

**Aryl Pyrazole Glucocorticoid Receptor Agonists: Probing Structure
Activity Relationships in the Expanded Ligand Binding Pocket**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
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DEDICATION

This work is dedicated to the family, friends, and mentors that have allowed this journey to be one of growth, resilience, and discovery. I would not be here without your unwavering support, guidance, and belief in me.

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ABSTRACT

Glucocorticoids (GCs) are a mainstay in modern medicine and one of the most frequently prescribed medications worldwide. They are used in the management of autoimmune-mediated conditions, organ transplantation, and some cancers. The transcriptional actions mediated by GCs include transrepression, proposed to be responsible for the anti-inflammatory activity, and transactivation, suggested to cause the unwanted side effects. A glucocorticoid receptor agonist (GRA) capable of dissociating the beneficial transrepression actions from the undesired transactivation activity is a highly sought-after target. In the presented studies, unique GRAs were designed, synthesized, biologically screened in assays using a rat insulinoma cell line, and evaluated through molecular docking experiments to probe structure activity relationships. The first study presents a series of aryl pyrazole glucocorticoid receptor agonists (APGRAs) with two modification sites. Compounds with an electron deficient aryl pyrazole moiety showed desirable activity with dissociated properties. Additionally, compounds 11aa and 11ab displayed anti-inflammatory efficacy comparable to dexamethasone (Dex) at 10 nM, with minimal transactivation activity and no reduction of insulin secretion in cultured rat 832/13 beta-cells. To build upon the conclusions of the first study, a set of novel, stereochemically pure, APGRAs were examined to recognize the stereochemical impacts of this scaffold. This study included an additional step to perform chiral separations via supercritical fluid chromatography, after which the stereochemically pure APGRAs were identified using two-dimensional nuclear magnetic resonance techniques. The isomers with an upward OH moiety was found to be approximately 70 percent more active on average than that with a downward OH. Finally, to explore the large space of the expanded ligand binding pocket in the human glucocorticoid receptor, the hydrocortisone (HC) scaffold was modified in two areas. The set of APGRAs derived from HC displayed potent activity, displaying greater than 95 percent the anti-inflammatory activity of Dex. Two compounds, 5 and 6, displayed exceptionally potent activity with up to 90 percent anti-inflammatory response at 0.1 nM while Dex only produced a 30 percent response at this concentration. This work

contributes to the understanding of the complex GC activities and reveals numerous compounds with potent anti-inflammatory effects and improved selectivity *in vitro*.

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INTRODUCTION

GLUCOCORTICOIDS

In modern medicine it is understood that medications come with certain risks known as side effects. All too often a medication is portrayed to solve some problem of ours only to expose a long warning of possible effects one could experience taking the medication. Due to today's rigorous prescreening of prescription drugs, many of these possible side effects are often labeled as acute and can be deemed worth the risk to solve a more severe problem. However, for millions of patients around the world needing glucocorticoid therapy, their medication is accompanied by a glaring list of side effects that in part includes: Cushing's disease, metabolic syndrome, osteoporosis, diabetes, skin and muscle atrophy, disordered fat metabolism, hypertension, mood disorders, osteoporosis, hyperglycemia, obesity, dyslipidemia, blurred vision, hair loss, and fatigue.¹⁻³ To treat one serious condition they must often risk developing another.

Glucocorticoids (GCs) are a class of lipophilic, hormone signaling compounds with anti-inflammatory and immunosuppressive properties. In 1948, patient X suffering from rheumatoid arthritis was injected with a synthetic version of an endogenous GC, hydrocortisone (cortisol), which is produced in the adrenal cortex.⁴ Within a matter of days, patient X was nearly asymptomatic.⁴ For this work, Philip S. Hench, Edward Kendall, and Tadeus Reichstein were awarded the Nobel Prize in Physiology or Medicine in 1950. The realm of synthetic GCs exploded and today it is estimated to be a yearly \$10 billion market that is continually growing.⁵ GCs are one of the most frequently prescribed medications worldwide as they treat a broad range of conditions such as asthma, rheumatoid arthritis, multiple sclerosis, allergic rhinitis, and ulcerative colitis.⁶ GCs are commonly used after organ transplantation as well, in which the anti-inflammatory actions aid the trauma of surgery, and the immunosuppressive properties combat organ rejection.⁴ GCs are even used in the treatment regimen of certain cancers such as leukemia, myeloma, and lymphoma.⁷⁻⁹

Given the serious conditions GCs are used to treat, for many, the complications of GC use are seemingly unavoidable. However, over the last several decades, scientists have

embarked on a search for a selective glucocorticoid receptor modulator (SGRM) that would ideally dissociate the undesired side effects from the therapeutic effects. The hunt to offer millions of patients improved treatment options has not yet been fruitful. While prospective SGRMs have been reported, to date none have been approved for clinical use.¹⁰⁻¹² This is in part due to the complex mechanistic actions of GCs, in which scientists are still working to uncover the full details of the intricate biological cascades prompted by GCs.

GLUCOCORTICOID MECHANISM

The glucocorticoid receptor (GR) is part of a diverse family of nuclear receptors. This ligand-initiated transcription factor exists in nearly all cell types and tissues.^{13, 14} Alternative splicing of the human GR generates two unique isoforms known as GR α and GR β , however, only the GR α isoform binds to GCs and initiates a majority of the activity observed with GC therapy.¹⁵ Since GR β doesn't bind to GCs, GR α is known primarily as the classical GR.¹⁶ Nuclear receptors, like the GR, are comprised of 3 major structural domains that are responsible for the activity of the receptor: a disordered N-terminal transactivation domain (NTD), a structured DNA binding domain (DBD), and a structured C-terminal ligand binding domain (LBD).¹⁷ The LBD folds into a three-layer helical domain forming a hydrophobic ligand binding pocket (LBP) at the base of the LBD.¹⁸

The unbound GR inhabits the cytoplasm of the cell as part of a multi-protein complex bound to heat shock proteins that keep the GR in a conformation associated with high affinity for ligand binding.¹⁹ Lipophilic GCs diffuse through the membrane of the cell and bind to the GR, inducing a conformational change that results in an activated complex.⁴ The activated GR is successively translocated to the nucleus where it regulates gene transcription through both activation and repression mechanisms.^{13, 20} Transactivation is in part caused by the dimerization of the activated GR which binds to specific glucocorticoid response elements (GREs). Transrepression results from interference of the monomer with several transcriptional factors involved in inflammation, such as nuclear factor κ B (NF- κ B) and activator protein 1 (AP-1) which later bind to DNA and regulate various

transcription factors including those that initiate immune cell response.^{13, 21-23} (Figure 1.1.1)

A mutant GR devoid of both the dimerization property and transcriptional activity (GR^{dim}) was found to retain both transrepressive and therapeutic activity. These findings gave rise to the current paradigm that transrepression is responsible for the therapeutic effects while transactivation provokes the undesired side effects.^{24, 25} It would be optimistic to assume the complex transcriptional behavior of the GR can be wholly explained by this simplistic dissociated model.²⁶ Yet, this is a start in the search for SGRMs and this philosophy set the stage for an expansive search of glucocorticoids that could distinguish between the transrepression and transactivation activity of the GR.²⁰ Although some structural modifications of GCs were found to result in improved therapeutic profiles, the intricacy of the GR transcriptional mechanisms makes it challenging to predict molecular changes that will provide an improved therapeutic profile. Expanding the scope of studied glucocorticoid receptor agonists (GRAs) with new compounds is essential to strengthen the understanding of properties associated with various structural components of the GRAs and ultimately lead to the development and discovery of potent SGRMs.

Among the SGRMs reported, deacylcortivazol (DAC) stands out as a favorable scaffold as it not only displayed selective performance but was also shown to be 40-fold more potent than dexamethasone (Dex), an effective GC that is commonly used in modern medicine.^{27, 28} However, the excitement surrounding DAC reduced when studies revealed DAC could not be used clinically due to its toxicity. While many GC alterations have been examined, DAC possesses one of the largest steroid modifications with a phenylpyrazole moiety attached to the A ring of the steroid framework. Interestingly, DAC was originally hypothesized to be a GR antagonist, and its potent agonistic activity confused scientists for decades as it was simply too large to fit within the understood steroidal LBP of the GR.²⁹ It wasn't until 2008 when Suino-Powell and coworkers²⁹ crystalized the DAC bound GR and revealed the aryl pyrazole moiety induced large structural changes that accommodated the oversized ligand, showcasing the vast flexibility of the GR LBP.

Even though DAC was only ~25% larger than Dex, it caused the LBP to nearly double in size, expanding from 540 Å³ to 1070 Å³. It was observed that DAC caused large

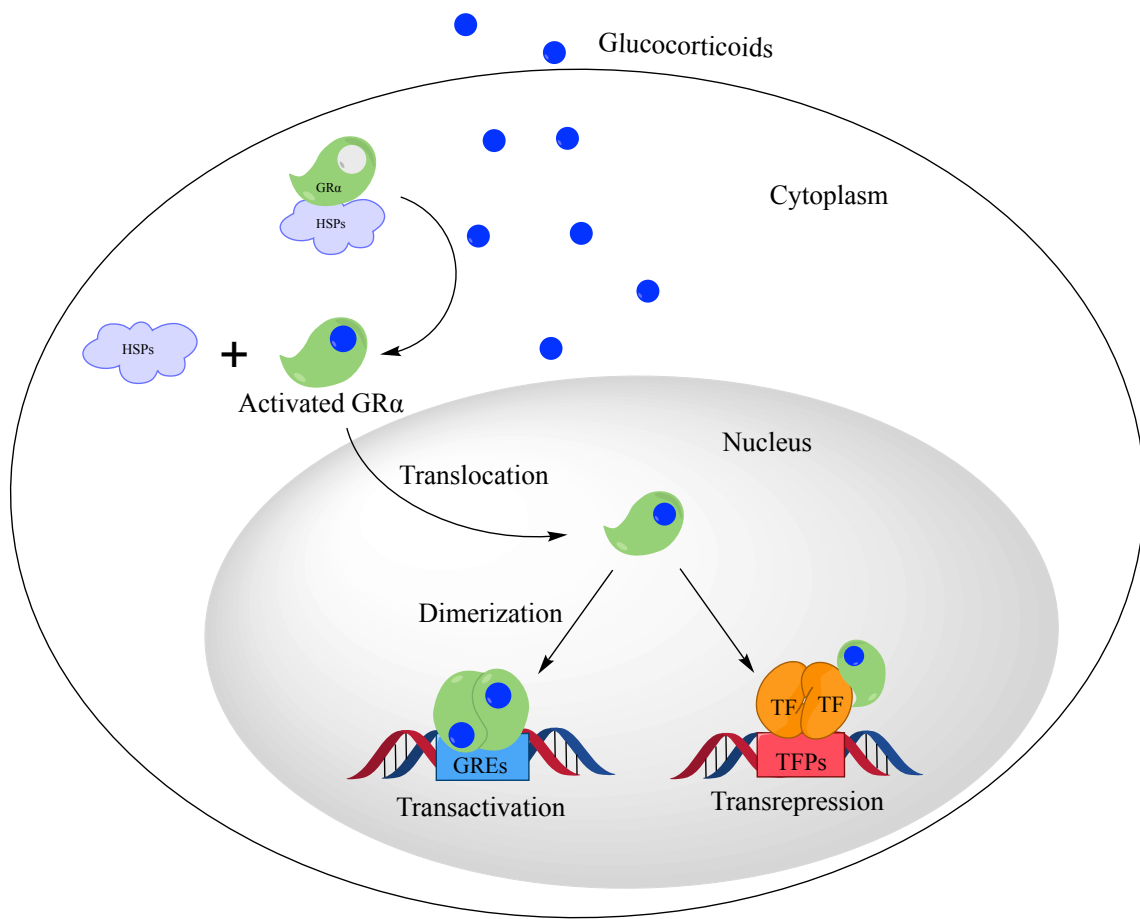


Figure 1.1.1 – Illustration of Glucocorticoid Receptor Mechanism

shifts in helices 3 and 5 in the GR LBD, exposing a hydrophobic channel with high affinity for the aryl pyrazole moiety.²⁹ Recently, the expansion of GR crystal structures in complex with a variety of ligands, has further displayed the flexibility of the GR LBP however, others pale in comparison to the vast expansion observed with DAC.³⁰ While the structure of DAC is among some of the largest GRAs reported, it still only occupies approximately 50% of the expanded LBP.²⁹ This leaves unlimited possibilities for creative exploration to reveal the true capabilities of this expanded ligand binding pocket in the search for SGRMs and a deeper understanding of the complex GR-GC mechanisms.

OUTLINE OF DISSERTATION

Herein, three studies have been designed to probe the capabilities of the expanded LBP of the human GR. Aryl pyrazole glucocorticoid receptor agonists (APGRAs) were designed using components thought to either increase potency and/or drive selective behavior. The APGRAs were then synthesized and examined using biological assays that measure transactivation and transrepression activities. Briefly, chapter one explores a truncated scaffold that mimics that A and B rings of steroidal GCs while including three different variants of the aryl pyrazole moiety observed with DAC. Further examination of top performing APGRAs was performed to understand the potential impact on β -cell function to study one of the severe side effects associated with GC use, steroid induced diabetes mellitus. All synthesized GRAs were further investigated for structure activity relationships (SARs) using molecular docking techniques. Chapter two expands on the work presented in the first study by investigating direct stereochemical impacts of this scaffold through a novel set of APGRAs. Chapter 3 probes deeper into the underutilized space of the expanded LBP with ligands that contain modifications to both the A and D ring of hydrocortisone, an endogenous GC. Biological and computational evaluation reveal trends that provide insight to selective GRA action.

**CHAPTER I: POTENT ANTI-INFLAMMATORY ARYL
PYRAZOLE-BASED GLUCOCORTICOID RECEPTOR AGONISTS
THAT DO NOT IMPAIR INSULIN SECRETION**

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Contributions:

AML and BJK contributed equally to the document. AML performed and analyzed molecular docking experiments using PDB ID: 3BQD, analyzed biological assays and calculated pharmacological values, produced most of the manuscript figures and all the tables, and assisted in synthesis, purification, characterization, and constructing supplementary information. BJK performed and analyzed molecular docking simulations using PDB ID: 5NFP, analyzed biological assays, compiled supplementary information, and performed synthesis, purification, and characterization. ARF analyzed biological assays and assisted synthesis, purification, characterization, and writing. CWP, LED, and RTS assisted with synthesis, purification, characterization, and reaction optimization. SJB completed *in vitro* testing of all compounds. JJK completed DFT calculations. KDV provided funding. SRC and JJC provided funding, designed the experiment, and helped write the document.

1.1 - ABSTRACT

Glucocorticoids (GCs) are widely used in medicine for their role in the treatment of autoimmune-mediated conditions, certain cancers, and organ transplantation. The transcriptional activities GCs elicit include transrepression, postulated to be responsible for the anti-inflammatory activity, and transactivation, proposed to underlie the undesirable side effects associated with long-term use. A GC analogue that could elicit only transrepression and beneficial transactivation properties would be of great medicinal value and is highly sought after. In this study, a series of 1-(4-substituted phenyl)pyrazole-based GC analogues were synthesized, biologically screened, and evaluated for SARs leading to the desired activity. Activity observed in compounds bearing an electron deficient aryl pyrazole moiety showed promise toward a dissociated steroid, displaying transrepression while having limited transactivation activity. In addition, compounds **11aa** and **11ab** were found to have anti-inflammatory efficacy comparable to dexamethasone at 10 nM, with minimal transactivation activity and no reduction of insulin secretion in cultured rat 832/13 beta-cells. (Figure 1.1.2)

1.2 - INTRODUCTION

Glucocorticoids (GCs) are steroid hormones synthesized endogenously in the adrenal glands, which operate by acting as ligands for the glucocorticoid receptor (GR). The GR is an intracellular receptor located in the cellular cytoplasm and bound to chaperone proteins in the non-ligand bound state. The GR is expressed by nearly all cell types, including pancreatic β -cells, and is the product of a single gene, NR3C1, that is post-translationally modified to produce functionally distinct subtypes of this transcriptional regulatory protein.³¹

GCs are a staple of modern medicine due to their anti-inflammatory and immunomodulatory effects. GC-based therapies have been successfully applied to treating a wide range of inflammatory conditions, autoimmune disorders, and cancers - in addition to aiding in organ and tissue transplantation.³²⁻³⁴ Despite the obvious beneficial properties of GCs, they pose a long list of serious side effects including Cushing's disease, muscle

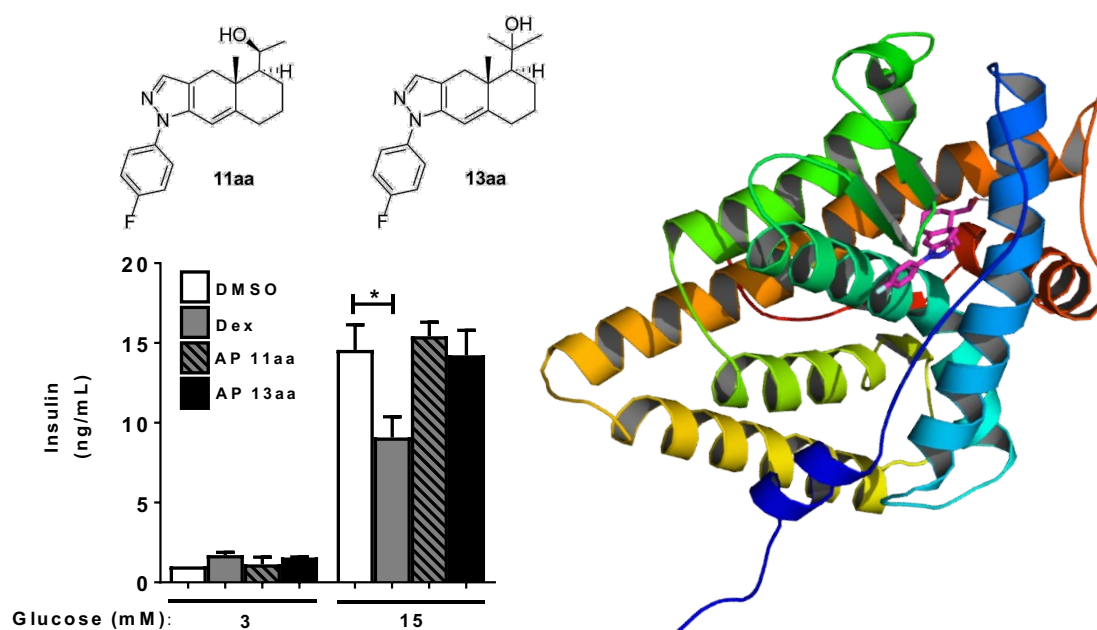


Figure 1.1.2 – Top candidates

Aryl pyrazole glucocorticoid receptor agonists show promise as anti-inflammatory agents with limited impact on β -cell function as compared to traditional glucocorticoid (GC) therapies (left). Synthesized compounds were computationally modeled within the human glucocorticoid receptor (3BQD) to observe possible structure-activity relationships (right; compound **11aa**).

atrophy, metabolic syndrome, osteoporosis, and diabetes.³⁵⁻³⁷ In recent years, reducing autoimmune-mediated inflammation in patients at risk for developing steroid induced diabetes mellitus (SIDM) has been of interest; however, current GC therapies are known to be diabetogenic by transcription-mediated mechanisms that modify the secretion of insulin from pancreatic β -cells, regulate β -cell proliferation, alter body composition, and promote insulin resistance.³⁸⁻⁴¹ The reduction in insulin sensitivity places extra stress on β -cells to produce increasing quantities of insulin, which increases the risk of β -cell failure.^{42,}

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The need for improved GC agonist compounds is due to side effects of current compounds upon chronic use, such as immunosuppressive agents for organ transplants. This long-term usage dramatically increases the risk for diabetes.^{44, 45} Diabetes is the seventh leading cause of death in the United States, affecting an estimated 34.2 million people or 10.5% of the U.S. population in 2018.^{46, 47} This disease also serves as a major contributor to heart disease and stroke and is the leading cause of kidney failure, non-traumatic lower limb amputations, and associated with cases of blindness in adults. The incidence of steroid induced diabetes mellitus (SIDM) among patients receiving traditional post-operative treatments for solid organ transplantation is relatively high in the case of lung (60%)⁴⁸, heart (29%)⁴⁹, liver (24%)⁵⁰, and kidney (17%)⁵¹ transplants. It should be noted that SIDM would likely be of great concern in pancreatic organ or islet transplantation, as these procedures manage post-operative care similarly.^{52, 53} These situations, as well as any condition requiring prolonged GC use, could greatly benefit from a synthetic GC analogue with a “dissociated” profile. (i.e., transrepression and desired transactivation activity with little or no unwanted effects). While it is difficult to summarize the cascade of intricate biological mechanisms initiated by GCs into a simplified model, the assumptions that transrepression elicits the desired anti-inflammatory effects while transactivation is the primary mode responsible for the undesired side effects still provides a framework to quickly assess the efficacy of GC analogues.^{5, 21, 54, 55}

Upon ligand-initiated GR activation, the GR-GC complex is translocated to the cellular nucleus where it can facilitate changes in gene transcription. Dimerization of the ligand activated GR binds to glucocorticoid-responsive elements (GREs) to regulate the

expression of target genes. GR action promotes both activation of genes which encode proteins that regulate inflammatory signals but can also directly repress the transcription of genes induced by pro-inflammatory cytokines, such as the cytokine IL-1 β .^{6, 56} The latter occurs through a monomeric GR-GC complex with transcription factors such as NF- κ B, or AP-1.

Current synthetic GCs suppress inflammation induced by IL-1 β , but also reduce insulin sensitivity and modulate insulin secretion in β -cells.^{8, 57-60} With the recently renewed interest in development of glucocorticoid receptor agonists⁶¹, this study provides novel insights into non-steroidal aryl pyrazole-based glucocorticoid receptor agonists (APGRAs) by investigating known⁶² and new molecules with more in-depth biochemical assays. These compounds were subjected to *in vitro* assays evaluating both transactivation and transrepression, and candidates displaying the most potent and/or selective properties were further evaluated to determine how insulin secretion was affected in cultured β -cells. Additionally, computational docking experiments were performed to explore whether the molecules bound in the normal steroidal site or the deacylcortivazol (DAC) expanded GC binding pocket, to assess which region of the expanded pocket the molecules accessed, and to determine structure-activity relationships (SARs) between the APGRA analogues and the GR.

1.3 - SYNTHETIC METHODS

The total synthesis of all APGRA analogues tested in this study were prepared by a previously described synthesis published by Merck scientists,⁶² with modifications made to the published method (Figure 1.3.1). A change of reagent is employed in the conversion of secondary alcohol **11** to ketone **12**, using a standard PCC oxidation in place of the costly oxidation with TPAP using NMO as a sacrificial oxidant. The molecules investigated by Ali and coworkers, focused on alterations to the R² moiety while leaving the 4-fluorophenyl R¹ group as a constant.⁶² Modifying the identity of R¹ was achieved through the addition of the appropriate phenylhydrazine reagent to β -ketoaldehyde **6**, thereby installing the desired R¹-arylpyrazole moiety. Additionally, to further enhance the scope of

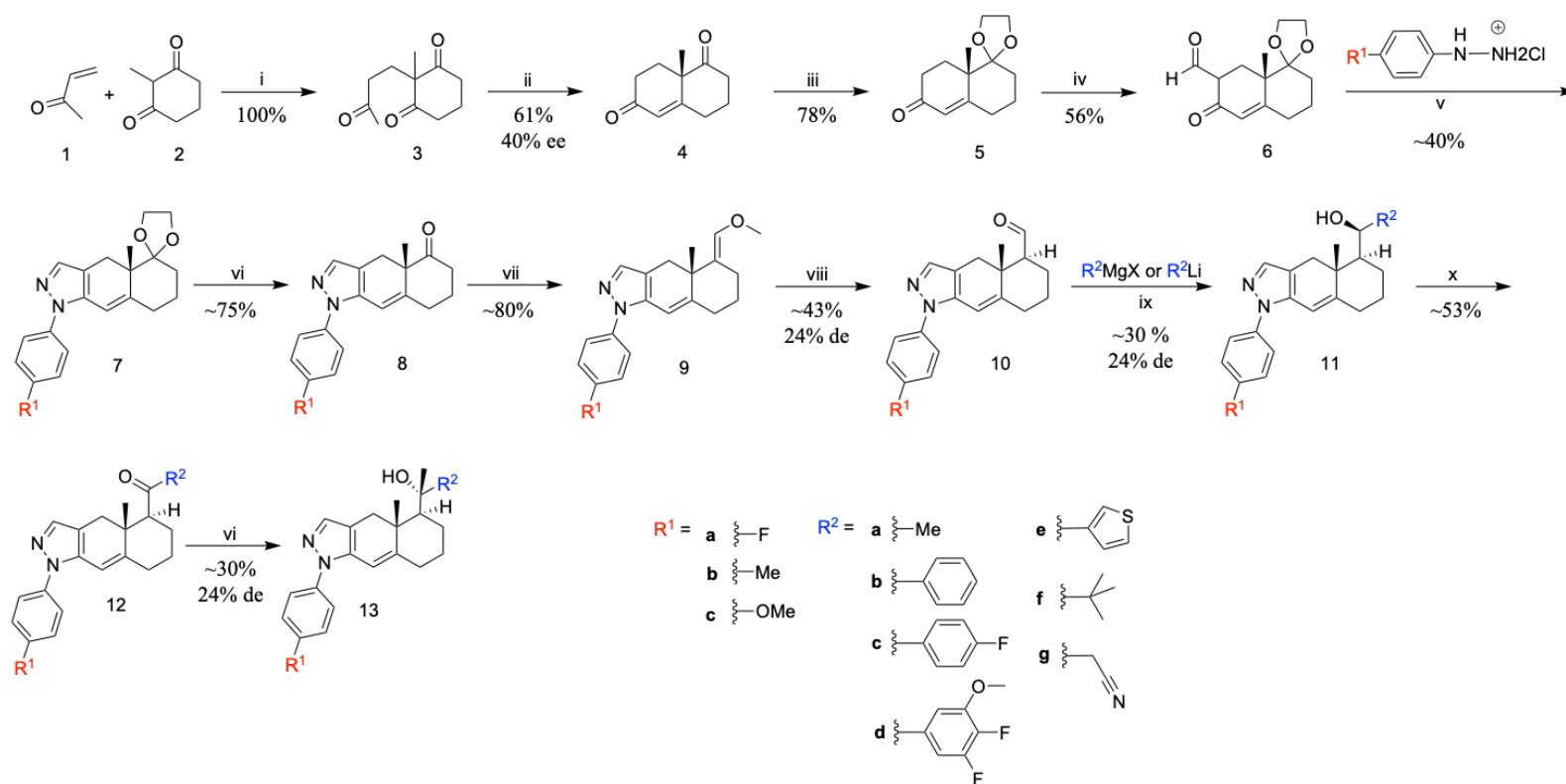


Figure 1.3.1 – Synthetic route

Reagents and conditions: (i) AcOH, H₂O, 75 °C, 1 h (ii) L-proline, DMF, rt, 120 h (iii) p-TsOH, 4Å mol sieves, ethylene glycol, rt, 30 min (iv) ethyl formate, NaH, toluene, 0 °C, 3 h (v) NaOAc, AcOH, rt, 3 h (vi) 6N HCl, THF, reflux, 3.5 h (vii) Ph₃PCH₂OCH₃Cl, KHMDS, THF, rt, 24 h (viii) 4N HCl, rt, 36 h (ix) THF, -78 °C, 1 h (x) PCC, 4Å mol sieves, DCM, rt, 6 h (xi) MeLi, THF, -78 °C, 1 h.

functionalities examined in our biological evaluation, a previously uninvestigated cyanomethylene unit was included as an R² analogue in the series.

Briefly, the total synthesis of the steroid core begins with a Robinson annulation of compounds **1** and **2** to form intermediate trione **3**, which upon asymmetric intramolecular cyclization gives Wieland-Miescher ketone **4**. Selective ethylene glycol protection of compound **4** leaves the α,β -unsaturated ketone available for enolate formation and subsequent addition to ethyl formate, giving β -ketoaldehyde **6**. Addition of the appropriate phenylhydrazine to **6** completes the core structure of the APGRAs as compounds **7a**, **7b**, and **7c**. The acid-catalyzed deprotection of acetal **7** provided synthon **8**, which was formylated via Wittig reaction to afford aldehyde **10**, which provided an intermediate that could be diversified to provide the analogues presented in this study. Separation of starting material from the product of the one-pot Wittig/hydrolysis sequence from ketone **8** to aldehyde **10** was futile and extra care was taken to obtain aldehyde **10** in high purity. This was done by isolating intermediate enol-ether **9**, which after purification was subjected to hydrolysis conditions to afford compound **10** in sufficient purity to proceed with the next reaction.

Insertion of the R² group into the GC scaffold was accomplished through generation of the appropriate alkyl lithium or Grignard reagent followed by addition of aldehyde **10**, resulting in a series of 2° alcohols (**11**) that were subjected to biological testing. Intermediate ketone **12** was obtained through a PCC oxidation of **11**, and after purification was treated with MeLi to afford a series of 3° alcohols (**13**), which were also assayed. Ali and coworkers found this reaction sequence led to stereoinduction in all subsequent transformations yielding a new chiral center at the formation of **4**.⁶² Based on these observations, the conformation shown for **11** and **13** is predicted to be the major product with a ~24% de (Figure 1.3.1).

1.4 – *IN VITRO* ASSAYS

It is well established that mouse, rat, and human beta cells secrete large amounts of insulin in response to glucose and reduce insulin secretion in response to GC exposure.^{56, 63-}

⁶⁵ Since there is excellent agreement across species, a model rat isolated cell line, 832/13,

was used in this study as it has been shown to recapitulate the key responses of human cell lines well.⁶⁶ Culture of 832/13 cells and measurements of insulin secretion using an insulin ELISA kit as previously described.⁶⁶ Six-point dose-response curves were used to generate EC₅₀ and saturating concentrations of each steroid compound. For GSIS, saturating concentrations of each commercial steroid or *N*-aryl pyrazole based steroid were used as indicated in the figure legends.

832/13 cells were grown in 24-well plates to 50% confluence and then transiently transfected with 25 ng of indicated plasmid per well using TransFectin Lipid Reagent (Bio-Rad) according to the manufacturer's instructions. Cell lysis, luciferase assays, and normalization to total protein content were carried out as described previously.⁵⁸ Briefly, transrepression is measured using a previously reported CCL2-promoter luciferase plasmid construct that is activated with known pro-inflammatory signal, IL-1 β , followed by GR agonism.^{53, 67} Ligand-activated transactivation is measured using a previously reported 3XGRE-promoter luciferase plasmid construct in which increases in luciferase activity are measured in response to GR agonists.^{57, 68}

1.5 - MOLECULAR MODELING

1.5.1 - Molecular Docking

Ligand-receptor interactions were modeled between the molecules subjected to *in vitro* testing and the extended pocket of the GR ligand-binding domain (LBD). (Figure 1.5.1) The crystal structure of the GR LBD bound to DAC, a potent APGRA₂, and the fourth LXXLL motif of steroid receptor coactivator 1 (SRC1-4; PDB ID: 3BQD) was used as the model for molecular docking calculations. The human GR in complex with budesonide (PDB ID: 5NFP) was used in molecular docking studies but was not chosen as the preferred model as the results corroborated those of the quantum chemical experiments.⁶¹ The 3BQD crystal structure was chosen as the best template as consistent trends in binding were observed, which validated previous work that assumed the enlarged ligand binding pocket

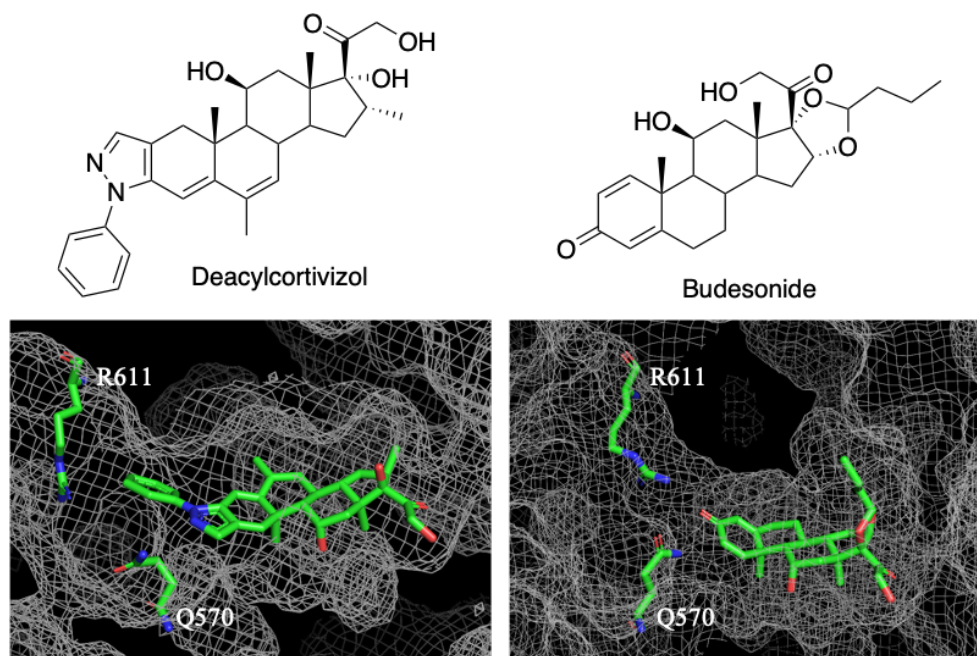


Figure 1.5.1 – Expanded ligand binding pocket

Expanded ligand binding pocket of APGRA₂ deacylcortivizol, bound to GR (left) compared to ligand binding pocket of steroidal GC, budesonide, bound to GR (right). Residues R611 and Q570 depicted to exhibit the conformational changes that lead to an expanded ligand binding pocket.

caused by the phenylpyrazole moiety would also house the smaller APGRA analogues (Figure 1.5.1).⁶⁹ The GR LBD was prepared for docking studies by removing the bound ligand and excess water molecules. Ligands were optimized in Chem3D (PerkinElmer) prior to docking, which was performed between a rigid 3BQD GR and flexible ligand using AutoDock Vina (The Scripps Research Institute).⁷⁰ The resulting docking conformations were viewed with PyMol (Schrödinger).⁷¹ Ligand-residue interactions were further analyzed with LigPlot+ (European Molecular Biology Laboratory).⁷² Relative binding affinities (AutoDock Vina) can be found in the appendix (section 1.9.6).

1.5.2 - DFT Calculations

The nature of the GC-GR interactions was further examined by means of density functional theory (DFT). The receptor pocket was modeled by including all main group atoms within 6 Å of the co-crystallized ligand (budesonide) with solvent molecules removed. All valences were capped with appropriate atoms based on geometry of the included fragments. Constrained geometry optimizations were performed on docking output orientations by relaxing all hydrogen atoms and the full glucocorticoid molecule within the pocket with atoms of the pocket frozen during convergence. The BP86 density functional was employed along with Grimme's D3 dispersion correction with the Becke-Johnson damping function (abbreviated as -D3(BJ)) and the def2-SVP basis set.⁷³⁻⁷⁷ The resolution of identity (RI) approximation was used for rapid integral evaluation.⁷⁸ Single-point calculations were performed on the BP86-D3(BJ)/def2-SVP optimized geometries at the B3LYP- D3(BJ)/def2-TZVPP level for higher accuracy.⁷⁹⁻⁸¹ All DFT computations were performed with the TURBOMOLE 7.2 quantum chemical software package.⁸² Additional information can be found in the appendix (section 1.9.7)

1.6 - RESULTS AND DISCUSSION

1.6.1 – Biological Evaluation

Previous work by Ali and coworkers reported the synthesis of APGRAs containing only F substitution on the aryl moiety in which compounds **11a(a-f)**, **13aa**, **13ac**, and **13ae** were originally reported as compounds **26**, **10**, **11**, **18**, **23**, **29**, **28**, **21**, and **25** respectively.⁶² Another limitation of this work was that the bioassays used, did not directly measure the ability of the compounds to impact transactivation and transrepression at the transcriptional level and instead relied on measures that could have impacted either pre-, during, or post-transcription. Therefore, this work aimed to investigate this promising class of anti-inflammatory compounds by expanding the library of APGRAs and using bioassays that directly measured the impact of these molecules on transcriptional outcomes. Although the difficulty in translating *in vitro* activity to *in vivo* success has been documented recently, this further validates the need to thoroughly investigate GR mechanisms with comprehensive biological assays to find avenues that better predict *in vivo* activity.⁸³⁻⁸⁵ Directly studying the ability of the ligand activated GR to dimerize and bind to the GRE allows for a more encompassing view on transactivation potential compared to traditional reporter gene assays such as tyrosine amino transferase (TAT) or glutamine synthase (GS) assay. The ability of the APGRAs to both reduce IL-1 β induced inflammation (CCL2 assay - transrepression) and enhance transcription of a synthetic GRE-containing promoter luciferase-reporter gene (3xGRE assay - transactivation) were tested and benchmarked against both dexamethasone (Dex) and hydrocortisone (HC).

The pIC₅₀ for transrepression by Dex was 8.2, and this compound also showed the highest activity in this assay. Transactivation occurred at nearly the same concentration (pEC₅₀ = 8.7) with Dex as well. Hydrocortisone showed 87.8% of the transrepression of Dex, and the pIC₅₀ for this compound was slightly lower as well (7.6). Interestingly, HC displayed a ~100 fold concentration difference between transrepression and transactivation activities, with the latter having a pEC₅₀ of 5.6; although this compounds transactivation potential was still 96.8% of Dex.

Two moieties on the APGRA scaffold were chosen to be manipulated for the purpose of identifying a ligand that displayed favorable ligand properties (Figure 1.3.1). Three different sets of APGRA derivatives were synthesized that varied in the substituent at R¹ leading to series of fluorophenyl-, tolyl-, and anisoyl-pyrazoles. Molecules in the fluorophenyl- series have been reported, and these molecules showed desirable properties.^{62, 86} The tolyl- and anisoyl-APGRAs have not been previously reported. The fluorophenyl- analogues showed a much-improved transrepressive profile over the tolyl- and anisoyl- series with the majority of the compounds having a pIC₅₀ > 8, and are therefore more likely to have useful anti-inflammatory activity. However, the tolyl- and anisoyl- analogues elicited lower transcription of the GRE-promoter luciferase gene fusion, indicating that they could be less likely to promote side effects (assuming that transactivation is the most likely cause of side effects).

Generally, compounds in series **13** bearing sidechains with 3° alcohols displayed better anti-inflammatory activity than those in series **11** with 2° alcohols in the sidechain, and the maximum activity for the 2° alcohols also often occurred at one to two orders of magnitude higher concentrations. Unfortunately, the more active compounds also tended to elicit more activation of the GRE as well (Table 1.6.1). Therefore, the slightly higher anti-inflammatory properties of the series **13** compounds must be weighed against benefits from the series **11** compounds that were more successful in differentiating transrepression from transactivation. If one assumes that the bulkiest substituent on the sidechain will be preferentially positioned away from the bridgehead methyl between the two rings, then the changes in activity may be more impacted by an apparent change in the stereochemistry of the alcohol that would be displayed on the top face for the 2° and the bottom face of the 3° alcohols, respectively, then by the addition of the methyl substituent (see the structures in Figure 1.3.1).

For each of the R¹ analogues, five different groups at the R² position on the APGRA scaffold were also explored (Figure 1.3.1). Two further R²s (cyanomethylene- and *t*-butyl-) were only prepared in the fluorophenyl- series, as these analogues lacked the ability to favor one transcription mode over the other and inclusion of these functional groups led to unexpected synthetic difficulties. The suite of molecules showed a broad variation in the

Table 1.6.1 - Biological analysis of transrepression and transactivation profiles.

Compd	R ¹	R ²	CCL2 ^{a,b}				3xGRE ^{a,c}			
			pIC ₅₀	E _{max}	% Dex ^d	% HC ^d	pEC ₅₀	E _{max}	% Dex ^d	% HC ^d
HC			7.6	75.0	87.8	100.0	5.6	10.5	96.8	100.0
Dex			8.2	85.5	100.0	113.8	8.7	10.9	100.0	103.3
11aa	F	Me	8.2	74.8	87.5	99.6	7.2	1.8	16.5	17.0
13aa	F	Me	7.4	67.6	79.1	90.1	7.2	9.3	85.2	88.0
11ab	F	Ph	7.4	67.3	78.7	89.6	7.0	2.8	26.1	27.0
13ab	F	Ph	8.1	80.8	94.6	107.7	8.4	6.6	60.5	62.5
11ac	F	4F-Ph	8.0	63.6	74.5	84.8	7.1	4.9	44.8	46.2
13ac	F	4F-Ph	8.3	73.4	85.9	97.8	7.8	9.0	82.5	85.2
11ad	F	2,3F-4Ani	7.8	58.1	68.0	77.4	7.2	13.3	122.8	126.8
13ad	F	2,3F-4Ani	8.7	74.7	87.4	99.5	8.2	10.6	97.3	100.5
11ae	F	3-Thioph	8.3	76.8	89.9	102.3	6.9	6.5	60.3	62.2
13ae	F	3-Thioph	8.2	84.7	99.1	112.8	8.4	8.4	77.7	80.3
11af	F	t-butyl	9.6	35.4	41.5	47.2	6.1	1.4	12.8	13.3
11ag	F	Cyano Me	5.8	51.0	59.7	68.0	5.6	1.2	11.3	11.6
11ba	CH ₃	Me	9.7	25.6	30.0	34.1	5.8	1.3	11.7	12.1
13ba	CH ₃	Me	7.2	53.5	62.6	71.3	9.4	0.8	7.7	8.0
11bb	CH ₃	Ph	6.4	39.4	46.1	52.4	5.0	1.9	17.9	18.4
13bb	CH ₃	Ph	7.5	42.6	49.9	56.8	5.9	2.2	19.8	20.5
11bc	CH ₃	4F-Ph	6.6	33.1	38.7	44.1	4.5	1.0	9.5	9.8
13bc	CH ₃	4F-Ph	6.2	42.0	49.2	56.0	6.5	0.9	8.1	8.3
11bd	CH ₃	2,3F-4Ani	6.8	16.9	19.7	22.5	4.0	1.5	13.9	14.4
13bd	CH ₃	2,3F-4Ani	8.1	45.7	53.5	61.0	5.7	2.0	18.2	18.8
11be	CH ₃	3-Thioph	7.4	36.7	42.9	48.9	5.0	1.1	10.2	10.5
13be	CH ₃	3-Thioph	7.0	56.3	65.9	75.0	5.8	1.1	10.4	10.7
11ca	OMe	Me	9.8	12.7	14.8	16.9	6.6	1.2	10.9	11.2
13ca	OMe	Me	5.6	53.7	62.8	71.5	7.1	1.0	9.6	9.9
11cb	OMe	Ph	5.9	31.3	36.6	41.7	5.0	1.2	10.8	12.0
13cb	OMe	Ph	6.3	62.4	73.0	83.1	5.3	1.2	10.7	11.0
11cc	OMe	4F-Ph	7.1	16.7	19.5	22.3	4.8	1.3	11.6	14.8
13cc	OMe	4F-Ph	6.6	48.4	56.7	64.5	6.9	0.9	8.5	8.8
11cd	OMe	2,3F-4Ani	8.5	39.1	45.7	54.0	8.1	1.6	14.3	15.3
13cd	OMe	2,3F-4Ani	5.9	31.1	36.4	41.4	5.0	2.3	21.5	22.2
11ce	OMe	3-Thioph	6.3	34.4	40.3	45.9	5.0	1.6	14.9	9.3
13ce	OMe	3-Thioph	5.8	56.4	66.0	75.2	5.0	1.3	11.8	12.1

a) Values are the mean of at least two experiments done in duplicate. *b)* CCL2-promoter-luciferase-reporter activity assay. *c)* 3xGRE-promoter-luciferase-reporter activity assay. *d)* Efficacy represented as percentage of maximal response of dexamethasone or hydrocortisone.

ability to dissociate their activity profile and differentiate anti-inflammatory activity from transactivation potential (Table 1.6.1). In most cases, the dominant factors driving the activities of the APGRAs seemed to be provided by the arylpyrazole moiety; however, the inclusion of an aryl group at R² was able to increase selectively the transrepression activity in several molecules from the tolyl- and anisoyl- series to ~66-75% that of Dex although higher concentrations were needed (see **13be**, **13cb**, and **13ce** in Table 1.6.1). It is noteworthy, that none of these compounds elicited transactivation activity above background levels. These results indicate that more exploration may provide further analogues of interest.

The most desirable compounds for this study have a profile with $\geq 75\%$ of the transrepression and $\leq 25\%$ of the transactivation activity of Dex, respectively, at similar concentrations. While more of these compounds are likely to be useful in further studies aimed towards understanding the mechanisms of action, only molecules **11aa** and **11ab** that contained F at R¹ and also bore a sidechain with a 2° alcohol with methyl or phenyl at R² displayed these properties (Figure 1.6.1 and Table 1.6.1). Therefore, these two compounds along with the corresponding molecules **13aa** and **13ab** containing 3° alcohols were further tested for their ability to limit insulin secretion (Figure 1.6.1c). All but **13ab** were able to perform better than Dex in the GSIS bioassays, with the 2° alcohols **11aa** and **11ab** and 3° alcohols **13aa** showing no reduction in insulin secretion at both 3 mM and 15 mM glucose concentrations. The fact that **13aa** was observed to elicit 85% of the transactivation potential of Dex in the 3xGRE assay is noteworthy, as this compound did not display GSIS inhibition as expected from the commonly accepted transrepression/transactivation model. Together, these results suggest that the design of anti-inflammatory compounds with decreased side effects is achievable and that our current understanding of the role of the GRE in physiological regulation is still incomplete and warrants further mechanistic study.

1.6.2 – Molecular Docking Observations

Further exploration into the binding of APGRAs to the GR was accomplished through computational experiments. Both molecular docking studies, performed using

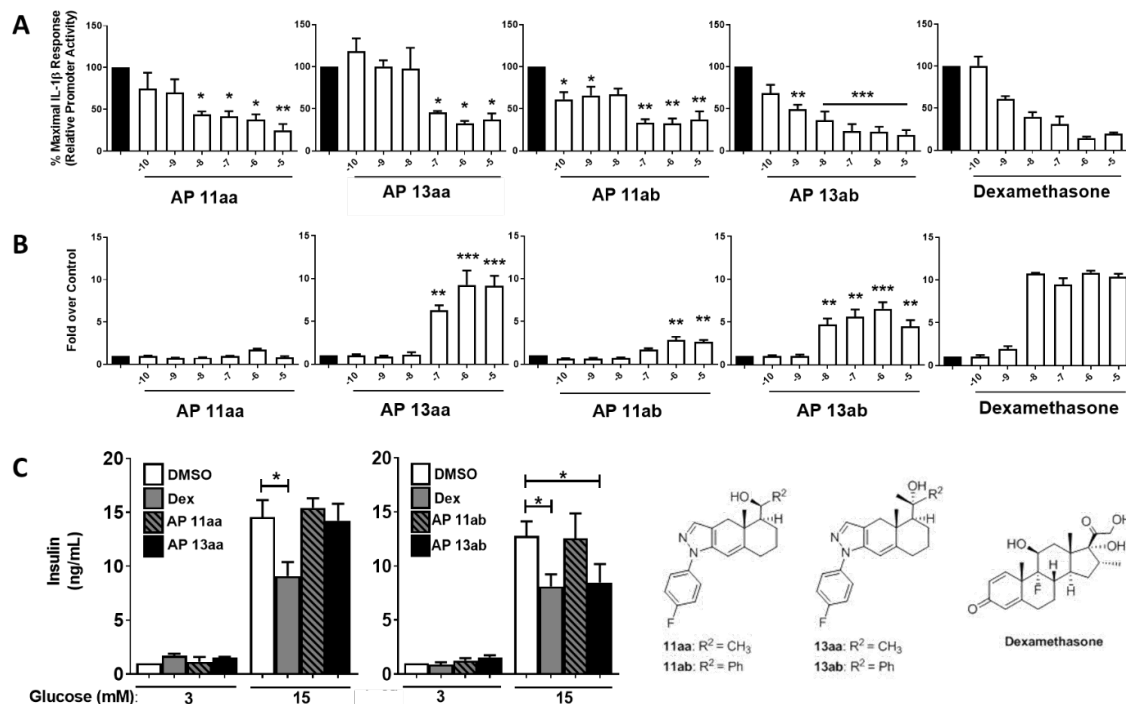


Figure 1.6.1 – Analysis of top candidates

Bioassay data showing GR agonist activity and impact on glucose-stimulated insulin secretion. (A) CCL2-promoter-luciferase-reporter activity assay; x-axis: log concentration (molar); y-axis: %-maximal IL-1 β response (relative promoter activity). (B) 3xGRE-promoter-luciferase-reporter activity assay; x-axis: log concentration (molar); y-axis: fold over control. (C) 832/13 cells were exposed to 100 nM of the indicated GR agonist compounds for 18 h, followed by measurements of glucose-stimulated insulin secretion

AutoDock Vina (The Scripps Research Institute)⁷⁰ and DFT calculations were used in this endeavor. A crystal structure of budesonide bound to the GR has been reported (PDB ID: 5NFP)⁶¹, and the coordinates from this structure were used to construct the truncated standard binding pocket for the experiments reported herein. Further, the binding of DAC to the GR elicits an expansion of the standard binding pocket to accommodate the extra bulk of the aryl pyrazole moiety appended to the intact steroidal core, and the crystal structure for the protein-ligand complex (PDB ID: 3BQD) was used to model an expanded binding pocket.²⁹ (Figure 1.5.1)

Previous studies modeling the interaction of APGRAs with structures similar to those reported herein have assumed that the pyrazole ring of the compounds would situate in the binding pockets at the location normally occupied by the A ring of the steroid ligands. This assumption always places the aryl pyrazole moiety of the APGRAs either within the expanded binding pocket or in the region of the natural binding pocket that would expand to accommodate the added steric bulk.^{29, 69} However, the APGRAs are smaller in size than typical steroids and could potentially reside fully in either pocket if the orientation of the molecule is not fully analogous to that of the steroidal ligands. Therefore, these efforts commenced with a comprehensive exploration of the interactions of these compounds with the standard binding site.

Computational docking of the APGRAs in the standard pocket led to random results in which multiple ligand orientations were possible, and many of these differed from the orientation that budesonide adopted in the original crystal structure (see appendix section 1.9.6). To overcome the limitations of a rigid receptor model, DFT calculations were also performed on a truncated 5NFP receptor pocket with the synthesized compounds. It was hypothesized that flexibility of the residues surrounding the ligands may lead to discovery of key residue interactions; however, results from these experiments also led to disordered protein-ligand interactions (see appendix section 1.9.7). For these experiments, a subset of molecules were modeled in the least sterically accessible conformation with respect to the orientation of the side chain, and the repositioning of the alcohol did not lead to more

reliable binding results. Therefore, it is unlikely that these compounds reside within the standard steroid binding pocket.

When performing molecular docking studies with the expanded GR pocket, the ligands all showed an analogous orientation, and clear trends in interactions with the receptor began to emerge. The orientation for the top calculated binding affinities mimicked that of DAC in the original crystal structure and key residue interactions that have been reported in previous studies were observed with a majority of the synthesized compounds.²⁹ Note, DFT calculations with the expanded binding pocket were not performed as the MM level of theory was deemed sufficient for this study.

Compared to the standard GR binding pocket, computational binding of the APGRAs to the expanded binding pocket resulted in reproducible orientations that showed defined interaction between the ligand and protein. (Figure 1.6.2) Hydrogen bonding between residue Q570 and the nitrogen atom in the aryl pyrazole moiety was observed, and the aryl moiety was tightly enclosed by nearby residues M604, A607, L608, R611, and F623. The Dex-bound structure shows similar hydrogen-bonding between the C3-ketone and residues Q570 and R611, with further contacts between residue N564 and the C11-hydroxyl group. Further, additional hydrophobic and π interactions between the protein and the aryl pyrazole moiety play a role in increasing binding affinity.⁸⁷ Overall, the fluorophenyl- series was able to reside deeper within the expanded binding pocket, while the tolyl- and anisoyl- derivatives tended to be shifted out of the expanded the binding pocket and further into the canonical binding pocket. This aligned the molecules further from helix-5 and closer to helix-10 in the GR LBD and weakened the critical hydrophobic and π interactions. This shift disrupts H-bonding to Q570 for the tolyl- and anisoyl-analogues. Molecules **13be**, **13cb**, and **13ce** gain a π interaction between the aromatic sidechain of the molecule and Y735, and this new contact may be responsible for the recovery of transactivation activity for these compounds (see appendix section 1.9.8).

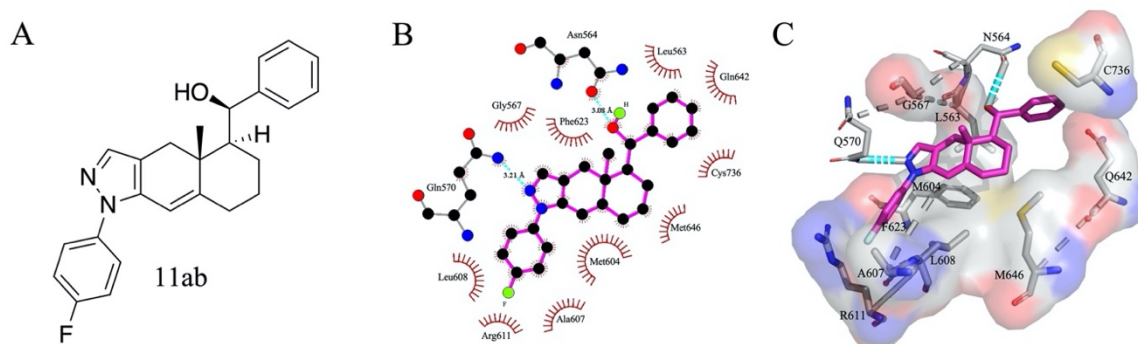


Figure 1.6.2 – Molecular docking of 11ab

Molecular docking experiment between glucocorticoid analogue **11ab** (structure shown in panel A) and GR LBD (3BQD). B) 2D representation of ligand-receptor interactions where a red semi-circle represents a hydrophobic interaction, and a cyan dotted line represents a hydrogen bond. C) 3D representation of ligand-receptor interactions with cyan dotted lines displaying hydrogen bonding.

1.7 - CONCLUSION

A reevaluation of previously reported and expansion of structures for a set of aryl pyrazole GC analogues was undertaken to determine whether transrepression and transactivation activity could be differentiated in order to decrease the side effects, such as inhibition of insulin secretion, of these potentially therapeutic entities. This work showed that members of the previously synthesized fluorophenyl series of analogues (**11aa**, **13aa**, **11ab**, and **13ab**) had anti-inflammatory activity approaching that of Dex and equal to that of HC, while **11aa** and **11ab** also displayed suppressed transactivation activity when compared to those drugs. All these molecules, except **13ab**, did not inhibit insulin secretion in the presence of glucose stimulation. These observations indicate that therapeutic potential of the 2° alcohols, **11aa** and **11ab**, has been overlooked previously. Further, the lack of insulin inhibitory side effects for **13aa** despite the noted transactivating properties highlights a knowledge gap concerning the nuanced regulatory mechanisms employed by the GRE. Similar knowledge gaps have been reported, promoting the need for further investigation of mechanisms effecting glucose metabolism.⁸³

Members of the tolyl- and anisoyl- series of APGRA analogues were not as potent as the fluorophenyl- series; however, several of these compounds (**13be**, **13cb**, **13ce**) had anti-inflammatory potentials that were $\sim \geq 66\%$ and $\sim \geq 75\%$ that of Dex and HC, respectively. These molecules also displayed no transactivation above background, which makes them entities of interest for further studies. The computational studies showed conclusively that the aryl pyrazoles with small substituent at the para- position of the aryl moiety fit tightly within the expanded binding pocket that was previously observed in crystals of the DAC-GRE complex. These studies also suggest that adjusting the placement of the ligands between the expanded and canonical steroid binding pocket will be useful for tuning the activity of future anti-inflammatory compounds that target the GRE.

GR signaling remains a complex area of study, in which this work highlights that efforts to reevaluate and further examine the phenomena that arise from the interplay of GRE transactivation and transrepression are still needed. Further studies into the SAR for therapeutically useful GC analogues, as well as *in vivo* investigations of the regulatory

mechanisms for ligand-GR complexes are underway with **11aa**, **11ab**, and **13aa**, since these compounds displayed a greater ability than DEX to separate transrepression and transactivation activity and have shown the potential to avoid inhibition of insulin secretion and potentially the debilitating drug induced diabetes that plagues current long-term GC active compounds.

1.8 - METHODS

1.8.1 - Materials

Reagents were purchased and used without further purification, except for methyl vinyl ketone (**1**), which was distilled immediately prior to use. Dry ethereal solvents were obtained by distillation from a potassium-benzophenone ketyl still, all other dry solvents were obtained via distillation over CaH₂. Thin-layer chromatography (TLC) was carried out using Sorbent Technologies silica G w/UV254 TLC plates. All purified compounds of interest were visualized as single spots using short-wave UV light; more permanent staining of TLC spots was achieved using vanillin, *p*-anisaldehyde, or ceric ammonium molybdate (Hannesian's) TLC staining solutions. ¹H NMR and ¹³C NMR spectra were recorded on either a Varian Mercury Vx 300 MHz or Varian Inova 500 MHz instrument (Palo Alto, CA) in solutions of CDCl₃. High resolution mass spectrometry (HRMS) was performed using either a JEOL DART source operating in positive ion mode coupled to a JEOL AccuTOF JMS-T100LC mass spectrometer (Akishima, Tokyo, Japan), an Applied Biosystems QStar Elite mass spectrometer (Foster City, CA) equipped with a Sciex Turboionspray ESI source (Framingham, MA) operating in positive ionization mode, or an Exactive™ Plus Orbitrap mass spectrometer with an ESI source operating in positive ionization mode. Optical rotation was measured using a Perkin-Elmer 241 polarimeter (Waltham, MA) equipped with a sodium source lamp. IR spectra were collected using a Thermo Scientific Nicolet™ iS5 FTIR spectrometer (Waltham, MA) as a dry film on single NaCl plates. LCMS analysis was conducted on a Thermo Scientific Dionex UltiMate™ 3000 UHPLC system coupled to an Exactive™ Plus Orbitrap mass spectrometer (Waltham,

MA) with an ESI source operating in positive ionization mode. NMR spectra of final compounds and bioassay data can be found in the appendix (sections 1.9.1 – 1.9.3).

1.8.2 – Synthetic Procedures and Characterization

All air/moisture sensitive reactions were done in an argon atmosphere under anhydrous conditions, unless otherwise stated. All solvents and reagents were used without further purification unless otherwise stated. All heated reactions were performed using oil baths. Analytical thin-layer chromatography was performed using Silica gel (normal phase) with a mobile phase consisting of EtOAc/hexanes unless otherwise stated. ¹H NMR chemical shifts were recorded in parts per million (ppm) Abbreviations used for ¹H NMR are: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd= doublet of doublets, td = triplet of doublets.

Compound **6** was synthesized as previously reported.^{62, 88} The general procedure used to generate compound **10a** was used to obtain both **10b** and **10c**, the tolyl- and anisoyl- pyrazole derivatives, which are novel aryl pyrazole compounds.

General procedure for GC R¹ installation - compound series 7: To a solution of **6** (5.0 g, 20 mmol) in glacial acetic acid (50 mL) was added the appropriate phenylhydrazine HCl salt (23 mmol) and sodium acetate (1.88 g, 23 mmol) and allowed to stir at room temperature for 2 h. The reaction mixture was then slowly and carefully poured into a cold (0-5 °C) and actively stirred solution of saturated NaHCO₃. The neutralized solution was then extracted with EtOAc (5x100 mL), washed with brine, dried with anhydrous MgSO₄, filtered, and concentrated in vacuo. Purification of the crude product was accomplished using flash chromatography (20% EtOAc in hexanes) to give a viscous orange oil.

1-(4-fluorophenyl)-4a-methyl-1,4,4a,6,7,8-hexahydrospiro[benzo[f]indazole-5,2'-[1,3]dioxolane] (7a): ¹H NMR (CDCl₃, 499.73 MHz) δ 1.21 (s, 3H), 1.65-1.82 (m, 4H), 2.27-2.30 (d, 1H), 2.42-2.52 (m, 2H), 3.14-3.17 (d, 1H), 3.97-4.10 (m, 4H), 6.20 (s, 1H), 7.14 (m, 2H), 7.41-7.44 (m, 3H).

4a-methyl-1-(p-tolyl)-1,4,4a,6,7,8-hexahydrospiro-[benzo[f]indazole-5,2'-[1,3]dioxolane] (7b): From **6** using p-tolylphenylhydrazine HCl. Yield = 44%. TLC R_f =

0.28 (30% EtOAc/hexanes). ¹H NMR (CDCl₃, 499.73 MHz) δ 1.21 (s, 3H), 1.65-1.83 (m, 4H), 2.28 (dt, 2H), 2.4 (s, 3H), 2.51 (d, 1H), 3.16 (d, 1H), 3.99-4.08 (m, 4H), 6.23 (s, 1H), 7.24-7.26 (d, 2H), 7.35-7.37 (d, 2H), 7.43 (s, 1H).

1-(4-methoxyphenyl)-4a-methyl-1,4,4a,6,7,8-hexahydrospiro[benzo[f]indazole-5,2'-[1,3]dioxolane] (7c): From **6** using p-methoxyphenylhydrazine HCl. Yield = 38%. TLC R_f = 0.27 (50% EtOAc/hexanes). ¹H NMR (CDCl₃, 499.73 MHz) δ 1.22 (s, 3H), 1.68-1.83 (m, 3H), 2.30-2.31 (d, 1H), 2.51-2.54 (d, 2H), 3.15-3.18 (d, 2H), 3.85 (s, 3H), 4.01-4.13 (m, 4H), 6.17 (s, 1H), 6.97-7.41 (m, 5H).

General acetal deprotection for preparation of ketone 8: To a solution of **7** (5.9 mmol) in THF (50 mL) was added 6 M HCl (3.9 mL, 23.6 mmol) and subjected to reflux for 3.5 h. The reaction mixture was then neutralized by the slow addition of saturated NaHCO₃ and extracted with ethyl acetate (4x100 mL). The pooled organic layer was washed with brine, dried with anhydrous MgSO₄, and concentrated in vacuo. Purification of the crude product was accomplished using flash chromatography (20% EtOAc in hexanes) to give a dull orange solid.

1-(4-fluorophenyl)-4a-methyl-1,4,4a,6,7,8-hexahydro-5H-benzo[f]indazol-5-one (8a): ¹H NMR (CDCl₃, 499.73 MHz) δ 1.25 (s, 3H), 1.63-1.72 (m, 1H), 2.06-2.12 (m, 1H), 2.51-2.57 (m, 2H), 2.60-2.69 (m, 2H), 2.87-2.91 (s, 2H), 6.29 (s, 1H), 7.15-7.18 (t, 2H), 7.44-7.47 (m, 2H), 7.48 (s, 1H).

4a-methyl-1-(p-tolyl)-1,4,4a,6,7,8-hexahydro-5H-benzo[f]indazol-5-one (8b): From **7b**. Flash column mobile phase - 15% EtOAc/hexanes. Yield = 72%. TLC R_f = 0.34 (30% EtOAc/hexanes). ¹H NMR (CDCl₃, 499.73 MHz) δ 1.25 (s, 3H), 1.62-1.71 (m, 1H), 2.06-2.11 (m, 1H), 2.40 (s, 3H), 2.51-2.57 (m, 2H), 2.60-2.67 (m, 2H), 2.90 (s, 2H), 6.33 (s, 1H), 7.26-7.28 (d, 2H), 7.35-7.38 (d, 2H), 7.48 (s, 1H).

1-(4-methoxyphenyl)-4a-methyl-1,4,4a,6,7,8-hexahydro-5H-benzo[f]indazol-5-one (8c): From **7c**. Flash column mobile phase - 20% EtOAc/hexanes. Yield = 77%. TLC R_f = 0.28 (40% EtOAc/hexanes). ¹H NMR (CDCl₃, 499.73 MHz) δ 1.24 (s, 3H), 1.60-1.73

(m, 1H), 2.04-2.11 (m, 1H), 2.49-2.69 (m, 4H), 2.93 (d, 2H), 3.85 (s, 3H), 6.26 (s, 1H), 6.95-7.47 (m, 5H).

General Procedure for the generation of enol ether 9 and synthesis of aldehyde

10: A commercially available solution of potassium bis(trimethylsilyl)amide in toluene (0.5 M, 33.12 mL, 16.5 mmol) was added dropwise with a syringe to an actively stirred suspension of methoxymethyltriphenylphosphonium chloride (6.81 g, 19.8 mmol) in THF (65 mL) cooled to -40 °C. The solution was warmed to 0 °C and stirred at that temperature for 15 min and a color change from pale yellow to dark red was observed. A solution of the appropriate ketone precursor **8** (6.6 mmol) in THF (16 mL) was transferred dropwise via canula to the chilled ylide. After stirring for 24 h, the reaction was quenched by the addition of 1:1 THF/MeOH (16 mL), diluted with three reaction volumes of EtOAc, washed with water and brine, dried over MgSO₄, filtered, concentrated in vacuo and subjected to flash chromatography (15% EtOAc in hexanes) to give a clear and colorless viscous oil. To a prepared solution of purified **9** in THF was added 4 M HCl and was allowed to stir at room temperature for 36 h. The reaction mixture was afterward diluted with EtOAc - washed with water, saturated NaHCO₃, and brine. The organic layer was dried over MgSO₄, filtered, and concentrated in vacuo to give crude aldehyde **10** as a beige solid. Purification of the crude product was accomplished using flash chromatography (15% EtOAc in hexanes) to give a white solid.

1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazole-5-carbaldehyde (10a): ¹H NMR (CDCl₃, 499.73 MHz) δ 1.25 (s, 3H), 1.35-1.45 (m, 2H), 1.68-1.77 (m, 1H), 1.89-1.95 (m, 2H), 2.32-2.42 (m, 3H), 2.87-2.90 (d, 1H), 3.07-3.10 (d, 1H), 6.18 (s, 1H), 7.12-7.16 (t, 2H), 7.41-7.44 (m, 3H), 9.93 (s, 1H).

4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazole-5-carbaldehyde (10b): Yield = 40.0 %. TLC R_f = 0.31 (25% EtOAc/hexanes). ¹H NMR (CDCl₃, 300.08 MHz) δ 1.12 (s, 3H), 1.30-1.40 (m, 2H), 1.70-1.78 (m, 2H), 1.90-1.96 (m, 1H), 2.32-2.44 (m, 2H), 2.40 (s, 3H), 2.91 (d, 1H), 3.10 (d, 1H), 6.21 (s, 1H), 7.26-7.28 (d, 2H), 7.35-7.37 (d, 2H), 7.44 (s, 1H), 9.90 (s, 1H)

1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazole-5-carbaldehyde (10c): Yield = 45.0 %. TLC R_f = 0.28 (25% EtOAc/hexanes). ^1H NMR (CDCl_3 , 499.73 MHz) δ 1.11 (s, 3H), 1.39-1.43 (m, 1H), 1.72-1.75 (m, 1H), 1.90-1.96 (m, 2H), 2.33-2.44 (m, 3H), 2.89-2.92 (d, 1H), 3.08-3.11 (d, 1H), 3.85-3.86 (s, 3H), 6.15 (s, 1H), 6.95-7.42 (m, 5H), 9.90 (s, 1H).

General Procedure for R^2 = methyl 2°OH analogues: A commercially available solution of 1.6 M MeLi in Et_2O (312 μL , 0.5 mmol) was transferred via syringe to a round bottom flask containing Et_2O (5 mL) and cooled to -78°C . A solution of the appropriate aldehyde **10** (0.05 mmol) in THF (5 mL) was added dropwise via canula, and the reaction was stirred at -78°C for 45 min. The reaction was quenched with isopropyl alcohol (IPA), poured into a separatory funnel containing saturated NH_4Cl , and extracted three times with EtOAc. The organic phase was washed with once each with water and brine, dried over anhydrous MgSO_4 , filtered, and concentrated in vacuo. Purification of the crude product was accomplished using flash chromatography (15% EtOAc in hexanes) to give a white solid.

1-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)ethan-1-ol (11aa): ^1H NMR (CDCl_3 , 499.73 MHz) δ 1.14 (s, 3H), 1.26-1.28 (d, 3H), 1.41-1.47 (dd, 2H), 1.63-1.68 (dd, 1H), 1.73-1.80 (m, 1H), 1.90-1.97 (m, 1H), 2.31-2.43 (m, 2H), 2.46-2.52 (d, 1H), 2.98-3.03 (d, 1H), 4.25 (s, 1H), 6.10 (s, 1H), 7.11-7.19 (m, 2H), 7.41-7.47 (m, 3H).

1-(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)ethan-1-ol (11ba): white solid obtained in 50% yield; mp 178.1 - 181.0°C ; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.16; $[\alpha]_{\text{D}}^{20}$ = -16 (c 1.0, MeOH); ^1H NMR (CDCl_3 , 499.73 MHz) (exchangeable proton not observed) δ 1.13 (s, 3H), 1.23-1.29 (m, 3H), 1.34-1.45 (m, 2H), 1.56-1.62 (m, 2H), 1.72-1.78 (m, 1H), 1.90-1.94 (m, 1H), 2.27-2.30 (m, 1H), 2.32-2.37 (m, 4H), 2.47-2.50 (d, 1H), 2.97-3.00 (d, 1H), 6.14 (s, 1H), 7.23-7.26 (d, 2H), 7.34-7.36 (d, 2H), 7.40 (s, 1H); ^{13}C NMR (CDCl_3 , 125.66 MHz) δ 19.21, 20.81, 24.34, 25.85, 33.32, 34.67, 41.14, 54.89, 56.00, 66.11, 109.50, 113.47, 123.47,

129.65, 136.81, 137.28, 137.60, 149.57; IR (NaCl, thin film) $\nu_{\max}$ (cm⁻¹): 3315 (OH), 3043 (w), 2962, 2936, 2862, 1620, 1518 (w), 1425, 1132, 820; HRMS (DART-RTOF) m/z: [M+H]⁺ Calcd for C₂₁H₂₆N₂O, 323.21179; found 323.21001.

1-(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)ethan-1-ol (11ca): white solid obtained in 58% yield; mp 156-159 °C; TLC (Silica G w/UV254) 25% EtOAc in hexanes, R_f = 0.11; [α]_D²⁰ = +25 (c 1.0, MeOH); ¹H NMR (CDCl₃, 499.73 MHz) (exchangeable proton not observed) δ 1.13 (s, 3H), 1.23-1.29 (m, 4H), 1.34-1.45 (m, 3H), 1.74-1.78 (d, 1H), 1.88-1.95 (d, 1H), 2.28-2.31 (d, 1H), 2.35-2.39 (m, 1H), 2.47-2.51 (d, 1H), 2.98-3.01 (d, 1H), 3.84-3.87 (s, 3H), 6.10 (s, 1H), 6.96-7.40 (m, 5H); ¹³C NMR (CDCl₃, 125.66 MHz) δ 19.21, 20.80, 24.36, 25.85, 33.31, 34.68, 41.16, 54.89, 55.49, 66.10, 109.38, 113.42, 114.25, 133.01, 136.73, 137.32, 149.51, 158.54; IR (NaCl, thin film) $\nu_{\max}$ (cm⁻¹): 3376 (OH), 3020 (w), 2962, 2931, 2862, 1612, 1517, 1250, 831; HRMS (ESI) m/z: [M+H]⁺ Calcd for C₂₁H₂₆N₂O₂, 339.20670; found 339.2067.

General Procedure for R² = phenyl 2°OH analogues: A commercially available solution of 1.9 M PhLi in di-n-butyl ether (3.42 mL, 6.5 mmol) was transferred to a round bottom flask containing a small portion of dry Et₂O and was cooled to -78 °C. A solution of **10** (0.65 mmol) in THF (2 mL) was added dropwise via canula, and the reaction was stirred at -78 °C for 45 min. The reaction was quenched with IPA, poured into a separatory funnel containing saturated NH₄Cl, and extracted three times with EtOAc. The organic phase was washed with once each with water and brine, dried over anhydrous MgSO₄, filtered, and concentrated in vacuo. Purification of the crude product was accomplished using flash chromatography (10% EtOAc in hexanes) to give a white solid.

(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)(phenyl)methanol (11ab): ¹H NMR (CDCl₃, 499.73 MHz) δ 1.18 (s, 3H), 1.52-1.58 (m, 2H), 1.60-1.63 (dd, 1H), 1.70-1.74 (m, 2H), 2.17-2.21 (m, 1H), 2.28-2.36 (m, 1H), 2.66-2.69 (d, 1H), 3.08-3.11 (d, 1H), 5.10 (d, 1H), 6.02 (s, 1H), 7.04-7.08 (m, 2H), 7.16-7.18 (m, 1H), 7.27-7.29 (m, 4H), 7.35-7.38 (m, 3H).

(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)(phenyl)methanol (11bb): white solid obtained in 28% yield; mp 184-187 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, $R_f = 0.20$; $[\alpha]_D^{20} = -12$ (c 1.0, MeOH); ^1H NMR (CDCl_3 , 499.73 MHz) (exchangeable proton not observed) δ 1.26 (s, 3H), 1.60-1.81 (m, 5H), 2.23-2.28 (d, 1H), 2.36-2.43 (m, 4H), 2.74-2.77 (d, 1H), 3.15-3.18 (d, 1H), 5.17 (s, 1H), 6.15 (s, 1H), 7.23-7.27 (d, 3H), 7.34-7.38 (m, 6H), 7.41 (s, 1H); ^{13}C NMR (CDCl_3 , 125.66 MHz) δ 19.90, 21.05, 25.79, 33.30, 35.18, 41.28, 56.66, 71.96, 99.08, 109.52, 113.68, 123.52, 125.39, 126.79, 128.18, 129.69, 137.61, 145.72, 149.79; IR (NaCl, thin film) ν_{max} (cm^{-1}): 3319 (OH), 3039 (w), 2929, 2868, 1610 (w), 1519 (m), 821; HRMS (DART-RTOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{26}\text{H}_{28}\text{N}_2\text{O}$, 385.22744; found 385.22612.

(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)(phenyl)methanol (11cb): beige solid obtained in 73% yield; mp 109.3-111.5 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, $R_f = 0.11$; $[\alpha]_D^{20} = -10$ (c 1.0, MeOH); ^1H NMR (CDCl_3 , 499.73 MHz) (exchangeable proton not observed) δ 1.27 (s, 3H), 1.56-1.81 (m, 6H), 2.24-2.27 (d, 1H), 2.36-2.44 (m, 1H), 2.75-2.78 (d, 1H), 3.17-3.20 (d, 1H), 3.85 (s, 3H), 5.20 (s, 1H), 6.11 (s, 1H), 6.96-7.44 (m, 9H); ^{13}C NMR (CDCl_3 , 125.66 MHz) δ 19.90, 25.78, 33.28, 35.23, 41.30, 56.65, 72.02, 114.27, 125.09, 125.36, 126.84, 128.20, 132.99, 145.61, 149.62, 158.58; IR (NaCl, thin film) ν_{max} (cm^{-1}): 3307 (OH), 3056 (w), 2932, 2868, 2835, 1611, 1518, 1250, 833; HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{26}\text{H}_{28}\text{N}_2\text{O}_2$, 401.22235; found 401.2224.

General Procedure for $\text{R}^2 = 4\text{-fluorophenyl } 2^\circ\text{OH}$ analogues: A commercially available solution of 2.0 M 4-fluorophenylmagnesium bromide in Et_2O was transferred to a round bottom flask and cooled to -78 °C. A solution of **10** (0.62 mmol) in THF (2 mL) was added dropwise via canula, and the reaction was stirred at -78 °C for 45 min. The reaction was quenched with IPA, poured into a separatory funnel containing saturated NH_4Cl , and extracted three times with EtOAc. The organic phase was washed with once each with water and brine, dried over anhydrous MgSO_4 , filtered, and concentrated in

vacuo. Purification of the crude product was accomplished using flash chromatography (10% EtOAc in hexanes) to give a white solid.

(4-fluorophenyl)(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)methanol (11ac): ^1H NMR (CDCl_3 , 300.08 MHz) δ 1.26 (s, 3H), 1.65-1.69 (m, 3H), 1.77-1.86 (m, 1H), 2.26-2.41 (m, 2H), 2.72-2.77 (d, 1H), 3.17-3.22 (d, 1H), 5.19-5.22 (s, 1H), 6.12 (s, 1H), 7.02-7.08 (t, 2H), 7.13-7.19 (t, 2H), 7.30-7.34 (dd, 2H), 7.44-7.48 (m, 3H).

(4-fluorophenyl)(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)methanol (11bc): white solid obtained in 11.5% yield; mp 188-191 °C; TLC (Silica G w/UV254) 25% EtOAc in hexanes, R_f = 0.24; $[\alpha]_D^{20}$ = -24 (c 1.0, MeOH); ^1H NMR (CDCl_3 , 499.73 MHz) (exchangeable proton not observed) δ 1.26 (s, 3H), 1.50-1.61 (m, 3H), 1.67-1.71 (m, 2H), 1.79-1.82 (d, 1H), 2.25-2.28 (d, 1H), 2.39 (s, 3H), 2.74-2.77 (d, 1H), 3.16-3.20 (d, 1H), 5.19 (s, 1H), 6.16 (s, 1H), 7.02-7.46 (m, 9); ^{13}C NMR (CDCl_3 , 125.66 MHz) δ 19.91, 21.04, 25.74, 33.25, 35.24, 41.24, 56.75, 71.48, 109.63, 113.55, 114.89, 115.06, 123.52, 126.87, 129.70, 136.70, 136.91, 137.25, 137.59, 141.25, 149.50, 160.71, 162.66; IR (NaCl, thin film) ν_{max} (cm^{-1}): 3312 (OH), 3041 (w), 2931, 2867, 2830, 1898, 1603, 1518, 1219, 819; HRMS (DART-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{26}\text{H}_{27}\text{FN}_2\text{O}$, 403.21802; found 403.21649.

(4-fluorophenyl)(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)methanol (11cc): white solid obtained in 69% yield; mp 178-182 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.26; $[\alpha]_D^{20}$ = +4 (c 4.0, MeOH); ^1H NMR (CDCl_3 , 499.73 MHz) (exchangeable proton not observed) δ 1.26 (s, 3H), 1.56-1.58 (m, 2H), 1.66-1.70 (m, 2H), 1.79-1.82 (m, 1H), 2.25-2.28 (m, 1H), 2.37-2.40 (m, 1H), 2.73-2.76 (d, 1H), 3.17-3.20 (d, 1H), 3.85 (s, 3H), 5.20 (d, 1H), 6.11 (s, 1H), 6.96-7.06 (m, 4H), 7.30-7.44 (m, 5H); ^{13}C NMR (CDCl_3 , 125.66 MHz) δ 19.84, 20.59, 21.04, 25.80, 29.68, 33.28, 35.05, 41.22, 55.69, 69.85, 109.63, 113.56, 119.81, 123.50, 125.51, 126.00, 129.68, 137.60, 147.31, 149.4; IR (NaCl, thin film) ν_{max} (cm^{-1}): 3399 (OH), 2930, 2850, 1602, 1512, 1223, 824; HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{26}\text{H}_{27}\text{FN}_2\text{O}_2$, 419.21293; found 419.2132.

General Procedure for R² = 2,3-difluoroanisyl 2°OH analogues: A solution of 5-bromo-2,3-difluoroanisole (4.6 mmol) in THF (10 mL) was cooled to -78 °C, to which a commercially available solution of 1.9 M t-BuLi in pentanes (4.6 mmol, 2.9 mL) was added dropwise via syringe. The reaction was stirred for 20 min at -78 °C, raised to 0 °C for 5-10 min until the solution transitioned from pale yellow to dark yellow-green, and then was immediately cooled back to -78 °C for 5 min. A solution of **10** (0.46 mmol) in THF (2 mL) was added dropwise via canula, and the reaction was stirred at -78 °C for 1 h. The reaction was quenched with IPA, poured into a separatory funnel containing saturated NH₄Cl, and extracted three times with EtOAc. The organic phase was washed with water and brine, dried over anhydrous MgSO₄, filtered, and concentrated in vacuo. Purification of the crude product was accomplished using flash chromatography (10% EtOAc in hexanes) to give a white solid.

(3,4-difluoro-5-methoxyphenyl)(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)methanol (11ad): From 10a using commercially available 5-bromo-2,3-difluoroanisole. Yield = 61.9 %. TLC R_f = 0.26 (25% EtOAc/hexanes). ¹H NMR (CDCl₃, 300.08 MHz) δ 1.26 (s, 3H), 1.54-1.57 (m, 1H), 1.64-1.72 (m, 1H), 1.82-1.86 (m, 2H), 2.28-2.46 (m, 3H), 2.72-2.77 (d, 1H), 3.17-3.22 (d, 1H), 3.92 (s, 3H), 5.12 (s, 1H), 6.12 (s, 1H), 6.72-6.80 (m, 2H), 7.15-7.21 (m, 2H), 7.45-7.54 (m, 3H).

(3,4-difluoro-5-methoxyphenyl)(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)methanol (11bd): white solid obtained in 76% yield; mp 97-101 °C; TLC (Silica G w/UV254) 25% EtOAc in hexanes, R_f = 0.22; [α]_D²⁰ = +31 (c 1.0, MeOH); ¹H NMR (CDCl₃, 499.73 MHz) (exchangeable proton not observed) δ 1.25 (m, 3H), 1.37-1.50 (m, 2H), 1.54-1.65 (m, 2H), 1.76-1.79 (d, 1H), 2.04 (s, 3H), 2.26-2.29 (d, 1H), 2.72-2.75 (d, 1H), 3.14-3.17 (d, 1H), 3.88 (s, 3H), 5.10 (s, 1H), 6.16 (s, 1H), 6.72-6.78 (m, 2H), 7.24-7.46 (m, 6H); ¹³C NMR (CDCl₃, 125.66 MHz) δ 14.18, 19.94, 21.04, 25.70, 33.17, 35.31, 41.20, 56.71, 60.38, 71.28, 105.52, 106.04, 106.19, 109.70, 113.35, 123.36, 123.51, 129.66, 136.67, 136.99, 137.17, 137.55, 149.25; IR (NaCl, thin film) ν_{max} (cm⁻¹): 3387 (OH), 3050 (w), 2927, 2850, 1623, 1522, 1454, 1426, 1339, 1226, 1097;

HRMS (DART-RTOF) m/z : $[M+H]^+$ Calcd for $C_{27}H_{28}F_2N_2O_2$, 451.21916; found 451.21790.

(3,4-difluoro-5-methoxyphenyl)(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)methanol (11cd): white solid obtained in 71% yield, mp 174.1-177.4 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.16; $[\alpha]_D^{20}$ = -50 (c 1.0, MeOH); 1H NMR ($CDCl_3$, 499.73 MHz) (exchangeable proton not observed) δ 1.26 (s, 3H), 1.52-1.56 (m, 2H), 1.66-1.69 (m, 2H), 1.80-1.84 (m, 2H), 2.26-2.29 (m, 1H), 2.73-2.76 (d, 1H), 3.15-3.18 (d, 1H), 3.85 (s, 3H), 3.91 (s, 3H), 5.12 (s, 1H), 6.11 (s, 1H), 6.72-6.79 (m, 2H), 6.96-6.99 (m, 2H), 7.37-7.44 (m, 3H); ^{13}C NMR ($CDCl_3$, 125.66 MHz) δ 20.00, 25.70, 33.15, 35.35, 41.23, 55.54, 56.72, 71.34, 106.04, 109.62, 113.09, 114.29, 125.09, 136.72, 137.36, 149.14, 158.65; IR (NaCl, thin film) ν_{max} (cm^{-1}): 3400 (OH), 2930, 2868, 1611, 1517, 1250, 833; HRMS (DART-RTOF) m/z : $[M+H]^+$ Calcd for $C_{27}H_{28}F_2N_2O_3$, 467.21408; found 467.21488.

General Procedure for R^2 = thiophenyl 2°OH analogues: A solution of 3-bromothiophene (0.6 mL, 6.4 mmol) in THF (10 mL) was cooled to -78 °C, to which a commercially available solution of 1.9 M *t*-BuLi in pentanes (6.8 mL, 12.8 mmol) was added dropwise via syringe. After the reaction stirred for 30 min at -78 °C, a solution of **10** (6.4 mmol) in THF (2 mL) was added dropwise via canula, and the reaction was allowed to gradually warm to room temperature over the course of 1.5 h. After returning the reaction to -78 °C, the reaction was quenched with IPA, poured into a separatory funnel containing saturated NH_4Cl , and extracted three times with EtOAc. The organic phase was washed with water and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. Purification of the crude product was accomplished using flash chromatography (10% EtOAc in hexanes) to give a white solid.

(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)(thiophen-3-yl)methanol (11ae): From 10a using commercially available 3-bromothiophene. Yield = 35.8%. TLC R_f = 0.26 (25% EtOAc/hexanes). 1H NMR ($CDCl_3$, 499.73 MHz) δ 1.24 (s, 3H), 1.65-1.93 (m, 5H), 2.27-2.48 (m, 2H), 2.67-2.72 (d, 1H), 3.08-

3.15 (d, 1H), 5.24 (s, 1H), 6.12 (s, 1H), 6.94-6.99 (m, 1H), 7.12-7.24 (m, 3H), 7.30-7.33 (m, 1H), 7.43-7.48 (m, 3H).

(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)(thiophen-3-yl)methanol (11be): white solid obtained in 36% yield; mp 109-114 °C; TLC (Silica G w/UV254) 25% EtOAc in hexanes, R_f = 0.26; $[\alpha]_D^{20}$ = -17 (c 1.0, MeOH); ^1H NMR (CDCl_3 , 499.73 MHz) (exchangeable proton not observed) δ 1.23 (s, 3H), 1.62-1.91 (m, 6H), 2.26-2.31 (m, 1H), 2.40 (s, 3H), 2.67-2.72 (m, 1H), 3.06-3.14 (m, 1H), 5.39 (s, 1H), 6.16 (s, 1H), 6.95-7.43 (m, 8H); ^{13}C NMR (CDCl_3 , 125.66 MHz) δ 19.20, 20.08, 21.05, 24.36, 25.85, 33.32, 34.66, 41.12, 54.89, 66.09, 109.51, 113.68, 123.47, 129.65, 136.66, 136.79, 137.54, 149.61; IR (NaCl, thin film) ν_{max} (cm^{-1}): 3319 (OH), 3106 (w), 3045 (w), 2928, 2856, 2836, 1611, 1540 (m); HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{24}\text{H}_{26}\text{N}_2\text{OS}$, 391.18386; found 391.1839.

(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)(thiophen-3-yl)methanol (11ce): white solid obtained in 79% yield; mp 171-173.6 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.17; $[\alpha]_D^{20}$ = -6.2 (c 1.0, MeOH); ^1H NMR (CDCl_3 , 499.73 MHz) (exchangeable proton not observed) δ 1.26 (m, 4H), 1.62-1.72 (m, 2H), 1.77-1.85 (m, 2H), 2.27-2.41 (m, 2H), 2.69-2.72 (d, 1H), 3.12-3.15 (d, 1H), 3.85 (s, 3H), 5.25 (s, 1H), 6.12 (s, 1H), 6.96-7.40 (m, 8H); ^{13}C NMR (CDCl_3 , 125.66 MHz) δ 19.87, 20.59, 25.80, 33.28, 35.04, 41.26, 55.54, 55.69, 69.83, 109.44, 113.31, 114.29, 119.82, 125.11, 125.51, 126.00, 137.26, 147.31, 158.65; IR (NaCl, thin film) ν_{max} (cm^{-1}): 3310 (OH), 3090 (w), 2920, 2860, 1610 (w), 1519, 1230, 830; HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{24}\text{H}_{26}\text{N}_2\text{O}_2\text{S}$, 407.1787; found 407.1788.

1-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-2,2-dimethylpropan-1-ol (11af): General procedure reported by Ali and coworkers in reference 36. ^1H NMR (CDCl_3 , 499.73 MHz) δ 0.97 (s, 9H), 1.08 (s, 3H), 1.38-1.45 (m, 1H), 1.59-1.67 (m, 1H), 1.72-1.79 (m, 2H), 1.83-1.87 (m, 1H), 2.30-2.35 (m, 1H), 2.36-2.44 (m, 1H), 2.45-2.48 (d, 1H), 2.82-2.85 (d, 1H), 3.50 (s, 1H), 6.13 (s, 1H), 7.13-7.17 (t, 2H), 7.41 (s, 1H), 7.43-7.47 (m, 2H).

3-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-3-hydroxypropanenitrile (11ag): A commercially available solution of 1.7 M t-BuLi in pentanes (0.9 mL, 1.53 mmol) was transferred to a round bottom flask containing THF (2 mL) and cooled to -78 °C. A solution of ACN (80 µL) in THF (2 mL) was added dropwise at -78 °C. Following addition, a solution of **10a** (400 mg, 1.3 mmol) in THF (2 mL) was added dropwise via canula, and the reaction was stirred at -78 °C for 1 h. The reaction was quenched with saturated NH₄Cl, acidified to a pH of 7 via dropwise addition of 0.5 M HCl, and extracted three times with Et₂O. The organic phase was washed with water and brine, dried over anhydrous MgSO₄, filtered, and concentrated in vacuo. Purification of the crude product was accomplished using flash chromatography (25% EtOAc/hexanes) to give a white solid. Yield = 45.5%. TLC R_f = 0.14 (25% EtOAc/hexanes). HRMS (DART-RTOF) m/z: [M+H]⁺ Calcd for C₂₁H₂₃FN₃O, 352.18197; found 352.18293. ¹H NMR (CDCl₃, 499.73 MHz) δ 1.04-1.13 (s, 3H), 1.25-1.46 (m, 2H), 1.61-1.71 (m, 2H), 1.78-1.97 (m, 2H), 2.30-2.42 (m, 2H), 2.47-2.72 (m, 3H), 2.93-3.21 (d, 1H), 4.16-4.40 (m, 1H), 6.12 (s, 1H), 7.14-7.44 (m, 5H). ¹³C NMR (CDCl₃, 125.66 MHz) δ 19.32, 20.91, 25.36, 26.20, 32.98, 34.64, 40.90, 52.82, 54.05, 66.63, 68.78, 109.77, 113.44, 115.97, 117.77, 125.34, 137.87, 148.61.

General method for the synthesis of intermediate 12: A solution of the appropriate 2°-OH precursor (**11**) in DCM was transferred dropwise via canula to a suspension of PCC (1.5 molar equivalents to compound being oxidized) and freshly activated 4 Å molecular sieves (an equivalent weight to compound being oxidized) in DCM. The reaction was stirred at room temperature until complete conversion was observed by TLC analysis (reaction times varied from 1-7 h). Upon complete conversion, the reaction mixture was immediately loaded onto a prepared florisil column (packed DCM slurry) and subjected to purification via flash chromatography (100% DCM flush) to give ketone **12** in sufficient purity to move forward with the synthesis.

1-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-2,2-dimethylpropan-1-one (12af): beige solid obtained in 14% yield; mp 192-194

°C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.54; $[\alpha]_D^{20}$ = +8.7 (c 1.0, MeOH); ^1H NMR (CDCl_3 , 499.73 MHz) δ 1.19 (s, 9H), 1.33 (s, 3H), 1.40-1.50 (m, 1H), 1.55 (s, 1H), 1.70-1.74 (dd, 1H), 2.27-2.33 (m, 1H), 2.42-2.53 (m, 1H), 2.56-2.68 (m, 2H), 3.08-3.13 (m, 1H), 6.11 (s, 1H), 7.12-7.18 (t, 2H), 7.36 (s, 1H), 7.42-7.46 (dd, 2H); ^{13}C NMR (CDCl_3 , 125.66 MHz) δ 19.22, 26.03, 27.02, 27.50, 31.99, 34.63, 41.82, 45.42, 53.04, 109.06, 113.60, 115.92, 119.70, 125.32, 135.81, 136.61, 137.77, 149.32, 160.46, 162.43, 218.05; IR (NaCl, thin film) ν_{max} (cm^{-1}) 2927, 2855, 1699, 1516, 1479, 1464, 1375, 1366, 1292, 1224, 1176, 1152, 1134, 1000; HRMS (DART-RTOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{23}\text{H}_{27}\text{FN}_2\text{O}$, 367.2180; found 367.2179.

3-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-3-oxopropanenitrile (12ag): White solid obtained from 11ag with PCC as the oxidizing agent. Yield = 16%. TLC R_f = 0.11 (30% EtOAc/hexanes). HRMS (DART-RTOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{21}\text{H}_{20}\text{FN}_3\text{O}$, 350.16632; found 350.16529. ^1H NMR (CDCl_3 , 499.73 MHz) δ 1.13 (s, 3H), 1.33-1.44 (m, 1H), 1.73-1.80 (t, 2H), 1.84-1.90 (d, 1H), 2.26-2.29 (m, 1H), 2.33-2.40 (m, 1H), 2.67-2.81 (m, 3H), 3.47-3.55 (d, 2H), 6.09 (s, 1H), 7.07-7.10 (t, 2H), 7.33-7.38 (m, 3H).

General method for the synthesis of compound 13 analogues: The appropriate ketone precursor (**12**) was dissolved in Et_2O and cooled to -40°C , and a commercially available solution of MeLi (1.9 M, 10 equivalents to ketone starting material) was added dropwise via syringe. The reaction was stirred under argon at -40° for 45 min. The reaction was quenched with IPA (10 equivalents to MeLi added), poured into a separatory funnel containing saturated NH_4Cl , and extracted with EtOAc. The organic phase was washed with water and brine, dried over anhydrous MgSO_4 , filtered, and concentrated in vacuo. Purification was accomplished using flash chromatography (10% EtOAc in hexanes) to give a white solid.

2-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)propan-2-ol (13aa): ^1H NMR (CDCl_3 , 300.08 MHz) δ 1.14 (s, 3H), 1.26-1.28 (d, 3H), 1.35-1.47 (m, 2H), 1.58 (s, 3H), 1.63-1.68 (dd, 1H), 1.73-1.80 (m, 1H), 1.89-1.98 (m, 1H),

2.26-2.42 (m, 2H), 2.46-2.52 (d, 1H), 2.98-3.03 (d, 1H), 6.10 (s, 1H), 7.12-7.18 (m, 2H), 7.41-7.47 (m, 3H).

1-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-1-phenylethan-1-ol (13ab): ¹H NMR (CDCl₃, 499.73 MHz) δ 1.27 (s, 3H), 1.56-1.65 (m, 2H), 1.67 (s, 3H), 1.74-1.80 (m, 1H), 2.02-2.08 (dd, 1H), 2.23-2.44 (m, 2H), 2.58-2.64 (d, 1H), 3.12-3.17 (d, 1H), 6.09 (s, 1H), 7.10-7.16 (t, 2H), 7.27-7.52 (m, 8H).

1-(4-fluorophenyl)-1-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)ethan-1-ol (13ac): ¹H NMR (CDCl₃, 499.73 MHz) δ 1.25 (s, 3H), 1.51-1.62 (m, 2H), 1.66 (s, 3H), 1.72-1.81 (m, 2H), 1.95-2.05 (m, 1H), 2.21-2.40 (m, 2H), 2.58-2.64 (d, 1H), 3.14-3.20 (d, 1H), 6.09 (s, 1H), 7.00-7.06 (t, 2H), 7.11-7.17 (t, 2H), 7.25-7.28 (m, 1H), 7.41-7.49 (m, 4H).

1-(3,4-difluoro-5-methoxyphenyl)-1-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)ethan-1-ol (13ad): ¹H NMR (CDCl₃, 499.73 MHz) δ 1.26 (s, 3H), 1.54-1.60 (m, 2H), 1.62 (s, 3H), 1.64-1.66 (m, 1H), 1.80-1.85 (m, 1H), 1.96-1.99 (dd, 1H), 2.24-2.30 (m, 1H), 2.34-2.42 (m, 1H), 2.57-2.60 (d, 1H), 3.07-3.11 (d, 1H), 3.91 (s, 3H), 6.10 (s, 1H), 6.87-6.91 (m, 2H), 7.12-7.16 (t, 2H), 7.28 (s, 1H), 7.42-7.45 (m, 2H); ¹³C NMR (CDCl₃, 125.66 MHz) δ 20.49, 25.61, 26.15, 28.85, 34.01, 35.69, 44.01, 56.37, 78.42, 106.26, 109.20, 115.26, 115.83, 116.13, 125.03, 125.15, 137.94, 151.52, 162.12; IR (NaCl, thin film) ν_{max} (cm⁻¹): 3381 (OH), 3101 (w), 2928, 2852, 1622, 1513 (m); mp 182.1-183.0 °C; HRMS (ESI) m/z: [M+H]⁺ Calcd for C₂₇H₂₈F₃N₂O₂, 469.2097; found 469.2098.

1-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-1-(thiophen-3-yl)ethan-1-ol (13ae): ¹H NMR (CDCl₃, 499.73 MHz) δ 1.27 (s, 3H), 1.52-1.62 (m, 3H), 1.68 (s, 3H), 2.01-2.06 (m, 1H), 2.24-2.43 (m, 3H), 2.64-2.69 (d, 1H), 3.24-3.30 (d, 1H), 6.10 (s, 1H), 7.14-7.17 (m, 3H), 7.27-7.30 (m, 1H), 7.33 (s, 1H), 7.42-7.47 (m, 3H).

2-((4aR)-4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)propan-2-ol (13ba): white solid obtained in 55% yield; mp 173-176 °C; TLC (Silica G w/UV254) 25% EtOAc in hexanes, R_f = 0.17; [α]_D²⁰ = -75 (c 1.0, MeOH); ¹H NMR

(CDCl₃, 499.73 MHz) (exchangeable proton not observed) δ 1.19 (s, 3H), 1.33-1.37 (s, 8H), 1.38-1.39 (d, 1H), 1.66-1.69 (d, 1H), 1.77-1.80 (dt, 1H), 1.84-1.87 (dt, 1H), 2.31 (m, 1H), 2.39 (s, 3H), 2.73-2.76 (d, 1H), 3.53-3.57 (d, 1H), 6.16 (s, 1H) 7.24-7.25 (d, 2H), 7.35-7.38 (d, 2H), 7.40 (s, 1H); ¹³C NMR (CDCl₃, 125.66 MHz) δ 19.65, 26.38, 27.48, 29.70, 33.91, 34.52, 36.19, 43.57, 57.61, 75.52, 109.76, 115.22, 123.26, 129.63, 136.59, 137.38, 150.95; IR (NaCl, thin film) ν_{max} (cm⁻¹): 3395 (OH), 2928, 2855, 1611 (w), 1519 (m); HRMS (ESI) m/z: [M+H]⁺ Calcd for C₂₂H₂₈N₂O, 337.2274; found 337.2275.

1-((4aR)-4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-1-phenylethan-1-ol (13bb): white solid obtained in 82% yield; mp 172-174.5 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.27; [α]_D²⁰ = -26 (c 1.0, MeOH); ¹H NMR (CDCl₃, 499.73 MHz) (exchangeable proton not observed) δ 1.27 (s, 3H), 1.62-1.66 (m, 2H), 1.68 (s, 3H), 1.74-1.79 (m, 2H), 2.04-2.07 (d, 1H), 2.24-2.28 (d, 1H), 2.33-2.40 (m, 4H), 2.60-2.63 (d, 1H), 3.10-3.13 (d, 1H), 6.14 (s, 1H), 7.23-7.29 (m, 4H), 7.33-7.36 (m, 4H), 7.49-7.52 (m, 2H); ¹³C NMR (CDCl₃, 125.66 MHz) δ 20.41, 21.02, 25.82, 26.11, 28.40, 29.68, 34.20, 35.90, 44.00, 56.78, 78.79, 109.52, 115.37, 123.26, 125.56, 126.74, 128.02, 129.62, 136.05, 136.53, 137.41, 137.72, 151.03; IR (NaCl, thin film) ν_{max} (cm⁻¹): 3365 (OH), 3054 (w), 2928, 2858, 1610 (w), 1519 (m), 821; HRMS (ESI) m/z: [M+H]⁺ Calcd for C₂₇H₃₀N₂O, 399.2430; found 399.2432.

1-(4-fluorophenyl)-1-((4aR)-4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)ethan-1-ol (13bc): white solid obtained in 80% yield; mp 188.5-191 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.25; [α]_D²⁰ = -9 (c 1.0, MeOH); ¹H NMR (CDCl₃, 499.73 MHz) (exchangeable proton not observed) δ 1.26 (s, 3H), 1.60-1.63 (m, 3H), 1.66 (s, 3H), 1.75-1.79 (d, 1H), 1.99-2.02 (d, 1H), 2.24-2.27 (d, 1H), 2.39 (s, 3H), 2.60-2.63 (d, 1H), 3.12-3.15 (d, 1H), 5.30 (s, 1H), 6.14 (s, 1H), 7.01-7.48 (m, 9H); ¹³C NMR (CDCl₃, 125.66 MHz) δ 20.40, 21.02, 25.82, 26.16, 28.46, 34.15, 36.03, 43.98, 56.89, 78.55, 109.60, 114.59, 115.25, 123.26, 127.24, 129.63, 136.58, 137.69, 150.94; IR (NaCl, thin film) ν_{max} (cm⁻¹): 3315 (OH), 3020 (w), 2928, 2860, 1600, 1519, 1222; HRMS (DART-RTOF) m/z: [M+H]⁺ Calcd for C₂₇H₂₉FN₂O, 417.23367; found 417.23232.

1-(3,4-difluoro-5-methoxyphenyl)-1-((4aR)-4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)ethan-1-ol (13bd): white solid obtained in 94% yield, mp 181-183 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.19; $[\alpha]_D^{20}$ = +13 (c 3.0, MeOH); ^1H NMR (CDCl_3 , 499.73 MHz) (exchangeable proton not observed) δ 1.22-1.35 (m, 6H), 1.58-1.62 (m, 4H), 1.79-1.83 (d, 1H), 1.96-1.99 (d, 1H), 2.25-2.28 (d, 1H), 2.34-2.44 (s, 3H), 2.57-2.61 (d, 1H), 3.05-3.08 (d, 1H), 3.91-3.96 (s, 3H), 6.15 (s, 1H), 6.87-6.92 (m, 2H), 7.23-7.30 (m, 4H), 7.36 (s, 1H); ^{13}C NMR (CDCl_3 , 125.66 MHz) δ 20.53, 25.64, 26.24, 28.90, 33.96, 35.77, 44.02, 56.34, 123.27, 129.65, 136.66; IR (NaCl, thin film) ν_{max} (cm^{-1}): 3348 (OH), 2927, 2853, 1622, 1520, 1451, 1420, 1334, 1228, 1101; HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{28}\text{H}_{30}\text{F}_2\text{N}_2\text{O}_2$, 465.2348; found 465.2349.

1-((4aR)-4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-1-(thiophen-2-yl)ethan-1-ol (13be): white solid obtained in 76% yield; mp 90-95 °C; TLC (Silica G w/UV254) 25% EtOAc in hexanes, R_f = 0.28; $[\alpha]_D^{20}$ = -12 (c 3.0, MeOH); ^1H NMR (CDCl_3 , 499.73 MHz) (exchangeable proton not observed) δ 1.25 (s, 3H), 1.40-1.41 (d, 1H), 1.57-1.59 (d, 1H), 1.65 (s, 3H), 1.69-1.70 (d, 2H), 1.76-1.78 (d, 1H), 2.03-2.06 (d, 1H), 2.26-2.28 (d, 1H), 2.39 (s, 3H), 2.66-2.69 (d, 1H), 3.22-3.25 (d, 1H), 6.15 (s, 1H), 7.12-7.37 (m, 8H); ^{13}C NMR (CDCl_3 , 125.66 MHz) δ 20.13, 21.02, 26.16, 28.19, 29.68, 34.31, 35.80, 43.88, 56.70, 109.66, 115.39, 119.69, 123.26, 125.64, 126.49, 129.63, 136.56, 137.73, 150.91; IR (NaCl, thin film) ν_{max} (cm^{-1}): 3381 (OH), 3105 (w), 3042 (w), 2928, 2859, 1611, 1518 (m), 821; HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{25}\text{H}_{28}\text{N}_2\text{OS}$, 405.1995; found 405.1996.

2-((4aR)-1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)propan-2-ol (13ca): white solid obtained in 58% yield; mp 194-197 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.08; $[\alpha]_D^{20}$ = -17 (c 1.0, MeOH); ^1H NMR (CDCl_3 , 499.73 MHz) (exchangeable proton not observed) δ 1.19 (s, 3H) 1.30-1.38 (d, 6H), 1.48-1.55 (m, 1H), 1.66-1.69 (d, 1H), 1.76-1.87 (dd, 2H), 2.27-2.37 (m, 3H), 2.73-2.76 (d, 1H), 3.53-3.57 (d, 1H), 3.85 (s, 3H), 6.11 (s, 1H) 6.95-7.41 (m, 5H); ^{13}C NMR (CDCl_3 , 125.66 MHz) δ 19.66, 26.38, 27.48, 28.39, 29.68, 33.91, 34.51, 36.20, 43.60, 55.52, 57.61, 75.49, 109.57, 114.24, 114.96, 124.86, 133.10, 136.29, 137.41,

150.90, 158.42; IR (NaCl, thin film) ν_{max} (cm⁻¹): 3349 (OH), 3018, 2928, 2857, 1612, 1517, 1248, 831; HRMS (ESI) m/z: [M+H]⁺ Calcd for C₂₂H₂₈N₂O₂, 353.2223; found 353.2222.

1-((4aR)-1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-1-phenylethan-1-ol (13cb): beige solid obtained in 100% yield, mp 105.1-108 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.20; $[\alpha]_D^{20}$ = -30 (c 1.0, CHCl₃); ¹H NMR (CDCl₃, 499.73 MHz) (exchangeable proton not observed) δ 1.27 (s, 3H), 1.55-1.63 (m, 3H), 1.68 (s, 3H), 1.75-1.77 (m, 1H), 2.04-2.07 (m, 1H), 2.23-2.26 (m, 1H), 2.33-2.38 (m, 1H), 2.60-2.63 (d, 1H), 3.10-3.13 (d, 1H), 3.84 (s, 3H), 6.08 (s, 1H), 6.95-7.51 (m, 10H); ¹³C NMR (CDCl₃, 125.66 MHz) δ 20.43, 25.82, 26.20, 28.38, 29.68, 34.19, 35.91, 44.03, 55.48, 56.79, 78.80, 109.33, 114.22, 115.10, 125.55, 128.01, 133.11, 136.12, 137.46, 151.03, 158.39; IR (NaCl, thin film) ν_{max} (cm⁻¹): 3338 (OH), 3020 (w), 2925, 2854, 1611, 1517, 1250, 833; HRMS (DART-RTOF) m/z: [M+H]⁺ Calcd for C₂₇H₃₀N₂O₂, 415.23800; found 415.23680.

1-(4-fluorophenyl)-1-((4aR)-1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)ethan-1-ol (13cc): white solid obtained in 49% yield; mp 182.1-185 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.28; $[\alpha]_D^{20}$ = -51 (c 1.0, MeOH); ¹H NMR (CDCl₃, 300.08 MHz) (exchangeable proton not observed) δ 1.25 (s, 3H), 1.53-1.62 (m, 2H), 1.67 (s, 3H), 1.73-1.78 (m, 1H), 1.99-2.02 (d, 1H), 2.24-2.36 (m, 3H), 2.60-2.63 (d, 1H), 3.12-3.15 (d, 1H), 3.85 (s, 3H), 6.09 (s, 1H), 6.95-7.49 (m, 9H); ¹³C NMR (CDCl₃, 499.73 MHz) δ 20.42, 25.82, 26.16, 28.46, 29.69, 34.14, 36.05, 44.02, 55.53, 56.90, 78.55, 109.48, 114.23, 114.59, 114.76, 124.84, 127.24, 136.09, 137.48, 150.91; IR (NaCl, thin film) ν_{max} (cm⁻¹): 3363 (OH), 2929, 2840, 1600, 1517, 1250, 834; HRMS (DART-RTOF) m/z: [M+H]⁺ Calcd for C₂₇H₂₉FN₂O₂, 433.22858; found 433.22897.

1-(3,4-difluoro-5-methoxyphenyl)-1-((4aR)-1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)ethan-1-ol (13cd): white solid obtained in 65% yield, mp 184-187 °C, TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.13; $[\alpha]_D^{20}$ = +55 (c 1.0, MeOH); ¹H NMR (CDCl₃, 499.73 MHz) δ 1.26 (s, 3H), 1.60-1.67 (m, 6H) 1.79-1.82(d, 1H), 1.96-1.99 (d, 1H), 2.24-2.39 (m, 2H), 2.57-2.60 (d,

1H), 3.05-3.08 (d, 1H), 3.85 (s, 3H), 3.91 (s, 3H), 6.10 (s, 1H), 6.87-7.01 (m, 4H), 7.32-7.45 (m, 3H); ¹³C NMR (CDCl₃, 125.66 MHz) δ 20.54, 25.65, 26.24, 28.89, 33.95, 35.79, 44.05, 55.53, 56.35, 56.78, 106.27, 106.78, 109.50, 114.25, 114.75, 124.85, 133.03, 137.42, 150.81; IR (NaCl, thin film) ν_{max} (cm⁻¹): 3329 (OH), 2932, 2850, 1622, 1518, 1251, 1100, 834; HRMS (DART-RTOF) m/z: [M+H]⁺ Calcd for C₂₇H₃₀FN₂O₂, 433.22858; found 433.22897.

1-((4aR)-1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-1-(thiophen-2-yl)ethan-1-ol (13ce): white solid obtained in 48% yield; mp 146-148.5 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.16; [α]_D²⁰ = +12 (c 1.0, MeOH); ¹H NMR (CDCl₃, 499.73 MHz) (exchangeable proton not observed) δ 1.25 (s, 3H), 1.59-1.65 (m, 2H), 1.68 (s, 3H), 1.73-1.78 (m, 2H), 2.03-2.06 (d, 1H), 2.25-2.36 (m, 2H), 2.65-2.69 (d, 1H), 3.22-3.25 (d, 1H), 3.84 (s, 3H), 6.10 (s, 1H), 6.94-7.40 (m, 8H); ¹³C NMR (CDCl₃, 125.66 MHz) δ 20.16, 26.16, 28.19, 34.31, 35.82, 43.91, 55.48, 56.71, 77.52, 109.47, 114.23, 115.13, 119.72, 124.88, 126.51, 133.11, 136.13, 137.47, 150.93, 152.99, 158.40; IR (NaCl, thin film) ν_{max} (cm⁻¹): 3365 (OH), 2930, 2850, 1610, 1517, 1250, 835; HRMS (ESI) m/z: [M+H]⁺ Calcd for C₂₅H₂₈N₂O₂S, 421.1944; found 421.1943.

APPENDIX

1.9.1 - ^1H NMR and ^{13}C NMR Spectra

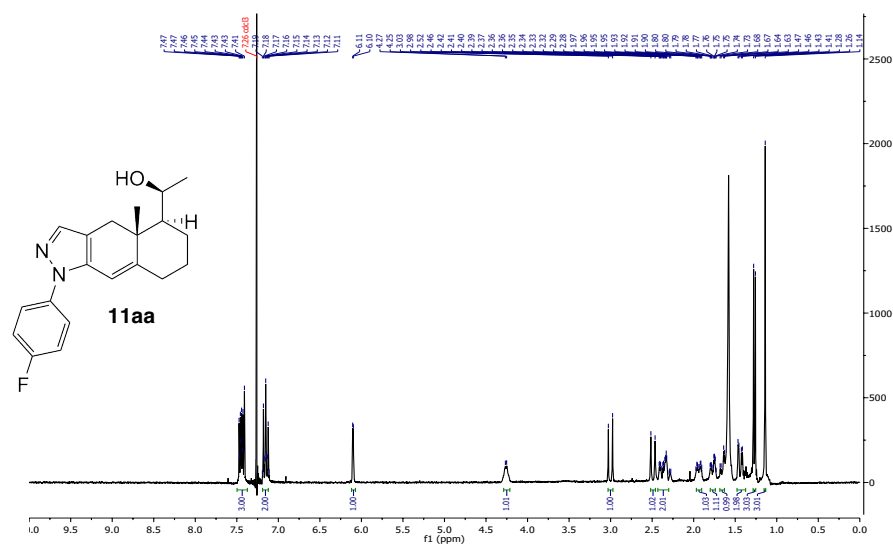


Figure 1.9.1 - ^1H NMR of **11aa**

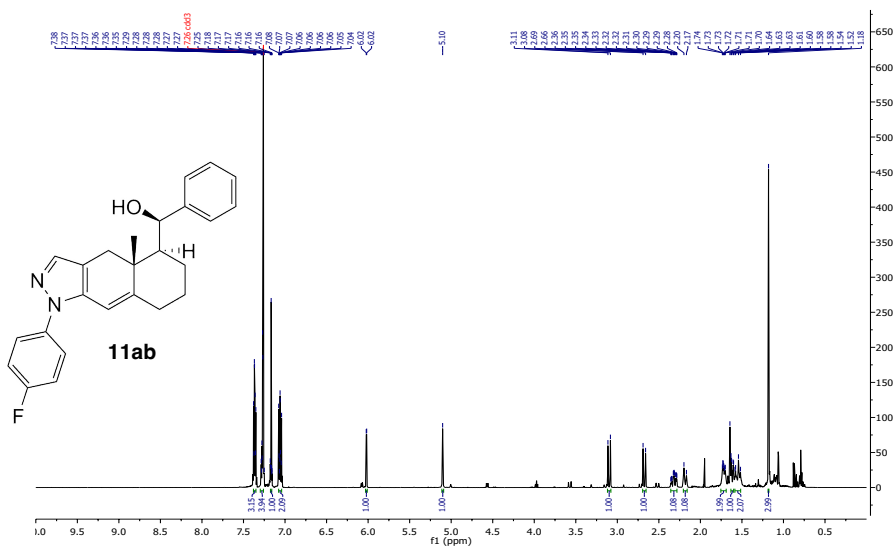


Figure 1.9.2 - ^1H NMR of **11ab**

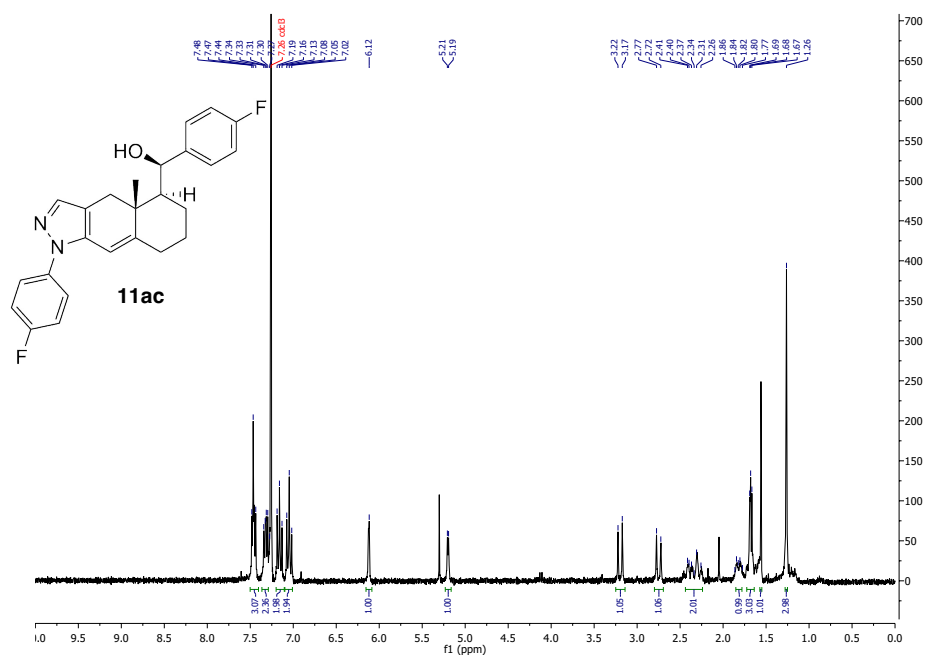


Figure 1.9.3 - ¹H NMR of **11ac**

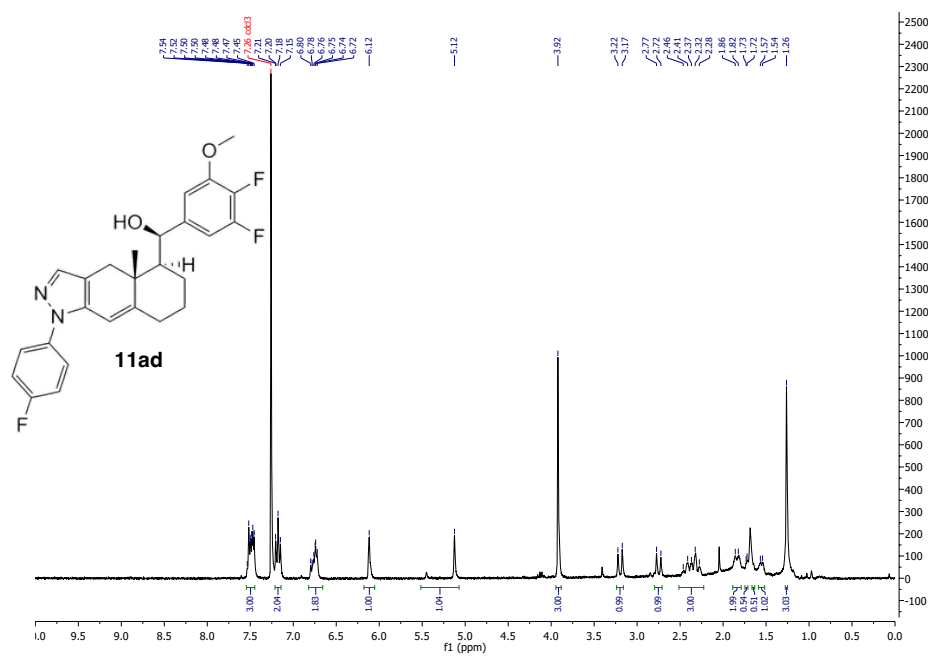


Figure 1.9.4 - ¹H NMR of **11ad**

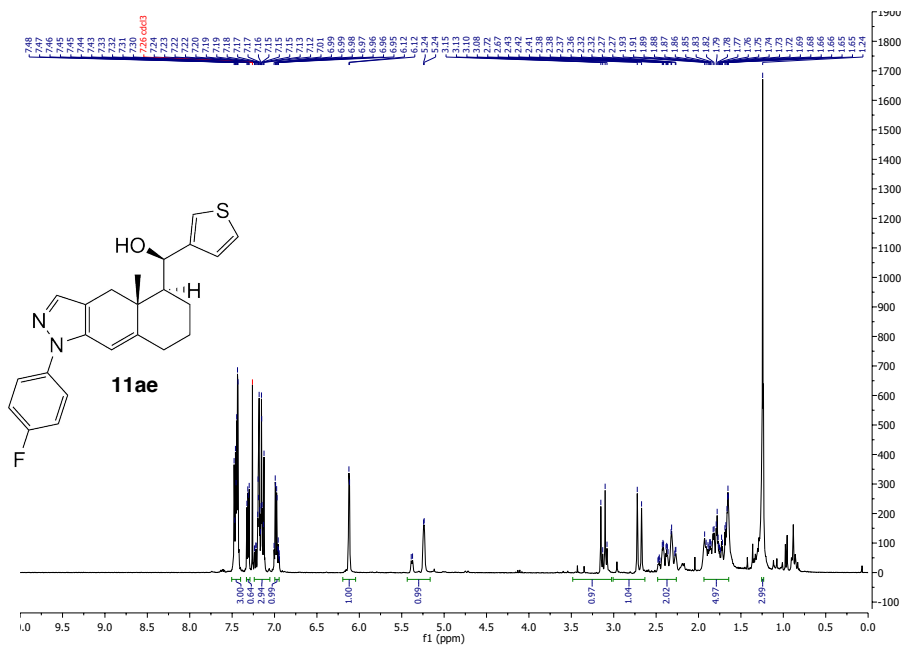


Figure 1.9.5 - ¹H NMR of **11ae**

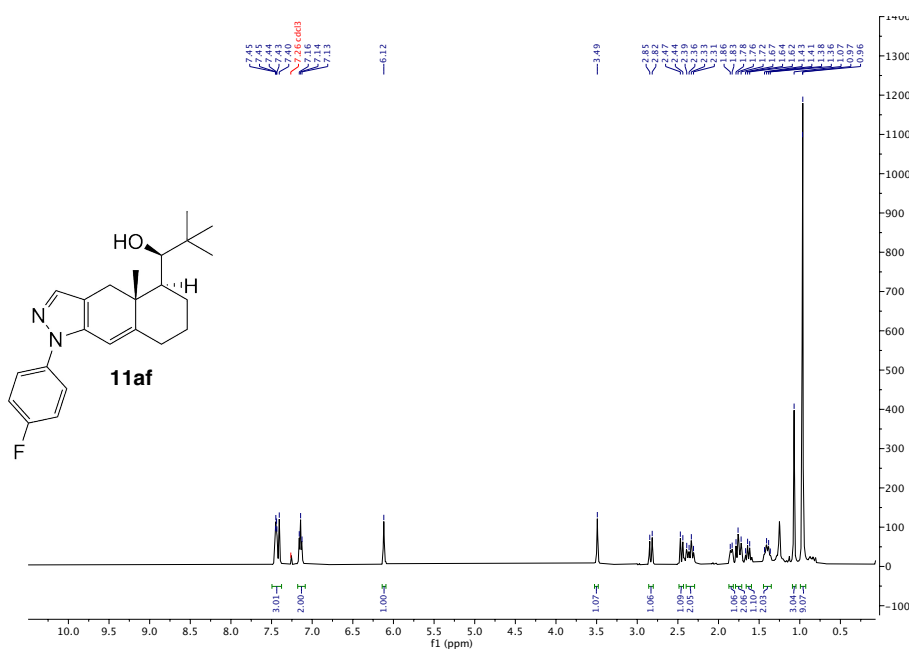


Figure 1.9.6 - ¹H NMR of **11af**

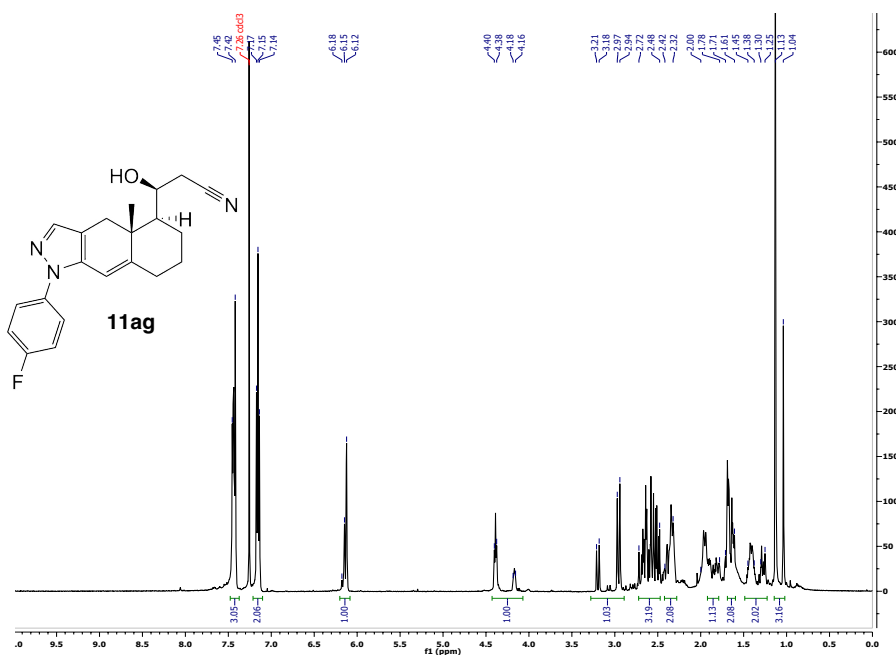


Figure 1.9.7 - ^1H NMR of **11ag**

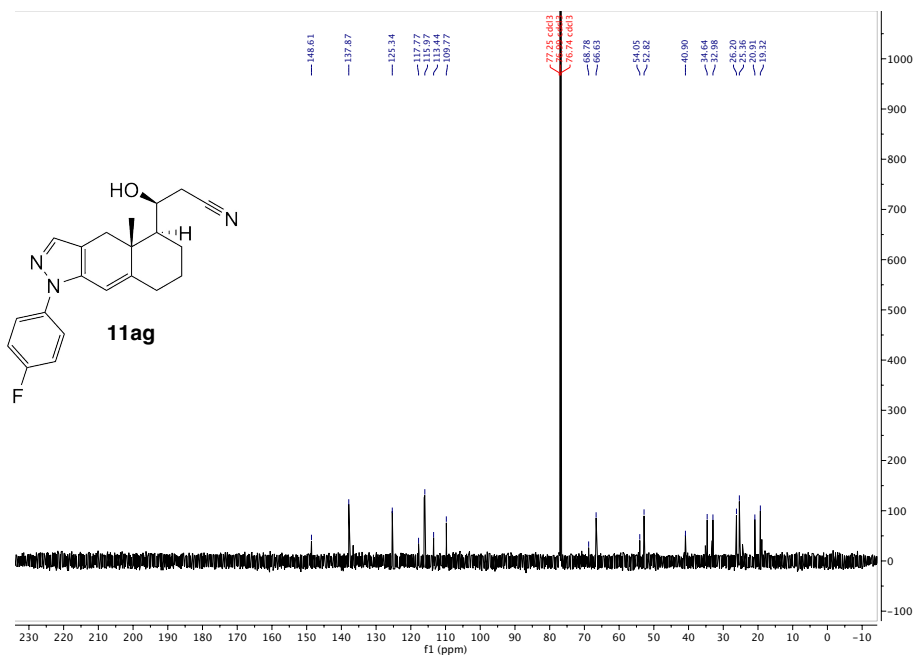


Figure 1.9.8 - ^{13}C NMR of **11ag**

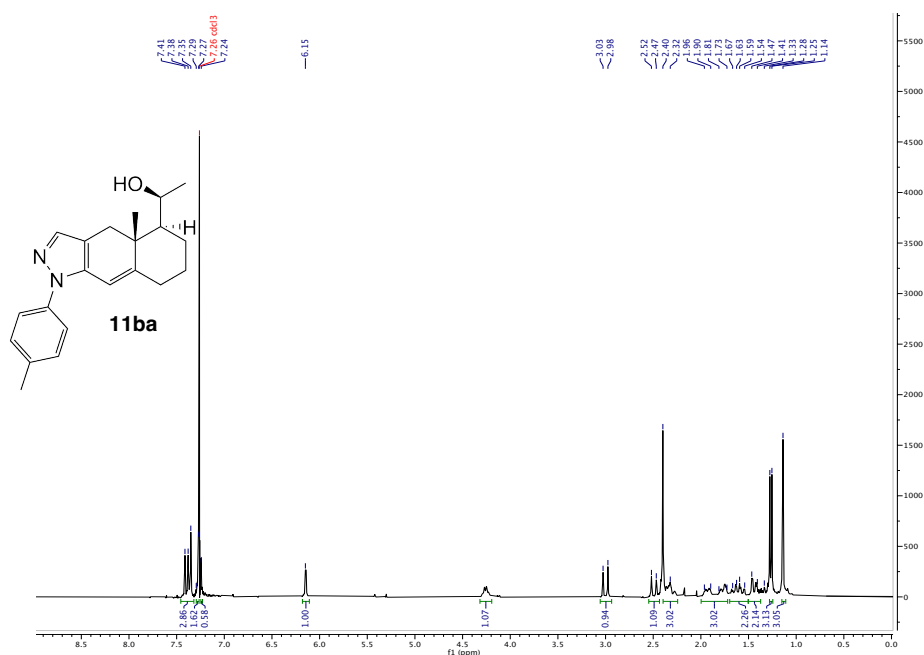


Figure 1.9.9 - ¹H NMR of **11ba**

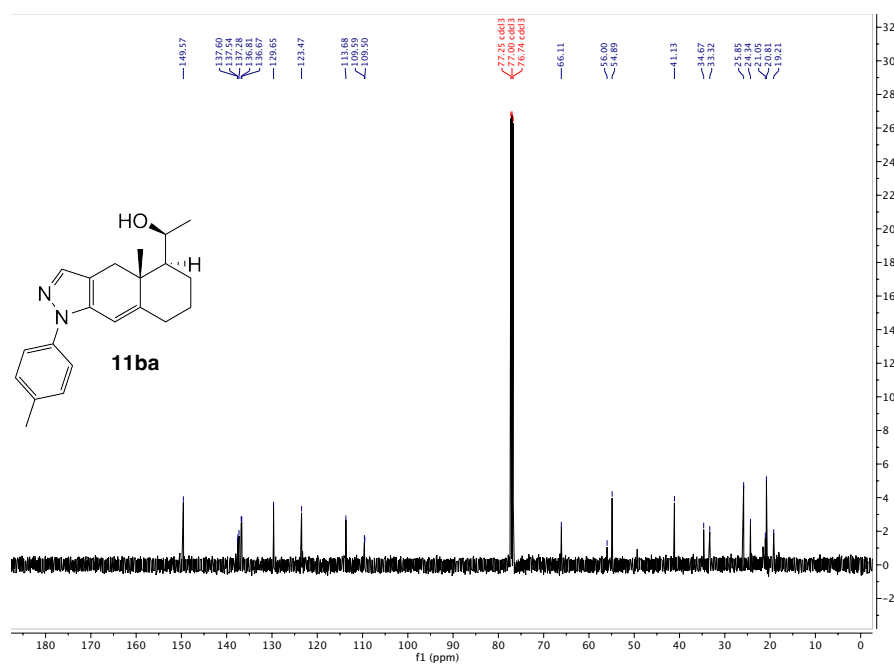


Figure 1.9.10 - ¹³C NMR of **11ba**

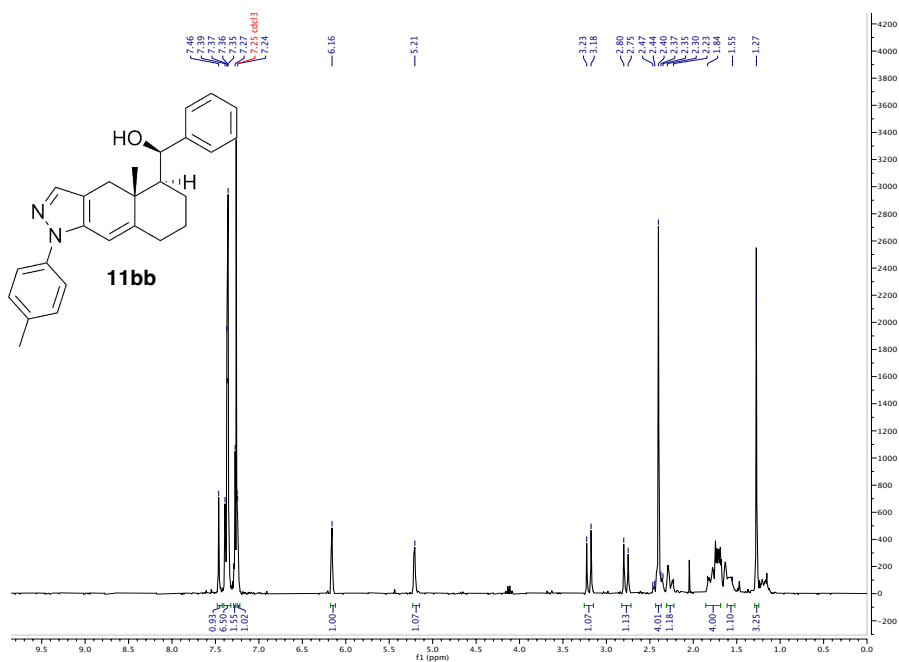


Figure 1.9.11 - ^1H NMR of **11bb**

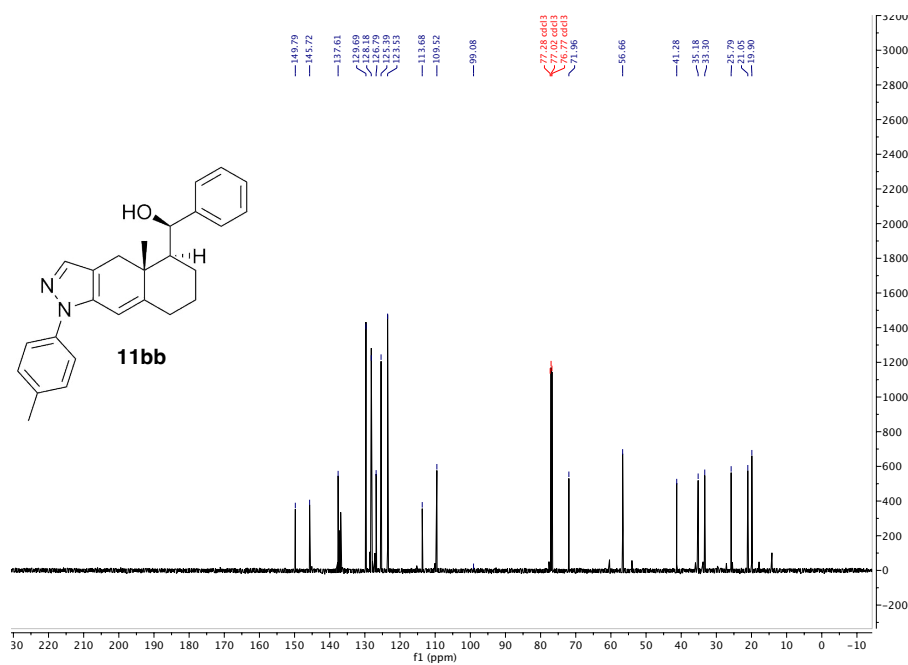
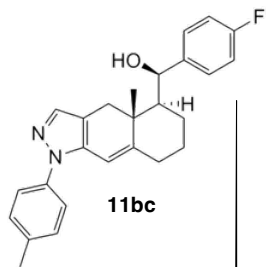


Figure 1.9.12 - ^{13}C NMR of **11bb**



Chemical structure of **11bc** is shown. The ¹H NMR spectrum (CDCl₃) displays peaks at the following chemical shifts (ppm): 162.67, 160.73, 149.50, 141.25, 137.29, 137.23, 136.91, 136.81, 129.70, 129.62, 128.87, 128.70, 123.52, 115.06, 114.49, 113.95, 108.63, 77.26 (CDCl₃), 77.00 (CDCl₃), 71.48, 56.75, 41.24, 35.34, 33.46, 25.74, 21.64, and 19.91.

50

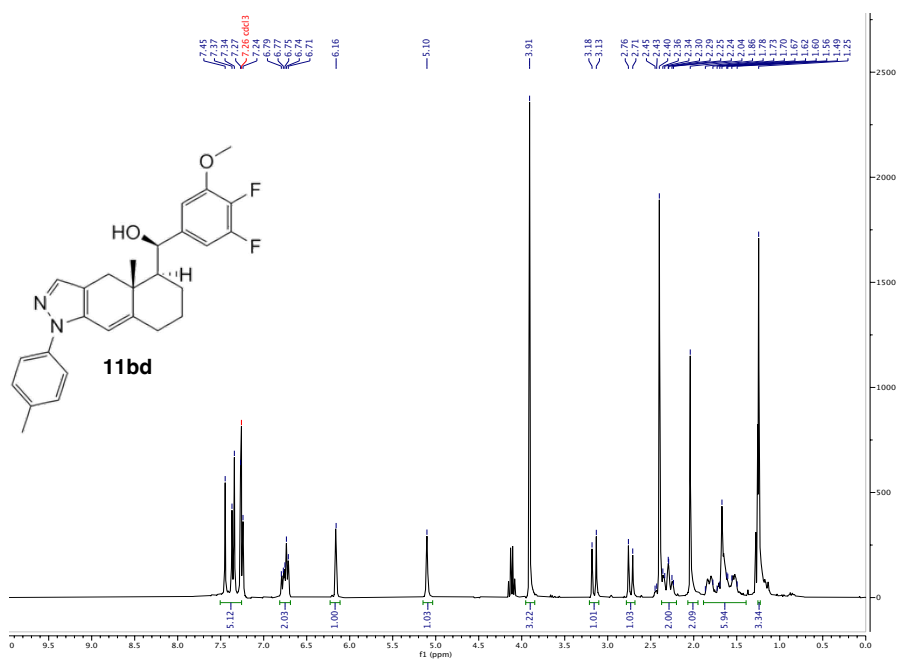


Figure 1.9.15 - ^1H NMR of **11bd**

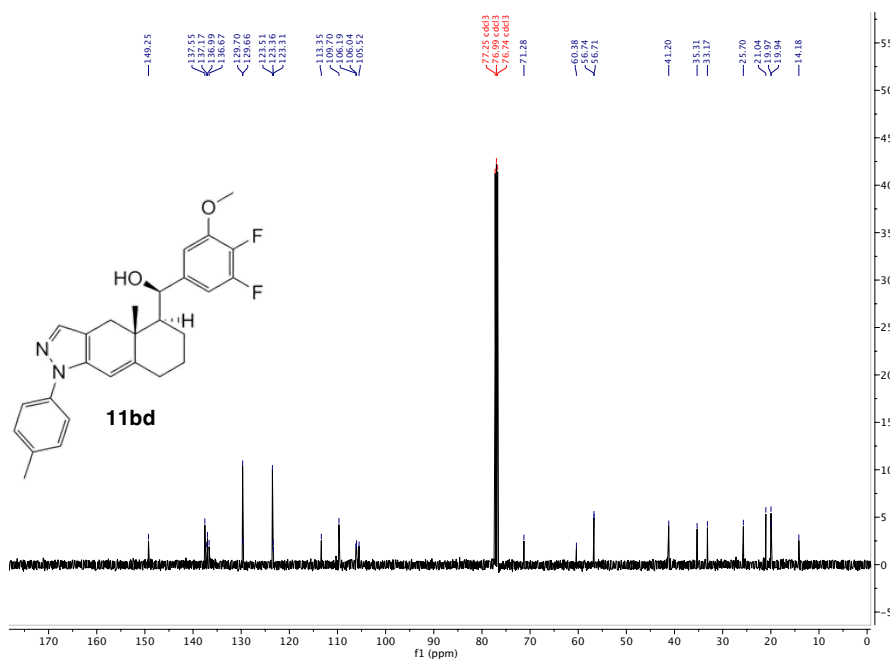


Figure 1.9.16 - ^{13}C NMR of **11bd**

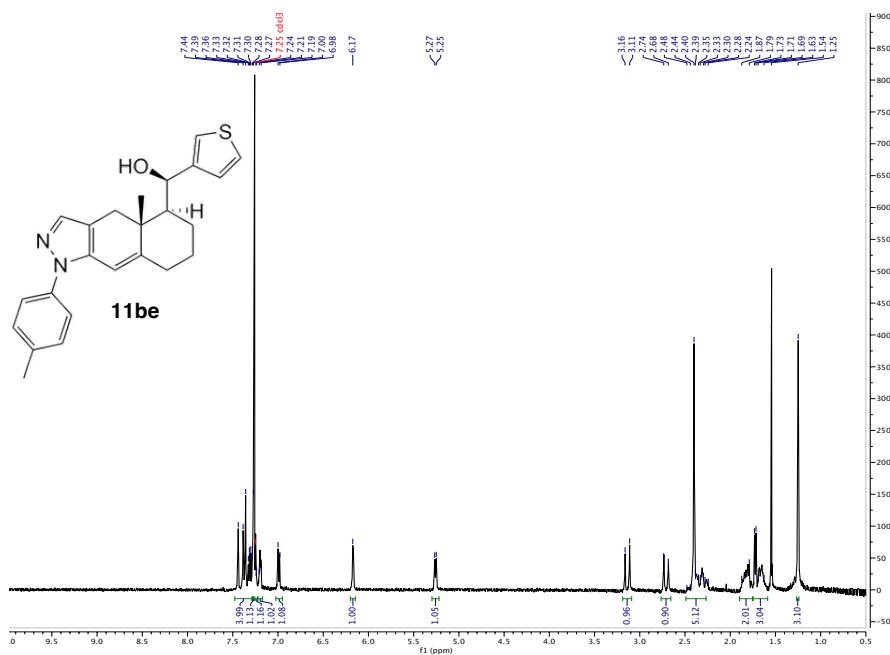


Figure 1.9.17 - ^1H NMR of **11be**

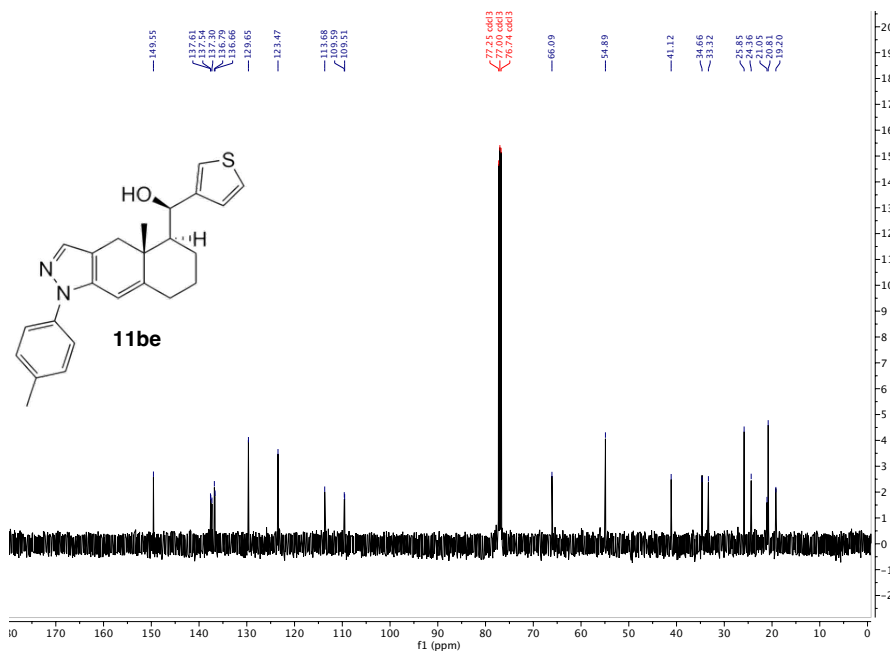
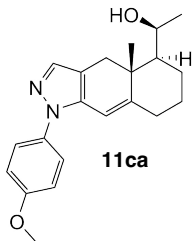


Figure 1.9.18 - ^{13}C NMR of **11be**



Chemical structure of **11ca** is shown. The ¹³C NMR spectrum (f1 (ppm)) displays peaks corresponding to the structure. The peak list is as follows:

Peak Value (ppm)
158.54
149.51
137.40
136.73
136.73
133.01
114.25
113.42
109.38
77.24
76.99
76.63
68.10
55.49
54.89
41.16
34.68
33.31
25.85
24.36
20.80
19.21

53

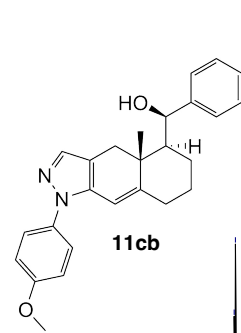


Figure 1.9.21 - ^1H NMR of **11cb**

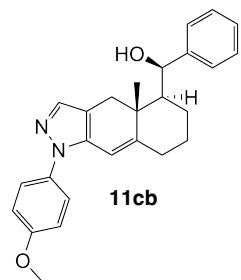
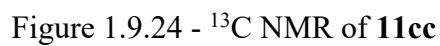
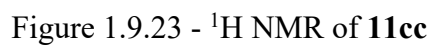


Figure 1.9.22 - ^{13}C NMR of **11cb**



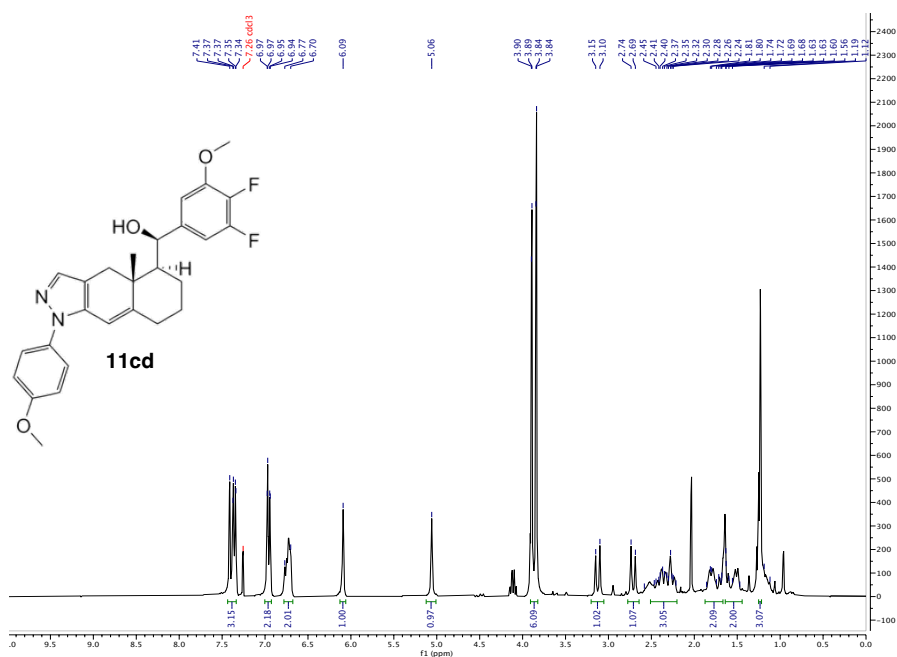


Figure 1.9.25 - ^1H NMR of **11cd**

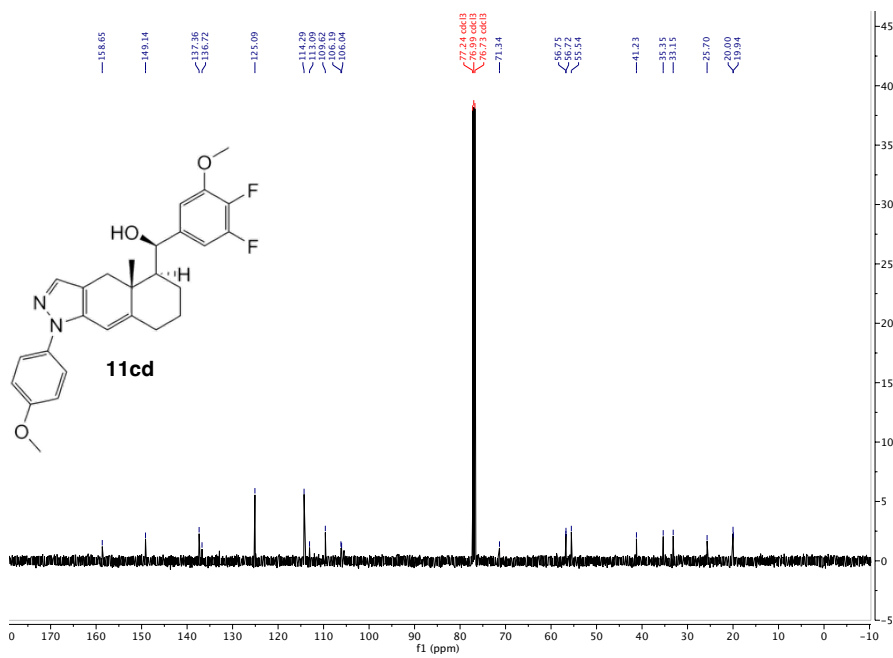


Figure 1.9.26 - ^{13}C NMR of **11cd**

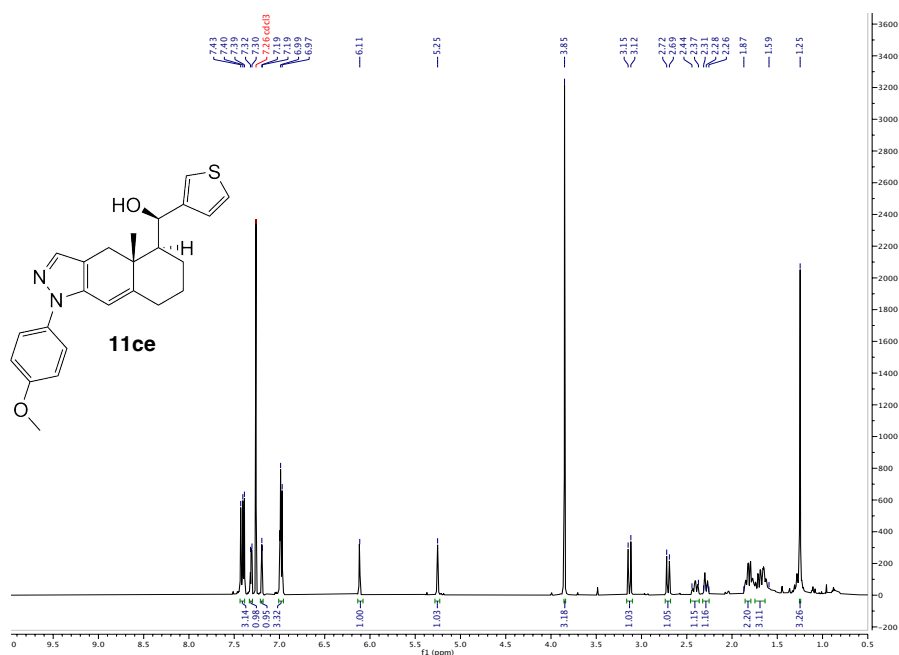


Figure 1.9.27 - ^1H NMR of **11ce**

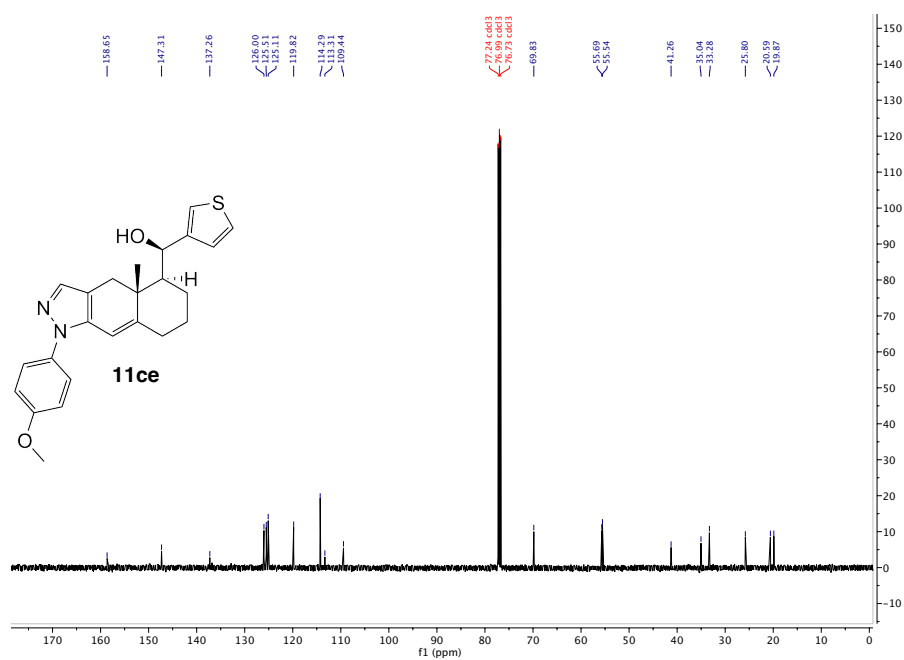


Figure 1.9.28 - ^{13}C NMR of **11ce**

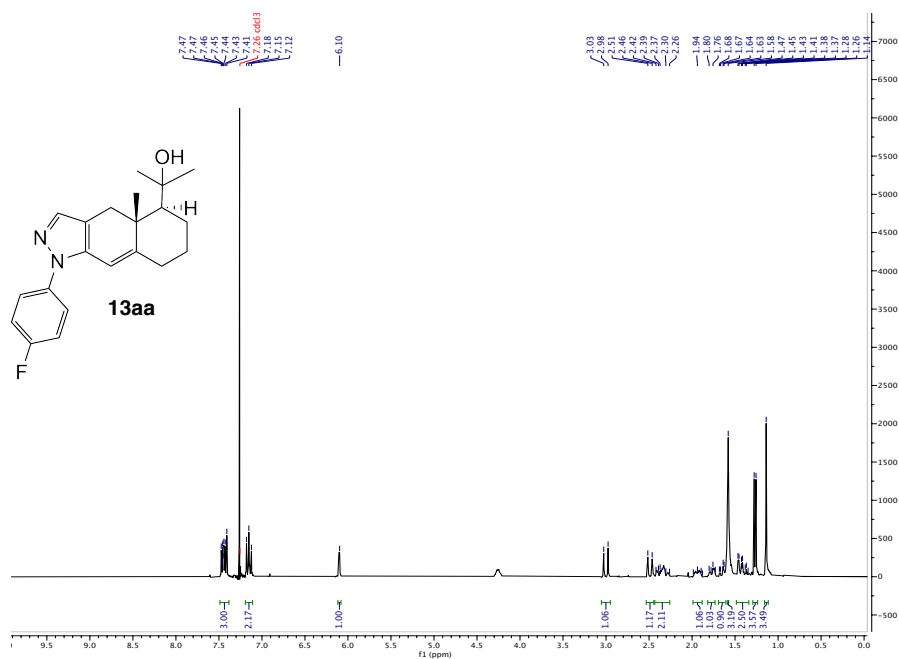


Figure 1.9.29 - ¹H NMR of **13aa**

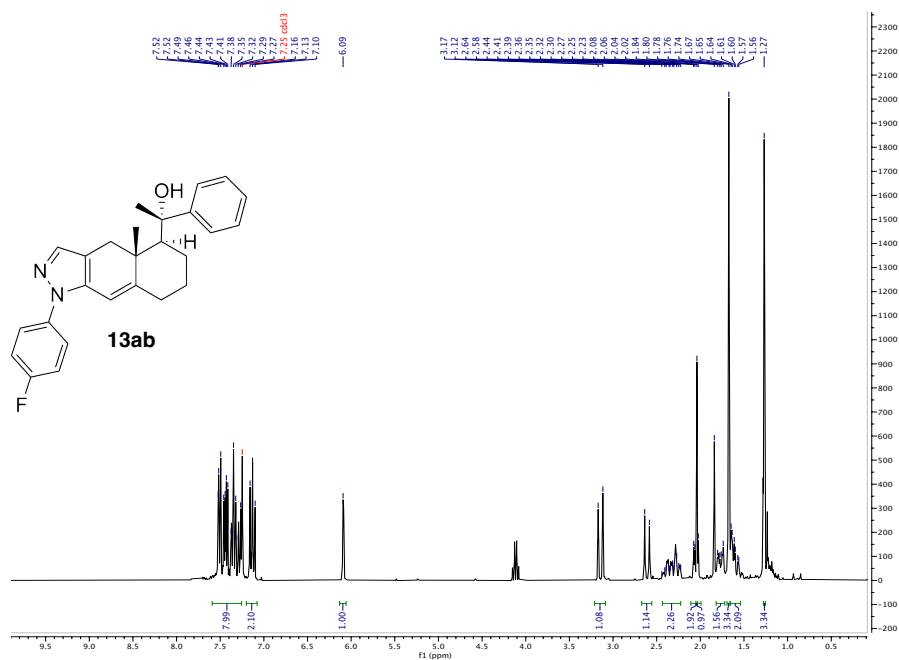


Figure 1.9.30 - ¹H NMR of **13ab**

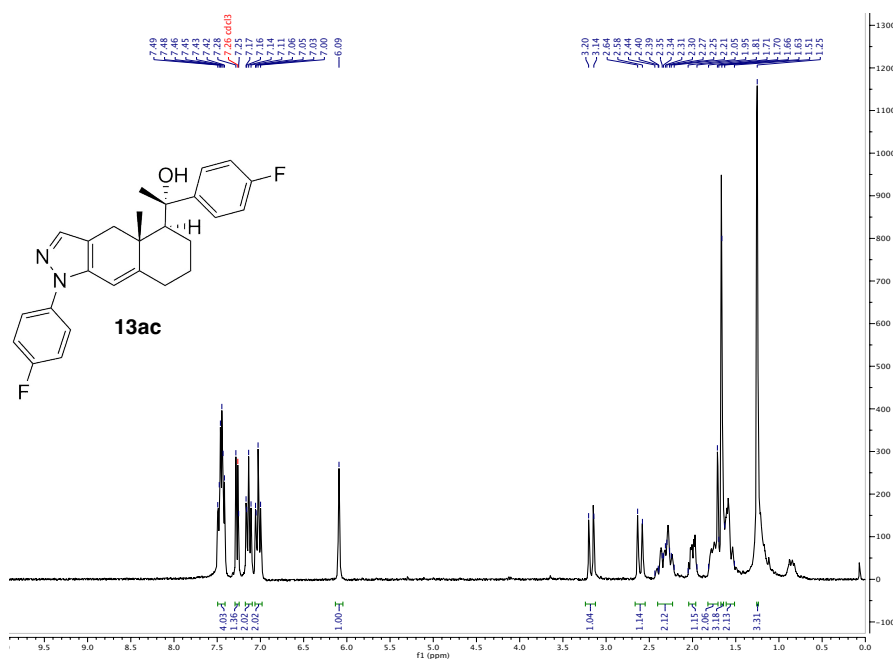


Figure 1.9.31 - ^1H NMR of **13ac**

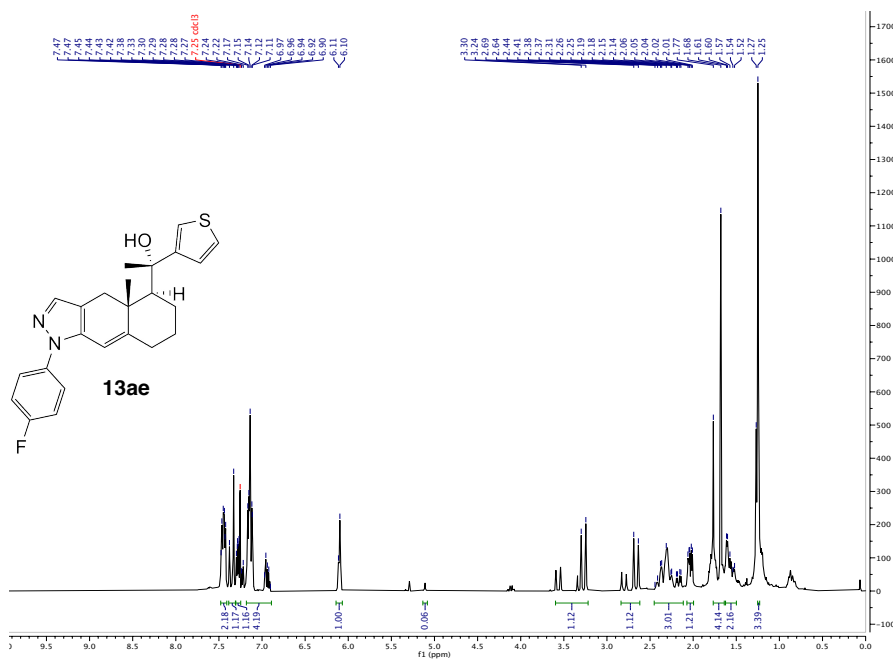


Figure 1.9.32 - ^1H NMR of **13ae**

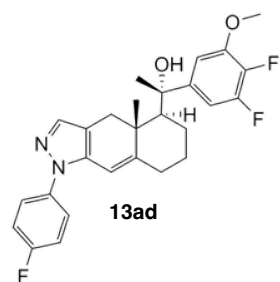


Figure 1.9.33 – ^1H NMR of **13ad**

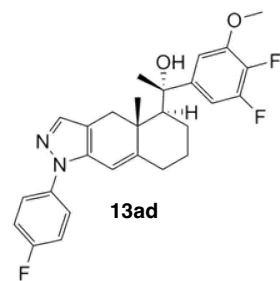


Figure 1.9.34 - ^{13}C NMR of **13ad**

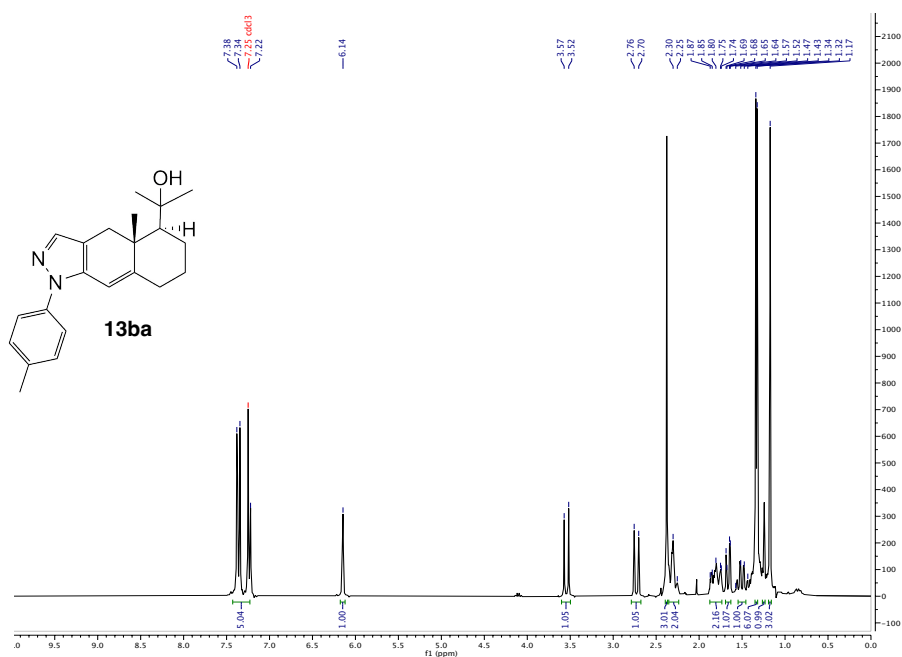


Figure 1.9.35 - ^1H NMR of **13ba**

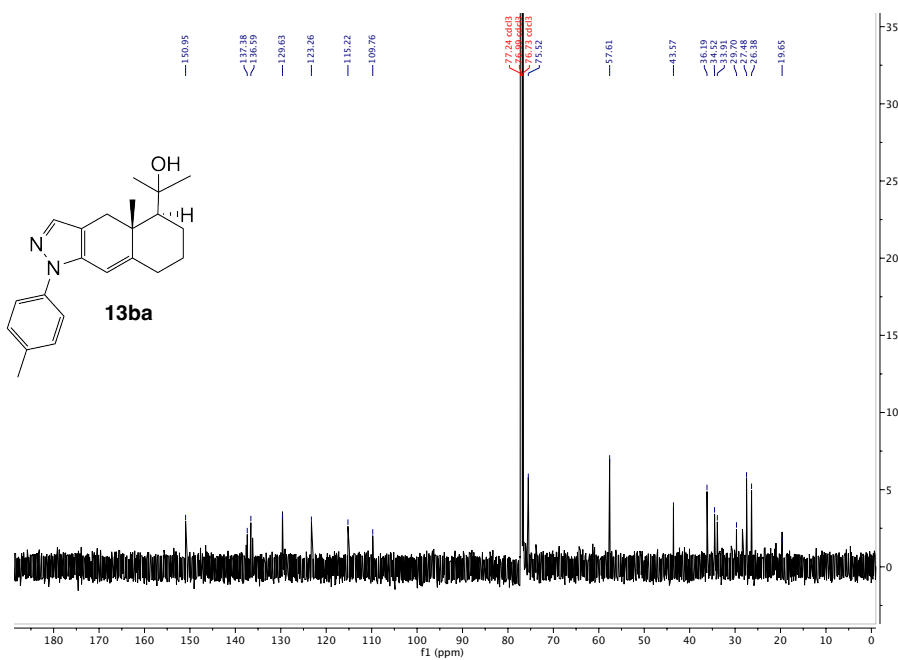
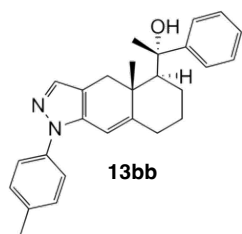


Figure 1.9.36 - ^{13}C NMR of **13ba**



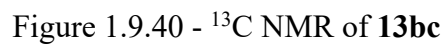
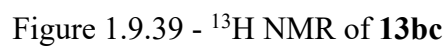
13bb

¹³C NMR spectrum (CDCl₃) of compound **13bb**. The chemical structure of **13bb** is shown above the spectrum. The spectrum displays peaks corresponding to the carbon atoms in the molecule, with the following chemical shifts (ppm) labeled on the right side:

- 151.03, 137.72, 136.51, 136.05, 128.02, 128.74, 123.26, 113.37, 109.52, 79.79, 77.23 (dcl3), 77.00 (dcl3), 76.74 (dcl3), 56.78, 44.00, 35.90, 34.20, 33.40, 28.40, 28.20, 26.11, 25.82, 25.40, 20.41.



13bb



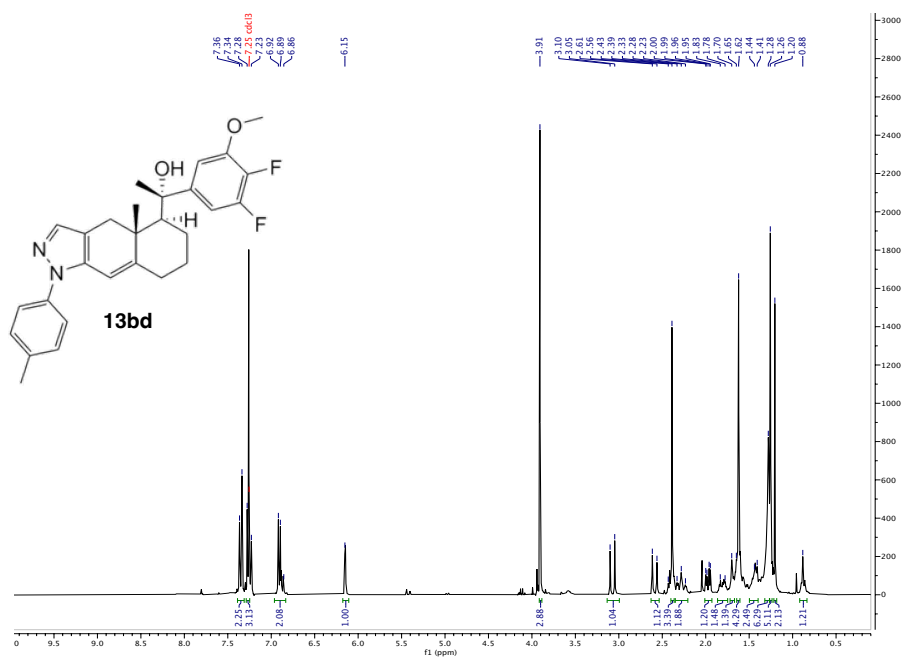


Figure 1.9.41 - ^1H NMR of **13bd**

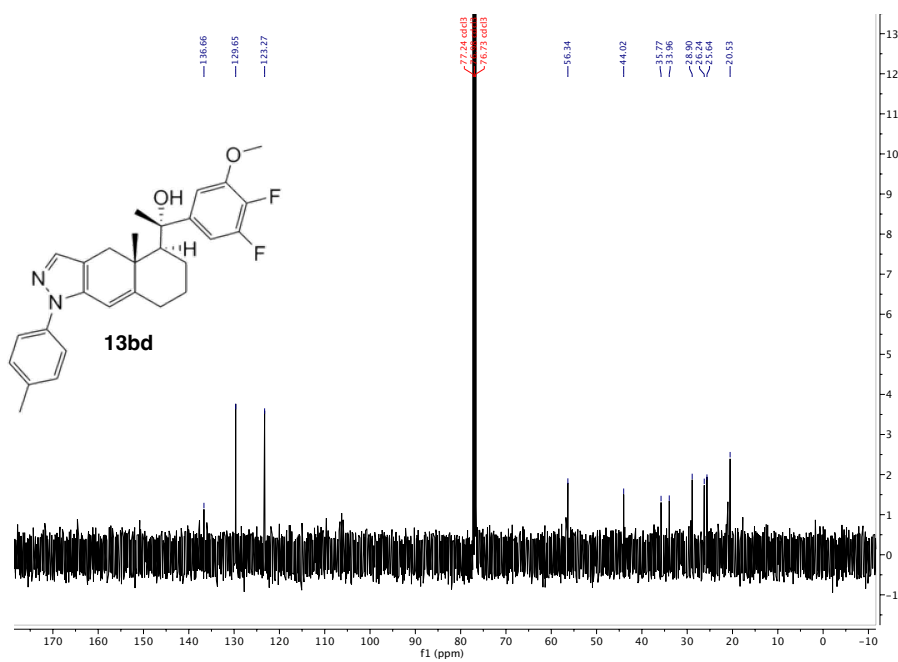
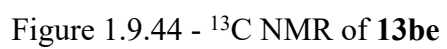
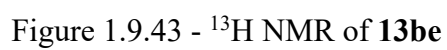


Figure 1.9.42 - ^{13}C NMR of **13bd**



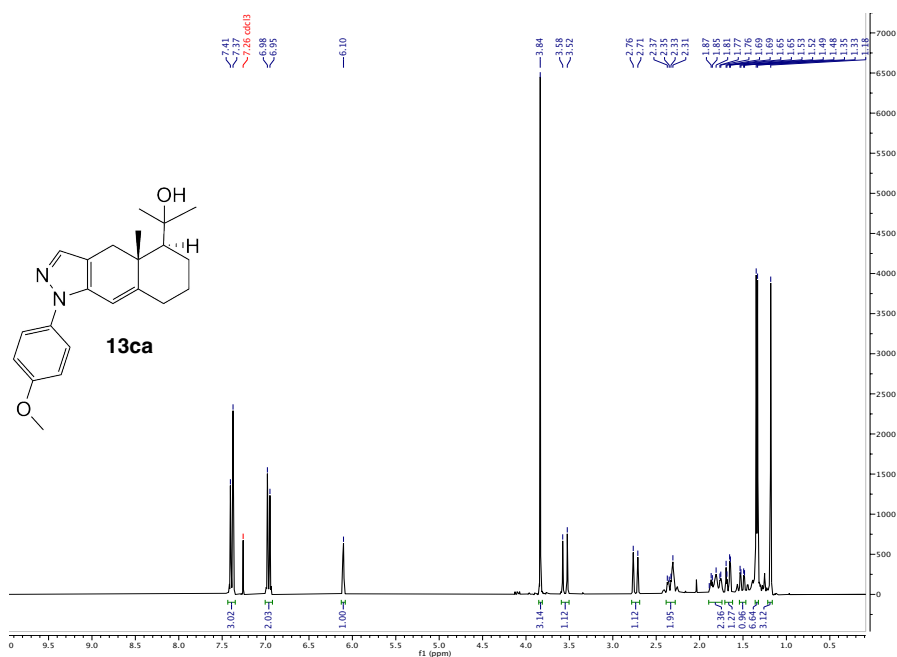


Figure 1.9.45 - ¹H NMR of **13ca**

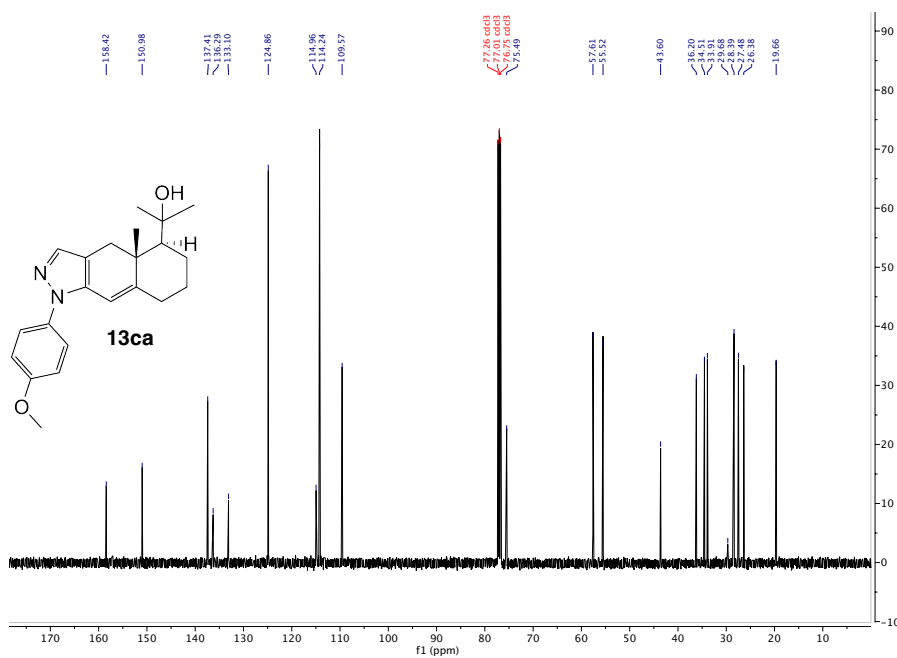
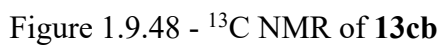
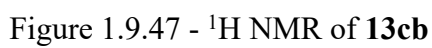


Figure 1.9.46 - ¹³C NMR of **13ca**



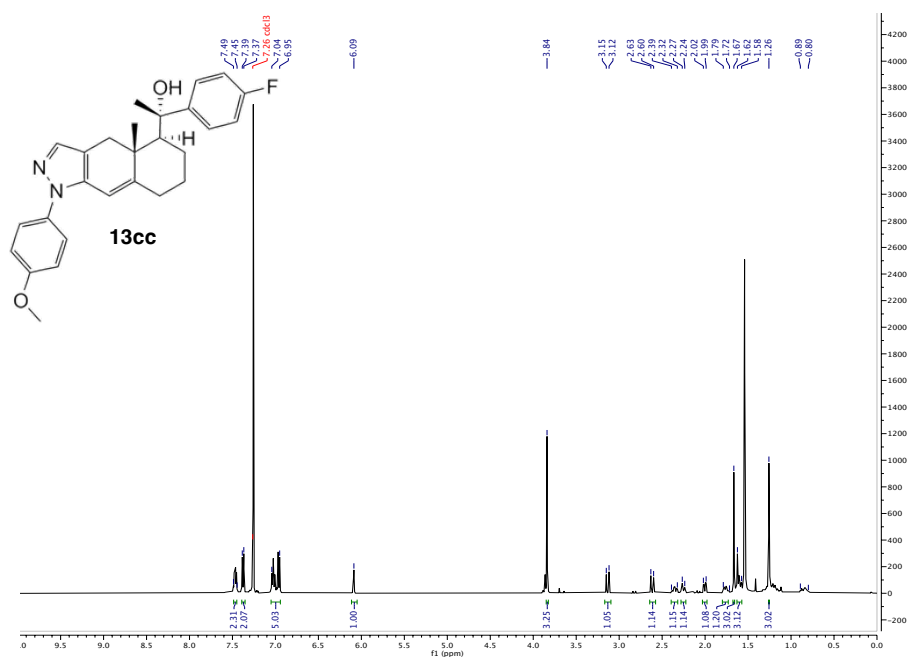


Figure 1.9.49 - ^1H NMR of **13cc**

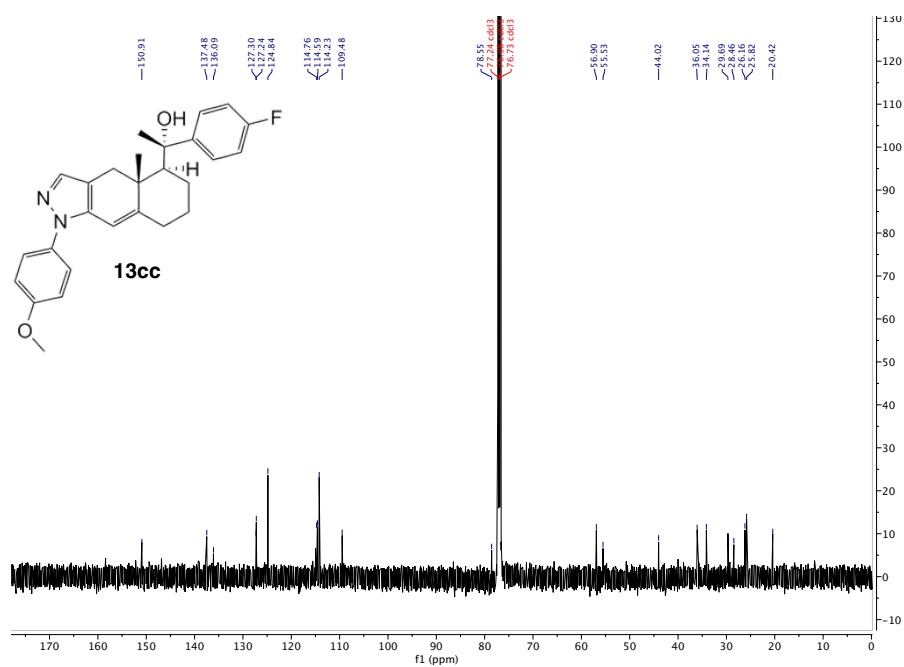


Figure 1.9.50 - ^{13}C NMR of **13cc**

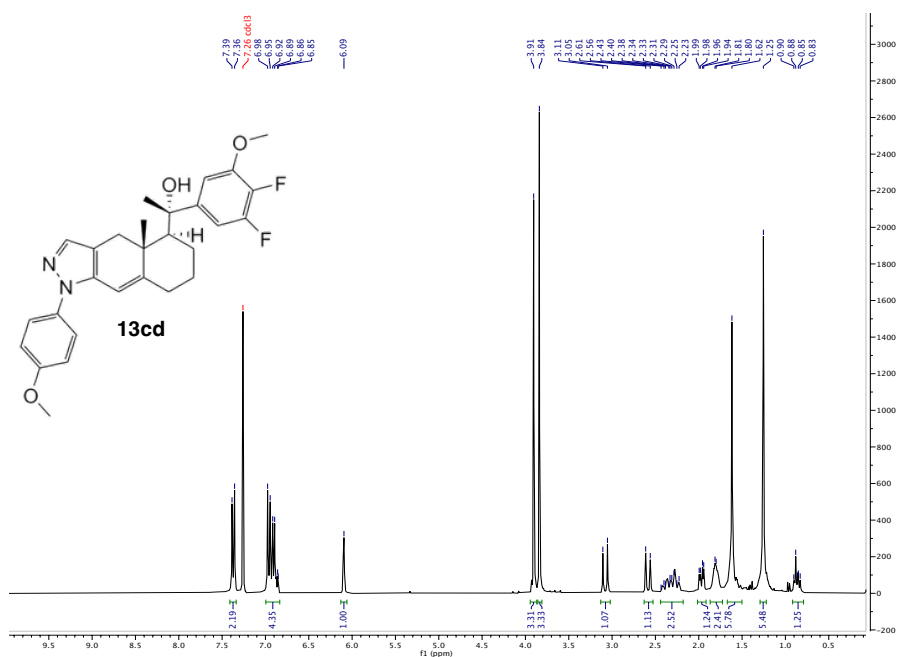


Figure 1.9.51 - ^1H NMR of **13cd**

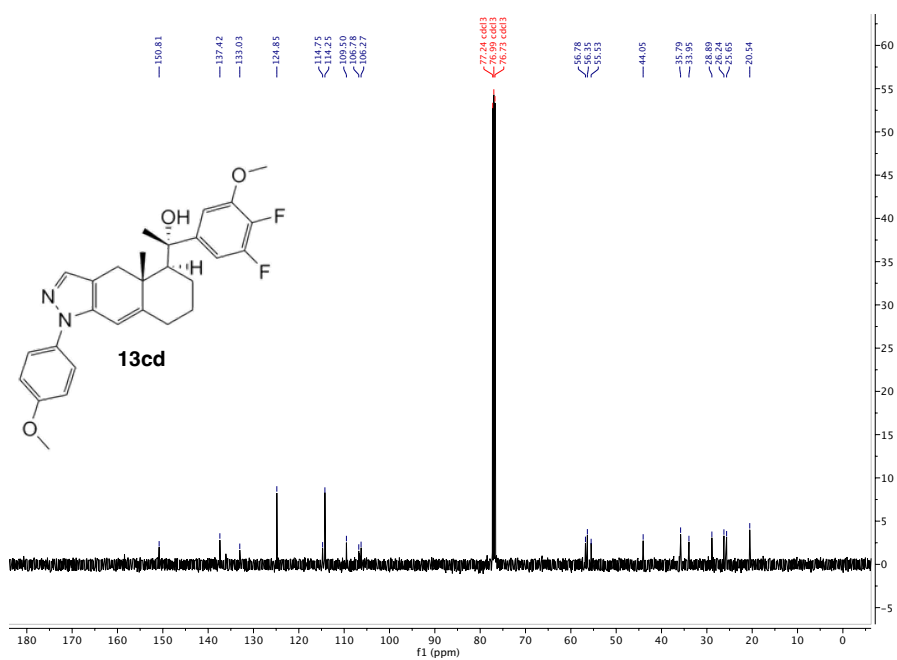
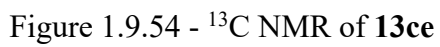
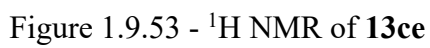


Figure 1.9.52 - ^{13}C NMR of **13cd**



1.9.2 – CCL2 Data

Table 1.9.1 – CCL2 assay raw data

Compd	CCL2 (%max IL-2 response)					
	Concentration of Compound (M)					
	1x10 ⁻¹⁰	1x10 ⁻⁹	1x10 ⁻⁸	1x10 ⁻⁷	1x10 ⁻⁶	1x10 ⁻⁵
HC	66.07 ± 10.08	75.67 ± 15.71	61.1 ± 5.17	74.12 ± 15.93	51.22 ± 19.25	24.95 ± 3.12
Dex	100.52 ± 19.07	60.96 ± 6.01	39.92 ± 9.41	31.21 ± 15.62	14.55 ± 2.77	19.58 ± 2.84
11aa	74.95 ± 32.8	70.29 ± 27.25	44.13 ± 6.22	41.87 ± 10.49	37.69 ± 11.15	25.24 ± 12.82
11ab	61.25 ± 14.67	65.76 ± 20.99	67.22 ± 11.94	33.22 ± 7.42	32.73 ± 11.4	37.23 ± 19.42
11ac	100.95 ± 7.31	81.55 ± 9.66	67.89 ± 10.82	42.93 ± 5.37	37.43 ± 8.63	36.37 ± 9.8
11ad	122.6 ± 34.88	117.8 ± 29.76	85.55 ± 23.32	62.05 ± 13.94	41.91 ± 11.59	42.47 ± 18.87
11ae	93.22 ± 23.16	72.37 ± 40.61	55.82 ± 31.05	36.35 ± 18.35	37.45 ± 27.92	23.22 ± 16.7
11af	125.49 ± 42.1	64.58 ± 30.47	83.82 ± 36.91	72.56 ± 40.27	66.87 ± 28.05	84 ± 65.86
11ag	98.24 ± 6.01	95.43 ± 6.27	89.58 ± 20.45	83.24 ± 3.83	74.6 ± 8.8	48.96 ± 1.82
11ba	74.37 ± 1.17	90.91 ± 12.19	86.81 ± 12.64	87.71 ± 5.63	80.92 ± 5.11	88.88 ± 8.28
11bb	97.55 ± 4.46	106.11 ± 17.07	90.71 ± 34.53	88 ± 23.02	74.87 ± 18.89	60.64 ± 16.44
11bc	96.86 ± 4.39	89.83 ± 3	93.26 ± 1.49	86.81 ± 5.56	70.9 ± 10.46	66.93 ± 18.14
11bd	123.7 ± 1.24	117.87 ± 12.47	128.85 ± 14.36	115.51 ± 3.62	83.14 ± 18.36	101.96 ± 3.42
11be	99.11 ± 12.69	97.8 ± 1.71	87.56 ± 5.57	77.25 ± 4.2	65.66 ± 6.11	63.3 ± 1.44
11ca	97.92 ± 0.17	90.62 ± 1.86	95.89 ± 6.46	93.36 ± 5.4	96.59 ± 21.81	87.33 ± 16.95
11cb	108.43 ± 12.98	94.89 ± 2.82	103.39 ± 5.5	100.43 ± 0.7	85.07 ± 5.81	68.7 ± 7.36
11cc	121.68 ± 12.98	112.05 ± 11.92	102.19 ± 7.58	100.13 ± 1.78	83.94 ± 9.93	83.3 ± 6.09
11cd	80.63 ± 17.8	82.71 ± 28.91	64.98 ± 15.59	61.74 ± 1.1	64.76 ± 9.12	60.94 ± 2.19
11ce	129.4 ± 29.02	106.66 ± 23.52	99.85 ± 0.42	100.88 ± 10.17	82.11 ± 3.99	65.56 ± 8.02
12aa	106.25 ± 19.18	73.92 ± 17.88	94.79 ± 26.32	58.64 ± 12.96	72.87 ± 13.82	75.36 ± 46.61
12ab	97.37 ± 5.81	81.54 ± 6.53	96.12 ± 12.7	108.48 ± 5.69	117.18 ± 64.2	73.54 ± 14.17
12ac	100.27 ± 12.97	91.55 ± 6.44	95.35 ± 27.36	71.88 ± 25.17	64.55 ± 15.72	39 ± 11.13
12ad	99.54 ± 12.14	81.06 ± 29.19	66.42 ± 15.56	40.21 ± 16.06	29.53 ± 5.72	42.49 ± 6.5
12ae	69.33 ± 5.52	72.88 ± 7.75	59.09 ± 30.83	41.93 ± 34.68	51.08 ± 49.1	8.19 ± 4.19
12af	75.83 ± 21.6	55.89 ± 23.97	59.78 ± 31.55	62.68 ± 27.42	57.64 ± 46.49	59.57 ± 41.09
12ag	97.34 ± 12.37	120.31 ± 13.47	119.56 ± 3.22	99.77 ± 14.51	95.66 ± 15.65	66.8 ± 8.81
13aa	118.44 ± 30.27	100.33 ± 14.56	97.95 ± 49.01	45.73 ± 2.66	32.38 ± 6.66	37.27 ± 14.34
13ab	65.94 ± 24.29	49.27 ± 12.26	36.59 ± 22.88	23.36 ± 19.04	22.44 ± 13.94	19.17 ± 12.58
13ac	91.51 ± 28.98	77.03 ± 25.75	51.52 ± 35.11	37.67 ± 35.69	30.06 ± 28.06	26.57 ± 19.66
13ad	105.05 ± 21.88	71.33 ± 30.94	49.03 ± 27.19	28.25 ± 7.99	27.71 ± 8.88	25.32 ± 7.33
13ae	57.82 ± 27.82	71.48 ± 25.66	25.82 ± 6.11	15.32 ± 3.64	18.82 ± 7.53	17.59 ± 5.48
13ba	94.99 ± 24.67	105.89 ± 44.44	91.97 ± 40.96	71.22 ± 17.2	69.64 ± 3.83	46.47 ± 3.01
13bb	101.51 ± 24.22	105.14 ± 35.56	88.58 ± 25.22	71.93 ± 20.85	57.39 ± 9.43	57.43 ± 19.41
13bc	98.94 ± 4.03	95.2 ± 10.96	91.59 ± 12.5	87.51 ± 7.04	71.77 ± 1.97	57.99 ± 0.39
13bd	107.78 ± 5.05	103.63 ± 8.49	87.73 ± 9.39	73.19 ± 3.09	54.27 ± 2.6	85.55 ± 29.74
13be	86.43 ± 10.52	100.31 ± 3.01	93.93 ± 6.33	68.44 ± 8.18	51.42 ± 0.07	43.71 ± 1.78
13ca	94.86 ± 19.02	92.8 ± 18.36	97.02 ± 15.97	78.58 ± 28.44	78.44 ± 19.29	46.33 ± 8.91
13cb	93.55 ± 6.6	87.76 ± 11.65	87.53 ± 6.25	74.7 ± 12.51	57.61 ± 5.32	37.6 ± 9.98
13cc	106.39 ± 9.56	101.72 ± 7.04	101.87 ± 3.47	86.81 ± 4.13	64.83 ± 5.89	51.57 ± 6.77
13cd	97.15 ± 4.88	93.24 ± 0.01	92.33 ± 4.63	93.59 ± 16.34	82.22 ± 9.67	68.91 ± 0.29
13ce	91.45 ± 6.27	91.25 ± 3.25	84.62 ± 22.05	72.44 ± 5.22	69.63 ± 5.64	43.56 ± 3.23

Note: % response values are the mean of all trials performed in duplicates. Standard deviation is calculated from these averages.

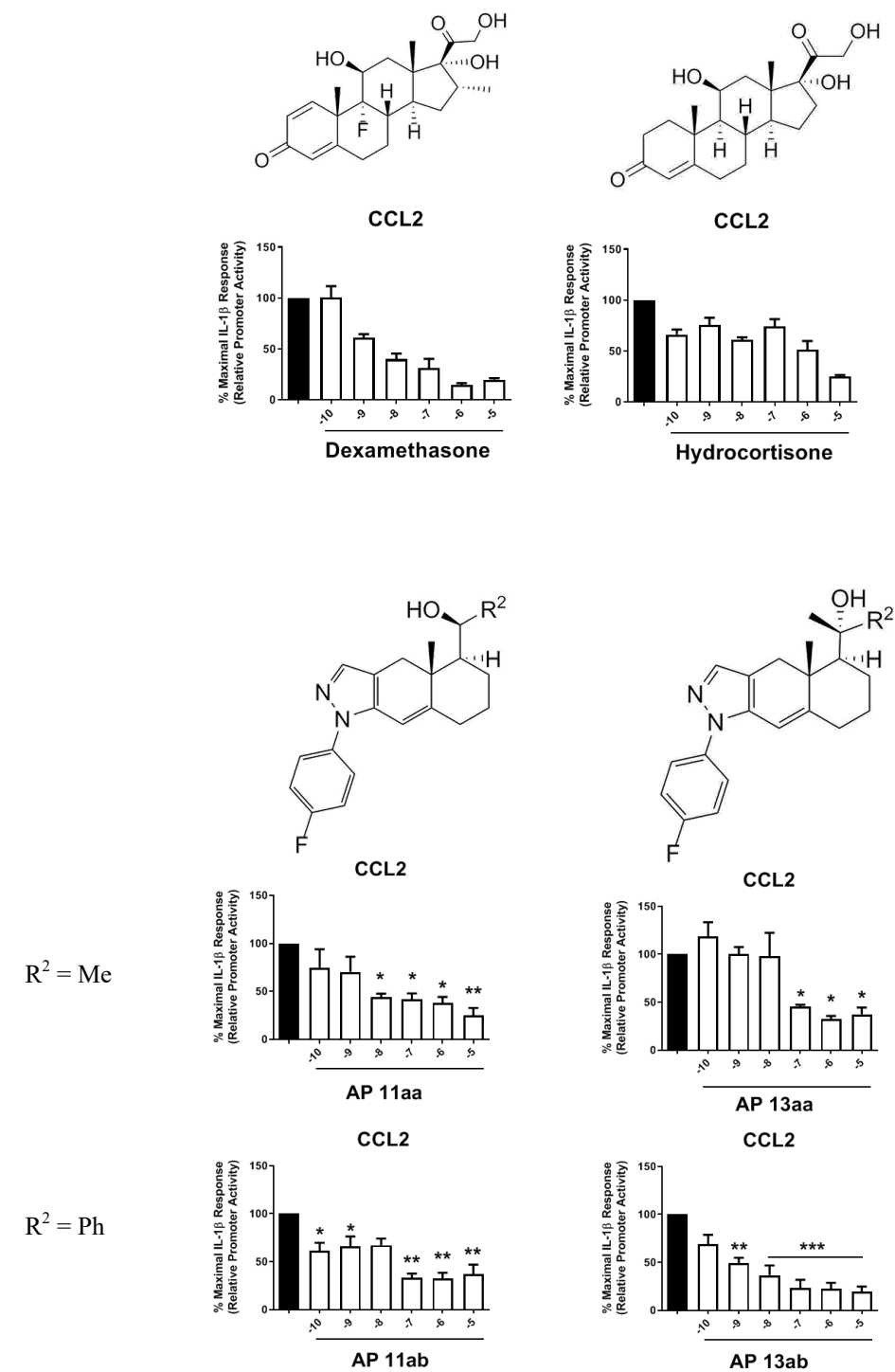


Figure 1.9.55 – CCL2 Data Analysis

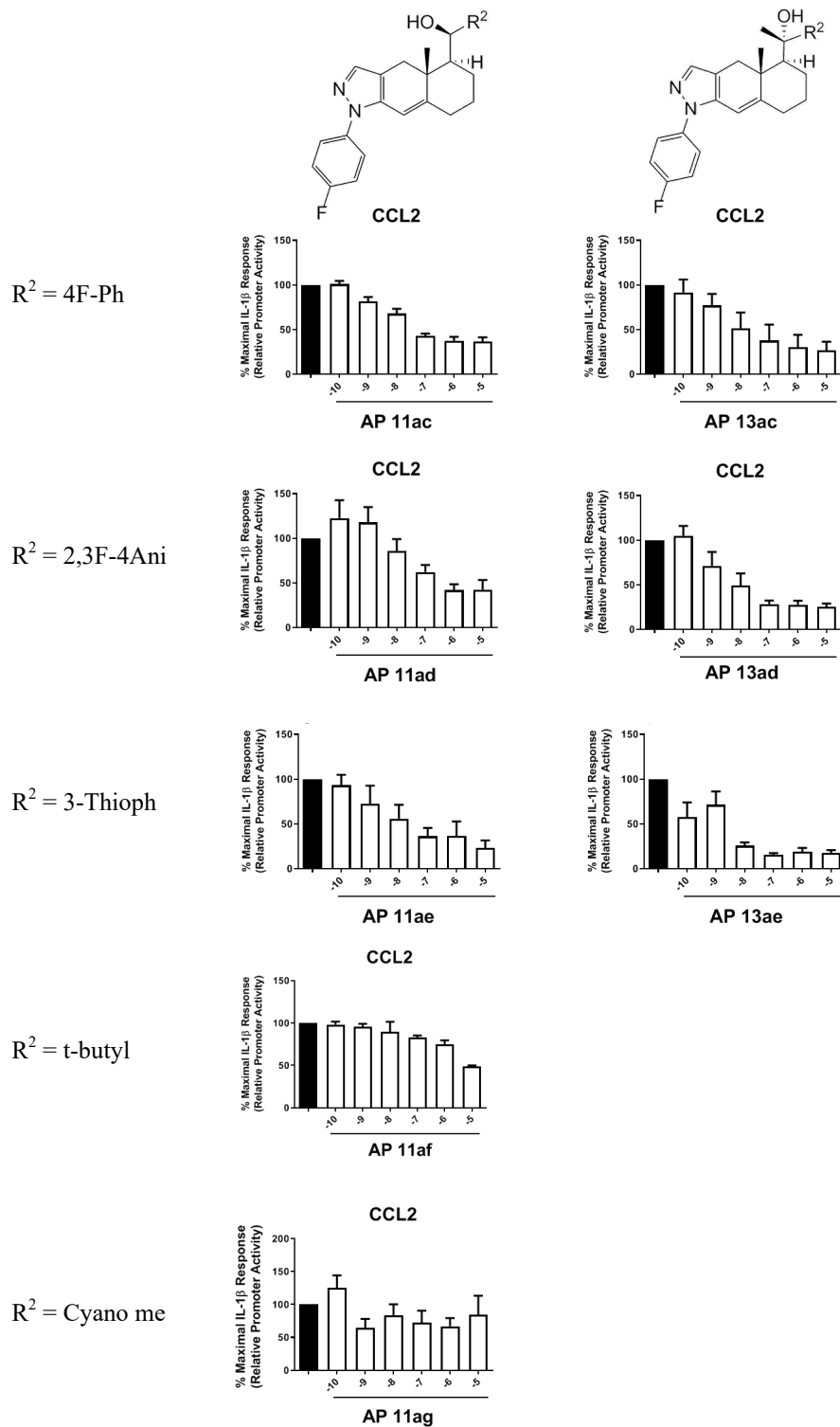


Figure 1.9.55 – Continued

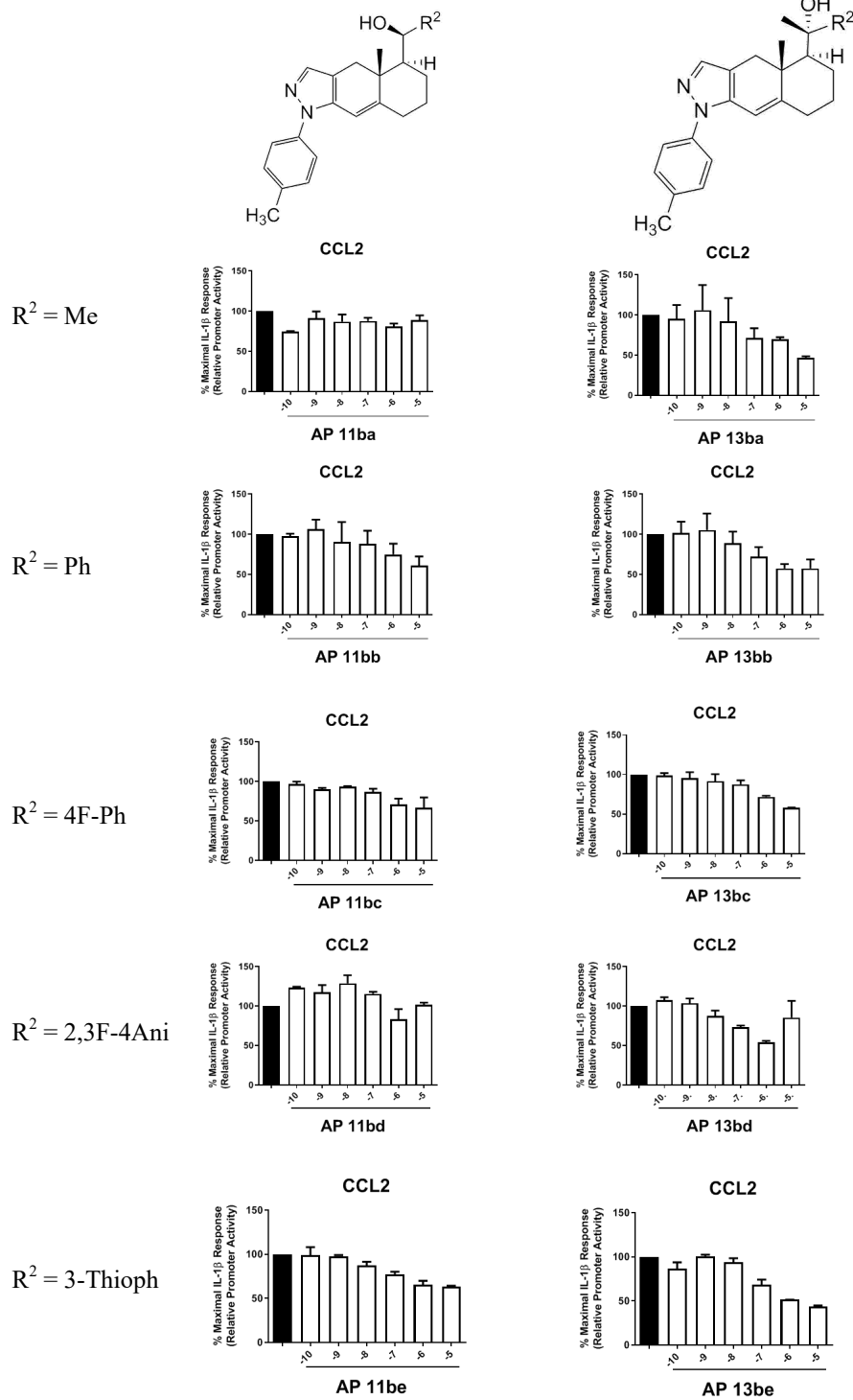


Figure 1.9.55 – Continued

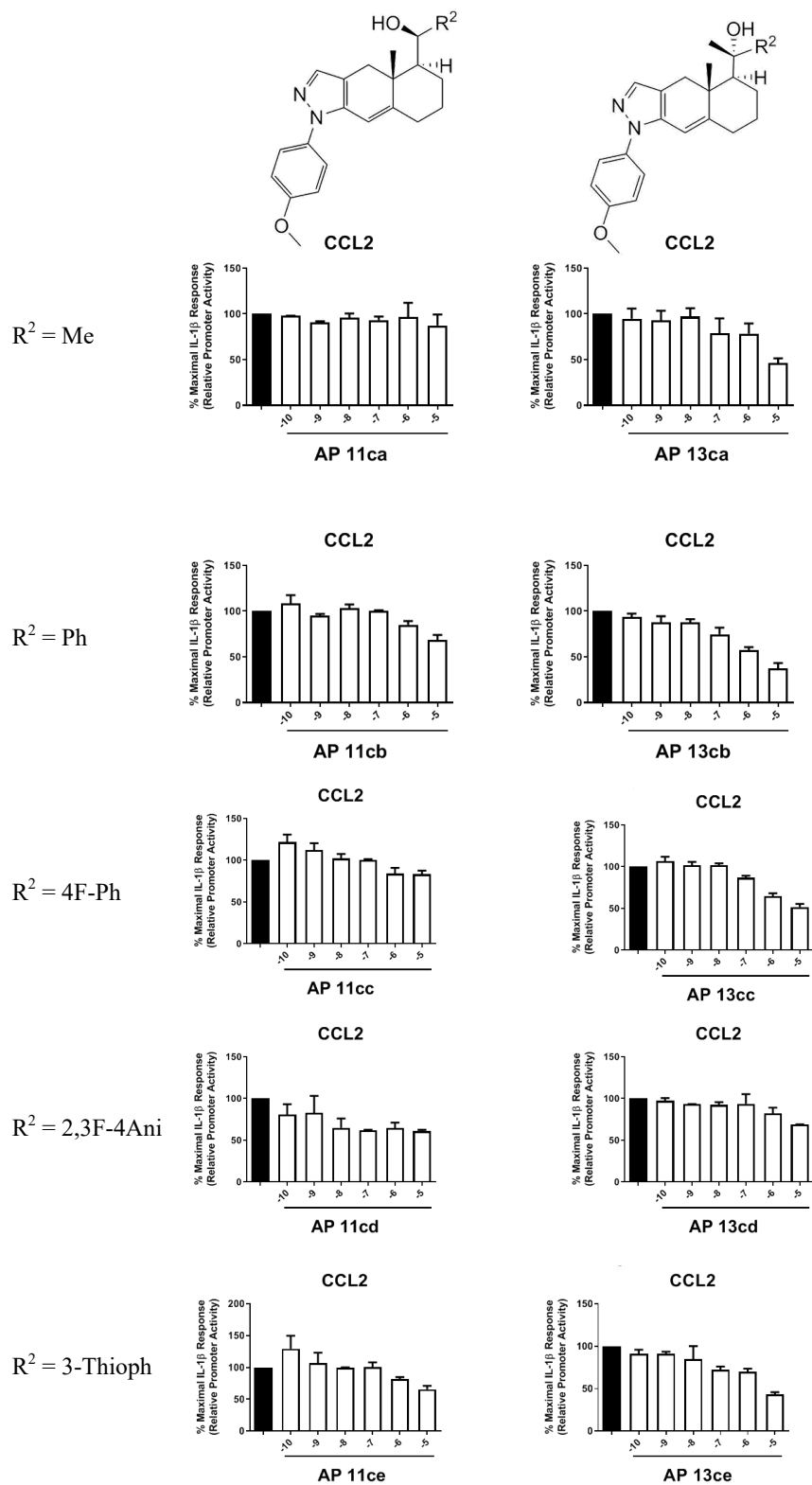


Figure 1.9.55 – Continued

1.9.3 – 3xGRE Data

Table 1.9.2 – 3xGRE assay raw data

3xGRE (Fold over control - DMSO)						
Compd	Concentration of Compound (M)					
	1x10 ⁻¹⁰	1x10 ⁻⁹	1x10 ⁻⁸	1x10 ⁻⁷	1x10 ⁻⁶	1x10 ⁻⁵
HC	0.84 ± 0.04	0.84 ± 0.04	3.27 ± 1.33	8.06 ± 0.43	8.58 ± 0.46	10.52 ± 0.87
Dex	1.05 ± 0.29	1.05 ± 0.29	10.76 ± 0.13	9.47 ± 1.26	10.86 ± 0.38	10.38 ± 0.59
11aa	1 ± 0.08	1 ± 0.08	0.8 ± 0.08	1.01 ± 0.04	1.79 ± 0.13	0.82 ± 0.3
11ab	0.64 ± 0.15	0.64 ± 0.15	0.72 ± 0.14	1.36 ± 0.74	2.83 ± 0.82	2.62 ± 0.45
11ac	0.91 ± 0.26	0.91 ± 0.26	0.84 ± 0.38	3.03 ± 1.21	4.87 ± 0.74	4.48 ± 1.42
11ad	1.21 ± 0.31	1.21 ± 0.31	1.16 ± 1.08	7.42 ± 2.56	13.34 ± 5.2	9.72 ± 0.58
11ae	1.13 ± 0.38	1.13 ± 0.38	2.13 ± 1.02	3.51 ± 1.08	5.72 ± 2.19	6.54 ± 4.28
11af	0.85 ± 0.27	0.85 ± 0.27	0.85 ± 0.13	0.8 ± 0.2	1.18 ± 0.31	1.39 ± 0.72
11ag	1.03 ± 0.07	1.03 ± 0.07	1.01 ± 0.12	1.03 ± 0.07	1.1 ± 0.09	1.22 ± 0.18
11ba	1.06 ± 0.23	1.06 ± 0.23	0.89 ± 0.08	0.91 ± 0.04	1.1 ± 0.04	1.27 ± 0.16
11bb	1.03 ± 0.09	1.03 ± 0.09	0.94 ± 0.04	0.99 ± 0.07	1.04 ± 0.09	1.94 ± 0.65
11bc	0.81 ± 0.08	0.81 ± 0.08	0.8 ± 0.09	0.81 ± 0.09	0.84 ± 0.14	1.03 ± 0.18
11bd	1.02 ± 0.05	1.02 ± 0.05	0.95 ± 0.16	0.96 ± 0.13	1.03 ± 0.12	1.51 ± 0.19
11be	0.99 ± 0.04	0.99 ± 0.04	0.83 ± 0.1	0.79 ± 0.1	0.75 ± 0.07	1.1 ± 0.21
11ca	0.68 ± 0	0.68 ± 0	0.79 ± 0.01	0.77 ± 0.03	1.18 ± 0.5	1.09 ± 0.19
11cb	0.74 ± 0.01	0.74 ± 0.01	0.84 ± 0.09	0.7 ± 0.07	0.72 ± 0.05	1.17 ± 0.39
11cc	0.96 ± 0.06	0.96 ± 0.06	0.88 ± 0.09	0.92 ± 0.24	0.96 ± 0.24	1.26 ± 0.02
11cd	1.29 ± 0.43	1.29 ± 0.43	1.43 ± 0.36	1.4 ± 0.28	1.51 ± 0.07	1.55 ± 0
11ce	0.9 ± 0.04	0.9 ± 0.04	0.87 ± 0.02	0.88 ± 0.13	0.84 ± 0	1.61 ± 0.55
12aa	0.89 ± 0.18	0.89 ± 0.18	0.88 ± 0.07	0.77 ± 0.08	0.97 ± 0.07	0.76 ± 0.07
12ab	0.7 ± 0.21	0.7 ± 0.21	0.78 ± 0.11	0.59 ± 0.44	0.68 ± 0.09	1.17 ± 0.21
12ac	1.02 ± 0.1	1.02 ± 0.1	1.02 ± 0.29	0.91 ± 0.17	0.93 ± 0.22	1.32 ± 0.6
12ad	0.92 ± 0.11	0.92 ± 0.11	1.1 ± 0.28	3.54 ± 1.79	4.52 ± 1.39	4.35 ± 0.65
12ae	0.97 ± 0.31	0.97 ± 0.31	0.93 ± 0.34	0.93 ± 0.34	0.9 ± 0.31	0.93 ± 0.36
12af	0.8 ± 0.18	0.8 ± 0.18	0.77 ± 0.07	0.78 ± 0.13	0.85 ± 0.15	0.85 ± 0.21
12ag	0.84 ± 0.02	0.84 ± 0.02	0.9 ± 0.05	0.94 ± 0.06	0.87 ± 0.09	1.19 ± 0.36
13aa	1.06 ± 0.15	1.06 ± 0.15	1.11 ± 0.51	6.29 ± 1.03	9.25 ± 2.9	9.15 ± 2.01
13ab	1.06 ± 0.11	1.06 ± 0.11	4.7 ± 1.24	5.61 ± 1.48	6.57 ± 1.29	4.47 ± 1.3
13ac	1.11 ± 0.5	1.11 ± 0.5	4.22 ± 1.6	7.84 ± 1.26	8.54 ± 1.75	8.96 ± 2.39
13ad	1.33 ± 0.44	1.33 ± 0.44	6.41 ± 2.49	10.57 ± 2.7	9.31 ± 2.09	9.63 ± 1.21
13ae	0.91 ± 0.08	0.91 ± 0.08	5.65 ± 0.52	8.44 ± 1.28	7.96 ± 1.49	6.23 ± 0.38
13ba	0.84 ± 0.09	0.84 ± 0.09	0.74 ± 0.02	0.69 ± 0.12	0.82 ± 0.04	0.71 ± 0.01
13bb	1.09 ± 0.31	1.09 ± 0.31	0.94 ± 0.11	1.07 ± 0.23	1.56 ± 1.02	2.15 ± 0.68
13bc	0.73 ± 0.08	0.73 ± 0.08	0.87 ± 0.2	0.84 ± 0.22	0.66 ± 0	0.76 ± 0.09
13bd	0.81 ± 0.09	0.81 ± 0.09	0.78 ± 0.05	0.85 ± 0.02	1.25 ± 0.09	1.97 ± 0.13
13be	0.9 ± 0.18	0.9 ± 0.18	0.83 ± 0.1	0.85 ± 0.16	0.99 ± 0.11	1.13 ± 0.25
13ca	1.03 ± 0.36	1.03 ± 0.36	1.04 ± 0.48	0.91 ± 0.38	0.82 ± 0.21	0.86 ± 0.11
13cb	0.91 ± 0.05	0.91 ± 0.05	0.84 ± 0.04	0.81 ± 0.03	0.93 ± 0.17	1.16 ± 0.04
13cc	0.83 ± 0.01	0.83 ± 0.01	0.75 ± 0.06	0.84 ± 0.04	0.92 ± 0.11	0.89 ± 0
13cd	1.19 ± 0.12	1.19 ± 0.12	1.18 ± 0.09	1.12 ± 0.12	1.14 ± 0.17	2.33 ± 0.72
13ce	0.82 ± 0.04	0.82 ± 0.04	0.79 ± 0	0.84 ± 0.01	0.84 ± 0.04	1.28 ± 0.09

Note: Fold change values are the mean of all trials performed in duplicates. Standard deviation is calculated from these averages.

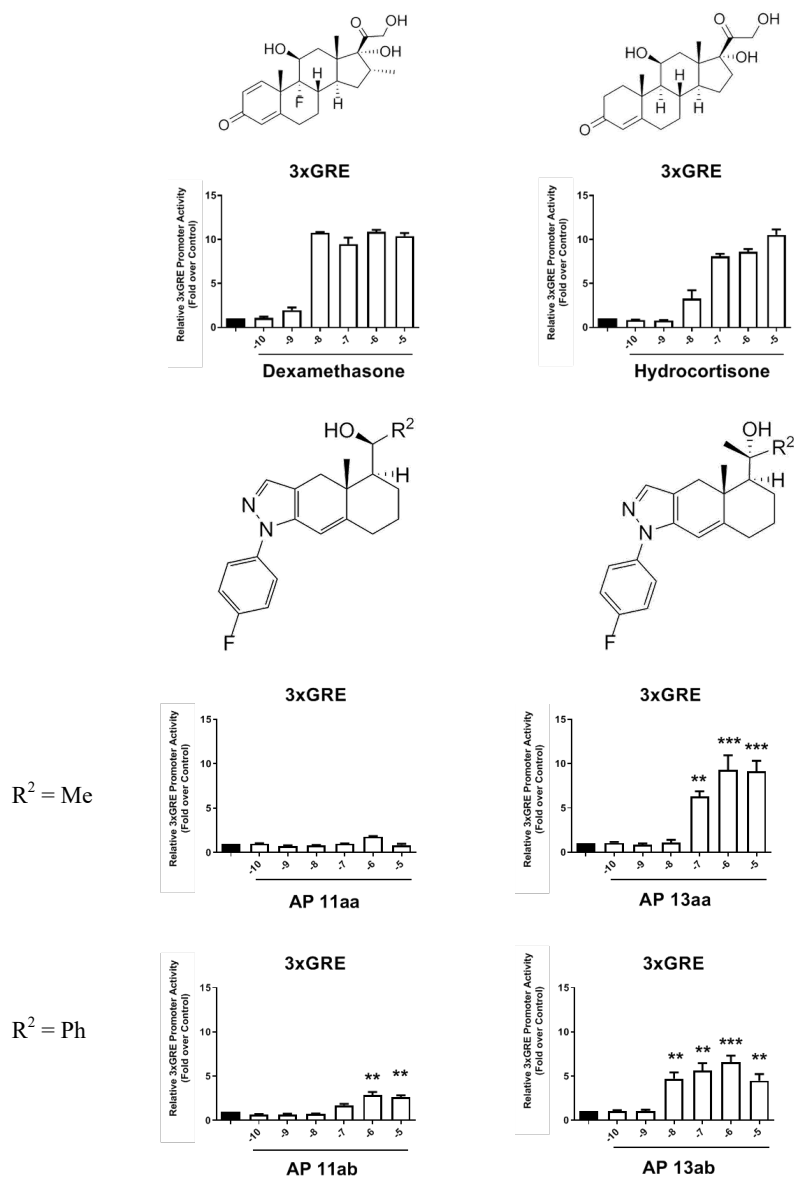


Figure 1.9.56 – 3xGRE Data Analysis

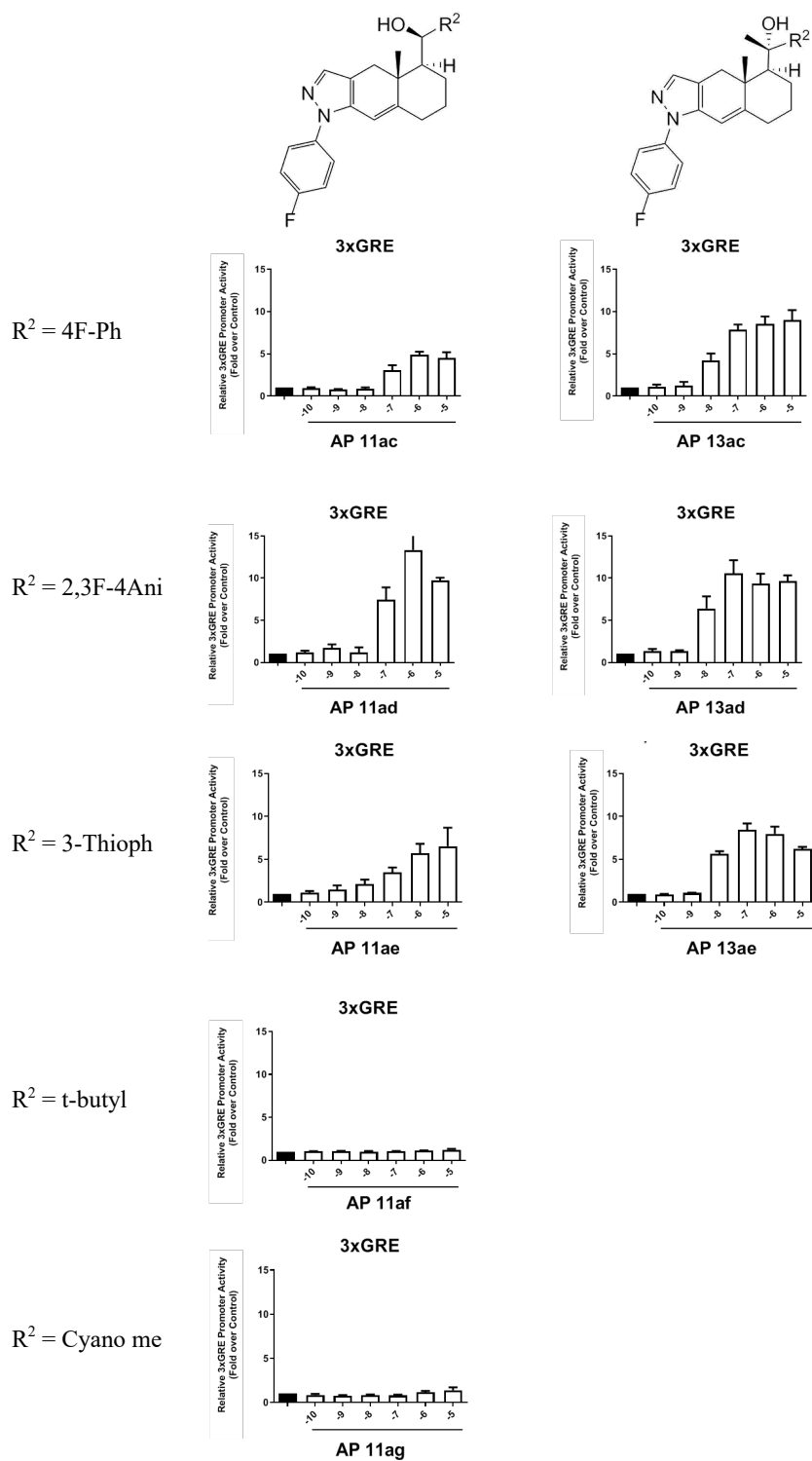


Figure 1.9.56 – Continued

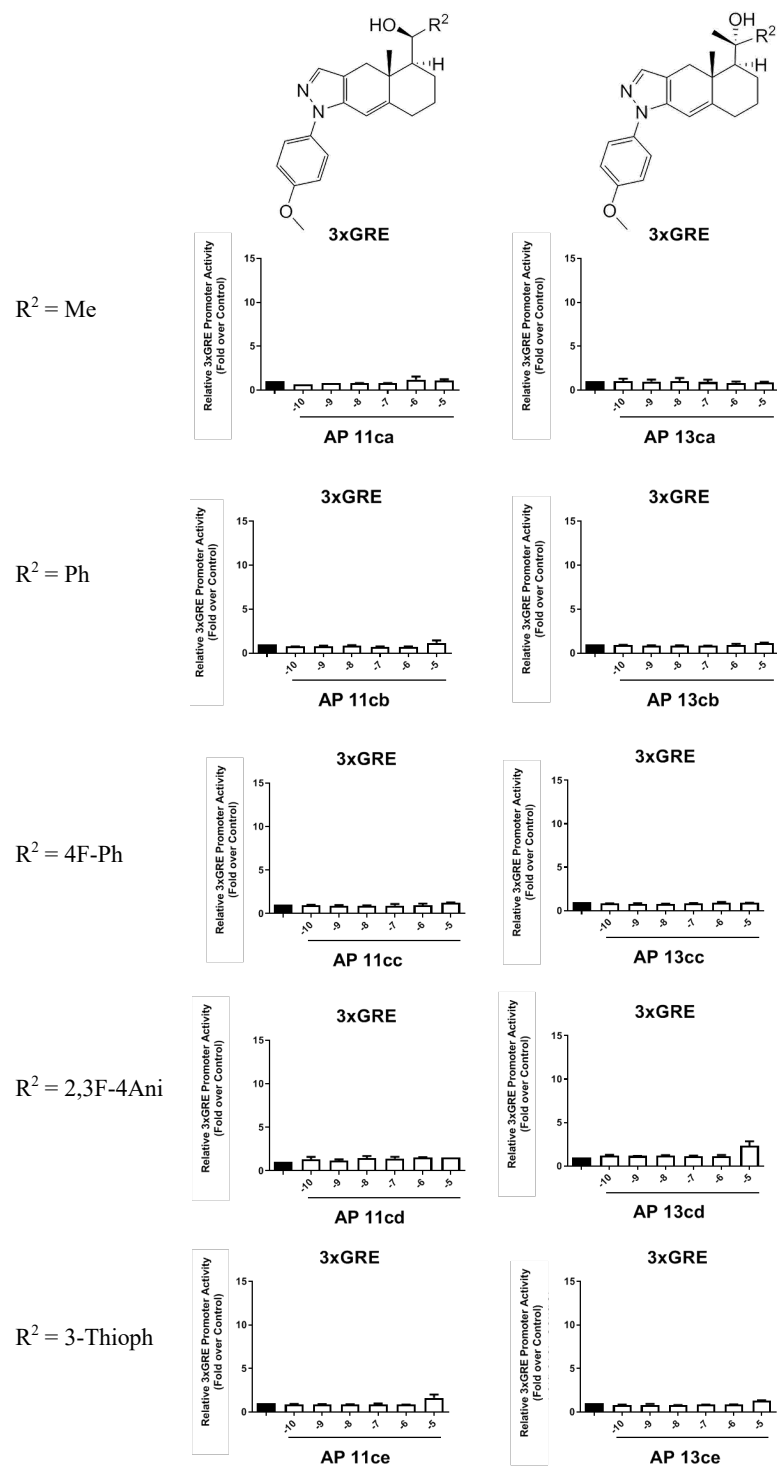


Figure 1.9.56 – Continued

1.9.4 – GSIS Data

Table 1.9.3 – GSIS assay raw data

GSIS		
Compd	Glucose (mM)	Insulin (ng/mL)
Dex	3	1.70 ± 0.31
	15	9.08 ± 2.23
11aa	3	1.13 ± 0.78
	15	15.42 ± 1.52
13aa	3	1.55 ± 0.07
	15	14.23 ± 2.71
Dex	3	0.87 ± 0.41
	15	8.11 ± 2.93
11ab	3	1.24 ± 0.45
	15	12.57 ± 4.58
13ab	3	1.55 ± 0.38
	15	8.45 ± 3.46

Note: Values are the mean of all trials performed in triplicates. Standard deviation is calculated from these averages.

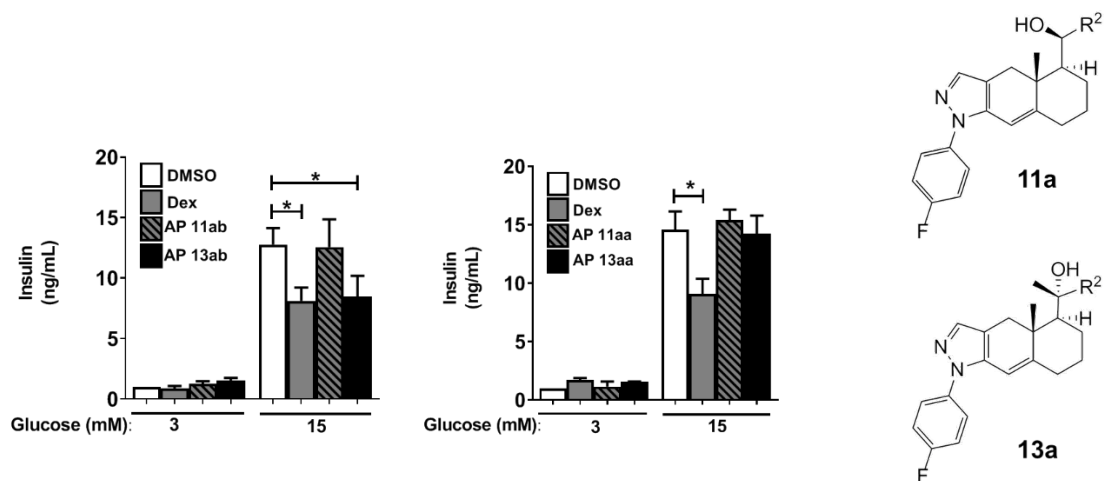


Figure 1.9.57 – GSIS Assay Analysis

1.9.5 – Ligand-Receptor Interactions for APGRAs in 5NFP

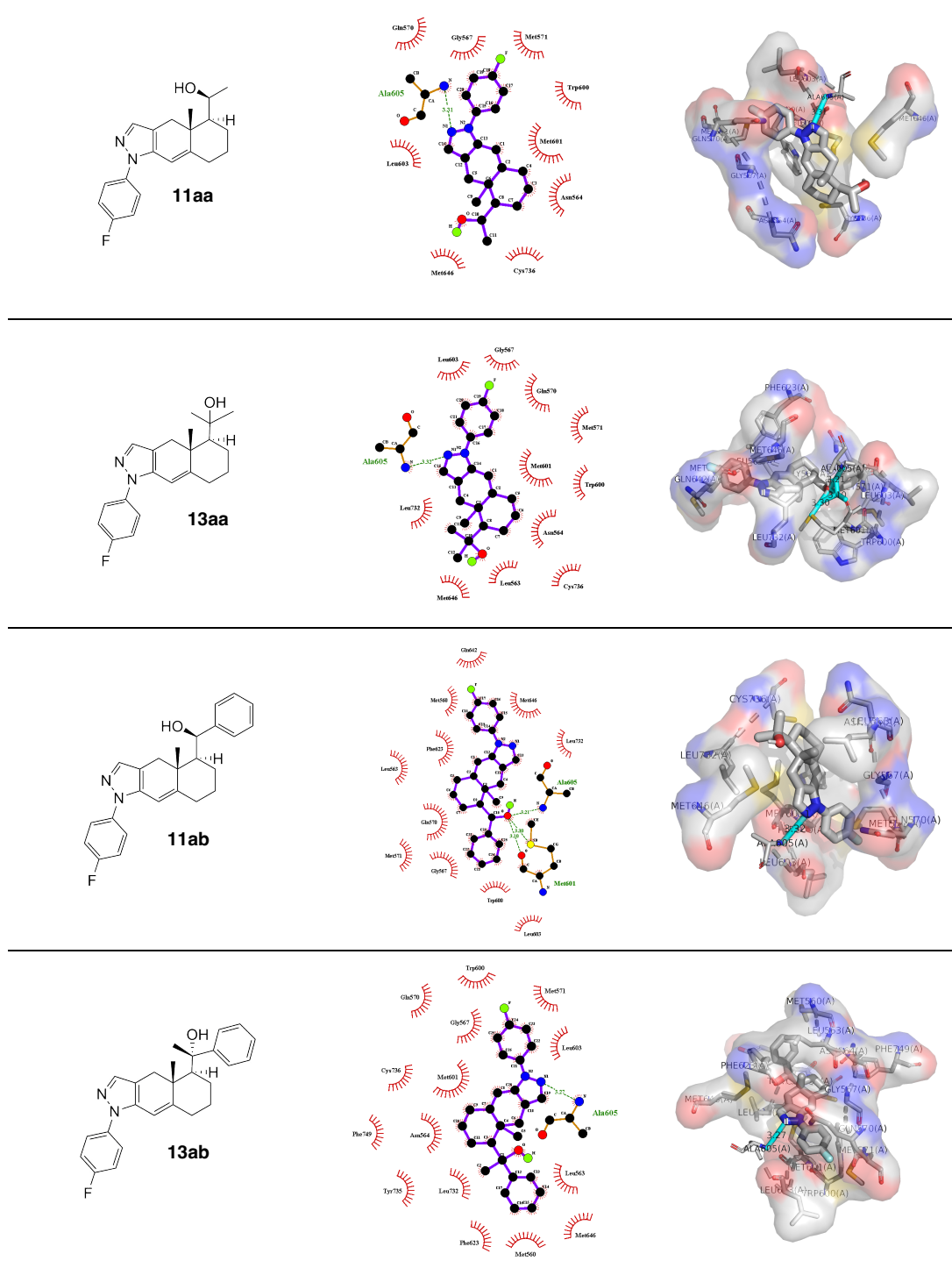
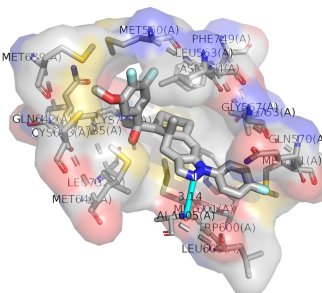
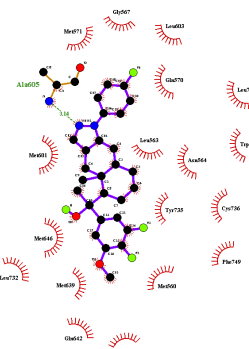
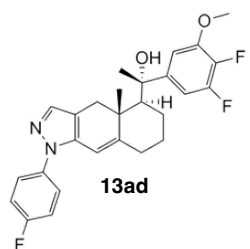
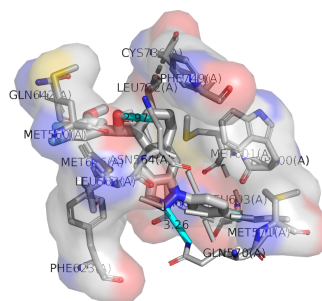
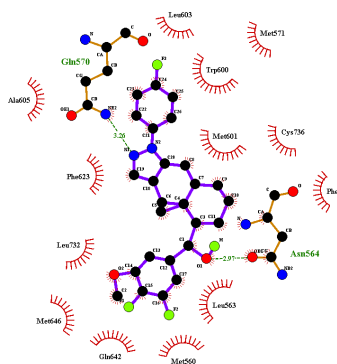
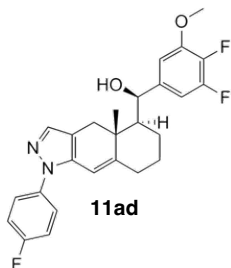
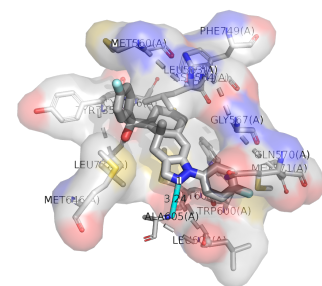
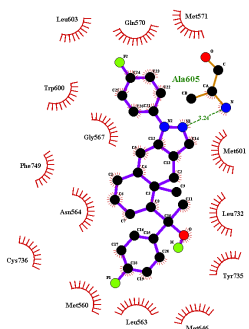
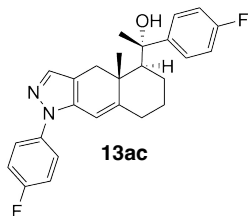
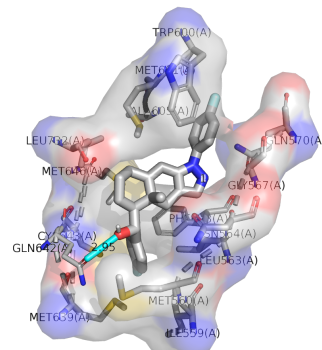
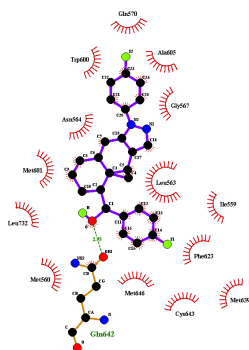
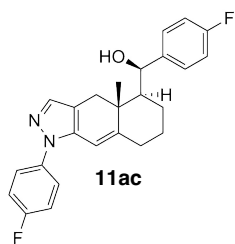


Figure 1.9.58 – Ligand – Receptor interactions for APGRAs in 5NFP



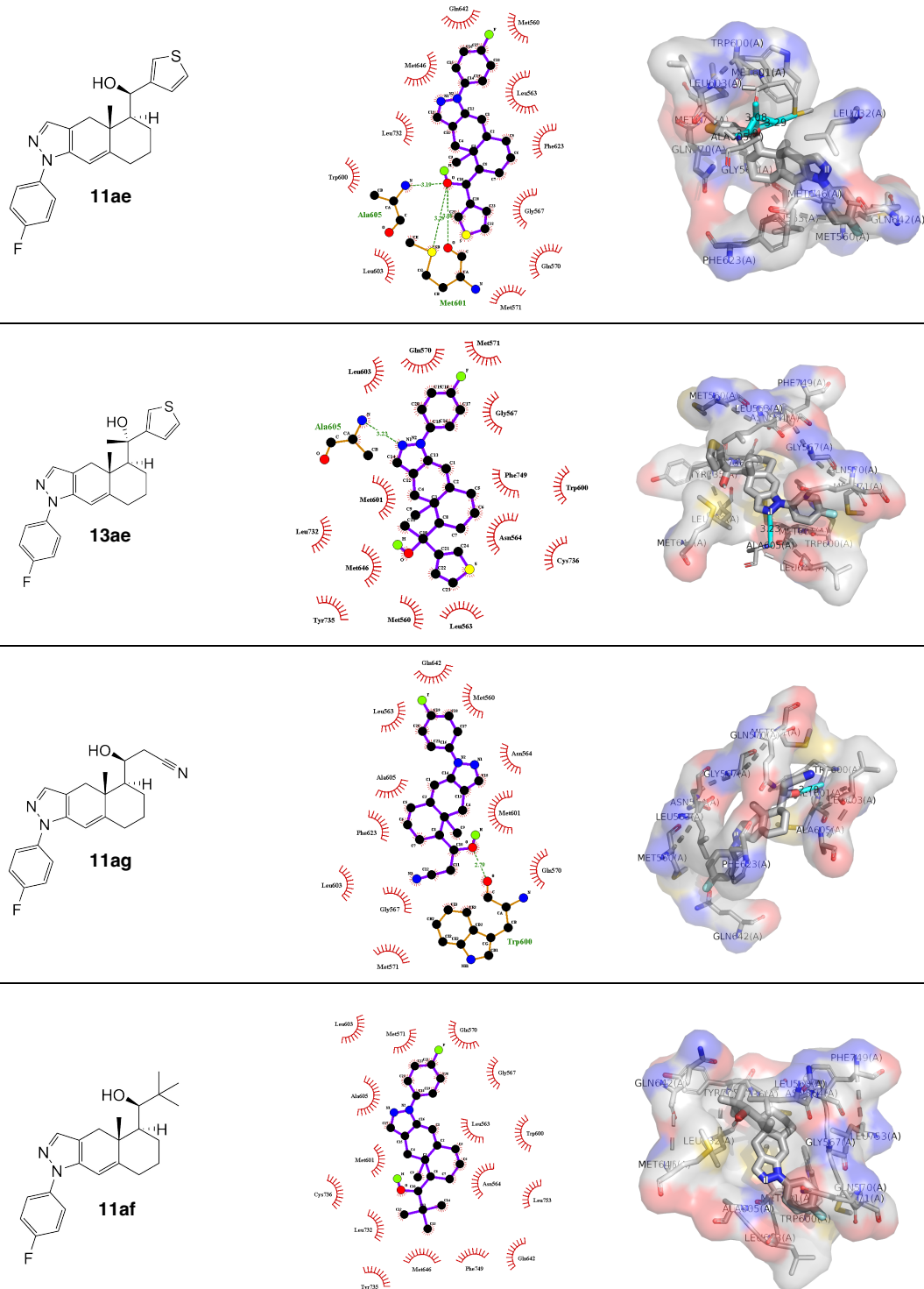
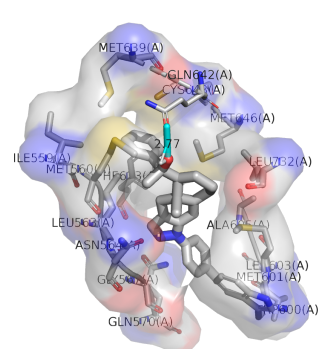
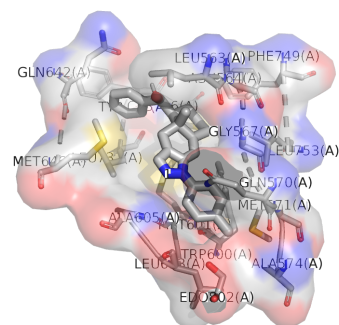
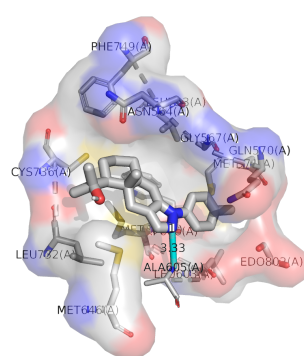
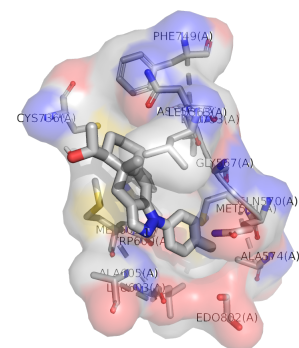


Figure 1.9.58 – Continued



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Figure 1.9.58 – Continued

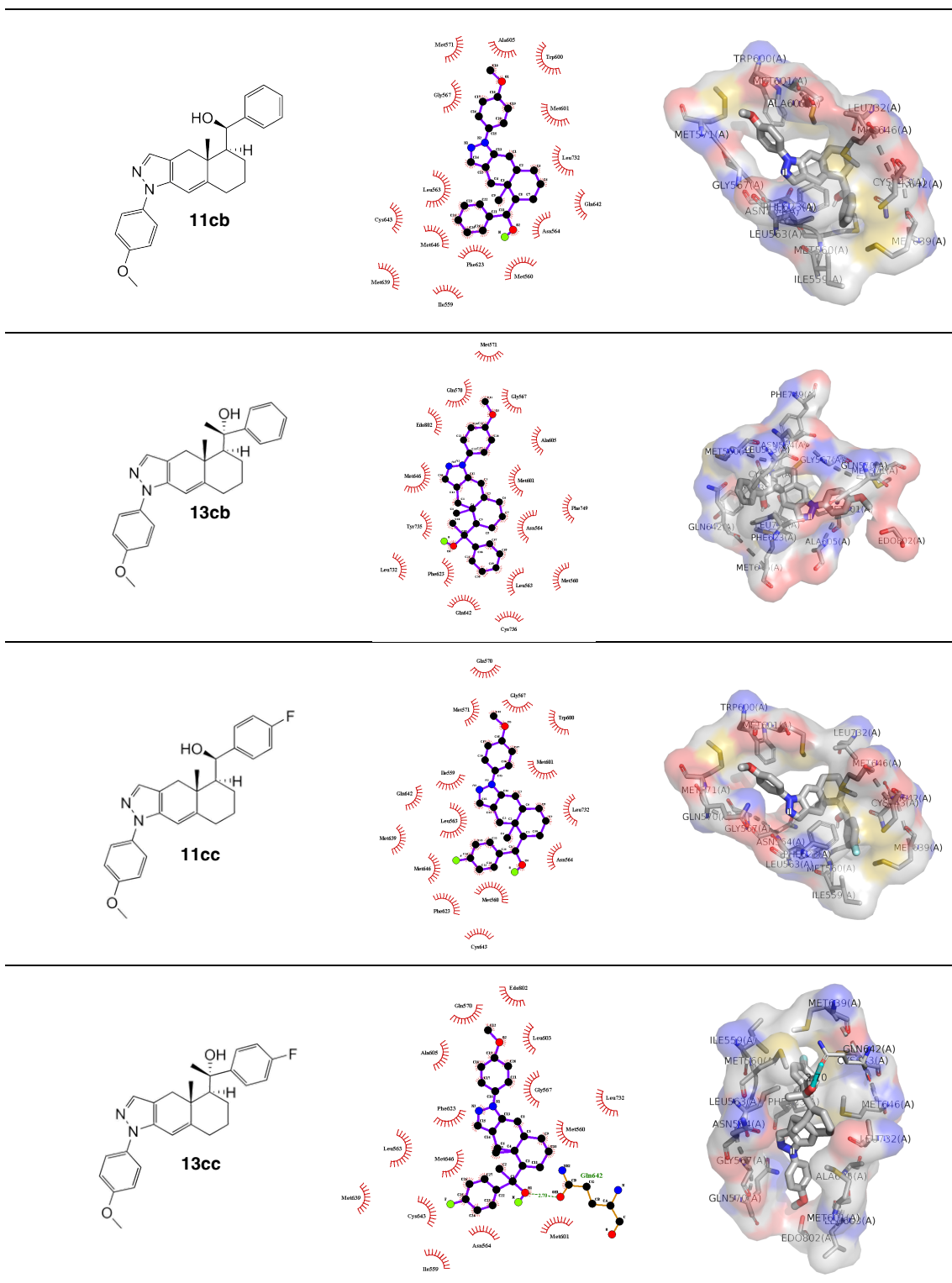


Figure 1.9.58 – Continued

1.9.6 – Ligand Orientation and Relative Binding Affinity in 5NFP

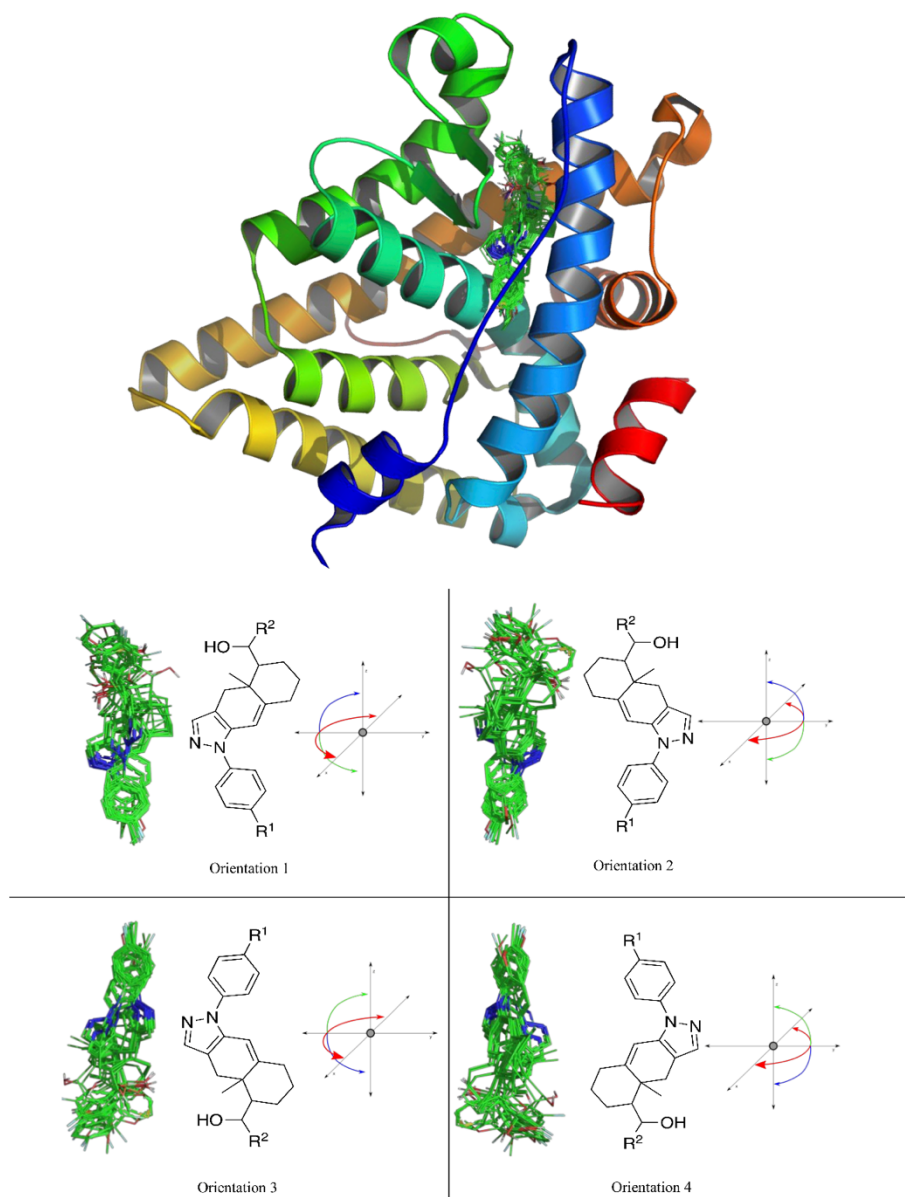


Figure 1.9.59 – APGRA docking orientation legend

Table 1.9.4 – Relative binding affinity in 5NFP

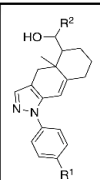
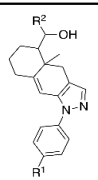
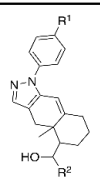
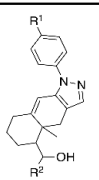
Glucocorticoid	Top binding conformations	binding affinity (kcal/mol)	orientation in 5NFP			
						
dexamethasone	1	-10.6		X		
	2	-9.0	X			
	3	-8.7			X	
	4	-8.7	X			
11aa	1	-9.5	X			
	2	-9.4	X			
	3	-9.3				X
	4	-9.0				X
13aa	1	-10.0	X			
	2	-9.9				X
	3	-9.7	X			
	4	-9.6	X			
11ab	1	-10.4				X
	2	-10.0	X			
	3	-9.7	X			
	4	-9.7	X			
13ab	1	-10.9	X			
	2	-10.4	X			
	3	-10.1				X
	4	-10.1	X			
11ac	1	-10.6	X			
	2	-10.1	X			
	3	-9.5				X
	4	-9.5				X
13ac	1	-11.3	X			
	2	-10.5	X			
	3	-10.4	X			
	4	-10.2				X
11ad	1	-9.6	X			
	2	-9.5	X			
	3	-7.4				X
	4	-7.2	X			
13ad	1	-11.2	X			
	2	-9.7	X			
	3	-9.7	X			
	4	-8.5				X

Table 1.9.4 - Continued

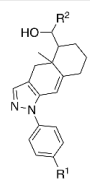
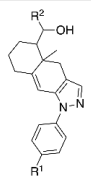
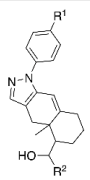
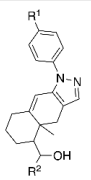
Glucocorticoid	Top binding conformations	binding affinity (kcal/mol)	orientation in 5NFP			
						
11ae	1	-10.0				X
	2	-9.8	X			
	3	-9.2	X			
	4	-9.1	X			
13ae	1	-10.3	X			
	2	-10.3	X			
	3	-9.8				X
	4	-9.8	X			
11af	1	-9.6				X
	2	-9.5	X			
	3	-9.2				X
	4	-9.1	X			
11ag	1	-10.4	X			
	2	-9.7	X			
	3	-9.5	X			
	4	-9.4	X			
11ba	1	-9.3	X			
	2	-9.3				X
	3	-9.1	X			
	4	-8.9	X			
13ba	1	-9.8	X			
	2	-9.7				X
	3	-9.5	X			
	4	-9.4	X			
11bb	1	-10.5	X			
	2	-10.2				X
	3	-10.2	X			
	4	-10.1				X
13bb	1	-10.6	X			
	2	-10.2	X			
	3	-9.5	X			
	4	-8.6	X			
11bc	1	-10.3	X			
	2	-10.2				X
	3	-10.2	X			
	4	-9.6	X			

Table 1.9.4 - Continued

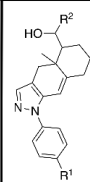
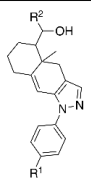
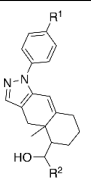
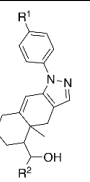
Glucocorticoid	Top binding conformations	binding affinity (kcal/mol)	orientation in 5NFP			
						
13bc	1	-10.8	X			
	2	-10.4	X			
	3	-10.3	X			
	4	-9.6	X			
11bd	1	-9.4	X			
	2	-9.3	X			
	3	-7.4		X		
	4	-7.1				X
13bd	1	-10.6	X			
	2	-9.4	X			
	3	n/a				
	4					
11be	1	-9.5				X
	2	-7.6				X
	3	-7.5				X
	4	n/a				
13be	1	-10.3	X			
	2	-10.2	X			
	3	-9.8	X			
	4	-9.3	X			
11ca	1	-9.6	X			
	2	-9.2				X
	3	-9.2	X			
	4	-8.4	X			
13ca	1	-9.5				X
	2	-9.5	X			
	3	-9.3	X			
	4	-7.5				X
11cb	1	9.8	X			
	2	-9.6	X			
	3	-9.3				X
	4	-9.1	X			
13cb	1	-10.2	X			
	2	-10.1	X			
	3	-9.6	X			
	4	-9.5	X			

Table 1.9.4 - Continued

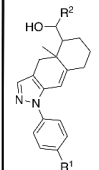
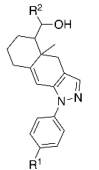
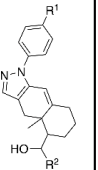
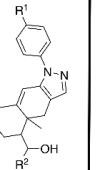
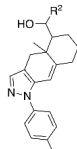
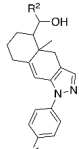
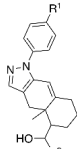
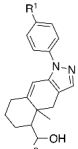
Glucocorticoid	Top binding conformations	binding affinity (kcal/mol)	orientation in 5NFP			
						
11cc	1	-10.2	X			
	2	-9.6	X			
	3	-9.5	X			
	4	-9.2	X			
13cc	1	-11	X			
	2	-10.4	X			
	3	-9.2	X			
	4	n/a				
11cd	1	-9.4	X			
	2	-8.6	X			
	3	-6.9		X		
	4	n/a				
13cd	1	-10.1	X			
	2	-9.6	X			
	3	-9.3	X			
	4	n/a				
11ce	1	-9.3	X			
	2	-9.3	X			
	3	-9.1				X
	4	-8.8	X			
13ce	1	-9.8	X			
	2	-9.7	X			
	3	-9.5	X			
	4	-9.2	X			
12aa	1	-9.9	X			
	2	-9.7	X			
	3	-9.6	X			
	4	-9.3				X
12ab	1	-11.1	X			
	2	-10.5	X			
	3	-10.2	X			
	4	-10.0				X
12ac	1	-11.5	X			
	2	-10.6	X			
	3	-10.5				X
	4	-10.3	X			

Table 1.9.4 - Continued

Glucocorticoid	Top binding conformations	binding affinity (kcal/mol)	orientation in 5NFP			
						
12ad	1	-11.0	X			
	2	-9.9	X			
	3	-8.6				X
	4	-8.5				X
12ae	1	-10.6	X			
	2	-10.3	X			
	3	-9.8	X			
	4	-9.8				X
12af	1	-10.1	X			
	2	-9.8	X			
	3	-9.7				X
	4	-9.5				X
12ag	1	-10.3	X			
	2	-9.8	X			
	3	-9.8				X
	4	-9.5	X			

1.9.7 – DFT Calculations with Truncated 5NFP Ligand Binding Pocket

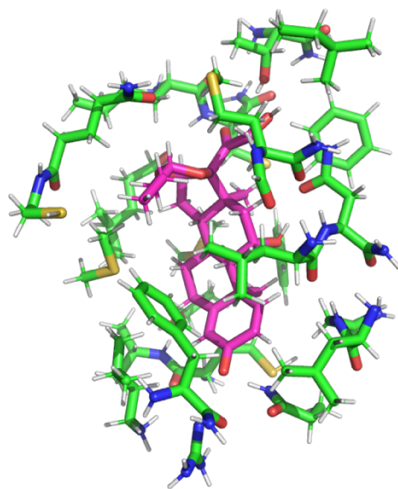


Figure 1.9.60 – Truncated ligand binding pocket with budesonide shown in magenta

Table 1.9.5 – DFT calculations

Compound	Total Energy (kcal/mol)	Orientation	RI-BP86- D3(BJ)/def 2-SVP	RI-B3LYP- D3(BJ)/def2- TZVPP	Relative B3LYP Energy (kcal/mol)	Binding Affinity (kcal/mol)
Pocket	-8966.70					
11aa	-1060.38	1	-10023.25	-10027.11	22.25	-18.62
		2	-10023.29	-10027.15	0.00	-40.87
		3	-10023.26	-10027.12	16.99	-23.87
		4	-10023.25	-10027.11	20.72	-20.14
11af	-1152.61	1	-10115.46	-10119.36	18.19	-29.53
		2	-10115.44	-10119.34	28.14	-19.58
		3	-10115.48	-10119.39	0.00	-47.73
		4	-10115.34	-10119.24	94.31	46.59
11ag	-1178.28	1	-10141.05	-10144.93	69.19	33.38
		2	-10141.11	-10145.00	25.03	-10.78
		3	-10141.15	-10145.04	0.00	-35.81
		4	-10141.07	-10144.96	54.16	18.35
11ba	-1000.44	1	-9963.33	-9967.13	65.75	8.68
		2	-9963.33	-9967.12	68.07	11.00
		3	-9963.43	-9967.23	0.00	-57.07
		4	-9963.43	-9967.22	3.75	-53.32
11bc	-1291.39	1	-10254.05	-10257.97	101.23	77.38
		2	-10254.15	-10258.09	28.14	4.29
		3	-10254.20	-10258.13	0.00	-23.85
		4	-10254.11	-10258.03	63.49	39.64
11be	-1512.89	1	-10475.72	-10479.58	27.07	6.15
		2	-10475.66	-10479.52	63.94	43.01
		3	-10475.75	-10479.60	15.17	-5.76
		4	-10475.76	-10479.63	0.00	-20.92
13ab	-1291.39	1	-10254.02	-10257.95	98.88	87.08
		2	-10254.08	-10258.00	67.24	55.43
		3	-10253.98	-10257.91	123.24	111.43
		4	-10254.18	-10258.11	0.00	-11.81
13ba	-1039.74	1	-10002.62	-10006.42	22.21	13.66
		2	-10002.65	-10006.45	5.58	-2.97
		3	-10002.66	-10006.46	0.00	-8.54
		4	-10002.65	-10006.45	0.73	-7.81
13bb	-1231.44	1	-10194.25	-10198.11	35.54	20.84
		2	-10194.30	-10198.16	0.00	-14.70
		3	-10194.19	-10198.04	76.16	61.46
		4	-10194.06	-10197.90	165.93	151.23
13be	-1552.19	1	-10515.04	-10518.92	7.60	-13.51
		2	-10515.01	-10518.89	23.87	2.76
		3	-10515.05	-10518.93	0.00	-21.11
		4	-10514.87	-10518.73	126.63	105.52

Table 1.9.5 – Continued

13ca	-1114.95	1	-10077.78	-10081.63	40.20	13.74
		2	-10077.81	-10081.65	23.17	-3.29
		3	-10077.83	-10081.68	4.51	-21.96
		4	-10077.84	-10081.69	0.00	-26.46
13cb	-1306.65	1	-10269.24	-10273.14	123.53	135.58
		2	-10269.17	-10273.03	188.64	200.69
		3	-10269.43	-10273.33	0.00	12.05
		4	-10269.42	-10273.32	6.30	18.35
13cc	-1405.90	1	-10368.54	-10372.52	0.00	46.68
		2	-10368.29	-10372.26	162.98	209.66
		3	-10368.43	-10372.41	70.47	117.15
		4	-10368.40	-10372.39	80.88	127.55
13ce	-1627.40	1	-10589.97	-10593.87	127.37	144.83
		2	-10589.96	-10593.85	137.78	155.24
		3	-10590.04	-10593.94	81.41	98.87
		4	-10590.16	-10594.07	0.00	17.46

1.9.8 – 2D Representation of Y735 Interaction in 3BQD Ligand Binding Pocket

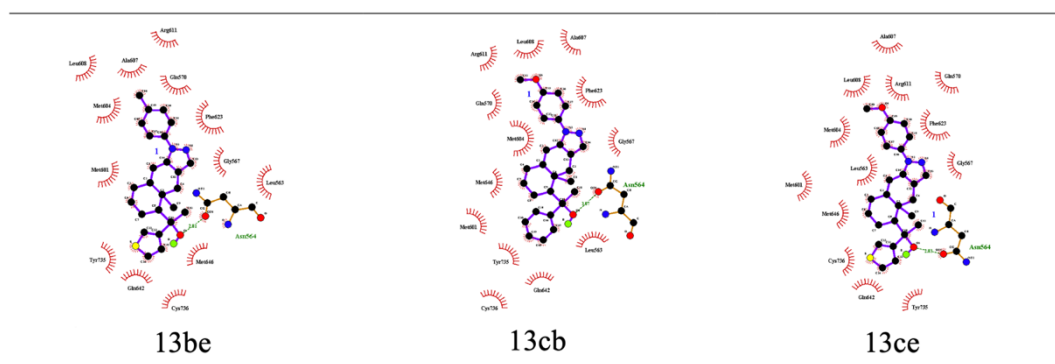


Figure 1.9.61 – 2D Ligplot Analysis of APGRA interaction with Y735

**CHAPTER II: STEREOISOMERS ELICIT DIFFERING ANTI-
INFLAMMATORY RESPONSE IN ARYL PYRAZOLE
GLUCOCORTICOID RECEPTOR AGONIST SCAFFOLD**

A version of this chapter is expected to be part of a future publication titled:

**Stereoisomers Elicit Differing Anti-inflammatory Response in Aryl Pyrazole
Glucocorticoid Receptor Agonist Scaffold**

Proposed Authors:

Lato, A. M.; Burke, S. J.; Ducote, M. P.; Kennedy, B. J.; Collier, J. J.; Campagna,
S. R.

Contributions:

AML wrote the manuscript, designed the experiment, performed synthesis and purification, collected all characterization, identified stereoisomers, performed and analyzed molecular docking experiments, calculated pharmacological values, and analyzed biological assays. SJB and MPD performed all *in vitro* assays. BJK performed chiral separation on all final compounds. JJC and SRC provided resources and funding.

2.1 – ABSTRACT

Glucocorticoids (GCs) are a mainstay in modern medicine, however, side effects associated with glucocorticoid use are well documented and often lead to serious complications with long term use. In general, GCs induce two primary modes of activation; dimer formation leads to transactivation (side effects) while monomeric interaction with co-regulators leads to transrepression (therapeutic effects). Previous work has shown this ligand scaffold produced two aryl pyrazole glucocorticoid receptor agonists (APGRAs) with potential therapeutic benefits, manipulating these two activation pathways to favor transrepression. In this study, a series of stereoisomers were thoroughly examined through synthesis, chiral purification, biological screening, and ultimately evaluated for structure activity relationships (SARs). The *si* isomer with an upward OH moiety was found to elicit ~70% greater anti-inflammatory response on average than that with a downward OH. Compound **AP12** displayed favorable selectivity eliciting 77% transrepression activity of hydrocortisone while producing less than 20% the transactivation activity of both hydrocortisone and dexamethasone. Additionally, **AP13** was found to elicit 79% transrepression activity of dexamethasone while eliciting less than half the transactivation response in 832/13 cells, a rat insulinoma cell line.

2.2 – INTRODUCTION

Glucocorticoids (GCs) remain one of the most frequently prescribed treatments worldwide due to their unrivalled anti-inflammatory and immunosuppressive benefits.^{89, 90} However, GC therapy is often accompanied by a number of adverse side effects including skin and muscle atrophy, disordered fat metabolism, hypertension, mood disorders, osteoporosis, and diabetes mellitus to name a few.^{26, 90, 91} GC usage continues to grow despite the ramifications, still, there is a well-defined need for improved therapies. Developing a GC treatment that retains the desired anti-inflammatory activity with a diminished side effect profile has been an ongoing effort for decades as it requires intricate

dissection of ligand-receptor structure activity relationships (SARs) to unravel the mechanistic details of the GCs promiscuous physiological and pharmacological effects.⁸⁹

The glucocorticoid receptor (GR) is a ligand-initiated transcription factor that regulates essential biological processes including metabolism, growth, and apoptosis.^{39, 92} Upon ligand activation, the GR-GC complex alters transcription either directly, through dimerization and binding to the appropriate DNA response elements, or indirectly through monomeric interactions with additional transcriptional factors such as NF- κ B. The current paradox is that monomeric activity is responsible for the therapeutic effects while dimer induced transactivation is at least in part responsible for the undesired side effects.^{24, 25, 93} While this model is simplified, a GC capable of inducing conformational changes in favor of the monomeric form that retains potent anti-inflammatory activity, is a highly coveted target. The GR is comprised of three domains: the N-terminal transactivation domain (NTD), DNA binding domain (DBD), and the C-terminal ligand binding domain (LBD).^{89, 94} The LBD is comprised of 4 small β sheets and 11 α helices (H1, H3 - H12) that form a three layer helical sandwich with H1 and H3 in front, H4, H5, H8, and H9 in the middle, and H7 and H10 in the back.⁹⁵ At the base of these helical layers, lies an enclosed hydrophobic ligand binding pocket (LBP).^{89, 95}

The LBD harbors a solvent-exposed dimerization interface and region for ligand driven transcription known as activation function 2 (AF-2).^{89, 96} The AF-2 domain, formed by residues in H3, H4, and H12, is a hydrophobic groove in which the end terminal helix, H12, may play a particularly vital role in spatial conformation and thus GR function.⁸⁹ Conformational changes to the AF-2 interface can drive selective interaction with coregulators and thus make way for the development of selective glucocorticoid receptor modulators (SGRMs).^{17, 97, 98}

The C11 hydroxyl group on the C ring of typical steroidal GCs is part of an important interaction with Asn564 in H3, effecting AF-2 stability and function.^{95, 99} The aryl pyrazole glucocorticoid receptor agonists (APGRAs) examined in this study imitate elements of the typical A, B and C rings of a steroidal GC, specifically the hydroxyl group on these non-steroidal ligands mimic the C11 OH on the steroidal framework. (Figure 2.2.1) In a previous study, molecular docking revealed the hydroxyl group of the APGRAs

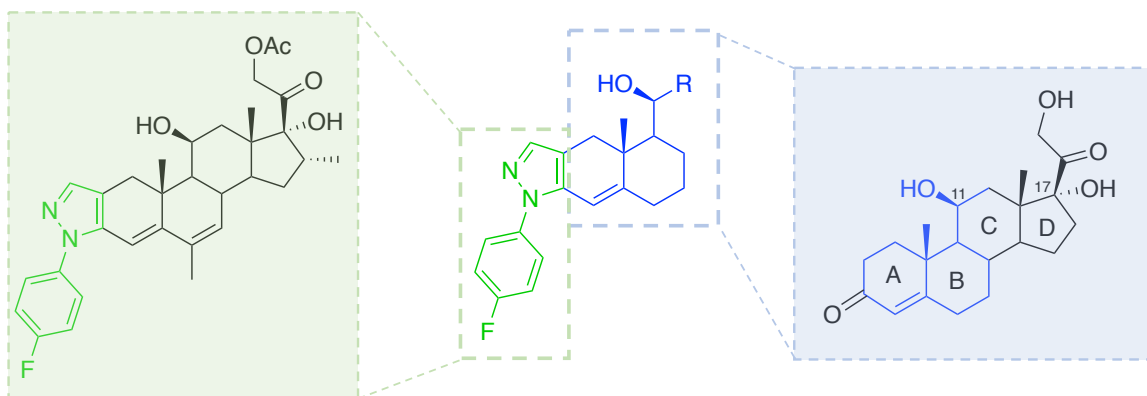


Figure 2.2.1 – APGRA Design

APGRA scaffold (center) formed from components of non-steroidal fluorocortivizol (green) and steroidal hydrocortisone (blue). Steroidal rings denoted A, B, C, and D with C11 and C17 hydroxyl moieties noted.

indeed contributes to this important interaction. However, given the flexible nature of this region on the APGRA scaffold, isomers **11A** and **11B** (Figure 2.3.1) have the potential to elicit differing responses, with both isomers capable of securing a polar interacting with Asn564.

Herein, a deeper investigation to the SARs of this previously developed ligand scaffold was provided by synthesizing a collection of novel APGRAs with a focus on examining the direct role of stereochemistry. Previous studies have found small alkyl and minimally substituted aromatic substituents performed most favorably.^{62, 100} Therefore, the current study expands the library of APGRAs to include small alkyl substituents, 3 and 5 carbon flexible ring substituents, and rigid aromatic moieties. To date, there is no known study directly investigating the stereochemical impact of this scaffold. Given the therapeutic potential of the methyl and phenyl derivative previously discovered¹⁰⁰, it is important to understand the stereochemical effects to achieve maximum benefit before proceeding to further analysis. These compounds were subjected to *in vitro* assays evaluating both transactivation and transrepression using in-depth biochemical assays. Furthermore, SARs were probed through molecular docking experiments using the deacylcortivazol (DAC) expanded ligand binding pocket.

2.3 – SYNTHETIC METHODS

All molecules for this study were synthesized with slight modifications of published synthetic routes.^{62, 100} Briefly, methyl vinyl ketone and 3-methyl-1,3-cyclohexedione are used to enantioselectively create Wieland - Mischer ketone **4** through an L-proline catalyzed Robinson Annulation.⁸⁸ Selective protection results in the ketal **5**, which is then formylated to produce **6**. Addition of p-fluorophenylhydrazine hydrochloride to β - ketoaldehyde **6** forms aryl pyrazole **7** which subsequently undergoes a deprotection to yield ketone **8**. A Wittig reaction allows for formation of enol **9** followed by hydrolysis to afford aldehyde **10**. A Grignard reaction utilizing Grignard and alkyl lithium reagents

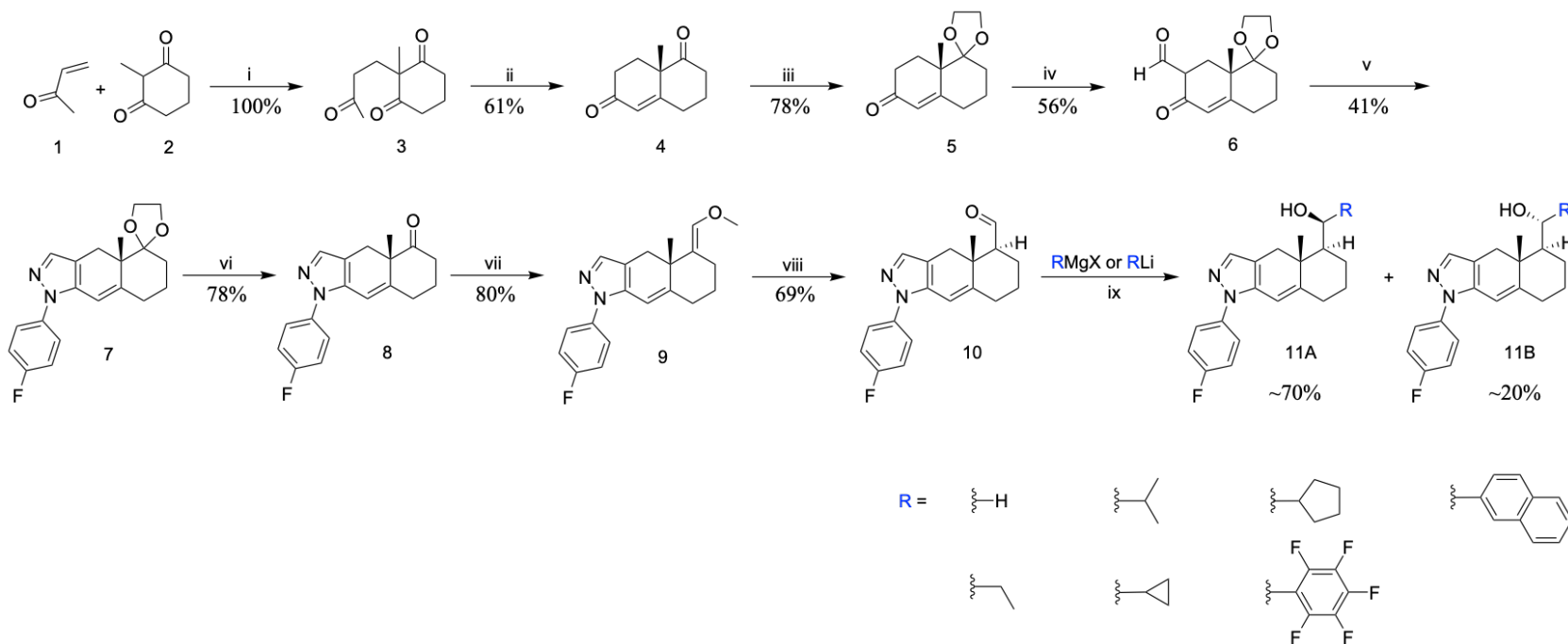


Figure 2.3.1 – Synthetic Route to APGRAs

Reagents and conditions: (i) AcOH, H₂O, 75 °C, 1 h (ii) L-proline, DMF, rt, 120 h (iii) p-TsOH, 4Å mol sieves, ethylene glycol, rt, 30 min (iv) ethyl formate, NaH, toluene, 0 °C, 3 h (v) NaOAc, AcOH, 4-Fluorophenylhydrazine Hydrochloride, rt, 3 h (vi) 6N HCl, THF, reflux, 3.5 h (vii) Ph₃PCH₂OCH₃Cl, KHMDS, THF, rt, 24 h (viii) 4N HCl, rt, 36 h (ix) THF, -78 °C, 1 h

allows for the incorporation of each substituent forming end products **11A** and **11B**. (Figure 2.3.1)

The final mixture of isomers underwent preparative supercritical fluid chromatography (SFC) separation. Compounds were analytically screened against 36 chiral separation conditions to determine the appropriate phase(s) to resolve the isomers of the compounds of interests (**11A** and **11B**) and to simultaneously determine conditions to remove unrelated achiral impurities. It was determined that the best phase for separation was ColumnTek EnantioCel[®] C4-5. For preparative separation of the isomers **11A** and **11B**, samples were dissolved in ethanol, sonicated, and filtered through a 0.2 μ m PVDF membrane syringe filter (Machery-Nagel; Düren, Germany) to a concentration of ~20 mg/mL. Enantiomers were separated on a ColumnTek EnantioCel[®] C4-5 (3 $\times$ 25 cm) cellulose-derived column, using 40% isopropanol / 60% CO₂ (100 bar backpressure) at a flowrate of 90 mL/min and detection at 220 nm. The sample was introduced onto the preparative system by stacked-injections every 250 second, resulting in a throughput of ~250 mg/hr, which yielded isomers at ~97% ee with minimal loss of sample (~8%). Finally, purified isomers were identified using 2D NMR techniques as described in the results.

2.4 – *IN VITRO* ASSAYS

The ability of the synthetic APGRAs to reduce the proinflammatory activity of IL-1 β was examined using a reporter gene assay consisting of a multimerized NF- κ B luciferase reporter construct that contains five copies of an NF- κ B element upstream of a luciferase gene (5X-NF- κ B-Luc).⁵⁷ 832/13 cells, a rat insulinoma cell line, were cultured as described previously⁶⁶ and determined to be free of mycoplasma contamination. Briefly, the 832/13 cells were grown in 24-well plates and transfected with the 5X-NF- κ B-Luc reporter plasmid. Twenty-four hours after transfection, the cells were exposed to each synthetic APGRAs for one hour, followed by culture for four hours with 1 ng/mL IL-1 β . Transactivation activity was determined by analyzing the ability of the synthetic APGRAs to drive transcriptional activity from plasmid construct containing three copies of a glucocorticoid response element (3xGRE).⁵⁷ The 832/13 cells were grown in 24-well plates

and transfected with the 3xGRE luciferase reporter plasmid. Twenty-four hours post-transfection, the cells were exposed to each synthetic APGRA for four hours. At the end of each respective treatment period, the cells were harvested using passive lysis buffer (Promega, Madison, WI) and luciferase activity measured in a Glomax luminometer (Promega, Madison, WI). Luciferase activity was normalized to total protein to account for differences in cell numbers between experiments.

2.5 – MOLECULAR DOCKING

Ligand-receptor interactions were modeled between the molecules subjected to *in vitro* testing and the extended pocket of the GR ligand-binding domain (LBD). The crystal structure of the GR LBD bound to DAC, a potent APGRA₂ and the fourth LXXLL motif of steroid receptor coactivator 1 (SRC1-4; PDB ID: 3BQD) was used as the model for molecular docking calculations.²⁹ The 3BQD crystal structure was chosen as the best template as consistent trends in binding were observed, which validated previous work that assumed the enlarged ligand binding pocket caused by the phenylpyrazole moiety would also house the smaller APGRA analogues.⁶⁹ The GR LBD was prepared for docking studies by removing the bound ligand and excess water molecules. Ligands were optimized in Chem3D (PerkinElmer) prior to docking, which was performed between a rigid 3BQD GR and a flexible ligand using AutoDock Vina (The Scripps Research Institute).⁷⁰ The resulting ligand poses were viewed with PyMol (Schrödinger).⁷¹ Ligand-residue interactions were further analyzed with LigPlot+ (European Molecular Biology Laboratory).⁷²

2.6 – RESULTS AND DISCUSSION

2.6.1 – Stereochemical Determination

After the final mixture of isomers underwent chiral separation via SFC, 1D and 2D nuclear magnetic resonance (NMR) techniques were employed to identify the

stereochemical configuration of each compound. Isomer identification was achieved using proton-proton nuclear Overhauser effect spectroscopy (^1H - ^1H NOESY), which provides information on spatial relationships, in conjunction with proton-proton coupling constants, which provide information on dihedral angles.¹⁰¹ All NMR experiments were performed at 25 °C using CDCl_3 as the solvent and reference signal.

After a standard ^1H NMR and ^{13}C NMR were obtained, heteronuclear single-quantum coherence (^1H - ^{13}C HSQC), heteronuclear multiple-bond correlation (^1H - ^{13}C HMBC), and ^1H - ^1H NOESY were collected to aid in identification. ^1H - ^{13}C HSQC shows proton - carbon single bond correlation in which the x axis contains the ^1H signals, and the y axis contains the ^{13}C signals. ^1H - ^{13}C HMBC correlates ^1H and ^{13}C nuclei through 2 to 3 bonds and occasionally 4 bonds.¹⁰² ^1H - ^1H NOESY measures cross-relaxation between ^1H nuclei within 2 to 4 angstroms of each other in space. ^1H - ^{13}C HMBC and ^1H - ^{13}C HSQC techniques provide connectivity information and allow precise identification of each ^1H and ^{13}C signal. ^1H - ^1H NOESY provides imperative insight to spatial proximity of ^1H nuclei, allowing isomers **11A** and **11B** to be distinguished from one another. Due to the rotatable nature of the stereocenter in question, the identification was often a result of minute differences between spectra. All 1D and 2D NMR can be found in the appendix (section 2.9.1).

Previously, Ali and coworkers establish this reaction sequence led to stereoiduction in all subsequent transformations yielding a new chiral center at the formation of **4**.^{62, 100} Based on this observation, isomer **11A** was predicted to be the major product and isomer **11B** predicted to be the minor product from the set of 8 possible stereoisomers. (Figure 2.3.1) Chiral separation yielded 2 final isomers for the ethyl, isopropyl, cyclopropyl, pentafluorophenyl, and naphthalene derivatives. Only one final isomer was isolated for the hydrogen and cyclopentyl derivatives.

To determine stereochemistry, all possible isomers were drawn and energy minimized in Chem3D (PerkinElmer). All distance and angle measurements were performed using PyMol (Schrödinger).⁷¹ Herein **AP13** will be discussed as an example, however, NMR spectra and characterization for all compounds can be found in the appendix (section 2.9.1). ^1H and ^{13}C NMR techniques were first used to assign peak shifts,

^1H - ^{13}C HSQC was then used to identify proton – carbon single bond connectivity. (Figure 2.6.1) ^1H - ^{13}C HMBC was then employed to ambiguate connectivity that could not be determined from HSQC. ^1H - ^1H NOESY in plane axial and equatorial correlations determined each unique proton signal, which led to the absolute structure illustrated in Figure 2.6.1. ^1H - ^1H NOESY correlations determined the orientation of the three stereocenters. (Figure 2.6.2) Correlation between the equatorial proton on C8 (2.96 ppm) and the protons of the methyl group of C18 (1.14 ppm) was used to assign the C9 stereocenter as the R configuration. Correlation between the axial proton on C8 (2.46 ppm) and the C10 proton signal (1.50 ppm) are representative of S configuration for the stereocenter at C10. Finally, the unique correlation between the C11 proton (3.86 ppm) and the C10 proton signal (1.50 ppm) is consistent with an S configuration for the stereocenter at C11. **AP14** shares the same correlations leading to R and S configuration for C9 and C10 respectively; however, the lack of a unique correlation between the C11 proton and C10 proton leads to the only possible isomer being that of R configuration at the C11 site. (See appendix section 2.9.1)

Conserved ^1H - ^1H NOESY interactions between the C8 protons, C10 proton, and C18 protons throughout all final compounds shows C9 R configuration, and C10 S configuration remain consistent, supporting the hypothesis that the major isomers of each derivative are regarding C11 chirality, yielding products with final structures represented by **11A** and **11B**. (Figure 2.3.1) The stereochemical determination for all remaining compounds was conducted in a similar manner.

2.6.2 – Biological Evaluation

To investigate this novel class of anti-inflammatory compounds, bioassays that directly measured the impact of transactivation and transrepression were chosen. Directly studying the ability of the ligand activated GR to activate transcription from glucocorticoid response elements (GREs), allows for an encompassing view of transactivation potential.

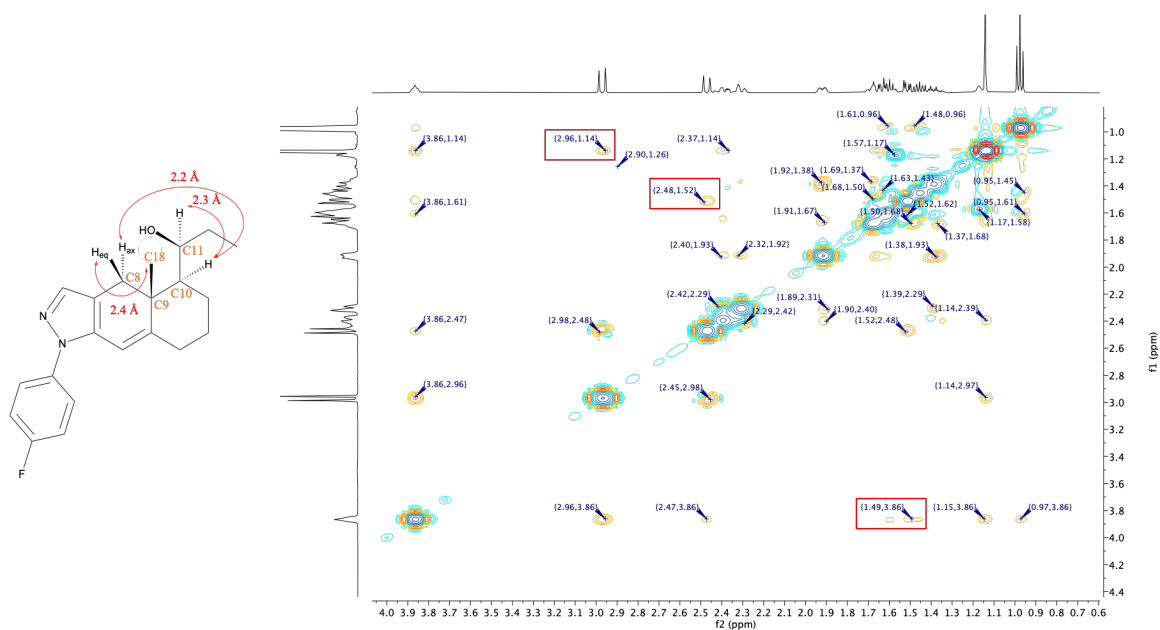


Figure 2.6.2 – ^1H - ^1H NOESY Correlations of AP13

^1H - ^1H NOESY NMR of **AP13** (in CDCl_3) with calculated distances between protons displayed on the left and their corresponding correlations boxed in red on the spectra to the right. Y axis (f1) and X axis (f2) displays proton signals from 0 to 4 ppm.

The ability of the APGRAs to reduce CCL2 activity induced by the pro-inflammatory cytokine IL-1 β (transrepression) and enhance transactivation of a synthetic GRE-driven promoter-luciferase construct (3xGRE assay - transactivation) were tested and benchmarked against both dexamethasone (Dex) and hydrocortisone (HC).

Among all the tested compounds, Dex displayed the greatest transrepression activity with a maximal response of 82% and a pIC₅₀ of 8.91. (Table 2.6.1) Hydrocortisone displayed a maximal response of 72% in the CCL2 assay and effective concentrations were shown to be ~250 fold greater than that for Dex with a pIC₅₀ of 6.53. Among the APGRAs tested, three compounds emerged as the top performing candidates. (Figure 2.6.3) **AP22** and **AP13** displayed activity above 78% that of Dex and above 88% that of HC in the CCL2 assay. While their effective concentrations were found to be 15 to 30 fold higher than Dex with pIC₅₀ values of 7.45 and 7.69 respectively, they were ~10 fold lower than HC. Additionally, **AP22** and **AP13** were shown to elicit half the transactivation activity than Dex and HC with E_{max} of 4.72 and 4.13 respectively. These compounds also showed desirable pEC₅₀ values in the micromolar range, 6.64 and 5.00 respectively.

AP12 also showed favorable activity when compared to HC, performing with 76% transrepression of HC in the CCL2 assay. This compound demonstrated a more desirable pIC₅₀ of 8.33 while the pIC₅₀ for HC was shown to be 6.53, nearly a 160-fold difference. Additionally, **AP12** displayed ~80% less transactivation activity in the 3xGRE assay compared to both Dex and HC, inducing less than a 1.5-fold change across all concentrations tested, whereas HC and Dex elicited a fold change >7 in that assay. HC is widely available and commonly used as a topical anti-inflammatory treatment. However, HC has long been known to be associated with side effects such as osteoporosis, altered collagen metabolism, and skin atrophy which often leads to permanent damage.¹⁰³ Ongoing efforts to find an alternative therapy without atrophogenic potential have been unsuccessful; however, a GC therapy with reduced transactivation activity such as that of **AP12**, may lead to a safer alternative.^{103, 104}

Comparison of the overall performance of si isomers (Table 2.6.1) and re isomers (Table 2.6.2) shared an interesting trend. In the cases in which both isomers were tested,

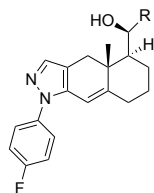


Table 2.6.1 - Biological analysis of si isomers with upward OH.

Compd	R	Relative Affinity 3BQD kcal/mol	CCL2 ^{a,b}				3xGRE ^{a,c}			
			pIC ₅₀	E _{max}	%Dex ^d	%HC ^d	pEC ₅₀	E _{max}	%Dex ^d	%HC ^d
				% Response				Fold Over Control		
HC		-8.8	6.53	72.50	88.49	100.00	6.96	7.60	146.70	100.00
Dex		-10.4	8.91	81.93	100.00	113.01	8.50	7.17	100.00	94.41
AP12	H	-10.3	8.33	55.53	67.77	76.58	7.48	1.43	20.00	18.88
AP13	Ethyl	-10.5	7.69	64.36	78.56	88.77	5.00	4.13	57.59	54.37
AP15	Isopropyl	-10.7	9.23	34.77	42.43	47.95	5.82	0.88	12.22	11.54
AP17	Cyclopropyl	-10.7	7.47	49.21	60.06	67.87	6.03	4.14	57.73	54.50
AP19	Cyclopentyl	-11.6	6.35	58.89	71.87	81.22	5.50	3.42	47.66	44.99
AP20	Naphthalene	-11.8	6.44	46.00	56.15	63.45	6.22	1.37	19.10	18.04
AP22	Penta-F Ph	-12.8	7.45	65.74	80.24	90.67	6.64	4.72	65.75	62.07

a) Values are the mean of at least two experiments done in duplicate. *b)* CCL2-promoter-luciferase-reporter activity assay. *c)* 3xGRE-promoter-luciferase-reporter activity assay. *d)* Efficacy represented as percentage of maximal response of dexamethasone or hydrocortisone.

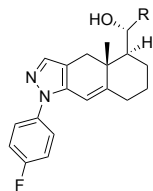


Table 2.6.2 - Biological analysis of re isomers with downward OH.

Compd	R	Relative Affinity	CCL2 ^{a,b}				3xGRE ^{a,c}			
			pIC ₅₀	E _{max}	%Dex ^d	%HC ^d	pEC ₅₀	E _{max}	%Dex ^d	%HC ^d
		3BQD kcal/mol	% Response				Fold Over Control			
AP14	Ethyl	-10.5	7.80	26.15	31.92	36.07	5.56	0.98	13.71	12.95
AP16	Isopropyl	-11.0	6.78	15.93	19.44	21.97	5.82	1.15	16.00	15.11
AP18	Cyclopropyl	-10.7	5.66	1.74	2.12	2.39	9.32	0.96	13.34	12.59
AP21	Naphthalene	-12.7	7.53	10.57	12.90	14.58	8.82	0.96	13.39	12.64
AP23	Penta-F Ph	-13.1	7.59	27.85	33.99	38.41	5.66	2.40	33.51	31.64

a) Values are the mean of at least two experiments done in duplicate. *b)* CCL2-promoter-luciferase-reporter activity assay. *c)* 3xGRE-promoter-luciferase-reporter activity assay. *d)* Efficacy represented as percentage of maximal response of dexamethasone or hydrocortisone.

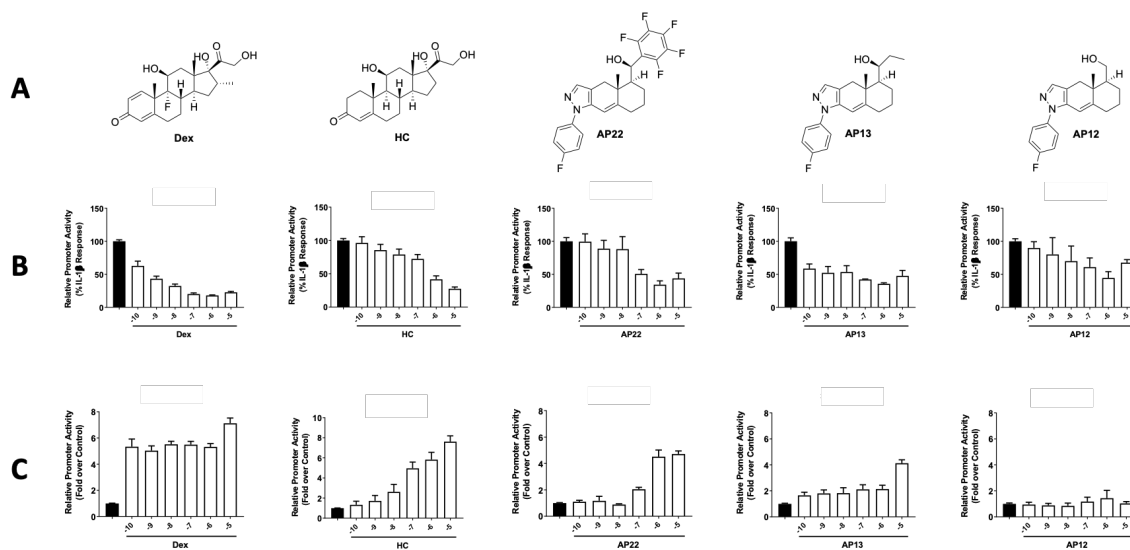


Figure 2.6.3 - Biological analysis of top performing compounds.

Bioassay data showing GR agonist activity and impact on glucose-stimulated insulin secretion. (A) Structures of tested compounds. (B) CCL2-promoter-luciferase-reporter activity assay; x-axis: log concentration (molar); y-axis: %-maximal IL-1 β response (relative promoter activity). (C) 3xGRE-promoter-luciferase-reporter activity assay; x-axis: log concentration (molar); y-axis: fold over control.

the re isomers showed on average ~70% less anti-inflammatory response of its counterpart in the CCL2 assay. The R and S cyclopropyl derivatives displayed the most drastic difference in which **AP18** was found to be nearly inactive, eliciting 96% less transrepression than its counterpart **AP17**. The most active compound among the re isomers was **AP23**. Still, this compound only produced 28% response in the CCL2 assay. Stereochemistry was found to play a large role in the transrepression activity in which si isomers were considerably more active and, in some cases, the only active isomer. This isomer most closely mimics traditional steroidal scaffolds, including cortisol which is naturally produced in the adrenal cortex.¹⁰⁵ For this reason, the upward OH was hypothesized to be the more desirable isomer.

2.6.3 – Computational Analysis

Computational experiments were used to probe the underlying interactions between the ligand and receptor. Molecular docking studies were performed using AutoDock Vina (The Scripps Research Institute)⁷⁰ through which the energy minimized ligand structures were allowed to freely interact within the expanded ligand binding pocket of the rigid receptor (PDB ID: 3BQD).²⁹ This technique allowed the ligand to assume various poses and computed the associated relative binding affinity. In this case, the top binding affinity posed the ligand in a configuration that matched that of DAC, displaying strong induction of the aryl pyrazole moiety within the hydrophobic channel observed in the expanded LBP when bound to DAC.²⁹ Previous studies have shown that using the expanded LBP to model this APGRA scaffold closely matched interactions seen with the host ligand, DAC, making it more suitable than the standard steroid LBP.¹⁰⁰ Because of the unique nature of GR activity in which both transactivation and transrepression are considered two separate modes of activation, the relative binding affinity does not necessarily correlate to overall ligand activity or selectivity. This requires a more intricate examination of ligand-residue integrations. LigPlot analysis of all tested compounds can be found in the appendix (Section 2.9.4).

Residue Asn564 in H3 was previously found to be a crucial interaction that may affect the stability of the AF-2 domain and potentially a key link between differentiating ligand activity.^{95, 99} While nearly all isomers conserved this ligand-residue interaction, there were some slight variations in the type of interaction detected. Compounds displaying polar interactions with the backbone of Asn564 rather than the side chain (**AP14, 20, 21, 23**) had relatively poor activity in the CCL2 assay, generating less than ~45% response. Contrarily, almost all compounds that retained a polar interaction with the C11 OH moiety and the side chain of Asn564 (**AP12, 13, 22**) performed relatively well with >55% response in the CCL2 assay, with the exception of **AP15**, which displayed this interaction but elicited only a 34% response in transrepression. The non-aromatic, cyclic substituents only possessed a hydrophobic interaction with Asn564 but performed moderately in the CCL2 assay with compounds **AP17** and **AP19** eliciting ~50% response.

Intriguingly, **AP12** displayed the highest ability to selectively mediate one mode of activation over the other with an E_{max} of 55% in the CCL2 assay and only a maximal 1.43 fold over control in the 3xGRE assay. **AP13** also displayed high selectivity with an E_{max} of 64% in the CCL2 assay and an approximate fold over control of 2 in the 3xGRE assay for all concentrations except the highest concentration where the activity abruptly doubles to a fold change of ~4. (Figure 2.6.3) These compounds share the lowest number of total residue interactions, which may facilitate a more fluid receptor structure. (See appendix section 2.9.4) Recent findings have shown that retaining high affinity while weakening interactions, particularly with H3, H4, and H12 that are involved with the AF-2 domain, results in a more selective ligand.⁹⁹ This correlation is observed in this study as well with compounds **AP12** and **AP13** displaying the most selectivity between transrepression and transactivation.

All non-aromatic substituents with the hydroxyl group backward also share an interaction with Trp600 and exhibited some of the least desirable activity in the biological assays with only 1% to 26% response in the CCL2 assay. Interestingly, compounds with an aromatic substituent (**AP20-23**), lead to an additional hydrophobic contact between Cys622 and the aryl pyrazole portion of the APGRA. All compounds with hydrophobic Cys736 contact (**AP14, 16, 18, 20, 21**) performed poorly in the CCL2 assay, producing

less than ~45% responses. It should be noted that all these compounds except **AP20** are the isomer with a downward OH.

Another explanation for the differing activity between isomers is offered by overlaying the ligands in the orientation associated with their highest binding affinity within the LBP. Looking at the ethyl derivatives as an example, (Figure 2.6.4) the ethyl moieties point in opposite directions in the LBP between isomers. The ethyl moiety of **AP13** (shown in blue) interacts more closely with H3, while the ethyl moiety of **AP14** (shown in magenta) is rotated 180° from that of **AP13** and interacts more closely with H11. **AP13** elicits ~60% higher transrepression response than **AP14** in the CCL2 assay, which may be due to the unique interaction between **AP13** and H3, which is part of the AF-2 domain. All non-aromatic derivatives displayed the same 180° rotation in R group positions when the top binding conformations are overlayed within the LBP, in which the si isomers have increased networks with H3.

2.7 – CONCLUSION

A class of novel, stereochemically pure, APGRAs was explored to gain deeper insight to the SARs using biological assays and molecular docking techniques. Although the compounds were not able to outperform Dex and HC in the CCL2 assay, they were able to show greater signs of selectivity with reduced transactivation activity while maintaining ~75% transrepression activity of Dex and/or HC. This work confirms previous finding that small alkyl and aromatic substituents perform most favorably on this scaffold with **AP12**, **AP13**, and **AP22** producing the most desired activity in the biological assays.⁶²

100

To our knowledge, this was the first study to directly examine the stereochemical impact on the activity of this non-steroidal framework. These analyses revealed the si isomer with an upward hydroxyl group to be more active than its counterpart, producing ~70% greater transrepression responses. Molecular docking revealed insight to this

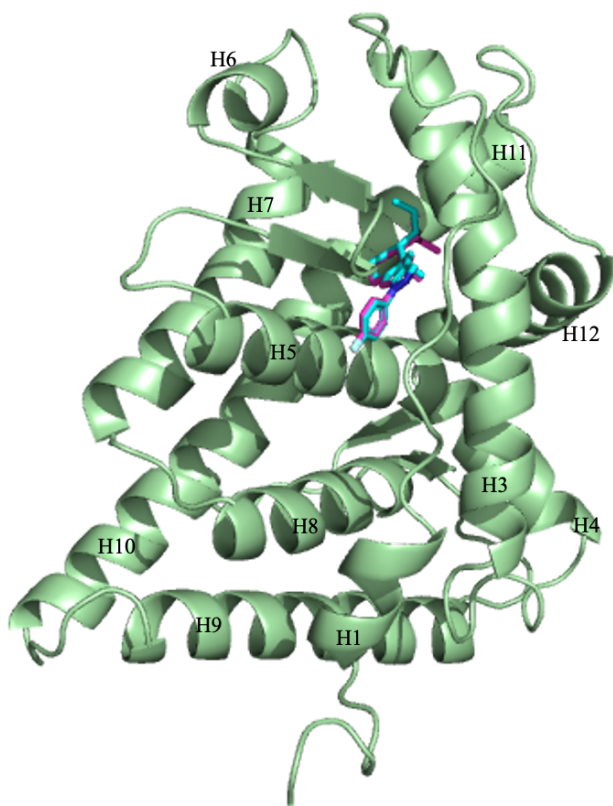


Figure 2.6.4 – Compounds AP13 and AP14 Docked in GR LBD

Top binding poses of **AP13** (shown in blue) and **AP14** (shown in magenta) superimposed in the LBP of the human glucocorticoid receptor (PDB ID: 3BQD, shown in green). Helixes labeled in black as H1 and H3 - H12.

observed activity, and two different interactions with H3, which is a key component of the AF-2 domain thought to drive selectivity between dimeric and co-regulating activity, may be responsible for the observed activity.^{89, 95} Interactions with Asn564 varied across isomers, with the top performing compounds possessing a polar interaction between the C11 OH and the side chain of Asn564, as seen in that of DAC and typical steroidal GCs.²⁹ Superimposing the stereoisomers in the LBP gave additional insight. Among the non-aromatic derivatives, the *si* isomer facilitated an interaction between the R moiety (Figure 2.3.1) and H3, whereas its counterpart showed this group was rotated 180°, resulting in an interaction with H11. The differing contacts with the AF-2 domain may cause more favorable interaction with co-regulators thus increasing transrepression.

This study further highlights the complex relationships between ligands and the GR, emphasizing the need for further investigation to unravel the complex GR-GC interactions. Moreover, the work presented here stresses the importance of achieving stereochemical purity prior to moving on to more in-depth *in vivo* studies. Methyl and phenyl derivatives of this scaffold were found to be the most desirable in a previous study, and efforts are currently underway to examine these stereochemically purified derivatives through *in vivo* studies.

2.8 – Methods

2.8.1 – Materials

All reactions were performed under inert atmosphere. Reagents were purchased from Fisher Scientific or VWR and used without further purification, except for methyl vinyl ketone (**1**), which was distilled immediately prior to use. Dry ethereal solvents were obtained by distillation from a potassium-benzophenone ketyl still, all other dry solvents were obtained via distillation over CaH₂. Thin-layer chromatography (TLC) was carried out using Sorbent Technologies silica G w/UV254 TLC plates. Final compounds were preparatively separated on a Berger Multigram II SFC equipped with two SD-1 Varian

pumps, a Knauer K-2501 Spectrophotometer set at 220 nm, a 6-ton bulk CO₂ tank with a built-in chiller and heater and G700 compressor (Mettler-Toledo, Newark, DE, USA). All purified compounds of interest were visualized as single spots using short-wave UV light; more permanent staining of TLC spots was achieved using vanillin. 1D and 2D NMR spectra were recorded on a Varian Inova 500 MHz instrument (Palo Alto, CA) in solutions of CDCl₃. High resolution mass spectrometry (HRMS) was performed using an Exactive™ Plus Orbitrap mass spectrometer with an ESI source operating in positive ionization mode. Optical rotation was measured using a Perkin-Elmer 241 polarimeter (Waltham, MA) equipped with a sodium source lamp. IR spectra were collected using a Thermo Scientific Nicolet™ iS5 FTIR spectrometer (Waltham, MA). All NMR spectra of final compounds and bioassay data can be found in the appendix.

2.8.2 – *Synthetic Procedures and Characterization*

((4aR,5S)-1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)methanol (AP12): Compound 10 was synthesized as previously reported.^{62, 100} Commercially available lithium aluminum hydride (1 mmol) was dissolved in dry THF (15 mL) and cooled to -78°C. Aldehyde 10 (1 mmol) was dissolved in THF (10 mL) and added to the reaction mixture dropwise via cannulation. The mixture was warmed to room temperature and monitored by TLC. After 24 hours the reaction was cooled to 0°C, diluted with diethyl ether and subsequently quenched with a slow addition of DI H₂O (40 µl), followed by 15% NaOH solution (40 µl), and finally an additional 120 µl of DI H₂O. After stirring at room temperature for 15 minutes, the reaction was dried over anhydrous MgSO₄, filtered, and concentrated in vacuo, and initially purified using flash chromatography (40% EtOAc/hexanes) before undergoing chiral separation using supercritical fluid chromatography. The final product was obtained as a white solid in 51% yield. Mp 184.7 - 186.4 °C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, R_f = 0.21; [α]_D²⁰ = 110.4 (c 1.0, MeOH); ¹H NMR (500 MHz, Chloroform-*d*) δ 7.45 (dd, 2H), 7.40 (s, 1H), 7.20 – 7.10 (m, 2H), 6.13 (s, 1H), 3.91 (dd, 1H), 3.59 – 3.48 (m, 1H), 2.96 (d, 1H),

2.66 (d, 1H), 2.44 – 2.27 (m, 2H), 2.02 (d, 1H), 1.94 – 1.85 (m, 1H), 1.71 – 1.62 (m, 1H), 1.39 (q, 3H), 0.96 (s, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 18.49, 25.66, 26.23, 33.37, 34.70, 39.99, 51.80, 64.20, 76.91, 77.16, 77.41, 109.53, 114.16, 116.07, 116.25, 125.44, 125.51, 136.04, 136.07, 136.80, 138.07, 149.79, 160.61, 162.57. Defining ^1H - ^1H NOESY Correlations (500 MHz, Chloroform-*d*) δ (2.94, 0.95), (2.64, 1.66). FT-IR ν_{max} (cm^{-1}): 3327 (OH), 3078, 2900, 2864, 2834, 1613, 1512, 1435, 1223, 841. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{19}\text{H}_{21}\text{FN}_2\text{O}$: 313.1711; found: 313.1709.

General Procedure for compound 11: Compound 10 was synthesized as previously reported.^{62, 100} A commercially available solution of the appropriate organolithium or Grignard reagent (10 mmol) was transferred to a round bottom flask with THF (15 mL). The solution was cooled to -78°C and a solution of aldehyde 10 (1 mmol) in THF (10 mL) was added dropwise via canula. The reaction was allowed to stir at -78°C for 1 hour before being quenched with isopropyl alcohol (IPA). The reaction was subsequently poured into a separatory funnel containing saturated NH_4Cl and extracted three times with EtOAc. The organic fractions were washed with water and brine, dried over anhydrous MgSO_4 , filtered, and concentrated in vacuo. Purification of the crude product was accomplished using flash chromatography (25-35% EtOAc/hexanes) followed by chiral separation via supercritical fluid chromatography.

(S)-1-((4aR,5S)-1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)propan-1-ol (AP13): Using a commercially available solution of ethylmagnesium bromide (1.0 M in THF), compound **AP13** was obtained as a white solid in 76% yield. Mp $168.8 - 169.5^\circ\text{C}$; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.42$; $[\alpha]_{\text{D}}^{20} = 97.8$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.45 (dd, 2H), 7.41 (s, 1H), 7.18 – 7.12 (m, 2H), 6.10 (s, 1H), 3.87 (s, 1H), 2.97 (d, 1H), 2.47 (d, 1H), 2.44 – 2.36 (m, 1H), 2.31 (d, 1H), 1.92 (d, 1H), 1.72 – 1.57 (m, 3H), 1.54 – 1.49 (m, 1H), 1.45 (dd, 1H), 1.42 – 1.34 (m, 1H), 1.17 (s, 1H), 1.14 (s, 3H), 0.98 (t, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 11.04, 19.55, 21.46, 26.04, 30.61, 33.52, 34.71, 41.11, 53.20, 72.15,

76.91, 77.16, 77.41, 109.33, 114.10, 116.06, 116.24, 125.42, 125.49, 136.09, 136.11, 136.97, 138.10, 150.36, 160.59, 162.56. Defining $^1\text{H} - ^1\text{H}$ NOESY Correlations (500 MHz, Chloroform-*d*) δ (2.96, 1.14), (2.46, 1.50), (3.86, 1.50). FT-IR ν_{max} (cm^{-1}): 3338 (OH), 2923, 2864, 1615, 1512, 1223, 845. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{21}\text{H}_{25}\text{FN}_2\text{O}$: 341.2024; found: 341.2025.

(*R*)-1-((4*aR*,5*S*)-1-(4-fluorophenyl)-4*a*-methyl-4,4*a*,5,6,7,8-hexahydro-1*H*-benzo[*f*]indazol-5-yl)propan-1-ol (AP14): Using a commercially available solution of ethylmagnesium bromide (1.0 M in THF), compound **AP14** was obtained as a white solid in 16% yield. Mp 164.9 - 166.7 °C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, R_f = 0.42; $[\alpha]_{\text{D}}^{20}$ = -78.3 (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.47 – 7.43 (m, 2H), 7.41 (s, 1H), 7.18 – 7.12 (m, 2H), 6.11 (d, 1H), 3.91 – 3.83 (m, 1H), 2.97 (d, 1H), 2.48 (d, 1H), 2.40 (m, 1H), 2.31 (m, 1H), 1.92 (m, 1H), 1.72 – 1.54 (m, 4H), 1.52 (dd, 1H), 1.49 – 1.42 (m, 1H), 1.42 – 1.34 (m, 1H), 1.15 (s, 3H), 0.98 (t, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 10.88, 19.39, 21.29, 25.88, 30.45, 33.35, 34.55, 40.95, 53.03, 72.00, 76.74, 76.99, 77.25, 109.17, 113.94, 115.90, 116.08, 125.26, 125.32, 135.92, 135.95, 136.81, 137.93, 150.19, 160.43, 162.39. Defining $^1\text{H} - ^1\text{H}$ NOESY Correlations (500 MHz, Chloroform-*d*) δ (2.96, 1.14), (2.46, 1.50). FT-IR ν_{max} (cm^{-1}): 3357 (OH), 2930, 2868, 1615, 1513, 1456, 1223, 833. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{21}\text{H}_{25}\text{FN}_2\text{O}$: 341.2024; found: 341.2025.

(*S*)-1-((4*aR*,5*S*)-1-(4-fluorophenyl)-4*a*-methyl-4,4*a*,5,6,7,8-hexahydro-1*H*-benzo[*f*]indazol-5-yl)-2-methylpropan-1-ol (AP15): Using a commercially available solution of isopropylmagnesium chloride (2.0 M in THF), compound **AP15** was obtained in 67% yield as a beige solid. Mp 110.1 - 111.2 °C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, R_f = 0.56; $[\alpha]_{\text{D}}^{20}$ = 51.7 (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.48 – 7.39 (m, 3H), 7.16 (d, 2H), 6.11 (s, 1H), 3.51 (d, 1H), 2.92 (d, 1H), 2.49 – 2.29 (m, 3H), 1.91 (d, 1H), 1.76 – 1.68 (m, 2H), 1.65 (d, 2H), 1.40 (d, 1H), 1.25 (s, 1H), 1.13 (s, 3H), 1.03 (d, 3H), 0.91 (d, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 19.30, 19.32, 19.98, 21.88, 25.92, 33.14, 33.37, 34.44, 40.94, 50.46, 75.96, 76.76, 77.02, 77.27, 109.19, 114.02, 115.92, 116.10, 125.35, 135.96, 136.84, 137.96, 150.33, 160.45, 162.41. Defining $^1\text{H} - ^1\text{H}$ NOESY Correlations (500 MHz, Chloroform-*d*) δ (2.90, 1.12), (2.47, 1.70), (3.50, 1.70),

(2.48, 0.91). FT-IR $\nu_{\max}$ (cm⁻¹): 3395 (OH), 2959 (w), 2223, 1700, 1512, 1464, 1223, 836. HRMS (ESI) m/z: [M+H]⁺ Calcd for C₂₂H₂₇FN₂O: 355.2180; found: 355.2179.

(R)-1-((4aR,5S)-1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-2-methylpropan-1-ol (AP16): Using a commercially available solution of isopropylmagnesium chloride (2.0 M in THF), compound **AP16** was obtained in 22% yield as a beige solid. Mp 84.5 - 86.3°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, R_f = 0.56; [α]_D²⁰ = -41.4 (c 1.0, MeOH); ¹H NMR (500 MHz, Chloroform-*d*) δ 7.48 – 7.40 (m, 3H), 7.14 (d, 2H), 6.10 (s, 1H), 3.50 (d, 1H), 2.92 (d, 1H), 2.49 – 2.27 (m, 3H), 1.90 (d, 1H), 1.76 – 1.62 (m, 4H), 1.41 (d, 2H), 1.13 (s, 3H), 1.03 (d, 3H), 0.91 (d, 3H). ¹³C NMR (500 MHz, Chloroform-*d*) δ 19.32, 19.98, 21.88, 25.92, 33.14, 33.37, 34.43, 40.94, 50.46, 75.96, 76.77, 77.02, 77.28, 109.18, 114.02, 115.92, 116.11, 125.35, 135.95, 136.85, 137.96, 150.35, 160.46, 162.42. Defining ¹H - ¹H NOESY Correlations (500 MHz, Chloroform-*d*) δ (2.90, 1.13), (2.47, 1.70). FT-IR $\nu_{\max}$ (cm⁻¹): 3373 (OH), 2961(w), 2250, 1696, 1513, 1464, 1223, 837. HRMS (ESI) m/z: [M+H]⁺ Calcd for C₂₂H₂₇FN₂O: 355.218; found: 355.2179.

(S)-cyclopropyl((4aR,5S)-1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)methanol (AP17): Using a commercially available solution of cyclopropylmagnesium bromide (0.5M in THF), compound **AP17** was obtained in 59% yield as a beige solid. Mp 96.1 - 97.4°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, R_f = 0.49; [α]_D²⁰ = 42.1 (c 1.0, MeOH); ¹H NMR (500 MHz, Chloroform-*d*) δ 7.47 – 7.37 (m, 3H), 7.16 (d, 2H), 6.10 (s, 1H), 3.06 (d, 1H), 2.87 (d, 1H), 2.43 (dd, 2H), 2.35 – 2.27 (m, 1H), 1.94 (d, 1H), 1.86 (d, 1H), 1.77 (s, 2H), 1.50 – 1.39 (m, 1H), 1.25 (s, 1H), 1.16 (s, 1H), 1.11 (s, 3H), 0.62 (d, 1H), 0.57 – 0.49 (m, 1H), 0.39 (t, 1H), 0.21 – 0.14 (m, 1H). ¹³C NMR (500 MHz, Chloroform-*d*) δ 3.24, 4.36, 18.42, 19.43, 21.88, 25.97, 33.37, 34.59, 40.64, 54.22, 75.97, 76.77, 77.02, 77.27, 109.09, 113.91, 115.92, 116.10, 125.36, 135.95, 136.87, 137.93, 150.34, 160.45, 162.42. Defining ¹H - ¹H NOESY Correlations (500 MHz, Chloroform-*d*) δ (2.88, 1.10), (2.43, 1.77), (2.46, 0.16). FT-IR $\nu_{\max}$ (cm⁻¹): 3380 (OH), 3079, 2930 (w), 2868, 2250, 2048, 1700, 1513, 1423, 1222, 835. HRMS (ESI) m/z: [M+H]⁺ Calcd for C₂₂H₂₅FN₂O: 353.2024; found: 353.2022.

(*R*)-cyclopropyl((4*aR*,5*S*)-1-(4-fluorophenyl)-4*a*-methyl-4,4*a*,5,6,7,8-hexahydro-1*H*-benzo[*f*]indazol-5-yl)methanol (AP18): Using a commercially available solution of cyclopropylmagnesium bromide (0.5M in THF), compound **AP18** was obtained in 17% yield as a white solid. Mp 111.7 - 113.4°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.49$; $[\alpha]_D^{20} = -51.4$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.48 – 7.37 (m, 3H), 7.13 (s, 2H), 6.10 (s, 1H), 3.06 (d, 1H), 2.87 (d, 1H), 2.42 (dd, 2H), 2.31 (d, 1H), 1.94 (d, 1H), 1.87 (d, 1H), 1.77 (d, 1H), 1.58 (s, 1H), 1.45 (d, 2H), 1.11 (s, 4H), 0.62 (t, 1H), 0.56 – 0.49 (m, 1H), 0.43 – 0.36 (m, 1H), 0.22 – 0.13 (m, 1H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 3.24, 4.36, 18.42, 19.43, 21.88, 25.97, 33.37, 34.59, 40.64, 54.22, 75.97, 76.77, 77.02, 77.27, 109.10, 113.91, 115.92, 116.10, 125.35, 135.97, 136.86, 137.94, 150.32, 160.45, 162.41. Defining ^1H - ^1H NOESY Correlations (500 MHz, Chloroform-*d*) δ (2.86, 1.08), (2.42, 1.77), (1.77, 1.15). FT-IR ν_{max} (cm^{-1}): 3380 (OH), 3079, 2929 (w), 2867, 2251, 2046, 1699, 1512, 1423, 1223, 835. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{22}\text{H}_{25}\text{FN}_2\text{O}$: 353.2024; found: 353.2023.

(*S*)-cyclopentyl((4*aR*,5*S*)-1-(4-fluorophenyl)-4*a*-methyl-4,4*a*,5,6,7,8-hexahydro-1*H*-benzo[*f*]indazol-5-yl)methanol (AP19): Using a commercially available solution of cyclopentylmagnesium chloride (2.0 M in diethyl ether), compound **AP19** was obtained in 17% yield as a white solid. Mp 200.8 - 202.4°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.63$; $[\alpha]_D^{20} = -28.8$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.47 – 7.40 (m, 3H), 7.13 (s, 2H), 6.11 (s, 1H), 3.64 (d, 1H), 2.96 (d, 1H), 2.49 (d, 1H), 2.45 – 2.35 (m, 1H), 2.30 (d, 1H), 2.03 (q, 1H), 1.90 (s, 2H), 1.66 (d, 7H), 1.59 (s, 3H), 1.37 (t, 2H), 1.26 (s, 2H), 1.14 (s, 3H), 1.10 (t, 1H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 10.90, 19.41, 21.31, 25.90, 30.47, 33.37, 34.57, 40.97, 53.05, 72.01, 76.77, 77.02, 77.27, 109.18, 113.96, 115.92, 116.10, 125.35, 135.97, 136.83, 137.95, 150.21, 160.45, 162.41. Defining ^1H - ^1H NOESY Correlations (500 MHz, Chloroform-*d*) δ (2.97, 1.13), (2.49, 1.62), (2.02, 1.62). FT-IR ν_{max} (cm^{-1}): 3353 (OH), 3079, 2932 (w), 2856, 2250, 2051, 1699, 1513, 1435, 1223, 836. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{24}\text{H}_{29}\text{FN}_2\text{O}$: 381.2337; found: 381.2336.

(*R*)-((4*aR*,5*S*)-1-(4-fluorophenyl)-4*a*-methyl-4,4*a*,5,6,7,8-hexahydro-1*H*-benzo[*f*]indazol-5-yl)(naphthalen-2-yl)methanol (AP20): Using a commercially

available solution of 2-naphthylmagnesium bromide (0.25M in MeTHF), compound **AP20** was obtained in 55% yield as a white solid. Mp 142.1 - 143.7°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.69$; $[\alpha]_D^{20} = -115.9$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.84 (dd, 4H), 7.52 – 7.38 (m, 6H), 7.17 (d, 2H), 6.12 (s, 1H), 5.36 (s, 1H), 3.26 (d, 1H), 2.85 (d, 1H), 2.48 – 2.37 (m, 1H), 2.28 (d, 1H), 1.94 – 1.54 (m, 5H), 1.32 (s, 3H), 1.22 – 1.09 (m, 1H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 19.89, 20.00, 25.77, 33.36, 35.25, 41.38, 56.48, 72.18, 76.77, 77.02, 77.28, 109.23, 113.88, 115.96, 116.14, 123.70, 124.03, 125.33, 125.77, 126.29, 127.68, 127.90, 128.00, 132.56, 133.17, 136.88, 138.01, 142.96, 150.23, 160.49, 162.45. Defining ^1H - ^1H NOESY Correlations (500 MHz, Chloroform-*d*) δ (3.24, 1.30), (2.83, 1.82), (7.88, 1.63). FT-IR ν_{max} (cm^{-1}): 3373 (OH), 3262, 3057, 2932, 2876, 2250, 1615, 1512, 1224, 832. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{29}\text{H}_{27}\text{FN}_2\text{O}$: 439.2180; found: 439.2180.

(S)-((4a*R*,5*S*)-1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1*H*-benzo[*f*]indazol-5-yl)(naphthalen-2-yl)methanol (AP21): Using a commercially available solution of 2-naphthylmagnesium bromide (0.25M in MeTHF), compound **AP21** was obtained in 16% yield as a white solid. Mp 106 - 107.4°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.69$; $[\alpha]_D^{20} = 111.2$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.88 – 7.79 (m, 4H), 7.53 – 7.43 (m, 5H), 7.40 (d, 1H), 7.16 (t, 2H), 6.12 (s, 1H), 5.36 (s, 1H), 3.26 (d, 1H), 2.87 (s, 1H), 2.48 – 2.38 (m, 1H), 2.28 (d, 1H), 1.94 – 1.72 (m, 3H), 1.62 (d, 2H), 1.32 (s, 3H), 1.26 (s, 1H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 19.89, 20.01, 25.77, 33.36, 35.25, 41.38, 56.48, 72.18, 76.77, 77.02, 77.28, 109.23, 113.88, 115.95, 116.14, 123.70, 124.03, 125.39, 125.77, 126.29, 127.68, 127.90, 128.00, 132.56, 133.17, 136.88, 138.01, 142.96, 150.23, 160.49, 162.45. Defining ^1H - ^1H NOESY Correlations (500 MHz, Chloroform-*d*) δ (3.23, 1.30), (2.85, 1.84), (7.39, 2.85). FT-IR ν_{max} (cm^{-1}): 3367 (OH), 3055, 2928, 2867, 2257, 2050, 1615, 1512, 1224, 833. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{29}\text{H}_{27}\text{FN}_2\text{O}$: 439.2180; found: 439.2181.

(R)-((4a*R*,5*S*)-1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1*H*-benzo[*f*]indazol-5-yl)(perfluorophenyl)methanol (AP22): Using a commercially available solution of pentafluorophenylmagnesium bromide (0.5 M in diethyl ether), compound **AP22** was obtained in 58% yield as a white solid. Mp 104.2 - 105.7 °C; TLC

(Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.65$; $[\alpha]_D^{20} = -55.0$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.44 (s, 3H), 7.16 (d, 2H), 6.13 (s, 1H), 5.53 (d, $J = 5$ Hz, 1H), 3.09 (d, 1H), 2.70 (d, 1H), 2.49 – 2.37 (m, 2H), 2.32 (d, 1H), 1.94 (d, 1H), 1.85 – 1.70 (m, 3H), 1.35 (d, 1H), 1.26 (s, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 19.59, 21.28, 25.49, 32.86, 34.30, 41.67, 55.44, 67.84, 76.74, 76.99, 77.25, 109.64, 113.68, 115.94, 116.13, 125.29, 125.36, 135.81, 135.83, 136.50, 137.98, 148.85, 160.49, 162.45. Defining ^1H - ^1H NOESY Correlations (500 MHz, Chloroform-*d*) δ (3.09, 1.21), (2.69, 1.73). FT-IR ν_{max} (cm^{-1}): 3351 (OH), 3055, 2932, 2868, 2266, 2050, 1615, 1513, 1224, 834. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{25}\text{H}_{20}\text{F}_6\text{N}_2\text{O}$: 479.1553; found: 479.1554.

(*S*)-((4*aR*,5*S*)-1-(4-fluorophenyl)-4*a*-methyl-4,4*a*,5,6,7,8-hexahydro-1*H*-benzo[*f*]indazol-5-yl)(perfluorophenyl)methanol (AP23): Using a commercially available solution of pentafluorophenylmagnesium bromide (0.5 M in diethyl ether), compound **AP23** was obtained in 19% yield as a white solid. Mp 81.8 - 82.5°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.65$; $[\alpha]_D^{20} = 41.3$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.45 (s, 3H), 7.15 (s, 2H), 6.12 (s, 1H), 5.52 (s, 1H), 3.09 (d, 1H), 2.69 (d, 1H), 2.38 (m, 3H), 1.92 (s, 1H), 1.78 (s, 3H), 1.34 (dt, 1H), 1.24 (s, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 19.59, 21.26, 25.48, 32.86, 34.29, 41.66, 55.44, 67.85, 76.73, 76.98, 77.24, 109.64, 113.67, 115.94, 116.12, 125.28, 135.80, 136.49, 137.97, 148.83, 160.48, 162.44. ^1H - ^1H NOESY Correlations (500 MHz, Chloroform-*d*) δ (3.07, 1.24), (2.68, 1.75). FT-IR ν_{max} (cm^{-1}): 3346 (OH), 2929, 2868, 2252, 2043, 1615, 1513, 1224, 835. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{25}\text{H}_{20}\text{F}_6\text{N}_2\text{O}$: 479.1553; found: 479.1553.

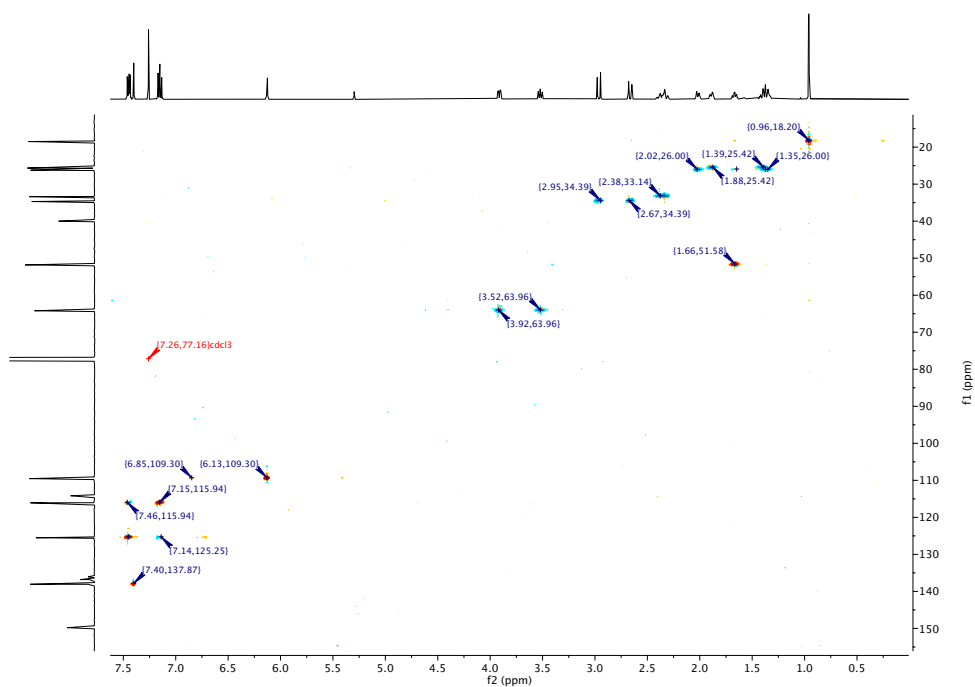


Figure 2.9.3 - ^1H - ^{13}C HSQC of AP12

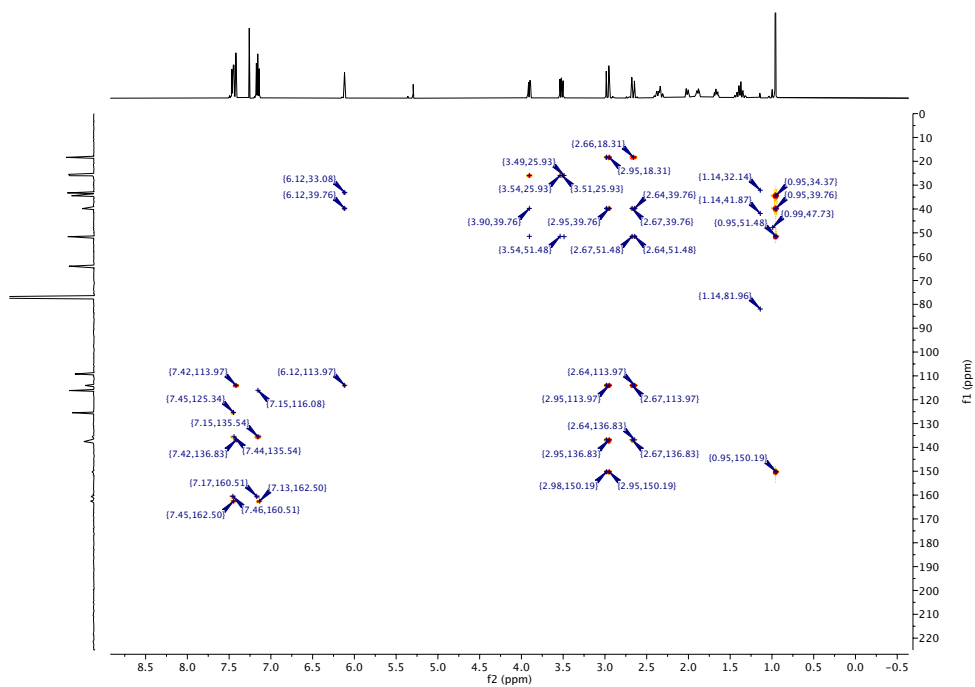
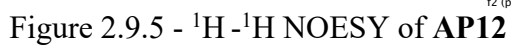


Figure 2.9.4 - ^1H - ^{13}C HMBC of AP12



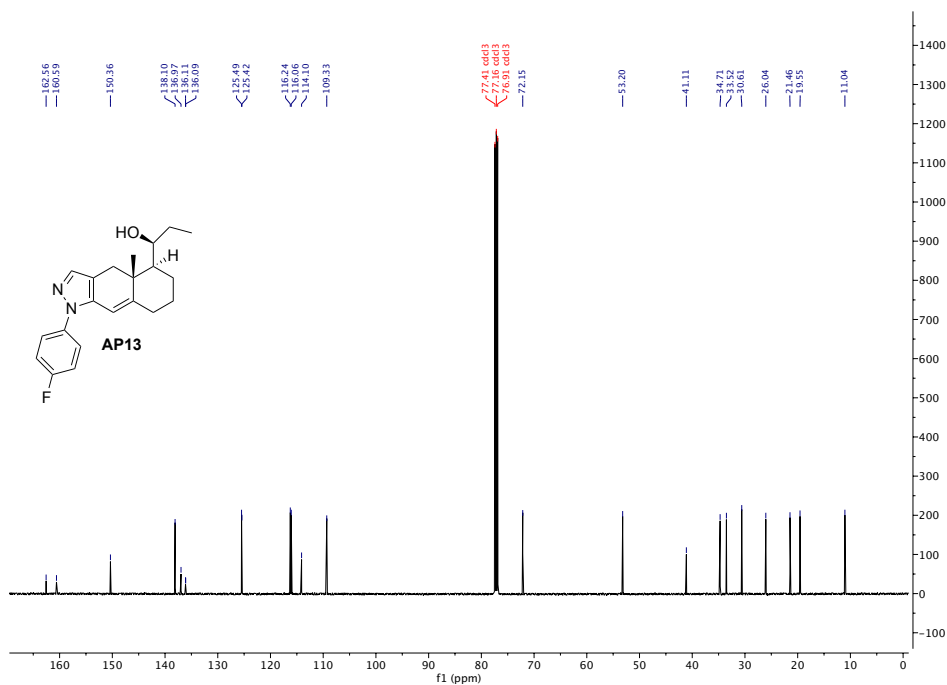


Figure 2.9.7 - ^{13}C NMR of AP13

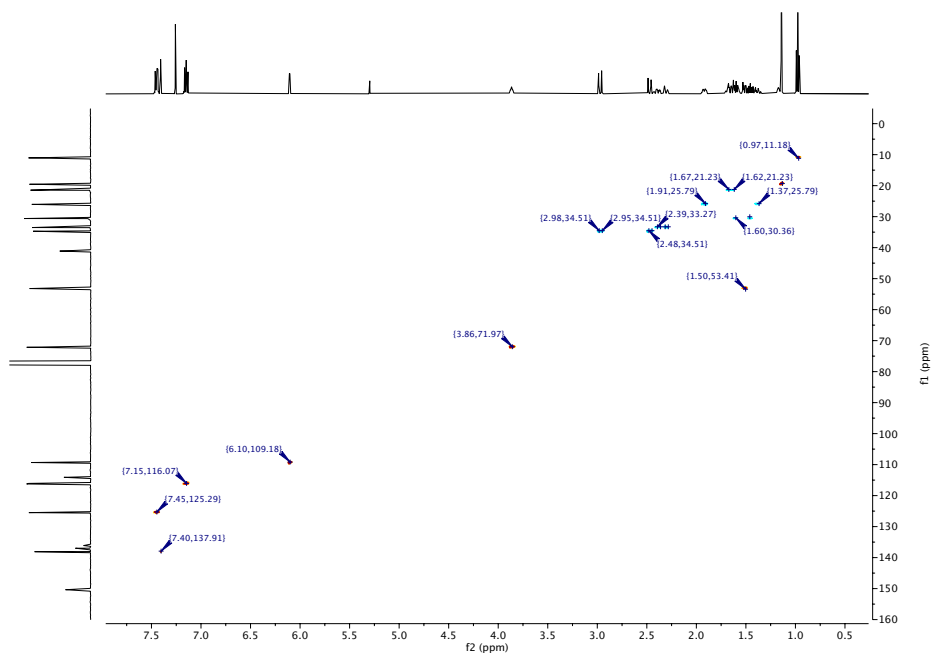


Figure 2.9.8 - ^1H - ^{13}C HSQC of AP13

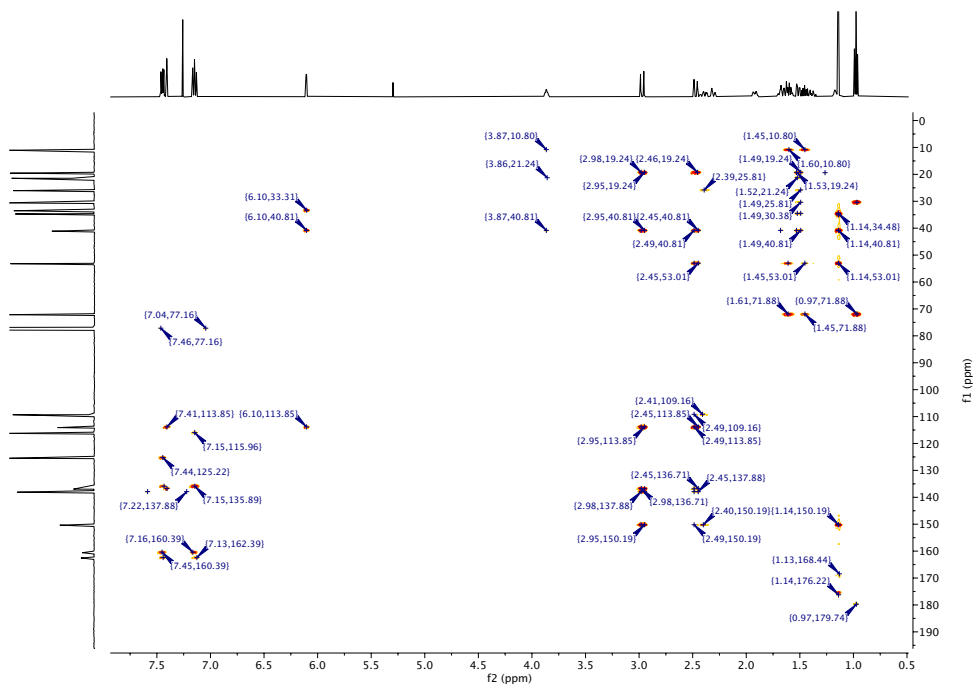


Figure 2.9.9 - ^1H - ^{13}C HMBC of AP13

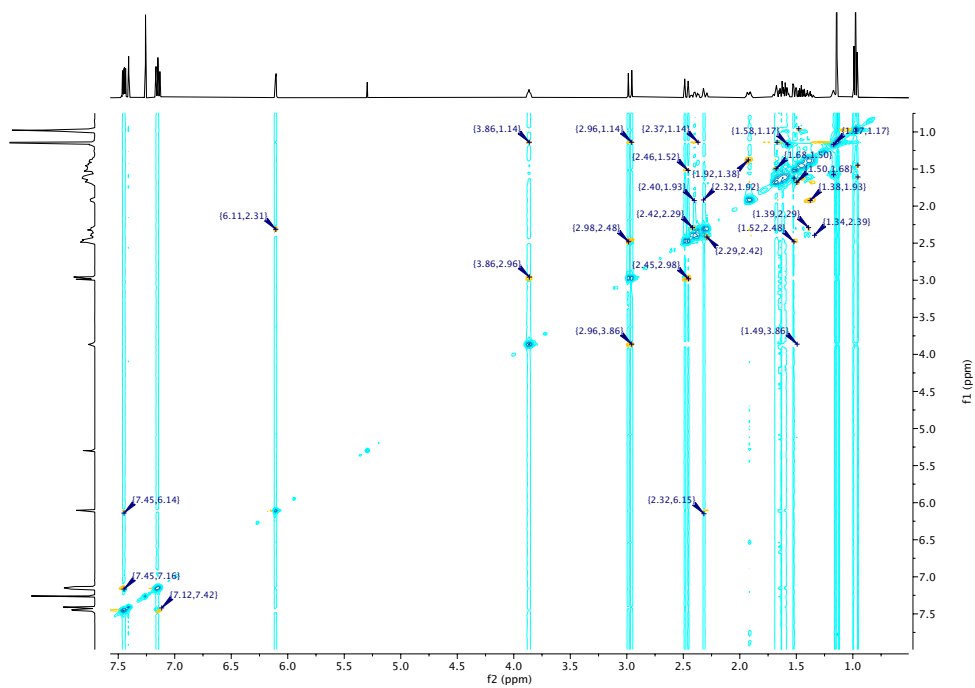


Figure 2.9.10 - ^1H - ^1H NOESY of AP13

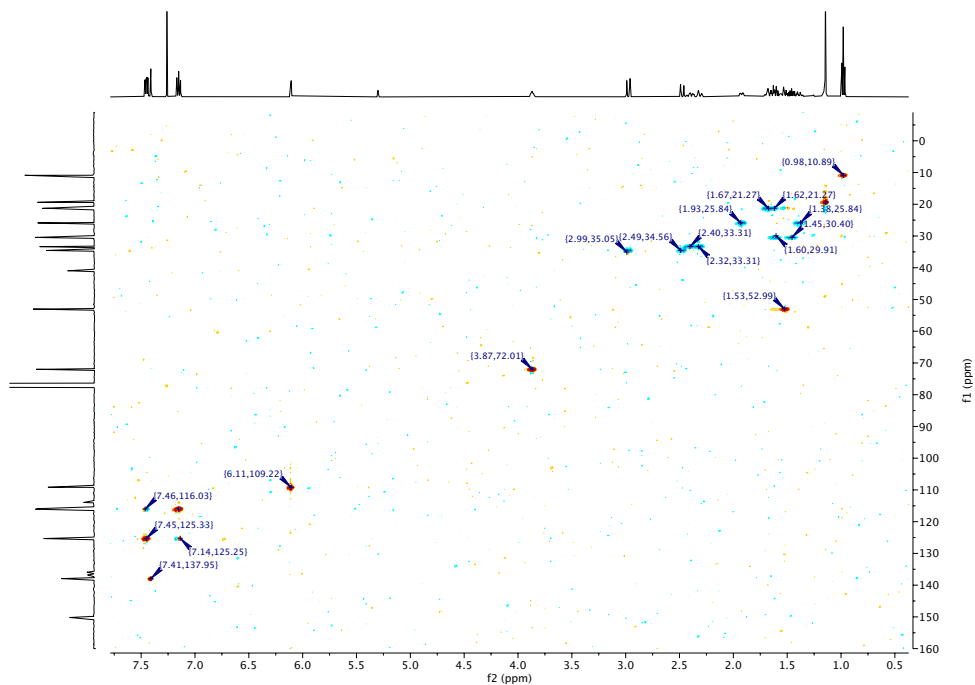


Figure 2.9.13 - ^1H - ^{13}C HSQC of AP14

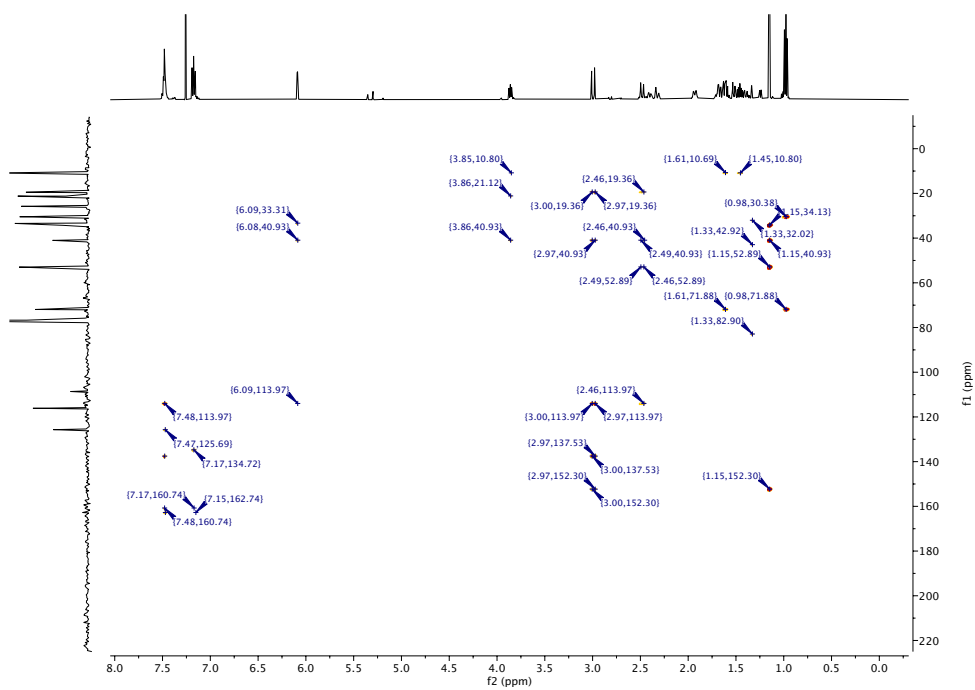


Figure 2.9.14 - ^1H - ^{13}C HMBC of AP14

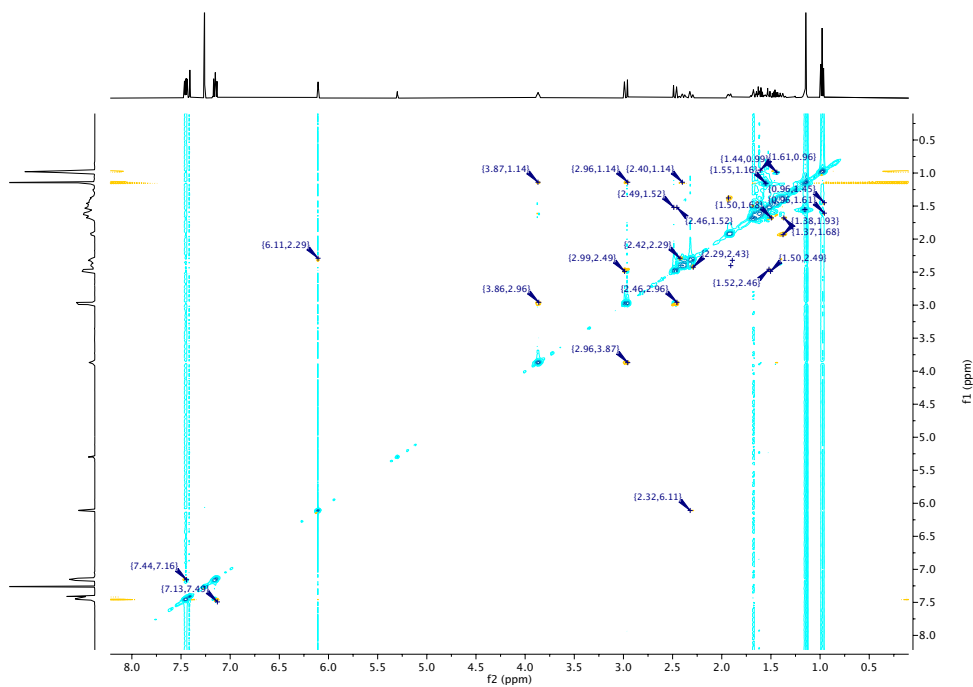


Figure 2.9.15 - ^1H - ^1H NOESY of AP14

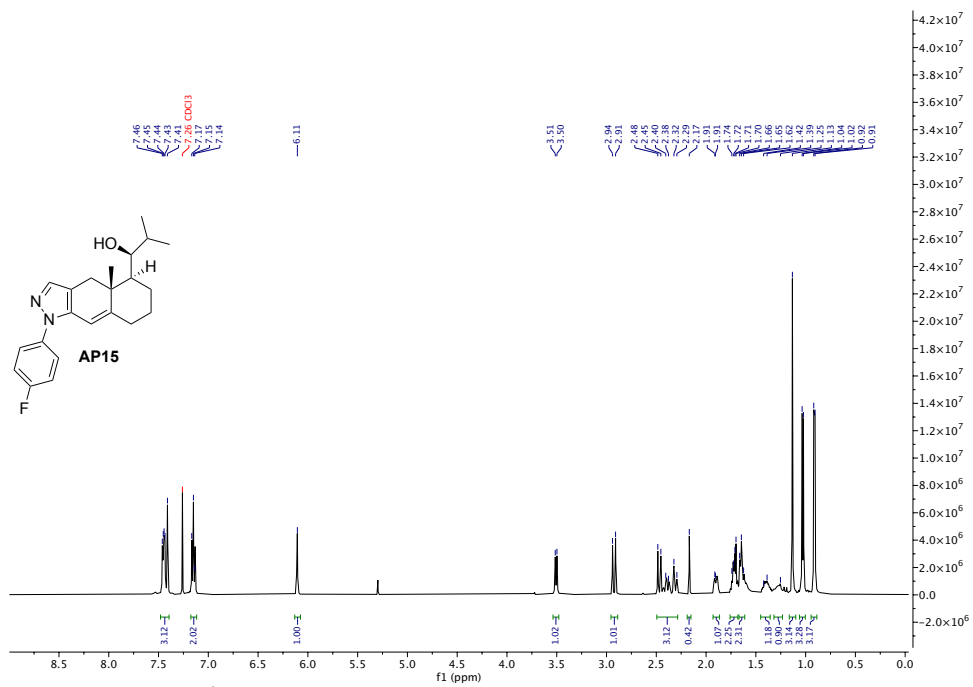


Figure 2.9.16 - ^1H NMR of AP15

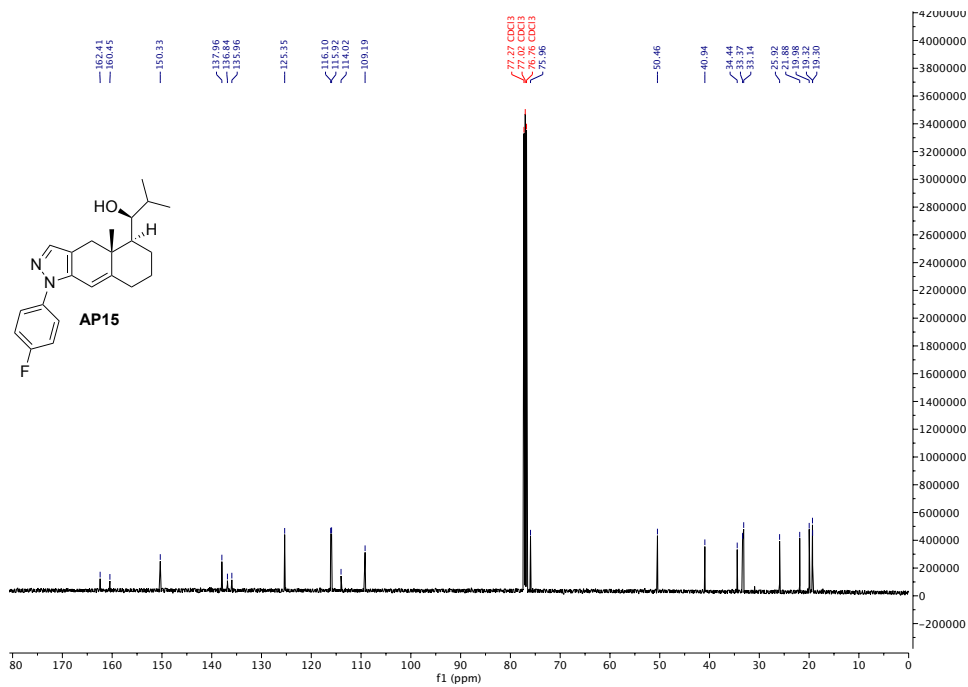


Figure 2.9.17 - ¹³C NMR of AP15

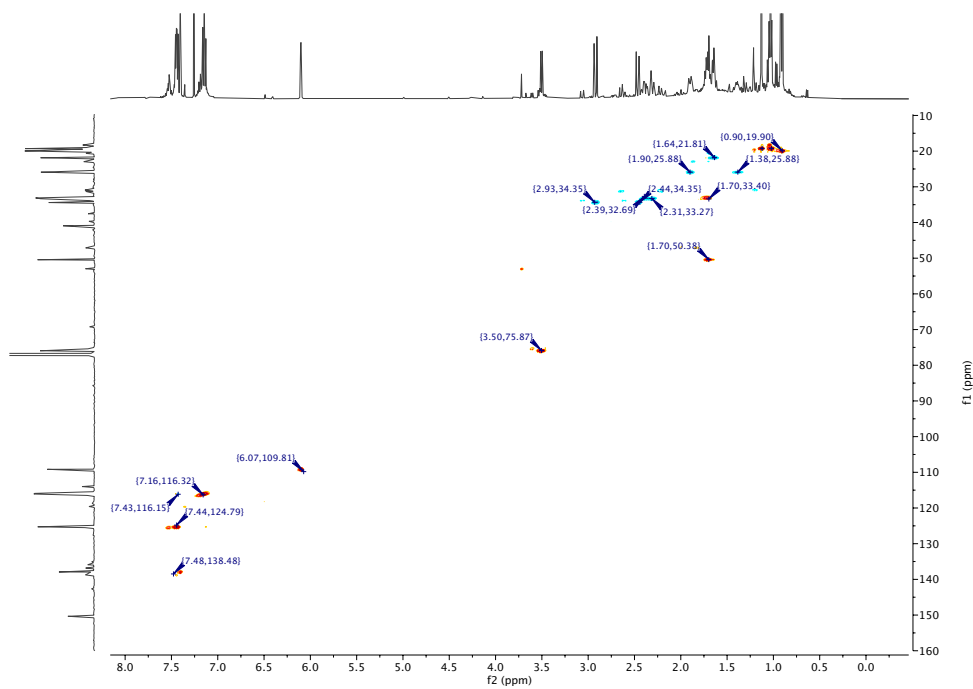


Figure 2.9.18 - ¹H-¹³C HSQC of AP15

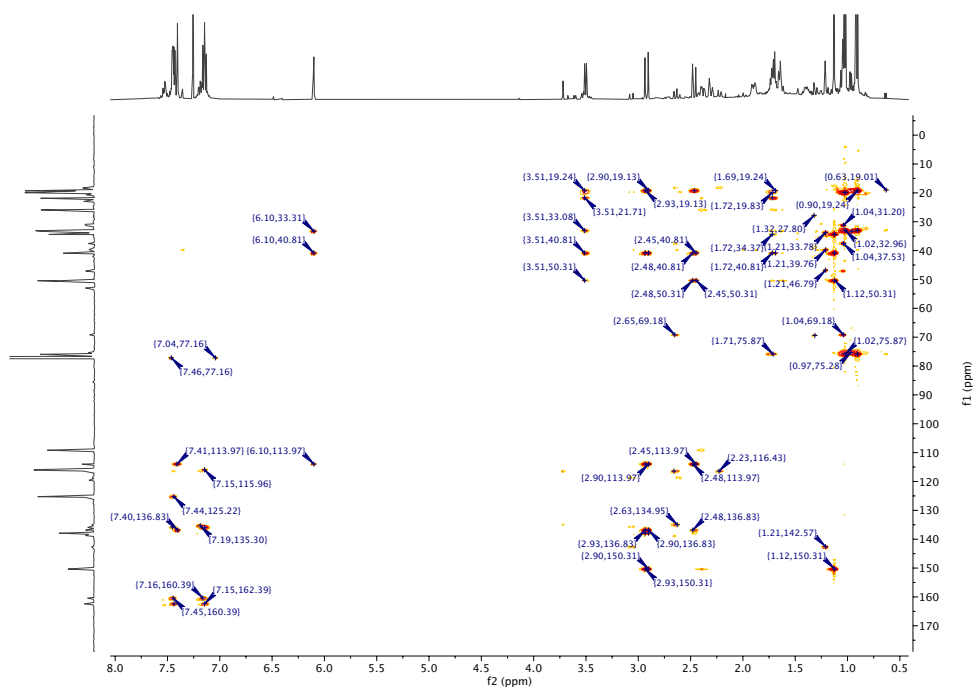


Figure 2.9.19 - ^1H - ^{13}C HMBC of AP15

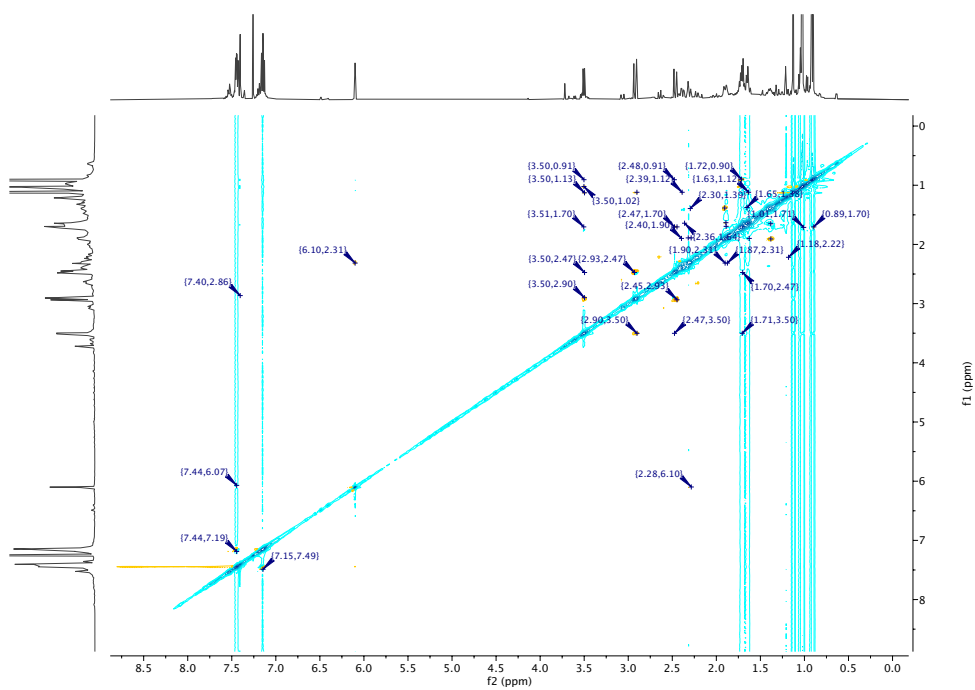


Figure 2.9.20 - ^1H - ^1H NOESY of AP15

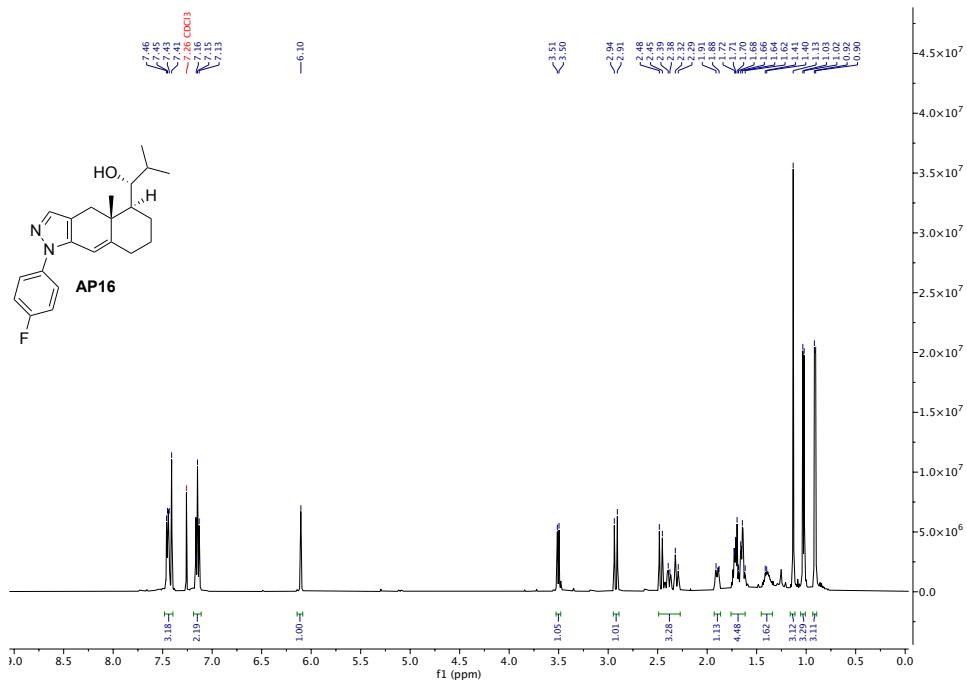


Figure 2.9.21 - ^1H NMR of AP16

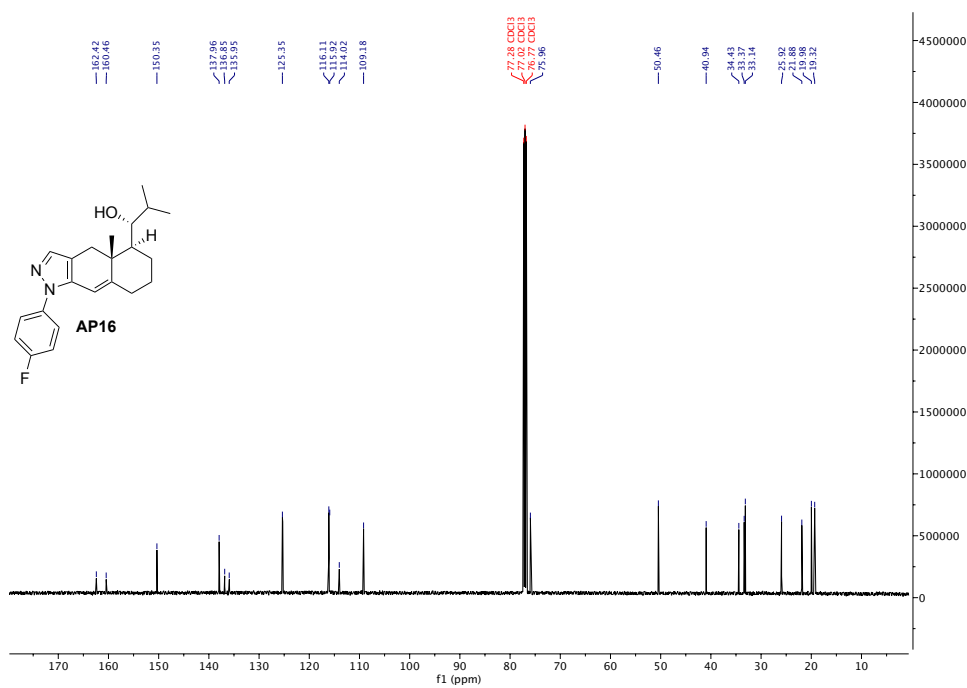


Figure 2.9.22 - ^{13}C NMR of AP16

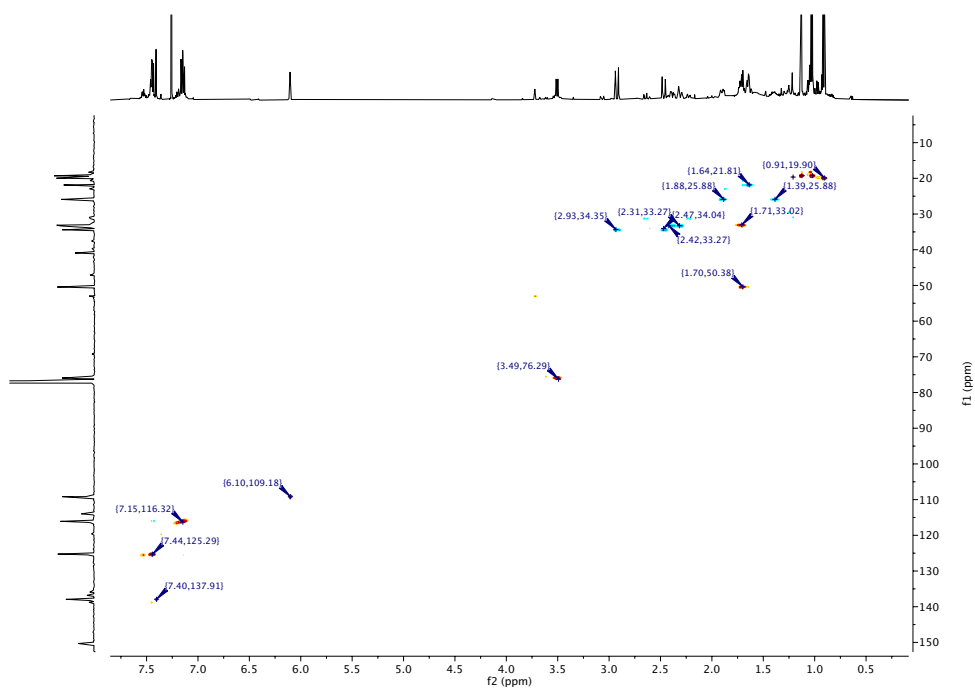


Figure 2.9.23 - ^1H - ^{13}C HSQC of AP16

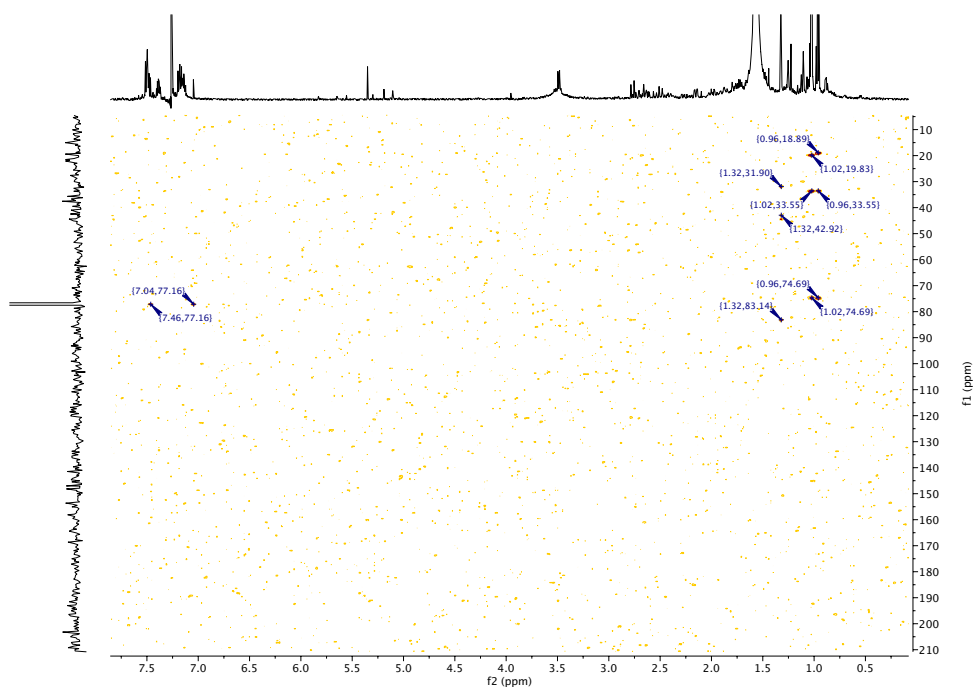


Figure 2.9.24 - ^1H - ^{13}C HMBC of AP16

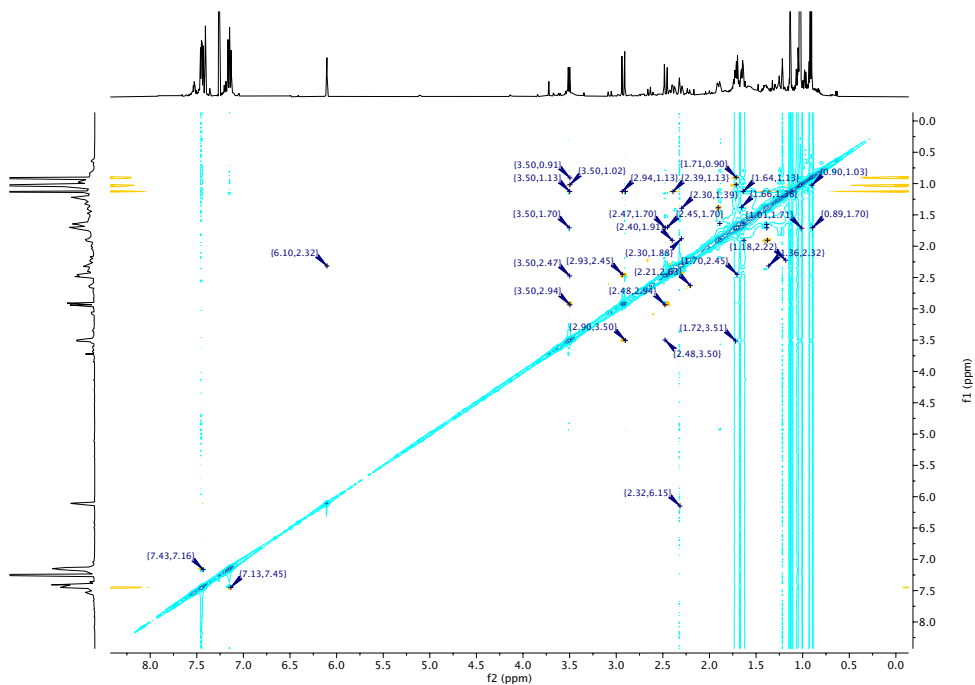


Figure 2.9.25 - ^1H - ^1H NOESY of AP16

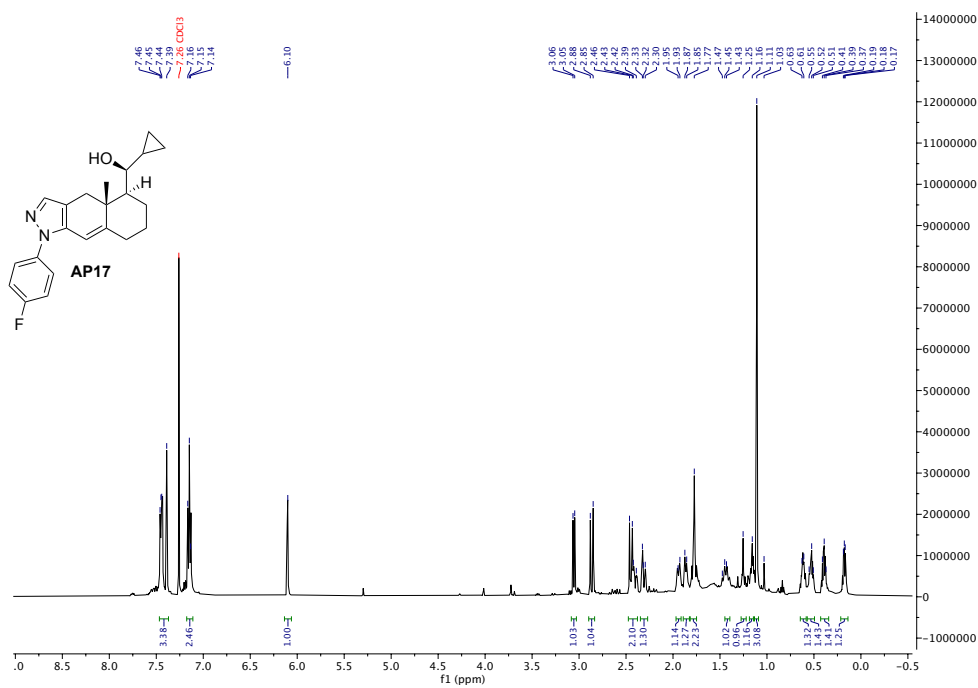


Figure 2.9.26 - ^1H NMR of AP17

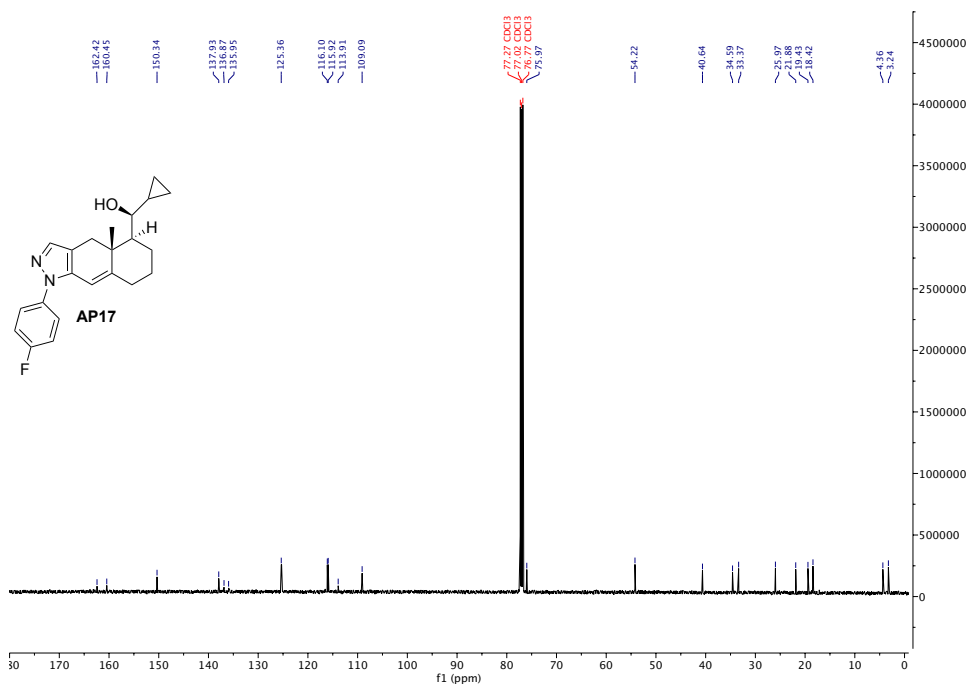


Figure 2.9.27 - ¹³C NMR of AP17

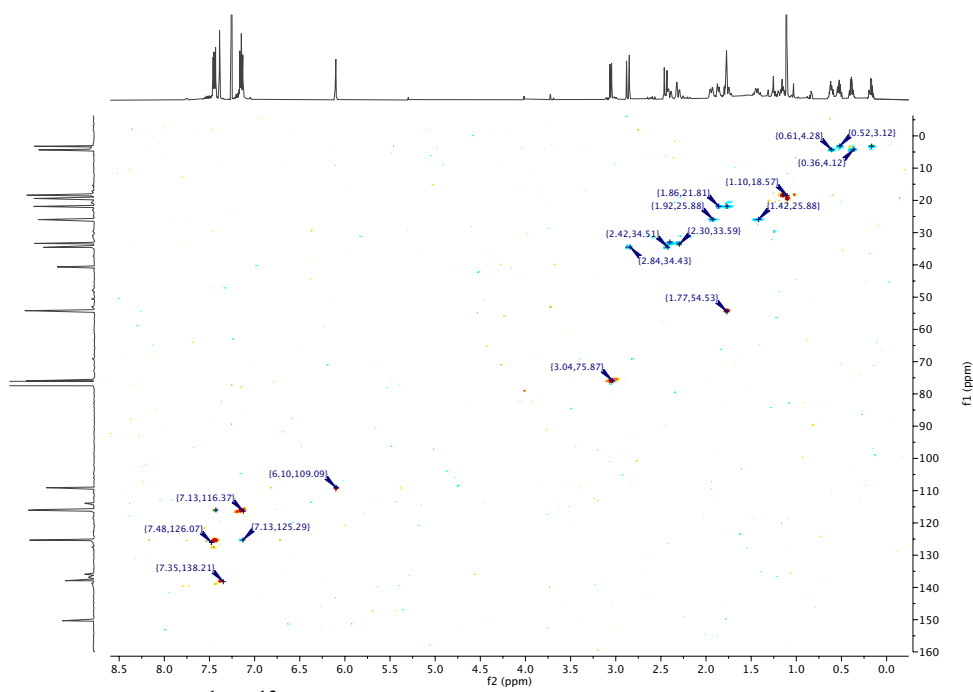


Figure 2.9.28 - ¹H-¹³C HSQC of AP17

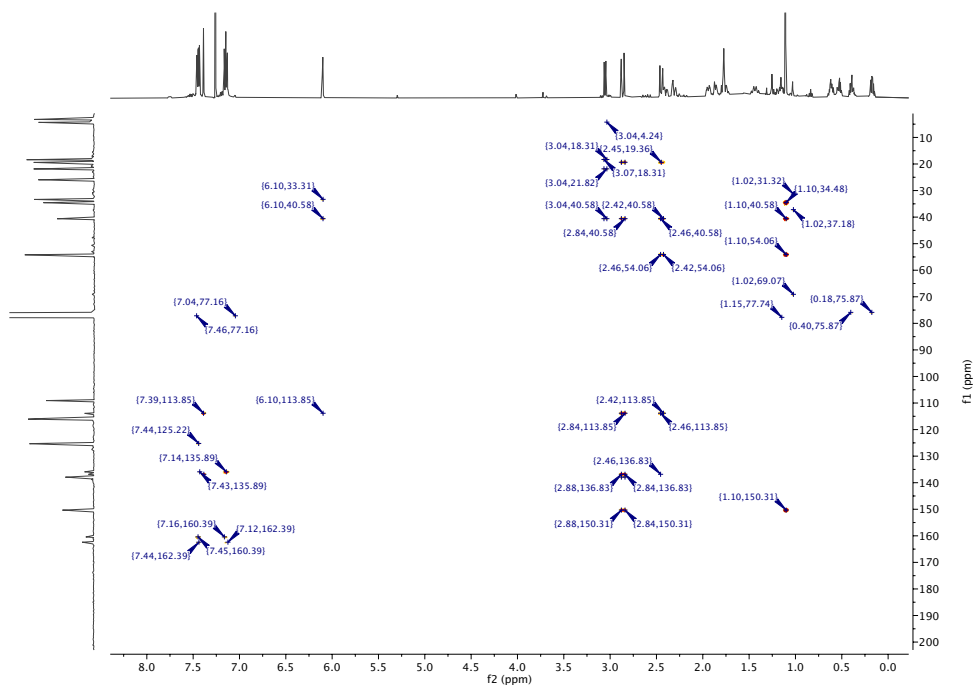


Figure 2.9.29 - ^1H - ^{13}C HMBC of AP17

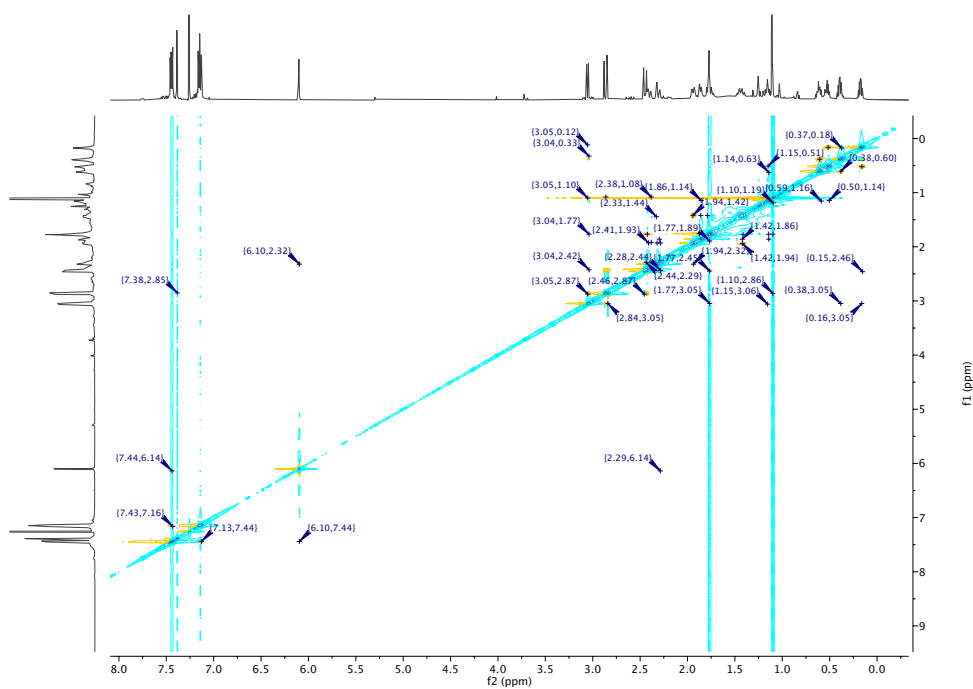


Figure 2.9.30 - ^1H - ^1H NOESY of AP17

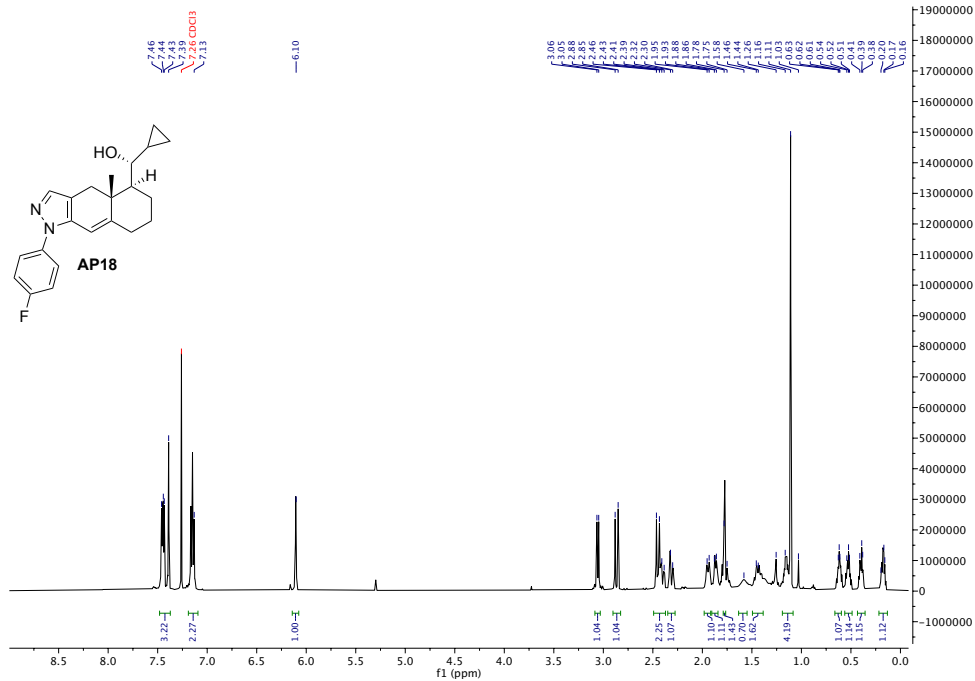


Figure 2.9.31 - ¹H NMR of AP18

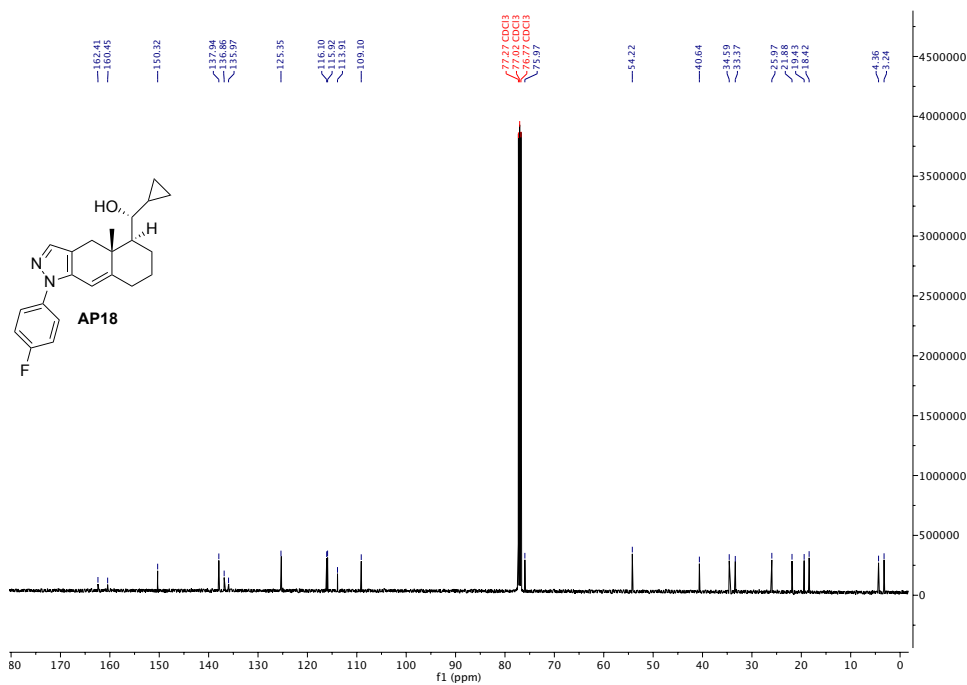


Figure 2.9.32 - ¹³C NMR of AP18

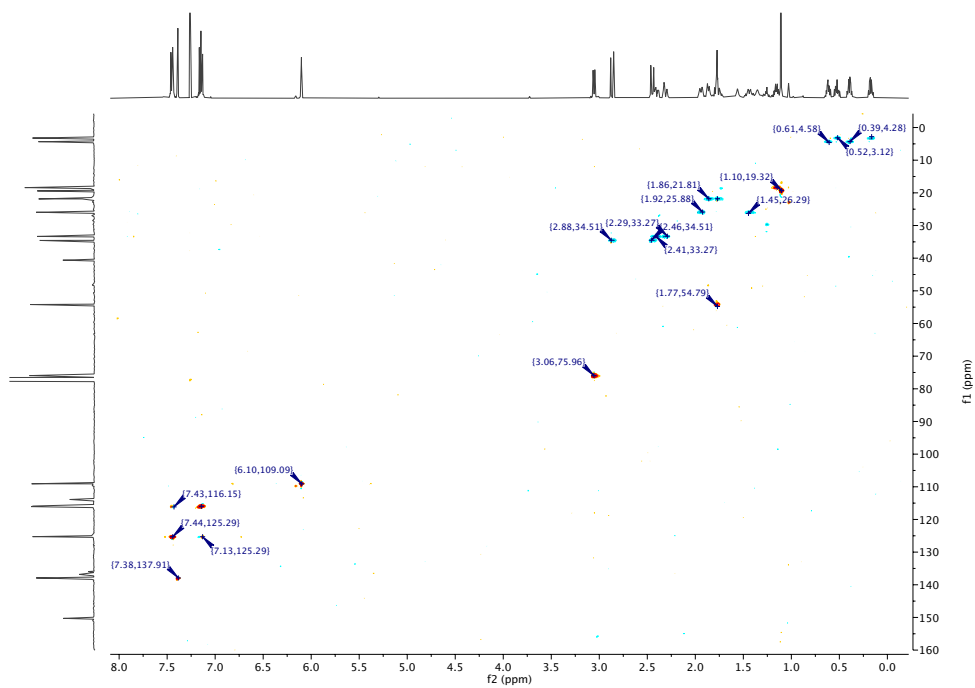


Figure 2.9.33 - ^1H - ^{13}C HSQC of AP18

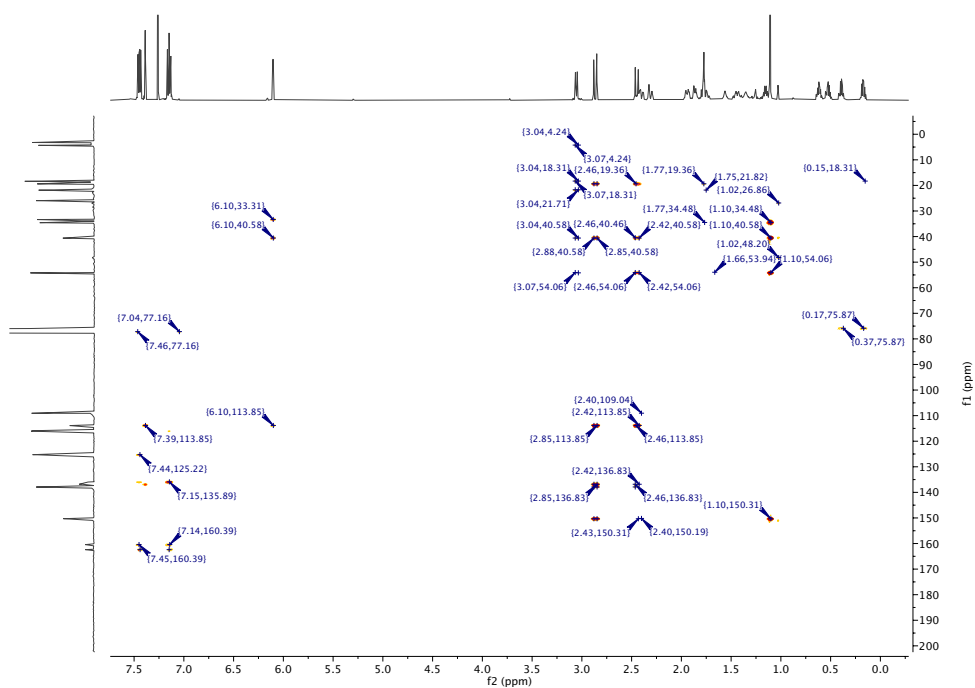
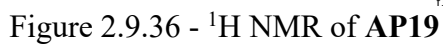
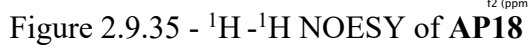


Figure 2.9.34 - ^1H - ^{13}C HMBC of AP18



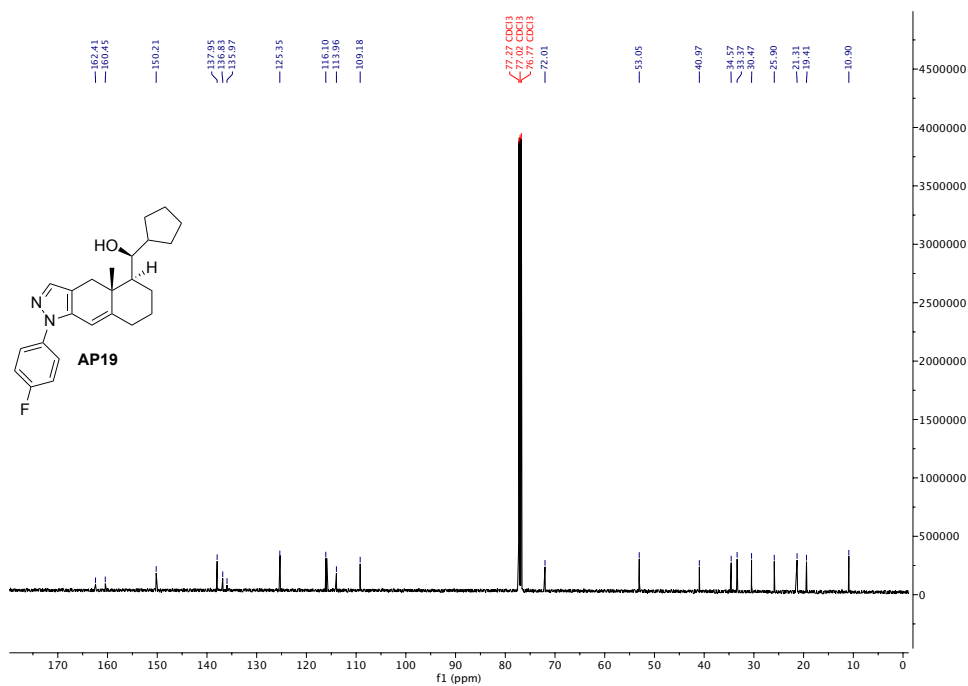


Figure 2.9.37 - ¹³C NMR of AP19

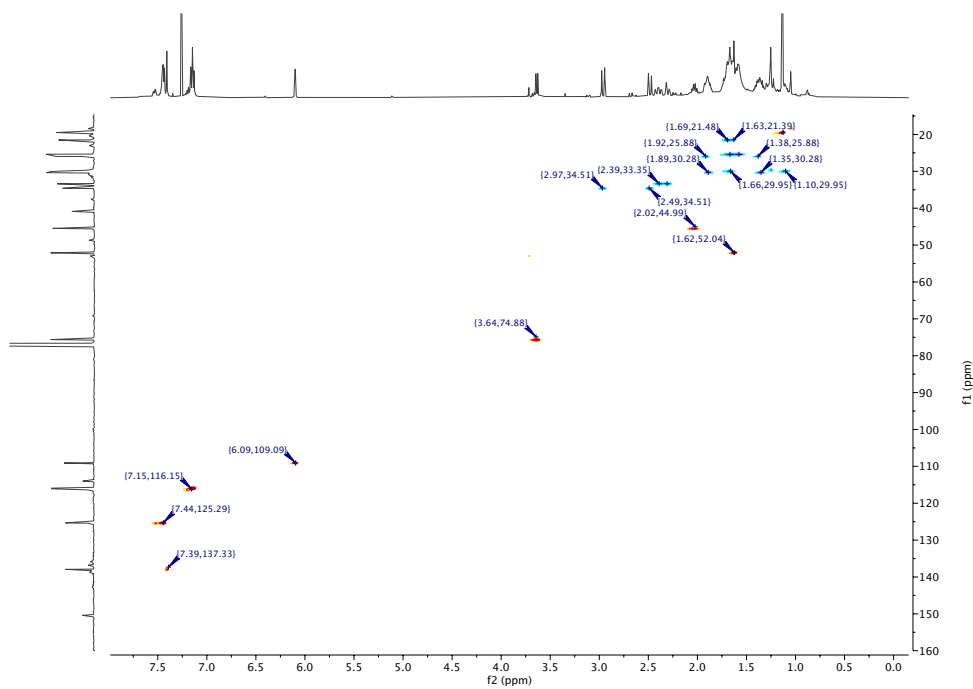


Figure 2.9.38 - ¹H-¹³C HSQC of AP19

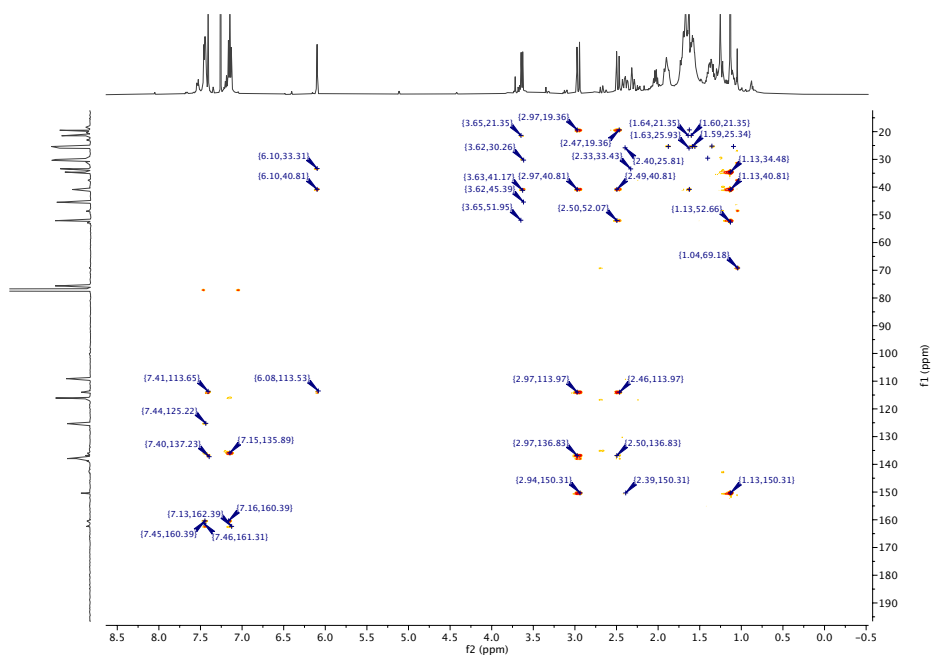


Figure 2.9.39 - ^1H - ^{13}C HMBC of AP19

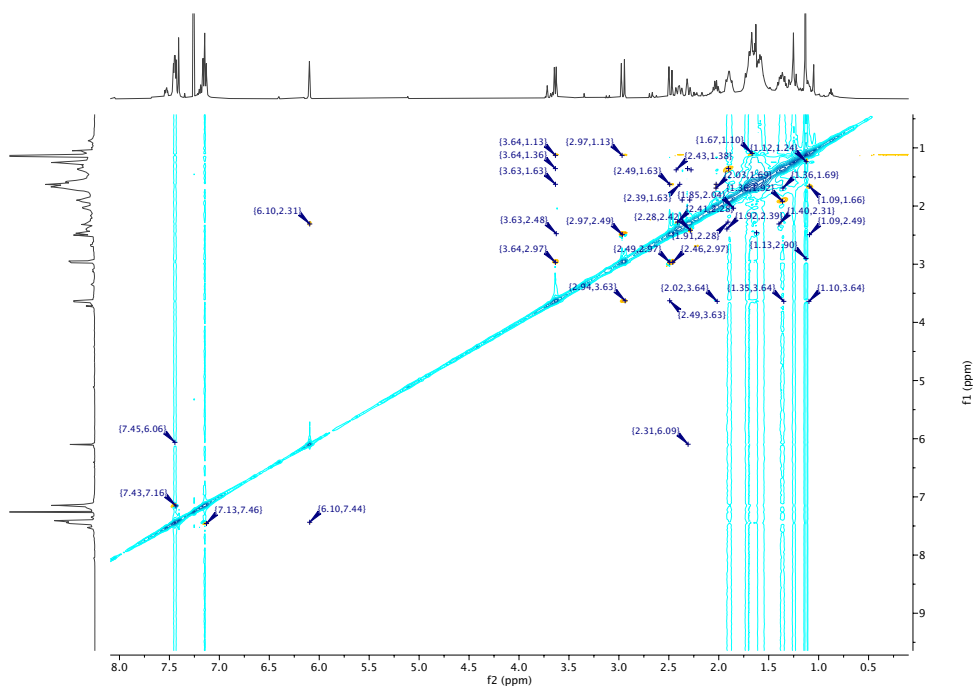


Figure 2.9.40 - ^1H - ^1H NOESY of AP19



Figure 2.9.42 - ^{13}C NMR of AP20

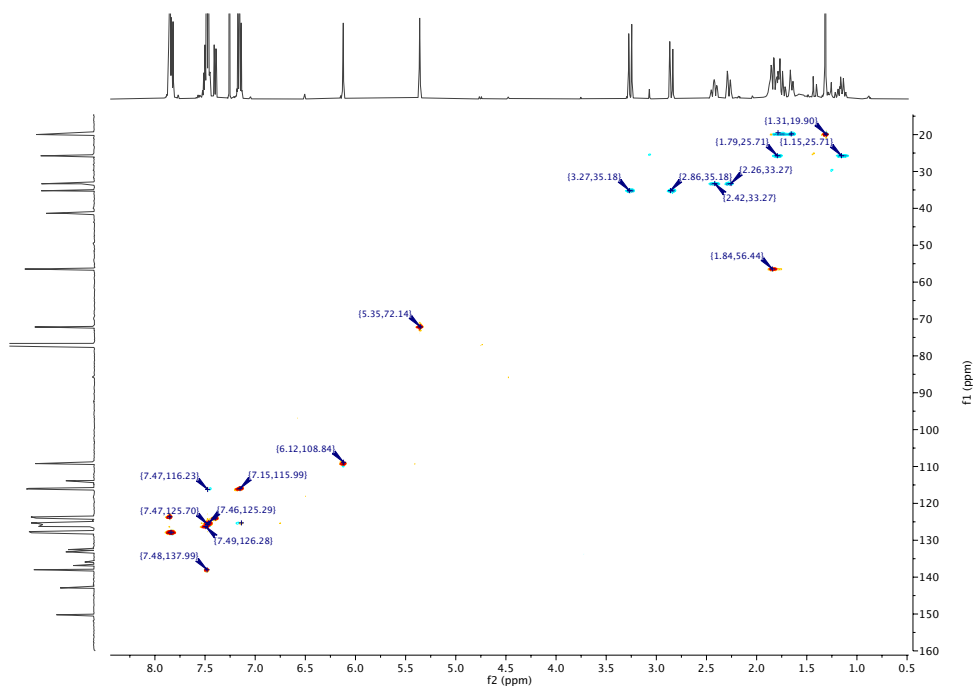


Figure 2.9.43 - ^1H - ^{13}C HSQC of AP20

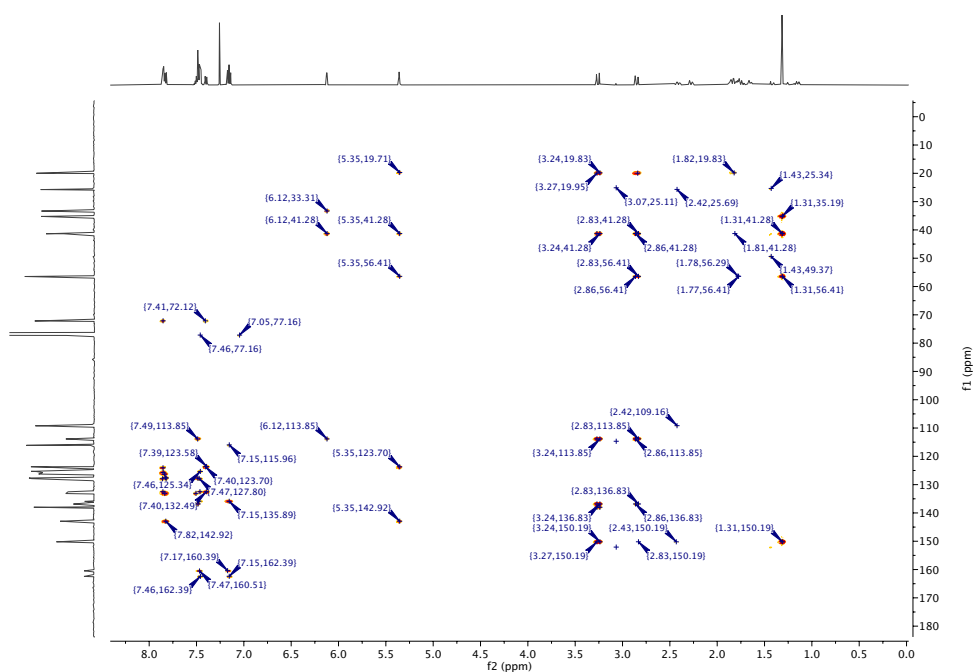


Figure 2.9.44 - ^1H - ^{13}C HMBC of AP20

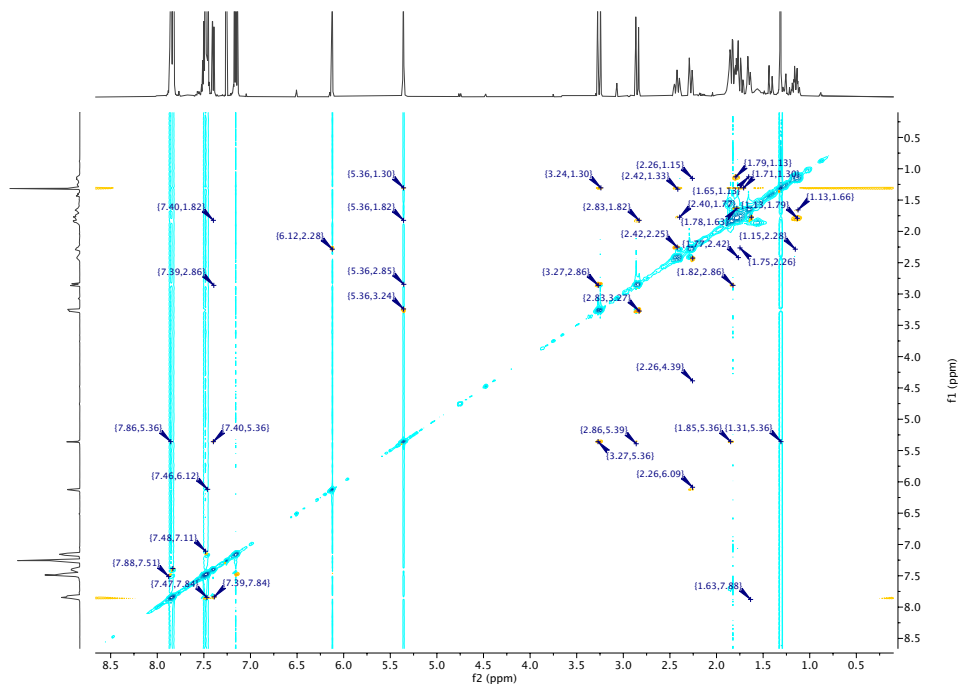


Figure 2.9.45 - ^1H - ^1H NOESY of AP20

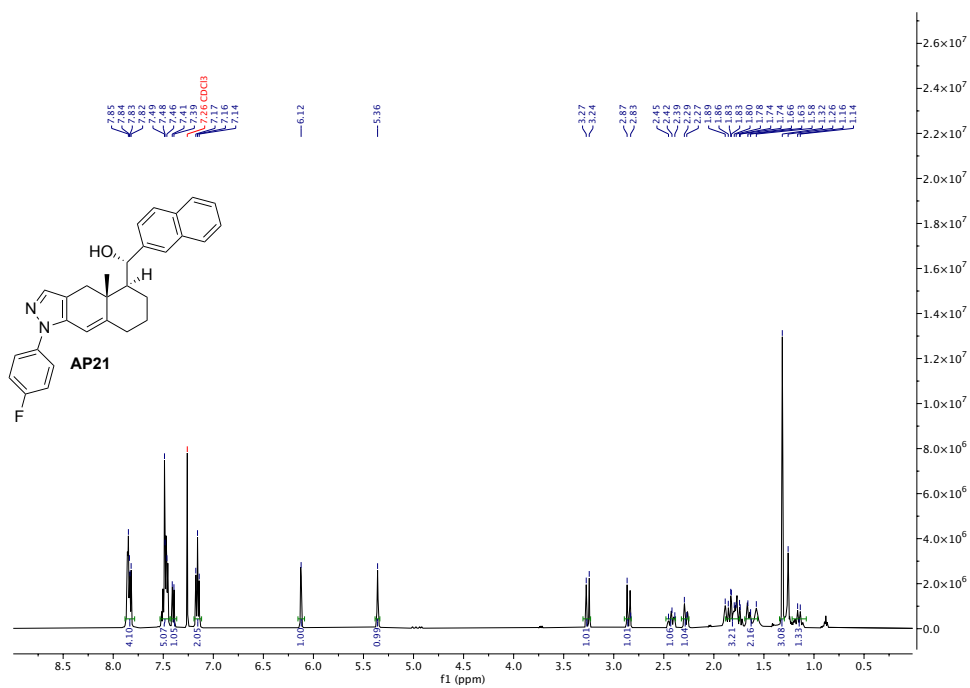


Figure 2.9.46 - ^1H NMR of AP21

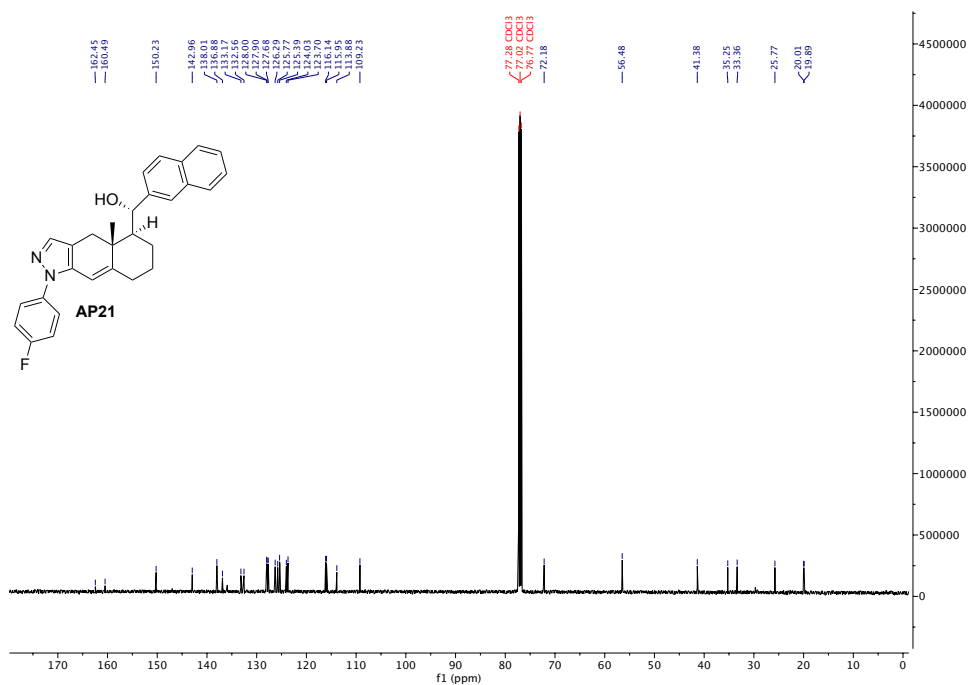


Figure 2.9.47 - ¹³C NMR of AP21

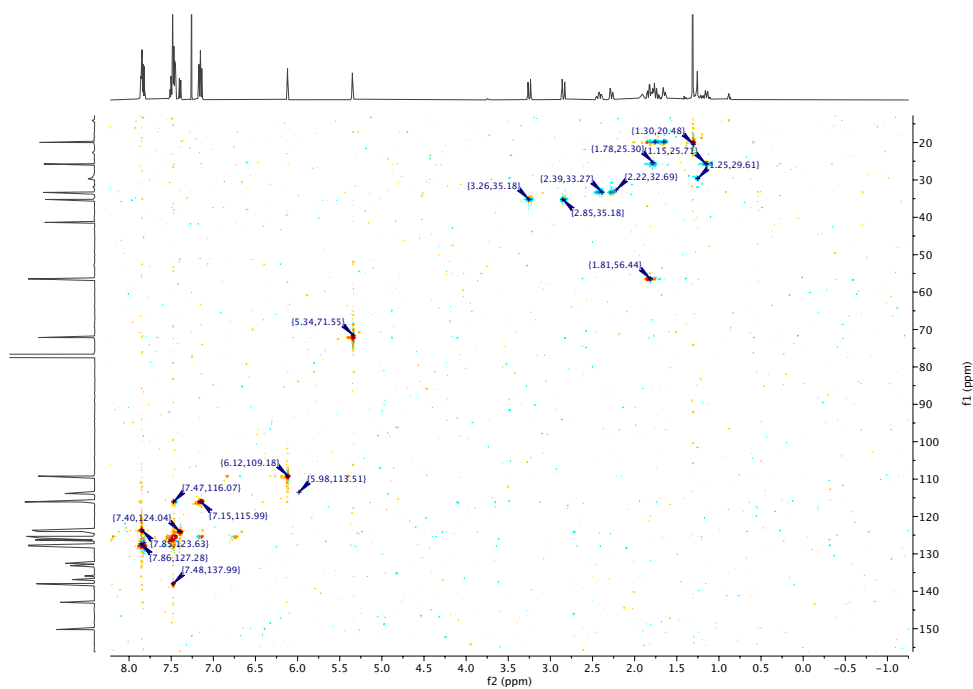


Figure 2.9.48 - ¹H-¹³C HSQC of AP21

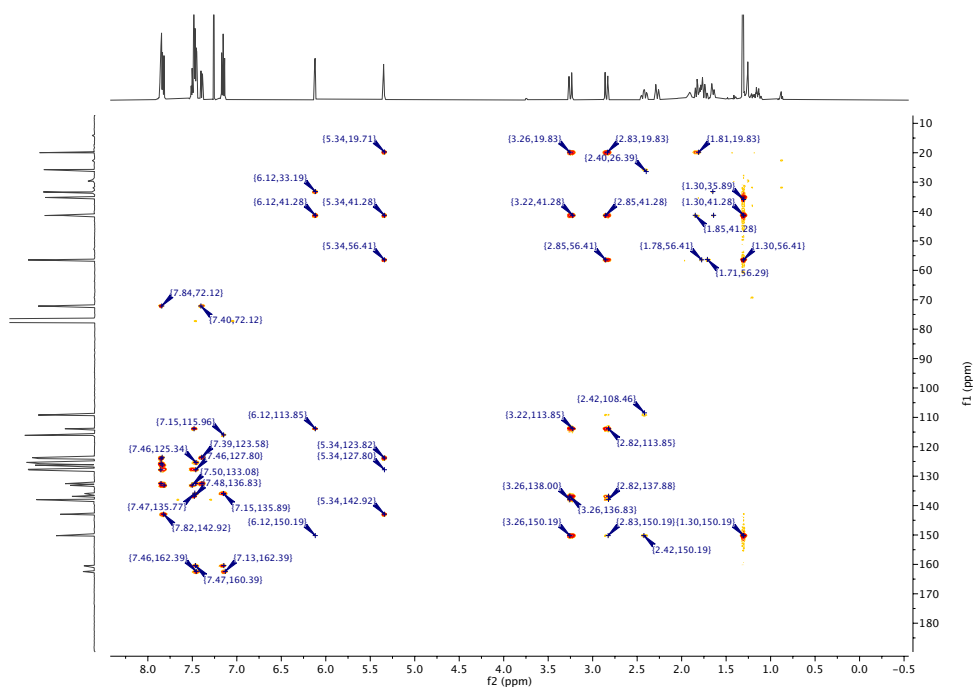


Figure 2.9.49 - ^1H - ^{13}C HMBC of AP21

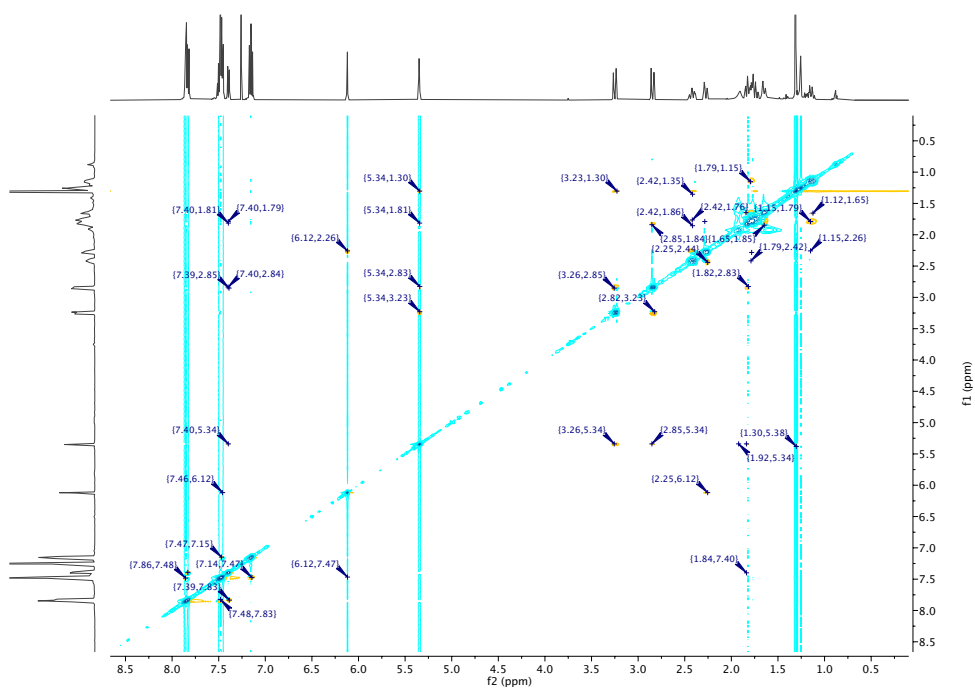


Figure 2.9.50 - ^1H - ^1H NOESY of AP21

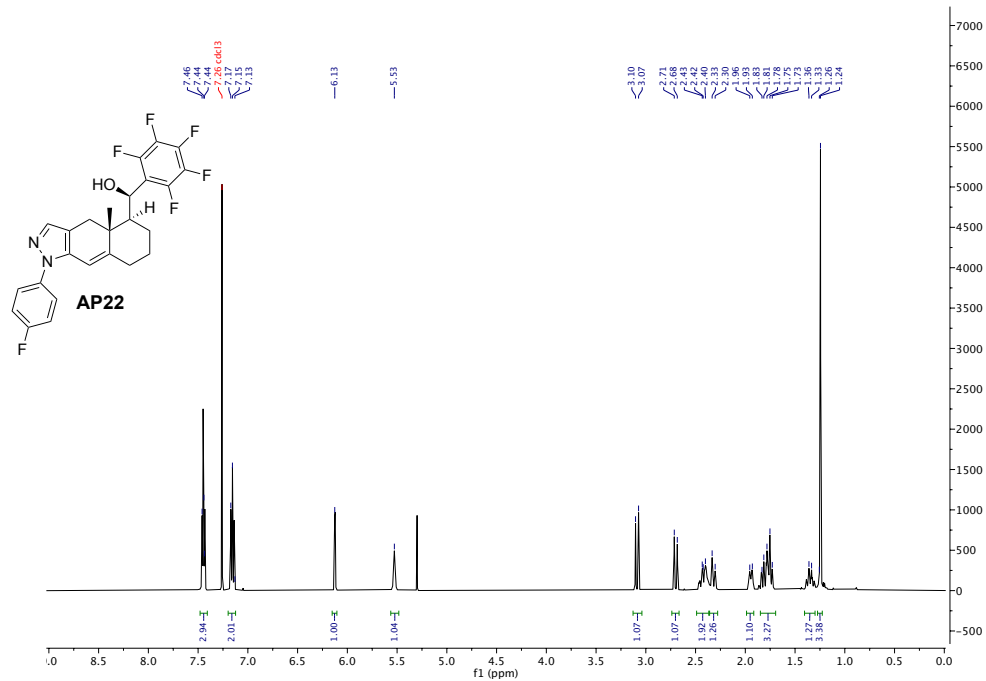


Figure 2.9.51 - ¹H NMR of AP22

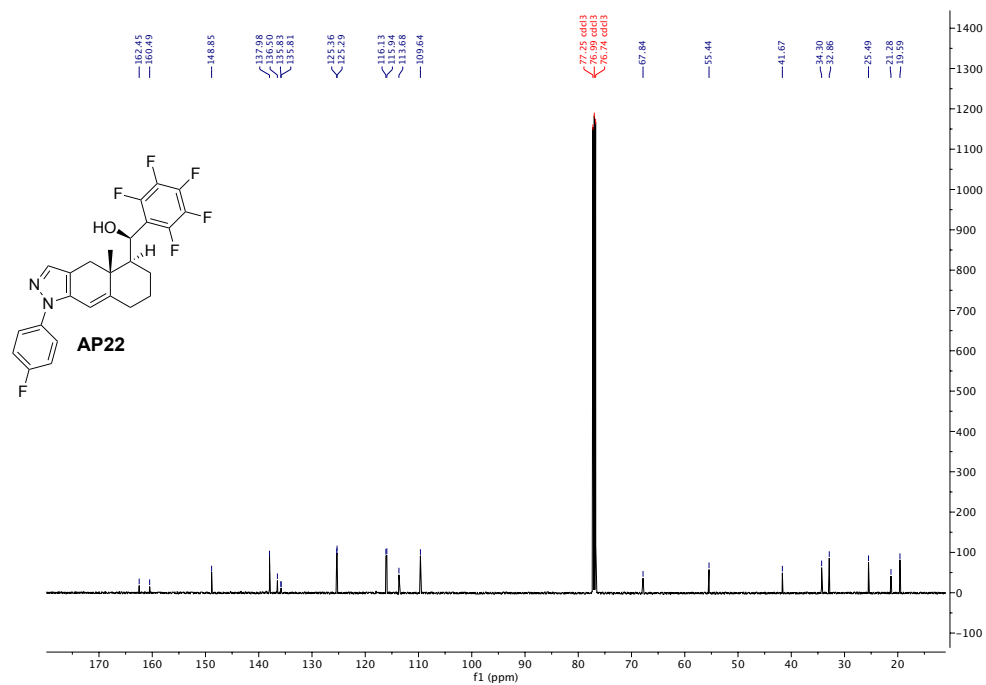


Figure 2.9.52 - ¹³C NMR of AP22

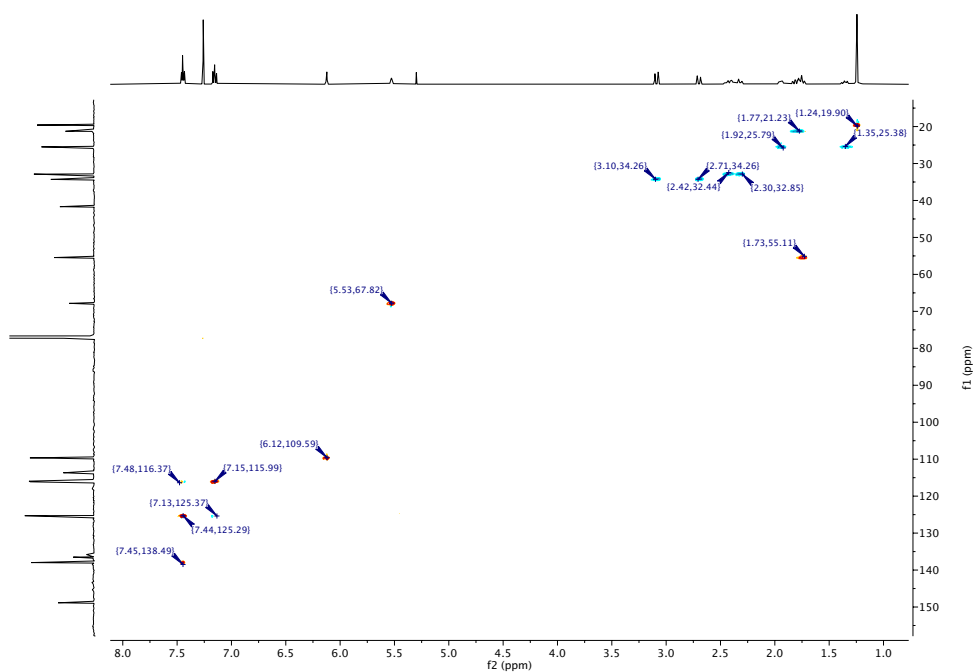


Figure 2.9.53 - ^1H - ^{13}C HSQC of AP22

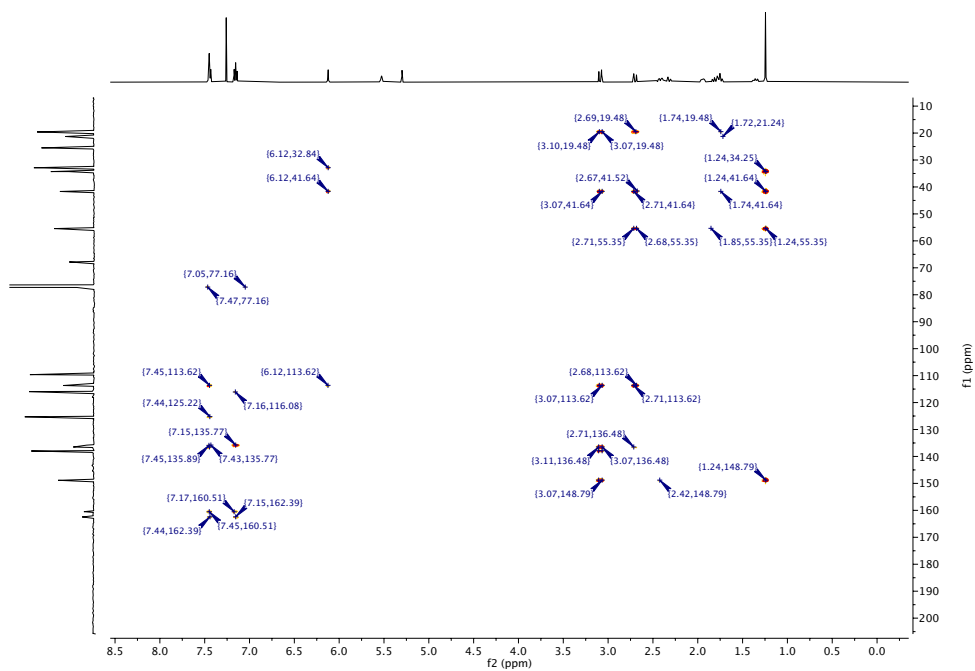
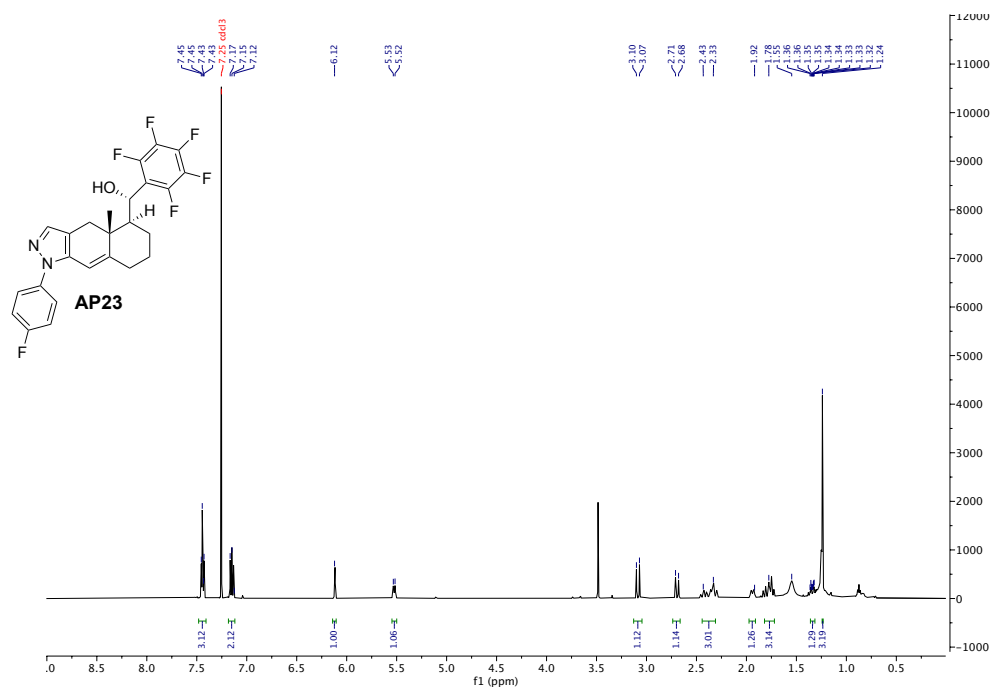
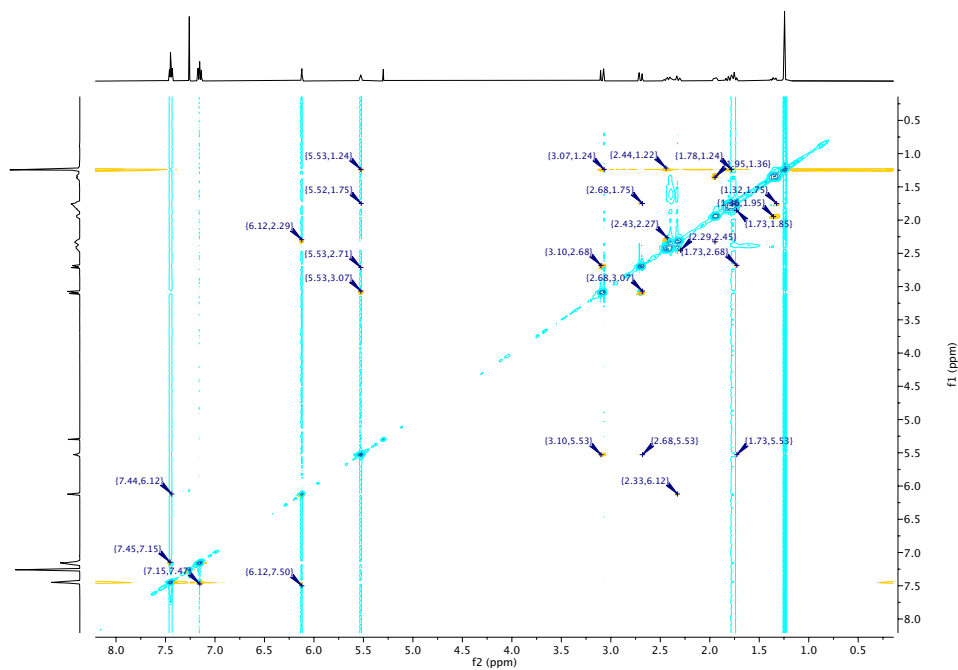


Figure 2.9.54 - ^1H - ^{13}C HMBC of AP22



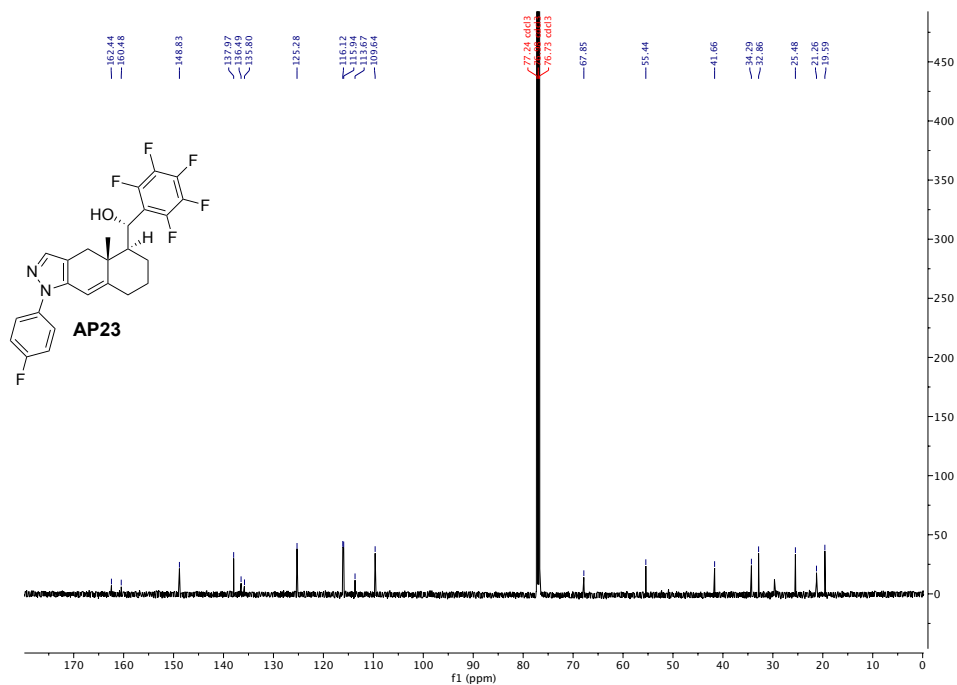


Figure 2.9.57 - ^{13}C NMR of AP23

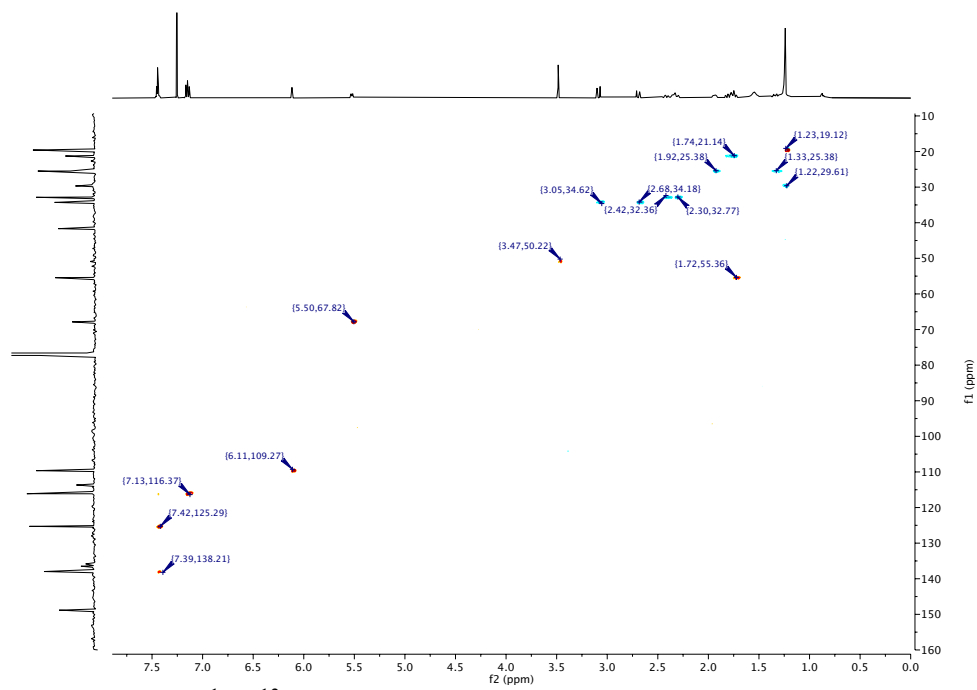


Figure 2.9.58 - ^1H - ^{13}C HSQC of AP23

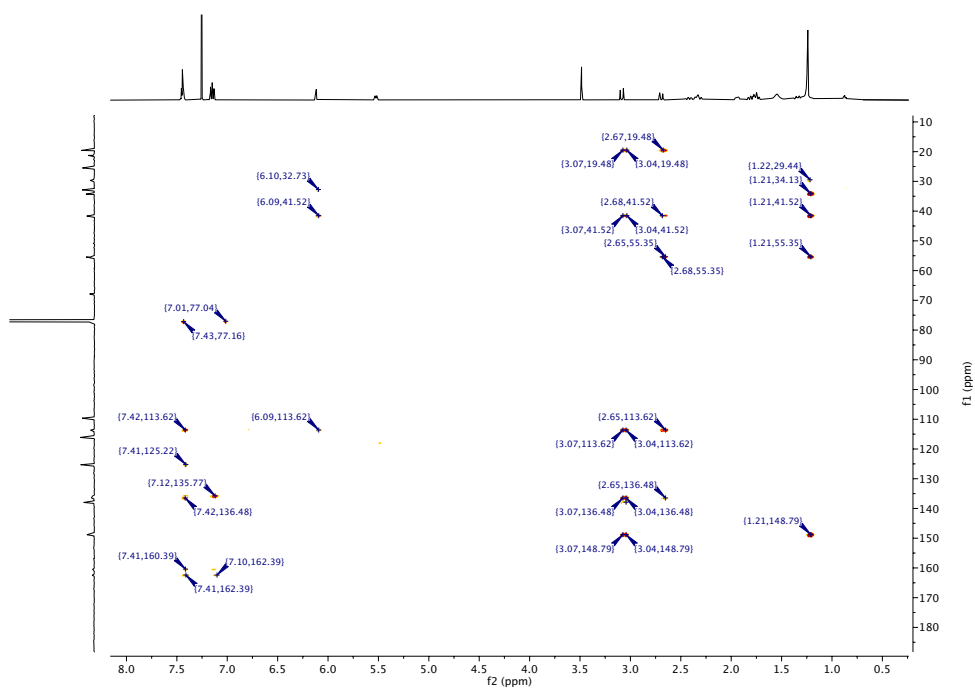


Figure 2.9.59 - ^1H - ^{13}C HMBC of AP23

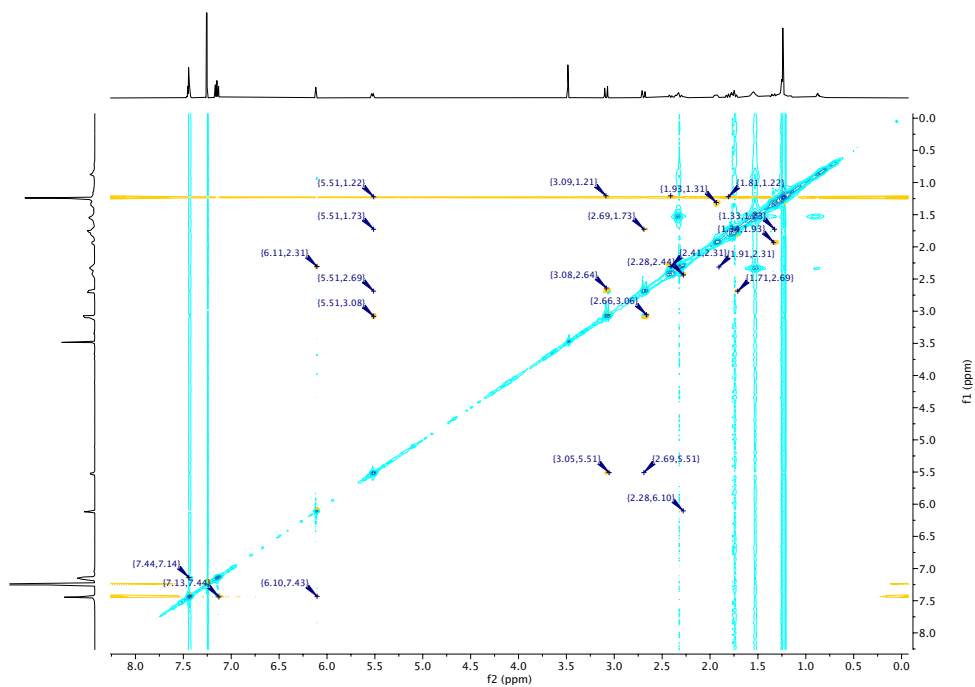


Figure 2.9.60 - ^1H - ^1H NOESY of AP23

2.9.2 – CCL2 Data

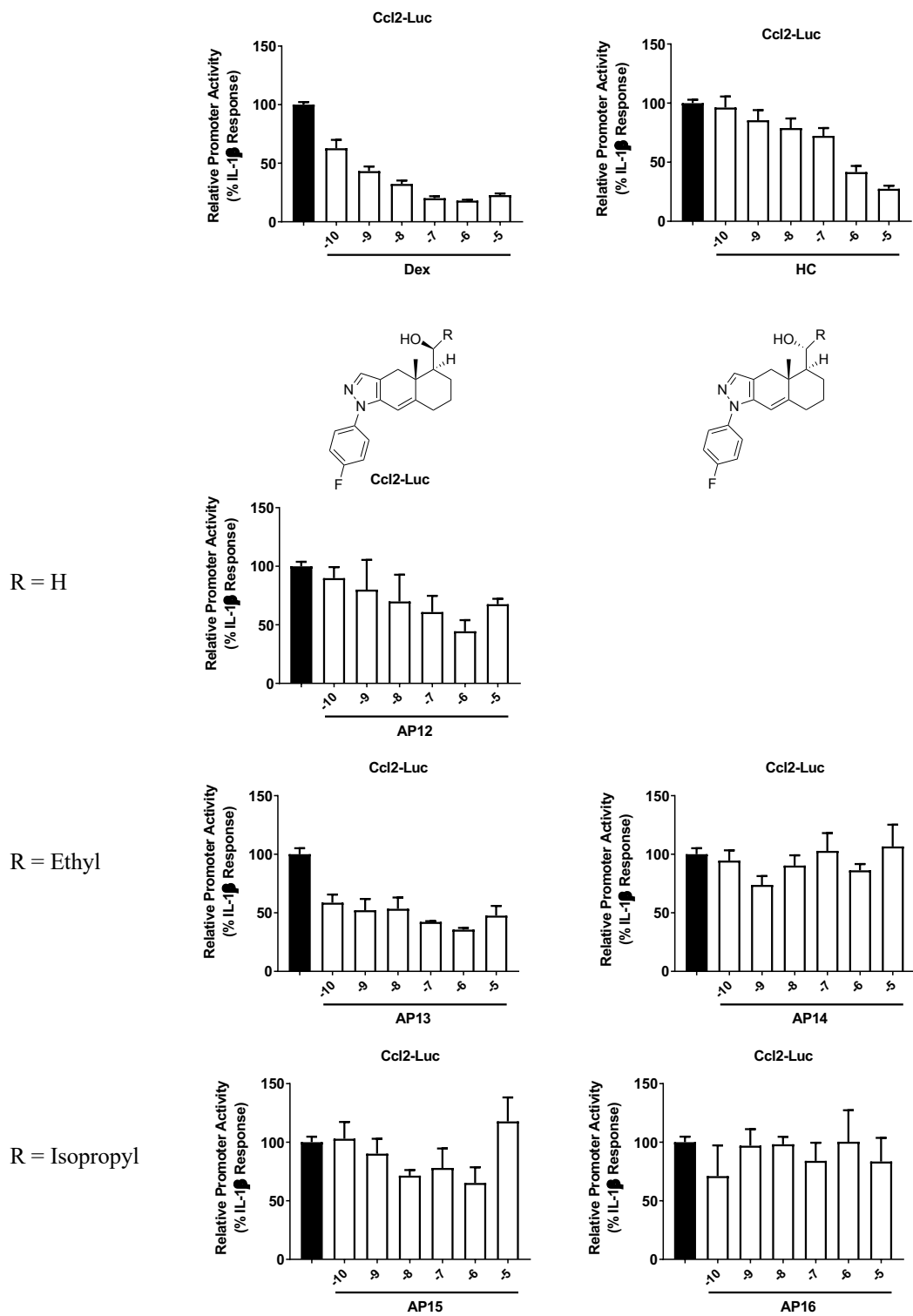


Figure 2.9.61 – CCL2 Data Analysis

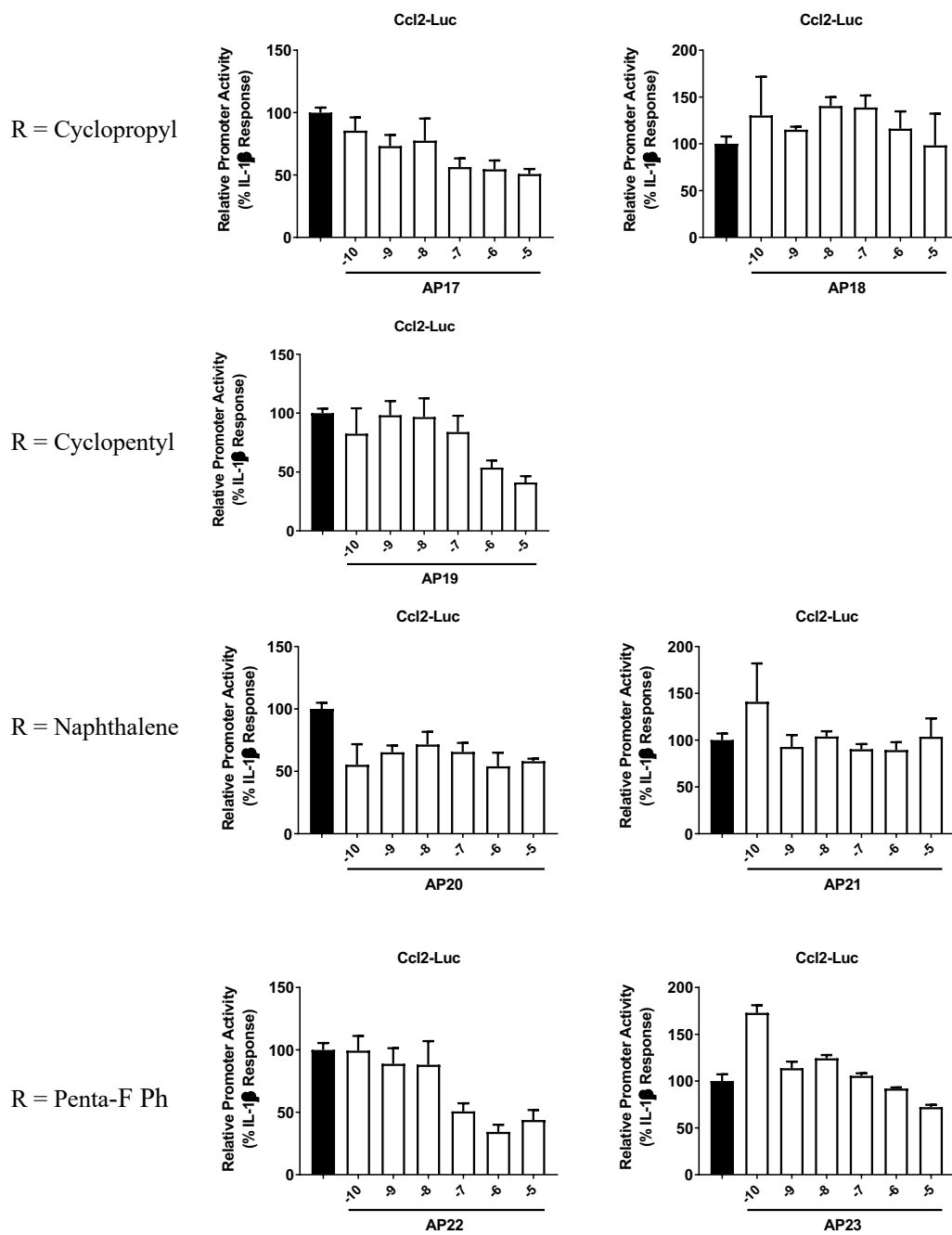


Figure 2.9.61 – Continued

2.9.3 – 3xGRE Data

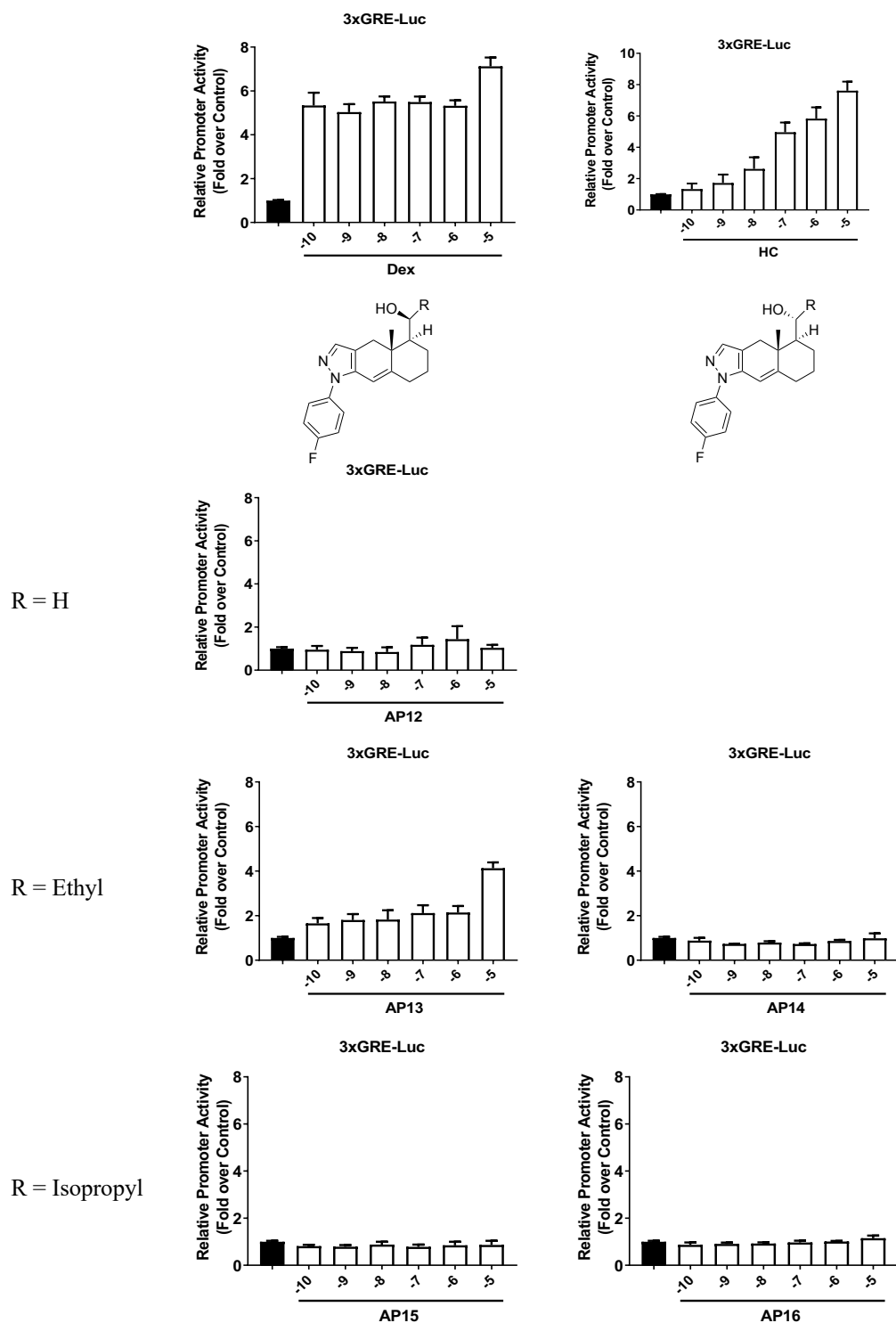
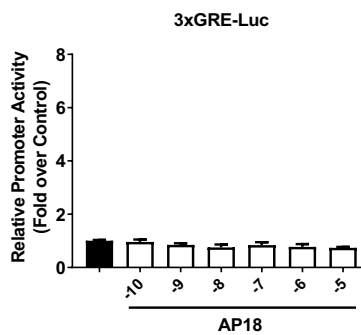
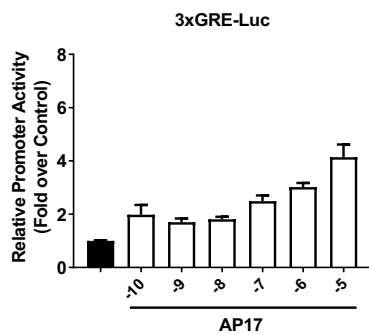
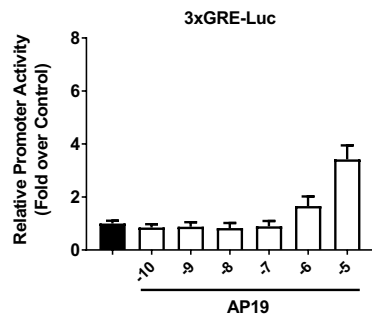


Figure 2.9.62 – 3xGRE Data Analysis

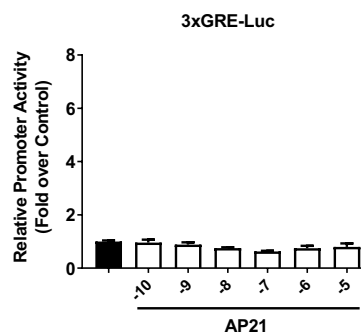
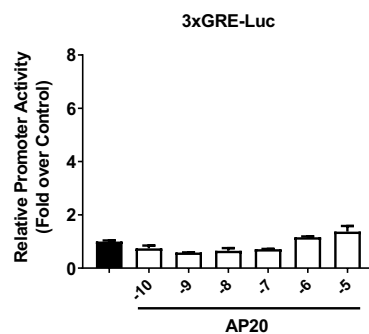
R = Cyclopropyl



R = Cyclopentyl



R = Naphthalene



R = Penta-F Ph

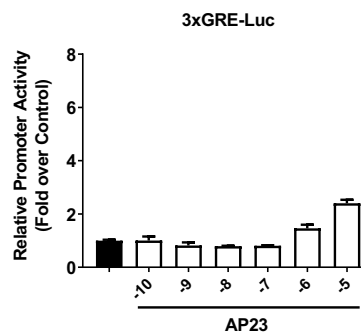
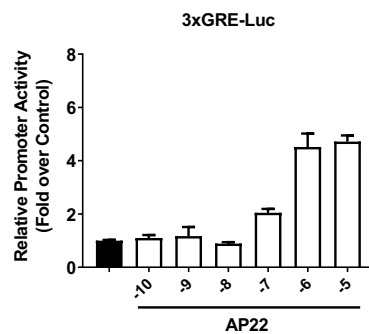


Figure 2.9.62 – Continued

2.9.4 – Ligplot Analysis in 3BQD

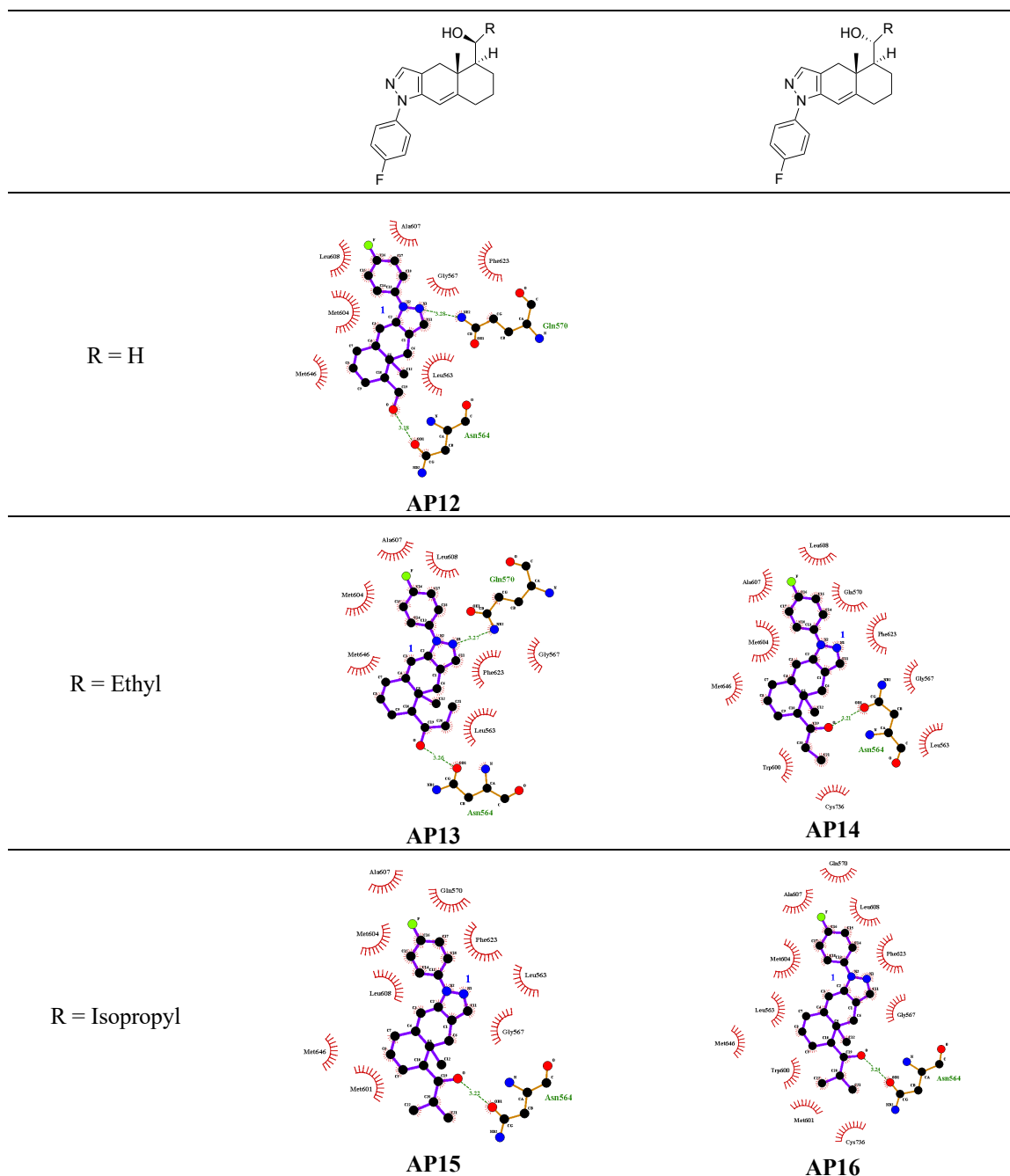
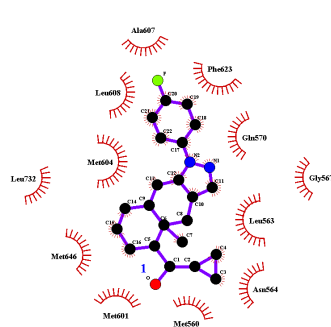
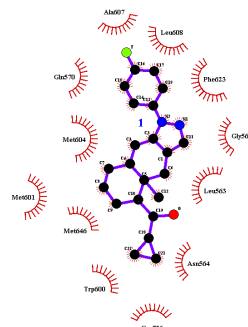


Figure 2.9.63 – Ligplot Analysis in 3BQD

R = Cyclopropyl

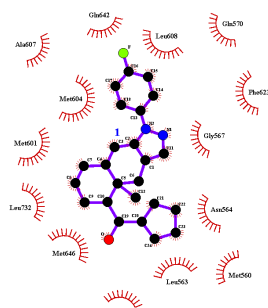


AP17



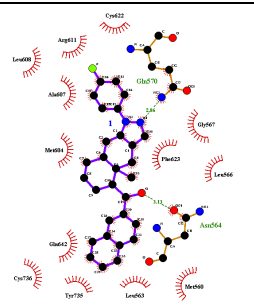
AP18

R = Cyclopentyl

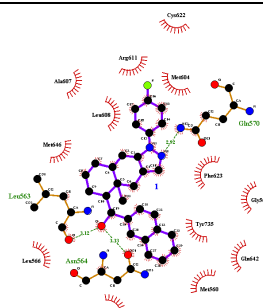


AP19

R = Naphthalene

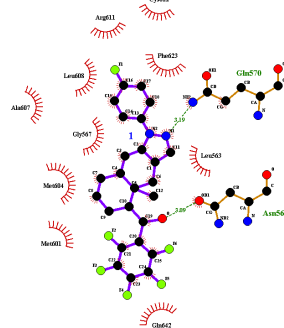


AP20

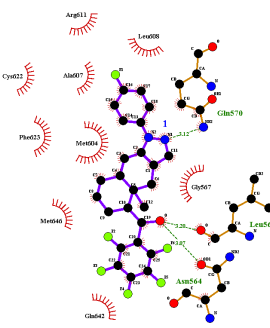


AP21

R = Penta-F Ph



AP22



AP23

Figure 2.9.63 – Continued

CHAPTER III: DISCOVERY OF POTENT ARYL PYRAZOLE GLUCOCORTICOID RECEPTOR AGONISTS DERIVED FROM HYDROCORTISONE

A version of this chapter is expected to be part of a future publication by:

Lato, A. M.; Seaton, W. B.; Burke, S. J.; Ducote, M. P.; Collier, J. J.; Campagna, S. R.

Contributions:

AML designed the experiment, performed synthesis and purification, collected all characterization, performed molecular docking experiments in PDB ID: 3BQD and analyzed all molecular docking experiments, calculated pharmacological values, and analyzed biological assays. WBS aided in the synthesis of compounds **1b-e** and performed molecular docking experiments in PDB ID: 5NFP for compounds **1a-e**. SJB and MPD performed all *in vitro* assays. JJC and SRC provided resources and funding.

3.1 – ABSTRACT

Glucocorticoids (GCs) are a class of hormone signaling molecules known to elicit potent anti-inflammatory and immunosuppressive benefits. GCs are one of the most prescribed medications worldwide, however, they have a long history of serious side effects. GCs bind to the glucocorticoid receptor (GR) and influence transcriptional activity to induce transrepression (beneficial effects) and/or transactivation (side effects). In the 1970s deacylcortivizol (DAC) was found to be a potent aryl pyrazole glucocorticoid receptor agonist (APGRA), however, its potent activity wasn't understood until 2008 when the crystal structure of the GR in complex with DAC revealed the aryl pyrazole moiety exposed a new hydrophobic channel and caused the ligand binding pocket to expand from 540 Å³ to 1070 Å³. To investigate the flexibility of the ligand binding pocket (LBP) and explore underutilized space in the expanded LBP, a series of derivatives formed from the hydrocortisone framework were thoroughly examined through design, synthesis, biological screening, and investigation of structure activity relationships (SARs) with the use of molecular docking techniques. The APGRAs in this study elicited potent activity, all performing >95% the anti-inflammatory response of dexamethasone (Dex) in 832/13 cells, a rat insulinoma cell line. Additionally, all APGRAs outperformed hydrocortisone in both the CCL2 and 3xGRE assay. Remarkably, compounds **5** and **6** displayed up to 90% anti-inflammatory response at 0.1 nM while Dex showed only 30% response at this concentration, showcasing the extreme potency of these APGRAs.

3.2 – INTRODUCTION

Glucocorticoids (GCs) are a class of signaling molecules with high affinity for the glucocorticoid receptor (GR).¹⁰⁶ GCs elicit anti-inflammatory and immunosuppressive responses by influencing transcriptional activity of the GR, specifically through binding with the ligand binding domain (LBD) and inducing conformational changes that lead to an activated GR. Upon ligand activation, the GR-GC complex translocates to the nucleus of the cell where it regulates transcription either directly, by binding to DNA, or indirectly,

though interaction with other transcription factors.¹⁰⁷ Dozens of different GCs are used clinically to treat diverse conditions such as asthma, rheumatoid arthritis, dermatitis, leukemia, autoimmune diseases, and after organ transplantation.^{30, 69} However, GC use is accompanied by a collection of side effects including osteoporosis, diabetes, skin and muscle atrophy, hyperglycemia, obesity, and hypertension.^{106, 108}

It has been shown that slight modifications to the steroidal scaffold of GCs can significantly improve potency.¹⁰⁹ However, an increase in therapeutic effects is normally accompanied with more severe side effects.^{69, 109} A novel GC that can dissociate the beneficial actions from the side effects has been under investigation for decades as it requires an in-depth understanding of GR-GC interactions and the mechanism induced by the ligand activated GR. Selective GR modulators (SGRMs) have been reported, however, to date none have been approved for clinical use.¹⁰⁻¹² Among the SGRMs reported, deacylcortivazol (DAC) stands out as a promising scaffold as it not only displayed selective performance but was also shown to be 40-fold more potent than dexamethasone (Dex), an effective GC that is commonly used in modern medicine.^{27, 28} Although powerful, the therapeutic potential of DAC was limited by its toxicity.

DAC possesses one of the largest steroid modifications with a phenylpyrazole moiety attached to the A ring of the standard steroid framework. Originally hypothesized to be a GR antagonist, the potent agonistic activity of DAC puzzled scientists for decades as it was too large to fit within the steroidal ligand binding pocket (LBP) of the GR.²⁹ It wasn't until 2008 when Suino-Powell and coworkers²⁹ crystalized the DAC bound GR and revealed the aryl pyrazole moiety caused large structural change. Even though DAC was only ~25% larger than Dex, it nearly doubled the size of the LBP as the aryl pyrazole moiety caused large shifts in helices 3 and 5, exposing a hydrophobic channel with high affinity for the aryl pyrazole moiety.²⁹ Recently, the expansion of GR crystal structures in complex with a variety of ligands, has further displayed the flexibility of the GR LBP.³⁰

Herein, the flexibility of the GR LBP is further investigated by modifying hydrocortisone (HC) with a series of mercapto- moieties at the C21 site. Additionally, the expanded LBP is explored through the synthesis of DAC-like ligands through the fusing of an aryl pyrazole moiety to the HC scaffold. While the aryl pyrazole moiety causes the

LBP to double in size, DAC only utilized 50% of the available space.²⁹ To probe deeper into the underutilized space of the expanded LBP, the aryl pyrazole HC scaffold was also further derivatized with a series of mercapto- moieties at the C21 site. These compounds were subjected to *in vitro* assays, evaluating both transactivation and transrepression using in-depth biochemical assays. Additionally, structure activity relationships (SARs) were explored through molecular docking experiments.

3.3 – SYNTHETIC METHODS

Compounds were synthesized according to previously published routes with modifications.^{57, 110, 111} A general synthetic procedure is outlined in Figure 3.3.1 and Figure 3.3.2. Briefly compounds **1a-e** were synthesized through mesylating commercially available hydrocortisone followed by a substitution reaction with the appropriate, commercially available, mercapto- reagent. (Figure 3.3.2)

Compounds **5**, **6**, and **7a-e** also began with commercially available hydrocortisone which was subject to a protection reaction in which a change of reagent from formaldehyde to trioxane was employed to minimize impurities. Formulation of ketal **2** was performed using sodium hydride and ethyl formate in toluene to yield enol **3**. Addition of p-fluorophenylhydrazine hydrochloride or phenylhydrazine hydrochloride produces aryl pyrazole **4** which subsequently underwent a deprotection in 50% formic acid to yield compounds **5** and **6** respectively. Changes in the workup procedure produced higher yields and simplified purification. Compound **5** was subject to mesylation followed by a substitution reaction with the appropriate, commercially available, mercapto- reagent to produce **7a-e**. (Figure 3.3.1) Detailed synthetic procedures and characterization data can be found in the methods section.

3.4 – *IN VITRO* ASSAYS

The ability of the compounds to reduce the proinflammatory activity of IL-1 β was examined using a reporter gene assay consisting of a multimerized NF- κ B luciferase

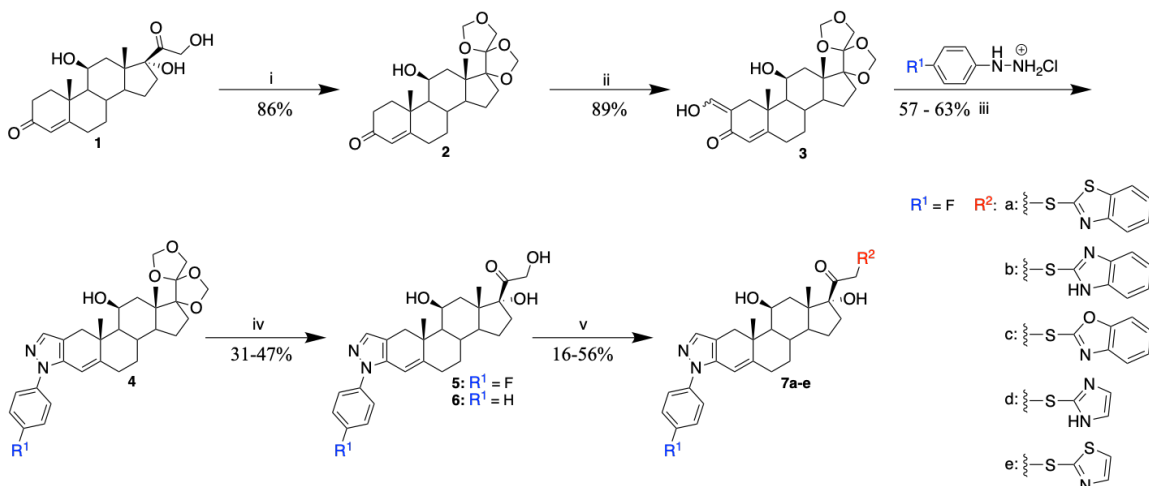


Figure 3.3.1 – Synthetic Route to APGRAs

Reagents and conditions: (i) Trioxane, conc. HCl, CHCl_3 , 4 h (ii) ethyl formate, NaH, toluene, rt, 16 h (iii) NaOAc, AcOH, rt, 24 h (iv) 50% formic acid, reflux, 40 min (v) 1. MsCl, DIPEA, DCM, 16h 2. K_2CO_3 , HS- R^2 , acetone, reflux, 1h.

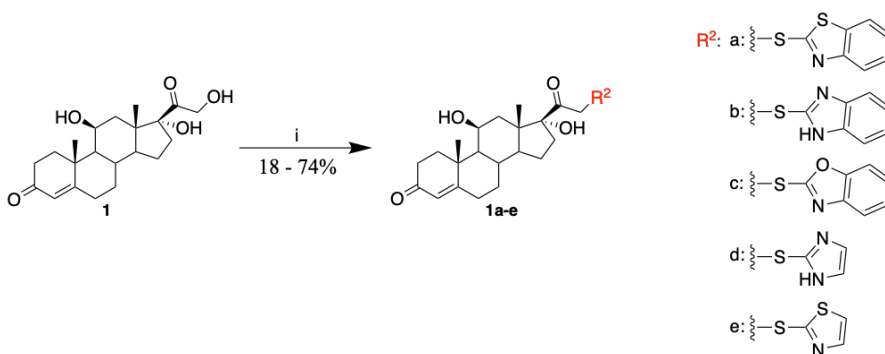


Figure 3.3.2 – Synthetic Route to HC Derivatives

Reagents and conditions: (i) 1. MsCl, DIPEA, DCM, 16h 2. K_2CO_3 , HS- R^2 , acetone, reflux, 1h.

reporter construct that contains five copies of an NF- κ B element upstream of a luciferase gene (5X-NF- κ B-Luc).⁵⁷ 832/13 cells, a rat insulinoma cell line, were cultured as described previously⁶⁶ and determined to be free of mycoplasma contamination. The 832/13 cells were grown in 24-well plates and transfected with the 5X-NF- κ B-Luc reporter plasmid. Twenty-four hours after transfection, the cells were exposed to each synthetic compound for one hour, followed by culture for four hours with 1 ng/mL IL-1 β . Transactivation activity was determined by analyzing the ability of the synthetic compounds to drive transcriptional activity from plasmid construct containing three copies of a glucocorticoid response element (3xGRE).⁵⁷ The 832/13 cells were grown in 24-well plates and transfected with the 3xGRE luciferase reporter plasmid. Twenty-four hours post-transfection, the cells were exposed to each synthetic compound for four hours. At the end of each respective treatment period, the cells were harvested using passive lysis buffer (Promega, Madison, WI) and luciferase activity measured in a Glomax luminometer (Promega, Madison, WI). Luciferase activity was normalized to total protein to account for differences in cell numbers between experiments.

3.5 – MOLECULAR DOCKING

Ligand-receptor interactions were modeled between the compounds subjected to *in vitro* testing and the extended pocket of the GR LBD. The crystal structure of the GR LBD bound to DAC, a potent APGRA₂ and the fourth LXXLL motif of steroid receptor coactivator 1 (SRC1-4; PDB ID: 3BQD) was used as the model for molecular docking studies.²⁹ The 3BQD crystal structure was chosen as the best template as compounds 5 through 7 most closely resemble that of DAC.²⁹ While compounds **1a-e** do not contain the aryl pyrazole moiety, they were modeled in the expanded LBP for consistency. This produced undesired results in which the ligand was flipped 180° from the orientation of the host ligand (DAC) resulting in the mercapto- portion of the ligand occupying the hydrophobic channel that normally houses the aryl pyrazole moiety. For this reason, compounds **1a-e** were also modeled in a standard steroidal LBP, the human GR in complex with budesonide (PDB ID: 5NFP).⁶¹ This was revealed to be a more suitable model as the

computed ligand poses more closely mimicked the orientation of the host ligand, budesonide. The GR LBD was prepared for docking studies by removing the bound ligand and excess water molecules. Ligands were optimized in Chem3D (PerkinElmer) prior to docking, which was performed between a rigid GR and flexible ligand using AutoDock Vina (The Scripps Research Institute).⁷⁰ The resulting docking conformations were viewed with PyMol (Schrödinger).⁷¹ Ligand-residue interactions were further analyzed with LigPlot+ (European Molecular Biology Laboratory).⁷²

3.6 – RESULTS AND DISCUSSION

3.6.1 – Biological Evaluation

To investigate this class of anti-inflammatory compounds, bioassays that directly measured the impact of transactivation and transrepression were chosen. The ability of the synthetic glucocorticoid receptor agonists (GRAs) to reduce CCL2 activity induced by the pro-inflammatory cytokine IL-1 β (transrepression) and enhance transactivation of a synthetic GRE-driven promoter-luciferase construct (3xGRE assay - transactivation) were tested and benchmarked against both dexamethasone (Dex) and hydrocortisone (HC). Several of the tested compounds outperformed Dex in both the CCL2 and 3xGRE assay (**5**, **6**, **7c**, and **7e**) and all the aryl pyrazole glucocorticoid receptor agonists (APGRAs) outperformed HC in both assays as well (**5**, **6**, **7a-e**). (Table 3.6.1)

All synthesized compounds are a derivative of the HC backbone. Looking at the first set of modifications (**1a-e**), compounds **1a** and **1c** slightly outperformed HC with up to 6% higher anti-inflammatory response in the CCL2 assay. Compound **1a** also proved to be more potent with a pIC₅₀ of 6.85 while HC has a pIC₅₀ of 6.53. Compounds **1a** and **1c** not only resulted in increased anti-inflammatory activity compared to HC, but also considerably improved ligand selectivity, eliciting less than 24% the transactivation activity than that of HC in the 3xGRE assay. (Table 3.6.2) Compound **1a** also showed an improved pEC₅₀ of 5.27 in the 3xGRE assay compared to HC with a pEC₅₀ of 6.96, nearly

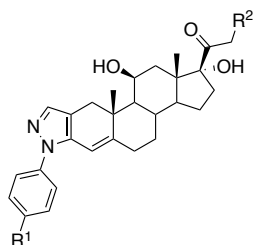


Table 3.6.1 - Biological analysis of APGRAs

Compd	R ¹	R ²	Relative Affinity	CCL2 ^{a,b}				3xGRE ^{a,c}			
				pIC50	E _{max}	%DEX ^d	%HC ^d	pEC50	E _{max}	%DEX ^d	%HC ^d
			3BQD kcal/mol	% Response				Fold Over Control			
HC			-8.8	6.53	72.50	88.49	100.00	6.96	7.60	105.92	100.00
Dex			-10.4	8.91	81.93	100.00	113.01	8.50	7.17	100.00	94.41
5	F	OH	-13.8	6.70	84.69	103.36	116.81	7.50	5.65	78.74	74.34
6	H	OH	-13.5	7.77	90.22	110.11	124.43	9.29	6.37	88.83	83.86
7a	F	Mercaptobenzothiazole	-9.2	7.18	78.30	95.56	107.99	6.30	4.05	56.50	53.34
7b	F	Mercaptobenzimidazole	-11.2	7.65	79.99	97.62	110.32	7.23	4.95	68.96	65.10
7c	F	Mercaptobenzoxazole	-10.8	7.91	82.13	100.24	113.27	7.89	2.60	36.23	34.21
7d	F	Mercaptoimidazole	-12.4	7.66	81.27	99.19	112.10	7.29	3.53	49.18	46.43
7e	F	Mercaptothiazole	-12.4	8.57	84.91	103.63	117.11	7.96	4.57	63.70	60.14

a) Values are the mean of at least two experiments done in duplicate. b) CCL2-promoter-luciferase-reporter activity assay. c) 3xGRE-promoter-luciferase-reporter activity assay. d) Efficacy represented as percentage of maximal response of dexamethasone or hydrocortisone.

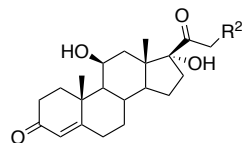


Table 3.6.2 - Biological analysis of HC derivatives

Compd	R ²	Relative Affinity	CCL2 ^{a,b}				3xGRE ^{a,c}			
			pIC50	E _{max}	%DEX ^d	%HC ^d	pEC50	E _{max}	%DEX ^d	%HC ^d
			3BQD kcal/mol	% Response				Fold Over Control		
HC		-8.8	6.53	72.50	88.49	100.00	6.96	7.60	105.92	100.00
Dex		-10.4	8.91	81.93	100.00	113.01	8.50	7.17	100.00	94.41
1a	Mercaptobenzothiazole	-9.0	6.85	77.01	93.99	106.22	5.27	1.77	24.63	23.25
1b	Mercaptobenzimidazole	-9.8	6.94	44.65	54.49	61.58	6.10	1.24	17.27	16.30
1c	Mercaptobenzoxazole	-9.7	5.69	74.54	90.97	102.80	7.25	1.65	22.94	21.66
1d	Mercaptoimidazole	-9.7	6.06	22.18	27.07	30.59	5.82	1.41	19.63	18.53
1e	Mercaptothiazole	-9.5	5.92	54.33	66.31	74.94	5.48	1.46	20.29	19.16

a) Values are the mean of at least two experiments done in duplicate. *b)* CCL2-promoter-luciferase-reporter activity assay. *c)* 3xGRE-promoter-luciferase-reporter activity assay. *d)* Efficacy represented as percentage of maximal response of dexamethasone or hydrocortisone.

a 50-fold difference, making it an attractive candidate for further studies. Modifications made to compounds **1b**, **1d**, and **1e** resulted in decreased activity in the CCL2 assay, eliciting less than 55% anti-inflammatory response.

The aryl pyrazole modification increased anti-inflammatory activity in all cases. Compounds **5** and **6** only differ from the HC scaffold through an addition of the aryl pyrazole moiety. Interestingly, they showed the greatest increase in anti-inflammatory response, with 80-90% response at 0.1 nM concentrations while HC elicits no anti-inflammatory response at this concentration and only achieves a maximal 72% response at a concentration of 10 μ M. Further testing is currently underway to discover the concentration at which anti-inflammatory response ceases for compounds **5** and **6**. The remaining APGRAs (**7a-e**) outperformed HC in the CCL2 assay showing 8-17% greater response. Compounds **7a-e** displayed greater potency than HC as well, with pIC_{50} values ranging from 7.18 to 8.57 while HC has a pIC_{50} of 6.53. Moreover, they all displayed greater selectivity eliciting maximal 2.60 to 4.95 fold over control in the 3xGRE assay while HC had an E_{max} of 7.60 fold over control. Compound **7e** displayed the most favorable activity in this group displaying a 110-fold difference in potency with a pIC_{50} of 8.57 compared to HC with a pIC_{50} of 6.53 in the CCL2 assay. Additionally, **7e** elicited approximately half the transactivation potential with an E_{max} of 4.47 fold over control. (Table 3.6.1)

Compounds **5** and **6** elicited the highest response in the CCL2 assay, displaying 80% to 90% anti-inflammatory response across all concentrations tested even at 0.1 nM while Dex displayed ~30% response at this concentration, exhibiting the extreme potency of these derivatives. (Figure 3.6.1) These compounds show slightly improved transactivation activity as well, eliciting ~20% lower response than Dex. However, ongoing testing to examine activity at lower concentrations may lead to a concentration with improved transactivation response while maintaining potent anti-inflammatory activity. The remaining APGRAs (**7a-e**) all performed 95% or greater than that of Dex in the CCL2 assay, in which Dex had a maximal 81.93% response. Compounds **7e** and **7c** displayed a greater anti-inflammatory response than that of Dex with E_{max} values of 84.91% and 82.13% respectively. Compound **7e** also displayed a comparable potency to Dex with a

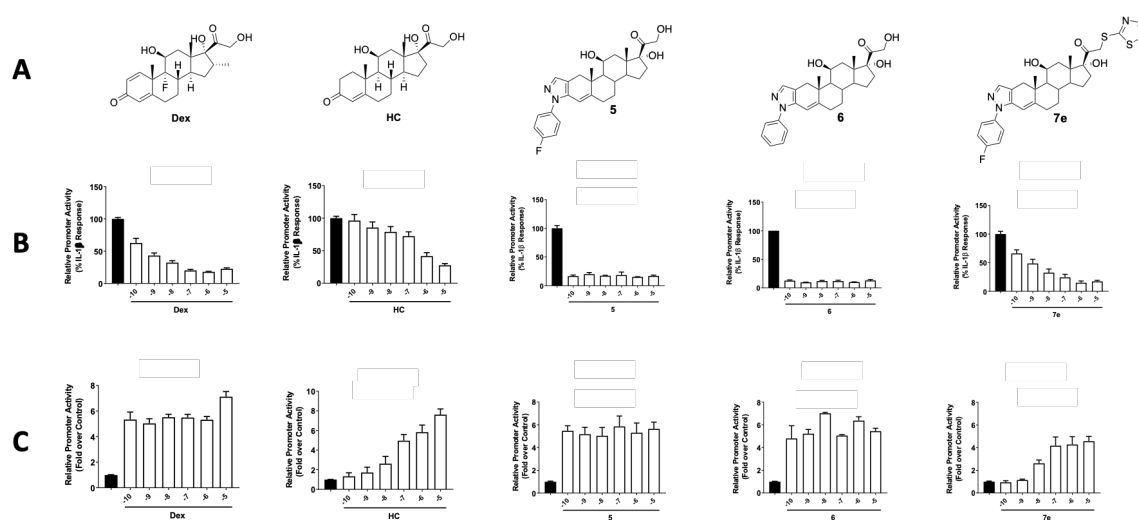


Figure 3.6.1 – Biological Analysis of Top Performing APGRAs

Bioassay data showing GR agonist activity and impact on glucose-stimulated insulin secretion. (A) Structures of tested compounds. (B) CCL2-promoter-luciferase-reporter activity assay; x-axis: log concentration (molar); y-axis: %-maximal IL-1 β response (relative promoter activity). (C) 3xGRE-promoter-luciferase-reporter activity assay; x-axis: log concentration (molar); y-axis: fold over control.

pIC₅₀ of 8.57 while Dex has a pIC₅₀ of 8.91, however, **7e** elicited ~40% less transactivation activity with an E_{max} of 4.57 fold over control while Dex had an E_{max} of 7.17 fold over control in the 3xGRE assay. Derivative **7c** displayed similar anti-inflammatory response to Dex, however, its potency was 10-fold greater with a pIC₅₀ of 7.91 making it slightly less desirable than other top performing compounds (**5**, **6**, and **7e**). However, it is noteworthy that **7c** elicited only ~35% transactivation of HC and Dex in the CCL2 assay, showing greater selectivity than **7e**.

When comparing the HC derivatives (**1a-e**) to the corresponding aryl pyrazole HC (APHC) derivatives (**7a-e**), only slight trends are observed. Overall APHC derivatives did show increased anti-inflammatory activity compared to its counterpart, however, the amount varied considerably. For instance, **7a** only showed a 1% increased anti-inflammatory response than **1a**, which had a maximal 77% response in the CCL2 assay. Additionally, **7a** exhibited a 2-fold increase in potency with a pIC₅₀ of 7.18 for while **1a** had a pIC₅₀ of 6.85. However, the mercaptothiazole derivatives displayed great differences in which **7e**, with an E_{max} of 84.91% response, increased anti-inflammatory response by 30% compared to **1e**, with an E_{max} of 54.33% response in the CCL2 assay. Additionally, **7e** proved to be substantially more potent with a pIC₅₀ of 8.57 while **1e** had a pIC₅₀ of 5.92, nearly a 900-fold difference. While the HC derivatives did perform better in the 3xGRE assay than the APHC derivatives, this can likely be attributed to HC derivatives possessing decreased agonistic activity in general as observed with their decreased activity in both the CCL2 and 3xGRE assay compared to the APHC derivatives.

3.6.2 – Computational Analysis

Molecular docking experiments were used to explore deeper into the underlying interactions between the ligand and receptor. Molecular docking studies were performed using AutoDock Vina (The Scripps Research Institute)⁷⁰ through which the energy minimized ligand structures were allowed to freely interact within the LBP of the rigid receptor. This molecular docking technique allows the ligand to take on various poses and

compute the associated relative binding affinity. Since the APGRAs were too large to be docked in the standard steroid LBP, the expanded LBP (PDB ID: 3BQD) was chosen as the best model.²⁹ However, after docking the HC derivatives (**1a-e**) an interesting trend emerged. Rather than positioning the ligand in an orientation that matches HC, compounds **1a-e** displayed a 180° flip in the LBP that positioned the various mercapto- moieties in the extended hydrophobic channel that normally houses the aryl pyrazole moiety of the host ligand, DAC. (Figure 3.6.2) This extended hydrophobic channel has only been proven to be exposed in the presence of a ligand with an aryl pyrazole moiety so the HC derivatives were docked in the standard steroid LBP as well (PDB ID: 5NFP).⁶¹ This resulted in compounds **1a-e** taking on the observed orientation of steroidal ligands, sharing well-known residue interactions such as the hydrogen bonding interaction between Arg611 and the C3 ketone on the A ring of the steroid. (Figure 3.6.3)

Because of the unique nature of GR activity in which ligands can initiate both transactivation and transrepression, it is difficult to use relative binding affinity as an indication of ligand activity or selectivity. However, there is a slight correlation between relative binding affinity in 3BQD and $E_{\max}$ in the CCL2 assay observed. Using the binding affinity data for **1a-e** in 5NFP also fits this correlation but for consistency, figure 3.6.4 displays the correlation using relative binding affinities in the expanded LBP, 3BQD. While binding affinity is useful in some instances, a more intricate examination of ligand-residue interactions are needed to attempt to understand the driving forces behind ligand selectivity. LigPlot analysis of all tested compounds can be found in the appendix.

Compounds **5** and **6** were by far the top performing compounds in the CCL2 assay eliciting 80-90% anti-inflammatory response at 0.1 nM. Interestingly, all the ligand-residue interactions seen in compounds **5** and **6** are also seen in DAC. DAC has additional hydrophobic interactions with Ala605, Ile747, and Met560 not observed in either of the synthesized APGRAs. Also noteworthy, is that compound **6** lacked an interaction with Thr739 which was seen with compound **5** and DAC, yet compound **6** was the most active in the CCL2 assay with ~90% response in all concentrations tested. Recent findings have shown that retaining high affinity while weakening interactions can result in a more selective ligand, especially in helix 12.⁹⁹ While Thr739 is part of helix 11, this lack of interaction

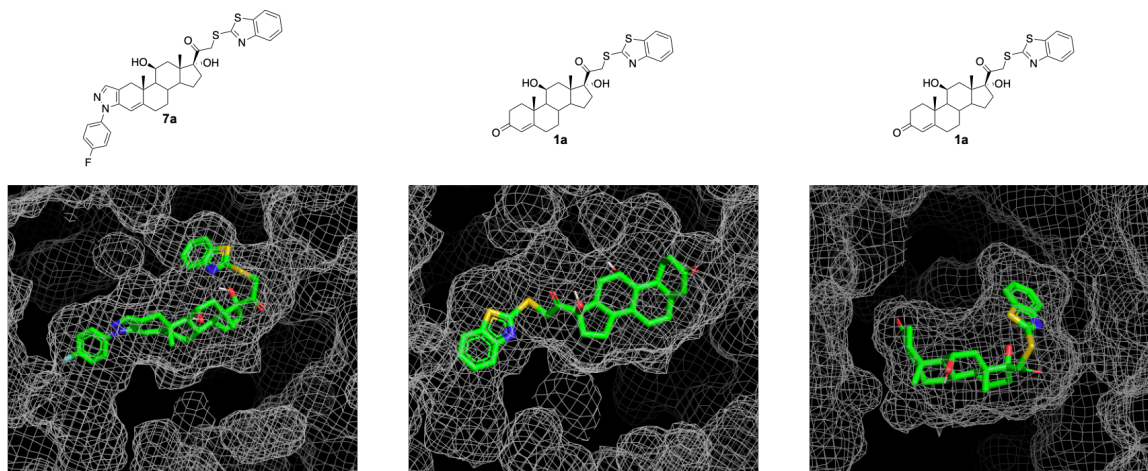


Figure 3.6.2 – Ligand Orientation in LBD

Orientation of the HC derivatives in expanded LBP and steroid LBP. (Left) APGRA **7a** in the expanded LBP (PDB ID: 3BQD) with the aryl pyrazole moiety occupying the extended hydrophobic channel. (Middle) HC derivative **1a** in the expanded LBP (PDB ID: 3BQD) with the mercapto- moiety occupying the extended hydrophobic channel, a 180° flip to the orientation of DAC and other APGRAs. (Right) HC derivative **1a** in the standard LBP (PDB ID: 5NFP), orientation mimics that of HC.

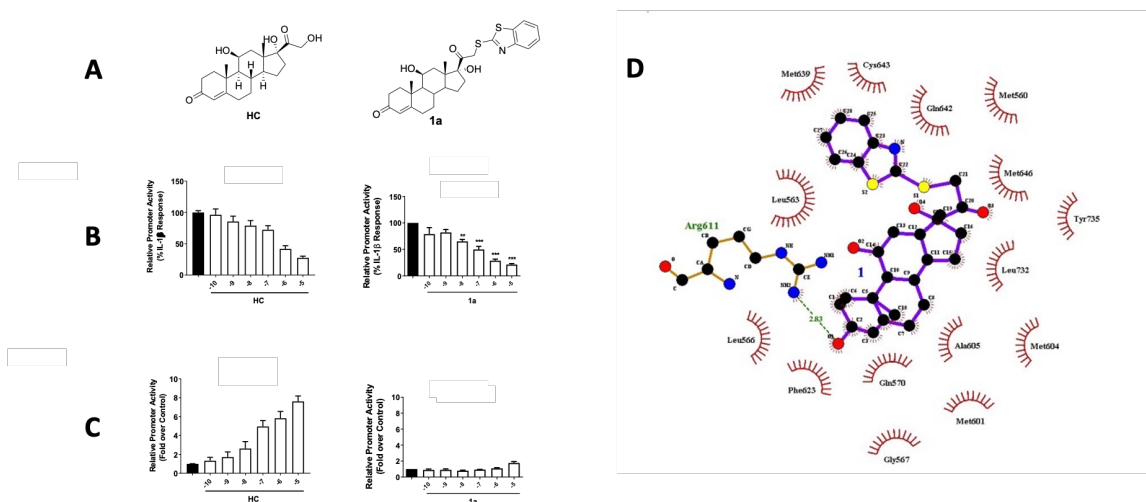


Figure 3.6.3 – Selectivity of Compound 1a

Bioassay data showing GR agonist activity and impact on glucose-stimulated insulin secretion. (A) Structures of tested compounds. (B) CCL2-promoter-luciferase-reporter activity assay; x-axis: log concentration (molar); y-axis: %-maximal IL-1 β response (relative promoter activity). (C) 3xGRE-promoter-luciferase-reporter activity assay; x-axis: log concentration (molar); y-axis: fold over control. (D) 2D LigPlot analysis of **1a** in human glucocorticoid receptor 5NFP where a red semi-circle represents a hydrophobic interaction and green dotted line represents a hydrogen bond.

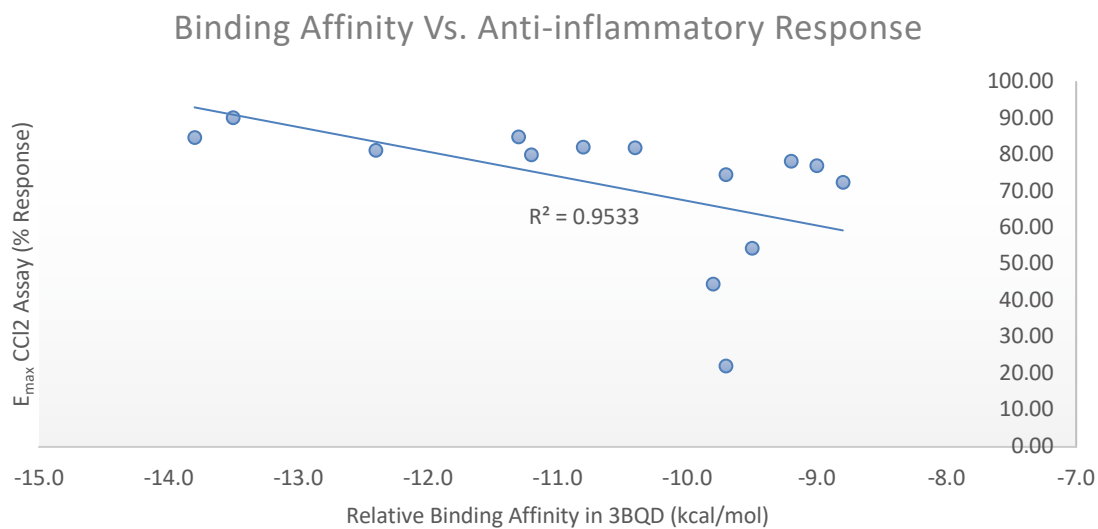


Figure 3.6.4 – Binding Affinity Correlation

Relative binding affinity of ligands modeled in expanded ligand binding pocket (3BQD) with values shown in kcal/mol (y axis). E_{max} from CCL2 assays for each compound shown in % response (x axis). Trendline shown with R^2 value.

may cause a more fluid receptor and therefore cause subsequent shifts in helix 12. Here it is observed that a decrease in residue interactions, leads to the most potent derivative, compound **6**.

In both the HC derivative and the APHC derivatives, imidazole containing derivatives had the most underwhelming activity in which compounds **1b** and **1d** elicited a maximal response of 22% and 45% in the CCL2 assay respectively. Although compounds **7b** and **7d** elicited ~80% response in the CCL2 assay, they were among the least active aryl pyrazole containing compounds. (Table 3.6.1) Interestingly, these compounds frequently possessed increased hydrogen bonding networks in which the imidazole portion formed a hydrogen bond with Gln642. (See appendix section 3.9.4 and section 3.9.5) This may lead to undesired receptor conformational changes that disrupt transrepression mechanisms. It is clear the APHC derivatives outperformed the HC derivatives in the CCL2 assay. Although the aryl pyrazole moiety doubles the size of the LBP, most of the interactions with the various R moieties are conserved between the HC and APHC derivatives. With this in mind, the enhanced affinity associated with the aryl pyrazole moiety appears to be driving this separation in activity. (See appendix section 3.9.4 and section 3.9.5)

Looking solely at the HC derivatives, compound **1a** stands out as the top performing compound reducing inflammatory response by 77% while eliciting less than 25% the transactivation activity of Dex and HC. (Table 3.6.2) Recently, residue Asn564 in helix 3 was found to be a crucial interaction that may affect the stability of the activation function - 2 (AF-2) domain and potentially a key link to achieving ligand selectivity.^{95, 99} Lui and coworkers⁹⁹ recently reported that disrupting the hydrogen bond with Asn564 weakens receptor dimerization and binding to co-activators but not co-repressors allowing transrepression to remain intact while reducing transactivation activity. Interestingly, although compound **1a** possesses the C11 OH group capable of hydrogen bonding with Asn564, the residue interaction is not observed and may be the reason behind the selective nature of **1a**. (Figure 3.6.3)

3.7 – CONCLUSION

The expanded LBP was investigated through the synthesis of a library of APGRAs and HC derivatives which were thoroughly examined through biological assays and molecular docking techniques. The expanded LBP was first discovered in 2008 when the human GR was crystalized with DAC.²⁹ Even though DAC was only 25% larger than Dex, the aryl pyrazole moiety caused the LBP to expand from 540 Å³ to 1070 Å³, nearly doubling in size.²⁹ However, DAC only utilizes about 50% of the expanded LBP with a ligand volume of 392 Å³. This study allowed an exploration into the underutilized space in the expanded LBP with a series of APGRAs that were up to 72 Å³ larger with various mercapto- moieties that extended into an open pocket in the expanded LBP.

Comparing compounds **7a-e** to **1a-e**, it is clear that the APGRAs outperformed the HC derivatives in the CCL2 assay with all APGRAs performing greater than 95% of Dex. (Table 3.6.1 and Table 3.6.2) The most striking exhibit of the aryl pyrazole moiety increasing activity is seen in compounds **5** and **6**. These compounds only differ from HC by the addition of the aryl pyrazole moiety, yet a substantial increase in activity was observed with compounds **5** and **6** eliciting an 80-90% anti-inflammatory response at 0.1 nM while HC elicits no anti-inflammatory response at this concentration and only achieves a maximal 72% response at a concentration of 10 µM, still ~20% less than compound **6**. Further testing at lower concentrations is needed to observe the true potency of these compounds, however, even in this maximally active state, some selectivity was observed with approximately 20% reduction in transactivation activity compared to Dex and HC in the 3xGRE assay. Even though compounds **5** and **6** show less selectivity between transrepression and transactivation, compounds with this high potency are highly desirable as well, as the lower doses needed to see benefits can also lead to less side effects and therefore improved therapeutic outcomes.

Interestingly, the greatest selectivity was seen with compound **1a** which displayed 106% and 94% the anti-inflammatory activity of HC and Dex respectively, in the CCL2 assay. However, while retaining the desired anti-inflammatory activity, a significant decrease in transactivation was observed. Compound **1a** elicited less than 25% the

transactivation response of Dex and HC, showing roughly 75% greater selectivity than some of the most widely used glucocorticoids while retaining the same beneficial transrepression mechanisms. Molecular docking reveals that this may be due to an interference with the AF-2 domain, in which a loss of hydrogen bonding with Asn564 may induce a conformational change that is less favorable to co-activator binding or dimerization and thus cause a decrease in transactivation.

This study emphasizes the powerful effects associated with the addition of an aryl pyrazole moiety to a steroidal scaffold and further displays the flexible nature of the GR LBD. The GR LBD was able to accommodate ligands ~40% larger than Dex and ~15% larger than DAC and still displayed powerful agonistic activity. While APGRAs with smaller moieties tended to perform most favorably (compounds **5**, **6**, and **7e**), further investigation into the underutilized space of the expanded LBP may lead to a better understanding the complex ligand-receptor communications and their induced mechanism.

3.8 – METHODS

3.8.1 – *Materials*

All reactions were performed under inert atmosphere. Reagents were purchased from Fisher Scientific, VWR, or Sigma Aldrich and used without further purification. Dry ethereal solvents were obtained by distillation from a potassium-benzophenone ketyl still, all other dry solvents were obtained via distillation over CaH₂. Thin-layer chromatography (TLC) was carried out using Sorbent Technologies silica G w/UV254 TLC plates. All purified compounds of interest were visualized as single spots using short-wave UV light; more permanent staining of TLC spots was achieved using vanillin. ¹H and ¹³C NMR spectra were recorded on a Varian Inova 500 MHz instrument (Palo Alto, CA) in solutions of CDCl₃ or CD₃OD. High resolution mass spectrometry (HRMS) was performed using an Exactive™ Plus Orbitrap mass spectrometer with an ESI source operating in positive ionization mode. Optical rotation was measured using a Perkin-Elmer 241 polarimeter (Waltham, MA) equipped with a sodium source lamp. IR spectra were collected using a Thermo Scientific Nicolet™ iS5 FTIR spectrometer (Waltham, MA). All synthetic

methods, annotated characterization data, including NMR spectra of final compounds, and bioassay data can be found in the appendix.

3.8.2 – *Synthetic Procedures and Characterization*

General synthesis for compound 1a-e: Synthesis for compounds **1a-e** was performed following a previously published method.⁵⁷ Briefly, hydrocortisone (5 mmol) was dissolved in 100 mL dichloromethane (DCM) and cooled to 0°C. Diisopropylethylamine (DIPEA) (4.8 mmol) was added via syringe followed by addition of mesylchloride (MsCl) (7.5 mmol). The reaction warmed to room temperature and was allowed to stir overnight (~16 hours). The reaction was concentrated *in vacuo*, before being redissolved in DCM (50 mL) and washed with saturated bicarbonate. The aqueous fraction was re-extracted with DCM (20 mL), combined organic fractions were washed twice with brine, dried with MgSO₄, filters, and concentrated *in vacuo* to yield a tan solid. Mesylated hydrocortisone (0.5 mmol) was dissolved in acetone to which potassium carbonate (5 mmol) and the appropriate merapto- reagent (1 mmol) was added. The reaction was refluxed for 1 hour, filtered through a short pad of silica, and concentrated *in vacuo*.

(10*R*,11*S*,13*S*,17*R*)-17-(2-(benzo[*d*]thiazol-2-ylthio)acetyl)-11,17-dihydroxy-10,13-dimethyl-1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one (1a): Using commercially available mercaptobenzothiazole, compound **1a** was obtain as a white solid in] 74% yield. TLC (Silica G w/UV254) 50% EtOAc in hexanes, R_f = 0.32; ¹H NMR (500 MHz, Chloroform-*d*) δ 7.77 (dd, 2H), 7.43 (t, 1H), 7.34 (t, 1H), 6.01 (s, 1H), 5.73 (d, 1H), 5.04 (s, 1H), 4.53 (s, 1H), 3.52 (s, 1H), 3.27 (d, 1H), 2.90 – 2.82 (m, 1H), 2.54 – 2.36 (m, 3H), 2.29 (d, 1H), 2.21 (dd, 1H), 2.14 – 1.84 (m, 5H), 1.68 – 1.57 (m, 3H), 1.48 (s, 3H), 1.28 – 1.10 (m, 3H), 0.99 (s, 3H). HRMS (ESI) m/z: [M+H]⁺ Calcd for C₂₈H₃₃NO₄S₂: 512.19238; found: 512.1927.

(10*R*,11*S*,13*S*,17*R*)-17-(2-((1*H*-benzo[*d*]imidazol-2-yl)thio)acetyl)-11,17-dihydroxy-10,13-dimethyl-1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one (1b): Using commercially available

mercaptobenzimidazole, compound **1b** was obtain as a pale yellow solid in 72% yield. Mp 210.1 - 212.9°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.12$; $[\alpha]_D^{20} = 101.4$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.59 (d, 1H), 7.39 (s, 2H), 7.18 – 7.08 (m, 2H), 5.70 (s, 1H), 5.08 – 4.90 (m, 1H), 4.46 (s, 1H), 3.11 (d, 1H), 2.80 (ddd, 1H), 2.53 – 2.44 (m, 2H), 2.38 (t, 2H), 2.25 (d, 1H), 2.16 (d, 1H), 2.07 – 2.00 (m, 2H), 1.99 – 1.92 (m, 1H), 1.91 – 1.81 (m, 2H), 1.59 (t, 2H), 1.44 (s, 4H), 1.29 – 1.06 (m, 4H), 0.94 (s, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 17.81, 21.10, 23.87, 31.51, 32.13, 32.63, 33.82, 34.42, 34.74, 35.04, 39.19, 40.75, 46.62, 52.15, 55.95, 68.61, 76.78, 77.00, 77.21, 90.95, 122.32, 122.66, 122.92, 149.81, 172.46, 199.73, 208.95. FT-IR ν_{max} (cm^{-1}): 3298 (OH), 2924 (w), 2360, 2338, 1705, 1615, 1513, 1222, 1128, 837. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{28}\text{H}_{34}\text{N}_2\text{O}_4\text{S}$: 495.2312; found: 495.2313.

(10*R*,11*S*,13*S*,17*R*)-17-(2-(benzo[*d*]oxazol-2-ylthio)acetyl)-11,17-dihydroxy-10,13-dimethyl-1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one (1c): Using commercially available mercaptobenzoxazole, compound **1c** was obtain as a tan solid in 18% yield. Mp 217.7 - 220.1°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.24$; $[\alpha]_D^{20} = 9.9$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.51 (d, 1H), 7.44 (d, 1H), 7.29 (d, 2H), 5.74 (s, 1H), 5.70 (s, 1H), 4.94 (d, 1H), 4.51 (s, 1H), 3.27 (s, 1H), 2.83 (t, 1H), 2.53 – 2.46 (m, 2H), 2.39 – 2.35 (m, 2H), 2.29 – 2.24 (m, 1H), 2.22 – 2.18 (m, 1H), 2.09 – 2.03 (m, 2H), 1.95 – 1.86 (m, 3H), 1.65 – 1.57 (m, 2H), 1.46 (s, 4H), 1.19 (d, 1H), 1.11 (d, 1H), 1.05 (s, 1H), 0.97 (s, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 17.72, 19.71, 21.11, 23.80, 29.69, 31.49, 31.95, 32.07, 32.65, 33.81, 34.66, 35.13, 35.42, 39.16, 40.65, 46.82, 52.11, 56.02, 68.59, 76.74, 76.99, 77.24, 90.84, 110.26, 117.75, 122.44, 124.51, 124.71, 140.55, 152.05, 165.24, 171.82, 199.34, 206.59. FT-IR ν_{max} (cm^{-1}): 3246 (OH), 2927 (w), 2336, 2323, 1707, 1623, 1513, 1220, 1109, 837. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{28}\text{H}_{33}\text{NO}_5\text{S}$: 496.21522; found: 496.2144.

(10*R*,11*S*,13*S*,17*R*)-17-(2-((1*H*-imidazol-2-yl)thio)acetyl)-11,17-dihydroxy-10,13-dimethyl-1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one (1d): Using commercially available mercapimidizole,

compound **1d** was obtained as a white solid in 22% yield. Mp 204.2 - 205.8°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.06$; $[\alpha]_D^{20} = 70.9$ (c 1.0, MeOH); ^1H NMR (500 MHz, Methanol- d_4) (exchangeable protons not observed) δ 7.04 (s, 2H), 5.65 (s, 1H), 4.38 (d, 2H), 3.91 (d, 1H), 2.75 – 2.69 (m, 1H), 2.59 – 2.45 (m, 2H), 2.33 – 2.18 (m, 3H), 2.08 – 2.00 (m, 3H), 1.91 – 1.83 (m, 1H), 1.76 (t, 2H), 1.65 (dd, 1H), 1.46 (s, 4H), 1.37 (d, 1H), 1.29 (d, 1H), 1.15 – 1.07 (m, 1H), 0.99 (dd, 1H), 0.82 (s, 3H). ^{13}C NMR (500 MHz, Methanol- d_4) δ 16.39, 19.98, 23.16, 29.23, 31.44, 31.86, 32.85, 32.92, 32.94, 33.18, 34.43, 39.28, 39.47, 41.49, 47.22, 47.40, 47.57, 51.97, 56.16, 67.28, 89.87, 115.58, 121.08, 139.34, 175.14, 201.05, 206.88, 206.93. FT-IR ν_{max} (cm^{-1}): 3520, 3137 (OH), 2974, 2941, 2873, 2648, 1357, 2331, 1694, 1615, 1575, 1381, 1229, 1084, 737. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{24}\text{H}_{32}\text{N}_2\text{O}_4\text{S}$: 445.21555; found: 445.2158.

(10*R*,11*S*,13*S*,17*R*)-11,17-dihydroxy-10,13-dimethyl-17-(2-(thiazol-2-ylthio)acetyl)-1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one (1e): Using commercially available mercaptothiazole, compound **1e** was obtained as a tan solid in 60% yield. Mp 178.8 - 180°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.18$; $[\alpha]_D^{20} = 57.1$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform- d) δ 7.50 (d, 1H), 7.20 (d, 1H), 5.80 (s, 1H), 5.69 (s, 1H), 4.86 (d, 1H), 4.49 (s, 1H), 3.24 (d, 1H), 2.84 – 2.75 (m, 1H), 2.50 – 2.44 (m, 2H), 2.39 – 2.30 (m, 2H), 2.27 – 2.17 (m, 2H), 2.05 (d, 2H), 1.90 – 1.82 (m, 3H), 1.61 – 1.49 (m, 2H), 1.44 (s, 4H), 1.21 – 1.07 (m, 3H), 0.94 (s, 3H). ^{13}C NMR (500 MHz, Chloroform- d) δ 17.74, 21.08, 23.75, 31.47, 32.07, 32.66, 33.80, 34.43, 35.09, 36.59, 39.16, 40.60, 46.73, 52.07, 56.01, 68.57, 76.74, 77.00, 77.25, 90.87, 119.31, 122.40, 141.11, 165.19, 171.97, 199.41, 207.16. FT-IR ν_{max} (cm^{-1}): 3517, 3138 (OH), 2912, 2872, 2650, 2360, 2341, 1692, 1634, 1483, 1230, 1059, 737. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{24}\text{H}_{31}\text{NO}_4\text{S}_2$: 462.17673; found: 462.1766.

(10*R*,11*S*,13*S*)-11-hydroxy-10,13-dimethyl-1,6,7,8,9,10,11,12,13,14,15,16-dodecahydrodispiro[cyclopenta[*a*]phenanthrene-17,4'-[1,3]dioxolane-5',4''-[1,3]dioxolan]-3(2*H*)-one (2): Hydrocortisone (2.5 g, 4 mmol) and trioxane (3.1 g, 34.5

mmol) were added to round bottom flask and dissolved in 50 mL of chloroform. Concentrated HCl (5 mL) was added, and the reaction stirred vigorously at room temperature for 4 hours. The reaction was diluted with chloroform (100 mL) and washed with water, saturated sodium bicarbonate, and brine. The organic layer was dried with MgSO₄, filter, and concentrated *in vacuo* to yield a white powder. The crude product was purified using flash chromatography (75% diethyl ether/hexanes) and obtained as a white powder in 86% yield. TLC (Silica G w/UV254) 100% diethyl ether, R_f = 0.55. ¹H NMR (500 MHz, Chloroform-*d*) δ 5.67 (d, 1H), 5.22 (s, 1H), 5.06 – 5.00 (m, 3H), 4.42 (p, 1H), 3.99 (q, 2H), 2.52 – 2.42 (m, 2H), 2.35 (dt, 1H), 2.26 – 2.16 (m, 2H), 2.11 – 1.96 (m, 4H), 1.90 – 1.82 (m, 2H), 1.77 – 1.71 (m, 2H), 1.67 – 1.62 (m, 1H), 1.44 (s, 4H), 1.12 (s, 3H), 1.03 – 0.99 (m, 1H). HRMS (ESI) m/z: [M+H]⁺ Calcd for C₂₃H₃₂O₆: 405.2272; found: 405.2277.

(10*R*,11*S*,13*S*)-11-hydroxy-2-(hydroxymethylene)-10,13-dimethyl-1,6,7,8,9,10,11,12,13,14,15,16-dodecahydrodispiro[cyclopenta[*a*]phenanthrene-17,4'-[1,3]dioxolane-5',4''-[1,3]dioxolan]-3(2*H*)-one (3): Compound 2 (2.05 g, 5 mmol) and NaH (60% dispersed in mineral oil) (2.1 g, 52.5 mmol) were added to a round bottom flask and dissolved in dry toluene (40 mL). The reaction was allowed to stir for one hour before adding freshly distilled ethyl formate (4.1 mL, 40 mmol) via syringe. After stirring 24 hours, the reaction was slowly acidified with 4N HCl (50 mL) and extracted with DCM until no more color transfer was observed (20 mL X 5). The organic fractions were combined and concentrated *in vacuo*. The remaining residue was redissolved in DCM (20 mL) and extracted with 1N NaOH until no color transfer was observed (20 mL X 6). The combined aqueous fractions were acidified with conc. HCl and subsequently re-extracted with DCM (30 mL X 5). The organic fractions were washed with brine, dried with MgSO₄, filtered, and concentrated *in vacuo* to obtain a yellow solid in 89% yield. The product was pushed to the next step without purification. ¹H NMR (500 MHz, Chloroform-*d*) δ 7.37 (s, 1H), 5.73 (d, 1H), 5.22 (d, 1H), 5.04 – 5.01 (m, 3H), 3.99 (d, 2H), 2.52 – 2.42 (m, 3H), 2.34 – 2.24 (m, 2H), 2.00 – 1.96 (m, 3H), 1.87 – 1.84 (m, 1H), 1.77 – 1.73 (m, 3H), 1.67 –

1.65 (m, 1H), 1.46 – 1.38 (m, 3H), 1.33 (d, 3H), 1.26 (d, 1H), 1.12 (s, 4H). HRMS (ESI) m/z : $[M+H]^+$ Calcd for $C_{24}H_{32}O_7$: 433.2221; found: 433.2229.

(10a*R*,11*S*,12a*S*)-7-(4-fluorophenyl)-10a,12a-dimethyl-3,3a,3b,4,5,7,10,10a,10b,11,12,12a-dodecahydro-2*H*-dispiro[cyclopenta[5,6]naphtho[1,2-*f*]indazole-1,4'-[1,3]dioxolane-5',4''-[1,3]dioxolan]-11-ol (4):

4-fluorophenyl hydrazine hydrochloride (0.28 g, 1.7 mmol) and sodium acetate (0.14 g, 1.7 mmol) were stirred in 10 mL H_2O and 5 mL glacial acetic acid. Once dissolved, this mixture was added to compound **3** (0.50 g, 1.1 mmol) in a solution of glacial acetic acid (20 mL) and stirred for 24 hours. The reaction was diluted with 1N HCl (100 mL) and extracted with DCM (3 x 50 mL). The organic extracts were combined and washed with saturated sodium bicarbonate (50 mL), brine (50 mL), dried with $MgSO_4$, filtered, and concentrated *in vacuo* to obtain compound **4** as a brown solid. The crude compound was purified using flash chromatography (30% ethyl acetate/hexanes) from which the purified product was obtained as an orange solid in 63% yield. 1H NMR (500 MHz, Chloroform-*d*) δ 7.45 (ddd, 3H), 7.17 – 7.11 (m, 2H), 6.05 (d, 1H), 5.25 – 5.12 (m, 2H), 5.08 – 5.00 (m, 2H), 4.43 (dq, 1H), 4.07 – 3.86 (m, 2H), 2.99 (dd, 1H), 2.67 (d, 1H), 2.54 – 2.44 (m, 1H), 2.27 (ddt, 1H), 2.13 – 2.05 (m, 1H), 2.02 – 1.89 (m, 3H), 1.88 – 1.81 (m, 1H), 1.80 – 1.65 (m, 3H), 1.50 – 1.34 (m, 2H), 1.32 (d, 3H), 1.24 – 1.16 (m, 1H), 1.12 (d, 4H). HRMS (ESI) m/z : $[M+H]^+$ Calcd for $C_{30}H_{35}FN_2O_5$: 523.2603; found: 523.2612.

1-((1*R*,10a*R*,11*S*,12a*S*)-7-(4-fluorophenyl)-1,11-dihydroxy-10a,12a-dimethyl-1,2,3,3a,3b,4,5,7,10,10a,10b,11,12,12a-tetradecahydrocyclopenta[5,6]naphtho[1,2-*f*]indazol-1-yl)-2-hydroxyethan-1-one (5): Compound **4** (0.83 g, 1.6 mmol) was dissolved in 50% formic acid (50 mL) and gently refluxed for 40 minutes. The reaction was diluted with water (10 mL) and extracted with DCM (3 x 25 mL). The combined organic fractions were washed with saturated sodium bicarbonate (20 mL), brine (20 mL), dried with $MgSO_4$, filtered, and concentrated *in vacuo* to obtain a yellow solid. The crude compound was purified using flash chromatography (3% MeOH/DCM) from which the purified product was obtained as a tan solid in 47% yield. 1H NMR (500 MHz, Chloroform-

d) δ 7.45 (dd, 2H), 7.40 (s, 1H), 7.15 (t, 2H), 6.06 (s, 1H), 4.66 (dd, 1H), 4.53 (s, 1H), 4.32 (dd, 1H), 3.08 (t, 1H), 2.99 (d, 1H), 2.75 – 2.70 (m, 1H), 2.68 (d, 1H), 2.48 (d, 1H), 2.28 (d, 1H), 2.04 – 1.93 (m, 3H), 1.91 – 1.86 (m, 1H), 1.78 – 1.71 (m, 1H), 1.58 (d, 2H), 1.31 (s, 3H), 1.24 (d, 2H), 1.13 – 1.05 (m, 2H), 0.97 (s, 3H). HRMS (ESI) m/z : $[M+H]^+$ Calcd for $C_{28}H_{33}FN_2O_4$: 481.24971; found: 481.2499.

1-((1*R*,10*aR*,11*S*,12*aS*)-1,11-dihydroxy-10*a*,12*a*-dimethyl-7-phenyl-1,2,3,3*a*,3*b*,4,5,7,10,10*a*,10*b*,11,12,12*a*-tetradecahydrocyclopenta[5,6]naphtho[1,2-*f*]indazol-1-yl)-2-hydroxyethan-1-one (6): Phenyl hydrazine hydrochloride (0.28 mL, 2.7 mmol) and sodium acetate (0.23 g, 2.7 mmol) were stirred in 10 mL H_2O and 5 mL glacial acetic acid. Once dissolved, this mixture was added to compound **3** (0.8 g, 1.8 mmol) in a solution of glacial acetic acid (20 mL) and stirred for 24 hours. The reaction was diluted with 1N HCl (100 mL) and extracted with DCM (3 x 50 mL). The organic extracts were combined and washed with saturated sodium bicarbonate (50 mL), brine (50 mL), dried with $MgSO_4$, filtered, and concentrated *in vacuo* to obtain the crude product as a brown solid. The crude compound was purified using flash chromatography (30% ethyl acetate/hexanes) from which the purified product was obtained as an orange solid in 57% yield.

The purified product was dissolved in 50% formic acid (50 mL) and gently refluxed for 40 minutes. The reaction was diluted with water (10 mL) and extracted with DCM (3 x 25 mL). The combined organic fractions were washed with saturated sodium bicarbonate (20 mL), brine (20 mL), dried with $MgSO_4$, filtered, and concentrated *in vacuo* to obtain a yellow solid. The crude compound was purified using flash chromatography (3% MeOH/DCM) from which the purified product was obtained as a tan solid in 31% yield. 1H NMR (500 MHz, Chloroform-*d*) δ 7.52 – 7.29 (m, 6H), 6.14 (d, 1H), 4.66 (d, 1H), 4.54 (s, 1H), 4.32 (d, 1H), 3.15 – 2.90 (m, 2H), 2.78 – 2.66 (m, 2H), 2.49 (t, 1H), 2.28 (d, 1H), 2.04 – 1.83 (m, 4H), 1.79 – 1.71 (m, 1H), 1.58 (d, 1H), 1.42 (s, 1H), 1.31 (d, 3H), 1.26 (t, 3H), 1.16 – 1.05 (m, 2H), 0.94 (d, 3H). HRMS (ESI) m/z : $[M+H]^+$ Calcd for $C_{28}H_{34}N_2O_4$: 463.2591; found: 463.2607.

General synthesis for compound 7a-e: Synthesis for compounds **7a-e** was performed following a previously published method.⁵⁷ Briefly, compound **5** (2 mmol) was dissolved in 100 mL dichloromethane (DCM) and cooled to 0°C. Diisopropylethylamine (DIPEA) (1.9 mmol) was added via syringe followed by addition of mesylchloride (MsCl) (3 mmol). The reaction warmed to room temperature and was allowed to stir overnight (~16 hours). The reaction was concentrated *in vacuo*, before being redissolved in DCM (50 mL) and washed with saturated bicarbonate. The aqueous fraction was re-extracted with DCM (20 mL), combined organic fractions were washed twice with brine, dried with MgSO₄, filters, and concentrated *in vacuo* to yield a tan solid. Mesylated aryl pyrazole hydrocortisone (0.5 mmol) was dissolved in acetone to which potassium carbonate (5 mmol) and the appropriate merapto- reagent (1 mmol) was added. The reaction was refluxed for 1 hour, filtered through a short pad of silica, and concentrated *in vacuo*.

2-(benzo[d]thiazol-2-ylthio)-1-((1*R*,10*aR*,11*S*,12*aS*)-7-(4-fluorophenyl)-1,11-dihydroxy-10*a*,12*a*-dimethyl-1,2,3,3*a*,3*b*,4,5,7,10,10*a*,10*b*,11,12,12*a*-tetradecahydrocyclopenta[5,6]naphtho[1,2-*f*]indazol-1-yl)ethan-1-one (7a): Using commercially available mercaptobenzothiazole, compound **7a** was obtain as a white solid in 35% yield. TLC (Silica G w/UV254) 50% EtOAc in hexanes, *R_f* = 0.41; ¹H NMR (500 MHz, Chloroform-*d*) δ 7.75 (dd, 2H), 7.49 – 7.43 (m, 2H), 7.40 (d, 2H), 7.33 – 7.28 (m, 1H), 7.16 (t, 2H), 6.07 (s, 1H), 5.96 (s, 1H), 5.01 (d, 1H), 4.56 (s, 1H), 3.26 (d, 1H), 3.00 (d, 1H), 2.85 (t, 1H), 2.70 (d, 1H), 2.50 (t, 1H), 2.40 (d, 1H), 2.30 (d, 1H), 2.04 – 1.90 (m, 4H), 1.68 (d, 1H), 1.61 – 1.57 (m, 1H), 1.48 – 1.40 (m, 1H), 1.33 (s, 4H), 1.17 (d, 2H), 0.98 (s, 3H). HRMS (ESI) *m/z*: [M+H]⁺ Calcd for C₃₅H₃₆FN₃O₃S₂: 630.22549; found: 630.2253.

2-((1*H*-benzo[d]imidazol-2-yl)thio)-1-((1*R*,10*aR*,11*S*,12*aS*)-7-(4-fluorophenyl)-1,11-dihydroxy-10*a*,12*a*-dimethyl-1,2,3,3*a*,3*b*,4,5,7,10,10*a*,10*b*,11,12,12*a*-tetradecahydrocyclopenta[5,6]naphtho[1,2-*f*]indazol-1-yl)ethan-1-one (7b): Using commercially available mercaptobenzimidazole, compound **7b** was obtain as an off white solid in 16% yield. Mp 225.8 - 226.9°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, *R_f* = 0.13; [α]_D²⁰ = 80.4 (c 1.0, MeOH); ¹H

NMR (500 MHz, Chloroform-*d*) δ 9.11 (s, 1H), 7.48 – 7.45 (m, 2H), 7.42 (s, 1H), 7.20 – 7.12 (m, 4H), 6.07 (d, 1H), 5.02 (d, 1H), 4.56 (s, 1H), 3.04 (dd, 2H), 2.81 (t, 1H), 2.70 (d, 1H), 2.48 (dd, 2H), 2.30 (d, 1H), 2.05 – 1.89 (m, 4H), 1.66 – 1.52 (m, 3H), 1.43 (d, 1H), 1.33 (s, 5H), 1.27 – 1.07 (m, 3H), 0.96 (s, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 17.97, 22.40, 24.00, 31.84, 32.13, 32.15, 34.07, 34.16, 34.76, 40.87, 41.40, 46.65, 52.46, 56.65, 60.39, 68.73, 76.74, 76.99, 77.25, 91.05, 107.55, 113.79, 115.92, 116.10, 123.52, 125.39, 125.46, 135.87, 136.95, 137.89, 149.83, 151.82, 160.47, 162.43, 208.76. FT-IR ν_{max} (cm^{-1}): 3190 (OH), 2932 (w), 2356, 2325, 1705, 1615, 1513, 1439, 1223, 1109, 837. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{35}\text{H}_{37}\text{FN}_4\text{O}_3\text{S}$: 613.26432; found: 613.2646.

2-(benzo[*d*]oxazol-2-ylthio)-1-((1*R*,10*aR*,11*S*,12*aS*)-7-(4-fluorophenyl)-1,11-dihydroxy-10*a*,12*a*-dimethyl-1,2,3,3*a*,3*b*,4,5,7,10,10*a*,10*b*,11,12,12*a*-tetradecahydrocyclopenta[5,6]naphtho[1,2-*f*]indazol-1-yl)ethan-1-one (7c): Using commercially available mercaptobenzoxazole, compound **7c** was obtained as an off white solid in 44% yield. Mp 186.9 - 188.2°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, R_f = 0.42; $[\alpha]_{\text{D}}^{20}$ = 123.0 (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.51 (d, 1H), 7.46 (s, 3H), 7.41 (s, 1H), 7.27 (s, 2H), 7.16 (d, 2H), 6.07 (s, 1H), 5.80 (s, 1H), 4.96 (d, 1H), 4.56 (s, 1H), 3.29 (d, 1H), 3.00 (d, 1H), 2.87 – 2.81 (m, 1H), 2.70 (d, 1H), 2.51 (d, 1H), 2.38 (d, 1H), 2.31 (s, 1H), 2.05 – 1.96 (m, 3H), 1.93 (d, 1H), 1.68 (d, 1H), 1.57 (t, 1H), 1.49 – 1.41 (m, 1H), 1.32 (s, 4H), 1.18 (s, 1H), 1.12 (s, 1H), 0.98 (s, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 17.85, 22.40, 23.92, 31.80, 32.11, 32.18, 34.07, 34.63, 35.48, 40.69, 41.41, 46.88, 52.42, 56.69, 68.63, 76.78, 76.99, 77.20, 89.82, 90.95, 107.62, 110.26, 113.76, 115.92, 116.07, 117.75, 124.49, 124.70, 125.36, 125.42, 130.83, 136.85, 137.90, 140.57, 151.59, 152.05, 165.29, 206.58. FT-IR ν_{max} (cm^{-1}): 3309 (OH), 2919, 2360, 2322, 1705, 1615, 1513, 1452, 1218, 1130, 836. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{35}\text{H}_{36}\text{FN}_3\text{O}_4\text{S}$: 614.24833; found: 614.2481.

2-((1*H*-imidazol-2-yl)thio)-1-((1*R*,10*aR*,11*S*,12*aS*)-7-(4-fluorophenyl)-1,11-dihydroxy-10*a*,12*a*-dimethyl-1,2,3,3*a*,3*b*,4,5,7,10,10*a*,10*b*,11,12,12*a*-tetradecahydrocyclopenta[5,6]naphtho[1,2-*f*]indazol-1-yl)ethan-1-one (7d): Using commercially available mercapimidazole, compound **7d** was obtained as a white solid in 56%

yield. Mp 188.4 - 190.1°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.04$; $[\alpha]_D^{20} = 96.6$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 9.00 (s, 1H), 7.46 (dd, 2H), 7.41 (s, 1H), 7.17 – 7.13 (m, 2H), 6.95 (s, 2H), 6.06 (d, 1H), 4.77 (d, 1H), 4.53 (s, 1H), 3.06 (d, 1H), 2.99 (d, 1H), 2.79 (t, 1H), 2.69 (d, 1H), 2.53 – 2.45 (m, 1H), 2.38 (d, 1H), 2.28 (d, 1H), 2.00 – 1.86 (m, 4H), 1.59 (d, 1H), 1.57 – 1.50 (m, 2H), 1.44 – 1.37 (m, 1H), 1.31 (s, 3H), 1.17 – 1.09 (m, 2H), 0.92 (s, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 17.91, 22.37, 23.87, 32.15, 34.36, 35.05, 40.79, 41.39, 46.69, 52.37, 56.68, 68.67, 76.74, 76.99, 77.25, 90.74, 107.55, 113.79, 116.08, 125.36, 125.43, 136.89, 137.89, 140.73, 151.73, 208.99. FT-IR ν_{max} (cm^{-1}): 3307 (OH), 2922, 2360, 2323, 1705, 1615, 1513, 1220, 1109, 836. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{31}\text{H}_{34}\text{FN}_3\text{O}_3\text{S}_2$: 563.24867; found: 563.2489.

1-((1*R*,10*aR*,11*S*,12*aS*)-7-(4-fluorophenyl)-1,11-dihydroxy-10*a*,12*a*-dimethyl-1,2,3,3*a*,3*b*,4,5,7,10,10*a*,10*b*,11,12,12*a*-tetradecahydrocyclopenta[5,6]naphtho[1,2-*f*]indazol-1-yl)-2-(thiazol-2-ylthio)ethan-1-one (7e): Using commercially available mercaptothiazole, compound **7e** was obtained as a white solid in 29% yield. Mp 138.7 - 140.5°C; TLC (Silica G w/UV254) 50% EtOAc in hexanes, $R_f = 0.25$; $[\alpha]_D^{20} = 113.0$ (c 1.0, MeOH); ^1H NMR (500 MHz, Chloroform-*d*) δ 7.50 (d, 1H), 7.46 (dd, 2H), 7.41 (s, 1H), 7.20 (d, 1H), 7.15 (t, 2H), 6.06 (s, 1H), 5.89 (s, 1H), 4.88 (d, 1H), 4.53 (s, 1H), 3.23 (d, 1H), 2.99 (d, 1H), 2.85 – 2.78 (m, 1H), 2.69 (d, 1H), 2.54 – 2.46 (m, 1H), 2.35 (d, 1H), 2.28 (d, 1H), 2.02 – 1.87 (m, 4H), 1.64 (d, 1H), 1.51 (d, 1H), 1.47 – 1.39 (m, 1H), 1.32 (s, 3H), 1.30 – 1.20 (m, 2H), 1.19 – 1.12 (m, 1H), 1.10 (d, 1H), 0.95 (s, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 17.90, 22.38, 23.86, 32.18, 34.06, 34.40, 36.52, 40.69, 41.40, 46.78, 52.37, 56.69, 68.63, 76.74, 76.99, 77.24, 90.99, 107.60, 113.77, 116.07, 119.28, 125.34, 125.41, 136.84, 137.90, 141.08, 151.60, 160.44, 165.28, 207.16. FT-IR ν_{max} (cm^{-1}): 3318 (OH), 2920, 2358, 2339, 1705, 1615, 1513, 1222, 1109, 836. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{31}\text{H}_{35}\text{FN}_4\text{O}_3\text{S}$: 580.20984; found: 580.2097.

APPENDIX

3.9.1 – ^1H and ^{13}C NMR Spectra

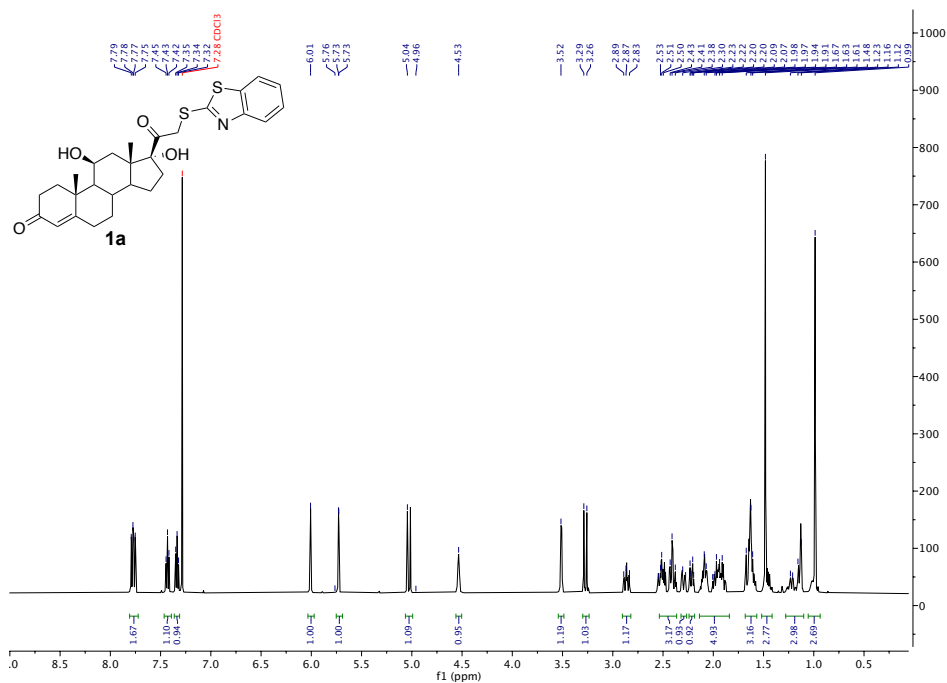


Figure 3.9.1 – ^1H NMR of **1a**

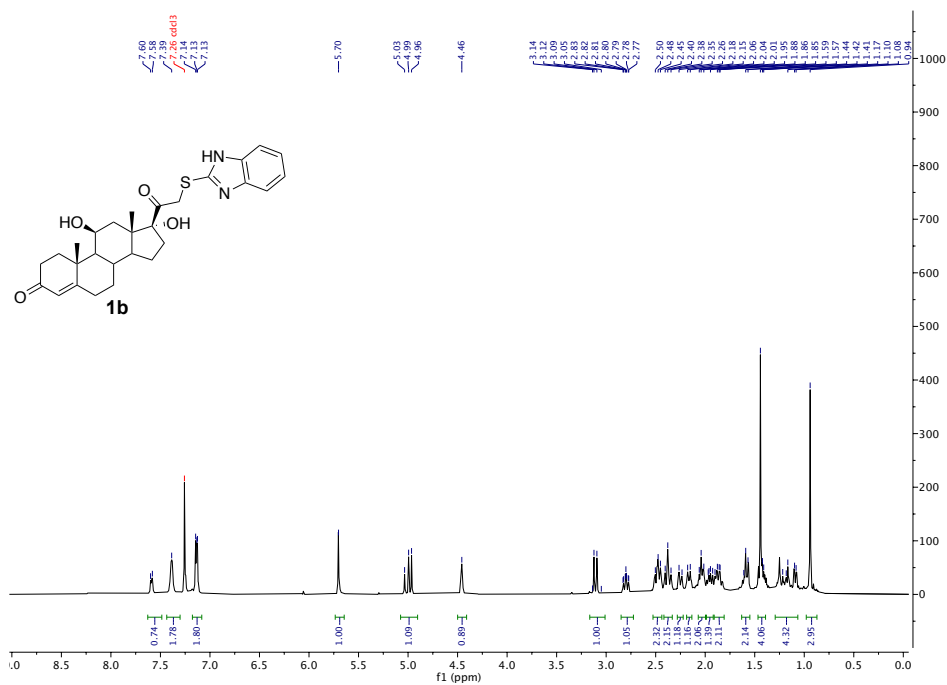


Figure 3.9.2 – ^1H NMR of **1b**

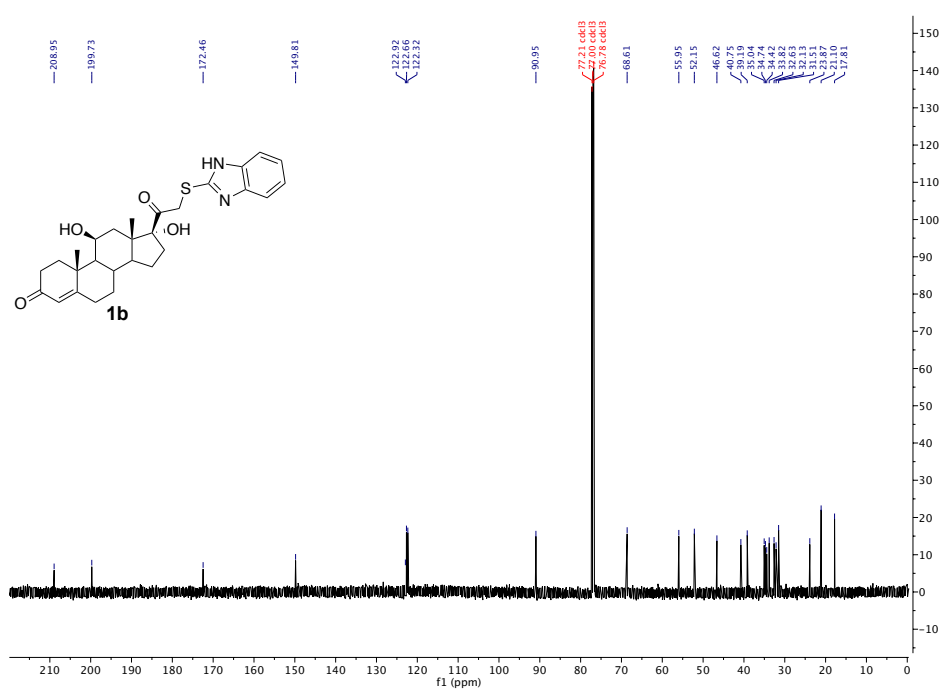


Figure 3.9.3 – ^{13}C NMR of **1b**

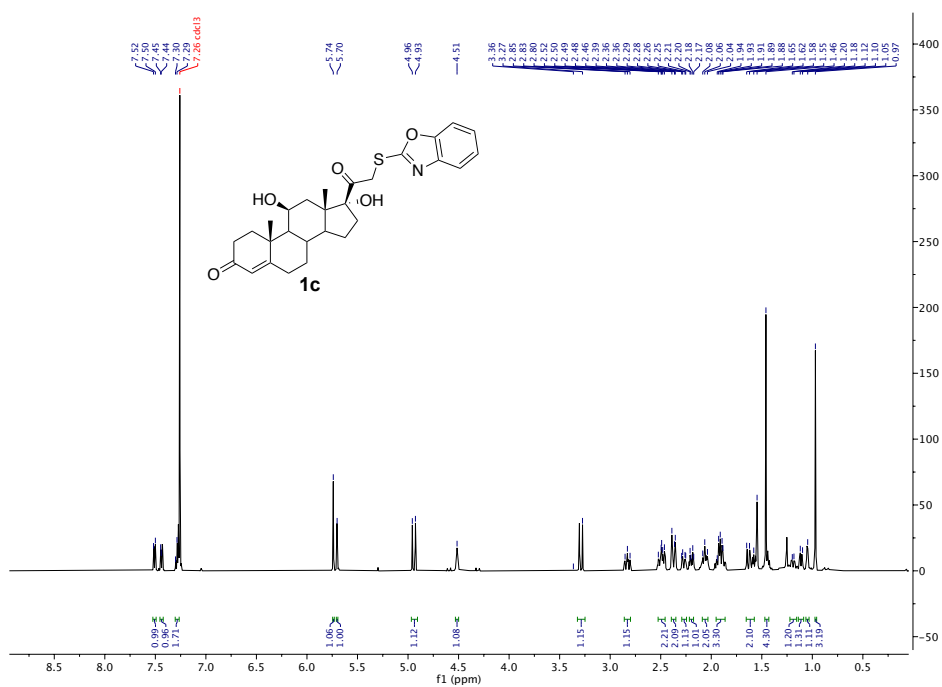
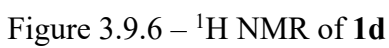
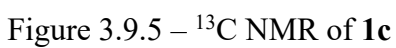


Figure 3.9.4 – ^1H NMR of **1c**



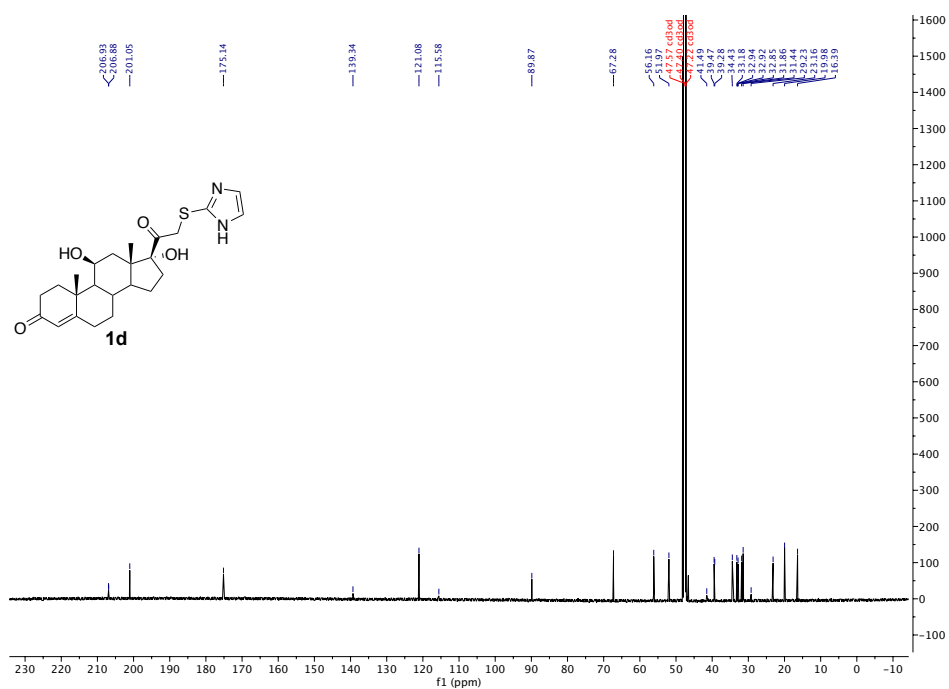


Figure 3.9.7 – ^{13}C NMR of **1d**

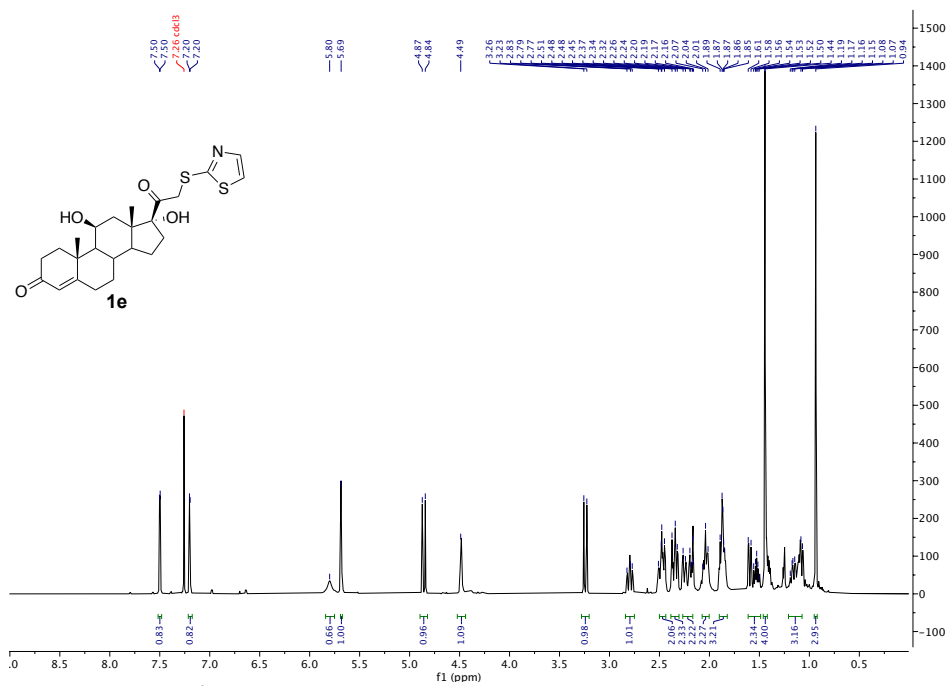


Figure 3.9.8 – ^1H NMR of **1e**

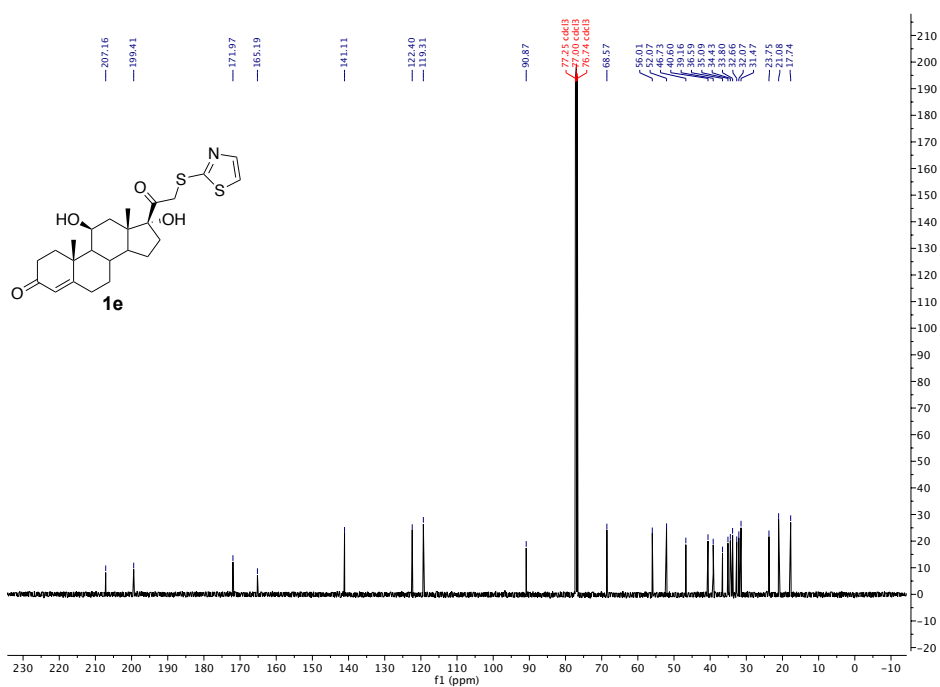


Figure 3.9.9 – ¹³C NMR of **1e**

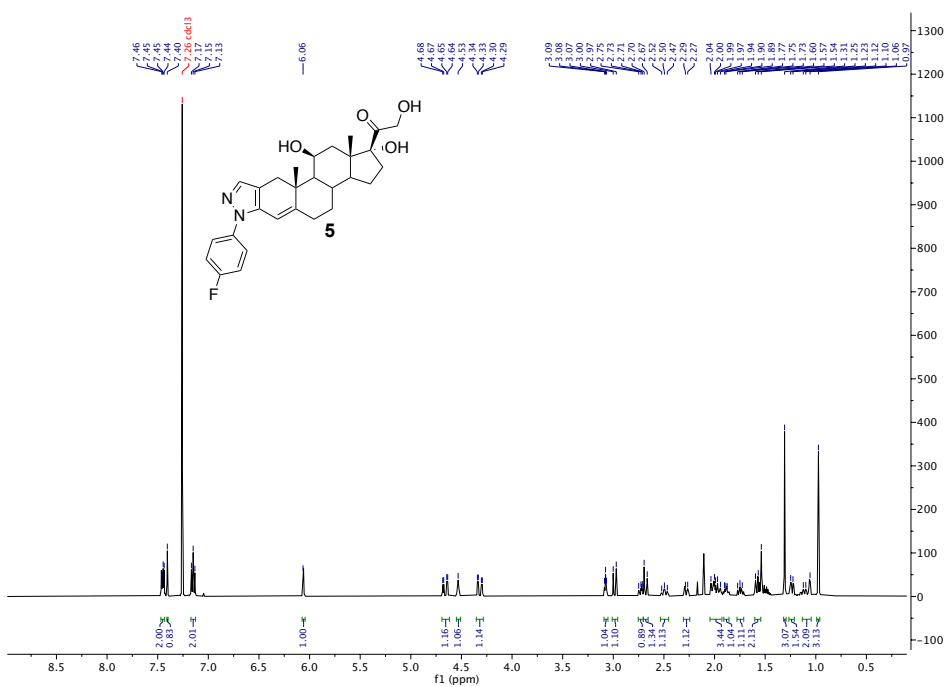


Figure 3.9.10 – ¹H NMR of **5**

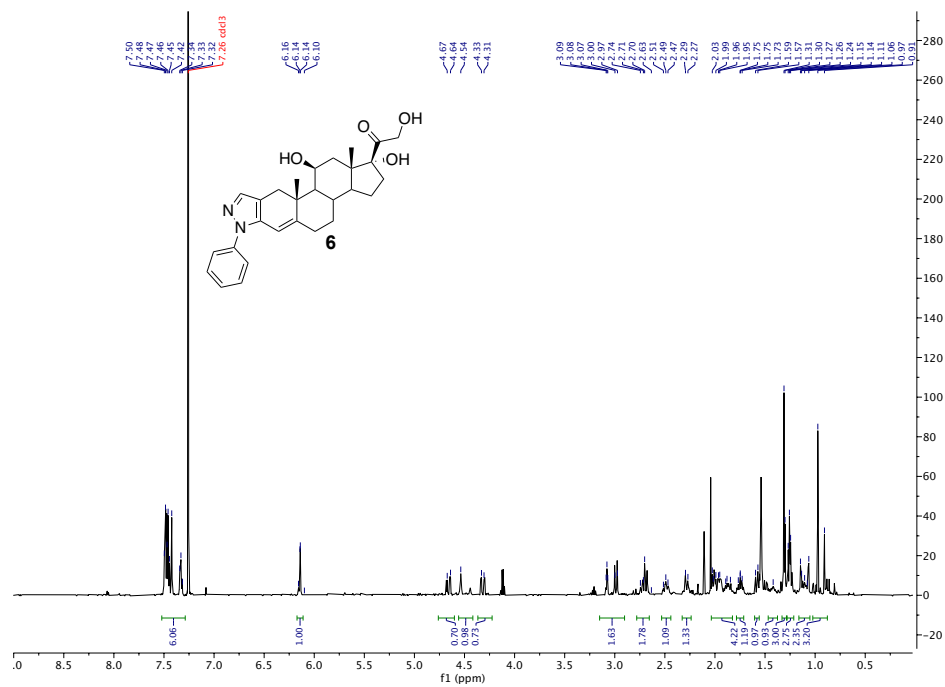


Figure 3.9.11 – ^1H NMR of **6**

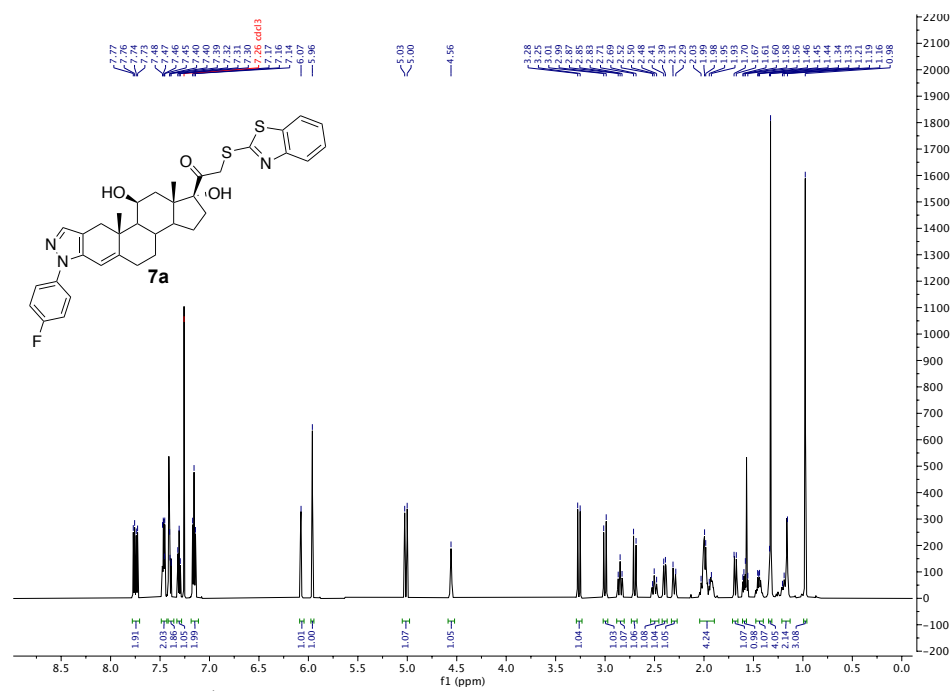


Figure 3.9.12 – ^1H NMR of **7a**

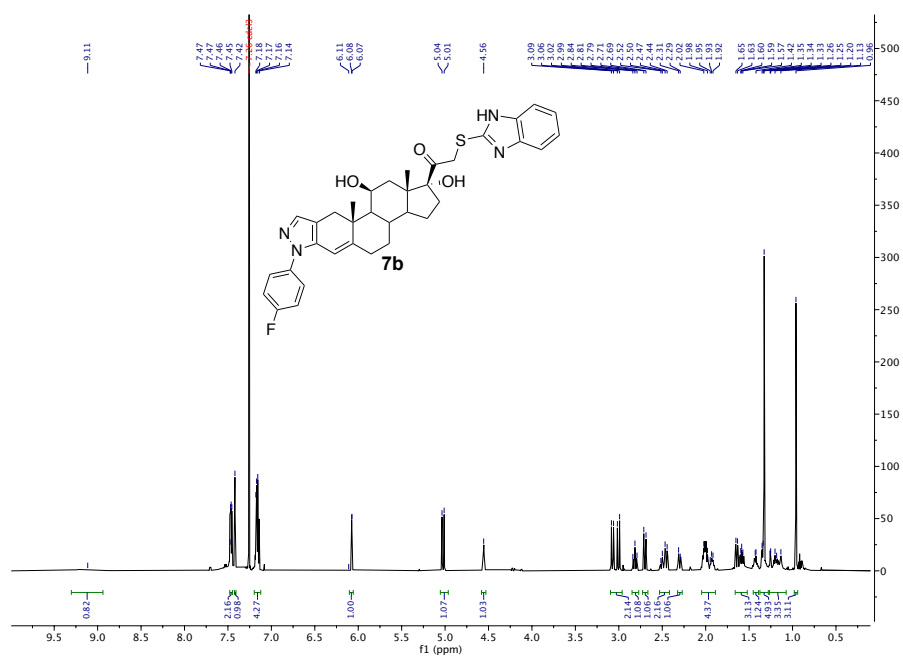


Figure 3.9.13 – ¹H NMR of **7b**

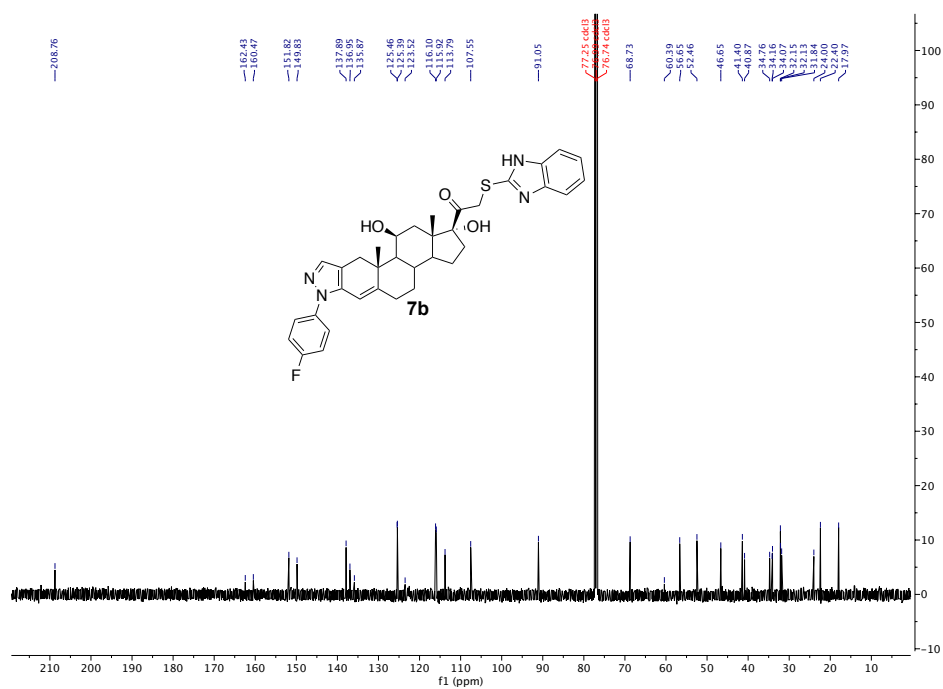


Figure 3.9.14 – ¹³C NMR of **7b**

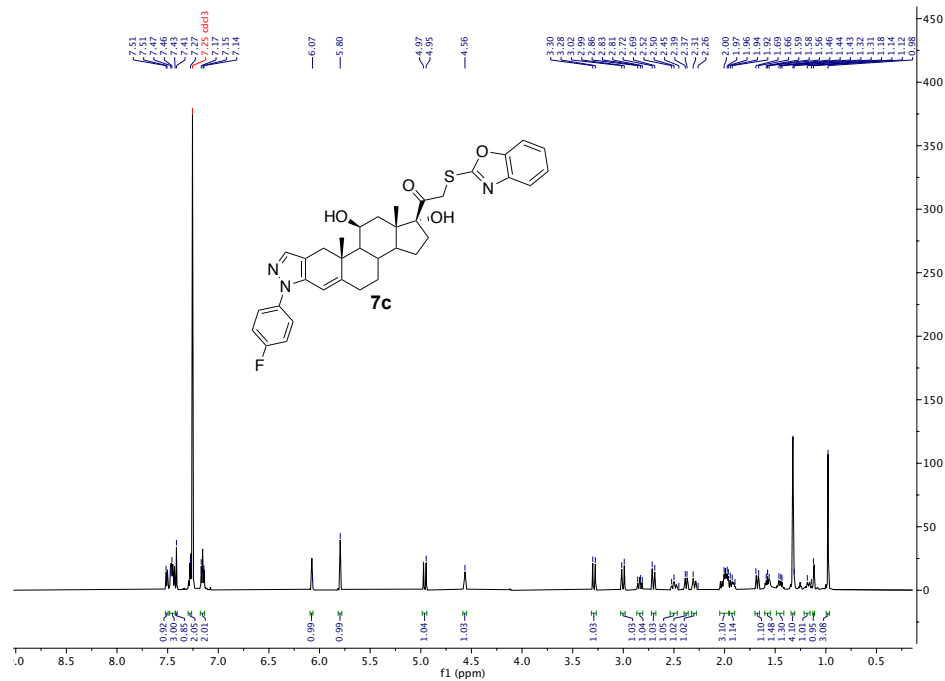


Figure 3.9.15 – ¹H NMR of **7c**

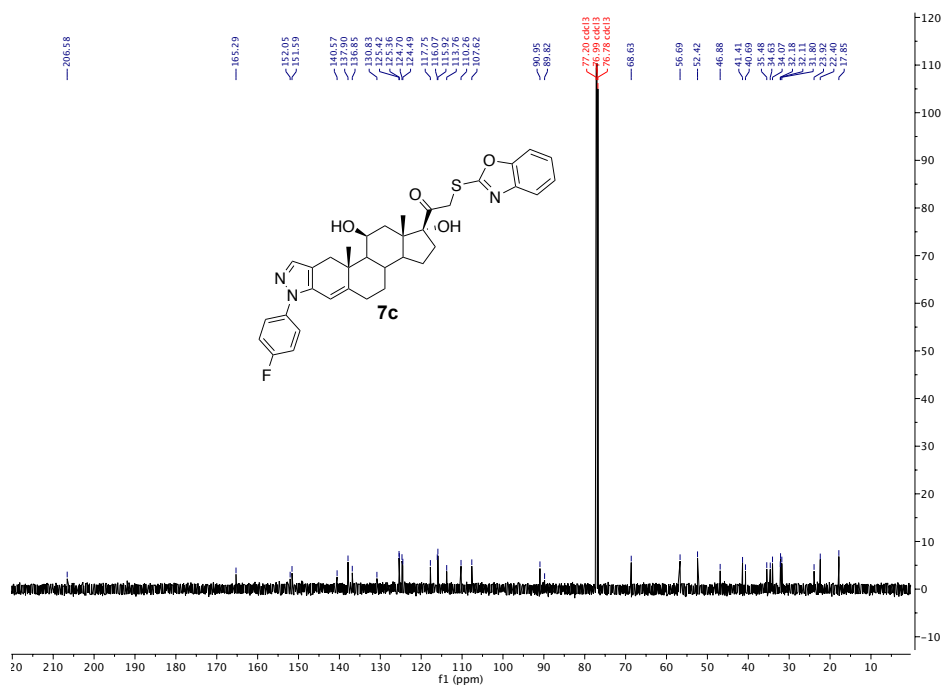


Figure 3.9.16 – ¹³C NMR of **7c**

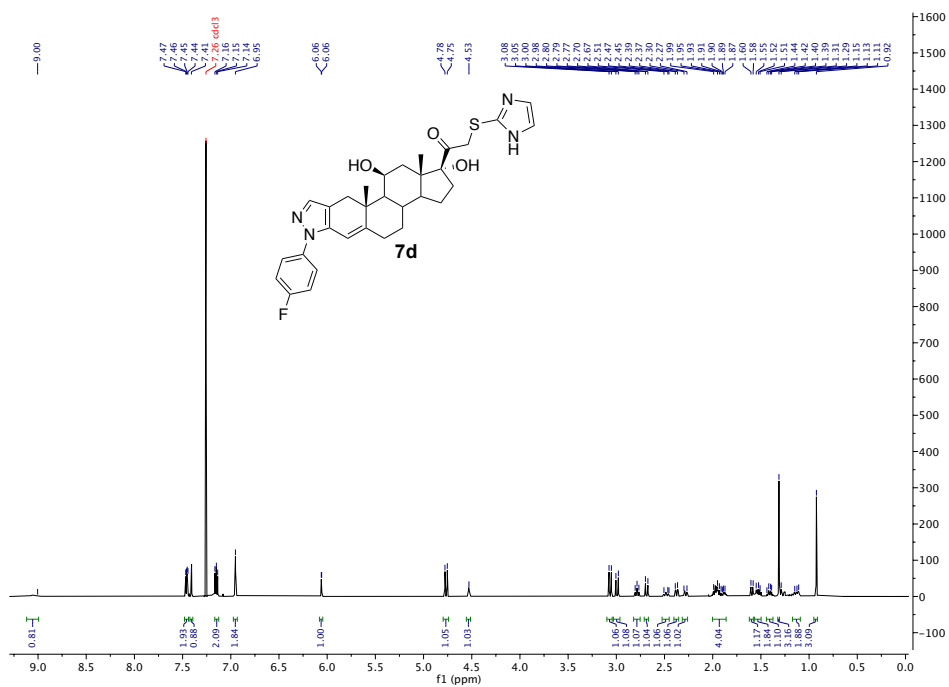


Figure 3.9.17 – ¹H NMR of **7d**

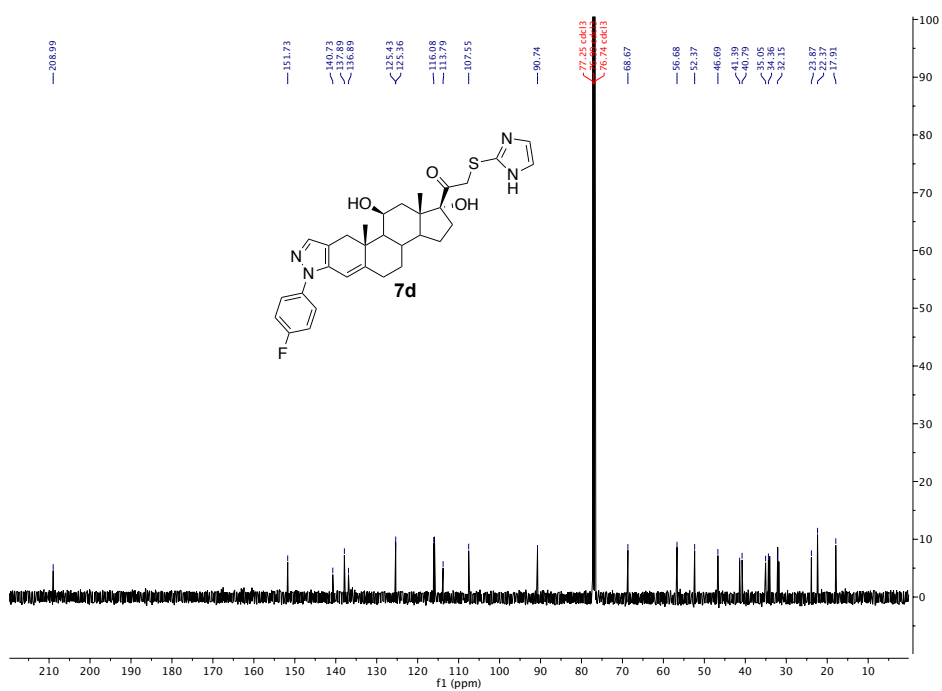


Figure 3.9.18 – ¹³C NMR of **7d**

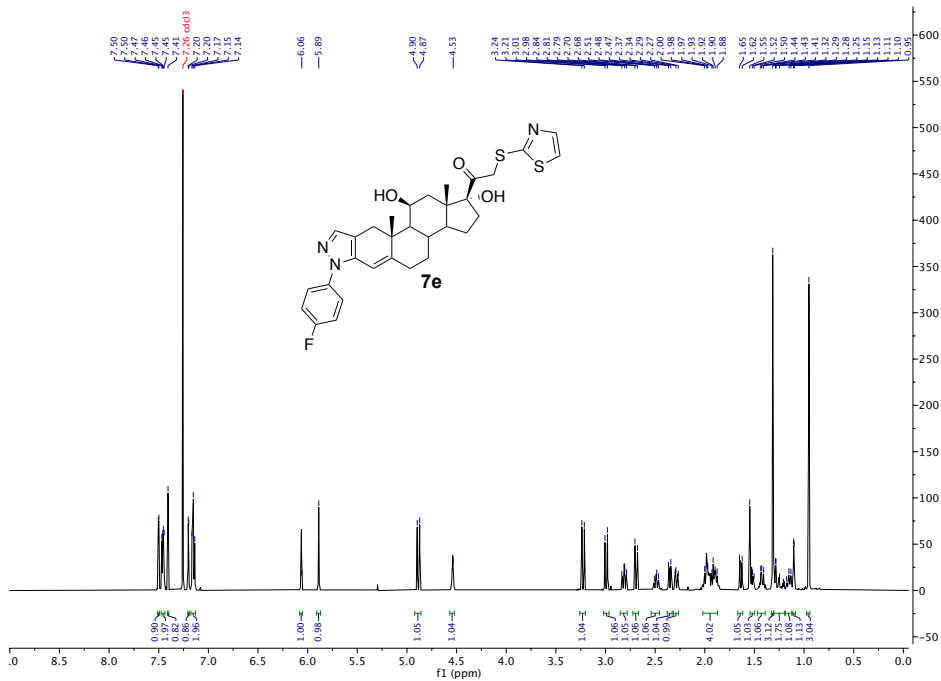


Figure 3.9.19 – ¹H NMR of **7e**

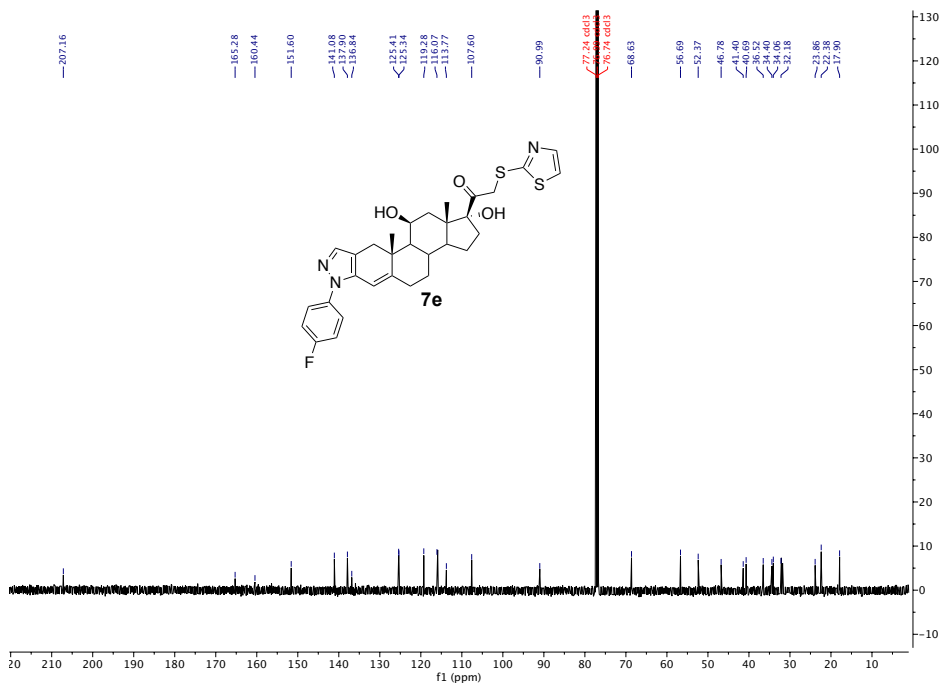


Figure 3.9.20 – ¹³C NMR of **7e**

3.9.2 – CCL2 Data

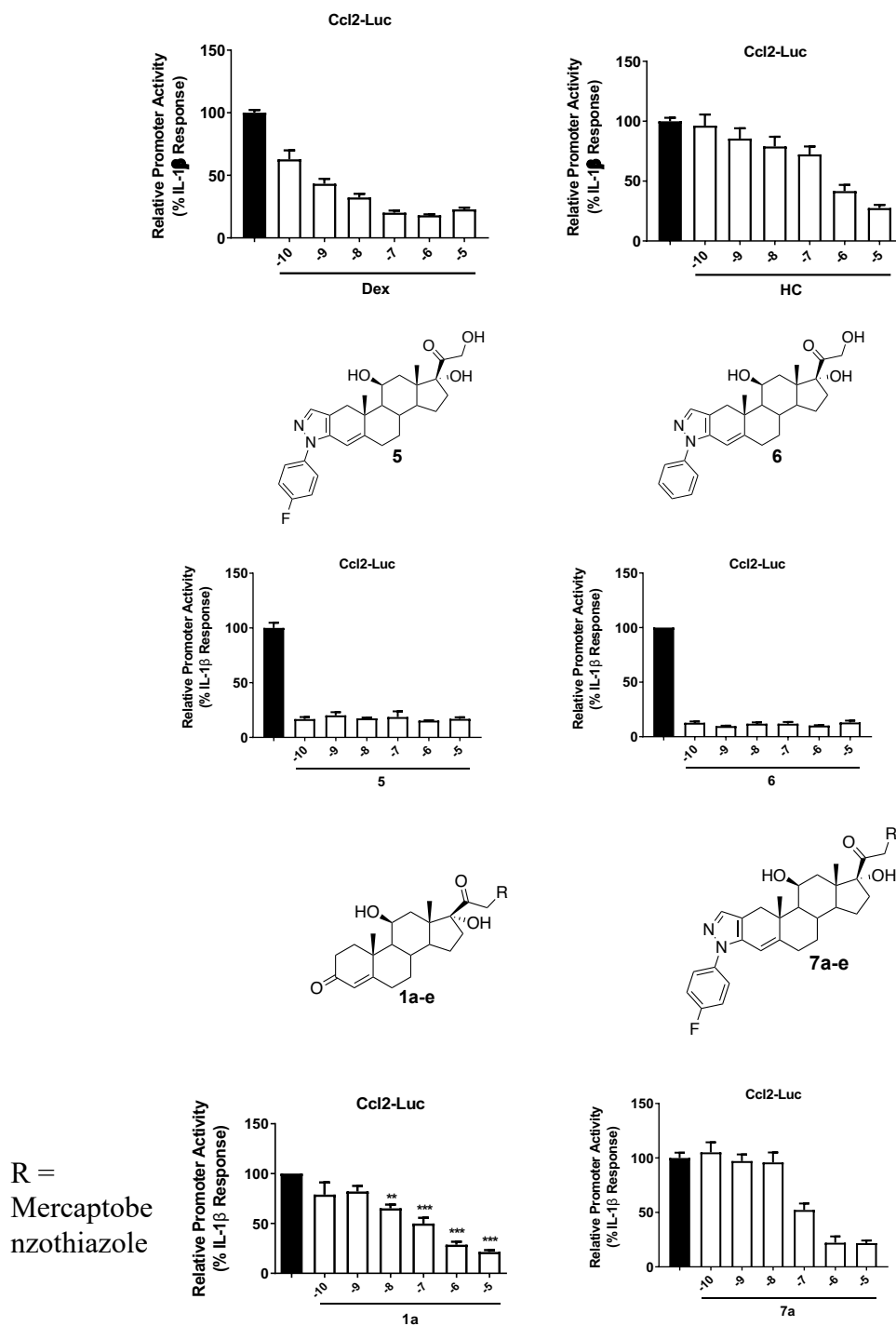
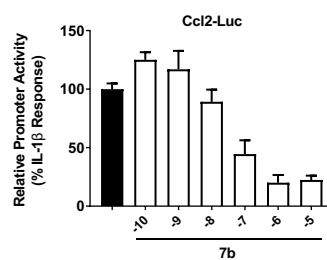
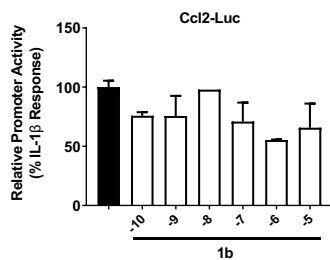
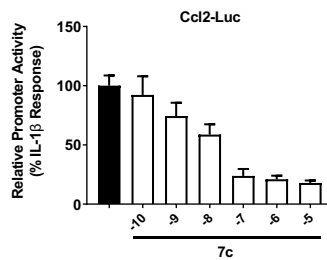
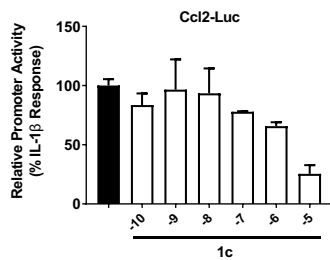


Figure 3.9.21 – CCL2 Data Analysis

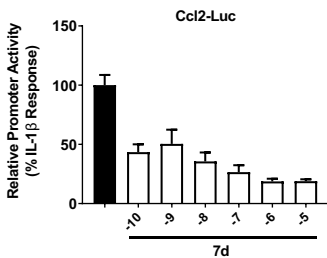
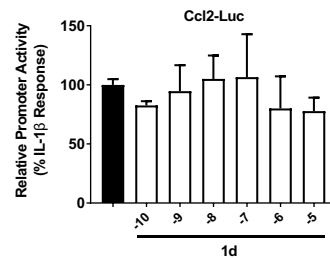
R =
Mercaptobenzimidazole



R =
Mercaptobenzoxazole



R =
Mercaptobenzimidazole



R =
Mercaptobenzthiazole

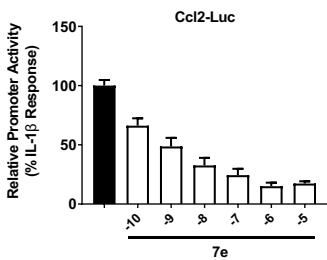
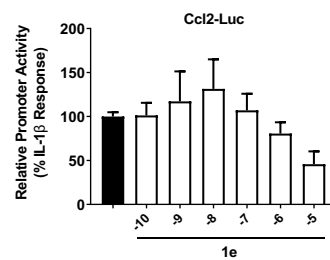


Figure 3.9.21 – Continued

3.9.3 – 3xGRE Data

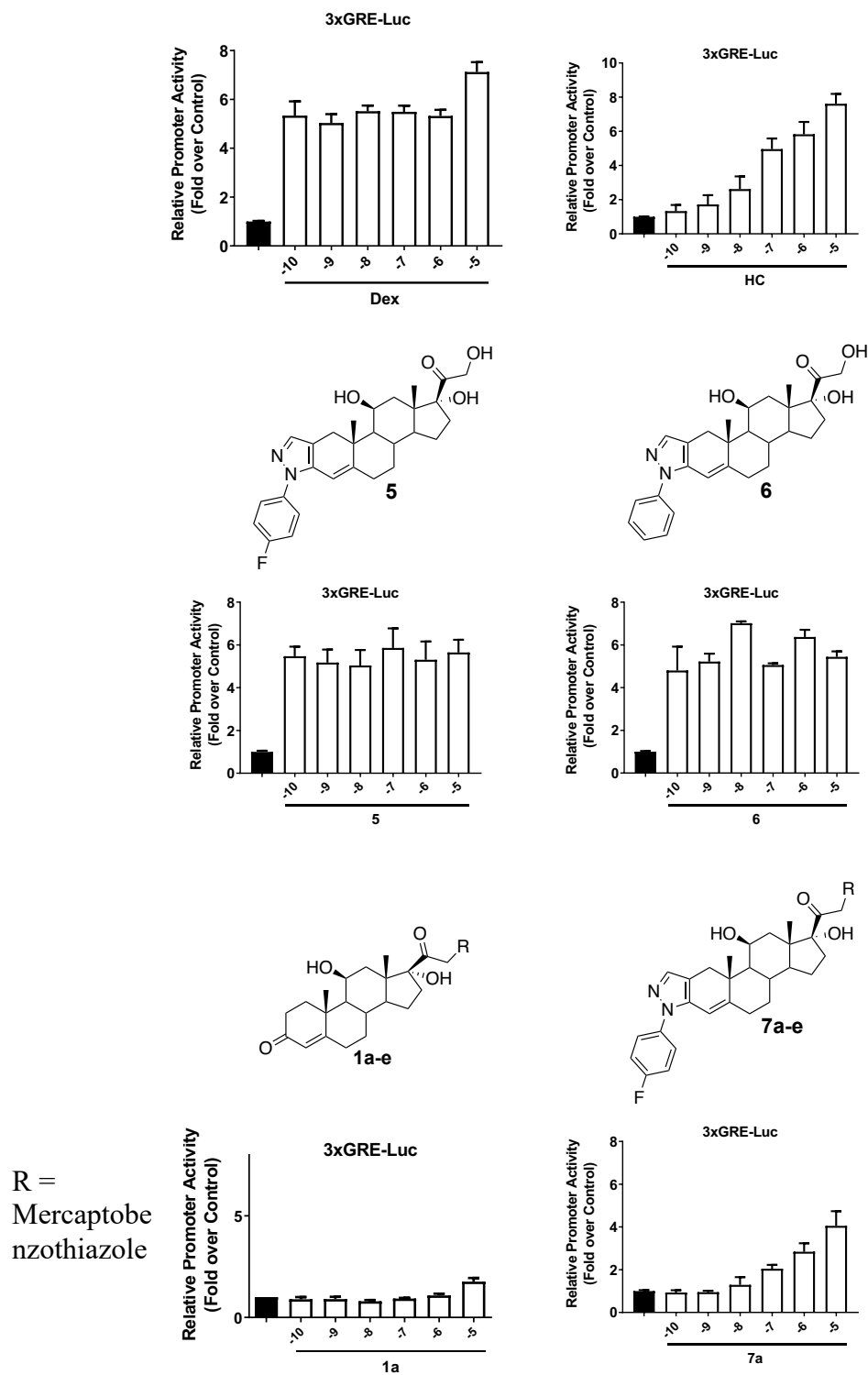
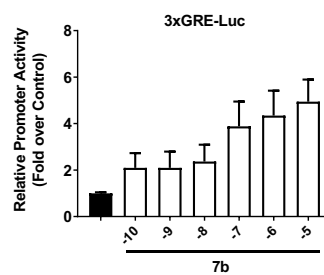
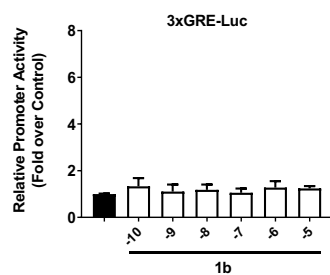
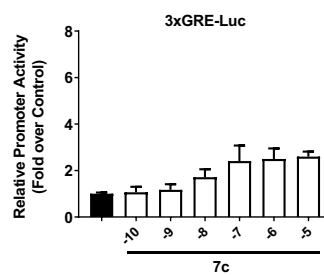
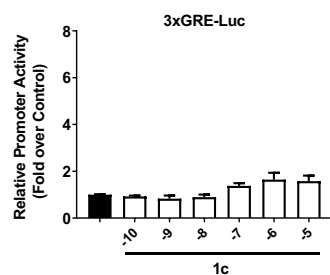


Figure 3.9.22 – 3xGRE Data Analysis

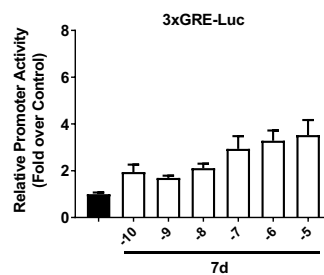
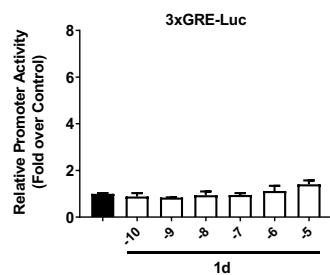
R =
Mercaptobenzimidazole



R =
Mercaptobenzoxazole



R =
Mercaptobenzimidazole



R =
Mercaptobenzthiazole

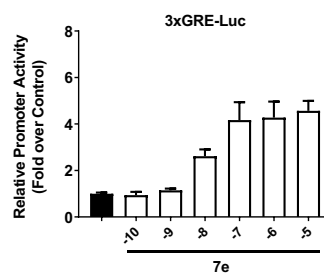
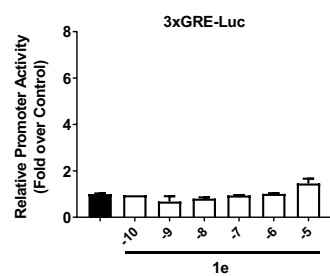
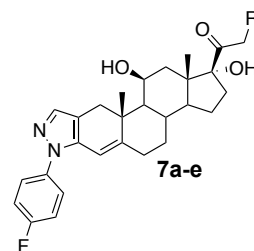
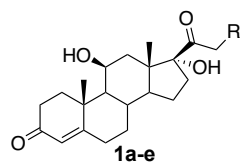
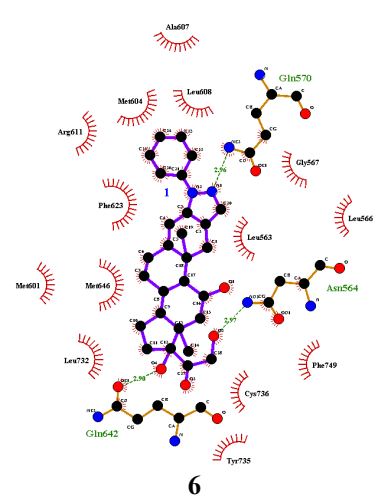
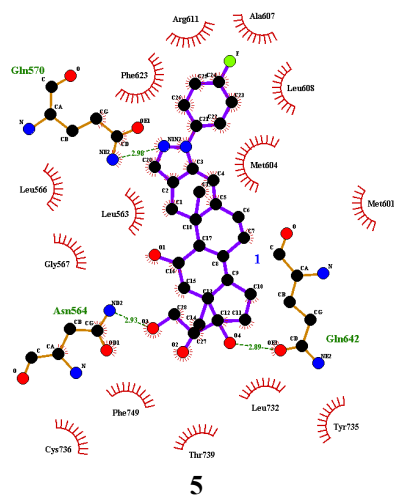


Figure 3.9.22 – Continued

3.9.4 – Ligplot Analysis in 3BQD

R Group

OH



Mercaptobenzo-
thiazole

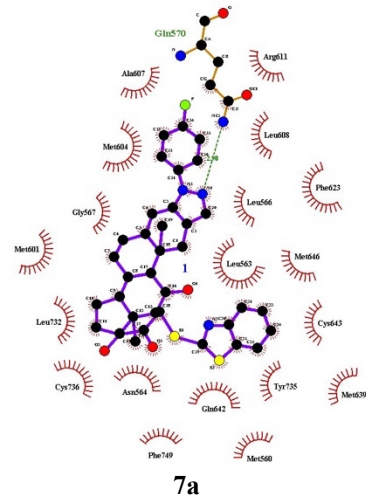
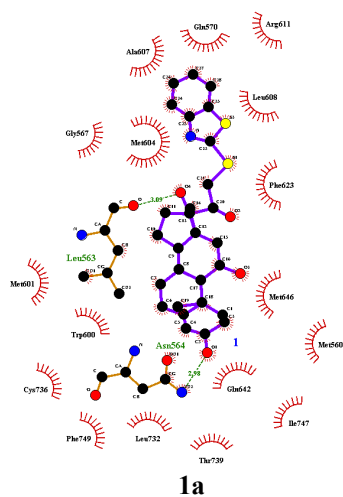
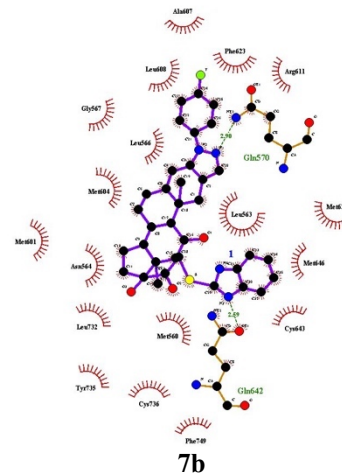
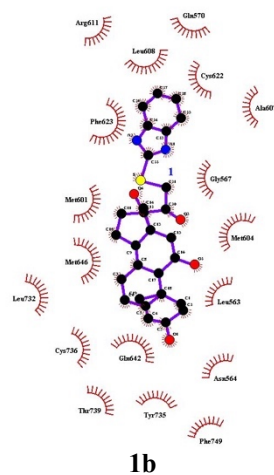
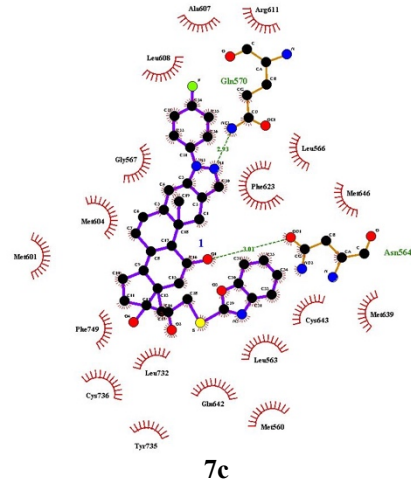
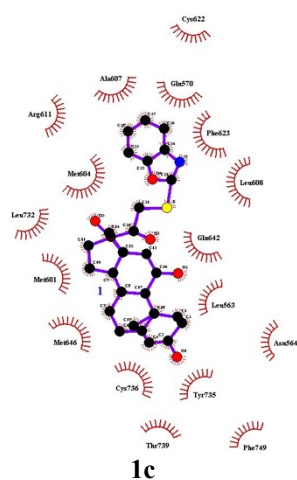


Figure 3.9.23 – Ligplot Analysis in 3BQD

Mercaptobenz-
imidazole



Mercaptobenz-
oxazole



Mercaptoimidazole

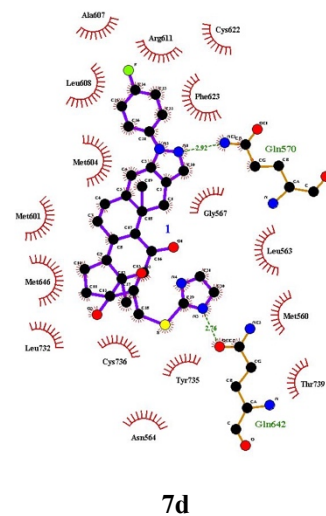
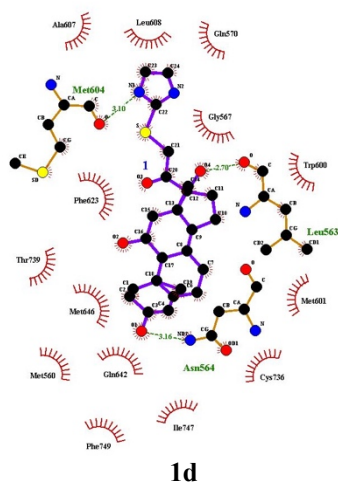
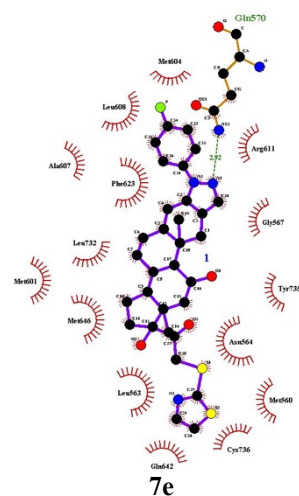
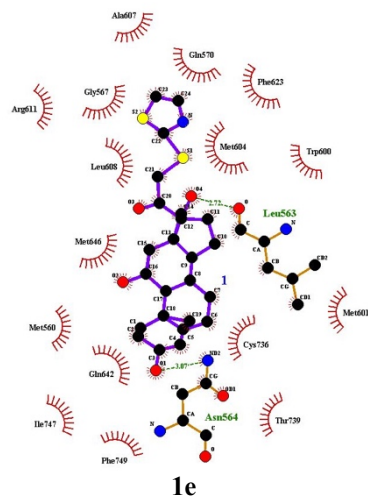
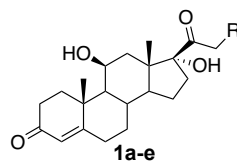


Figure 3.9.23 – Continued

Mercaptothiazole



3.9.5 – Ligplot Analysis in 5NFP



R Group	Relative Binding Affinity
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Mercaptobenzo-
thiazole

-7.0 kcal/mol

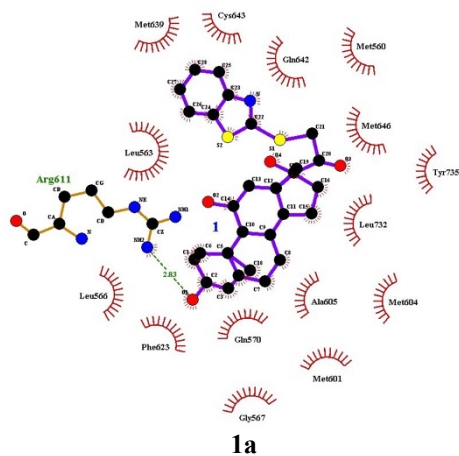
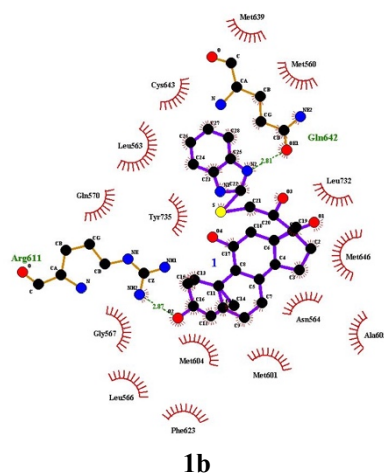


Figure 3.9.24 – 5NFP Ligplot Analysis

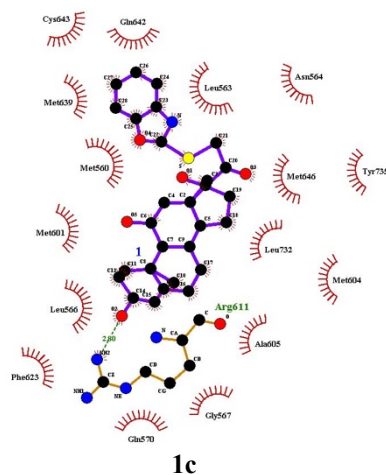
Mercaptobenz-
imidazole

-8.3 kcal/mol



Mercaptobenz-
oxazole

-7.8 kcal/mol



Mercaptoimidazole

-9.3 kcal/mol

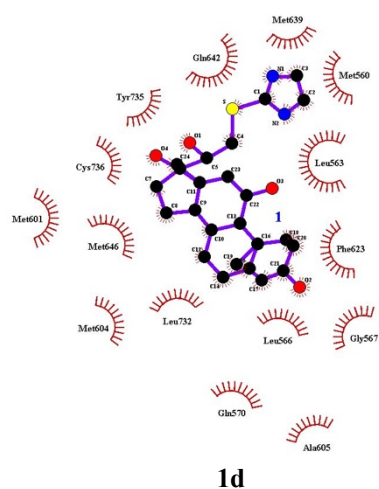


Figure 3.9.24 – Continued

Mercaptothiazole

-8.4 kcal/mol

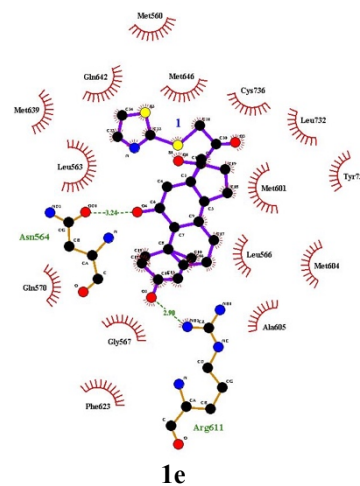


Figure 3.9.24 – Continued

CONCLUSION

The search for an improved glucocorticoid (GC) treatment remains a steep uphill battle. While complex biological mechanisms, multifactorial transcription activity, a widely accommodating ligand binding pocket, and intricate ligand-receptor interactions all makes for a grueling challenge, it is a necessary one. Millions of people around the globe would benefit from improved treatment options and so, any step toward uncovering the enigmatic mechanisms of the GR-GC system, is a step forward toward the development of GCs with improved therapeutic profiles. This work investigates a wide range of aryl pyrazole glucocorticoid receptor agonists (APGRAs), uncovering unique trends and leading to the discovery of ligands with increased potency and selectivity *in vitro*.

The first study describes an expansive list of APGRAs that allowed an in-depth inspection of modifications made to two sites of the unique truncated APGRA scaffold, including modifications to the aryl pyrazole moiety. In this study, it was observed that an electron deficient aryl pyrazole moiety elicited desirable activity. It was also shown that compounds with small alkyl and aromatic moieties at the second modification site led to improved activity. Moreover, three compounds were revealed to have no impact on insulin secretion in the presence of glucose stimulation in cultured rat 832/13 beta-cells. Demonstrating potential toward a GC therapy with decreased risk of developing steroid induced diabetes mellitus; a serious side effect associated with long term GC usage.

The following study expands upon these conclusions to design a novel set of APGRAs with potential to display improved therapeutic activity. A second component of this experiment investigated the direct stereochemical impacts of this APGRA scaffold. This work demonstrated a staggering 70% average difference in activity between stereoisomers which alluded to the intricate ligand-receptor interactions. Computational analysis of structure activity relationships suggests the observed difference in isomeric activity may be in part due to differing interactions with helix three of the ligand binding domain, which is thought to play an important role in co-regulator binding. Ultimately, this study highlighted the importance of stereochemical purity prior to moving on to more in-

depth studies. Scale up synthesis and chiral purification of top APGRAs is currently underway to further investigate the therapeutic properties *in vivo*.

Finally, investigation to the spacious expanded ligand binding pocket was performed through the design and synthesis of hydrocortisone derivatives with two modification sites. The glucocorticoid receptor not only accommodated ligands up to 40% larger than dexamethasone (Dex) but many were also observed to outperform Dex in both the transactivation and transrepression assays. These compounds were also tested against hydrocortisone in which all modifications to the set of APGRAs presented, led to improved activity in both biological assays as well. The discovery of numerous potent APGRAs in this study displays the impressive potential of the underexplored expanded ligand binding pocket and sets a strong base for which additional modifications may lead to greater selectivity.

In conclusion, these studies contribute to the understanding of GR-GC interactions and the consequential activity. However, the complexity of GC mediated processes is not to be underestimated. In the last several decades, great progress has been made toward uncovering pathways to selective GCs. However, this work emphasizes the need for further studies to continue unraveling the intricate network of GR-GC mechanisms with the goal of bringing improved therapy options to millions of people and the generations to follow.

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VITA

Ashley Starck, formerly Ashley Lato, was born February 21st, 1995, and grew up in the small town of Thorp, Wisconsin where she found a deep fascination for chemistry her junior year of high school. Her high school chemistry teacher, Mrs. Teri Steinbach, broadened her view on the many career opportunities available through studying chemistry and so, Ashley attended the University of Wisconsin – Eau Claire to pursue a Bachelor of Science degree in chemistry. It was in college that Ashley's curiosity rapidly expanded, and she found a passion for research. In her sophomore year of college, she acquired her first undergraduate research position with Dr. Roslyn Theisen synthesizing model complexes for the metalloenzyme Quercetin Dioxygenase. After her first year of research, Ashley became a Ronald E. McNair postbaccalaureate achievement scholar and was awarded funding to pursue undergraduate research with Dr. Jennifer Dahl. Ashley spent her junior and senior year of college synthesizing biocompatible nanoparticles for protein dynamic studies and due to Dr. Dahl's mentorship, she decided to pursue graduate research opportunities. After graduating college, Ashley joined Dr. Shawn R. Campagna's research group at the University of Tennessee – Knoxville. During her time in the Campagna lab, Ashley studied synthetic organic chemistry, probing various biological systems through a chemical approach with a focus in medicinal chemistry and signaling molecules.