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## Bioreversible Derivatization of Drug Molecules with a Pyridine Ring for Improved Water Solubility: Model Studies

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Bioreversible Derivatization of Drug Molecules with a Pyridine Ring for Improved Water  
Solubility: Model Studies

by

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Under the Direction of Binghe Wang, PhD

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

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## ABSTRACT

A prodrug is a modified drug molecule having no significant biological activity but converts to the parent drug in the biological system. Conventionally, the prodrug is designed and synthesized by modifying a functional group that serves as a 'synthetic handle'. However, certain drugs do not have any functional group for easy manipulations. For these drugs, it becomes challenging to design or synthesize a prodrug. Herein, we examine a series of methods for bioreversible derivatization of drugs with a pyridine ring to improve their physicochemical properties including water solubility and conjugation with a targeting moiety. Several model prodrugs were synthesized and examined for solubility, stability, and bioreversible conversion. Three water-soluble prodrug models (**BW-HIF-352**, **BW-HIF-354**, and **BW-HIF-355**) showed significant amount of drug activation under physiological conditions. The same method should be applicable to the preparation of prodrugs of other drugs that contain a pyridine ring or even a quinoline ring.

**INDEX WORDS:** Prodrug, Synthetic handle, Bioreversible, Water solubility.

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Solubility: Model Studies

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## 1 INTRODUCTION

### 1.1 Prodrugs

Prodrugs are molecules which have no significant biological activity but convert to the drug molecules in the biological system. Prodrug preparation often involves derivatization of a drug molecule via a functional group/handle, which affords bioreversibility *in vivo*. The conversion of a prodrug molecule to its parent drug can be accomplished by either an enzymatic reaction or a chemical reaction, or by a combination of these two.<sup>1, 2</sup> Prodrug strategies are often used for drugs having pharmaceutical issues including poor water solubility (e.g., corticosteroids, Figure 1), poor absorption (e.g., ampicillin, Figure 1), poor targeting or bad site selectivity, high toxicity, instability (e.g., dopamine, Figure 1), high first-pass metabolism (e.g., propranolol, Figure 1), and bad taste/smell, among other properties. These problems limit drug development in multiple ways, from formulation to poor pharmacokinetics.<sup>3, 4</sup> Prodrug strategies can also be used for targeting through conjugation with a targeting moiety.

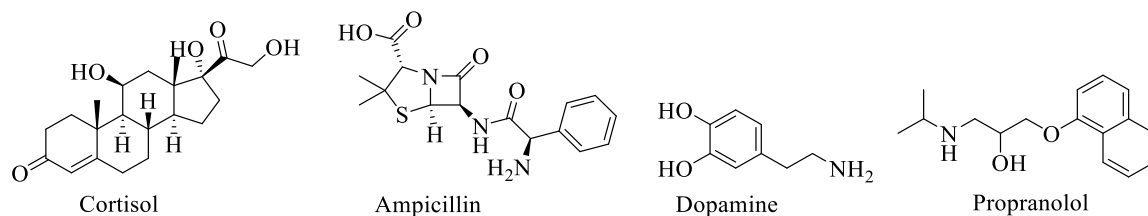


Figure 1. Structures of cortisol (corticosteroid class), ampicillin, dopamine, and propranolol.

For instance, the antiviral drug tenofovir (TFV) has an  $EC_{50}$  of 1400 nM,  $CC_{50}$  of  $>50 \mu\text{M}$ , and a selectivity index (SI) of 36 (Figure 2). The issue with TFV is its low absorption, and by prodrug modification, the absorption was improved, resulting in an FDA approved prodrug tenofovir alafenamide fumarate (TAF) having an  $EC_{50}$  of 6.4 nM,  $CC_{50}$  of  $35 \mu\text{M}$ , and SI of 5469 (Figure 2).<sup>5</sup>

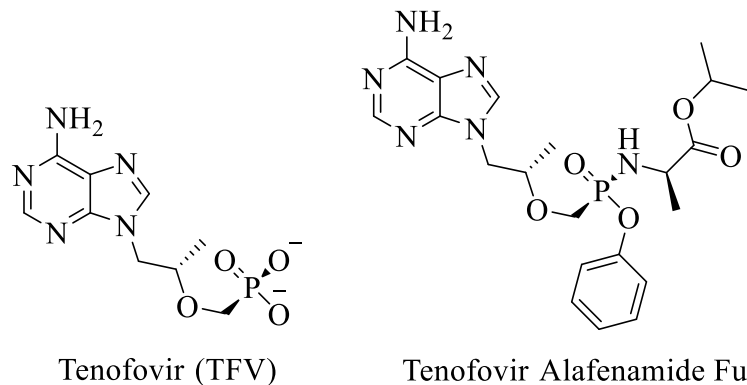


Figure 2. Structures of antiviral drug tenofovir (TFV) and its FDA approved prodrug tenofovir alafenamide fumarate (TAF).

## 1.2 Natural Prodrugs

There are prodrugs available naturally from the plant, animal, microbial and marine sources, which are FDA approved, including (i) butyrin which occurs naturally in butter and is a prodrug of butyric with the ability to enhance the antiproliferative effects of dihydroxycholecalciferol in human colon cancer cells acid (Figure 3);<sup>6</sup> (ii) psilocybin which is isolated from *Psilocybe mexicana* and *Stropharia cubensis* and is a prodrug of psilocin (Figure 3);<sup>7</sup> (iii) melatonin which is a prodrug of N1-acetyl-5-methoxykynuramine (AMK) with the ability to protect the mitochondria and occurs naturally in animals and plants (Figure 3);<sup>8</sup> and (iv) baicalin which is a prodrug of baicalein and is used to treat schizophrenia and related neuropsychiatric disorders (Figure 3).<sup>9</sup>

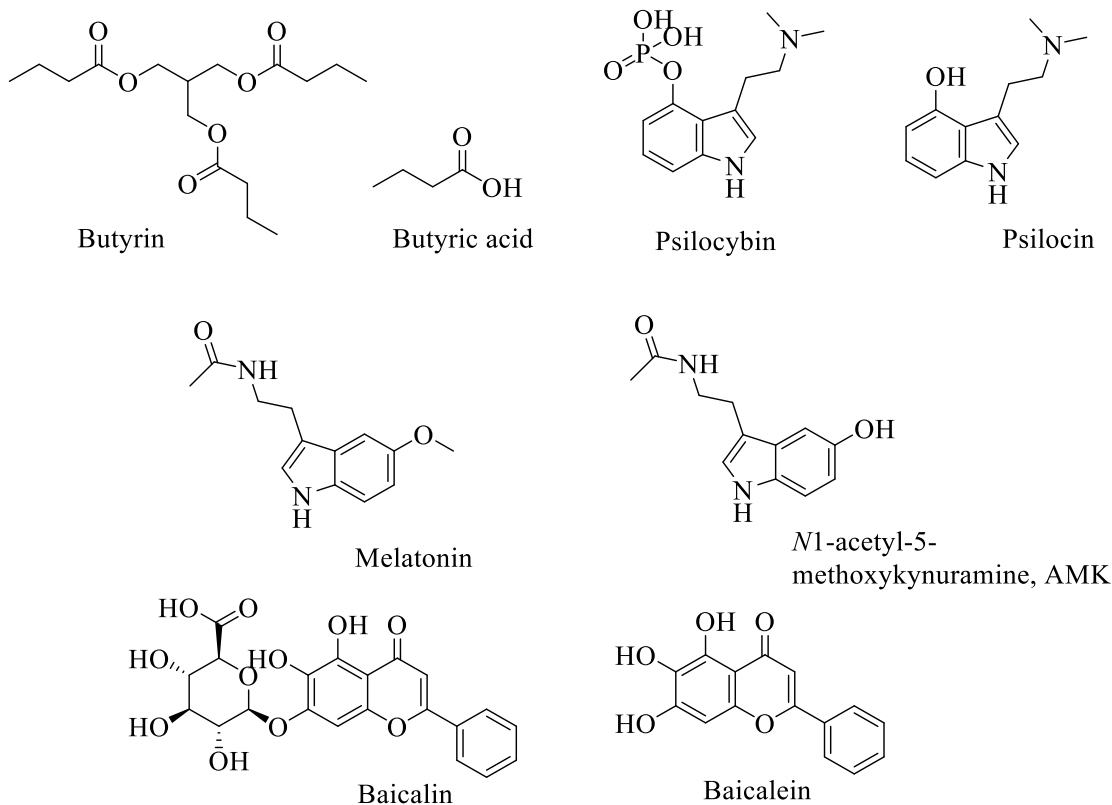


Figure 3. Structures of (i) butyryn and butyric acid, (ii) psilocybin and psilocin, (iii) melatonin and *N*1-acetyl-5-methoxykynuramine (AMK), and (iv) baicalin and baicalein.

### 1.3 Conventional Approaches to Synthetic Prodrugs

Synthetic prodrugs are structurally modified with the aim to endow improved pharmaceutical properties. In the past decade, the US Food and Drug Administration (FDA) has approved more than 30 prodrugs, constituting more than 10% of its overall drug approvals.<sup>11</sup> Structural modifications for prodrug preparation are generally achieved at a functional group of the active drug, including hydroxyl, carboxyl, amino, phosphate, phosphonate, guanidyl, and amidinyl groups. These functional groups are referred to as synthetic handles for a prodrug design. For example, an ionizable and polar carboxyl group can be converted to an ester or amide derivative to improve membrane permeability. For modified properties, a hydroxyl group can also be converted to an ester, acetal/ketal, or labile ether group. The prodrug can be converted to active

drug form by either enzymatic or chemical reactions. Such reactions may include hydrolysis, reduction, or oxidation, among other possibilities.<sup>1, 10, 11</sup> Below, some specific examples are described.

The hydroxyl groups can be modified into more lipophilic groups including ester, alkoxy ester, carbonate, and phosphate. These prodrugs convert back to their parent drug by esterase, amidase, alkaline phosphatase, or simple chemical hydrolysis (Figure 4).<sup>12</sup>

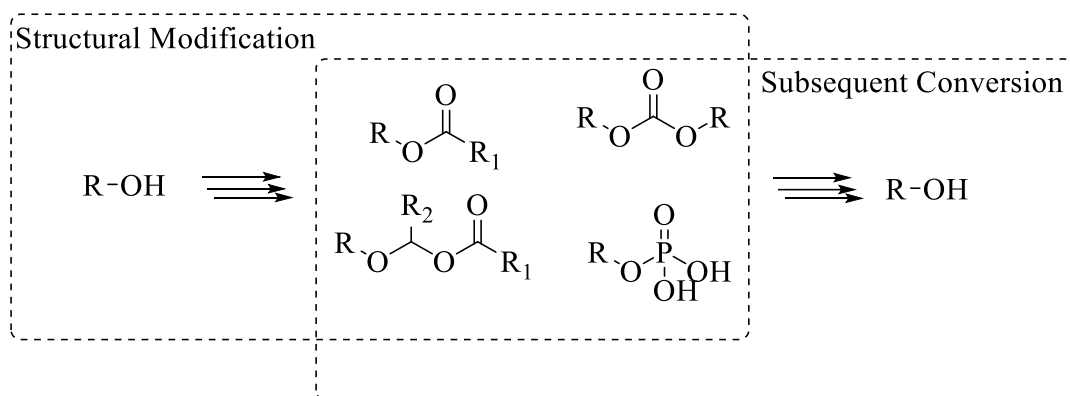


Figure 4. Structural modifications of a hydroxy group for prodrug preparation and the subsequent conversion to the parent drug.

The carboxylic acid group is mainly modified into the ester, which converts back to its original drug by an esterase or chemical hydrolysis. A carboxylic group can also be modified into an amide or an acylsulfonamide which can be converted back to its parent drug either by an esterase or amidase (Figure 5).<sup>13</sup>

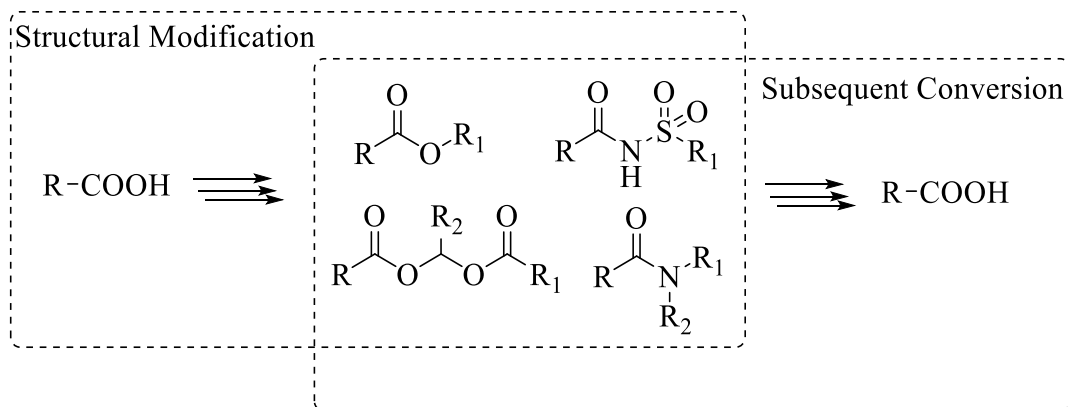


Figure 5. Structural modifications of a carboxyl group for prodrug preparation and the subsequent conversion to the parent drug

Amines give more flexibility towards modification as they can be modified into amides, *N*-Mannich adducts, *N*-acyloxyalkylamines, imines, azo compounds, and phosphoramidates which convert back to the parent drug by amidase, esterase, RSH, or alkaline phosphatase (Figure 6).<sup>14</sup>

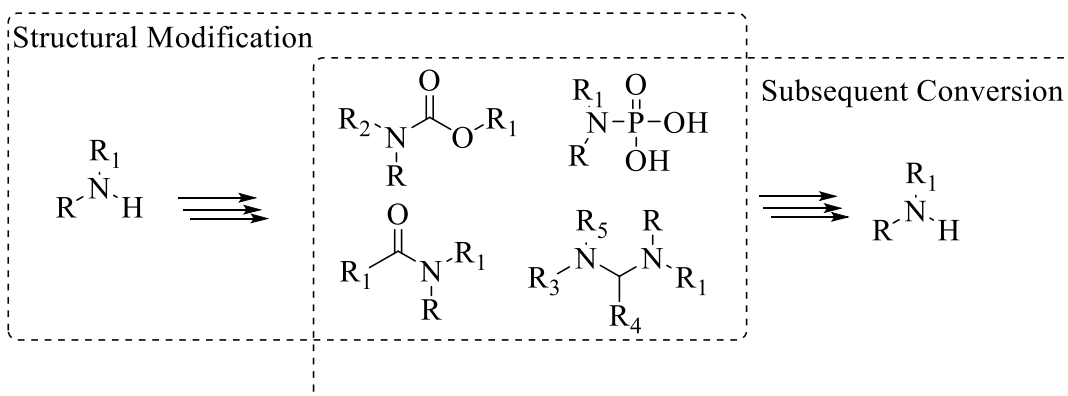


Figure 6. Structural modifications of an amino group for prodrug preparation and the subsequent conversion to the parent drug.

Phosphate groups are usually modified to produce a charge neutral compound with increased lipophilicity. These prodrugs convert back to their original form by esterase, CYP450, and phospholipase (Figure 7).<sup>15</sup>

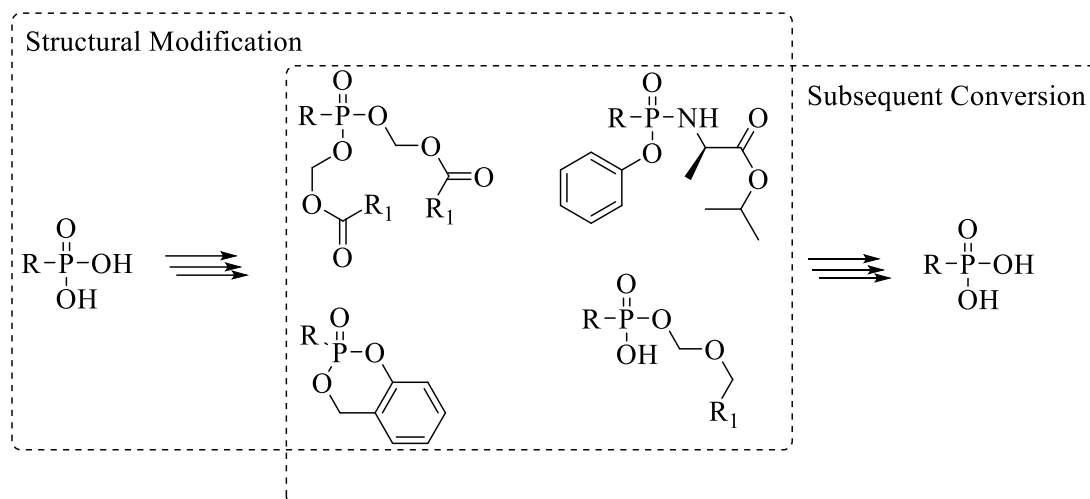


Figure 7. Structural modifications of phosphate/phosphonates group for prodrug preparation and the subsequent conversion to the parent drug.

#### 1.4 An example of a target compound

Even with the years of research in prodrug, there is still a lack of methods for bioreversible derivatization of some commonly encountered structural moieties. For example, the pyridyl group is very commonly seen in approved drugs and in drug research.<sup>16, 17</sup> In our own research, we have also encountered similar structural scaffolds. Herein, we use one specific example to describe the issues related to compounds with a pyridyl moiety. Specifically, we are interested in prodrugs of a pharmacologically validated HIF-1 inhibitor, **64B** (Figure 8) for improved water solubility, which is critical for formulation for oral and parenteral routes of administration. Cancer is one of the biggest contributors of deaths in the US, with 1.8 million new cancer cases diagnosed and 606,520 cancer deaths in the United States in 2020.<sup>18</sup> The rapid growth of the cells forms a tumor and outgrows the supply of blood and thus oxygen in the cells or tissue and creates a hypoxia condition, i.e., partial pressure of oxygen less than the physiological pressure.<sup>18-20</sup> Hypoxic conditions may lead to or augment resistance to most available chemotherapy and radiation treatments.<sup>21, 22</sup> The hypoxia-inducible factors (HIF) are helix-loop-helix heterodimers consisting

of an oxygen-sensitive HIF-1 $\alpha$  subunit and HIF-1 $\beta$ .<sup>23</sup> Under hypoxic conditions, HIF-1 $\alpha$  is stable and accumulates, then translocate to the nucleus where then it interacts with the HIF-1 $\beta$  to form the active transcription factor HIF-1.<sup>24, 25</sup> HIF-1 binds to the hypoxia-responsive elements (HRE) on the gene regulatory DNA sequence and specifically activates the transcription of over 100 genes.<sup>24, 25</sup> Tumor angiogenesis is majorly driven by these HIF-1-related genes as they trigger red blood cell production and anaerobic glycolysis. Due to these reasons, the HIF pathway has been exploited previously by our lab to develop some small-molecule inhibitors for the development of new cancer therapies.<sup>26</sup>

Specifically, **64B** is a HIF-1 inhibitor with an IC<sub>50</sub> of 280 nM.<sup>26</sup> In the tumor cell lines originated from lungs, breasts, pancreas, skin (melanoma), and brain (glioblastoma), **64B** showed IC<sub>50</sub> in the low micromolar range. In animal-model experiments, **64B** was found to suppresses tumor growth and metastasis in mouse models of triple-negative breast cancer and lung cancer.<sup>27</sup> Additionally, **64B** was also found to be cytotoxic against tumor cells independent of tissue origin or oncogenic mutation.<sup>27</sup> When **64B** was tested against Uveal melanoma (UM), the most lethal intraocular malignancy and prevalent in the adults. it was found to potently inhibit UM tumor growth, its invasiveness, ability to extravasate, and metastatic potential. **64B** was found to work by disrupting the hypoxia-inducible factor (HIF) transcription complex by interfering in the HIF-1 $\alpha$  recruitment of p300/CBP co-factors which is essential for their activation under hypoxia.<sup>26, 28</sup>

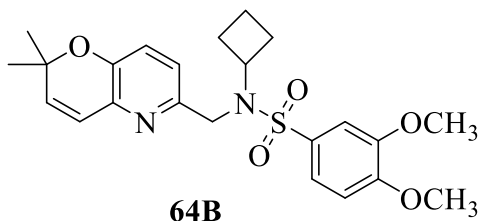


Figure 8. Structure of **64B**.

## 1.5 Issue with HIF Inhibitor 64B

The main issue with **64B** is its poor water solubility. In animal model studies, formulation of **64B** was achieved by suspending it in a cremophor EL: ethanol (1:1) by heating at 90 °C and extensive vortexing.<sup>27, 28</sup> Hence, we decided to make prodrugs of **64B** to improve its water solubility. However, this molecule does not have a functional group/handle commonly used for prodrug derivatization. The only “functionalizable” group for prodrug preparation seems to be the pyridyl ring. Therefore, we were interested in various bioreversible functionalization of the pyridyl nitrogen via model studies and then application in **64B**. In executing the project, we plan to first develop chemical methods for bioreversible derivatization of the pyridyl unit. Later, we will work on applying such strategies to **64b** and possibly other drugs with a pyridyl moiety in need of improved water solubility. We also hope to use similar approaches for conjugation with a targeting molecule.

## 1.6 Design

For designing bioreversible derivatization of the pyridyl nitrogen for improved water solubility, we thought of introducing a permanent positive charge via either *N*-oxidation or *N*-alkylation. For *N*-oxidation, we have designed **BW-HIF-345** (Figure 9) with the idea that the polar nature of an *N*-oxide group and the permanent positive charge on the nitrogen would help improve water solubility. Further, there have been literature reports of reducing an *N*-oxide group at the tumor site by CYP-450-mediated processes.<sup>29</sup> The latter point suggests the likelihood of bioreversible regeneration of **64B**.

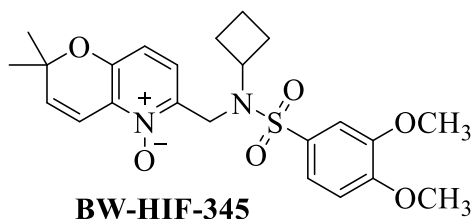


Figure 9. Structure of **BW-HIF-345**.

The second method is via alkylation, which creates a permanent charge for improved water solubility. In doing so, there is the need for an alkyl group that can be cleaved *in vivo*. For this, we designed four different types of linkers, as shown in Table 1, using pyridine as a model compound. The first three take advantage of the commonly used quinone methide<sup>30, 31</sup> (Table 1, Entry 1-3) chemistry for removing the linker. Specifically, the first case relies on reductive activation (Table 1, Entry 1). It is well known that hypoxia creates a reductive environment for activation of various prodrugs for tumor-targeting.<sup>32-34</sup>

Along this line, a nitro group is commonly used because it is prone to reduction in tumor and there are multiple reports of utilizing a nitro group for hypoxia-activated prodrugs.<sup>35, 36</sup> Here is a specific example, by Korner *et al.* of a hypoxia-activated prodrug via reduction of a nitro group in the hypoxia environment. After activation, the drug was released by quinone methide chemistry.<sup>35</sup> The mechanistic studies of nitro group reduction under hypoxic conditions are also reported by Zong *et al.*<sup>37</sup> Collectively this makes a strong case of having a hypoxia-activated prodrug system for a HIF inhibitor drug.

The second case relies on hydrolysis activation and then quinone methide chemistry to regenerate the original drug (Table 1, Entry 2). This prodrug contains an amide bond which usually provides high stability and allows for synthesis of stable prodrugs. There are multiple reports of

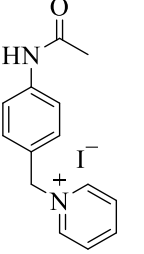
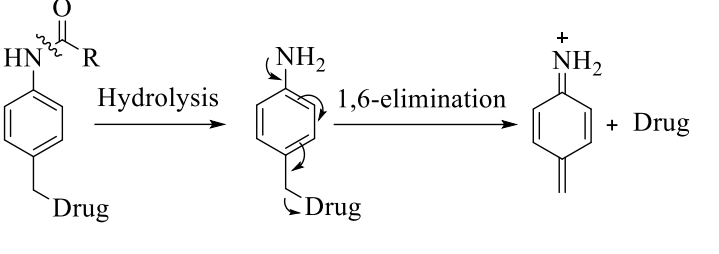
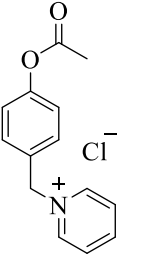
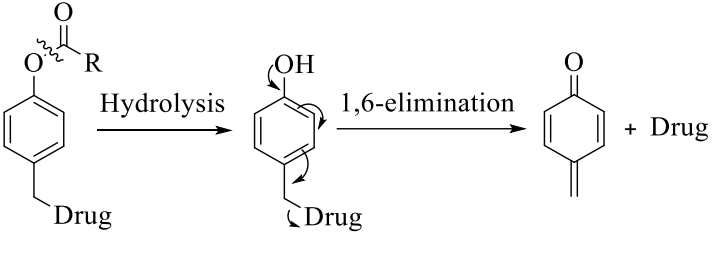
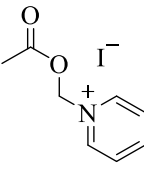
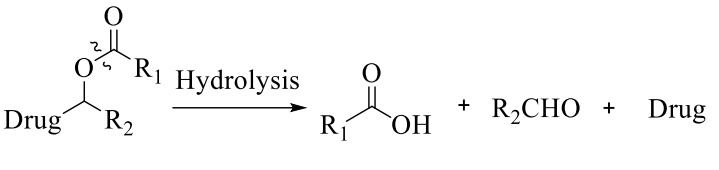
amides getting hydrolyzed by enzymes.<sup>38</sup> Specifically, Pillow *et al.* reported examples of drug release by 1,6-elimination after the hydrolysis of an amide.<sup>39</sup>

Amides does provide high stability in the biological systems but are generally considered as hard to hydrolyze. There are multiple reports where harsh conditions were utilized to break an amide bond.<sup>40-42</sup> This can create hardships for the activation of a prodrug and can ultimately slow the release of a drug. Keeping that in mind, an ester group was introduced to ease the hydrolysis and to achieve facile drug release. So, the third case contains an ester which upon hydrolysis releases the drug by quinone methide chemistry (Table 1, Entry 3). Nishio *et al.* reported the release of alcohols by the quinone methide chemistry after activation via hydrolysis of an ester.<sup>43</sup> Additionally, esterase-sensitive prodrugs are also previously reported by our lab for glutathione persulfide (GSSH), hydrogen sulfide (H<sub>2</sub>S), and carbon monoxide (CO) deliveries.<sup>44-47</sup>

The fourth case relies on a masked geminal “aminoalcohol” to create an esterase-sensitive trigger to regenerate the original drug (Table 1, Entry 4). In a quinone methide chemistry the drug is released over time as the intermediates formed are somewhat stable. Whereas in the fourth case (Table 1, Entry 4), the intermediates formed after hydrolysis are highly unstable and decomposes spontaneously to generate the original drug. Such chemistry creates a good case for a prodrug design system where the drug release is fast and of high yield. Dudley *et al.* reported the half-life of such compounds being from 8 to 28 min in human liver microsome.<sup>48</sup>

Table 1. Structures of model prodrug molecules and their activation chemistry

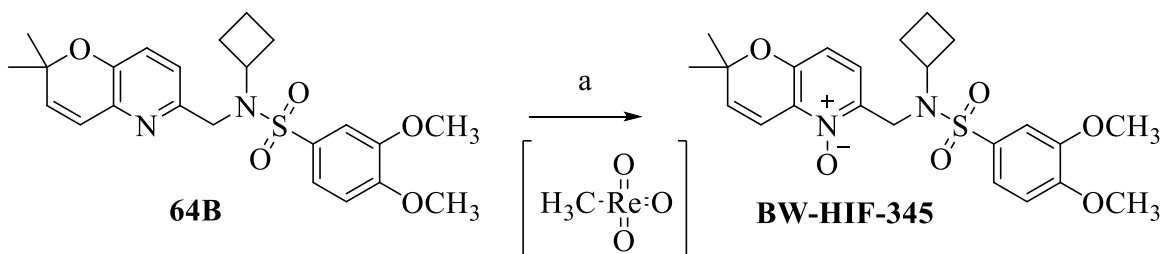
#	Compound ID	Models	Activation Chemistry	Ref
1	BW-HIF-352			37

2	BW-HIF-353			39
3	BW-HIF-354			49, 50
4	BW-HIF-355			48

## 2 RESULTS AND DISCUSSION

### 2.1 Synthesis

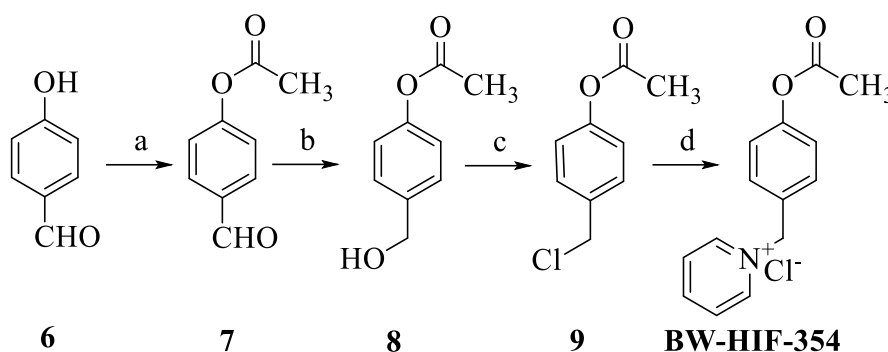
The prodrug **BW-HIF-345** was synthesized by oxidizing **64B** using  $\text{H}_2\text{O}_2$  as an oxidant in the presence of a catalyst methyltrioxorhenium (VII) in DCM at room temperature for 12 h to yield the desired product in 82% yield (Scheme 1).



Scheme 1. Synthesis of prodrug **BW-HIF-345**. Reagents and conditions: (a)  $\text{H}_2\text{O}_2$ , methyltrioxorhenium (VII), 21 °C, 12 h, 82%.

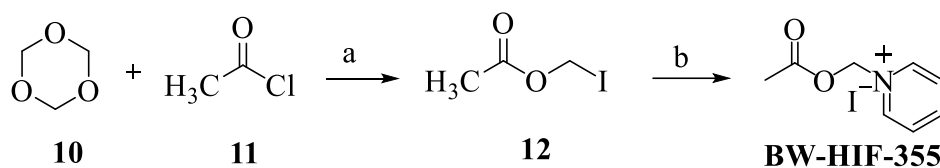


For the synthesis of prodrug **BW-HIF-354**, 4-hydroxybenzaldehyde **6** was reacted with acetic anhydride for 3 h to yield ester functionalized compound **7** in 88%. In the next step, compound **7** was then reduced by NaBH<sub>4</sub> in 2 h to obtain compound **8** in quantitative yield. Compound **8** then reacted with SOCl<sub>2</sub> to substitute the hydroxyl group by chloride to yield compound **9** in quantitative yield. Compound **9** was then reacted with pyridine in 2 h to yield the desired prodrug **BW-HIF-354** in 84% yield (Scheme 4).



Scheme 4. Synthesis of prodrug **BW-HIF-354**. Reagents and conditions: (a) acetic anhydride, triethylamine, 90 °C, 3 h, 88%; (b) NaBH<sub>4</sub>, DCM, reflux, 2 h, 98%; (c) SOCl<sub>2</sub>, DCM, 0 °C to 35 °C, 1 h, 98%; (d) pyridine, sealed tube, 90 °C, 2 h, 84%.

For prodrug **BW-HIF-355**, compound **12** was synthesized by reacting paraformaldehyde **10** with an acetyl chloride **11** in the presence of sodium iodide, aluminum chloride, and iodine as describe by a previously reported method.<sup>51</sup> Before the next reaction, the stability of the synthesized compound **12** was tested in acetone-*d*<sub>6</sub> by NMR spectroscopy. It was found to be stable after one day (Figure 10). Then, Compound **12** was reacted with pyridine in 30 min to yield prodrug **BW-HIF-355** in 55% yield (Scheme 5).



Scheme 5. Synthesis of prodrug **BW-HIF-355**. Reagents and conditions: (a) NaI, AlCl<sub>3</sub>, I<sub>2</sub>, DCM, r.t., 12 h; (d) pyridine, sealed tube, 90 °C, 30 min, 55%.

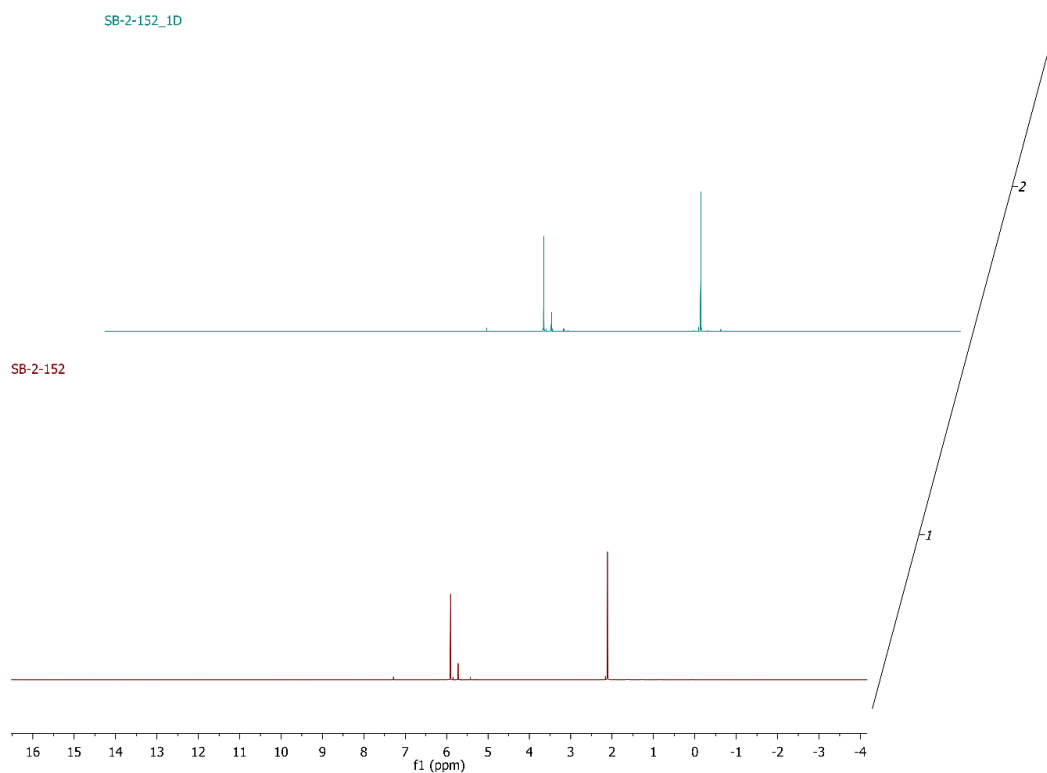
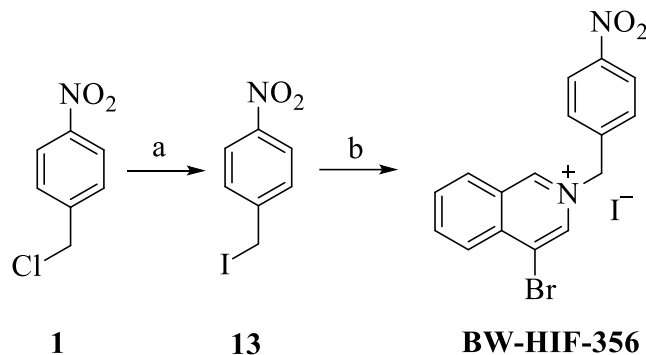


Figure 10. Stability studies of the compound **12** by NMR in acetone- $d_6$ . After one day at 21°C, the H-NMR spectrum remained unchanged, indicating the stable nature of compound **12** in acetone.

To synthesize prodrug **BW-HIF-356**, firstly 4-nitrobenzyl iodide **13** was synthesized from the 4-nitrobenzyl chloride **1** by refluxing with NaI in acetone for 1 h to yield 4-nitrobenzyl iodide **13** in 98% yield (Scheme 6). Then, 4-bromoisoquinoline was reacted with 4-nitrobenzyl iodide **13** in a sealed tube for 1 h to yield the desired prodrug **BW-HIF-356** in 60% yield (Scheme 6).



Scheme 6. Synthesis of **BW-HIF-356**. Reagents and conditions: (a) NaI, acetone, reflux, 1 h, 98%; (b) 4-bromoisoquinoline, sealed tube, 90 °C, 1 h, 60%.

## 2.2 Assessment

### 2.2.1 Stability Studies

To assess the feasibility of using the strategies described for prodrug preparation, we first need to study their chemical stability under near physiological conditions. Therefore, we studied their stability firstly in phosphate buffer saline (PBS) at pH 7.4. Specifically, the stability studies of prodrugs in PBS were carried for up to 24 h using HPLC. It was found that prodrug **BW-HIF-352** is stable in PBS under physiological conditions for the duration of the study 24 h (Figure 11). Similarly, Prodrug **BW-HIF-353** was found to be stable in PBS under physiological conditions for the duration of the study 24 h (Figure 12). Prodrug **BW-HIF-354** on the other hand showed some degradation starting from the 1-h time point (Figures 13 and 14). Using the peak area at 0 min time point as 100%, it was observed that at degradation was <2% at 1-h time point and ~8% at the 5-h time point (Figures 13 and 14). After 24 h, 30% degradation of the prodrug was observed. Further, pyridine release was observed after degradation as confirmed by running an external standard. After 24 h, 70% prodrug remained in its original form, indicating the half-life of prodrug **BW-HIF-354** being >24 h (Figures 13 and 14). Such results suggested the feasibility of using the esterase-sensitive prodrug approached exemplified by **BW-HIF-354**, but attention is needed to keep exposure to an aqueous solution to the minimal before the intended activation by hydrolysis.

Prodrug **BW-HIF-355** upon incubating in PBS showed degradation at a higher rate (Figure 15) compared to prodrug **BW-HIF-354** (Figure 13). Keeping 0 min time point as 100%, the prodrug degraded ~12% in first hour, 27% in 2 h, 38% in 3 h, >50% in 5 h, and >90% after 24 h via pyridine release as confirmed by running an external standard (Figures 15 and 16). The prodrug **BW-HIF-355** degraded more than 50% within 5 h, indicating the half-life being less than 5 h (Figure 16). Prodrug **BW-HIF-355** showed the least stability among synthesized prodrugs, raising questions about the suitability of this approach for preparing the intended prodrug. Table 2 shows the half-life of each prodrug. Again, prodrugs **BW-HIF-352** and **BW-HIF-353** remained stable within 24 h in PBS (Table 1, Entries 1 and 2). Prodrug **BW-HIF-354** showed some stability issues for the intended conjugation chemistry while **BW-HIF-355** is not expected to be stable enough for the intended application.

Table 2. Stability studies of the prodrugs by HPLC.

Entry	Prodrug	Half Life*
1	<b>BW-HIF-352</b>	Stable
2	<b>BW-HIF-353</b>	Stable
3	<b>BW-HIF-354</b>	$t_{1/2} = >24$ h
4	<b>BW-HIF-355</b>	$t_{1/2} = <5$ h

\*Based on single run

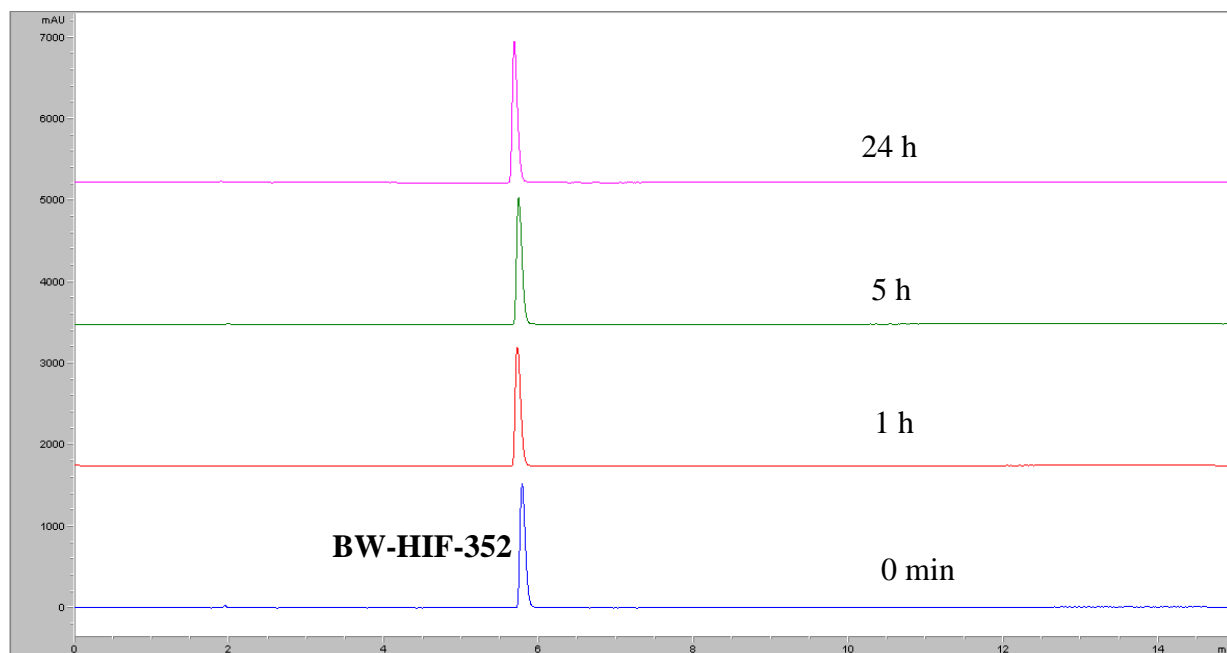


Figure 11. HPLC studies showing the stability of **BW-HIF-352** in PBS under physiological conditions.

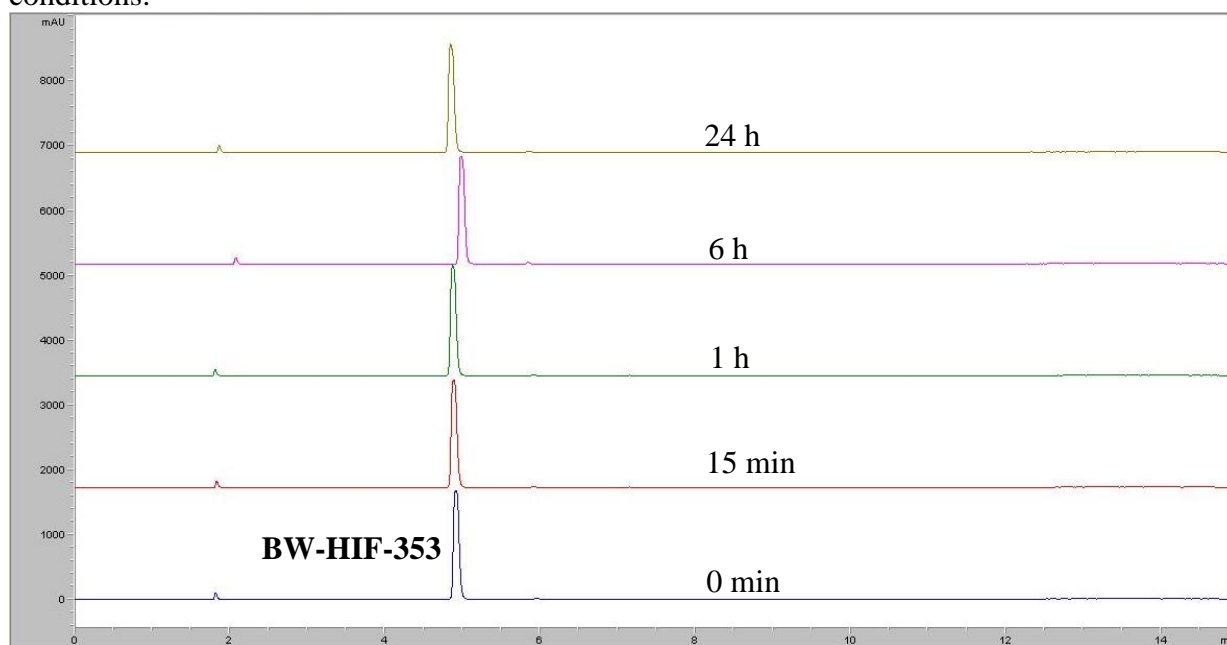


Figure 12. HPLC studies showing the stability of **BW-HIF-353** in PBS under physiological conditions.

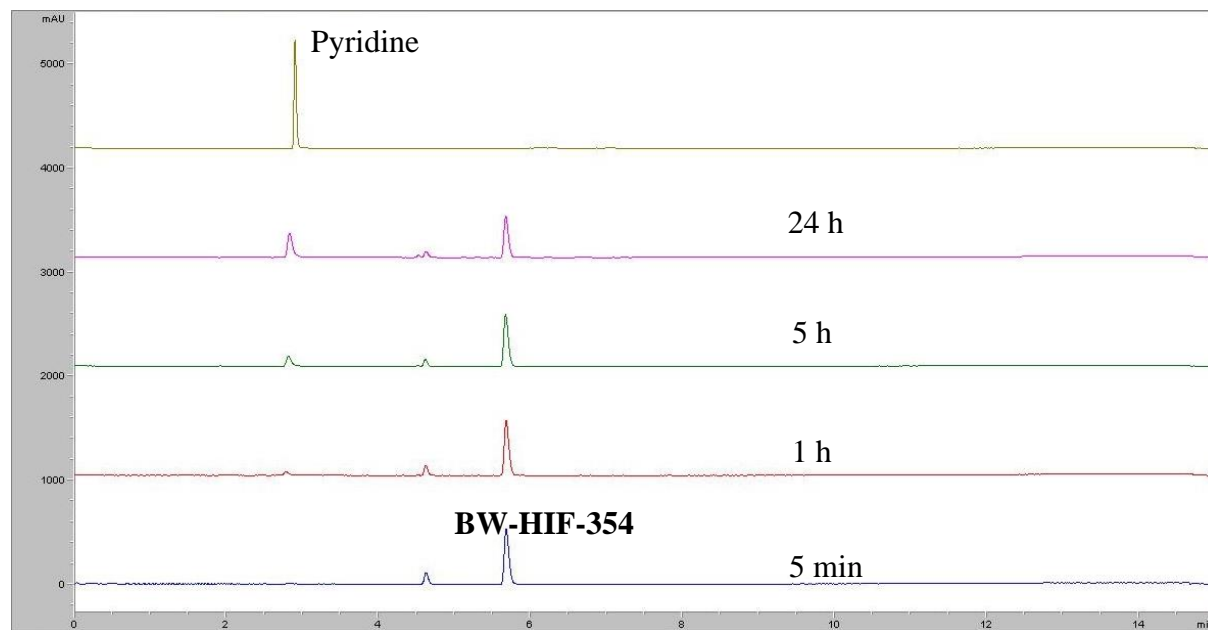


Figure 13. HPLC studies showing the stability of **BW-HIF-354** in PBS under physiological conditions. Increase in the pyridine peak shows that the prodrug does degrade in the PBS.

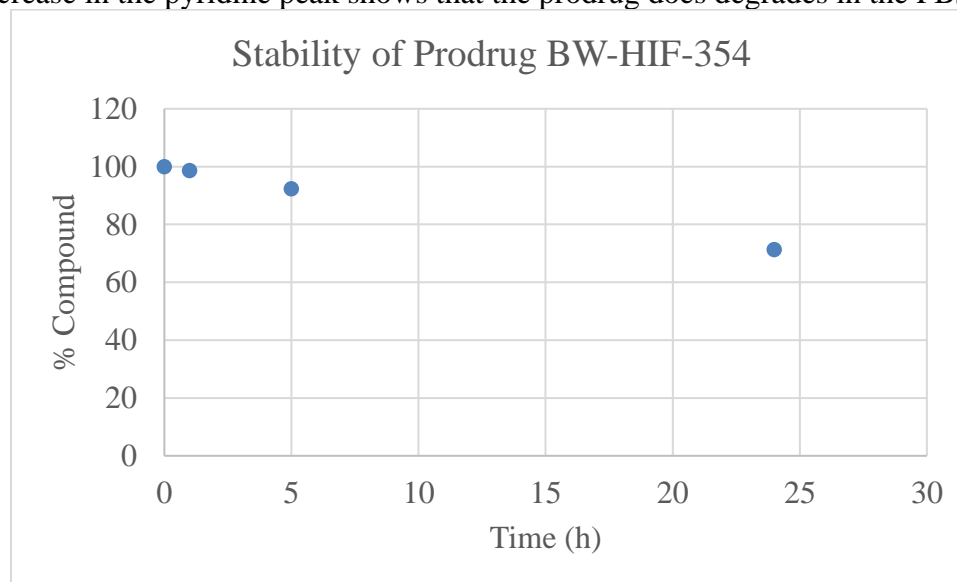


Figure 14. Stability of prodrug **BW-HIF-354** in PBS under physiological conditions.

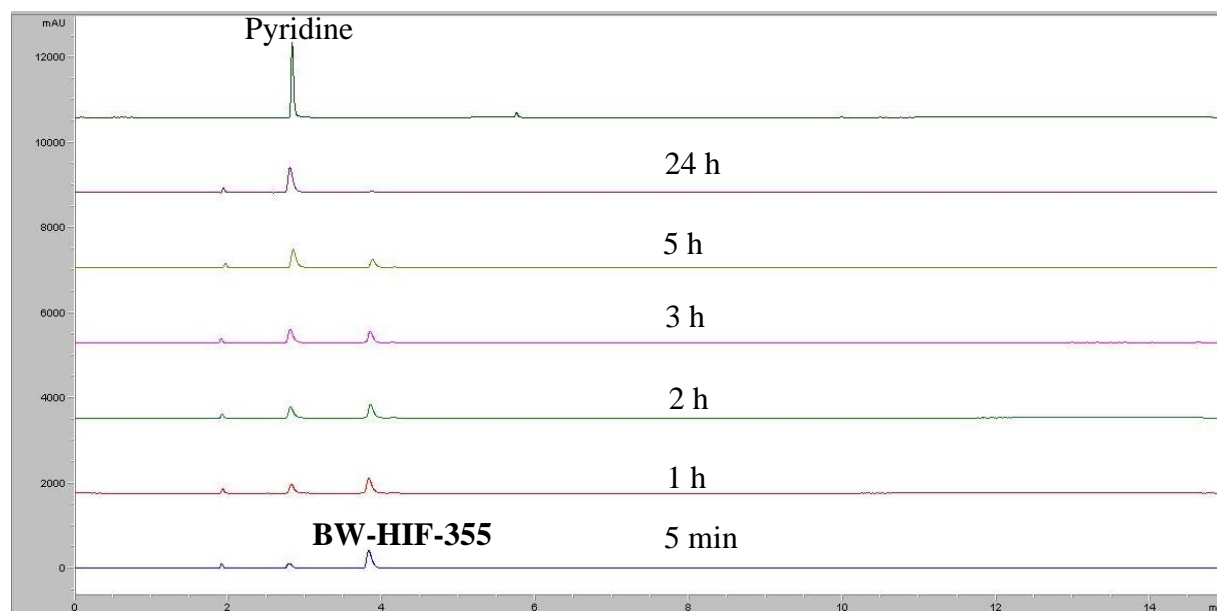


Figure 15. HPLC studies showing the stability of **BW-HIF-355** in PBS under physiological conditions.

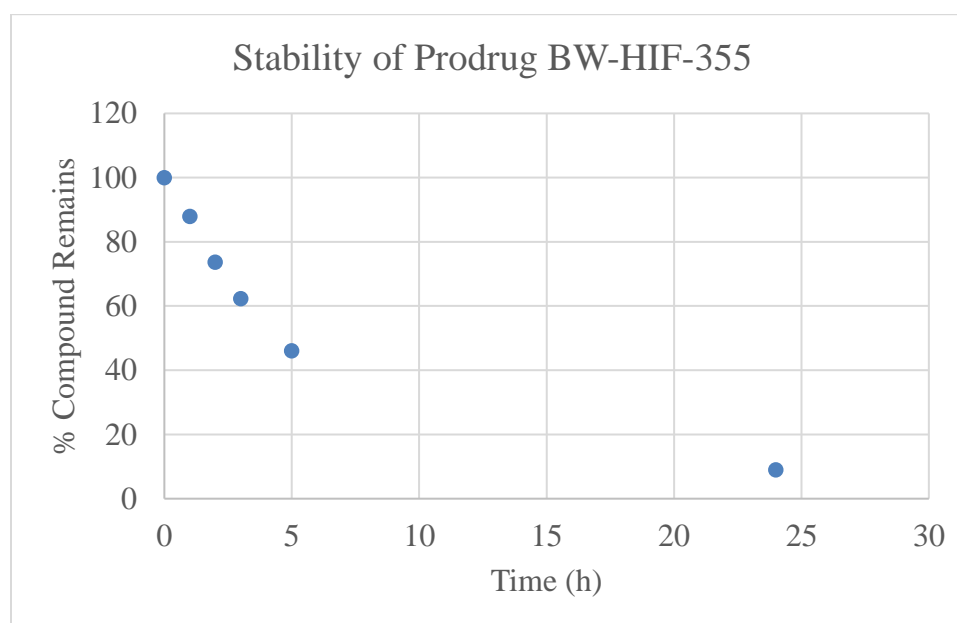


Figure 16. Stability of prodrug **BW-HIF-355** in PBS under physiological conditions.

### 2.2.2 Solubility Studies

The major reason for the prodrug approaches described is to improve water solubility, so we also measured the water solubility of these model compounds. We used HPLC to do such

measurements by following a previously reported method.<sup>52</sup> To determine the solubility, we needed to first prepare a saturated solution of compound in water. Then from the supernatant of the saturated solution, the solubility can be determined as reported previously.<sup>52</sup> To do so we added 100 mg of each prodrug in 100  $\mu$ L water. When 100 mg of the prodrug **BW-HIF-354** was added in 100  $\mu$ L of water, we observed some insoluble particles, which were removed by vortexing for 5 min and centrifugation at 14000 rpm for 10 minutes. Then, from the supernatant, a 10- $\mu$ L aliquot was diluted 1000 times and analyzed with HPLC. The solubility was then calculated from a standard curve of the prodrug **BW-HIF-354** in water ranging from 0.125 mg/mL to 2 mg/mL (Figure 27) and converted back to the initial concentrations by multiplication by 1000 (Table 3, Entry 1). When 100 mg of prodrug **BW-HIF-352** or prodrug **BW-HIF-355** was added to 100  $\mu$ L of water, they gave a clear solution indicating a solubility of at least 1 g/mL. However, in these cases, there was nothing to compare with in terms of the improvement in solubility after prodrug derivatization, because pyridine itself is miscible with water. In order to provide an assessment of the prodrug's ability to improve water solubility, we also selected a poor water-soluble compound, 4-bromoisoquinoline, for prodrug derivatization. The prodrug of 4-bromoisoquinoline was synthesized in a way similar to prodrug **BW-HIF-352** (Scheme 6). For both the 4-bromoisoquinoline and its prodrug **BW-HIF-356**, the solubility was measured in the same way of prodrug **BW-HIF-354**. First the standard curves were generated in water (Figures 28 and 29). Then the saturated solution of 4-bromoisoquinoline or its prodrug **BW-HIF-356** was made in water by adding 20 mg in 500  $\mu$ L of water. The resultant mixture was vortexed for 5 min and centrifuged at 14000 rpm for 10 minutes so that the insoluble particles were removed. Then, a 10- $\mu$ L aliquot of the supernatant was diluted and analyzed by HPLC. First the concentration was calculated from the standard curve and then was converted to the concentration of the undiluted

solution by multiplying with the dilution factor. It was found that 4-bromoisoquinoline has a solubility of  $454.4 \pm 31.1 \mu\text{g/mL}$  (Table 3, Entry 3) and its prodrug **BW-HIF-356** has a solubility of  $2329.4 \pm 101.6 \mu\text{g/mL}$  (Table 3, Entry 2). This study gave us a comparison of solubilities as prodrug **BW-HIF-356** is ~5 times more soluble than its parent compound 4-bromoisoquinoline. Such results suggest the feasibility of using this prodrug approach to improve water solubility for drugs having poor water solubility.

Table 3. Solubility studies of the prodrugs by HPLC.

Entry	Prodrug	Solubility*
1	<b>BW-HIF-354</b>	$457.5 \pm 3.1 \text{ mg/mL}$
2	<b>BW-HIF-356</b>	$2329.4 \pm 101.6 \mu\text{g/mL}$
3	<b>Compound 13</b>	$454.4 \pm 31.1 \mu\text{g/mL}$

\*Tripllicated results

### 2.2.3 Bio-reduction of prodrug **BW-HIF-345**

Prodrug **BW-HIF-345** was designed based on activation through reduction of the *N*-oxide moiety. The reduction of *N*-oxides under hypoxic conditions are well documented.<sup>53, 54</sup> However, the reported examples are mainly of aliphatic tertiary amines,<sup>55</sup> whereas our designed prodrug contains a heterocyclic *N*-oxide. Therefore, we assessed the initial feasibility of bioreduction of our prodrug **BW-HIF-345** in cells culture. Specifically, LN-229 cells (human glioblastoma) were used for bio-reduction of **BW-HIF-345**. This part was conducted by Dr. Wen Lu, a postdoc in the lab. Because of reports available on tumor selective prodrugs activation,<sup>54</sup> two parallel experiments were performed one under hypoxia (0.1% pO<sub>2</sub>) conditions and the other under normoxia (21% pO<sub>2</sub>) conditions. The analysis of reduced product was performed by lysing the cells followed by extraction with ACN and finally analysis of the ACN extracts by LCMS. The expectation was to have selective reduction of prodrug **BW-HIF-345** under hypoxia conditions but it was found that

only 1.5% of the prodrug was reduced to **64B**, the parent drug, under both hypoxic and normoxia conditions. Such results indicate that the prodrug **BW-HIF-345** is stable in cell culture under either hypoxic or normoxic conditions. More studies are needed in animal models of solid tumor to truly assess the feasibility of reductive activation.

#### **2.2.4 Chemical reduction of prodrug BW-HIF-352**

The key factor in a prodrug approach is the bioreversibility of the prodrug. A prodrug should release or convert back to its parent drug under near physiological conditions with or without a trigger. Prodrug **BW-HIF-352** is based on reduction activation (Table 1, Entry 1). We chose to study the activation feasibility by first subject the nitro group-based prodrugs using chemical reduction before moving into cell culture and/or animal model.<sup>35</sup> For such chemical reduction, zinc reduction has been widely utilized.<sup>35</sup> Other methods of reduction including H<sub>2</sub>/Pd can cleave the benzylic bond, which would give us a competitive side reaction. Therefore, we chose to test prodrug **BW-HIF-352** first for the chemical reduction using zinc metal. This chemical reduction would provide us preliminary results for both reduction of the nitro group and the drug release. When the prodrug **BW-HIF-352** was stirred with zinc metal (10 eq.) and NH<sub>4</sub>Cl (2.5 eq.) in water at 21 °C, pyridine release was observed over time. Pyridine release was confirmed by both running an external standard and spiking experiments (Figure 17). It was found that prodrug **BW-HIF-352** released 31% of pyridine at the 6-h point and 42% pyridine at the 26-h point (Figure 17). This chemical reduction established an initial proof of concept that pyridine can be released through reduction of the nitro group as designed, should the reduction work *in vitro* or *in vivo*.

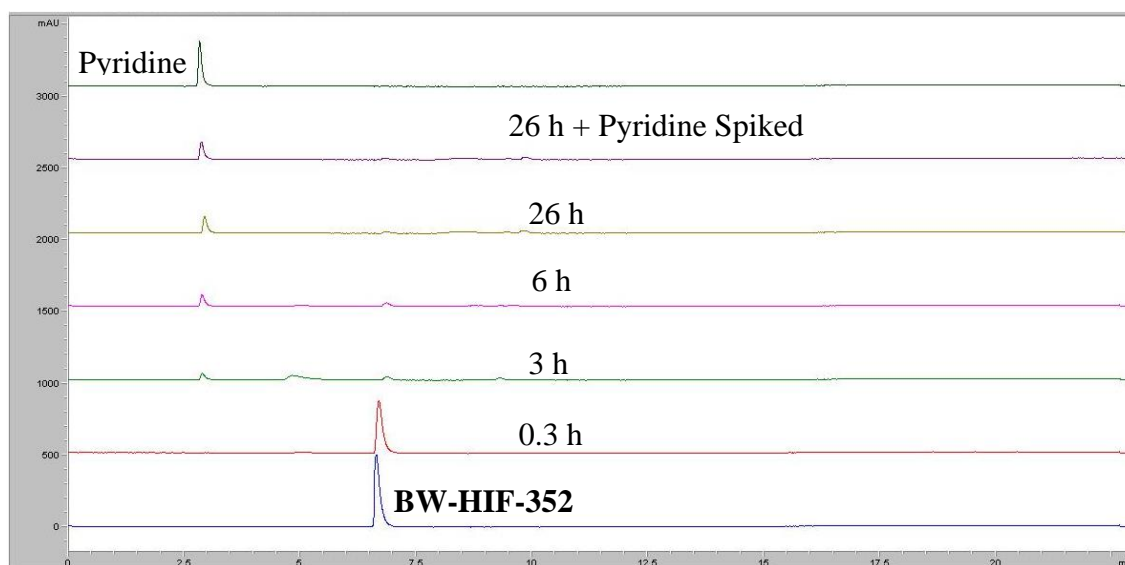


Figure 17. HPLC studies showing chemical reduction of **BW-HIF-352** by zinc in H<sub>2</sub>O in the presence of NH<sub>4</sub>Cl at 37 °C. At zero time point it showed 100% of the prodrug and over time it showed the consumption of starting material and increases in the peak intensity of the product, which was confirmed to be pyridine by both comparing with an external standard and spiking with pyridine.

### 2.2.5 Enzymatic hydrolysis of prodrug **BW-HIF-353**

Prodrug **BW-HIF-353** was designed based on hydrolysis activation (Table 1, Entry 2). In general, amides are stable in aqueous environments. Our stability studies described in an earlier section 2.2.1 showed the stability of **BW-HIF-353** in PBS under near physiological conditions (Figure 12). We thought of testing prodrug **BW-HIF-353** first with porcine liver esterase because of the known sensitivity of some amides to esterases.<sup>38</sup> Interestingly when we incubated the prodrug with 20 units/ mL porcine liver esterase we did not observe any pyridine release within 24 h (Figure 18) but we observed two peaks from the same prodrug in HPLC. We ran multiple experiments to show that indeed the two peaks were from the same compound. At this time, we are unsure whether it was because of the presence of porcine liver esterase and/or the different solvent composition (we used a mixture of PBS-ACN, 1:3, for the protein precipitation while preparing sample for HPLC injection in enzyme hydrolysis experiments (further details in section

3.4.1)) that led to the separation of a fraction of the prodrug from its native form. However, when we were tested the stability of this prodrug in PBS with an enzyme, we observed only one peak (Figure 12). Further, when dissolved prodrug **BW-HIF-353** in ACN, we also only observed one peak (data not shown). Then we tested the hydrolysis of this prodrug by other enzymes as amide hydrolysis have been reported with multiple enzymes.<sup>39, 56</sup> These enzymes included pancreatin (1 mg/mL) (Figure 19), lysozyme (2200 U/mL, Figure 20), amidase (100 U/mL, Figure 21), and trypsin (0.25%, Figure 22). Unexpectedly, we found no prodrug activation and pyridine release. For the drug release purpose, the designed prodrug **BW-HIF-353** showed exceptional stability with the enzymes tested. This exceptional stability can be utilized in the conjugation chemistry with a different activator including the ROS sensitive prodrugs<sup>57</sup> as the desired compound should also show similar kind of stability.

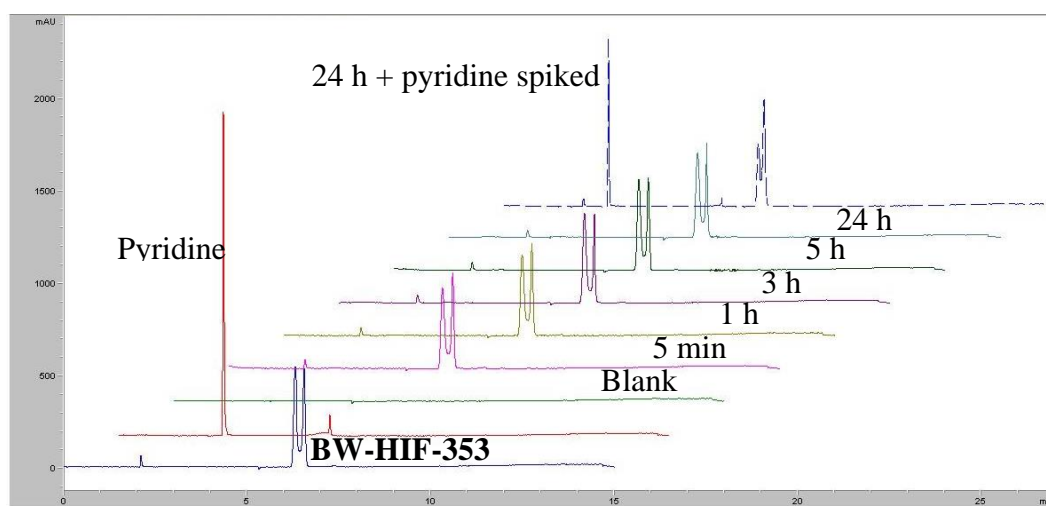


Figure 18. HPLC studies showing hydrolysis of prodrug **BW-HIF-353** by esterase (20 units/mL) under physiological conditions.

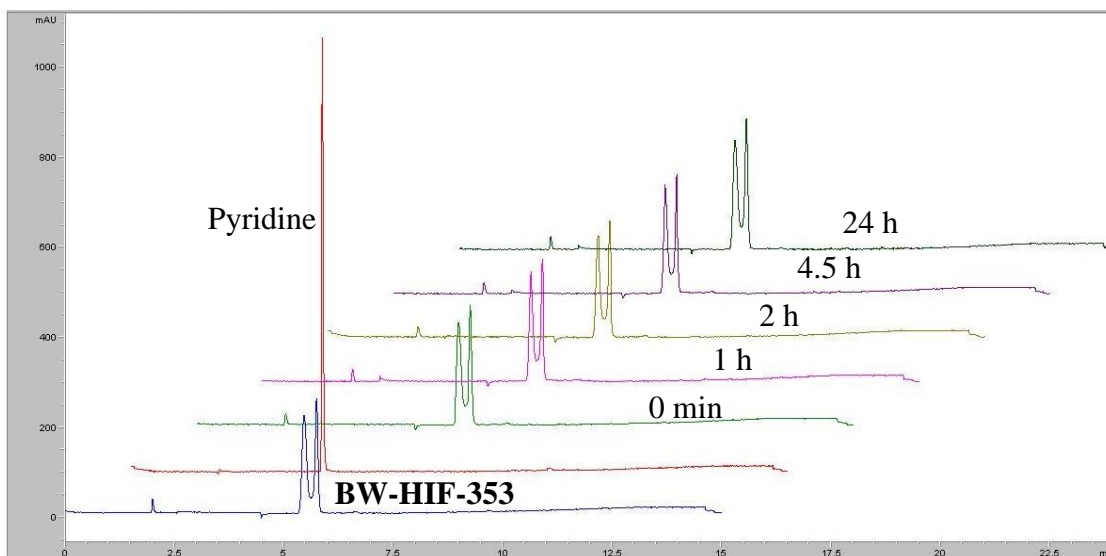


Figure 19. HPLC studies showing hydrolysis of prodrug **BW-HIF-353** by pancreatin (1 mg/mL) in PBS at pH 6.9 at 37 °C.

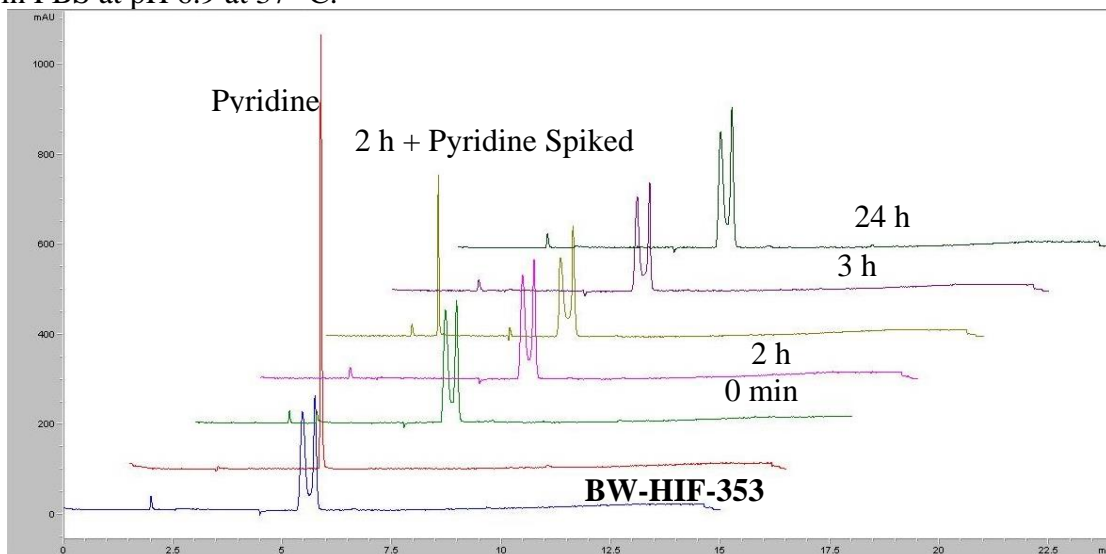


Figure 20. HPLC studies showing hydrolysis of prodrug **BW-HIF-353** by lysozyme (2200 units/mL) under physiological conditions.

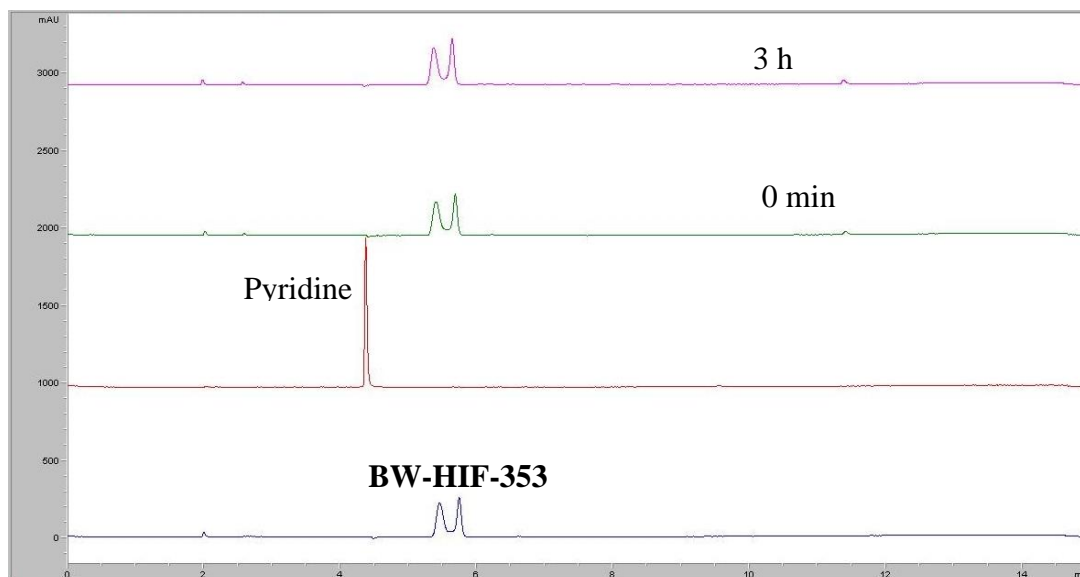


Figure 21. HPLC studies showing hydrolysis of prodrug **BW-HIF-353** by amidase (100 units/mL) under physiological conditions.

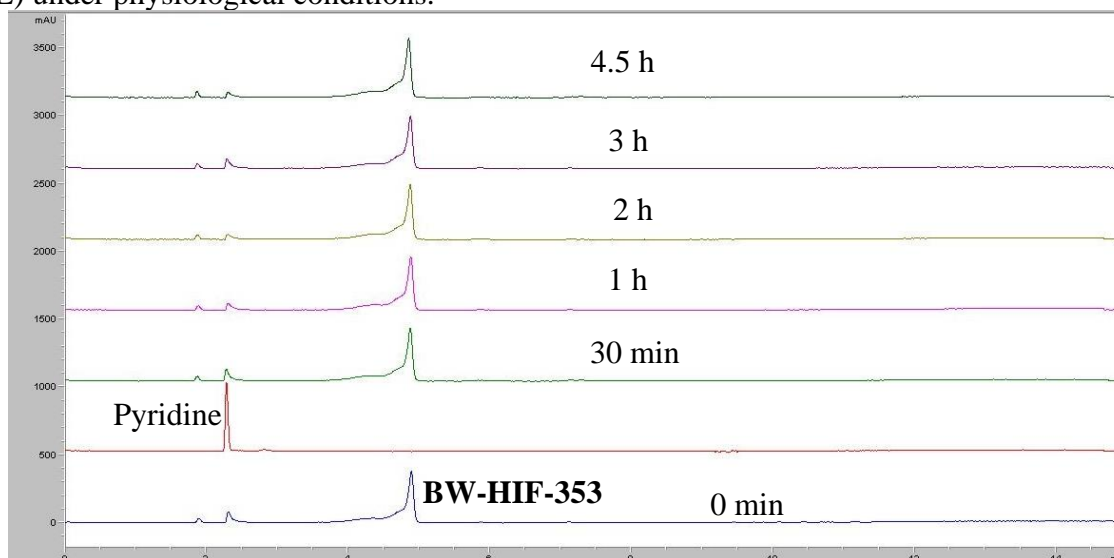


Figure 22. HPLC studies showing hydrolysis of prodrug **BW-HIF-353** by 0.25% trypsin under physiological conditions.

### 2.2.6 Enzymatic hydrolysis of prodrug **BW-HIF-354**

Prodrug **BW-HIF-354** was designed to for activation through ester hydrolysis. Esterase-sensitive prodrugs have been previously reported.<sup>44-47</sup> Due to that reason enzymatic hydrolysis of prodrug **BW-HIF-354** was tested with esterase. When prodrug **BW-HIF-354** was incubated with porcine liver esterase (20 units/mL) in PBS under near physiological

conditions, extensive prodrug hydrolysis was observed within the first 5 minutes (Figure 23). At the 5-minute point, three new peaks were observed with one being pyridine (Figure 23). We reasoned that the other two peaks were intermediates or byproducts. However, their identity was not established. The esterase hydrolysis was found to be fast as the prodrug **BW-HIF-354** gets hydrolyzed within minutes (Figures 23 and 24). The stability of the intermediates was expected to be pH dependent. To probe this pH-dependency, we conducted the hydrolysis experiments at pH 8.5. Because the enzyme hydrolysis is very fast (Figure 23), the elimination step was thought to be rate-limiting. Indeed, it was found that the pyridine release was increased by 1.5-folds at pH 8.5 (Figure 24). Such results suggest the facilitation of the elimination step at elevated pH. Overall, the esterase-sensitive prodrug approach seems to be a very promising method for prodrug preparations of pyridine-containing compounds.

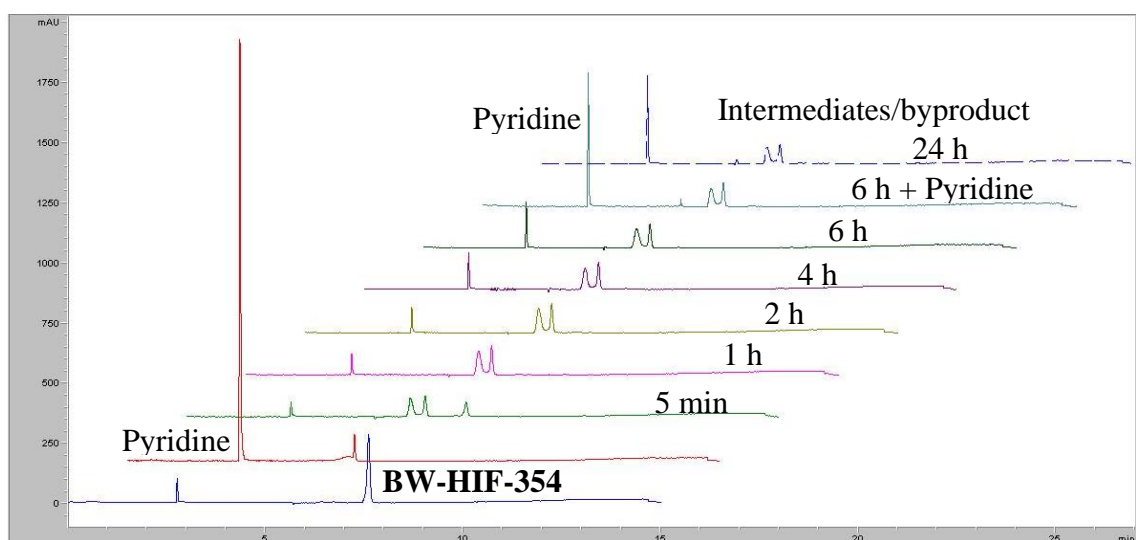


Figure 23. HPLC studies showing hydrolysis of prodrug **BW-HIF-354** by esterase (20 units/mL) under physiological conditions.

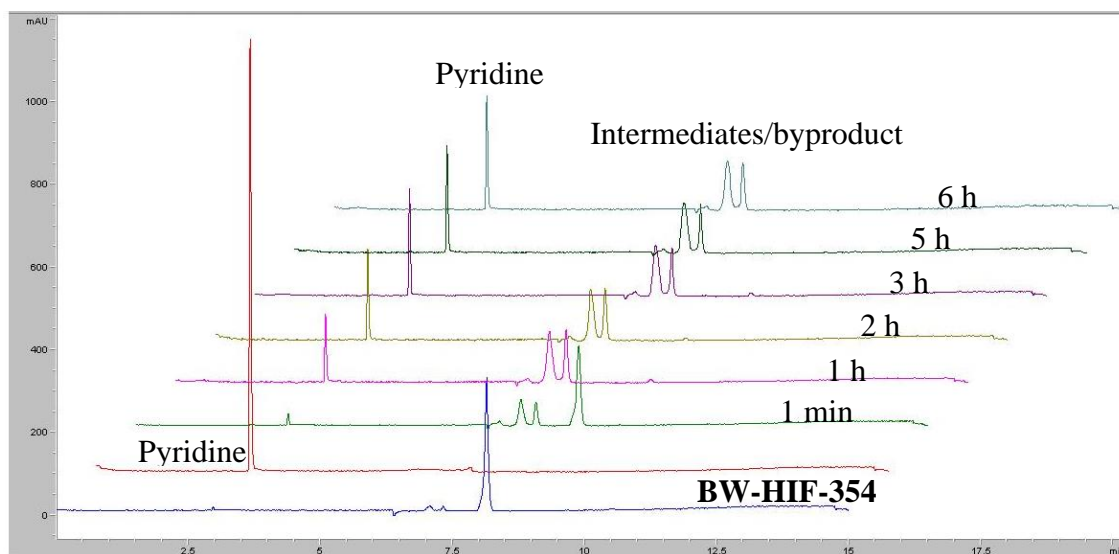


Figure 24. HPLC studies showing hydrolysis of prodrug **BW-HIF-354** by esterase (20 units/mL) in PBS at pH 8.5 at 37 °C.

### 2.2.7 Esterase-catalyzed hydrolysis of prodrug **BW-HIF-355**

Prodrug **BW-HIF-355** showed less stability (Figure 15) compared to other prodrugs. For understanding the basic chemistry behind this class of prodrugs, we were interested in seeing whether incubation with an esterase would further enhance the hydrolysis rate and thus facilitate pyridine release. Consequently, **BW-HIF-355** was incubated with porcine liver esterase in PBS (10 units/mL) in PBS under near physiological conditions. For the HPLC sample preparation, the protein was precipitated by the addition of 3-fold ACN (further details in section 3.4.1) so as to stop the reaction and remove the protein from the samples to be analyzed with HPLC. The standards were also prepared in with the same solvent composition of PBS: ACN (1:3). When the prodrug **BW-HIF-355** standard was prepared in the PBS: ACN (1:3) and injected in HPLC two peaks were observed (Figure 25) with one being the same as pyridine in retention time. From the stability studies, we know that prodrug **BW-HIF-355** degrades in the PBS (Figure 15) but does have a half-life of 4.4 h (Figure 15). In the enzyme hydrolysis experiments, ACN was only used to precipitate the protein so other solvents can also be utilized to precipitate the protein. We chose methanol for the protein precipitate. So, the prodrug **BW-HIF-355** standard was prepared in the

PBS: methanol (1:3) and injected in HPLC, two peaks were observed (Figure 25) with one being the same as pyridine in retention time. Although the prodrug **BW-HIF-355** degrades at a slower rate upon the addition of methanol than the ACN but cannot be used to precipitate the protein. To resolve the issue of protein precipitation 1% acetic acid was utilized as prodrug **BW-HIF-355** does not degrade in 1% acetic acid (data not shown).

After finalizing the utilization of 1% acetic acid for the protein precipitation, the enzymatic hydrolysis of prodrug **BW-HIF-355** was carried out using the similar procedure of other prodrugs. Therefore, prodrug **BW-HIF-355** was incubated with 10 U/mL porcine liver esterase under physiological conditions (Figure 26) and it showed the pyridine release similar to stability studies (Figure 15). This similarity in esterase hydrolysis and PBS stability can be because of either slow esterase hydrolysis or can be from degradation. Although the drug release is high with this prodrug but the degradation with ACN and methanol would increase difficulty in synthesis and handling of this prodrug **BW-HIF-355**.

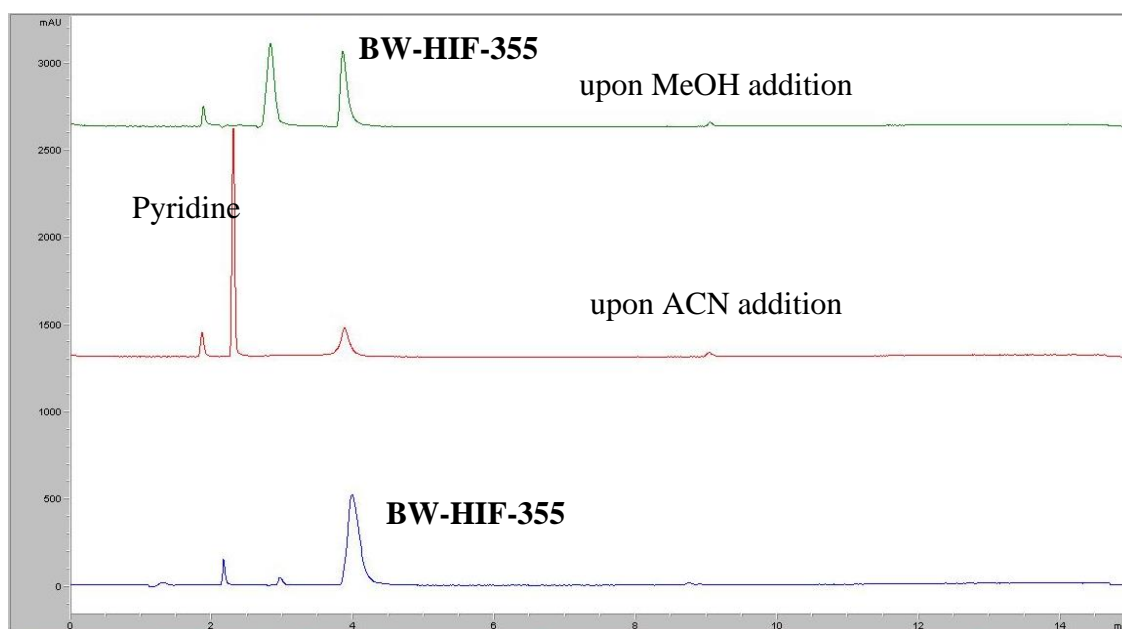


Figure 25. HPLC studies showing the degradation of prodrug **BW-HIF-355** in H<sub>2</sub>O: ACN (1:3), H<sub>2</sub>O: MeOH (1:3) at 37 °C.

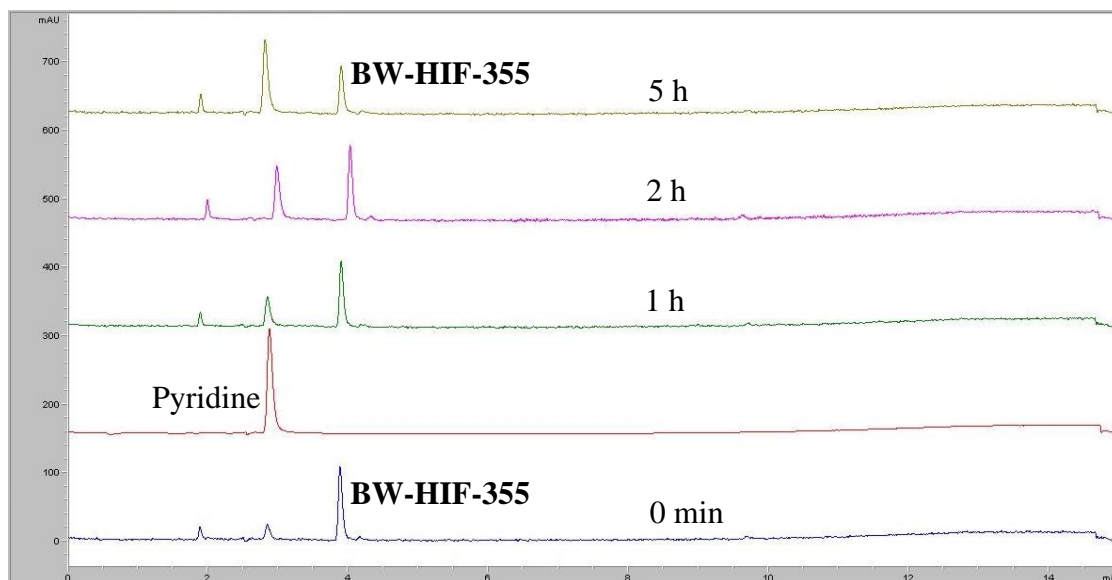


Figure 26. HPLC studies showing hydrolysis of prodrug **BW-HIF-355** by esterase (10 units/mL) in PBS under near physiological conditions. HPLC studies showed pyridine release of more than 50% within 5 h.

### 3 EXPERIMENTAL

All reagents and solvents were of reagent grade and purchased from Sigma-Aldrich (Massachusetts, USA) or Oakwood Products, Inc. (South Carolina, USA). Porcine liver esterase (E0319-20KU) and penicillin amidase from *Escherichia coli* (76427) were purchased from Sigma-Aldrich (Massachusetts, USA). Pepsin (470301-944), pancreatin (470301-922), and lysozyme (470301-618) were purchased from Ward's science (Ontario, Canada). Corning™ 0.25% Trypsin, 2.21 mM EDTA, 1x [-] sodium bicarbonate was purchased from Thermo Fisher Scientific (USA). Column chromatography was carried out using silica gel (Sorbent Technologies (Georgia, USA) 230–400 mesh). TLC analyses were conducted on silica gel plates (Sorbent Technologies (Georgia, USA) Silica XHL TLC plates w/UV254). <sup>1</sup>H NMR (400 MHz) and <sup>13</sup>C NMR (100 MHz) spectra were recorded on a Bruker Avance 400MHz NMR spectrometer in deuterated solvent from Cambridge Isotope Laboratories, Inc. Chemical shifts were reported as  $\delta$  values (ppm). TMS ( $\delta = 0.00$  ppm) or residual peaks of the deuterated solvent were used as the internal reference. Mass

spectrometric analyses were conducted by the Georgia State University Mass Spectrometry Facilities. The milligram scale quantities were weighed on C-33 microbalance (CAHN instruments Inc., California, USA). HPLC was performed on Shimadzu LC-20AT or Agilent HPLC system. Mobile phase used was made of A: H<sub>2</sub>O (0.1% TFA) and B: ACN (0.1% TFA). Either of these two gradient methods were used: (i) 95% B, 0-15 min; 95% B, 15-20 min; 5% B, 20-20.2 min; 5% B, 20.2-23. (ii) (a) 95% B, 0-10 min; 95% B, 10-12 min; 5% B, 12-12.1 min; 5% B, 12.1-15. Column: C18, 5  $\mu$ m, 4.6  $\times$  150 mm; detector: DAD monitored at 254 nm.

### 3.1 Stability Studies

Stability studies of the prodrugs were carried out using HPLC. A 1-mM solution of each prodrug (**BW-HIF-352**, **BW-HIF-353**, **BW-HIF-354**, or **BW-HIF-355**) was prepared in PBS at pH 7.4 and kept at 37 °C. 20  $\mu$ L was injected in HPLC at different intervals of time. Peak area vs time was plotted in excel keeping the 100% peak area at 0 min time point.

### 3.2 Solubility Studies

Solubility of each compound (**BW-HIF-354**, 4-bromoisoquinoline, and **BW-HIF-356**) was tested by following our previously reported HPLC method.<sup>52</sup> First, for prodrugs **BW-HIF-352**, **BW-HIF-354** and **BW-HIF-355**, 100 mg prodrug was added to 100  $\mu$ L ultra-pure water; and for prodrug **BW-HIF-356** and 4-bromoisoquinoline **13**, 20 mg was added to 500  $\mu$ L in 1.5 ml Eppendorf tube and vortexed for 5 min at 21°C. The Eppendorf was then centrifuged at 14,000 rpm for 15 min. 10  $\mu$ L of supernatant from prodrug **BW-HIF-354** was diluted first 100-fold then that solution was diluted again 10-fold. 20  $\mu$ L of that solution was injected in the HPLC and concentration was calculated by plotting the peak area vs concentration standard curve. The standard curve of prodrug **BW-HIF-354** was prepared by making serial dilutions with water. The concentration range used for preparing the standard curve of **BW-HIF-354** was 0.125 mg/mL to

2 mg/mL (Figure 27). The concentration range used for preparing the standard curve of 4-bromoisquinoline **13** was 0.012 mg/mL to 0.1 mg/mL (Figure 28). The concentration range used for preparing the standard curve of **BW-HIF-356** was 0.025 mg/mL to 0.4 mg/mL (Figure 29).

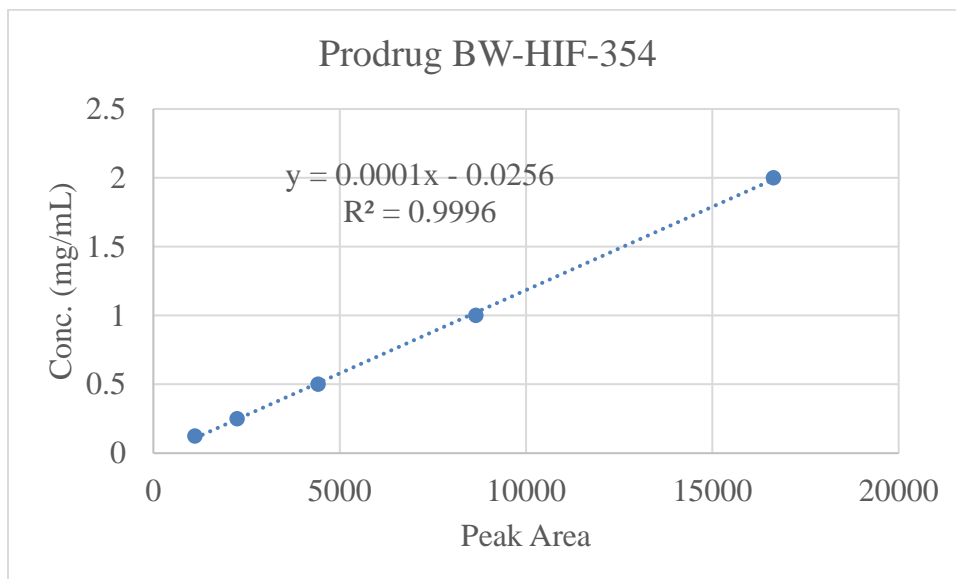


Figure 27. Standard curve of prodrug **BW-HIF-354**.

Concentration of injected sample was calculated from the equation as  $\text{Conc. (mg/mL)} = 0.0001(\text{Peak Area}) - 0.0256$ , which then multiplied by 1000 to get the final solubility of prodrug **BW-HIF-354**.

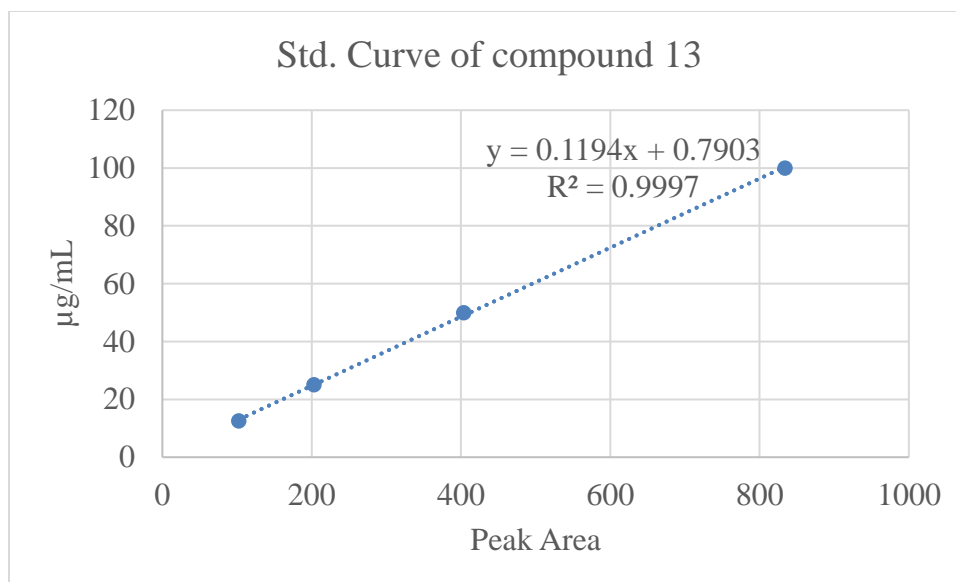


Figure 28. Standard curve of compound **13**.

Concentration of injected sample was calculated from the equation as  $\text{Conc. } (\mu\text{g/mL}) = 0.1194(\text{Peak Area}) + 0.7903$ . which then multiplied by 10 to get the final solubility of compound **13**.

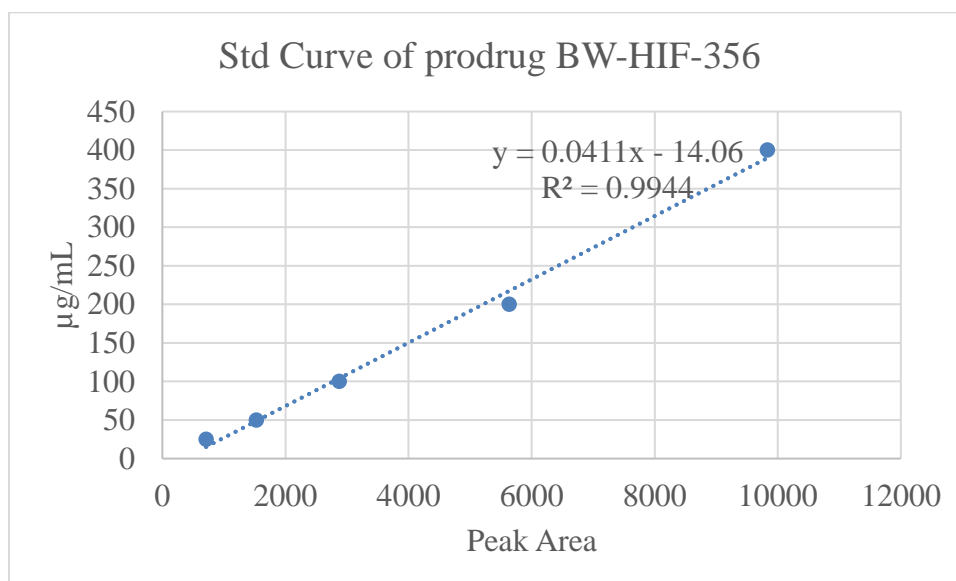


Figure 29. Standard curve of prodrug **BW-HIF-356**.

Concentration of injected sample was calculated from the equation as  $\text{Conc. } (\mu\text{g/mL}) = 0.0411(\text{Peak Area}) - 14.06$ . which then multiplied by 10 to get the final solubility of prodrug **BW-HIF-356**.

Table 5 summarizes the peak area, concentration of injected sample and solubility of saturated solution from each trail.

Table 4. Plot of concentration vs peak area.

Compound	Trail	Peak Area	Concentration	Solubility
<b>BW-HIF-354</b>	1	4810	0.4554 mg/mL	455.4 mg/mL
<b>BW-HIF-354</b>	2	4816	0.456 mg/mL	456 mg/mL
<b>BW-HIF-354</b>	3	4867	0.4661 mg/mL	466.1 mg/mL
<b>Compound 13</b>	1	403.3	48.94 $\mu\text{g/mL}$	489.4 $\mu\text{g/mL}$
<b>Compound 13</b>	2	364.9	44.35 $\mu\text{g/mL}$	443.5 $\mu\text{g/mL}$
<b>Compound 13</b>	3	353.7	43.02 $\mu\text{g/mL}$	43.02 $\mu\text{g/mL}$
<b>BW-HIF-356</b>	1	6293	244.586 $\mu\text{g/mL}$	2445.9 $\mu\text{g/mL}$
<b>BW-HIF-356</b>	2	5789	225.867 $\mu\text{g/mL}$	2258.7 $\mu\text{g/mL}$
<b>BW-HIF-356</b>	3	5850	228.375 $\mu\text{g/mL}$	2283.8 $\mu\text{g/mL}$

### 3.3 Bio-reduction of BW-HIF-345

Bio-reduction of **BW-HIF-345** was performed by Dr. Wen Lu. LN-229 cells (human glioblastoma) (ATCC, Virginia, USA) were seeded in 6-well plate which then treated with 100  $\mu\text{M}$  of **BW-HIF-345**. Then, the treated cells we kept under hypoxic (0.1%  $\text{pO}_2$ ) or normoxic (21%  $\text{pO}_2$ ) conditions for 24 h. After that the cells were incubated for another 24 h under normoxia conditions. After that, cells were lysed and extracted by ACN. Then, the ACN extracts were centrifuged at 14,000 rpm for 10 minutes and the supernatant was directly analyzed by LC-MS. Extracted ion chromatogram (EIC) was used to evaluate the ratio of reduced compound (**64B**) and N-oxide.

### 3.4 General procedure for enzymatic hydrolysis by HPLC

#### 3.4.1 General procedure of sample preparation for HPLC

At different time points, 200  $\mu$ L of reaction mixture was taken and transferred to a centrifugation tube containing 600  $\mu$ L ACN (for prodrug **BW-HIF-353** and **BW-HIF-354**) or 1% acetic acid (for prodrug **BW-HIF-355**) and kept at -78 °C. After 15 min, the mixture was centrifuged at 14,000 rpm for 5 min, and the supernatant was collected and injected in HPLC. Mobile phase used was made of A: H<sub>2</sub>O (0.1% TFA) and B: ACN (0.1% TFA). Either of these two gradient methods were used: (i) 95% B, 0-15 min; 95% B, 15-20 min; 5% B, 20-20.2 min: 5% B, 20.2-23. (ii) (a) 95% B, 0-10 min; 95% B, 10-12 min; 5% B, 12-12.1 min: 5% B, 12.1-15. Column: C18, 5  $\mu$ m, 4.6  $\times$  150 mm; detector: DAD monitored at 254 nm.

#### 3.4.2 Porcine liver esterase-mediated hydrolysis for Prodrug **BW-HIF-353** and **BW-HIF-354**

10 mM stock solution of prodrugs was prepared in PBS as by dissolving the 2.545 mg (weighed using C-33 microbalance) in 3.240 mL for prodrug **BW-HIF-354** or 4.291 mg in 1.2115 mL for prodrug **BW-HIF-353**. Esterase stock solution of 24 units/mL was made in PBS by dissolving 5.184 mg (weighed using C-33 microbalance) of the enzyme in 6 mL of PBS. Then 1 mL of compound solution was added to 4 mL of the esterase solution and the mixture was kept at 37 °C. The final concentration of prodrug was 2 mM and esterase were 20 units/mL in PBS. HPLC samples were prepared according to the procedure mentioned in section 3.4.1.

#### 3.4.3 Pancreatin hydrolysis for Prodrug **BW-HIF-353**

1 mg/mL solution of pancreatin was prepared in PBS at pH 6.5 by dissolving 5.768 mg (weighed using C-33 microbalance) in 5.768 mL. Then 0.688 mg (weighed using C-33 microbalance) of prodrug **BW-HIF-352** was weighed and added to a 1.942 mL of PBS at pH 6.5 in

6 mL vial to give a concentration of 1 mM of prodrug. Then, the reaction mixture was kept at 37 °C. HPLC sample was prepared according to the procedure mentioned in section 3.4.1.

#### ***3.4.4 Lysozyme hydrolysis for Prodrug BW-HIF-353***

2200 units/mL solution of lysozyme was prepared in PBS at pH 7.4 by dissolving 0.568 mg (weighed using C-33 microbalance) in 5.68 mL. Then 0.732 mg (weighed using C-33 microbalance) of prodrug **BW-HIF-352** was weight and added to a 2.066 mL of PBS at pH 7.4 in a 6 mL vial to give a concentration of 1 mM of prodrug. Then, the reaction mixture was kept at 37 °C. HPLC sample was prepared according to the procedure mentioned in section 3.4.1.

#### ***3.4.5 Amidase hydrolysis for Prodrug BW-HIF-353***

100 units/mL solution of amidase was prepared by diluting the amidase (976 U/mL) solution in PBS. Then 0.383 mg (weighed using C-33 microbalance) of prodrug **BW-HIF-352** was weight and added to a 6 mL vial and then 0.540 mL of amidase solution was added to give a concentration of 2 mM of prodrug. Then, the reaction mixture was kept at 37 °C. HPLC sample was prepared according to the procedure mentioned in section 3.4.1.

#### ***3.4.6 Trypsin hydrolysis for Prodrug BW-HIF-353***

For trypsin hydrolysis, 0.25% trypsin solution (Corning) was used directly. Then 1.064 mg (weighed using C-33 microbalance) of prodrug **BW-HIF-353** was weight and added to 1.502 mL in a 6 mL vial to give a 2 mM concentration. Then, the reaction mixture was kept at 37 °C. HPLC sample was prepared according to the procedure mentioned in section 3.4.1.

#### ***3.4.7 Esterase hydrolysis for Prodrug BW-HIF-355***

For esterase hydrolysis of prodrug **BW-HIF-355**, First a 2 mM stock solution of prodrug was prepared by dissolving 3.130 mg (weighed on C-33 microbalance) in 5.607 mL of PBS. Then, esterase stock solution of 20 units/mL was made in PBS. Then 2 mL of compound solution was

added to 2 ml of esterase solution and the reaction mixture was kept at 37 °C giving a final concentration of prodrug 1 mM and esterase 10 units/mL. HPLC sample was prepared according to the procedure mentioned in section 3.4.1.

### 3.5 Chemistry

**6-(((N-cyclobutyl-3,4-dimethoxyphenyl)sulfonamido)methyl)-2,2-dimethyl-2H-pyrano[3,2-*b*]pyridine 5-oxide (BW-HIF-345).** To a solution of **64B** (22 mg, 0.05 mmol) in 125  $\mu$ L of DCM in a 6 mL vial was added H<sub>2</sub>O<sub>2</sub> (100  $\mu$ L). To this mixture methyltrioxorhenium (VII) (0.6 mg, 5 mol%) was added and stirred overnight. Then, the reaction mixture was concentrated, and the residue recrystallized from MeOH to yield a white solid. Isolated yield: 82%. <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  7.57 (d, *J* = 8.8 Hz, 1H), 7.46 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.29 (d, *J* = 2.7 Hz, 2H), 7.17 (d, *J* = 8.8 Hz, 1H), 7.10 (d, *J* = 10.4 Hz, 1H), 6.97 (d, *J* = 8.5 Hz, 1H), 6.11 (d, *J* = 10.4 Hz, 1H), 4.64 (s, 2H), 4.60 – 4.44 (m, 1H), 3.96 (d, *J* = 5.4 Hz, 6H), 2.09 – 1.84 (m, 4H), 1.65 – 1.49 (m, 8H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  152.94, 150.34, 149.31, 143.98, 138.86, 134.50, 130.37, 123.21, 121.25, 121.13, 113.52, 110.77, 109.56, 78.77, 56.31, 56.21, 52.56, 42.99, 28.61, 27.93, 14.73. HRMS calculated for C<sub>23</sub>H<sub>29</sub>N<sub>2</sub>O<sub>6</sub>S [M+H]: *m/z* 461.1746 and found 461.1764.

**1-(4-nitrobenzyl)pyridin-1-ium chloride (BW-HIF-352).** In a sealed tube 4-nitrobenzyl chloride (0.171 g, 1 mmol) was added and pyridine (0.161 mL, 2 mmol) was added. The tube was sealed and heated at 90 °C for 15 min. Then, the crude was washed several times with diethyl ether to give a white solid. Isolated yield: 86%. <sup>1</sup>H NMR (CD<sub>3</sub>OD)  $\delta$  9.23 (d, *J* = 5.8 Hz, 2H), 8.72 (t, *J* = 7.8 Hz, 1H), 8.31 (d, *J* = 8.7 Hz, 2H), 8.24 (t, *J* = 7.1 Hz, 2H), 7.81 (d, *J* = 8.7 Hz, 2H), 6.11 (s, 2H). <sup>13</sup>C NMR (CD<sub>3</sub>OD)  $\delta$  148.61, 146.43, 145.10, 140.12, 129.92, 128.65, 124.10, 63.02. HRMS calculated for C<sub>12</sub>H<sub>11</sub>N<sub>2</sub>O<sub>2</sub><sup>+</sup> [M+]: *m/z* 215.0821 and found 215.0822.

***N*-(4-(hydroxymethyl)phenyl)acetamide (3).** To a solution of 4-aminobenzyl alcohol (0.615 g, 2 mmol) and triethylamine (0.278 mL, 2 mmol), acetic anhydride (0.19 mL, 2 mmol) was added. The reaction mixture was heated at 90 °C for 3 h. Then, EA was added to the reaction mixture and the organic layer was washed with 0.5 N HCl. The organic layer was then dried over NaSO<sub>4</sub> for 30 min. The organic layer was concentrated and purified by silica gel column (hexane/EtOAc) to give a golden yellow solid. Isolated yield: 75%. <sup>1</sup>H NMR (DMSO-D<sub>6</sub>) δ 9.89 (s, 1H), 7.52 (d, *J* = 8.4 Hz, 2H), 7.23 (d, *J* = 8.4 Hz, 2H), 5.09 (t, *J* = 5.7 Hz, 1H), 4.43 (d, *J* = 5.7 Hz, 2H), 2.03 (s, 3H). <sup>13</sup>C NMR (DMSO-D<sub>6</sub>) δ 168.60, 138.42, 137.54, 127.38, 119.18, 63.10, 24.43. HRMS calculated for C<sub>9</sub>H<sub>10</sub>NO<sub>2</sub> [M-H]: m/z 164.0712; found 164.0716.

***N*-(4-(chloromethyl)phenyl)acetamide (4).** To a solution of compound **1** (0.330 g, 2 mmol) in DCM (2 mL) was added thionyl chloride (0.145 mL, 2 mmol). The mixture was gently heated to refluxing for 1 h. The organic layer was washed with water multiple times and dried over NaSO<sub>4</sub> for 30 min. Then, the reaction mixture was concentrated to give a yellow solid. Isolated yield: 98%. <sup>1</sup>H NMR (DMSO-D<sub>6</sub>) δ 10.02 (s, 1H), 7.57 (d, *J* = 8.4 Hz, 2H), 7.36 (d, *J* = 8.5 Hz, 2H), 4.71 (s, 2H), 2.07 (d, *J* = 18.4 Hz, 3H). <sup>13</sup>C NMR (101 MHz, DMSO-D<sub>6</sub>) δ 168.87, 139.86, 132.47, 129.93, 119.40, 46.71, 24.49. HRMS calculated for C<sub>9</sub>H<sub>9</sub>ClNO [M-H]: m/z 182.0451 and found 182.0280.

***N*-(4-(iodomethyl)phenyl)acetamide (5).** To a solution of compound **2** (0.367 g, 2 mmol) in acetone NaI (0.6 g, 4 mmol) was added and refluxed for 1 h. Then, the reaction mixture was concentrated and purified by silica gel column (DCM) to yield a yellow solid. Isolated yield: 98%. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.43 (d, *J* = 8.5 Hz, 2H), 7.34 (d, *J* = 8.5 Hz, 2H), 4.45 (s, 2H), 2.17 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 168.17, 137.41, 135.10, 129.52, 119.98, 24.69, 5.61. HRMS calculated for C<sub>9</sub>H<sub>11</sub>INO [M+H]: m/z 275.9885 and found 275.9882

**1-(4-acetamidobenzyl)pyridin-1-ium iodide (BW-HIF-353).** Compound **3** (0.165 g, 0.6 mmol) was added to a vial containing pyridine (0.160 g, 2 mmol) then the vial was sealed and heated at 90 °C for 2 h. Then, the mixture was washed with diethyl ether multiple times to yield a yellow solid. Isolated yield: 84%. <sup>1</sup>H NMR (CD<sub>3</sub>OD) δ 9.05 (d, *J* = 5.7 Hz, 2H), 8.64 (t, *J* = 7.8 Hz, 1H), 8.15 (t, *J* = 7.1 Hz, 2H), 7.67 (d, *J* = 8.6 Hz, 2H), 7.53 (d, *J* = 8.6 Hz, 2H), 5.84 (s, 2H), 2.17 (s, 3H). <sup>13</sup>C NMR (CD<sub>3</sub>OD) δ 171.18, 146.04, 144.29, 139.85, 129.74, 128.48, 128.43, 120.74, 64.12, 22.71. HRMS for calculated C<sub>14</sub>H<sub>15</sub>N<sub>2</sub>O<sub>1</sub><sup>+</sup> [M<sup>+</sup>]: *m/z* 227.1192 and found 227.1129.

**4-formylphenyl acetate (7).** To a mixture of 4-hydroxy benzaldehyde (0.244 g, 2 mmol) and triethylamine (0.278 mL, 2 mmol) was added acetic anhydride (0.188 mL, 2 mmol) and stirred at 90 °C for 3 h. DCM was added to the reaction mixture, and the organic layer was washed with water. The organic layer was then dried over NaSO<sub>4</sub> for 30 min. After concentrating the reaction mixture was purified by a silica gel column (Hexane/EA) to yield yellow liquid. Isolated yield: 88%. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 9.99 (s, 1H), 7.92 (d, *J* = 8.6 Hz, 2H), 7.28 (d, *J* = 8.5 Hz, 2H), 2.33 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 190.93, 168.71, 155.34, 133.99, 131.20, 122.38, 21.14. The compound has been reported before.<sup>58,59</sup>

**4-(hydroxymethyl)phenyl acetate (8).** To a solution of compound **4** (0.327 g, 2 mmol) in DCM (4 mL) NaBH<sub>4</sub> (0.5 g, 4 mmol) was added. The temperature was gently raised to 35 °C and stirred for 2 h. Then, water was added to the reaction mixture, and the product was extracted with DCM. The organic layer was washed with water several times and then dried over NaSO<sub>4</sub> for 30 min. The crude was then purified by a silica gel column (Hexane/EA) to yield a light-yellow liquid. Isolated yield: 98%. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.20 (d, *J* = 8.4 Hz, 2H), 6.94 (d, *J* = 8.4 Hz, 2H), 4.45 (s, 2H), 2.18 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 169.86, 149.86, 138.70, 128.04, 121.54, 64.29, 21.10. The compound has been reported before.<sup>60</sup>

**4-(chloromethyl)phenyl acetate (9).** To a solution of compound 5 (0.33 g, 2 mmol) in DCM (4 mL), thionyl chloride (0.29 mL, 4 mmol) was added. The temperature of the reaction mixture was gently raised to 35 °C and stirred for 1 h. Then, water was added to the reaction mixture, and the product was extracted with ethyl acetate. The organic layer was then washed with saturated NaHCO<sub>3</sub> and water several times and then dried over NaSO<sub>4</sub> for 30 min. The crude was purified by a silica gel column (Hexane/EA) to yield white liquid. Isolated yield: 98%. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.31 (d, *J* = 8.5 Hz, 2H), 7.00 (d, *J* = 8.5 Hz, 2H), 4.48 (s, 2H), 2.20 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 169.34, 150.62, 135.08, 129.80, 121.93, 45.58, 21.13. The compound has been reported before.<sup>61</sup>

**1-(4-acetoxybenzyl)pyridin-1-ium chloride (BW-HIF-354).** Compound 5 (0.369 g, 2 mmol) was added to a vial containing pyridine (0.322 g, 4 mmol) then the vial was sealed and heated for 2 h. Then, diethyl ether was added to the mixture, and a yellow liquid was separated, then centrifuged at 3000 rpm for 5 min. The supernatant was discarded, and the process was repeated washed multiple times to yield a yellow liquid that solidifies upon keeping in a vacuum for 3 days. Isolated yield: 84%. <sup>1</sup>H NMR (CD<sub>3</sub>OD) δ 9.12 (d, *J* = 5.9 Hz, 2H), 8.64 (t, *J* = 7.8 Hz, 1H), 8.16 (t, *J* = 6.9 Hz, 2H), 7.60 (d, *J* = 8.4 Hz, 2H), 7.24 (d, *J* = 8.4 Hz, 2H), 5.90 (s, 2H), 2.30 (s, 3H). <sup>13</sup>C NMR (CD<sub>3</sub>OD) δ 169.48, 152.07, 145.98, 144.60, 130.72, 130.21, 128.38, 122.75, 63.63, 19.48. HRMS calculated for C<sub>14</sub>H<sub>14</sub>NO<sub>2</sub><sup>+</sup> [M<sup>+</sup>]: *m/z* 228.1014 and found 228.1025.

**iodomethyl acetate (12).** The iodomethyl acetate (7) was synthesized by following the previously reported method.<sup>1</sup> <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 5.91 (s, 2H), 2.11 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 169.02, 49.78, 30.42, 21.09.

**1-(acetoxymethyl)pyridin-1-ium iodide (BW-HIF-355).** Compound 7 (0.6 g, 3 mmol) was added to a vial containing pyridine (0.161 mL, 2 mmol) then the vial was sealed and heated

for 30 min. Then, the mixture was washed with diethyl ether multiple times. The crude was then dissolved in acetone (1 mL) and filtered. The filtrate was then concentrated to yield a yellow solid. Isolated yield: 55%.  $^1\text{H}$  NMR (Acetone- $d_6$ )  $\delta$  9.33 (d,  $J = 5.5$  Hz, 2H), 8.78 (t,  $J = 7.8$  Hz, 1H), 8.28 (t,  $J = 7.1$  Hz, 2H), 6.61 (s, 2H), 2.07 (s, 3H).  $^{13}\text{C}$  NMR (Acetone- $d_6$ )  $\delta$  169.60, 148.29, 145.40, 128.43, 79.65, 19.66. HRMS calculated for  $\text{C}_8\text{H}_{10}\text{NO}_2^+$  [M $^+$ ]:  $m/z$  157.0712 and found 157.0710.

**1-(iodomethyl)-4-nitrobenzene (13).** To a solution of 4-nitrobenzyl chloride 13 (0.343 g, 2 mmol) in acetone (2 mL) was added NaI (0.6 g, 4 mmol) and the mixture was refluxed for 1 h. Then, the mixture was concentrated and purified by a silica gel column (DCM) to yield a whiteish solid. Isolated yield: 98%.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  8.14 (d,  $J = 8.7$  Hz, 2H), 7.52 (d,  $J = 8.7$  Hz, 2H), 4.48 (s, 2H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  147.20, 146.81, 129.63, 124.11, 2.24. HRMS calculated for  $\text{C}_7\text{H}_7\text{INO}_2$  [M+H]:  $m/z$  263.9516 and found 263.9524.

**BW-HIF-356.** Compound 13 (0.264 g, 1 mmol) was added to a vial containing 4-bromoisoquinoline (0.250 g, 1.2 mmol) then the vial was sealed and heated at 90 °C for 1 h. Then, the mixture was washed with diethyl ether multiple times to yield a yellow solid. Isolated yield: 60%.  $^1\text{H}$  NMR ( $\text{DMSO-}d_6$ )  $\delta$  10.36 (s, 2H), 9.40 (s, 2H), 8.61 (d,  $J = 8.2$  Hz, 2H), 8.44 (t,  $J = 7.5$  Hz, 3H), 8.38 (d,  $J = 8.3$  Hz, 1H), 8.51 – 7.90 (m, 12H), 8.24 – 8.15 (m, 2H), 8.25 – 7.90 (m, 4H), 7.88 (d,  $J = 8.6$  Hz, 4H), 6.12 (s, 4H).  $^{13}\text{C}$  NMR ( $\text{DMSO-}d_6$ )  $\delta$  151.21, 148.41, 141.21, 139.60, 136.57, 136.62, 132.74, 132.41, 130.86, 128.08, 126.51, 124.53, 122.73, 62.74. HRMS calculated for  $\text{C}_{16}\text{H}_{12}\text{BrN}_2\text{O}_2^+$  [M $^+$ ]:  $m/z$  343.0077 and found 343.0181.

#### 4 CONCLUSION AND FUTURE OUTLOOK

In search for prodrug approach to improve water solubility of drugs with a pyridine moiety, four model prodrugs have been designed, synthesized, and assessed. All the four model prodrugs were designed and synthesized for the drugs having no functional group/handle. The synthesized prodrugs were assessed broadly in three aspects. First, the stability of prodrugs in PBS under near physiological conditions. Second, the aqueous solubility of the prodrugs and difference in solubility of parent drug and the synthesized prodrug. Third, the drug release from the prodrug upon activation in near physiological conditions. In the stability studies, three prodrugs (**BW-HIF-352**, **BW-HIF-353** and **BW-HIF-354**) showed high stability in PBS under near physiological conditions. In the solubility studies, all the developed prodrugs showed high water solubility. Further, to get a comparative value of solubility between parent drug and the prodrug. A prodrug **BW-HIF-356** of 4-bromoisoquinoline was synthesized. Prodrug **BW-HIF-356** showed a 5-fold improvement in aqueous solubility over its parent drug. This significant improvement in the aqueous solubility indicated that these prodrug approaches can be utilized to improve aqueous solubility of drugs. In the drug release assessment, three prodrugs (**BW-HIF-352**, **BW-HIF-354**, **BW-HIF-355**) showed a significant pyridine release under near physiological conditions, demonstrating the initial proof of concepts. Prodrug **BW-HIF-353** did not show any drug release with the tested enzymes under near physiological conditions. Indicating that other ways of activation need to be developed in future. Collectively, the developed prodrugs give high water solubility, stability, and drug release. These general strategies can be employed to prepare prodrugs of other drugs containing a pyridine ring or a quinoline ring.

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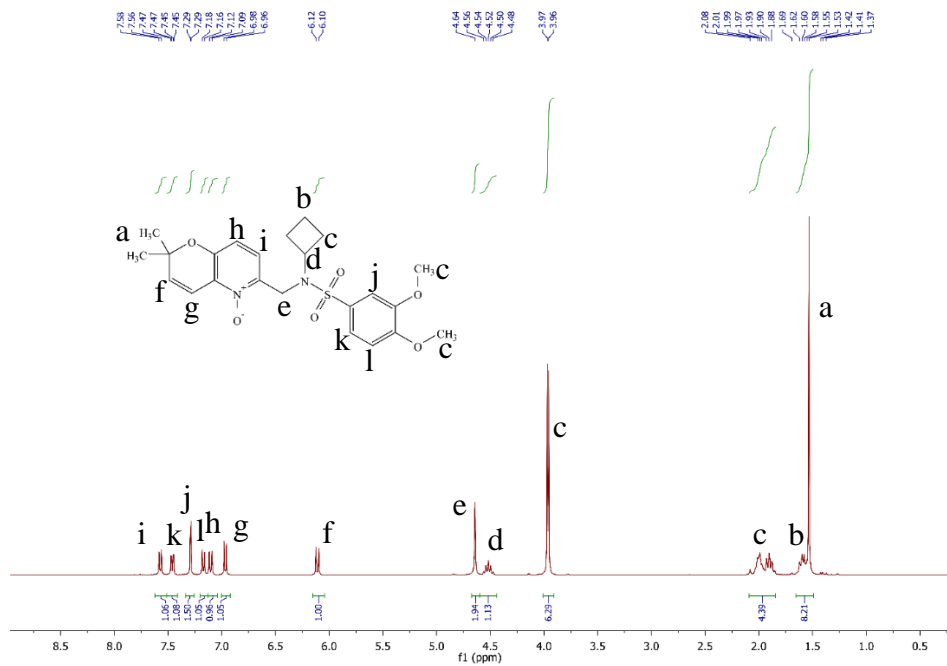
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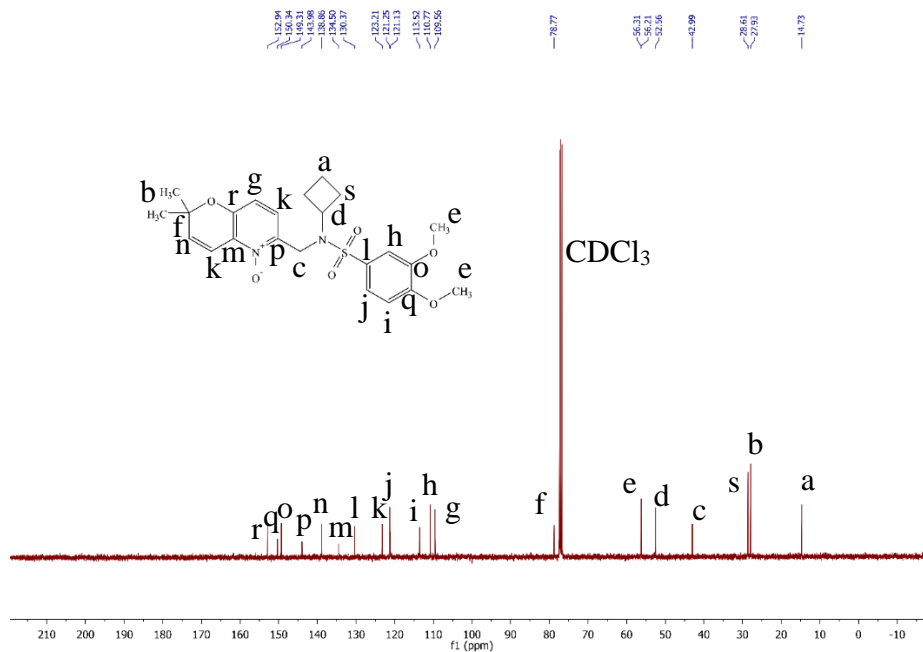
## APPENDICES

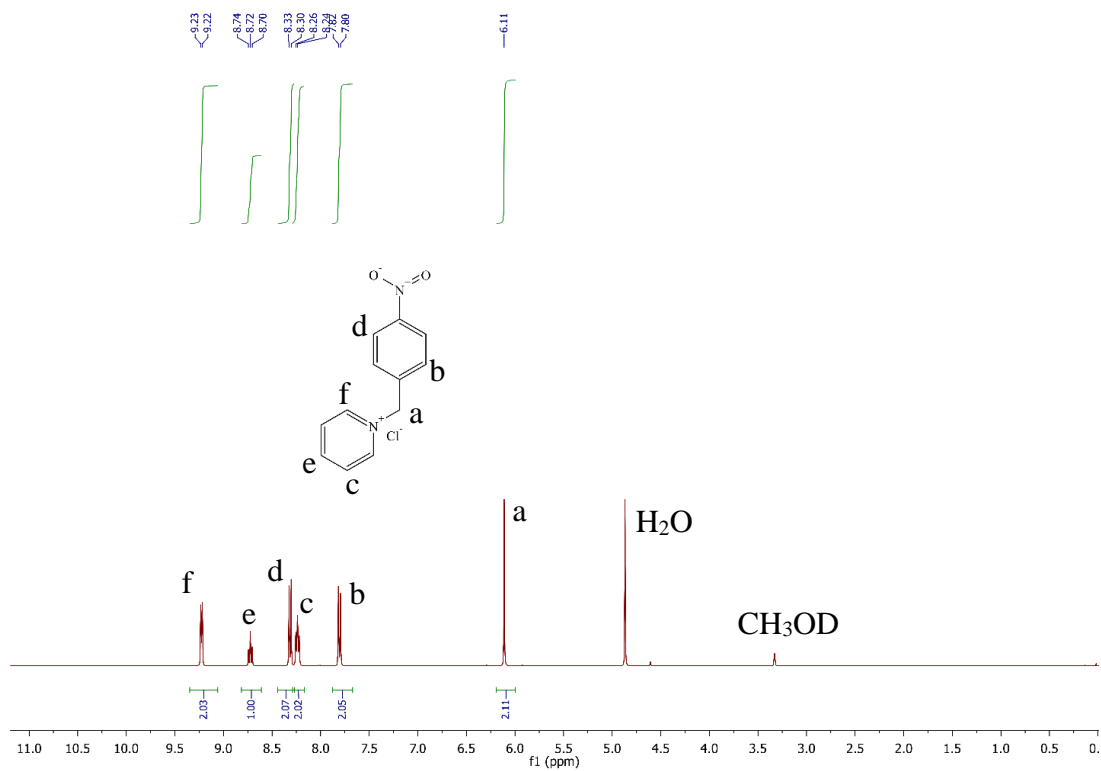
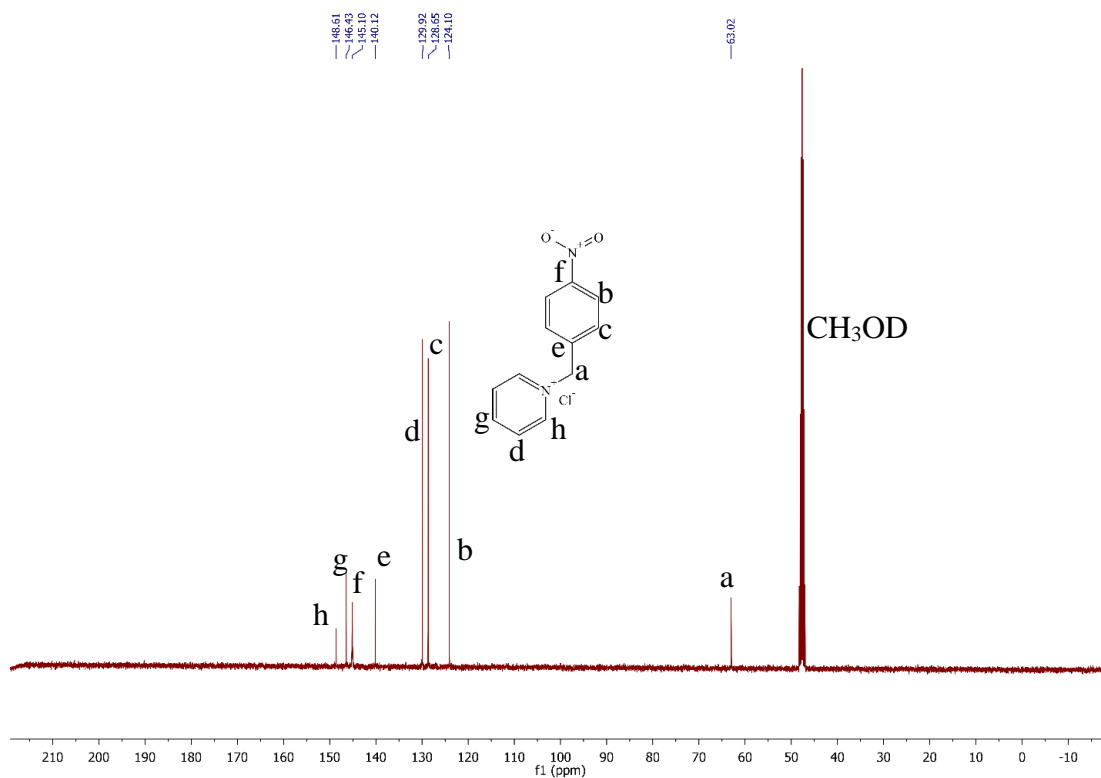
## NMR Data

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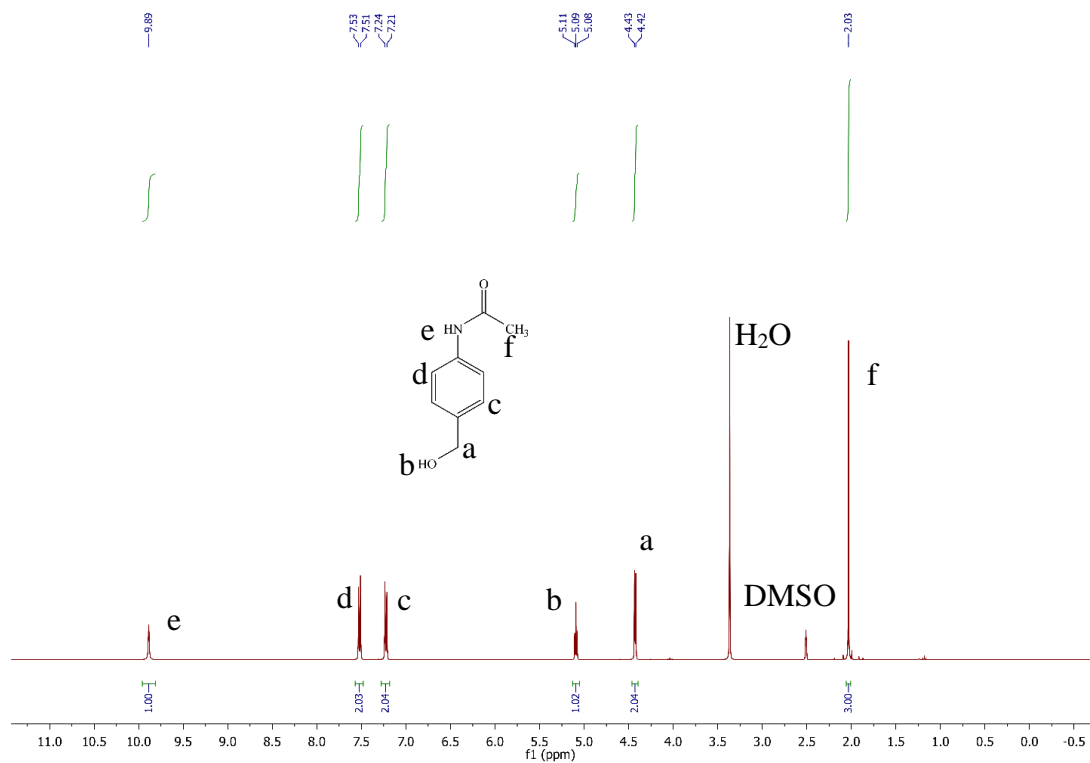


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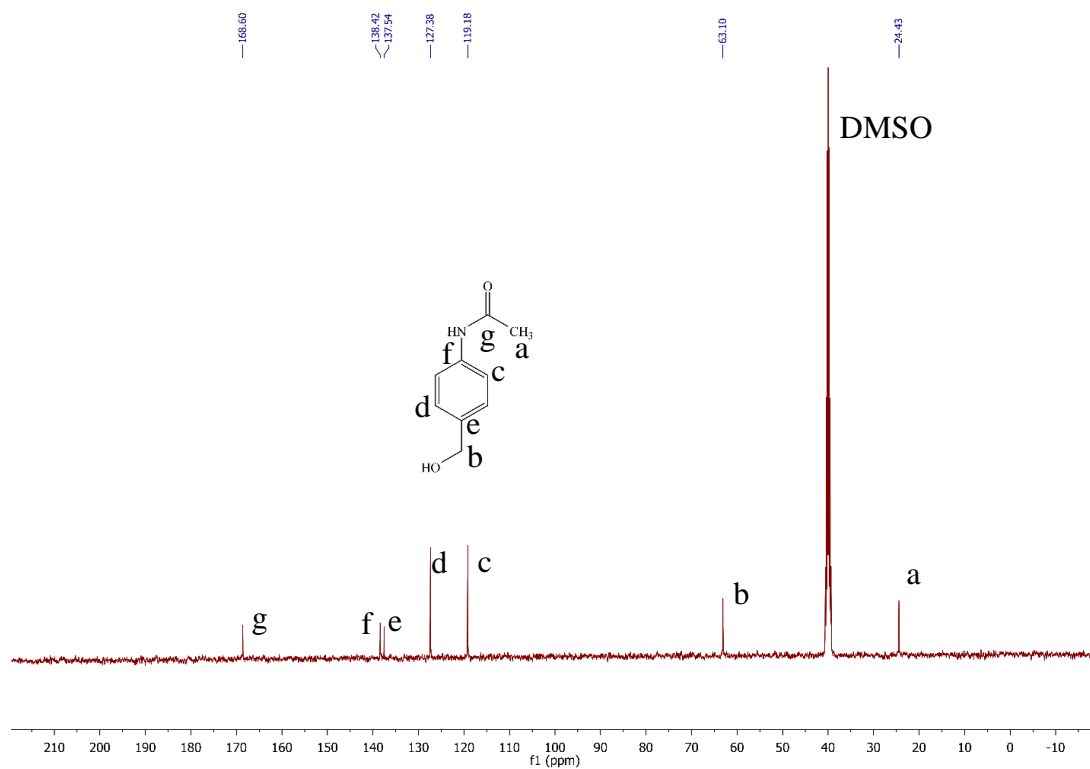


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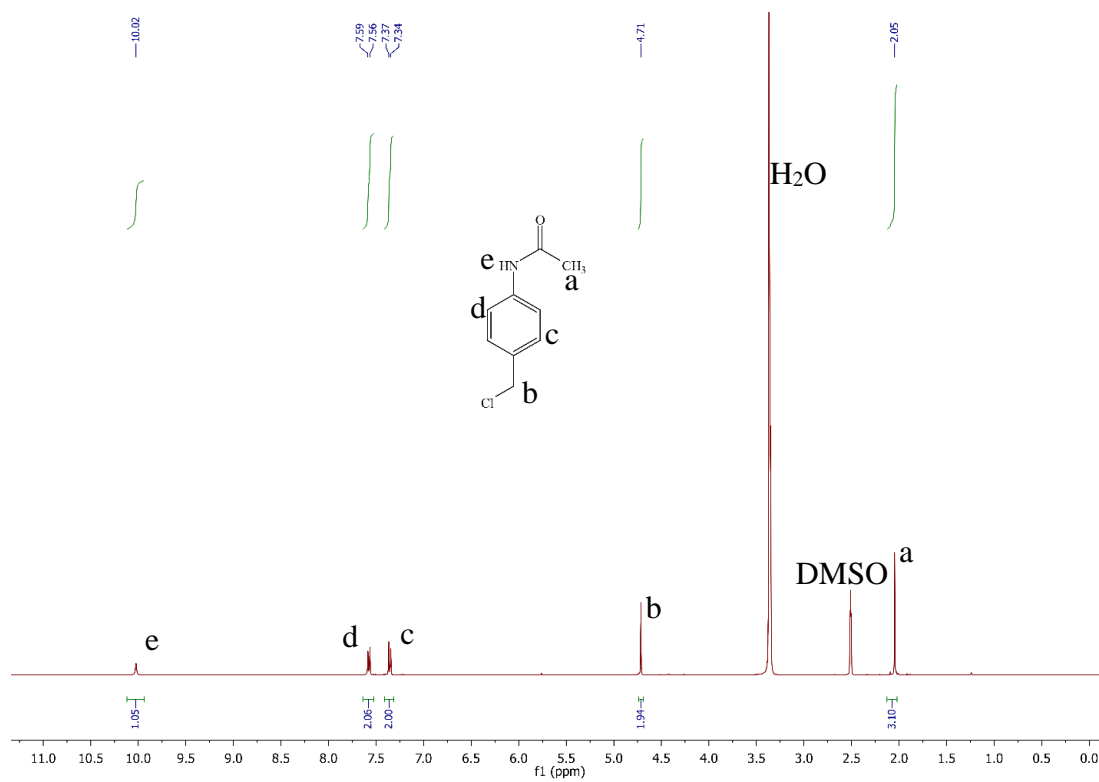
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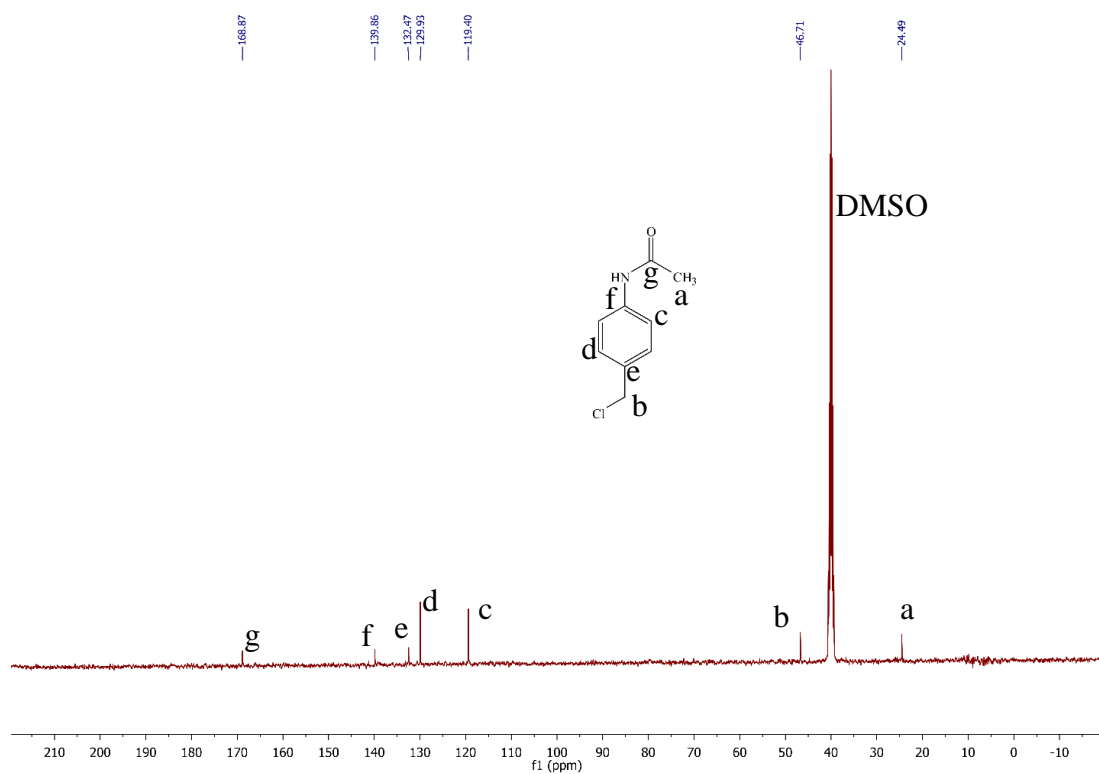
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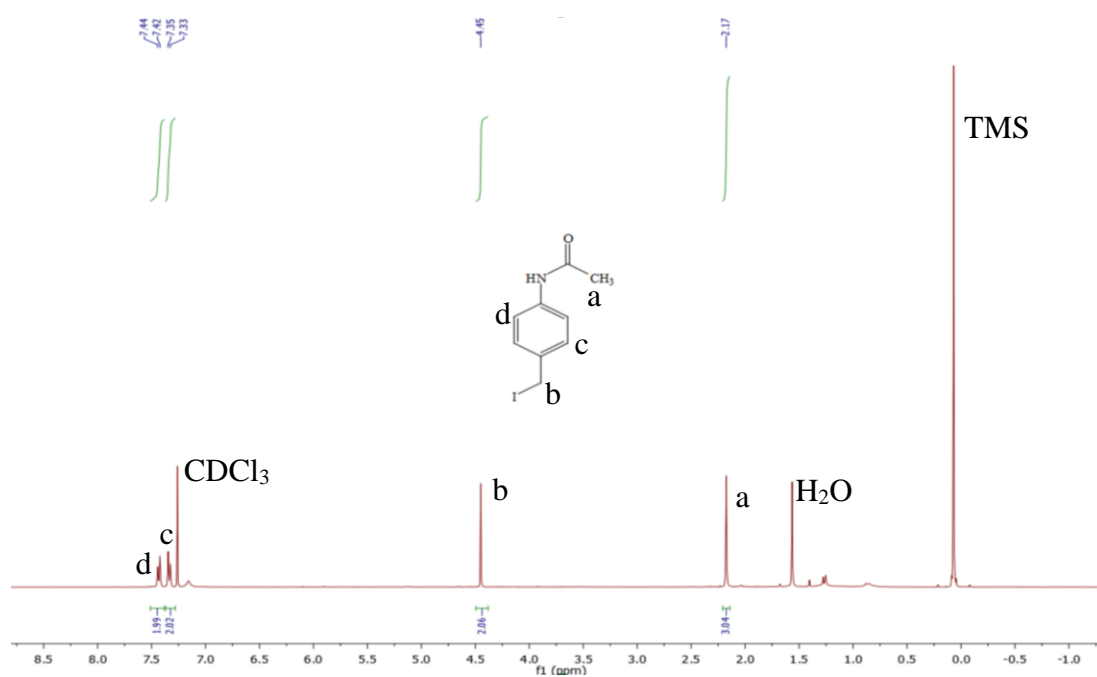
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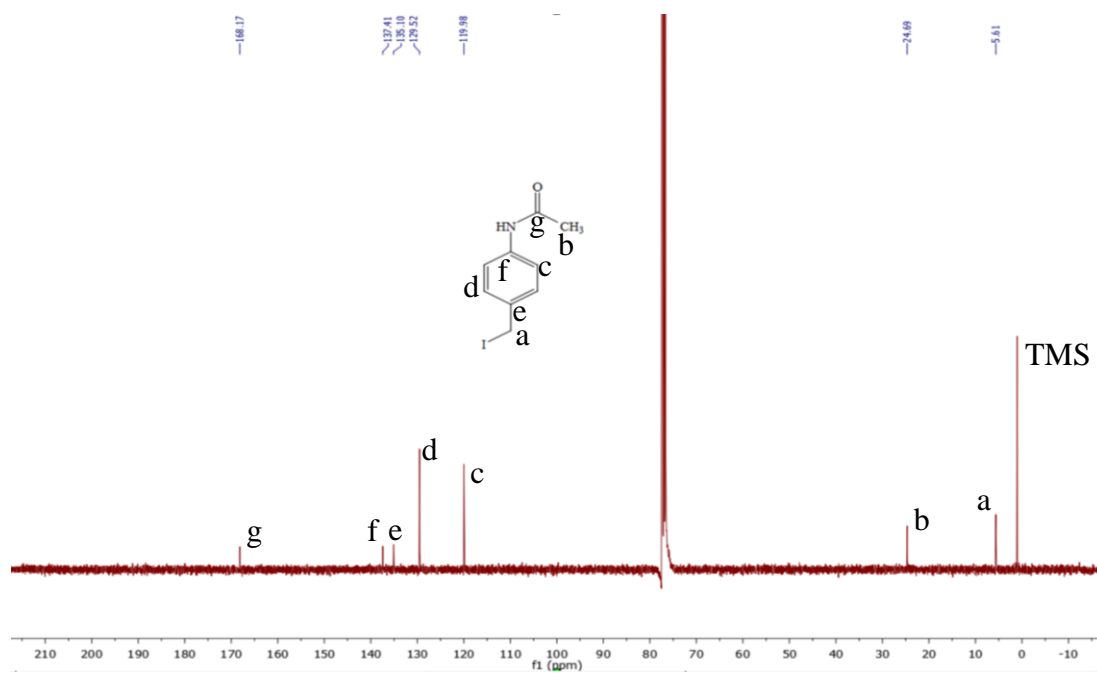
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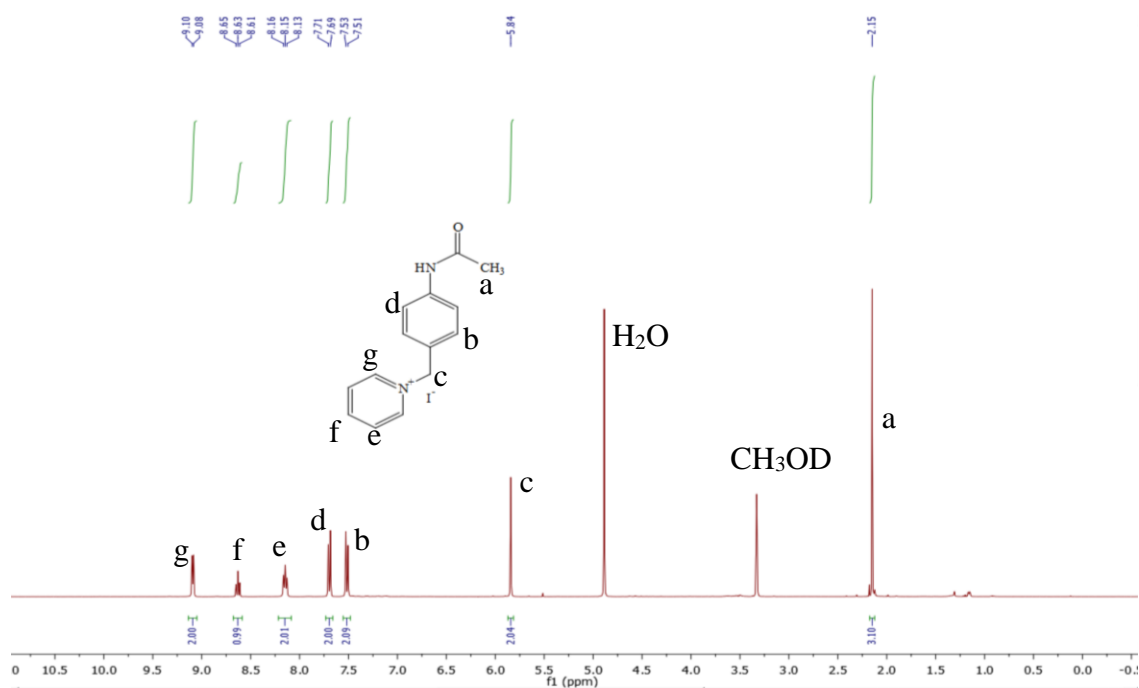
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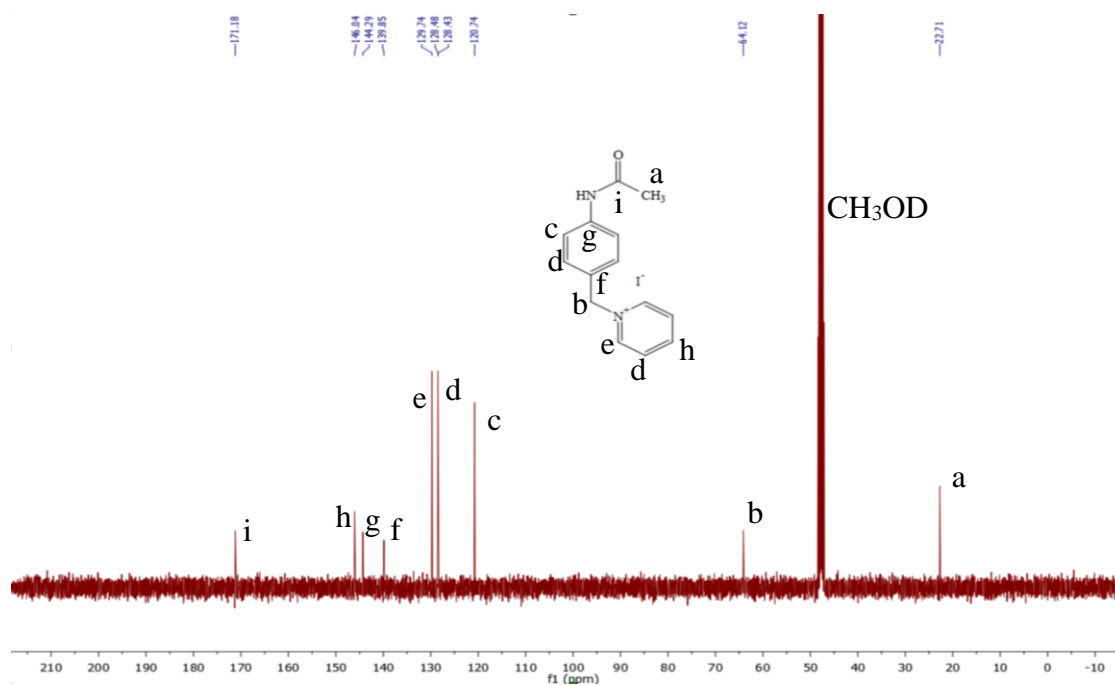
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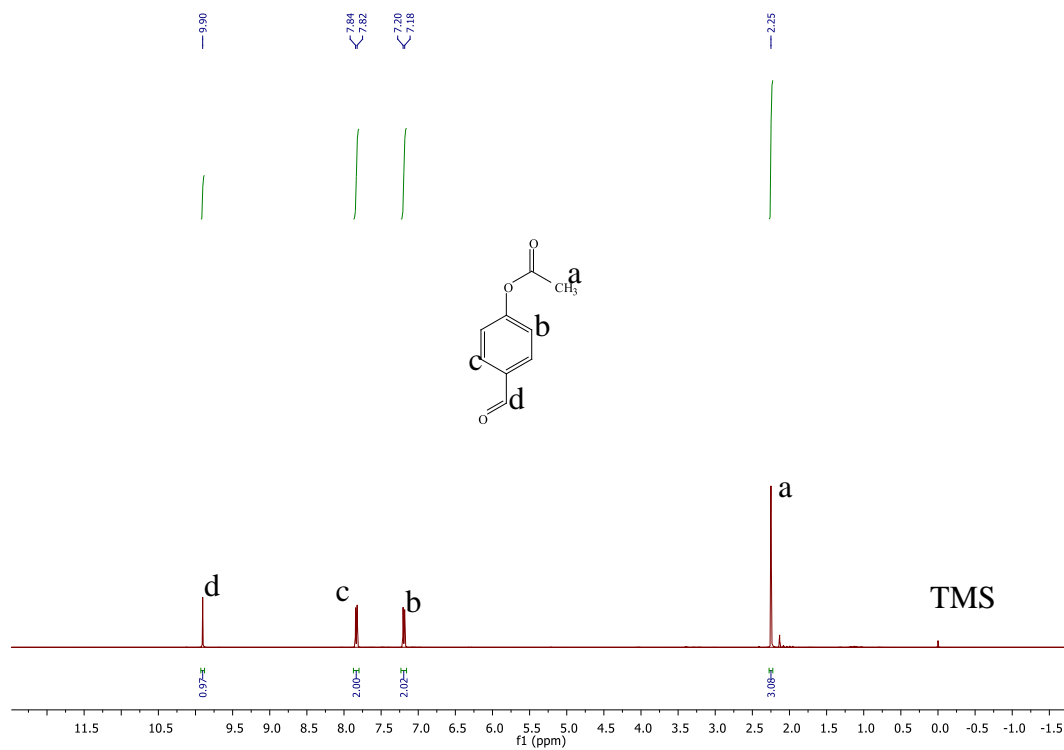
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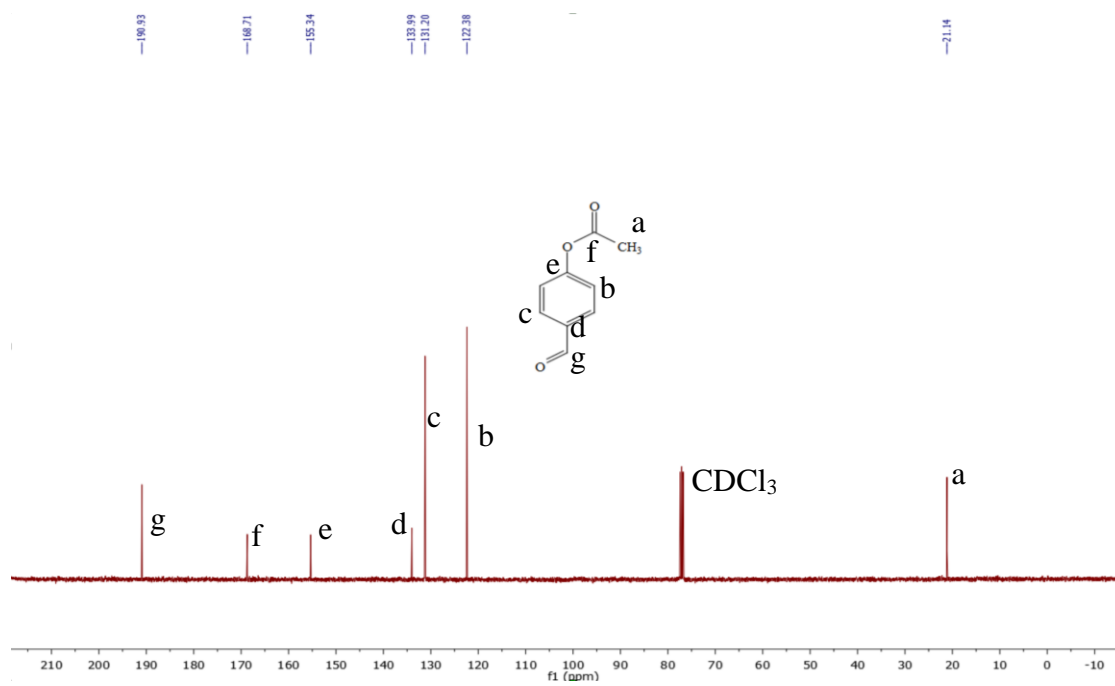
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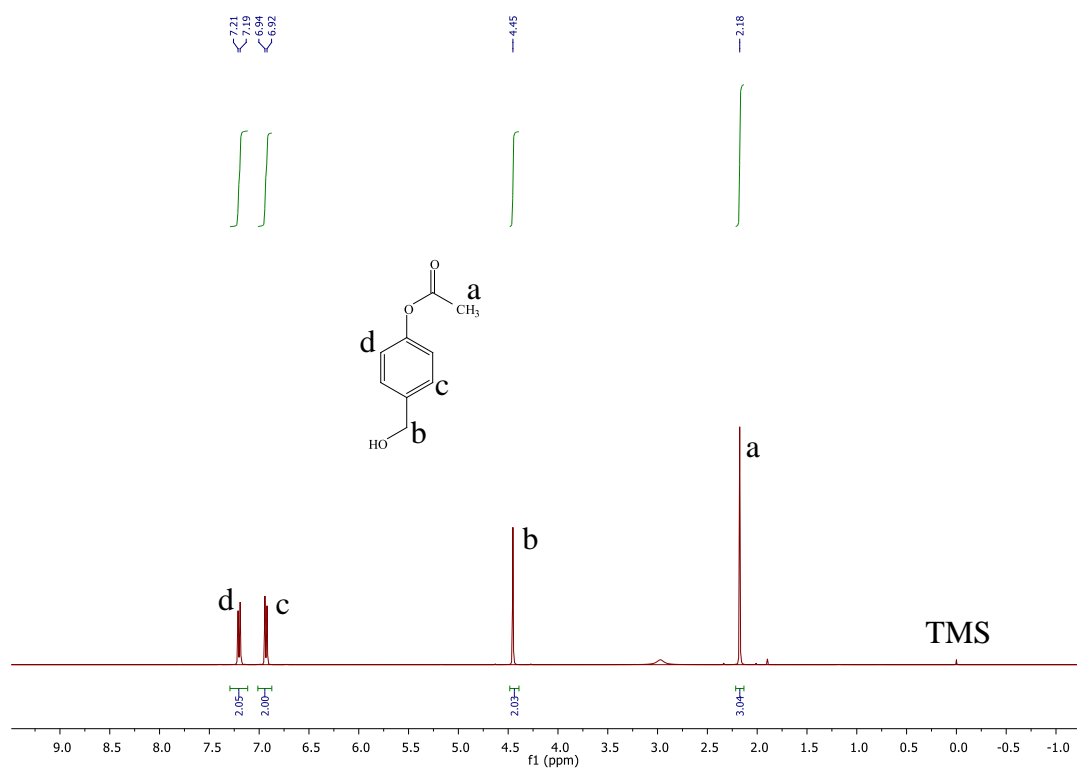
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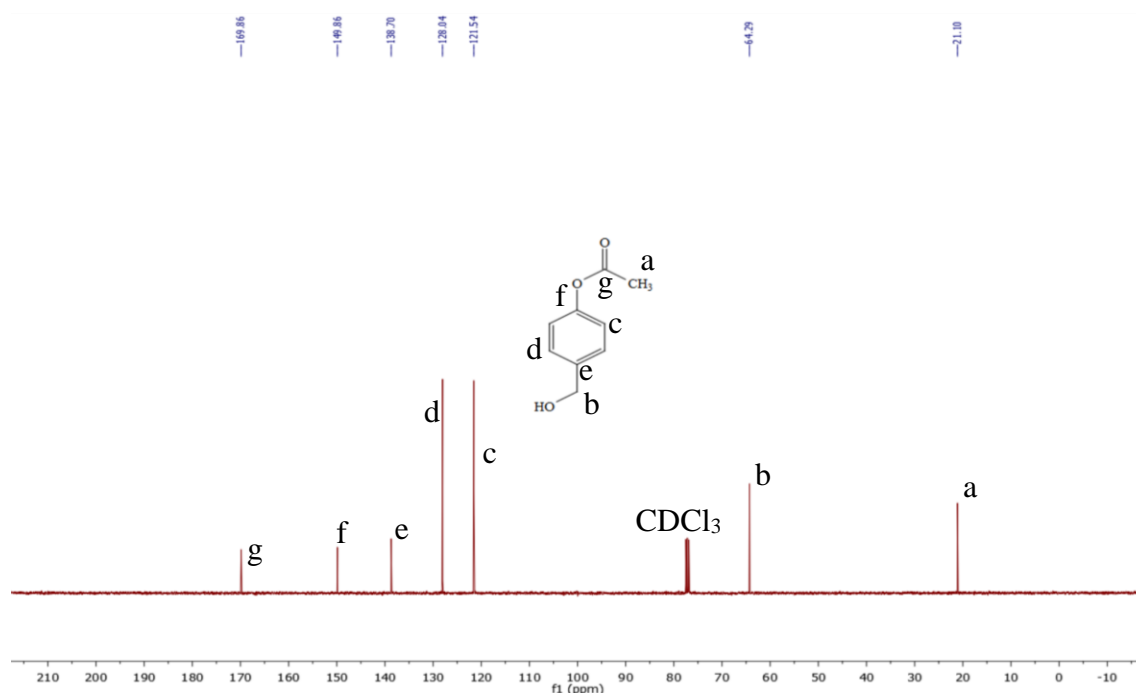
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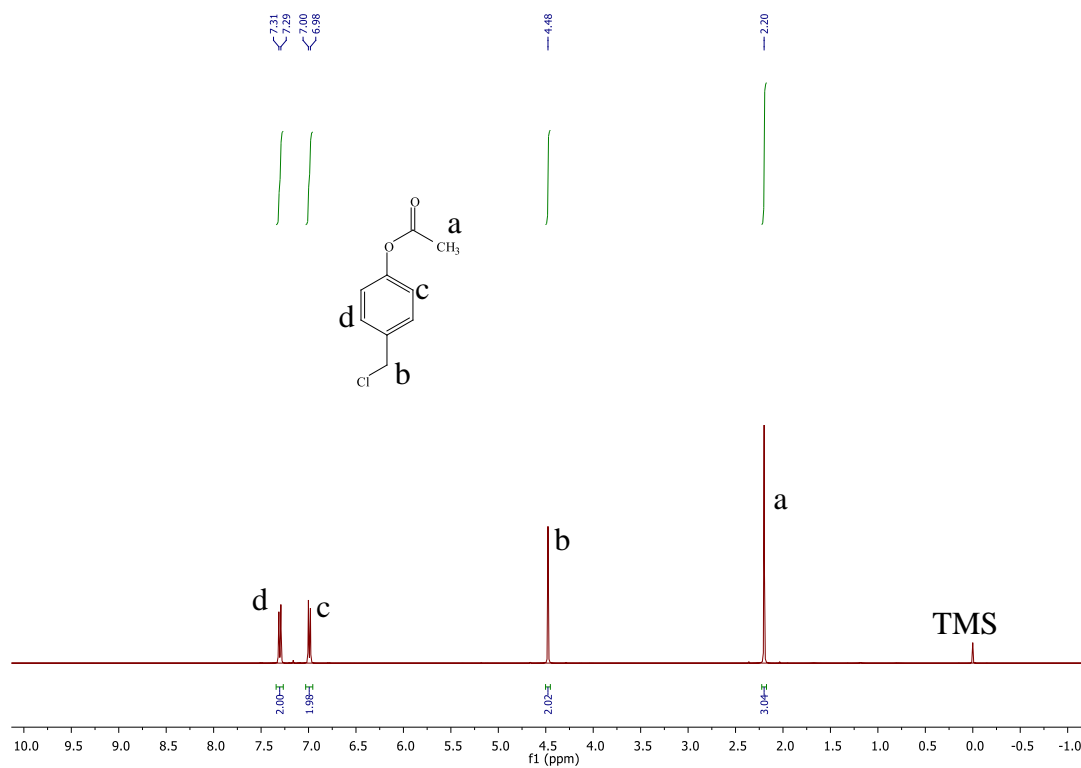
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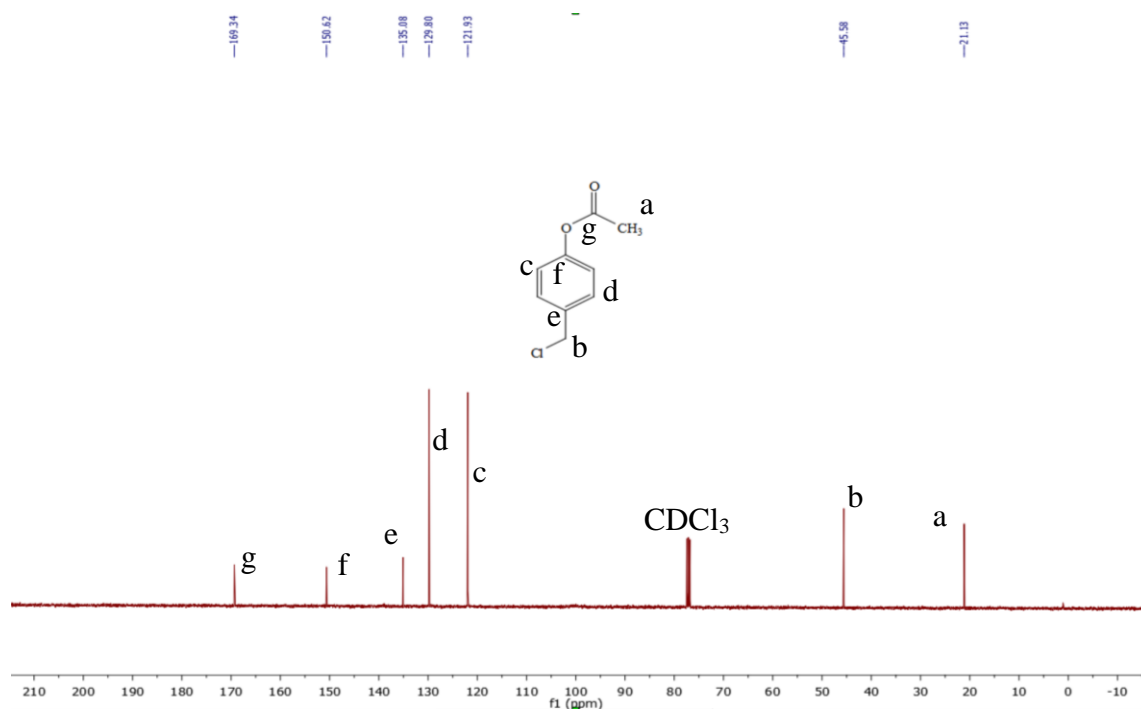
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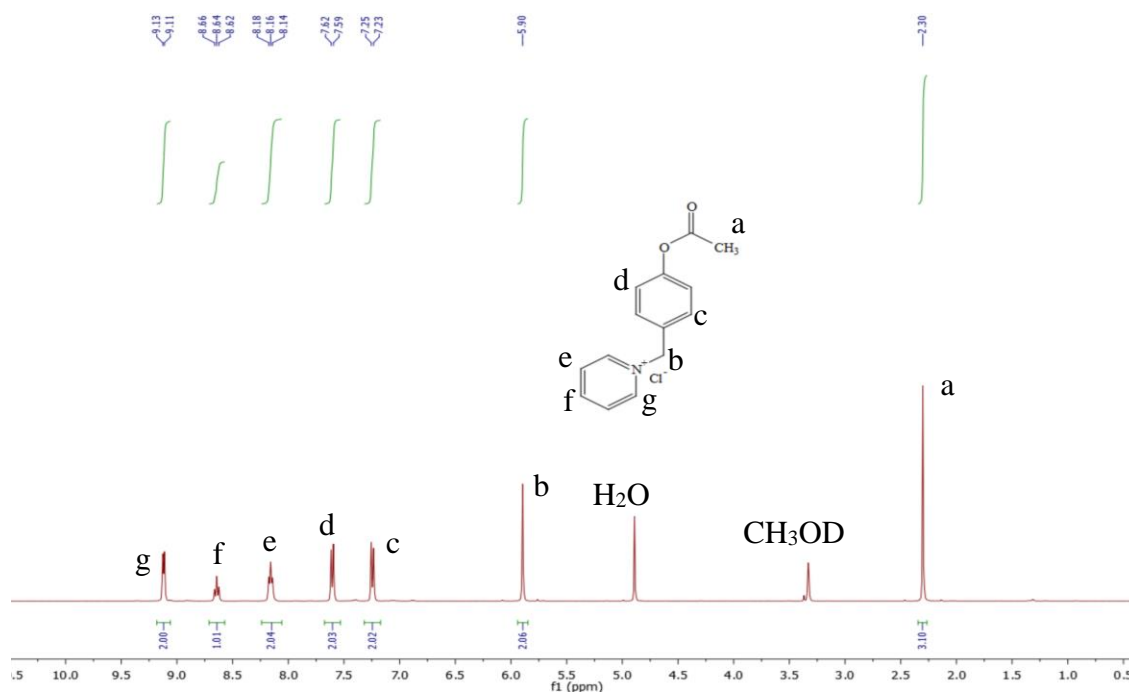
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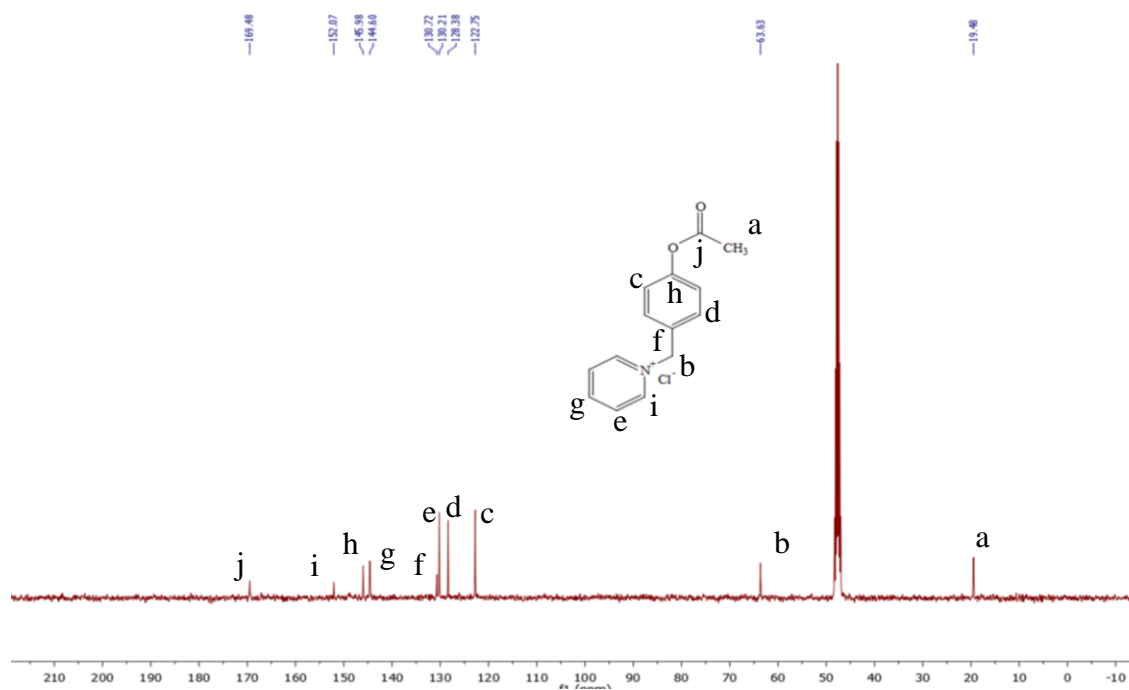
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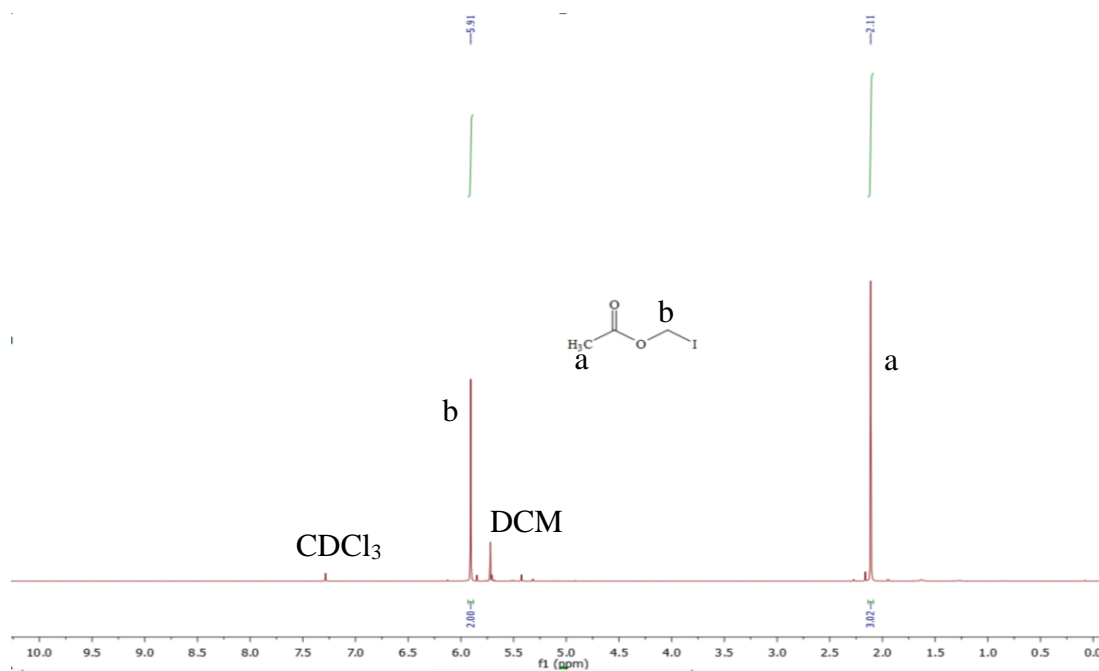
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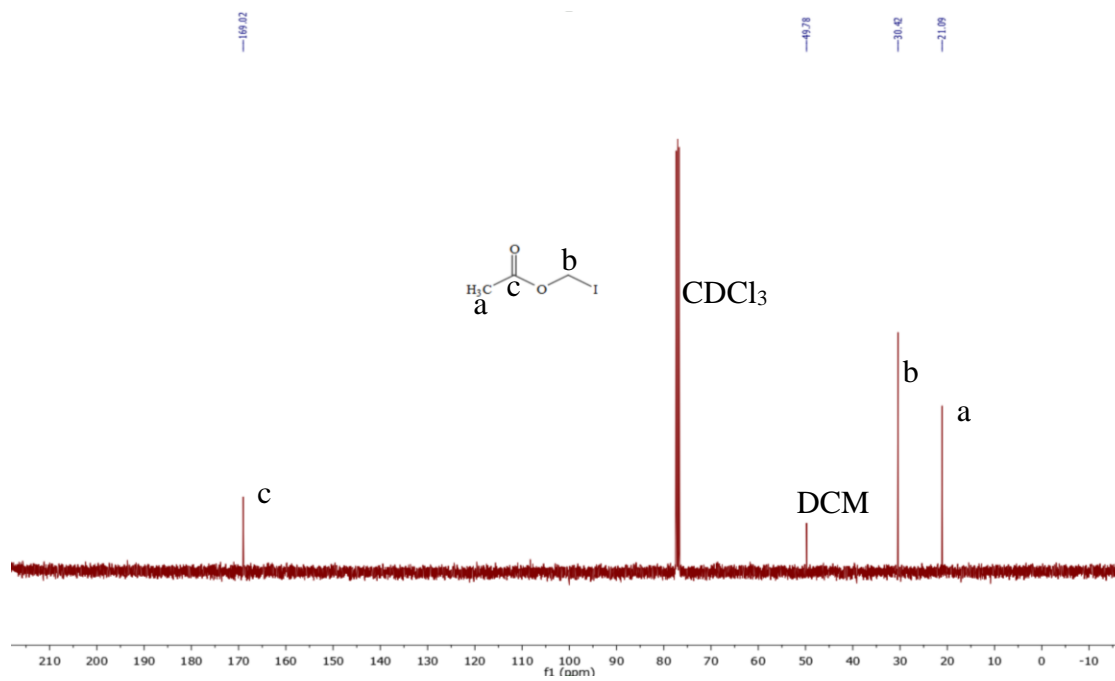
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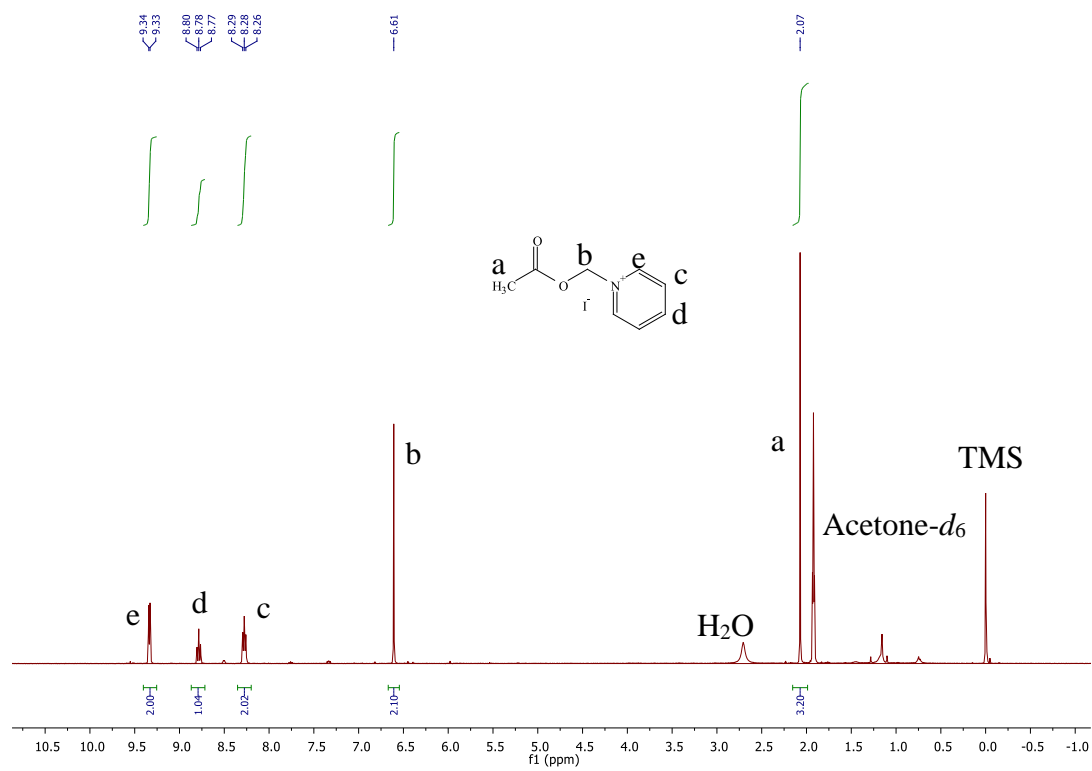
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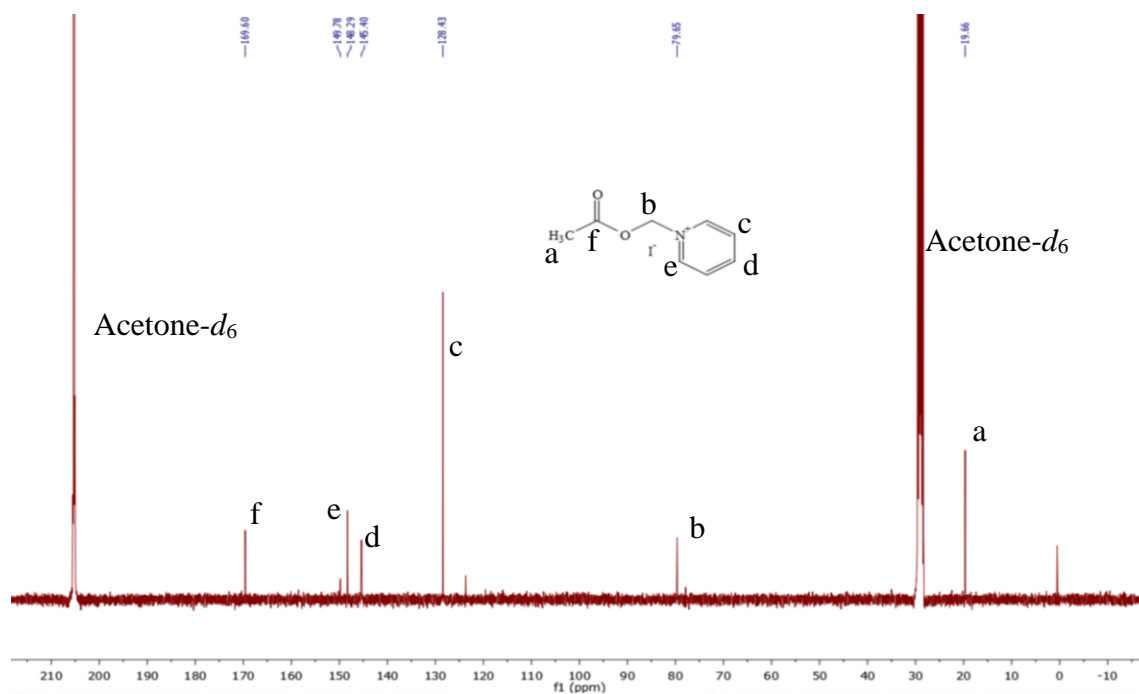
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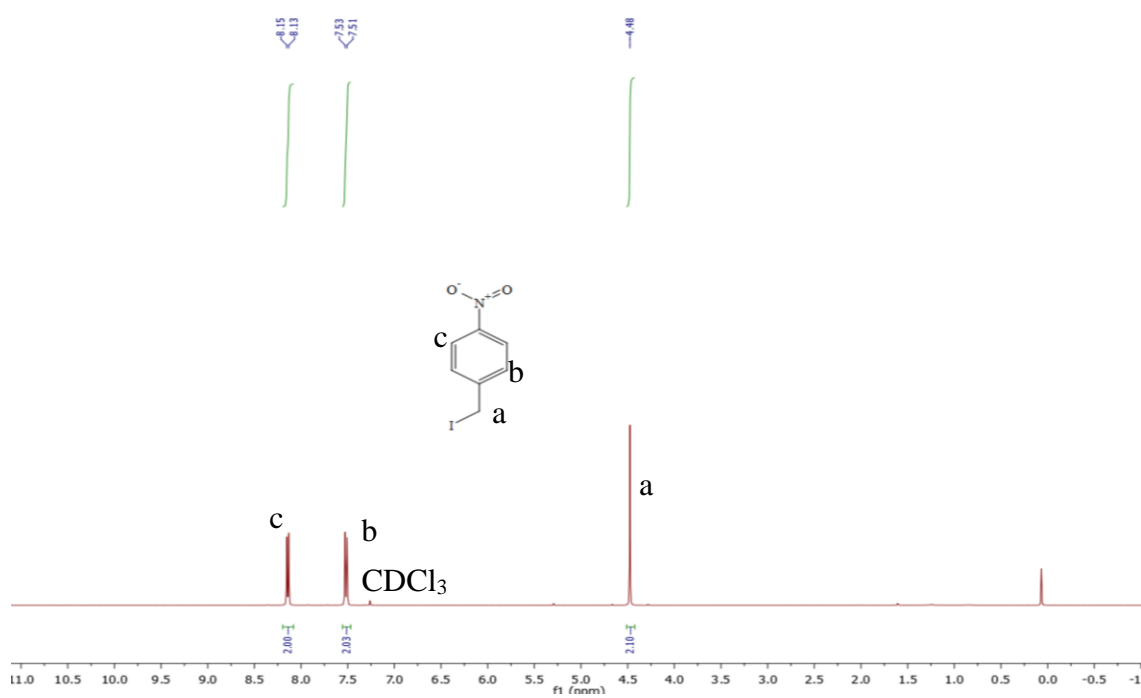
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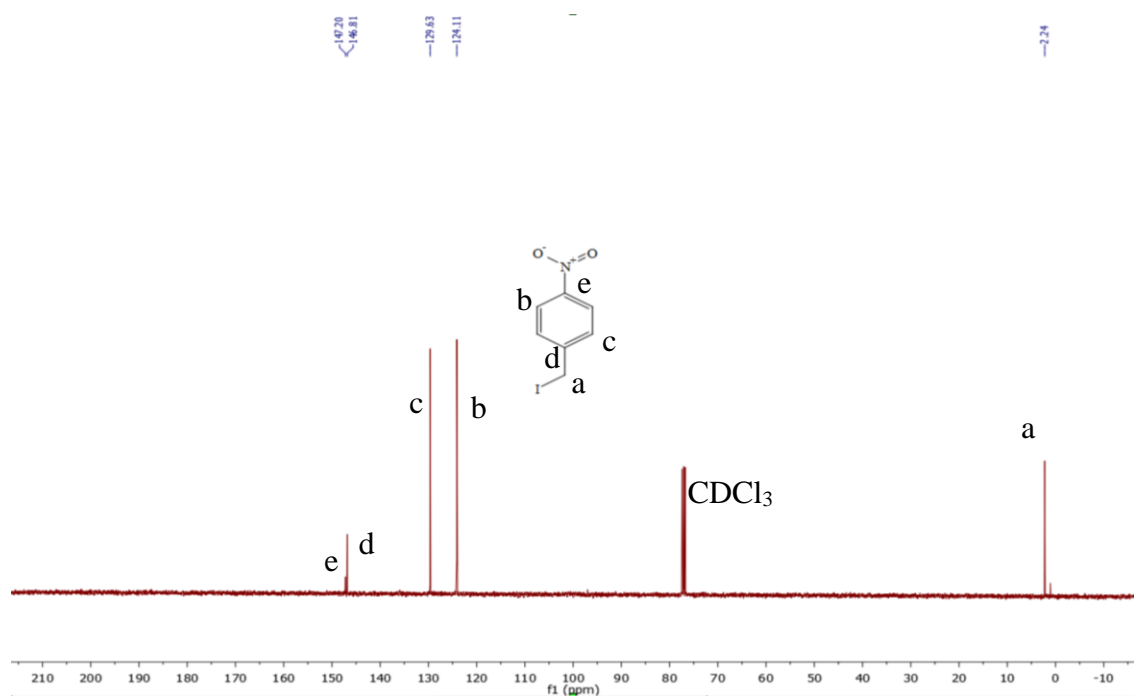
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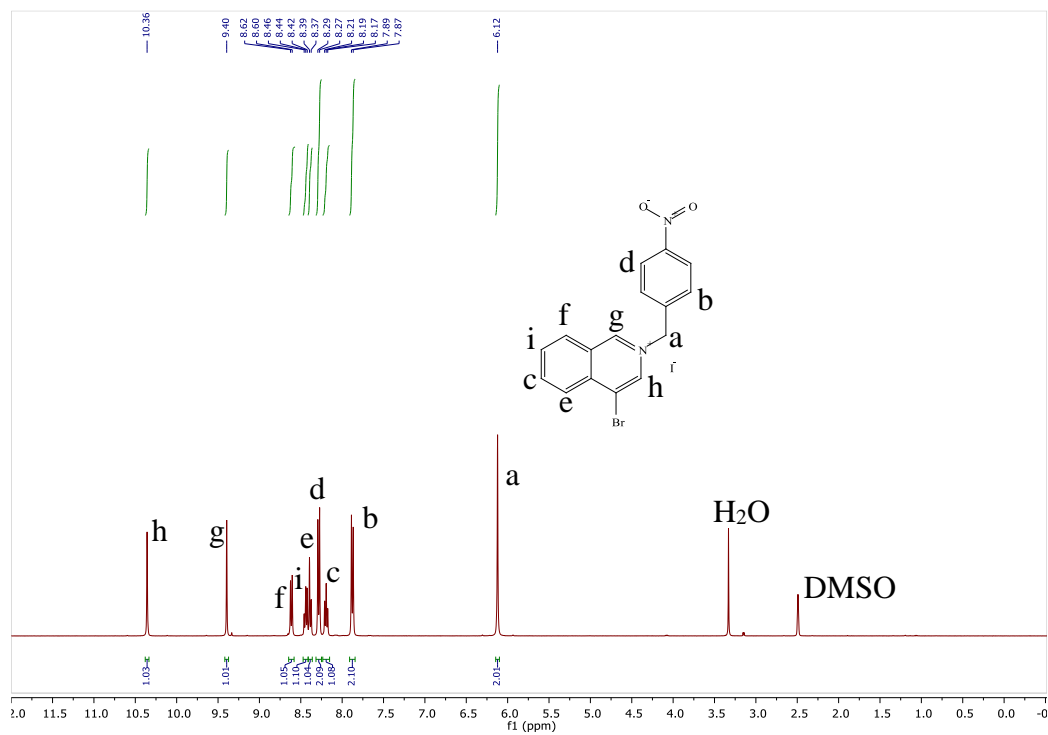


Compound 13:



Compound 13:



Prodrug **BW-HIF-356**:Prodrug **BW-HIF-356**: