 2H) 2.34 (s, 3H); $^{13}$C NMR (DMSO-$d_6$, 100.62 MHz) δ 159.86, 149.90, 137.80, 137.50, 136.56, 132.34, 128.40, 128.35, 125.38, 124.26, 121.54, 93.23, 55.34, 14.93; IR (film): $\nu_{\text{max}}$ 3258.1, 1595.6, 1306.2, 1113.4 cm$^{-1}$; MS (ESI$^+$) calcd. for C$_{12}$H$_{10}$F$_3$INOS 399.94 (MH$^+$), found 399.99. Anal. calcd. for C$_{12}$H$_9$F$_3$INOS: C, 36.11; H, 2.27; N, 3.51. Found: C, 36.28; H, 2.44; N, 3.38.
**tert-butyl {3-[5-[5-(hydroxymethyl)-4-methyl-1,3-thiazol-2-yl]-2-(trifluoromethyl)phenyl]prop-2-yn-1-yl}carbamate (65)**

To a flame-dried and degassed schlenk flask was charged 64 (500 mg, 1.3 mmol), 22 (583 mg, 3.8 mmol), DIEA (1.1 mL, 6.3 mmol) and Pd(PPh₃)₂Cl₂ (44 mg, 5 mol%) in THF (30 mL). The mixture was then degassed under argon and Cul (24 mg, 10 mol%) was added. The reaction mixture was degassed once more and stirred vigorously for 45 min at 22 °C. The mixture was then poured into H₂O (50 mL) and extracted with EtOAc (3 x 25 mL). The combined organics were dried over Na₂SO₄ and concentrated to give a red oil that was purified by column chromatography eluting with acetone/CH₂Cl₂ (1:7) to afford 65 as a tan oil (472 mg, 85%).

1H NMR (CDCl₃, 400.13 MHz) δ 7.85 (s, 1H), 7.65 (d, J = 9.2 Hz, 1H), 7.46 (d, J = 8.4 Hz, 1H), 5.32 (bs, 1H), 4.45 (s, 2H), 4.07-4.06 (m, 2H), 2.67 (s, 3H), 1.37 (s, 9H); 13C NMR (CDCl₃, 100.62 MHz) δ 162.45, 155.49, 149.96, 136.11, 133.84, 131.74, 131.43, 131.06, 126.23, 126.18, 125.06, 121.63, 121.50, 91.97, 79.94, 56.13, 30.92, 30.59, 28.06, 14.67; IR (film): νmax 3337.5, 2978.5, 1693.9, 1504.1 cm⁻¹; MS (ESI⁺) calcd. for C₂₀H₂₂F₃N₂O₃S (TOF ESI⁺) 427.1, found 427.1. Anal. calcd. for C₂₀H₂₁F₃N₂O₃S: C, 56.33; H, 4.96; N, 6.57. Found: C, 55.97; H, 5.10; N, 6.25.
tert-butyl {3-[5-[5-(chloromethyl)]-4-methyl-1,3-thiazol-2-yl]-2-(trifluoromethyl)phenyl}prop-2-yn-1-yl}carbamate (66)

To a stirring solution of 65 (440 mg, 1.0 mmol) in THF (20 mL) at 0 °C was added thionyl chloride (0.10 mL, 1.2 mmol). The reaction was stirred for 20 min, poured into saturated aqueous NaHCO₃ (30 mL) and extracted with CH₂Cl₂ (30 mL). The organics were dried over Na₂SO₄ and concentrated to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (3:7) to give the title compound as a tan oil (379 mg, 85%). ¹H NMR (CDCl₃, 400.13 MHz) δ 7.89 (s, 1H), 7.69 (d, J = 8.2 Hz, 1H), 7.49 (d, J = 8.3 Hz, 1H), 5.26 (bs, 1H), 4.66 (s, 2H), 4.09-4.03 (m, 2H), 2.34 (s, 3H), 1.37 (s, 9H) ; ¹³C NMR (CDCl₃, 100.62 MHz) δ 163.29, 155.22, 152.91, 135.82, 132.10, 131.79, 131.20, 129.14, 126.24, 126.19, 125.10, 124.29, 121.67, 121.64, 121.57, 92.34, 79.61, 36.75, 30.88, 28.02, 14.62; IR (film): ν_max 2928.8, 1705.8, 1314.2, 1053.1 cm⁻¹; MS (ESI⁺) calcd. for C_{20}H_{21}F₃ClN₂O₂S (MH⁺) 445.1, found 445.1. Anal. calcd. for C_{20}H_{20}F₃ClN₂O₂S: C, 53.99; H, 4.53; N, 6.30. Found: C, 54.21; H, 4.91; N, 6.12.
**tert-butyl {3-[5-(5-{[(4-hydroxy-3-methylphenyl)thio]methyl}-4-methyl-1,3-thiazol-2-yl)-2-(trifluoromethyl)phenyl]prop-2-yn-1-yl}carbamate (68)**

Compounds 66 (379 mg, 0.85 mmol) and 67 (143 mg, 1.0 mmol) were combined with Cs$_2$CO$_3$ (333 mg, 1.0 mmol) in CH$_3$CN (20 mL) and stirred at 22 °C for 12 h. The reaction mixture was diluted with H$_2$O (30 mL) and extracted with EtOAc. The organics were dried over Na$_2$SO$_4$ and concentrated to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (2:3) to give the product as a tan oil (379 mg, 85%). $^1$H NMR (CDCl$_3$, 400.13 MHz) δ 8.02 (s, 1H), 7.83 (d, $J$ = 8.4 Hz, 1H), 7.59 (d, $J$ = 8.3 Hz, 1H), 7.29-7.24 (m, 2H), 6.79-6.68 (m, 1H), 5.20 (bs, 1H), 5.09 (s, 2H), 4.16-4.11 (m, 2H), 2.45 (s, 3H), 2.16 (s, 3H), 1.43 (s, 9H); $^{13}$C NMR (CDCl$_3$, 100.62 MHz) δ 163.38, 156.09, 155.36, 151.43, 136.04, 133.99, 132.78, 132.05, 131.75, 131.29, 130.07, 129.19, 128.86, 128.83, 128.58, 128.14, 127.02, 126.32, 126.27, 125.33, 125.18, 124.38, 121.66, 115.18, 111.71, 92.12, 79.94, 62.23, 30.98, 28.11, 20.77, 16.07, 15.72; IR (film): $\nu_{\text{max}}$ 2928.8, 1705.8, 1314.2, 1053.1 cm$^{-1}$; MS (ESI$^+$) calcd. for C$_{27}$H$_{28}$F$_3$N$_2$O$_3$S$_2$ (MH$^+$) 549.15, found 549.22. Anal. calcd. for C$_{27}$H$_{27}$F$_3$N$_2$O$_3$S$_2$: C, 59.11; H, 4.96; N, 5.11. Found: C, 58.86; H, 5.19; N, 4.71.
methyl {4-[[2-[3-[[tert-butoxycarbonyl]amino]prop-1-yn-1-yl]-4-(trifluoromethyl)phenyl]-4-methyl-1,3-thiazol-5-yl]methyl}thio]-2-methylphenoxy)acetate (70)

Compound 68 (367 mg, 0.67 mmol) was combined with methyl bromoacetate (0.07 mL, 0.80 mmol) and Cs$_2$CO$_3$ in CH$_3$CN (15 mL). The reaction mixture was stirred at 22 °C for 12 h, diluted with H$_2$O (30 mL) and extracted with EtOAc. The organics were dried over Na$_2$SO$_4$ and concentrated to give an oil that was dissolved in THF:MeOH (3:1, 25 mL) and treated with aqueous 1M LiOH (1.0 mL). The reaction was stirred for 1 h at 22 °C, diluted with H$_2$O (20 mL) and acidified to pH = 2 with aqueous 2M HCl. The aqueous layers were extracted with EtOAc (3 x 15 mL), dried over Na$_2$SO$_4$, and concentrated to give an oil that was purified by column chromatography eluting with MeOH/CH$_2$Cl$_2$ (1:9) to give 70 as a slightly impure yellow solid, which was taken on without further purification (241 mg, 78%). $^1$H NMR (CDCl$_3$, 400.13 MHz) δ 8.08 (s, 1H), 7.84 (d, $J = 8.3$ Hz, 1H), 7.59 (d, $J = 8.2$ Hz, 1H), 7.29-7.25 (m, 1H), 6.79-6.70 (m, 1H), 6.68-6.62 (m, 1H), 5.21 (bs, 1H), 5.20 (s, 2H), 4.63 (s, 2H), 4.12-4.17 (m, 2H), 2.47 (s, 3H), 2.18 (s, 3H), 1.46 (s, 9H); $^{13}$C NMR (CDCl$_3$, 100.62 MHz) δ HRMS (TOF ESI$^+$) m/z 607.1578 (MH$^+$, C$_{29}$H$_{30}$F$_3$N$_2$O$_5$S$_2$ requires 607.1548).

\[
\text{\textbf{70}}
\]
Trifluoroacetic acid (2 mL) was added to a stirring solution of Compound 70 (30 mg, 0.05 mmol) in “wet” CH$_2$Cl$_2$ (20 mL) and the reaction was stirred at 22 °C for 1 h. The reaction was concentrated and the crude amine was combined with PA Green OSu$^{55}$ (24 mg, 0.05 mmol) in DMF (1.5 mL). DIEA (20 uL, 0.12 mmol) was added and the reaction was stirred in the dark at 22 °C for 12 h. The solvent was removed in vacuo and the residue was purified via preparative reverse-phase HPLC (gradient: 20% CH$_3$CN, 80% H$_2$O, and 0.1% TFA to 99% CH$_3$CN, 1% H$_2$O, and 0.1% TFA over 25 min; retention time = 19.9 min) afforded 71 as an orange solid (20 mg, 46%). $^1$H NMR (DMSO-d$_6$, 400.13 MHz) δ 10.91 (bs, 1H), 8.12 (s, 1H), 7.92 (d, $J = 7.1$ Hz, 2H), 7.86 (d, $J = 8.2$ Hz, 1H), 7.59 (d, $J = 8.3$ Hz, 1H), 7.30-7.24 (m, 1H), 6.94-6.70 (m, 2H), 6.67-6.70 (m, 3H), 6.41-6.38 (m, 2H), 5.23 (bs, 1H), 5.21 (s, 2H), 4.61 (s, 2H), 4.26-4.11 (m, 4H), 2.47 (s, 3H), 2.18 (s, 3H), 2.00 (s, 3H); $^{13}$C NMR (DMSO-d$_6$, 100.62 MHz) δ 173.21, 171.47, 166.81, 163.72, 163.37, 159.71, 156.11, 155.29, 154.23, 151.76, 150.02, 139.19,
5-(chloromethyl)-2-[3-iodo-4-(trifluoromethyl)phenyl]-4-methyl-1,3-thiazole (72)

To a stirring solution of 64 (300 mg, 0.70 mmol) in THF (30 mL) at 0 °C was added thionyl chloride (0.06 mL, 0.84 mmol). The reaction was stirred for 0.5 h, poured into saturated aqueous NaHCO₃ (30 mL) and extracted with CH₂Cl₂ (30 mL). The organics were dried over Na₂SO₄ and concentrated to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (3:7) to give the title compound as a tan solid (283 mg, 91%). ¹H NMR (CDCl₃, 400.13 MHz) δ 8.46 (s, 1H), 7.93 (d, J = 8.2 Hz, 1H), 7.73 (d, J = 8.4 Hz, 1H), 5.09 (s, 2H), 2.41 (s, 3H); ¹³C NMR (CDCl₃, 100.62 MHz) δ 161.84, 153.19, 138.08, 136.83, 133.19, 132.88, 132.58, 132.28, 130.39, 128.32, 128.26, 128.21, 126.86, 125.63, 124.14, 121.42, 93.26, 37.41, 14.72; IR (film): ν_max 3517.2, 2812.3, 1710.5, 1594.1, 1029.1 cm⁻¹; HRMS (FAB⁺) m/z 871.1579 (MH⁺, C₄₅H₃₂F₅N₂O₇S₂ requires 871.1571).
Anal. calcd. for C_{12}H_{8}F_{3}ClNS: C, 34.51; H, 1.93; N, 3.35. Found: C, 34.80; H, 1.83; N, 3.23.

![Chemical structure of compound 73](image)

{4-[(2-[3-iodo-4-(trifluoromethyl)phenyl]-4-methyl-1,3-thiazol-5-yl)methyl]thio}-2-methylphenoxy)acetic acid (73)

Compounds 72 (283 mg, 0.64 mmol) and 67 (107 mg, 0.76 mmol) were combined with Cs_{2}CO_{3} (249 mg, 0.76 mmol) in CH_{3}CN (20 mL) and stirred at 22 °C for 12 h. Methyl bromoacetate (69) (0.07 mL, 0.76 mmol) was then added and the reaction was stirred at 22 °C for an additional 12 h. The reaction mixture was diluted with H_{2}O (30 mL) and extracted with EtOAc. The organics were dried over Na_{2}SO_{4} and concentrated to give an oil that was dissolved in THF:MeOH (3:1, 25 mL) and treated with aqueous 1M LiOH (1.0 mL). The reaction was stirred for 6 h at 22 °C, diluted with H_{2}O (20 mL) and acidified to pH = 2 with aqueous 2M HCl. The aqueous layers were extracted with EtOAc (3 x 15 mL), dried over Na_{2}SO_{4}, and concentrated to give an oil that was purified by column chromatography eluting with EtOAc/Hexanes (3:7) followed by MeOH/CH_{2}Cl_{2} (1:9) to give 73 as a white solid (217 mg, 59%). ^{1}H NMR (CDCl_{3}, 400.13 MHz) δ 8.54 (s, 1H), 7.90 (d, J = 8.2 Hz, 1H), 7.64 (d, J = 8.3 Hz, 1H), 7.28-7.22 (m, 2H), 6.62 (d, J = 8.5 Hz, 1H), 5.16 (s, 2H), 4.64 (s, 2H), 2.48 (s, 3H), 2.19 (s, 3H); ^{13}C NMR (CDCl_{3},
10.62 MHz) δ 171.50, 161.84, 156.11, 153.19, 138.08, 136.83, 133.19, 132.88,
132.58, 132.28, 130.39, 128.60, 128.32, 128.26, 128.21, 127.03, 126.86, 125.63,
2924.3, 1488.3, 1311.5, 1135.7 cm⁻¹; MS (ESI⁻) calcd. for C_{21}H_{16}F_{3}NO_{3}S_{2} (MH⁻)
578.0, found 578.0. Anal. calcd. for C_{21}H_{17}F_{3}NO_{3}S_{2}: C, 43.53; H, 2.96; N, 2.42.
Found: C, 43.72; H, 2.82; N, 2.31.

3.10.3 Cell Culture

Chinese hamster ovary (CHO-K1) cells (ATCC #CCL-61) were maintained
in F-12K media supplemented with fetal bovine serum (FBS, 10%), penicillin
(100 units / mL), and streptomycin (100 µg / mL). Cells were propagated in a
humidified 5 % CO₂ incubator at 37°C.

3.10.4 Transfection and Labeling

The day before transfection, exponentially growing CHO-K1 cells (5-8 x
10^4 cells/ml) were seeded on glass coverslips in a 6-well plate. The cells were
transfected using Effectene Transfection Reagent kit (Qiagen, Inc.) as per
manufacturer’s specifications. For each transfection, 600 ng plasmid DNA
(alpha, beta or gamma, courtesy of Jerry Thompson) were used with a
plasmid:Effectene ratio of 1:30. After 5-6 hours the cells were rinsed (2 x 1mL) with Dulbecco’s phosphate buffered saline (dPBS) and fresh media (1 mL) was added to each well containing the appropriate compound(s) with the DMSO concentration content kept at 1%. The cells were incubated overnight (16-18 hours) at 37 °C, rinsed 3 x with dPBS (2 mL) and imaged by confocal laser scanning and differential interference contrast (DIC) microscopy. The Pennsylvania Green probes were used at 20 μM and the unlabeled probes used for competition were at 50 μM.

3.10.5 Confocal Microscopy

An inverted Zeiss LSM 5 Pascal confocal laser-scanning microscope fitted with a Plan Apochromat oil-immersion objective (63 X) was employed. Fluorophores were excited with the 488 spectral line of an argon laser and emitted photons were collected through a 505 nm LP filter. For cellular imaging, coverslips bearing living cells were carefully removed from the 6-well plate and mounted on microscope slides bearing a press-to-seal silicone isolater filled with media to preserve cell viability during the course of the analysis.
3.11 References


43. Rossi, R.; Carpita, A.; Bellina, F., Palladium-Mediated and/or Copper-Mediated Cross-Coupling Reactions between 1-Alkynes and Vinyl, Aryl, 1-


Chapter 4

Artificial Cell Surface Receptors Designed to Span Plasma Membranes of Living Mammalian Cells

4.1 Introduction

Many receptors on cell surfaces span plasma membranes. For example, the low-density lipoprotein receptor (LDLR), one of the most extensively characterized internalizing cell surface receptors,\(^1\) \(^2\) is a membrane-spanning protein, consisting of an extracellular domain, a transmembrane domain, and a cytoplasmic domain. This receptor enables cells to internalize cholesterol-laden LDL particles (Figure 4.1). These particles, composed of the protein apolipoprotein B-100 (Apo-B), cholesteryl esters, phospholipids, cholesterol, and triglycerides, bind the LDLR through interactions with Apo-B, promoting endocytic uptake of exogenous cholesterol.\(^3\) Given the fact that cholesterol is a key building block for the biosynthesis of steroid hormones, bile acids, and cellular plasma membranes, the LDLR is often overexpressed on rapidly proliferating cancer cells, and this receptor provides a target for the delivery of anticancer therapeutics.\(^4\) Mechanistically, LDL undergoes receptor-mediated endocytosis by binding the LDLR clustered in pits on the cell surface that are coated by the intracellular protein clathrin. Clathrin controls the endocytosis of many cell surface receptors, including, at least in part, small natural cell surface receptors such as ganglioside GM1, which is responsible for the endocytosis of the protein.
During the initial steps of endocytosis, clathrin coated pits containing LDLR/LDL complexes fold and break off to form intracellular membrane-sealed endocytic vesicles. These vesicles fuse and are acidified by activation of proton pumps, resulting in the formation of larger acidic endosomes. The LDLR dissociates from LDL in these compartments, allowing the released

Figure 4.1. Illustration of an LDL particle (Panel A), its cell surface receptor (Panel B) and endocytosis of LDL mediated by the LDL receptor (Panel C). Figure adapted from Peterson, B.R.³
LDLR to cycle back to the cell surface and initiate another round of delivery. The endosomes containing free LDL fuse with lysosomes, which liberate cholesterol and amino acid nutrients for utilization by the cell. (Figure 4.1).¹

Over the past several years, the Peterson laboratory has created artificial cell surface receptors derived from 3β-cholesterylamines that enable endocytic delivery of cell-impermeable macromolecules and therapeutics into mammalian cells.⁶ These compounds are similar in size to the glycolipid ganglioside GM1, and appear to be internalized at least in part by clathrin mediated endocytosis. Based on these results, we postulated that dimers related to 3β-cholesterylamines might function as artificial cell surface receptors with the potential to span cellular membranes and mimic functions of natural transmembrane proteins, such as the LDLR. Moreover, related non-symmetrical dimers could potentially engage molecules in the cytosol and provide a new method for controlling cell signaling.

4.2 Design of Membrane Spanning Cell Surface Receptors

Initially, we sought to construct a variety of fluorescent symmetrical dimers that vary the distance between two steroids to investigate the optimal distance that would allow insertion into cellular membranes as shown in Figure 4.2. We chose acetylene-like dimeric 3β-cholesterylamines because of the wide scope of copper and palladium-mediated reactions that might allow access to such
Figure 4.2. Structures of symmetrical and asymmetrical dimers related to 3β-cholesterylamine.

compounds via convergent syntheses. In addition, reduction of these analogues to provide saturated compounds of similar length would allow us to compare the
effects of rigidity on biological activity. The fluorophore Oregon Green was chosen as a head group based on previous studies of monomeric cholesterylamines bearing this polar green fluorescent moiety.

Figure 4.3. A strategy for controlling cell signaling via artificial transmembrane receptors. A: The receptor inserts into the outer leaflet in a U-shaped conformation. B: The less polar biotinylated motif slides across the plasma membrane and forms a stable transmembrane geometry that projects biotin into the cytosol. C and D: Streptavidin binds biotin at the inner leaflet and forms a non-covalent complex. E: Proteins fused to streptavidin influence signaling and uptake pathways. Figure courtesy of B. R. Peterson.
The fluorescent and biotinylated asymmetrical dimer (80) was hypothesized to initially incorporate into the outer leaflet of the plasma membrane in a U-shaped conformation (Figure 4.3). Since one of the two steroids comprises a non-protonatable 3β amide tethered to biotin, this less polar side of the dimer should passively diffuse across the plasma membrane outer leaflet and provide a membrane spanning artificial receptor. Treatment of genetically modified cells that express red fluorescent streptavidin with this dimer should allow recruitment of this protein to the inner leaflet of the membrane, which can be visualized through fluorescence microscopy. This represents a fundamentally new strategy for controlling cellular signaling and endocytosis.

4.3 Synthesis of Symmetrical Dimers Related to 3β-Cholesterylamine

To synthesize a symmetrical di-acetylene analogue of 3β-cholesterylamine we initially silylated methyl hyodeoxycholate (81) at C-3 and C-6 with tert-butyl-dimethylsilyl chloride/imidazole in DMF/pyridine solution (Figure 4.4).\(^8\),\(^9\) Disilylated 82 was reduced to the corresponding alcohol (83) using lithium aluminum hydride (Figure 4.4). Alcohol 83 was then treated with iodine and triphenylphosphine to provide the desired iodide (84), which was displaced by trimethylsilyl acetylide to give 85. The C-3 and C-6 silyl ethers were desilylated under acidic conditions to afford diol 86. Mitsunobu conditions\(^10\)\(^-\)\(^12\) employing excess hydrazoic acid and triphenylphosphine at elevated temperature provided the 3β-azide and C-5 to C-6 olefin of 87. The azide was
reduced via a Staudinger reaction\textsuperscript{13-15} and the primary amine mono-alkylated with 3-bromopropyl phthalimide at moderate temperature to give the secondary amine, which was immediately protected as the Boc-carbamate (88). This advanced intermediate (88) provides a key building block for the construction of symmetrical and asymmetrical dimers due to its robust nature and terminal alkyne suitable for palladium-mediated reactions.

**Figure 4.4.** Synthesis of key intermediate 88 for subsequent palladium couplings.
As shown in Figure 4.5, intermediate 88 was dimerized using newly developed methods for palladium-mediated homodimerization of alkynes\textsuperscript{16} to afford 89. Further elaboration by cleavage of the phthalimide protecting groups with hydrazine afforded the free amine, which was immediately acylated with 90.

**Figure 4.5.** Synthesis of the symmetrical Oregon Green diyne dimer 74.
to afford the di-amino acid linkage in 91. The Fmoc carbamates were cleaved under basic conditions, the resulting free amine acylated with Oregon Green OSu, and the Boc-carbamates cleaved under acidic conditions to give the final symmetrical Oregon Green labeled diyne dimer 74.

Figure 4.6. Synthesis of butane-linked Oregon Green butane-linked dimer 75.
Utilizing chemistry developed by Fu et al.,\textsuperscript{17} we sought to investigate the effect of linker length between the two acetylenes on cellular association of these compounds. In this regard, intermediate 88 was combined with 1,4-diiodobutane in a palladium mediated Sp-Sp\textsuperscript{3} coupling to afford the butane-linked diyne 92. Subsequent phthalimide cleavage followed by acylation with 90 lead to the β-alanine derivative 93. Fmoc cleavage with piperidine, acylation with Oregon Green OSu, and Boc removal with TFA yielded the symmetrical Oregon Green butane-linked diyne dimer 75 (Figure 4.6).

4.4 Alternative Methodology for Syntheses of Symmetrical Dimers

Alternative synthetic methodology was sought to facilitate the future synthesis of asymmetrical steroidal dimers. We desired two distinct monomeric scaffolds that can be readily attained and are tolerant of conditions that would allow effective hetero-dimerization. Second, each monomeric steroid needs to contain orthogonal protecting groups for further elaboration of the 3β-amine side chains on each end of the molecule. Finally, the best route must easily adapt to subtle changes with regards to amino acid linkers, fluorophores, and different carbon lengths between the two bridging D rings.
4.4.1 Wittig Methodology

We explored the concept of using Wittig chemistry\textsuperscript{18-20} where one steroidal aldehyde could be combined with a steroidal ylide to generate an asymmetrical dimer. Simple catalytic hydrogenation could then afford an alkane linked asymmetrical dimer that would allow us to further study the effects of linker rigidity. The attempted synthesis of alkenes via this route is outlined in Figure 4.7.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure47.png}
\caption{Figure 4.7. Attempted Wittig route.}
\end{figure}
The acetate di-bromide of stigmasterol (94) was obtained via a literature procedure.\textsuperscript{21} Ozonolysis of 94 employing trimethylphosphite to quench the ozonide was achieved through modification of an earlier reported method to afford 95 in poor yield.\textsuperscript{22} Treatment of 95 with NaI in acetone gave compound 96 in moderate yield. A Wittig reaction employing the phosphonium salt derived from 8-bromo-1-octanol with 97 provided 98 in low yield. The corresponding alcohol was converted to the phosphonium ylide in an attempted Wittig reaction with 99. However, after various synthetic attempts and modified reaction conditions, the desired product was never attained in sufficient yield for complete characterization. Moreover, the fact that we could not optimize earlier yields lead us to abandon this route and search for alternative methods.

\textbf{4.4.2 Click Methodology}

We also investigated the use of the regiospecific and mild copper (I)-catalyzed Huisgen 1,3-cycloaddition of fully elaborated azides (105) and terminal alkynes (104) to prepare dimeric cholesterylamine analogues (Figure 4.8)\textsuperscript{23, 24} In this case, a 1,4-disubstituted triazole linkage would be installed between the two steroids.
This approach shortened our earlier synthesis (Figure 4.4) by accessing azide ester 100 from the Mitsunobu reaction followed by a double reduction to generate amino alcohol 101. Selective mono alkylation of the primary amine was achieved and Boc protection gave the highly robust phthalimide-Boc alcohol 102.

Figure 4.8. Synthesis of “Click” dimer intermediate.
Iodination of 102 was achieved through the use of triphenylphosphine and iodine to give 103, whereas treatment of 102 with hydrazoic acid provided the elaborated azide 105. Palladium mediated coupling between 1,7 octadiyne and iodide 103 provided 104 in respectable yield. A microwave-assisted 1,3-
cycloaddition of 105 with 104 in DMF:H$_2$O furnished the 1,4-triazole 106 in moderate yield. Subsequent phthalimide cleavage followed by acylation with 90 lead to the $\beta$-alanine derivative 107. Fmoc cleavage with piperidine, acylation with Oregon Green OSu, and Boc removal with TFA yielded the pseudo-symmetrical Oregon Green “Click” dimer 76 (Figure 4.9).

### 4.4.3 Sonagashira Methodology via a Takai Reaction

The amino alcohol 101 was envisioned as a more versatile building block for the construction of dimeric steroids. Conversion of 101 into vinyl iodide 108 was proposed to enable coupling with commercially available alkynes such as 109 and 110 via Sonagashira reactions (Figure 4.10). This could allow us to create structurally similar motifs using a better synthetic route for the eventual synthesis of asymmetrical analogues.

Initially, intermediate 102 was subjected to Swern oxidation$^{25, 26}$ to provide aldehyde 97. A modified Takai reaction$^{27, 28}$ utilizing the fairly air-stable chromium (III) chloride and zinc dust as the reducing agent lead to the desired vinyl iodide 108 (first generated by Paul Munson), which was then subjected to Songashira reaction with alkynes 109 and 110 to give enyne 111 and 112 respectively. Catalytic hydrogenation employing palladium on carbon furnished the saturated alkane version 113. Phthalimide cleavage of 111, 112, and 113 was achieved by treatment with hydrazine followed by acylation of the free amines with 90 to provide the $\beta$-alanine derivatives. Fmoc cleavage with piperidine, acylation with
Oregon Green OSu, and Boc removal with TFA yielded the symmetrical Oregon Green diyne dimers 77 and 78 and the saturated dimer 79 (Figure 4.10).

Figure 4.10. Synthesis of symmetrical steroids via Sonagashira routes.
4.5 Examination of the Spectral Properties of Symmetrical Dimers

As shown in Figure 4.12 (Panel A), the concentrations of all of the symmetrical dimers were normalized using UV spectroscopy to allow comparison in other experiments. Fluorescence emission data was also obtained to examine if differences in structure would be observed (Panel B). Interestingly, each dimer showed significant differences in emission, which we hypothesize could be related to linker rigidity and fluorescence quenching through HOMO-FRET effects. In compounds 75, 77, 78, and 79 the length between the steroids should be approximately the same. However, compound 78 shows the greatest fluorescence emission. This compound (78) also contains the most rigid linker between the two steroids, which should maintain the fluorophores distant from one another. In contrast, we postulate that the flexible linker of the saturated dimer 79 may allow folding or stacking of the two steroids by placing the two fluorophores in close proximity, which would allow fluorescence quenching via a

Figure 4.11. Panel A: Normalized absorbances of symmetrical dimers (20 µM) in PBS. Panel B: Fluorescence emission data for symmetrical dimers (200 nM) in PBS. Data credited to Ewa Maddox.
HOMO-FRET mechanism. In addition, compounds 77 and 78 are red shifted indicating that there may be electronic coupling between the enynes and the fluorophores. Although the di-acetylene of 74 and triazole of 76 are fairly rigid, these functional groups may quench fluorescence, given that these dimers exhibit very low levels of fluorescence emission. The low fluorescence of 74 and 76 needs further investigation to definitively establish the mechanism of quenching.

4.6 Analysis of Symmetrical Dimers on Living Cells

Living Jurkat lymphocytes were treated with compounds 74, 75, 76, 77, 78 and 79 and examined by confocal laser scanning microscopy. This human T-cell line was treated with the compounds for 1 h and 4 h, centrifuged, washed to remove unincorporated probes, and cellular fluorescence was imaged as shown in Figure 4.12. Diyne 74, which has the shortest length between the two steroidal scaffolds resulted in very little cellular fluorescence even after 4 h. However, the more extended butane-linked dimer 75 engendered high levels of cellular fluorescence after only 1 h. Moreover, we also found that the cells had a preference for carbon-linked chains rather than amide-like heterocycles such as 76 of similar length. A more dramatic comparison is observed between enynes 77 and 78 where the addition of a single carbon atom in 77 dramatically affected the cellular results. Enyne 78 and its fully saturated version 79 both showed high
**Figure 4.12.** Confocal laser scanning (left) and differential interference contrast (right) micrographs of living Jurkat lymphocytes treated with dimers (10 μM) in RPMI media for 1 h and 4 h at 37 °C. Images contributed by Ewa Maddox.
levels of cellular association, and gratifyingly, these were the most synthetically accessible.

We further used flow cytometry to quantify the amount of whole cell fluorescence engendered by the Oregon Green derived symmetrical dimers (Figure 4.13). A high correlation was observed between the qualitative confocal micrographs and the mean fluorescence quantified in this way. Comparison of diyne 74 with the butane-linked dimer 75 revealed that the butane linker enhanced fluorescence by 20-fold at 1 h treatment. The “click” dimer 76 and enyne 77 showed essentially no cellular fluorescence. The intrinsically low fluorescence emission of 76 might explain these results with this particular

![Cellular Uptake of Fluorescent Dimers](image)

**Figure 4.13.** Whole cell fluorescence of dimers measured by flow cytometry. Living cells were treated with compounds (10 uM) for the times shown. Flow data credited to Ewa Maddox.
compound. However, this compound may not load at all into cellular membranes given the sensitivity of cellular fluorescence to the structure of the linker region. The high sensitivity of cells to linker structure is exemplified by the low level of cellular fluorescence observed with enyne 77, which differs only by a single CH₂ group from 75. Both enyne 78 and the fully saturated 79 gave comparable results to 75 in regards to the magnitude of fluorescence and the trends after 1 h and 4 h. This data combined with its advantageous synthesis allowed us to conclude that enyne 78 would be the optimal candidate for generation of an asymmetrical dimer.

4.7 Synthesis of an Asymmetrical Dimer

Adapted from the synthesis of enyne 112 we began the synthesis of the asymmetrical dimer 80 by protecting the C-3 amine of 101 as the Fmoc carbamate followed by Swern oxidation of the alcohol provided aldehyde 115 (Figure 4.14). A Songashira reaction between intermediate 108 and di-acetylene 110 generated the mono-acetylated product 114. A Takai reaction provided vinyl iodide 116, which was combined with the mono-acetylated product 114 under Sonagashira conditions to yield the asymmetrical dimer precursor 117 in 57% yield.

The Fmoc carbamate of dimer 117 was removed under basic conditions and the free amine was acylated with Fmoc-β-Ala-OSu (118) to provide 119. Sequential deprotection and acylation with D-Biotinamidocaproate NHS ester
(Biotin-ε-Ahx-OSu) gave the biotinylated dimer 120. Phthalimide cleavage of 120 was achieved with treatment with hydrazine followed by acylation of the free amine with 90 to afford the β-alanine derivative. Fmoc cleavage with piperidine, acylation with Oregon Green OSu, and Boc removal with TFA yielded the asymmetrical Oregon Green enyne dimer 80 (Figure 4.15).

**Figure 4.14.** Synthesis of asymmetrical dimer precursor 119.
Figure 4.15. Synthesis of asymmetrical dimer 80.
4.8 Conclusions

We report the synthesis and biological activity of a series of symmetrical 3β-cholesterylamine dimers bearing Oregon Green derived headgroups. Some of these compounds become incorporated into membranes of living Jurkat lymphocytes. Our findings suggest that only C-C bonds of a certain length between the two steroidal motifs are acceptable substrates for incorporation into cellular membranes. In addition, it appears as though the more rigid enynes exhibit higher overall fluorescence possibly due to the lack of fluorescence quenching through a HOMO-FRET mechanism. Based on its more convergent synthesis, and its high biological activity, we generated the asymmetrical transmembrane receptor 80 based on this scaffold. Future work is needed to develop a more economically sound synthesis, such as utilizing Pennsylvania Green, a cheaper and more hydrophobic fluorophore, as an alternative to Oregon Green. Once a feasible synthetic route is developed it can be expanded to investigate a subsequent generation of asymmetrical transmembrane receptors that may allow control of cellular signaling.

4.9 Experimental Section

4.9.1 General

All reactions, except those in aqueous media, were performed under a nitrogen atmosphere using standard techniques for the exclusion of moisture. All
commercial grade reagents for synthesis were purchased from Aldrich, Acros, TCI, or Alfa Aesar and used without further purification. Anhydrous solvents were obtained after passage though a drying column of a solvent purification system from GlassContour (Laguna Beach, CA). Thin layer chromatography (TLC) was performed on silica gel 60 F254 plates (EM Science, 0.25 mm). ICN SiliTech Silica Gel (40—63 µm) was employed for column chromatography. Purification by preparative reverse phase HPLC employed an Agilent 1100 preparative pump / gradient extension instrument equipped with a Hamilton PRP-1 (polystyrene-divinylbenzene) reverse phase column (7 mm particle size, 21.5 mm x 25 cm). The HPLC flow rate was increased from 10 mL/min ($t = 0$ min) to 20 mL/min ($t = 1$ min) and maintained at 20 mL/min for the remainder of the run. All $^1$H and $^{13}$C NMR Spectra were recorded using Bruker DPX-300, AMX-360, DRX-400, and AMX-2-500 MHz spectrometers at ambient temperature (22 °C). NMR signals were referenced to internal CDCl$_3$ ($\delta_H$ 7.27) and CDCl$_3$ ($\delta_C$ 77.23) or d$_6$-DMSO ($\delta_H$ 2.50) and d$_6$-DMSO ($\delta_C$ 39.5) peaks in parts per million (ppm). Mass spectral data was obtained from either The University of Texas at Austin Mass Spectrometry Facility or The Pennsylvania State University Mass Spectrometry Facility (ESI and CI). Infrared spectra were obtained with a Perkin Elmer 1600 Series FTIR. Elemental analyses were performed by Midwest Microlab, LLC (Indianapolis, IN). Melting points are uncorrected.
4.9.2 Synthetic Procedures and Compound Characterization

methyl(3α,6α,8ξ,9ξ,14ξ)-3,6-bis{[tert-butyl(dimethyl)silyl]oxy}cholan-24-oate (82)\textsuperscript{29}

Methyl Hyodeoxycholate (38 g, 0.09 mol) was combined with imidazole (64 g, 930 mmol) and pyridine (25 mL, 210 mmol) in DMF (115 mL). tert-Butylchloro-dimethylsilane (35 g, 230 mmol) was added and the reaction was stirred at 50 °C for 1.5 h. The reaction was then poured into ice-water (500 mL) and extracted with Et\textsubscript{2}O (2 x 300 mL). The combined organics were dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:9) to give the product as a white foam (59 g, 99%). mp 124-125 °C; \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400.13 MHz) \( \delta \) 3.94 (bs, 1H), 3.61 (s, 3H), 3.50-3.48 (m, 1H), 2.31-1.99 (m, 2H), 1.99-0.83 (m, 48H), 0.59 (s, 3H), 0.03 (s, 6H), 0.01 (s, 6H); \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 100.61 MHz) \( \delta \) 174.63, 72.99, 68.70, 56.23, 56.04, 51.46, 49.67, 42.93, 40.09, 39.72, 36.03, 35.53, 35.45, 34.97, 31.71, 31.14, 31.08, 29.90, 28.21, 26.10, 25.97, 24.31, 23.65, 20.89, 18.47, 18.34, 18.17, 14.24, 12.14, -4.40, -4.53, -4.66, -4.69; IR (film): \( \nu_{\text{max}} \)
(3α,6α,8ξ,9ξ,14ξ)-3,6-bis[tert-butyl(dimethyl)silyl]oxy]cholan-24-ol (83)

A solution of 82 (59 g, 90 mmol) in THF (1 L) was slowly cannulated over 45 min into a stirring mixture of Lithium aluminum hydride (5.3 g, 140 mmol) and the resulting solid mixture was allowed to stand at 22 °C for 1.5 h. Crushed-ice was carefully added in small portions until the H2 gas ceased followed by H2O (300 mL). The aqueous layer was extracted with Et2O (2 x 300 mL) and the combined organics were dried over Na2SO4 to give the product as a white foam (55 g, 96%). mp 125-128 °C; 1H NMR (CDCl3, 400.13 MHz) δ 3.97-3.93 (m, 2H), 3.56 (t, J = 6.5 Hz, 2H), 3.43-3.51 (m, 2H), 2.25-0.85 (m, 48H), 0.61 (s, 3H), 0.03 (s, 6H), 0.01 (s, 6H) ; 13C NMR (CDCl3, 100.62 MHz) δ 73.11, 68.76, 63.44, 56.36, 56.28, 49.69, 42.93, 40.14, 39.76, 36.07, 35.80, 35.57, 35.00, 32.02, 31.73, 31.12, 29.57, 28.38, 26.15, 26.02, 24.37, 23.68, 20.93, 18.78, 18.53, 18.21, 14.30, 12.19, -4.34, -4.50, -4.61, -4.66; IR (film): νmax 3460.4, 1260.7, 1090.2 cm⁻¹; HRMS (Cl⁺) m/z 607.4947 (MH⁺, C36H71O3Si2 requires 607.4942).
(3α,6α,8ξ,9ξ,14ξ)-3,6-bis[[tert-butyl(dimethyl)silyl]oxy]-24-iodocholane (84)

Iodine (26 g, 100 mmol) was added to a stirring solution of 83 (48 g, 80 mmol), triphenylphosphine (27 g, 100 mmol) and imidazole (7.0 g, 100 mmol) in THF (700 mL) at 0 °C. The reaction was stirred at 0 °C for 0.5 h and 10% aqueous sodium thiosulfate pentahydrate was added. The aqueous layer was extracted with Et₂O (2 x 300 mL) and the combined organics were dried over Na₂SO₄. Concentration gave an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:18) to give the product as a white foam (52 g, 91%). mp 62-63 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 3.99-3.96 (m, 1H), 3.53-3.49 (m, 1H), 3.20-3.08 (m, 2H), 1.95-0.87 (m, 50H), 0.63 (s, 3H), 0.03 (s, 6H), 0.02 (s, 6H); ¹³C NMR (CDCl₃, 100.62 MHz) δ 73.15, 68.84, 56.32, 49.77, 43.04, 40.15, 39.78, 37.05, 36.17, 36.12, 35.63, 35.33, 30.08, 31.23, 30.62, 30.00, 28.45, 26.22, 26.10, 24.42, 23.75, 20.97, 18.88, 18.65, 18.32, 12.24, 8.08, -4.28, -4.42, -4.54, -4.58; IR (film): ν_max 2956.4, 1470.5, 1373.1, 1251.6 cm⁻¹; HRMS (Cl⁺) m/z 717.3952 (MH⁺, C₃₆H₇₀I₂O₂Si₂ requires 717.3959).
[(3R,6S,10R,13R,17R)-10,13-dimethyl-17-{[(1R)-1-methyl-6(trimethylsilyl)hex-5-yn-1-yl]hexadecahydro-1Hcyclopenta[a]phenanthrene-3,6-diyl}bis(oxy)]bis[tert-butyl(dimethyl)silane] (85)

To a flame dried and degassed schlenk was trimethylsilylacetylene (14.5 mL, 100 mmol) in THF:HMPA (30:1, 500 mL) at -78 °C. n-Butyllithium (29 mL, 2.4 M in hexanes) was added and the solution was stirred at -78 °C for 20 min. A solution of 84 (49 g, 70 mmol) in THF (300 mL) was cannulated into the reaction over 30 min and the reaction was brought to 22 °C over 1.5 h. The reaction was diluted with saturated ammonium chloride (200 mL) and the aqueous layer was extracted with Et₂O. The combined organics were dried over Na₂SO₄ and concentrated to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:17) to give the product as a white foam (38 g, 81%). mp 135-136 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 3.99-3.96 (m, 1H), 3.55-3.49 (m, 1H), 2.18-0.88 (m, 52H), 0.63 (s, 3H), 0.14 (s, 9H) 0.03 (s, 6H), 0.02 (s, 6H); ¹³C NMR (CDCl₃, 100.62 MHz) δ 107.94, 84.48, 73.14, 68.88, 56.39, 56.33, 49.83, 43.02, 40.24, 39.90, 36.18, 35.68, 35.32, 35.20, 35.13, 31.81, 31.29, 30.04, 28.38, 26.22, 26.11, 25.46, 24.44, 23.78, 21.03, 20.36, 18.86, 18.58, 18.29,
Concentrated HCl (10 mL) was slowly added to a stirring suspension of 85 (38 g, 55 mmol in absolute EtOH (690 mL). The reaction was stirred at 22 °C for 2 h, concentrated to half the volume and poured into H₂O (100 mL). The aqueous layer was extracted with Et₂O and the combined organics were dried over Na₂SO₄. Removal of solvent in vacuo gave an oil that was purified by column chromatography eluting with acetone/CH₂Cl₂ (1:4) followed by acetone/CH₂Cl₂ (1:1) to give the product as a white foam (38 g, 87%). mp 111-112 °C; ¹H NMR (CDCl₃, 360.14 MHz) δ 4.06-3.96 (m, 1H), 3.61-3.50 (m, 1H), 3.28 (bs, 2H), 2.15-0.86 (m, 34H), 0.61 (s, 3H), 0.11 (s, 9H); ¹³C NMR (CDCl₃, 90.56 MHz) δ 107.91, 86.12, 84.45, 71.63, 68.12, 56.49, 56.29, 48.73, 42.99, 40.22, 40.16, 36.11, 35.87, 31.72, 30.24, 29.51, 28.35, 25.40, 24.40, 23.74, 20.98, 20.31, 18.82, 12.18, 0.34; IR (film): ν_max 3400.2, 3280.7, 2120.9 cm⁻¹; HRMS (Cl⁺) m/z 459.3657 (MH⁺, C₂₉H₅₁O₂Si requires 459.3658).
(3S,10R,13R,17R)-10,13-dimethyl-17-[(1R)-1-methylhex-5-yn-1-yl]-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl azide (77)

To a stirring mixture of 86 (11.3 g, 25 mmol) and triphenylphosphine (19.4 g, 74 mmol) in benzene (200 mL) was added a solution of hydrazoic acid in benzene\(^3\) (58 mL) over 3 portions. A solution of diethylazodicarboxylate (36 mL, 74 mmol) in benzene (100 mL) was added over 10 min and the reaction was heated to 80 °C and stirred for 3 h. The reaction mixture was concentrated to an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:9) to give a crude mixture of silated and desilated product as a yellow oil. This oil was combined with THF (100 mL) and cooled to 0 °C. Tetrabutylammonium fluoride (27 mL, 1M in THF) was added and the reaction was stirred at 0 °C for 20 min. The reaction was poured into H\(_2\)O, extracted with Et\(_2\)O (2 x 40 mL) and dried over Na\(_2\)SO\(_4\). Concentration gave an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:9) to give the product as an off-white solid (6.7 g, 68%). mp 60-62 °C; \(^1\)H NMR (CDCl\(_3\), 299.87 MHz) \(\delta\) 5.31 (d, \(J = 4.3\) Hz, 1H), 3.18-3.07 (m, 1H), 2.23-2.20 (m, 2H), 2.08-1.97 (m, 2H), 1.93-1.78 (m, 6H), 1.51-0.87 (m, 24H), 0.62 (s, 3H); \(^{13}\)C NMR (CDCl\(_3\), 75.41 MHz) \(\delta\) 139.49, 122.18, 84.33, 68.00, 60.78, 56.43, 55.70, 49.81, 42.44, 42.03, 39.46,
37.91, 37.37, 36.29, 35.11, 34.87, 34.40, 31.60, 31.51, 27.92, 27.69, 24.92, 24.02, 20.76, 19.01, 11.58; IR (film): \( \nu_{\text{max}} \) 2938.5, 2094.0, 1463.9, 1252.6 cm\(^{-1}\); HRMS (Cl\(^+\)) \( m/z \) 366.3156 (M - N\(_2\)H\(^+\), C\(_{26}\)H\(_{40}\)N requires 366.3161).

**tert-butyl \{[(3S,10R,13R,17R)-10,13-dimethyl-17-[(1R)-1-methylhex-5-yn-1-yl]-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H cyclopenta [a] phenanthren-3-yl][3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]carbamate (78)**

Compound 87 (667 mg, 1.6 mmol) was combined with triphenylphosphine (630 mg, 2.4 mmol) in THF (30 mL) and the reaction was stirred at 22 °C for 12 h. H\(_2\)O (2 mL) was added and the reaction was stirred at 22 °C for an additional 24 h. The solvent was removed *in vacuo* to give the crude amine as an oil, which was taken on without further purification. This amine was combined with N-(3-Bromo-propyl)phthalimide (499 mg, 1.9 mmol) and K\(_2\)CO\(_3\) (584 mg, 4.2 mmol) in DMF (10 mL). The reaction mixture was stirred at 60 °C for 16 h and filtered. The filtrate was concentrated to give a solid that was resuspended in CH\(_2\)Cl\(_2\) (25 mL) and DIEA (0.7 mL, 4.3 mmol). Di-tert-butyl dicarbonate (928 mg, 4.3 mmol) was directly added and the reaction was stirred at 22 °C for 3 h. The reaction mixture
was concentrated to an oil that was purified by column chromatography eluting with EtOAc/hexanes (3:7) followed by EtOAc/hexanes (1:1) to give the product as a white solid (452 mg, 43%). mp 140-141 °C; \(^1\)H NMR (CDCl\(_3\), 300.13 MHz) \(\delta\) 7.71-7.24 (m, 4H), 5.18 (s, 1H), 3.71-3.54 (m, 2H), 3.31-3.04 (m, 2H), 2.28-0.80 (m, 46 H), 0.54 (s, 3H); \(^{13}\)C NMR (CDCl\(_3\), 75.47 MHz) \(\delta\) 167.86, 154.86, 140.63, 133.62, 131.69, 122.85, 121.02, 84.36, 78.98, 77.19, 67.99, 56.59, 56.34, 49.74, 42.05, 39.40, 38.05, 36.47, 36.37, 35.59, 35.30, 34.98, 34.78, 31.37, 29.55, 28.15, 27.87, 26.40, 24.83, 23.96, 20.67, 19.09, 18.52, 18.38, 11.57; IR (film): \(\nu_{\text{max}}\) 2939.0, 1714.3, 1686.5, 1396.1 cm\(^{-1}\); HRMS (TOF ESI\(^+\)) \(m/z\) 655.4462 (MH\(^+\), C\(_{42}\)H\(_{59}\)N\(_2\)O\(_4\) requires 655.4475).

di-tert-butyl \{(2R,13R)-tetradeca-6,8-diyne-2,13-diylbis[(3S,10R,13R,17R)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-17,3-diyl]bis[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]carbamate} - methane (89)
To a flame dried and degassed schlenk flask was charged 1,4-
diazabicyclo[2.2.2]octane (10 mg, 0.09 mmol), Pd(PPh$_3$)$_2$Cl$_2$ (1 mg, 0.002 mmol),
and Cul (0.3 mg, 0.002 mmol) in THF (5 mL). 88 (50 mg, 0.07 mmol) was added
followed by ethyl bromoacetate (5.1 uL, 0.05 mmol) and the resulting yellow
mixture was stirred at 22 °C for 16 h. The reaction was poured into H$_2$O (20 mL),
extracted with Et$_2$O (3 x 15 mL) and the combined organics were dried over
Na$_2$SO$_4$. Concentration gave an oil that was purified by column chromatography
eluting with EtOAc/hexanes (1:9) followed by EtOAc/hexanes (1:1) to give the
product as a yellow solid (41 mg, 90%). mp 132-133 °C; $^1$H NMR (CDCl$_3$, 299.87
MHz) $\delta$ 7.82-7.79 (m, 4H), 7.71-7.67 (m, 4H), 5.29-5.28 (m, 2H), 3.67 (t, $J$ = 7.0
Hz, 4H), 3.23-3.19 (m, 4H), 2.18-0.86 (m, 90H), 0.63 (s, 6H); $^{13}$C NMR (CDCl$_3$,
100.62 MHz) $\delta$ 168.14, 155.15, 140.75, 133.80, 131.97, 123.05, 121.23, 79.33,
77.40, 65.23, 60.22, 56.55, 55.79, 49.95, 42.28, 42.18, 40.85, 39.60, 38.22,
36.68, 36.46, 35.81, 35.26, 35.08, 34.25, 31.74, 31.45, 30.72, 29.57, 28.32,
28.04, 26.60, 24.95, 24.15, 24.00, 22.52, 20.90, 20.85, 19.52, 19.26, 18.79,
18.51, 14.09, 14.01, 11.73; IR (film): $\nu_{max}$ 3400.4, 1714.5, 1462.1 cm$^{-1}$; HRMS
(TOF ESI$^+$) m/z 1323.9031 (MH$^+$, C$_{85}$H$_{114}$N$_4$O$_8$ requires 1323.9028).
To a suspended mixture of Fmoc-β-alanine (15 g, 48 mmol), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (12 g, 62 mmol), and 1-hydroxybenzotriazole (8 g, 53 mmol) in CH₂Cl₂ (60 mL) was added a mixture of β-alanine ethyl ester hydrochloride (7.4 g, 48 mmol) and TEA (7.4 mL) in CH₂Cl₂ (20 mL). The resulting solution was stirred at 22 °C for 6 h and the reaction was then washed with aqueous 1M H₃PO₄ (40 mL) and H₂O (40 mL). The organics were dried over Na₂SO₄ and purified by column chromatography eluting with acetone/CH₂Cl₂ (1:9) followed by acetone/CH₂Cl₂ (3:7) to afford the methyl ester as a white solid, which was taken on without further purification. A stirring solution of the above ester (5.0 g, 12.0 mmol) in THF (200 mL) was treated with aqueous 0.2M LiOH (183 mL) and stirred for 1 h at 0 °C. The reaction mixture was and acidified to pH = 2 with aqueous 2M HCl. The aqueous layers were extracted with EtOAc (3 x 50 mL), dried over Na₂SO₄, and concentrated in vacuo to give the free acid as a pale yellow solid. This acid was combined with 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (1.8 g, 7.0 mmol), and N-hydroxysuccinimide (1.1 g, 9.0 mmol) in CH₂Cl₂ (40 mL) and the suspension was stirred at 22 °C for 10 h. The reaction was diluted with CHCl₃ (50 mL), washed with H₂O (50 mL), concentrated onto silica gel and purified by column
chromatography eluting with MeOH/CH$_2$Cl$_2$ (1:19) to give the activated ester as a white solid, which was used without further purification.

di-tert-butyl[(2R,13R)-tetradeca-6,8-diyne-2,13-diylbis[(3S,10R,13R,17R)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-17,3-diyl]]bis{[15-(9H-fluoren-9-yl)-5,9,13-trioxo-14-oxa-4,8,12-triazapentadec-1-yl]carbamate} (91)

To a stirring solution of 89 (97 mg, 0.07 mmol) in THF:EtOH (1:5, 15 mL) was added hydrazine (35 uL, 1.1 mmol). The reaction was stirred at 22 °C for 16 h and filtered. The filtrate was concentrated to give the crude di-amine, which was taken on without further purification. This di-amine was combined with 90 (91 mg, 0.22 mmol) and DIEA (39 uL, 0.22 mmol) in CH$_2$Cl$_2$ and the cloudy reaction mixture was stirred at 22 °C for 3 h. The reaction was concentrated and the residue was purified by column chromatography eluting with Acetone/CH$_2$Cl$_2$ (1:9) followed by MeOH/CH$_2$Cl$_2$ (1:19) to give the product as an off-white solid (98 mg, 79%). mp 141-143 °C; $^1$H NMR (CDCl$_3$, 300.13 MHz) δ 7.72 (d, $J = 7.4$ Hz, 4H), 7.56 (d, $J = 7.3$ Hz, 4H), 7.36 (t, $J = 7.1$ Hz, 4H), 7.27 (t, $J = 7.2$ Hz, 4H), 5.29-5.27 (m, 2H), 4.31 (d, $J = 7.0$ Hz, 4H), 4.17 (t, $J = 6.9$ Hz, 2H), 3.50-3.47 (m, 8H), 3.23-3.19 (m, 4H), 2.49-2.13 (m, 8H), 1.98-0.85 (m, 94H), 0.64 (s, 6H); $^{13}$C
NMR (CDCl₃, 75.48 MHz) δ 206.98, 171.44, 156.44, 143.92, 143.85, 141.18, 127.56, 126.94, 125.10, 121.34, 119.85, 79.86, 77.52, 77.20, 66.62, 65.27, 61.53, 58.24, 56.61, 55.81, 53.73, 53.38, 49.94, 47.14, 42.22, 41.31, 39.63, 38.32, 37.16, 36.62, 35.91, 35.29, 35.11, 31.76, 31.68, 30.87, 29.63, 29.19, 28.46, 28.08, 26.79, 24.97, 24.96, 24.20, 20.90, 19.58, 19.37, 18.65, 18.55, 11.77; IR (film): ν max 2938.4, 1715.3, 1394.3 cm⁻¹; HRMS (Cl⁺) m/z 1776.1459 (MH⁺, C₁₁₀H₁₅₁N₈O₁₂ requires 1776.1451).

3,3’-{(2R,13R)-tetradeca-6,8-diyne-2,13-diylbis[(3S,8R)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-17,3-diyl]ammoniopropane-3,1-diylimino(3-oxopropane-3,1-diyl)imino(3-oxopropane-3,1-diyl)iminocarbonyl]bis[6-(2,7-difluoro-6-oxido-3-oxo-3H-xanthen-9-yl)benzoate] (74)

Piperidine (0.37 mL) was added to a stirring mixture of compound 91 (14 mg, 0.01 mmol) in DMF (1.5 mL). The resulting solution was stirred at 22 °C for 20 min and the solvent was removed in vacuo to give the crude diamine as a white film, which was taken on without further purification. This diamine was combined with Oregon Green OSu (10 mg, 0.02 mol) and DIEA (0.1 mL, 5.7 mmol) in DMF (5 mL). The reaction was stirred at 22 °C for 12 h and the solvent was removed in vacuo. The residue was suspended in trifluoroacetic acid / "wet"
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\text{CH}_2\text{Cl}_2 \ (3:47, \ 10 \text{ mL}) \text{ and stirred at } 22 ^\circ \text{C for } 3 \text{ h. The reaction mixture was concentrated and the residue was purified via preparative reverse-phase HPLC (gradient: 20\% CH}_3\text{CN, 80\% H}_2\text{O, and 0.1\% TFA to 99\% CH}_3\text{CN, 1\% H}_2\text{O, and 0.1\% TFA over 20 min to afford 74 as an orange solid. (8.3 mg, 43\%). Mp 129-130 ^\circ \text{C; } ^1\text{H NMR (DMSO-}\text{d}_6, \ 400.13 \text{ MHz}) \delta 8.93 \text{ (bs, 4H), 8.72 (t, J = 5.7 Hz, 2H), 8.30 (t, J = 5.9 Hz, 2H), 8.14-8.07 (m, 4H), 8.00 (bs, 2H), 7.49 (d, J = 7.8 Hz, 2H), 6.90 (bs, 4H), 6.74 (s, 2H), 6.72 (s, 2H), 5.29-5.27 (m, 2H), 3.50-3.47 (m, 8H), 3.24-3.19 (m, 4H), 2.51-2.14 (m, 8H), 1.99-0.87 (m, 76H), 0.66 (s, 6H); } ^{13}\text{C NMR (DMSO-}\text{d}_6, \ 100.61 \text{ MHz}) \delta 172.74, 171.52, 166.69, 163.67, 159.62, 154.52, 150.09, 139.68, 137.13, 136.72, 135.63, 130.34, 130.01, 125.82, 123.42, 115.08, 79.86, 77.52, 77.20, 65.49, 61.53, 58.25, 56.62, 55.80, 53.73, 53.38, 49.92, 47.14, 42.21, 41.30, 39.64, 38.32, 37.16, 36.64, 35.92, 35.30, 35.11, 31.76, 31.69, 30.86, 29.63, 29.19, 28.46, 28.10, 26.80, 25.00, 24.97, 24.20, 20.91, 19.58, 19.37, 18.65, 18.55, 11.79; \text{ IR (film): } \nu_{\text{max}} 3300.6, 1714.8, 1263.2 \text{ cm}^{-1}; \text{ HRMS (Cl}^+\text{) m/z 1923.9947 (MH}^+, \text{ C}_{112}\text{H}_{135}\text{F}_4\text{N}_8\text{O}_{16} \text{ requires 1923.9932).}
\]
di-tert-butyl {((2R,17R)-octadeca-6,12-diyne-2,17-diylbis[(3S,10R,13R,17R)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-17,3-diyl]bis[[3-(1,3-dioxo-1,3-dihydro-2H-isoinol-2-yl)propyl]carbamate} (92)

To a flame dried and degassed schlenk flask was charged 1,3-Bis(1-adamantyl)imidazolium chloride (3 mg, 0.008 mmol), Allylpalladium chloride dimer (1 mg, 0.004 mmol), Cul (2 mg, 0.01 mmol) and cesium carbonate (70 mg, 0.21 mmol) in DMF:Et₂O (1:2, 10 mL). Compound 88 (100 mg, 0.15 mmol) was added followed by the addition of 1,4-diiodobutane (10 uL, 0.076 mmol) and the reaction was stirred at 40 °C for 12 h. The reaction mixture was concentrated to give a black oil that was purified by column chromatography eluting with EtOAc/hexanes (1:9) followed by EtOAc/hexanes (3:7) to give the product as a thin white film (61 mg, 60%). mp 112-114 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 7.83-7.81 (m, 4H), 7.70-7.68 (m, 4H), 5.30-5.29 (m, 2H), 3.68 (t, J = 7.1 Hz, 4H), 3.25-3.14 (m, 4H), 2.27-0.91 (m, 98H), 0.65 (s, 6H); ¹³C NMR (CDCl₃, 100.62 MHz) δ 170.37, 167.71, 154.92, 140.32, 133.51, 131.73, 122.74, 83.72, 80.27, 79.08, 78.84, 68.21, 65.40, 59.86, 56.31, 55.59, 53.19, 49.72, 41.89, 39.36,
36.18, 35.51, 34.99, 34.81, 31.47, 28.05, 27.81, 27.69, 21.17, 25.33, 23.90, 20.62, 20.58, 18.99, 18.77, 18.35, 17.89, 17.60, 14.94, 13.87, 11.48; IR (film): \( \nu_{\text{max}} \) 2939.4, 1813.9, 1689.4 cm\(^{-1}\); HRMS (Cl\(^+\)) \( m/z \) 1363.9342 (MH\(^+\), C\(_{88}H_{123}N_4O_8\) requires 1363.9341).

di-\text{tert}-\text{butyl} \{(2R,17R)-octadeca-6,12-diyn-2,17-diylbis[(3S,10R,13R,17R)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-17,3-diyl]bis[(15-(9H-fluoren-9-yl)-5,9,13-trioxo-14-oxa-4,8,12-triazapentadec-1-yl]carbamate\} (93)

To a stirring solution of 92 (52 mg, 0.04 mmol) in THF:EtOH (1:5, 10 mL) was added hydrazine (6 uL, 0.19 mmol). The reaction was stirred at 22 \( ^\circ \)C for 16 h and filtered. The filtrate was concentrated to give the crude di-amine, which was taken on without further purification. This di-amine was combined with 90 (91 mg, 0.22 mmol) and DIEA (39 uL, 0.22 mmol) in CH\(_2\)Cl\(_2\) and the cloudy reaction mixture was stirred at 22 \( ^\circ \)C for 3 h. The reaction was concentrated and the residue was purified by column chromatography eluting with Acetone/CH\(_2\)Cl\(_2\) (1:9) followed by MeOH/CH\(_2\)Cl\(_2\) (1:19) to give the product as an off-white solid (60 mg, 82%). mp 152-153 \( ^\circ \)C; \(^1\)H NMR (CDCl\(_3\), 300.13 MHz) \( \delta \) 7.72 (d, \( J = 7.4 \) Hz, 4H), 7.56 (d, \( J = 7.3 \) Hz, 4H), 7.36 (t, \( J = 7.3 \) Hz, 4H), 7.27 (t, \( J = 7.1 \) Hz, 4H),
5.30-5.29 (m, 2H), 4.32 (d, J = 7.1 Hz, 4H), 4.18 (t, J = 7.0 Hz, 2H), 3.53-3.49 (m, 8H), 3.23-3.20 (m, 4H), 2.60-2.41 (m, 8H), 2.14-0.88 (m, 102H), 0.64 (s, 6H); $^{13}$C NMR (CDCl$_3$, 75.48 MHz) δ 174.17, 171.41, 156.44, 143.94, 143.84, 141.19, 127.56, 126.94, 125.10, 121.37, 119.86, 79.85, 77.20, 69.44, 68.82, 66.62, 56.64, 56.03, 53.38, 49.99, 47.16, 42.22, 41.34, 39.66, 38.34, 37.17, 36.63, 36.26, 35.92, 35.67, 32.96, 31.79, 30.87, 30.10, 29.67, 29.64, 29.20, 28.47, 28.16, 27.19, 26.79, 26.02, 25.36, 24.26, 20.92, 19.38, 18.66, 16.01, 11.78; IR (film): $\nu_{\text{max}}$ 3304.5, 1714.7, 1402.1 cm$^{-1}$; HRMS (Cl$^+$) $m/z$ 1832.2075 (MH$^+$, C$_{114}$H$_{159}$N$_8$O$_{12}$ requires 1832.2077).

3,3'-(2R,17R)-octadeca-6,12-diyn-2,17-diylbis[((3S,8R)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-17,3-diyl]ammoniopropane-3,1-diylimino(3-oxopropane-3,1-diyl)imino(3-oxopropane-3,1-diyl)iminocarbonyl)]bis[6-(2,7-difluoro-6-oxido-3-oxo-3H-xanthen-9-yl)benzoate] (75)

Piperidine (0.37 mL) was added to a stirring mixture of compound 93 (14 mg, 0.01 mmol) in DMF (1.5 mL). The resulting solution was stirred at 22 °C for 20 min and the solvent was removed in vacuo to give the crude diamine as a white film, which was taken on without further purification. This diamine was combined with Oregon Green OSu (10 mg, 0.02 mol) and DIEA (0.1 mL, 5.7
mmol) in DMF (5 mL). The reaction was stirred at 22 °C for 12 h and the solvent was removed \textit{in vacuo}. The residue was suspended in trifluoroacetic acid / “wet” CH$_2$Cl$_2$ (3:47, 10 mL) and stirred at 22 °C for 3 h. The reaction mixture was concentrated and the residue was purified via preparative reverse-phase HPLC (gradient: 20% CH$_3$CN, 80% H$_2$O, and 0.1% TFA to 99% CH$_3$CN, 1% H$_2$O, and 0.1% TFA over 20 min to afford 75 as an orange solid (9.1 mg, 46%). Mp 137-138 °C; $^1$H NMR (DMSO-$d_6$, 400.13 MHz) δ 8.96 (bs, 4H), 8.72 (t, $J = 5.8$ Hz, 2H), 8.30 (t, $J = 5.9$ Hz, 2H), 8.12-8.07 (m, 4H), 8.00 (bs, 2H), 7.49 (d, $J = 7.7$ Hz, 2H), 6.90 (bs, 4H), 6.75 (s, 2H), 6.72 (s, 2H), 5.31-5.29 (m, 2H), 3.53-3.49 (m, 8H), 3.23-3.20 (m, 4H), 2.61-2.41 (m, 8H), 2.16-0.86 (m, 84H), 0.65 (s, 6H); $^{13}$C NMR (DMSO-$d_6$, 100.61 MHz) δ 172.72, 171.52, 166.70, 163.67, 159.62, 154.52, 150.09, 139.69, 137.13, 136.72, 135.63, 130.33, 129.99, 125.82, 123.42, 115.09, 79.82, 77.19, 68.83, 66.67, 56.64, 56.03, 53.38, 49.99, 47.16, 42.22, 41.34, 39.66, 38.34, 37.17, 36.63, 36.26, 35.92, 35.67, 32.96, 31.79, 30.87, 30.10, 29.67, 29.64, 29.20, 28.47, 28.16, 27.19, 26.79, 26.02, 25.36, 24.26, 20.92, 19.39, 18.66, 16.01, 11.78; IR (film): $\nu_{\text{max}}$ 3379.6, 1714.9, 1466.7 cm$^{-1}$; HRMS (Cl$^+$) m/z 1980.0560 (MH$^+$, C$_{116}$H$_{143}$F$_4$N$_8$O$_{16}$ requires 1980.0558).

![Absorbance spectrum](image)

Absorbance wavelength = 254 nm

Analytical reverse-phase HPLC of compound 75 after purification (retention time = 14.34 min).
(3S,5R,6R,10R,13S,17R)-5,6-dibromo-10,13-dimethyl-17-[(1S)-1-methyl-2-oxoethyl]hexadecahydro-1H-cyclopenta[a]phenanthren-3-yl acetate (95)

A stream of oxone was bubbled through a solution of 94 \(^{21}\) (500 mg, 0.8 mmol) in \(\text{CH}_2\text{Cl}_2:\text{EtOH} (1:1, 20 \text{ mL})\) at -78 °C for ca 20 min or until a light blue coloration was observed. \(\text{N}_2\) was then bubbled through the reaction to remove any excess ozone and trimethyl phosphite (0.8 mL) was added. The reaction was stirred at -78 °C for 0.5 h and then allowed to warm to 22 °C over 30 min. The reaction was concentrated and purified by column chromatography eluting with EtOAc/hexanes (1:9) to give the product as an oil (97 mg, 23%). \(^1\)H NMR (CDCl\(_3\), 299.87 MHz) \(\delta 9.53 (s, 1\text{H}), 5.47-5.39 (m, 1\text{H}), 4.79-4.76 (m, 1\text{H}), 2.64-0.89 (m, 30\text{H}), 0.69 (s, 6\text{H});\) \(^{13}\)C NMR (CDCl\(_3\), 75.41 MHz) \(\delta 204.76, 170.30, 87.74, 71.85, 55.73, 54.34, 50.87, 49.32, 47.16, 43.21, 41.81, 41.77, 39.16, 37.08, 36.80, 36.42, 30.76, 26.90, 24.33, 21.25, 21.13, 20.03, 13.37, 12.08; IR (film): \(\nu_{\max} 2341.1, 1706.0, 1458.2 \text{ cm}^{-1};\) HRMS (TOF ESI\(^+\)) \(m/z 532.1179 (M + 2\text{H}^+,\) \(\text{C}_{24}\text{H}_{38}\text{Br}_2\text{O}_3\) requires 532.1188).
(3S,10R,13S,17R)-10,13-dimethyl-17-[(1S)-1-methyl-2-oxoethyl]-2,3,4,7,8,9,
10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl
acetate (96)

Sodium iodide (371 mg, 2.5 mmol) was added over 3 portions to a stirring
solution of 95 (376 mg, 0.71 mmol) in acetone (20 mL) at 0 °C. The reaction was
allowed to warm to 22 °C and stirred for 6 h. The reaction was filtered, diluted
with Et₂O (50 mL), and the organics were washed with 10% aqueous sodium
thiosulfate pentahydrate. The organics were dried over Na₂SO₄ and concentrated
to give an oil that was purified by column chromatography eluting with
EtOAc/hexanes (1:9) to give the product as a yellow solid (179 mg, 68%). mp
117-118 °C; ¹H NMR (CDCl₃, 299.87 MHz) δ 9.47 (s, 1H), 5.27-5.26 (m, 1H),
4.53-4.46 (m, 1H), 2.26-2.20 (m, 3H), 1.93-0.76 (m, 27H), 0.62 (s, 6H); ¹³C NMR
(CDCl₃, 100.63 MHz) δ 205.58, 170.35, 139.60, 122.28, 56.03, 51.77, 49.88,
48.68, 42.81, 41.97, 39.29, 37.97, 36.49, 36.46, 31.66, 30.06, 26.90, 26.32,
23.77, 21.27, 20.41, 19.18, 14.00, 12.03; IR (film): νₘₐₓ 2941.4, 1731.5, 1373.7,
1244.9 cm⁻¹; HRMS (TOF ESI⁺) m/z 373.2745 (MH⁺, C₁₁₆H₃₇O₃ requires
373.2743).
8-bromo-1-octanol (0.8 mL, 4.8 mmol) was combined with triphenylphosphine (1.4 g, 5.3 mmol) in CH$_3$CN and the reaction was allowed to reflux for 48 h. The reaction was concentrated and hexanes (20 mL) was added. The mixture was brought to a boil and the hexanes layer was decanted. The resulting residue was triturated with Et$_2$O and the solvent removed *in vacuo* to give the phosphonium salt as an oil, which was used without further purification. To a flamed dried and degassed schlenk flask was charged the phosphonium salt (80 mg, 0.17 mmol) in THF (15 mL) at -78 °C. n-butyllithium (2.2M in hexanes, 0.12 mL) was added and the reaction mixture was warmed to 0 °C and stirred for 20 min. A solution of 97 (100 mg, 0.16 mmol) in THF (10 mL) was cannulated in over 15 min and the reaction was brought to 22 °C and stirred for 0.5 h. Saturated aqueous ammonium chloride (30 mL) was added and the aqueous layer was extracted with EtOAc (3 x 15 mL). The combined organics were dried over Na$_2$SO$_4$ and concentrated to give an oil that was purified by
column chromatography eluting with EtOAc/hexanes (3:7) followed by EtOAc/hexanes (1:1) to give the product as an off-white solid (38 mg, 30%). mp 152-154 °C; ¹H NMR (CDCl₃, 300.13 MHz) δ 7.82-7.80 (m, 2H), 7.71-7.67 (m, 2H), 5.31-5.27 (m, 2H), 4.45 (bs, 1H), 3.60-3.57 (m, 3H), 3.37-3.29 (m, 2H), 2.27-2.25 (m, 1H), 1.99-1.77 (m, 12H), 1.53-0.83 (m, 44H), 0.62 (s, 3H); ¹³C NMR (CDCl₃, 100.63 MHz) δ 168.22, 155.15, 140.65, 133.80, 131.98, 130.92, 130.40, 129.69, 121.73, 79.13, 68.05, 63.50, 62.99, 62.97, 62.87, 60.53, 56.82, 56.14, 50.20, 42.45, 42.42, 39.85, 38.04, 36.64, 36.12, 35.62, 34.03, 32.81, 32.70, 32.00, 29.80, 29.38, 28.56, 25.88, 25.72, 24.40, 24.03, 22.80, 21.09, 19.45, 14.25, 11.98; IR (film): νmax 3401.2, 1802.6, 1505.3 cm⁻¹; HRMS (Cl⁺) m/z 757.5522 (MH⁺, C₄₈H₇₃N₂O₅ requires 757.5519).

methyl (3β,8ξ,9ξ,14ξ)-3-azidochol-5-en-24-oate (100)

To a stirring mixture of Methyl Hyodeoxycholate (5.0 g, 12.3 mmol) and triphenylphosphine (10.0 g, 38.1 mmol) in benzene (75 mL) was added a solution of hydrazoic acid in benzene (28 mL) over 3 portions. A solution of diisopropylazodicarboxylate (7.1 mL, 38.1 mmol) in benzene (50 mL) was added over 10 min and the reaction was heated to 80 °C and stirred for 3 h. The reaction mixture was concentrated to an oil that was purified by column
chromatography eluting with EtOAc/hexanes (1:8) to give the product as a white foam (5.0 g, 98%). mp 76-77 °C; \( ^1H \) NMR (CDCl\textsubscript{3}, 400.13 MHz) \( \delta \) 5.33-5.29 (m, 1H), 3.48 (s, 3H), 3.17-3.12 (m, 4H), 1.96-1.79 (m, 6H), 1.29-0.84 (m, 21H), 0.62 (s, 3H); \( ^{13}C \) NMR (CDCl\textsubscript{3}, 100.62 MHz) \( \delta \) 174.50, 139.67, 122.34, 60.98, 56.53, 55.94, 51.31, 49.91, 42.21, 39.79, 38.01, 37.43, 36.45, 35.22, 31.69, 31.65, 31.08, 30.88, 27.96, 27.80, 24.73, 21.65, 19.13, 18.12, 11.90; IR (film): \( \nu_{\text{max}} \) 2940.4, 2093.8, 1739.9, 1252.7 cm\textsuperscript{-1}; MS (ESI\textsuperscript{+}) calcd. for C\textsubscript{25}H\textsubscript{40}N\textsubscript{3}O\textsubscript{2} (MH\textsuperscript{+}) 414.31, found 414.32. Anal. calcd. for C\textsubscript{25}H\textsubscript{39}N\textsubscript{3}O\textsubscript{2}: C, 72.60; H, 9.50. Found: C, 72.34; H, 9.34.

(3β,8ξ,9ξ,14ξ)-3-aminochole-5-en-24-ol (101)

Lithium aluminum hydride (1.5 g, 39.2 mmol) was carefully added in small portions to a stirring solution of 100 (5.4 g, 13.1 mmol) in THF (150 mL). The solidified reaction was allowed to stand at 22 °C for 2 h. Crushed ice was carefully added followed by H\textsubscript{2}O (75 mL) and the aqueous layer was extracted with EtOAc (150 mL). The organics were dried over Na\textsubscript{2}SO\textsubscript{4} and the solvent removed \textit{in vacuo} to give the product as a white solid (4.5 g, 96%). mp 158-159 °C; \( ^1H \) NMR (CDCl\textsubscript{3}:MeOD, 3:1, 400.13 MHz) \( \delta \) 4.99-4.92 (m, 1H), 3.08-3.16 (m, 2H), 2.19-2.03 (m, 1H), 1.75-1.18 (m, 6H), 1.16-0.67 (m, 25H), 0.46 (s, 3H); \( ^{13}C \)
NMR (CDCl₃, 100.62 MHz) δ 140.59, 120.20, 56.16, 55.47, 50.89, 49.64, 41.96, 41.62, 41.59, 39.17, 37.44, 35.80, 35.01, 31.29, 31.14, 31.03, 28.43, 27.46, 23.53, 20.87, 20.32, 18.45, 10.93; IR (film): νmax 3359.9, 2934.0, 1771.3, 1370.4 cm⁻¹; MS (ESI⁺) calcd. for C₂₄H₄₂NO (MH⁺) 360.33, found 360.32. Anal. calcd. for C₂₄H₄₁NO: C, 80.16; H, 11.49. Found: C, 80.23; H, 11.34.

**tert-butyl [3-(1,3-dioxo-1,3-dihydro-2H-isooindol-2-yl)propyl][3β,8ξ,9ξ,14ξ)-24-hydroxychol-5-en-3-yl]carbamate (102)**

Compound 101 (3.7 g, 10.3 mmol) was combined with N-(3-Bromo-propyl)phthalimide (3.2 g, 12.3 mmol) and K₂CO₃ (3.3 g, 23.7 mmol) in DMF (20 mL). The reaction mixture was stirred at 60 °C for 16 h and filtered. The filtrate was concentrated to give a solid that was resuspended in CH₂Cl₂ (40 mL) and DIEA (4.5 mL, 25.7 mmol). Di-tert-butyl dicarbonate (5.6 g, 25.7 mmol) was directly added and the reaction was stirred at 22 °C for 3 h. The reaction mixture was concentrated to an oil that was purified by column chromatography eluting with EtOAc/hexanes (3:7) followed by EtOAc/hexanes (1:1) to give the product as a white solid (3.5 g, 52%). mp 182-183 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 7.79-7.77 (m, 2H), 7.67-7.63 (m, 2H), 5.21-5.19 (m, 1H), 3.71-3.54 (m, 2H), 3.31-3.04 (m, 2H), 1.92-0.80 (m, 46H), 0.59 (s, 3H); ¹³C NMR (CDCl₃, 100.62 MHz) δ
167.86, 154.86, 140.63, 133.62, 121.02, 85.07, 79.32, 63.40, 56.59, 55.92, 49.98, 42.22, 36.65, 36.48, 35.85, 35.51, 35.30, 34.78, 34.98, 31.76, 31.14, 29.34, 28.36, 28.13, 27.72, 27.32, 24.26, 23.96, 20.89, 19.30, 18.60, 11.78; IR (film): \(v_{\text{max}} = 2937.9, 1714.9, 1395.9, 1121.5\ \text{cm}^{-1}\); MS (ESI\(^{+}\))
calcd. for C\textsubscript{40}H\textsubscript{59}N\textsubscript{2}O\textsubscript{5} (MH\(^{+}\)) 647.44, found 647.44. Anal. calcd. for C\textsubscript{40}H\textsubscript{58}N\textsubscript{2}O\textsubscript{5}: C, 74.27; H, 9.04. Found: C, 74.37; H, 8.86.

tert-butyl [3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl][3\(\beta,8\xi,9\xi,14\xi\)-24-iodochol-5-en-3-yl]carbamate (103)

Iodine (88 mg, 0.35 mmol) was added to a stirring solution of 102 (203 mg, 0.31 mmol), triphenylphosphine (91 mg, 0.35 mmol) and imidazole (24 mg, 0.35 mmol) in THF (15 mL) at 0 °C. The reaction was stirred at 0 °C for 0.5 h and 10% aqueous sodium thiosulfate pentahydrate was added. The aqueous layer was extracted with Et\textsubscript{2}O (2 x 30 mL) and the combined organics were dried over Na\textsubscript{2}SO\textsubscript{4}. Concentration gave an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:18) to give the product as a white foam (198 mg, 87%). mp 77-78 °C; \(^1\text{H} NMR (CDCl\textsubscript{3}, 400.13 MHz) \delta 7.78-7.77 (m, 2H), 7.67-7.65 (m, 2H), 5.26 (bs, 1H), 3.63 (t, \(J = 6.9 \text{ Hz}, 2H), 3.14-3.04 (m, 4H), 1.97-0.87 (m, 14H), 1.34-1.22 (m, 2H), 0.78-0.65 (m, 6H).
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43H), 0.59 (s, 3H); $^{13}$C NMR (CDCl$_3$, 100.62 MHz) δ 168.15, 155.02, 133.82,
131.96, 123.07, 121.15, 85.22, 79.26, 56.51, 55.71, 49.88, 42.20, 39.57, 36.60,
36.46, 35.90, 35.82, 34.93, 31.72, 30.20, 28.40, 28.30, 27.74, 24.08, 23.92,
20.83, 19.35, 19.21, 18.72, 18.57, 11.84, 7.76; IR (film): $\nu_{\text{max}}$ 2937.2, 1714.4,
1395.9, 1171.4 cm$^{-1}$; HRMS (TOF ESI$^+$) m/z 757.3464 (MH$^+$, C$_{40}$H$_{58}$N$_2$O$_4$
requires 757.3441).

tert-butyl ([(3S,10R,13R,17R)-10,13-dimethyl-17-[(1R)-1-methyldeca-5,9-diyn-
1-yl]-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]
phenanthren-3-yl][3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]
carbamate (104)

To a flame dried and degassed schlenk flask was charged 1,3-Bis(1-
adamantyl)imidazolium chloride (5 mg, 0.01 mmol), Allylpalladium chloride dimer
(2.4 mg, 0.007 mmol), Cul (4 mg, 0.02 mmol) and cesium carbonate (120 mg,
0.36 mmol) in DMF:Et$_2$O (1:2, 10 mL). Compound 103 (200 mg, 0.26 mmol) was
added followed by the addition of 1,7 octadiyne (0.1 mL, 0.79 mmol) and the
reaction was stirred at 40 °C for 12 h. The reaction mixture was concentrated to
give a black oil that was purified by column chromatography eluting with
EtOAc/hexanes (1:9) followed by EtOAc/hexanes (3:7) to give the product as a
clear oil (120 mg, 63%). \(^1\)H NMR (CDCl\(_3\), 400.13 MHz) \(\delta\) 7.67-7.63 (m, 2H), 7.56-7.54 (m, 2H), 5.15-5.13 (m, 1H), 3.52 (t, \(J = 7.1\) Hz, 2H), 3.30-3.25 (m, 2H), 2.03-0.75 (m, 30H), 0.61 (s, 3H); \(^{13}\)C NMR (CDCl\(_3\), 75.48 MHz) \(\delta\) 170.41, 167.81, 154.29, 133.50, 131.77, 122.74, 83.71, 80.30, 78.83, 68.21, 65.40, 59.87, 56.31, 55.60, 53.19, 49.77, 41.90, 39.37, 36.19, 35.50, 34.96, 34.80, 31.47, 28.06, 27.81, 27.70, 27.17, 25.33, 23.91, 20.92, 20.58, 18.90, 18.76, 17.90, 17.61, 14.95, 13.81, 11.44; IR (film): \(\nu_{\text{max}}\) 2938.7, 1714.4, 1400.4 cm\(^{-1}\); HRMS (TOF ESI\(^+\)) \(m/z\) 735.5120 (MH\(^+\), C\(_{48}\)H\(_{67}\)N\(_2\)O\(_4\) requires 735.5101).

tert-butyl [(3b,8x,9x,14x)-24-azidochol-5-en-3-yl][3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]carbamate (105)

To a stirring solution of 102 (203 mg, 0.31 mmol) and triphenylphosphine (91 mg, 0.35 mmol) in benzene (20 mL) was added a solution of hydrazoic acid in benzene\(^3\) (3.9 mL) over 3 portions. A solution of diisopropylazodicarboxylate (0.5 mL, 0.35 mmol) in benzene (5 mL) was added and the reaction was stirred at 22 °C for 1 h. The reaction mixture was concentrated to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:9) to give the product as a pale yellow solid (1.1 g, 92%) mp 137-138°C; \(^1\)H NMR (CDCl\(_3\), 300.13 MHz) \(\delta\) 7.78-7.77 (m, 2H), 7.67-7.64 (m, 2H), 5.25 (s, 1H), 3.64 (t, \(J = 6.9\) Hz, 2H).
Hz, 2H), 3.14-3.03 (m, 3H), 2.23-1.72 (m, 7H), 1.54-0.83 (m, 37H), 0.58 (s, 3H);

$^{13}$C NMR (CDCl$_3$, 75.48 MHz) $\delta$ 167.86, 159.64, 140.30, 133.64, 131.70, 122.91, 121.23, 78.48, 73.95, 56.42, 55.62, 51.63, 49.81, 42.05, 39.48, 39.39, 37.70, 36.20, 35.20, 32.67, 31.58, 29.17, 28.20, 27.96, 25.26, 24.00, 21.31, 20.73, 19.09, 18.40, 11.60; IR (film): $\nu_{\text{max}}$ 2934.8, 2094.9, 1683.3, 1519.4 cm$^{-1}$; HRMS (TOF ESI$^+$) m/z 644.4431 (M – N$_2$H$^+$, C$_{40}$H$_{58}$N$_3$O$_4$ requires 644.4427).

tert-butyl [(3R,10S,13S,17R)-17-((1R)-4-[(10R)-10-((3S,10R,13R,17R)-3-{{(tert-butoxycarbonyl)[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]amino}-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]undec-5-yn-1-yl]-1H-1,2,3-triazol-1-yl]-1-methylbutyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl][3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]carbamate (106)

To a mixture of 104 (344 mg, 0.48 mmol) and 105 (347 mg, 0.72 mmol) in DMF:H$_2$O (4:1, 5 mL) was added (+)-Sodium L-ascorbate (38 mg, 0.20 mmol) followed by Copper(II) sulfate pentahydrate (6 mg, 0.02 mmol). The reaction
mixture was then microwave irradiated at 300W, 80 °C for 8 min and poured into H₂O (20 mL). The aqueous layer was extracted with EtOAc (2 x 15 mL) and the combined organics were dried over Na₂SO₄. Concentration gave an oil that was purified by column chromatography eluting with EtOAc/hexanes (3:7) followed by EtOAc/hexanes (1:1) to give the product as a white foam (265 mg, 46%). mp 79-80 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 7.72-7.70 (m, 2H), 7.61-7.59 (m, 2H), 7.19 (s, 1H), 5.30-5.25 (m, 3H), 4.61-4.00 (m, 3H), 3.99-3.97 (m, 2H), 3.57 (t, J = 6.7 Hz, 2H), 3.13-3.04 (m, 2H), 2.60 (t, J = 7.4 Hz, 2H), 2.35-0.82 (m, 85 H), 0.53 (s, 6H); ¹³C NMR (CDCl₃, 100.62 MHz) δ 168.22, 155.15, 154.97, 140.77, 140.40, 137.27, 135.29, 133.82, 131.98, 123.59, 121.23, 111.32, 80.43, 79.61, 78.76, 72.78, 68.62, 56.49, 55.99, 55.54, 53.30, 50.42, 49.88, 49.41, 42.63, 42.15, 39.55, 39.47, 37.79, 36.37, 35.79, 35.23, 35.11, 34.72, 31.68, 28.47, 27.99, 26.87, 25.74, 25.64, 25.01, 24.06, 23.40, 20.79, 20.64, 19.18, 19.04, 18.52, 18.47, 18.42, 18.25, 18.24, 17.94, 11.89, 11.69; IR (film): νmax 2938.8, 1713.5, 1395.8, 1171.6 cm⁻¹; HRMS (TOF ESI⁺) m/z 1406.9508 (MH⁺, C₈₈H₁₄N₇O₈ requires 1406.9511).
9H-fluoren-9-ylmethyl {12-[(3R,10S,13S,17R)-17-((1R)-4-{(4-[(10R)-10-
((3S,10R,13R,17R)-3-{{tert-butoxycarbonyl}[15-(9H-fluoren-9-yl)-5,9,13-
trioxo-14-oxa-4,8,12-triazapentadec-1-yl]amino}-10,13-dimethyl-2,3,4,7,
8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-
17-yl)undec-5-yn-1-yl}-1H-1,2,3-triazol-1-yl]-1-methylbutyl]-10,13-dimethyl-
2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta [a]phe
nanthren-3-yl]-15,15-dimethyl-3,7,13-trioxo-14-oxa-4,8,12-triazahexadec-1-
yl}carbamate (107)

To a stirring solution of 106 (100 mg, 0.71 mmol) in THF:EtOH (1:5, 20
mL) was added hydrazine (11 uL, 0.35 mmol). The reaction was stirred at 22 °C
for 16 h and filtered. The filtrate was concentrated to give the crude di-amine,
which was taken on without further purification. This di-amine was combined with
90 (85 mg, 0.17 mmol) and DIEA (37 uL, 0.21 mmol) in CH₂Cl₂ and the cloudy
reaction mixture was stirred at 22 °C for 3 h. The reaction was concentrated and
the residue was purified by column chromatography eluting with Acetone/CH₂Cl₂
(1:4) followed by MeOH/CH₂Cl₂ (1:19) to give the product as a white solid (103
mg, 77%). mp 100-101 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 7.75 (d, J = 7.4 Hz,
4H), 7.59 (d, J = 7.4 Hz, 4H), 7.38 (t, J = 7.3 Hz, 4H), 7.32-7.26 (m, 6H), 5.34-5.30 (m, 2H), 4.32 (d, J = 7.1 Hz, 4H), 4.16 (t, J = 7.0 Hz, 2H), 3.53-3.49 (m, 8H), 3.25-3.21 (m, 4H), 2.64-2.44 (m, 8H), 2.14-0.88 (m, 107H), 0.64 (s, 6H); 13C NMR (CDCl₃, 75.48 MHz) δ 174.19, 171.45, 156.40, 143.94, 143.82, 143.22, 141.20, 134.78, 127.54, 127.14, 124.89, 121.37, 119.86, 79.82, 77.25, 69.56, 69.00, 66.68, 56.59, 56.12, 53.38, 50.04, 47.26, 42.25, 41.38, 39.78, 38.46, 37.22, 36.54, 36.29, 35.87, 35.67, 32.96, 31.79, 30.97, 30.30, 29.57, 29.44, 29.20, 28.47, 28.16, 27.22, 26.89, 26.55, 25.46, 24.76, 20.89, 19.35, 18.76, 16.22, 11.90; IR (film): νmax 3328.2, 2937.8, 1648.3, 1541.1 cm⁻¹; HRMS (TOF ESI⁺) m/z 1875.2242 (MH⁺, C₁₁₄H₁₆₀N₁₁O₁₂ requires 1875.2248).

5-{{3-{{3-{{3S,8R)-17-((1R)-10-{1-[(4R)-4-[(3S,8R)-3-{{3-{{3-carboxylato-4-(2,7-difluoro-6-oxyd-3-oxo-3H-xanthen-9-yl)benzoyl]amino}propanoyl]amino}propanoyl]amino}propyl]ammonio}-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentyl]-1H-1,2,3-triazol-4-yl]-1-methyldec-5-yn-1-yl]-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl]ammonio}propyl]amino]-3-oxopropyl}amino)-3-oxopropyl]amino}carbonyl)-2-(2,7-difluoro-6-oxyd-3-oxo-3H-xanthen-9-yl)benzoate (76)
Piperidine (0.37 mL) was added to a stirring mixture of compound 107 (19 mg, 0.01 mmol) in DMF (1.5 mL). The resulting solution was stirred at 22 °C for 20 min and the solvent was removed *in vacuo* to give the crude diamine as a white film, which was taken on without further purification. This diamine was combined with Oregon Green OSu (10 mg, 0.02 mol) and DIEA (0.1 mL, 5.7 mmol) in DMF (5 mL). The reaction was stirred at 22 °C for 12 h and the solvent was removed *in vacuo*. The residue was suspended in trifluoroacetic acid / “wet” CH₂Cl₂ (3:47, 10 mL) and stirred at 22 °C for 3 h. The reaction mixture was concentrated and the residue was purified via preparative reverse-phase HPLC (gradient: 20% CH₃CN, 80% H₂O, and 0.1% TFA to 99% CH₃CN, 1% H₂O, and 0.1% TFA over 20 min to afford 76 as an orange solid (9.7 mg, 48%). Mp 161-163 °C; ¹H NMR (DMSO-d₆, 400.13 MHz) δ 8.96 (bs, 4H), 8.71 (t, J = 5.8 Hz, 2H), 8.30 (t, J = 5.7 Hz, 2H), 8.11-8.08 (m, 4H), 8.01 (bs, 2H), 7.49 (d, J = 7.6 Hz, 2H), 7.19 (s, 1H), 6.90 (bs, 4H), 6.75 (s, 2H), 6.72 (s, 2H), 5.34-5.30 (m, 2H), 3.53-3.49 (m, 8H), 3.26-3.20 (m, 4H), 2.64-2.43 (m, 8H), 2.21-0.91 (m, 84H), 0.66 (s, 6H); ¹³C NMR (DMSO-d₆, 100.61 MHz) δ 172.75, 171.56, 166.70, 163.67, 159.62, 154.52, 150.09, 143.22, 139.68, 137.13, 136.72, 135.63, 134.78, 130.34, 130.01, 125.82, 123.42, 115.08, 79.82, 77.25, 68.98, 66.68, 56.59, 56.13, 53.38, 50.02, 47.26, 42.25, 41.38, 39.79, 38.46, 37.22, 36.54, 36.29, 35.87, 35.67, 32.96, 31.79, 30.97, 30.30, 29.60, 29.44, 29.20, 28.49, 28.16, 27.22, 26.89, 26.57, 25.46, 24.76, 20.89, 19.35, 18.77, 16.22, 11.90; IR (film):
ν_max 2989.4, 1713.2, 1687.5, 1482.1 cm⁻¹; HRMS (TOF ESI⁺) m/z 2023.0781 (MH⁺, C₁₁₆H₁₄₄F₄N₁₁O₁₆ requires 2023.0729).

Analytical reverse-phase HPLC of compound 76 after purification (retention time = 7.04 min).

tert-butyl [3-(1,3-dioxo-1,3-dihydro-2H-isouindol-2-yl)propyl][3β,8ξ,9ξ,14ξ)-24-oxochol-5-en-3-yl]carbamate (97)

To a solution of oxalyl chloride (4.8 mL, 9.5 mmol) in CH₂Cl₂ (30 mL) at -78 °C was added dimethyl sulfoxide (0.95 mL, 13.4 mmol) over 10 min. The reaction was stirred at -78 °C for 15 min and a solution of 102 (2.8 g, 4.3 mmol) in CH₂Cl₂ (30 mL) was added dropwise over 20 min. The reaction was stirred at -
78 °C for 1.5 h, warmed to room temperature and washed with saturated aqueous ammonium chloride (30 mL). The organic layer was dried over Na₂SO₄ and the solvent removed in vacuo to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (3:7) to give the product as a white solid (2.4 g, 87%). mp 88-89 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 9.74 (s, 1H), 7.79-7.77 (m, 2H), 7.67-7.62 (m, 2H), 5.26 (bs, 1H), 3.72-3.63 (m, 2H), 3.38-3.35 (m, 2H), 3.01 (s, 1H), 1.95-0.87 (m, 43H), 0.66 (s, 3H); ¹³C NMR (CDCl₃, 100.62 MHz) δ 182.21, 168.22, 134.00, 133.00, 133.87, 131.99, 123.25, 123.11, 121.02, 85.07, 79.32, 63.40, 56.60, 55.92, 50.00, 47.19, 42.22, 39.65, 36.48, 35.85, 35.51, 34.69, 31.76, 31.14, 29.34, 28.36, 28.13, 27.72, 27.32, 24.16, 28.89, 19.30, 18.60, 11.78; IR (film): ν_max 2937.4, 1713.8, 1688.0, 1395.9 cm⁻¹; MS (ESI⁺) calcd. for C₄₀H₅₇N₂O₅ (MH⁺) 645.43, found 645.40. Anal. calcd. for C₄₀H₅₆N₂O₅: C, 74.50; H, 8.75. Found: C, 74.12; H, 8.52.
**tert-buty 1 [3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]{(3S,10R,13R, 17R)-17-[(1R,4E)-5-iodo-1-methylpent-4-en-1-yl]-10,13-dimethyl-2,3,4,7,8, 9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl}carbamate (108)**

A neat mixture of chromium (III) chloride\(^{27}\) (6.2 g, 39.1 mmol), zinc powder (1.3 g, 19.5 mmol) and sodium iodide (4.5 g, 32.5 mmol) was heated under vacuum at 100 °C for 5 min. The mixture was allowed to cool to room temperature and THF (30 mL) was added. The mixture was stirred for 10 min and cannulated into a solution of 97 (2.8 g, 4.3 mmol) and iodoform (4.3 g, 10.8 mmol) in THF (30 mL). The resulting black mixture was stirred at 22 °C for 10 h and aqueous 1M HCl (20 mL) was added. The aqueous layer was extracted with \(\text{Et}_2\text{O}\) (2 x 40 mL) and the combined organics were washed with saturated aqueous NaCl (40 mL) and dried over \(\text{Na}_2\text{SO}_4\). Concentration gave a brown solid that was purified by column chromatography eluting with EtOAc/hexanes (1:9) followed by EtOAc/hexanes (3:7) to give the product as a pale yellow solid (2.9 g, 67%). mp 131-132 °C; \(^1\)H NMR (CDCl\(_3\), 400.13 MHz) \(\delta\) 7.79-7.76 (m, 2H), 7.68- 7.64 (m, 2H), 6.52-6.45 (m, 1H), 5.95 (d, \(J = 14.2\) Hz, 1H), 5.35-5.33 (m, 1H), 3.49-3.47 (m, 1H), 2.11-1.81 (m, 4H), 1.56-0.90 (m, 42H), 0.66 (s, 3H); \(^{13}\)C NMR
(CDCl₃, 100.62 MHz) δ 167.89, 154.90, 147.06, 140.61, 133.59, 131.71, 122.87, 121.03, 81.98, 74.05, 66.32, 56.58, 55.81, 51.29, 49.97, 47.24, 42.27, 39.64, 37.78, 36.44, 35.23, 34.98, 34.78, 34.53, 32.83, 31.76, 31.31, 30.27, 29.65, 29.31, 28.13, 24.18, 23.92, 20.89, 19.29, 18.42, 11.82; IR (film): νmax 2936.5, 1700.1, 1527.9, 1449.4 cm⁻¹; HRMS (Cl⁺) m/z 769.3447 (MH⁺, C₄₁H₅₈N₂O₄ requires 769.3441).

tert-butyl {(3R,10S,13S,17S)-17-[(1S,4E,13E,17R)-17-((3S,10R,13R,17S)-3-((tert-butoxycarbonyl)[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]amino)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-1-methyloctadeca-4,13-diene-6,11-diyn-1-yl]-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl][3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]carbamate (111)

To a degassed schlenk flask was charged 108 (255 mg, 0.3 mmol), 1,6 heptadiyne (20 uL, 0.17 mmol), DIEA (0.2 mL, 1.0 mmol) and Pd(PPh₃)₂Cl₂ (12 mg, 5 mol%) in THF (15 mL). The mixture was then degassed under nitrogen and Cul (6 mg, 10 mol%) was added. The reaction mixture was degassed once
more and stirred vigorously for 3 h at 22 °C. The mixture was then poured into H₂O (20 mL) and extracted with EtOAc (3 x 25 mL). The combined organics were dried over Na₂SO₄ and concentrated to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:8) followed by EtOAc/hexanes (3:7) to afford 111 as an oil (157 mg, 69%). ^1H NMR (CDCl₃, 400.13 MHz) δ 7.81-7.79 (m, 4H), 7.69-7.68 (m, 4H), 6.07-5.99 (m, 2H), 5.40 (d, J = 15.4 Hz, 2H), 5.27 (bs, 2H), 4.13-4.05 (m, 6H), 3.69 (t, J = 7.0 Hz, 4H), 3.14-3.12 (m, 4H), 2.46-0.92 (m, 86H), 0.62 (s, 6H); ^13C NMR (CDCl₃, 100.62 MHz) δ 168.26, 155.87, 144.73, 133.90, 132.05, 123.15, 121.29, 109.97, 109.23, 86.64, 79.35, 77.33, 75.27, 66.60, 60.33, 56.62, 55.93, 50.02, 42.29, 39.69, 38.28, 36.54, 35.89, 33.36, 34.97, 31.81, 30.59, 29.70, 28.19, 28.13, 26.67, 24.19, 20.97, 20.94, 19.77, 19.64, 19.34, 19.25, 19.22, 19.17, 19.06, 19.10, 18.47, 16.71, 14.19, 11.82; IR (film): νₘₐₓ 2933.5, 1713.2, 1360.4, 1171.7 cm⁻¹; HRMS (TOF ESI⁺) m/z 1373.9189 (MH⁺, C₈₉H₁₂₁N₄O₈ requires 1373.9184).
tert-butyl \{(3S,10R,13R,17R)-17-[(1R,4E,12E,16S)-16-((3S,10R,13R,17S)-3-\{(tert-butoxycarbonyl)\}[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]amino]-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[\alpha]phenanthren-17-yl]-1-methylheptadeca-4,12-diene-6,10-diyn-1-yl]-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl\}[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]carbamate (112)

To a degassed schlenk flask was charged 108 (175 mg, 0.23 mmol), 1,5-hexadiyne (18 uL, 0.11 mmol), DIEA (0.2 mL, 1.1 mmol) and Pd(PPh\textsubscript{3})\textsubscript{2}Cl\textsubscript{2} (8 mg, 5 mol\%) in THF (15 mL). The mixture was then degassed under nitrogen and Cul (4 mg, 10 mol\%) was added. The reaction mixture was degassed once more and stirred vigorously for 3 h at 22 °C. The mixture was then poured into H\textsubscript{2}O (20 mL) and extracted with EtOAc (3 x 25 mL). The combined organics were dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:8) followed by EtOAc/hexanes
(3:7) to afford 112 as a thin film (157 mg, 69%). $^1$H NMR (CDCl$_3$, 400.13 MHz) $\delta$ 7.81-7.79 (m, 4H), 7.69-7.68 (m, 4H), 6.06-5.99 (m, 2H), 5.39 (d, $J = 16$ Hz, 2H), 5.28 (bs, 2H), 4.11-4.02 (m, 6H), 3.66 (t, $J = 7.0$ Hz, 4H), 3.14-3.12 (m, 4H), 2.46-0.92 (m, 84H), 0.62 (s, 6H); $^{13}$C NMR (CDCl$_3$, 100.62 MHz) $\delta$ 168.26, 155.86, 144.73, 133.89, 132.04, 123.14, 121.30, 109.97, 109.21, 86.63, 79.35, 77.32, 75.26, 66.59, 60.33, 56.62, 55.93, 50.02, 42.29, 39.68, 38.27, 36.54, 35.89, 33.36, 34.97, 31.80, 30.58, 29.71, 28.20, 28.13, 26.67, 24.19, 20.99, 20.93, 19.74, 19.64, 19.34, 19.25, 19.22, 19.17, 19.06, 19.03, 18.45, 14.19, 11.83; IR (film): $\nu$$_{max}$ 2933.7, 1713.9, 1395.9, 1170.3 cm$^{-1}$; HRMS (TOF ESI$^+$) $m/z$ 1381.8948 (M + Na$^+$, C$_{88}$H$_{118}$N$_4$O$_8$ requires 1381.8847).
tert-butyl \{\text{3S,10R,13R,17R}\}-17-[(\text{1R,16S})-16-(\text{3S,10R,13R,17S})-3-\{\text{tert-butoxycarbonyl}\}3-(\text{1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl})propyl]amino\}-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-1-methylheptadecyl]-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl\}[3-(\text{1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl})propyl]carbamate (113)

To a schlenk flask was charged a catalytic amount 10% palladium on activated carbon and the flask was degassed under hydrogen. A solution of 112 (77 mg, 0.06 mmol) in toluene (10 mL) was syringed in and the reaction was allowed to stir under ~ 1 atmosphere of hydrogen at 22 °C for 3 h. The reaction mixture was filtered through a pad of Celite and concentrated to afford 113 as a tan solid (74 mg, 89%). mp 125-126 °C; \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 299.87 MHz) \(\delta\) 7.83-7.79 (m, 4H), 7.70-7.67 (m, 4H), 5.32 (bs, 2H), 3.67 (t, \(J = 7.0\) Hz, 4H), 3.14-3.12 (m, 4H), 2.46-0.92 (m, 106H), 0.62 (s, 6H); \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75.41 MHz) \(\delta\) 168.15, 155.02, 133.82, 131.96, 123.14, 121.22, 79.32, 56.63, 55.93, 50.02,
5-[(3-[(3-([(3S,8R)-17-[(1R,4E,13E,17S)-17-((3R,8S)-3-([3-((3-carboxy-4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoyl]amino)propanoyl)amino)[propanoyl]amino)propyl]ammonio)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-1-methyloctadeca-4,13-diene-6,11-diyn-1-yl]-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl]ammonio)propyl]amino]-3-oxopropyl]amino]-3-oxopropyl]amino)carbonyl]-2-(2,7-difluoro-6-oxido-3-oxo-3H-xanthen-9-yl)benzoate (77)

To a stirring solution of 111 (43 mg, 0.03 mmol) in THF:EtOH (1:5, 10 mL) was added hydrazine (5 uL, 0.16 mmol). The reaction was stirred at 50 °C for 6 h and filtered. The filtrate was concentrated to give the crude di-amine, which was taken on without further purification. This di-amine was combined with 90 (38 mg, 0.07 mmol) and DIEA (22 uL, 0.13 mmol) in CH₂Cl₂ and the cloudy reaction
mixture was stirred at 22 °C for 3 h. The reaction was concentrated and the residue was combined with piperidine (0.37 mL) in DMF (1.5 mL). The resulting solution was stirred at 22 °C for 20 min and the solvent was removed in vacuo to give the crude diamine as a white film, which was taken on without further purification. This diamine was combined with Oregon Green OSu (10 mg, 0.02 mol) and DIEA (0.1 mL, 5.7 mmol) in DMF (5 mL). The reaction was stirred at 22 °C for 12 h and the solvent was removed in vacuo. The residue was suspended in trifluoroacetic acid / “wet” CH₂Cl₂ (3:47, 10 mL) and stirred at 22 °C for 3 h. The reaction mixture was concentrated and the residue was purified via preparative reverse-phase HPLC (gradient: 20% CH₃CN, 80% H₂O, and 0.1% TFA to 99% CH₃CN, 1% H₂O, and 0.1% TFA over 20 min to afford 77 as an orange solid (8.3 mg, 14%). Mp 101-103 °C; ¹H NMR (DMSO-d₆, 400.13 MHz) δ 8.98 (bs, 4H), 8.72 (t, J = 5.9 Hz, 2H), 8.30 (t, J = 5.7 Hz, 2H), 8.13-8.08 (m, 4H), 8.00 (bs, 2H), 7.49 (d, J = 7.7 Hz, 2H), 6.90 (bs, 4H), 6.75 (s, 2H), 6.72 (s, 2H), 6.06-5.98 (m, 2H), 5.41 (d, J = 15.6 Hz, 2H), 5.31-5.29 (m, 2H), 3.53-3.49 (m, 8H), 3.23-3.20 (m, 4H), 2.61-2.41 (m, 8H), 2.16-0.86 (m, 80H), 0.66 (s, 6H); ¹³C NMR (DMSO-d₆, 100.62 MHz) δ 172.72, 171.52, 166.70, 163.67, 159.62, 154.52, 150.09, 139.69, 137.13, 136.72, 135.63, 133.90, 130.33, 129.99, 125.82, 123.42, 115.09, 109.21, 86.64, 79.35, 77.32, 75.25, 66.60, 60.33, 56.63, 55.93, 50.02, 42.29, 39.69, 38.28, 37.18, 36.55, 35.89, 34.99, 33.36, 31.81, 30.59, 29.70, 28.19, 28.13, 27.10, 26.67, 24.19, 20.97, 20.95, 19.77, 19.64, 19.34, 19.24, 19.22, 19.17, 19.06, 19.10, 18.47, 16.72, 14.19, 11.90; IR (film): ʋmax
2930.4, 1715.6, 1374.1 cm$^{-1}$; HRMS (TOF ESI$^+$) m/z 1990.0423 (MH$^+$, C$_{117}$H$_{141}$F$_4$N$_8$O$_{16}$ requires 1990.0402).

Analytical reverse-phase HPLC of compound 77 after purification (retention time = 11.94 min).
To a stirring solution of 112 (50 mg, 0.04 mmol) in THF:EtOH (1:5, 10 mL) was added hydrazine (7 uL, 0.2 mmol). The reaction was stirred at 50 °C for 8 h and filtered. The filtrate was concentrated to give the crude di-amine, which was taken on without further purification. This di-amine was combined with 90 (48 mg, 0.10 mmol) and DIEA (22 uL, 0.13 mmol) in CH₂Cl₂ and the cloudy reaction mixture was stirred at 22 °C for 3 h. The reaction was concentrated and the residue was combined with piperidine (0.37 mL) in DMF (1.5 mL). The resulting solution was stirred at 22 °C for 20 min and the solvent was removed in vacuo to give the crude diamine as a white film, which was taken on without further purification. This diamine was combined with Oregon Green OSu (10 mg, 0.02
mol) and DIEA (0.1 mL, 5.7 mmol) in DMF (5 mL). The reaction was stirred at 22 °C for 12 h and the solvent was removed *in vacuo*. The residue was suspended in trifluoroacetic acid / “wet” CH₂Cl₂ (3:47, 10 mL) and stirred at 22 °C for 3 h. The reaction mixture was concentrated and the residue was purified via preparative reverse-phase HPLC (gradient: 20% CH₃CN, 80% H₂O, and 0.1% TFA to 99% CH₃CN, 1% H₂O, and 0.1% TFA over 20 min to afford 78 as an orange solid (15.1 mg, 21%). Mp 110-111 °C; ¹H NMR (DMSO-d₆, 400.13 MHz) δ 8.98 (bs, 4H), 8.72 (t, J = 6.0 Hz, 2H), 8.30 (t, J = 5.7 Hz, 2H), 8.14-8.09 (m, 4H), 8.00 (bs, 2H), 7.50 (d, J = 7.6 Hz, 2H), 6.90 (bs, 4H), 6.75 (s, 2H), 6.72 (s, 2H), 6.05-5.97 (m, 2H), 5.40 (d, J = 15.9 Hz, 2H), 5.31-5.29 (m, 2H), 3.53-3.49 (m, 8H), 3.23-3.20 (m, 8H), 2.61-2.41 (m, 8H), 2.18-0.87 (m, 74H), 0.64 (s, 6H); ¹³C NMR (DMSO-d₆, 100.62 MHz) δ 172.72, 171.53, 166.70, 163.67, 159.62, 154.54, 150.08, 139.69, 137.13, 136.72, 135.63, 133.90, 130.33, 129.99, 125.82, 123.42, 115.10, 109.22, 86.64, 79.36, 77.35, 75.28, 66.60, 60.33, 56.63, 55.93, 50.02, 42.29, 39.69, 38.28, 37.18, 36.55, 35.89, 34.99, 33.36, 31.81, 30.59, 29.70, 28.19, 28.13, 27.10, 26.67, 24.19, 20.95, 19.77, 19.64, 19.34, 19.24, 19.22, 19.17, 19.06, 19.10, 18.47, 16.70, 14.19, 11.88; IR (film): νₑₓₚₑ 2929.4, 1714.5, 1396.6 cm⁻¹; HRMS (TOF ESI⁺) m/z 1962.0100 (MH⁺, C₁₁₅H₁₃₇F₄N₈O₁₆ requires 1962.0089).
To a stirring solution of 113 (34 mg, 0.02 mmol) in THF:EtOH (1:5, 10 mL) was added hydrazine (4 uL, 0.1 mmol). The reaction was stirred at 50 °C for 12 h and filtered. The filtrate was concentrated to give the crude di-amine, which was
taken on without further purification. This di-amine was combined with 90 (24 mg, 0.05 mmol) and DIEA (22 uL, 0.13 mmol) in CH₂Cl₂ and the cloudy reaction mixture was stirred at 22 °C for 3 h. The reaction was concentrated and the residue was combined with piperidine (0.37 mL) in DMF (1.5 mL). The resulting solution was stirred at 22 °C for 20 min and the solvent was removed *in vacuo* to give the crude diamine as a white film, which was taken on without further purification. This diamine was combined with Oregon Green OSu (10 mg, 0.02 mol) and DIEA (0.1 mL, 5.7 mmol) in DMF (5 mL). The reaction was stirred at 22 °C for 12 h and the solvent was removed *in vacuo*. The residue was suspended in trifluoroacetic acid / “wet” CH₂Cl₂ (3:47, 10 mL) and stirred at 22 °C for 3 h. The reaction mixture was concentrated and the residue was purified via preparative reverse-phase HPLC (gradient: 20% CH₃CN, 80% H₂O, and 0.1% TFA to 99% CH₃CN, 1% H₂O, and 0.1% TFA over 20 min to afford 79 as an orange solid (7.7 mg, 16%). Mp 128-130 °C; ¹H NMR (DMSO-d₆, 400.13 MHz) δ 8.96 (bs, 4H), 8.71 (t, J = 5.9 Hz, 2H), 8.30 (t, J = 5.8 Hz, 2H), 8.14-8.09 (m, 4H), 8.00 (bs, 2H), 7.50 (d, J = 7.6 Hz, 2H), 6.90 (bs, 4H), 6.76 (s, 2H), 6.72 (s, 2H), 5.30 (bs, 2H), 3.53-3.49 (m, 8H), 3.22-3.19 (m, 4H), 2.61-2.41 (m, 8H), 2.23-0.86 (m, 90H), 0.67 (s, 6H); ¹³C NMR (DMSO-d₆, 100.62 MHz) δ 172.72, 171.53, 166.70, 163.67, 159.62, 154.54, 150.08, 139.69, 137.13, 136.72, 135.63, 130.33, 129.99, 125.82, 123.42, 115.10, 56.63, 55.93, 50.02, 42.29, 39.69, 38.28, 37.18, 36.55, 36.30, 36.27, 35.89, 34.99, 33.36, 32.24, 31.81, 30.59, 29.70, 28.62, 28.19, 28.13, 27.10, 26.67, 24.19, 20.95, 19.77, 19.64, 19.34, 19.24, 19.22,
19.17, 19.06, 19.10, 18.47, 16.70, 14.19, 11.88; IR (film): \( \nu_{\text{max}} \) 2931.2, 1709.3, 1402.4 cm\(^{-1}\); HRMS (TOF ESI\(^+\)) \( m/z \) 1988.1182 (MH\(^+\), C\(_{116}\)H\(_{151}\)F\(_4\)N\(_8\)O\(_{16}\) requires 1988.1184).

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\text{Absorbance wavelength} = 254 \text{ nm}
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Analytical reverse-phase HPLC of compound 79 after purification (retention time = 12.78 min).

**9H-fluoren-9-ylmethyl [(3\(\beta\),8\(\xi\),9\(\xi\),14\(\xi\))-24-oxochol-5-en-3-yl]carbamate (115)**

To a stirring mixture of amino alcohol 101 (1.9 g, 5.3 mmol) in THF (40 mL) was added sodium bicarbonate (890 mg, 10.5 mmol) and 9-fluorenylmethyl chloroformate (1.5 g, 5.8 mmol). The mixture was stirred at 22 °C for 0.5 h, diluted with H\(_2\)O (50 mL) and extracted with EtOAc (2 x 50 mL). The combined organics were dried over Na\(_2\)SO\(_4\) and the solvent removed *in vacuo* to give an off-white solid that was purified by column chromatography eluting with
EtOAc/hexanes (3:7) to give the alcohol as a slightly impure white solid. To a solution of oxalyl chloride (2.3 mL, 4.5 mmol) in CH₂Cl₂ (30 mL) at -78 °C was added dimethyl sulfoxide (0.45 mL, 6.3 mmol) over 10 min. The reaction was stirred at -78 °C for 15 min and a mixture of the above alcohol (1.2 g, 2.0 mmol) in CH₂Cl₂ (30 mL) was added dropwise over 20 min. The reaction was stirred at -78 °C for 1.5 h, warmed to room temperature and washed with saturated aqueous ammonium chloride (30 mL). The organic layer was dried over Na₂SO₄ and the solvent removed in vacuo to give an oil that was purified by column chromatography eluting with EtOAc/hexanes (3:7) to give the product as a white solid (802 mg, 69%). mp 112-113 °C; ¹H NMR (CDCl₃, 400.13 MHz) δ 9.70 (s, 1H), 7.72 (d, J = 7.5 Hz, 2H), 7.58 (d, J = 7.3 Hz, 2H), 7.35 (t, J = 7.4 Hz, 2H), 7.27 (t, J = 7.4 Hz, 2H), 5.30 (bs, 1H), 4.31 (q, J = 5.4 Hz, 2H), 4.16 (t, J = 6.0 Hz, 2H), 3.36 (s, 1H), 2.26-0.91 (m, 30H), 0.67 (s, 3H); ¹³C NMR (CDCl₃, 100.62 MHz) δ 171.39, 155.91, 143.48, 140.77, 140.05, 127.11, 126.49, 124.51, 121.05, 119.35, 104.71, 104.64, 65.86, 60.07, 56.17, 55.33, 52.10, 50.82, 49.61, 46.73, 41.76, 39.21, 38.68, 37.40, 35.97, 35.00, 31.32, 31.26, 29.96, 28.56, 27.63, 23.68, 20.42, 20.12, 18.60, 17.98, 13.33, 11.18; IR (film): νmax 2938.3, 1698.2, 1449.8, 757.6 cm⁻¹; MS (ESI⁺) calcd. for C₃₉H₅₀NO₃ (MH⁺) 580.38, found 580.30. Anal. calcd. for C₃₉H₄₉NO₃: C, 80.79; H, 8.52. Found: C, 80.40; H, 8.58.
9H-fluoren-9-ylmethyl \{(3S,10R,13R,17R)-17-[(1R,4E)-5-iodo-1-methylpent-4-en-1-yl]-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl}carbamate (116)

A neat mixture of chromium (III) chloride\(^{27}\) (2.0 g, 12.9 mmol), zinc powder (422 mg, 6.4 mmol) and sodium iodide (1.6 g, 10.7 mmol) was heated under vacuum at 100 °C for 5 min. The mixture was allowed to cool to room temperature and THF (30 mL) was added. The mixture was stirred for 10 min and cannulated into a solution of 115 (831 mg, 1.4 mmol) and iodoform (1.4 g, 3.6 mmol) in THF (30 mL). The resulting black mixture was stirred at 22 °C for 10 h and aqueous 1M HCl (20 mL) was added. The aqueous layer was extracted with \(\text{Et}_2\text{O}\) (2 x 40 mL) and the combined organics were washed with saturated aqueous NaCl (40 mL) and dried over \(\text{Na}_2\text{SO}_4\). Concentration gave a brown solid that was purified by column chromatography eluting with EtOAc/hexanes (1:4) to give the product as a yellow solid (750 mg, 76%). mp 102-103 °C; \(^1\text{H NMR}\) (CDCl\(_3\), 400.13 MHz) \(\delta\) 7.74 (d, \(J = 7.5\) Hz, 2H), 7.64 (d, \(J = 7.3\) Hz, 2H), 7.38 (t, \(J = 7.4\) Hz, 2H), 7.29 (t, \(J = 7.4\) Hz, 2H), 6.52-6.45 (m, 1H), 5.96 (d, \(J = 14.2\) Hz, 1H), 5.34 (bs, 1H), 4.37 (d, \(J = 7.7\) Hz, 2H), 4.20 (t, \(J = 6.6\) Hz, 1H), 3.59-3.48
(m, 1H), 2.11-1.80 (m, 4H), 1.56-0.85 (m, 27H), 0.66 (s, 3H); $^{13}$C NMR (CDCl$_3$, 100.62 MHz) $\delta$ 147.06, 143.96, 141.29, 140.25, 127.57, 126.94, 124.99, 121.81, 119.89, 81.98, 74.05, 66.32, 56.58, 55.82, 51.29, 49.97, 47.24, 42.27, 39.64, 37.78, 36.44, 35.23, 34.53, 34.53, 32.83, 31.76, 30.27, 29.65, 29.31, 28.13, 24.18, 20.90, 19.29, 18.42, 11.82; IR (film): $\nu_{\text{max}}$ 2937.1, 1701.4, 1526.9, 1031.9 cm$^{-1}$; HRMS (TOF ESI$^+$) $m/z$ 704.2951 (MH$^+$, C$_{40}$H$_{51}$INO$_2$ requires 704.2965).

tert-butyl ((3S,10R,13R,17R)-10,13-dimethyl-17-[(1R,4E)-1-methylundec-4-ene-6,10-diyn-1-yl]-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]carbamate – methane (114)

To a degassed schlenk flask was charged 108 (330 mg, 0.43 mmol), DIEA (0.2 mL, 1.3 mmol) and Pd(PPh$_3$)$_2$Cl$_2$ (15 mg, 5 mol%) in THF (25 mL). The mixture was then degassed under nitrogen and Cul (8 mg, 10 mol%) was added. The reaction mixture was stirred for 20 min at 22 °C and 1,5 hexadiyne (0.4 mL, 2.1 mmol) was added. The reaction mixture was then stirred vigorously for 12 h at 22 °C and poured into H$_2$O (50 mL). The aqueous layer was extracted with EtOAc (3 x 25 mL) and the combined organics were dried over Na$_2$SO$_4$. 
Concentrated gave an oil that was purified by column chromatography eluting with EtOAc/hexanes (1:9) followed by EtOAc/hexanes (1:4) to afford **114** as an orange foam (220 mg, 71%). mp 87-88 °C; $^1$H NMR (CDCl$_3$, 400.13 MHz) $\delta$ 7.77-7.74 (m, 2H), 7.65-7.62 (m, 2H), 6.01-5.94 (m, 1H), 5.35 (d, $J$ = 14.7 Hz, 1H), 5.23 (bs, 1H), 3.62 (t, $J$ = 7.1 Hz, 2H), 3.08 (bs, 2H), 2.49-0.82 (m, 50H), 0.63 (s, 3H); $^{13}$C NMR (CDCl$_3$, 100.62 MHz) $\delta$ 168.04, 144.37, 133.74, 131.90, 123.11, 122.98, 121.16, 116.26, 109.05, 108.61, 86.05, 82.61, 80.11, 79.16, 69.12, 56.48, 55.79, 49.88, 42.15, 42.14, 39.54, 38.15, 36.40, 35.74, 35.30, 35.21, 34.84, 31.66, 29.55, 28.27, 28.00, 26.65, 24.07, 20.80, 19.48, 19.29, 19.20, 18.79, 18.67, 18.42, 18.30, 11.71; IR (film): $\nu_{\text{max}}$ 2937.2, 1714.2, 1396.0, 1170.9 cm$^{-1}$; MS (ESI$^+$) calcd. for C$_{47}$H$_{62}$N$_2$O$_4$ (MH$^+$) 719.48, found 719.42. Anal. calcd. for C$_{47}$H$_{62}$N$_2$O$_4$: C, 78.51; H, 8.69. Found: C, 78.70; H, 8.52.
To a degassed Schlenk flask was charged 116 (220 mg, 0.3 mmol), 114 (215 mg, 0.31 mmol), DIEA (0.16 mL, 1.0 mmol) and Pd(PPh$_3$)$_2$Cl$_2$ (11 mg, 5 mol%) in THF (15 mL). The mixture was then degassed under nitrogen and CuI (6 mg, 10 mol%) was added. The reaction mixture was degassed once more and stirred vigorously for 12 h at 22 °C. The mixture was then poured into H$_2$O (50 mL) and extracted with EIOAc (3 x 25 mL). The combined organics were dried over Na$_2$SO$_4$ and concentrated to give an oil that was purified by column chromatography eluting with EIOAc/hexanes (1:18) followed by EIOAc/hexanes (3:7) to afford 117 as a tan oil (230 mg, 57%).

$^1$H NMR (CDCl$_3$, 400.13 MHz)
7.82-7.80 (m, 2H), 7.53 (d, J = 7.5 Hz, 2H), 7.69-7.67 (m, 2H), 7.56 (d, J = 7.3 Hz, 2H), 7.37 (t, J = 7.4 Hz, 2H), 7.28 (t, J = 7.2 Hz, 2H), 6.06-6.00 (m, 2H), 5.42 (d, J = 16.0 Hz, 2H), 5.29-5.28 (m, 2H), 4.45-4.43 (m, 1H), 4.35 (d J = 6.4 Hz, 2H), 4.18-4.12 (m, 1H), 3.67 (t, J = 6.9 Hz, 2H), 2.52-0.88 (m, 81H), 0.64 (s, 6H); 

$^{13}$C NMR (CDCl$_3$, 75.48 MHz) δ 203.24, 168.29, 155.51, 144.52, 144.50, 144.42, 143.96, 143.81, 141.24, 140.25, 133.90, 131.96, 128.57, 127.58, 126.95, 124.99, 123.29, 123.16, 121.84, 121.32, 111.90, 86.65, 80.09, 79.99, 79.37, 78.21, 77.20, 56.59, 55.91, 49.99, 47.23, 42.30, 42.27, 41.21, 40.86, 39.65, 39.53, 39.45, 38.27, 37.78, 36.75, 36.65, 36.57, 36.51, 36.45, 35.88, 35.43, 35.36, 35.27, 34.88, 31.76, 30.57, 29.71, 29.65, 29.32, 28.40, 28.13, 27.39, 26.80, 26.65, 24.64, 24.20, 23.96, 22.65, 21.02, 20.98, 20.90, 19.92, 19.84, 19.72, 19.63, 19.34, 19.29, 19.06, 18.50, 18.40, 18.35; IR (film): $\nu$ max 2937.0, 1713.6, 1365.7 cm$^{-1}$; HRMS (TOF ESI$^+$) m/z 1316.8374 (M + Na$^+$, C$_{87}$H$_{111}$N$_3$O$_6$ requires 1316.8371).

Compound 117 (64 mg, 0.05 mmol) was combined with piperidine (0.37 mL) in DMF (1.5 mL). The resulting solution was stirred at 22 °C for 20 min and the solvent was removed in vacuo to give the crude amine as a film. This amine was combined with 118 (20 mg, 0.05 mmol) and DIEA (20 uL, 0.98 mmol) in CH₂Cl₂ and the reaction mixture was stirred at 22 °C for 8 h. The reaction was concentrated and residue was purified by column chromatography eluting with Acetone/CH₂Cl₂ (1:4) followed by MeOH/CH₂Cl₂ (1:19) to give the product as a yellow oil (59 mg, 86%). ¹H NMR (CDCl₃, 400.13 MHz) δ 7.97 (s, 1H), 7.71 (d, J =
7.4 Hz, 2H), 7.54 (d, J = 7.4 Hz, 2H), 7.44-7.33 (m, 4H), 7.24 (t, J = 7.5 Hz, 2H),
7.18 (d, J = 6.9 Hz, 1H), 6.06-6.05 (m, 2H), 5.80-5.76 (m, 1H), 5.67-5.64 (m, 1H),
5.41 (d, J = 15.7 Hz, 2H), 5.29-5.25 (m, 2H), 4.30 (d, J = 7.0 Hz, 2H), 4.16 (t, J =
7.0 Hz, 1H), 3.66-0.89 (m, 87H), 0.63 (s, 6H); $^{13}$C NMR (CDCl$_3$, 75.48 MHz) δ
169.92, 162.46, 156.52, 144.42, 143.96, 143.88, 141.19, 140.07, 132.05, 131.95,
130.49, 128.50, 128.37, 127.58, 127.56, 126.95, 125.05, 121.89, 121.22, 119.86,
109.19, 92.36, 86.61, 80.61, 77.20, 56.56, 55.91, 49.99, 49.95, 49.69, 47.15,
42.55, 42.27, 42.26, 39.63, 39.12, 37.75, 37.10, 36.61, 36.44, 36.40, 36.00,
35.33, 34.97, 33.07, 31.78, 31.74, 31.35, 30.84, 29.68, 29.61, 29.19, 28.99,
18.47, 18.38, 11.80; IR (film): $\nu_{\text{max}}$ 2935.0, 1664.2, 1540.9, 1255.9 cm$^{-1}$; HRMS
(TOF ESI$^+$) m/z 1365.8951 (MH$^+$, $C_{90}H_{117}N_4O_7$ requires 1365.8922).

2,5-dioxopyrrolidin-1-ylN-[(9H-fluoren-9-ylmethoxy)carbonyl]-b-alaninate
(118)

To a suspended mixture of Fmoc-β-ala-OH (5.0g, 16 mmol) and N-
hydroxysuccinimide (2.4 g, 21 mmol) in CH$_2$Cl$_2$ (100 mL) was added EDC
methiodide (6.2 g, 21 mmol). The reaction mixture was stirred at 22 ºC for 12 h
and washed with aqueous 1M H$_3$PO$_4$ (50 mL), H$_2$O (50 mL) and the combined
organics were dried over Na$_2$SO$_4$. Concentration gave an oil that was purified by
column chromatography eluting with acetone/CH$_2$Cl$_2$ (1:4) to give the product as a white solid, which was used without further purification.

tert-butyl[(3S,10R,13R,17S)-17-((1S,4E,12E,16R)-16-((3S,10R,13R,17R)-10,13-dimethyl-3-[[6-((3aR,4R,6aS)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoyl]amino]hexanoyl]amino)propanoyl]amino]-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-1-methylheptadeca-4,12-diene-6,10-diyn-1-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl][3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]carbamate (120)

Compound 119 (43 mg, 0.03 mmol) was combined with piperidine (0.37 mL) in DMF (1.5 mL). The resulting solution was stirred at 22 °C for 20 min and the solvent was removed in vacuo to give the crude amine as a film. This amine was combined with Biotinamidohexanoic acid N-hydroxysuccinimide ester (14 mg, 0.03 mmol) and DIEA (10 uL, 0.6 mmol) in DMF and the reaction mixture was stirred at 22 °C for 8 h. The reaction was concentrated and residue was
purified by column chromatography eluting with MeOH/CH₂Cl₂ (1:9) to give the product as an off-white gel (38 mg, 85%). ¹H NMR (CDCl₃/MeOD 3:1, 300.13 MHz) δ 7.65-7.55 (m, 2H), 7.21-7.12 (m, 2H), 5.68-5.61 (m, 2H), 5.00 (d, J = 15.8 Hz, 2H), 4.94 (bs, 2H), 4.11-4.07 (m, 1H), 3.91-3.87 (m, 1H), 3.11 (t, J = 6.9 Hz, 2H), 2.93-2.90 (m, 16H), 2.58-0.70 (m, 90H), 0.51 (s, 6H); ¹³C NMR (CDCl₃/MeOD 3:1, 75.48 MHz) δ 173.88, 173.26, 171.58, 163.90, 162.74, 142.80, 139.42, 131.29, 131.15, 128.30, 127.99, 126.32, 120.19, 118.87, 85.74, 61.37, 59.58, 56.58, 56.07, 55.05, 61.37, 59.58, 56.07, 55.05, 49.53, 46.27, 43.76, 42.15, 41.64, 39.42, 38.40, 35.86, 35.65, 35.24, 35.02, 34.81, 34.71, 34.62, 33.89, 33.56, 33.12, 32.01, 31.97, 31.88, 31.65, 32.38, 30.37, 28.25, 27.84, 27.61, 27.54, 27.50, 27.46, 26.87, 26.53, 26.21, 26.01, 27.42, 25.89, 25.80, 25.68, 24.98, 24.87, 24.62, 24.48, 24.36, 23.82, 23.62, 23.56, 21.94, 21.44, 17.47; IR (film): ν_max 2941.2, 1706.5, 1620.4, 1408.5 cm⁻¹; HRMS (TOF ESI⁺) m/z 1482.9852 (MH⁺, C₉₁H₁₃₂N₇O₈S requires 1482.9858).
2-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-((3-{(3-{((3S,8R)-17-((1R,4E,12E,15S)-15-{(3R,8S)-10,13-dimethyl-3-{[6-((5-{(3aR,4R,6aS)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl]pentanoyl}amino)hexanoyl]amino}propanoyl]amino}-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-1-methylhexadeca-4,12-diene-6,10-diyn-1-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl]amino)propyl]amino]-3-oxopropyl}amino)-3-oxopropyl]amino)carbonyl)benzoic acid (80)

To a stirring solution of 120 (38 mg, 0.03 mmol) in THF:EtOH (1:5, 10 mL) was added hydrazine (7 uL, 0.2 mmol). The reaction was stirred at 50 °C for 6 h and filtered. The filtrate was concentrated to give the crude amine, which was taken on without further purification. This di-amine was combined with 90 (22 mg, 0.04 mmol) and DIEA (22 uL, 0.13 mmol) in CH₂Cl₂ and the cloudy reaction mixture was stirred at 22 °C for 8 h. The reaction was concentrated and the residue was combined with piperidine (0.37 mL) in DMF (1.5 mL). The resulting solution was stirred at 22 °C for 20 min and the solvent was removed in vacuo to give the crude diamine as a white film, which was taken on without further purification. This diamine was combined with Oregon Green OSu (10 mg, 0.02 mol) and DIEA (0.1 mL, 5.7 mmol) in DMF (5 mL). The reaction was stirred at 22
°C for 12 h and the solvent was removed in vacuo. The residue was suspended in trifluoroacetic acid / “wet” CH₂Cl₂ (3:47, 10 mL) and stirred at 22 °C for 3 h. The reaction mixture was concentrated and the residue was purified via preparative reverse-phase HPLC (gradient: 10% CH₃CN, 80% H₂O, and 0.1% TFA to 99% CH₃CN, 1% H₂O, and 0.1% TFA over 20 min to afford 80 as an orange solid (6 mg, 13%). ¹H NMR (CDCl₃/MeOD 3:1, 299.87 MHz) δ 7.97-7.87 (m, 1H), 7.42 (d, J = 8.0 Hz, 2H), 7.05-6.98 (m, 2H), 6.58-6.50 (m, 2H), 6.10-6.09 (m, 2H), 5.38 (d, J = 15.9 Hz, 2H), 4.98 (bs, 2H), 4.12-4.10 (m, 1H), 3.92-3.88 (m, 1H), 3.65-3.51 (m, 3H), 3.49-3.10 (m, 30H), 2.82-2.57 (m, 26H), 2.35-0.85 (m, 68H), 0.55 (s, 6H); IR (film): ν max 2930.4, 1713.6, 1370.2 cm⁻¹; HRMS (TOF ESI⁺) m/z 1777.0324 (MH⁺, C₁₁₇H₁₄₁F₄N₈O₁₆ requires 1777.0310).

Analytical reverse-phase HPLC of compound 80 after purification (retention time = 12.15 min).
4.9.3 Cell Culture

Jurkat lymphocytes (human acute T-cell leukemia, ATCC #TIB-152) were maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with fetal bovine serum (FBS, 10%), penicillin (100 units / mL), and streptomycin (100 µg / mL). RPMI media used for cell culture and wash steps contained antibiotics and FBS unless otherwise noted.

4.9.4 Concentration Normalization of 77

Compound 77 was used to generate a 6-point calibration curve (concentration ranges 0 µM to 40 µM). Absorbances were obtained with a Cary Scan UV-Vis spectrophotometer in phosphate-buffered saline (PBS)/1% DMSO. Absorbance values at 507 nm were used to generate a calibration curve; whereby, the slope of the line was used to normalize the concentration for all dimers at 507 nm. Subsequently, all dimer compounds were diluted to 1 mM in PBS/1% DMSO for flow cytometry analysis, fluorescence emission spectra and analysis by confocal microscopy.
Table 4.1 Absorbance Measurements of Compound 77 for construction of a standard curve.

<table>
<thead>
<tr>
<th>Concentration 77 µM (x)</th>
<th>Absorbance 507 (y)</th>
<th>Absorbance (-PBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.135</td>
<td>0.000</td>
</tr>
<tr>
<td>2.5</td>
<td>0.174</td>
<td>0.039</td>
</tr>
<tr>
<td>5.0</td>
<td>0.210</td>
<td>0.075</td>
</tr>
<tr>
<td>10.0</td>
<td>0.278</td>
<td>0.143</td>
</tr>
<tr>
<td>20.0</td>
<td>0.394</td>
<td>0.259</td>
</tr>
<tr>
<td>40.0</td>
<td>0.584</td>
<td>0.449</td>
</tr>
</tbody>
</table>

**Figure 4.16.** Calibration curve used to normalize the concentration of dimers based on the absorbance of 77.
4.8.5 Fluorescence Emission

Emission spectra for each dimer was obtained with a PTi MD-5020 fluorimeter in phosphate-buffered saline (PBS)/1% DMSO at pH = 7.4. Normalized dimers were diluted to 200 nM, excited at 500 nm and the integrated fluorescence emission from 510 nm to 650 nm was quantified (a 1 cm path length quartz cuvette was used).

4.9.6 Analysis by Flow Cytometry

Jurkat lymphocytes (5 x 10^4 cells/mL) in RPMI media (1 mL) were treated with 10 µM of each dimer for 1 and 4 hours. Jurkat cells were incubated with the dimer compounds at 37 °C for 1 hour, a 500 µL aliquot was removed and the remainder of the cells were incubated for a total of 4 hours. Upon completion of the 1 hour or 4 hour timepoints, the cells were washed with RPMI (0.4 mL) to remove excess compound and the cells were maintained on ice. Just prior to flow cytometry analysis, samples were suspended in cold RPMI (0.5 mL). Analyses were performed with a Beckman-Coulter XL-MCL bench-top flow cytometer. Forward-scatter (FS) and side-scatter (SSC) dot plots gave the cellular physical properties of size and granularity for gating of live cells. After gating, 10,000 cells were counted. Oregon green was excited at 488 nm with a 15 mW air-cooled argon-ion laser, the emission was split with a 550 nm dichroic and filtered through a 510 nm long pass filter and 525 nm band pass filter. The
PMT voltage for this instrument was set to 806 for detection. Samples were prepared in duplicate.

4.9.7 Imaging by Confocal Microscopy

Jurkat lymphocytes (5 x 10^4 cells/mL) in RPMI media (1 mL) were treated with 10 μM of each dimer for 1 and 4 hours. Jurkat cells were incubated with the dimer compounds at 37 °C for 1 hour, a 500 μL aliquot was removed and the remainder of the cells were incubated for a total of 4 hours. Upon completion of the 1 or 4 hour timepoints, the cells were washed with RPMI (0.4 mL) to remove excess compound and suspended in 200 μL RPMI. For imaging, 30 μL of the prepared cells were pipetted onto a microscope slide mounted with a press-to-seal silicone isolater (to preserve cell viability during the course of the analysis) and covered with a glass coverslip. An inverted Zeiss LSM 5 Pascal confocal laser-scanning microscope fitted with a Plan Apochromat oil-immersion objective (63 X) was employed. Fluorophores were excited with the 488 spectral line of an argon laser and emitted photons were collected through a 505 nm LP filter.
4.10 References


VITA

Michael J. DeGrazia

Mike DeGrazia was born in Edison, NJ. He attended Middlesex County College where he received an A.S. in Biology and then received his B.S. in Chemistry from the University of the Sciences in Philadelphia. In 2001, he enrolled in the graduate program at the Pennsylvania State University where he pursued a Ph.D. degree in organic chemistry under the supervision of Professor Blake R. Peterson. Upon graduation Mike will be working for Finnegan, Henderson, Farabow, & Dunner, LLC as a technical advisor. Through compensation from the firm, he plans to attend Harvard Law School in Fall 2009.