

The Discovery, Synthesis, and Characterization of Biological Signaling Molecules and Signaling Disruptors

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ABSTRACT

Chemical biology is a multidisciplinary field, combining perspectives and techniques from biology and chemistry to study biological systems. Often, these multidisciplinary endeavors hinge on the application of the powerful tools of synthetic and analytical chemistry to solve problems or test an interesting hypothesis in a biological system. The viewpoint of biology offers a larger scope with the potential for more immediately applicable results. Application of organic synthesis and analytical techniques identify biological signals or signal disrupters. Following, perturbation of a signaling pathway via a synthetic signaling molecule modulates the transcription of a desired gene produced correlated to a phenotype of interest or aids in the identification of an unknown component in the biological degradation of a chemical signal. Finally, implementation of nuclear magnetic resonance (NMR) and/or high performance liquid chromatography- mass spectrometry (HPLC-MS) contributes to elucidating the structure, annotating the identity, or quantitating biological signals or signal disrupters. In the first study, a library of 1-(4-substitutedphenyl)pyrazole-based glucocorticoid (GC) analogues were synthesized and evaluated for their ability to reduce inflammation with a reduced impact on insulin production. Compounds 11aa and 11ab at 1 μ M [micromolar] reduced inflammation to a similar extent to dexamethasone 10 nM with improved transactivation and reduced impact on insulin production. In the process of screening, structure-activity relationships were also shown to be correlated to the desired biological activity. In the second series of studies, 4-methylphenol (4MP), a putative anoxic, abiotic, mineral-mediated degradation product of bisphenol A (BPA), was shown to not be a BPA degradation product. Additionally, it was shown that BPA is not inert under anoxic conditions and that MnO₂ [magnesium oxide]-mediated coupled abiotic-biotic processes may be relevant for controlling the fate and longevity of BPA in sediments and aquifers. In the third study, the *N*-acyl homoserine lactone (AHL) production, gene transcription, and phenotype expression of *Phaeobacter* sp. strain Y4I was quantitated to ultimately describe the regulation of indigoidine biosynthesis by two quorum sensing gene sequences. In the fourth study, AHLs are shown to be present in coral black band disease (BBD) and their production, enhanced by temperature, may control the structuring of infected and healthy coral microbial communities.

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INTRODUCTION

The four chapters of this dissertation each represent a study or pair of sequential studies focusing on the study of biological signaling molecules or signaling disruptors. The first chapter features non-steroidal glucocorticoid (GC) analogues. GCs, are steroids, possessing both anti-inflammatory and metabolic effects, synthesized endogenously in the adrenal glands.¹ Their potential for being a possible type 1 diabetes mellites therapy was explored. The second study features Bisphenol A (BPA) and studies its degradation in abiotic, anoxic freshwater sediment. BPA is a high production volume chemical used to manufacture polycarbonate, epoxy resins, flame retardants, and lacquer coatings on food cans, as well as other products.² The third and fourth chapters feature *N*-acyl homoserine lactones (AHL), a bacterial cell-to-cell signaling molecules used to coordinate population size, colonization, virulence, and antibiotic production s³. The third chapter describes the regulation of the antimicrobial indigoidine by two AHL cell-to-cell communication systems. The fourth chapter showed how AHLs and autoinducer 2 (AI-2), another bacterial cell-to-cell signaling molecule, may play a role in structuring the microbial communities of both a polymicrobial infection and the healthy coral microbiome as well as the pathobiology of coral black band disease (BBD).

The focus of this dissertation is an overview of studies in the field of chemical biology, which often applies the tools of chemistry to test biological hypothesis. Each chapter focuses on the chemist's contribution to each study in the areas of discovery, synthesis, and characterization. These contributions are made possible by the tools of chemistry, generally in these studies, organic synthesis, high performance liquid chromatography-mass spectrometry (HPLC-MS), and nuclear magnetic resonance spectroscopy (NMR). Synthesis of biological signaling molecules, signaling disruptors, and analogues allow perturbation of biological systems, confirmation of structures during the process of structural elucidation, and ability to afford internal calibration and quantitation during HPLC-MS experiments. HPLC-MS and its variants employed in these studies allow aids in discovery and characterization via determining molecular formula and some structural information, as well as quantitative measurements. NMR aids in discovery and characterization provides very detailed information about the structure of a molecule as well as its chemical environment.

**CHAPTER I. EVALUATION OF THE STRUCTURAL VARIATIONS OF A
SERIES OF NON-STEROIDAL 1-(4-SUBSTITUTEDPHENYL)PYRAZOLE-
BASED GLUCOCORTICOID ANALOGUES THAT LEAD TO ANTI-
INFLAMMATORY ACTIVITY WITH REDUCED SIDE-EFFECTS ON BETA
CELL FUNCTION**

The work outlined in this chapter is currently in the final stages of preparation for publication. The work was accomplished by Carson W. Prevatte¹, Susan J. Burke^{2,3}, Brandon J. Kennedy¹, Alex R. Fisch¹, Russel T. Smith¹, Lee E. Dunlap¹, Jason J. Collier^{2,3}, and Shawn R. Campagna¹. This chapter represents the major work of CWP in this dissertation. This chapter was written by CWP under the supervision of SRC and he is the primary contributor in terms of the work performed and intellectual input in each section apart from the generation of experimental bioassay data by SJB.

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JJC and SRC conceived the study. The synthesis of all 36 glucocorticoid (GC) analogs evaluated were synthesized by CWP with assistance from RTS and LED. Modifications and optimizations to the previously reported synthesis were performed by CWP.⁴ Characterization was performed by CWP, BJK and ARF. This includes synthesis of some analogues that were in insufficient quantities to characterize after being subjected to bioassay evaluation. Bioassay evaluation of the GC analogues was performed by SJB, and the generation of the experimental data was accomplished by SJB and JJC. The modifications and optimizations of the previously reported synthesis are discussed by CWP. CWP and BJK contributed to the discussion of the structure-activity relationships determined from the bioassay results.

CWP contribution

Technical skill, fundamental knowledge of organic chemistry, and the ability to combine chemical and biological results came together in a discussion of new structure-activity relationships and reevaluation of previously reported glucocorticoid (GC) analogues. Previously reported by Merck (2004), 1-(4-fluorophenyl)pyrazole-based GC analogues were shown to act as ligands for the glucocorticoid receptor (GR). Their ability to reduce inflammation without accompanying side effects was measured using general bioassays. Our study took it a step further, by synthesizing a larger, more structurally diverse library of GC analogues with the Merck scaffold and evaluating them with specific bioassays relevant to determine their preliminary potential for treating type 1 diabetes mellitus (T1DM). First,

The previously reported⁴ library which possessed analogues with a single, electron-withdrawing 1-(4-fluorophenyl)pyrazole. In contrast, this study reports three series of 1-(4-substitutedphenyl)pyrazoles each possessing an electron-withdrawing, electron donating, and neutral substituent at the 4-position. Second, the previously reported bioassays produced structure activity relationships correlating structure to general anti-inflammatory and transactivation activity while this study correlates its wider scope of structures to anti-inflammatory, transactivation, and insulin production *in vivo* using rat beta cells containing luciferase gene reporter assays and the glucose stimulated insulin secretion assay (GSIS).

Despite the synthesis of 1-(4-fluorophenyl)pyrazole-based analogues being previously reported, the two most significant steps in the synthetic scheme did not generate the desired products in high purity and yield using the reported conditions. The optimizations for these two reactions made up the most significant contribution in terms of effort and allowed efficient synthesis of the aldehyde point of differentiation and secondary (2°) alcohol analogues. Additionally, the synthesis of two additional, novel analogue series with different 4-substituted headgroups expanded the previously reported study by Merck. Precedence for the headgroups exists in their application in other analogue scaffolds containing a 1-(4-substitutedphenyl)pyrazole and allows modulation of the electronics that can generate new structure-activity relationships. Finally, for this chapter, the discussion consists of two sections. First, a discussion of the optimization of a Wittig reaction and two organolithium additions to carbonyl compounds is discussed. Generally, discussion of the optimization of these reactions serves to educate the reader in regards to the general process of reaction optimization driven by a decision-making process based on the fundamental principles of organic chemistry. More specifically, the measures taken, and the reasoning behind them, can be applied to the same or similar reaction types. Second, the discussion for the dissertation, as opposed to the publication for this project, is written solely by CWP and focuses on identifying trends in structure-activity relationships. From this, reevaluating the potential of the GC analogues with the Merck scaffold in application to treating T1DM will also be discussed.

1.1 Abstract

Glucocorticoids (GCs), acting via the glucocorticoid receptor (GR) ubiquitously expressed in mammalian tissues, are commonly implemented in managing inflammatory,

autoimmune, and allergic disorders, certain cancers, and transplantation of organs and tissues. Previously reported 1-(4-fluorophenyl)pyrazole-based GC analogues acting as GR agonists have been shown to reduce interleukin 6 (IL-6)-induced inflammation (transrepression), but were often accompanied by an unwanted increase in transcription of genes associated with type 1 diabetes mellitus (T1DM), muscle wasting, and osteoporosis (transactivation). Development of an anti-inflammatory GC-based molecule with a biological activity that minimizes transactivation could greatly contribute to the prevention and treatment of T1DM, which is characterized as an autoimmune disorder caused by inflammation and subsequent autoimmune-mediated destruction of the insulin-producing beta cells. In this study, a series of diverse 1-(4-substitutedphenyl)pyrazole-based GC analogues were synthesized and screened to determine the structure-activity relationships that lead to the desired biological activity. Luciferase bioreporter assays, including chemokine ligand 2 (CCL2) promoter-luciferase promoter, glucocorticoid response element (GRE) promoter-luciferase promoter, and glucose stimulated insulin secretion (GSIS), were used to measure repression of interleukin 1 beta (IL-1 β)-induced inflammation, activation of pro-inflammatory genes, and the effect on insulin production, respectively. Repression of IL-1 β -induced inflammation by compounds **11aa** and **11ab** at 1 μ M were comparable to dexamethasone at 10 nM with significantly reduced GRE activation. Compounds **11aa** and **11ab** also exhibited a reduced impact on insulin production relative while dexamethasone lowered insulin production about 40%.

1.2 Introduction

Glucocorticoids (GCs) are steroids, possessing both anti-inflammatory and metabolic effects, synthesized endogenously in the adrenal glands. GCs operate by acting as the ligand for the glucocorticoid receptor (GR), which resides in the cytoplasm bound to chaperone proteins such as heat shock protein 90 (hsp90). The GR is expressed by nearly all cell types, including beta cells, and is the product of a single gene, NR3C1, that is post-translationally modified to produce functionally distinct subtypes of the GR.¹ The GR has six modes of activation at the cellular level. The ligand-bound GR (GR-GC) can translocate to the nucleus whereby it can bind a glucocorticoid response element (GRE), a palindromic gene sequence within the promotor, as a dimer to (1) induce or (2) suppress gene expression⁵. Additionally, while in the nucleus, GR-GC can (3) facilitate or (4) hinder the actions of other transcription factors. Alternatively, the GR-GC can remain in the

cytoplasm and (5) increase or (6) decrease the activity of a protein in the cytoplasm.⁶ Due to the anti-inflammatory and metabolic-altering effects of GCs, along with the ubiquity of the GR, GC-based therapies have been successfully applied to treating a wide range of inflammatory, auto-immune disorders and cancers as well aiding in organ and tissue transplants.

T1DM is characterized as an autoimmune disorder characterized by inflammation and subsequent autoimmune-mediated destruction of the insulin-producing beta cells.⁷ One manner of autoimmune beta cell death commonly associated with T1DM occurs through insulinitis, which is the process by which beta cell damage and death occur via infiltration by macrophages and T cells along with accumulation of pro-inflammatory cytokines such as IL-1 β , interferon gamma (IFN- γ), and tumor necrosis factor alpha (TNF- α).⁸⁻¹⁰ Cytokine-mediated inflammation of beta cells causes the expression of chemokine ligands 9 and 10 (CXCL9 and CXCL10), which signal the recruitment of T cells chemokine receptor 3 (CXCR3).^{8, 11-12} Two of the six modes of activation for the GR are often attributed to contributing to the deleterious and salutary effects. The deleterious mode of activation is termed transactivation which describes the translocation of the GR-GC to the nucleus to increase transcription of pro-inflammatory genes via binding GRE. The metabolic deregulation caused by the direct binding of the GR-GC to GREs is thought to result in the multitude of side effects previously mentioned, as well as hypertension and hyperglycemia. The salutary mode of activation is termed transrepression which describes the translocation of the GR-GC to the nucleus to inhibit the action of pro-inflammatory cytokines, such as IL-1 β , AP-1, and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). These cytokines serve as transcription factors that can generally influence immune system regulation and response. The repression of various pro-inflammatory genes via binding directly to transcription factors is attributed to conferring the sought-after anti-inflammatory effects.¹³⁻²⁷ More recently, however, the GR-GC has been shown to suppress transcription of pro-inflammatory genes by binding to GRE. Additionally, it has been determined that GR-GC binding conformation is effected by the type of GRE, which suggests that one a GR-GC subtype that cannot bind one GRE may be able to bind another based on the specific target gene.²⁸⁻²⁹ Furthermore, since the types and ratios of transcriptional cofactors vary among cell or tissue types, the interaction between the GR-GC and transcription factors is dependent upon the activity and dissociative properties of the GR-GC in terms of tissue type.²⁹⁻³⁴

Application of synthetic GCs as a possible anti-inflammatory therapeutic for T1DM and immunosuppressive agent for organ transplants is a significant field of research. In the US, diabetes is the 7th leading cause of death, affecting 29.1 million people or 9.3% of the U.S. population in 2016, 86 million US adults have prediabetes³⁵. Diabetes is the leading cause of kidney failure, non-traumatic lower-limb amputations, and new cases of blindness in adults and significantly contributes to heart disease and stroke³⁵. Estimated cost of diabetes in the US in 2014 was \$176 billion in direct and \$69 billion indirect³⁶. The ideal GC-based T1DM therapeutic would aim to address the inflammation that initiates the autoimmune response that ultimately destroys the beta cells. Moreover, synthetic GCs are designed to exhibit a therapeutic profile possessing enhanced anti-inflammatory activity with reduced side effects. Despite incredible beneficial properties of GCs, they pose a long list of serious side effects including Cushing's disease, Metabolic Syndrome, osteoporosis, and diabetes. In fact, the most common cause of drug-induced diabetes is clinical administration of GCs, and the incidence of GC-induced diabetes continues to rise.

Synthetic GCs have been shown to improve beta cell development by reducing inflammation at the transcription-level by reducing the expression of interleukin 1 (IL-1) and TNF- α -induced expression of IL-6.^{37-38,39} However, they have also been shown to reduce adult beta cell mass.^{40,41} A study published by Merck Research Laboratories in 2004 reported the synthesis of non-steroidal 1-(4-fluorophenyl)pyrazole-based GC analogues and biological evaluation of bioassays measuring general transactivation and transrepression.⁴ The study showed that two tertiary (3°) alcohol derivatives of 1-(4-fluorophenyl)pyrazole-based GC analogues possessing 3-thiophenyl- or 4-fluorophenyl-modifications resulted in potent IL-6 inhibitors relative to dexamethasone as evaluated by the IL-6-release assays using the human A549 lung epithelial cell line and isolated mouse thioglycolate-elicited peritoneal exudate cells. Ultimately, the 3-thiophenyl 3° alcohol analogue was evaluated *in vivo* in the mouse LPS challenge model producing an ED₅₀ of 4.0 mg/kg relative to 0.5 mg/kg for prednisolone in the same assay.

First, the library of previously reported analogues possessed structurally diverse side chains 1-(4-fluorophenyl)pyrazole-based scaffold. Our study reports a three-analogue series of the same scaffold possessing an electron-withdrawing, electron donating, and neutral substituent in the 4-position. Second, the previous reported bioassays producing general structure activity relationships correlating structure to general anti-inflammatory/transactivation activity while this study correlates its wider scope of

structures to anti-inflammatory, transactivation, and insulin production. Testing the transactivation and anti-inflammatory (transrepression) properties of GC was accomplished *in vivo* using rat beta cells containing luciferase gene reporter assays and the glucose stimulated insulin secretion assay (GSIS).

This study is an extension of the previously reported study and possess three main areas of innovation.⁴ First, whereas the Merck study screened for more general anti-inflammatory and transactivation activity, the study presented herein uses bioassay more specific to determining the potential of the ability of these non-steroidal GR ligands to treat T1DM. A compound library possessing 36 1-(4-substitutedphenyl)pyrazole-based GC analogues were synthesized and subjected biological evaluation (**Table 1.1**). Of these 36, the 21 possessing methyl or methoxy headgroups are previously unreported. This study measured the anti-inflammatory, transactivation, and insulin suppression actives *in vivo* using rat beta cells, the cells possessing the chemokine ligand 2 (CCL2) promoter-luciferase promoter, measuring IL-1 β -mediated transrepression, and the GRE promoter-luciferase promoter, measuring the transactivation of pro-inflammatory genes. The best top performing analogues for the luciferase gene reporter assays were subjected to the glucose-stimulated insulin secretion assay (GSIS) to determine the impact on insulin production. Second, the scope of analogues screened is expanded, including the previously reported 1-(4-fluorophenyl)pyrazole-based GC analogues as well as 4-methyl and 4-methoxy analogues. This will provide three series of analogues possessing a 1-(4-substitutedphenyl)pyrazole with a fluoro, methoxy, and methyl group that provide a series with an electron-withdrawing, electron-donating, and relatively neutral substituent. Ultimately, this will generate new structure-activity relationships among a relatively larger scope of 1-(4-substitutedphenyl)pyrazole-based GC analogues by comparing structure to a very specific selection of bioassays relevant to testing the potential for acting as a T1DM therapeutic.

1.3 Materials and Methods

Chemistry materials and instrumentation. Chemical reagents were purchased commercially and used without further purification. Dry ethereal solvents were obtained by distillation from a potassium benzophenone ketyl still. All other dry solvents were distilled from CaH₂(s). Thin-layer chromatography (TLC) was performed using Sorbent

Table 1.1. All 2° and 3° alcohol 1-(4-substituted-substituted phenyl)pyrazole-based GC analogues synthesized.

	R_2	R_1					
		2° ROH			3° ROH		
		F	Me	OMe	F	Me	OMe
		a	b	c	a	b	c
a	Me	11aa	11ba	11ca	13aa	13ba	13ca
b	Ph	11ab	11bb	11cb	13ab	13bb	13cb
c	4-fluorophenyl	11ac	11bc	11cc	13ac	13bc	13cc
d	1-(3,4-difluoro-5-methoxyphenyl)	11ad	11bd	11cd	13ad	13bd	13cd
e	3-thiophenyl	11ae	11be	11ce	13ae	13be	13ce
f	CH ₂ CN	11af					
g	t-Bu	11ag	11bg	11bg			

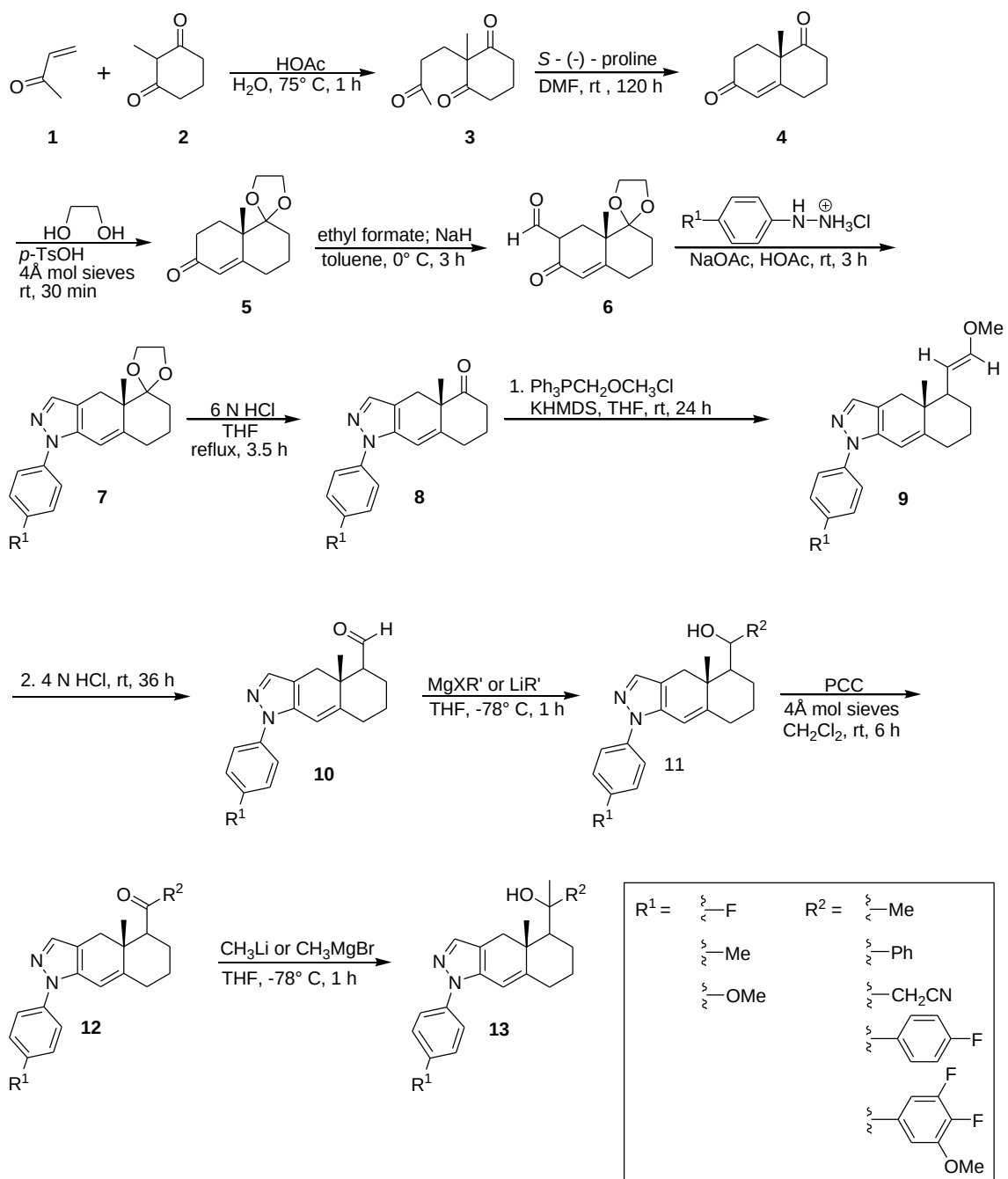
Previously reported
 Previously unreported, prepared using previously reported method
 Previously reported, prepared using optimized method
 Previously unreported, prepared using optimized method
 undesired t-Bu adduct byproduct

A total of 31 2° and 3° alcohols were subjected to biological evaluation. Compounds highlighted in green were previously reported and were synthesized by a previously reported addition to an aldehyde or ketone. Compounds highlighted in yellow were previously unreported and were synthesized in the same manner as those in green. Compounds highlighted in orange were previously reported 2° alcohols generated using significantly modified versions of a previously reported organolithium addition. Compounds highlighted in red were previously unreported 2° alcohols generated using significantly modified versions of a previously reported organolithium addition. Compounds in purple were an undesired t-Bu adduct sometimes obtained during the generation of 1-(3,4-difluoro-5-methoxyphenyl)-modified 2° alcohols.

Technologies silica G F₂₅₄ TLC plates. All compounds of interest were visualized as single spots using short-wave UV light; more permanent staining of TLC spots was achieved using *p*-anisaldehyde, vanillin, or ceric ammonium molybdate (Hannesian's stain) TLC staining solutions. Silica gel with a 60 Å pore size and a particle diameter of 40-63 µm was used for flash chromatography.

¹H-NMR spectra were obtained on either an Oxford 300 MHz or Varian InNova 500 MHz NMR spectrometer in solutions of CDCl₃. All ¹³C-NMR spectra were recorded on a Varian InNova 500 MHz NMR spectrometer in solutions of CDCl₃. High resolution mass spectrometry (HRMS) for characterization of previously unreported compounds was performed using a JEOL DART source in positive ion mode coupled to a JEOL AccuTOF JMS-T100LC mass spectrometer. HRMS for characterization of crude mixtures was performed using the JEOL DART source in positive ion mode coupled to a JEOL AccuTOF JMS-T100LC mass spectrometer or a Sciex Turboionspray electrospray ionization source in positive mode coupled to an Applied Biosystems QStar Elite mass spectrometer. Optical rotation was measured using a Perkin-Elmer 241 polarimeter equipped with a sodium source lamp. IR spectra were collected using a Nicolet iS5 spectrophotometer.

Synthesis of 1-(4-substitutedphenyl)pyrazole-based GC analogues. The total synthesis of all 1-(4-substitutedphenyl)pyrazole-based GC analogues tested in this study were prepared as previously described or by a slightly modified version of a published synthesis for 1-(4-fluorophenyl)pyrazole-based GC analogues (**Scheme 1.1, Table 1.1**).⁴ First, the previously described one-pot Wittig-hydrolysis reaction employed to obtain the homologous aldehyde **10** from ketone **8** was performed in two steps via isolation and purification of the intermediate enol ether **9**. Following, reaction conditions were significantly modified for reactions of aldehyde **10** to secondary (2°) alcohol **11** requiring *in situ* generation and addition of organolithium compounds. Next, an entirely different method for the oxidation of secondary alcohol **11** to ketone **12** was employed using a PCC oxidation rather than the previously described method using N-Methylmorpholine-N-Oxide (NMO) with TPAP (tetrapropylammonium perruthenate). Additionally, the synthetic scheme also differs from the previously described synthesis by exchanging 4-fluorophenyl hydrazine for the appropriate 4-substituted phenylhydrazine used to generate previously unreported intermediates and GC analogues containing a 4-methyl- or 4-methoxy-substituents. This creates three different analogue series with different "headgroups."



Scheme 1.1. Synthetic route to 1-(4-substitutedphenyl)pyrazole-based GC analogues.

Furthermore, chiral HPLC was not employed in the purification of the GC analogues as previously reported, but rather standard bench-top flash chromatography and trituration techniques were employed. Consequently, purification of some crude mixtures was not feasible. Thus, the synthetic methodology was ultimately modified to prevent generating crude mixtures containing components bearing identical R_f values by TLC analysis.

The total synthesis of the steroid core begins with a Robinson annulation conducted over two steps. A Michael addition between methyl vinyl ketone and 2-methylcyclohexane-1,3-dione, **1** and **2**, using acetic acid and water heated to 75° for one hour to form intermediate trione **3**. Intramolecular cyclization is accomplished by stirring **3** with L-proline in DMF for 120 h at room temperature to generate primarily the (*S*)-isomer of Wieland-Miescher ketone **4** (WMK)⁴. Further resolution of **4** was not performed. Next, selective protection of **4** with ethylene glycol performed neat with catalytic *p*-toluenesulfonic acid and 4 Å molecular sieves for 30 min or until complete via TLC monitoring generates intermediate **5** which possesses an α-β-unsaturated ketone available for enolate formation. Subsequent addition to ethyl formate in toluene over 3 h to generate β-ketoaldehyde **6**. Addition of the appropriate 4-substituted phenylhydrazine to **6** in acetic acid for 2 h or until complete via TLC monitoring completes the core structure of the 1-(4-substitutedphenyl)pyrazole-based GCs, **7**. The acid-catalyzed deprotection of acetal **7** using 6 N HCl in THF for 3 h under reflux provides intermediate ketone **8**. A Wittig-hydrolysis-type homologation of ketone **8**, conducted over two steps via intermediate enol ether **9**, affords aldehyde **10**, the essential intermediate for the divergent synthesis of all analogues presented in this study. To obtain aldehyde **10** in high purity, the intermediate enol-ether **9** was isolated from the Wittig reaction, purified via flash chromatography, and subsequently subjected to hydrolysis conditions. A series of 2° alcohols **11** was achieved by adding aldehyde **10** to a solution of the appropriate organolithium or Grignard reagent in THF at -78° C with various conditions depending on reaction partners. Intermediate ketone **12** was obtained through a PCC oxidation of **11** in dichloromethane for 2 h or complete via TLC monitoring, and after purification via flash chromatography, was treated with MeLi in ether at -40° C to afford 3° alcohols **13**.

Purification Methods. Throughout the synthetic procedure are purification steps requiring silica-based flash chromatography. Difficulty was encountered during the purification of most 2° alcohol GC analogues. TLC analysis typically done using a 25%

solution of EtOAc in hexanes resulted in visualization of resolved compounds using short-wave UV light revealed separations that do not seem particularly challenging, but the same solvent system applied to a flash chromatography by routine methods resulted in poor separation. Dissolution of the compounds in halogenated solvents such as dichloromethane allow for wet-loading very concentrated solutions to the top of the column as is typically employed in standard benchtop flash chromatography. However, the intermediate polarity of these polar aprotic solvents contributes to increasing unwanted band-broadening and poor separation, especially during equilibration after loading between the loading solvent and solvent system. Unfortunately, all the compounds purified in this synthesis would only dissolve in mostly polar solvents that would obscure the chromatography and result in poor separation. It was found that the best chromatographic resolution can be obtained by loading compounds onto silica, wet-packing the silica column, carefully placing a thin layer of sand to maintain column integrity during compound loading, and finally loading the vacuum-dried compound-adsorbed silica on top of the sand layer. This procedure mimics that of a typical TLC analysis where the compound is loaded onto a small section of silica, solvent is allowed to evaporate, and then the whole chromatographic system is subjected to the desired mobile phase composition. Despite careful solvent system design and column technique, many of the 2° alcohol GC analogues were subjected to column chromatography multiple times in order to obtain an adequate amount of high purity material to subject to bioassay evaluation as well as move forward in the synthesis.

Trituration was employed to remove trace impurities that were not removed with flash chromatography as well as in specific application to separating up to 0.11 ratio mixtures of ketones **8** and aldehyde **10** resulting after incomplete conversion of **8** to **10** using the previously reported one-pot Wittig reaction⁴. In either application, trituration was accomplished by sonication of the preliminarily flash chromatography-purified, often colored, crude mixture in diethyl ether or THF. Removal of the colored supernatant containing the impurity or starting ketone, leaves the purified product as a white powder. In the case of the separation of ketones **8** and aldehydes **10**, several triturations may be necessary.

Cell Culture, Islet Isolation, Primary Hepatocytes, and Glucose-stimulated Insulin Secretion (GSIS). Insulin secretion was measured as previously described using

832/13 rat insulinoma cells.⁴²⁻⁴³ Isolation of rat islets was performed as previously reported.¹² Primary hepatocytes from Prague-Dawley rats were supplied from Triangle Research Labs. The age of the rats was 8–10 week, and their weights were between 150 and 200 g when the islets were harvested. The saturating concentrations of the commercially available steroid or GC analogue used in GSIS measurements as indicated in each figure.⁴³

Cell Viability Assays. 832/13 rat insulinoma cells were exposed to the commercially available steroid or GC analogue as indicated in each figure. Viability was assessed using both 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and adenylate kinase (ADK) assays. These procedures have been previously validated using the clonal 832/13 beta cell line.¹²

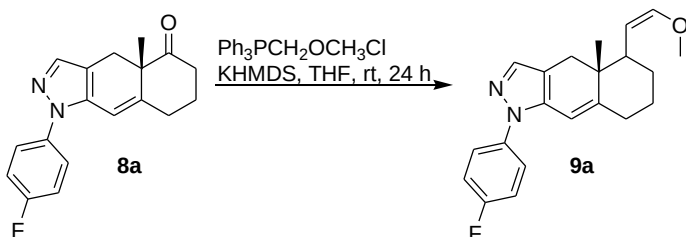
Luciferase Assays, ELISA, and Real-time PCR Analysis of mRNA. 832/13 rat insulinoma grown to 50% confluence in 24-well plates were transiently transfected with 25 ng of the appropriate plasmid using TransFectin Lipid Reagent (Bio-Rad) per the manufacturer's protocol. Following, the results were normalized to total protein content according to a reported previously method⁴¹. Rat Quantikine (CCL2) ELISA kits (R&D Systems, Inc., Minneapolis, MN) was used to detect the CCL2 concretions in the media. Total RNA isolation, cDNA synthesis, and quantification of mRNA abundance measured with real-time RT-PCR was performed using a previously reported method.^{11,44}

1.4 Results and Discussion

Optimization of the Wittig-hydrolysis reaction to obtain aldehyde 10. Despite the thorough details of the previously reported synthesis of 1-(4-fluorosubstituted phenyl)pyrazole-based GC analogues (Merck Research Laboratories 2004), application in converting ketones **8** to aldehydes **10** via the one-pot Wittig-hydrolysis sequence did not consistently result in a complete conversion. The TLC-determined R_f value between all ketones **8** and aldehydes **10** was less than 0.10 in all solvent systems tested. Solvent systems tested included solutions of EtOAc in hexane ranging from 10-40%, MeOH in DCM ranging from 0.5-2%, and EtOAc in DCM ranging from 10-20%. Furthermore, the developed TLC plate often contained the two as overlapping spots. Thus, application of standard benchtop flash chromatography techniques was ill-suited for separation of these two

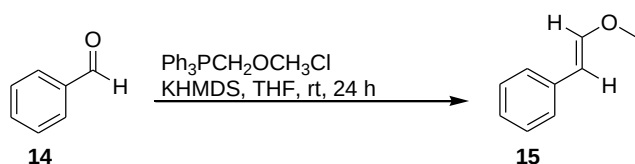
components. This posed a significant problem since the intermediate aldehyde is the point of differentiation from which all 1-(4-substitutedphenyl)pyrazole-based GC analogues were derived. Separation of the two-component aldehyde-ketone mixture, up to a 0.11 ratio by NMR, was accomplished by subjecting the orange-brown crude product to flash chromatography followed by several successive triturations in diethyl ether. It was discovered that ketones **8** are marginally more soluble in diethyl ether than aldehydes **10**. This method of successive triturations was exclusively used to obtain aldehyde **10a** of fair purity that could be used to make the product GC analogues. However, it was detrimental to the yield. For the synthesis of aldehyde **10b** and **10c**, an optimization of the previously reported method⁴ was conducted, and this new, optimized method was ultimately employed, as opposed to trituration, to ultimately access these aldehydes.

Initially, a series of test reactions were run to determine which stage or component to optimize. In the first test reaction, ketone **8a** was subjected to the previously reported Wittig reaction conditions⁴ with the phosphonium salt of interest, (methoxymethyl)triphenylphosphonium chloride ($\text{Ph}_3\text{PCH}_2\text{OCH}_3\text{Cl}$) (**Scheme 1.2**). The reaction was immediately quenched by the addition of 1:1 THF/MeOH, diluted with EtOAc, and washed with water and brine. After concentration, crude aldehyde **9a** was analyzed via NMR to determine the extent to which the ketone was being converted to the enol ether. Though a spectrum of conversion for each run varied, an incomplete conversion was observed among all runs as evidenced by a peak at 6.27-6.28 ppm corresponding to the vinyl proton of ketone **8a**. This suggested that the previously reported one-pot, two-step Wittig reaction required optimization. The hydrolysis of enol ether **9a** to generate aldehyde **10a** was not suspected to result in incomplete conversion as the equilibrium between an enol ether and an aldehyde under acidic, aqueous hydrolysis conditions greatly favors the forward reaction.



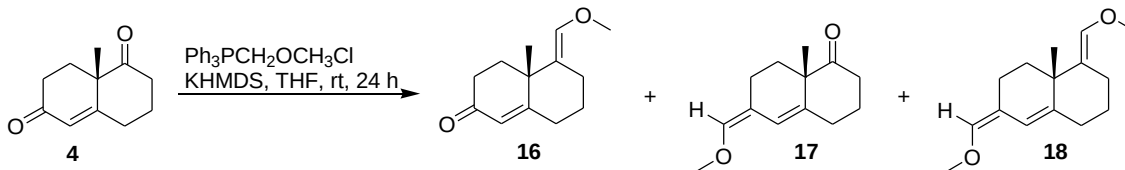
Scheme 1.2. Previously reported one-pot Wittig hydrolysis reaction quenched after the Wittig reaction. An incomplete conversion of ketone **8a** to enol ether **9a** suggested that it was the Wittig reaction component of the previously reported one-pot, two step homologation reaction which required optimization.

For the second test reaction, a Wittig reaction between benzaldehyde and Ph₃PMOMCl was performed using previously reported conditions (**Scheme 1.3**)⁴. Complete conversion of the aldehyde was observed in the NMR spectrum of the crude product as evidenced by the absence of an aldehyde peak in the 9.5-10.5 ppm range and two sets of vinyl and methoxy protons for both alkene stereoisomers. Though this reaction was run for the full 24 hours, it was likely completed in a much shorter time because the carbonyl component of the Wittig reaction was a more reactive, unhindered aldehyde. The successful reaction between the ylide generated from Ph₃PMOMCl and the more reactive model carbonyl compound, benzaldehyde, reduced the concern of the reactivity of the carbonyl reacting partner of the Wittig reaction. This suggested that the ylide was being efficiently produced from the phosphonium salt of interest and attention should be focused on the carbonyl component.



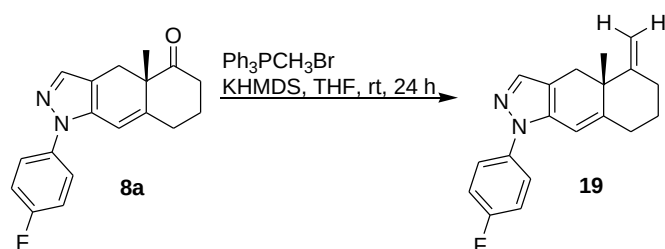
Scheme 1.3. Wittig reaction between benzaldehyde **14** and Ph₃PMOMCl **15**. Complete conversion was observed in the NMR spectrum for the Wittig reaction between the ylide generated from Ph₃PMOMCl and a carbonyl compound more reactive relative to ketone **8**.

For the third test reaction, two reactions between WMK ketone **4** and Ph₃PMOMCl were conducted to study a Wittig reaction with a ketone sterically analogous to ketone **8** (**Scheme 1.4**). The reaction conditions were the same as those previously reported, but a complete conversion of the starting ketone was observed by TLC after 7.5 h. Full conversion was observed in the NMR spectrum of the crude product as evidenced by the absence of a peak at 5.85 ppm corresponding to vinyl proton of WMK **4** as well as the presence of new vinyl and methoxy protons. The product distribution consisted of three sets of enol ether protons, **16-18**, which resulted from reactions at each or both ketones.



Scheme 1.4. Wittig reaction between WMK **4** and Ph₃PMOMCl. Complete conversion was observed between two runs of this test reaction. These results suggested that the sterics of the ketone of interest, **8a**, were not a significant factor in the incomplete conversion observed in the reaction of interest.

The final test Wittig reaction was conducted by way of the first step in an alternative sequence that was attempted to obtain the aldehyde. Successful Wittig reactions were conducted between ketone **8a** and either methyltriphenylphosphonium bromide ($\text{Ph}_3\text{PCH}_3\text{Br}$) or methyltriphenylphosphonium iodide ($\text{Ph}_3\text{PCH}_3\text{I}$) using previously reported conditions (**Scheme 1.5**).⁴⁵ Complete conversion of the starting ketone **8a** was observed by TLC after 6 h. This was observed in the NMR spectrum for the crude product by the absence of a peak at 6.28 ppm corresponding to the vinyl proton of the starting ketone **8a**. Product **19** was subjected to flash chromatography, and its structure was confirmed by NMR. The percent yield for the reaction was 76.8 %.



Scheme 1.5. Wittig reaction between ketone **8a** and $\text{Ph}_3\text{CH}_3\text{Br}$. Complete conversion was observed by TLC after 7.5 h. Complete conversion was also observed in the NMR spectrum. Product **19** was subjected to flash chromatography, and pure product **19** was isolated in 76.8 % yield. These results suggest that it is the relatively lengthy time of the reaction of interest that is correlated to its incomplete conversion.

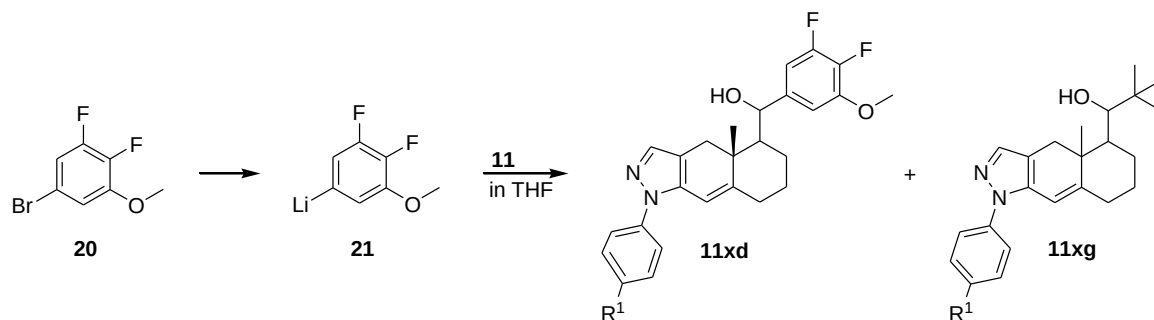
The results of the test reactions showed that the previously reported Wittig reaction conditions efficiently generated an ylide from two different phosphonium salts and resulted in complete conversion with their aldehyde or ketone reaction partner. However, since the same conditions resulted in incomplete conversion for the reaction of interest, attention was drawn to the lengthy reaction time it required, which was the most significant difference between the reaction of interest and the test reactions. All test reactions were all complete in less than 24 h except for the reaction with benzaldehyde, which would have likely been observed to have a shorter reaction if the reaction had been monitored by TLC. It was hypothesized that because the stir time was so long, the reaction would be more likely to succumb to trace amounts of water that could enter either from the nitrogen line that provided the inert atmosphere for the reaction or from the crude needle and septum setup commonly employed in academic research labs. Use of a drying tube packed with anhydrous CaSO_4 directly before the reaction flask did not result in a consistent improvement in conversion. Additionally, swapping out the needle and septum setup for a

ground glass vacuum adapter immediately after the reaction setup was complete, which likely allowed air to enter during the exchange, also did not result in an improvement.

Rather than optimizing the reaction conditions or purification, an intermediate workup and purification step between the Wittig and hydrolysis steps was envisioned to give a crude reaction product with a mixture of enol ether **9** and ketone **8** which could be separated using standard benchtop chromatography. Good separation on TLC between the two stereoisomers of the enol ether and the ketone was observed and provided the promise for successful flash chromatography. As an example, for enol ether **9c**, the R_f values for the pair of enol ether diastereomers was 0.63 and 0.67 while the starting ketone **8c** was 0.57 when developed with 30% EtOAc in hexanes. The reaction was performed as previously reported and immediately quenched by the addition of 1:1 THF/MeOH, diluted with EtOAc, washed with water and brine, concentrated in vacuo, and subjected to a column eluted with 15% EtOAc in hexanes. The successfully isolated enol ether stereoisomers were concentrated and subjected to the previously reported hydrolysis conditions⁴. The yield of **9c** over these two steps with intermediate purification was 71%. Though not optimizing the reaction or purification directly, this reliably produced aldehyde product of high purity and good yield while allowing the remaining starting ketone to be recovered.

Optimization the synthesis of 1-(3,4-difluoro-5-methoxyphenyl)-modified 2° alcohols 11xd (Table 1.2). Application of the previously reported general method describing the generation and addition of organolithium nucleophiles to the aldehyde precursor of 1-(4-substitutedphenyl)pyrazole-based GC analogues did not result in efficient conversion when applied to the generation and addition of 5-lithio-2,3-difluoroanisole and 3-lithiothiophene to aldehydes **10** in this study. Because of this, optimization of these two organolithium additions was conducted as each 1-(4-substitutedphenyl)pyrazole-based GC analogue was synthesized (Tables 1.2). The reactions could be optimized as analogues were successfully synthesized because despite most reaction conditions resulting in a low yield, each condition produced product in adequate quantities and purity for bioassay evaluation. here was no difference in reactivity expected or observed among aldehydes **10** with a fluoro, methyl, or methoxy 4-substituted phenyl headgroup. Thus, a single condition may have contained runs carried about among different aldehydes. Additionally, the previously reported method was described on single milligram scale with the final purification of the 2° alcohols **11** sometimes requires purification by single milligram-

Table 1.2. Optimization of the generation and addition of 5-lithio-1,2-difluoro-3-methoxybenzene to aldehyde **10** to obtain 2° alcohol **11** was performed.

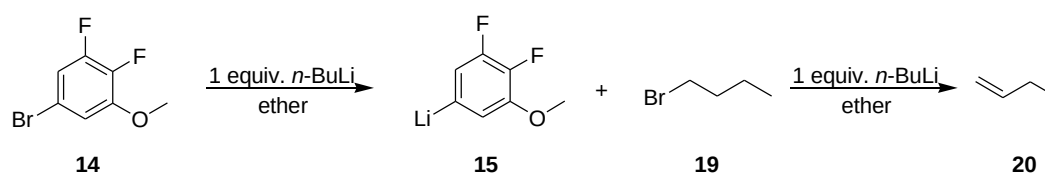


Condition	Organolithium formation	Addition to aldehyde	Color change
1	20 min at -78° C	45 min at -78° C	
2	5 min at -78° C	60 min at -78° C	dark yellow
	warmed to 0° C for 5 min returned to -78° C for 5 min		
3	warmed to -40° C for 20 min	60 min at -78° C	avoided color
4	5 min at -78° C	60 min at -78° C	
	-40° C to r.t. over 55 min, no dry ice added so allowed to warm some		yellow-green
5	5 min at -78° C 5 min at 0° C 5 min at -78° C	60 min at -78° C	

scale chiral HPLC. The optimizations of 2° alcohol analogues reported herein is a method for generating alcohols **11xd** and **11xe**, discussed in the following section, in gram quantities and purification was accomplished exclusively by standard benchtop chromatography with occasional post work-up via trituration. With a larger amount of molecule in hand, a larger scale studies subjecting the molecules to more bioassays or perhaps larger numbers of organisms for *in vivo* evaluation can be accomplished.

In application to the synthesis of 1-(3,4-difluoro-5-methoxyphenyl)-modified analogues, the previously reported method for organolithium addition conditions called for a large excess of 5-Bromo-2,3-difluoroanisole and *t*-BuLi, 10 and 20 equiv. respectively. Because this equivalency was initially thought to be excessive, more mild conditions were initially used in conditions 1 and 2. Condition 1 used 2.0 equiv. *n*-BuLi and 5.0 equiv. of aryl bromide (ArBr) and resulted in a low yield, 21.3%. The results of condition 1 were not thoroughly investigated. Condition 2 used 3.0 equiv. *n*-BuLi and 1.5 equiv. 5-Bromo-2,3-difluoroanisole and resulted in a 13.1% of the adduct between *n*-BuLi and aldehyde **10a**. It should be noted that at the time conditions 1 and 2 were performed, the optimization of the Wittig reaction was not complete. Thus, these reactions were conducted with starting material containing 5-10% of the ketone **8a**. Despite this, the NMR and mass spectra of the crude product generated from condition 2 provided data constructive to the end of determining the point of inefficiency for this organolithium addition. Beginning with WMK **4**, all intermediate products, and the 1-(4-substitutedphenyl)pyrazole-based GC analogue products all contained a vinyl proton with a signature chemical shift. The vinyl proton peak indicated of the number and identity of components in the crude mixture which included the product of interest, undesired products, or the unreacted starting material. The NMR spectra for the crude product obtained from condition 2 contained a complex mixture of components of interest as evidenced by the four peaks in the alkene region. The vinyl proton corresponding to enol ether **9a** (6.12 ppm, 1.05 H) was accompanied by three other vinyl protons corresponding to ketone **8a** unreacted from the previous Wittig reaction (6.28 ppm, 1.71 H), the adduct between *n*-BuLi and ketone **8a** (6.15 ppm, 2.80 H), and the adduct between *n*-Bu and the aldehyde **10a** (6.10 ppm, 2.00 H). High resolution mass spectrometry analysis of the crude product showed four peaks corresponding to the four components identified in the NMR spectrum. Peaks with mass-to-charge ratios (*m/z*) of 454.72186, 369.23278, 355.29356, and 297.53081 correspond to the [M+H]⁺ ions for the product of interest, the adduct between *n*-BuLi and aldehyde **10a**, the adduct between *n*-BuLi and ketone **8a**, and ketone **8a**.

The presence of the *n*-BuLi adducts in the NMR and mass spectra of the crude product generated from condition 2 suggests that the conversion of 5-Bromo-2,3-difluoroanisole to 5-lithio-2,3-difluoroanisole was incomplete. Rather than reacting with 5-Bromo-2,3-difluoroanisole, the excess *n*-BuLi was available to add to the aldehyde **10a**. This was unexpected because 2 equiv. of *n*-Bu relative to 5-Bromo-2,3-difluoroanisole was employed. This is the standard equivalency required since one equivalent is responsible for the halogen-lithium exchange while the other is necessary to quench the alkyl halide byproduct (**Scheme 1.6**). Elimination of the alkyl halide by-product ensures the reverse halogen-lithium exchange does not occur. Comparing the mass and NMR spectra of the crude product generated from condition 2, the ratio between the product of interest, unreacted ketone, the adduct between *n*-BuLi and the **8a**, and the adduct between *n*-BuLi and the **10a** was 1.05:1.71:2.80:2.00 per the integration values for the vinyl protons obtained from the NMR spectrum for the crude product and about 11:35:38:4 per the intensities in the mass spectrum for the crude product, respectively. This provided difficulty at the time of analysis because the four components in the NMR spectrum of the crude product could not be easily correlated to the components of the crude mass spectrum by matching the relative integrations to the relative intensities of each component. The lower intensity of the product of interest in the mass spectrum made it seem like a more minor component than it was. This was likely due to a difference in the ionization potential between the *n*-Bu adducts and 1-(3,4-difluoro-5-methoxyphenyl) adducts. The crude mixture was subjected to flash chromatography, and the main product generated by condition 2 was determined to be the adduct between *n*-BuLi and aldehyde **10a** by the presence of a single peak with a *m/z* of 369.23370 for the corresponding [M+H]⁺ ion from an accurate mass measurement taken of the main component eluted from the column.



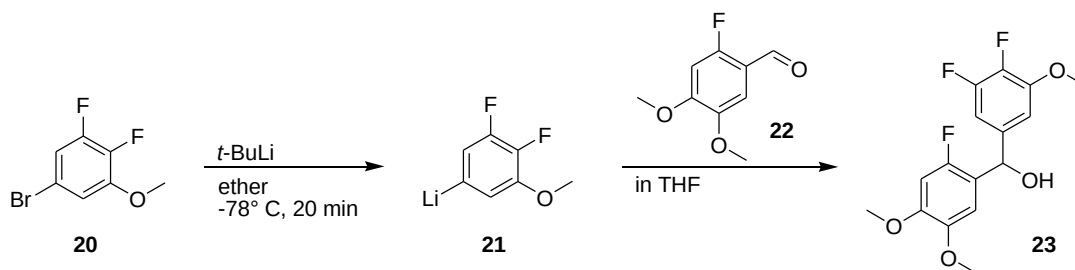
Scheme 1.6. Halogen-lithium exchange requires two equivalents of RLi. One equivalent is responsible for the generation of **15** from **14** while the other is necessary to eliminate the **19** by generation of **20** via E2 elimination so that the reverse halogen-lithium exchange does not occur.

Condition 3 used the same 10 equiv. 5-Bromo-2,3-difluoroanisole as the previously reported synthesis⁴ and a slight excess of 10.5 equiv. *t*-BuLi. These conditions were successful in producing **11ad** from **11a** as the predominate product, but also resulted in formation of small amounts of the adduct between *t*-BuLi and the aldehyde. Purification using flash chromatography was especially challenging since the difference between the R_f values of the components was less than 0.10 in all solvent systems tested. Solvent systems tested included solutions of EtOAc in hexane ranging from 10-40%, MeOH in DCM ranging from 0.5-2%, and EtOAc in DCM ranging from 10-20%. Furthermore, the developed TLC plate often contained two overlapping spots corresponding to the *t*-BuLi adduct and **11ad**. Because complete separation of these two components was never achieved, only a few fractions collected from each column possessed pure product of interest, and in many cases, flash chromatography was performed more than once for the same crude mixture. For any given column, most fractions collection from the column possessed the *t*-Bu adduct which was observed in both NMR and mass spectra. For the NMR, peaks at 6.05 and 6.11 ppm correlated to the vinyl protons for **11ad** and the *t*-Bu adduct, respectively. A discrepancy existed between the intensities of the *t*-Bu adduct ($m/z = 381.26541$) and **11ad** ($m/z = 467.21434$) in the mass spectrum and the integrations in the NMR spectrum. Each impure fraction possessed a mass spectrum with a ratio of about 1:1 for the **11ad** to *t*-Bu adduct while the ratio of the relative integrations in the NMR spectrum was 10:1. Because only a few of the final fractions could be isolated that contained pure **11ad**, the yield was a mere 10.5%.

For condition 4, the exact equivalents and conditions of the previously published method were conducted except for THF in place of ether as the solvent for the organolithium formation. These conditions resulted in an improved yield, 45.41%, averaged over five reactions including one reaction conducted using ether as the solvent for the organolithium formation. It was observed that using THF in place of ether did not have a significant effect on the yield, with the four runs conducted in THF averaging a 47.40% yield and the single ether run producing 37.44% yield. The difference of 10% yield was not considered significant given the range of yields observed for the reactions run in THF. Purification of the crude mixture was again difficult, with the major side product again being the *t*-Bu adduct evidenced by a peak at 6.14 ppm in the NMR of crude **11bd** generated by condition 4. The ratio of the product to *t*-Bu adduct ranged from 1.00:0.08 to 1.00:0.15 as determined by integration of the protons corresponding to the vinyl protons.

Since application of the previously reported conditions were also produced significant *t*-BuLi adduct, five organolithium additions between veratraldehyde and 5-Bromo-2,3-difluoroanisole were performed using the previously reported equivalents while varying temperature and stir time to determine the condition that best ensures the complete conversion the aryl halide to the aryl lithium while producing no observable *t*-BuLi adduct (**Table 1.3**). Condition 4 successfully achieved full conversion with no observable *t*-BuLi adduct in the NMR or mass spectrum of the crude product. The NMR spectrum showed no peaks from 9-10 ppm corresponding to the starting aldehyde nor a peak at 0.94 ppm observed in other conditions for the methyl groups of the *t*-Bu group of the *t*-BuLi adduct. In condition 4, after the initial addition of *t*-BuLi at -78 °C, the dry ice-acetone bath was exchanged for a dry ice-acetonitrile bath to achieve the -42 °C temperature and allowed to warm to room temperature while observing the color change. The pale-yellow solution transitioned to a dark green as the reaction warmed up over 55 minutes and a dry ice-acetone bath was reinstalled to lower the temperature back to -78 °C for the addition of the aldehyde. This color change was interpreted to suggest a point where complete conversion of 5-Bromo-2,3-difluoroanisole to 5-lithio-2,3-difluoroanisole had occurred. With the lessons learned from the previous conditions and the test reaction between veratraldehyde and 5-Bromo-2,3-difluoroanisole, condition 5 was employed using 10 equiv. of 5-Bromo-2,3-difluoroanisole and *t*-BuLi, reduced from the 20 equiv. *t*-BuLi with the intention to reduce the amount of *t*-Bu adduct formed. Adjustments to the organolithium formation reaction time were also made. After addition of the *t*-BuLi at -78 °C, the solution was stirred for 5 min followed by raising the temperature to 0 °C for 5 min. After 5 min at 0 °C the solution assumed a dark green color, and the solution was then returned to -78 °C for 5 min before the addition of the aldehyde. Increasing the temperature to 0 °C for 5 min rather than the -40 °C used in the test reaction was done to more quickly achieve the dark green color. This resulted in a yield of 41.7% and a 1.00:0.14 ratio of product to *t*-Bu adduct determined by the integrations in the NMR spectrum of the crude product. The percentage *t*-BuLi adduct was similar to condition 4, however, when the NMR spectra of the crude product between conditions 4 and 5 were compared, peaks at 6.980, 6.75, 6.74, and 3.897 ppm corresponding to 2,3-difluoroanisole were observed. The peaks at 6.980, 6.75, 6.74, and 3.897 ppm matched the spectrum for 2,3-difluoroanisole published by the Spectral Database for Organic Compounds (SDBS)⁴⁶. Additionally, peaks in the electron rich aromatic/alkene region from 6.88-6.98 ppm and the methoxy region were observed, which likely correspond to side products derived from additional reactions with the unreacted 5-

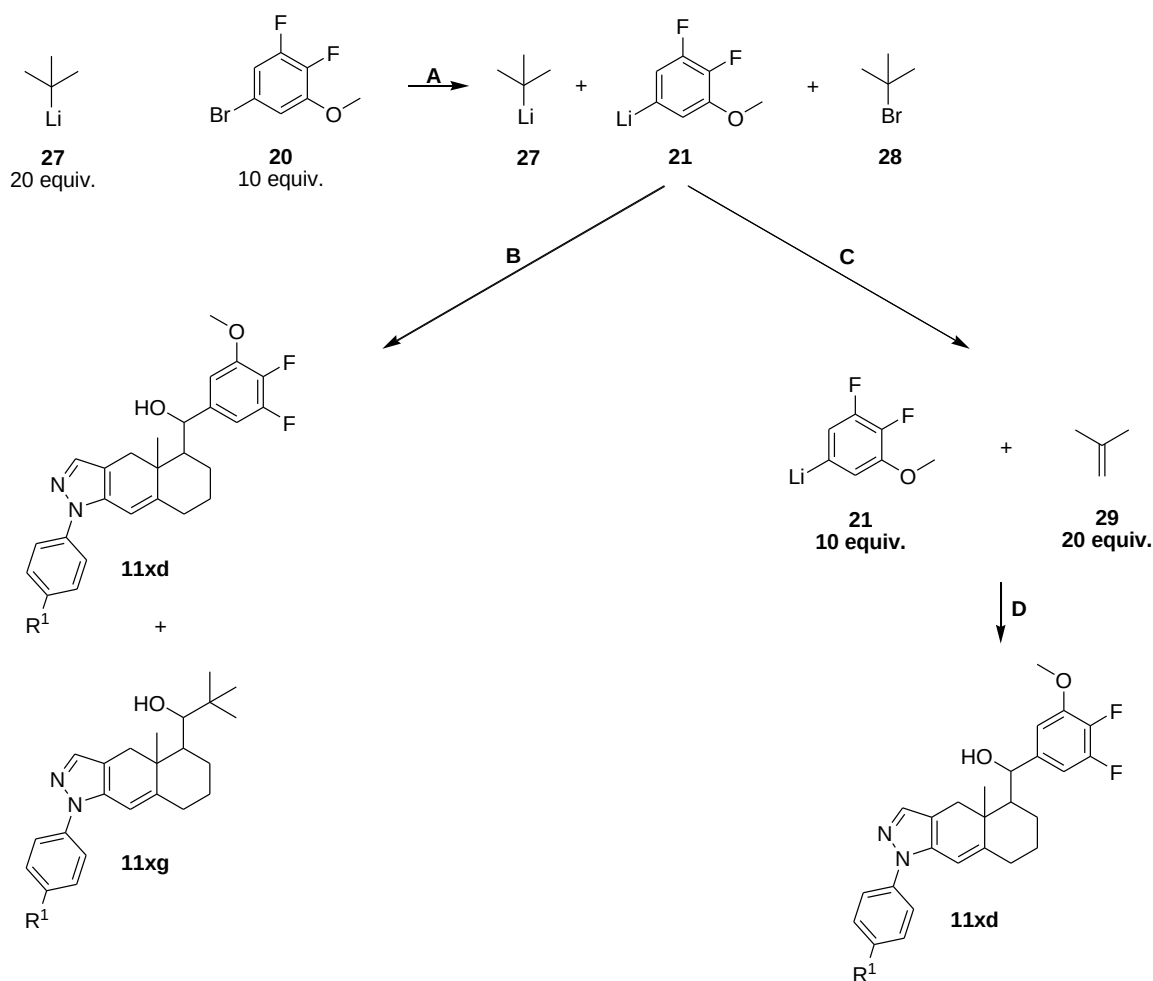
Table 1.3. Optimization of generation and addition of **21** to **22**.



Condition	Organolithium formation	Addition to aldehyde	Color change
1	20 min at -78°C	45 min at -78°C	
2	5 min at -78°C warmed to 0°C for 5 min returned to -78°C for 5 min	60 min at -78°C	dark yellow
3	warmed to -40°C for 20 min	60 min at -78°C	avoided color
4	5 min at -78°C -40°C to r.t. over 55 min, no dry ice added so allowed to warm some	60 min at -78°C	yellow-green
5	5 min at -78°C 5 min at 0°C 5 min at -78°C	60 min at -78°C	

This was performed to determine a temperature gradient to ensure the complete conversion the **20** to the **21** while producing no observable $t\text{-BuLi}$ adduct.

Bromo-2,3-difluoroanisole. The additional side reactions were likely due to the use of a 1:1 equiv. of ArBr:*t*-BuLi rather than the typical 1:2 equiv. ArBr:*t*-BuLi. It was at this point hypothesized that full conversion 5-Bromo-2,3-difluoroanisole was achieved, but the 5 min stir time at the elevated temperature of 0° was not sufficient for reaction complete reaction between *t*-BuLi and *t*-butyl bromide (*t*-BuBr) (**Scheme 1.7**). The aldehyde was exposed to both 5-lithio-2,3-difluoroanisole and *t*-BuLi so the possibility of adducts between both organolithium species was possible. Furthermore, the formation of 2,3-difluoroanisole should have always occurred if any excess 5-lithio-2,3-difluoroanisole existed in solution after addition to the aldehyde. Its observance suggested that reactions conducted using previous conditions employing stir times at -78 °C were very poor at converting 5-Bromo-2,3-difluoroanisole to 5-lithio-2,3-difluoroanisole since none was previously observed. Using 10 equiv. 5-Bromo-2,3-difluoroanisole 15 equiv. *t*-BuLi, condition 6 resulted in a lower yield than condition 5, 31.2% vs 41.7%, but possessed significantly less *t*-Bu adduct relative to previous conditions, a ratio of 1.00:0.05 product:*t*-Bu adduct based on the integrations from the NMR spectrum of the crude product. After addition of the *t*-BuLi at -78 °C, the solution was allowed to warm to 0 °C over the course of 25-30 min upon which a color change from pale to dark yellow was observed and held at 0 °C for 5 min. After 5 min at 0 °C, the solution was returned to -78 °C for 5 min before addition of the aldehyde. The vinyl proton corresponding to the *t*-Bu adduct at 6.21-6.22 ppm was not present in the NMR spectrum of the crude product for condition 6 as it was in condition 5. The darkening of the yellow color at nearly 30 minutes was interpreted as an indication of complete conversion to the aryl lithium. Condition 7, the final optimization, used the same 10 equiv. 5-Bromo-2,3-difluoroanisole and 20 equiv. *t*-BuLi, a standard ratio of 1:2 ArBr:*t*-BuLi, employed in the previously reported synthesis along with an optimized temperature gradient to obtain a



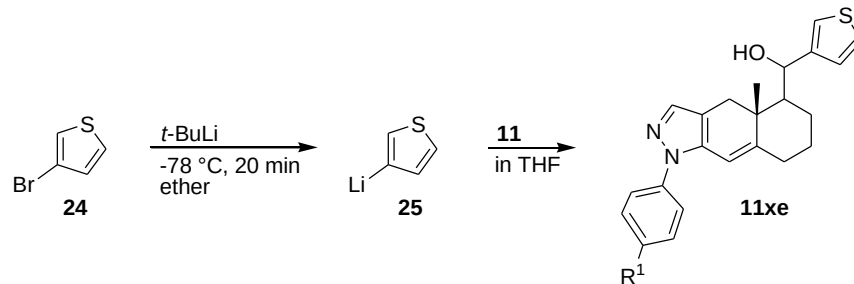
Scheme 1.7. Adequate stir time at elevated temperatures above -78° are hypothesized to be required for the full conversion of **20** to **21** and to ensure no **27** remains upon addition of the aldehyde. **A.** Inadequate stir time between 20 equiv. **27** and 10 equiv. **20**, will create a distribution of species contains two nucleophiles, **27** and **21**. **B.** If aldehyde is added at this point, then two possible product adducts, **11xd** and **11xg**, can be observed. **C.** Additional stir time ensures a complete reaction between **27** and **28**. **D.** Addition of aldehyde after adequate stir time ensures generation of only the adduct of interest, **11xd**.

74.1% yield averaged over two runs with no *t*-Bu adduct observed. After addition of the *t*-BuLi at -78 °C, the reaction was stirred for 20 min at this temperature, raised to 0 °C for 10 min upon which the solution transitioned from pale to dark yellow as in condition 6, then immediately lowered back to -78 °C for 5 min before addition of the aldehyde. The previously observed vinyl peak at 6.21-6.22 ppm the corresponded to the *t*-Bu adduct in conditions 5 and 6 was absent in the NMR spectrum of the crude **11bd** generated from condition 7. The trend of increasing the stir time at elevated reaction temperatures succeeded in avoiding formation of the *t*-BuLi adduct. However, the higher temperatures resulted in an increase of the total number of components as evidenced by the small peaks slightly above the baseline in the vinyl region. The crude mass spectrum also showed the mass for the product of interest with no *t*-Bu adduct present.

Optimization the synthesis of 3-thiophenyl-modified 2° alcohols 11xe (Table 1.4).

In contrast to the difficulty encountered in generating 5-lithio-2,3-difluoroanisole from 5-Bromo-2,3-difluoroanisole, the generation of the 3-lithiothiophene from 3-bromo-thiophene proceeded smoothly using the previously reported conditions. However, the previously reported conditions were insufficient to provide complete addition of 3-lithiothiophene to the aldehyde. Fortunately, the resolution between the product and other components in the crude mixture, including *t*-Bu adducts, could be easily accomplished with standard benchtop flash chromatography. Reaction optimization was conducted, ultimately achieving complete addition after allowing the reaction to warm from -78 °C to 0 °C over the course of 1.5 h (**Table 1.4**). The modification of the previously reported method began after the solution of aldehyde in THF was added to the prepared 3-lithiothiophene at -78 °C. In condition 1, the reaction was performed as previously reported. The average yield over two runs was only 30.7 %, with the NMR spectrum of crude **11ae** depicting incomplete addition of 3-lithiothiophene to aldehyde **10a** as evidenced by peaks at 9.90 and 9.99 ppm which corresponded to unreacted aldehyde. Additionally, other minor, yet significant vinyl protons were observed corresponding to the precursor ketone **8a** (6.28 ppm) and *t*-Bu adduct (6.15-6.17 ppm). It should be noted that at the time of condition 1, the optimization of the Wittig reaction was not complete. Thus, these reactions were conducted with aldehyde starting material containing 5-10% of the ketone **8a** precursor to aldehyde **10a**. Condition 2 called for removal of the ice bath after 45 min at -78 °C, allowing the reaction to warm to room temperature over 20 min. However, the marginally improved yield of 35.8% was likely due to the use of pure aldehyde starting material. Condition 3 saw a significant improvement

Table 1.4. Optimization of the organolithium addition between **24** and **10** was performed.



Conditions	Aldehyde addition	Yield	Product:aldehyde
1	45 min $-78\text{ }^{\circ}\text{C}$	30.7	1.00:0.02
2	45 min at -78 , remove ice bath and stir for 20	35.8	1.00:0.02
3	allowed to warm to r.t. over 1.5 h	79.5	1.00:0.00

in conversion and yield, 79.5%. The NMR spectrum of **11be** suggested complete conversion of the aldehyde since no protons corresponding to aldehyde **10c** starting material was observed. Condition 3 employed a more aggressive temperature gradient. Proceeding addition of the solution of aldehyde dissolved in THF to the prepared 3-lithiothiophene, the dry ice bath was immediately removed, and the reaction was allowed to warm from -78 °C to r.t. over 1.5 h.

Bioassay results. The 36-analogue compound library was tested for their ability to reduce IL-1 β -induced inflammation, an example of transrepression activity, and their propensity for enhancing transcription of pro-inflammatory cytokines via GRE, transactivation, using CCL2 promoter-luciferase promoter, GRE promoter-luciferase promoter, respectively (**Figures 1.1-1.11**). The best candidates from these two bioassays, **11aa** and **13ab**, were further subjected to the GSIS to determine the influence of these molecules on insulin production (**Figures 1.12 and 1.13**). For all CCL2 and GRE bioassay bar graphs, the x-axis reflects the concentration of the analogue on log scale from 0.1 nM to 10 μ M. The CCL2 promoter-luciferase promoter measures the ability of an analogue to represses CCL2 expression induced by the pro-inflammatory cytokine IL-1 β . CCL2 expression has been shown to increase during inflammation, and the CCL2 chemokine is tissue responsible for recruitment of monocytes, macrophages, and T-lymphocytes^{47 48-49}. The bar graphs for this assay depict the gene expression of CCL2, with the black bar being the maximum expression by 1ng/mL IL-1 β , and the white bars represent the repression of the analogue increasing in concentration along the x-axis. The GRE promoter luciferase plasmid is used to measure the ability of each analogue to activate transcription of a GRE. Control in this case is no GC analogue added, represented by the black bars. The for the analogues, represented by the white bars, are expressed as fold over control. All compounds are benchmarked against dexamethasone which at 10 nM, reduces IL-1 β stimulated CCL2 activity by ~70% and produces 10-15 fold activation of the GRE promoter. Among the GC analogues evaluated, four significant structure-activity relationships were discovered. First, ketone analogues with R¹ = F, **11a**, generally possessed poor activity or an undesirable biological activity in terms of reducing inflammation without enhancing transactivation. Second, the 1-(4-fluorophenyl)pyrazole-based series were both more active and possessed analogues with the best biological activity relative to the 4-methyl or 4-methoxy series. Third, 3° alcohol analogues also enhanced transactivation relative to the corresponding 2° alcohol. Additionally, it should be noted that the analogues tested in this study were not single

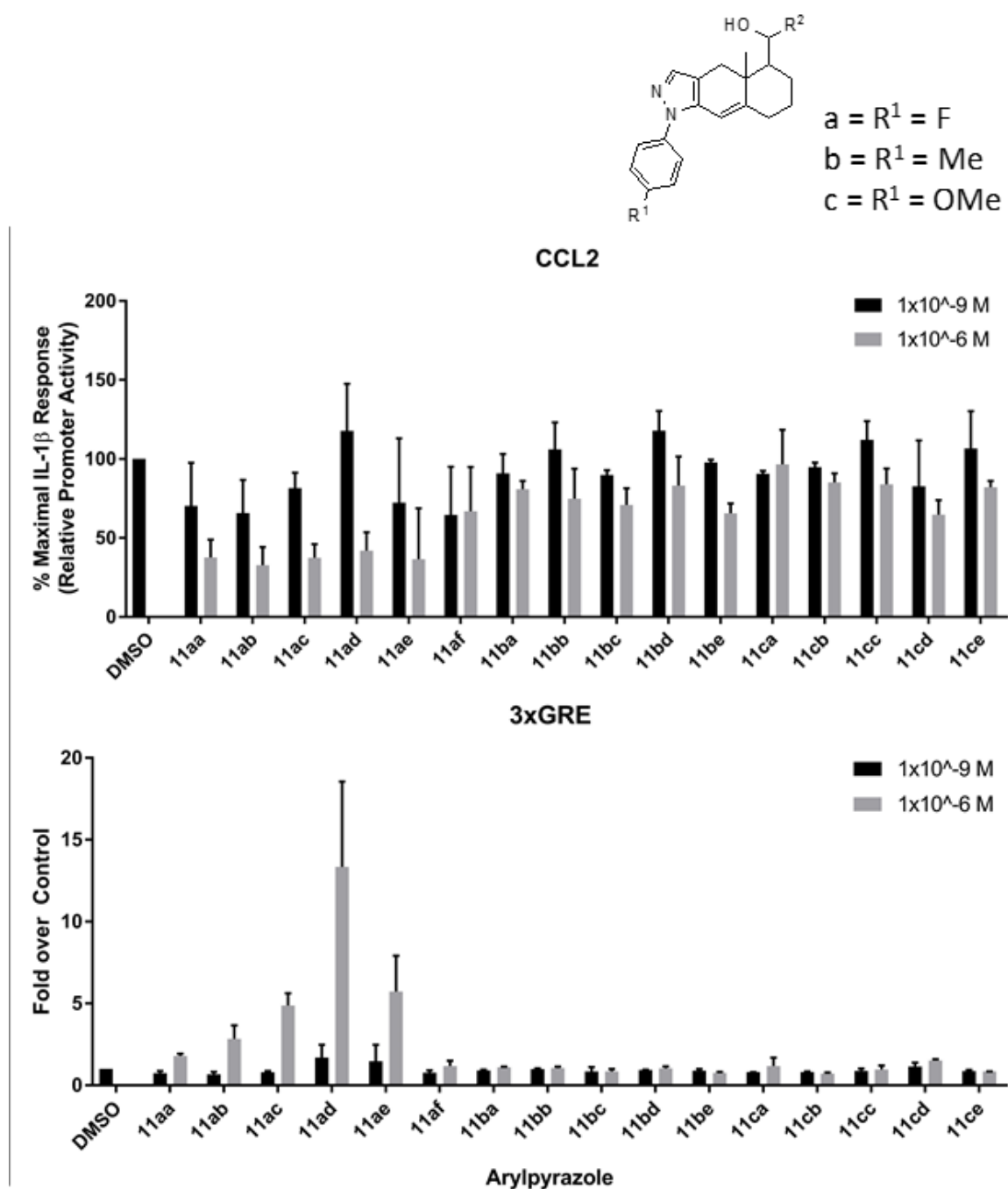


Figure 1.1. CCL2 and GRE promoter-luciferase promoter results for all 2° alcohol analogues 11.

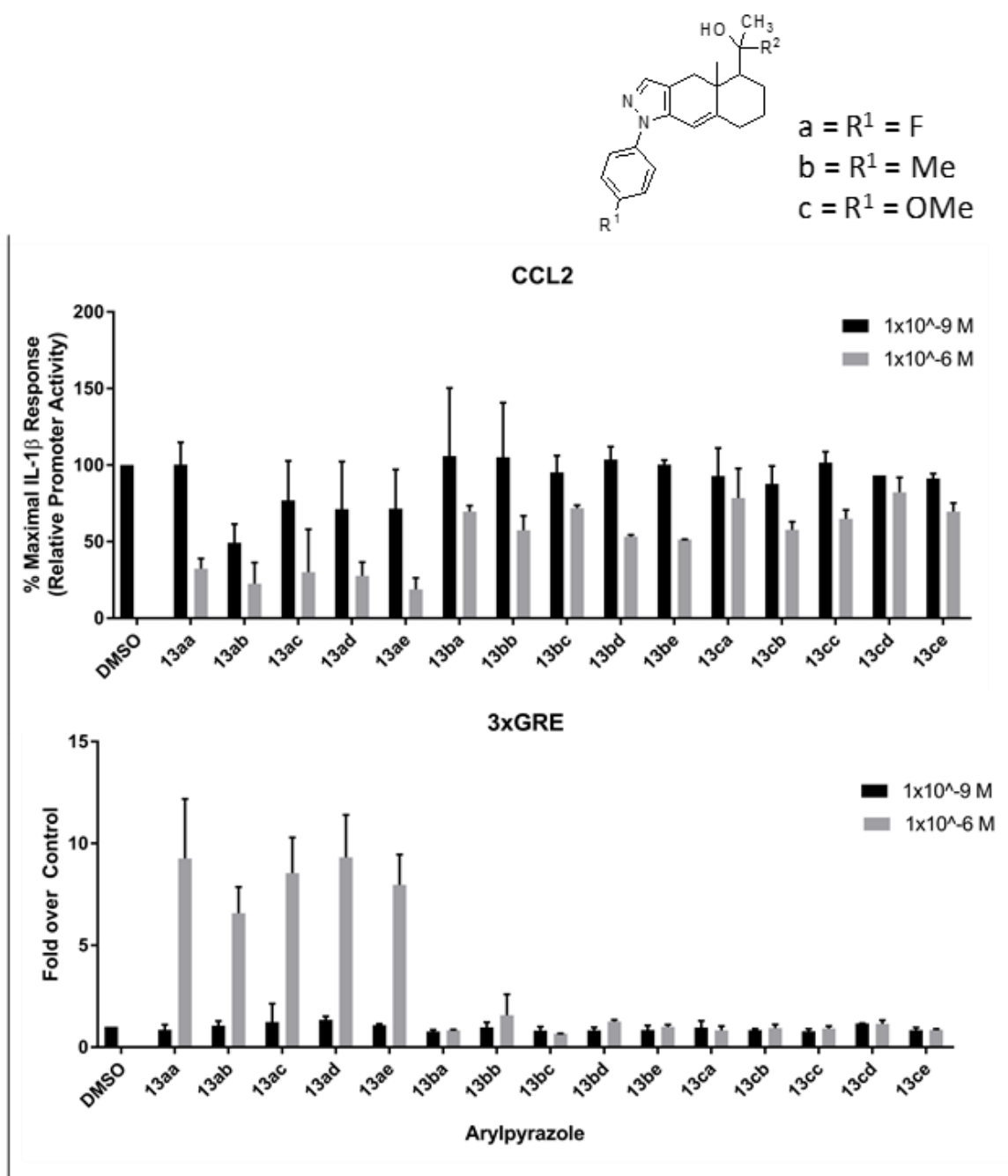


Figure 1.2. CCL2 and GRE promoter-luciferase promoter results for all 3° alcohol analogues 13.

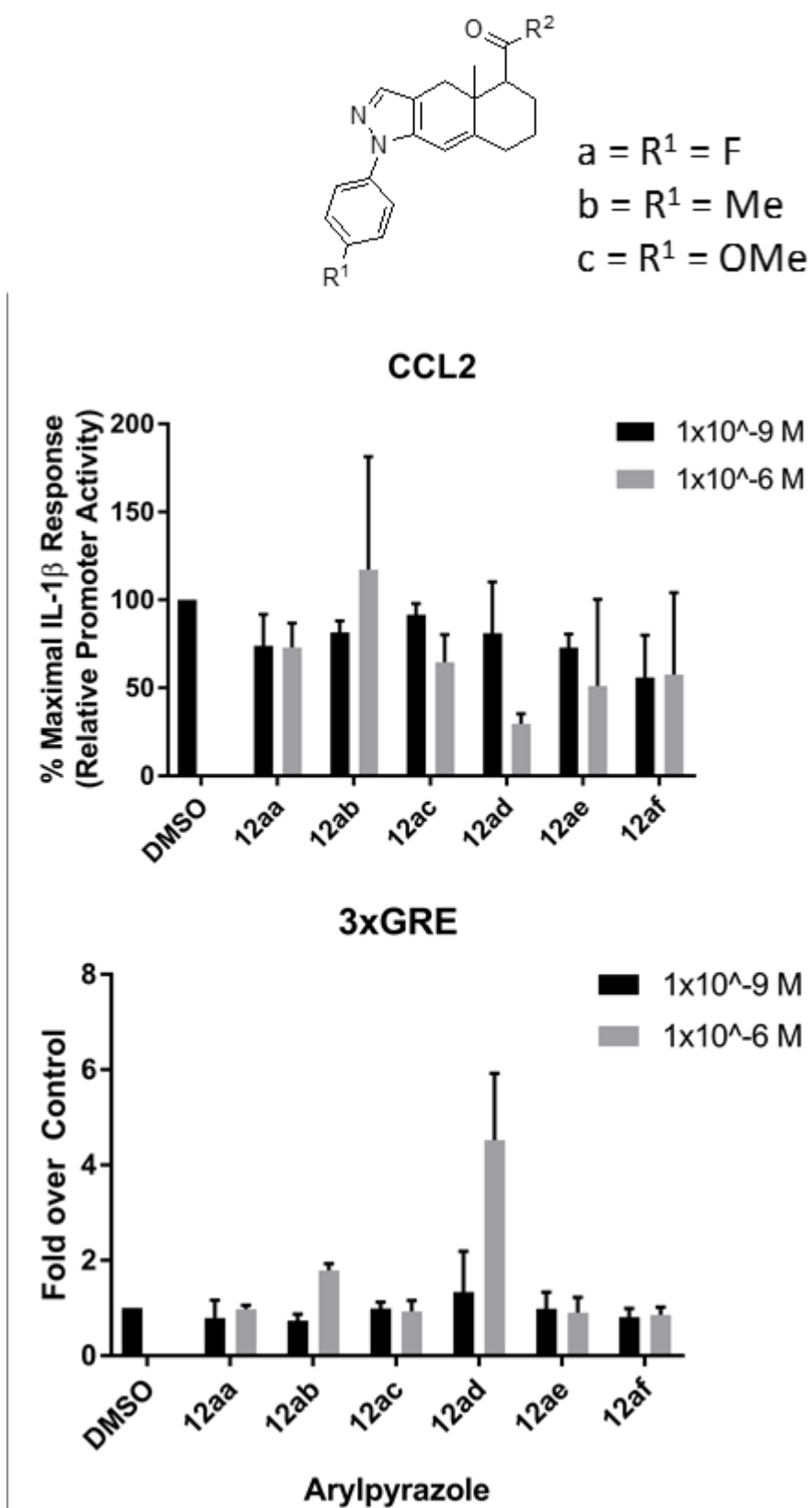
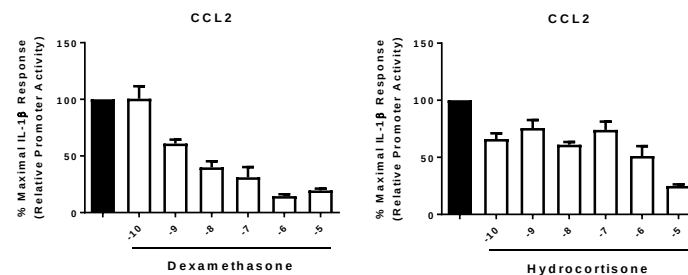
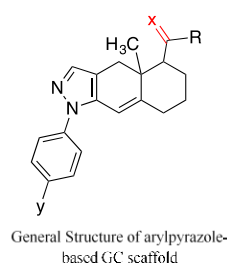


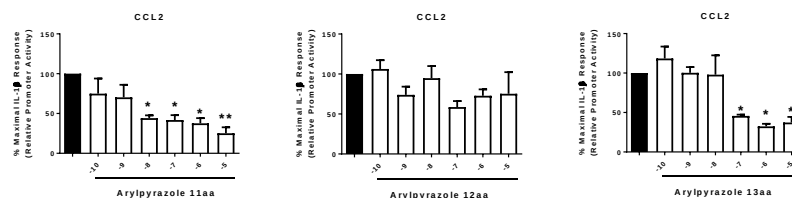
Figure 1.3. CCL2 and GRE promoter-luciferase promoter results for all ketone analogues 12a.



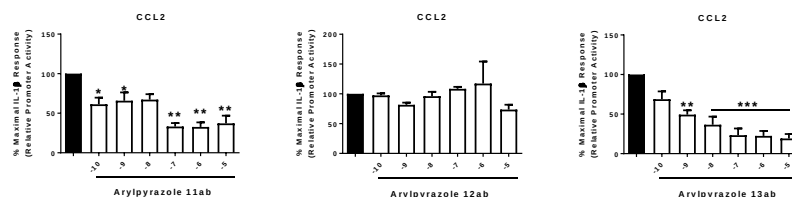
GC Analog



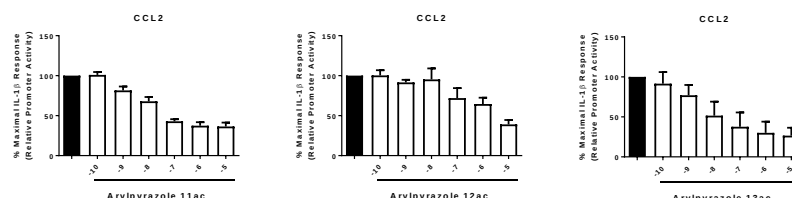
y = fluoro
R = methyl



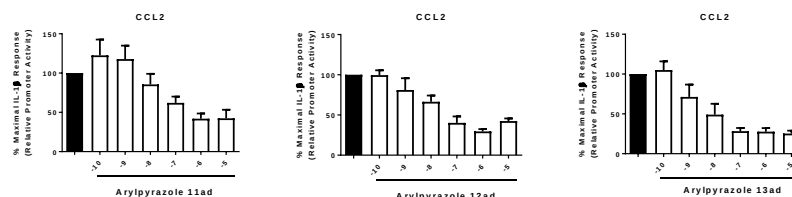
y = fluoro
R = phenyl



y = fluoro
R = 4-fluorophenyl



y = fluoro
R = 2,3-difluoroanisoyl



y = fluoro
R = 3-thiophenyl

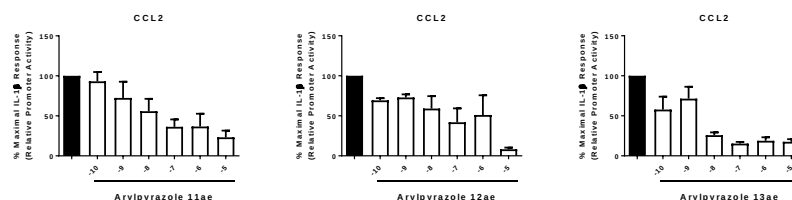


Figure 1.4. Comparison of CCL2 promoter-luciferase promoter results among dexamethasone, hydrocortisone, 2° alcohols 11aa-11ae, ketones 12aa-12ae, and 3° alcohols 13aa-13ae.

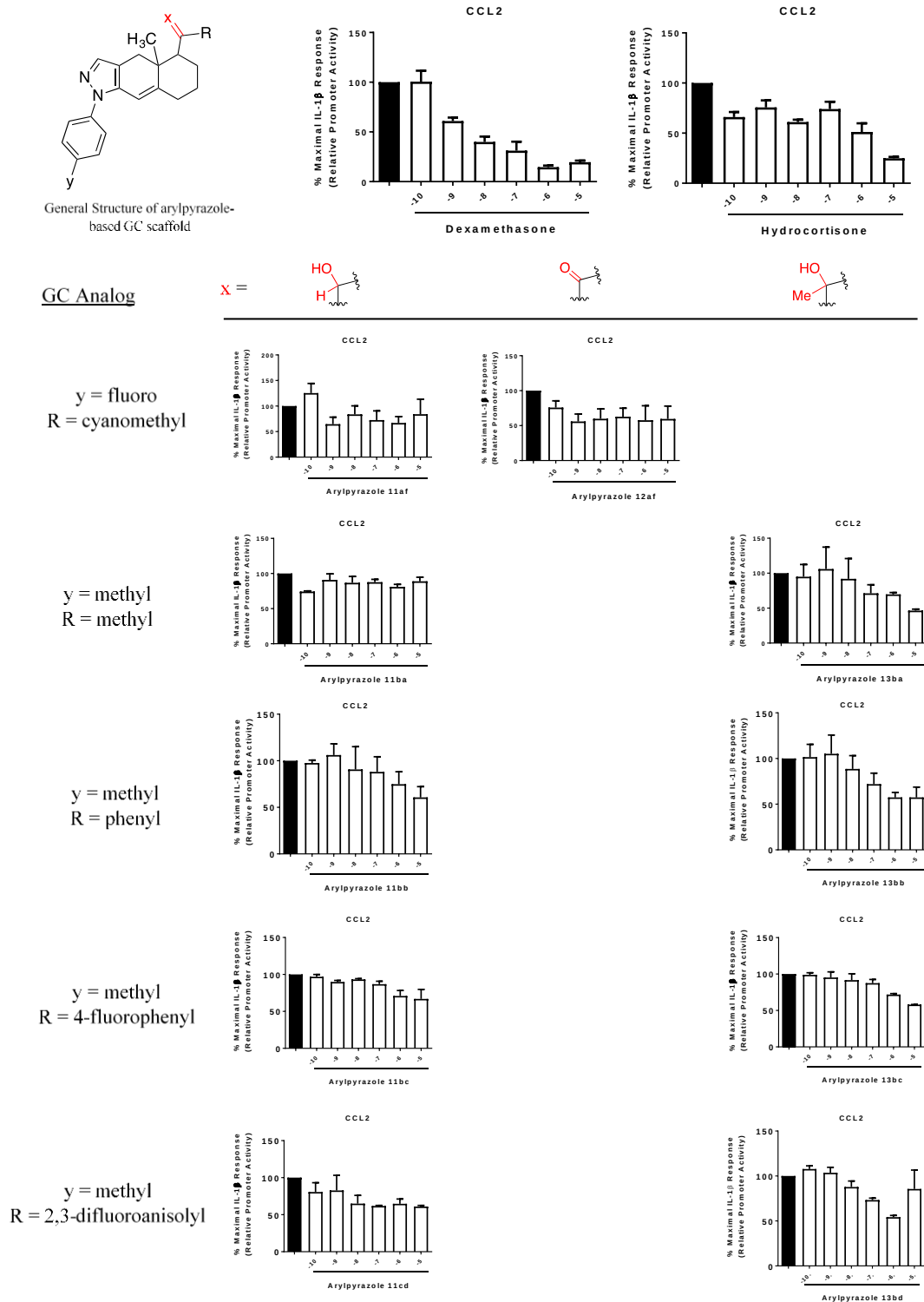
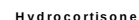
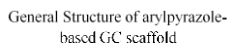
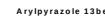
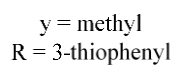


Figure 1.5. Comparison of CCL2 promoter-luciferase promotor results among dexamethasone, hydrocortisone, 2° alcohols **11af-11bd**, ketone **11af**, and 3° alcohols **13af-13bd**.

GC Analog

y = methoxy
R = methyl



y = methoxy
R = phenyl



y = methoxy
R = 4-fluorophenyl



y = methoxy
R = 2,3-difluoroanisoyl

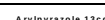


Figure 1.6. Comparison of CCL2 promoter-luciferase promoter results among dexamethasone, hydrocortisone, 2° alcohols 11b and 11ca-11cd, and 3° alcohols 13b and 13ca-13cd.

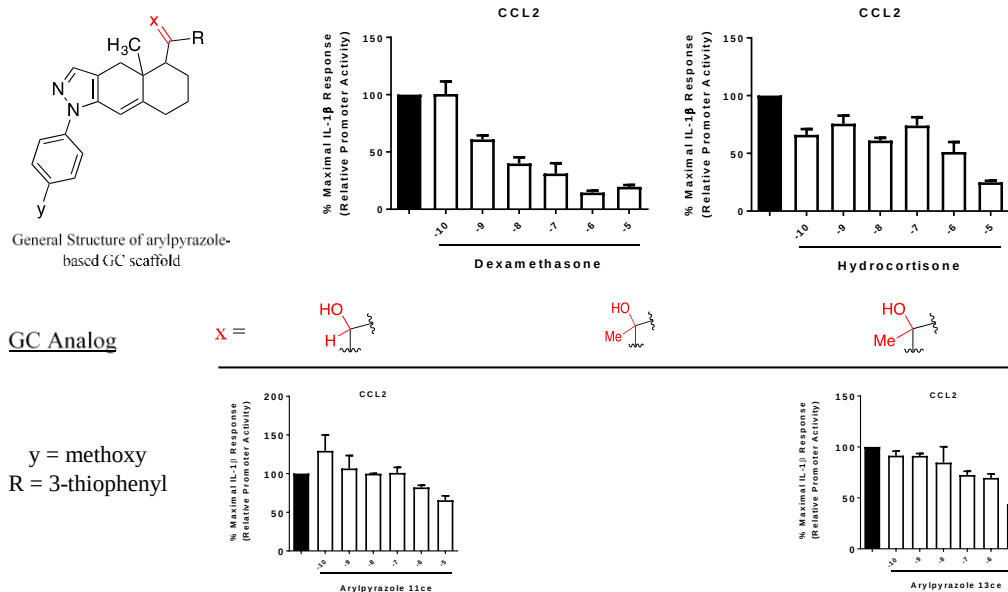


Figure 1.7. Comparison of CCL2 promoter-luciferase promotor results among dexamethasone, hydrocortisone, 2° alcohol **11ce** and 3° alcohol **13ce**.

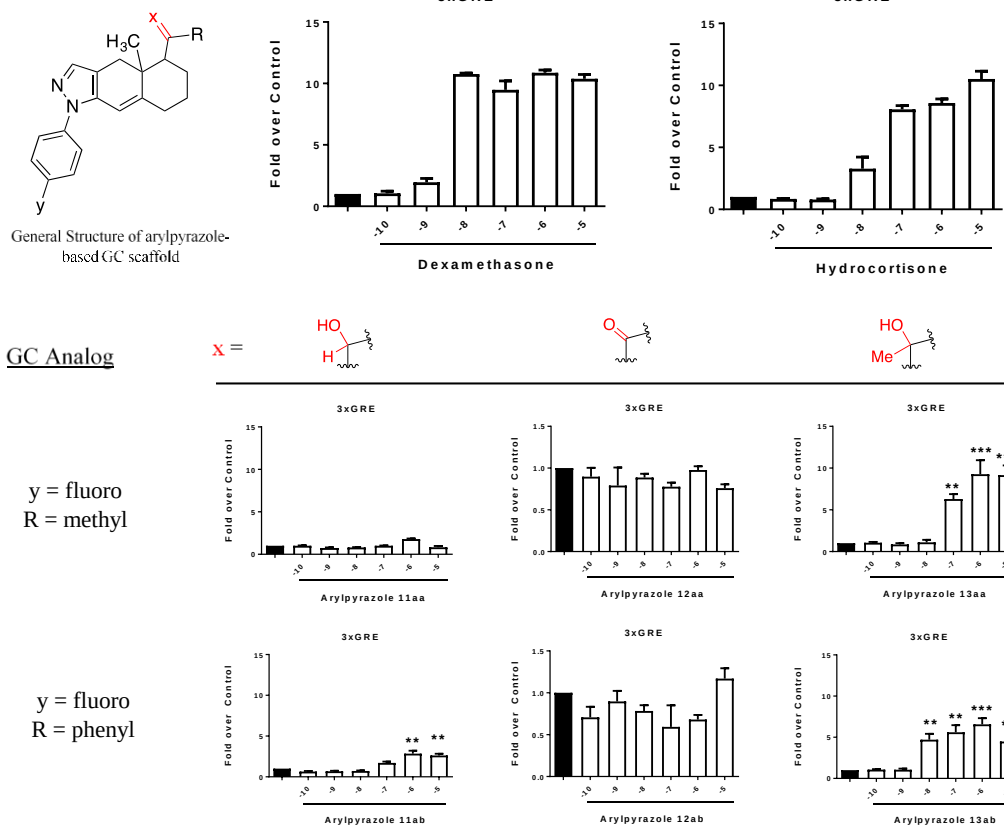
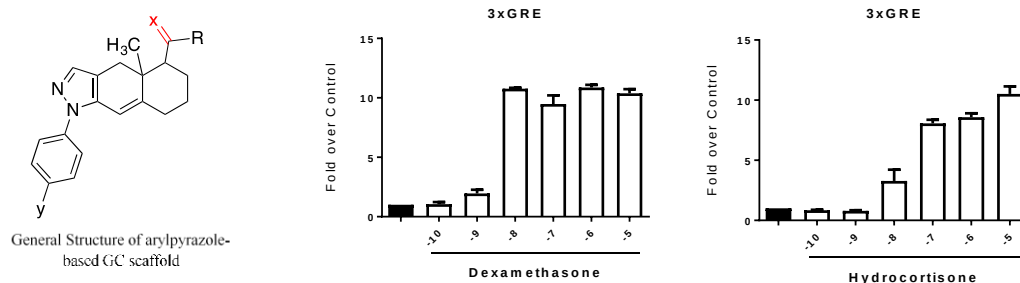


Figure 1.8. Comparison of GRE promoter-luciferase promoter results among dexamethasone, hydrocortisone, 2° alcohols **11aa** and **11ab**, ketones **12aa** and **12ab** and 3° alcohols **13aa** and **13ab**.

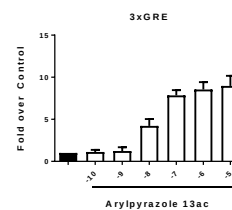
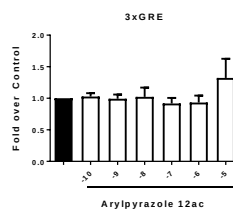
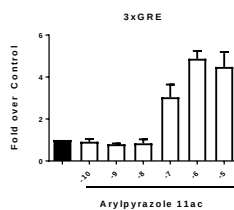


GC Analog

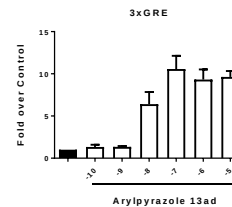
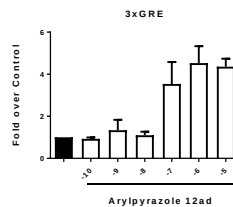
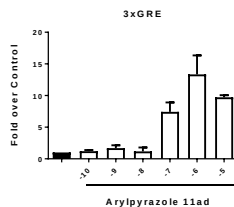
X =



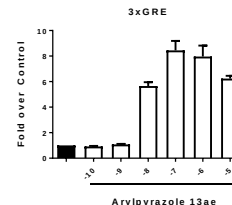
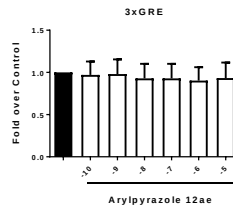
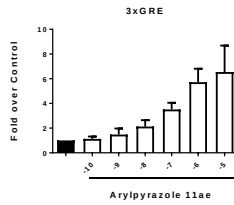
y = fluoro
R = 4-fluorophenyl



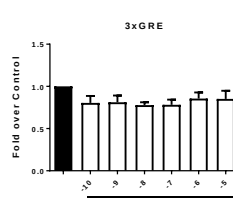
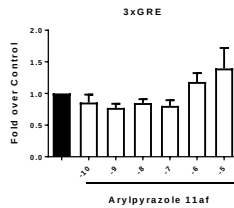
y = fluoro
R = 2,3-difluoroanisyl



y = fluoro
R = 3-thiophenyl



y = fluoro
R = cyanomethyl



y = methyl
R = methyl

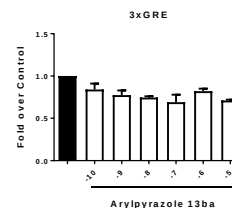
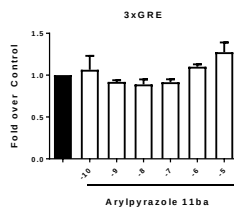
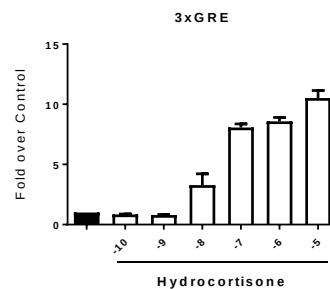
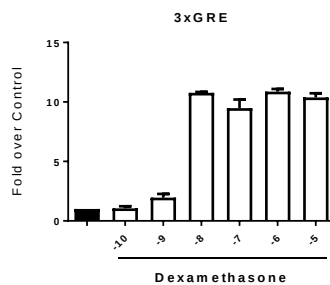
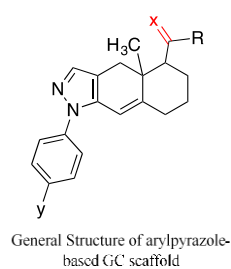


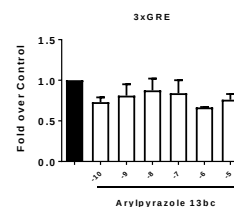
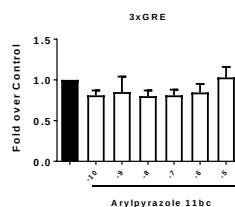
Figure 1.9. Comparison of GRE promoter-luciferase promoter results among dexamethasone, hydrocortisone, 2° alcohols **11ac-11af** and **11ba**, ketones **12ac-12af** and 3° alcohols **13ac-13ae**, and **13ba**.



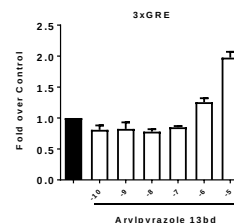
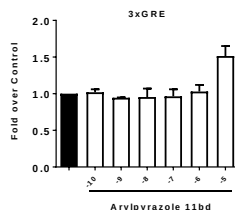
GC Analog



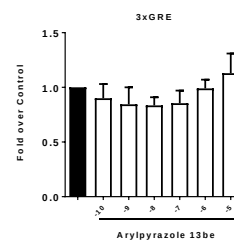
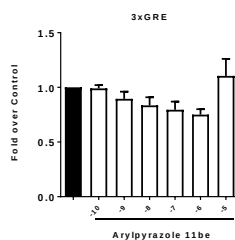
y = methyl
R = 4-fluorophenyl



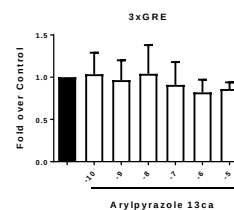
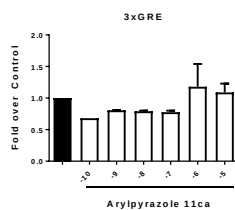
y = methyl
R = 2,3-difluoroanisyl



y = methyl
R = 3-thiophenyl



y = methoxy
R = methyl



y = methoxy
R = phenyl

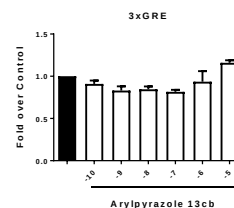
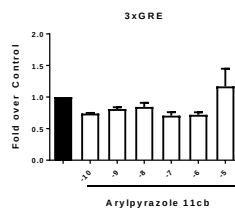
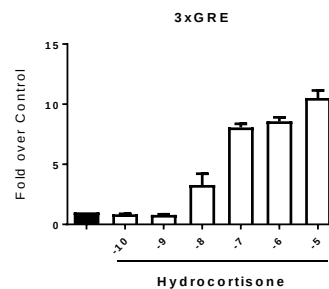
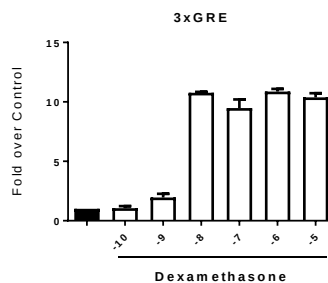
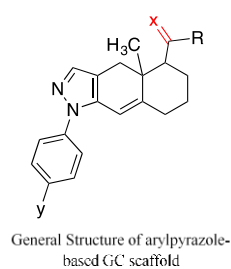


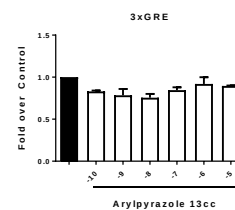
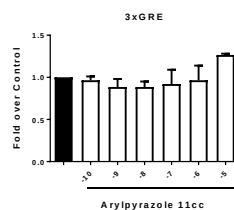
Figure 1.10. Comparison of GRE promoter-luciferase promotor results among dexamethasone, hydrocortisone, 2° alcohols **11bc-11be**, **11ca**, and **11cb** and 3° alcohols **11bc-11be**, **11ca**, and **11cb**.



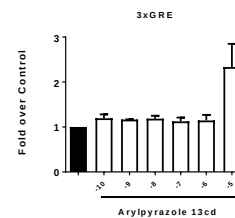
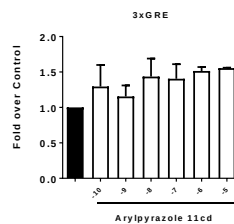
GC Analog



y = methoxy
R = 4-fluorophenyl



y = methoxy
R = 2,3-difluoroanisyl



y = methoxy
R = 3-thiophenyl

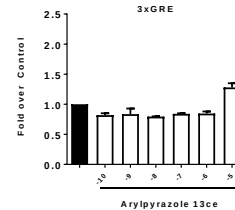
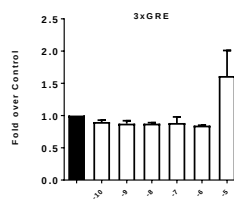


Figure 1.11. Comparison of GRE promoter-luciferase promotor results among dexamethasone, hydrocortisone, 2° alcohols **11cc-11ce** and 3° alcohols **11bc-11be**, **11ca**, and **11cb**.

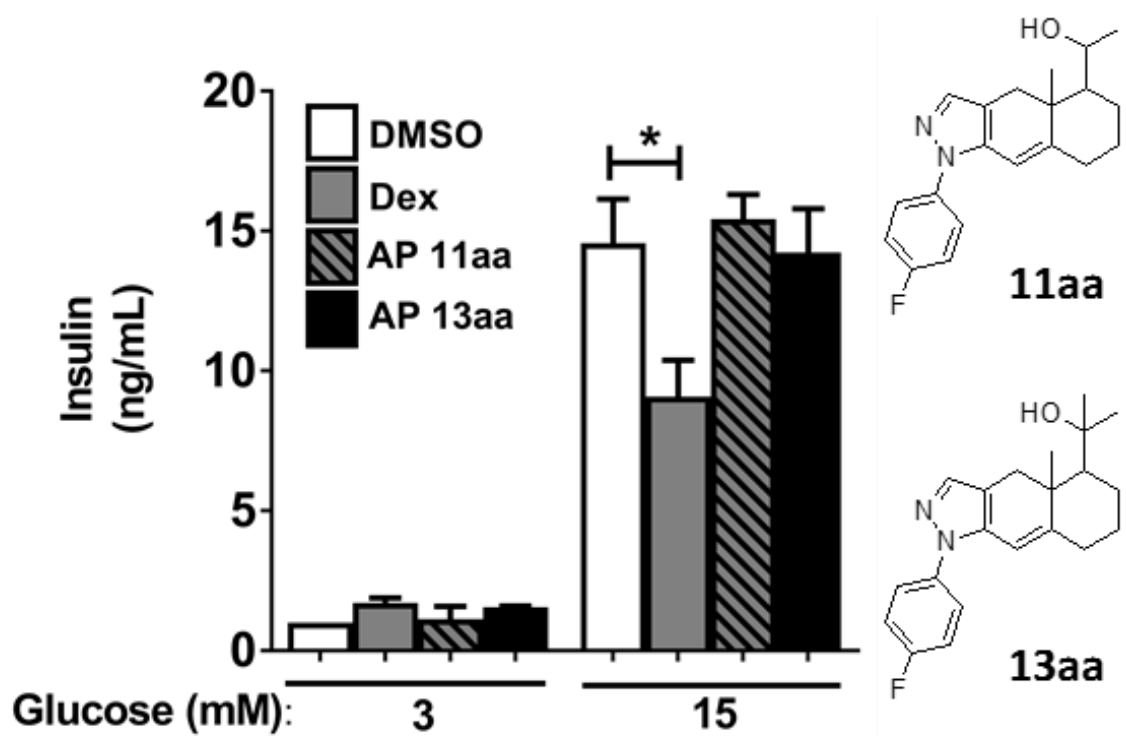


Figure 1.12. GSIS is improved in mouse islets treated with GC analogues **11aa** and **11ab** relative to dexamethasone for islets cultured overnight in the presence of 1 μ M of **11aa** or 10 nM of dexamethasone followed by static incubation in 15 mM glucose.

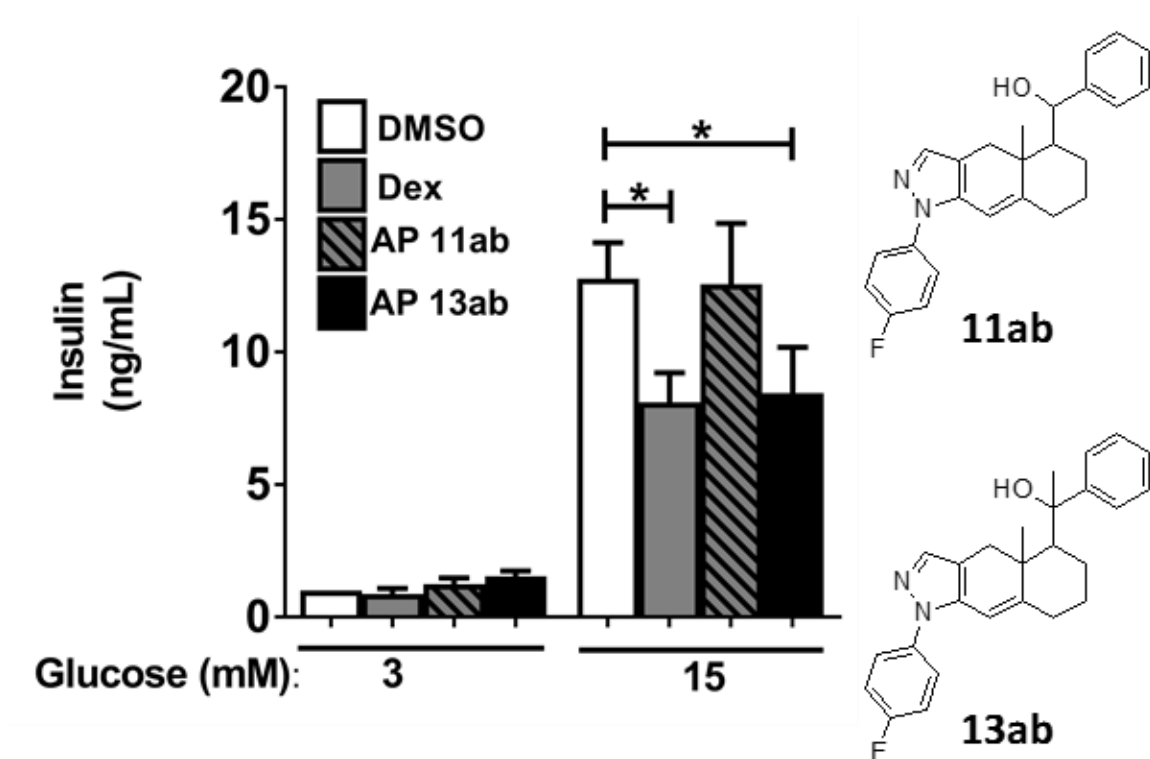


Figure 1.13. GSIS is improved in mouse islets treated with GC analogues **11ab** relative to dexamethasone for islets cultured overnight in the presence of 1 μ M of **11ab** or 10 nM of dexamethasone followed by static incubation in 15 mM glucose.

enantiomers. The previously reported study by Merck did not rely on synthetic innovation to obtain single enantiomers during the generation of the aldehyde 10 and the secondary alcohols 11, each reaction introducing a stereocenter. 10 was obtained as an 8:1 mixture of diastereomers. For the 11, the researchers at Merck reported that small amounts of minor diastereomers were removed using standard benchtop flash chromatography while in other cases, chiral HPLC was necessary to remove diastereomeric impurities. This means that the compounds produced in this study were similarly tainted and tested with small amounts of the diastereomeric impurity.

Ketone analogues with $R^1 = F$, 11ax, generally possessed the least desirable biological activity (Figure 1.3). Ketone analogues with $R^1 = F$, 11a, represent the group that generally possesses the least desirable biological activity. Analogues 12aa, 12ab, 12ac, 12ae, and 12af did not significantly reduce IL-1 β -induced CCL2 expression relative to control at 1 nM. Increasing concentration to 1 μ M did not significantly improve CCL2 expression, but increased transactivation of 12ab and 12ad was observed. Analogue 12ad was more active at 1 μ M in both transrepression and transactivation assays. Because the activities of ketone analogues 12aa-12af showed little promise, ketones in the 4-methyl and 4-methoxy series were not subjected to biological evaluation. It seems that the active site of the GR requires sp^3 -geometry to accomplish coordination of the alcohol and R^2 substituent to the GR active site to elicit a dissociated profile.

Analogues with $R^1 = F$ were the most active and analogues possessed the most desired biological activity (Figures 1.1 and 1.2). For both 2° and 3° alcohol analogues, 11aa-11af and 13aa-13ae, respectively, $R^1 = F$ was correlated to an increase in activity for both transrepression and transactivation assays. This structural feature is the most significant in terms of improving activity. Most notably, it was only the 2° alcohol analogues with $R^1 = F$ that reduced CCL2 expression at 1 nM. At 1 μ M, CCL2 expression was further reduced with an accompanying enhancement of transactivation. The increase in transactivation for 2° alcohols $R^1 = F$ is significantly higher when $R^2 = 3$ -thiophenyl or 1-(3,4-difluoro-5-methoxyphenyl), which were also found to be most active in the general functional assays of transrepression and transactivation in the previously reported study conducted by Merck Research Laboratories.⁴ Both 4-methyl and 4-methoxy series 2° and 3° alcohol analogues did not significantly reduce CCL2 expression at 1 nM, but possessed marginally improved activity at 1 μ M. A lesser correlation between inclusion of an $R^2 = 3$ -

thiophenyl or 1-(3,4-difluoro-5-methoxyphenyl) and increased activity also exists for the 4-methyl and 4-methoxy series. This difference in activity compared to the R^1 = fluorophenyl series may be attributable to a structure-activity relationship or perhaps a difference in solubility between the analogues being that fluorinated drugs are known for their increased lipid-solubility making them more easily transported throughout the cell⁵⁰. Thus, the 4-fluoro analogue series may have a greater partial agonist profile for the GR or may just be more easily transported throughout the cell. Ultimately, 2° alcohol analogues with R^1 = F possessed analogues the greatest reduction of CCL2 expression, about 50% or less maximal IL-1 β response, response relative to enhancement of transactivation, which was about 5-fold or less enhancement of gene production at the GRE relative to control. However, this dissociated activity was only observed at micromolar concentrations. Further modifications that reduced CCL2 expression did so at the cost of greatly enhancing transactivation, which is not the desired biological activity.

3° alcohol analogues enhanced activity relative to the corresponding 2° alcohol (Figures 1.1 and 1.2). Nearly every 3° alcohol analogue, except for 13ad, possessed enhanced transrepression activity relative to the corresponding 2° alcohol analogue at both 1 nM and 1 μ M. For 3° alcohol analogues with R^1 = methyl or methoxy, the improvement was significant, accompanied by minimal enhancement of transactivation, but a marginal reduction of CCL2 expression could only be obtained with micromolar concentrations. At micromolar concentrations, the 3° alcohol analogues possessing R^1 = F reduced CCL2 gene expression to the greatest extent, less than 50% maximal IL-1 β response, but not without significant enhancement of transactivation, which was approaching 10-fold. The additional steric bulk around the alcohol introduced by a methyl group in 3° alcohol analogues seems to increase activity at the cost of selectivity for 3° alcohol analogues with R^1 = F, which makes 3° alcohol analogue candidates corresponding to the already active 2° alcohols with R^1 = F undesirable.

Compounds 11aa and 11ab possessed the most desirable biological activity (Figures 1.12 and 1.13). Of the molecules screened for their biological activity, two of the compounds synthesized displayed a markedly better directionality of therapy. Reduction of IL-1 β -induced CCL2 gene expression by Compounds 11aa and 11ab at 1 μ M were comparable to dexamethasone at 10 nM while increasing gene activation only 2-3 fold relative to 10-15 fold for dexamethasone. Both compounds 11aa and 11ab maintained

insulin levels at the same level as the control, whereas dexamethasone reduced insulin levels about 40%. However, these activities of 11aa and 11ab are at two orders of magnitude higher than dexamethasone.

Conclusions, implications, and future directions. This results of this study suggest that previously reported non-steroidal 1-(4-fluorophenyl)pyrazole-based glucocorticoid analogues may possess a greater efficacy than previously thought in terms of the ability of specifically reducing inflammation of beta cell islets without an accompanying enhancement of undesired gene production or reduction of insulin secretion. These activities indicate that this class of molecules may be potential candidates for a T1DM therapy. Additionally, these compounds may prove useful alongside other treatments more generally aiming to reduce beta cell inflammation with reduced side effects. This study provides synthetic optimizations of the previously reported synthesis which allow gram-scale generation of a more diverse series of 1-(4-fluorophenyl)pyrazole-based glucocorticoid analogues. However, one limitation was that the desired activities were observed at micromolar scale, which increases the potential for undesired activities. Further studies should be conducted using different bioassays to determine the extent of side effects other than the two measured in this study, both in terms of side effects on the beta cell, such as promoting insulin resistance, as well as the effects on the immune cells that are also associated in the process of beta cell inflammation, dysfunction, and death. Measurements such as these, and the desire to evaluate the effect of these compounds in mice. Furthermore, a greater scope of R^1 and R^2 modification of the 1-(4-fluorophenyl)pyrazole-based scaffold will be synthesized in an attempt to synthesize compounds with enhanced anti-inflammatory activities.

1.5 Experimental synthetic procedures

Chemistry. Chemical reagents were purchased commercially and used without further purification. Dry ethereal solvents were obtained by distillation from a potassium benzophenone ketyl still. All other dry solvents were distilled from $\text{CaH}_{2(s)}$. Thin-layer chromatography (TLC) was carried out using Sorbent Technologies silica G F₂₅₄ TLC plates. All compounds of interest were visualized as single spots using short-wave UV light; more permanent staining of TLC spots was achieved using p-anisaldehyde, vanillin, or ceric ammonium molybdate (Hannesian's) TLC staining solutions. Silica gel with a 60A pore size and a particle diameter of 40-63 μm was used for flash chromatography.

^1H -NMR spectra were obtained on either an Oxford 300 MHz or Varian InNova 500 MHz NMR spectrometer in solutions of CDCl_3 . All ^{13}C -NMR spectra were recorded on a Varian InNova 500 MHz NMR spectrometer in solutions of CDCl_3 . High resolution mass spectrometry (HRMS) for characterization of previously unreported compounds was performed using a JEOL DART source in positive ion mode coupled to a JEOL AccuTOF JMS-T100LC mass spectrometer. HRMS for characterization of crude mixtures was performed using the JEOL DART source in positive ion mode coupled to a JEOL AccuTOF JMS-T100LC mass spectrometer or a Sciex Turboionspray electrospray ionization source in positive mode coupled to an Applied Biosystems QStar Elite mass spectrometer. Optical rotation was measured using a Perkin-Elmer 241 polarimeter equipped with a sodium source lamp. IR spectra were collected using a Nicolet iS5 spectrophotometer. All characterization data can be found in the supplemental information.

Synthetic methods. Synthetic methods, chromatographic methods, and characterization data for intermediates and the previously reported 1-(4-fluorophenyl)pyrazole-based GC analogues will not be shown except for GC analogues which were subjected to biological testing.

Intermediate 6: The synthesis was performed up to this point using the previously reported synthesis of 1-(4-fluorophenyl)pyrazole-based GC analogues ⁴.

General method for the addition of the 4-substituted headgroup to obtain compound series (7): To a solution of **6** (5.0 g, 20 mmol) in glacial acetic acid (50 mL) was added the

appropriate 4-substituted phenyl hydrazine HCl (23 mmol) and sodium acetate (1.88 g, 23 mmol) and allowed to stir for 2 h. The reaction mixture was then slowly and carefully poured into an actively stirred solution of saturated sodium bicarbonate cooled to 0 °C. 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 to give a viscous orange oil.

4a-methyl-1-(p-tolyl)-1,4,4a,6,7,8-hexahydrospiro[benzo[f]indazole-5,2'-[1,3]dioxolane]

(7a): Flash chromatography conditions, 20% EtOAc/hexanes. Yield = 44%. TLC R_f = .28 (30% EtOAc/hexanes). HRMS [M+H]⁺ = . ¹H-NMR (CDCl₃, 500 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]

(7b): Flash chromatography conditions, 25% EtOAc/hexanes. Yield = 38%. TLC R_f = .27 (50% EtOAc/hexanes). HRMS [M+H]⁺ = . ¹H-NMR (CDCl₃, 500 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 method for the acetal deprotection for compound series (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 poured into saturated sodium bicarbonate, 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 to give a dull orange solid.

4a-methyl-1-(p-tolyl)-1,4,4a,6,7,8-hexahydro-5H-benzo[f]indazol-5-one **(8b)**: Flash chromatography conditions, 15% EtOAc/hexanes. Yield = 72%. R_f = 0.34 (30% EtOAc/hexanes). HRMS [M+H]⁺ = . ¹H-NMR (CDCl₃, 500 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)**: Flash chromatography conditions, 20% EtOAc/hexanes. Yield = 77%. R_f = 0.28 (40%

EtOAc/hexanes). HRMS $[M+H]^+$ = . $^1\text{H-NMR}$ (CDCl_3 , 500 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 method for the Wittig-hydrolysis sequence to obtain aldehyde compound series

(10): A commercially available solution of 0.5 M 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 triphenylphosphonium chloride (6.81 g, 19.8 mmol) in THF (65 mL) cooled to $-40\text{ }^\circ\text{C}$. The solution was warmed to $0\text{ }^\circ\text{C}$ 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 **8** (6.6 mmol) in THF (16 mL) was transferred dropwise via canula. 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, concentrated in vacuo, and subjected to flash chromatography (15% EtOAc/hexanes). Fractions containing enol **9** either were concentrated in a round bottom flask, and dissolved in THF (60 mL). A solution of 4 M HCl (16 mL) was slowly added to the prepared solution of **9** as it was being actively stirred. After the reaction was allowed to stir at room temperature for 36 h, the reaction mixture was diluted with EtOAc (100 mL), washed with water, saturated NaHCO_3 , and brine. The pooled organic layer was dried over MgSO_4 , filtered, and concentrated in vacuo to give crude aldehyde **10** as a beige solid. Purification of the crude product was accomplished using flash chromatography.

4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzoflindazole-5-carbaldehyde (10b):

Flash chromatography conditions, 15% EtOAc/hexanes. Yield = 40.0 %. R_f = 0.31 (25% EtOAc/hexanes). HRMS $[M+H]^+$ = . $^1\text{H-NMR}$ (CDCl_3 , 300 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-benzoflindazole-5-

carbaldehyde (10c): Flash chromatography conditions, 20% EtOAc/hexanes. Yield = 79.1 % R_f = 0.28 (40% EtOAc/hexanes). HRMS $[M+H]^+$ = . $^1\text{H-NMR}$ (CDCl_3 , 500 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 method for the preparation of R₂ = methyl 2° OH analogues (**11aa**, **11ba**, **11ca**): A commercially available solution of 1.6 M MeLi in diethyl ether (312 μ L, 0.5 mmol) was transferred to a round bottom flask and cooled to -78 °C. A solution of the appropriate aldehyde **10** in THF 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 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 to give a white solid.

1-(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzof[*l*]indazol-5-yl)ethan-1-ol (**11ba**): Flash chromatography conditions, 15% EtOAc/hexanes. Yield = 50%. R_f = 0.16 (30% EtOAc/hexanes). HRMS (DART-RTOF) m/z: [M+H]⁺ Calcd for C₂₁H₂₇N₂O, 323.21179; found 323.21001. ¹H-NMR (CDCl₃, 500 MHz): 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)

1-(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*l*]indazol-5-yl)ethan-1-ol (**11ca**): Flash chromatography conditions, 15% EtOAc/hexanes. Yield = 58%. R_f = 0.11 (25% EtOAc/hexanes). HRMS (DART-RTOF) m/z: [M+H]⁺ Calcd for C₂₁H₂₇N₂O₂, 339.20670; found 339.20344. ¹H-NMR (CDCl₃, 500 MHz): 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).

General method for the preparation of R₂ = phenyl 2° OH analogues (**11ab**, **11bb**, **11cb**): A commercially available solution of 1.9 M PhLi (3.42 mL, 6.5 mmol) in di-butyl ether was transferred to a round bottom flask and cooled to -78 °C. A solution of the appropriate aldehyde **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.

(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)(phenyl)methanol (**11bb**). From 21 using PhLi (1.9 M solution in di-n-butyl ether). Flash column conditions, 15% EtOAc/hexanes, to give a white solid. Yield = 28%. TLC R_f = 0.20 (30% EtOAc/hexanes). HRMS (DART-RTOF) *m/z*: [M+H]⁺ Calcd for C₂₆H₂₉N₂O, 385.22744; found 385.22612. ¹H-NMR (CDCl₃, 500 MHz): 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).

(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)(phenyl)methanol (**11cb**): From 21 using PhLi (1.9 M solution in di-n-butyl ether). Flash column mobile phase – 20% EtOAc/hexanes, to give a beige solid. Yield = 73 %. TLC R_f = 0.11 (30% EtOAc/hexanes). HRMS (DART-RTOF) *m/z*: [M+H]⁺ Calcd for C₂₆H₂₉N₂O₂, 401.22235; found 401.21952. ¹H-NMR (CDCl₃, 500 MHz): 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).

General method for the preparation of R₂ = 4-fluorophenyl 2° OH analogues (**11ac**, **11bc**, **11cc**): A commercially available solution of 2.0 M 4-fluorophenylmagnesium bromide in diethyl ether was transferred to a round bottom flask and cooled to -78 °C. A solution of the appropriate aldehyde **10** in THF 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 to give a white solid.

(4-fluorophenyl)(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)methanol (**11bc**): Flash chromatography conditions, 10% EtOAc/hexanes. Yield = 11.5 %. R_f = 0.24 (25% EtOAc/hexanes). HRMS (DART-RTOF) *m/z*: [M+H]⁺ Calcd for C₂₆H₂₈FN₂O, 403.21802; found 403.21649. ¹H-NMR (CDCl₃, 500 MHz): 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).

(4-fluorophenyl)(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*f*]indazol-5-yl)methanol (**11cc**): Flash chromatography (15% EtOAc/hexanes). Yield = 69%. R_f = 0.26 (30% EtOAc/hexanes). HRMS (DART-RTOF) m/z : $[M+H]^+$ Calcd for $C_{26}H_{28}FN_2O_2$, 419.21293; found 419.20660. ^1H -NMR ($CDCl_3$, 500 MHz): 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).

General method for the preparation of R^2 = 2,3-difluoroanisoyl 2°OH analogues (**11ad**, **11bd**, **11cd**): A solution of 5-Bromo-2,3-difluoroanisole (4.6 mmol) was prepared in either THF (10 mL) or diethyl ether, cooled to $-78^\circ C$, 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 $-78^\circ C$, raised to $0^\circ C$ for 5-10 min until the solution transitioned from pale yellow to dark yellow-green, and then immediately lowered back to $-78^\circ C$ for 5 min. A solution of the appropriate aldehyde **10** (0.46 mmol) in THF (2 mL) was added dropwise via canula, and the reaction was stirred at $-78^\circ C$ for 1 h. 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 to give a white solid.

(3,4-difluoro-5-methoxyphenyl)(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*f*]indazol-5-yl)methanol (**11ad**): Flash chromatography, 30% EtOAc/hexanes. Yield = 61.9 %. R_f = (% EtOAc/hexanes). ^1H -NMR ($CDCl_3$, 300 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-benzof[*f*]indazol-5-yl)methanol (**11bd**): Flash chromatography conditions, 10% EtOAc/hexanes. Yield = 76.7 %. R_f = 0.22 (25% EtOAc/hexanes). HRMS (DART-RTOF) m/z : $[M+H]^+$ Calcd for $C_{27}H_{29}F_2N_2O_2$, 451.21916; found 451.21790. ^1H -NMR ($CDCl_3$, 500 MHz) 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).

(4-fluorophenyl)(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*h*]indazol-5-yl)methanol (11cd): Flash chromatography conditions, 15% EtOAc/hexanes. Yield = 71.4 %. R_f = 0.16 (30% EtOAc/hexanes). HRMS (DART-RTOF) m/z : $[M+H]^+$ Calcd for $C_{27}H_{29}F_2N_2O_3$, 467.21408; found 467.21488. ^1H -NMR ($CDCl_3$, 500 MHz) 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).

General method for the preparation of R^2 = 3-thiophenyl 2°OH analogues (11ae, 11be, 11ce): A solution of 3-bromo thiophene (0.6 mL, 6.4 mmol) was prepared in either THF (10 mL) or diethyl ether, cooled to $-78^\circ C$, a commercially available solution of 1.9 M *t*-BuLi (6.8 mL, 12.8 mmol) in pentanes was added dropwise via syringe. After the reaction stirred for 30 min at -78° , a solution of the appropriate aldehyde **10** in THF (6.4 mmol) 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 $-78^\circ C$, 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 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 to give a white solid.

(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*h*]indazol-5-yl)(thiophen-3-yl)methanol (11ae): Flash chromatography condition, 10% EtOAc/hexanes. Yield = 35.8 %. R_f = 0.26 (25% EtOAc/hexanes). ^1H -NMR ($CDCl_3$, 500 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-benzof[*h*]indazol-5-yl)(thiophen-3-yl)methanol (11be): Flash chromatography conditions, 10% EtOAc/hexanes. Yield = 35.8 %. R_f = 0.26 (25% EtOAc/hexanes). HRMS (DART-RTOF) m/z : $[M+H]^+$ Calcd for $C_{24}H_{27}N_2OS$, 391.18386; found 391.13976. ^1H -NMR ($CDCl_3$, 500 MHz) 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).

(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)(thiophen-3-yl)methanol (**11ce**): Flash chromatography, 15% EtOAc/hexanes. Yield = 79.5 %. R_f = 0.17 (30% EtOAc/hexanes). HRMS (ESI-RTOF) m/z : $[M+H]^+$ Calcd for $C_{24}H_{27}N_2O_2S$, 407.1787; found 407.1720. ^1H -NMR ($CDCl_3$, 500 MHz) 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).

3-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-3-hydroxypropanenitrile (**11af**): A commercially available solution of 1.7 M *t*-BuLi (0.9 mL, 1.53 mmol) and 2 mL of THF was transferred to a round bottom flask and cooled to -78 °C. A solution of 80 μ L of ACN in 2 mL of THF was added dropwise at -78 °C. Following, a solution of 400 mg of Compound **12** $R_1 = F$ in 1 mL THF was added dropwise via canula, and the reaction was stirred at -78 °C for 1 h. The reaction was quenched with saturated NH_4Cl , acidified to a pH of 7 via dropwise addition of HCl, and extracted three times with diethyl ether. 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 (25% EtOAc/hexanes). Yield = 45.5%. R_f = 0.14 (25% EtOAc/hexanes). HRMS (DART-RTOF) m/z : $[M+H]^+$ Calcd for $C_{21}H_{23}FN_3O$, 352.18197; found 352.18293. ^1H -NMR ($CDCl_3$, 500 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).

General method for the synthesis of ketones (**12**): A solution of the appropriate 2° alcohol precursor (**11**) in dichloromethane was transferred dropwise via canula to a suspension of pyridinium chlorochromate (1.5 equiv.) and 4 Å molecular sieves (1:1 m/m) in dichloromethane. The reaction was stirred at room temperature until complete conversion was observed by TLC analysis. Reaction times ranged from 1-7 h. The reaction mixture was immediately loaded onto a silica column (25% EtOAc/hexanes) and subjected to purification via flash chromatography to afford the ketone **12**.

3-(1-(4-fluorophenyl)-4,4a,5,6,7,8-hexahydro-4a-methyl-1H-benzof[f]indazol-5-yl)-3-oxopropanenitrile (12af): Flash chromatography conditions, 20% EtOAc/hexanes. White solid. Yield = 16%. TLC R_f = 0.11 (30% EtOAc/hexanes). HRMS (DART-RTOF) m/z : $[M+H]^+$ Calcd for $C_{21}H_{21}FN_3O$, 350.16632; found 350.16529. ^1H -NMR ($CDCl_3$, 500 MHz) δ 1.20 (s, 3H), 1.45-1.49 (m, 2H), 1.82-1.87 (t, 2H), 1.93-1.97 (d, 1H), 2.32-2.44 (m, 3H), 2.74-2.77 (d, 1H), 2.82-2.88 (m, 2H), 3.53-3.61 (s, 2H), 6.15 (s, 1H), 7.14-7.44 (m, 5H).

General method for the synthesis of 3° methyl alcohol GC analogues (13): The appropriate ketone 12 was dissolved in diethyl ether and cooled to -40 °C, and a commercially available solution of MeLi (1.9 M, 10 equiv.) was added dropwise via syringe. The reaction was stirred at -40° for 45 min. The reaction was quenched with IPA (10 equiv.), 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*.

Annotated characterization data of novel compounds. This following entries contain the characterization data required by the American Chemical Society publication guidelines for all previously unreported compounds at the time of writing. ^1H and ^{13}C nuclear magnetic resonance spectra (NMR), high resolution mass spectra (HRMS), and infrared spectra (IR) obtained at the time of writing for all compounds are in the Appendix.

1-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[f]indazol-5-yl)ethan-1-ol (11aa): ^1H -NMR ($CDCl_3$, 500 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).

2-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[f]indazol-5-yl)propan-2-ol (13aa): ^1H -NMR ($CDCl_3$, 300 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-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[f]indazol-5-yl)(phenyl)methanol (11ab): ^1H -NMR ($CDCl_3$, 500 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).

1-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*f*]indazol-5-yl)-1-phenylethan-1-ol (**13ab**): ¹H-NMR (CDCl₃, 500 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).

(4-fluorophenyl)(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*f*]indazol-5-yl)methanol (**11ac**): ¹H-NMR (CDCl₃, 300 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).

1-(4-fluorophenyl)-1-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*f*]indazol-5-yl)ethan-1-ol (**13ac**): ¹H-NMR (CDCl₃, 500 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).

(3,4-difluoro-5-methoxyphenyl)(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*f*]indazol-5-yl)methanol (**11ad**): ¹H-NMR (CDCl₃, 300 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).

1-(3,4-difluoro-5-methoxyphenyl)-1-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*f*]indazol-5-yl)ethan-1-ol (**13ad**): ¹H-NMR (CDCl₃, 500 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).
(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*f*]indazol-5-yl)(thiophen-3-yl)methanol (**11ae**): ¹H-NMR (CDCl₃, 500 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).

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**): $^1\text{H-NMR}$ (CDCl_3 , 500 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).

3-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-3-hydroxypropanenitrile (**11af**): white solid obtained in 46% yield; mp 121-123.8 °C; TLC (Silica G w/UV254) 25% EtOAc in hexanes, R_f = 0.14; $[\alpha]_D^{20}$ = +60 (c 10.0, MeOH); $^1\text{H-NMR}$ (CDCl_3 , 500 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); $^{13}\text{C-NMR}$ (CDCl_3 , 500 MHz) δ 19.32, 20.91, 25.37, 26.20, 32.98, 33.19, 34.64, 52.82, 66.62, 109.76, 115.96, 116.14, 125.32, 137.86; IR (NaCl, thin film) ν_{max} (cm^{-1}): 3335 (OH), 3030 (w), 2934, 2867, 2249 (CN), 1619, 1518, 1225, 840; HRMS (DART-RTOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{21}\text{H}_{23}\text{FN}_3\text{O}$, 352.18197; found 352.18293.

3-(1-(4-fluorophenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzo[f]indazol-5-yl)-3-hydroxybutanenitrile (**12af**): white solid obtained in 16% yield; mp 192-194 °C; TLC (Silica G w/UV254) 30% EtOAc in hexanes, R_f = 0.11; $[\alpha]_D^{20}$ = +60 (c 10.0, CHCl_3); $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) δ 1.20 (s, 3H), 1.45-1.49 (m, 2H), 1.82-1.87 (t, 2H), 1.93-1.97 (d, 1H), 2.32-2.44 (m, 3H), 2.74-2.77 (d, 1H), 2.82-2.88 (m, 2H), 3.53-3.61 (s, 2H), 6.15 (s, 1H), 7.14-7.44 (m, 5H); $^{13}\text{C-NMR}$ (CDCl_3 , 500 MHz) δ 18.53, 25.21, 25.61, 31.72, 33.85, 34.75, 41.16, 59.60, 110.18, 112.91, 113.49, 116.00, 135.61, 136.29, 137.72, 146.60, 160.56, 162.53, 199.38; IR (NaCl, thin film) ν_{max} (cm^{-1}) 2955, 2920, 2850, 1723 (m), 1514 (m), 1462, 1377, 1222; HRMS (DART-RTOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{21}\text{H}_{21}\text{FN}_3\text{O}$, 350.16632; found 350.16529.

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; $[\alpha]_D^{20}$ = -16 (c 10.0, MeOH); $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) 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); ¹³C-NMR (CDCl₃, 500 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) ν_{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 C₂₁H₂₇N₂O, 323.21179; found 323.21001.

2-((4aR)-4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzof[*l*]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 10.0, MeOH); ¹H-NMR (CDCl₃, 500 MHz) 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, 500 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-RTOF) *m/z*: [M+H]⁺ Calcd for C₂₂H₂₉N₂O, 337.2274; found 337.2200.

(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzof[*l*]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; [α]_D²⁰ = -12 (c 10.0, MeOH); ¹H-NMR (CDCl₃, 500 MHz) 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); ¹³C-NMR (CDCl₃, 500 MHz) δ 19.90, 21.05, 25.79, 41.28, 56.66, 71.95, 109.51, 113.67, 123.52, 125.38, 126.78, 128.16, 129.68, 137.60, 145.70, 149.77; IR (NaCl, thin film) ν_{max} (cm⁻¹): 3319 (OH), 3039 (w), 2929, 2868, 1610 (w), 1519 (m), 821; HRMS (DART-RTOF) *m/z*: [M+H]⁺ Calcd for C₂₆H₂₉N₂O, 385.22744; found 385.22612.

1-((4aR)-4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzof[*l*]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 10.0, MeOH); ¹H-NMR (CDCl₃, 500 MHz) 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, 500 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) $\nu_{\max}$ (cm⁻¹): 3365 (OH), 3054 (w), 2928, 2858, 1610 (w), 1519 (m), 821; HRMS (ESI-RTOF) m/z : [M+H]⁺ Calcd for C₂₇H₃₁N₂O, 399.2430; found 399.2368.

(4-fluorophenyl)(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzof[*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 10.0, MeOH); ¹H-NMR (CDCl₃, 500 MHz) 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); ¹³C-NMR (CDCl₃, 500 MHz) δ 19.19, 21.04, 25.74, 33.25, 35.24, 41.24, 56.75, 71.47, 109.62, 113.54, 114.58, 123.50, 126.92, 129.69, 136.68, 136.90, 137.23, 137.58, 141.26, 149.48, 160.71, 162.66; IR (NaCl, thin film) $\nu_{\max}$ (cm⁻¹): 3312 (OH), 3041 (w), 2931, 2867, 2830, 1898, 1603, 1518, 1219, 819; HRMS (DART-RTOF) m/z : [M+H]⁺ Calcd for C₂₆H₂₈FN₂O, 403.21802; found 403.21649.

1-(4-fluorophenyl)-1-((4aR)-4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzof[*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; $[\alpha]_D^{20}$ = -9 (c 1.0, MeOH); ¹H-NMR (CDCl₃, 500 MHz) 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, 500 MHz) δ 20.40, 21.02, 25.83, 26.16, 28.41, 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) $\nu_{\max}$ (cm⁻¹): 3315 (OH), 3020 (w), 2928, 2860, 1600, 1519, 1222; HRMS (DART-RTOF) m/z : [M+H]⁺ Calcd for C₂₇H₃₀FN₂O, 417.23367; found 417.23232.

(3,4-difluoro-5-methoxyphenyl)(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzof[*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; $[\alpha]_D^{20}$ = +31 (c 10.0, MeOH); ¹H-NMR (CDCl₃, 500 MHz) 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, 500 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, 109.70, 113.35, 123.36, 129.66, 136.67, 136.99, 137.17, 137.55, 149.25; IR

(NaCl, thin film) $\nu_{\max}$ (cm⁻¹): 3387 (OH), 3050 (w), 2927, 2850, 1623, 1522, 1454, 1426, 1339, 1226, 1097; HRMS (DART-RTOF) m/z : [M+H]⁺ Calcd for C₂₇H₂₉F₂N₂O₂, 451.21916; found 451.21790.

1-(3,4-difluoro-5-methoxyphenyl)-1-((4aR)-4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzof[*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 (CDCl₃, 500 MHz) 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); ¹³C-NMR (CDCl₃, 500 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) $\nu_{\max}$ (cm⁻¹): 3348 (OH), 2927, 2853, 1622, 1520, 1451, 1420, 1334, 1228, 1101; HRMS (ESI-RTOF) m/z : [M+H]⁺ Calcd for C₂₈H₃₁F₂N₂O₂, 465.2348; found 465.2283.

(4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzof[*f*]indazol-5-yl)(thiophen-2-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 10.0, MeOH); ¹H-NMR (CDCl₃, 500 MHz) 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); ¹³C-NMR (CDCl₃, 500 MHz) δ 19.07, 21.03, 21.64, 23.60, 24.35, 25.83, 33.32, 33.51, 34.67, 35.08, 40.64, 41.13, 54.89, 56.02, 66.12, 67.89, 109.57, 114.13, 123.42, 129.65, 136.46, 137.59, 149.61; IR (NaCl, thin film) $\nu_{\max}$ (cm⁻¹): 3319 (OH), 3106 (w), 3045 (w), 2928, 2856, 2836, 1611, 1540 (m); HRMS (DART-RTOF) m/z : [M+H]⁺ Calcd for C₂₄H₂₇N₂OS, 391.18386; found 391.13976.

1-((4aR)-4a-methyl-1-(p-tolyl)-4,4a,5,6,7,8-hexahydro-1H-benzof[*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 (CDCl₃, 500 MHz) 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); ¹³C-NMR (CDCl₃, 500 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) $\nu_{\max}$

(cm⁻¹): 3381 (OH), 3105 (w), 3042 (w), 2928, 2859, 1611, 1518 (m), 821; HRMS (ESI-RTOF) *m/z*: [M+H]⁺ Calcd for C₂₅H₂₉N₂OS, 405.1995; found 405.1899.

1-(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*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 10.0, MeOH); ¹H-NMR (CDCl₃, 500 MHz) 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, 500 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) *v*_{max} (cm⁻¹): 3376 (OH), 3020 (w), 2962, 2931, 2862, 1612, 1517, 1250, 831; HRMS (DART-RTOF) *m/z*: [M+H]⁺ Calcd for C₂₁H₂₇N₂O₂, 339.20670; found 339.20344.

2-((4aR)-1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*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; [α]_D²⁰ = -17 (c 10.0, MeOH); ¹H-NMR (CDCl₃, 500 MHz) 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); ¹³C-NMR (CDCl₃, 500 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) *v*_{max} (cm⁻¹): 3349 (OH), 3018, 2928, 2857, 1612, 1517, 1248, 831; HRMS (ESI-RTOF) *m/z*: [M+H]⁺ Calcd for C₂₂H₂₉N₂O₂, 353.2223; found 353.2184.

(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*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; [α]_D²⁰ = -10 (c 10.0, MeOH); ¹H-NMR (CDCl₃, 500 MHz) 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); ¹³C-NMR (CDCl₃, 500 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) *v*_{max} (cm⁻¹): 3307 (OH), 3056 (w), 2932, 2868, 2835, 1611, 1518, 1250, 833; HRMS (DART-RTOF) *m/z*: [M+H]⁺ Calcd for C₂₆H₂₉N₂O₂, 401.22235; found 401.21952.

1-((4aR)-1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*h*]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 10.0, CHCl₃); ¹H-NMR (CDCl₃, 500 MHz) 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, 500 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) $\nu_{\max}$ (cm⁻¹): 3338 (OH), 3020 (w), 2925, 2854, 1611, 1517, 1250, 833; HRMS (DART-RTOF) m/z : $[M+H]^+$ Calcd for C₂₇H₃₁N₂O₂, 415.23800; found 415.23680.

(4-fluorophenyl)(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*h*]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 (CDCl₃, 500 MHz) 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); ¹³C-NMR (CDCl₃, 500 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) $\nu_{\max}$ (cm⁻¹): 3399 (OH), 2930, 2850, 1602, 1512, 1223, 824; HRMS (DART-RTOF) m/z : $[M+H]^+$ Calcd for C₂₆H₂₈FN₂O₂, 419.21293; found 419.20660.

1-(4-fluorophenyl)-1-((4aR)-1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*h*]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 10.0, MeOH); ¹H-NMR (CDCl₃, 300 MHz) 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, 500 MHz) δ 20.42, 25.82, 26.18, 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, 151.60; IR (NaCl, thin film) $\nu_{\max}$ (cm⁻¹): 3363 (OH), 2929, 2840, 1600, 1517, 1250, 834; HRMS (DART-RTOF) m/z : $[M+H]^+$ Calcd for C₂₇H₃₀FN₂O₂, 433.22858; found 433.22897.

(3,4-difluoro-5-methoxyphenyl)(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[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 10.0, MeOH); $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) 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); $^{13}\text{C-NMR}$ (CDCl_3 , 500 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 : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{27}\text{H}_{29}\text{F}_2\text{N}_2\text{O}_3$, 467.21408; found 467.21488.

1-(3,4-difluoro-5-methoxyphenyl)-1-((4aR)-1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[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 10.0, MeOH); $^1\text{H-NMR}$ (CDCl_3 , 500 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); $^{13}\text{C-NMR}$ (CDCl_3 , 500 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 : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{27}\text{H}_{30}\text{FN}_2\text{O}_2$, 433.22858; found 433.22897.

(1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[f]indazol-5-yl)(thiophen-2-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 10.0, MeOH); $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) 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); $^{13}\text{C-NMR}$ (CDCl_3 , 500 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-RTOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{24}\text{H}_{27}\text{N}_2\text{O}_2\text{S}$, 407.1787; found 407.1720.

1-((4aR)-1-(4-methoxyphenyl)-4a-methyl-4,4a,5,6,7,8-hexahydro-1H-benzof[*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; $[\alpha]_D^{20}$ = +12 (c 10.0, MeOH); $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) 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); $^{13}\text{C-NMR}$ (CDCl_3 , 500 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-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{25}\text{H}_{29}\text{N}_2\text{O}_2\text{S}$, 421.1944; found 421.1907.

**CHAPTER II. INSIGHTS INTO THE DEGRADATION OF BISPHENOL
A AND THE MNO_2 -MEDIATED DEGRADATION PRODUCT, 4-
HYDROXYCUMYL ALCOHOL, BY FRESHWATER SEDIMENT
MICROCOSMS**

The work outlined in this chapter below was performed by Jeongdae Im^{1,2}, Carson W. Prevatte³, Hong G. Lee⁴, Shawn R. Campagna³, and Frank E. Löffler^{1,2,5,6}. JI, under the supervision of FEL, is the primary author in terms of the introduction, biological experimentation methods, results, and conclusions. CWP, along with other members of the Campagna laboratory and combined research team, authored sections of the original manuscript containing chemical data and methods contributions. Two publications have been merged without changes to the original content of the articles. The abstract, introductions, and methods sections were merged in order to reduce redundancy between the articles and edited to present the two articles as two parts of a single, overarching project. Additions were also made in the methods section to highlight the synthesis and analytical methods implemented to elucidate and confirm unknown structures in the two degradation studies. The results and discussion were merged and are as originally published.

Im, J., Prevatte, C. W., Campagna, S. R., & Löffler, F. E. Identification of 4-hydroxycumyl alcohol as the major MnO₂-mediated bisphenol A transformation product and evaluation of its environmental fate. *Environmental science & technology* 2015 49 (10) 6214-21.

Im, J., Prevatte, C. W., Lee, H. G., Campagna, S. R., & Löffler, F. E. 4-Methylphenol produced in freshwater sediment microcosms is not a bisphenol A metabolite. *Chemosphere* 2014 117:521-526.

Affiliations: ¹Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996, United States; ²Center for Environmental Biotechnology, University of Tennessee, Knoxville, Tennessee 37996, United States; ³Department of Chemistry, University of Tennessee, Knoxville, Tennessee 37996, United States; ⁴ Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, United States; ⁵Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, Tennessee 37996, United States; ⁶University of Tennessee and Oak Ridge National Laboratory (UT-ORNL) Joint Institute for Biological Sciences (JIBS) and Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, United States

The Löffler lab, FEL and JI, conceived the study. The experimental work was performed by JI, CWP, and HGL. JI and HGL will be referred to as collaborators. CWP and collaborators all contributed to the identification, characterization, and the experiments probing the degradation reactions of BPA and 4-hydroxycumyl alcohol (HCA) under different conditions. Collaborators setup microcosms for both freshwater sediment amended with different redox active salts as well as freshwater sediment amended with MnO_2 . Collaborators used gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) and a diode array detector (DAD) and fluorescence detector to detect and quantitate BPA and the putative degradation intermediates, 4-methylphenol (4-MP) and HCA. CWP synthesized ^{13}C -labelled BPA to determine the role of 4-MP in BPA degradation in anoxic microcosms established with freshwater sediment amended with redox active salts. Additionally, CWP synthesized and characterized the putative MnO_2 -induced BPA degradation intermediate HCA to elucidate the structure of this previously unreported intermediate for this degradation pathway. Collaborators determined the physiochemical properties of HCA, the kinetics of BPA and HCA degradation by MnO_2 , and described the HCA degradation in freshwater sediment under oxic and anoxic conditions. The data was generated, analyzed by collaborators. Collaborators, CWP, and SRC contributed to the discussion, with CWP contributing to the discussion of the elucidation of the structure of HCA.

CWP contribution

The contribution by CWP was vital for each study, applying organic synthesis and analytical chemistry characterization methods to facilitate testing each hypothesis and better understand the mineral-mediated degradation of bisphenol A (BPA) in microcosms containing freshwater sediment under anoxic conditions. The first study initially sought to determine the degradation mechanism of BPA induced by various mineral salts acting as electron acceptors. 4-methylphenol (4-MP), an intermediate in the degradation of BPA under other conditions*, was initially detected and thought to be an intermediate under the conditions of interest for this study. CWP synthesized two different versions of ^{13}C -labelled BPA, one with both methyl groups labelled and one with all non-aromatic carbons labelled using an optimized version of a previously described method, including an entirely new flash chromatography method⁵¹ (**Scheme 2.1**). Synthesis was vital to determining if 4-MP was a degradation product of BPA. Initially, the $^{13}\text{C}_2$ -labelled version was synthesized to specifically

track the methyl groups. Though the methyl groups in BPA were most likely to account for the methyl group in 4-MP through a Wagner–Meerwein rearrangement* (Scheme 2.3), the 4-MP isolated did not contain ^{13}C suggesting that 4-MP was not from BPA. The second $^{13}\text{C}_3$ -labelled BPA was made to confirm this and microcosms amended with it also resulted in the presence of unlabeled 4-MP. The synthesis of these ^{13}C -labelled BPA molecules by CWP, and their amendment into the microcosm, proved to be the most important experiment in this study and generated the result which most significantly supports the final conclusions of this study.

The second study set out to understand MnO_2 -induced BPA degradation in microcosms containing freshwater sediment under anoxic conditions by identifying, characterizing, and synthesizing the major degradation products to understand the environmental fate of BPA and the resulting degradation products of BPA. A putative BPA degradation product was initially identified by HPLC-fluorescence detection of a peak that emerged in the chromatogram as the peak for BPA was consumed, the absence of this peak in microcosms not amended with MnO_2 , and a similar UV absorption spectrum between this unknown intermediate and BPA. CWP was tasked with elucidating the structure of the unknown intermediate. High-resolution MS analysis resulted in a base peak with an accurate mass of 135.08011, suggesting a most likely molecular formula of $\text{C}_9\text{H}_{11}\text{O}^+$. When combined with NMR experiments that suggested p-substituted phenol and 2-substituted isopropyl moiety and the knowledge of the tendency for 3° benzylic hydroxy groups to be lost during ionization, HCA was tentatively assigned as the structure of the unknown transformation product. One step further, CWP contributed to the final confirmation of the tentative assignment by synthesizing and characterizing HCA. The accurate mass measurement and the NMR spectrum were identical to the isolated BPA transformation product. The synthetic HCA also allowed collaborators to determine the physiochemical properties, the kinetics of MnO_2 -mediated degradation under oxic and anoxic conditions, and describe more broadly the implication the HCA degradation in freshwater sediment.

2.1 Abstract

Bisphenol A (BPA), an environmental contaminant with weak estrogenic activity, resists microbial degradation under anoxic conditions but is susceptible to abiotic transformation. In an initial study, 4-methylphenol (4-MP), a putative BPA degradation intermediate, was

detected at concentrations reaching 2.1 mg L⁻¹ in anoxic microcosms containing 10 mg L⁻¹ BPA and 5 g of freshwater sediment material collected from four geographically distinct locations and amended with nitrate, nitrite, ferric iron, or bicarbonate as electron acceptors. 4-MP accumulation was transient, and 4-MP degradation was observed under all redox conditions tested. 4-MP was not detected in microcosms not amended with BPA. Unexpectedly, incubations with ¹³C-labeled BPA failed to produce ¹³C-labeled 4-MP suggesting that 4-MP was not derived from BPA. The detection of 4-MP in live microcosms amended with lactate, but not containing BPA corroborated that BPA was not the source of 4-MP. A further study was conducted outlining MnO₂-induced degradation of BPA. BPA degradation followed pseudo-first-order kinetics with a rate constant of 0.96 (±0.03) min⁻¹ in the presence of 2 mM MnO₂ (0.017% w/w) at pH 7.2. 4-hydroxycumyl alcohol (HCA) was the major transformation product, and, on a molar basis, up to 64% of the initial amount of BPA was recovered as HCA. MnO₂ was also reactive toward HCA, albeit at 5-fold lower rates, and CO₂ evolution (i.e., mineralization) occurred. In microcosms established with freshwater sediment, HCA was rapidly biodegraded under oxic, but not anoxic conditions. With a measured octanol–water partition coefficient (Log K_{ow}) of 0.76 and an aqueous solubility of 2.65 g L⁻¹, HCA is more mobile in saturated media than BPA (Log K_{ow} = 2.76; aqueous solubility = 0.31 g L⁻¹), and therefore more likely to encounter oxic zones and undergo aerobic biodegradation. These findings corroborate that BPA is not inert under anoxic conditions and suggest MnO₂-mediated coupled abiotic–biotic processes may be relevant for controlling the fate and longevity of BPA in sediments and aquifers. Furthermore, the transient formation of 4-MP as a possible BPA degradation intermediate must be interpreted cautiously, as microbial activity in streambed microcosms may generate 4-MP from sediment-associated organic material.

2.2 Introduction

Bisphenol A, 2,2-bis(4-hydroxyphenyl)propane (BPA), a high production volume chemical, is used to manufacture polycarbonate, epoxy resins, flame retardants, and lacquer coatings on food cans, as well as other products². The global demand of BPA was estimated to exceed 7 million tons in 2013 and has been predicted to grow at a compound annual growth rate of 4.7% from 2014 to 2020., making BPA one of the highest production volume chemicals in the world⁵². As a consequence of high capacity use, BPA often has been detected in environmental systems, ranging from 0.5–343 µg kg⁻¹ in fresh water sediments,

0.5–776 ng L⁻¹ in surface water bodies, 0.02–149.2 µg L⁻¹ in sewage effluents, and 1.3–17 200 µg L⁻¹ in landfill leachate⁵³⁻⁵⁹. Bearing structural resemblance to estrogens, BPA can bind to the estrogen receptor and is considered weakly estrogenic. This, along with high volume production, have triggered interest in the environmental fate of BPA in recent years. Several aerobic BPA degraders have been reported that use BPA as the sole source of carbon and energy⁶⁰⁻⁶⁴. On the other hand, a number of studies concluded that BPA undergoes little or no microbial degradation in the absence of oxygen^{61, 65-67}.

Based on the identification of intermediates, a few different aerobic BPA degradation pathways have been proposed^{60, 62, 64}. A recent survey of 107 soil samples demonstrated aerobic BPA degradation in 85 samples, and 26 BPA-degrading isolates were obtained belonging to the genera *Pseudomonas*, *Klebsiella*, *Pandoraea*, *Alcaligenes*, *Enterobacter*, *Serratia*, *Bacillus*, *Bordetella*, and *Sphingomonas*⁶⁸. These findings suggest that diverse bacterial groups are capable of degrading BPA under oxic conditions. A significant mass of BPA resides in anoxic sediments^{53, 55}, but very little is known about the fate of BPA under anoxic conditions. Several studies investigating microbial BPA degradation under anoxic conditions have concluded that BPA is recalcitrant and undergoes “little or no” biodegradation in the absence of oxygen^{61, 67}. No microbial BPA degradation was observed in anoxic microcosms established with freshwater sediment⁶⁹, marine sediments⁷⁰, and soil⁷¹. Halogenated BPAs were reductively dehalogenated to BPA in estuarine sediment microcosms, but no further degradation was observed under different redox conditions⁶⁷. Experimental evidence supporting anaerobic BPA degradation is scarce. Chiou (2010) reported a 14% of loss in BPA concentration after a 120 d incubation period with anoxic river sediment⁷². Similarly, Kang and Kondo (2002) reported a 10% loss of initial BPA added to anoxic river water microcosms, and (Patterson et al., 2010) reported BPA removal under denitrifying conditions⁷³. At best, these studies demonstrated BPA disappearance, but BPA degradation intermediates and end-products were not identified or quantified. To predict the environmental fate of BPA in anoxic environments, more detailed studies of BPA degradation under anoxic conditions are needed.

This study detected 4-MP in anoxic sediment microcosms as a possible BPA degradation intermediate but experiments using ¹³C-labeled BPA implicated that 4-MP was derived from another source, presumably sediment-associated organic matter, emphasizing the need for careful results interpretation regarding the environmental fate of BPA or similar phenolic

compounds. To further understand the anoxic degradation of BPA, a further study was performed to identify and characterize major MnO₂-mediated BPA transformation product(s), evaluate the degradation of the intermediate(s), and assess the potential consequences of MnO₂-mediated oxidation on the environmental fate of BPA and the transformation product(s).

Manganese and iron can form reactive minerals that affect the fate and transport of organic contaminants via sorption, hydrolysis and/or oxidative transformation⁷⁴⁻⁷⁶. In particular, manganese oxides, such as MnO₂, are abundant in soils, as well as freshwater and marine sediments^{74-75, 77-78}. MnO₂ is the most common manganese mineral in soils—185 $\mu\text{mol cm}^{-3}$ of MnO₂ have been reported in marine sediments of the Panama Basin, the Skagerrak, the Black Sea and the northern Barents Sea⁷⁹⁻⁸¹. Values up to 1–15% (dry wt., $\sim 300\text{--}4000 \mu\text{mol cm}^{-3}$) of MnO₂ have been observed in Lake Zürich sediments⁸². In Lake Charlotte, Nova Scotia, MnO₂ comprised as much as 2.5% (dry wt., $750 \mu\text{mol cm}^{-3}$) of the sediment⁷⁹. MnO₂ is a strong oxidant and serves not only as respiratory electron acceptor in microbial metabolism but also as a reactive mineral to remove many phenolic compounds, including BPA^{75-76, 83-89}. Lin and co-workers proposed 11 BPA transformation products generated in aqueous systems amended with MnO₂ based on the fragmentation patterns obtained by gas chromatography–mass spectrometry methods following derivatization with N,O-bis(trimethylsilyl)-trifluoroacetamide⁸⁶. However, a quantitative assessment was not performed and no major pathway was established.

2.3 Materials and methods

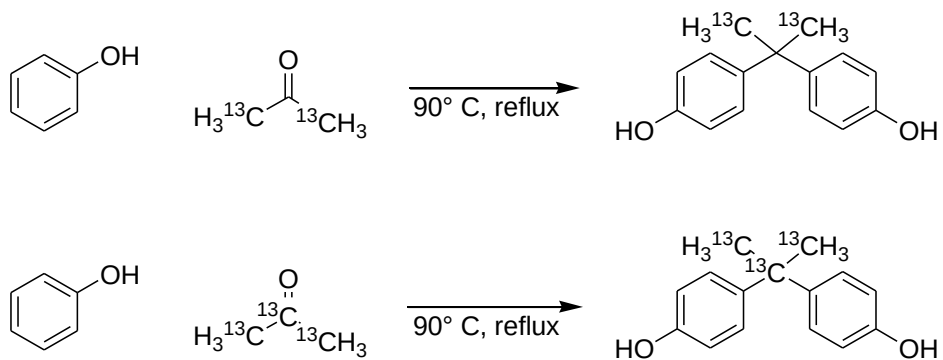
Chemicals. BPA (>99% purity), p-acetoxyacetophenone, and 3.3 M solution of methylmagnesium iodide in diethyl ether was obtained from Sigma Aldrich (St. Louis, MO), 4-MP (>99% purity), Amberlyst 15 was purchased from Rohm and Haas (Philadelphia, PA), and L-tyrosine (99% purity) was purchased from MP Biomedicals (Chicago, IL). The ¹³C-labelled acetone (>99% purity) and the BPA containing ¹³C-labelled phenyl groups, 2,2-bis(4-hydroxy[¹³C₆]phenyl) propane (¹³C₆-BPA, >99% purity), were purchased from Cambridge Isotopes Laboratories (Andover, MA). Dry diethyl ether employed in the synthesis of HCA was obtained by distillation from a potassium benzophenone ketyl still. Thin-layer chromatography (TLC) was carried out using Sorbent Technologies silica G F₂₅₄ TLC plates. All compounds of interest were visualized as single spots using short-wave UV

light; more permanent staining of TLC spots was achieved using p-anisaldehyde, vanillin, or ceric ammonium molybdate (Hannesian's) TLC staining solutions. Silica gel with a 60Å pore size and a particle diameter of 40-63 µm was used for flash chromatography.

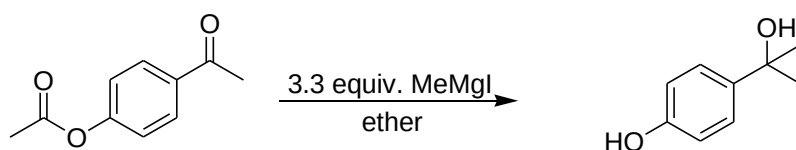
Synthesis of ^{13}C -labelled BPA and HCA. Specifically labeled versions of BPA, 2,2-bis(4-hydroxyphenyl)[1,3- $^{13}\text{C}_2$]propane ($^{13}\text{C}_2$ -BPA) and 2,2-bis(4-hydroxyphenyl)[$^{13}\text{C}_3$]propane ($^{13}\text{C}_3$ -BPA), were synthesized by condensation of [1,3- $^{13}\text{C}_2$] or [$^{13}\text{C}_3$]acetone, respectively, with five equivalents of phenol, neat, in the presence of a cation exchange resin catalyst, Amberlyst 15 using an optimized version of a previously described method (**Scheme 2.1**)⁵¹. BPA synthesized by this method was obtained in 21% yield after purification by flash chromatography eluted with 10% EtOAc in hexanes. HCA was not commercially available and was synthesized using a previously described method (**Scheme 2.2**)⁹⁰. Briefly, 7 mL of 3.0 M methylmagnesium iodide in diethyl ether was added dropwise to 20 mL of 0.33 M p-acetoxyacetophenone in diethyl ether under a nitrogen atmosphere at room temperature, and the resulting solution was stirred for 5 h. After addition of 100 mL saturated aqueous ammonium chloride, the reaction mixture was extracted three times with 30 mL ethyl acetate. The organic phase was washed with 75 mL saturated NaCl solution, dried over anhydrous magnesium sulfate, filtered, and concentrated in vacuo to yield a slightly yellow solid. HCA was obtained in 21% yield after purification by flash chromatography eluted with 25% EtOAc in hexanes.

MnO₂ preparation. MnO₂ was synthesized according to a previously reported method⁹¹. Briefly, 2.96 g of KMnO₄ in 74.1 mL of distilled water was brought to 90 °C on a hot plate under continuous stirring. With the solution at 90 °C, 3.71 mL of 5 M NaOH was added, and then 5.55 g of MnCl₂ dissolved in 27.8 mL distilled water was slowly added. The formed MnO₂ particles were allowed to settle at room temperature and the suspension was washed five times with distilled water by centrifugation at 6,000 g for 10 min. The precipitate was suspended in 100 mL deoxygenated distilled water to achieve a nominal concentration of 0.4 M. The average surface area of MnO₂ particles was determined by the Brunauer–Emmett–Teller method using an Autosorb-1 surface area analyzer (Quantachrome Instruments, Syosset, NY)⁹².

Oxidative Transformation of BPA and HCA by MnO₂. All glassware was soaked in a 5% nitric acid bath overnight, and thoroughly rinsed with high purity Milli-Q water (Millipore Milli-Q, Bedford, MA) prior to use. Experiments were conducted in 160 mL glass serum



Scheme 2.1. Synthesis of 2,2-bis(4-hydroxyphenyl)[1,3- $^{13}\text{C}_2$]propane ($^{13}\text{C}_2$ -BPA) and 2,2-bis(4-hydroxyphenyl)[$^{13}\text{C}_3$]propane ($^{13}\text{C}_3$ -BPA).



Scheme 2.2. Synthesis of HCA.

bottles in a total volume of 100 mL containing 44 μM of BPA (10 mg L^{-1}). The following buffers were used (10 mM each): phosphate (pH 2), sodium acetate (pH 4.3 and 5.5), 4-morpholinepropanesulfonic acid (MOPS) (pH 6.5, 7.2 and 8), and 2-(cyclohexylamino)ethanesulfonic acid (CHES) (pH 9 and 10). An appropriate amount of sodium chloride was added to maintain a constant ionic strength of 10 mM for each buffer. All solutions were deoxygenated by boiling and cooling under an oxygen-free nitrogen atmosphere. The effect of the initial MnO_2 loading on BPA transformation rates and extent was examined by adding different amounts of MnO_2 to achieve initial nominal concentrations of 0.2, 0.5, 1, and 2 mM at pH 7.2. To test the effect of pH on BPA transformation, the reaction vessels received 44 μM BPA and 0.5 mM of MnO_2 . The conversion efficiency of BPA to HCA, on a molar basis, was calculated by dividing the amount (μmol) HCA formed by the amount BPA removed. To test possible mineralization, CO_2 evolution was examined in reaction vessels containing 2 mM MnO_2 and 44 μM BPA or 44 μM HCA in phosphate buffer (pH 2). The bottles were incubated on a rotary shaker at 100 rpm at room temperature in the dark. The reactions were initiated by adding MnO_2 from a 0.4 M stock suspension. Aliquots (0.5 mL) of the reaction mixtures were periodically collected, and transferred to 2 mL glass HPLC vials containing 20 μL of L-ascorbic acid solution (50 mg mL^{-1}) and immediately vigorously mixed. Ascorbic acid quenches the reaction and converts any remaining MnO_2 to Mn(II) ions and liberates any sorbed BPA and reaction products^{89, 93}. The extent of BPA and HCA sorption onto the MnO_2 surface was measured by comparing BPA and HCA concentrations in ascorbic acid-treated samples versus untreated samples that were filtered through a 0.22 μm membrane (Nalgene, Rochester, NY). The pseudo-first-order rate constants were obtained by plotting the natural log of BPA concentrations as a function of time using four early time points, where linear relationships were observed. Additional vials containing 4.4, 11, 22, and 44 μM BPA and ascorbic acid were prepared in triplicates to construct standard curves (**Figure 2.1**). The transformation of HCA by MnO_2 and the effect of the initial MnO_2 loading were examined as described for BPA in vessels containing 44 μM HCA (6.7 mg L^{-1}) at pH 7.2. Different amounts of MnO_2 were added to achieve nominal concentrations of 0.2, 0.5, 1, and 2 mM. Vials containing 0.5 mL of 6.6, 16.4, 32.9, and 65.7 μM HCA and 20 μL ascorbic acid were prepared in triplicate to construct standard curves (**Figure 2.1**).

Physical-chemical properties of HCA. The aqueous solubility of HCA was determined at 20 °C based on the modified EPA Office of Prevention, Pesticides, and Toxic Substances

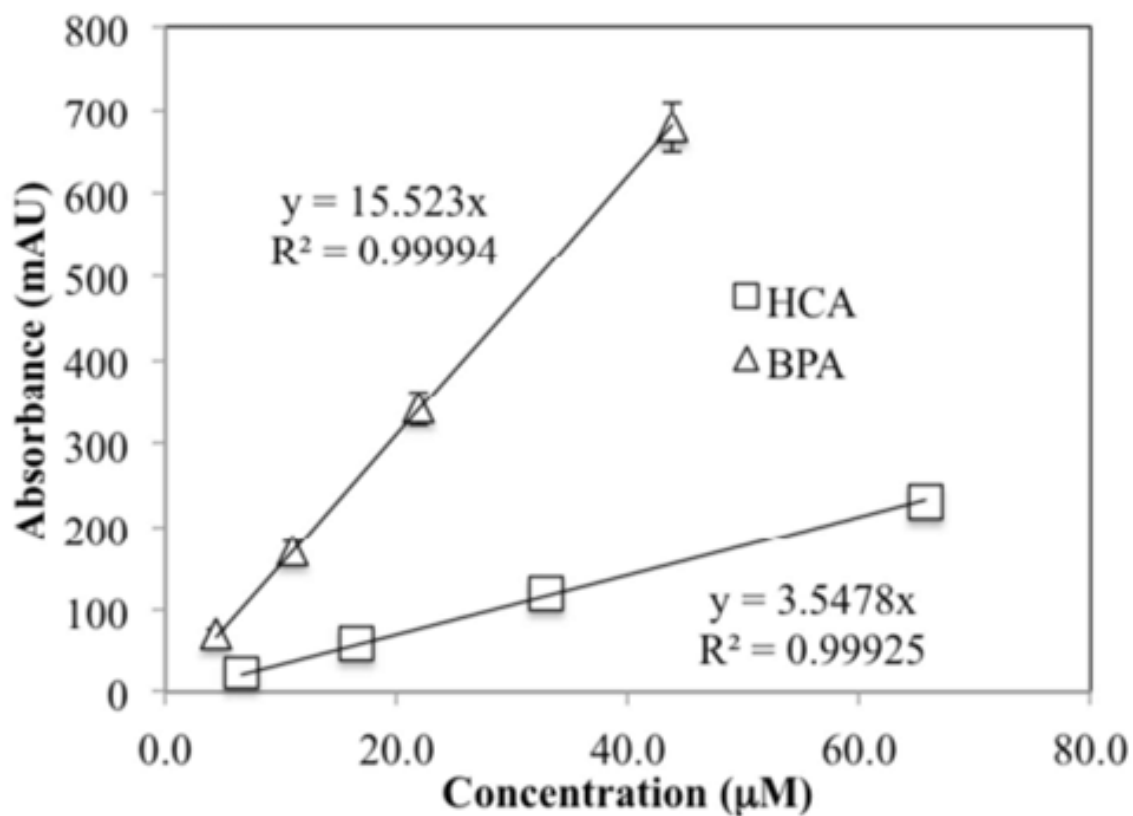


Figure 2.1. Exemplary standard curves for BPA and HCA constructed using HPLC-FLD measurements. Standard curves were prepared with different buffers or mineral salts medium, as necessary, to have the same matrix in samples and standards. Error bars smaller than the symbol size are not depicted.

(OPPTS) 830.7840 guidelines by stirring an excess amount of HCA in 10 mL Milli-Q water with a magnetic stirrer⁹⁴. An aliquot of the mixture was centrifuged at 8,000 g for 5 min to remove any undissolved HCA, and the concentration of HCA in the saturated solution was determined by repeated HPLC analysis over the course of 1 week to confirm that equilibrium had been reached. The experiments were performed in quintuplicate. The octanol-water partitioning coefficient was measured at 20 °C following the modified EPA OPPTS 830.7550 guidelines⁹⁵. In brief, 10 mL of Milli-Q water containing 50 µg of HCA was mixed with 10 mL of *n*-octanol in ten 20 mL glass vials. The vials were sealed with Teflon-lined rubber septa, and shaken by inverting 100 times for 5 min. After quiescence for 24 hours and phase separation, the HCA concentration in the lower aqueous phase was measured by HPLC. Measurement of HCA concentrations after an additional 24-hour incubation period confirmed that chemical equilibrium had established. The molar extinction coefficient of HCA was determined by measuring the S3 absorbance of HCA solutions at room temperature using a PerkinElmer Lambda 35 UV–Vis spectrophotometer (PerkinElmer, Waltham, MA).

Microcosm setup for initial 4-MP study. Microcosms were established in 60 mL serum bottles closed with black butyl rubber stoppers (Geo-Microbial Technologies, Ochelata, OK). Sediment samples were collected from four geographically distinct locations (latitude, longitude), including the Third Creek (35.949284, -83.939861), the Partnach Gorge (47.459198, 11.122777), the Neckar River (48.780193, 9.245422), and the Hainbach Creek (48.776559, 9.300439). The Third Creek location has a history of contamination of chlorinated solvents, the Neckar River flows along industrial areas, and the Partnach Gorge and Hainbach Creek are considered pristine water bodies. Each serum bottle received 5 g of sediment material (wet weight) and reduced (0.2 mM L-cysteine and 0.2 mM sodium sulfide) mineral salts medium to achieve a total volume of 30 mL in each vessel as well as a single addition of 2 mM of lactate as a readily fermentable substrate to ensure rapid establishment of anoxic conditions. BPA was added to the microcosms to a final aqueous phase concentration of 10 mg L⁻¹ (44 µM). Autoclaved control microcosms with BPA, and live microcosms without BPA, electron acceptor, or lactate were established for each sediment sample. All manipulations were performed inside an anoxic chamber (Coy Laboratory Products, Ann Arbor, MI) containing a nitrogen/hydrogen (97/3; v/v) atmosphere. The microcosms were incubated at room temperature in the dark without shaking. To establish different redox conditions, electron acceptors including nitrate (2 mM), nitrite (0.5 mM), amorphous ferric oxyhydroxide (FeOOH, 10 mM, nominal concentration), ferric citrate

(5 mM), sulfate (10 mM), and bicarbonate (30 mM) were added from anoxic, sterilized stock solutions at the concentrations indicated in parentheses. FeOOH was prepared following an established procedure⁹⁶. Electron acceptors were replenished by syringe when depleted. To test BPA degradation under oxic conditions, microcosms were established using the same mineral salts medium except that the bicarbonate buffer system was replaced with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (10 mM, pH 7.0) and the reductants and lactate were omitted. These microcosms were incubated in the dark, and the headspace was purged with filter-sterilized air every second day. Additional microcosms were prepared with the Third Creek sediment to assess the biodegradability of 4-MP. Ten mg L⁻¹ of 4-MP and 2 mM of lactate were added and electron acceptors were replenished as above.

Microcosm Setup for the MnO₂-induced BPA degradation study. Sediment samples used for microcosm setup were collected from four geographically distinct locations (latitude, longitude), including the Third Creek in Knoxville, TN (35.949284, -83.939861), the Axton Cross brown field site located alongside the Housatonic River in CT (47.459198, 11.122777), and the Shady Valley, a restored wetland area in TN (36.519554, -81.933327). An additional Third Creek sample was collected upstream (35.950664, -83.941932) of the site with reported chlorinated solvent contamination. Microcosms were established in 160 mL glass serum bottles containing 100 mL of reduced (0.2 mM L-cysteine and 0.2 mM sodium sulfide), 30 mM bicarbonate-buffered basal salts medium and sealed with black butyl rubber stoppers (Geo-Microbial Technologies, Ochelata, OK). Each serum bottle received 5 g (wet wt.) of sediment material. HCA was added to the microcosms from a filter-sterilized 1 g L⁻¹ stock solution to a final aqueous phase concentration of 5 mg L⁻¹ (33 µM). Autoclaved microcosms with HCA, live microcosms without HCA, and autoclaved, sediment-free medium with HCA served as controls. All manipulations were performed inside an anoxic chamber (Coy Laboratory Products, Ann Arbor, MI) containing a nitrogen/ hydrogen (97/3; v/v) atmosphere. To establish different redox conditions, electron acceptors including nitrate (2 mM), sulfate (10 mM), and amorphous ferric oxyhydroxide (FeOOH, 10 mM, nominal concentration) were added from anoxic, sterilized stocks at the concentrations indicated in parentheses. To examine HCA degradation under oxic conditions, microcosms were established using the same mineral salts medium except that the bicarbonate buffer system was replaced with phosphate buffer (5 mM, pH 7.0) and no reductants were added. The microcosms were incubated at room temperature in the dark without shaking, and the

electron acceptors were replenished by syringe when depleted. FeOOH was prepared following an established procedure⁹⁷.

Analytical procedures. The amount of BPA associated with the sediments was determined after sampling by an ultrasonic solvent extraction method with some modifications⁹⁸. In brief, 5 g of sediment was mixed with 10 mL of acetone/ethyl acetate (50:50, v/v), sonicated at 20 kHz for 15 min using a Branson Sonifier 250 (Branson Ultrasonic, Danbury, CT), and centrifuged at 5000g for 10 min. The extracts from three consecutive solvent extractions were combined and evaporated to dryness under a stream of filtered (0.25 μ m) nitrogen. The residue was dissolved in 10 mL water/acetonitrile (50:50, v/v) and subjected to HPLC analysis. The adsorption capacity of BPA on Third Creek sediment was also evaluated. Triplicate 60 mL vessels that received 5 g of autoclaved sediment, 30 mL of mineral salt medium, and 300 μ g (10 mg L⁻¹) of BPA each were incubated for 5 d. The slurry was removed from the vessels and transferred into 30 mL glass centrifuge tubes. The supernatants obtained after centrifugation at 5000g for 10 min and analyzed by HPLC.

An Agilent 1200 Series HPLC equipped with a diode array detector (DAD) and fluorescence detector (FLD) was used for the detection and quantification of BPA, its potential metabolites, and L-tyrosine. Separation was achieved on an Agilent Eclipse XDB C18 column (4.6 mm $\times$ 150 mm, 5 μ m) using isocratic elution with 1 mL min⁻¹ of acetonitrile/water (50:50, v/v). The FLD was set at excitation and emission wavelengths of 226 and 310 nm, respectively. For the initial 4-MP study, the DAD recorded the entire UV/Vis absorption spectra of peaks ranging from 190 to 400 nm. For the MnO₂-induced BPA degradation study, UV spectra from 220 to 400 nm were recorded to detect possible aromatic reaction intermediates.

Ferric and ferrous iron were measured using the ferrozine assay⁸⁶, and other electron acceptors were monitored by ion chromatography using a Dionex ICS 2100 system equipped with an 4 mm $\times$ 250 mm Ion-Pac AS18 hydroxide-selective anion exchange column (Sunnyvale, CA) with 23 mM potassium hydroxide solution as the eluent at a flow rate of 1 mL min⁻¹ and an ERS 500 suppressor (4 mm) set at a current of 57 mA.

To identify putative BPA transformation products in the initial 4-MP study, liquid–liquid extraction coupled with GC–MS was used. Three water-immiscible solvents including dichloromethane, ethyl ether, and ethyl acetate were employed for separation and purification of the metabolites. For metabolite extraction, a 2 L Pyrex glass vessel received 1 L of bicarbonate-buffered medium and 20 g of Third Creek sediment, and was incubated inside the anoxic chamber. After 30 d of incubation, 18 mL of sample was withdrawn from the vessel, and mixed with 2 mL of each solvent. The mixtures were shaken for 1 h and allowed to settle for 10 min. The organic phases were separated using a glass pipette and evaporated under a stream of nitrogen. The residues were dissolved in 100 μ L acetonitrile for GC–MS and HPLC analyses. Compound identity was confirmed by comparing HPLC retention times, UV/Vis absorption spectra, and mass spectra with authentic standards.

MnO₂-mediated degradation of BPA and HCA to carbon dioxide (CO₂) was monitored using a Micro-GC (Agilent 3000A, Agilent Technologies, Santa Clara, CA) equipped with a thermal conductivity detector using a PLOT Q column. A dimensionless Henry's constant of 1.24 was used to calculate the total amount of CO₂ in the incubation vessels⁹⁹.

¹H-NMR spectra were obtained on a Varian InNova 500 MHz NMR spectrometer in solutions of CDCl₃. All ¹³C-NMR spectra were recorded on a Varian InNova 500 MHz NMR spectrometer in solutions of CDCl₃.

Accurate mass measurements were performed on an Exactive Plus Orbitrap MS (Thermo Fisher Scientific, Waltham, MA) via ESI with a spray voltage of 4,000 V. The samples were direct injected as solutions in methanol using a Hamiltonian 250- μ L syringe (Hamiltonian Company, Reno, NV) at a flow rate of 20 μ L/min. Detection was done in positive-ion mode with nitrogen as the sheath gas.

2.4 Results and discussion

BPA associated with sediment. No BPA was detected in extracts from the untreated sediment samples indicating that no extractable BPA was associated with any of the sediments used in this study. Following the addition of BPA, 20–50% sorbed to the solids depending on the origin of the sediment (**Table 2.1**). The ultrasonic solvent extraction

method efficiently desorbed BPA from the sediments with recoveries of 96–100% (**Table 2.1**).

Table 2.1. Adsorption and recovery of BPA from spiked sediment samples.

	Third Creek	Partnach Gorge	Neckar River	Hainbach Creek
% Adsorption	53 (4)	22 (3)	34 (3)	50 (4)
% Recovery	98 (1)	99 (1)	97 (2)	100 (3)

Values in parenthesis represent the standard deviations of three replicates.

Fate of BPA in sediment microcosms. Aqueous phase BPA concentration profiles in microcosms established with the Third Creek sediment demonstrated an initial decrease in BPA concentrations, which was expected based on the sorption experiments (**Figure 2.2**). Under oxic conditions, aqueous phase BPA was completely degraded within 1 week of incubation. Aerobic BPA degradation has been demonstrated previously^{61, 68-69, 100} and these findings indicate that microorganisms capable of oxygen-dependent BPA catabolism are present in freshwater sediments. In the absence of oxygen, none of the live microcosms showed a statistically significant loss of aqueous phase BPA compared to negative control incubations ($p > 0.05$, **Figure 2.2**), although the mean concentrations were slightly lower than those of the negative control incubations. Interestingly, a putative BPA transformation product was detected in all microcosms except those amended with sulfate or incubated under oxic conditions (**Figure 2.3**). The same peak was observed in BPA-amended microcosms established with the other three freshwater sediment materials after a 1 month incubation period under the same conditions. The transformation product was not detected in live control microcosms without BPA suggesting that this compound was a possible BPA transformation product. The formation of this putative BPA metabolite did not coincide with a decrease in aqueous BPA concentrations, which remained near 5 mg L⁻¹. We initially concluded that the rates of BPA desorption from the sediment exceeded the BPA degradation rates, or that sorbed BPA was preferentially degraded.

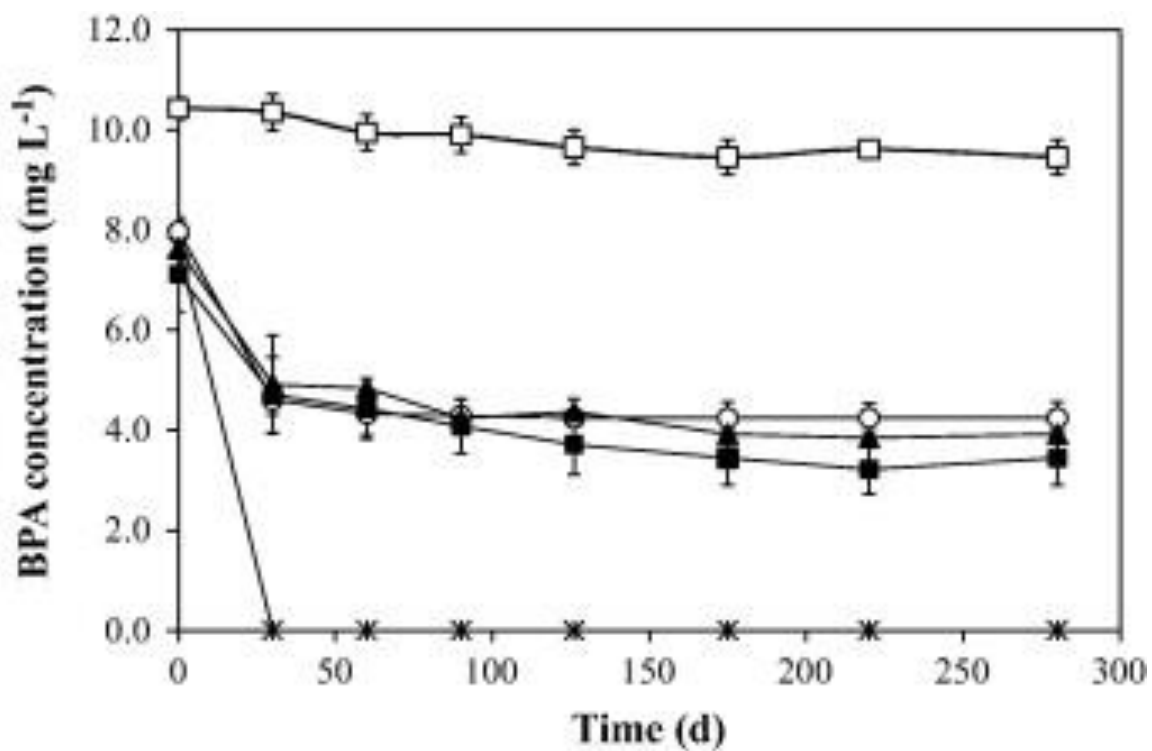


Figure 2.2. Concentration profiles of BPA in the microcosm established with the Third Creek sediment under nitrate-reducing (N), Fe (III)-reducing (FeCit-amended, j), and oxic conditions (□), and two autoclaved negative controls in the absence (h) or presence (s) of sediment. All data points are the means of three replicates. Error bars represent the standard deviations.

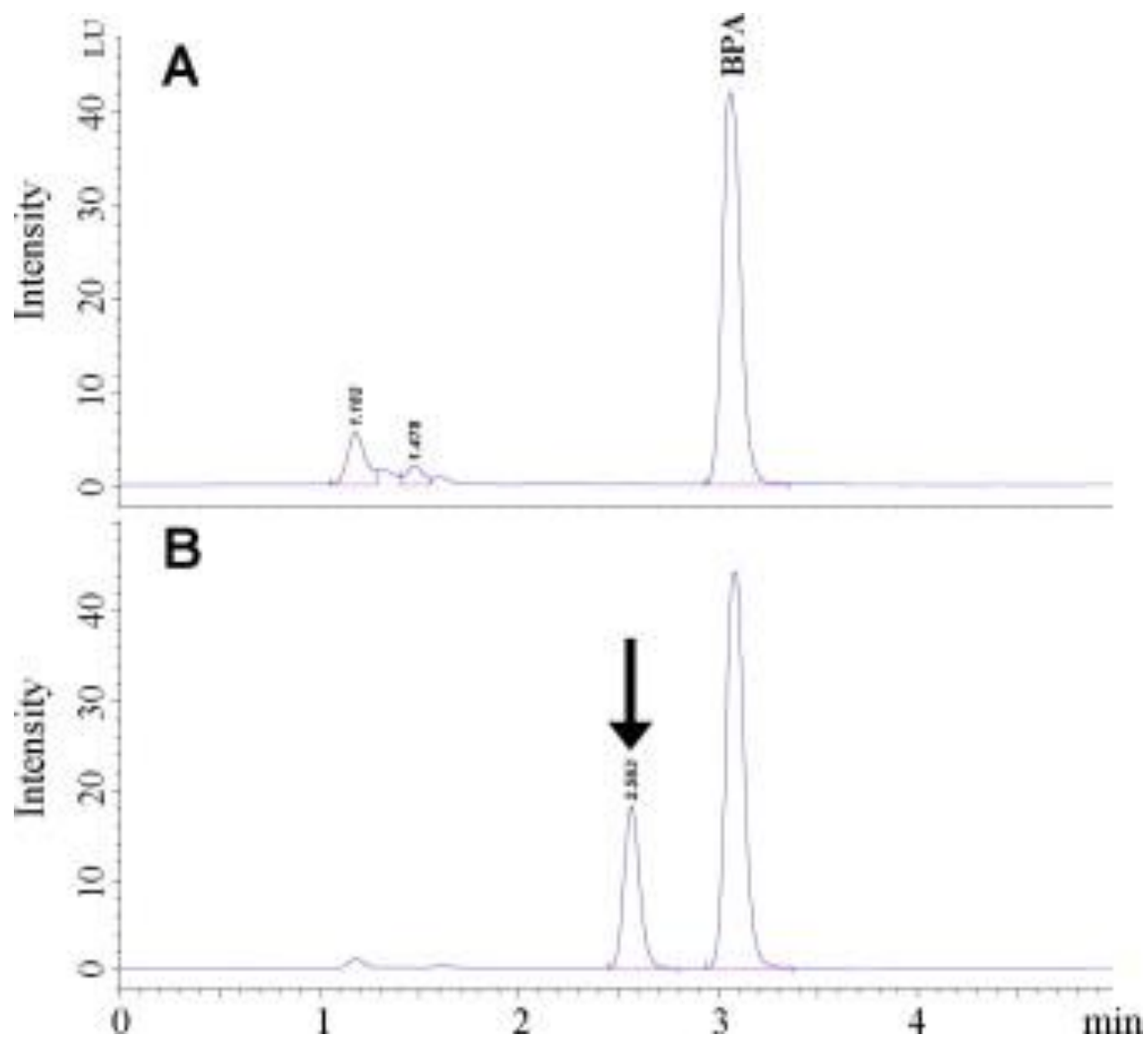


Figure 2.3. Representative HPLC chromatograms obtained from a negative control microcosm (A) and a microcosm established with the Third Creek sediment amended with nitrate after 30 d of incubation (B). The black arrow indicates the retention time of the putative BPA metabolite.

After a 1 year incubation period, each vessel was sacrificed to determine the total amount of BPA remaining (**Table 2.2**). In the microcosms incubated under oxic conditions, no BPA was detected associated with the sediment indicating complete removal. In contrast, BPA was detected in all microcosms incubated without oxygen. A 2-tailed t-test was used to determine the statistical significance of the discrepancies between live treatments and negative controls in each microcosm (SPSS 20.0, SPSS Chicago, IL). Based on the analyses, the decreases in BPA amounts extracted from live microcosms were significantly larger compared to the negative controls ($p < 0.01$, **Table 2.2**) suggesting a process that reduced the amount of extractable BPA was present in live incubations only. For example, aging and the formation of covalently bound residues can affect the extraction efficiency¹⁰¹⁻¹⁰³. Alternatively, reactive mineral-mediated⁸⁶ or microbial BPA transformation may have occurred.

Table 2.2. Mass balance of BPA after 1 year of incubation period.

Third Creek sediment				Partnach Gorge sediment		
	BPA-S	BPA-L	Discrepancy	BPA-S	BPA-L	Discrepancy
Nitrate	107.1	128.2	64.7 (5.5)	122.9	125.7	51.4 (5.6)
Nitrite	103.9	136.4	59.7 (8.2)	126.3	124.2	49.5 (7.7)
Sulfate	111.7	133.7	54.6 (12.2)	127.6	126.7	45.6 (8.6)
Methanogenic	120	129.1	50.9 (10.0)	121.9	129.9	48.2 (9.2)
FeOOH	108.3	128	63.7 (6.6)	131.5	119.4	49.1 (9.3)
FeCit	104.3	137.2	58.5 (9.2)	119.4	126.5	54.0 (9.8)
Negative	115.9	141.5	42.7 (8.8)	133.1	134.3	32.6 (9.8)

Neckar River sediment				Hainbach Creek sediment		
	BPA-S	BPA-L	Discrepancy	BPA-S	BPA-L	Discrepancy
Nitrate	123.4	138.9	37.7 (5.5)	138.2	110	51.8 (2.6)
Nitrite	129	132.4	38.6 (8.2)	146	103.9	50.1 (9.2)
Sulfate	131.4	144.8	23.8 (12.2)	139.7	101.7	58.6 (9.7)
Methanogenic	126.7	140.6	32.7 (10.1)	119.1	120	60.9 (8.1)
FeOOH	128.2	129.7	42.1 (6.6)	158	100.4	41.6 (12.2)
FeCit	116.7	138.1	45.1 (9.2)	137.2	104.3	58.5 (7.2)
Negative	132	145.5	22.4 (14.7)	151.5	115.9	32.7 (8.3)

The unit is μg . Values in parenthesis represent the standard deviations of duplicate samples. BPA-S represents the total amount of BPA extracted from the solid phase, and BPA-L represents that of BPA extracted from the liquid phase. Discrepancy was obtained by subtracting BPA-S and BPA-L from the initial amount (300 μg) added to each bottle.

Identification of a putative BPA metabolite. In order to identify the putative BPA degradation intermediate, liquid–liquid extraction procedures were evaluated. Among the solvents used for the extraction, ethyl ether showed the best extraction yields. The GC–MS analysis of the extract identified the putative metabolite as 4-MP (**Figure 2.4**). The identity of 4-MP was confirmed by comparing the HPLC retention times, UV–Vis absorption spectra, and mass spectra with authentic 4-MP. The maximum 4-MP concentrations detected in the Third Creek sediment microcosms ranged from 6.3×10^{-1} to $20.8 \times 10^{-1} \text{ mg L}^{-1}$ depending on the electron acceptor provided. A hypothetical pathway to explain the formation of 4-MP

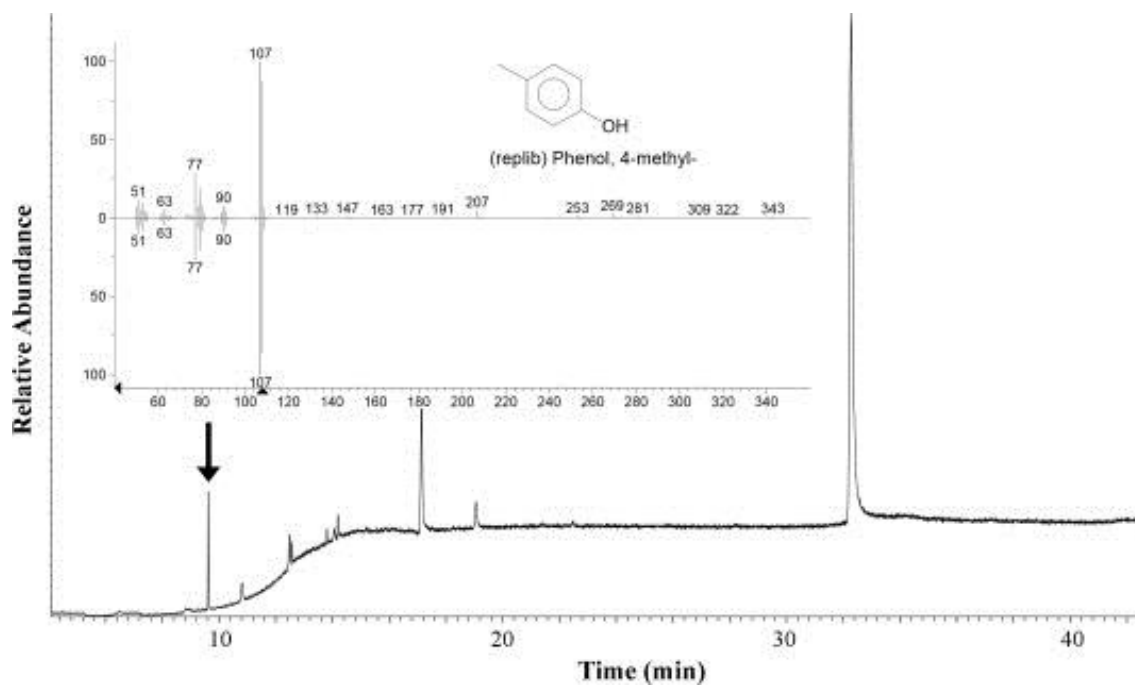
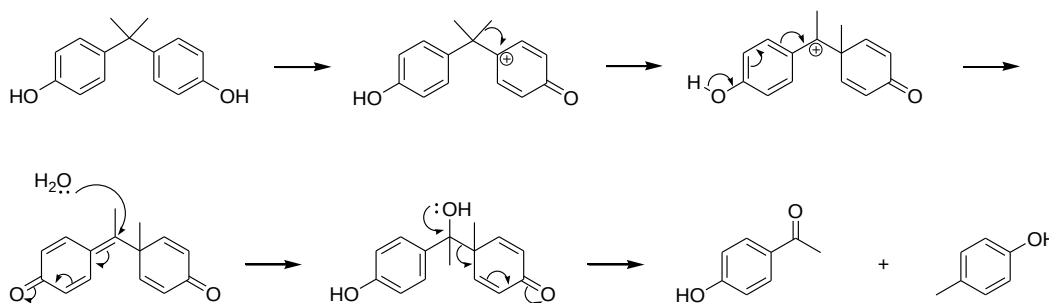


Figure 2.4. Full scan GC–MS chromatogram and the mass spectrum of 4-MP. The arrow indicates the retention time of 4-MP.

from BPA is shown (**Scheme 2.3**). The oxidation of one of the phenol rings can lead to a cationic intermediate, which undergoes a methyl shift (i.e., Wagner–Meerwein rearrangement). Subsequent C–C bond cleavage and rearomatization can form the putative intermediate 4-MP. To test if this pathway contributes to 4-MP formation in the microcosms, $^{13}\text{C}_2$ -BPA with only methyl carbons labeled, $^{13}\text{C}_3$ -BPA with all non-aromatic carbons labeled, and $^{13}\text{C}_{12}$ -BPA with all aromatic carbons labeled were added to Third Creek sediment microcosms. Material labeled only outside of the aromatic rings was not commercially available, and $^{13}\text{C}_2$ -BPA and $^{13}\text{C}_{12}$ -BPA were synthesized from phenol and ^{13}C -acetone with yields of 90–92%. The formation of 4-MP was observed in the microcosms supplied with ^{13}C -labeled BPA; however, no label was detected in 4-MP indicating that BPA was not the source of 4-MP. A series of control experiments demonstrated that 4-MP did not originate from laboratory plastic materials (e.g., syringes) chemicals used for medium preparation, and implicated the sediments as possible sources. To assess if the sediments were the source of 4-MP, microcosms without organic amendments and electron acceptors were monitored. None of these microcosms produced 4-MP. Another set of microcosms received 2 mM of lactate but no BPA or electron acceptors. Unexpectedly, after a 1 month incubation period, the formation of 4-MP was observed in these microcosms. This finding indicated that 4-MP formation from the sediment required the addition of an electron donor (e.g., lactate). No 4-MP was detected in the lactate stock solution or the sediment, indicating that 4-MP was formed during microcosm incubation. 4-MP was not detected in the live microcosms amended with sulfate (**Figure 2.5**) suggesting that (i) 4-MP was not generated from the sediment-associated organic matter in the presence of sulfate or that (ii) 4-MP was rapidly degraded in the presence of sulfate and evaded detection. The microcosms amended with 4-MP showed that this molecule was degraded under all redox conditions tested (**Figure 2.6**), as has been demonstrated previously¹⁰⁴⁻¹⁰⁷.



Scheme 2.3. Putative anaerobic microbial transformation of BPA leading to 4-MP as an intermediate.

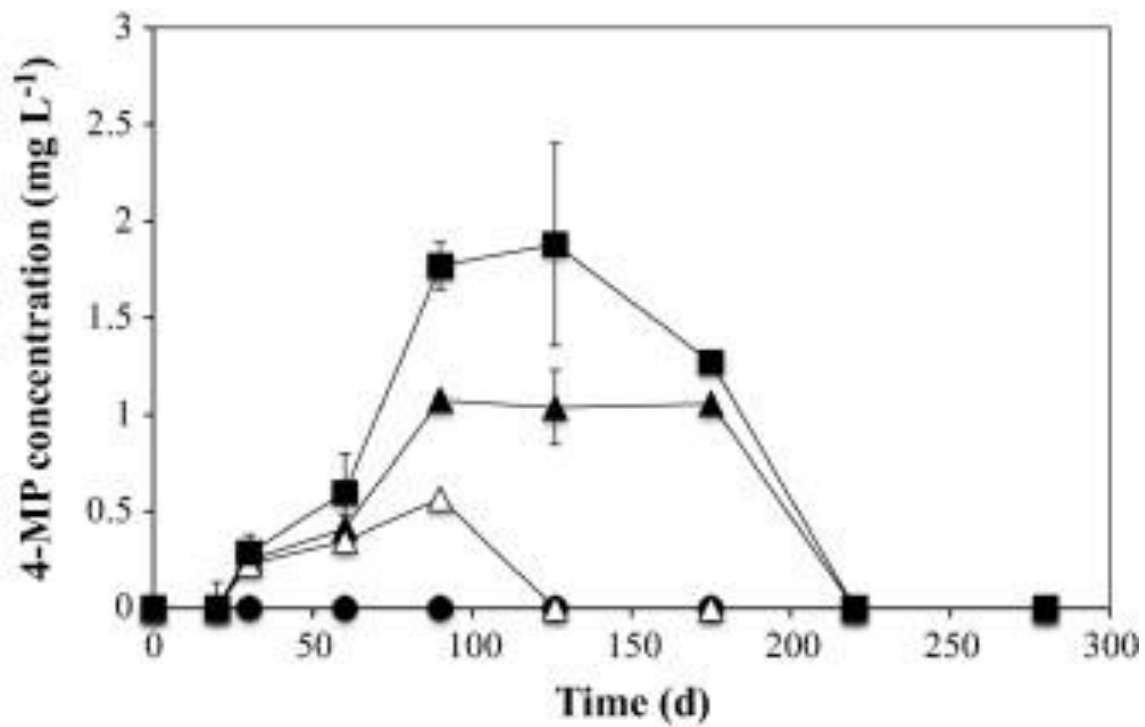


Figure 2.5. Concentration profiles of 4-MP in Third Creek microcosms under different redox conditions, including nitrate-reducing (●), Fe (III)-reducing (△), sulfate-reducing (▲), and methanogenic conditions (■). All data points are the means of three replicates. Error bars represent the standard deviations.

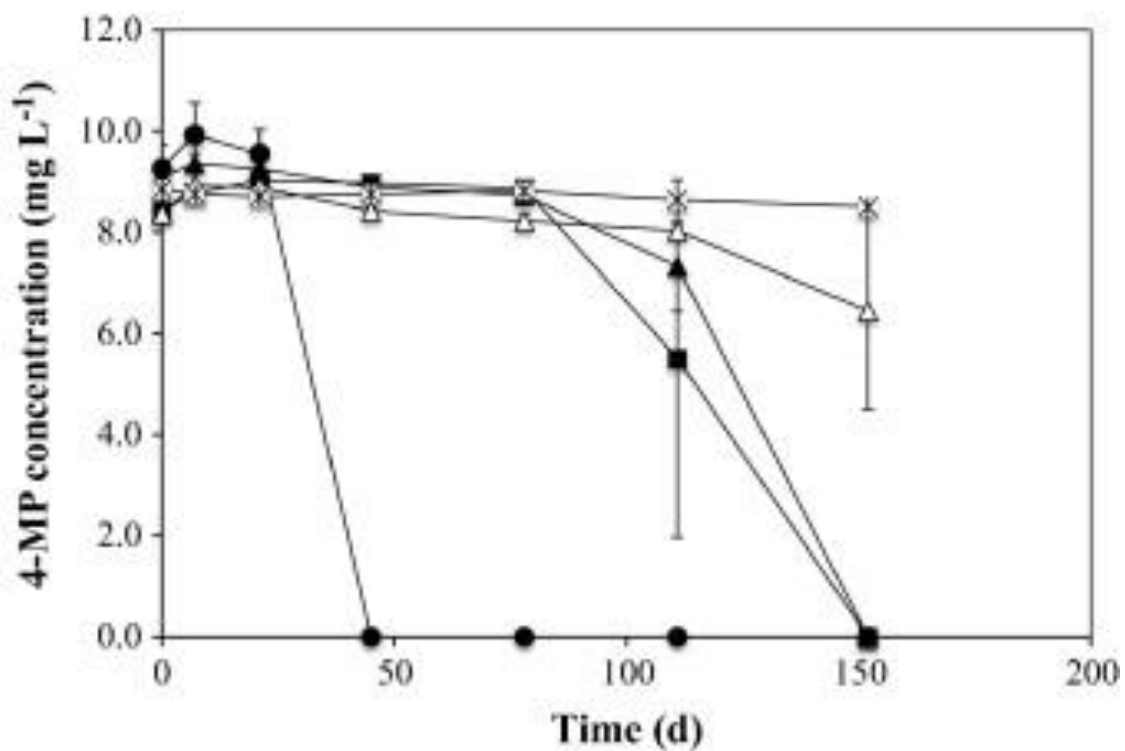


Figure 2.6. Concentration profiles of 4-MP in the microcosms established with the Third Creek sediment under selected redox conditions, including nitrate-reducing (●), Fe (III)-reducing (△), sulfate-reducing (▲), and methanogenic (■) and autoclaved negative controls in the presence of sediment (×). All data points are the means of three replicates. Error bars represent the standard deviations.

The rates of 4-MP degradation varied depending on the electron acceptor provided, and the degradation was significantly faster in nitrate compared to sulfate-amended microcosms (**Figure 2.6**). This observation suggested that 4-MP formation from sediment organic matter was repressed in the presence of sulfate, possibly due to sulfide toxicity¹⁰⁸.

Several anaerobic intestinal bacteria such as *Lactobacillus* sp. and *Clostridium difficile* can form 4-MP from L-tyrosine via decarboxylation in the absence of oxygen, but 4-MP formation from soils or sediments has not been reported¹⁰⁹⁻¹¹⁰. Furthermore, L-tyrosine was not observed in any microcosm (detection limit of 50 $\mu\text{g L}^{-1}$) (data not shown). Lactate addition and microbial activity were essential for 4-MP formation in the microcosms, suggesting microbial biomass or sediment-associated organic matter as possible sources of 4-MP; however, the detailed formation mechanism(s) remain to be determined. Since the formation of 4-MP was observed in microcosms established with sediments collected from different geographical locations, it is likely that the mechanisms and pathways leading to 4-MP formation are common to freshwater sediments. The ¹³C-labeling experiments excluded BPA as a source of 4-MP, and our findings emphasize that the detection of possible aromatic BPA degradation products such as 4-MP in sediments or sediment-derived microcosms must be interpreted carefully to avoid erroneous conclusions regarding the fate of BPA or similar compounds.

Detection of a Major MnO₂-generated BPA Transformation Intermediate. BPA disappearance was observed in abiotic incubations with MnO₂ as oxidant. The average surface area of the MnO₂ particles used in these experiments was 529 ± 14 ($n = 3$) $\text{m}^2 \text{g}^{-1}$. Typical BPA concentration profiles correlated with the initial MnO₂ loading and are shown in (**Figure 2.7**). No significant (< 0.01) suggesting that BPA loss due to sorption to the glass surface or the rubber stopper was negligible. In the presence of MnO₂, BPA transformation gave rise to a new peak in HPLC chromatograms with a shorter retention time of 1.7 min (**Figure 2.8**). The peak area representing the unknown transformation product increased concomitantly with BPA consumption. The similarity between the UV spectra of the unknown transformation product ($\lambda_{\text{max}1} = 220 \text{ nm}$, $\lambda_{\text{max}2} = 273$) and BPA ($\lambda_{\text{max}1} = 225 \text{ nm}$, $\lambda_{\text{max}2} = 277$) (**Figure 2.9**), the correlation of the area increase of the new peak with BPA decrease (**Figure 2.7**), and the absence of this peak in control incubations suggested that this peak represented a transformation product of MnO₂-mediated BPA oxidation. Throughout the incubation, UV spectra were recorded over a range of 220–400 nm but neither the DAD nor

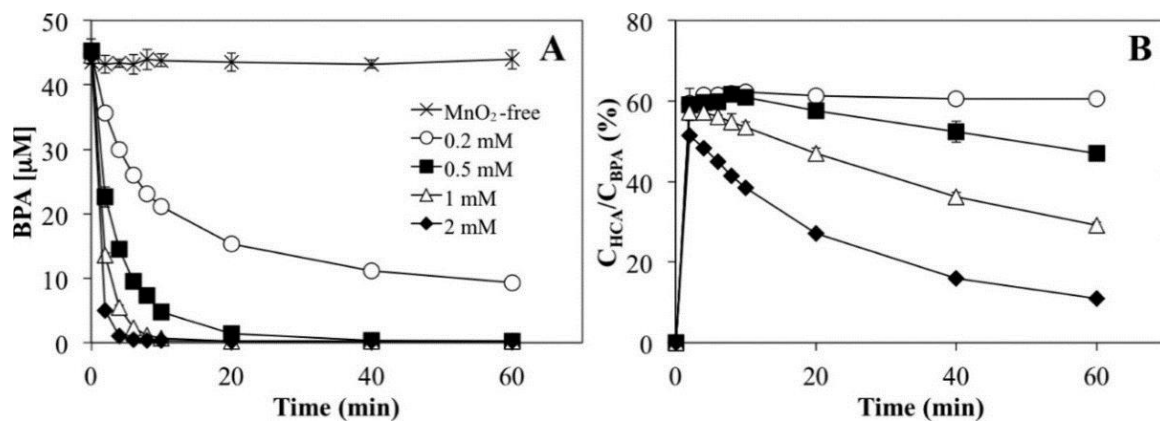


Figure 2.7. Effect of MnO₂ loading on BPA degradation (A) and conversion efficiency of BPA to HCA (B) in MOPS buffer (pH 7.4) initially containing 44 μM (10 mg L⁻¹) BPA. The legend shown in panel A also applies to panel B. Error bars represent the *standard* deviation of triplicate samples. MnO₂ is expressed as nominal concentration. Error bars smaller than the symbol size are not depicted.

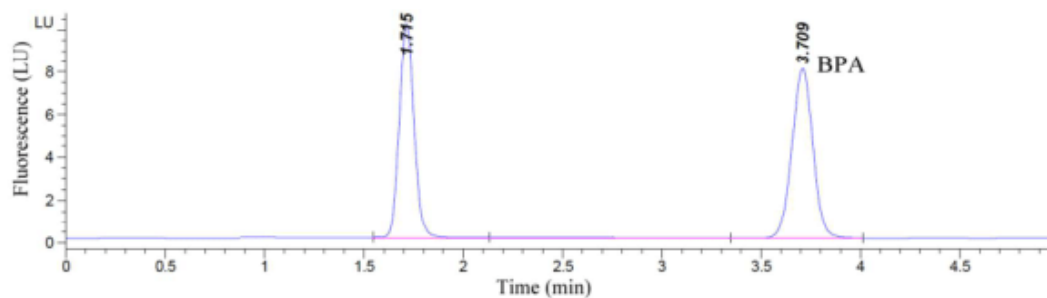


Figure 2.8. HPLC-FLD chromatogram of 44 μM of BPA after incubation for 1 hour with 0.2 mM nominal concentration of MnO₂ at pH 7. LU refers to luminescence units.

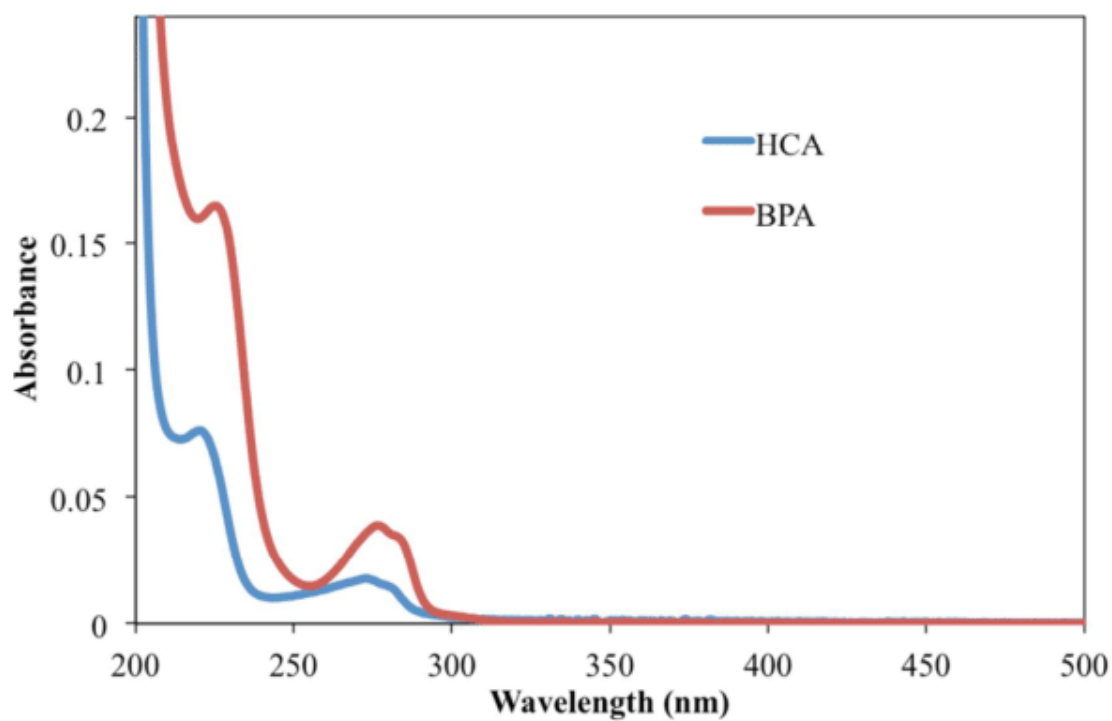


Figure 2.9. Comparison of UV spectrum of BPA and HCA (10 μ M, each) at pH 7 in high purity Milli-Q water (Millipore Milli-Q, Bedford, MA) analyzed by a PerkinElmer Lambda 35 UV-vis spectrophotometer.

the FLD generated signals indicative for the formation of other aromatic compounds.

Identification of the BPA Transformation Product. High-resolution MS analysis resulted in a base peak with an accurate mass of 135.08011 (**Figure 2.10A**). The most likely molecular formula containing only C, H, and O for this ion was predicted to be C₉H₁₁O⁺. ¹H NMR spectra also were obtained from several samples of the transformation product, originally in CDCl₃ and later in CD₃OD, in order to determine the probable chemical environment of protons in the molecule (**Figure 2.11A and 2.11C**). In CDCl₃ at 500 MHz, three peaks were observed: a singlet integrating for 6 H at δ 1.53, a doublet integrating for 2 H at δ 6.80, and a doublet integrating for 2 H at δ 7.37. The two doublets were coupled with a J value of 8.6 Hz. The chemical shift and coupling for the doublets at δ 6.80 and δ 7.37 are indicative of p-substituted phenol, whereas the chemical shift and integrations for the singlet at δ 1.53 is suggestive of a 2-substituted isopropyl moiety. These observations led to HCA being assigned as the tentative structure of the BPA transformation product. The discrepancy between the expected mass-to-charge ratio (*m/z*) of 153.09101 for the [M + H]⁺ ion of HCA and the observed *m/z* of 135.08011 suggested that the 2-position substituent had likely been lost during ionization, and the chemical shift of the methyl groups of the isopropyl moiety fell in a range that would not unambiguously report the nature of this functional group. The accurate mass measurement of synthetic HCA displayed an identical base peak (135.0801 *m/z*) to that of the BPA transformation product (**Figure 2.10B**). The NMR spectra of synthetic HCA and the BPA transformation product, both in CD₃OD, revealed matching spectra (**Figure 2.11B and 2.11C**). Further, the BPA transformation product and HCA shared identical HPLC retention times and UV–Vis spectra. Agreement between MS data, ¹H NMR data in CD₃OD, retention times and UV–Vis spectra confirmed that HCA was the major BPA transformation product.

Physicochemical Properties of HCA. Several physicochemical properties including octanol-water partition coefficient (*K*_{ow}), aqueous solubility, and UV–Vis spectral properties for HCA were determined (**Table 2.3, Figures 2.9 and 2.12**). An HCA partition coefficient Log *K*_{ow} of 0.76 ± 0.04 (*n* = 10) and an aqueous solubility of 2.65 ± 0.04 g L⁻¹ at 25 °C (*n* = 5) were measured. For BPA, the same procedures determined a Log *K*_{ow} of 2.76 ± 0.08 (*n* = 10) and an aqueous solubility of 0.31 ± 0.02 g L⁻¹ at 25 °C (*n* = 5), and both of these measurements were in the range of literature values reported for BPA (Log *K*_{ow} = 2.2–3.82; aqueous solubility 0.12–0.3 g L⁻¹)^{2, 111–112}. Compared to BPA, the *K*_{ow} of HCA is at least 2

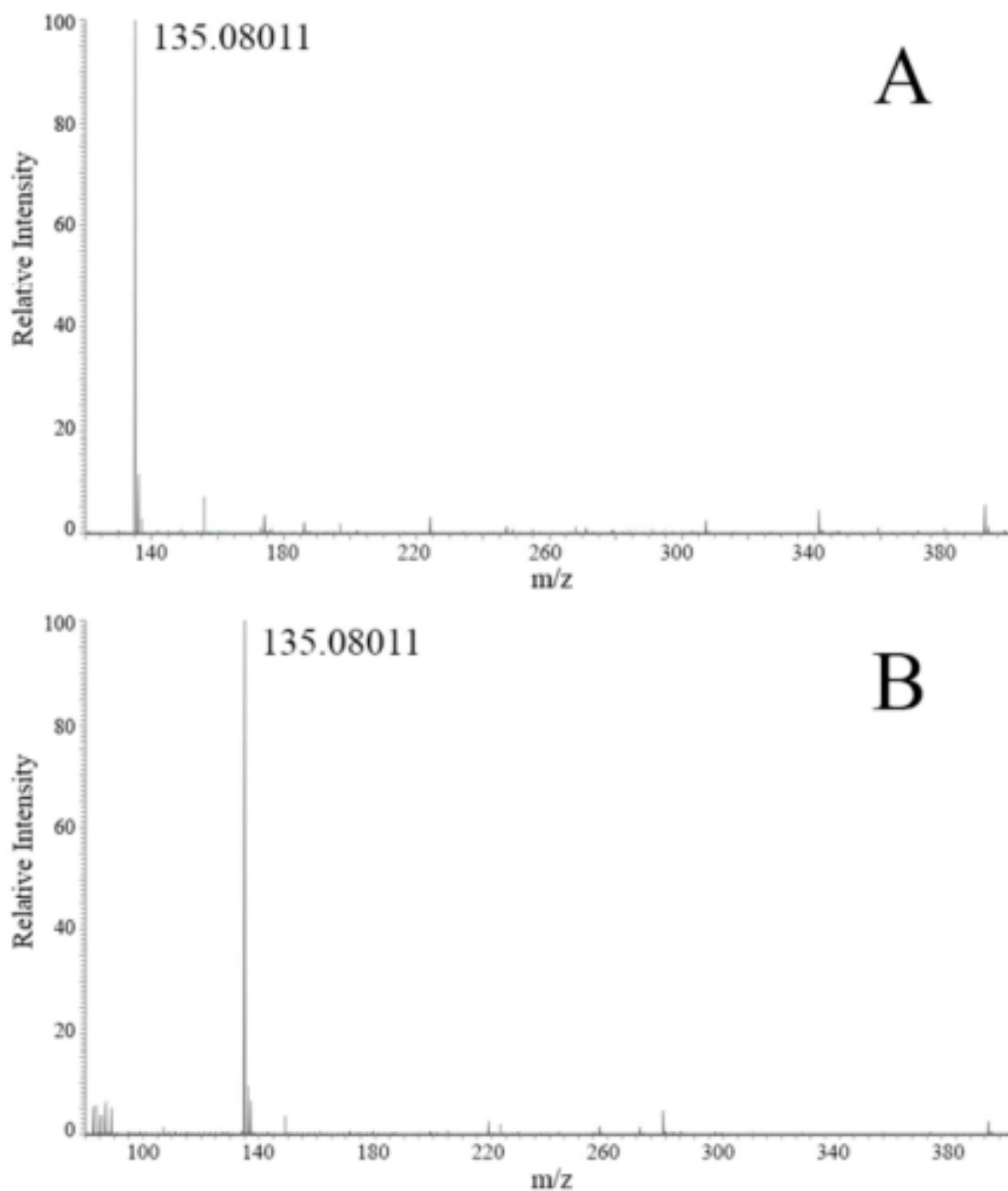


Figure 2.10. Electrospray ionization mass spectrum of the BPA transformation product (A) and synthesized authentic HCA (B). An accurate mass measurement of $m/z = 135.08011$ was obtained.

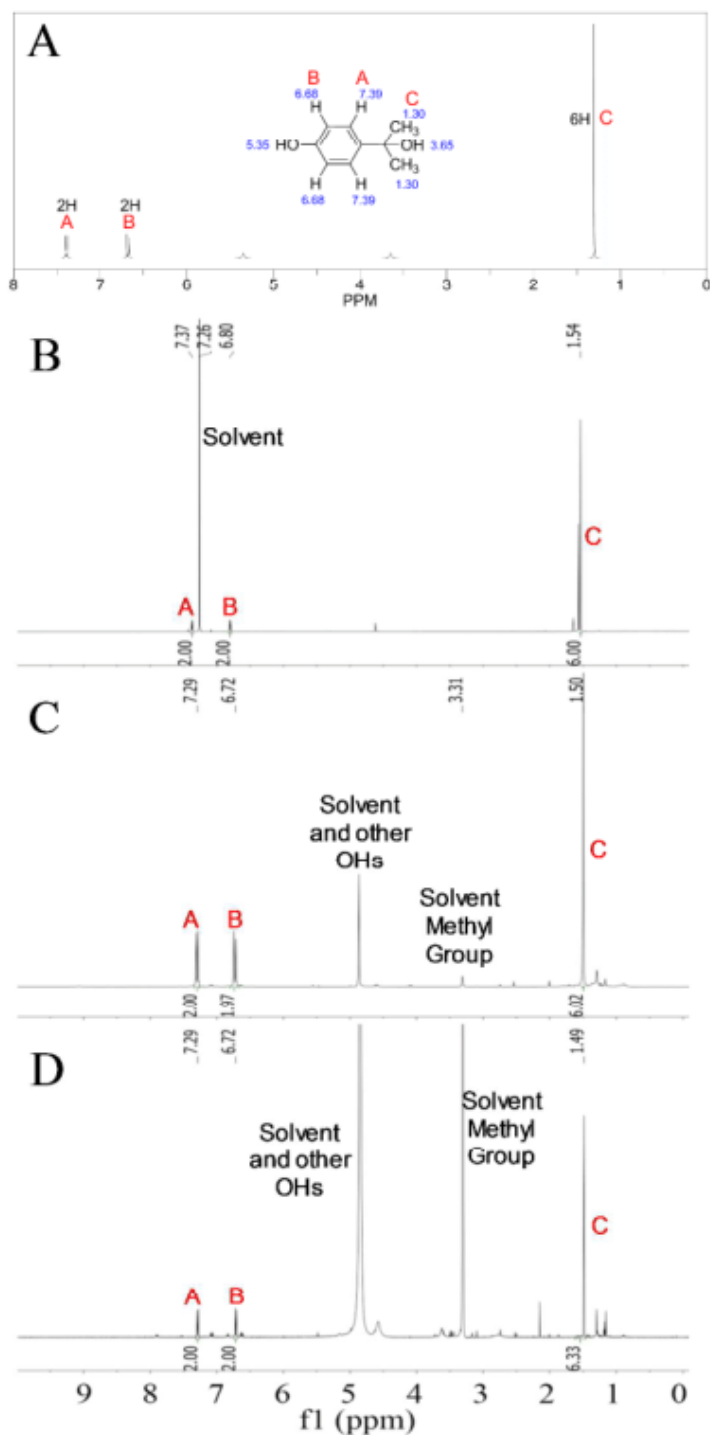


Figure 2.11. ^1H NMR spectrum of HCA predicted using ChemBioDraw Ultra 12.0 (A); 500 MHz ^1H NMR spectrum of the BPA transformation product in CDCl_3 , δ 7.37 (d, $J=8.5$ Hz, 2H), 6.80 (d, $J=8.5$ Hz, 2H), 1.53 (s, 6H) (B); 500 MHz ^1H NMR spectrum of synthesized authentic HCA in CD_3OD , δ 7.29 (d, $J=8.8$ Hz, 2H), 6.72 (d, $J=8.8$ Hz, 2H), 1.50 (s, 6H) (C); 500 MHz ^1H NMR spectrum of the BPA transformation product in CD_3OD , δ 7.29 (d, $J=8.8$ Hz, 2H), 6.72 (d, $J=8.8$ Hz, 2H), 1.49 (s, 6H) (D).

Table 2.3. Physicochemical properties of HCA and BPA determined in this study.

Compound	octanol-water partition coefficient (Log K _{ow})	aqueous solubility ^b (g/L)	molar extinction coefficient (1/mM*cm)	
			$\lambda_{\max 1}^c$	$\lambda_{\max 2}^d$
HCA	0.76 ± 0.04	2.65 ± 0.02	7.6	2.1
	(NA) ^a	(NA)	(NA)	(NA)
BPA	2.76 ± 0.08	0.31 ± 0.02	16.5	3.7
	(2.2-3.82)	(0.12-0.3)	16	4

Literature values are reported in parenthesis. Not available (NA)^a. measured at 25 °C^b. HCA = 220 nm, BPA = 225 nm^c. HCA = 273 nm, BPA = 277 nm^d.

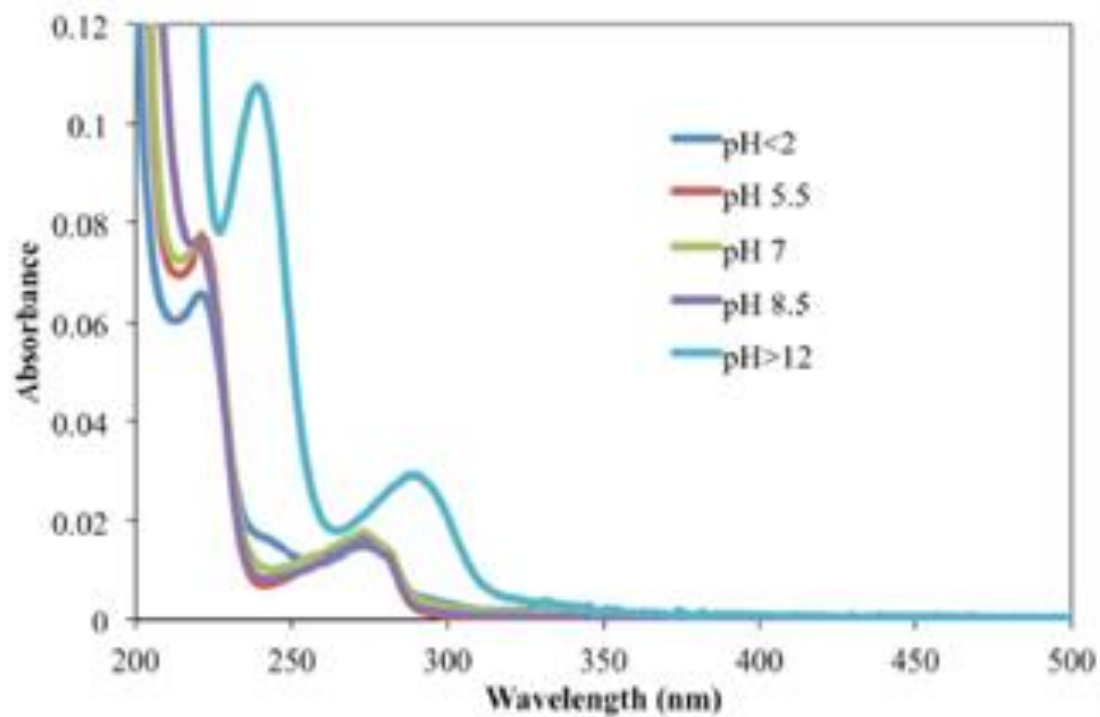


Figure 2.12. UV spectrum of HCA (10 μ M) at different pH analyzed by a PerkinElmer Lambda 35 UV–vis spectrophotometer. For pH < 2 and pH > 12, 0.02 M of HCl and NaOH was used, respectively, and for pH 5.5– 8.5, 1 mM Tris buffer was used.

orders of magnitude lower and the aqueous solubility about 10-fold higher, suggesting that HCA has increased mobility in saturated environmental systems. HCA had more than 2-fold lower molar extinction coefficients than BPA at each λ_{max} (**Table 2.3, Figure 2.10**). The UV spectrum maxima obtained with HCA at circumneutral pH shifted to higher wavelengths at alkaline pH (>pH 12) (**Figure 2.12**). Under acidic conditions (pH 2), no spectral shifts were observed compared to pH 7 spectra; however, the absorbance at $\lambda_{\text{max}1}$ decreased (**Figure 2.12**). Comparable absorbance maxima at both λ_{max} were observed in the pH range of 5.5 to 8.5 (**Figure 2.12**).

Kinetics of BPA and HCA Degradation by MnO₂. When 44 μM BPA was incubated with 0.5 mM (nominal concentration) MnO₂ at pH 7.2, 39.2 ± 0.4 μM BPA was consumed and 25.1 ± 0.3 μM HCA was formed in 10 min. A BPA removal rate of 0.10 ± 0.01 min⁻¹ was measured with 0.2 mM MnO₂, and a 10-fold higher rate of 0.96 min⁻¹ was observed with 2 mM MnO₂. The conversion efficiency of BPA to HCA reached 64% with 0.2 mM MnO₂ (**Figure 2.7**). With higher MnO₂ loadings (i.e., > 0.5 mM), HCA concentrations decreased after reaching a maximum (**Figure 2.13**) suggesting that HCA was also susceptible to MnO₂-mediated oxidation.

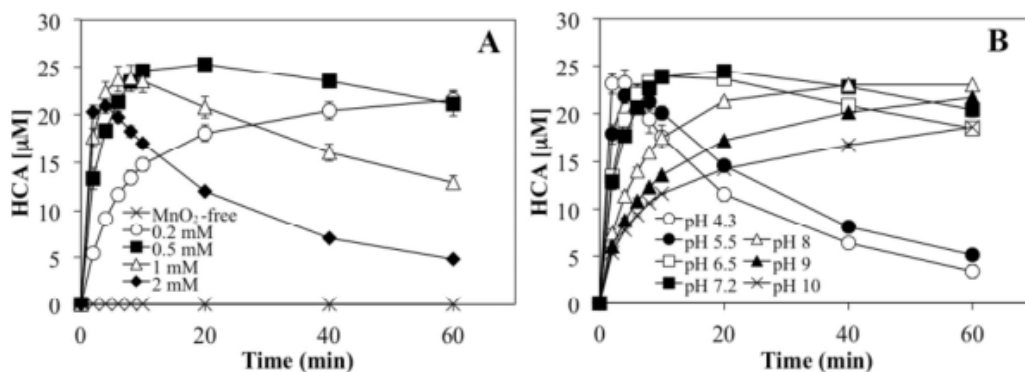


Figure 2.13. Effect of MnO₂ loading (A) and pH (B) on formation of HCA in MOPS buffer (pH 7.4) initially containing 44 μM (10 mg L⁻¹) BPA. Error bars represent the standard deviation of triplicate samples. MnO₂ is expressed as nominal concentration. Error bars smaller than the symbol size are not depicted.

The conversion efficiency of BPA to HCA with 0.2 mM MnO₂ did not decrease over time (**Figure 2.7**), indicating that MnO₂ preferentially reacted with BPA over HCA. An independent experiment with 44 μM HCA in MOPS buffer (pH 7.2) (no BPA present) demonstrated that HCA was indeed degraded by MnO₂ (**Figure 2.14**), but the reaction rates were

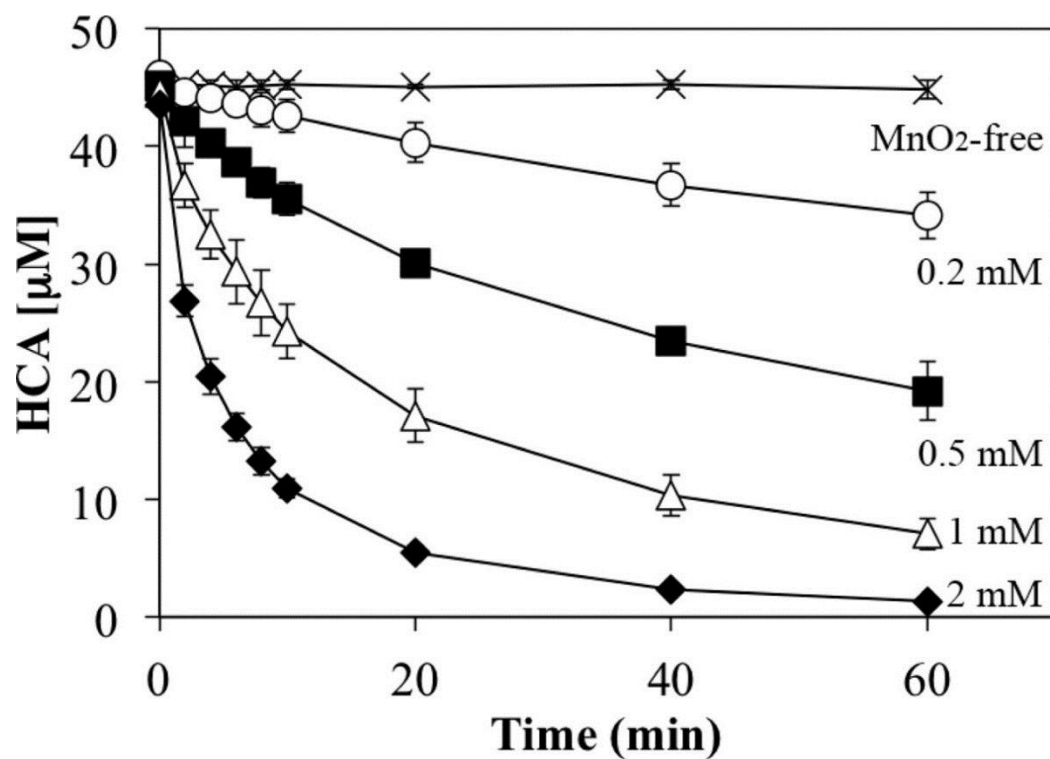


Figure 2.14. Effect of MnO₂ loading on HCA degradation in MOPS buffer (pH 7.4) initially containing 44 μM HCA. Error bars represent the standard deviation of triplicate samples. MnO₂ is expressed as nominal concentration. Error bars smaller than the symbol size are not depicted.

approximately 5-fold lower than those measured for BPA (**Figure 2.15**). During the early stages of the reaction (up to 10 min), the BPA and HCA concentration decreases followed pseudo-first order reaction kinetics, but the rates slowed down in the later stage of the reaction (**Figure 2.16**).

Thus, to evaluate the reaction kinetics, the initial rate constants, k_{init} (min^{-1}), were calculated from four early time points (i.e., < 10 min) where a linear relationship was maintained (**Figure 2.16**). A linear relationship between the initial rates and the loading of MnO_2 was observed for both BPA and HCA (**Figure 2.15**). Solution pH affected the BPA and HCA degradation reaction kinetics, and higher rate constants were observed with decreasing pH over the tested pH range from 4.5 to 10 (**Figures 2.15 and 2.17**). The solution pH did not significantly affect the conversion efficiency of BPA to HCA (**Figure 2.17**), suggesting that the pathway leading to the formation of HCA occurs over a broad pH range. CO_2 evolution was observed during the incubations with BPA and HCA, suggesting that BPA and HCA were mineralized (**Figure 2.18**). When 44 μM BPA was incubated with 2 mM MnO_2 at pH 2, both BPA and its degradation intermediate HCA were completely consumed within 10 min of incubation, and up to 73% (mol/mol) of the initial amount of BPA was recovered as CO_2 during a 24 day incubation period (**Figure 2.18**). Under the same conditions, 44 μM HCA was consumed within 10 min and up to 69% of the HCA carbons were recovered as CO_2 over a 24 day incubation period (**Figure 2.18**). The extent of BPA and HCA sorption onto MnO_2 was examined. When 44 μM BPA was incubated with 0.5 mM MnO_2 for 10 min at pH 7.2, 4.8 ± 0.4 μM BPA was detected in the ascorbic acid-treated samples. However, no BPA was detected in the filtrates of untreated samples, indicating that all BPA had sorbed to the MnO_2 particles. Under the same reaction conditions, similar amounts of 37 ± 0.5 μM and 35 ± 0.6 μM HCA were detected in the ascorbic acid-treated and in the filtrates of untreated samples, respectively (**Figure 2.14**), indicating that HCA had a lower sorption tendency to MnO_2 than BPA.

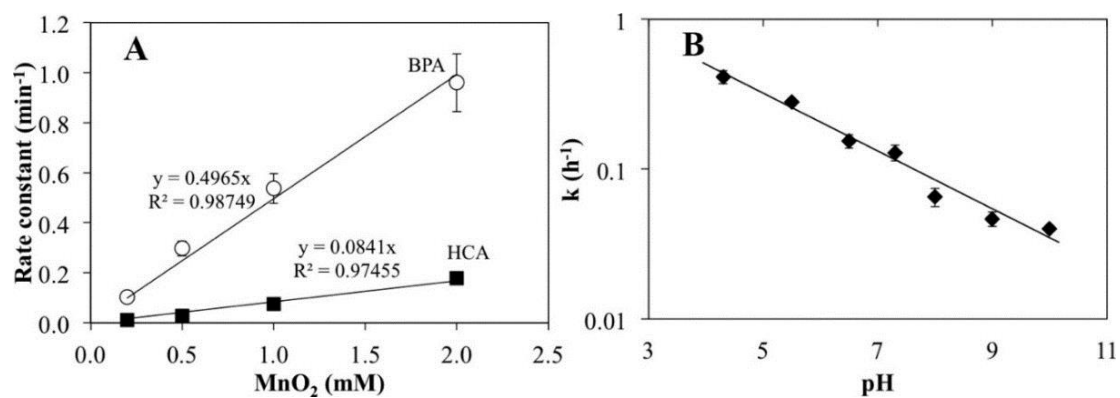


Figure 2.15. Effect of MnO_2 loading on BPA and HCA degradation rate constants (A) and effects of pH on BPA degradation rate constants (B). Error bars represent the standard deviation of triplicate samples. Error bars smaller than the symbol size are not depicted.

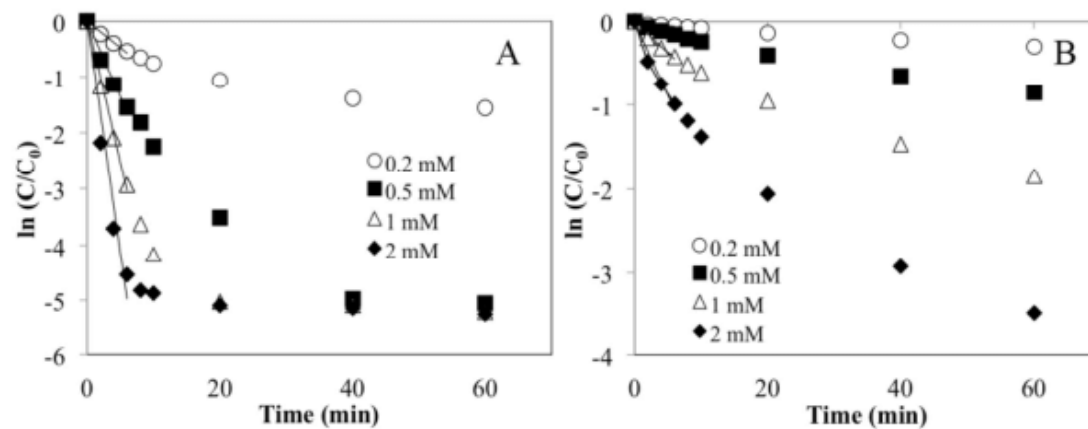


Figure 2.16. BPA (A) and HCA (B) concentrations in incubations (10 mM MOPS buffer, pH 7.4) with different amounts (shown are the nominal concentrations) of MnO₂.

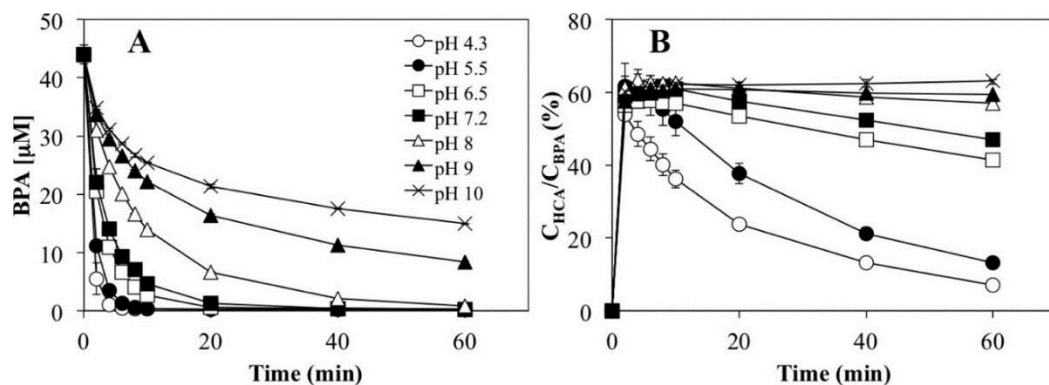


Figure 2.17. Effect of pH on BPA degradation (A) and conversion efficiency of BPA to HCA (B) in MOPS buffer (pH 7.4) initially containing $44 \mu\text{M}$ (10 mg L^{-1}) BPA. The legend shown in panel A also applies to panel B. Error bars represent the standard deviation of triplicate samples. Error bars smaller than the symbol size are not depicted.

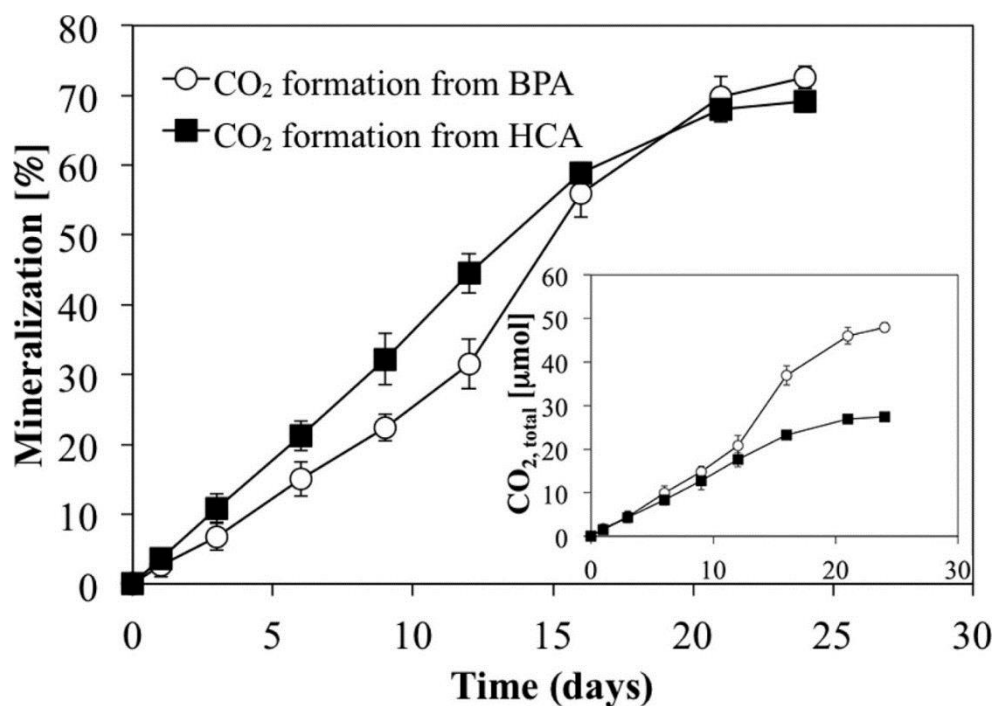


Figure 2.18. Percent mineralization of BPA and HCA in phosphate buffer (pH 2) initially containing $4.4 \mu\text{mol}$ BPA and HCA, respectively. Inset: the release of CO_2 from BPA and HCA over time.

Biodegradation of HCA. Aqueous phase HCA concentration profiles in microcosms established with Third Creek sediment demonstrated that sorption of HCA was not significant after several days of incubation (**Figure 2.19**). In contrast, 50% of BPA instantaneously sorbed to the same sediment material⁶⁵. In oxic microcosms established with sediment materials from four distinct locations, HCA was completely removed within 3 weeks of incubation at slightly different rates depending on the origin of the sediment (**Figure 2.20**). No degradation products were observed suggesting that mineralization occurred. Repeated transfers from oxic Third Creek microcosms to fresh medium yielded a sediment-free, HCA-degrading enrichment culture. In the presence of oxygen, this mixed culture consumed HCA with a concomitant increase in optical density at 600 nm indicating microbial growth (data not shown). In anoxic incubations, nitrate, sulfate, ferric iron, and CO₂ reduction occurred as evidenced by electron acceptor depletion and/or reduced product formation. Although active nitratereducing, sulfidogenic, ferric iron-reducing, and methanogenic conditions established in the microcosms, no HCA disappearance was observed over a 5 month incubation period (**Figure 2.19**). Similar results were obtained with microcosms established with Shady Valley and Axton Cross sediment materials, and Third Creek sediment collected from an upstream location (data not shown).

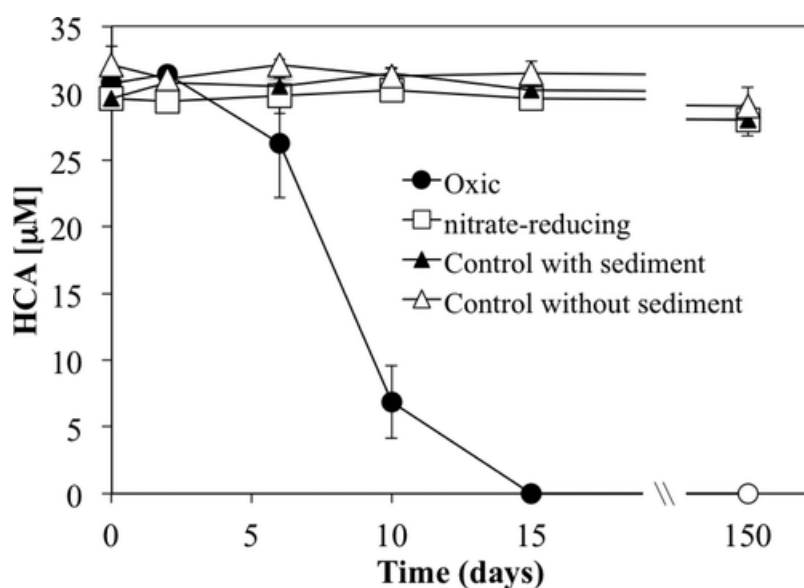


Figure 2.19. Concentration profiles of HCA in microcosms established with Third Creek sediment. HCA concentration profiles of microcosms incubated under sulfidogenic, Fe(III)-reducing, and methanogenic conditions were similar to those incubated under nitrate-reducing conditions (data not shown for clarity). The error bars represent the range of duplicate samples. Error bars smaller than the symbol size are not depicted.

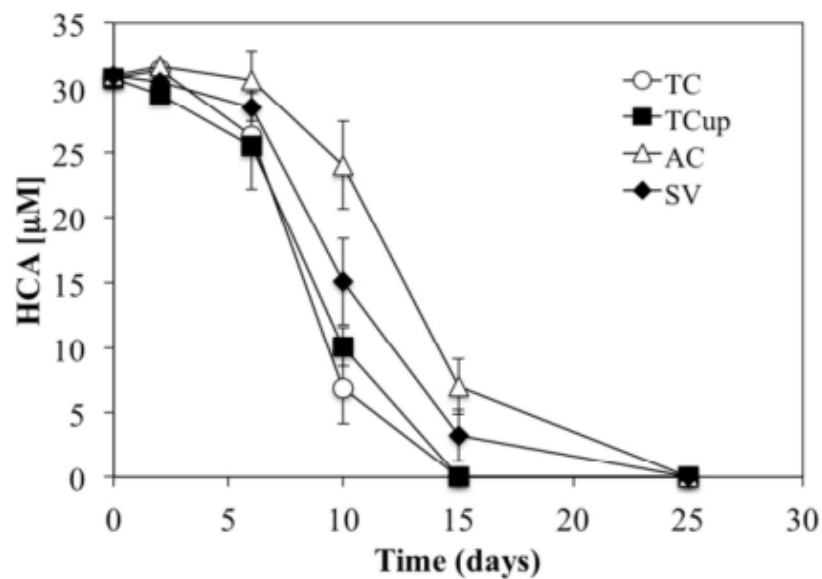


Figure 2.20. Concentration profiles of HCA in microcosms incubated under oxic conditions using sediment materials obtained from two Third Creek (TC) locations, and the Axton Cross (AC) and the Shady Valley (SV) sampling sites. The error bars represent the range of duplicate samples. No significant HCA decrease was observed in microcosms incubated under nitrate-reducing, sulfidogenic, Fe(III)-reducing, and methanogenic conditions compared to negative control incubations (see **Figure 2.19**).

Discussion. Manganese oxides are strong, naturally occurring oxidants, and play relevant roles in the biogeochemical cycling of carbon and other elements. Abiotic and microbial processes have been implicated in the formation of manganese oxides; however, the full range of pathways and mechanisms is not understood¹¹³. Mn(II) ions can be abiotically oxidized in aqueous solution via mineral surface-catalyzed oxidation, but microbial oxidation of Mn(II) to Mn(III/IV) is likely a major process leading to the formation of manganese oxides⁷⁵. The average total manganese amount was reported to range between 40 and 900 mg kg⁻¹ in U.S. soils¹¹⁴. Manganese oxides are also abundant in marine and freshwater sediments, for example, 25–185 $\mu\text{mol mL}^{-1}$ of MnO₂ have been detected in marine sediments, and 300– 4000 $\mu\text{mol mL}^{-1}$ of MnO₂ have been observed in freshwater sediments^{79, 81-82}. Our experiments used nominal MnO₂ concentrations of 0.2 to 2 $\mu\text{mol mL}^{-1}$, which is in the range of MnO₂ amounts observed in natural soils and sediments. Thus, the abiotic transformation of BPA by MnO₂ leading to HCA formation could be an environmentally relevant pathway for BPA attenuation.

The particle size or surface area of metal oxides is known to affect the reactivity, and smaller particle size generally more reactive resulting in higher rates. The average surface area of MnO₂ prepared with conventional preparative methods has been reported to range from 128 to 270 m² g⁻¹, which is in the range of naturally occurring MnO₂^{92, 115-116}. Our experiments used slightly finer MnO₂ particles with an average surface area of 529 $\pm$ 14 m² g⁻¹, and it is likely that nanoparticulate MnO₂ (i.e., smaller particles) will result in higher rates.

Oxidative transformation of BPA by MnO₂ has been demonstrated previously using synthetic MnO₂ and MnO₂-coated sand^{85-86, 117}. In a previous study, 11 reaction intermediates were proposed, including HCA, but a mass balance approach was not applied and no quantitative assessment was provided. In our experiments, up to 64% (mol/mol) of BPA was recovered as HCA, suggesting that HCA was the major reaction intermediate over a broad pH range⁸⁶. HCA has an over 2-fold lower molar extinction coefficient than BPA at each λ_{max} (**Figure 2.8**), and HCA may have been overlooked due to the low response to UV light and associated high detection limits with the UV detector. Compared with UV detection, an approximately 10-fold higher response was obtained with the fluorescence detector, which enabled HCA detection and quantification. Further, HCA may have evaded detection in previous studies that used high MnO₂:BPA ratios because HCA is also susceptible to

degradation by MnO_2 resulting in merely transient formation of low amounts of HCA (**Figure 2.14**).

The identification of HCA as the major BPA transformation product was not straightforward. One of the obstacles was a m/z difference of 18.01057 between the observed mass of 135.08011 and the mass of HCA (153.09068). It is not uncommon for molecules to lose water during electrospray ionization, and the discrepancy accounts for the fragmentation of the C–OH bond at the benzylic position generating a tertiary, resonance stabilized carbocation with a m/z of 135.08011. It should also be noted that samples of the BPA transformation product possessed minimal impurities (**Figure 2.11A and 2.11C**), suggesting that other minor transformation products were formed.

HCA was proposed as a hypothetical BPA degradation intermediate during aerobic degradation by *Sphingomonas* sp. strain TTNP3 and by *Cupriavidus basilensis* strain JF1^{60, 62}. Kolvenbach et al. speculated that a P450-type enzyme system could be involved in the degradation process. HCA was detected as an intermediate in mammalian metabolism of BPA, and microsomal cytochrome P450 catalyzed BPA transformation to HCA via ipso substitution⁹⁰. Hydroquinone was formed in addition to HCA in these microsomal cytochrome P450 assays, suggesting the other phenol ring yielded hydroquinone. However, we were unable to detect this compound in any of the incubations suggesting the MnO_2 -mediated BPA transformation does not follow the microsomal cytochrome P450-catalyzed reaction pathway. An additional experiment demonstrated that MnO_2 was less reactive toward hydroquinone than toward HCA (data not shown), suggesting that hydroquinone was not a reaction intermediate. A previous study identified reaction intermediates including HCA in BPA incubations with MnO_2 ^{66, 86}. For example, 4-(2-(4-(4-hydroxyphenoxy)phenyl)propan-2-yl)phenol was detected and proposed to contain the remaining phenolic moiety of BPA⁸⁶. However, HCA was the only major BPA transformation product in incubations with MnO_2 and no other aromatic compounds were observed in DAD 3-D scans (220–400 nm). These observations suggest that the missing C6 moiety intermediate is susceptible to rapid reaction with MnO_2 , thereby evading detection, or is an aliphatic compound.

Based on the percent mineralization of BPA and HCA, **Figure 2.21** schematically depicts the carbon flux during MnO_2 -mediated degradation of BPA. BPA ($\text{C}_{15}\text{H}_{16}\text{O}_2$) is converted to

HCA (C₉H₁₂O₂) and an unidentified, presumably aliphatic C6 compound(s). HCA contains 60% of the BPA carbons and the data depicted in **Figure 2.18** indicate that 69% of the HCA carbons were recovered as CO₂, indicating that 41% (60% × 69%) of the BPA carbons were mineralized via HCA as an intermediate. As shown in **Figure 2.18**, a total of 73% of the BPA carbons were recovered as CO₂. Thus, the remaining 32% of BPA carbon atoms recovered as CO₂ could be attributed to the mineralization of the unidentified C6 compound(s). MnO₂-mediated ring cleavage and mineralization have been previously reported for compounds such as 2-mercaptobenzothiazole and sulfadiazine¹¹⁸⁻¹¹⁹.

The results depicted in **Figure 2.7** demonstrate that complete BPA oxidation was achieved with ≥0.5 mM (nominal concentration) MnO₂, suggesting that most of the experiments in this study were performed with excess amounts of MnO₂. Independent of the amount of MnO₂ added, the initial (i.e., up to 10 min) BPA and HCA transformation rates were high but then declined and deviated from the pseudo-first-order kinetics (**Figure 2.9**). Such MnO₂-mediated reaction patterns have been observed with other organic compounds, and this reaction pattern has been attributed to the blockage of MnO₂ surface area by reactants and reaction products over time^{89, 92-93, 116, 120}.

A linear decrease of log_{k_{init}, BPA} was observed with increasing pH (**Figure 1.19**), similar to what has been described for the MnO₂-mediated oxidation of triclosan, oxytetracycline, and 4-chloroaniline^{92, 120}. This pH dependence has been explained by the effect of pH on the sorption of organic compounds to metal oxide surfaces (i.e., precursor complex formation) and/or the redox potential of the reactants^{89, 92, 116, 120}. Most metal oxide surfaces carry negative charges over a wide pH range and the protonated forms of phenolic organic compounds are more susceptible to complex with MnO₂. The first and second acid dissociation constants (pK_{a1}, pK_{a2}) of BPA are 9.6 and 10.2, suggesting that the effect of deprotonation of BPA was negligible over the pH range tested (**Figure 2.18**). Meanwhile, lower pH increases the reduction potential of MnO₂; for example, the reduction potential of MnO₂ increases from 0.76 to 0.99 V when the pH decreases from 8 to 4.51. According to MnO₂(s) + 4H⁺ + 2e⁻ → Mn²⁺(aq) + 2H₂O, 50 protons are required for the reduction of MnO₂ to Mn(II), which may explain why BPA degradation rates increased with decreasing pH.

The daughter products generated from contaminant transformation may still have potential to adversely impact ecosystem and human health. Some BPA transformation products have

been reported to have increased estrogenic activity relative to the parent compound^{90, 121-122}. For example, the in vitro estrogen receptor binding assay suggested that HCA has 100-fold higher binding affinity to the estrogen receptor than BPA⁹⁰. Therefore, understanding the fate of HCA is relevant for predicting environmental concerns associated with BPA. Although HCA was further degraded by MnO₂, the degradation rate constants of HCA were 5-fold lower than those of BPA suggesting that BPA can be a source of HCA. The lower MnO₂ reaction rates with HCA compared to BPA may be a consequence of differential adsorption to the MnO₂ surface, and the higher affinity of BPA resulting in preferential degradation (**Figure 2.7, Figure 2.13**). We determined that HCA has higher aqueous solubility and a lower K_{ow} than BPA, suggesting that HCA has a lower tendency for sorption (**Table 2.3, Figure 2.19**) and is potentially more mobile than BPA. The microcosms study suggested that rapid microbial HCA degradation under anoxic conditions cannot be expected; however, HCA was readily metabolized in the presence of oxygen in microcosms established with four different sediment materials, suggesting that aerobic HCA degraders may be common members of freshwater sediment microbial communities. Environmental Implications.

Environmental Implications. BPA is recalcitrant to microbial degradation under anoxic conditions, and its high partition coefficient (K_{ow}) indicates that BPA is relatively immobile in sediments and soils^{2, 65-67, 101, 123}. Not surprisingly, up to 343 µg/kg of BPA has been detected in anoxic sediments^{53, 55}. Given the prevalence of MnO₂ in soils and sediments, MnO₂-mediated BPA oxidation leading to HCA formation and mineralization could be an environmentally relevant process. HCA is more mobile than BPA suggesting that MnO₂-mediated BPA oxidation could lead to faster environmental distribution. However, with enhanced mobility, HCA is more likely to encounter oxic environments where it is susceptible to microbial degradation. We suggest that such coupled abiotic-biotic processes may be relevant for controlling the fate and longevity of BPA in sediments and aquifers.

**CHAPTER III. *PHAEOBACTER* SP. STRAIN Y4I UTILIZES TWO
SEPARATE CELL-TO-CELL COMMUNICATION SYSTEMS TO
REGULATE PRODUCTION OF THE ANTIMICROBIAL INDIGOIDINE**

The work outlined in this chapter was originally published by ¹Nathan W. Cude, Carson W. Prevatte, ¹Mary K. Hadden, Amanda L. May, Russell T. Smith, Caleb L. Swain, Shawn R. Campagna, and ¹Alison Buchan. NWC, under the supervision of AB, is the primary author in terms of the introduction, biological experimentation methods, results, and conclusions. CWP, along with other members of the Campagna laboratory and combined research team, authored sections of the original manuscript containing chemical data and methods contributions. The content of this article has been kept as originally submitted with modifications to the format only.

Cude, W. N., Prevatte, C. W., Hadden, M. K., May, A. L., Smith, R. T., Swain, C. L., Campagna, S. R., & Buchan, A. *Phaeobacter* sp. strain Y4I utilizes two separate cell-to-cell communication systems to regulate production of the antimicrobial indigoidine. *Applied And Environmental Microbiology* 2015 81 (4), 1417-1425.

Affiliations: ¹Department of Microbiology, University of Tennessee, Knoxville, Tennessee, USA; ²Department of Chemistry, University of Tennessee, Knoxville, Tennessee, USA

WNC and AB conceived the study. WNC and CWP performed the majority of the experimental work. WNC was assisted by MKH, and CWP was assisted by RTS and CLS. WNC and MKH will be referred to as the Buchan lab. Growing *Phaeobacter* sp. strain Y4I cultures was performed by the Buchan lab. Culture extraction and processing was performed by WNC and CWP. CWP performed the identification and quantitation of the AHLs using isotope dilution high-performance liquid chromatography-tandem mass spectrometry (ID-HPLC-MS/MS). CWP also synthesized the dideuterated (D₂) N-acyl homoserine lactones (AHLs) used as internal standards. WNC conducted the attached-biomass and motility assays and AHL and indigoidine quantification. WNC and CWP generated and analyzed the data for their respective experimental contributions. WNC, ALM, SRC, and AB contributed to the discussion.

CWP contribution

This chapter is the first of two chapters involving similar contributions by CWP, in which dideuterated (D₂) N-acylhomoserine lactones (AHLs) are synthesized to facilitate quantitation or annotation using isotope dilution high-performance liquid chromatography-

tandem mass spectrometry (ID-HPLC-MS/MS) of these bacterial cell-to-cell signaling molecules to correlate AHL production to bacterial phenotype, community behavior, and exclusive to this chapter, expression of the genes responsible regulation of AHL synthesis. This study was an extension of previous work by the Buchan lab which had reported that marine roseobacter *Phaeobacter* sp. strain Y4I possessed two *luxRI* homologues responsible for regulating the production of AHLs that was shown to likely contribute to the regulation of the blue antimicrobial secondary metabolite indigoidine, motility, and biofilm density. CWP performed an initial identification of the AHLs Y4I produced. A standard mixture of (D₂)AHLs was added to extracts of liquid cultures from early log and stationary phase to measure retention time match the AHL gene products of the two *luxRI* homologues. (D₂)AHLs containing acyl chains that were 4 to 20 carbons in length and included masses for acyl chains with either hydroxy or keto modifications at position 3. Two AHLs were initially identified as C₈-HSL and *N*-(3-oxododecenoyl)-homoserine lactone (3OC₁₂-HSL). The confirmation of C₈-HSL was easily accomplished by comparison to the (D₂)C₈-HSL internal standard present in the standard mixture (**Figure 3.1A**). The standard mixture possessed (D₂)C₄-HSL, (D₂)3OHC₄-HSL, (D₂)C₆-HSL, (D₂)C₃OC₆-HSL, (D₂)C₇-HSL, (D₂)C₈-HSL, (D₂)C₁₂-HSL, and (D₂)C₁₄-HSL. Since a deuterated standard for 3OC₁₂-HSL was not present in the standard mixture, the tentative assignment was made based on the ionized parent mass of 298 *m/z* = 298, the retention time of 5.80-6.20 min, and a knowledge of the commonly occurring sidechains. Synthesis of 3OC₁₂-HSL was accomplished and comparison of the retention times between the standard and naturally occurring AHL revealed a marginal but significant difference between the two (**Figure 4.1C**). Ultimately, an accurate mass measurement using an Orbitrap mass analyzer, knowledge of the impact of structure on retention time, and direct injection of (D₂)3OHC_{12:1}-HSL internal standard, verified the final structural assignment as 3OHC_{12:1}-HSL (**Figures 4.1D-4.1F**). Though the location and chirality of the double bond could not be determined by these analyses, 3OHC_{12:1}-HSL with one unit of unsaturation at any position of the sidechain represents a previously unreported AHL. A simple synthesis was also developed to access this previously unreported AHL. The identification and synthesis of deuterated internal standards by CWP made quantitation of the AHLs produced by Y4I possible. This was a significant contribution to the final experiment in which Y4I was grown on agar plates over a 48 h time period quantitating AHL concentration, AHL and indigoidine gene production, and indigoidine concentration. CWP developed the AHL extraction and processing method that allowed AHLs to be extracted from the agar plates with high efficiency. Ultimately, quantitation at the

levels of transcription, cell-to-cell chemical signaling, and phenotype over the 48 h time period, along with previous gene knock-out data, was used to describe the interplay among *pgaRI* and *phaRI*, responsible for synthesis and regulation of C₈-HSL and 3OHC_{12:1}-HSL respectively, local AHL concentration, indigoidine biosynthesis, and growth-phase-dependent motility and biofilm size.

3.1 Abstract

The marine roseobacter *Phaeobacter* sp. strain Y4I synthesizes the blue antimicrobial secondary metabolite indigoidine when grown in a biofilm or on agar plates. Prior studies suggested that indigoidine production may be, in part, regulated by cell-to-cell communication systems. *Phaeobacter* sp. strain Y4I possesses two *luxR* and *luxI* homologous *N*-acyl-L-homoserine lactone (AHL)-mediated cell-to-cell communication systems, designated *pgaRI* and *phaRI*. We show here that Y4I produces two dominant AHLs, the novel monounsaturated *N*-(3-hydroxydodecenoyl)-L-homoserine lactone (3OHC_{12:1}-HSL) and the relatively common *N*-octanoyl-L-homoserine lactone (C₈-HSL), and provide evidence that they are synthesized by *phal* and *pgal*, respectively. A Tn5 insertional mutation in either genetic locus results in the abolishment (*pgaR*::Tn5) or reduction (*phaR*::Tn5) of pigment production. Motility defects and denser biofilms were also observed in these mutant backgrounds, suggesting an overlap in the functional roles of these systems. Production of the AHLs occurs at distinct points during growth on an agar surface and was determined by isotope dilution high-performance liquid chromatography–tandem mass spectrometry (ID-HPLC-MS/MS) analysis. Within 2 h of surface inoculation, only 3OHC_{12:1}-HSL was detected in agar extracts. As surface-attached cells became established (at ~10 h), the concentration of 3OHC_{12:1}-HSL decreased, and the concentration of C₈-HSL increased rapidly over 14 h. After longer (>24-h) establishment periods, the concentrations of the two AHLs increased to and stabilized at ~15 nM and ~600 nM for 3OHC_{12:1}-HSL and C₈-HSL, respectively. In contrast, the total amount of indigoidine increased steadily from undetectable to 642 µM by 48 h. Gene expression profiles of the AHL and indigoidine synthases (*pgal*, *phal*, and *igiD*) were consistent with their metabolite profiles. These data provide evidence that *pgaRI* and *phaRI* play overlapping roles in the regulation of indigoidine biosynthesis, and it is postulated that this allows *Phaeobacter* sp. strain Y4I to coordinate production of indigoidine with different growth-phase-dependent physiologies.

3.2 Introduction

Bacteria are in constant competition for niches in the environment, and the ability to coordinate gene expression may provide a competitive advantage¹²⁴. Some bacteria are able to synchronize gene expression in a cell-density-dependent manner by sensing and responding to small diffusible molecules synthesized by individuals; in proteobacteria, members of the broadly distributed and chemically diverse class of *N*-acyl-L-homoserine lactones (AHLs) are among the best-characterized signaling molecules³. Proteobacterial AHL synthases are fairly well conserved and form the LuxI protein family. Members of the LuxR protein family are cytoplasmic transcriptional regulators that can modulate gene expression, most typically when bound to the corresponding cognate AHL¹²⁵⁻¹³⁰. It has been shown that *luxI-luxR* (*luxIR*) pairs are frequently colocalized within chromosomes and constitutively expressed at low concentrations when bacterial densities are low^{126, 129}. Thus, bacteria possessing *luxIR* homologs produce and release AHLs into the external environment¹²⁹. At high cell densities, AHL concentrations can reach a threshold concentration, bind to the corresponding cognate cytoplasmic LuxR proteins, and affect transcription of various genetic loci¹³⁰⁻¹³³. AHLs have also been shown to activate regulators other than the corresponding cognate LuxR, suggesting that cross talk may play an important role in intra- and interspecies interactions¹³⁴⁻¹³⁹. Furthermore, AHL concentrations have been shown to change throughout the growth cycle of some bacteria, suggesting that the type and concentration of an AHLs may also convey information about the metabolic state of the population¹⁴⁰⁻¹⁴¹. Examples of physiologies mediated by AHLs include biofilm formation, suppression of immune responses during infection, exoenzyme production, swarming motility, colonization of host by a symbiont, and antimicrobial production^{131, 142-144}. Roseobacters are a biogeochemically active group of marine alphaproteobacteria, within the *Rhodobacteraceae* family, that are prevalent in the coastal ocean¹⁴⁵⁻¹⁴⁸. The *Roseobacter* clade currently contains more than 50 described genera¹⁴⁹, including *Phaeobacter*, *Sulfitobacter*, and *Ruegeria*. The prevalence of *luxRI* homologs in *Roseobacter* genomes suggests that AHL-based cell-to-cell communication is a core trait of this successful lineage of aquatic bacteria¹⁵⁰⁻¹⁵¹. Representative members of the *Roseobacter* clade have been shown to produce multiple AHLs, and these compounds have been linked to biofilm formation, motility, and antimicrobial production in a limited number of strains¹⁵²⁻¹⁵⁸. Relatively few *Roseobacter* AHLs have been linked to the LuxI homologs that mediate their syntheses, and many *Roseobacters* possess multiple *luxRI* systems,

suggesting the presence of complex AHL-mediated regulatory schemes in these strains that have not been explored¹⁵³. Furthermore, the presence of non-AHL quorum sensing systems has been characterized within other *Roseobacter* strains, including *Ruegeria* sp. strain TM1040, which uses at least two different chemical signals to regulate the physiologies associated with its symbiosis with an algal host¹⁵⁹⁻¹⁶⁰.

Roseobacter representative strain Y4I produces the blue antimicrobial secondary metabolite indigoidine, which suppresses the growth of other marine bacteria. 16S rRNA gene analysis places strain Y4I within the *Phaeobacter* genus; however, the researchers who published a recent genome-scale study¹⁶¹ suggested that a formal reclassification of this genus may be forthcoming. We have previously demonstrated that indigoidine production is an exclusively surface-attached phenotype in Y4I and that its production allows this strain to outcompete *Vibrio fischeri* for surface niches in coculture¹⁵⁰. In addition, the Y4I genome carries two *luxRI* systems, and transposon insertional mutations in either of those loci alter indigoidine production and, subsequently, the ability of Y4I to inhibit *V. fischeri*^{150, 153}. In this study, we sought to identify the dominant AHLs produced by *Phaeobacter* sp. strain Y4I, determine the genes mediating their synthesis, and examine the contribution of the two *luxRI* systems to the regulation of indigoidine biosynthesis during surface-attached growth.

3.3 Materials and methods

Growth conditions, strains, and media. The following culture conditions were used, unless otherwise noted: *Phaeobacter* sp. strain Y4I and Tn5 derivatives were maintained in YTSS broth (per liter, 2.5 g yeast extract, 4 g tryptone, 15 g Sea Salts [Sigma-Aldrich, St. Louis, MO]) at 30°C, with shaking (200 rpm). YTSS agar (1.5%) plates were used for streaking and isolation and were also incubated at 30°C. Y4I transposon Tn5 insertional mutants were isolated from a previously generated transposon mutant library and maintained with 50 µg/ml kanamycin¹⁵⁰. Kanamycin was not, however, included in the assays described below.

AHL identification and deuterated (D₂) AHL synthesis. Triplicate Y4I cultures were grown in 200 ml of YTSS broth and analyzed at 5 h (early log phase) and 24 h (stationary phase) following inoculation. At the end of the incubation period, the entire culture was

pelleted by centrifugation at $3,700 \times g$ for 20 min at 4°C using a Sorvall Legend RT centrifuge (Thermo Fisher, Waltham, MA). The supernatant was filtered through a 47-mm-diameter 0.45- μ m-pore-size nylon filter (GE Osmonics, Feasterville Trevose, PA). The filtrate was extracted twice with 150 ml of 1% (vol/vol) acetic acid–ethyl acetate (EtOAc). The organic layer was collected and concentrated *in vacuo* to give an off-white solid, which was reconstituted with 300 μ l EtOAc and transferred to an autosampler vial.

AHL identification was accomplished using a previously developed high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) method¹⁶². Briefly, 10 μ l of the concentrated extract was injected onto a reverse-phase C₁₈ core-shell column (Phenomenex Kinetex, Torrance, CA) (2.6- μ m pore size, 100-Å particle size, 100 by 2.1 mm) by the use of a Surveyor Autosampler Plus autosampler (Thermo Electron, Waltham, MA) at 25°C. LC was performed with an electron surveyor MS pump at a flow rate of 200 μ l/min, and the effluent was introduced into a TSQ Quantum Discovery Max triple-quadrupole mass spectrometer (MS) for ion detection (Thermo Electron, Waltham, MA) via electrospray ionization (ESI) with a spray voltage of 4,500 V. Samples were analyzed by the use of selected-reaction monitoring (SRM) with a scan time of 0.05 s and a scan width of 0.1 m/z . Detection was done in positive-ion mode with nitrogen as a sheath gas and argon as the collision gas. AHLs were identified using the chromatographic retention time as well as the characteristic parent m/z -fragment m/z pair. The screened parent masses corresponded to AHLs containing acyl chains that were 4 to 20 carbons in length and also included masses for acyl chains with either hydroxy or keto modifications at position 3. Fragment masses of 102 and 104 m/z were used, which corresponded to the conserved HSL core in the naturally occurring and deuterated AHLs, respectively. Detection was done in positive-ion mode with nitrogen as the sheath gas and argon as the collision gas.

Accurate mass measurements were performed on an Exactive Plus Orbitrap MS (Thermo Fisher Scientific, Waltham, MA) via ESI with a spray voltage of 4,000 V. For these measurements, 300 μ l of Y4I culture–YTSS taken 4 h after inoculation was direct injected using a Hamiltonian 250- μ l syringe (Hamiltonian Company, Reno, NV) at a flow rate of 20 μ l/min. Detection was done in positive-ion mode with nitrogen as the sheath gas.

The deuterated AHL standards *N*-octanoyl-homoserine lactone [(D₂)C₈-HSL] and *N*-(3-hydroxy-6*E*-dodecenoyl)-homoserine lactone [(D₂)3OHC_{12:1}-HSL] were synthesized for quantitation of AHLs as previously reported¹⁶²⁻¹⁶³. Following accurate identification of the AHLs in question, subsequent experiments were performed with a TSQ Quantum Discovery Max triple-quadrupole MS as it provided more-accurate quantitation.

Attached-biomass and motility assays. Attached biomass was quantified for wild-type Y4I and for the *pgaR*::Tn5 and *phaR*::Tn5 strains using a crystal violet assay¹⁶⁴. Briefly, each culture was diluted to 10⁶CFU/ml and 100-μl aliquots were transferred to a 96-well polypropylene plate (Costar; Corning Incorporated, Corning, NY) in octuplet. The plates are incubated at 27°C for 18 h. A total of 25 μl of crystal violet was added to each well and left at room temperature for 15 min. The wells were rinsed three times with deionized water, and the crystal violet was solubilized in 95% ethanol for 1 h. Absorbance readings were measured using a plate reader (BioTek, Winooski, VT) at 600 nm.

Motility was assessed using semisolid (0.35%) 1/10 YTSS agar plates (per liter, 0.25 g yeast extract, 0.4 g tryptone, 15 g Sea Salts, and 3.5 g agar). A 20-μl volume of stationary-phase liquid cultures was spotted to the center of a motility agar plate in triplicate. The plates were incubated at 30°C for 48 h, after which the diameter of the swim circle was measured.

AHL and indigoidine quantification. A 47-mm-diameter 0.45-μm-pore-size nylon filter (GE Osmonics, Feasterville Trevose, PA) was placed onto 60-mm-diameter petri dishes containing 10 ml of YTSS agar medium. A 100-μl volume of early-stationary-phase wild-type Y4I culture (~10⁸ CFU/ml) was inoculated on the membrane, and the plates were incubated at 30°C. Plates were harvested in sextuplet 13 times over 48 h of growth. For each set of six plates, three filters were removed and placed in 4-ml cryovials and flash frozen in liquid nitrogen for reverse transcription-quantitative PCR (RT-qPCR) analysis. Indigoidine concentrations were determined for the remaining three filters by first placing them in 10 ml of dimethyl sulfoxide (DMSO) and then incubating in the dark with frequent vortex mixing until the indigoidine was solubilized. The tubes were centrifuged at 6,000 × *g* for 5 min. A 1-ml volume of the supernatant was removed, and the absorbance was measured at 612 nm. Indigoidine quantitation was achieved with a calibration curve determined using extracted indigoidine derived from 20 lawns of a hyperpigmented Y4I

mutant on YTSS agar plates as previously described¹⁵⁰. The mass of the extracted indigoidine was recorded, and the indigoidine was solubilized in 1 ml of DMSO. Quarter-log dilutions were performed, and the absorbance at 612 nm was plotted to generate a calibration curve.

After the filters were removed, an internal standard was added to the cell-free agar plates via addition of 90 μ l of a solution containing 3.30 μ M (D₂)C₈-HSL and 0.299 μ M (D₂)3OHC_{12:1}-HSL and was allowed to diffuse into the plate. The 10-ml volume of agar was minced and transferred to a 250-ml Erlenmeyer flask. AHL extraction from the agar was performed by addition of 100 ml of EtOAc followed by stirring for 3 h. Afterward, the EtOAc was decanted and concentrated *in vacuo* to give an off-white solid, which was reconstituted with 300 μ l EtOAc and transferred to an autosampler vial for analysis via isotope dilution high-performance liquid chromatography–tandem mass spectrometry (ID-HPLC-MS/MS). AHL concentrations in the plate were calculated using the previously described ID-HPLC-MS/MS methodology¹⁶². Briefly, the concentrations of the endogenous AHLs were determined by integrating the area under the peak and comparing it to the stable isotope-labeled internal standards using the following formula: [area of endogenous AHL/area of (D₂)AHL] $\times$ [final concentration of (D₂)AHL in the plate]. As the cells were physically separated from the agar surface by a nylon filter, the AHLs measured are only those that diffused across the membrane into the agar. As the rate of diffusion for these AHLs in agar is unknown, the measured concentrations could be lower than those in the bacterial biomass. It should be noted, however, that the rate of diffusion of many nutrients in agar has been reported to be relatively high¹⁶⁵⁻¹⁶⁶.

RT-qPCR for *igiD*, *phal*, and *pgal* expression. *igiD*, *phal*, and *pgal* gene expression was analyzed for the wild-type cells grown on nylon filters on agar plates in the time course described above and for the *phaR*::Tn5 and *pgaR*::Tn5 strains on agar plates with nylon filters at 24 h. Primers were designed to amplify products ranging in size between 160 and 240 bp of three reference genes (*alaS*, *map*, and *rpoC*) as well as of the *igiD* gene encoding the indigoidine nonribosomal peptide synthetase (NRPS)¹⁵⁰, a 216-bp portion of *phal* (forward, 5'-CAGCCTTGCGCCTCGGAAA-3'; reverse, 5'-TGCAGCGGAGCGAATTGCGA-3'), and a 155-bp portion of *pgal* (forward, 5'-GGCGGCTCCATGCGGTTTCT-3'; reverse, 5'-CCTGCTGCGATCCTGCCCTG-3'). Frozen filters were thawed in the cryovials, and 640 μ l of 200 μ M zirconium grinding beads

(OPS Diagnostics, Lebanon, NJ) was added with 1.5 ml of 1:100 β -mercaptoethanol–buffer RLT (Qiagen, Germantown, MD). The vial was subjected to vortex mixing for 10 min at $13,000 \times g$ and incubated for 5 min at 70°C. After a brief centrifugation, 700 μ l of the liquid phase was transferred to a microcentrifuge tube. A 700- μ l volume of 100% ethanol was added, and the lysate was transferred to an RNeasy Mini spin column. RNA was isolated per the RNeasy minikit protocol (Qiagen, Germantown, MD). All time points were relativized to the initial time point, ~15 min after plating of the liquid culture, and data were normalized to the reference gene data. In all cases, RT-qPCR data analysis and the normalized relative transcript quantity were calculated using the qBASE method¹⁶⁷ (45).

Statistical analysis. Analysis of variance (ANOVA) mean separations were done using the Holm-Sidak method in SigmaPlot 11.0 (Systat Software, Inc., Chicago, IL).

3.4 Results

Phaeobacter sp. strain Y4I produces two dominant AHLs. Isotope dilution high-performance liquid chromatography-tandem mass spectrometry (ID-HPLC-MS/MS) was used to characterize the AHLs produced by *Phaeobacter* sp. strain Y4I. Two AHLs were consistently recovered from broth cultures with ionized parent masses of 228 m/z and 298 m/z and retention times of 5.80 to 6.00 min and 5.80 to 6.20 min, respectively. Given these characteristics, the AHLs were initially predicted to be C8-HSL and N-(3-oxododecenoyl)-homoserine lactone (3OC12-HSL). The C8-HSL structure was verified by comparison to a (D2)C8-HSL internal standard (Figure 1A and B). Comparisons of the retention times of the 298 m/z endogenous AHL to an added 3OC12-HSL standard, however, revealed a marginal but significant difference between the two (Figure 1C). As the mass accuracy of the triple-quadrupole instrument alone was not sufficient to fully confirm a molecular formula(e) or differentiate 3O C12-HSL from other isobaric candidate structures, such as C13-HSL, a high-resolution, accurate mass measurement was obtained using electrospray ionization and an Orbitrap mass analyzer. This analysis revealed a value of m/z of 298.20129. The two most likely molecular formulae for the AHLs based on the low-resolution mass data were C₁₆H₂₈NO₄, which corresponds to either 3OC12-HSL or 3OHC12:1-HSL, and C₁₇H₂₃NO₃, which corresponds to C13-HSL. These formulae have calculated m/z values of 298.20128 (3OC12-HSL or 3OHC12:1-HSL) and 298.23822 (C13-HSL), resulting in mass errors of 0.034 ppm and 123.827 ppm for the two

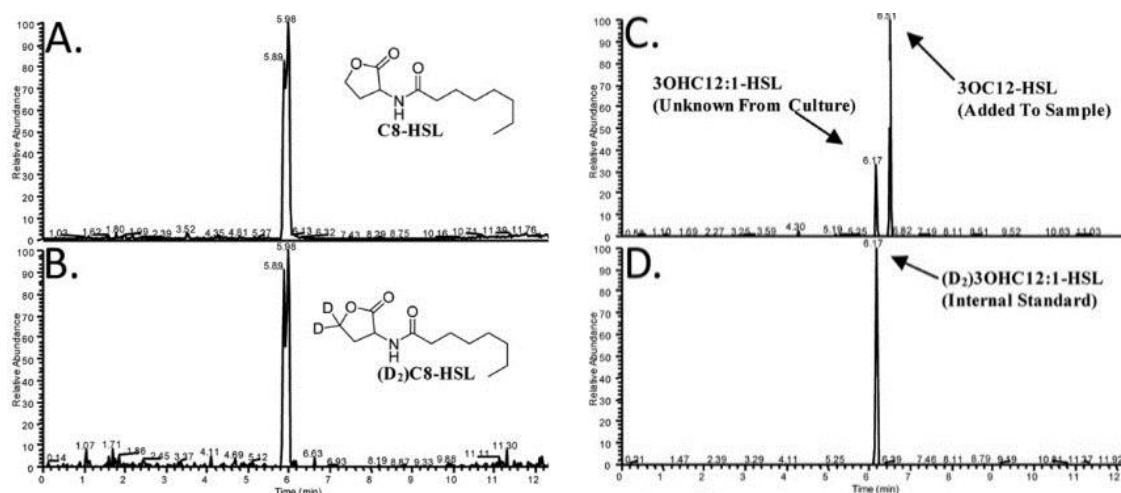


Figure 3.1. ID-HPLC-MS/MS of extracts from *Phaeobacter* sp. strain Y4I grown in YTSS for 4 to 5 h with added standards to identify and confirm AHL identities. (A) Extracted ion chromatogram (EIC) for C₈-HSL with a parent *m/z* of 228 and fragment *m/z* of 102. (B) EIC for the deuterated C₈-HSL internal standard with a parent *m/z* of 230 and fragment *m/z* of 104. (C) EIC for the parent-fragment pair of 298 *m/z* to 102 *m/z* with an added 3OC₁₂-HSL standard. (D) EIC for the deuterated 3OHC₁₂-HSL internal standard with a parent *m/z* of 300 and fragment *m/z* of 104. The retention times for the endogenous 3OHC_{12:1}-HSL and the added (D₂)3OHC_{12:1}-HSL were identical, indicating that this is the most probable structure for the unknown AHL. (E) EIC for 3OHC_{12:1}-HSL with a parent *m/z* of 298 and fragment *m/z* of 102. (F) EIC for the deuterated 3OHC_{12:1}-HSL internal standard with a parent *m/z* of 300 and fragment *m/z* of 104. The peak at 1.99 min represents a medium component that is typical of samples. The chemical structure for each AHL is shown next to its chromatogram. The location of the double bond in 3OHC_{12:1}-HSL cannot be determined by this analysis, and its position in the structure is based on that of a structurally similar AHL found in a *Rhizobium* species.

candidates, respectively. Following these results, the 3OHC12:1-HSL isomer was predicted as it had a chemical formula consistent within the 5-ppm mass accuracy of the Orbitrap MS, and this structure was verified by direct comparison to a (D2)3OHC12:1-HSL internal standard (Figure 1D to F). The location of the double bond and the chirality of the 3OHC12:1-HSL could not be determined by these analyses. The position and chirality depicted in the structure is based on that of a structurally similar AHL found in a *Rhizobium* species¹⁶⁸. The concentrations of these AHLs in stationary-phase broth cultures were $11.2 \pm 3.5 \mu\text{M}$ and $226.5 \pm 48.7 \text{ nM}$ for C₈-HSL and 3OHC_{12:1}-HSL, respectively.

This bacterium possesses two separate *luxRI* cassettes within its genome, termed *pgaRI* (RBY4I_1689 and RBY4I_3631) and *phaRI* (RBY4I_1027 and RBY4I_3464) (Figure 2). In order to gain insight into the contributions of the *PgaRI* and *PhaRI* systems to the phenotypes predicted to be influenced by AHL-mediated cell-to-cell communication systems, we analyzed previously generated Tn5 derivatives of Y4I with interruptions in either *pgaR* or *phaR* (28). Both the *pgaR*:Tn5 strain and the *phaR*:Tn5 strain showed a significant alteration in indigoidine production. It should be noted that transposon insertions in both of these loci could be polar to downstream genes, most notably, the *luxI* homologs that are immediately downstream of these genes and are expected to be cotranscribed (Figure 2). Pigment production is completely abolished in the *pgaR*:Tn5 strain, while the *phaR*:Tn5 mutant produces pigment later (~3 days following inoculation on an agar plate) than the wild type and to a lesser degree. Consistent with these observed indigoidine phenotypes, expression of these genes was significantly diminished in both mutants ($P = <0.001$) relative to the wild-type level. The *pgaR*:Tn5 mutant yielded the fewest transcripts of the three strains. The expression of both *luxI* homologs was also depressed in both of the mutant strains (Figure 3A). In addition, the LuxR-deficient strains showed an enhanced ability to grow in biofilms (Figure 3B) as well as defects in motility ($P = <0.001$). Moreover, the *pgaR*:Tn5 mutant was significantly less motile than the *phaR*:Tn5 mutant ($P = <0.001$) (Figure 3C). In contrast, the growth dynamics on agar surfaces were indistinguishable from the wild-type growth dynamics for both mutants. All strains showed a 50-fold increase in cell numbers during the first 24 h following plate inoculation, and the cell numbers did not change over the subsequent 24-h period (data not shown). The concentrations of the two identified AHLs were determined from agar extracts for

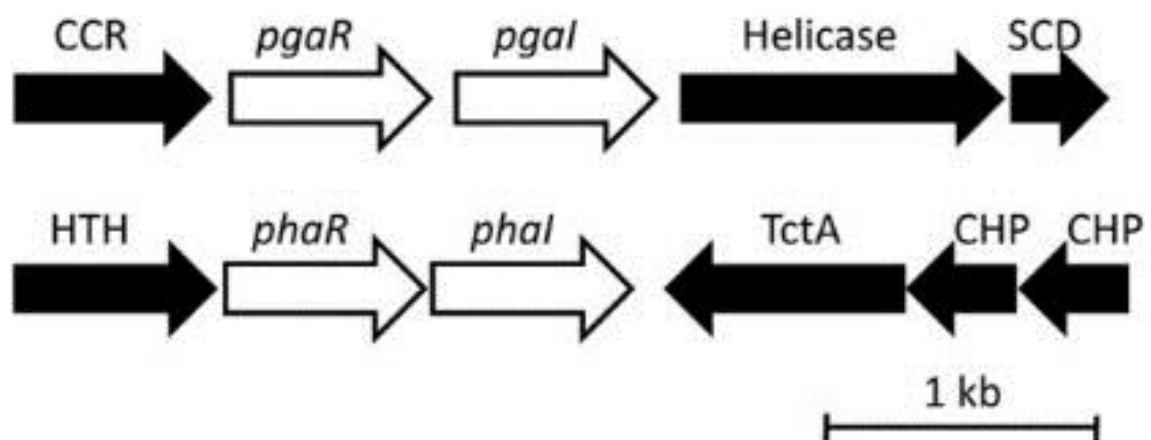


Figure 3.2. Gene orientation for the two *luxRI* loci in *Phaeobacter* sp. strain Y4I. White arrows indicate *luxR* and *luxI* homologs. Gene designations are listed above these arrows. Black arrows represent adjacent open reading frames, and putative protein annotations are indicated above each arrow. Abbreviations used: CCR, crotonyl coenzyme A (CoA) reductase; CHP, conserved hypothetical protein; SCD, short-chain dehydrogenase; TctA, tripartite tricarboxylate transporter family; HTH, uncharacterized protein containing a helix-turn-helix motif.

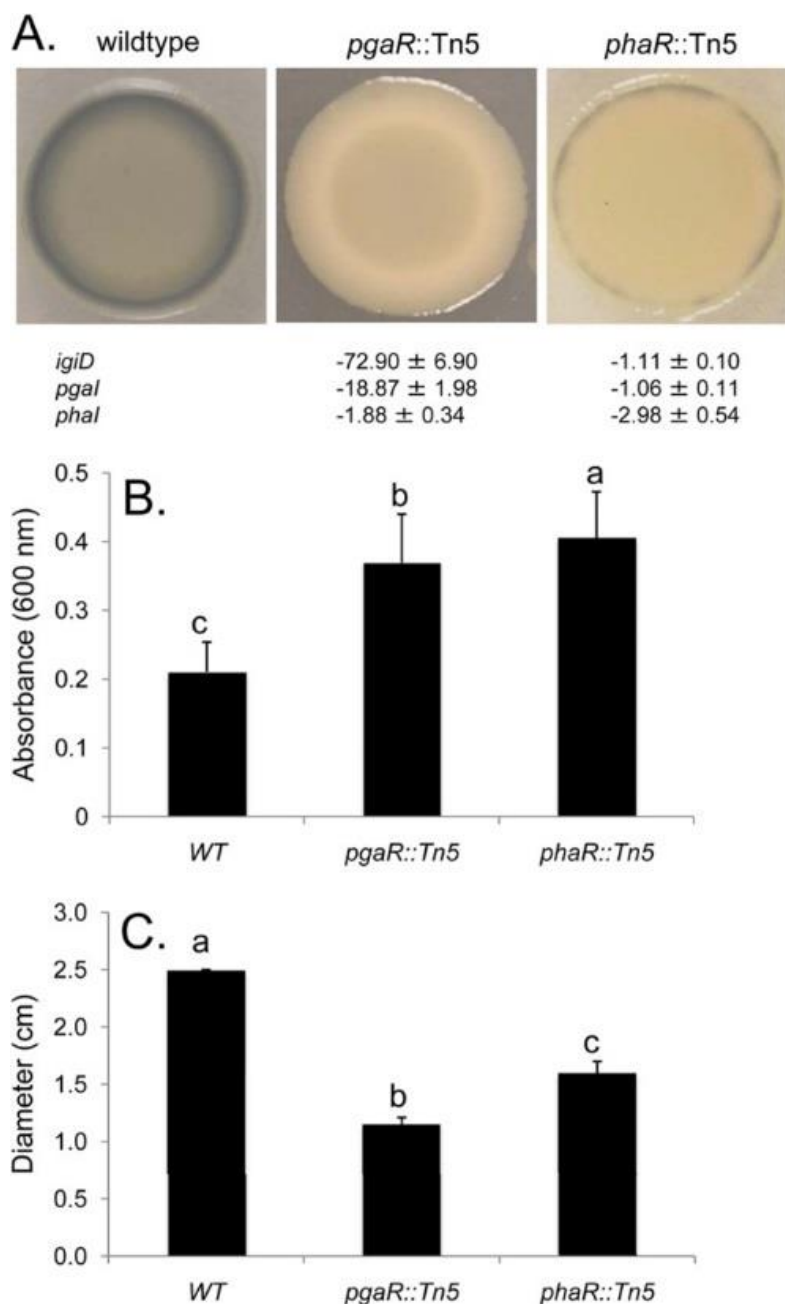


Figure 3.3. Genotypes and phenotypes of wild-type Y4I and *luxR* mutants. (A) Indigoidine pigmentation and synthase gene expression. The images show colony pigmentation in the wild-type, *pgaR::Tn5*, and *phaR::Tn5* strains 6 days following inoculation on agar plates. Normalized transcript quantities for the indigoidine synthase (*igiD*) and two *luxI* homologs (*pgaI* and *phaI*) are shown for the two mutant strains below the images. The gene expression assay was performed on colonies 24 h following inoculation on agar plates, and the values are relativized to wild-type transcript values for bacteria grown in parallel. Standard errors of the means of the results of triplicate biological and technical experiments are shown. (B) Total attached biomass for Y4I and the *luxR* mutants after 18 h. Data represent absorbance of solubilized crystal violet. Error bars represent the standard deviations of the results determined in octuplet experiments. Letters represent significantly different values ($P \leq 0.003$). WT, wild type. (C) Motility of wild-type Y4I and the *luxR* mutants. Error bars represent the standard deviations of the results of triplicate biological experiments. Lowercase letters above the columns represent significantly different values ($P < 0.001$).

the *luxR*-like insertional mutants and the wild-type strain at two time points during growth on an agar plate, 6 h and 24 h post-inoculation, where relatively high concentrations of each AHL were observed in a preliminary, coarse temporal experiment performed using the wild-type strain (data not shown). The highest concentrations of C₈-HSL were 5-fold higher than those of 3OHC_{12:1}-HSL, and the former displayed a cell density dependence not apparent for 3OHC_{12:1}-HSL. The (~10 nM) concentrations of 3OHC_{12:1}-HSL in wild-type *Phaeobacter* sp. strain Y4I plate cultures were statistically indistinguishable at the two time points analyzed. In contrast, C₈-HSL was not detected at the early time point but was present at a relatively high (>500 nM) concentration by the 24-h time point. The *phaR*::Tn5 strain produced significantly less 3OHC_{12:1}-HSL and significantly more C₈-HSL than the wild type at both time points (Figure 4; $P < 0.001$). In contrast, the 3OHC_{12:1}-HSL concentration for the *pgaR*::Tn5 strain was indistinguishable from the concentration seen with the wild type at the 6-h time point but was considerably less than the concentration seen with the wild type at 24 h (Figure 4A). The ability of the *pgaR*::Tn5 strain to produce C₈-HSL at both time points was significantly diminished (Figure 4B; $P < 0.001$).

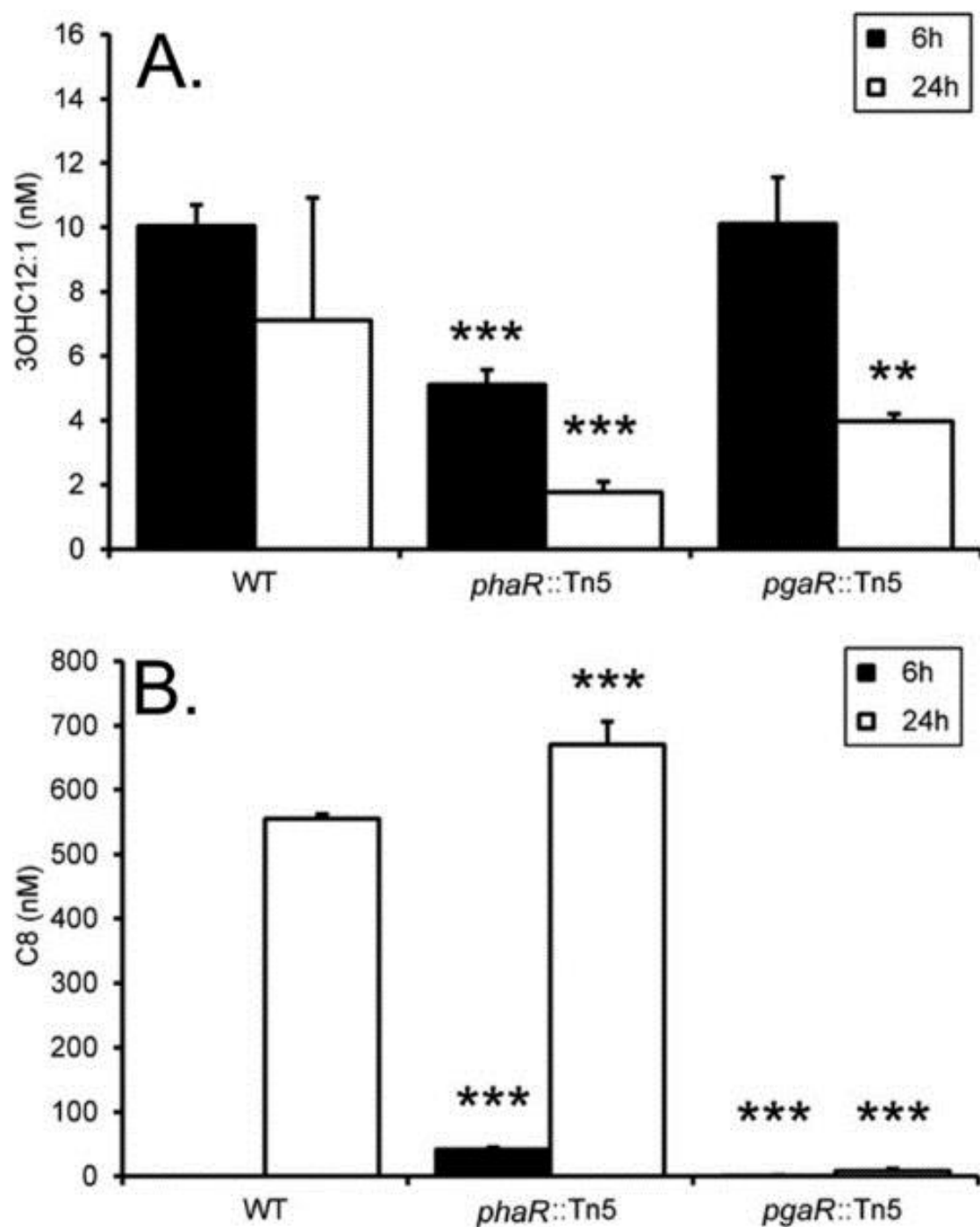


Figure 3.4. 3OHC_{12:1}-HSL (A) and C₈-HSL (B) concentrations for *Phaeobacter* sp. strain Y4l and *luxR* mutants on agar plates at 6 and 24 h postinoculation. Values and error bars represent averages and standard deviations of the results from three biological replicate experiments and two MS injection replicate experiments. Stars represent values significantly different from the wild-type values; three stars indicate $P < 0.001$, and two stars indicate $P = 0.005$.

The distinct temporal patterns of the AHL concentrations identified in the previous experiment prompted us to undertake a higher-resolution temporal analysis of synthesis of the two characterized AHLs when the strain was grown on agar plates over 48 h. The terminal time point was selected to capture AHL concentrations in populations in which growth had slowed considerably, as viable counts were static between 24 and 48 h. To complement these data, we also monitored the gene expression of the two putative AHL synthases, *pgaI* and *phal*. In addition, the indigoidine concentration was quantified, and the expression of an indigoidine biosynthetic gene (*igiD*) was profiled over the course of the experiment. Only 3OHC12:1-HSL was detected in the intermediate stages of growth (~2 to 10 h) on the agar plate at a maximum concentration of 13 ± 2 nM. After 10 h, the concentration of 3OHC12:1-HSL dropped, and the C8-HSL concentration concomitantly increased steadily until it reached a maximum of 795 ± 44 nM at 24 h. At this point, the concentration of C8-HSL dropped ~30% (to 607 ± 28 nM), and the concentration remained relatively stable thereafter. By 15 h, the 3OHC12:1-HSL concentration again began to slowly increase, reaching a maximum of 18 ± 1.5 nM at 48 h (Figure 5A). The C8-HSL concentration correlated strongly with the *pgaR* gene expression profile ($r^2 = 0.99$). In contrast, the expression of *phal* was out of sync with the 3OHC12:1-HSL concentrations during the first 12 h ($r^2 = -0.60$) but showed better agreement at the later time points when the concentration of this AHL was maximal (i.e., 36 and 48 h; Figure 5). Concentrations of indigoidine, extracted from microbial biomass, increased in a nearly linear fashion over the course of 48 h. In contrast, the expression of the indigoidine NRPS (*igiD*) showed some variability, particularly within the first 12 h. In fact, *igiD* expression levels correlated better with the 3OHC12:1-HSL concentration ($r^2 = 0.81$) than with the indigoidine concentration ($r^2 = 0.69$) (Figure 5).

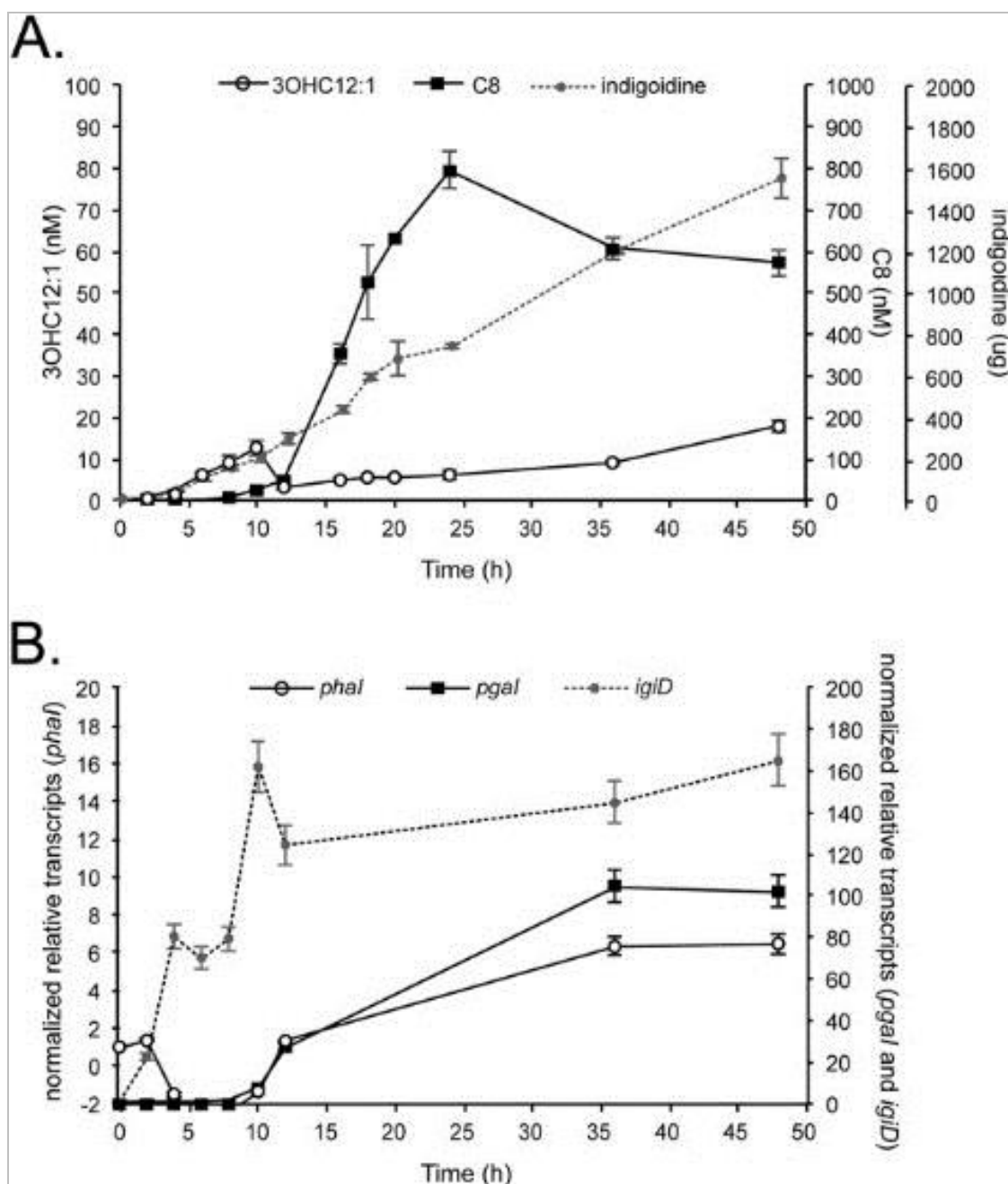


Figure 3.5. Concentrations of AHLs and indigoidine (A) and the gene expression profiles of their synthases (B) from *Phaeobacter* sp. strain Y4I over 48 h of growth on an agar plate. Error bars represent standard deviations (A) or standard errors of the means (B) of the results of biological and technical replicate experiments.

3.5 Discussion

While Roseobacters are known to produce long-chain AHLs with various degrees of oxidation and side-chain saturation^{133, 152, 157-158, 169}, the full complement of AHLs synthesized by lineage members and the contribution of these chemical signals to roseobacter ecology are not yet known. Here we identified a novel AHL, 3OHC_{12:1}-HSL, in *Phaeobacter* sp. strain Y4I that is most structurally similar to a 14-carbon HSL from the soil alphaproteobacterium *Rhizobium leguminosarum*¹⁶⁸. The 3OHC_{14:1}-HSL is at the top of a hierarchical regulatory network of four different AHL-based quorum sensing systems in this plant-associated bacterium¹⁷⁰. In contrast, the other AHL identified in *Phaeobacter* sp. strain Y4I, C₈-HSL, has previously been reported in *Roseobacter* species, as well as in many other proteobacteria^{131, 171-176}. There are several lines of evidence providing support for the conclusion that *Pgal* and *Phal* synthesize C₈-HSL and 3OHC_{12:1}-HSL, respectively. The concentrations of each AHL produced by the *phaR*::Tn5 and *pgaR*::Tn5 regulatory mutants support this assignment (**Figure 4**). Furthermore, the concentration of C₈-HSL correlates strongly with the *pgal* gene expression profile (**Figure 5**), which is consistent with the bacterium's growth dynamics on plates (i.e., the cell abundance increases until 24 h, after which it levels off; data not shown). The correlation between 3OHC_{12:1}-HSL and *phal* transcript abundance is not as strong due to a temporal lapse in the correlation between *phal* transcript abundance and the AHL concentration during the first 12 h following inoculation onto the agar surface (**Figure 5**). These inconsistencies may be the result of a carryover of gene expression from the stationary-phase broth culture inoculum. It is feasible that *phal* expression was already induced in the initial inoculum and continued to result in the production of AHLs during the initial growth period on the agar surface. Indeed, it has been shown in *Vibrio harveyi* that feedback mechanisms ensure a graded, rather than abrupt, response to AHL concentrations¹⁷⁷. Alternatively, these findings may suggest that 3OHC_{12:1}-HSL is an important signal when the bacterial cell density is relatively static, a state that is to be expected for cultures during the lag phase.

The contribution of AHL-mediated signals to the regulation of indigoidine biosynthesis in *Phaeobacter* sp. strain Y4I is undoubtedly complex. The strain appears to utilize both *luxRI*-like systems for full induction of indigoidine, though the contributions of the two systems are not equivalent (see, e.g., **Figure 3A**) and though high levels of these AHLs alone are not sufficient to induce indigoidine expression. Instead, surface-attached growth

is a prerequisite for indigoidine biosynthesis¹⁵⁰. Given that the production of secondary metabolites diverts energy from cellular division¹⁷⁸⁻¹⁸⁰, utilization of quorum sensing in connection with surface colonization may provide to Y4I a means of tightly controlling indigoidine production under the various environmental conditions that the strain encounters. In addition, the two *luxRI* systems appear to modulate other physiologies in this strain. Again, though, the contributions of the two systems to these behaviors do not appear to be equivalent, which is further suggestive of the presence of a hierarchical regulatory network or of one that is modulated by a combinational response to multiple AHL signals¹⁸¹.

Both *luxR*-like mutants show enhanced biofilm growth and limited motility (**Figure 3B and C**) Similar phenotypes have been reported in another roseobacter, *Ruegeria* sp. strain KLH11; it has been suggested that quorum sensing may control the lifestyle switch between the sessile (biofilm) and planktonic forms of that strain^{158, 182}. An analogous cell-to-cell communication may be used to induce dispersion of *Phaeobacter* sp. strain Y4I bacteria once the biofilm reaches a threshold concentration or level of maturity. This may be a mechanism to control the biofilm structure such that it is maintained at an optimal state, particularly with respect to microscale oxygen gradients, which have been shown to influence bacterial activities within biofilms¹⁸³⁻¹⁸⁴. Indeed, the reduced form of indigoidine (leucoindigoidine) is predicted to be the antimicrobial form of the molecule, and it spontaneously oxidizes to the nonactive form in the presence of atmospheric oxygen^{150, 185-186}. Thus, oxygen concentrations within biofilms may need to be low enough to maintain the antimicrobial form of indigoidine, in order to suppress colonization of competing organisms, and yet high enough to facilitate the aerobic respiration of Y4I. Sequential and multitiered quorum sensing regulatory networks have been described for several proteobacterial strains, including *Vibrio fischeri*, in which quorum sensing is used to modulate intermediate and late symbiotic colonization of the *Euprymna scolopes* light organ and bioluminescence^{134, 139, 187-188}. *V. fischeri* has two AHL-based quorum sensing systems that synthesize different AHLs at different stages of squid colonization^{134, 139, 187}. Moreover, it has been shown that the response regulator that directly controls bioluminescence (LuxR) can respond to the two AHLs at different binding efficiencies, thus inducing bioluminescence at different cell densities and stages of colonization^{127, 189}. This type of sequential activation of quorum sensing systems, and cross talk between these systems, may be similar to the induction of indigoidine biosynthesis in Y4I at the intermediate and late stages of biofilm colonization. These systems are not completely analogous, however. Unlike the *V.*

fischeri bioluminescence operon, a TetR repressor homolog is encoded directly upstream of the indigoidine biosynthesis operon in Y4I and likely directly mediates expression of that gene cluster¹⁵⁰. While bioluminescence in *V. fischeri* is directly activated by the quorum sensing-induced LuxR protein, in Y4I, quorum sensing of other stimuli, such as surface sensing, likely inactivates this repressor to induce indigoidine biosynthesis. Indeed, the underlying mechanism(s) that facilitates recognition of a surface and signals cells to switch from a motile lifestyle to a sessile lifestyle, the latter of which includes indigoidine biosynthesis, is unknown in this strain. We speculate that flagellum-mediated mechanosensing of surfaces¹⁹⁰ results in a signal transduction process evidently involving a master transcriptional regulatory circuit, perhaps the two-component CckA-CtrA master regulator of flagellar and motility genes in several alphaproteobacteria, including *Ruegeria* sp. strain TM1040¹⁶⁰. This master regulatory circuit likely contributes to the hierarchical regulatory network seen in *Phaeobacter* sp. strain Y4I, and this is an area ripe for further investigation.

We have demonstrated that *Phaeobacter* sp. strain Y4I synthesizes 3OHC_{12:1}-HSL during the initial colonization of a surface by the strain and that C₈-HSL is synthesized during later stages of growth. Therefore, 3OHC_{12:1}-HSL may activate either PhaR or PgaR to initiate a regulatory cascade that leads to indigoidine production. Insertional mutations in *phaR* significantly impair 3OHC_{12:1}-HSL production and result in delayed pigmentation, suggesting that *PhaR* may induce expression of the quorum sensing regulon during intermediate colonization and initiate pigment production. Further, insertional mutations in *pgaR* lead to the abolishment of indigoidine production in the strain, implying a key role for this regulator and cross talk between the systems (**Figure 2 and 3**). Utilizing both systems at different stages of growth could lead to antimicrobial production both during intermediate colonization and after establishment. In this way, the bacterium may be best able to ensure its success as not only a pioneering colonizer but also an enduring resident of marine surfaces.

The complexity of AHL-mediated signaling is enhanced when interspecies interactions are considered. Indeed, we have shown here that Y4I produces C₈-HSL, a trait that it shares with a number of other proteobacteria^{131, 171-176}, including *V. fischeri*, a bacterium outcompeted in coculture by indigoidine-producing Y4I strains¹⁵⁰. Given the prevalence of this signal among bacteria, it can be predicted that Y4I may use this signal to acquire

information concerning not only its own population status but also that of a variety of potentially competing proteobacterial populations in the ocean. In contrast, 3OHC_{12:1}-HSL may represent a species-specific signal for Y4I. Thus, these two molecules likely provide Y4I cells with distinct information on metabolic status as well as on local population size and structure (e.g., *Phaeobacter* sp. strain Y4I versus the total proteobacterial population size), and Y4I populations mediate gene expression accordingly.

**CHAPTER IV. BLACK BAND DISEASE-ASSOCIATED QUORUM
SENSING SIGNAL PRODUCTION, MICROBIAL INTERACTIONS,
AND TEMPERATURE-ENHANCED ACYLHOMOSERINE
LACTONE PRODUCTION BY VIBRIOS**

The work outlined in this chapter was originally performed by ^{1,2}Beth L. Zimmer, ³Amanda L. May, ¹Chinmayee D. Bhedi, ³Stephen P. Dearth, ³Carson W. Prevatte, ¹Zoe Pratte, ³Shawn R. Campagna, and ¹Laurie L. Richardson reported in one publication and ¹Chinmayee D. Bhedi, ³Carson W. Prevatte, ³Maggie S. Lookadoo, ¹Patricia A. Waikel, ⁴Patrick M. Gillevet, ⁴Masoumeh Sikaroodi, ³Shawn R. Campagna, and ¹Laurie L. Richardson and reported in a second publication. BLZ and CDB, under the supervision of LLR, is the primary author in terms of the introduction, biological experimentation methods, results, and conclusions. CWP, along with other members of the Campagna laboratory and combined research team, authored sections of the original manuscript containing chemical data and methods contributions. The two publications are listed below. The articles have been merged without changes to the original content of the articles. The abstract, introductions, and methods sections were merged in order to reduce redundancy between the articles and edited to present the two articles two parts of a single, overarching project. The results and discussion were merged and are as originally published.

Zimmer, B. L., May, A. L., Bhedi, C. D., Dearth, S. P., Prevatte, C. W., Pratte, Z., Campagna, S. R., & Richardson, L. L. Quorum sensing signal production and microbial interactions in a polymicrobial disease of corals and the coral surface mucopolysaccharide layer. *PLoS one* 2014 9 (9), e108541.

Bhedi, C. D., Prevatte, C. W., Lookadoo, M. S., Waikel, P. A., Gillevet, P. M., Sikaroodi M., Campagna, S. R., Richardson, L. L. Elevated temperature enhances short- to medium-chain acyl homoserine lactone production by black band disease-associated vibrios. *FEMS Microbiology Ecology* 2017 93 (3).

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The Richardson lab and SRC and ALM of the Campagna lab conceived the study. BLZ, ALM, CDB, SPD, CWP, ZP, MSL, PAW, PMG, and MS all performed the experimental work. The field sampling, sample preparation, quorum sensing (QS) signal reporter strain assays, 16S rRNA gene sequencing of isolates and metagenomics experiments were performed by

the Richardson lab and collaborators. The Campagna lab contributed to sample preparation and high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis of the QS signals. CWP and MSL synthesized the (D₂)AHLs used to annotate the (*N*-acyl homoserine lactone) AHL signals reported. SPD and ALM synthesized the ¹³C-labelled DPD used to quantitate DPD. Each lab generated the data for their respective experimental contributions. The Richardson lab analyzed the data for their respective experimental contributions. SPD and CWP analyzed the data for 4,5-dihydroxy 2,3-pentanedione (DPD) quantitation and AHL annotation, respectively. BLZ, CDB, ALM, SRC, LLR contributed to the discussion.

CWP contribution

In both studies, CWP performed the extraction, sample preparation and high performance liquid chromatography-tandem mass spectrometry HPLC-MS/MS analysis of isolates grown over a time course. The *N*-acyl homoserine lactones (AHLs) produced by these isolates, which had previously tested positive for AHLs in bioassays performed by collaborators, were detected, annotated, and identified using a modified version of a previously described HPLC-MS/MS. This method screened for an expanded number of masses, including AHLs possessing chain lengths ranging from 4 to 20 carbons with a single hydroxyl or ketone group at the 3-position as well as one unit of unsaturation in the chain. The AHL extraction and processing method for agar plates developed by CWP was used in the first study. CWP synthesized dideuterated (D₂) AHLs that were used as internal standards that allowed easy identification of many AHLs through retention time matching. Internal standards included (D₂)C₄-HSL, (D₂)3OHC₄-HSL, (D₂)C₆-HSL, (D₂)C3OC₆-HSL, (D₂)C₇-HSL, (D₂)C₈-HSL, (D₂)C₁₂-HSL, and (D₂)C_{12:1}-HSL, and (D₂)C₁₄-HSL. However, the mass accuracy of the triple-quadrupole instrument used cannot differentiate some isobaric product ions. Additionally, the possibility of the production of an AHL of one of the 19 isolates analyzed not included among the internal standards employed is likely. As a result, annotation of many unknown structures for which standards were not available. This was accomplished by comparing the unknown AHL retention to that of the known using knowledge of how structure and chain length effect retention time and biological information, such as precedence for the compound in literature. Identification of the AHLs produced by the 19 isolates screened was a very significant contribution in demonstrating that AHLs are present in an active coral

disease and the production of them is enhanced above the temperature threshold of black band disease (BBD) of corals.

4.1 Abstract

Black band disease (BBD) of corals is a globally distributed disease composed of a complex horizontally migrating, pathogenic, polymicrobial mat community active above a temperature threshold of 27.5 °C. BBD is considered to be a threat to coral reef health, as it can lead to mortality of massive reef-building corals. The BBD community is dominated by gliding, filamentous cyanobacteria with a highly diverse population of heterotrophic bacteria. Microbial interactions such as quorum sensing (QS) and antimicrobial production may be involved in BBD disease pathogenesis. In the first in a series of two studies, BBD (whole community) samples, as well as 199 bacterial isolates from BBD, the surface mucopolysaccharide layer (SML) of apparently healthy corals, and SML of apparently healthy areas of BBD-infected corals were initially screened for the production of acylhomoserine lactones (AHLs) and for autoinducer-2 (AI-2) activity using three bacterial reporter strains. AHLs were detected in all BBD (intact community) samples tested and in cultures of 5.5% of BBD bacterial isolates. Over half of a subset (153) of the isolates were positive for AI-2 activity. AHL-producing isolates were further analyzed using LC-MS/MS to determine the identity of AHLs present and the concentration of (S)-4,5-dihydroxy-2,3-pentanedione (DPD), the biosynthetic precursor of AI-2. C6-HSL was the most common AHL variant detected, followed by 3OC4-HSL. In addition to QS assays, 342 growth challenges were conducted among a subset of the isolates, with 27% of isolates eliciting growth inhibition and 2% growth stimulation. 24% of BBD isolates elicited growth inhibition as compared to 26% and 32% of the bacteria from the two SML sources. With one exception, only isolates that exhibited AI-2 activity or produced DPD inhibited growth of test strains. In the following study of the series of two studies, bacterial isolates of the same sample type were preliminarily screened for production of acylhomoserine lactones (AHLs) using the *Chromobacterium violaceum* CV026 reporter strain. Of 229 bacterial isolates tested, 27 produced AHLs and 23 of these were from BBD. Eight AHLs were identified using LC-MS/MS, with 3OHC4-HSL the most commonly produced followed by C6-HSL. Exposing AHL-producing isolates to three temperatures (24°, 27°, 30 °C) revealed that production of four AHLs (C6, 3OHC4, 3OHC5, and 3OHC6) significantly increased at 30 °C when compared to 24°C. 16S rRNA gene sequencing revealed that all

but one of the AHL producing BBD isolates were vibrios. Metagenomic analysis of the BBD community showed the presence of AHL (and AI-2) genes, most of which are known to be associated with vibrios. The first study demonstrates for the first time that AHLs are present in an active coral disease. Proceeding the first study, it was hypothesized that AI-2 production and quorum sensing among BBD and coral SML bacteria may be involved in the structuring the microbial communities of both a polymicrobial infection and the healthy coral microbiome as well as the pathobiology of BBD. The results of the second study corroborate this hypothesis due to the enhanced production of AHLs above the temperature threshold of BBD.

4.2 Introduction

There has been a significant decline in coral cover and diversity on tropical and subtropical reefs worldwide, and one of the foremost contributing factors has been coral disease¹⁹¹. Disease could affect the coral animal itself or one or more of its associates, including bacteria, fungi, endolithic algae, zooxanthellae, protozoa, and other partners, collectively termed the coral holobiont¹⁹²⁻¹⁹³. The diverse and host specific coral-associated microbial community is harbored within the tissue, skeleton, and protective surface mucopolysaccharide layer (SML) secreted by the coral epidermal mucus cells¹⁹⁴⁻¹⁹⁵. Interactions within this microbial community have been proposed to be cooperative as well as antagonistic in nature and its members have been proposed to be potentially pathogenic and/or probiotic¹⁹⁶⁻¹⁹⁷. The composition and taxonomy of microbial communities of the SML layer of apparently healthy 57 corals have been well researched¹⁹⁸⁻²⁰⁹. Such studies have shown that there is a shift in the heterotrophic microbial population within the SML when corals are stressed, including in the case of disease^{198, 200, 204-205, 210}. One of the most destructive and intricate coral diseases is black band disease (BBD)²¹¹. It is also one of the most widely distributed diseases, affecting sixty-four species of corals and exhibiting coral host and geographic specificity^{212-215, 216-217}. BBD is easily distinguished in the reef environment (**Figure 4.1**), manifesting as a dark pigmented band (phycoerythrin) separating healthy coral tissue from recently exposed coral skeleton.

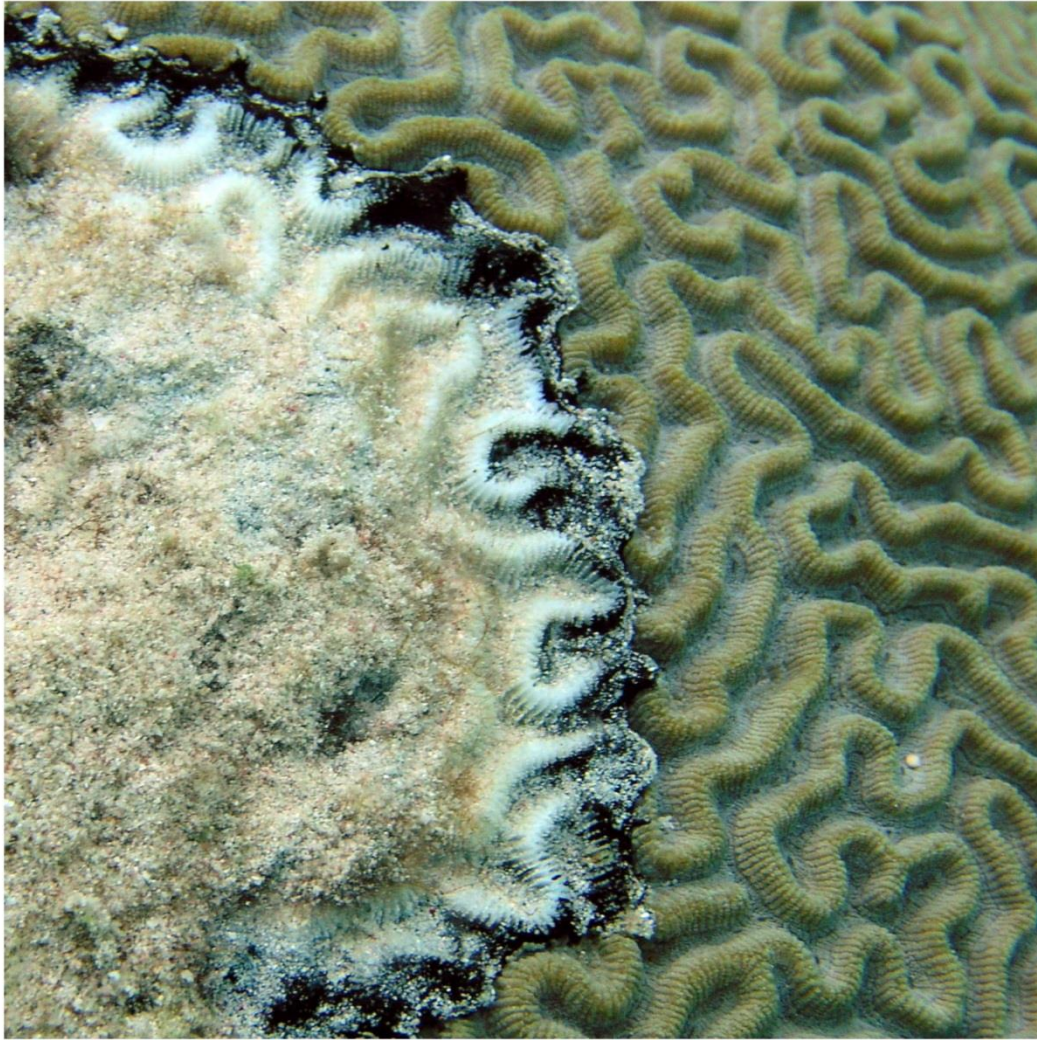


Figure 4.1. Black band disease infection on a colony of *Diploria strigosa* on a reef of Curaçao. The dark-colored black band disease microbial mat separates apparently healthy coral tissue from white, denuded coral skeleton. Photograph provided by Abigail Brownell.

The band migrates horizontally across the surface of a coral colony, causing coral tissue necrosis at a rate of approximately 3.0 mm per day²¹⁸. The tissue loss on an individual colony infected with BBD can be substantial and can result in total colony mortality²¹⁹⁻²²¹. Massive reef-building corals are susceptible to BBD which exacerbates the impact of the disease on reef ecology and function^{213, 218, 220, 222}. The BBD mat consists of a microbial consortium volumetrically dominated by a dense population of the gliding, filamentous cyanobacterium *Roseofilum reptotaenium*²²³⁻²²⁴, and consists of a highly variable polymicrobial consortium of microbes that contains four known functional groups: photoautotrophs (cyanobacteria)²¹⁸, sulfate-reducing bacteria²²⁵, sulfide oxidizing bacteria²²⁶, and heterotrophs²²⁵. By far, the heterotrophic bacteria have been found to be the most taxonomically rich and varied community within the disease consortium²¹⁵. Several research groups have proposed that this diverse set of microbes includes potential primary, as well as secondary, pathogens^{200, 203, 210, 227-228}. A number of studies have targeted BBD heterotrophic bacteria^{221, 226-227, 229-230}. Mechanisms of BBD pathogenicity include anoxic conditions within the BBD mat in combination with sulfide production by BBD sulfate reducing bacteria, and BBD cyanobacterial toxin (microcystin) production²³¹⁻²³⁵. A recent microscopic study documented cellular necrosis (i.e., loss of tissue confluence, cell-to-cell adhesion, cytoplasmic disintegration, nuclear breakdown, and the presence of autophagous bodies, pyknotic nuclei, and apoptotic bodies) in the coral tissue surrounding cyanobacterial filaments in active BBD infections²³⁶. These microscopic observations support the previous studies demonstrating the role cyanobacterial toxins in BBD pathogenicity. BBD infections occur on the surface tissues of infected coral colonies, which are also known to be microbially diverse. In particular, the coral surface mucopolysaccharide layer (SML) supports a dynamic microbial community which is believed to play an important role in coral disease resistance²³⁷⁻²³⁹. Interactions between several bacterial coral pathogens and the microbial community of the coral SML have been the subject of multiple studies, and the coral SML and its associated microbes have been shown to produce antimicrobial agents and biofilm inhibitors that may be acting to protect corals from pathogen growth²³⁹⁻²⁴⁴. However, relatively little is currently known about the interactions between microbes within the SML. Bacterial 16S rRNA gene clone libraries demonstrated shifts in bacterial ribotypes during transitions from cyanobacterial patches (CP) shown to develop into BBD on corals on the Great Barrier Reef (GBR)²²⁹. In these studies, α -proteobacteria-affiliated sequences were dominant in CP libraries, whereas γ -proteobacterial ribotypes became more abundant after transition to the BBD community²²⁹. The CP-to-BBD transition has only been seen on reefs

of the GBR. A meta-analysis of 87 published BBD clone libraries from the Caribbean and Indo-Pacific detected a common cyanobacterial sequence, recently characterized as *Roseofilum reptotaenium* gen. et sp. nov., as present in 78% of the clone libraries^{215, 223}. In contrast to the low diversity of BBD cyanobacterial taxa, the meta-analysis also revealed an extremely high diversity of heterotrophic BBD bacteria, with 73% of all sequences detected present as singletons (only 1 copy in the 87 clone libraries). Very little is known about the role of BBD heterotrophic bacteria, the one exception being that BBD has a well-documented and very active sulfur cycle generated by sulfate reducing bacteria within the disease consortium²⁴⁵⁻²⁴⁶. The meta-analysis also found α -proteobacteria to be the most diversely represented group of bacteria in a meta-analysis of clone libraries produced from 87 BBD samples collected on reefs world-wide²¹⁵. While the gene sequence corresponding to the dominant BBD cyanobacterium is by far the most common in BBD clone libraries, the second most common sequence detected has been *Roseovarius crassostreae*^{203, 215, 227-229, 247}, an α -proteobacterium which is the causative agent of *Roseovarius* Oyster Disease in juvenile oysters²⁴⁸⁻²⁴⁹. *R. crassostreae* has also been detected in clone libraries constructed from several other coral diseases such as white band disease²⁵⁰, white plague-like disease²⁵¹ and more recently, Australian subtropical white syndrome²⁵². A second bacterium of potential interest is *Ferrimonas* sp., a selenite and iron reducing genus which has also been detected in 93 BBD clone libraries²⁰³. Since iron reduction is a known virulence factor in a number of bacteria,²⁵³ *Ferrimonas* is of particular interest for targeted study of the BBD consortium. BBD most actively infects corals when the sea water temperature rises above 27.5 °C^{219, 222, 254-255}. Since bacterial species and communities have stringent tolerance thresholds regarding the environment (including temperature), any change in surroundings surpassing a threshold may lead to a perturbation of the delicate relationship with a host. Environmental factors can also lead to decreased host health and fitness, and an increase in temperature has been shown to cause a switch from a symbiotic to a pathogenic role in some host associated microbes²⁵⁶. In several cases this transition has been shown to be controlled by quorum sensing²⁵⁷.

Quorum sensing is a type of pheromone-based cell to cell communication system that is often density regulated and that allows bacteria to converse with the aid of chemically synthesized signals called autoinducers (AIs)²⁵⁸⁻²⁵⁹. Different bacteria synthesize different AIs and release them into the environment. While different bacteria can synthesize unique AIs, many different bacteria can also synthesize the same AIs, allowing the potential for

intra- and inter-species species signaling and coordination between different bacterial genera as well as species¹²⁹. After extracellular accumulation of AI molecules reaches a threshold (the "quorum" in quorum sensing), bacteria regulate their behavior to coordinate expression of specific genes to adapt and thrive in that specific environment. This is achieved by inducing production of an array of physiological and functional adaptations^{129, 257, 260-264}. Quorum sensing as a phenomenon is responsible for regulating a wide range of bacterial behaviors that include virulence, bioluminescence, biofilm maintenance and maturation, motility, symbiosis, antibiotic synthesis, exoenzyme production, and swarming^{127, 265-269}.

Chemical signaling in tropical and subtropical coastal environments is a relatively unexplored area that may be important in coral health and disease. It has been noted that QS systems in *Vibrios* often integrate other information, including environmental parameters, with cell density when using these networks²⁷⁰. Quorum sensing is one mechanism by which coral pathogenic bacteria and SML bacteria may be interacting¹²⁵. Acylhomoserine lactones (AHLs) and autoinducer-2 (AI-2) are two of the more well-characterized groups of QS signaling molecules²⁷¹. The latter (AI-2) is a family of related molecules derived from (S)-4,5-dihydroxy-2,3-pentanedione (DPD)²⁷¹. AHLs are considered to be intraspecies signaling molecules²⁷², although it has been shown that cross-talk between bacteria using these molecules can occur²⁷³⁻²⁷⁵. AHL production has been well documented among members of the proteobacteria^{257, 276} a group that is commonly detected in BBD clone libraries^{200, 203, 227-229}, making AHL production a prime target for BBD research. AI-2 signaling is widely recognized as having an important role in interspecies communication^{269, 277-279}, since DPD production is common in both Gram-negative and Gram-positive bacteria²⁸⁰⁻²⁸¹. AI-2 signaling may also play a role in the complex bacterial communities of both BBD and coral SML.

Gram negative bacteria specifically produce AHLs which are constituted of a fatty acid (acyl) side chain attached to a homoserine lactone ring¹³¹. The side chains vary on the basis of their length, saturation and presence/absence of functional groups, creating a suite of AHL molecules that bacteria use to communicate in complex environments¹³¹. As the side chain length increases, the solubility of the signaling molecule decreases while the stability increases²⁸²⁻²⁸³. Various factors such as temperature, pH, oxygen availability, and redox state have been known to affect QS regulation and QS regulatory gene expression^{170, 284}.

The structural integrity of the homoserine lactone ring has been observed to be reversibly susceptible to alkaline conditions, affecting long chain AHLs to a lesser extent^{282-283, 285} which is significant in terms of environmental influence since lactone ring hydrolysis disables the AHL molecule from signaling²⁸³.

Overall, QS is associated with a wide range of interactive social responses in bacteria^{269, 286} and has been shown to regulate virulence in both Gram-positive and Gram-negative pathogens²⁷⁷, including upregulation of antibiotic biosynthesis²⁸⁷⁻²⁸⁸. AHL production has been observed in coral-associated bacteria isolated from the SML of healthy corals²⁸⁹, isolates from other marine invertebrates and their endosymbiotic dinoflagellates²⁴⁰, and from the tissues of 10 cnidarian species (all healthy) examined recently²⁹⁰. *Vibrio* spp. isolated from the mucus of healthy and diseased corals have been shown to produce both AHLs and DPD²⁹¹. In one study, QS was proposed to play a major role in the pathogenicity of the coral pathogen *Vibrio coralliitycus*²⁹². This study extends the research on BBD to examine the variation of QS (AHL) molecules produced by BBD and coral-associated bacteria in response to environmental (temperature) changes, including a comparison of AHLs produced across different coral health states. As mentioned above, BBD is typically observed in summers when sea water temperatures exceed 27.5 °C^{222, 254}. Therefore, we examined AHL production at temperatures close to, above, and below this threshold. The QS experiments were performed in conjunction with assays to assess antimicrobial activity of the isolates to further broaden our understanding of bacterial interactions within BBD and the coral SML. Metagenomics analysis was done to assess the presence and nature of quorum sensing genes in the BBD mat in addition to culture dependent methods.

The first study of the two-study series demonstrates the *in situ* presence of AHLs in an active coral disease for the first time. Following the hypothesis that AHL production among BBD and coral SML bacteria may structure both the microbial communities of a polymicrobial infection as well as the native coral microbiome, the second study suggests that quorum sensing may be involved in BBD pathobiology and community structure due to enhanced production of AHLs above the temperature threshold of this globally distributed coral disease.

4.3 Materials and Methods

Field sampling and isolation of bacteria for the initial AI screening study. BBD, apparently healthy SML from BBD infected coral (BSML) and SML from apparently healthy coral (HSML) samples were collected from reefs in Ft. Lauderdale, Florida under Florida Fish and Wildlife Conservation Commission sampling license SAL-11-1344-SRP and from Florida Keys reefs under permits FKNMS-2007-026 and FKNMS 2009-045-A3. Freshly collected BBD mat as well as both bacterial (N=191) and cyanobacterial (N=8) isolates from BBD and coral mucus samples were examined for production of autoinducers in this study (**Table 4.1**). The bacterial isolates included 182 newly isolated strains cultured from colonies of *Montastraea cavernosa*, *Colpophyllia natans*, and *Diploria strigosa* located on the Florida Reef Tract [Ft. Lauderdale, Florida, USA (26° 11.35' N, 80° 5.49' W); Horseshoe Reef, Florida Keys, USA (25° 08.362' N, 80° 17.641'); and Algae Reef, Florida Keys, USA (25° 08.799' N, 80° 17.579' W)] (**Table 4.1**). Thirty-nine of the 191 bacteria were newly isolated from *Diploria strigosa* on a reef of Curaçao (Water Factory 3, 12° 06.779' N, 68° 57.662' W). Samples were collected using sterile, needleless 10 ml or 60 ml syringes. SML from apparently healthy areas of BBD-infected colonies (designated BSML) was sampled at the farthest distance (minimum of 20 cm) from the BBD mat. SML samples were also collected from apparently healthy colonies (HSML) of the same coral species located in the near vicinity of BBD-infected corals. To collect SML, the coral surface was gently agitated using the syringe tip (to cause mucus secretion) and the resulting mucus aspirated into the syringe. The BBD mat was easily collected by syringe as it is loosely attached to the coral surface.

Syringes with freshly collected samples were held in coolers at ambient seawater temperature during transport to the laboratory, where the SML sample syringes were inverted to allow sampled mucus to settle to the syringe tip for collection. BBD samples clumped within the syringe shortly after aspiration (a behavioral response of the filamentous cyanobacteria) and each clump was directly collected and transferred into sterile 2.0 ml cryovial. Each SML sample was extruded into a sterile 2.0 ml cryovial to limit the amount of seawater in the sample. Autoclaved artificial seawater was then added to each cryovial. Samples were mixed by vortexing and spread plated, after a standard dilution series, onto plates containing the following media: Difco marine agar (MA), 1/10 strength MA, Thiosulfate Citrate Bile Salts Sucrose (TCBS; *Vibrio*-specific, BD) agar, and Sea Water Tryptone (SWT)

Table 4.1. Sampling and bacterial isolate designations.

Site Name and Location	Date of Collection	Water Depth (m)	Water Temp (°C)	Colony No.	Host Coral	Sample Type(s) ^a	Bacterial Isolate Designations ^{b,c,d}	No. of Isolates Examined
Ft. Lauderdale, Broward County, FL, USA; 26° 11.35' N, 80° 5.49' W	17-May-08	3.7	28	FTL 1		BBD	BBD-FTL-1x	109 (41 BBD, 23 BSML, 45 HSML)
	27-Jun-08	4.6	28	FTL 2	<i>Montastraea cavernosa</i>	BBD	BBD-FTL-2x	
	27-Jun-08	4.3	28	FTL 3	<i>M. cavernosa</i>	BBD	BBD-FTL-3x	
	27-Jun-08	4	28	FTL 4	<i>M. cavernosa</i>	BBD	BBD-FTL-4x	
	27-Jun-08	3.3	28	FTL 5	<i>M. cavernosa</i>	BBD	BBD-FTL-5x	
	1-Aug-09	4.6	29	FTL 6	<i>M. cavernosa</i>	BBD, BSM	BBD-FTL-6x, BSM-FTL-6x	
	31-Jul-10	3.7	30	FTL 7	<i>M. cavernosa</i>	BBD, BSM	BBD-FTL-7x, BSM-FTL-7x	
	31-Jul-10	4.3	30	FTL 8	<i>M. cavernosa</i>	BBD, BSM	BBD-FTL-8x, BSM-FTL-8x	
	31-Jul-10	4.6	30	FTL 9	<i>Diploria strigosa</i>	HSML	HSML-FTL-9x	
	31-Jul-10	4.3	30	FTL 10	<i>M. cavernosa</i>	HSML	HSML-FTL-10x	
	31-Jul-10	4.3	30	FTL 11	<i>M. cavernosa</i>	HSML	HSML-FTL-11x	
Algae Reef, Florida Keys, FL, USA; 25° 08'36.2" N, 80° 17.579' W	24-Oct-09	6.7	25	FLK 1	<i>D. strigosa</i>	BBD, BSM	BBD-FLK-1x, BSM-FLK-1x ⁵	27 (13 BBD, 14 BSML)
Horseshoe Reef, Florida Keys, FL, USA; 25° 08.3629' N, 80° 17.6419' W	7-Sept-12, 30-Sept-12	3.7	29	FLK 2 ^f	<i>Colpophyllia natans</i>	BBD	BBD-FLK-1Mx, BSM-FLK-15Mx, 7 BBD-FLK-2Mx, BSM-FLK-25Mx	
Water Factory 3, Curacao; 12° 06.7799' N, 68° 57.6629' W	23-Feb-13	2.5	27	CUR 1	<i>D. strigosa</i>	BBD	BBD-CUR-3Mx, BBD-CUR-3Sx	39
Horseshoe Reef, Lee Stocking Island, Bahamas; 23° 46.309' N, 76° 5.339' W	9-Jul-04	8.2	29	216	<i>Siderastrea siderea</i>	BBD	BBD-216-x	5
		7.3	28	217	<i>S. siderea</i>	BBD	BBD-217-x	4

BBD samples were obtained from a BBD mat sample; BSML samples were obtained from the SML of an apparently healthy area of a BBD-infected colony; HSML samples were obtained from the SML of an apparently healthy colony^a. The "x" in the isolate designation refers to an individual isolate from the source colony [e.g. BBD-FTL-1a, BBD-FTL-1b, etc.]^b. The "M" in the isolate designation refers to isolates that were selected from a marine agar plate^c. The "S" in the isolate designation refers to isolates that were selected from a sea water tryptone agar plate^d. Includes BBD-216-1b, BBD-216-2d, BBD-216-3d, BBD-216-4a, BBD-214-4e, BBD-217-2b, BBD-217-2d, BBD-217-2g, and BBD-217-3m^e. Two BBD samples were collected from the single colony of *C. natans* on separate days. Sample 1 and Sample 2 were collected on 27-Sept-12 and 30-Sept-12, respectively.

agar. Plates were incubated at room temperature ($\sim 23^{\circ}\text{C}$) and unique colonies were selected on the basis of color and morphology for further isolation and evaluation. In addition to the above isolates, 17 previously isolated BBD bacteria (N=9) and cyanobacteria (N=8), collected in the Florida Keys, Bahamas, and Philippines were tested for QS signal production (**Table 4.1**). The nine bacterial cultures (strains BBD-216-1b, BBD-216-2d, BBD-216-3d, BBD-216-4a, BBD-216-4e, BBD-217-2b, BBD-217-2d, BBD-217-2g, and BBD-217-3m, all documented in^{203, 228} were maintained on MA slants at room temperature. The eight cyanobacterial cultures (strains BBD 1991, HS 217, HS 223, W-1, FLK BBD1, Phil 2b-2, 102a-1, and 96-2) are all members of the genera *Geitlerinema* and *Leptolyngbya* and were collected on various reefs in the Florida Keys, Bahamas, and the Philippines^{222, 230, 232, 234, 293-294}. The cyanobacterial cultures were maintained in 125 ml Erlenmeyer flasks in algal mineral media ASNIII or marine BG11 at 26°C under a 12:12 h light:dark cool white fluorescent light regime at an intensity of $20\ \mu\text{E m}^{-2}\ \text{s}^{-1}$.

Field sampling and isolation of bacteria for the AHL temperature enhancement study. BBD, BSML and HSML samples were collected from reefs of the northern Florida Keys, USA (Algae Reef and Horseshoe Reef) and the Water Factory in Curaçao, Netherlands Antilles, from two scleractinian coral species, *Montastraea cavernosa* and *Pseudodiploria strigosa*. Sterile needleless 60 ml syringes were used for sampling. BBD mat and SML were suctioned off of the coral host with slight agitation, after which the syringes were capped. BSML samples were collected at a minimum distance of 10 cm from the BBD mat. HSML samples were collected from adjacent (healthy) colonies of the same coral species. Once on the boat, syringes with samples were held in coolers containing sea water at ambient temperature and transported to the shore. BBD mat and SML were then transferred to artificially sterilized sea water (ASW) to minimize contamination from sea water bacteria. Isolation of bacteria from three coral health states Heterotrophic bacteria were isolated from samples of each of the three coral health conditions from both reef systems and coral host species. Dilution series (10⁻¹ to 10⁻¹⁰) of BBD, HSML and BSML samples were prepared using both ASN III medium and filtered sea water, and incubated at both room temperature and 30°C under a 12 hours light:12 hours dark regime. 100 μl from dilution tubes 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ were plated every day for a period of seven days and incubated 183 at 30°C . Direct streaking from the original mat and SML samples was also carried out. All plating was onto Difco marine agar (MA) and sea water tryptone (SWT) agar plates, in triplicate. Uniquely distinct colonies

were chosen based on colony color and morphology followed by streaking for purification by plating onto the same medium.

Processing Cell-free culture filtrates. Cell-free culture filtrates (CFs) from the bacterial isolates were collected for use in QS assays. Isolates were grown in sterile Difco marine broth (MB) that was filtered twice (Whatman 1 paper filters, 5.5 cm) to remove the medium precipitate. The cultures were grown to stationary phase at 29°C with shaking. This temperature is ecologically relevant, since BBD is most active in the wider Caribbean when surface water temperatures are above approximately 28°C^{222, 254, 295-297}. Bacterial cell concentrations were monitored by measuring the optical density at 600 nm (OD₆₀₀) using either a Modulus Microplate Multimode Reader (Turner BioSystems, Sunnyvale, CA, USA) or a Thermo UV1 Spectrophotometer (Thermo Electron Ltd., Cambridge, UK) with sterile MB as a zero/blank.

At early stationary phase, CF samples were prepared by centrifugation at 12,000 *g* for 10 min, and the supernatant was divided into two, 1.0 ml aliquots. Because alkaline conditions have been shown to result in AHL lactonolysis^{282, 285, 298}, prior to division of the sample the pH of the supernatant was measured using a Jenco Model 60 Digital pH meter (Jenco Electronics, Ltd., Taipei, Taiwan). Since AHLs have been shown to remain stable for extended time periods at pH 5.0–6.0²⁹⁹, the pH of the supernatant to be used in the AHL assays was adjusted to this pH range using a sterile HCl solution (1 N). Each CF was then filter sterilized (0.22 µm membrane, Millipore, Billerica, MA, USA) and the CFs were stored at –20°C. For the cyanobacterial strains, CFs were prepared by obtaining a 2.0 ml aliquot from an active cyanobacterial culture. The culture was vortexed and cells were removed using a combination of centrifugation (12,000 *g* for 10 min) and filter sterilization. The cyanobacterial CFs were used immediately in the QS assays (with no acidification).

AHL reporter strain assays. *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTL4(pZLR4) reporter strains were used to detect the presence of short-chain and medium- to long-chain AHLs, as indicated (**Table 4.2**). The *C. violaceum* CV026 assay used in this study follows a previously reported protocol with slight modification³⁰⁰. *C. violaceum* CV026 was cultured in sterile Difco Luria-Bertani (LB) broth overnight at 30°C and used to prepare the assay plates. The assay plates (triplicates) contained a base of 1.5% Difco LB agar with an overlay of 100 µl of the *C.*

Table 4.2. Quorum sensing reporter strains and positive control strains used in this study.

Name	Strain characteristics
<i>Chromobacterium violaceum</i>	
CV026 ^a	Detects short-chain AHLs (i.e. C4-AHL, C6-AHL, C6-3-oxo-AHL, C8-AHL, C8-3-oxo-AHL).
<i>Agrobacterium tumefaciens</i> NTL4(pZLR4) ^b	Detects medium- to long-chain AHLs (i.e. C6-AHL, C10-AHL, C12-AHL, C14-AHL, C6-3-hydroxy-AHL, C8-3-hydroxy-AHL, C10-3-hydroxy-AHL, all 3-oxo-AHLs).
<i>Agrobacterium tumefaciens</i> NT1(pTIC58accR) ^b	Synthesizes C8-3-oxo-AHL which is recognized by <i>A. tumefaciens</i> NTL49pZLR4). Culture filtrates of this strain were used as a positive control in the <i>A. tumefaciens</i> NTL4(pZLR4) assay.
<i>Vibrio harveyi</i> BB170 ^c	Detects the DPD and the CAI-1 ^d signals.
<i>Vibrio harveyi</i> BB152 ^c	Synthesizes the DPD signal. Culture filtrates of this strain were used as a positive control in the DPD assay.

Strain obtained from K. Mathee, Florida International University^a. Strains obtained from S.K. Farrand, University of Illinois^b. *V. harveyi* strains BB170 (ATCC BAA-1117) and BB152 (ATCC BAA-1119) obtained from the American Type Culture Collection^c. CAI-1 is the *Vibrio*-specific QS signal (S)-3-hydroxytridecan-4-one^d.

violaceum CV026 culture ($OD_{600}=1.0$) in 5.0 ml of 0.7% LB agar. Samples tested for the presence of short-chain AHLs consisted of both CFs and biomass (colonies from a plate) of the 199 bacterial and cyanobacterial isolates, as well as freshly collected (full community) BBD mat (**Table 4.3**).

To test the bacterial CFs, wells were punched in the assay plates using the wide end of a sterile pipette tip. To each experimental well, 75 μ l of the appropriate CF was added. To test the BBD mat material, a portion of each BBD sample was placed in 1.0 ml of sterile seawater. The mixture was shaken on a vortexer and 75 μ l of the solution was placed into an experimental well in each assay plate. Each assay plate contained a positive control (i.e., 0.01 μ l of 50 mM *N*-hexanoyl-L-homoserine lactone spotted on the agar surface) and a negative control (i.e., a well containing 75 μ l of sterile MB for the CF assays or a well containing 75 μ l of sterile seawater for the BBD mat assays). The assay plates were incubated for 24 h at 30°C and then examined for purple coloration (violacein) surrounding the wells which indicated a positive result. For the bacterial isolate patch tests, each isolate was streaked onto a plate containing MA and incubated at 29°C until colonies were visible. Colonies were collected using a sterile loop and transferred to the surface of *C. violaceum* CV026 assay plates. In the case of the cyanobacterial strains, clumps of cyanobacteria were selected from the culture flask using sterile forceps and transferred to the surface of the assay plates. Each experiment was conducted in triplicate with positive and negative controls, incubated for 24 h at 30°C, and then assessed for violacein presence. The *Agrobacterium tumefaciens* NTL4(pZLR4) assay used in this study follows a previously reported protocol with slight modification³⁰¹. *A. tumefaciens* NTL4(pZLR4) from frozen glycerol stock was streaked on a plate containing autoinducer bioassay minimal (AB_{At}) agar (i.e., 3 g/L K₂HPO₄, 1 g/L NaH₂PO₄, 1 g/L NH₄Cl, 0.3 g/L MgSO₄·7H₂O, 0.15 g/L KCl, 0.01 g/L CaCl₂, 0.0025 g/L FeSO₄·7H₂O)³⁰², along with 5 g/L mannitol (0.5%) and gentamicin (30 μ g/ml) and grown at 28°C until colonies were visible. A single colony was transferred via sterile loop to 1.0 ml of AB_{At} medium with gentamicin (30 μ g/ml) and grown overnight at 28°C with shaking. The day of the assay, a fresh solution of AB_{At} medium with gentamicin (3 μ g/ml) was prepared, inoculated with 50 μ l of the overnight culture, and grown to late exponential phase at 28°C with shaking. The assay plates contained a base of 1.5% AB_{At} agar (0.5% mannitol) with an overlay that included 500 μ l of the *A. tumefaciens* NTL4(pZLR4) culture in 5.0 ml of 0.7% water agar with gentamicin (30 μ g/ml) and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal; 40 μ g/ml). As above (for

Table 4.3. Quorum sensing assays conducted in this study.

Site Name and Location	No. of Isolates Examined	<i>Chromobacterium violaceum</i> CV026 Assay ^a			<i>Agrobacterium tumefaciens</i> NTL4(pZLR4) Assay ^a		<i>Vibrio harvey</i> BB170 Assay ^a	
		Patch Test	CF Test	BBD Mat Test	Patch Test	CF Test	BBD Mat Test	Cultured Isolates
Ft. Lauderdale	109	+	+	+	+	+	+	+
Algae Reef	27	+	+	+	+	+	+	+
Horseshoe Reed, Florida Keys	7	+	NT	NT	NT	NT	NT	NT
Water Factory 3, Curacao	39	+	NT	NT	NT	NT	NT	NT
Horseshoe Reed, Lee Stocking Island	9	+	+	+	+	+	+	+
BBD cyanobacteria	8	+	+	+	+	+	+	+

"+" indicates that the assay was conducted; NT indicates that the assay was not conducted. Patch Test and CF Test refer to isolates and BBD Mat Test refers to intact, freshly collected BBD Mat Test refers to intact, freshly collected BBD mat, all tested using the CV026 and the NTL4 reporter strains. The BB170 assay utilized actively growing cultures of isolates only (see methods section)^a.

the *C. violaceum* CV026 reporter strain), assay plates were used to test both CFs and biomass (colonies) of the 199 bacterial isolates, as well as freshly collected BBD mat (**Table 4.3**). As a positive control, *A. tumefaciens* NTL4(pTiC58 Δ *accR*), which produces C8-3-oxo-AHL, was grown in AB_{At} broth at 28°C with shaking. An aliquot of the culture was centrifuged at 12,000 *g* for 10 min, and the resulting supernatant was filter sterilized. The negative

control well on each plate contained either 75 μ l of sterile MB for the CF assays or 75 μ l of sterile seawater for the BBD mat assays. The assay plates were incubated for 24 h at 28°C and then assessed for the presence of blue coloration (X-gal cleavage) surrounding the wells, which would indicate a potential positive result. To detect if any of the isolates produced an extracellular factor that hydrolyzes X-Gal, isolates that produced a blue coloration in the patch test were retested on a plate containing only AB_{At} agar (0.5% mannitol) with X-Gal (40 μ g/L). Isolates producing blue coloration on these plates were considered a false-positive result in the *A. tumefaciens* NTL4(pZLR4) assay and the result was scored as negative. AHLs were extracted from those isolates that tested positive in at least one AHL reporter strain assay. Extracted AHLs were then analyzed using liquid chromatography–tandem mass spectrometric (LC-MS/MS), which allows for the determination of the AHL chemical structure. The extraction and LC-MS/MS methods are described below.

The *V. harveyi* BB170 reporter strain was used to screen for the presence of AI-2 (**Table 4.2**) in 153 bacterial CFs (**Table 4.3**). The AI-2 assay used in this study follows a previously reported protocol developed by with slight modification³⁰³. A modified autoinducer bioassay (AB_{Vh}) medium was prepared (1 L solution of 0.3 M NaCl, 0.05 M MgSO₄, and 0.2% casamino acids) and adjusted to pH 7.5. This solution was autoclaved (121°C) and the following components were added to the cooled solution from sterile stocks: 0.05 M K₂HPO₄ (pH 7.0), 0.001 M L-arginine, and glycerol (to 1.0%). Fresh AB_{Vh} medium was inoculated with an aliquot of the *V. harveyi* BB170 frozen glycerol culture, which was then grown (30°C with aeration, shaking) until turbid and showing obvious luminescence in a darkroom. This culture was then diluted 1:5,000 in fresh AB_{Vh} medium and used to prepare the assay plates. The CF from the *V. harveyi* BB152 mutant strain served as a positive control in the AI-2 assay (**Table 4.2**). *V. harveyi* BB152 was cultured overnight in AB_{Vh} medium (30°C with aeration, shaking), centrifuged (12,000 g, 10 min), and the supernatant was filter sterilized and stored at –20°C. AI-2 assays were conducted in sterile 96-well microtiter plates (BD Falcon 353219 - black plate with a clear, flat bottom and lid). Each assay plate contained triplicate experimental wells, control wells, and reference wells, all with final well volumes of 100 μ l. The prepared plates were incubated at 30°C with shaking over the course of the 7 h assay. Optical density (OD₆₀₀) and luminescence (490 nm) readings were conducted every 15 min using a BioTek Synergy HT Multi-Mode Microplate Reader linked directly to a PC with Gen5 software (BioTek Instruments, Inc.,

Winooski, VT, USA). Because some growth media components have been shown to induce luminescence in the *V. harveyi* BB170 reporter strain (e.g., borate and glucose³⁰⁴⁻³⁰⁶, medium control wells were included to monitor possible growth media effects on reporter strain luminescence. Media reference wells were monitored during the AI-2 assays to determine background luminescence output for each of the media tested. AI-2 activity was determined at the optimal time point (OTP) of the *V. harveyi* BB170 assay, defined as the time point immediately preceding self-induction by the *V. harveyi* BB170 reporter strain³⁰³. The OTP represents the time when the mean luminescence of the medium control wells was lowest during the course of the assay. The percentage of AI-2 (% AI-2) activity is expressed as a percentage of the positive control luminescence at the OTP³⁰⁷. The % AI-2 activity was calculated as the fold induction of the sample, i.e., the fold change between the sample luminescence and the corresponding reference luminescence (MB for the bacterial CFs and BG11 for the cyanobacterial CFs) divided by the fold induction of the positive control (the fold change between the positive control luminescence and the corresponding reference luminescence, which was AB_{Vh} medium for the positive control CF). The luminescence at the OTP for each sample was used to calculate the induction of luminescence, which is expressed as a fold induction of the sample in comparison to the luminescence of the positive control. The induction of luminescence was calculated by dividing the sample luminescence by the positive control luminescence at the OTP^{303, 308}.

AHL extraction from isolates in marine broth cultures. Fresh 25 ml aliquots of half strength MB in 125 ml Erlenmeyer flasks were inoculated with 500 µl of cultures grown overnight in the same medium. Duplicate cultures were then grown at 25°C for 24 h with shaking (200 rpm). At this point, 10 ml aliquots of each culture were filtered through 0.22 µm nylon filters (GE Magna) in duplicate to generate a total of four samples per isolate. The filtrate was transferred to a separatory funnel, and the flask was washed with ~1 ml water and added to the funnel. The filtrate was then extracted twice with 5 ml of 1.0% acetic acid in ethyl acetate (H⁺/EtOAc). The combined organic layers were then dried with MgSO₄ and filtered. The filtrate was concentrated *in vacuo* and the resulting oil was then resuspended in 300 µl of H⁺/EtOAc and transferred to an autosampler vial. Samples were immediately analyzed via LC-MS/MS¹⁶².

Determination of antimicrobial production. Antimicrobial production capabilities of BBD, BSML and HSML isolates were tested against *Roseovarius crassostreae* CV919-312 and *Ferrimonas* sp. EF3B-B688 using the agar overlay method. Test plates consisting of a basal layer of MA were overlaid with 5 ml molten MA and 100 μ l of a freshly grown broth culture of either *R. crassostreae* or *Ferrimonas* sp. The plates were allowed to dry for 15 min after which bacterial isolates to be tested for activity were spotted onto the plates, followed by incubation at room temperature for 48 hours with visual monitoring for zones of inhibition.

Chromobacterium violaceum CV026 AHL reporter strain assay. QS signal production capabilities were tested using the reporter strain *Chromobacterium violaceum* CV026 using a similar agar overlay method. This strain is used to detect the presence of short to medium chain AHLs, particularly C4-AHL, C6-3-oxo-AHL, C8-AHL and C8-3-oxo-AHL³⁰⁰. Test isolates were freshly grown overnight on MA at room temperature. The *C. violaceum* CV026 biosensor strain was grown overnight in 5 ml Luria Bertan (LB) broth at 30 °C at 35 rpm. Test plates consisted of a basal layer of LB medium overlaid with 5 ml of molten LB with 100 μ l of a freshly grown overnight broth culture of *C. violaceum* CV026. For a positive control 0.01 μ l of 50 mM synthetic N-hexanoyl-L-homoserine lactone solution in ethanol was used while sterile LB broth served as negative control. All assays were carried out in triplicate. All results were analyzed statistically by doing the proportion t-test for significance ($p \leq 0.05$).

Subjection of AHL-positive isolates to three different temperatures. Isolates that tested positive for production of AHLs using the *C. violaceum* CV026 assay were further tested for the effect of temperature on AHL production by exposing isolates to three different temperatures (24 °C, 27 °C and 30 °C). *R. crassostreae* CV919-312 was also tested in these experiments. For each temperature, 25 ml aliquots of freshly made half strength marine broth (MB) in 125 ml Erlenmeyer flasks were inoculated in duplicate with 500 μ l of culture previously grown overnight at each respective temperature (24 °C, 27 °C and 30 °C) with shaking (250 rpm). 1ml of sample was aliquoted every two hours from each flask at $t = 0, 2, 4, 6, \text{ and } 8, \text{ and } 10$ hours. At every time point for every sample, OD was measured (600 nm) and 500 μ l of sample was added to a microcentrifuge tube containing deuterated AHL internal standards that consisted of (D2)C4, (D2)3OHC4, (D2)C6, (D2)C3OC6, (D2)C7, (D2)C8, (D2)C12, (D2)3OHC12:1 and (D2)C14 that were synthesized as

previously described in¹⁶². This was done in duplicate to generate a total of four samples per isolate per time point per temperature. The eppendorf tubes were then centrifuged for 1 min at 209 ×g to spin down bacterial cells, after which 500 µl of the supernatant were placed in autosampler vials and kept at -80 °C until further processing.

AHL extraction from isolates on marine agar plates. Fresh 25 ml aliquots of half strength MB in 125 ml Erlenmeyer flasks were inoculated with 500 µl of cultures grown overnight in the same medium. Triplicate cultures were then shaken at 200 rpm at 25°C for 1 h. At this time, a 5.0 ml aliquot of each culture was filtered through a 0.45 µm nylon filter (GE Magna), which was placed cell side up on half strength MA plates and incubated at 25°C for 24 h, after which the filter was removed and the agar was minced and transferred to a 250 ml Erlenmeyer flask. A 100 ml aliquot of ethyl acetate (EtOAc) was added to each flask, and the suspension was stirred for 3 h. Once extraction was complete, the organic layer was collected. The flask was rinsed once more with 5.0 ml EtOAc, which was added to the previous organic layer. The combined organic fractions were then dried *in vacuo* and the resulting oils were redissolved in 300 µl of EtOAc, transferred to an autosampler vial, and immediately analyzed via LC-MS/MS¹⁶².

LC-MS/MS analysis of AHLs. AHL analysis was performed using an LC-MS/MS method optimized for AHL detection¹⁶² using previously reported methods³⁰⁹ with slight modifications¹⁶². Briefly, each sample was kept in an autosampler (Thermo Electron Surveyor) at 4°C before injection of 10 µl of onto a reverse-phase C18 core-shell column ((Phenomenex Kinetex, Torrance, CA, USA) (5 µm pore size, 100 Å particle size, 100 mm x 2 mm or 2.1 µm pore size, 100 Å particle size, 100 mm x 2 mm) via a Thermo Electron Surveyor autosampler (Thermo Fisher Scientific, Waltham, MA, USA). Separation was obtained using a gradient of 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile at a flow rate of 200 µl/min. The eluent was introduced into a TSQ Quantum Ultra Triple Stage Quadrupole mass spectrometer (Thermo Scientific) using electrospray ionization, and multiple reaction monitoring (MRM) in positive ion mode was used for compound detection, annotation, and identification. This method screened for 54 unique parent *m/z*–fragment *m/z* pairs based on neutral loss of the acyl chain giving common 102 *m/z* fragment corresponding to the common homoserine lactone of all AHLs. The method includes masses corresponding to chain lengths ranging from 4 to 20 carbons with the possibility of one hydroxyl or ketone at the 3-position as well as one double bond in the

chain. Note: MRM alone is unable to differentiate between compounds with the same parent m/z -fragment m/z pairs (e.g., 3OC6-HSL and C7-HSL). To circumvent this, further chemical (such as LC retention time) and biological precedence for the compound in the literature) information was used to annotate structures for AHLs for which standards were not available as previously reported³⁰⁹. Unique parent m/z -fragment m/z pairs were screened to confirm the presence of AHL molecules. An EIC extraction window of 200 ppm was used to envision peaks, and the area for each AHL was integrated, which was further used to detect relative abundance and percentage of each detected AHL. Files collected from the instrument were converted from RAW to.mzML files using Proteowizard's MSconvert algorithm³¹⁰. The files were then imported into MAVEN to detect and report peak intensities and to generate extracted ion chromatograms (EICs) for each MRM³¹¹. An EIC extraction window of 200 ppm was used, and the relative percentage for each detected AHL was calculated from the integrated areas.

LC-MS/MS analysis of DPD. Briefly, duplicate 300 μ l aliquots of culture supernatants were transferred to 1.5 ml centrifuge tubes containing 10 μ l of a ^{13}C -labeled DPD (^{13}C -DPD) internal standard, prepared using a previously reported method³¹². Each tube was mixed thoroughly by vortexing for 5 s and then centrifuged for 1.5 min at 16.1 rcf to pellet cells. The supernatants (260 μ l) were transferred to new 1.5 μ l centrifuge tubes containing 25 μ l of a 5 mg/ml DPD derivatizing solution³¹³. The contents were thoroughly mixed by vortexing and allowed to react for 45 to 60 min. The resulting solutions were then extracted twice with 130 μ l of EtOAc and the combined extracts were transferred to 300 μ l autosampler vials. Samples were placed in an autosampler tray cooled to 4°C and 10 μ l of each was injected onto a Kinetex 5 μ C18 100 Å 100 × 2.10 mm column. Separation was performed with an isocratic gradient of 95% 0.1% acetic acid in HPLC grade water and 5% HPLC grade Acetonitrile with a flow rate of 200 μ l/min for 4 min. The eluent was introduced into a TSQ Quantum Ultra Triple Stage Quadrupole mass spectrometer (Thermo Scientific) using electrospray ionization, and a positive mode selected reaction monitoring protocol was used for detection³¹³. The parent m/z -fragment m/z pairings used for endogenous DPD and ^{13}C -DPD were 381 m/z -202 m/z and 382 m/z -203 m/z , respectively. A collision energy of 43 was used for both. The peaks were automatically integrated with manual adjustments using the Xcalibur software (Thermo Electron Corporation). A correction factor was applied to account for natural isotopes³¹³, and DPD

concentration was then calculated by comparing the peak areas of endogenous DPD to the peak areas of the ^{13}C -DPD and multiplying by the internal standard concentration.

16S rRNA gene sequencing. Genomic DNA was extracted from those isolates that tested positive for AHL production using at least one of the reporter strains assays either by placing a single colony in 100 μl of sterile phosphate buffered saline (PBS), heating to either 99°C in the first study or 95°C in the second study for 10 min, and centrifuging at 5,000 g for 10 min, or alternatively by bead-beating using the FastDNA Spin Kit for Soil (Qbiogene, Vista, CA, USA) according to manufacturer's protocol. DNA was then PCR-amplified using the universal bacterial primers 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R 5'-TAC GGY TAC CTT GTT ACG ACT T-3'³¹⁴; Integrated DNA technologies, Coralville, IA, USA). The final concentration for PCR reactions was 1x PCR Buffer, 2.5 mM MgCl_2 , 0.25 mM of each deoxynucleotide triphosphate, 0.5 μM of each forward and reverse primer, 0.25 U GoTaq Flexi DNA Polymerase (Promega), 0.1% bovine serum albumin, DNA-grade sterile water, and 1–2 ng genomic DNA. PCR was carried out in a Peltier Thermal Cycler (PTC-200, MJ Research, Waltham, MA, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 30 s, with a final soak at 72°C for 20 min.

The amplified bacterial 16S rDNA was cleaned using an ExoSAP-IT PCR cleanup kit (USB Corp., Cleveland, OH, USA) and sequenced with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) at the DNA Core Facility at Florida International University using the BigDye Terminator version 3.1 (Applied Biosystems) with the 27F and 1492R primers. The sequences were trimmed, cleaned, aligned, and assembled using DNA Baser Sequence Assembler (v3.2.5). The nearly full-length sequences (covering the V1–V9 variable regions of the 16S rRNA gene) were then analyzed using the BLAST queuing system³¹⁵ to identify their closest relatives in NCBI GenBank. Sequences analyzed in the first study were submitted to the GenBank database under accession numbers BBD-CUR-3M8 (KF494427), BBD-CUR-3S11 (KF494426) and *Ferrimonas* sp. EF3B-B688 (KC545309). Sequences analyzed in the second study were submitted to GenBank database under the accession numbers KX146440- KX146449 and KX353776-KX353778, and cultures.

Growth challenge assays. To determine whether a subset of BBD and SML isolates produce secondary metabolites that impact the growth of other BBD and SML bacteria, CFs from 19 isolates were used to challenge the growth of the other members of this group of isolates. The 19 isolates consisted of nine that tested positive for AHL in at least one of the *C. violaceum* CV026 and *A. tumefaciens* NTL4(pZLR4) reporter strain assays, and an additional 10 isolates that were randomly selected from the pool of isolates that tested negative in both assays. These isolates were also examined for synthesis of DPD. A total of 342 growth challenges was conducted (i.e., each of the 19 CFs were tested against the 18 other isolate cultures). The bacterial growth challenges were conducted in sterile 96-well microtiter plates (BD Falcon 351172). Each isolate was grown in sterile MB overnight at 29°C (shaking) and added to the appropriate wells. Assay plates contained triplicate experimental wells, control wells, and blank wells, with a single reference well for each CF tested to ensure that the CFs were sterile. The final volume in each well was 100 µL. Plates were incubated at 29°C (shaking) and OD₆₀₀ readings were conducted every 30–60 min using the Modulus Microplate Multimode Reader until the bacterial isolate culture control reached stationary phase. The mean growth rate constant and mean generation time were calculated over the linear portion of the growth curve for the untreated control culture and the experimental treatments. Only statistically significant effects on culture growth will be discussed (t-test, p<0.05).

Metagenomic analysis. Samples of BBD for metagenomics analysis were collected from BBD infected *Colpophyllia natans* (Horseshoe Reef, northern Florida Keys, August 2013) and *Pseudodiploria strigosa* (Curaçao, off shore of the CARMABI research station, May 2013). Samples were collected with sterile 10 ml syringes and placed in RNA-later, frozen at -80° C and sent for whole genome sequencing via Illumina HiSeq to Patrick Gillevet's laboratory at George Mason University, Fairfax, Virginia. We obtained an average of 12 million reads per sample with a read length of 150 base pairs. The metagenomics datasets were uploaded for analysis on the Metagenomics RAST (MG-RAST) server (<http://metagenomics.anl.gov/>). MG-RAST is a public resource for the automatic phylogenetic and functional analysis of metagenomes (Meyer et al. 2008). The presence of QS genes was assessed by using the subsystems with MG-RAST (Max. e-value cutoff= 10⁻⁵, Min. % identity cutoff = 60%, Min. alignment length cutoff = 40 amino 273 acids).

AHL and antimicrobial production detected in the second study. A total of 229 pure cultures of heterotrophic bacteria were isolated from four BBD infected colonies of two

coral species. Of these, 155 were from BBD, 38 were from apparently healthy coral (HSML) and 36 were from the apparently healthy part of BBD infected coral (BSML). Each isolate was tested for both QS signal production, using the *Chromobacterium violaceum* CV026 bioassay, and for antimicrobial activity.

4.4 Results

AHL production identified using reporter strains in the first study. Nine BBD mat (full community) samples, collected from two Caribbean coral species (*Montastraea cavernosa* and *Diploria strigosa*), were examined using both the *C. violaceum* CV026 and *A. tumefaciens* NTL4(pZLR4) patch test assays. Each test was positive, revealing that both short-chain and medium- to long-chain AHLs are present in freshly collected, active BBD mat. A total of 199 bacterial isolates (BBD and SML) were examined using the *C. violaceum* CV026 assay. Of these, four (2.0%) tested positive for the patch test and none for the CF test (**Table 4.4**). A subset of isolates (N=153) was also examined using the *A. tumefaciens* NTL4(pZLR4) assay and of these, nine (5.9%) tested positive for either the patch or CF test (**Table 4.4**).

In total, 11 (5.5%) of the 199 isolates strains were positive for at least one of the two AHL reporter strain assays, and two of these isolates (i.e., BBD-FTL-6j and BBD-FTL-8c) tested positive in both assays. These two isolates were both positive in the patch test with the *C. violaceum* CV026 assay and the CF test with *A. tumefaciens* NTL4(pZLR4), indicating that they produce short-chain AHLs when grown in benthic form (on MA) and medium- to long-chain AHLs when grown in planktonic form (in MB). The two isolates that tested positive for both assays were each a 99% (16S rRNA gene) sequence match in GenBank to one strain of *Vibrio rotiferianus*; however, they were obtained from BBD infections on two separate coral colonies and exhibited different colony morphologies when grown on agar. Five of the 11 isolates tested positive in both the patch and CF assays with *A. tumefaciens* NTL4(pZLR4), indicating that these isolates produced medium- to long-chain AHLs when growing in planktonic or benthic form. Two isolates were positive only for the *A. tumefaciens* NTL4(pZLR4) patch tests. Of the 11 AHL-positive isolates, five were from BBD samples (collected from five separate BBD-infected coral colonies), two from BSML samples, and four from HSML samples. None of the eight BBD cyanobacterial strains tested positive in either AHL reporter stain assay.

Table 4.4. Reporter assay results and sequencing data for the eleven bacterial isolates that tested positive for AHL production.

<i>Chromobacterium violaceum</i> CV026 Assay ^a			<i>Agrobacterium tumefaciens</i> NT14(pZLR4) Assay ^a		GenBank Accession No.	Sequence Length (bp)	Closest Relative in GenBank (% Similarity), Accession No.
BBD and Coral Isolates	Patch test	CF Test	Patch Test	CF Assay			
BBD-FTL-6j	+	-	-	+	KF148598	1393	^b <i>Vibrio rotiferianus</i> strain : LMG 21460 (99%), NR042081
BBD-FTL-8c	+	-	-	+	KF148599	1415	^b <i>Vibrio rotiferianus</i> strain : LMG 21460 (99%), NR042081
BBD-FTL-1d	-	-	+	+	KF148596	1326	<i>Nautella italica</i> Istrain : LMG 24365 (99%), NR042673
BBD-FTL-3MB	+	NT ^c	NT ^d	NT ^d	KF494427	1391	<i>Vibrio harveyi</i> strain : NB0903 (99%), HM008704
BBD-FTL-3S11	+	NT ^c	NT ^d	NT ^d	KF494426	1396	<i>Vibrio harveyi</i> strain : BD1 (99%), KC756840
BSML-FTL-6w	-	-	+	-	KF48595	1326	<i>Ruegeria scottomollicae</i> : LMG 24367 (96%), KC756840
BSML-FTL-7l	ND ^e	-	+	-	KF148600	1385	<i>Pseudoalteromonas phenolica</i> strain O-BC30 (98%), NR028809
HSML-FTL-9c	-	-	+	+	KF148601	1414	<i>Vibrio harveyi</i> strain NCIMB 1280 (99%), NR043165
HSML-FTL-9e	-	-	+	+	KF148602	1398	<i>Aliagarivorans marinus</i> strain AAM1 (95%), NR044585
HSML-FTL-9i	-	-	+	+	KF148603	1345	<i>Pseudoruegeria aquimaris</i> strain SW-255 (96%), NR043932
HSML-FTL-10a	ND ^e	-	+	+	KF148597	1387	<i>Pseudoalteromonas luteoviolacea</i>

"+" indicates a positive assay result and a "-" indicates a negative result^a. Although BBD-FTL-6j and BBD-FTL-8c shared the same closest relative in GenBank, these isolates showed differing colony morphologies and were isolated from two different BBD samples on three separate coral colonies^b. Culture fluids of isolates not tested with the *C. violaceum* CV026 reporter strain^c. Isolates not tested with the *A. tumefaciens* NT14(pZLR4) reporter strain^d. Not detectable. These isolates inhibited growth of the *C. violaceum* CV026 reporter strain in the patch tests, eliciting an area of clear, cell-free agar in the assay plate surrounding the bacterial patch. Additionally, isolate HSML-FTL-10a produced a dark purple pigment^e.

AI-2 Production identified using reporter strains from the first study. More than half (84, or 55%) of the 153 CFs tested positive in the AI-2 reporter strain assay (**Table 4.5**). **Figure 4.2** shows results of a representative experiment using the CFs of four isolates, including two that tested positive (BSML-FTL-7m and BSML-FTL-7q) and two that tested negative (BSML-FTL-7h and BSML-FTL-7k). The positive results indicate that isolates produced DPD-like molecules (or a CAI-1 signal specific to certain *Vibrio* spp.) that can be detected by the *V. harveyi* BB170 reporter strain used in this study.

The AI-2 activity induced by CFs from the positive isolates ranged widely, from approximately 28% to 346% of the positive control luminescence (**Table 4.6**) with the triplicate readings within 30% of the mean luminescence values, expressed in relative light units (rlu). The induction of luminescence over the positive control was calculated for each CF tested and ranged from 0.22–2.8 fold. 70% of the BBD CFs, 68% of the BSML CFs and 33% of the HSML CFs tested positive for AI-2 production (**Table 4.5**). None of the cyanobacterial CFs tested positive in the AI-2 assay.

The media used in this study did not in any case stimulate light production in the AI-2 assay prior to self-induction by the *V. harveyi* BB170 reporter strain (**Figure 4.3**). The slight rise in luminescence near the end of the experiment is likely the result of light contamination from adjacent wells (**Figure 4.3**). The medium control curves indicate that the three growth media used in this study did not stimulate light production prior to self-induction by the *Vibrio harveyi* BB170 reporter strain. The luminescence of the medium reference wells, which contained sterile growth media, remained minimal over the course of the AI-2 reporter assay, although some minor increases in light were measured in these wells at the end of the assay due to light contamination from adjacent wells. Arrow indicates time of self-induction by the reporter strain, after which the luminescence of the reporter strain increases rapidly.

Table 4.5. Autoinducer-2 activity detected using the *Vibrio harveyi* BB170 reporter strain presented according to isolate type.

Isolate Type	No. CFs Tested	No. and % Positive CFs	Percentage of Total Positive Results
BBD	63	44 (70%)	52%
BSML	37	25 (68%)	30%
HLML	45	15 (33%)	18%
Cyanobacteria	8	0	0%
Totals	153	84 (55%)	100%

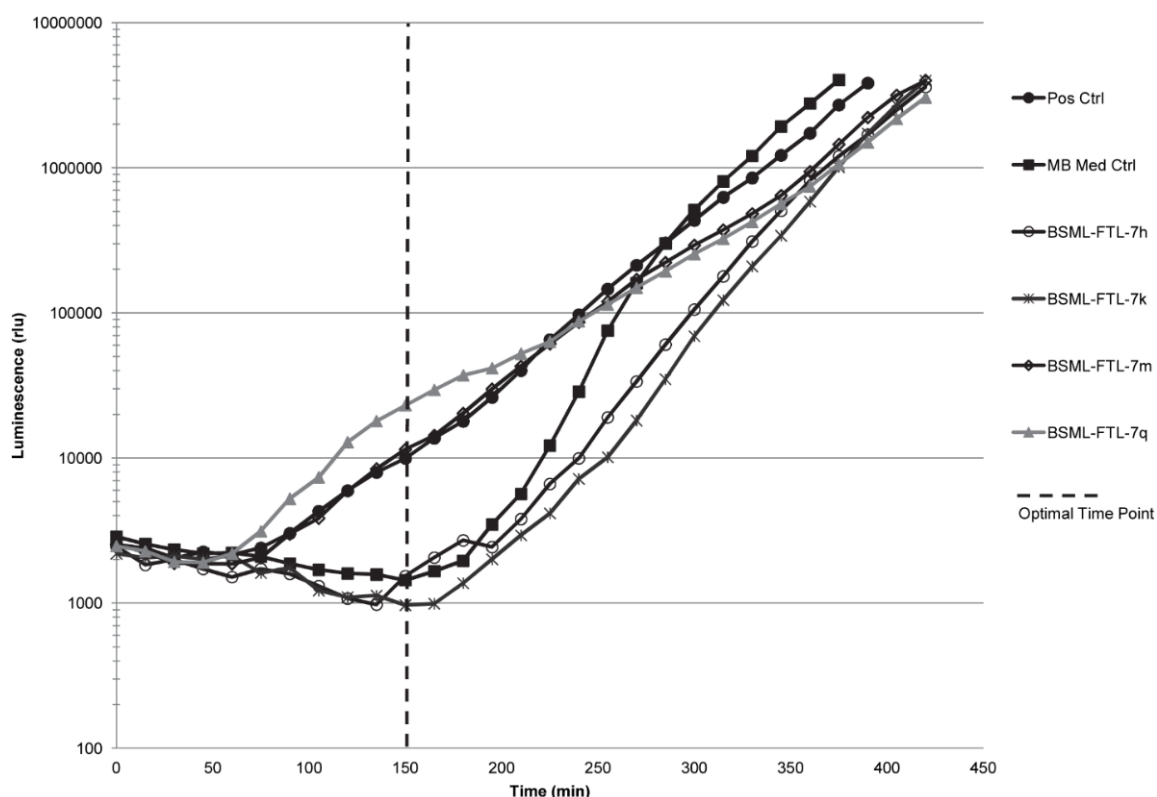


Figure 4.2. Representative experiment showing light production over time in the AI-2 reporter strain assay. The curves generated by the cell-free culture filtrates (CFs) from BSML-FTL-7m and BSML-FTL-7q (both positive in the assay) follow the positive control curve. The curves generated by the CFs from BSML-FTL-7h and BSML-FTL-7k (both negative in the assay) follow the Marine Broth (MB) medium control curve. Dashed vertical line indicates the optimal time point (OTP) of the assay, which is the time point immediately preceding self-induction by the *V. harveyi* BB170 reporter strain and the time when the mean luminescence of the medium control wells was lowest during the course of the assay. Data at the OTP is used to calculate the percentage of AI-2 (% AI-2) activity and the induction of luminescence over the positive control.

Table 4.6. Percentage of AI-2 activity ($\pm$ standard deviation) and induction of luminescence values for the 84 CFs that tested positive in the AI-2 assay.

Isolate CF	% AI-2 Activity ($\pm$ SD)	Induction of Luminescence	Isolate CF	% AI-2 Activity ($\pm$ SD)	Induction of Luminescence	Isolate CF	% AI-2 Activity ($\pm$ SD)	Induction of Luminescence
BBD-216-1b	345.84 $\pm$ 21.6	2.7	BBD-FTL-6c	120.89 $\pm$ 21.4	0.94	BSML-FLK-1h	65.307 $\pm$ 6.42	0.65
BBD-FTL-6j	275.70 $\pm$ 50.3	2.15	BSML-FLK-1i	117.61 $\pm$ 11.8	1.17	BSML-FLK-1a	65.153 $\pm$ 2.62	0.65
HSML-FTL-11b	275.28 $\pm$ 8.36	2.81	BBD-FTL-1g	116.64 $\pm$ 4.84	1.4	BSML-FLK-1l	63.282 $\pm$ 2.79	0.63
BBD-FLK-1h	271.58 $\pm$ 53.2	2.12	BSML-FTL-7m	115.50 $\pm$ 20.8	1.15	BSML-FTL-8b	60.889 $\pm$ 10.0	0.61
BBD-FLK-1k	260.04 $\pm$ 31.1	2.03	BSML-FLK-1k	115.34 $\pm$ 11.2	1.15	BBD-FTL-5f	60.073 $\pm$ 11.6	0.58
HSML-FTL-10bb	256.29 $\pm$ 14.8	2.62	BBD-FTL-6m	111.42 $\pm$ 11.3	1.07	BSML-FLK-1m	59.759 $\pm$ 9.66	0.6
BSML-FTL-7q	232.11 $\pm$ 12.8	2.32	BBD-FTL-6b	111.03 $\pm$ 27.9	0.87	BBD-FTL-8c	57.091 $\pm$ 3.90	0.68
BSML-FLK-1c	226.54 $\pm$ 12.2	2.26	BBD-216-2d	110.58 $\pm$ 7.23	1.07	BBD-FTL-7a	55.744 $\pm$ 4.52	0.54
HSML-FTL-9f	213.76 $\pm$ 16.1	1.7	BBD-FLK-1n	109.27 $\pm$ 15.5	0.85	BBD-FTL-4b	54.912 $\pm$ 5.43	0.43
HSML-FTL-9d	200.67 $\pm$ 11.9	1.6	BSML-FTL-7d	108.65 $\pm$ 19.7	1.3	BBD-FTL-6k	51.745 $\pm$ 2.51	0.5
HSML-FTL-11i	191.23 $\pm$ 10.6	1.96	BBD-FLK-1l	108.07 $\pm$ 13.1	0.84	BBD-FLK-1j	49.431 $\pm$ 9.76	0.39
HSML-FTL-10z	188.15 $\pm$ 5.13	1.92	BBD-FLK-1c	101.00 $\pm$ 6.31	0.79	BSML-FTL-6w	48.672 $\pm$ 6.20	0.47
HSML-FTL-10aa	173.52 $\pm$ 12.7	1.77	BBD-216-4e	99.694 $\pm$ 8.49	0.96	BBD-217-2d	47.873 $\pm$ 6.88	0.46
BBD-FLK-1b	173.11 $\pm$ 26.4	1.35	BSML-FTL-7o	97.875 $\pm$ 5.70	0.98	BBD-FLK-1e	45.179 $\pm$ 1.91	0.44
BBD-FTL-6g	167.57 $\pm$ 16.5	1.31	BBD-FTL-6p	95.795 $\pm$ 8.34	0.92	BBD-216-4a	42.636 $\pm$ 4.45	0.51
HSML-FTL-9m	156.74 $\pm$ 7.91	1.25	BSML-FTL-6u	95.241 $\pm$ 7.32	1.14	BBD-FTL-1m	42.118 $\pm$ 0.30	0.5
BBD-FLK-1m	148.82 $\pm$ 22.8	1.16	BBD-FTL-6n	90.130 $\pm$ 11.8	0.87	HSML-FTL-10r	39.907 $\pm$ 4.77	0.32
HSML-FTL-9a	146.16 $\pm$ 11.0	1.16	BSML-FLK-1o	86.974 $\pm$ 6.15	0.87	BBD-FTL-7b	39.287 $\pm$ 1.19	0.38
HSML-FTL-9b	144.32 $\pm$ 14.4	1.15	BSML-FLK-1n	83.783 $\pm$ 5.23	0.84	BBD-FTL-3h	37.647 $\pm$ 2.27	0.36
BSML-FTL-7e	143.00 $\pm$ 17.9	1.71	BBD-216-3d	83.109 $\pm$ 1.18	0.8	BBD-FTL-1h	37.159 $\pm$ 6.77	0.44
BBD-FTL-3b	140.38 $\pm$ 4.35	1.1	BSML-FLK-1b	82.627 $\pm$ 13.8	0.82	HSML-FTL-11l	36.328 $\pm$ 5.64	0.37
BSML-FLK-1e	132.75 $\pm$ 7.79	1.32	BBD-FTL-1f	79.148 $\pm$ 19.5	0.95	BBD-FTL-1e	35.451 $\pm$ 2.36	0.42
BBD-FTL-8b	127.81 $\pm$ 9.08	1.53	BBD-217-2b	74.398 $\pm$ 19.0	0.89	BSML-FLK-1f	35.182 $\pm$ 8.99	0.35
HSML-FTL-9j	127.21 $\pm$ 8.97	1.01	BSML-FTL-6r	74.102 $\pm$ 6.47	0.89	HSML-FTL-9i	33.598 $\pm$ 3.87	0.27
BBD-FTL-3j	122.89 $\pm$ 5.15	1.18	BSML-FLK-1d	73.342 $\pm$ 3.42	0.73	BBD-FTL-6q	32.479 $\pm$ 1.54	0.31
BBD-FTL-1j	122.04 $\pm$ 3.40	1.46	BSML-FTL-7n	71.458 $\pm$ 14.4	0.71	HSML-FTL-9c	31.191 $\pm$ 5.50	0.25
BSML-FTL-8c	121.85 $\pm$ 6.18	1.22	BBD-FTL-6o	66.858 $\pm$ 5.12	0.64	BBD-FTL-6l	30.549 $\pm$ 2.75	0.29
BSML-FTL-8a	121.47 $\pm$ 4.81	1.21	BBD-FTL-1b	65.542 $\pm$ 2.36	0.63	BBD-FTL-6h	28.236 $\pm$ 3.21	0.22

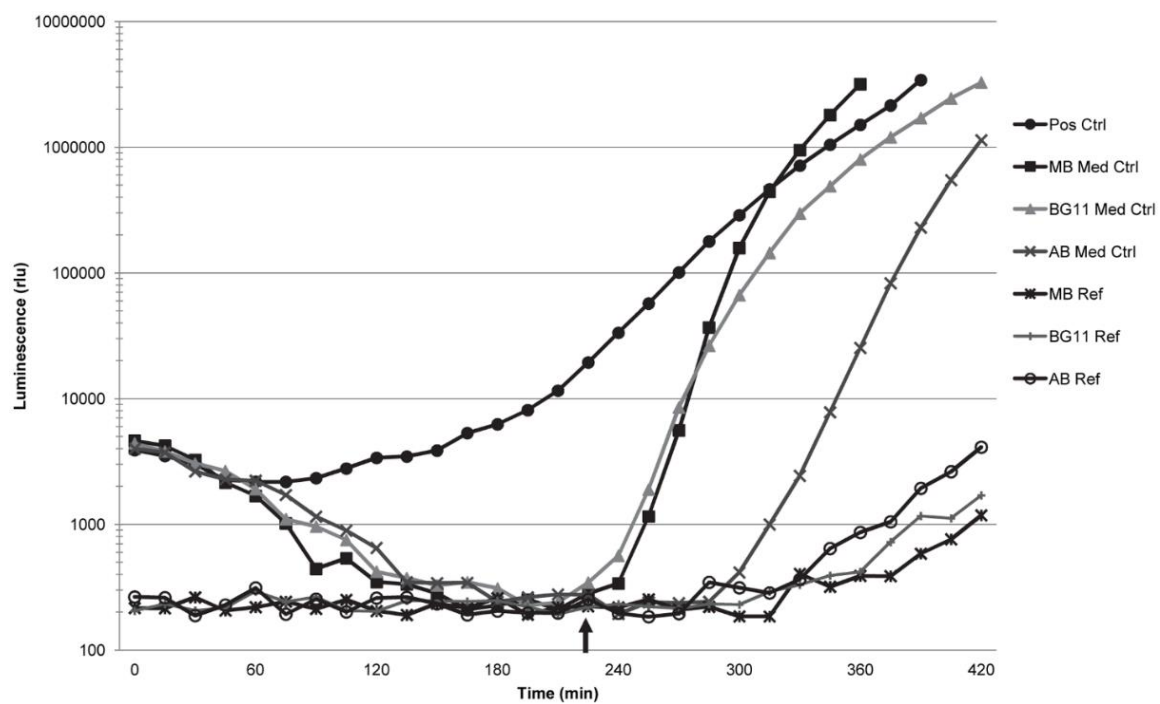


Figure 4.3. Light production of the media control wells and media reference wells in the AI-2 reporter assay.

Among the 229 isolates, 27 tested positive in the QS assay, consisting of 23 BBD isolates and two each from HSML and BSML. HSML and BSML isolates had similar percentages of isolates that tested positive for QS in the CV026 assay, 5.3% (2 out of 38) and 5.6% (2 out of 36) respectively. The percentage of QS positive isolates from BBD in comparison was approximately three times higher at 14.8% (24 of 155). When comparing QS producing isolates between BBD vs. HSML and BBD vs. BSML, a significant ($p \leq 0.05$) difference in proportions was detected. The isolates from the different health states were also tested for antimicrobial activity against two ecologically relevant test strains, *Roseovarius crassostreae* CV919-312 and *Ferrimonas* sp. EF3B-B688 (**Tables 4.7 and 4.8**). In the case of *R. crassostreae* CV919-312, a higher percentage of BSML (13.9%) isolates caused inhibition in comparison to the HSML (10.5%) and BBD (9.0%) isolates. In contrast, the HSML isolates had the highest percentage that inhibited *Ferrimonas* sp. EF3B-B688 (23.7%), followed by 293 5.6% of BSML isolates and 2.6% of BBD isolates. 23.7% isolates from HSML and 19.4% isolates from BSML also inhibited *C. violaceum* CV026. When viewing the isolates as a whole, of the 229 that were tested 23 (14.8%) inhibited *R. crassostreae* CV919-312, 15 (6.6%) inhibited the growth of *Ferrimonas* sp. EF3B-B688, and 16 (6.9%) inhibited the QS reporter strain.

Varieties and relative abundance of AHLs in the first study. With one exception (BSML-FTL-7I), each of the 11 isolates that were examined for identification of AHLs by LC-MS/MS produced AHLs under both planktonic (MB) and benthic (MA) conditions (**Table 4.9**), a result that is in contrast to the variable results obtained using the reporter strains. Note that of these 11 strains, 10 were discussed previously in terms of reporter strain results (**Table 4.4**).

One strain (HSML-FTL-10a) was not analyzed by LC-MS/MS because this strain failed to grow at the time of the LC-MS/MS analysis. Also, note the inclusion of an additional isolate (BBD-FLK-1M2) not previously discussed (**Table 4.9**). Though this isolate tested negative for AHL production using the reporter assays, it is the subject of current physiological studies of BBD bacteria (data not included here), and thus was included in the LC-MS/MS analysis. The most common AHL variant produced among the 11 isolates was C6-HSL, produced by five (45%) of the strains in liquid culture and seven (64%) of the strains grown on plates (**Table 4.9**). This signal accounted for 50–92% of the AHLs produced (per strain) and was produced by all of the AHL-positive strains isolated from BBD and two strains from the SML

Table 4.7. Sampling dates and locations and bacterial isolates used in this study.

Isoaltes	AHLs Detected in Liquid Culture ^{a,c,d}	AHLs Detected on Agar ^{b,c,d}	DPD Conc (μM) ^{1,5}
BBD-FTL-6j	C6 (78%), 3OC4 (13%), 3 others (9%)	C6 (74%), 3OC4 (11%), 3 others	0.99±0.32
BBD-FTL-8c	C6 (70%), 3OC4 (11%), 6 others (9%)	C6 (50%), 3OC6 (21%), C4 (13%), 4 others (16%)	0.94±0.09
BBD-FTL-1d	C6 (69%), 3OHC10 (9%), 9 others (22%)	C6 (61%), 7 others (39%)	1.08±0.28
BBD-FTL-3MB	C6 (92%), 3 others (8%)	C6 (69%), COC4 (31%)	1.10±0.49
BBD-FTL-3S11	C6 (86%), 3 others (14%)	C6 (80%), 3 others (20%)	1.38±0.19
BSML-FTL-6w	C14 (45%), C10 (26%), 3OHC4 (23%), 3OC20 (5%)	C10 (39%), 3OHC8 (23%), 3OC4 (18%), 3others (20%)	0.32±0.13
BSML-FTL-7l	26 AHLs measured (ranging from 0.6-14%)	ND ^f	0.48±0.09
HSML-FTL-9c	3OC4 (52%), C14(31%), 3OC18:1 (12%), 3OC20 (5%)	3OHC9:1 (79%), 3OHC4 (21%)	1.41±0.16
HSML-FTL-9e	3OHC10 (75%), 7 others (15%)	C6 (65%), 3OHC10 (22%), 4 others (13%)	0.37±0.02
HSML-FTL-9i	3OHC10 (70%), 3OHC18 (14%), 2 others (16%)	C6 (51%), 3OHC10 (36%), 3OC4:1 (14%)	0.31±0.08
HSML-FTL-1M2 ^g	3OC6 (29%), 3OC18:1 (19%), C14 (19%), 3OHC10 (12%), 7 others (21%)	3OHC10 (100%)	1.19±0.12

BBD = black band disease mat sample, HSML= sample taken from apparently healthy coral of the same host species, BSML= sample taken from the apparently healthy part of the BBD infected coral.

Table 4.8. Isolates from the three coral health states that were positive for QS signal production tested with the *C. violaceum* CV026 biosensor strain, and antimicrobial activity against *R. crassostreae* CV919-312, *Ferrimonas* sp. EF3B-B688, and *C. violaceum* CV026.

Isolate Source	No. of isolates positive in CV026 QS assay	No. of isolates inhibiting <i>R. crassostreae</i> CV919-312	No. of isolates inhibiting <i>Ferrimonas</i> sp. EF3B-B688	No. of isolates inhibiting <i>C. violaceum</i> CV026	Total no. of isolates tested
HSML	2 (5.3%)	4 (10.5%)	9 (23.7%)	9 (23.7%)	38
BSML	2 (5.6%)	5 (13.9%)	2 (5.6%)	7 (19.4%)	36
BBD	23 (14.8%)	14 (9.0%)	4 (2.6%)	0 (0%)	155
Total	27 (11.8%)	23 (14.8%)	15 (6.6%)	16.9 (6.9%)	229

Table 4.9. AHLs detected via LC-MS/MS after 24 hour incubation.

Location	Date of sample collection	Coral host	Sample type	No. of isolates
Horseshoe Reef, Florida Keys, FL, USA (N 25° 08.362' W 80° 17.641')	9/30/2012	<i>Pseudodiploria strigosa</i>	BBD	8
Water Factory, Curacao, Netherlands Antilles (N 12° 06.779' W 68° 57.662')	2/23/2013	<i>P. strigosa</i>	BBD	44
Water Factory, Curacao, Netherlands Antilles (N 12° 06.779' W 68° 57.662')	5/25/2013	<i>P. strigosa</i>	BBD	67
Algae Reef, Florida Keys, FL, USA (N 25° 08.799' W 80° 17.579')	8/19/2013	<i>Montastraea cavernosa</i>	BBD, HSML, BSML	36, 38, 36
Total number of isolates isolated and tested				229

Results are an average of four samples (two cultures sampled twice)^a. Results are an average of six samples (two cultures injected twice)^b. Percentages represent relative abundance within the culture^c. AHLs are comprised of a homoserine lactone ring attached to an acyl side chain (generally between 4-20 carbons in length) and may have a keto or hydroxy substituent at the C3-position. Abbreviations: CX - AHL contains "X" carbon molecules in the acyl chain; 3OCX-AHL has a keto substituent at the C3-position; 3OHCX-AHL has a hydroxyl substituent at the C3-position^d. Error is reported substituent at the C3-position^e. Not detected^f. Isolate BBD-FLK-1M2 was identified through 16S rRNA gene sequencing as a 100% match to *Ferrimonas* sp. EF3B-B688 (Accession No. KC54309.1). This isolate tested negative in the *Chromobacterium violaceum* CV026 patch test and was not tested using the *Agrobacterium tumefaciens* NTL4(pZLR4) assay^g.

of (two) apparently healthy corals (HSML samples). The second most common AHL was 3OC4-HSL, detected in five (45%) of the strains (isolated from 3 BBD, 1 BSML, and 1 HSML sample). This variant accounted for a maximum of 52% of AHL detected for strain HSML-FTL-9c (range of 11–52%). Additional AHLs were produced by fewer strains, but in some cases were the majority of the variant detected (e.g., for strains HSML-FTL-9e and HSML-FTL-9i grown in broth culture, 3OHC10-HSL accounted for 75% and 70% of AHLs produced, respectively). Other AHLs were produced in smaller amounts (see **Table 4.9**), including 3OC20-HSL which constituted 5% of AHLs produced by two isolates. To our knowledge, this AHL has not been reported in the literature.

DPD Quantitation from the first study. LC-MS/MS was also used to assess the presence and concentration of DPD produced by the 11 isolates discussed above. All of the isolates produced DPD at concentrations that ranged from 0.31 to 1.41 μM (**Table 4.9**). All six of the BBD isolates produced high amounts of DPD (average of 1.11 μM) while, with one exception, the SML isolates (both HSML and BSML) produced much less (approximately one third to one half of the amount produced by BBD isolates). The anomalous isolate (HSML-FTL-9c), which produced the highest concentration of DPD detected for any strain independent of source, was a 99% match to *Vibrio harveyi*, a well-known AI-2 signaling species. The other five isolates produced an average of 0.37 μM DPD.

Variety and relative abundance of AHLs across different temperatures from the second study. A subset of isolates (23 of the 27 that tested positive for AHL signal production), 298 plus the 299 ATCC culture of *R. crassostreae*, were investigated further (**Tables 4.10 and 4.11**). Data are reported for only one of the two BSML isolates that tested positive in the *C. violaceum* assay since the second did not grow during the additional experiments. The isolates tested were identified by 16S rRNA gene sequencing (**Table 4.10**) and analyzed using LC-MS/MS to determine the identity and relative abundance of specific AHLs (**Table 4.11**). Nearly all the isolates were found to be *Vibrio* species. It should also be noted that several *Vibrio harveyi* and *Vibrio* spp. are different strains (**Table 4.11**). One isolate, *Pseudoalteromonas ruthenica* strain D2-5M, did not yield any identifiable AHLs although this strain was positive in the QS reporter assay. The identity and relative abundance of AHLs produced by the subset of isolates were across three temperatures (24 °C, 27 °C and 30 °C) across six time points ($t = 0, 2, 4, 6, 8$, and 10

Table 4.10. Identification of AHL producing isolates used in this study based on 16S rRNA gene sequencing.

Strain no.	Isolate	Source	Genbank Accession Number	Closest relative in Genbank	Percentage Similarity of relative	Accession number of closest relative in GenBank
1	BBD-CUR-3M8	BBD	KF494427	<i>Vibrio harveyi</i> strain NB0903	99%	HM008704
2	BBD-CUR-3S11	BBD	KF494426	<i>Vibrio rotiferianus</i> strain DBI	99%	KC756840
3	3-BBD-S26	BBD	KX353778	Uncultured proteobacterium clone ATIntfc2_RS175	99%	KM018571
4	4-BBD-S11	BBD	KX353777	<i>Vibrio</i> sp. PaD3.29b	100%	GQ406662
5	5-BBD-S1	BBD	KX353776	<i>Vibrio harveyi</i> strain QH141026D	99%	KU245732
6	5-BBD-S5	BBD	NA	NA	NA	NA
7	5-BBD-S8	BBD	KX146443	<i>Vibrio harveyi</i> strain X11XC19	99%	KM881533
8	5-BBD-S11	BBD	KX146444	<i>Vibrio</i> sp. PMS13	99%	EU372931
9	5-BBD-S21	BBD	NA	NA	NA	NA
10	5-BBD-S22	BBD	KX146442	<i>Vibrio harveyi</i> strain QH141026D	99%	KU245732
11	5-BBD-S24	BBD	NA	NA	NA	NA
12	5-BBD-M2	BBD	KX146440	<i>Vibrio harveyi</i> strain QH141026D	99%	
13	5-BBD-M5	BBD	NA	NA	NA	NA
14	5-BBD-M6	BBD	KX146441	<i>Vibrio harveyi</i> strain QH141026D	99%	KU245732
15	5-BBD-M10	BBD	KX146445	<i>Vibrio harveyi</i> strain bb4	99%	KC455398
16	5-BBD-M12	BBD	KX146446	<i>Vibrio</i> sp. sw0106-05	100%	KT276382
17	4-BBD-M29	BBD	KX146447	<i>Pseudoalteromonas ruthenica</i> strain D2-5M	99%	AB617562
18	<i>Roseovarius crassostreae</i> CV919-312	ATCC	NA	NA	NA	NA
19	<i>Ferrimonas</i> sp. EF3B-B688	BBD	KC545309	NA	NA	NA
20	4-BBD-M13	BBD	NA	NA	NA	NA
21	5-BBD-M4	BBD	KX146448	<i>Vibrio tubiashii</i> strain T33	99%	KP329558
22	5-BBD-S17	BSML	KX146449	<i>Vibrio</i> sp. PaH2.06c	99%	GQ406738
23	5-BBD-S16	HSML	NA	NA	NA	NA
24	5-BBD-S1	HSML	NA	NA	NA	NA

ATCC = American Type Culture Collection. 'M' in isolate designation refers to culture isolated using marine agar medium; 'S' in isolate designation refers to culture isolated using sea water tryptone agar medium. NA = not applicable, "-" = cultures that failed to grow for 16S rRNA gene identification.

Table 4.11. Percentages of AHLs produced by each isolate across three temperatures and six time points (pooled and averaging replicate sample values) as analyzed by LC-MS/MS.

Strain no.	Designation of isolate	Source of isolate	3OHC4	C5	3OHC5	3OC5:1	C6	3OHC6	3OHC8	3OC12
1	BBD-CUR-3M8	BBD	5.10%	3.40%	-	-	91.50%	-	-	-
2	BBD-CUR-3S11	BBD	-	-	-	-	100%	-	-	-
3	3-S26	BBD	-	1.90%	10.20%	-	87.90%	-	-	-
4	4-S11	BBD	-	-	-	-	100%	-	-	-
5	5-BBD-S1	BBD	2.60%	-	-	-	97.40%	-	-	-
6	5-BBD-S5	BBD	-	1.50%	-	2.70%	95.80%	-	-	-
7	5-BBD-S8	BBD	-	-	-	-	100%	-	-	-
8	5-BBD-S11	BBD	98.70%	-	1.30%	-	-	-	-	-
9	5-BBD-S21	BBD	6.50%	-	-	-	93.50%	-	-	-
10	5-BBD-S22	BBD	96.40%	-	3.60%	-	-	-	-	-
11	5-BBD-S24	BBD	98.30%	-	1.70%	-	-	-	-	-
12	5-BBD-M2	BBD	45.40%	-	-	-	54.60%	-	-	-
13	5-BBD-M5	BBD	37.90%	-	-	-	62.10%	-	-	-
14	5-BBD-M6	BBD	98.60%	-	1.40%	-	-	-	-	-
15	5-BBD-M10	BBD	98.60%	-	1.40%	-	-	-	-	-
16	5-BBD-M12	BBD	31.20%	-	-	-	-	68.80%	-	-
17	4-BBD-M29	BBD	No AHL detected							
18	Roseovarius crassostreae CV919-312	ATCC	-	-	-	-	100%	-	-	-
19	Ferrimonas sp. EF3B-B688	BBD	87.60%	-	-	-	12.40%	-	-	-
20	4-BBD-M13	BBD	94.50%	-	2.70%	-	2.80%	-	-	-
21	5-BBD-M4	BBD	66.70%	-	-	-	-	32.00%	1.30%	-
22	5-BSML-S17	BSML	99.20%	-	-	-	-	0.80%	-	-
23	5-HSML-S16	HSML	12.90%	-	-	-	-	78.90%	-	8.20%
24	5-HSML-S1	HSML	75.70%	-	-	-	-	3.80%	-	20.50%
Number of times the corresponding AHL is present (out of 24)			17	3	7	1	13	5	1	2

hours). Data are reported in amounts of relative abundance of AHLs per isolate (**Table 4.11**). 3OHC4 was found to be produced by the highest number of isolates (17 out of 24), followed by C6 (13 out of 24), 3OHC5 (6 out of 24) and 3OHC6 (5 out of 24). Comparing relative quantities (per isolate), the production of 3OHC4 was approximately 10-fold that of the next most abundantly produced AHL, C6 (data not shown), followed by 3OHC5 and 3OHC6 (produced by seven and five isolates respectively). C5 was produced by three isolates. The longest chain AHL (3OC12) detected in this study was produced by two isolates, both from HSML. 3OC5:1 and 3OHC8 were the rarest, each with one isolate producing it in combination with other AHLs. Of the 24 isolates, four isolates produced only one AHL, identified as C6 in all cases (**Table 4.11**). Twelve isolates produced two AHLs and seven isolates produced three AHLs. The two HSML isolates produced the same three AHLs (3OHC4, 3OHC6 and 3OC12) but in varying percentages. Most of the AHL production by the sole BSML isolate tested was contributed by 3OHC4 (99.2%). At each sampling, the OD for each isolate was measured to assess any correlation between AHL production and the stage of growth of that isolate. The growth curves were found to be standard across all isolates and there was no correlation between AHL production and growth (data not shown).

Table 4.12 compiles statistically significant results from an ANOVA multivariate analysis of all isolates that produced a particular AHL together with results of ANOVA repeated measure analyzing all isolates individually for each AHL at three different temperatures. For both of these statistical tests, AHL production values across all time-points were taken together across all replicates ($p \leq 0.05$). Twelve of the 24 isolates tested exhibited significant differences in AHL production in terms of temperature, and of these 12, ten were isolated from BBD and were vibrios. Isolates with non-significant differences in AHL production across temperature, and temperature comparisons that were non-significant for a particular AHL, are not shown (**Table 4.12**). AHLs 3OHC4, 3OHC5 and 3OHC6 had significant differences across all three temperature comparisons for some of the isolates, while 3OC5:1 had a significant difference only when comparing temperatures 24 °C vs. 27 °C (**Table 4.12**). However, it is noteworthy that these results pertained to only one isolate that produced this AHL (isolate no. 6).

Table 4.12. Results of ANOVA repeated measures analysis of all isolates individually for AHL production, and ANOVA multivariate analysis comparing each AHL individually across all isolates.

AHL	Temperatures compared	Isolate ID												Overall significance across isolates
		1	6	8	10	11	14	15	16	20	21	23	24	
3OHC4	24 °C vs. 27 °C	0.05	-	NS	NS	NS	NS	NS	0.001	NS	NS	NS	0.014	0.017
	27 °C vs. 30 °C	NS	-	NS	NS	NS	NS	NS	0	NS	0.045	0.001	0	0.017
	24 °C vs. 30 °C	NS	-	NS	NS	NS	NS	NS	NS	NS	NS	0.001	NS	0
	24 °C vs. 27 °C	-	-	NS	NS	NS	0.012	0.036	-	0	-	-	-	0
3OHC5	24 °C vs. 27 °C	-	-	NS	NS	NS	0.002	0.008	-	NS	-	-	-	0
	27 °C vs. 30 °C	-	-	NS	NS	NS	0.002	0.008	-	NS	-	-	-	0
	24 °C vs. 30 °C	-	-	0.001	0.002	0	0	0	-	0	-	-	-	0
	24 °C vs. 27 °C	NS	NS	-	-	-	-	-	-	0	-	-	-	NS
C6	24 °C vs. 27 °C	NS	NS	-	-	-	-	-	-	0	-	-	-	NS
	27 °C vs. 30 °C	NS	NS	-	-	-	-	-	-	0	-	-	-	NS
	24 °C vs. 27 °C	-	-	-	-	-	-	-	0.01	-	0.001	0	NS	0
	27 °C vs. 30 °C	-	-	-	-	-	-	-	NS	-	0.004	0.02	NS	0.011
3OHC6	24 °C vs. 27 °C	-	-	-	-	-	-	-	NS	-	NS	0	NS	0.006
	27 °C vs. 30 °C	-	-	-	-	-	-	-	NS	-	NS	0	NS	0.006
	24 °C vs. 30 °C	-	-	-	-	-	-	-	NS	-	NS	0	NS	0.006
	24 °C vs. 27 °C	-	0.011	-	-	-	-	-	-	-	-	-	-	0.011

All time-points were pooled across all replicates ($p \leq 0.05$). Comparisons of AHL quantitation were done in pairs (24 °C vs. 27 °C, 27 °C vs. 30 °C, 24 °C vs. 30 °C). “-” = absence of AHL production, NS = non-significant AHL production, *0.000 indicates that the p values were very small or very significant. Only data for AHLs that yielded a significant difference per temperature comparison are shown.

Table 4.13 shows relative AHL production among isolates (all time-points pooled) when comparing production at 24 °C vs 30 °C (below and above the temperature threshold of BBD on the reef). Only data that were significant ($p \leq 0.05$) are shown, representing seven of the 24 tested. Of these seven, six were from BBD (all vibrios) and one from HSML (isolate no. 23). In each case AHL production was relatively higher at 30 °C when compared to 24 °C. This elevated production above the BBD temperature threshold was seen for four AHLs, with 3OHC5 by far the most common.

Table 4.13. Comparison of AHL production by isolates at 24 °C vs 30 °C.

3OHC4		C5		3OHC5		3OC5:1		C6		3OHC6		3OHC8		3OC12	
24 °C	30 °C	24 °C	30 °C	24 °C	30 °C	24 °C	30 °C	24 °C	30 °C	24 °C	30 °C	24 °C	30 °C	24 °C	30 °C
NS	NS	-	-	Lower	Higher	-	-	-	-	-	-	-	-	-	-
NS	NS	-	-	Lower	Higher	-	-	-	-	-	-	-	-	-	-
NS	NS	-	-	Lower	Higher	-	-	-	-	-	-	-	-	-	-
NS	NS	-	-	Lower	Higher	-	-	-	-	-	-	-	-	-	-
NS	NS	-	-	Lower	Higher	-	-	-	-	-	-	-	-	-	-
NS	NS	-	-	Lower	Higher	-	-	Lower	Higher	-	-	-	-	-	-
Lower	Higher	-	-	-	-	-	-	-	-	Lower	Higher	-	-	NS	NS

Only results that were statistically significant ($p < 0.05$) are presented. “-” = absence of AHL production, NS = non-significant AHL production.

Comparison of AHLs across three coral health states from the second study.

BBD isolates, in addition to constituting the sample group with the highest number of QS producing isolates (**Table 4.8**), produced the largest variety of AHLs when compared to isolates from the other two coral health states (**Table 4.14**). AHLs 3OHC4 and 3OHC6 were produced by isolates from all three health states. These AHLs were also two of the four that varied significantly in terms of temperature (**Table 4.9**) and, as noted above, 3OHC4 was the most commonly produced AHL among all isolates (**Table 4.14**).

Table 4.14. Comparison of AHLs produced by isolates in terms of isolate origin across three coral health states.

Isolate Source		
BBD infected coral (BBD) (n=20)	SML of healthy coral (HSML) (n=2)	SML from healthy part of BBD infected coral (BSML) (n=1)
3OHC4	3OHC4	3OHC4
3OHC6	3OHC6	3OHC6
C5	3OC12	
3OHC5		
3OC5:1		
C6		
3OHC8		

Growth Challenge Assays. Of the 342 growth challenges, 92 (27%) resulted in significant (see methods) inhibition and eight (2.3%) resulted in significant stimulation of isolate growth (**Table 4.15**). When considered by isolate source and the number of isolates tested per source, the CFs of HSML isolates inhibited the most growth at 32% of challenges (29 of 90 tests), followed by the CFs of BSML isolates (26%, or 28 of 108), and CFs of BBD isolates (24%, or 35 of 144). Growth stimulation by CFs occurred for approximately 4% of challenges with BSML isolates, 2% of BBD isolates, and 1% of HSML isolates (**Table 4.15**). Independent group t-tests revealed that there was no significant difference ($p>0.05$) between the numbers of cultures inhibited or stimulated by CFs from any combination of the three isolate types. The 19 isolates examined in the growth challenges included nine AHL-positive isolates (**Table 4.15**). Five of these nine also tested positive for AI-2 activity using the *V. harveyi* BB170 reporter strain assay, and eight produced DPD detected by LC-MS/MS (**Table 4.9**). Of the 10 AHL-negative isolates, nine tested positive for AI-2 activity. **Table 4.16** examines the correlation between the presence/absence of AHL, AI-2 activity, and DPD among isolates that caused inhibition of growth. Strain HSML-FTL-10a (which inhibited seven strains and stimulated one; **Table 4.15**) was not included in this data set because it did not grow during the LC-MS/MS

Table 4.15. Isolates that elicited inhibition and stimulation of growth according to isolate source and production of AHLs or AI-2 activity based on reporter strain data and DPD (LC-MS/MS).

Isolate Source	CF Tested	AHL	AI-2	DPD	No. Cultures Inhibited	No. Cultures Stimulated
BBD (144 challenges)	BBD-FTL-6j	+	+	+	6	0
	BBD-FTL-8c	+	+	+	3	0
	BBD-FTK-1d	+	-	+	3	0
	BBD-FTL-1h ^a	-	+	NT	4	0
	BBD-FTL-6d ^a	-	-	NT	6	1
	BBD-FTL-6n ^a	-	+	NT	5	0
	BBD-FTL-6p ^a	-	+	NT	6	1
	BBD-FTK-1e ^a	-	+	NT	2	1
	N and % of Challenges ^b				35 (24%)	3 (2.1%)
BSML (108 challenges)	BSML-FTL-6w	+	+	+	9	1
	BSML-FTL-7l	+	-	+	3	0
	BSML-FTL-6u ^a	-	+	NT	5	1
	BSML-FTL-7d ^a	-	+	NT	4	2
	BSML-FTL-7q ^a	-	+	NT	4	0
	BSML-FTL-1d ^a	-	+	NT	28 (26%)	0
	N and % of Challenges ^b					4 (3.7%)
HSML (90 challenges)	HSML-FTL-9c	+	+	+	3	0
	HSML-FTL-9e	+	+	+	4	0
	HSML-FTL-9i	+	+	+	4	0
	HSML-FTL-10a ^a	+	+	NT	11	0
	HSML-FTL-10r ^a	-	-	NT	7	1
	N and % of Challenges ^b				29 (32%)	1 (1.1%)
Total Challenges (342)					92 (27%)	

These strains denoted were not analyzed for DPD production using LC-MS/MS (refer to Table 6)^a. The percent values denoted represent the percent of the total challenges (144 BBD strains, 108 BSML strains, and 90 HSML strains) that resulted in inhibition or stimulation of growth in each isolate source category.

Table 4.16. Percent of 81 assays that resulted in growth inhibition.

Source of Isolate and Autoinducer Produced	No. Isolates that Elicited Inhibition (No. Test Strains Inhibited)			% of Inhibition ^a
Source of Isolate	BBD	BSML	HSML	49.4
AI-2/DPD	4 (17)	4 (16)	1 (7)	13.2
AHL and AI-2/DPD	3 (12)	2 (12)	3 (11)	7.4
Neither	1 (6)	0	0	100%
Total	8 (35)	6(28)	4 (18)	

Isolate HSML-FTL-10a not included (see text)^a.

experiments. Although HSML-FTL-10a was negative for AI-2 activity using the reporter strain, three additional strains that also tested negative for the reporter strain assay produced detectable DPD (**Table 4.9**), thus we cannot rule out that HSML-FTL-10a also produces DPD. The resulting data set therefore consists of 81 challenges (using 18 isolates) that resulted in inhibition of growth. Of these, nine strains produced AI-2/DPD (positive for at least one of these assays) but no AHL, and were responsible for 49% of the 81 cases of growth inhibition. Eight strains produced both AI-2/DPD and AHL, and inhibited 43% of the 81 cases. One strain (BBD-FTL-6d) did not produce AI-2/DPD or AHL and inhibited six isolates. There was no statistically significant difference (independent group t-tests, $p>0.05$) between the number of cultures inhibited by isolates producing AI-2/DPD only and those that produced both AHL and AI-2-DPD.

Metagenomics analysis. **Table 8** presents all proteins and their ascribed functions associated with QS genes that were identified in BBD metagenomes from the Florida Keys and Curacao samples under the subsystem category of regulation and cell signaling within MG-RAST. The light grey colored cells in the first column represent the Florida Keys sample, and the Curacao sample is represented by the dark grey cells. Rows that are shaded throughout represent protein products from QS genes that are unique in that particular sample. As is evident, more than 50% of the identified QS genes were found to be those associated with vibrios. The *luxI*, *rhIL* and *luxR* genes identified under the subset of 'AHL autoinducer QS' are not associated with any particular bacterial genus. QS associated functional sequences were differentially found within the two BBD metagenomes. Both *luxI* and *luxR* genes associated with AHL synthesis were found in both samples, while *rhIL*, another AHL synthase, was found to be present only in the Florida Keys BBD metagenome. Four *Vibrio*-associated QS genes were found exclusively in the Florida Keys metagenome, specifically *cqsA*, *luxP*, *luxM* and *hapR*. Other QS genes (*luxI*, *luxR*, *luxO*, *luxS* and *uvrY*) were found in both metagenomes. A particular N-homoserine lactone hydrolase (associated with QS in *Yersinia*) was uniquely present in the Curacao metagenome. One *Pseudomonas* associated QS gene was exclusively present in either of the two metagenomes (*vfr* in Curacao metagenome and *rhIR* in Florida Keys metagenome). The results of the QS genes in the BBD metagenomes correlated with the identification of the AHL-producing isolates used in this study (mainly vibrios) in that a high number of *Vibrio*-associated QS genes were present in the metagenomes.

4.5 Discussion for the first study

Quorum Sensing Signals in BBD and Coral-Associated Bacteria. This study revealed the presence of AHLs in nine freshly collected BBD microbial mat samples from two Caribbean coral species. In each case, the full BBD community was present. The immediate questions that rise from these results are: 1) what microbial constituents within this pathogenic polymicrobial community are synthesizing AHLs?; 2) are the AHL producers in BBD also present in the coral microbiome?; 3) what are the structures of the AHLs produced?; and 4) do BBD and SML bacteria also produce AI-2? We addressed each of these questions in the present study.

The observation that all (full community) BBD samples examined using the AHL reporter strains were positive for the presence of AHLs is in agreement with previous studies on QS signal production in cyanobacterial-dominated communities in other environments. These include *Trichodesmium* colonies in the North Atlantic³¹⁶, marine stromatolites in the Bahamas²⁸³, and cyanobacterial mats in Swiss alpine wetland ponds³¹⁷. On the other hand, although cyanobacterial isolates have been reported to produce AHLs in the laboratory³¹⁸, none of the eight BBD cyanobacterial isolates examined in this study tested positive in either AHL reporter strain assay. We note that our cyanobacterial culture medium had a pH of ~8 (or higher during photosynthesis), and that alkaline pH has been demonstrated to degrade AHL signals^{282, 298}. Furthermore, it is possible that the AHL reporter strain assays yielded false-negative results for a variety of other reasons (e.g., the AHL produced was undetectable by the reporter strains, AHL concentrations were below the detectable levels of the reporter strains, AHL production was inhibited by the laboratory conditions used in this study, the isolate was also producing quorum quenching molecules that inhibited detection of AHLs, and/or other abiotic factors besides pH degraded the AHL signal). These same scenarios could explain false negative results in any of the reporter strain assays used to detect QS signals in this study.

To investigate the identity of culturable BBD bacteria that are capable of producing AHLs (as well as DPD), we tested bacterial isolates from the complex microbial communities that constitute BBD, the apparently healthy SML of BBD-diseased colonies, and the SML of healthy colonies. We found that 11 of 199 (5.5%) isolates, all bacterial, from BBD, BSML, and HSML can produce QS signals under laboratory conditions. This result is similar to the

4% observed in a study²⁴⁰ in which more than 300 bacterial cultures isolated from marine invertebrates (i.e., coral SML, other marine invertebrates, and dinoflagellate symbionts) were screened using a variety of reporter strains that detect AHLs of varied chain lengths. We also showed that 55% of CFs tested positive for AI-2 activity (or the CAI-1 signal specific to some *Vibrio* spp.), as detected by the *V. harveyi* BB170 reporter strain. The high percentage of isolates capable of AI-2 activity is not unexpected, as this molecule is considered to be an interspecies signal molecule^{279, 319}. It should be noted that AHL and AI-2 production in some bacteria may be specific to a certain portion of the bacterial growth phase³²⁰⁻³²¹. For the bacterial isolates investigated in this study, all CFs were collected during early stationary phase. Thus, an isolate producing QS signals during a different growth phase may have produced a negative result in the reporter strain assays. Based on subsequent 16S rRNA gene sequencing, all 11 AHL-producing isolates most closely aligned with members of the *Alpha*- and *Gamma*-*proteobacteria* (**Table 4.4**). Five of these were closely related to *Vibrio* spp. *Vibrio fischeri* and *V. harveyi* represent the archetypal system for AHL- and AI-2-regulated gene expression^{257, 322}, and a variety of *Vibrio* spp. have been documented to produce AHLs³²³⁻³²⁵. *Vibrios* have been routinely detected in BBD^{228, 247, 326}, and it has been suggested that proteolytic activity by *Vibrio* spp. might play a role in BBD pathology²⁴⁷. *Vibrio* strains isolated from both healthy and diseased corals have also been shown to produce AHLs²⁹¹.

Two AHL-producing isolates (BBD-FLK-1d and BSML-FTL-6w) were most closely related to strains belonging to the *Rhodobacteraceae*, also commonly detected in BBD and coral SML communities^{203, 228}. AHL production by members of the *Roseobacter-Ruegeria* subgroup of the *Alpha*-*proteobacteria* has been previously demonstrated^{3, 327-328}. Isolates BSML-FTL-7I and HSML-FTL-10a were most closely related to the genus *Pseudoalteromonas*, also previously documented to produce AHLs³²⁹. Isolate HSML-FTL-10a was most closely related to *P. luteoviolacea* strain NCIMB 1893 (99% similarity) and, consistent with the description of this strain as a producer of violacein³³⁰, isolate HSML-FTL-10a produced a dark purple pigment. *P. luteoviolacea* has been documented to have antibacterial activity³³⁰, and antibiotic activity has also been demonstrated for violacein³³¹.

QS Signals in the BBD Mat and Coral SML. Within the BBD mat, an exopolysaccharide (EPS) matrix surrounds the diverse members of the BBD microbial community. High concentrations (>600 μ M) of AHLs have been measured within *Pseudomonas*

aeruginosa biofilms³³², suggesting that bacterial EPS may effectively sequester QS signals. Because the surface of the BBD mat is exposed to seawater, and thus diffusion, advection, and dilution³³³, accumulation of QS signals would likely be greater in the depths of the BBD mat than on the mat surface. In a similar manner, coral mucus forms a protective coating over the coral surface and is present as a thin layer that can be considered as a biofilm. While the chemical composition of coral mucus varies between species, the basic structure of the SML consists of an insoluble, hydrated glycoprotein^{226, 334}. The gel-like nature of coral mucus would likely concentrate particularly hydrophobic signal molecules similar to EPS.

Within BBD, the persistence of QS signals may be impacted by the local chemical and physical microenvironment to a greater extent than within non-cyanobacterial associated biofilms. Alkaline conditions may result in hydrolysis of the AHL lactone ring^{282, 298} with short-chain (< C10) AHLs appearing to be more susceptible²⁸²⁻²⁸³. The BBD mat contains pronounced biogeochemical microgradients of oxygen, pH, and sulfide²⁴⁵⁻²⁴⁶ which fluctuate based on changes in the predominant metabolic pathways occurring within the BBD mat. During daylight, when oxygenic photosynthesis dominates, the pH in the BBD mat ranges from 7.58–8.13²⁴⁶, while in darkness, when predominant metabolic pathways shift to respiration and fermentation, pH values drop to 7.33–7.49²⁴⁶. Based on the relative stability of AHLs in terms of pH²⁸³⁻²⁸⁴, microbial mats in general could use both short- and longer-chain AHL signaling during the night and longer-chain AHL signaling during the day. Coral mucus itself is acidic (pH of 5.5 to 7.7) in comparison to surrounding seawater³³⁵; thus, AHL signals within the coral SML would likely remain stable enough for signaling to occur both night and day. We found both short- and long-chain AHLs in this study, including variant 3OC20-HSL produced by two isolates. To our knowledge this AHL has not been reported in the literature.

The implications of abiotic and biotic influences on AI-2 signaling and the impact of the natural environment are not well understood at this time. Like AHLs, production of DPD may be dependent on the particular strain, the growth phase, or the culture medium^{303, 336}. DPD appears to be relatively stable in comparison to the AHLs³³⁷. We found that 70% of the CFs from BBD isolates and 68% of the CFs from BSML isolates tested positive for AI-2 activity, compared to only 33% of the CFs from HSML isolates. All of the 11 isolates we examined for DPD production produced detectable amounts of this precursor to AI-2, with BBD isolates

producing two to three times as much as the SML isolates (**Table 4.9**). It is possible that AI-2 signaling may be occurring more frequently within the BBD microbial consortium as compared to the coral SML, if AI-2 signaling is indeed occurring within these environments.

Interactions among Isolates from BBD and SML. Results of the growth challenges revealed that 27% of 342 challenges resulted in significant inhibition of bacterial growth. While others have detected increased antimicrobial activity by the mucus of healthy corals, as opposed to diseased corals²⁴¹⁻²⁴², this study found no significant difference in antimicrobial production based on isolate source (BBD, BSML, or HSML). Toxins have been documented previously to be produced by BBD cyanobacteria^{232-234,235} and antimicrobials by coral-associated bacteria^{242-243, 338-339}, and it is likely that antimicrobial production contributes to the temporal and spatial dynamics occurring in BBD³⁴⁰. Interestingly, only 2% of the 342 growth challenges exhibited significant growth stimulation by the culture filtrates. The vast majority (~88%) of these stimulatory CFs were from BBD isolates. Thus, cooperation between microbes may be more common in interactions among members of the BBD than among SML bacteria.

Almost all (~93% of cases) of the growth inhibition documented in the challenge study was caused by the CFs of isolates that were positive for AI-2 activity and/or production of DPD. AI-2 is widely recognized for its involvement in interspecies signaling and has been linked to the upregulation of antibiotic biosynthesis^{288, 341}. Regulation of bacterial relationships within complex microbial communities is another proposed role of DPD in the natural environment²⁷⁷. Our findings are in complete agreement with this proposal. For example, isolates that produced AI-2/DPD (with some also producing AHLs) were cultured from BBD, BSML, and HSML, and were shown to inhibit growth of isolates from all three of these sources. Thus, if present and active within these natural environments, it is possible that AI-2 could regulate both pathogenic and probiotic microbial populations with communication between such populations. Only one strain that caused growth inhibition produced neither AI-2/DPD nor AHL. Further research is needed to determine if this is a pattern that holds true for bacterial members of complex communities in general.

Potential Roles of QS Signal Production in Coral Health and Disease. This is the first report of the presence of AHLs in samples freshly collected from *in situ* coral disease. Only one previous study²⁹¹ has directly and empirically addressed the potential role of QS

in coral health and disease. Similar to our study, Tait *et al.*²⁹¹ used reporter strains (as well as thin layer chromatography) to detect AHLs and AI-2 production by bacteria (*Vibrio* spp. and *Photobacterium rosenbergii* strains) isolated from or near corals. Twenty-nine isolates were tested, of which 17 produced AHLs and 29 (100%) produced AI-2. Six of the 29 strains were isolated from the tissue of “diseased” corals, although the disease was not identified; of these, three were positive for AHL production. Three strains were isolated from bleached corals (two from tissue, one from mucus), with both tissue samples positive for AHL production and the mucus sample negative. An additional 11 samples are identified as from the water in association with specific coral species; however, the distance of the sample from the coral surface is not provided; of these, six were positive for AHLs. Finally, of nine isolates from tissue or mucus samples associated with presumably healthy corals (only one identified as such), five (two tissue and three mucus) produced AHLs. These results suggest that QS may be a process in the coral microbiome (and coral environment), and our findings support their conclusion²⁹¹.

Despite nearly 40 years of research, the etiology, pathogenesis, and pathobiology of BBD remain unresolved. Mechanisms of BBD mat formation and organization, the mode of microbial recruitment to the BBD community, and the factors leading to initiation of the pathogenic, migrating band are not understood. It is, however, well known that AHLs produce and control similar virulence factors in other systems, including in laboratory experiments using the coral pathogen *Serratia marcescens*³⁴². Although there has been little work on DPD production by coral-associated bacteria, AI-2 signaling is also known to be associated with disease function in pathogenic bacteria in general (reviewed in^{277, 279}).

If QS is indeed occurring within BBD, the potential roles of AHL and/or AI-2 signals with regard to virulence, biofilm structure, and community composition makes QS an important aspect of future study for BBD as well as other coral diseases. QS between potential pathogens and the healthy coral microbiota may contribute to disease processes as well. Future research to elucidate the roles that QS signaling molecules play in coral health and disease will require thorough investigations targeting specific effects of QS signals on regulatory and physiological systems, as well as gene expression.

4.6 Discussion for the second study

One of the most interesting findings of this study was the fact that, by far, isolates from BBD had a significantly higher proportion of members that produced AHLs when compared to isolates from the SML of apparently healthy and BB-diseased corals. Only four of 74 (5.4%) isolates from BSML and HSML combined tested positive using the reporter strain whereas 23 of 155 (14.8%) BBD isolates did so. This suggests that quorum sensing is more active, and presumably more important, in BBD bacterial communities as compared to SML bacterial communities. Overall BBD is a pathogenic polymicrobial consortium and the coral SML is believed to have probiotic characteristics. Both of these types of microbial communities (pathogenic and probiotic) have been proposed to involve QS on a functional basis. A second very interesting result of this study was that 12 of the 24 AHL producing isolates exhibited a significant difference in AHL production in terms of temperature. In particular, seven of the twelve isolates were significant when comparing AHL production above and below the threshold temperature of BBD (27.5 °C). In each case, there was increased AHL production at the higher temperature (30 °C) compared to the lower temperature (24 °C). Four of the eight AHLs detected in this study showed this pattern, and six of the seven isolates that exhibited the temperature threshold response were from BBD, which further suggests that quorum sensing is potentially important in BBD etiology. Previous studies of AHL production in terms of temperature and disease revealed varying effects for different AHLs. For example, AHL production by some sub-species of *Erwinia*, which causes soft rot in plants above a threshold temperature of 20 °C, was increased at 34.5 °C compared to 28 °C, whereas production decreased at the elevated temperature for other, non-pathogenic sub-species of the same genus³⁴³. *Pecobacterium atrosepticum*, which causes soft rot in potatoes at and above 20 °C, produces four AHLs which all increase from 4 °C to 24 °C, but then decrease at 28 °C³⁴⁴. These findings, along with the results of this study, implicate temperature as an environmental factor that influences the role of QS in disease. AHLs are affected by other environmental factors in addition to temperature. These include pH, redox state and oxygen concentration^{170, 333}. Alkaline pH causes degradation of AHLs rendering the molecule incapable of signaling²⁸². In general, it has been shown that the longer the AHL chain length, the more stable the molecule is to external factors and the lower the turnover rate^{282, 316}. The pH of sea water is around 8.2, hence one can argue that marine AHL producing bacteria must have long chain AHLs to be capable of signaling in an alkaline pH³²⁹. However, several studies have found that both short and long

chain AHLs are produced by bacteria in marine environments^{154, 157, 240, 289-290, 309, 328, 345-347}. It may be that the small chain AHLs are produced at high quantities to counteract breakdown due to external factors. Decho *et al.* (2009) proposed that differential degradation of AHLs likely affects signal reception by bacteria, and that this could result in variation of chronological windows in which signaling can occur²⁸³. A greater abundance of AHLs has been observed in buffered media as compared to unbuffered media, which could be the result of increased pH as a growing culture becomes more alkaline²⁸². Although buffered media were not utilized in the present study, growth periods were short (on the order of hours) and samples were extracted and immediately frozen at - 20 °C to minimize any breakdown and/or degradation of AHL molecules. In terms of BBD, the results of this study showed that temperature influenced the relative abundance of AHLs produced by BBD isolates with more AHL produced above the BBD temperature threshold. Considering the polymicrobial nature of BBD, the observed variable effect of temperature on specific AHLs would likely affect the interactions within the BB-disease microbial community, which may lead to maintenance of the disease process either during initiation or progression. In addition to temperature, pH is an important environmental factor in BBD in that it fluctuates widely over each 24-hour period. The pH in the band is 7.6 during the night due to release of CO₂ during respiration and 8.2 during the day due to CO₂ uptake during photosynthesis^{245-246, 283}. This may indicate that only long chain AHLs are available during the day and both short and long chain AHLs at night, which would in turn affect gene transcription and regulation over the 24-hour period²⁸³. In the current experiment pH was not manipulated, but it would be interesting in future studies to assess the role of pH on AHL production among the BBD (and SML) isolates. Nearly all isolates that produced AHLs in this study were identified to be *Vibrio* species, which is interesting in that many vibrios exhibit QS. *Vibrio* species are also known pathogens in several coral diseases. For example, *Vibrio coralliilyticus* and *Vibrio shiloi* both cause coral bleaching³⁴⁸⁻³⁵⁰, *Vibrio harveyi* and other vibrio species have been implicated in white syndrome disease³⁵¹⁻³⁵² and *V. alginolyticus* is a proposed pathogen of yellow blotch/band disease³⁵³. Several studies have found *Vibrio* species to be present in higher abundance in diseased corals as compared to healthy corals^{291, 354-355} and vibrios have been proposed to be, in general, opportunistic pathogens of corals³⁵⁶, including BBD²⁴⁷. To date, all of the vibrios that have caused disease in corals have done so at a temperature of above 26 °C, close to the temperature threshold of BBD. In our study all six of the BBD isolates 435 that produced significantly more ALHs at 30°C compared to 24°C were vibrios. The presence of many well-known and common QS genes in the BBD

metagenome dataset indicates a very high potential for active quorum sensing within the black band mat. Both AHL and AI-2 genes were found to be present in the BBD metagenome, many of which are vibrio associated, which agrees with the high proportion of vibrios among the BBD isolates tested. Overall, a greater variety of QS genes were exclusively present in the Florida Keys metagenome. The antimicrobial assays conducted in this study revealed that, when comparing bacterial isolates from the three coral health states, BBD isolates had the fewest number of isolates that exhibited growth inhibition of the potentially important BBD bacteria *Roseovarius* and *Ferrimonas*. This result, although based on tests using only two strains, does suggest that isolates from BBD in general are less competitive than bacterial members within the SML. It may be that interactions within polymicrobial pathogenic communities minimize antagonism among the individual community members, in contrast to enhanced competition that would be expected in a nutrient and carbon rich environment (such as the coral SML on coral reefs). In summary, our findings reveal that temperature has a significant effect on the production of specific quorum sensing signal molecules (AHLs) produced by isolates of BBD bacteria and that production of half of these (four of eight detected) is increased above the BBD temperature threshold. Furthermore, we found that when comparing QS signal production by BBD bacteria vs. bacteria isolated from coral SML, the proportion of QS sensing by BBD bacteria was significantly higher. Our results further imply a particular importance of BBD vibrios in the functioning of BBD community quorum sensing, both in the laboratory experiments using bacterial isolates and in our investigation into the BBD metagenome. Whether vibrios and specific quorum sensing signal production capabilities are directly involved in BBD disease etiology necessitates further investigation targeting specific mechanisms of BBD pathobiology that are linked to QS.

CONCLUSION

This dissertation consisted of six studies spread over three chapters, each describing the activities of three different biological signaling molecules in terms of therapeutic potential on an *in vivo* system, environmental degradation, and transcription-level control of phenotype of a species and consortium of bacteria. Each of these studies was in the realm of chemical biology and relied on the powerful tools of synthetic and analytical chemistry in application to identification, synthesis, and characterization of each biological signaling molecules in each study. The primary chemist contribution of CWP in each study was significant in terms of the generation and analysis of data and discussion thereof. Through contribution and experience in these inherently collaborative projects, mastery of a type of scientific method was accomplished. First, the hypothesis is generated from the presumption that a single, group, or class of signaling molecules are responsible for an activity of interest. If the molecule is unknown or poorly defined, the tools of biology and chemistry, such as NMR and MS can be applied to this end. From this, organic synthesis can generate a synthetic version or analogue of the signaling molecule(s) to modulate the activity of interest in order to understand the activity or alter the activity in a desired fashion. Finally, evaluation of the activities through biological methods such as quantitating gene transcripts of products associated with the activity of interest, such as a luciferase reporter gene assay or qPCR assay, and chemistry methods capable of quantitating the amount of molecules of interest, such as ID-HPLC-MS/MS, provide data to be analyzed to test and refine the hypothesis. Mastery of this method may allow a chemist working in the field of chemical biology to contribute to the conception of a study similar to those discussed herein, which studies the activities or degradation of a biological signaling molecule or signal disruptor.

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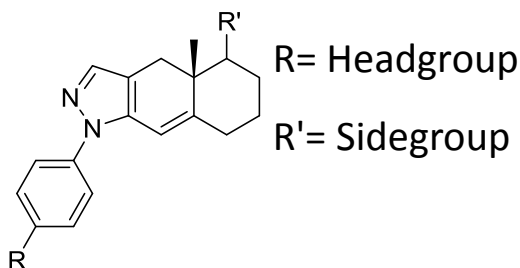
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APPENDIX

Supplemental Figures



Hyperlinks- hold down Ctrl and click a compound to be taken to its corresponding spectra

Headgroup-Sidegroup-Oxidation (#)

[Methyl Acetal \(7b\)](#)

[Methyl Ketone \(8b\)](#)

[Methyl Aldehyde \(10b\)](#)

[Methyl Methyl 2OH \(11ba\)](#)

[Methyl Methyl 3OH \(13ca\)](#)

[Methyl Phenyl 2OH \(11bb\)](#)

[Methyl Phenyl 3OH \(13bb\)](#)

[Methyl Anisole 2OH \(11bd\)](#)

[Methyl Anisole 3OH \(13bd\)](#)

[Methyl Fluorophenyl 2OH \(11bc\)](#)

[Methyl Fluorophenyl 3OH \(13bc\)](#)

[Methyl Thiophene 2OH \(11be\)](#)

[Methyl Thiophene 3OH \(13be\)](#)

[Fluoro Nitrile 2OH \(11af\)](#)

[Fluoro Nitrile Ketone \(12af\)](#)

[Methoxy Acetal \(7c\)](#)

[Methoxy Ketone \(8c\)](#)

[Methoxy Aldehyde \(10c\)](#)

[Methoxy Methyl 2OH \(11ca\)](#)

[Methoxy Methyl 3OH \(13ca\)](#)

[Methoxy Phenyl 2OH \(11cb\)](#)

[Methoxy Phenyl 3OH \(13cb\)](#)

[Methoxy Anisole 2OH \(11cd\)](#)

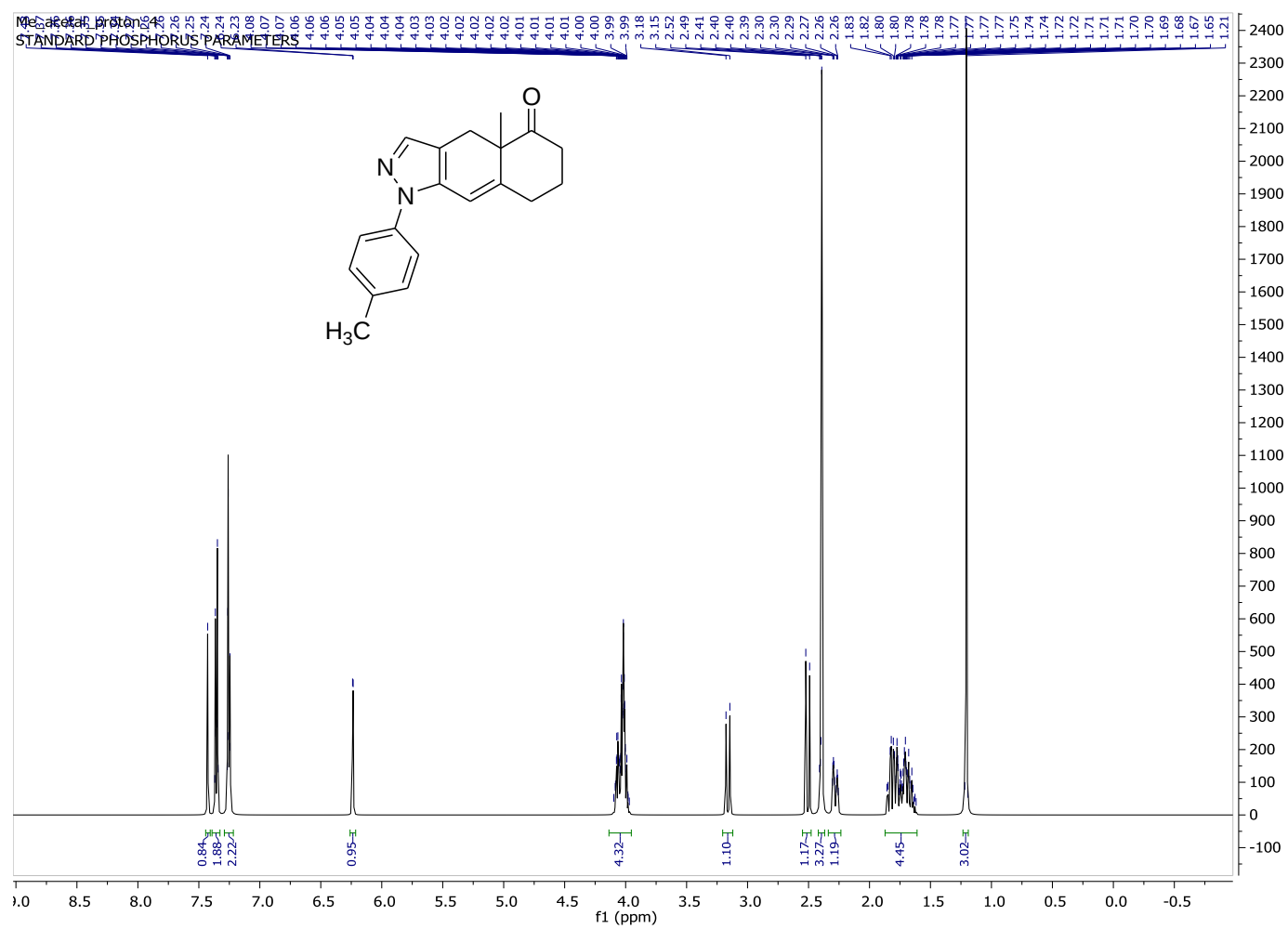
[Methoxy Anisole 3OH \(13cd\)](#)

[Methoxy Fluorophenyl 2OH \(11cc\)](#)

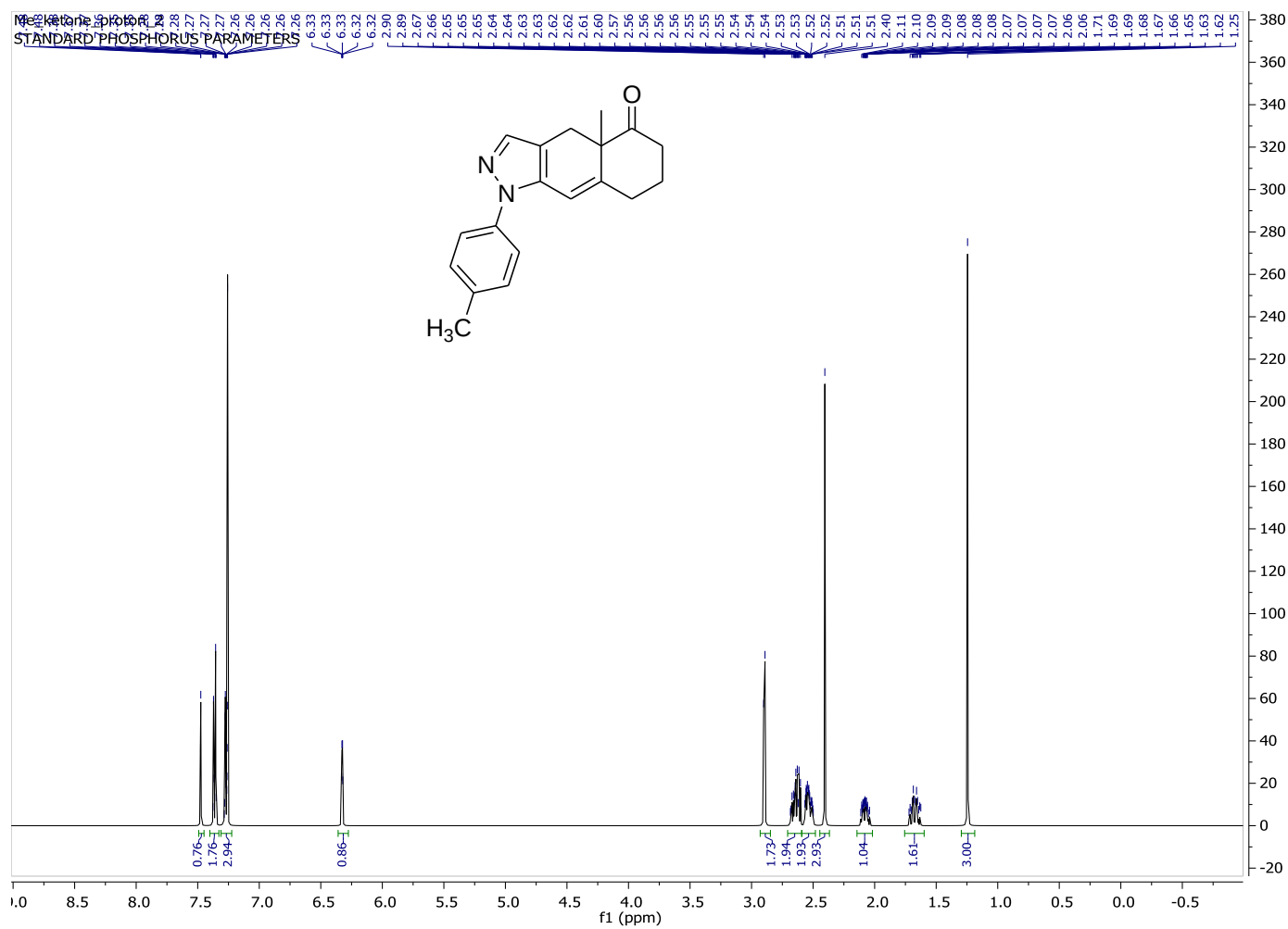
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[Methoxy Thiophene 2OH \(11ce\)](#)

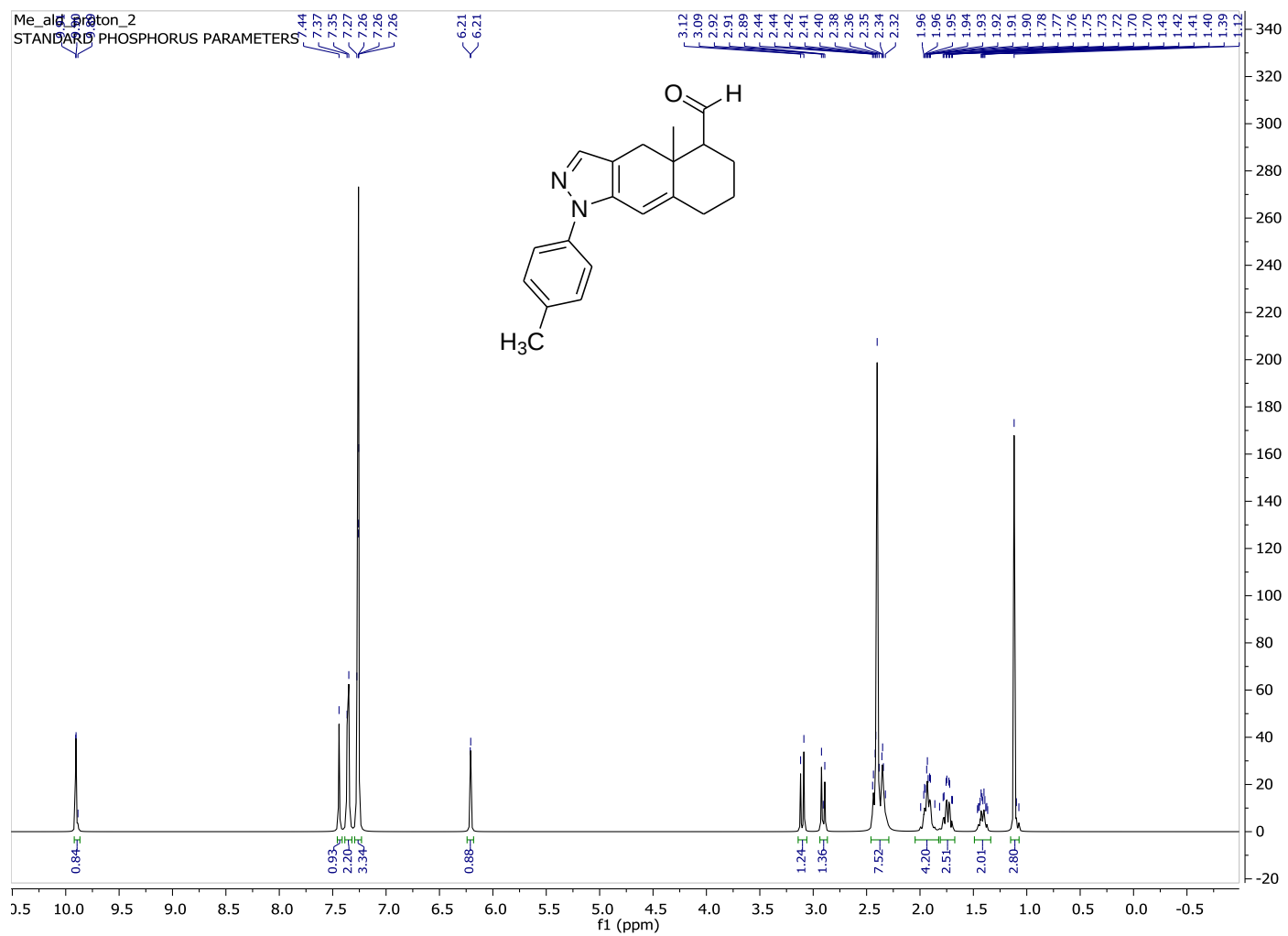
[Methoxy Thiophene 3OH \(13ce\)](#)



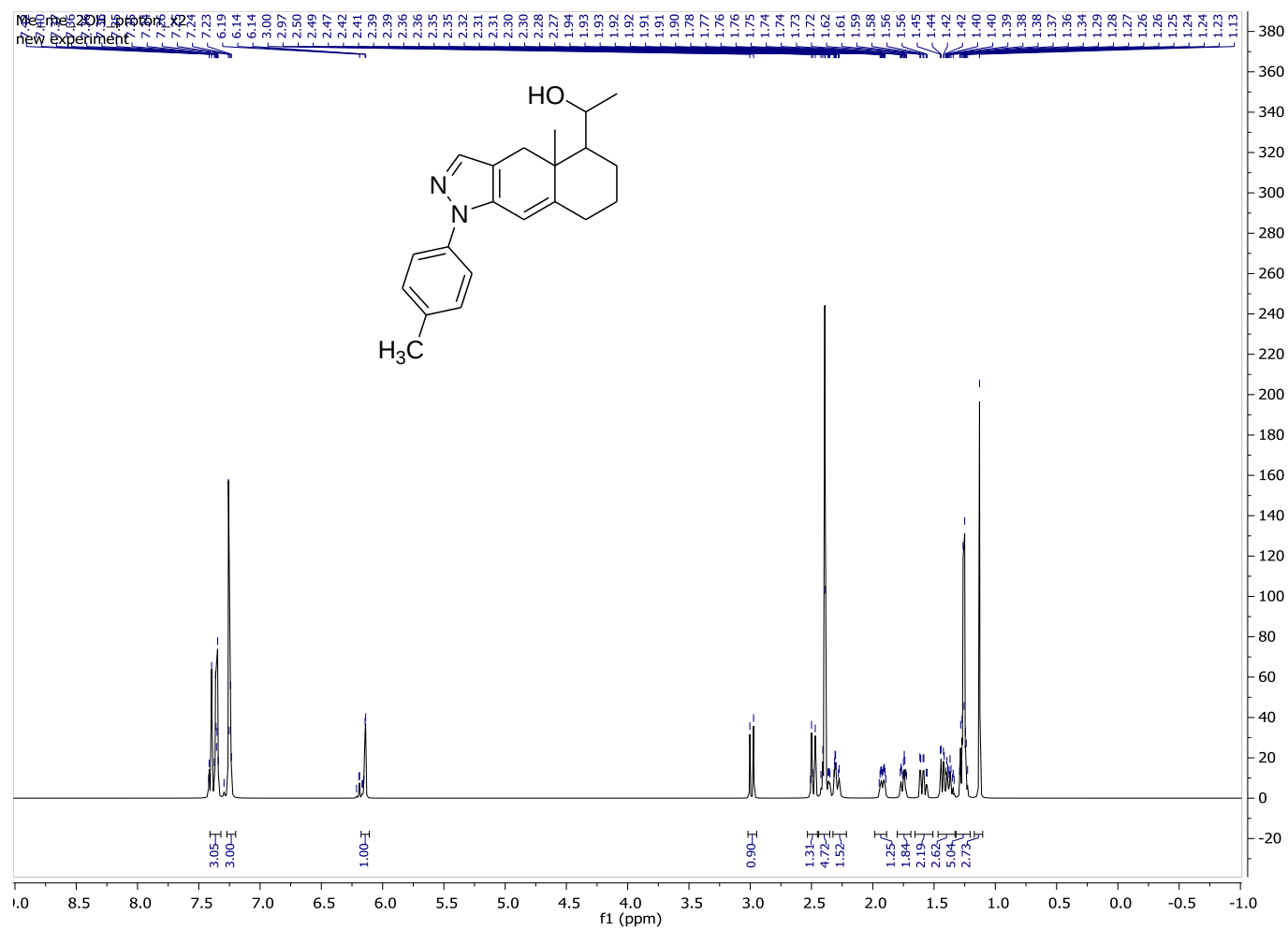
1. ¹H NMR spectrum of compound **7b**.



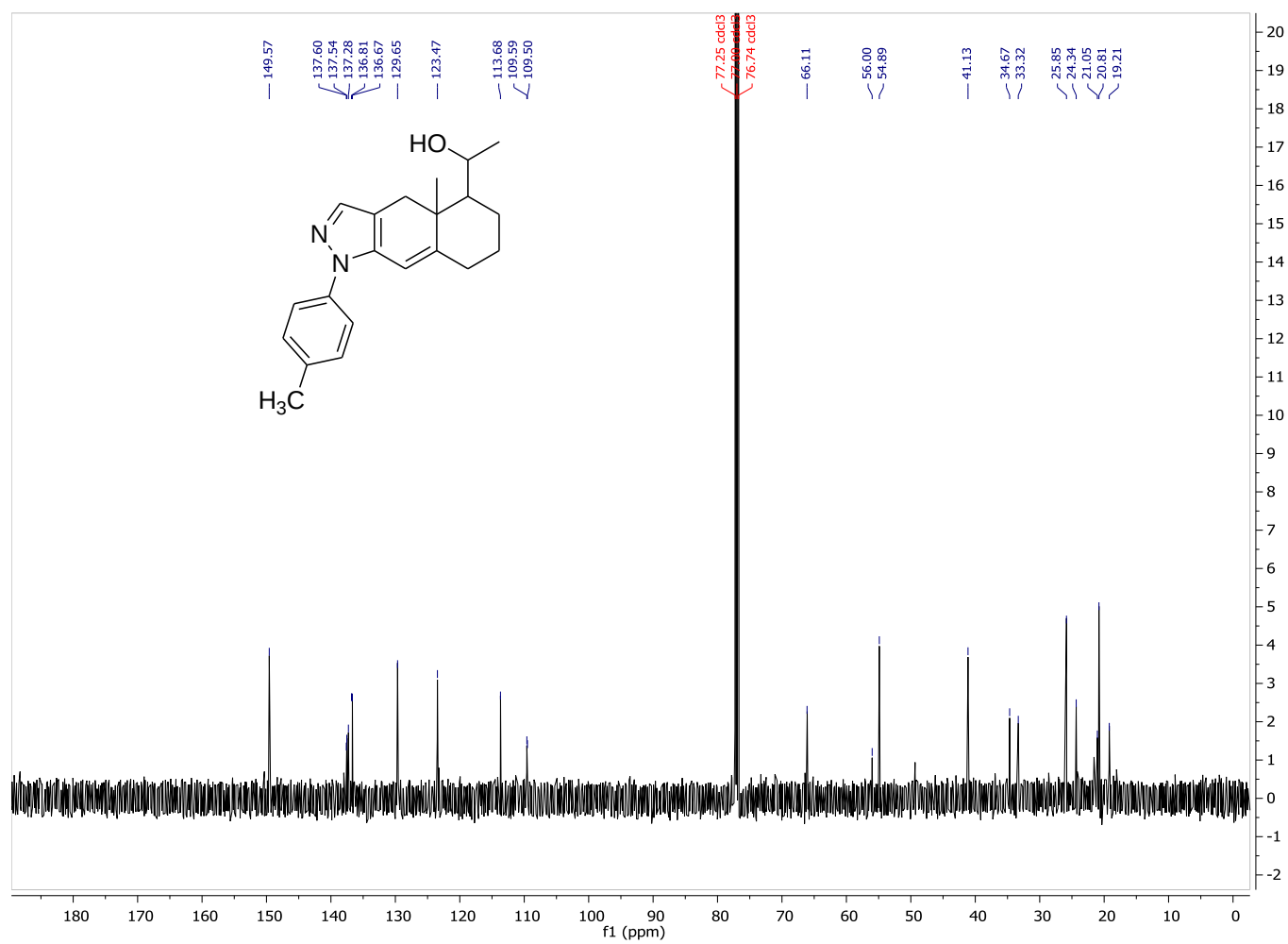
2. ¹H NMR spectrum of compound 8b.



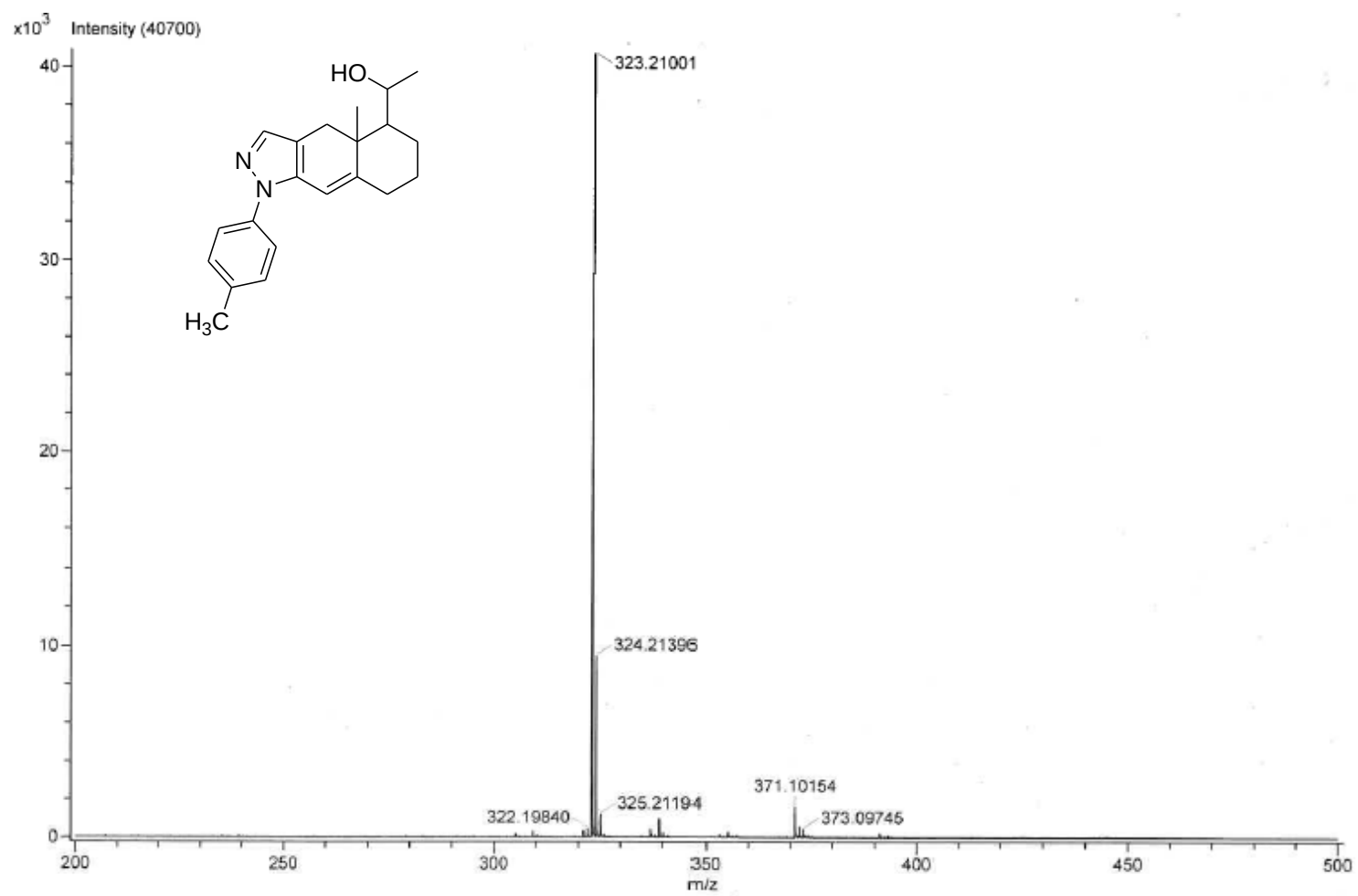
3. ¹H NMR spectrum of compound **10b**.



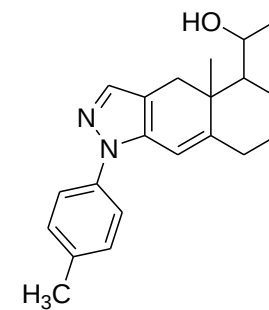
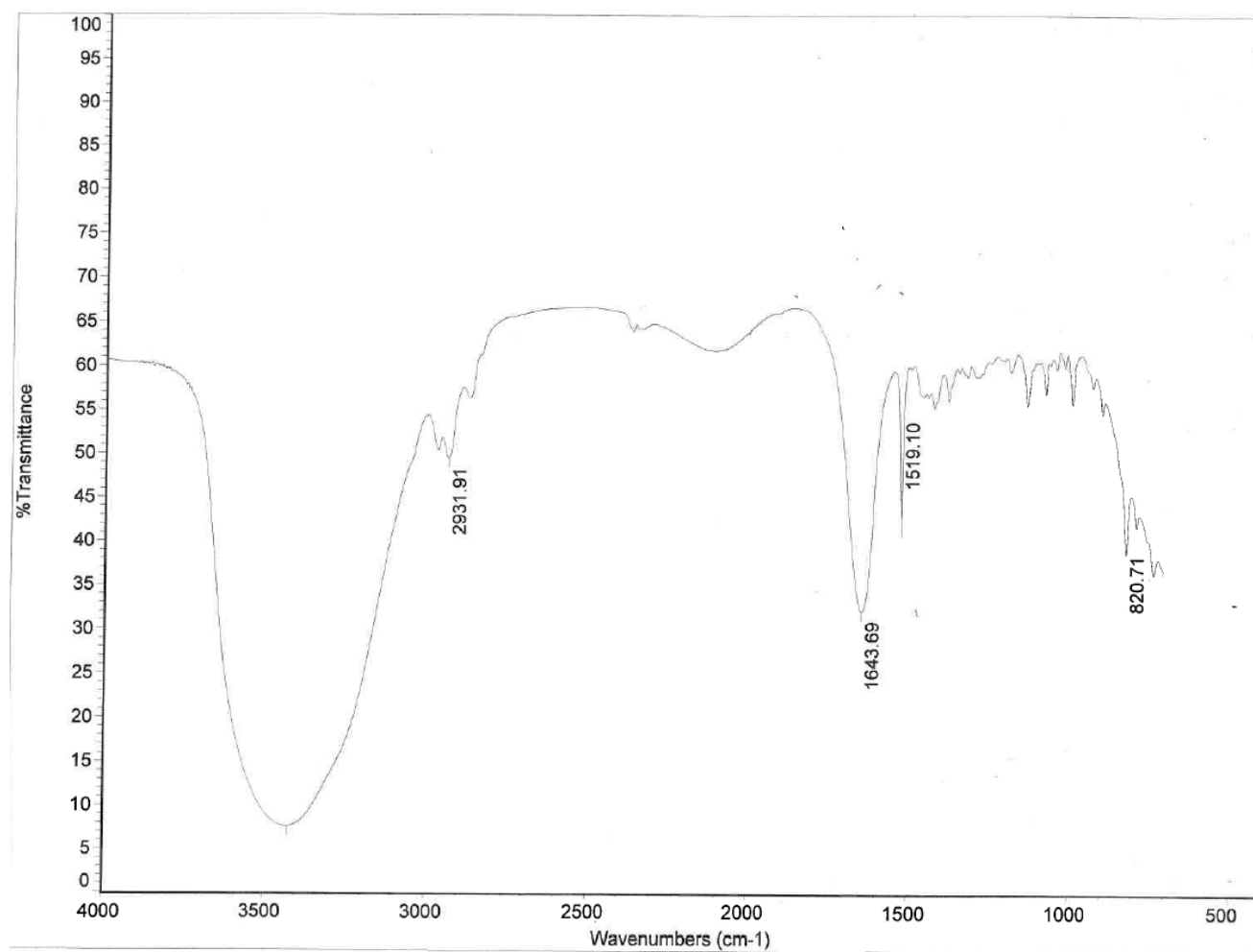
4. ¹H NMR spectrum of compound **11ba**.



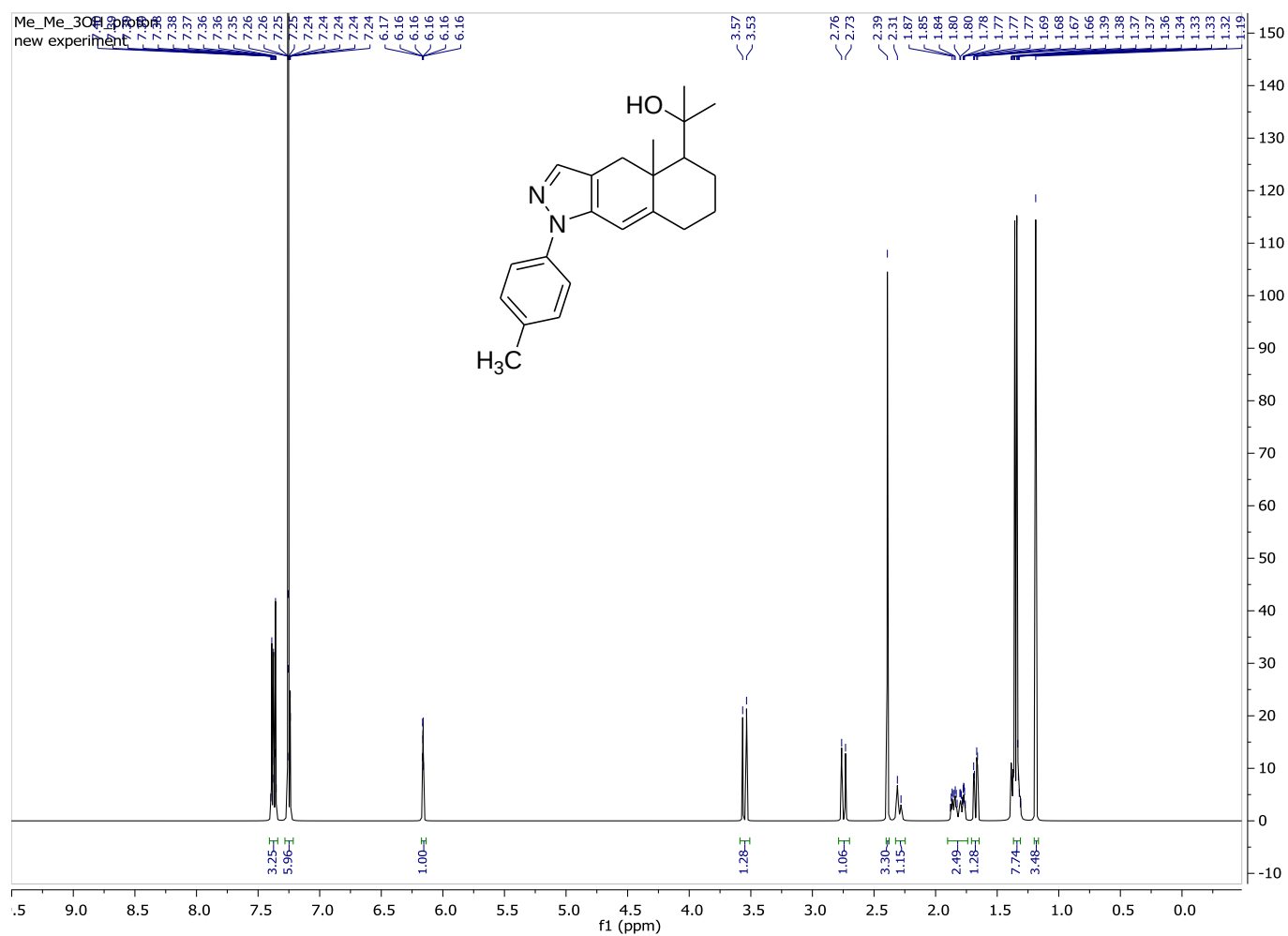
5. ¹³C NMR spectrum of compound **11ba**.



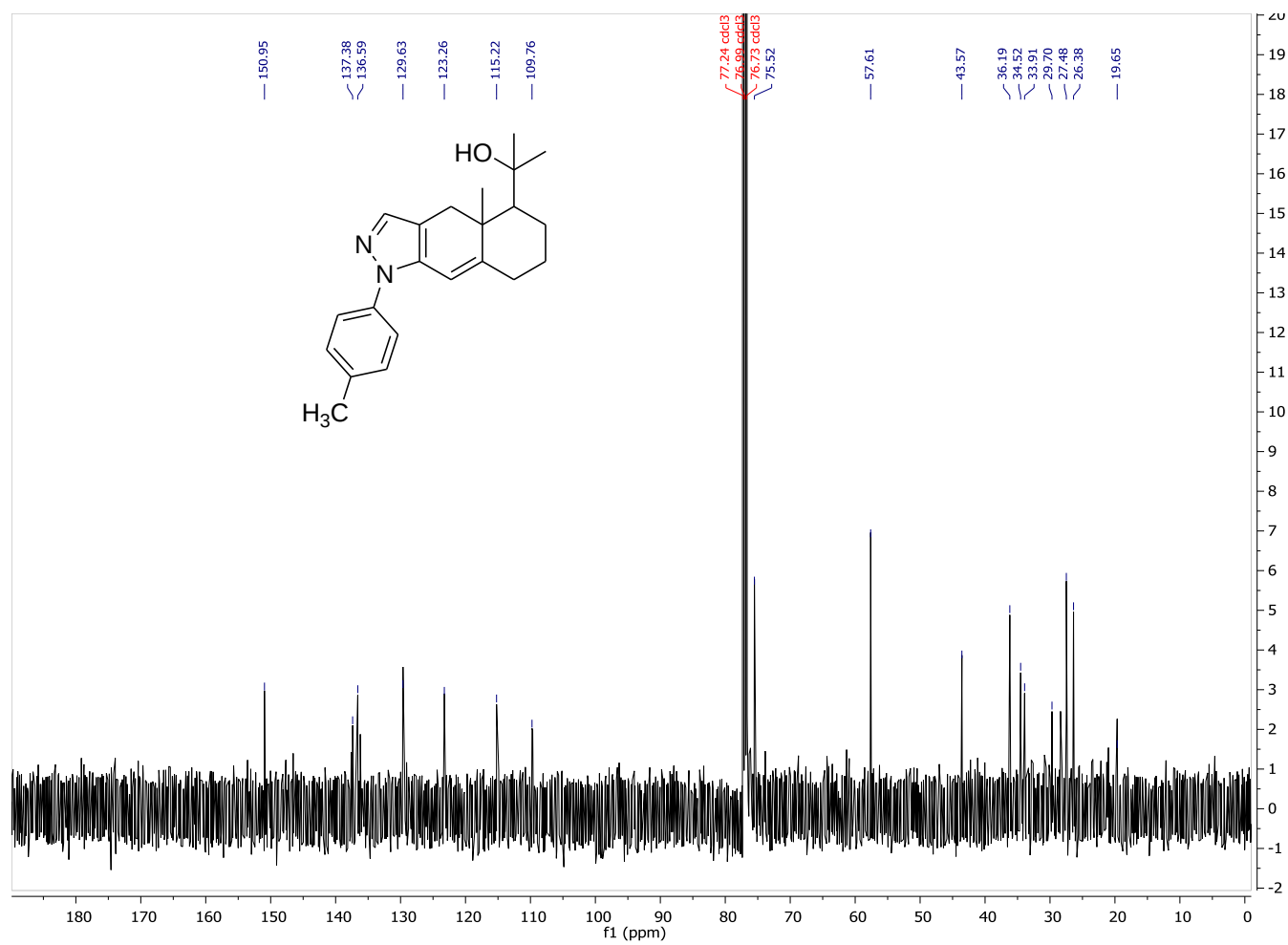
6. HRMS spectrum of compound **11ba**.



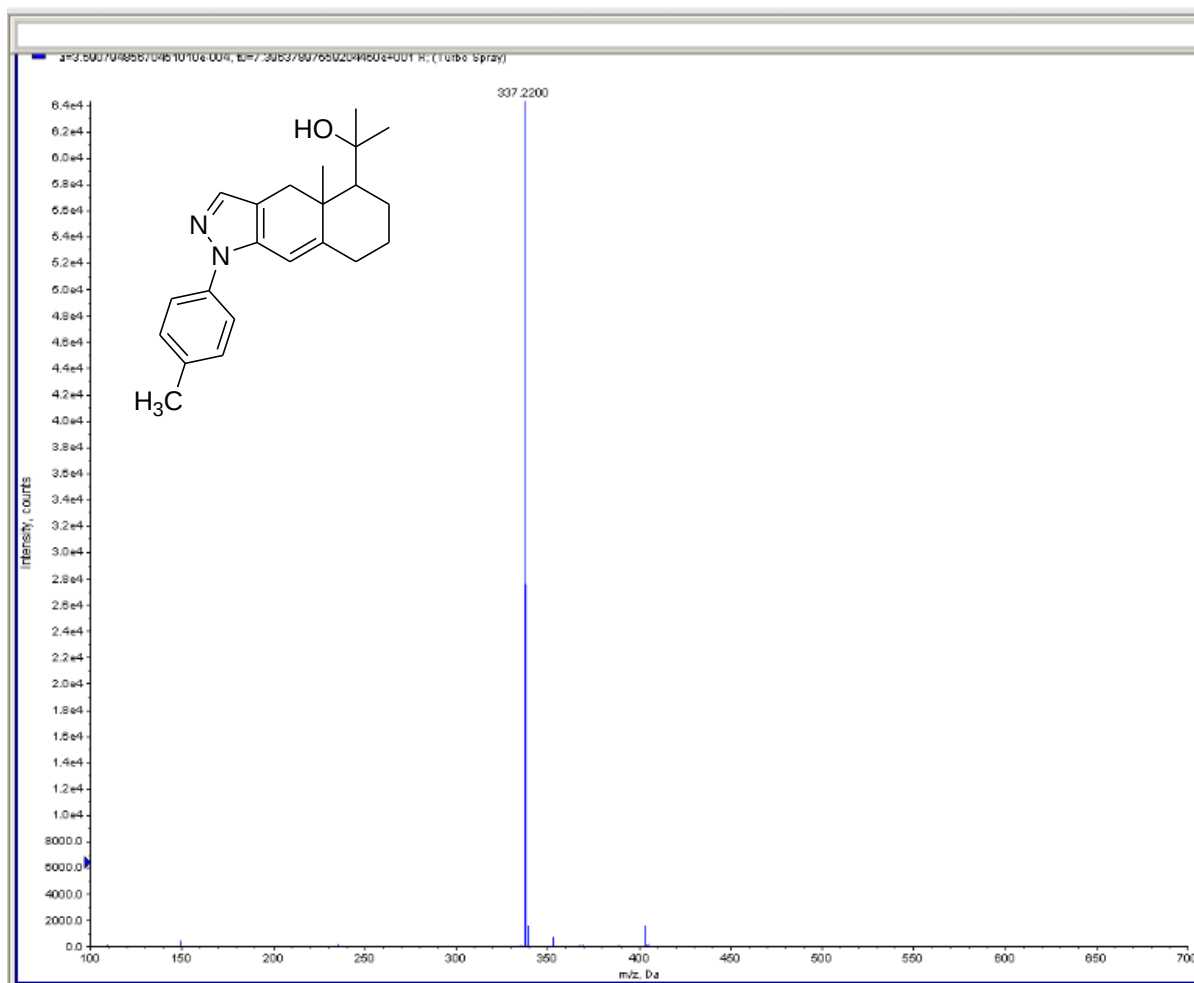
7. IR spectrum of compound **11ba**.



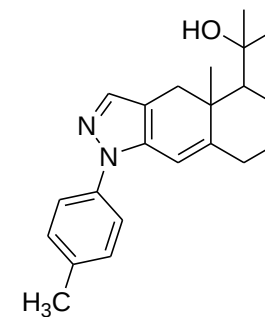
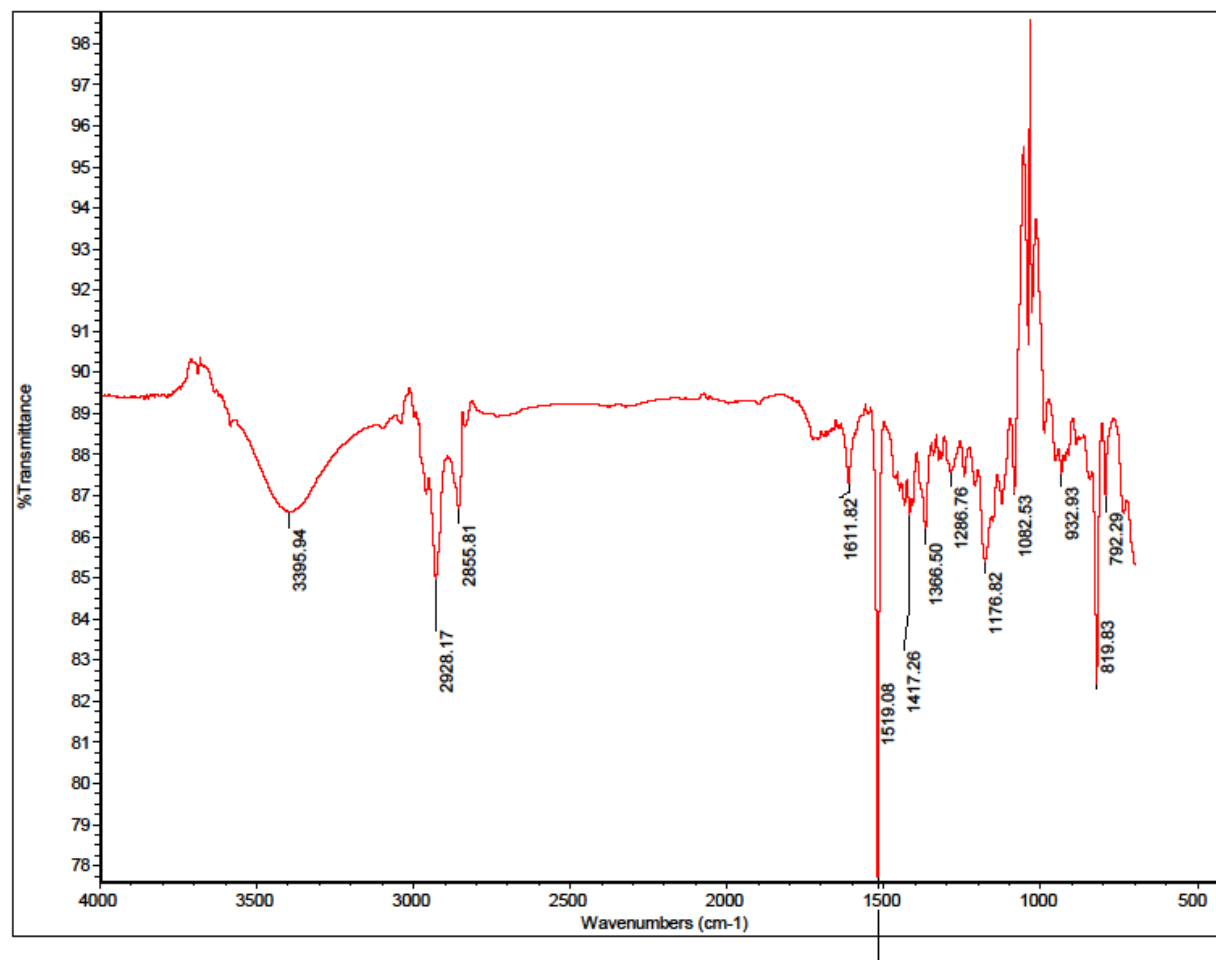
8. ¹H NMR spectrum of compound **13ba**.



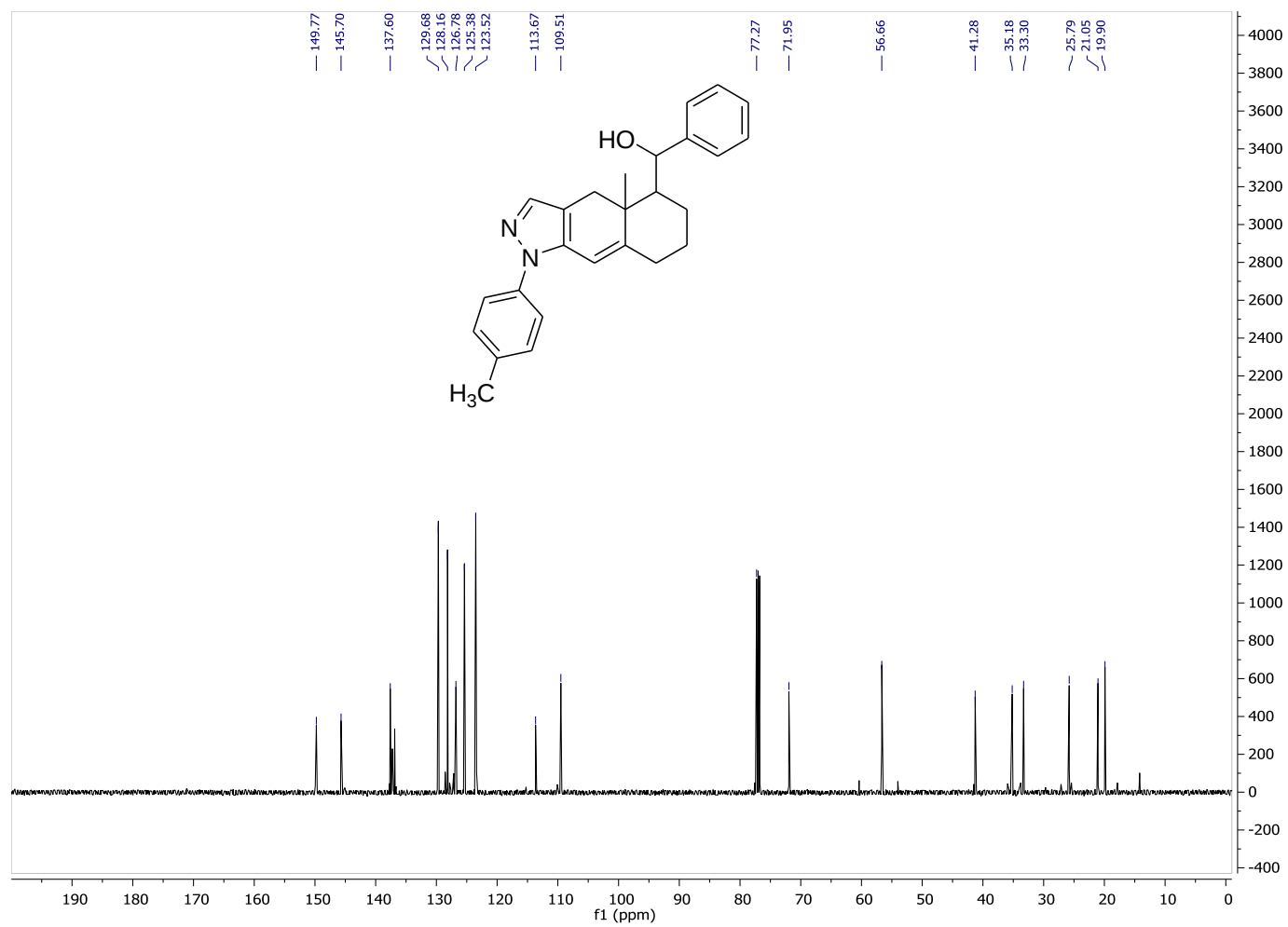
9. ¹³C NMR spectrum of compound **13ba**.



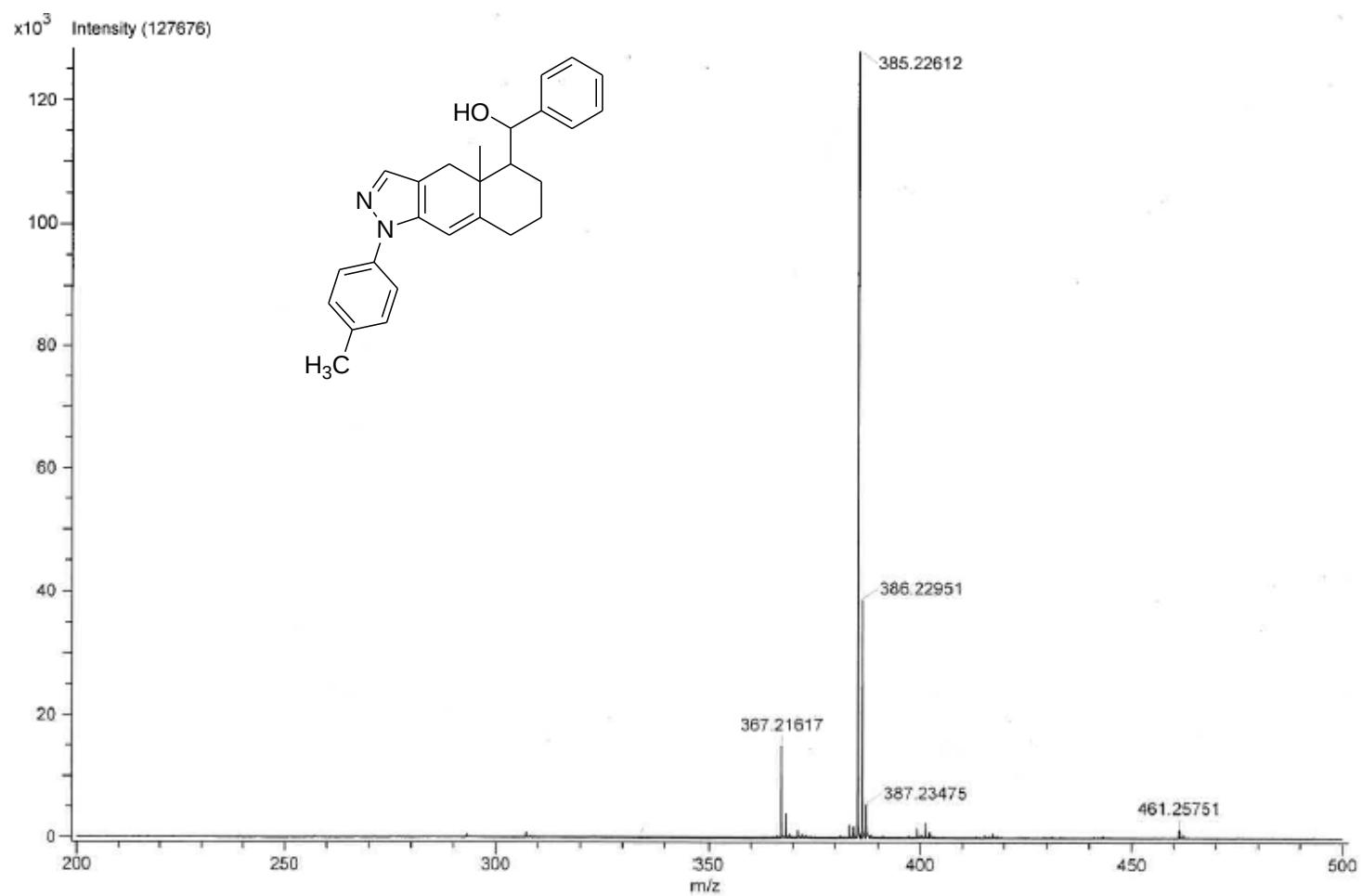
10. HRMS spectrum of compound **13ba**.



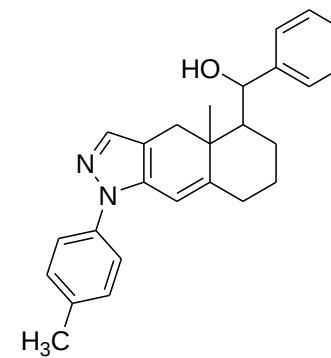
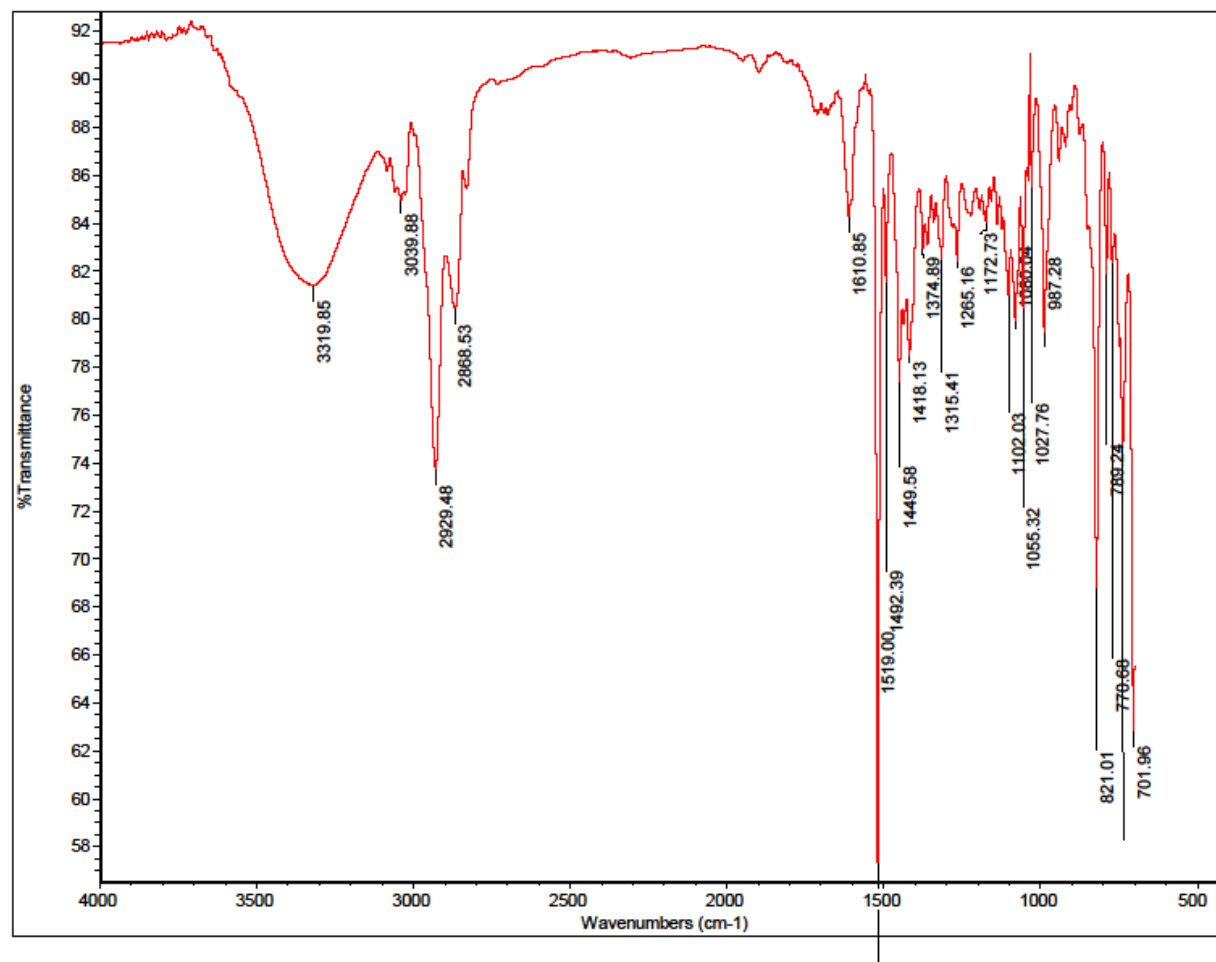
11. IR spectrum of compound 13ba.



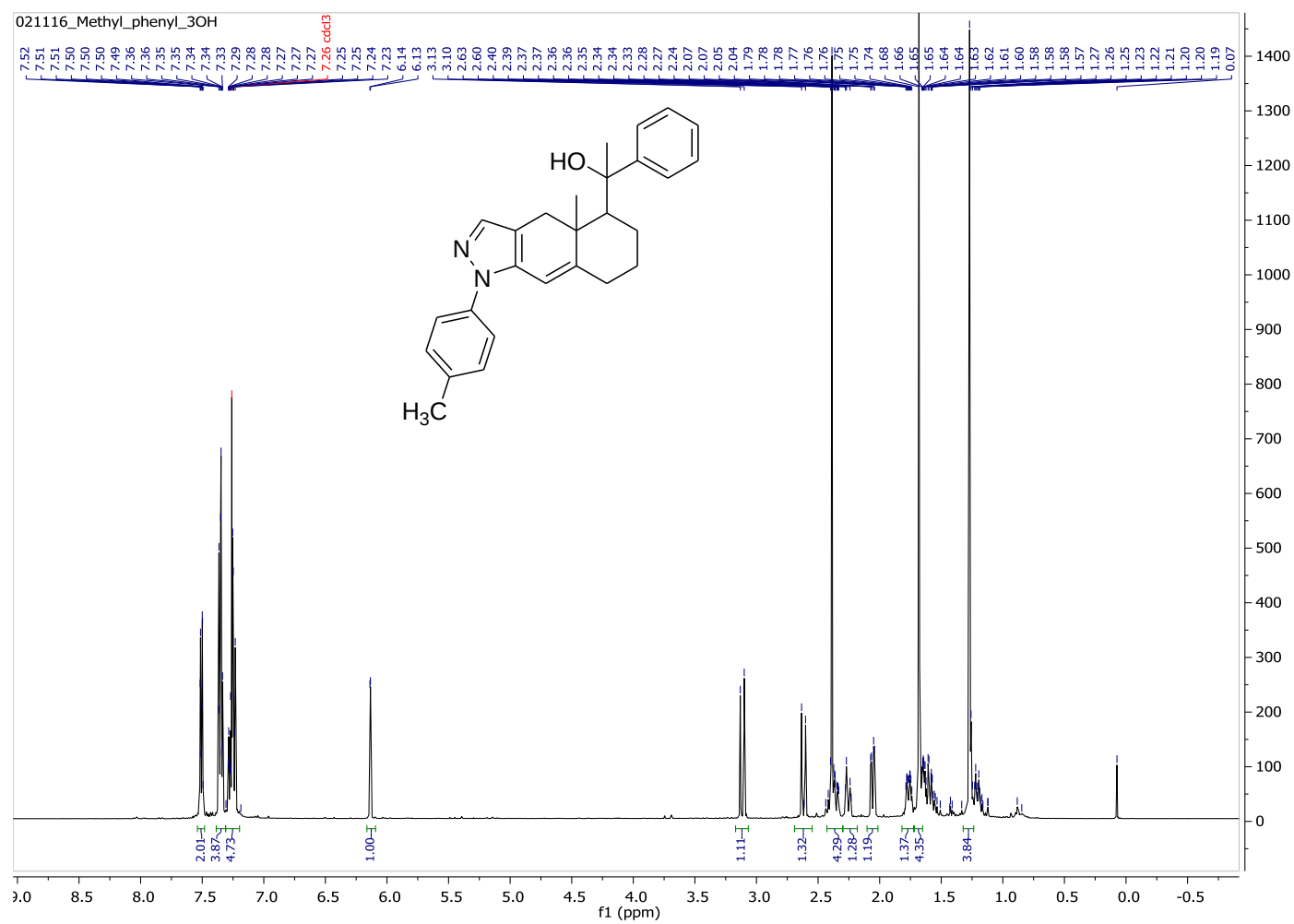
13. ^{13}C NMR spectrum of compound 11bb.



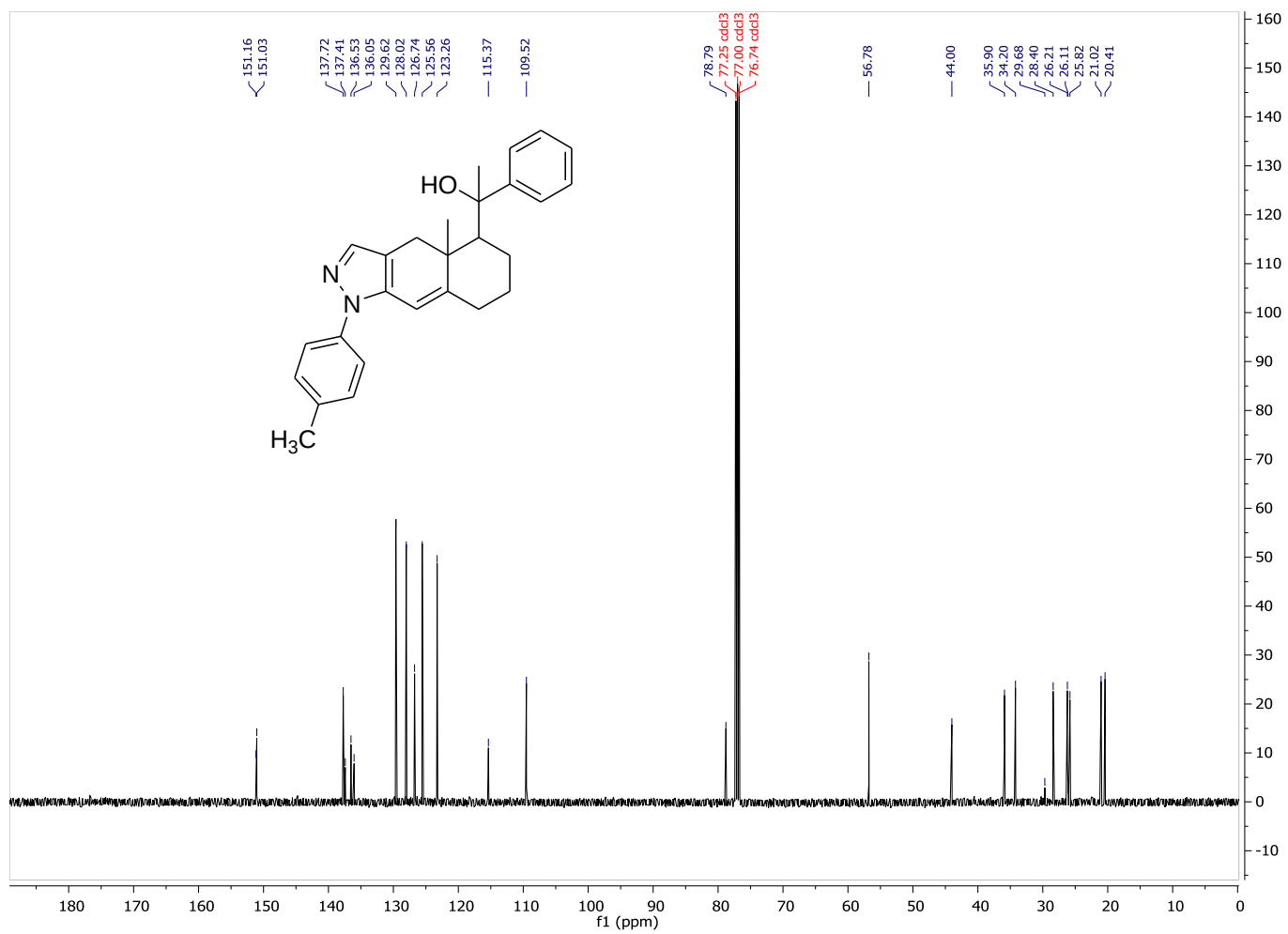
14. HRMS spectrum of compound **11bb**.



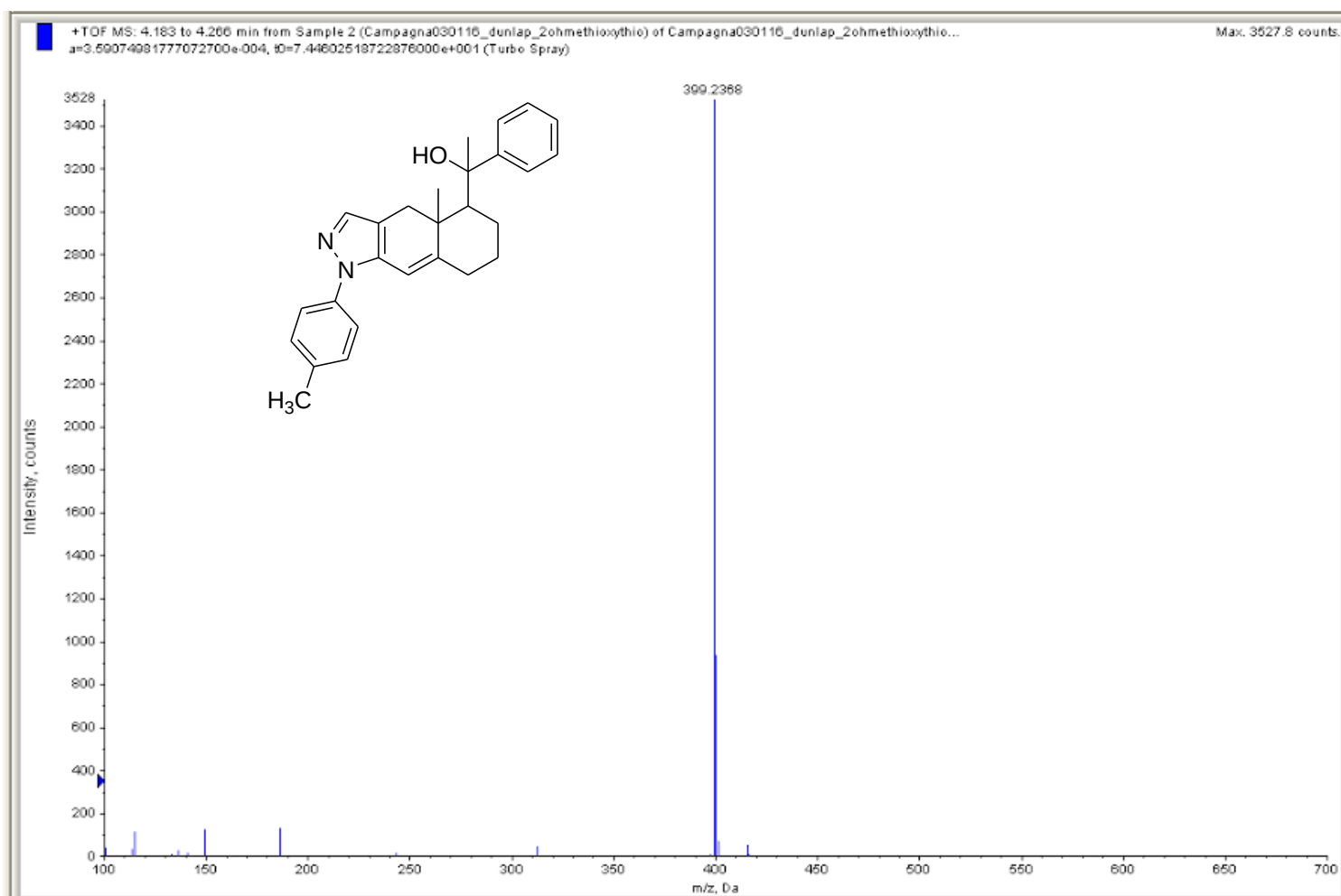
15. IR spectrum of compound **11bb**.



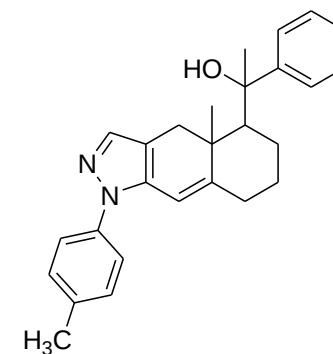
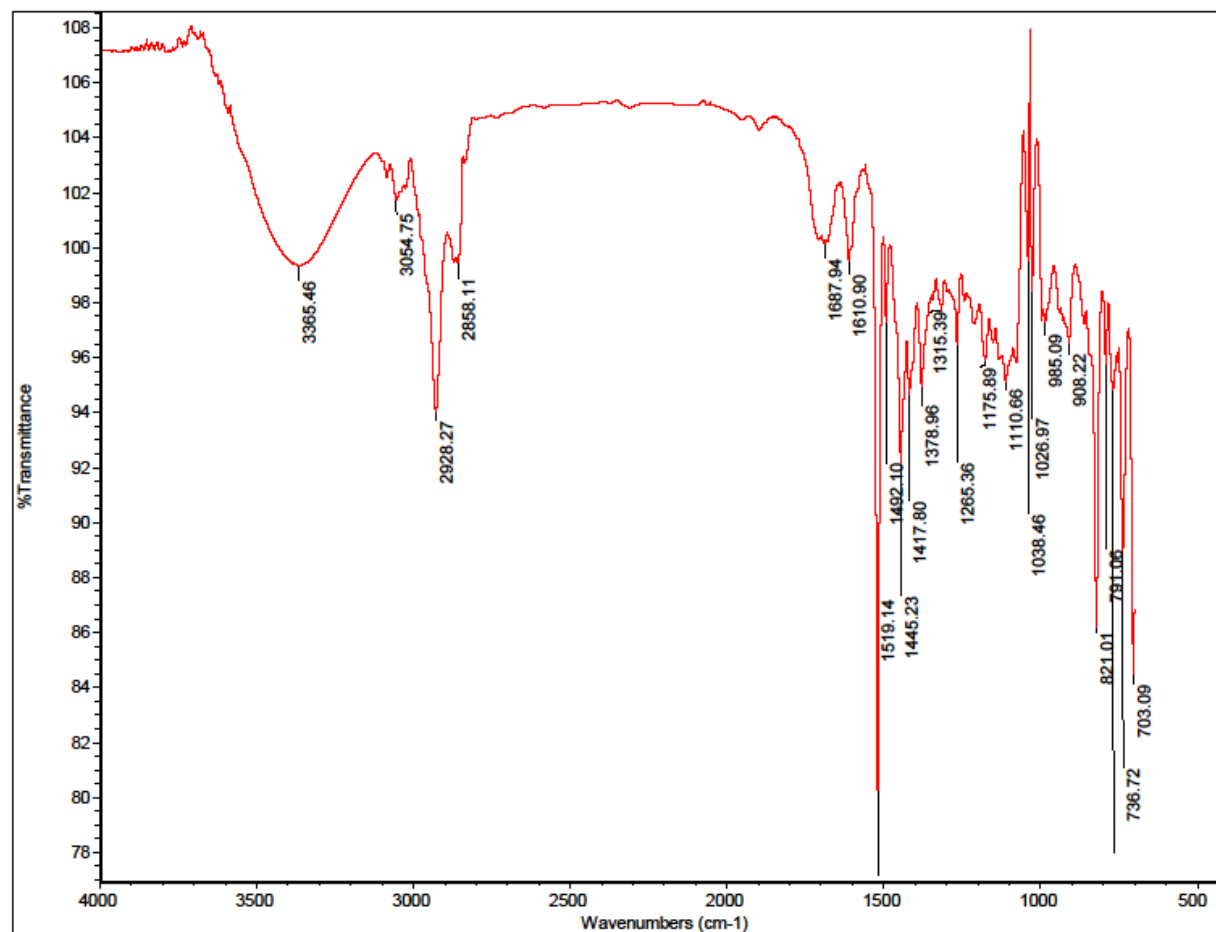
16. ¹H NMR spectrum of compound **13bb**.



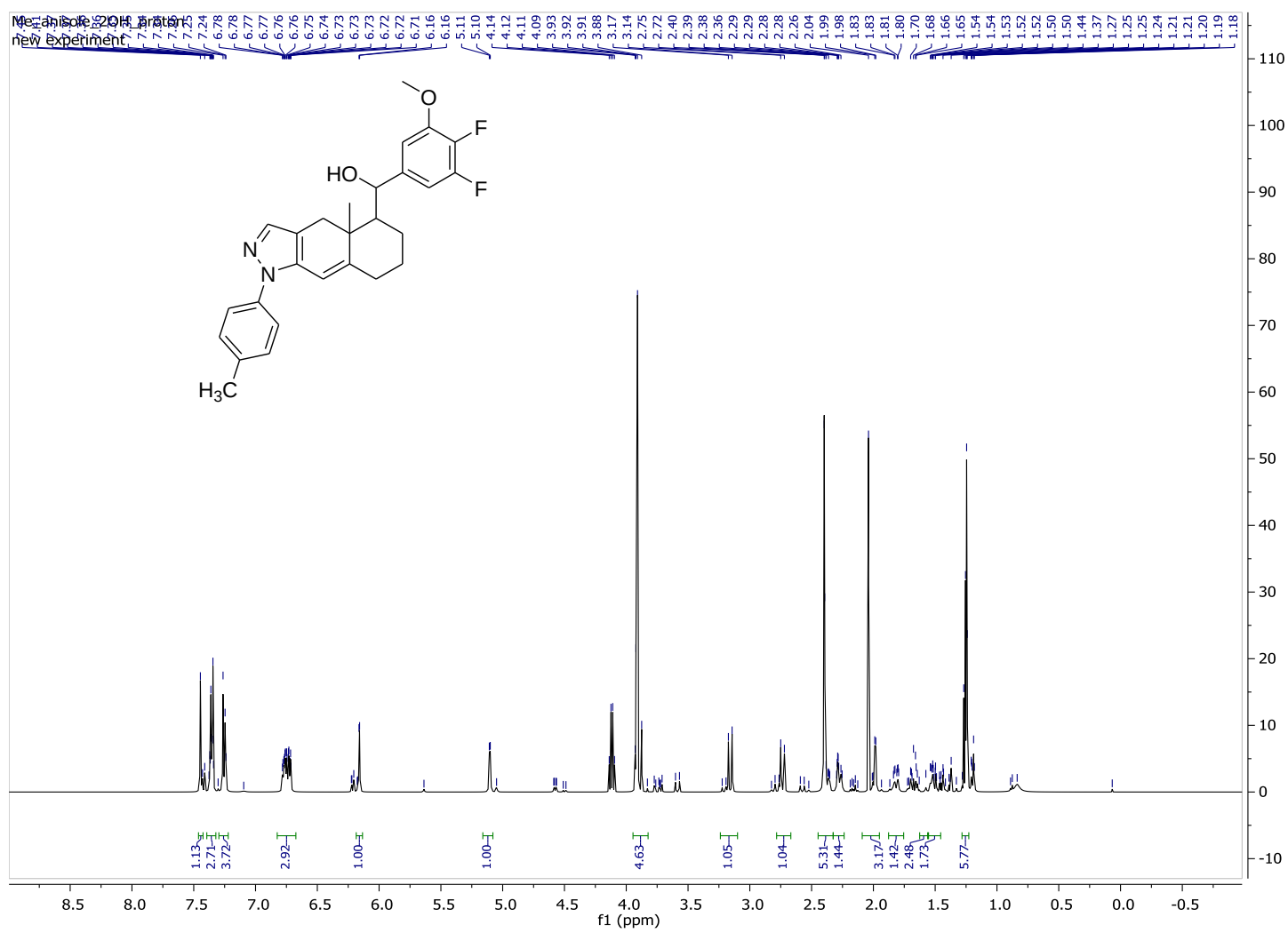
17. ¹³C NMR spectrum of compound 13bb.



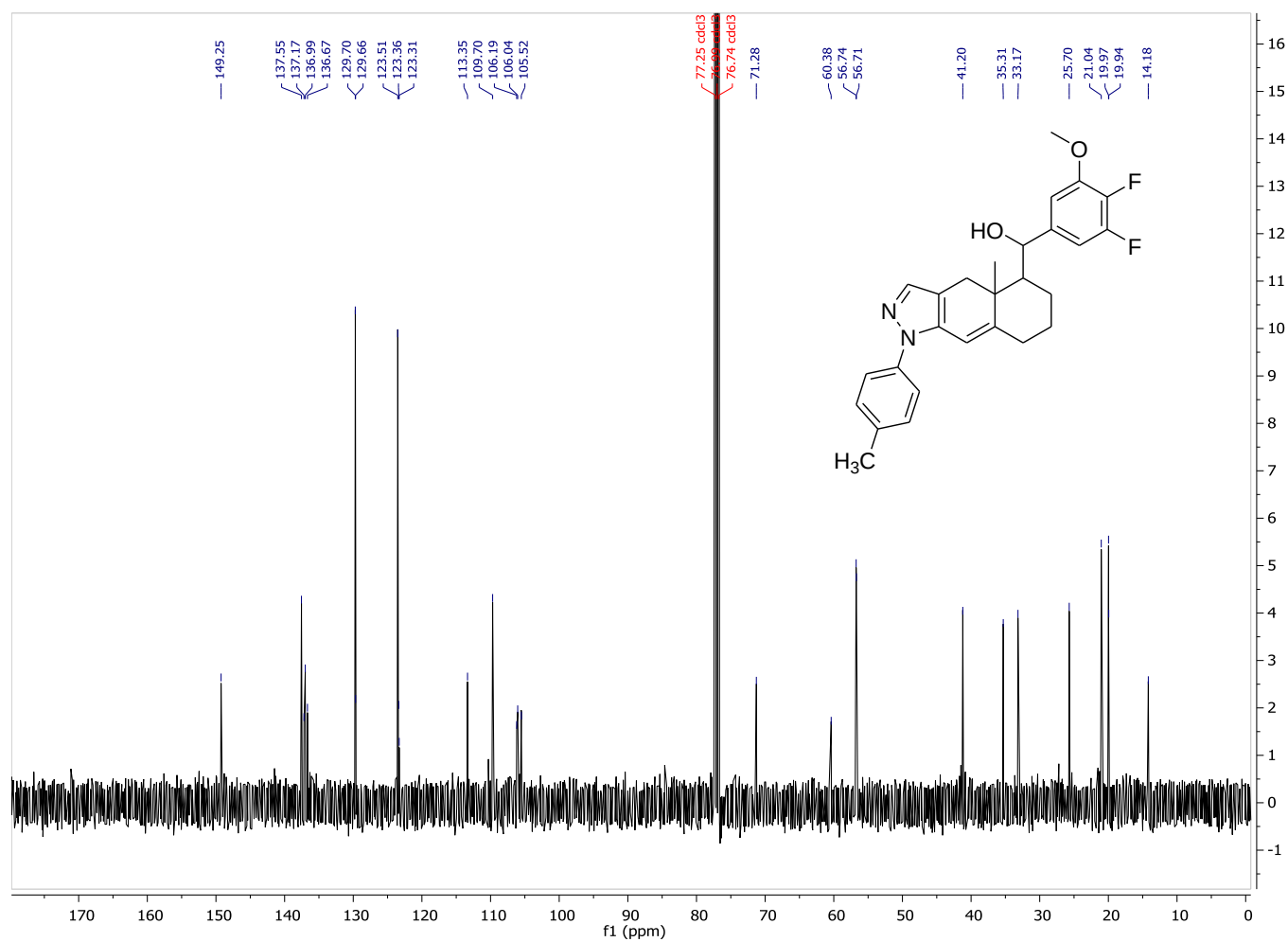
18. HRMS spectrum of compound **13bb**.



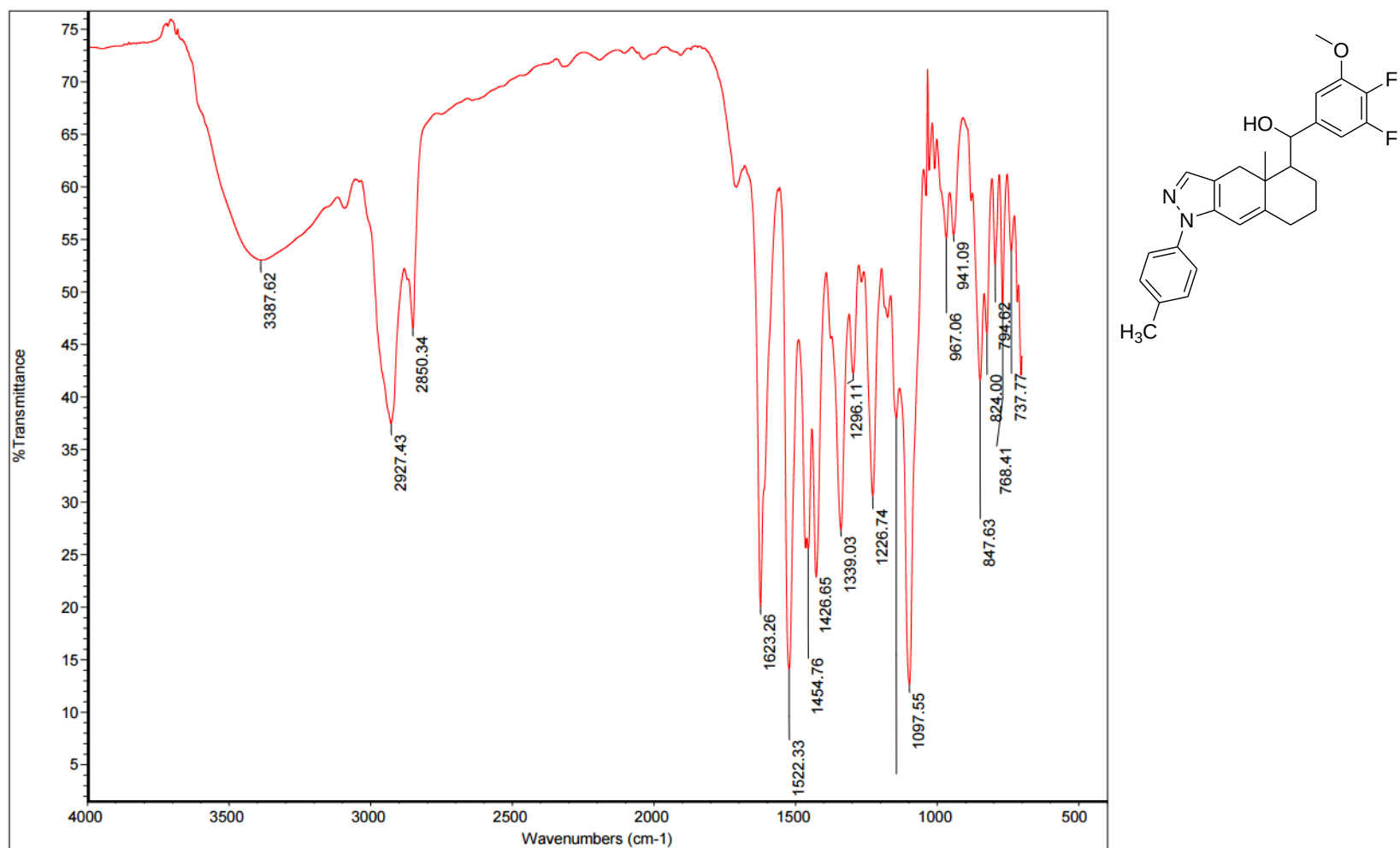
19. IR spectrum of compound **13bb**.



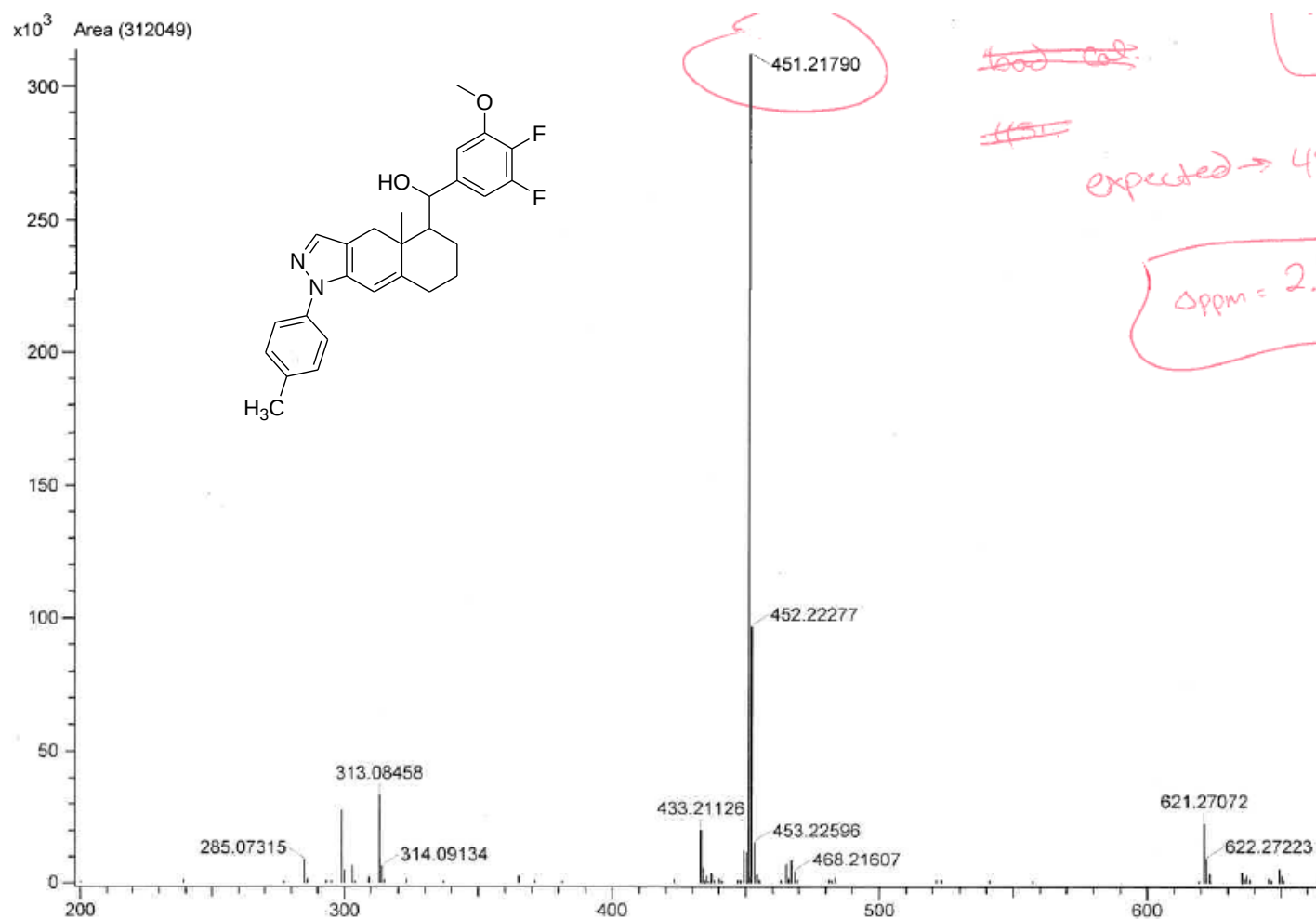
20. ^1H NMR spectrum of compound **11bd**.



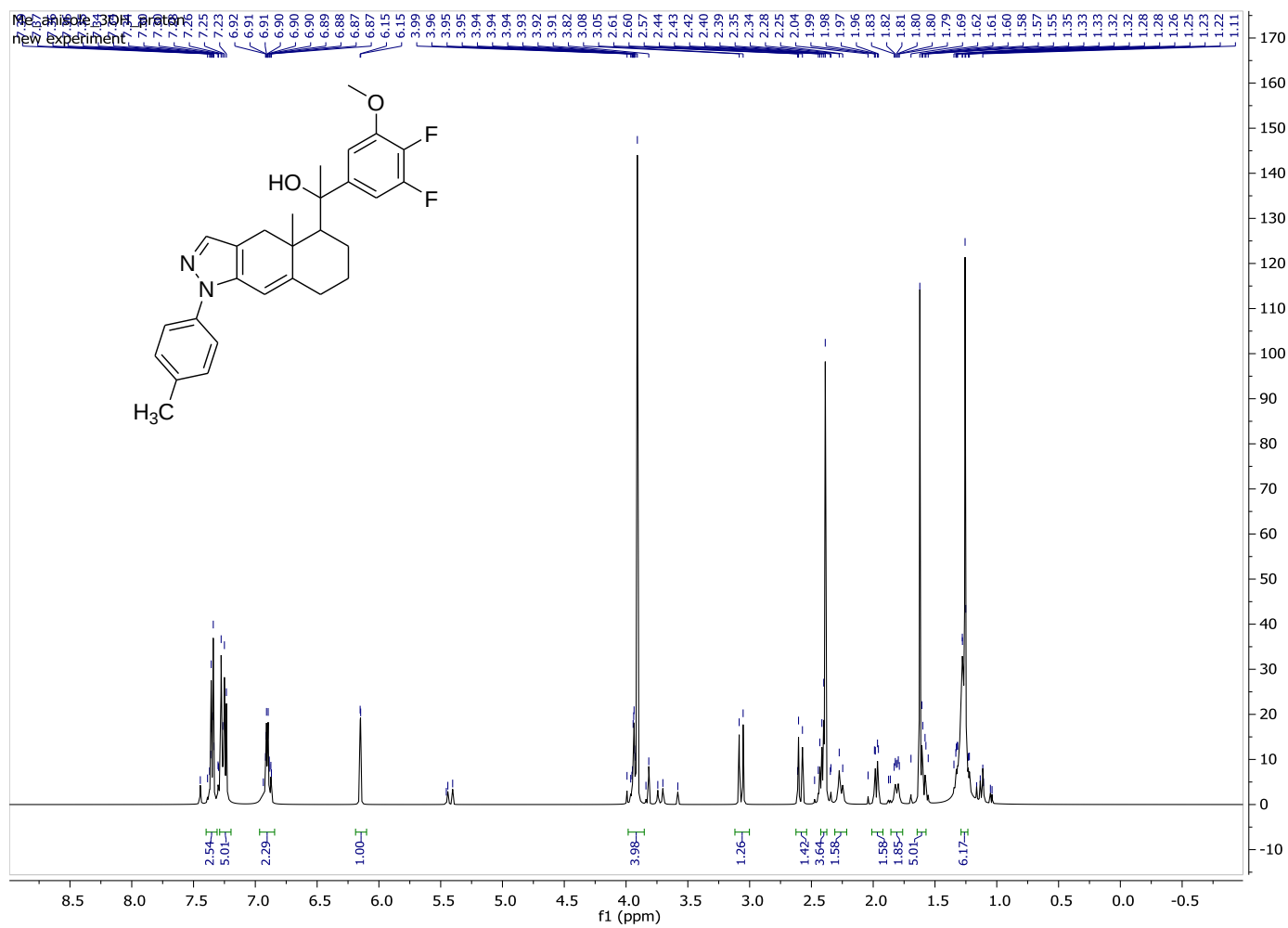
21. ¹³C NMR spectrum of compound 11bd.



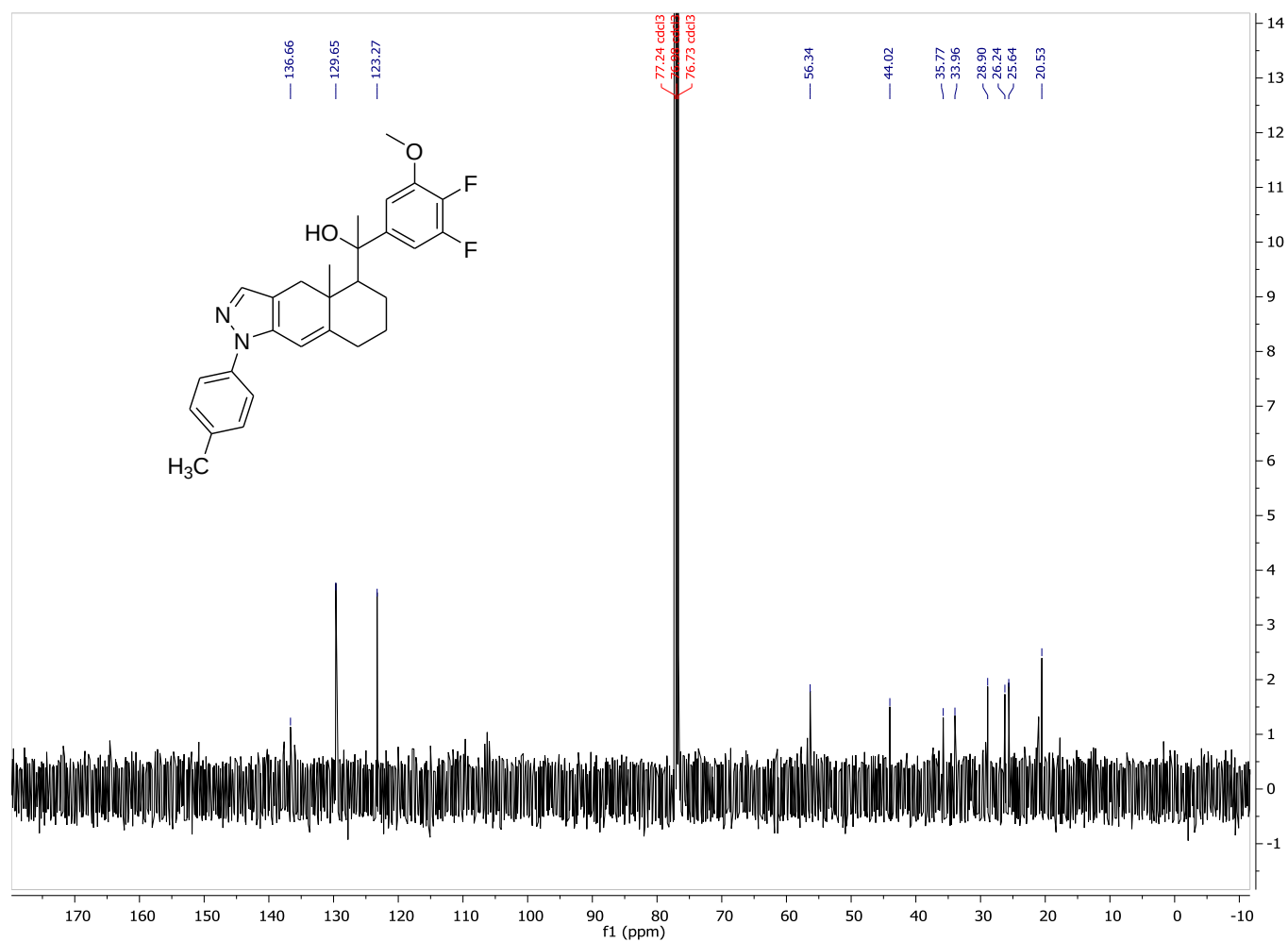
22. IR spectrum of compound **11bd**.



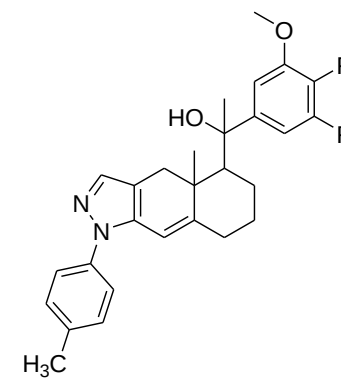
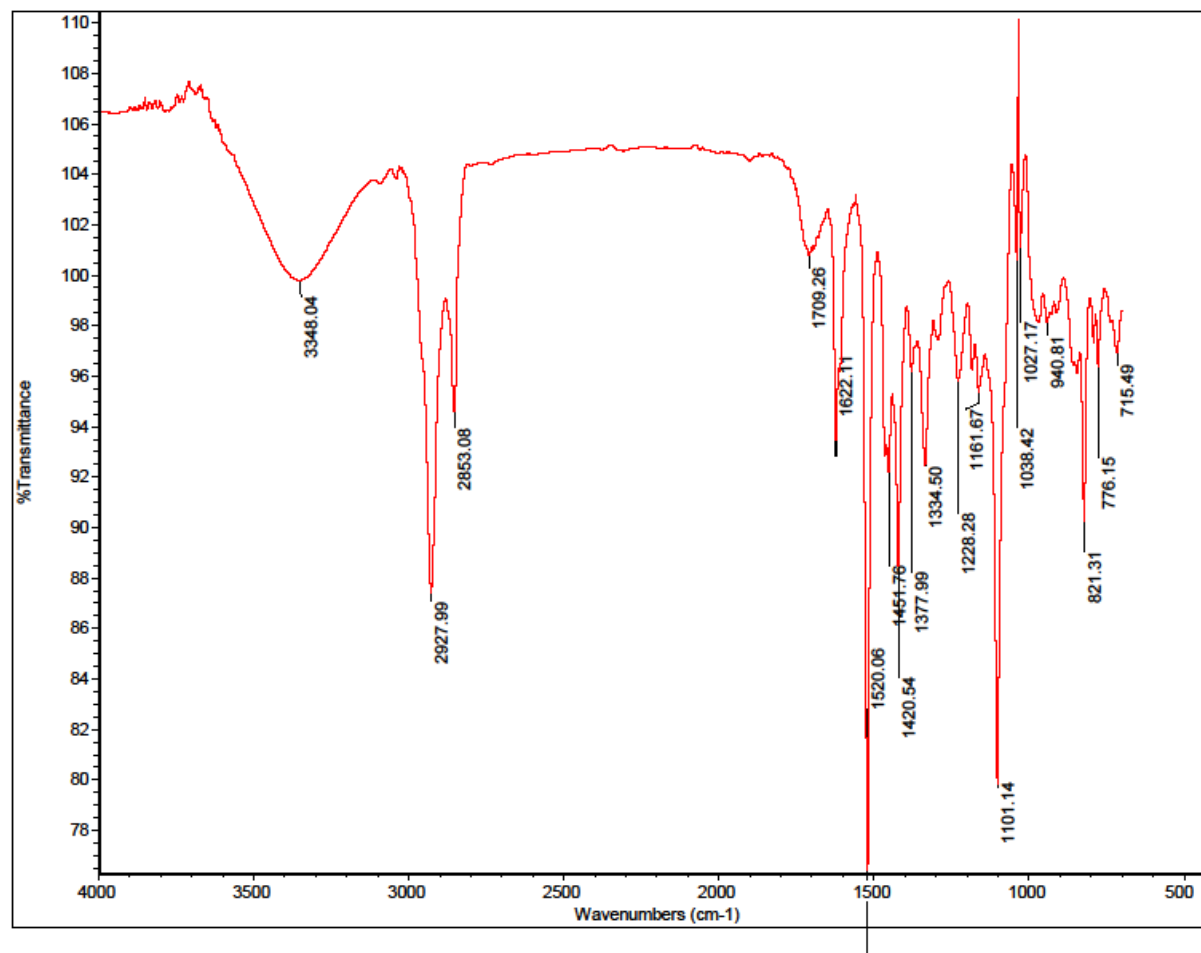
23. HRMS spectrum of compound **11bd**.



24. ^1H NMR spectrum of compound **13bd**.



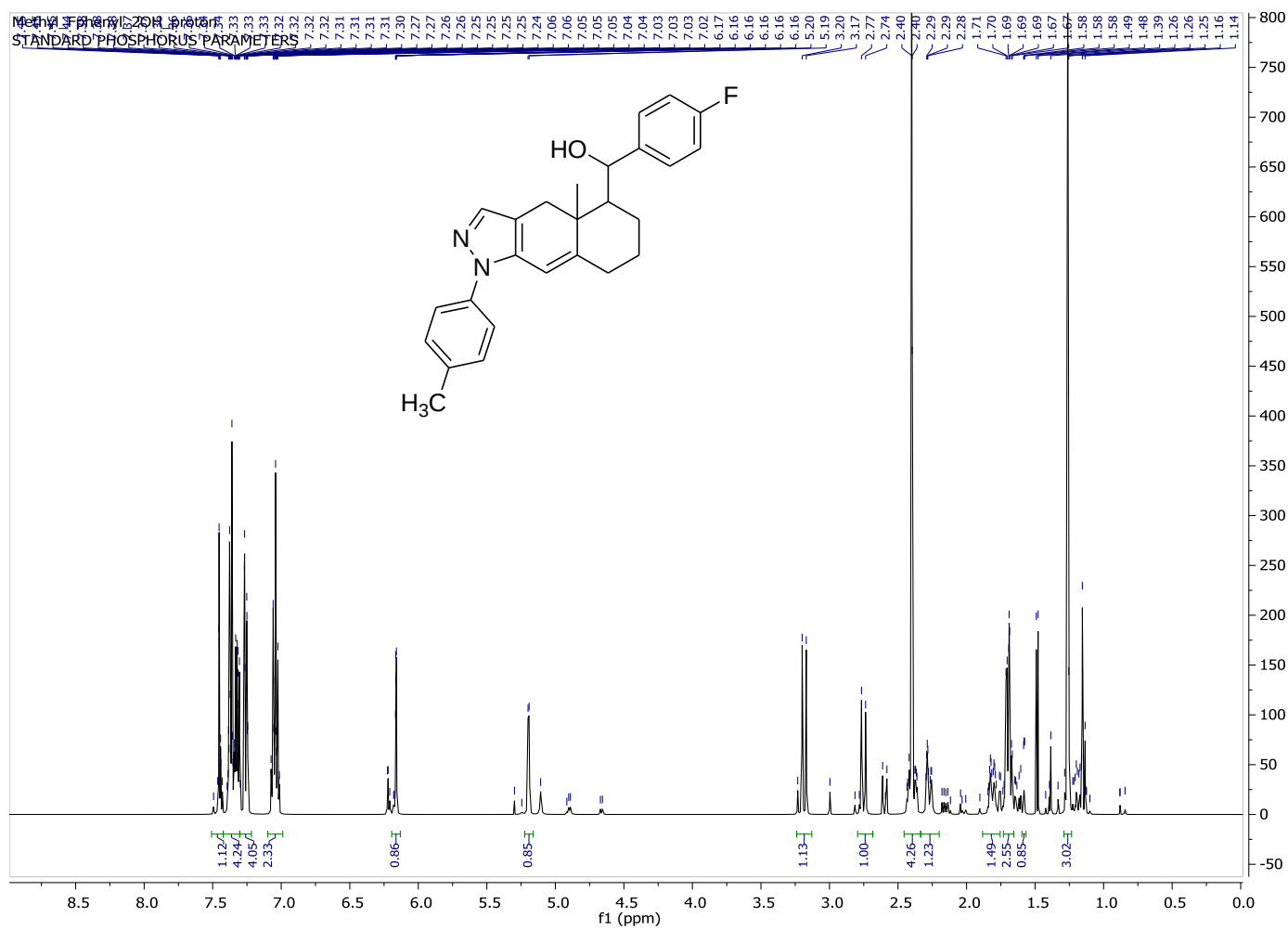
25. ¹³C NMR spectrum of compound 13bd.



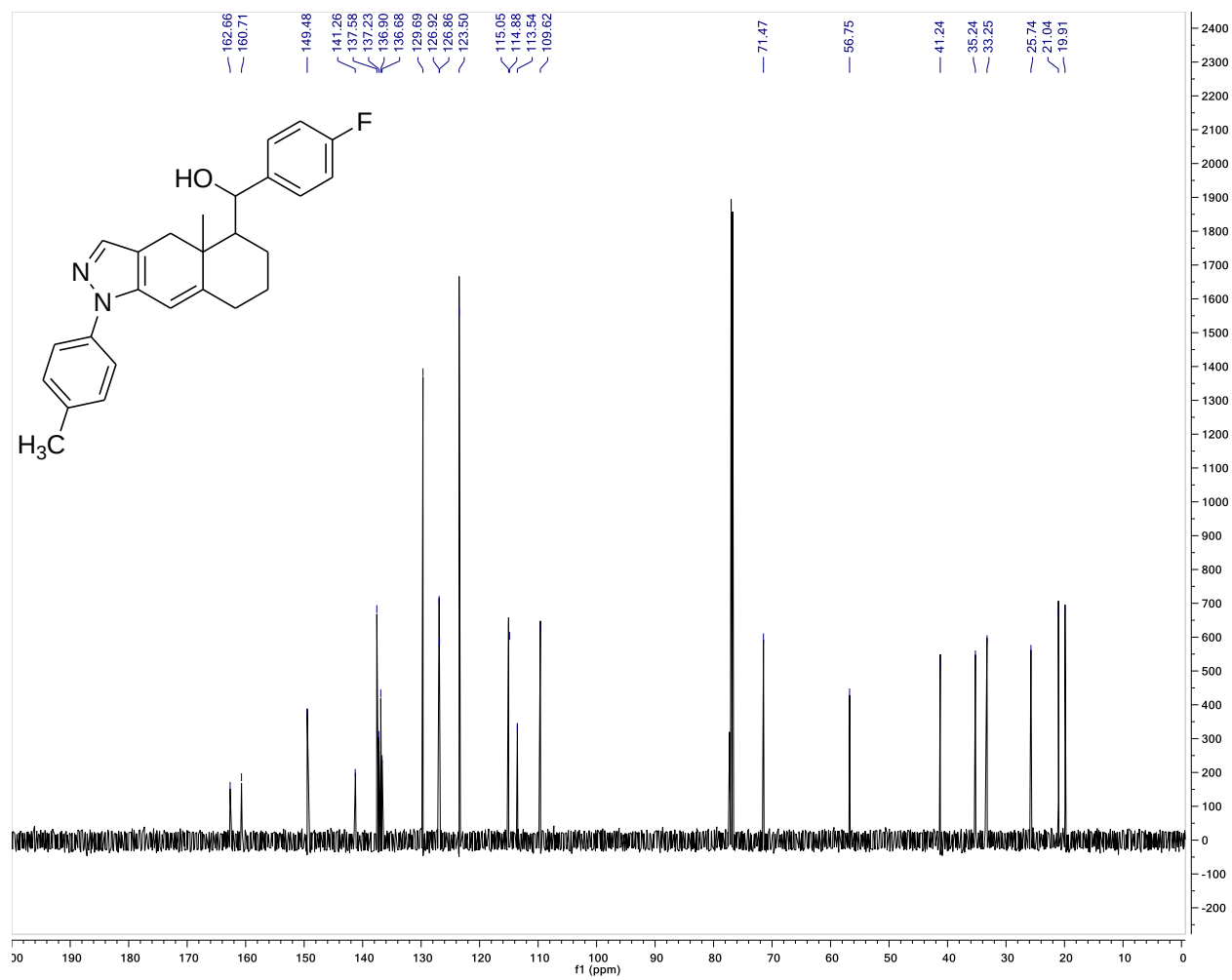
26. IR spectrum of compound **13bd**.



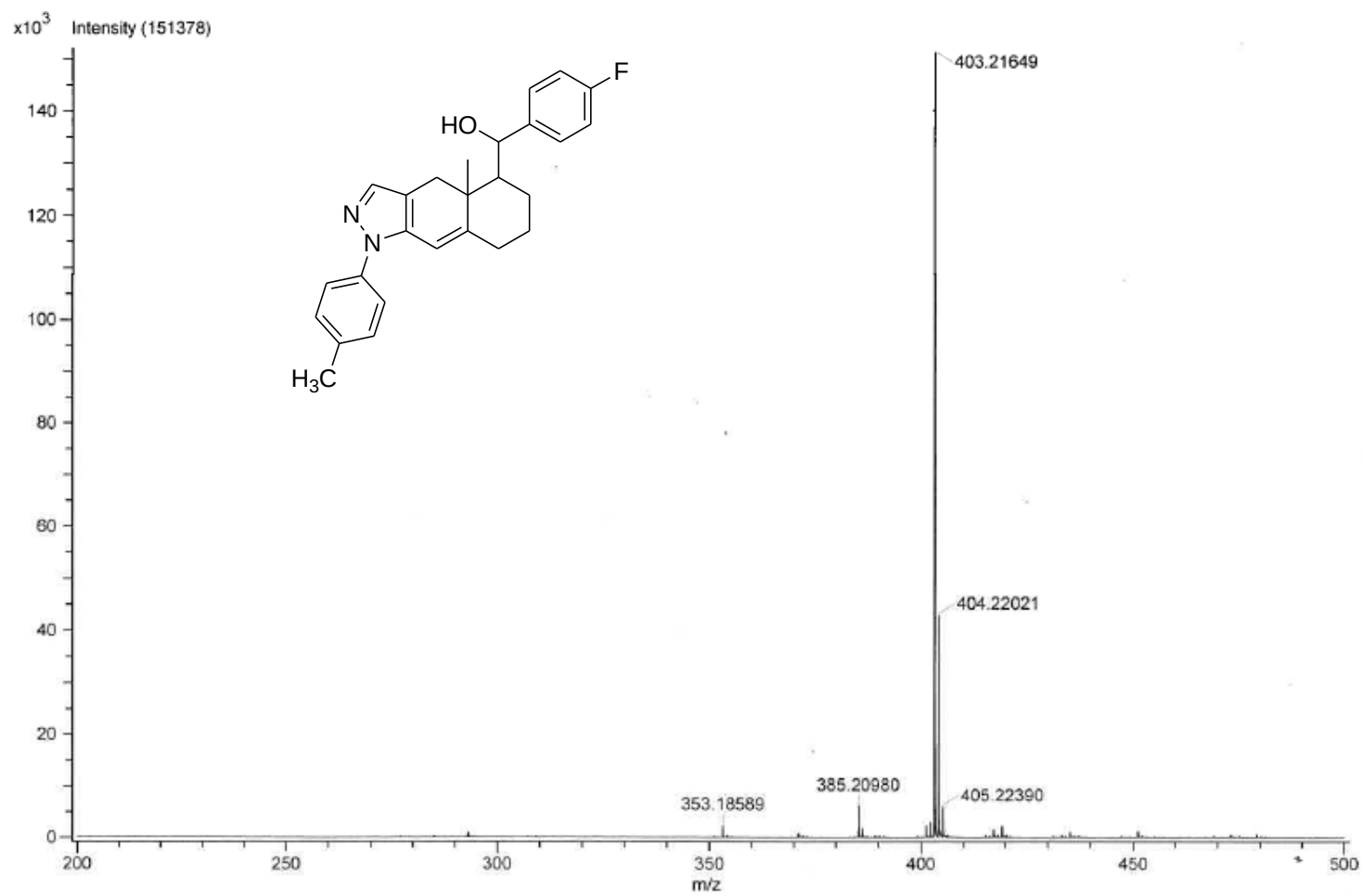
27. HRMS spectrum of compound **13bd**.



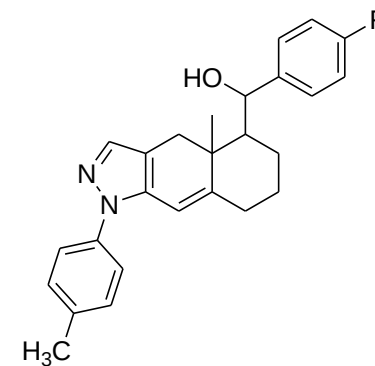
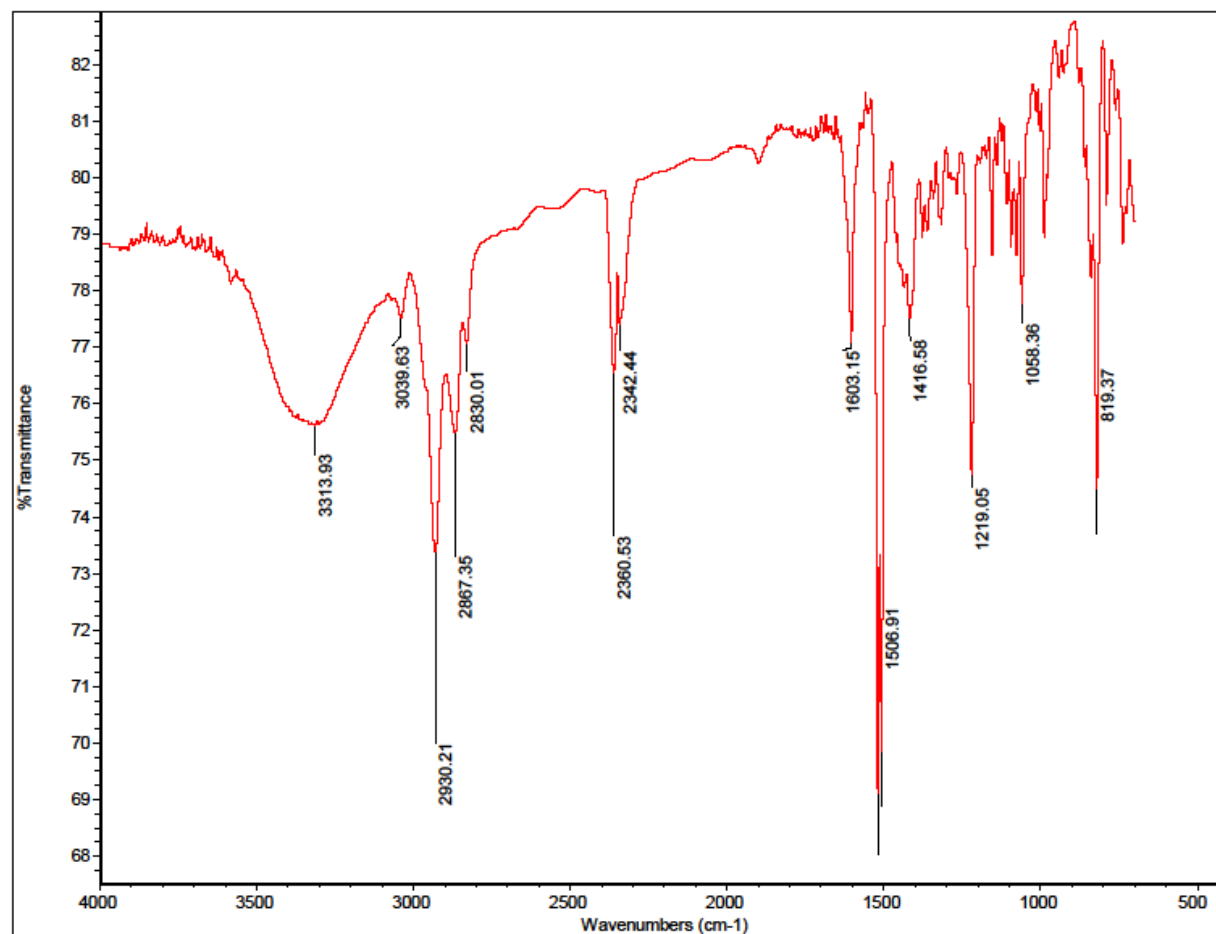
28. ^1H NMR spectrum of compound **11bc**.



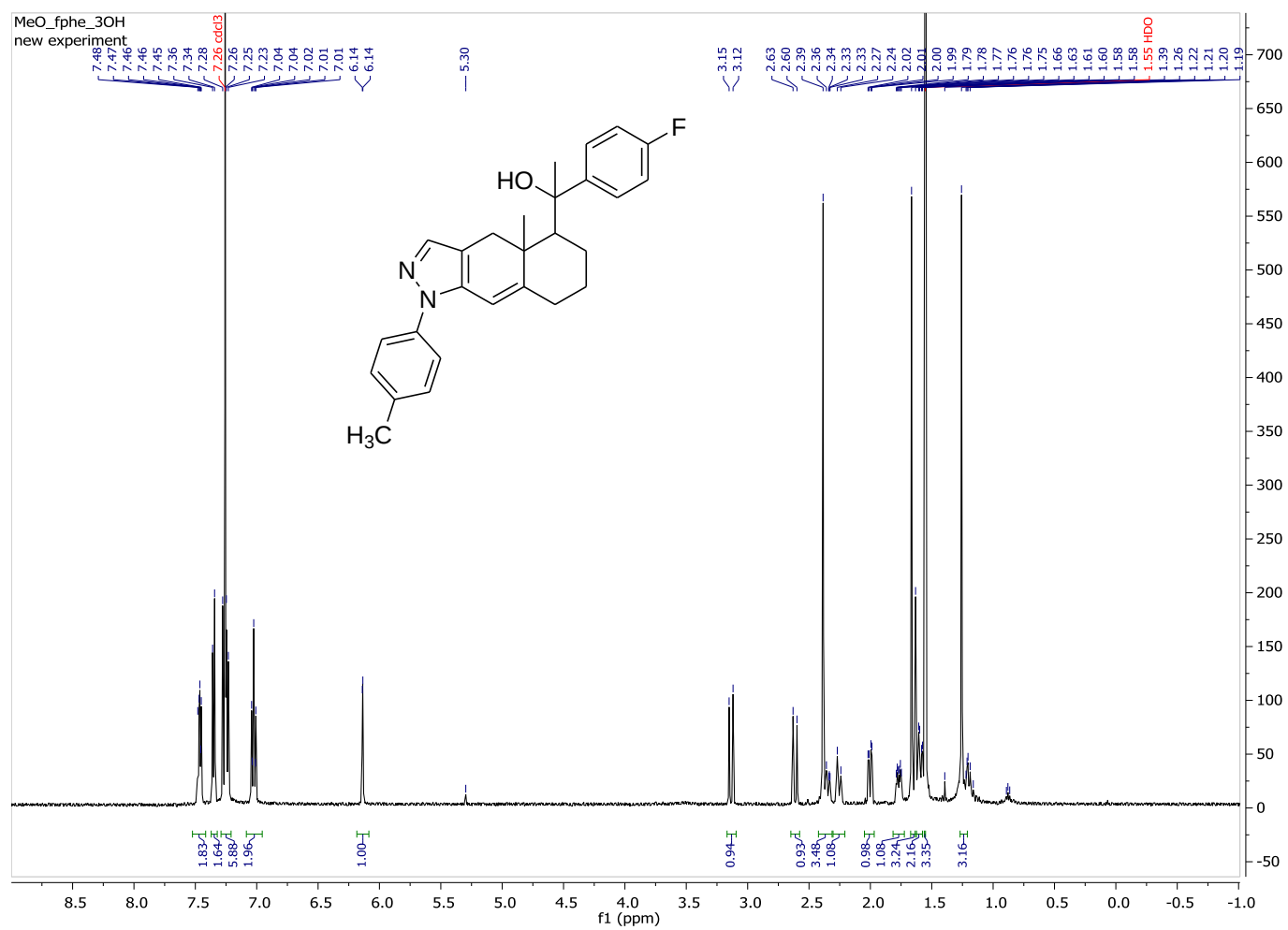
29. ^{13}C NMR spectrum of compound **11bc**.



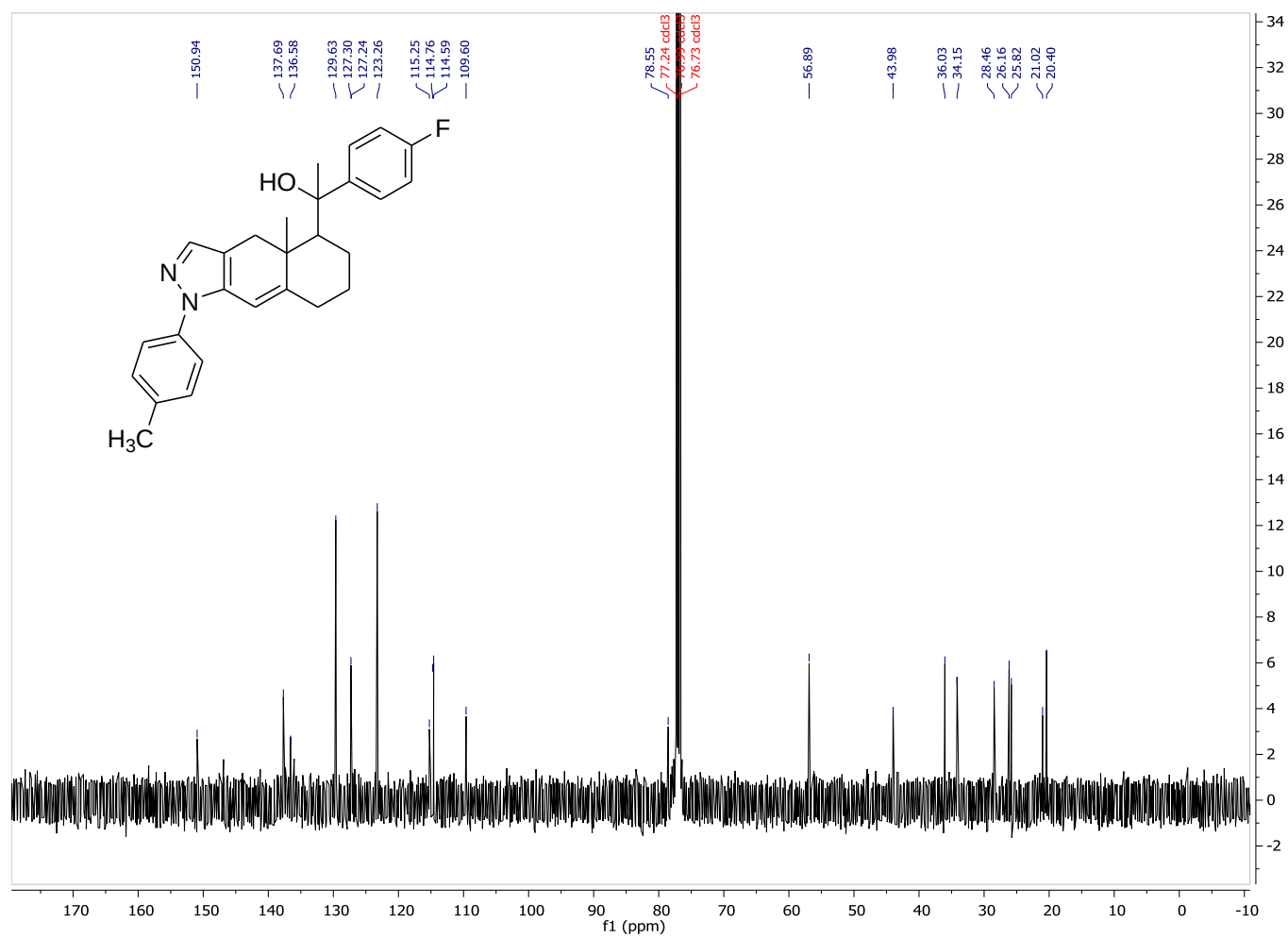
30. HRMS spectrum of compound **11bc**.



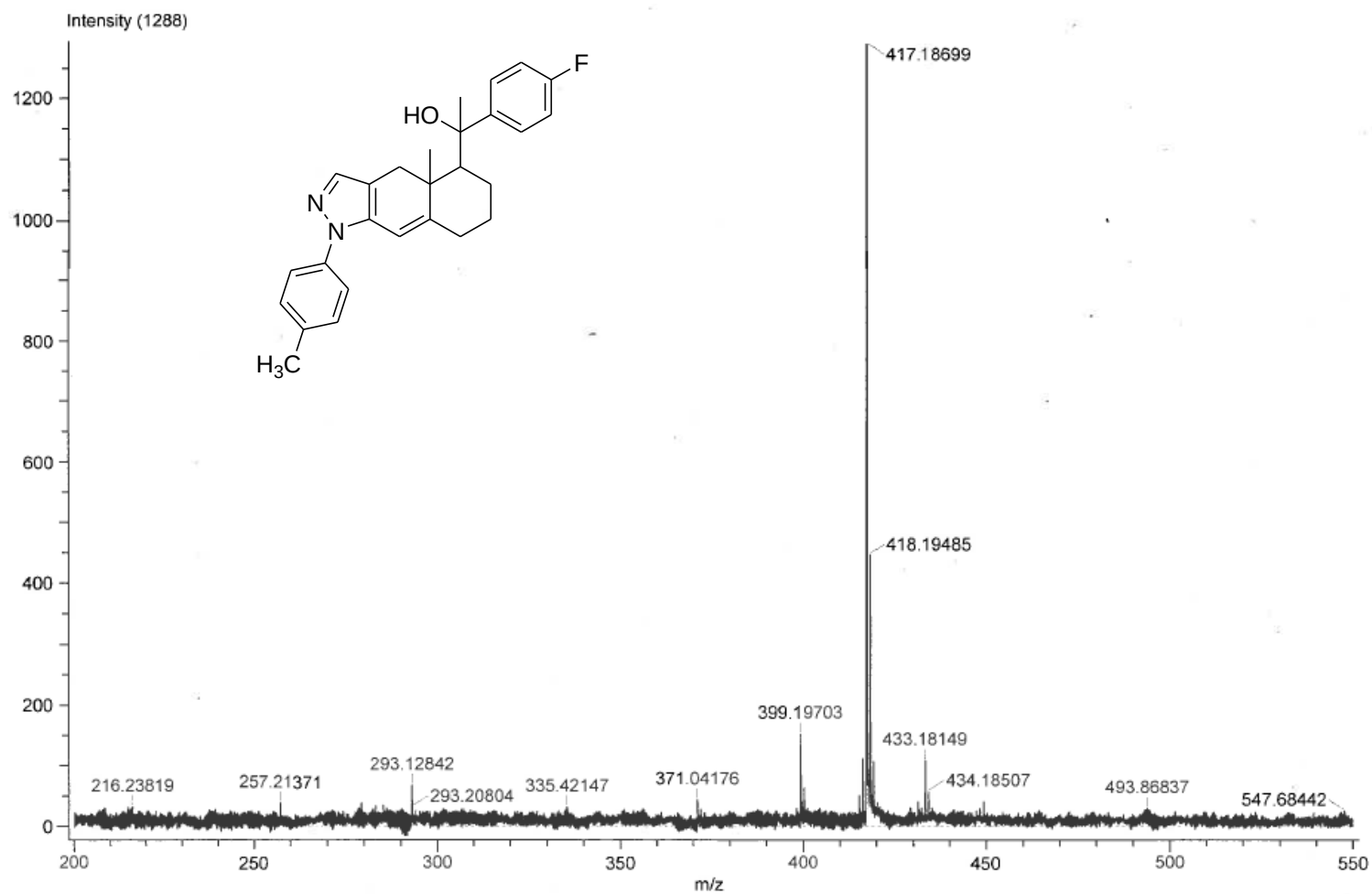
31. IR spectrum of compound **11bc**.



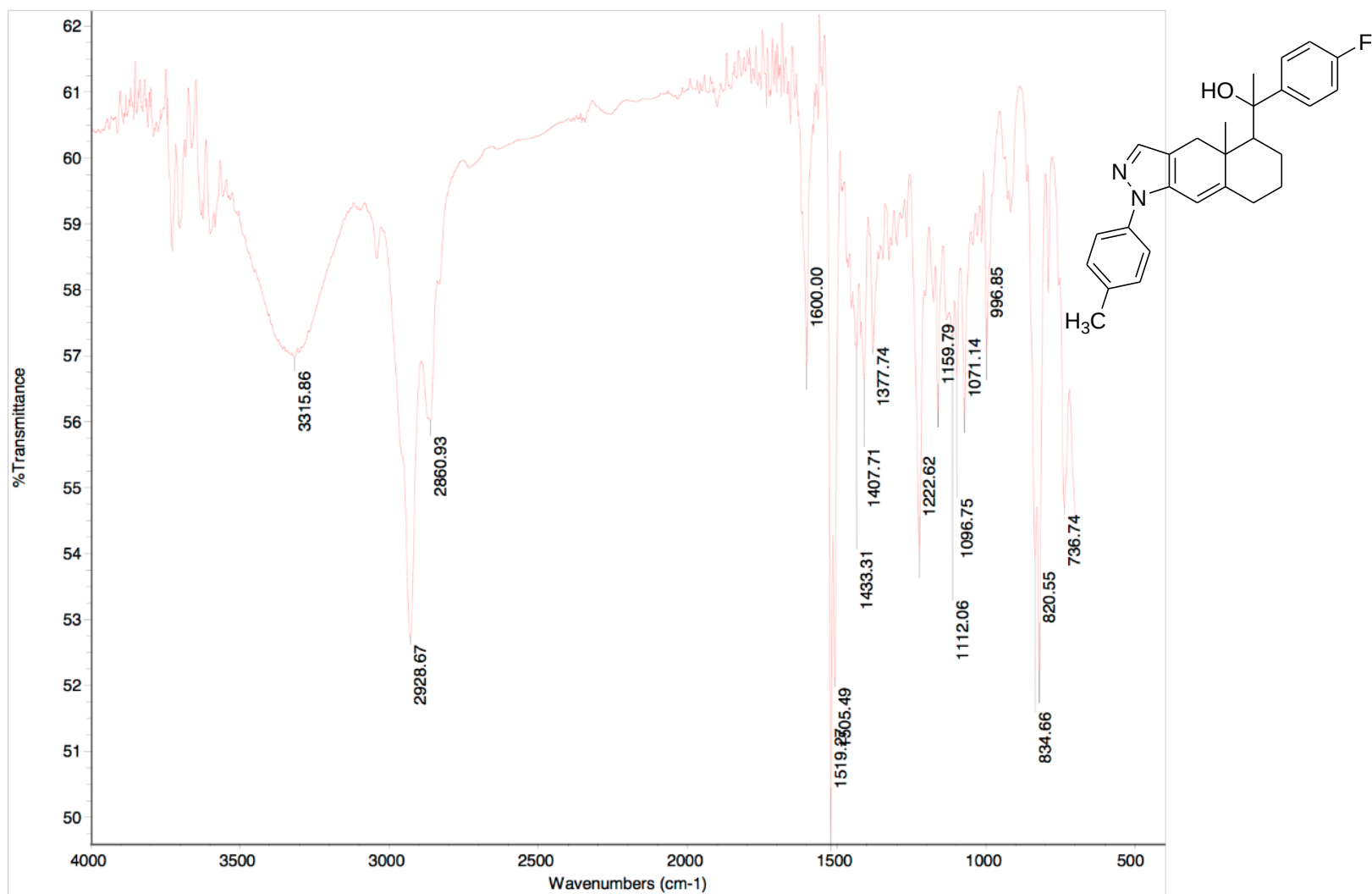
32. ^1H NMR spectrum of compound **13bc**.



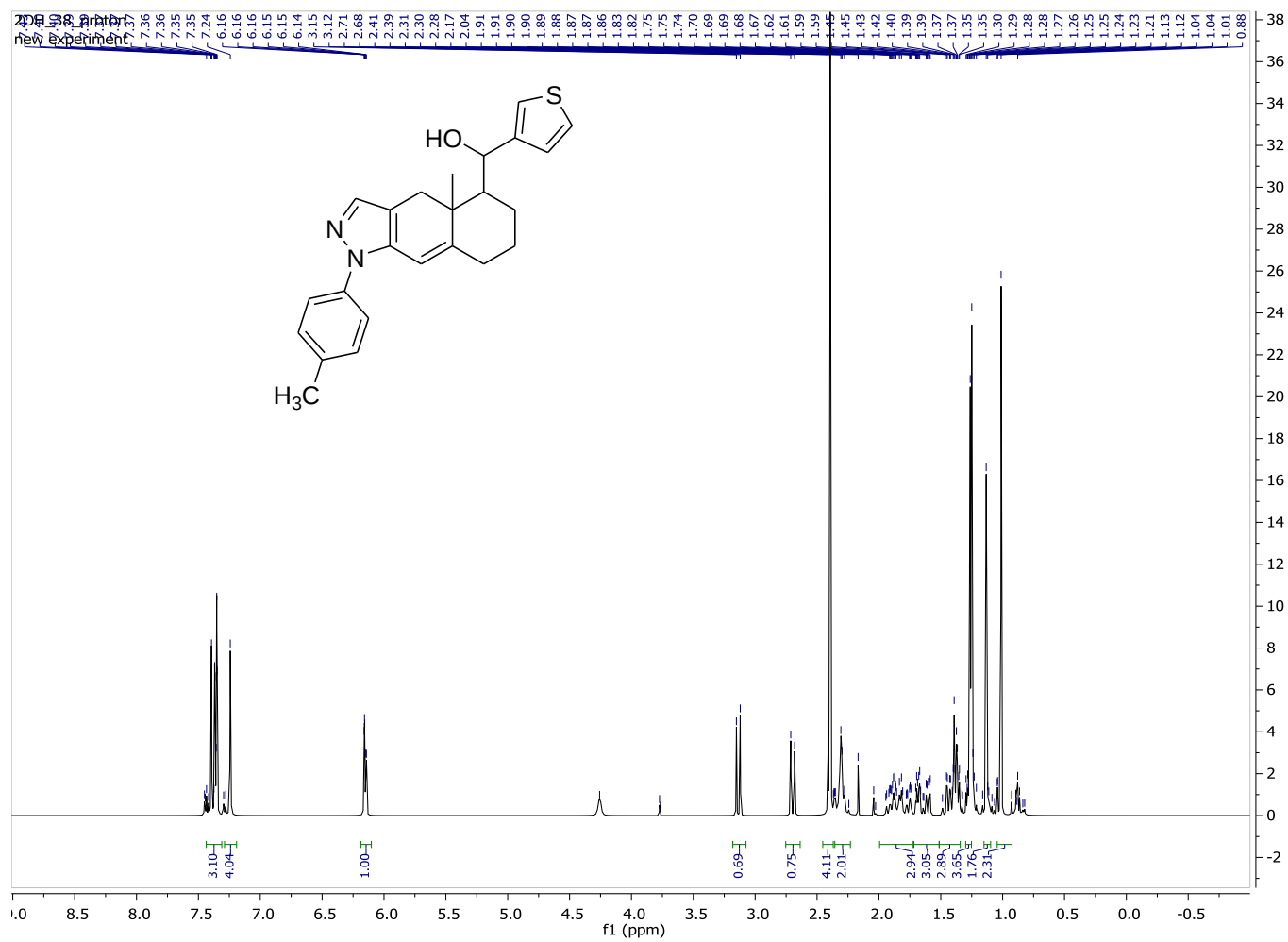
33. ¹³C NMR spectrum of compound **13bc**.



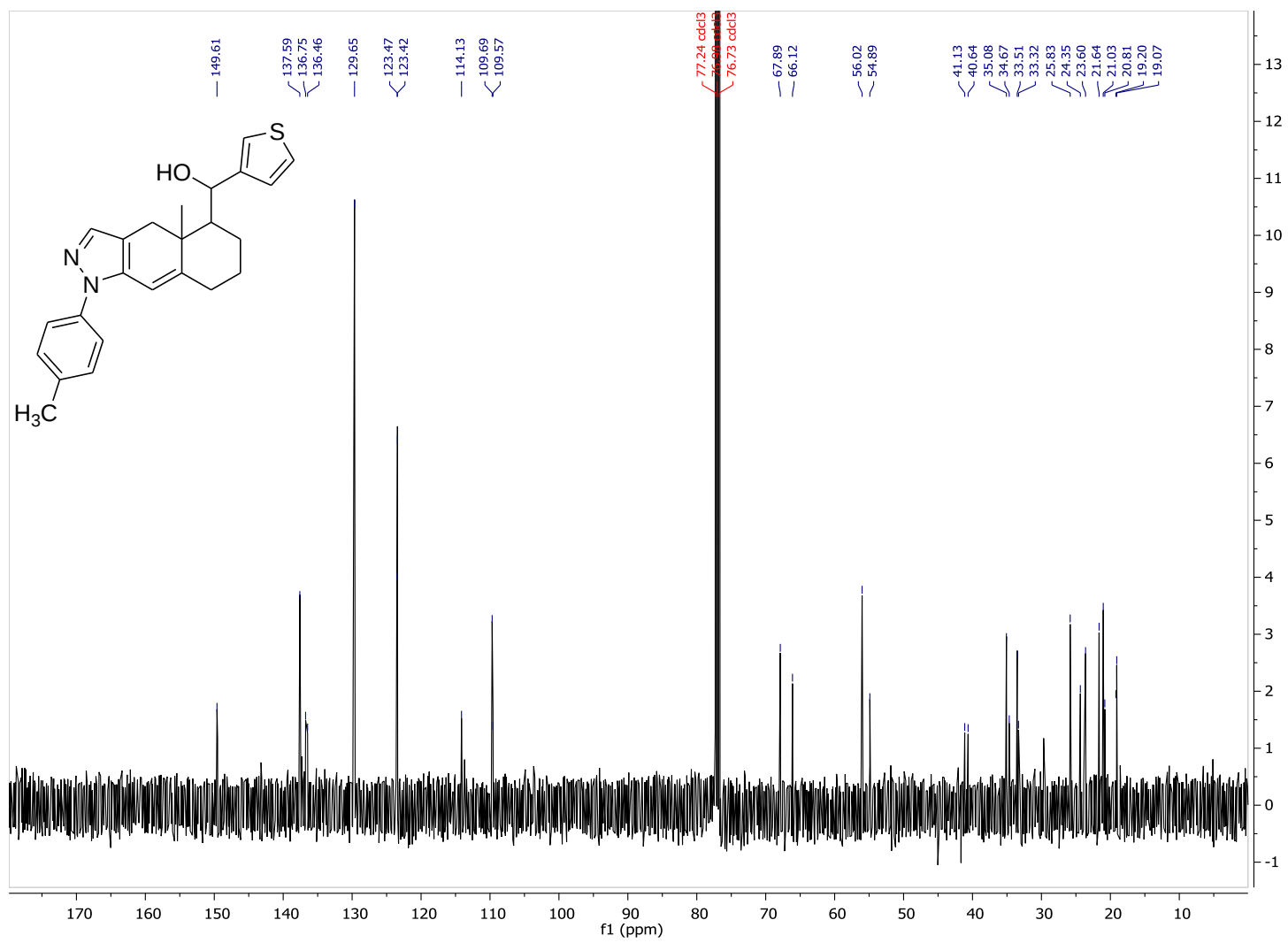
34. HRMS spectrum of compound **13bc**.



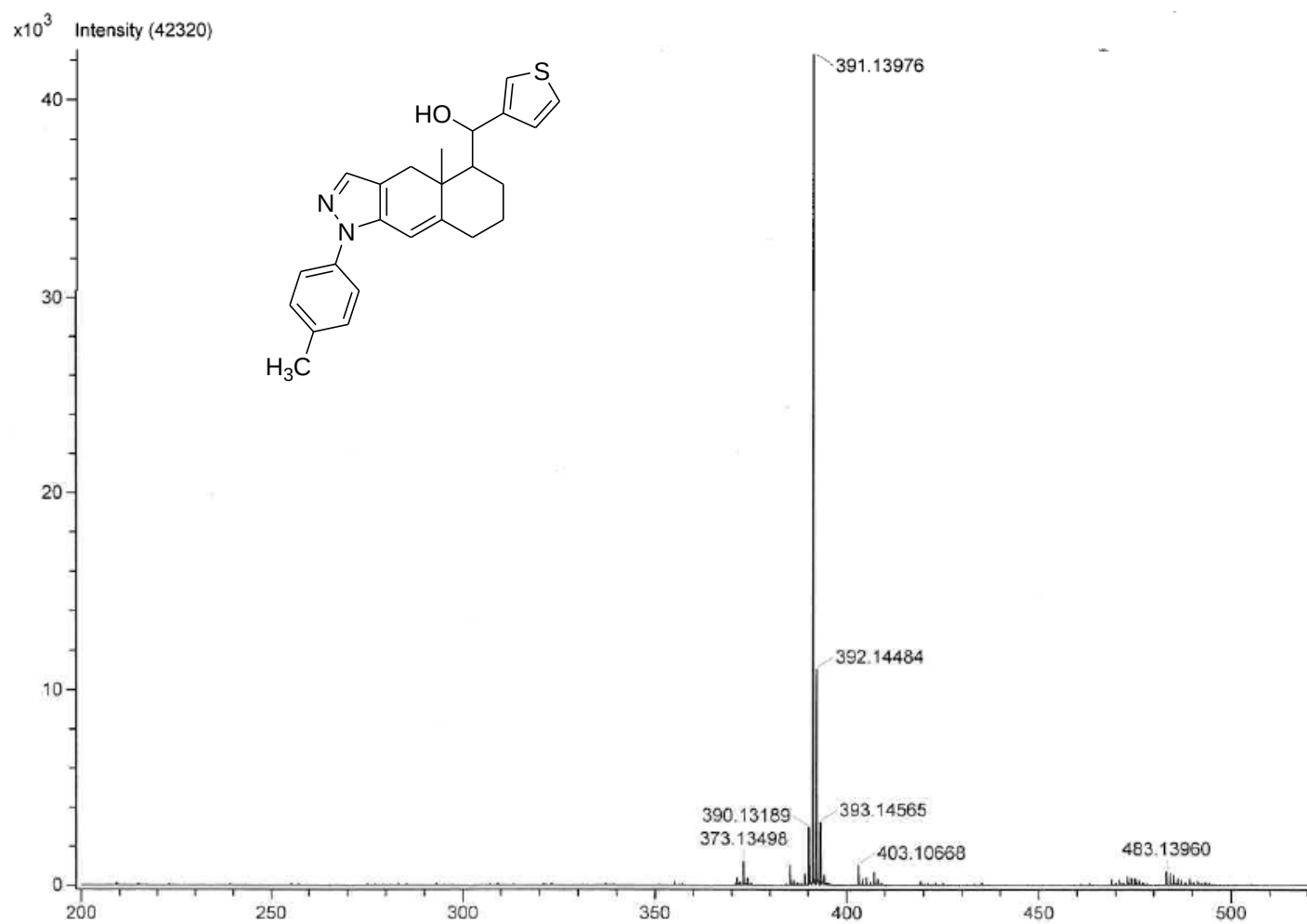
35. IR spectrum of compound **13bc**.



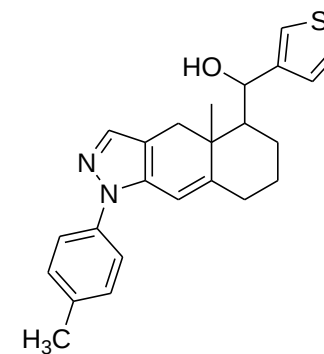
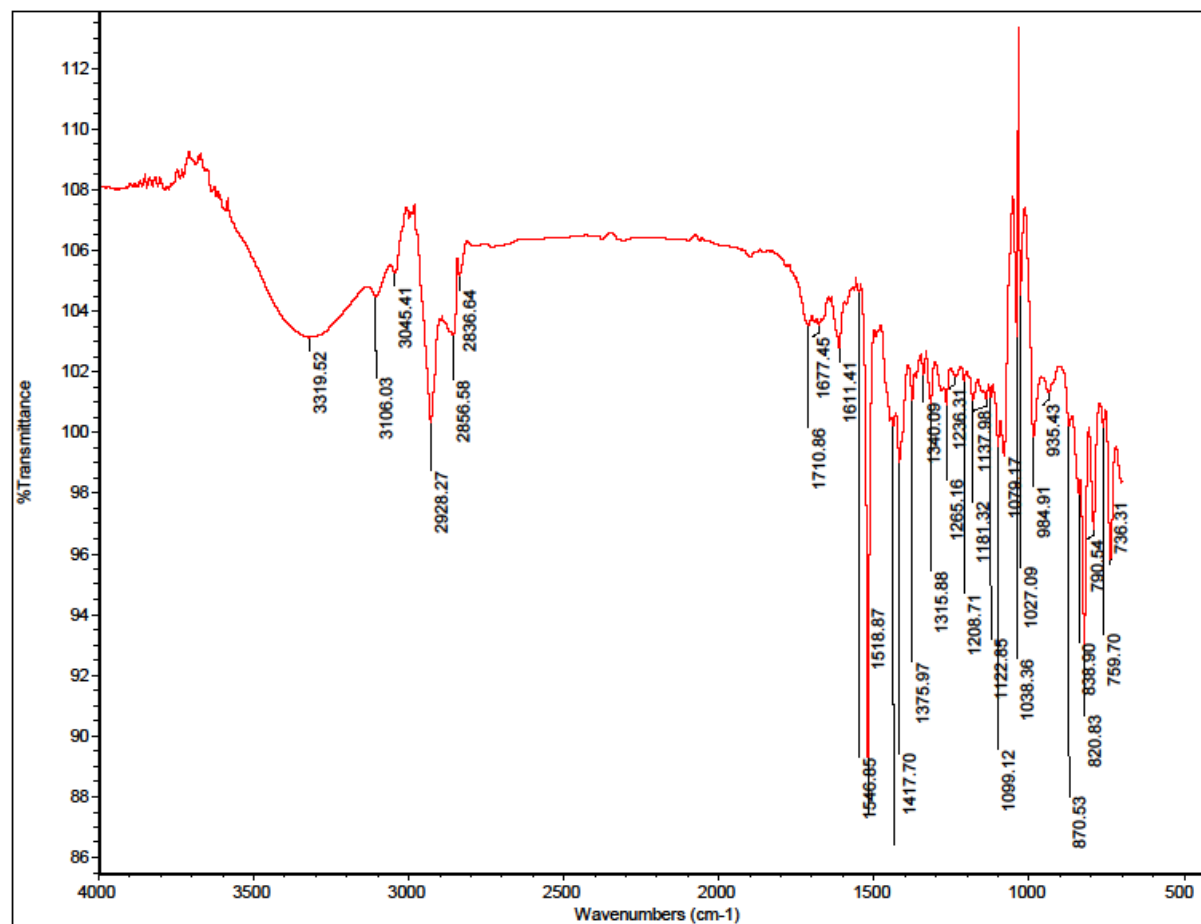
36. ^1H NMR spectrum of compound **11be**.



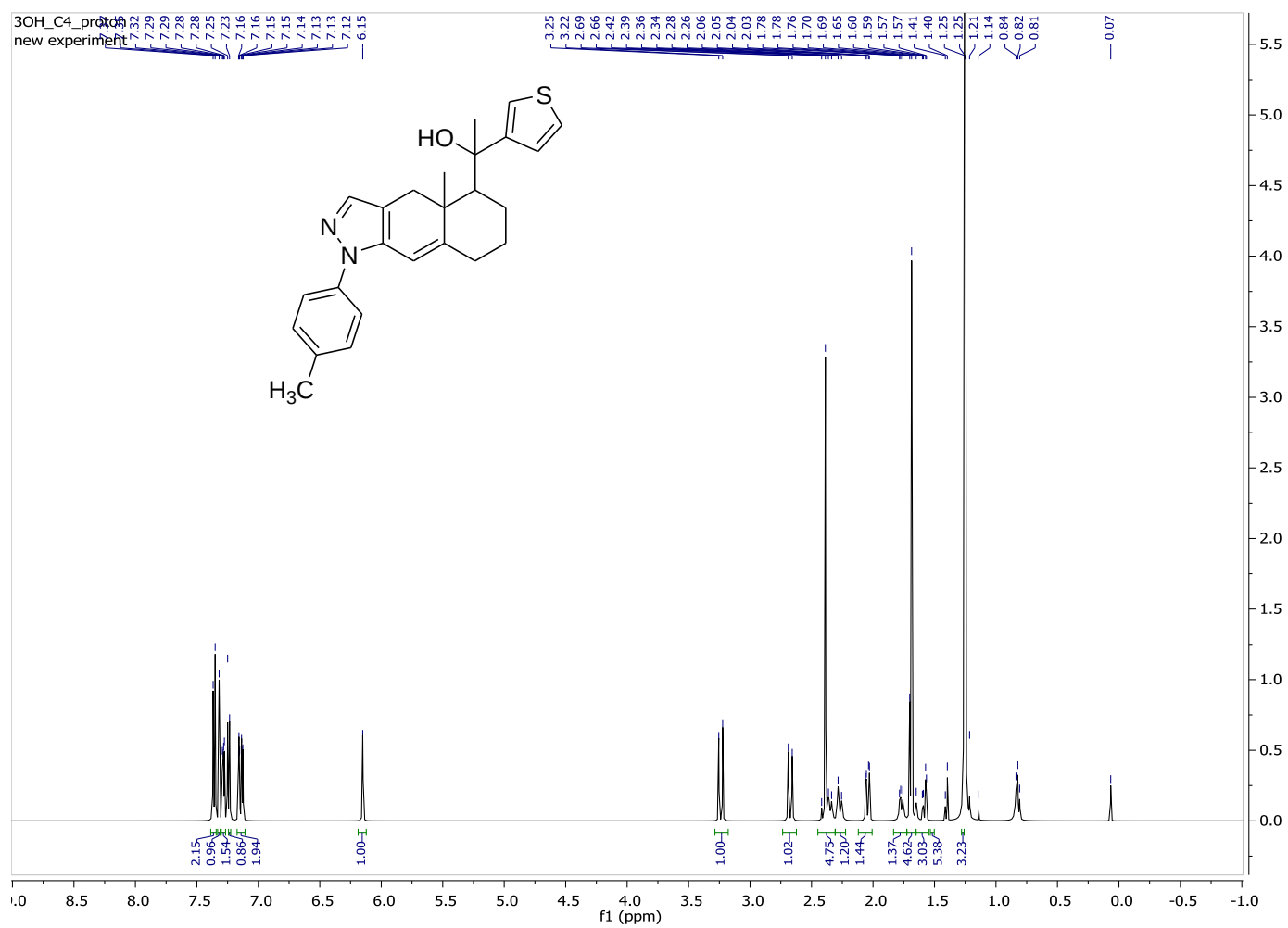
37. ¹³C NMR spectrum of compound **11be**.



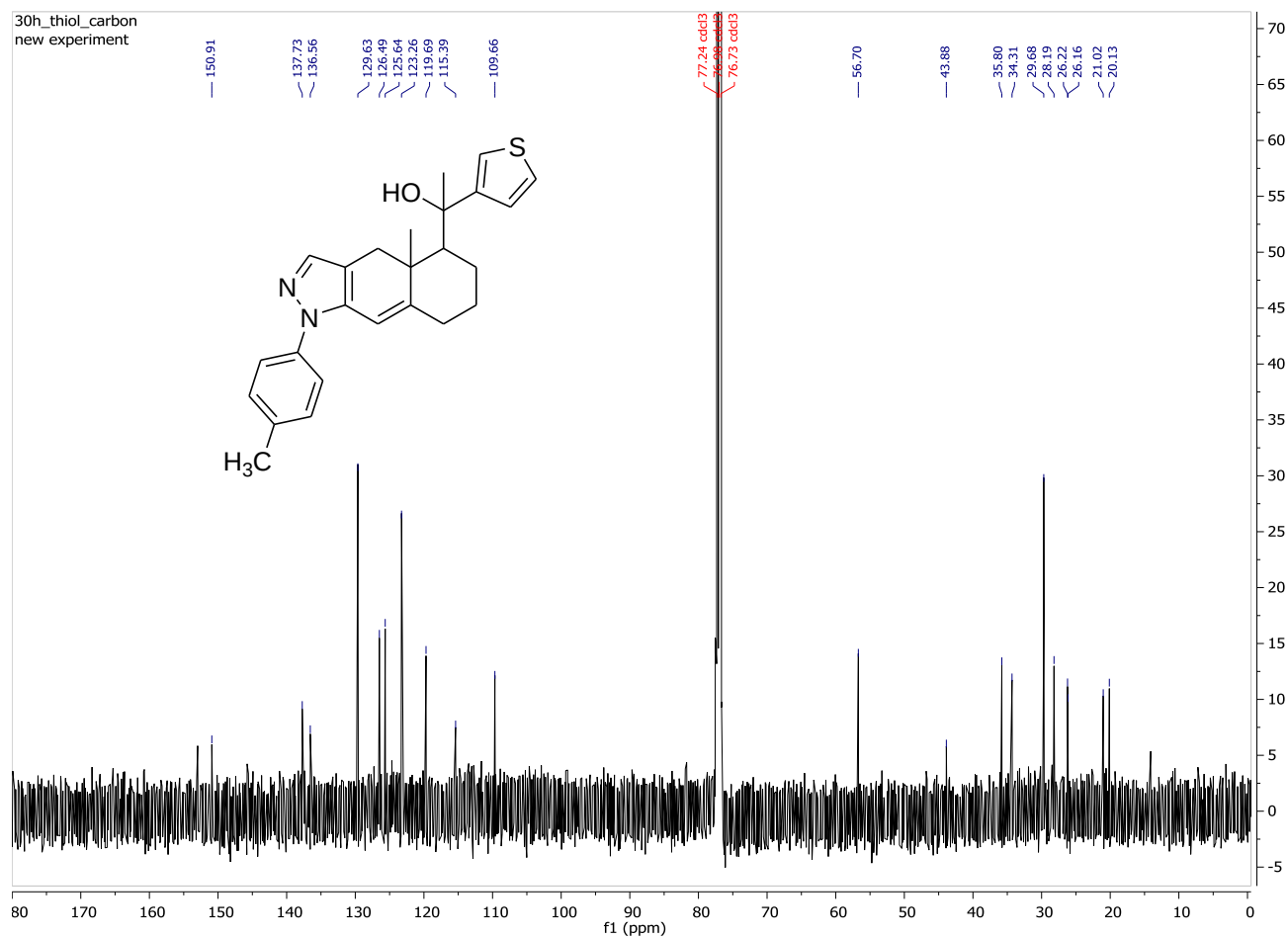
38. HRMS spectrum of compound **11be**.



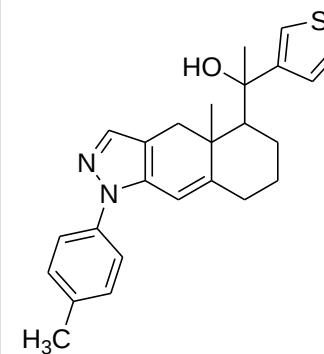
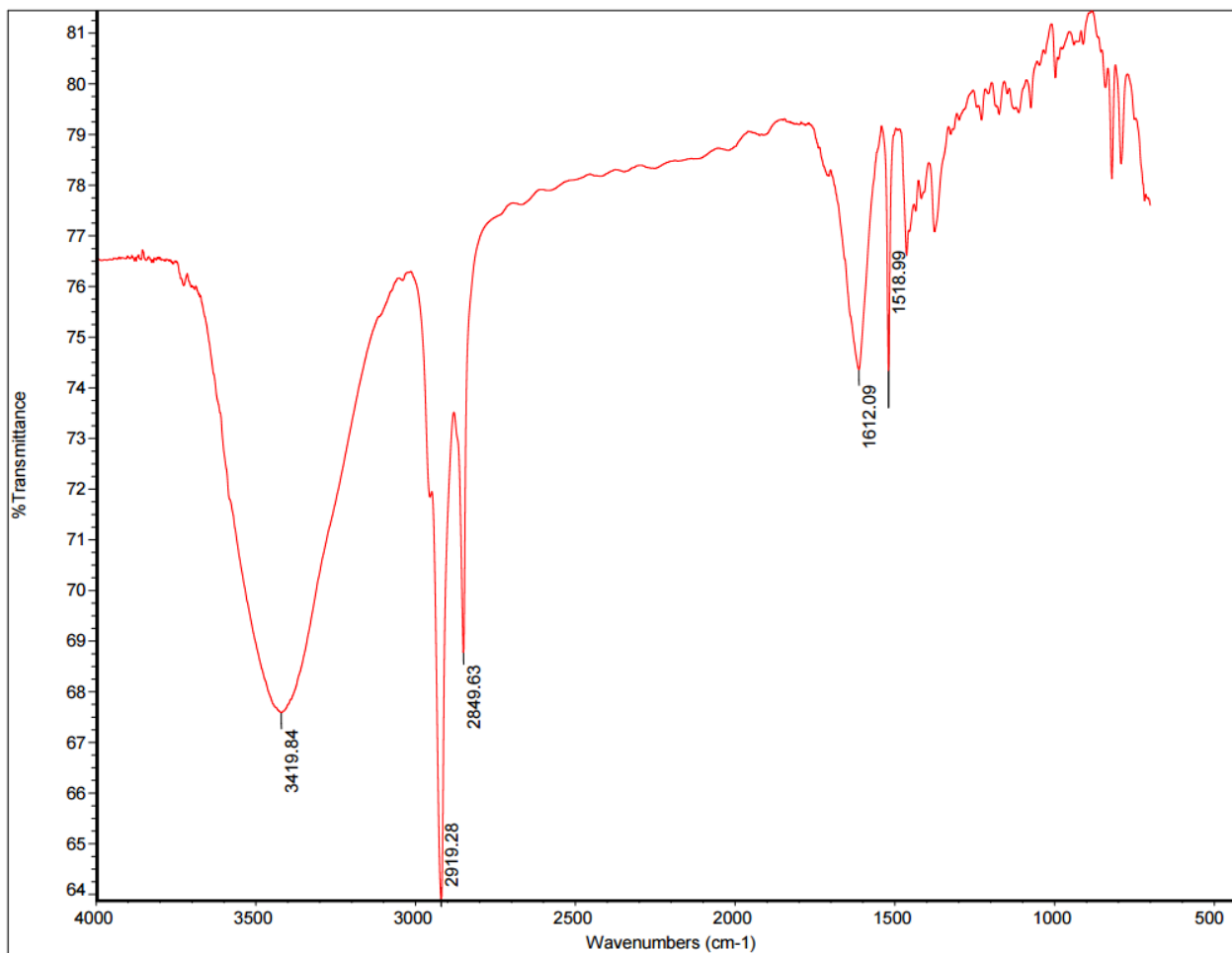
39. IR spectrum of compound **11be**.



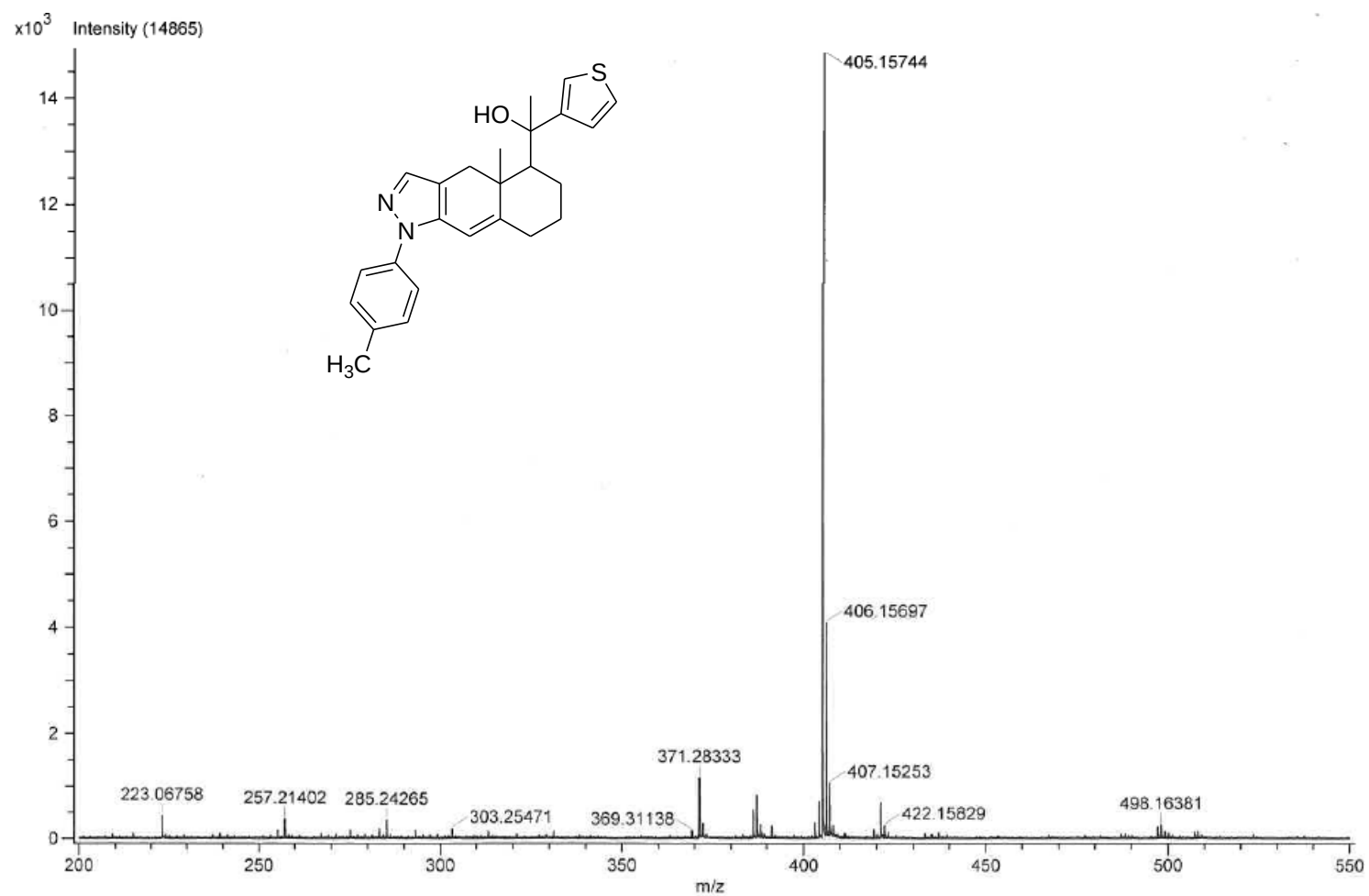
40. ^1H NMR spectrum of compound **13be**.



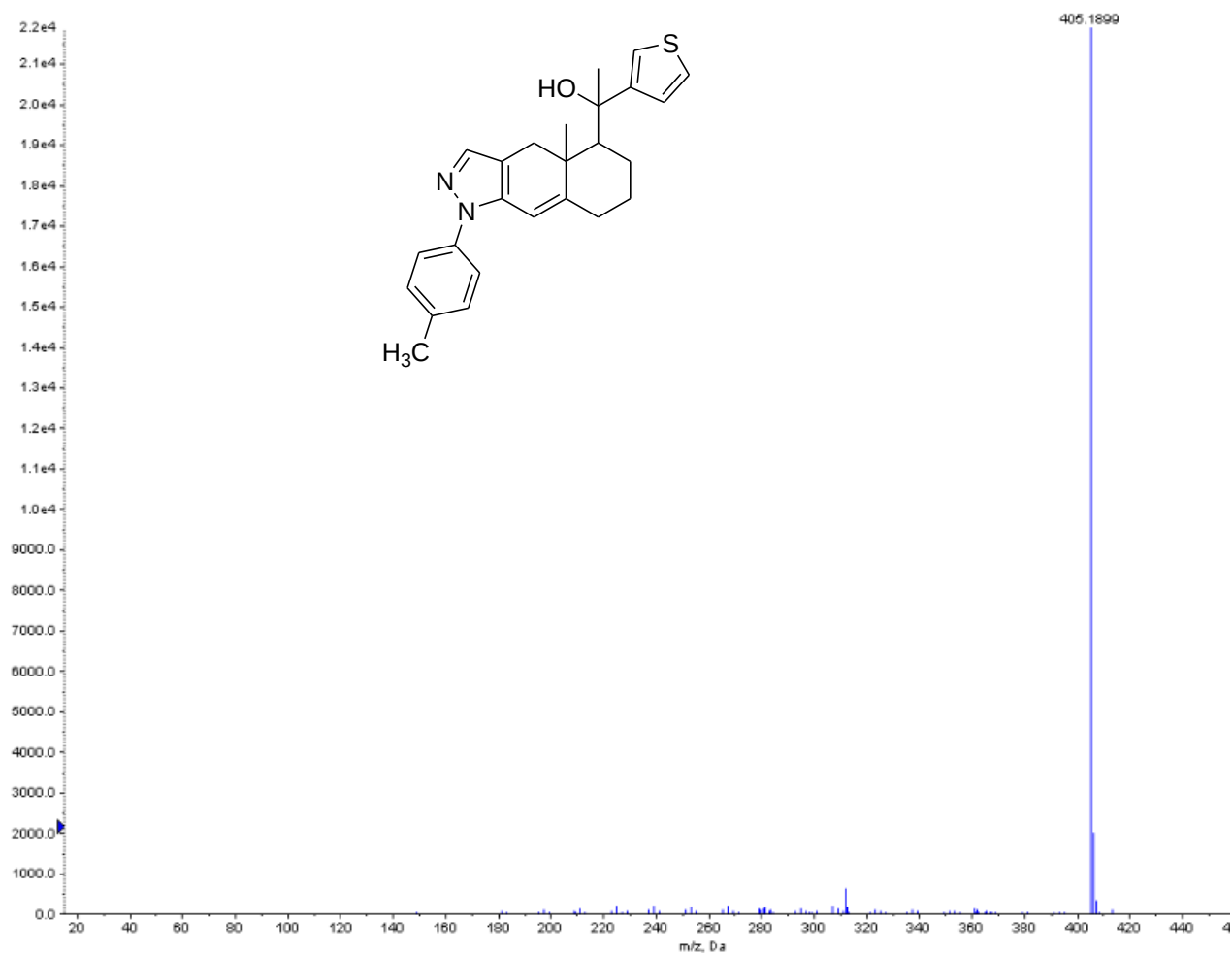
41. ¹³C NMR spectrum of compound 13be.



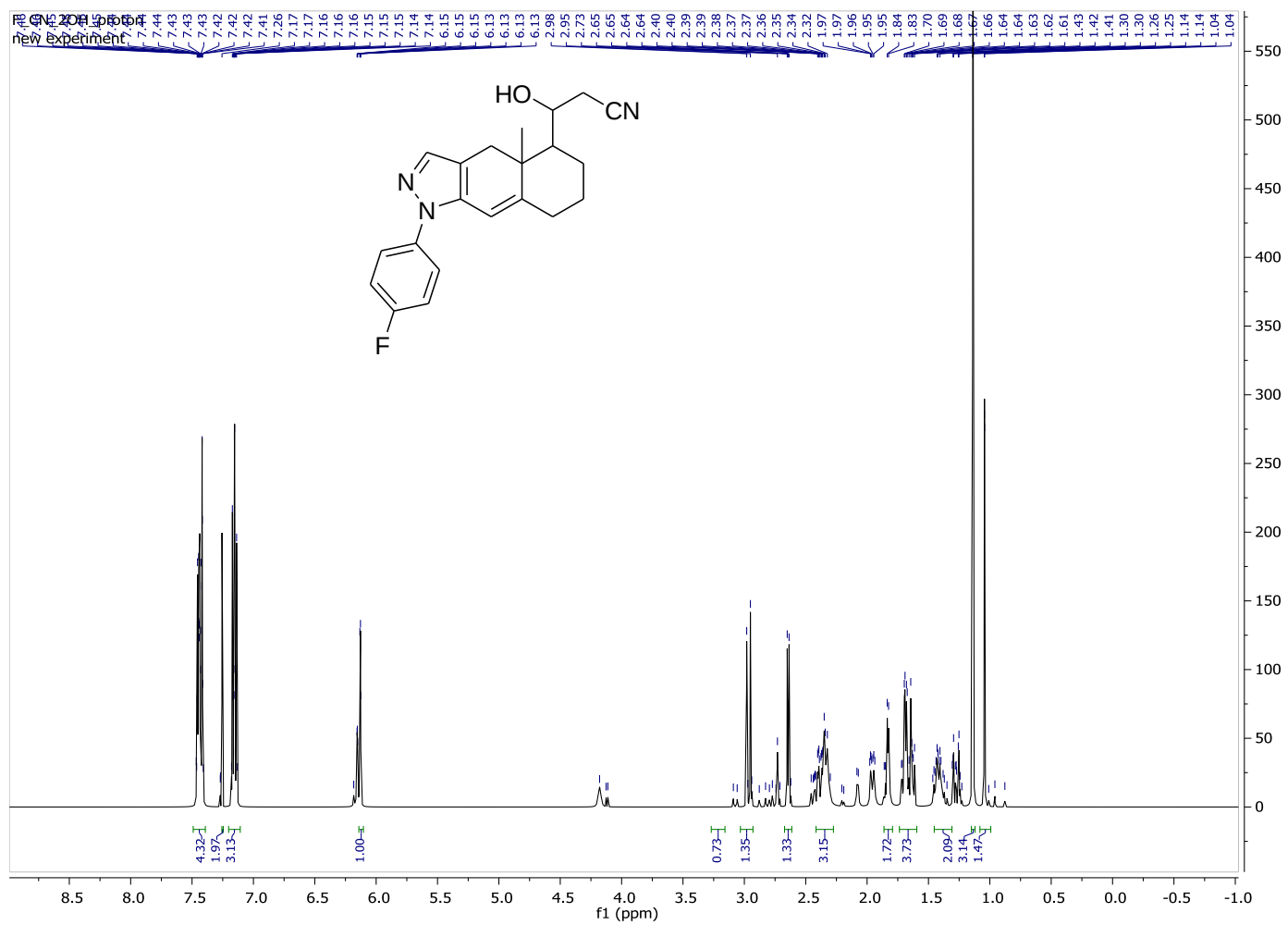
42. IR spectrum of compound 13be.



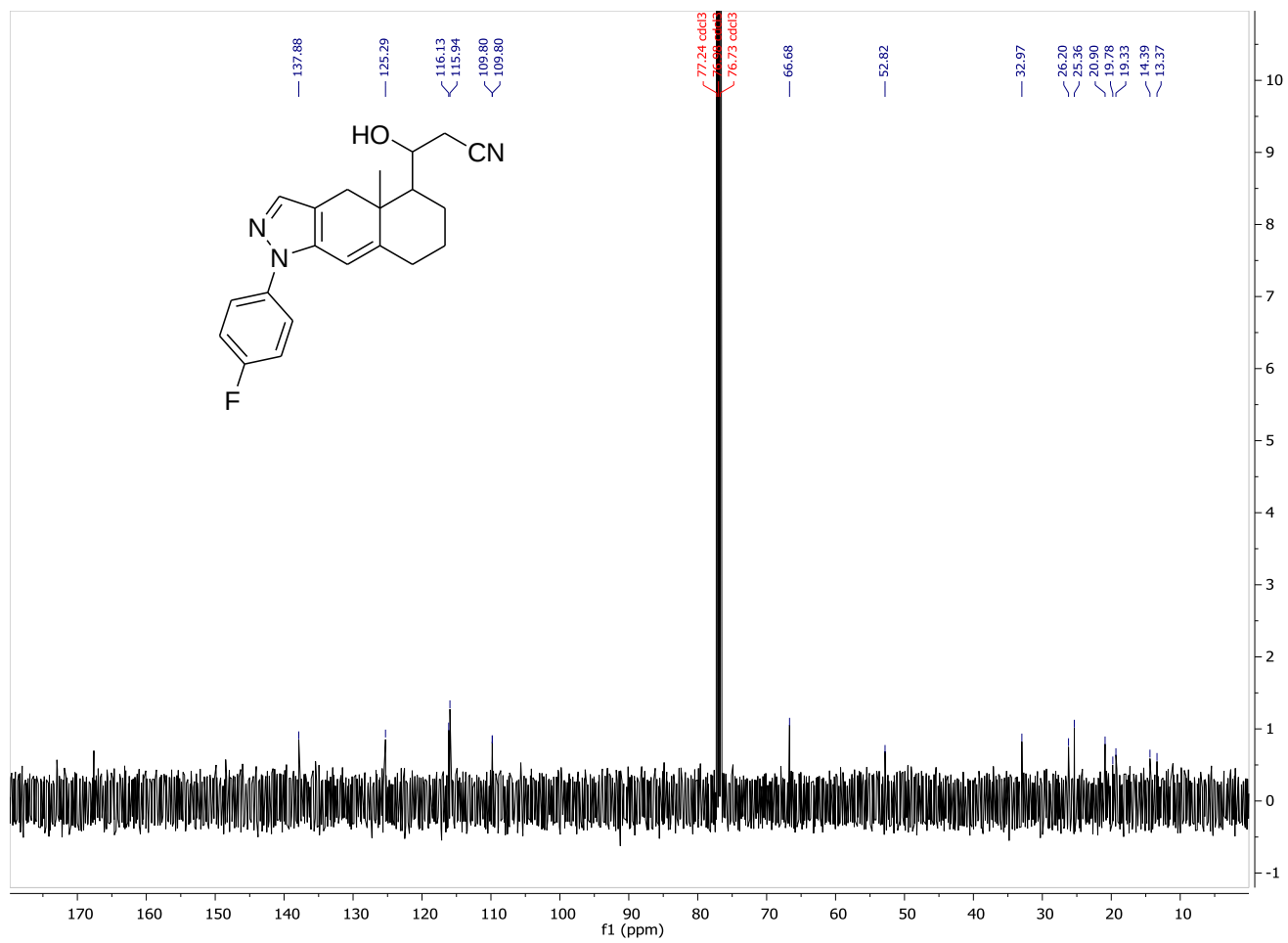
43. HRMS spectrum of compound **13be**.



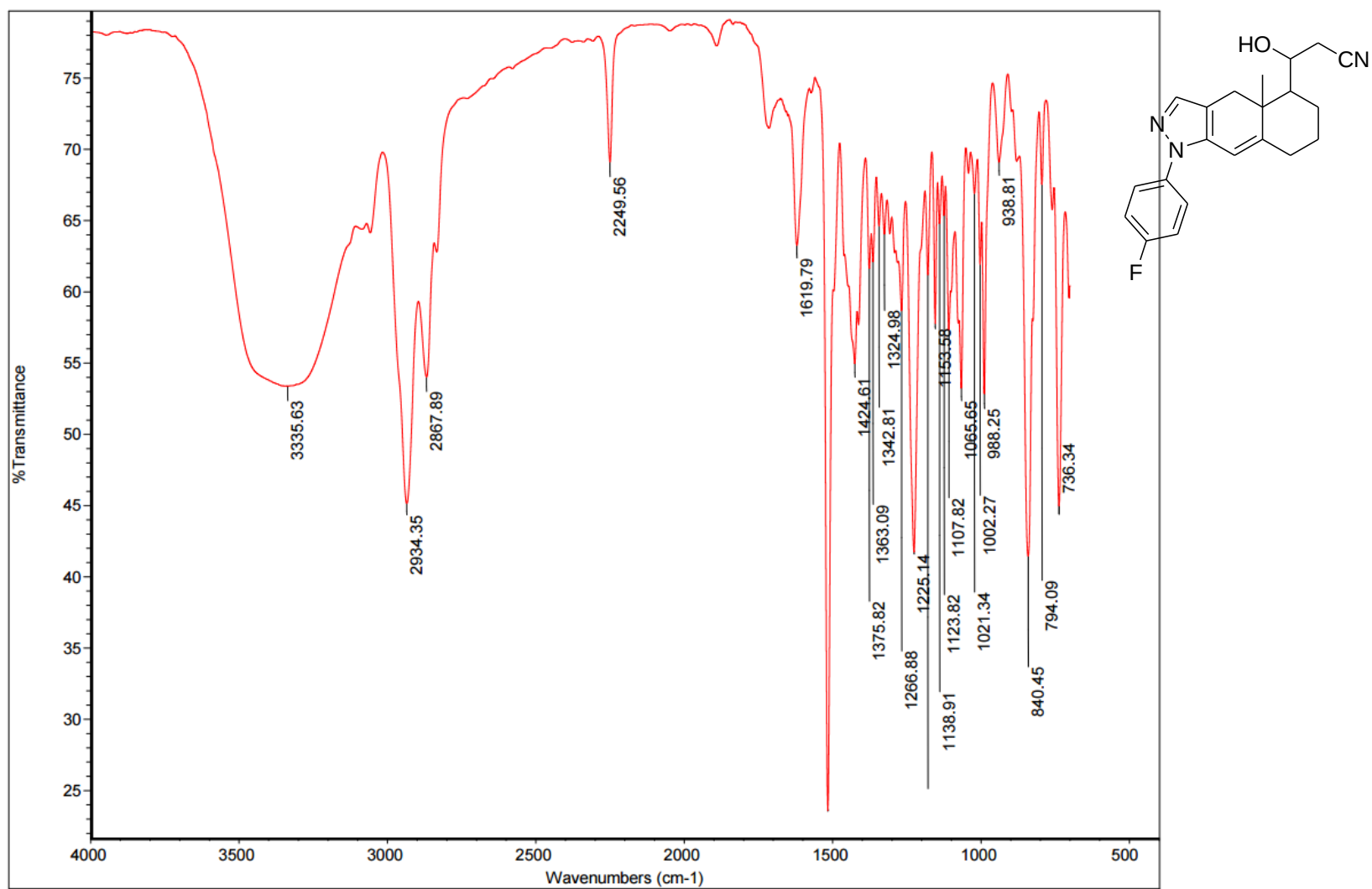
44. HRMS spectrum of compound **13be**.



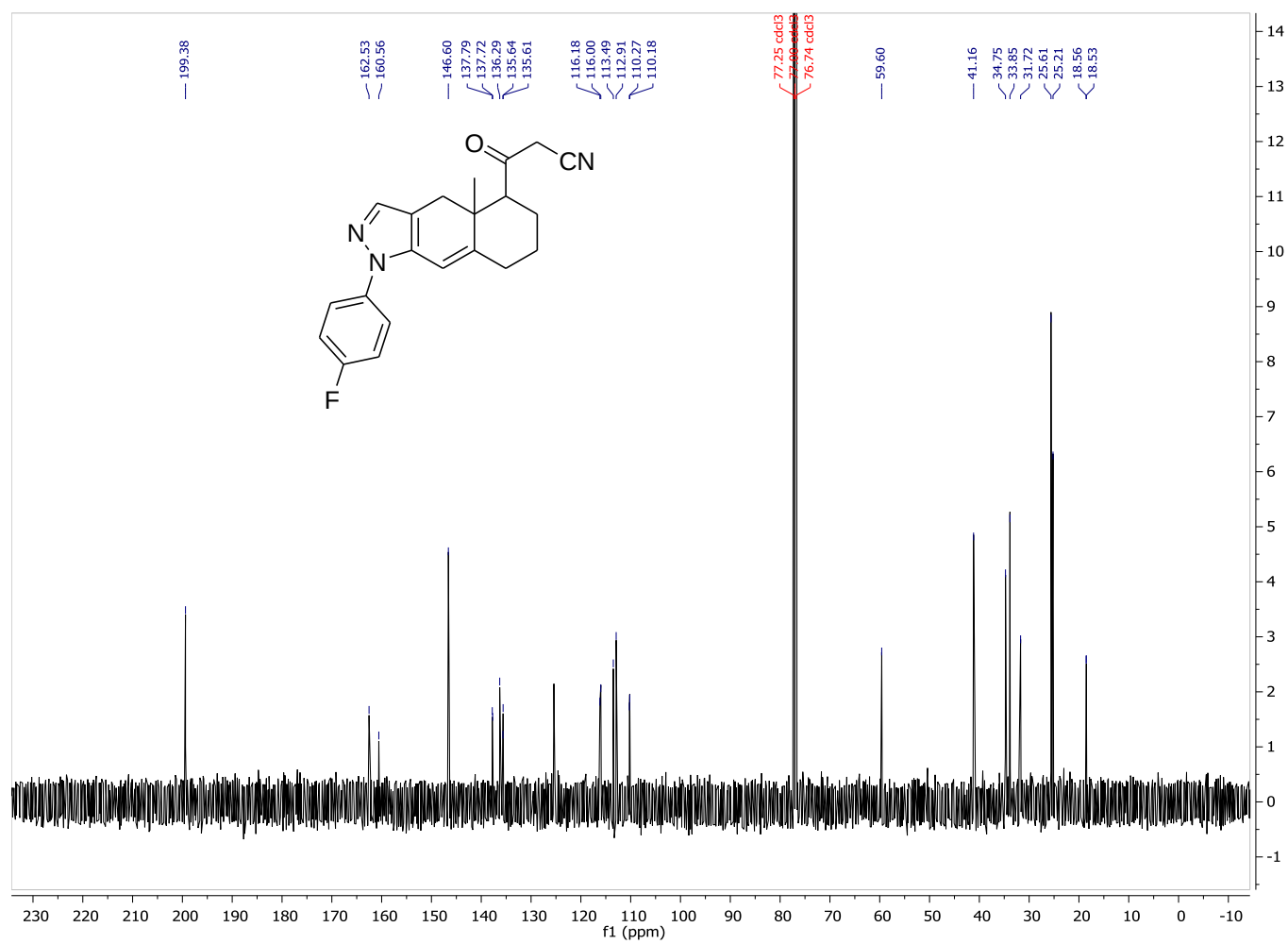
45. ¹H NMR spectrum of compound 11af.



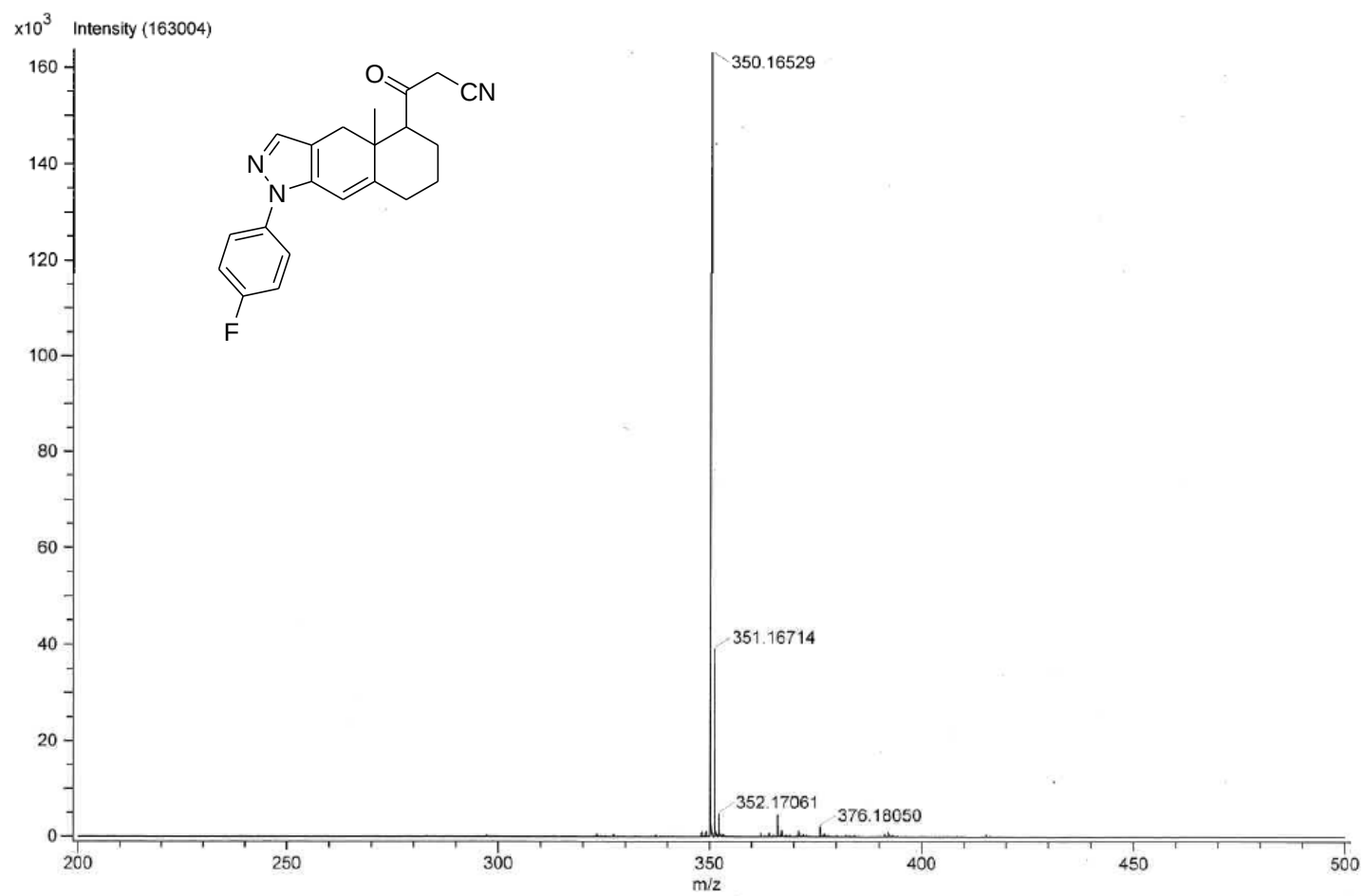
46. HRMS spectrum of compound **11af**.



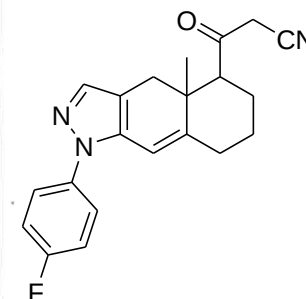
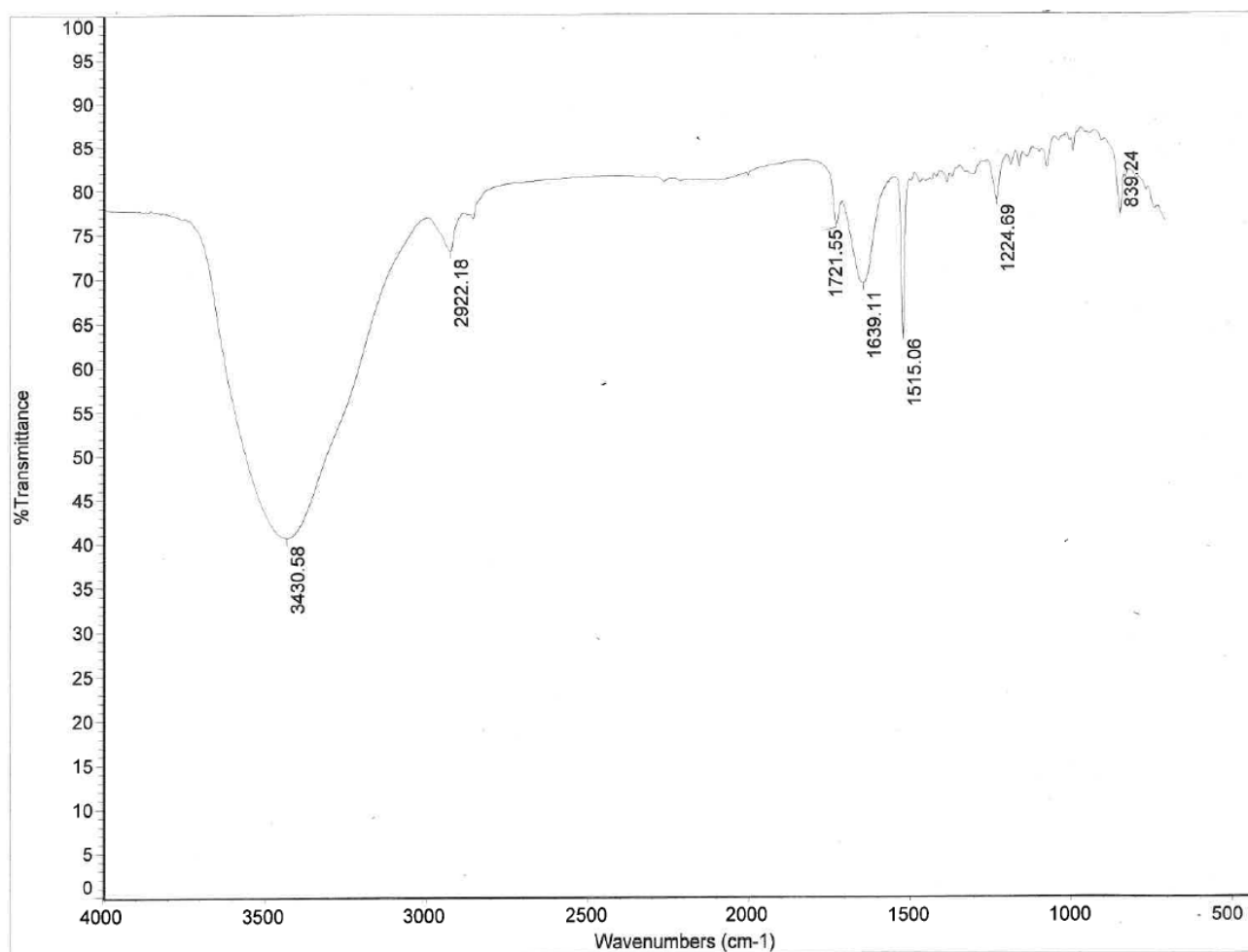
47. IR spectrum of compound **11af**.



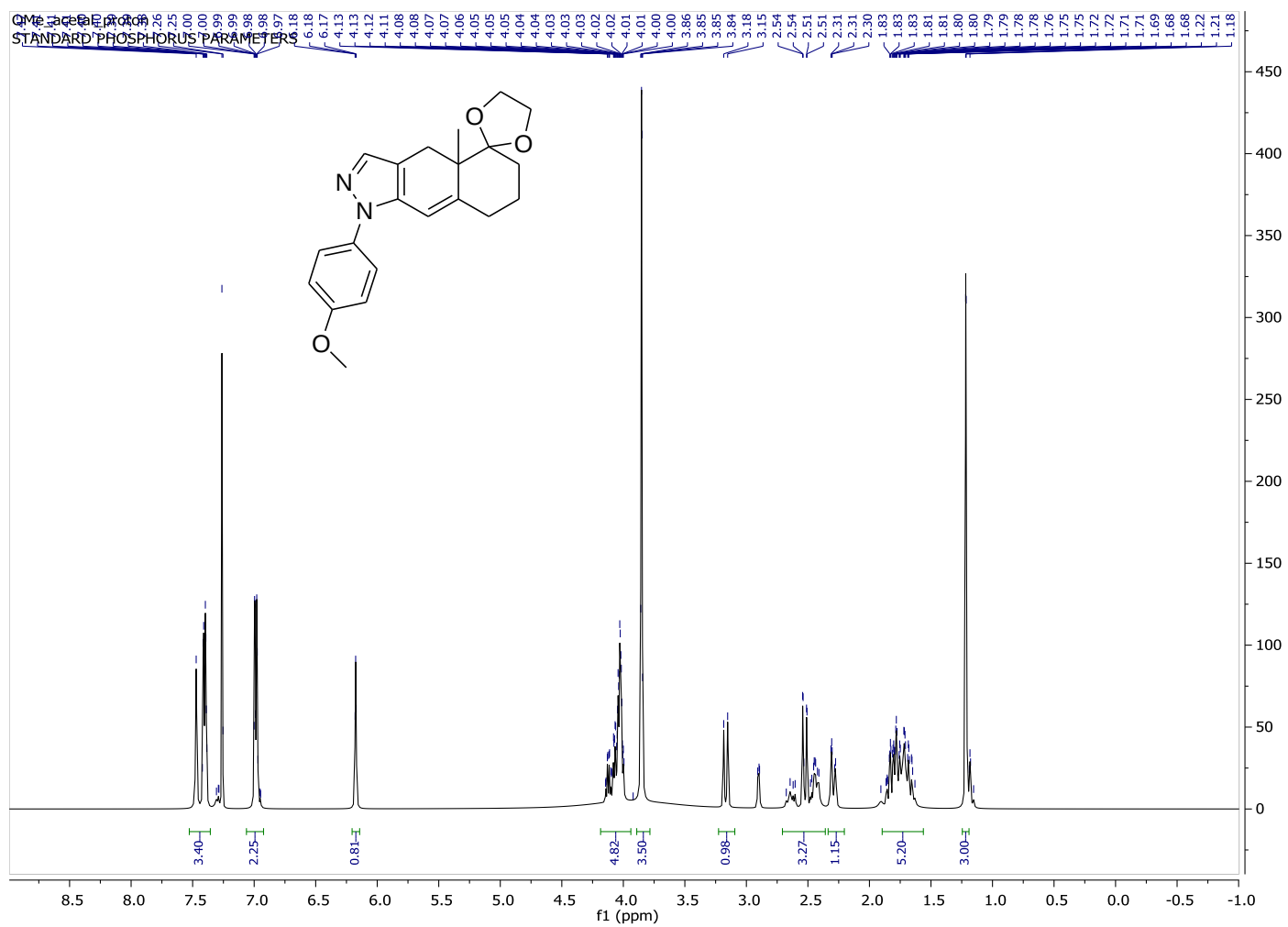
49. ¹³C NMR spectrum of compound 12af.



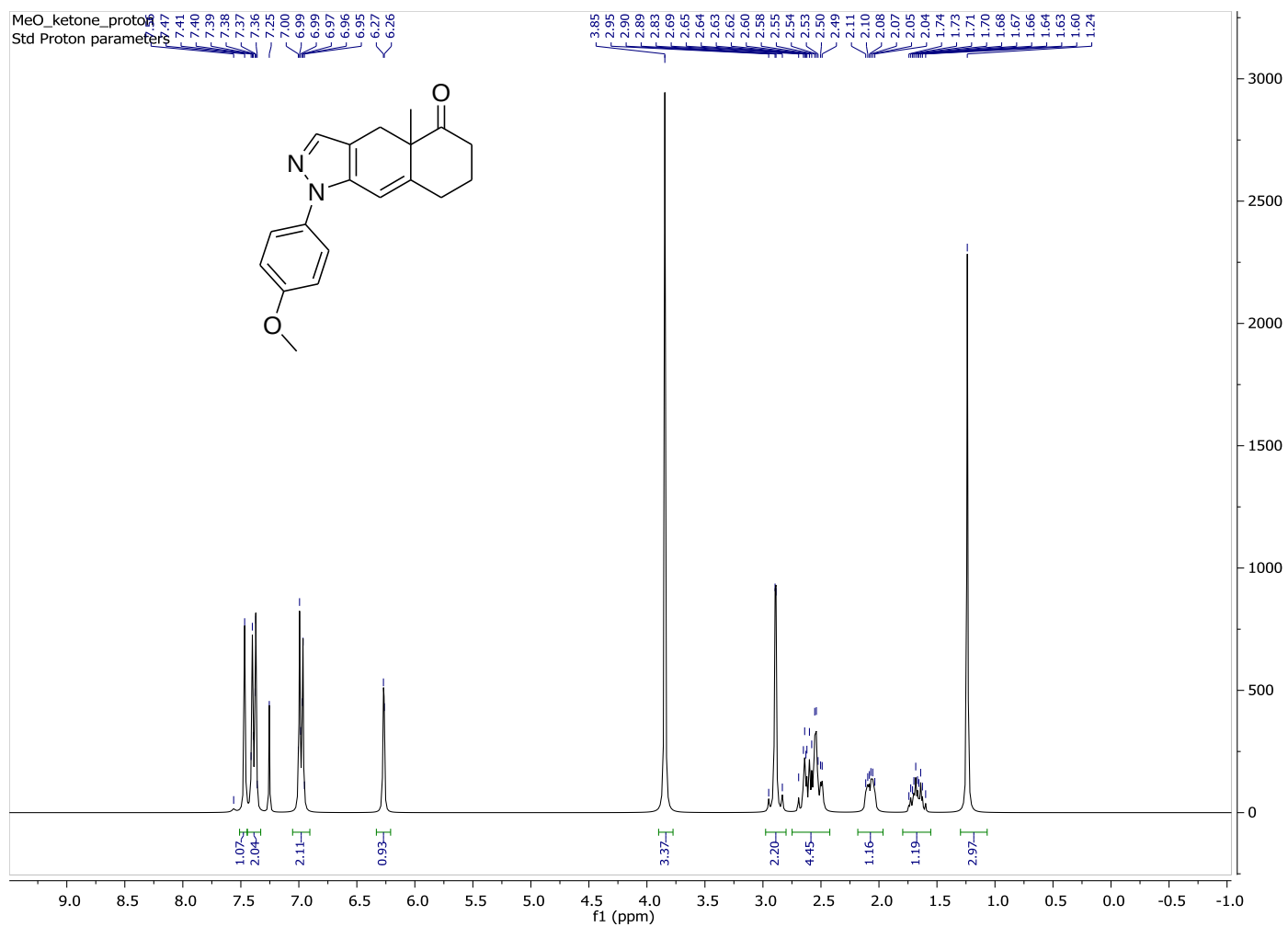
50. HRMS spectrum of compound **12af**.



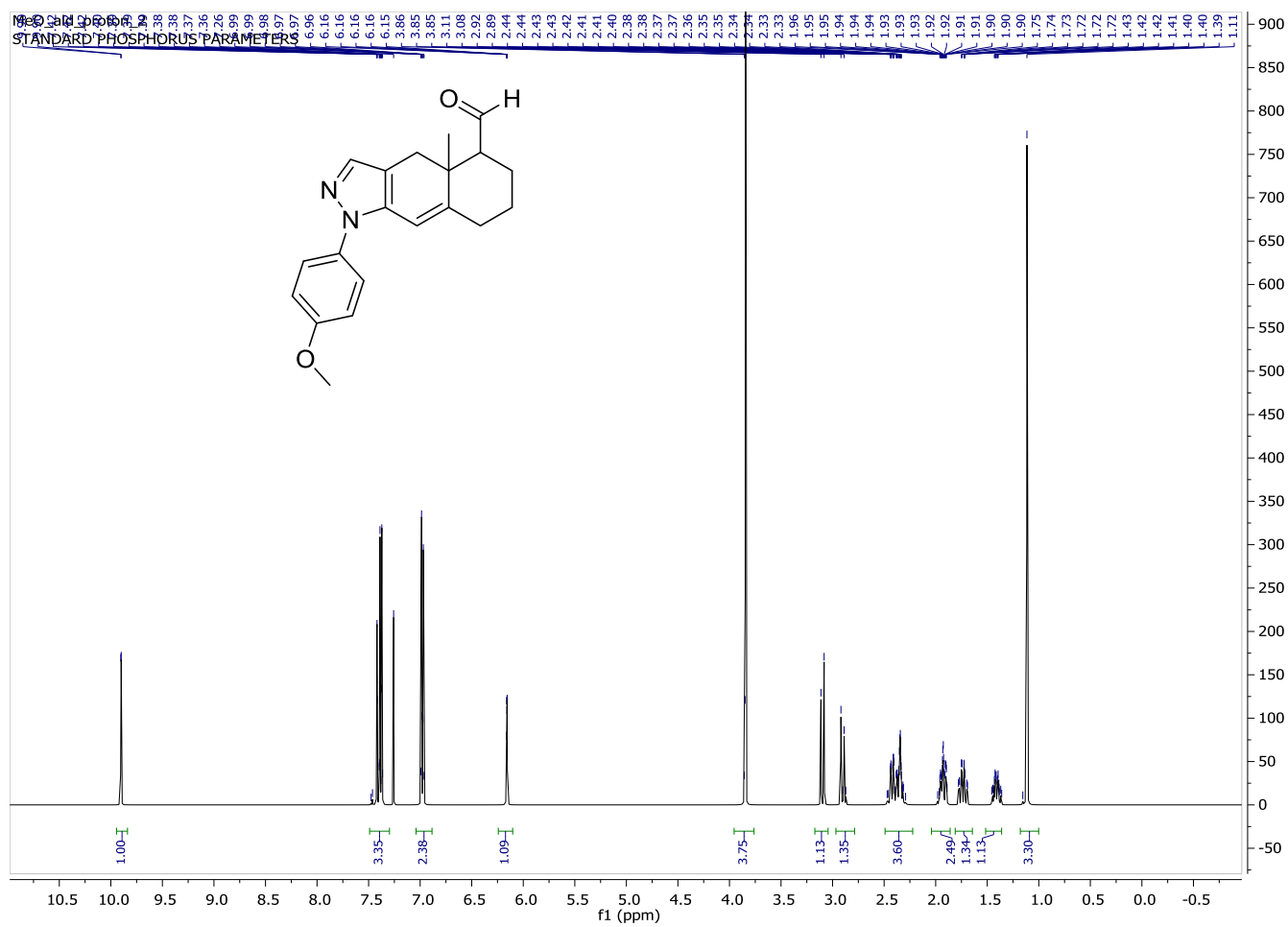
51. IR spectrum of compound 12af.



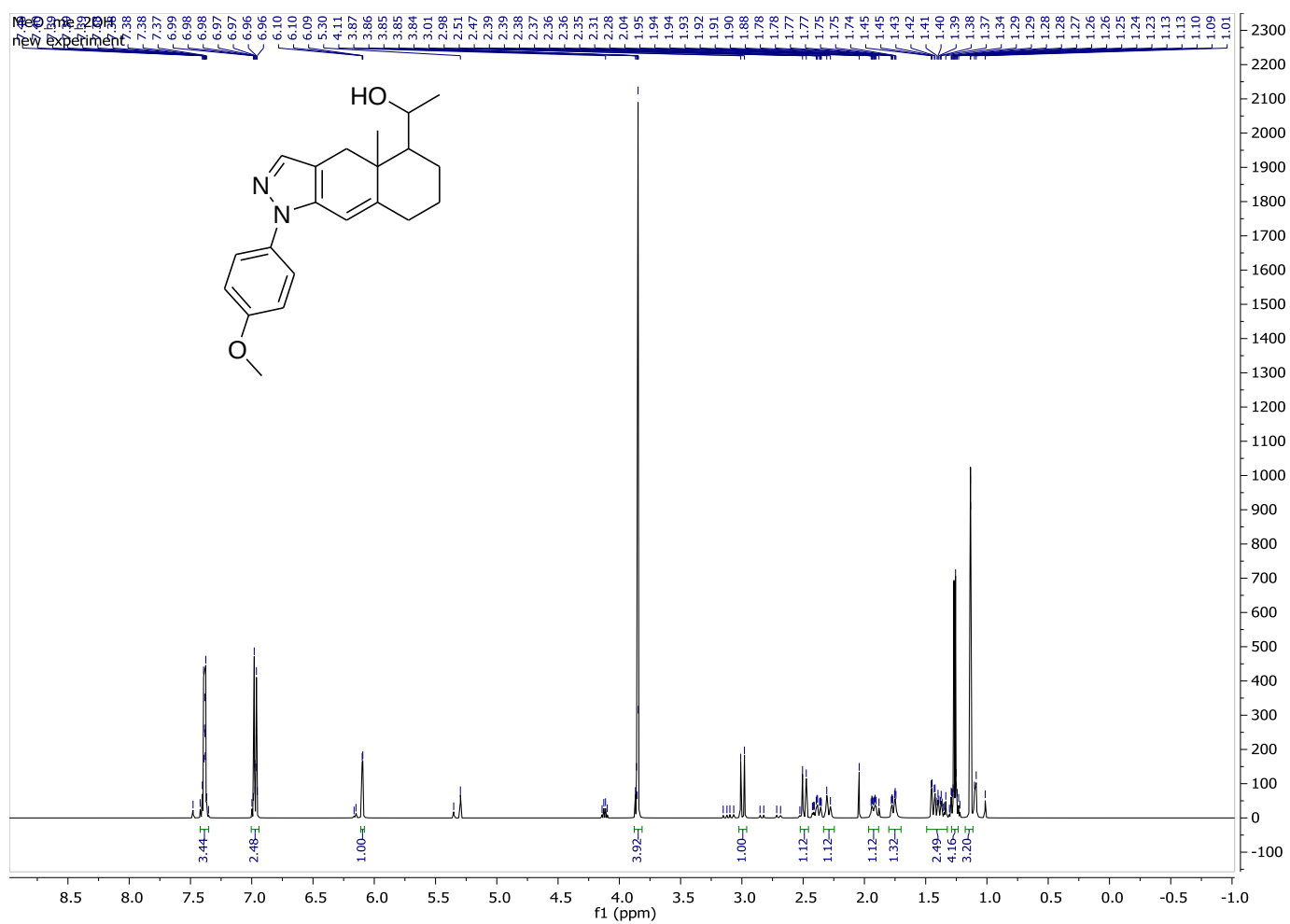
52. ^1H NMR spectrum of compound **7c**.



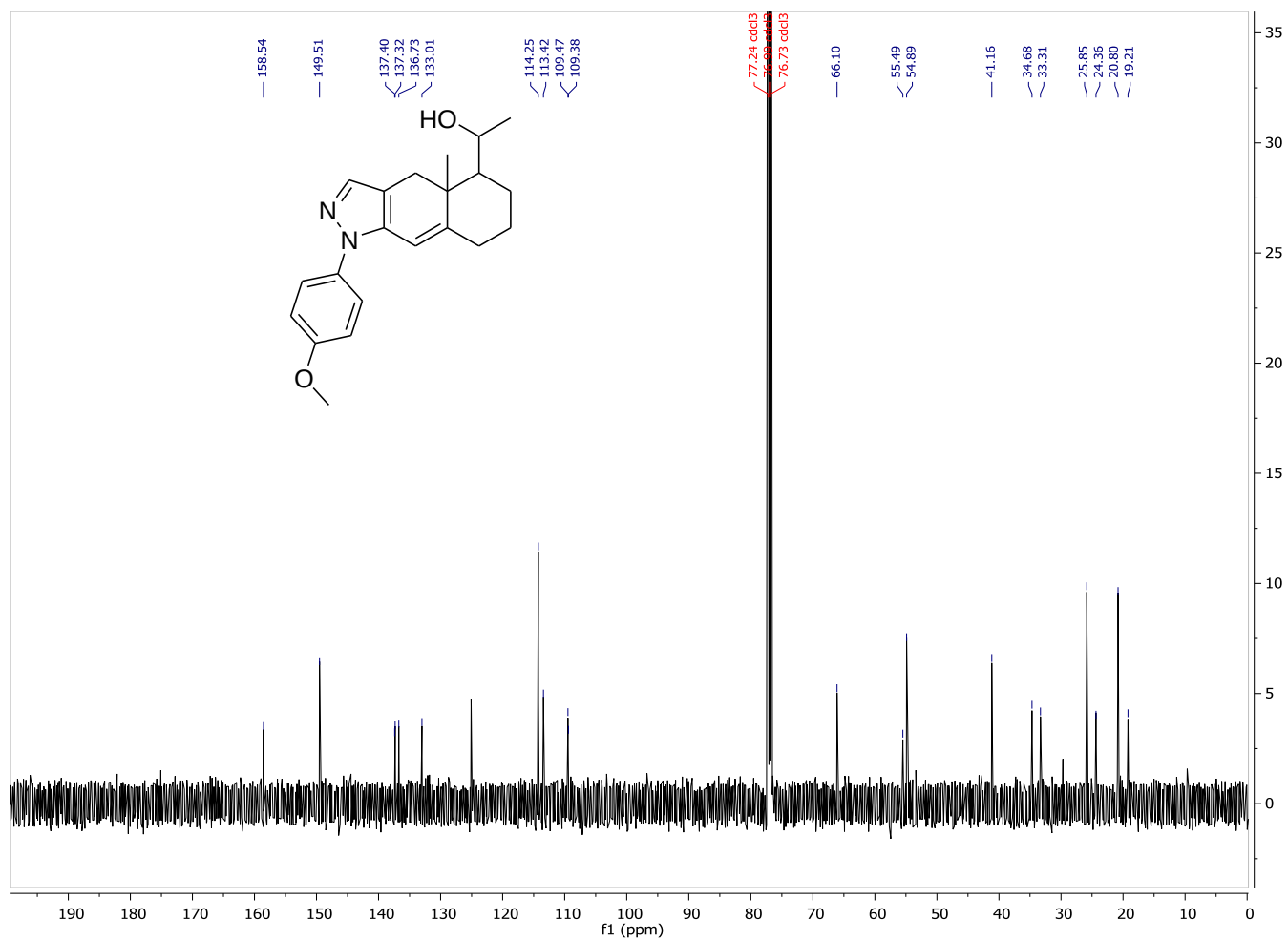
53. ^1H NMR spectrum of compound **8c**.



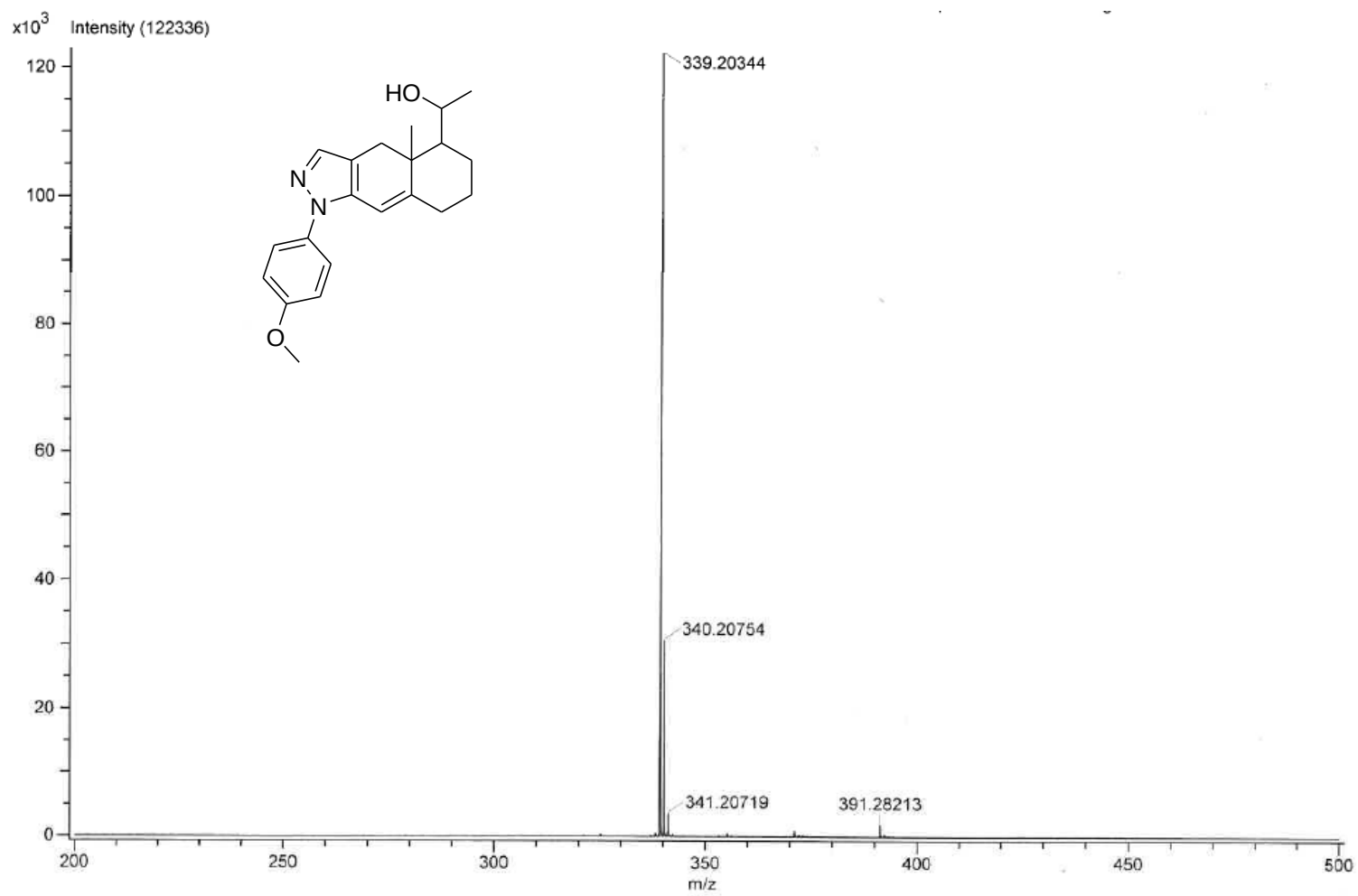
54. ¹H NMR spectrum of compound 10c.



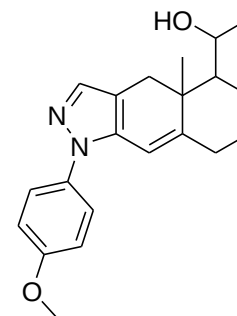
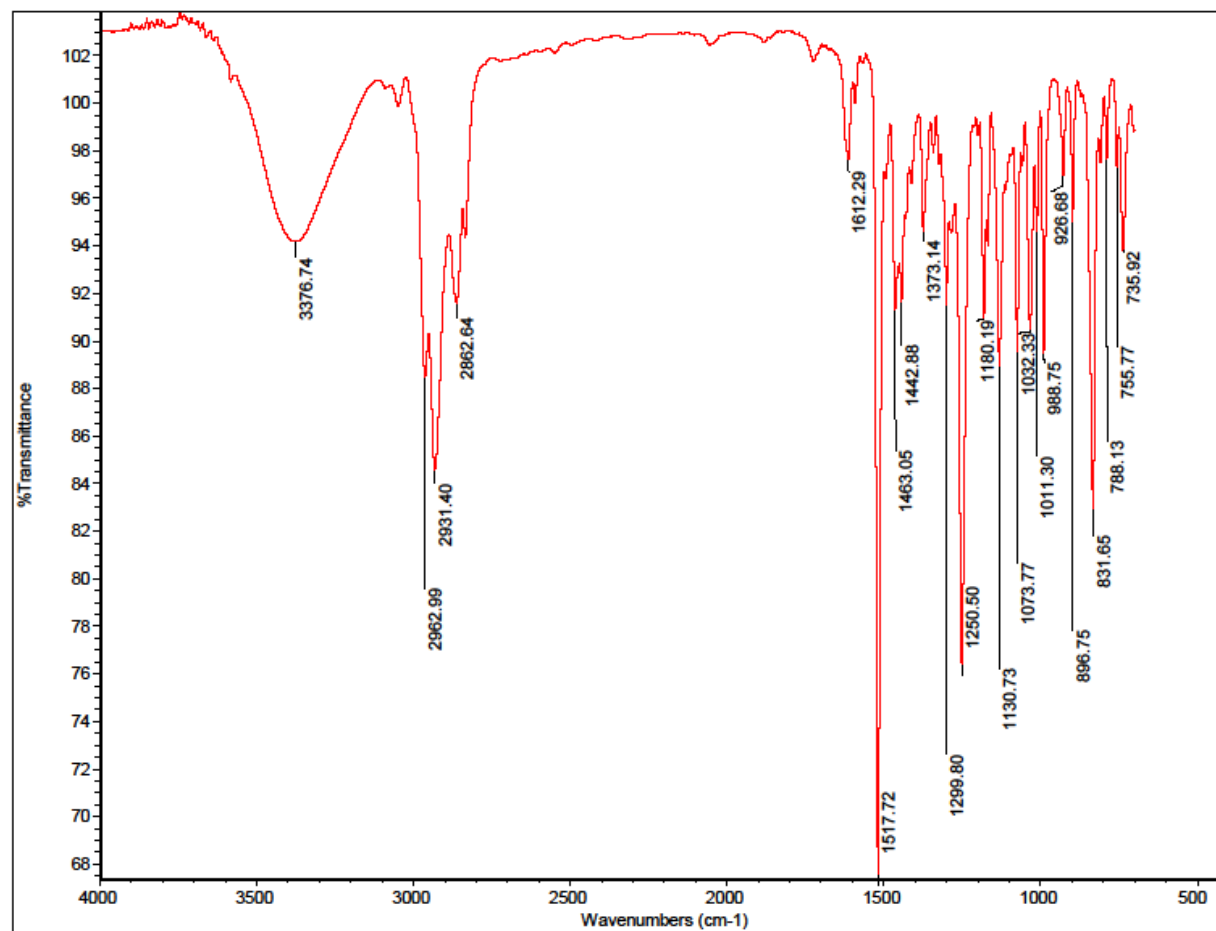
55. ^1H NMR spectrum of compound **11ca**.



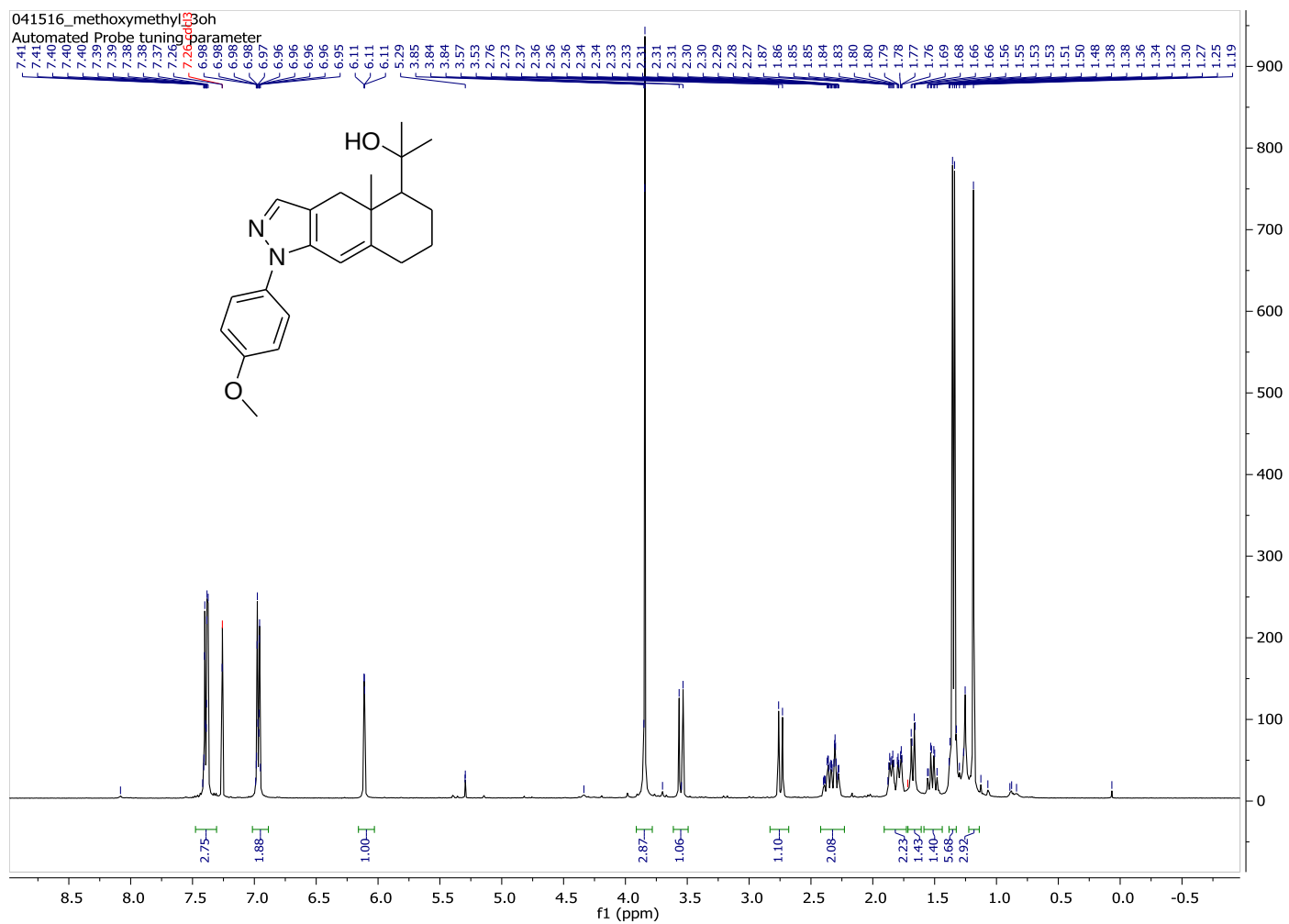
56. ^{13}C NMR spectrum of compound 11ca.



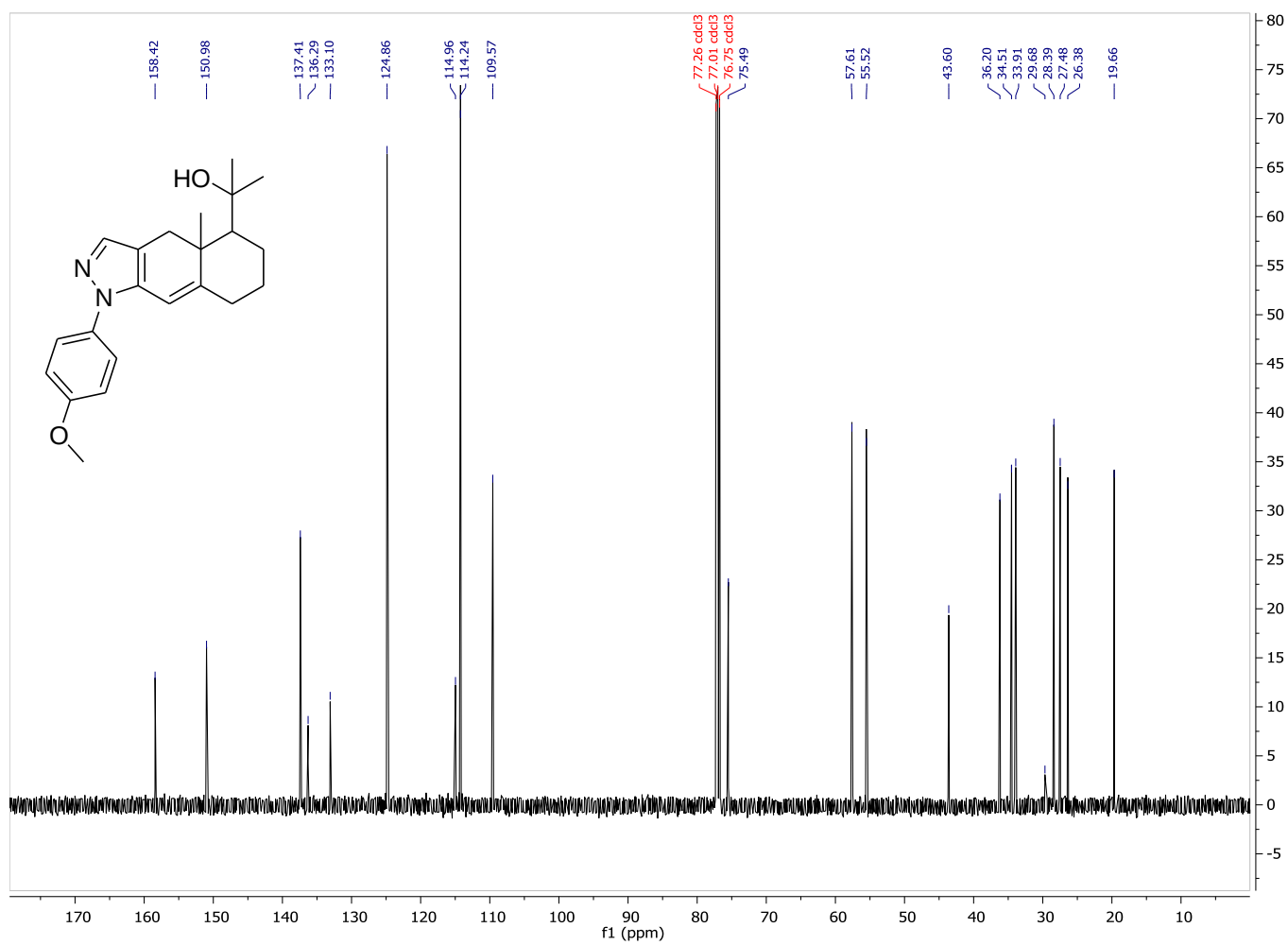
57. HRMS spectrum of compound **11ca**.



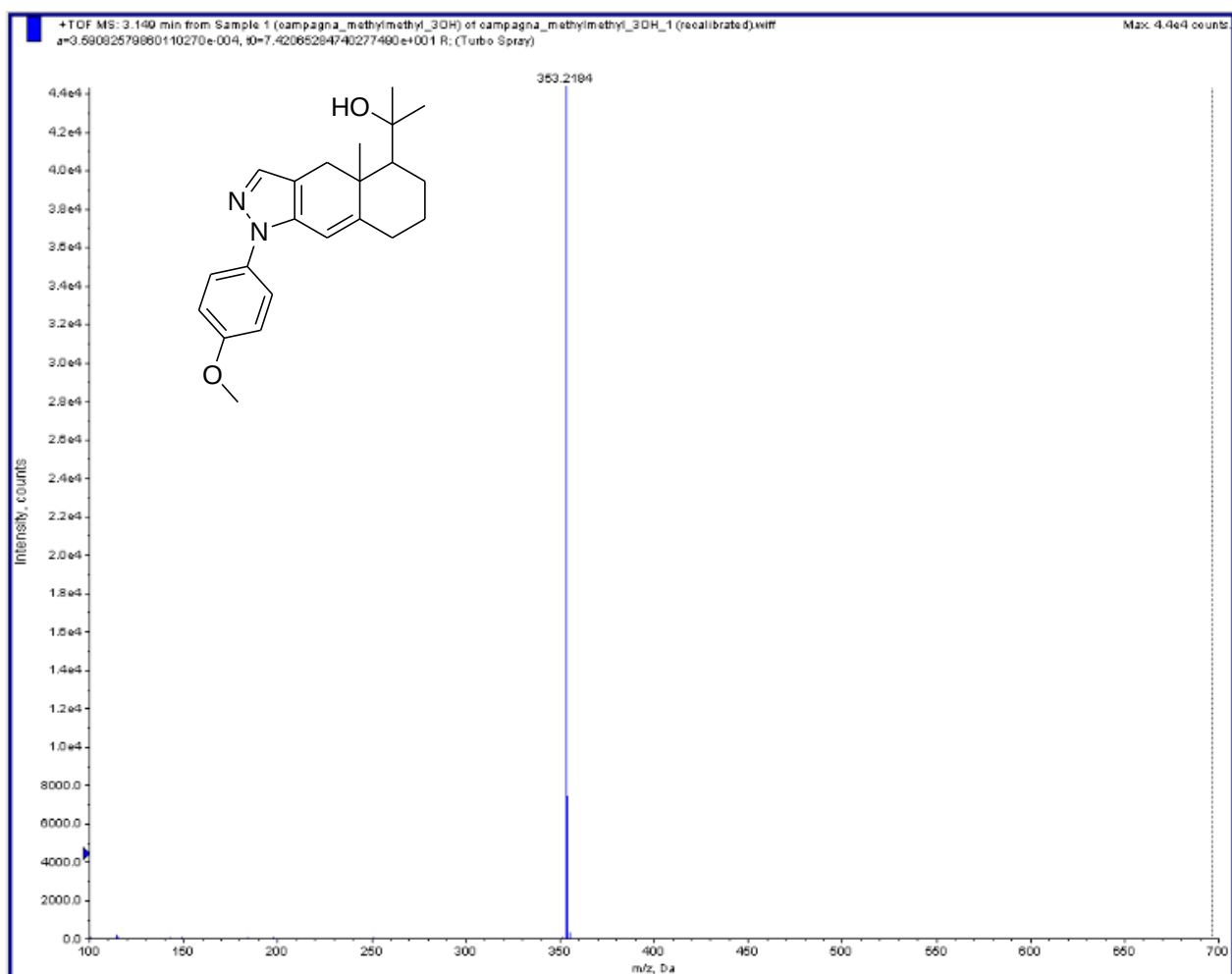
58. IR spectrum of compound **11ca**.



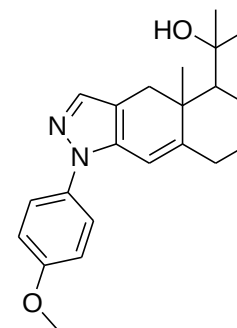
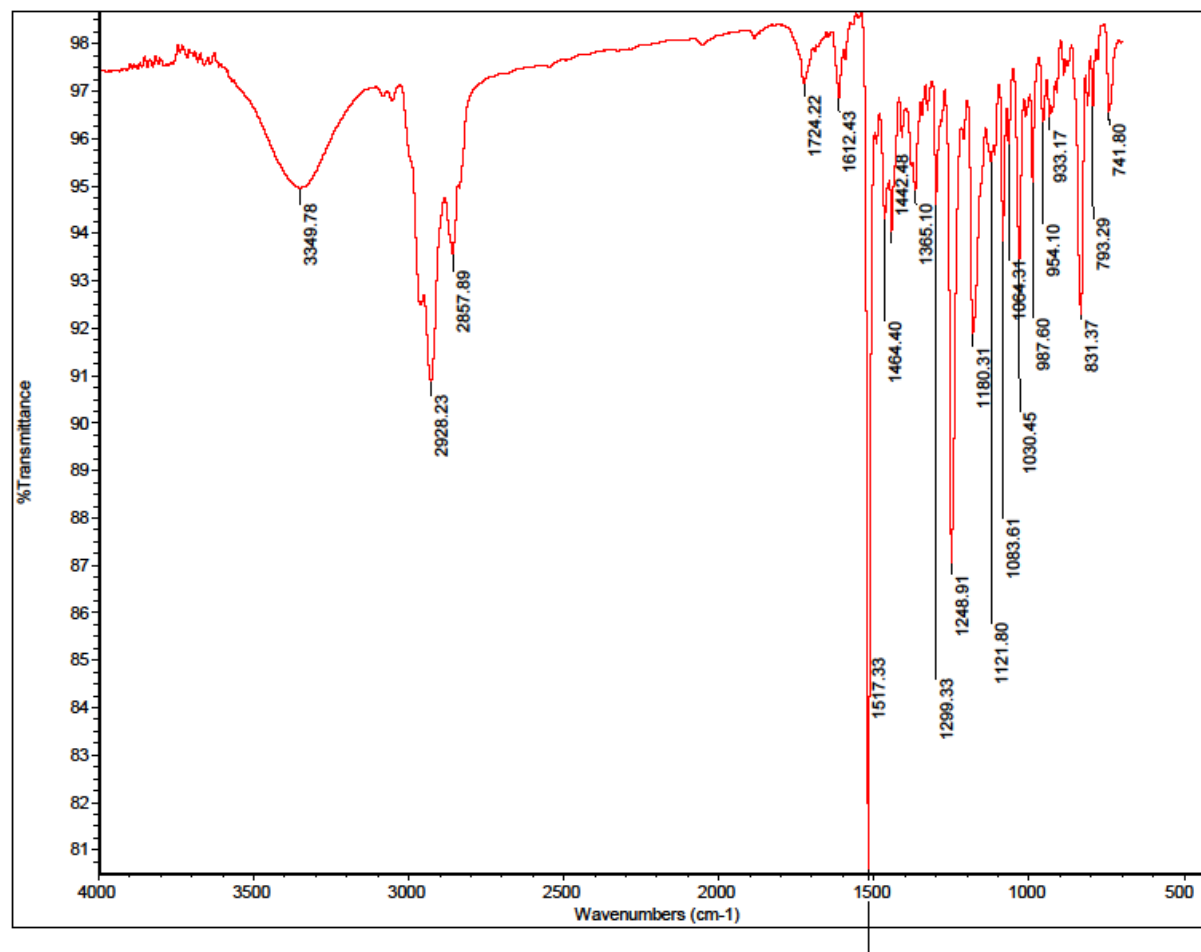
59. ^1H NMR spectrum of compound 13ca.



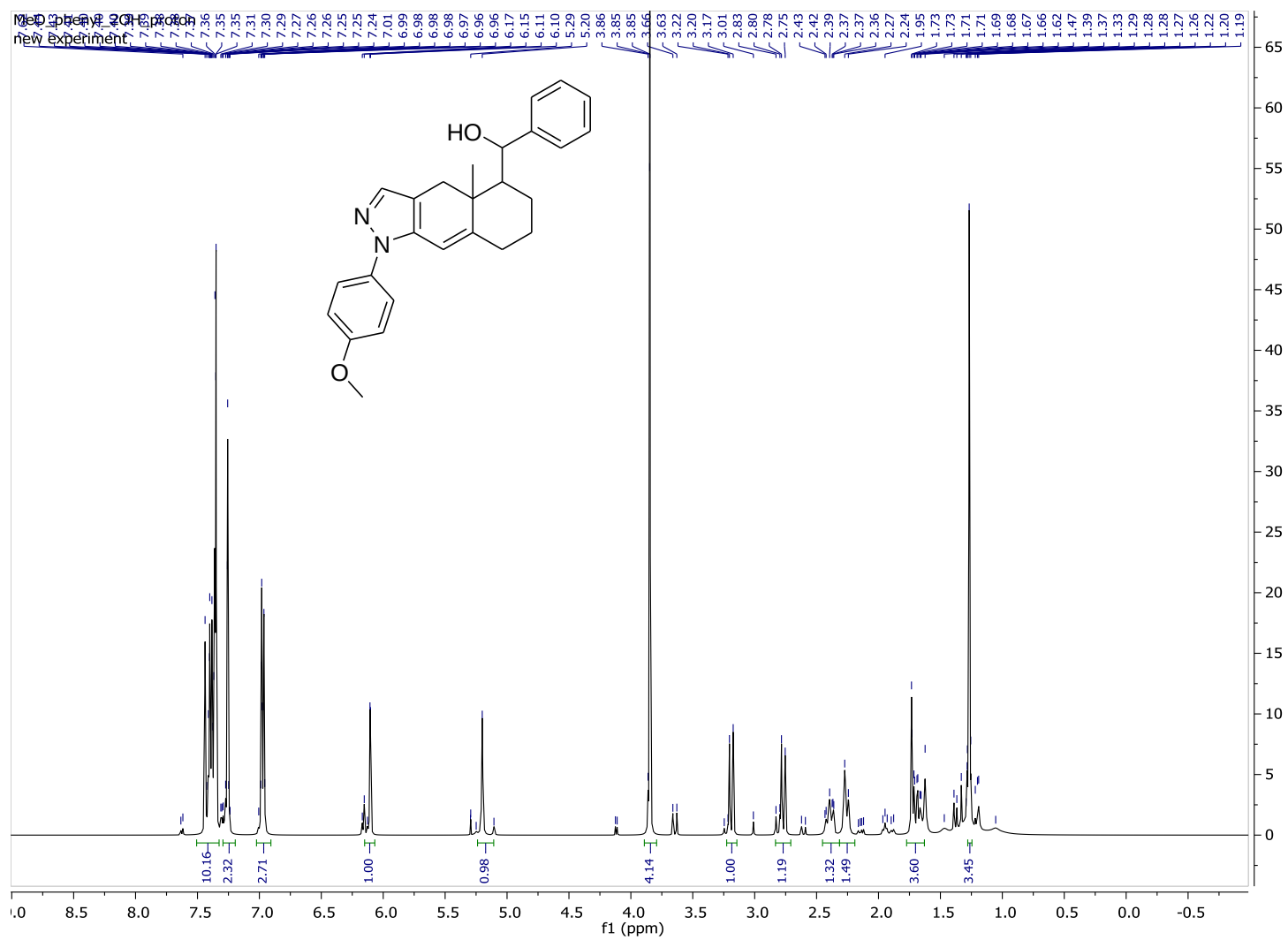
60. ¹³C NMR spectrum of compound 13ca.



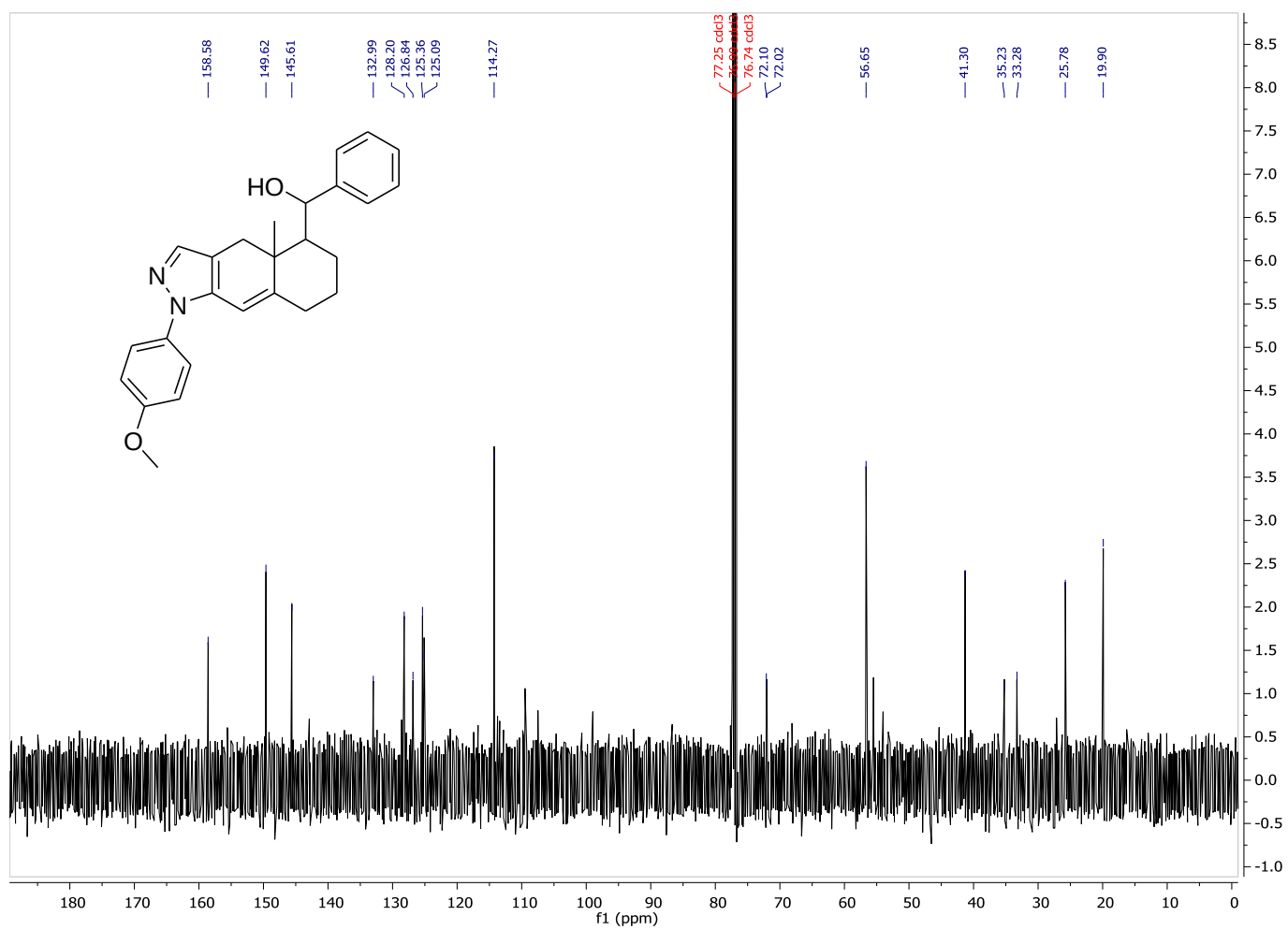
61. HRMS spectrum of compound **13ca**.



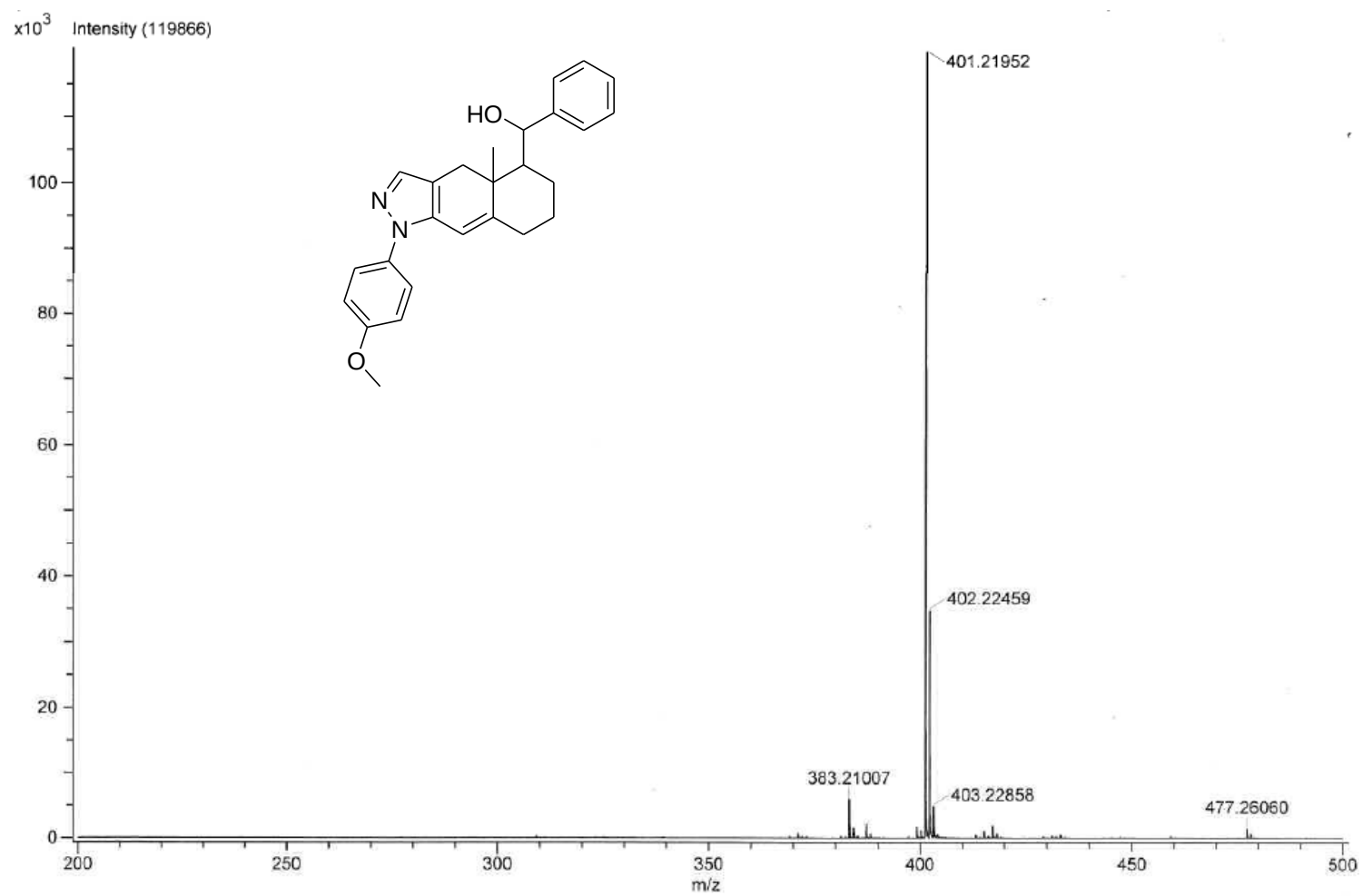
62. IR spectrum of compound **13ca**.



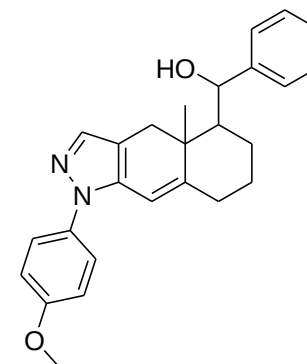
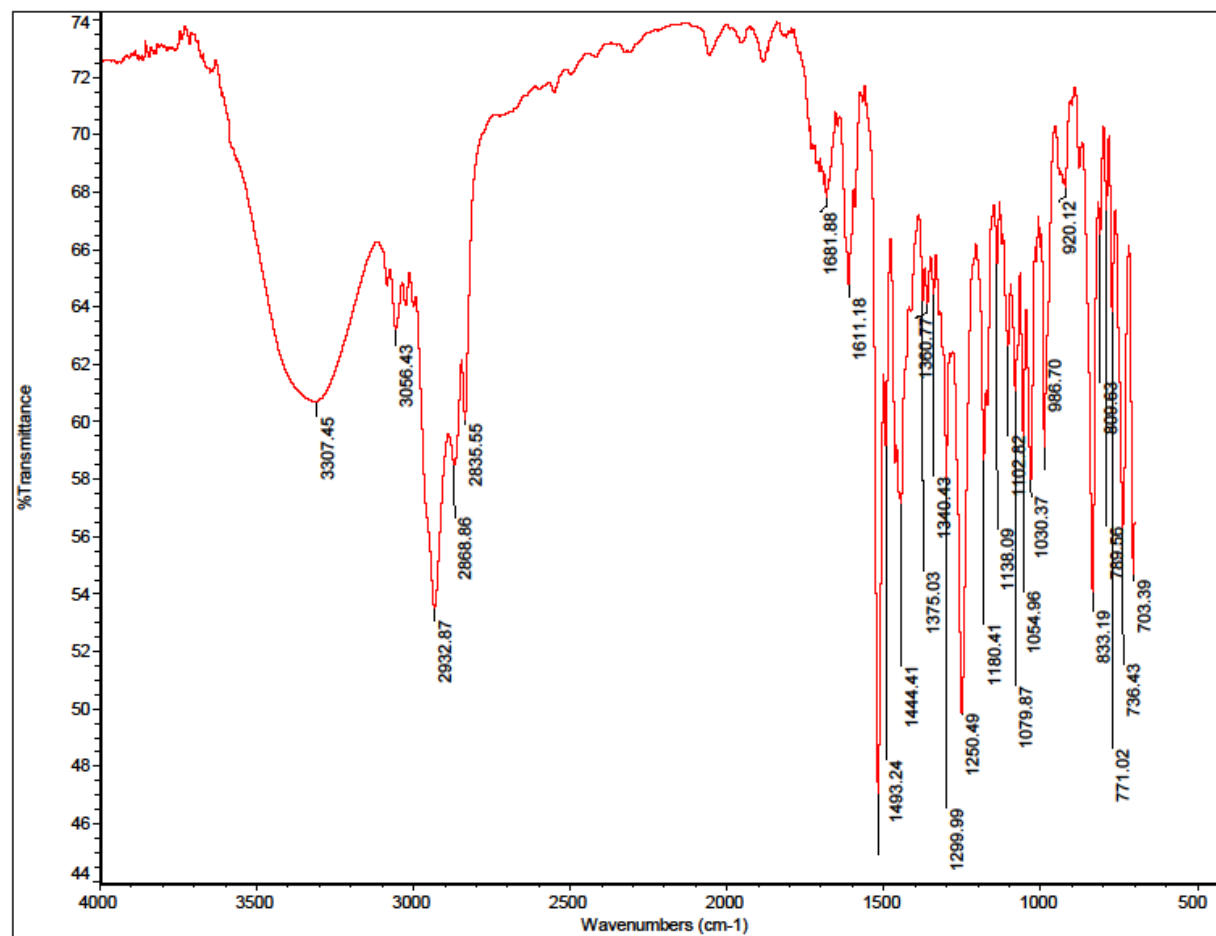
63. ¹H NMR spectrum of compound **11cb**.



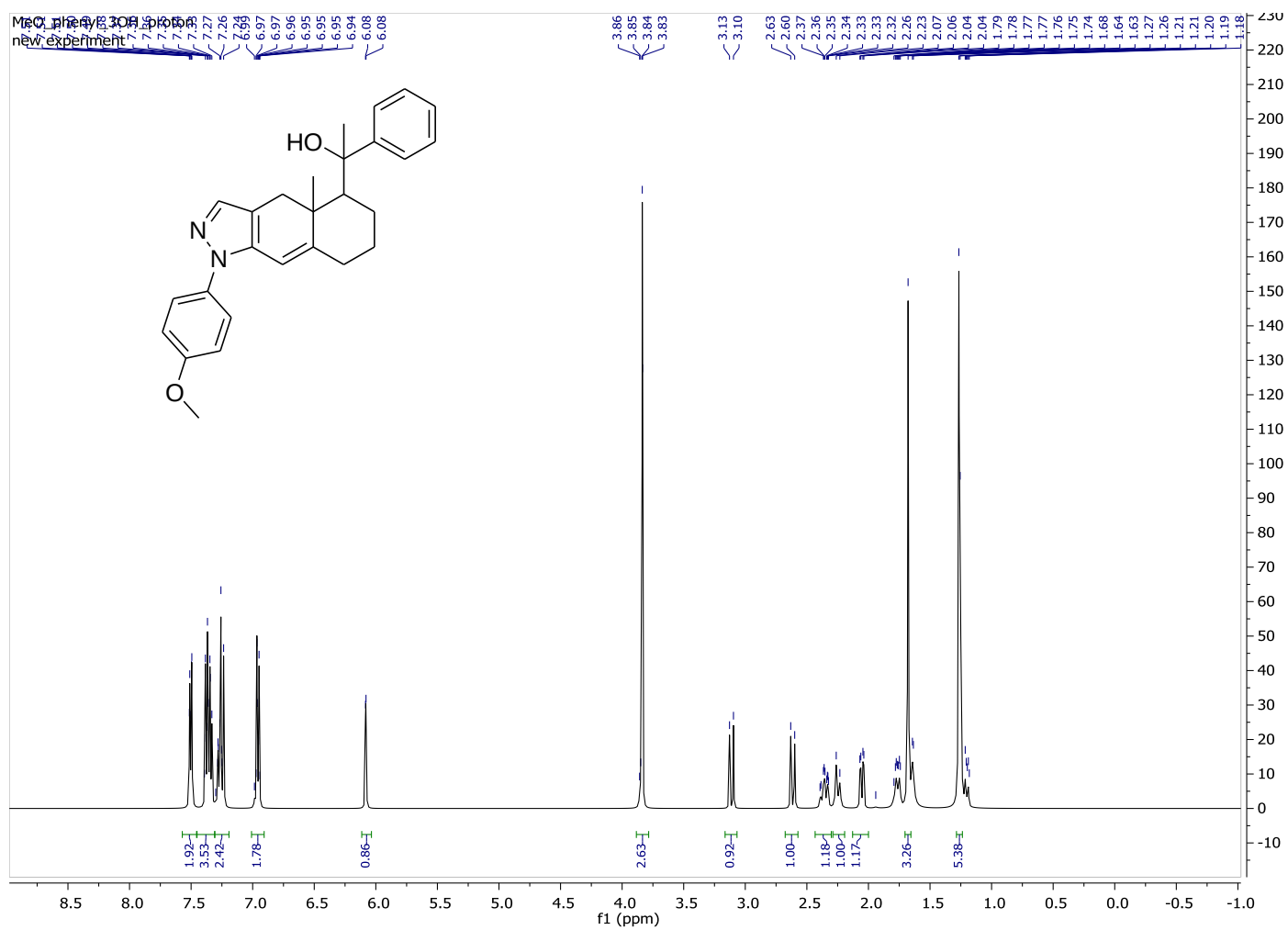
64. ^{13}C NMR spectrum of compound **11cb**.



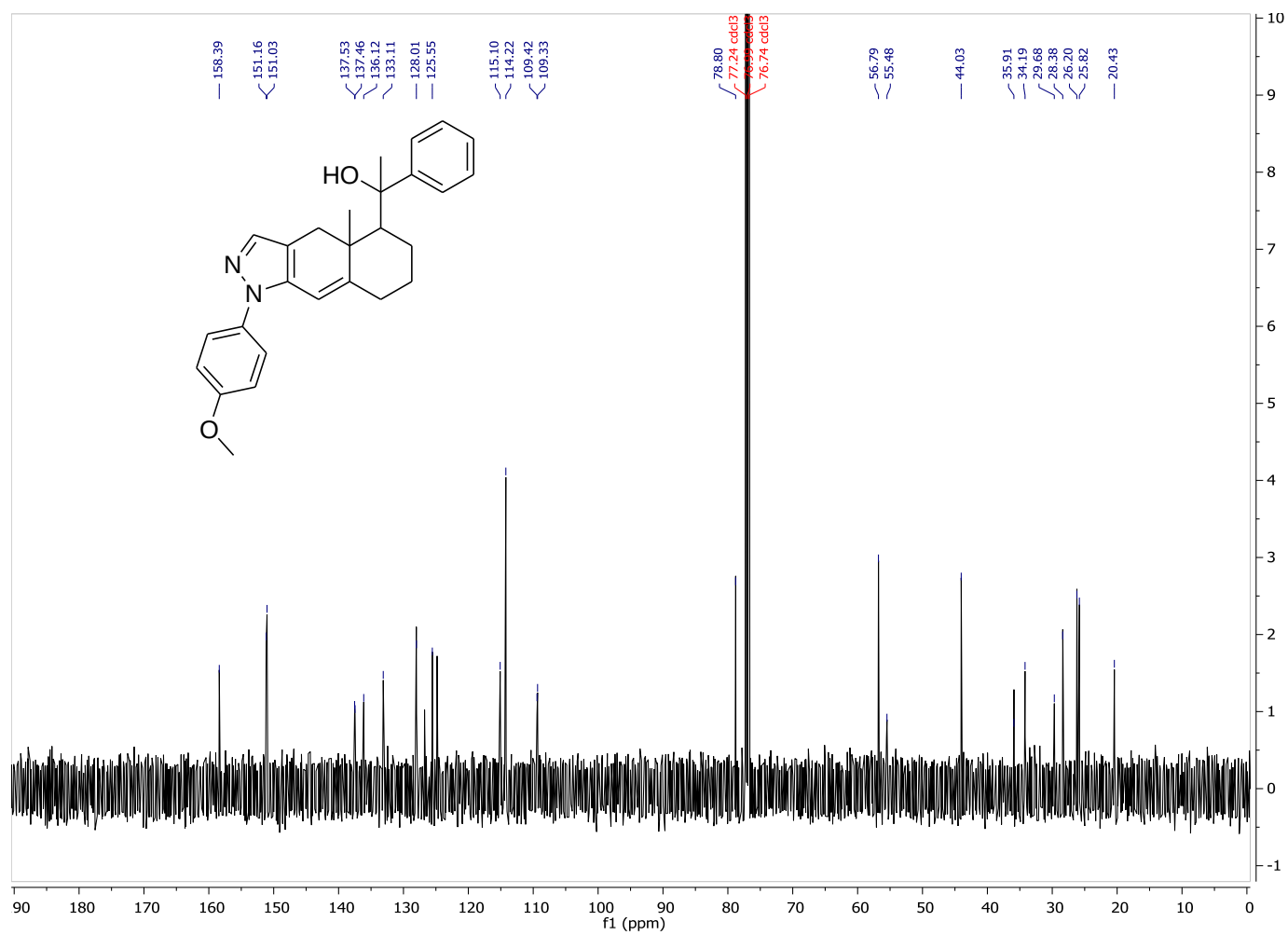
65. HRMS spectrum of compound **11cb**.



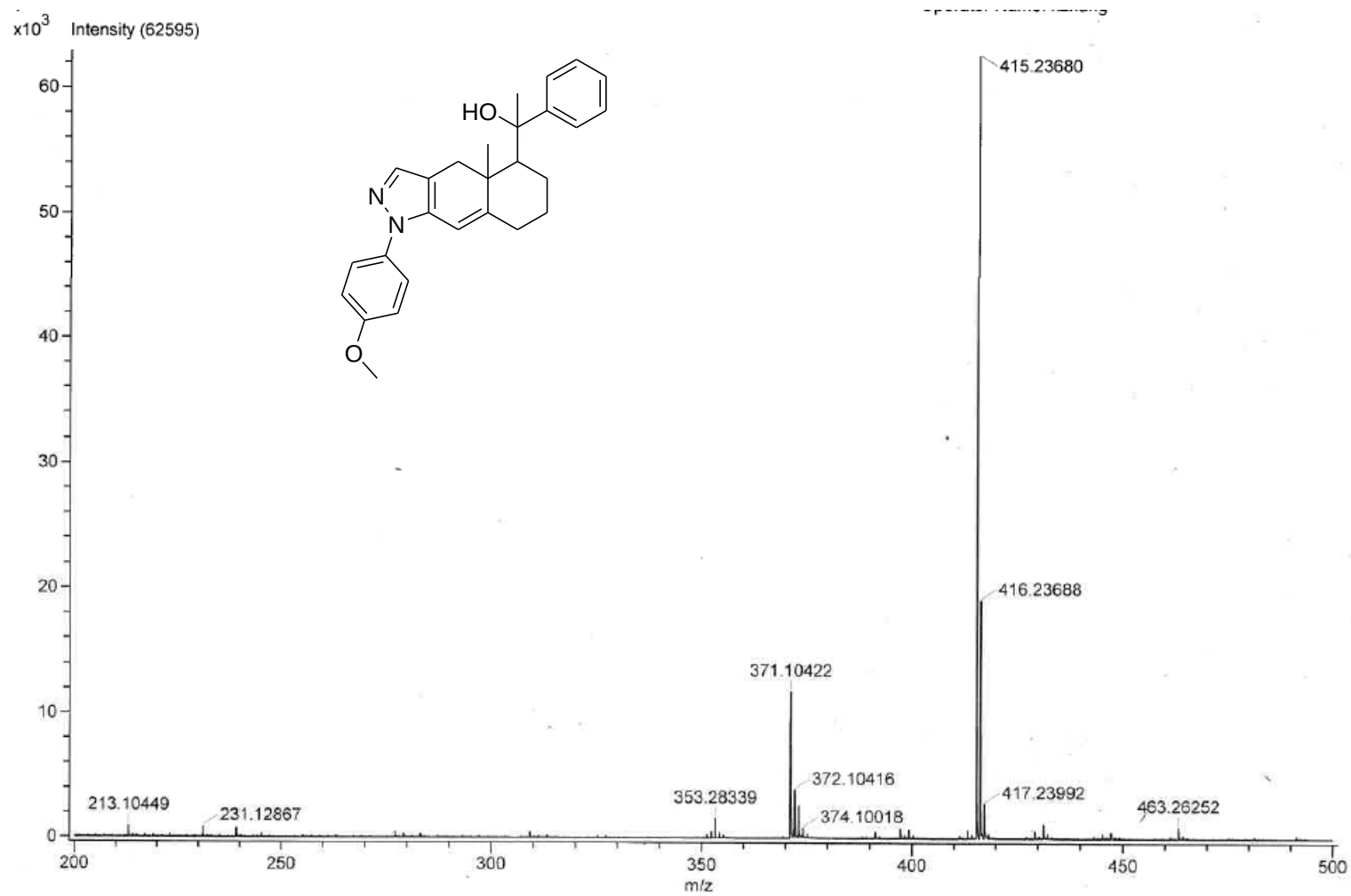
66. IR spectrum of compound **11cb**.



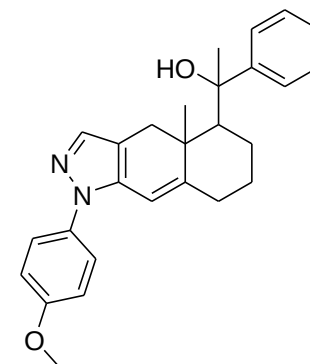
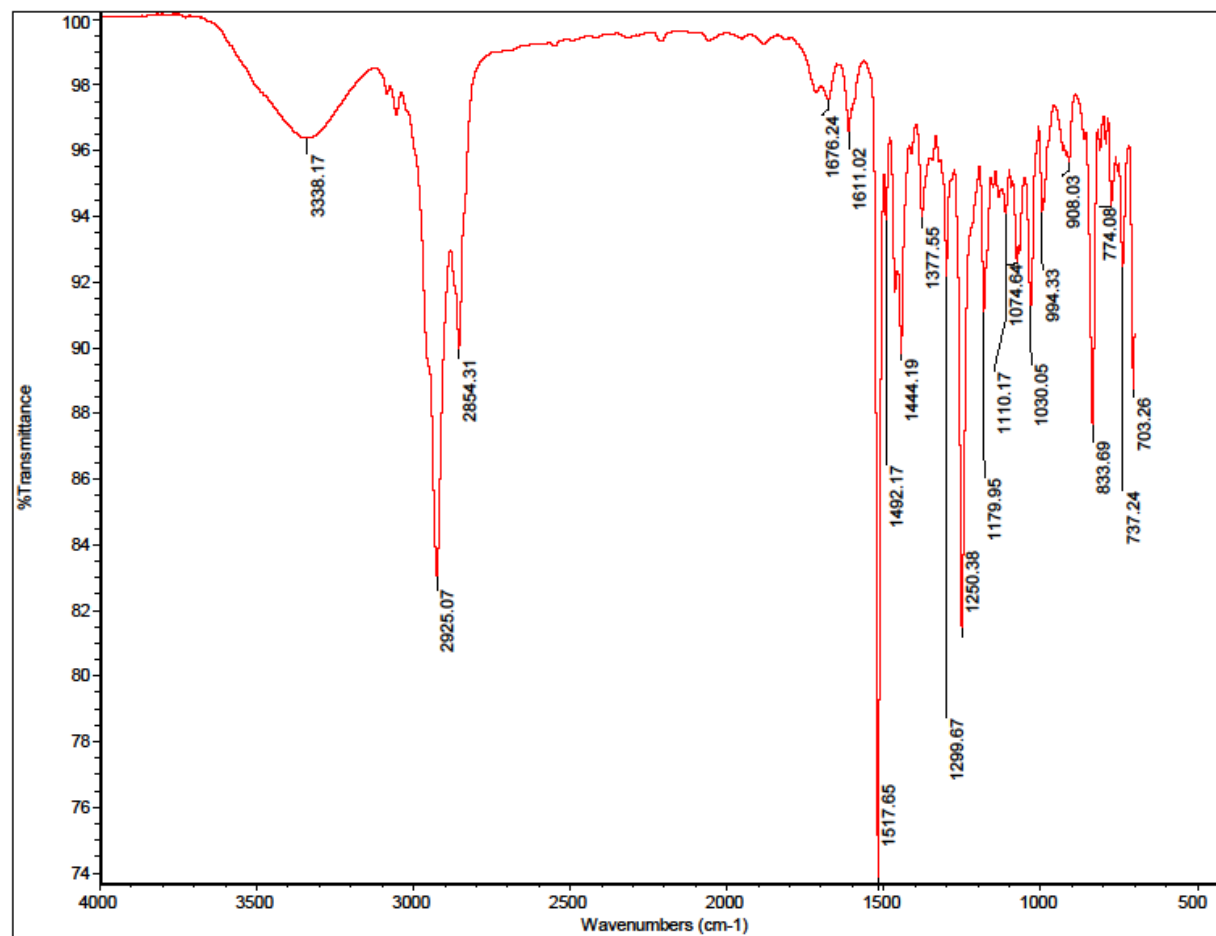
67. ¹H NMR spectrum of compound **13cb**.



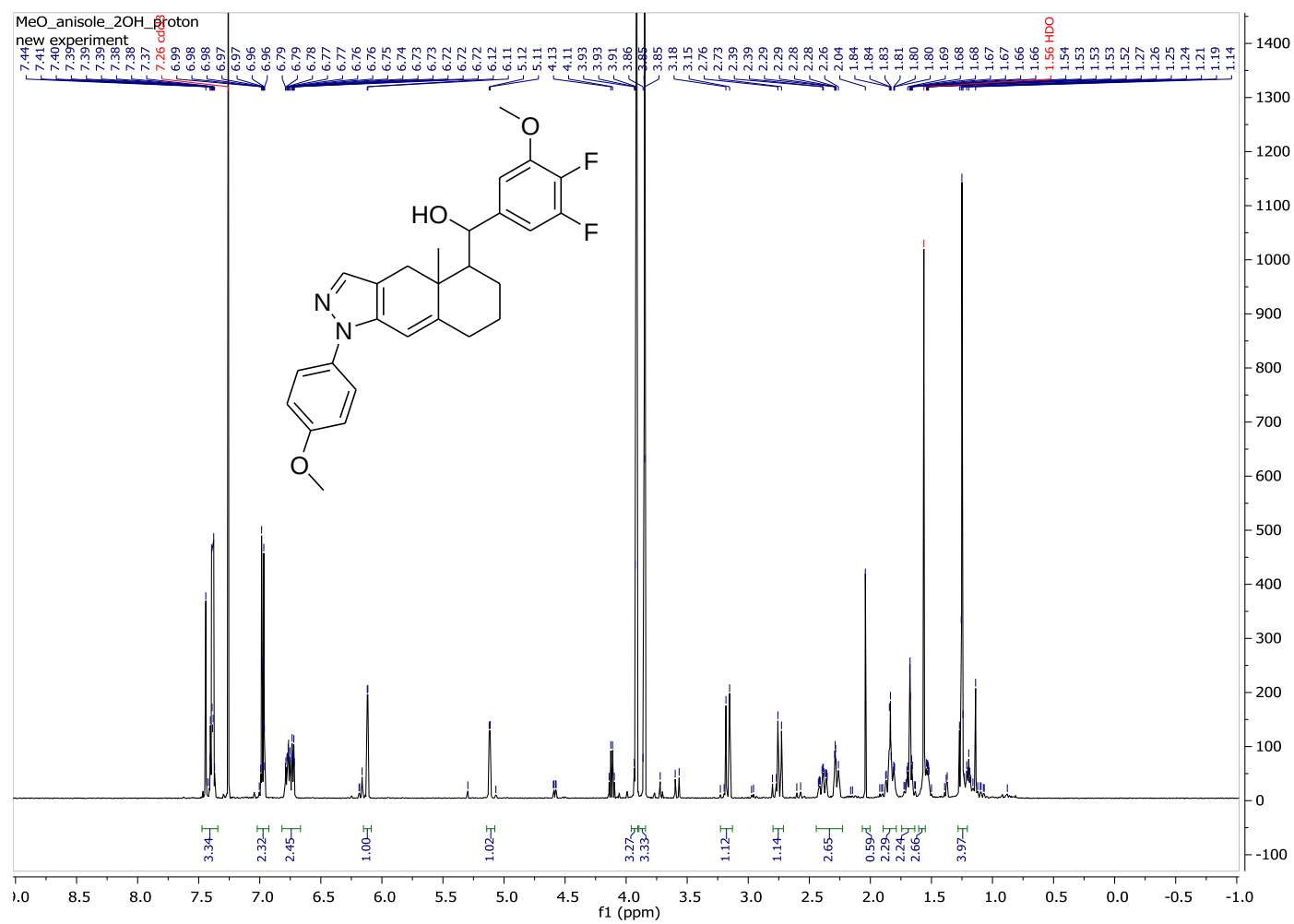
68. ^{13}C NMR spectrum of compound **13cb**.



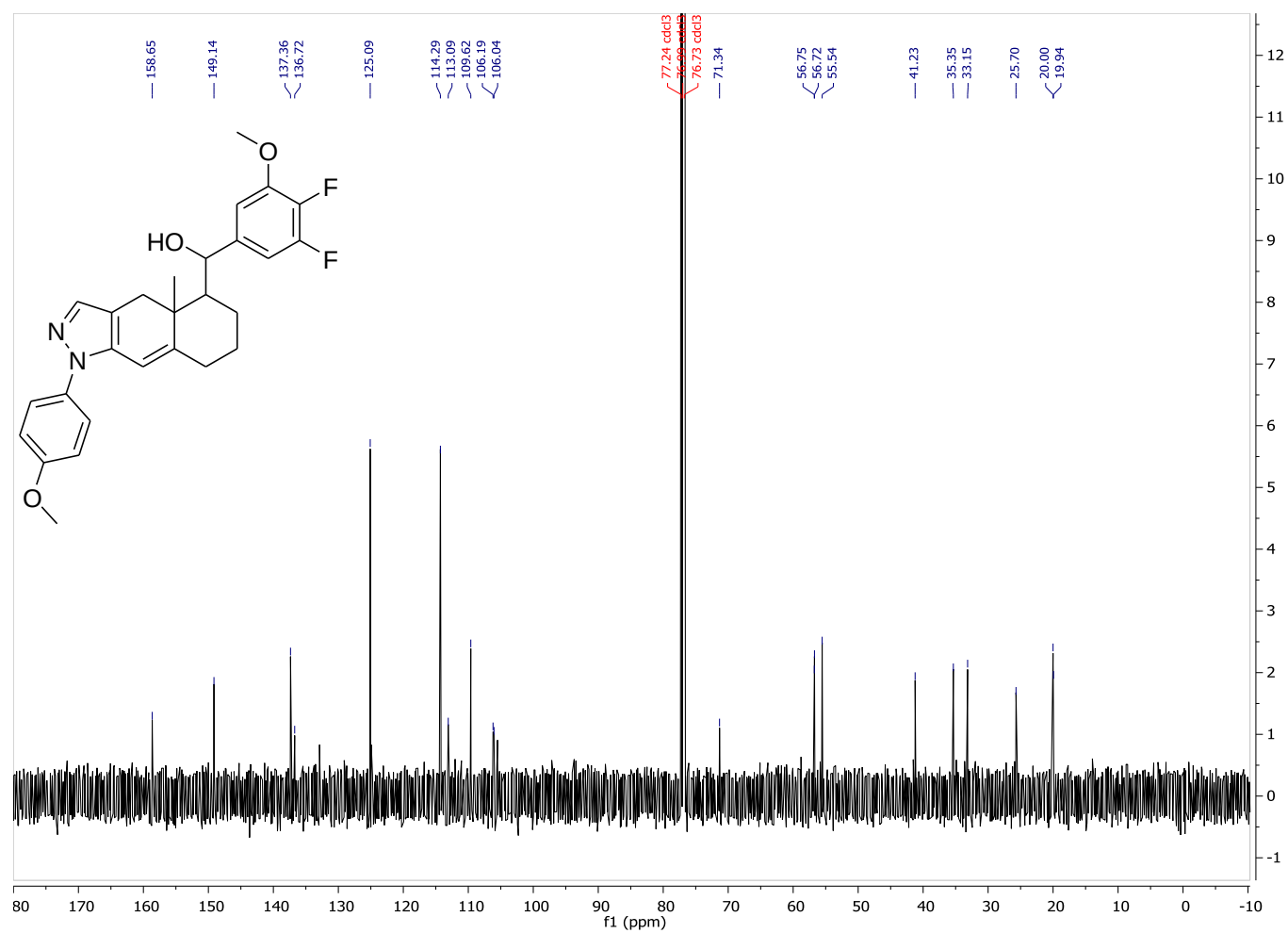
69. HRMS spectrum of compound 13cb.



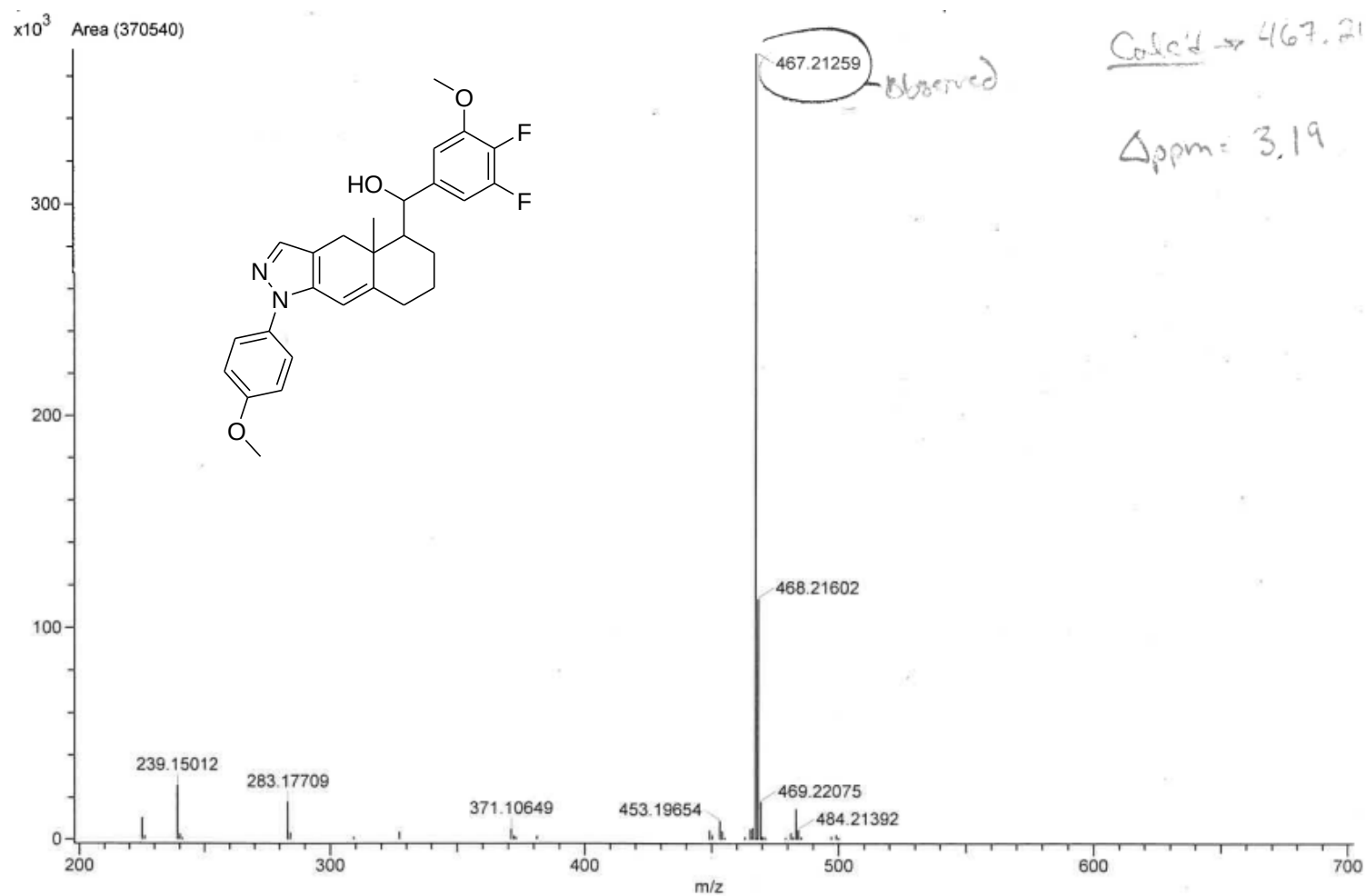
70. IR spectrum of compound **13cb**.



71. ^1H NMR spectrum of compound **11cd**.



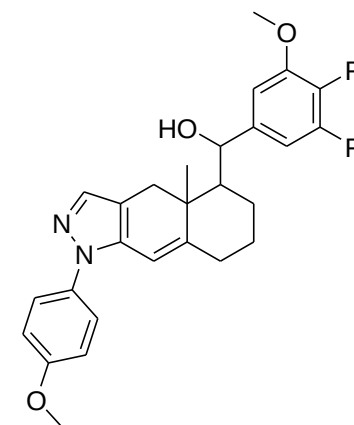
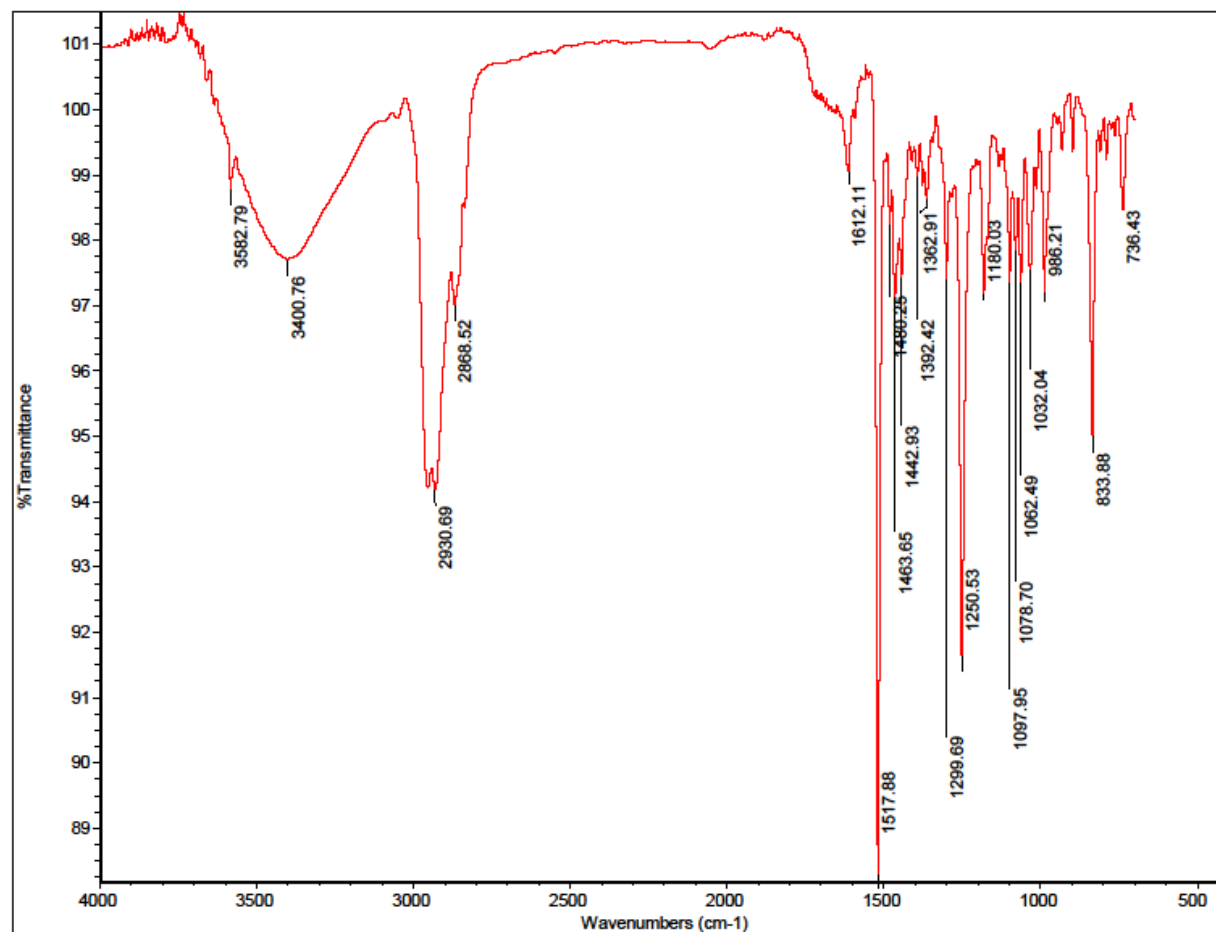
72. ^{13}C NMR spectrum of compound **11cd**.



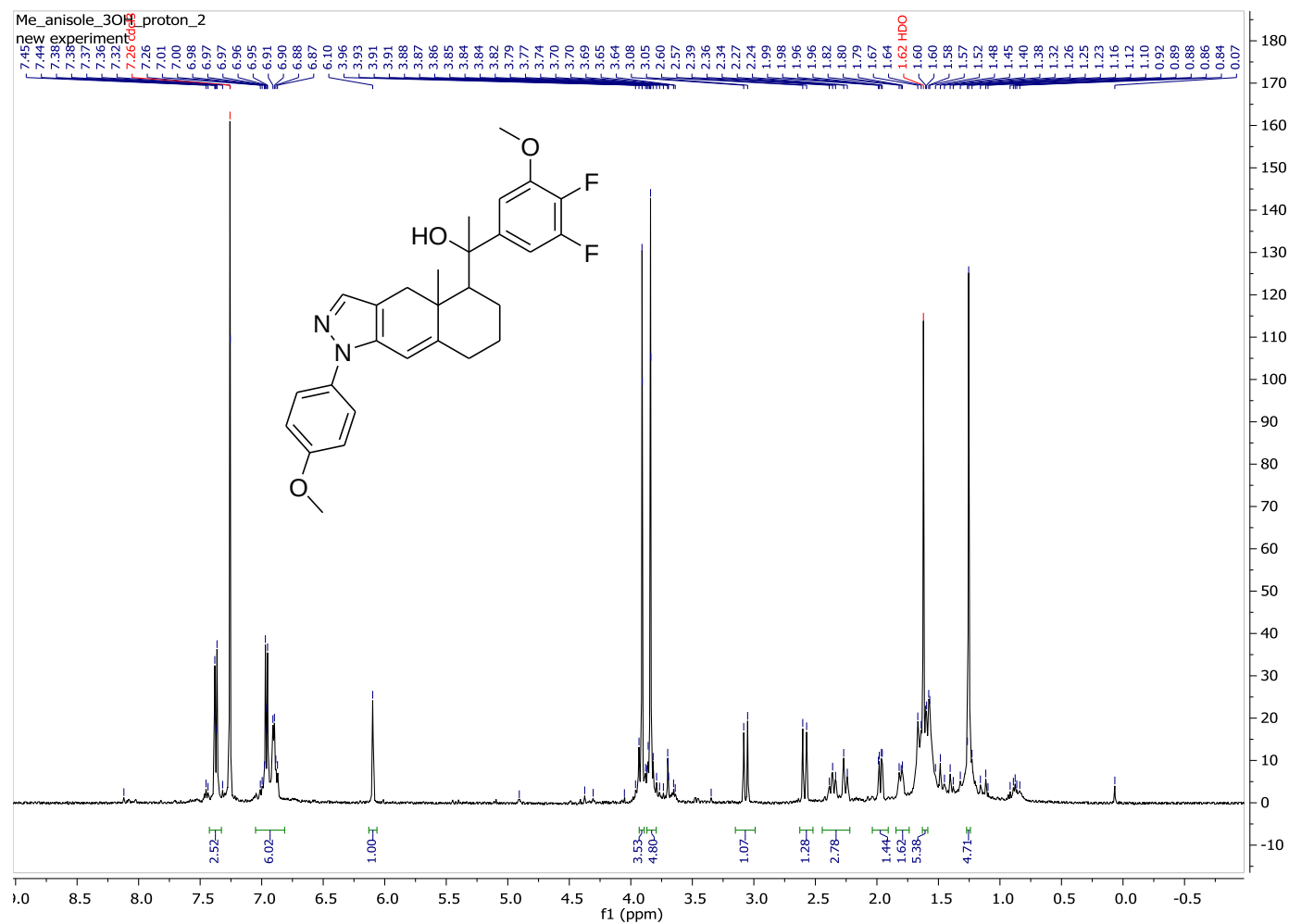
Calcd $\rightarrow$ 467.21478

Δ ppm = 3.19

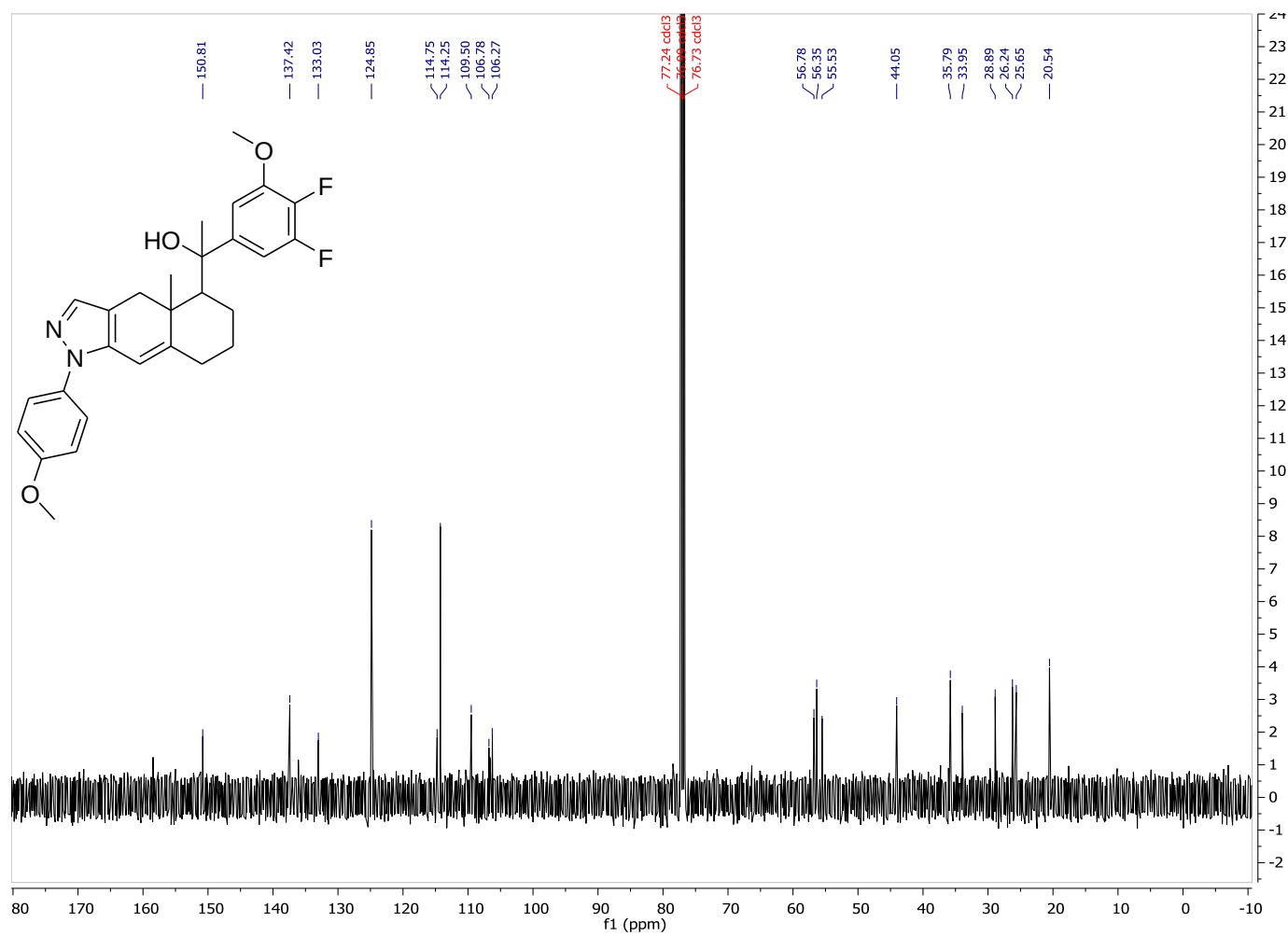
73. HRMS spectrum of compound **11cd**.



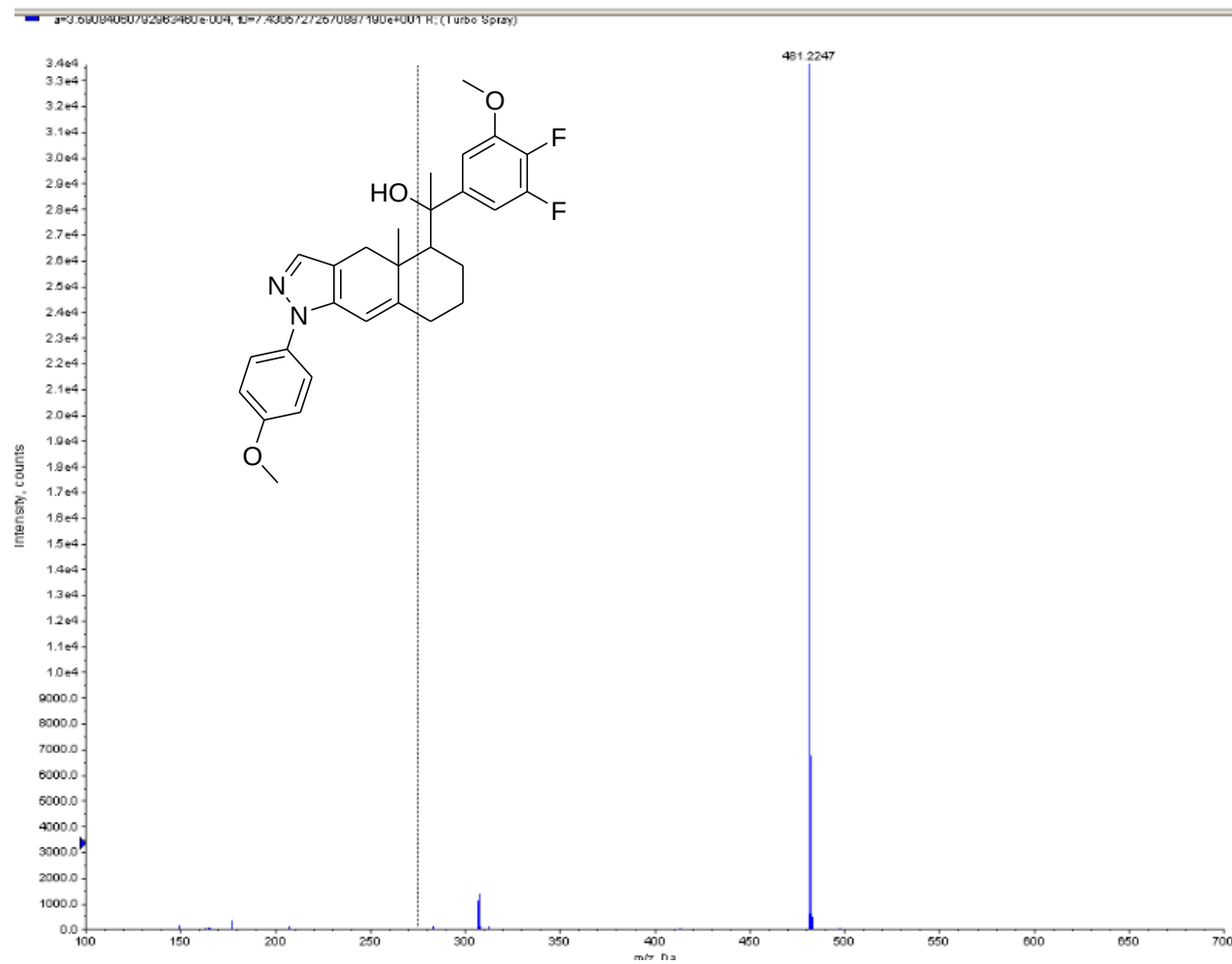
74. IR spectrum of compound **11cd**.



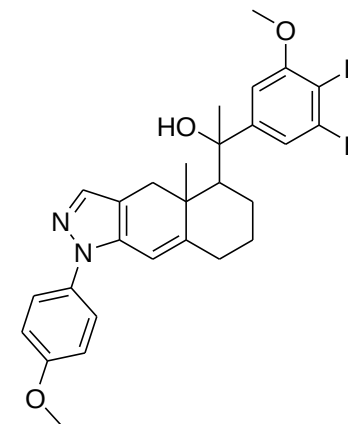
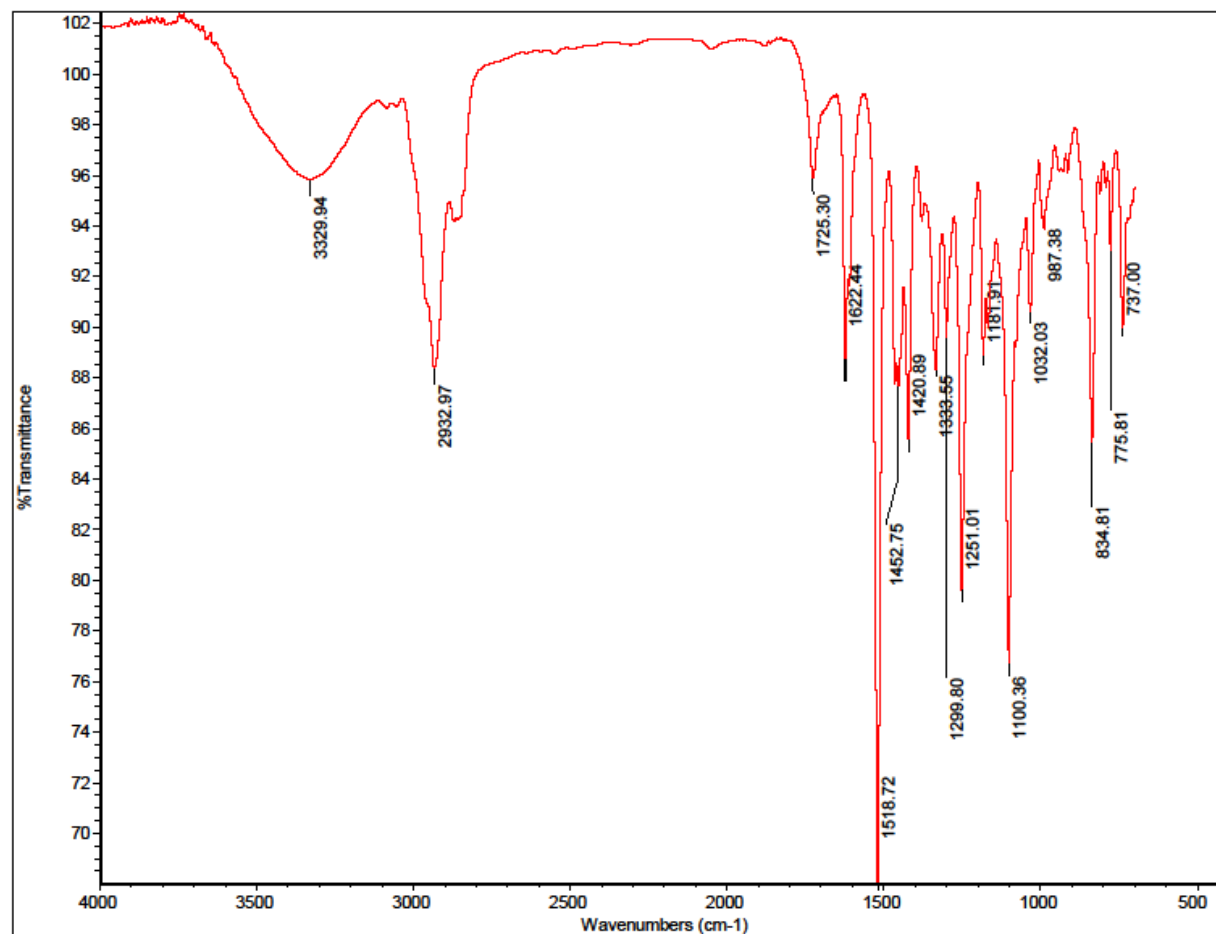
75. ¹H NMR spectrum of compound 13cd.



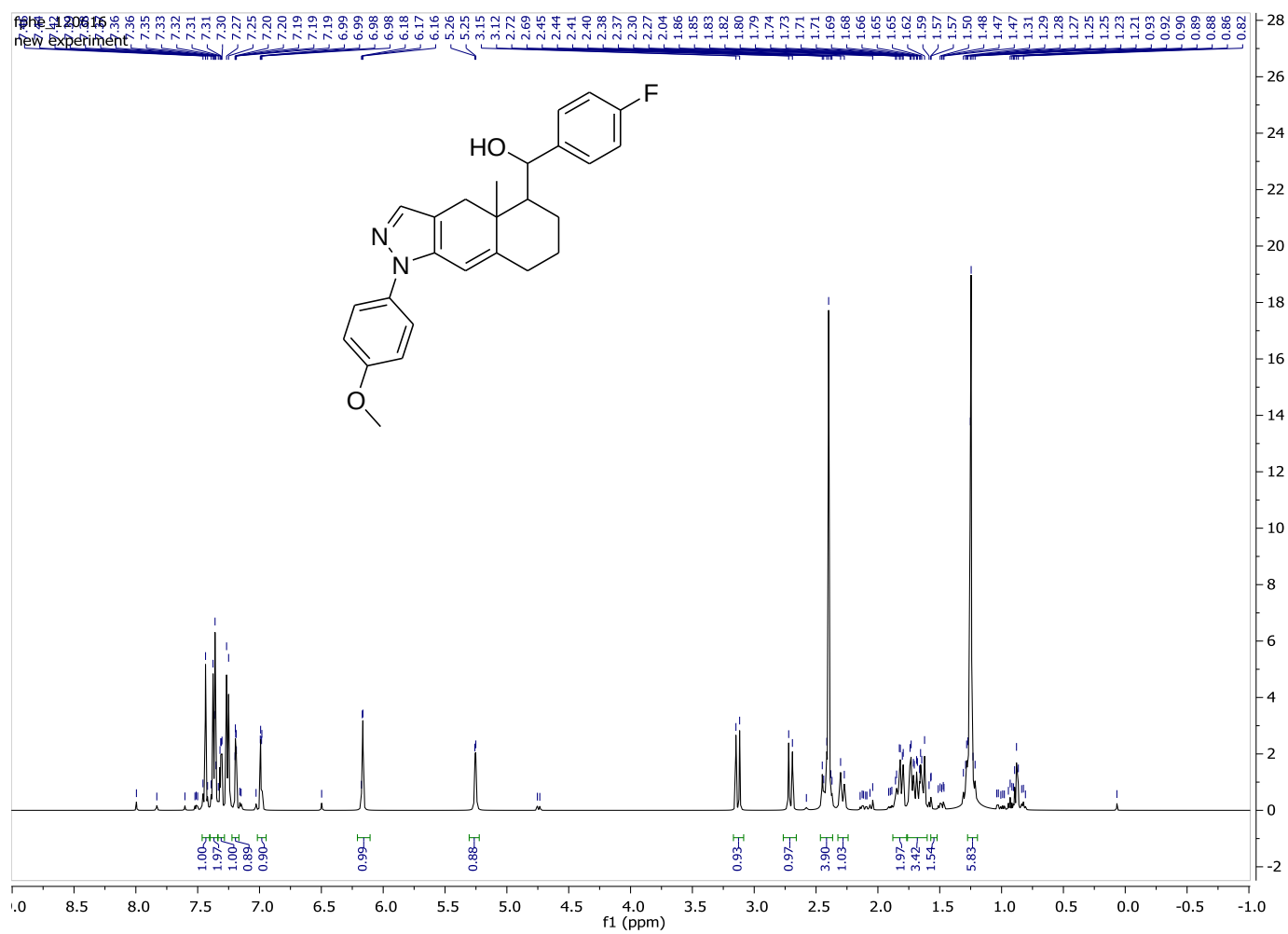
76. ¹³C NMR spectrum of compound 13cd.



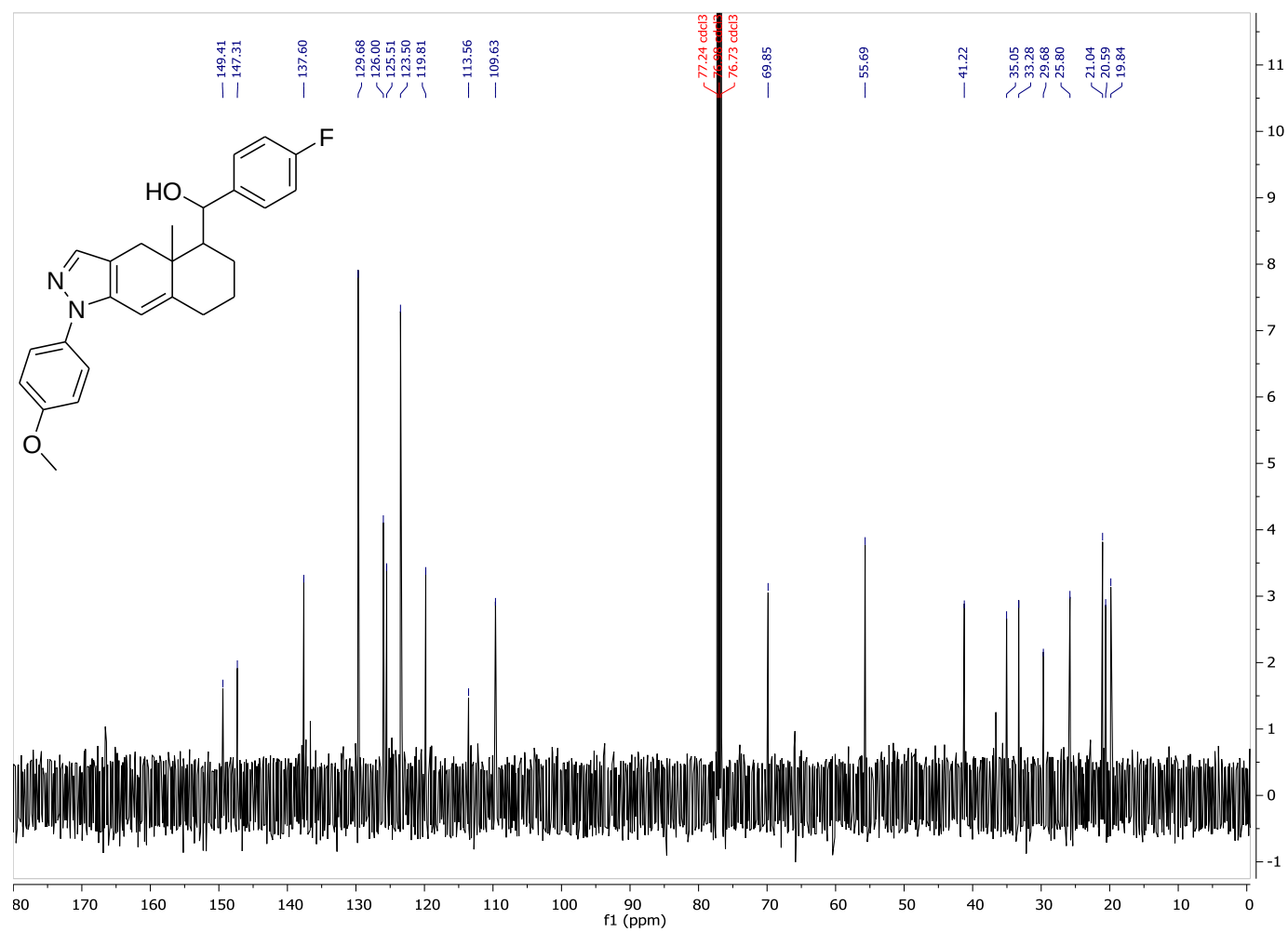
77. HRMS spectrum of compound **13cd**.



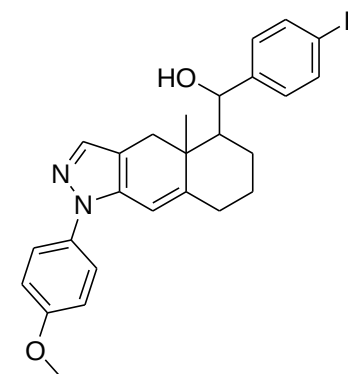
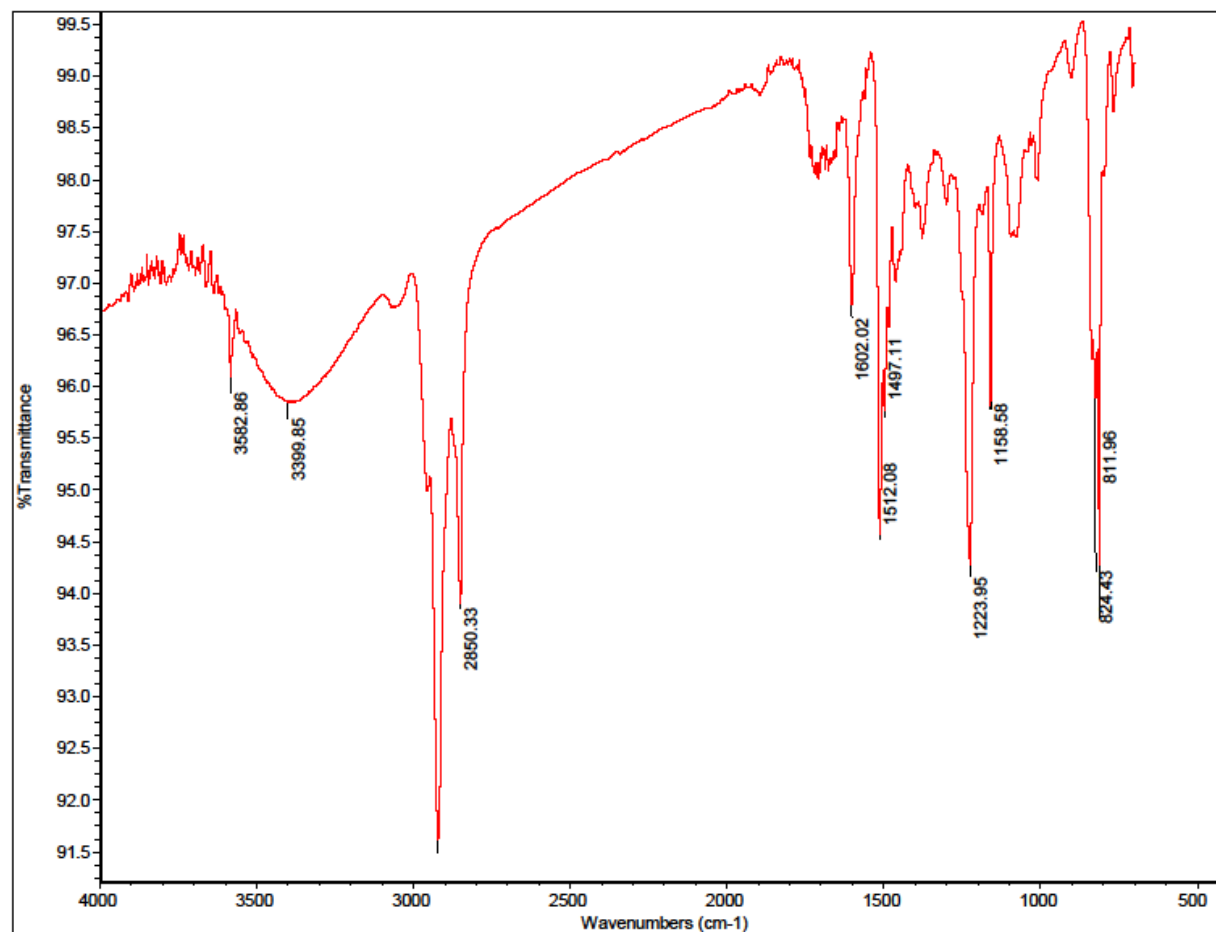
78. IR spectrum of compound 13cd.



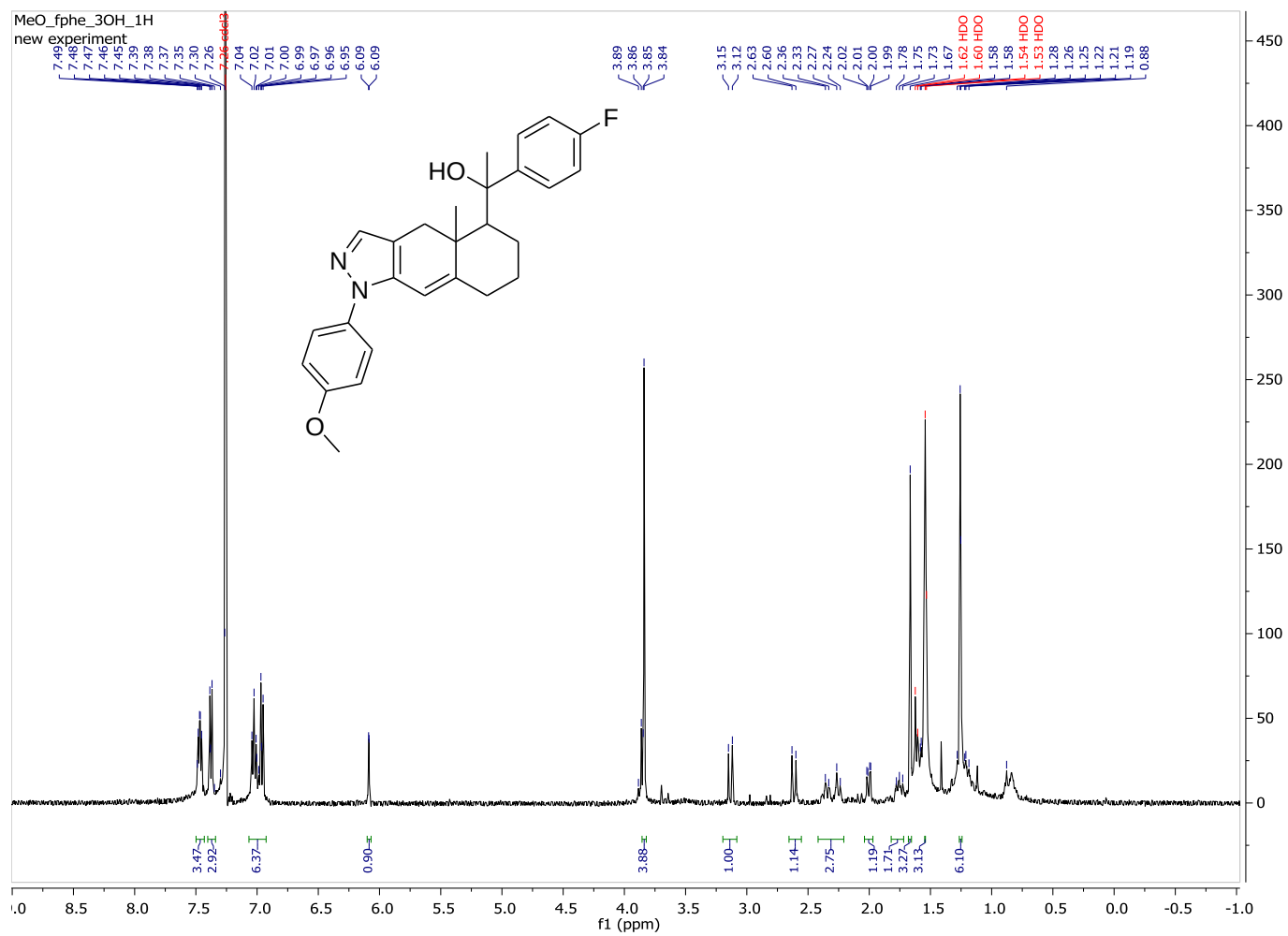
79. ¹H NMR spectrum of compound 11cc.



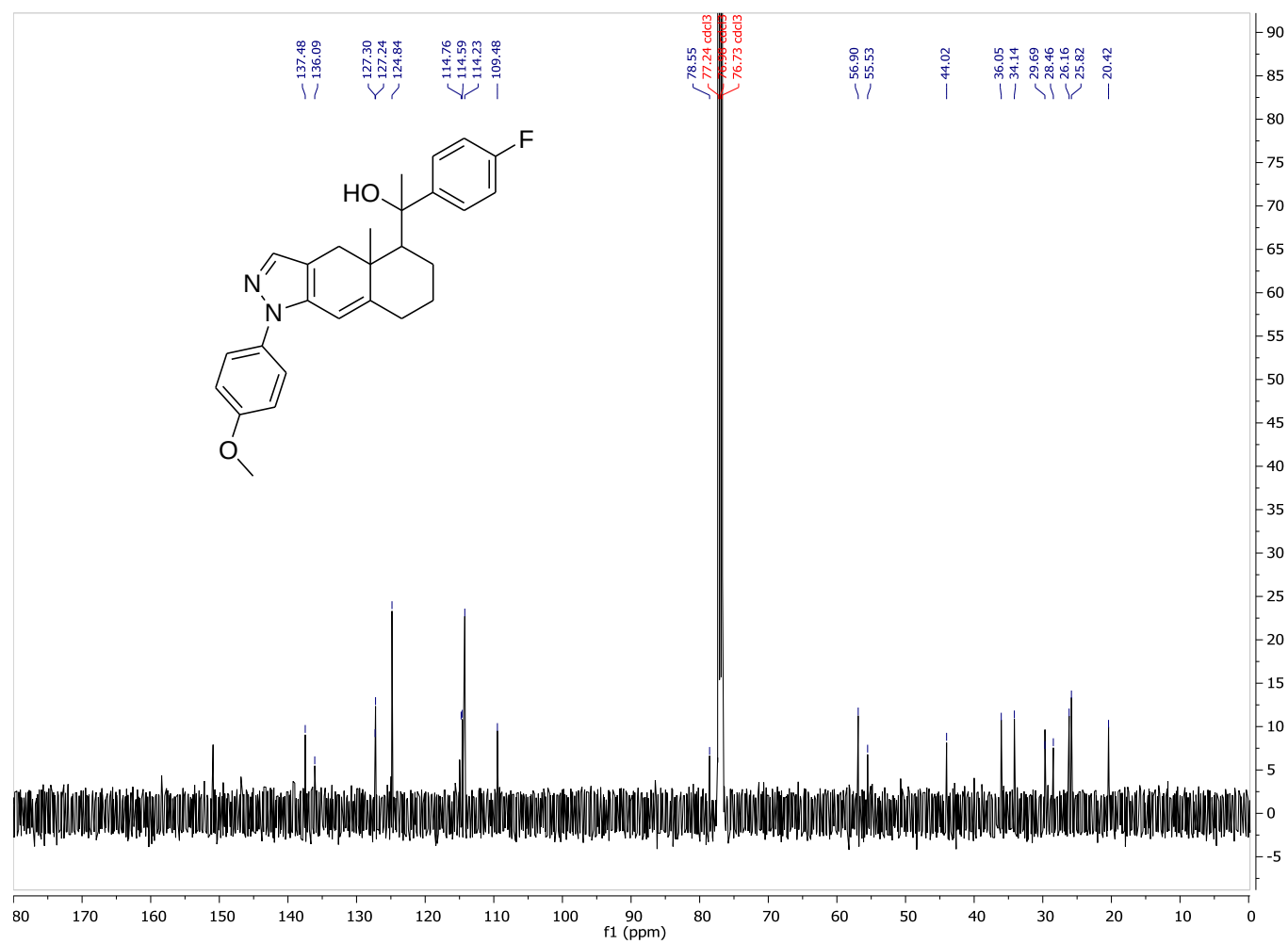
80. ¹³C NMR spectrum of compound 11cc.



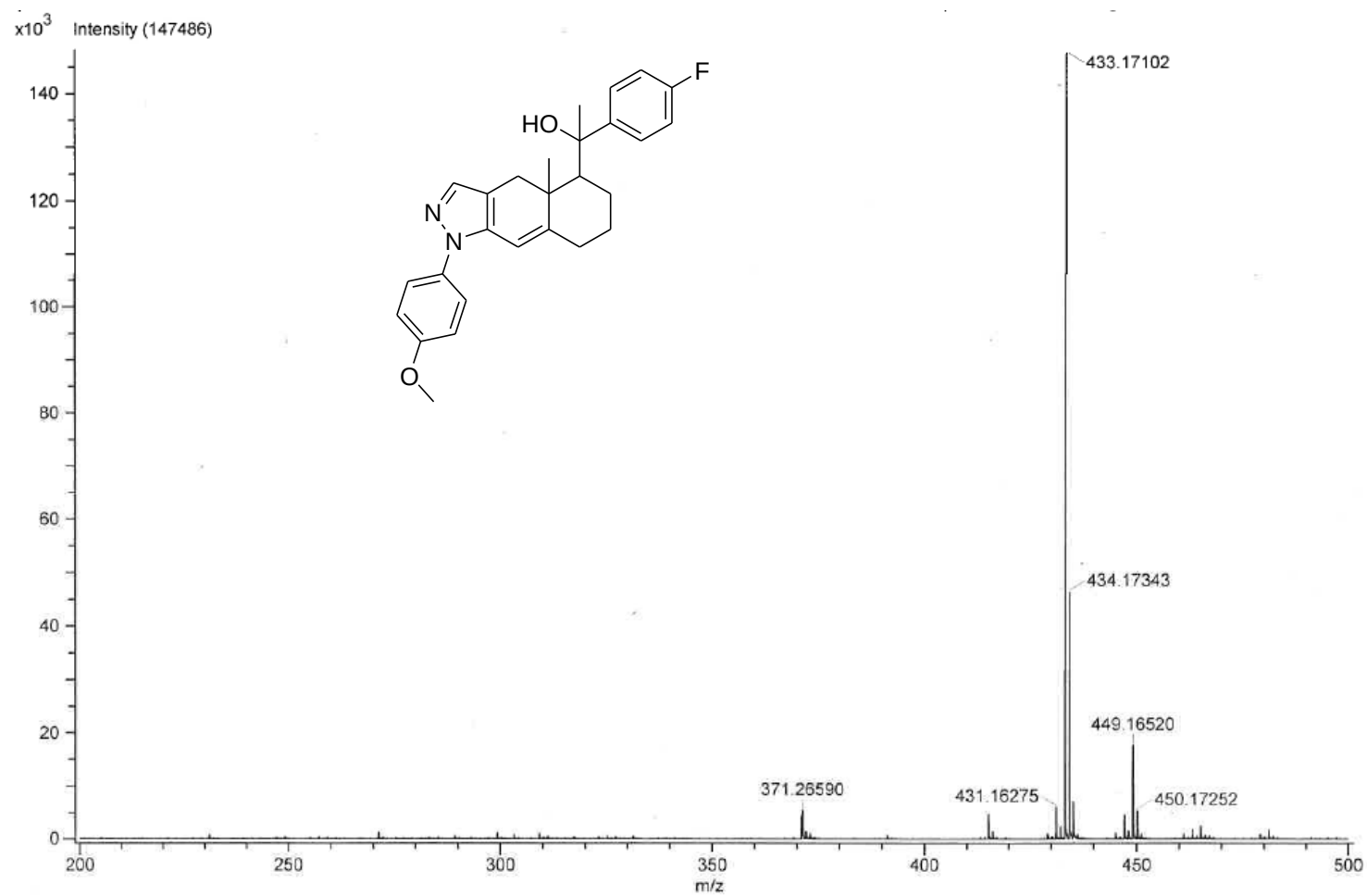
81. IR spectrum of compound **11cc**.



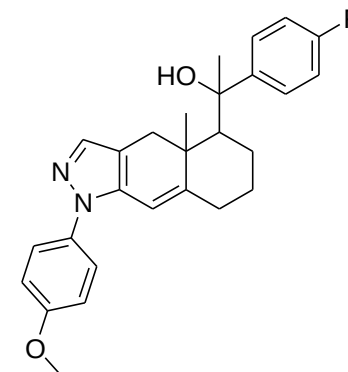
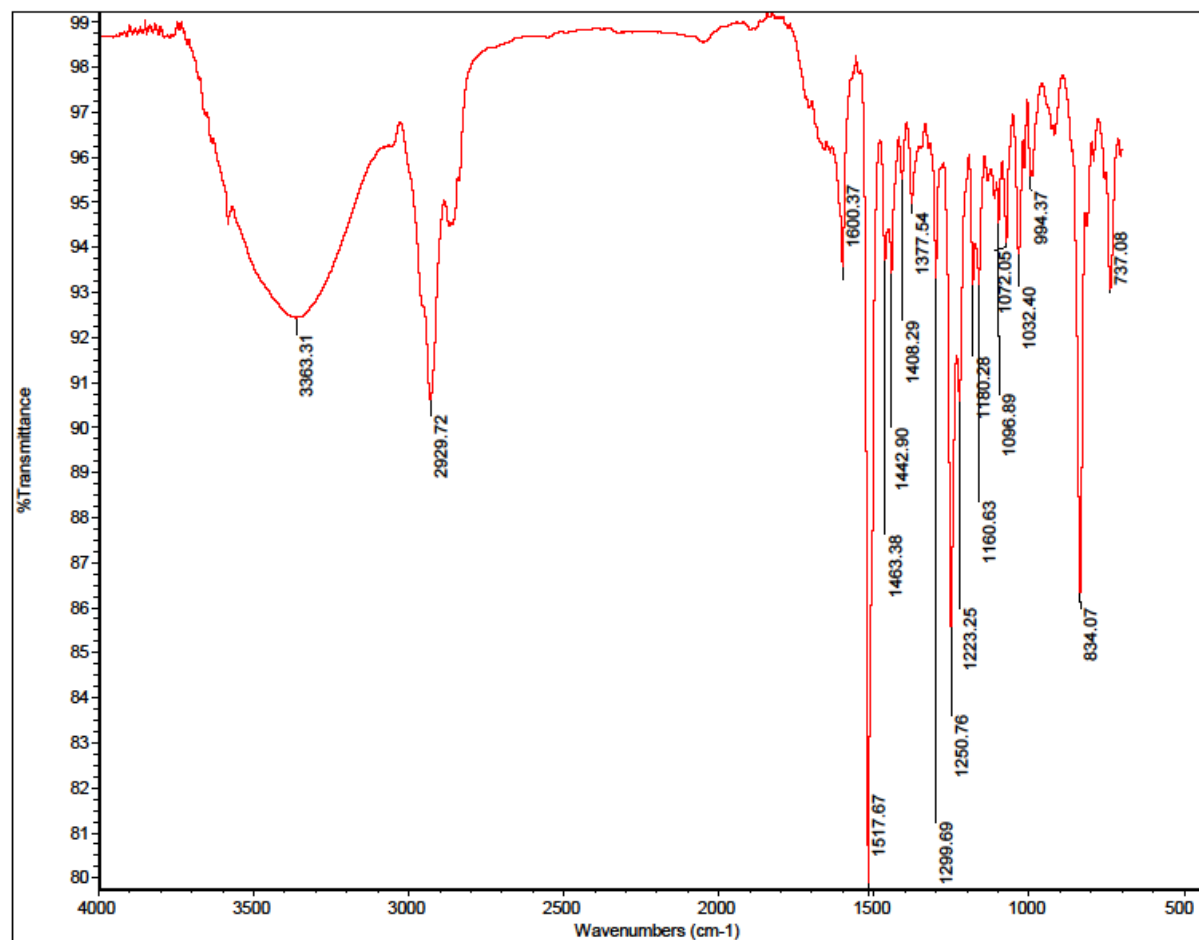
82. ^1H NMR spectrum of compound 13cc.



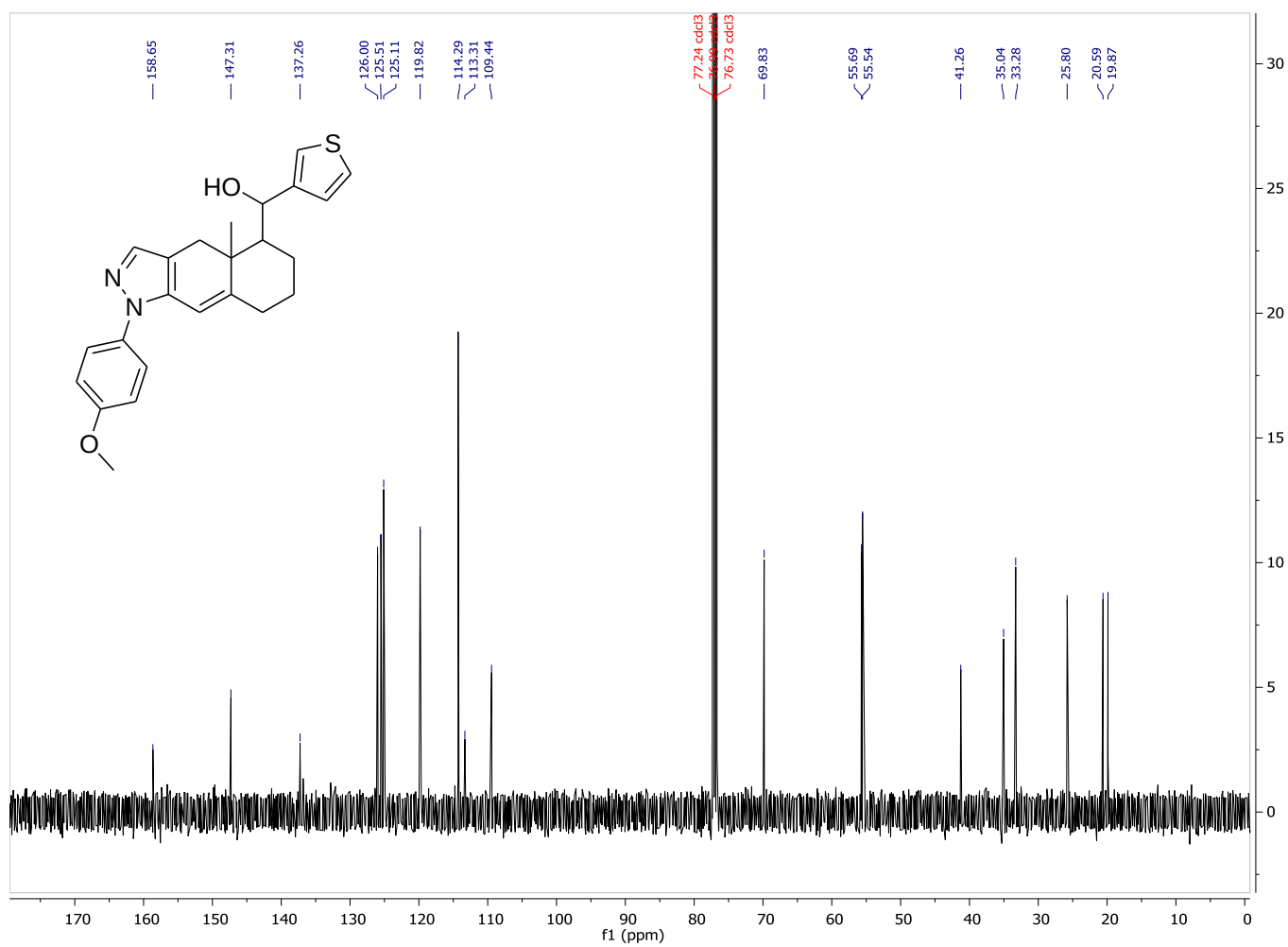
83. ^{13}C NMR spectrum of compound 13cc.



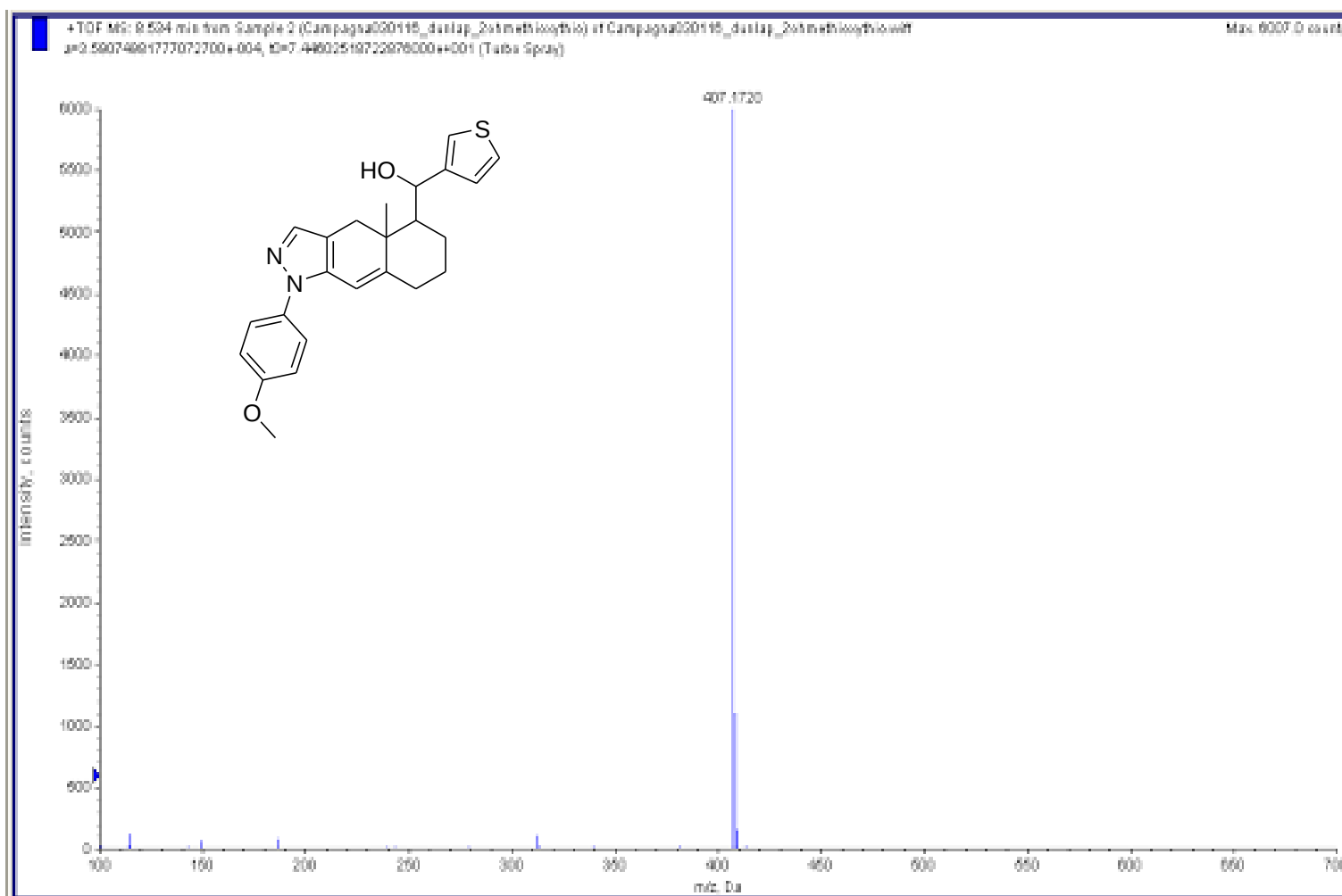
84. HRMS spectrum of compound **13cc**.



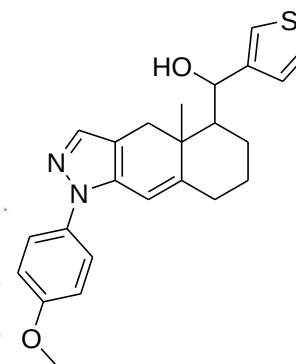
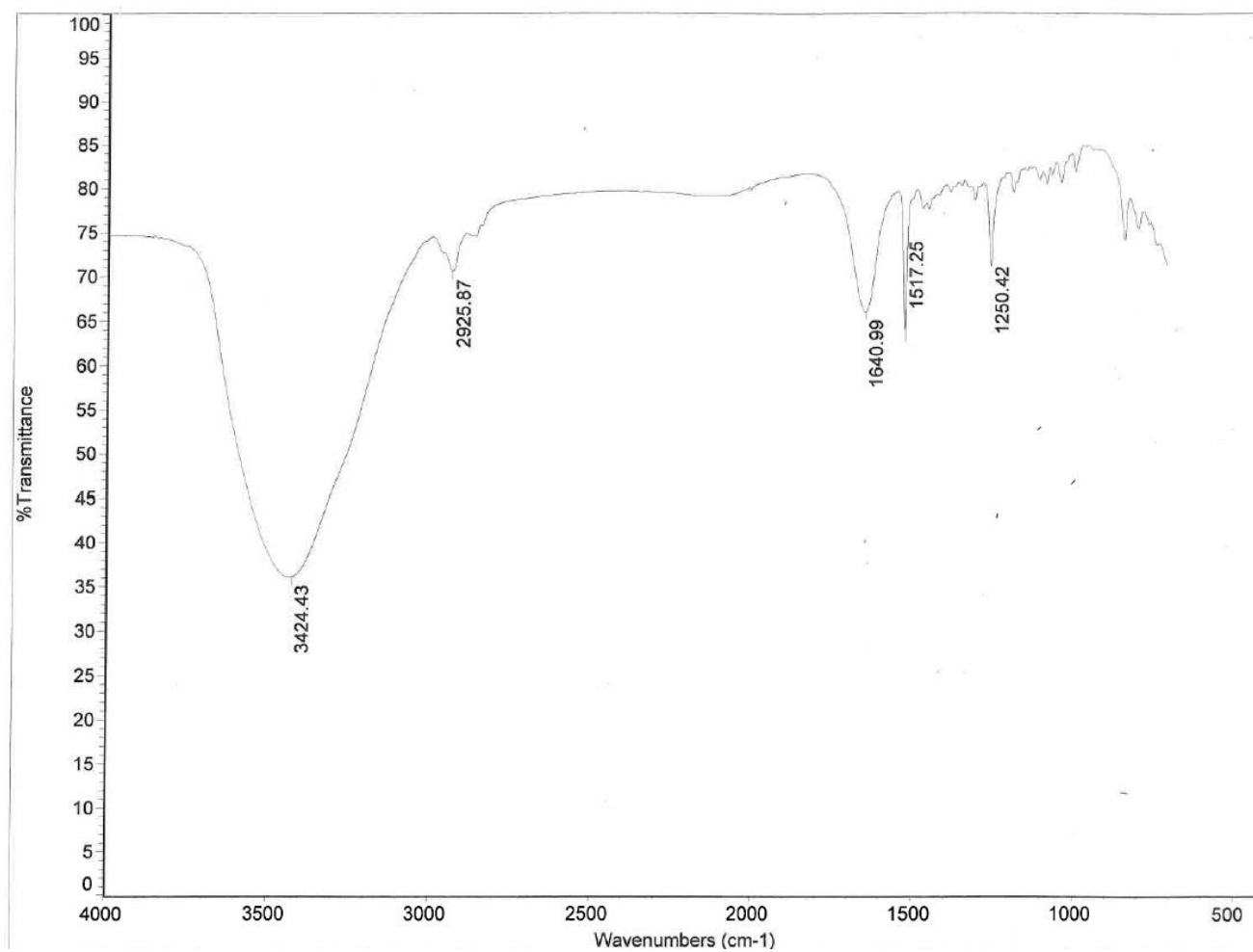
85. IR spectrum of compound 13cc.



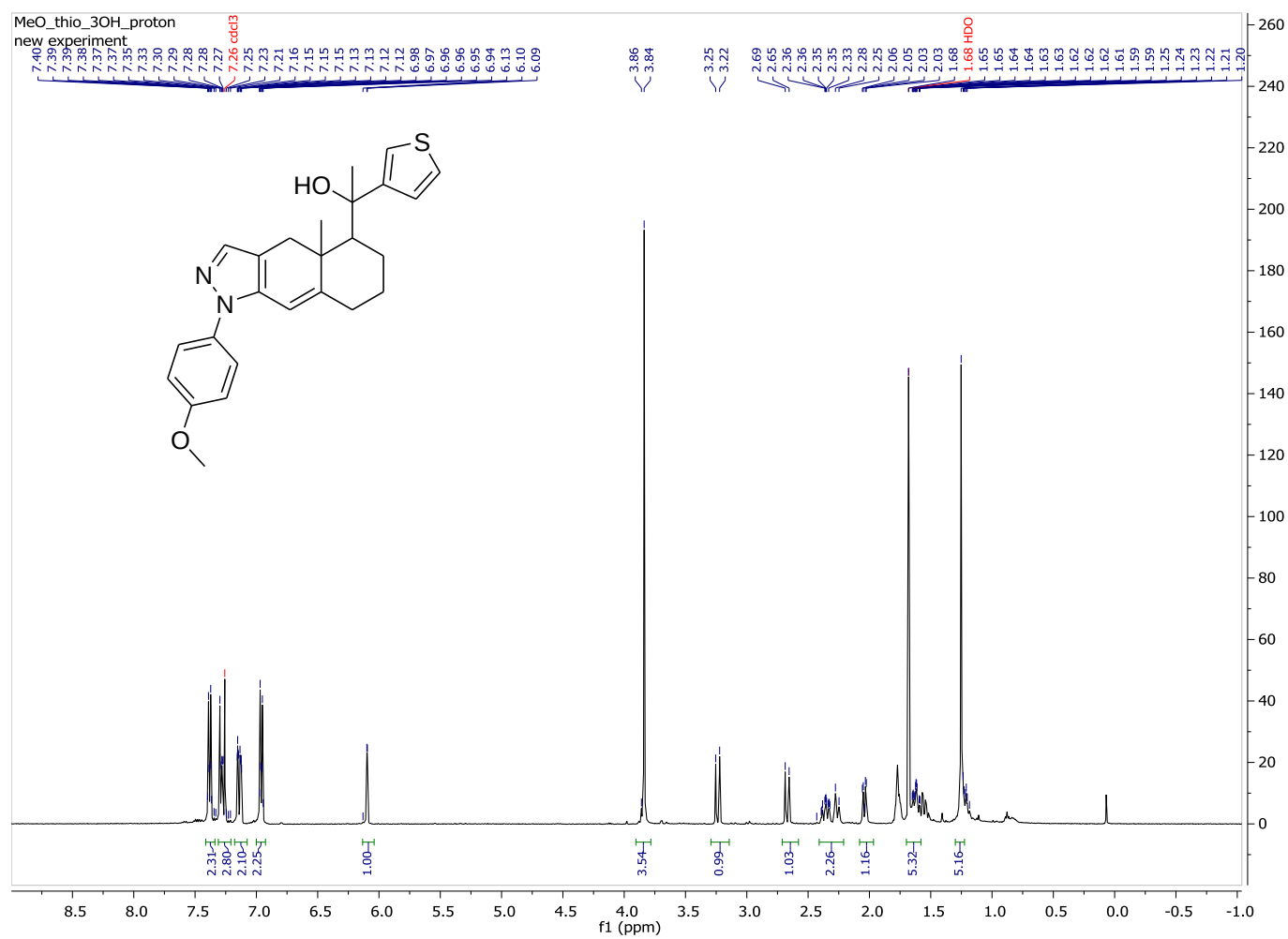
87. ^{13}C NMR spectrum of compound 11ce.



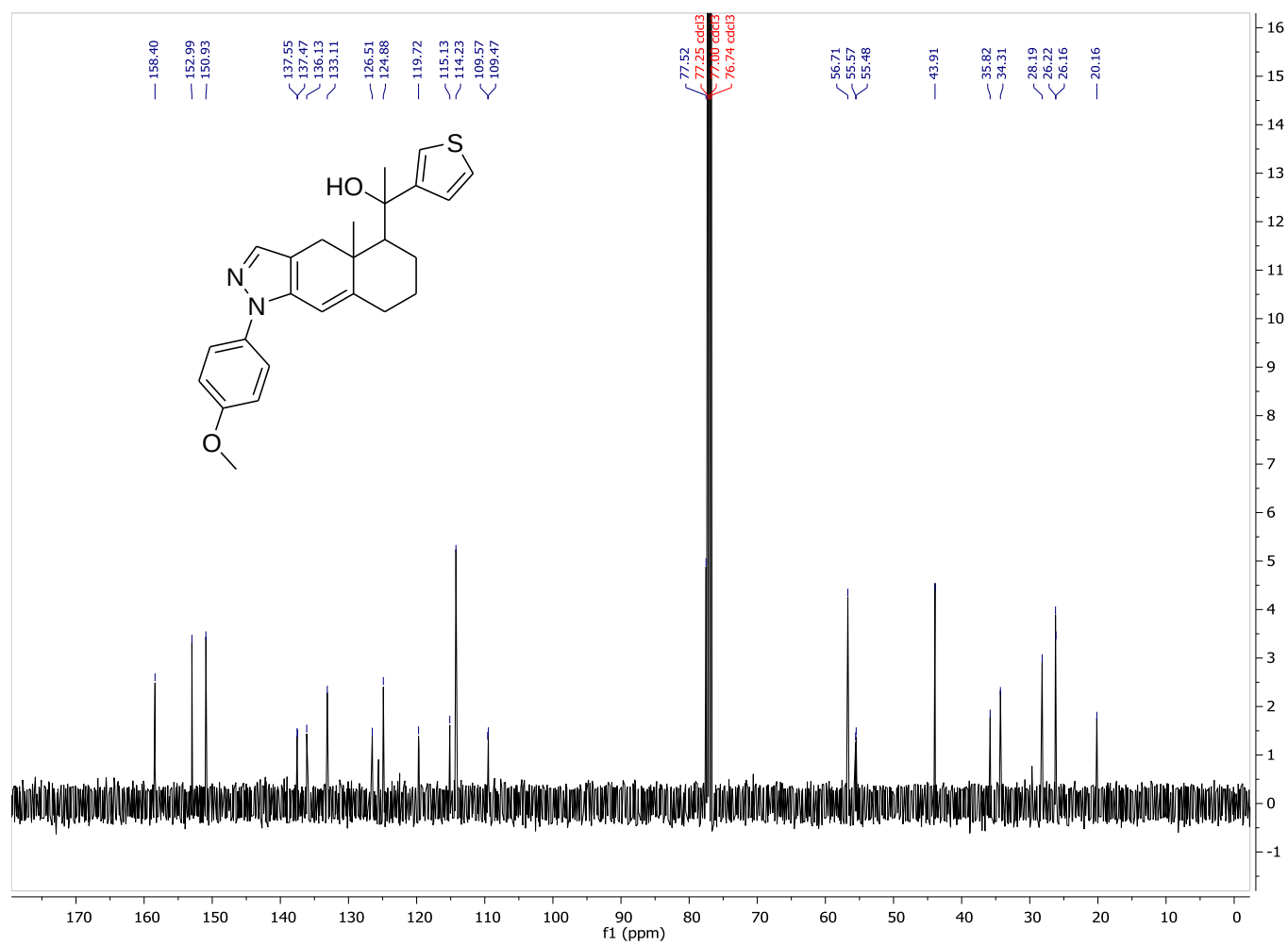
88. HRMS spectrum of compound 11ce.



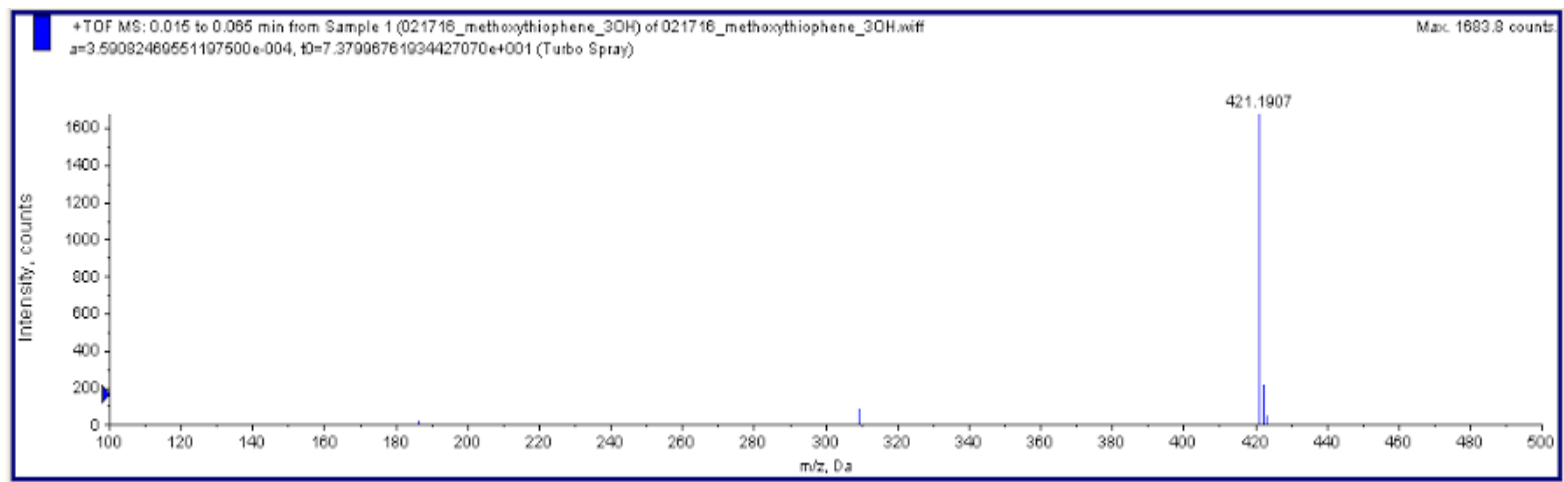
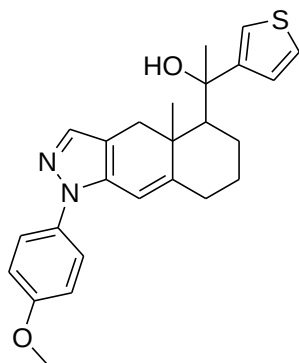
89. IR spectrum of compound **11ce**.



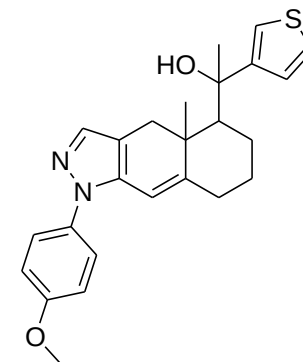
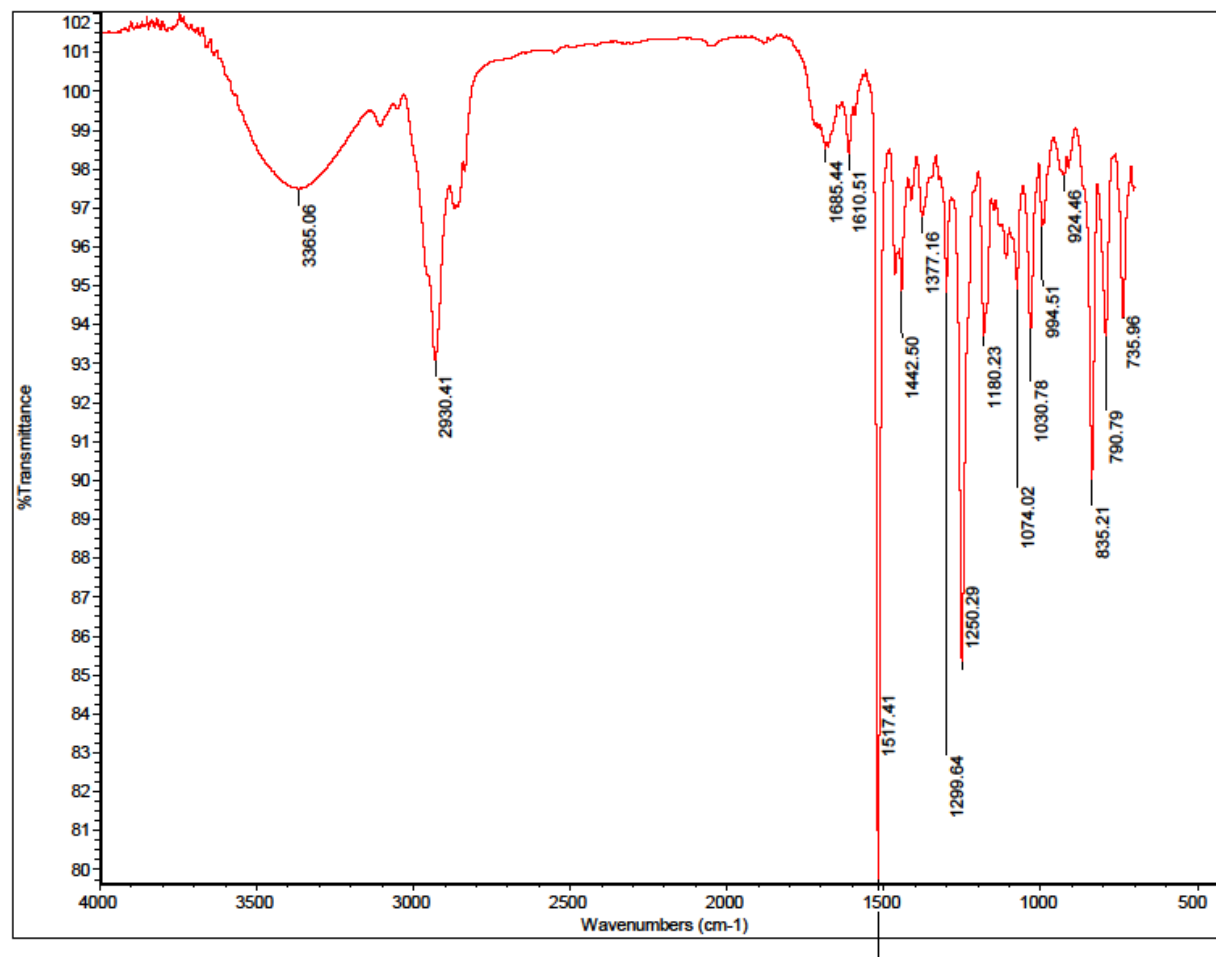
90. ^1H NMR spectrum of compound **13ce**.



91. ^{13}C NMR spectrum of compound 13ce.



92. HRMS spectrum of compound **13ce**.



93. IR spectrum of compound 13ce.

VITA

Carson Wayne Prevatte obtained a B.S. degree in biochemistry at Tennessee Technological University in 2011. He is currently working in the lab of Dr. Shawn Campagna at the University of Tennessee, Knoxville utilizing the techniques of synthetic and analytical chemistry to discover, synthesize, and characterize various biological signaling molecules and signaling disruptors in applications such as non-steroidal anti-inflammatory drug discovery and studies of BPA degradation, and marine bacterial communication.